Quantitative LC-HRMS determination of selected cardiovascular drugs, in dried blood spots, as an indicator of adherence to medication

Authors: Dennis Bernieh, Graham Lawson, Sangeeta Tanna*

Affiliations: Leicester School of Pharmacy, Faculty of Health and Life Sciences, De Montfort University, Leicester LE1 9BH, UK

*Corresponding author: Sangeeta Tanna, Email: stanna@dmu.ac.uk

Author email contact addresses:
Dennis Bernieh, Email: P13064053@my365.dmu.ac.uk
Graham Lawson. Email: glawson@dmu.ac.uk

Conflict of interest: none

Abstract

Dried blood spot (DBS) sampling was investigated as a means of obtaining micro-volume blood samples for the quantitative analyses of ten commonly UK prescribed cardiovascular drugs as an indicator of medication adherence. An 8 mm disc was punched out from each DBS from calibration, quality control and volunteer samples and extracted using methanol containing the internal standard. Each extract was evaporated to dryness, the residue reconstituted in methanol:water (40:60 v/v) containing 0.1% formic acid and analysed by LC-HRMS. Chromatography was performed using gradient elution on a Zorbax Eclipse C18 HD 100 mmx2.1 mm, 1.8 µm pore size column with the column oven temperature at 40˚C. Flow rate of the mobile phase was 0.6ml/min with a run time of 2.5 min. Electrospray positive ionization was used for MS detection. Drug recoveries from spiked blood spots were 68% for simvastatin and ≥ 87% for all other target drugs. Compound specificity was obtained operating the MS with a 5ppm mass window. The LC-HRMS method was validated, with results for accuracy and precision within acceptable limits; analytes were stable at room temperature for at least 10 weeks and different blood spot volumes and haematocrit values had no significant effect. The LC-HRMS assay was used to analyse DBS samples from volunteers, some of whom were prescribed one or more of the target drugs. In results from 37 volunteers the assay successfully identified volunteers who were known to be either adherent or nonadherent; confirmed the correct drug/drugs for multiple prescriptions; demonstrated no false positives from other cardiovascular drugs; revealed several examples of unsuspected non-adherence. These results indicated that the developed assay was suitable for trials with patients.

Key Words

Microsampling, Dried blood spot (DBS), Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS), Medication adherence, therapeutic drug monitoring

Introduction

Cardiovascular disease (CVD) involving disorders of the heart and blood vessels remains the number one cause of death globally [1]. It affects an estimated 7 million people in the UK and is responsible for about 155,000 deaths each year. The economic burden of CVD is large with healthcare costs alone estimated at £11 billion every year in the UK [2]. An essential component of managing cardiovascular diseases properly and ensuring treatment success is to ensure patients take the prescribed medication. The drug selected and the dose prescribed should produce therapeutic drug levels in the patient's blood stream. Patient adherence to the prescription helps ensure that the blood concentration of the drug is within the therapeutic limits in order to improve treatment outcomes [3]. However a World Health Organisation (WHO) report [4] stated that about 50% of all patients do not adhere to their treatment regimen. Evidence suggests that >50% of heart disease patients do not adhere to their prescription treatment [5]. In the UK, for example, about 370 million prescriptions were dispensed for heart diseases in 2014 and half of these were believed to be wasted because patients did not take their medicines as prescribed [6]. According to a National Institute of Clinical Excellence (NICE) guideline on medication adherence, wasted (unused) medicines cost the UK National Health Service (NHS) up to £4 billion annually [7, 8]. This level of non-adherence results in poor clinical outcomes, increased cost of care, hospital readmission, and sometimes death [9].

There is currently no gold standard measurement tool for assessing adherence to prescription medication in routine clinical practice [10]. Current methods to assess medication adherence involves patient self-report, pill counts, pharmacy refill or claims, data logs or electronic monitors. None of these can confirm the patient ingested the medication and therefore only capture a part of the information needed for accurate assessment of medication adherence and consequently may lead to optimistic results [11, 12]. Sensors are now available that can document ingestion but patient security and cost may be of concern [13, 14].

Therapeutic drug levels are conventionally monitored using either whole blood or plasma samples. Urine samples can only confirm that particular drugs were ingested based on the detection of either the drug or its metabolite. Urine analysis has been used to investigate the presence of prescribed CVD drugs for patients exhibiting 'resistant hypertension' [15, 16] but this approach provides no information of the drug levels in the patient's blood. Data obtained from the routine 10ml liquid blood samples or the more recently developed dried blood spot (DBS) samples can confirm satisfactory adherence to medication by confirming a therapeutic level of the drug in the patient's blood [17]. In addition, as the population ages and patients are given more prescriptions (polypharmacy) factors such as individual variation in drug metabolism and possible drug-drug interactions become more important [18]. Hence monitoring therapeutic drug levels by direct analyses of patient blood samples can offer clinicians very valuable information about possible drug-drug interactions, side effects occurring from the co-administration of several cardiovascular drugs [19] and a patient's adherence to a complex prescribed medication regimen.

The quantitative determination of target cardiovascular drugs in plasma using either liquid chromatography – tandem mass spectrometry (LC-MS/MS) [20] or LC-MS [21] has been reported. However, these investigations required large sample volumes (1 – 10ml) of blood which would not be suitable for routine non-clinical testing. Dried blood spot (DBS) sampling is an alternative

approach to measuring CVD drug concentrations [22] and since it requires only a micro blood volume (<30µl) it has great potential in overcoming the barriers associated with blood collection using venepuncture [23]. DBS sample collection can be undertaken by the patients themselves or by parents/guardians at home. This allows for convenient monitoring at any desired sampling time [24].

Tanna et al [25-27] have reported the ease of use and low cost of the DBS micro-sampling platform which makes it ideal for assessing adherence to selected CVD medication.

This article describes a method for fast and simple quantification of ten (10) commonly UK prescribed cardiovascular drugs from DBS samples using liquid chromatography – high resolution mass spectrometry (LC-HRMS) analyses. The target drugs studied were atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin, and valsartan. The developed and validated method was used to assess adherence to prescribed cardiovascular medication using blood spot samples taken from volunteers; some prescribed with no medication and others who were prescribed with one or more of the target drugs investigated. It was envisaged that this group would provide a challenge to the capabilities of the system developed.

2. Experimental

2.1 Chemicals and Materials

Reference drug samples: atenolol (R-(+), 99%), atenolol d_7, atorvastatin calcium salt, bisoprolol hemifumarate salt, diltiazem hydrochloride, doxazosin mesylate salt, lisinopril, losartan potassium salt, ramipril, simvastatin and valsartan were purchased from Sigma–Aldrich (Poole, UK). LC–MS grade acetonitrile, methanol and water were also obtained from Sigma–Aldrich (Poole, UK). 903 specimen collection paper, polyethylene bags, microcentrifuge tubes (1.5 ml), pipette tips and volumetric pipettes were purchased from Fisher Scientific (Loughborough, UK). Autosampler vials with 250µl inserts, vial caps and formic acid were obtained from Agilent Technologies (Cheshire, UK). Heparin coated blood collection tubes were purchased from International Scientifique Supplies Ltd. (Bradford, UK). An 8 mm diameter punch was acquired from Maun Industries Ltd. (Nottingham, UK).

Following De Montfort University's Ethics Protocols, fresh blank blood was obtained from informed volunteers.

2.2 Preparation of standard stock and working solutions for the 10 cardiovascular drugs

Atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan standard stock solutions were prepared in methanol at a concentration of 1mg/ml. Multicomponent working solutions for each target drug were prepared freshly by diluting the stock solutions with methanol/water (70:30, v/v).

For the preparation of spiked blood standards, several samples of fresh blank blood (900 µl) were spiked with 100 µl of one of each multicomponent working solution to produce final blood target drug concentrations. The haematocrit of the blood was 45%. 100 µl of methanol/water (70:30, v/v)

was spiked into 900µl of fresh blank blood to produce a zero (blank) blood sample. Internal standard, atenolol D_7 stock solution was prepared in methanol at a concentration of 10µg/ml and diluted further with methanol/water (70:30, v/v) to produce an extraction solvent containing 20 ng/ml of IS. Whilst it is generally recommended to use 5% solvent when preparing DBS calibration and quality control (QC) standards, 10% solvent was used in this assay. Work in this laboratory [27, 28] has shown that the use of a 10% solvent standard did not produce any changes to the blood spot spreading.

