THE DEVELOPMENT AND VALIDATION OF ASSAY METHODS FOR THE QUANTITATIVE DETERMINATION OF DRUGS AND THEIR METABOLITES IN BIOLOGICAL SPECIMENS (PIROXICAM AND NABUMETONE)

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Co-study leader: Dr KJ Swart

DECLARATION

It is herewith declared that this dissertation for the degree Master of Medical Science at the University of the Free State is the independent work of the undersigned and has not previously been submitted to another university or faculty for a degree. In addition, copyright of the dissertation is hereby ceded in favour of the University of the Free State.

Andrew David de Jager

16 August 2001

Date

Declaration certifying the candidate's personal contribution towards the research which is the subject of this M. Med. Sc. (Bioanalytical Chemistry)

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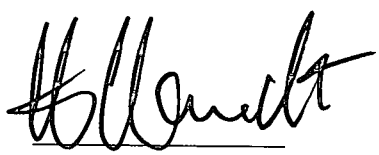
Title: THE DEVELOPMENT AND VALIDATION OF ASSAY METHODS FOR THE QUANTITATIVE DETERMINATION OF DRUGS AND THEIR METABOLITES IN BIOLOGICAL SPECIMENS (PIROXICAM AND NABUMETONE)

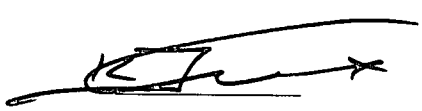
Study leader: Professor HKL Hundt

Co-study leader: Doctor KJ Swart

We, the undersigned, declare that under our supervision, Mr Andrew David de Jager performed the development and validation of the two assay methods contained in this dissertation, as well as the sample assays of the said research projects. Under our supervision, Mr. de Jager personally prepared and submitted full length papers dealing with the assay methods described in the dissertation for publication in the Journal of Chromatography B. Furthermore, the work pertaining to piroxicam was presented orally at the 1998 annual congress of the SA Pharmacological Society. Mr de Jager personally typed and compiled the dissertation in its present form.



Prof HKL Hundt



Dr KJ Swart

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1 Introduction

The development and validation of bioanalytical methods which are used to generate data for pharmacokinetic and bioavailability studies has become a highly regulated science. Over the years, most national regulatory authorities have laid down guidelines as to what represents acceptable analytical procedures. This has led to a number of workshops, symposia and conferences during which consensus was sought on the matter of method validation, with

special reference to bioanalytical assay methods developed for the purpose of assaying drugs and their metabolites in biological specimens, in support of submissions to such authorities. [8]. It is for this reason that method development should be undertaken bearing in mind the minimum validation requirements that regulatory authorities demand. Method development and method validation can thus not be disconnected from one another and should be approached as a loosely defined unit.

2 Method development

2.1 Introduction

If the perfect research facility were to exist, it would possess every type of state-of-the-art detector, sample preparation instrument, data management system, expensive reagent and general gadgetry that one could desire. The analyst would have available to him or her, an unlimited amount of sample on which to perform quantitation, unlimited time and unlimited money. Detectors would be infinitely sensitive, deadlines would be meaningless and tedious extraction procedures would all be automated.

But since this Utopian environment does not exist, the bioanalytical chemist must begin by carefully taking stock of the task at hand and the resources available, and often the analyst is forced to exercise the next-best option. In essence the analytical process is the means by which chemical information is obtained from a sample [1].

2.2 The Literature Survey

The prudent analyst will begin method development with a comprehensive search of the literature available on the analyte. In an environment of electronic communication, databases and information sharing, it seems senseless to rediscover facts that have already been documented. Information regarding facts such as drug stability in plasma, absorption maxima and pK_a values can spare the analyst many hours in an industry where there is never enough time.

While analytical literature is generally the primary source of information, it is usually necessary to cover a broader spectrum. Clinical literature, for instance, will indicate maximal plasma concentrations that can be expected following a particular dose. This will immediately give the analyst insight into the range that a calibration curve will have to span. When no data are available on the analyte, data on similar compounds can often be useful. Having collected relevant information, it is useful to make a summary of the literature and to pay attention to, *inter alia*, the following questions:

- Is the analyte stable in the matrix under investigation? If not, what precautions were taken?
- Is the information regarding stability likely to be acceptable to regulatory authorities?
- At what temperature should samples be stored, and for how long can they be stored without significant degradation?
- What types of detectors have been used to determine the analyte?
- Is it possible to detect the analyte in the original form, or should it first be derivatized?

- Is it possible to quantify the analyte itself, or is it better to quantify a metabolite (so-called pro-drug)?
- How have other authors extracted the analyte from the matrix?
- What type of analytical columns proved successful in resolving the compound?
- What is the nature of the mobile phase used for separation?
- What are the possible metabolites, and could they interfere with the assay?
- Should the metabolites, if any, be quantified simultaneously?
- Have any of the authors noted novel problems that should be monitored?

Finally, despite the valuable information gained from literature, it is not wise to be blinded by such data. The analyst should always strive to improve on existing methods, and it is important to view literature as a source of insight only. For example, new types of detection that have not yet been used in the literature could significantly enhance the method.

2.3 Formulation of an analytical plan

Having gained as much information as possible, it is worthwhile to spend some time taking stock of the particular analytical situation and formulating a plan of action.

Ideally, what is already known (represented by knowledge gained from the literature) should be combined with what is required (represented by the requirements of the study) and what is available (represented by resources, equipment, expertise, etc.), and formulated into a general method development strategy. The aim of this strategy is firstly to reliably quantify samples with as few post-validation problems as possible, and secondly to satisfy the stringent international validation criteria.

For example, the analyst may not have a wide selection of analytical detectors available, and may be required to improvise. If a particular study will generate a large number of samples, the analyst should envisage an extraction procedure that requires minimal sample preparation. If, on the other hand, sensitivity of the assay method is of the utmost importance, a procedure involving sample concentration and optimal recovery should be favoured.

In sections that will follow, method development strategies for two analytes (piroxicam and 6-methoxy-2-naphthylacetic acid) were devised, which will serve as examples of the formulation of an analytical plan.

Having said this, even the most well planned and systematic of approaches are, from time to time, thwarted by the sheer complexity of this science. Occasionally, the analyst must abandon what appears to be the perfect strategy on paper and to adopt whatever works.

2.4 Consideration of analytical variables

2.4.1 Matrix

From an analytical point of view, there are three important factors that must be taken into account with respect to the matrix in which the analyte resides:

- How to get the analyte into the matrix (preparation of calibration standards)?
- How to remove the analyte from the matrix (extraction)?
- How stable the analyte is in the matrix (matrix stability)?

Most commonly, plasma is used for drug measurement in humans. However, urine, saliva, cerebrospinal fluid, faeces, hair, nails, tissue, semen, bronchial secretions and vaginal fluid *inter alia* are all possible media of measurement [2]. A key factor to remember is that most of the above-mentioned matrices represent aqueous media, and it is often necessary to exploit this characteristic.

2.4.1.1 Introduction of analyte into the matrix

As opposed to study samples, where the analyte is introduced into the medium of measurement (predominantly plasma) by the body, the analyte must be introduced into the matrix artificially when preparing calibration standards, quality controls or general plasma solutions of analyte used for method development. It is for this reason that the characteristics of the matrix be well understood. The most common way of introducing a known amount of analyte into plasma is by using a stock solution, preferably prepared in water, as plasma is akin to water. However, not all analytes are soluble in water and it is often necessary to prepare stock solutions in organic solvents such as methanol or acetonitrile. Introduction of these solvents into plasma will have implications. Firstly, this could result in the precipitation of plasma proteins, and care should be taken to ensure that analyte actually dissolves in the matrix by way of proper shaking. Secondly, introduction of organic solvents into the matrix will change the characteristics of the matrix, which implies that study samples and calibration standards are no longer identical. The only option in this instance is to spike as small a volume as possible into the plasma pool, using strong spiking solutions. It is recommended that no more than 1% (v/v) be added to the matrix pool. It is also possible, where high concentrations are required and the analyte is water-soluble, to dissolve the

analyte directly in the plasma, but care should be taken to ensure that the mixture is well shaken to ensure complete dissolution. Buick et al. [12] report that matrices become more difficult to spike with analyte the less fluid they are and consequently analytical results may have more error associated with them, and that solid matrices cause even greater problems. Shah [11] proposes that whenever possible, the same biological matrix as that in the intended samples should be used for validation. However, for tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices may suffice.

2.4.1.2 Extraction of the analyte from the matrix

Before a sample can be introduced into an instrument, the analyte must be removed from the matrix and re-dissolved in a solvent that is compatible with the analytical system. This procedure is known as extraction. In general, two extraction procedures, namely liquid-liquid extraction (LLE) and solid phase extraction (SPE) are commonly used in the bioanalytical laboratory. A diverse array of other procedures such as ultrafiltration and dialysis, to name but two, have been described but are less frequently used for bioanalytical applications.

It is occasionally unnecessary to remove the analyte from the matrix, but rather to modify the matrix by way of protein precipitation. A detailed discussion on each of these procedures falls outside of the scope of this dissertation. However, what is of importance to the analyst is the judicious selection of the extraction/sample preparation procedure that is most likely to be successful.

The following represent broad selection criteria with respect to the selection of a suitable sample preparation procedure during the method development phase:

When to attempt a protein precipitation procedure

Removal of protein by denaturation or precipitation is an effective method of sample preparation that is often used on plasma and whole blood samples. It should be remembered that dilution occurs during protein precipitation and if no further sample preparation is undertaken it may result in a lower sensitivity of the assay method. The main reason is to remove proteins that can precipitate when in contact with the mobile phase causing clogging of the chromatographic system. This procedure can be considered when the following conditions exist:

- The expected matrix concentrations are high (C_{max} in the order of 2μg/ml).
- It is not essential (for the sake of detector functioning and physical design) that samples be clean (eg. the cell of a UV detector is not easily damaged or soiled by dirty samples, while the electrodes of an electrochemical detector will rapidly become poisoned by even a few dirty samples).
- Compatible detection modes have a relatively high degree of specificity (eg. a λmax that is above 300nm, fluorescence or MS detection). In the case of the highly specific MS/MS detection, very often even low concentrations of analyte can be assayed.
- High sample throughput is a priority.

The main advantages of this technique are the speed at which samples can be prepared and its simplicity, while the main disadvantage is that there may be loss of the analyte by occlusion in the precipitate.

How to select between a LLE and a SPE procedure

Whether to use LLE or SPE is often a matter of preference. However, there is an international trend to favour SPE for the preparation of bioanalytical samples. In the main, this is due to the need for automated sample preparation procedures which increase sample throughput, and this has given rise to the swift growth in 96-well SPE technology. General criteria for discrimination between the two are listed below:

- If the analyte is amphoteric, use SPE. If the analyte is not amphoteric, either of the two procedures may be suitable
- If the analyte is relatively polar, SPE is more likely to be successful. If the analyte tends towards non-polarity, either procedure may be suitable.
- If high throughput is required, use SPE as it lends itself to automation.

2.4.1.3 Stability of the analyte in the matrix

Before samples are assayed, it is necessary to determine the conditions under which they can be safely stored. If samples rapidly degenerate, a long delay between sample collection and assay will result in dramatic errors in data generated [8,9]. Often, analytical literature contains reliable data with respect to suitable storage conditions of a particular analyte. In recent times, however, regulatory authorities have become less inclined to accept such references and require laboratories to generate such stability data in house.

If it is evident (either from literature or experimentation) that the analyte is unstable in the matrix under investigation, appropriate action will have to be taken. These measures include storing at lower temperatures, addition of antioxidants or enzyme inhibitors to the collection vessels and processing immediately after collection [12].

2.4.2 Internal/external standardisation

The internal standard technique is very common in bioanalytical methodology [14]. The rationale for the use of an internal standard is that the partition characteristics of the analyte and internal standard are very similar. According to Curry and Whelpton, however, the only appropriate uses of non-isotopic analogue internal standards are to serve as qualitative markers, to monitor detector stability, and to correct for errors in dilution and pipetting [3].

Internal standards are usually beneficial for classical instrumentation and manual sample pre-treatment. Modern equipment and automation, however, can provide extremely reproducible response measurements.

An internal standard is used to minimise the effects of human and analytical errors that occur from time-to-time in analytical laboratories. These errors include inaccurate pipetting, sample spillage and inconsistent injection volumes. The common practice is to use the so-called internal standard ratio method, whereby the detector response generated by the analyte is divided by the internal standard response (an equal amount of internal standard is added to each sample). This ratio is then used for quantitation. If, for instance, 15% of the sample is spilled (after the addition of internal standard), the detector response for both analyte and internal standard will be some 15% lower but the ratio, and thus the analytical result, will remain unchanged. The focus of internal standardisation is to render analytical methods more

robust. Internal standards are not considered mandatory by regulatory authorities, but more and more pressure is being placed on laboratories to use them if at all possible. However, Pachla *et al.* [9] caution that even though an internal standard may correct for minor recovery imprecision, imprecise and erratic recovery of the internal standard itself may introduce additional analytical error [4] and further bias data interpretation. The internal standard technique will not inevitably improve, nor will it always adversely affect, the precision of an analytical method [14].

The characteristics of a good internal standard for HPLC* quantitation are as follows:

1. It must be eluted in a vacant spot on the chromatogram.
2. It must be completely resolved from the neighbouring peaks.
3. It must have a k' value similar to the k' value of the analyte peak.
4. It must be chemically similar to the analyte of interest.
5. It must be added at a concentration similar to the analyte of interest.
6. It must be stable and available in a highly pure form.

In the case of a UV detector, in which absorbance is measured, the omission of an internal standard is not as problematic as when using LC-MS/MS with electrospray ionisation (ESI) for instance. Large variations in sensitivity within batches of even 100 –200 samples have

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* *These characteristics apply to classical detectors such as UV, fluorescence, electrochemical (ECD) and refractive index (RI) detectors. Characteristics of a suitable internal standard for a mass spectrometric assay method differ slightly, but this falls outside of the scope of this discussion.*

been documented when using LC-MS/MS ESI. If an internal standard cannot be found for such an assay, problems with quantitation will be encountered. The internal standard of choice for LC-MS/MS ESI analysis is an isotopically labelled form of the analyte. However, these labelled internal standards are not always readily available, and another internal standard must often be used.

If it is not possible to get an internal standard to track the extraction, it may be necessary to add an external standard, the purpose of which is to compensate for variations in injection volume and variable sensitivity. An external standard is a chemical entity added to a sample after extraction and like an internal standard, is added in equal quantities to every sample. Here too, a ratio of analyte to internal standard response is used for quantitation.

2.4.3 Detection

The analyst must know what type of detection the drug is predisposed to, based on the physico-chemical properties, and whether or not such equipment is available. If sensitivity of the assay procedure is of primary importance (eg. the study involves the tracking of a drug following a very low dosage), then the most sensitive detector available to the analyst will have to be used.

2.4.4 Sample preparation

If, as mentioned above, sensitivity is the foremost consideration, the sample preparation procedure will have to be geared to optimising sensitivity, and a procedure that results in concentration, rather than dilution of the sample will have to be optimised.

If, however, a large number of samples will be generated during the study, and throughput is the determining factor rather than sensitivity, a rapid sample preparation procedure, or a procedure which lends itself to automation is best. From time-to-time it is necessary to prepare samples rapidly owing to instability of the analyte. The analyst may often be forced to develop a compromise assay procedure that is suited to more than one analyte simultaneously (eg. a drug and one or more of its metabolites).

The isolation and measurement of organic compounds in a biological matrix, especially at low concentrations, may present a significant analytical challenge. The primary objectives of a sample preparation scheme can be summarised as follows [1]:

- Removal of unwanted protein or non-protein material that would interfere with analyte determination.
- Removal of material if the resolving power of the chromatographic system is insufficient to separate all the components in the sample (or in a time that is reasonable).
- Removal of material that would affect chromatographic resolution or reproducibility (this is particularly significant in LC-MS/MS, where the so called matrix effect is an important factor [5,6].
- Suspension of compounds to enable injection under the initial chromatographic conditions.
- Concentration of the analyte(s) to within the detection capabilities of the analytical detector.
- Dilution to reduce solvent strength, or to avoid solvent incompatibility.

- Removal of material that could block the chromatograph tubing, valve(s), column or frit(s).

A balance should be struck between the specificity obtained from the sample preparation scheme and that obtained from the instrumentation [1]. Insufficient sample clean-up may result in interference with the analyte, but too great a sample preparation effort may result in low sample throughput.

Huber and Zech [7] view sample preparation schemes as a collection of unit operations, as summarised in Table 1. A thorough discussion of all sample preparation schemes falls outside the scope of this dissertation, but the so-called unit operations of SPE, LLE and protein precipitation are summarised as follows:

Table 1: Comparison of the unit operations required for some sample preparation schemes, adapted from McDowall [1]

Liquid-liquid Extraction	Solid phase Extraction	Protein precipitation
Aliquot sample	Centrifuge sample	Aliquot sample
Add internal standard	Aliquot sample	Add precipitant
Add buffer	Add internal standard	Mix
Add organic phase	Mix	Centrifuge
Mix sample	Activate cartridge (phase 1)	Transfer into vial
Centrifuge	Activate cartridge (phase 2)	HPLC analysis
Collection of organic phase[a]	Apply sample	
Dry organic phase	Wash cartridge	
Reconstitute sample	Elute analyte(s)	
Mix	Transfer into vial[aa]	
Transfer into vial	HPLC analysis	
HPLC analysis		

[a] This may either be aspiration or merely decanting (following aqueous phase freezing), depending on the relative densities of the two phases.

[aa] In certain cases it may be necessary to first evaporate the eluent to dryness and reconstitute the sample in a more suitable solvent before injection.

3 Validation

Before an analytical method can be used to quantify samples, it must be demonstrated that all aspects of the procedure are fit to do so, against the backdrop of internationally accepted norms. Analytical method validation includes all the procedures recommended to demonstrate that a particular method for the quantitative measurement of an analyte(s) in a given biological matrix, such as blood, plasma, serum or urine, is reliable and reproducible. [8].

3.1 Pre-study validation

Pre-study validation is performed at the end of the method development phase, when the analyst has satisfied him or herself that the method is acceptable for use on clinical samples. This pre-study validation is carefully scrutinised against documented acceptance criteria, usually in the form of a Standard Operating Procedure (SOP). If the method is shown to be acceptable, then the analyst (or laboratory technician) may proceed with assaying clinical samples. When an analyst is at the point performing a pre-study validation, detailed knowledge of the following is important:

3.1.1 Stability in the matrix

To obtain reliable data, the drug must be stable from the time of sample collection to the completion of sample analysis [9], and in particular, stability should be demonstrated in the biological media under storage. Without sound stability information, all subsequent pharmacokinetic data are questionable and this could eventually lead, in the worst case, to misleading information concerning the clinical use of the drug [10]. The stability of an

analyte in a particular matrix and container should not be extrapolated to other matrices and containers [8]. Generally, long-term matrix stability is determined at –20°C and –70°C, in order to determine if there is significant degradation of the analyte in the matrix. Most often this is done by comparing, at various concentrations, pre-prepared samples of analyte in matrix to samples that have been freshly prepared. If there is no significant difference (a decrease of more than 10% is considered significant [10]), then the analyte can be deemed stable in the matrix for at least the interval between the two preparation dates. Although the above-mentioned procedure is most commonly adopted, more complex statistical procedures have been reported [10]. If it is clear that there is degradation, measures will have to be taken in order to minimise analyte loss. These measures include the addition of antioxidants or enzyme inhibitors to the collection vessels or processing immediately after sampling [12].

3.1.2 Freeze-thaw stability

If a biological sample is going to be subjected to multiple freeze-thaw cycles, it should be demonstrated that this will not influence the analytical result that the sample produces [11]. High, medium and low concentration samples should be kept at the intended storage temperature for 24 hours. The sample should then be allowed to thaw unassisted at room temperature. When completely thawed, the sample should be transferred to the original freezer and kept frozen for 12 – 24 hours. The cycle of thawing and re-freezing should be repeated two more times, and the sample analysed on the third cycle in order to ascertain freeze-thaw stability [11,12].

3.1.3 Stability of compounds in stock solution

If stock solutions are going to be used repeatedly (eg. to prepare fresh samples or internal standard solutions on an on-going basis), is should be shown that the compound of interest is stable in stock solution for the period over which it is to be used. If this is not the case, fresh stock solutions will have to be prepared. More often than not, such solutions are prepared in an organic solvent such as methanol, or some aqueous buffer and stored at 4°C. If solutions prove to be unstable under these conditions, a lower storage temperature, or different solvents should be investigated.

3.1.4 On-instrument stability

After preparation, samples generally reside on an autosampler (automatic sampling device) in batches before being injected onto the chromatographic system. It must be proved that samples do not degrade on the autosampler while awaiting injection. Stability should be assessed over the anticipated batch duration to be used during sample processing [8]. If the analyte and/or internal standard is not acceptably stable on the instrument, smaller batches will have to be processed.

3.1.5 Selectivity/Specificity

Often, the terms selectivity and specificity are used interchangeably [13,14]. The term specific, however, implies that a method produces a response for a single analyte only. The term selective refers to a method that provides responses for a group of chemical entities, which may or may not be distinguishable [15]. In practice, the analyst must ensure that only the drug of interest produces a response, that is to say no interference exists. Sources of interference include concomitant medication and metabolites, but most often, interference

from compounds naturally present in the matrix itself represents the most significant problem. Furthermore, it cannot be assumed that the level of interference in a blank measurement will be equal to that in a measured sample, and therefore cannot be compensated for by subtraction [14]. The simplest way to establish specificity is to demonstrate a lack of response in blank biological matrix from a number of different sources. One limitation of this approach is that if the blank sample originates from a volunteer that has not been exposed to the drug of interest, possible interference due to the presence of metabolites will not be observed. A further test of specificity is the degree to which the intercept of the calibration curve differs from zero, with a large deviation indicating interference [16].

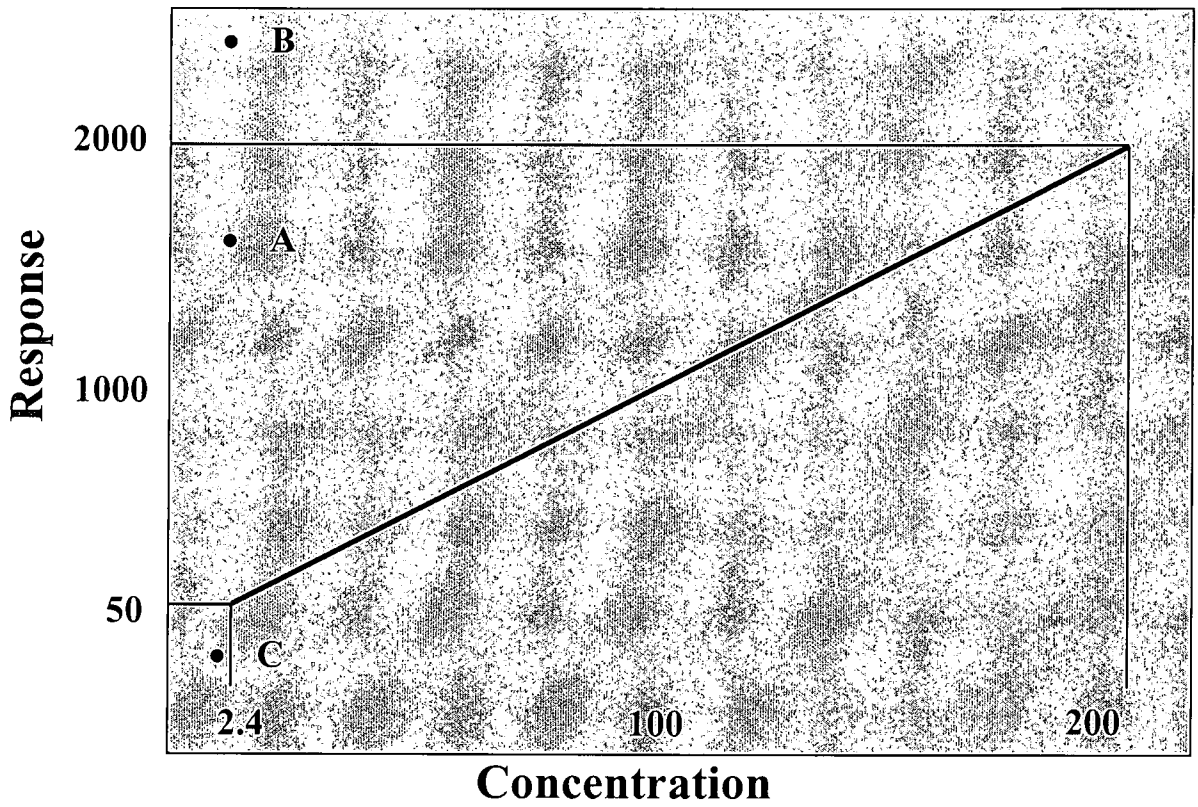
3.1.6 Recovery of analyte from the matrix

Recovery is the fraction of analyte removed from the sample by the extraction procedure. If, for example, 12μg of an analyte is present in a 1ml sample aliquot, and 9μg is extracted during the procedure, then the recovery is 75%. Not only should the recovery of analyte be determined, but that of the internal standard as well, if one is used. In practice, recovery should be determined by comparing analytical results from extracted samples with unextracted samples (in appropriate solvent) that represent 100% recovery. Since it cannot be assumed that extraction characteristics are the same over a given concentration range, recoveries should be determined at high, medium and low concentration [8]. There are varying opinions as to what constitutes acceptable recovery. While some authors feel that recovery should at least be in the order of 75% [9], it has been argued (and is generally accepted) that recovery may be as low as 50%, provided that the recovery is reproducible [8].

3.1.7 Range and linearity

From literature that is available, the analyst must obtain as much information as possible regarding the concentrations that can be expected in study samples. Let us say, for example, that an analyst is required to develop a method to determine pharmacokinetic parameters, following a 40mg dose of trimetazidine. If the analyst validates such an analytical method between 0.2 and 12μg/ml, then the assay method would be extremely inappropriate to quantify the samples generated. The reason is that literature reflects that the maximum plasma concentration such a regimen should produce is approximately 100ng/ml [17], and thus a range between 200 and 0.2ng/ml would be the appropriate range to validate. The upper level of the calibration range is dictated by the maximum concentration in the study samples (C_{max}), while the lower limit of the calibration range, the so-called lower limit of quantification (LLOQ), is more often than not determined by the sensitivity of the assay method. The LLOQ should be such that the area beneath the concentration versus time curve extrapolated from the last measurable time point to infinity should not be greater than 15% of the total area beneath the curve. Ideally, the concentrations of all study samples should fall within the validated calibration range.

Figure 1: Illustration of a calibration range showing responses (A) within the range, (B) above the range and (C) below the limit of quantitation.



3.1.8 System suitability

As part of the entire validation process, the analyst must demonstrate that the equipment used is suitable for the intended purpose. Before a method can be validated, each analytical component (eg. analytical pump, autosampler, UV detector, analytical column, etc.) must be

tested and evaluated against documented criteria, in order to determine whether or not it is suitable for use in sample analysis. This is known as the operational qualification and performance verification of the analytical set-up (OQ/PV). Generally, manufacturers of analytical equipment document such criteria in the instrument manual and users follow these acceptance criteria, but it is not unusual for users to compile documents containing their own criteria. As most modern equipment is software driven, modules are tested using self-diagnostic software and a standard instrument configuration. With an autosampler, for example, precision and reproducibility are the most important performance parameters, whereas for a UV detector, wavelength accuracy and minimal baseline inconsistency is important. Most often, these systems are tested using well-characterised solutions of suitable compounds. The integration of Good Laboratory Practice (GLP) into the pharmaceutical laboratory implies that detailed, traceable records of instrument testing should be kept, and that instrument usage should be logged in a suitable database.

3.1.9 The pre-study validation procedure

First, calibration standards that are used to construct a calibration line, should be prepared in the same matrix as the clinical samples. The number of calibration standards required depends largely on the concentration range it is required to span, that is to say, the wider the calibration range, the more calibration standards required.

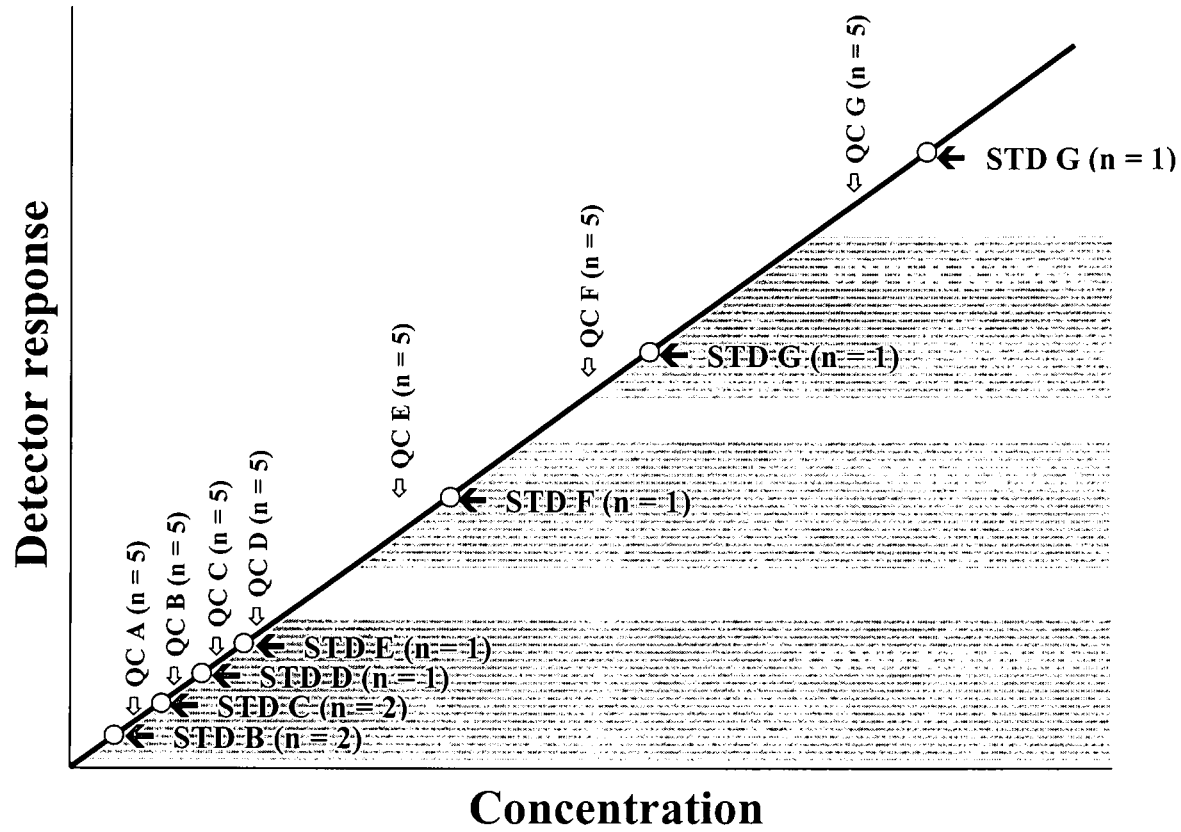
Table 2: Example of appropriately constructed calibration line

Concentration	Calibration standards	Quality controls	Concentration
LLOQ[1]	STD $B_{(dupl)}$		
		← QC $A_{(five\text{-}fold)}$	1.2×LLOQ
2×LLOQ	STD $C_{(dupl)}$		
		← QC $B_{(five\text{-}fold)}$	2.4×LLOQ
3×LLOQ	STD D		
		← QC $C_{(five\text{-}fold)}$	3.6×LLOQ
4×LLOQ	STD E		
		← QC $D_{(five\text{-}fold)}$	4.8×LLOQ
		← QC $E_{(five\text{-}fold)}$	0.4 – 0.5×Cmax
$0.5\times C_{max}$	STD F		
		← QC $F_{(five\text{-}fold)}$	0.8×Cmax
C_{max}[2]	STD G		
		← QC $G_{(five\text{-}fold)}$	1.6×Cmax
$2\times C_{max}$	STD H		

[1] The LLOQ is defined as the lowest concentration in prepared plasma samples that can firstly be detected at a signal-to-noise ratio of at least 5:1 and secondly perform with acceptable precision (CV% less than 20%).

[2] The C_{max} is defined as the maximum concentration that can be expected in plasma samples. This information is usually obtained from clinical and analytical literature.

Figure 2: Distribution of calibration standards and quality controls in a typical intra-day validation



As depicted above, a good calibration line is populated at the lower, middle and upper sections of the expected concentration range. The four lower calibration standards (STD B – E) have been dispersed at the lower section of the calibration line at regular incremental intervals (the lower two prepared in duplicate). This has been engineered in such a way that the LLOQ (which will necessarily be the lowest calibration standard used) can be raised to the level of the next calibration standard, should this become necessary.

In the example above, STD F would be a point in the mid-section of the calibration line, while STD G and STD H represent C_{max} and $2 \times C_{max}$ respectively.

Furthermore, quality controls (which are used to verify the calibration line) are interspersed throughout the calibration line. The lower quality controls (QC A – D) have been prepared 20% above consecutive calibration standards, also engineered to facilitate raising the LLOQ, should it be necessary. The upper quality controls have also been wedged between the middle and upper quality controls. This is to ensure that the entire calibration line is monitored.

In contrast, the FDA of America recommends that only four quality controls be used in the three pre- study validations that they require [8].

LLOQ QC sample:	same concentration as the lowest non-zero sample.
Low QC sample:	≤3×LLOQ.
Medium QC sample:	approximately midway between the high and low QC concentrations.
High QC sample:	75 to 90% of the highest calibration standard.

Preparation of a typical validation batch

In order to perform pre-study method validation, the required calibration standards, quality controls, blanks (plasma containing no analyte or internal standard), a zero sample (plasma containing internal standard only), response standard (a solution of analyte and internal standard in suitable solvent) and on-instrument stability samples are prepared according to the method that the analyst has optimised during method development. These samples are

then processed in a single batch, which is then subjected to pre-study validation criteria to determine whether or not the method can be considered valid.

Table 3: Typical validation batch structure

1. RESPONSE STANDARD	**21. STD F**	**41. STD C**
2. STD H	22. BLANK 3	**42. STD C**
3. BLANK 1	23. STAB 2	43. BLANK 5
4. STAB 1	24. QC G	44. QC G
5. QC G	25. QC F	45. QC F
6. QC F	26. QC E	46. QC E
7. QC E	27. QC D	47. QC D
8. QC D	28. QC C	48. QC C
9. QC C	29. QC B	49. QC B
10. QC B	30. QC A	50. QC A
11. QC A	**31. STD E**	**51. STD B**
12. STD F	32. BLANK 4	**52. STD B**
13. BLANK 2	33. QC G	53. BLANK 6
14. QC G	34. QC F	54. STAB 3
15. QC F	35. QC E	55. STAB 4
16. QC E	36. QC D	56. STAB 5
17. QC D	37. QC C	57. STAB 6
18. QC C	38. QC B	58. STAB 7
19. QC B	39. QC A	59. STAB 8
20. QC A	**40. STD D**	60. ZERO
		61. RESPONSE STANDARD

Once the validation batch has been prepared and injected, the batch as an entity is scrutinised and subjected to acceptance criteria, which must be satisfied before the method can be considered validated.

3.2 Pre-study validation batch acceptance criteria

Green [18] proposes that the first step in the method development and validation cycle is to set minimum requirements, which are essentially acceptance specifications for the method. Green further states that a complete list should be agreed upon by the developer and the end user before the method is developed.

However, most bioanalytical laboratories find it more practical to cast their net as wide as possible by attempting to satisfy minimum acceptance criteria set by most regulatory authorities. If, for instance, a laboratory caters for the American market only, then the criteria set out by the FDA would naturally be the benchmark for acceptance criteria. However, if that same laboratory also did intermittent work for a European clientele, acceptance criteria laid out by the European authorities would be relevant. These are frequently combined into in-house standard operating procedures (SOPs) pertaining to pre-study validation batch acceptance criteria.

There is no universally agreed upon set of acceptance criteria, but generally the criteria set out below are considered to be the kernel acceptance criteria.

3.2.1 Performance parameters

3.2.1.1 Specificity

Blank plasmas obtained from no less than six different volunteers, are prepared without internal standard (see Table 3, samples 3, 13, 22, 32, 43 and 53). Each blank sample must be free of interference when using the proposed extraction procedure. It is not sufficient to test only one source of blank matrix, or to choose one from many that were tested [14]. Any sample with significant interference (ie. a peak in excess of 20% of the response produced by

the lowest calibration standard) must be rejected. If more that 10% of the blank samples tested exhibit interference, then additional blank samples must be tested [8]. If 10% of the subsequent group of blank samples still show interference, then the method can not be considered valid, and the method will have to be re-developed in order to improve specificity. At this point, caution should be taken not to confuse a lack of specificity with carry-over, which can easily be interpreted as interference. During the method development phase, it must be established that no carry-over from sample to sample is occurring, and instrumental parameters will have to be adjusted accordingly.

3.2.1.2 Calibration curve

A calibration curve should be prepared in the same biological matrix as the samples in the intended study. Care should be taken to avoid precipitation while spiking the biological matrix. A calibration curve should consist of a blank sample, a zero sample, and five to eight non-zero samples covering the expected range [8]. The blank and zero samples are not used in the calibration function, but serve only to evaluate interference.

- *Lower limit of quantitation (LLOQ)*

The lower limit of quantification (LLOQ) is the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision, and variability [11]. Shah *et al.* [11] believe that the LLOQ should be proven by assaying at least five samples independent of the standards but at the same concentration as the lowest standard and determining their coefficient of variation. This is also the approach advocated in the FDA draft Guidance for Industry [8] and is therefore probably the method used by most bioanalytical laboratories.

At FARMOVS-PAREXEL Bioanalytical Services Division® (FBSD) we have always used a slightly modified approach since we consider the described approach to be slightly flawed. Instead of preparing the LLOQ samples at the same concentration as the lowest non-zero calibration standard, these samples are prepared at concentrations of approximately 20 to 40% above the lowest non-zero calibration standard. The chances of having to extrapolate half the LLOQ samples below the lowest non-zero calibrator (and by definition below the LLOQ) are therefore considerably reduced, and this practice of extrapolation is in fact not allowed. While this implies that the LLOQ is technically proven at about 20 to 40% above the lowest non-zero standard, the inexact nature of the LLOQ would, in our opinion, nevertheless allow one to peg the LLOQ at the lowest non-zero standard. We certainly consider this to be a more acceptable practice than accepting determinations below the LLOQ during the validation of the assay procedure, which, in the process of assaying of the actual study samples, paradoxically, become unacceptable.

The two criteria that these five samples must meet (see Table 3, samples 11, 20, 30, 39 and 50) is that the coefficient of variation must be less than 20%, and have an accuracy (calculated from the calibration line) of 80 – 120%. Some laboratories further apply minimum signal-to-noise criteria (most commonly 5:1) to these samples.

If samples at the LLOQ do not meet the above-mentioned criteria, then the LLOQ will have to be raised to the next lowest calibration standard.

- *Regression*

The simplest workable regression equation should be fitted to the calibration line, with as little weighting as possible [8]. In the main, regulatory authorities agree that a calibration curve should meet the following criteria [8]:

1. ≤20% deviation at the LLOQ (ie. samples 51 and 52, Table 3) [11].
2. ≤15% deviation of standards other than the LLOQ [11].
3. At least 67% of non-zero samples must meet the above criteria, and the 67% must include a LLOQ sample and the highest calibration standard.
4. A 0.95 or better correlation coefficient.

- *Quality control samples*

At FBSD, quality controls are included at seven levels (ie. QC A – G, see Table 3). Listed below are the acceptance criteria applied to quality control samples from such a pre-study validation batch:

1. The CV% for the five replicates, determined at each quality control level, should be less than 15% (n = 5).
2. The mean precision of each quality control level should be between 85 and 115%.
3. At least 60% (ie. three out of five) of the quality control samples on each level should have a precision between 80 and 120%.
4. Overall, no more than 15% of the quality controls included in the batch may be lost to one of the above- mentioned criteria

- *Recovery*

Although recovery determination is usually done in the method development phase, prior to pre-study validation, the documentation generated forms part of the data necessary for method validation. Recovery is determined by comparing detector response from an amount of analyte added to and recovered from the biological matrix, to detector response obtained from the pure authentic standard [8].

Recovery determination should be done at high, medium and low regions of the expected calibration range. Although values of not less than 50, 80 and 90% have all been used as acceptable limits, it is more important that recovery be reproducible [14]. For this reason, recovery at each concentration is preferably determined in five-fold, in order to scrutinise the reproducibility. Although it is desirable to obtain recovery close to 100%, there is no universally accepted value for minimum recovery. As there are no prescriptive criteria as such, it is vital that the manner in which recovery was determined is well documented and included in an analytical report.

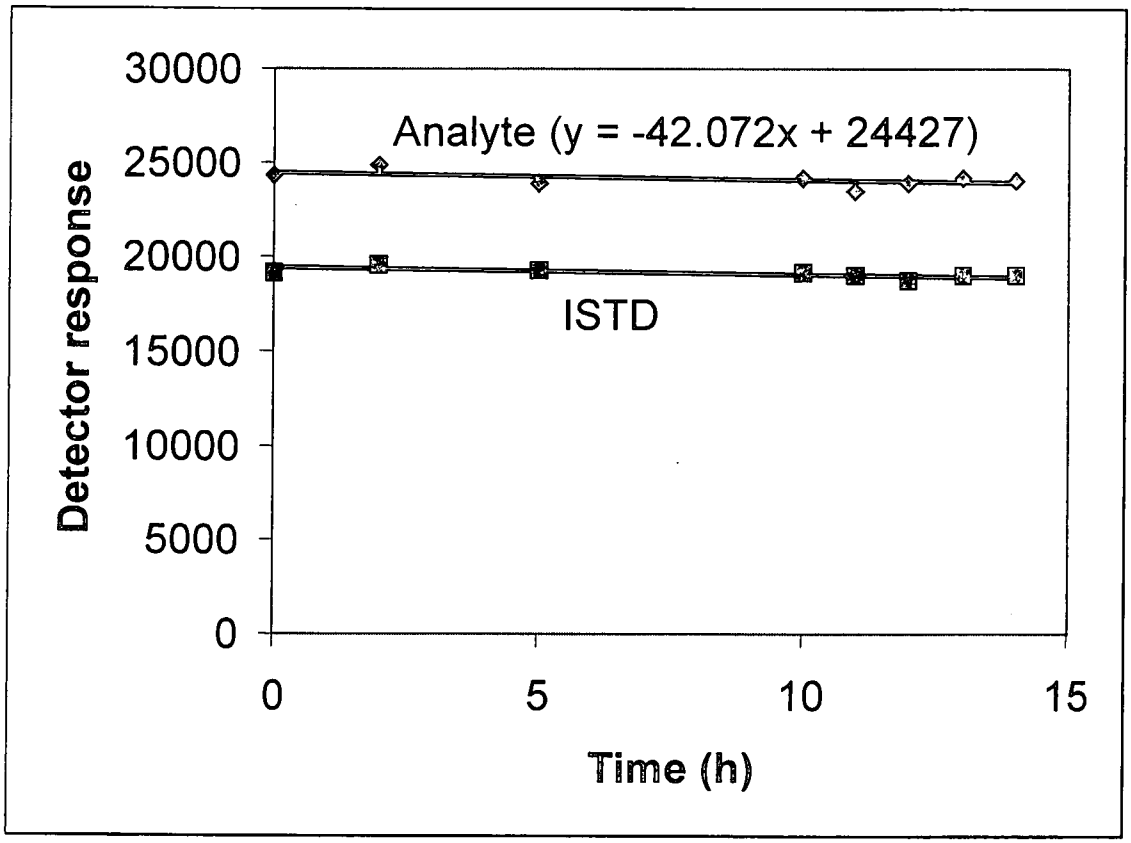
- *On-instrument stability*

It is necessary to demonstrate that samples are stable on the analytical instrument while awaiting injection. The most common reasons for instability on an analytical instrument are thermal instability and degradation due to light exposure. To remedy this, samples are usually kept on a cooled autosampler in darkly coloured vials.

