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# Captopril Determination in Dried Blood Spot Samples with LC-MS and LC-HRMS: A Potential Method for Neonate Pharmacokinetic Studies

**Graham Lawson[1], Hussain Mulla[2] and Sangeeta Tanna[1]***

[1]Leicester School of Pharmacy, Faculty of Health and Life Sciences, De Montfort University, Leicester, LE1 9BH, UK
[2]Centre for Therapeutic Drug Evaluation of Drugs in Children, University Hospitals of Leicester NHS Trust, Glenfield Hospital, Groby Road, Leicester, LE3 9QP, UK

## Abstract

The use of blood spot collection cards was investigated as a means of obtaining small volume samples for the analysis of therapeutic drugs for the purpose of neonatal pharmacokinetic studies. We describe the development of two micro-analytical methods for the determination of captopril extracted from dried blood spots (DBS). Firstly a liquid chromatography ion-trap mass spectrometry method with selected ion monitoring (LC-MS(SIM)) of target ion at *m/z* 218.0 was developed to determine captopril levels in 8mm discs punched from each DBS. This was compared in terms of specificity and sensitivity to a simple accurate mass liquid chromatography high resolution TOF mass spectrometry (LC-HRMS) method in which MS detection was carried out in electrospray positive ion mode for target ions at *m/z* 218.0845 for captopril and 377.2084 for the IS. Dithiothreitol was used both to pre-treat the sampling cards and as part of the extraction medium in order to stabilise the DBS extracted captopril. Drug extraction efficiency from spiked blood spots was demonstrated to be 90 ± 10% and the drug was stable in DBS for at least 12 weeks. Validation of both micro-analytical methods showed good precision and accuracy and the LC-HRMS method was linear within the tested calibration range 10-400 ng/ml for captopril and had improved sensitivity and specificity compared to the LC-MS(SIM) method. This method was applied to blood spots on sampling card from a neonate patient previously administered 1 mg/kg captopril orally. The amount of captopril in the DBS was 88 ng/ml. Requiring only a micro volume (30 µl) blood sample for analysis, the developed DBS based micro-analytical method has the potential to facilitate pharmacokinetic studies of captopril in children.

**Keywords:** Dried blood spot (DBS); Captopril, LC-MS; Guthrie card; LC-HRMS; Accurate mass; Pediatrics

**Abbreviations:** PK: Pharmacokinetics; DBS: Dried Blood Spot; LC-HRMS: Liquid Chromatography-High Resolution Mass Spectrometry

## Introduction

Captopril is an angiotensin-converting enzyme (ACE) inhibitor widely used in pediatric cardiology for the treatment of congestive heart failure. Despite its widespread administration, pharmacokinetic (PK) data about captopril is limited in young infants and no data are available in premature infants [1-3]. To account for the variability in absorption, distribution, metabolism and elimination of drugs in the pediatric population, PK data for captopril are needed to develop an age-appropriate dosing regimen. No such studies are normally performed in children and instead doses are extrapolated from adults. Neonates may be more susceptible to adverse effects associated with ACE inhibitors, including acute renal failure and hypotension and therefore conservative initial doses of captopril such as 0.1-0.3 mg/kg/dose for full-term neonates and 0.01-0.05 mg/kg/dose for premature neonates are initially recommended for oral administration [2,4]. Indeed, there is a lack of pharmaceutical information and appropriate formulations to support the administration of many medicines to children leading to possible unwanted side effects or under dosing with sub optimal drug efficacy. There has been an emergence of new pediatric regulations around the world to stimulate research in this area and captopril has been identified as one of the drugs for which pediatric data are required. For instance, the European Medicines Agency's (EMEA) priority list of off-patent medicines includes captopril with the specific requirements for data on pharmacokinetics, efficacy and safety and age-appropriate formulations [5]. One of the major reasons for the lack of appropriate PK information in the pediatric population is the practicality of performing such studies in children and in particular in preterm and term neonates. Repeated, large volume (5-10 ml) blood sampling in children with a small circulating volume (<250 ml) is unethical, impractical and also unacceptable to parents. To acquire this much needed PK information and to develop age-appropriate drug dosing regimens it is necessary to determine the concentrations of drug in blood plasma, over a period of time after administration. In this preliminary investigation the feasibility of determining captopril levels in dried blood spots (DBS) on Whatman 903 sampling cards was investigated as a means for measuring captopril levels from neonatal patients.

To determine captopril or its metabolites in blood or plasma, several analytical methods have been described including HPLC with UV and fluorescence detection [6-10], liquid chromatography-mass spectrometry (LC-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS) [11-14], gas chromatography-mass spectrometry (GC-MS) [15,16], radioimmunoassay (RIA) [6] and enzyme immunoassay [6] with the HPLC methods most widely investigated. Although the analytical literature seems abundant for the determination of captopril in human blood or plasma, a method using standard analytical equipment providing a sensitive and simple micro-analytical method for determining captopril from a small blood

***Corresponding author:** Sangeeta Tanna, Leicester School of Pharmacy, Faculty of Health and Life Sciences, De Montfort University, The Gateway, Leicester LE1 9BH, UK, Tel: +44 (0)116 2078274; Fax: +44 (0)116 2577287; E-mail: stanna@dmu.ac.uk

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volume has not been addressed and was therefore investigated in the present study.

Captopril is a thiol containing a sulphydryl group (Figure 1) and readily forms disulphides with endogenous thiol-containing compounds (cysteine, glutathione), as well as self dimerisation. It also binds to albumin and other proteins. For captopril only the free thiol is pharmacologically active. *In vivo*, the formation of the inactive disulphides is reversible because intracellularly, these disulphides are reduced to the pharmacologically active free thiol and as such they can act as a reservoir for free captopril and contribute to a longer duration of action than predicted by the blood concentrations of free captopril. Captopril's propensity to rapidly undergo oxidation to pharmacologically inactive disulphide dimers can occur *in vitro* during sample preparation and analysis and indeed in this study captopril was found to dimerise virtually completely within a matter of hours after sample preparation. Therefore the measurement of free or unchanged captopril concentration needs to be preceded by the addition of the stabilizer, 1,4-dithiothreitol (DTT), to biological or *in vitro* samples in order to prevent disulphide formation [12,14]. In a routine clinical setting it would not be possible to add a chemical stabilizer to the blood sample before it is sampled on a sampling card. For this reason the use of sampling card pre-treated with the chemical stabilizer was investigated in this study in addition to investigating the effect of adding the chemical stabilizer to the extraction solvent.

