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

Clarithromycin, Carbamazepine and Carbamazepine-10, 11-epoxide

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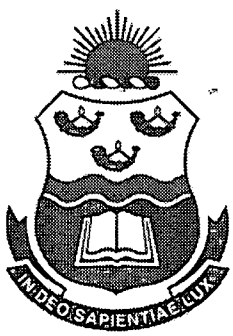

This dissertation meets the requirements for the qualification M.Med.Sc. in the Faculty of Medicine, Department of Pharmacology, at the University of the Free State

May 2003

Supervisor, Doctor Ken Swart

Joint supervisor, Professor Hans Hundt

I declare that the dissertation hereby handed in for the qualification M.Med.Sc. at the University of the Free State, is my own independent work and that I have not previously submitted the same work for a qualification at/in another university/faculty.

G.F. van Rooyen

I would like to say a big thank you to the following people:

My supervisor, Doctor Ken Swart

Joint supervisor, Professor Hans Hundt

To them my thanks are due for suggestions, advice, encouragement, expert opinions, and other such things.

All concentrations have been rounded off to three significant figures and have been converted to nanograms per millilitre (ng/ml), where appropriate.

The Harvard reference system has been used.

ABSTRACT

The projects described in this dissertation use cutting edge technology to quantitate various drugs in plasma. Highly sensitive assay methods for the quantification of clarithromycin, carbamazepine and its major metabolite, carbamazepine-10, 11-epoxide in human plasma were developed and validated. The application of these methods to analyse samples generated during pharmacokinetic studies is also discussed where up to 230 samples were analysed per day. The methods were sensitive enough to quantitate the analytes for at least 5 half-lives of the drug after an oral dose to human volunteers.

A sensitive method for the determination of clarithromycin, a macrolide antibiotic, using high-performance liquid chromatographic separation with tandem mass spectrometry, and the method development thereof, are described in this dissertation. Samples were prepared using liquid-liquid extraction and were separated on a C_{18} column with a mobile phase consisting of acetonitrile, methanol and acetic acid. Detection was performed by a mass spectrometer in the multiple reaction monitoring (MRM) mode, using TurboIonSpray ionisation. The mean recovery of clarithromycin was 87.3%, with a lower limit of quantification of 2.95 ng/ml when using 300µl plasma. This high-throughput method was used to quantify 230 samples per day, and is sufficiently sensitive to be employed in pharmacokinetic studies.

Carbamazepine, an anticonvulsant, and its major metabolite, carbamazepine-10, 11-epoxide, were quantified using tandem liquid chromatography-mass spectrometry, with electrospray ionisation. Both these analytes were extracted from 500µl of human plasma, using a liquid-liquid extraction method that showed recoveries greater than 95% for both these analytes. Concentrations as low as 0.722 ng/ml for carbamazepine and 5.15 ng/ml for carbamazepine-10, 11-epoxide could be quantified by using this method.

These are the first LC-MS/MS assay methods described for the quantification of carbamazepine and its metabolite, carbamazepine-10, 11-epoxideas well as clarithromycin and their applications to pharmacokinetic studies.

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INTRODUCTION

An appropriate response to a drug requires the appropriate concentration of drug at the site of action. The dosage regimen required to attain and maintain the appropriate concentration depends on the pharmacokinetic parameters of the drug. The appropriate concentration and dosage regimen depend on the patient's clinical state, severity of the disorder, presence of concurrent disease, use of other drugs, and other factors.

Because of individual differences, drug administration must be based on each patient's needs, traditionally, by empirically adjusting dosage until the therapeutic objective is met. This approach is frequently inadequate because optimal response may be delayed or serious toxic reactions may occur. Alternatively, a drug can be administered according to its expected absorption and disposition in a patient, and dosage can be adjusted by monitoring plasma drug concentration and drug effects. This approach requires knowledge of the drug's pharmacokinetics as a function of the patient's age and weight and the kinetic consequences of concurrent diseases (e.g., renal, hepatic, or cardiovascular disease or a combination of diseases).

Directly measuring the desired therapeutic effect is not always possible. Monitoring plasma drug concentration is an easier, faster way to estimate dosage requirements than observing drug effects only. Monitoring can determine when a target concentration range is reached and help ensure that it is maintained. A strategy for drug administration can sometimes be guided primarily by plasma drug concentrations. Whether plasma drug concentration monitoring is used may depend on the drug or on the clinical situation. Frequency of monitoring depends on the drug, accuracy of previous measurements, and presumed changes in factors affecting drug response.

To determine the drug's pharmacokinetic parameters, e.g. elimination half-life, absorption half-life, distribution etc., one must be able to construct a good concentration-time profile of the drug in the plasma, at least up to five half-life times. In certain drug pharmacokinetic-profile constructions, the assay method is not sensitive or specific enough to construct or determine the concentration-time profile up to five half-life times. To reach the necessary sensitivity and specificity, analysts usually need to increase their assay times, causing an increase in the time to market for the drug. No pharmaceutical company can afford the competitive disadvantage of having any aspect of its drug development slowed down by the use of analytical procedures, which do not posses the necessary sensitivity and specificity requirements.

The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed.

The first step in the method development and validation cycle should be to set minimum requirements, which are essentially acceptance specifications for the method. These performance criteria include specificity, selectivity, limit of detection and quantitation, linearity, range, sensitivity, precision, accuracy and ruggedness [1, 2]. One can say linearity;

sensitivity and limit of detection/quantitation are aspects of equipment suitability, while accuracy and recovery assess the sample preparation process. Precision and ruggedness cover both these aspects [3].

Two approaches can be utilised to determine these parameters. One can either search the literature for the desired methodology or examine the published guidelines by organisations like the Food and Drug Administration (FDA) [4].

Validation is a continuous process [1] and the results of the validation apply only to the concentration range of the analyte and the equipment used during the validation. Any change in the method should be evaluated in the revalidation of the method [5].

1. CLARITHROMYCIN *

1.1. Introduction

Clarithromycin is a semi-synthetic 14-membered macrolide. Macrolide antibiotics are macrocyclic lactones; isolated first from *Streptomyces* spp. Macrolide antibiotics are effective against Gram-positive and some Gram-negative bacteria and are useful in the treatment of *Mycoplasma* species, H*aemophilus* species, *Clamydia* species, *Campylobacter* species and *Legionella pneumophilia* [6, 7]. Macrolide antibiotics commonly have a 12 to 16-membered lactone ring with glycosidic linked neutral and/or amino sugars. Erythromycin, a 14-membered macrolide antibiotic, was the most commonly used macrolide antibiotic since its discovery in 1952 [7]. However, due to its instability in an acid environment, erythromycin had very poor absorption characteristics in the gastrointestinal track after oral administration, resulting in unpredictable plasma concentrations.

A recent review article [6] lists many assay methods, for macrolide antibiotics in general, including clarithromycin.

Chen and Chiou published one of the first papers on the determination of a macrolide antibiotic with high-performance liquid chromatography in 1983, using electrochemical detection [8].

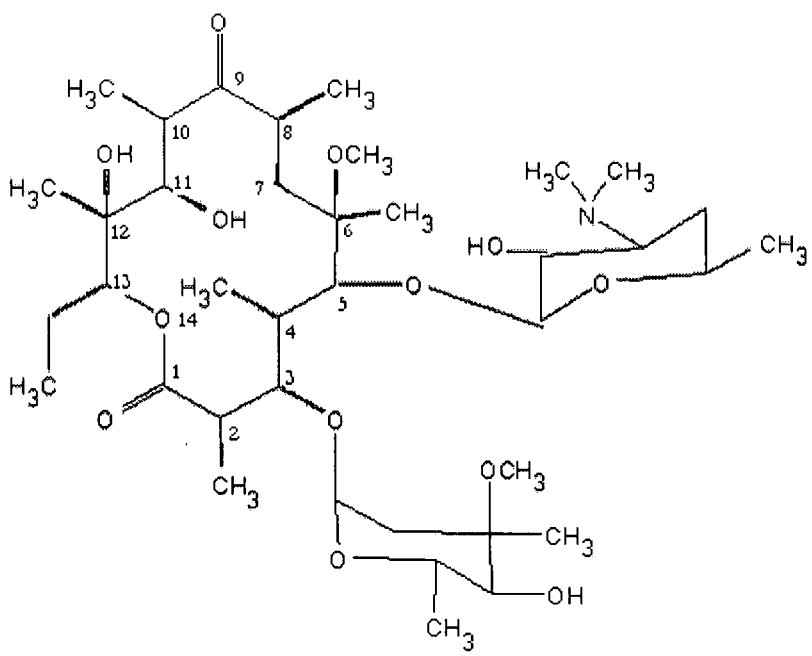


Figure 1: Structure of clarithromycin.

* See appendix I

Clarithromycin (6-*O*-methylerythromycin)($C_{38}H_{69}NO_{13}$)(M_r: 747.96) is a semi-synthetic 14-membered macrolide, which displays a wide antibacterial spectrum. The effect of combining clarithromycin with a variety of other drugs for the treatment and prevention of disseminated *Mycobacterium avium* infection in patients with AIDS is currently under investigation [9, 10, 11, 12].

Clarithromycin was first discovered and patented by Thaiso Pharmaceutical Co. Ltd., Japan [13].

The chemical structure of clarithromycin, a semi-synthetic derivate of erythromycin A, is identical to that of erythromycin, except that the 0-methyl group has been substituted for a hydroxyl group at position six of the lactone. This semi synthetic derivative of erythromycin is more stable in an acidic environment and therefore exhibits a better oral bioavailability and thus a more favourable pharmacokinetic profile.

Previous literature [13] stated that clarithromycin has a serum half-life of 4.9 hours, and exhibits peak serum concentrations (C_{max})(2 510 ng/ml) within two hours after a single 500mg oral dose. Steady-state plasma concentrations were reached after 2 to 3 days of administering a 250mg dose every 12 hours.

Clarithromycin is metabolised in the liver by cytochrome P450 enzymes, mainly by hydroxylation at the 14 positions, and by oxidative N-demethylation. Clarithromycin and its principal metabolites are excreted through faeces, urine and non-renal mechanisms. 20 to 30% of clarithromycin is excreted unchanged via these routes of elimination [13].

1.2. Method Development and Validation

1.2.1. Literature Survey (*Available Approaches*)

Morgan *et al.*, [14] investigated the effect of mobile phase composition and pH[ᶜᵒ], using reverse-phase chromatography with a UV detector set at 205nm, on clarithromycin and several other related compounds.

They injected 50µl of a working standard solution (containing 210 µg/ml clarithromycin) prepared in acetonitrile: water (1:3, v/v). The mobile phase consisted of acetonitrile and KH_2PO_4 (0.033M)(48: 52, v/v, pH 5.5). A flow-rate of 1 ml/min was used, while the column was maintained at 50°C. Several different columns were investigated, including a Nucleocil (C_{18}, 5µm, 150 x 4.6mm and 250 x 4.6mm), a Zorbax Golden Series (C_8, 3µm, 80 x 6.2mm), an IBM (C_8, 3µm, 100 x 4.5mm) and a YMC (C_{18}, 5µm, 150 x 4.6mm and 250 x 4.6mm) column. The best performance was given by the YMC column (A-303, 5µm, 120-Å, C_{18}, 250 x 4.6mm, YMC, Japan). The YMC column was also compared to 5µm, 250 x 4.6mm columns with the following stationary phases: Phenomenex Carbosphere, Whatman ODS, TSK Gel ODS and Regis ODS II, but were found not to be equivalent to the YMC column.

They found that the separation process was largely dependent on the organic-aqueous ratio of the mobile phase and, in contrast with erythromycin, almost unaffected by temperature and pH, although an elevated column temperature was necessary to maintain peak symmetry and the desired resolution.

Chu *et al.*, [15] determined clarithromycin and one of its major metabolites, 14(*R*)-hydroxyclarithromycin, simultaneously in plasma and urine using HPLC with electrochemical detection.

To 500µl of plasma was added the internal standard (erythromycin A 9-*O*-methyloxime), sodium carbonate (0.1M, 200µl), and 3ml of ethyl acetate: hexane (1:1, v/v). The sample was vortex-mixed and centrifuged. The organic layer was transferred to a clean tube, and evaporated to dryness at 45°C under a stream of air. The residue was dissolved in acetonitrile: water (1:1, v/v)(200 to 400µl) and 20 to 80µl was injected onto the column. An octyl-bonded (C_8) reverse-phase HPLC column (250 x 4.6mm, 5µm, Alltech Assoc.) was used, although several octyl-bonded (C_8) reverse-phase columns, including 5µm Spherisorb, Nucleosil (Alltech) and Sepralyte (Analytichem International) were suitable. The mobile phase consisted of acetonitrile: methanol: water (39:9:52, v/v/v) and contained NaH_2PO_4 (0.04M) and sodium hydroxide, to maintain a pH at 6.8. The flow-rate was set between 1.2 and 1.4 ml/min. Injections were approximately 35 minutes apart, with a retention time of ~20 minutes for clarithromycin.

Chu *et al.*, claimed to have a recovery of more than 85%. The calibration curve had a correlation coefficient (r^2) of 0.999 (weighted ($^1/_{Concentration}$) linear), showing good linearity. The limit of quantification (LOQ) was determined at 30.0 ng/ml.

This assay method was used to determine the mean plasma concentration vs. time profile, showing a C_{max} (maximum concentration) of ~2 500 ng/ml after two hours, after the fifth 500mg (taken twice daily) oral dose, of clarithromycin.

[ᶜᵒ] pH (potential of Hydrogen)

Borner *et al.*, [16] also determined clarithromycin and 14(*R*)-hydroxyclarithromycin in human serum and urine by HPLC, using electrochemical detection.

Serum (500µl) was spiked with internal standard (erythromycin A 9-*O*-methyloxime), mixed with sodium carbonate (0.1M, 100µl), and extracted with ethyl acetate: hexane (1:1, v/v, 3ml). After centrifugation, 2ml of the organic phase was evaporated to dryness. The residue was dissolved in 500µl of mobile phase (sodium acetate (8.2g), repurified water (300ml), methanol (78ml), acetonitrile (370ml), filled up with re-distilled water up to 1000ml), adjusted to pH 6.8. 12 µl was injected onto the Nucleocil © 120-3 C_8 column (125 x 3mm)(30°C) preceded by a guard column (15 x 3mm, Macharey & Nagel). Clarithromycin had a retention time of ~17 minutes at a flow rate of 0.7 ml/min. The limit of detection was reported to be 50 ng/ml from 500µl of serum, while the limit of quantification was set at 100 ng/ml.

The operation of an electrochemical detector is very laborious, and requires extremely pure reagents and solvents and high cleanliness in all manipulations.

Hedenmo and Eriksson [17] published the first liquid chromatography method, with electrochemical detection (LC-ED) for roxithromycin and clarithromycin, using solid-phase cyano-material for on-line sample clean up, followed by chromatography on C_{18} material.

100µl of plasma was pipetted into a glass vial, whereafter 100µl of the internal standard (roxithromycin) in phosphate buffer (pH 10.5), with 10% acetonitrile, was added. The sample was then placed onto the autosampler and a volume of 20 to 100µl was injected. The solid-phase extraction (SPE) cartridges (10 x 2mm) contained a 20mg CN-packing (30 to 40µm). The cartridges were conditioned with 100% methanol (2ml) followed by 10% methanol in water (2ml) and phosphate buffer (pH 10.5, 4ml) with 10% of acetonitrile. They found that increase in the column temperature resulted in long retention times, which improved the peak shape, thus the analytical column (Hypersil BDS C_{18} (100 x 4.6mm, 3µm, Shandon)) was maintained at a temperature of 55°C. The mobile phase (buffered at pH 7) consisted of NaH_2PO_4 (4.5mM), Na_2HPO_4 (6.8mM) and 54% acetonitrile. Clarithromycin had a retention time of ~6 minutes with a flow rate of 1 ml/min. They reported a 99% $\pm$ 5.3 recovery for clarithromycin.

Hedenmo and Eriksson found that their on-line sample clean up showed good comparison with liquid-liquid extraction methods.

Erah *et al.*, [18] published an ion-pair high-performance liquid chromatographic assay method for the assessment of clarithromycin stability in aqueous solution and in gastric juice. The parent and major degradation products were separated on reverse phase material with an ion-pairing agent incorporated into the mobile phase.

500µl of gastric juice was diluted to 2.5ml using deionised water. The sample was vortex-mixed and filtered through a nylon membrane filter. The UV detector was set at a wavelength of 210nm. 50µl of the sample was injected onto a Hypersil ODS (5µm, 150 x 4.6mm, Hypersil Runcorn) column, protected by a guard column (20 x 2mm, 5µm, Hypersil ODS). The mobile phase consisted of acetonitrile: aqueous phosphate buffer (0.05M), containing octane sulphuric acid (5mM, pH 4.6) (1:1, v/v). The flow-rate of the mobile phase was 1 ml/min and clarithromycin had a retention time of ~6 minutes with the column temperature at 50°C.

The limit of detection (LOD) was found to be 400 ng/ml (aqueous solution) and 780 ng/ml (gastric juice). The recovery was 98.5% ± 2.9.

Erah *et al.*, found this method to be simple, sensitive and specific enough to apply in stability studies.

Kees *et al.*, [7] determined several macrolides (clarithromycin, roxithromycin and azithromycin) in several biological matrices with high-performance liquid chromatography, using electrochemical detection.

To 500µl of plasma was added the internal standard (roxithromycin), sodium carbonate (Na_2CO_3)(0.1M, 200µl) and *tert*-butyl methyl ether (3ml). The sample was shaken and centrifuged, after which the aqueous phase was frozen. The organic phase was then transferred to a clean tube and evaporated to dryness. The residue was reconstituted in methanol: water (1:1, v/v) (250µl). 20 to 50µl was injected onto the column. They used a cyanopropyl silica column (150 x 4.6mm, 5 µm, Zorbax SB CN), while the mobile phase consisted of mixtures of NaH_2PO_4: Na_2HPO_4 (50 mM): acetonitrile: methanol (pH 6.5 to 7.5) and was pumped through the system at a flow-rate of 1 ml/min. Clarithromycin had a retention time of ~10 minutes.

Kees *et al.*, reported a recovery of 80 to 90%. Their assay showed good linearity ($r > 0.9996$ for clarithromycin) over a range from 100 to 5000 ng/ml. A limit of quantification (LOQ) of 30 ng/ml for clarithromycin was reported.

This assay method was used in several pharmacokinetic studies, in humans for clarithromycin and roxithromycin.

Sastre Torano and Guchelaar [19] published a paper on the determination of several macrolide antibiotics, including clarithromycin in serum via high-performance liquid chromatography, using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) and fluorescence detection.

They added internal standard (roxithromycin) to 1ml of serum containing clarithromycin, and added warmed saturated sodium carbonate solution (0.5 g/ml, pH ~12, 200µl). The sample solution was vortex-mixed, and shaken after the addition of diethyl ether (6ml).

After the sample was centrifuged, the aqueous phase was frozen on an ethanol bath. The organic layer was transferred to a new tube, evaporated under nitrogen (40°C), and the residue dissolved into 200µl acetonitrile. The solution was centrifuged and quantitatively transferred to a reaction vial. 100µl of an FMOC-Cl solution (2.5 mg/ml in acetonitrile) and 75µl phosphate buffer (0.1M in water, pH 7.5,) was added. The reaction vial was incubated in a water bath at 40°C for 40 minutes. After derivitization, 50µl was injected onto the base-deactivated Supelcosil silica C_{18} column (125 x 4.6mm, 5µm, Supelco), protected by a guard column (C_{18}, 20 x 4.6mm, 5µm, Supelco). The mobile phase consisted of acetonitrile: potassium dihydrogenphosphate (0.05M) and triethylamine (600: 400: 0.5, v/v/v). The pH was adjusted to 7.5 with 10% potassium hydroxide. The pump delivered the mobile phase at a flow-rate of 2 ml/min, while the column was maintained at 50°C.

Spiked serum concentrations, stored at 4°C and –20°C and assayed daily, were found to be stable over a one-week period. The LOQ (S/N = 5) for clarithromycin was set at 200 ng/ml.

This assay method appeared not to be robust enough, since the derivatization reaction appeared to be highly dependent on pH, reaction temperature and reaction time, but was applied in a pharmacokinetic study.

Taninaka *et al.*, [20] developed a high-performance liquid chromatographic method for the quantitative determination of various macrolide antibiotics in rat plasma with amperometric detection.

They pipetted 150µl of the internal standard solution into a centrifuge-tube and evaporated it to dryness. To this was added 150µl plasma, sodium hydroxide (1M, 10µl) and *tert*-butyl methyl ether (2ml). The tube was vortexed and centrifuged. The upper organic layer was transferred into another tube and evaporated to dryness. The residue was dissolved in 50µl of mobile phase (acetonitrile: phosphate buffer (pH 7.2)) (43: 57, v/v). 20µl of the sample was injected onto the analytical column (RP YMC-pack ODS-AP, 250 x 6mm, 5µ, YMC), maintained at 30°C. The mobile phase was pumped through the system at a flow rate of 1.7 ml/min, and clarithromycin had a retention time of approximately 14 minutes.

Clarithromycin had a recovery of 106%. The calibration curve for clarithromycin ranged from 30.0 ng/ml (LOQ) to 3 000 ng/ml. Taninaka *et al.*, found this extraction method suitable to investigate the pharmacokinetic parameters of clarithromycin and roxithromycin in rats.

Clarithromycin was used as internal standard by Araujo *et al.*, [21] to determine minocycline in human plasma by high-performance liquid chromatography, coupled to tandem mass spectrometry. A 200 ng/ml solution of clarithromycin in EDTA buffer (10mM) was used. Diethyl ether and dichloromethane (70:30, v/v, 4ml) were added and the sample vortex-mixed. The upper organic phase was transferred to a clean tube and evaporated under nitrogen (37°C), the sample was reconstituted in mobile phase (acetonitrile: water: trifluoroacetic acid)(800: 199: 1, v/v/v, 200µl) and 40µl was injected onto the analytical column (Zorbax RX-C_8, 15 x 4.6mm, Hewlett-Packard) while it was maintained at 40°C. A Micromass Quattro Ultima monitored the transition of the parent ion, *m/z* 748.4 to the daughter ion, *m/z* 157.8 for clarithromycin, using electrospray ionisation in the positive mode. A recovery of 82.9% $\pm$ 5.6 for clarithromycin was reported.

Pappa-Louisi *et al.*, [22] published a paper in 2001, aiming to explore the effects of organic modifier and column temperature on the electrochemical detector response, as well as on the loss of sensitivity, in order to find the optimal conditions for analysis for macrolide antibiotics, clarithromycin and roxithromycin, in reverse-phase high-performance liquid chromatography.

Plasma samples were prepared by mixing 500µl of drug-free plasma and 500µl of a solution containing roxithromycin and clarithromycin in a phosphate buffer (pH 7) with 80% methanol and 1 ml of acetonitrile. After the sample was vortex-mixed, it was centrifuged in order to compact the precipitated plasma proteins. The organic solvent was evaporated and the supernatant was transferred to the extraction columns (a series of several packing materials from different manufacturers were investigated)(Alltech C_8, Supelco, Baker and/or Rigas Labs C_{18}, Water Oasis HLB). The extraction cartridge was activated with methanol (2 x 3ml) and washed with water (2 x 3ml). After sample loading, the cartridge was washed with water or phosphate buffer (pH 7). The analytes were eluted with methanol, evaporated to dryness and reconstituted in 500µl of mobile phase (phosphate buffer (pH 7): methanol, 2: 8, v/v). 50µl was injected onto the analytical column (MZ-Analytical Inertsil ODS-3, 250 x 4mm, 5 µm), which was maintained at 40°C.

The limit of detection (LOD) was 100 ng/ml (S/N = 3.4) with a correlation coefficient greater than 0.998. The recovery for clarithromycin was 93.4% $\pm$ 3.8 (n = 7) at 1000 ng/ml. The average retention time for clarithromycin was

~17.1 minutes (depending on the mobile phase used), but late eluting peaks at 38 and 43 minutes were reported, delaying injection time.

Pappa-Louisi *et al.*, demonstrated that the choice of eluent and column temperature is crucial in the assaying of clarithromycin. They made the interesting observation that the rate of passivation of the ED electrode depended on the organic modifier in the mobile phase. Thus the sensitivity decreased by 50% within a working day when the mobile phase contained 50% acetonitrile, while a mobile phase containing 80% methanol could be used for 15 days before re-polishing of the electrode was necessary.

Pappa-Louisi *et al.*, demonstrated that increased attention has to be paid to eluent composition and column temperature to ensure sensitive and reproducible responses.

Using two HPLC pumps (one pumping a "washing" solvent consisting of acetonitrile: methanol: potassium phosphate (0.05M, pH 7.0)(5: 2: 93, v/v/v) at 0.8 ml/min, and one pumping a separation mobile phase consisting of acetonitrile: methanol: potassium phosphate (0.05M, pH 7), 41: 6: 53, v/v/v) at 0.8 ml/min, and one high pressure switching valve with some innovative solvent switching, Choi *et al.*, [23] were able to develop an in-line extraction HPLC determination of clarithromycin in human plasma with electrochemical detection.

The plasma sample (120µl) was mixed with "washing" solvent (120µl), containing the internal standard (roxithromycin), whereafter it was filtered with disposable low protein binding membrane syringe filters (0.22µm). By using a sample column switching procedure, the filtered plasma sample (200µl) was first loaded onto the precolumn (AC-ODS, 4 x 8mm, 5µ, Eicom) with the "washing" solvent. After washing the precolumn with the "washing" solvent for 4 minutes (1 ml/min), the solvents were switched so that the analytes were back-flushed out of the precolumn onto the analytical column (Luna C_{18} (2), 150 x 4mm, 5 µm, Phenomenex) maintained at 25°C, protected by a Luna 2 C_{18} guard cartridge (4 x 3mm, Phenomenex), for five minutes with the separation mobile phase. At this point the solvents switched again, allowing the loading of the next sample with the "washing" solvent and the chromatography of the analytes with the separation mobile phase to proceed. Clarithromycin had a retention time of approximately 16 minutes with a total analysis time of 30 minutes.

Using only 100µl of plasma, the assay method had a LOQ of 100 ng/ml.

Their pharmacokinetic profile for clarithromycin, after a single oral dose (500mg), showed a C_{max} of 2 640 $\pm$ 400 ng/ml, a $t_{1/2}$ (half-life time) of 3.31 $\pm$ 0.98 hours and a t_{max} (time to maximum concentration) of 1 hour.

This extraction method has many advantages, such as simplicity, sensitivity, reliability and speed, and was successfully applied in a pharmacokinetic study.

1.2.1.1. Literature Summary

Table 1: Summary of literature survey for clarithromycin.

	Plasma used (µl)	Inj. Vol. (µl)	LOD (ng/ml)	LOQ (ng/ml)	~Recovery (%)	Retention times (minutes)	Dose (mg)	C_{max} (ng/ml)
Chu et al.,	500	20-80		38	85	20.0	600	2 500
Borner et al.,	500	12	50	100	110	17.0	250	
Hedenmo and Eriksson	100	25		373	99	6.0		
Erah et al.,	500	50	400		98	6.0		
Kees et al.,	500	20-50		30	85	10.0	1 200	
Sastre Torano and Guchelaar		50		200	104	5.7	20mg/kg	4 660
Taninaka et al.,		40		30	83	14.0		
Araujo et al.,	500	50			83	2.1		
Pappa-Louisi et al.,	500	50	100		94	16.0		
Choi et al.,	120	200		100	87	16.0	500	2 640

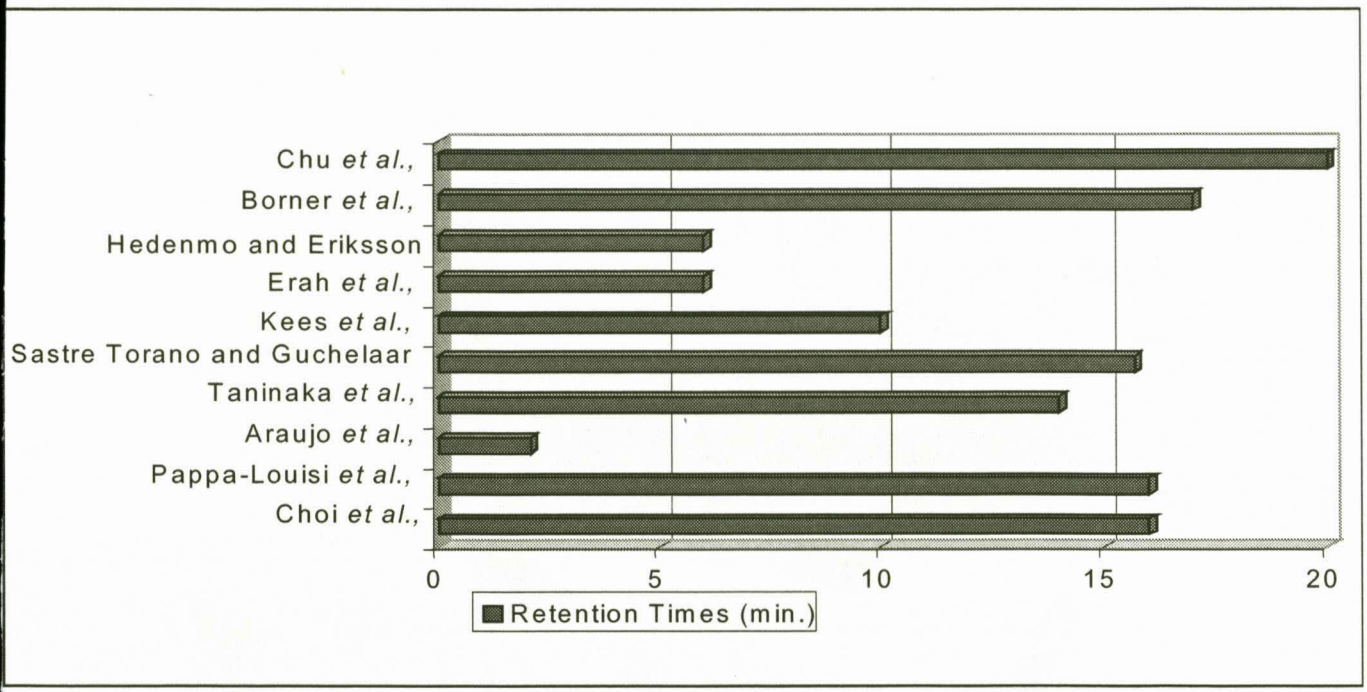


Figure 2: Retention times of reported references.

Various analytical procedures are described for the quantification of clarithromycin in plasma, and were used in several pharmacokinetic studies. Most of the above mentioned methods have very long turn-around times. Longer retention times were required to increase their specificity and resolution, causing a decrease in production. Using the method of Chu *et al.*, [15] the turn-around time of 30 minutes allows one to assay very few samples per day.

Although Borner *et al.*, [16] claimed to have a fairly selective method; none of the published analytical methods has the selective, or the sensitive capabilities of a LC-MS/MS instrument.

The literature survey, clearly indicated that an assay procedure with less critical sample preparation procedures and a much faster turn-around time in the chromatographic process would be advantageous in clinical studies generating large numbers of samples. Although LC-MS/MS would be a natural first choice, the only reference to an assay method for clarithromycin, using mass spectrometry as mode of detection, was Araujo *et al.*, [21], where clarithromycin was used as internal standard.

1.2.2. Instrumentation

An Agilent 1100 quaternary pump was used to deliver the mobile phase. For sample injection, an Agilent 1100 autosampler was used (Agilent, Palo Alto, CA, USA). An Applied Biosystems Sciex API 2000 triple-quadropole mass spectrometer interfaced to an electrospray ionisation (ESI) TurboIonSpray source (Applied Biosystems Sciex, Ontario, Canada) was used for the detection of clarithromycin.

Sensitive quantification of clarithromycin requires sample cleanup and enrichment of the analyte. Although most existing quantitation methods made use of electrochemical detection [16, 17, 23], it is a known fact that the operation of an electrochemical detector requires extremely pure reagents and solvents and high cleanliness in all manipulations.

1.2.3. Mass Spectrometry

For optimum ionisation of a basic drug, using electrospray ionisation (ESI), the mobile phase or infusion solution should ideally be at least two pH units below the pKa value of the drug (figure 3).

Hedenmo and Eriksson [17] reported a pKa value of approximately 9, meaning ~100% ionisation at pH 7 and lower for clarithromycin (base), using ESI. Isam Ismail Salem [13] also found that the solubility of clarithromycin is significantly increased at lower pH values. It was further found that clarithromycin was soluble in acetone, slightly soluble in methanol and ethanol and practically insoluble in water [13], therefore the stock solutions for clarithromycin (500 ng/ml) and roxithromycin (500 ng/ml) were prepared in acetonitrile: 0.1% formic acid (1: 1, v/v) for tuning.

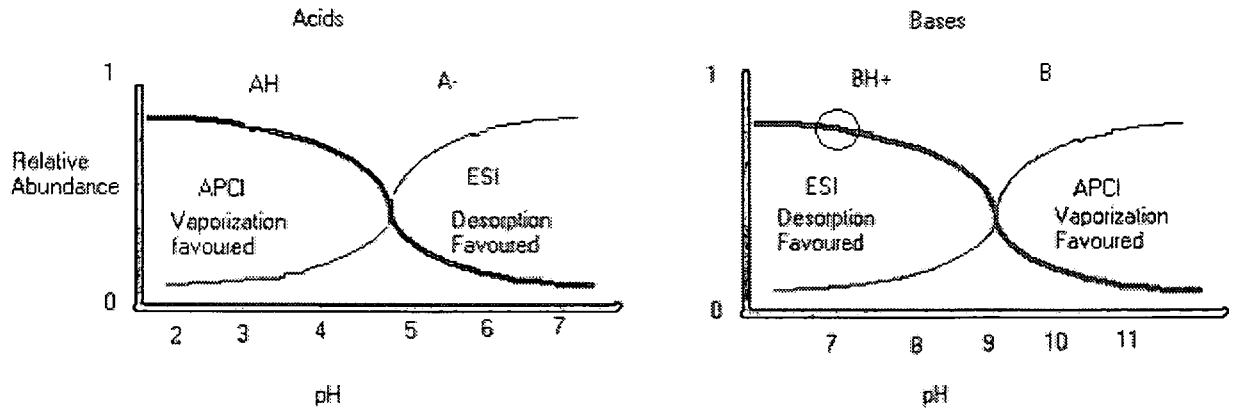


Figure 3: pH values vs. relative abundance for acidic and basic drugs, using different ion sources

The ionisation source was connected by a fused silica capillary to the syringe pump (10µl/min) for tuning and the instrument response for clarithromycin and roxithromycin were optimised using flow injection (10 µl/min). Electrospray ionisation was performed in the positive mode with the nebulizing gas (nitrogen), curtain gas and turbospray set at 70, 50 and 70 units, respectively. Optimal response was obtained with a declustering potential setting of 61V, a focusing potential of 230V, entrance potential and exit potential of -8.0V and 8V, respectively. The TurboIonSpray temperature was set at 400°C. The pause time was set at 5 ms and the dwell time at 150 ms. The relative collision energy was set at 39 units for both analytes. The mass spectrometer was interfaced to a computer workstation running Analyst ™ software, version 1.0.

The Applied Biosystems Sciex API 2000 LC-MS/MS was operated at unit resolution in the multiple reaction monitoring (MRM) mode and the transitions of the protonated molecular ion for clarithromycin at *m/z* 748.5 to the predominant product ion *m/z* 158.2 (figure 4), and for roxithromycin *m/z* 837.6 to *m/z* 679.5 (figure 5) were monitored. The product ion at *m/z* 679.5 was chosen to avoid any possibility of "cross-talk" [d].

The use of high-speed automated analytical procedures such as LC-MS/MS in support of FDA, data submissions has markedly increased over the past five years. Instrument through-put can easily exceed several hundred result records per analytical run, creating the need for software capable of performing real-time post-acquisition data processing and report generation. Results were calculated using the PhIRSt[1] chromatographic data-reporting package. Peak heights/areas are electronically read automatically from the batch summary report files generated by the Analyst ™ acquisition software. Data are automatically summarised, calibration curves calculated according to pre-set regression equations and concentrations interpolated by the program. Results are presented in printed ordered tables with

[d] Cross-talk occurs when the same product ions (same *m/z*) for various analytes are monitored.
[1] Product of Phoenix International Life Sciences, Montreal, Canada.

performance statistics per batch and later summarised to give overall study statistics. The manufacturer has validated this package in Canada to FDA requirements.

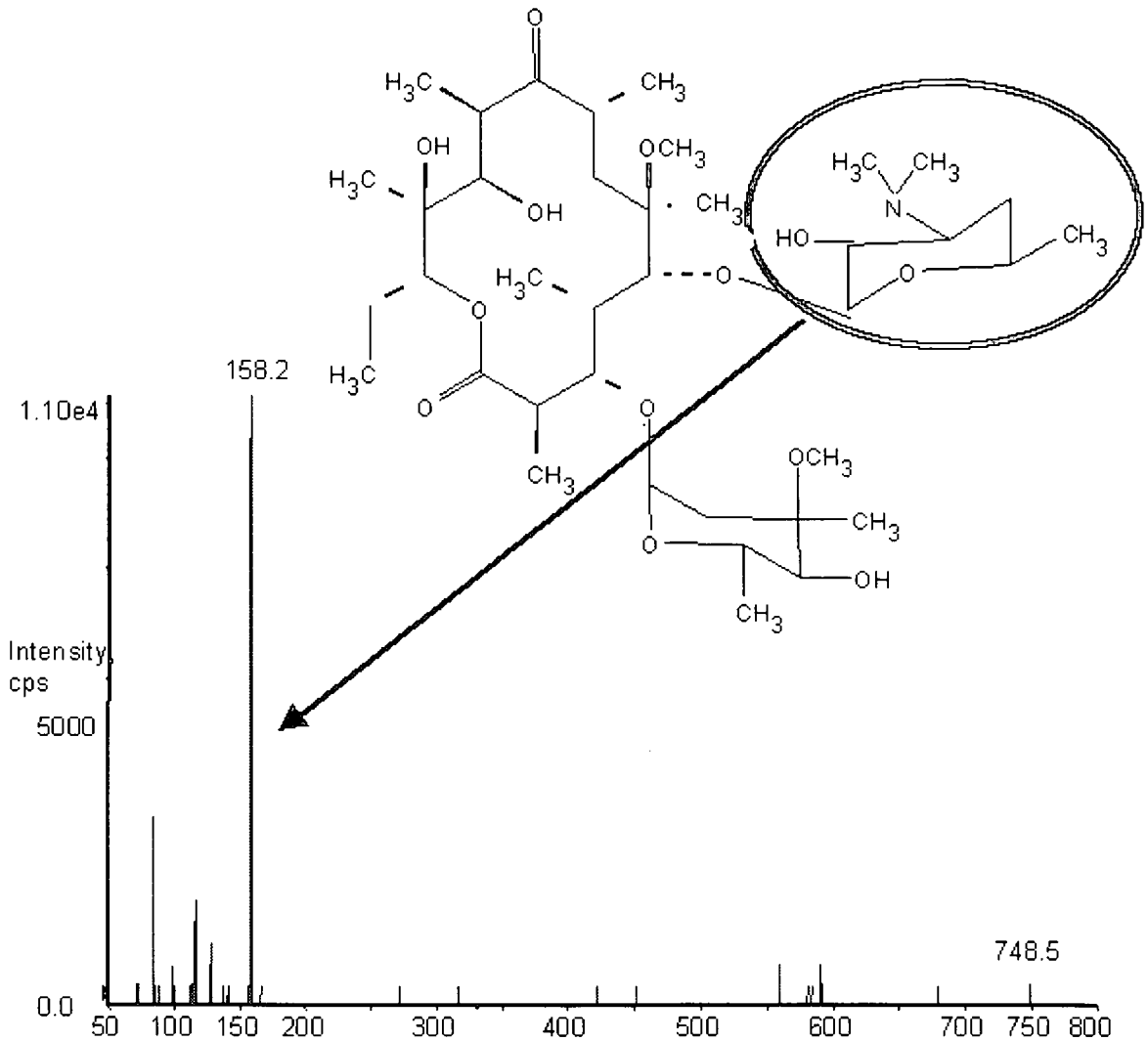


Figure 4: Full scan spectrum (MS/MS) of a pure solution of clarithromycin in acetonitrile: 0.1% formic acid (1:1, v/v). The parent $[M+1]^+$ ion with m/z 748.5 and the predominant product ion m/z 158.2 is shown.

