THE DEVELOPMENT AND VALIDATION OF QUANTITATIVE METHODS FOR THE DETERMINATION OF STAVUDINE AND ALFUZOSIN IN PLASMA AND MONIC ACID IN URINE

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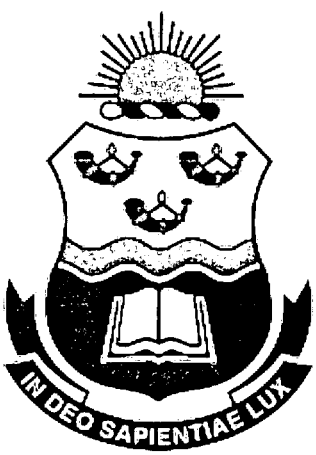

DECLARATION

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

Candidate: Mr. Joachim Lubbe Wiesner (B. Sc. Hons)

Title: The development and validation of quantitative methods for the determination of stavudine and alfuzosin in plasma and monic acid in urine

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We, the undersigned, declare that under our supervision, Mr. Wiesner performed the development and validation of the three assay methods contained in this dissertation, as well as the sample assays of the said research projects. Under our supervision, Mr. Wiesner personally prepared and submitted full length papers dealing with the assay methods described in this dissertation for publication in the Journal of Chromatography B. Mr. Wiesner personally compiled and typed the dissertation in its present form.

Dr KJ Swart

7 Aug 03
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07 Aug 2003
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LIST OF ABBREVIATIONS

% nom	Percentage of Nominal Concentration
AIDS	Acquired Immunodeficiency Syndrome
APCI	Atmospheric Pressure Chemical Ionisation
AUC	Area Under Curve
CID	Collision Induced Dissociation
C_{max}	Maximum Expected Concentration
CUR	Curtain Gas
CV %	Coefficient of Variation
ECD	Electrochemical Detector
ESI	Electrospray Ionisation
FID	Flame Ionisation Detector
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
ISTD	Internal Standard
LC	Liquid Chromatography
LLOQ	Lower Limit of Quantification with a signal to noise ratio greater than 5
LOD	Limit of Detection with a signal to noise ratio greater than 3
LTAT	Long turn-around time
min.	Minutes
MP	Mobile Phase
MRM	Multi Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Mass Spectrometry/Mass Spectrometry
NNRTI's	Non-nucleoside Reverse Transcriptase Inhibitors
NPD	Nitrogen Phosphorus Detector
NRTI's	Nucleoside Reverse Transcriptase Inhibitors
PI's	Protease Inhibitors
PPG	Polypropylene Glycol

QC	Quality Control Standard
SD	Standard Deviation
sec.	Seconds
SPE	Solid Phase Extraction
SPVS	System Performance Verification Standard
STAB	Stability
STD	Calibration Standard
TBA	Tetrabutyl-Ammonium Bromide
TBME	Tert.-Buthyl Methyl Ether
UV	Ultra Violet

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1. INTRODUCTION

Pharmacokinetic and bio-equivalence studies require very precise and accurate assay methods that are well validated to quantify drugs in biological samples. The assay methods have to be sensitive enough to determine the biological sample concentrations of the drug and/or its metabolite(s) for a period of about five elimination half-lives after dosage of the drug. The assay methods also have to be very selective to ensure reliable data, free from interference of endogenous compounds and possible metabolites in the biological sample. In addition, methods have to be as robust and cost-effective as possible, making them of particular importance to bio-equivalence studies. Above all, the assay methods must be able to withstand the scrutiny of national drug registration authorities who judge them on the basis of criteria established by international consensus.

Currently, there is a need in the pharmaceutical environment to develop analytical methods for the determination of stavudine and alfuzosin in human plasma and monic acid in human urine in support of clinical trials involving stavudine, alfuzosin and mupirocin. These drugs have very different molecular characteristics, so that the approaches for method development (extraction, chromatography and ion production in the mass spectrometer's source) will be different. Alfuzosin is a basic compound, monic acid an acidic compound and stavudine a "more" neutral molecule. Since no published assay method could be traced for monic acid (the main metabolite of mupirocin) in urine, the main aim is to develop a new assay method for monic acid in urine, while for alfuzosin and stavudine the aim will be to achieve more selectivity, sensitivity and more rapid assay methods than have been previously described. The developed methods could then be applied to clinical trials to obtain more accurate pharmacokinetic parameters in human plasma.

To achieve the stated objective a mass spectrometer with MS/MS capabilities will be used as a detector for all the assay procedures in tandem with LC. (a UV detector might be used during the initial development stages of the project to optimise chromatography and extraction). This will also allow for shorter sample preparation and chromatography time that would make the methods more cost-effective. Different analytical columns and mobile phases will be tested for optimal chromatography and ionisation (positive and negative ESI and/or positive and negative APCI).

Different buffers, organic solvents and SPE cartridges will be used to optimise the extraction procedure.

2. METHOD DEVELOPMENT

2.1. Introduction

Method development should begin with a comprehensive literature search. Valuable information could be gained with such a search, but it is important that one should strive to improve existing methods. After the literature search has been completed it is time to formulate an action plan, and this plan should include the following steps: choice of instrumentation, choice of chromatography system and choice of extraction technique. After the plan has been formulated the analyst must do all the experiments that were described in this plan. This will result in an analytical method, but before it can be used to quantify samples, it must demonstrate that all aspects of the international criteria are met.

2.2. Literature survey

A comprehensive literature survey is needed to obtain as much information as possible about published assay methods for the drug to be assayed. Electronic databases such as *Micromedex CCIS* and *Analytical Abstacts* were used for the literature searches. Analytical literature is generally the most important source of information, but it is also important to search clinical literature which could be useful to obtain information of a drug's pharmacokinetic data such as AUC, C_{max}, $T_{1/2,}$ etc. When no data is available on the specific analyte, data on other similar compounds may be useful. The information that was gained from the literature should be summarised and great attention should be given to the following questions:

- What type of detectors were used for analyte detection?
- Which chromatography systems were used for analyte separation?
- Which extraction techniques were used?
- Were the analytes stable in solution, in matrix, on instrument, when exposed to light and when exposed to high temperatures?

The literature study is a starting point of the method development phase of the project and should be used in that context, while the analyst should always strive to improve existing methods.

2.3. Action plan

By now the analyst has gained much knowledge about the physical and chemical properties of the drug and this information should now be transformed into a plan of action, which would include the choice of detection, chromatography, extraction techniques, matrix effect testing and to evaluate the robustness of the method.

2.3.1. Detection instrumentation

Various detection techniques are available which include the following: UV, fluorescence, electrochemical, MS, MS/MS in the case of HPLC and NPD, ECD, FID, MS and MS/MS in the case of GC. The physical and chemical properties of the drug should guide the analyst during decision making, and whether or not such equipment is available. The most sensitive and selective detector should always be the detector of choice.

2.3.2. Chromatographic systems

The analyst has to decide on an appropriate chromatography system depending on the availability of instruments (HPLC, GC, electrokinetic chromatography, liquid chromatography, adsoption chromatography, electrochromatography, ion exchange chromatography, etc.), but normally either HPLC or GC and the chosen system has to be optimised with respect to column types, mobile - and stationary phases and environmental conditions.

2.3.3. Extraction techniques

The analyst has a number of options to consider for analyte extraction out of a complex biological matrix, which include the following: liquid-liquid extraction, solid phase extraction, protein precipitation, ultra-filtration, microwave assisted liquid-liquid extraction, counter current, etc. The chosen system has to be optimised with respect to pH, sorbents, solvents, filter types etc.

After the plan has been formulated many experiments will be performed to optimise the method. This will hopefully result in an analytical method that could be used to quantify samples, but before it can be used it must demonstrate that all aspects of the method will pass all of the criteria set by the international drug administration authorities.

2.3.4. Matrix effect

It has been noted that coeluting, undetected endogenous matrix components may reduce/enhance the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LC/MS-MS assay (especially when the ESI source is used).[1] In order to determine whether this effect (called the Matrix Effect) is present or not, 10 different plasma pools must be extracted and spiked with a known concentration of analyte. These samples will be injected and peak areas compared. The reproducibility of the peak areas will be an indication of the presence or absence of the matrix effect.

2.3.5. Robustness of the method

The evaluation of robustness depends on the type of procedure under investigation. It should show reliability of an analysis with respect to deliberate variations in method parameters, such as: stability of analytical solutions, extraction time, influence of variations of pH in a mobile phase, influence of variations in mobile phase composition, different columns (different lots and/or suppliers), temperature and flow-rates.

3. METHOD VALIDATION

This process is the final test to demonstrate that the developed method is fit to be used as a "tool" to quantify samples. The validation process is also performed to objectively demonstrate the specificity, reliability, sensitivity and suitability of the assay method for the purposes of assaying samples of unknown concentrations.

Van Zoonen *et. al.,* described the importance of method validation in the analytical laboratory.[2] Method validation is the key element in both the elaboration of reference methods and the assessment of a laboratory's competence in producing reliable analytical data. The principal product of an analytical chemical laboratory is information about the chemical composition of material systems. The validation process measures the quality of this information.

Shah *et. al.,* described the fundamental parameters for a bioanalytical method validation.[3] Accuracy, precision, selectivity, sensitivity, reproducibility and stability are the key parameters for the validation process. According to the FDA Guidance [4] the following should be determined during this process: selectivity, accuracy, precision, recovery, linearity of the calibration curve and the stability of the analytes in solution and matrix.

3.1. Parameters for the validation process

3.1.1. Selectivity

This is the ability of the method to differentiate and quantify the analyte in the presence of other components in the sample. To observe the selectivity of the method, six blank sources of the appropriate biological matrix (plasma, urine, or other matrix) would be screened to test for interferences, and the selectivity should be ensured at the lower limit of quantification (LLOQ). There are potential interfering substances in a biological matrix that include endogenous matrix components, metabolites, decomposition products, and in the study-sample, concomitant medication and other xenobiotics. Each analyte in the assay should be tested to ensure that there is

no interference.

3.1.2. Accuracy

Accuracy is described as the closeness of mean test results obtained by the method to the true value of the analyte. The mean value should be within 15 % of the actual value except at LLOQ, where it should not deviate by more than 20 %. The deviation of the mean from the true value serves as the measure of accuracy. Accuracy should be measured using at least six determinations per concentration.

3.1.3. Precision

Precision is described as the closeness of individual measurements of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. At least six determinations per concentration should be used to measure precision. The coefficient of variation of the precision determination at each concentration level should not exceed 15 %, except at the LLOQ, where it should not exceed 20 %. A minimum of three concentration levels ranging from low to high should be tested.

3.1.4. Recovery

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. The recovery should be consistent, precise and reproducible, and need not to be 100 %. These experiments should be performed at three concentration levels (low, medium and high).

3.1.5. Calibration / Standard Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte, and should be generated for each analyte. A sufficient number of standards should be used (at least five levels) to adequately define the relationship between concentration and response. The standards should be prepared in the same biological matrix as the samples in the intended study. The concentration of the standards should be chosen on the basis of the concentration range expected in a particular study. The LLOQ should be at least 5 times the response if compared to blank response, and should be reproducible with a precision of 20 % and accuracy of 80 – 120 %.

The FDA Guidance for industry [4] also indicates that the simplest model that adequately describes the concentration-response relationship should be used and when weighting or complex regression equations are used it should be justified. The following conditions should be met in developing a calibration curve: deviation of the LLOQ from nominal concentration and deviation of standards other than LLOQ from nominal concentrations should not exceed 20 % and 15 % respectively. At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the highest standard. Those that are excluded should not change the regression model used.

3.1.6. Stability

Stability information is assessed to ensure that all necessary precautions are taken to ensure that the analyte concentration are not affected by internal and external conditions such as matrix-interactions, chemical properties, storage conditions of the drug and the container system. These stability procedures should evaluate the stability of the analyte during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term strorage, and after going through freeze and thaw cycles and the analytical process. These experiments should reflect situations likely to be encountered during actual sample handling and analysis.

3.1.6.1. Freeze and Thaw Stability

The FDA Guidance [4] suggested that three freeze- and thaw cycles should be determined to ensure analyte stability. They also indicated that at least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. After the samples have been thawed completely, it should be refrozen for 12 to 24 hours at the same conditions. This cycle should be repeated two more times, and analysed after the third cycle. If it is found that an analyte is unstable at the intended temperature, these stability samples should be frozen at –70 °C and tested again as described above.

3.1.6.2. Long Term Stability

Long term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The time that the samples are stored should exceed the time between the date of first sample collection and the date of last sample analysis.

3.1.6.3. Stock Solution Stability

The FDA Guidance [4] states that the stability of stock solutions of drug and internal standard should be evaluated at room temperature for at least 6 hours. When stock solutions are refrigerated or frozen, stability of the relevant period should be tested and documented. Stock solutions will be used immediately to spike the matrix and therefore no stock solution stability will be tested during this project.

3.1.6.4. Post Preparative Stability

The stability of the drug and the internal standard should be assessed over the anticipated run time

of sample batches. This time is usually the time that samples are kept on the autosampler while awaiting injection.

3.2. Validation Process

3.2.1. Preparation of calibration standards and quality control standards in biological fluids

Calibration standards (STDs) will be prepared with the purpose of setting up a calibration curve from which the concentrations of the unknown samples will be calculated. Quality control standards (QCs) will also be prepared, but the purpose of these standards is to monitor the performance of the assay procedure. Both STDs and QCs will be prepared by weighing of the biological fluids, thereby avoiding as much as possible the use of volumetric equipment. This is done to increase the accuracy with which standards are prepared. The reference material will be weighed accurately and dissolved in an appropriate solvent to obtain a stock solution of known concentration. This stock solution will be used to spike a pool of biological matrix (plasma or urine) to obtain a pool of biological matrix with known concentration. The concentration of this pool must be in the 2 times expected highest concentration range (2 x C_{max}) and will be used as the highest concentration standard. This standard will be serially diluted (1:1) with blank matrix until the LLOQ standard is reached, resulting in standards that will be used to construct calibration curves. The same methodology will be followed when preparing the QCs that will be used to monitor the accuracy of the calibration curve. The lowest QC should be between 1.2 and 1.3 times the LLOQ standard and the highest QC should be in the order of 1.8 times the C_{max} STD.
These standards will be stored under the same conditions as the study samples.

3.2.2. Process of validating the assay method

Repeated analysis of the calibration and quality control standards in three (one intra- and two inter–batches) consecutive batches are performed to demonstrate intra– and inter-batch accuracy and precision over the entire concentration range. Quantification models based on peak heights, peak

height ratios, peak areas and peak area ratios will be assessed to determine which model performed the best. The statistical analysis of the accuracy and precision of the intra-batch and inter-batch results would indicate if the calibration range is valid and would also determine the LLOQ.

3.2.3. Preparation of a typical calibration batch

The analyst will construct a batch sequence and perform the intra-batch validation according to the method that was optimised during the method development phase of the project. The two lower STDs will be performed in duplicate (in case the LLOQ has to be raised). The QCs will be interspersed throughout the calibration curve (STDs) and repeated six times. Table 1 is an example of such a batch list (for this illustration only six STD levels and five QC levels are used).

Table 1 Typical intra-validation batch

No.	Sample	No.	Sample	No.	Sample
1	SPVS	20	BLANK 3	39	QC E
2	STD F	21	STAB 3	40	QC D
3	BLANK 1	22	QC E	41	QC C
4	STAB 1	23	QC D	42	QC B
5	ZERO 1	24	QC C	43	QC A
6	QC E	25	QC B	44	STD B
7	QC D	26	QC A	45	BLANK 6
8	QC C	27	STD C	46	STAB 6
9	QC B	28	BLANK 4	47	QC E
10	QC A	29	STAB 4	48	QC D
11	STD E	30	QC E	49	QC C
12	BLANK 2	31	QC D	50	QC B
13	STAB 2	32	QC C	51	QC A
14	QC E	33	QC B	52	STD B
15	QC D	34	QC A	53	STAB 7
16	QC C	35	STD C	54	STAB 8
17	QC B	36	BLANK 5	55	ZERO 2
18	QC A	37	STAB 5	56	SPVS
19	STD D	38	SPVS		

The performance of the analytical system is monitored by the three system performance verification standard (SPVS) samples, one at the beginning, one at the middle and one at the end of the batch. These three samples will monitor whether the instrument response was stable during the run or not. Six blank samples (matrix that contains no analyte or internal standard) are placed after the calibration standards to serve as indicators for possible carry-over in the system and for selectivity/specificity purposes. The two zero samples (matrix containing ISTD only) will indicate if the ISTD contribute to the analyte's response in the system. The stability samples (STAB) will indicate whether or not the analyte and ISTD are stable on-instrument. The calibration standards will be used to construct a calibration curve and the quality control standards will monitor the calibration curve. Other stability samples such as the freeze and thaw, and matrix stability samples

may also be interspersed (not shown in this example) throughout the validation batch, or could be tested before the intra-batch as a separate batch.

After completion of the intra-batch validation, two inter-batch validations have to be performed. The same methodology will be performed as was for the intra-validation. Low, medium and high QCs will be selected and used during these batches. Table 2 illustrates the selection of such a batch. QC A is selected as the lower QC, if STD B (from intra-batch) is found to be the standard defining the LLOQ (S/N > 5).

Table 2 Typical inter-batch validation list

No.	Sample	No.	Sample
1	SPVS	**22**	STAB 12
2	STD F	**23**	QC E
3	BLANK	**24**	QC C
4	STAB 9	**25**	QC B
5	QC E	**26**	QC A
6	QC C	**27**	STD C
7	QC B	**28**	STAB 13
8	QC A	**29**	QC E
9	STD E	**30**	QC C
10	STAB 10	**31**	QC B
11	QC E	**32**	QC A
12	QC C	**33**	STD B
13	QC B	**34**	STAB 14
14	QC A	**35**	QC E
15	STD D	**36**	QC C
16	STAB 11	**37**	QC B
17	QC E	**38**	QC A
18	QC C	**39**	STD B
19	QC B	**40**	STAB 15
20	QC A	**41**	Stab 16
21	STD C	**42**	SPVS

QC A = QC at LLOQ
QC B = low QC
QC C = medium QC
QC E = high QC

3.3. Performing the validations

The three validations will be performed over a three day period. The samples will be prepared according to the optimised extraction method and introduced into the LC-system and finally filtered, measured and quantified by the mass spectrometer (or other detector) and computer system. The data obtained from these validations will be interpreted by the analyst and quantification models will be constructed (peak heights, peak height ratios, peak area and peak area ratios) and the best model will be used as a "tool" for quantifying study samples.

4. METHOD DEVELOPMENT AND VALIDATION OF AN ANALYTICAL ASSAY METHOD FOR THE DETERMINATION OF STAVUDINE IN HUMAN PLASMA

4.1. Objective

A sensitive, accurate, specific, precise and robust method was needed to quantitatively determine stavudine concentrations in plasma samples to follow the concentration vs. time profile for at least five half lives of the drug after a single 40 mg oral dose of stavudine was given to healthy adult male human subjects, and heparinised blood samples were obtained at the following time periods: 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12 and 24 hours. The samples were centrifuged and duplicate plasma samples were stored at – 20 °C until analysed.

4.2. Physico-chemical information

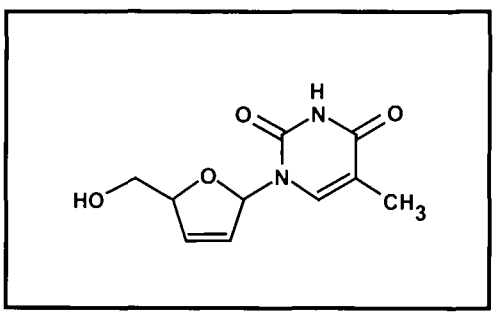


Figure 1 Chemical structure of stavudine

Stavudine (figure 1) is a colourless, granular, solid recrystallised from ethanol/benzene with a melting point of 165-166 °C. It is also reported to form crystals from ethanol-ether with a melting point of 174 °C.[5]

Chemical name: 2',3'-Didehydro-3'-deoxythymidine or 1-(2,3-dideoxy-β-glycero-pent-2-enofuranosyl)thymine

Additional name(s):	3'-deoxy-2'-thymidinene and D4T
Trade name:	Zerit (Bristol-Myers Squibb)
Molecular formula:	$C_{10}H_{12}N_2O_4$
Chemical composition:	C 53.57%, H 5.39%, N 12.49%, O 28.54%
Molecular weight:	224.22
Monoisotopic mass:	224.0797

4.3. Literature survey

4.3.1. Clinical information

Human Immunodeficiency Virus (HIV), the causative agent of the Acquired Immunodeficiency Syndrome (AIDS), encodes at least three enzymes: protease, reverse transcriptase and endonuclease. To inhibit the viral replication, three therapeutic classes have been developed:

- Nucleoside Reverse Transcriptase Inhibitors (NRTI's): abacavir, didanosine, dideoxycytidine, lamivudine, stavudine and zidovudine
- Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI's): delaviridine, efavirenz and nevirapine
- Protease Inhibitors (PI's): amprenavir, indinavir, nelfinavir, ritonavir and saquinavir

Therapeutic strategy regimens require the combination of these drugs. Some of these combinations gave very promising results in decreasing the levels of HIV RNA, and increasing CD4 cell counts, and preventing AIDS and death. [6]

Stavudine is a thymidine analogue with *in vitro* and *in vivo* activity against HIV. It is a reverse transcriptase inhibitor whose mode of action is similar to that of other nucleoside analogues and is active at concentrations that are generally 100-fold below the levels that are cytotoxic. Following phosphorylation by cellular kinases, d4t-triphosphate is produced, which preferentially inhibits HIV-1 reverse transcriptase activity. [7,8,9,10]

4.3.2. Analytical information

Kaul *et al.*, described an HPLC assay method for the quantification of stavudine in rat and monkey plasma. [11] A UV detector was used to detect stavudine and the internal standard (thymidine oxetane), and was set at 254 nm. An Apex octadecyl (250 x 4.6 mm, 5 µm) analytical column was used for analyte separation and a guard column packed with ODS, 37 – 53 µm preceded the analytical column. The mobile phase consisted of methanol and 0.05 M potassium phosphate buffer solution (20:80, v/v) and was delivered at a flow-rate of 1.0 ml/min. Extraction was performed on 1.0 ml Bond Elut C_{18} columns as follows: The columns were activated with 2 column volumes each of methanol and with water. After the conditioning of the columns the plasma sample (0.25 ml) and ISTD solution (0.1 ml, 125 µg/ml) were transferred to the columns and allowed to pass through the bed with minimal suction. The columns were then washed with 2 column volumes of water. The analyte and ISTD were eluted from the columns with 1.0 ml methanol and the eluate was evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residue was reconstituted in 200 µl of the mobile phase and 50 µl injected onto the analytical column. The assay was linear over the concentration range of 0.1 – 100 µg/ml with a LOD of 0.05 µg/ml and a LLOQ of 0.1 µg/ml, and no difference between the standard curves prepared in rat, and those in monkey plasma were observed. The retention times were 6 and 8 min. for stavudine and the ISTD, respectively. Stavudine was found to be stable at – 20 °C for at least 21 days. It was also found to be stable when put through 3 freeze- and thaw cycles. Both stavudine and the ISTD were found to be stable in the injection solvent (mobile phase) for at least 70 hours at ambient temperature. The recoveries for stavudine and the ISTD were 86 and 82 %, respectively. This method however has two limitations:

- The method is not very sensitive (LLOQ of 0.1 µg/ml) and would not be suitable for the method yet to be developed. The literature survey indicated that an LLOQ of about 0.02 µg/ml would be required in order to be able to quantify stavudine in plasma for a period of 5 elimination half-lives after a single 40 mg dose of stavudine.
- It also lacks selectivity and a more selective detector like a mass spectrometer would definitely increase the selectivity.

Burger *et al.*, also described an HPLC assay method for the determination of stavudine in human plasma. [12] They found that the most acceptable HPLC method was reported by Kaul *et al.* [11] who

used C_{18} SPE techniques to extract stavudine out of rat and monkey plasma. Human plasma samples, that were extracted and analysed according to these methods, gave many interferences, therefore silica gel columns were tested and major improvements were accomplished with these extraction columns. The phenyl analytical column used also improved the chromatography.

Detection was performed on a UV detector set at 265 nm (for the determination of stavudine and didanosine). Chromatography was carried out on a phenyl column with a mobile phase consisting of phosphate buffer (5 mM, pH 6.8) and methanol (90:10, v/v) and was delivered at a constant flow-rate of 1 ml/min. The rentention times were ~ 8 min. for stavudine and ~ 9.5 min. for the internal standard (Didanosine). Solid phase extraction (SPE) was performed on C_{18} (3 ml capacity; Bakerbond SPE, J.T. Baker, Phillipsburg, NT, USA) and silica gel columns (3 ml capacity; Bond Elut, Analytichem International, Rotterdam, Netherlands). The columns were conditioned with 2 ml of methanol and rinsed with 2 ml of water. Plasma samples (500 µl) were applied to the columns using reduced pressure. The columns were then washed with 1 ml water. The absorbed analytes were eluted with 1 ml methanol and evaporated to dryness under a gentle stream of nitrogen at 60 °C. The mobile phase (200 µl) was used to redissolve the residues, and 100 µl was injected. Recoveries were tested at three different levels and the average was ~ 96 %. The LOD was 10 ng/ml (S/N = 3) and a linear regression equation was used. The correlation coefficients of all the curves were greater than 0.994 and showed low variability. Stavudine was stable in human plasma for 30 min. at 60 °C, 24 hours at 25 °C, 7 days at 4 °C and 21 days at -30 °C. This method however has two shortcomings:

- The detector used is a relatively non-selective detector.
- The LOD was set at 10 ng/ml, with a S/N ratio of 3. LOD is not acceptable to be used as an LLOQ. It is therefore likely that this method lacks the sensitivity required to quantify stavudine in plasma for a period of 5 elimination half-lives after a single 40 mg dose of stavudine.

Janiszewski *et al.*, developed an HPLC method for the determination of stavudine in human plasma and urine. [13]

A UV detector was used to monitor the column effluent and was set at 266 nm. An Apex octadecyl column (250 mm x 4.6 mm, 5 µm) was used for chromatographic separation at a flow-rate of 0.8 ml/min. The mobile phase consisted of 10 mM ammonium phosphate and acetonitrile (9:1, v/v) with 7.2 mM triethylamine added, and 85 % phosphoric acid was used to adjust the pH to 2.5. The retention times for stavudine were 7 and 7.5 min., and for the ISTD analogue 9 and 10.5 min. in the

plasma and urine matrices, respectively. For the plasma samples, SPE was performed on 1-ml Bond Elut columns using a vacuum system. The columns were activated by consecutive rinses with methanol and water. The plasma sample (0.5 ml) and ISTD (50 µl) were then aspirated through the column and the column rinsed with two column volumes of water. The absorbed analyte and ISTD were eluted with 1 ml methanol and the eluate evaporated to dryness under a stream of nitrogen at 37 °C. The samples were reconstituted in 125 µl mobile phase and 100 µl injected on the analytical column. For the urine samples 3 ml phenyl solid phase extraction columns (Bakerbond SPE, J.T. Baker, Phillipsburg, NJ, USA) were used. The columns were activated with one column volume (3 ml) of methanol followed by two column volumes of 20 mM potassium phosphate (pH 8). The urine samples (0.5 ml) and 50 µl of the ISTD were loaded onto the columns and aspirated, the columns were washed with one column volume each of 20 mM potassium phosphate (pH 4.1), 20 mM potassium phosphate (pH 8.0) and water. The analyte and ISTD were eluted with two steps each using 500 µl of elution solvent (methanol and water, 7:3, v/v with 1.4 mM TEA). The collected eluate was diluted with 500 µl of 20 mM potassium phosphate (pH 7.2) and 75 µl injected on the analytical column. The LLOQ's were set at 25 and 500 ng/ml for plasma and urine assays, respectively.

These assay methods have got the following limitations:

- The cost-effectiveness of these methods can be questioned due to the relatively long chromatography time.
- A non-specific UV detector is used for monitoring stavudine and the ISTD in the extracted matrix.
- The LLOQ of the assay that was developed in plasma was 25 ng/ml, and would not be sensitive enough for the assays in this project for the same reason stated before.

Stancato *et al.*, described a method where the effect of temperature was tested on the chromatographic separation of stavudine and didanosine. They concluded that chromatographic analysis at lower temperatures may permit the simultaneous monitoring of stavudine and didanosine in human plasma. [14]

Detection was performed with a UV detector and was set at 254 nm. Two Brownlee analytical columns (4.6 x 30 mm, 5 µm and 4.6 x 220 mm, 5 µm) were used for chromatographic separation. The 30 mm column could not resolve the stavudine and didanosine peaks, even at the -15 °C tested. The mobile phase consisted of 15 % methanol or 15 % methanol with 3 % acetonitrile in 40 mM

monobasic potassium phosphate buffer containing 0.2 % triethylamine (pH 4, using 85 % phosphoric acid) and was delivered at a constant flow-rate of 0.7 ml/min. The addition of the 3 % acetonitrile decreased the chromatography runtime with about 10 min. This article examines the effect of temperature on the chromatographic system, and it was concluded that peak resolution (stavudine and didanosine) can be improved when column temperature is lowered. The use of longer analytical columns also resulted in better peak resolution.

Jarugula and Boudinot described an HPLC method where stavudine was used as the ISTD to quantify 5-fluorouracil, tegafur and 4-deoxy-5-fluorouracil in rat plasma. [15]
The UV detector was set at 254 nm for 5-fluorouracil, tegafur and stavudine and at 313 nm for 4-deoxy-5-fluorouracil. The mobile phase was delivered at a flow-rate of 1.5 ml/min and consisted of tetrabutyl ammonium hydroxide (5 mM, pH 11.1) solution and acetonitrile (84:16, v/v). A Hamilton PRP-1 column (250 x 4.1 mm, 10 µm) was used for compound separation. Sample clean-up was performed by precipitating the plasma proteins with acetonitrile. Ice-cold acetonitrile (1 ml) was added to 200 µl plasma and 50 µl ISTD solution (stavudine). The samples were mixed and centrifuged at 9000 G for 7 min. and to the supernatant was added excess crystalline magnesium sulphate. The samples were mixed for 2 min. and centrifuged for 10 min. at 9000 G. The supernatant was evaporated to dryness under a stream of nitrogen gas and reconstituted in 200 µl mobile phase. Volumes that ranges from 15 µl to 150 µl were injected on the analytical column depending on the expected drug and pro-drug concentrations. Stavudine was only used as the internal standard, therefore interpretations of the pharamacokinetic data would not be relevant. The sample clean-up procedure may however be useful when developing the extraction procedure.

Specific radioimmunoassays have been developed for the measurement of stavudine in human plasma and urine by Kaul *et al.* [16]
The previously developed HPLC (with UV detection) methods for the determination of stavudine in human plasma were considered as inadequate for providing meaningful pharmacokinetic profiles at low doses in adult and paediatric patients. They claim that this RIA method is more sensitive and specific if compared with the HPLC methods. The standard calibration ranges were 2.5 – 100 ng/ml and 5 – 1 000 ng/ml in plasma and urine respectively (LLOQ in plasma: 2.5 ng/ml; LLOQ in urine: 5 ng/ml). The half-life in human patients is approximately 1 hour and stavudine could be quantified in plasma for a period of about 5 elimination half-lives as required. Stavudine was found

to be stable (at 5.5 and 80 ng/ml) for 4 days at room temperature and 4 °C, and for at least 1 year at – 20 °C.

This method is a vast improvement in relation to sensitivity (LLOQ of 2.5 ng/ml in plasma), but claims about specificity of RIA assay methods are always questionable due to the possibility of cross-reactions that may occur with metabolites. However, since we do not have the facilities to develop RIA methods, this assay method was not considered as a possible candidate for development.

Aymard *et al.*, described a reversed-phase HPLC method for the determination of twelve antiretroviral agents in human plasma. [17]

They used two different HPLC systems. The first system was used to assay PI's and efavirenz. The second one was used to assay NRTI's and nevirapine. In the first system they used two detectors that were connected in-line (UV and fluorescence). The spectrophotometer was set at 261 nm between 0 and 9 min., at 241 between 9 and 20 min. and at 254 nm between 20 and 32 min.. The fluorescence detector was set at 305 and 425 nm for excitation and emission wavelengths, respectively. A Symmetry C_{18} column (250 x 4.6 mm, 5 µm) was used for analyte separation. The mobile phase was composed of a Na_2HPO_4 buffer (0.04 M) with 4 % (v/v) octane sulphonic acid and acetonitrile (50:50, v/v) and was delivered at a flow-rate of 1.3 ml/min.

A UV detector was used in the second system and was set at 260 nm. A Symmetry Shield C_{18} column (250 x 4.6 mm, 5 µm) was used for analyte separation. Three mobile phases (MP's) were prepared using KH_2PO_4 buffer (M/15) with 1 % OSA and different acetonitrile proportions (v/v): 5 % for MP1 delivered at a flow-rate of 1 ml/min, 20 % for MP2 delivered at the same flow-rate and 71 % for MP3 delivered at a flow-rate of 1.2 ml/min. Three pumps with three mobile phases were used in this system, and were connected to a switching valve. Switch 1 was connected to an AI 406 module interface, programmed by a Beckman Gold 2 software system. The second switch was connected to a Waters autosampler injector and programmed in step function. The first pump was connected through a six-way switching valve to the analytical column. When the sample was injected, switch 1 was in position 1 and the eluent from MP1 was directed to the column; the second switch was in position 2 and MP 2 and 3 were directed to waste. Switching valve 1 was activated to position 2 between 12 and 35 min and MP1 was directed to the waste position, valve 2 was in position 1 and MP2 was directed to the column through valve 1. At 30 min., switching valve 2 was set back to position 2 and MP3 was directed to the column to rinse it. Between 35 min. and 40

min., the column was re-equilibrated with MP1 before the next sample would be injected. After each chromatographic session, the symmetry column was washed with methanol-water (50:50, v/v) and acetonitrile-water (80:20, v/v); the Symmetry Shield column was rinsed with water and methanol. The HPLC column switching system is presented in figure 2.

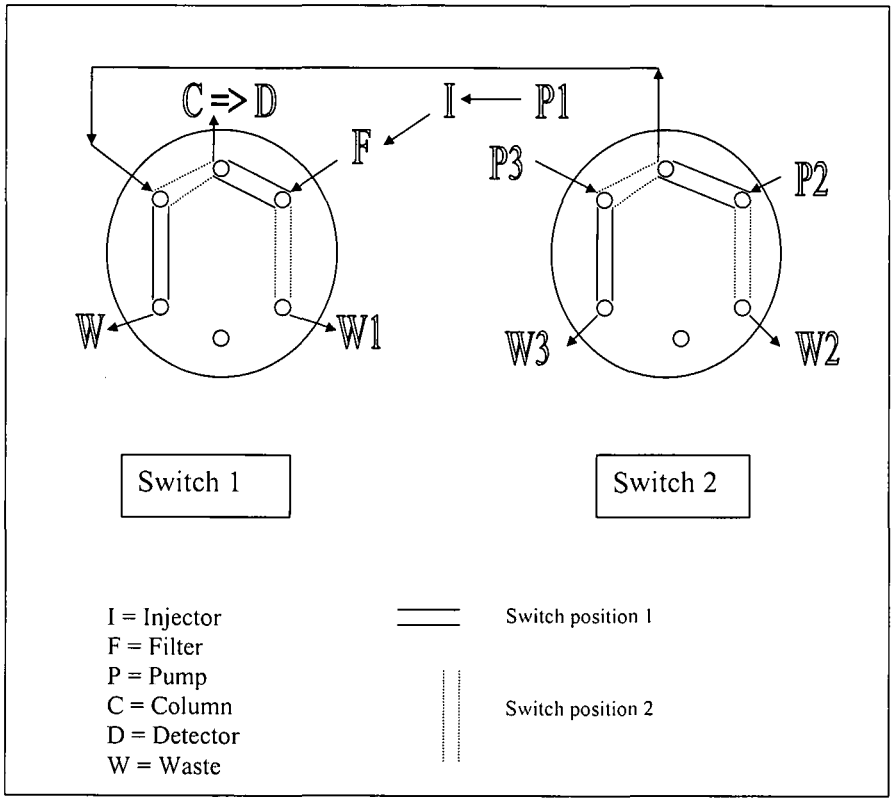


Figure 2 Block diagram of the HPLC column-switching system

Extraction was performed on C_{18} solid-phase columns (J.T. Baker, Deventer, The Netherlands). The columns were activated with methanol (3 ml) and water (3 ml). The plasma samples (1 ml) were loaded onto the columns and pressed into them by applying pressure. The columns were washed with water (2 ml) followed by vacuum suction for 1 min. Methanol (2.6 ml) was used to elute the analytes and the recovery of stavudine was found to be greater than 70 %. Therapeutic agents most likely to be encountered in the plasma of HIV positive patients were tested for interference with the twelve antiretroviral agents and no interference was found. Stavudine was found to be stable in human plasma for 6 months at –20 °C. The calibration range for stavudine fitted a linear least-squares regression and had a coefficient of determination greater than 0.998, with an LLOQ of 10 ng/ml. This complex HPLC assay method that measured twelve antiretroviral

agents in human plasma still lacks the required sensitivity for stavudine (LLOQ of 10 ng/ml) and the very long turn-around time makes it unsuitable to assay a large number of samples.

Sarasa *et al.*, developed an HPLC method for the determination of stavudine in human plasma and urine using a reduced sample volume. [18]

Detection was performed on a UV detector that was set at 266 nm. A Waters Nova Pak C_{18} (150 x 3.9 mm, 5 μm) was used for analyte separation. The mobile phase consisted of a mixture of acetonitrile and 10 mM potassium phosphate buffer (3:97, v/v), triethylamine (1 %), was added and the pH adjusted to 2.5 with orthophosphoric acid.

Extraction of the plasma samples was performed on solid-phase extraction cartridges (Waters Oasis®, 1 ml, 30 mg). The columns were conditioned with methanol (1 ml) and water (1 ml), the plasma samples (200 μl) loaded onto the cartridges and allowed to pass through the bed with minimal suction. The columns were rinsed with two 1 ml water aliquots and the bed was then suctioned dry. The analyte was eluted with methanol (1 ml) and the eluent was evaporated to dryness under a stream of nitrogen at ambient temperature. The residue was reconstituted with 50 μl of mobile phase and 40 μl injected into the HPLC system. No internal standard was used during this extraction.

To the urine samples (10 μl) were added 20 μl of the internal standard solution (tymidine oxetane, 100 μg/ml) and 970 μl of HPLC water, and 100 μl injected into the HPLC system.

The calibration range for the plasma assay was 25 - 2 500 ng/ml, fitted a linear least-squares regression and showed a coefficient of determination greater than 0.999. The LOD was 11.6 ng/ml and the LLOQ was 24.6 ng/ml. The calibration curves for the urine assay also fitted a linear least-squares regression. The calibration range was 2 - 100 μg/ml with a LOD of 1.33 μg/ml and a LLOQ of 1.97 μg/ml. Stavudine was found to be stable in human plasma for 30 min. at 60 °C and 24 hours at 25 °C. It was also found to be stable for three freeze- and thaw cycles.

This well described method still lacks selectivity, sensitivity and the runtime on-instrument is relatively long (turn-around time of about 15 minutes).

Moore *et al.*, described an HPLC tandem mass spectrometry method for the simultaneous quantitation of the 5'-triphosphate metabolites of zidovudine, lamivudine and stavudine in peripheral mononuclear blood cells of HIV infected people. [19]

They used a PE Sciex API-III triple quadrupole mass spectrometer for analyte detection. A C_{18} Phenomenex (100 x 1 mm, 5 μm) column was used for separation. The mobile phase consisted of 10 mM ammonium acetate and acetonitrile (86:14, v/v) and was delivered at a flow-rate of 0.05 ml/min. The column was connected to an Ionspray interface of a PE Sciex API III triple quadrupole mass spectrometer and the acquisitions were performed in positive ionisation mode.

Mononuclear cells were isolated from whole blood using several centrifugation and washing steps and suspended in 60 % methanol. The suspended cells were stored at – 80 °C until analysed after a complex sample preparation procedure, the metabolites were analysed by LC-MS/MS.

This method, set up for the determination of the 5'-triphosphate metabolites and not for the determination of the pro-drugs indicates the advantages of the use of a mass-selective detector instead of a UV detector. The use of a triple quadrupole mass spectrometer will definitely increase the selectivity and sensitivity of the method and may also result in shorter chromatography times.

4.3.3. Literature summary

The methods that were described in the literature are summarised in table 3. These methods will only be used as a starting point to construct the "action plan" for the method development phase of the project.

The methods that have been described in the literature have got certain limitations, the HPLC with UV detection methods lack sensitivity and specificity and the LC-MS/MS method described by Moore *et al.*, [19] was used to determine triphosphate metabolites. The aim of this project is to improve the sensitivity and selectivity as well as reducing the turn-around time of the assay method that will be developed for the determination of stavudine in human plasma.

Table 3 Summary of analytical methods that were found in the literature

Reference	Detector	Analytical column	Extraction method	LLOQ or LOD	Limitations
Kaul *et. al.*	UV (254 nm)	Apex octadecyl	SPE using Bond Elut C_{18}	LOD: 0.05 ug/ml LLOQ: 0.1 ug/ml	Specificity ? Sensitivity ? Long turn-around time (LTAT) ?
Burger *et. al.*	UV (265 nm)	Phenyl column	SPE using silica gel columns	LOD: 10 ng/ml	Specificity ? Sensitivity ? LTAT ?
Janiszewski *et. al.*	UV (266 nm)	Apex octadecyl	SPE using Bont Elut	LLOQ in plasma: 25 ng/ml LLOQ in urine: 500 ng/ml	Specificity ? Sensitivity ? LTAT ?
Stancato *et. al.*	UV (254 nm)	C_{18}	n/a	n/a	Specificity ? LTAT ?
Jarugula and Boudinot	UV (254 nm)	Hamilton PRP-I column	Protein precipitation with acetonitrile	n/a	Specificity ? LTAT ?
Aymard *et. al.*	UV (260 nm)	C_{18}	SPE using C_{18} J.T. Baker® columns	LLOQ: 10 ng/ml	Specificity ? Sensitivity ? LTAT ?
Sarasa *et. al.*	UV (266 nm)	C_{18}	SPE using Oasis® columns	LLOQ in plasma: 24.6 ng/ml LLOQ in urine: 1.97 µg/ml	Specificity ? Sensitivity ? LTAT ?
Moore *et. al.*	PE Sciex API- III mass spectrometer	C_{18}	SPE using ion exchange and Waters C_{18} columns	n/a (tested metabolites)	triphosphate metabolites were determined

4.4. Method development and discussion

It was originally decided to start the development phase on HPLC (with UV detection) to sort the chromatography and extraction systems out, and to test the effectiveness of the system in respect to sensitivity, selectivity and run-time on instrument.

4.4.1. HPLC (with UV detection) development

4.4.1.1. Instrumentation, chemicals and materials used during the method development stage (HPLC with UV detection)

An Agilent 1100 Series variable wavelength (UV) detector (Agilent, Palo Alto, CA, USA) was connected to an Agilent Series 1100 pump and an Agilent Series 1100 autoampler. Different columns were tested for separation of the analytes from interfering peaks which included phenyl, cyano and C_{18} from different manufacturers. Methanol and acetonitrile (Burdick and Jackson, High Purity) were obtained from Baxter chemicals (USA); sodium hydroxide, triethylamine and ammonium acetate from Fluka chemicals (Buchs, Switzerland), and orthophosphoric acid (85%) was obtained from Merck (Darmstadt, Germany). All chemicals were used as received.

Water was purified by a Millipore Elix 5 reverse osmosis and Milli-Q® (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA).

Stavudine ($C_{10}H_{12}N_2O_4$) was supplied by Cipla Ltd., Mumbai Central, India. Metronidazole, fluconazole, nevirapine and theophylline were obtained from the FARMOVS-PAREXEL® reference substance library.

4.4.1.2. Chromatography and extraction development, using HPLC with UV detection

4.4.1.2.1. Chromatography and solid phase extraction development (testing didanosine as a possible internal standard)

An HPLC system was set up and detection was performed on a UV detector that was set at 266 nm. Initially a Phenomenex Phenyl-Hexyl column (150 x 2 mm, 5 µm) was used for analyte separation

in the chromatography system. Various ratios of acetonitrile and a phosphate buffer (10 mM, 1 % triethylamine added) were tested to optimise the mobile phase, and it was found that a ratio of 5:95 (v/v), gave the best resolution when it was delivered at a flow-rate of 0.3 ml/min. The rentention times were ~ 7 min. for stavudine and ~ 5.5 min. for the internal standard (didanosine). The resolution, peak shape and retention on the column were satisfactory and the extraction development could start. Figure 3 is an example of a chromatogram of a SPVS sample that was prepared in the mobile phase at a concentration of about 700 ng/ml for both stavudine and didanosine.

