CHAPTER 2

ANALYTICAL METHODOLOGY FOR CAPTOPRIL

2.1 INTRODUCTION

Analytical methods for the quantitation of drugs or its metabolites in biological samples play an important role in the evaluation of bioavailability, bioequivalence and pharmacokinetic data. A wide range of assay techniques has been applied to the analysis of captopril involving a large range of detectors and extraction procedures. The various assays differ in their time and equipment requirements and in the method of sample preparation. The assays also vary in their sensitivity, specificity and reproducibility. This chapter reviews all the aspects related in analytical methodology for the assay of captopril including the published methods, method development for this study and method validation for the developed assay. Development of rapid, sensitive and reproducible method for the quantitation of captopril had not been easy. This is due to some important criteria which needs to be considered. These criteria are laid out below.

2.1.1 The existence of a sulphydryl group in the captopril structure

Captopril is a sulphydryl compound, and because of high reactivity of sulphydryl compounds, captopril is unstable in biological fluid or in any aqueous media. The unstability is due to oxidation of captopril and the rapid formation of disulphides (Funke *et al.*1980). Quantitation of captopril has therefore been difficult and several approaches have been utilised to assay captopril accurately. The initial development of suitable procedures for captopril assay were the use of antioxidants, chelating agents and derivatising agents. The most commonly used were derivatising agents, an example of which is N- ethylmaleimide (NEM) (Figure 2.1), which was first used by Funke *et al.*(1980) to stabilise captopril in blood and urine. The unchanged captopril and captopril disulphides dimer metabolite was found to be stable in

biological fluids and was unaffected by the addition of NEM. The use of NEM resulted in the formation of a captopril – NEM adduct which was then derivatised to another product for analysis by GCMS.

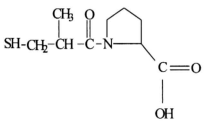
2.1.2 Chemical structure for the detection of captopril by GCMS

The chemical forms of the compound is important in order to achieve good detection by GCMS. In the case of captopril, the presence of the -COOH group makes the compound not volatile enough to be detected by GCMS. Therefore it has to be derivatised to form another product which can be easily analysed by GCMS. Derivatisation techniques involve reacting a compound of interest with an agent that can improve the characteristic of the compound for analysis by gas chromatography. There are several techniques of derivatisation. From the literature review, the most popular technique for derivatisation of captopril is esterification. Esterification typically involves condensation of the carboxyl group of an acid and the hydroxyl group of an alcohol, with the elimination of water. Pentafluorobenzylbromide (PFBB) (Figure 2.1) was found to be a suitable derivatising agent for captopril.

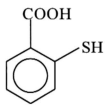
2.1.3 Internal standard

In order to minimise the contributions of variation in sample preparation and in injection volume to the final result, an internal standard was used. The selection of an internal standard is also important. The internal standard should possess the same functional group with captopril so that during the derivatisation process, the compound to be detected and the internal standard will be derivatised at the same time. Several internal standard had been used recently for the quantitation of captopril. The use of thiosalysilic acid (TSA) (Figure 2.1) as an internal standard proved to be suitable as it contained both functional groups which are

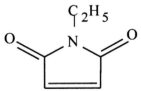
present on captopril and both are involved in the stabilisation reaction with NEM and with the derivatisation reaction with PFBB. (Franklin M.E. *et* al.1998)



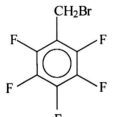
Captopril



Thiosalysilic acid



N-ethyl maleimide



Pentafluorobenzylbromide

Figure 2.1 Chemical structure for captopril and related substances

2.2 PUBLISHED METHODS FOR CAPTOPRIL ANALYSIS

Severals method have been used for the measurement of captopril and their metabolites. All the methods have been performed largely using chromatography techniques like thin – layer chromatography, High Performance Liquid Chromatography (HPLC), Gas – Liquid Chromatography (GLC) and Gas Chromatography Mass Spectrometry (GCMS). The initial difficulties encountered in the development of the chemical assay for captopril in blood necessitated the development of an assay using thin layer radiochromatography (TLRC) (Migdalof *et al.*1980), and this type of assay was used in a variety of early studies to assess the absorption and disposition of the drug. With this method, the lowest limit of quantitation of unchanged captopril was about 5 μg/ml. The use of this technique was limited by the fact that only the radiolabeled drug could be administered.

After intensive investigation, several chemical assays were developed. The most popular techniques and the most widely used currently are HPLC and GCMS methods . These two methods consistently show a high sensitivity and accuracy.

2.2.1 High performance liquid chromatography

A number of HPLC procedures have been described for the determination of captopril in biological samples. Jarrot *et al.* (1980), and Pereira *et al.* (1988) used ascorbic acid and adetate to prevent captopril being oxidised upon blood sampling. The internal standard used was [(4R)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid and N-(1-Pyrene)-maleimide was used to derivatise the captopril and the internal standard to the fluorescent adducts. The extraction procedure used was liquid-liquid extraction. The limit of quantitation by Jarrot *et al.* was 150 pmoles/ml and by Pereira *et al.* was 10 ng/ml.