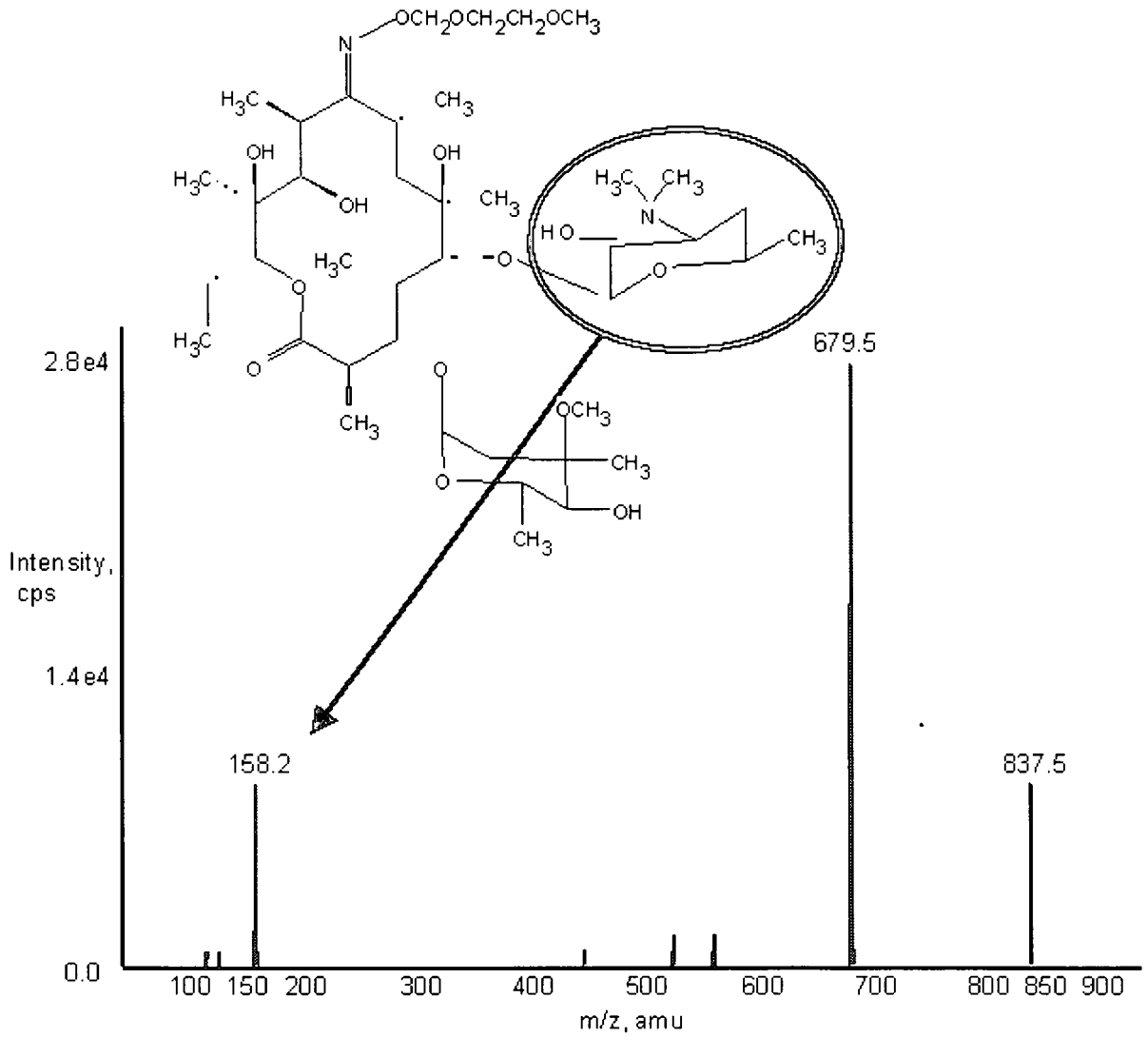


Figure 5: Full scan spectrum (MS/MS) of a pure solution of roxithromycin (internal standard) in acetonitrile: 0.1% formic acid (1:1, v/v). The parent $[M+1]^+$ ion with m/z 837.5 and predominant product ion m/z 679.5 is shown.

1.2.4. Liquid Chromatography

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products, process impurities, etc.) is compared to the response of a solution containing only the analyte. The analyte peak is evaluated for peak purity and resolution from the nearest eluting peak. Once acceptable resolution is obtained for the analyte and the potential sample components, the chromatographic parameters, such as column type, mobile phase composition, flow-rate and detection mode, are considered set.

Chromatography is performed to increase the specificity of the method for identity bases on a unique retention time, to increase the selectivity of the method by isolating the analyte from other sample components, and to enrich the analyte and thus enhance sensitivity.

Thus far, one has considered pH for ionisation/evaporation and for the optimisation of the mass spectrometer. One can also use pH for separation. Using figure 6, one can determine that clarithromycin (base) in a basic environment, would be retained more than in an acidic environment.

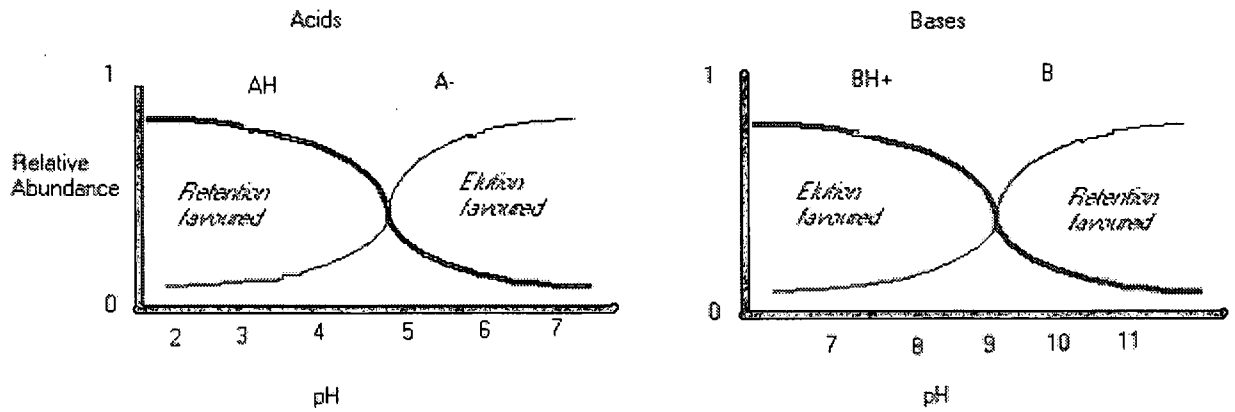


Figure 6: Favoured regions for retention and elution for acids/bases and their conjugated bases/acids on a reverse phase column.

A stock solution of clarithromycin was prepared in acetonitrile: 0.1% formic acid (1:1, v/v)(20 000 ng/ml). Several injections of this stock solution were made onto the system to optimise chromatography. Although Morgan *et al.*, [14] investigated a number of columns, and recommend a YMC, C_{18}, 5µm, 250 x 4.6mm column, the Supelco Discovery C_{18}, 5µm, 150 x 2.1mm, column was the column of choice. Although no other column-type was tried, it proved to be adequate.

Pappa-Louisi *et al.*, demonstrated that increased attention has to be paid to eluent composition and column temperature to ensure sensitive and reproducible responses [22]. By adjusting the solvent strength, composition, column selection, and buffer conditions, one can achieve required separation. Various compositions of the mobile phase, consisting of organic solvent and an acid (for optimum ionisation, using ESI) were used to accomplish the desired retention, peak shape en resolution.

Various references quoted that an elevated column temperature increased peak shape [14, 17, 22]. This was confirmed when the chromatography was optimised, and a column temperature maintained at 60°C proved to give the optimum response and desired chromatography for clarithromycin in the given system.

Optimum conditions for both clarithromycin and roxithromycin were attained by using the Supelco Discovery C_{18}, 5µm, 150 x 2.1mm, column (Supelco Park, Bellefonte, USA), heated at a constant temperature of 60°C in a Shimadzu CTO - 6A column oven (Shimadzu, Kyoto, Japan).

Injection volumes higher than 5µl resulted in peak tailing, indicating a too large amount of analyte on the column. Symmetry was obtained when injecting 5µl onto the column. Chromatography was performed at a flow-rate of 0.26 ml/min (t_r for clarithromycin: 1.97 - 2.05 minutes (RSD_{mean} = 0.30 %) and t_r for roxithromycin: 1.92 - 1.96 minutes (RSD_{mean} = 0.30 %)) with acetonitrile: methanol: acetic acid (0.1% in water) (25: 25: 50; v/v/v) as mobile phase. All chromatographic mobile phases were sparged with helium before use.

1.2.5. Internal Standard

During sample preparation, an accurately known amount of a known compound (internal standard) is added to the sample at the earliest possible stage [24], in the expectation that any procedural loss of sample will be accompanied by an equivalent loss of internal standard. The ratio of the detector response (peak height or area) for the drug and the internal standard is then used in the calibration and assay. The use of an internal standard, however, does not lessen the importance of careful analytical work. Internal standardisation is preferred by most methods in LC-MS. Stabile-isotope labelled standards are the internal standards of choice, because they behave exactly like the analyte. Chemical analogues are the next best approach if they could simulate the behaviour of the analyte.

Ideally, an internal standard should have physicochemical and chromatographic properties close to those of the test compound, so that it can elute close to the analyte of interest. Also, it should be stable and easily available. If a derivative of the analyte is to be prepared, the internal standard should be capable of producing a similar derivative. For these reasons, the internal standards are compounds structurally similar to the drug to be assayed. If a mass spectrometer is used as detector, it is always wise to use a stable isotope labelled form of the drug as an internal standard.

Internal standard technique offers better precision. It is also advisable that the internal standard elute after the analyte (with reversed-phase chromatography), so that the possible interference with faster eluting more polar compounds be excluded [24].

Using an internal standard is very common in bio-analytical methodology. According to Karnes *et al.*,[25] the most common false assumption made is that the partition characteristics of the analyte and the internal standard are the same. The only appropriate use of a non-isotopic internal standard is to monitor detector stability, serve as a quality marker and to correct for errors in dilutions and pippetting.

Since roxithromycin is closely related to clarithromycin in structure, and was available from the FARMOVS-PAREXEL Bioanalytical Services pure reference material library, and most of the references used roxithromycin as internal standard, it was decided to use roxithromycin as internal standard.

Clarithromycin (6-*O*-methylerythromycin)($C_{38}H_{69}NO_{13}$) and roxithromycin were obtained from the FARMOVS-PAREXEL Bioanalytical Services pure reference material library.

1.2.6. Determining the Range/Linearity

The range of an analytical method is the concentration interval over which acceptable accuracy, linearity and precision are obtained. A procedure's range is linked to its linearity. In practice, the range is determined using data from the linearity and accuracy studies [26].

According to the protocol, a single 500mg oral dose of clarithromycin was given to healthy volunteers and blood samples were collected at specific time intervals up to 36 hours after dose. Two articles describing similar dose studies, reported half-life times and C_{max} (maximum plasma concentration) of 4.9 hours, 2 510 ng/ml [23] and 3.31 hours, 2 640 ng/ml [13], respectively.

An acceptable pharmacokinetic profile should include at least five half-lives, in the case of clarithromycin, at least up to 24.5 hours (using the longer half-life). An estimate limit of quantification (LOQ) can therefore be calculated as follow:

- $LOQ_{estimate}$ = the expected C_{max} divided five times by 2 (elimination half-life)
- $LOQ_{estimate}$ = (2 500 ng/ml / (2)*5) = 78.1 ng/ml after 24.5 hours

Thus, to follow a validated pharmacokinetic profile for clarithromycin from the literature, the analytical method should be able to determine, with acceptable linearity and accuracy, the concentration of clarithromycin down to approximately 70 ng/ml. From the literature available (Table 1), it is clear that only 3 analytic methods were able to determine the concentration of clarithromycin up to five half-lives, with the lowest limit of quantification reported in the literature as 30 ng/ml [7, 15, 20]. Chu *et al.*, [15] reported a retention time for clarithromycin over 20 minutes, while Kees *et al.*,[7] and Taninaka *et al.*, [20] showed retention times of over 14 minutes. It was obvious that a method with a shorter run time would increase productivity.

Linearity implies that the sample concentrations are in a concentration range where the analytes response is linearly proportional to concentration. For assay methods, this study is generally performed by preparing standard solutions at at least six concentration levels, since five levels are required to allow detection of curvature in the plotted data [3].

A linear calibration equation is usually presented as:

$$y = bx + a$$

Equation 1: Calibration equation.

where **y** = measured response (absorbance, peak area, peak height); **x** = concentration, **b** = slope of the calibration curve (sensitivity) and **a** = intercept [1].

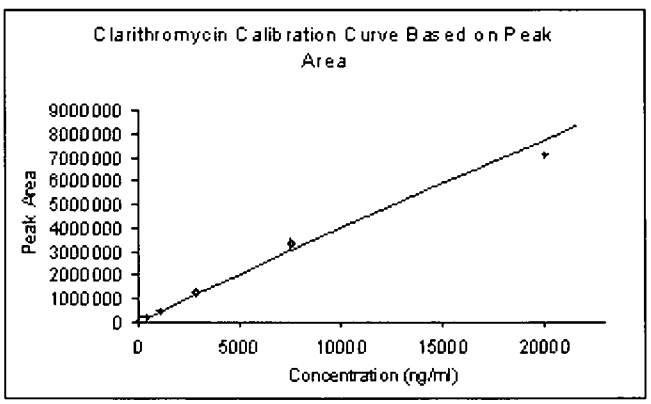


Figure 7: Wagner regression line for the response versus concentration plot.

Calibration Standards used:	STD B - STD K (10 standards)
Calibration Range:	2.95 – 20 016 ng/ml
Regression Equation:	Wagner: $\ln y = a(\ln x)^2 + b(\ln x) + c$
a:	-0.00809
b:	1.02
c:	4.55
r^2:	0.999

Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of greater than 0.997 [1] is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level.

1.2.7. Extraction Procedures and Recovery

When dealing with aqueous samples, an extraction method is required to isolate the analyte and internal standard from the matrix components. Liquid-liquid and solid-phase extraction methods are the most common.

One should always keep in mind that in the case of drug molecules one is often dealing with acids and bases. Figure 8, is a presentation of the pH dependency of extraction efficiency. If one is extracting acids from an aqueous matrix by means of an organic solvent or solid-phase extraction, it is necessary to neutralise the molecule by lowering the pH value approximately 2 pH units below the drug's pKa value, if possible.

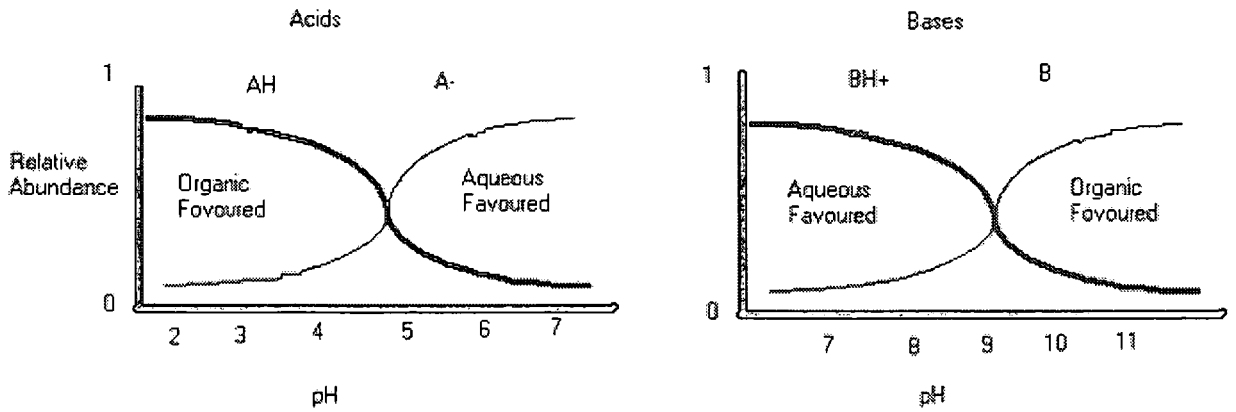


Figure 8: Favoured regions for acidic and basic drugs.

Initially protein precipitation was investigated as a possible sample preparation procedure for the quantification of clarithromycin. 400ml of acetonitrile was added to 200ml of plasma in a microfuge tube. The sample was vortex mixed for 30 seconds and centrifuged at 3000g for 5 minutes at 10°C. The supernatant aqueous layer ($\pm$ 200ml) was transferred to an autosampler vial and 5µl was injected onto the HPLC column. A LOQ of 8.00 ng/ml could be reached with this precipitation method.

For any molecule to be extracted via a liquid-liquid extraction, it is required to be as close to 100% unionised as possible for it to move to the organic phase, taking into account the polarity.

Sometimes, to reach your limit of detection or limit of quantification, you have to concentrate or enrich your sample. Enrichment is accomplished by a number of techniques. With liquid-liquid extraction one can back-extract from a large sample volume into a smaller volume, or evaporate, and reconstitute in an even smaller volume. Alternatively, you can concentrate on-column, with either solid-phase extraction or by utilising the analytical column (online extraction).

Optimum conditions for the liquid-liquid extraction of both analytes were as follows:
To 300µl plasma, in a 10ml glass ampoule, was added 200µl (0.1M) $Na_2CO_3/NaHCO_3$ buffer solution (to suppress ionisation and to favour the partitioning into the organic medium). 100µl of roxithromycin (internal standard) solution (3.76 ng/ml in water) was added. Hexane: ethyl acetate (1:1, v/v) (3ml) was added and the sample was vortex-mixed to increase the surface area, resulting in a maximum partitioning towards the organic phase. The sample was then centrifuged to separate the organic phase and the aqueous phase. The aqueous phase (bottom) was frozen on a Fryka

Polar KP250 cooling plate (Kältetechnik, Esslingen) at –30°C, whereafter the organic phase was decanted into a clean 5ml glass ampoule and evaporated to dryness in a Savant SpeedVac (Savant, Holbrook, NY, USA) rotary vacuum evaporator at ambient temperature. The extract was reconstituted in 300µl of acetonitrile: water (1: 9, v/v), vortex-mixed to ensure dissolution, transferred to an autosampler vial, and injected (5µl) onto the HPLC column.

The liquid-liquid extraction procedure described was the method of choice. The limit of quantification (LOQ) was found to be 2.95 ng/ml (s/n = 27) (~10 times lower than any reference found). This relatively high LOQ could have been much lower if it were not for carry-over (equivalent to an absolute amount of about 5 picograms clarithromycin injected) inherent in the chromatographic instrument system, which yielded a signal equivalent to an extract of an approximately 0.30 ng/ml sample. We observed carry-over problems with different analytes on several occasions. Although it appears to be analyte-specific in many cases, the exact nature of the carry-over does not appear to be the same in all the cases that we have encountered. This phenomenon is the subject of continuous investigation in our laboratory. In the case of the clarithromycin, an LOQ of about 0.40 ng/ml (100 times lower than any reference found) was reached when an extensive needle wash step with a stronger needle-wash solution was introduced to reduce carry-over substantially. However, this procedure would have increased the turn-around time for each analysis by an unacceptable margin while the LOQ, which was attained (2.95 ng/ml), was totally acceptable considering the nature of the study and the range of concentrations studied.

Table 2: Data for plotting Concentration vs. Peak Area Ratios.

Concentration (ng/ml)	***Peak Area Ratio***	
20 016	38.000	
7 506	15.000	
2 818	4.340	
1 058	1.710	
397	0.654	
149	0.241	
55.8	0.106	Previous reported LOQ
20.9	0.040	
7.86	0.013	
2.95	0.006	Current LOQ
1.11	0.003	
0.416	0.001	LOQ with intense needle wash

Linear Regression Statistics	
Slope:	0.002
Intercept:	-0.088
R - squared:	0.999

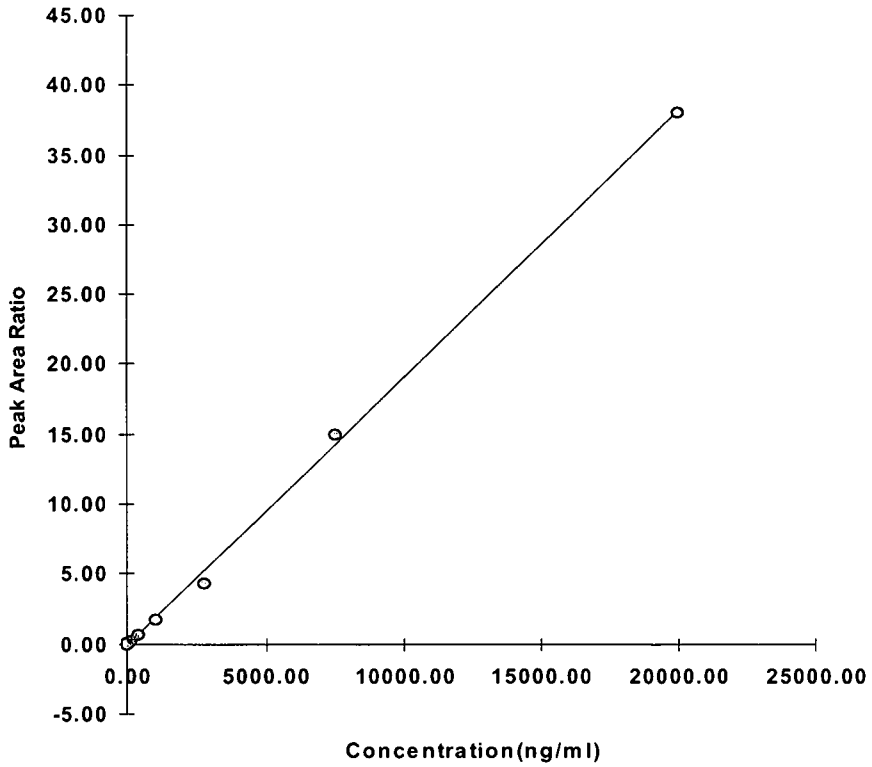


Figure 9: Concentration vs. Peak area ratio plot to determine LOQ of clarithromycin.

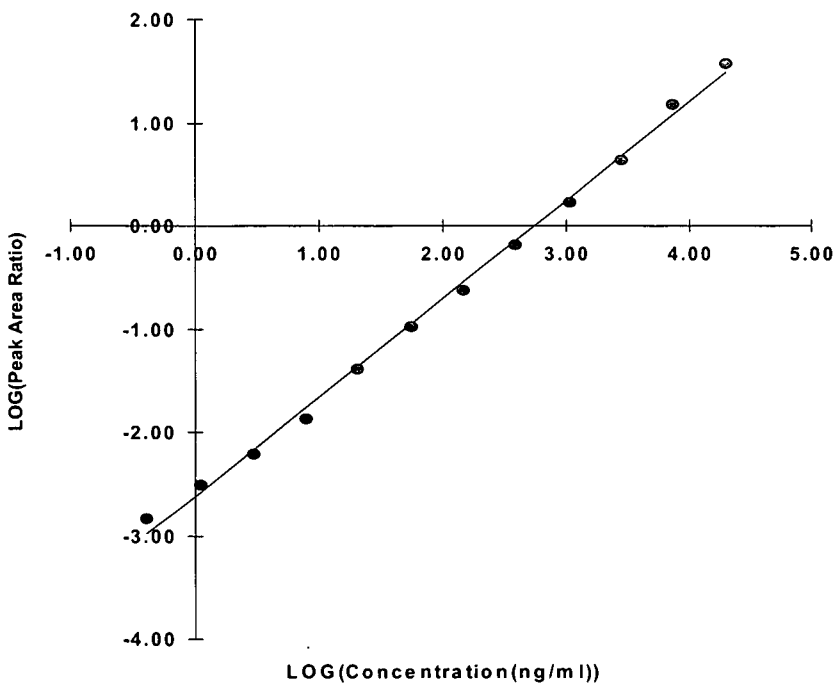


Figure 10: Log Concentration vs. Log Peak area ratio plot for clarithromycin.

Recovery experiments are particularly useful in testing the various stages of an analytical method and for assessing losses in sample processing, e.g., in solvent extraction. Extraction conditions should always be optimised by using the relevant biological fluid. Ideally, several recovery experiments should be performed by adding varying amounts of authentic standard to a blank specimen [25]. Spiking should cover the highest and lowest concentrations expected in the unknowns, because recovery may vary with concentration. Recovery is calculated as follow:

$$\text{Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100\%$$

Equation 2: Recovery.

Absolute recovery can be determined by comparing the average peak height or area for extracted plasma and or urine samples at each standard concentration of the drug, with that for un-extracted samples of identical concentrations made up in reconstitution fluid (preferably the mobile phase in the case of HPLC).

The accuracy, precision and LOD (limit of detection) of the method are greatly influenced by the recovery of the drug to be determined. The overall recovery for a standard taken through the entire assay procedure should be reproducible. Recovery values in the region of 50% are acceptable, although it is desirable to attain a recovery as close to 100% as possible [25]. If recovery is low but reproducible, it may still be acceptable. If, however, it is low, variable, and unpredictable, the reason should be investigated and eliminated, or an alternative approach to isolate the drug should be sought. Good recovery is aided by efficient solvent extraction, and by preventing adsorption losses.

Absolute recovery for clarithromycin was determined in triplicate at high, medium and low concentrations in normal plasma by extracting drug free plasma samples fortified with clarithromycin. Recovery was calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted analyte standards, representing 100% recovery.

The recovery of the internal standard should be measured independently.

Table 3: Absolute recovery of clarithromycin using chromatographic peak areas.

	ANALYTE	MEAN OF PEAK AREAS		RECOVERY	CV
	(ng/ml)	AFTER EXTRACTION	THEORETICAL VALUES	(%)	n = 6 (%)
RC_{max}	6 737	2657400	3303220.65	80.45	6.91
RC_{ave}	964	429320	472659.15	90.83	6.44
RC_{min}	8.78	3896	4304.92	90.51	12.27

Table 4: Absolute recovery of roxithromycin using chromatographic peak areas.

ISTD	MEAN OF PEAK AREAS		RECOVERY	CV
	AFTER	THEORETICAL		n = 6
(ng/ml)	EXTRACTION	VALUES	(%)	(%)
1 888	96020	188750	50.87	2.92

1.2.8. Sensitivity and Limit of Detection (LOD)

The term sensitivity is sometimes confused with the term; limit of detection (LOD). A method is said to be sensitive if a small change in concentration (**c**) causes a large change in instrumental response (**x**) [1]. Sensitivity is thus defined as the slope of the calibration line and, provided the plot is linear, can be measured at any point on it. The value for **a**, the intercept (see equation 1) of the calibration line on the y-axis (known as *Standard A*), can be used as the blank since it gives a more accurate estimate of the blank than a single measured value does. On the other hand, the limit of detection (LOD) of the method is calculated by using the section of plot near the origin and is arbitrarily defined as the lowest concentration or amount of analyte which can be distinguished from the blank with a stated degree of confidence. The detection limit (LOD) of an analytical method is the lowest analyte concentration that produces a response detectable above the noise [2] level of the system, typically three times the noise level [5]. The blank is also known as the background or noise. This interpretation is presented in figure 11 and expressed mathematically by the following equation:

$$C_L = \frac{k\sigma_B}{X_A} \times C_O$$

Equation 3: Detection Limit.

where C_L = detection limit concentration, X_A = analyte signal, **k** = constant (2-3), σ_B = the background standard deviation (noise), signal-to-noise = k/σ_B, C_O the concentration yielding the analyte signal (X_A) [1]. The constant **k** (2-3) represents the limit of detection (LOD). ♦

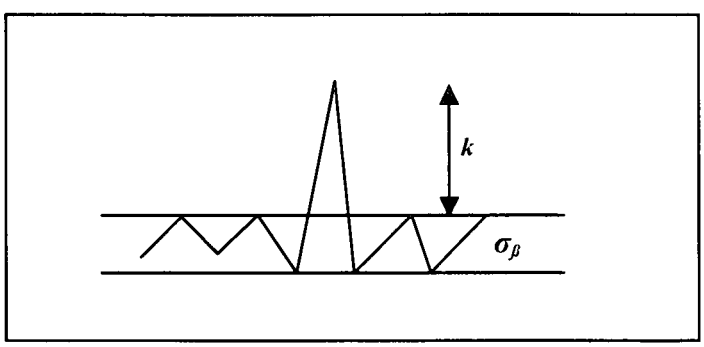


Figure 11: Analyte peak at detection limit.

♦ The noise measured should be the method noise, not only instrument noise.

The volume of sample needed to achieve the stated limit of detection should always be given in the publication to put the LOD in perspective with other methods. The limit of detection varies with different batches of blank control (plasma, urine, etc.), type of sample (e.g., presence of other drugs), and type and condition of instrument. Thus, for these reasons the limit of detection should not be rigidly fixed at the time the method is validated. It is necessary to reassess it whenever changes in conditions affecting it are suspected.

It is seldom advisable to use an instrument at its highest sensitivity setting or to work near the limit of detection of a method. High sensitivity of the instrument cannot always achieve better detection limits, because an increase in noise level would result in only a small increase in overall signal-to-noise ratio. The limit of detection value can often be improved by a different choice of experimental method, by adjusting the size of the sample, the reconstitution solvent, or the injection volume.

Limit of detection (LOD) should not be confused with limit of quantification (LOQ). Krull *et al.*, [27], defines limit of detection as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantitated, while limit of quantification is defined as the lowest concentration of the analyte that can be quantified with acceptable precision and accuracy. The limits of LOD and LOQ are determined from signal-to-noise ratios (s/n). The detection limit of an analytical method is typically three times the noise level [2, 3, 28], while for limit of quantification it is ten times the noise level as rule of thumb [1, 2, 27, 28].

"The practice of pushing the LOQ to the limits of the LOD in order to compete with other more sensitive methods should be avoided" - Dadgar *et al.*,[24]

1.2.9. Preparation of Calibration Standards

Clarithromycin stock solutions were prepared in methanol and used immediately to fortify blank plasma. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal plasma to a known concentration and then serially diluting it with normal blank plasma to attain the desired concentration range (20 016 ng/ml - 0.416 ng/ml).

Table 5: Preparation of Stock Solution SA for spiking STD M.

Solvent used	SG solvent	Mass analyte	Mass solvent	Volume solvent	Volume spiked	Concentration analyte
		(mg)	(g)	(ml)	(µl)	(µg/ml)
MeOH	0.791	11.762	5.891	7.448	500	1 579

Table 6: Preparation of standards from spiking solution SA.

Sample Code & No.	Source Solution	A	B	C	D (ng/ml)
STD M	Stock SA	108.613	148.613		20 016
STD L	STD M	105.916	125.912	137.910	7 506
STD K	STD L	105.166	125.163	137.180	2 818
STD J	STD K	106.661	126.664	138.695	1 058
STD I	STD J	104.013	124.017	136.011	397
STD H	STD I	106.864	126.869	138.879	149
STD G	STD H	106.894	126.888	138.889	55.8
STD F	STD G	107.104	127.104	139.119	20.9
STD E	STD F	105.423	125.420	137.427	7.86
STD D	STD E	105.767	125.760	137.766	2.95
STD C	STD D	105.917	125.906	137.905	1.11
STD B	STD C	105.430	125.432	137.471	0.416

∇

A = Mass of empty container.
B = Mass of container and normal biological fluid.
C = Total mass of container plus normal plus spiked biological fluid.
D = Concentration (ng/ml).

∇ Mass of biological fluid (g) is converted to volume (ml). SG = 1.0269 for plasma.

1.2.10. Preparation of Quality control Standards

Table 7: Preparation of stock QA for spiking QC K.

Solvent used	SG solvent	Mass analyte	Mass solvent	Volume solvent	Volume spiked	Concentration analyte
		(mg)	(g)	(ml)	(µl)	(µg/ml)
MeOH	0.791	8.029	4.010	5.070	450	1 584

Table 8: Preparation of quality controls from spiking solution QA.

Sample	Source	A	B	C	D
Code & No.	**Solution**				(ng/ml)
QC K	Stock SA	56.362	96.339		18 098
QC J	QC K	56.768	76.801	88.679	6 737
QC I	QC J	55.186	75.127	87.164	2 536
QC H	QC I	56.963	76.942	89.193	964
QC G	QC H	57.547	77.788	89.805	359
QC F	QC G	57.373	77.356	89.365	135
QC E	QC F	57.990	83.894	89.315	23.3
QC D	QC E	57.569	77.534	89.579	8.78
QC C	QC D	56.776	76.473	87.338	3.12
QC B	QC C	57.849	77.804	89.852	1.17
QC A	QC B	58.928	79.093	91.040	0.437

A = Mass of empty container.
B = Mass of container and normal biological fluid.
C = Total mass of container plus normal plus spiked biological fluid.
D = Concentration (ng/ml).

Since it was decided to lift the limit of quantification (from 0.416 to 2.95 ng/ml), all the standards and quality controls were renamed, because the quantitation software that FARMOVS-PAREXEL is using allows for only 9 concentration levels to be entered, so that QC A = QC C (*old*) = 3.12 ng/ml. The same was done for the calibration standards. The prepared calibration standards and quality control standards were aliquoted into polypropylene tubes and stored in a freezer at -20°C pending analysis.

1.2.11. Accuracy

The accuracy (BIAS) of the method is the closeness of the measured value to the true value [3, 4]. Accuracy is usually determined in a couple of ways. First, analysing a sample of known concentration, and comparing the measured value to the true value can assess accuracy. Another approach is to compare test results from the new method with results from an existing alternative method that is known to be accurate [3, 29].

According to the Food and Drug Administration Centre for Drug Evaluation and Research [30], accuracy should be measured using a minimum of five determinations per concentration. Usually three concentrations (a low, medium and high value of the analytical range) are tested to determine whether or not accuracy is concentration-dependant. At least six replicate assays are required to show accuracy according to Jenke [4]. The mean value should be within 15% of the actual value except at LOQ, where it should not deviate by more than 20% [5]. The deviation from the true values serves as a record of the method's accuracy.

"The information provided by an analysis method can only be considered as useful if the difference between the mean measurement result and the real analyte concentration, which is called the BIAS, is acceptably small." – Penninkx [29]

Bruce *et al.*, [1] tabulated some of the possible error sources causing a biased measurement:

(1) Sampling
- Analyte is not representative
- The sample is contaminated
- The analyte is degraded or lost during sampling/storage

(2) Sample preparation
- Analyte unstable in solutions
- Analyte does not extract from matrix
- Incomplete removal of disturbing components
- Contamination via impurities
- Incomplete derivative reaction

(3) Calibration
- Wrong calibration technique
- Sample concentrations does not coincide with method linear range
- Unsuitable internal standard
- Unreliable integration technique
- Random error of measurement (noise)

(4) Sample analysis
- Matrix effect
- Instability of the analysis system

1.2.12. Precision

The precision or reproducibility of an analytical method is the amount of scatter in the results obtained from multiple analysis of a homogeneous sample [3]. To be meaningful, the precision study must be performed using the exact sample and standard preparation procedures that will be used in the final method.

According to the United States Department of Health and Human Services Food and Drug Administration (FDA) [30], the precision determined at each concentration level should not exceed 15% (CV), except for the limit of quantification (LOQ), where the CV should not exceed 20%.

It must be emphasised that good precision does not necessarily imply good accuracy: a systematic error may lead to precise but inaccurate results. The bigger the standard deviation (RSD), the poorer the precision of the measurement. The standard deviation is an indication of how stable the equipment is at the actual measurement.

Repeatability and reproducibility is just a further indication of the method's precision. A method is repeatable if the same sample is analysed with the same procedure, using the same instrument by the same analyst and producing the same result. Whereas a method is reproducible if the result is produced by the same procedure, a different instrument, at different times and by a different analyst and producing the same result [1].

According to Hokanson [3], precision could be divided into three components; system precision, method precision (intraday validation) and day-to-day precision (interday validation). A poor system precision will lead to poor reproducibility, and it is important to assess system suitability daily. To assess method precision/reproducibility, at least six of the same homogeneous samples (6 QC samples per level) should be tested. For day-to-day precision (interday validation), the same samples used for method precision (intraday validation) should be used (freshly prepared). The analysis should be carried out by the same analyst, using the same equipment, but using fresh samples, standards, mobile phase and reagents [3, 26].

1.2.13. Specificity

The specificity of the assay of the drug and its major metabolites should be thoroughly investigated in the method development phase. The constituents of the biological fluids and the presence of other drugs, if any, should not interfere with the determination. A method producing a response for only a single analyte can be considered specific, while a method producing responses for several analytes, but able to distinguish between them, can be called selective [1].

Specificity can be incorporated into a work-up step (selective extraction) and/or the analytical technique (chromatographic separation and use of specific detectors). The specificity of the method should be determined with at least six independent sources of the same matrix [5].

The most important parameters describing the method's specificity is described by Bruce *et al.*, [1] as resolution, relative retention, symmetry and the number of theoretical plates.

It has been noted that co-eluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy [1] of the LC-MS/MS assay. Interfering compounds may strengthen or weaken the signal, and the magnitude may also depend on the concentration. To determine whether this effect (called the matrix effect) is present or not, 10 different blank plasma pools were extracted and the reconstituted extracts then spiked with a known concentration of analyte. These samples were assayed and their peak areas compared (table 9). If no matrix without the analyte is available (blank plasma), one can compare the slopes of the standard addition curves.

Table 9: Matrix effect data for clarithromycin and roxithromycin (ISTD).

Plasma Pool	Clarithromycin Peak Area (1 579 ng/ml)	ISTD Peak Area (1 315 ng/ml)
1	1069000	334400
2	1067000	323600
3	1027000	322800
4	1173000	349900
5	1150000	340400
6	1157000	348600
7	1353000	388800
8	1134000	334900
9	1108000	321600
10	1206000	335900
Mean	1144400	340090
CV%	7.58	5.53

The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect. The data in table 9 show that no significant matrix effect (CV% = 7.58 and 5.53 for clarithromycin and internal standard, respectively) were present at the tested concentrations.

The very high specificity of a 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. Due to this high specificity of MS/MS detection, no interfering or late eluting peaks were found when assaying blank plasma extracts from six different sources when taking special precautions to avoid carry-over. As mentioned earlier, carry-over was a problem when time consuming special precautions were not taken.

1.2.14. Stability

During method development and literature survey, the method developer gains some information on the stability of the analyte, reagents, mobile phases and solutions. For routine testing in which many samples are prepared and analysed each day, it is often essential that solutions be stable enough to allow for delays such as instrument breakdowns or overnight analyses using autosamplers. Standards and samples should be tested over at least a 48-hour period, and quantitation of components should be determined by comparison to freshly prepared standards. If the solutions are not stable over the 48-hour period, storage conditions or additives should be identified that can improve stability [24].

Acceptable stability is less than 20% change in standard or sample response at the limit of quantification (LOQ), relative to freshly prepared standards. The mobile phase is considered to have acceptable stability if aged mobile phase produces equivalent chromatography and if impurity results at the limit of quantification are within 20% of the values obtained with fresh mobile phase [24].

- Freeze and Thaw Stability

According to the FDA, analyte stability should be determined after three [Ω] freeze and thaw cycles [30]. Aliquots at each of the low and high concentrations should be stored at the intended storage temperature and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen. The freeze-thaw cycle should be repeated two more times. If an analyte is unstable at the intended storage temperature, the samples should be frozen at -70°C [♠ 24].