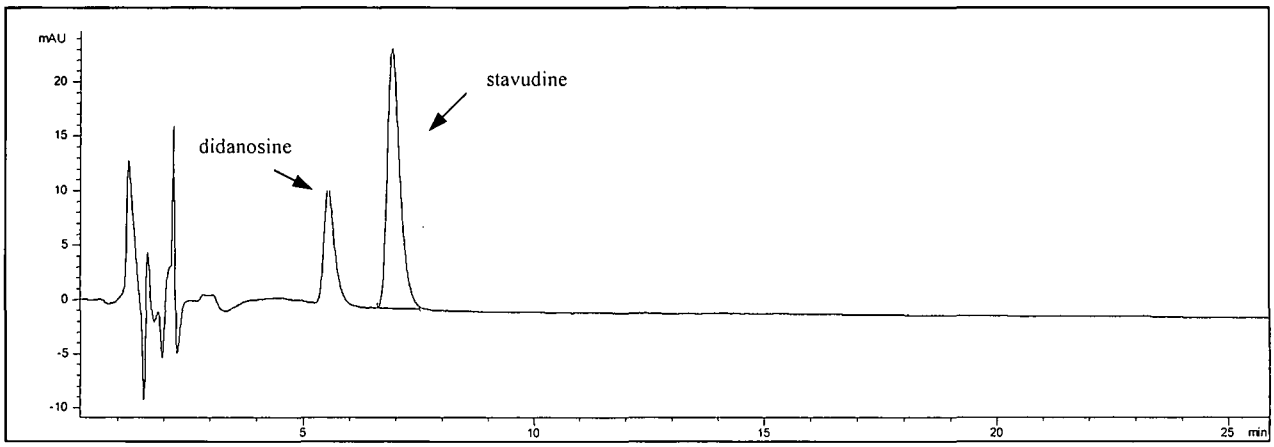


Figure 3 High performance liquid cromatogram of an SPVS sample of stavudine (~ 700 ng/ml) and didanosine (~ 700 ng/ml)

It was decided to start extraction development on C_{18} Waters (1 ml) solid phase columns. The columns were conditioned with methanol (1 ml) and water (1 ml) and the plasma samples loaded onto the columns. The columns were washed with water (2 ml) and the analytes were eluted from the columns with methanol (1 ml). The methanol was evaporated under a stream of nitrogen at 45 °C until dry and the dry extracts were reconstituted with mobile phase solution (200 µl). The recovery for stavudine was 92 % with no interference under these HPLC conditions, but interference with didanosine was observed.

Figure 4 illustrates the problem with the peak that would interfere with didanosine. Figure 5 is an example of a chromatogram of a sample extract spiked with stavudine (~ 1500 ng/ml).

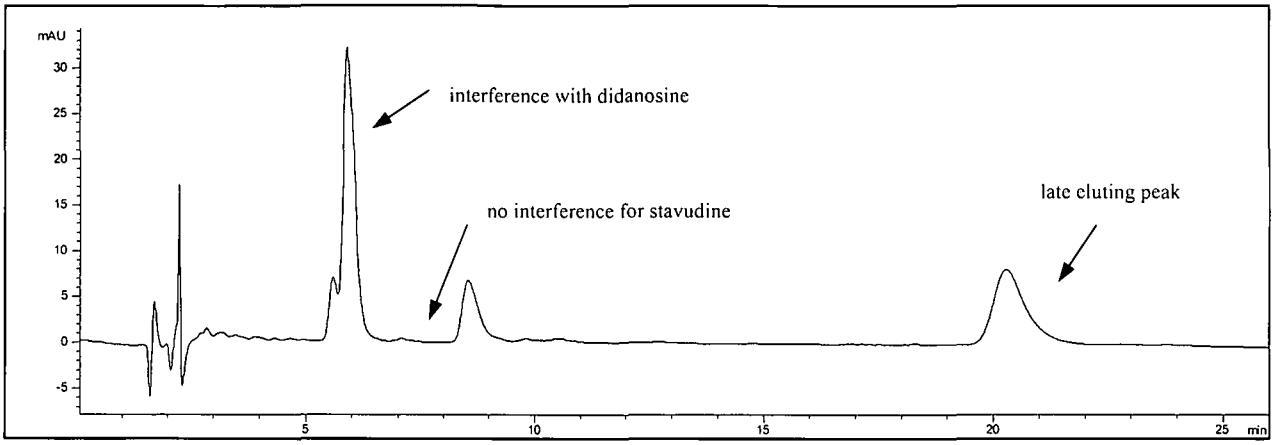


Figure 4 High performance liquid chromatogram of a blank extract (SPE)

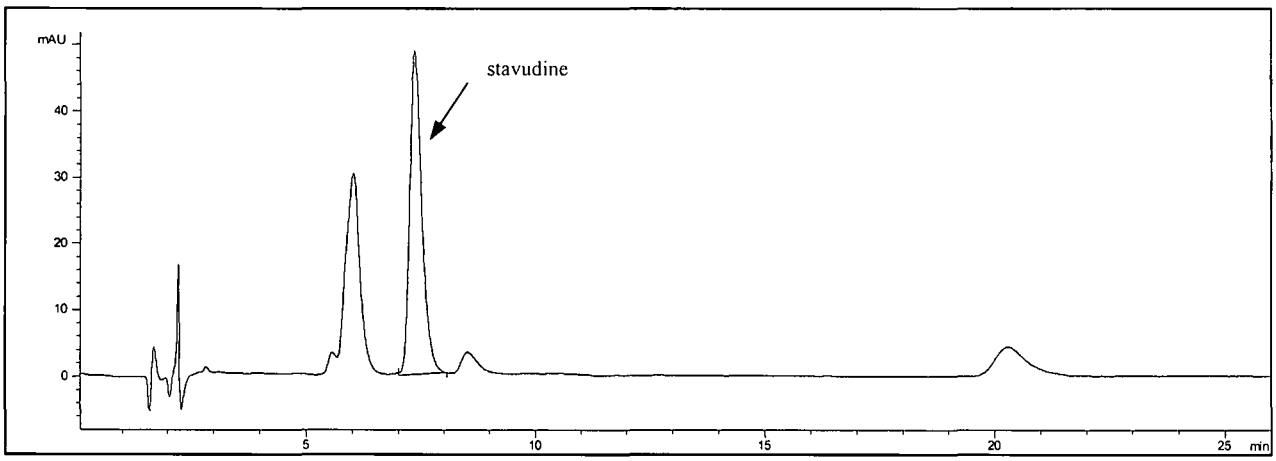


Figure 5 High performance liquid chromatogram of a spiked sample extract (SPE)

4.4.1.2.2. Chromatography and sample clean-up using protein precipitation

An alternative to solid phase extraction is protein precipitation that could have certain advantages if compared with SPE (more cost-effective, less time-consuming) and therefore it was decided to test this sample clean-up method before the SPE method would be optimised.

Ice-cold acetonitrile (1 ml) was added to the spiked sample (200 µl) at a concentration of about 800 ng/ml, vortexed for 30 sec. and centrifuged for 3 min. at 1 300 G. The supernatent layer was transferred to a polypropylene tube and excess $MgSO_4$ was added after which the sample was vortexed for 2 min. and centrifuged for 10 min. at 1 300 G. The supernatant layer was transferred to a clean polypropylene tube and the acetonitrile evaporated under a stream of nitrogen at 45 °C until dry. The dry extract was reconstituted with mobile phase (200 µl). The recovery for stavudine was 86 %, with little interference (figure 7), but interference was still observed at the retention time of the internal standard (figure 6).

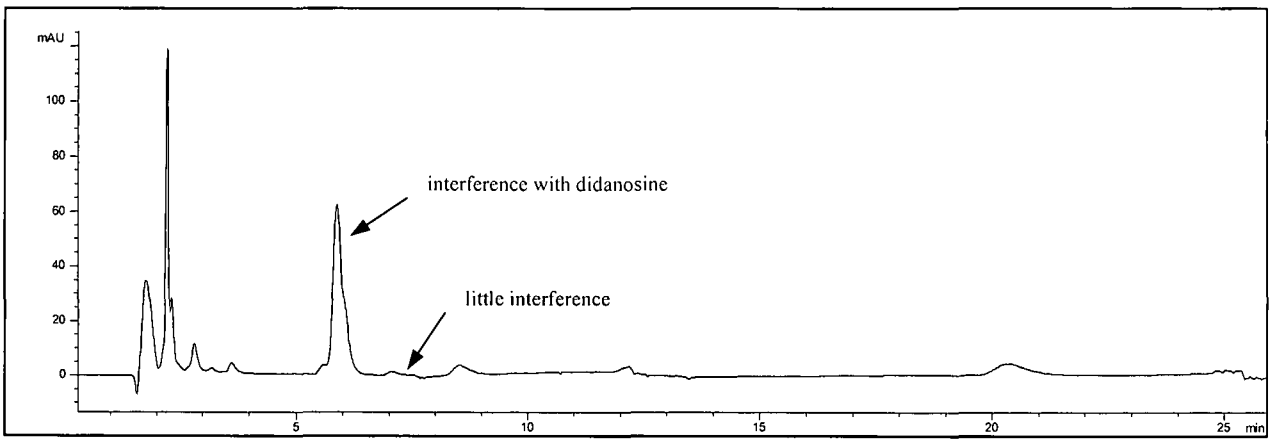


Figure 6 High performance liquid chromatogram of a blank sample (protein precipitaion)

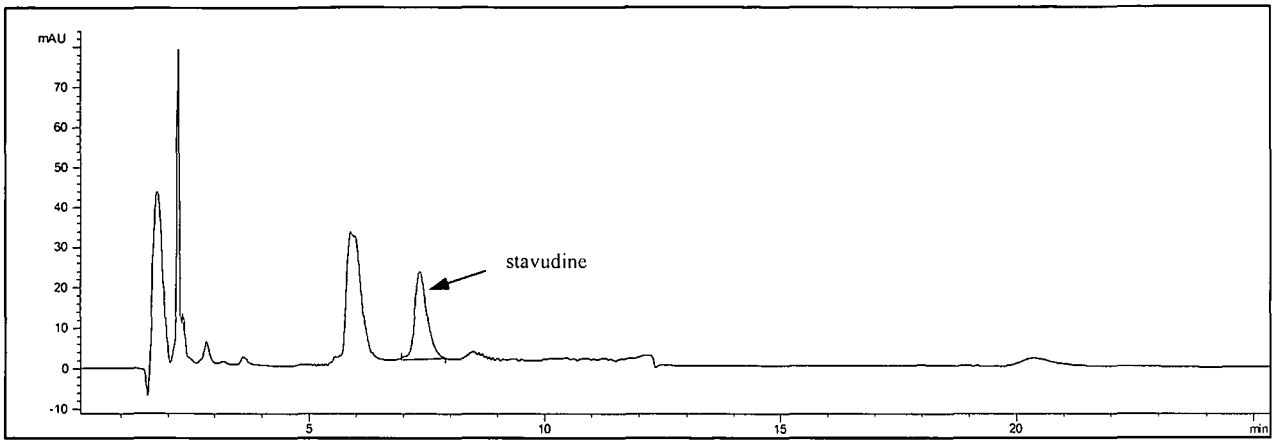


Figure 7 High performance liquid chromatogram of a spiked sample (protein precipitation)

The protein precipitation technique was not an improvement if compared to the SPE method, interfering peaks were still observed. It was clear that the SPE method was the better technique to optimise; alternative internal standards could be tested, the washing steps could be optimised as an attempt to decrease the interfering peaks, and the chromatography-system can be optimised to shift interfering peaks.

4.4.1.2.3. Alternative internal standards tested using the SPE extraction sample clean-up method

It was necessary to test alternative internal standards due to the interference problem encountered with didanosine. Metronidazole (figure 8), fluconazole, nevirapine and theophylline (figure 9) were tested, but interference with plasma peaks was still observed for metronidazole and theophylline. The retention time for nevirapine was 83 min., while fluconazole had no retention on the column. The same chromatography conditions were used for the alternative internal standard tests, as were optimised for stavudine.

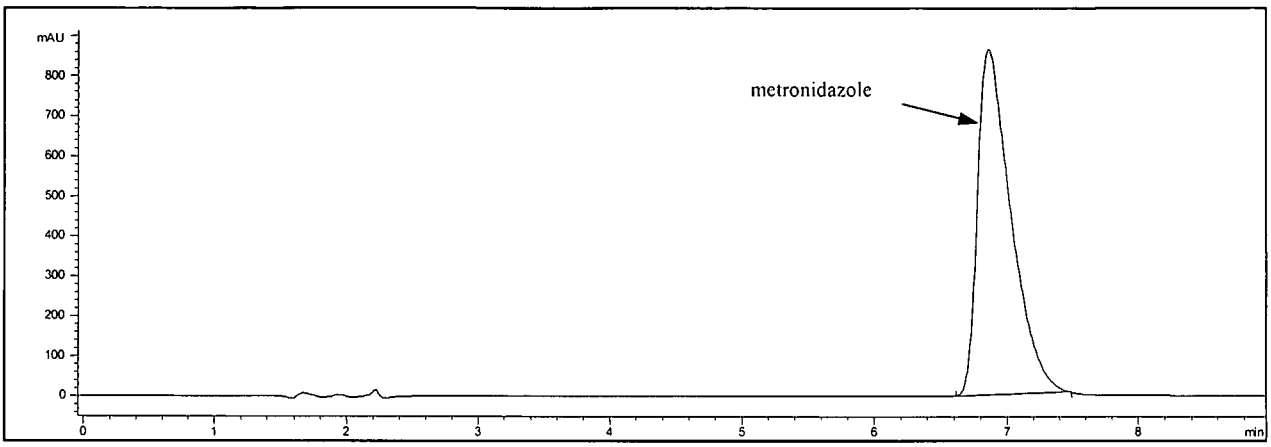


Figure 8 High performance liquid chromatogram of metronidazole

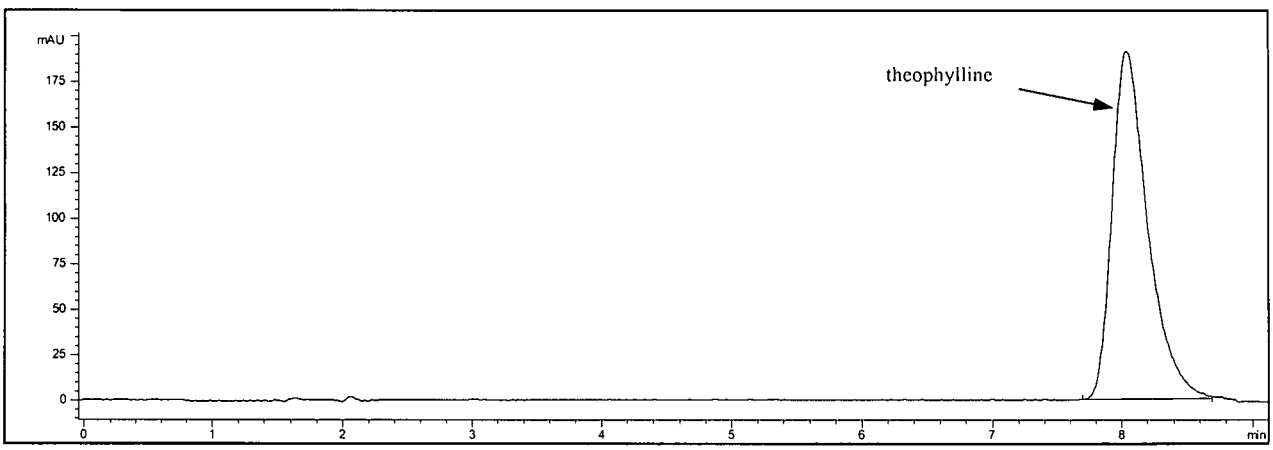


Figure 9 High performance liquid chromatogram of theophylline

Various compounds were tested, but as a suitable internal standard could not be established, it was decided to develop an assay method without one.

4.4.1.2.4. Optimisation of solid phase extraction and chromatography

The SPE was optimised and finalised as follow:

The plasma samples were thawed in a water bath at approximately 37 °C, vortexed for 5 sec. and centrifuged for 5 min. at 1 300 G.

- *SPE conditioning:* methanol (1ml)
  water (1 ml)
  phosphate buffer (1 ml, 0.05 M, pH 7)
- *Sample loading:* phosphate buffer (0.5 ml, 0.05 M, pH 7)
  plasma sample (0.5 ml)
- *Washing:* phosphate buffer (1 ml, 0.05 M, pH 7)
  water (1 ml)
- *Elution:* methanol (0.5 ml)

The methanol was evaporated at ~ 60 °C until dryness was achieved, using a savant Speedvac® rotary concentrator. The sample residues were reconstituted in water by vortex mixing for 15 sec. The mobile phase consisted of a phosphate buffer (10 mM, 1% triethylamine was added) and acetonitrile (95:5, v/v), and was delivered at a constant flow-rate of 0.3 ml/min. Chromatography was performed on a phenyl column. The retention time for stavudine was ~ 8 min. (figures 11 and 12) and no peak that would interfere with stavudine was observed in the blank extract (figure 10).

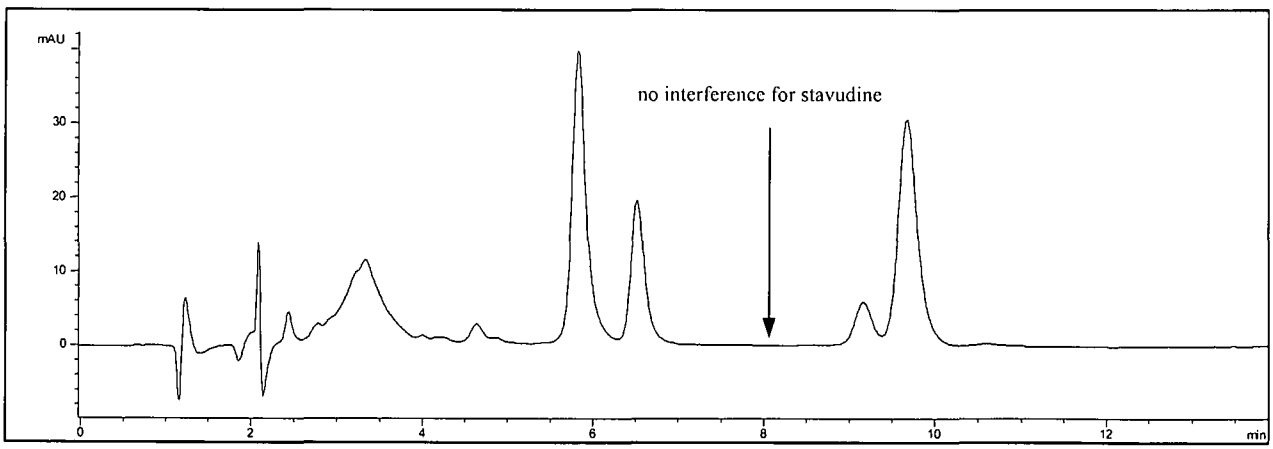


Figure 10 High performance liquid chromatogram of a blank extract (SPE)

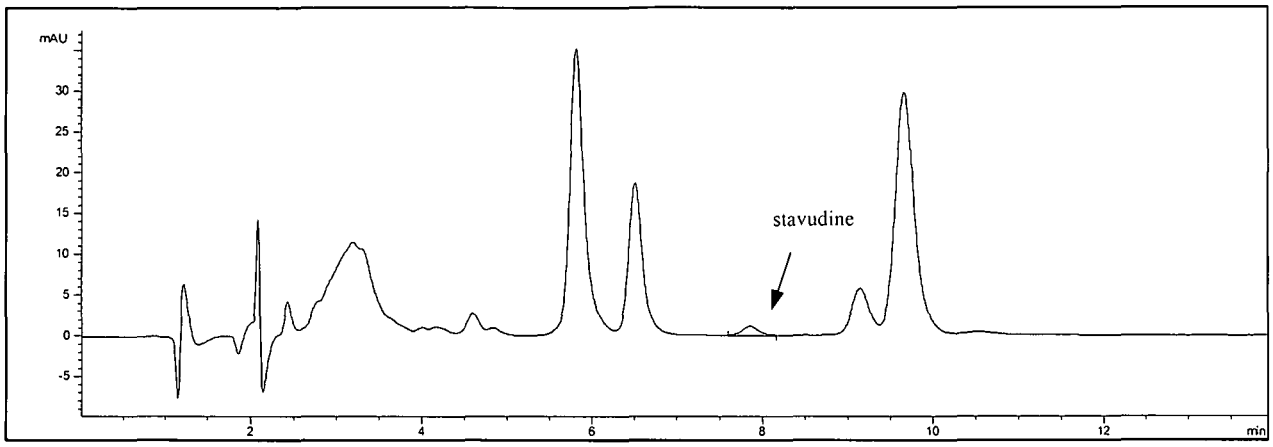


Figure 11 High performance liquid chromatogram of a spiked extract (~ 37.5 ng/ml) (SPE)

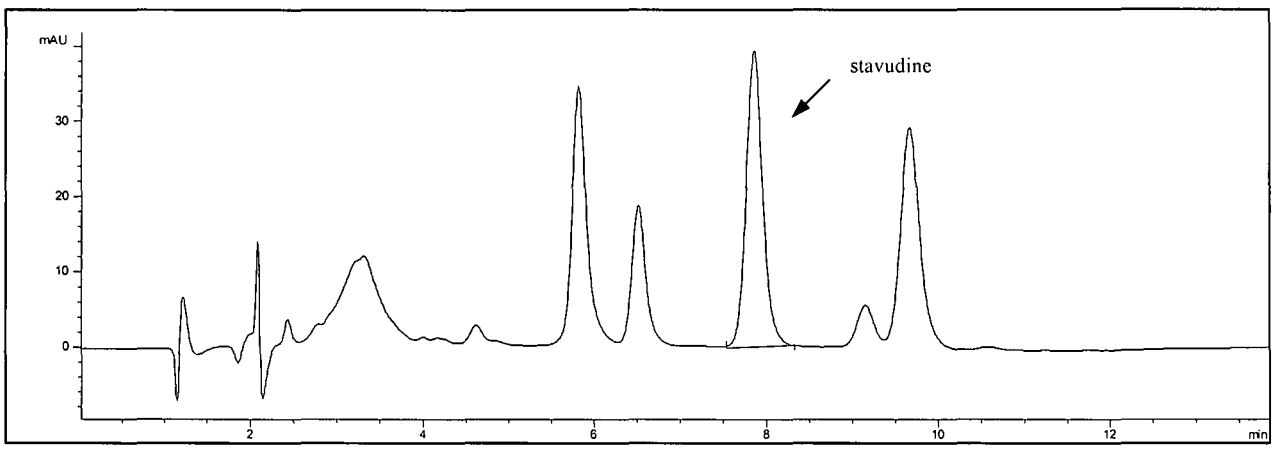


Figure 12 High performance liquid chromatogram of a spiked extract (~ 1 200 ng/ml) (SPE)

A standard calibration sequence was prepared in plasma, spanning a range of 4.7 – 2 400 ng/ml, extracted and analysed. These data are summarised in table 4 and figure 13 illustrates the linearity of the method (r^2 = 0.9999, slope is 0.033 with an intercept of – 0.308) . This method performed well but was very time-consuming.

Table 4 Summary of stavudine's standard calibration data

Concentration (ng/ml)	Peak height
4.70	0.143
9.40	0.294
18.8	0.502
37.5	1.107
75.0	2.370
150	4.259
300	9.108
600	19.449
1200	39.324
2400	79.660

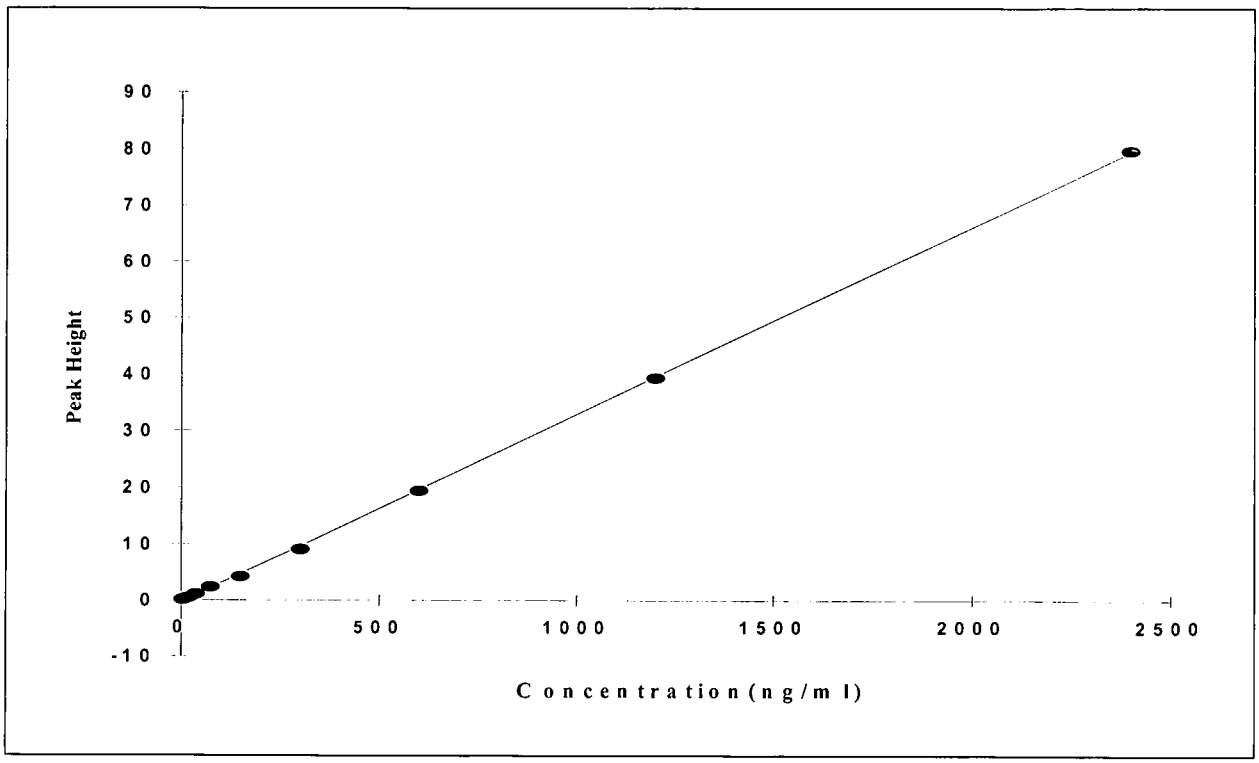


Figure 13 Standard calibration curve of stavudine

4.4.1.2.5. Summary of chromatography development done on HPLC

Initially an HPLC method using UV detection (266 nm) was developed. Due to the highly polar nature of stavudine a very aqueous mobile phase had to be used, leading to many late eluting peaks. Different columns were tested with a phenyl column giving the best results (retention, peak shape). Under these conditions the chromatography time had to be in excess of 20 min. (due to the late eluting peaks) which made the method very time-consuming and not very productive. A more rapid method was needed to analyse the large number of samples (~700) and it was decided that LC-MS/MS technology should be used.

4.4.2. Chromatography and extraction development, using HPLC with MS/MS detection

4.4.2.1. Mass spectrometry optimisation

An Applied Biosystems API 2000 LC-MS/MS detector was set up for ion detection. The mass spectrometer was calibrated by using a PPG Standard solution in the positive and negative ionisation mode. The instrument response was optimised for stavudine by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column.

Different concentrations of ammonium acetate were tested for optimum ionisation of the analyte and it was found that 10 mM ammonium acetate gave the best result. Stavudine also gave a much higher (10 fold) response with APCI than with ESI.

APCI was performed in the negative ionisation mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 20 and 40 (arbitrary values), respectively. The heated nebulizer temperature was set at 450 °C and the nebulizer current on -3.0 μA. The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) set at 3 (arbitrary value).

The mass spectrometer was operated at unit resolution in the MRM mode, monitoring the transition of the deprotonated molecular ion m/z 223.1 to the product ion m/z 42.01.

Figure 14 shows the single parent (m/z 223.1) to product ion mass spectrum of stavudine. The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.1 software.

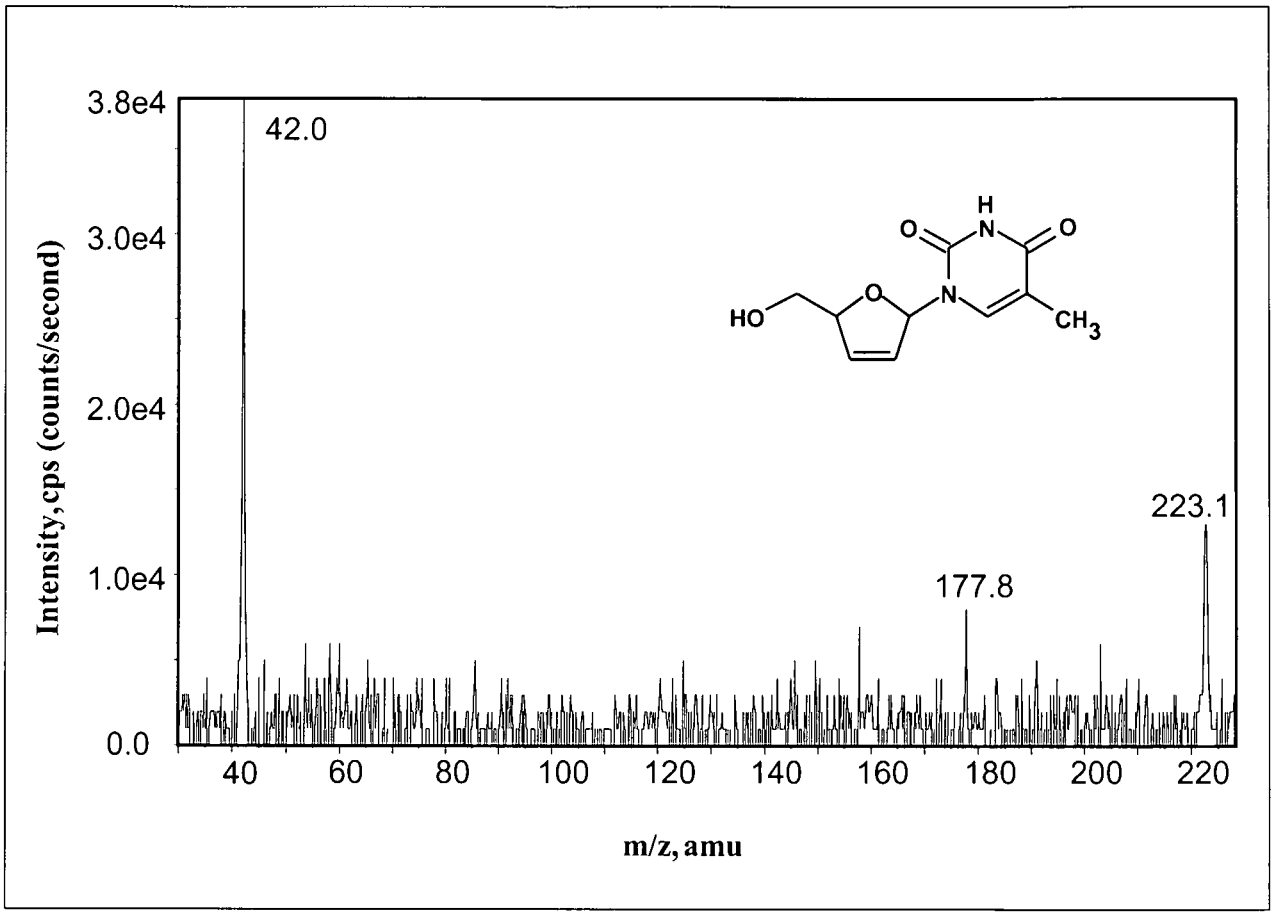


Figure 14 Product ion mass spectrum of the deprotonated stavudine molecular ion (m/z 223.1, molecular structure given) and the principal product ion formed at m/z 42.01 after collision (MS/MS)

4.4.2.2. Chromatography development on LC-MS/MS

Because of the high selectivity of the mass spectrometer it was decided to use a column that would give short chromatography time. A Supelco Discovery C_{18} column was used with a mobile phase consisting of ammonium acetate (0.01M): acetonitrile: methanol (800 : 100 : 100, v/v/v) at a flow-rate of 0.3 ml/min at ambient temperature. The retention time for stavudine was 1.97 min. (figure 15). A total chromatography run time of 4 min. made it possible to analyse large batches of samples (215 samples) per day.

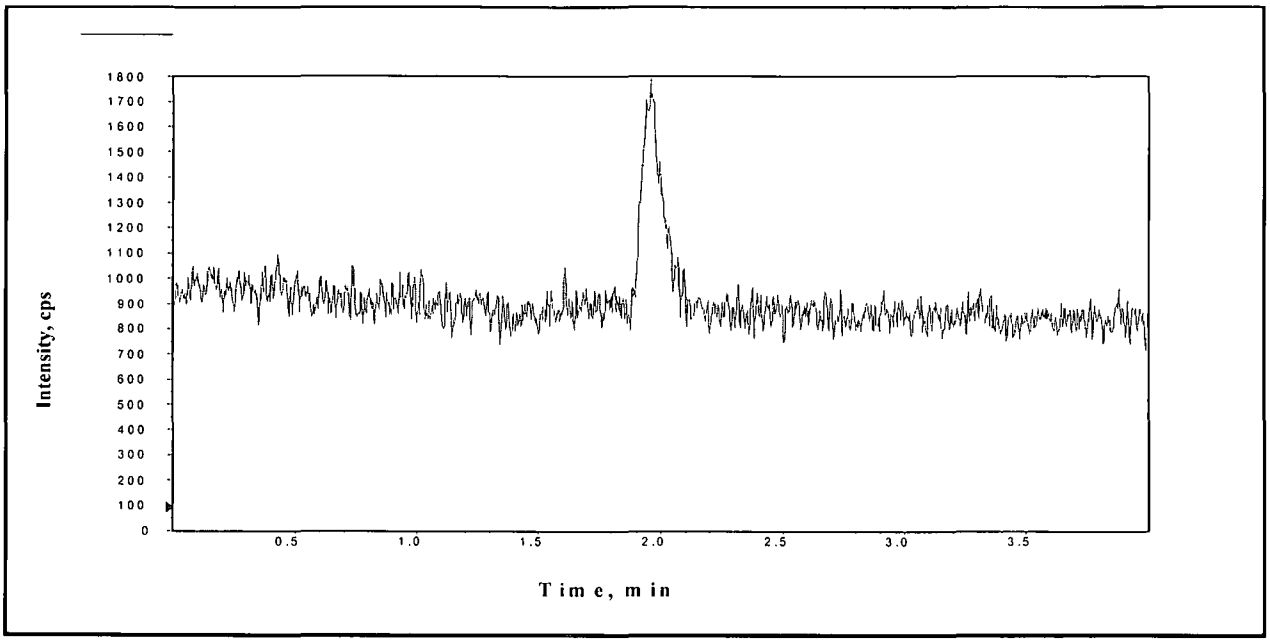


Figure 15 A chromatogram of stavudine at a concentration of 4 ng/ml

4.4.2.3. Extraction development

SPE and protein precipitation was fully tested as sample clean-up techniques during the HPLC-method development phase of the project. Additional liquid-liquid extraction experiments were done during the LC-MS/MS development phase. Various experiments were performed without success. Of the different organic solvents (hexane, ethyl ether, ethyl acetate and a mixture (1:1, v/v) of hexane and iso-amyl alcohol) tested, ethyl acetate gave the best recovery (32 %). These extractions were performed as follows: 200 µl of a 0.1 M sodium hydroxide solution was added to 200 µl plasma and the organic liquid (4 ml) added. The samples were vortexed for 60 sec. and centrifuged for 3 min. at 1300 G. The aqueous phase was frozen in an alcohol-freezing bath and the organic phase was transferred to a 5 ml ampoule. The samples were evaporated until dry with a stream of nitrogen and reconstituted in mobile phase or back extracted in 2 % aqueous formic acid.

Although the internal standard technique is widely used in chromatography, this does not inevitably improve the precision of an assay method; in fact it may even impair the precision of the assay,[20] if the internal standard is not judiciously chosen. However, when using a mass spectrometer as a

detector, stable isotope labelled analogues of the analyte to be analysed can be employed as ideal internal standards as far as their physical and chemical properties of the analyte are concerned.
Due to the unavailability of a stable isotope labelled analogue of stavudine many alternative compounds were tested as internal standards but none were found to be suitable. Consequently it was decided to develop the assay without an internal standard.

4.4.2.4. Matrix effect

It has been noted that coeluting undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LC/MS-MS assay.[1] In order to determine whether this effect (called the Matrix Effect) is present or not, 10 different plasma pools were extracted and spiked with a known concentration (762 ng/ml) of analyte. These samples were injected and peak areas compared. The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect. The summarised data (table 5) shows no significant matrix effect (CV% = 3.49) at the tested concentration.

Table 5 Tested plasma pools

	Analyte Peak Area
Plasma Pool	**762 ng/ml**
1	442000
2	403000
3	425000
4	412000
5	449000
6	429000
7	411000
8	421000
9	414000
10	411000
Mean	421700
SD	14735.07
CV%	3.49

The peak areas of the 10 reconstituted samples had a coefficient of variation of 3.49 % indicating that the extracts were "clean" with no co-eluting compounds influencing the ionisation of the analyte.

4.5. Analytical method validation and discussion

4.5.1. Extraction procedure

The plasma samples were thawed in a water bath at approximately 37 °C, vortexed for 5 sec. and centrifuged for 5 min. at 1 300 G. The SPE procedure was performed manually on C_{18} columns (Waters, Sep-Pak®Vac, 100 mg):

- *Conditioning of columns:* methanol (1 ml)
  water (1 ml)
  phosphate buffer (1 ml, 0.05 M, pH 7)
- *Loading of sample:* samples were diluted with the phosphate buffer (1:1, v/v),
  1 ml of the mixture was loaded
- *Washing of columns*: phosphate buffer (1 ml)
  water (1 ml)
- *Sample elution:* methanol (0.5 ml)

The samples were evaporated at high temperatures (~ 60 °C) until dryness was achieved (Savant Speedvac®rotary concentrator) and the sample residues were reconstituted in water (0.25 ml) by vortex mixing the sample for 15 sec. The sample (20 µl) was injected onto the HPLC column.

4.5.2. Instrumental and chromatography conditions

Chromatography was performed on a Supelco Discovery C_{18} (150 x 2.0 mm, 5 µm) stainless steel column. The mobile phase consisted of ammonium acetate (10 mM) : acetonitrile : methanol (800:100:100, v/v/v) and was delivered at a constant flow rate of 0.3 ml/min. Detection was performed on an API 2000 mass spectrometer (APCI in the negative ionisation mode, MRM) and the settings on the apparatus are summarised in tables 6 and 7.

Table 6 APCI settings

Nebulizer gas	70
Turbo spray	20
Curtain gas	40
Heated nebulizer (°C)	450
Nebulizer current	-3

Table 7 MS/MS settings

Mono-isotopic molecular mass	224.08
Deprotonated molecular ion (m/z)	223.10
Dwell time (ms)	150
Product ion (m/z):	42.01
Declustering potential (V)	-21
Focusing potential (V)	-310
Entrance potential (V)	9.5
Collision cell entrance potential (V)	-12
Collision energy (eV)	-30
Ccollision cell exit potential (V)	-8
Collision activated dissociation gas	3
Scan type	MRM
Polarity	negative
Pause time	5 ms

4.5.3. Preparation of calibration standards

C_{max} was expected to be ~ 1000 ng/ml, therefore it was decided to validate the method between 2x C_{max} and the LLOQ (S/N > 5). The LLOQ should be sensitive enough to determine stavudine in plasma for a period of at least 5 elimination half-lives after a 40 mg dose of stavudine. The method was expected to be sufficiently sensitive with an LLOQ of about 4 ng/ml.

The calibration standards were prepared in human plasma. A stock solution with a concentration of 160 µg/ml was prepared in water as indicated in table 8. A pool of normal plasma (STD K) was spiked with the stock solution (1000 µl) and was serially diluted with normal plasma to attain the desired concentrations (table 9). STD J represents C_{max} with a concentration of 1015 ng/ml and the LLOQ is represented by STD B (3.97 ng/ml). The calibration standards were aliquoted (1.2 ml) into polypropylene tubes and stored at approximately -20°C.

Table 8 Preparation of Stock Solution SA for Spiking STD K

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Water	1.000	0.960	5.996	5.996	1000	160

Table 9 Preparation of Calibration Standards

Calibration Standard	Source Solution	A	B	C	D (ng/ml)
STD K	*Stock SA*	106.303	186.303	-	2029
STD J	*STD K*	105.788	145.799	185.819	1015
STD I	*STD J*	106.931	146.940	186.965	507
STD H	*STD I*	105.944	145.954	186.021	254
STD G	*STD H*	109.563	149.571	189.586	127
STD F	*STD G*	108.630	148.635	188.644	63.5
STD E	*STD F*	114.429	154.424	194.433	31.7
STD D	*STD E*	104.212	144.218	184.232	15.9
STD C	*STD D*	105.198	145.191	185.184	7.94
STD B	*STD C*	105.312	145.318	185.340	3.97

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

4.5.4. Preparation of quality control standards

Quality control standards were prepared in human plasma (same methodology as for the preparation of the standards). The preparation of the stock solution is shown in table 10. A pool of normal plasma was spiked with the stock solution and was serially diluted with normal plasma to attain the desired concentrations (table 11). The quality control standards were stored under the same conditions as were the standards.

Table 10 Preparation of Stock Solution QA for Spiking QC I

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Water	1.000	0.682	4.265	4.265	1000	160

Table 11 Preparation of Quality Control Standards

Quality Control Standard	Source Solution	A	B	C	D (ng/ml)
QC I	*Stock QA*	106.949	206.947	-	1625
QC H	*QC I*	105.468	155.467	205.465	813
QC G	*QC H*	105.660	155.660	205.675	406
QC F	*QC G*	116.567	166.567	216.567	203
QC E	*QC F*	106.978	156.977	206.980	102
QC D	*QC E*	104.021	154.021	204.018	50.8
QC C	*QC D*	105.556	167.558	205.560	19.3
QC B	*QC C*	105.726	155.728	205.733	9.65
QC A	*QC B*	105.974	155.973	205.971	4.83

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

4.5.5. Intra-batch accuracy and precision

The method was validated by analysing plasma quality control samples six times at nine different stavudine concentrations, i.e. 1625, 813, 406, 203, 102, 50.8, 19.3, 9.65 and 4.83 ng/ml, to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing ten different concentrations spanning the concentration range (2029 - 3.97 ng/ml). Calibration graphs were constructed using a weighted linear regression ($1/concentration^2$) of the drug peak-area of the product ions versus nominal drug concentrations. Several regression types were tested and the weighted linear regression ($1/concentration^2$) was found to be the simplest regression, giving the best results.

Intra-batch accuracy and precision are assessed by the assay of all the calibration standards in duplicate to produce one calibration curve and 6 replicates of all the prepared quality control standards in a single batch of assays. The intra-batch accuracy and precision of the assay procedure are assessed by calculating the regression equations and constructing the calibration curves based on both peak heights and peak areas to result in two different quantification methods.

Accuracy is expressed as recovery of the analyte as % nominal while the precision is expressed as the CV %. For a valid method the intra-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nominal should be between 85 % - 115 %) over most of the range, and within 20 % of nominal concentration at the LLOQ. For a valid method the intra-batch precision is required to be less than 15 % (i.e. CV% should be less than 15 %) over most of the range, and less than 20 % at the LLOQ.

The method performed well using both quantitations (peak height and peak area). The peak area quantitation method gave the best results and was used for the two inter-batch validations. A linear regression weighted $1/c^2$ was the simplest equation giving the best results and was used for the statistical analyses of the inter-batch validations. The calibration range was validated between 3.97 and 2029 ng/ml for both peak heights and peak areas.

QC I was diluted (1:1) with blank plasma and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

The results of the intra-batch validation are summarised in tables 12 to 15.

4.5.5.1. Quantitation by peak height

Table 12 Back-calculated concentrations of stavudine based on peak heights

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD K	2029	2176.961	7.3
STD K	2029	2220.502	9.4
STD J	1015	1066.667	5.1
STD J	1015	1211.804	19.4
STD I	507	528.211	4.2
STD I	507	539.822	6.5
STD H	254	237.938	-6.3
STD H	254	260.434	2.5
STD G	127	119.652	-5.8
STD G	127	130.537	2.8
STD F	63.5	51.365	-19.1
STD F	63.5	59.420	-6.4
STD E	31.7	31.336	-1.1
STD E	31.7	29.087	-8.2
STD D	15.9	16.750	5.3
STD D	15.9	14.065	-11.5
STD C	7.94	7.113	-10.4
STD C	7.94	7.462	-6.0
STD B	3.97	4.189	5.5
STD B	3.97	4.247	7.0

Calibration Range: 3.97 - 2029 ng/ml
Regression Equation: Linear (1/concentration^2)
Intercept: 11.79
Slope: 137.80
r^2: 0.9890

Table 13 Summary of intra-batch quality control results based on peak heights

Code	QC I 1625 ng/ml	QC I Dil 1625 ng/ml	QC H 813 ng/ml	QC G 406 ng/ml	QC F 203 ng/ml	QC E 102 ng/ml	QC D 50.8 ng/ml	QC C 19.3 ng/ml	QC B 9.65 ng/ml	QC A 4.83 ng/ml
1	1748.808	1668.898	877.990	402.668	211.088	106.590	56.082	22.628	11.090	4.951
2	1683.497	1567.302	827.192	449.112	198.751	116.749	56.663	20.741	10.800	5.089
3	1930.229	1509.248	914.274	422.987	175.530	116.749	50.785	21.177	10.800	5.604
4	1806.863	1639.871	936.044	435.324	203.105	105.864	53.252	16.823	9.566	5.132
5	1632.699	1639.871	798.165	417.182	191.495	79.014	45.778	19.508	9.131	5.234
6	1727.038	1567.302	805.422	407.022	202.380	97.882	46.358	19.218	10.001	4.922
MEAN	**1754.86**	**1598.75**	**859.85**	**422.38**	**197.06**	**103.81**	**51.49**	**20.02**	**10.23**	**5.16**
%nom	**108.0**	**98.4**	**105.8**	**104.0**	**97.1**	**101.8**	**101.4**	**103.7**	**106.0**	**106.7**
CV%	**5.4**	**3.5**	**6.2**	**3.8**	**5.7**	**12.4**	**8.3**	**9.1**	**7.0**	**4.4**

4.5.5.2. Quantitation by peak area

Table 14 Back-calculated concentrations of stavudine based on peak areas

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD K	2029	2066.619	1.9
STD K	2029	2014.630	-0.7
STD J	1015	990.444	-2.4
STD J	1015	1119.117	10.3
STD I	507	480.950	-5.1
STD I	507	538.138	6.1
STD H	254	239.200	-5.8
STD H	254	254.797	0.3
STD G	127	118.976	-6.3
STD G	127	133.922	5.5
STD F	63.5	58.408	-8.0
STD F	63.5	63.737	0.4
STD E	31.7	33.843	6.8
STD E	31.7	31.244	-1.4
STD D	15.9	17.077	7.4
STD D	15.9	15.517	-2.4
STD C	7.94	7.589	-4.4
STD C	7.94	7.511	-5.4
STD B	3.97	4.015	1.1
STD B	3.97	4.067	2.4

Calibration Range: 3.97 - 2029 ng/ml
Regression Equation: Linear (1/concentration^2)
Intercept: -38.73
Slope: 769.39
r^2: 0.9963

Table 15 Summary of intra-batch quality control results based on peak areas

Code	QC I 1625 ng/ml	QC I Dil 1625 ng/ml	QC H 813 ng/ml	QC G 406 ng/ml	QC F 203 ng/ml	QC E 102 ng/ml	QC D 50.8 ng/ml	QC C 19.3 ng/ml	QC B 9.65 ng/ml	QC A 4.83 ng/ml
1	1585.720	1544.179	818.880	397.767	195.010	102.859	54.249	20.716	9.954	4.755
2	1676.701	1396.010	801.983	413.364	208.007	112.737	55.029	19.546	10.435	4.846
3	1806.674	1463.595	861.771	418.563	174.214	109.747	49.310	20.066	10.253	5.093
4	1767.682	1603.966	853.972	421.163	208.007	109.358	54.899	18.117	9.200	4.976
5	1611.714	1637.759	803.283	402.966	196.309	86.742	50.480	19.806	10.071	4.807
6	1754.684	1663.754	821.479	408.165	210.606	106.108	47.490	19.546	10.071	5.028
MEAN	1700.53	1551.54	826.89	410.33	198.69	104.59	51.91	19.63	10.00	4.92
%nom	104.6	95.5	101.7	101.1	97.9	102.5	102.2	101.7	103.6	101.8
CV%	4.8	6.2	2.8	2.0	6.3	8.2	5.7	4.0	3.9	2.5

4.5.6. Inter-batch accuracy and precision

Inter-batch accuracy and precision are assessed by assaying two separate consecutive batches, each consisting of one set of calibration standards designated for use in the assay of samples of unknown concentrations, and 6 replicates of each of the quality control standards designated for use in the assay of samples of unknown concentrations. The intra-batch accuracy and precision of each of the batches is assessed separately by calculating the regression equation and constructing the calibration curve based on the best performing quantification method, and must pass the criteria for intra-batch acceptance. The inter-batch accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 in total). Accuracy is expressed as the % difference between the nominal and calculated value or expressed as % nominal of the analyte, while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra- and inter-batch accuracy is required to be within 15 % of the nominal concentration (ie. % nom should be between 85 % - 115 %) over most of the range and within 20 % of nominal concentration at the LLOQ. For a valid method the intra- and inter-batch precision is required to be less than 15 % (i.e. CV % should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The method performed well during the two inter-batch validations with a highest variation of 7.7 % (QC B, second inter-batch).