The analysis of dried blood spots (DBS) using tandem mass spectrometry or MS/MS techniques is an established technique used routinely for newborn screening purposes where a low volume blood sample is obtained via a simple heelstick prick procedure [17-19]. The ease of sample storage and transmission between sites has led to a surge in the use of this methodology for various applications including therapeutic drug monitoring, toxicokinetic (TK) and pediatric pharmacokinetic (PK) studies [20-31]. High Resolution Mass Spectrometry (HRMS) offers an alternative route to the compound specificity provided by the MS/MS instrument [19].

Instead of the multiple reaction monitoring (MRM) approach the HRMS instrument intrinsically measures the *m/z* values of all the ions to an accuracy of ca 1ppm in mass and thus uniquely identifies the target species based on its accurate mass. Both the reflectron TOF and the Orbitrap provide this HRMS capability with the added advantage that since the TOF is a non-scanning instrument a total ion chromatogram (TIC) is produced for every analysis which can be stored and reprocessed for additional data subsequently. There is increasing demand to rapidly identify and quantify therapeutic drugs in DBS samples and the use of high-resolution, accurate mass spectrometry detection coupled to liquid chromatography separation has the potential to offer improved selectivity and resolution [19,32]. Here we demonstrate the benefit of the accurate mass LC-HRMS TOF based system in the study of the analytically challenging compound captopril.

In the present study we report on a simple optimized sample preparation technique for the quantitative and reproducible recovery of captopril from blood spots collected on sampling card. A liquid-chromatography-mass spectrometry with selected ion monitoring (LC-MS(SIM)) method for subsequent quantification is described and compared with a simple accurate mass liquid-chromatography high resolution TOF mass spectrometry (LC-HRMS) method for the quantification of captopril in DBS.

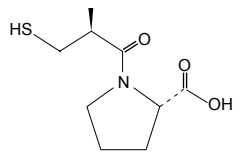


**Figure 1:** Structure of captopril.

## Materials and Methods

### Chemicals and materials

Acetonitrile, methanol, isopropanol and water, LC-MS grade, were purchased from Fisher Scientific (Loughborough, UK). Formic acid (≥ 98%), specimen collection filter paper type 903, autosampler vials with 0.3ml inserts with caps, microcentrifuge tubes (1.5ml), volumetric pipettes, pipette tips and polyethylene bags were also obtained from Fisher Scientific (Loughborough, UK). Captopril, enalapril maleate and 1,4 dithiothreitol were from Sigma-Aldrich (Poole, UK). Polyethylene bags for storage of blood spot cards were obtained from Richardsons of Leicester (Leicester, UK). An 8mm diameter punch was obtained from Maun Industries Ltd. (Nottingham, UK). Lithium heparin coated blood collection tubes were purchased from International Scientific Supplies Ltd. (Bradford, UK). Blank human blood was ethically obtained from healthy adult volunteers following informed consent.

### Preparation of captopril standard stock and working solutions

A standard stock solution of captopril was prepared in methanol:water (60:40, v/v) at a concentration of 10µg/ml. Captopril working solutions for spiked blood spots were prepared in methanol:water (60:40, v/v) by appropriate dilution of the stock solution at the following concentrations: 4000; 2000; 1000; 500; 200; 100 ng/ml. All working solutions were prepared on the day of analysis. Spiked blood standards were prepared by spiking different samples of fresh blood with 100 µl of one of the above mentioned working solutions to yield final blood captopril concentrations of 400; 200; 100; 50; 20; 10 ng/ml. A zero (blank) captopril blood sample was prepared by spiking with 100 µl of methanol:water (60:40, v/v).

Addition of an internal standard (IS) solution (enalapril maleate) was investigated. This IS was selected as its use had been documented previously for the LC-MS analysis of captopril [12, 14]. A stock solution of IS was prepared by dissolving in methanol:water (60:40, v/v) to produce a 10 µg/ml concentration. The stock solution was further diluted with methanol:water (60:40, v/v) to produce an extraction solvent containing 100 ng/ml IS.

### Pre-treatment of Whatman 903 sampling paper with 1,4 dithiothreitol (DTT)

The Whatman 903 sampling paper was cut into pieces of 5 cm x 5 cm size. Each piece was placed separately in a plastic dish and 3500 µl of 200 mM DTT solution added ensuring that the sampling paper was completely soaked. These were left for 2 hours at room temperature after which the DTT solution was decanted and the sampling paper pieces were left to dry at room temperature. After the DTT pre-treated paper was dry it was stored in sealable plastic bags until use.

### Preparation of calibration standards and validation samples

Thirty microlitres of captopril calibration standards in blood across the concentration range 400, 200, 100, 50, 20, 10 and 0 ng/ml were spotted directly onto the DTT pre-treated sampling paper type 903 using a volumetric pipette. The spotted samples were allowed to air dry

overnight at room temperature prior to process. A 30 µl volume applied onto the sampling paper gave a spot of size of ~9.5 mm in diameter.

### Dried blood spot analyte solvent extraction

An 8 mm disc (approximately 20 µl of blood) was punched from the centre of the DBS sample and transferred to a 1.5 ml microcentrifuge tube. A 200 µl volume of extraction solvent consisting of methanol/water (60:40, v/v) containing 10% v/v 200 mM DTT plus 100 ng/ml of IS was added to this. Sample tubes were then vortexed for 1 min and sonicated for 30 min. Thereafter they were centrifuged for 10 min at 13200 x g and each extract was transferred to an auto sampler vial for analysis by LC-iontrap MS (SIM) or LC-HRMS.