Chu *et al.*, reported that clarithromycin is stable in plasma and urine for at least five (5) freeze-thaw cycles [15].

To ascertain freeze-thaw stability, aliquots of quality control samples at 6 829 ng/ml (QC G) and 8.73 ng/ml (QC B) were allowed to thaw and then immediately re-frozen. These quality controls were assayed the following day together with a set of the same quality controls (QC G and QC B) that had not been subjected to this additional freeze-thaw cycle. The results are summarised in table 10, where a freeze-thaw-freeze-thaw (F/T/F/T) cycle was compared to a freeze-thaw (F/T) cycle, using the area ratios of the analyte/internal standard at a high concentration (6 829 ng/ml) and a low (8.73 ng/ml) concentration. From these results we conclude that clarithromycin is stable in plasma over at least two freeze-thaw cycles.

Ω FDA serves as a guide only.

Table 10: Freeze thaw stability of clarithromycin

High Concentration (6 829 ng/ml)	(F/T/F/T)	(F/T)	Low Concentration (8.73 ng/ml)	(F/T/F/T)	(F/T)
1	6.677546	6.955363	*1*	0.025530	0.024311
2	6.892744	6.963494	*2*	0.024299	0.027144
3	6.772616	6.648668	*3*	0.023705	0.028253
4	6.841626	7.105839	*4*	0.025438	0.025809
5	6.801128	7.133946	*5*	0.026400	0.027661
Mean	6.797132	6.961462		0.025074	0.026636
% Diff.	2.36			5.86	

- Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis [24].

Chu *et al.*, reported that frozen clarithromycin plasma samples are stable up to two years [15]. Borner *et al.*, [16] reported that clarithromycin is stable at –80°C,* without additives for at least 6 months, while Hedenmo and Eriksson [17] reported clarithomycin to be stable at –20°C and –70°C for at least 2 months. Serum samples containing clarithromycin were reported to be stable for seven days at –20°C by Sastre Torano and Guchelaar [19].

Table 11: Matrix stability of clarithromycin.

Matrix Stability					
C_{max} Nominal Conc.	Measured Conc. (ng/ml) C_{max}		$0.2C_{max}$ Nominal Conc.	Measured Conc. (ng/ml) $0.2C_{max}$	
(ng/ml)	(-20°C)	(-70°C)	(ng/ml)	(-20°C)	(-70°C)
100	75	90	***20***	16	20
100	80	80	***20***	15	17
100	85	100	***20***	14	23
100	87	100	***20***	17	20
100	80	100	***20***	18	20
Mean	81	94	***Mean***	16	20
Std.Dev.	4.72	8.94	***Std.Dev.***	1.58	2.12
CV%	5.80	9.52	***CV%***	9.88	10.61
%Nom.	81.40	94.00	***%Nom.***	80.00	100.00

Table 11 shows the data for long-term frozen stability (2 months) of clarithromycin. As shown, stability was tested at C_{max} and 0.2 x C_{max}, both at –20°C and –70°C. From these data it is apparent that clarithromycin is stable for two months at –70°C, but not at –20°C.

* According to the Arrhenius equation, stability is assured at very low temperatures, with activation energies 10 000 times lower as at –20°C.

Although Sastre Torano and Guchelaar [19] found that the stock solutions prepared in acetonitrile were stable for at least 21 days, the stock solutions were freshly prepared in methanol and used immediately for the fortification of normal biological fluid. The stock solutions were not retained for further use.

- Post-Preparative Stability (On-instrument stability)

The stability of processed samples, for both analyte and internal standard should be determined to assess the resident time in the autosampler. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards[24].

Chu *et al.*, [15] reported that clarithromycin is on-instrument stable at ambient temperature for at least 30 hours, while Hedenmo and Eriksson [17] reported that clarithromycin is stable for at least 24 hours while refrigerated (4°C).

To assure post-preparative stability, sixteen stability samples of the same concentration were extracted. The extracts were combined, re-aliquoted and injected at intervals during the validation batch to simulate the time of a batch run. The peak area ratios were plotted against the cumulative time as depicted in the graph below. By regression analysis of the tabulated data below it can be established that the clarithromycin to internal standard response tends to increase by 6.66% over a period of 9.37 hours while awaiting injection on-instrument (figure 12). This trend is deemed insignificant and the extracts are considered to be stable on-instrument for at least 9 hours (duration of a batch).

Table 12: Determining on-instrument stability.

Replicates	Injection Time	Time Difference	Cumulative Time (hr)	Analyte Peak Area	IS Peak Area	Peak Area Ratio
1	3:19		0.00	245500.00	135700	1.809
2	3:56	0:37:00	0.62	231500.00	124600	1.858
3	4:38	0:42:00	1.32	229200.00	124800	1.837
4	5:23	0:45:00	2.07	225600.00	124900	1.806
5	6:00	0:37:00	2.68	282000.00	155000	1.819
6	6:37	0:37:00	3.30	303400.00	164400	1.845
7	7:23	0:46:00	4.07	201900.00	116800	1.729
8	7:27	0:04:00	4.13	211400.00	123700	1.709
9	8:34	1:07:00	5.25	242500.00	123300	1.967
10	9:11	0:37:00	5.87	230300.00	120900	1.905
11	9:52	0:41:00	6.55	230900.00	118300	1.952
12	10:38	0:46:00	7.32	226900.00	117800	1.926
13	11:15	0:37:00	7.93	230700.00	119000	1.939
14	11:52	0:37:00	8.55	244600.00	127400	1.920
15	12:37	0:45:00	9.30	241300.00	127800	1.888
16	12:41	0:04:00	9.37	231400.00	124000	1.866
			Mean	238068.75	128025.00	1.86
			Std Dev	24437.70	13308.17	0.08
			CV	10.26%	10.39%	4.04%

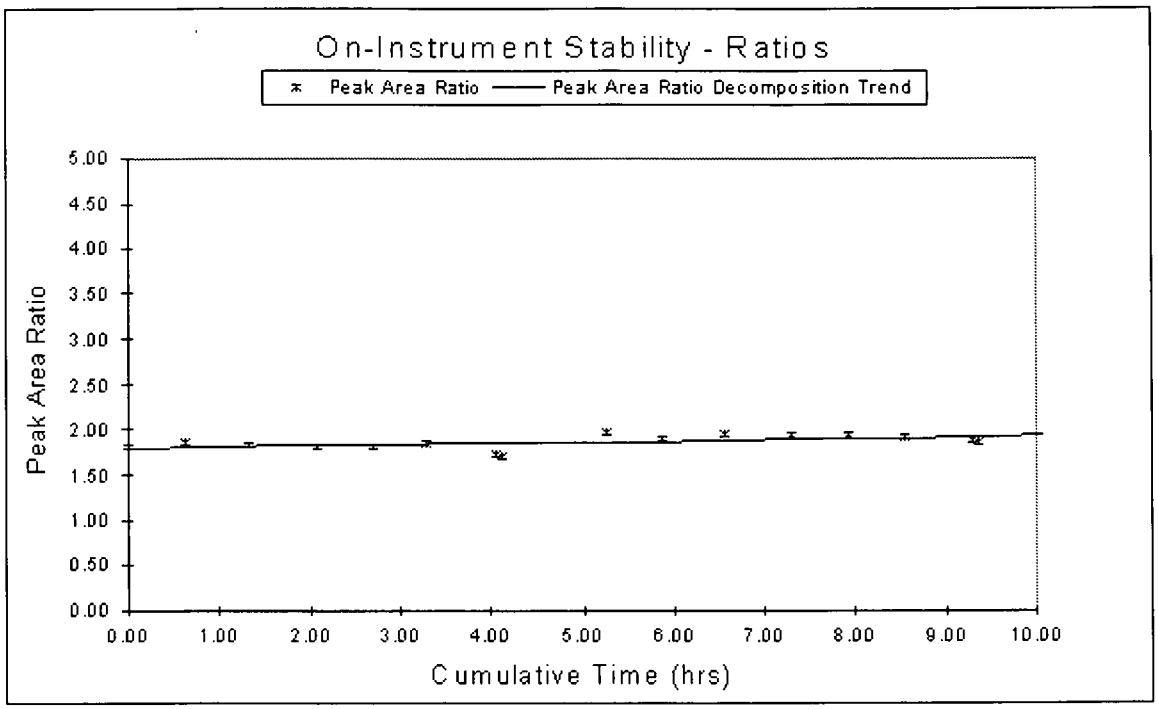


Figure 12: On-instrument stability plot of clarithromycin-roxithromycin area ratios

1.2.15. Ruggedness or Robustness [♥]

Ruggedness or robustness is merely another term for stability of the entire method. Hokanson regards robustness as a fourth phase of precision, changing equipment, analyst, mobile phase etc. Changing laboratories (inter-laboratory validation) is regarded as the ultimate ruggedness test [3].

1.2.16. Actual Validation

The method was validated by assaying clarithromycin plasma quality control samples in six-fold at 3.12, 8.78, 23.3, 135, 359, 964, 2 536, 6 737 and 18 098 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve, composed of nine different concentrations, to assess the methods accuracy and precision, spanning the concentration range 2.95 - 20 016 ng/ml for clarithromycin. Calibration graphs were constructed using peak area ratios of the relevant product ions versus nominal concentrations using Wagner [$\ln y = a(\ln x)^2 + b(\ln x) + c$] calibration curves.

[♥] British English

Intra-Batch Accuracy and Precision

Intra-batch accuracy and precision was assessed by the assay of two sets of all the calibration standards 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 was 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. Quantification by peak height ratios or peak area ratios requires using the internal standard, whereas quantification by peak heights or peak areas requires omission of the internal standard.

Table 13: A typical batch structure for an intra-day validation.

Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample
1	SYS	21	STD K	41	STD H	61	STAB 4	81	QC K DIL	101	QC I
2	STD M	22	STD K	42	STD H	62	blank 4	82	QC J	102	QC H
3	STD M	23	STD J	43	STAB 3	63	QC K	83	QC I	103	QC G
4	STD L	24	STD J	44	BLANK 3	64	QC K DIL	84	QC H	104	QC F
5	STD L	25	STAB 2	45	QC K	65	QC J	85	QC G	105	QC E
6	STAB 1	26	BLANK 2	46	QC K DIL	66	QC I	86	QC F	106	QC D
7	BLANK 1	27	QC K	47	QC J	67	QC H	87	QC E	107	QC C
8	ZERO 1	28	QC K DIL	48	QC I	68	QC G	88	QC D	108	QC B
9	QC K	29	QC J	49	QC H	69	QC F	89	QC C	109	QC A
10	QC K DIL	30	QC I	50	QC G	70	QC E	90	QC B	110	STD B
11	QC J	31	QC H	51	QC F	71	QC D	91	QC A	111	STD B
12	QC I	32	QC G	52	QC E	72	QC C	92	STD D	112	SYS
13	QC H	33	QC F	53	QC D	73	QC B	93	STD D	113	ZERO 2
14	QC G	34	QC E	54	QC C	74	QC A	94	STAB 6	114	STAB 7
15	QC F	35	QC D	55	QC B	75	STD E	95	BLANK 6	115	STAB 8
16	QC E	36	QC C	56	QC A	76	STD E	96	STD C		
17	QC D	37	QC B	57	STD G	77	STAB 5	97	STD C		
18	QC C	38	QC A	58	STD G	78	SYS	98	QC K		
19	QC B	39	STD I	59	STD F	79	BLANK 5	99	QC K DIL		
20	QC A	40	STD I	60	STD F	80	QC K	100	QC J		

Accuracy is expressed as either percentage deviation from nominal (BIAS%) or as percentage of nominal. Precision is expressed as coefficient of variation (CV %).

- Quantification by Peak Height Ratios

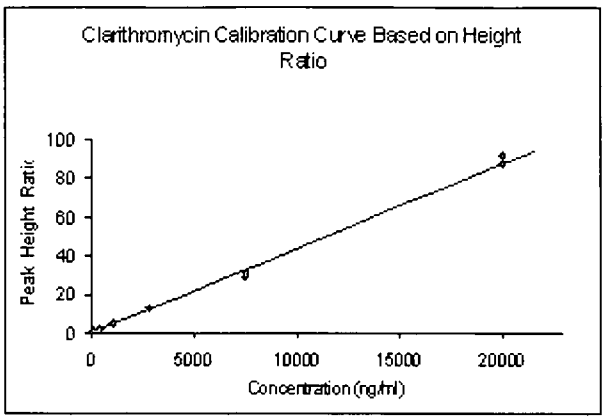


Figure 13: Quantification by Peak Height Ratios: Clarithromycin.

Calibration Standards used: STD B - STD K
Calibration Range: 2.95 – 20 016 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: 0.004428
b: 0.918210
c: -5.057637
r^2: 0.999557

Table 14: Quantification by Peak Height Ratios: Clarithromycin.

STD Code	Nominal Conc (ng/ml)	Back-calculated Conc (ng/ml)	% Bias
STD K	20 016	20 090	0.4
STD K	20 016	21 045	5.1
STD J	7 506	6 597	-12.1
STD J	7 506	7 221	-3.8
STD I	2 818	2 911	3.3
STD I	2 818	2 952	4.8
STD H	1 058	1 089	2.9
STD H	1 058	1 049	-0.9
STD G	397	410	3.3
STD G	397	396	-0.2
STD F	149	163	9.7
STD F	149	154	3.4
STD E	55.8	52.5	-5.9
STD E	55.8	57.3	2.6
STD D	20.9	20.0	-4.3
STD D	20.9	19.1	-8.5
STD C	7.86	7.90	0.6
STD C	7.86	7.06	-10.2
STD B	2.95	3.23	9.5
STD B	2.95	3.06	3.8

Table 15: Summary of Intra-batch Quality Control Results, using peak height ratios.

Code	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	18 098	6 737	2 536	964	359	135	23.3	8.78	3.12
Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
1	15229.67	6323.21	2769.87	1028.07	409.19	140.26	23.99	10.8	3.61
2	15778.28	6277.48	2506.65	1031.41	352.60	144.42	22.76	7.71	3.34
3	15241.95	6025.19	2539.35	1006.76	397.45	138.04	23.07	7.73	3.29
4	15257.79	6002.67	2354.56	965.90	346.45	140.10	21.84	8.57	3.15
5	15485.86	6258.81	2498.45	886.01	351.97	146.34	23.70	8.90	3.71
6	13597.52	5350.63	2235.99	944.95	369.72	128.50	25.24	8.07	3.36
MEAN	15098.51	6039.67	2484.15	977.18	371.23	139.61	23.43	8.62	3.41
%nom	83.4	89.6	98.0	101.4	103.4	103.4	100.6	98.2	109.3
CV%	4.6	5.5	6.7	5.3	6.5	4.1	4.5	12.1	5.6

- Quantification by Peak Area Ratios

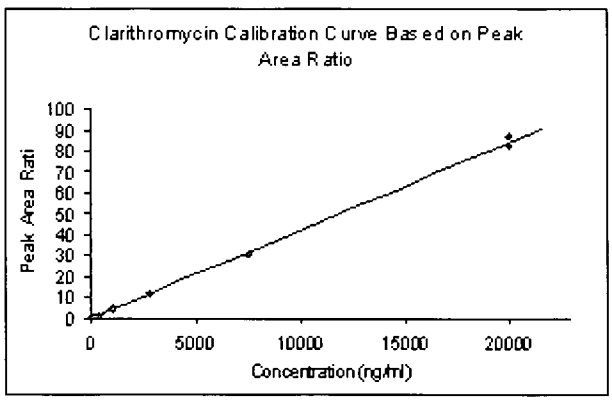


Figure 14: Quantification by Peak Area Ratios: Clarithromycin

Calibration Standards used: STD B - STD K
Calibration Range: 2.95 – 20 016 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: 0.000955
b: 0.979998
c: -5.366874
r^2: 0.999714

Table 16: Quantification by Peak Area Ratios: Clarithromycin.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD K	20 016	19 604	-2.1
STD K	20 016	20 562	2.7
STD J	7 506	7 220	-3.8
STD J	7 506	7 315	-2.5
STD I	2 818	2 776	-1.5
STD I	2 818	2 822	0.1
STD H	1 058	1 136	7.4
STD H	1 058	1 080	2.1
STD G	397	408	2.6
STD G	397	407	2.6
STD F	149	157	5.3
STD F	149	147	-1.2
STD E	55.8	51.8	-7.3
STD E	55.8	57.2	2.5
STD D	20.9	21.0	-1.6
STD D	20.9	19.2	-8.4
STD C	7.86	8.14	3.5
STD C	7.86	7.19	-8.5
STD B	2.95	3.25	10.0
STD B	2.95	2.95	0.1

Table 17: Summary of Intra-batch Quality Control Results, using peak area ratios.

Code	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	18 098	6 737	2 536	964	359	135	23.3	8.78	3.12
Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
1	17176.56	6454.05	2722.06	1039.87	394.37	139.01	25.21	9.84	3.31
2	16440.00	6616.30	2644.04	1055.22	359.53	143.26	23.12	8.19	3.27
3	16499.76	6404.71	2549.73	946.56	371.61	135.83	22.08	7.56	3.06
4	16836.02	6474.69	2393.71	1002.74	368.84	141.90	22.41	8.29	3.46
5	16315.37	6235.33	2460.43	921.06	358.94	141.21	23.60	9.68	3.46
6	15093.19	5574.62	2305.43	987.98	357.75	129.97	24.19	8.12	3.44
MEAN	16393.48	6293.28	2512.57	992.24	368.51	138.53	23.44	8.61	3.33
%nom	90.6	93.4	99.1	102.9	102.6	102.6	100.6	98.1	106.8
CV%	4.0	5.4	5.7	4.8	3.4	3.3	4.5	9.8	4.3

- Quantification by Peak Heights (without ISTD)

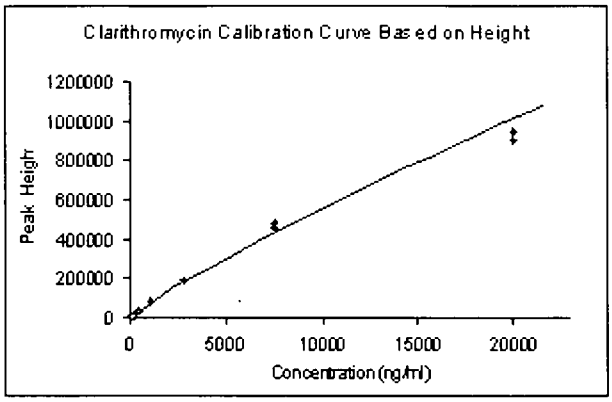


Figure 15: Quantification by Peak Heights: Clarithromycin

Calibration Standards used: STD B - STD K
Calibration Range: 2.95 – 20 016 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.008090
b: 1.016308
c: 4.554554
r^2: 0.999317

Table 18: Quantification by Peak Heights: Clarithromycin.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD K	20016	17 480	-12.7
STD K	20016	18 500	-7.6
STD J	7506	7 939	5.8
STD J	7506	8 487	13.1
STD I	2818	2 951	4.7
STD I	2818	2 971	5.4
STD H	1058	1 121	6.0
STD H	1058	1 048	-0.9
STD G	397	395	-0.5
STD G	397	390	-1.6
STD F	149	155	3.7
STD F	149	153	3.0
STD E	55.8	48.9	-12.4
STD E	55.8	55.3	-0.9
STD D	20.9	20.2	-3.3
STD D	20.9	20.9	0.2
STD C	7.86	7.72	-1.7
STD C	7.86	6.82	-13.2
STD B	2.95	3.18	7.9
STD B	2.95	3.26	10.6

Table 19: Summary of Intra-batch Quality Control Results, using peak heights.

Code	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	18098	6737	2536	964	359	135	23.3	8.78	3.12
Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
1	14953.15	7751.21	3002.08	897.34	377.02	131.18	19.55	9.14	3.12
2	17015.34	7100.74	2739.58	1098.87	319.67	113.16	17.87	6.43	3.16
3	18916.64	7601.74	3012.57	1008.30	346.93	139.49	21.84	7.40	3.48
4	17967.62	7086.95	2651.65	973.95	333.68	140.13	23.42	8.56	3.21
5	19256.16	7454.69	2610.40	992.56	335.32	137.98	23.11	8.91	3.73
6	17019.84	6845.29	2933.98	1054.47	347.34	119.54	23.04	7.29	3.51
MEAN	17521.46	7306.77	2825.04	1004.25	343.33	130.25	21.47	7.96	3.37
%nom	96.8	108.5	111.4	104.2	95.6	96.5	92.2	90.6	108.0
CV%	8.2	4.4	5.8	6.3	5.2	8.0	9.6	12.3	6.6

- Quantification by Peak Areas (without ISTD)

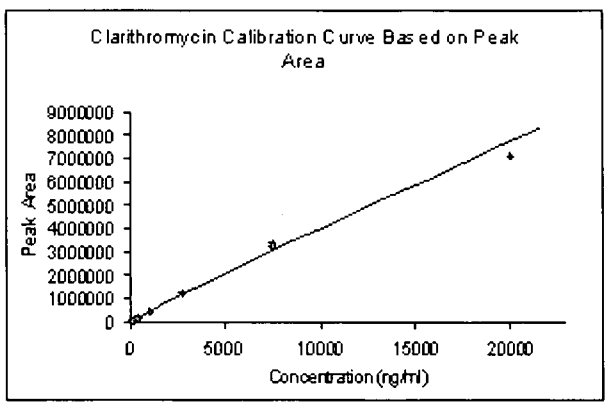


Figure 16: Quantification by Peak Areas: Clarithromycin

Calibration Standards used: STD B - STD K
Calibration Range: 2.95 – 20 016 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.004932
b: 1.039081
c: 6.055410
r^2: 0.999503

Table 20: Quantification by Peak Areas: Clarithromycin.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD K	20016	17 480	-12.7
STD K	20016	18 500	-7.6
STD J	7506	7 939	5.8
STD J	7506	8 486	13.1
STD I	2818	2 951	4.7
STD I	2818	2 970	5.4
STD H	1058	1 121	6.0
STD H	1058	1 048	-0.9
STD G	397	395	-0.5
STD G	397	390	-1.6
STD F	149	154	3.7
STD F	149	153	3.0
STD E	55.8	48.7	-12.4
STD E	55.8	55.3	-0.9
STD D	20.9	20.2	-3.3
STD D	20.9	20.9	0.2
STD C	7.86	7.72	-1.7
STD C	7.86	6.82	-13.2
STD B	2.95	3.18	7.9
STD B	2.95	3.26	10.6

Table 21: Summary of Intra-batch Quality Control Results, using peak areas.

Code	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal	18098	6737	2536	964	359	135	23.3	8.78	3.12
Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
1	15416.75	7333.43	2926.24	982.40	415.17	149.45	23.88	9.99	3.27
2	15047.71	6553.75	2667.40	1091.98	336.89	119.75	20.39	7.38	3.19
3	15548.89	6311.17	2616.26	911.40	338.46	139.43	22.15	7.50	3.36
4	14434.86	6102.22	2300.33	927.68	342.96	141.19	24.77	8.65	3.46
5	14910.48	6030.97	2258.02	945.59	327.22	133.64	23.19	9.45	3.43
6	13997.78	5728.67	2497.13	974.71	316.44	120.52	23.15	7.56	3.55
MEAN	14892.75	6343.37	2544.23	972.29	346.19	134.00	22.92	8.42	3.38
% nom	82.3	94.2	100.3	100.9	96.4	99.3	98.4	95.9	108.2
CV%	3.6	8.0	8.9	6.1	9.3	8.1	6.0	12.1	3.6

Inter-Batch Accuracy and Precision

Inter-batch accuracy and precision was assessed by the assay of two separate consecutive batches, each consisting of two sets 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 as determined by the first intra-batch accuracy and precision measurements. Each of the inter-batch validation batches must pass the criteria for intra-batch acceptance. Calculating the accuracy and precision statistics over the intra- and inter-batch validation batches (3 in total) assesses the inter-batch accuracy and precision of the assay procedure.

Table 22: A typical batch structure for the first inter-batch validation.

Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample
1	SYS	**21**	QC K	**41**	STD G	**61**	QC H	**81**	SYS
2	STD M	**22**	QC J	**42**	STD G	**62**	QC F	**82**	STAB 15
3	STD M	**23**	QC H	**43**	STD F	**63**	QC D	**83**	STAB 16
4	STD L	**24**	QC F	**44**	STD F	**64**	QC B		
5	STD L	**25**	QC D	**45**	STAB 12	**65**	QC A		
6	STAB 9	**26**	QC B	**46**	BLANK 4	**66**	STD D		
7	BLANK 1	**27**	QC A	**47**	QC K	**67**	STD D		
8	QC K	**28**	STD I	**48**	QC J	**68**	STAB 14		
9	QC J	**29**	STD I	**49**	QC H	**69**	BLANK 6		
10	QC H	**30**	STD H	**50**	QC F	**70**	STD C		
11	QC F	**31**	STD H	**51**	QC D	**71**	STD C		
12	QC D	**32**	STAB 11	**52**	QC B	**72**	QC K		
13	QC B	**33**	BLANK 3	**53**	QC A	**73**	QC J		
14	QC A	**34**	QC K	**54**	STD E	**74**	QC H		
15	STD K	**35**	QC J	**55**	STD E	**75**	QC F		
16	STD K	**36**	QC H	**56**	STAB 13	**76**	QC D		
17	STD J	**37**	QC F	**57**	SYS	**77**	QC B		
18	STD J	**38**	QC D	**58**	BLANK 5	**78**	QC A		
19	STAB 10	**39**	QC B	**59**	QC K	**79**	STD B		
20	BLANK 2	**40**	QC A	**60**	QC J	**80**	STD B		

- Inter-batch Validation 1 (using peak area ratios)

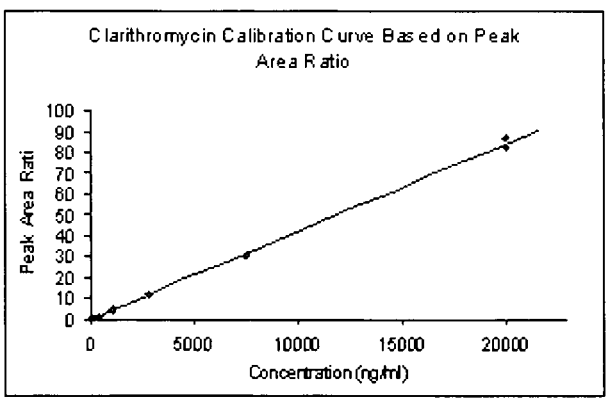


Figure 17: Inter-batch Validation 1

Calibration Standards used: STD B - STD K
Calibration Range: 2.95 – 20 016 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: 0.002187
b: 0.951214
c: -5.372676
r^2: 0.999831

Table 23: Inter-batch Validation 1.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD J	7 506	7 457	-0.6
STD J	7 506	7 359	-1.9
STD I	2 818	2 823	0.2
STD I	2 818	2 862	1.6
STD H	1 058	1 048	-0.9
STD H	1 058	1 082	2.3
STD G	397	393	-1.0
STD G	397	394	-0.6
STD F	149	155	4.0
STD F	149	148	-0.2
STD E	55.8	59.1	5.9
STD E	55.8	55.4	-0.6
STD D	20.9	20.9	0.2
STD D	20.9	19.8	-5.3
STD C	7.86	7.39	-6.0
STD C	7.86	7.46	-5.1
STD B	2.95	3.08	4.3
STD B	2.95	3.09	4.8

Table 24: Summary of Quality Control Results obtained for inter batch 1.

Code	QC H	QC G	QC F	QC B	QC A
Nominal	6737	2536	964	8.78	3.12
Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
1	6823.37	2623.07	1046.46	8.18	3.60
2	6658.07	2726.97	962.07	10.03	3.48
3	6497.35	2487.91	957.92	8.60	2.95
4	6719.58	2442.59	945.62	10.02	3.05
5	6417.23	2619.90	998.93	8.71	3.21
6	6444.82	2516.57	958.95	8.56	3.52
MEAN	6593.40	2569.50	978.33	9.02	3.30
%nom	97.9	101.3	101.5	102.7	105.8
CV%	2.3	3.8	3.5	8.1	7.5

Table 25: A typical batch structure for the second inter-batch validation.

Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample	Inj. no.	Sample
1	SYS	21	QC H	41	STD F	61	STD D
2	STD M	22	QC F	42	BLANK 4	62	STD D
3	STD M	23	QC D	43	QC K	63	BLANK 6
4	STD L	24	QC B	44	QC J	64	STD C
5	STD L	25	QC A	45	QC H	65	STD C
6	BLANK 1	26	STD I	46	QC F	66	QC K
7	QC K	27	STD I	47	QC D	67	QC J
8	QC J	28	STD H	48	QC B	68	QC H
9	QC H	29	STD H	49	QC A	69	QC F
10	QC F	30	BLANK 3	50	STD E	70	QC D
11	QC D	31	QC K	51	STD E	71	QC B
12	QC B	32	QC J	52	SYS	72	QC A
13	QC A	33	QC H	53	BLANK 5	73	STD B
14	STD K	34	QC F	54	QC K	74	STD B
15	STD K	35	QC D	55	QC J	75	SYS
16	STD J	36	QC B	56	QC H		
17	STD J	37	QC A	57	QC F		
18	BLANK 2	38	STD G	58	QC D		
19	QC K	39	STD G	59	QC B		
20	QC J	40	STD F	60	QC A		

- Inter-batch Validation 2 (using peak area ratios)

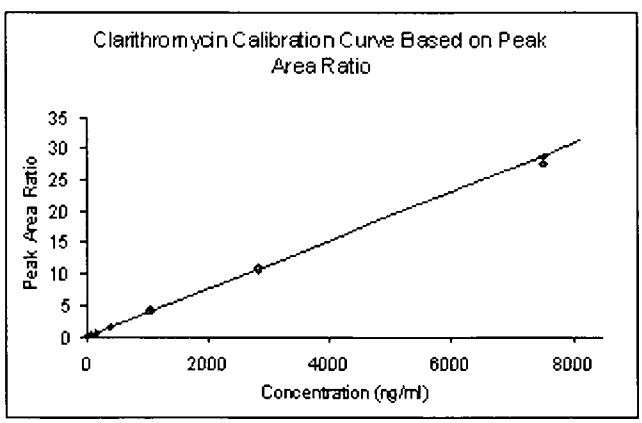


Figure 18: Inter-batch Validation 2

Calibration Standards used: STD B - STD K
Calibration Range: 2.95 – 20 016 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: 0.004174
b: 0.932155
c: -5.284745
r^2: 0.999636

Table 26: Inter-batch Validation 2.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD J	7 506	7 461	-0.6
STD J	7 506	7 182	-4.3
STD I	2 818	2 782	-1.2
STD I	2 818	2 866	1.7
STD H	1 058	1 070	1.2
STD H	1 058	1 113	5.2
STD G	397	408	2.8
STD G	397	408	3.0
STD F	149	147	-0.9
STD F	149	147	-1.3
STD E	55.8	55.3	-0.9
STD E	55.8	56.0	0.4
STD D	20.9	20.8	-0.2
STD D	20.9	20.2	-3.1
STD C	7.86	7.19	-8.6
STD C	7.86	7.61	-3.2
STD B	2.95	3.45	16.9
STD B	2.95	2.81	-4.7

Table 27: Summary of Quality Control Results obtained for inter-batch validation 2.

Code	QC H	QC G	QC F	QC B	QC A
Nominal	6 737	2 536	964	8.78	3.12
Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
1	6974.78	2534.99	1016.89	10.70	4.27
2	6385.85	2559.68	1018.70	9.40	3.46
3	6293.02	2450.20	1028.39	9.47	3.28
4	6644.60	2615.21	985.63	9.43	2.85
5	6498.34	2503.00	981.51	9.28	3.33
6	6351.47	2559.28	975.69	9.16	3.55
MEAN	6524.68	2537.06	1001.14	9.57	3.46
%nom	96.8	100.0	103.9	109.0	110.8
CV%	3.5	2.0	2.1	5.4	12.3

Cumulative Statistics of Quality Control Results

Table 28: The combined quality control results of the intra-batch and inter-batch validations based on peak area ratios.

Validation batch	Code	QC H	QC G	QC F	QC B	QC A
	Nominal	6 737	2 536	964	8.78	3.12
	Replicates	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
Intra-batch Validation	1	6454.05	2722.06	1039.87	9.84	3.31
	2	6616.30	2644.04	1055.22	8.19	3.27
	3	6404.71	2549.73	946.56	7.56	3.06
	4	6474.69	2393.71	1002.74	8.29	3.46
	5	6235.33	2460.43	921.06	9.68	3.46
	6	5574.62	2305.43	987.98	8.12	3.44
Inter- batch Validation 1	1	6823.37	2623.07	1046.46	8.18	3.60
	2	6658.07	2726.97	962.07	10.03	3.48
	3	6497.35	2487.91	957.92	8.60	2.95
	4	6719.58	2442.59	945.62	10.02	3.05
	5	6417.23	2619.90	998.93	8.71	3.21
	6	6444.82	2516.57	958.95	8.56	3.52
Inter- batch Validation 2	1	6974.78	2534.99	1016.89	10.70	4.27
	2	6385.85	2559.68	1018.70	9.40	3.46
	3	6293.02	2450.20	1028.39	9.47	3.28
	4	6644.60	2615.21	985.63	9.43	2.85
	5	6498.34	2503.00	981.51	9.28	3.33
	6	6351.47	2559.28	975.69	9.16	3.55
	MEAN	6470.454	2539.709	990.566	9.068	3.364
	% nom	96.0	100.1	102.8	103.3	107.8
	CV%	4.4	4.2	3.8	9.0	9.0

For the assignment of a valid calibration range percent deviation from nominal, or percent BIAS is taken as measure of accuracy. Coefficient of variation (CV %) is taken as measure of precision.

Inter-batch accuracy and precision for a valid range must be within 15%, but within 20% at the limit of quantification [30, 5]. Results from the inter-batch validation assays above indicate a valid calibration range from 2.95 to 20 016 ng/ml for clarithromycin. The limit of quantification (LOQ) was preliminarily set at 2.95 ng/ml for clarithromycin.

A common phenomenon during sample analysis is that less than the validated sample volume is available for analysis, and a partial sample volume must be used. To circumvent this problem, one needs to validate partial samples by diluting known quality controls by known factors, e.g. 2, 4, or 5. These diluted samples are then analysed during validation to demonstrate that the dilution is still accurate and within the precision and accuracy criteria. Dilutions are sometimes necessary if the determined values of unknown samples are above the validated range.

When it is necessary to make changes to the system, or the extraction method, a revalidation may be necessary. A guideline to revalidation is given by Dadgar *et al.*, [24] in table 29.

Table 29: Guidelines for revalidation.

Method Parameter Changed	Parameters to Revalidate
Extraction solvent	Linearity
Buffer	Recovery
Back extraction matrix	Selectivity
Injection solvent	LOQ
	Intra-batch validation
	Accuracy
	On-instrument stability
Chromatography column	Linearity
Mobile phase	Selectivity
Detector	Intra-batch validation
	Accuracy
	LOQ
Changing the range	Linearity
	LOQ (if reduced)
	Intra-batch precision
	Accuracy
Internal standard	Selectivity
	Intra-batch validation
	Accuracy
	Recovery

1.3. Routine Analysis

In order to exclude a possible time effect or degrading of standards and quality controls, and generate an accurate calibration curve independent of degrading, the standards and quality controls are freshly extracted with each batch.

A standard curve is generated for each analytical run, and is used to calculate the concentration of analyte in the unknown samples assayed [5]. Once the assay has been established for routine use, its performance is regularly monitored to update information regarding its characteristics and to ensure that it continues to work satisfactorily. This is done by using an internal quality control (QC) scheme, which is defined as the long-term and continuous assessment of accuracy and precision of an assay for a particular drug with a view to minimising within-laboratory variation and to improve assay precision. The QC samples are used to accept or reject the run.

Quality controls (QC) are usually run in duplicate. The sample results are acceptable if the QC results are within 10-25% of the known values [25]. Shah *et al.*, [5] criteria stipulate that at least 67% of the QC samples must be within the 20% of their respective nominal values and that 33% of the QC samples (not both at the same concentration) may be outside the 20% respective nominal value.

Table 30: A typical batch structure.

Inj No	Sample	Inj No	Sample	Inj No	Sample	Inj No	Sample	Inj No	Sample	Inj No	Sample
1	SYS	**39**	5,1.0,1	**77**	4,2.5,3	**115**	4,4.0,1	**153**	3,8.0,3	**191**	3,16,2
2	STD C	**40**	5,1.0,2	**78**	5,2.5,1	**116**	4,4.0,2	**154**	4,8.0,1	**192**	3,16,3
3	1,0,1	**41**	5,1.0,3	**79**	5,2.5,2	**117**	4,4.0,3	**155**	4,8.0,2	**193**	4,16,1
4	1,0,2	**42**	QC G	**80**	5,2.5,3	**118**	5,4.0,1	**156**	4,8.0,3	**194**	4,16,2
5	1,0,3	**43**	1,1.5,1	**81**	STD G	**119**	5,4.0,2	**157**	5,8.0,1	**195**	4,16,3
6	3,0,1	**44**	1,1.5,2	**82**	1,3.0,1	**120**	5,4.0,3	**158**	5,8.0,2	**196**	5,16,1
7	3,0,2	**45**	1,1.5,3	**83**	1,3.0,2	**121**	STD I	**159**	5,8.0,3	**197**	5,16,2
8	3,0,3	**46**	3,1.5,1	**84**	1,3.0,3	**122**	1,5.0,1	**160**	QC G	**198**	5,16,3
9	4,0,1	**47**	3,1.5,2	**85**	3,3.0,1	**123**	1,5.0,2	**161**	1,10,1	**199**	STD E
10	4,0,2	**48**	3,1.5,3	**86**	3,3.0,2	**124**	1,5.0,3	**162**	1,10,2	**200**	QC B
11	4,0,3	**49**	4,1.5,1	**87**	3,3.0,3	**125**	3,5.0,1	**163**	1,10,3	**201**	1,24,1
12	5,0,1	**50**	4,1.5,2	**88**	4,3.0,1	**126**	3,5.0,2	**164**	3,10,1	**202**	1,24,2
13	5,0,2	**51**	4,1.5,3	**89**	4,3.0,2	**127**	3,5.0,3	**165**	3,10,2	**203**	1,24,3
14	5,0,3	**52**	5,1.5,1	**90**	4,3.0,3	**128**	4,5.0,1	**166**	3,10,3	**204**	3,24,1
15	QC F	**53**	5,1.5,2	**91**	5,3.0,1	**129**	4,5.0,2	**167**	4,10,1	**205**	3,24,2
16	1,0.5,1	**54**	5,1.5,3	**92**	5,3.0,2	**130**	4,5.0,3	**168**	4,10,2	**206**	3,24,3
17	1,0.5,2	**55**	STD H	**93**	5,3.0,3	**131**	5,5.0,1	**169**	4,10,3	**207**	4,24,1
18	1,0.5,3	**56**	1,2.0,1	**94**	QC B	**132**	5,5.0,2	**170**	5,10,1	**208**	4,24,2
19	3,0.5,1	**57**	1,2.0,2	**95**	1,3.5,1	**133**	5,5.0,3	**171**	5,10,2	**209**	4,24,3
20	3,0.5,2	**58**	1,2.0,3	**96**	1,3.5,2	**134**	QC H	**172**	5,10,3	**210**	5,24,1
21	3,0.5,3	**59**	3,2.0,1	**97**	1,3.5,3	**135**	1,6.0,1	**173**	STD D	**211**	5,24,2
22	4,0.5,1	**60**	3,2.0,2	**98**	3,3.5,1	**136**	1,6.0,2	**174**	1,12,1	**212**	5,24,3
23	4,0.5,2	**61**	3,2.0,3	**99**	3,3.5,2	**137**	1,6.0,3	**175**	1,12,2	**213**	QC A
24	4,0.5,3	**62**	4,2.0,1	**100**	3,3.5,3	**138**	3,6.0,1	**176**	1,12,3	**214**	STD B
25	5,0.5,1	**63**	4,2.0,2	**101**	4,3.5,1	**139**	3,6.0,2	**177**	3,12,1	**215**	1,36,1
26	5,0.5,2	**64**	4,2.0,3	**102**	4,3.5,2	**140**	3,6.0,3	**178**	3,12,2	**216**	1,36,2
27	5,0.5,3	**65**	5,2.0,1	**103**	4,3.5,3	**141**	4,6.0,1	**179**	3,12,3	**217**	1,36,3
28	STD B	**66**	5,2.0,2	**104**	5,3.5,1	**142**	4,6.0,2	**180**	4,12,1	**218**	3,36,1
29	QC A	**67**	5,2.0,3	**105**	5,3.5,2	**143**	4,6.0,3	**181**	4,12,2	**219**	3,36,2
30	1,1.0,1	**68**	QC H	**106**	5,3.5,3	**144**	5,6.0,1	**182**	4,12,3	**220**	3,36,3
31	1,1.0,2	**69**	1,2.5,1	**107**	STD J	**145**	5,6.0,2	**183**	5,12,1	**221**	4,36,1
32	1,1.0,3	**70**	1,2.5,2	**108**	BLANK	**146**	5,6.0,3	**184**	5,12,2	**222**	4,36,2
33	3,1.0,1	**71**	1,2.5,3	**109**	1,4.0,1	**147**	STD F	**185**	5,12,3	**223**	4,36,3
34	3,1.0,2	**72**	3,2.5,1	**110**	1,4.0,2	**148**	1,8.0,1	**186**	QC F	**224**	5,36,1
35	3,1.0,3	**73**	3,2.5,2	**111**	1,4.0,3	**149**	1,8.0,2	**187**	1,16,1	**225**	5,36,2
36	4,1.0,1	**74**	3,2.5,3	**112**	3,4.0,1	**150**	1,8.0,3	**188**	1,16,2	**226**	5,36,3
37	4,1.0,2	**75**	4,2.5,1	**113**	3,4.0,2	**151**	3,8.0,1	**189**	1,16,3	**227**	STD C
38	4,1.0,3	**76**	4,2.5,2	**114**	3,4.0,3	**152**	3,8.0,2	**190**	3,16,1	**228**	QC A
										229	SYS

[8]

STD = Calibration Standard
QC = Quality Control Standard
SYS = System Performance Verification Sample

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

When a batch of samples is analysed, calibration standards are included in the run (table 30) to allow a standard curve to be constructed. This will secure meaningful results even if the instrument response changes for some reason.

