Volumetric absorptive microsampling (VAMS) coupled with high-resolution, accurate-mass (HRAM) mass spectrometry as a simplified alternative to dried blood spot (DBS) analysis for therapeutic drug monitoring of cardiovascular drugs

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Abstract

Here, volumetric absorptive microsampling (VAMS), used for the measurement of cardiovascular drugs, is compared against conventional dried blood spot (DBS) card sampling to evaluate adherence to prescribed medication. Volumetric absorptive microsampling (VAMS) is an attractive alternative to plasma sampling for routine drug monitoring and potentially overcomes haematocrit issues associated with quantitative bioanalysis of conventional dried blood spots. A quantitative VAMS-based LC-HRAM MS assay for atenolol, lisinopril, simvastatin and valsartan was developed and validated. The assay demonstrated acceptable linearity, selectivity, accuracy, precision, recovery and insignificant matrix effects with no impact of haematocrit on assay accuracy. Volunteers provided both VAMS and DBS 903 card samples (the current standard) to allow comparison of the two methods and demonstrate the potential utility of VAMS. Analysis of VAMS samples correctly identified drugs in volunteers known to be adherent, and found no false positives from volunteers known to be taking no medication. There was a strong correlation between the two sampling systems confirming the utility of VAMS. Therapeutic drug monitoring (TDM) can assist clinicians in deciding how to proceed with treatment in the event of poor improvement in patient health. VAMS could offer a potentially more efficient method of sample collection, with fewer rejected samples than the DBS approach.

Key Words

Volumetric absorptive microsampling (VAMS), Dried blood spot (DBS), Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS), Medication adherence, compliance, therapeutic drug monitoring, Atenolol, Lisinopril, Simvastatin, Valsartan

Abbreviations: Cardiovascular disease (CVD); Coefficient of variation (CV); Dried blood spot (DBS); Extracted ion chromatogram (EIC); Haematocrit (Hct); Internal standard (IS); Limit of quantification (LOQ); Liquid chromatography (LC); Liquid chromatography–high resolution, accurate mass mass spectrometry (LC-HRAM MS); Liquid chromatography-mass spectrometry (LC-MS); Liquid chromatography-tandem mass spectrometry (LC-MS/MS); Quadrupole Time of Flight (QTOF); Quality control (QC); Relative error (RE); Therapeutic drug monitoring (TDM); Volumetric absorptive microsampling (VAMS)

Highlights

- VAMS was used for the quantification of four CVD therapy drugs to assess adherence.
- LC-HRAM MS was used to quantify atenolol, lisinopril, simvastatin and valsartan.
- VAMS showed no haematocrit effect because there is fixed volume sampling.
- VAMS and DBS gave comparable drug concentrations for each individual volunteer.
- The VAMS based assay has good potential for routine TDM for self-collected samples.

Introduction

Cardiovascular disease (CVD) is a major global killer, responsible for 17.7 million deaths every year [1]. In the UK, around 7 million people are affected with an estimated 155,000 deaths each year. CVD is a major economic burden with annual UK healthcare costs estimated at £11 billion [2]. The current medical care of CVD patients uses a combination of cardiovascular therapy drugs to treat high blood pressure and lower cholesterol [2]. The drug(s) selected and the dose(s) prescribed are structured to achieve and maintain a therapeutic drug concentration in the blood, with the goal of improving patient status [3]. However, it is reported that >50% of CVD patients do not adhere to their prescribed drug therapy [4, 5]. Medication nonadherence can result in poor clinical outcomes, hospital readmission with unnecessary additional care costs, and sometimes death. Nonadherence is, therefore, a growing concern to clinicians, other healthcare professionals and health service providers [6,7].

Determination of therapeutic drug concentration in the blood can indicate the extent of the patient's adherence to prescribed drug therapy [3]. The quantitative determination of cardiovascular drugs in plasma and serum using either liquid chromatography (LC) – tandem mass spectrometry (MS/MS) or LC-MS is documented [8-11]. However, these investigations required large biosample volumes (e.g., 1 – 10 mL), which can be a challenge to obtain from any patient in repeat routine testing scenarios, such as for therapeutic drug monitoring [3].

Dried blood spot (DBS) microsampling is an alternative approach to conventional liquid blood sample collection for quantitative bioanalysis and provides numerous advantages, for example, self-sampling is possible, the sample has long-term stability and is readily transportable [12]. These advantages, in combination with improved analytical instrument capability, have led to a surge in the use of DBS in various healthcare applications [13, 14]. Our research group has reported on the potential for using a DBS-based micro-sampling assay to assess adherence to selected CVD medications [15-18]. However, it is acknowledged that DBS-based assays present critical issues and challenges that affect result quality; the primary issue being the influence of haematocrit (Hct) on

the accuracy of the quantitative assay [19]. Abu-Rabie et al [19] identified two potential Hct-based assay biases, namely area bias and recovery bias. The area bias, where blood with high Hct is more viscous and leads to the formation of smaller blood spots on DBS cards, is well-documented in the literature [20]. The Hct based recovery bias is less well recognised, but has the potential to cause significant assay bias, particularly for DBS assays with lower (e.g., 60-40%) recovery [19]. The Hct range varies with age for adult males and females and is typically 40-54% and 36-48%, respectively [20]. This Hct-related uncertainty has prompted research into alternative microsampling platforms and has led to the development and introduction of novel collection devices, including volumetric absorptive microsampling (VAMS) [21]. VAMS microsampling devices were designed to provide the advantages of DBS, but circumvent its Hct-driven biases. VAMS facilitates the easy collection and drying of an accurate volume of blood (i.e., 10 μl or 20 μl) on an absorbent white tip attached to a plastic sample handler. This methodology eliminates the volumetric Hct effect associated with conventional DBS sampling when a punched disk is used, whereas VAMS collects a precise volume sample and the entire sample is extracted. VAMS has been used successfully in quantitative bioanalytical assays for therapeutic drug monitoring (TDM) and the determination of pharmacokinetic parameters [22].

