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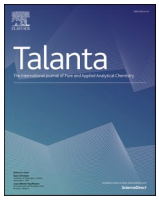

Capillary blood microsampling and LC–Orbitrap analysis for adherence monitoring of cardiovascular drugs: method development, cross-validation, and patient proof-of-concept using VAMS and Capitainer-B

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ABSTRACT

Microsampling enables decentralized adherence monitoring by capturing defined volumes of capillary blood. We developed, validated, and cross-evaluated a volumetric absorptive microsampling (VAMS) and Capitainer-B workflow for 15 cardiovascular drugs using high-resolution liquid chromatography Orbitrap analysis. Amlodipine, atenolol, atorvastatin, bisoprolol, carvedilol, clopidogrel, diltiazem, lercanidipine, metoprolol, nebivolol, prasugrel, rosuvastatin, salicylic acid, simvastatin hydroxy acid, and verapamil were included. Sampling by VAMS and Capitainer-B should be evaluated and compared. VAMS tips and Capitainer-B disks loaded with 10 µL whole blood were extracted with 200 µL methanol, shaken and centrifuged, evaporated to dryness, reconstituted, and injected for analysis. The method met international validation criteria for most analytes, with quantification and selectivity achieved for all except prasugrel. Capitainer-B generally exhibited higher matrix effects than VAMS, while accuracy and precision were within acceptance limits except for lercanidipine across both devices and for atorvastatin on Capitainer-B. Under long-term storage, VAMS showed >15 % loss for carvedilol, lercanidipine, and simvastatin hydroxy acid after two weeks, whereas Capitainer-B showed no degradation. In a proof-of-concept involving 30 patients, finger-prick concentrations from VAMS and Capitainer-B were interchangeable, supporting the suitability of these adherence workflows but reference ranges for capillary blood remain necessary to enable routine clinical interpretation. In summary, this work established a reliable, LC-Orbitrap-based microsampling platform for monitoring adherence and identified stability issued amongst others that guide future method selection and development.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, accounting for an estimated 20.5 million deaths in 2021 [1]. Coronary artery disease (CAD), a major subset of CVDs, affected approximately 315 million people in 2022 worldwide [2]. Current international guidelines recommend beta-blockers, and ACE inhibitors for heart failure, calcium channel blockers, ACE-inhibitors and beta-blockers for hypertension and oral antiplatelet agents, and statins for primary and secondary prevention to prevent complications in CADs [3,4]. The need for multiple drugs increases the risk of medication nonadherence and rapid onset of complications [5]. Adherence is the extent to which a patient correctly follows recommendations agreed with healthcare providers, such as taking medications, maintaining a particular diet, or making lifestyle modifications [6]. However, especially medication adherence is essential to improve the medical outcome of the therapy. Failure to adhere to prescribed medications can result in adverse clinical outcomes, increased rates of hospitalization, avoidable healthcare expenditures, and increased mortality [7]. Consequently, medication nonadherence has become a significant concern for

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clinicians, healthcare providers, and healthcare systems worldwide [8,9] and adherence monitoring was shown to improve treatment adherence [10]. Drug concentrations in blood can provide insights into adherence to prescribed drug therapy [11]. Such quantitative analysis of cardiovascular drugs in blood plasma or serum using liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS) has been extensively documented [12,13]. However, venous blood collection can cause discomfort and anxiety, and must be performed by medical professionals. Additionally, infection risk is increased and the transport of venous blood often requires specific conditions, such as centrifugation to separate plasma or serum, and/or storage at temperatures below 4 °C [14]. An alternative to venous blood sampling is the use of dried matrix microsamples, such as dried blood spots (DBS). After adequate training, patients are able to use a lancet to perform a finger prick and collect small amounts of capillary blood themselves. The resulting blood drops, typically of unknown volume, are applied to filter paper, dried, and then mailed to a laboratory [15]. This method has also been successfully demonstrated for various CVD medication [16–18]. However, DBS have certain limitations, including imprecise sample volume and variability due to hematocrit effects [19]. Hematocrit-related variability in DBS sampling prompted research into alternative microsampling techniques such as volumetric absorptive microsampling (VAMS) and the Capitainer-B device. Unlike conventional DBS, where punched disks may contain variable amounts of blood due to hematocrit effects, VAMS and Capitainer-B are expected to enable for precise volume collection and full extraction of the sample [20,21]. VAMS devices feature a porous, hydrophilic tip attached to a plastic sample handler, capable of absorbing a defined volume of capillary blood (most commonly used are 10 µL or 20 µL) through passive wicking. VAMS have been used in various applications, including therapeutic drug monitoring [22], personalized pharmacotherapy [23], metabolomic studies [24], and adherence monitoring [25]. The Capitainer-B device consists of a combination of paper, polymer-based microfluidics, and thin water-soluble membranes to accurately meter a fixed volume of blood. Upon application of blood to the inlet, a metering channel within the device automatically fills, directing the sample to a pre-cut DBS paper disc for collection [26]. Capitainer-B were used e.g., for proteome profiling [27], therapeutic drug monitoring [28] and metabolomic and lipidomic studies [29].

This study aimed to develop and validate two microsampling-based analytical strategies for adherence monitoring of 15 drugs in the context of CVDs. Sampling using both VAMS and Capitainer-B devices was to be evaluated and compared. Amlodipine, atenolol, atorvastatin, bisoprolol, carvedilol, clopidogrel, diltiazem, lercanidipine, metoprolol, nebivolol, prasugrel, rosuvastatin, salicylic acid, simvastatin hydroxy acid, and verapamil were to be included. The analysis was performed using LC-high-resolution tandem mass spectrometry (HRMS/MS) and the entire quantitative workflow needed to be validated in compliance with international guidelines [30,31]. Furthermore, a proof-of-concept study was conducted to demonstrate the clinical applicability of the method and to identify potential limitations associated with the different sampling strategies.

2. Experimental

2.1. Chemicals and other materials

Amlodipine besylate (CAS 111470-99-6) and diltiazem hydrochloride (CAS 33286-22-5) were obtained from Pfizer (Karlsruhe, Germany), atenolol (CAS 29122-68-7) from Imperial Chemical Industries (London, UK), amlodipine-d_4 (CAS 1185246-14-3), atorvastatin calcium salt (CAS 134523-03-8), bisoprolol-d_5 (CAS 1189881-87-5), carvedilol (CAS 72956-09-3), clopidogrel hydrogen sulfate (CAS 135046-48-9), racemic clopidogrel-d_4 hydrogen sulfate (CAS 1219274-96-0), diltiazem-d_4 hydrochloride (CAS 1217769-52-2), prasugrel hydrochloride (CAS 389574-19-0), rosuvastatin-d_6 sodium salt (CAS 2070009-41-3), salicylic acid-d_4 (CAS 78646-17-0), verapamil hydrochloride (CAS 152-11-4) and verapamil-d_6 hydrochloride (CAS 1185032-80-7) from LGC (Luckenwalde, Germany), bisoprolol (CAS 66722-44-9), metoprolol tartrate (CAS 56392-17-7), nebivolol hydrochloride (CAS 152520-56-4) and salicylic acid (CAS 69-72-7) from Sigma Aldrich (St. Louis, MO, USA), lercanidipine hydrochloride (CAS 132866-11-6), rosuvastatin calcium salt (CAS 147098-20-2) and simvastatin hydroxy acid ammonium salt (CAS 139893-43-9) from Toronto research chemicals (Toronto, ON, Canada) and metoprolol-d_6 tartrate (CAS 96849-43-3) from Cayman Chemical (Ann Arbor, MI, USA). Methanol (LC-MS grade) and all other chemicals were purchased from VWR (Darmstadt, Germany). Mitra VAMS with a 10 µL absorbing tip were obtained from Neoteryx (Torrance, CA, USA) and Capitainer-B with a 10 µL absorbing disk from Capitainer AB (Solna, Sweden). Blank whole blood stabilized with ethylenediaminetetraacetic acid (EDTA) was used for the development and validation of the method. This was obtained from the authors' laboratory for regular toxicological analysis and handled in accordance with institutional protocol and regulations for data protection and sample handling. Finger prick blood- (FPB) loaded VAMS and FPB-loaded Capitainer-B were sampled for the proof-of-concept study from volunteers with CVDs between September 2024 and May 2025 during their regular consultation at Saarland University Hospital, Homburg, Germany. Samples were stored at −20 °C until analysis. Along with the samples, medication plans were collected. Written informed consent was obtained from all patients involved in the study, which was approved by the local ethics committees (NCT01888315).

2.2. Calibrators, quality controls, and internal standards

The stock solutions for all compounds were prepared in methanol at a concentration of 1 mg/mL except for salicylic acid (10 mg/mL). The internal standard (IS) solution for spiking the samples consisted of 11 ng/mL amlodipine-d_4, 75 ng/mL bisoprolol-d_5, 5 ng/mL clopidogrel-d_4, 120 ng/mL diltiazem-d_4, 250 ng/mL metoprolol-d_6, 12 ng/mL rosuvastatin-d_6, 10 µg/mL salicylic acid-d_4, and 90 ng/mL verapamil-d_6 in methanol. Six quality control (QC) working solutions were prepared by diluting the stock solutions with methanol. All solutions were stored in amber glass vials at −20 °C.