In addition to this a number of QC samples are analysed along with actual samples at intervals depending on the total number of actual samples [25]. As a rough guide one control every ten samples should suffice. Once the number of controls per batch has been decided, it should be maintained.

It is true that quality control standards increase workload, but in the long run it does improve the quality of results, and increases the individual's confidence in the assay.

Typical intra-batch retention times for clarithromycin were 1.97 - 2.05 minutes (RSD_{mean} = 0.30 %) and for roxithromycin were 1.92 - 1.96 minutes (RSD_{mean} = 0.30 %). A turn-around time of 3.0 minutes made it possible to analyse ±230 samples per day. Figure 19 shows representative chromatograms of clarithromycin obtained at 20 016 ng/ml (**A**) and 2.95 ng/ml (LOQ)(**B**), while figure 20 depicts chromatograms from a calibration standard containing 2.95 ng/ml (**A**) of clarithromycin, and a blank plasma extract (showing carry-over)(**B**), magnified.

Another observation was that the source of the Applied Biosystems Sciex was "saturating" over a period of 12 hours, which caused a drop in response down to a certain constant level of sensitivity. This was dealt with by injecting a series of 100 samples before the start of the analysis of the first study samples (getting the response to a constant level) and keeping on injecting samples in between batches (to keep the response at that level). This phenomenon is also presently the subject of intense investigations and will be reported on as soon as we have definitive results.

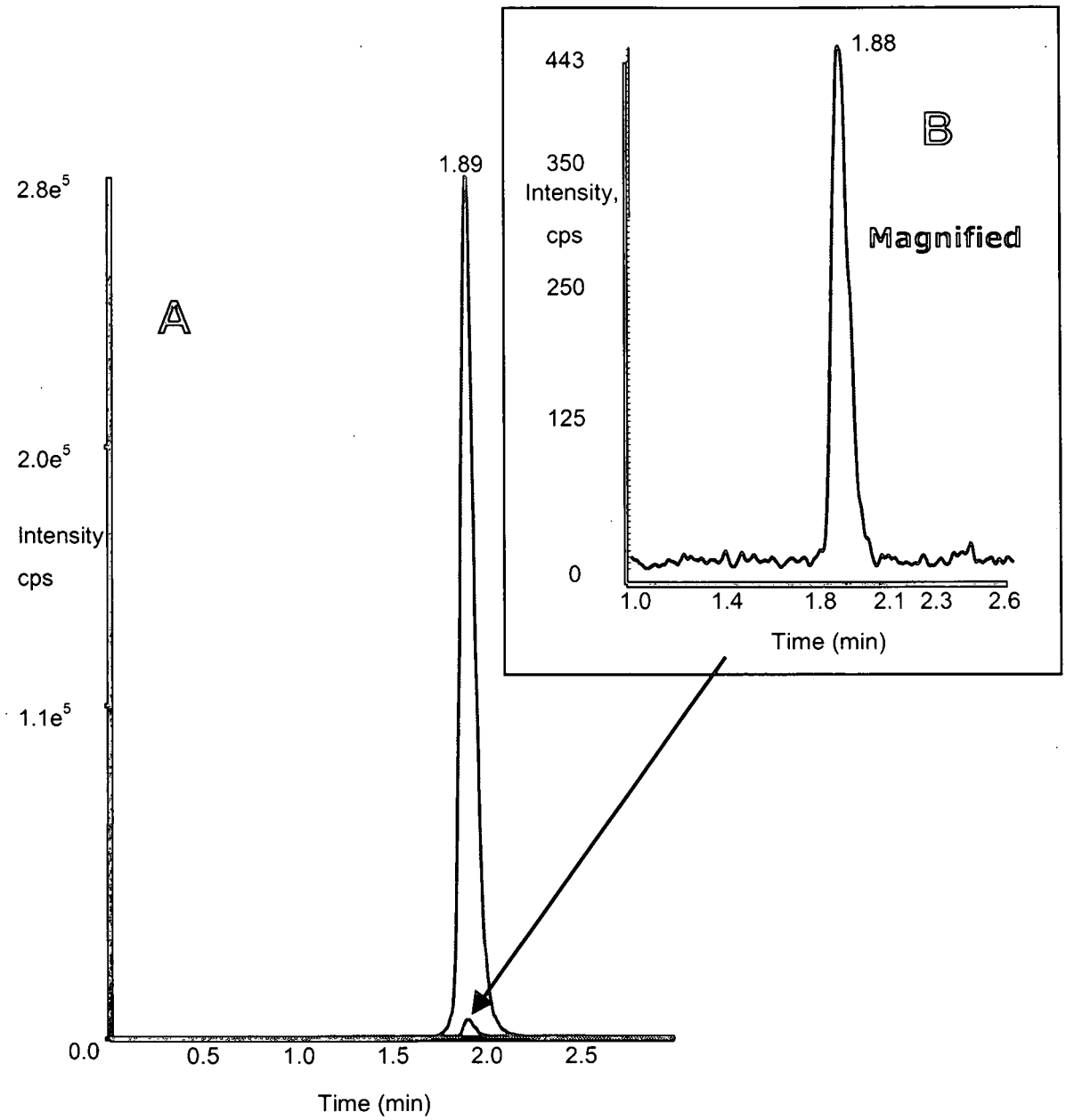


Figure 19: Chromatograms of calibration standards, containing 20 016 ng/ml (A) and 2.95 ng/ml (B), respectively, of clarithromycin in plasma.

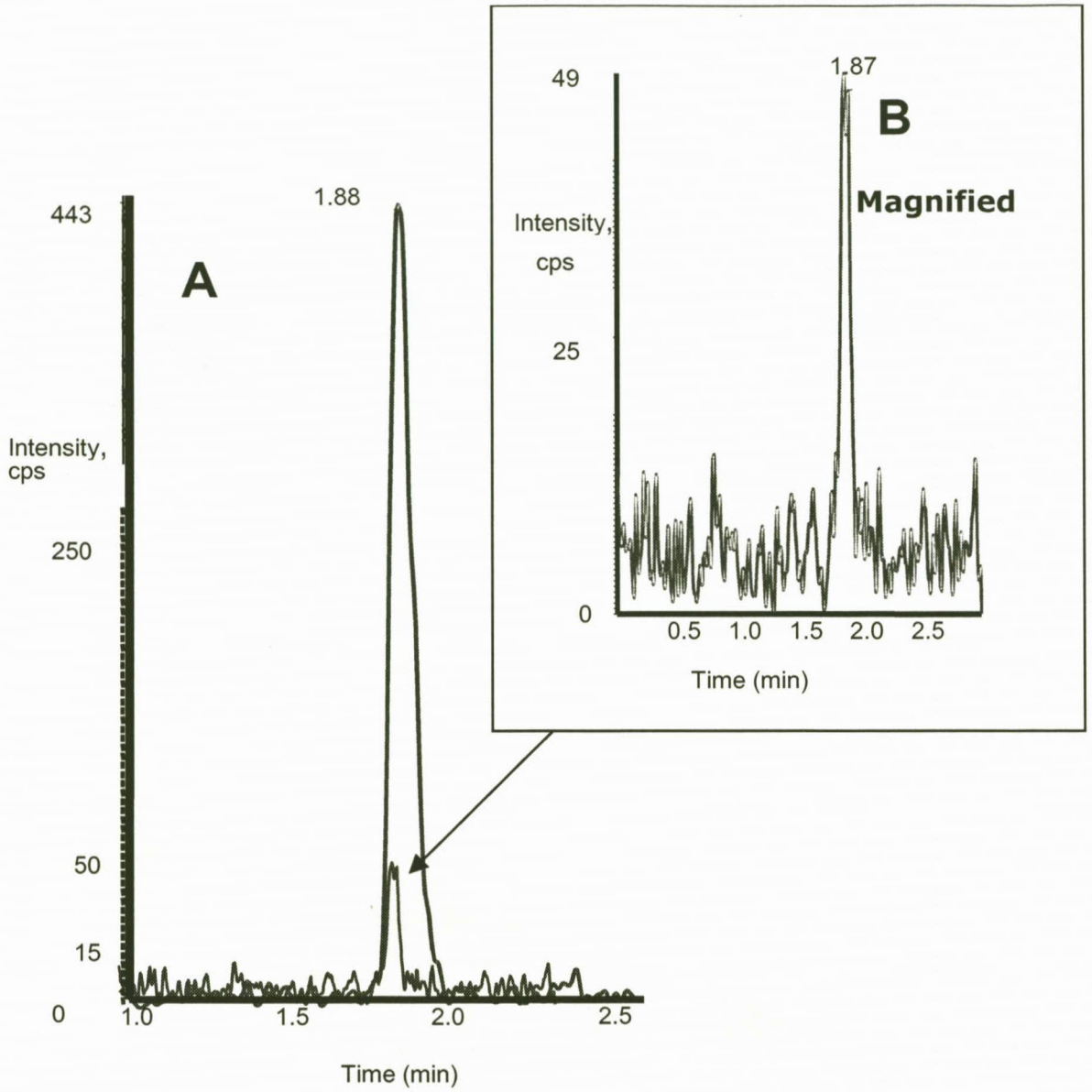


Figure 20: Chromatograms of calibration standard containing 2.95 ng/ml of clarithromycin (A) in plasma and blank plasma extract (B), showing carry-over.

Repeat assays are identified in each batch during the study. The analyst or data processing analysts identifies analyses which do not pass the chromatographic acceptance criteria as poor chromatograms and samples analyses deemed unacceptable due to technical errors such as equipment failure, excessive internal standard aberrations or accidental sample loss or destruction, as lost in process. These samples should be re-analysed and since no acceptable original result is obtained, only the result of the re-assay is reported [24].

With pharmacokinetic outliers, the re-analysis is conducted in duplicate. Based on the criteria, one can decide to report all values, the mean between the two duplicate values, or the mean between the original and the duplicate.

When the calculated concentration of a study sample exceeds the concentration of the highest nominal calibration standard the sample is coded as being outside the calibration range. The sample is diluted with drug-free matrix to bring the concentration within the standard curve range and the analysis is repeated. The dilution factor is then used to calculate and report the concentration before dilution [24].

If the calibration range is narrowed by the loss of either the highest or lowest calibration standards, samples of which the concentrations would have fallen within the validated calibration range (if the standards were not missing) are coded as lying outside the calibration range. Such samples are re-analysed in single fold [24].

At the end of the study, after all subject samples have been assayed, the repeat assays are all processed together as a normal batch with the usual set of calibration and quality control standards. Repeat assays were always done using the duplicate sample aliquot prepared during the clinical execution of the study, thus the samples were not exposed to an additional freeze-thaw cycle.

1.4. Conclusion

A highly selective and sensitive method for the quantification of clarithromycin in human plasma was developed and validated. Due to the high specificity of the LC-MS/MS instrument, no interference of other drugs, metabolites or substances was observed.

With previously reported LOQs (figure 21), one would have been able to quantify clarithromycin in treatment 3 only up to 15 hours, treatment 1 only up to 25 hours and treatment 2 only up to 30 hours, providing too few significant data points to construct a good pharmacokinetic profile for each treatment. Plasma concentrations of clarithromycin could be quantified from 2.95 ng/ml (making this assay method 10 times more sensitive than the lowest published LOQ) to 20 016 ng/ml using this developed assay method, making it possible to analyse samples up to 36 hours after a single oral dose of 500mg of clarithromycin to human volunteers.

A turn-around time of 3.0 minutes made it possible to analyse ±230 samples per day, increasing production by more than 400%.

This is the first LC-MS/MS assay method described for the quantification of clarithromycin in human plasma [φ].

Figure 21 represents the mean pharmacokinetic profiles obtained after a single dose of 500mg clarithromycin of three different formulations of clarithromycin tested in 9 healthy human volunteers. The maximum clarithromycin plasma concentrations obtained varied between 450 and 1 800 ng/ml.

In figure 21, treatment 2 showed a rapid absorption and some secondary absorption after 5 hours of administration, while treatment 1 showed a very efficient absorption and elimination.

[φ] See appendix IV

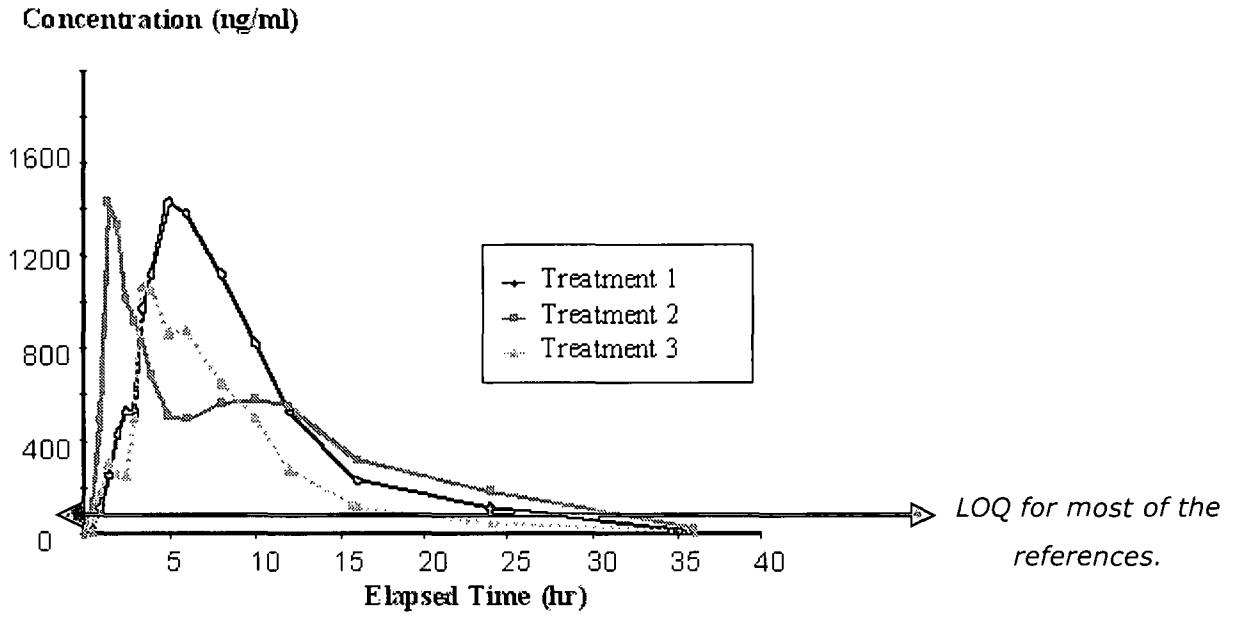


Figure 21: Representative mean clarithromycin plasma concentration/time profile (n = 9), as obtained after a single 500mg oral dose of clarithromycin to human volunteers.

Table 31: Comparison between the available literature and the developed method.

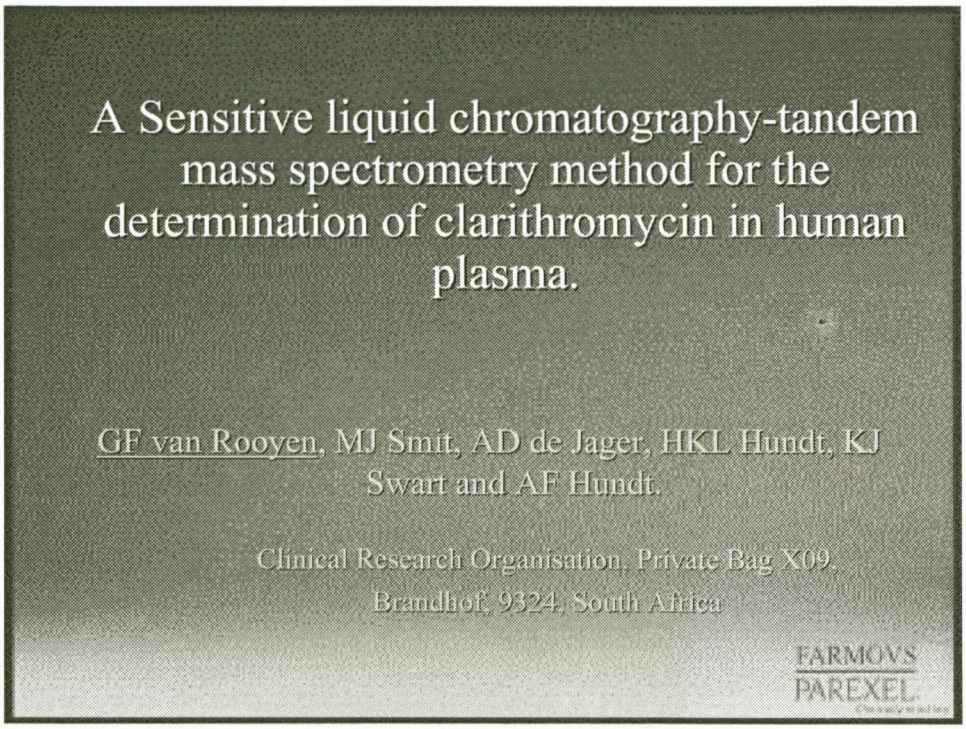
	Amount used	Inj. Vol.	LOD	LOQ	~Recovery	Retention times	Dose	C_{max}
	(µl)	(µl)	(ng/ml)	(ng/ml)	(%)	(minutes)	(mg)	(ng/ml)
Chu et al.,	500	20-80		38	85	20.0	600	2500
Borner et al.,	500	12	50	100	110	17.0	250	
Hedenmo and Eriksson	100	25		373	99	6.0		
Erah et al.,	500	50	400		98	6.0		
Kees et al.,	500	20-50		30	85	10.0	1200	
Sastre Torano and Guchelaar		50		200	104	15.7	20mg/kg	4660
Taninaka et al.,		40		30	83	14.0		
Araujo et al.,	500	50			83	2.1		
Pappa-Louisi et al.,	500	50	100		94	16.0		
Choi et al.,	120	200		100	87	16.0	500	2640
van Rooyen et al.,	**300**	**5**	**0.40**	**2.95**	**87**	**2.0**	**500**	**1100**

By comparing the literature with the developed method, one can easily see that a more sensitive and productive method has been developed. By injecting only 5µl, one could easily quantify clarithromycin at 2.95 ng/ml, providing enough data points on the elimination phase of the drug to construct a good pharmacokinetic profile, thus providing more accurate and precise pharmacokinetic data. This assay method contributed to more accurate and precise pharmacokinetic data, meaning a better understanding of the drug's elimination, absorption, half-life, etc.

Proven pharmacokinetic strategies significantly reduce the time and expense required for proper and safe drug development. As pharmacokinetic information reveals drug concentration and effect relationships, clinical outcomes can be predicted with greater certainty. If one has a good understanding of drug concentration and effect through pharmacokinetics, one can measure surrogate markers and predict clinical outcomes. Clinical applications of pharmacokinetics result in optimal trial design and affect all phases of clinical drug development, resulting in improvements in drug utilization that ultimately benefit the patient. Therefore, it is imperative that pharmacokinetic scientists keep current with tools and innovations that lead to greater predictive power.

1.5. Poster Presentation

- This poster was presented at the Immuno-Pharmacology Congress held at Sun City, in September 2001.
- This poster presentation won the award for **"Best Junior Poster Presentation (Laboratory)"** [3] at the annual Faculty Forum (2001) of the School of Health Sciences at the University of the Free State.



[3] See appendix III

Introduction

- A sensitive method for the determination of clarithromycin in plasma is described, using high-performance liquid chromatographic separation with tandem mass spectrometric detection.
- Clarithromycin displays a wide antibacterial spectrum. The effect of combining clarithromycin with a variety of other drugs for the treatment and prevention of disseminated *M. avium* infection in patients with AIDS is under investigation. Abbott markets clarithromycin under the proprietary names "Biaxin", "Klacid" and "Klaricid".
- The sample to sample turn-around time in the published assay methods for clarithromycin are of the order of 12 to 30 minutes.

FARMOVS PAREXEL

Extraction

300 µl plasma
200 µl 0.1M $Na_2CO_3/NaHCO_3$ buffer (pH 9.21)
100 µl roxithromycin (ISTD)(3.76 ng/ml in water)
Hexane: ethyl acetate (1:1) (3ml)

Vortex (60 seconds) and centrifuge at 2500 G for 3 minutes

Freeze aqueous phase
Decant organic phase into a 5ml glass ampoule

Evaporate to dryness
Reconstitute in 300µl of acetonitrile: water (1: 9)

FARMOVS PAREXEL

Table 1: Intra-day Accuracy & Precision
(Quality controls; n=6)

Clarithromycin (n=6)			
Added concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	Accuracy Bias%
3.12	3.33	4.3	-6.7
8.78	8.61	9.8	1.9
135	138.53	3.3	-2.6
359	368.51	3.4	-2.6
964	992.24	4.8	-2.9
6737	6293.28	5.4	6.6

FARMOVS
PAREXEL

Conclusion

A highly selective method for the quantification of clarithromycin in human plasma has been developed and validated.

Plasma concentrations of clarithromycin could be quantified from 2.95 ng/ml to 20016 ng/ml.

Possible to analyse samples up to 36 hours after a single oral dose of 500 mg of clarithromycin to human volunteers.

This is the first LC-MS/MS assay method described for the quantitation of clarithromycin in plasma

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1.6. Publication

Also see Appendix I.

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Sensitive liquid chromatography–tandem mass spectrometry method for the determination of clarithromycin in human plasma

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Abstract

A sensitive method for the determination of clarithromycin in plasma is described, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. Samples were prepared using liquid–liquid extraction and separated on a Supelco Discovery® C_{18} column with a mobile phase consisting of acetonitrile, methanol and acetic acid. Detection was performed by a PE SCIEX API 2000 mass spectrometer in the multiple reaction monitoring (MRM) mode (LC–MS–MS) using TurboIonSpray ionization and monitoring the transition of the protonated molecular ion for clarithromycin at m/z 748.5 (M+1) to the predominant product ion of m/z 158.2. The mean recovery of clarithromycin was 87.3%, with a lower limit of quantification of 2.95 ng/ml when using 0.3-ml plasma. This high-throughput method was used to quantify 230 samples per day, and is sufficiently sensitive to be employed in pharmacokinetic studies.

Keywords: Clarithromycin

1. Introduction

Clarithromycin (6-*O*-methylerythromycin) ($C_{38}H_{69}NO_{13}$) (Mr.=747.96) (Fig. 1) is a semi-synthetic 14-membered macrolide which displays a wide antibacterial spectrum. The effect of combining clarithromycin with a variety of other drugs for the treatment and prevention of disseminated *M. avium* infection in patients with AIDS is under investigation [7–10]. Abbott markets clarithromycin under the proprietary names "Biaxin", "Klacid" and "Klaricid". Its structure is identical to that of erythromycin, except that the *O*-methyl group has been substituted for a hydroxyl group at position six of the lactone [1]. Several methods for the determination of clarithromycin in plasma have been published [2–4], and a recent review article [5] lists many assay methods for the macrolide antibiotics in general. As is the case with many of the macrolide antibiotics, high-performance liquid chromatography (HPLC) with electrochemical detection was used [2–4] for the assay of clarithromycin because the molecule lacks a suitable chromophore which would make it amenable to UV detection. Although selective and highly sensitive, assay procedures making use of electrochemical detection is often very time consuming, both in the sample preparation steps and the chromatography. Thus, the sample to sample turn-around time in the published assay methods [2–4] for clarithromycin are of the order of 14, 12

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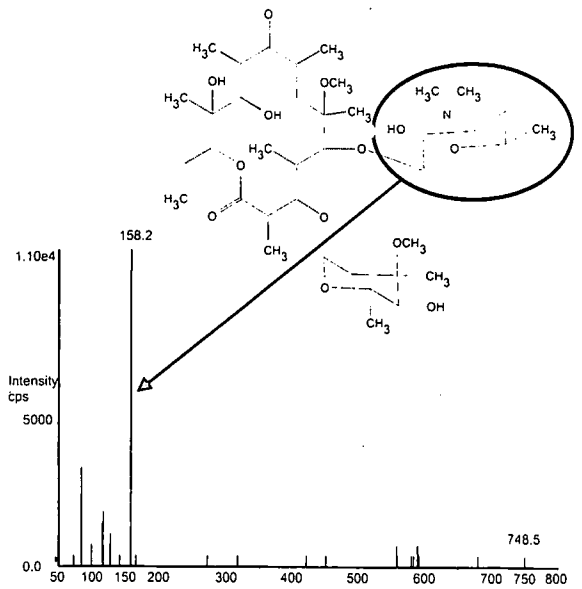

Fig. 1. A full scan spectrum (MS–MS) of a pure solution of clarithromycin in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ion with m/z 748.5 and the predominant product ion m/z 158.2 is shown.

and 30 min, respectively, if one allows half a minute for the automated sample injection between each sample. Clearly, an assay procedure requiring less critical sample preparation procedures and a much faster turn-around time in the chromatographic process would be advantageous in clinical studies generating large numbers of samples. Although LC–MS–MS springs to mind immediately, no reference to any assay methods for clarithromycin using mass spectrometry as mode of detection could be found in the literature. This paper thus represents the first quantitative LC–MS–MS method for the determination of clarithromycin in human plasma using a relatively simple sample preparation procedure and a short turn-around time of only 3 min.

2. Experimental

2.1. Materials and chemicals

A Supelco Discovery® C_{18}, 5 μm, 150×2.1 mm, column (Supelco Park, Bellefonte, USA), heated at a constant temperature of 60 °C with a Shimadzu CTO-6A column oven (Shimadzu, Kyoto, Japan), was used for separation at a flow-rate of 0.26 ml/min (5 μl was injected onto the column). An Agilent 1100 quaternary pump delivered the mobile phase and the samples injected with an Agilent 1100 autosampler (Agilent, Palo Alto, CA). Detection was performed by a P-E Sciex API 2000 triple-quad mass spectrometer interfaced to an electrospray ionisation (ESI) TurboIonSpray source (Applied Biosystems Sciex, Ontario, Canada).

Acetic acid (Pro-Analysi) was obtained from Merck (Darmstadt, Germany); acetonitrile and methanol (BandJ High Purity) was obtained from Baxter (Muskegon, USA). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

Clarithromycin (6-*O*-methylerythromycin) (Fig. 1) ($C_{38}H_{69}NO_{13}$) was supplied by ALPHARMA (Dalslandgade 11, Copenhagen, Denmark) and roxithromycin was obtained from the FARMOVS-PAREX-EL® Bioanalytical Services pure reference material library.

2.2. Preparation of calibration standards

Clarithromycin stock solutions (1579 μg/ml) were prepared in methanol and used immediately to spike blank plasma. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal plasma to a known concentration and then serially diluting it with normal blank plasma to attain the desired concentration range (20 016–2.95 ng/ml). The prepared calibration standards and quality control standards were pipetted (500 μl) into polypropylene tubes and stored at −20 °C pending analysis.

2.3. Extraction procedure

To 300 μl plasma in a 10 ml glass ampoule was added 200 μl (0.1 *M*) $Na_2CO_3/NaHCO_3$ buffer solution (pH 9.2) and 100 μl roxithromycin internal standard solution (3.76 ng/ml in water). Hexane–ethyl acetate (1:1) (3 ml) was added, the samples vortexed for 60 s, and then centrifuged at 2500 *g* for 3 min at 10 °C. The aqueous phase was frozen on a Fryka Polar KP 250 cooling plate (Kältetechnik, Esslingen) at −30 °C for 3 min, the organic phase

decanted into a clean 5 ml glass ampoule and evaporated to dryness in a Savant SpeedVac® (Savant, Holbrook, NY, USA) rotary vacuum evaporator at ambient temperature. The extracts were reconstituted in 300 μl of acetonitrile–water (1:9), by vortexing for 30 s, transferred to autosampler vials, and 5 μl injected onto the HPLC column.

2.4. Liquid chromatography

Chromatography was performed at 60 °C, at a flow-rate of 0.26 ml/min with acetonitrile–methanol–acetic acid (0.1% in water) (25:25:50, v/v/v) as mobile phase. All chromatographic solvents were sparged with helium before use.

2.5. Mass spectrometry

Electrospray ionisation was performed in the positive mode with the nebulizing gas (nitrogen), curtain gas and turbospray set at 70, 50 and 70 l/h, respectively. The ionisation source was connected by a fused-silica (375 μm) capillary to the syringe pump for tuning, and the instrument response for clarithromycin was optimised using flow injection. Optimal response was obtained with a declustering potential setting of 61 V, a focusing potential of 230 V, entrance potential and exit potential of −8.0 and 8 V, respectively. The TurboIonSpray temperature was set at 400 °C.

The P-E Sciex API 2000 LC–MS–MS was operated at unit resolution in the multiple reaction monitoring (MRM) mode and the transitions of the protonated molecular ion for clarithromycin at m/z 748.5 to the predominant product ion m/z 158.2, and for roxithromycin m/z 837.6 to m/z 679.5 were monitored. The pause time was set at 5 ms and the dwell time at 150 ms. The relative collision energy was set at 39 eV. The mass spectrometer was interfaced to a computer workstation running Analyst™ software, version 1.0.

2.6. Validation

The method was validated by assaying clarithromycin plasma quality control samples in 6-fold at 3.12, 8.78, 23.3, 135, 359, 964, 2536, 6737 and 18 098 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve composed of nine different concentrations spanning the concentration range 2.95–20 016 ng/ml for clarithromycin. Calibration graphs were constructed using peak area ratios of the relevant product ions versus nominal concentrations using Wagner ($\ln(y) = a(\ln(x)^2) + b(\ln(x)) + c$) ($r^2 = 0.9997$) (mean BIAS% = 0.1%) calibration curves.

2.7. Matrix effects

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 [6]. To determine whether this effect (called the matrix effect) is present or not, 10 different blank plasma pools were extracted and the reconstituted extracts then spiked with a known concentration of analyte. These samples were assayed and their peak areas compared. The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect. The following data show that no significant matrix effect was observed in the samples assayed (RSD% = 2.36% for clarithromycin and 0.64% for roxithromycin) at the tested concentration of 1580 ng/ml for clarithromycin and 1315 ng/ml for roxithromycin.

2.8. Recovery

Absolute recovery of the analyte was determined in triplicate at high, medium and low concentrations in normal plasma by extracting drug free plasma samples spiked with clarithromycin. Recovery was calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted analyte standards, representing 100% recovery.

3. Results and discussion

Initially protein precipitation was investigated as a possible sample preparation procedure. Then, 400 μl of acetonitrile was added to 200 μl of plasma in a microfuge tube. The samples were vortex mixed for

Table 1
Intra-day quality control results of clarithromycin

($n=6$)	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	18 098	6737	2536	964	359	135	23.3	8.78	3.12
Mean	16 393	6293	2513	992	369	139	23.4	8.61	3.33
% nom	90.6	93.4	99.1	102.9	102.6	102.6	100.6	98.1	106.8
C.V.%	4.0	5.4	5.7	4.8	3.4	3.3	4.5	9.8	4.3

30 s and centrifuged at 3000 *g* for 5 min at 10 °C. The supernatant aqueous layer (±200 μl) was transferred to an autosampler vial and 5 μl injected onto the HPLC column. An LLOQ of 8 ng/ml could be reached with this precipitation method.

The liquid–liquid extraction procedure described, however, was the method of choice. Although the LLOQ was found to be only 2.95 ng/ml (signal-to-noise (*S/N*) ratio = 27), this relatively high LLOQ was the result of carry-over (equivalent to an absolute amount of about 5 pg clarithromycin injected) inherent in the chromatographic instrument system which yielded a signal equivalent to an extract of an approximately 0.3 ng/ml sample. We have observed carry-over problems with different types of analytes on several occasions. Although it appears to be analyte-specific in many cases, the exact nature of the carry-over does not appear to be the same in all the cases that we have encountered. This phenomenon is the subject of continuous investigation in our laboratory. In the case of the clarithromycin, an LLOQ of about 0.4 ng/ml was reached when an extensive needle wash step with a stronger needle-wash solution was introduced to reduce carry-over (±2 ng/ml, *S/N* ratio = 16.0) substantially. However, this procedure would have increased the turn-around time for each analysis by an unacceptable margin while the LLOQ, which was attained (2.95 ng/ml), was totally acceptable considering the nature of the study and the range of concentrations studied (i.e. LLOQ to 1800 ng/ml).

Another observation was that the source of the P-E Sciex was "saturating" over a period of 12 h, which caused a drop in response down to a certain constant level of sensitivity. This was dealt with by injecting a series of 100 samples before the start of the analysis of the first study samples (getting the response to a constant level) and keeping on injecting samples in between batches (to keep the response at that level). This phenomenon is also presently the subject of intense investigations and will be reported on as soon as we have definitive results.

The mean absolute recoveries of clarithromycin, determined in triplicate at 8.78, 964 and 6737 ng/ml were 80.5, 90.8 and 90.5%, respectively. According to Kees et al. (1998) [11], the recovery of clarithromycin from water and plasma using liquid–liquid extraction was 80–90%.

Results presented in Table 1 indicate a valid calibration range of 2.95–20 016 ng/ml for clarithromycin. Table 2 depicts the quality control results obtained during the assaying of the study samples. On-instrument stability was inferred from intra-day quality control data obtained during the pre-study

Table 2
Inter-day quality control results of clarithromycin

	QC H	QC G	QC F	QC B	QC A
Nominal (ng/ml)	6829	2601	970	8.73	3.25
Mean	6677	2616	1023	8.61	3.07
C.V.%	5.7	2.5	4.6	6.8	0.8
n	6	6	6	5	6
% nom	97.8	100.6	105.4	98.7	94.6

validation. The ratio analyte/internal standard of 16 stability samples were compared during validation and inter-day validation, and no significant degradation could be detected in the cooled samples left on the autosampler for at least 21.1 h. To ascertain freeze–thaw stability, aliquots of quality control samples at 6829 ng/ml (QC G) and 8.73 ng/ml (QC B) were allowed to thaw and then immediately re-frozen. These quality controls were assayed the following day together with a set of the same quality controls (QC G and QC B) that had not been subjected to this additional freeze–thaw cycle. The results are summarised in Table 3. From these results we conclude that clarithromycin is stable in plasma over at least two freeze–thaw cycles.

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when assaying blank plasma extracts from six different sources when taking special precautions to avoid carry-over. As mentioned earlier, carry-over was a problem when time consuming special precautions was not taken.

Fig. 1 represents the single parent to product ion mass spectrum (MS–MS) of clarithromycin acquired with the most abundant product ion at m/z 158.2. A strong daughter ion with m/z 158.2 was also observed for roxithromycin, but to avoid cross talk, the transition to the even more abundant daughter ion with m/z 679.5 (Fig. 2) was optimised and monitored instead.

The P-E Sciex 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 ions m/z 748.5 and 837.6 to the principal product ions m/z 158 and 679.5 for clarithromycin and roxithromycin, respectively.

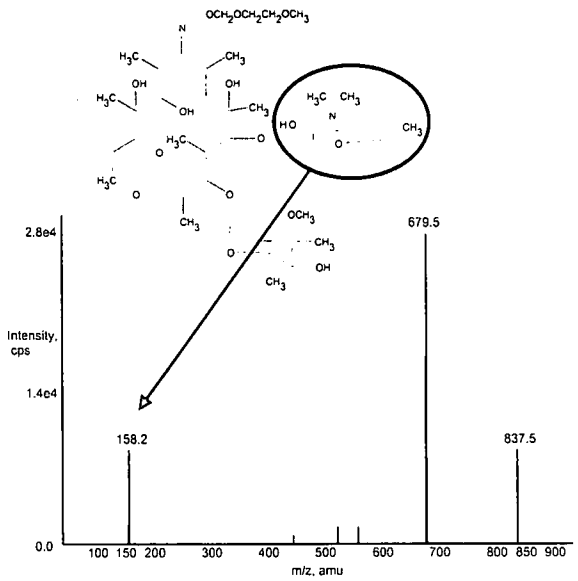

Fig. 2. A full scan spectrum (MS–MS) of a pure solution of roxithromycin (internal standard) in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ions with m/z 837.5 and predominant product ion m/z 679.5 are shown.

Typical intra-batch retention times for clarithromycin were 1.87–1.92 min (mean RSD=0.30%) and for roxithromycin were 1.97–2.05 min (mean RSD=0.30%). A turn-around time of 3 min made it possible to analyse 230 samples per day. It took two analysts approximately 4–5 h to prepare the samples for analysis.

Table 3
Freeze–thaw stability, comparing a freeze–thaw–freeze–thaw (F/T/F/T) cycle to a freeze–thaw (F/T) cycle, using the ratio analyte/internal standard (ng/ml) at a high and a low concentration control

High (6737 ng/ml)	(F/T/F/T) (ng/ml)	(F/T) (ng/ml)	Low (8.73 ng/ml)	(F/T/F/T) (ng/ml)	(F/T) (ng/ml)
1	6678	6955	1	8.75	8.81
2	6893	6963	2	8.64	8.74
3	6773	6649	3	8.68	8.69
4	6842	7106	4	8.71	8.82
5	6801	7134	5	8.72	8.72
Mean	6797	6961		8.70	8.76
% Diff.	2.36			0.64	

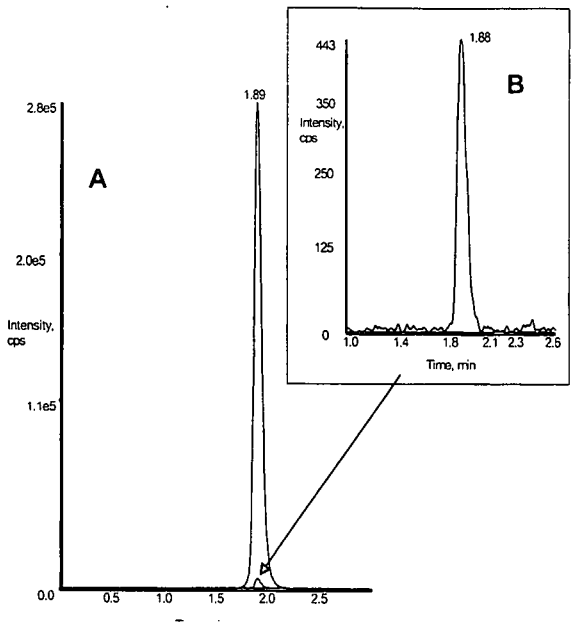

Fig. 3. Chromatograms of calibration standards, containing 20 016 ng/ml (A) and 2.95 ng/ml (B), respectively, of clarithromycin in plasma.