Here, we evaluate the performance of VAMS using liquid chromatography – high resolution accurate mass (LC-HRAM) MS-based quantitative determination of four CVD drugs commonly prescribed in the UK. Additionally, we quantitatively compared the VAMS method against a previously published DBS-based [15] method on atenolol, lisinopril, simvastatin, and valsartan. The performance of VAMS was evaluated over a Hct range of 35-55% to confirm the absence of an Hct effect for each of the four drugs. This study describes a novel approach based on a VAMS based LC-HRAM MS assay for the simultaneous quantification of four CVD drugs in human blood with the potential for assessment of adherence.

2. Materials & Methods

2.1 Chemicals and Materials

Atenolol (R-(+), 99%), atenolol d_7, lisinopril, 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). VAMS (Mitra®) devices (10 μL tip size) were purchased from Neoteryx (Torrance, CA, USA). 903 specimen collection paper, polyethylene bags, microcentrifuge tubes (1.5 mL), pipette tips and volumetric pipettes were all purchased from Fisher Scientific (Loughborough, UK). Autosampler vials with 250 μL inserts, vial caps and formic acid were obtained from Agilent Technologies (Cheshire, UK). Heparin-coated blood collection tubes were purchased from International Scientific Supplies Ltd. (Bradford, UK).

Both the collection of fresh blank blood and VAMS and DBS samples from informed volunteers received ethical approval from the De Montfort University Research Ethics Committee. Informed consent was obtained from all participants following the provision of participant information leaflets.

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

Standard stock solutions of atenolol, lisinopril, simvastatin and valsartan were prepared at a concentration of 1mg/mL in methanol. Multicomponent working solutions for each target drug were freshly prepared by diluting the stock solutions with methanol/water (i.e., 70:30, v/v).

Spiked blood standards were prepared by spiking different samples of 950 µL fresh blank blood with 50 µL of one of each multicomponent working solution to yield final blood target drug concentrations of 10, 20, 50, 100, 200, 500, 1000 and 1500 ng/mL for atenolol, 0.1, 0.5, 1, 5, 10, 25, 50, 100 ng/mL for lisinopril and simvastatin and 50, 100, 250, 500, 1000, 2000, 3000, 4000 ng/mL for valsartan. The Hct of the blood was 45%. 50 µL of methanol/water (70:30, v/v) was spiked into 950 µL of fresh blank blood to produce a zero (blank) blood sample. An internal standard (IS), atenolol D_7, stock solution was prepared in methanol at a concentration of 10 µg/mL and diluted further with methanol/water (70:30, v/v) to produce an extraction solvent containing 20 ng/mL of IS.

2.3 Preparation of calibration standards and validation samples

VAMS calibration samples were prepared following the manufacturer's instructions using the 10 µL tip size devices. The upper part of the tip was dipped into a volume of spiked whole blood and blank blood. Care was taken not to completely immerse the tip into the blood in order to prevent overfilling.

DBS calibration samples were prepared as detailed in our previously published work [15].

2.4 Solvent extraction of analytes from VAMS and DBS

Each VAMS tip was separated from the handler and transferred to a 1.5 mL microcentrifuge tube. A 300 µL volume of methanol containing IS (20 ng/mL of atenolol D_7) was used for the extraction of the target drugs because of its optimum extraction efficiency and reduced interference, as shown in previously published work using DBS [15]. Tubes were vortexed for 1 minute, sonicated for 30 minutes in a temperature controlled ultrasonic bath at 40°C and centrifuged at 13200 rpm for 10 minutes. 270 µL of each supernatant was transferred into a new microcentrifuge tube and dried under a gentle stream of nitrogen 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-HRAM MS analyses.

For the 903 sampling paper 30 µL of each prepared standard was spotted onto the sampling paper and allowed to dry for at least 3 hours. Solvent extraction of target analytes from an 8 mm punched disc was carried out using the protocol detailed by Bernieh et al [15].

2.5 LC-HRAM MS conditions

LC-HRAM MS analyses were performed on an Agilent 1290 LC on-line to an Agilent G6530A QTOF mass spectrometer following the previously described conditions with DBS samples [15].

2.6 Validation studies

To demonstrate that the developed bioanalytical method was fit for purpose, validation experiments were conducted in accordance with international guidelines [23, 24]. The validation process includes an assessment of the selectivity, linearity, sensitivity, intra and inter-assay accuracy and precision, limit of quantification (LOQ), matrix effects, and Hct effects for the analytical process. The stability of atenolol, lisinopril, simvastatin and valsartan for extended periods within the analytical procedure were also examined. These experiments were carried out using appropriate low, medium and high concentrations of each analyte in the manner detailed for the DBS samples by Bernieh et al [15].

2.6.1 Selectivity

Conventionally, in analyses of this type, selectivity is obtained via the use of multiple reaction monitoring where the characteristic fragmentation pathways have been previously identified. In this work, the accurate mass of the target analyte ion, measured to within 5 ppm, was used to demonstrate the necessary selectivity. This was achieved by comparison of data from blank VAMS tips and tips spiked with the analytes. The extracted ion chromatograms for the protonated species of atenolol (m/z 267.1703), lisinopril (m/z 406.2336) and valsartan (m/z 436.2343) and the sodium adduct for simvastatin (m/z 441.2611) were used for this comparison.