For the preparation of the spiked QC samples, 10 µL of working solution A containing all analytes of interest except of salicylic acid and 5 µL of working solution B containing salicylic acid were added to 185 µL whole blood and incubated for 30 min at 1500 rpm and 37 °C. For loading the VAMS, their tips were dipped into spiked blood until they were soaked completely, held for two extra seconds and dried for at least 3 h at room temperature (24 °C) [25,32]. For loading the Capitainer-B, 40 µL of spiked blood were added to the sampling spot, the capillary system was automatically filled with 10 µL blood and released to the sampling disk, the samples were dried for at least 4 h at room temperature (24 °C) [33]. The final concentrations of analytes in whole blood used to load VAMS and Capitainer-B devices are summarized in Table 1.

2.3. Sample preparation – VAMS

The dried VAMS-tips were transferred to a 2 mL plastic reaction tube. The samples were extracted by adding 190 µL of methanol and 10 µL of the IS solution. After 30 min of shaking at 1500 rpm and 37 °C, they were centrifuged at 15,000×g and −10 °C for 15 min. The supernatant was evaporated to dryness under a gentle stream of nitrogen at 45 °C and reconstituted in 40 µL of methanol. After transferring to an amber LC vial, a volume of 5 µL was injected into the LC-HRMS/MS system and analyzed as described in the instrumental settings.

2.4. Sample preparation – Capitainer-B

The dried Capitainer-B-disks were transferred to a 2 mL plastic reaction tube. The samples were extracted by adding 190 µL of methanol

Table 1
Final concentrations (ng/mL) of analytes used for quality controls (QC). (lev: level; LLOQ: lower limit of quantification; ULOQ: upper limit of quantification).

Analyte	QC LLOQ	QC low	QC lev 3	QC lev 4	QC mid	QC ULOQ
Amlodipine	3	8	15	23	23	45
Atenolol	60	150	600	1000	1500	3000
Atorvastatin	6	15	60	100	150	300
Bisoprolol	8	20	40	60	60	120
Carvedilol	20	50	100	150	150	300
Clopidogrel	0.7	2	3	5	5	10
Diltiazem	20	50	100	150	150	300
Lercanidipine	0.2	0.5	2	3	5	10
Metoprolol	16	40	160	270	400	800
Nebivolol	1	3	10	17	25	50
Prasugrel	10	25	120	200	250	500
Rosuvastatin	3	8	15	23	23	45
Salicylic acid	16,000	40,000	80,000	120,000	120,000	240,000
Simvastatin hydroxy acid	0.8	2	4	6	6	12
Verapamil	9	23	90	150	230	450

and 10 μL of the IS solution. After 30 min of shaking at 1500 rpm and 37 °C, they were centrifuged at 15,000×*g* and −10 °C for 15 min. The supernatant was evaporated to dryness under a gentle stream of nitrogen at 45 °C and reconstituted in 40 μL of methanol. After transferring to an amber LC vial, a volume of 5 μL was injected into the LC-HRMS/MS system and analyzed as described in the instrumental settings.

2.5. Instrumental settings

Samples were analyzed using a ThermoFisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 Rapid Separation UHPLC system consisting of a degasser, a quaternary pump, an UltiMate autosampler, coupled to a TF Q-Exactive Plus mass spectrometer system equipped with a heated electrospray ionization (HESI)-II source. Gradient elution was performed on a TF Accucore Phenyl Hexyl column (100 mm × 2.1 mm, 2.6 μm particle size). Eluent A [2 mM aqueous ammonium formate containing formic acid (0.1 %, *v/v*, pH 3)] and eluent B [2 mM aqueous ammonium formate with acetonitrile:methanol (50:50, *v/v*) plus formic acid (0.1 %, *v/v*), and water (1 %, *v/v*)] were used as mobile phases. The gradient was set as follows: 0–1 min from 1 % B to 20 % B, 1–3.5 min from 20 % B to 38 % B, 3.5–8 min hold 38 % B, 8–10 min from 38 % B to 99 % B, 10–11.5 min hold 99 % B, 11.5–13.5 min hold 1 % B. The flow rate was programmed as follows: 0–10 min 0.5 mL/min, 10–11.5 min from 0.5 to 0.8 mL/min, 11.5–13.5 from 0.8 to 0.5 mL/min. Chromatographic separation was performed at 40 °C. The HESI-II source conditions were as follows: ionization mode, positive or negative polarity (separate runs); sheath gas flow rate, 60.0 arbitrary units (AU); auxiliary gas flow rate, 10.0 AU; spray voltage 4.00 kV (positive polarity) and −3.20 kV (negative polarity); auxiliary gas heater temperature, 320 °C; ion transfer capillary temperature, 320 °C; and S-lens RF level 50.0. Full scan analysis (scan range mass-to-charge ratio value, *m/z*, 130–930) was used to determine the ion mass (*m/z*) and retention time of all analytes. For all analysis targeted selected ion monitoring (t-SIM) was performed with an inclusion list containing masses of interest and expected retention times. The settings for t-SIM were as follows: resolution 35,000; microscans 1; automatic gain control target 5e4; maximum injection time 250 ms; isolation window 1.0 *m/z*. Ion masses (*m/z*) and expected retention times with their time window used for the inclusion list are represented in Table 2. TF Xcalibur Qual Browser software version 4.6 was used for data handling. Extracted ion chromatograms of the analytes were used for quantification. The accepted mass accuracy for all analytes was set at 5 ppm for solvents and extracted samples.

2.6. Method validation

Method validation for VAMS and Capitainer-B devices was based on international guidelines including the ICH guideline M10 on bioanalytical method validation and study sample analysis published by the European Medicines Agency (EMA) and the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) guideline for Development and Validation of Dried Blood Spot-Based Methods for Therapeutic Drug Monitoring [30,31].The statistical evaluation was performed with Microsoft (Redmond, WA, USA) Excel version 16.

Table 2
Precursor ion masses, used ionization modes (+, positive; -, negative), retention times and method time windows of the analytes and internal standards (IS).

Analyte	Ionization mode and precursor ion mass, *m/z*	Retention time, min	Time window, min
Amlodipine	+409.1537	6.5	5.6–7.4
Amlodipine-d_4 (IS)	+413.1791	6.5	5.6–7.4
Atenolol	+267.1713	1.7	0.8–2.5
Atorvastatin	−557.2427	10.1	9.1–11.1
Bisoprolol	+326.2325	3.6	2.7–4.7
Bisoprolol-d_5 (IS)	+331.2630	3.6	2.7–4.7
Carvedilol	+407.1974	5.6	4.7–6.7
Clopidogrel	+322.0652	9.7	8.7–11.0
Clopidogrel-d_4 (IS)	+326.0926	9.7	8.7–11.0
Diltiazem	+415.1690	5.1	4.5–6.5
Diltiazem-d_4 (IS)	+419.1949	5.1	4.5–6.5
Lercanidipine	+612-3041	10.0	9.2–11.0
Metoprolol	+268.1912	2.8	2.0–4.0
Metoprolol-d_6 (IS)	+274.2277	2.8	2.0–4.0
Nebivolol	+406.1840	7.1	6.2–8.2
Prasugrel	+374.1222	5.4	4.2–6.2
Rosuvastatin	−480.1613	9.2, 9.7	8.5–10.5
Rosuvastatin-d_6 (IS)	−486.1988	9.2, 9.7	8.5–10.5
Salicylic acid	−137.0237	3.4	2.5–4.5
Salicylic acid-d_4 (IS)	−141.0488	3.4	2.5–4.5
Simvastatin hydroxy acid	−435.2744	10.3	9.5–11.5
Verapamil	+455.2906	6.2	5.5–7.2
Verapamil-d_6 (IS)	+461.3295	6.2	5.5–7.2

Ion suppression or enhancement by co-eluting analytes was tested for all analytes with a corresponding deuterated IS in methanol at a concentration of 50 ng/mL for amlodipine, 50 ng/mL for amlodipine-d_4, 150 ng/mL bisoprolol, 75 ng/mL bisoprolol-d_5, 50 ng/mL clopidogrel, 50 ng/mL clopidogrel-d_4, 300 ng/mL diltiazem, 120 ng/mL diltiazem-d_4, 1000 ng/mL metoprolol, 250 ng/mL metoprolol-d_6, 50 ng/mL rosuvastatin, 50 ng/mL rosuvastatin-d_6, 240 μg/mL salicylic acid, 10 μg/mL salicylic acid-d_4, 500 ng/mL verapamil and 90 ng/mL verapamil-d_6 (n = 3). Peak areas were analyzed in presence and in absence of coeluting substances. Suppression and enhancement of the coeluting substances should not be more than ±15 % [34].

For investigating matrix-induced ion suppression or enhancement,

the matrix factor (MF) and the IS-normalized matrix factor (NMF) were investigated using six matrix samples of different donors at QC level (lev) 3 and QC upper limit of quantification (ULOQ) (see Table 1) not containing the analytes of interest but other frequently prescribed drugs (listed in Table 3). The MF was calculated as ratio of the peak area in the presence of matrix (blank matrix spiked after extraction), to the peak area in the absence of matrix (pure analyte solution) according to Equation (1). The NMF was defined as the ratio of the MF of the analyte to the MF of the IS according to Equation (2). NMF was calculated only for analytes with corresponding isotope-labeled IS. The coefficients of variation (CVs) of the MF and the NMF should be ≤ 15 % [30].

Equation 1

Equation for the calculation of the matrix factor (MF)

$$MF = \frac{peak\ area\ of\ blank\ matrix\ spiked\ after\ extraction}{peak\ area\ of\ pure\ analyte\ solution} * 100\%$$

Equation 2

Equation for the calculation of the normalized matrix factor (NMF) (A: analyte; IS: internal standard; MF: matrix factor)

$$NMF = \frac{MF_A}{MF_{IS}} * 100\%$$

Table 3
Listing of analytical validation parameters tested, number of whole blood samples used for the indicated validation parameters, drugs contained in the whole blood sampled, and their hematocrit (%).