Data for on-instrument stability should span an interval at least as long as a typical sample batch. At FBSD, data for on-instrument stability is generated as follows:

Plasma is spiked with analyte to a concentration of approximately C_{max}. These samples are then extracted along with the pre-study validation batch (see samples 4, 23, 54, 55, 56, 57, 58 and 59, Table 3). Before injecting these eight samples, the extracts are pooled in a single container, vortexed and then re-distributed to eight separate sample vials. The reason for this is to eliminate any variability that may result from sample preparation. The first two samples (samples 4 and 23, Table 3) are injected using the standard analytical method, while the last six samples (54, 55, 56, 57, 58 and 59, Table 3) are injected using a slightly modified method. This method has been modified by lengthening the time between consecutive injections to ninety minutes. The rationale behind this is to collect stability data over a period that is at least as long as a typical sample batch. The analyte and internal standard peak areas are then graphically plotted against time.

Figure 3: Graphical method used to calculate maximum batch duration



No more than a 10% decrease may be observed for either analyte or internal standard during the batch. The maximum batch duration, for either analyte or internal standard, can be calculated as follows:

- *Calculation of maximum batch duration*

Given that the equation for the decomposition trend of the analyte is given by y = -42.072x + 24427, it remains only to calculated the x-co-ordinate for which the y-value equal to 21984 (10% decrease in the y-intercept). This substitution produces the following equation:

$$21984 = -42.072x + 24427$$

Solving this equation reveals that the maximum batch duration allowable is approximately 58 hours. For a labile analyte however, a maximum batch duration of 5 – 6 hours may be observed, and in that case, samples will have to be processed in smaller batches so the maximum batch duration is not exceeded. It is important to note that batch length must be shortened to compensate for the drug entity that is most labile. If the internal standard is found to labile, the batch will have to be truncated in the same fashion or a more suitable internal standard sought.

3.3 Recent developments regarding pre-study validation

In recent times, there has been an international drive to more adequately demonstrate the suitability of an analytical method prior to sample processing. This movement has been spearheaded chiefly by the FDA of America, who propose that a single pre-study validation is insufficient to adequately show that a method is suitable for the intended purpose.

It is felt that at least three pre-study validations must be performed before any single sample is assayed. These three batches are then scrutinised individually, and as a unit and then subjected to inter- and intra- batch acceptance criteria.

Each of the three batches should consist of a calibration curve (as described in section 3.2), quality control at the LLOQ, (n = 5), low quality control[†] (n = 5), medium quality control (n = 5) and high quality control (n = 5). Furthermore, a blank, a zero and a response standard

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[†] *According to the FDA, a low QC sample is quality control sample not exceeding three times the concentration of the lowest non-zero calibration standard (so-called LLOQ) used to construct the calibration line.*

should also be included. All of the acceptance criteria discussed in section 3.2 are applicable, with the addition of the following acceptance criteria:

1. *Precision* The between-batch CVs for low, medium and high concentrations should be ≤15%, and ≤20% for the LLOQ quality controls, using a minimum of three batches.
2. *Accuracy* The between-batch mean value should be within ±15% of the nominal value at the low, medium and high quality control concentrations, and between ±20% at the LLOQ quality control.
3. *Sensitivity* The lowest standard should be accepted as the limit of quantitation of the method if the between-batch CV at the LLOQ is ≤20%.
4. *Specificity* The responses of interfering peaks at the retention time of the analyte should be less than 20% of the response of the LLOQ standard. Responses of interfering peaks at the retention time of the internal standard should be ≤5% of the response of the internal standard, at the concentration of internal standard to be used in the study.

In a further development, it has become necessary to demonstrate that it is possible to dilute any sample that may be above the validated calibration range. At FBSD, this is done by diluting the highest quality control (1.8× C_{max}) with blank matrix (1:1), and preparing and including this diluted quality control in five- fold in one of the pre-study validation batches. These quality controls are then calculated from the calibration line and multiplied with a dilution factor of two. If there is close agreement (≤ 10% difference) between the diluted and

undiluted quality control at this high level, then it is understood that samples that fall outside of the calibration can be diluted and quantified. In the opinion of the author, it would probably be more meaningful to prepare a quality control that is indeed above the validated calibration range and dilute this quality control, rather than dilute a quality control that already lies within the calibration range.

Furthermore, it is necessary to demonstrate that samples are stable on the analytical instrument and do not decompose while awaiting injection. At FBSD, this is done by including stability samples in two of the three validation batches (there is an interval of one day between these batches). In total, 16 stability samples (at a single concentration) are extracted with the first of the two said batches. These 16 extracts are then pooled and re-divided in order to exclude variability that may be introduced by extraction. The first eight are injected together with the first validation batch and the remaining eight together with the second. It is important to note that the second group of stability samples must reside on the autosampler during both analytical batches, that is to say eight autosampler positions are occupied during the first batch, but the samples will in fact only be injected together with the second validation batch. The resulting chromatograms are used to plot response versus time. The resulting data is then used to calculate maximum batch duration (section 3.1.4)

3.4 Batch acceptance criteria

The purpose of pre-study validation is that samples can be assayed with confidence. Ensuring that reliable and accurate data are obtained during routine sample analysis is necessary, even though the method has been adequately characterised during the pre-study validation [9].

It is for this reason that minimum acceptance criteria for sample batches, as is the case with pre- study validation, should be established. If these criteria are not met, the source of error should be determined and corrected, and the batch repeated.[‡] It is also vital that all samples generated should be assayed within the time period for which matrix stability data are available [8].

In general, analysis of biological samples can be done with a single determination if precision and accuracy variables routinely fall within acceptable tolerance levels. However, difficult procedures with labile analytes may require duplicate or even triplicate analysis.

A standard curve should be generated for each analytical run (for each analyte if multiple analytes are being quantified) and used to calculate the concentrations of the analyte(s) in the unknown samples [11]. Estimation of unknown samples by extrapolation either above or below the validated calibration line is not recommended. Instead it is suggested that the standard curve be re-determined, or the samples be diluted with blank matrix[§] and re-assayed in the case of samples above the validated calibration range. It is further recommended that all study samples from a study subject should be assayed in the same batch.

At FBSD, it is policy to include a calibration line of at least five non-zero calibration standards and a blank (usually injected after the highest calibration standard). The two lowest calibration standards (ie. the LLOQ calibration standard and the calibration standard

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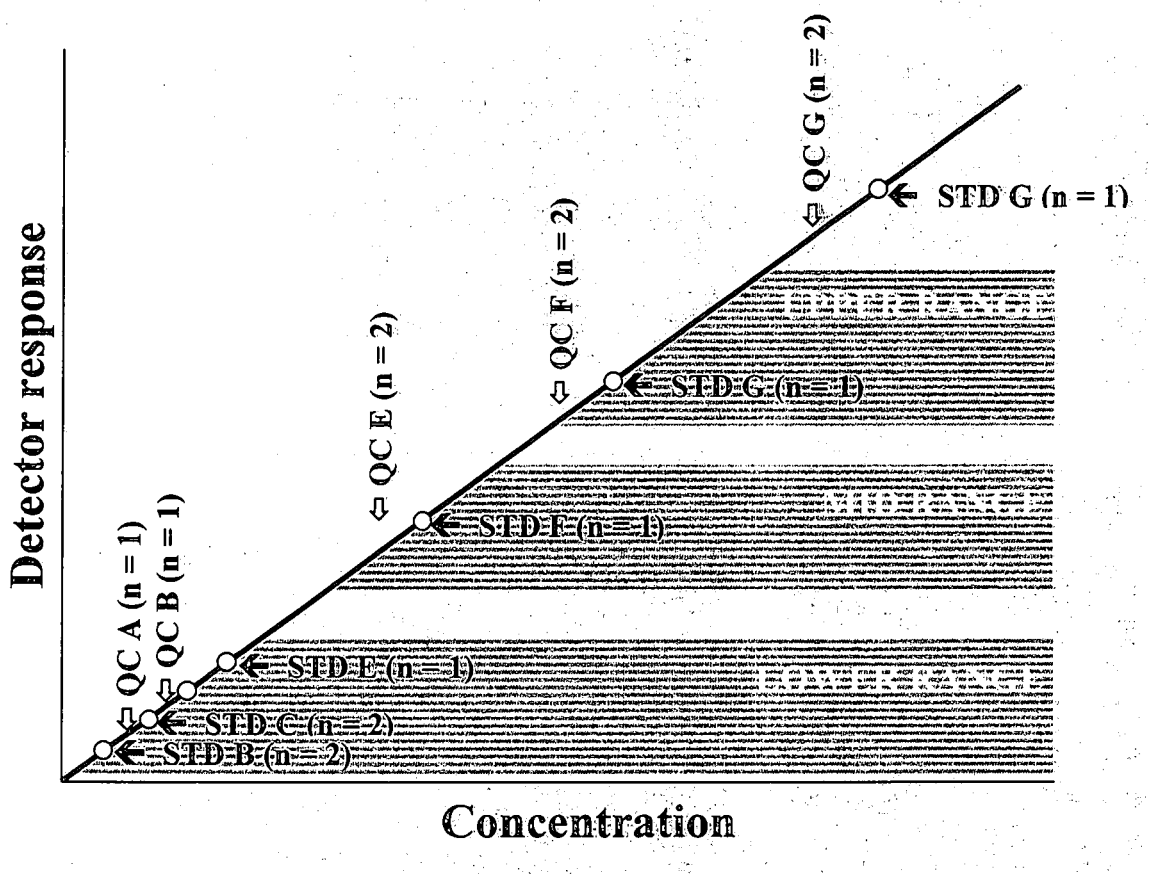
[‡] *It is for this reason that upon collection, samples are divided into two and sometimes three aliquots and stored in separate sample tubes. This is to circumvent multiple freeze-thaw cycles. The alternative is to store the sample as a single aliquot, but it is then necessary to investigate multiple freeze-thaw cycles, should it be necessary to repeat any sample(s).*

[§] *If at all possible, it is best to use a particular subject's own blank matrix in the form of a pre-dosing sample.*

immediately above) are included in duplicate, while the remainder are included in single-fold. As discussed in section 3.1, FBSD has devised a system whereby the LLOQ of a single batch can be raised if necessary. If, for example, the chromatography at STD B has become unacceptable, it is possible to raise the batch LLOQ to STD C (the next calibration standard, see Table 2), and the next quality control (QC B) will become the LLOQ quality control. However, two important conditions apply to doing this. Firstly, the pre-study validation(s) will have to be reviewed in order to demonstrate that the method did indeed validate without the lowest calibration standard. Secondly, any study sample lying below the new batch LLOQ (STD C) will have to be repeated in a subsequent batch, after the problem has been rectified and the original LLOQ (STD B) restored. If it is not possible to restore the original LLOQ, STD C will become the new study LLOQ, and any samples below STD C will be reported as being below the LLOQ. Quality controls are included in all sample runs and must constitute at least 5% of the batch.

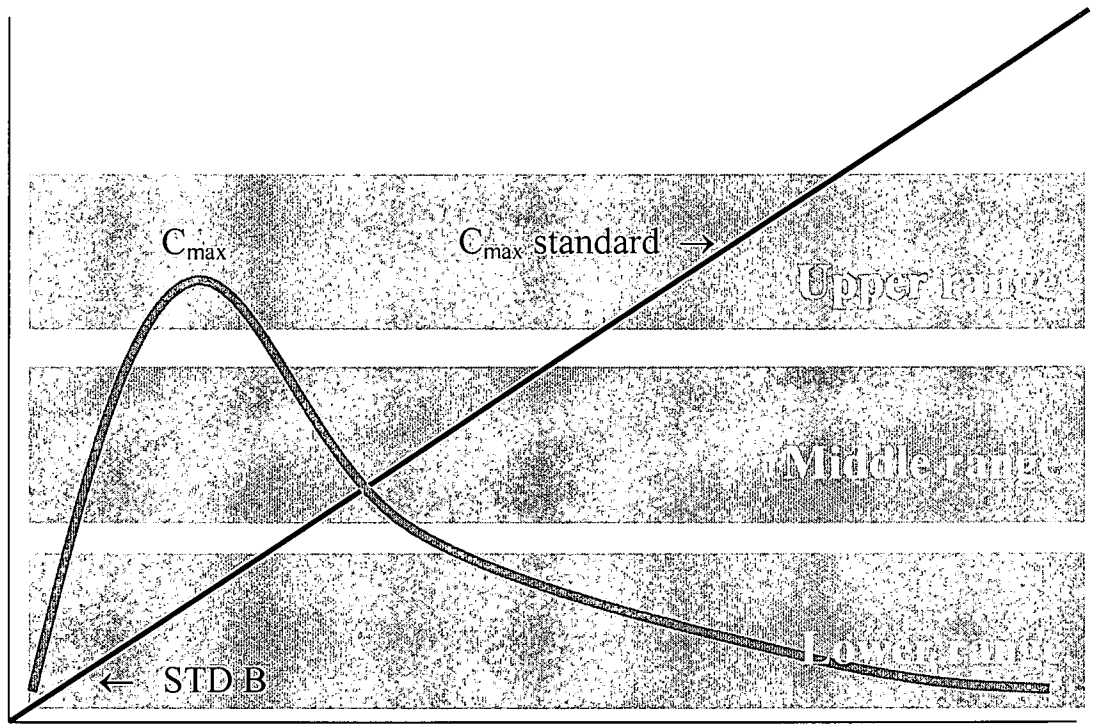
Figure 4: Distribution of calibration standards and quality controls in sample processing batches



Until recently, high (ca. 1.8 × Cmax), medium (ca. ½ × Cmax) and low range (ca. 3 × LLOQ) quality controls (see Fig. 2) were included in duplicate while two lower quality controls (ca. 1 × LLOQ and ca. 2 × LLOQ) in single-fold were included in each batch of study samples processed. This number of quality controls was more than the number proposed by Shah et al. [11] which included duplicate quality controls at high, medium and low concentrations, where the low concentration quality control was defined as being "close

to the LLOQ". The closeness to the LLOQ was never specified, but generally considered to be near 2 to 3 × LLOQ. The procedure used by FBSD thus included quality controls which were used to monitor the performance of the assay method at the LLOQ throughout the assaying of the study samples while the procedure of Shah et al. [11] assumed that the assay method performed acceptably at the LLOQ throughout the study. The procedure used at FBSD therefore predated the procedure now being recommended by the FDA in their Guidance to Industry [8] albeit in a slightly different form. This Guidance suggests that quality controls at high, medium and low (≤ 3 × LLOQ) should be included in duplicate in each batch as well as a control at the LLOQ, also in duplicate. While the controls at high medium and low concentrations are to be used to determine the acceptance of each batch, the control at LLOQ is to be used to monitor the performance of the assay method at its LLOQ. Based on the results obtained with these quality controls processed in each batch, a batch is considered to be acceptable if at least four of the six determinations of the quality controls at high medium and low concentration are between 80 and 120 % of their nominal values. Further, it is required that at least one control from a level is within this acceptance range, that is to say that it is not permitted that both controls on a single level be outside the said acceptance range. Alternatively this can be stated as follows: A batch is considered acceptable if not more than two of the six determinations of the quality controls at high, medium and low concentrations deviate by more than 20% from their nominal values, provided these two controls are not at the same level. A batch that is not considered acceptable when applying these criteria, must be repeated.

Figure 5: Appropriate calibration line



At FBSD, the same criteria are applied, with the exception that at least five quality controls are included in each run, with the upper three being included in duplicate.

At FBSD calibration standards and quality controls are interspersed between study samples (see Table 4), which is considered to be more appropriate than the common practice of running the calibration samples and controls before any of the study samples.

Table 4: A typical sample batch structure. Samples are designated in the run sheet table by a three digit code separated by commas consisting of *subject number, sampling time (hr),period.* SYS denotes a response standard.

Injection No.	Sample	Injection No.	Sample	Injection No.	Sample
1	SYS	31	1,6.0,2	61	1,18.0,1
2	1,0,1	32	2,6.0,1	62	1,18.0,2
3	1,0,2	33	2,6.0,2	63	2,18.0,1
4	2,0,1	34	QC F	64	2,18.0,2
5	2,0,2	35	1,7.0,1	65	QC E
6	STD J	36	1,7.0,2	66	1,24.0,1
7	STD B	37	2,7.0,1	67	1,24.0,2
8	1,1.5,1	38	2,7.0,2	68	2,24.0,1
9	1,1.5,2	39	QC D	69	2,24.0,2
10	2,1.5,2	40	STD E	70	1,48.0,1
11	2,1.5,2	41	1,8.0,1	71	1,48.0,2
12	QC A	42	1,8.0,2	72	2,48.0,1
13	STD C	43	2,8.0,1	73	2,48.0,2
14	1,3.0,1	44	2,8.0,2	74	QC B
15	1,3.0,2	45	QC D	75	1,72.0,1
16	2,3.0,1	46	1,10.0,1	76	1,72.0,2
17	2,3.0,2	47	1,10.0,2	77	2,72.0,1
18	STD H	48	2,10.0,1	78	2,72.0,2
19	1,4.0,1	49	2,10.0,2	79	1,96.0,1
20	1,4.0,2	50	QC E	80	1,96.0,2
21	2,4.0,1	51	1,12.0,1	81	2,96.0,1
22	2,4.0,2	52	1,12.0,2	82	2,96.0,2
23	STD K	53	2,12.0,1	83	STD C
24	1,5.0,1	54	2,12.0,2	84	1,120,1
25	1,5.0,2	55	STD G	85	1,120,2
26	2,5.0,1	56	1,14.0,1	86	2,120,1
27	2,5.0,2	57	1,14.0,2	87	2,120,2
28	STD I	58	2,14.0,1	88	STD B
29	BLANK	59	2,14.0,2	89	1,144,1
30	1,6.0,1	60	QC F	90	1,144,2
				91	2,144,1
				92	2,144,2

3.5 *Data auditing and repeating samples*

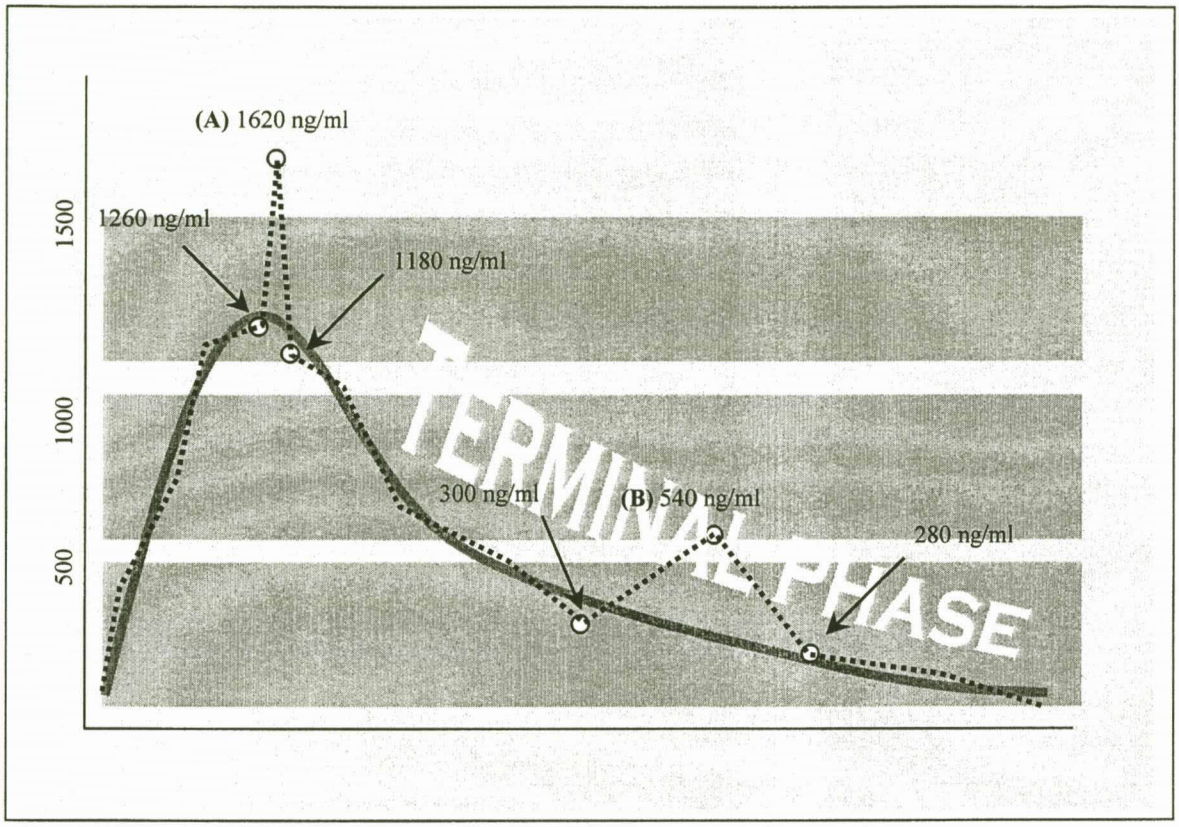
It is necessary to scrutinise data generated by sample batches on an ongoing basis. The policy of FBSD is to prepare a sample batch only when the previous batch has been evaluated and accepted. If a sample batch has not met the batch acceptance criteria, the possible cause must be established and the fault must first be corrected before sample production can resume.

Single- dose concentration versus time profiles (see Fig. 6) are used to calculate pharmacokinetic parameters. If any point(s) on these profiles are aberrant, it could bias these parameters. No rigid guidelines exist with respect to identifying these so-called suspected pharmacokinetic outliers. Shah *et al.* [11] propose that a protocol for repeat analysis be established *a priori*, and state that cautious use of 'pharmacokinetic fit' methodology may call for repeat analysis of some study samples, but that the reasons for such repeats should be well documented. This cautioning is sensible as selection of repeat samples can become a subjective matter. It is for this reason that it is preferable that the so-called data auditing be done by an analyst other than the one performing batch analysis. The following standard procedure is in place at FBSD:

- *Values at and near the* C_{max}

Any point that appears unusual is compared to the point immediately before and after. If the value of the point differs by more that 30% from the mean of the two points on either side, that point (sample) will be repeated in duplicate. For example, the value at (A) in Fig. 6 is ensconced between two values of which the average is 1220 ng/ml. As (A) differs by 32.8%

Figure 6: Concentration versus time profile



from this mean, it is repeated in duplicate. As pointed out in section 3.1.2, a separate aliquot is taken for the purpose of repeating samples, as no multiple freeze-thaw cycles are permitted.

- *Values on the terminal slope of the profile*

Any point that appears unusual is compared to the point immediately before and after. If the value of the point differs by more that 50% from the mean of the two points on either side, that sample will be repeated in duplicate. As depicted in Fig. 6, (B) is ensconced between two values with a mean of 290 ng/ml. As (B) differs by 86% from this mean, it will be repeated in duplicate.

- *Miscellaneous repeats*

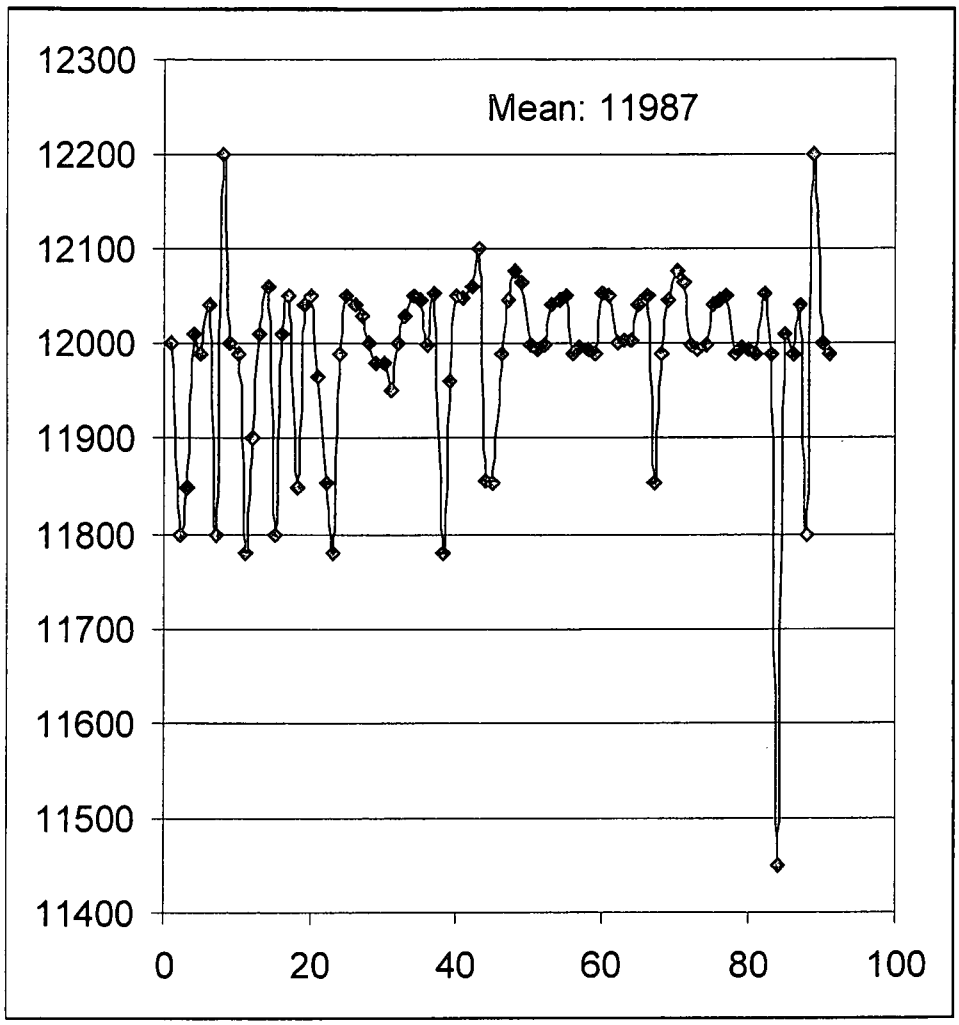
There are certain samples that are repeated as a matter of course, for reasons other than being pharmacokinetic outliers.

Sample lost in process:	Samples are repeated (single- fold) if they are lost during sample preparation for technical reasons such as spillage and sample tubes breaking. Only a single value is available is available and is accepted, unless it appears to be a pharmacokinetic outlier in which case it will probably become not reportable.
Poor chromatography:	Samples are repeated (single- fold) if the chromatography for an isolated sample appears too poor to quantify with confidence.
Internal standard deviation:	If the internal standard (if used) value for a particular sample differs by more than 50% from the mean internal standard value for the entire batch, then that sample is repeated in single- fold.

- *The internal standard plot*

A plot of internal standard response over an entire batch has proved to be a useful tool in monitoring the performance of assay methods at FBSD, and thus deserves a brief discussion.

Figure 7: Internal standard plot over an entire sample batch



It is often the case that scrutiny of such an internal standard plot can be used to detect analytical problems. Potential problems to be on the lookout for include:

- A general downward trend in the plot indicates decreasing detector sensitivity, perhaps due to a failing lamp (in the case of a fluorescence detector) or a dirty interface (in the case of LC-MS/MS ESI)

- Marked differences in internal standard response between study samples, and calibration standards and quality controls. Such a phenomenon could indicate the presence of so called matrix effects [5], or interference with the internal standard as a result of metabolites generated *in situ*, which are not present in the calibration standards and quality controls.
- The precision of the internal standard response over an entire batch should preferably be high. A CV% smaller than 10% is preferable for any single batch. Poor precision may suggest a problematic autosampler or an internal standard that is in fact adding additional bias to the assay method (see section 2.4.2).
- Globally, internal standard plots between batches should be compared. A single batch having an anomalous plot should be investigated, or the internal standard at least monitored more closely in subsequent batches.

3.6 Documentation

Documentation of the successful validation of an analytical method should be provided in an assay validation report [8]. All analytical experiments, which led to pre- study validation should be bound and recorded in an analytical notebook. These notebooks should be signed by the analyst and inspected by the laboratory supervisor. All data should be available for data audit and inspection.

The FDA of America propose that the documentation should include the following:

Pre- study validation

- An approved description of the analytical method that has been decided upon during method development (this must remain at the workbench where the analyst will prepare all samples).
- A description of all experiments done, establishing analyte stability. Clear conclusions should be drawn from these experiments.
- Description and summary of experiments determining accuracy, precision, recovery, specificity, linearity and limit of quantitation.
- Tables of intra- and inter- day accuracy and precision (inter- day data is inferred from the three validations performed).
- Evidence of the purity of all the reference materials used in validation experiments (usually in the form of a certificate of analysis supplied with the reference material).
- Any deviations from SOPs and justifications for these deviations.

In- study assay performance

- Calibration curve data (such as gradients, r and r^2 values) should be summarised and tabulated for inspection, involving all the batches that were assayed.
- Summaries on inter-day accuracy and precision of quality control samples included in all assay batches.
- A protocol giving clear reasons for re-assay of samples. This should include acceptance criteria for re-assayed samples.
- Reasons for missing samples.

- Any deviations from the analytical protocol or SOP, with justification for these deviations.
- Some authorities require that 20% of the chromatograms generated by study samples be supplied to regulatory authorities. These should be in the form of continuous batches and not arbitrarily selected.
- All SOPs, raw data, calculations of concentration and repeat batches.

4 Assay method development – 6-methoxy-2-naphthylacetic acid (metabolite of nabumetone)

4.1 Background

Early in 1997, a bioequivalence study was requested involving nabumetone, a non-steroidal anti-inflammatory drug (NSAID). Nabumetone (a naphthylalkanone) is a prodrug which undergoes extensive first pass metabolism. The major circulating metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA), is an effective prostaglandinsynthetase inhibitor [19]. An already registered formulation of the drug (Relifex®) was used as the reference product to which the new formulation of the drug would be compared with a view to registration of the generic formulation. A novel assay method was developed and study samples generated during the study assayed using this method. In 1999, the sponsor was approached with a view to publishing not only the analytical method, but also the pharmacokinetic data generated during the study. A paper was prepared and submitted to the Journal of Chromatography B in August 1999 and accepted, following minor revision, in February 2000.

4.2 Summary of analytical literature survey

Relatively little literature existed at the time regarding the quantitation of this drug in human plasma by HPLC. Nabumetone (Fig. 8), a prodrug used to treat rheumatoid and osteoarthritis, rapidly undergoes first-pass metabolism, yielding the active metabolite 6-

MNA (Fig. 9), which is believed to be largely responsible for the pharmacological activity of the drug [19]. While some authors dealt with the quantitation of the prodrug and the active metabolite simultaneously in human plasma [20,21], others focussed on the active metabolite only [22,23]. For the purposes of the study conducted by FBSD, the relevant regulatory authority required that only 6-MNA be quantified.

Figure 8: Nabumetone

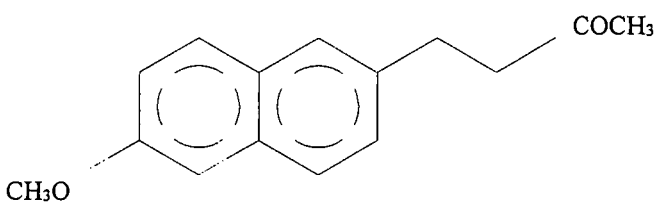
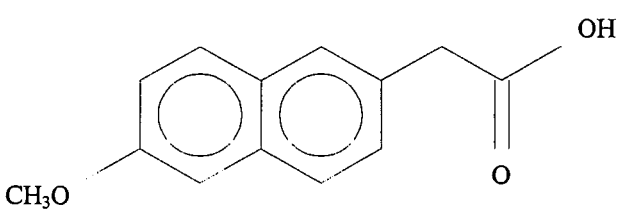


Figure 9: 6-Methoxy-2-naphthlyacetic acid (6-MNA)

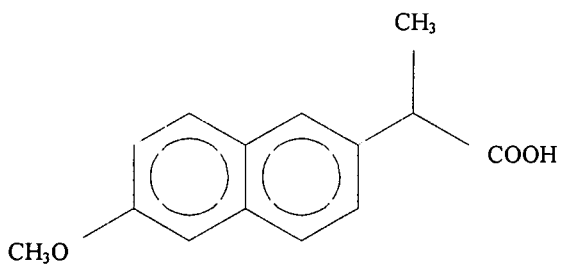


What became evident from the above-mentioned literature was that, analytically speaking, this was not a particularly difficult drug to quantify. Firstly, plasma levels associated with the dose used in this study (1000mg) are high. Kendall *et al.* [19] reported that C_{max} concentrations in the order of 20μg/ml could be expected, following the dose that this

particular study involved. Secondly, the literature reflected that 6-MNA possesses good chromophores, and readily lends itself to both UV [21,22] and fluorescence detection [20], without derivatization of the analyte. Thirdly, both SPE and LLE had been successfully employed to extract 6-MNA from human plasma and finally, reversed-phase chromatography had been successfully employed in separating 6-MNA from endogenous matrix components.

Furthermore, selected analytical literature on naproxen, a structurally similar drug, was also consulted. Naproxen (Figure 10), which is used for similar conditions in humans, has been successfully quantified in human plasma following a simple protein precipitation procedure, using UV detection [24].

Figure 10: Naproxen



4.3 Formulation of an analytical strategy, based on literature

On consideration of what the analytical and clinical literature revealed, the method appeared to be the perfect candidate on which to attempt a protein precipitation procedure, even though no such procedure had yet been published. This was owing to the high plasma

concentrations and relative ease of detection. What further supported this notion was the fact that such a procedure had been successful in quantifying naproxen, which from both a pharmacological and a structural point-of-view, is very similar to 6-MNA.

The envisaged *modus operandi* for the development of the method was as follows:

- Set up a reversed-phase HPLC system with a UV detector monitoring at 280nm, as the literature reflected this to be an optimal wavelength.
- Install a reversed-phase analytical column (either C8 or C18).
- Optimise a mobile phase that would be suitable for both 6-MNA and naproxen, which would, in all probability, be a suitable internal standard.
- Prepare and inject a series dilution of the analyte and proposed internal standard in mobile phase in order to establish linearity of the system prior to sample preparation.
- If the results of the experiment above show the method to be linear prior to sample preparation, then a plasma sample containing 6-MNA and naproxen will be prepared.
- A number of protein precipitation procedures will be performed on the above-mentioned sample and injected on the HPLC system.
- If the chromatography is not acceptable due to interfering peaks resulting from endogenous plasma components, the mobile phase and/or column will have to be adjusted in order to obtain acceptable resolution.

- Potential interference from other metabolites of nabumetone generated *in vivo* will have to tested by assaying small quantities of plasma left over from the collection process of actual study samples.

- Determine analyte and internal standard recovery from the matrix.

- Once the chromatography is acceptable, prepare a series 1:1 dilution of the analyte in plasma that covers the concentration range expected in the samples. At the same time, it will be possible to get an idea of how sensitive the method will be, by observing how far it is possible to serially dilute before the signal is too small to be adequately detected.

Once all of the above procedure have been completed and found to be acceptable, calibration standards and quality controls will be prepared and the method validated.

4.4 Excecution of method development - 6-MNA

The following section is a summary, together with explanations where necessary, of the assay method development process:

- *Preparation of stock solutions*

Solutions of 6-MNA and naproxen, which were used for development, were prepared as follows:

- 0.464mg of 6-MNA was weighed using an analytical balance and then dissolved in 74.375g methanol, yielding a spiking solution of 4.935μg/ml.
- Similarly, 0.703mg naproxen was dissolved in 79.327g methanol yielding a spiking solution of 5.61μg/ml. It was noted that the analytical reference standard used to prepare this solution was an expired standard that was approximately 80% pure. It was noted that, according to the certificate of analysis, the reference standard used to prepare the solution, had expired some time ago. However, since this was the only reference standard available at the time, and the initial development of the assay method does not involve critical quantitative steps, it was decided to proceed with the assay development in order to save time and a new reference standard was ordered in the meantime. These solutions were stored at 4°C when not in use.

- *Preparation of mobile phase*

A mobile phase was prepared which was based on that used by Ray and Day [20].

2.11g citric acid was made up to 1litre using water (solution A). 1.41g Na_2HPO_4 was made up to 0.5 litre using water (solution B). To 1000cm^3 of solution A was added 175cm^3 solution B

To 600cm^3 of this buffer was added 400cm^3 acetonitrile, and the apparent pH adjusted to 3.1 using *o*-phosphoric (85%) acid. This mobile phase was used as a starting point for mobile phase optimisation.

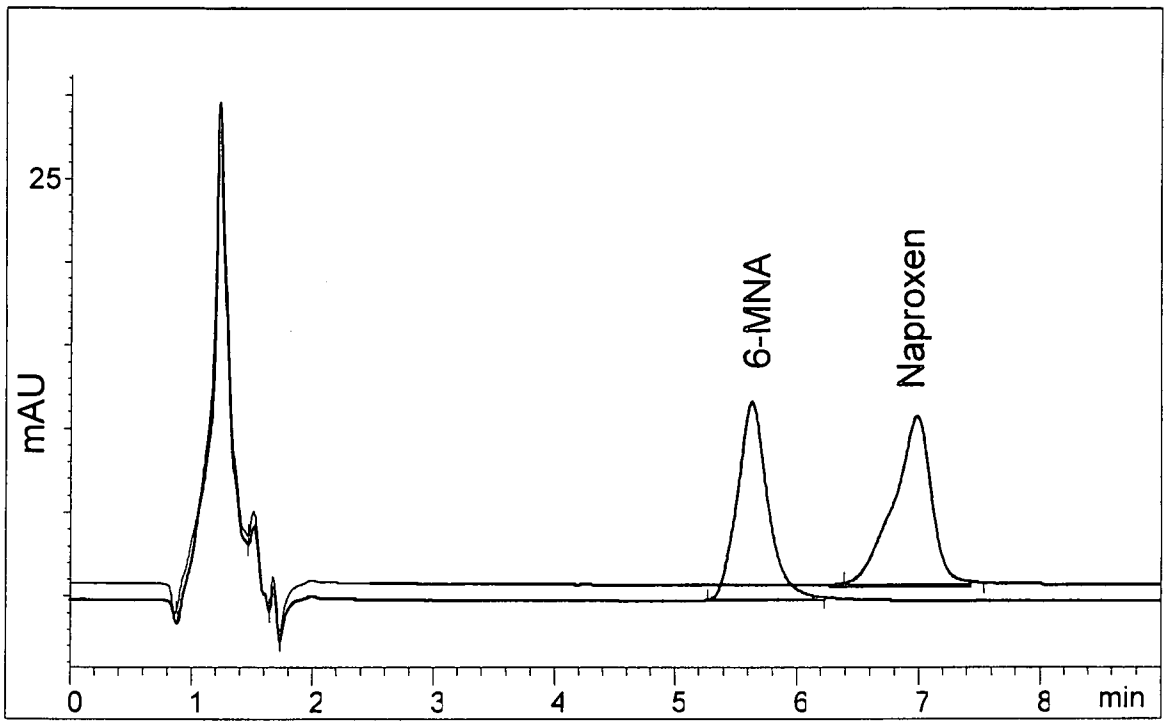
- *Instrumentation and chromatographic conditions.*

An HPLC instrument stack was set up based on relevant information gleaned from the literature consulted. The stack consisted of an analytical pump (Hewlett-Packard series 1100), a UV detector monitoring at 280nm (Hewlett-Packard series 1100 VWD) and an automatic sample injector (Hewlett-Packard series 1100 autosampler). A Hewlett-packard lichrosphere® RP8 (5μ , 150×4.2mm ID) analytical column was installed on the system, and preceded by a manually packed precolumn (Upchurch 20 × 2mm), dry-filled with Perisorb® RP18 (30 - 40μm) packing. The mobile phase was pumped through the system at 1ml/min and allowed to equilibrate for 2 hours. The two solutions were then injected onto the column (10μl) under the same chromatographic conditions.

- *Chromatography*

Upon injection, Naproxen exhibited poor peak symmetry.

Figure 11: Initial injection of 6-MNA and naproxen (in methanol), showing poor naproxen peak symmetry

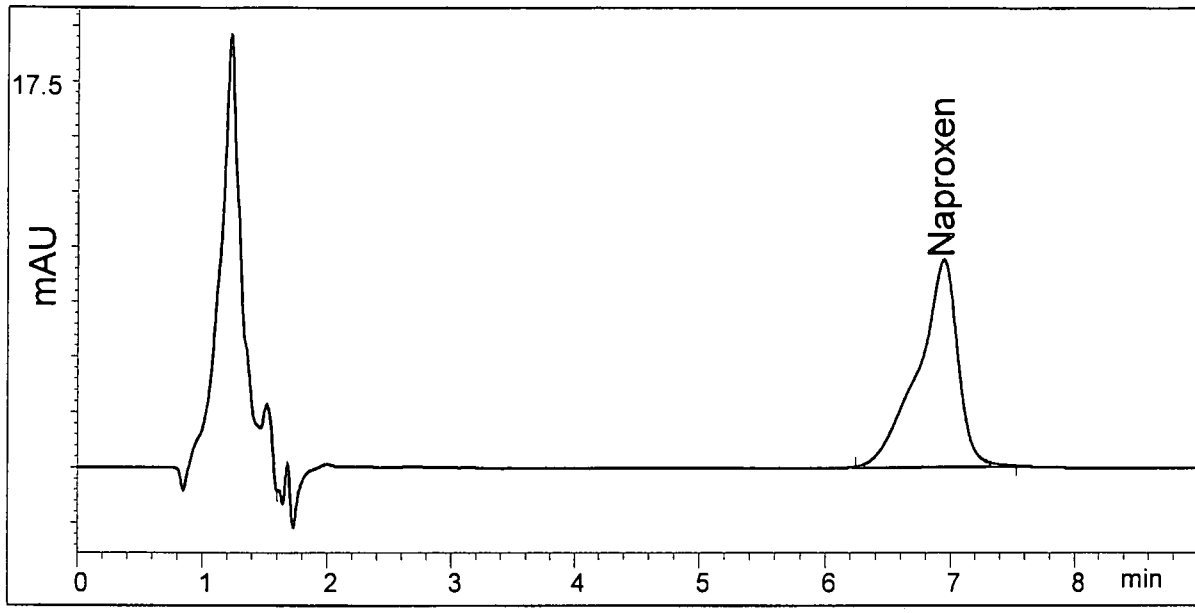


It was speculated that impurities in the expired reference standard could explain the poor naproxen peak shape. In order to test this, a fresh solution of naproxen, which was prepared from a pure reference standard (recently obtained), was prepared as follows:

- 0.239mg of naproxen (100% pure) was dissolved in 20.400g methanol, yielding a solution of 9.267µg/ml.

This solution was then injected onto the column, but the peak shape remained as poor as before.

Figure 12: Fresh solution of naproxen injected, failing to improve chromatography

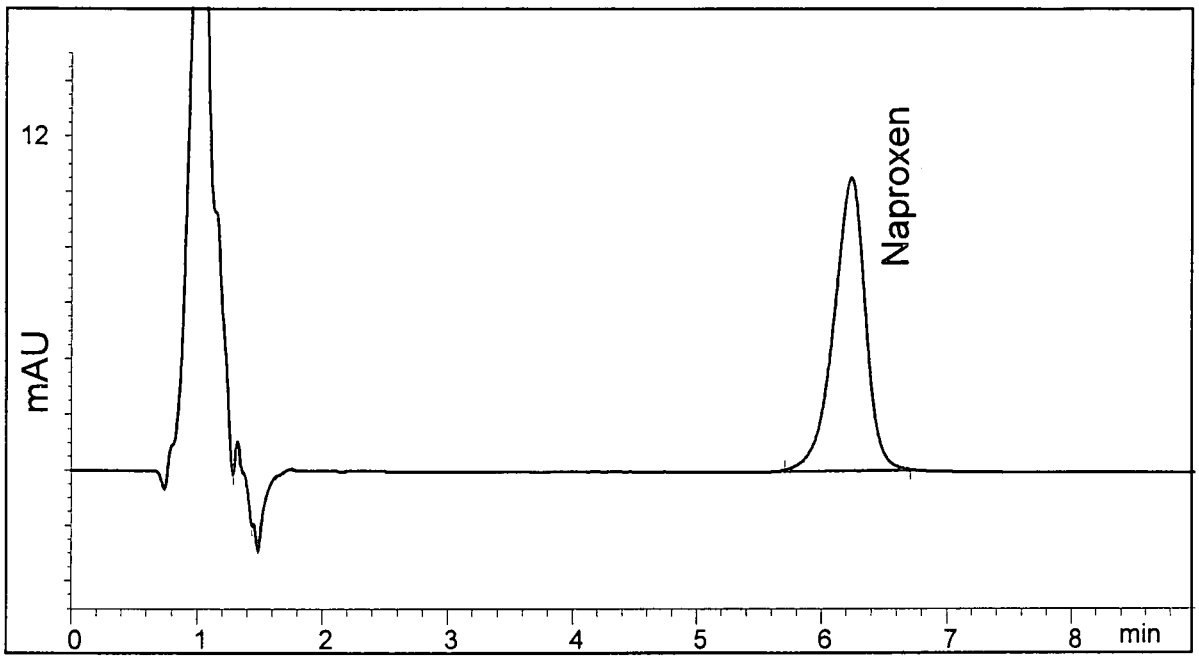


It was concluded that suspect purity of the reference standard was not the critical factor responsible for the poor naproxen peak shape. Fortunately the new reference standard was obtained shortly after the assay method development had started so that not much time was lost. However, a valuable lesson was learnt; that, although from a quantitative point of view, the purity of the reference standard is not so critical during the early development stage of assay method development, qualitative aspects can play a significant role which should not be underestimated. In this case, the suspect reference standard was shown to be in order, but doubts about it were an early confounding factor during the initial assay method development stage.