2.6.2 Linearity and sensitivity

Calibration standards were prepared in replicate (n = 6) and analysed on three separate 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. A signal-to-noise ratio of ≥ 10 was used in order to determine the limit of quantification (LOQ) for atenolol, lisinopril, simvastatin and valsartan in the VAMS extracts. The coefficient of variation at the (LOQ) was determined for each target drug (n = 6) and was shown to be within the ≤20% limit.

2.6.3 Accuracy and precision

The inter- and intra-day accuracy and precision of the reported methodology was assessed by replicate (n = 6) analyses of quality control (QC) samples at the low, medium and high concentrations for each drug. Accuracy was expressed as the relative error (RE%) and precision as the coefficient of variation (CV%). With reference to international guidelines [23,24], a RE and CV of ≤15% was considered acceptable.

2.6.4 Matrix effects

The matrix effects on drug detection at appropriate low, medium and high concentrations, due to constituents within VAMS were assessed in the manner detailed by Bernieh et al [15] using blood samples collected from three different sources. Replicate (n = 6) samples of the four target analytes spiked in blank blood extracts from VAMS and 903 DBS sampling paper [15] to represent low, medium and high concentrations were prepared. The prepared samples were compared with standards of equal concentration spiked into methanol/water (40:60%, v/v) containing 0.1% formic acid for the four target analytes. The matrix effect was calculated using the formula (B/A -1) x 100. Where A represents the ratio of the Target Analyte/IS response from analyte spiked into pure solvent and B represents the ratio of the Target Analyte/IS response from analyte spiked into extracted blank whole blood.

2.6.5 Determination of the recovery of the four target analytes from VAMS

The extraction efficiency, or the recovery, of atenolol, lisinopril, simvastatin and valsartan from spiked VAMS tips was determined using the DBS-based methods detailed in Bernieh et al [15]. It was determined using replicate (n=6) samples prepared at the low, medium and high concentrations for the four target drugs from spiked VAMS. Recovery was determined by comparing the ratios of analyte to IS response from VAMS extracts with those obtained from blank blood spot extracts spiked with solution standards of equal concentration. The recovery was calculated using the formula: % recovery = (analyte-to-IS response of VAMS extract)/(analyte to IS response of post extraction blank VAMS spiked extract) x 100.

2.6.6 Evaluation of the effects of different Hct levels

The effects of different Hct levels on the DBS assay performance have been previously evaluated at the low, medium and high concentrations of each target drug (n = 6) with an adjusted Hct of 35, 45 and 55% [15]. In the present study, the performance of VAMS was evaluated over the same Hct range for the four target drugs. The Hct reference samples were prepared from blank human whole blood centrifuged at 10,000 g for 12 minutes and the plasma generated transferred into a clean microcentrifuge tube. The red blood cell suspension and plasma were mixed in proportions of (35:65, v/v), (45:55, v/v) and (55:45, v/v) to achieve an adjusted whole blood Hct of 35%, 45% and 55%, respectively [25]. These were used to prepare calibration samples on both 903 DBS sampling paper [15] and the VAMS device for the four target analytes at the blank, low, medium and high concentration ranges.

2.6.7 Sample stability on VAMS

The stability of VAMS samples during storage for eight weeks at room temperature was determined by analysing replicate (n=6) VAMS extracts spiked with atenolol, lisinopril, simvastatin and valsartan at low, medium and high concentrations. This was to investigate if batch-wise preparation and subsequent storage would be possible.

2.7 Application of method to volunteer VAMS and DBS samples

The developed LC–HRAM MS analytical methodology was applied to a series of both VAMS and DBS samples collected from two different groups of informed volunteers. The first group of volunteers were all receiving a course of one or more of the target drugs atenolol, lisinopril, simvastatin and valsartan. The second group of volunteers were not prescribed any of the target drugs and the samples collected from these volunteers were used as reference blanks for the analytical process. Where relevant, volunteers were asked to specify the time delay between taking their medication and the time the sample was collected. Typically, four DBS samples (i.e., 4 x 30 µL), and four VAMS samples (i.e., 4 x 10 µL) were collected as quickly as possible from each volunteer. These were collected from either one or two fingerprick samples per patient, as necessary.

Prior to sample collection, it was confirmed that each volunteer had read the information sheet and was willing to progress by signing the consent documentation.