### LC-iontrap (SIM) analyses

The instrument used was an Agilent 1100 LC/MSD Ion Trap mass spectrometer (Agilent Technologies). Captopril was analysed on a Zorbax Eclipse Plus C8 column (Agilent Technologies, Chesire UK, 150 mm x 3.0 mm i.d., 3.5 µm particle pore size) with a C8 Security Guard column 4mm x 3mm (Phenomenex, Macclesfield, UK). The analytical column was maintained at 35°C. The sample injection volume was 25 µl. The mobile phase consisted of acetonitrile containing 0.1% v/v formic acid (eluent A), water containing 0.1% v/v formic acid (eluent B) and isopropanol (eluent C). This was delivered at 0.5 ml/min with gradient elution. The gradient elution programme maintained 2% C throughout the programme. It started with 38% A and held at this composition until 1.3 min. It was then increased linearly to 98% A in 2.2 min and held until 2.6 min and then returned to the initial composition in 0.5 min. The gradient elution programme was then held for 3.5 min to re-equilibrate the column prior to the next injection.

Operation of the mass spectrometer was in electrospray positive ion mode and set to carry out selected ion monitoring (SIM) for the protonated molecular ion $[MH]^+$ of captopril at *m/z* 218.0. The MS source and chamber conditions were optimized as follows: Capillary: 32 nA; Nebuliser: 50.0 psi; Dry Gas: 10.0 l/min; Dry Temperature: 350°C.

LC-MS data were acquired using the 6300 Series Ion Trap LC/MS software version 6.1 and processed (integrated) using Quant Analysis for 6300 Series Ion Trap LC/MS software version 1.8.

### LC-high resolution MS analyses

The chromatographic system was different from the above. This consisted of an Agilent 1290 LC which was coupled to an Agilent 6530 TOF mass spectrometer. The target drug was analysed on a Zorbax Eclipse C18 column (Agilent Technologies, Chesire UK, 100 mm x 2.1 mm i.d., 3.5 µm particle pore size) which was preceded by a 0.3 µm inline filter. The column oven temperature was set to 40°C. The sample injection volume was 20 µl. The mobile phase consisted of water containing 0.1% v/v formic acid (eluent A) and methanol containing 0.1% v/v formic acid (eluent B) and was delivered at 0.5 ml/min with gradient elution. The mobile phase was initiated at 10% B and maintained for 1.0 min before increasing to 100% B by 4.0 min and held until 5.0 min before returning to 10% B. The gradient elution programme was then held for 3.0 min to re-equilibrate the column prior to the next injection.

Operation of the mass spectrometer was in electrospray positive ion mode. The MS source and chamber conditions were optimised as follows: Fragmentor voltage: 150V; Skimmer: 65V; Gas Temperature: 300°C; Dry Gas: 5 l/min; Nebuliser: 50.0 psig; Sheath Gas Temperature: 350°C; Sheath Gas Flow: 12 l/min. Scan Range: 100-1000 *m/z*; Scan Rate: 1 Hz. Reference Masses: 121.0508 *m/z* and 922.0987 *m/z*.

Mass Hunter Workstation Software (Agilent Technologies) was used to operate the system and acquire all data and the data processed using Qualitative Analysis B.02.00 and Quantitative Analysis B.01.04 software (Agilent Technologies). External calibration of the TOF mass spectrometer was performed daily before starting the analysis.

### Validation

**Selectivity:** To demonstrate the selectivity of the two micro-analytical methods blank blood spots and captopril spiked blood spots were analysed using both methods and subsequently processed. Representative extracted ion chromatograms at *m/z* 218.0 were generated from the LC-MS (SIM) data. Representative extracted ion chromatograms from the LC-HRMS data for protonated captopril using mass tolerance filters at (a) *m/z* 218.0 ± 0.5 (i.e. ± 500 ppm) (b) *m/z* 218.0 ± 0.1 (i.e. ± 100 ppm) and (c) *m/z* 218.084 ± 0.001 (i.e. ± 1 ppm) were generated to show that other components that could be present in the sample matrix or from the sampling paper were removed from the target analyte.

**Linearity and sensitivity**: A standard curve was prepared for captopril spiked blood spots for five replicates of each standard using both micro-analytical methods. For calculation of the standard curve from the LC-MS (SIM) data, a plot of peak areas against concentrations was used whereas for the LC-HRMS data a calibration plot of analyte/IS peak area ratio against nominal captopril concentration was produced and an equally weighted linear regression applied.

The quantification and detection limits of captopril in the DBS extracts were determined. The limit of detection (LOD) was based a signal-to-noise ratio of 3 in a 0.5 min window around the elution time of the analyte in an analytical blank and the limit of quantification (LOQ) was based a signal-to-noise ratio of 10.

**Accuracy and precision:** Intra-day precision and accuracy for both analytical methods were calculated from the 8 mm fixed size DBS disc data obtained for six replicates from a 30 µl blood spot volume. Three whole blood concentrations were chosen from the high, medium and low captopril concentration range. These were 400 ng/ml, 100 ng/ml and 50 ng/ml. Precision was determined by calculating the relative standard deviation (RSD) for intraday replicates. Accuracy was assessed by calculating the percent deviation from the theoretical concentration and was expressed as the % bias. A precision (RSD) and accuracy (% bias) of less than 15% were considered satisfactory.

**Matrix-effects:** Replicate (n=5) samples of analyte spiked in extracted blank whole blood to 50, 100 and 400 ng/ml were produced to evaluate suppression or enhancement of the detector response due to constituents within dried blood spots. The samples prepared were compared to standards of the same concentration spiked into methanol: water (60:40, v/v). The matrix effect was calculated by (B/A-1) × 100. Where A represents captopril spiked into pure solvent and B represents captopril spiked into extracted blank whole blood. This was investigated for both analytical methods.

**Recovery of captopril from dried blood spot:** The extraction efficiency or the recovery of captopril from the spiked dried blood spots was determined by comparing peak area ratios from DBS with those obtained by direct injection of captopril in the extraction solvent at the

same concentration as the spiked blood spots. Recovery was calculated using the following equation: % recovery = (peak area of dried blood spot extract/peak area of standard captopril solution) × 100. The recovery of captopril was determined at three concentrations (50, 100 and 400 ng/ml). All determinations were repeated six times.

**Blood spot size:** The need for quantitative reproducibility raises the question of the physical size of the sample taken from the DBS for subsequent extraction. The approach adopted was to punch a disk from within the DBS with the logical compromise of the largest diameter disks consistent with a typical DBS from normal clinical practice. The purpose of this series of experiments was to show that the results obtained were independent of the size of the blood spot collected. The analysis of a fixed sample size disc should produce extract data which is directly related to the concentration of captopril in the original blood sample, assuming the spot has spread uniformly throughout the paper.