Kawahara *et al.* (1981) developed another HPLC procedure using p-bromophenacyl bromide to trap captopril, and the addition product were then separated by HPLC. This method was able to detect unchanged captopril levels of 5 ng/ml in whole blood.

The method by Edward Bald *et al.* (1997) in the HPLC analysis of captopril is more advanced. The method involved a pre-column derivatisation of the drug via its sulphydryl group with 1- benzyl – 2 – chloropyridinium bromide followed by solid phase extraction and reversed-phase high performance liquid chromatography separation with ultraviolet detection. This method would minimise the oxidation of captopril by derivatising blood samples at the time of collection. This is performed by drawing blood directly into tubes containing solutions of 1 – benzyl – 2 – chloropyridinium bromide. Using this method, the limit of quantitation was 10 ng/ml.

Another application of HPLC for captopril analysis was developed by Robert J. Kok *et al.* (1997). Two methods were developed in order to obtain the best method. The method used pre- and post-column labeling. The first method was based on pre-column derivatisation of captopril with the fluorescent label monobromobimane (MBB). The derivatising agent was added to the plasma before extraction and after that injected into the HPLC. The second method was based on a post-column reaction with the fluorescent reagent *o*- phthaldialdehyde (OPA). In this method, the derivatising agent was added as a post-column reagent in the HPLC system at a certain flow rate. The OPA method can be used for measurement of free and total captopril. For total captopril tributylphosphine (TBP) was used to reduce the disulphides. Since the TBP interferes with the MBB, the MBB method is not suitable for total captopril analysis. The limit of quantitation for free captopril by MBB method is 12.5 ng/ml and by OPA method is 25 ng/ml.

Another method for HPLC analysis were performed by C. Arroyo *et al.* (1997). As with the previous method by Jarrot *et al.*, ascorbic acid and EDTA were used to prevent the oxidation of captopril and N – (1-Pyrenylmaleimide) [NPM] was also used as a derivatising agent but the internal standard used was N-Acetyl-L-cysteine. The extraction procedure used was liquid – liquid extraction. On the other hand, Jankowski A. *et al.* 1995, used p – bromophenacyl bromide as the derivatising agent and nitrazepam was used as the internal standard. The extraction procedure was liquid – liquid extraction and the limit of quantitation was 30 ng/ml.

2.2.2 Gas chromatography-mass spectrometry

Gas chromatography – mass spectrometry (GC-MS) has also been utilised for the analysis of captopril in plasma and widely used currently. The first method utilising GCMS was developed by Funkee *et al.* (1980) and involved the formation of a captopril – NEM derivative. This derivative was methylated and measured as the methyl ester. The lower limit of quantitation of unchanged captopril in blood was initially 16 ng/ml. Following this, Bathala *et al.* (1983) used N-ethylmaleimide (NEM) as the stabilising agent and captopril was further derivatised to hexafluoroisopropyl ester using hexafluoroisopropanol. The internal standard used was 4-ethoxyproline. The quantitation of captopril was done using GC with electron capture detection but for the confirmation of the structure a GC-MS was used. The lower limit of quantitation were 20 ng/ml for captopril in blood and 50 ng/ml for captopril and its disulphides metabolites in plasma.

Drummer *et al.* (1984) used GCMS to quantitate captopril and its sulphur-conjugated metabolites in plasma and urine. NEM was used as the stabilising agent. Hexafluoroisopropanol and perfluorobutyric anhydride were used to derivatise the captopril

to hexafluoroisopropyl ester agent. The extraction procedure was performed using liquid – liquid extraction. The limit of quantitation was 10 ng/ml.

A method by Ito *et al.* (1987) also used NEM as the stabilising agent. While S-benzylcaptopril was used as the internal standard. The derivatisation of captopril and internal standard were performed using pentafluorobenzylbromide (PFBB) whereas the captopril and internal standard was converted into bis-PFB derivative. The extraction procedure was performed using liquid-liquid extraction. The quantitation limit was 10 ng/ml.

In another method, Hans Jorg Leis *et al.* (1990) used gas chromatography – negative – ion chemical ionization mass spectrometry to quantitate the captopril, while [$^{18}O_2$]captopril was used as internal standard. The stabilising agent and derivatising agent was the same as that used by Ito *et al.* (1987). By using these method the limit of detection was 0.5 ng/ml.

Franklin M.E. *et al.* (1998) developed a rapid and sensitive method for the quantitation of captopril by GCMS. The stabilising and derivatising agent was the same as that used by Ito *et al.* (1987) but the internal standard used was thiosalysilic acid (TSA). Liquid-liquid extraction was used as the extraction process. Captopril and TSA were detected as bis-pentafluorobenzyl derivatives. The quantitation limit was 10 ng/ml.