Analytical validation parameter	Number of whole blood samples, not containing the analytes of interest	Drugs contained	Hematocrit, %
Selectivity	1	Mirtazapine, pipamperone	35
	2	Midazolam	38
	3	None	55
	4	Clonazepam, ketamine	50
	5	Citalopram, diphenhydramine, zopiclone	48
	6	Paracetamol	39
	7	Metamizole, mirtazapine, omeprazole	47
	8	Amisulpride, quetiapine, sertraline	50
	9	Cafedrine, mepivacaine	58
	10	Amitriptyline, nortriptyline	45
	11	None	51
	12	Paracetamol	47
Carry-over	1	None	51
Accuracy and precision	1	None	45
	2	Paracetamol	47
Matrix factor, normalized matrix factor	1	Baclofen, clonazepam, lacosamide, levetiracetam	45
	2	Prothipendyl, sertraline	40
	3	Amphetamine, diazepam, nordazepam	50
	4	Mirtazapine, opipramol, zolpidem	38
	5	Amphetamine, methadone	44
	6	Olanzapine, pipamperone	49
Autosampler stability	1	Doxepin, nordoxepin	52
Freezer stability	1	Doxepin, nordoxepin	52
Long-term stability	1	None	51

To test selectivity, EDTA whole blood samples from 12 different donors were tested and analyzed for possible signal interferences with IS or analytes. These samples did not contain the analytes of interest, but other frequently prescribed drugs (listed in Table 3). Interfering signals in the samples should be ≤ 20 % of the lower limit of quantification (LLOQ) and ≤5 % of the IS [31].

For carry-over testing, the QC ULOQ (see Table 1) was injected directly followed by two blank matrix samples (n = 3). Interfering signals in the blank matrices should be ≤ 20 % of the LLOQ [30].

The quantification was based on the relative response factor (RRF) without the need for frequent external calibration [35]. The RRF of each analyte was determined using the corresponding isotope-labeled IS or an isotope-labeled IS with a similar chemical structure. The RRF was calculated for QC LLOQ, QC lev 3, QC lev 4, and QC ULOQ (see Table 1) (n = 3) using Equation (3). The concentration of the analytes was calculated using the analyte/IS peak area ratio and the RRF according to Equation (4) [36]. The RRF for VAMS and for Capitainer-B are listed in Table 4. For rosuvastatin and its corresponding IS, the sum of the peak areas of the two diastereomers was used. The mean value of all RRFs of each device and each QC level was used to calculate the concentrations.

Equation 3

Determination of the relative response factor (RRF) using peak areas of an analyte (A) and an isotope-labeled internal standard (IS) at known concentrations (c)

$$RRF = \frac{area_A}{area_{IS}} * \frac{c_{IS}}{c_A}$$

Equation 4

Calculation of the concentration (c) of an analyte (A) using the relative response factor (RRF) of the corresponding isotope-labeled internal standard (IS) or an isotope-labeled IS with a similar chemical structure

$$c_A = \frac{area_A}{area_{IS}} * \frac{c_{IS}}{RRF}$$

Accuracy and precision were evaluated for the QC LLOQ, QC low, QC mid, and QC ULOQ (concentrations see Table 1). The back calculated concentrations for all QC levels were compared to their expected concentrations. For the within-day assessment, five sample replicates of each level were analyzed in a single run. For the between-day assessment five sample replicates of each level in three runs on three different days were analyzed. For determining the accuracy, back calculated mean concentrations should not be more than ±15 % of their actual value (±20 % for LLOQ). In case of precision, the CV should not be more than 15 % (20 % for LLOQ) [31].

Twenty cycles of freeze-thaw stability of the stock solutions at 100 ng/mL were tested (n = 3). The measured peak areas should be within ±15 % of the first measured peak area (t_0). Autosampler stability (36 h, 10 °C, n = 3) and freezer stability (36 h, −20 °C, n = 3) of extracted samples at QC lev 3 and QC ULOQ were tested (concentrations see Table 1). Long-term stability of loaded devices at QC lev 3 and QC ULOQ were tested (1 and 2 weeks, 24 °C, n = 3). The calculated concentrations should not be >±15 % of the calculated value at t_0 [30].

2.7. Proof-of-concept

Samples from 30 patients were analyzed. From each patient one VAMS sample and one Capitainer-B sample, each soaked with 10 μL of FPB, and a medication plan were collected. 30 VAMS and 24 Capitainer-B were extracted and analyzed. Due to sampling difficulties, not all Capitainer-B could be used for analysis but all VAMS. Seventeen patients were prescribed with acetylsalicylic acid, eight patients with amlodipine, 15 patients with atorvastatin, 17 patients with bisoprolol, four patients with carvedilol, seven patients with clopidogrel, three patients with lercanidipine, six patients with metoprolol, one patient with nebivolol, five patients with prasugrel, 11 patients with rosuvastatin,

Table 4
Listing of the reference therapeutic plasma concentration ranges of the analytes (ng/mL), corresponding isotope-labeled internal standards (IS), quantification ranges (ng/mL), relative response factors (RRF) for volumetric absorptive microsampling (VAMS), and RRF for Capitainer-B. (-: no data).

Analyte	Reference therapeutic plasma range, ng/mL [50]	IS	Quantification range, ng/mL	RRF VAMS	RRF Capitainer-B
Amlodipine	3–15	Amlodipine-d_4	3–45	1.23	1.38
Atenolol	100–2000	Metoprolol-d_6	60–3000	0.49	0.54
Atorvastatin	7–250	Rosuvastatin-d_6	6-300 (VAMS) 6-150 (Capitainer-B)	0.62	0.75
Bisoprolol	10–100	Bisoprolol-d_5	8–120	0.89	0.74
Carvedilol	20–300	Bisoprolol-d_5	20–300	0.48	0.36
Clopidogrel	1–6	Clopidogrel-d_4	0.7–10	0.51	0.35
Diltiazem	30–250	Diltiazem-d_4	20–300	0.81	0.56
Lercanidipine	0.1–10	Amlodipine-d_4	5–10	12.62	6.83
Metoprolol	20–600	Metoprolol-d_6	16–800	0.91	0.59
Nebivolol	1–60	Metoprolol-d_6	1–50	0.31	0.32
Prasugrel	–	Clopidogrel-d_4	–	–	–
Rosuvastatin	6–20	Rosuvastatin-d_6	3–45	0.71	0.77
Salicylic acid	20,000–200,000	Salicylic acid-d_4	16,000–240,000	0.91	0.81
Simvastatin hydroxy acid	1-9 [38]	Rosuvastatin-d_6	0.8–12	1.14	1.07
Verapamil	10–400	Verapamil-d_6	9–450	0.55	0.39

and two patients with simvastatin. Atenolol, diltiazem and verapamil were not prescribed. After sampling, VAMS and Capitainer-B were stored at −20 °C until extraction and analysis as described earlier (maximum nine months). The analytical batch included two QC samples (QC lev 3, QC ULOQ). If a batch contained more than ten patient samples, the QCs must be reinjected after every ten patient samples.

To confirm adherence, the measured concentration in blood for both devices should be higher than the calculated cut-off concentration ($c_{cut\text{-}off}$) [37]. The trough plasma concentration (c_{min}) 24 h post-dose was calculated according to Equation (5), using pharmacokinetic parameters, including the elimination half-life ($t_{1/2}$), peak plasma concentration (c_{max}), and time to peak concentration (t_{max}), as described by Baselt [38].

Equation 5

Calculation of the trough plasma concentration (c_{min}) 24 h (h) post-dose using the peak plasma concentration (c_{max}), the elimination half-life ($t_{1/2}$), and time to peak concentration (t_{max})

$$c_{min} = c_{max} * 0.5^{\frac{24h - t_{max}}{t_{1/2}}}$$

For all calculations, the shortest reported $t_{1/2}$ was used. A 20 % reduction from c_{min} led to $c_{cut\text{-}off}$.

3. Results and discussion

Using serum drug concentrations to evaluate medication adherence is comprehensive to urine analysis alone, particularly if a dose-related concentration approach is used [39]. This is especially relevant for drugs with low oral bioavailability, extensive metabolic transformation, or limited renal excretion, where qualitative urine testing may fail to reliably detect recent intake [40]. In such cases, serum-based assessment provides a more accurate and quantitative measure of adherence than urine analysis alone [41]. An alternative to venous blood sampling is microsampling from FPB. Therefore, we aimed to develop and validate a VAMS- and Capitainer-B based analytical approach for the simultaneous quantification of a selection of beta-blockers, calcium channel blockers, oral antiplatelet agents, and statins commonly used for the treatment of CVDs with a particular focus on adherence monitoring. Two different microsampling devices were chosen to compare their performance directly, including differences in analysis, validations results and application on patient samples.

Both devices, VAMS and Capitainer-B, were found to be suitable for sampling, extraction and analysis. For loading the devices more blood was required to fill the Capitainer-B devices, as the metering channel had to be filled first before the blood could be released on the sampling disk. With VAMS, the tips could be loaded directly with blood, making this sampling method faster as well. The extraction method was identical for both devices. The centrifugation step was particularly important for VAMS, as small dried blood pieces were found more frequently in the extraction solution. The extracts for both devices were clear without any discoloration. There was no difference between the two devices of chromatographic separation and subsequent analysis.