The results are summarised in tables 16 to 19.

4.5.6.1. Inter-batch 1 accuracy and precision

Table 16 Back-calculated concentrations of stavudine

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD K	2029	1904.101	-6.2
STD J	1015	1008.401	-0.7
STD I	507	497.380	-1.9
STD H	254	263.555	3.8
STD G	127	125.900	-0.9
STD F	63.5	66.690	5.0
STD E	31.7	31.144	-1.8
STD D	15.9	15.965	0.4
STD C	7.94	8.054	1.4
STD C	7.94	8.196	3.2
STD B	3.97	3.595	-9.5
STD B	3.97	4.245	6.9

Calibration Range:	3.97 - 2029 ng/ml
Quantification Method:	Peak Area
Regression Equation:	Linear (1/concentration^2)
Intercept:	467.54
Slope:	1060.62
r^2:	0.9968

Table 17 Summary of quality control results for inter-batch 1

Code	QC I 1625 ng/ml	QC H 813 ng/ml	QC G 406 ng/ml	QC C 19.3 ng/ml	QC B 9.65 ng/ml	QC A 4.83 ng/ml
1	1460.965	757.605	355.011	17.096	8.535	4.321
2	1762.675	802.861	402.153	17.945	8.790	4.179
3	1724.961	852.832	407.810	19.830	8.799	4.424
4	1743.818	832.089	404.039	19.547	8.922	4.537
5	1696.676	828.318	416.296	20.490	9.459	4.688
6	1706.104	858.489	428.553	20.019	9.930	4.424
MEAN	1682.53	822.03	402.31	19.15	9.07	4.43
%nom	103.5	101.1	99.1	99.2	94.0	91.7
CV%	6.0	4.1	5.7	6.3	5.2	3.6

4.5.6.2. Inter-batch 2 accuracy and precision

Table 18 Back-calculated concentrations of stavudine

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD K	2029	2091.803	3.1
STD J	1015	1063.102	4.7
STD I	507	480.460	-5.2
STD H	254	254.837	0.3
STD G	127	115.660	-8.9
STD F	63.5	64.398	1.4
STD E	31.7	34.142	7.7
STD D	15.9	16.334	2.7
STD C	7.94	8.044	1.3
STD C	7.94	7.136	-10.1
STD B	3.97	4.232	6.6
STD B	3.97	3.826	-3.6

Calibration Range:	3.97 - 2029 ng/ml
Quantification Method:	Peak Area
Regression Equation:	Linear (1/concentration^2)
Intercept:	204.55
Slope:	1156.80
r^2:	0.9949

Table 19 Summary of quality control results for inter-batch 2

Code	QC I 1625 ng/ml	QC H 813 ng/ml	QC G 406 ng/ml	QC C 19.3 ng/ml	QC B 9.65 ng/ml	QC A 4.83 ng/ml
1	1659.576	811.546	375.861	17.804	9.678	4.509
2	1694.154	729.423	387.963	20.397	10.542	4.915
3	1754.666	805.495	394.879	18.495	8.814	5.174
4	1547.197	807.224	375.861	16.766	8.286	4.889
5	1529.908	663.724	387.099	17.890	9.073	5.278
6	1694.154	767.459	419.948	19.533	9.678	5.019
MEAN	1646.61	764.15	390.27	18.48	9.35	4.96
%nom	101.3	94.0	96.1	95.8	96.8	102.8
CV%	5.0	7.0	3.8	6.5	7.7	4.9

4.5.7. Summary of the combined quality control results for the 3 validations

The combined quality control results are summarised in table 20. The method performed well during all three validations with the highest coefficient of variation of 7.1 %.

Table 20 Summary of the combined quality control results for the 3 validations

Validation Batch	Nominal Replicates	1625 ng/ml	813 ng/ml	406 ng/ml	19.3 ng/ml	9.65 ng/ml	4.83 ng/ml
Intra-batch Validation	1	1585.720	818.880	397.767	20.716	9.954	4.755
	2	1676.701	801.983	413.364	19.546	10.435	4.846
	3	1806.674	861.771	418.563	20.066	10.253	5.093
	4	1767.682	853.972	421.163	18.117	9.200	4.976
	5	1611.714	803.283	402.966	19.806	10.071	4.807
	6	1754.684	821.479	408.165	19.546	10.071	5.028
Inter-batch Validation 1	1	1460.965	757.605	355.011	17.096	8.535	4.321
	2	1762.675	802.861	402.153	17.945	8.790	4.179
	3	1724.961	852.832	407.810	19.830	8.799	4.424
	4	1743.818	832.089	404.039	19.547	8.922	4.537
	5	1696.676	828.318	416.296	20.490	9.459	4.688
	6	1706.104	858.489	428.553	20.019	9.930	4.424
Inter-batch Validation 2	1	1659.576	811.546	375.861	17.804	9.678	4.509
	2	1694.154	729.423	387.963	20.397	10.542	4.915
	3	1754.666	805.495	394.879	18.495	8.814	5.174
	4	1547.197	807.224	375.861	16.766	8.286	4.889
	5	1529.908	663.724	387.099	17.890	9.073	5.278
	6	1694.154	767.459	419.948	19.533	9.678	5.019
	MEAN	1676.56	804.36	400.97	19.09	9.47	4.77
	%nom	3843.6	4240.2	11169.1	2807.3	3266.1	3669.3
	CV%	5.5	6.0	4.6	6.2	7.1	6.4

4.5.8. Stability assessment

4.5.8.1. Stability in matrix

Stavudine is reported to be stable for at least 4 days at room temperature and at 4 °C and for at least 1 year at - 20 °C. [16] This data was taken as conclusive evidence of the stability of stavudine in plasma at – 20 °C, and no further long-term stability testing was therefore undertaken. Samples, calibration standards and quality control standards are kept frozen at - 20 °C until assayed.

4.5.8.2. Freeze and thaw stability

Spiked solutions of stavudine in plasma at two different concentrations (813 and 203 ng/ml) were frozen at -20°C and put through two freeze- and thaw cycles. These samples were analysed during the intra-batch validation. Peak areas and means as well as the calculated differences between the two sets of aliquots are summarised in table 21.

Only two freeze- and thaw cycles were performed to obtain some data on the freeze- and thaw stability of stavudine, but no trial samples were reanalysed using samples that were previously thawed. All re-analyses of samples were performed using the frozen duplicate samples.

Table 21 Freeze and thaw stability measured at 813 and 203 ng/ml

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
813	824.08	101.36
813	729.20	89.69
813	770.79	94.81
813	800.68	98.48
813	830.58	102.16
Mean	791.00	97.30
Std.Dev.	41.78	5.14
CV%	5.28	
%Nom.	97.30	

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
203	210.61	103.75
203	201.51	99.27
203	205.41	101.19
203	200.21	98.63
203	209.31	103.11
Mean	205.4	101.2
Std.Dev.	4.60	2.26
CV%	2.24	
%Nom.	101.19	

By comparing measured concentrations against the nominal concentrations of the tabulated data, a correlation coefficient of 0.96 (figure 16) at -20 °C was obtained for stavudine, indicating that the effect of two freeze-thaw cycles on the measured stavudine concentrations is negligible.

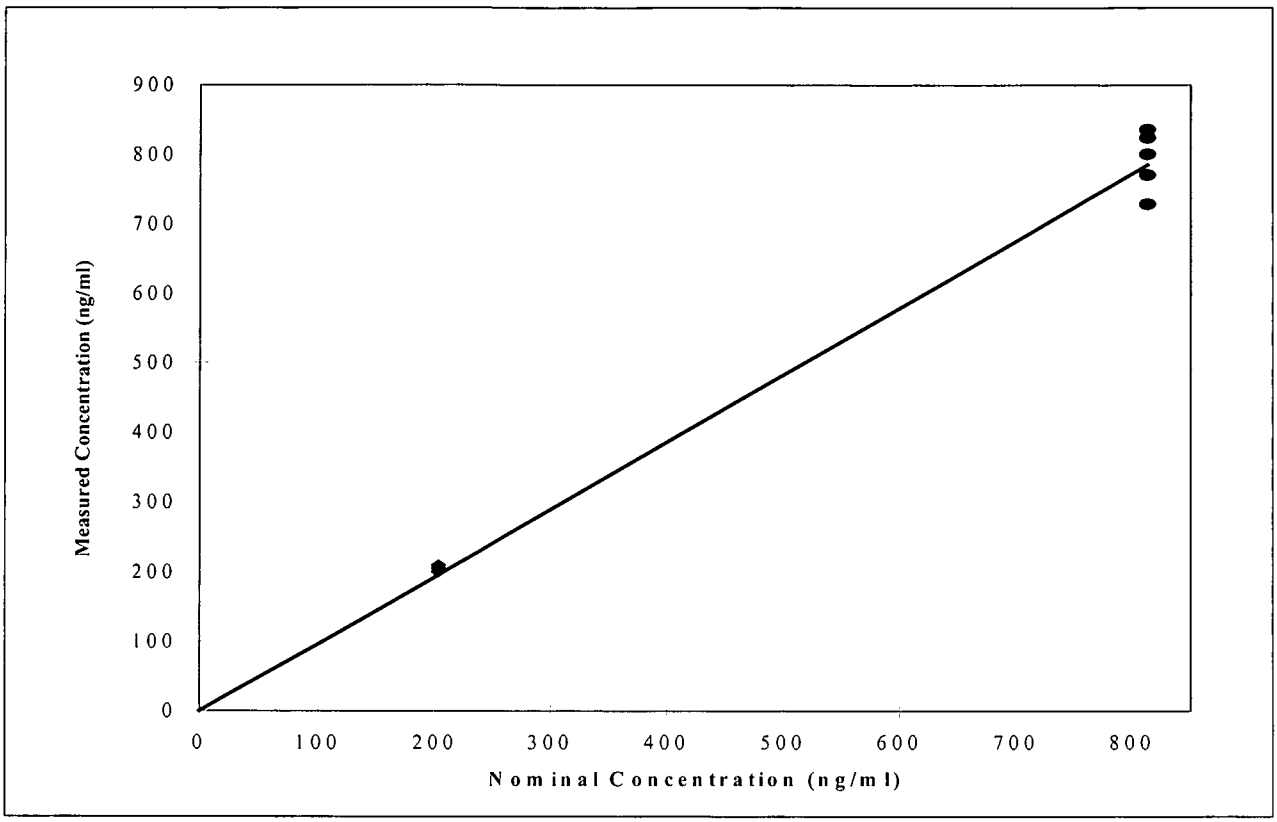


Figure 16 Freeze-Thaw stability correlation of measured vs. nominal concentrations

4.5.8.3. On-Instrument stability

Sixteen stability samples of the same concentration were extracted; the extracts combined and re-aliquoted, and injected at intervals during the first two validation batches to simulate the time of a batch run. The measured peak areas, injection times and cumulative time are summarised in table 22.

Table 22 Stability data of sixteen stability samples injected at different intervals

Replicates	Injection Time	Time Difference	Cumulative Time (hr)	Analyte Peak Area
1	17:04	-	0.00	369000
2	17:47	0:43:00	0.72	412000
3	18:30	0:43:00	1.43	426000
4	19:17	0:47:00	2.22	438000
5	20:00	0:43:00	2.93	433000
6	20:43	0:43:00	3.65	448000
7	21:45	1:02:00	4.68	440000
8	21:50	0:05:00	4.77	449000
9	13:15	15:25:00	20.18	453000
10	13:58	0:43:00	20.90	454000
11	14:41	0:43:00	21.62	456000
12	15:28	0:47:00	22.40	428000
13	16:11	0:43:00	23.12	410000
14	16:54	0:43:00	23.83	451000
15	17:56	1:02:00	24.87	471000
16	18:01	0:05:00	24.95	469000
			Mean	437938
			Std Dev	25481
			CV	5.82%

The peak areas of stavudine are seen to be relatively stable within a batch run. By regression analysis of the peak areas against the cumulative time tabulated above it can be established that the peak area of stavudine tends to increase by 7.49 % over a period of 24.95 hours over two batches while awaiting injection on-instrument (figure 17). This trend is deemed insignificant and the extracts are considered to be stable on-instrument for at least 25 hours.

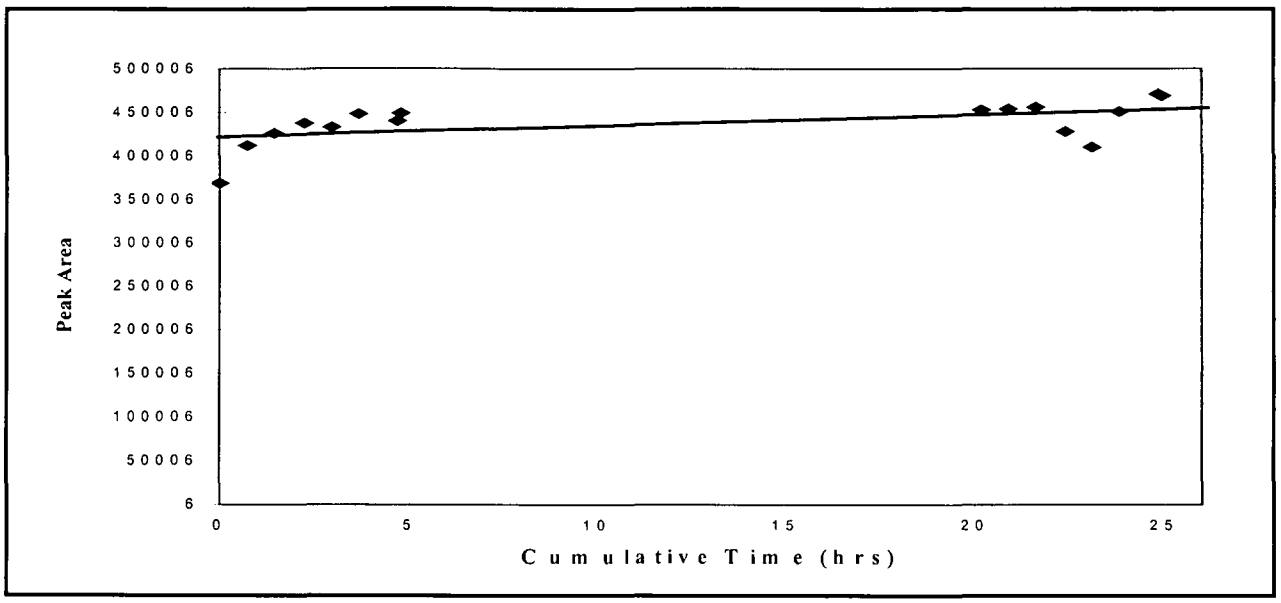


Figure 17 On-Instrument Stability

4.5.9. Specificity

Due to the high specificity of MS/MS detection, no interfering or late eluting peaks were found when chromatographing blank plasma extracts from six different sources. Figure 18 is an example of a chromatogram of a blank plasma extract.

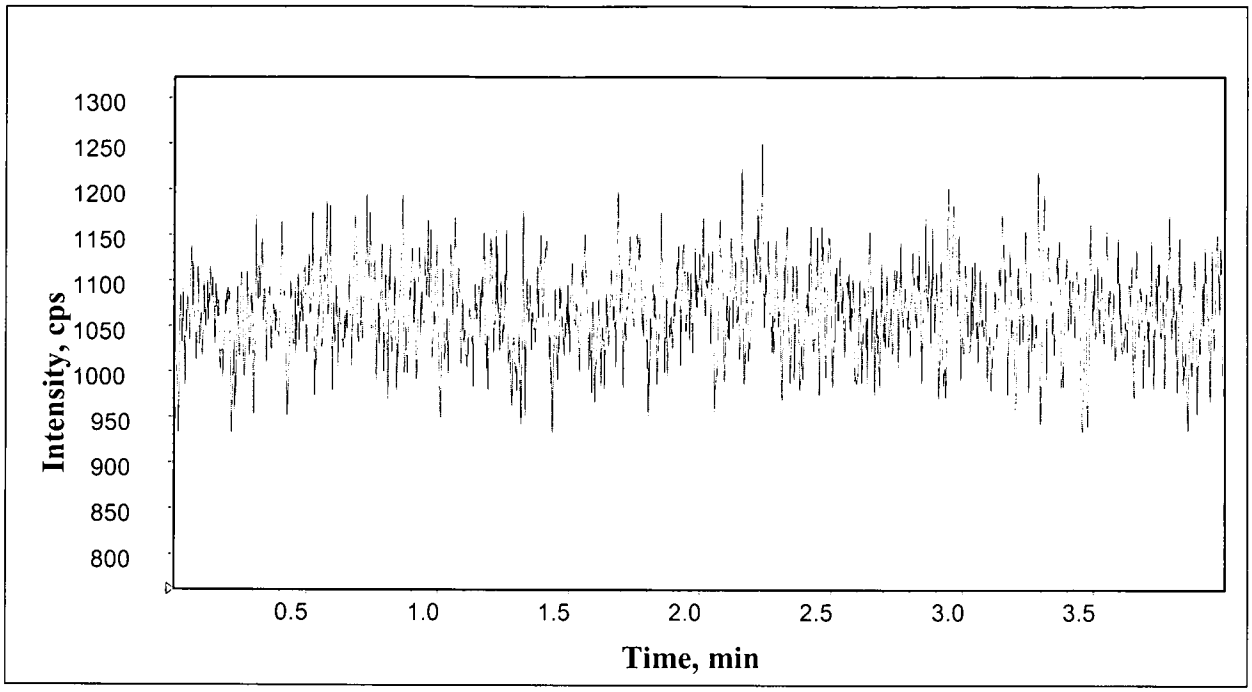


Figure 18 Chromatogram of a blank plasma extract

4.5.10. Sensitivity

The LLOQ, defined as that concentration of stavudine which can still be determined with acceptable precision (CV % < 20) and accuracy (bias < 20 %), was found to be 4 ng/ml with a signal to noise ratio of ~ 4 (figure 19). Results of the intra-batch and inter-batch validation assays indicate a valid calibration range of 4 - 2029 ng/ml for stavudine.

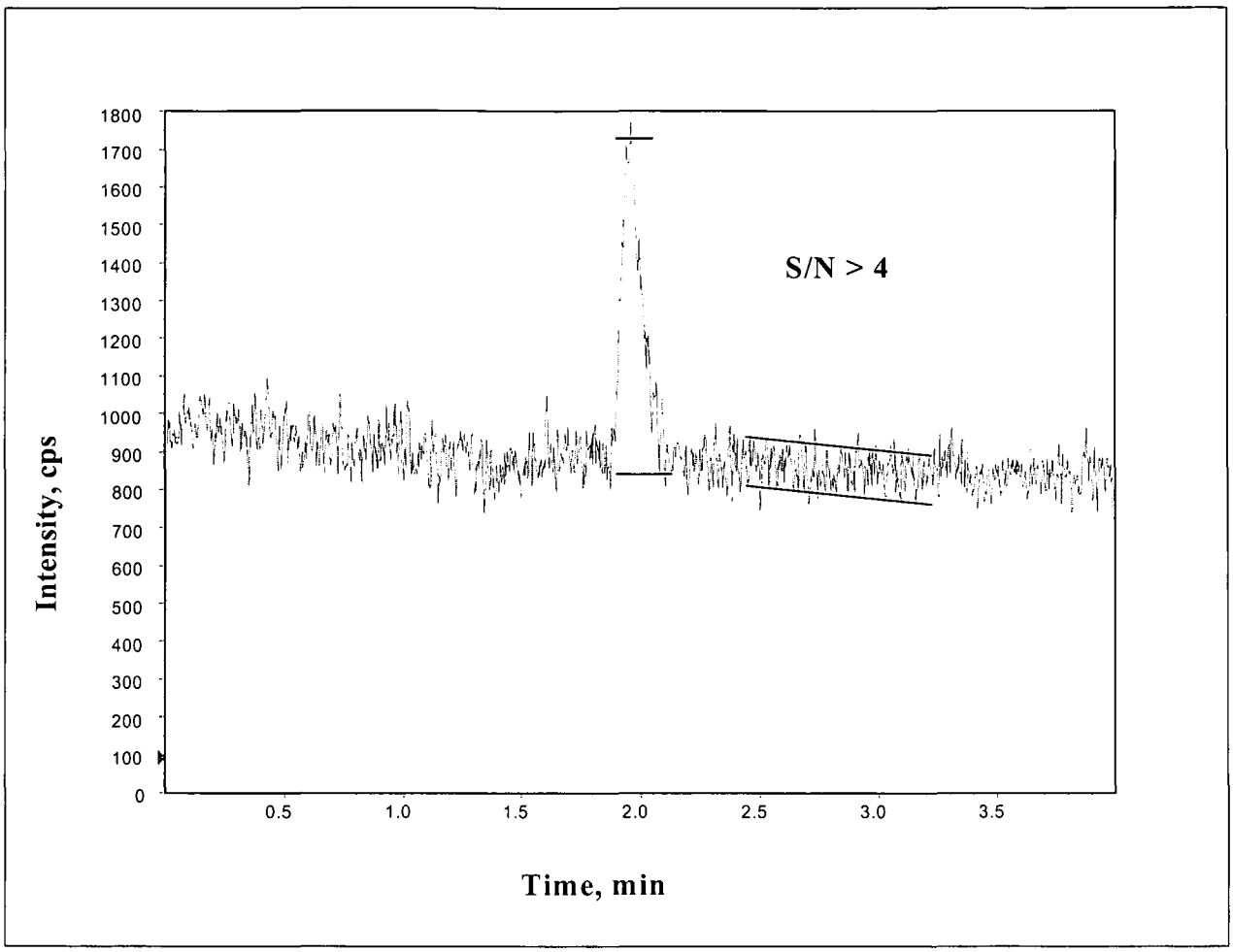


Figure 19 Chromatogram of the LLOQ with a signal to noise greater than 4

Figure 20 illustrates a chromatogram of a calibration standard at the LLOQ (I) containing 4 ng/ml stavudine, and that of a study sample (II) close to the LLOQ at the late elimination phase of the pharmacokinetic profile of the analyte.

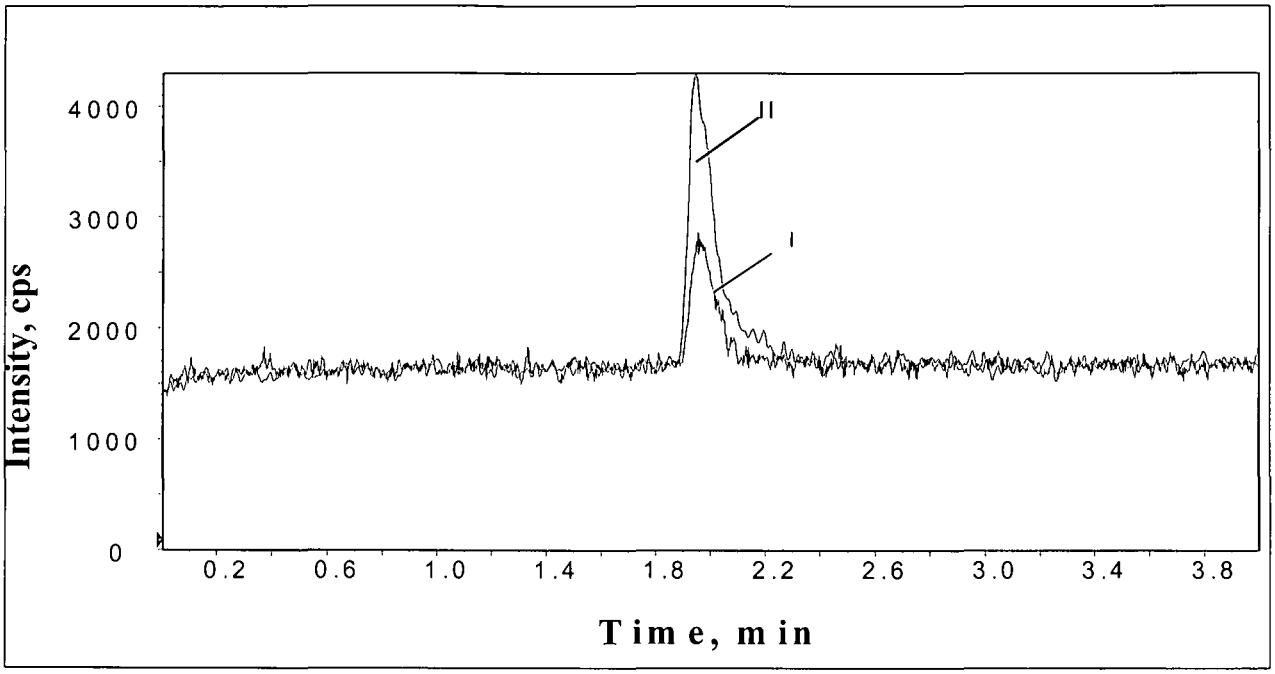


Figure 20 High performance liquid chromatograms of the calibration standard at the LLOQ (I) containing 4 ng/ml stavudine and of a study sample (II) close to the LLOQ at the late elimination phase of the pharmacokinetic profile (~ 12 ng/ml)

4.5.11. Recovery

Recovery is the measure of the analyte(s) losses incurred during sample processing, and is defined as: **Recovery (%) = (peak area of QC/peak area of SPVS) x 100**

Peak areas of 3 different quality control concentrations, and theoretical peak areas obtained from die system performance verification standard (SPVS) samples are used in calculating the recovery of the analyte(s) according to the above mentioned formula. Absolute recoveries of the analyte were determined in triplicate at high, medium and low concentrations of the analytes in plasma and are summarised in table 23.

Table 23 Absolute recovery of analyte using response factor areas

ANALYTE ng/ml	AFTER EXTRACTION	THEORETICAL VALUES	ABSOLUTE RECOVERY (%)	CV (%)
1625	1300000	1359143	95.65	5.08
406	316000	339577	93.06	2.19
102	80200	85312	94.01	8.97

The results indicate a recovery for stavudine of ~ 94 % with a coefficient of variation of ~ 5 % over the entire range of the analyte concentration.

4.6. Study application

This method was used to analyse plasma samples generated during a pharmacokinetic study where a single 40 mg oral dose was given to 24 healthy volunteers. Table 24 is an example of a batch list indicating the arrangement of the study samples and the STDs and QCs.

Results of the back-calculated calibration standards and quality control standards processed together with the batches of study samples are summarised in tables 25 and 26.

Table 24 Batch list

No.	Sample	No.	Sample	No.	Sample	No.	Sample	No.	Sample	No.	Sample
1	SYS	41	P1,1.5,1	81	P2,6.0,1	121	P6,0.25,1	161	P4,2.5,1	201	P5,12,1
2	STD H	42	P1,1.5,2	82	P2,6.0,2	122	P6,0.25,2	162	P4,2.5,2	202	P5,12,2
3	BLANK	43	QC A	83	QC A	123	STD C	163	STD G	203	P6,12,1
4	P1,0,1	44	P2,1.5,1	84	P3,6.0,1	124	P4,0.5,1	164	P5,2.5,1	204	P6,12,2
5	P1,0,2	45	P2,1.5,2	85	P3,6.0,2	125	P4,0.5,2	165	P5,2.5,2	205	P4,24,1
6	P2,0,1	46	P3,1.5,1	86	P1,9.0,1	126	P5,0.5,1	166	P6,2.5,1	206	P4,24,2
7	P2,0,2	47	P3,1.5,2	87	P1,9.0,2	127	P5,0.5,2	167	P6,2.5,2	207	P5,24,1
8	STD B	48	STD F	88	STD B	128	QC G	168	QC G	208	P5,24,2
9	P3,0,1	49	P1,2.0,1	89	P2,9.0,1	129	P6,0.5,1	169	P4,3.0,1	209	P6,24,1
10	P3,0,2	50	P1,2.0,2	90	P2,9.0,2	130	P6,0.5,2	170	P4,3.0,2	210	P6,24,2
11	P1,0.25,1	51	P2,2.0,1	91	P3,9.0,1	131	P4,0.75,1	171	P5,3.0,1	211	SYS
12	P1,0.25,2	52	P2,2.0,2	92	P3,9.0,2	132	P4,0.75,2	172	P5,3.0,2		
13	QC H	53	QC H	93	STD C	133	STD D	173	STD I		
14	P2,0.25,1	54	P3,2.0,1	94	P1,12,1	134	P5,0.75,1	174	P6,3.0,1		
15	P2,0.25,2	55	P3,2.0,2	95	P1,12,2	135	P5,0.75,2	175	P6,3.0,2		
16	P3,0.25,1	56	P1,2.5,1	96	P2,12,1	136	P6,0.75,1	176	P4,4.0,1		
17	P3,0.25,2	57	P1,2.5,2	97	P2,12,2	137	P6,0.75,2	177	P4,4.0,2		
18	STD C	58	STD G	98	P3,12,1	138	QC B	178	QC B		
19	P1,0.5,1	59	P2,2.5,1	99	P3,12,2	139	P4,1.0,1	179	P5,4.0,1		
20	P1,0.5,2	60	P2,2.5,2	100	P1,24,1	140	P4,1.0,2	180	P5,4.0,2		
21	P2,0.5,1	61	P3,2.5,1	101	P1,24,2	141	P5,1.0,1	181	P6,4.0,1		
22	P2,0.5,2	62	P3,2.5,2	102	P2,24,1	142	P5,1.0,2	182	P6,4.0,2		
23	QC G	63	QC G	103	P2,24,2	143	STD E	183	STD J		
24	P3,0.5,1	64	P1,3.0,1	104	P3,24,1	144	P6,1.0,1	184	P4,6.0,1		
25	P3,0.5,2	65	P1,3.0,2	105	P3,24,2	145	P6,1.0,2	185	P4,6.0,2		
26	P1,0.75,1	66	P2,3.0,1	106	SYS	146	P4,1.5,1	186	P5,6.0,1		
27	P1,0.75,2	67	P2,3.0,2	107	STD H	147	P4,1.5,2	187	P5,6.0,2		
28	STD D	68	STD I	108	BLANK	148	QC A	188	QC A		
29	P2,0.75,1	69	P3,3.0,1	109	P4,0,1	149	P5,1.5,1	189	P6,6.0,1		
30	P2,0.75,2	70	P3,3.0,2	110	P4,0,2	150	P5,1.5,2	190	P6,6.0,2		
31	P3,0.75,1	71	P1,4.0,1	111	P5,0,1	151	P6,1.5,1	191	P4,9.0,1		
32	P3,0.75,2	72	P1,4.0,2	112	P5,0,2	152	P6,1.5,2	192	P4,9.0,2		
33	QC B	73	QC B	113	STD B	153	STD F	193	STD B		
34	P1,1.0,1	74	P2,4.0,1	114	P6,0,1	154	P4,2.0,1	194	P5,9.0,1		
35	P1,1.0,2	75	P2,4.0,2	115	P6,0,2	155	P4,2.0,2	195	P5,9.0,2		
36	P2,1.0,1	76	P3,4.0,1	116	P4,0.25,1	156	P5,2.0,1	196	P6,9.0,1		
37	P2,1.0,2	77	P3,4.0,2	117	P4,0.25,2	157	P5,2.0,2	197	P6,9.0,2		
38	STD E	78	STD J	118	QC H	158	QC H	198	STD C		
39	P3,1.0,1	79	P1,6.0,1	119	P5,0.25,1	159	P6,2.0,1	199	P4,12,1		
40	P3,1.0,2	80	P1,6.0,2	120	P5,0.25,2	160	P6,2.0,2	200	P4,12,2		

Example: P6,1.0,2
P6 = Subject no. 6
1.0 = 1 hour after dose
2 = phase 2

Table 25 Summary of the back-calculated calibration standard concentrations of stavudine

Stavudine concentration added (ng/ml)	Stavudine mean concentration found (ng/ml)	Precision (RSD, %)	% nom.	n
3.97	4.02	6.0	101.3	18
7.94	7.76	6.3	97.8	18
15.9	15.88	7.0	99.9	9
31.7	31.4	5.9	99.1	9
63.5	62.3	4.3	98.0	9
127	124	3.0	97.4	9
254	258	6.2	101.6	9
507	514	4.9	101.3	9
1015	1046	4.0	103.1	9
2029	2083	4.6	102.7	5

Table 26 Summary of the quality control standard concentrations of stavudine

Stavudine concentration added (ng/ml)	Stavudine mean concentration found (ng/ml)	Precision (RSD, %)	% nom.	n
4.83	4.80	7.5	99.3	17
9.65	9.48	5.0	98.2	17
406	414	3.4	102.0	17
813	836	5.8	102.9	17
1625	1683	4.3	103.6	17

4.7. Pharmacokinetic data

The average stavudine plasma concentrations as well as the standard deviation data are summarised in table 27 (24 x 2 subjects x 14 sample times = 672 samples). Stavudine was very rapidly absorbed, leading to maximum plasma concentrations being reached within 1 hour. The elimination half-life of stavudine was ~ 2.25 hours.

Table 27 Mean stavudine plasma concentration obtained from 24 subjects

Time (hours)	Mean (ng/ml)	STDEV
0.00	0	0
0.25	227	307
0.50	613	353
0.75	603	199
1.00	546	126
1.50	429	90
2.00	339	60
2.50	274	46
3.00	232	54
4.00	162	35
6.00	73	17
9.00	24	6
12.0	9	2
24.0	0	0

Concentration vs time profiles were constructed for up to 12 hours for the analyte (figure 21).

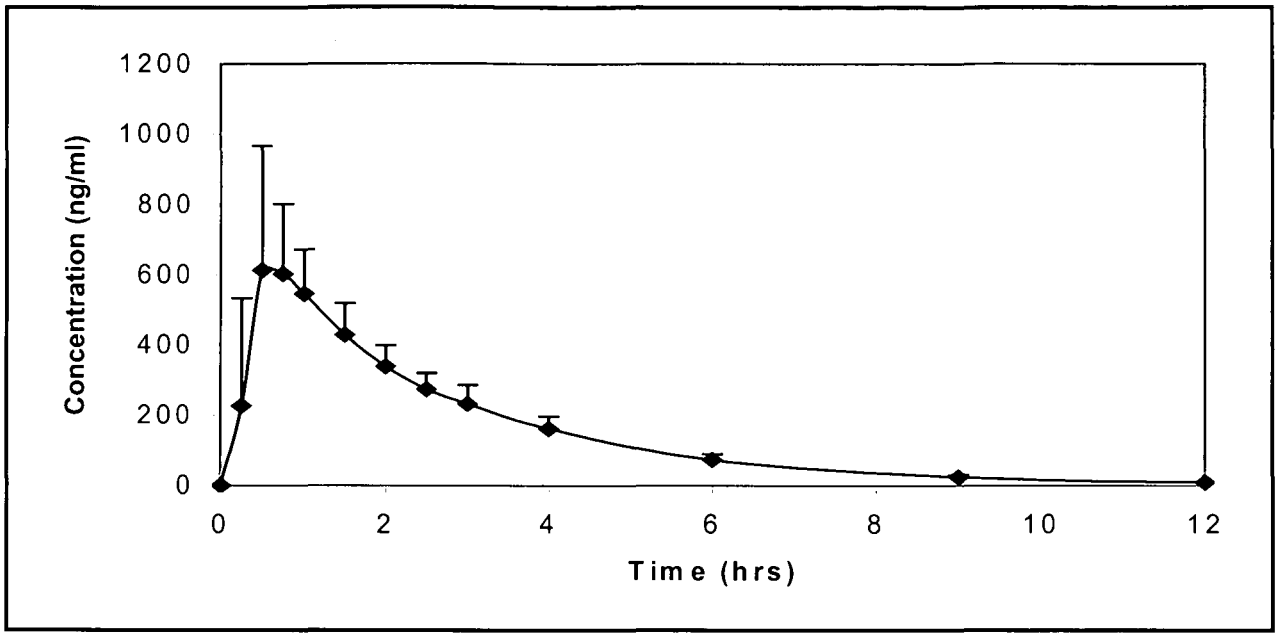


Figure 21 Representative stavudine plasma concentrations vs. time profiles as obtained after a single 40 mg oral dose of stavudine (24 subjects)

4.8. Conclusion

A sensitive method for the determination of stavudine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted from plasma with Waters, Sep-Pak®Vac, 100 mg, tC_{18}® SPE columns. Chromatography was performed on a Supelco Discovery® C_{18}, 5 µm, 150 x 2 mm column with a mobile phase consisting of ammonium acetate (0.01 M) : acetonitrile : methanol (800:100:100, v/v/v) at a flow rate of 0.3 ml/min. Detection was achieved by an Applied Biosystems API 2000 mass spectrometer (LC-MS/MS) set at unit resolution in the MRM mode. APCI was used for ion production in the negative ionisation mode. The mean recovery for stavudine was 94 % with a lower limit of quantification set at 4 ng/ml. This assay method makes use of the increased sensitivity and selectivity of mass spectrometric (MS/MS) detection to provide a more rapid (extraction and chromatography) and selective method for the determination of stavudine in human plasma than has previously been described. The new method is compared to those that were found in the literature in table 28. The new method is definitely more selective and sensitive when compared to those in literature.

Table 28 Comparison between methods that were found in the literature and the newly developed one

Reference	Detector	Analytical column	Extraction method	LLOQ or LOD	Limitations
Kaul *et. al.*	UV (254 nm)	Apex octadecyl	SPE using Bond Elut C_{18}	LOD: 0.05 ug/ml LLOQ: 0.1 ug/ml	Specificity ? Sensitivity ? LTAT ?
Burger *et. al.*	UV (265 nm)	Phenyl column	SPE using silica gel columns	LOD: 10 ng/ml	Specificity ? Sensitivity ? LTAT ?
Janiszewski *et. al.*	UV (266 nm)	Apex octadecyl	SPE using Bont Elut	LLOQ in plasma: 25 ng/ml LLOQ in urine: 500 ng/ml	Specificity ? Sensitivity ? LTAT ?
Stancato *et. al.*	UV (254 nm)	C_{18}	n/a	n/a	Specificity ? LTAT ?
Jarugula and Boudinot	UV (254 nm)	Hamilton PRP-I column	Protein precipitation with acetonitrile	n/a	Specificity ? LTAT ?
Aymard *et. al.*	UV (260 nm)	C_{18}	SPE using C_{18} J.T. Baker® columns	LLOQ: 10 ng/ml	Specificity ? Sensitivity ? LTAT ?
Sarasa *et. al.*	UV (266 nm)	C_{18}	SPE using Oasis® columns	LLOQ in plasma: 24.6 ng/ml LLOQ in urine: 1.97 μg/ml	Specificity ? Sensitivity ? LTAT ?
Moore *et. al.*	PE Sciex API-III mass spectrometer	C_{18}	SPE using ion exchange and Waters C_{18} columns	n/a (tested metabolites)	triphosphate metabolites were determined
NEW METOD	**PE Sciex API-2000 mass spectrometer**	**C_{18}**	**SPE C_{18} Waters, Sep-Pak®Vac, 100 mg columns**	**LLOQ: 4 ng/ml**	**Selectivity √ Sensitivity √ Short turn-around time √**

4.9. Publication in peer reviewed scientific journal

This assay method was submitted for publication to the Journal of Chromatography on 13/11/2001, accepted for publication on 28/02/02 and was published in the Journal of Chromatography B, 773 (2002) 129 – 134 with the title: Sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of stavudine in human plasma

Authors: JL Wiesner*, FCW Sutherland, MJ Smit, GH van Essen, HKL Hundt, KJ Swart and AF Hundt.

See Appendix 1

5. METHOD DEVELOPMENT AND VALIDATION OF AN ASSAY METHOD FOR THE DETERMINATION OF ALFUZOSIN IN HUMAN PLASMA

5.1. Objective

A sensitive, accurate, specific, precise and robust analytical method was needed to quantitatively determine alfuzosin concentrations in plasma samples to follow the concentration vs. time profile for 24 hours after a 5 mg multiple-dose at steady state of alfuzosin was given to 40 healthy, caucasian male subjects. Blood samples were drawn before administration of study medication and thereafter at the following time periods: 0, 0, 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 7, 9, 12, 13, 13.5, 14, 14.5, 15, 15.5, 16, 17, 19, 21 and 24 hours. The samples were centrifuged and duplicate plasma samples stored at – 20 °C until analysed.

5.2. Physico-chemical information

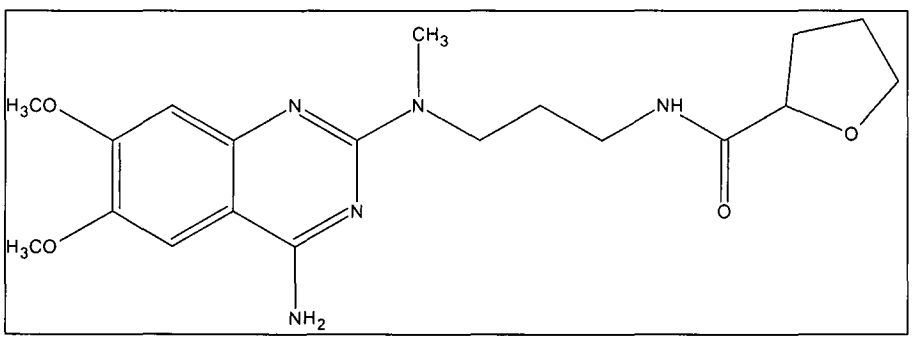


Figure 22 Chemical structure of alfuzosin

Alfuzosin crystals are obtained from ethanol and ether with a melting point of 225 °C with a pKa value of 8.13. [5]

Chemical name:	N-[3-[(4-Amino-6,7-dimethoxy-2-quinazolinyl)methylamino]propyl]tetrahydro-2-furancarboxamide
Additional name(s):	N1-(4-amino-6,7-dimethoxyquinazol-2-yl)-N1-methyl-N2-(tetrahydrofuroyl-2)-propylenediamine
Trade name:	Alfoten (Synthelabo), Urion (Zambon) and Xatral Synthelabo
Molecular formula:	$C_{19}H_{27}N_5O_4$
Chemical composition:	C 58.60 %, H 6.99 %, N 17.98 % and O 16.43 %.
Molecular weight:	389.45
Monoisotopic mass:	389.2063

5.3. Literature survey

5.3.1. Clinical information

Alfuzosin, N-{3-[(4-amino-6,7-dimethoxy-2-quinazolinyl)methylamino]propyl}tetrahydro-2-furancarboxamide, is an antagonist of α_1 post-synaptic adrenergic receptors, showing some myorelaxant effects. Alfuzosin was introduced in therapeutics as an antihypertensive agent [21], and more recently for the treatment of benign prostate hypertrophy. [24]

5.3.2. Analytical information

Guinebault *et al.*, described an HPLC method for the determination of alfuzosin in biological fluids with fluorimetric detection and large-volume injection, and indicate that alfuzosin is a basic compound with a pKa value of 8.13, and is stable at pH 1-13 under normal conditions of temperature and light. [22]

Two detectors were used in their system: A Schoeffel detector with excitation wavelength set at 314 nm and the emission was cut off by a KV 370 filter, and a Kontron SFM 23 detector with excitation wavelength set at 334 nm and the emission wavelength set at 378 nm. A Spherisorb ODS column (15 cm x 4.6 mm, 5 μm) was used for analyte separation, and the mobile phase

consisted of acetonitrile and a 0.02 M (pH 2.5) phosphate buffer (2:3, v/v), delivered at a flow-rate of 1 ml/min.