To assess the effect of blood volume collected on captopril quantification 20, 30 and 40 µl blood spots at 50, 100 and 400 ng/ml were prepared in replicate (n=6). Eight millimetre discs (approximately 20 µl of blood) were sampled from the centre of the 20, 30 and 40 µl volume DBS of 50, 100 and 400 ng/ml captopril. These spots were of different diameters corresponding to the volume of sample deposited. The captopril was extracted as described above and analysed as specified above. The concentrations of extracts were determined using the linear regression equation generated from a calibration produced from 30 µl dried blood spots.

**Stability of captopril dried blood spots:** The stability of dried blood spot samples during storage for 12 weeks at room temperature was determined by analysing blood spots spiked with captopril at the level of 50 ng/ml, 100 ng/ml and 400 ng/ml (n=6). The aim of this was to investigate if batch wise preparation and subsequent storage would be possible. Eight millimetre discs (approximately 20 µl of blood) were punched from the centre of the 30 µl volume DBS of 50, 100 and 400 ng/ml captopril. The captopril was extracted as described above and analysed as stated above.

**Application of the LC-MS method:** The clinical applicability of the developed LC-MS based micro-analytical method was evaluated using 2 samples collected from a neonatal patient. Two single drops of blood, from a neonatal patient admitted in the pediatric cardiology ward at UHL, Glenfield Hospital Leicester, were collected on DTT pre-treated 903 sampling card. This patient had been administered 1 mg/kg captopril orally 2 hours prior to sample collection. The drops of blood were taken by heel-prick with a lancet. Dried in the same conditions as standards and controls, the collection cards were placed in a resealable plastic bag and stored at room temperature. The actual method of application to the sampling card was not recorded.

Two 8mm diameter circles were punched from each dried blood spot and the punched disks cut into six pieces and placed in a labelled 1.5 ml snap cap vial. The samples were extracted and then analysed as described above.

Ethical approval for this research project was obtained from the NHS-NRES and De Montfort University Ethics Committees.

## Results and Discussions

### LC- MS SIM and HRMS-method development

The inclusion of the internal standard (IS) (enalapril maleate) as part of the extraction solvent was found to worsen the captopril signal in the LC-MS(SIM) analyses and was therefore precluded from further use in this method of analysis.

Multiple reaction monitoring (MRM) is used in MS/MS analyses to provide enhanced specificity and the iontrap mass spectrometer was used in this mode for the protonated molecular ion $[MH]^+$ of captopril at *m/z* 218.0 and *m/z* 116.0 for the product ion. The product ion, *m/z* 116.0, for captopril was extracted for quantification. The overall sensitivity of this method was found to be lower than that needed to monitor therapeutic levels of captopril and this instrument was used in the SIM detection mode.

### Selectivity

Under the LC-MS (SIM) assay conditions described above, captopril, from standard solutions, was well resolved with a retention time of 1.9 min. However, this method lacked selectivity for endogenous compounds as there appeared to be a small interfering peak in the blank DBS extracted ion chromatogram when compared with the captopril spiked sample (Figure 2). Analysis of blank sampling paper extracts under identical conditions confirmed that this small interfering signal resulted from the from the specimen collection paper (data not shown). Figure 2, which compares the extracted ion chromatograms for blank DBS and captopril spiked DBS, demonstrates the contribution of this endogenous peak.

Using the accurate mass liquid-chromatography high resolution TOF mass spectrometry (LC-HRMS) method the accurate masses of the $[MH]^+$ ions for captopril and the IS were determined from the analysis of reference solutions in methanol/ water (60:40, v/v). The results obtained were: captopril *m/z* 218.0845 and for the IS *m/z* 377.2084.

The benefits of the LC-HRMS method for the separation of captopril from dried blood spots is shown in (Figure 3) where the increasing selectivity towards captopril, as the mass window decreases to 1 ppm, is clearly shown. The significant contribution to the expected signal from the paper shown (black) in the traces for the 500 and 100 ppm mass windows is eliminated by the 1 ppm mass window. Indeed the extracted ion chromatograms showed there were no interferences at the same retention time of captopril (3.07 min) when the mass spectrometer extracted data with less than a 50 ppm mass window. (Figure 4) shows the data obtained (TIC, EIC *m/z* 218.0845 and EIC

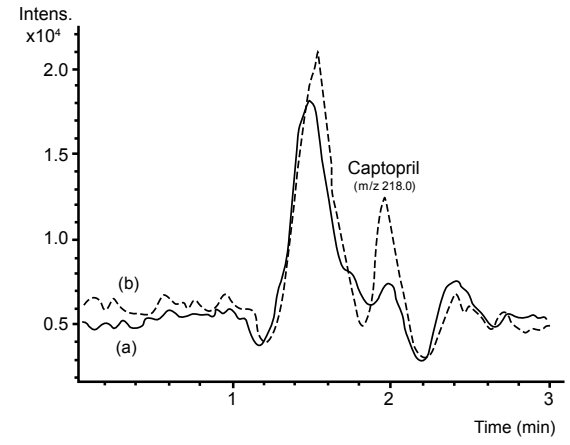


**Figure 2:** Overlay extracted ion chromatograms (EIC) for captopril at *m/z* 218.0 of a blank DBS (a) and DBS spiked with captopril at 100ng/ml (b).

*m/z* 377.2084) for a DBS blank extract (Figure 4a) and sample spiked with 100 ng/ml captopril (Figure 4b). The EIC traces on (Figure 4a) show no captopril signal (*m/z* 218.0845) at the expected retention time whilst the IS (*m/z* 377.2084) is detected. Similarly on the data for the spiked sample (Figure 4b) both the captopril and the IS are detected. The 'humps' on the TIC trace at short retention times are a consequence of the addition of DTT to the sample to stabilize the captopril.