2.3 Preparation of calibration standards and validation samples

The calibration ranges were chosen to cover the concentration ranges in (Table 1) for the selected drugs. A minimum of 7-point calibration curve was prepared by spotting 30µl of calibration standards including blanks directly onto the 903 sampling paper using a volumetric pipette. The prepared samples were dried at room temperature for at least 3h prior to processing. A 30 µl volume produced a spot of size of ~9.5 mm in diameter on the sampling paper.

2.4 Solvent extraction of analytes from dried blood spot

An 8 mm disc (~20 µl of blood) was punched from the centre of each DBS sample and transferred to a 1.5 ml micro-centrifuge tube. A 300 µl volume of methanol containing IS (20 ng/ml), atenolol D_7, was used for the extraction of atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan because of its optimum extraction efficiency and less interference. Tubes were vortexed for 1 min, sonicated for 30 mins in a temperature controlled ultrasonic bath at 40°C and centrifuged at 13200rpm for 10mins. 270 µl of each supernatant was transferred into a new microcentrifuge tube and dried under a gentle stream of N_2 gas. Dried residue was reconstituted with 150 µl of methanol/water (40:60, v/v) containing 0.1% formic acid. The final extracts were transferred into auto-sampler vials for LC-HRMS analyses.

2.5 LC-High Resolution MS analyses

Chromatographic and mass spectrometry conditions were optimized for better chromatographic separation and sensitivity for the 10 cardiovascular drugs. Analyses were performed on an Agilent 1290 LC on-line to an Agilent G6530A QTOF mass spectrometer, operated in the TOF mode with a 5 parts-per-million mass to charge window. Separation of the ten target drugs was achieved using a Zorbax Eclipse Plus C18 rapid resolution HD column (100 mm x 2.1 mm i.d., 1.8 µm particle pore size) Agilent Technologies, Cheshire, UK which was preceded by a security guard ultra-cartridge (Phenomenex, Macclesfield, UK. The LC injector was maintained at 4^{o}C, the injection volume was 20 µl and the column oven was maintained at 40^{o}C. The mobile phases used were water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B) delivered at a flow rate of 0.6 ml/min with gradient elution. The mobile phase was initiated at 4% B and held for 0.5 min before increasing to 65% B for 1.0 min and then to 95% B by 1.5 min and maintained until 2.5 min before returning to 4% B. Column re-equilibration was achieved by holding the gradient elution programme for 1.5 min prior to the next injection.

The mass spectrometer was operated in electrospray positive ion mode. Calibration of the TOF mass spectrometer was performed daily before analyses. The optimum MS source and chamber conditions were: fragmentor voltage: 150 V; skimmer: 65 V; drying gas temperature: 350°C; drying gas flow: 10 l/min; nebuliser: 45.0 psig; sheath gas temperature: 400°C; sheath gas flow: 12 l/min. Mass range: 100–1000 m/z; recording rate: 1 Hz. HRMS reference masses: 121.0508 m/z and 922.00979 m/z. MassHunter Workstation Acquisition Software for TOF/Q-TOF version B.04.00 (Agilent Technologies) was used to operate the system and acquire all data. The data was processed using Qualitative Analysis B.04.00 and Quantitative Analysis B.05.00 SP02 software (Agilent Technologies).

2.6 Validation studies

For the purposes of validation studies, three concentrations were chosen for the independent preparation of quality control samples (QCs) at low, medium and high concentration levels for each target drug and run alongside calibration standards as detailed in Table 2. To demonstrate that the developed bioanalytical method was fit for purpose, validation was conducted based upon international guidelines [29, 30]. The selectivity, linearity, sensitivity, intra and inter-assay accuracy and precision, limit of quantification (LOQ), matrix effects, haematocrit effects and stability were determined for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan.

2.6.1 Selectivity

Possible interference from the matrix was investigated by the analyses of blank blood spots and target analyte spiked blood spots and the data processed. A mass window of 5 ppm was used to generate extracted ion chromatograms (EIC) for protonated species of atenolol at m/z 267.1703, atorvastatin at m/z 559.2610, bisoprolol at m/z 326.2326, diltiazem at m/z 415.1686, doxazosin at m/z 452.1928, lisinopril at m/z 406.2336, losartan at m/z 423.1695, ramipril at m/z 417.2384, and valsartan at m/z 436.2343. For simvastatin, the sodium adduct ion with a 5 ppm mass extraction window gave the highest intensity signal at m/z 441.2611 and was used for quantification.

2.6.2 Linearity and sensitivity

Replicate (n = 6) analyses of calibration standards were run per day over the three days. A calibration plot for each target analyte/IS peak area ratio against nominal analyte concentration was produced and an equally-weighted linear regression was applied. The limit of quantification of atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan in the DBS extracts was determined using a signal-to-noise ratio of ≥ 10. The coefficient of variation at the limit of quantification (LOQ) determined for each target drug (n = 6) was within the ≤20% limit.

2.6.3 Accuracy and precision

Replicate (n = 6) analyses of (QCs) samples at the low, medium and high concentration levels of the ten target drugs, were analysed to evaluate the inter and intra-day accuracy and precision. Accuracy was expressed as the relative error (RE%) and precision as the coefficient of variation (CV%). With reference to FDA and EU guidelines, a RE and CV of ≤15% at all tested concentrations was considered acceptable.

197 2.6.4 Matrix effects

198 To assess the effect of matrix due to constituents within the dried blood spot, blood samples were
199 collected from three different sources. Replicate (n = 6) samples of the ten target analytes spiked in
200 blank blood spot extracts to represent low, medium and high concentrations were prepared to
201 evaluate suppression or enhancement of the detector response. The prepared samples were
202 compared with standards of equal concentration spiked into methanol/water (40:60, v/v) containing
203 0.1% formic acid for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan,
204 ramipril, simvastatin and valsartan. The matrix effect was calculated using the formula (B/A − 1) x
205 100. Where A represents the ratio of the target analyte/I.S response from analyte spiked into pure
206 solvent and B represents the ratio of target analyte/I.S response from analyte spiked into extracted
207 blank whole blood.

208 2.6.5 Recovery of the 10 target analytes from dried blood spots

209 Extraction efficiency was determined using replicate (n = 6) samples prepared at the (low, medium
210 and high) concentrations for the ten target drugs from spiked DBS. Recovery was assessed by
211 comparing the ratios of analyte to I.S response from DBS extracts with those obtained from blank
212 blood spot extracts spiked with solution standards of equal concentration. Recovery was calculated
213 using the formula: % recovery = (analyte to I.S response of dried blood spot extract/analyte to I.S
214 response of post extraction blank DBS spiked extract) x 100.

215 2.6.6 Blood spot size

216 This investigation was conducted to demonstrate that after selection of a disc size for analyses, the
217 quantitative results obtained were not affected by the volume of blood deposited or the size of the
218 blood spot presuming there is uniformity in the spread of the spot on filter paper. To investigate the
219 blood volume effect on the quantification of the ten target analytes, replicate analyses (n = 6) were
220 performed at medium and high concentrations for the target drugs using prepared 20, 30 and 40 µl
221 blood spots. These spots had different diameters directly proportional to sample volume deposited.
222 8mm discs (approximately 20 µl of blood) were punched from the centre of the already prepared 20,
223 30 and 40 µl volume DBS standards. Extraction of the target drugs was performed using the
224 procedure described in Section 2.4 prior to LC-HRMS analyses. Using a linear regression equation
225 obtained from a calibration generated with 30 µl volume DBS, the analyte concentration of the
226 extracts were determined.

227 2.6.7 Evaluation of Haematocrit effects

228 The haematocrit (Hct) level represents the relative volume of red blood cells (RBC) in blood. It has a
229 direct effect on the viscosity of blood, which in turn affects the spread of blood on cellulose based
230 paper. Hence permeability of a DBS card is influenced by the haematocrit of blood [31, 32]. Blood
231 with high Hct (due to the high cellular composition) is more viscous and leads to the formation of
232 small spots on DBS cards. The Hct range varies according to age for healthy adult males and females.
233 It is 40 – 54% and 36 – 48% respectively [33]. Hct values may however deviate from these ranges in
234 certain disease states e.g. anaemia and polycythaemia. An Hct value of 45% was chosen to represent
235 the average value expected in the target population planned for this study. The bias caused by the
236 haematocrit variability of the DBS sample has been considered a critical parameter impacting on

quantitative DBS analyses [34, 35]. Hence the influence of haematocrit on assay performance was evaluated at the low, medium and high concentrations of each target drug (n = 6) using 30µl spots with an adjusted Hct of 35, 45 and 55% to cover the range for the target population.