Liquid-liquid extraction was performed using diethyl ether (7 ml) as the organic phase. An analogue of alfuzosin was used as the internal standard (10 µl of a 5 µg/ml stock solution). Sodium hydroxide solution (0.1 M) was used to buffer the pH of the plasma or blood sample (1 ml). pH values between 9 and 12, were all found to be suitable for extraction. Alfuzosin was then extracted with 7 ml diethyl ether using a shaker for 30 min. and centrifuged at 1 000 G for 5 min. 6.5 ml of the upper organic layer was transferred into a second tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 870 µl of the injection solvent (phosphate buffer (0.02 M, pH 2.5) : acetonitrile, (1:9, v/v)), and injected onto the analytical column.

The regression curve was linear between 0.5 and 100 ng/ml in the blood plasma and 0.05 – 10 µg/ml in urine. Alfuzosin was found to be stable in plasma for 24 h at 37 °C and at – 20 °C for 6 months.

This method is quite sensitive (LLOQ of about 0.5 ng/ml in plasma) with relatively high selectivity. However, a more selective detector like a mass spectrometer would increase the selectivity of the method.

Krstulovic and Vende described a direct enantioselective separation of the enantiomers of alfuzosin on a second generation α_1-AGP column. [23]

A Jasco spectrofluorimeter was used for analyte detection and the excitation and emission wavelengths were set at 265 and 400 nm respectively. A Chiral-AGP column (100 x 4.0 mm, 5 µm) was used for analyte separation, the mobile phase consisted of tetrabutylammonium bromide solution which was adjusted to pH 7.4 with a sodium hydroxide solution (1 M) and acetonitrile (94:6, v/v) and was delivered at a flow rate of 0.9 ml/min.

The plasma samples (1 ml) were spiked with an analogue of alfuzosin in methanolic solution (2 mg/ml) and sodium hydroxide solution (0.1 M, 1 ml) was added. A mixture of dichloromethane and ethyl ether (7 ml, (3:4, v/v)) was added to each tube and the samples were stirred for 25 min. and centrifuged at 1 000 G for 5 min. at 4 °C. The organic phase was transferred to another tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 80 µl of the mobile phase, and 20 µl injected onto the column.

The calibration range was between 5 to 50 ng/ml with an LOD (for each enantiomer) of 1 ng/ml. The enantiomers of alfuzosin in mobile phase were found to be stable for at least 48 hours at room temperature and for several months at -20 °C.

Rouchouse *et al.*, developed an HPLC system for the determination of the enantiomers of alfuzosin in plasma on a second-generation α_1-acid glycoprotein chiral column. [24]

Detection of the analytes was performed on a spectrofluorimeter (265 nm for excitation - and 400 nm for emission wavelengths). They used a chiral-AGP column (100 mm x 4.0 mm, 5 μm) for analyte retention, the mobile phase consisted of phosphate buffer (0.025 M) containing tetrabutyl-ammonium bromide (TBA) (0.025 M) and acetonitrile (94:6, v/v), with a pH of 7.4, and was delivered at a flow rate of 0.9 ml/min.

An analogue of alfuzosin was used as the internal standard. The same extraction method was used as described by Krstulovic and Vende. [23]

They used a calibration range of 1 to 50 ng/ml for each isomer. Different uncharged modifiers were tested for optimal resolution (2-propanol, acetonitrile, methanol and ethanol) and the best results were achieved with acetonitrile as organic modifier. They tested the effect of pH and a cationic modifier TBA on the capacity factor and the resolution of the alfuzosin enantiomers. The influence of pH was more drastic than the effect of the cationic modifier. They found that the capacity factor and the resolution improved with an increase of pH.

Desager *et al.*, studied the effect of cimetidine on the pharmacokinetics of a single oral dose of alfuzosin [25] and used the HPLC method described by Guinebault [22] for the determination of alfuzosin in plasma.

The pharmacokinetics of alfuzosin were not greatly affected by cimetidine, and no statistically significant effect on the disposition of alfuzosin could be determined. The slight enhancement of AUC and C_{max} may indicate that the specific enzymes involved in the biotransformation of the two drugs do not belong to the same sub-family of cytochrome P450.

Carlucci *et al.*, determined alfuzosin in human plasma, using HPLC with column-switching. [26]

Alfuzosin was detected with a fluorescence detector that was set at 265 nm for the excitation wavelength and at 400 nm for the emission wavelength.

A column switching procedure was used to isolate the drug (no off-line extraction was used). A LiChrosorb C_{18} (50 x 4.6 mm, 10 µm) column was used as the clean-up column. The analytical column used for analyte separation was a Spherisorb S5W, cyanopropyl column (25 cm x 4.6 mm, 5 µm). A clean-up solvent (methanol : water (5:95 v/v)) was pumped through the clean-up column by one pump. A second pump delivered the mobile phase (methanol, phosphate buffer (0.05 M, pH 2.5) and acetonitrile (60 : 2 : 38 v/v/v)) through the analytical column at a flow rate of 1 ml/min.

The plasma sample (50 µl) was injected into the clean-up column, which was equilibrated previously with clean-up solvent. The column was then washed for 1 min. with the clean-up solvent at a flow-rate of 1 ml/min. The analytical mobile phase was used to introduce the substance absorbed on the clean-up column to the analytical column by switching the six-port valve to back flush mode for 2 min. The six-port valve was then returned to its initial position. The analytical column was disengaged from the clean-up column, and the latter was equilibrated with clean-up solvent ready for the next injection. The extraction efficiency was approximately 87 %.

The retention time for alfuzosin was 6.2 min. The calibration curve for alfuzosin was linear over a range of 2 to 150 ng/ml with a LOD of 1 ng/ml.

The LLOQ (about 2 ng/ml) of the assay would not be sensitive enough for the assays in this project.

5.3.3. Literature summary

The methods that were described in the literature are summarised in table 29. Fluorescence detection lent good selectivity and relatively high sensitivity to all the assay methods. However, the rather long turn-around time per sample analysis prompted us to investigate the possibility of developing an assay method that would allow us to assay a larger number of samples per day than was possible with the published assay methods.

Table 29 Summary of analytical methods that were found in the literature

Reference	Detection method	Analytical column	Extraction method	LLOQ or LOD	Limitations
Guinebault *et. al.*	Fluorimetric	Spherisorb ODS	Liquid-liquid	LLOQ in blood: 0.5 ng/ml LLOQ in urine: 0.05 µg/ml	Long turn-around time (LTAT) ?
Krstulovic and Vende	Fluorimetric	Chiral-α_1-AGP	Liquid-liquid	LLOQ: 5 ng/ml for each enantiomer LOD: 1 ng/ml for each enantiomer	LTAT ?
Rouchouse *et. al.*	Fluorimetric	Chiral-α_1-AGP	Liquid-liquid	LLOQ: 1 ng/ml for each enantiomer	LTAT ?
Carlucci *et. al.*	Fluorimetric	Spherisorb S5W cyanopropyl	Column switching Clean-up	LLOQ: 2 ng/ml LOD: 1 ng/ml	LTAT ?

5.4. Method development and discussion

As a sensitive, rapid and highly selective analytical method was needed to determine alfuzosin in plasma samples, it was decided to start with the method development phase using LC with MS/MS detection. Owing to the similar chemical properties of prazosin and alfuzosin, the possible use of prazosin as an ISTD was investigated.

5.4.1. Mass Spectrometry optimisation

An Applied Biosystems API 2000 LC-MS/MS detector was set up for ion detection. The mass spectrometer was calibrated by using a PPG Standard solution in the positive and negative ionisation mode.

Alfuzosin is a basic compound with a pKa value of 8.13.[5] In an acidic vicinity, alfuzosin will have a high affinity for protons and would probably form positive ions, therefore ESI was performed in the positive ion mode. Nitrogen as the nebulizing gas, turbo spray gas and curtain gas was set at optimum values of 70, 90 and 50 (respective arbitrary values). The optimal temperature for ion production was tested at 400, 450 and 500 °C and the best results were obtained when the heated nebulizer was set at 400 °C. Stock solutions of alfuzosin (173 ug/ml in methanol) and prazosin (182 ug/ml in methanol) were prepared. These stock solutions were used to spike the infusion solutions (0.2 % aqueous formic acid : acetonitrile (50:50, v/v)) to obtain the following concentrations: alfuzosin: 577 ng/ml and prazosin: 607 ng/ml. The instrument response was optimised for alfuzosin by infusing the infusion solution at a constant flow. The same methodology was used to optimise the response of the instrument for the ISTD. The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) was set at 3 (arbitrary value).

Both alfuzosin and prazosin ionised well and the formation of product ions was excellent.

The Applied Biosystems API 2000 LC-MS/MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 390.2 to the product ion m/z 71.2 for alfuzosin, and also monitoring the transition of the protonated molecular ion m/z 384.2 to the product ion m/z 95.0 for the ISTD.

Presented in figure 23 is the product ion mass spectrum of protonated alfuzosin showing the M+1 ion (m/z 390.2, molecular structure given) and the principal product ion at m/z 71.2 formed by CID.

Presented in figure 24 is the product ion mass spectrum of protonated prazosin showing the M+1 ion (m/z 384.2, molecular structure given) and the principal product ion at m/z 95 formed by CID. Plausible fragmentation patterns presented in figures 23 and 24 are suggested but not proven.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.1 software.

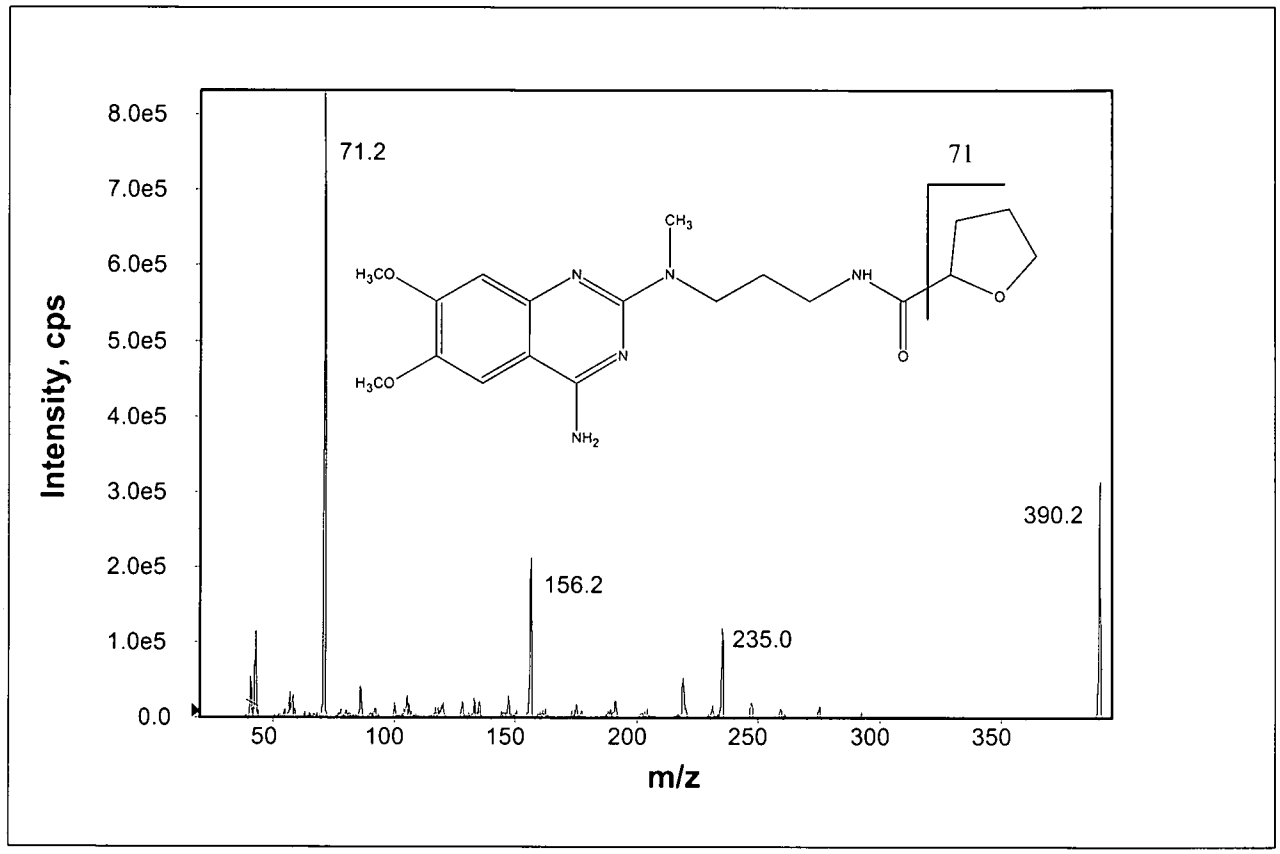


Figure 23 Product ion mass spectrum of protonated alfuzosin showing the (M+1) ion (m/z 390.2, molecular structure given) and the principal product ion at m/z 71.2 formed by CID

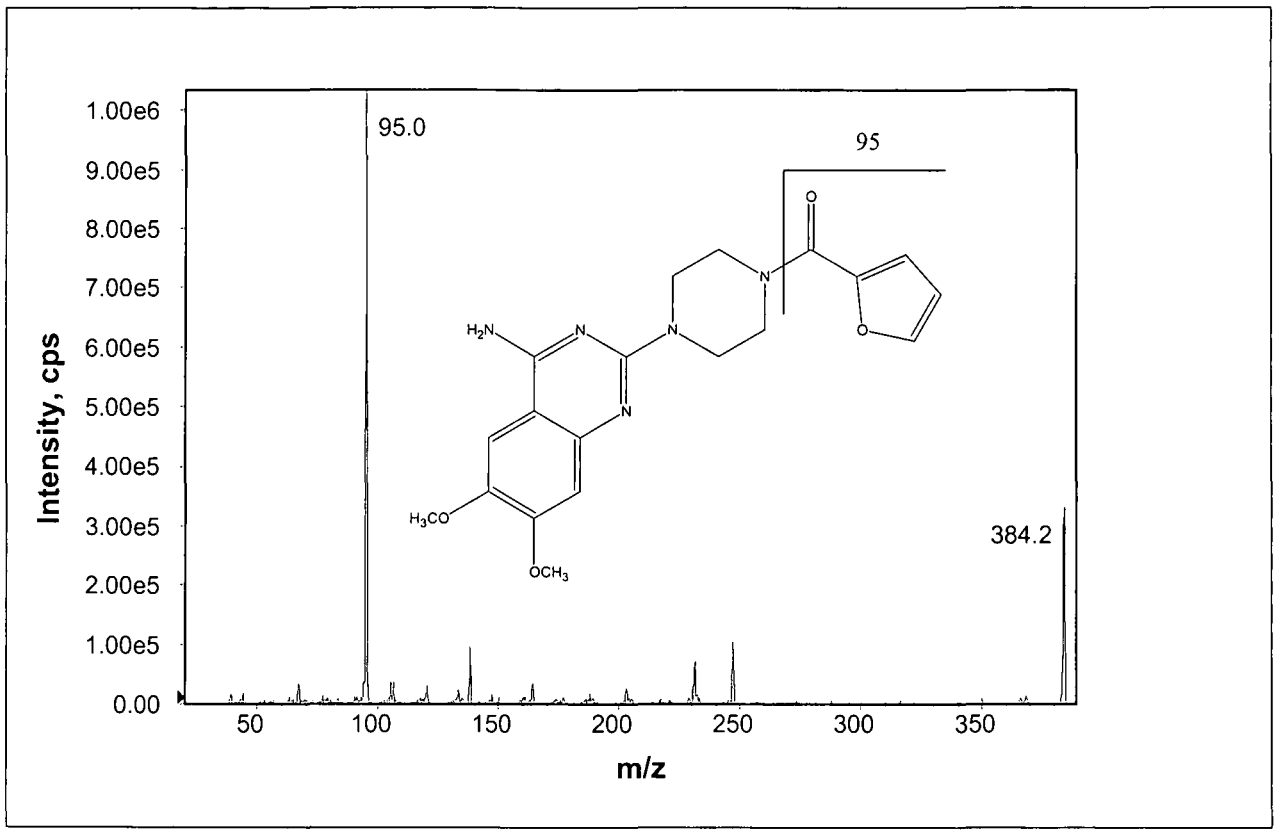


Figure 24 Product ion mass spectrum of protonated prazosin showing the (M+1) ion (m/z 384.2, molecular structure given) and the principal product ion at m/z 95.0 formed by CID

5.4.2. Chromatography development

A SPVS-solution was prepared in 2 % aqueous formic acid. The concentration was 58 ng/ml for alfuzosin and 61 ng/ml for prazosin. Mobile phase was prepared consisting of acetonitrile and a 0.2 % formic acid solution (50:50, v/v). A Discovery® C_{18} column was set up and equilibrated for 1 hour by pumping the mobile phase at a constant flow rate of 0.2 ml/min through the column. The injection volume was 10 μl. Both peaks (from alfuzosin and prazosin) came out on the front and showed no retention on the column under these conditions (figure 25).

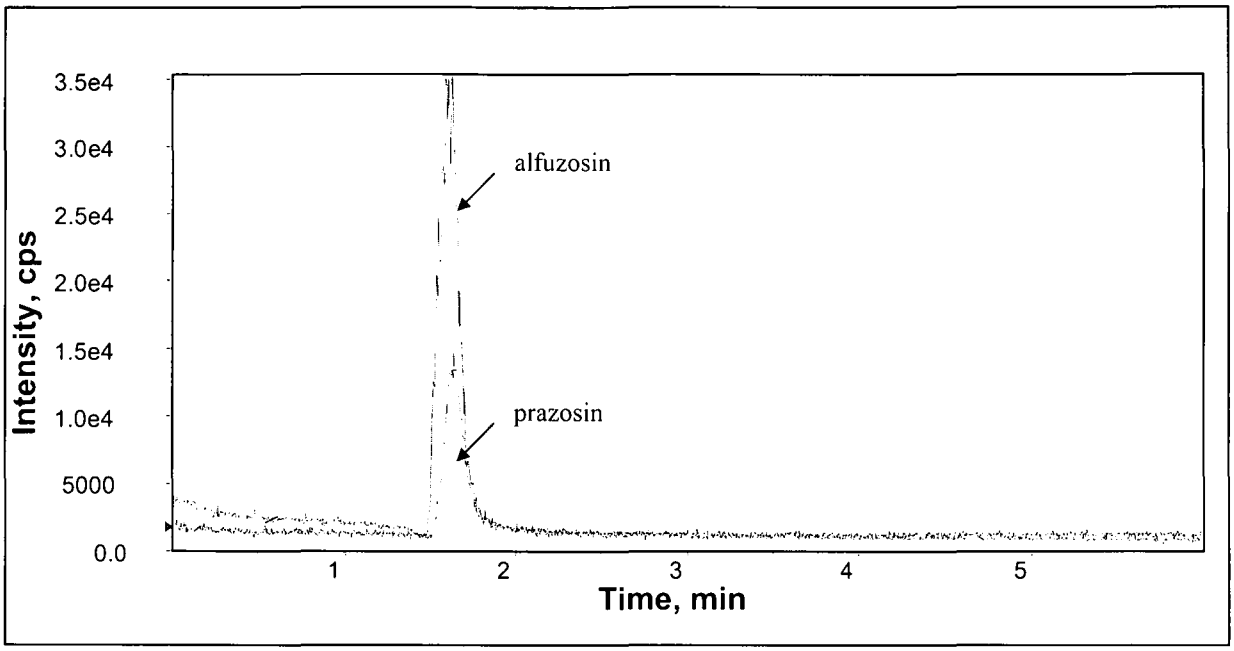


Figure 25 Chromatogram of alfuzosin and prazosin

A more polar mobile phase would theoretically increase the retention time of the analyte and the following three different mobile phase solutions were prepared:

Mobile phase 1: methanol : 0.2% formic acid solution (20:80, v/v)

Mobile phase 2: methanol : 0.2% formic acid solution (40:60, v/v)

Mobile phase 3: methanol : acetonitrile : 0.2% formic acid solution (40:10:50, v/v)

These mobile phases were tested and the third mobile phase gave the best retention (~ 2.6 min. for both analytes) for the analytes on column (figure 26).

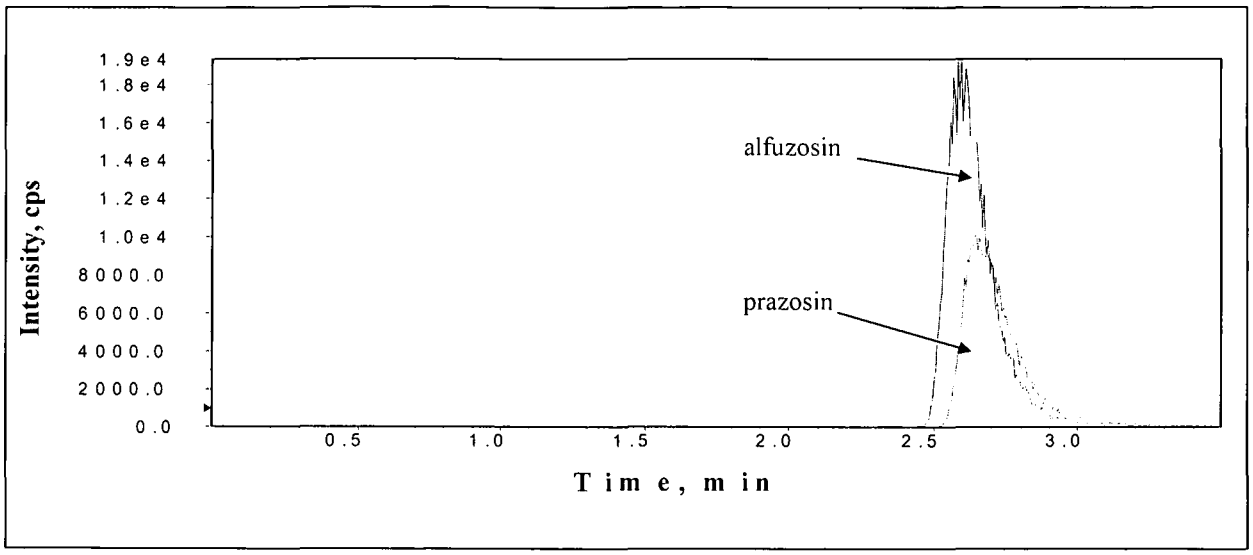


Figure 26 Chromatogram of alfuzosin and prazosin (mobile phase 3)

Different injection solutions were tested, which included 2 % formic acid, 1 % formic acid, 2 % acetic acid and 0.1 % acetic acid. The best peak shape was obtained with a 2 % formic acid solution.

Various injection volumes were tested and 10 μl was found to be the best volume to inject onto the column.

More retention was needed on the column to decrease the chance for interference with endogenous matrix components which could increase or decrease ion formation in the ESI source, therefore the mobile phase was changed as follows: methanol : acetonitrile : 0.2 % formic acid solution (35:10:55, v/v/v).

This resulted in better retention on column for both alfuzosin and prazosin, but too much tailing was observed (figure 27).

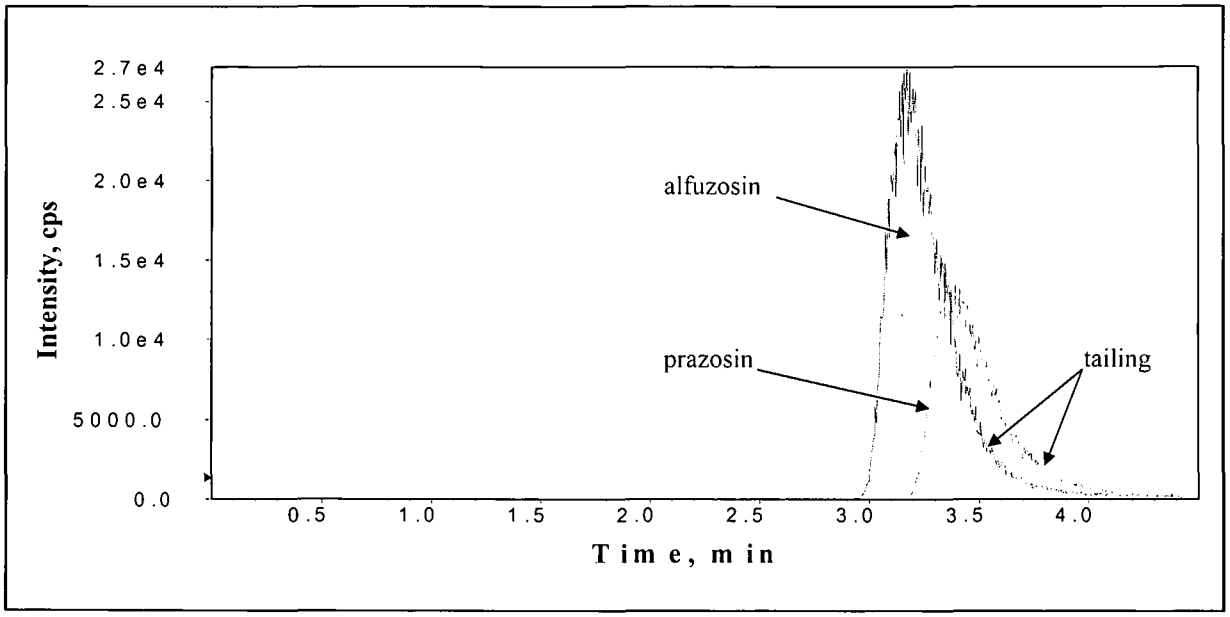


Figure 27 Chromatogram of alfuzosin and prazosin

A dilution sequence of pure solutions under these conditions was tested. The range was linear from 0.460 - 58 ng/ml with a r^2 greater than 0.99. An LLOQ of 0.460 ng/ml could be reached in pure solutions.

The mobile phase was adapted as follows to attempt to improve the peak shape of both alfuzosin and prazosin: methanol : acetonitrile : 0.2% formic acid solution (20:20:60, v/v/v). This mobile phase resulted in very good chromatography. The retention time was ~ 2.9 min. for alfuzosin and ~ 3.1 min. for prazosin (figure 28).

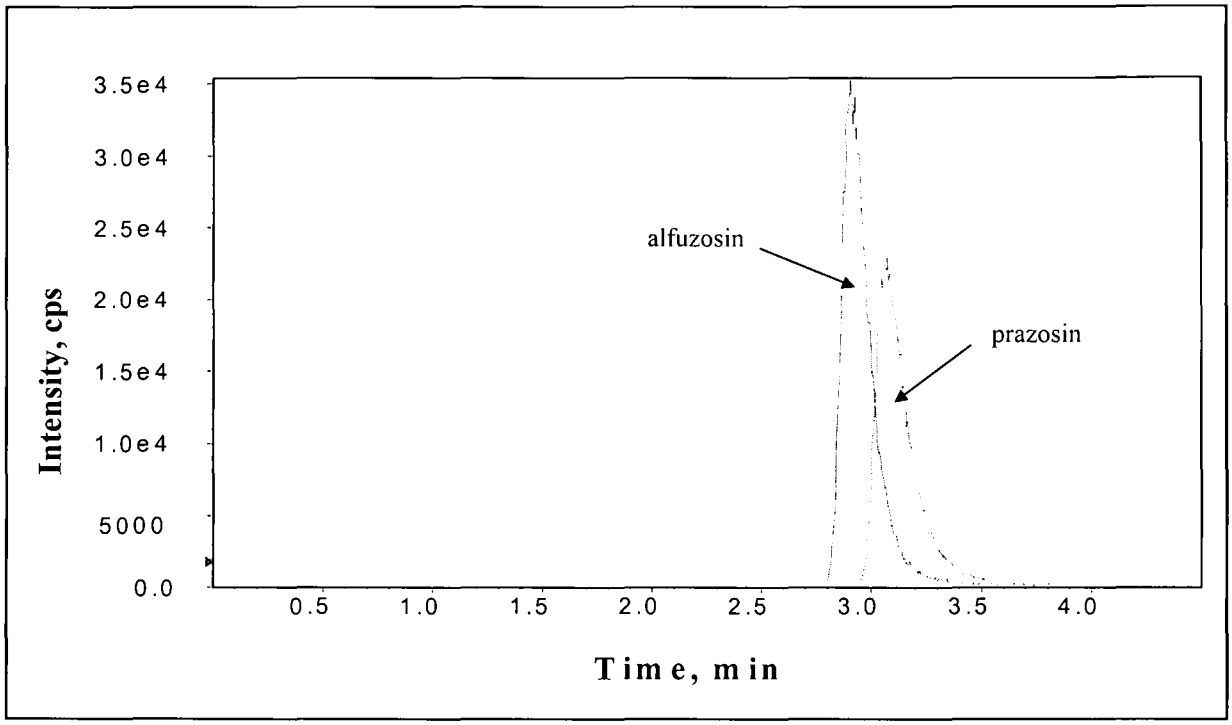


Figure 28 Chromatogram of alfuzosin and prazosin

Chromatography development was completed, the final mobile phase consisting of acetonitrile, methanol and aqueous formic acid (0.2 %), (20:20:60, v/v/v), and delivered at a flow-rate of 0.2 ml/min.

5.4.3. Extraction development

Information gained from the literature discussion indicates that liquid-liquid extraction should be the method of choice when developing the extraction method. With a pKa value of 8.13 a basic buffer with a pH greater than 10.1 should be used to shift the equilibrium equation of alfuzosin to the non-ionised side. The non-ionised molecules will be able to dissolve into the organic phase. Ethyl ether was used as the organic solvent in most of the literature. A mixture of dichloromethane : diethyl ether (3:4, v/v), diethyl ether and TBME were tested. TBME gave the best recovery.

Different buffers were tested: 0.1 M carbonate buffers (pH 9, 9.5,10,10.5 and 11) as well as a 0.1 M NaOH solution, and a 1 M NaOH solution.

The optimum buffer was found to be the 0.1 M carbonate buffer with a pH of 10.5. A back

extraction into 2 % formic acid solution was tested and gave promising results.

The next step was to investigate the repeatability of the extraction method. A pool of plasma was spiked with alfuzosin to give a concentration of 58 ng/ml. Ten of these samples were extracted using the following method:

To 0.5 ml plasma (in a 10 ml ampoule) was added 250 μl of the IS solution (prazosin in H_2O, ~ 61 ng/ml), 0.5 ml of a carbonate buffer (0.1 M, pH of 10.5) and 5 ml TBME. The samples were vortexed for 1 min. and centrifuged for 1 min. at 1300 G. After centrifugation the aqueous phase was frozen in an alcohol freezing bath at –25 °C and the organic layer transferred to a 5 ml ampoule. 2 % Formic acid solution (250 μl) was added and the samples were vortexed for 1 min. and centrifuged for 1 min. at 1300 G. The aqueous phase was frozen and the organic phase discarded. The residual organic phase that remained in the aqeous phase was evaporated under a gentle stream of nitrogen at 45 °C for 2 min. The final extract was transferred to an autosampler vial insert and 10 μl was injected onto the column.

The method performed well, the CV % for alfuzosin (n = 10) was 2.2 and for prazosin 2.3. The CV % for the ratio was 2.4. The recoveries were 80 % and 86 % for alfuzosin and prazosin respectively. The extraction appeared to be repeatable.

The C_{max} was suspected to be about 20 ng/ml, therefore an LLOQ of at least 0.625 ng/ml should be reached to be able to determine concentration levels after five elimination half-lives of the drug. A dilution sequence was prepared in plasma, using the spiked plasma pool (58 ng/ml) and serially diluting it 1:1 down to a concentration of 0.460 ng/ml. These standards were processed and the resulting calibration fitted a quadratic regression. The S/N at 0.460 ng/ml was 28.

The extraction method was repeatable and sensitive enough, therefore the extraction development part of the project was considered to be adequate.

5.4.4. Matrix effect

The matrix effect was tested using the methodology described by Matuszewski *et al.* [1] The data presented in table 30 show no significant matrix effect.

Table 30 Tested plasma pools for matrix effects

Plasma Pool	Analyte area	ISTD area	Ratio
1	495000	242000	2.05
2	529000	263000	2.01
3	539000	256000	2.11
4	505000	242000	2.09
5	559000	258000	2.17
6	539000	259000	2.08
7	535000	253000	2.11
8	513000	248000	2.07
9	539000	253000	2.13
10	536000	253000	2.12
Mean	528900	252700	2.09
STDEV	19070	6961	0.04
CV%	3.61	2.75	2.13

The method development part was optimised and the next phase in this project was the validation of the newly developed method.

5.5. Analytical method validation and discussion

5.5.1. Extraction procedure

The plasma samples were thawed in a waterbath at 37 °C, briefly vortexed and centrifuged for 3 min. at 1300 G. Plasma samples (500 µl) were pipetted into 10 ml amber glass ampoules. Sodium carbonate buffer (500 µl, 0.1 M, pH 10.5), internal standard solution (250 µl, 60 ng prazosin/ml water) and TBME (5 ml) were added and the samples vortexed for 60 sec. After centrifugation at 1300 G for 60 sec., the aqueous phase was frozen in an alcohol freezing bath at –25 °C and the organic phase then decanted into 5 ml amber glass ampoules. Formic acid solution (250 µl, 2 %) was added and the samples were vortexed for 60 sec. and centrifuged at 1300 G for 60 sec. The aqueous phase was frozen in an alcohol freezing bath at -25 °C and the organic phase discarded. The remaining organic phase was evaporated under a gentle stream of nitrogen at 45 °C for 2 min., the aqueous extract transferred to an autosampler vial containing a micro glass insert, and 10 µl injected onto the HPLC column.

5.5.2. Instrumental and chromatographic conditions

Chromatography was performed on a Supelco Discovery C_{18} (150 x 2.0 mm, 5 µm) stainless steel column. The mobile phase consisted of acetonitrile : methanol : formic acid solution (0.2% v/v), (20:20:60; v/v/v) and was delivered at a constant flow rate of 0.2 ml/min. A Perkin Elmer Series 200 autosampler injected 10 µl onto the HPLC column. The sampling needle was rinsed with a methanol/water solution (50:50, v/v), 3 pre- and 3 post injection rinses of 500 µl. The autosampler was fitted with a Peltier cooling device keeping the samples at 5°C.

Detection was performed on an API 2000 mass spectrometer (ESI in the positive ion mode, MRM) and the settings on the apparatus are summarised in tables 31 and 32. The first 2.3 min. of the run was diverted to waste with a switching valve to keep the source of the mass spectrometer "clean".

Table 31 ESI settings

Nebulizer gas	70
Turbo spray	90
Curtain gas	50
Heated nebulizer (°C)	400
Ionspray voltage (V)	5000

Table 32 MS/MS settings

	Alfuzosin	**Prazosin**
Monoisotope molecular mass	389.206	383.159
Protonated molecular ion (m/z)	390.314	384.255
Dwell time (ms)	150	150
Product ion (m/z):	71.120	95.049
Declustering potential (V)	61	66
Focusing potential (V)	360	360
Entrance potential (V)	-11.5	-11
Collision cell entrance potential (V)	20	22
Collision energy (eV)	59	75
Ccollision cell exit potential (V)	8	12
Collision activated dissociation gas	3	3
Scan type	MRM	MRM
Polarity	positive	positive
Pause time	5 ms	5 ms

5.5.3. Preparation of calibration standards

Calibration standards were prepared in human plasma. A stock solution with a concentration of 111 μg/ml was prepared in methanol as indicated in table 33. A pool of normal plasma (STD I) was spiked with the stock solution (50 μl) and was serially diluted with normal plasma to attain the desired concentrations (table 34). STD H represents C_{max} with a concentration of 19.0 ng/ml and the LLOQ is represented by STD B (0.298 ng/ml). The calibration standards were aliquoted into polypropylene tubes and stored at approximately -20°C.

Table 33 Preparation of Stock Solution SA for Spiking STD I

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (μl)	Concentration analyte (μg/ml)
Methanol	0.791	1.344	9.550	12.073	50	111

Table 34 Preparation of Calibration Standards

Calibration Standard	Source Solution	A	B	C	D (ng/ml)
STD I	Stock SA	44.529	194.520	-	38.1
STD H	STD I	45.540	120.537	195.518	19.0
STD G	STD H	44.483	119.486	194.474	9.52
STD F	STD G	45.501	120.515	195.513	4.76
STD E	STD F	44.504	119.493	194.505	2.38
STD D	STD E	44.488	119.484	194.492	1.19
STD C	STD D	44.546	119.549	194.560	0.595
STD B	STD C	44.622	119.626	194.652	0.298

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

5.5.4. Preparation of quality control standards

Quality control standards were prepared in human plasma (same methodology as for the preparation of the standards). The preparation of the stock solution is shown in table 35. A pool of normal plasma was spiked with the stock solution and was serially diluted with normal plasma to attain the desired concentrations (table 36). The quality control standards were stored under the same conditions as were the standards.

Table 35 Preparation of Stock Solution QA for Spiking QC I

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	1.573	9.901	12.517	50	126

Table 36 Preparation of Quality Control Standards

Quality Control Standard	Source Solution	A	B	C	D (ng/ml)
QC G	*Stock QA*	44.604	244.603		32.3
QC F	*QC G*	45.304	145.314	245.329	16.1
QC E	*QC F*	45.396	145.412	245.415	8.06
QC D	*QC E*	44.696	144.698	245.247	4.04
QC C	*QC D*	44.033	168.039	246.383	1.57
QC B	*QC C*	45.432	145.427	245.443	0.783
QC A	*QC B*	45.325	145.326	245.331	0.391

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

5.5.5. Intra-batch accuracy and precision

Intra-batch accuracy and precision are assessed by assaying all the calibration standards in duplicate to produce one calibration curve and 6 replicates of all the prepared quality control standards in a single batch of assays. The intra-batch accuracy and precision of the assay procedure are assessed by calculating the regression equations and constructing the calibration curves based on both peak heights and peak areas both with and without the use of the internal standard to result in four different quantification methods. Quantitation by peak height ratios or peak area ratios requires using the internal standard, whereas quantification by peak heights or peak areas requires not using the internal standard.

Accuracy is expressed as the concentration of analyte found as a percentage of the nominal concentration (% nom) while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra-batch accuracy is required to be within 15 % (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % at the LLOQ. For a valid method the intra-batch precision is required to be less than 15 % (i.e. CV % should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The method performed well during the intra-batch validation and passed all of the criteria for both peak heights and areas both with and without the use of the internal standard. A Wagner regression was fitted to the calibration line and the calibration range was validated between 0.298 and 38.1 ng/ml.

The results of the intra-batch validation are summarised in tables 37 to 44.

5.5.5.1. Quantitation by peak height

Table 37 Back-calculated concentrations of alfuzosin based on peak heights

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD I	38.100	38.893	2.1
STD I	38.100	35.268	-7.4
STD H	19.000	20.776	9.3
STD H	19.000	19.293	1.5
STD G	9.520	9.312	-2.2
STD G	9.520	9.719	2.1
STD F	4.760	4.360	-8.4
STD F	4.760	4.816	1.2
STD E	2.380	2.327	-2.2
STD E	2.380	2.507	5.3
STD D	1.190	1.181	-0.7
STD D	1.190	1.203	1.1
STD C	0.595	0.591	-0.7
STD C	0.595	0.591	-0.7
STD B	0.298	0.344	15.4
STD B	0.298	0.261	-12.3

Calibration Standards used:	STD B – STD I
Calibration Range:	0.298 – 38.1 ng/ml
Regression Equation:	Wagner
a:	-0.001910
b:	0.986346
c:	7.513698
r^2:	0.998389

Table 38 Summary of intra-batch quality control results based on peak heights

Code	QC G 32.3 ng/ml	QCG (Dil) 32.3 ng/ml	QC F 16.1 ng/ml	QC E 8.06 ng/ml	QC D 4.04 ng/ml	QC C 1.57 ng/ml	QC B 0.783 ng/ml	QC A 0.391 ng/ml
1	31.173	32.794	16.751	8.211	4.303	1.822	0.910	0.435
2	29.252	34.682	16.220	8.384	4.320	1.800	0.838	0.391
3	28.952	28.321	15.808	7.748	4.086	1.559	0.811	0.405
4	26.916	30.791	15.101	7.575	4.098	1.537	0.794	0.384
5	28.472	31.733	15.808	7.517	4.172	1.649	0.855	0.391
6	25.182	31.380	15.101	7.575	3.694	1.509	0.789	0.370
MEAN	28.32	31.62	15.80	7.84	4.11	1.65	0.83	0.40
%nom	87.7	97.9	98.1	97.2	101.8	104.8	106.4	101.3
CV%	7.3	6.7	4.1	4.7	5.5	8.3	5.5	5.6

Note: QC G was diluted (1:1) with blank plasma and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

5.5.5.2. Quantitation by peak height-ratios

Table 39 Back-calculated concentrations of alfuzosin based on peak height-ratios

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD I	38.100	37.414	-1.8
STD I	38.100	36.921	-3.1
STD H	19.000	19.890	4.7
STD H	19.000	19.764	4.0
STD G	9.520	9.195	-3.4
STD G	9.520	9.798	2.9
STD F	4.760	4.722	-0.8
STD F	4.760	4.752	-0.2
STD E	2.380	2.213	-7.0
STD E	2.380	2.494	4.8
STD D	1.190	1.176	-1.1
STD D	1.190	1.153	-3.1
STD C	0.595	0.592	-0.4
STD C	0.595	0.640	7.5
STD B	0.298	0.306	2.7
STD B	0.298	0.285	-4.5

Calibration Standards used:	STD B – STD I
Calibration Range:	0.298 – 38.1 ng/ml
Regression Equation:	Wagner
a:	-0.006170
b:	0.973465
c:	-2.427700
r^2:	0.999406

Table 40 Summary of intra-batch quality control results based on peak height-ratios

Code	QC G 32.3 ng/ml	QCG (Dil) 32.3 ng/ml	QC F 16.1 ng/ml	QC E 8.06 ng/ml	QC D 4.04 ng/ml	QC C 1.57 ng/ml	QC B 0.783 ng/ml	QC A 0.391 ng/ml
1	30.228	30.276	14.757	7.567	4.001	1.633	0.804	0.394
2	30.811	32.965	16.421	8.172	4.354	1.592	0.764	0.359
3	33.453	30.908	16.144	7.678	4.033	1.608	0.777	0.413
4	31.333	32.855	16.785	8.665	4.145	1.522	0.757	0.419
5	34.550	34.970	17.059	8.995	4.396	1.516	0.878	0.403
6	36.549	35.306	16.020	8.211	3.783	1.596	0.748	0.378
MEAN	32.82	32.88	16.20	8.21	4.12	1.58	0.79	0.39
%nom	101.6	101.8	100.6	101.9	101.9	100.5	100.6	100.9
CV%	7.5	6.2	5.0	6.7	5.6	3.0	6.1	5.7

Note: QC G was diluted (1:1) with blank plasma and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

5.5.5.3. Quantitation by peak area

Table 41 Back-calculated concentrations of alfuzosin based on peak areas

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD I	38.100	38.161	0.2
STD I	38.100	36.080	-5.3
STD H	19.000	19.727	3.8
STD H	19.000	19.958	5.0
STD G	9.520	9.681	1.7
STD G	9.520	9.294	-2.4
STD F	4.760	4.488	-5.7
STD F	4.760	4.883	2.6
STD E	2.380	2.435	2.3
STD E	2.380	2.419	1.6
STD D	1.190	1.140	-4.2
STD D	1.190	1.202	1.0
STD C	0.595	0.591	-0.6
STD C	0.595	0.580	-2.4
STD B	0.298	0.335	12.5
STD B	0.298	0.273	-8.3

Calibration Standards used:	STD B – STD I
Calibration Range:	0.298 – 38.1 ng/ml
Regression Equation:	Wagner
a:	0.000386
b:	0.999585
c:	9.464854
r^2:	0.999095

Table 42 Summary of intra-batch quality control results based on peak areas

Code	QC G 32.3 ng/ml	QCG (Dil) 32.3 ng/ml	QC F 16.1 ng/ml	QC E 8.06 ng/ml	QC D 4.04 ng/ml	QC C 1.57 ng/ml	QC B 0.783 ng/ml	QC A 0.391 ng/ml
1	31.532	32.966	16.869	8.830	4.286	1.706	0.884	0.422
2	31.840	33.893	17.024	8.289	4.201	1.752	0.845	0.418
3	29.835	28.948	16.251	8.134	4.124	1.613	0.798	0.410
4	29.141	30.648	15.710	7.693	4.124	1.613	0.814	0.383
5	28.910	32.348	16.251	7.445	4.248	1.714	0.829	0.373
6	25.361	32.966	15.710	7.747	3.845	1.527	0.773	0.383
MEAN	29.44	31.96	16.30	8.02	4.14	1.65	0.82	0.40
%nom	91.1	99.0	101.3	99.5	102.4	105.4	105.2	101.8
CV%	7.9	5.7	3.4	6.2	3.8	5.1	4.7	5.3

Note: QC G was diluted (1:1) with blank plasma and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

5.5.5.4. Quantitation by peak area-ratios

Table 43 Back-calculated concentrations of alfuzosin based on peak area-ratios

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD I	38.100	37.638	-1.2
STD I	38.100	37.675	-1.1
STD H	19.000	19.184	1.0
STD H	19.000	19.640	3.4
STD G	9.520	9.518	-0.0
STD G	9.520	9.293	-2.4
STD F	4.760	4.760	0.0
STD F	4.760	4.905	3.0
STD E	2.380	2.361	-0.8
STD E	2.380	2.388	0.3
STD D	1.190	1.142	-4.0
STD D	1.190	1.156	-2.9
STD C	0.595	0.601	1.0
STD C	0.595	0.629	5.7
STD B	0.298	0.308	3.3
STD B	0.298	0.284	-4.6

Calibration Standards used:	STD B – STD I
Calibration Range:	0.298 – 38.1 ng/ml
Regression Equation:	Wagner
a:	-0.002777
b:	0.991210
c:	-2.549990
r^2:	0.999699

Table 44 Summary of intra-batch quality control results based on peak area-ratios

Code	QC G 32.3 ng/ml	QCG (Dil) 32.3 ng/ml	QC F 16.1 ng/ml	QC E 8.06 ng/ml	QC D 4.04 ng/ml	QC C 1.57 ng/ml	QC B 0.783 ng/ml	QC A 0.391 ng/ml
1	32.784	32.086	15.450	8.093	4.145	1.609	0.805	0.392
2	32.524	33.397	16.975	8.315	4.286	1.514	0.778	0.376
3	31.735	32.144	16.377	8.108	4.129	1.663	0.788	0.424
4	31.761	32.584	17.145	8.411	4.180	1.593	0.799	0.403
5	32.534	34.440	17.525	8.584	4.334	1.616	0.809	0.372
6	35.071	34.897	16.609	8.152	3.733	1.625	0.758	0.380
MEAN	32.73	33.26	16.68	8.28	4.13	1.60	0.79	0.39
%nom	101.3	103.0	103.6	102.7	102.3	102.1	100.8	100.0
CV%	3.7	3.6	4.3	2.4	5.1	3.1	2.4	5.0

Note: QC G was diluted (1:1) with blank plasma and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

5.5.6. Inter-batch accuracy and precision

Inter-batch accuracy and precision are assessed by assaying two separate consecutive batches, each consisting of one set of calibration standards designated for use in the assay of samples of unknown concentrations and 6 replicates of each of the quality control standards designated for use in assaying samples of unknown concentrations. The intra-batch accuracy and precision of each of the batches is assessed separately by calculating regression equations and constructing calibration curves based on the best performing quantification method and must pass the criteria for intra-batch acceptance.