2.3 METHOD DEVELOPMENT FOR CAPTOPRIL FOR THE PRESENT STUDY

2.3.1 Introduction

This method development for the assay of captopril was based on a previous methods (Franklin M.E. *et al.* 1998) but some modifications were made in order to improve the analytical procedure such as the extraction process and the chromatographic conditions. All

the chemical reagents used for the whole assay process were similar with the previous methods (Franklin M.E. *et al.* 1998).

Stock solutions of captopril were prepared in phosphate buffer (0.2M, pH 7.0) and stored in -4°C. Since captopril is unstable in blood and plasma *ex vivo* due to rapid formation of disulphides, N-ethylmaleimide was used to avoid the oxidation. A 0.5% (w/v) of powdered NEM was put into a phosphate buffer solution (0.2M, pH 7.0) when preparing the standard, and directly weighed into the blood tubes when collecting the samples. Thiosalysilic acid (TSA) was used as the internal standard as its contain the same functional group as with captopril. TSA were prepared in the same buffer solution without NEM and was spiked together with captopril in plasma for the standard or sample preparation and the same extraction process for the quantitation of captopril was then used.

Most of the previous methods used liquid – liquid extraction procedures for the extraction of captopril from biological fluid. In the present method, extraction was initially carried out by liquid-liquid extraction but subsequently solid phase extraction (SPE) was chosen as the better technique for this study. Solid phase extraction involves the separation process that takes place between a liquid and a solid phase. Even though liquid – liquid extraction can also give the same results compared to SPE, the liquid-liquid extraction procedure was longer and not suitable for analysing a large number of samples as with bioequivalence study. The extraction procedure was performed using solid phase extraction with ENV$^+$ column (Figure 2.2). The ENV$^+$ column was packed with a high capacity and highly crosslinked polystyrene based polymer. The columns capable of retaining analytes of a wide range of polarities.

To enhance the volatility and GC elution properties of the captopril-NEM adduct and the internal standard, the carboxylic acid group was esterified using pentafluorobenzyl bromide through a derivatisation procedure. Pentafluorobenzylation of captopril-NEM occurred in the presence of sodium hydroxide which act as a catalyst when placed in methanol. The proposed derivatisation method proved to be satisfactory with respect to simplicity, sensitivity and reliability for the determination of low levels of captopril in biological fluids.To confirm that captopril was fully derivatised, the derivatisation was performed with different amounts of derivatising agent and under different derivatisation conditions in term of time and temperature. After performing according to the above conditions, the temperature of 60°C and the period of 1 hour was found to be the optimum condition for the derivatisation of captopril. The amount of the derivatising agent used in this assay was the optimum amount whereby increasing the amount did not increase the peak area of the chromatogram, but decreasing the amount reduced the peak area of the chromatogram. It should be noted that pentafluorobenzylation of captopril-NEM provides solely the bis-pentafluorobenzyl (PFB) derivative without the artificial formation of diasterioisomers (Ito *et al.*1987) (Figure 2.3 and 2.4).

Gas chromatographic - mass spectrometric method was used for the separation and detection of captopril. The column used was capillary column BPX35MS [stationary phase; fused silica, BP1(non polar)], manufactured by SGE, Australia. Captopril and the internal standard were detected as pentafluorobenzyl derivatives. The mass spectra of bis-pentafluorobenzyl derivative of captopril and TSA is shown in Figure 2.5 . Selected ion monitoring (SIM) with selected ions of 333 and 514 for TSA and 294 and 396 for captopril were used for the quantitation of captopril by GCMS. The total ion chromatogram of captopril and TSA is shown in Figure 2.6.

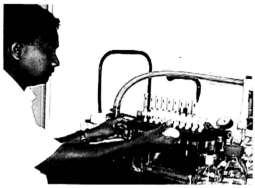


Figure 2.2 Extraction of plasma using solid phase extraction

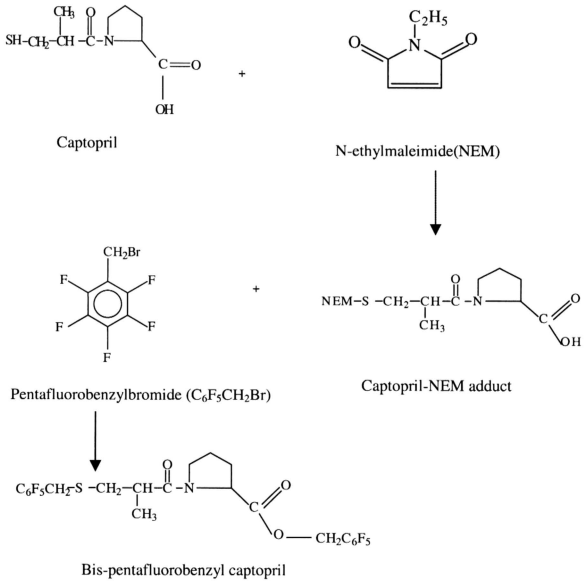
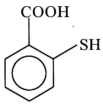
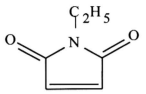