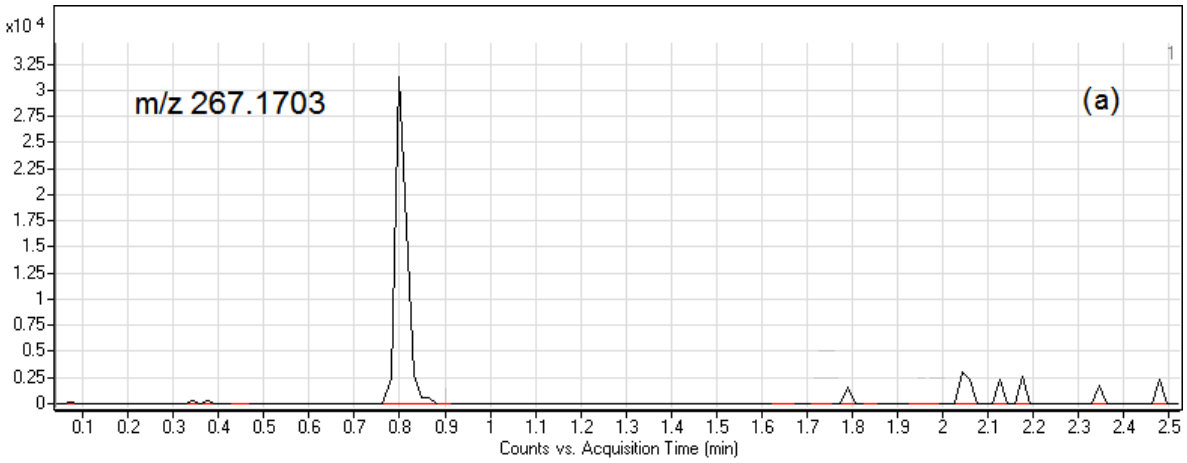
2.8 DBS Summary

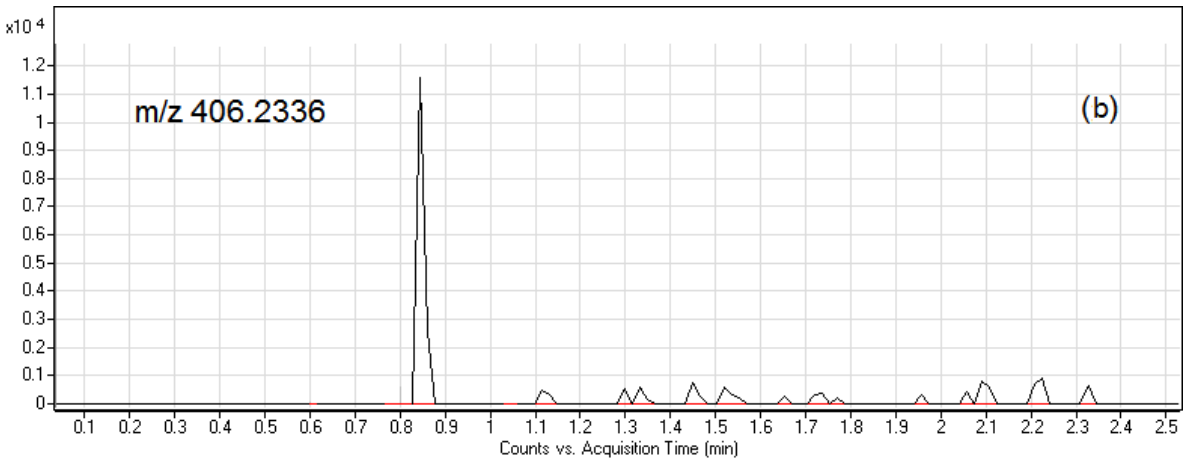
DBS samples were prepared, collected and analysed as detailed in the previously published work by Bernieh et al[15].

3. Results and Discussion

3.1 Selectivity

Using the accurate masses determined for the four cardiovascular drugs and internal standard, selectivity was evaluated by comparing extracted ion chromatograms (EICs) derived at the limit of quantification from a VAMS calibration standard for each target analyte and the internal standard, with those obtained from blank VAMS samples. Selectivity enhancement was obtained by narrowing the m/z extraction window, as demonstrated previously [16]. Using a 5 ppm window for each target drug gave the most intense signal with no other interfering compounds and, therefore, improved selectivity. Representative EICs at the LOQ for each analyte and internal standard are shown in Figure 1(a) – (e). The protonated molecule $[M+H]^+$ gave a high response for atenolol at m/z 267.1703, lisinopril at m/z 406.2336 and valsartan at m/z 436.2343. The sodium adduct ion $[M+Na]^+$ showed the highest signal intensity for simvastatin at m/z 441.2611. The DBS based LC-HRAM MS method also showed strong selectivity, as the EICs revealed no interfering peaks at the retention times for each of the four drugs and IS[15].





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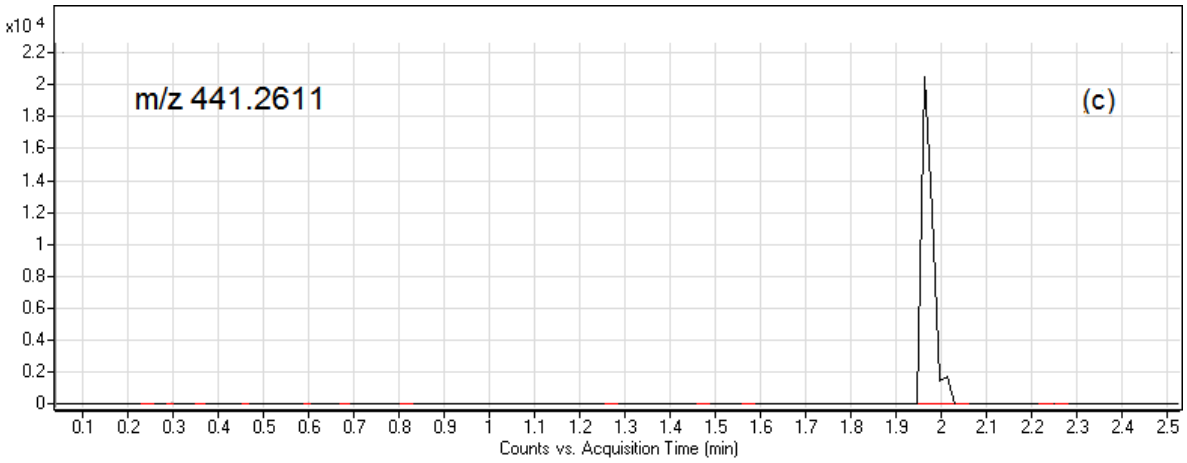