The second possible cause for the poor peak shape was that the injection solvent (methanol) was not compatible with the mobile phase, resulting in poor peak symmetry.

It was decided to first rinse the entire HPLC system with methanol, pack a fresh pre-column, and then re-inject the samples before investigating the preparation of the analyte and internal standard in a more suitable solvent (usually the mobile phase itself). This resulted in improved symmetry for naproxen (Fig 13).

Figure 13: Improved naproxen peak symmetry



What actually corrected the poor peak symmetry was not clear, but as a result, incompatibility of the injection solvent was not investigated further.

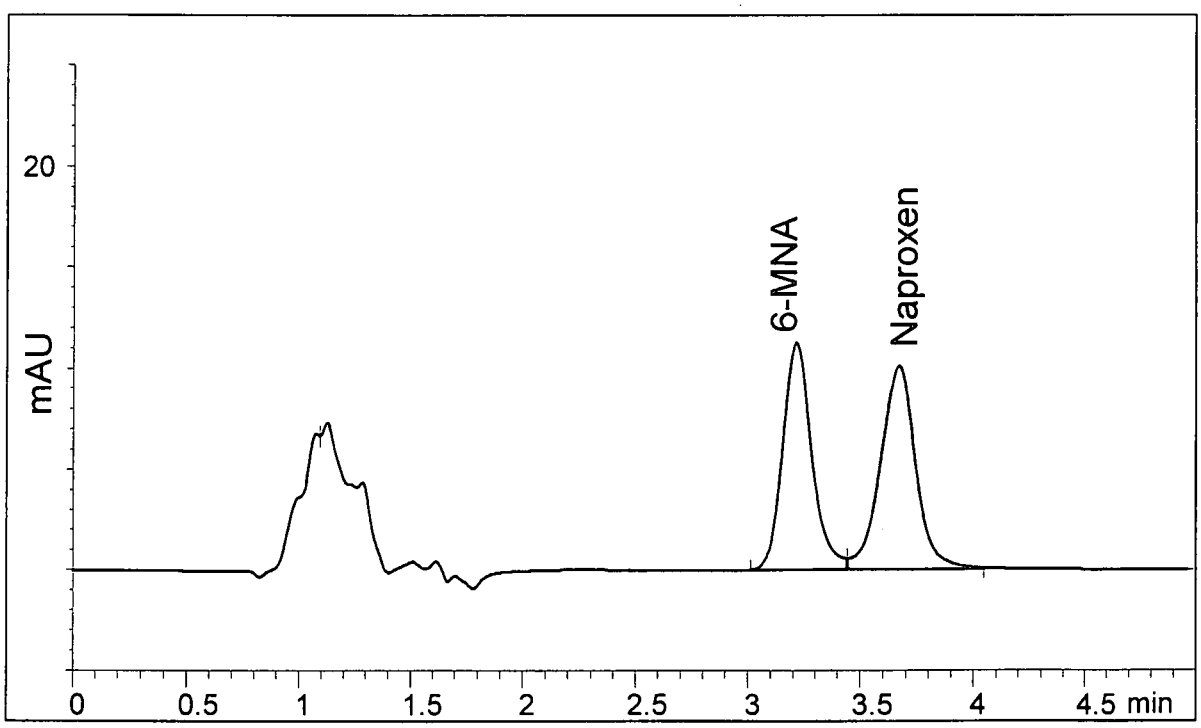
In order to assess resolution between the analyte and proposed internal standard, the 6-MNA and naproxen solutions were mixed (1:1) and injected onto the HPLC system. Good resolution between the two was obtained.

- *Optimising the mobile phase*

In an attempt to shorten the chromatography time of the analyte and internal standard, which eluted at 5.3 and 6.4 minutes respectively (at a flow rate of 0.8 ml/min), it was necessary to adjust the mobile phase. This was owing to the fact that the study required rapid sample processing, and relatively long chromatographic runs would impact negatively on sample throughput.

500cm^3 Acetonitrile was added to 500cm^3 20mM citric acid buffer. The apparent pH of the mobile phase was adjusted to 3.1 using *o*-phosphoric acid (85%) and 1-heptanesulphonic acid (200mg/l) was added in an attempt to sharpen the peaks. The sample was then re-injected with this new mobile phase at 1.0ml/min and the analyte and internal standard were found to now elute at ca. 3.2 and 3.7 minutes respectively.

Figure 14: Mixture of 6-MNA and naproxen injected with the newly adjusted mobile phase



- *Sample preparation*

The next step was to ascertain whether or not injections of prepared plasma samples (extracts) would give similar results to injections of the analyte and internal standard in methanol.

Taking into account the expected concentrations in the plasma samples, it was decided, as a first approach to sample preparation, to investigate the feasibility of a simple protein precipitation procedure. Precipitation with acetonitrile was chosen since previous experience with an ibuprofen assay in plasma had been excellent and because such an assay procedure for naproxen using acetonitrile precipitation of plasma proteins had already been published by Karidas *et al.* [24].

- *Preparation of precipitation solution containing internal standard*

1.386mg Naproxen was directly dissolved in 14.042g acetonitrile, yielding a solution of 77.2µg/ml. This solution would be used to precipitate the plasma containing 6-MNA. This solution was stored at 4°C when not in use.

- *Preparation of 6-MNA plasma calibrators*

23.873mg 6-MNA was dissolved in 12.138g methanol, yielding a spiking solution of 1556µg/ml.

This spiking solution was used to spike blank plasma to a concentration of 25.4µg/ml representing the highest calibration standard S1. Dilutions of this standard were performed with blank plasma as follows:

S1 25.4µg/ml

S2 13.1µg/ml (1:1 dilution of S1)

S3 6.60µg/ml (1:1 dilution of S2)

S5 1.32μg/ml (1+4 dilution of S3)

S4 3.30μg/ml (1:1 dilution of S3)

These five plasma samples (**S1 – S5**) were all precipitated with the internal standard solution described above, in the following manner:

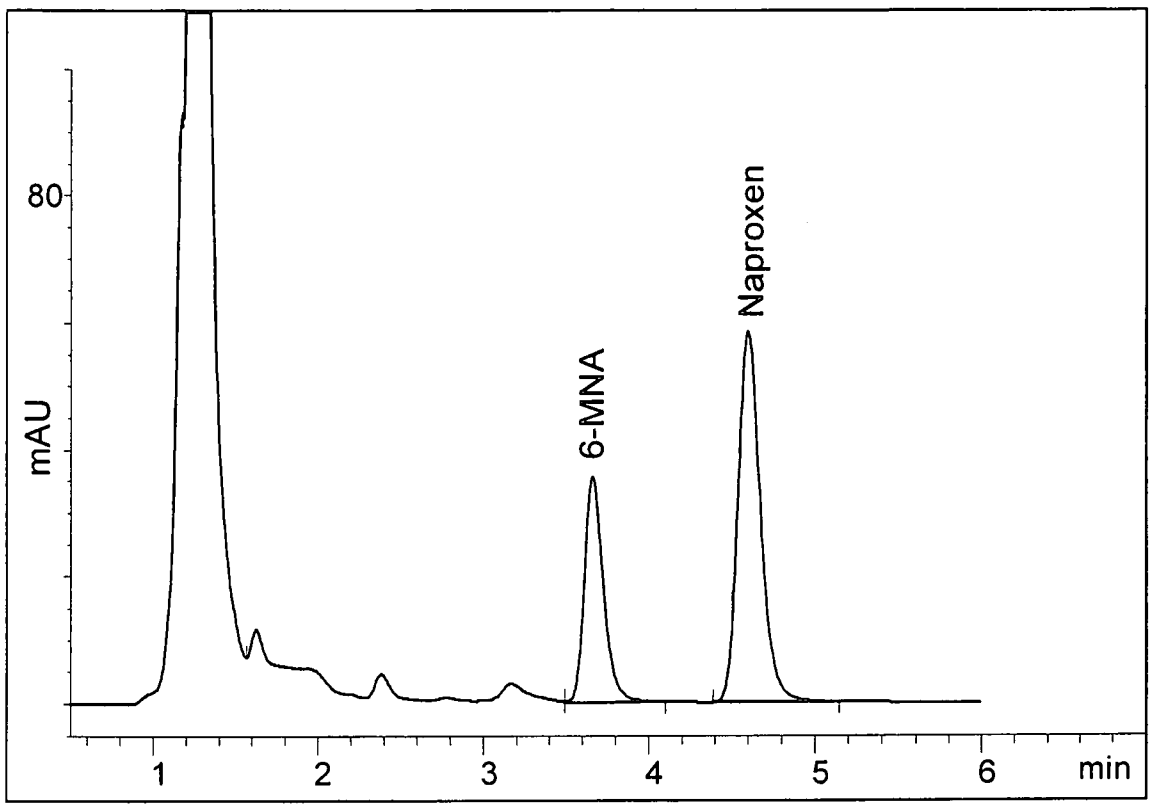
- *Sample preparation*

To 200μl plasma in a 1.5ml microfuge tube, 200μl of the precipitation solution was added. The sample was then vortexed for 1 minute and centrifuged for 5 minutes (6800 *g*).
In addition, a blank plasma sample was precipitated with acetonitrile containing no naproxen in order to determine whether or not there were potential endogenous interference for both analyte and internal standard. 10μl of the supernatant layer was injected onto the HPLC column.

- *Results of precipitated samples*

The results proved to be promising. Not only was the range tested linear, but the chromatography obtained with plasma samples was as good as when using pure solutions of methanol. The retention time of 6-MNA and naproxen were now 3.6 and 4.6 minutes respectively (Fig. 15) and no interfering endogenous components were observed at these retention times.

Figure 15: Chromatography resulting from precipitated samples. Note the change in the so-called solvent front



The lowest of the five plasma concentrations (1.32μg/ml) produced a strong signal (s/n = 120). It was clear that a full order of magnitude reduction in analyte concentration would still result in an acceptable signal-to-noise ratio (at least 5:1).

Figure 16: Established linearity (from extracts) between 25.4 and 1.32μg/ml

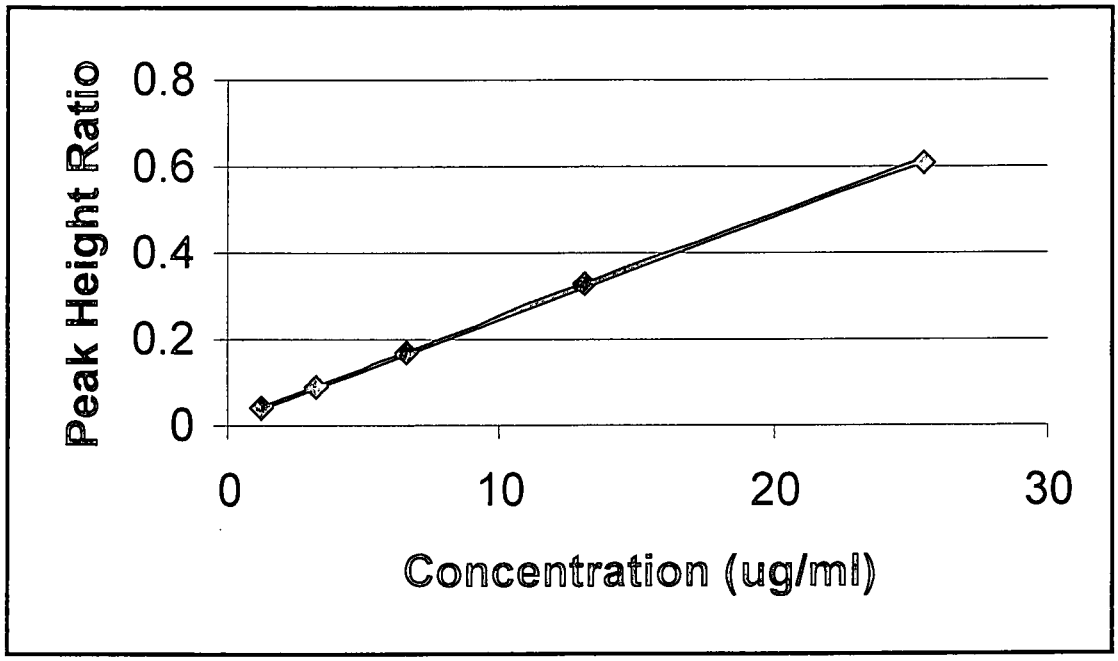
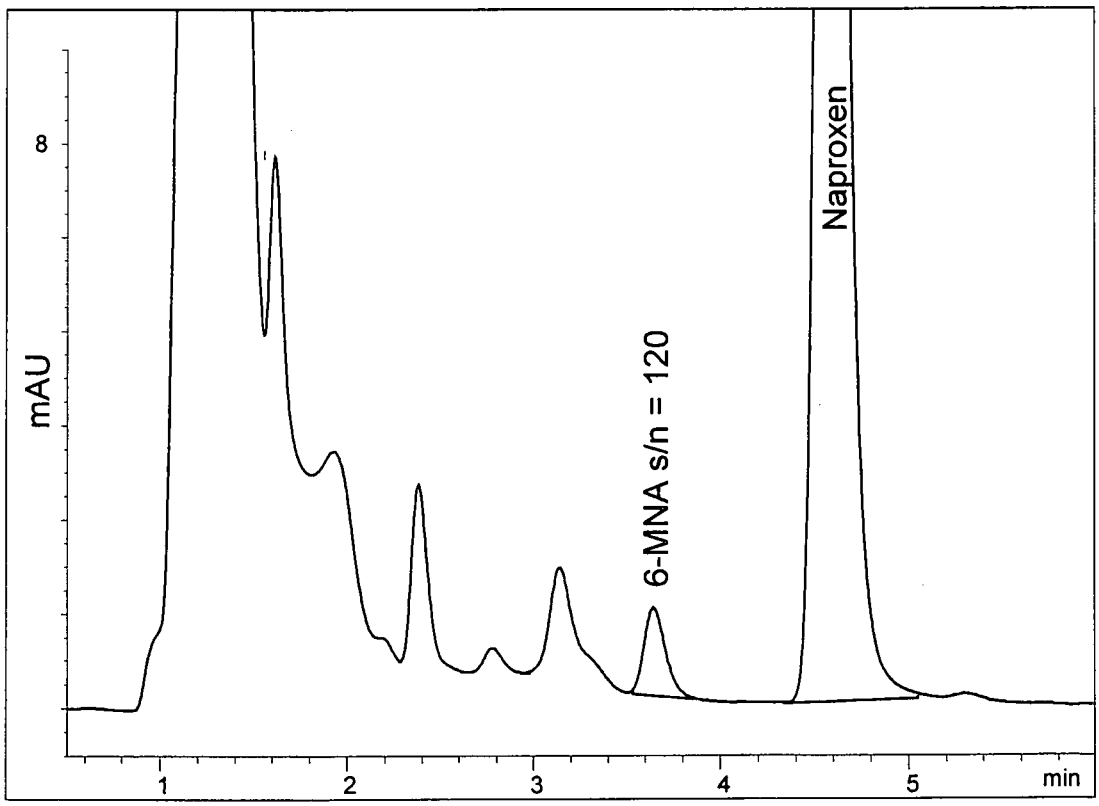


Figure 17: Chromatography resulting from a 1.32 μg/ml plasma standard



On examination of the chromatography, the existing mobile phase was further optimised as follows:

- *Adjusted mobile phase*

450cm^3 acetonitrile was added to 550cm^3 20mM citric acid buffer. The apparent pH of the mobile phase was adjusted to 3.1 using *o*-phosphoric acid (85%) and 1-heptane-sulphonic acid (300mg/l) was added to the mobile phase.

- *Determination of the theoretical lower limit of quantification (tLLOQ)*

Since linearity had been established between 25.4 and 1.32µg/ml (approximate range that Kendall *et al.* [19] revealed could be expected), the next step was to determine the LLOQ. For this purpose a second plasma serial dilution, which would span a lower section of the expected calibration line, was prepared as follows:

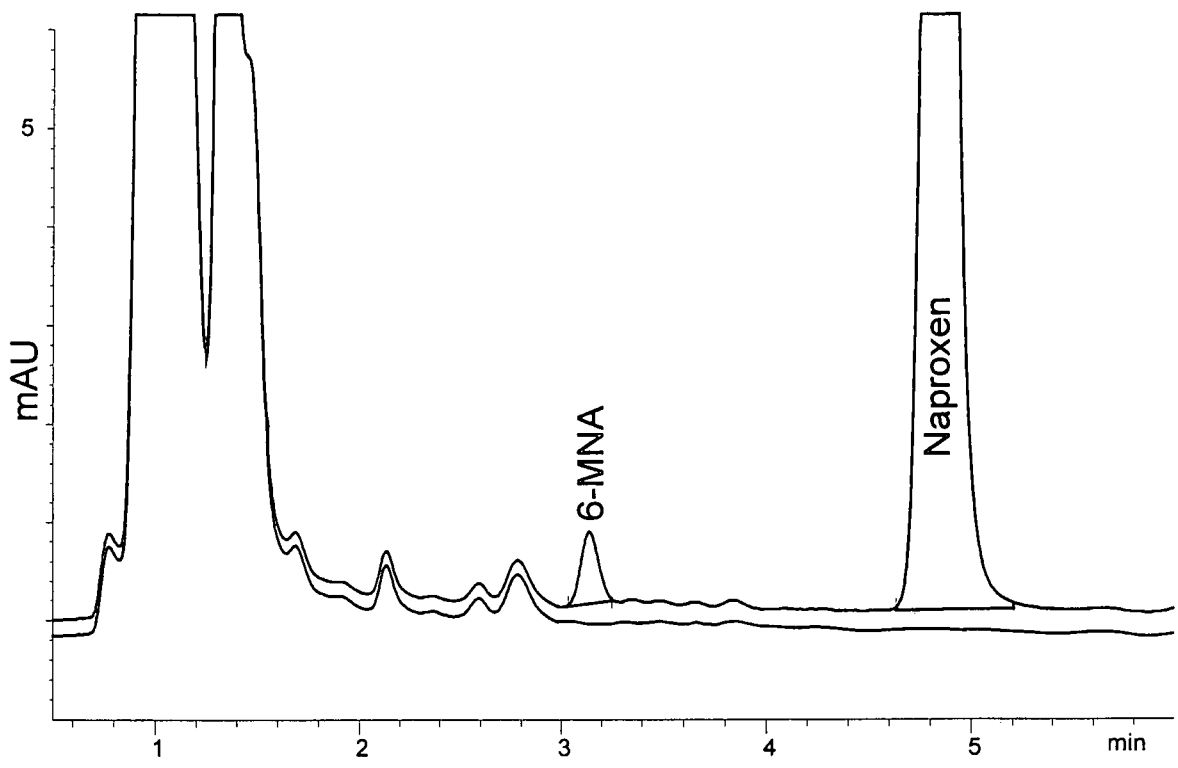
25µl Of the 1556µg/ml 6-MNA stock solution was spiked into 18.215g blank plasma yielding a calibration standard of 2.16 µg/ml. This sample was serially diluted with blank plasma (1:1 v/v), yielding the following nine plasma standards:

S6.	2.19µg/ml	**S11.**	0.068µg/ml
S7.	1.09µg/ml	**S12.**	0.034µg/ml
S8.	0.547µg/ml	**S13.**	0.017µg/ml
S9.	0.274µg/ml	**S14.**	0.009µg/ml
S10.	0.137µg/ml		

These nine standards, together with a blank plasma sample, were precipitated with the acetonitrile solution containing internal standard.

Injection of the above-mentioned samples revealed that the first six provided sufficient detector response, with the 0.068μg/ml sample in this range having a signal-to-noise ratio of approximately 8:1.

Figure 18: Chromatogram of a 0.068μg/ml plasma standard, precipitated (1:1) with acetonitrile containing naproxen

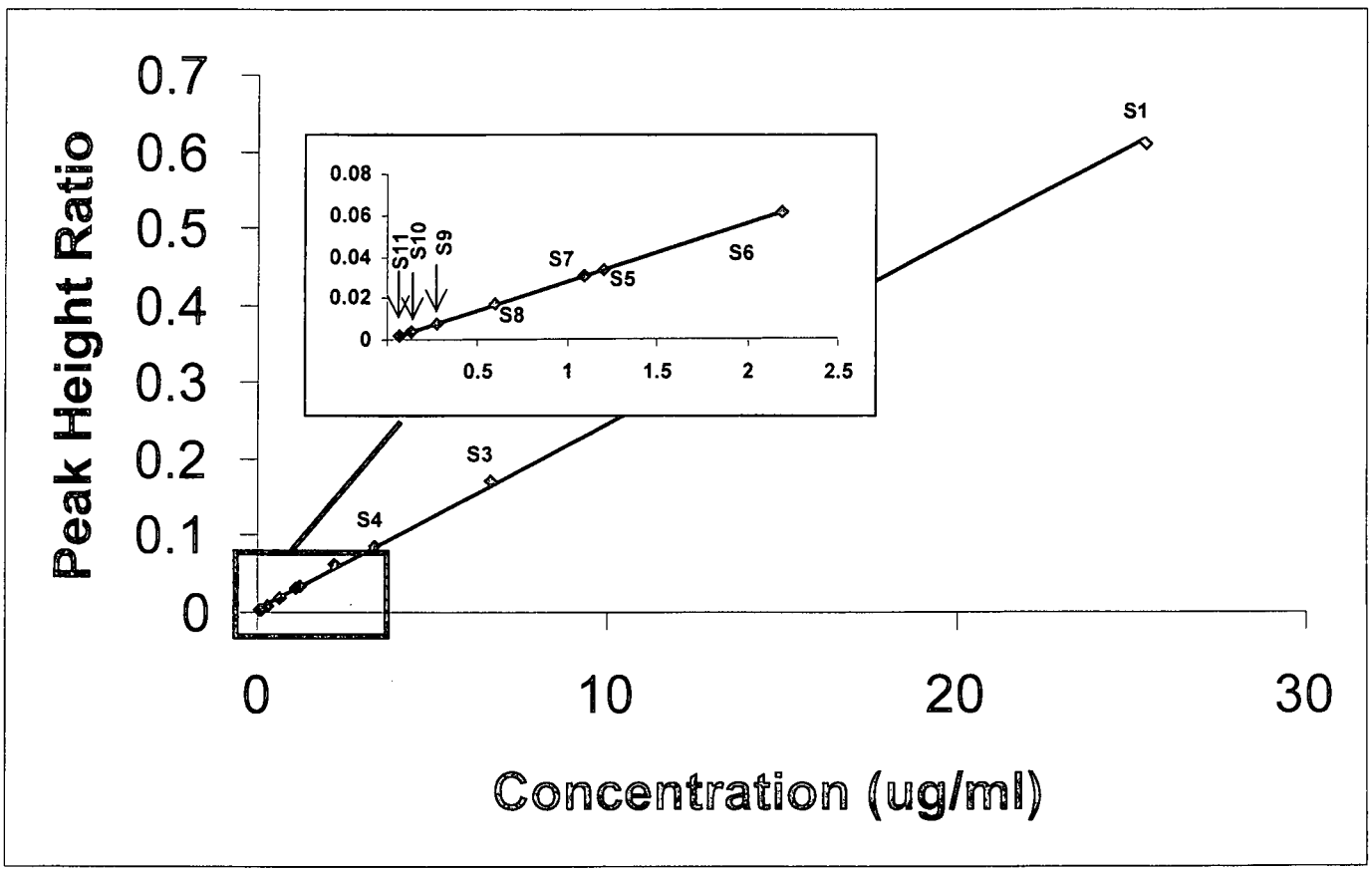


The analyte signal in the last three of the samples was too weak to be used with any confidence. Moreover, this lower section of the calibration range formed a continuous line

with the upper section that had been extracted the day before (Fig 19). Thus, linearity over the expected calibration range had been successfully established, using plasma samples.

At this stage, the assay method was considered to be developed and optimised to such an extent that a validation could be undertaken.

Figure 19: Lower calibration standards (zoom) together with previous calibrators



4.5 Assay method validation

4.5.1 Preparation for assay method validation

- *Screening of blank plasma*

The plasma used for preparing calibration standards and quality controls was obtained from volunteer donors. Usually, three to four volunteers donate blood on any given day, and this blood is then processed and combined into a single pool of plasma. Although volunteers donating blood are required to be drug-free and actually give such an undertaking verbally, this is not a guarantee. It is for this reason that plasma used for preparing calibration standards and quality controls should be screened for components that could interfere chromatographically with either the analyte or the internal standard. This is particularly necessary when using UV detection, which can be considered relatively non-specific when working at a wavelength of 280nm.

Plasma collected on the following six dates was screened:

1.	13 Dec 1996	4.	25 Apr 1997
2.	29 Jan 1997	5.	26 May 1997
3.	21 Feb 1997	6.	29 May 1997

Aliquots of these plasma pools were precipitated with acetonitrile containing no internal standard. And the chromatograms run for 30 minutes to also gauge the effects of possible late eluting peaks. It was found that two of the plasma sources (21 Feb 1997 and 13 Dec

1996) contained peaks that were unresolved from the internal standard, and these plasma pools were not used to prepare calibration standards and quality controls**.

- *Preparation of appropriate response standard*

A judiciously prepared solution of analyte and internal standard in an appropriate solution fulfils two important functions:

- It can be injected daily to monitor the entire HPLC system (so-called system performance verification standard).
- If made in the appropriate solution, it can be combined with data from the validation batch and used to determine analyte and internal standard recovery.

Ideally, the concentration of the analyte in the response standard should be in the neighbourhood of the expected C_{max} , while the internal standard should be at approximately the same concentration that would be in the samples after sample preparation.
In this case, the ideal solution in which to prepare the response standard is the supernatant layer of blank plasma that has been precipitated with acetonitrile, as per the precipitation

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** *It is important to note that this does not vitiate the analytical method with respect to specificity, as this particular study was a controlled, two way cross-over bioequivalence study. This necessarily implies that trial subjects included in the study were required to exclude any other medication during the study. This would not be the case for a patient study for example, where specificity with respect to each concomitant medication would have to be established.*

procedure. For this reason, a large volume of blank plasma was precipitated, centrifuged and the supernatant solution retained for this purpose.

The response standard was prepared by dissolving both analyte (0.392g 6-MNA) and internal standard (0.626g naproxen) directly in 15ml of supernatant layer. This response standard containing 26.1μg/ml 6-MNA and 41.7 μg/ml naproxen was stored at 4°C while not in use.

- *Planning of calibration standards and quality controls*

Sufficient of calibration standards and quality controls have to be prepared to perform method validation as well as process all samples generated during the study. Validation of the accuracy and precision of the assay method requires quality controls to be prepared in five-fold, while calibration standards, with the exception of the two lowest, are prepared in single-fold (see Fig. 2).

This particular study involved 30 healthy volunteers, receiving two formulations of nabumetone, with a wash out period of two weeks in between. Each of these so-called profile days was associated with 18 sampling times. This implied that 1080 samples would have to be assayed.

- *Quantities of standards and quality controls*

It is prudent to prepare standards and quality controls for three validations, in the event of instrumental error, or problems with the analytical procedure that the analyst has not yet discovered[††] Thus, as each validation requires each quality control be prepared in five-fold,

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[††] *It is possible that an analyst can attempt method validation without the method being suitable. In fact, method validation is the very act of demonstrating that a method is fit for use.*

a total of fifteen aliquots of each quality control, and three of each calibration standard (with the exception of the lower two which are extracted in duplicate, which implies six aliquots) is required for method validation. Considering that both profiles of two volunteers could be processed by a single analyst in a single day, approximately fifteen sample batches would have to be processed. This implied thirty aliquots of the higher quality controls, and fifteen of the lower were required for batches, while fifteen aliquots of the upper calibration standards and thirty of the lower were required. The quantities required can be summarised as follows

Level	**Number of aliquots required**
Upper quality controls (QE – QG)	45
Lower quality controls (QA – QD)	30
Upper calibration standards (SD – SM)	18
Lower calibration standards (SB – SC)	36

It is wise to prepare 10 – 20% more calibration standards and quality controls than required, in the event of unforeseen problems. Further, any excess calibration standards and quality controls that remain after completion of the study are stored (at -20°C and -70°C) and could be used to investigate long term stability in the matrix, should a study involving the same analyte be requested by a sponsor at a later stage.

4.5.2 Preparation of Calibration Standards and Quality Controls

Calibration standards and quality controls were prepared using the pure (99.7%)) reference material supplied by the sponsor. Calibration standards were prepared in biological fluid by the analyst who performed the assays on the trial samples by dissolving an accurately determined mass of analyte in a pool of normal biological fluid which was serially diluted with normal biological fluid to attain the desired concentrations. All volumetric operations were performed by weighing and the masses of biological fluid were converted to volumes when calculating concentrations. The upper range of quality controls were prepared in biological fluid by an analyst other than the analyst who performed the study assays by the same method as used for the calibration standards. The lower range of quality controls were prepared in biological fluid by the preparation of a stock solution, using a suitable solvent, which was spiked into a pool of normal biological and serially diluted with normal biological fluid to attain the desired concentrations. The calibration standards were aliquoted into tubes and stored under the same conditions as the trial samples; approximately -20°C and normally in the same freezer.

- *Calibration standards*

Mass of analyte weighed into STD M: 5.091mg

Sample Code & No.	Source Solution	A	B	C	D ug/ml
STD M	*Dissolved*	126.819	162.879		145
STD L	*STD M*	109.378	127.439	145.380	72.2
STD K	*STD L*	103.066	121.080	139.040	36.1
STD J	*STD K*	105.010	123.080	141.070	18.0
STD I	*STD J*	108.434	126.460	144.400	8.98
STD H	*STD I*	109.989	128.190	146.170	4.46
STD G	*STD H*	111.131	129.150	147.260	2.24
STD F	*STD G*	110.930	128.950	146.970	1.12
STD E	*STD F*	55.473	73.460	91.620	0.562
STD D	*STD E*	66.986	84.970	103.140	0.282
STD C	*STD D*	57.590	75.600	93.440	0.140
STD B	*STD C*	64.390	82.400	100.480	0.070

Note: Mass of biological fluid (g) is converted to volume (ml), using SG = 1.0269 for plasma.

- *Quality controls*

The high concentration controls were prepared by a different analyst, using the same methodology used to prepare the calibration standards.

Mass of analyte weighed into QC G: 3.901mg

Sample Code & No.	Source Solution	A	B	C	D ug/ml
QC G	*Dissolved*	103.405	176.804		54.6
QC F	*QC G*	64.387	96.387	136.494	30.4
QC E	*QC F*	58.063	82.073	114.074	17.3

The lower range quality controls were prepared using slightly different methodology. A spiking solution of 6-MNA was prepared in methanol and used immediately to spike a pool of blank plasma to 0.330µg/ml (QC D). This quality control was then serially diluted with blank plasma to produce the three lowest quality controls. Care was taken not to spike excessive volumes of methanol into the pool of plasma (QC D), in order to minimise

modification of the matrix. As this spiking solution was not retained for further use, the stability of 6-MNA is stock solution was not tested.

Preparation of Stock Solution QA for Spiking QC D:

Solvent used	SG solvent (kg/l)	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	2.472	11.126	14.066	200	175.75

Preparation of lower range Quality Control Standards:

Sample Code & No.	Source Solution	A	B	C	D ug/ml
QC D	*Stock QA*	64.958	174.059		0.330
QC C	*QC D*	62.811	74.826	110.835	0.248
QC B	*QC D*	65.979	90.993	115.996	0.165
QC A	*QC B*	64.920	81.447	98.046	0.083

Note: Mass of biological fluid (g) is converted to volume (ml), using SG = 1.0269 for plasma.
A = Mass of empty container.
B = Mass of container + normal biological fluid.
C = Total mass of container + normal + spiked biological fluid
D = Concentration of analyte in the biological fluid

Aliquots of 0.4ml were immediately placed in 1.5ml microfuge tubes, and stored at -20°C.

Figure 20: Certificate of analysis for 6-MNA supplied by the sponsor

R124

Sekhsaria Chemicals Limited

CERTIFICATE OF ANALYSIS

PRODUCT	2-(6-METHOXY NAPHTHYL) ACETIC ACID
BATCH NO.	MNA # 02
DATE OF MANUFACTURING	APRIL 1997
NAME OF THE TEST	ANALYTICAL RESULTS
DESCRIPTION	ALMOST WHITE POWDER
MELTING RANGE	171 C - 174 C
RELATED SUBSTANCE BY TLC	NOT DETECTABLE
ASSAY (CHEMICAL)	99.7%
ASSAY BY HPLC	98.7%

ANALYSED BY Parathe 17/4/97 QUALITY CONTROL Purohit 17/4/97 QUALITY ASSURANCE M J Shah. 17/4/97

DATE DATE DATE

A fresh internal solution was prepared by dissolving 19.206mg naproxen in 163.07g acetonitrile, giving a concentration of 93.16µg/ml. A fresh batch of mobile phase was also prepared.

- *Confirming the calibration range and specificity*

Quite often when an assay method needs to be developed for a clinical study, there exists a paucity of information regarding the actual plasma concentrations that will be encountered. This means that assay methods often have to be developed and validated over a very wide range of concentrations. While the lower limit of quantitation is determined by the sensitivity of the assay method, the upper limit to which the assay method should be validated is open ended and quite often a matter of conjecture. This is not a trivial problem, since the wrong choice of upper concentration calibrator could lead to repercussions, the least of which would be that the validation of the assay method might have to be repeated. For this reason alone it is imperative that everything possible should be done to ascertain the most probable highest concentration that will need to be measured. An extensive literature search for studies in which the analyte may have been assayed is mandatory. In this particular case a good reference [19] was obtained, which indicated that the maximum concentration to be expected, could vary between 34 and 74 μg/ml depending on the age of the subjects since elderly subjects attained higher C_{max} values that younger ones. Notwithstanding such information, it is standard practice in the FBSD to combine small aliquots (ca 50 to 100 μl) of the serially collected plasma samples during bioequivalence studies at the specific time intervals in order to be able to gain some insight into the probable mean C_{max} concentration in that particular study. In addition, this is to identify peaks in the chromatograms which can be attributed to metabolites of the drug, which are formed *in vivo.* By their nature these metabolites are often quite closely related to the parent drug and could elute very closely to the parent drug or even at the same retention time as the internal standard. These so-called "specificity samples" are therefore assayed, before the final

validation is to be undertaken in case the chromatography procedure needs further adjustment to avoid possible interference by these metabolites. Accordingly, such samples were combined in this study and assayed using the, up to now, unvalidated Preliminary Assay Procedure.

From the results obtained with these assays, it was concluded that firstly, there was no interference from metabolites generated *in vivo* and secondly, judging from the relative height of the samples to the response standard, the calibration range prepared in plasma was indeed appropriate.

4.5.3 Processing the validation batch

At this stage, it was appropriate to proceed with the pre-study validation. The analyst felt confident that a pre- study validation batch would meet the acceptance criteria (see section 3.2).

At the end of method development, the following Preliminary Assay Procedure (PAP) method had been decided upon by the analyst:

- *Sample preparation procedure*

  To 200µl of plasma was added 200µl of acetonitrile containing naproxen (ca. 80 µg/ml) as internal standard. The sample was then vortexed for 30 seconds and immediately centrifuged at 6800g for 5 minutes. Of the supernatant layer, 10µl was injected onto the HPLC column.

- *Instrumental and chromatographic conditions*

Analytical column

The chromatographic column used was a Hewlett-Packard LiChrospher®100 RP8 (5 μm) stainless steel column fitted with an Upchurch precolumn (20×2 mm) dry-filled with Perisorb®RP-18 (30-40 μm) packing.

Mobile phase

Acetonitrile : citric acid buffer (20 mM) (450 + 550). 1-Heptane sulphonic acid was added to the solution (300 mg/L) and the apparent pH of the mobile phase is adjusted to pH 3.1 using concentrated o-phosphoric acid. The mobile phase was not recycled during batches.

Pump and Flow Rates

Hewlett-Packard series 1100 pump delivering 1.0 ml/min at ambient temperature.

Sample injection

Hewlett-Packard series 1100 autosampler injecting 10 μl onto the HPLC column.

Detection

Hewlett-Packard series 1100 variable wavelength detector set at 280 nm.

Recording and integration

Hewlett-Packard series 1100 detector interfaced to a computer workstation running Hewlett-Packard HPLC^{2D} ChemStation® version A.04.01 software

The pre-study validation batch was prepared according to the procedure above and processed as a single batch presented in the following table:

Project	: 36/96\AHP01						
Operator	: A.D. de Jager	Date Extracted	: 11-06-1997	Date Injected	: 11-06-1997	Subjects	: None
Period	: N/A	Plasma Set	: N/A				
Analytical Pump	: HP 1100	No	: DE 52700182	Flow Rate	: 1.0 ml/min	Pressure	: 56 bar
Column	: LiChrospher	Serial No.	: 310	Program (Y/N)	: N	MP Prep date\Batch	: 9-06-1997
Auxilliary Pump	: -	No	: -	Flow Rate	: -	Pressure	: -
Column	: -	Serial No.	: -	Program (Y/N)	: -	MP Prep date	: -
Switch Valve No.	: -	Time 1	: -	Time 2	: -	Solvent Select No	: -
A %	: -	B%	: -	C%	: -	D%	: -
Program (Y/N)	: -	Column Heater No.	: -	Temperature	: -		
Integrator\AD Box	: -	Channel No.	: -	Chart Speed	: -	Attenuation	: -
Input Voltage	: -	Run Time	: -				
Auto Injector	: HP 1100	No.	: DE 54900636	Inject Volume	: 10 ul	Runtime	: 7 min
Wash Solution	: Mob. phase	Program (Y/N)	: N				
Ultraviolet Det	: HP 1100	No.	: JP 64201933	Wavelength	: 280 nm	Range	: -
Rise Time	: -	Output Voltage	: 220 V	Program (Y/N)	: N		
Fluorescence Det	: -	No.	: -	Wavelength	: -	PMT Voltage	: -
Attenuation	: -	Range	: -	Response	: -	Output Voltage	: -
Program (Y/N)	: -	Misc	: -				

Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF
1 R. STD		21 QC A		41 QC A		61		81		101		121		141	
2 STD M		22 STD I		42 STD E		62		82		102		122		142	
3 STD L		23 STD H		43 STD D		63		83		103		123		143	
4 BLANK 1		24 BLANK 3		44 BLANK 5		64		84		104		124		144	
5 QC G		25 QC G		45 QC G		65		85		105		125		145	
6 QC F		26 QC F		46 QC F		66		86		106		126		146	
7 QC E		27 QC E		47 QC E		67		87		107		127		147	
8 QC D		28 QC D		48 QC D		68		88		108		128		148	
9 QC C		29 QC C		49 QC C		69		89		109		129		149	
10 QC B		30 QC B		50 QC B		70		90		110		130		150	
11 QC A		31 QC A		51 QC A		71		91		111		131		151	
12 STD K		32 STD G		52 STD C		72		92		112		132		152	
13 STD J		33 R. STD		53 STD B		73		93		113		133		153	
14 BLANK 2		34 BLANK 4		54 BLANK 6		74		94		114		134		154	
15 QC G		35 QC G		55 STD F		75		95		115		135		155	
16 QC F		36 QC F		56 R. STD		76		96		116		136		156	
17 QC E		37 QC E		57		77		97		117		137		157	
18 QC D		38 QC D		58		78		98		118		138		158	
19 QC C		39 QC C		59		79		99		119		139		159	
20 QC B		40 QC B		60		80		100		120		140		160	

Samples loaded and System Checked by :
Sample Position Verified by :
Approval :
Notes :

Date :
Date :
Date : 12/06/1997

The following represents the data for the pre-study validation calibration line, generated by PhIRSt

Project : 36/96.AHP
Analyte : 6-MNA
Curve : AHP01
Quant : Height
Method : Hp Asterix

CurveFit : 1/Concentration² Linear
Conc units : ng/ml
LLOQ : 0.070 ~~ng/ml~~ ng/ml
Data : h:\36frm96\analytic\inpdata\ahp01\1-01.D\REPORT.TXT
Calculation: Individual response

Rev :PO VO MO RO
Tables were not updated
Date of Injection : 11-06-1997
Process : Read from Raw Data

12-Jun-1997
14:46
Page - 1

###	STD ID	Drug Peak	IS Peak	Ratio	Actual	Calc	% Dev	RT Dg	RT IS	RRT	Time	Rep	Code
2	STD M	79.963	28.887	2.7681	145	133	-8.3	3.60	4.73	0.76	10:49	Asterix	
3	STD L	39.780	28.196	1.4108	72.2	67.8	-6.1	3.59	4.72	0.76	10:57	Asterix	
12	STD K	20.637	27.919	0.7392	36.1	35.5	-1.7	3.58	4.71	0.76	12:10	Asterix	
13	STD J	10.180	26.478	0.3845	18.0	18.5	2.6	3.58	4.71	0.76	12:19	Asterix	
22	STD I	5.129	27.654	0.1855	8.98	8.90	-0.9	3.57	4.69	0.76	13:32	Asterix	
23	STD H	2.599	27.047	0.0961	4.46	4.61	3.3	3.57	4.70	0.76	13:40	Asterix	
32	STD G	1.350	27.337	0.0494	2.24	2.36	5.5	3.57	4.69	0.76	14:54	Asterix	
55	STD F	0.751	27.967	0.0268	1.12	1.28	14.4	3.58	4.71	0.76	18:53	Asterix	
42	STD E	0.325	27.851	0.0117	0.562	0.553	-1.6	3.57	4.69	0.76	17:07	Asterix	
43	STD D	0.160	28.297	0.0056	0.282	0.264	-6.5	3.57	4.69	0.76	17:15	Asterix	
52	STD C	0.084	28.209	0.0030	0.140	0.136	-2.9	3.58	4.70	0.76	18:29	Asterix	
53	STD B	0.046	28.055	0.0016	0.070	0.072	2.2	3.57	4.71	0.76	18:37	Asterix	

r = .997646 r² = .995297 Slope = 0.020817 Intercept = 0.000156 n = 12

###	QC ID	Drug Peak	IS Peak	Ratio	Actual	Calc	% Dev	RT Dg	RT IS	RRT	Time	Rep	Code
5	QC G	30.963	27.428	1.1289	54.6	54.2	-0.7	3.59	4.72	0.76	11:13	Asterix	
15	QC G	30.818	27.674	1.1136	54.6	53.5	-2.0	3.58	4.71	0.76	12:35	Asterix	
25	QC G	31.866	28.402	1.1220	54.6	53.9	-1.3	3.57	4.69	0.76	13:57	Asterix	
35	QC G	35.811	30.513	1.1736	54.6	56.4	3.2	3.57	4.69	0.76	15:18	Asterix	
45	QC G	30.846	29.113	1.0595	54.6	50.9	-6.8	3.57	4.70	0.76	17:32	Asterix	
6	QC F	17.123	27.215	0.6292	30.4	30.2	-0.6	3.59	4.72	0.76	11:22	Asterix	
16	QC F	17.417	26.599	0.6548	30.4	31.4	3.4	3.58	4.71	0.76	12:43	Asterix	
26	QC F	17.542	27.659	0.6342	30.4	30.5	0.2	3.57	4.69	0.76	14:05	Asterix	
36	QC F	19.832	30.606	0.6480	30.4	31.1	2.4	3.56	4.69	0.76	15:26	Asterix	
46	QC F	17.222	28.549	0.6033	30.4	29.0	-4.7	3:57	4.70	0.76	17:40	Asterix	
7	QC E	9.749	27.420	0.3555	17.3	17.1	-1.3	3.59	4.72	0.76	11:30	Asterix	
17	QC E	9.851	27.488	0.3584	17.3	17.2	-0.5	3.58	4.70	0.76	12:51	Asterix	
27	QC E	10.189	27.968	0.3643	17.3	17.5	1.1	3.57	4.69	0.76	14:13	Asterix	
37	QC E	10.885	28.892	0.3767	17.3	18.1	4.6	3.57	4.69	0.76	16:26	Asterix	
47	QC E	9.821	28.488	0.3448	17.3	16.6	-4.3	3.58	4.70	0.76	17:48	Asterix	
8	QC D	0.183	27.360	0.0067	0.330	0.314	-4.8	3.59	4.71	0.76	11:38	Asterix	
18	QC D	0.193	26.940	0.0072	0.330	0.337	2.2	3.58	4.70	0.76	12:59	Asterix	
28	QC D	0.199	27.767	0.0072	0.330	0.337	2.1	3.56	4.69	0.76	14:21	Asterix	
38	QC D	0.210	28.297	0.0074	0.330	0.349	5.8	3.56	4.69	0.76	16:34	Asterix	
48	QC D	0.219	29.049	0.0075	0.330	0.354	7.4	3.57	4.70	0.76	17:56	Asterix	
9	QC C	0.145	27.516	0.0053	0.248	0.246	-0.9	3.59	4.71	0.76	11:46	Asterix	