3.1. Method validation

HRMS/MS analysis was used in this method because this strategy shows high mass accuracy and excellent selectivity. The chromatographic separation of analytes and their detection using positive mode is shown in Fig. 1. The chromatographic separation of analytes and their detection in negative mode is shown in Fig. 2. Separating the runs in positive and negative mode resulted in better signal intensity and a lower LLOQ, especially for analytes in negative mode compared to a non-separated run. All analytes showed a baseline separation or less than 25 % overlapping peak areas. This ensured that the analytes did not influence each other due to chromatographic separation [42]. All analytes in solvents and extracted samples met the predefined mass accuracy of 5 ppm.

Amlodipine, bisoprolol, clopidogrel, diltiazem, metoprolol, rosuvastatin, salicylic acid, and verapamil coeluted with their corresponding isotope-labeled IS, for which ion suppression and enhancement was tested. Verapamil showed 18 % ion suppression and salicylic acid-d_4 17 % enhancement. All other analytes and IS showed suppression or enhancement between −7 and +13 % [34].

The MFs determined for VAMS and Capitainer-B and their corresponding CVs are shown in Table 5. A MF of 50 % indicates that the peak area of the analyte in the matrix is 50 % lower than the peak area in the pure analyte solution. A MF of 150 % indicates that the peak area of the analyte in the matrix is 50 % higher than the peak area in the pure analyte solution. The MFs for VAMS varied between 64 % (atorvastatin) and 192 % for atenolol for QC lev 3, except for diltiazem with 523 % and diltiazem-d_4 with 518 %. Corresponding CVs ranged up to 21 % (amlodipine, simvastatin hydroxy acid), except for clopidogrel, metoprolol and metoprolol-d_6 (28 %, 29 %, and 28 %, respectively). At QC ULOQ, the MFs for VAMS ranged from 50 % (verapamil) to 207 % (diltiazem-d_4) with CVs up to 12 % (rosuvastatin-d_6) except for atorvastatin, salicylic acid, and salicylic acid-d_4 (23 %, 31 %, 37 %, respectively). The MFs for Capitainer-B varied from 32 % (clopidogrel) to 204 % (atenolol) for QC lev 3 except for diltiazem and diltiazem-d_4 (524 % and 525 %, respectively). Corresponding CVs ranged up to 21 % (atorvastatin, clopidogrel-d_4) except for salicylic acid and salicylic acid-d_4 (44 %, 52 %). At QC ULOQ, the MFs for Capitainer-B ranged from 36 % (clopidogrel) to 286 % (atorvastatin). Corresponding CVs reached up to 16 % (rosuvastatin-d_6) except for atorvastatin, salicylic acid and

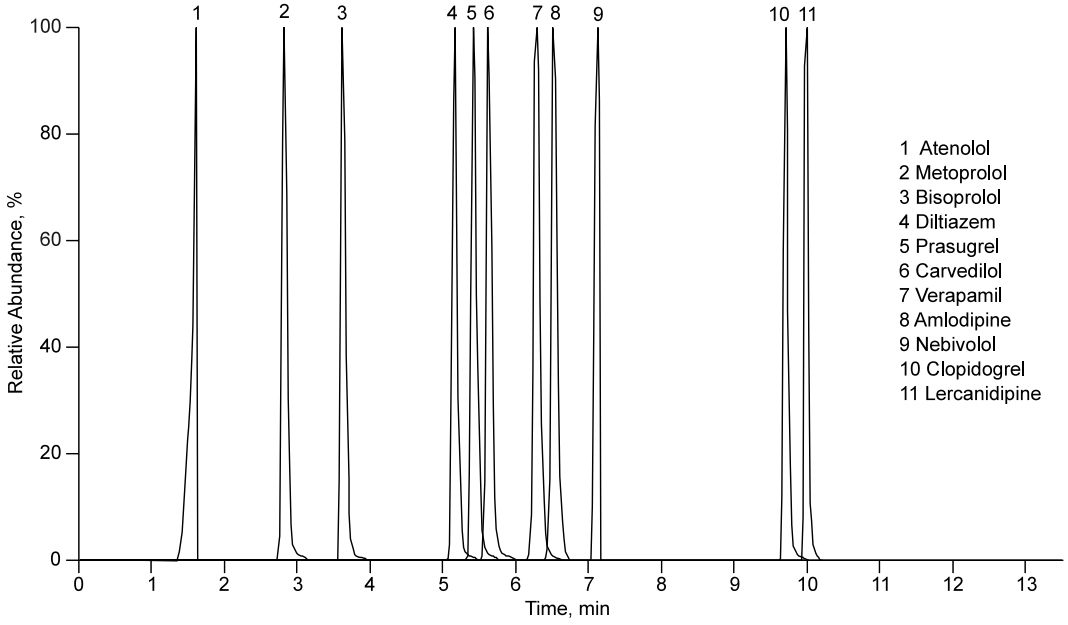

Fig. 1. Extracted ion chromatograms of analytes after positive ionization. All *m/z* at 100 % relative abundance.

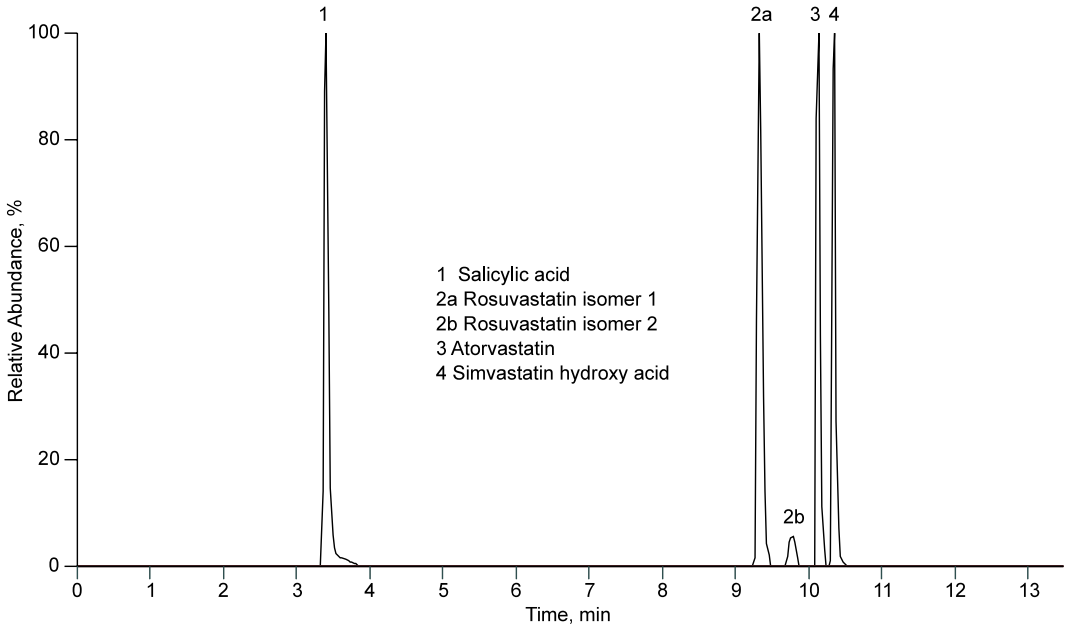

Fig. 2. Extracted ion chromatograms of analytes after negative ionization. All *m/z* at 100 % relative abundance.

salicylic acid-d_4 (28 %, 35 %, 41 %, respectively). For the analytes with a corresponding isotope-labeled IS, the NMF was calculated, represented in Table 6. At QC lev 3, the NMF for VAMS varied from 93 % (clopidogrel) to 106 % (amlodipine) with CVs up to 8 % (rosuvastatin). At QC ULOQ, the NMF ranged from 91 % (clopidogrel) to 102 % (amlodipine), except for verapamil (41 %). Corresponding CVs reached up to 13 % (rosuvastatin). At QC lev 3, the NMF for Capitainer-B varied from 87 % (rosuvastatin) to 115 % (amlodipine) with CVs up to 15 % (rosuvastatin). At QC ULOQ, the NMF ranged from 79 % (rosuvastatin) to 110 % (amlodipine) except for verapamil (41 %). Corresponding CVs reached up to 15 % (rosuvastatin). The large variability of the matrix effect for analytes with a corresponding isotope-labeled IS is compensated by the calculation of the NMF, so that the CVs are $\leq$15 % for both concentration levels and both devices, especially evident for salicylic acid. The matrix effects for Capitainer-B were higher for most analytes compared to VAMS at both concentration levels. The difference in MF is greatest for atorvastatin with 65 % at QC lev 3 and 107 % at QC ULOQ for VAMS and 154 % at QC lev 3 and 286 % at QC ULOQ for Capitainer-B. The great difference is also visible for lercanidipine with 79 % at QC lev 3 and 116 % at QC ULOQ for VAMS and 41 % at QC lev 3 and 56 % at QC ULOQ for Capitainer-B. This can also be seen with clopidogrel with 71 % (QC lev 3) and 101 % (QC ULOQ) for VAMS and 32 % (QC lev 3) and 36 % (QC ULOQ) for Capitainer-B. The two different devices and sampling strategies have different influences on the MF. The hematocrit of the blank whole blood samples, not containing the analytes of interest, varied between 38 and 50 % (see Table 3). Matrix effects were not determined at specific hematocrit levels (e.g., 20 %, 40 % and 60 %), as previous studies did not observe a hematocrit effect at different hematocrit levels for both sampling devices [43,44].