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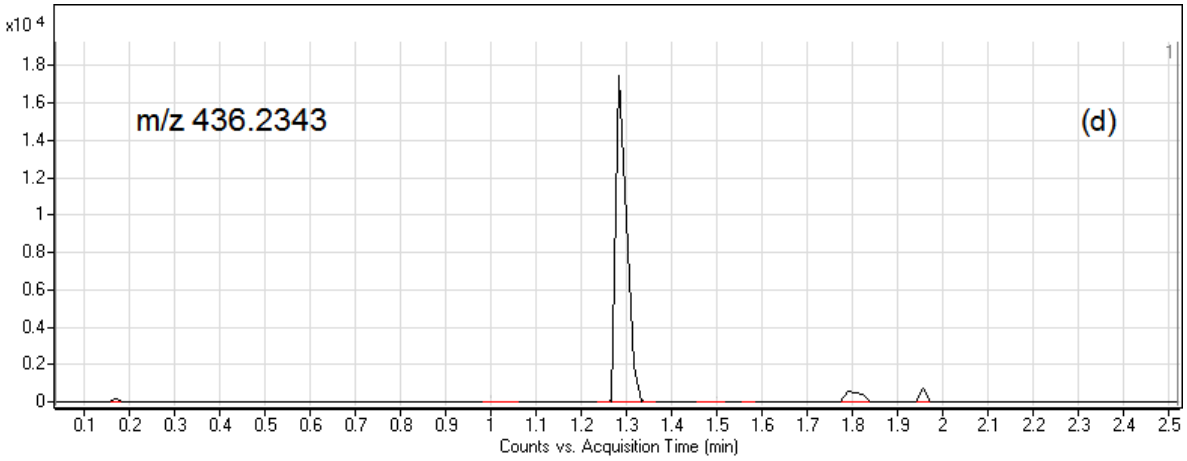

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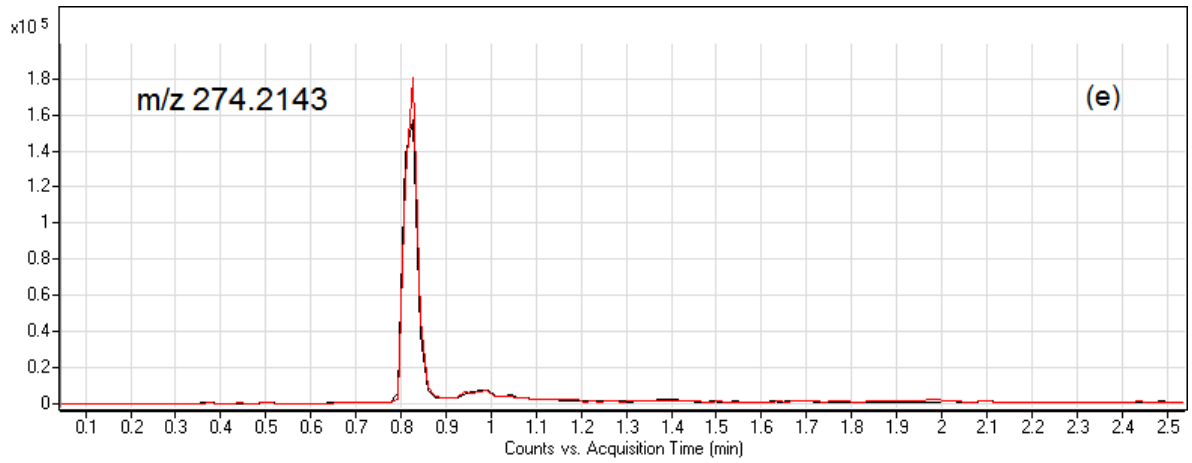

Figure 1. Representative LC-HRAM MS extracted ion chromatograms of a blank VAMS tip extract (red) and a calibration standard at the LLOQ spiked with the four target drugs (black). A narrow mass extraction window (5 ppm) was used for (a) atenolol at m/z 267.1703 (b) lisinopril at m/z 406.2336 (c) simvastatin at m/z 441.2611 (d) valsartan at m/z 436.2343 (e) atenolol d7 (internal standard) at m/z 274.2143.

3.2 Linearity and sensitivity

The calibration plots for the four target analytes in VAMS were generated in replicate (n = 6) using a plot of Target Analyte/IS peak area ratio against nominal analyte concentration. An equally weighted linear regression was applied and the data (i.e., slope, intercept and the mean correlation coefficient R^2) is presented in Table 1. Back calculations indicated a relative error of less than 15% (typically between 2 and 10%) over the appropriate calibration range for each drug. The limit of quantification (LOQ) with a signal to noise ratio of ≥10 and the required assay accuracy and precision was 10 ng/mL for atenolol, 0.1 ng/mL for lisinopril, 0.1 ng/mL for simvastatin and 50 ng/mL for valsartan in whole dried blood.

Table 1. Linearity and sensitivity data for the four cardiovascular drugs

Drug	Range (ng/mL)	y = ax + b	R2	LOQ (ng/mL)
Atenolol	10 - 1500	y = 0.0074x - 0.136	0.992 ± 0.001	10
Lisinopril	0.1 - 100	y = 0.0013x + 0.021	0.985 ± 0.004	0.1
Simvastatin	0.1 - 100	y = 0.016x + 0.215	0.988 ± 0.003	0.1
Valsartan	50 - 4000	y = 0.006x + 0.125	0.992 ± 0.001	50

3.3 Accuracy and precision

The accuracy and precision of the developed LC-HRAM MS method were determined by intra- and inter-day replicate analyses of six spiked VAMS QC samples containing the four target analytes at the low, medium and high concentration levels on three separate days. Accuracy was expressed as the mean relative error (RE %) and precision was expressed as the coefficient of variation (CV %) and data obtained for both were within the predefined 15% limit [23, 24] for all concentrations in each run for all the target drugs. The overall variation in data between runs was also ≤15% for all target drugs. A summary of the results is presented in Table 2.