Verified by : _____
PhReg 2.1-024

Approved by : _____

Project : 36/96.AHP
Analyte : 6-MNA
Curve : AHP01
Quant : Height
Method : Hp Asterix

CurveFit : 1/Concentration² Linear
Conc units : ng/ml
LLOQ : 0.070 ~~ng/ml~~ ng/ml
Data : h:\36frm96\analytic\inpdata\ahp01\1-01.D\REPORT.TXT
Calculation: Individual response

Rev :P0 V0 M0 R0
Tables were not updated
Date of Injection : 11-06-1997
Process : Read from Raw Data

12-Jun-1997
14:46

### QC ID	Drug Peak	IS Peak	Ratio	Actual	Calc	% Dev	RT Dg	RT IS	RRT	Time	Rep	Code
19 QC C	0.141	26.870	0.0053	0.248	0.245	-1.1	3.58	4.70	0.76	13:07	Asterix	
29 QC C	0.148	27.243	0.0054	0.248	0.253	2.2	3.57	4.69	0.76	14:29	Asterix	
39 QC C	0.162	27.891	0.0058	0.248	0.271	9.4	3.57	4.69	0.76	16:43	Asterix	
49 QC C	0.175	28.362	0.0062	0.248	0.289	16.7	3.57	4.70	0.76	18:04	Asterix	
10 QC B	0.095	27.198	0.0035	0.165	0.160	-3.0	3.59	4.71	0.76	11:54	Asterix	
20 QC B	0.095	26.790	0.0036	0.165	0.163	-1.0	3.57	4.70	0.76	13:16	Asterix	
30 QC B	0.100	27.732	0.0036	0.165	0.166	0.4	3.57	4.69	0.76	14:37	Asterix	
40 QC B	0.100	27.306	0.0037	0.165	0.169	2.5	3.57	4.69	0.76	16:51	Asterix	
50 QC B	0.097	28.032	0.0035	0.165	0.159	-3.8	3.57	4.70	0.76	18:13	Asterix	
11 QC A	0.054	27.238	0.0020	0.083	0.089	6.6	3.58	4.71	0.76	12:02	Asterix	
21 QC A	0.062	28.446	0.0022	0.083	0.097	17.3	3.58	4.70	0.76	13:24	Asterix	
31 QC A	0.055	27.237	0.0020	0.083	0.089	7.2	3.56	4.69	0.76	14:46	Asterix	
41 QC A	0.058	28.689	0.0020	0.083	0.089	7.0	3.56	4.69	0.76	16:59	Asterix	
51 QC A	0.054	28.391	0.0019	0.083	0.084	0.9	3.57	4.70	0.76	18:21	Asterix	

### Sample ID	Drug Peak	IS Peak	Ratio	Calc	RT Dg	RT IS	RRT	Time	Rep	Code

Verified by : _____
PhReg 2.1-024

Approved by : _____

Project : 36/96.AHP	CurveFit : 1/Concentration² Linear	Rev :P0 V0 M0 R0	12-Jun-1997
Analyte : 6-MNA	Conc units : ng/ml	Tables were not updated	14:46
Curve : AHP01	LLOQ : 0.070 ng/ml	Date of Injection : 11-06-1997	Page - 3
Quant : Height	Data : h:\36frm96\analytic\inpdata\ahp01\1-01.D\REPORT.TXT		
Method : Hp Asterix	Calculation: Individual response	Process : Read from Raw Data	

###	Misc ID	Drug Peak	IS Peak	Ratio	Calc	RT Dg	RT IS	RRT	Time	Rep	Code
33	R. STD	33.204	42.321	0.7846	37.7	3.56	4.68	0.76	15:02	Asterix	
56	R. STD	32.692	41.750	0.7831	37.6	3.58	4.71	0.76	19:02	Asterix	
57		No Peak	No Peak	N/ap	BLQ	N/ap	N/ap	N/ap	N/ap	N/ap	
1	R. STD	32.592	41.499	0.7854	37.7	3.60	4.73	0.76	10:08	Asterix	
4	BLANK 1	No Peak	No Peak	N/ap	BLQ	3.66	4.95	0.74	11:05	Asterix	
14	BLANK 2	No Peak	No Peak	N/ap	BLQ	3.66	4.95	0.74	12:27	Asterix	
24	BLANK 3	No Peak	No Peak	N/ap	BLQ	3.66	4.95	0.74	13:48	Asterix	
34	BLANK 4	No Peak	No Peak	N/ap	BLQ	3.66	4.95	0.74	15:10	Asterix	
44	BLANK 5	No Peak	No Peak	N/ap	BLQ	3.66	4.95	0.74	17:24	Asterix	
54	BLANK 6	No Peak	No Peak	N/ap	BLQ	3.66	4.95	0.74	18:45	Asterix	

Summary statistics

6-MNA		Calibration STDs		System Suitability	
Retention Time		Response		Response	
Mean:	3.575		0.0000		0.0000
SD :	0.0095		0.00000		0.00000
%CV :	0.3		0.0		0.0
n :	47		0		0
High:	3.600		0.0000		0.000
Low :	3.560		0.0000		0.000
Internal Standard		**Internal Standard**		**Calibration Curve**	
Retention Time		Response		IS Response	
Mean:	4.701		27.9		27.8
SD :	0.0107		0.86		0.63
%CV :	0.2		3.1		2.3
n :	47		47		12
High:	4.730		30.6		28.9
Low :	4.690		26.5		26.5

Verified by : _____ Approved by : _____

PhReg 2.1-024

4.5.4 Analytical report – 6-MNA

The validation of the assay method and subsequent assay of the study samples culminated in the following analytical report:

- *Intra-day Accuracy and Precision*

Calibration Curve

Calibration Standards used: STD B - STD M

Calibration Range: 0.070 - 145 µg/ml

Regression Equation: Linear (1/Concentration^2)

Slope: 0.020817

Intercept: 0.000156

r^2: 0.995297

STD Code	Nominal Conc. (µg/ml)	Back-calculated Conc. (µg/ml)	% Bias
STD M	145	133	-8.3
STD L	72.2	67.8	-6.1
STD K	36.1	35.5	-1.7
STD J	18.0	18.5	2.6
STD I	8.98	8.90	-0.9
STD H	4.46	4.61	3.3
STD G	2.24	2.36	5.5
STD F	1.12	1.28	14.4
STD E	0.562	0.553	-1.6
STD D	0.282	0.264	-6.5
STD C	0.140	0.136	-2.9
STD B	0.070	0.072	2.2

- *Summary of Intra-day Quality Control Results*

Accuracy is measured as % bias and precision is measured as coefficient of variation (CV %)

Code	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	54.5	30.4	17.3	0.330	0.248	0.165	0.083
Replicates							
1	54.20	30.20	17.10	0.314	0.246	0.163	0.089
2	53.50	31.40	17.20	0.337	0.245	0.160	0.097
3	53.90	30.50	17.50	0.337	0.253	0.166	0.089
4	56.40	31.10	18.10	0.349	0.271	0.169	0.089
5	50.90	29.00	16.60	0.354	0.289	0.159	0.084
MEAN	53.78	30.44	17.30	0.34	0.26	0.16	0.09
BIAS	-1.5%	0.3%	-0.3%	2.4%	5.1%	-1.0%	7.7%
CV	3.3%	2.7%	2.9%	4.1%	6.5%	2.3%	4.7%

- *Calibration Range*

For the assignment of a valid calibration range bias is taken as measure of accuracy and coefficient of variation (CV %) is taken as measure of precision. Intra-day accuracy and precision for a valid range must be within 15% but within 20% at the lower limit of quantification. Results from the intra-day validation assays above indicate a valid calibration range of 0.070 - 145 µg/ml. The lower limit of quantification (LLOQ) was preliminarily set at 0.070 µg/ml.

- *Extraction Efficiency*

Absolute recoveries of the analyte and internal standard are determined from the comparison of a theoretically extrapolated peak height, based on the response factor of a suitable analyte solution of precisely determined concentration, to the mean analyte peak height of an extracted Quality Control Standard. Extraction efficiency of the analyte is determined at high, moderate and low concentrations of analyte in triplicate. The extraction efficiency of the internal standard is determined from the mean of ten values.

ABSOLUTE RECOVERY OF ANALYTE

ANALYTE: **6-Methoxy-2-naphthylacetic acid**

SAMPLE	ANALYTE µg/ml	MEAN OF PEAK AREAS		ABSOLUTE RECOVERY (%)	CV (%)
		AFTER EXTRACTION	THEORETICAL VALUES		
RCmax	30.4	17.36	19.10	90.91	1.01
RCave	0.330	0.19	0.21	92.46	3.44
RCmin	0.083	0.054	0.052	103.57	6.24
Mean Recovery:				95.6%	

ABSOLUTE RECOVERY OF INTERNAL STANDARD

INTERNAL STANDARD: **Naproxen**

ISTD µg/ml	MEAN OF PEAK AREAS		ABSOLUTE RECOVERY (%)	CV (%)
	AFTER EXTRACTION	THEORETICAL VALUES		
93.16	29.81	46.08	64.70	2.99

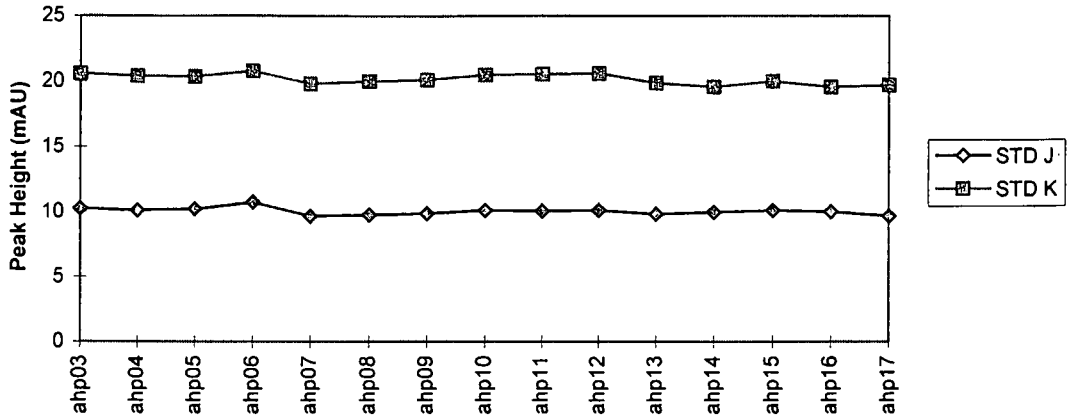
- *Stability*

Stock Solutions

Standard solutions were made up in methanol, used immediately to spike plasma and discarded thereafter. Stock solutions were not retained for further use.

Stability in the Matrix

When stored at -20°C, the analyte displayed no significant deterioration in analyte response over the 21 days in which the batches were completed. The analyte can thus be considered stable at -20°C for at least three weeks.

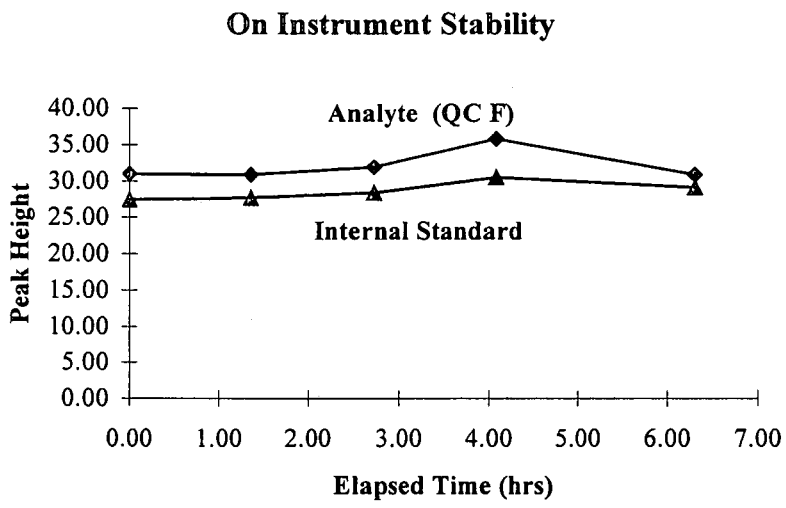


Freeze-thaw Stability

All samples were thawed once only shortly before analysis. Sample remnants were discarded and not retained for further analysis. No multiple freeze-thaw cycles were permitted.

On-instrument Stability

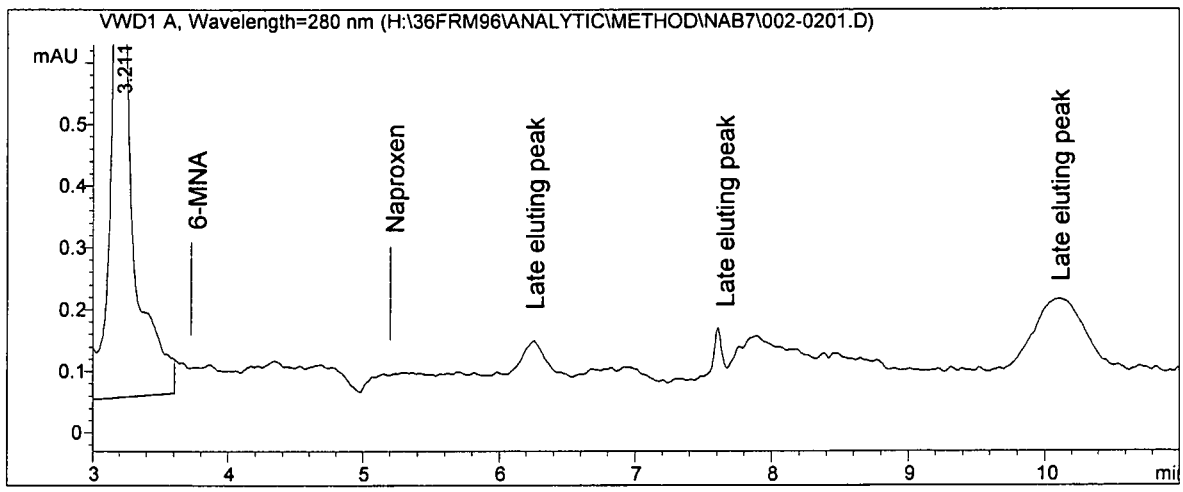
Samples were shown to be stable on the instrument for at least 7 hours.



- *Specificity*

Specificity is determined by analysing "blank" biological fluids from six different sources without the addition of the internal standard. The chromatograms are inspected for peaks which may interfere with the analyte and the internal standard. In the case of high performance liquid chromatographic procedures the chromatograms are run for 30 minutes to determine the presence of late eluting peaks which may cause interference in subsequent chromatograms.

Late eluting peaks were found at ~6.2 min, 8.5 min and 10 min. The run time was lengthened appropriately to exclude interference in subsequent chromatograms.



4.5.5 Within-Study Assay Performance

- *Study Execution*

Samples are assayed in batches, consisting of calibration standards (usually 6 to 10), quality controls (at high, medium and low concentrations), and study samples. The number of samples that can be assayed in a batch depends on factors such as the stability of the analyte in the biological fluid or in the extraction solvent and the length of the chromatographic runs.

Attempts are always made to process complete profiles of a subject for each treatment in a batch. Thus in a given batch, profiles of treatments are alternated whenever possible. The calibration standards and quality controls are interspersed among the study samples in a predetermined manner. The quality controls which are processed in each batch comprise duplicates near the maximum, near the mean concentration and near 3 × LLOQ (where LLOQ represents the lower limit of quantification determined during the validation of the assay method) as well as two controls respectively near LLOQ and 2 × LLOQ. After the batch has been run the chromatograms are inspected and checked against documented acceptance criteria.

The calibration curves are plotted, regression equations determined, and the quality controls calculated as unknowns using the regression equation giving the best overall results throughout the study.

- *Preparation of Calibration Standards and Quality Controls*

Sufficient calibration standards and quality control standards are prepared during the pre-study validation to serve as calibration standards and quality control standards for the assaying of study samples. The preparation of these standards and quality controls has already been presented under the Pre-Study Validation section of this report.

- *Typical Batch Structure*

Samples are designated in the run sheet table by a three digit code separated by commas consisting of *subject number, sampling time(hr),period*

23-Jun-1997 HPLC2 Run Sheet

Project	: 36/96\AHP02						
Operator	: A.D. de Jager	Date Extracted	: 23-06-1997	Date Injected	: 23-06-1997	Subjects	: POOLED PLASMA
Period	: TR A & B	Plasma Set	: POOL				
Analytical Pump	: HP 1100	No	: DE 52700182	Flow Rate	: 1.0 ml/min	Pressure	: 56 bar
Column	: LiChrospher	Serial No.	: 310	Program (Y/N)	: N	MP Prep date\Batch	: 9-06-1997
Auxilliary Pump	: -	No	: -	Flow Rate	: -	Pressure	: -
Column	: -	Serial No.	: -	Program (Y/N)	: -	MP Prep date	: -
Switch Valve No.	: -	Time 1	: -	Time 2	: -	Solvent Select No	: -
A %	: -	B%	: -	C%	: -	D%	: -
Program (Y/N)	: -	Column Heater No.	: -	Temperature	: -		
Integrator\AD Box	: -	Channel No.	: -	Chart Speed	: -	Attenuation	: -
Input Voltage	: -	Run Time	: -				
Auto Injector	: HP 1100	No.	: DE 54900636	Inject Volume	: 10 ul	Runtime	: 7 min
Wash Solution	: Mob. phase	Program (Y/N)	: N				
Ultraviolet Det	: HP 1100	No.	: JP 64201933	Wavelength	: 280 nm	Range	: -
Rise Time	: -	Output Voltage	: 220 V	Program (Y/N)	: N		
Fluorescence Det	: -	No.	: -	Wavelength	: -	PMT Voltage	: -
Attenuation	: -	Range	: -	Response	: -	Output Voltage	: -
Program (Y/N)	: -	Misc	: -				

Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF
1 SYS		21 50,7.0,1		41 50,48.0,1		61		81		101		121		141	
2 50,0,1		22 50,7.0,2		42 50,48.0,2		62		82		102		122		142	
3 50,0,2		23 50.8.0,1		43 STD G		63		83		103		123		143	
4 50,1.5,1		24 50.8.0,2		44 QC F		64		84		104		124		144	
5 50,1.5,2		25 STD J		45 50,72.0,1		65		85		105		125		145	
6 BLANK		26 QC F		46 50,72.0,2		66		86		106		126		146	
7 STD B		27 50,10.0,1		47 QC E		67		87		107		127		147	
8 QC A		28 50,10.0,2		48 50,96.0,1		68		88		108		128		148	
9 50,3.0,1		29 QC D		49 50,96.0,2		69		89		109		129		149	
10 50,3.0,2		30 50,12.0,1		50 50,120,1		70		90		110		130		150	
11 STD C		31 50,12.0,2		51 50,120,2		71		91		111		131		151	
12 50,4.0,1		32 50,14.0,1		52 QC B		72		92		112		132		152	
13 50,4.0,2		33 50,14.0,2		53 STD C		73		93		113		133		153	
14 50,5.0,1		34 STD E		54 50,144,1		74		94		114		134		154	
15 50,5.0,2		35 QC D		55 50,144,2		75		95		115		135		155	
16 STD H		36 50,18.0,1		56 STD B		76		96		116		136		156	
17 STD K		37 50,18.0,2		57		77		97		117		137		157	
18 50,6.0,1		38 QC E		58		78		98		118		138		158	
19 50,6.0,2		39 50,24.0,1		59		79		99		119		139		159	
20 STD I		40 50,24.0,2		60		80		100		120		140		160	

Samples loaded and System Checked by : Date :
Sample Position Verified by : Date : 24-06-1997
Approval : Date :
Notes :

- *Calculation of Results*

Results are calculated using the PhIRSt[‡‡] chromatographic data reporting package. Peak heights/areas are electronically read automatically from the report files generated by Hewlett-Packard HPLC^{2D} ChemStation. Data are automatically summarised, calibration curves calculated according to pre-set regression equations and concentrations interpolated by the program. Results are presented in printed ordered tables with performance statistics per batch and later summarised to give overall study statistics. This package has been validated in Canada by the manufacturer to FDA requirements.

[‡‡] *Product of Phoenix International Life Sciences, Montreal, Canada.*

- *Inter-day Accuracy and Precision*

Accuracy is measured as % bias and precision is measured as coefficient of variation (CV%)

Back Calculated Calibration Standards Concentrations

Curve code	STD B	STD C	STD E	STD G	STD H	STD H	STD J	STD K
Nominal Conc (ug/ml)	**0.070**	**0.140**	**0.562**	**2.24**	**4.46**	**8.98**	**18.0**	**36.1**
AHP03	0.070 0.069	0.143 0.137	0.557	2.21	4.64	9.03	17.20	36.70
AHP04	0.077 0.063	0.142 0.141	0.540	2.15	4.50	9.35	17.20	38.20
AHP05	0.073 0.068	0.137 0.142	0.544	2.17	4.48	9.49	18.00	35.90
AHP06	0.063 0.078	0.128 0.148	0.559	2.24	4.51	8.82	17.60	37.90
AHP07	0.067 0.072	0.141 0.146	0.556	2.23	4.55	8.66	17.30	37.10
AHP08	0.069 0.07	0.133 0.153	0.532	2.29	4.52	8.73	17.70	37.30
AHP09	0.065 0.082	0.122 0.132	0.543	2.21	4.66	9.29	18.40	37.30
AHP10	0.078 0.071	0.121 0.124	0.534	2.26	4.64	9.20	18.50	38.40
AHP11	0.069 0.069	0.143 0.148	0.525	2.19	4.41	8.79	18.40	38.00
AHP12	0.063 0.08	0.134 0.136	0.558	2.24	4.52	9.05	18.00	37.50
AHP13	0.069 0.071	0.141 0.139	0.558	2.20	4.52	8.97	18.10	36.50
AHP14	0.068 0.073	0.13 0.147	0.535	2.29	4.46	9.19	18.00	36.50
AHP15	0.081 0.072	0.115 0.113	0.580	2.24	4.64	9.17	19.20	37.10
AHP16	0.072 0.074	0.121 0.135	C	2.21	4.67	9.44	18.10	36.00
AHP17	R 0.076	0.131 0.125	0.565	2.24	4.63	9.00	18.10	37.30
Mean **CV%** **N** **%Nom**	0.0714 7.2 29 102.0	0.1349 7.6 30 96.4	0.5496 2.9 14 97.8	2.225 1.8 15 99.3	4.551 1.9 15 102.0	9.079 2.8 15 101.1	18.00 2.9 15 100	37.18 2.0 15 103.0

R = Rejected

C = Poor Chromatogram

- *Quality Control Results*

Curve code	QC A	QC B	QC D	QC E	QC F
Nominal Conc (ug/ml)	**0.083**	**0.165**	**0.33**	**17.3**	**30.4**
AHP03	B	0.164	0.33 0.31	17.5 16.8	30.9 29.7
AHP04	0.088	0.15	0.33 0.31	15.3 15.4	28.6 28.6
AHP05	0.083	0.144	0.30 0.31	15.6 16.2	29.6 28.5
AHP06	0.073	0.17	0.36 0.32	17.1 15.7	29.3 31.0
AHP07	0.083	0.173	0.33 0.31	16.6 16.0	30.9 29.6
AHP08	0.087	0.131*	0.30 0.31	17.1 17.4	31.9 27.9
AHP09	0.077	0.152	0.31 0.32	17.2 17.1	30.9 30.4
AHP10	0.08	0.142	0.37 0.37	16.9 17.8	31.3 31.0
AHP11	0.077	0.161	0.33 0.30	16.8 17.5	31.0 29.4
AHP12	0.077	0.151	0.31 0.31	17.6 16.6	30.0 29.6
AHP13	0.083	0.16	0.33 0.33	17.1 17.3	30.5 30.1
AHP14	0.077	0.151	0.30 0.36	17.2 17.4	30.4 29.5
AHP15	F	0.148	0.32 0.33	17.5 17.6	30.5 31.5
AHP16	0.078	0.143	0.32 C	16.7 17.3	31.0 31.5
AHP17	0.071	0.162	0.31 0.32	17.2 16.9	31.0 31.1
Mean	0.0796	0.1535	0.3216	16.89	30.22
CV%	6.5	7.5	5.8	4.0	3.2
N	13	15	29	30	30
%Nom	95.9	93.0	97.5	97.6	99.4

* = Response outside acceptance range

C = Poor Chromatogram

F = Outside range

B = Lost in Process

- *Lower Limit of Quantification*

The lower limit of quantification (LLOQ) is initially determined from the data obtained for the assayed quality controls during Pre-Study Validation, since these data often include determinations of the analyte at concentrations close to the limit of detection. The LLOQ is defined as that concentration of the analyte which can still be determined with acceptable precision (CV% < 20) and accuracy (bias < 20 %) for the purposes of the particular application. This limit is reappraised during the performance of the assay with actual clinical study samples. After all the clinical study samples have been analysed the limit of quantification is finally set at a value which is determined by the performance of the procedure with the quality controls which are processed with each batch of samples run. This is considered to be a more objective reflection of the assay performance under clinical study conditions than the validation data alone.

LLOQ: 0.070 µg/ml

Figure 21: Chromatography obtained at the LLOQ quality control

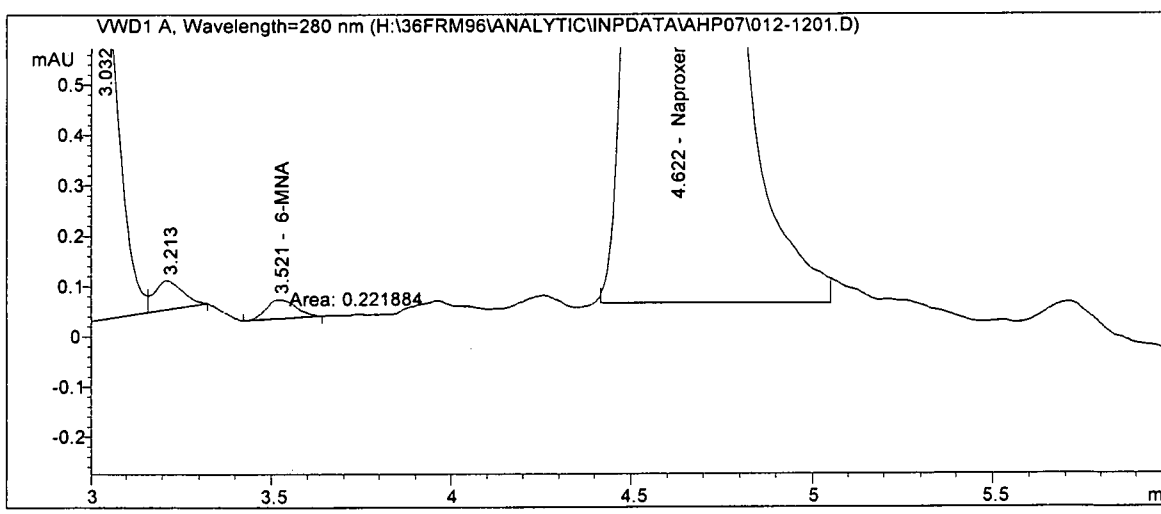


Figure 22: A study sample, 5 hours after a 1000 mg dose of nabumetone. The calculated sample concentration was 25.9 μg/ml

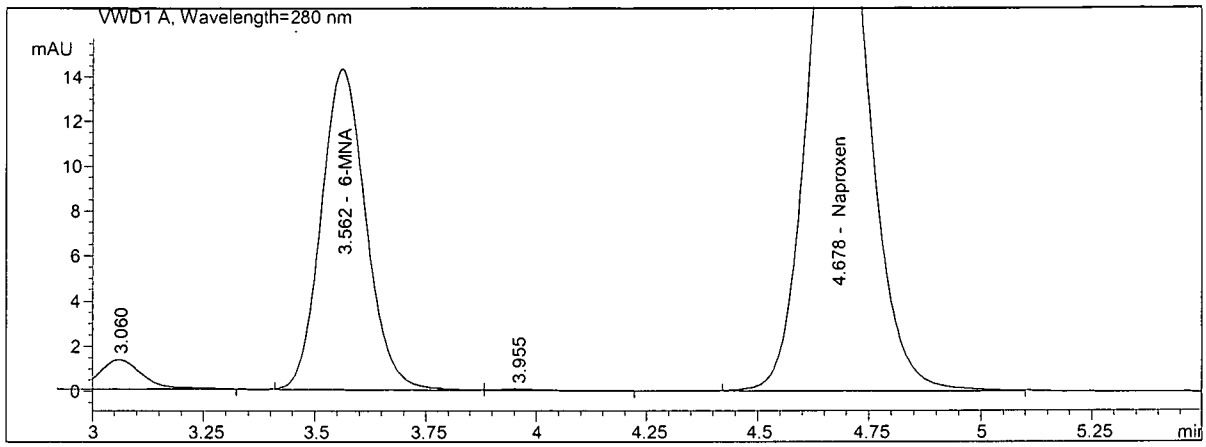
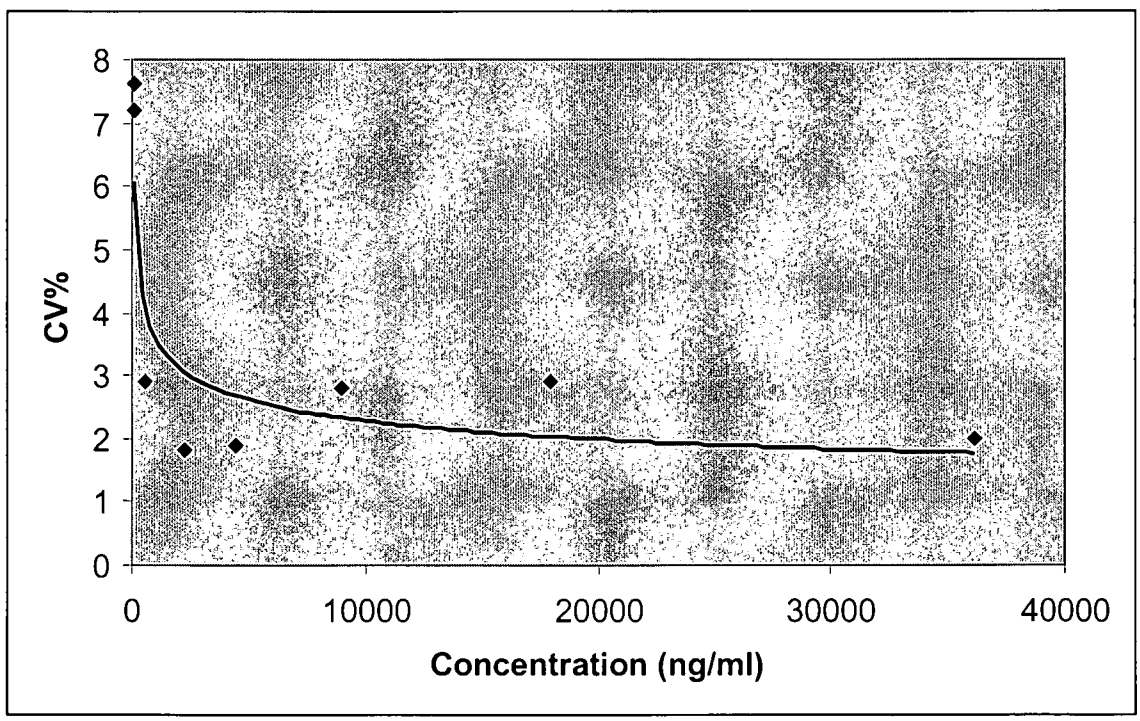


Figure shows concentration plotted against CV%. Precision was high at the upper end of the calibration range and decreased slightly as the LLOQ was approached, which is a typical pattern of such an analytical method [18].

Figure 23: Concentration versus CV% over the entire study



As a further measure of performance, the equation of the calibration line (linear with $1/c^2$ weighting) used for each batch was tabulated (Table 5). The Low CV% with respect to r, r^2 and m were interpreted by the author as an indication of method robustness, as there was little change in the calibration line from batch–to-batch.

Table 5: Summary and statistics of calibration lines used to validate and complete sample analysis.

Batch	R	r^2	Gradient (m)	Intercept (b)
Validation	0.999559	0.999117	0.018434	0.000272
1	0.997527	0.995061	0.017947	0.000089
2	0.999272	0.998545	0.018366	0.000166
3	0.997062	0.994132	0.017228	-0.000005
4	0.999231	0.998463	0.018822	-0.000214
5	0.998548	0.997099	0.017347	-0.000296
6	0.995189	0.990401	0.018943	-0.000422
7	0.995552	0.991123	0.019908	-0.000598
8	0.998959	0.997919	0.020427	0.000271
9	0.997044	0.994097	0.020562	0.000072
10	0.999893	0.999786	0.020753	-0.000032
11	0.998820	0.997641	0.020551	-0.000316
12	0.991642	0.983355	0.019704	-0.000210
13	0.997193	0.994393	0.019813	0.000186
Sundry repeats	0.997853	0.995711	0.020209	0.000023
N	15	15	15	15
Mean	0.9975563	0.9951229	0.0192676	-0.0000706
CV%			6.2	

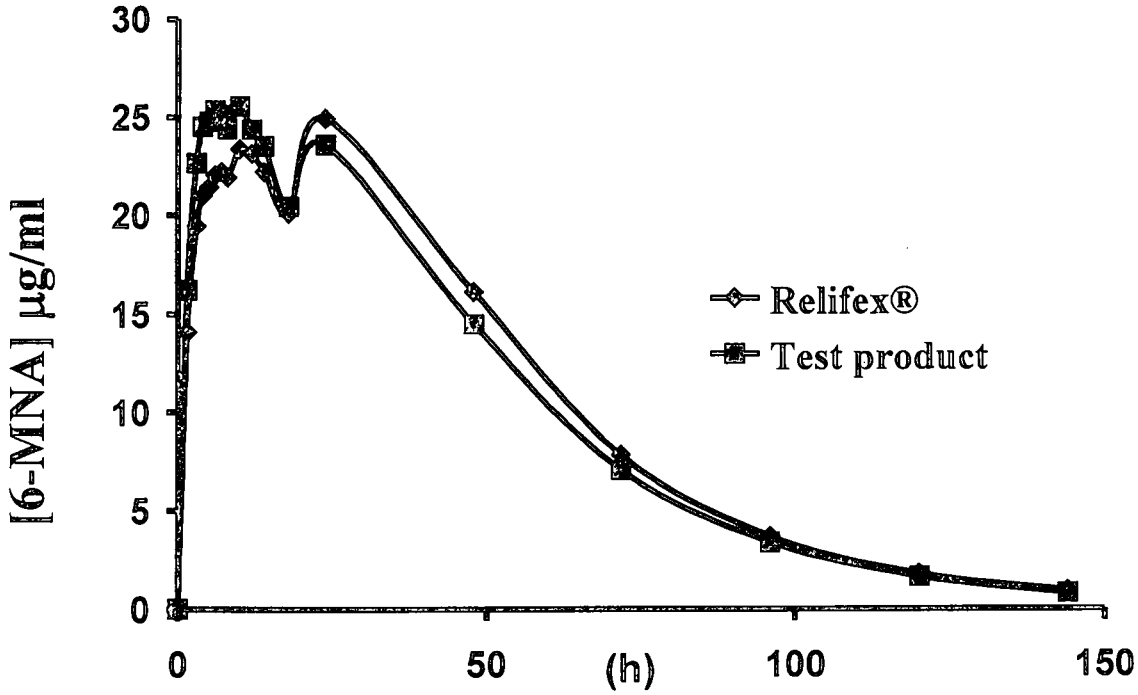
Of the 1044 samples assayed, a total of 8 samples required re-assaying (see section 3.5). These repeat samples were assayed in a single repeat batch, which included a full set of calibration standards and quality controls. This amounts to 0.77% of the samples requiring repetition (see Table 6).

Table 6: Summary of samples re-assayed

Reason for re-assay	Number	% of total
Sample lost in extraction process	1	< 0.1
Poor chromatography	0	0
Pharmacokinetic outlier	5	< 0.5
Sample outside of calibration range	2	< 0.2

The study samples that were assayed during the study were used to generate plasma concentration versus time profiles and used to calculate pharmacokinetic parameters required to ascertain oral bioavailability between the two formulations. These parameters include C_{max} T_{max} (time to maximum concentration), $t_{1/2}$ and $AUC_{(0-\infty)}$ (area under curve extrapolated to infinity).

Figure 24: Mean profiles of test and reference product (Relifex®) following a 1000mg oral dose of nabumetone

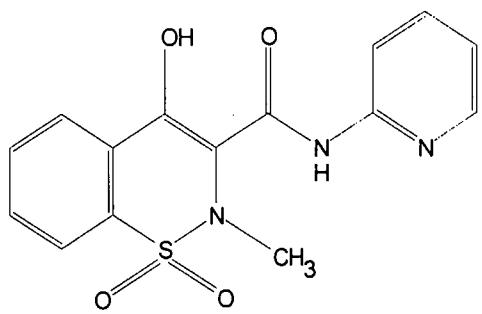


5 Assay method development - Piroxicam

5.1 Background

Piroxicam [4-hydroxy-2-methyl-*N*-(2-pyridyl)-*2H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide] is a non-steroidal anti-inflammatory drug (NSAID), which has been used chiefly to treat various arthropathies and inflammatory diseases in humans [25].

Figure 25: Piroxicam



A request to quantify piroxicam in plasma, sub-cutaneous tissue (SCT), synovial capsule (SC) and synovial fluid (SF) after repeated topical application of a gel formulation to the knee prior to surgery gave rise to the development of this interesting assay procedure. In the seven days prior to knee surgery, 8 g of a gel containing 40 mg piroxicam was applied twice daily to the knee for 7 days and on the eighth day, surgery was performed.

An analytical method was developed and validated and approximately 160 unknown samples and 130 calibration standards and quality controls were processed in order to generate the data required by the sponsor.

The analytical work, as well as certain aspects of the data generated were prepared and submitted for publication, in consultation with the sponsor. The paper was submitted in

October 1998 and published in revised form in the Journal of Chromatography B, 729 (1999) 183 - 189.

5.2 Summary of analytical literature survey

Owing to the nature of the study, maximal sensitivity and a means of extracting the analyte from solid samples were identified as the two most important features to search for in the analytical literature.

A survey of the literature available at the time revealed that a study of this exact nature had not yet been attempted. A fair number of HPLC methods was described [26, 27, 28, 29, 30, 31] for the determination of piroxicam in biological samples using UV detection. Even though UV detection is not optimal with respect to sensitivity, this is a good detection modality for the quantitation of piroxicam in plasma, owing to the fact that it is sufficiently sensitive to detect levels associated with common oral dosage regimens.

Hundal *et al.* [32] describe a solid phase extraction procedure, followed by UV detection for the quantitation of piroxicam in plasma and synovial fluid, and used the ratio between plasma and synovial fluid concentrations to draw clinical conclusions. Although this publication seemed to point to the fact that SPE would be a suitable extraction modality, the problem lay in the fact that sub- cutaneous tissue and synovial capsule are solids, unlike plasma and synovial fluid, which are liquids. In an attempt to widen the base of analytical literature consulted, some literature dealing with the determination similar compounds in biological fluids was consulted. Radhofer-Welte and Dittrich [33] published a paper describing the determination of lornoxicam in plasma and synovial fluid, using off-line solid phase extraction. In the above-mentioned work, it was only possible to prepare a calibration

line in plasma, and then to quantify the synovial fluid using the said calibration line. This was due to the difficulty associated with preparing calibration standards and quality controls in a matrix that is particularly difficult to obtain and it was clear that FBSD would probably have to adopt a similar approach with the piroxicam study. Mason and Hobbs [34] reported on a method for the determination of tenoxicam in human plasma. This paper was consulted to get insight into possible mobile phases and appropriate HPLC columns. Kazemifard and Moore [35], in a paper dealing with relative sensitivities of detection modes, suggested that electrochemical detection (ECD) would be preferable to the commonly used UV detection, if sensitivity was to be the foremost consideration.

5.3 Formulation of analytical strategy based on analytical literature

- *Extraction*

Due to the nature of the study it was clear that a very sensitive assay method would have to be developed. This meant that the most sensitive detection mode available to us would have to be used. Since it was clear that electrochemical detection would be the detection mode of choice the most important requirements of the extraction procedure were that it should be efficient and that it should yield an exceptionally clean extract.

Based on the analytical literature consulted, it was decided from the outset to try to optimize a liquid-liquid extraction procedure for all four matrices. This was further justified by the fact that very few samples would be generated during this study. For the solid tissue samples, it would be probably be necessary to soak samples in an appropriate solution and then

homogenize them. Owing to the fact that blank SF, SCT and SC are not easily obtained, most of the initial method development work would have to be done on plasma. Once a reasonable procedure had been developed for plasma, this would serve as a starting point for the remaining three matrices. It was further reasoned that SF, SC and SCT were in all likelihood 'cleaner' matrices with respect to endogenous compounds, and that if chromatographic resolution from endogenous plasma components could be obtained it would, in all probability, be suitable for the remaining three matrices.

- *Chromatography*

As many authors had achieved good chromatography when using reversed-phase chromatography (C_{18}), this was seen as a starting point for chromatographic optimization. Furthermore, acidic buffers (pH in the region of 3.5) together with organic modifiers (either methanol or acetonitrile) were typical mobile phases used to separate piroxicam and chemically similar compounds.

- *Detection*

The work done by Kazemifard and Moore [35] suggested that amperometry should be the choice in detection, chiefly due to the fact that high sensitivity was a priority with this particular study.

5.4 *Excecution of method development - piroxicam*

The following section is a summary, together with explanations where necessary, of the assay method development process:

- *Preparation of stock solutions*

A spiking solution of piroxicam was prepared by dissolving 2.251mg piroxicam reference material (supplied by the sponsor) in 9.704g methanol. This produced a spiking solution of ca. 183.5 μg/ml which was stored at 4°C pending use.

A response standard was prepared by spiking 10 μl of the piroxicam spiking solution into 15 cm^3 of the starting mobile phase, of which the concentration was 122.2 ng/ml.

Furthermore, solutions of a number of possible internal standards were prepared by dissolving reference material in methanol and is tabulated below (these solutions were all kept at 4°C until used.

Table 7: Solutions of possible internal standards prepared in methanol

Compound	Mass of reference material weighed (mg)	Mass of methanol (μg/ml)	Concentration (μg/ml)
Ketoprofen	1.647	12.018	110.2
Eltenac	1.848	12.094	120.9
Flurbiprofen	0.621	8.056	60.97
Ibuprofen	0.847	8.059	83.13
Fenoprofen	1.494	14.564	81.14
Diclofenac	0.911	8.061	89.39
Fenclofenac	2.824	14.613	152.9

- *Preparation of mobile phase*

At the outset of method development, the following mobile was prepared as follows:

A 0.015M H_3PO_4 solution prepared, and 670ml of this solution was added to 330 ml methanol. To this mixture was added 150mg KCl (which is necessary for the functioning of a electrochemical detector). This mobile phase was then adjusted to pH 4.03 using 4N NaOH, and stored at 4°C until used.