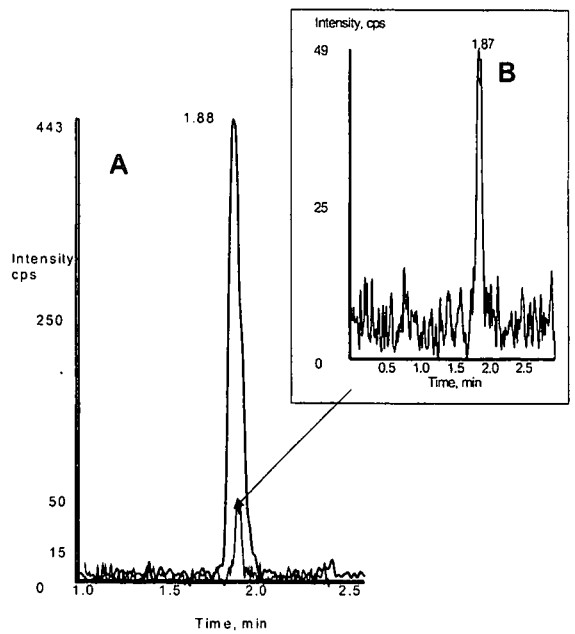

Fig. 4. Chromatograms of calibration standard containing 2.95 ng/ml of clarithromycin (A) in plasma and blank plasma extract (B), showing carry-over.

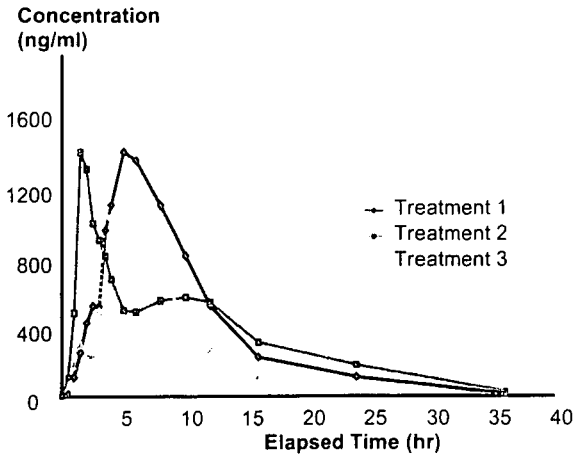

Fig. 5. Representative mean clarithromycin plasma concentration–time profile ($n=9$), as obtained after a single 500 mg oral dose of clarithromycin to human volunteers.

Fig. 3 shows representative chromatograms of clarithromycin obtained at 20 016 ng/ml and 2.95 ng/ml (LLOQ), while Fig. 4 depicts chromatograms from a calibration standard containing 2.95-ng/ml of clarithromycin, and a blank plasma extract (showing carry-over), magnified as an inset.

Fig. 5 represents the mean pharmacokinetic profiles obtained after a single dose of 500-mg clarithromycin of three different formulations of clarithromycin tested in nine healthy human volunteers. The maximum clarithromycin plasma concentrations obtained varied between 450 and 1800 ng/ml.

4. Conclusion

A highly selective method for the quantification of clarithromycin in human plasma has been developed and validated. Plasma concentrations of clarithromycin could be quantified from 2.95 to 20 016 ng/ml, making it possible to analyse samples up to 36 h after a single oral dose of 500 mg of clarithromycin to human volunteers. This is the first LC–MS–MS assay method described for the quantitation of clarithromycin in human plasma.

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2. CARBAMAZEPINE AND CARBAMAZEPINE-10, 11-EPOXIDE [℘]

2.1. Introduction

Carbamazepine is an established drug for the control of *grand mal* and psychomotor epilepsy and it is also effective in the treatment of trigeminal neuralgia. Furthermore, carbamazepine has proven to be effective against mania-depressive illness [31]. It is predominantly eliminated in the liver, where it is metabolised to the active metabolite, carbamazepine-10, 11-epoxide as well as other metabolites.

Over 30 metabolites of carbamazepine have been isolated, with the major metabolites being carbamazepine-10, 11-epoxide, iminostilbene and trans-10, 11-dihydroxy-10, 11-dihydro-carbamazepine [32, 33]. Hydrolase enzymes easily degrades carbamazepine-10, 11-epoxide [34], leading to carbamazepine-diol, which has no anticonvulsive activities. Carbamazepine-10, 11-epoxide shows a low percentage protein binding (~50%), with carbamazepine at almost 80% [35].

It is advisable to determine the plasma concentrations of carbamazepine-10, 11-epoxide during therapeutic drug monitoring, due to the fact that the metabolic rate of carbamazepine depends on the presence of enzyme inducers, which are common in anti epileptic therapy.

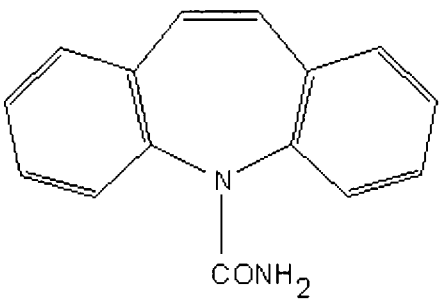

Figure 22: Structure of carbamazepine.

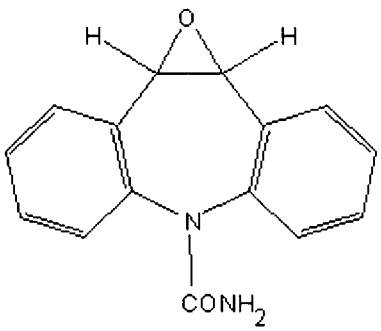

Figure 23: Structure of carbamazepine-10, 11-epoxide.

[℘] See appendix II

Adverse events such as nystagmus, dizziness, disturbance of vision and ataxia may occur within the therapeutic range (3 000 to 12 000 ng/ml)[36].

Carbamazepine is absorbed very slowly through the gastrointestinal tract after oral administration, causing a pseudo-steady-state plasma concentration. Induction of diarrhoea increases the motility of the gastrointestinal tract, which favours absorption and a faster time (t_{max}) to the maximum plasma concentration (C_{max}), reaching the therapeutic window in a shorter period [34].

2.2. Method Development and Validation

Since this method is a multi-component method, each analyte was treated independently with regard to acceptance or rejection and re-analysis.

2.2.1. Literature Survey

Mihaly *et al.*, [37] described a high-performance liquid chromatography method for the determination of carbamazepine and its epoxide metabolite and compared it to GC and immunoassays.

To 1ml of plasma was added 1ml of sodium phosphate buffer (0.3M, pH 4.3) and 5ml chloroform, containing cyheptamide (50 µg). Each sample was vortex-mixed and centrifuged, whereafter the organic (lower) layer was transferred to another tube and 1ml of phosphate buffer (0.2M, pH 11) was added. The sample was again vortex-mixed and centrifuged, and the aqueous phase discarded. The remaining chloroform was evaporated under nitrogen (50°C). The residue was reconstituted into 50µl of methanol, and 10µl was injected onto the HPLC column (RP µBondapak, C_{18}, 300 x 3.9mm). The mobile phase (methanol: water, 55:45, v/v) was pumped through the system at a flow rate of 1.3 ml/min. Carbamazepine had a retention time of ~6.5 minutes, while carbamazepine-10, 11-epoxide had a retention time of ~4 minutes.

They reported to have an 85% recovery for both analytes and a limit of detection (LOD) at 50.0 ng/ml for carbamazepine-10, 11-epoxide and 16.7 ng/ml for carbamazepine. Their calibration curves were in the concentration range of 500 to 20 000 ng/ml for carbamazepine and 50.0 to 10 000 ng/ml for carbamazepine-10, 11-epoxide.

Plasma concentrations for carbamazepine-10, 11-epoxide in epileptic patients receiving chronic administration of carbamazepine, 0.3 – 1.2 g/day, ranged between 520 ng/ml to 3 880 ng/ml, with a mean concentration of 1 500 ng/ml. They stated that the use of both acidic and basic clean-up steps during extraction was unavoidable. They found a highly significant correlation between results by the three different procedures (HPLC, GC and immunoassay) for the quantification of carbamazepine, which was highlighted by the near-unity values for the slopes of the curves of the three methods, although it was stressed that accurate measurements of carbamazepine via GC-MS was very difficult, because of the compound's heat-instability. The HPLC method was also the only method that would quantify carbamazepine-10, 11-epoxide in plasma samples.

Kumps [32] published a liquid extraction method for the determination of carbamazepine and oxcarbamazepine (a 10,11-dihydro-10-keto derivate of carbamazepine), and their respective metabolites from serum.

To 500µl of serum/plasma was added 50µl of sodium hydroxide (1M) and ethyl acetate (2.5ml), containing the internal standard, 9-hydroxy-methyl-10-carbamoylacridane. The sample was vortex-mixed and centrifuged, whereafter 2ml of the organic phase was transferred to a centrifuge tube and evaporated to dryness at 50°C with nitrogen. 100µl of mobile phase (water: methanol: acetonitrile, 55: 40: 5, v/v/v) was added to the residue, vortexed and injected (40µl) onto the analytical column (RP Spherical C_{18}, 150 x 3.9mm, 5 µm, Waters Ass.). The mobile phase was pumped through the

system at 0.9 ml/min. The ultraviolet (UV) detector was fixed at 254nm. Carbamazepine had a retention time of 13.8 minutes, while carbamazepine-10, 11-epoxide had a retention time of 6.1 minutes.

Several extraction solvents were investigated, including ethyl acetate, methyl-isobutylketone, dichloromethane, ethyl ether, di-isopropylether, and several mixtures of these solvents. Methyl-isobutylketone and dichloromethane yield clean chromatograms and did not extract phenobarbital and phenytoin, but had a poor recovery for the analytes of interest.

Kumps reported a limit of detection (LOD) of 50.0 ng/ml for carbamazepine and 100 ng/ml for carbamazepine-10, 11-epoxide and showed excellent separation between the drugs with no interference from other anticonvulsants or endogenous constituents from the samples. This method was applied in a pharmacokinetic study.

Contin *et al.*, [38] determined the total and the free plasma carbamazepine concentrations by enzyme-multiplied immunoassay technique (EMIT) in 1985. Their study was designed to evaluate the results obtained from HPLC methods and EMIT methods.

The HPLC method that they used was adapted from Mihaly *et al.*, [37]. Contin *et al.*, extracted 300µl of plasma (or plasma filtrate) with chloroform (4ml) in an alkaline medium (6N, 50µl, sodium hydroxide). The dried extract was dissolved in mobile phase (methanol: water, 55: 45, v/v) and injected onto a reverse-phase column (Lichrosorb C_{18}, 250mm, 10 µm) and detected at 210nm.

They reported a recovery for carbamazepine of 99% and for carbamazepine-10, 11-epoxide of 97%. The EMIT values were, on average, 35% higher than the HPLC values for the unbound carbamazepine. They reported total carbamazepine-10, 11-epoxide concentrations to be 5,4 $\pm$ 2,1 µmol/l (ranging from 1,6 to 13,2 µmol/l) and free carbamazepine-10, 11-epoxide concentrations at 2,0 $\pm$ 0,8µmol/l (ranging from 0,55 to 5,0µmol/l) in clinical specimens from patients receiving constant therapy of carbamazepine for at least 2 months.

Contin *et al.*, noted that the observed differences between the two methods in accuracy are of no serious consequence, provided that the same technique is constantly used in a given clinical study, and that the methodological discrepancies reported, are taken into account in the interpretation of drug monitoring data.

Post *et al.*, [31] reported no extraction or analytical method, nor any condition under which carbamazepine was determined, but gave valuable insight on the average blood level of carbamazepine, 9 500 ng/ml (ranging from 7 800 to 11 700 ng/ml) after an average dose of 925 mg/day.

Post *et al.*, suggested that carbamazepine does not act by blocking dopamine receptors and thus measured the dopamine metabolite homovanillic acid (HVA) in the cerebrospinal fluid.

Vree *et al.*, [34] determined carbamazepine and two of its metabolites, including carbamazepine-10, 11-epoxide, in humans, after an overdose, via high-performance liquid chromatography.

To determine carbamazepine and carbamazepine-10, 11-epoxide, they added sodium hydroxide (1N, 50µl) and diethyl ether (1.5ml) to 100µl of plasma or urine. After mixing and centrifugation, they evaporated 1ml of the organic phase to dryness under nitrogen (70°C). The residue was dissolved in mobile phase (methanol: water, 55: 45, v/v)(400µl) and injected (100µl) onto the analytical column (RP CpSpher C_8, 250 x 4.6mm, 5µm, Chrompack), protected by a guard

column (CpSpher C_8, 20 µm, Chrompack). A spectrophotometer was used for detection (215nm). The mobile phase was pumped through the system at a flow rate of 1.6 ml/min with a retention factor [Δ] for carbamazepine of 6.6 and for carbamazepine-10, 11-epoxide of 13.9.

Vree *et al.*, reported a limit of detection (LOD) for both analytes at 100 ng/ml, with a recovery of 88% ± 5.

The C_{max} for carbamazepine ranged from 12 000 to 77 000 ng/ml with a t_{max} from 1.5 to 18.0 hours. The half-life ($t_{1/2}$) reported for carbamazepine ranged from 11.0 to 38.0 hours. For carbamazepine-10, 11-epoxide, the C_{max} ranged from 3 000 to 34 000 ng/ml and the t_{max} from 10.0 to 30.0 hours. The half-life ($t_{1/2}$) reported for carbamazepine-10, 11-epoxide ranged from 11.0 to 48.0 hours.

Vree *et al.*, suggested that an induction of diarrhoea would increase the motility of the gastrointestinal tract, which favours absorption and a faster time (t_{max}) to the maximum plasma concentration (C_{max}), reaching the therapeutic window in a shorter period.

Mendez-Alvarez *et al.*, [39] determined the long-term stability for carbamazepine and carbamazepine-10, 11-epoxide in plasma.

They diluted 100µl of plasma with 100µl of water, containing the internal standard, 10, 11-dihydrocarbamazepine. To this mixture, they have added 2.5ml dichloromethane. They completely deproteinised the samples with the addition of solid ammonium sulphate (200mg), whereafter the sample was vortex-mixed, centrifuged and the aqueous phase discarded. Two millilitres of the organic phase were evaporated to dryness under air (45°C). The residue was dissolved in 50µl of mobile phase (acetonitrile: water, 28: 72, v/v) and 20µl was injected onto the analytical column (Spherisorb ODS, 250 x 4.9mm, 5 µm, Kontron AG), preceded by a 50 x 4.9 mm i.d. guard-column, fitted with Co: Pell ODS (Whatman Inc.). The ultraviolet detector detected carbamazepine at ~9.0 minutes and carbamazepine-10, 11-epoxide at ~5.0 minutes, respectively. Recoveries of 99% for carbamazepine and 98% for carbamazepine-10, 11-epoxide were reported. Mendez-Alvarez *et al.*, detected an unidentified compound with a peak which eluted faster than carbamazepine-10, 11-epoxide. This peak did not interfere with their assay. They also gave valuable insight on the stability of both carbamazepine and carbamazepine-10, 11-epoxide in plasma.

Deng *et al.*, [36] wrote an article on carbamazepine toxicity and compared enzyme-multiplied immunoassay techniques and high-performance liquid chromatography methods.

To 400µl of plasma was added internal standard (diazepam), and dichloromethane (5ml), whereafter the sample was shaken and centrifuged. The aqueous layer was removed by aspiration and an aliquot (4ml) of the organic phase was evaporated under reduced pressure at ambient temperature. The residue was dissolved in 250µl of mobile phase (methanol: water, 55: 45, v/v), after which 100µl was injected onto the column (RP Bondapak C_{18}, 300 x 3.9mm, Waters Ass.). The mobile phase was pumped through the system at a flow rate of 0.75 ml/min. The variable wavelength detector (254nm) detected carbamazepine and carbamazepine-10, 11-epoxide at ~8.0 and ~6.0 minutes, respectively.

[Δ] Also known as: Capacity factor = $\frac{\text{(Elution time of retained component – elution time of unretained components (solvent front))}}{\text{Elution time of unretained components (solvent front)}}$

Peak carbamazepine plasma concentrations were at 30 000 ng/ml after 14.0 hours, and for carbamazepine-10, 11-epoxide at 14 500 ng/ml after 28.5 hours, after a ~2000mg oral dose. The elimination half-life for carbamazepine was reported to be 16.5 hours and for carbamazepine-10, 11-epoxide to be 9.8 hours.

The high-performance liquid chromatography method proved to be superior over the enzyme-multiplied immunoassay techniques, since the enzyme-multiplied immunoassay techniques can only determine carbamazepine concentrations due to antibody cross-reactivity with structural similar metabolites.

Chelberg *et al.*, [33] determined carbamazepine and various metabolites from plasma and urine, using a variable-wavelength detector set at 212nm.

They used 2-methylcarbamazepine as internal standard. To 250µl of plasma was added sodium hydroxide (0.1M, 250µl) and methyl-*t*-butyl ether (3ml). The sample was vortex-mixed, shaken and centrifuged. The top layer was transferred to a clean tube, put into a waterbath (20°C) and evaporated to dryness under nitrogen. The residue was reconstituted in mobile phase (acetonitrile: water, 28: 72, v/v) and 20µl was injected onto the analytical column (RP Rainin C_{18}, 150 x 4.6mm, 5 µm, Rainin Instrument Co.), maintained at 37°C. The pump delivered a flow rate of 2.0 ml/min. Retention times for carbamazepine and carbamazepine-10, 11-epoxide were 4.95 and 2.48 minutes, respectively.

A recovery of 100% for both analytes was reported with a limit of detection (LOD) at 10.0 ng/ml. The method was validated from 1000 ng/ml to 16 000 ng/ml for carbamazepine, and from 2 000 ng/ml to 10 000 ng/ml for carbamazepine-10, 11-epoxide.

Chelberg *et al.*, using the described chromatography conditions, could distinguish between 3-hydroxycarbamazepine, 2-hydroxycarbamazepine and carbamazepine-10, 11-epoxide, although no 3-hydroxycarbamazepine was detected in the unknown plasma samples.

Martens and Banditt [40] validated and compared a HPLC method with an EMIT and a GC-procedure.

Sodium hydroxide (1.5M, 400µl) and sodium chloride (100mg) was added to 400µl of serum. 1,3-dimethyl-7-benzylxanthine was used as internal standard. The analytes were extracted with ethyl acetate: chloroform (1:1, v/v) (4ml), shaken, centrifuged and evaporated (organic). The residue was reconstituted in 200µl of hexane (GC) or mobile phase (acetonitrile: water, 3: 7, v/v)(HPLC).

The HPLC analysis was performed at a flow-rate of 1.1 ml/min. 20µl of the reconstitute was injected onto the column (Spherisorb ODS-2, 250 x 4mm, 5 µm, LKB Pharmacia), preceded by a guard-column (10 x 4mm, 5 µm, LKB Pharmacia). For the HPLC method, detection was performed with a UV-detector set at 210nm. Carbamazepine was detected at 10.9 minutes, while carbamazepine-10, 11-epoxide was detected at 5.6 minutes.

The GC-system was a Hewlett-Packard HP 5890A fitted with a flame ionisation detector, using a HP-17 capillary column (10m x 0.53mm). The immunoassay was a SYVA carbamazepine assay run on a SYVA autolab System.

Reported limits of detection (LOD), using HPLC, for carbamazepine and carbamazepine-10, 11-epoxide were 80.0 ng/ml and 7.00 ng/ml, with limits of quantification (LOQ) at 270 ng/ml and 25.0 ng/ml, respectively.

The analysis of carbamazepine-10, 11-epoxide unfortunately could not be done via GC, nor the enzyme-multiplied immunoassay technique, but the HPLC method and the enzyme-multiplied immunoassay technique showed good

comparison for carbamazepine concentrations. The correlation between the high-performance liquid chromatography method and gaschromatography -procedures was quite poor.

Liu *et al.*, [41] determined various antiepileptic drugs and their metabolites from serum, saliva and urine with high-performance liquid chromatography, using a photodiode-array detector.

They precipitated the proteins by adding acetonitrile (200µl) to 100µl of serum. The sample was vortex-mixed and centrifuged. 2µl of the supernatant was injected onto the column (ODS-Hypersil, 250 x 2mm, 3 µm, Keystone Scientific) maintained at 40°C and preceded by a guard column (ODS-Hypersil, 20 x 2mm, Keystone Scientific). The mobile phase consisted of potassium phosphate buffer (0.01M, pH 7): acetonitrile: methanol (110: 50: 30, v/v/v) and was pumped through the system at a flow-rate of 0.2 ml/min. A HP 1040A photodiode-array detector, set at 200nm, detected carbamazepine at ~13.0 minutes, and carbamazepine-10, 11-epoxide at ~8.0 minutes. Nitrazepam was used as internal standard, initially, but Liu *et al.*, showed that the use of an internal standard with this assay method was not necessary, and discontinued the use of the internal standard in subsequent quantitation studies.

The dependence of carbamazepine on different pH of the potassium phosphate buffer in the mobile phase was investigated. Carbamazepine showed no dependence on pH at the three pH levels (6.7, 7.0 and 7.3) that were investigated, with a retention factor of ~5.4.

Calibration ranges for carbamazepine stretched from 780 ng/ml to 50 000 ng/ml, and for carbamazepine-10, 11-epoxide from 390 to 25 000 ng/ml. Recoveries of 97.2% $\pm$ 2.5 were reported for carbamazepine, and 98.5% $\pm$ 2.0 for carbamazepine-10, 11-epoxide.

Liu *et al.*, assayed hundreds of samples from patients on a wide variety of drug regimens with this assay method, and found no substance, compound or metabolite that interfered with their analysis.

Kouno and Ishikura [42] described a HPLC method, using a syringe-type mini-column for the extraction of three antiepileptic drugs (AED) in therapeutic drug monitoring. The analytes were injected and extracted onto a silica-gel column using a syringe-type minicolumn, Extrashot-silica™ from Kusano Scientific.

The syringe-type minicolumn was preconditioned with 200µl ethanol and dichloromethane. The remaining dichloromethane was pushed out of the syringe-type minicolumn with 500µl of air, using a syringe. 5µl of serum was introduced onto the surface of the support material of the syringe-type minicolumn with a microsyringe, whereafter the syringe-type minicolumn was connected to the HPLC system through the injector.

Dichloromethane (130µl) was gently introduced onto the syringe-type minicolumn, through the injector. The mobile phase (*n*-hexane: acetic acid: ethanol: dichloromethane, 82.8: 0.2: 2.0: 15.0, v/v/v/v) was pumped through the system at a flow-rate of 1 ml/min. The analytical column that was used on the HPLC system was a LiChrosorb Si60 (125 x 4mm, 5 µm) from Merck, Darmstadt, Germany. The ultraviolet (UV) detector was set at 240nm, with a retention time of 14.7 minutes for carbamazepine.

Carbamazepine could be detected from 1 000 to 20 000 ng/ml, with a mean recovery of 96.6% $\pm$ 2.4. The analytical accuracy of their method was confirmed by comparison with EIA, although they reported slightly higher concentrations of carbamazepine as compared to EIA. This assay procedure is clearly not an automated high-throughput method, and

would be very laborious and impractical to implement in a large pharmacokinetic study, but has the advantage of requiring only 5µl serum.

Romanyshyn *et al.*, [43] used high-performance liquid chromatography to simultaneously determine various antiepileptic drugs from more than 20 000 human plasma samples.

To 100µl of plasma was added 1ml of sodium hydroxide (1N, saturated with ammonium sulphate) and 8ml organic (ethyl acetate: methyl t-butyl ether, 2: 98, v/v). The sample was rotated and centrifuged, whereafter 7ml of the organic phase was evaporated under reduced pressure at 50°C. The residue was reconstituted in 200µl of mobile phase. The mobile phase contained buffer: acetonitrile: methanol (70: 16: 14, v/v/v), where the buffer consisted of dibasic potassium phosphate trihydrate (5.71 mg/ml in water) and monobasic potassium phosphate (3.43 mg/ml in water), adjusted to pH 6.9 with dibasic potassium phosphate trihydrate.

The mobile phase heated to column temperature (40-50°C) was delivered at a flow-rate of 1 ml/min. 20µl was injected onto the Spherisorb ODS2 column (150 x 4.6mm, 3 µm, YMC Inc. or Keystone Scientific Inc.), maintained between 40°C and 50°C. An ultraviolet (UV) detector, set at 210nm, detected carbamazepine after 20.7 minutes and carbamazepine-10, 11-epoxide after 9.1 minutes.

Romanyshyn *et al.*, reported recoveries of 89.7% $\pm$ 0.7 for carbamazepine, and 87.3% $\pm$ 1.3 for carbamazepine-10, 11-epoxide. The calibration lines were linear with correlation coefficients (r^2) of 0.99998 (ranging from 195 to 100 000 ng/ml) for carbamazepine and 0.99981 (ranging from 49.0 to 25 000 ng/ml) for carbamazepine-10, 11-epoxide.

The doses given by Romanyshyn *et al.*, were up to 3 000 mg/day for carbamazepine, divided into three doses.

Matar *et al.*, [44] published an article that determined several antiepileptic drugs and their metabolites using high-performance liquid chromatography in plasma.

100µl of plasma was mixed with 20 µl of methanol, containing the internal standard (9-hydroxymethyl-10-carbamyl-acridan), and 1ml of diethyl ether. The sample was vortex-mixed, shaken and centrifuged. The organic phase was evaporated to dryness at room temperature with nitrogen after phase separation. The residue was dissolved in mobile phase, comprising of K_2HPO_4 (0.01M), methanol and acetonitrile (65: 18: 17, v/v/v). 20µl was injected onto the column, a Supelcosil LC-18 stainless steel column (150 x 4.6mm, 5µm, Supelco), preceded with a C_{18} guard column (Waters). The mobile phase was pumped through the system at 1 ml/min and carbamazepine and carbamazepine-10, 11-epoxide was detected by a dual wavelength absorbance detector (220nm) after 10.2 and 4.5 minutes, respectively.

Matar *et al.*, reported a mean recovery of ~98% for carbamazepine-10, 11-epoxide and ~99% for carbamazepine over a range of concentrations.

They also gave valuable insight on the stability of both carbamazepine and carbamazepine-10, 11-epoxide in plasma.

Speed *et al.*, [45] determined six anticonvulsant drugs with a Hewlett-Packard 5890 series II gas-chromatograph with an HP 5970 mass spectrometer, fitted with a fused-silica capillary column (SGE).

For determining carbamazepine with GC-MS, Speed *et al.*, needed to derivatise carbamazepine. To 200µl of whole blood, they have added 1.6ml of a sodium dihydrogen orthophosphate solution (1M). This mixture was sonicated and centrifuged. The Bond Elute columns, which combine a C_{18} sorbent with a cation exchange phase, were conditioned

with 2ml of methanol, followed by 2ml of phosphate solution (1M). The blood sample was applied and washed with 1ml of sodium dihydrogen orthophosphate (1M) and 1ml of acetic acid (0.01M). The column was dried and elution was achieved with 2ml of acetone. The extract was butylated by adding 100µl of tetramethyl ammonium hydroxide (2.5%) and 50µl of butyl iodide to the extract. The mixture was heated to 65°C and evaporated with nitrogen to between 200 and 500µl. Saturated sodium chloride (1ml), bicarbonate buffer (0.1M, 150µl) and ethyl acetate (200µl) were added. The mixture was vortex-mixed and centrifuged, whereafter the organic phase (ethyl acetate) was transferred to an autosampler vial and 3µl was injected onto the fused-silica capillary column (12m x 0.15mm) with a cross-linked (5% phenyl)-methylpolysiloxane coating of 0.4µm thickness, using splitless injection.

Although no details regarding the method's performance were given, Speed *et al.*, reported an approximate recovery of ~158%, from 500 to 60 000 ng/ml (r^2 = 0.998). Speed *et al.*, suggested that this recovery of carbamazepine indicates that the breakdown product was formed to a larger extent in the extracted standard. They also stated that this value does not reliably represent the extraction efficiency.

Fedorova *et al.*, [46] applied micro-column (Milichrom A-02) high-performance liquid chromatography to determine carbamazepine and phenobarbital in serum. Chromatography was accomplished with a C_{18} column, thermostated at 35°C and a gradient elution solvent, consisting of lithium perchlorate and acetonitrile, pumped through the system at 0.15 ml/min. Detection was done by UV-absorption at 210, 220, 230 and 240nm.

To extract the analytes, 1ml of hexane was added to 120µl of serum. The mixture was shaken and centrifuged, whereafter the organic phase, which contained the extracted lipids, was discarded. To 50µl of the aqueous layer was added 50µl of an acetonitrile solution of lithium perchlorate-acetic acid (0.6M, 1%) to precipitate the remaining plasma proteins. The sample was again shaken and centrifuged and 5 to 10µl was injected onto the microbore column (Nucleocil 100-5 C_{18}, 2 x 75mm, Macherey-Nagel).

Carbamazepine had a retention time in the order of 14 minutes. The limit of detection for this extraction method was set at 50.0 ng/ml, using 50 µl of serum. The range for carbamazepine was from 2 000 to 16 000 ng/ml.

The authors considered an important feature of this method to be the low costs involved: one analysis with two parallel serum samples is done with only 1ml of hexane and 2ml of acetonitrile.

Queiroz *et al.*, [47] compared high-resolution gas chromatography (HRGC) with high-performance liquid chromatography for the determination of carbamazepine.

For HPLC, they precipitated the plasma proteins by adding acetonitrile (200µl), containing the internal standard, 4-methyl primidone, to 100µl plasma. The mixture was vortex-mixed and centrifuged. An aliquot of the supernatant (100µl) was transferred to a clean vial, and the organic solvent was evaporated with nitrogen. The residue was reconstituted in 100µl of water and 20µl was injected onto the column.

Separation was at room temperature, using a RP 18 (125 x 4mm, 5µm, Lichrocart-Merck) column, with a guard column with the same packing material, and a mobile phase consisting of potassium phosphate buffer (0.01M, pH 6.5): acetonitrile: methanol (65: 18: 17, v/v/v), pumped through the system at 1 ml/min.

Carbamazepine and carbamazepine-10, 11-epoxide had retention times of 10.9 and 6.1 minutes, respectively. The HPLC method was more sensitive, with a LOQ for carbamazepine at 100 ng/ml, compared to 250 ng/ml using HRGC.

Carbamazepine was quantified from 250 to 20 000 ng/ml and was linear with the correlation coefficient greater than 0.999. The relative recovery for carbamazepine was reported to be ~101%.

Kuldvee and Thormann [48] proved that micellar electrokinetic capillary chromatography (MEKC) in the absence of electro-osmosis is suitable for the determination of carbamazepine and carbamazepine-10, 11-epoxide.
They have added 25µl of *p*-bromoacetanilide (internal standard), 50µl of sodium hydroxide (0.5M) and 1ml of ethyl acetate to 250µl of plasma. After the mixture was shaken, it was centrifuged and the organic phase was transferred to a clean vial. The organic phase was evaporated to dryness at 40°C with nitrogen and the residue was dissolved in 60µl of 10-times diluted running buffer, containing 5% methanol instead of 2-propanol. The running buffer was composed either of $Na_2B_4O_7/NaHPO_4$ or $H_3PO_4/NaOH$, where the sodium dodecyl sulphate concentration varied between 10 to 75 mM and the 2-propanol content between 0 and 5% v/v.
Analyses were performed in a BioFocus 3000 capillary electrophoresis system (BioRad Laboratories), equipped with an untreated fused silica capillary (50/375 µm ID/OD, 50 cm, Polymicro Technologies). A voltage between 20 and 25 kV was applied over the capillary, while it was maintained at 25°C. For spectral analysis, the detector was operated in the fast scanning mode between 195 and 320 nm, with 5 nm increments.
They reported a recovery of 92% for carbamazepine and a 95% recovery for carbamazepine-10, 11-epoxide. Carbamazepine and carbamazepine-10, 11-epoxide had retention times of 4.2 and 2.0 minutes, respectively.
Kuldvee and Thormann concluded that an improved coating stability is required such that the attractive features of the described MEKC assay in the absence of electroosmosis can be adopted for routine use.

Sanchez *et al.*, [49] demonstrated the good correlation between carbamazepine free serum concentrations obtained by ultrafiltration and protein precipitation with sulphosalicylic acid.
To determine the free serum concentration, they filtered 1ml of serum through the ultrafiltration device (Centrifree), whereafter the serum was centrifuged at 25°C. For the protein precipitation method, they used 200µl of serum to which they have added 200µl sulphosalicylic acid as a 5% solution in methanol: water (1:1, v/v). The mixture was vortex-mixed and centrifuged.
Detection was by fluorescent polarisation immunoanalysis (TDx, Abbott) for both techniques, using 100µl of the supernatant serum.
The mean carbamazepine serum concentration found was 7 100 ng/ml with an IV dose of 14.2±5 mg/kg BD. No significant difference was found between the two extraction methods.

Ternes *et al.*, [50] determined various neutral drugs, including carbamazepine, from water using GC/MS and LC-MS/MS.
They used solid phase extraction as an enrichment step. The solid phase material (C_{18}-end capped) was conditioned with 3 x 2ml hexane, followed by 3 x 2ml methanol and 1ml water (pH 7.5) and 2ml tetramethylammoniumhydroxide. After washing the cartridges with 5 x 2ml of water (pH 7.5), 1000ml of sample (drinking water) was applied to the solid phase cartridges. The cartridges were dried under reduced pressure and the analytes were eluted with 5 x 1ml of methanol. For the detection by LC-MS/MS, the extracts were evaporated to about 20µl and diluted to 1ml with phosphate buffer (20mM, pH 6). 50µl was injected onto the LiChrospher (125 x 3mm, 5µm, Merck) RP-C_{18} column.

The mobile phase was pumped through the system at 0.4 ml/min and consisted of water: acetonitrile (68.5: 31.5, v/v), containing ammonium acetate (10mmol/l) at pH 5.7. Carbamazepine was detected after 11.1 minutes.
They monitored the transition from *m/z* 237.2 to *m/z* 179.2 for carbamazepine with a Perkin Elmer API III, using electrospray ionisation in the positive ion mode. The detection limit in drinking water for carbamazepine was 0.01 ng/ml, using LC-MS/MS. The carbamazepine recovery was 99%.

Gholami *et al.*, [51] determined carbamazepine and carbamazepine-10, 11-epoxide in serum with a protein precipitation method, using UV detection (214nm).
To 500µl of serum, they added acetonitrile (1ml) containing the internal standard (benzophenone). The mixture was vortex-mixed and centrifuged. 20µl of the supernatant was injected onto a µ-Bondapack phenyl column (300 x 3.9mm, 4µm). The mobile phase consisted of methanol: water (1:1, v/v) and was pumped through the system at a flow rate of 1.5 ml/min. An UV detector (214nm) detected carbamazepine after 5.2 minutes and carbamazepine-10, 11-epoxide after 7.1 minutes. The carbamazepine and carbamazepine-10, 11-epoxide was linear over a very wide range up to 10 000 ng/ml. The recoveries reported for carbamazepine range from 80 to 85% and for carbamazepine-10, 11-epoxide from 95 to 98%. The limit of detection (LOD) was reported to be 10.0 ng/ml for both analytes.
This method proved to be simple and precise, but not sensitive enough.

2.2.1.1. Literature Summary

Table 32: Summery of most important literature.

	Volume of serum/ plasma used	Inj. Vol.	LOD		LOQ		~Recovery		Retention times		Dose	C_{max}	
			CBZ	CBZ-epox	CBZ	CBZ-epox	CBZ	CBZ-epox	CBZ	CBZ-epox		CBZ	CBZ-epox
	(µl)	(µl)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(%)	(%)	(min)	(min)	(mg)	(ng/ml)	(ng/ml)
Mihaly et al.,	1000		50	16.67	500	50	85	85	7.0	5.0			1 500
Kumps	500	40	50	100					13.8	6.1		8 500	2 780
Post et al.,											925	9 500	
Vree et al.	100	100	100	100			88	88			1000	77 000	34 000
Mendez-Alvarez et al.,	100	20					99	98	9.0	5.0			
Deng et al.,	400	100									2000	30 000	14 500
Chelberg et al.,	250	20	10	10	1000	2000	100	100	5.0	2.5		16 000	10 000
Martens and Banditt	400	20	80	7	270	25			10.9	5.6			
Liu et al.,	100	2			780	390	97	98	13.0	8.0		50 000	25 000
Kouno and Ishikura	5	5			1000		96		14.7			20 000	
Romanyshyn et al.,	100	20			195	49	89	87	20.7	9.1			
Gholami et al.,	500	20	10	10			83	96	5.2	7.1			
Sanchez et al.,	200											7 100	
Matar et al.,	100	20	200	200	2000	500	98	99	10.2	4.5			
Speed et al.,	200				500		158						
Fedorova et al.,	120	5	50						14.0				
Queiros et al.,	100	20			100		98		10.9	6.1			
Kuldvee and Thormann	250						92	95	4.2	2.0			

2.2.2. Justification for the use of LC-MS/MS

To analyse the large number of samples that are generated in a clinical study in the shortest possible time, an assay procedure with better specificity and a much faster turn-around time, without compromising the sensitivity, had to be developed.

To achieve the desired sensitivity and specificity, the use of an LC-MS/MS was imperative. In our study, the mobile phase was delivered and the samples injected by a Waters-Alliance 2790 System (Water Corporation, Milford, USA). A Micromass Quattro Ultima™ mass spectrometer (Micromass UK Ltd, Altricham, Cheshire, UK) interfaced to an electrospray ionisation (ESI) source, performed detection. The mass spectrometer operated in the multiple reaction monitoring (MRM) mode, using positive ions.

2.2.3. Mass Spectrometry

Mass spectrometry using electrospray ionisation (ESI) was optimised and performed in the positive mode with the nebulizing gas (nitrogen), cone gas and desolvation gas set at 80, 135 and 640 L/h, respectively. The ionisation source was connected by a fused silica capillary to the syringe pump for tuning, and the instrument response for carbamazepine and carbamazepine-10, 11-epoxide were optimised using flow injection. Optimal responses were obtained with a cone voltage setting of 40V, a heated capillary voltage of 2.8 kV and a source block temperature of 100°C. The desolvation temperature was set at 400°C.

The transitions of the protonated molecular ions for carbamazepine at *m/z* 237 and carbamazepine-10, 11-epoxide at *m/z* 253 to the predominant ions were *m/z* 194 and 180, respectively. The isolation width was 2amu and the relative collision energy was set at 30%, for both analytes. The mass spectrometer was interfaced to a computer workstation running MassLynx version 3.3 software.

Figure 24 shows the product ion mass spectrum (MS/MS) of carbamazepine and figure 25 the product ion mass spectrum (MS/MS) of carbamazepine-10, 11-epoxide, acquired with the abundant product ions at *m/z* 194 and 180, respectively. A stronger product ion with *m/z* 210 was observed for carbamazepine-10, 11-epoxide, but the production formed at *m/z* 180 was more stable. Figure 26 shows the product ion mass spectrum (MS/MS) of nitrazepam.