## Linearity and sensitivity

The lower quantification limits of both analytical methods was estimated by extracting captopril from the 8mm diameter punch disks across the concentration range 400, 200, 100, 50, 20, 10 and 0 ng/ml for captopril (n = 5 at all concentration levels). The punched blood spots were extracted as described above. The data was then plotted as peak area (signal output) from the extracted ion chromatogram for the protonated molecular ion $[MH]^+$ of captopril at *m/z* 218.0 for the LC-MS(SIM) method. For the LC-HRMS method the calibration curves were produced in replicate (n=5) by plotting captopril (*m/z* 218.0845)/IS peak area ratio against nominal captopril concentration and an equally weighted linear regression applied. Accuracy (RE%) and precision (CV%) were within the pre-defined 15% limit for all concentrations determined using both analytical methods. A linear calibration graph with a coefficient of determination ($R^2$) of 0.991 for captopril was obtained using the LC-HRMS method. The calibration curve between the range of 10 ng/ml-400 ng/ml for the LC-MS(SIM) method was also linear ($R^2$ = 0.990). For this analytical method the limit of quantification with a signal-to-noise ratio of 10 or greater was 50 ng/ml in whole blood; this compared to 20 ng/ml in whole blood using the LC-HRMS method.

## Accuracy and precision

Method precision and accuracy were evaluated using the captopril extraction data from the 8 mm discs sampled from the centre of the 20, 30 and 40 µl volume DBS of 50, 100 and 400 ng/ml captopril. Intra day precision and accuracy of the LC-MS(SIM) and LC-HRMS methods was determined using 6 determinations for each concentration level and are shown in (Table 1). All values for accuracy and precision were less than 15% and therefore considered acceptable.

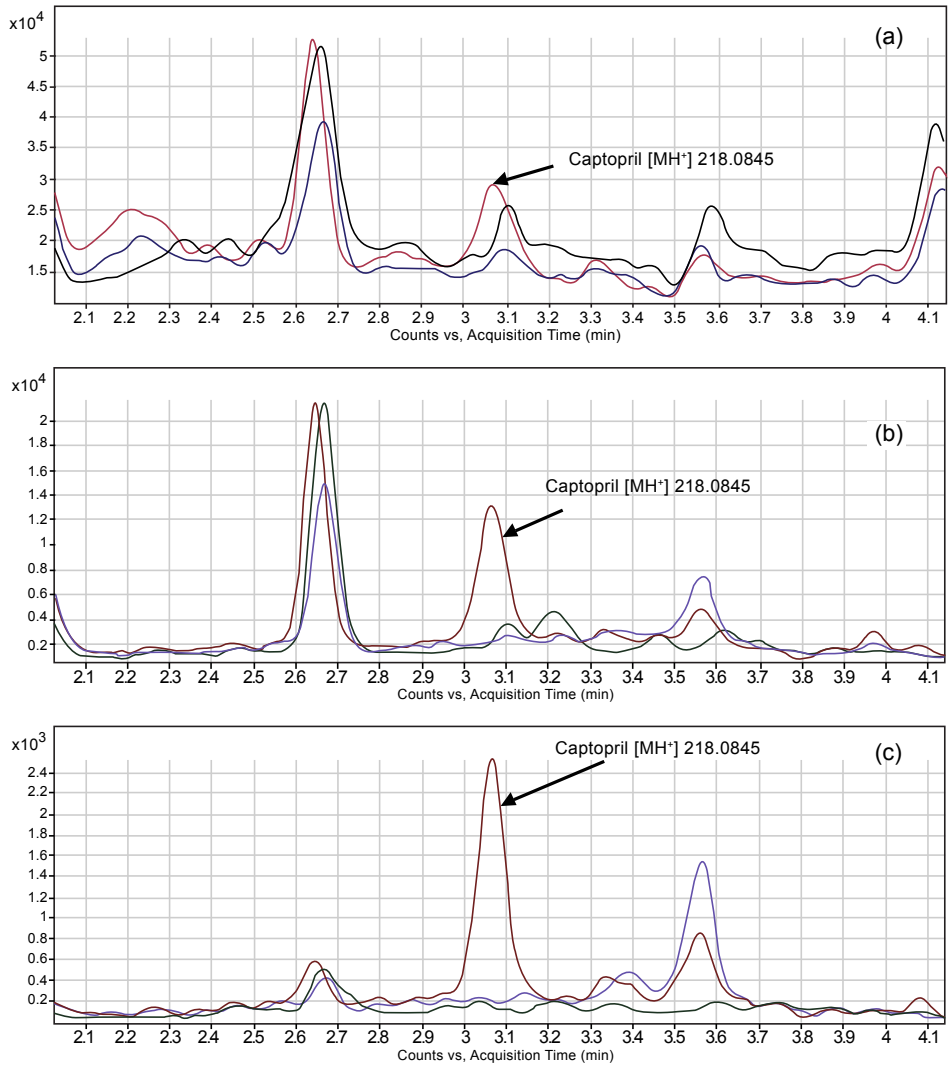


**Figure 3:** Demonstration of enhanced selectivity resulting from the use of narrow mass tolerance windows for 100ng/ml captopril DBS (red), blank DBS (blue), and 903 sampling card extract (black). EIC of the protonated captopril at m/z 218.0845 using mass tolerance filters of (a) ± 500ppm (b) ± 100ppm and (c) ±1ppm.