Figure 2.3 Derivatisation reaction of captopril

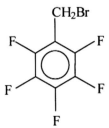


Thiosalysilic acid (TSA)

+

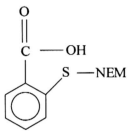


N-ethylmaleimide(NEM)

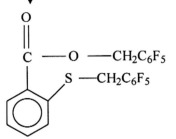


Pentafluorobenzylbromide($C_6F_5CH_2Br$)

+



TSA-NEM adduct



Bis-pentafluorobenzyl TSA

Figure 2.4 Derivatisation reaction of TSA

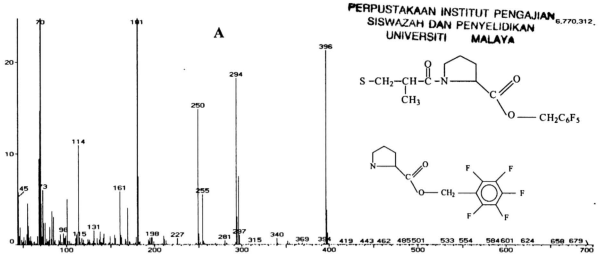


Figure 2.5 Mass spectra of (A) bis-pentafluorobenzyl captopril and (B) bis-pentafluorobenzyl TSA

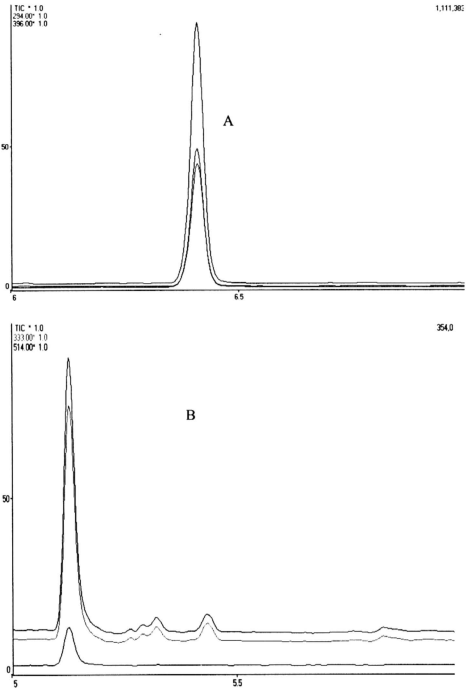


Figure 2.6 Total ion chromatogram for (A) captopril and (B) thiosalysilic acid

2.3.2 Extraction method of captopril

A schematic diagram of the extraction procedure is shown in Figure 2.7 and 2.8. A 1000 µl volume of serum sample containing captopril and internal standard was transferred using a plastic disposable transfer pipette, to the ENV$^+$ column placed at a solid phase extraction manifold. The plasma was allowed to flow under vacuum condition at 1 inHg until finished. 1 ml of phosphate buffer solution (0.01 M, pH 2.5) was added to the column twice under the same vacuum condition until finished. This step which is called the interference elution is to clean out any endogenous compounds, which are trapped at the packing material. Following that, the column was vacuumed at 10 in.Hg for five minutes. To elute the captopril out of the column, 3 ml of acetone:ethyl acetate (3:1) was added to the column and collected in a labelled glass tube. The contents of the glass tube was then evaporated to dryness under nitrogen stream at 40°C (this process usually takes about 15 minutes). The samples was then derivatised by the addition of 0.5 ml pentafluorobenzylbromide (2% in acetone) in the presence of 0.1 ml of 0.5 M sodium hydroxide in methanol. The tubes were then capped and incubated at 60°C for 1 hr. Excess derivatising agent was evaporated to dryness under nitrogen stream at 40°C (this process takes about 3 to 5 minutes). Finally the residue was reconstituted in 0.2 ml chloroform for analysis by GCMS. Detailed GCMS procedure and conditions will be explained in section 2.4.5.