Table 2. Intra- and inter-day accuracy and precision data for the four target cardiovascular drugs in VAMS extracts (n = 6 at each concentration level, for 3 days)

Drug	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (RE) %	Coefficient of variation (%)	
				Intra day	Inter day
Atenolol	50	51.58	3.16	1.71	2.07
	500	499.02	-0.20	6.04	0.97
	1500	1518.72	1.25	2.99	1.71
Lisinopril	1	1.06	4.49	5.66	2.53
	25	25.16	0.63	9.18	1.75
	100	100.32	0.32	7.82	0.75
Simvastatin	1	1.05	5.18	7.28	0.97
	25	25.15	0.59	6.25	1.30
	100	99.84	-0.16	5.16	0.22
Valsartan	250	250.34	0.14	5.27	1.19
	2000	1971.85	-1.41	3.05	1.55
	4000	4086.14	2.15	7.76	0.29

3.4 Matrix effect

Matrix effect caused by blood constituents, as well as from the VAMS device material itself [26], was examined to ensure that the sensitivity and precision of the developed method was not compromised. The matrix effect data obtained for each target analyte investigated at the low, medium and high concentration levels of the calibration curve is presented in Table 3. No significant (i.e., >10%) matrix effects on the analyte signal due to endogenous components of blood or the VAMS sampling material was observed at the three tested concentrations of each target drug. These results demonstrate the robustness of the extraction procedure, and of the ionisation mechanism, for these target analytes. Comparison of the results from 903 sampling paper [15] versus VAMS revealed mean matrix effects for lisinopril and simvastatin were significantly higher for 903 sampling paper. This could be attributed to the extraction of constituents from within the 903 sampling paper that cause ionisation competition with lisinopril and simvastatin at the ESI source of the MS.

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

Drug	Nominal conc. (ng/mL)	Matrix effect % (mean)	Precision (CV%)
Atenolol	50	0.88	1.34
	500	3.39	2.52
	1500	2.20	3.81
Lisinopril	1	0.99	2.74
	25	1.46	3.52
	100	3.66	4.76
Simvastatin	1	2.70	3.56
	25	0.66	2.86
	100	0.51	2.80
Valsartan	250	-1.17	5.87

	2000	1.01	0.02
	4000	0.99	0.08

302

303 3.5 Recovery

304 Mean extraction recoveries of atenolol, lisinopril and simvastatin, and valsartan at the low, medium
305 and high concentration levels of the calibration curve were 102%, 88%, 67% and 47%, respectively,
306 from VAMS, and 89%, 88%, 68% and 96%, respectively, from 903 paper reported by Bernieh et al
307 [15]. Possible reasons for the difference may be poor extraction of valsartan from the VAMS
308 substrate or poor uptake of valsartan on the VAM absorptive tip. Recovery data from VAMS for each
309 target analyte at the low, medium and high concentration levels is summarised in Table 4.

310 **Table 4** Recovery data for atenolol, lisinopril, simvastatin and valsartan extracted from VAMS at the low,
311 medium and high concentration levels (n = 6 at each concentration level)

Drug	Nominal conc. (ng/mL)	Recovery (%)	Standard Deviation (SD)	Precision (CV)
Atenolol	50	103.31	6.36	5.61
	500	106.17	2.65	2.34
	1500	98.95	1.26	1.28
Lisinopril	1	88.71	11.97	13.40
	25	84.16	11.33	9.92
	100	91.66	9.43	9.46
Simvastatin	1	62.87	13.18	8.09
	25	71.95	6.06	8.42
	100	66.94	1.91	2.85
Valsartan	250	39.89	5.80	3.82
	2000	53.02	3.68	1.47
	4000	48.44	2.55	2.43

312

313 3.6 Haematocrit (Hct) evaluation

314 The VAMS device has been reported to be independent of the Hct effect [27-29]. To confirm this, the
315 influence of Hct on assay performance for the quantitative analysis of atenolol, lisinopril, simvastatin
316 and valsartan was evaluated at the low, medium and high concentrations with an adjusted Hct of 35,
317 45 and 55% to cover the range for the target population. Concentrations of extracts were
318 determined using a linear regression equation generated from calibration data produced from
319 standards prepared with the 45% Hct on VAMS. The results for the Hct investigation are presented in
320 Table 5 and show that the VAMS device delivered accuracy (RE%) and precision (CV%) values within
321 the pre-defined limit of ≤ 15% [20] at all Hct levels for each tested analyte concentration. As
322 expected for VAMS, the differences between the Hct levels are much reduced in comparison with
323 previously published work using DBS [15]. This demonstrates that quantitative analytical data
324 collected on the VAMS device is not likely to be affected by inter-individual variability in Hct values
325 for the Hct range investigated for the four target analytes.