- *Instrumentation and chromatographic conditions*

An HPLC instrument stack was set up which included an autosampler (Hewlett-Packard series 1050), an analytical pump (Hewlett-Packard series 1100) and an electrochemical detector (Hewlett-Packard series 1049A). The system was fitted with a Higgins Haisil 120 BD C_{18} , 120 × 3.0 mm ID., 5μm analytical column. The mobile phase was pumped through the entire system overnight in order to allow the system equilibrate (1.0 ml/min). During this time, the oxidation potential of the electrochemical cell was maintained at 0.8V.

- *Chromatography*

In order to assess the chromatography using the starting mobile phase, a solution of piroxicam in the mobile phase (ca. 50 ng/ml) was prepared and 20 μl injected onto the column. This produced a fairly symmetrical peak that eluted at ± 3.1 min.

- *Sample preparation*

In order to perform extraction method development, the following plasma samples were prepared:

1. Blank plasma (collected 28/11/1997)
2. Blank plasma (collected 27/06/1997)
3. Blank plasma (collected 08/07/1997)
4. 373 ng piroxicam/ml plasma (prepared in plasma collected 08/07/1997) – ISTD Ketoprofen
5. 373 ng piroxicam/ml plasma (prepared in plasma collected 08/07/1997) – ISTD Eltenac

6. 373 ng piroxicam/ml plasma (prepared in plasma collected 08/07/1997) – ISTD Flurbiprofen
7. 373 ng piroxicam/ml plasma (prepared in plasma collected 08/07/1997) – ISTD Ibuprofen
8. 373 ng piroxicam/ml plasma (prepared in plasma collected 08/07/1997) – ISTD Diclofenac

Liquid-liquid extraction using tert-butyl-methyl ether (TBME) was performed on 0.2ml plasma samples at two pH values, resulting in a total of 18 samples. To samples 5 – 9 was added the internal standard (indicated above) to be tested (±50 ng). No internal standard was added to the first three samples. The procedures tested were as follows:

Extraction procedure - 1

200 µl plasma + 100 µl 0.1M HCl + 3 ml TBME. Vortex the sample for 1 minute and centrifuge at 1300G (1 min). Freeze the aqueous layer and collect the TBME in a fresh ampoule. Evaporate the sample to dryness and reconstitute the sample in 200 µl mobile phase.

Extraction procedure – 2

The second procedure was identical to the first, with the exception that a pH 7.5 phosphate buffer was used in stead of the 0.1 M HCl.

These reconstituted extracts were injected onto the HPLC system (20µl), and the following insight was gained. Firstly, the extracted obtained with the pH 7.5 phosphate buffer adjusted samples failed to produce a piroxicam peak as piroxicam was probably ionised at this pH. This, in all probability, was the reason why piroxicam did not partition into the TBME. Secondly, the chromatograms of the reconstituted extracts obtained with the 0.1M HCl adjusted samples contained many chromatographic peaks (probably endogenous matrix compounds). It was speculated that this could either be due to the oxidation potential of the electrochemical detector being too high, or non-selectivity of the TBME extraction at the pH used.

- *Optimising the extraction procedure*

Since Kazemifard and Moore [35] found an oxidation potential of 0.9V to be suitable in their assay method, it was clear that the first approach would be to perform a back-extraction procedure, modelled on the first tested procedure, in an attempt to accomplish better sample clean-up.

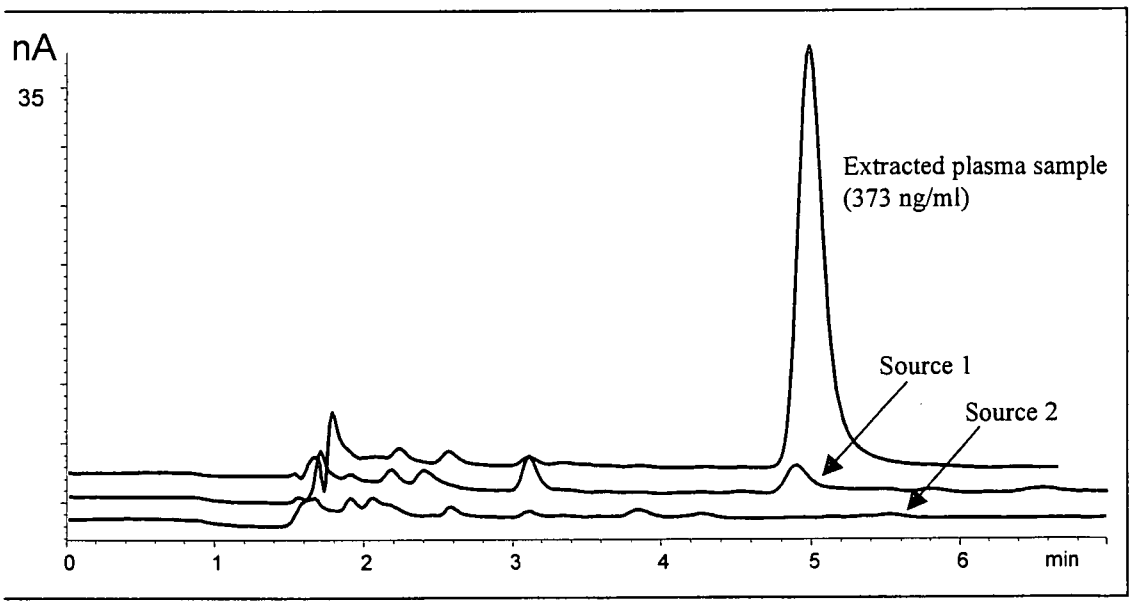
The following double back-extraction procedure was therefore performed on a 373 ng/ml piroxicam plasma standard and a blank plasma sample:

0.2ml plasma + 0.2 ml 0.1M HCl + 3 ml TBME. The sample was vortexed (1 min), centrifuged (1300G, 1 min.) and the aqueous layer frozen on a cooling plate. The TBME was decanted into a fresh 5ml ampoule, and 0.1M NaOH added (0.2 ml). The sample was vortexed (1 min), centrifuged (1300G, 1 min.) and the aqueous layer frozen on a cooling plate and the organic phase (which no longer contained the piroxicam) was discarded. To the

remaining aqueous phase was added 0.1M HCl (0.5ml) which once again rendered the aqueous phase acidic. 3ml TBME was added and the sample vortexed (1 min), centrifuged (1300G, 1 min.) and the aqueous layer frozen on a cooling plate. The organic phase was then decanted into a fresh ampoule, and evaporated to dryness under a gentle stream of nitrogen. The sample was reconstituted in mobile phase (0.2 ml) and 20 µl injected onto the HPLC column.

The chromatograms obtained were considerably 'cleaner'. However, when the blank sample was overlaid with the 373 ng/ml sample, it was clear that there was a still a measure of interference at the retention time of piroxicam, resulting from endogenous plasma components. This was only present in one of two the blank plasmas tested.

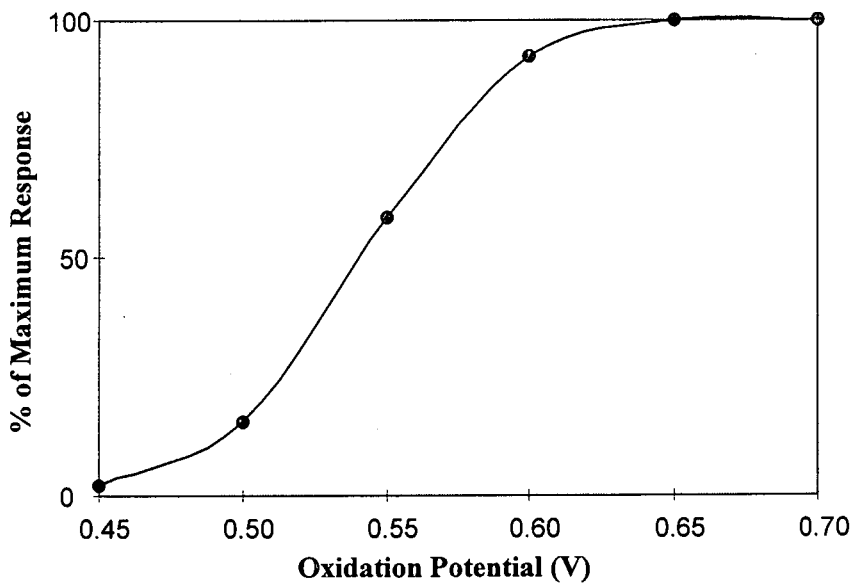
Figure 26: Overlaid chromatograms of a piroxicam standard (373ng/ml) extracted using a double-back extraction procedure, and two blank plasmas obtained from different sources.



- *Optimising chromatography and detector conditions*

In an attempt to remedy this, the mobile phase was adjusted to pH 4.6 with a view to affecting resolution from the interfering peak. Furthermore, seven instrumental methods were prepared and saved on the computer workstation operating the HPLC instruments. These methods were all identical to the one that was being used, but each monitored at different oxidation potentials. These potentials were 0.45, 0.50, 0.55, 0.60, 0.65, 0.70 and 0.75 V. 5 µl Of the extracted plasma sample was injected seven times, each at a different oxidation potential.

Figure 27: Voltamogram obtained from injection of extracted plasma samples at various oxidation potentials



When using an electrochemical detector, the ideal oxidation potential is the minimum potential that provides a stable signal. From Figure it is clear that 0.65V is the most suitable

oxidation potential, as a potential below is still on the slope, while a potential above 0.65V will result in unnecessary noise. This is owing to the fact that the higher the oxidation potential, the greater the chance that compounds other than the analyte (and particularly those not eliminated by the extaction procedure) will undergo oxidation.

Figure 28: Overlaid chromatograms of piroxicam at various oxidation potentials

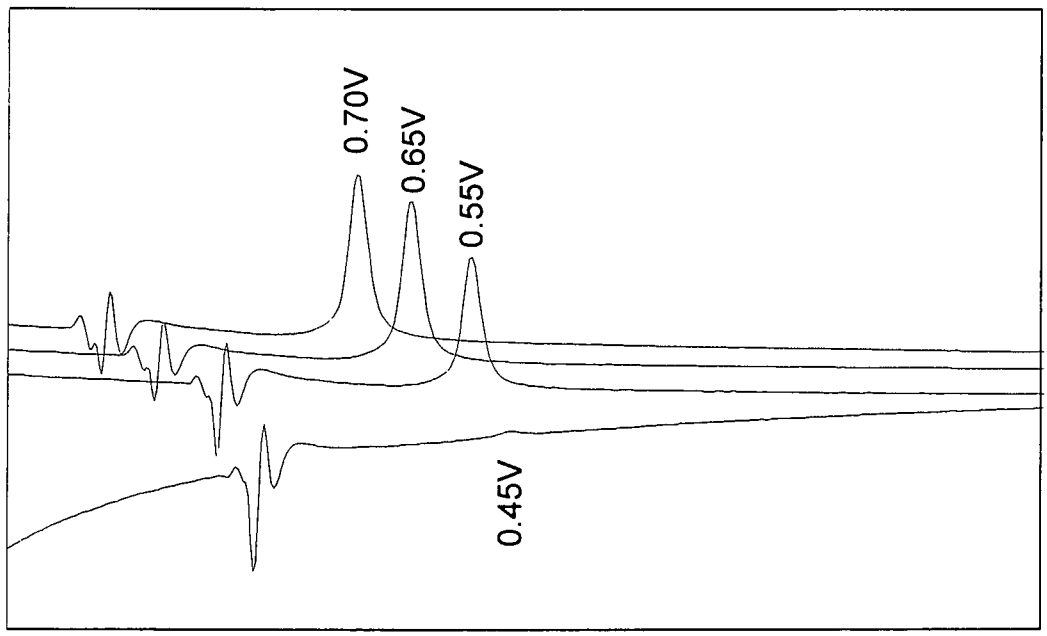
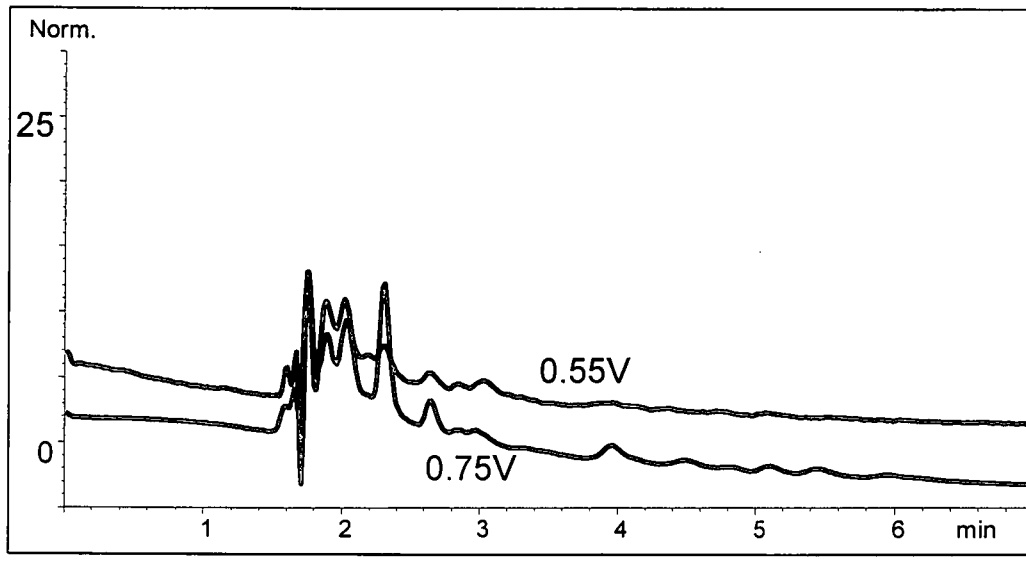


Figure 29: Overlaid chromatograms of a single plasma source extracted using the double back-extraction procedure with TBME.



- *Optimising chromatography and extraction*

Several iterative changes in chromatographic conditions were made and the extraction solvent changed to a mixture of hexane:dichloromethane (4:1) to try to resolve the problem of the interfering endogenous components. Thus the following mobile phases were tried:

a) *H_3PO_4 (0.015 M) + methanol + acetonitrile (440 + 400 + 200 v/v) + 150 mg KCl and pH adjusted to 3.54 with 4 N NaOH*

b) H_3PO_4 (0.015 M) + methanol + acetonitrile (430 + 320 + 25 v/v) + 120 mg KCl without pH adjustment

c) H_3PO_4 (0.015 M) + methanol + acetonitrile (440 + 400 + 200 v/v) + 150 mg KCl and pH adjusted to 3.75 with 4 N NaOH

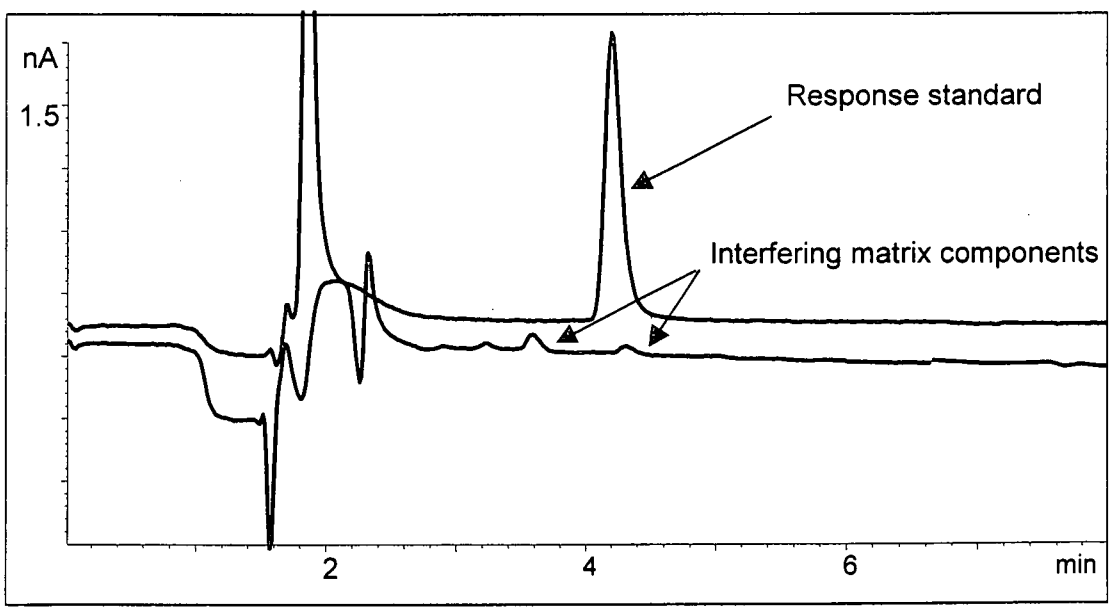
d) H_3PO_4 (0.015 M) + methanol + acetonitrile (440 + 400 + 200 v/v) + 150 mg KCl and pH adjusted to 320 with 4 N NaOH

e) H_3PO_4 (0.015 M) + methanol + acetonitrile (440 + 350 + 200 v/v) + 150 mg KCl and pH adjusted to 3.20 with 4 N NaOH

The type of column which up to now had been a Higgins Haisil 120 BD C_{18}, 120 × 3.0 mm, 5 µm particle size stainless steel column was replacedby a PhaseSep®Spherisorb, C_{18}, ODS B, 150 × 4.6 mm, 5 µm particle size stainless steel column and several of the mobile phases adjusted but all to no avail.

Again several mobile phase conditions were tried on the original Haisil column with tetrahydrofuran (THF) as additional modifier and a higher ionic strength buffer:

Figure 30: Response standard overlaid with a freshly extracted blank

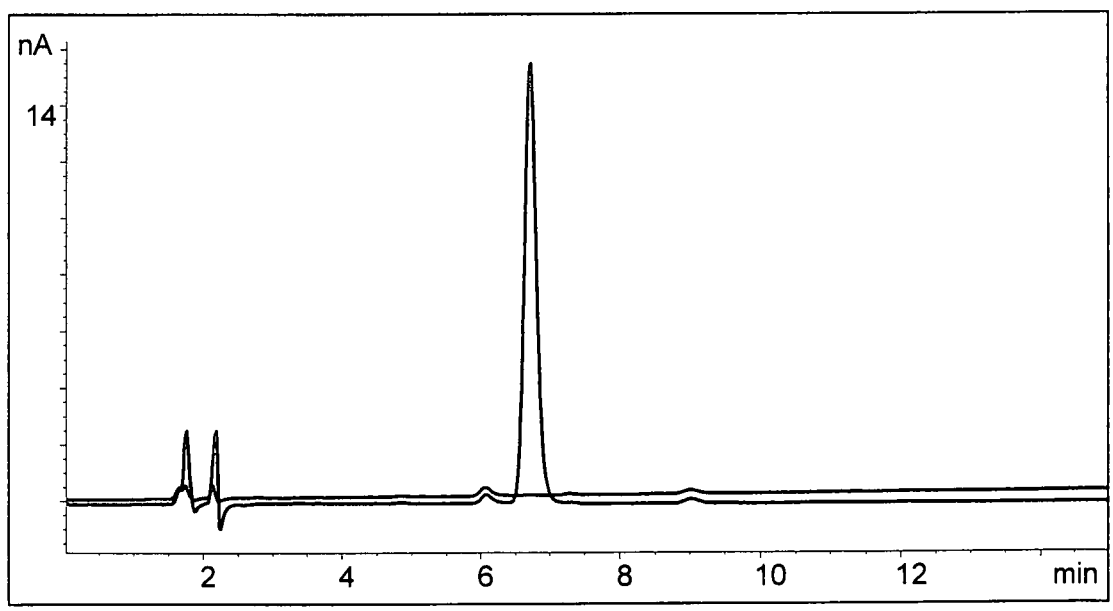


Following injection of a response standard and an extracted blank plasma extract, the following mobile phase was used to ascertain interference:

H_3PO_4 (0.030 M) + methanol + THF (600 + 320 + 80 v/v) + 150 mg KCl and pH adjusted to 2.42 with 4 N NaOH

The response standard together with the blank plasma extract was then re-injected with this newly adjusted mobile phase (Figure 31).

Figure 31: Blank plasma extract overlaid with response standard showing complete resolution between piroxicam and endogenous plasma compounds



Following the successful separation of piroxicam from plasma components, it was felt that the next step was to investigate the chromatography that would result from the remaining three matrices.

- *Sample preparation of SC and SCT*

As an initial approach, it was decided that tissue samples would have to be soaked in NaOH and ultrasonicated for a full hour before an extraction of any sort could be attempted. A further complication was introducing piroxicam into the solid matrices. As a compromise, the tissue samples were very briefly dipped in an aqueous solution of piroxicam, and in so doing, an unspecified amount of piroxicam would be present on the tissue sample to be extracted (this sample was not rinsed before preparation). Following this, approximately 0.2g of a SC and SCT sample that was obtained for method development (kindly supplied by the UOFS medical faculty), was placed in a polypropylene tube containing 1ml NaOH (0.1M), sealed with parrafilm®, and ultrasonicated for a full hour. This sample was then homogenised for 1 minute at high speed using a T 25 Ultra Turrax fitted with an IKA® UT disperser (O.D. 8mm). This produced an homogenate of slightly higher viscosity than plasma. As was the case with the plasma extraction, 0.5 ml of this aqueous phase was then acidified (using 200µl 1M HCl to override the 0.1M NaOH that had already been added to the sample) and 3 ml of the organic extraction solvent (hexane : dichloromethane, 1:4) added. From this point on, the sample was treated as were the plasma extracts, applying the same double-back-extraction procedure described.

- *Sample preparation of SF*

In the method development phase, SF (which had been spiked with piroxicam) was diluted 1:1 with 0.1M NaOH and also ultrasonicated for an hour. At this point, 250µl of this diluted SF was acidified using 200µl 1.0M HCl (it was only possible to use 250µl of the diluted SF as very little could be obtained for the purposes of method development). Following this, 3 ml of the extraction solvent (hexane : dichloromethane, 1:4) was added and the rest of the double-back-extraction procedure was applied, as described.

Inspection of the resulting chromatograms (Figure 32, 33 and 34) revealed piroxicam to be separated from endogenous matrix components, as was now the case with plasma, using this newly optimised mobile phase. As was speculated earlier, blank extracts from SC and SCT were indeed far 'cleaner' than plasma extracts. Notably, the SF extract was very similar to the plasma extract with respect to endogenous components being detected.

Figure 32: Blank SC extract overlaid with the extract of SC that had been dipped in a solution of piroxicam

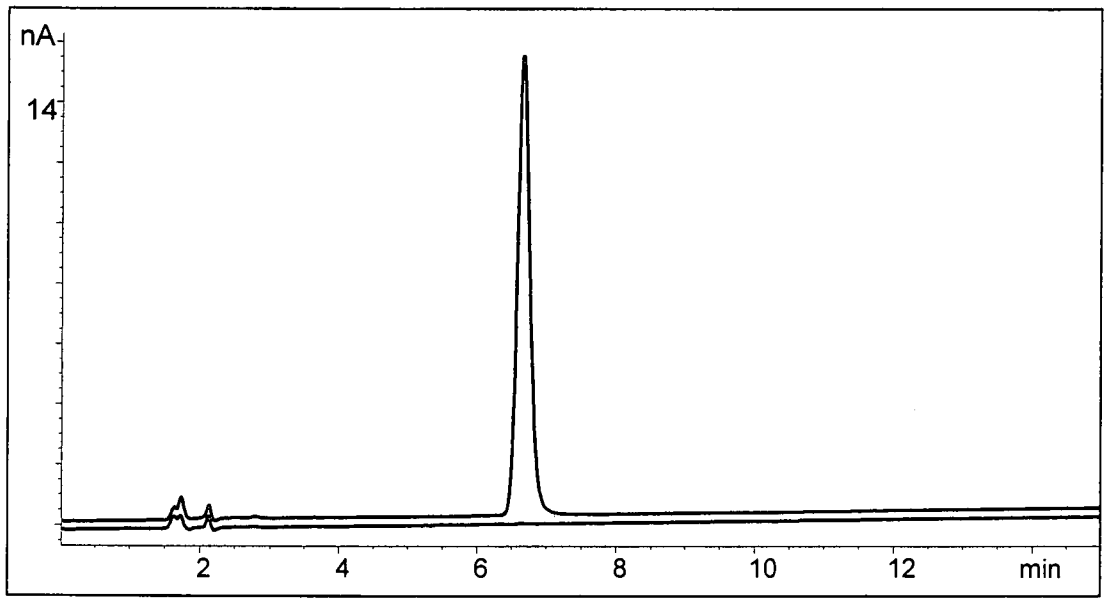


Figure 33: Blank SCT extract overlaid with the extract of SC that had been dipped in a solution of piroxicam

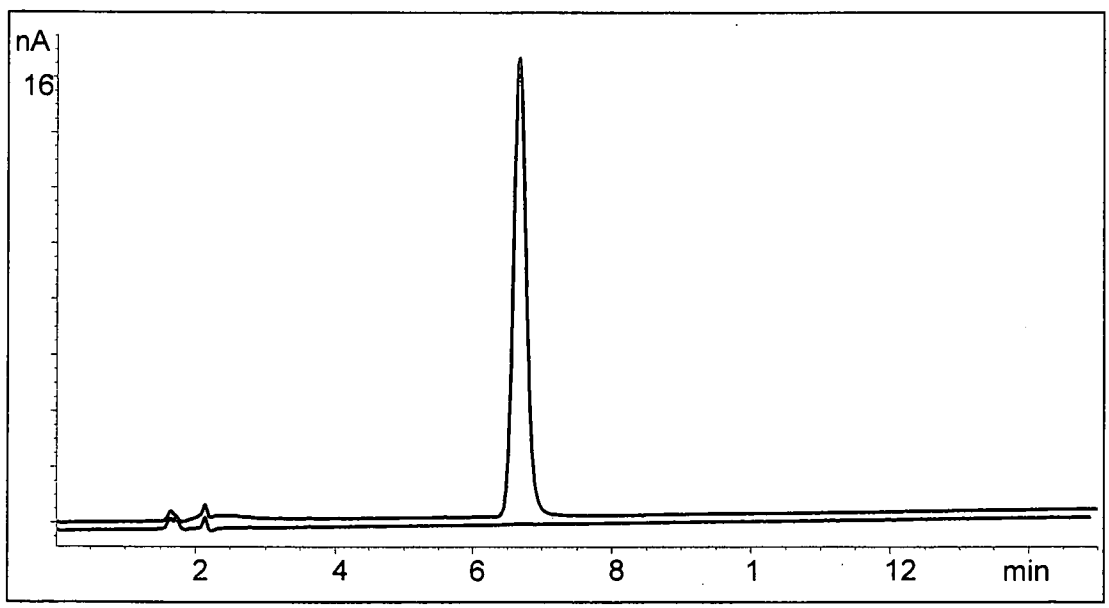
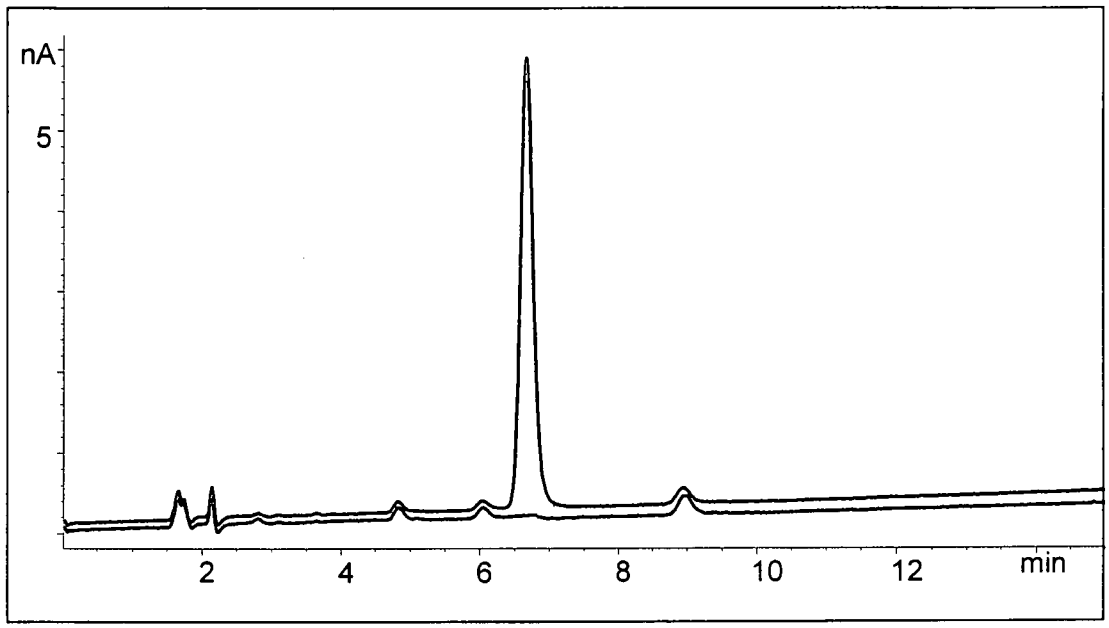


Figure 34: Blank SF extract overlaid with the extract of spiked SF



5.5 Assay method validation

5.5.1 Preparation for assay method validation

As mentioned previously, it was decided that calibration standards and quality controls would only be prepared in plasma as a surrogate for the matrices because of the difficulty of obtaining sufficient tissue to prepare calibration standards and quality controls. The protocol of the study was such that each individual's SF, SC and SCT piroxicam concentrations was to be compared with his or her own plasma piroxicam concentrations. Although it is clearly not ideal to quantify samples using a calibration line prepared in a different matrix, there was no choice. This is supported by the fact that other authors have found it necessary to follow similar procedures [33].

- *Screening of blank plasma*

Blank plasma collected on the following dates was tested:

1. 12/11/97
2. 05/05/97 – caffeine free
3. 21/01/98

Of these three blank plasmas, 21/01/98 exhibited some interference. It was speculated that the reason for this could be that topical formulations of piroxicam are commonly used by active young males, particularly those actively participating in sport. As this fits the profile of the blood donors most commonly used by FBSD, it was not inconceivable that the interference was in fact piroxicam, or at least a similar compound. This, however, was not investigated further and the two plasma sources that did not exhibit interference were set aside to prepare calibration standards and quality controls.

- *Determination of recovery*

At this stage, it was necessary to determine the recovery of analyte from the four matrices. At this point, synovial fluid was diluted 1:1 with 0.1M NaOH to form a homogenate, which was then extracted as if it were plasma. In an attempt to determine recovery, this homogenate was spiked to a known piroxicam concentration, extracted in triplicate and then compared to an appropriate response standard. This experiment revealed that the recovery was approximately 60%, but that recovery from the homogenate was not reproducible.

A subsequent discussion with the laboratory director led to the conclusion that the irreproducibility was probably due to the fact that the homogenate was far more viscous than was plasma. This immediately led the analyst to suspect that the irreproducibility was probably due to differences in the vortexing characteristics.

The immediate conclusion was that the SF would have to be further diluted in order to get reproducible recovery. It was clear that recovery experiments would have to be repeated at a later stage.

Meanwhile the following approach was fixed upon for handling the SC and SCT samples:

1. The tissue sample would be weighed (and trimmed if necessary) to obtain a sample as close to 0.2g as possible (exact mass not required).
2. To the sample would be added 0.1M NaOH so that the mass of sample (g) to volume NaOH added (ml) ratio would be exactly 1+5 (m/v).
3. The sample would then be sealed with parrafilm and ultrasonicated for a full hour.
4. The sample would then be homogenised (at high speed) using an Ultra Turrax.
5. The sample would be transferred to an eppendorf microfuge tube and centrifuged at high speed (8500g) for 5 minutes in order to separate discreet layers.
6. 0.5 ml of the aqueous layer was then transferred to a 5 ml amber ampoule, acidified with 1M HCl (0.2ml). The aqueous layer was found beneath a supernatant layer of congealed matter (cream in colour) and a layer of underlying protein. The rest of the extraction was performed as per the double back-extraction procedure fixed upon in the earlier stages of method development.

In the above procedure, a reasonably high dilution ratio (1+5) was chosen in an attempt to try to mimic the viscosity of plasma as much as possible. The reason behind this was the initial recovery experiments with SF seemed to suggest that a lower homogenate viscosity was desirable. In the case of SF, the following revised procedure was fixed upon:

1. Prior to pipetting, the sealed sample would be ultrasonicated for 20 minutes, rendering it more manageable when using an air-interface pipette.
2. To 0.2ml SFwould be added 0.1M NaOH (1ml).
3. The sample would then be sealed with parrafilm® and ultrasonicated for a full hour.
4. The sample would then be briefly vortexed to homogenise.
5. 0.5 ml of the homogenate would then be transferred to a 5mlamber ampoule, acidified with 1.0M HCl (0.2ml) and the rest of the extraction performed as per the double back-extraction procedure fixed upon in the earlier stages of method development.

Now that an extraction procedure had been fixed upon for SF, SC and SCT, it was decided to repeat the recovery experiments. The recovery was determined at a single concentration only (owing to the poor availability of tissue for method development) by spiking homogenates of SC, SCT and SF to known concentrations. These homogenates were then extracted as described above, and compared to a response standard prepared in mobile phase in order to calculate preliminary analyte recovery (Table 8). This was later confirmed during the pre-study method validation.

Table 8: Preliminary recoveries from matrix homogenates

Matrix	Concentration	Mean recovery n = 3 (%)
SCT homogenate	454	64.9
SC homogenate	454	57.8
SF homogenate	127	67.8

5.5.2 Preparation of Calibration Standards and Quality Controls

Calibration standards were prepared in plasma by the analyst who performed the assays on the trial samples by preparation of a stock solution in a suitable solvent and spiking a pool of normal plasma which was serially diluted with normal plasma to attain the desired concentrations. All volumetric operations were performed by weighing and the masses of plasma were converted to volumes when calculating concentrations. Quality controls were prepared in plasma by an analyst other than the analyst who performed the study assays, by the same method as used for the calibration standards. The calibration standards were aliquoted into tubes and stored under the same conditions as the trial samples; approximately -20 °C and normally in the same freezer.

- *Calibration standards*

Preparation of Stock Solution SA for Spiking STD L:

Solvent used	SG Solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume Spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	3.244	13.524	17.097	100	189.74

Preparation of Calibration Standards:

Sample Code & No.	Source Solution	A	B	C	D ng/ml
STD N	*Stock SA*	103.390	135.770		600
STD M	*STD N*	81.110	85.900	99.780	446

Preparation of Stock Solution SB for Spiking STD J:

(Dilution of Stock Solution SA)

Solvent used	SG Solvent	Mass Stock SA (g)	Mass solvent (g)	Volume solvent (ml)	Volume Spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	3.478	2.826	7.970	150	104.68

Preparation of Calibration Standards:

Sample Code & No.	Source Solution	A	B	C	D ng/ml
STD L	*Stock SB*	114.520	168.320		299
STD K	*STD L*	112.720	130.390	165.660	199
STD J	*STD K*	115.560	120.160	134.560	151
STD I	*STD K*	119.910	140.590	161.150	99.3
STD H	*STD I*	118.090	141.070	163.950	49.5
STD G	*STD H*	110.410	136.610	154.130	19.8
STD F	*STD G*	112.380	137.420	162.390	9.91
STD E	*STD F*	126.840	156.870	186.730	4.94
STD D	*STD E*	88.610	118.720	148.760	2.47
STD C	*STD D*	125.910	150.950	176.070	1.24
STD B	*STD C*	111.700	125.780	145.040	0.71

- *Quality controls*

Preparation of Stock Solution QA for Spiking QC G:

Solvent used	SG Solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume Spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	2.342	7.205	9.109	150	257.12

Preparation of Quality Controls:

Sample Code & No.	Source Solution	A	B	C	D ng/ml
QC G	*Stock QA*	109.160	182.570		538
QC F	*QC G*	112.520	144.440	184.520	300
QC E	*QC F*	109.890	133.910	157.890	150

Preparation of Stock Solution QB for Spiking QC D:

(Dilution of Stock Solution QA)

Solvent Used	SG Solvent	Mass Stock QA (g)	Mass solvent (g)	Volume solvent (ml)	Volume Spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	1.006	13.008	17.717	20	18.46

Preparation of Quality Controls:

Sample Code & No	Source Solution	A	B	C	D ng/ml
QC D	*Stock QB*	113.620	222.750		3.47
QC C	*QC D*	112.580	124.570	160.560	2.61
QC B	*QC D*	111.210	136.220	161.240	1.74
QC A	*QC B*	118.550	135.180	151.630	0.86

Note: Mass of biological fluid (g) is converted to volume (ml).
A = Mass of empty container.
B = Mass of container + normal biological fluid.
C = Total mass of container + normal + spiked biological fluid
D = Concentration of analyte in the biological fluid

5.5.3 Processing the validation batch

The assay method development phase was now considered to be concluded and it was decided to proceed with the formal assay method validation using the following Preliminary Assay Procedure (PAP)

- *Sample preparation procedure - plasma*

    1. Thaw the plasma sample in a water bath set at approximately 37 °C for ten minutes.
    2. Vortex the sample briefly and then centrifuge at 650 *G* for five minutes.

3. Pipette sample (500 µl) into a 5 ml ampoule.
4. Add HCl (200 µl, 0.1M) to the sample and vortex briefly to homogenise.
5. Add hexane : dichloromethane ((1 + 4), 4 ml) to the sample and vortex for 1 minute.
6. Centrifuge the sample at 650 *G* for 1 minute.
7. Freeze the aqueous layer in an alcohol bath at -30 °C and decant the supernatant layer into a second 5 ml ampoule containing NaOH (200 µl, 0.1 M).
8. Vortex the sample for 1 minute and centrifuge at 650 *G* for 1 minute.
9. Freeze the aqueous layer in an alcohol bath at -30 °C and discard the organic layer. The ampoule is shaken vigorously to remove as much organic layer as possible.
10. Thaw the aqueous layer in a water bath (approximately 37 °C) for 1 minute.
11. Add HCl (500 µl, 0.1 M) to the sample and vortex briefly to homogenise.
12. Add hexane : dichloromethane ((1 + 4), 3 ml) to the sample and vortex for 1 minute.
13. Centrifuge the sample at 650 *G* for 1 minute.
14. Freeze the aqueous layer in an alcohol bath at -30 °C and decant the supernatant layer into a third 5 ml ampoule and evaporate to dryness using a Savant Speed Vac® rotary concentrator.
15. Reconstitute the sample in mobile phase (200 µl) and inject 20 µl onto the HPLC column.

- *Sample preparation - synovial capsule and sub-cutaneous tissue*

    1. Thaw the sample in a water bath set at approximately 37 °C for ten minutes.
    2. In a polypropylene ASPEC tube (12 × 55 mm), add NaOH (1 ml, 0.1 M) to sample (0.2 g). In the event of the sample not weighing exactly 0.2 g, the amount of 0.1 M NaOH added is proportionately adjusted in order to maintain a constant sample mass (g) to volume NaOH (ml) ratio of 1 : 5.
    3. Ultrasonicate the sample for 1 hour.
    4. Homogenise the samples for 1 minute at 22000 rpm using a Jankel & Kunkel T 25 Ultra-Turrax, fitted with an IKA® UT disperser (8 mm OD).
    5. Centrifuge the samples at 8500 *G* for 5 min.
    6. Pipette 500 µl of the clear aqueous layer into a 5 ml ampoule.
    7. Add HCl (200 µl, 1.0 M) to the sample and vortex briefly to homogenise.
    8. Add hexane : dichloromethane ((1 + 4), 4 ml) to the sample and vortex for 1 minute.
    9. Centrifuge the sample at 650 *G* for 1 minute.
    10. Freeze the aqueous layer in an alcohol bath at -30 °C and decant the supernatant layer into a second 5 ml ampoule containing NaOH (200 µl, 0.1 M).
    11. Vortex the sample for 1 minute and centrifuge at 650 *G* for 1 minute.

12. Freeze the aqueous layer on an alcohol bath at -30 °C and discard the organic layer. The ampoule is shaken vigorously to remove as much organic layer as possible.
13. Thaw the aqueous layer in a water bath (approximately 37 °C) for 1 minute.
14. Add HCl (500 µl, 0.1 M) to the sample and vortex briefly to homogenise.
15. Add hexane : dichloromethane ((1 + 4), 3 ml) to the sample and vortex for 1 minute.
16. Centrifuge the sample at 650 *G* for 1 minute.
17. Freeze the aqueous layer in an alcohol bath at -30 °C and decant the supernatant layer into a third 5 ml ampoule and evaporate to dryness using a Savant Speed Vac® rotary concentrator.
18. Reconstitute the sample in mobile phase (200 µl) and inject 20 µl onto the HPLC column.

- *Sample preparation - Synovial Fluid (SF)*

1. Thaw the samples in a water bath set at approximately 37 °C for ten minutes.
2. Ultrasonicate the sample for 20 min.
3. Vortex the sample briefly to homogenise.
4. In a polypropylene ASPEC tube (12 × 55 mm), add NaOH (1 ml, 0.1 M) to sample (200 µl). In the event of there being less than 200 µl sample,

the volume of 0.1 M NaOH added is proportionately adjusted in order to maintain a constant sample volume (ml) to volume NaOH (ml) ratio of 1 : 5.

5. Ultrasonicate the sample for 1 hour.
6. Homogenise the samples for 1 minute at 22000 rpm using a Jankel & Kunkel T 25 Ultra-Turrax, fitted with an IKA® UT disperser (8 mm OD).
7. Pipette 500 µl homogenate into a 5 ml ampoule.
8. Add HCl (200 µl, 1.0 M) to the sample and vortex briefly to homogenise.
9. Add hexane : dichloromethane ((1 + 4), 4 ml) to the sample and vortex for 1 minute.
10. Centrifuge the sample at 650 *G* for 1 minute.
11. Freeze the aqueous layer in an alcohol bath at -30 °C and decant the supernatant layer into a second 5 ml ampoule containing NaOH (200 µl, 0.1 M).
12. Vortex the sample for 1 minute and centrifuge at 650 *G* for 1 minute.
13. Freeze the aqueous layer in an alcohol bath at -30 °C and discard the organic layer. The ampoule is shaken vigorously to remove as much organic layer as possible.
14. Thaw the aqueous layer in a water bath (approximately 37 °C) for 1 minute.
15. Add HCl (500 µl, 0.1 M) to the sample and vortex briefly to homogenise.
16. Add hexane : dichloromethane ((1 + 4), 3 ml) to the sample and vortex for 1 minute.

17. Centrifuge the sample at 650 *G* for 1 minute.
18. Freeze the aqueous layer in an alcohol bath at -30 °C and decant the supernatant layer into a third 5 ml ampoule and evaporate to dryness using a Savant Speed Vac® rotary concentrator.
19. Reconstitute the sample in mobile phase (200 µl) and inject 20 µl onto the HPLC column.

- *Instrumental and Chromatographic Conditions*

Analytical Column

Higgins Haisil 120 BD C18 5 µm stainless steel column, 15 x 3 mm, fitted with an Upchurch stainless steel precolumn (2 × 20 mm) dry filled with Perisorb® RP 18 pellicular packing (30 - 40 µm), kept at 30 °C in a Shimadzu CTO-6A column heater.

Mobile Phase

30 mM *o*-Phosphoric acid : methanol : tetrahydrafuran (600 + 320 + 80). Add KCl (150 mg/L). Adjust the final pH of the mobile phase to 2.60 using 4 M NaOH.

Pump and Flow Rates

Hewlett-Packard series 1100 isocratic pump delivering 0.5 ml/min.

Sample Injection

Hewlett-Packard series 1050 autosampler injecting 20 µl onto the HPLC column. While on the autosampler, the samples are cooled to 5 °C using a Lauda RM 6 water circulating cooling system.

Detection

Hewlett-Packard series 1049A programmable electrochemical detector, in amperometric mode set at an oxidation potential of 0.650 V.

Recording and Integration

Hewlett-Packard series 1049A programmable electrochemical detector interfaced to a computer workstation running Hewlett-Packard HPLC^{2D} ChemStation version A.04.01 software. All chromatograms and reports are printed out in hardcopy and stored in electronic form on the workstation hard disk drive.