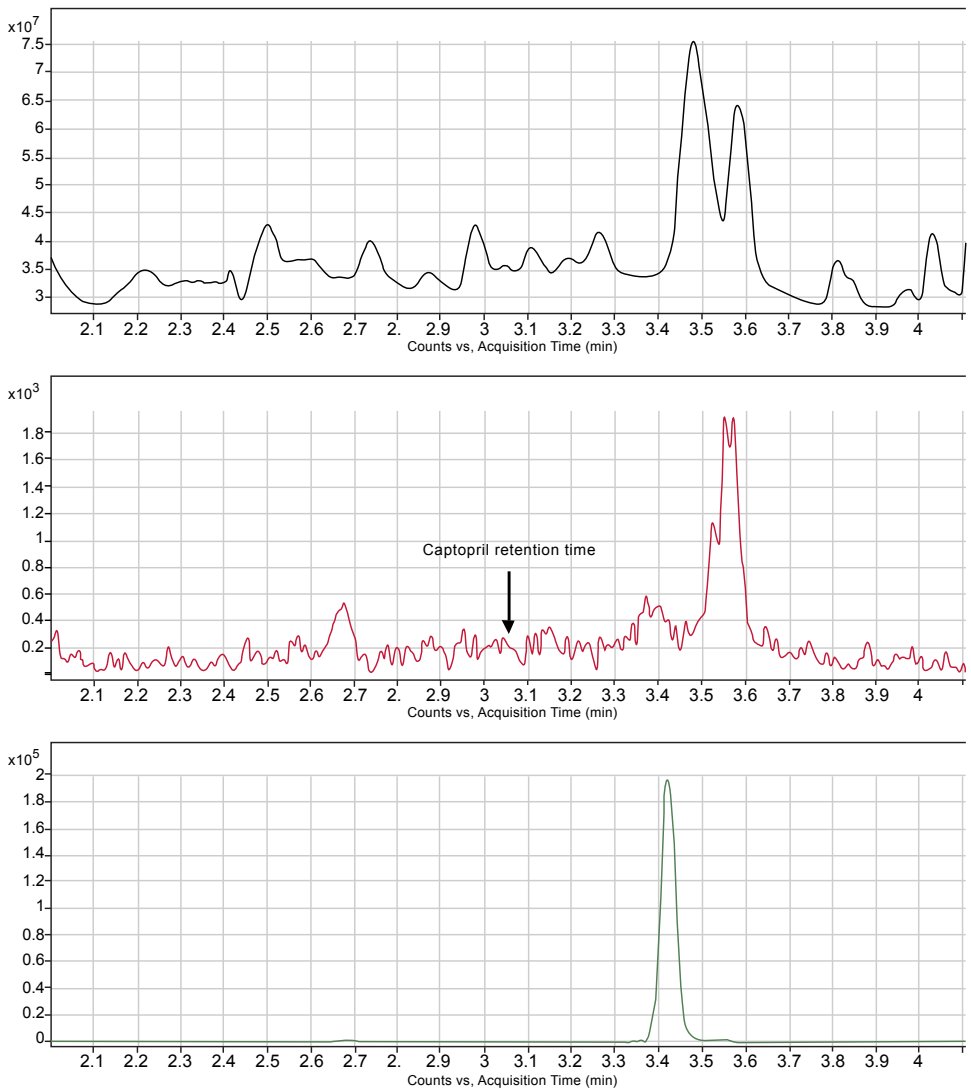


**Figure 4(a):** Total ion chromatogram (TIC) for blank DBS extract (black). Extracted ion chromatogram (EIC) for captopril at m/z 218.0845 (red). EIC for IS at m/z 377.2084 (green).

## Matrix effect

No significant (<5%) ion suppression or enhancement of the analyte signal due to endogenous components of blood or the sampling paper was observed at the three tested captopril concentrations using the two analytical methods (Table 2). The three concentrations were chosen from the low, medium and high range of the calibration curve (50, 100 and 400 ng/ml). These results provide assurance on the selectivity of the extraction procedure and the ionisation method.

## Recovery

Recovery data of captopril from 8 mm discs punched from spiked dried blood spots at three concentrations and three blood spot volumes are listed in (Table 3).

The mean captopril extraction recovery was 90 ± 10%.

The RSD of replicate recovery experiments (n = 6) was found to be less than 15% for all results. Differences in extraction efficiencies between 8 mm DBS disks of the same concentration could be attributed to differences in the weights of the disks i.e. different paper thickness.

## Blood spot size

Method precision and accuracy were evaluated using the captopril extraction data from the 8 mm discs sampled from the centre of the 20, 30 and 40 µl volume DBS of 50, 100 and 400 ng/ml captopril. Intra-day precision and accuracy of the method was determined using 6 determinations for each concentration level and are shown in (Table 4). All values for accuracy and precision were less than 15% and therefore considered acceptable.

The purpose of this series of experiments was to show that the results obtained were independent of the size of the blood spot collected. The analysis of a fixed sample size disc should produce extract data which is directly related to the concentration of captopril in the original blood sample. The hypothesis here is that each blood spot will spread evenly and uniformly across the sampling card. The results in (Table 4) show that within experimental error for the therapeutically relevant