The best quantification method was peak area ratio and it was used for the two inter-batch validations. The inter-batch accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 in total). Accuracy is expressed as concentration of the analyte found as a percentage of the nominal concentration (% nom), while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra- and inter-batch accuracy is required to be within 15 % (ie. % nom should be between 85 % - 115 %) over most of the range and within 20 % of nominal concentration at the LLOQ. For a valid method the intra- and inter-batch precision is required to be less than 15 % (ie. CV % should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The highest variation was 6.4 % (QC A, first inter-batch). This illustrates that the method performed very well during the two inter-batch validations.

The results are summarised in tables 45 to 48.

5.5.6.1. Inter-batch 1 accuracy and precision

Table 45 Back-calculated concentrations of alfuzosin

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD I	38.100	38.759	1.7
STD I	38.100	37.249	-2.2
STD H	19.000	17.702	-6.8
STD H	19.000	20.159	6.1
STD G	9.520	9.529	0.1
STD G	9.520	9.711	2.0
STD F	4.760	4.811	1.1
STD F	4.760	4.809	1.0
STD E	2.380	2.412	1.4
STD E	2.380	2.302	-3.3
STD D	1.190	1.207	1.5
STD D	1.190	1.183	-0.6
STD C	0.595	0.580	-2.6
STD C	0.595	0.588	-1.2
STD B	0.298	0.282	-5.4
STD B	0.298	0.323	8.4

Calibration Standards used:	STD B – STD I
Calibration Range:	0.298 – 38.1 ng/ml
Quantification by:	Peak Area Ratios
Regression Equation:	Wagner
a:	-0.007942
b:	1.013370
c:	-2.548241
r2:	0.999452

Table 46 Summary of quality control results for inter-batch 1

Code	QC G 32.3 ng/ml	QC F 16.1 ng/ml	QC E 8.06 ng/ml	QC D 4.04 ng/ml	QC B 0.783 ng/ml	QC A 0.391 ng/ml
1	31.331	15.641	7.700	4.101	0.742	0.399
2	31.973	15.705	7.823	3.867	0.783	0.424
3	32.447	16.270	8.097	4.264	0.806	0.381
4	31.885	16.062	8.244	4.143	0.769	0.362
5	32.507	15.804	7.984	4.054	0.803	0.402
6	33.369	16.005	8.095	4.157	0.780	0.430
MEAN	32.25	15.91	7.99	4.10	0.78	0.40
%nom	99.9	98.8	99.1	101.4	99.7	102.2
CV%	2.2	1.5	2.5	3.2	3.0	6.4

5.5.6.2. Inter-batch 2 accuracy and precision

Table 47 Back-calculated concentrations of alfuzosin

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD I	38.100	36.210	-5.0
STD I	38.100	36.362	-4.6
STD H	19.000	19.941	5.0
STD H	19.000	18.854	-0.8
STD G	9.520	10.430	9.6
STD G	9.520	9.767	2.6
STD F	4.760	4.941	3.8
STD F	4.760	4.855	2.0
STD E	2.380	2.377	-0.1
STD E	2.380	2.421	1.7
STD D	1.190	1.091	-8.4
STD D	1.190	1.089	-8.5
STD C	0.595	0.536	-9.9
STD C	0.595	0.616	3.5
STD B	0.298	0.307	3.2
STD B	0.298	0.323	8.6

Calibration Standards used:	STD B – STD I
Calibration Range:	0.298 – 38.1 ng/ml
Quantification by:	Peak Area Ratios
Regression Equation:	Wagner
a:	-0.012573
b:	1.034418
c:	-2.570849
r^2:	0.998639

Table 48 Summary of quality control results for inter-batch 2

Code	QC G 32.3 ng/ml	QC F 16.1 ng/ml	QC E 8.06 ng/ml	QC D 4.04 ng/ml	QC B 0.783 ng/ml	QC A 0.391 ng/ml
1	33.992	15.964	7.443	4.157	0.827	0.400
2	32.164	15.319	8.217	4.183	0.870	0.450
3	34.786	16.234	8.074	4.086	0.822	0.430
4	32.199	16.274	8.327	4.007	0.840	0.421
5	33.946	16.777	8.690	4.095	0.849	0.419
6	32.220	15.516	7.882	3.969	0.825	0.426
MEAN	33.22	16.01	8.11	4.08	0.84	0.42
%nom	102.8	99.5	100.6	101.1	107.1	108.5
CV%	3.5	3.3	5.2	2.0	2.2	3.8

5.5.7. Summary of the combined quality control results for the 3 validations

The combined quality control results are summarised in table 49. The method performed well during all three validations with a highest coefficient of variation of 5.9 % for the lowest QC.

Table 49 Summary of the combined quality control results for the 3 validations

Validation Batch	Nominal Replicates	32.3 ng/ml	16.1 ng/ml	8.06 ng/ml	4.04 ng/ml	0.783 ng/ml	0.391 ng/ml
Intra-batch Validation	1	32.78	15.45	8.09	4.15	0.81	0.39
	2	32.52	16.98	8.32	4.29	0.78	0.38
	3	31.74	16.38	8.11	4.13	0.79	0.42
	4	31.76	17.15	8.41	4.18	0.80	0.40
	5	32.53	17.53	8.58	4.33	0.81	0.37
	6	35.07	16.61	8.15	3.73	0.76	0.38
Inter-batch Validation 1	1	31.33	15.64	7.70	4.10	0.74	0.40
	2	31.97	15.71	7.82	3.87	0.78	0.42
	3	32.45	16.27	8.10	4.26	0.81	0.38
	4	31.89	16.06	8.24	4.14	0.77	0.36
	5	32.51	15.80	7.98	4.05	0.80	0.40
	6	33.37	16.01	8.10	4.16	0.78	0.43
Inter-batch Validation 2	1	33.99	15.96	7.44	4.16	0.83	0.40
	2	32.16	15.32	8.22	4.18	0.87	0.45
	3	34.79	16.23	8.07	4.09	0.82	0.43
	4	32.20	16.27	8.33	4.01	0.84	0.42
	5	33.95	16.78	8.69	4.10	0.85	0.42
	6	32.22	15.52	7.88	3.97	0.83	0.43
	MEAN	32.73	16.20	8.12	4.11	0.80	0.41
	%nom	101.3	100.6	100.8	101.6	102.5	103.6
	CV%	3.2	3.7	3.6	3.4	4.0	5.9

5.5.8. Stability assessment

5.5.8.1. Stability in matrix

In order to determine stability in matrix, spiked solutions of alfuzosin in plasma at two different concentrations (16.1 and 1.57 ng/ml) were stored at –20 °C for 125 days. These samples were analysed together with a freshly prepared set of standards and quality control standards. The results of the measured concentrations of the samples tested are summarised in table 50.

Table 50 Matrix stability

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)
16.1	15.3
16.1	16.8
16.1	14.8
16.1	16.0
16.1	15.9
Mean	15.76
Std.Dev.	0.78
CV%	4.94
%Nom	97.88

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)
1.57	1.53
1.57	1.58
1.57	1.47
1.57	1.59
1.57	1.57
Mean	1.55
Std.Dev.	0.05
CV%	3.24
%Nom	98.51

By comparing the measured concentration against the nominal concentrations of the tabulated data, a correlation coefficient of 0.98 was obtained, indicating that no significant degradation of the sample tested could be detected.

Alfuzosin was found to be stable in plasma for at least 125 days at –20 °C.

5.5.8.2. Freeze and thaw stability

In order to ascertain freeze-thaw stability, spiked solutions of alfuzosin in plasma at two different concentrations were frozen at –20 °C, and put through two freeze- and thaw cycles. These samples were analysed during the intra-validation batch. Peak areas and means as well as the calculated recoveries (% of nominal concentration) for the two sets of aliquots are tabulated below.
Only two freeze- and thaw cycles were performed to obtain some data on the freeze- and thaw stability of alfuzosin, but no trial samples were reanalysed using samples that were previously thawed. All re-analyses of samples were performed using the frozen duplicate samples.

Table 51 Freeze and thaw stability measured at 16.1 and 4.04 ng/ml

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
16.1	15.742	97.78
16.1	16.221	100.75
16.1	16.243	100.89
16.1	16.412	101.94
16.1	15.713	97.60
Mean	16.07	99.79
Std.Dev.	0.32	1.98
CV%	1.98	N/A

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
4.04	3.858	95.50
4.04	4.089	101.21
4.04	3.923	97.10
4.04	4.057	100.42
4.04	3.972	98.32
Mean	3.98	98.5
Std.Dev.	0.09	2.35
CV%	2.38	N/A

Regression analysis of measured concentrations against nominal concentrations showed a gradient of 0.9971 indicating that there was no significant difference between the measured and nominal concentrations after two freeze - and thaw cycles.

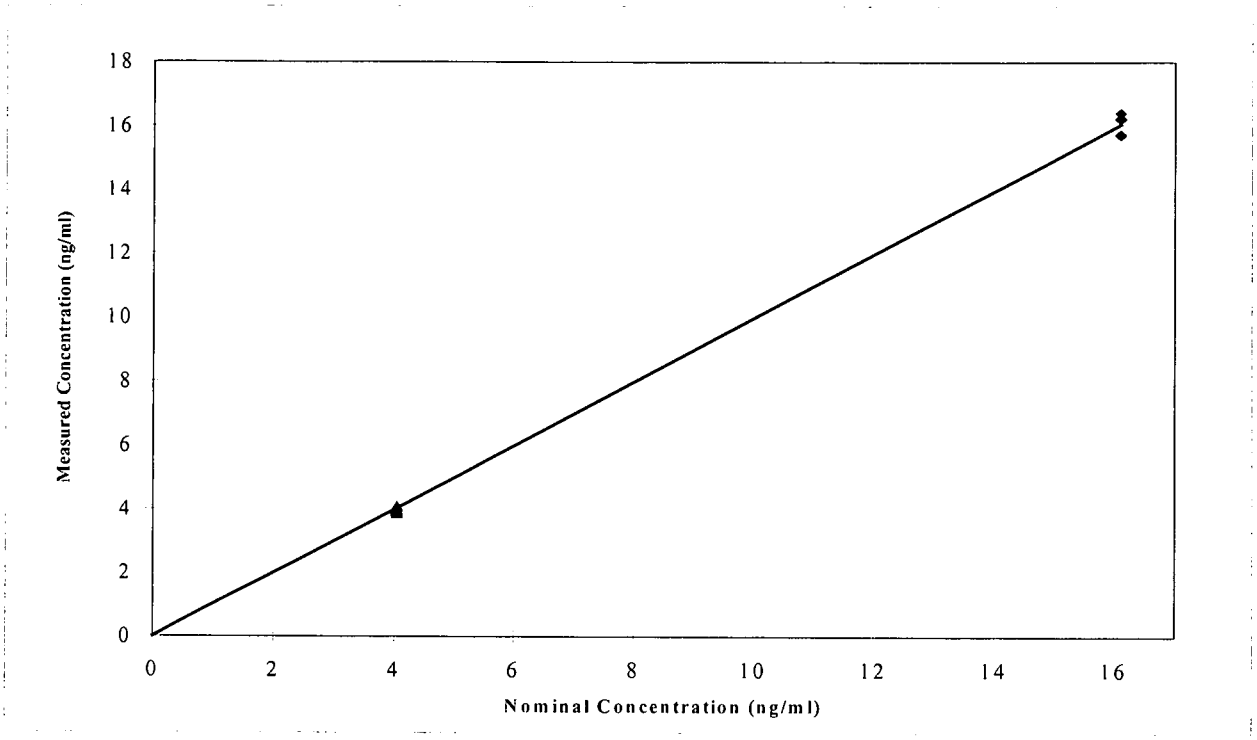


Figure 29 Freeze-Thaw stability correlation of measured vs. nominal concentrations

5.5.8.3. On-Instrument stability

Sixteen stability samples of the same concentration were extracted, the extracts combined and re-aliquoted, and injected at intervals during the first two validation batches to simulate the time of a batch run. The measured peak areas, injection times, cumulative time and peak area ratios are summarised in table 52.

Table 52 Stability data of sixteen stability samples injected at different intervals

Replicates	Injection Time	Time Difference	Cumulative Time (hr)	Analyte Peak Area	IS Peak Area	Peak Area Ratios
1	13:17		0.00	116000	185000	0.63
2	14:24	1:07:00	1.12	111000	172000	0.65
3	15:30	1:06:00	2.22	111000	177000	0.63
4	16:37	1:07:00	3.33	104000	157000	0.66
5	17:38	1:01:00	4.35	108000	172000	0.63
6	18:40	1:02:00	5.38	107000	171000	0.63
7	19:36	0:56:00	6.32	103000	161000	0.64
8	19:41	0:05:00	6.40	104000	157000	0.66
9	12:39	16:58:00	23.37	102000	164000	0.62
10	13:25	0:46:00	24.13	98500	154000	0.64
11	14:11	0:46:00	24.90	104000	165000	0.63
12	14:57	0:46:00	25.67	97200	157000	0.62
13	15:43	0:46:00	26.43	99400	156000	0.64
14	16:29	0:46:00	27.20	99400	162000	0.61
15	17:15	0:46:00	27.97	98100	155000	0.63
16	17:20	0:05:00	28.05	103000	156000	0.66
			Mean	104100	1638123	0.64
			Std Dev	5332	9152	0.02
			CV%	5.12	5.59	2.39

By regression analysis of the peak areas against the cumulative time tabulated, it can be established that the peak area of alfuzosin decreased by 9.5 % over a period of 28 hours, while the internal standard tended to decrease by 8.5 % over a period of 28 hours, resulting in a net decrease of the peak area ratio by 1.2 % over the batch while awaiting injection on-instrument (figure 30). The observed trends are more likely to be trends in detector sensitivity than in analyte stability, with the trend of the analyte being well compensated for by the parallel trend of the internal standard. The extracts are therefore considered to be stable on-instrument for at least 28 hours.

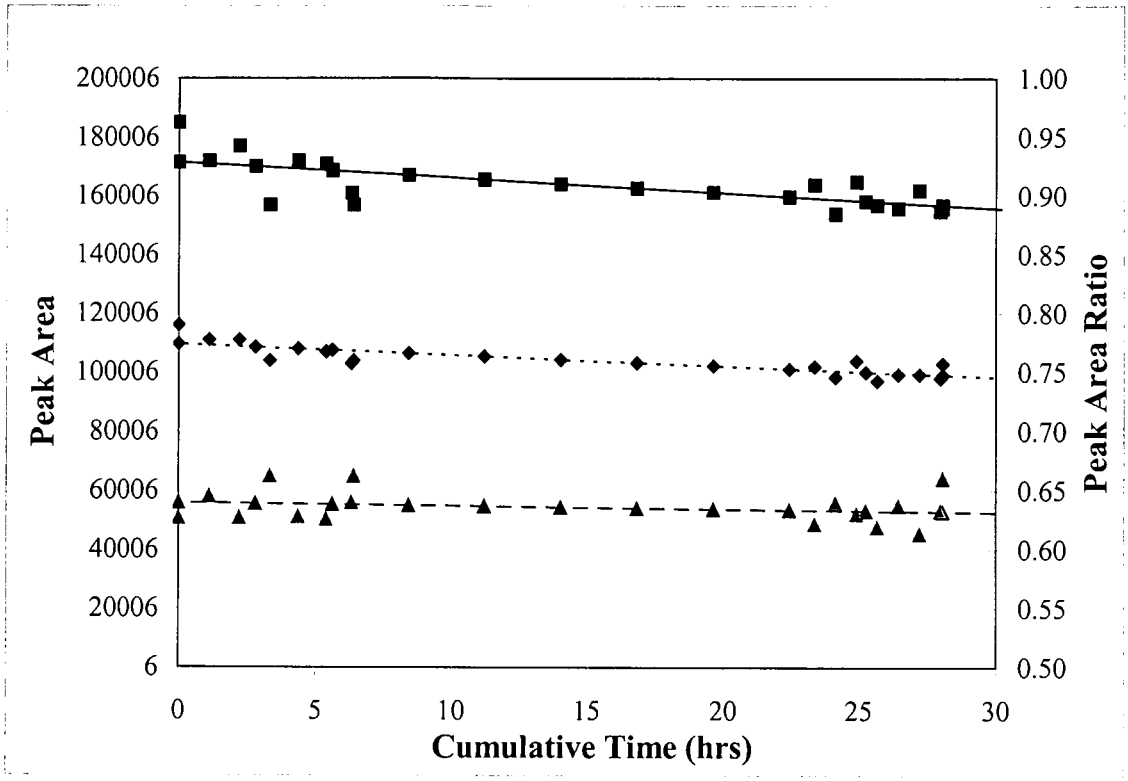


Figure 30 On-Instrument Stability

5.5.9. Specificity

The very high specificity of the LC-MS/MS assay procedure precludes the detection of any compounds that do not possess the capability to produce the specific parent ion followed by formation of the specific product ion produced and monitored in the mass spectrometer.

Blank sample extracts were positioned in the injection sequence immediately after the highest calibration standard in order to assess possible carry-over effects. Small peaks present in the blank sample extracts indicated that some carry-over was present in spite of the fact that autosampler needle flushing was done after each injection. The magnitude of the carry-over peak after the highest concentration calibration standard was consistently less than 20 % of the LLOQ standard (figure 31). This carry-over was therefore deemed to be insignificant. Figure 32 is an example of a chromatogram of a blank plasma extract.

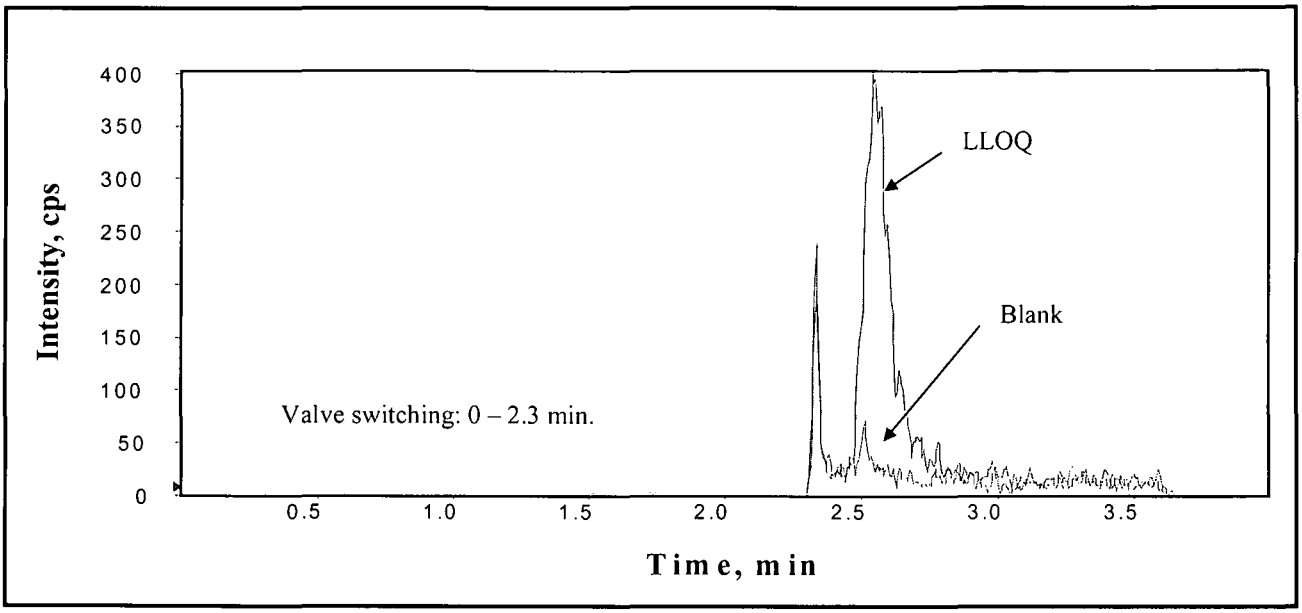


Figure 31 Chromatograms of a blank plasma extract and the LLOQ

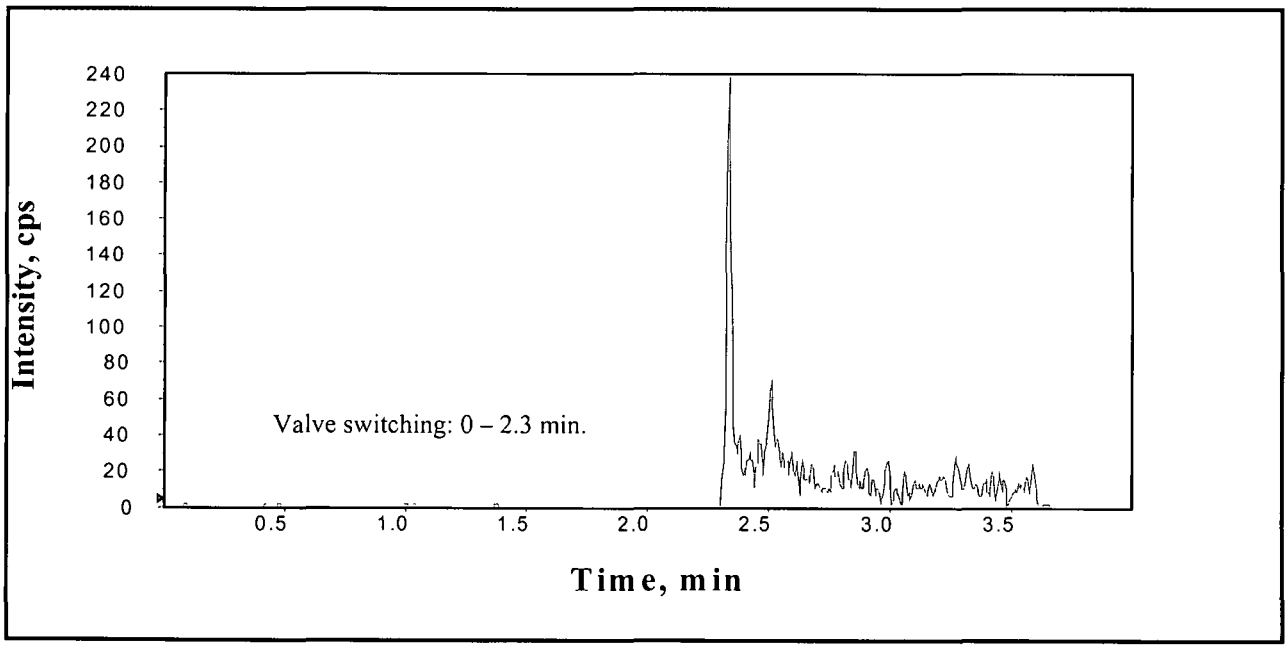


Figure 32 Chromatogram of a blank plasma extract

5.5.10. Sensitivity

The lower limit of quantification (LLOQ) of this method is 0.298 ng/ml (concentration of lowest STD), which enables the quantification of the analyte for at least 24 hrs (approximately 5 elimination half-lives) after a multiple oral dose of 5 mg alfuzosin to human volunteers. The signal to noise ratio was > 20 (figure 33).

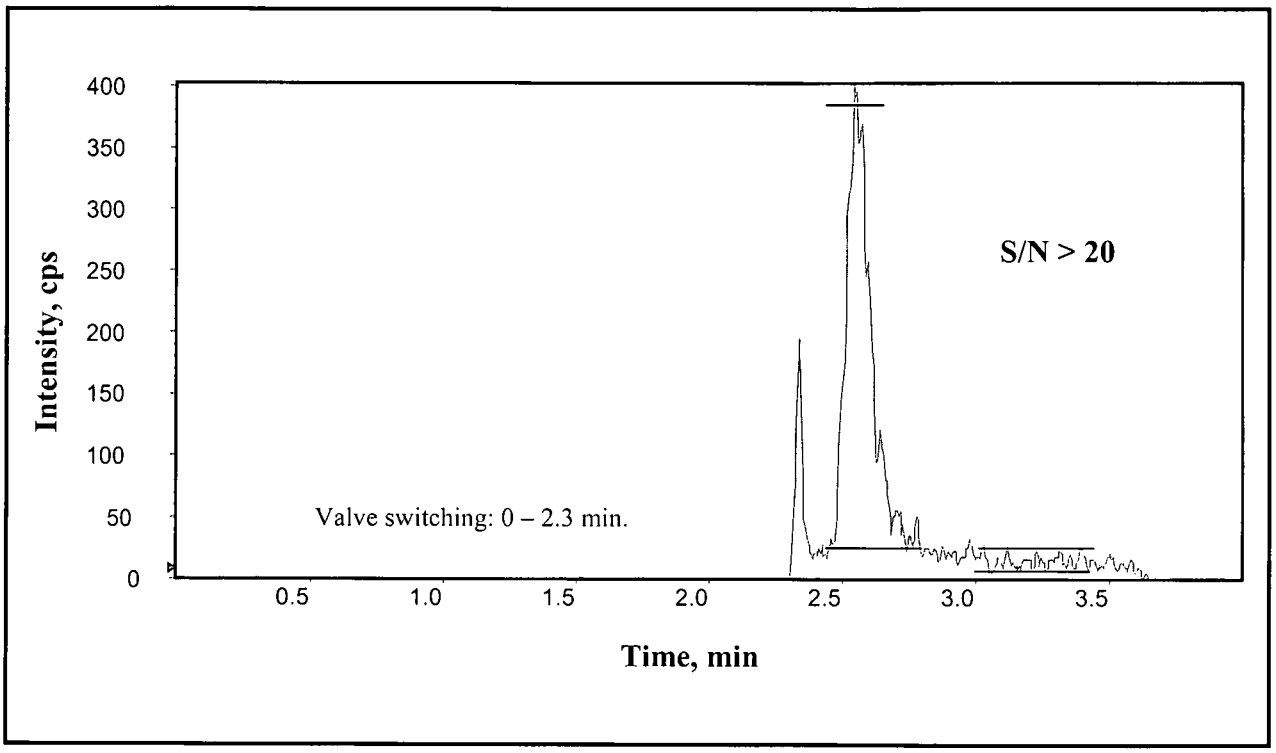


Figure 33 Chromatogram of the LLOQ with a signal to noise greater than 20

5.5.11. Recovery

Recovery is the measure of the analyte(s) losses incurred during sample processing, and is defined as: **Recovery (%) = (peak area of QC/peak area of SPVS) x 100**

Peak areas of 3 different quality control concentrations, and theoretical peak areas obtained from die system performance verification standard (SPVS) samples are used in calculating the recovery of the analyte(s) according to the above mentioned formula. Absolute recoveries of the analyte

were determined in triplicate at high, medium and low concentrations of the analytes in plasma and are summarised in table 53.

Table 53 Absolute recovery of alfuzosin using response factor areas

Analyte ng/ml	Mean areas (after extraction)	Mean areas (theoretical values)	Absolute recovery (%)	CV (%)
16.1	212200	260931	81.32	2.90
4.04	54140	65476	82.69	1.56
0.783	10760	12690	84.79	3.51

The average recovery for alfuzosine was ~ 83 % with a coefficient of variation of ~ 3 %. The absolute recovery for the ISTD was determined at one concentration and was found to be 77 %.

5.6. Study application

This method was used to analyse plasma samples generated during a pharmacokinetic study where a 5 mg multiple-dose at steady state of alfuzosin was given to 40 healthy, caucasian male subjects. Table 54 is an example of a batch list indicating the arrangement of the study samples and the STDs and QCs.

Results of the back-calculated calibration standards and quality control standards processed together with the batches of study samples are summarised in tables 55 and 56.

Table 54 Batch list

No.	Sample	No.	Sample	No.	Sample	No.	Sample	No.	Sample
1	SYS 1	41	P1,2.0,1	81	P3,3.33,1	121	STD H	161	P2,24,2
2	STD B	42	P1,2.0,2	82	P3,3.33,2	122	P1,8.0,1	162	P3,24,1
3	STD I	43	P2,2.0,1	83	P4,3.33,1	123	P1,8.0,2	163	P3,24,2
4	BLANK	44	P2,2.0,2	84	P4,3.33,2	124	P2,8.0,1	164	P4,24,1
5	P1,0.0,1	45	P3,2.0,1	85	STD F	125	P2,8.0,2	165	P4,24,2
6	P1,0.0,2	46	P3,2.0,2	86	P1,3.67,1	126	P3,8.0,1	166	STD B
7	P2,0.0,1	47	P4,2.0,1	87	P1,3.67,2	127	P3,8.0,2	167	SYS 2
8	P2,0.0,2	48	P4,2.0,2	88	P2,3.67,1	128	P4,8.0,1		
9	P3,0.0,1	49	STD G	89	P2,3.67,2	129	P4,8.0,2		
10	P3,0.0,2	50	P1,2.33,1	90	P3,3.67,1	130	QC D		
11	P4,0.0,1	51	P1,2.33,2	91	P3,3.67,2	131	P1,10,1		
12	P4,0.0,2	52	P2,2.33,1	92	P4,3.67,1	132	P1,10,2		
13	QC D	53	P2,2.33,2	93	P4,3.67,2	133	P2,10,1		
14	P1,0,1	54	P3,2.33,1	94	QC A	134	P2,10,2		
15	P1,0,2	55	P3,2.33,2	95	P1,4.0,1	135	P3,10,1		
16	P2,0,1	56	P4,2.33,1	96	P1,4.0,2	136	P3,10,2		
17	P2,0,2	57	P4,2.33,2	97	P2,4.0,1	137	P4,10,1		
18	P3,0,1	58	QC B	98	P2,4.0,2	138	P4,10,2		
19	P3,0,2	59	P1,2.67,1	99	P3,4.0,1	139	STD C		
20	P4,0,1	60	P1,2.67,2	100	P3,4.0,2	140	P1,12,1		
21	P4,0,2	61	P2,2.67,1	101	P4,4.0,1	141	P1,12,2		
22	QC E	62	P2,2.67,2	102	P4,4.0,2	142	P2,12,1		
23	P1,1.0,1	63	P3,2.67,1	103	STD C	143	P2,12,2		
24	P1,1.0,2	64	P3,2.67,2	104	P1,5.0,1	144	P3,12,1		
25	P2,1.0,1	65	P4,2.67,1	105	P1,5.0,2	145	P3,12,2		
26	P2,1.0,2	66	P4,2.67,2	106	P2,5.0,1	146	P4,12,1		
27	P3,1.0,1	67	STD D	107	P2,5.0,2	147	P4,12,2		
28	P3,1.0,2	68	P1,3.0,1	108	P3,5.0,1	148	QC A		
29	P4,1.0,1	69	P1,3.0,2	109	P3,5.0,2	149	P1,16,1		
30	P4,1.0,2	70	P2,3.0,1	110	P4,5.0,1	150	P1,16,2		
31	STD E	71	P2,3.0,2	111	P4,5.0,2	151	P2,16,1		
32	P1,1.5,1	72	P3,3.0,1	112	QC F	152	P2,16,2		
33	P1,1.5,2	73	P3,3.0,2	113	P1,6.0,1	153	P3,16,1		
34	P2,1.5,1	74	P4,3.0,1	114	P1,6.0,2	154	P3,16,2		
35	P2,1.5,2	75	P4,3.0,2	115	P2,6.0,1	155	P4,16,1		
36	P3,1.5,1	76	QC E	116	P2,6.0,2	156	P4,16,2		
37	P3,1.5,2	77	P1,3.33,1	117	P3,6.0,1	157	QC B		
38	P4,1.5,1	78	P1,3.33,2	118	P3,6.0,2	158	P1,24,1		
39	P4,1.5,2	79	P2,3.33,1	119	P4,6.0,1	159	P1,24,2		
40	QC F	80	P2,3.33,2	120	P4,6.0,2	160	P2,24,1		

Example: P1,8.0,2
P1 = Subject no. 1
8.0 = 8 hours after dose
2 = phase 2

Table 55 Summary of the back-calculated calibration standard concentrations of alfuzosin

Alfuzosin concentration added (ng/ml)	Alfuzosin mean concentration found (ng/ml)	Precision (RSD, %)	% nom	n
0.298	0.302	5.3	101.3	29
0.595	0.593	6.0	99.6	32
1.19	1.16	4.9	97.7	16
2.38	2.34	3.9	98.5	16
4.76	4.90	3.5	102.9	16
9.52	9.37	4.9	98.4	16
19.0	20.2	3.9	106.4	16
38.1	36.6	2.7	96.0	16

Table 56 Summary of the quality control standard concentrations of alfuzosin

Alfuzosin concentration added (ng/ml)	Alfuzosin mean concentration found (ng/ml)	Precision (RSD, %)	% nom	n
0.391	0.407	5.8	104.0	31
0.783	0.808	6.4	103.2	31
4.04	3.99	5.9	98.7	31
8.06	8.24	6.9	102.3	31
16.1	15.9	4.5	98.7	31

5.7. Pharmacokinetic data

The mean alfuzosin plasma concentrations as well as the standard deviation data are summarised in table 57 (40 subjects x 2 phases x 25 sample times = 2000 samples).

Table 57 Mean alfuzosin plasma concentration obtained from 40 subjects

Time (hours)	Mean (ng/ml)	STDEV	Time (hours)	Mean (ng/ml)	STDEV
0.0	5.7	2.8	12.0	5.1	2.1
0.0	5.4	2.7	13.0	6.4	2.5
0.0	6.2	3.2	13.5	7.2	2.9
1.0	9.7	4.2	14.0	7.9	2.8
1.5	10.2	4.0	14.5	8.6	3.0
2.0	10.9	4.6	15.0	9.0	3.3
2.5	11.7	4.5	15.5	9.2	3.4
3.0	12.4	5.6	16.0	8.9	3.2
3.5	12.3	5.7	17.0	8.0	3.1
4.0	11.7	5.2	19.0	6.9	2.8
5.0	10.0	4.2	21.0	5.3	2.2
7.0	8.1	3.6	24.0	5.1	2.0
9.0	6.9	2.7			

Concentration vs. time profiles were constructed for up to 24 hours (figure 34). Error bars are shown.

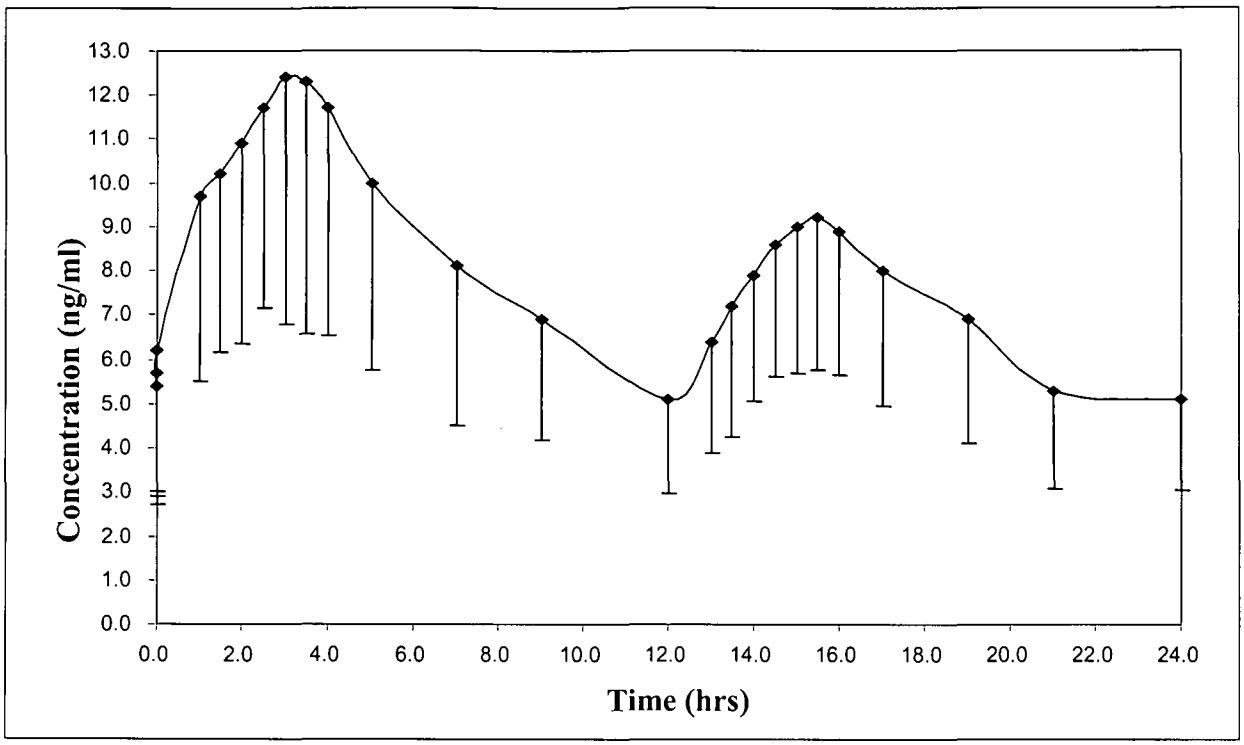


Figure 34 Representative alfuzosin plasma concentrations vs. time profiles as obtained after multiple-dose (5 mg bd.) study at steady state (40 subjects)

5.8. Conclusion

A selective, sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of alfuzosin in plasma was developed. A PE Sciex API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using TurboIonSpray (TIS) with positive ionisation was used. Using prazosin as an internal standard, liquid-liquid extraction was followed by C_{18} reversed phase liquid chromatography and tandem mass spectrometry. The mean recovery for alfuzosin was 82.9 % with a lower limit of quantification set at 0.298 ng/ml, the calibration range being between 0.298 and 38.1 ng/ml. This assay method makes use of the increased sensitivity and selectivity of mass spectrometric (MS/MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of alfuzosin in human plasma than has previously been described. The assay method was used to quantify alfuzosin in human plasma samples generated in a multiple-dose (5 mg bd.) study at steady state.

The new method is compared to those that were found in the literature in table 58. The main advantage of this assay method is the much shorter turn-around time (~ 4 to 5 minutes) with concomitant higher specificity and sensitivity enabling the assaying of large numbers of study samples in a much shorter time.

Table 58 Comparison between methods that were found in the literature and the newly developed one

Reference	Detection method	Analytical column	Extraction method	LLOQ or LOD	Limitations
Guinebault *et. al.*	Fluorimetric	Spherisorb ODS	Liquid-liquid	LLOQ in blood: 0.5 ng/ml LLOQ in urine: 0.05 µg/ml	LTAT ?
Krstulovic and Vende	Fluorimetric	Chiral-α_1-AGP	Liquid-liquid	LLOQ: 5 ng/ml for each enantiomer LOD: 1 ng/ml for each enantiomer	LTAT ?
Rouchouse *et. al.*	Fluorimetric	Chiral-α_1-AGP	Liquid-liquid	LLOQ: 1 ng/ml for each enantiomer	LTAT ?
Carlucci *et. al.*	Fluorimetric	Spherisorb S5W cyanopropyl	Column switching Clean-up	LLOQ: 2 ng/ml LOD: 1 ng/ml	LTAT ?
NEW METOD	**PE Sciex API-2000 mass spectrometer**	**C_{18}**	**Liquid-liquid**	**LLOQ: 0.298 ng/ml**	**Short turn-around time √ Selectivity √ Sensitivity √**

5.9. Publication in peer reviewed scientific journal

This assay method was submitted for publication to the Journal of Chromatography on 25/07/2002, accepted for publication on 08/01/2003 and was published in the Journal of Chromatography B, 788 (2003) 361 – 368 with the title: Selective, Sensitive and Rapid Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Alfuzosin in Human Plasma

Authors: JL Wiesner*, FCW Sutherland, GH van Essen, HKL Hundt, KJ Swart and AF Hundt.

See Appendix 1

6. METHOD DEVELOPMENT AND VALIDATION OF TWO ASSAY METHODS FOR THE DETERMINATION OF MONIC ACID IN HUMAN URINE

6.1. Objective

To develop and validate an accurate and precise analytical method for the rapid and convenient determination of monic acid concentrations in human urine in order to detect and quantify the absorption of mupirocin after multiple topical applications of 0.5 g of a 2 % ointment. Mupirocin is rapidly hydrolysed by non-specific esterase to monic acid in human blood [27]. A schematic diagram of this reaction is presented in figure 35.

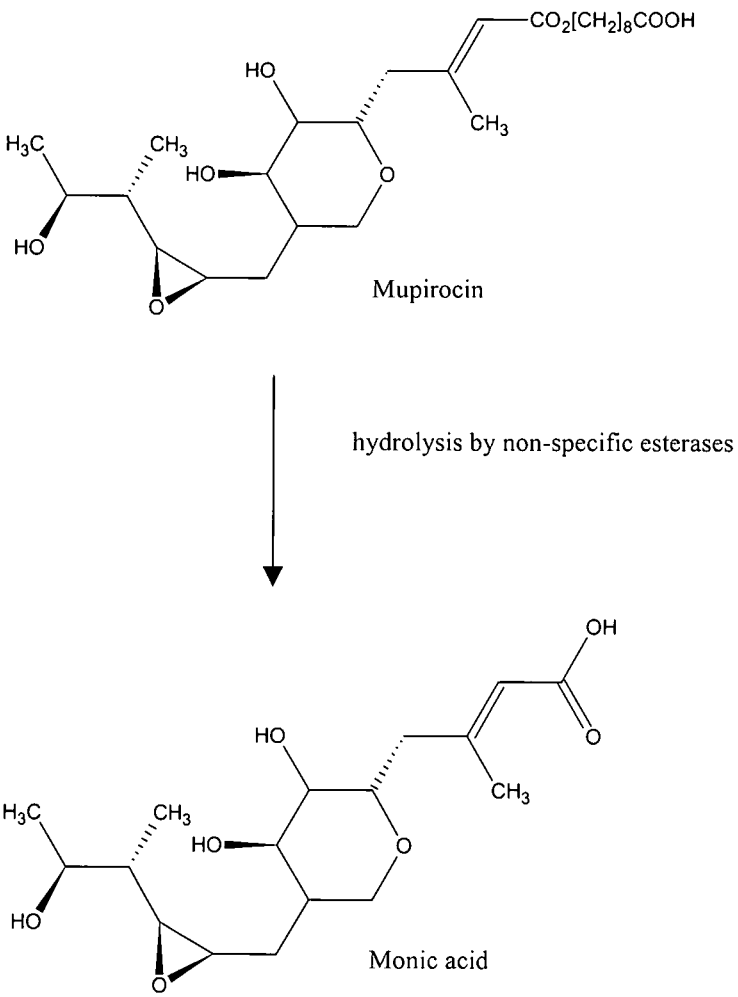


Figure 35 Schematic presentation of the formation of monic acid from mupirocin in blood

Since mupirocin is rapidly metabolised to monic acid following minimal absorption of mupirocin after topical application, and since monic acid is excreted almost exclusively by the kidneys, excretion of monic acid in urine is a good quantitative measure of the absorption of mupirocin if it was absorbed through the skin after topical application.

A monic acid assay method which could measure down to 100 ng/ml monic acid in urine, would be able to quantify 200 micrograms of monic acid dissolved in two litres of urine. If one assumes an average excretion of 2 litres of urine in 24 hours by a healthy subject, and takes into account that monic acid is excreted almost exclusively by the kidneys, one would therefore be able to quantify the absorption of about 290 micrograms of mupirocin by such an assay method. Since this would constitute minimal absorption (about 2.9 % of an application of 0.5 g of a 2 % ointment) of mupirocin, it was decided to develop and validate an assay method for monic acid in urine with a lower limit of quantification (LLOQ) of about 50 to 100 ng/ml. Since absorption of mupirocin after topical application to the skin is known to be very low, it was decided to validate the assay method over a range of about 50 to 1000 ng/ml of monic acid in urine.

6.2. Physico-chemical information

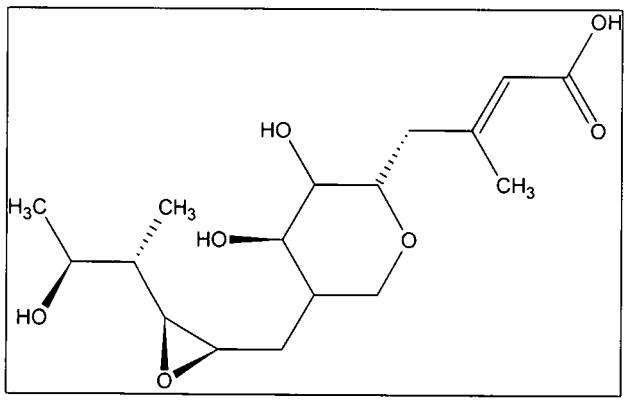


Figure 36 Chemical structure of monic acid

Molecular formula: $C_{17}H_{28}O_7$

Molecular weight: 344.2

Monoisotopic mass: 344.1835

6.3. Literature survey

6.3.1. Clinical information

Mupirocin is a topical antibacterial agent which inhibits bacterial protein and RNA synthesis. It has excellent *in vitro* activity against *Staphylococci* and most *Strepococci*, but has less activity against other Gram-positive and most Gram-negative bacteria. [27]

Mupirocin has a unique chemical structure unlike any other antibiotic. The molecule contains a short fatty acid side-chain (9-hydroxy nonanoic acid) linked to a larger molecule, monic acid, by an unsaturated ester linkage. [28]

After topical application mupirocin is minimally absorbed systemically (less than 1 %), with no detectable concentrations appearing in faecal or urinary output. Mupirocin is slowly metabolised by the skin to the antimicrobially inactive metabolite monic acid. [29]

6.3.2. Analytical information

No analytical methods for the quantification of monic acid in urine were found in the literature.