2.6.7.1 Preparation of DBS with adjusted Hct of 35, 45 and 55%

Blank human whole blood was centrifuged at 10,000g for 12 minutes [36, 37]. The plasma generated was transferred into a clean eppendorf tube. The RBC suspension and plasma were mixed in proportions (35:65, v/v), (45:55, v/v) and (55:45, v/v) to give whole blood with an adjusted Hct of 35, 45 and 55% respectively. These were used to prepare calibration DBS samples for the ten target analytes at the blank, low, medium and high concentration ranges. 30µl of each prepared standard were spotted on 903 sampling papers and allowed to dry for 3 hours. 8mm disc were punched from the centre of each spot and extracted using the procedure described in section 2.4.

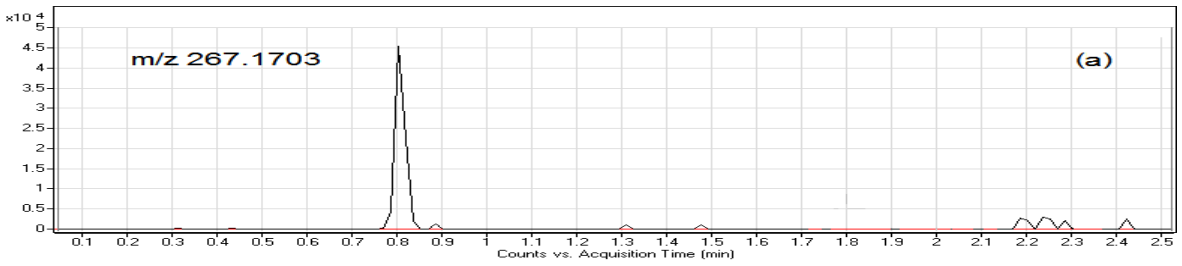
2.6.8 Stability of dried blood spots

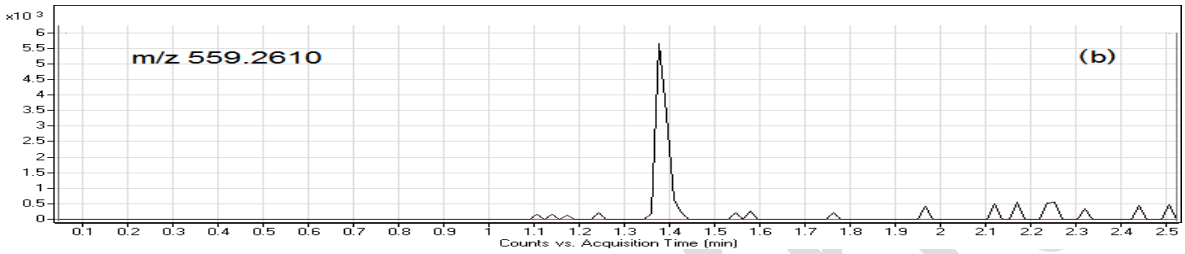
Stability experiments were performed for the DBS samples during storage at room temperature for 10 weeks, demonstrating the possibility to prepare DBS samples in batches followed by storage. This was done by the replicate analyses (n = 6) of blood spots containing atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan at the low, medium and high concentrations. Using the extraction procedure described in Section 2.4, 8mm diameter discs were punched from the DBS calibration standards at the low, medium and high concentrations of the 10 target drugs and analysed.

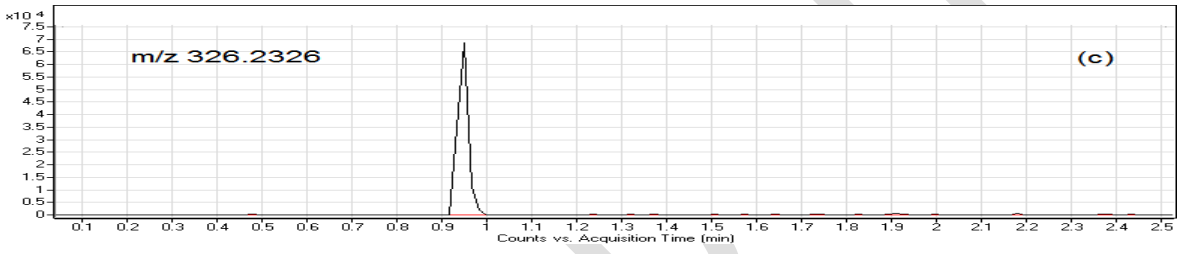
2.7 Application of method to volunteer blood spot samples

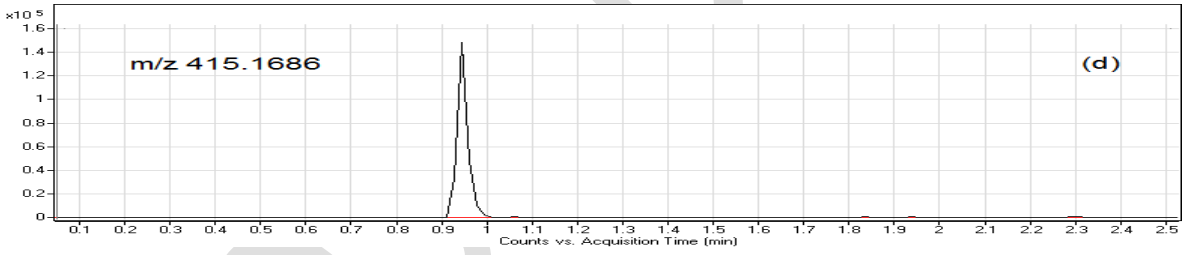
The developed DBS based LC–HRMS method was applied to a series of dried blood spot samples collected from selected healthy volunteers. These volunteers were all prescribed with one or more of the target drugs atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, lisinopril, losartan, ramipril, simvastatin and valsartan. Samples were taken between 0.5 and 24 h after the oral intake of the drugs. A series of blank control DBS samples were taken from a second group of volunteers not prescribed any of the target drugs. The study has received ethical approval from the De Montfort University Research Ethics Committee.