The pre-study validation batch was prepared according to the procedure above and processed as a single batch presented in the following table:

Project	: 71/98\AJ006						
Operator	: A. D. DE JAGER	Date Extracted	: 06/06/98	Date Injected	: 06/06/98	Subjects	: N/A
Period	: N/A	Plasma Set	: N/A				
Analytical Pump	: HP 1100	No	: DE52700183	Flow Rate	: 0.5 ml/min	Pressure	: 100 bar
Column	: HAISIL	Serial No.	: 417	Program (Y/N)	: N	MP Prep date\Batch	: 05/06/98
Auxilliary Pump	: -	No	: -	Flow Rate	: -	Pressure	: -
Column	: -	Serial No.	: -	Program (Y/N)	: -	MP Prep date	: -
Switch Valve No.	: -	Time 1	: -	Time 2	: -	Solvent Select No	: -
A %	: -	B%	: -	C%	: -	D%	: -
Program (Y/N)	: -	Column Heater No.	: ASD 39	Temperature	: 30 deg C		
Integrator\AD Box	: -	Channel No.	: -	Chart Speed	: -	Attenuation	: -
Input Voltage	: -	Run Time	: -				
Auto Injector	: HP 1050	No.	: 2394G00666	Inject Volume	: 20 ul	Runtime	: 9 min
Wash Solution	: None	Program (Y/N)	: N				
Ultraviolet Det	:	No.	:	Wavelength	:	Range	:
Rise Time	:	Output Voltage	:	Program (Y/N)	:		
Fluorescence Det	:	No.	:	Wavelength	:	PMT Voltage	:
Attenuation	:	Range	:	Response	:	Output Voltage	:
Program (Y/N)	:	Misc	: HP 1046 ECD				

Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF	Sample	DF
1	SYS	21	QC C	41	QC F	61	SYS	81		101		121		141	
2	STAB 1	22	QC B	42	QC E	62	STAB 3	82		102		122		142	
3	STD N	23	QC A	43	QC D	63	STAB 4	83		103		123		143	
4	STD M	24	STD I	44	QC C	64	STAB 5	84		104		124		144	
5	BLANK 1	25	STD H	45	QC B	65	STAB 6	85		105		125		145	
6	QC G	26	BLANK 3	46	QC A	66	STAB 7	86		106		126		146	
7	QC F	27	SYS	47	STD D	67	STAB 8	87		107		127		147	
8	QC E	28	STAB 2	48	STD C	68		88		108		128		148	
9	QC D	29	QC G	49	STD C	69		89		109		129		149	
10	QC C	30	QC F	50	BLANK 5	70		90		110		130		150	
11	QC B	31	QC E	51	QC G	71		91		111		131		151	
12	QC A	32	QC D	52	QC F	72		92		112		132		152	
13	STD L	33	QC C	53	QC E	73		93		113		133		153	
14	STD K	34	QC B	54	QC D	74		94		114		134		154	
15	STD J	35	QC A	55	QC C	75		95		115		135		155	
16	BLANK 2	36	STD G	56	QC B	76		96		116		136		156	
17	QC G	37	STD F	57	QC A	77		97		117		137		157	
18	QC F	38	STD E	58	STD B	78		98		118		138		158	
19	QC E	39	BLANK 4	59	STD B	79		99		119		139		159	
20	QC D	40	QC G	60	BLANK 6	80		100		120		140		160	

Samples loaded and System Checked by : [signature] Date : 06/06/98

Sample Position Verified by : [signature] Date : 05-06-98

Approval :

Notes :

The following represents the data for the pre-study validation calibration line, generated by PhRSt

Project : 71/98.AJO	CurveFit : 1/Concentration² Linear		Rev :P0 V0 M0 R0		08-Jun-1998
Analyte : Piroxicam P	Conc units : ng/ml		Tables were not updated		15:47
Curve : AJO06	LLOQ : 0.71 ng/ml		Date of Injection : 06/06/98		Page - 1
Quant : Area	Data : H:\71FRM98\ANALYTIC\INPDATA\AJO06\101-0101.D\Area.TXT				
Method : Hp Asterix	Calculation: Individual response		Process : Read from Raw Data		

###	STD ID	Drug Peak	Actual	Calc	% Dev	RT	Dg	Time	Rep	Code
3	STD N	819.938	600.00	630.81	5.1	6.61		15:45	Asterix	
4	STD M	567.902	446.00	436.87	-2.0	6.61		15:59	Asterix	
13	STD L	413.443	299.00	318.02	6.4	6.60		18:04	Asterix	
14	STD K	255.882	199.00	196.78	-1.1	6.59		18:17	Asterix	
15	STD J	199.226	151.00	153.19	1.4	6.59		18:31	Asterix	
24	STD I	131.099	99.30	100.76	1.5	6.55		20:35	Asterix	
25	STD H	64.688	49.50	49.66	0.3	6.54		20:49	Asterix	
36	STD G	26.561	19.80	20.32	2.7	6.54		23:21	Asterix	
37	STD F	11.504	9.91	8.74	-11.8	6.53		23:34	Asterix	
38	STD E	6.482	4.94	4.87	-1.3	6.54		23:48	Asterix	
47	STD D	3.267	2.47	2.40	-2.8	6.55		01:52	Asterix	
48	STD C	1.806	1.24	1.28	2.9	6.54		02:06	Asterix	
49	STD C	1.712	1.24	1.20	-2.9	6.55		02:20	Asterix	
58	STD B	1.076	0.71	0.71	0.7	6.57		04:24	Asterix	
59	STD B	1.079	0.71	0.72	1.0	6.55		04:37	Asterix	

r = 0.9987 r² = 0.9975 Slope = 1.2996 Intercept = 0.1473 n = 15

###	QC ID	Drug Peak	Actual	Calc	% Dev	RT	Dg	Time	Rep	Code
6	QC G	659.522	538.00	507.37	-5.7	6.61		16:27	Asterix	
17	QC G	687.660	538.00	529.02	-1.7	6.58		18:59	Asterix	
29	QC G	706.984	538.00	543.89	1.1	6.53		21:44	Asterix	
40	QC G	730.575	538.00	562.04	4.5	6.54		00:16	Asterix	
51	QC G	747.039	538.00	574.71	6.8	6.56		02:47	Asterix	
7	QC F	351.770	300.00	270.56	-9.8	6.60		16:41	Asterix	
18	QC F	374.449	300.00	288.02	-4.0	6.57		19:13	Asterix	
30	QC F	432.261	300.00	332.50	10.8	6.53		21:58	Asterix	
41	QC F	417.046	300.00	320.79	6.9	6.53		00:30	Asterix	
52	QC F	387.412	300.00	297.99	-0.7	6.56		03:01	Asterix	
8	QC E	176.365	150.00	135.59	-9.6	6.59		16:55	Asterix	
19	QC E	187.222	150.00	143.95	-4.0	6.56		19:26	Asterix	
31	QC E	193.312	150.00	148.64	-0.9	6.54		22:12	Asterix	
42	QC E	188.869	150.00	145.22	-3.2	6.54		00:43	Asterix	
53	QC E	197.562	150.00	151.91	1.3	6.56		03:15	Asterix	
9	QC D	3.790	3.47	2.80	-19.2	6.60		17:08	Asterix	
20	QC D	4.774	3.47	3.56	2.6	6.55		19:40	Asterix	
32	QC D	5.236	3.47	3.92	12.8	6.54		22:26	Asterix	

Verified by : _____ Approved by : _____

PhReg 2.1-024

Project : 71/98.AJO	CurveFit : 1/Concentration² Linear		Rev :P0 V0 M0 R0		08-Jun-1998
Analyte : Piroxicam P	Conc units : ng/ml		Tables were not updated		15:47
Curve : AJO06	LLOQ : 0.71 ng/ml		Date of Injection : 06/06/98		Page - 2
Quant : Area	Data : H:\71FRM98\ANALYTIC\INPDATA\AJO06\101-0101.D\Area.TXT				
Method : Hp Asterix	Calculation: Individual response		Process : Read from Raw Data		

###	QC ID	Drug Peak	Actual	Calc	% Dev	RT	Dg	Time	Rep	Code
43	QC D	4.513	3.47	3.36	-3.2	6.54		00:57	Asterix	
54	QC D	4.799	3.47	3.58	3.2	6.55		03:28	Asterix	
10	QC C	3.060	2.61	2.24	-14.1	6.60		17:22	Asterix	
21	QC C	3.649	2.61	2.69	3.2	6.55		19:54	Asterix	
33	QC C	3.564	2.61	2.63	0.7	6.54		22:39	Asterix	
44	QC C	3.557	2.61	2.62	0.5	6.54		01:11	Asterix	
55	QC C	3.577	2.61	2.64	1.1	6.55		03:42	Asterix	
11	QC B	2.503	1.74	1.81	4.2	6.59		17:36	Asterix	
22	QC B	2.543	1.74	1.84	6.0	6.55		20:08	Asterix	
34	QC B	2.481	1.74	1.80	3.2	6.54		22:53	Asterix	
45	QC B	2.653	1.74	1.93	10.8	6.55		01:25	Asterix	
56	QC B	2.749	1.74	2.00	15.1	6.55		03:56	Asterix	
12	QC A	1.248	0.86	0.85	-1.5	6.62		17:50	Asterix	
23	QC A	1.226	0.86	0.83	-3.5	6.55		20:22	Asterix	
35	QC A	1.323	0.86	0.90	5.2	6.53		23:07	Asterix	
46	QC A	1.206	0.86	0.81	-5.3	6.56		01:38	Asterix	
57	QC A	1.429	0.86	0.99	14.7	6.57		04:10	Asterix	

###	Sample ID	Drug Peak	Calc	RT	Dg	Time	Rep	Code

Verified by : _____

Approved by : _____

PhReg 2.1-024

Project : 71/98.AJO	CurveFit : 1/Concentration² Linear		Rev :P0 V0 M0 R0	08-Jun-1998
Analyte : Piroxicam P	Conc units : ng/ml		Tables were not updated	15:47
Curve : AJO06	LLOQ : 0.71 ng/ml		Date of Injection : 06/06/98	Page - 3
Quant : Area	Data : H:\71FRM98\ANALYTIC\INPDATA\AJO06\101-0101.D\Area.TXT			
Method : Hp Asterix	Calculation: Individual response		Process : Read from Raw Data	

###	Misc ID	Drug Peak	Calc	RT	Dg	Time	Rep	Code
65	STAB 6	244.971	188.38	6.58		06:06	Asterix	
28	STAB 2	243.766	187.46	6.54		21:31	Asterix	
63	STAB 4	246.115	189.27	6.57		05:33	Asterix	
64	STAB 5	243.457	187.22	6.57		05:49	Asterix	
66	STAB 7	246.715	189.73	6.57		06:22	Asterix	
2	STAB 1	235.565	181.15	6.61		15:31	Asterix	
62	STAB 3	243.440	187.21	6.57		05:19	Asterix	
68		No Peak	BLQ	N/ap	N/ap	N/ap		
67	STAB 8	245.992	189.17	6.58		06:39	Asterix	
1	SYS	21.762	16.63	6.61		15:17	Asterix	
27	SYS	22.462	17.17	6.54		21:17	Asterix	
61	SYS	22.189	16.96	6.57		05:05	Asterix	
5	BLANK 1	No Peak	BLQ	6.75		16:13	Asterix	
16	BLANK 2	No Peak	BLQ	6.75		18:45	Asterix	
26	BLANK 3	No Peak	BLQ	6.75		21:03	Asterix	
39	BLANK 4	No Peak	BLQ	6.75		00:02	Asterix	
50	BLANK 5	No Peak	BLQ	6.75		02:33	Asterix	
60	BLANK 6	No Peak	BLQ	6.75		04:51	Asterix	

Verified by :_____ Approved by :_____

PhReg 2.1-024

5.5.4 Analytical report – piroxicam

The validation of the assay method and subsequent assay of the study samples culminated in the following analytical report documenting two pre-study validations. This was unusual at the time bearing in mind that FBSD had not yet established the use of multiple pre- study validation. The first validation was extracted on 18 March 1998, and the second on 6 June 1998. The fact that these two validations proved to be so similar with respect to performance, considering that there was roughly three months between the two validations, can be taken as in indication of method robustness:

- *Intra-day Accuracy and precision*

Calibration Curve

Calibration Standards used: STD B - STD N

Calibration Range: 0.71 - 600 ng/ml

Regression Equation: Linear (Weighted 1/Concentration^2)

Slope: 0.6458

Intercept: 0.0834

r^2: 0.9964

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD N	600	613.72	2.3
STD M	446	R	N/A
STD L	299	324.27	8.5
STD K	199	201.43	1.2
STD J	151	149.71	-0.9
STD I	99.3	96.79	-2.5
STD H	49.5	50.38	1.8
STD G	19.8	19.21	-3.0
STD F	9.91	9.53	-3.8
STD E	4.94	5.00	1.2
STD D	2.47	R	N/A
STD C	1.24	1.24	-0.3
STD C	1.24	1.10	-11.3
STD B	0.71	0.77	8.4
STD B	0.71	0.70	-1.6

R - Rejected

- *Summary of Intra-day Quality Control Results*

Accuracy is measured as % bias and precision is measured as coefficient of variation (CV%).

Code	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	**538.37**	**299.69**	**149.72**	**3.47**	**2.61**	**1.74**	**0.86**
Replicates							
1	487.47	242.06	127.25	3.13	2.28	1.59	1.00
2	488.68	275.23	148.56	3.02	2.32	1.84	0.80
3	492.52	284.93	141.41	3.62	2.48	1.64	1.11
4	511.75	301.04	138.03	2.91	2.28	1.53	0.90
5	505.26	274.65	144.54	3.00	2.34	1.62	0.79
MEAN	497.14	275.58	139.96	3.14	2.34	1.64	0.92
BIAS	-7.7%	-8.0%	-6.5%	-9.7%	-10.2%	-5.3%	6.5%
CV	1.9%	7.0%	5.2%	8.0%	3.2%	6.4%	13.2%

- *Calibration Range*

For the assignment of a valid calibration range bias is taken as measure of accuracy and coefficient of variation (CV %) is taken as measure of precision. Intra-day accuracy and precision for a valid range must be within 15% but within 20% at the lower limit of quantification. Results from the intra-day validation assays above indicate a valid calibration range of 0.71 - 600 ng/ml in plasma. The lower limit of quantification (LLOQ) in plasma was preliminarily set at 0.71 ng/ml.

Since the sub-cutaneous tissue, synovial capsule and synovial fluid samples were diluted 6 times the LLOQ for sub-cutaneous tissue and synovial capsule was preliminarily set at 4.26 ng/g of tissue and the synovial fluid at 4.26 ng/ml.

- *Intra-day Accuracy and Precision: (Validation 2)*

Calibration Curve

Calibration Standards used: STD B - STD N

Calibration Range: 0.71 - 600 ng/ml

Regression Equation: Linear (1/Concentration^2)

Slope: 1.2996

Intercept: 0.1473

r^2: 0.9975

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD N	600	630.81	5.1
STD M	446	436.87	-2.0
STD L	299	318.02	6.4
STD K	199	196.78	-1.1
STD J	151	153.19	1.4
STD I	99.3	100.76	1.5
STD H	49.5	49.66	0.3
STD G	19.8	20.32	2.7
STD F	9.91	8.74	-11.8
STD E	4.94	4.87	-1.3
STD D	2.47	2.40	-2.8
STD C	1.24	1.28	2.9
STD C	1.24	1.20	-2.9
STD B	0.71	0.71	0.7
STD B	0.71	0.72	1.0

R - Rejected

- *Summary of Intra-day Quality Control Results (Validation 2)*

Accuracy is measured as % bias and precision is measured as coefficient of variation (CV%).

Code	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	**538.37**	**299.69**	**149.72**	**3.47**	**2.61**	**1.74**	**0.86**
Replicates							
1	507.37	270.56	135.59	2.80	2.24	1.81	0.85
2	529.02	288.02	143.95	3.56	2.69	1.84	0.83
3	543.89	332.50	148.64	3.92	2.63	1.80	0.90
4	562.04	320.79	145.22	3.36	2.62	1.93	0.81
5	574.71	297.99	151.91	3.58	2.64	2.00	0.99
MEAN	543.41	301.97	145.06	3.44	2.56	1.88	0.88
BIAS	0.9%	0.8%	-3.1%	-0.8%	-1.6%	8.0%	1.4%
CV	4.4%	7.4%	3.8%	10.7%	6.4%	4.1%	7.3%

- *Extraction Efficiency*

Absolute recovery of piroxicam in plasma was determined in triplicate at high, medium and low concentrations. The recoveries of the sub-cutaneous tissue, synovial capsule and synovial fluid were determined at one concentration only due to the lack of availability of the above mentioned normal tissues.

Absolute recovery of analyte based on theoretical areas calculated by way of response standards

ANALYTE: Piroxicam

SAMPLE	ANALYTE ng/ml	MEAN OF PEAK AREAS		ABSOLUTE RECOVERY (%)	CV (%)
		ACTUAL AREA	THEORETICAL AREA		
Plasma (max)	538	492.2	821.6	59.9	4.1
Plasma (ave)	150	136.4	229.1	59.5	5.8
Plasma (min)	261	2.35	3.99	58.9	3.3
Sub-cutaneous tissue	454*	215	331.3	64.9	3.8
Synovial capsule	454*	191.3	331.3	57.7	1.7
Synovial fluid	127*	64.2	95.9	66.9	8.0

* Final homogenate was spiked to precise concentration.

- *Stability*

Stock Solutions

Stock solutions were freshly prepared in methanol and used immediately for the spiking of normal biological fluid. The stock solutions are not retained for further use.

Stability in the Matrix

The response factors of quality control samples analysed in triplicate, at high and medium concentration prepared during the pre-study validation and stored at -20 °C, were compared with the response factor of a freshly prepared calibration standard analysed in triplicate. All these samples were extracted and analysed as a single batch. As can be seen from the tables below, piroxicam is stable in human plasma for at least 17 weeks when stored at -20 °C.

Fresh Calibration Standard: Preparation date: 7-Jul-98

Nominal Conc. (ng/ml)	Area (nA*s)	Response Factor
299.9	331.5	1.11
299.9	300.1	1.00
299.9	316.3	1.05
	Mean	1.05
	Std. Dev.	0.05
	CV %	4.96

High Quality Control samples stored at -20 °C since 12-Mar-98

Nominal Conc. (ng/ml)	Area nA*s	Response Factor
538	585.1	1.09
538	557.8	1.04
538	557.4	1.04
	Mean	1.05
	Std. Dev.	0.03
	CV %	2.80

Medium Quality Control samples stored at -20 °C since 12-Mar-98

Nominal Conc. (ng/ml)	Area nA*s	Response Factor
150	183.8	1.23
150	167.2	1.11
150	175.0	1.17
	Mean	1.17
	Std. Dev.	0.06
	CV %	4.74

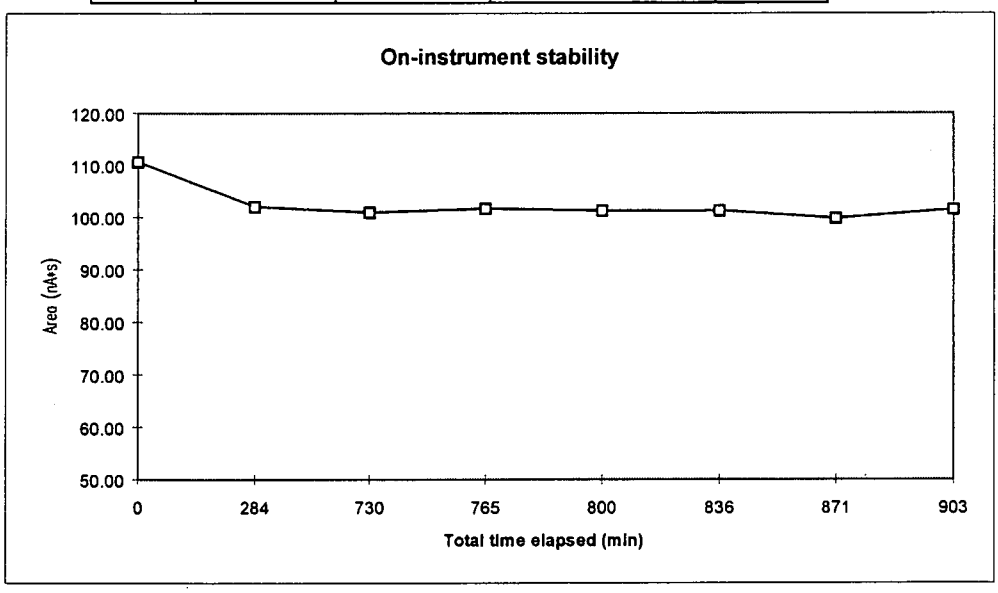
- *Freeze-thaw Stability*

Samples were not subjected to multiple freeze-thaw cycles and were assayed immediately after thawing. Sample residues were discarded after analysis.

- *On-instrument Stability*

Eight stability samples of the same concentration prepared in plasma were analysed and injected at intervals during the validation batch to simulate the time of a batch run. The analyte peak area was plotted against the cumulative time as can be seen in the graph below. The analyte was deemed stable on the instrument for the duration of a batch run (approximately 15 hours).

Sample no.	Injection time	Elapsed time (min)	Total elapsed time (min)	Area nA*s
1	4:52 PM	0	0	110.62
2	9:36 PM	284	284	102.02
3	5:02 AM	446	730	100.93
4	5:37 AM	35	765	101.60
5	6:12 AM	35	800	101.18
6	6:48 AM	36	836	101.21
7	7:23 AM	35	871	99.70
8	7:55 AM	32	903	101.40



- *Specificity*

Specificity is determined by analysing "blank" plasma from six different sources. Samples from synovial fluid, synovial capsule and sub-cutaneous tissue were extracted once for specificity due to the lack of availability of normal tissues. The chromatograms were inspected for peaks which may interfere with the analyte. In the case of high performance liquid chromatographic procedures the chromatograms are run for 30 minutes to determine the presence of late eluting peaks which may cause interference in subsequent chromatograms.

No interfering peaks were found at the retention time of the analyte.

5.5.5 Within-Study Assay Performance

Samples are assayed in batches consisting of calibration standards (usually 6 to 10), quality controls (at high, medium and low concentrations), and study samples. The number of samples that can be assayed in a batch depends on factors such as the stability of the analyte in the biological fluid or in the extraction solvent and the length of the chromatographic runs. Attempts are always made to process complete profiles of a subject for each treatment in a batch. Thus in a given batch, profiles of treatments are alternated whenever possible. The calibration standards and quality controls are interspersed among the study samples in a predetermined manner. The quality controls which are processed in each batch comprise duplicates near the maximum, near the mean concentration and near 3 × LLOQ (where LLOQ represents the lower limit of quantification determined during the validation of the

assay method) as well as two controls respectively near LLOQ and 2 × LLOQ. After the batch has been run the chromatograms are inspected and checked against documented acceptance criteria.

The calibration curves are plotted, regression equations determined, and the quality controls calculated as unknowns using the regression equation giving the best overall results throughout the study.

- *Preparation of Calibration Standards and Quality Controls*

Sufficient calibration standards and quality controls are prepared during the re-instatement validation to serve as calibration standards and quality controls for the assay of the study samples. The preparation of these standards and controls has already been presented under the Pre-Study Validation section of this report.

5.5.6 Typical Batch Structure

Samples are designated in the run sheet table by a three digit code separated by commas consisting of *subject number, sampling time(hr), period*

Inj. No.	Sample	Inj. No.	Sample	Inj. No.	Sample
1	SYS	17	P5,2,1	33	P6,3,1
2	QC E	18	QC E	34	QC C
3	STD E	19	QC B	35	T6,4,1
4	P4,1,1	20	P5,3,1	36	SYS
5	P4,2,1	21	STD G	37	STD C
6	P4,3,1	22	T5,4,1	38	C6,5,1
7	QC F	23	QC D	39	F6,6,1
8	STD L	24	C5,5,	40	P7,1,1
9	T4,4,1	25	STD N	41	P7,2,1
10	C4,5,1	26	BLANK	42	P7,3,1
11	STD H	27	STD C	43	T7,4,1
12	QC D	28	F5,6,1	44	C7,5,1
13	STD D	29	STD D	45	F7,6,1
14	STD J	30	P6,1,1	46	SYS
15	P5,1,1	31	QC F	47	
16	STD K	32	P6,2,1	48	

P = Plasma sample
T = Sub-cutaneous tissue sample
F = Synovial fluid sample
C = Synovial capsule sample

- *Calculation of Results*

Results are calculated using the PhIRSt chromatographic data reporting package. Peak heights/areas are electronically read automatically from the report files generated by Hewlett-Packard HPLC^{2D} ChemStation. Data are automatically summarised, calibration curves calculated according to pre-set regression equations and concentrations interpolated by the program. Results are presented in printed ordered tables with performance statistics per batch and later summarised to give overall study statistics. This package has been validated in Canada by the manufacturer to FDA requirements.

Inter-day Accuracy and Precision

Back-Calculated Calibration Standards Concentrations

Curve code	STD C	STD D	STD E	STD G	STD H	STD J	STD K	STD L	STD N
Nominal (ng/ml)	1.24	2.47	4.94	19.8	49.5	151	199	299	600
AJO04	1.27 1.14	2.74	4.91	21.37	51.82	141.36	194.69	311.00	593.77
AJO05	1.39 1.03	2.76 2.40	R	21.23	49.41	135.30	196.46	293.57	614.92
AJO08	R 1.28	2.46	4.34	20.08	50.23	162.49	202.15	305.10	571.71
AJO09	1.25 R	2.60	4.15	22.64	48.73	159.84	191.30	296.39	577.68
AJO10	1.26 1.26	2.31	5.02	19.87	47.81	141.20	227.71	304.48	572.35
AJO11	1.24 1.17	2.80	4.75	18.72	53.39	151.93	183.76	305.47	591.19
AJO12	1.21 1.10	2.89	5.92	20.39	47.30	146.10	186.66	284.52	555.63
AJO13	1.19 1.39	2.12	4.65	22.23	47.55	150.20	195.56	318.69	597.18
Mean	1.23	2.56	4.82	20.81	49.53	148.55	197.29	302.40	584.30
CV%	8.1	10.1	11.8	6.2	4.4	6.4	6.9	3.5	3.2
N	14	9	7	8	8	8	8	8	8
%Nom	98.9	103.8	97.6	105.1	100.1	98.4	99.1	101.1	97.4

R – Rejected

Quality Control Results

Curve code	QC B	QC C	QC D	QC E	QC F
Nominal (ng/ml)	1.74	2.61	3.47	150	300
AJO04	1.62	2.38	3.86	137.36 131.62	290.51 297.73
AJO05	2.01	2.18	3.46 3.52	127.97 130.41	288.49 318.85
AJO08	1.76	2.65	3.98 3.27	124.73 134.50	251.77 336.62
AJO09	1.83	2.92	3.60 3.64	79.91* 121.06	271.90 308.74
AJO10	1.60	2.30	3.72 3.03	128.17 138.10	309.29 291.03
AJO11	1.60	2.61	3.44 3.36	163.24 148.83	306.01 292.67
AJO12	1.74	2.20	3.45 3.18	152.38 133.02	269.13 270.67
AJO13	1.52	2.26	3.24 3.18	146.27 139.43	301.52 259.16
Mean	1.71	2.44	3.46	133.56	291.51
CV%	9.3	10.8	7.7	13.5	7.8
N	8	8	15	16	16
%Nom	98.3	93.4	99.8	89.0	97.2

- *Lower Limit of Quantification*

The lower limit of quantification (LLOQ) is initially determined from the data obtained for the assayed quality controls during Pre-Study Validation, since these data often include determinations of the analyte at concentrations close to the limit of detection. The LLOQ is defined as that concentration of the analyte which can still be determined with acceptable precision (CV% < 20) and accuracy (bias < 20 %) for the purposes of the particular application. This limit is reappraised during the performance of the assay with actual clinical study samples. After all the clinical study samples have been analysed the limit of quantification is finally set at a value which is determined by the performance of the assay procedure with the calibration standards and quality controls which are processed with each

batch of samples run. This is considered to be a more objective reflection of the assay performance under clinical study conditions than the validation data alone. The LLOQ is set to the value of the lowest calibration standard used throughout the study that met the acceptance criteria.

LLOQ (Plasma):	1.24 ng/ml
LLOQ (Sub-cutaneous tissue):	7.44 ng/g
LLOQ (Synovial capsule):	7.44 ng/g
LLOQ (Synovial fluid):	7.44 ng/ml

- *Selected study chromatograms*

Figure 35: Calibration Standard L (Piroxicam: 299 ng/ml)

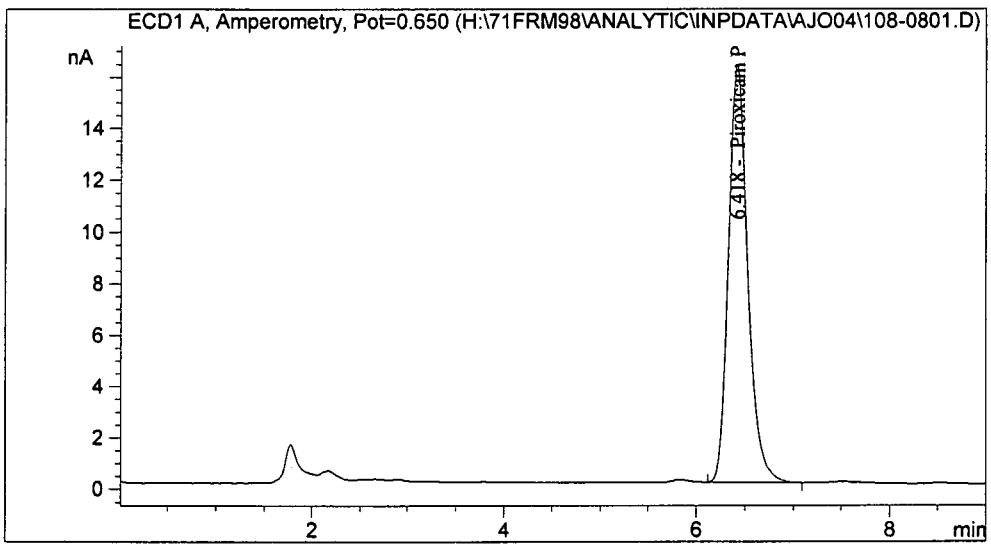


Figure 36: Calibration Standard H (Piroxicam: 49.5 ng/ml)

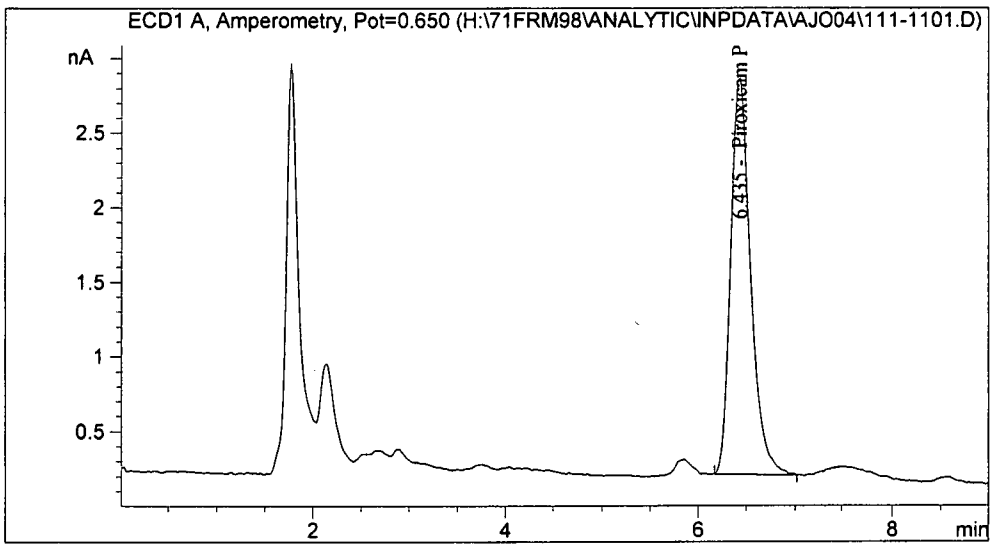


Figure 37: Calibration Standard C 1 (Piroxicam: 1.24 ng/ml)

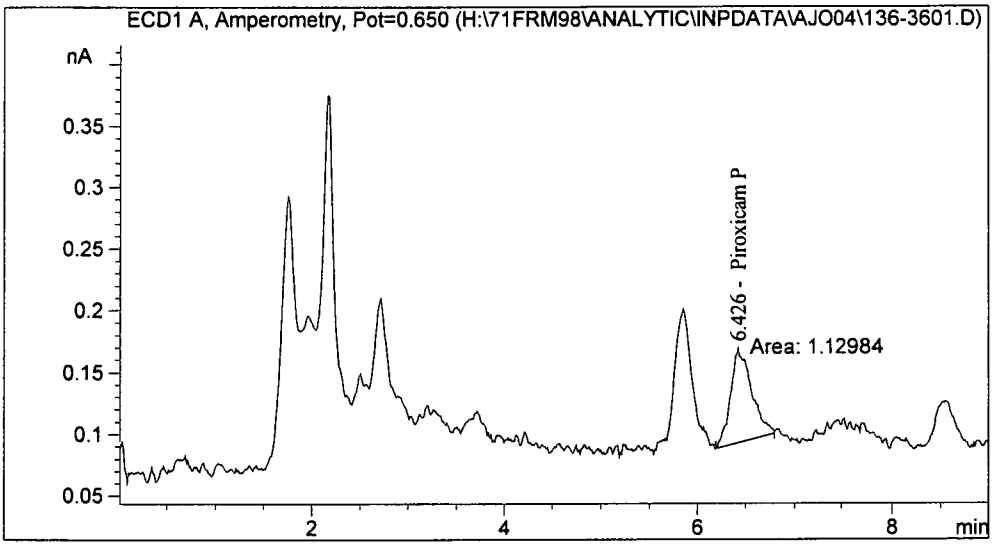


Figure 38: Subject 1, Plasma Day 8 (Piroxicam: 144.39 ng/ml)

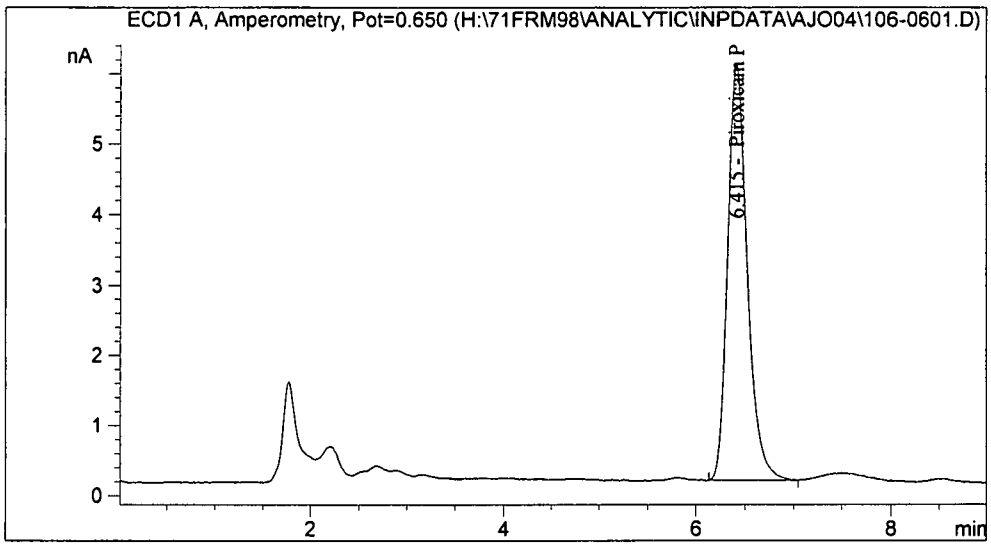


Figure 39: Subject 1, Synovial Fluid (Piroxicam: 75.24 ng/ml after dilution factor is applied)

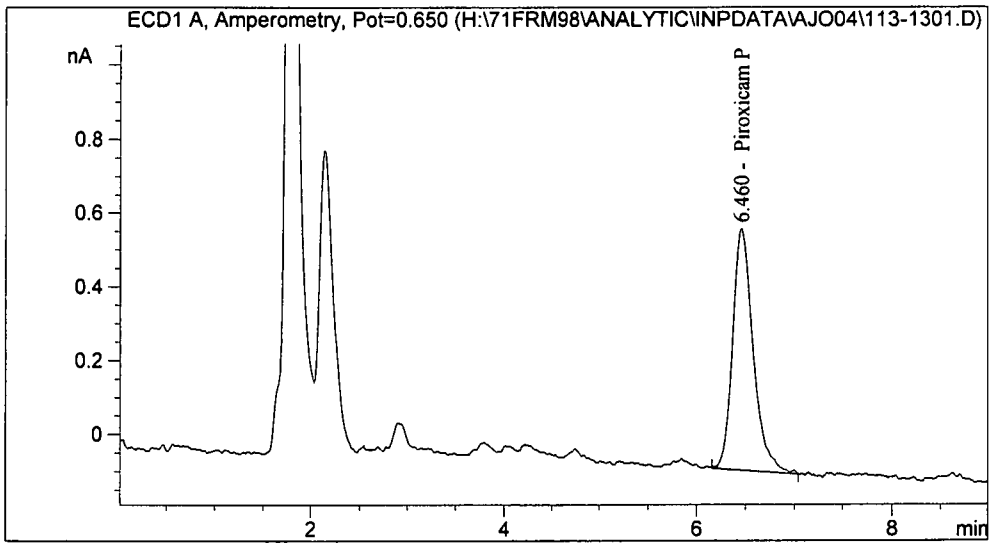


Figure 40: Subject 1, Sub-cutaneous Tissue (Piroxicam: 791.72 ng/g after dilution factor is applied)

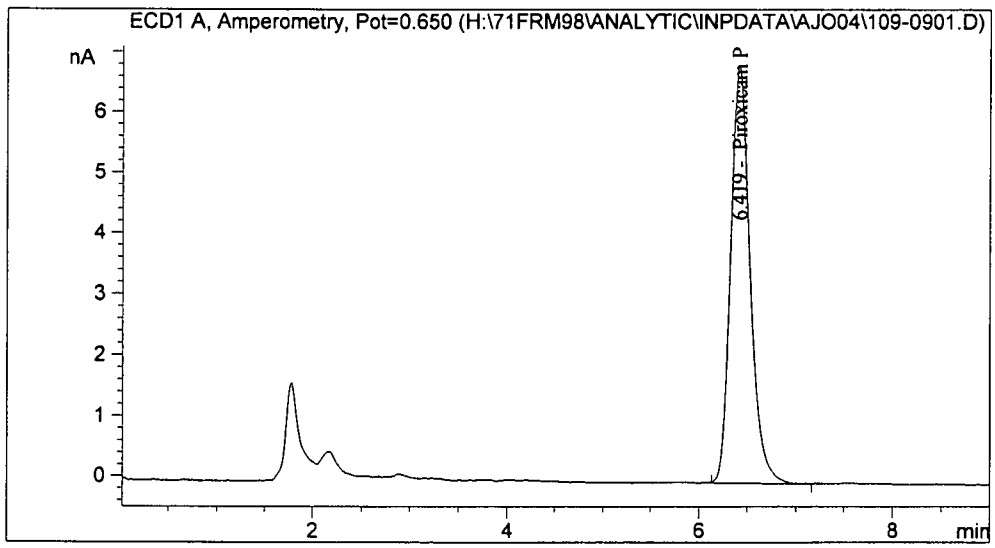


Figure 41: Subject 1, Synovial Capsule (Piroxicam: 207.54 ng/g after dilution factor is applied)

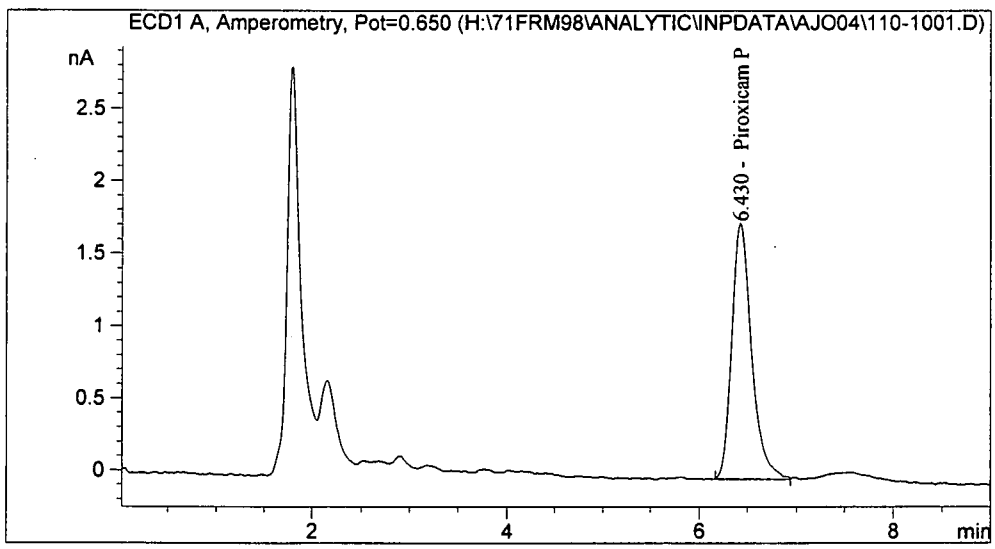


Figure 42: Blank Plasma

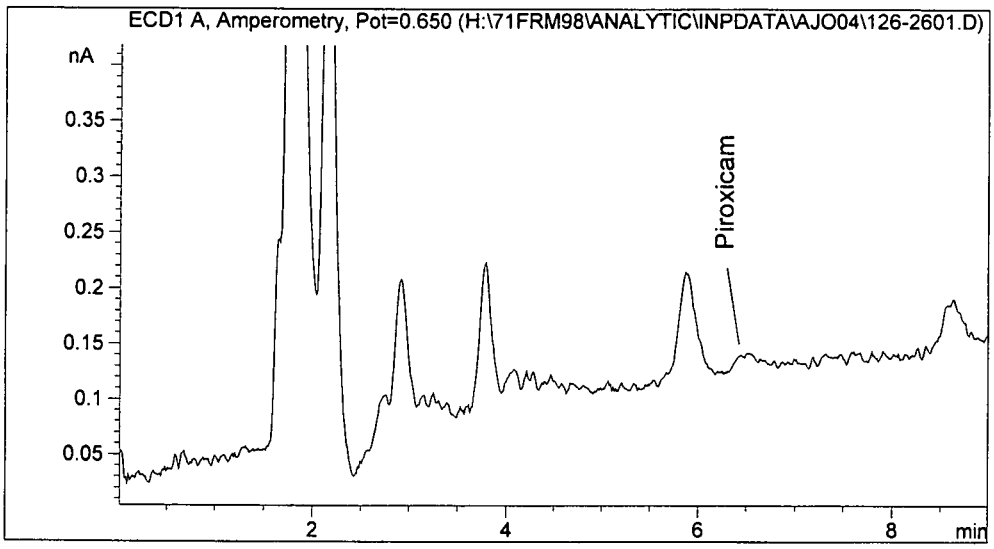
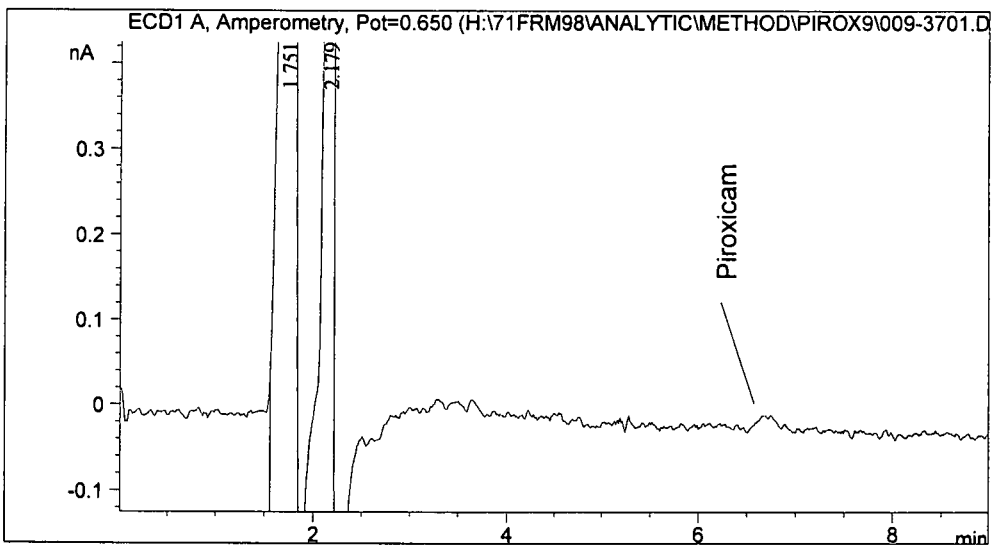


Figure 43: Blank Synovial Capsule



5.5.7 Observations and discussion

When comparing the method developed for 6-MNA with the above-mentioned piroxicam method, a number of things are evident. Firstly, the 6-MNA was a reasonably simple method, without much in the way of novelty. The piroxicam method, on the other hand, was

particularly novel, and far more complex, as a study of this exact nature had not yet been done to the best of the author's knowledge. Secondly, while the 6-MNA required minimal sample preparation, the nature of the piroxicam study necessitated a labour intensive sample preparation procedure. This implies that should the piroxicam method be used for a simple bioequivalence study, in which large numbers of plasma samples (and not tissue samples) are usually generated, the method would probably have to be truncated somewhat.