Table 5. Influence of Hct on the accuracy (RE %) of analyte quantification for VAMS 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 at each concentration and Hct level)

		Haematocrit (Hct)		
Drug	Nominal conc. (ng/mL)	35%	45%	55%
Atenolol	50	-6.52% (4.03)	Normalized (7.15%)	-5.80 (4.13)
	500	5.19% (4.27)	Normalized (4.38%)	5.10 (3.15)
	1500	-1.13 (8.45)	Normalized (4.72%)	-2.19 (4.74)
Lisinopril	1	-0.93% (10.03%)	Normalised (13.44%)	2.93% (13.60%)
	25	4.35% (10.17%)	Normalised (3.37%)	3.65% (8.45%)
	100	-5.90% (9.32%)	Normalised (4.64%)	-0.95% (3.89%)
Simvastatin	1	1.13% (3.68%)	Normalised (8.16%)	-0.21% (9.89%)
	25	5.03% (6.08)	Normalised (3.7%)	-2.70% (6.6%)
	100	-2.42% (2.9%)	Normalised (3.2%)	-5.70% (4.6%)
Valsartan	250	-1.45% (2.28%)	Normalised (1.15%)	-1.05% (2.07%)
	2000	-0.52% (3.37%)	Normalised (3.48%)	-2.81% (3.08%)
	4000	-0.43% (3.97%)	Normalised (2.61%)	-0.98% (2.23%)

3.7 Stability

The results shown in Table 6 demonstrate that the spiked samples of the selected drugs were stable in VAMS for 8 weeks storage at room temperature. This implies that VAMS microsampling methodology could be applicable in resource limited areas where samples may have to be collected in remote areas and there is a time delay before analysis can be completed.

Table 6. Accuracy, precision and quantification of the LC-HRAM MS VAMS assay at the low, medium and high concentrations for atenolol, lisinopril, simvastatin and valsartan after 8 weeks of storage at room temperature (n = 6 at each concentration level)

Drug	Concentration in whole blood (ng/mL)	Mean concentration found ±SD (ng/mL) (n=6)	Accuracy (RE%)	Precision (CV%)
Atenolol	50	55.71 ± 2.34	11.42	4.21
	500	523.31 ± 46.34	4.66	8.85
	1500	1516.29 ± 82.00	1.09	5.41
Lisinopril	1	0.99 ± 0.06	-1.01	6.41
	25	27.05 ± 3.66	8.19	13.55
	100	107.65 ± 10.07	7.65	9.36
Simvastatin	1	1.08 ± 0.10	7.96	9.35
	25	25.35 ± 1.82	1.39	7.19
	100	94.20 ± 7.30	-5.80	7.75
Valsartan	250	253.57 ± 30.06	1.43	11.86
	2000	1921.63 ± 19.91	-3.92	1.04
	4000	4039.78 ± 173.14	5.99	8.80

3.8 Application of method to volunteer VAMS samples and comparison with DBS drug concentrations

The developed and validated LC-HRAM MS method was successfully applied to the quantitative analysis of atenolol, lisinopril, simvastatin and valsartan for VAMS samples obtained from volunteers undergoing cardiovascular drug therapy treatment with one or more of the target drugs. These volunteer VAMS samples were collected opportunistically after a supposedly single dose of the target drug(s). A series of blank control VAMS samples were also taken from healthy volunteers not prescribed any of the target drugs, and analysed. The requirements for a bioanalytical method to monitor adherence to prescribed pharmacotherapy are to be able to determine the residual levels of drug up to 24 hours after the initial dose and to ascertain if the calculated drug levels are within the therapeutic window [3]. VAMS samples were self-collected from seventeen volunteers and were analysed with the developed LC-HRAM MS method; the VAMS concentrations obtained are shown in Table 7.

Table 7. VAMS concentrations compared with DBS concentrations [15, 30] of the studied CVD drugs from volunteers prescribed one or more of the CVD drugs investigated

N	Sex	Administered Drug	Time after Oral intake (hrs)	VAMS Concentration (ng/mL) ± (s.d)	DBS Concentration (ng/mL) ± (s.d)
1	M	Valsartan 160mg	4	503.11 ± 5.12	493.72 ± 8.78
2	F	Simvastatin 20mg	13	2.81 ± 0.30	2.90 ± 0.77
3	F	Simvastatin 20mg	15	1.44 ± 1.19	1.79 ± 0.74
4	M	Valsartan 160mg	4	412.31 ± 11.68	407.16 ± 14.73
5	M	Simvastatin 40mg	6	< LOQ	< LOQ
6	M	Lisinopril 20mg	?	29.59 ± 5.31	37.02 ± 8.59
7	M	Lisinopril 20mg	3.5	7.94 ± 1.35	8.02 ± 3.68
8	M	Simvastatin 20mg	10	1.29 ± 0.86	1.32 ± 0.42
9	M	Simvastatin 20mg	11	0.69 ± 0.77	0.85 ± 0.55
10	M	Valsartan 80mg	4	136.45 ± 2.62	131.49 ± 0.88
11	M	Atenolol 100mg	13	254.16 ± 5.37	248.30 ± 9.12
12-14	F	Controls - None	N/A	<LOQ	<LOQ
15-17	M	Controls - None	N/A	<LOQ	<LOQ