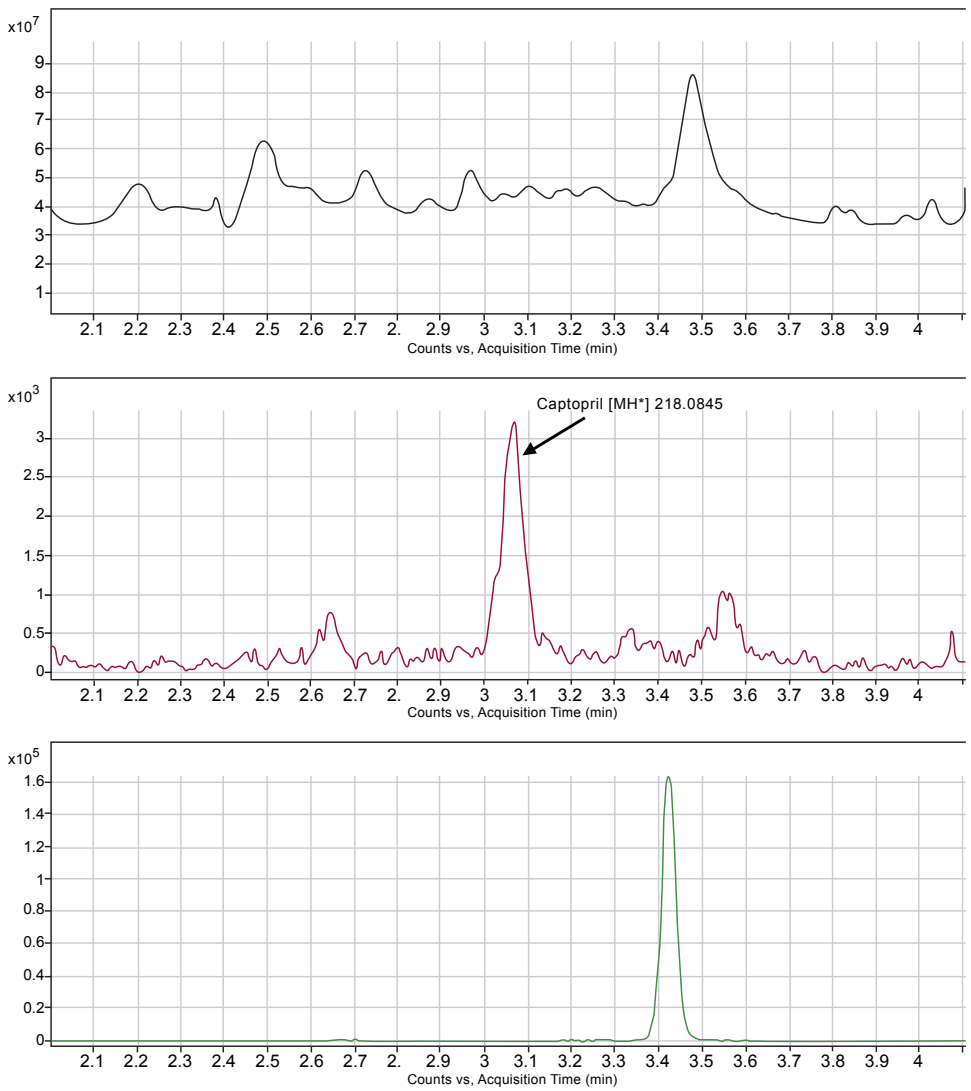


**Figure 4(b):** Total ion chromatogram (TIC) for 100ng/ml captopril DBS extract (black). Extracted ion chromatogram (EIC) for captopril at m/z 218.0845 (red). EIC for IS at m/z 377.2084 (green).

concentration range the data output is the same for each sample volume chosen.

## Stability

The mean and standard deviation for the extraction recoveries carried out after a 12 week interval for captopril from a DBS sample (n=6) was 91 ± 7%. (Table 5) shows the captopril concentrations from the dried blood spots after this time period. These results indicate that captopril is stable in the dried blood spots for at least 2 months when stored at room temperature. Further tests are underway to determine long-term stability of analyte on the sampling paper.

## Application of LC-MS(SIM) method

(Figure 5) shows a representative extracted ion chromatogram for ion of *m/z* 218.0 of a DBS sample from a neonatal patient.

The neonate patient extracted ion chromatograms showed a peak at 1.9 min, corresponding to 88 ng/ml captopril in disc 1 and 55 ng/ml captopril in disc 2 punched from the two dried blood spots. The peaks for captopril were within the limit of quantification of the method. The levels of captopril in the patient blood spots were calculated from the response in the LC-MS(SIM) analysis and the calibration carried out previously.

The work of Pereira et al. [3] can be used to provide an indication of the likely levels of captopril to be determined following oral administration of captopril at 1 mg/kg. For a cohort of 10 infants (aged 6.8 +/- 4.6 months) they found the maximum captopril concentration (C(max)) to be 350 +/- 184 ng/ml with the time to C(max) of 1.6 +/- 0.4 h. Whilst these results can only be taken as indicative the data obtained in this investigation is in very close agreement and is therefore considered to be realistic.

The precision for the duplicate samples was not good. This could be explained by the blood not saturating the sampling card for DBS disc 2. This led us to investigate more closely the gravimetric data for the blood spots. A series of blank discs i.e. no blood were punched from the sampling card and the mean weight of these discs were subtracted from the values obtained for the 8 mm dried blood spot discs (data not

| Captopril concentration in whole blood (ng/ml) | Analytical Method | Mean concentration found ±SD (ng/ml) (n=6) | Precision (% RSD) | Accuracy (%bias) |
|---|---|---|---|---|
| 400 | LC-MS(SIM) | 424 ± 18 | 4.3 | 6.0 |
| | LC-HRMS | 398 ± 16 | 0.7 | 9.7 |
| 100 | LC-MS(SIM) | 98 ± 8 | 8.5 | -2.0 |
| | LC-HRMS | 101 ± 5 | 0.4 | 4.1 |
| 50 | LC-MS(SIM) | 49 ± 5 | 10.2 | -2.0 |
| | LC-HRMS | 52 ± 5 | 7.7 | 8.7 |

**Table 1:** Accuracy and precision of the quantification of captopril in the DBS samples.

| **LC-MS(SIM)** | | | |
|---|---|---|---|
| **Nominal conc. (ng/ml)** | 50 | 100 | 400 |
| Matrix effect % (mean) | 3.9 | 3.2 | 4.4 |
| Precision (CV%) | 3.7 | 1.8 | 3.1 |
| **LC-HRMS** | | | |
| Nominal conc. (ng/ml) | 50 | 100 | 400 |
| Matrix effect % (mean) | 2.8 | 1.7 | 3.9 |
| Precision (CV%) | 3.1 | 2.9 | 2.3 |

**Table 2:** Matrix effect data at three concentrations of captopril (n=5).

| | 40µl volume DBS (%) | 30µl volume DBS (%) | 20µl volume DBS (%) |
|---|---|---|---|
| Replicate 1 | 98.88 | 92.47 | 83.98 |
| Replicate 2 | 106.81 | 97.23 | 88.08 |
| Replicate 3 | 75.99 | 101.97 | 95.50 |
| Replicate 4 | 95.62 | 93.55 | 87.67 |
| Replicate 5 | 106.17 | 100.37 | 92.16 |
| Replicate 6 | 74.33 | 92.65 | 97.76 |
| Average | 92.97 | 96.37 | 90.69 |
| RSD % | 14.45 | 4.12 | 5.35 |

Recovery of captopril from 8mm diameter dried blood spots by the extraction procedure

**Table 3a:** 400ng/ml captopril dried blood spots spotted at 20, 30 and 40µl.

| | 40µl volume DBS (%) | 30µl volume DBS (%) | 20µl volume DBS (%) |
|---|---|---|---|
| Replicate 1 | 96.62 | 90.28 | 92.94 |
| Replicate 2 | 72.62 | 83.77 | 94.09 |
| Replicate 3 | 110.78 | 97.04 | 74.81 |
| Replicate 4 | 108.48 | 78.03 | 99.31 |
| Replicate 5 | 88.66 | 89.20 | 80.93 |
| Replicate 6 | 94.33 | 97.76 | 68.98 |
| Average | 95.25 | 89.35 | 85.18 |
| RSD % | 14.66 | 8.52 | 14.16 |

Recovery of captopril from 8mm diameter dried blood spots by the extraction procedure