Thirdly, the author would have liked to have had more control over the way in which the tissue samples were collected and for this reason, the initial search for an internal standard for the piroxicam assay method was abandoned. This was owing to the fact that there was scant information available regarding concomitant medication, and it was speculated that it was preferable not to introduce the possibility of internal standard interference. This particular study was a multi-centre study in which a variety of surgeons collected tissue samples, with little control over the way in which the samples were handled and stored. Naturally this would introduce a certain measure of doubt as to whether or not the tissue samples could have been impregnated with the analyte upon contact with blood during surgery. It is the opinion of the author that this is not the case, as the levels of piroxicam found in the tissues were far superior to those in the plasma, for each individual subject. Simply stated, it is not possible for a sample of lower concentration, in a short space of time, to contaminate one of higher concentration.

What further supports the notion that the concentrations found in tissues are a true reflection of absorption is that there was a clear relationship between concentration and depth below the epidermis. It was found that the highest concentration of piroxicam was to be found just

below the skin (SCT), while concentrations in SF and SC were somewhat lower, but still superior to those found in plasma.

Table 9: Plasma levels (ng/ml) associated with topical application of piroxicam to the knee.

Subj. no	Sampling time		
	Plasma D1	Plasma D3	Plasma D8
1	BLQ	47.57	114.39
2	BLQ	7.47	38.22
3	BLQ	56.95	161.11
4	BLQ	33.91	41.77
5	BLQ	BLQ	3.34
6	BLQ	46.33	160.68
7	BLQ	10.58	16.29
8	BLQ	32.27	139.43
9	BLQ	14.35	13.23
10	BLQ	4.75	51.24
12	BLQ	30.57	59.22
13	BLQ	14.96	51.10
14	BLQ	13.67	64.61
15	BLQ	70.10	355.95
16	BLQ	2.90	29.25
17	BLQ	5.97	53.05
20	BLQ	27.70	81.73
21	BLQ	26.08	58.53
22	BLQ	28.43	83.49
23	BLQ	12.03	10.22
24	BLQ	16.38	16.07
25	BLQ	202.96	317.99
26	BLQ	291.00	469.29
27	BLQ	26.24	21.57
28	BLQ	16.24	27.07
29	BLQ	10.76	30.02
30	BLQ	5.35	25.59
31	BLQ	28.83	74.96
32	BLQ	10.12	33.83
CV%	N/A	156.2	121.7

Codes: BLQ = Value below the limit of quantification
D1: Pre- dosing
D3: 3 days into the dosing regimen
D8: 8 days into the dosing regimen

Table 10: Concentrtions (ng/ml) determined in SF, SCT and SC.

Subject no.	SF Concentration	SCT Concentration	SC Concentration
1	75.24	791.72	207.54
2	N/S	2136.3	298.99
3	47.19	1137.19	34.3
4	N/S	43.93	30.3
5	22.08	11.35	7.82
6	55.68	25.32	19.7
7	26.15	7.85	NR
8	N/S	38.03	NR
9	NR	40.32	NR
10	34.96	9.65	12.79
12	N/S	1196.08	N/S
13	60.16	12.65	13.05
14	57.31	857.81	30.6
15	303.8	1124.85	N/S
16	12.26	24.39	34.05
17	20.07	733.34	NR
20	49.03	408.81	87.92
21	28.19	35.58	15.47
22	36.86	78.77	13.15
23	N/S	191.94	90.62
24	56.03	1490.96	164.86
25	338.79	300.03	124.32
26	N/S	343.45	118.28
27	22.93	1072.16	20.47
28	30.59	26.07	19.98
29	28.44	14.05	10.53
30	24.44	30.62	10.83
31	51.97	114.3	64.85
32	103.68	611.29	75.11
CV%	101.1	124.5	107.9

Codes: N/S = No sample delivered
NR = Not reportable

Following quantitation, ratios of SC, SF and SCT were calculated relative to a particular subjects own plasma concentration on day eight.

Table 11: Calculated ratios

Ratio expressed	Mean ratio	CV%	N
SC/plasma	3.02	136	22
SF/plasma	3.04	77.4	21
SCT/plasma	20.9	150	28

The ratios above suggest that there is significant accumulation of piroxicam below the skin, with decreasing concentrations with an increase in tissue depth.

6 Summary

The development and validation of bioanalytical assay methods suitable for the quantitation of drugs in biological matrices is discussed, as well as general principles applicable to this particular aspect of drug development. Relevant literature is consulted, with a view to exemplifying what constitutes good assay method development strategy, as well as to reflect current international policy in this field, with particular reference to bioequivalence studies. Comparisons are made between international practices and those in place at FARMOVS-PAREXEL Bioanalytical Services Division®. Attention is given *inter alia* to detector selection, chromatographic optimisation, extraction procedures and method validation, with reference to new assay methods for two drugs in particular that have been developed and validated according internationally acceptable standards. In the first instance, a high-performance liquid chromatographic method with ultraviolet detection was developed for the determination of 6-methoxy-2-naphthylacetic acid (6-MNA, the active metabolite of nabumetone). The sample preparation involved a simple but effective protein precipitation procedure. Reversed-phase liquid chromatography was optimised, and full resolution between the analyte and endogenous matrix peaks achieved in a chromatographic runtime of five minutes. The assay method was validated over a range of plasma concentrations between 0.070 and 145µg/ml. 1242 Plasma samples generated during a comparative bioequivalence study were then assayed and the performance of the assay method shown to be well within accepted international norms. The coefficient of variation for quality control

standards over the range of 0.18 to 39 μg/ml processed during the assaying of the study samples, varied between 3.6 and 8.1 %.

In the second instance, a novel method for the determination of piroxicam in four biological matrices (sub-cutaneous tissue (SCT), synovial fluid (SF), synovial capsule (SC) and plasma) was developed. A double back-extraction procedure was followed by reversed-phase liquid chromatography and electrochemical detection (ECD). Extracts from all four biological matrices were injected onto a single HPLC system. Ratios between plasma and the three remaining matrices were used to characterise transdermal absorption of two topical preparations of piroxicam when applied to the knee. Low systemic levels associated with topical formulations necessitated the development and validation of a highly sensitive assay method. Plasma was used as a surrogate matrix for all the processed tissue samples and the assay method was validated over a range of plasma concentrations between 1.24 and 600 ng/ml. 168 Samples generated during a multi-centre study involving knee replacement surgery, were assayed and the performance of the assay method shown to be well within accepted international norms. The coefficient of variation for quality control standards over the range of 1.74 to 300 ng/ml processed during the assaying of the study samples, varied between 7.7 and 13.5 % which can be considered excellent in the light of the complexity of the sample preparation process.

Analytical data generated during the above-mentioned two research projects are discussed, with novelties and improvements to existing assay methods being elucidated. Both assay methods were presented and accepted for publication in peer reviewed scientific journals. Both full-length publications are included in an appendix in this dissertation, together with the correspondence entered into with journal editors and referees. Furthermore, a section

containing copies of the slides used to present the latter HPLC assay method as an oral presentation at the 1998 Annual Congress of the South African Pharmacological Society, is included.

Keywords

Method development, validation, drug development, bioequivalence, high-performance liquid chromatography, plasma, sub-cutaneous tissue synovial capsule, synovial fluid.

Die ontwikkeling en validering van bioanalitiese metodes wat vir die kwantifisering van geneesmiddels in biologiese monsters toepaslik is, asook algemene beginsels wat van toepassing is op hierdie aspek van geneesmiddelontwikkeling, word bespreek. Relevante literatuur is geraadpleeg met die doel om voorbeelde van sinvolle strategieë vir die ontwikkeling van bioanalitiese metodes uit te lig asook om huidige internasionale praktyke, veral ten opsigte van bioekwivalensiestudies, te reflekteer. In die verhandeling word vergelykings tussen bogenoemde praktyke en dié by FARMOVS-PAREXEL Bioanalytical Services Division® getref. Onder andere word aandag aan die keuse van die detektor, chromatografiese optimisering, monster voorbereiding en validering van twee nuwe analitiese metodes gewy wat albei ontwikkel en valideer is volgens internasionaal aanvaarde standaarde. Eerstens is 'n hoë-verrigting vloeistofchromatogra-fiese metode met ultravioletdeteksie vir die bepaling van 6-metoksie-2-naftiel asynsuur (6-MNA, die aktiewe metaboliet van nabumetoon) ontwikkel . Die monstervoorbereiding behels 'n eenvoudige

maar baie effektiewe proteïen presipitasie van plasmamonsters gevolg deur omgekeerde vloeistofchromatografie. Die sisteem is geoptimiseer en skeiding tussen die analiet en endogene plasmakomponente geskied binne die bestek van 'n vyf minute chromatografielopie. Die metode is oor 'n bereik van 0.070 en 145 µg/ml plasmakonsentrasies gevalideer. 1242 Plasmamonsters wat gedurende ,n vergelykende biobeskibaarheidsstudie verkry is, is dan geanaliseer en die verrigting van die analitiese metode was gemaklik binne aanvaarde internasionale norme. Die variasiekoëffisiënt van die kwaliteitskontroles wat in die studie saam met die studiemonsters geproseseer is, en wat oor 'n konsentrasiebereik van 0.070 en 145 µg/ml gestrek het, het gevariëer tussen 3.6 en 8.1 %. Die tweede navorsingsstuk behels die bepaling van piroksikam in vier verskillende biologiese matrikse (subkutane weefsel, sinoviale kapsel, sinoviale vog en plasma). 'n Dubbele terug-ekstraksie, gevolg deur omgekeerde fase vloeisstof-chromatografie en elektrochemiese deteksie was angewend om piroksikam te kwantifiseer. Ekstrakte uit elk van die vier verskillende matrikse is op 'n enkele HPLC sisteem ingespuit. Verhoudings tussen plasmakonsentrasie en die konsentrasie van piroksikam in die drie oorblywende matrikse is bereken om die transdermale absorpsie van piroksikam uit twee topikaal, op die knie aangewende preparate, te karakteriseer. Aangesien die aanwending van topikale piroksikam gelpreparate maar baie lae sistemiese piroksikamkonsentrasies lewer, was dit nodig om 'n baie sensitiewe analitiese te ontwikkel. Plasma was gebruik as 'n surrogaatmatriks vir die geproseseerde weefselmonsters en die analitiese metode was gevalideer oor 'n plasmakonsentrasie-bereik van 1.24 tot 600 ng/ml. 168 Monsters wat ontvang is uit 'n multisentriese studie waarby knievervangssjirurgie betrokke was, is geanaliseer en die verrigting van die bepalingsmetode was gemaklik binne die internasionaal aanvaarde norme.

Die variasiekoëffisiënt van die kwaliteitskontroles wat in die studie saam met die studiemonsters geproseseer is, en wat oor 'n konsentrasiebereik van 1.70 en 300 ng/ml gestrek het, het gevariëer tussen 7.7 en 13.5 % wat as uitstekend gereken kan word in die lig van die kompleksiteit van die monstervoorbereiding.

Analitiese gegewens wat gedurende die twee bogenoemde navorsingsprojekte versamel is , word bespreek, en veral verbeteringe en nuwighede ten opsigte van bestaande bepalingsmetodes word uitgelig. Beide bepalingsmetodes is vir publikasie in aanstaande wetenskaplike joernale aanvaar en beide vollengte publikasies verskyn as bylae tot hierdie verhandeling tesame met alle korrespondensie wat met die journaal redakteur en referente aangegaan is. Verder is 'n bylaag ingesluit wat die skyfies bevat wat tydens die 1998 Jaarlikse Kongres van die Suid Afrikaanse Farmakologievereniging gebruik is in 'n mondelinge voordrag oor die piroksikam bepalingsmetode.

---

7 Appendix 1 Publication of analytical methods

Both of the assay methods discussed in this dissertation have been submitted and accepted as full-length publications in the Journal of Chromatography B. In so-doing, both pieces of research have been subjected to peer review. Correspondence entered into with the journal referees together with copies of the full-length articles have been included in this final section of the dissertation in the form of appendices.

7.1 6-Methoxy-2-naphthylacetic acid

DIE UNIVERSITEIT VAN DIE ORANJE-VRYSTAAT

Departement Farmakologie
Fakulteit Geneeskunde

✉ 339 BLOEMFONTEIN 9300 REPUBLIEK VAN SUID-AFRIKA
(051) 4013097 FAKS (051) 4471779 SA

August 12 1999

The Editor
Journal of Chromatography

Dear Sir

We hereby give permission that the article: 'Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by HPLC' may be published in the Journal of Chromatography.

Professor B.H. Meyer

Chief Executive Officer

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
Pharmacology and Drug Development
P.O. Box 339
Bloemfontein
9300 South Africa

Re: I 1427

Amsterdam, 24 August 1999

Dear Dr. De Jager,

On behalf of the editors of the *Journal of Chromatography B*, I have the pleasure of acknowledging receipt of the manuscript entitled: *Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography*, by De Jager, A.D., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

Your manuscript has been forwarded to the referees. You will be informed of their comments as soon as possible.

Yours sincerely,
EDITORIAL OFFICE

Trees Roof-Kramer

Street address: Molenwerf 1, 1014 AG Amsterdam, The Netherlands
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Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

3-page fax

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
Pharmacology and Drug Development
P.O. Box 339
Bloemfontein
9300 South Africa

Re: I 1427

Amsterdam, November 18, 1999

Dear Dr. De Jager,

On behalf of the editor handling your manuscript, Professor I.W. Wainer, I am writing to you in reference to your manuscript entitled: *Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography*, by De Jager, A.D., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

As you will see from the enclosed comments, a revision has been requested. I should be grateful if you would revise the paper in accordance with these recommendations. Also, please provide a detailed letter, replying to each point raised, and on one of the copies of the revised manuscript please underline in red the changes made in response to these comments.

We encourage submission of electronic manuscripts, which facilitates typesetting and proofreading. Therefore, please find enclosed a concise guide on the preparation of electronic manuscripts, which should be submitted together with three copies of the print-out. I look forward to receiving, in due course, three copies of a suitably amended version of your manuscript along with the letter of response. Please note that all correspondence concerning this manuscript should be addressed as follows: Journal of Chromatography B, Editorial Office, P.O. Box 681, NL-1000 AR Amsterdam, The Netherlands; fax: +31-20-4852304; e-mail: chrom-eo@elsevier.nl.

Yours sincerely,
EDITORIAL OFFICE

TRRoof

Trees Roof-Kramer

Street address: Molenwerf 1, 1014 AG Amsterdam, The Netherlands
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JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Reference's report Manuscript No.: I1427

Recommendations and comments should be sent within two weeks to the Editor. Please type your name and sign original only and return both copies.

Author : A.D. de Jager, M.L. Hundt, K.J. Swart, A.F. Hundt

Title : Extractionless determination of 6-MNA ...

1. Is the subject matter suitable for publication in the Journal of Chromatography B: Biomedical Applications? YES/NO

2. Is the paper acceptable
- ☐ in its present form
- ☐ with minor revision
- ☒ with revision
- ☐ with major revision
- ☐ with revision and condensation
- ☐ not at all

3. Is it clearly presented and well organized? YES/NO

4. Does it give adequate references to related work? YES/NO

5. Is the English satisfactory? YES/NO

Comments:

In the paper the authors have described a HPLC method for the determination of 6-methoxy-2-naphtylacetic acid, a major metabolite of nabumetone in human plasma. The developed method was applied for the comparative bio-equivalence study. I recommend this manuscript for publishing in your journal. However, it requires some revision before being accepted for final publication.

The comments are following:

1. In the paper authors mentioned that relatively few HPLC methods have been described for the determination of 6-MNA in human plasma. Short review of those methods would be desirable.

2. In *Reagents and materials* the phrase: "6-MNA and naproxen were kindly supplied by the sponsor" is not quite informative. The information about the manufacturer of these compounds is essential.

3. The information about preparation of the calibration standards and quality control samples is too general; more details are necessary.

4. No details are given about how the bio-equivalence study was done. Which two market brands of nabumetone were selected for the study? What subjects and how many were involved in that study? How were the blood samples collected?

5. In the paper authors presented the table with pharmacokinetic data without specifying how those data were calculated. The details about how the pharmacokinetic parameter were determined are necessary.

6 Please do not use abbreviations in the title

7 Reference list: delete comma after journals; delete issue numbers; Anal. Lett.; J. Chromatogr.; Int. J. Clin. Pharmacol. Ther. Toxicol.

DIE UNIVERSITEIT VAN DIE ORANJE-VRYSTAAT

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FARMOVS Research Centre for Clinical Pharmacology and Drug development
University of the Orange Free State
PO Box 339 (G6)
Bloemfontein
9300
South Africa

23 November 1999

Dear sir/madam.

This letter is in response to the queries raised by the referee, as requested

1. A short review of the relevant cited literature has now been included in the introduction.
2. The supplier of the analyte and internal standard has been specified in the text.
3. The 'preparation of calibration standards' section now includes a more detailed explanation of how standards and quality controls were prepared.
4. A brief overview of the study design, number of subjects and sampling conditions has been included in the introduction, as well as the name of the reference product.
5. The methodology used to calculate the cited pharmacokinetic parameters has been included in a section entitled 'calculation of pharmacokinetic variables'.
6. The abbreviation 'HPLC' in the title has been replaced by 'high-performance liquid chromatography'.
7. The reference list has been corrected accordingly.

Yours sincerely

Andrew David de Jager

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
Pharmacology and Drug Development
P.O. Box 339
Bloemfontein
9300 South Africa

Re: I 1427

Amsterdam, December 13, 1999

Dear Dr. De Jager,

On behalf of the editors, I acknowledge with thanks the safe receipt of the revised manuscript entitled: *Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography*, by De Jager, A.D., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

The manuscript has been sent back to the editor for re-evaluation. The decision concerning acceptance of your contribution will be communicated to you as soon as possible.

Yours sincerely,
EDITORIAL OFFICE

p/o

Trees Roof-Kramer

Street address: Molenwerf 1, 1014 AG Amsterdam, The Netherlands
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Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
Pharmacology and Drug Development
P.O. Box 339
Bloemfontein
9300 South Africa

Re: I 1427

Amsterdam, January 27, 2000

Dear Dr. De Jager,

On behalf of the editor handling your revised manuscript, Professor I.W. Wainer, I am pleased to inform you about the acceptance of the manuscript entitled: *Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography*, by De Jager, A.D., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

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Yours sincerely,

EDITORIAL OFFICE

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Journal of Chromatography B, 740 (2000) 247–251

JOURNAL OF CHROMATOGRAPHY B

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Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography

A.D. de Jager,[a,*], H.K.L. Hundt[a], A.F. Hundt[a], K.J. Swart[a], M. Knight[b], J. Roberts[b]

[a]*FARMOVS Research Centre for Clinical Pharmacology and Drug Development, University of the Orange Free State, PO Box 339 (G6), Bloemfontein 9300, South Africa*

[b]*Arthur H. Cox & Co. Ltd., Whiddon Valley, Barnstaple, Devon EX32 8NS, UK*

Received 28 August 1999; received in revised form 10 December 1999; accepted 1 February 2000

Abstract

Following oral administration of the prodrug nabumetone, the major metabolite 6-methoxy-2-naphthylacetic acid (6-MNA) was determined in human plasma. Minimal sample preparation was followed by reversed-phase liquid chromatography and UV detection, affording high sample throughput. The lower limit of quantification (LLOQ) was 70 ng/ml, at a signal-to-noise ratio of 8:1. The assay method displayed good correlation (r=0.997), and can be readily employed in pharmacokinetic and bioequivalence studies.

Keywords: 6-Methoxy-2-naphthylacetic acid; Nabumetone

1. Introduction

Nabumetone is a relatively new non-steroidal anti-inflammatory drug (NSAID) which has proved effective in the treatment of rheumatoid and osteoarthritis. This prodrug undergoes extensive first-pass metabolism, yielding 6-MNA, which is believed to be largely responsible for the pharmacological activity [1]. Relatively few HPLC methods have been described for the determination of nabumetone and/or 6-MNA in human plasma [2–5].

Jang et al. [3] and Ray et al. [5] both described liquid–liquid extractions, following acidification of drug-containing plasma. Jang et al. acidified using a of combination 0.1 *M* HCl and 0.5 *M* citrate buffer (pH 3), while Ray et al. used 1.5 *M* HCl only. Both authors then extracted the analyte into an organic phase [ether and *n*-hexane–ethyl acetate (50:50), respectively] and evaporated to dryness under a gentle stream of nitrogen, after which the samples were reconstituted and injected onto normal-phase HPLC columns. While Jang et al. detected fluorometrically (excitation=284 nm, emission=320 nm), Ray et al. made use of UV detection at 280 nm, fluorometric detection having a higher sensitivity (0.1 μg/ml). The extraction procedure described by Al-Momani et al. [2] appears to be loosely based on the method described by Ray et al., while using UV detection (270 nm). Jang and Al-Momani used approximately 1 ml of serum/plasma, while Ray et al. used 0.5 ml of plasma.

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00074-8

The present paper describes an HPLC assay of 6-MNA in which samples are prepared by protein precipitation, using naproxen as internal standard. Since a protein precipitation assay method has not yet been described, there is good motivation for developing such a procedure, considering the large number of samples generated by bioequivalence studies. The present paper describes a procedure that is well suited to rapid sample processing, requiring only a small volume of plasma (200 μl). 6-MNA was separated from the internal standard and endogenous plasma components using a Hewlett-Packard LiCrospher® 100 RP8 (5 μm) stainless-steel column (Hewlett-Packard, Palo, Alto, CA, USA), fitted with an Upchurch guard column, dry filled with Perisorb® RP-18 pellicular packing (Upchurch, WA, USA).

This assay method was used to quantify samples that were generated during a single-dose, two-way cross-over study with a wash out period of 14 days between 2 clinic days, following a 1000 mg oral dose of nabumetone. The study was designed to ascertain oral bioavailability between the reference product (Relifex®), and an as yet unnamed test product. The study was conducted on 30 healthy subjects (14 males and 16 females), aged between 19 and 29 years.

Venous blood samples were collected in heparinised glass tubes just prior to dosing and 1.5, 3, 4, 5, 6, 7, 8, 10, 12, 14, 18, 24, 48, 72, 96, 120 and 144 h thereafter. All samples were immediately handled on ice and centrifuged at 1200 *g* at 4°C for 10 min within 1 h of collection. Plasma was then transferred into sample tubes and stored at −20°C until analysis.

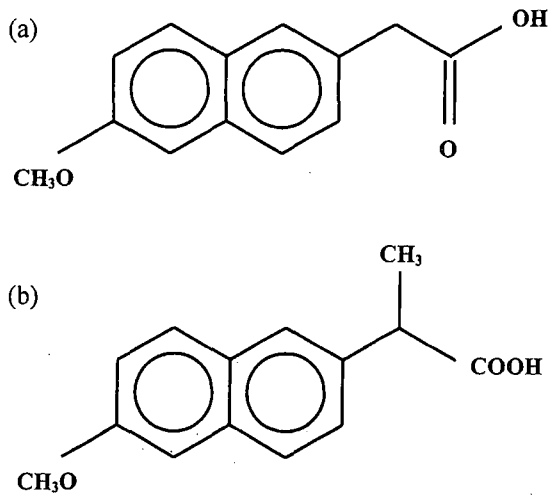

Fig. 1. (a) 6-MNA, (b) naproxen.

2. Experimental

2.1. Reagents and materials

6-MNA (Fig. 1a) was supplied by Wessex Fine Chemicals Ltd. (Billingshurst, West Sussex, UK) and naproxen (Fig. 1b) obtained from Syntex Inc. (Palo, Alto, CA, USA). Acetonitrile and methanol (B & J Brand™) were obtained from Baxter (Muskegon, MI, USA). Orthophosphoric acid (85%) and di-sodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Citric acid (Fluka, Buchs, Switzerland) and 1-heptane sulphonic acid (Saarchem, Krugersdorp, South Africa) were used without further purification. All water used was purified by RO 20SA reverse osmosis system and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

2.2. Apparatus

UV detection was performed by a Hewlett-Packard series 1100 variable wavelength detector monitoring at 280 nm. Separation was achieved on a LiCrospher® 100 RP8 (5 μm) stainless-steel column (Hewlett-Packard). The mobile phase consisted of acetonitrile–citric acid buffer (450:550, 20 m*M*, pH 2.8). 1-Heptane sulphonic acid (300 mg/l) was added and the apparent pH of the mobile phase adjusted to 3.1 using concentrated orthophosphoric acid. Mobile phase was delivered by a series 1100 isocratic pump (Hewlett-Packard, Palo, Alto, CA, USA) at 1.0 ml/min and at ambient temperature. A Hewlett-Packard series 1100 autosampler injected 10 μl onto the HPLC column. High speed centrifuging of microfuge tubes (Eppendorf, Hamburg, Germany) was done in a centrifuge 5416 (Eppendorf).

2.3. Preparation of calibration standards

6-MNA (5.091 mg) was weighed and directly dissolved in 36.06 g of blank plasma, contained in a

stoppered Erlenmeyer flask. This calibration standard was then shaken for 2 h using a GFL sample shaker (Bugwedel, Germany). This produced the highest calibration standard (145 μg/ml). Plasma dilution (1:1) of this calibration standard and subsequent standards yielded a total of 12 calibration standards, spanning a concentration range of 0.070–145 μg/ml.

Upper range quality controls (54.6, 30.4 and 17.3 μg/ml) were prepared in a similar fashion by dissolving 3.901 mg of 6-MNA in 73.40 g of blank plasma and preparing two serial dilutions from this highest quality control. The lower range quality controls were prepared by dissolving 2.472 mg of 6-MNA in 11.126 g of methanol and spiking 100 μl of this solution into 109.10 g of blank plasma. This quality control (0.330 μg/ml) was then serially diluted with plasma to produce the remaining quality controls (0.248, 0.165 and 0.083 μg/ml). The calibration standards and quality controls were stored at −20°C until assayed. Sufficient calibration standards and quality controls were prepared to develop and validate the assay method, as well as to assay all study samples. A new calibration line, along with quality controls, was included in each assay batch.

2.4. Sample preparation

Plasma (200 μl) was pipetted into a microfuge tube and an equal volume of acetonitrile containing naproxen (80 μg/ml) added. The microfuge tube was then sealed, vigorously vortexed (1 min), and centrifuged (6800 *g*, 5 min). An aliquot of 10 μl of the supernatant layer was injected onto the HPLC column.

3. Results and discussion

3.1. Sample preparation

Due to the large number of samples generated during comparative bioequivalence studies, it was thought beneficial to investigate extractionless sample preparation as an alternative to liquid–liquid and solid-phase extraction, with a view to maximising sample throughput. Furthermore, plasma concentrations following a single 1000-mg oral dose are appreciable [1] and it was speculated that the loss in sensitivity, traded off against rapid sample handling, would not be significant enough to render protein precipitation inappropriate for single-dose bioequivalence studies.

3.2. Method validation

Based on the ratio between analyte and internal standard peak height, the calibration line was found to be linear over the range 0.070–145 μg/ml and was characterised by the equation $y=(0.017$ to $0.021)x+(-0.0006$ to $0.0003)$, with a mean calibration curve slope C.V. of 6.2% ($n=15$) over a period of 3 weeks when using a $1/\text{concentration}^2$ weighting.

3.3. Chromatography

Analyte and internal standard were well separated from endogenous plasma components (Fig. 2), affording good chromatographic results.

3.4. Recovery

The analyte was fully recovered (~100%) in the supernatant layer, and the internal standard recovery was found to be 70%. Analyte recovery was determined in five-fold at high (30.4 μg/ml), medium (0.330 μg/ml) and low (0.083 μg/ml) concentration.

3.5. Sensitivity

The LLOQ was set at 70 ng/ml at a signal-to-noise ratio of 8:1, which was sufficient to detect 6-MNA in human plasma up to 144 h ($6\times t_{1/2}$), following a single 1000-mg dose of nabumetone. Over a time period of 144 h in which study samples were collected and assayed during a bioequivalence study, no sample was found to have a 6-MNA concentration below this LLOQ.

3.6. Inter and intra-day accuracy and precision

The intra- and inter-day accuracy and precision of the assay method (Tables 1 and 2) showed the assay method to be robust over the 21 days in which 15 batches, each consisting of 72 samples, 10 cali-

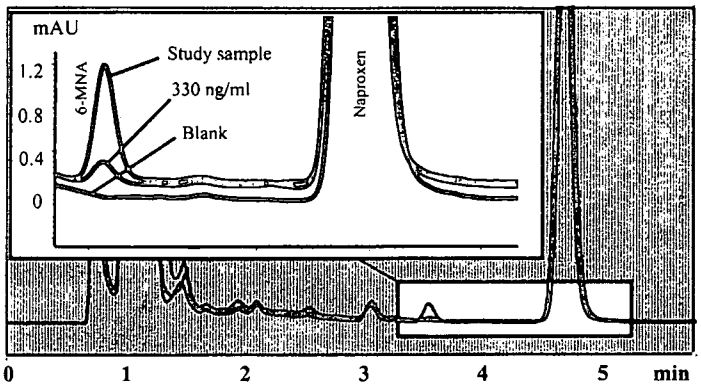

Fig. 2. Enlargement of overlaid chromatograms, showing good resolution between 6-MNA and endogenous plasma components. The study sample (96 h after the ingestion of 1000 mg of nambumetone) produces a peak roughly five-times larger than a 330 ng/ml calibration standard.

Table 1
Intra-day accuracy and precision of quality controls ($n=5$)

Nominal concentration (ng/ml)	Found concentration (ng/ml)	n	C.V. (%)
30 400	30 400	5	2.7
17 300	17 300	5	2.9
330	334	5	4.1
248	254	5	6.5
165	165	5	2.3
83.0	91.0	5	4.7

bration standards and 8 quality controls were processed.

3.7. Matrix stability

Calibration standard and quality control response factors were monitored daily and compared to freshly prepared standard solutions of 6-MNA in the mobile phase. 6-MNA was found to be stable in human plasma for at least 3 weeks when stored at −20°C.

Table 2
Inter-day accuracy and precision of assay batch quality controls, completed over 21 days

Nominal concentration (ng/ml)	Found concentration (ng/ml)	n	C.V. (%)
30 400	30 220	30	3.2
17 300	16 890	30	4.0
330	322	29	5.8
165	154	15	7.5
83.0	79.6	13	6.5

3.8. Specificity

Plasma from six different sources was extracted and no peak was found to co-elute with 6-MNA or the internal standard. In addition, pre-dosing samples from the 30 subjects assayed were included in the batches and no interference was observed.

3.9. On-instrument stability

6-MNA was found to be stable in the final reconstituted solution for the period during which samples were on the instrument (approximately 10 h).

3.10. Application

This assay method was employed in a single-dose bioequivalence study of two film-coated oral formulations of nabumetone. Good chromatographic results were obtained in spite of minimal sample preparation. There was good concordance between the calculated parameters and existing literature [1], as reflected in Table 3.

Table 3
Comparison between experimentally determined pharmacokinetic data and existing literature

Reference	n	Dose (mg)	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC(0–∞) (μg h/ml)
Relifex®	29	1000	25.1 (10.1–38.5)	24.0 (1.5–48.0)	22.8 (13.8–32.1)	1444 (537–2436)
Test product	29	1000	26.5 (10.3–46.3)	10.0 (3.0–24.0)	23.1 (16.0–33.1)	1412 (513–2213)
Kendall et al. [1]	12	1000	21.9	13	26.3	1120

3.11. Calculation of pharmacokinetic variables

The maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were read directly from the observed concentrations. The apparent terminal half-life ($t_{1/2}$) was calculated by fitting the concentration versus time data of the terminal phase to a single exponential function (Ce^{-kt}) using sum of least squares regression analysis. The terminal half-life was then calculated as $t_{1/2}=0.693/k$, where k is the terminal rate constant. The area under the plasma concentration versus time data, extrapolated to infinity [AUC(0–∞)], was calculated by adding $C(t_{last})/k$ (the plasma concentration at the last sampling time divided by the terminal rate constant) to the total area beneath the plasma concentration versus time curve at the last sampling time (AUC(0–t_{last})). Thus $AUC(0–\infty) = AUC(0 - t_{last}) + C(t_{last})/k$. The mean profiles (Fig. 3) display good agreement between the test and reference product. The financial viability of processing all the samples generated during the study was first assessed by the comparison of pooled samples from each treatment phase [6], producing mean treatment profiles. On the strength of the pooled plasma results, samples were individually processed. The mean 6-MNA concentration versus time profiles (Fig. 3) compare well with those reported by Kendall et al. [1]

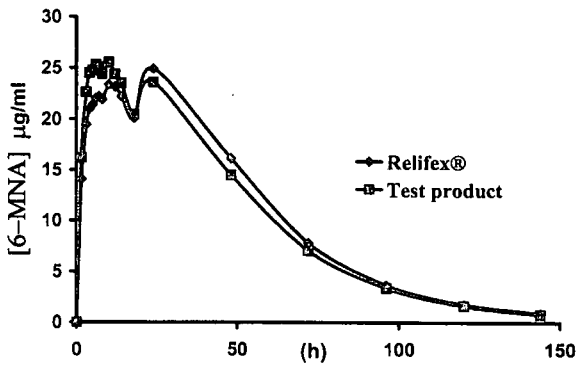

Fig. 3. Mean profiles of the test and reference product (Relifex), following a 1000-mg oral dose of nabumetone.

References

[1] M.J. Kendall, M.C. Chellingsworth, R. Jubb, A.R. Thawley, N.A. Undre, D.C. Kill, Eur. J. Clin. Pharmacol. 36 (1989) 299.
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Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
Pharmacology and Drug Development
P.O. Box 339
Bloemfontein
9300 South Africa

Re: E 52

Amsterdam, 19 October 1998

Dear Dr. De Jager,

On behalf of the editors of the *Journal of Chromatography B*, I have the pleasure of acknowledging receipt of the manuscript entitled: *High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues*, by De Jager, A.D., Ellis, H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

Your manuscript has been forwarded to the referees. You will be informed of their comments as soon as possible.

Yours sincerely,
EDITORIAL OFFICE

Trees Roof-Kramer

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
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P.O. Box 339
Bloemfontein
9300 South Africa

Re: E 52

Amsterdam, 11 December 1998

Dear Dr. De Jager,

On behalf of the editor handling your manuscript, Prof. Dr. G.J. de Jong, I am writing to you in reference to your manuscript entitled: *High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues*, by De Jager, A.D., Ellis, H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

As you will see from the enclosed comments, a revision has been requested. I should be grateful if you would revise the paper in accordance with these recommendations. Also, please provide a detailed letter, replying to each point raised by the referees, and on one of the copies of the revised manuscript please underline in red the changes made in response to these comments.

We encourage submission of electronic manuscripts, which facilitates typesetting and proofreading. Therefore, please find enclosed a concise guide on the preparation of electronic manuscripts, which should be submitted together with three copies of the print-out. I look forward to receiving, in due course, three copies of a suitably amended version of your manuscript along with the letter of response to the referee comments. Please note that all correspondence concerning this manuscript should be addressed as follows: Journal of Chromatography, Editorial Office, P.O. Box 681, NL-1000 AR Amsterdam, The Netherlands; fax: (+31-20) 4852304; e-mail: chrom-eo@elsevier.nl.

Yours sincerely,
EDITORIAL OFFICE

Trees Roof-Kramer

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES & APPLICATIONS

Manuscript No.: E52

Recommendations and comments should be sent within two weeks to the Editor.
Please type your name and sign original only and return both copies.

Author: A.D. de Jager, H. Ellis et al.

Title: High performance liquid chromatographic determination with amperometric ...

1. Is the subject matter suitable for publication in the Journal of Chromatography B: Biomedical Sciences and Applications? YES/~~NO~~

2. Is the paper acceptable
   - ☐ in its present form
   - ☒ with minor revision
   - ☐ with revision
   - ☐ with major revision
   - ☐ with revision and condensation
   - ☐ not in present form
   - ☐ not at all

3. Is it clearly presented and well organized? YES/~~NO~~

4. Does it give adequate references to related work? ~~YES~~/NO

5. Is the English satisfactory? YES/~~NO~~

Comments:

The figures and tables must be referenced in the text.
If figures 2 and 3 are made smaller in order to fit into the page size of the journal, are they still readible?
The recovery of piroxicam was determined only at a relatively high concentration level. For quantitation it is further assumed that there are no differences in recovery between the sample types. Is this further proven?

For comparison reasons the lay-out of the tables II and III should be the same.
There is no discussion given about the intra- and inter-laboratory accuracy and reproducibility data.

Ratios were determined between steady-state plasma concentrations and the levels in the other biological matrices. Table IV shows in all cases mean ratios larger than 1. However, the relative standard deviations are rather high (about 100%). Since plasma levels may vary considerably during a day, I would like to know how these experiments were carried out. Were plasma samples taken over a certain time period and the values found averaged? Is anything known in literature about these ratios, e.g. Hundak, Kvien et al.: Total and free plasma and total synovial fluid piroxicam concentrations: relationships to antiinflammatory effects in patients with reactive arthitis and other arthitis. Scanadian journal of reumatology 22(4), 1993, 183-187.

The literature references are rather old. I think more recent references are available, e.g. M. Amanlou et al.: Rapid method for the determination of piroxicam in rat plasma using HPLC. J. Chromatography B, 696(2), 1997, 317-319.

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES & APPLICATIONS

Manuscript No.: E52

Recommendations and comments should be sent within two weeks to the Editor.
Please type your name and sign original only and return both copies.

Author: A. D. de Jager et al

Title: HPLC determination with amperometric detection of piroxicam in human...

1. Is the subject matter suitable for publication in the Journal of Chromatography B: Biomedical Sciences and Applications? YES/NO

2. Is the paper acceptable
   - ☐ in its present form
   - ☒ with minor revision
   - ☐ with revision
   - ☐ with major revision
   - ☐ with revision and condensation
   - ☐ not in present form
   - ☐ not at all

3. Is it clearly presented and well organized? YES/NO

4. Does it give adequate references to related work? YES/NO

5. Is the English satisfactory? YES/NO

Comments:

This is a clearly written article which describes a HPLC method which allows the low-level determination of piroxicam in plasma and knee tissues after topical application. It should be published after some minor alterations:

1. The reference list is rather dated, there are other more recent discussions in the literature on the determination of piroxicam, including at least one study where plasma levels were determined after topical administration.

2. Some minor formatting comments – the Figures should be referred to and discussed in the "results and discussion" section, rather than being placed in the "introduction" or "experimental" parts as they are now. The figures could be printed with heavier weight lines, otherwise they may not reproduce very well in the journal.

3. Please give details of the calibration line, i.e. the equation of the line, the standard errors in slope and intercept, n, etc.

Minor points: p. 3, please give a supplier location for the column, I am not familiar with this particular phase; p. 5, "200 µl" and "0.2 ml" are used in the same paragraph, please standardize; Tables II and III, in one the nominal concentration goes from high to low reading across the page, in the other it reads from low to high – please make them consistent

For minor editorial comments, see manuscript pages enclosed

Please return these pages along with the revised version

Figure 1

EXPERIMENTAL

Reagents and materials

Piroxicam was supplied by Francochim, France. HPLC grade tetrahydrofuran, methanol, hexane and dichloromethane (Burdick & Jackson) were obtained from Baxter (Muskegon,USA). Potassium chloride (Merck, Darmstadt, Germany) and 85% ortho-phosphoric acid (Fluka, Buchs, Switzerland), were used without further purification. All water used was purified by RO 20SA reverse osmosis system and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

Apparatus

Mobile phase was delivered by a series 1100 isocratic pump (Hewlett-Packard, Palo, Alto, CA) at 0.5 ml/min. The mobile phase consisted of 30 mM ortho-phosphoric acid : methanol : tetrahydrofuran (600 : 320 : 80). Potassium chloride (150 mg/L) was added to the mobile phase and the apparent pH adjusted to 2.70 using 4 M NaOH. Electrochemical detection was by way of a Hewlett-Packard 1049A programmable electrochemical detector, in amperometric mode, set at an oxidation potential of +0.650 V. A Hewlett-Packard series 1050 autosampler injected 20 µl onto the HPLC column. While on the autosampler, samples were cooled to 4 °C using a Lauda RM 6 circulatory cooling system (Lauda, Königshofen). A Higgins Haisil 120 BD C18 (120 mm × 3.0 mm I.D., 5 µm particle size) column was used, and was maintained at 30 °C using a CTO 6A column oven (Shimadzu, Kyoto, Japan). Where necessary, sample ultrasonication was done using a

Sonorex RK 100 ultrasonicator (Bandelin). Homogenisation of sample was done using a T25 Ultra-Turrax (Janke & Kunkel, Germany), fitted with an IKA® UT disperser (O.D. 8 mm). The centrifuging of ampoules was done in a Megafuge 1.0R (Heraeus , Hanau, Germany). High speed centrifuging of microfuge tubes was performed by a Centrifuge 5416 (Eppendorf - Hamburg, Germany). Sample freezing was done on a Fryka Polar KP 250 cooling plate (Kältetechnik , Esslingen, Germany). A Speed Vac® concentrator (Savant , Holbrook, New York) was used for sample concentration.

Preparation of calibration standards

Spiking solutions of piroxicam were prepared in methanol, and calibration standards were prepared by spiking blank plasma, and then making a series of plasma dilutions that yielded 13 calibration standards, spanning a concentration range of 0.72 ng/ml - 600 ng/ml. A set of 7 quality controls, that spanned the same range, were independently made using the same methodology, and used to verify the intra-day and inter-day assay method performance. The calibration standards and quality controls were stored at -20°C until assayed.

As the extraction recovery of piroxicam from all four matrices was similar (ca. 60%), calibration standards and quality controls were prepared in plasma only, and all samples were quantified from the plasma calibration line, applying appropriate multiplication factors where necessary.

Sufficient calibration standards and quality controls were prepared to develop and validate the assay method, and to assay all study samples. A new calibration line, along with quality controls, was included in each assay batch.

Sample preparation

Plasma extraction

Step 1 : To 0.5 ml plasma, in a 5 ml amber ampoule, was added 200 µl 0.1M HCl. ***Step 2*** : 4 ml Dichloromethane : hexane (1 + 4) was added to the sample, vortexed for 1 minute, and centrifuged for 1 minute at 650 g , 4 °C. ***Step 3*** : The aqueous layer was frozen and the organic layer decanted into a second 5 ml amber ampoule containing 0.2 ml 0.1 M NaOH. ***Step 4*** : The sample was vortexed for 1 minute and centrifuged for 1 minute (650 g, 4 °C). ***Step 5*** : The aqueous layer was frozen, and the organic supernatant discarded. ***Step 6*** : To the remaining aqueous layer was added 0.5 ml 0.1 M HCl and the sample placed in a water bath (37 °C) for 1 minute. ***Step 7*** : 3 ml Dichloromethane : hexane (1 + 4) was added and the sample vortexed for 1 minute and centrifuged (650 g , 4 °C) for 1 minute. ***Step 8*** : The final aqueous layer was frozen, the organic layer decanted into a third 5 ml ampoule, and the sample evaporated to dryness. ***Step 9*** : The residue was reconstituted in 200 µl mobile phase, vortexed for 10 seconds, and 20 µl injected onto the HPLC column.

Sub-cutaneous tissue and synovial capsule extraction

Step A : To approximately[1] 0.2 g tissue sample (accurately weighed) in a polypropylene tube (12 × 55 mm) was added 0.1 N NaOH (volume equivalent to 5 × the mass of the tissue sample), and the sample ultrasonicated for 1 hour. Parafilm "M" ® (Chicago, IL,) was used to seal the polypropylene tube while in the ultrasonicator, to circumvent sample loss by spluttering and evaporation. ***Step B*** : The sample was homogenized at 22000 rpm for 1 minute ***Step C*** : The sample was transferred into an Eppendorf microfuge tube and centrifuged at 8500 g for 5 minutes.