Twelve samples each for VAMS and Capitainer-B, loaded with blank

Table 5
Matrix factor (MF) (n = 6) of analytes for volumetric absorptive microsampling (VAMS) and Capitainer-B devices. (-: not detectable; CV: coefficient of variation; lev: level; QC: quality control; ULOQ: upper limit of quantification).

Analyte	MF, %; CV, %			
	VAMS		Capitainer-B	
	QC lev 3	QC ULOQ	QC lev 3	QC ULOQ
Amlodipine	89; 21	135; 6	97; 0	144; 0
Amlodipine-d_4	84; 20	132; 9	86; 17	132; 14
Atenolol	192; 20	154; 8	204; 10	153; 7
Atorvastatin	64; 14	107; 23	154; 21	286; 28
Bisoprolol	99; 19	132; 3	121; 15	160; 11
Bisoprolol-d_5	98; 18	143; 3	126; 15	179; 6
Carvedilol	96; 14	128; 4	102; 13	139; 8
Clopidogrel	71; 28	101; 7	32; 8	36; 10
Clopidogrel-d_4	78; 0	111; 0	36; 8	42; 12
Diltiazem	523; 13	198; 5	524; 21	197; 9
Diltiazem-d_4	518; 0	207; 0	525; 20	213; 6
Lercanidipine	–	116; 3	–	56; 6
Metoprolol	86; 29	123; 5	111; 13	141; 10
Metoprolol-d_6	87; 28	131; 9	117; 13	152; 11
Nebivolol	107; 20	135; 3	113; 13	140; 10
Prasugrel	–	–	–	–
Rosuvastatin	94; 16	109; 5	140; 14	169; 13
Rosuvastatin-d_6	90; 20	114; 12	163; 16	216; 16
Salicylic acid	92; 16	91; 31	70; 44	93; 35
Salicylic acid-d_4	97; 18	95; 37	71; 52	99; 41
Simvastatin hydroxy acid	83; 21	112; 7	81; 0	111; 0
Verapamil	89; 17	50; 4	94; 13	54; 9
Verapamil-d_6	92; 16	121; 1	100; 14	133; 4

Table 6
Normalized matrix factor (NMF) (n = 6) of analytes for volumetric absorptive microsampling (VAMS) and Capitainer-B. (CV: coefficient of variation; lev: level; QC: quality control; ULOQ: upper limit of quantification).

Analyte	NMF, %; CV, %			
	VAMS		Capitainer-B	
	QC lev 3	QC ULOQ	QC lev 3	QC ULOQ
Amlodipine	106; 7	102; 6	115; 8	110; 10
Bisoprolol	101; 3	92; 4	96; 3	89; 8
Clopidogrel	93; 4	91; 3	88; 2	85; 7
Diltiazem	101; 4	96; 4	100; 3	92; 8
Metoprolol	98; 3	95; 6	95; 3	92; 7
Rosuvastatin	105; 8	97; 13	87; 15	79; 15
Salicylic acid	95; 3	99; 10	104; 10	98; 11
Verapamil	96; 3	41; 4	93; 5	41; 5

whole blood, were analyzed and examined for interfering signals from endogenous compounds or false positive results. Selectivity was given and the combination of retention times and extracted precursor ion masses (see Table 2) was suitable for unambiguous analyte identification for each compound except for prasugrel. Consequently, prasugrel cannot be clearly identified using the current analytical method.

In the blank matrices injected after the QC ULOQ for VAMS and Capitainer-B samples, no carry-over effect exceeding 15 % of the LLOQ was observed. Only carvedilol (VAMS) and verapamil (Capitainer-B) were detected in the first blank matrix, while nothing was detected in the second blank matrix. Anyway, after injecting a sample with a higher concentration, the following sample should be reanalyzed after one or more wash-out runs using extracted blank samples. Wash-out runs must be performed until no analyte carry-over is detectable in subsequent sample injections.

The quantification was based on a previously determined RRF with an isotope-labeled IS. The use of an isotope-labeled IS instead of a non-deuterated IS was particularly important for a quantification using the RRF. This approach was expected to help correct differences in recovery and matrix effects and compensate for ion suppression or enhancement, extraction variability, and instrument variations. [45]. Furthermore, the use of a non-deuterated IS might carry the risk that this analyte may be present in genuine patient samples, whereas the probability of isotope-labeled IS being present in patient samples appeared to be rather low. As no external calibration (curve) was required to quantify each analyte, this method was designed to be time-saving and sustainable. To also work cost-efficiently, no corresponding isotope-labeled IS for each analyte was used, but an isotope-labeled IS with a similar chemical structure. This strategy allowed for the quantification of all analytes except for prasugrel, since prasugrel could not be detected. The spiked blood samples did not contain prasugrel anymore, probably due to severe stability reasons in blood. Stability studies have shown a rapid, complete hydrolytic degradation of the acetoxy group of prasugrel under basic conditions [46], and blood has slightly basic conditions. Further validations focused on quantifying its active metabolite, R-138727 [47], but it was not possible to detect R-138727 in the required concentrations.

The results for within- and between-day accuracy and precision are shown in Table 7 (VAMS) and Table 8 (Capitainer-B). The results for within- and between-day accuracy for VAMS were found to be acceptable according to the EMA guidelines with mean concentrations within ±15 % of the nominal values for the QC low, medium, and ULOQ samples (−15 to 15 %) and within ±20 % for the QC LLOQ samples (−20 to 11 %) for all analytes [31], except for lercanidipine. For the within- and between day precision in case of VAMS, all analytes met the required criteria with CV values within ±15 % for the QC low, medium, and ULOQ samples (0–15 %) and within ±20 % for the QC LLOQ samples (0–15 %) for all analytes, except for lercanidipine. Accuracy and precision for lercanidipine could not be determined for the previously defined QC LLOQ and QC low, which were based on the therapeutic range, as no reproducible peaks were detected at these concentrations. The specified LLOQ for lercanidipine was raised to 5 ng/mL for this validated method, meaning the therapeutic range (0.1–10 ng/mL) cannot be fully covered with this method for VAMS. The results for within- and between-day accuracy for Capitainer-B were found to be acceptable according to the EMA guidelines with mean concentrations within ±15 % of the nominal values for the QC low, medium, and ULOQ samples (−15 to 15 %) and within ±20 % for the QC LLOQ samples (−20 to 14 %) for all analytes [31], except for atorvastatin (within-day accuracy, QC ULOQ -24 %; between-day accuracy QC ULOQ 3 %) and lercanidipine. For the within- and between day precision for Capitainer-B all analytes met the required criteria with CV values within ±15 % for the QC low, medium, and ULOQ samples (0–15 %) and within ±20 % for the QC LLOQ samples (0–13 %) for all analytes, except for atorvastatin (within-day precision, QC ULOQ 20 %; between-day precision QC ULOQ 27 %) and lercanidipine. Accuracy and precision for lercanidipine could not be determined for the previously defined QC LLOQ and QC low, which were based on the therapeutic range, as no reproducible peaks were detected at these concentrations. The specified LLOQ for lercanidipine was raised to 5 ng/mL for this validated method, so that the therapeutic range (0.1–10 ng/mL) cannot be fully covered with this method for Capitainer-B. For atorvastatin the therapeutic range (7–250 ng/mL) cannot be covered completely with Capitainer-B (quantification range 6–150 ng/mL), but sampling with VAMS (quantification range 6–300 ng/mL).

The freeze-thaw stability of the stock solutions of all analytes and IS at 100 ng/mL was tested over twenty cycles. Measured peak areas of the analytes and IS were within ±15 % of the first measured peak area (t_0) except for lercanidipine (−16 %), simvastatin hydroxy acid (−24 %), and prasugrel (−34 %). The results for autosampler and freezer stability are shown in Table 9 (VAMS) and Table 10 (Capitainer-B). Extracted samples stored for 36 h at 10 °C in the autosampler showed no analyte degradation of more than ±15 % compared to the concentration at t_0 for VAMS for QC low and ULOQ levels. For Capitainer-B, all analytes met the required criteria except for amlodipine (−17 %). These results indicate adequate autosampler stability for extended analytical runs.

Table 7
Within- and between-day accuracy and precision for 4 quality controls (QC) (n = 5 at three different days) for volumetric absorptive microsampling (VAMS). (-: not detectable; CV: coefficient of variation; LLOQ: lower limit of quantification; ULOQ: upper limit of quantification).

Analyte	Relative mean concentration (accuracy), %; CV (precision), %							
	Within-day				Between-day			
	QC LLOQ	QC low	QC mid	QC ULOQ	QC LLOQ	QC low	QC mid	QC ULOQ
Amlodipine	85; 13	89; 12	87; 9	96; 8	84; 10	90; 9	96; 7	96; 9
Atenolol	108; 10	102; 9	112; 14	115; 12	113; 9	110; 9	104; 12	107; 15
Atorvastatin	108; 15	112; 8	112; 5	109; 11	114; 14	110; 10	111; 7	108; 13
Bisoprolol	91; 14	103; 11	86; 4	87; 4	90; 12	96; 11	86; 5	87; 9
Carvedilol	80; 6	90; 6	86; 4	85; 5	91; 11	105; 12	95; 7	96; 10
Clopidogrel	90; 3	110; 4	103; 4	110; 6	90; 8	111; 8	105; 8	108; 7
Diltiazem	105; 2	115; 7	113; 3	115; 6	107; 4	114; 7	113; 5	113; 7
Lercanidipine	–	–	86; 6	86; 6	–	–	87; 5	88; 10
Metoprolol	107; 4	90; 6	94; 5	89; 5	105; 7	98; 11	94; 10	95; 11
Nebivolol	90; 9	88; 7	89; 11	86; 6	104; 15	98; 13	100; 15	100; 15
Prasugrel	–	–	–	–	–	–	–	–
Rosuvastatin	95; 3	114; 9	106; 8	108; 11	99; 12	110; 11	108; 12	109; 9
Salicylic acid	98; 3	90; 5	94; 3	90; 7	95; 6	93; 7	96; 6	94; 8
Simvastatin hydroxy acid	113; 5	108; 9	113; 6	114; 9	112; 10	108; 10	112; 8	113; 11
Verapamil	106; 2	97; 3	115; 4	111; 3	111; 7	104; 8	113; 4	113; 5

Table 8
Within- and between-day accuracy and precision for 4 quality controls (QC) (n = 5 at three different days) for Capitainer-B. (-: not detectable; CV: coefficient of variation; LLOQ: lower limit of quantification; ULOQ: upper limit of quantification).