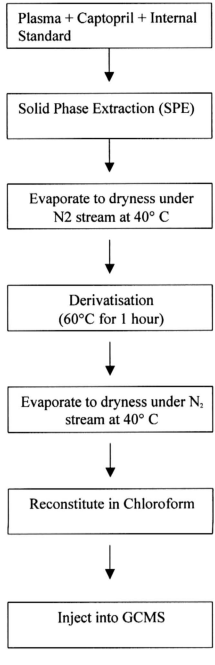


Figure 2.7 Schematic diagram for extraction procedure of captopril

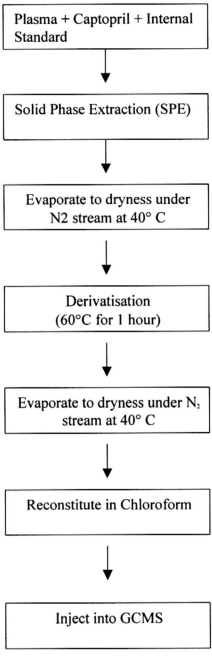


Figure 2.7 Schematic diagram for extraction procedure of captopril

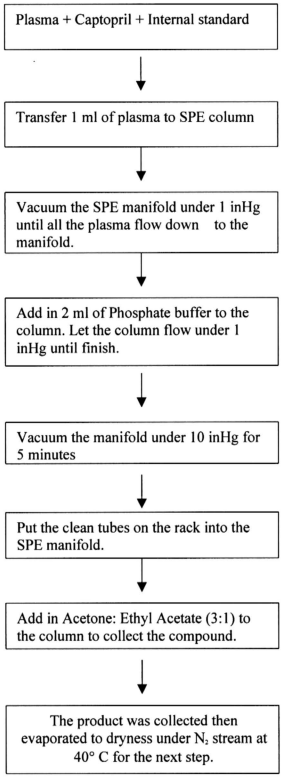


Figure 2.8 Schematic diagram for SPE

2.4 EXPERIMENTAL

2.4.1 Chemicals

Captopril (USP Reference standard) was obtained from Hubert Lendo, USA. Thiosalysilic acid (TSA), N- ethylmaleimide and pentafluorobenzylbromide was obtained from Acros Organics, Geel, Belgium. Potassium phosphate (for the preparation of buffer) was from Sigma Chemical, St Louise USA. Sodium hydroxide was purchased from BDH Chemicals while methanol, chloroform, ethyl acetate and acetone (HPLC grade) were purchased from Fisher Scientific.

2.4.2 Stock solutions

Stock solutions (1 mg/ml) of captopril and its appropriate dilutions were prepared in phosphate buffer (0.2 M, pH 7.0) and stored at 4° C. Powdered NEM was added to the phosphate buffer to make a final solution of 0.5 % NEM in the buffer to prevent oxidation of captopril. TSA was diluted in the same buffer solution without NEM.

2.4.3 Standard / control solutions

Standard and control solutions of captopril and the internal sandard were prepared as described in Appendix A1.1 (Laboratory working manual).

2.4.4 Sample preparation

The samples to be analysed were taken out from the freezer (-20°C) and thawed at room temperature. After complete thawing, a 1000µl samples were transferred to a clean glass tube. A fix volume of internal standard was spiked into each glass tube as described in Appendix A1.1(Laboratory working manual). The samples then underwent an extraction procedure (as

described in section 2.3.2) and then chromatographed under the chromatographic conditions as described in section 2.4.5 below.

2.4.5 Gas Chromatograph – Mass spectrometer

A Shimadzu QP 5000 gas chromatograph – mass spectrometer was used for the quantitation of captopril. The column used was a capillary column BPX35 (SGE, Australia) with 25 m (l), 0.25 mm (ID) and 0.22 μm thickness. The operating conditions for the Gas Chromatograph are as follows:

Oven temperature: Initial temperature 160°C

Ramp 35°C/min. to 330°C and hold for 5 min.

Injector temperature: 320°C

Interface temperature: 330°C

Carrier gas: Helium (Highly purified)

Carrier gas pressure: 69.0 kPa

Column flow: 0.9 ml/min

Injection mode: Split less

Injection volume: 1 μl

Mass spectrometer:

The detection was done using quadrupole detector with electron ionization mode of detection.

Data acquisition mode: Selected Ion Monitoring (SIM)

Ion monitored :Thiosalysilic acid: 333 and 514; Captopril: 294 and 396

2.4.6 Method Validation for the captopril assay

2.4.6.1 Specificity

Specificity is the ability of an analytical method to differentiate and quantitate the analyte in the presence of other constituents in the sample. It refers to the ability of a method to produce a response for a single analyte. Six samples of blank plasma and six samples of plasma spiked with captopril and internal standard were prepared and the same extraction procedure was carried out with the chromatographic condition as described before. The concentration of captopril and internal standard were 1 ng/ml (limit of quantitation) and 20 ng/ml respectively. The result obtained for the quantitation of captopril in plasma was compared with the result of blank plasma. The responses of interfering peaks at the retention time of captopril should be less than 20% of the response of an LOQ standard. Responses of interfering peaks at the retention time of the internal standard should be less than or equal to 5% of the response of the internal standard at the concentration used. For assays utilizing GCMS, specificity was further determined by comparing the peaks for both ion sets in selected ion monitoring (SIM) of captopril and internal standard from human plasma with the working standard solution. The identity of responses is considered positive if both ion sets appear at the same retention time.