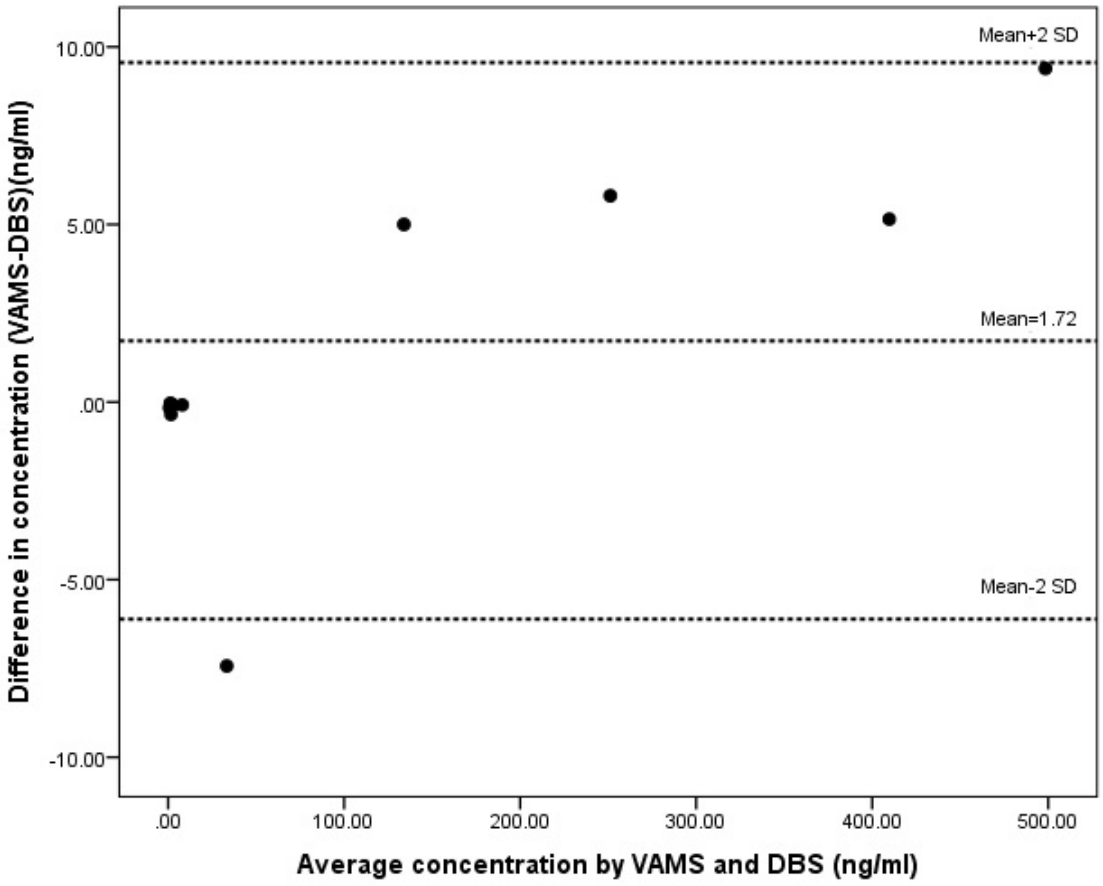

Figure 2. Bland-Altman plot comparing VAMS and DBS concentrations for atenolol, lisinopril, simvastatin and valsartan in volunteer samples.

The drug concentrations found for VAMS were also compared to those obtained by DBS [15, 30] from the same volunteers, sampled at approximately the same time. The volunteer samples are "dynamic *in vivo*" samples obtained from a constantly changing drug pharmacokinetics environment within the body. To compare the two microsampling methods we used a Bland Altman plot (Figure 2), which showed good concordance in the drug concentrations for VAMS and DBS because the difference was less than 2 SD from the mean. The results confirmed acceptable reproducibility between the two microsampling methods and demonstrate that the microsampling methodologies produce comparable quantitative results. However, significant differences in both patient ease-of-use and cost of the sampling device should be considered before use in routine applications.

Adherence to a prescribed drug therapy is indicated by the drug level in the blood being between the published C_{max} concentration and 5.25% of C_{max} i.e. the drug concentration after 5 half-lives, when it is considered to be therapeutically inactive. Conversely, non-adherence is indicated by absence of the drug in the volunteer's blood sample or if the level is outside its therapeutic window [3]. On this basis, the results showed that for all volunteers, except volunteer 5, the calculated drug levels indicated adherence to prescribed drug therapy. For volunteer 5, there was no detectable simvastatin after 6 hours following oral intake. *In vivo*, simvastatin hydrolyses to simvastatin acid and the collected HRAM MS data for volunteer 5 was therefore re-interrogated for the $[M+H]^+$ and $[M+Na]^+$ ions for simvastatin acid at m/z 437.2898 and m/z 459.2727, which were shown not to be present. For volunteer 3 the experiments show that simvastatin can be measured at *m/z* 441.2611 directly up to 15 hours after administration, indicating that the levels are within the limit of quantification of the assay at this time period.

Volunteer feedback was obtained on their experience using self-sampling with VAMS and 903 paper and particularly regarding ease of use of both microsampling methods. All volunteers commented that it was easier and quicker to self-collect a micro-volume blood sample with VAMS. This advantage could translate in the adoption of this methodology and enhance the implementation of TDM in routine clinical practice [31].

4. Conclusion

The feasibility of a VAMS based LC-HRAM MS method for the simultaneous quantification of atenolol, lisinopril, simvastatin and valsartan in 10 μL of whole has been demonstrated. The LC-HRAM MS method developed was rapid, in terms of instrument time, and provided the sensitivity required for the determination of the four target cardiovascular drugs. The method was validated following international guidelines and has shown to be precise and accurate at all tested concentrations and also exhibited appropriate specificity and linearity. The VAMS samples were shown to be stable at room temperature for at least 8 weeks. This offers the possibility of batch-wise preparation and also allows time for the transport of samples to laboratories with appropriate analytical facilities. As expected, the target drug concentrations from the VAMS samples were not influenced by Hct. These advantages indicate VAMS, therefore, has great potential for routine therapeutic adherence monitoring by home sampling, however, the costs of purchasing these microsampling devices are an important consideration for healthcare service provision. This method has the potential to assist clinicians in monitoring patient adherence to prescribed drug therapy, and with that data, to optimize treatment. However, before implemention for routine therapeutic drug monitoring it will be necessary to correlate whole blood drug concentrations with plasma levels and conduct an extensive patient study.

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