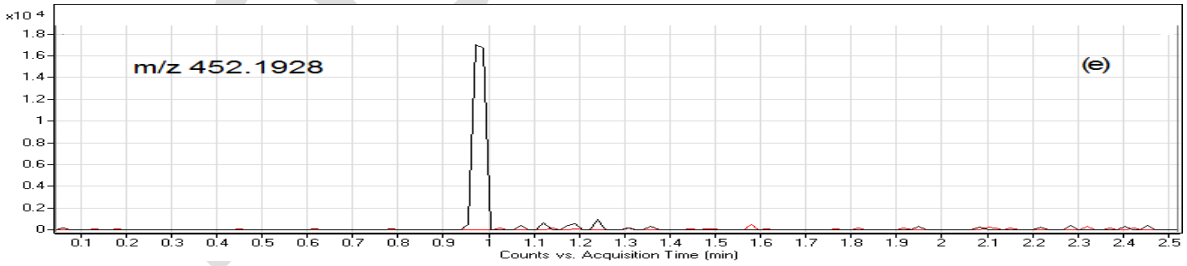
3. Results and discussion

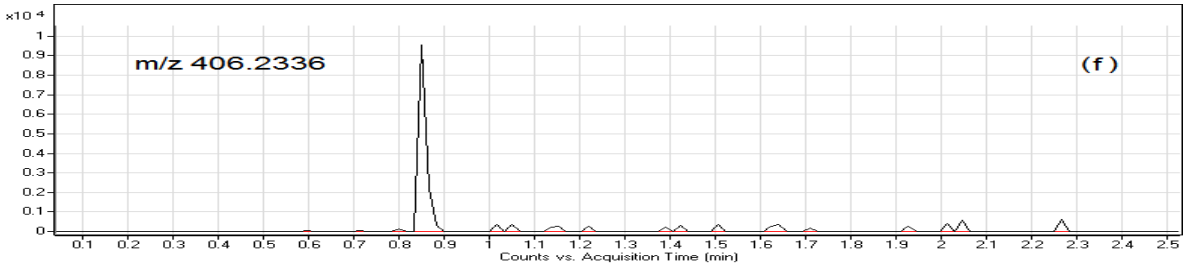












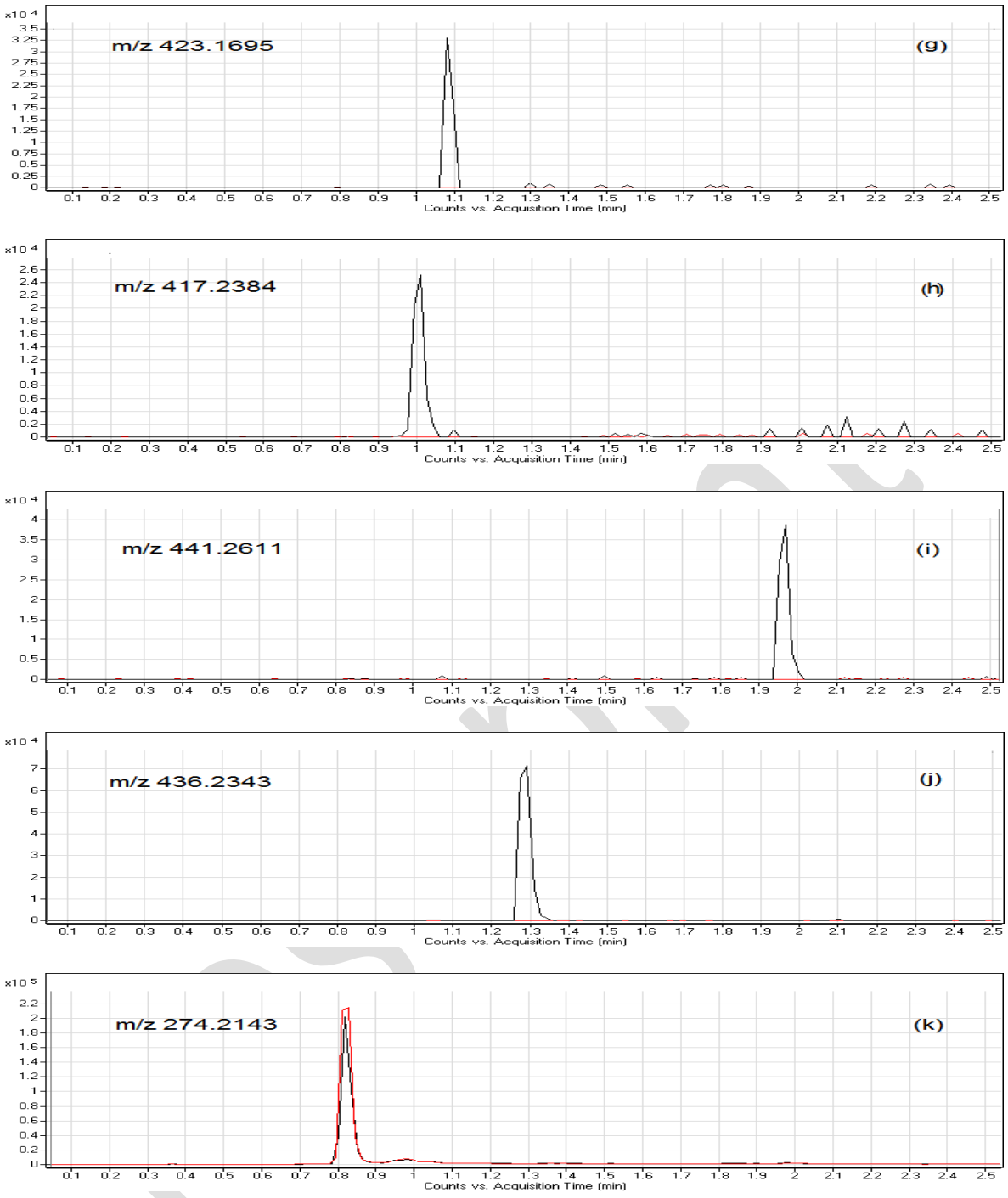


Figure 1. Representative LC-HRMS extracted ion chromatograms of an extracted blank blood spot (red) and a calibration standard at the LOQ spiked with the ten target drugs (black). A narrow mass extraction window (5ppm) was used for (a) atenolol at m/z 267.1703 (b) atorvastatin at m/z 559.2610 (c) bisoprolol at m/z 326.2326 (d) diltiazem at m/z 415.1686 (e) doxazosin at m/z 452.1928 (f) lisinopril at m/z 406.2336 (g) losartan at m/z 423.1695 (h) ramipril at m/z 417.2384 (i) simvastatin at m/z 441.2611 (j) valsartan at m/z 436.2343 (k) atenolol d7 (internal standard) at m/z 274.2143.

Table 1
Linearity and sensitivity data for the ten cardiovascular drugs

Drug	Range (ng/ml)	y = ax + b	R2	LOQ (ng/ml)
Atenolol	10 - 1500	y = 0.0044x - 0.047	0.997 ± 0.001	10
Atorvastatin	0.5 - 100	y = 0.0014x + 0.0244	0.986 ± 0.013	0.5
Bisoprolol	0.1 - 100	y = 0.019x + 0.034	0.994 ± 0.003	0.1
Diltiazem	0.5 - 600	y = 0.016x + 0.053	0.997 ± 0.002	0.5
Doxazosin	0.1 - 100	y = 0.016x + 0.033	0.992 ± 0.005	0.1
Lisinopril	0.1 - 100	y = 0.002x + 0.031	0.978 ± 0.007	0.1
Losartan	5 - 1000	y = 0.004x + 0.0713	0.995 ± 0.002	5
Ramipril	0.1 - 100	y = 0.025x + 0.018	0.997 ± 0.002	0.1
Simvastatin	0.1 - 100	y = 0.013x + 0.081	0.996 ± 0.003	0.1
Valsartan	50 - 4000	y = 0.002x - 0.139	0.994 ± 0.003	50

Table 2
Intra and inter-day accuracy and precision data for the ten target cardiovascular drugs in DBS samples (n = 6 at all concentration levels, for 3 days)

Drug	Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Coefficient of variation (%)	
			Intra day	Inter day
Atenolol	50	51.87	4.00	1.37
	500	498.02	4.14	1.36
	1500	1517.51	2.22	1.24
Atorvastatin	1	1.05	4.06	5.93
	25	25.23	7.54	2.45
	100	100.69	7.19	2.41
Bisoprolol	1	1.09	2.63	3.50
	25	25.54	6.10	4.14
	100	102.42	3.21	2.76
Diltiazem	5	5.29	5.95	0.83
	100	98.64	6.41	1.06
	600	611.85	2.03	1.49
Doxazosin	1	1.07	9.23	1.03
	25	25.59	3.74	3.58
	100	99.24	3.89	2.78
Lisinopril	1	1.04	9.14	1.37
	25	24.91	6.55	1.89
	100	100.31	6.61	2.19
Losartan	25	25.25	3.08	0.54
	250	248.57	5.03	0.59
	1000	1014.66	5.99	1.62
Ramipril	1	1.01	4.29	2.60
	25	25.23	6.17	2.92
	100	101.76	4.60	3.28
Simvastatin	1	1.06	10.01	6.81
	25	25.13	6.43	0.86
	100	99.85	3.98	2.11
Valsartan	250	242.75	3.71	1.44
	2000	2078.29	3.32	3.44
	4000	4060.6	1.17	0.44

Table 3
Matrix effect results obtained for the ten target drugs studied at the low, medium and high concentration levels. (n = 6 for each concentration).

Drug	Nominal conc. (ng/ml)	Matrix effect % (mean)	Precision (CV%)
Atenolol	50	-1.94	5.59
	500	0.84	2.03
	1500	-1.86	1.72
Atorvastatin	1	2.41	1.65
	25	1.25	1.93
	100	1.95	1.29
Bisoprolol	1	-1.39	2.17
	25	0.41	2.73
	100	0.67	0.98
Diltiazem	5	1.43	2.75
	100	0.06	3.03
	600	1.49	1.33
Doxazosin	1	0.60	2.76
	25	0.73	1.69
	100	-0.85	2.01
Lisinopril	1	8.91	4.55
	25	5.99	1.60
	100	2.54	2.33
Losartan	25	0.94	1.72
	250	2.07	1.51
	1000	0.51	0.93
Ramipril	1	0.35	2.86
	25	0.54	2.94
	100	1.98	0.34
Simvastatin	1	7.01	6.23
	25	-3.62	5.43
	100	-4.56	5.68
Valsartan	250	-1.12	2.71
	2000	-1.70	2.97
	4000	-2.84	1.50

Table 4
Recovery data for the 10 target drugs extracted from DBS at the low, medium and high concentration levels (n = 6).