2.4.6.2 Calibration curve

A calibration curve is a relationship between known concentrations of the analyte and the response of the instrumentation. The standard samples used for a calibration curve consists of known concentrations of captopril and TSA (internal standard). The calibration curve should be constructed using at least five to eight values from the expected range of concentrations. In this study the expected range is from 1 to 160 ng/ml covering six concentration points. Six standard solutions of captopril were prepared: 8000, 4000, 2000, 1000, 500 and 250 ng/ml as

explained in Appendix A1.1. A 20 µl volume of each standard solutions were spiked into six glass tubes containing 980 µl of blank plasma each to give a final concentration of 160, 80, 40, 20, 10 and 1 ng/ml. A 10 µl volume of 2000 ng/ml internal standard was spiked into each tube giving the final concentration of 20 ng /ml in all the samples. All the samples after that follow the same extraction procedure with the same chromatographic conditions. From the chromatograms, the peak area of captopril was divided by the peak area of the internal standard to obtain a value for the peak area ratio. The calibration curve plotted was of the concentrations of captopril against the peak area ratio. The calibration curve must be linear with R^2 value of greater than 0.95.

The limit of quantitation (LOQ) for captopril in this assay was 1 ng/ml, this being the lowest concentration of captopril that can be quantitated with the variability of 7.9%. The CV % at this concentration should be within 20 %. The peak at this concentration is five times higher than the interference peak at the retention time of captopril.

2.4.6.3 Precision, accuracy and recovery

Precision

Precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. Precision was measured using a minimum of six determinations per concentration. Three concentrations were chosen, these concentrations being within the range of the calibration curve. The three concentrations of captopril identified as being low, medium and high were 1.5, 75 and 150 ng/ml. Precision is further subdivided into within-day and between-day. For the within-day precision, each concentration was injected six times on the same day and the ratio of captopril/internal standard was measured for every sample

injected. For the between-day precision, the samples were prepared everyday for five days continuously i.e. 5 working days and injected once each day, at the same time. The coefficient of variation (C.V.) was measured for every concentration and is measured as standard deviation (SD)/mean. The precision around the mean value should not exceed 15% of C.V. for low, medium and high concentrations and should not exceed 20% for the LOQ.

Accuracy

The accuracy of an analytical method describes the closeness of test results obtained by the method to the true value of the analyte. The values are back-predicted from the equation y = A1X + A0 which is obtained from the calibration curve. The mean value for low , medium and high concentration should be within 15% of the actual value and not more than 20% at the LOQ

Recovery

The recovery of an analyte is the result obtained from an amount of the analyte added to and extracted out from the biological matrix, compared to the result obtained from the pure standard. Six extracted and six non-extracted samples of captopril were prepared for low (1.5 ng/ml), medium (75 ng/ml) and high concentrations (150 ng/ml). From the chromatogram, the peak area of the extracted samples were divided by the area of non- extracted.

$$\text{Recovery} = \frac{\text{Area of extracted samples}}{\text{Area of non-extracted samples}} \times 100\%$$

2.4.6.4 Stability

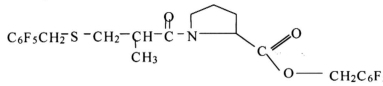
Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. The type of stability test performed in this study is long-term stability test. For the stability determinations, a set of

standard samples prepared from freshly-made stock solution were spiked in captopril and TSA-free plasma. The standards were kept in cryo vials in the same condition as the samples to be analysed.

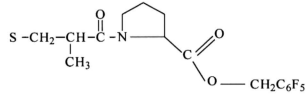
Two aliquots at low (1.5 ng/ml) and high (150 ng/ml) concentrations of captopril were stored at -20°C. The aliquots were analysed before freezing, and after 2 days, 7 days, 1 month, 2 months and 5 months after freezing. Long term stability was performed only for the maximum period of sample storage from the time the samples were collected until analysis is completed.

2.5 RESULT AND DISCUSSION

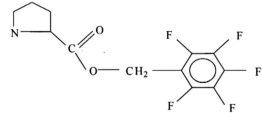
From the mass spectra of bis-pentafluorobenzyl captopril, M^+ for captopril derivative at m/z 577 was not observed. The observed signals for captopril were m/z 396 and 294 resulting from the respective losses of one PFB moiety and of the side chain following cleavage of the C-N bond (Figure 2.9). For bis-pentafluorobenzyl TSA an M^+ of 514 was observed, as was a signal at m/z 333 representing loss of a PFB moiety (Figure 2.10). The signal at m/z 181 in both mass spectra resulted from free pentafluorobenzyl groups (Figure 2.11).