Analyte	Relative mean concentration (accuracy), %; CV (precision), %							
	Within-day				Between-day			
	QC LLOQ	QC low	QC mid	QC ULOQ	QC LLOQ	QC low	QC mid	QC ULOQ
Amlodipine	80; 7	86; 7	85; 10	86; 6	85; 13	91; 10	86; 10	86; 10
Atenolol	86; 7	85; 5	86; 10	86; 5	90; 11	89; 11	87; 11	87; 10
Atorvastatin	108; 5	97; 8	96; 13	76; 20	111; 10	108; 10	108; 13	103; 27
Bisoprolol	85; 3	92; 2	86; 5	86; 3	83; 5	89; 8	86; 4	86; 6
Carvedilol	80; 4	94; 9	85; 6	86; 9	84; 10	89; 10	86; 6	87; 8
Clopidogrel	86; 5	106; 2	101; 3	105; 2	89; 5	108; 6	101; 6	106; 9
Diltiazem	102; 2	115; 2	110; 4	111; 4	100; 6	115; 4	109; 5	114; 6
Lercanidipine	–	–	89; 10	86; 6	–	–	87; 11	86; 5
Metoprolol	99; 3	85; 4	85; 8	86; 5	104; 4	86; 4	86; 6	86; 9
Nebivolol	87; 8	85; 8	87; 10	86; 6	85; 12	86; 9	87; 10	86; 9
Prasugrel	–	–	–	–	–	–	–	–
Rosuvastatin	109; 12	92; 11	86; 15	88; 13	108; 10	97; 12	89; 15	95; 15
Salicylic acid	85; 2	86; 6	90; 7	93; 2	85; 2	87; 5	92; 7	94; 8
Simvastatin hydroxy acid	96; 13	88; 8	95; 1	85; 6	93; 12	91; 9	93; 9	89; 10
Verapamil	111; 3	97; 2	115; 2	110; 3	110; 4	98; 3	113; 4	112; 6

Table 9
Autosampler stability for 36h at 10 °C (n = 3) and freezer stability for 36h at −20 °C (n = 3) for volumetric absorptive microsampling (VAMS). (-: not detectable; CV: coefficient of variation; lev: level; QC: quality control; ULOQ: upper limit of quantification).

Analyte	Relative mean concentration, %; CV, %			
	QC lev 3		QC ULOQ	
	36h 10 °C	36h −20 °C	36h 10 °C	36h −20 °C
Amlodipine	104; 7	93; 11	99; 10	100; 5
Atenolol	98; 20	104; 5	101; 16	108; 5
Atorvastatin	99; 1	97; 8	98; 3	87; 7
Bisoprolol	101; 2	103; 3	99; 8	99; 3
Carvedilol	99; 1	99; 5	100; 9	96; 1
Clopidogrel	102; 5	101; 3	100; 11	99; 4
Diltiazem	101; 3	98; 3	105; 8	101; 4
Lercanidipine	–	–	111; 12	101; 6
Metoprolol	103; 5	98; 5	100; 9	105; 5
Nebivolol	96; 20	114; 4	97; 18	113; 3
Prasugrel	–	–	–	–
Rosuvastatin	101; 15	102; 7	93; 1	98; 2
Salicylic acid	96; 7	98; 3	99; 13	102; 6
Simvastatin hydroxy acid	95; 9	90; 4	95; 4	87; 14
Verapamil	102; 3	101; 5	101; 8	101; 3

Table 10
Autosampler stability for 36h at 10 °C (n = 3) and freezer stability for 36h at −20 °C (n = 3) for Capitainer-B. (-: not detectable; CV: coefficient of variation; lev: level; QC: quality control; ULOQ: upper limit of quantification).

Analyte	Relative mean concentration, %; CV, %			
	QC lev 3		QC ULOQ	
	36h 10 °C	36h −20 °C	36h 10 °C	36h −20 °C
Amlodipine	83; 11	97; 3	91; 4	113; 4
Atenolol	101; 6	100; 3	101; 7	106; 3
Atorvastatin	98; 1	112; 7	94; 2	105; 6
Bisoprolol	99; 1	98; 3	98; 2	101; 1
Carvedilol	96; 1	103; 4	101; 2	104; 3
Clopidogrel	96; 1	100; 3	112; 3	98; 1
Diltiazem	103; 2	101; 2	99; 1	104; 2
Lercanidipine	–	–	103; 4	111; 0
Metoprolol	100; 2	104; 4	103; 1	100; 2
Nebivolol	93; 5	113; 3	103; 8	106; 1
Prasugrel	–	–	–	–
Rosuvastatin	95; 4	98; 7	96; 3	93; 7
Salicylic acid	92; 14	96; 2	101; 2	107; 1
Simvastatin hydroxy acid	99; 7	89; 15	97; 2	86; 8
Verapamil	98; 2	103; 4	99; 1	100; 2

However, since stability is only confirmed up to 36 h, batch durations should not exceed this time period. Extracted samples stored for 36 h at −20 °C in the freezer showed no analyte degradation more than ±15 % compared to the concentration at t_0 for VAMS and Capitainer-B for QC low and ULOQ levels. The results for long-term stability are shown in Table 11 (VAMS) and Table 12 (Capitainer-B). Most of the analytes showed acceptable long-term stability in the devices under the tested conditions. After one week of storage at 24 °C, no analyte showed degradation of more than ±15 % compared to the concentration at t_0 for VAMS for QC low and ULOQ levels. After two weeks of storage under the same conditions, carvedilol showed degradation of more than 15 % for QC lev 3 (19 %), lercanidipine for QC ULOQ (16 %), and simvastatin hydroxy acid for QC lev 3 and QC ULOQ (both 17 %). All other analytes met the required criteria. For Capitainer-B, all analytes showed no degradation of more than ±15 % compared to the concentration at t_0 after one and after two weeks of storage at 24 °C.

3.2. Proof-of-concept

As a proof-of-concept, matching samples from 30 patients were collected using VAMS and Capitainer-B devices. Unfortunately, samples containing atenolol, diltiazem and verapamil were unavailable. Thirty VAMS tips soaked with FPB were analyzed. For patients 5, 7, 10, 11, 12, and 14, no Capitainer-B disks soaked with FPB were available as it was not possible to collect the necessary amount of blood from these patients to completely fill the measuring channel, meaning it was not possible to collect 10 µL of whole blood with the disk. For loading the Capitainer-B device, more blood was required, as the metering channel had to be filled first before the blood could be released on the sampling disk. With VAMS, the tips could be loaded directly with blood, which meant that less blood was required.

For method validation, the quantification range was based on the therapeutic plasma range. The therapeutic plasma range was not suitable for assessing adherence, especially in patients with different daily doses, as different concentration levels were to be expected. For that, a dose-specific $c_{cut\text{-}off}$ was calculated. Table 13 shows the quantitative results after analyzing VAMS tips and Capitainer-B disks as well as the assessment of adherence. To confirm adherence, the measured concentration in blood for both devices should be higher than the calculated $c_{cut\text{-}off}$. The pharmacokinetic parameters used for calculation and calculated $c_{cut\text{-}off}$ are shown in Table 14. Ideally, drug concentrations in capillary blood should be used for the calculation, as drug concentrations in capillary blood may differ from those in venous blood [48,49]. But therapeutic reference ranges established for venous blood plasma were chosen for the calculation for the assessment of adherence, as to our knowledge no therapeutic values for capillary blood are available for the selected substances. For bisoprolol, data were only available for 5 mg, not for 2.5 mg and 1.25 mg. For the assessment of bisoprolol adherence, $c_{cut\text{-}off}$ for 5 mg was used for all dosages. For acetylsalicylic acid data was only available for 75 mg, not for 100 mg. For the assessment of acetylsalicylic acid adherence, $c_{cut\text{-}off}$ for 75 mg was used. For metoprolol data was only available for 200 mg, not for 47.5 mg sustained release formulation. For the assessment of metoprolol adherence, $c_{cut\text{-}off}$ for 200 mg was used.

Table 11
One- and two-week stability in the sampling device at 24 °C (n = 3) for volumetric absorptive microsampling (VAMS). (-: not detectable; CV: coefficient of variation; lev: level; QC: quality control; ULOQ: upper limit of quantification).