6.4. Method development and discussion: direct urine injection procedure

Faced with the prospect of having to assay a relatively polar acidic compound with a weak chromophore in urine in which many acidic compounds abound, the most likely assay procedure to succeed was considered to be LC-MS/MS. Unlike the other two cases in which the LLOQ was dictated by literature references to the maximum plasma concentrations attained after specific doses, the main aim in this case was to be able to quantify monic acid in urine down to about 50 ng/ml based on a totally different criterion. Preliminary experiments with liquid-liquid extraction of monic acid from water did not look promising. Thus, to avoid complications inherent in extraction methods, it was decided to investigate the development of an extractionless assay procedure. It was

argued that provided monic acid could be made to ionise effectively by any one of the ionisation procedures available with our MS ion sources i.e. positive or negative ESI or APCI, the required LLOQ could be achieved by injection of a suitable volume of the urine samples directly onto the HPLC column. Thus, if one could detect 1 ng of monic acid in a chromatographic peak with a signal/noise ratio of at least 5, it should be possible to attain the required LLOQ on injecting only 20 μl of a urine sample containing 50 ng/ml monic acid. Moreover, an extractionless assay method also has the advantage of not requiring an internal standard providing the injection volume precision (autosampler injection reproducibility) is high.[20]

If at some stage a more sensitive assay method were required, a solid phase extraction (SPE) procedure was to be investigated.

6.4.1. Mass Spectrometry optimisation

An Applied Biosystems API 2000 LC-MS/MS detector was set up for ion detection. The mass spectrometer was calibrated by using a PPG Standard solution in the positive and negative ionisation mode.

Electrospray ionisation (ESI) was performed in the positive ionisation mode with nitrogen as the nebulizing gas, turbo spray gas and curtain gas with the optimum values set at 75, 75 and 55 (respective arbitrary values). The heated nebulizer temperature was set at 400 °C. The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) was set at 3 (arbitrary value). The Applied Biosystems API 2000 LC-MS/MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 345.2 to the product ion m/z 327.0 for monic acid. Although the selected product ion only constitutes a loss of water it was necessary to choose this product ion to obtain the desired sensitivity. The product ion proved to be stable and did not appear to influence the selectivity. Other product ions needed a higher collision energy but this caused the parent and subsequent product ions to become unstable and form a large number of small fragment ions.

The instrument response was optimised for monic acid by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. Figure 37 shows the product ion mass spectrum of the protonated monic acid molecular ion (m/z 345.2, molecular structure given) and the principal product ion formed at m/z 327.0 after collision (MS/MS).

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.1 software.

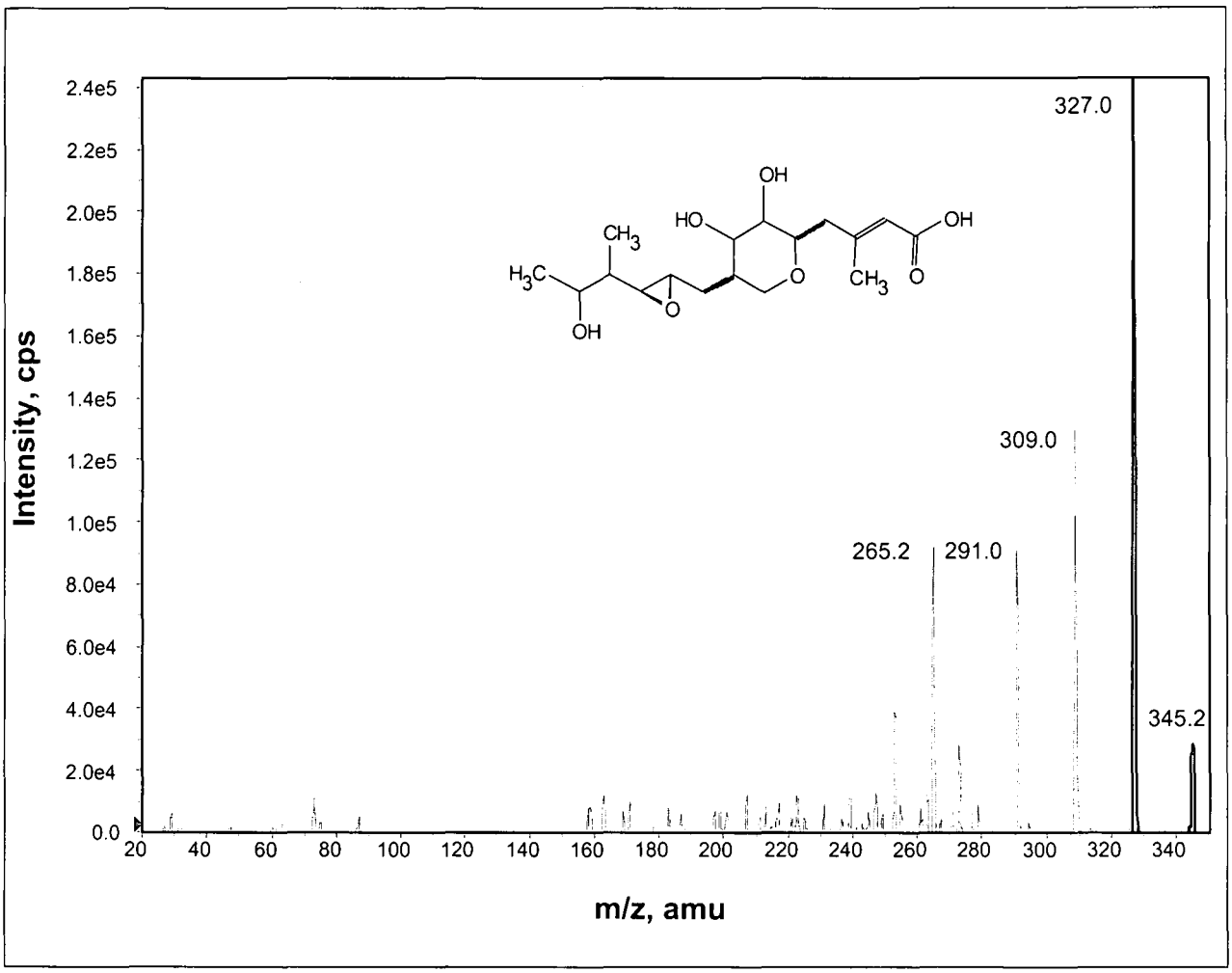


Figure 37 Product ion mass spectrum of the protonated monic acid molecular ion (m/z 345.2, molecular structure given) and the principal product ion formed at m/z 327.0 after collision (MS/MS)

6.4.2. Chromatography development

An SPVS solution was prepared in water (1 μg/ml) and mobile phase consisting of methanol and a 0.2 % aqueous acetic acid solution (25:75, v/v) was delivered at a constant flow of 0.3 ml/min through a Discovery C_{18} (150 x 2 mm, 5 μm) column. This resulted in good chromatography (figure 38). The organic component of the mobile phase was increased to 30 % and the retention

time shifted to 3.8 min. (figure 39). The first 3.3 min. of the run was diverted to waste using a switching valve. Chromatography development was deemed adequate with this mobile phase.

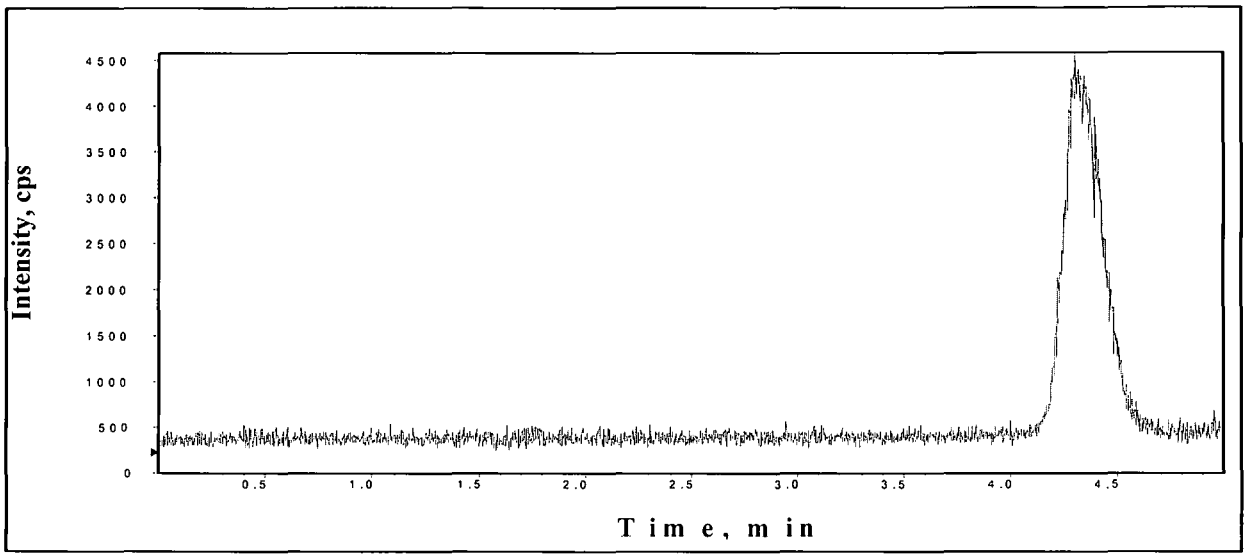


Figure 38 Chromatogram of monic acid using a mobile phase consisting of methanol and 0.2 % aqueous acetic acid solution (25:75, v/v)

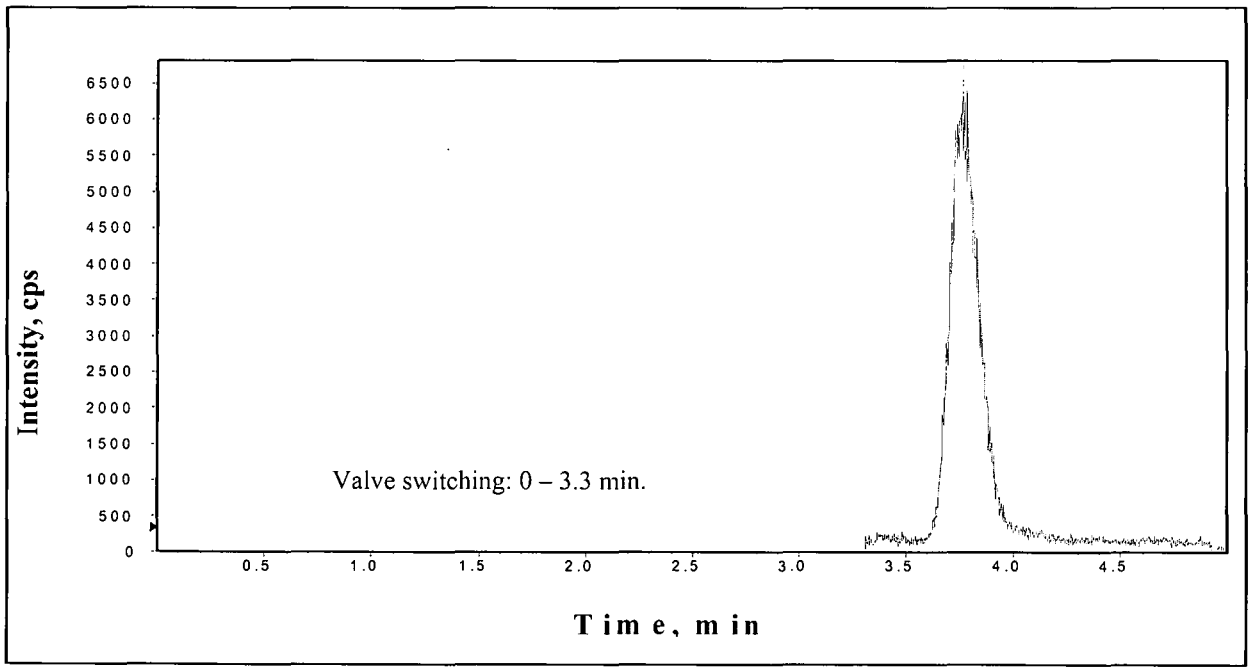


Figure 39 Chromatogram of monic acid using a mobile phase consisting of methanol and 0.2 % aqueous acetic acid solution (30:70, v/v)

6.4.3. Sample preparation

The urine samples were thawed in a water bath at approximately 37 °C, vortexed for 5 sec. and centrifuged at 1500 g for 5 *min*. Approximately 200 µl of the sample was transferred to an autosampler vial containing a glass insert and 20 µl was injected onto a Supelco Discovery C_{18} (150 × 2 mm, 5 µm) HPLC column.

6.4.4. Instrumental and chromatographic conditions

All chromatographic solvents were degassed with helium before use. Chromatography was carried out at ambient temperature with a mobile phase consisting of methanol and aqueous acetic acid solution (0.2 %), (30:70, v/v) at a flow-rate of 0.3 ml/min.

6.4.5. Matrix effect

The matrix effect was tested as described by Matuzevski *et al.* [1] Matrix effect was present but was deemed insignificant (table 59).

Table 59 Tested urine pools

Urine Pool	Analyte area
1	15500
2	16000
3	16300
4	16900
5	15500
6	18700
Mean	16483
STDEV	1207
CV%	7.0

6.5. Analytical method validation and discussion

6.5.1. Preparation of calibration standards

Calibration standards were prepared by dissolving monic acid in water, and spiking 400µl of this stock solution (table 60) into a pool of blank urine (STD H). This resulted in the highest calibration standard (1001 ng/ml). This calibration standard was then serially diluted with blank urine (1:1) six times, which resulted in a calibration standard range between 1001 and 50.1 ng/ml (table 61). This entire calibration range was validated.

Table 60 Preparation of Stock Solution SA for Spiking STD H

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Water	1.000	1.939	7.717	7.717	400	251

Table 61 Preparation of Calibration Standards

Calibration Standard	Source Solution	A	B	C	D (ng/ml)
STD H	*Stock SA*	20.584	120.589	-	1001
STD G	*STD H*	20.541	70.532	120.543	501
STD F	*STD G*	20.630	40.641	120.656	400
STD E	*STD F*	20.600	45.609	120.600	300
STD D	*STD E*	20.435	53.741	120.476	200
STD C	*STD D*	20.658	70.654	120.661	100
STD B	*STD C*	20.553	70.559	120.574	50.1

A = Mass of empty container.
B = Mass of container + normal plasma.
C = Total mass of container + normal + spiked plasma.
D = Concentration of analyte.

6.5.2. Preparation of quality control standards

Similarly, quality control standards were prepared (using the same methodology) spanning a range between 900 and 60.7 ng/ml (tables 62 and 63).

Table 62 Preparation of Stock Solution QA for Spiking QC F

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Water	1.000	3.608	8.003	8.003	400	451

Table 63 Preparation of Quality Control Standards

Quality Control Standard	Source Solution	A	B	C	D (ng/ml)
QC F	*Stock QA*	44.469	244.474	-	900
QC E	*QC F*	44.560	137.553	244.684	482
QC D	*QC E*	45.271	95.290	245.491	361
QC C	*QC D*	44.480	111.479	244.624	240
QC B	*QC C*	44.619	144.634	244.887	120
QC A	*QC B*	46.003	146.033	247.935	60.7

A = Mass of empty container.
B = Mass of container + normal plasma.
C = Total mass of container + normal + spiked plasma.
D = Concentration of analyte.

Sufficient calibration standards and quality controls were prepared to validate the method and to serve as standards and controls during the assay of all study sample batches. These were stored together with the study samples at -20 °C until used for sample processing.

6.5.3. Intra-batch accuracy and precision

Intra-batch accuracy and precision are assessed by assaying all the calibration standards in duplicate to produce one calibration curve and 6 replicates of all the prepared quality control standards in a single batch of assays. The intra-batch accuracy and precision of the assay procedure are assessed by calculating the regression equations and constructing the calibration curves based on both peak heights and peak areas.

Accuracy is expressed as recovery of the analyte as a percentage of the nominal concentration (% nom) while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % of nominal concentration at the LLOQ. For a valid method the intra-batch precision is required to be less than 15 % (i.e. CV% should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The method performed well during the intra-batch validation and passed all of the criteria set for both peak area and peak heights. A linear regression weighted $1/c^2$ was fitted to the calibration line and the calibration range was validated between 50.1 and 1001 ng/ml.

The results of the intra-batch validation are summarised in tables 64 to 67.

6.5.3.1. Quantitation by peak height

Table 64 Back-calculated concentrations of monic acid based on peak heights

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1001	1131.2	13.0
STD H	1001	1021.9	2.1
STD G	501	491.73	-1.8
STD G	501	496.39	-0.9
STD F	400	382.44	-4.4
STD F	400	405.70	1.4
STD E	300	296.40	-1.2
STD E	300	287.10	-4.3
STD D	200	198.73	-0.6
STD D	200	203.61	1.8
STD C	100	85.256	-14.8
STD C	100	103.62	3.6
STD B	50.1	69.898	R
STD B	50.1	53.155	6.1

R = Rejected

Calibration Standards used:	STD B – STD H
Calibration Range:	50.1 - 1001 ng/ml
Regression Equation:	Linear: $y = mx + c$ (weighted $1/c^2$)
m:	4.3002
c:	-14.58
r^2:	0.992394

Table 65 Summary of quality control results based on peak heights for intra-batch validation

Code	QC F 900 ng/ml	QCF (Dil) 900 ng/ml	QC E 482 ng/ml	QC D 361 ng/ml	QC C 240 ng/ml	QC B 120 ng/ml	QC A 60.7 ng/ml
1	833.583	816.044	470.810	345.234	245.239	114.547	54.782
2	808.003	867.204	435.927	363.838	210.589	118.035	58.736
3	826.607	890.459	487.088	331.281	214.077	115.477	58.038
4	805.678	829.996	482.437	366.163	214.077	110.129	54.550
5	835.909	816.044	440.578	335.932	219.426	120.128	58.503
6	780.097	857.902	482.437	359.187	247.564	112.222	56.178
MEAN	814.98	846.27	466.55	350.27	225.16	115.09	56.80
%nom	90.6	94.0	96.8	97.0	93.8	95.9	93.6
CV%	2.6	3.6	4.8	4.2	7.4	3.2	3.3

Note: QC F was diluted (1:1) with blank urine and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

6.5.3.2. Quantitation by peak area

Table 66 Back-calculated concentrations of monic acid based on peak areas

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1001	1154.3	15.3
STD H	1001	1089.1	8.8
STD G	501	464.92	-7.2
STD G	501	513.83	2.6
STD F	400	399.71	-0.1
STD F	400	406.70	1.7
STD E	300	294.90	-1.7
STD E	300	273.94	-8.7
STD D	200	197.32	-1.3
STD D	200	202.21	1.1
STD C	100	84.364	-15.6
STD C	100	92.982	-7.0
STD B	50.1	59.211	18.2
STD B	50.1	47.101	-6.0

Calibration Standards used:	STD B – STD H
Calibration Range:	50.1 – 1001 ng/ml
Regression Equation:	Linear: $y = mx + c$ (weighted $1/c^2$)
m:	42.937
c:	-162.37
r^2:	0.984264

Table 67 Summary of quality control results based on peak areas for intra-batch validation

Code	QC F 900 ng/ml	QCF (Dil) 900 ng/ml	QC E 482 ng/ml	QC D 361 ng/ml	QC C 240 ng/ml	QC B 120 ng/ml	QC A 60.7 ng/ml
1	853.859	850.654	474.235	334.497	235.282	109.284	54.320
2	832.898	859.970	455.604	371.760	227.829	127.218	48.964
3	844.543	925.181	464.920	329.839	228.761	111.148	55.019
4	814.266	850.654	467.249	355.457	217.815	105.791	56.184
5	856.188	813.390	457.933	343.813	241.337	127.450	55.485
6	800.293	776.126	490.538	348.471	233.186	117.203	55.019
MEAN	833.67	846.00	468.41	347.31	230.70	116.35	54.17
%nom	92.6	94.0	97.2	96.2	96.1	97.0	89.2
CV%	2.7	5.9	2.7	4.4	3.5	8.0	4.8

Note: QC F was diluted (1:1) with blank urine and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

6.5.4. Inter-batch accuracy and precision

Inter-batch accuracy and precision are assessed by assaying two separate consecutive batches, each consisting of one set of calibration standards designated for use in the assay of samples of unknown concentrations and 6 replicates of each of the quality control standards designated for use in the assay of samples of unknown concentrations. The intra-batch accuracy and precision of each of the batches is assessed separately by calculating the regression equation and constructing the calibration curve based on the best performing quantification method, and must pass the criteria for intra-batch acceptance. The inter-batch accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 in total). Accuracy is expressed as recovery of the analyte as a percentage of the nominal concentration (% nom) while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra- and inter-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % of nominal concentration at the LLOQ. For a valid method the intra- and inter-batch precision is required to be less than 15 % (ie. CV % should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The highest variation was 6.8 % (QC B from the first inter-batch validation). This information illustrates that the method performed well during both inter-batch validations.

The results are summarised in tables 68 to 71.

6.5.4.1. Inter-batch 1 accuracy and precision

Table 68 Back-calculated concentrations of monic acid

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1001	1043.3	4.2
STD H	1001	1027.4	2.6
STD G	501	491.87	-1.8
STD G	501	489.60	-2.3
STD F	400	405.64	1.4
STD F	400	412.45	3.1
STD E	300	308.06	2.7
STD E	300	298.99	-0.3
STD D	200	190.52	-4.7
STD D	200	197.10	-1.5
STD C	100	94.753	-5.2
STD C	100	97.023	-3.0
STD B	50.1	53.907	7.6
STD B	50.1	48.688	-2.8

Calibration Standards used:	STD B – STD H
Calibration Range:	50.1 – 1001 ng/ml
Regression Equation:	Linear: $y = mx + c$ (weighted $1/c^2$)
m:	44.0675
c:	-275.538
r^2:	0.99744

Table 69 Summary of quality control results for inter-batch 1 validation

Code	QC F 900 ng/ml	QC E 482 ng/ml	QC D 361 ng/ml	QC C 240 ng/ml	QC B 120 ng/ml	QC A 60.7 ng/ml
1	878.890	485.064	335.294	218.881	107.461	56.176
2	862.056	455.564	337.563	227.958	119.488	59.807
3	854.692	441.948	339.832	233.177	101.561	50.957
4	857.848	432.871	321.678	219.108	121.984	60.488
5	847.327	462.371	337.563	210.258	113.361	57.538
6	888.358	416.986	342.101	217.293	110.411	52.999
MEAN	864.86	449.13	335.67	221.11	112.38	56.33
%nom	96.1	93.2	93.0	92.1	93.6	92.8
CV%	1.8	5.3	2.2	3.7	6.8	6.7

6.5.4.2. Inter-batch 2 accuracy and precision

Table 70 Back-calculated concentrations of monic acid

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1001	1049.4	4.8
STD H	1001	1026.8	2.6
STD G	501	504.61	0.7
STD G	501	499.59	-0.3
STD F	400	401.67	0.4
STD F	400	389.11	-2.7
STD E	300	306.26	2.1
STD E	300	303.75	1.2
STD D	200	193.52	-3.2
STD D	200	195.28	-2.4
STD C	100	100.63	0.6
STD C	100	91.336	-8.7
STD B	50.1	54.176	8.1
STD B	50.1	48.402	-3.4

Calibration Standards used:	STD B – STD H
Calibration Range:	50.1 – 1001 ng/ml
Regression Equation:	Linear: y = mx +c (weighted $1/c^2$)
m:	39.8284
c:	2.2450
r^2:	0.99695

Table 71 Summary of quality control results for inter-batch 1 validation

Code	QC F 900 ng/ml	QC E 482 ng/ml	QC D 361 ng/ml	QC C 240 ng/ml	QC B 120 ng/ml	QC A 60.7 ng/ml
1	879.420	469.458	361.495	242.233	115.690	60.453
2	889.082	479.501	379.070	237.211	118.703	58.947
3	861.169	494.565	379.070	261.064	127.742	60.704
4	822.519	482.012	356.473	230.683	122.720	54.930
5	789.238	482.012	389.113	233.445	108.660	57.440
6	854.727	474.479	333.876	221.394	109.664	57.691
MEAN	849.36	480.34	366.52	237.67	117.20	58.36
%nom	94.4	99.7	101.5	99.0	97.7	96.1
CV%	4.4	1.8	5.5	5.6	6.3	3.7

6.5.5. Summary of the combined quality control results for the 3 validations

The combined quality control results are summarised in table 72. The method performed well during all three validations (the highest coefficient of variation was only 6.9 %).

Table 72 Summary of the combined quality control results for the 3 validations

Validation Batch	Nominal	900	482	361	240	120	60.7
	Replicates	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
Intra-batch Validation	1	854	474	334	235	109	54.3
	2	833	456	372	228	127	49.0
	3	845	465	330	229	111	55.0
	4	814	467	355	218	106	56.2
	5	856	458	344	241	127	55.5
	6	800	491	348	233	117	55.0
Inter-batch Validation 1	1	879	485	335	219	107	56.2
	2	862	456	338	228	119	59.8
	3	855	442	340	233	102	51.0
	4	858	433	322	219	122	60.5
	5	847	462	338	210	113	57.5
	6	888	417	342	217	110	53.0
Inter-batch Validation 2	1	879	469	361	242	116	60.5
	2	889	480	379	237	119	58.9
	3	861	495	379	261	128	60.7
	4	823	482	356	231	123	54.9
	5	789	482	389	233	109	57.4
	6	855	474	334	221	110	57.7
	MEAN	849.3	466.0	349.8	229.7	115.3	56.3
	%nom	94.4	96.7	96.9	95.7	96.1	92.7
	CV%	3.3	4.4	5.5	5.1	6.9	5.8

6.5.6. Stability assessment

6.5.6.1. Stability in matrix

Stability in matrix samples were prepared by spiking stock solutions of monic acid in urine to obtain 2 different urine concentrations (900 and 120 ng/ml). These samples were kept at –20 °C for 6.5 months and were then analysed together with a freshly prepared set of standards and quality control standards. The results of the stability samples are summarised in table 73.

Table 73 Matrix stability

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)
900	924.704
900	811.347
900	808.108
900	795.153
900	754.669
Mean	818.80
Std.Dev.	63.36
CV%	7.74
%Nom.	90.98

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)
120	107.239
120	94.932
120	95.256
120	96.066
120	103.677
Mean	99.43
Std.Dev.	5.66
CV%	5.69
%Nom.	82.86

By comparing the measured concentrations against the nominal concentration of the tabulated data, a correlation coefficient of 0.91 was obtained, indicating that some degradation of the sample did occur. The duration of a study was approximately 2 months. Samples were analysed within this period, and therefore the degradation for this time period would be insignificant and one can assume

that monic acid was stable in matrix for the duration of the study.

6.5.6.2. Freeze and thaw stability

Freeze-thaw stability was determined with QC E (482 ng/ml) and QC B (120 ng/ml) over three freeze-thaw cycles. Originally frozen QC E and QC B samples were allowed to thaw to room temperature (Table 74), frozen to –20 °C and thawed again to room temperature (Table 75), frozen to -20 °C and thawed again to room temperature (Table 76).

Table 74 Freeze-thaw cycle 1

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
482	474	98.39
482	456	94.52
482	465	96.46
482	467	96.94
482	458	95.01
482	491	101.77
Mean	468	96.26
Std.Dev.	12.73	1.55
CV%	2.72	
%Nom.	97.18	

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
120	109.3	91.07
120	127.2	106.02
120	111.1	92.62
120	105.8	88.16
120	127.5	106.21
120	117.2	97.67
Mean	116.3	96.8
Std.Dev.	9.28	8.64
CV%	7.98	
%Nom.	96.96	

Table 75 Freeze-thaw cycle 2

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
482	475	98.57
482	478	99.16
482	458	95.02
482	392	81.42
482	420	87.04
Mean	445	92.24
Std.Dev.	37.33	7.74
CV%	8.40	
%Nom.	92.24	

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
120	86.4	72.01
120	104.7	87.22
120	91.8	76.53
120	92.4	77.00
120	101.1	84.25
Mean	95.3	79.4
Std.Dev.	7.43	6.19
CV%	7.79	
%Nom.	79.40	

Table 76 Freeze-thaw cycle 3

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
482	435	90.29
482	414	85.85
482	467	96.79
482	447	92.66
482	445	92.36
Mean	441	91.59
Std.Dev.	19.19	3.98
CV%	4.35	
%Nom.	91.59	

Nominal Concentration (ng/ml)	Measured Concentration (ng/ml)	Calculated % of nominal
120	97.2	81.04
120	91.7	76.41
120	97.5	81.28
120	97.1	80.92
120	99.8	83.18
Mean	96.7	80.6
Std.Dev.	3.00	2.50
CV%	3.10	
%Nom.	80.56	

Although the above data appeared to indicate some measure of instability during freeze-thaw cycling, the data are not conclusive since there was no difference in the concentrations between the samples assayed after the second and the third freeze-thaw cycles. This does suggest that monic acid is stable under the freeze-thaw conditions tested. No trial samples were reanalysed using samples that were previously thawed. All re-analyses of samples were performed using the frozen duplicate samples.

6.5.6.3. On-Instrument stability

Sixteen stability samples of the same concentration were injected at intervals during the first two validation batches to simulate the time of a batch run. The measured peak areas, injection times and cumulative time are summarised in table 77.

Table 77 Stability data of sixteen stability samples injected at different intervals

Replicates	Injection Time	Time Difference	Cumulative Time (hr)	Analyte Peak Area
1	1:19		0.00	19400
2	2:24	1:05:00	1.08	19000
3	3:24	1:00:00	2.08	19200
4	4:35	1:11:00	3.27	20300
5	5:40	1:05:00	4.35	18600
6	6:45	1:05:00	5.43	21700
7	9:47	3:02:00	8.47	19500
8	9:53	0:06:00	8.57	19500
9	1:13	15:20:00	23.90	19800
10	2:06	0:53:00	24.78	20400
11	2:59	0:53:00	25.67	20000
12	3:59	1:00:00	26.67	21000
13	4:52	0:53:00	27.55	19900
14	5:45	0:53:00	28.43	18500
15	6:27	0:42:00	29.13	18900
16	6:33	0:06:00	29.23	18200
			Mean	19619
			Std Dev	932
			CV	4.75%

The peak areas of monic acid are seen to be stable within a batch run.

By regression analysis of the peak areas of the cumulative time tabulated above it can be established that the peak area decreased by 0.75 % over a period of 30 hours over two batches while awaiting injection on-instrument (figure 40). This trend is deemed insignificant and the extracts are considered to be stable on-instrument for at least 30 hours.

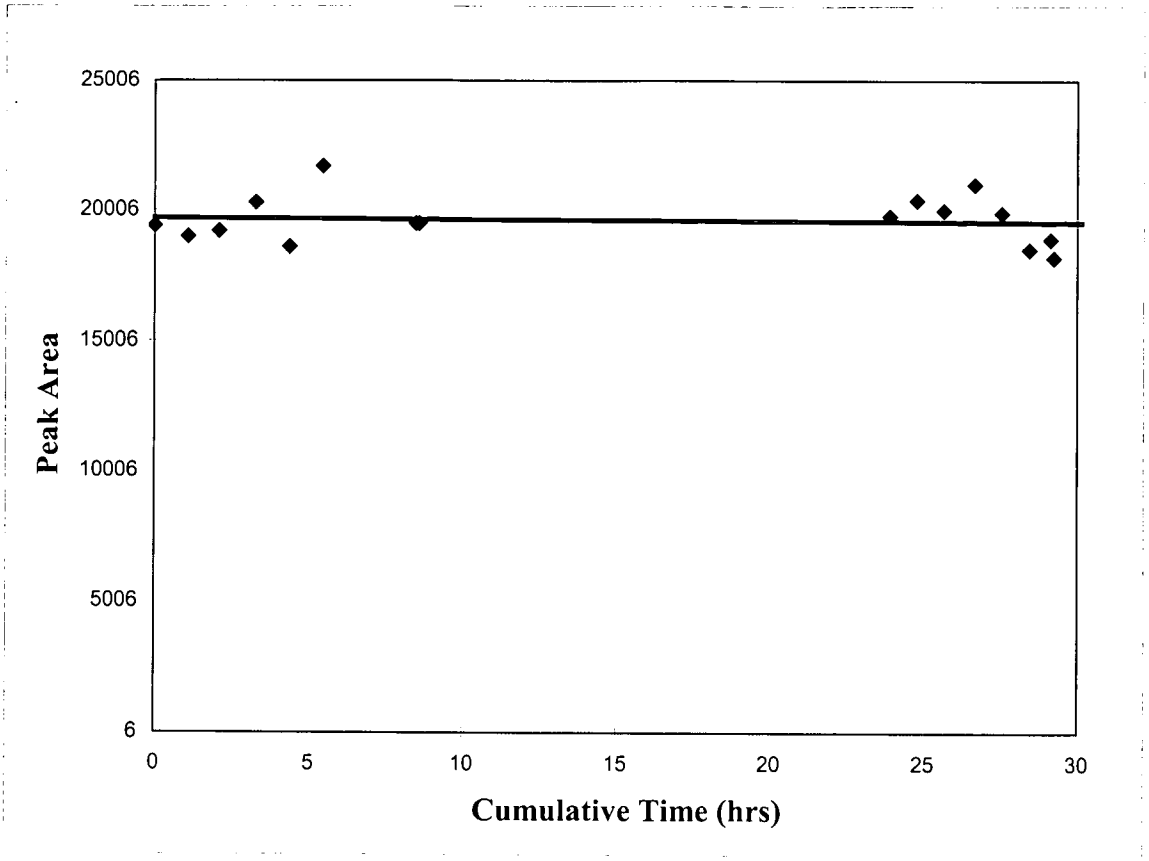


Figure 40 On-Instrument Stability

6.5.7. Specificity

Specificity is determined by analysing blank normal human urine from six different sources. 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 min. to determine the presence of late eluting peaks which may cause interference in subsequent chromatograms. The very high specificity of the LC/MS-MS technique precludes the detection of compounds that do not produce the same parent and product ions as the analyte of interest. No chromatographic peaks were observed in blank normal human urine injected from six different subjects.

Figure 42 is an example of a chromatogram of a blank urine injection and figure 41 is an overlay of the LLOQ over a blank urine injection. No interference was observed. The mobile phase was diverted to waste between 0.6 and 3.3 min., using a switching valve.

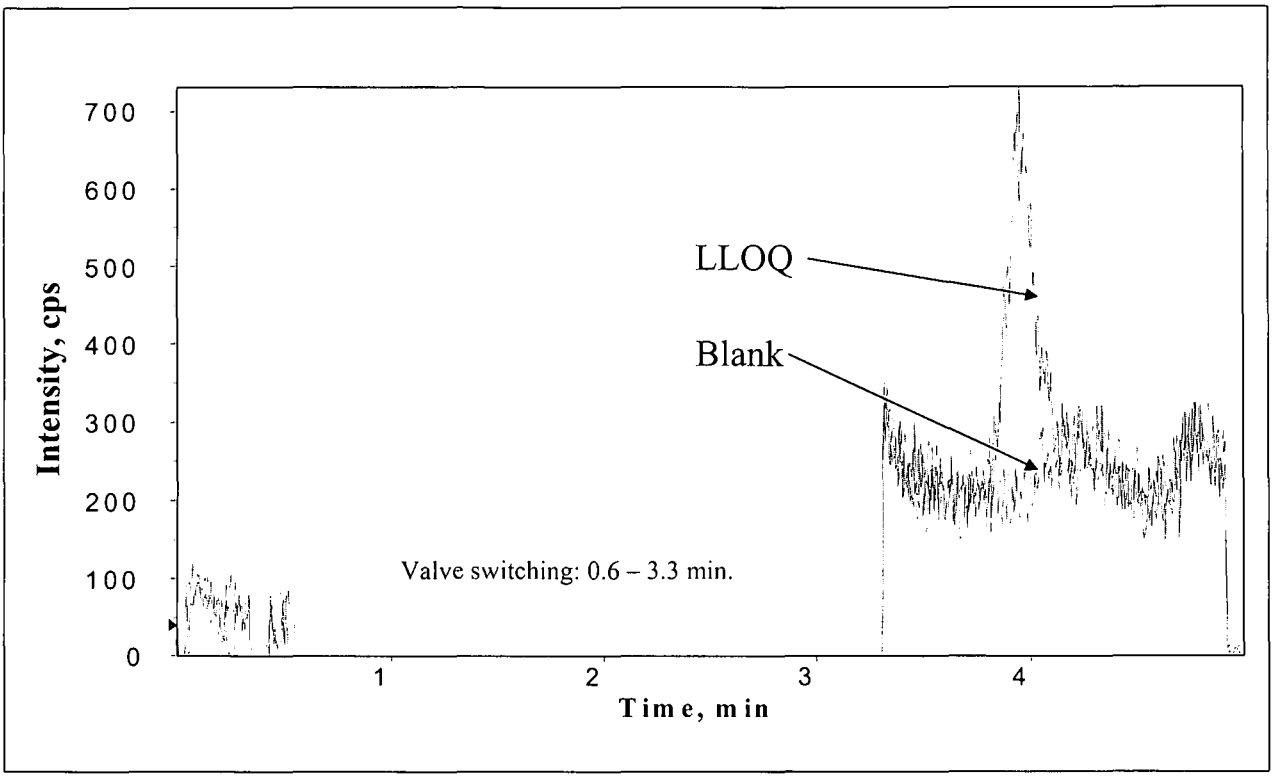


Figure 41 Overlay of the chromatograms of a blank urine injection and an injection of urine containing 50.1 ng/ml monic acid

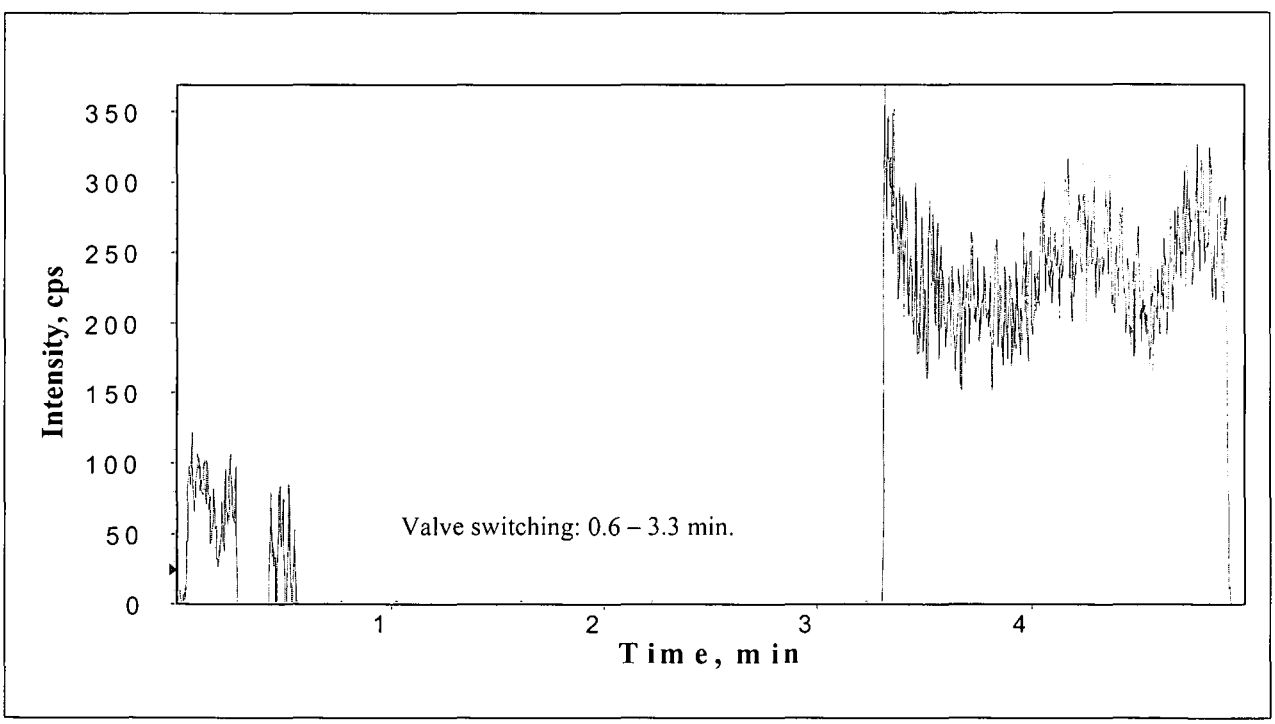


Figure 42 Chromatogram of a blank urine injection

6.5.8. Sensitivity

The lower limit of quantification (LLOQ) of this method is 50 ng/ml (figure 43) which is considered to be sensitive enough to monitor the low absorption of mupirocin expected in this clinical study.

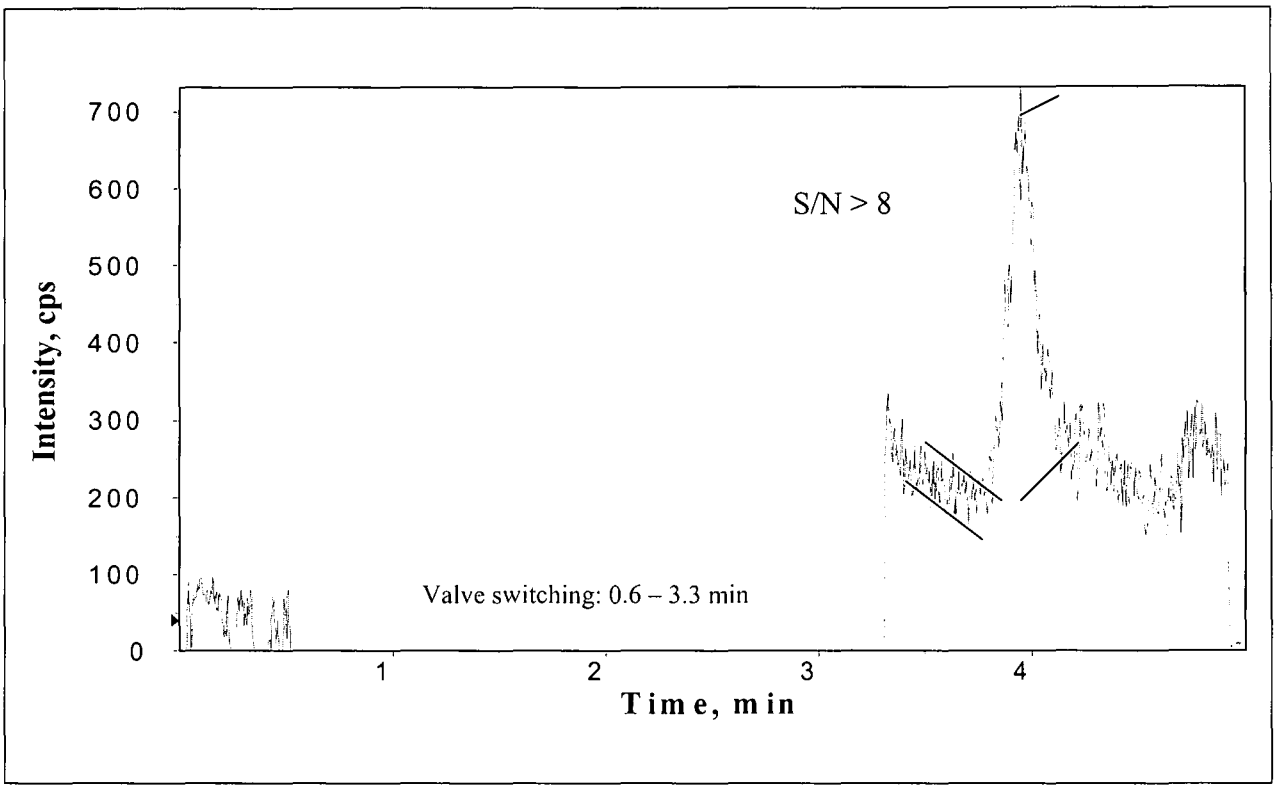


Figure 43 Chromatogram of a urine injection containing 50 ng/ml monic acid

6.6. Study Application

Table 78 is an example of a batch list indicating the arrangement of the study samples and the STDs and QCs (direct injection method).