Drug	Nominal conc. (ng/ml)	Recovery (%)	Standard Deviation (SD)	Precision (CV%)
Atenolol	50	89.13	6.53	7.32
	500	82.54	7.60	9.21
	1500	93.16	3.69	3.96
Atorvastatin	1	101.09	10.24	10.13
	25	95.43	7.25	7.60
	100	99.76	1.64	1.64
Bisoprolol	1	101.65	11.34	11.16
	25	99.19	5.68	5.73
	100	89.53	5.52	6.16
Diltiazem	5	98.08	12.42	12.67
	100	88.92	4.24	4.77
	600	85.05	1.80	2.11
Doxazosin	1	97.86	7.07	7.23
	25	97.37	5.00	5.14
	100	94.89	6.19	6.52
Lisinopril	1	97.43	9.08	9.32
	25	90.51	7.88	8.71
	100	75.39	4.65	6.17
Losartan	25	97.34	4.03	4.14
	250	94.27	10.25	10.88
	1000	87.1	4.61	5.30
Ramipril	1	97.08	7.15	7.37
	25	89.94	5.38	5.98
	100	92.96	3.36	3.62
Simvastatin	1	67.88	4.26	6.28
	25	64.74	5.97	9.22
	100	70.81	3.96	5.59
Valsartan	250	100.66	3.44	3.41
	2000	97.35	2.29	2.35
	4000	88.67	9.11	10.28

Table 5

Impact of dried blood spot size on accuracy and precision of assay at the medium and high concentrations for each target drug (n = 6)

Atenolol concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
500	40	523.84 ± 9.03	4.77	1.72
	30	489.10 ± 19.27	2.18	3.94
	20	494.26 ± 17.82	1.15	3.61
1500	40	1492.36 ± 129.02	0.51	8.65
	30	1456.05 ± 12.75	2.93	0.88
	20	1590.79 ± 16.73	6.05	1.05
Atorvastatin concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
25	40	24.33 ± 2.25	2.26	9.24
	30	24.55 ± 2.06	1.81	8.39
	20	24.80 ± 3.11	0.79	12.54
100	40	100.94 ± 3.90	0.94	3.86
	30	98.32 ± 2.83	1.68	2.88
	20	100.35 ± 2.75	0.35	2.74
Bisoprolol concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
25	40	25.41 ± 2.62	1.65	10.33
	30	22.96 ± 0.71	8.17	3.07
	20	25.25 ± 1.07	0.99	4.22
100	40	99.93 ± 1.41	0.07	1.42
	30	101.52 ± 7.10	1.52	6.99
	20	105.27 ± 2.95	5.27	2.8
Diltiazem concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
100	40	92.51 ± 5.40	7.49	5.84
	30	93.18 ± 6.23	6.82	6.69
	20	91.70 ± 5.59	8.3	6.1
600	40	595.19 ± 34.09	0.8	5.73
	30	590.04 ± 10.84	1.66	1.84
	20	615.61 ± 4.35	2.6	0.71
Doxazosin concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
25	40	25.37 ± 1.19	1.46	4.68
	30	26.26 ± 0.96	5.03	3.64
	20	25.71 ± 1.04	2.83	4.05
100	40	100.77 ± 5.74	0.77	5.69
	30	98.96 ± 2.17	1.04	2.2
	20	103.19 ± 1.69	3.19	1.63

334

Table 5 continued

Lisinopril concentration in whole blood (ng/ml)	DBS volume (µl)	Mean concentration found ±SD (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
25	40	24.01 ± 1.02	3.96	4.27
	30	26.47 ± 2.39	5.87	9.04
	20	25.81 ± 2.18	3.25	8.44
100	40	102.00 ± 7.91	2	7.75
	30	100.21 ± 5.04	0.21	5.03
	20	107.93 ± 3.41	7.93	3.16
Losartan concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
250	40	251.40 ± 3.90	0.56	1.55
	30	251.87 ± 2.51	0.75	1
	20	250.16 ± 6.41	0.07	2.56
1000	40	1012.38 ± 43.75	1.24	4.32
	30	987.23 ± 20.32	1.28	2.06
	20	1017.71 ± 14.84	1.77	1.46
Ramipril concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
25	40	24.80 ± 1.06	0.81	4.26
	30	25.84 ± 0.95	3.36	3.69
	20	24.67 ± 0.82	1.33	3.31
100	40	101.18 ± 4.86	1.18	4.81
	30	99.59 ± 1.09	0.41	1.1
	20	102.95 ± 2.18	2.95	2.12
Simvastatin concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
25	40	25.46 ± 1.77	1.82	6.95
	30	25.57 ± 0.88	2.27	3.44
	20	25.14 ± 0.54	0.58	2.16
100	40	105.55 ± 6.18	5.55	5.86
	30	100.84 ± 3.11	0.84	3.08
	20	100.91 ± 1.87	0.91	1.86
Valsartan concentration in whole blood (ng/ml)	**DBS volume (µl)**	**Mean concentration found ±SD (ng/ml) (n=6)**	**Accuracy (RE%)**	**Precision (CV%)**
2000	40	1942.50 ± 17.02	2.87	0.88
	30	1943.26 ± 11.80	2.84	0.61
	20	1988.18 ± 83.18	0.59	4.18
4000	40	4038.38 ± 77.57	0.96	1.92
	30	4075.53 ± 83.71	1.89	2.05
	20	4149.79 ± 26.93	3.74	0.65

335

336

Table 6

Influence of Haematocrit on the accuracy (RE %) of analyte quantification presented as the difference from the analyte/internal standard peak area ratio at the 45% Hct level. Precision (CV %) values for each tested concentration are shown in brackets (n = 6).

		Haematocrit		
Drug	Concentration (ng/ml)	35%	45% (Normalized)	55%
Atenolol	50	-7.4% (4.1%)	(5.9%)	8.8% (3.5%)
	500	-7.6% (1.5%)	(2.6%)	14.5% (5.0%)
	1500	-8.4% (3.6%)	(1.9%)	6.4% (2.1%)
Atorvastatin	1	-4.1% (6.04%)	(10.1%)	-4.0% (12.8%)
	25	-15.3% (2.67%)	(6.6%)	12.5% (7.7%)
	100	-14.6% (3.65%)	(3.0%)	-2.2% (2.6%)
Bisoprolol	1	-10.2% (9.2%)	(5.1%)	11.2% (10.5%)
	25	-12.4% (4.6%)	(15.1%)	13.8% (5.5%)
	100	-14.4% (7.3%)	(7.0%)	7.9% (4.7%)
Diltiazem	5	-9.4% (6.3%)	(10.1%)	13.1% (5.5%)
	100	-7.1% (10.6%)	(6.6%)	13.9% (2.8%)
	600	-12.3% (2.4%)	(3.0%)	10.5% (1.5%)
Doxazosin	1	-14.1% (5.2%)	(10.3%)	3.1% (7.8%)
	25	-3.0% (4.6%)	(3.9%)	2.8% (2.1%)
	100	-7.9% (4.2%)	(5.5%)	5.7% (3.3%)
Lisinopril	1	-10.7% (10.3%)	(10.1%)	8.5% (6.1%)
	25	-12.8% (4.7%)	(6.6%)	3.4% (8.7%)
	100	-6.6% (10.5%)	(3.0%)	10.3% (10.1%)
Losartan	25	-14.3% (7.0%)	(5.0%)	7.14% (6.6%)
	250	-9.8% (2.2%)	(7.9%)	10.9% (6.0%)
	1000	-9.3% (5.6%)	(6.1%)	2.7% (1.9%)
Ramipril	1	-10.6% (14.2%)	(6.1%)	12.8% (7.8%)
	25	-10.1% (4.1%)	(5.9%)	7.2% (6.2%)
	100	-9.1% (1.7%)	(6.2%)	1.4% (1.37%)
Simvastatin	1	1.5% (12.3%)	(10.1%)	(-13.4%) (3.8%)
	25	-13.3% (6.0%)	(6.6%)	11.5% (7.4%)
	100	-3.1% (2.9%)	(3.0%)	9.5% (8.9%)
Valsartan	250	-11.5% (5.5%)	(1.6%)	-5.4% (8.2%)
	2000	-7.6% (7.2%)	(8.2%)	13.6% (11.5%)
	4000	-11.4% (6.0%)	(12.5%)	11.6% (3.7%)