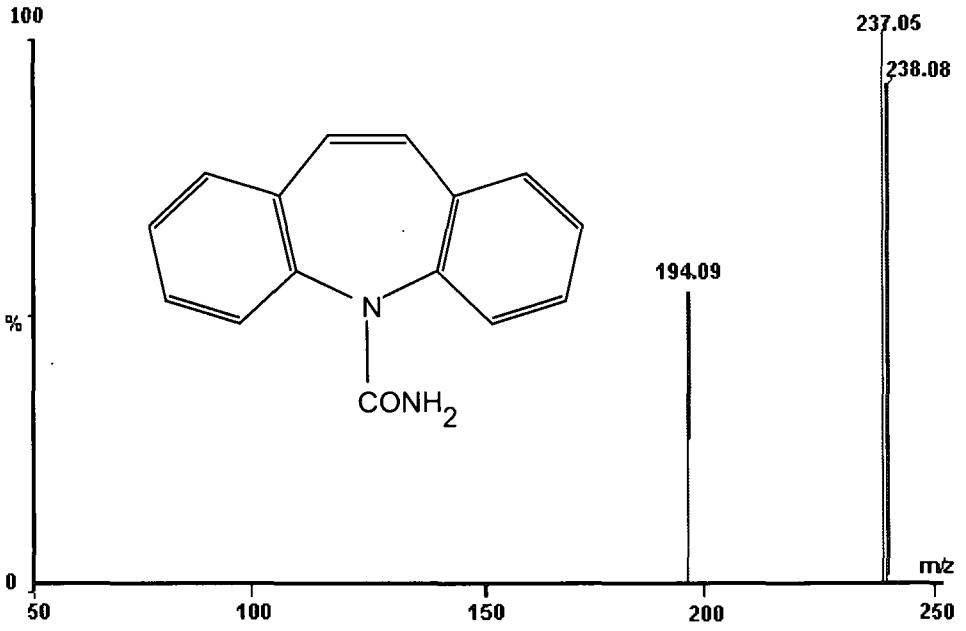

Figure 24: A full scan spectrum (MS/MS) of a pure solution of carbamazepine in acetonitrile: 0.1% formic acid (1:1, v/v). The parent [M+1] ion with m/z 237 and the predominant product ion m/z 194 is shown.

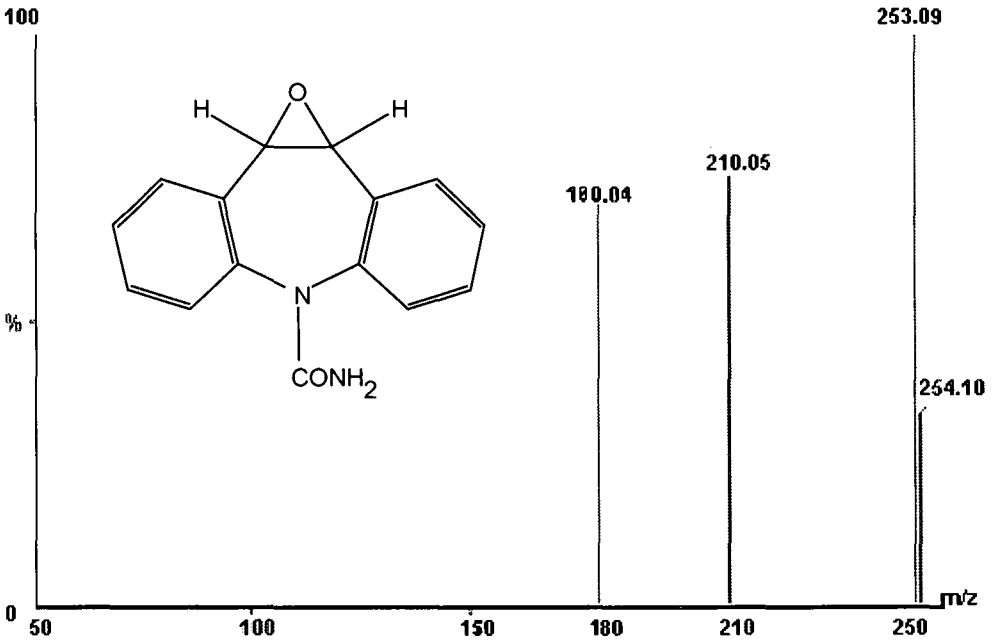

Figure 25: A full scan spectrum (MS/MS) of a pure solution of carbamazepine-epoxide in acetonitrile: 0.1% formic acid (1:1, v/v). The parent [M+1] ions with m/z 253 and predominant product ions m/z 180 and m/z 210 are shown.

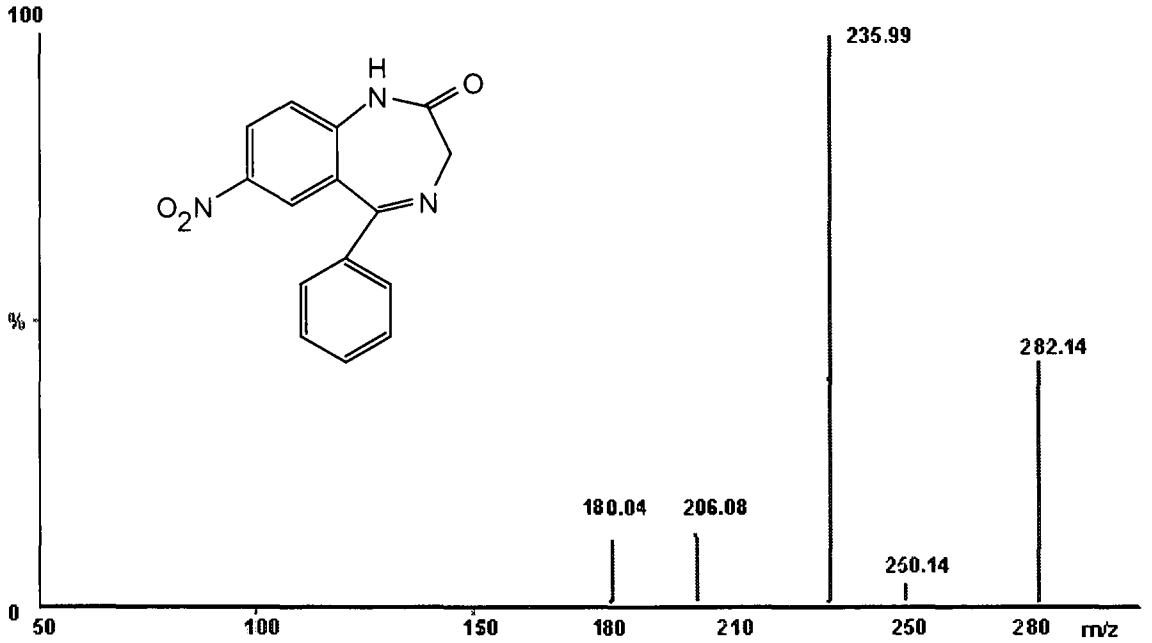

Figure 26: A full scan spectrum (MS/MS) of a pure solution of nitrazepam in acetonitrile: 0.1% formic acid (1:1, v/v). The parent [M+1] ions with m/z 283.14 and the predominant product ions with m/z *235.99.*

Quantitation was achieved at unit resolution in full scan MS/MS mode, scanning the product ion spectrum from *m/z* 50-260 and monitoring the transition of the protonated molecular ion at *m/z* 237 for carbamazepine, to the largest product ion, *m/z* 194 and the protonated molecular ion at *m/z* 253 for carbamazepine-10, 11-epoxide to the more stable product ion, *m/z* 180. The transition of the protonated molecular ion at *m/z* 282 for nitrazepam, to the largest product ion, *m/z* 236, was monitored.

2.2.4. Liquid Chromatography

For optimum ionisation of basic drugs, using electrospray ionisation (ESI), the mobile phase should ideally be at least two pH units below the pKa value of the drug (figure 3), thus the stock solutions of carbamazepine and carbamazepine-10, 11-epoxide were prepared in acetonitrile: 0.1% formic acid (2 000 ng/ml). Several injections of this stock solution were made onto the system to optimise chromatography. A Phenomenex Luna C_{18} (5μ), 150 x 2mm column (Phenomenex, Torrance, CA, USA) gave the desired resolution and symmetry and was the column of choice, although no other types were tried, it proved to be adequate.

Some references [41] quoted that an elevated column temperature was used for chromatography. This seemed unnecessary and the desired chromatography was obtained without an elevated column temperature.

Separation was achieved at a flow rate of 0.25 ml/min with acetonitrile: methanol: formic acid (0.1% in water)(10: 70: 20; v/v/v) as mobile phase. 20µl was injected onto the column. The mobile phase was delivered and the samples injected by a Waters-Alliance 2790 System, using parallel injection to shorten the in-between injection time, decreasing turn-around time. All chromatographic solvents were sparged with helium before use.

Typical retention times for carbamazepine were between 2.65 - 2.84 minutes (RSD_{mean} = 0.62%) and for carbamazepine-10, 11-epoxide between 2.73 - 2.87 minutes (RSD_{mean} = 0.60%). A chromatography time of 4 minutes made it possible to analyse ~212 samples per day with ease.

2.2.5. Internal Standard

Various internal standards have been used for the determination of carbamazepine and its 10, 11-epoxide metabolite. Since Liu *et al.*, [41] initially used nitrazepam as internal standard, and as nitrazepam resembles carbamazepine and carbamazepine-10, 11-epoxide in chromatography, it was decided to use nitrazepam as internal standard, since it was easily obtainable.

Nitrazepam was obtained from FARMOVS - PAREXEL Bioanalytical Services internal reference library. VIS Farmaceutici (Hamburg, Germany) supplied carbamazepine. Carbamazepine-10, 11-epoxide was supplied by Sigma - Aldrich (Sigma-Aldrich Corporation, 3050 Spruce Street, St. Louis, MO 63103, USA).

2.2.6. Determining the Range

A 400mg oral dose of carbamazepine was given to 22 human volunteers each. Samples were taken up to 144 hours after the dose, implying that a sensitive method would be needed to determine both carbamazepine and carbamazepine-10, 11-epoxide from human plasma up to 144 hours after the dose.

2.2.7. Extraction Procedure and recovery

Advantages of a protein precipitation method is the low cost involved and the simplicity of the procedure with an almost 100% recovery. On the other hand, the disadvantages of a protein precipitation method is that one is actually diluting the samples, instead of concentrating them, causing a less sensitive extraction method with a higher limit of detection (LOD). Another shortcoming is the potential of interfering substances, since all water-soluble impurities are not precipitated and injected onto the column, decreasing the column's life.

At first, plasma containing carbamazepine and carbamazepine-10, 11-epoxide was extracted on C_{18} solid phase extraction columns. The columns were washed with ammonium hydroxide (0.01M) and the analytes eluted with acetonitrile. The eluant was dried in a Savant Speedvac evaporator under reduced pressure, and the analytes were reconstituted in the mobile phase. The extracts were found to be dirty with poor chromatography and an increase in column pressure was observed after just 5 injections.

A liquid-liquid extraction was then tried with sodium hydroxide (0.1M) as buffer, using ethyl acetate (5 ml) and diethyl ether (5 ml), respectively, as organic solvents in 10ml ampoules. The samples were vortex-mixed and centrifuged. The aqueous phases were frozen on a freezerbath at –30°C whereafter the organic phases were poured into 5ml ampoules and dried under a gentle stream of nitrogen at 40°C. The analytes were re-dissolved in mobile phase, vortexed briefly to homogenise and injected onto the HPLC column. The extraction with ethyl acetate gave the best results and it was decided to optimise this extraction.

The extraction method that proved to give the greatest recovery, accuracy and repeatability was a liquid-liquid extraction, where sodium hydroxide (0.1M, 500µl), nitrazepam (internal standard)(100µl, 800 ng/ml in water) and ethyl acetate (3ml) were added to 500µl of plasma. The sample was vortex-mixed and centrifuged. The aqueous phase was frozen on a freezerbath at –30°C, whereafter the organic phase was poured into a clean ampoule and dried under a gentle stream of nitrogen at 40°C. The analytes were re-dissolved in 200µl of mobile phase (acetonitrile: methanol: formic acid (0.1% in water)(10: 70: 20; v/v/v)), vortexed briefly to homogenise and 20µl was injected onto the HPLC column (Luna C_{18} (5µ), 150 x 2mm).

Concentrations as low as 0.722 ng/ml for carbamazepine and 5.15 ng/ml for carbamazepine-10, 11-epoxide could be determined by using this liquid-liquid extraction method.

To assess the method's extraction efficiency, the analytes were extracted in triplicate at a high, medium and low concentration in normal plasma by extracting drug-free plasma samples spiked with carbamazepine and carbamazepine-10, 11-epoxide. Recovery (tables 33 and 34) was calculated by comparison of the analyte peak-areas of the extracted samples with those of the un-extracted analyte standards, representing 100% recovery.

Table 33: Absolute recovery of carbamazepine using response factor areas.

	ANALYTE	MEAN OF PEAK AREAS		RECOVERY	CV
	(ng/ml)	AFTER EXTRACTION	THEORETICAL VALUES	(%)	(%)
RC_{max}	3 27	1586.80	1566.92	101	3.46
RC_{ave}	411	185.80	196.70	94.5	3.15
RC_{min}	105	45.08	50.25	89.7	4.45

Table 34: Absolute recovery of carbamazepine-10, 11-epoxide, using response factor areas.

SAMPLE	ANALYTE	MEAN OF PEAK AREAS		RECOVERY	CV
	(ng/ml)	AFTER EXTRACTION	THEORETICAL VALUES	(%)	(%)
RC_{max}	418	241.80	236.04	102	2.98
RC_{ave}	107	63.13	60.42	104	2.91
RC_{min}	26.7	14.50	15.08	96.2	10.7

2.2.8. Preparation of Calibration Standards

Since carbamazepine had a low aqueous solubility [34], the carbamazepine and carbamazepine-10, 11-epoxide stock solutions were prepared in methanol and used immediately to spike blank plasma. Calibration standards and quality control standards were prepared in normal human plasma by spiking plasma to a known concentration and then serially diluting it with normal blank plasma to attain the desired concentration range (10 644 ng/ml to 0.722 ng/ml for carbamazepine and 1 204 to 5.15 ng/ml for carbamazepine-10, 11-epoxide). The prepared calibration standards and quality control standards (tables 37 and 40) were aliquoted into microfuge tubes and stored at –20°C pending analysis.

Carbamazepine-10, 11-epoxide stock solution had to be prepared in a polypropylene scintillation vial, since the aluminium in the lid of the glass scintillation vial reacted as a catalyst in the presence of formic acid, causing reduction of the carbamazepine-10, 11-epoxide dissolved in the methanol.

Table 35: Preparation of Carbamazepine Stock Solution SA for Spiking STD O.

Solvent used	SG solvent	Mass analyte	Mass solvent	Volume solvent	Volume spiked	Concentration analyte
		(mg)	(g)	(ml)	(µl)	(µg/ml)
Methanol	0.791	7.579	9.473	11.976	500	632.85

Table 36: Preparation of Carbamazepine-10, 11-Epoxide Stock Solution SA for Spiking STD J.

Solvent used	SG solvent	Mass analyte	Mass solvent	Volume solvent	Volume spiked	Concentration analyte
		(mg)	(g)	(ml)	(µl)	(µg/ml)
Methanol	0.791	1.050	11.594	14.657	500	71.64

[0] Note: "Volume spiked" represents the volume of stock solution SA spiked into the highest concentration Std O plasma pool.

Table 37: Preparation of Calibration Standards.

Sample Code & No.	Source Solution	A	B	C	Carbamazepine (ng/ml)	CBZ-Epoxide (ng/ml)
STD O	Stock SA	56.900	86.913		10 644	-
STD N	STD O	117.483	132.484	147.508	5 326	-
STD M	STD N	108.398	123.403	138.855	2 702	-
STD L	STD M	112.175	127.126	142.122	1 353	-
STD K	STD L	62.094	77.107	92.128	677	-
Dilution 1	STD K	61.609	76.612	91.607	338	-
STD J	Dilution 1	55.994	71.046	86.039	169	1 204
STD I	STD J	62.558	77.555	92.550	84.4	602
STD H	STD I	62.108	77.111	92.120	42.2	301
STD G	STD H	63.000	78.009	93.024	21.10	151
STD F	STD G	66.963	81.968	96.977	10.60	75.3
STD E	STD F	61.322	76.327	91.332	5.28	37.6
STD D	STD E	61.574	76.540	91.557	2.64	18.8
STD C	STD I	116.588	146.082	146.588	1.42	10.2
STD B	STD H	62.575	92.097	92.611	0.722	5.15

· No carbamazepine-epoxide was spiked in STD K - STD O.
· Dilution 1 was not included in the validation calibration range and was used mainly to dilute STD K.
· A = Mass of empty container.
· B = Mass of container + normal plasma.
· C = Total mass of container + normal + spiked plasma.

2.2.9. Preparation of Quality Control Standards

Table 38: Preparation of Carbamazepine Stock Solution QA for Spiking QC O.

Solvent used	SG solvent	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Volume spiked (µl)	Concentration analyte (µg/ml)
Methanol	0.791	11.431	11.424	14.442	1000	791.48

Table 39: Preparation of Carbamazepine-10, 11-Epoxide 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	1.270	11.424	14.442	1000	87.94

» Note: "Volume spiked" represents the volume of stock solution QA spiked into the highest concentration QC O plasma pool.

Table 40: Preparation of Quality Control Standards.

Sample Code & No.	Source Solution	A	B	C	Carbamazepine (ng/ml)	CBZ-Epoxide (ng/ml)
QC O	Stock SA	62.091	122.100		13 316	-
QC N	QC O	62.108	92.119	122.443	6 693	-
QC M	QC N	56.898	86.891	116.904	3 347	-
QC L	QC M	62.806	92.813	122.826	1 674	-
QC K	QC L	61.570	91.582	121.612	837	-
QC J	QC K	66.960	96.967	126.994	419	-
QC I	QC J	116.167	146.181	176.319	210	1 476.00
QC H	QC I	61.609	91.619	121.629	105	738.00
QC G	QC H	62.347	92.359	122.445	52.5	369.00
QC F	QC G	61.322	91.329	121.340	26.3	185.00
QC E	QC F	111.166	141.177	171.169	13.1	92.30
QC D	QC E	117.773	147.769	177.780	6.56	46.20
QC C	QC D	112.085	142.092	172.088	3.28	23.10
QC B	QC H	109.702	139.704	170.306	1.66	11.70
QC A	QC G	116.498	146.493	176.604	0.830	5.84

· No carbamazepine - epoxide was spiked in QC J - QC O.
· A = Mass of empty container.
· B = Mass of container + normal plasma.
· C = Total mass of container + normal + spiked plasma.

The number of standards to be used in a validation is determined by the range. Five to eight standards may define the curve (excluding the blank) [5]. For non-linear relationships, more than eight standards should be used to detect curvature. The quality control samples are used to determine the accuracy and precision of the method, and must be determined at at least three different concentrations across the range (high, medium and low). The LOQ should be determined by at least five replicate samples.

2.2.10. Specificity (Matrix Effect)

It has been noted that co-eluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of an LC-MS/MS assay. In order to determine whether this effect (called the "*Matrix Effect*" [52]) is present or not, 6 different plasma pools were extracted and then spiked with a known concentration of the analyte. These samples were injected and the peak areas compared. The calculated CV is used as measurement of reproducibility of the peak areas, and a CV greater than 10% is considered to be an indication of the presence of matrix effects.

Table 41: Matrix effect data for both analytes.

Replicates	Carbamazepine (Peak Area)	Epoxide (Peak Area)
1	4120000	1280000
2	4230000	1220000
3	4080000	1200000
4	4270000	1170000
5	4200000	1240000
6	4220000	1180000
MEAN	4186667	1215000
CV%	1.6	3.1

The data in table 41 show no significant matrix effects (CV% = 1.6 for carbamazepine and 3.1 for carbamazepine-10, 11-epoxide) at the tested concentration for these two analytes.

2.2.11. Stability

- Freeze-thaw Stability

Freeze-thaw stability is not relevant in this study since samples were not subjected to multiple freeze-thaw cycles and were assayed immediately after thawing. Sample residues were discarded after analysis. Repeat assays were performed on the duplicate aliquots, which remain frozen until assayed.

Matar *et al.*, reported that both carbamazepine and carbamazepine-10, 11-epoxide are stable through five freeze-thaw cycles (-20°C to room temperature) [44].

- Long term stability

Although Liu *et al.*, reported that the working standard solutions for carbamazepine and carbamazepine-10, 11-epoxide are stable for at least 45 days when kept at –80°C [41], the stock solutions were freshly prepared in methanol and used immediately for the spiking of normal biological fluid. The stock solutions were not retained for further use. Queiros *et al*, [47] reported that the stock solutions prepared in methanol are stable at –20°C for at least 45 days.

Medez-Alvarez *et al.*, [39] published a paper on the stability of carbamazepine and carbamazepine-10, 11-epoxide in plasma and reported that carbamazepine was stable up to three months at room temperature (±20°C), 4°C and –20°C. They also noted that carbamazepine-10, 11-epoxide concentrations decreased after the first month of storage at room temperature, possibly due to bacterial growth. They stated that an increase in the carbamazepine-10, 11-epoxide reactivity was noticed in the presence of an acid. We found that carbamazepine-10, 11-epoxide breakdown in methanol was caused by the aluminium in the lid of the glass scintillation vial that reacted as a catalyst in the presence of formic acid. It is not clear in what type of vials Medez-Alvarez *et al.*, made up their solutions. Medez-Alvarez *et al.*, reported

that carbamazepine-10, 11-epoxide was stable up to three months [39] at 4°C and –20°C. Matar *et al,* reported that both analytes are stable at –20°C for 4 weeks [44].

- On-instrument stability

Matar *et al.,* [44] reported that the reconstituted samples were stable for a 24-hour period at room temperature and Fedorova *et al.,* [46] reported that the extracts are stable up to 3 days at 3°C.

To assess post-preparative stability, ten stability samples at the same concentration were extracted. The extracts were combined, re-aliquoted and injected at intervals during the validation batch to simulate the time of a batch run. The analyte and internal standard peak areas were plotted against the cumulative time depicted in figure 27. By regression analysis of the tabulated data below it was established that the carbamazepine and carbamazepine-10, 11-epoxide responses tended to increase by 3.16% and decrease by 0.91%, respectively over a period of 7.4 hours, while the internal standard response tended to increase by 8.15% while awaiting injection on-instrument (figure 27). This trend was deemed insignificant and the extracts were considered to be stable on-instrument for at least 7.4 hours.

Table 42: On-instrument stability data.

Replicates	*Injection Time*	*Time Difference*	*Cumulative Time*	*CBZ Peak Area*	*CBZ-Epoxide Peak Area*	*IS Peak Area*
			(hr)			
1	12:38		0.00	1583452	47811	87486
2	13:14	0:36:00	0.60	1813011	52687	90617
3	14:14	1:00:00	1.60	1641420	45675	93348
4	14:39	0:25:00	2.02	1578881	44380	94555
5	15:39	1:00:00	3.02	1314499	38699	97053
6	17:00	1:21:00	4.37	1297036	38743	94750
7	17:25	0:25:00	4.78	1343747	40891	94793
8	18:25	1:00:00	5.78	1440136	44866	95012
9	19:41	1:16:00	7.05	1467872	46674	93984
10	20:01	0:20:00	7.38	1536858	49155	100637
			Mean	1501691.20	44958.10	94223.50
			Std. Dev.	162319.22	4517.07	3491.21
			CV	10.81%	10.05%	3.71%

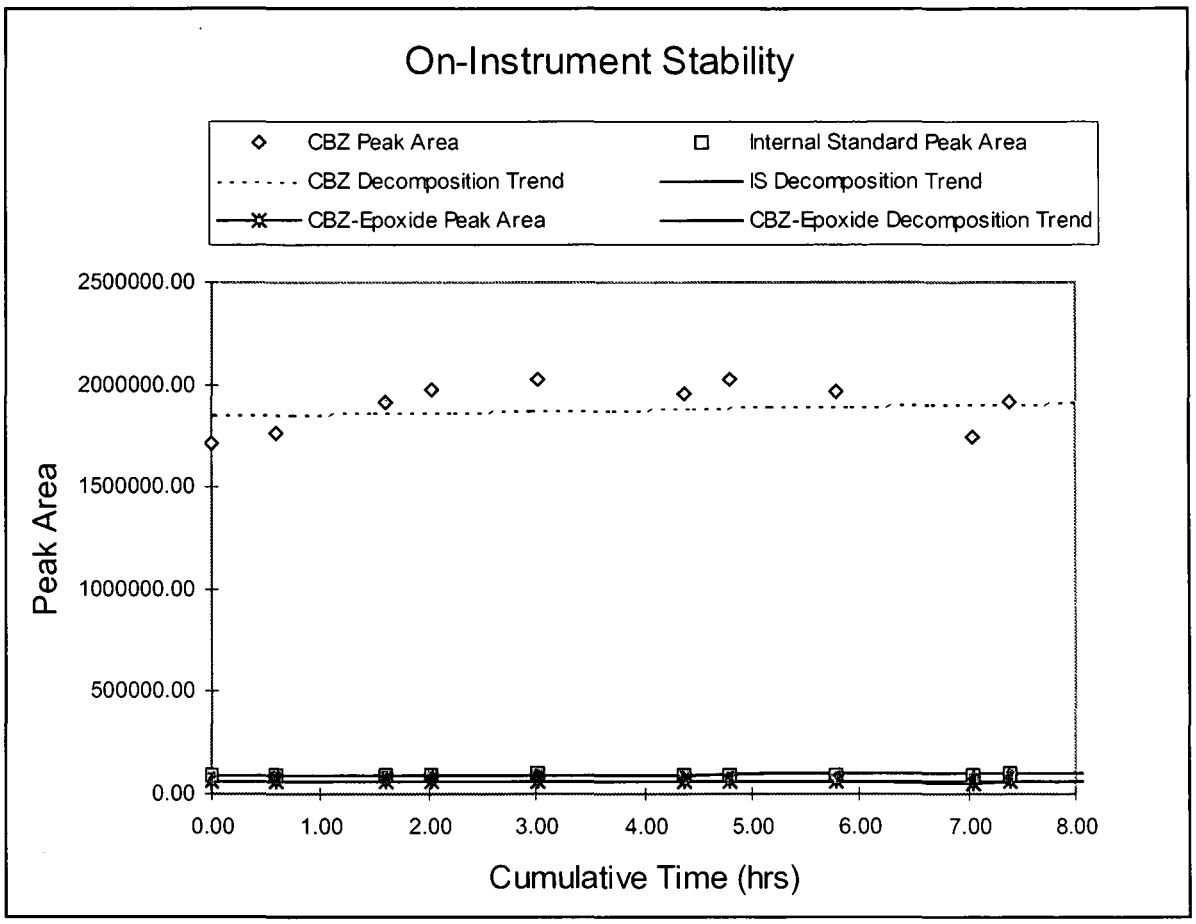

Figure 27: Regression analysis of the tabulated data to show on-instrument stability

2.2.12. Actual Validation

The inter-day accuracy and precision of the assay procedure were assessed with and without the use of the internal standard using calibration curves and calculations based on both peak heights and peak areas. Quantitation by peak height ratios or peak area ratios required using the internal standard whereas quantification by peak heights or peak areas required not using the internal standard.

The method was validated assaying plasma quality control samples (n = 5) at 0.830, 1.66, 3.28, 13.1, 26.3, 52.5, 210 and 837 ng/ml for carbamazepine and at 5.84, 11.7, 23.1, 46.2, 92.3, 185, 369 and 738 ng/ml for carbamazepine-10, 11-epoxide, to determine the accuracy and precision of the method. Quality control values were calculated from a standard regression curve containing eight different concentrations spanning the concentration range from 0.722 to 1 353 ng/ml for carbamazepine, and from 5.15 to 1 204 ng/ml for carbamazepine-10, 11-epoxide.

For the assignment of a valid calibration range, percentage deviation from nominal or percentage BIAS is taken as measurement of accuracy, while coefficient of variation (CV %) is taken as measurement of precision. Intra-day accuracy and precision for a valid range must be within 15%, but within 20% at the limit of quantification.

Results from the intra-day validation assays indicate a valid calibration range from 0.722 to 1 353 ng/ml for carbamazepine and from 5.15 to 1 204 ng/ml for carbamazepine-10, 11-epoxide. The limit of quantification (LOQ) was preliminarily set at 0.722 ng/ml for carbamazepine and at 5.15 ng/ml for carbamazepine-10, 11-epoxide.

- Quantification by Peak Height Ratios: Carbamazepine

Calibration Standards used: STD B - STD O
Calibration Range: 0.722 - 10 644 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.005802
b: 0.995121
c: -2.554738
r^2: 0.999795

Table 43: Quantification by Peak Height Ratios: Carbamazepine calibration curve.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD O	10 644	10 950.37	2.9
STD N	5 326	5 208.71	-2.2
STD M	2 702	2 561.77	-5.2
STD L	1 353	1 439.44	6.4
STD K	677	668.55	-1.2
STD J	169	161.81	-4.3
STD I	84.4	85.64	1.5
STD H	42.2	43.98	4.2
STD G	21.1	22.08	1.7
STD F	10.6	10.52	-0.8
STD E	5.28	4.86	-7.9
STD D	2.64	2.54	-3.6
STD C	1.42	1.44	1.6
STD C	1.42	1.53	8.1
STD B	0.722	R	-
STD B	0.722	0.70	-2.6

Code: R = Rejected

Table 44: Summary of Intra-day Quality Control Results, using peak height ratios.

Code	QC J	QC J (Dil)	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	6693	6693	3347	1674	210	105	52.5	13.1	3.28	1.66	0.830
Replicates											
1	7113.58	6868.79	3285.39	1641.64	203.04	103.04	46.94	14.24	3.11	1.81	0.910
2	6946.32	6686.62	3178.22	1688.77	200.96	101.01	51.41	11.93	3.19	1.75	0.990
3	7358.82	6872.45	3608.51	1714.53	203.52	108.51	52.83	13.06	3.24	1.60	0.940
4	7368.15	6814.72	3443.45	1771.37	201.63	107.31	50.02	12.67	2.87	1.58	0.860
5	6938.40	6593.57	3757.66	1559.30	192.60	96.61	46.12	11.74	2.71	1.43	0.940
MEAN	7145.05	6767.23	3454.65	1675.12	200.35	103.30	49.46	12.73	3.02	1.63	0.928
%nom	106.8	101.1	103.2	100.1	95.4	98.4	94.2	97.2	92.2	98.4	111.8
CV%	2.6	1.6	6.1	4.3	2.0	4.2	5.2	7.0	6.7	8.2	4.6

- Quantification by Peak Heights (without Internal Standard): Carbamazepine

Calibration Standards used: STD B - STD O
Calibration Range: 0.722 - 10 644 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.017143
b: 1.049574
c: 9.726863
r^2: 0.999409

Table 45: Quantification by Peak Heights without Internal Standard: Carbamazepine calibration curve

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD O	10644	9755.78	-8.3
STD N	5326	5180.20	-2.7
STD M	2702	2829.98	4.7
STD L	1353	1523.65	12.6
STD K	677	715.19	5.6
STD J	169	158.78	-6.0
STD I	84.4	77.34	-8.4
STD H	42.2	44.63	5.8
STD G	21.1	22.47	6.5
STD F	10.6	10.27	-3.1
STD E	5.28	5.05	-4.4
STD D	2.64	2.41	-8.9
STD C	1.42	1.31	-7.4
STD C	1.42	1.61	13.1
STD B	0.722	R	-
STD B	0.722	0.76	5.1

Code: R = Rejected

Table 46: Summary of Intra-day Quality Control Results, using peak heights.

Code	QC J	QC J (Dil.)	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	6693	6693	3347	1674	210	105	52.5	13.1	3.28	1.66	0.830
Replicates											
1	7354.87	7160.08	3451.04	1750.45	193.65	94.66	41.96	13.40	2.95	1.73	0.890
2	7366.64	7469.02	3426.99	1840.98	195.88	97.82	46.51	10.89	2.92	1.75	0.810
3	7689.71	7307.08	3874.48	1821.33	207.09	119.39	57.44	13.85	3.52	1.76	1.040
4	7827.96	7843.74	3965.94	1995.37	205.21	107.39	50.06	12.30	2.69	1.53	0.890
5	7381.66	7002.56	3773.95	1769.54	193.07	107.68	49.81	12.69	2.85	1.56	1.010
MEAN	7524.17	7356.50	3698.48	1835.53	198.98	105.39	49.16	12.63	2.99	1.67	0.928
%nom	112.4	109.9	110.5	109.6	94.8	100.4	93.6	96.4	91.0	100.4	111.8
CV%	2.6	3.9	6.0	4.7	3.0	8.2	10.3	8.1	9.4	6.0	9.2

Θ

Θ QC J 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.

- Quantification by Peak Area Ratios: Carbamazepine

Calibration Standards used: STD B - STD O
Calibration Range: 0.722 - 10 644 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.001110
b: 0.946032
c: -2.443848
r^2: 0.999534

Table 47: Quantification by Peak Area Ratios: Carbamazepine - calibration curve.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD O	10 644	10 366.85	-2.6
STD N	5 326	5 220.39	-2.0
STD M	2 702	2 658.34	-1.6
STD L	1 353	1 461.24	8.0
STD K	677	690.88	2.1
STD J	169	165.66	-2.0
STD I	84.4	87.44	3.6
STD H	42.2	43.26	2.5
STD G	21.1	20.36	-3.5
STD F	10.6	10.38	-2.1
STD E	5.28	5.11	-3.2
STD D	2.64	2.33	-11.9
STD C	1.42	1.60	12.6
STD C	1.42	1.44	1.7
STD B	0.722	0.82	13.9
STD B	0.722	0.64	-11.7

Table 48: Summary of Intra-day Quality Control Results, using peak area ratios.

Code	QC J	QC J (Dil.)	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	6693	6693	3347	1674	210	105	52.5	13.1	3.28	1.66	0.830
Replicates											
1	6949.81	6986.38	3308.83	1733.19	206.00	106.09	47.01	14.28	2.73	1.69	0.720
2	6793.36	6754.84	3244.71	1767.28	202.01	104.37	50.68	11.59	3.20	1.67	0.850
3	7168.35	7036.42	3674.66	1788.40	206.33	110.40	52.45	12.72	2.91	1.38	0.820
4	7053.51	6823.46	3556.71	1817.54	209.77	107.36	50.44	12.75	2.49	1.25	0.830
5	6787.10	6657.36	3752.48	1625.73	199.05	100.55	47.46	11.83	2.33	1.20	0.820
MEAN	6950.43	6851.69	3507.48	1746.43	204.63	105.75	49.61	12.63	2.73	1.44	0.808
%nom	103.8	102.8	104.8	104.3	97.4	100.7	94.5	96.4	83.3	86.6	97.3
CV%	2.1	2.1	5.7	3.8	1.8	3.1	4.2	7.5	11.2	14.3	5.6

- Quantification by Peak Areas (without Internal Standard): Carbamazepine.

The assay procedure did not validate using peak areas without internal standard.

- Quantification by Peak Height Ratios: Carbamazepine-10, 11-epoxide

Calibration Standards used: STD B - STD J
Calibration Range: 5.15 - 1 204 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.016433
b: 1.117770
c: -4.212301
r^2: 0.999587

Table 49: Quantification by Peak Height Ratios: Carbamazepine-10, 11-epoxide - calibration curve.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD J	1 204	1 142.16	-5.1
STD I	602	610.40	1.4
STD H	301	322.45	7.1
STD G	151	157.08	4.0
STD F	75.3	74.45	-1.1
STD E	37.6	34.99	-6.9
STD D	18.8	18.65	-0.8
STD C	10.2	10.25	0.5
STD C	10.2	10.02	-1.8
STD B	5.15	5.32	3.4
STD B	5.15	5.16	0.2

Table 50: Summary of Intra-day Quality Control Results, using peak area ratios.

Code	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	738	369	92.3	23.1	11.7	5.84
Replicates						
1	750.58	356.91	99.21	21.45	12.09	5.20
2	712.67	372.01	88.99	23.95	11.88	6.63
3	758.80	368.80	92.26	22.35	11.28	5.72
4	740.14	351.66	92.57	22.65	11.09	5.52
5	659.51	332.59	82.88	21.21	10.53	5.74
MEAN	724.34	356.39	91.18	22.32	11.37	5.76
%nom	98.1	96.6	98.8	96.6	97.2	98.7
CV%	5.0	3.9	5.8	4.4	4.9	8.3

- Quantification by Peak Heights (without Internal Standard): Carbamazepine-10, 11-epoxide

Calibration Standards used: STD B - STD J
Calibration Range: 5.15 - 1 204 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.025807
b: 1.190402
c: 7.979266
r^2: 0.998842

Table 51: Quantification by Peak Heights without Internal Standard: Carbamazepine-10, 11-epoxide.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD J	1 204	1 156.55	-3.9
STD I	602	566.66	-5.9
STD H	301	337.43	12.1
STD G	151	163.79	8.5
STD F	75.3	73.94	-1.8
STD E	37.6	36.60	-2.6
STD D	18.8	17.64	-6.2
STD C	10.2	9.28	-9.0
STD C	10.2	10.41	2.1
STD B	5.15	5.34	3.7
STD B	5.15	5.43	5.4

Table 52: Summary of Intra-day Quality Control Results, using peak heights.

Code	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	738	369	92.3	23.1	11.7	5.84
Replicates						
1	710.33	328.19	95.14	20.42	11.54	5.02
2	711.42	345.80	82.73	21.97	11.83	5.42
3	865.86	414.03	99.79	24.32	12.31	6.24
4	763.80	362.38	91.63	21.25	10.66	5.63
5	761.27	371.04	91.25	22.26	11.37	6.05
MEAN	762.54	364.29	92.11	22.04	11.54	5.67
%nom	103.3	98.7	99.8	95.4	98.6	97.1
CV%	7.4	7.9	6.1	5.9	4.7	7.7

- Quantification by Peak Area Ratios: Carbamazepine-10, 11-epoxide

Calibration Standards used: STD B - STD J
Calibration Range: 5.15 - 1 204 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.016443
b: 1.130090
c: -4.300546
r^2: 0.999734

Table 53: Quantification by Peak Area Ratios: Carbamazepine-10, 11-epoxide - calibration curve.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD J	1 204	1 169.49	-2.9
STD I	602	599.33	-0.4
STD H	301	318.44	5.8
STD G	151	156.65	3.7
STD F	75.3	73.12	-2.9
STD E	37.6	35.50	-5.6
STD D	18.8	19.09	1.6
STD C	10.2	10.34	1.3
STD C	10.2	10.06	-1.4
STD B	5.15	5.20	1.0
STD B	5.15	5.16	0.3

Table 54: Summary of Intra-day Quality Control Results, using peak area ratios.

Code	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	738	369	92.3	23.1	11.7	5.84
Replicates						
1	750.36	356.47	95.68	22.18	11.87	5.78
2	716.56	362.81	88.51	23.34	12.34	6.40
3	760.51	364.49	91.46	22.56	11.72	5.52
4	723.78	350.99	93.28	22.11	11.13	5.83
5	672.97	338.36	82.94	21.18	10.98	6.02
MEAN	724.84	354.62	90.37	22.27	11.61	5.91
%nom	98.2	96.1	97.9	96.4	99.2	101.2
CV%	4.2	2.7	4.9	3.1	4.3	4.9

- Quantification by Peak Areas (without Internal Standard): Carbamazepine-10, 11-epoxide

Calibration Standards used: STD B - STD J
Calibration Range: 5.15 - 1 204 ng/ml
Regression Equation: Wagner: ln y = a(ln x)2 + b(ln x) + c
a: -0.023195
b: 1.180754
c: 7.096805
r^2: 0.999035

Table 55: Quantification by Peak Areas without Internal Standard: Carbamazepine-10, 11-epoxide - calibration curve.