Analyte	Relative mean concentration, %; CV, %			
	QC lev 3		QC ULOQ	
	1 week 24 °C	2 weeks 24 °C	1 week 24 °C	2 weeks 24 °C
Amlodipine	112; 12	109; 13	104; 6	105; 8
Atenolol	86; 15	86; 13	111; 10	97; 14
Atorvastatin	108; 19	92; 5	109; 4	90; 15
Bisoprolol	90; 5	98; 4	101; 4	100; 9
Carvedilol	108; 11	81; 5	113; 4	91; 1
Clopidogrel	91; 8	91; 3	96; 2	90; 8
Diltiazem	99; 2	95; 2	103; 6	93; 7
Lercanidipine	–	–	96; 8	84; 5
Metoprolol	91; 2	98; 2	98; 5	96; 6
Nebivolol	99; 13	100; 13	112; 6	104; 3
Prasugrel	–	–	–	–
Rosuvastatin	93; 16	86; 12	86; 6	86; 3
Salicylic acid	114; 2	113; 1	113; 8	115; 12
Simvastatin hydroxy acid	91; 12	83; 10	93; 4	83; 5
Verapamil	97; 4	98; 2	102; 1	100; 8

Table 12
One- and two-week stability in the sampling device at 24 °C (n = 3) for Capitainer-B. (-: not detectable; CV: coefficient of variation; lev: level; QC: quality control; ULOQ: upper limit of quantification).

Analyte	Relative mean concentration, %; CV, %			
	QC lev 3		QC ULOQ	
	1 week 24 °C	2 weeks 24 °C	1 week 24 °C	2 weeks 24 °C
Amlodipine	100; 4	100; 12	114; 4	113; 8
Atenolol	113; 9	102; 1	85; 6	96; 15
Atorvastatin	101; 4	98; 1	85; 3	89; 5
Bisoprolol	98; 2	99; 4	98; 4	100; 3
Carvedilol	114; 11	89; 7	110; 5	89; 1
Clopidogrel	101; 2	97; 4	102; 6	106; 4
Diltiazem	103; 2	99; 4	101; 4	97; 4
Lercanidipine	–	–	114; 14	90; 7
Metoprolol	97; 6	103; 3	85; 1	90; 4
Nebivolol	114; 4	109; 5	113; 10	110; 1
Prasugrel	–	–	–	–
Rosuvastatin	95; 4	88; 6	89; 4	85; 3
Salicylic acid	111; 4	114; 3	112; 5	115; 3
Simvastatin hydroxy acid	102; 9	89; 7	104; 7	90; 6
Verapamil	104; 4	112; 4	105; 2	112; 6

The determined concentrations in FPB sampled with VAMS and in FPB sampled with Capitainer-B were comparable between the two sampling methods. In 24 matched samples, the assessment of adherence differed only twice between VAMS and Capitainer-B. For patient 1, the concentration of metoprolol was <16 ng/mL in VAMS, which was not assessable, and 18 ng/mL in Capitainer-B, which was assessed as adherent. In case of patient 13, the concentration of amlodipine was 3 ng/mL in VAMS, which was assessed as adherent, and <3 ng/mL in Capitainer-B, which was not assessable. This was because $c_{cut\text{-}off}$ used to assess adherence was lower than the LLOQ, and it is not possible to assess adherence or nonadherence for concentrations below the LLOQ.

Of a total of 96 drug intakes for VAMS, 67 were assessed as adherent (70 %), four as nonadherent (4 %) and 25 as not assessable (26 %), and of a total of 77 drug intakes for Capitainer-B, 52 were classified as adherent (67 %), two as nonadherent (3 %) and 23 as not assessable (30 %). Drug intakes that could not be evaluated included prasugrel, as the validation results have shown, that analyte detection in whole blood is not possible. For other drug intakes, $c_{cut\text{-}off}$ was below the LLOQ, making it impossible to assess adherence or nonadherence for measured concentrations below the LLOQ. For acetylsalicylic acid, out of a total of 17 drug intakes for VAMS, six patients showed adherence and 11 patients were not evaluable. Of the total of 15 drug intakes for Capitainer-B, four patients showed adherence and 11 patients were not evaluable. The inability to assess adherence or not is due to the non-detection of salicylic acid in these samples. The calculated cut-off concentration for assessing adherence is 2 ng/mL, which is well below the LLOQ (16,000 ng/mL). Therefore, adherence cannot be definitively excluded, as the expected concentrations for assessment are not fully covered by this method.

Excluding the not assessable drug intakes for the assessment of

Table 13
Quantification of coronary artery disease drugs (ng/mL) using finger prick blood sampled by volumetric absorptive microsampling (VAMS) and Capitainer-B. Prescribed medication and mode of intake provided by medication plans. Mode of intake prescribed as number of tablets taken in the morning-noon-evening-night. The dose used for calculation of the cut-off concentration given in parentheses if it was different from the patient's prescribed daily dose. (-: not available; ↑: classified as adherent; ↓: classified as nonadherent; ?: classification not possible; n.d.: not detectable; r: sustained release formulation).

Patient	Medication	Mode of intake	Concentration in VAMS, ng/mL	Concentration in Capitainer-B, ng/mL
1	Acetylsalicylic acid 100 mg	0-1-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Atorvastatin 80 mg	1-0-0-0	>300 ↑	>150 ↑
	Clopidogrel 75 mg	1-0-0-0	>10 ↑	>10 ↑
	Metoprolol 47.5 mg r	1-0-1-0	<16 ? (200 mg)	18 ↑ (200 mg)
2	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Atorvastatin 40 mg	1-0-0-0	18 ↑	20 ↑
	Bisoprolol 2.5 mg	1-0-0-0	<8 ? (5 mg)	<8 ? (5 mg)
	Prasugrel 10 mg	1-0-0-0	n.d. ?	n.d. ?
3	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Atorvastatin 40 mg	1-0-0-0	86 ↑	75 ↑
	Bisoprolol 2.5 mg	1-0-1-0	23 ↑ (5 mg)	25 ↑ (5 mg)
	Prasugrel 10 mg	1-0-0-0	n.d. ?	n.d. ?
4	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Bisoprolol 2.5 mg	1-0-0-0	13 ↑ (5 mg)	12 ↑ (5 mg)
	Clopidogrel 75 mg	1-0-0-0	6 ↑	7 ↑
	Rosuvastatin 40 mg	1-0-0-0	12 ↑	9 ↑
5	Bisoprolol 5 mg	1-0-1-0	60 ↑	–
	Rosuvastatin 10 mg	1-0-0-0	17 ↑	–
6	Acetylsalicylic acid 100 mg	1-0-0-0	<16,000 ↑ (75 mg)	<16,000 ↑ (75 mg)
	Bisoprolol 5 mg	1-0-1-0	22 ↑	24 ↑
	Rosuvastatin 10 mg	1-0-0-0	10 ↑	7 ↑
7	Atorvastatin 80 mg	1-0-0-0	>300 ↑	–
	Amlodipine 5 mg	1-0-0-0	17 ↑	–
	Carvedilol 12.5 mg	1-0-1-0	<20 ?	–
8	Amlodipine 5 mg	1-0-1-0	18 ↑	15 ↑
	Carvedilol 12.5 mg	1-0-1-0	<20 ?	<20 ?
	Rosuvastatin 40 mg	1-0-0-0	31 ↑	27 ↑
9	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Atorvastatin 40 mg	1-0-0-0	10 ↓	8 ↓
	Bisoprolol 2.5 mg	1-0-0-0	<8 ? (5 mg)	<8 ? (5 mg)
	Prasugrel 10 mg	1-0-0-0	n.d. ?	n.d. ?
10	Acetylsalicylic acid 100 mg	1-0-0-0	<16,000 ↑ (75 mg)	–
	Amlodipine 5 mg	2-0-0-0	5 ↑	–
	Atorvastatin 40 mg	1-0-0-0	28 ↑	–
	Bisoprolol 2.5 mg	1-0-1/2-0	18 ↑ (5 mg)	–
	Prasugrel 10 mg	1-0-0-0	n.d. ?	–
11	Amlodipine 10 mg	1-0-0-0	23 ↑	–
	Atorvastatin 80 mg	1-0-0-0	6 ↓	–
	Bisoprolol 2.5 mg	1-0-1-0	68 ↑ (5 mg)	–
12	Bisoprolol 5 mg	1-0-1/2-0	69 ↑	–
	Rosuvastatin 10 mg	0-0-1-0	7 ↑	–
13	Amlodipine 5 mg	1-0-0-0	3 ↑	<3 ?
	Nebivolol 5 mg	1-0-0-0	<1 ?	<1 ?
	Rosuvastatin 20 mg	0-0-1-0	<3 ?	<3 ?
14	Acetylsalicylic acid 100 mg	1-0-0-0	<16,000 ↑ (75 mg)	–
	Atorvastatin 40 mg	1-0-0-0	<6 ↓	–
	Bisoprolol 2.5 mg	1-0-0-0	16 ↑ (5 mg)	–
	Clopidogrel 75 mg	1-0-0-0	3 ↑	–
15	Atorvastatin 10 mg	0-0-1-0	n.d. ↓	n.d. ↓
	Bisoprolol 5 mg	1-0-1-0	19 ↑	22 ↑
	Lercanidipine 10 mg	1-0-0-0	<5 ↑	<5 ↑
16	Acetylsalicylic acid 100 mg	1-0-0-0	<16,000 ↑ (75 mg)	<16,000 ↑ (75 mg)
	Amlodipine 10 mg	1-0-0-0	41 ↑	37 ↑
	Atorvastatin 40 mg	1-0-0-0	114 ↑	107 ↑
	Carvedilol 12.5 mg	1-0-1-0	<20 ?	<20 ?
17	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Amlodipine 5 mg	1-0-0-0	17 ↑	14 ↑
	Atorvastatin 40 mg	1-0-0-0	197 ↑	>150 ↑
18	Bisoprolol 1.25 mg	1-0-0-0	15 ↑ (5 mg)	15 ↑ (5 mg)
	Lercanidipine 10 mg	1-0-0-0	6 ↑	5 ↑
	Simvastatin 20 mg	0-0-1-0	6 ↑	7 ↑
19	Acetylsalicylic acid 100 mg	1-0-0-0	<16,000 ↑ (75 mg)	<16,000 ↑ (75 mg)
	Clopidogrel 75 mg	1-0-0-0	1 ↑	1 ↑
	Lercanidipine 10 mg	1-0-0-0	<5 ↑	<5 ↑
20	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Bisoprolol 5 mg	1-0-1-0	63 ↑	56 ↑
	Rosuvastatin 10 mg	1-0-0-0	<3 ?	<3 ?
21	Clopidogrel 75 mg	0-1-0-0	8 ↑	7 ↑
	Metoprolol 47.5 mg r	1-0-1-0	130 ↑ (200 mg)	123 ↑ (200 mg)
	Simvastatin 20 mg	0-0-1-0	5 ↑	6 ↑
22	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Atorvastatin 40 mg	0-0-2-0	209 ↑	>150 ↑
	Bisoprolol 2.5 mg	1-0-0-0	31 ↑ (5 mg)	25 ↑ (5 mg)
23	Atorvastatin 40 mg	0-0-1-0	75 ↑	68 ↑
	Metoprolol 47.5 mg r	1-0-1-0	77 ↑ (200 mg)	71 ↑ (200 mg)
24	Bisoprolol 2.5 mg	1-0-0-0	25 ↑ (5 mg)	23 ↑ (5 mg)