**Table 3b:** 100ng/ml captopril dried blood spots spotted at 20, 30 and 40µl.

shown). The mass of blood measured lay between 4 and 6 mg i.e. a mean of 5.0 ± 1.0 mg. This will introduce a significant level of uncertainty in the final drug concentration measured and consequently will have its own effects on the level of the target drug measured. Although the sampling card with the two blood spots was visually examined prior to extraction and showed that blood had penetrated through the card and was dark coloured on both sides of the sampling card, it is also possible that there was an asymmetric spreading of the blood spot around the centre sampling zone. The hole for DBS disc 2 may have sampled a region of lower blood density and would therefore be expected to give the lower concentration observed. This may be associated with the uniformity of the sampling card itself. This might be remedied by punching smaller discs in the centre of the blood spot or by better sampling by the clinician. The precision with which the card is punched could provide another source of variation. Other researchers in this field have also identified that the uniformity of the DBS sample spots is a major issue to ensure sample to sample reproducibility [33]. Further work is therefore underway to address this pertinent issue before this method can be used on a routine basis in a clinical setting.

## Conclusion

The LC-MS(SIM) and accurate mass LC-HRMS micro-analytical methods developed in this study are the most sensitive reported for the determination of captopril from small volume blood samples (20 µl) collected as dried blood spots on sampling paper. The advantages of using accurate mass LC-HRMS analyses of captopril from DBS samples have been demonstrated for samples at therapeutic concentrations in neonates. The developed LC-HRMS method also showed improved sensitivity and specificity for captopril in DBS and its potential for use in quantitative DBS analysis has been highlighted. In this study, with the pre-treatment of the sampling card with the chemical stabiliser 1,4 dithiothreitol (DTT) and with this stabiliser present in the extraction solvent, the extraction efficiency is increased to enable determinations of low levels of captopril in pediatric blood samples. The development of these novel micro-analytical methods would facilitate the much needed PK studies for captopril and other drugs in the paediatric population in addition to facilitating assessment of medication adherence and preventing toxicity in a routine clinical setting in neonates and children. The accurate mass LC-HRMS methodology described in this preliminary investigation has combined the advantage

| | 40µl volume DBS (%) | 30µl volume DBS (%) | 20µl volume DBS (%) |
|---|---|---|---|
| Replicate 1 | 67.18 | 105.40 | 78.33 |
| Replicate 2 | 95.86 | 82.01 | 101.82 |
| Replicate 3 | 83.39 | 79.91 | 78.69 |
| Replicate 4 | 68.78 | 91.29 | 86.26 |
| Replicate 5 | 97.67 | 87.67 | 95.48 |
| Replicate 6 | 77.56 | 86.26 | 74.80 |
| Average | 83.40 | 88.76 | 87.56 |
| RSD % | 13.62 | 10.26 | 10.70 |

Recovery of captopril from 8mm diameter dried blood spots by the extraction procedure

**Table 3c:** 50ng/ml captopril dried blood spots spotted at 20, 30 and 40µl.

| Captopril concentration in whole blood (ng/ml) | DBS volume (µl) | Mean concentration found ±SD (ng/ml) (n=6) | Precision (% RSD) | Accuracy (%bias) |
|---|---|---|---|---|
| 400 | 40 | 405 ± 59 | 14.7 | 1.3 |
| | 30 | 424 ± 18 | 4.3 | 6.0 |
| | 20 | 399 ± 23 | 5.9 | -0.3 |
| 100 | 40 | 102 ± 9 | 9.2 | 2.0 |
| | 30 | 98 ± 8 | 8.5 | -2.0 |
| | 20 | 94 ± 13 | 14.1 | -6.0 |
| 50 | 40 | 48 ± 4 | 9.3 | -4.0 |
| | 30 | 49 ± 5 | 10.2 | -2.0 |
| | 20 | 48 ± 6 | 11.5 | -4.0 |

**Table 4:** Accuracy and precision of the quantification of captopril in the DBS samples.

| Captopril concentration in whole blood (ng/ml) | Mean concentration found ±SD (ng/ml) (n=6) | Precision (% RSD) | Accuracy (%bias) | Extraction efficiency ±SD (%) |
|---|---|---|---|---|
| 400 | 406 ± 45 | 11.13 | 1.5 | 92.3 ± 10.3 |
| 100 | 98 ± 4 | 4.4 | -2.0 | 89.4 ± 4.0 |
| 50 | 50 ± 3 | 6.5 | 0 | 92.2 ± 6.0 |

**Table 5:** Accuracy, precision and quantification of captopril in DBS samples after 12 weeks of storage.

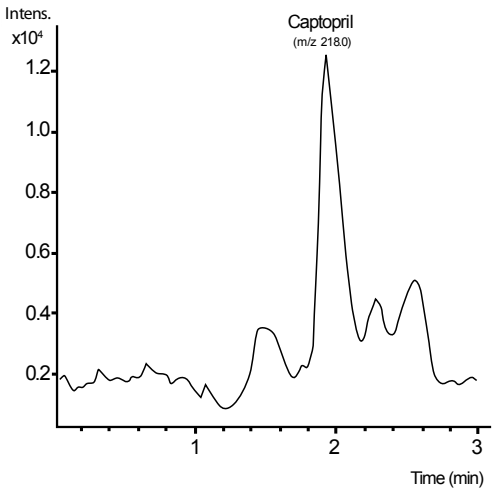


**Figure 5:** Extracted ion chromatogram showing the result of captopril extraction from a neonate patient DBS.

of micro sampling, inherent in blood collection on sampling card, with an accurate, precise and selective mass spectrometric technique. The small volume of blood required combined with the simplicity of the extraction procedure and analytical technique has the potential for making this a useful procedure for monitoring captopril concentrations in routine clinical settings in neonates and children. There is no doubt that the triple quadrupole tandem mass spectrometer will remain the primary instrument used in newborn screening investigations where the target analytes are readily predetermined and new electronic developments will allow many more compounds to be monitored by the MRM process during a single LC-MS/MS run. It perhaps in the areas of clinical pediatric investigations, where samples are often very difficult to obtain, that the accurate mass HRMS systems with the potential for re-interrogation of data already collected will make a major contribution. The capability to search data already collected for new target species without having to re-run the sample could have significant impact on areas as diverse as drug discovery, food safety, forensic and *apriori* clinical investigations.

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