Table 78 Batch list

No.	Sample	No.	Sample	No.	Sample	No.	Sample	No.	Sample
1	SYS 1	41	P8,0,2	81	QC A	121	P3,8.0,1	161	P4,18,1
2	STD B	42	P1,1.0,1	82	P2,4.0,1	122	P3,8.0,2	162	P4,18,2
3	STD H	43	P1,1.0,2	83	P2,4.0,2	123	QC D	163	P5,18,1
4	BLANK	44	P2,1.0,1	84	P3,4.0,1	124	P4,8.0,1	164	P5,18,2
5	P1,0.0,1	45	P2,1.0,2	85	P3,4.0,2	125	P4,8.0,2	165	QC A
6	P1,0.0,2	46	STD E	86	P4,4.0,1	126	P5,8.0,1	166	P6,18,1
7	P2,0.0,1	47	P3,1.0,1	87	P4,4.0,2	127	P5,8.0,2	167	P6,18,2
8	P2,0.0,2	48	P3,1.0,2	88	STD H	128	P6,8.0,1	168	P7,18,1
9	P3,0.0,1	49	P4,1.0,1	89	P5,4.0,1	129	P6,8.0,2	169	P7,18,2
10	P3,0.0,2	50	P4,1.0,2	90	P5,4.0,2	130	STD E	170	P8,18,1
11	QC F	51	P5,1.0,1	91	P6,4.0,1	131	P7,8.0,1	171	P8,18,2
12	P4,0.0,1	52	P5,1.0,2	92	P6,4.0,2	132	P7,8.0,2	172	STD B
13	P4,0.0,2	53	QC C	93	P7,4.0,1	133	P8,8.0,1	173	P1,24,1
14	P5,0.0,1	54	P6,1.0,1	94	P7,4.0,2	134	P8,8.0,2	174	P1,24,2
15	P5,0.0,2	55	P6,1.0,2	95	QC F	135	P1,12,1	175	P2,24,1
16	P6,0.0,1	56	P7,1.0,1	96	P8,4.0,1	136	P1,12,2	176	P2,24,2
17	P6,0.0,2	57	P7,1.0,2	97	P8,4.0,2	137	QC C	177	P3,24,1
18	STD G	58	P8,1.0,1	98	P1,6.0,1	138	P2,12,1	178	P3,24,2
19	P7,0.0,1	59	P8,1.0,2	99	P1,6.0,2	139	P2,12,2	179	P4,24,1
20	P7,0.0,2	60	STD D	100	P2,6.0,1	140	P3,12,1	180	P4,24,2
21	P8,0.0,1	61	P1,2.0,1	101	P2,6.0,2	141	P3,12,2	181	P5,24,1
22	P8,0.0,2	62	P1,2.0,2	102	STD G	142	P4,12,1	182	P5,24,2
23	P1,0,1	63	P2,2.0,1	103	P3,6.0,1	143	P4,12,2	183	P6,24,1
24	P1,0,2	64	P2,2.0,2	104	P3,6.0,2	144	STD D	184	P6,24,2
25	QC E	65	P3,2.0,1	105	P4,6.0,1	145	P5,12,1	185	P7,24,1
26	P2,0,1	66	P3,2.0,2	106	P4,6.0,2	146	P5,12,2	186	P7,24,2
27	P2,0,2	67	QC B	107	P5,6.0,1	147	P6,12,1	187	P8,24,1
28	P3,0,1	68	P4,2.0,1	108	P5,6.0,2	148	P6,12,2	188	P8,24,2
29	P3,0,2	69	P4,2.0,2	109	QC E	149	P7,12,1	189	SYS 2
30	P4,0,1	70	P5,2.0,1	110	P6,6.0,1	150	P7,12,2		
31	P4,0,2	71	P5,2.0,2	111	P6,6.0,2	151	QC B		
32	STD F	72	P6,2.0,1	112	P7,6.0,1	152	P8,12,1		
33	P5,0,1	73	P6,2.0,2	113	P7,6.0,2	153	P8,12,2		
34	P5,0,2	74	STD C	114	P8,6.0,1	154	P1,18,1		
35	P6,0,1	75	P7,2.0,1	115	P8,6.0,2	155	P1,18,2		
36	P6,0,2	76	P7,2.0,2	116	STD F	156	P2,18,1		
37	P7,0,1	77	P8,2.0,1	117	P1,8.0,1	157	P2,18,2		
38	P7,0,2	78	P8,2.0,2	118	P1,8.0,2	158	STD C		
39	QC D	79	P1,4.0,1	119	P2,8.0,1	159	P3,18,1		
40	P8,0,1	80	P1,4.0,2	120	P2,8.0,2	160	P3,18,2		

Example: P3,8.0,1
P3 = Subject no. 3
8.0 = 8 hours after dose
1 = phase 1

The direct injection method was used to quantify samples for two studies, 400 study samples were processed in four batches during the first study, each including a seven point calibration line and twelve quality controls (determined at six levels), and 110 samples were processed in one batch during the second study.

Most of the samples had concentration levels above the LLOQ, indicating some absorption of monic acid. The significance of this data has still to be determined before any conclusions can be made.

6.7. Method development and discussion: solid phase extraction procedure

In the initial study it was found that the monic acid concentrations in urine was lower than expected due to the poor absorption of mupirocin through the skin. We therefore decided to try and improve the sensitivity of the method if in future a more sensitive method was required to obtain a better picture of the mupirocin kinetics.

6.7.1. Mass Spectrometry optimisation

The same procedure was followed as described in section 6.4.1.

6.7.2. Extraction development

Extraction development was performed on tC_{18} SPE Waters, Oasis® columns. The columns were conditioned with methanol (1 ml) and water (1 ml). The optimum pH for extraction had to be determined, and therefore different pH-ranges of citrate buffer (50 mM citrate buffer) were tested, which included the following: pH 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75 and 4. The best recovery (74%) was obtained with the pH 2 buffer. Different washing steps were tested that included the following solutions: H_2O (1 ml), H_2O (2 ml), a 5 % aqueous methanol solution (1 ml), a 10 % aqueous methanol solution (1 ml) and a 15 % aqueous methanol solution (1 ml). The recovery yield for all of the above-mentioned washing solutions was approximately 74 % and this information indicated that the sample can be rinsed with at least a 15 % aqueous methanol solution. The analyte was eluted with methanol (0.5 ml). The methanol was evaporated under a gentle stream of nitrogen at 45 °C. Various injection solutions were tested for optimum retention on the analytical column. The tested pH-ranges were: 5.5, 6, 6.5, 7, 7.5 and 8 (50 mM formate buffer). The retention time for all the tested conditions was approximately 4.6 min.

6.7.3. Sample preparation

The urine samples were thawed in a water bath at approximately 37 °C, vortexed for 5 sec. and centrifuged for 5 min. at 1 300 G. The SPE procedure was performed manually on C_{18} columns (Waters, Sep-Pak®Vac, 100 mg):

- *Conditioning:* methanol (1 ml)
  H_2O (1 ml)
  50 mM, pH 2 citrate buffer (0.5 ml)
- *Retention:* 2 ml of the following mixture was loaded onto the columns: 1.1 ml sample and 1.1 ml of the 50 mM, pH 2 citrate buffer
- *Washing:* H_2O (1 ml)
  15 % methanol solution (1 ml)
- *Elution:* methanol (0.5 ml)

The methanol was evaporated under a gentle stream of nitrogen at 45 °C and the dry extracts were reconstituted with 200 µl of a 50 mM, pH 6.5 formate buffer. Approximately 200 µl of the sample was transferred to an autosampler vial containing a glass insert and 20 µl was injected onto a Supelco Discovery C_{18} (150 × 2 mm, 5 µm) HPLC column.

6.7.4. Instrumental and chromatographic conditions

The same instumental and chromatographic conditions were used as described in section 6.4.4.

6.7.5. Matrix effect

The same methodology was performed as for the "direct injection method" and led to similar results (table 79).

Table 79 Tested urine pools

Urine Pool	Analyte area
1	23800
2	25400
3	22500
4	20700
5	21300
6	22500
Mean	22700
STDEV	1705
CV%	7.5

6.8. Analytical method validation and discussion

6.8.1. Preparation of calibration standards

Calibration standards were prepared by dissolving monic acid in water, and spiking 250 µl of this stock solution (table 80) into a pool of blank urine (STD H). This resulted in the highest calibration standard (1013 ng/ml). This calibration standard was then serially diluted with blank urine (1:1) six times, which resulted in a calibration standard range between 1013 and 15.8 ng/ml (table 81). This entire calibration range was validated.

Table 80 Preparation of Stock Solution SA for Spiking STD H

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Water	1.000	2.275	6.993	6.993	250	325.33

Table 81 Preparation of Calibration Standards

Calibration Standard	Source Solution	A	B	C	D (ng/ml)
STD H	*Stock SA*	20.505	100.510	-	1013
STD G	*STD H*	20.532	60.542	100.547	507
STD F	*STD G*	20.587	60.598	100.607	253
STD E	*STD F*	20.562	60.566	100.572	127
STD D	*STD E*	20.587	60.594	100.597	63.3
STD C	*STD D*	20.462	60.471	100.471	31.7
STD B	*STD C*	20.540	60.540	100.554	15.8

A = Mass of empty container.
B = Mass of container + normal plasma.
C = Total mass of container + normal + spiked plasma.
D = Concentration of analyte.

6.8.2. Preparation of quality control standards

Similarly, quality control standards were prepared (using the same methodology) spanning a range between 906 and 20.3 ng/ml (tables 82 and 83).

Table 82 Preparation of Stock Solution QA for Spiking QC F

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Water	1.000	3.466	9.510	9.510	250	364.46

Table 83 Preparation of Quality Control Standards

Quality Control Standard	Source Solution	A	B	C	D (ng/ml)
QC F	*Stock QA*	20.618	120.936	-	906
QC E	*QC F*	20.707	70.732	120.473	452
QC D	*QC E*	20.641	70.763	121.170	226
QC C	*QC D*	20.642	84.672	120.670	81.5
QC B	*QC C*	20.517	70.786	120.730	40.6
QC A	*QC B*	20.477	70.614	120.636	20.3

A = Mass of empty container.
B = Mass of container + normal plasma.
C = Total mass of container + normal + spiked plasma.
D = Concentration of analyte.

Sufficient calibration standards and quality controls were prepared to validate the method. These were stored at -20 °C until used for the validations.

6.8.3. Intra-batch accuracy and precision

Intra-batch accuracy and precision are assessed by assaying all the calibration standards in duplicate to produce one calibration curve and 6 replicates of all the prepared quality control standards in a single batch of assays. The intra-batch accuracy and precision of the assay procedure are assessed by calculating the regression equations and constructing the calibration curves based on both peak heights and peak areas.

Accuracy is expressed as recovery of the analyte as a percentage of the nominal concentration (% nom) while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % of nominal concentration at the LLOQ. For a valid method the intra-batch precision is required to be less than 15 % (i.e. CV% should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The method performed well during the intra-batch validation and passed all of the criteria set for both peak area and peak heights. A linear regression weighted 1/c was used for the statistical analyses. The calibration range was validated between 15.8 and 1013 ng/ml.

The results of the intra-batch validation are summarised in tables 84 to 87.

6.8.3.1. Quantitation by peak height

Table 84 Back-calculated concentrations of monic acid based on peak heights

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1013	L	-
STD H	1013	1067.039	5.3
STD G	507	482.081	-4.9
STD G	507	496.782	-2.0
STD F	253	236.801	-6.4
STD F	253	249.955	-1.2
STD E	127	132.344	4.2
STD E	127	122.285	-3.7
STD D	63.3	61.158	-3.4
STD D	63.3	60.307	-4.7
STD C	31.7	33.613	6.0
STD C	31.7	34.154	7.7
STD B	15.8	L	-
STD B	15.8	16.281	3.0

Calibration Standards used:	STD B – STD H
Calibration Range:	15.8 - 1013 ng/ml
Regression Equation:	Linear: y = mx + c (weighted 1/c)
m:	12.9
c:	9.6
r^2:	0.997162

L = lost sample

Table 85 Summary of quality control results based on peak height for intra-batch validation

Code	QC F 906 ng/ml	QCF (Dil) 906 ng/ml	QC E 452 ng/ml	QC D 226 ng/ml	QC C 81.5 ng/ml	QC B 40.6 ng/ml	QC A 20.3 ng/ml
1	943.239	514.579	493.719	240.669	81.276	42.898	21.001
2	881.338	501.425	489.076	240.669	85.919	43.749	B
3	966.451	B	499.909	261.561	88.240	39.184	22.858
4	912.288	479.760	499.909	236.801	79.729	39.958	17.983
5	943.239	495.235	503.004	253.050	83.597	44.291	21.078
6	950.976	485.176	470.506	233.706	89.014	47.308	23.090
MEAN	932.92	495.24	492.69	244.41	84.63	42.90	21.20
%nom	103.0	109.6	109.0	108.1	103.8	105.7	104.4
CV%	3.3	2.8	2.4	4.4	4.4	7.0	9.6

B = outlier

Note: QC F was diluted (1:1) with blank urine and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

6.8.3.2. Quantitation by peak area

Table 86 Back-calculated concentrations of monic acid based on peak areas

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1013	L	-
STD H	1013	1069.266	5.6
STD G	507	493.849	-2.6
STD G	507	499.301	-1.5
STD F	253	236.126	-6.7
STD F	253	243.560	-3.7
STD E	127	128.576	1.2
STD E	127	116.681	-8.1
STD D	63.3	57.702	-8.8
STD D	63.3	62.162	-1.8
STD C	31.7	33.466	5.6
STD C	31.7	34.308	8.2
STD B	15.8	L	-
STD B	15.8	17.804	12.7

L = lost sample

Calibration Standards used:	STD B – STD H
Calibration Range:	15.8 – 1013 ng/ml
Regression Equation:	Linear: y = mx + c (weighted 1/c)
m:	201.8
c:	-742.3
r^2:	0.996857

Table 87 Summary of quality control results based on peak area for intra-batch validation

Code	QC F 906 ng/ml	QCF (Dil) 906 ng/ml	QC E 452 ng/ml	QC D 226 ng/ml	QC C 81.5 ng/ml	QC B 40.6 ng/ml	QC A 20.3 ng/ml
1	960.229	496.327	504.962	242.569	86.448	39.265	22.017
2	930.491	509.213	466.304	242.569	81.492	44.617	B
3	1034.572	B	486.129	245.542	84.961	36.836	22.711
4	900.754	456.677	461.348	214.814	80.005	38.273	19.291
5	940.404	479.476	493.067	232.161	81.492	42.288	20.976
6	925.535	479.476	479.190	222.744	86.448	42.536	23.008
MEAN	948.66	484.23	481.83	233.40	83.47	40.64	21.60
%nom	104.7	107.1	106.6	103.3	102.4	100.1	106.4
CV%	4.9	4.1	3.4	5.3	3.4	7.3	7.0

B = outlier

Note: QC F was diluted (1:1) with blank urine and assayed in the validation batch in order to validate the dilution of unknown sample concentrations that do not otherwise fall within the undiluted validated range. The tabulated results are adjusted by a dilution factor of 2 in order to arrive at the correct nominal concentration.

6.8.4. Inter-batch accuracy and precision

Inter-batch accuracy and precision are assessed by assaying two separate consecutive batches, each consisting of one set of calibration standards designated for use in the assay of samples of unknown concentrations and 6 replicates of each of the quality control standards designated for use in the assay of samples of unknown concentrations. The intra-batch accuracy and precision of each of the batches is assessed separately by calculating the regression equation and constructing the calibration curve based on the best performing quantification method, and must pass the criteria for intra-batch acceptance. The inter-batch accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 in total). Accuracy is expressed as recovery of the analyte as a percentage of the nominal concentration (% nom) while the precision is expressed as the coefficient of variation (CV %). For a valid method the intra- and inter-batch accuracy is required to be within 15 % of the nominal concentration (i.e. % nom should be between 85 % - 115 %) over most of the range and within 20 % of nominal concentration at the LLOQ. For a valid method the intra- and inter-batch precision is required to be less than 15 % (ie. CV % should be less than 15 %) over most of the range and less than 20 % at the LLOQ.

The highest variation was 6.6 % (QC E from the second inter-batch validation). This information illustrates that the method performed well during both inter-batch validations.

The results are summarised in tables 88 to 91.

6.8.4.1. Inter-batch 1 accuracy and precision

Table 88 Back-calculated concentrations of monic acid

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1013	1061.193	4.8
STD G	507	481.201	-5.1
STD F	253	237.514	-6.1
STD E	127	119.267	-6.1
STD D	63.3	60.369	-4.6
STD C	31.7	33.392	5.3
STD C	31.7	32.088	1.2
STD B	15.8	L	-
STD B	15.8	17.476	10.6

L = lost sample

Calibration Standards used:	STD B – STD H
Calibration Range:	15.8 – 1013 ng/ml
Regression Equation:	Linear: y = mx + c (weighted 1/c)
m:	222.4
c:	27
r^2:	0.996688

Table 89 Summary of quality control results for inter-batch 1 validation

Code	QC F 906 ng/ml	QC E 452 ng/ml	QC D 226 ng/ml	QC B 40.6 ng/ml	QC A 20.3 ng/ml
1	840.886	458.720	237.963	48.319	20.848
2	930.807	472.208	215.033	44.902	23.321
3	854.374	437.589	211.437	42.924	24.445
4	854.374	429.046	224.925	41.620	23.636
5	831.894	423.201	205.142	44.273	23.861
6	849.878	439.387	216.832	44.722	22.287
MEAN	860.37	443.36	218.56	44.46	23.07
%nom	95.0	98.1	96.7	109.5	113.6
CV%	4.1	4.2	5.3	5.1	5.6

6.8.4.2. Inter-batch 2 accuracy and precision

Table 90 Back-calculated concentrations of monic acid

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Dev.
STD H	1013	1073.692	6.0
STD G	507	472.788	-6.7
STD F	253	234.669	-7.2
STD E	127	118.076	-7.0
STD D	63.3	59.779	-5.6
STD C	31.7	33.232	4.8
STD C	31.7	32.245	1.7
STD B	15.8	16.774	6.2
STD B	15.8	17.043	7.9

L = lost sample

Calibration Standards used:	STD B – STD H
Calibration Range:	15.8 – 1013 ng/ml
Regression Equation:	Linear: y = mx + c (weighted 1/c)
m:	223
c:	-430.7
r^2:	0.995093

Table 91 Summary of quality control results for inter-batch 2 validation

Code	QC F 906 ng/ml	QC E 452 ng/ml	QC D 226 ng/ml	QC B 40.6 ng/ml	QC A 20.3 ng/ml
1	934.677	490.726	238.257	39.869	20.138
2	939.161	468.304	224.804	40.586	21.707
3	889.833	436.017	214.938	37.627	18.882
4	818.084	429.290	220.768	37.313	21.393
5	853.958	442.295	222.113	38.748	20.003
6	885.349	408.662	214.490	41.394	19.510
MEAN	886.84	445.88	222.56	39.26	20.27
%nom	97.9	98.6	98.5	96.7	99.9
CV%	5.3	6.6	3.9	4.2	5.4

6.8.5. Summary of the combined quality control results for the 3 validations

The combined quality control results are summarised in table 92. The method performed well during all three validations with the highest coefficient variation of 7.9 %.

Table 92 Summary of the combined quality control results from the 3 validations

Validation Batch	Nominal	906	452	226	40.6	20.3
	Replicates	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
Intra-batch Validation	1	960.229	504.962	242.569	39.265	22.017
	2	930.491	466.304	242.569	44.617	B
	3	1034.572	486.129	245.542	36.836	22.711
	4	900.754	461.348	214.814	38.273	19.291
	5	940.404	493.067	232.161	42.288	20.976
	6	925.535	479.190	222.744	42.536	23.008
Inter-batch Validation 1	1	840.886	458.720	237.963	48.319	20.848
	2	930.807	472.208	215.033	44.902	23.321
	3	854.374	437.589	211.437	42.924	24.445
	4	854.374	429.046	224.925	41.620	23.636
	5	831.894	423.201	205.142	44.273	23.861
	6	849.878	439.387	216.832	44.722	22.287
Inter-batch Validation 2	1	934.677	490.726	238.257	39.869	20.138
	2	939.161	468.304	224.804	40.586	21.707
	3	889.833	436.017	214.938	37.627	18.882
	4	818.084	429.290	220.768	37.313	21.393
	5	853.958	442.295	222.113	38.748	20.003
	6	885.349	408.662	214.490	41.394	19.510
	MEAN	898.63	457.02	224.84	41.45	21.65
	%nom	99.2	101.1	99.5	102.1	106.6
	CV%	6.2	6.0	5.4	7.6	7.9

6.8.6. Stability assessment

6.8.6.1. On-Instrument stability

Eight stability samples of the same concentration were injected at intervals during the first validation batch to simulate the time of a batch run. The measured peak areas, injection times and cumulative time are summarised in table 93.

Table 93 Stability data of eight stability samples injected at different intervals

Replicates	Injection Time	Time Difference	Cumulative Time (hr)	Analyte Peak Area
1	11:44	-	0.00	23700
2	12:45	01:01:00	1.02	21900
3	13:59	01:14:00	2.25	24300
4	15:20	01:21:00	3.60	24000
5	18:49	03:29:00	7.08	23600
6	20:03	01:14:00	8.32	24300
7	21:25	01:22:00	9.68	22000
8	21:32	00:07:00	9.80	21100
			Mean	23112.50
			Std Dev	1250.64
			CV	5.41%

The chromatographic peak areas of monic acid are seen to be stable within a batch run.

By regression analysis of the peak areas against the cumulative time tabulated above it can be established that the peak area decreased by 4.7 % over a period of about 10 hours (figure 44). This trend is deemed insignificant and the extracts are considered to be stable on-instrument for at least 10 hours.

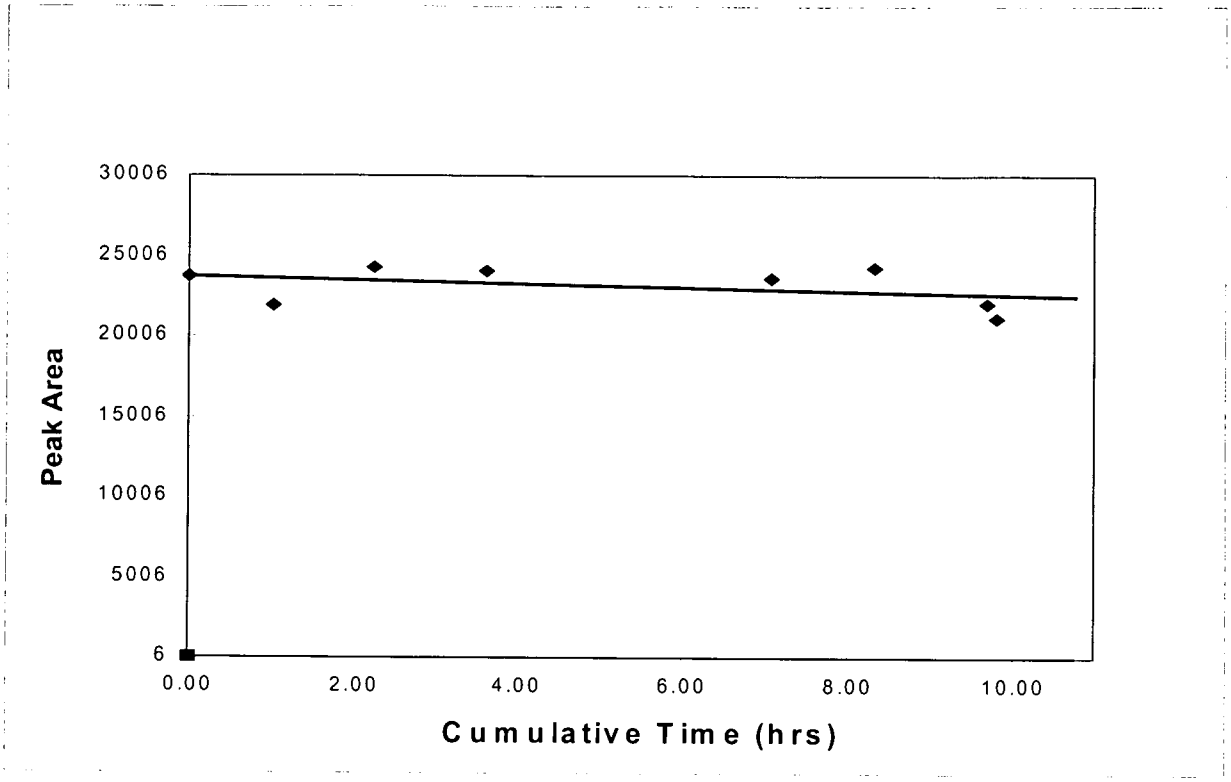


Figure 44 On-Instrument Stability

6.8.7. Specificity

The same methodology was performed as was for the direct injection method. No chromatographic peaks were observed in blank normal human urine injected from six different subjects.

Figure 45 is an example of a chromatogram of a blank plasma extract and figure 46 is an overlay of the LLOQ over a blank extract. No interference was observed. The first 4 min. of the run was diverted to waste, using a switching valve.

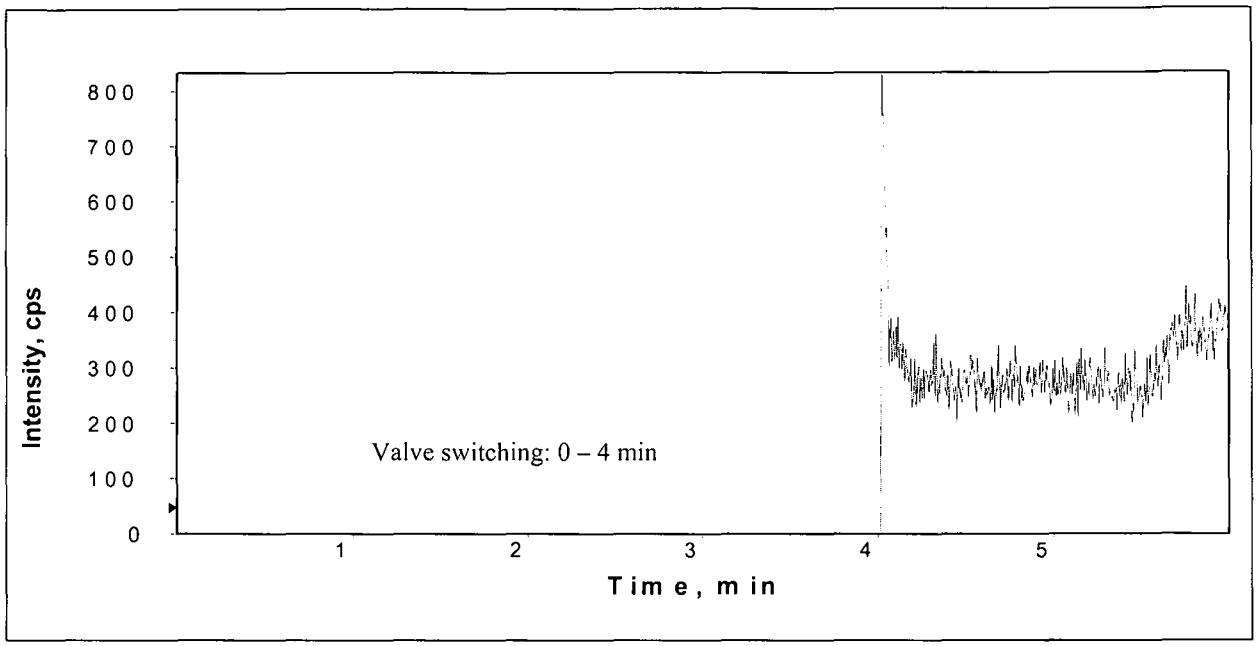


Figure 45 Chromatogram of a blank plasma extract

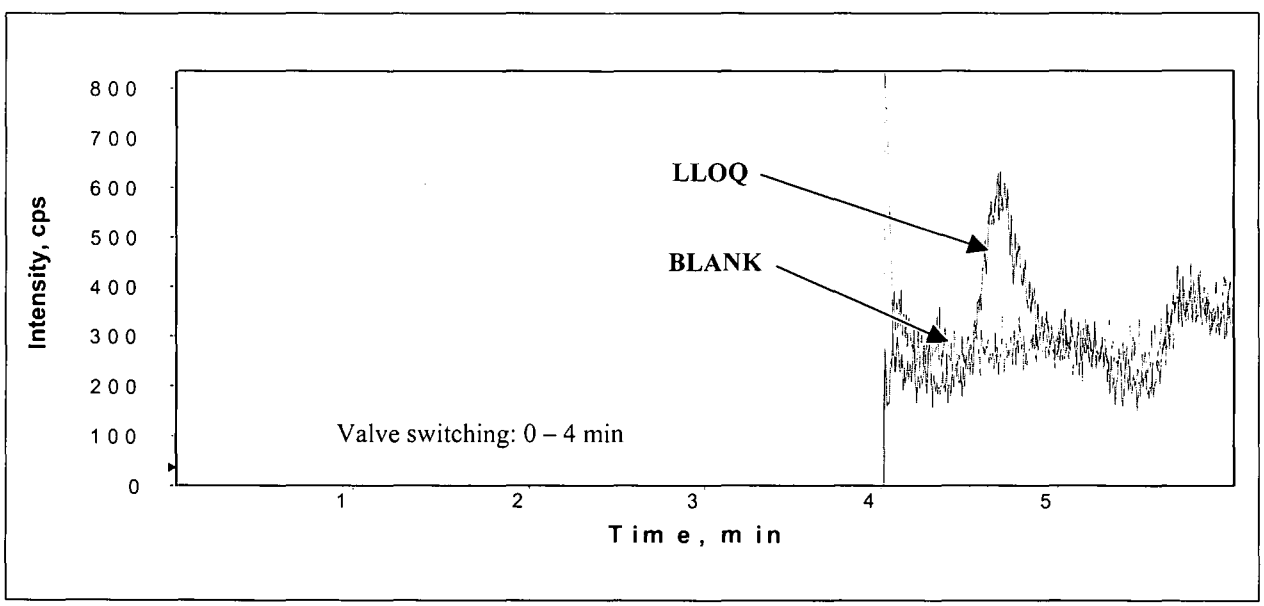


Figure 46 Overlay of the LLOQ over a blank extract

6.8.8. Sensitivity

The lower limit of quantification (LLOQ) of this method is 15.8 ng/ml (figure 47) which is considered to be sensitive enough to monitor the low absorption of mupirocin expected in a clinical study.

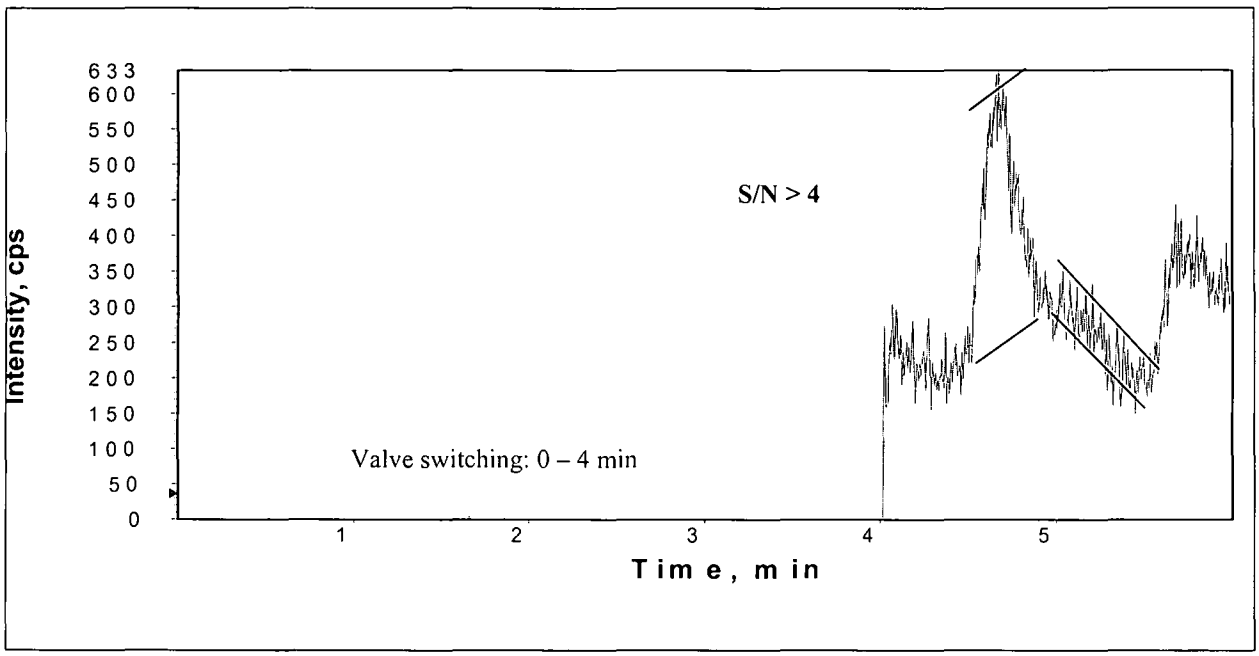


Figure 47 Chromatogram of the LLOQ with a signal to noise greater than 4

6.8.9. Recovery

Recovery is the measure of the analyte(s) losses incurred during sample processing, and is defined as: **Recovery (%) = (peak area of QC/peak area of SPVS) x 100**

Peak areas of 3 different quality control concentrations, and theoretical peak areas obtained from the system performance verification standard (SPVS) samples are used in calculating the recovery of the analyte(s) according to the above mentioned formula. Absolute recoveries of the analyte were determined in triplicate at high, medium and low concentrations of the analyte in urine and are summarised in table 94.

Table 94 Absolute recovery of analyte using response factor areas

ANALYTE ng/ml	AFTER EXTRACTION	THEORETICAL VALUES	ABSOLUTE RECOVERY (%)	CV (%)
906	191600	281464	68.07	4.73
226	46780	70210	66.63	4.88
40.6	7380	12613	58.51	7.71

The results indicate a recovery for monic acid of ~ 64 % with a coefficient of variation of ~ 6 % over the entire range of the analyte concentration.

6.9. Conclusion

Two selective, accurate and precise bioassay methods for the determination of monic acid in urine were developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The direct injection method covers a suitable concentration range (50 - 1001 ng/ml) for the assay of urine samples in which monic acid is expected to be excreted after multiple topical applications of 0.5 g of a 2 % mupirocin ointment. The SPE method is an improvement to the direct injection method due to the lower LLOQ. Both methods however, are excellent analytical options for quick screening and quantification of monic acid in human urine. Because of the short chromatography time and minimal sample preparation the method is very rapid and cost – effective.

7. SUMMARY

The development and validation of bio-analytical assay methods suitable for the quantification of stavudine in plasma, alfuzosin in plasma and monic acid in urine is discussed. A short summary of these methods are given:

- A sensitive method for the determination of stavudine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted from plasma with Waters, Sep-Pak®Vac, 100 mg, tC_{18}® solid phase extraction (SPE) columns. Chromatography was performed on a Supelco Discovery® C_{18}, 5 µm, 150 × 2 mm column with a mobile phase consisting of ammonium acetate (0.01 M) : acetonitrile : methanol (800:100:100, v/v/v) at a flow rate of 0.3 ml/min. Detection was achieved by an Applied Biosystems API 2000 mass spectrometer (LC-MS/MS) set at unit resolution in the multiple reaction monitoring mode (MRM). Atmospheric pressure chemical ionization (APCI) was used to obtain deprotonated ions (molecular ion m/z 223.1 to the product ion m/z 42.01). The mean recovery for stavudine was 94 % with a lower limit of quantification set at 4 ng/ml. This assay method makes use of the increased sensitivity and selectivity of mass spectrometric (MS/MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of stavudine in human plasma than has previously been published. The assay metod was used to quantitatively determine stavudine concentrations in plasma samples to follow the concentration vs. time profile for at least five half lives of the drug after a single 40 mg oral dose of stavudine was given to healthy adult male human subjects.

- A selective, sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of alfuzosin in plasma was developed. An Applied Biosystems API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using TurboIonSpray (TIS) with positive ionisation was used (molecular ion of alfuzosin m/z 390.2 to the product ion m/z 71.2; molecular ion of prazosin m/z 384.2 to the product ion m/z 95.0). Using prazosin as an internal standard, liquid-liquid extraction was followed by C_{18} reversed phase liquid chromatography and tandem mass spectrometry. The mean recovery for alfuzosin

was 82.9 % with a lower limit of quantification set at 0.298 ng/ml, the calibration range being between 0.298 and 38.1 ng/ml. This assay method makes use of the increased sensitivity and selectivity of tandem mass spectrometric (MS/MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of alfuzosin in human plasma than has previously been published. The assay method was used to quantify alfuzosin in human plasma samples generated in a multiple-dose (5 mg bd.) study at steady state.

- Two selective methods for the determination of monic acid (a metabolite of mupirocin) in urine were developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. An Applied Biosystems API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using TurboIonSpray (TIS) with positive ionisation, was used (molecular ion of monic acid m/z 345.2 to the product ion m/z 327.0) The minimal sample preparation and short chromatography time (retention time ~ 3.8 min.) makes these methods suitable for the assay of large numbers of samples per day. Linearity (weighted 1/concentration^2) was established from 50.1 to 1001 ng/ml for the direct injection method, and linearity (weighted 1/concentration) was established from 15.8 to 1013 ng/ml for the SPE method. The assay method was used for the determination of monic acid concentrations in human urine in order to detect and quantify the absorption of mupirocin after multiple topical applications of 0.5 g of a 2 % ointment.

Analytical data that were generated during these three research projects are discussed in this dissertation, improvements and novelties to existing methods are elucidated. The methods for the determination of stavudine and alfuzosin have been published. Both full-length publications are included in this dissertation, together with correspondense between myself and the journal editors and referees. The assay methods for monic acid will soon be submitted for publication in the Journal of Chromatography B.

Keywords:
method development, validation, high-performance liquid chromatography, mass spectrometry, stavudine, alfuzosin and monic acid

Die ontwikkeling en validering van bioanalitiese metodes vir die kwantifisering van geneesmiddels (stavudien in plasma, alfuzosin in plasma en "monic acid" in urien) word bespreek. 'n Kort opsomming van hierdie metodes word bespreek:

- 'n Sensitiewe metode vir die bepaling van stavudien in plasma is ontwikkel deur gebruik te maak van hoë-verrigting vloeistofchromatografiese skeiding en massa-spektrometriese bepaling. Die analiet is uit plasma geëkstraheer deur gebruik te maak van Waters, Sep-Pak® Vac, 100 mg, tC_{18}® soliede fase ekstraksiekolomme. Chromatografie is uitgevoer op 'n Supelco Discovery® C_{18}, 5 µm, 150 × 2 mm kolom met 'n mobiele fase wat bestaan uit ammonium asetaat (0.01 M) : asetonitriel : metanol (800:100:100, v/v/v) teen 'n vloei-tempo van 0.3 ml/min. Die bepaling van stavudien is gedoen met 'n Applied Biosystems API 2000 massaspektrometer (LC-MS/MS) wat op eenheid resolusie in die meervoudige reaksiebepalings modus (MRM) gestel is (molekulêre ioon m/z 223.1 na die produkioon m/z 42.01). Atmosferiese druk-chemiese ionisering (APCI) is as ioniseringsproses gebruik om gedeprotoneerde ione te produseer. Die gemiddelde opbrengs vir stavudien was 94 % met 'n kwantifiseringslimiet van 4 ng/ml. In hierdie metode word 'n massa-spektrometriese bepaling met verhoogde sensitiwiteit en selektiwiteit beskryf. Daar word dus voorsiening gemaak vir 'n vinniger en meer selektiewe metode as wat voorheen gepubliseer is vir die bepaling van stavudien in menslike plasma. Die metode is aangewend vir die bepaling van stavudien konsentrasies in plasma monsters om die konsentrasie-tyd profiel van die middel vir ten minste vyf halfleeftye te bepaal, nadat 'n 40 mg stavudien dosering mondelings aan gesonde, volwasse, manlike proefpersone toegedien is.

- 'n Selektiewe, sensitiewe en vinnige vloeistofchromatografiese-tandem-massa-spektrometriese metode is ontwikkel vir die bepaling van alfuzosin in plasma. 'n Applied Biosystems API 2000 massaspektrometer met 'n turbo-ioonsproeibron is gebruik. Die spektrometer is in die positiewe ioniseringsmodus gestel en meervoudige reaksiebepalings is gedoen (molekulêre ioon van alfuzosin m/z 390.2 na die produkioon m/z 71.2; molekulêre ioon van prazosin m/z 384.2 na die produkioon m/z 95.0). 'n Vloeistof-vloeistof ekstraksieprosedure is uitgevoer en prazosin is as interne standaard gebruik. Chromatografiese skeiding is op 'n C_{18} omgekeerde fase kolom uitgevoer. Die gemiddelde opbrengs vir alfuzosin was 82.9 % met 'n kwantifiseringslimiet van 0.298 ng/ml. Die kalibrasie reikwydte was tussen 0.298 en 38.1 ng/ml. In hierdie metode word 'n tandem massa-spektrometriese bepalingsmetode met verhoogde sensitiwiteit en selektiwiteit

beskryf. Daar word dus voorsieninig gemaak vir 'n metode wat vinniger en meer selektief is as metodes wat voorheen gepubliseer is vir die bepaling van alfuzosin in menslike plasma. Hierdie metode is aangewend om alfuzosin in menslike plasma te kwantifiseer nadat veelvoudige doserings (5 mg) aan die proefpersone toegedien is.

- Twee selektiewe metodes vir die bepaling van "monic acid" (metaboliet van mupirocin) in urien is ontwikkel deur gebruik te maak van hoë-verrigting vloeistofchromatografiese skeiding en massa-spektrometriese bepaling. 'n Applied Biosystems API 2000 massaspektrometer met 'n turbo-ioonsproei bron is gebruik. Die instrument is in die positiewe ioniseringsmodus gebruik en bepalings is in die meervoudige reaksiebepalingsmodus gedoen (molekulêre ioon van "monic acid" m/z 345.2 na die produkioon m/z 327.0). Die minimale monstervoorbereiding en kort chromatografietyd (retensie-tyd ~ 3.8 min.) maak hierdie metode uiters geskik vir die bepaling van 'n groot aantal monsters per dag. Die direkte-inspuitingsmetode het 'n lineêre kromme ($1/c^2$) tussen 50.1 en 1001 ng/ml gelewer en die soliede-fase-metode het 'n lineêre kromme (1/c) tussen 15.8 en 1013 ng/ml gelewer.

Analitiese data wat in die drie navorsingsprojekte gegenereer is word bespreek en verbeteringe sowel as nuwighede word uitgelig. Die metodes vir die bepaling van stavudien en alfuzosin is reeds as vollengte artikels gepubliseer. Beide die twee gepubliseerde artikels tesame met die korrespondensie wat plaasgevind het tussen myself en die redakteurs en beoordelaars is ingesluit in die verhandeling. Die metodes vir die bepaling van "monic acid" sal binnekort vir publikasie aan die "Journal of Chromatography B" voorgelê word.

APPENDIX 1

The assay methods of Stavudine and Alfuzosin were submitted and accepted as full-length publications in the Journal of Chromatography B. The assay methods for monic acid will soon be submitted for publication in the Journal of Chromatography B. Copies of the two published articles and the correspondence with the journal editors and referees have been included in this section of the dissertation.

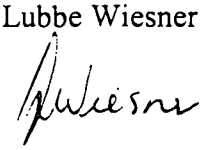
Editorial Office
P.O. Box 681
NL-1000 AR Amsterdam
The Netherlands

25 October 2001

Dear Editor,

I would like to submit the following article to the **Journal of Chromatography**: *A sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of stavudine in human plasma.*

Yours sincerely,
Lubbe Wiesner



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BIOMEDICAL APPLICATIONS

Reference's report Manuscript No.: I1814

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 : J.L. Wiesner .....

Title : A sensitive and rapid LC-MS/MS ....

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

2. Is the paper acceptable
   - ☐ in 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 reference to related work? (YES) NO

5. Is the English satisfactory? (YES) NO

Comments:

This manuscript describes the development of a LC-MS/MS method suitable for the determination of stavudine in human plasma. While there is nothing novel in the method itself but a straightforward application of well known MS methodology to a particular drug, the manuscript is clearly written and the method is properly validated. Furthermore, it may be a useful contribution to the study of the bioavailability of this drug and thus can be recommended for publication after consideration of the following comments.

Authors should consider the mentioning the reference from Moore et al. in J.Am. Soc. Mass Spectrom. 11, 1134 (2000) as it refers to the determination of metabolites from this stavudine by LC-MS/MS.

Also, I would suggest adding in the structure depicted in Figure 1 an indication of the loss producing the ion at m/z 42

Title: Please delete "A"

Experimental: please add "USA" to the American addresses

Abbreviation that should be used: s for seconds, min for minutes

Reference list: ① Please give all authors (not et al.)
② journals should be abbreviated (check recent issue)

Editorial Office
P.O. Box 681
NL-1000 AR Amsterdam
The Netherlands

21 February 2002

Dear Editor

I would like to submit the revised manuscript (Sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of stavudine in human plasma) for publication. I have made the changes that Professor I.W. Wainer recommended.

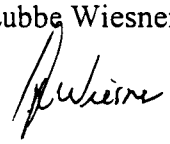
Recommendations by Professor I.W. Wainer:

1.) Mentioning of Moore et al.
2.) Structure for product ion m/z 42.
3.) Delete "A" in title.
4.) Add USA to the American addresses.
5.) Abbreviation: s for seconds and min for minutes.
6.) References: give all authors and journals should be abbreviated.

Author replying to points that were raised by Professor I.W. Wainer:

1.) Moore et al. is mentioned – see reference [8].
2.) A plausible fragmentation mechanism resulting in the strong low mass negative product ion of m/z 42 monitored in this assay method is presently under investigation (extract of page 8).
3.) Did the deletion of the "A" in the title.
4.) Did add USA to all the American addresses.
5.) Did the abbreviations that were suggested.
6.) Did update the reference list.

Yours sincerely
Lubbe Wiesner



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Sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of stavudine in human plasma

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Abstract

A sensitive method for the determination of stavudine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted from plasma with Waters, Sep-Pak®Vac, 100 mg, $tC_{18}^{®}$ solid-phase extraction (SPE) columns. Chromatography was performed on a Supelco Discovery® C_{18}, 5 μm, 150×2 mm column with a mobile phase consisting of ammonium acetate (0.01 *M*)–acetonitrile–methanol (800:100:100, v/v/v) at a flow-rate of 0.3 ml/min. Detection was achieved by an Applied Biosystems API 2000 mass spectrometer (LC–MS–MS) set at unit resolution in the multiple reaction monitoring mode (MRM). Atmospheric pressure chemical ionization (APCI) was used for ion production. The mean recovery for stavudine was 94% with a lower limit of quantification set at 4 ng/ml. This assay method makes use of the increased sensitivity and selectivity of mass spectrometric (MS–MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of stavudine in human plasma than has previously been described.

Keywords: Stavudine

1. Introduction

Stavudine (2′,3′-didehydro-3′ deoxythymidine, d4T, Zerit®) is a thymidine analogue with in vitro and in vivo activity against the human immunodeficiency virus (HIV). It is a reverse transcriptase inhibitor whose mode of action is similar to that of other nucleoside analogues and is active at concentrations that are generally 100-fold below the levels which are cytotoxic. Following phosphorylation by cellular kinases, d4t-triphosphate is produced, which preferentially inhibits HIV-1 reverse transcriptase activity [1–4]. Moore et al. [8] describe a sensitive LC–MS–MS method for the simultaneous measurement of the intracellular nucleoside 5′-triphosphate anabolites of zidovudine, lamivudine and stavudine in peripheral blood mononuclear cells which are the sites of HIV replication and drug action. An oral dose of stavudine (40 mg) leads to a maximum plasma stavudine concentration of around

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PII: S1570-0232(02)00136-8

876 ng/ml after 90 min [6]. In this study we determined the pharmacokinetics of stavudine up to 24 h after a single oral dose of 40 mg stavudine.