Table 7

Accuracy, precision and quantification of DBS assay at the low, medium and high concentrations for each target drug after 10 weeks of storage at room temperature (n = 6)

Drug	Concentration in whole blood (ng/ml)	Mean concentration found (ng/ml) (n=6)	Accuracy (RE%)	Precision (CV%)
Atenolol	50	59.9	12.06	1.11
	500	464.47	0.52	2.58
	1500	1572.7	-0.69	0.85
Atorvastatin	1	1.2	-1.34	11.69
	25	27.64	0.17	8.34
	100	91.51	-1.58	2.10
Bisoprolol	1	1.19	4.77	9.57
	25	28.21	-2.13	2.68
	100	116.01	4.50	5.74
Diltiazem	5	4.7	4.51	4.68
	100	109.97	1.93	5.64
	600	631.98	-3.95	2.64
Doxazosin	1	1.11	10.74	6.68
	25	27.93	3.47	5.52
	100	100.45	-0.50	0.61
Lisinopril	1	1.13	13.0	9.01
	25	29.13	3.46	6.71
	100	106.95	-2.06	4.21
Losartan	25	23.9	4.40	7.93
	250	259.25	-0.47	2.85
	1000	1111.52	1.66	0.91
Ramipril	1	1.12	12.41	3.66
	25	21.33	5.12	2.09
	100	94.96	2.28	3.13
Simvastatin	1	1.2	4.30	5.45
	25	23.62	-0.89	3.00
	100	95.28	-1.09	2.70
Valsartan	250	242.62	-0.85	6.47
	2000	1972.39	7.35	8.62
	4000	4221.61	-3.20	4.35

Table 8

DBS concentrations of the studied cardiovascular drugs from volunteers prescribed with one or more of the CVD drugs investigated.

N	Sex	Administered Drug	Time after Oral intake (h)	Concentration (ng/ml) ±(SD)	Cmax (ng/ml)
1	M	Bisoprolol 2mg	4	41.78 ± 1.99	37 - 87
		Doxazosin 4mg	4	32.74 ± 1.04	18 - 48
		Valsartan 160mg	4	493.72 ± 8.78	879 - 3874
2	M	Atorvastatin 10mg	11	8.88 ± 0.99	3.2 -10.5
		Losartan 50mg	11	28.95 ± 1.93	89 - 306
3	F	Losartan 75mg	22	20.60 ± 5.65	263 - 783
4	F	Simvastatin 20mg	13	2.90 ± 0.77	5.1 - 40.1
5	F	Ramipril 1.25mg	5	3.11 ± 0.37	<11.1 - 31.1
6	F	Losartan 100mg	5.5	11.60 ± 1.51	469 - 1131
7	M	Losartan 5mg	7	6.25 ± 3.41	89 -306
8	M	Atorvastatin (lowest)	16	6.11 ± 2.21	3.2 -10.5
9	F	Atorvastatin 20mg	17	6.77 ± 3.84	5.0 -20.5
10	M	Ramipril 5mg	15	5.22 ± 0.31	<11.1 - 31.1
		Simvastatin 20mg	15	1.79±0.74	5.1 - 40.1
11	M	Atorvastatin 10mg	14	5.21±1.99	3.2 -10.5
12	M	Bisoprolol 2mg	4	34.32±12.87	37 - 87
		Doxazosin 4mg	4	32.40±2.13	18 - 48
		Valsartan 160mg	4	407.16±14.73	879 - 3874
13	M	Simvastatin	11	0.85±0.55	5.1 - 40.1
		Ramipril 10mg	2.5	9.37±1.04	11.1 - 31.1
14	F	Atorvastatin 10mg	17	2.86±1.72	3.2 -10.5
		Losartan 100mg	7	65.48±3.72	469 - 1131
15	F	Losartan 100mg	6	74.76±8.03	469 - 1131
16	M	Atenolol 50mg	6	456.01±23.20	240 - 1370
		Simvastatin 40mg	6	<LOQ	5.1 - 40.1
17	F	Ramipril 10mg	18	<LOQ	11.1 - 31.1
18	F	Atorvastatin 20mg	14	14.01±2.39	5.0 -20.5
		Bisoprolol 5mg	3	23.58±1.94	37 - 87
19	M	Lisinopril 20mg	?	37.02±8.59	50 - 88
20	M	Ramipril 10mg	4	5.29±0.84	11.1 - 31.1
		Simvastatin 20mg	10	1.32±0.42	5.1 - 40.1
21	F	Ramipril 5mg	2.5	5.63±0.54	<11.1 - 31.1
22	M	Atorvastatin 40mg	> 48	<LOQ	13.2 -44.3
		Lisinopril 2.5mg	3.5	8.02±3.68	<50 - 88
23	F	Losartan 12.5mg	12	37.57±2.54	43.6 - 125.4
24	F	Bisoprolol 1.25mg	0.3	9.28±0.55	17 - 87
25	F	Ramipril 10mg	4	7.03±0.39	11.1 - 31.1
26	F	Ramipril 2.5mg	3	6.49±0.96	<11.1 - 31.1
27	F	Atorvastatin 40mg	15	18.36±7.20	13.2 - 44.3
		Bisoprolol 5mg	8	24.46±5.70	37 - 87
28-32	F	None - Controls	N/A	<LOQ	
33-37	M	None - Controls	N/A	<LOQ	

353 3.1 Selectivity

354 Using the accurate masses determined for the 10 cardiovascular drugs and internal standard,
355 selectivity was evaluated by comparing extracted ion chromatograms (EICs) derived at the limit of
356 quantification from a DBS calibration standard for each target analyte and the internal standard with
357 those obtained from blank DBS samples. A narrow mass extraction window of 5ppm was used to
358 obtain enhanced selectivity. Representative EICs at the LOQ for each analyte and internal standard is
359 shown in Figure 1(a) – (k). The protonated molecule $[M+H]^+$ gave a high response for atenolol at m/z
360 267.1703, atorvastatin at m/z 559.2610, bisoprolol at m/z 326.2326, diltiazem at m/z 415.1686,
361 doxazosin at m/z 452.1928, lisinopril at m/z 406.2336, losartan at m/z 423.1695, ramipril at m/z
362 417.2384, and valsartan at m/z 436.2343. The sodium adduct ion $[M+Na]^+$ showed the highest signal
363 intensity for simvastatin at m/z 441.2611. The DBS based LC-HRMS method showed good selectivity
364 because the EICs revealed that no interfering peaks were observed at the retention times for each of
365 the ten drugs and IS.

366 3.2 Linearity and sensitivity

367 The calibration curves for the ten target analytes were generated in replicate (n = 6) using a plot of
368 target analyte/IS peak area ratio against nominal analyte concentration. An equally weighted linear
369 regression was applied. Back calculations gave relative errors less than 15% (typically between 2 and
370 10% over the appropriate calibration range for each drug. The data (slope, intercept and the mean
371 correlation coefficient R^2) for each drug is presented in Table 1. The limit of quantification (LOQ)
372 with a signal to noise ratio of ≥10 and the required assay accuracy and precision was 10ng/ml for
373 atenolol, 0.5ng/ml for atorvastatin, 0.1ng/ml for bisoprolol, 0.5ng/ml for diltiazem, 0.1ng/ml for
374 doxazosin, 0.1ng/ml for lisinopril, 5ng/ml for losartan, 0.1ng/ml for ramipril, 0.1ng/ml for
375 simvastatin, 50ng/ml for valsartan.

376 3.3 Accuracy and precision

377 The accuracy and precision of the developed LC-HRMS method were determined by intra and inter-
378 day replicate analyses of six spiked DBS (QC) samples containing the 10 target analytes at the low,
379 medium and high concentration levels on three separate days. Accuracy was expressed as the mean
380 relative error (RE %) and precision was expressed as the coefficient of variation (CV %) and data
381 obtained for both were within the predefined 15% limit for all concentrations in each run for all the
382 target drugs. The overall variation in data between runs was also ≤15% for all target drugs. A
383 summary of the results is presented in Table 2.