M^+:m/z 577

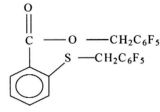


m/z 396

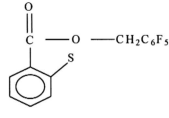


m/z 294

Figure 2.9 Chemical structure of fragment ions for bis-pentafluorobenzyl captopril

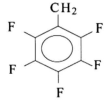


M^+ : m/z 514



m/z 333

Figure 2.10 Chemical structure of fragment ion for bis-pentafluorobenzyl TSA



m/z 181

Figure 2.11 Chemical structure of free pentafluorobenzyl group

Specificity

The use of selected ion monitoring (SIM) resulted in high signal to noise ratios for both compounds as evident in the total ion chromatogram of a plasma extract shown in Figure 2.6. Using the described chromatographic condition, the retention times for captopril and TSA during the assay development period were 6.3 – 6.5 min and 5.1 – 5.3 min respectively. Both ions for captopril (294 and 396) and internal standard (333 and 514) from plasma appeared at the same retention time as that of working standard solution.

The responses of interfering peaks at the retention time of captopril was 11% and that of the internal standard (TSA) was 0.3% (Figure 2.12).

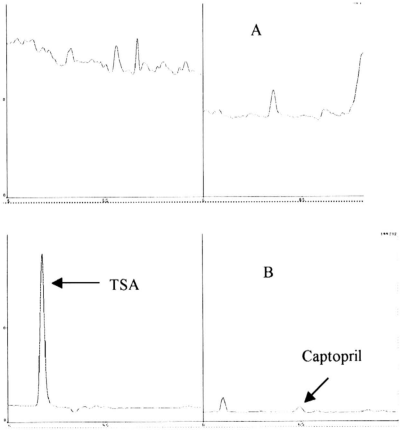


Figure 2.12 GCMS chromatograms of plasma extracts: (A) blank plasma, (B) plasma spiked with 20 ng/ml IS (TSA) and 1 ng/ml captopril (LOQ)

Calibration curve

Linear model calibration was used, in which the independent variable (X), is the concentration of captopril and the dependant variable (Y) is detector response (peak area ratio) . The standard curve based on peak area ratio of captopril and internal standard was linear from 1 to 160 ng/ml giving a linear regression (r^2) value of 0.9959 with y – intercepts not significantly different from zero (figure 2.13).

Concentration (X) (ng/ml)	Peak area ratio (Y)					
	Y1	Y2	Y3	Y4	Y5	Y6
160	5.47	6.64	5.83	5.46	6.05	5.57
80	3.05	3.77	3.26	2.87	2.7	3.37
40	1.59	1.61	1.62	1.57	1.6	1.62
20	0.8	0.91	1.09	0.87	0.8	0.92
10	0.44	0.41	0.33	0.45	0.4	0.45
1	0.04	0.09	0.05	0.04	0.07	0.07

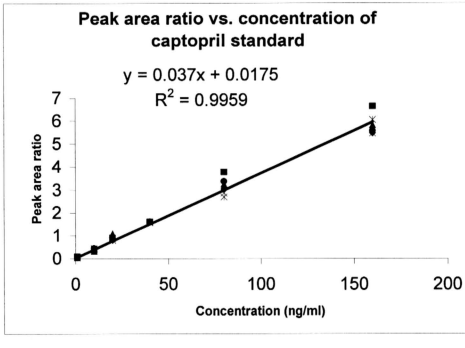


Figure 2.13 Standard curve for captopril from 1 to 160 ng/ml in plasma

Limit of Quantitation (LOQ)

The limit of quantitation (LOQ), was found to be 1 ng/ml during throughout the period of assay for the pharmacokinetic studies.

Precision, accuracy and recovery

Mean coefficients of variation for within- and between-day precision value were 2.9% (Table 2.1) and 7.1% (Table 2.2) respectively for low, medium and high concentration. For the LOQ the CV was 7.9% for within-day precision (Table 2.1) and 10% for between-day precision (Table 2.2). The mean accuracy value for low, medium and high concentration was 6.4% and at LOQ was 7.6% (Table 2.3). The mean recoveries for low, medium and high concentrations of captopril was 102% (Table 2.4), and for internal standard was 99% (Table 2.5). For the medium concentration of 75 ng/ml one aliquot shows the recovery of 141 % which was quite high compared to the others. This may be due to exogenous contamination from the SPE cartridge or from the glasswares used. It could also be due to baseline disturbances.

Table 2.1 Within-day precision for the assay of captopril in plasma using TSA as the internal standard

	High concentration 150 ng/ml	Medium concentration 75 ng/ml	Low concentration 1.5 ng/ml	LOQ 1 ng/ml
	5.11	2.82	0.047	0.042
	5.04	2.83	0.045	0.039
	5.14	2.82	0.042	0.035
	5.03	2.89	0.042	0.040
	5.05	2.73	0.042	0.038
	5.14	2.79	0.040	0.045
Mean	5.09	2.81	0.04	0.04
SD	0.05	0.05	0.003	0.003
CV%	1.0	1.9	5.9	7.9

Table 2.2 Between-day precision for the assay of captopril in plasma using TSA as the internal standard

	High concentration 150 ng/ml	Medium concentration 75 ng/ml	Low concentration 1.5 ng/ml	LOQ 1 ng/ml
Day 1	5.03	2.89	0.042	0.040
Day 2	4.51	2.88	0.042	0.047
Day 3	4.67	2.48	0.045	0.050
Day 4	4.92	2.32	0.050	0.040
Day 5	4.90	2.72	0.046	0.050
Mean	4.81	2.66	0.045	0.045
SD	0.21	0.25	0.003	0.005
CV%	4.4	9.5	7.4	10.0

Table 2.3 Accuracy for assay of captopril in plasma using TSA as internal standard.