Several methods have been described for the determination of stavudine in plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with ultraviolet detection achieving lower limits of quantification (LLOQ) of around 10–25 ng/ml [6,7]. Kaul et al. [5] achieved the best sensitivity with an LLOQ of 2.5 ng/ml for stavudine using a radioimmunoassay (RIA) method. We initially developed an assay method using HPLC with UV detection but due to a very aqueous mobile phase and a common UV detection wavelength, too much interference from endogenous components was observed. To overcome this problem, very long chromatography times were required to prevent late eluting peaks from interfering with the analyte of interest. This made the method impractical for the determination of large numbers of samples. It was therefore decided to develop a new method involving the use of a mass-selective detector with mass spectrum–mass spectrum (MS–MS) capabilities in tandem with liquid chromatography (LC) to increase the selectivity which would allow for more rapid chromatography and sample clean-up. This report describes an LC–MS–MS method for the determination of stavudine in plasma using a simple solid-phase extraction procedure. With a total turnaround of 4 min between sample injections, the analyst is able to assay a large number of samples per day. The LLOQ of 4 ng/ml is also sensitive enough to do pharmacokinetic studies after a 40-mg oral dose of stavudine.

2. Experimental

2.1. Materials and chemicals

A Supelco Discovery® C_{18} 5 μ, 2.1×150 mm column (Supelco, Bellefonte, PA, USA) was used for separation at a flow-rate of 0.3 ml/min and injecting 20 μl onto the column. The mobile phase was delivered by an Agilent Series 1100 pump (Agilent, Palo Alto, CA, USA) and the samples injected by an Agilent Series 1100 autosampler. Detection was performed by an Applied Biosystems API-2000 detector (Applied Biosystems, Ontario, Canada) using atmospheric pressure chemical ionisation (APCI) for ion production.

Methanol and acetonitrile (Burdick and Jackson, High Purity) were obtained from Baxter chemicals, USA; sodium hydroxide and ammonium acetate were obtained from Fluka chemicals (Buchs, Switzerland). Orthophosphoric acid (85%) was obtained from Merck (Darmstadt, Germany). All chemicals were used as received. Water was purified by Millipore Elix 5 reverse osmosis and Milli-Q® (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA). Phosphate buffer (0.05 *M*) was prepared from 0.05 *M* phosphoric acid and adjusted to pH 7 with sodium hydroxide (5 *M*).

Stavudine ($C_{10}H_{12}N_2O_4$) was supplied by Cipla Ltd., Mumbai Central, India.

2.2. Extraction procedure

A stavudine standard solution was made up in water and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal human plasma which was then serially diluted with normal blank plasma to attain the desired concentrations (4–2029 ng/ml for the calibration standards and 5–1625 ng/ml for the quality control standards). The calibration standards and quality control standards were aliquoted into Cellstar® Cryo.s tubes and stored under the same conditions as the trial samples; at −20 °C. Stavudine has been shown to be stable at −20 °C for at least 1 year [5].

The plasma samples were thawed in a waterbath at ~37 °C, mixed for 5 s on a vortex mixer and centrifuged for 5 min at 3000 *g*. A solid-phase extraction (SPE) procedure was then performed manually, using Waters, Sep-Pak®Vac, 100 mg, $tC_{18}^{®}$ SPE columns and a SPEEDISK® 48 plate positive pressure system. The SPE columns were conditioned with methanol (1 ml), water (1 ml) and a phosphate buffer (1 ml, 0.05 *M*, pH 7). The samples were then loaded (0.5 ml plasma diluted with 0.5 ml phosphate buffer) onto the SPE columns. The columns were rinsed with phosphate buffer (1 ml, 0.05 *M*, pH 7) and water (1 ml). The analyte was eluted with methanol (0.5 ml) and the eluate evaporated to dryness using a Savant SpeedVac® rotary concentrator (~60 °C) for ~90 min. The residual extracts

were redissolved in water (0.25 ml) by mixing on a vortex mixer for 30 s. The samples were transferred into autosampler vials and 20 μl were injected onto the HPLC column.

2.3. Liquid chromatography

All chromatographic solvents were degassed with helium before use. Chromatography was carried out at ambient temperature with a mobile phase consisting of ammonium acetate (0.01 *M*)–acetonitrile–methanol (800:100:100, v/v/v) at a flow-rate of 0.3 ml/min.

2.4. Mass spectrometry

Atmospheric pressure chemical ionization was performed in the negative ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 20 and 40 (respective arbitrary values). The heated nebulizer temperature was set at 450 °C and the nebulizer current on −3.0 μA. The instrument response was optimised for stavudine by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. The pause time was set at 5 ms and the dwell time at 150 ms.

Atmospheric pressure chemical ionization (APCI) was used for ion production and the collision gas (N_2) set at 3 (arbitrary value).

The Applied Biosystems API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the deprotonated molecular ion *m/z* 223.1 to the product ion *m/z* 42.01. Fig. 1 shows the single parent (*m/z* 223.1) to product ion MS–MS of stavudine. The molecular structure for the analyte is also indicated in this figure.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.0 software.

2.5. Validation

The method was validated by analysing plasma quality control samples six times at nine different

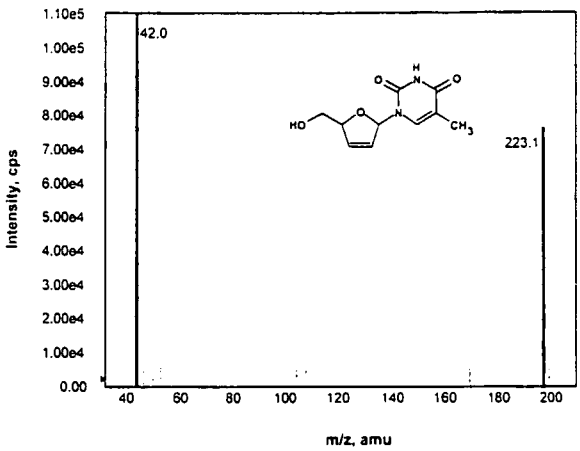

Fig. 1. Full mass spectrum of the deprotonated stavudine molecular ion (*m/z* 223.1, molecular structure given) and the principal product ion formed at *m/z* 42.01 after collision (MS–MS).

stavudine concentrations, i.e. 1625, 813, 406, 203, 102, 50.8, 19.3, 9.65 and 4.83 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing 10 different concentrations spanning the concentration range (2029–3.97 ng/ml). Calibration graphs were constructed using a weighted linear regression (1/concentration^2) of the drug peak-area of the product ions versus nominal drug concentrations. Several regression types were tested and the weighted linear regression (1/concentration^2) was found to be the simplest regression, giving the best results.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated by extracting "blank" normal human plasma from 10 different sources, reconstituting the final extract in injecting solvent containing a known amount of the analyte, analysing the reconstituted extracts and then comparing the peak areas of the analytes.

Absolute recoveries of the analyte were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with stavudine. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted system performance verification standard mixtures (prepared in the injection vehicle) representing 100% recovery.

Table 1
Intra-day quality control results of stavudine

Nominal concentration (ng/ml)	Stavudine ($n=9$) Mean concentration found (ng/ml)	RSD (%)	% Nominal
1625	1701	4.8	104.6
813	827	2.8	101.7
406	410	2.0	101.1
203	199	6.3	97.9
102	105	8.2	102.5
50.8	51.9	5.7	102.2
19.3	19.6	4.0	101.7
9.65	10.0	3.9	103.6
4.83	4.92	2.5	101.8

3. Results and discussion

The mean absolute recoveries of stavudine determined in triplicate at 1625, 406 and 102 ng/ml were 96% (RSD 5.1%), 93% (RSD 2.2%) and 94% (RSD 9.0%), respectively. No matrix effect for stavudine was observed for 10 different plasma pools tested. The peak areas of the 10 reconstituted samples had a coefficient of variation of 3.5% indicating that the extracts were "clean" with no co-eluting compounds influencing the ionisation of the analyte.

Initially a HPLC method using UV detection was developed. Due to the highly polar nature of stavudine a very aqueous mobile phase had to be used leading to many late eluting peaks. Different columns were tested with a phenyl-hexyl column giving the best results (retention, peak shape). Under these conditions the chromatography time had to be in excess of 25 min which made the method very time-consuming and not very productive. The much higher selectivity of MS–MS detection allowed the development of a very specific and rapid method for the determination of stavudine in plasma.

The LLOQ, defined as that concentration of stavudine which can still be determined with acceptable precision (C.V.%<20) and accuracy (bias <20%) was found to be 4 ng/ml. Results of the intra-day and inter-day validation assays presented in Tables 1–3 indicate a valid calibration range of 2029–4 ng/ml for stavudine.

On-instrument stability was inferred from stability samples which were prepared and included in the validation batch. No significant degradation could be detected in the samples (ambient temperature) left on the autosampler for at least 49 h.

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Several extraction procedures were tested which included protein precipitation, solid-phase and liquid–liquid extraction methods. A solid-phase ex-

Table 2
Inter-day 1 quality control results of stavudine

Nominal (ng/ml)	4.83	9.65	19.3	406	813	1625
Mean	4.43	9.07	19.2	402	822	1683
RSD	3.6	5.2	6.3	5.7	4.1	6.0
% Nominal	91.7	94.0	99.2	99.1	101.1	103.5
n	6	6	6	6	6	6

Table 3
Inter-day 2 quality control results of stavudine

Nominal (ng/ml)	4.83	9.65	19.3	406	813	1625
Mean	4.96	9.35	18.5	390	764	1646
RSD	4.9	7.7	6.5	3.8	7.0	5.0
% Nominal	102.8	96.8	95.8	96.1	94.0	101.3
n	6	6	6	6	6	6

traction procedure proved to be the most successful. The extracts were clean with high recovery rates.

Different concentrations of ammonium acetate were tested for optimum ionisation of the analyte and it was found that 10 m*M* ammonium acetate gave the best result. Stavudine also gave a much higher (10-fold) response with APCI than with electrospray ionisation (ESI).

A plausible fragmentation mechanism resulting in the strong low mass negative product ion of m/z 42 monitored in this assay method is presently under investigation.

Due to the unavailability of a deuterated analogue of stavudine or other suitable internal standards, it was decided to work without an internal standard. Two sets of calibration and quality control standards were used (one set in the first half and one set in the second half) in each batch of 215 samples arranged to compensate for any temporal change in the ionisation response. The ionisation response, monitored by injecting a system performance verification standard at the beginning and at the end of each batch indicated that the system response remained stable with the response not varying more than 5% within each batch.

The retention time for stavudine was 1.97 min. A total chromatography run time of 4 min made it possible to analyse large batches of samples (215 samples) per day. Fig. 2 shows a representative chromatogram obtained of stavudine at a concentration of 4 ng/ml (the LLOQ) and of a study sample close to the limit of quantification at the late elimination phase of the pharmacokinetic profile for the analyte.

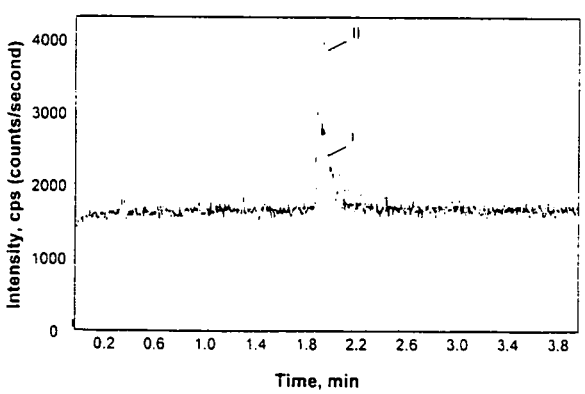

Fig. 2. High performance liquid chromatograms of the calibration standard at the limit of quantification (I) containing 4 ng/ml stavudine and of a study sample (II) close to the limit of quantification at the late elimination phase of the pharmacokinetic profile of the analyte.

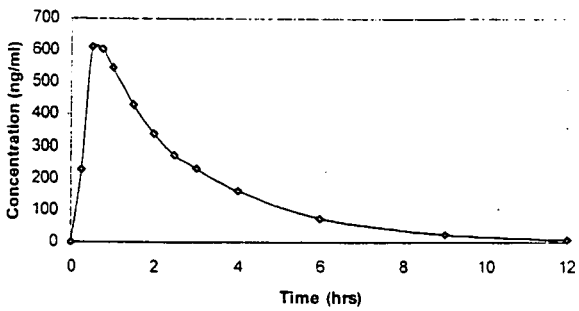

Fig. 3. Representative stavudine plasma concentrations vs. time profiles as obtained after a single 40 mg oral dose of stavudine (24 subjects).

The method was employed to analyse plasma samples containing stavudine obtained after a single oral dose of 40 mg stavudine per treatment phase in 24 healthy volunteers. Concentration vs. time profiles were constructed for up to 24 h for the analyte (Fig. 3).

The maximum stavudine plasma concentrations obtained varied between 399 and 1435 ng/ml. Stavudine was very rapidly absorbed leading to maximum plasma concentrations being reached within 1 h. The elimination half-life of stavudine was 2.25 h.

4. Conclusion

A rapid, sensitive and highly selective method for the determination of stavudine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. With an LLOQ of 4 ng/ml, pharmacokinetic profiles of the drug could be constructed for up to 24 h after a single oral dose of 40 mg stavudine. The method is more selective than previously described methods and allows for a much higher sample throughput due to the short chromatography time (4 min) and relatively simple sample preparation.

References

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Editorial Office
P.O. Box 681
NL-1000 AR Amsterdam
The Netherlands

8 July 2002

Dear Editor,

I would like to submit the following article to the **Journal of Chromatography B**:
Selective, sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of alfuzosin in human plasma.

Yours sincerely,
Lubbe Wiesner

Reprints should be sent to:
JL Wiesner
FARMOVS-PAREXEL® Clinical Research Organisation
Private Bag X09
Brandhof
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Fax: +27 (051) 444-3841
E-mail: Lubbe.Wiesner@farmovs-parexel.com

Journal of Chromatography B

Biomedical Sciences and Applications

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

Dr. J.L. Wiesner
FARMOVS-PAREXEL
Clinical Research Organisation
Private Bag X09, Brandhof
Bloemfontein
9324 South Africa

Re: DL 387

Amsterdam, October 28, 2002

Dear Dr. Wiesner,

On behalf of the editor handling your manuscript, Dr. D.K. Lloyd, I am writing to you in reference to your manuscript entitled: *Selective, sensitive and rapid liquid chromatographic-tandem mass spectrometric method for the determination of alfuzosin in human plasma*, by Wiesner, J.L., Sutherland, F.C.W., Van Essen, G.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, 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.com.

Yours sincerely,
EDITORIAL OFFICE

Trees Roof-Kramer

Journal of Chromatography B
Biomedical Sciences and Applications

Memorandum

Editor: Dr. D.K. Lloyd

Manuscript No.: DL
387

First author:
J. Wiesner et al

Additional Editor's comments:

In addition to the reviewer's comments:

1. Refs. 4 and 5 discuss chiral methods. What is the justification for developing an achiral method? Have the pk, biological activity, etc, of the enantiomers of alfuzosin been proven to be equivalent?
2. final page numbers are not needed for the references.
3. Please add error bars to the data in Fig. 5.
4. The referee asks for more information on the calibration lines. Please provide this in the form of calibration equations (including error estimates), not in the form of figures.

JOURNAL OF CHROMATOGRAPHY B
Analytical Technologies in the Biomedical and Life Sciences

Manuscript No. DL-387

Recommendations and comments should be sent within two weeks to the Editor.

Author: J. Wiesner, F. Sutherland, G. vanEeden, H. K. I. Hundt, K. Swart and A. F. Hundt
Title: Selective, sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of alfuzosin in human plasma

1. Is this subject matter suitable for publication in the Journal of Chromatography B — YES NO

2. In the paper acceptable:
   - as in its present form
   - ~~publication as soon~~
   - 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: See attached sheet.

his manuscript describes a method for the determination of alfuzosin in human plasma. The methodology employed (multiple ages of liquid/liquid extraction followed by isocratic LC/MS/MS) is widely used for bioanalytical analysis. The manuscript generally well organized and clearly written (with a couple of exceptions noted below).

he authors have chosen not to include the calibration curve and its associated figures of merit (slope, intercept, R^2) and though the results from the QC samples indicate that the method produces highly accurate and precise results, the calibration urve should never the less be included in the manuscript. Also the calibration graphs were created using a Wagner regression. ince this is an atypical type of regression for this type of data the authors should reference its use and provide an explanation to why a simple linear regression (weighted or unweighted) was not suitable.

the manuscript there are several mentions of the method robustness and its suitability for the analysis of large batches of inical samples. The author may wish to quantify the degree of robustness by adding a brief discussion of the number of jections that are typically possible before it is necessary to perform some sort of procedure to restore system performance (for xample flushing or changing the HPLC column or cleaning the ion source of the mass spectrometer) .

inor corrections and suggestions to improve the clarity of the manuscript

ge 2 Paragraph 1 line 1
ake the "s" in the word selective lowercase

ge 2 Paragraph 1 line 8
dd the word "tandem" before the word "mass"

ge 3 Paragraph 5
ssibly replace the with the following:
is new method makes use of a tandem mass spectrometer in conjunction with liquid chromatography...

ge 5 Paragraph 1 line 8-10
first reading, I did not understand the procedure that was used. After subsequent readings it became clear that the emaining organic phase" referred to the residual organic solvent present in the vial with the aqueous fraction. The sentence ould be rewritten to improve its clarity.

ge 6 Paragraph 2 line 5-9
e wording should be changed to conform to the standard terminology used to describe product ion mass spectra. The llowing would be a more standard description: Presented in figure 1 is the product ion mass spectrum of protonated fuzosin showing the M+1 ion (m/z 390.2, molecular structure given) and the principal product ion at m/z 71.2 formed by llision-induced dissociation (CID). A similar rewording should be made to the figure legends.

ge 6 Paragraph 4 line 5-7
e meaning of the sentence beginning "Calibration graphs were...." is not clear. What is being described is the production calibration graphs using the ratio of the analyte peak area to IS peak area versus the nominal analyte concentration. There is need to mention product ions in the description of calibration graphs.

g 1 and 2:
mu is no longer in use and should be removed from the legend. The structural assignments of the m/z 94 and 71 product ions ould be shown.

Dr. D.K. Lloyd
Journal of Chromatography B
Editorial Office
P.O. Box 681
NL-1000 AR Amsterdam
The Netherlands

Re: DL 387

Bloemfontein, November 14, 2002

Dear Dr. Lloyd,

Thank you for the handling of my manuscript entitled: *Selective, sensitive and rapid liquid chromatography-tandem mass spectrometry method for the determination of alfuzosin in human plasma.* I found the recommendations to be very helpful. Please find enclosed four copies of the revised manuscript (the changes have been underlined in red on one of the copies) and a 3.5-inch disk containing the manuscript entitled "alfuzosin" (MS Word '97).

In the next section of this letter I will reply to each point raised by the editor and the referee:

Editor:

1. Alfuzosin is dosed as a racemate of which the R- and S- isomers are known to have similar pharmacological activity. Since the analytical method was required to assay plasma samples collected during a comparative bioequivalence study in which both products contained the racemate, the use of an enantiospecific bioanalytical method was not required.

2. The final page numbers were deleted for the references.

3. Error bars were added to the data in figure 5.

4. See comment: Referee 1

Referee:

1. Non-linearity in calibration lines covering a wide range of concentrations is quite common, especially in the very low concentration range. The Wagner regression which is a log transformed quadratic regression of the form $\ln Y = a(\ln X)^2 + b(\ln X) + C$ is often found to result in a better overall fit to the calibration data than a weighted linear regression. We found this to be the case with the alfuzosin calibration curves.

2. The degree of robustness is now discussed as requested in the conclusion section of the revised document.

3. Changed the "s" to lower case (page 2 paragraph 1 line 1).

4. Added "tandem" before "mass" (page 2 paragraph 1 line 8).

5. Changed page 3 paragraph 5 as suggested.

6. Changed page 5 paragraph 1 lines 8 to10 as follow: The residual organic phase that remained in the aqueous phase was evaporated under a gentle stream of nitrogen at 45 °C for 2 min. The final extract was transferred to an autosampler vial containing a micro glass insert, and 10 μl injected onto the HPLC column.

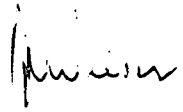
7. Changed page 6 paragraph 2 lines 5 – 9 and the figure legends as was suggested.

8. The sentence on page 6 paragraph 4 lines 5 – 7 was changed to make it more clear: Calibration graphs were constructed using a Wagner regression of the drug peak-area ratios of the analyte to the internal standard versus nominal analyte concentrations.

9. Amu is removed from the figure legends and the suggested fragmentation patterns are also indicated the figures.

I hope that these revisions will meet your collective approval.

Yours Sincerely,



JL Wiesner

Re: DL387

Dear Dr. Wiesner,

On behalf of the editors, I acknowledge with thanks the safe receipt of the revised manuscript entitled:

"Selective, sensitive and rapid liquid chromatographic--tandem mass spectrometric method for the determination of alfuzosin in human plasma"
by Wiesner, J.L., Sutherland, F.C.W., Van Essen, G.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,
Trees Roof-Kramer
=

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=

Re: DL387

Dear Dr. Wiesner,

On behalf of the editor handling your revised manuscript, Dr. D.K. Lloyd, I am pleased to inform you about the acceptance of the manuscript entitled:

"Selective, sensitive and rapid liquid chromatographic--tandem mass spectrometric method for the determination of alfuzosin in human plasma" by Wiesner, J.L., Sutherland, F.C.W., Van Essen, G.H., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

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Selective, sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of alfuzosin in human plasma

J.L. Wiesner*, F.C.W. Sutherland, G.H. van Essen, H.K.L. Hundt, K.J. Swart, A.F. Hundt

FARMOVS-PAREXEL Clinical Research Organisation, Private Bag X09, Brandhof 9324, South Africa

Received 25 July 2002; received in revised form 30 December 2002; accepted 8 January 2003

Abstract

A selective, sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of alfuzosin in plasma was developed. A PE Sciex API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using TurboIonSpray with positive ionisation was used. Using prazosin as an internal standard, liquid–liquid extraction was followed by C_{18} reversed-phase liquid chromatography and tandem mass spectrometry. The mean recovery for alfuzosin was 82.9% with a lower limit of quantification set at 0.298 ng/ml, the calibration range being between 0.298 and 38.1 ng/ml. This assay method makes use of the increased sensitivity and selectivity of tandem mass spectrometric (MS–MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of alfuzosin in human plasma than has previously been described. The assay method was used to quantify alfuzosin in human plasma samples generated in a multiple-dose (5 mg bd.) study at steady state.

Keywords: Alfuzosin

1. Introduction

Alfuzosin, *N*-{3-[(4-amino-6,7-dimethoxy-2-quinazolinyl) methylamino] propyl} tetrahydro - 2 - furancarboxamide hydrochloride, is an antagonist of α_1 post-synaptic adrenergic receptors, showing some myorelaxant effects [1,2]. Alfuzosin is a basic compound with a pK_a value of 8.13 and is stable under normal conditions of temperature and light [3].

Several methods have been described for the determination of alfuzosin in plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with fluorimetric and luminescence detection, achieving lower limits of quantification (LLOQ) of around 1 ng/ml [3–7]. Carlucci et al. described a high-performance liquid chromatography method using a column-switching procedure without extraction to isolate the drug from the biological matrix. Their method was linear from 2 to 150 ng/ml [7]. Krstulovic et al. and Rouchouse et al. developed assay methods for the determination of the enantiomers of alfuzosin [4,5]. Guinebault et al. used a liquid–liquid extraction and large volume injection technique for the quantitation of alfuzosin in biological fluids [3].

The aim of this study was to develop and validate

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E-mail address: lubbe.wiesner@farmovs-parexel.com (J.L. Wiesner).

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a more selective and sensitive assay method than previously described with minimal sample preparation and short chromatography time. This new method makes use of a tandem mass spectrometer in conjunction with liquid chromatography to increase the selectivity, which allows for more rapid chromatography and sample cleanup and is well suited for pharmacokinetic studies involving large numbers of samples.

2. Experimental

2.1. Materials and chemicals

A Supelco Discovery C_{18} 5 μm, 2.1×150 mm column (Supelco, Bellefonte, PA, USA) was used for separation at a flow-rate of 0.2 ml/min and injecting 10 μl onto the column. The mobile phase was delivered by a Perkin-Elmer series 200 Micropump (Perkin-Elmer, Foster City, CA, USA) and the samples injected by an Agilent Series 1100 autosampler (Agilent, Palo Alto, CA, USA). Detection was performed by an Applied Biosystems API-2000 detector (Applied Biosystems, Ontario, Canada) fitted with a TurboIonSpray source.

Methanol and acetonitrile (Burdick and Jackson, High Purity) were obtained from Baxter (USA). Anhydrous sodium carbonate and sodium bicarbonate were obtained from Fluka (Buchs, Switzerland). Formic acid (BDH, UK) was used without further purification. *tert.*-Butyl methyl ether was obtained from Aldrich (USA). Water was purified by a Millipore Elix 5 reverse osmosis and a Milli-Q (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA). Alfuzosin ($C_{19}H_{27}N_5O_4$) was supplied by Heumann. Prazosin was obtained from the FARMOVS-PAREXEL BSD pure substance reference library.

2.2. Preparation of standards and quality control samples

Calibration standards (STD) were prepared by dissolving alfuzosin in methanol to obtain a stock solution. By spiking an aliquot of this stock solution into a pool of blank human plasma and by serially diluting it with blank human plasma (1:1, v/v) seven times, a calibration standard range between 38.1 and 0.298 ng/ml was prepared. Similarly, quality control standards (QC) were prepared (using the same methodology) spanning a range between 32.3 and 0.391 ng/ml. Sufficient calibration standards and quality controls were prepared to validate the method and assay all the study samples. Aliquots of the standards and quality controls were stored together with the study samples at −20 °C until used for sample processing.

2.3. Extraction procedure

Plasma samples (500 μl) were pipetted into 10 ml amber glass ampoules. Sodium carbonate buffer (500 μl, 0.1 *M*, pH 10.5), internal standard solution (250 μl, 60 ng prazosin/ml water) and *tert.*-butyl methyl ether (5 ml) were added and the samples vortexed for 60 s. After centrifugation at 1300 *g* for 60 s, the aqueous phase was frozen in an alcohol freezing bath at −25 °C and the organic phase then decanted into 5 ml amber glass ampoules. Formic acid solution (250 μl, 2%) was added and the samples were vortexed for 60 s and centrifuged at 1300 *g* for 60 s. The aqueous phase was frozen in an alcohol freezing bath at −25 °C and the organic phase discarded. The residual organic phase that remained in the aqueous phase was evaporated under a gentle stream of nitrogen at 45 °C for 2 min. The final extract was transferred to an autosampler vial containing a micro glass insert, and 10 μl injected onto the HPLC column.

2.4. Liquid chromatography

Chromatography was carried out at ambient temperature with a mobile phase consisting of acetonitrile, methanol and aqueous formic acid (0.2%), (20:20:60, v/v) at a flow-rate of 0.2 ml/min. All chromatographic solvents were degassed with helium before use.

2.5. Mass spectrometry

Electrospray ionisation (ESI) was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 90 and 50 (respective arbitrary

values). The heated nebulizer temperature was set at 400 °C. The instrument response was optimised for alfuzosin by infusing a solution of the drug dissolved in mobile phase at a constant flow. The same methodology was used to optimise the response of the instrument for the internal standard (I.S.). The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) was set at 3 (arbitrary value).

The Applied Biosystems API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 390.2 to the product ion m/z 71.2 for alfuzosin and also monitoring the transition of the protonated molecular ion m/z 384.2 to the product ion m/z 95.0 for the I.S. Presented in Fig. 1 is the product ion mass spectrum of protonated alfuzosin showing the [M+1] ion (m/z 390.2, molecular structure given) and the principal product ion at m/z 71.2 formed by collision-induced dissociation (CID). Presented in Fig. 2 is the product ion mass spectrum of protonated prazosin showing the [M+1] ion (m/z 384.2, molecular structure given) and the principal product ion at m/z 95 formed by CID. Plausible fragmentation patterns presented in Figs. 1 and 2 are suggested but not proven.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.1 software.

2.6. Validation

The method was validated by analysing plasma quality control samples six times at six different alfuzosin concentrations, i.e. 32.3, 16.1, 8.06, 4.04, 0.783 and 0.391 ng/ml, to determine the accuracy and precision of the method. The quality control values were interpolated from a calibration curve containing eight different concentrations spanning the concentration range of 38.1 to 0.298 ng/ml. Calibration graphs were constructed using a Wagner regression of the drug peak-area ratios of the analyte to the internal standard versus nominal drug concentrations. Several regression types were tested and the Wagner regression [log transformed quadratic regression curve; $\ln Y = a(\ln X)^2 + b(\ln X) + C$] was found to be most suited for the specific range (Fig. 3). As we often optimise our systems for the lowest possible LLOQ we often find a curving at the higher

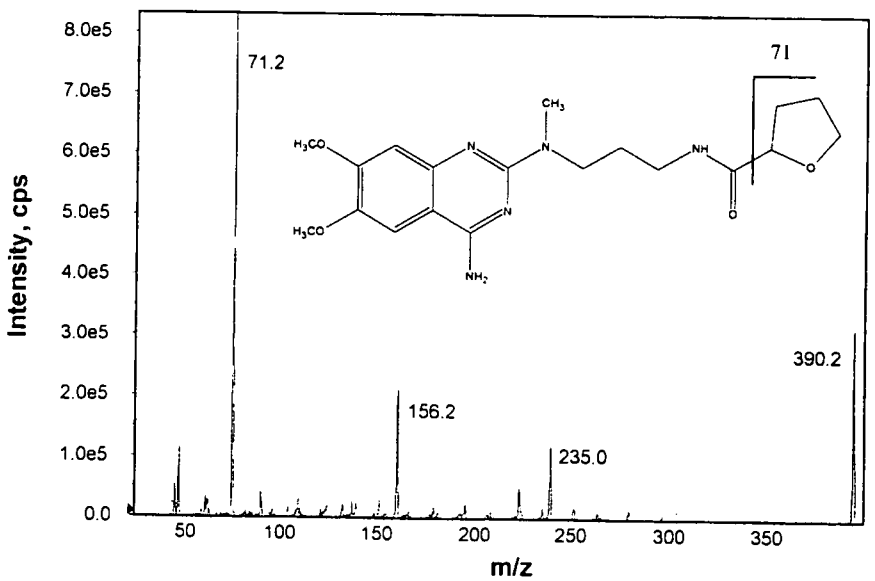

Fig. 1. Product ion mass spectrum of protonated alfuzosin showing the [M+1] ion (m/z 390.2, molecular structure given) and the principal product ion at m/z 71.2 formed by CID.

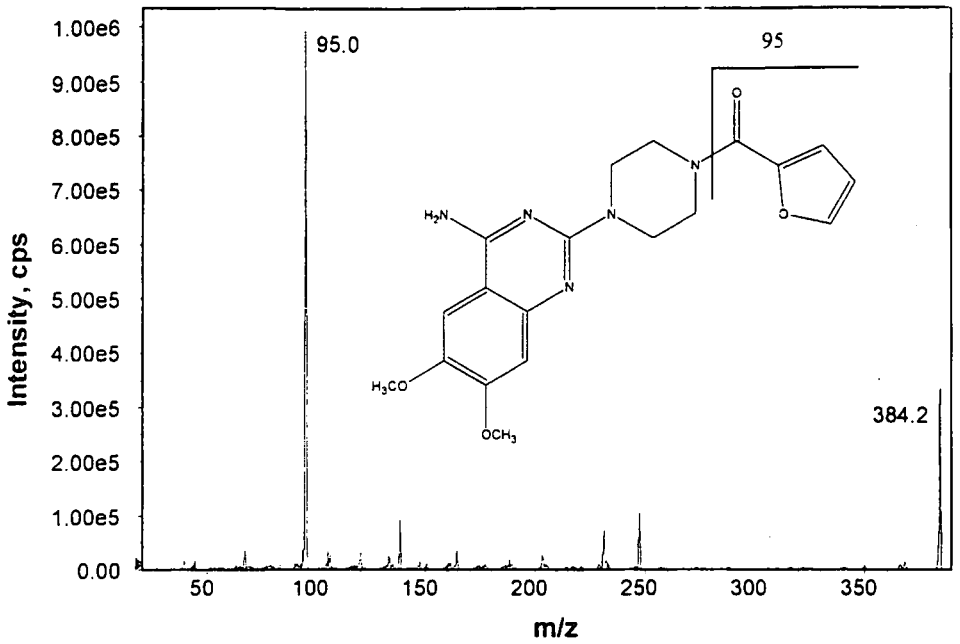

Fig. 2. Product ion mass spectrum of protonated prazosin showing the [M+1] ion (*m/z* 384.2, molecular structure given) and the principal product ion at *m/z* 95.0 formed by CID.

and lower concentration ranges on the MS systems thereby making an atypical regression like the Wagner regression more suitable than other simpler regressions like linear or weighted linear.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated using the procedure described by Matuszewski et al. [8].

Absolute recoveries of the analyte were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with alfuzosin. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the non-extracted system performance verification standard mixtures (prepared in the injection vehicle) representing 100% recovery.

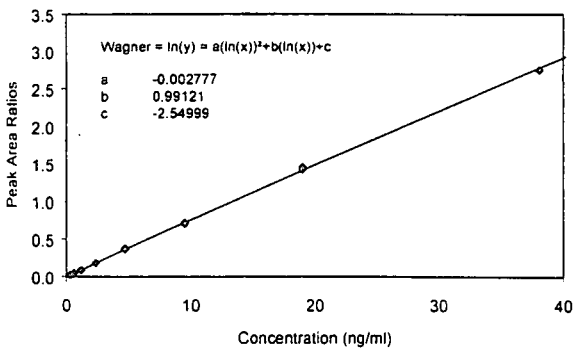

Fig. 3. Calibration curve based on peak-area ratios as obtained from the intra-batch validation.

3. Results and discussion

The mean absolute recoveries of alfuzosin determined in triplicate at 16.1, 4.04 and 0.783 ng/ml were 81.3, 82.7 and 84.8%, respectively. The mean absolute recovery of prazosin was 77.1%.

No significant matrix effect for alfuzosin was observed for 10 different plasma pools tested. The peak areas of the 10 reconstituted samples had a coefficient of variation of 3.6% for alfuzosin and 2.8% for the I.S., indicating that the extracts were "clean" with no co-eluting compounds influencing the ionisation of the analyte and I.S.

The much higher selectivity of MS–MS detection

allowed the development of a very specific and rapid method for the determination of alfuzosin in plasma.

The LLOQ, defined as that concentration of alfuzosin which can still be determined with acceptable precision (C.V.% <20) and accuracy (bias <20%), was found to be 0.298 ng/ml (concentration of the lowest calibration standard) with a signal-to-noise ratio of 20. Results of the intra-batch and inter-batch validation assays presented in Table 1 indicate a valid calibration range of 0.298–38.1 ng/ml for alfuzosin. The calibration parameters for 16 batches are summarised in Table 2, indicating a reproducible and reliable method. The quality control values found during the processing of 16 batches of study samples over a period of 16 days are summarised in Table 3 and attest to the excellent inter-day performance of the assay method.

Table 3
Summary of the back-calculated quality control standard concentrations of alfuzosin (16 batches) showing the repeatability of the method (inter-day variation)

Nominal (ng/ml)	0.391	0.783	4.04	8.06	16.1
Mean	0.407	0.808	3.986	8.243	15.897
C.V.%	5.8	6.4	5.9	6.9	4.5
N	31	32	31	32	32
% Nom	104	103.2	98.7	102.3	98.7

On-instrument stability was inferred from stability samples that were prepared and included in the first intra-day validation batch. No significant degradation

Table 1
Summary of intra- and inter-batch quality control results

Validation batch	Nominal replicates	32.3 ng/ml	16.1 ng/ml	8.06 ng/ml	4.04 ng/ml	0.783 ng/ml	0.391 ng/ml
Intra-batch validation	1	32.78	15.45	8.09	4.15	0.81	0.39
	2	32.52	16.98	8.32	4.29	0.78	0.38
	3	31.74	16.38	8.11	4.13	0.79	0.42
	4	31.76	17.15	8.41	4.18	0.80	0.40
	5	32.53	17.53	8.58	4.33	0.81	0.37
	6	35.07	16.61	8.15	3.73	0.76	0.38
Inter-batch validation 1	1	31.33	15.64	7.70	4.10	0.74	0.40
	2	31.97	15.71	7.82	3.87	0.78	0.42
	3	32.45	16.27	8.10	4.26	0.81	0.38
	4	31.89	16.06	8.24	4.14	0.77	0.36
	5	32.51	15.80	7.98	4.05	0.80	0.40
	6	33.37	16.01	8.10	4.16	0.78	0.43
Inter-batch validation 2	1	33.99	15.96	7.44	4.16	0.83	0.40
	2	32.16	15.32	8.22	4.18	0.87	0.45
	3	34.79	16.23	8.07	4.09	0.82	0.43
	4	32.20	16.27	8.33	4.01	0.84	0.42
	5	33.95	16.78	8.69	4.10	0.85	0.42
	6	32.22	15.52	7.88	3.97	0.83	0.43
	Mean	32.73	16.20	8.12	4.11	0.80	0.41
	% Nom	101.3	100.6	100.8	101.6	102.5	103.6
	C.V.%	3.2	3.7	3.6	3.4	4.0	5.9

Table 2
Summary of the calibration parameters as obtained from 16 batches

	r	r^2	*a*	*b*	*c*
Mean	0.9994420	0.9988843	−0.0167805	1.0144447	−2.9108783
SD	0.00019136	0.00038251	0.008068818	0.02945442	0.12543491
C.V.%	0.0	0.0	−48.1	2.9	−4.3

could be detected in the samples (cooled at 5 °C on the autosampler while awaiting injection) left on the autosampler for at least 28 h.

Due to the high specificity of MS–MS detection, no interfering or late-eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Various liquid–liquid extraction procedures were tested which included different organic solvents [a mixture of dichloromethane and diethyl ether (3:4, v/v), diethyl ether and *tert.*-butyl methyl ether], buffers [sodium hydroxide solutions (0.1 and 1 *M*) and carbonate buffers (0.1 *M*; pH 9, 9.5, 10, 10.5 and 11)] and back-extracting solutions [formic acid and acetic acid solutions (1 and 2%)]. The best results were obtained with *tert.*-butyl methyl ether, carbonate buffer (0.1 *M*, pH 10.5) and 2% formic acid solution. Average recoveries above 77% were found for both analytes.

Mobile phase solutions with varying concentrations of formic acid were tested for optimum ionisation of the analytes and it was found that 0.2% formic acid gave the best result. The best resolution and chromatographic peak shape were obtained with a mobile phase consisting of acetonitrile, methanol and 0.2% formic acid (20:20:60, v/v).

Prazosin is structurally related to alfuzosin and was tested as a possible internal standard. Ionisation, retention and extraction characteristics were found to be similar to that of alfuzosin. The retention times for alfuzosin (I) and prazosin (II) were 2.5 and 2.6 min, respectively (Fig. 4). The total chromatography run time of 4 min made it possible to analyse a large number of samples in a batch. Fig. 5 shows a representative chromatogram obtained of an alfuzosin (I) calibration standard at a concentration of 0.298 ng/ml in plasma (the LLOQ) and of a study sample (II) at the late elimination phase (24 h) of the pharmacokinetic profile for the analyte.

This assay method was employed to analyse plasma samples containing alfuzosin obtained during a multiple oral dose study of 5 mg alfuzosin in 40 healthy volunteers. Concentration vs. time profiles were constructed for up to 24 h after the last dose (Fig. 6).

4. Conclusion

A rapid, sensitive and highly selective method for the determination of alfuzosin in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. This newly developed assay method was

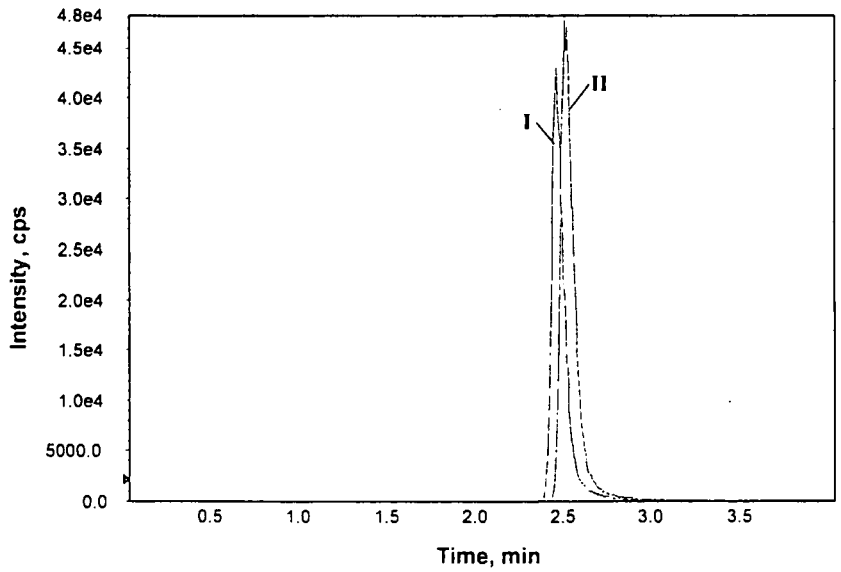

Fig. 4. Representative chromatogram illustrating the retention times of alfuzosin (I) and of the internal standard, prazosin (II).

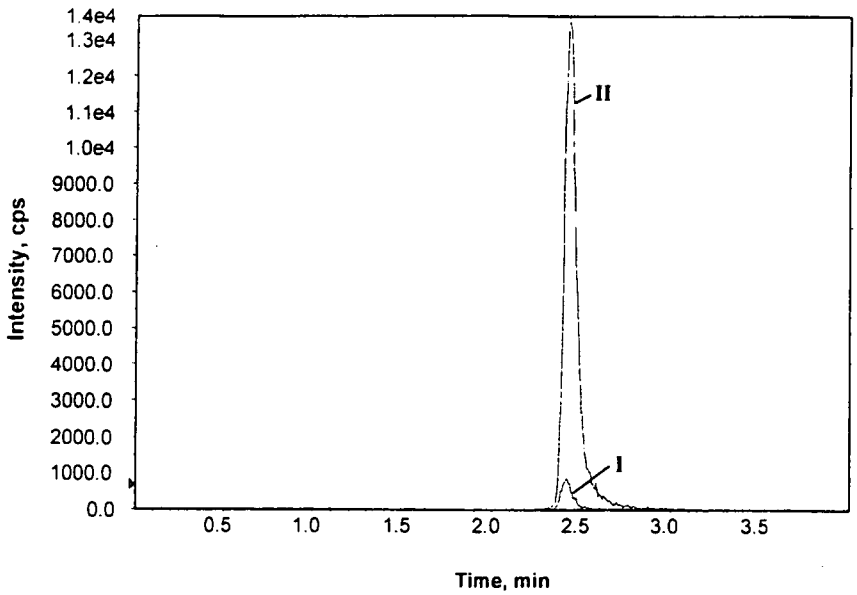

Fig. 5. High-performance liquid chromatograms of the calibration standard at the limit of quantification (I) containing 0.298 ng/ml alfuzosin and of a study sample (II) at the late elimination phase (24 h after dose) of the pharmacokinetic profile for the analyte.

used in a clinical study in which 40 healthy volunteers were each given 5 mg multiple oral doses of alfuzosin. The assay method is more selective than previously described methods and allows for a much higher sample throughput due to the short chromatography time (4 min) and simple sample preparation. Robust LC–MS–MS instrument performance was observed, with only slight variations in the instrument response within batches. It was not necessary to clean the ion source during the entire

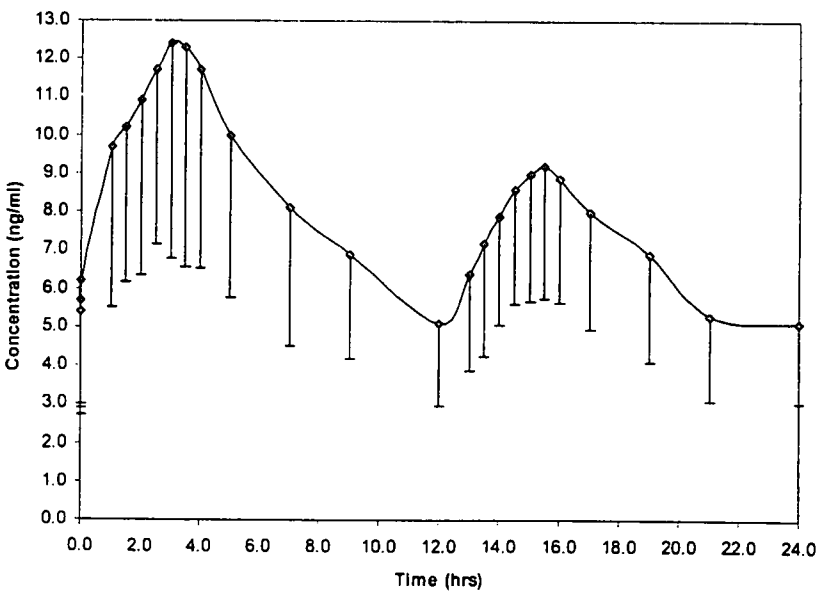

Fig. 6. Alfuzosin plasma concentrations vs. time profile as obtained after a multiple 5 mg oral dose of alfuzosin (average of 40 subjects).

study and a single analytical column was used to chromatograph more than about 2400 extracts without significant deterioration of the column performance. This attests to the clean nature of the final extracts injected onto the column. This method is an excellent analytical option for rapid quantification of alfuzosin in human plasma.

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