384 3.4 Matrix effect

385 The effect of matrix arising from ionization competition between analytes of interest and co-eluents
386 [38] was examined to ensure that the sensitivity and precision of the developed method was not
387 compromised. The matrix effect data obtained for each target analyte investigated at the low,
388 medium and high concentration levels of the calibration curve is presented in Table 3. No significant
389 (<10%) matrix effects on the analyte signal due to endogenous components of blood or the sampling
390 paper was observed at the three tested concentrations of each target drug. These results
391 demonstrate the robustness of the extraction procedure and the ionisation mechanism for these

target analytes. The introduction of several compounds as I.S could also lead to ionization competition with the analytes of interest at the ESI source resulting in additional matrix effects.

3.5 Recovery

The extraction recoveries of the ten target analytes from DBS samples at the low, medium and high concentration levels of the calibration curve were obtained. Recoveries for atenolol, atorvastatin, bisoprolol, diltiazem, doxazosin, losartan, ramipril and valsartan were consistent, with values between 87 and 98%. The high recoveries observed indicate analyte stability under the extraction conditions applied and good extraction. The overall mean recovery for simvastatin was the lowest at 68%. Recovery data for each target analyte at the low, medium and high concentration levels is summarised in Table 4.

3.6 Blood spot size

Method precision and accuracy were assessed using extraction data from an 8 mm discs, sampled from the centre of the 20, 30 and 40 µl volume DBS prepared at the medium and high concentration levels for the ten target analytes. Table 5 shows the intra-day precision and accuracy of the method evaluated using 6 determinations for each concentration level. Results obtained for accuracy and precision were less than 15% and therefore considered acceptable. These experiments were performed to demonstrate that results obtained were not dependent on the size of the blood spot collected. Analysing a fixed sample size disc should produce extract data which is directly proportional to the concentration of the target analyte in the original blood sample assuming that each blood spot will spread evenly and uniformly across the sampling card. The results in Table 5 affirm that within experimental error for each concentration range the data from 8 mm discs is the same regardless of sample volume chosen.

3.7 Haematocrit (Hct) evaluation

Concentrations of extracts were determined using a linear regression equation generated from a calibration produced from standards prepared with the 45% Hct. A decrease in size of spots formed was observed with increasing Hct value across the range of 35% to 55% investigated. The results from the haematocrit investigation, shown in Table 6, gave accuracy (RE%) and precision (CV%) values within the pre-defined limit of ≤ 15% [32] at all haematocrit levels for each tested analyte concentration, except for atorvastatin at the 35% Hct where accuracy was 15.3%. This demonstrates the acceptability of the developed DBS based LC-HRMS method for quantitative analyses. The results also demonstrate the robustness of the extraction procedure, as different haematocrits do not result in differences in matrix effects.

3.8 Stability

The stability of dried blood spot samples after 10 weeks of storage at room temperature was determined by analysing blood spots prepared at the low, medium and high concentration levels for the ten target drugs. No significant changes in concentrations were observed at the low, medium and high concentration levels of target drugs as shown in Table 7. These results demonstrate that for spiked samples the ten target drugs are stable in DBS for 2 and half months when stored at room temperature. Studies in this laboratory have shown similar stability for atenolol, bisoprolol, simvastatin and valsartan in ‘real’ DBS samples from volunteers. It also affirms the feasibility of using

DBS microsampling methodology in resource limited areas for example Africa. This is because samples may have to be collected in remote areas of the country and will take several days to be transported back to the laboratory for analyses.

3.9 Application of method to volunteer DBS samples

Volunteers were chosen either because they were prescribed one or more of the target medications or they were receiving no medication at all. DBS samples from volunteers not prescribed any of the target drugs were analysed and used as blank reference samples. DBS samples were obtained from each volunteer by gently massaging the fingertip to encourage blood flow. The finger was pricked with a retractable lancet and the first drop of blood wiped away with a sterile gauze. Subsequent drops were deposited onto marked sections on a Whatman 903 sampling card and allowed to dry. The spot sizes were sufficient to allow the use of an 8mm punch without compromising the DBS sample. Samples of smaller spot sizes were rejected. The validated DBS based LC-HRMS method was successfully used for the identification and quantification of 10 target cardiovascular drugs in 146 dried blood spot samples obtained from a group of volunteers. No false signals were detected from DBS samples from volunteers receiving no medication. Where adherent volunteer samples were analysed the anticipated drug was detected. Furthermore there were no false positive signals for volunteers taking chemically related drugs, for example, atenolol and bisoprolol.

The measured DBS drug concentrations obtained are presented in Table 8. The eclectic Cmax data from the literature for the individual drugs has also been included in Table 8 to provide reference values against which volunteer data can be compared. Values similar to, but lower than, the Cmax concentration would be anticipated from volunteers who are adherent to prescribed medication. On this basis the data in Table 8 would suggest that concern might be raised over the results from:

- volunteer 16 - where atenolol was detected but there was no detectable simvastatin
- volunteer 17 – no detectable ramipril signal
- volunteer 22 - no detectable atorvastatin signal but the anticipated lisinopril was detected

Data from volunteer 16 raised concern initially because both drugs were stated to have been taken at the same time whereas simvastatin should be taken in the evening. It may be that the patient was distracted and took two atenolol tablets rather than one simvastatin tablet. This would lead to a DBS atenolol level corresponding to a 100mg dose as actually observed by the correlation between the measured concentration and the Cmax data for a 100mg dose [39]. Non detectable simvastatin suggests that the patient was non-adherent bearing in mind that volunteers 4, 10 and 20 took simvastatin at a lower dose of 20mg and which was still detected after 10 hours. Data from volunteer 17 showed no detectable level of ramipril, the prescribed drug but, according to the volunteer, the sample was collected 18 hours after the dose was taken and might not be detectable. In this case the dose was 10mg and as can be seen for volunteer 10, prescribed a 5mg dose, levels of ramipril were detected 15 hours after taking a dose. This would suggest that volunteer 17 needs to discuss this situation with the clinician and it should be remembered that pharmacogenetics effects may lead to unexpected changes in drug levels in the blood. Several studies have demonstrated a significant link between angiotensin converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism and cardiovascular outcomes. However, the impact of this genetic polymorphism on ACE inhibitor response is not well understood [40, 41].

When asked about the data obtained volunteer 22 freely admitted not taking atorvastatin tablets for several days and was clearly non-adherent to the prescribed medication. These results clearly indicate areas where a clinician would be unaware of an adverse clinical condition which they would be able to rectify to improve the individuals healthcare. This also demonstrates the robustness of the developed DBS based LC-HRMS method. This approach can also identify the situation where a dose is taken because a test is anticipated (white coat syndrome). This is comparable to a single dose trial and the pharmacokinetics would lead to a rapid increase followed by a decrease in the drug concentration in the blood, rather than a steady state situation. A comparison of drug concentrations in two DBS samples collected several hours apart, from the same volunteer, would clarify the situation. Significantly less in the second sample would indicate that the dose was taken in anticipation of the test whereas a comparable level is indicative of a steady state as a result of adherence to prescription.

4. Conclusion

The developed and validated DBS based LC–HRMS method offers fast analyses time and the sensitivity required for the determination of the ten cardiovascular drugs in DBS samples. The method gave accuracy (RE) and precision (CV) values of ≤ 15% at all tested concentrations for the ten target drugs. Stability of the ten analytes in DBS following storage at room temperature was shown to be 10 weeks. This offers the possibility of batch wise preparation and also allows time for the transportation of samples from remote or resource limited areas to the laboratory for analyses. Haematocrit effects was observed but was not significant as accuracy (RE%) and precision (CV%) values obtained were with ≤ 15% limit at all haematocrit levels for each tested analyte concentration. The method has great potential in aiding clinicians indicate adherence to prescribed medication to enable treatment to be optimised for patients. The method is currently being extended to study adherence to prescribed cardiovascular medication in a multi-ethnic inner city community.

References

[1] World Health Organization, Cardiovascular diseases (CVDs). http://www.who.int/mediacentre/factsheets/fs317/en/, 2016 (accessed 13.07.16).