Accuracy Assay

Concentration Level	Expected (ng/ml)	Predicted (ng/ml)	% different(inaccuracy) (%)
High concentration (150 ng/ml)	150	140	6.7
	150	154	2.7
	150	148	1.3
	150	137	8.7
	150	136	9.3
		Mean	**5.7**
		SD	3.2
Medium concentration (75 ng/ml)	75	81	8.0
	75	80	6.7
	75	69	8.0
	75	65	13.0
	75	76	1.3
		Mean	**7.4**
		SD	3.7
Low concentration (1.5 ng/ml)	1.5	1.47	2.0
	1.5	1.55	3.3
	1.5	1.42	5.3
	1.5	1.30	13.3
	1.5	1.60	6.7
		Mean	**6.1**
		SD	3.9
Limit of quantitation (LOQ) (1 ng/ml)	1.0	1.03	3
	1.0	1.00	0
	1.0	1.11	11
	1.0	1.11	11
	1.0	1.13	13
		Mean	**7.6**
		SD	5.1

Table 2.4 Percentage recovery at 3 concentrations of captopril

Concentration Level	Non extracted (area)	Plasma extracted (area)	% recovery (%)
High concentration (150 ng/ml)	375201	350730	93
	548923	541264	99
	501391	502634	100
	494878	492803	100
	527379	517212	98
		Mean	**98**
		SD	2.6
Medium concentration (75 ng/ml)	264683	264931	100
	407106	378269	93
	278972	278610	100
	222942	202679	91
	199878	282814	141
		Mean	**105**
		SD	18.4
Low concentration (1.5 ng/ml)	5099	5424	106
	7495	7873	105
	9300	8909	96
	8252	8904	108
		Mean	**104**
		SD	4.6

Table 2.5 Percentage Recovery of the Internal Standard

Concentration level	Non extracted (area)	Plasma extracted (area)	% recovery (%)
20 ng/ml	102473	123839	121
	194713	159637	82
	121892	96746	79
	100469	75270	75
	81700	110852	136
		Mean	**98.6**
		SD	25.0

Stability

Captopril and TSA were found to be stable in plasma when stored frozen at -20°C for a period of at least five months. The stability test for low concentration gave the inaccuracy value of 1.7% with 95% confidence limit from –13.6 to 10.2%. For high concentrations, inaccuracy is 0.25% with 95% confidence limit from –5.6 to 6.1% (Table 2.6).

Table 2.6 Long-term stability test of captopril at concentrations in the lower and higher part of the concentration range determined from mean of two readings.

Time	Concentration (ng/ml)			
	Nominal conc.:1.5		Nominal conc.:150	
	Conc. Measured	inaccuracy %	Conc. Measured	inaccuracy %
Day 0	1.49	-0.7	144.93	-3.38
Day 2	1.495	0.33	154.77	3.18
Day 7	1.51	0.7	137.66	-8.23
1 month	1.53	2	148.21	-1.19
2 month	1.15	23.3	160.65	7.1
5 month	1.66	10.7	156.06	4.04
Mean,		-1.7		0.25
95% confidence limit		-13.6 to 10.2%		-5.6 to 6.1%

2.6 CONCLUSION

In conclusion, this assay provided adequate sensitivity, specificity, linearity, recovery, accuracy and precision for the determination of captopril in plasma. The Limit of Detection (LOD) for this assay was 1 ng/ml. The comparison of some validated parameters for this study with the published method were tabulated in the Table 2.7 below. The solid phase extraction procedure that was developed was relatively rapid when compared to the published methods utilizing liquid-liquid extraction. The liquid-liquid extraction method developed by Franklin M.E. (1998) took about 25 minutes to complete the extraction of a set of 14 samples, but using our SPE method, the extraction for fourteen samples took about 15 minutes. Hence, this method takes a shorter period to analyse a large number of samples as in the case with bioequivalence studies.

Table 2.7 Published data of the values of accuracy, precision and LOD.

	Inaccuracy (%)	Precision (CV %)		LOD (ng/ml)
		Intraday	Interday	
Franklin M.E. *et al.* (1998)	5.93	3.73	3.47	-
Hans Jorg Leis *et al.* (1990)	-	2.13	1.15	0.5
Drummer *et al.* (1983)	-	3.65	8.90	1
Present study, Zamri *et al.*(2000)	6.40	2.93	7.10	1

- = No result presented