STD Code	Nominal Conc. (ng/ml)	Back-calculated Conc. (ng/ml)	% Bias
STD J	1 204	1 176.14	-2.3
STD I	602	561.47	-6.7
STD H	301	330.85	9.9
STD G	151	166.34	10.2
STD F	75.3	73.23	-2.8
STD E	37.6	35.74	-4.9
STD D	18.8	18.23	-3.1
STD C	10.2	9.45	-7.3
STD C	10.2	10.42	2.1
STD B	5.15	5.24	1.8
STD B	5.15	5.41	5.0

Table 56: Summary of Intra-day Quality Control Results.

Code	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	738	369	92.3	23.1	11.7	5.84
Replicates						
1	712.57	328.73	92.43	21.33	11.43	5.51
2	706.31	342.00	82.68	21.81	12.29	5.22
3	854.47	407.27	100.59	24.40	12.82	6.05
4	751.34	360.57	92.28	21.06	10.85	5.94
5	757.05	371.40	92.59	22.31	11.77	6.22
MEAN	756.35	361.99	92.11	22.18	11.83	5.79
%nom	102.5	98.1	99.8	96.0	101.1	99.1
CV%	7.0	7.5	6.2	5.4	5.8	6.4

Inter-Batch Accuracy and Precision

The inter-batch accuracy and precision for carbamazepine and carbamazepine-10, 11-epoxide, were assessed by the calibration standards and quality control standards used in the assays of unknown samples. 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 as determined by the intra-batch accuracy and precision measurements. The inter-batch accuracy and precision must pass the criteria for intra-batch acceptance. Calculating the accuracy and precision statistics over the intra- and inter-batch validation batches assesses the inter-batch accuracy and precision of the assay procedure.

Table 57: Back-Calculated Calibration Standards Concentrations: Carbamazepine (using peak height ratios).

Nominal	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J	STD K	STD L
(ng/ml)	0.722	1.42	2.64	5.28	10.6	21.1	42.2	84.4	169	677	1353
Batch 1	0.751	1.39	R	5.62	10.85	18.96	38.72	78.75	R	606.46	1547.82
	0.714	1.68	2.16	R	10.37	21.57	45.30	96.41	178.59	733.90	1231.88
Batch 2	0.699	1.71	2.15	5.18	10.78	22.29	43.18	86.08	198.48	719.51	1237.43
	0.702	1.50	R	4.74	10.00	19.27	47.55	78.37	R	673.57	1255.85
	-	-	-	-	-	-	-	-	170.72	-	-
	-	-	-	-	-	-	-	-	R	-	-
Batch 3	0.726	R	2.62	5.05	10.97	23.36	44.89	83.74	166.06	717.86	1257.42
	0.720	1.56	2.97	5.00	10.61	19.53	47.52	R	190.15	683.05	1270.05
	-	1.45	-	-	10.52	19.81	-	-	-	-	-
	-	1.25	-	-	10.80	16.91	-	-	-	-	-
Batch 4	0.700	1.41	2.39	5.18	9.81	21.17	41.20	91.51	R	710.07	1339.78
	0.750	1.62									
Batch 5	0.731	1.58	R	4.82	9.71	20.85	39.49	89.90	189.89	691.86	1366.48
	0.715	R									
Mean	0.721	1.52	2.46	5.08	10.44	20.37	43.48	86.40	182.32	692.03	1313.34
CV%	5.8	9.3	14.0	5.7	4.3	9.1	7.9	7.7	6.9	5.8	8.1
n	10	10	5	7	10	10	8	7	6	8	8
%Nom	99.8	106.7	93.1	96.3	98.5	96.6	103.0	102.4	107.9	102.2	97.1

Note: The extra replicates of STD J in batch 2 and STDs C, F and G in batch 3 were inadvertently included in the respective batches.
Codes: R = Rejected

Table 58: Quality Control Results: Carbamazepine (using peak height ratios).

Nominal	QC A	QC B	QC C	QC D	QC E	QC F	QC G	QC H
(ng/ml)	0.830	1.66	3.28	13.1	26.3	52.5	210	837
Batch 1	0.839	1.96	-	12.29	-	B	B	-
	0.791	2.07*	-	12.93	-	56.38	216.35	-
	0.830	1.66	-	12.50	-	53.91	198.92	-
	0.845	1.66	-	17.90*	-	61.93	224.00	-
	0.841	2.33*	-	15.79*	-	65.05*	219.93	-
Batch 2	0.850	1.52	B	13.98	24.04	51.96	206.38	879.49
	B	1.66	3.18	12.57	25.04	52.78	175.68	908.24
	0.830	1.53	-	12.23	-	50.00	191.49	-
	0.822	F	-	15.04	-	52.90	214.51	-
	0.799	1.51	-	11.26	-	51.01	175.38	-
Batch 3	0.831	1.51	2.76	11.72	27.23	-	215.42	886.93
	F	F	2.46*	12.25	27.24	-	173.28	851.80
Batch 4	0.811	1.51	2.95	13.14	29.88	-	210.48	889.39
	0.841	F	3.65	B	24.46	-	241.47	795.39
Batch 5	0.834	1.55	3.06	13.14	27.89	-	213.86	942.49
	0.825	1.42	B	13.32	23.98	-	208.50	834.97
Mean	0.830	1.68	3.01	13.34	26.22	55.10	205.71	873.59
CV%	4.3	15.0	13.4	13.0	8.2	9.3	9.5	5.2
n	14	13	6	15	8	9	15	8
%Nom	100	101.5	91.8	101.8	99.7	105.0	98.0	104.4

Note: The extra replicates of STD J in batch 2 and STDs C, F and G in batch 3 were inadvertently included in the respective batches.
Codes: B = Sample lost in process
F = Outside range
* = Response outside acceptance range

Table 59: Back-Calculated Calibration Standards Concentrations: Carbamazepine-10, 11-epoxide (using peak height ratios).

Nominal	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
(ng/ml)	5.15	10.2	18.8	37.6	75.3	151	301	602	1204
	5.05	10.19	R	37.13	81.80	136.54	273.18	558.42	R
Batch 1	5.15	10.37	17.05	46.25	72.07	146.27	319.14	642.57	1267.43
	5.41	10.52	17.45	37.11	81.81	158.94	310.83	608.83	1292.98
Batch 2	5.33	9.95	17.11	35.64	75.58	149.17	333.28	544.92	1092.40
	5.69	10.07	18.54	36.09	75.40	164.57	304.61	594.02	1184.15
	5.21	10.92	20.17	33.97	77.68	148.55	341.39	468.33	1357.84
	5.77	10.01			78.58	163.87			
Batch 3	4.37	8.90			79.59	132.47			
	5.01	10.08	18.02	37.14	77.66	155.27	282.53	613.55	1210.76
Batch 4	5.18	10.93							
	4.99	10.10	19.64	40.53	72.40	147.73	288.03	605.44	1236.16
Batch 5	5.21	10.04							
Mean	5.20	10.17	18.28	37.98	77.26	150.34	306.62	579.51	1234.53
CV%	6.9	5.2	6.7	10.1	4.4	7.1	7.9	9.4	6.9
N	12	12	7	8	10	10	8	8	7
%Nom	100.9	99.7	97.3	101.0	102.6	99.6	101.9	96.3	102.5

Note: The extra replicates STDs B, C, F and G in batch 3 were inadvertently included.
Codes: R = Rejected

Table 60: Quality Control Results: Carbamazepine-10, 11-epoxide (using peak height ratios).

Nominal	QC A	QC B	QC C	QC D	QC E	QC F	QC G
(ng/ml)	5.84	11.7	23.1	92.3	185	369	1476
	5.39	13.50		87.19		B	
	6.16	11.48		91.53		351.05	
		10.99		87.49		377.69	
		11.07		[120.70]*		418.20	
Batch 1		12.34		105.00		411.48	
	5.91	12.22	B	98.73	168.93	365.04	1389.44
	6.30	11.14	21.67	94.77	200.97	375.86	1197.16
		12.22		94.15		337.97	1344.87
		11.19		107.52		351.49	1334.58
Batch 2		10.43		87.20		348.32	1170.70*
	5.80	11.26	21.71	85.07	195.22		1494.48
Batch 3	5.88	10.06	17.77*	90.09	198.38		1157.13*
	5.84	11.85	23.85	95.80	193.15		1141.05*
Batch 4	5.91	10.62	26.15	[128.90]*	175.02		1372.74
	6.23	12.61	22.51	101.62	197.30		1250.91
Batch 5	5.46	11.18	22.85	94.96	168.66		1296.97
Mean	5.89	11.51	22.36	94.37	187.20	370.79	1286.37
CV%	5.1	7.7	11.4	7.3	7.4	7.6	8.8
n	10	16	7	14	8	9	11
%Nom	100.8	98.4	96.8	102.2	101.2	100.5	87.2

Note: The extra replicates of STD J in batch 2 and STDs C, F and G in batch 3 were inadvertently included in the respective batches.
Codes: B = Sample lost in process
* = Response outside acceptance range
[ ] = Outlier not included in statistics

2.3. Routine Analysis

A standard curve is generated for each analytical run and for each analyte, and is used to calculate the concentration of the analyte in the unknown samples assayed within that run [5]. Once the assay has been established for routine use, its performance has been regularly monitored to update information regarding its characteristics and to ensure that it continued to work satisfactorily. This was done by using an internal quality control (QC) scheme, which is defined as the long-term and continuing assessment of accuracy and precision of an assay for a particular drug with a view to minimising within-laboratory variation and improving assay precision. The QC samples are used to accept or reject the run.

Controls are usually run in duplicate. The sample results are acceptable if the QC results are within 10-25% of the known values [25]. Shah *et al.*, criteria stipulate that at least 67% of the QC samples must be within the 20% of their respective nominal values and that 33% of the QC samples (not both at the same concentration) may be outside the 20% respective nominal value.

Table 61: A typical batch structure.

Inj. No.	Sample	Inj. No.	Sample	Inj. No.	Sample	Inj. No.	Sample	Inj. No.	Sample	Inj. No.	Sample
1	SYS	37	20,6.0,2	73	20,20,1	109	22,0,1	145	22,8.0,1	181	QC C
2	SYS	38	STD N	74	20,20,2	110	22,0,2	146	22,8.0,2	182	22,36,1
3	SYS	39	BLANK	75	QC A	111	23,0,1	147	23,8.0,1	183	22,36,2
4	SYS	40	18,7.0,1	76	18,24,1	112	23,0,2	148	23,8.0,2	184	23,36,1
5	SYS	41	18,7.0,2	77	18,24,2	113	QC C	149	STD I	185	23,36,2
6	18,0,1	42	20,7.0,1	78	20,24,1	114	22,1.0,1	150	22,9.0,1	186	STD M
7	18,0,2	43	20,7.0,2	79	20,24,2	115	22,1.0,2	151	22,9.0,2	187	22,48,1
8	20,0,1	44	18,8.0,1	80	18,36,1	116	23,1.0,1	152	23,9.0,1	188	22,48,2
9	20,0,2	45	18,8.0,2	81	18,36,2	117	23,1.0,2	153	23,9.0,2	189	23,48,1
10	STD J	46	20,8.0,1	82	20,36,1	118	22,2.0,1	154	22,10,1	190	23,48,2
11	18,1.0,1	47	20,8.0,2	83	20,36,2	119	22,2.0,2	155	22,10,2	191	QC B
12	18,1.0,2	48	QC G	84	STD F	120	23,2.0,1	156	23,10,1	192	22,72,1
13	20,1.0,1	49	18,9.0,1	85	18,48,1	121	23,2.0,2	157	23,10,2	193	22,72,2
14	20,1.0,2	50	18,9.0,2	86	18,48,2	122	STD G	158	QC B	194	23,72,1
15	QC H	51	20,9.0,1	87	20,48,1	123	22,3.0,1	159	22,12,1	195	23,72,2
16	18,2.0,1	52	20,9.0,2	88	20,48,2	124	22,3.0,2	160	22,12,2	196	STD C
17	18,2.0,2	53	18,10,1	89	18,72,1	125	23,3.0,1	161	23,12,1	197	22,96,1
18	20,2.0,1	54	18,10,2	90	18,72,2	126	23,3.0,2	162	23,12,2	198	22,96,2
19	20,2.0,2	55	20,10,1	91	20,72,1	127	22,4.0,1	163	22,14,1	199	23,96,1
20	QC E	56	20,10,2	92	20,72,2	128	22,4.0,2	164	22,14,2	200	23,96,2
21	18,3.0,1	57	STD L	93	STD C	129	23,4.0,1	165	23,14,1	201	QC D
22	18,3.0,2	58	18,12,1	94	18,96,1	130	23,4.0,2	166	23,14,2	202	22,120,1
23	20,3.0,1	59	18,12,2	95	18,96,2	131	STD K	167	STD E	203	22,120,2
24	20,3.0,2	60	20,12,1	96	20,96,1	132	22,5.0,1	168	22,16,1	204	23,120,1
25	18,4.0,1	61	20,12,2	97	20,96,2	133	22,5.0,2	169	22,16,2	205	23,120,2
26	18,4.0,2	62	18,14,1	98	QC E	134	23,5.0,1	170	23,16,1	206	STD B
27	20,4.0,1	63	18,14,2	99	18,120,1	135	23,5.0,2	171	23,16,2	207	22,144,1
28	20,4.0,2	64	20,14,1	100	18,120,2	136	22,6.0,1	172	22,20,1	208	22,144,2
29	STD D	65	20,14,2	101	20,120,1	137	22,6.0,2	173	22,20,2	209	23,144,1
30	18,5.0,1	66	STD B	102	20,120,2	138	23,6.0,1	174	23,20,1	210	23,144,2
31	18,5.0,2	67	18,16,1	103	STD H	139	23,6.0,2	175	23,20,2	211	QC G
32	20,5.0,1	68	18,16,2	104	18,144,1	140	QC H	176	QC D	212	SYS
33	20,5.0,2	69	20,16,1	105	18,144,2	141	22,7.0,1	177	22,24,1		
34	18,6.0,1	70	20,16,2	106	20,144,1	142	22,7.0,2	178	22,24,2		
35	18,6.0,2	71	18,20,1	107	20,144,2	143	23,7.0,1	179	23,24,1		
36	20,6.0,1	72	18,20,2	108	QC A	144	23,7.0,2	180	23,24,2		

ϕ

Key:
STD = Calibration Standard
QC = Quality Control Standard
SYS = System Performance Verification Sample

When a batch of samples is analysed, calibration standards are included in the run (table 61) to allow a standard curve to be constructed. This will secure meaningful results even if the instrument response changes for some reason.

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

In addition to this, a number of QC samples are analysed along with actual samples at intervals depending on the total number of actual samples [25].

Typical retention times for carbamazepine were 2.65 - 2.84 minutes (RSD_{mean} = 0.62%) and for carbamazepine-10, 11-epoxide were 2.73 - 2.87 minutes (RSD_{mean} = 0.6%). A chromatography time of 4 minutes made it possible to analyse ~212 samples per day.

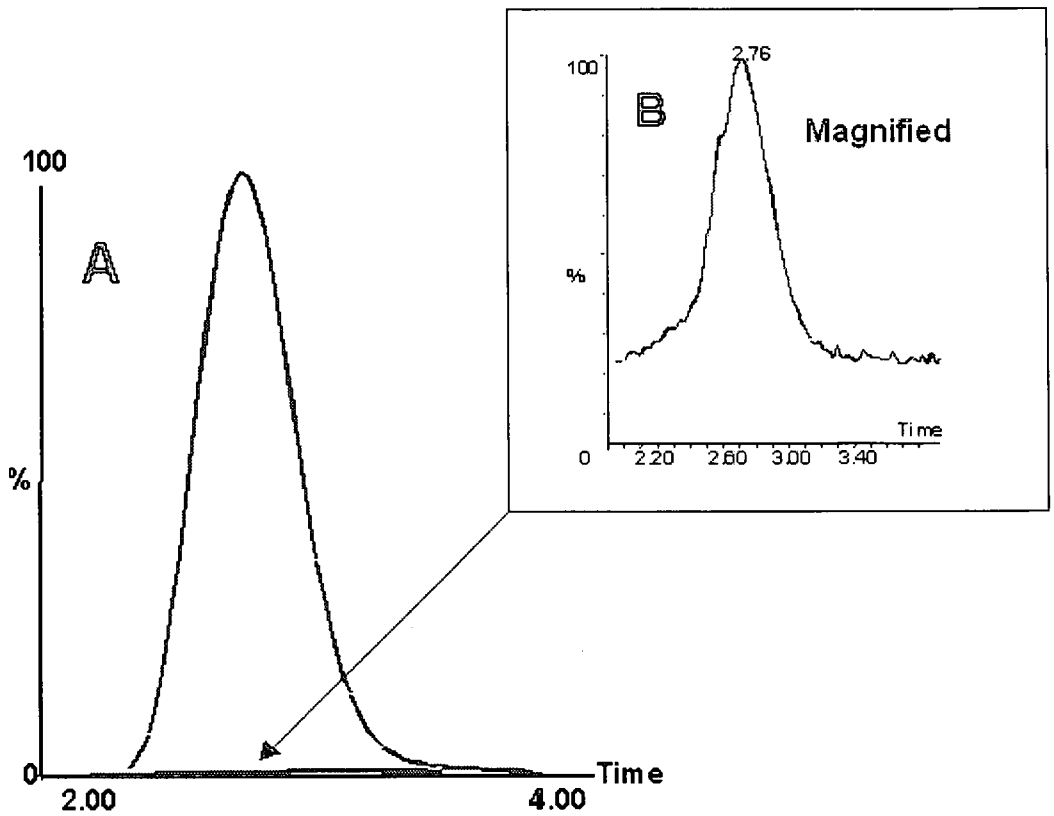

Figure 28: Chromatograms of calibration standards, containing 10 644 ng/ml (A) and 0.722 ng/ml (B), respectively, of carbamazepine in plasma.

Figure 28 shows representative chromatograms of carbamazepine obtained at 10 644 ng/ml (**A**) and at 0.722 ng/ml (LOQ) (**B**), and figure 29 shows representative chromatograms of carbamazepine-10, 11-epoxide obtained at 602 ng/ml (**A**) and at 5.84 ng/ml (**B**), while figure 30 depicts chromatograms from a subject sample (both carbamazepine and carbamazepine-10, 11-epoxide) (**A** & **C**) and a blank plasma extract (**B**), magnified to show the absence of any interference.

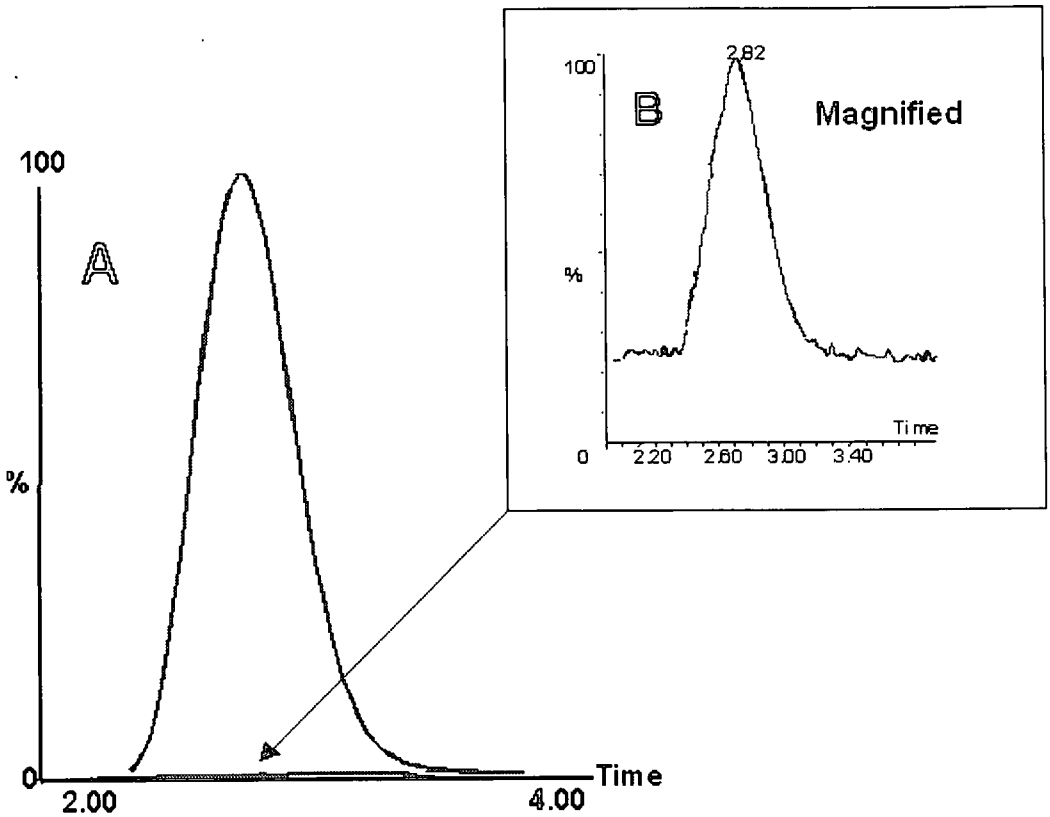

*Figure 29: Chromatograms of calibration standards, containing 602 ng/ml (**A**) and 5.84 ng/ml (**B**), respectively, of carbamazepine-10, 11-epoxide in plasma.*

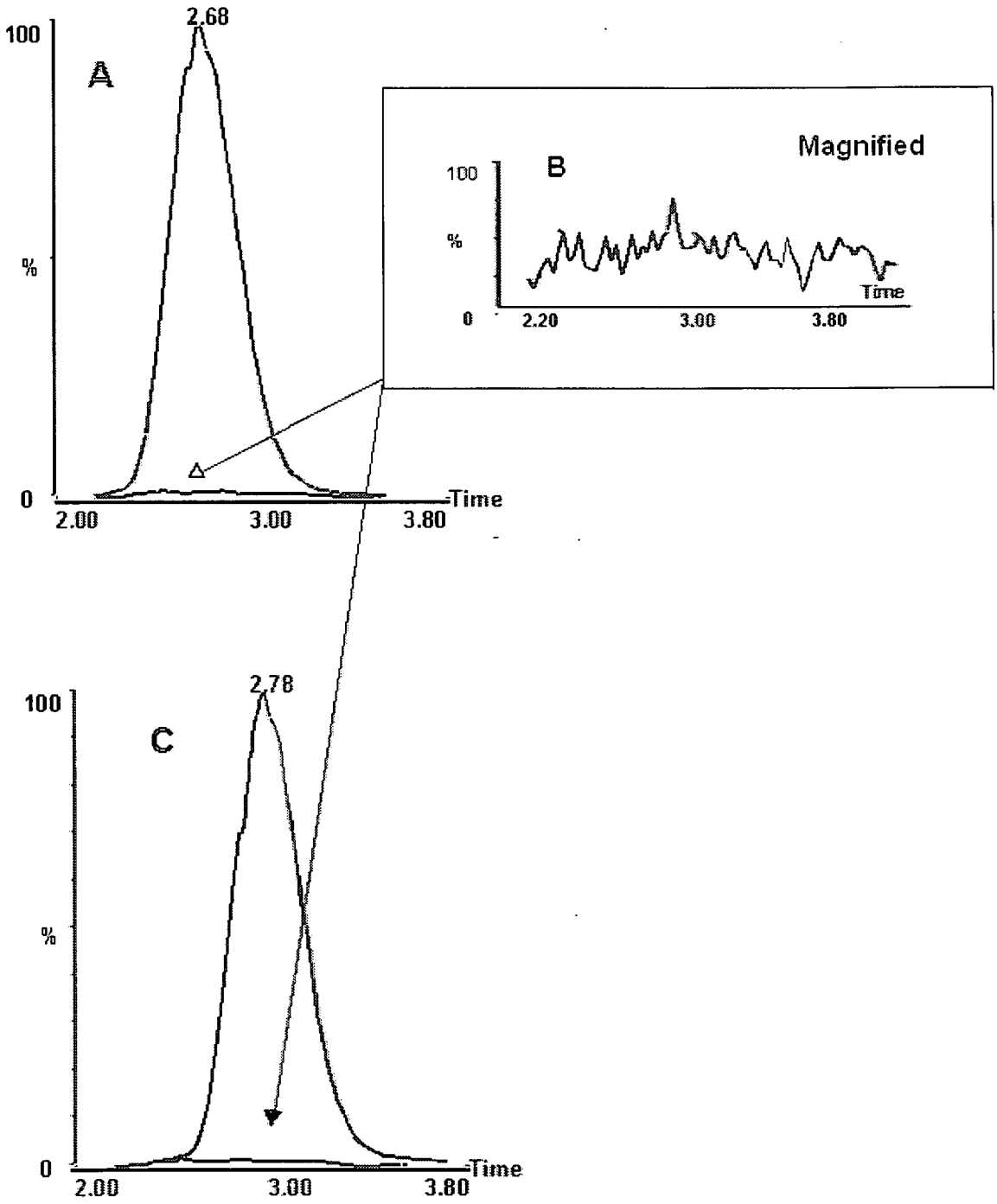

*Figure 30: Chromatograms of a subject sample containing 225 ng/ml (**A**) of carbamazepine in plasma, 74.6 ng/ml (**C**) of carbamazepine-10, 11-epoxide in plasma, and blank plasma extract (**B**).*

2.4. Conclusion

Carbamazepine, one of the most often used antiepileptic drugs, undergoes enzyme biotransformation through epoxidation with the formation of its metabolite, carbamazepine-10,11-epoxide (carbamazepine epoxide). The determination of carbamazepine-10, 11-epoxide is clinically significant in therapeutic drug monitoring as it decreases the risk of toxic reactions and increases the possibility of reaching the expected therapeutic result.

The simultaneous determination of serum levels of carbamazepine-10, 11-epoxide and carbamazepine offers the possibility of measuring the total drug concentration, as well as that of its metabolite, while considering the results of clinical response and the special features of carbamazepine's enzymatic biotransformation through epoxidation with the formation of its metabolite.

The aim of this study was to introduce a LC method with mass spectrometric detection to simultaneously determine the serum levels of carbamazepine and carbamazepine-10, 11-epoxide. The method was employed to analyse plasma samples containing carbamazepine and carbamazepine-10, 11-epoxide obtained after a single 400mg oral dose of carbamazepine in 22 healthy volunteers.

The maximum plasma concentrations obtained varied between 1 200 and 1 000 ng/ml for carbamazepine and between 85.0 and 75.0 ng/ml for carbamazepine-10, 11-epoxide. Figures 31 and 32 represent the mean pharmacokinetic profiles, for carbamazepine and carbamazepine-10, 11-epoxide, respectively, for 22 human volunteers after each receiving a 400mg oral dose of carbamazepine.

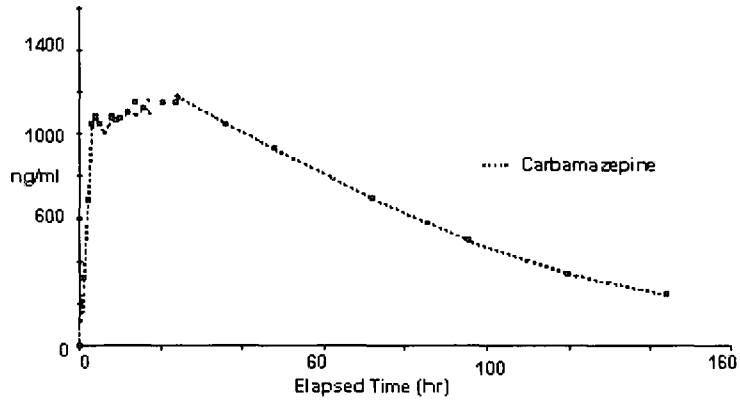

Figure 31: Representative mean carbamazepine plasma concentration/time profile (n=22) as obtained after a single 400mg oral dose of carbamazepine to human volunteers.

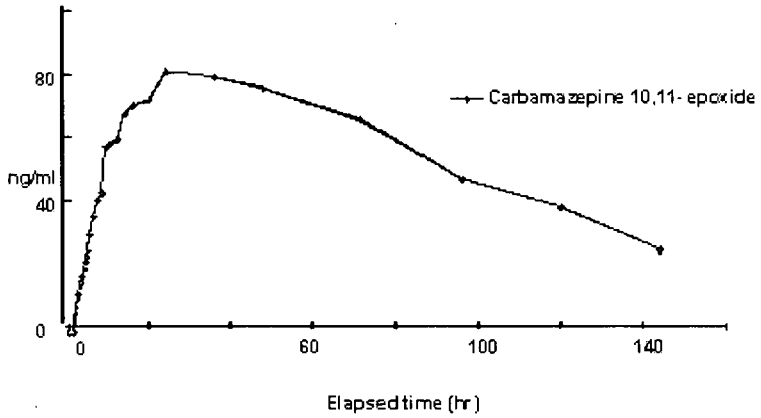

Figure 32: Representative mean carbamazepine-10, 11-epoxide plasma concentration/time profile (n=22) as obtained after a single 400mg oral dose of carbamazepine to human volunteers.

A highly sensitive and selective method for the quantification of carbamazepine and its metabolite, carbamazepine-10, 11-epoxide in human plasma has been developed and validated (table 62) [ϖ]. Plasma concentrations of carbamazepine and carbamazepine-10, 11-epoxide could be quantified from 0.722 ng/ml to 10 644 ng/ml for carbamazepine and from 5.15 ng/ml to 1 204 ng/ml for carbamazepine-10, 11-epoxide, making it possible to analyse samples longer than 144 hours after a single oral dose of 400mg of carbamazepine to human volunteers.

This is the first chromatographic method for the quantification of carbamazepine and carbamazepine-10, 11-epoxide in plasma described using liquid chromatography, with tandem mass spectrometry.

[ϖ] See appendix IV

Table 62: Comparison between the available literature and the developed method.

	Volume of serum/plasma	Inj. Vol.	LOD		LOQ		~Recovery		Retention times		Dose	C_{max}	
			CBZ	CBZ-epox	CBZ	CBZ-epox	CBZ	CBZ-epox				CBZ	CBZ-epox
	(µl)	(µl)	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)	(%)	(%)		(µl)	(µl)	(ng/ml)	(ng/ml)
Mihaly et al.,	1000		50	16.67	500	50	85	85	7.0	5.0			1 500
Kumps	500	40	50	100					13.8	6.1		8 500	2 780
Post et al.,											925	9 500	
Vree et al.,	100	100	100	100			88	88			1000	77 000	34 000
Mendez-Alvarez et al.,	100	20					99	98	9.0	5.0			
Deng et al.,	400	100									2000	30 000	14 500
Chelberg et al.,	250	20	10	10	1000	2000	100	100	5.0	2.5		16 000	10 000
Martens and Banditt	400	20	80	7	270	25			10.9	5.6			
Liu et al.,	100	2			780	390	97	98	13.0	8.0		50 000	25 000
Kouno and Ishikura	5	5			1000		96		14.7			20 000	
Romanyshyn et al.,	100	20			195	49	89	87	20.7	9.1			
Gholami et al.,	500	20	10	10			83	96	5.2	7.1			
Sanchez et al.,	200											7 100	
Matar et al.,	100	20	200	200	2000	500	98	99	10.2	4.5			
Speed et al.,	200				500		158						
Fedorova et al.,	120	5	50						14.0				
Queiros et al.,	100	20			100		98		10.9	6.1			
Kuldvee and Thormann	250						92	95	4.2	2.0			
van Rooyen et al.,	**500**	**20**			**0.722**	**5.15**	**95**	**100**	**2.7**	**2.8**	**400**	**1 200**	**80**

2.5. Publication

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Determination of carbamazepine and carbamazepine 10,11-epoxide in human plasma by tandem liquid chromatography–mass spectrometry with electrospray ionisation

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Abstract

A sensitive method for the determination of carbamazepine and carbamazepine 10,11-epoxide in plasma is described, using high-performance liquid chromatographic separation with tandem mass spectrometry. Samples were purified using liquid–liquid extraction and separated on a Phenomenex® Luna C_{18} 5 μm, 150×2 mm column with a mobile phase consisting of acetonitrile, methanol and formic acid (0.1%) (10:70:20, v/v). Detection was performed by a Micromass Quattro Ultima™ mass spectrometer in the MRM mode (LC–MS–MS) using electro spray ionisation (ESI+), monitoring the transition of the protonated molecular ion for carbamazepine at *m/z* 237.05 and carbamazepine 10,11-epoxide at *m/z* 253.09 to the predominant ions of *m/z* 194.09 and 180.04, respectively. The mean recovery was 95% for carbamazepine and 101% for carbamazepine 10,11-epoxide, with a lower limit of quantification of 0.722 ng/ml for carbamazepine and 5.15 ng/ml for carbamazepine 10,11-epoxide, when using 0.5 ml plasma. This high-throughput method was used to quantify 230 samples per day, and is sufficiently sensitive to be employed in pharmacokinetic studies.

Keywords: Carbamazepine; Carbamazepine 10,11-epoxide

1. Introduction

Carbamazepine is an established drug for the control of grand mal and psychomotor epilepsy and it is also effective in the treatment of trigeminal neuralgia. Furthermore, it is presently used in bipolar depression. It is predominantly eliminated in the liver, where it is metabolised to carbamazepine 10,11-epoxide and other derivatives. Carbamazepine 10,11-epoxide seems to have antiepileptic properties as well as carbamazepine itself.

Chelberg et al. [1] and Martens and Banditt [2] both published liquid–liquid extraction methods followed by HPLC and UV-detection to determine carbamazepine. Liu et al. [3] described a precipitation method with acetonitrile for the quantitation of carbamazepine in serum with a photodiode-array detector at 200 nm, while Romannyshyn et al. [4] described a liquid–liquid extraction followed by isocratic HPLC analysis with UV detection at 210 nm in a range of 0.5–20 μg/ml for carbamazepine and 0.25–15 μg/ml for carbamazepine 10,11-epox-

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ide. Yukimitsu and Chiyoji [5] published a solid-phase extraction method from serum followed by HPLC and UV-detection at 240 nm. Reported run-times for the determination of carbamazepine and carbamazepine 10,11 epoxide were between 10 and 27 min [2–4].

Our described LC–MS–MS method made it possible to inject the samples 4 min apart, increasing the through-put up to six times. The lowest LOQ (with high precision) from the references was 1 μg/ml [1], whereas our method has an LOQ of 0.722 ng/ml.

This paper thus represents the first quantitative LC–MS–MS method for the determination of carbamazepine and carbamazepine 10,11-epoxide in human plasma. A sensitive assay method, in the lower ng/ml levels in plasma, was optimised on a Micromass Quattro Ultima™ mass spectrometer with mass spectrum–mass spectrum (MS–MS) capabilities in tandem with liquid chromatography (LC).

2. Experimental

2.1. Materials and chemicals

A Phenomenex® Luna C_{18} 5 μm, 150×2 mm column (Phenomenex, Torrance, CA, USA) was used for separation at a flow-rate of 0.25 ml/min and 20 μl was injected onto the column. The mobile phase was delivered and the samples injected by a Waters-Alliance 2790 system. Detection was performed by a Micromass Quattro Ultima™ mass spectrometer interfaced to an electrospray ionisation (ESI) source.

Acetic acid (Pro-Analysi) was obtained from Merck (Darmstadt, Germany), acetonitrile (B&J High Purity) was obtained from Baxter (Muskegon, USA) and formic acid (98/100%) was obtained from BDH Laboratory Supplies (Dorset, UK). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

Carbamazepine (5*H*-dibenz[*b*,*f*]azepine-5-carboxamide, $C_{15}H_{12}N_2O$) (Fig. 1a) was supplied by VIS Farmaceutici (Hamburg, Germany) and carbamazepine 10,11-epoxide (10,11-epoxy-5*H*-dibenz[*b*,*f*]-azepine-5-carboxamide, $C_{15}H_{12}N_2O_2$) (Fig. 1b) was supplied by Sigma–Aldrich (St. Louis, MO, USA).

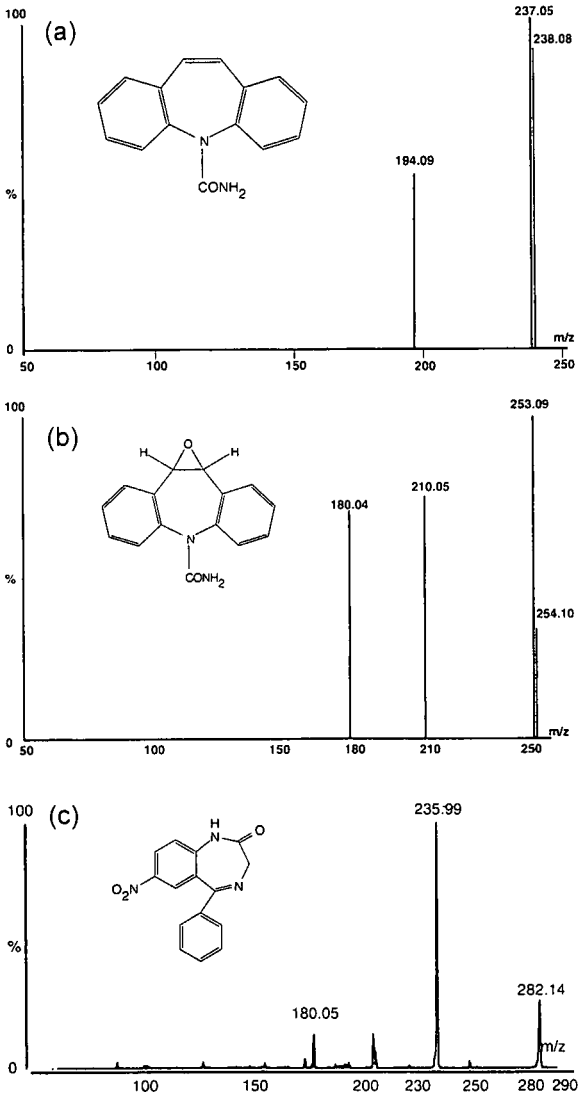

Fig. 1. (a) A full scan spectrum (MS–MS) of a pure solution of carbamazepine in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ion with *m/z* 237.05 and the predominant product ion *m/z* 194.09 are shown. (b) A full scan spectrum (MS–MS) of a pure solution of carbamazepine 10,11-epoxide in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ions with *m/z* 253.09 and predominant product ion *m/z* 180.04 are shown. (c) A full scan spectrum (MS–MS) of a pure solution of nitrazepam in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ions with *m/z* 282.14 and predominant product ion *m/z* 235.99 are shown.

Nitrazepam was obtained from FARMOVS–PAREXEL Bioanalytical Services internal reference library.

2.2. Calibration standard preparation

Carbamazepine and carbamazepine 10,11-epoxide stock solutions were prepared in methanol and used immediately to spike blank plasma. The solutions were not retained for further use. Calibration standards and quality control standards were prepared in normal human plasma by spiking plasma to a known concentration and then serially diluting it with normal blank plasma to attain the desired concentration range. The prepared calibration standards and quality control standards were aliquoted into microfuge tubes and stored at −20 °C until analysis.

2.3. Extraction procedure

To 500 μl of plasma was added NaOH (0.1 *M*, 500 μl) and nitrazepam (internal standard) (100 μl, 800 ng/ml in water) in a 5-ml ampoule. Ethyl acetate (3 ml) was added and the samples were vortexed for 45 s and centrifuged at 3000 *g* for 3 min at 10 °C. The aqueous phase was frozen on a freezerbath at −30 °C for 3 min, the organic phase poured into another 5-ml ampoule and dried under a gentle stream of nitrogen at 40 °C. The analytes were redesolved in 200 μl of mobile phase and vortexed briefly to homogenise. The Waters Alliance series 2790 autosampler injected 20 μl onto the HPLC column. The samples were cooled to 4 °C on the autosampler.

2.4. Instrumental conditions

Chromatography was performed at ambient temperature, at a flow-rate of 0.25 ml/min with acetonitrile:methanol:formic acid (0.1%) (10:70:20, v/v) as mobile phase. All chromatographic solvents were sparged with helium before use.