[2] Anon British Heart Foundation Statistics Database UK. Economic cost. Coronary Heart Disease, economic cost. https://www.bhf.org.uk/, 2013 (accessed 13.07.16).

[3] S. Tanna, G. Lawson, Analytical Chemistry for Assessing Medication Adherence. 1st Edition, Elsevier, New York, (2016) pp. 13-16.

[4] E. Sabate, Adherence to Long – Term Therapies: Evidence for Action. Switzerland, World Health Organization. http://www.who.int/chp/knowledge/publications/adherence_report/en/ (2003) (assessed 13.07.16).

[5] I.M. Kronish, S. Ye, Adherence to cardiovascular medications: lessons learned and future directions, Prog Cardiovasc Dis. 55(6), (2013) 590-600.

[6] British Heart Foundation UK, Cardiovascular Disease Statistics. https://www.bhf.org.uk/publications/statistics/cvd-stats-2015/, 2015 (assessed 13.07.16).

[7] National Institute for Clinical Excellence UK, Costing statement: Medication adherence. https://www.nice.org.uk/guidance/cg76/resources/costing-statement-242056477 (accessed 13.07.16).

[8] A.O. Iuga, M.J. McGuire, Adherence and health care costs, Risk Manag Healthc Policy. 7 (2014) 35 - 44.

[9] A. La Caze, G. Gujral, W.N. Cottrell, How do we better translate adherence research into improvements in patient care, Int J Clin Pharm. 36(1) (2014) 10-14.

[10] A. Lehmann, P. Aslani, R. Ahmed, J. Celio, A. Gauchet, P. Bedouch, M.P. Schneider, Assessing medication adherence: options to consider, Int J Clin Pharm. 36(1) (2014) 55-69.

[11] M.B. Neiheisel, K.J. Wheeler, M.E. Roberts, Medication adherence part one: Understanding and assessing the problem, J Am Assoc Nurse Pract. 26(1) (2014) 49-55.

[12] S. Tanna, G. Lawson, Dried blood spot analysis to assess medication adherence and to inform personalization of treatment, Bioanalysis. 6(21) (2014) 2825-2838.

[13] H. Hooman, T.L. Robertson, G.D. Moon, K.Y Au-Yeung, M.J. Zdeblick, G.M. Savage. An ingestible sensor for measuring medication adherence. IEEE Trans Biomed Eng. 62(1) (2015) 99-109.

[14] J.C. Kvedar, T. Nesbitt, J.G. Kvedar, A. Darkins. E-patient connectivity and the near-term future. J Gen Intern Med. 26(2) (2011) 636-638.

[15] I. Hamdidouche, V. Jullien, E.M. Billaud, P. Boutouyrie, M. Azizi, S. Laurent, 7B. 06: Routine urinary detection of antihypertensive drugs for estimation of adherence to treatment: a cross sectional study, J Hypertens. 33 (2015) e93.

[16] M. Tomaszewski, C. White, P. Patel, N. Masca, R. Damani, J. Hepworth, N.J. Samani, P. Gupta, W. Madira, A. Stanley, B. Williams, High rates of non-adherence to antihypertensive treatment revealed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) urine analysis, Heart. 100 (2014) 855-861.

[17] A. Morrison, M. Stauffer, A. Kaufman, Defining medication adherence in individual patients, Patient Prefer Adherence. (2015) 893 - 897.

[18] O. González, N. Ferreirós, M. Blanco, R. Alonso, Cardiovascular drug determination in bioanalysis: an update, Bioanalysis. 7(18) (2015) 2399-2417.

[19] O. Gonzalez, R.M. Alonso, N. Ferreirós, W. Weinmann, R. Zimmermann, S. Dresen, Development of an LC–MS/MS method for the quantitation of 55 compounds prescribed in combined cardiovascular therapy, J Chromatogr B. 879(3) (2010) 243-252.

[20] O. Gonzalez, G. Iriarte, E. Rico, N. Ferreiros, M. ItxasoMaguregui, R. Maria Alonso, R. Maria Jimenez, LC-MS/MS method for the determination of several drugs used in combined cardiovascular therapy in human plasma, J Chromatogr B Analyt Technol Biomed Life Sci. 878(28) (2010) 2685-2692.

[21] E. Dias, B. Hachey, C. McNaughton, H. Nian, C. Yu, B. Straka, R.M. Caprioli, An LC–MS assay for the screening of cardiovascular medications in human samples, J Chromatogr B. 937 (2013) 44-53.

[22] A. Sharma, S. Jaiswal, M. Shukla, J. Lal, Dried blood spots: Concepts, present status, and future perspectives in bioanalysis, Drug Test Anal. 6(5) (2014) 399-414.

[23] A. Deep, P. Kumar, A. Kumar, A. Thakkar, Dry blood spot technique: A review, Int J Pharm Sci Rev Res. 15(2) (2012) 90-94.

[24] N. Spooner, A dried blood spot update: still an important bioanalytical technique? Bioanalysis. 5(8) (2013) 879-883.

[25] G. Lawson, E. Cocks, S. Tanna, Quantitative determination of atenolol in dried blood spot samples by LC-HRMS: A potential method for assessing medication adherence, J Chromatogr B Analyt Technol Biomed Life Sci. 897 (2012) 72-79.

[26] G. Lawson, E. Cocks, S. Tanna, Bisoprolol, ramipril and simvastatin determination in dried blood spot samples using LC-HRMS for assessing medication adherence, J Pharm Biomed Anal. 81-82 (2013) 99-107.

[27] G. Lawson, H. Mulla, S. Tanna, Captopril determination in dried blood spot samples with LC-MS and LC-HRMS: A potential method for neonate pharmacokinetic studies, J Bioanal Biomed. 4 (2012) 016-025.

[28] P. Patel, An investigation into the use of dried blood spot analysis in pharmacokinetic studies, Doctoral Thesis (2011). De Montfort University, Leicester UK. p 59.

[29] Draft guidance for industry: bioanalytical method validation, US Department of Health and Human Services, Food and Drugs Administration. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf/, 2013 (accessed 15.07.16).

[30] Guideline on bioanalytical method validation, European Medicine Agency, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf/, 2011 (accessed 15.07.16).

[31] P. Denniff, N. Spooner, The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs, Bioanalysis. 2(8) (2010) 1385-1395.

[32] R. De Vries, M. Barfield, N. Van de Merbel, B. Schmid, C. Siethoff, J. Ortiz, P. Timmerman, The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium, Bioanalysis. 5(17) (2013) 2147-2160.

[33] H.K. Walker, W.D. Hall, and J.W. Hurst, Clinical Methods, The History, Physical and Laboratory examinations, Boston, Butterworths 1990, Chapter 151, p

[34] M. O'mara, B. Hudson-Curtis, K. Olson, Y. Yueh, J. Dunn, N. Spooner, The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples, Bioanalysis. 3(20) (2011) 2335-2347.

[35] L. Fan, J.A. Lee, Managing the effect of hematocrit on DBS analysis in a regulated environment, Bioanalysis. 4(4) (2012) 345-347.

[36] R.A. Koster, J.W.C. Alffenaar, R. Botma, B. Greijdanus, D. J. Touw, D.R. Uges, J.G. Kosterink, What is the right blood hematocrit preparation procedure for standards and quality control samples for dried blood spot analysis? Bioanalysis. 7(3) (2015) 345-351.

[37] Hettich Laboratory Application note, determination of the hematocrit value by centrifugation. http://www.hettichlab.com/documents/22402/225be2b3-ef94-487b-a4b3-532e0c766f40/, (accessed 12.09.16).

[38] L. Tang, P. Kebarle, Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution, Anal Chem. 65(24) (1993) 3654-3668.

[39] F. Wu, P. Chen, Y. Lee, R. Chen, Comparative pharmacokinetics of two atenolol products. J Food Drug Anal. 11(1), (2003) 4-7.

[40] F. Cambien, The angiotensin converting enzyme (ACE) genetic polymorphism: its relationship with plasma ACE level and myocardial infarction. Clin Genet. 46 (1994) 94–101.

[41] P.G. Joseph, P. Guillaume, R. Stephanie, R. Roberts, S. S. Anand, Pharmacogenetics in Cardiovascular Disease: The Challenge of Moving from Promise to Realization. Clin Cardiol. 37(1) (2014) 48-56.