Step C : 0.5 ml Of the aqueous layer[2] was transferred into a 5 ml amber ampoule. The sample was acidified by adding 0.2 ml 1.0 M HCl, and the remainder of the extraction performed as per *steps 2-9*, described in the *plasma extraction* section.

Synovial fluid extraction

Step I : Prior to initial pipetting, the sealed sample was ultrasonicated for 20 minutes, rendering it more manageable when using a conventional air-interface pipette. ***Step II*** : 0.2 ml Sample was pipetted into a polypropylene tube, and 1 ml 0.1 M NaOH added. ***Step III*** : The tube was briefly vortexed, sealed with Parafilm "M"®, and ultrasonicated for 1 hour. ***Step IV*** : The sample was again briefly vortexed, 0.5 ml transfered into a 5 ml amber ampoule, and acidified by adding 0.2 ml 1.0 M HCl. The remainder of the extraction was performed as per *steps 2-9*, described in the *plasma extraction* section.

Representative chromatograms

Figure 2

Figure 3

[1] When adding 0.1 N NaOH to the sample, the volume was adjusted so that the ratio of sample (g) to volume 0.1 N NaOH added (ml) remained constant at 1 + 5.

[2] The aqueous layer was found between a supernatant layer of congealed matter, that was cream in colour, and an underlying layer of protein. This congealed, supernatant layer was carefully removed with a wooden applicator, exposing the aqueous layer.

TABLE II (n=5)

INTRA-DAY ACCURACY AND PRECISION OF PLASMA QUALITY CONTROLS.

Nominal (ng/ml)	538.37	299.69	149.72	3.47	2.61	1.74	0.86
n	5	5	5	5	5	5	5
Mean	543.41	301.97	145.06	3.44	2.56	1.88	0.88
CV%	4.4	7.4	3.8	10.7	6.4	4.1	7.3

I would propose the following layout.

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	CV (%)
538	543.4	4.4
300	etc	etc
150		
3.5		
2.6		
1.7		
0.9		

Please check the number of significant figures

TABLE III

INTER-DAY ACCURACY AND PRECISION OF PLASMA CALIBRATION STANDARDS.

Nominal (ng/ml)	1.24	2.47	4.94	19.8	49.5	151	199	299	600
n	14	9	7	8	8	8	8	8	8
Mean	1.23	2.56	4.82	20.81	49.53	148.55	197.29	302.50	584.3
CV%	8.1	10.1	11.8	6.2	4.4	6.4	6.9	3.5	3.2

For layout, see Table II

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Analytical Services Division

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26 January 1999

Dear sir/madam.

This letter is in response to the queries raised by the referees, as requested. As a small point of order, I must inform you that I am Mr. and not Dr. de Jager, as per the destination address of your response.

Referee 1

1. Tables and figures are now referred to in the text, instead of just being included. Some discussion on these tables has also been included.
2. The recovery of piroxicam from plasma was determined at high (454ng/ml) and two relatively low (3.47ng/ml, 0.86ng/ml) concentrations. The recoveries at these extremes were the same, and the values were then averaged, producing the quoted plasma recovery. The recovery of piroxicam from SC, SF and SCT homogenate was done at a single, relatively high concentration. The recovery determination for each of these three matrices was done in triplicate. Owing to the fact that it is difficult to obtain these three matrices, it was assumed that the recovery from these three matrices was the same over a wider range, as was proven to be the case with plasma. Separate recovery determinations were done for each of the four matrices, and it was not arbitrarily assumed that the recovery from the three matrices was the same as for plasma. All the recoveries quoted were experimentally determined.
3. Tables II and III have been altered as requested, and an additional table included presenting the inter-day performance of the method over a period of three months.
4. The reported ratios were produced as follows: piroxicam was repeatedly applied to the knee joint in the specified number of days preceding surgery. Plasma drawn just prior to surgery was considered to reflect the steady-state level. During

surgery, the various tissues were obtained. The concentrations in all four matrices were then analytically determined, and the ratios produced as follows: for each individual subject, piroxicam concentrations in SC, SCT an SF were compared to the subject's own steady-state plasma concentration to produce the three ratios. This process was repeated for each individual subject. A mean of these ratios was then produced and quoted, as per the text.

5. Clearly, there is great variability in these ratios, in fact the steady-state plasma levels themselves varied greatly. Hundal et al. quoted very different ratios, which were in fact smaller than 1, which too showed a large degree of variation, admittedly not as large as ours. I think the explanation lies in the fact that their determinations were done following oral administration of piroxicam, while ours were done following topical application. Marks, R., Dykes, P., (Skin Pharmacology. 7(6) 340-4, 1994) found that high levels of piroxicam were to be found just beneath the skin following topical application, with the levels decreasing with depth in experimental skin biopsies. They concluded that piroxicam rapidly permeates the stratum corneum into the epidermis/dermis after application of the gel. The levels that they found in plasma were often undetectable with their analytical method.
6. I have since consulted the literature of Amanlou et al. and made reference to it in the text of the article.

Referee 2

1. More recent literature has been consulted and included. I have made reference to a study that determined piroxicam in plasma following topical application of piroxicam.
2. The formatting errors have been corrected, and figures have been printed with heavier lines.
3. Details of the calibration line have been included in the text.
4. Details of the column manufacturer have been included in the text. The inconsistencies in the units of volume on p. 5 have been corrected, and the nominal concentrations of tables II and III are now both in descending order.

Yours sincerely

Andrew David de Jager

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
Pharmacology and Drug Development
P.O. Box 339
Bloemfontein
9300 South Africa

Re: E 52

Amsterdam, 5 February 1999

Dear Dr. De Jager,

On behalf of the editors, I acknowledge with thanks the safe receipt of the revised manuscript entitled: *High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues*, by De Jager, A.D., Ellis, H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

The manuscript has been sent back to the editor for re-evaluation. The decision concerning acceptance of your contribution will be communicated to you as soon as possible.

Yours sincerely,
EDITORIAL OFFICE

Trees Roof-Kramer

Street address: Molenwerf 1, 1014 AG Amsterdam, The Netherlands
Telefax: 020-4852304 - Telephone: 020-4852796 - E-mail: chrom-eo@elsevier.nl

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
FARMOVS Research Centre for Clinical
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P.O. Box 339
Bloemfontein
9300 South Africa

Re: E 52

Amsterdam, 3 March 1999

Dear Dr. De Jager,

On behalf of the editor handling your revised manuscript, Prof. Dr. G.J. de Jong, I am writing to you in reference to your manuscript entitled: *High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues*, by De Jager, A.D., Ellis, H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

I am pleased to inform you that the revised paper has been favourably received and that publication after minor revision is recommended (see enclosure). I should be grateful if you would revise the paper in accordance with these recommendations. Also, please provide a detailed letter, replying to each point raised by the referees, and on one of the copies of the revised manuscript please underline in red the changes made in response to these comments.

We encourage submission of electronic manuscripts, which facilitates typesetting and proofreading. Therefore, please find enclosed a concise guide on the preparation of electronic manuscripts, which should be submitted together with three copies of the print-out. I look forward to receiving, in due course, three copies of a suitably amended version of your manuscript along with the letter of response to the referee comments. Please note that all correspondence concerning this manuscript should be addressed as follows: Journal of Chromatography, Editorial Office, P.O. Box 681, NL-1000 AR Amsterdam, The Netherlands; fax: (+31-20) 4852304; e-mail: chrom-eo@elsevier.nl.

Yours sincerely,
EDITORIAL OFFICE

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Trees Roof-Kramer

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
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P.O. Box 339
Bloemfontein
9300 South Africa

Re: E 52

Amsterdam, 3 March 1999

Dear Dr. De Jager,

On behalf of the editor handling your revised manuscript, Prof. Dr. G.J. de Jong, I am writing to you in reference to your manuscript entitled: *High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues*, by De Jager, A.D., Ellis, H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

I am pleased to inform you that the revised paper has been favourably received and that publication after minor revision is recommended (see enclosure). I should be grateful if you would revise the paper in accordance with these recommendations. Also, please provide a detailed letter, replying to each point raised by the referees, and on one of the copies of the revised manuscript please underline in red the changes made in response to these comments.

We encourage submission of electronic manuscripts, which facilitates typesetting and proofreading. Therefore, please find enclosed a concise guide on the preparation of electronic manuscripts, which should be submitted together with three copies of the print-out. I look forward to receiving, in due course, three copies of a suitably amended version of your manuscript along with the letter of response to the referee comments. Please note that all correspondence concerning this manuscript should be addressed as follows: Journal of Chromatography, Editorial Office, P.O. Box 681, NL-1000 AR Amsterdam, The Netherlands; fax: (+31-20) 4852304; e-mail: chrom-eo@elsevier.nl.

Yours sincerely,
EDITORIAL OFFICE

TRRoof

Trees Roof-Kramer

EDITOR's COMMENTS:

E 52 revised

- Only a mean of the concentration has to be given in Table 4.
- The ratio of ref. 10 is not clear and a comparison with the ratio found in the present paper is missing.
- The legends of figures 1 and 2 do not contain sufficient details.

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18 March 1999

Dear sir/madam.

This letter is in response to the queries raised by the referee, as requested.

1. Table 4 now contains only mean values.
2. A comparison between the work done by Hundal et al and our findings has been included in the *application* section of the text.
3. Some elucidation has been included in the legends of fig. 1 & 2.

Yours sincerely

Andrew David de Jager

Journal of Chromatography B

Biomedical Sciences and Applications

EDITORIAL OFFICE: P.O. BOX 681, 1000 AR AMSTERDAM, THE NETHERLANDS

Dr. A.D. De Jager
University of Orange Free State
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Re: E 52

Amsterdam, 30 March 1999

Dear Dr. De Jager,

On behalf of the editor handling your revised manuscript, Prof. Dr. G.J. de Jong, I am pleased to inform you about the acceptance of the manuscript entitled: *High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues*, by De Jager, A.D., Ellis, H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

Transfer of copyright form and galley proofs will be sent to you in due course by the publisher.

For questions with regard to proofs, publication date or reprints of your article, please contact our Author Support Department (fax: +31-20-4853752; e-mail: authorsupport@elsevier.nl).

Yours sincerely,
EDITORIAL OFFICE

Trees Roof-Kramer

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JOURNAL of CHROMATOGRAPHY B

Journal of Chromatography B, 729 (1999) 183–189

High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues

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Received 19 October 1998; received in revised form 30 March 1999; accepted 30 March 1999

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JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic determination with amperometric detection of piroxicam in human plasma and tissues

A.D. de Jager*, H. Ellis, H.K.L. Hundt, K.J. Swart, A.F. Hundt

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Received 19 October 1998; received in revised form 30 March 1999; accepted 30 March 1999

Abstract

After repeated topical application of a piroxicam gel preparation to the knee, piroxicam was quantified in plasma, subcutaneous tissue, synovial capsule and synovial fluid, using specimens obtained during knee surgery. Electrochemical detection was used and the limit of quantification (LOQ) was 0.72 ng/ml in plasma at a signal-to-noise ratio of 10:1. The chromatographic method was optimised to determine piroxicam in all four matrices, and the analyte was quantified using a calibration line constructed from plasma calibration standards. Levels in subcutaneous tissue, synovial capsule and synovial fluid were compared to plasma steady-state levels and expressed as a ratio, in order to ascertain bioavailability.

Keywords: Piroxicam

1. Introduction

Piroxicam [4-hydroxy-2-methyl-*N*-(2-pyridyl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide] is a non-steroidal anti-inflammatory drug (NSAID) that has been used to treat various arthropathies and inflammatory diseases in humans [1].

A number of high-performance liquid chromatography (HPLC) methods have been employed to determine piroxicam in biological samples [1–8], and while electrochemical detection (ED) of piroxicam is by no means novel [2], UV detection is most commonly employed [1,3–8], as it is sufficiently sensitive to detect levels associated with common oral dosage regimens. As is the case with some commonly used NSAIDs, the detection limit of piroxicam improves by between five- and 20-fold when ED is used instead of UV detection [2]. Low systemic concentrations, associated with topical dosage regimens, necessitated the development of a more sensitive assay method.

The present paper describes a multiple liquid–liquid extraction using dichloromethane–hexane (1:4). While the extraction efficiency from the four matrices concerned (ca. 60% from all the matrices) was inferior to that of most existing methods, the benefits of a clean extract far outweighed the disadvantages of inferior recovery and a laborious extraction procedure. The purity of the extracts allowed for the determination of piroxicam in all four matrices, without any adjustments to the mobile phase or HPLC column.

Considering the small amounts of biological sam-

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PII: S0378-4347(99)00169-3

ple ethically attainable during surgery, and the lower piroxicam levels associated with topical applications, electrochemical detection was considered the method of choice, using an oxidation potential of +0.650 V. A much improved limit of quantitation (LOQ) of 0.72 ng/ml in plasma was attained at a signal-to-noise ratio of 10:1.

2. Experimental

2.1. Reagents and materials

Piroxicam was supplied by Francochim (Blagnac, France). HPLC grade tetrahydrofuran, methanol, hexane and dichloromethane (B&J brand) were obtained from Baxter (Muskegon, MI, USA). Potassium chloride (Merck, Darmstadt, Germany) and 85% orthophosphoric acid (Fluka, Buchs, Switzerland), were used without further purification. All water used was purified by RO 20SA reverse osmosis system and Milli-Q polishing system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Mobile phase was delivered by a series 1100 isocratic pump (Hewlett-Packard, Palo Alto, CA, USA) at 0.5 ml/min. The mobile phase consisted of 30 m*M* orthophosphoric acid–methanol–tetrahydrofuran (600:320:80). Potassium chloride (150 mg/l) was added to the mobile phase and the apparent pH adjusted to 2.70 using 4 *M* NaOH. Electrochemical detection was by way of a Hewlett-Packard 1049A programmable electrochemical detector, in amperometric mode, set at an oxidation potential of +0.650 V. A Hewlett-Packard series 1050 autosampler injected 20 μl onto the HPLC column. While on the autosampler, samples were cooled to 4°C using a Lauda RM 6 circulatory cooling system (Lauda, Königshofen, Germany). A Higgins Haisil 120 BD C_{18}, 120 mm×3.0 mm I.D., 5 μm column was used (Higgins Analytical, CA, USA), and was maintained at 30°C using a CTO 6A column oven (Shimadzu, Kyoto, Japan). Where necessary, sample ultrasonication was done using a Sonorex RK 100 ultrasonicator (Bandelin, Berlin, Germany). Homogenisation of sample was done using a T25 Ultra-Turrax (Janke and Kunkel, IKA Labortechnik, Staufen, Germany), fitted with an IKA UT disperser (O.D. 8 mm). The centrifuging of ampoules was done in a Megafuge 1.0R (Heraeus, Hanau, Germany). High speed centrifuging of microfuge tubes was performed by a centrifuge 5416 (Eppendorf, Hamburg, Germany). Sample freezing was done on a Fryka Polar KP 250 cooling plate (Kältetechnik, Esslingen, Germany). A Speed Vac concentrator (Savant, Holbrook, NY, USA) was used for sample concentration.

2.3. Preparation of calibration standards

Spiking solutions of piroxicam were prepared in methanol, and calibration standards were prepared by spiking blank plasma, and then making a series of plasma dilutions that yielded 13 calibration standards, spanning a concentration range of 0.72–600 ng/ml.

A set of seven quality controls, that spanned the same range, were independently made using the same methodology, and used to verify the intra-day and inter-day assay method performance. The calibration standards and quality controls were stored at −20°C until assayed.

As the extraction recovery of piroxicam from all four matrices was similar (ca. 60%), calibration standards and quality controls were prepared in plasma only, and all samples were quantified from the plasma calibration line, applying appropriate multiplication factors where necessary.

Sufficient calibration standards and quality controls were prepared to develop and validate the assay method, and to assay all study samples. A new calibration line, along with quality controls, was included in each assay batch.

2.4. Sample preparation

2.4.1. Plasma extraction

Step 1: To 0.5 ml plasma, in a 5-ml amber ampoule, was added 0.2 ml 0.1 *M* HCl. Step 2: 4 ml dichloromethane–hexane (1:4) was added to the sample, vortexed for 1 min, and centrifuged for 1 min at 650 *g*, 4°C. Step 3: The aqueous layer was frozen and the organic layer decanted into a second 5-ml amber ampoule containing 0.2 ml 0.1 *M* NaOH.

Step 4: The sample was vortexed for 1 min and centrifuged for 1 min (650 *g*, 4°C). Step 5: The aqueous layer was frozen, and the organic supernatant discarded. Step 6: To the remaining aqueous layer was added 0.5 ml 0.1 *M* HCl and the sample placed in a water bath (37°C) for 1 min. Step 7: 3 ml dichloromethane–hexane (1:4) was added and the sample vortexed for 1 min and centrifuged (650 *g*, 4°C) for 1 min. Step 8: The final aqueous layer was frozen, the organic layer decanted into a third 5-ml ampoule, and the sample evaporated to dryness. Step 9: The residue was reconstituted in 0.2 ml mobile phase, vortexed for 10 s, and 0.02 ml injected onto the HPLC column.

2.4.2. Subcutaneous tissue (SCT) and synovial capsule (SC) extraction

Step A: To approximately[1] 0.2 g tissue sample (accurately weighed) in a polypropylene tube (55× 12 mm) was added 0.1 *M* NaOH (volume equivalent to five-times the mass of the tissue sample), and the sample ultrasonicated for 1 h. Parafilm "M" (Chicago, IL, USA) was used to seal the polypropylene tube while in the ultrasonicator, to circumvent sample loss by spluttering and evaporation. Step B: The sample was homogenised at 22 000 rpm for 1 min. Step C: The sample was transferred into an Eppendorf microfuge tube and centrifuged at 8500 *g* for 5 min. Step C: 0.5 ml of the aqueous layer[2] was transferred into a 5-ml amber ampoule. The sample was acidified by adding 0.2 ml 1.0 *M* HCl, and the remainder of the extraction performed as per steps 2–9, described in Section 2.4.1.

2.4.3. Synovial fluid (SF) extraction

Step I: Prior to initial pipetting, the sealed sample was ultrasonicated for 20 min, rendering it more manageable when using a conventional air–interface pipette. Step II: 0.2 ml sample was pipetted into a polypropylene tube, and 1 ml 0.1 *M* NaOH added. Step III: The tube was briefly vortexed, sealed with Parafilm "M", and ultrasonicated for 1 h. Step IV: The sample was again briefly vortexed, 0.5 ml transfered into a 5-ml amber ampoule, and acidified by adding 0.2 ml 1.0 *M* HCl. The remainder of the extraction was performed as per steps 2–9, described in Section 2.4.1.

[1]When adding 0.1 *M* NaOH to the sample, the volume was adjusted so that the ratio of sample (g) to volume 0.1 *M* NaOH added (ml) remained constant at 1:5.

[2]The aqueous layer was found between a supernatant layer of congealed matter, that was cream in colour, and an underlying layer of protein. This congealed, supernatant layer was carefully removed with a wooden applicator, exposing the aqueous layer.

3. Results and discussion

3.1. Extraction

Given the nature of the matrices, a decision was made from the outset to optimise a liquid–liquid extraction procedure for plasma and tissue homogenates. Although piroxicam contains a variety functional groups that predispose the molecule to amphoteric tendencies, the homogenisation of tissue samples was done in a basic, aqueous medium.

A number of extraction solvents were investigated, and although *tert.*-butyl methyl ether and dichloromethane gave good recovery, samples were not sufficiently clean. Various combinations of *tert.*-butyl methyl ether and hexane were investigated, but a combination of hexane–dichloromethane (4:1) proved to be the most successful compromise between recovery and a clean extract.

3.2. Method validation

Based on piroxicam peak area, the calibration line was found to be linear over the plasma concentration range (0.72–600 ng/ml). Due to the fact that little data exist regarding the expected tissue concentrations of piroxicam associated with such a regimen, a calibration range was chosen which was wider than literature would suggest, regarding the expected plasma concentrations. The calibration line produced from nine calibration standards was characterised by the equation $y=(0.727 \text{ to } 1.439)x+(-0.197 \text{ to } 0.574)$, with a mean $r^2=0.992$ and a relative standard deviation (RSD) of 24.7% for $n=8$, over a period of three months, when using a 1/concentration^2 weighting.

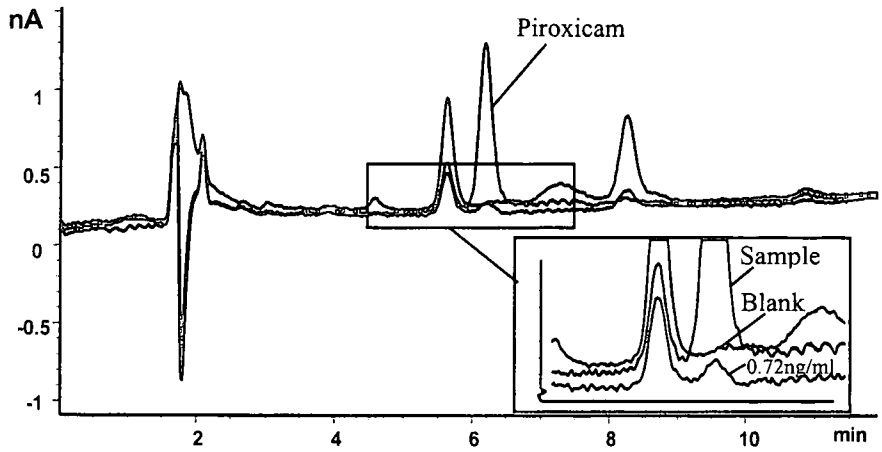

Fig. 1. Overlaid chromatograms of plasma extracts, showing good resolution between endogenous plasma components and piroxicam. A calibration standard of 0.72 ng/ml produces a peak that is well defined from a blank plasma extract. The sample reflects steady-state plasma levels, following repeated topical application of piroxicam.

3.3. Chromatography

Good chromatographic results were obtained from the extracts of all four matrices (Figs. 1 and 2). In none of the four matrices assayed was any endogenous peak found to co-elute with piroxicam. Although it was not possible to obtain blank tissue samples from each volunteer, blank SC, SCT and SF were obtained and assayed prior to commencing with batches. No interference was observed in these three matrices. A blank plasma sample from each volunteer, obtained prior to dosing, was assayed with the post-dosage samples and no interference was observed.

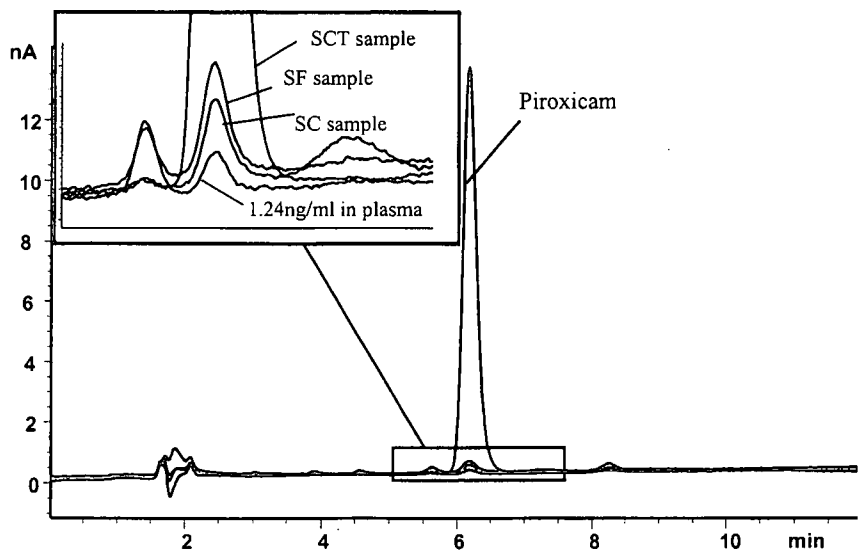

Fig. 2. Overlaid chromatograms of mixed plasma and tissue extracts. Peak areas obtained from SCT, SC and SF, which are all larger than a 1.24 ng/ml calibration standard peak, are yet to be multiplied by approximately the same multiplication factor, in order to obtain absolute concentrations. The large peak area obtained from the SCT sample, and roughly equivalent areas for SC and SF, suggest significant accumulation of piroxicam beneath the skin, and a decrease in tissue concentrations with an increase in tissue depth.

3.4. Recovery

Plasma was spiked with a known amount of piroxicam and then extracted. Recovery experiments with SCT and SC were done by spiking the basic tissue homogenate, obtained in step B of the subcutaneous tissue and synovial capsule extraction section with known amounts of piroxicam, and extracting as described. Recovery from SF was determined by spiking the basic homogenate obtained in step II of the synovial fluid extraction section, and extracting as described. Response factors of extracts were compared to those of appropriate response standard solutions and expressed as a percentage, as set out in Table 1.

Table 1
Extraction recoveries of piroxicam for plasma, SCT, SC and SF

Matrix	Concentration (ng/ml)	Mean recovery, $n=3$ (%)
Plasma	454, 3.47, 0.86	64.3
SCT homogenate	454	64.9
SC homogenate	454	57.8
SF homogenate	127	67.8

3.5. Sensitivity

Compared to previously published methods [1–8], sensitivity was greatly improved. This was due to the fact that ED was employed and optimised (Fig. 3). The LOQ for the method was 0.72 ng/ml in plasma and homogenate, with a signal-to-noise ratio of 10:1. Due to the dilutions necessitated for the assay of the tissue homogenates, this translates into 4.32 ng/g tissue, if 0.2 g tissue is available, and 4.32 ng/ml SF if 0.2 ml SF is obtained.

3.6. Inter- and intra-day accuracy and precision

Within a single batch, the method was found to be both accurate and precise (Table 2). In addition, comparison of calibration standard and quality control data from the various batches (Tables 3 and 4), which were completed over three months, shows a high degree of day-to-day reproducibility.

3.7. Matrix stability

Prior to method development, plasma stability standards were prepared at high, medium and low concentration, and stored at −20°C. After an interval of 16 weeks, fresh standards were prepared in plasma, and assayed together with the pre-prepared stability standards. Response factors of the freshly made standards were compared to those of the 16-week-old standards and no significant difference was observed. It was concluded that when stored at −20°C, piroxicam is stable in human plasma for at least 16 weeks.

3.8. Specificity

Chromatograms from samples obtained from 30 subjects assayed were scrutinised, and no interference was detected. In addition, plasma from six different sources was extracted and no peak was found to elute near the retention time of piroxicam. Similarly, blank SC, SCT and SF from a single source were extracted and no interference was observed.

3.9. On-instrument stability

Piroxicam was found to be stable in the reconstituted extract for the period during which samples were on the instrument (approximately 16 h).

3.10. Application

This assay method, which could be employed in comparative bioavailability studies, was used to determine piroxicam levels in plasma, SCT, SC and SF, following repeated topical application of piroxicam gel to the knee joint (40 mg, applied twice daily, in the seven days preceding knee surgery). Bioavailability was ascertained from the three ratios produced between steady-state plasma concentration, and concentration in the remaining three matrices. Results regarding these ratios are presented in Table 5.

Following the dosage regimen used in this study, a determinable amount of piroxicam is found at the suspected sites of action and it is evident that there is significant accumulation of piroxicam in subcutaneous tissue, with an even distribution of piroxicam between the synovial capsule and synovial fluid.

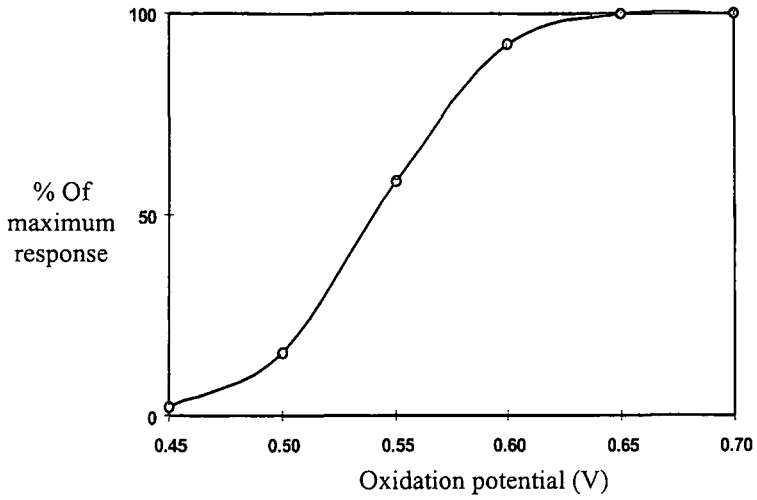

Fig. 3. Piroxicam voltammogram.

Table 2
Intra-day accuracy and precision ($n=5$) of plasma quality controls

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)
538	543	4.4
300	302	7.4
150	145	3.8
3.47	3.44	10.7
2.61	2.56	6.4
1.74	1.88	4.1
0.86	0.88	7.3

We could find no directly comparable data reported in the literature. Marks and Dykes [9] reported on cutaneous concentrations following topical application of piroxicam gel. They found the highest levels of piroxicam in superficial skin surface biopsies (80–320 μg/g of tissue), with the lowest tissue levels recorded in skin surface biopsies nearest the viable epidermis. Low and often undetectable plasma levels were observed in this study.

Hundal et al. [10] reported on synovial fluid concentrations after oral administration of piroxicam. The levels were in the order of 0.3–4.6 μg/ml of

Table 3
Inter-day accuracy and precision of plasma calibration standards

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	n
600	584	3.2	8
299	303	3.5	8
199	197	6.9	8
151	148	6.4	8
49.5	49.5	4.4	8
19.8	20.8	6.2	8
4.94	4.82	11.8	7
2.47	2.56	10.1	9
1.24	1.23	8.1	14

Table 4
Inter-day accuracy and precision of plasma quality controls

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	RSD (%)	*n*
300	291	7.8	16
150	137	13.5	15
3.47	3.46	7.7	15
2.61	2.44	10.8	8
1.74	1.71	9.3	8

fluid, while steady-state total plasma concentrations ranged between 0.5–8.3 μg/ml. The ratio between total synovial fluid and total plasma concentration ranged between 0.39–0.9 with an average synovial fluid/plasma quotient of 0.51 (synovial fluid/plasma 1:1.96). The ratio determined by Hundal et al. differs appreciably from the synovial/plasma ratio of 3.04:1 found in this study (Table 5). This is understandable as Hundal et al. quantified piroxicam in synovial fluid following oral ingestion of piroxicam, while the present study involved the topical application.

4. Conclusion

This assay method is sufficiently sensitive to quantify piroxicam in plasma, SC, SCT and SF, following the repeated topical application of a piroxicam gel formulation. In addition to being sensitive, it is accurate and precise between 0.72 and 600 ng/ml.

Table 5
Ratios of SC, SF and SCT, relative to steady-state plasma concentrations

Ratio expressed	Mean ratio	RSD (%)	*n*
SC/plasma	3.02	136	22
SF/plasma	3.04	77.4	21
SCT/plasma	20.9	150	28

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[2] A.G. Kazemifard, D.E. Moore, J. Chromatogr. 533 (1990) 131.
[3] J. Macek, J. Vacha, J. Chromatogr. 420 (1987) 445.
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8 Appendix 2 Congress presentation

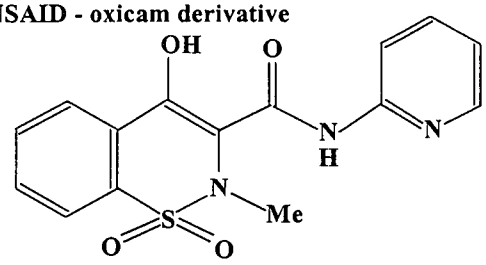
The assay method developed for the quantitation of piroxicam in the four said matrices was presented at the 32nd annual congress of the South African Pharmacological Society held on board 'The Symphony', from 16 – 19 October 1998. As a result, an abstract of the method appeared in the South African Journal of Science Vol. 94 September 1998. Included below are printouts of the slides used in an oral presentation of the assay method.

FARMOVS Research Centre
for Clinical Pharmacology and Drug Development
Analytical Services Division

High-Performance Liquid Chromatographic determination of piroxicam in human plasma and tissues, following topical application, using electrochemical detection.

A. D. de Jager*, H. Ellis, A. F. Hundt,
H. K. L. Hundt, K. J. Swart.

Piroxicam ($C_{15}H_{13}N_3O_4S$)
NSAID - oxicam derivative



4-Hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide

Indications

- Chronic inflammation of joints
- Exacerbated joint disease
- Tendonitis
- Sprains

Piroxicam formulations

- **Oral**
- **Parenteral**
- **Rectal**
- **Topical** (percutaneous absorption)

Theoretical advantages of topical formulations

- Target site of action
- Lower systemic concentrations
- Potentially less adverse events

Study Dosage Regimen

- **40 mg (topical), applied b.d. to the knee joint, in the seven days preceding surgery.**

Plasma

Sub-cutaneous tissue

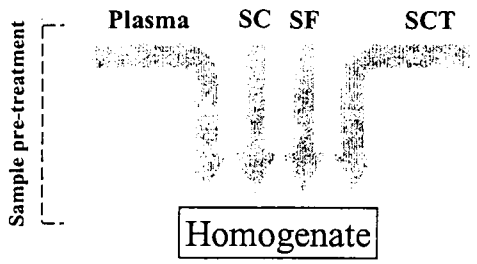
Synovial capsule

Synovial fluid

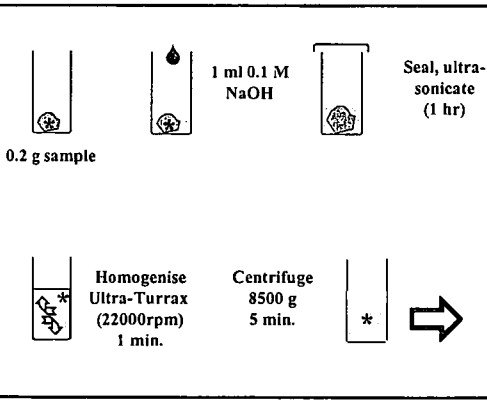
Abbreviations:

- SCT = Sub-cutaneous tissue
- SC = Synovial capsule
- SF = Synovial fluid
- QC = Quality Control
- S = Calibration Standard

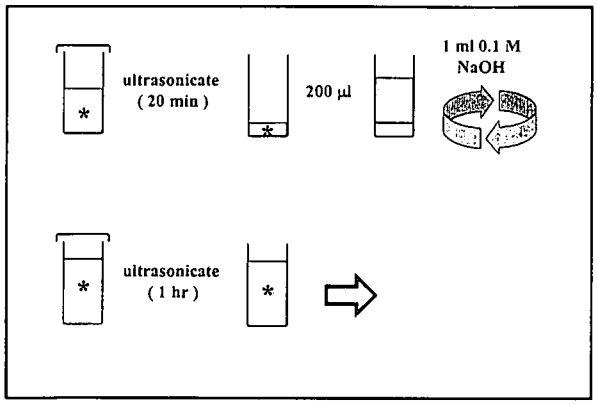
Sample preparation



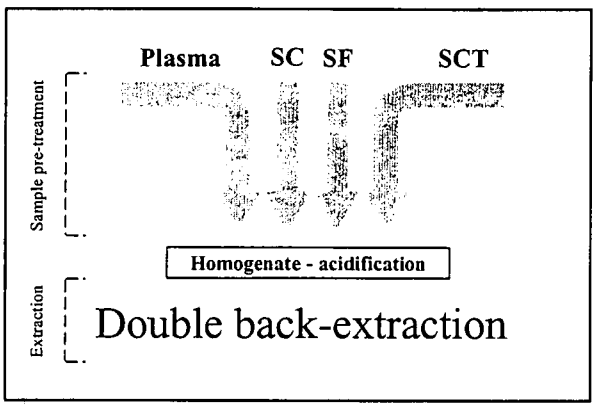
Pre-treatment (SCT and SC)

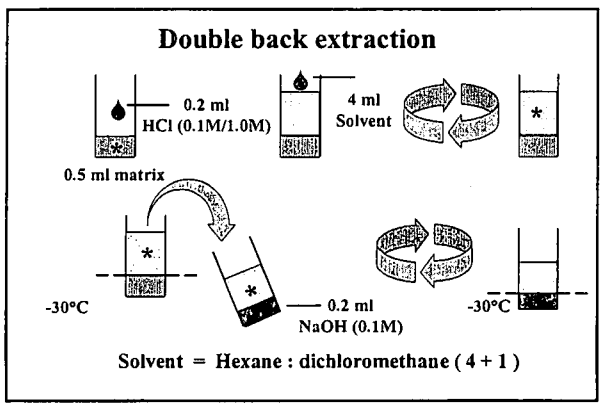


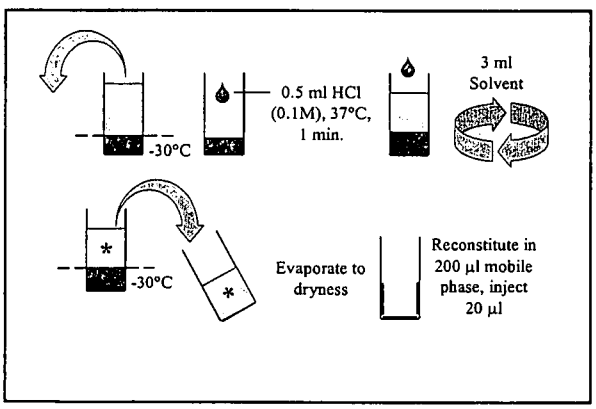
Pre-treatment (SF)



Extraction







Extraction efficiency

Matrix	Concentration (ng/ml)	Mean recovery, n = 3 (%)
Plasma	454	64.3
SCT homogenate	454	64.9
SC homogenate	454	57.8
SF homogenate	127	67.8

- Plasma calibration line

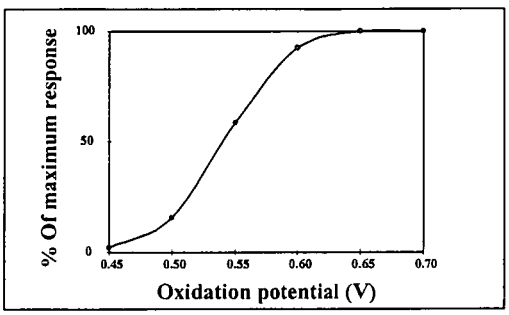
Chromatographic conditions

Mobile phase

30 mM *o*-Phosphoric acid : MeOH : THF
(600 + 320 + 80)
KCl (150mg/L)
Apparent pH 2.70 (4M NaOH)

HPLC system

Pump (isocratic)
0.5 ml/min
Autosampler
20 µl
Column
Higgins Haisil 120 BD (150 × 3mm) C18 (5µm), maintained at 30 °C
Detection
Amperometry + 0.650 V



Piroxicam voltammogram

Detection

- **Detection - UV**
- **Detection - ECD**

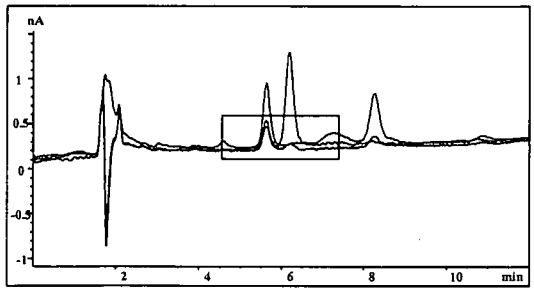
(5 - 20 × improvement in sensitivity[1])

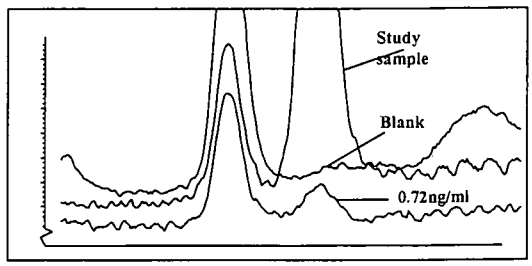
[1] A. G. Kazerifard, D. E. Moore., J. Chromatogr. 533 (1990) 131

Plasma	-	**1.24 ng/ml**	(0.72 ng/ml)
SCT and SC	-	**7.44 ng/g**	(3.72 ng/ml)
SF	-	**7.44 ng/ml**	(3.72 ng/ml)

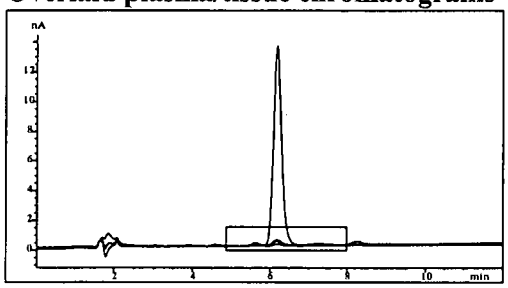
Chromatography

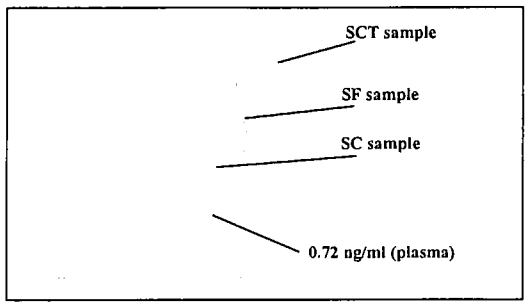
Overlaid plasma chromatograms





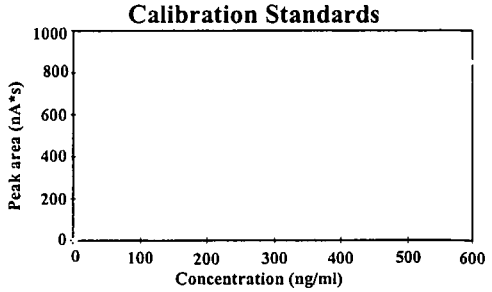
Overlaid plasma/tissue chromatograms



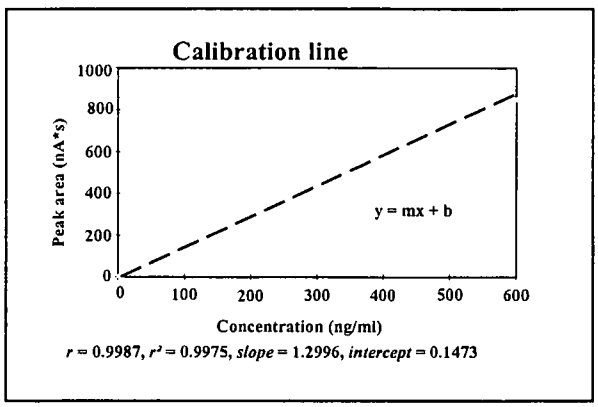


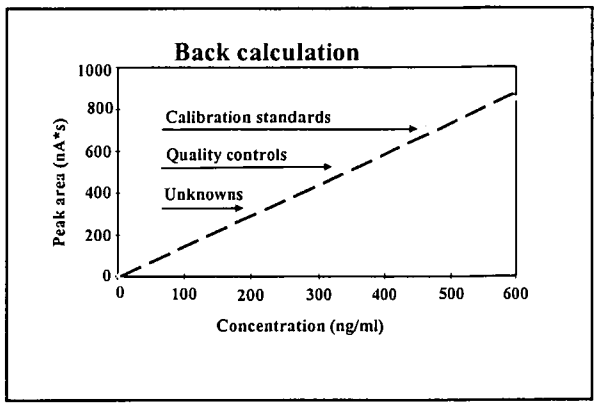
Calculation of results

Calibration Standards



PhIRSt - Phoenix International Life Sciences, Montreal, Canada





Results

- Calibration Standard Summary

[1] ng/ml

- Quality Control Summary

[1] ng/ml

- Data - Study Samples

[1] ng/ml [2] ng/g [3] < 1.24 ng/ml

Method discussion

Advantages

- Single HPLC system (Plasma, SCT, SC and SF)
- Sensitivity

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- [5] P. A. Milligan, J. Chromatogr. 576 (1992) 121.
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Sensitivity improvement

- Topical application
- ca. 10 × improvement [2] - plasma only
- ca. 100 × improvement [1,6] - tissue

Quantification:

89.7 % plasma samples	(98.3 %)
51.7 % SCT samples	(All quantified)
21.7 % SC samples	(All quantified)
13.6 % SF samples	(All quantified)

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