2.5. Mass spectrometry

Electrospray ionisation was performed in the positive mode with the nebulizing gas (nitrogen), cone gas and desolvation gas set at 80, 135 and 640 l/h, respectively. The ionisation source was connected by a fused-silica (375 μm) capillary to the syringe pump for tuning and the instrument responses for carbamazepine and carbamazepine 10,11-epoxide were optimised using flow injection. Optimal responses were obtained with a cone voltage setting of 40 V, a heated capillary voltage of 2.8 kV and temperature at 100 °C.

The transition of the protonated molecular ion for carbamazepine at *m/z* 237.05 and carbamazepine 10,11-epoxide at *m/z* 253.09 to the predominant ions were *m/z* 194.09 and 180.04, respectively. The transition of the protonated molecular ion for nitrazepam at *m/z* 282.14 to the predominant ion was *m/z* 235.99. The isolation width was 2 a.m.u. and the relative collision energy set at 30% for all three analytes. The mass spectrometer was interfaced to a computer workstation running MassLynx version 3.3 software.

2.6. Validation

The method was validated assaying plasma quality control samples ($n=5$) at 0.83, 1.66, 3.28, 13.10, 52.50, 105.00, 210.00, 1674, 3347 and 6693 ng/ml for carbamazepine and 5.84, 11.70, 23.10, 92.30, 369.00 and 738.00 ng/ml for carbamazepine 10,11-epoxide, to determine the accuracy and precision of the method. Quality control values were calculated from a standard regression curve, constructed from the ratio of analyte to internal standard peak areas, containing eight different concentrations, spanning the concentration range 0.83–6693.00 ng/ml for carbamazepine (Wagner $(\ln(y) = a(\ln(x)^2) + b(\ln(x)) + c$, a: −0.001110, b: 0.946032, c: −2.443848, r^2: 0.999534, mean RSD (%) 7.43), and 5.84–738.00 ng/ml for carbamazepine 10,11-epoxide (Wagner $(\ln(y) = a(\ln(x)^2) + b(\ln(x)) + c$, a: −0.016443, b: 1.130090, c: −4.300546, r^2: 0.999734, mean RSD (%) 7.18).

2.7. Matrix effects

Matrix effects were determined by analysing blank biological fluids from six different sources to determine possible interference.

2.8. Recovery

Absolute recovery of the analyte was determined in triplicate at high, medium and low concentrations in normal plasma by extracting drug free plasma samples spiked with carbamazepine and carbamazepine 10,11-epoxide. Recovery was calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted analyte standards, representing 100% recovery.

3. Results and discussion

At first, plasma containing carbamazepine and carbamazepine 10,11-epoxide were extracted on C_{18} solid-phase extraction columns. The columns were washed with NH_3OH (0.01 *M*) and the analytes eluted with acetonitrile. The elute was dried in a Savant speedvac evaporator and the analytes reconstituted in mobile phase. The extracts were found to be dirty with poor chromatography and an increase in column pressure was observed after just five injections. Liquid–liquid extractions was performed with NaOH (0.1 *M*) as pH modifier, using ethyl acetate (5 ml) and diethyl ether (5 ml), respectively, as organic solvents in a 10-ml ampoule. The samples were vortexed and centrifuged at 3000 *g*. The aqueous phases were frozen on a freezerbath at −30 °C, the organic phases poured into 5-ml ampoules and dried under a gentle stream of nitrogen at 40 °C. The analytes were redesolved in mobile phase, vortexed briefly to homogenise and injected onto the HPLC column. The extraction with ethyl acetate gave the best results and it was decided to optimise this extraction.

The carbamazepine 10,11-epoxide stock solution had to be prepared in a polypropylene scintillation vial, since the aluminium in the lid of the glass scintillation vial reacted as a catalyst, causing a breakdown of the carbamazepine 10,11-epoxide dissolved in the methanol. The mean absolute recoveries of carbamazepine, determined in triplicate at 105, 411 and 3274 ng/ml, were 89.71, 94.46 and 101.27%, respectively. The mean absolute recoveries of carbamazepine 10,11-epoxide, determined in triplicate at 26.7, 107 and 418 ng/ml, were 96.19, 104.48 and 102.44%, respectively.

Results from the intra-day validation assay indicate a valid calibration range of 0.722–10 644 ng/ml for carbamazepine and 5.15–1204 ng/ml for carbamazepine 10,11-epoxide. Table 1 depicts the quality control data obtained during the validation of the method for carbamazepine and carbamazepine 10,11-epoxide, while Table 2 depicts the intra-day back calculated quality controls for carbamazepine and carbamazepine 10,11-epoxide. On-instrument stability was inferred from intra-day quality control data obtained during the pre-study validation. No significant degradation could be detected in the cooled samples (4 °C) left on the autosampler for at least 35 h for carbamazepine, 121 h for carbamazepine 10,11-epoxide and 13.5 h for nitrazepam (internal standard). The batches were divided into groups of

Table 1
Summary of intra-day quality control results for carbamazepine ($n=5$) and carbamazepine 10,11-epoxide ($n=5$)

(a) Carbamazepine

	QC J	QC J (Dil.)	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	6693	6693	3347	1674	210	105	52.5	13.1	3.28	1.66	0.830
Mean	7145	6767	3454	1675	200	103	49	12	3	1.63	0.928
%Nom	106.8	202.2	103.2	100.1	95.4	98.4	94.2	97.2	92.2	98.4	111.8
RSD (%)	2.6	1.6	6.1	4.3	2.0	4.2	5.2	7.0	6.7	8.2	4.6

(b) Carbamazepine 10,11-epoxide

	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	738	369	92.3	23.1	11.7	5.84
Mean	762.54	364.29	92.11	22.04	11.54	5.67
%Nom	103.3	98.7	99.8	95.4	98.6	97.1
RSD (%)	7.4	7.9	6.1	5.9	4.7	7.7

Table 2
Summary of back calculated quality control concentrations of carbamazepine and carbamazepine 10,11-epoxide (inter-day variation) showing the repeatability of the method

(a) Carbamazepine

	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B
Nominal (ng/ml)	3347	837	210	52.5	26.3	13.1	3.28	1.66
Mean	3229.97	873.59	205.71	55.10	26.33	13.34	3.01	1.68
RSD%	4.7	5.2	9.5	9.3	8.2	13.0	13.4	16.0
n	10	8	15	9	8	15	6	13
%Nom	96.5	104.4	98.0	105.0	99.7	101.8	91.8	101.5

(b) Carbamazepine 10,11-epoxide

	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	1476	369	185	92.3	23.1	11.7	5.84
Mean	1286.37	370.79	187.20	94.37	22.36	11.51	5.89
RSD%	8.8	7.6	7.4	7.3	11.4	7.7	5.1
n	11	9	8	14	7	16	10
%Nom	87.2	100.5	101.2	102.2	96.8	98.4	100.8

samples (two groups of ~115 samples each) which were extracted consecutively and loaded onto the autosampler ~4 h apart to ensure that samples did not remain on-instrument awaiting injection for longer than the tested on-instrument stability times. Each group of 100 assays contained an equal proportion of the calibration and quality control standards processed in the batch.

Freeze–thaw stability was not relevant in this study since samples were not subjected to multiple freeze–thaw cycles and were assayed immediately after thawing. Sample residues were discarded after analysis. Repeat assays were performed on the duplicate aliquots, which remained frozen until assayed. Carbamazepine and carbamazepine-10,11-epoxide are reported to be stable when frozen in serum for at least 4 months [6].

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

Fig. 1a shows the single parent to product ion mass spectrum (MS–MS) of carbamazepine and Fig. 1b the single parent to product ion mass spectrum (MS–MS) of carbamazepine 10,11-epoxide acquired with the abundant product ions at m/z 194.09 and 180.04, respectively. A strong daughter ion with m/z 210.05 was observed, but the daughter formed at m/z 180.04 was more stable.

Fig. 1c shows the single parent to product ion mass spectrum (MS–MS) of nitrazepam acquired with the abundant product ion at m/z 235.99.

Quantitation was achieved at unit resolution in full scan MS–MS mode scanning the product ion spectrum from m/z 50 to 250 and monitoring the transition of the protonated molecular ion at m/z 237.05 for carbamazepine, to the largest product ion m/z 194.09 and the protonated molecular ion at m/z 253.09 for carbamazepine 10,11-epoxide to the largest product ion m/z 180.04.

Typical retention times for carbamazepine were 2.65–2.84 min (mean RSD 0.62%), for carbamazepine 10,11-epoxide were 2.73–2.87 min (mean RSD 0.60%) and for the internal standard were 2.33–2.47 min (RSD 0.66%). A chromatography time of 4 min made it possible to analyse 230 samples per day. In order to test for the presence of matrix effects [7], six different plasma pools were extracted and then spiked with a known concentration of the analyte. These samples were injected and peak areas compared. The calculated RSD was used as measurement of reproducibility with a RSD of greater than 10% suggesting the presence of matrix effects [7]. The data showed no significant matrix effects either for carbamazepine (RSD 1.6%), carbamazepine 10,11-epoxide (RSD 3.1%) or nitrazepam at the tested concentrations.

Fig. 2a shows representative chromatograms of

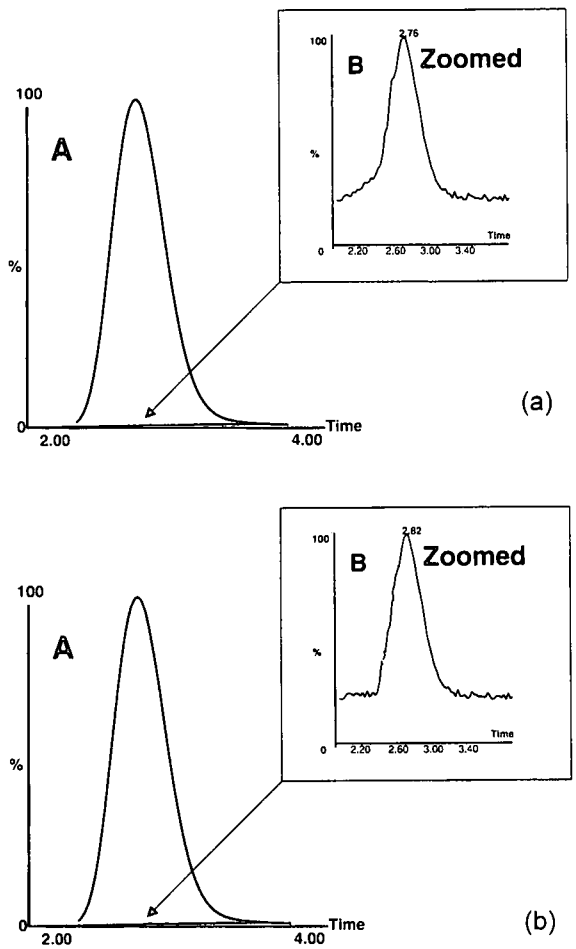

Fig. 2. (a) Chromatograms of calibration standards, containing 10 644 ng/ml (A) and 0.722 ng/ml (B), respectively, of carbamazepine in plasma. (b) Chromatograms of calibration standards, containing 602 ng/ml (A) and 5.84 ng/ml (B), respectively, of carbamazepine 10,11-epoxide in plasma.

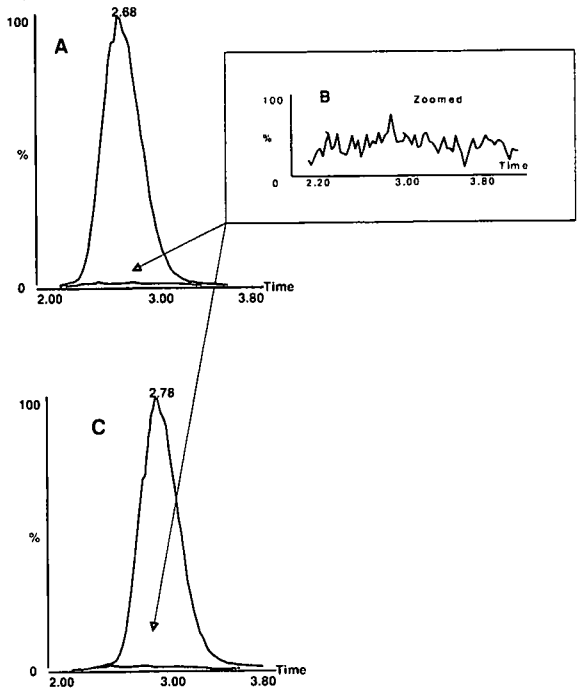

Fig. 3. Chromatograms of a subject sample containing 224.69 ng/ml (A) of carbamazepine in plasma, 74.59 ng/ml (C) of carbamazepine 10,11-epoxide in plasma, and blank plasma extract (B).

carbamazepine obtained at 10 644 ng/ml and 0.722 ng/ml (LLOQ), and Fig. 2b shows representative chromatograms obtained at 602 and 5.84 ng/ml, while Fig. 3 depicts chromatograms from a subject sample (both carbamazepine and carbamazepine 10,11-epoxide) and a blank plasma extract, zoomed in to show the absence of any interference.

The method was employed to analyse plasma samples containing carbamazepine and carbamazepine 10,11-epoxide obtained after a single 400-mg oral dose of carbamazepine in 22 healthy volunteers. The maximum plasma concentrations obtained varied between 1200 and 1000 ng/ml for carbamazepine and between 85 and 75 ng/ml for carbamazepine 10,11-epoxide (quantified using peak area ratios). Fig. 4a,b represents the mean pharmacokinetic profiles for carbamazepine and carbamazepine 10,11-epoxide for 22 human volunteers after receiving a 400-mg oral dose of carbamazepine each.

4. Conclusion

A highly sensitive and selective method for the quantification of carbamazepine and its metabolite, carbamazepine 10,11-epoxide, in human plasma has been developed and validated. Plasma concentrations of carbamazepine and carbamazepine 10,11-epoxide could be quantified from 0.722 to 10 644 ng/ml making it possible to analyse samples up to 144 h after a single oral dose of 400 mg of carbamazepine

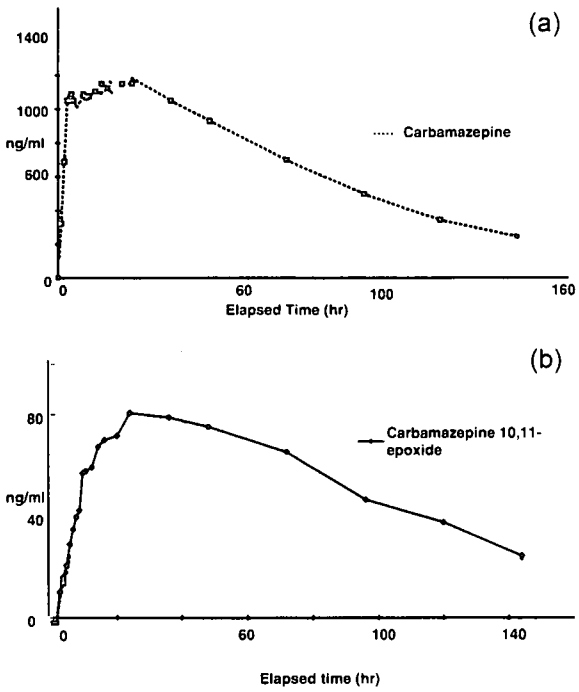

Fig. 4. (a) Representative mean carbamazepine plasma concentration/time profile ($n=22$) as obtained after a single 400-mg oral dose of carbamazepine to human volunteers. (b) Representative mean carbamazepine 10,11-epoxide plasma concentration/time profile ($n=22$) as obtained after a single 400-mg oral dose of carbamazepine to human volunteers.

to human volunteers. This LC–MS–MS method is far more sensitive and precise than any other method described. Radioimmunoassays [4] are much more sensitive, but due to cross-reactivity, it is advisable to determine only one analyte at a time.

This is the first chromatographic method for the quantitation of carbamazepine and carbamazepine 10,11-epoxide in plasma described using liquid chromatography, with tandem mass spectrometry.

References

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3. CONCLUSION

Ira Krull and Michael Swartz [27, 28] have recently completed excellent texts, which should be a mandatory reference for every methods development lab. One important point that the authors emphasize is that validation is not confined to only the LC or the LC/MS method, but also relates to the software, hardware, method, and system suitability. This is of particular importance to the method developer when they have to deal with sample complexities, multiple components and integration issues relating to LC/MS.

Validating bio-analytical methods is a crucial component of successful product development and quality. There are a variety of product types that require some level of evaluation and testing either at the raw material, intermediate, or final product level. Critical decisions may be made based on these results, making it imperative that pharmaceutical companies ensure their accuracy and reproducibility to remain compliant with FDA and ICH guidelines in the current climate of increased regulatory enforcement.

To meet the ever-increasing demands on the pharmaceutical industries to speed products to the market, companies have recognized the need to devise creative strategies for more effective method analysis without compromising their compliance with the existing regulatory compliance guidelines.

The speed and sensitivity of bio-analysis has many commercial implications within the pharmaceutical sector. Drug companies that are working in the forefront of new drug development are highly attuned to any aspect of the drug development process, which could reduce time to market. No pharmaceutical company can afford the competitive disadvantage of having any aspect of its drug development slowed down by the use of analytical procedures, which are any less than state-of-the art.

Contract research organisations (CRO) that provide bio-analytical support services presently find themselves with a number of issues when meeting the demands of sponsors. One of the most difficult commercial risks for a clinical research organisation to manage is the estimated time dedicated to method development.

The historical achievement of mass measuring intact bio-molecular ions has established mass spectrometry as a valuable tool in biotechnology. It is the combination of attributes such as sensitivity, mass range, accuracy, and the ability to obtain structural information that has brought mass spectrometry to the forefront of biotechnological research and which propels its further development as an important analytical tool for both chemists and biologists. The enhanced speed of LC-MS/MS systems enables contract research organisations to offer the more flexible and reactive service which is necessary to meet the expectations of sponsors and to ensure that the time taken for the bio-analysis does not cause unnecessary delays.

Ten years ago, the techniques most widely used in biopharmaceutical analysis were high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). High performance liquid chromatography could be coupled to a range of detectors such as ultra violet, fluorescence or electrochemical detectors. However, the techniques still require that the analyte, internal standard and any matrix interference are chromatographically resolved, and this can result in quite lengthy run-times. GC-MS, although a more selective technique, often requires more extensive sample clean up and derivatization of extracts to render the analytes more volatile and less thermally labile.

Towards the end of the 1980s, there were major advances in the design of interfaces between HPLC (LC) and mass spectrometry (MS). This lead to the establishment of LC-MS as a robust, sensitive and quantitative analytical technique.

Generic companies looking to develop drugs, frequently demand the use of HPLC or GC-MS methodologies that were described in the literature some years ago. In many cases these methods are perfectly adequate in providing the bio-equivalence data, which is required. Analysis by these methods will be more economic, because they are less capital intensive, but inevitably ignore the advances in speed, sensitivity and specificity available with LC-MS/MS techniques. In studies with less than say 800 samples, HPLC and GC-MS methods may be preferred financially, especially if there are already validated methods available. However, as sample numbers increase, and in cases where old methodologies are technically demanding and time consuming, the balance increasingly swings towards LC-MS/MS.

These two projects, determining clarithromycin, carbamazepine and its metabolite, carbamazepine-10, 11-epoxide from human plasma, respectively, were successfully designed as high-throughput methods, causing an increase in production and a more accurate, sensitive and specific quantitation from human plasma, while they still remain compliant with FDA and ICH guidelines.

List of Figures

List of Tables

Appendix I

Journal of Chromatography B

Biomedical Sciences and Applications

Dr. G.F. Van Rooyen
FARMOVS - PAREXEL

Re: 1 1773 Amsterdam, October 10, 2001

Dear Dr. Van Rooyen,

On behalf of the editor handling your manuscript, Professor I.W. Wainer, I am writing to you in reference to your manuscript entitled: Sensitive liquid chromatographic-tandem mass spectrometric method for the determination of clarithromycin in human plasma, by Van Rooyen, G.F., Smit, M.J., De Jager, A.D., Hundt, H.K.L., Swart, K.J. and Hundt, A.F.

I regret to inform you that your manuscript was not found acceptable for publication in its present form. We would be prepared, however, to reconsider the manuscript if it can be revised to meet the objections detailed in the enclosed comments.

I look forward to receiving, in due course, three copies of a suitably revised manuscript and on one of these copies please underline in red the changes made in response to the comments. Also, please provide a detailed letter, responding to each point raised and indicating where the changes were made. 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. Please note that 0 correspondence concerning your manuscript should be addressed as follows: Journal of Chromatography B, Editorial Office, P.O. Box 68 1, NL- I 000 AR Amsterdam, The Netherlands; fax: 20-4852304; e-mail: chrom-eo@elsevier.nl.

Yours sincerely,
EDITORIAL OFFICE

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

Reviewer's Copy
Manuscript No.
I1773

Recommendations and comments should be sent directly to the Editor.
Please note your name can either only be typed or printed.

Author: G.F. van Rooyen et al
Title: Sensitive LC-tandem MS method for the determination of clarithromycin in human plasma

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

2. Is the paper acceptable
- [ ] in its present form
- [ ] with minor changes
- [ ] with revision
- [x] with major revision
- [ ] with revision and condensation
- [ ] not at all

3. Is the theory 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 NEXT PAGE

J. Chrom. B **11773**

G.F. van Rooyen *et al*
Sensitive liquid chromatography – tandem mass spectrometry method.....clarithromycin..

1 Suitable? **Yes**

2 Acceptable **with revision** ***I'd be willing to look at a revised manuscript.***

3 Is it well organized and presented **Yes**

4 References to related work **Yes**

5 Is the English satisfactory **Yes**

Comments:
The manuscript describes an HPLC-MS/MS method for plasma clarithromycin (C) which uses a related antibiotic roxithromycin (R) as the internal standard.

Major deficiencies:
1. In the "Preparation of calibration standards", no details are given concerning the amounts of C and R used to make these standards, volumes of plasma, not even the number of calibrating samples used.
2. No calibrating data of any kind are presented; no typical calibrating plot (response vs quantity) or even an equation of best fit are apparent. It is impossible then to judge the errors and reproducibility encountered in repeat injections of the same sample.

Minor comments:
1. Figure 5 represents mean PK profiles following 500 mg doses of 3 formulations of C in 9 volunteers. Does this mean 3 volunteers/formulation? If these are "mean", what are the SD's? Is the 'hump' at 10 hr for treatment 2 real?
2. "HP" is now Agilent. The address for the autosampler should be for Agilent.
3. Page 4, 2 lines up: there is no English verb "to aliquot" it is an adjective and a noun. "Measured" or "pipetted" perhaps.
4. P5, L5 up: gas flows of 70, 50 and 70 "units" are meaningless to someone wishing to duplicate the method on other instruments.
5. P5 last line: are these potentials in volts? Positive or negative?
6. P10, L8: "zoomed in"? How about "magnified as an insect"
7. Are references 7 – 10 single page articles?
8. Table 1 and 2: Tabel?
9. P10, L1: RT's for C are stated as 1.97 – 2.05 min. Yet fig's 3 & 4 show the RT to be 1.89 and 1.88 min resp.

Reply on comments

Major deficiencies:

Comment 1. See page 4, paragraph 4 and page 6, paragraph 3. All the requested data are there.

Comment 2. Added to article.

Minor Comments:

Comment 1. The mean pharmacokinetic profile given in figure 5 is after 3 different formulations in 9 healthy volunteers.

Comment 2. Done

Comment 3. Corrected

Comment 4. Corrected

Comment 5. Corrected

Comment 6. Corrected

Comment 7. Corrected

Comment 8. Changed.

Comment 9. Corrected in the manuscript.

From: "Author Support IE (ELS)" <AuthorSupport@elsevier.ie>

To: "Van Rooyen, GF, Gert" <Gert.vanRooyen@farmovs-parexel.com>

Subject: RE: CHROMB 11896 VOL. 768/2 PPS. 223-229

Date sent: Thu, 28 Feb 2002 11:38:29 -0000

Dear Dr. van Rooyen,

We are pleased to inform you that your paper has been published in volume 768, issue 2, on pages 223 - 229, cover date February 2002.

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Appendix II

Journal of Chromatography B

Dr. G.F. van Rooyen
FARMOVS - PAREXEL

Re: DL 252 Amsterdam, September 17,2001

Dear Dr. Van Rooyen,

On behalf of the editor handling your manuscript, Dr. D.K. Lloyd, I am writing to you in reference to your manuscript entitled: Determination of carbamazepine and carbamazepine 10, 11-epoxide in human plasma by liquid chromatography-tandem mass spectrometry with electrospray ionisation, by Van Rooyen, G.F., Badenhorst, D., Swart, K.J., Hundt, H.K.L., Scanes, T. and Hundt, A.F.

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

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

Yours sincerely,

EDITORIAL OFFICE

NAL OF CHROMATOGRAPHY B:
EDICAL SCIENCES & APPLICATIONS

Manuscript No. DL-252

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

Author: GF van Rooyen etal.

Title: Determination of carbamazepine and carbamazepine 10,11-epoxide in human plasma by tandem liquid chromatography-mass spectrometry with electrospray ionisation.

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

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

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

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

5. Is the English satisfactory? YES/NO

Comments:

1. The authors state that the internal standard (nitrazepam) is stable for ~13.5 hours and yet a typical run (230 samples) should last at least 19 hours (~15 hours for the sample run and 1 minute per sample for injection [about typical for a Waters 2790 injector]. How did they compensate for this reported lack of stability? Also, no data on the internal standard was reported (reproducibility, retention time, chromatography).
2. Why were the n's (number of samples) for the inter-day reproducibility(Tables 2a and 2b) different for each quality control sample? Were not all of the QC's run every day? If not, how were the QC's to be run chosen? Were some of the data omitted because they were "less than optimal"?
3. The authors imply that the samples placed in the autosampler were cooled. What was the sample chamber temperature maintained at?
4. Do the authors know the freezer stability of the samples (how long can the plasma samples be kept in the freezer prior to analysis)? Likewise, do they know the freeze-thaw stability of the samples?
5. There are some minor "typo" and spelling errors.

JOURNAL OF CHROMATOGRAPHY B:
BIOMEDICAL SCIENCES & APPLICATIONS

Manuscript No. DL-252

Comments:

6. 1. Generally, the English language needs to be improved in the manuscript. Many spelling and grammer mistakes are in the text.
7. 2. The introduction needs to be expanded to give the readers more details about the scientific novelty of the work in comparison to the already existing methods.
8. 3. The references are not sufficient in the text. They need also to be organized according to list of references given.
9. 4. Titles, subtitles, text units, tables and figures should be in the style of Journal. — also references
10. 5. The proposed method should be fully validated. The authors should report on linearity of the method (equations for calibration curves, RSD values of slopes, regression coefficient.).
11. 6. The authors gave no details on nitrazepam as IS. Data regarding full-MS/MS and MRM spectra of IS should be reported.
12. 7. The quantitation procedures is not clear, as no data were provided regarding IS.
13. 8. No pharmacokinetic data were provided, although large number of samples were analyzed. Detailed clinical study should be also reported in the Experimental section.
14. 9. The values of mass ions (m/z) and the concentration values of carbamazepine and carbamazepine metabolite in tables / figures should be in the same decimal figures as in the text. At the same time, the decimal figures used in the manuscript should be unified.
15. 10. The authors should give reasons for selecting the concentration ranges of carbamazepine and carbamazepine metabolite in standard curves and quality tests, at these levels. The concentration data in the manuscript showed the same concentration levels for both compounds, athough it is well-known that the concentration of metabolite is much smaller.
16. 11. The authors should reports in the manuscript about the advantages of LC-MS/MS over other LC/MS and radioimmunoassays.

Reply on comments

Comment 1. The Waters 2790 injector has a parallel injection setting, which takes about 4 seconds in between injections. Thus for 230 samples at a runtime of 4 minutes (including in between sample time), 230 samples will take approximately 15.3 hours.

The batches were divided into groups of samples (2 groups of approximately 115 samples) which were extracted consecutively and loaded onto the autosampler approximately 4 hours apart to ensure that samples did not remain on-instrument awaiting injection for longer than the tested on-instrument stability times. Each group of 100 assays contained an equal proportion of the calibration and quality control standards processed in the batch.

This has been added to the article.

Comment 2. There were extra replicates of STD J in batch 07 and STDs C, D, F and G (STD B were done in duplicate according to our SOP) in batch 08 accidentally included in the respective batches. Because the batches are split into 2 (115 each) and done by two different analysts, some STDs were included by mistake. For statistical reasons, it was decided to include all extras into the calculations for a more accurate estimate, thus the difference in *n*.

Comment 3. The samples were cooled at 4°C, and it is corrected in the manuscript.

Comment 4. Added to article.

Comment 5. Corrections made.

Comment 6. Corrections made.

Comment 7. Done

Comment 8. The references have been organised according to list of references.

Comment 9. Done

Comment 10. The curve-type and Slope-RSD that was used to quantify the samples have been added to the manuscript.

Comment 11 & 12. More data on the internal standard (nitrazepam) has been added to the document.

Comment 13. Due to confidentiality of this data, we are not allowed to publish any data without prior permission of our sponsors. Unfortunately, we have not permission to publish any pharmacokinetic data other than that reported in the manuscript. These data will be published at a later stage.

Comment 14. All figures have been rounded off to 2 decimal places in the manuscript.

Comment 15. The wrong concentration ranges where accidentally entered into the manuscript: This has been corrected.

Comment 16. Done

From: "Author Support Global (ELS)" <AuthorSupport@elsevier.com>

To: "Van Rooyen, GF, Gert" <Gert.VanRooyen@farmovs-parexel.com>

Subject: RE: support - CHROMB 11916

Date sent: Fri, 15 Mar 2002 16:38:27 -0000

Dear Dr. Van Rooyen,

We are pleased to inform you that your paper has been published in volume 769, issue 1, on pages 1-7, cover date 25 March 2002.

The 50 offprints of your paper will be despatched to you within the next few weeks to the following address:

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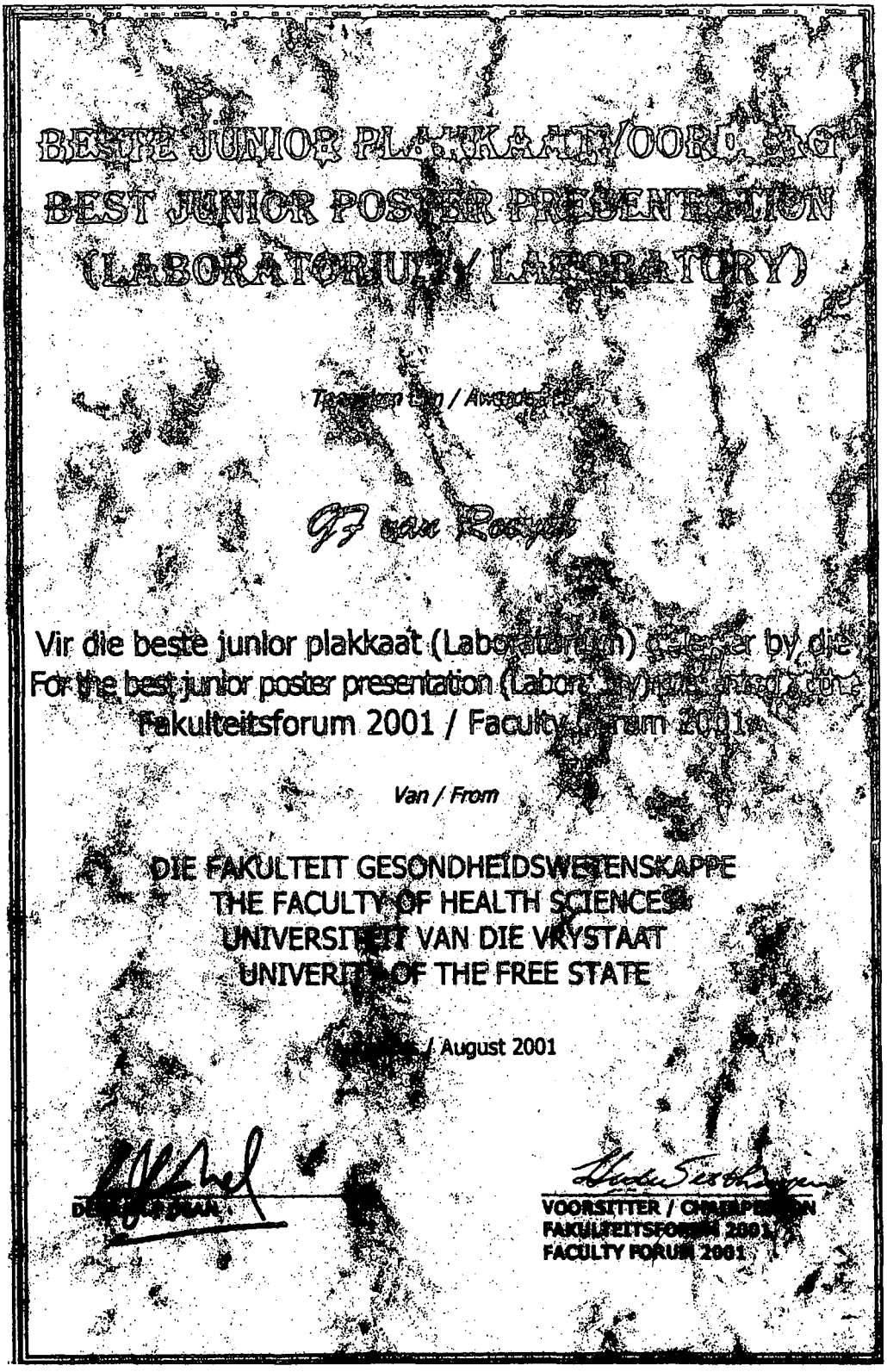
Yours sincerely,

Maeve Butler

Appendix III



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Appendix IV

From: Parvez Haris <pharis@dmu.ac.uk>

To: gert.vanrooyen@farmovs-parexel.com

Subject: First International Conference on Biomedical Spectroscopy: From Molecules to Men

Date sent: Thu, 18 Apr 2002 12:13:38 +0100

Dear Dr Van Rooyen,

I have read with interest some of your work on the subject of spectroscopic characterisation of biological systems. I am organising the First International Conference on Biomedical Spectroscopy: From Molecules to Men, 7-10 July, 2002 Cardiff, Wales, United Kingdom. I would like to invite you to make a lecture presentation on your interesting work on tandem liquid chromatography-mass spectrometry with electrospray ionisation approach for the rapid characterisation of molecules in plasma.

We would also like you to submit an article for publication in Spectroscopy - An International Journal. Unfortunately, due to the limited funding of the conference (due to lack of sponsorship) we are in the unfortunate situation of not being able to provide you with any financial support. In this regard, we hope that you will be able to find other sources of funding to cover your registration fee, and travel expenses.

The registration fee of 400 pounds covers your accommodation, local transportation (transfer from Cardiff Central train station or Cardiff International Airport) lunch, tea, and conference dinner. If you can attend, please send me an Abstract, and your lecture title, and please complete the registration form included below.

I look forward to hearing from you.

Best wishes,

Dr P.I. Haris

Editor-in-chief: Spectroscopy - An International Journal

Editor: Biochemical Journal

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ABSTRACT

The projects described in this dissertation use cutting edge technology to quantitate various drugs in plasma. Highly sensitive assay methods for the quantitation of clarithromycin, carbamazepine and its major metabolite, carbamazepine-10, 11-epoxide in human plasma were developed and validated. The application of these methods to analyse samples generated during pharmacokinetic studies is also discussed where up to 230 samples were analysed per day. The methods were sensitive enough to quantitate the analytes for at least 5 half-lives of the drug after an oral dose to human volunteers.

A sensitive method for the determination of clarithromycin, a macrolide antibiotic, using high-performance liquid chromatographic separation with tandem mass spectrometry, and the method development thereof, are described in this dissertation. Samples were prepared using liquid-liquid extraction and were separated on a C_{18} column with a mobile phase consisting of acetonitrile, methanol and acetic acid. Detection was performed by a mass spectrometer in the multiple reaction monitoring (MRM) mode, using TurboIonSpray ionisation. The mean recovery of clarithromycin was 87.3%, with a lower limit of quantification of 2.95 ng/ml when using 300µl plasma. This high-throughput method was used to quantify 230 samples per day, and is sufficiently sensitive to be employed in pharmacokinetic studies.

Carbamazepine, an anticonvulsant, and its major metabolite, carbamazepine-10, 11-epoxide, were quantified using tandem liquid chromatography-mass spectrometry, with electrospray ionisation. Both these analytes were extracted from 500µl of human plasma, using a liquid-liquid extraction method that showed recoveries greater than 95% for both these analytes. Concentrations as low as 0.722 ng/ml for carbamazepine and 5.15 ng/ml for karbamasepien-10, 11-epoxide could be quantified by using this method.

These are the first LC-MS/MS assay methods described for the quantification of carbamazepine and its metabolite, karbamasepien-10, 11-epoxideas well as carbamazepine and their applications to pharmacokinetic studies.

UITTREKSEL

Die projekte wat beskryf word in die verhandeling maak gebruik van die nuutste tegnologie om verskeie geneesmiddels kwantitatief in plasma te bepaal. Sensitiewe analitiese metodes vir die kwantifisering van klaritromisien, karbamasepien en een van sy belangrikste metaboliete, karbamasepien-10, 11-epoksied, is ontwikkel en beproef. Die aanwending van die metodes om plasmamonsters kwantitatief te analiseer vir die geneesmiddels gegenereer tydens farmakokinetiese studies, is ook beskryf. Dit was moontlik om tot soveel as 230 monsters per dag te analiseer, en die metodes was sensitief genoeg om die geneesmiddels vir ten minste vyf leef-tye, na 'n orale dosis, te kwantifiseer.

Die verhandeling beskryf die metodeontwikkeling vir asook die finale analitiese metode vir die kwantifisering van klaritromisien, 'n makrolied-antibiotikum. Die metode maak gebruik van hoë verrigtings vloeistofchromatografiese skeiding en massaspektrometriese bepaling. Die monsters is voorberei deur gebruik te maak van vloeistof-vloeistof ekstraksie, waarna skeiding op 'n C_{18} kolom, met 'n mobiele fase wat bestaan uit asetonitriel, metanol en asynsuur, plaasvind. Die bepalings is gedoen op 'n massaspektrometer deur gebruik te maak van veelvuldige reaksiemonitering en "TurbolonSpray" om die geneesmiddel te ioniseer. 'n Gemiddeld van 87% van die geneesmiddel is herwin vanuit net 300µl plasma, met die laagste kwantifiseerbare konsentrasie van 2.95 ng/ml. Die hoogsproduktiewe metode is gebruik om tot soveel as 230 monsters per dag te analiseer. Dit is ook gevind dat die analitiese metode sensitief genoeg was om monsters te analiseer wat gegenereer is tydens 'n farmakokinetiese studie met klaritromisien.

Die metodeontwikkeling vir die bepaling van karbamasepien, 'n anti-konvulsant, en een van sy belangrikste metaboliete, karbamasepien-10, 11-epoksied, asook die finale analitiese metode wat gebruikmaak van hoë verrigtings vloeistofchromatografie en massaspektrometrie, word ook in die verhandeling beskryf. Die massaspektrometer maak gebruik van elektronsproei-ionisasie om die analiete te ioniseer. Beide geneesmiddels is geëkstraheer vanuit 500µl menslike plasma deur middel van 'n vloeistof-vloeistof ekstraksiemetode. Dié metode kon ongeveer 95% van die geneesmiddels herwin. Konsentrasies so laag as 0.722 ng/ml vir karbamasepien en 5.15 ng/ml vir karbamasepien-10, 11-epoksied kon gekwantifiseer word deur dié metode.

Dit is die eerste massaspektrometriese metode wat beskryf is vir die kwantifisering van beide karbamasepien en een van sy belangrikste metaboliete, karbamasepien-10, 11-epoksied, asook klaritromisien in menslike plasma en die toepassing van die metodes om die geneesmiddels kwantitatief te bepaal in monsters wat gegenereer is tydens farmakokinetiese studies.

KEY WORDS

Method development, validation, bioequivalence, high-performance liquid chromatography, plasma, mass spectrometry, carbamazepine, carbamazepine -10, 11-epoxide, clarithromycin.