(*continued on next page*)

Table 13 (*continued*)

Patient	Medication	Mode of intake	Concentration in VAMS, ng/mL	Concentration in Capitainer-B, ng/mL
	Rosuvastatin 5 mg	0-0-1-0	11 ↑	10 ↑
25	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Amlodipine 5 mg	1-0-0-0	20 ↑	19 ↑
	Metoprolol 47.5 mg r	1-0-1-0	74 ↑ (200 mg)	78 ↑ (200 mg)
26	Acetylsalicylic acid 100 mg	1	<16,000 ↑ (75 mg)	<16,000 ↑ (75 mg)
	Atorvastatin 40 mg	1	110 ↑	120 ↑
	Bisoprolol 5 mg	1	39 ↑	42 ↑
	Clopidogrel 75 mg	1	>10 ↑	>10 ↑
27	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Atorvastatin 40 mg	1-0-0-0	12 ↑	13 ↑
	Bisoprolol 5 mg	1-0-0-0	32 ↑	32 ↑
	Prasugrel 10 mg	1-0-0-0	n.d. ?	n.d. ?
28	Clopidogrel 75 mg	1-0-0-0	>10 ↑	>10 ↑
	Metoprolol 47.5 mg r	1-0-1-0	101 ↑ (200 mg)	110 ↑ (200 mg)
	Rosuvastatin 10 mg	1-0-0-0	21 ↑	19 ↑
29	Carvedilol 25 mg	1-0-0-0	115 ↑	109 ↑
	Rosuvastatin 20 mg	1-0-0-0	5 ↑	5 ↑
30	Acetylsalicylic acid 100 mg	1-0-0-0	n.d. ? (75 mg)	n.d. ? (75 mg)
	Metoprolol 47.5 mg r	1-0-1-0	283 ↑ (200 mg)	287 ↑ (200 mg)
	Rosuvastatin 20 mg	0-0-1-0	7 ↑	6 ↑

Table 14
Patient medications, daily doses and pharmacokinetic parameters including elimination half-lives ($t_{1/2}$), time to peak concentration (t_{max}), and peak plasma concentration (c_{max}) according to Baselt [38], and calculated dose-dependent cut-off concentrations ($c_{cut\text{-}off}$). (-: not available).

Medication	Daily dose, mg	$t_{1/2}$, h	t_{max}, h	c_{max}, ng/mL	$c_{cut\text{-}off}$, ng/mL
Acetylsalicylic acid as salicylic acid	75	2–20	1	4300	2
Amlodipine	5	30–70	5.4	2.7	2
	10	30–70	5.4	5.5	3
Atenolol	100	4–12	9.2	540	34
Atorvastatin	10	11–24	2.0–2.5	7.4	2
	40	11–24	2.0–2.5	67	12
	80	11–24	2.0–2.5	252	45
Bisoprolol	5	7–15	1.9	21	2
Carvedilol	12.5	4–7	1.5	52	1
	25	4–7	1	108	2
Clopidogrel	75	1.4–3.6	1.0–1.4	2.0	0.00002
Diltiazem	60	2.8–9.2	3	130	0.6
Lercanidipine	10	6–10	1.8	3.0	0.2
Metoprolol	200	2.5–7.5	4–6	175	0.6
Nebivolol	5	8–14	1	1.5	0.2
Prasugrel	10	–	–	–	–
Rosuvastatin	5	12–32	2.0–3.4	8.3	2
	10	12–32	3.5	6.0	2
	20	12–32	2.0–3.4	20	5
	40	12–32	3.0	37	9
Simvastatin as simvastatin hydroxy acid	20	2–4	5.8	4.2	0.01
Verapamil	80	3–7	0.5	55	0.2

patient adherence, of a total of 30 patients sampled with VAMS, 26 were assessed as fully adherent (87 %), three as partially adherent (10 %), and one as fully nonadherent (3 %). Of a total of 23 patients sampled with Capitainer-B, 21 were classified as fully adherent (91 %), one as partially adherent (4 %), one as completely nonadherent (4 %).

3.3. Limitations

Selectivity was not given for prasugrel, and it was also not possible to detect prasugrel in blood samples. Therefore, this method could not be validated for prasugrel. Quantification was possible for most analytes, except for prasugrel. Only for lercanidipine it was not possible to cover the entire therapeutic plasma range with the method for both devices. For atorvastatin, the entire therapeutic plasma range could only be covered with VAMS, but not with Capitainer-B.

No reference ranges for FPB or capillary blood were available for assessing adherence, so dose-dependent $c_{cut\text{-}off}$ were calculated based on reference ranges for venous blood. A more reliable assessment of adherence requires reference ranges for FPB, which must be clinically validated. It was possible to collect patient samples for all analytes of interest, but not for atenolol, diltiazem and verapamil. These analytes have therefore not been analyzed in patient samples yet. For acetylsalicylic acid and lercanidipine in particular, $c_{cut\text{-}off}$ used to assess adherence was below the LLOQ. For these analytes, it is not possible to assess adherence or nonadherence for measured concentrations below the LLOQ. For most of the analytes, the measured concentration was above the LLOQ, so an assessment based on $c_{cut\text{-}off}$ was possible. In addition, LC-HRMS based adherence monitoring cannot rule out white coat adherence, where patients only take their medication in anticipation of a scheduled monitoring event. Nevertheless, follow-up visits of all patients are necessary to assess long-term adherence for the selected medication.

Several factors must be considered for successful clinical implementation. Both devices offer various advantages. VAMS and Capitainer-B were used to collect FPB samples, a smaller sample volume, which could also improve patient comfort. Both are therefore expected to be suitable for at-home sampling and decentralized collection. However, clinicians and patients must be thoroughly instructed in the correct sampling procedures to minimize the risk of inaccurate results due to sampling errors. In some patients, sample collection can be difficult, as observed in six patients for Capitainer-B disk, who were unable to fill the disk completely due to circulation pathologies. The development of standardized training protocols could be helpful in addressing differences between patients. A standardized workflow should be implemented for at-home sampling and decentralized collection, including storage and transport conditions for collected samples. Costs remain an important factor for a successful clinical implementation. The price per device and the necessary consumables may exceed those of established venous blood collection, although bulk purchases can help offset expenses. In addition, successful implementation depends on laboratories that adopt high-throughput workflows being able to process large volumes of samples using VAMS and Capitainer-B.

4. Conclusions

A quantitative method for the simultaneous analysis of 15 drugs used in the treatment of CVDs was successfully developed and validated, except for prasugrel. Sampling of 10 µL of FPB using VAMS and Capitainer-B devices was possible. After simple sample preparation, chromatographic separation of the target analytes was achieved. Quantification was possible for all analytes, except for prasugrel. VAMS and Capitainer-B as a sampling strategy had the advantage of stability in dried matrix for most analytes. A proof-of-concept was carried out in which 164 concentrations were determined in 30 different patients and two microsampling devices. When taking samples from patients, VAMS

performed better than Capitainer-B. Adherence assessment was possible for all analytes except for acetylsalicylic acid and prasugrel. However, the current results of this study demonstrate that VAMS and Capitainer-B are a promising tool for adherence monitoring for CVDs drugs.

CRediT authorship contribution statement

Diana Kretschmer: Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Mert Tokcan:** Writing – review & editing, Resources. **Philipp Markwirth:** Writing – review & editing, Resources. **Lea Wagmann:** Writing – review & editing, Supervision, Conceptualization. **Felix Mahfoud:** Writing – review & editing, Resources. **Michael Böhm:** Writing – review & editing, Resources. **Markus R. Meyer:** Writing – review & editing, Supervision, Resources, Conceptualization.

Code availability

Not applicable.

Ethical disclosure and source of biological material

For investigations involving human subjects, informed consent has been obtained from the participants involved and an ethics committee approved the study (NCT01888315).

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Data availability

Data will be made available on request.

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