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Screening of cardiovascular agents in plasma with LC-MS/MS: A valuable tool for objective drug adherence assessment

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ABSTRACT

Adherence to cardiovascular preventive agents is important to prevent short and long term cardiovascular events. Recently, qualitatively compound screening using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has gained interest for drug adherence assessment in patients at high risk of cardiovascular events. Therefore, we developed and tested an assay including 52 compounds and metabolites, covering over 95% of the antihypertensive and antithrombotic agents available worldwide. Trichloroacetic acid was used as simple and fast method for protein precipitation. The assay was validated for lower limit of quantification (LLOQ), linearity, stability for freeze/thaw, room temperature, autosampler and matrix effects. The LLOQ for each compound was targeted under the population trough concentration (PTC) as reported in literature to assure high sensitivity for adherence detection. This was accomplished for 50 of 52 compounds with a LLOQ equal or lower compared to the PTC. Linearity was confirmed for all compounds ($r^2 > 0.995$), except for acetylsalicylic acid ($r^2 = 0.991$). For room temperature stability, 12 compounds showed degradation over 20% after 20 h. 3 compounds suffer from matrix effect with recoveries < 50%. After analytical validation, blood samples from 91 patients with difficult-to-treat hypertension were analyzed. Patients were unaware of adherence assessment. Adherence varied largely per agent and per concentration ratio (CR) (ratio of the detected concentration with LC-MS/MS and the PTC) cut-off value. Additionally, stratification by adherence group showed that the percentage of patients classified as non-adherent increased from 6.6% for qualitative analysis (pos/neg) to 19.8% for a CR cut-off of 0.5. The data imply that using the CR cut off values has a significant and relevant effect on patient adherence classification.

1. Introduction

Treatment with lipid modifying drugs, blood pressure lowering and antiplatelet agents reduce the risk of cardiovascular events. Cardiovascular disease is the leading cause of death worldwide [1]. However, 80% of patients do not adhere to their prescribed medical therapy when patients are unaware of monitoring and therefor remain at high risk for cardiovascular events [2]. Several methods are used in clinical studies and practice to assess medication adherence. Most of these are subjective, such as questionnaires [3], and amongst other methods like pharmacy dispense records, are unreliable [4]. Recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has gained interest for compound screening in drug adherence assessment [2,5,6], as a means to objective measure adherence to prescribed medication. The majority of LC-MS/MS methods studying adherence to cardiovascular drugs used qualitative assays in urine [7,8]. Albeit advantages over questionnaires, such as higher objectivity, at major disadvantage of urine screening is misclassification of drug adherence and the elaborate manner of collection the specimens [9]. Compounds with a short half-life and/or low assay sensitivity could yield false negative classification, while compounds with a long half-life and/or high assay sensitivity could yield false positive classification for adherence. An alternative is quantitative analysis in plasma with optimized specificity and sensitivity for clinical adherence assessment, which creates the opportunity to link drug exposure to the phenotype like blood pressure when sampling is accompanied by blood pressure measurement. As

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such, screening methods with LC-MS/MS for detection of limited number (< 22) of cardiovascular agents in plasma have been reported [10,11]. A study that tested 34 cardiovascular compounds as described by Dias et al. [12] did a large clinical validation in hospitalized patients. In another study, the assay covered a large number of 55 compounds [13], however the clinical validation was limited to a small number of patients. More importantly, stability testing was not performed in previous studies [12,13], covering a large scope of antihypertensive agents. Furthermore, compound sensitivity, stability and matrix effects are important factors affecting the limits of quantification, introducing false negative or false positive results. Control of these parameters in relation to the population trough concentrations (PTC) is essential for the determination of specificity and sensitivity of adherence classification. Therefore, we developed and tested an assay including 52 compounds and metabolites, covering over 95% of the antihypertensive and antithrombotic agents available worldwide. Secondly, we investigated the effect of quantitative reporting on adherence classifications in patients with difficult-to-treat hypertension.

2. Material and methods

2.1. Chemicals and reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All analytes were purchased from Bio-connect (Huissen, Netherlands). Gibco Newborn Calfs Plasma, heat inactivated, was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Drug Free Plasma was obtained from Bio-Rad (Hercules, CA, USA). Blank bovine plasma was obtained from Drug analysis and toxicology studies (SKML, Nijmegen, Netherlands). Blank patient plasma samples were randomly selected from residual material obtained in routine therapeutic drug monitoring (TDM) specimens with patients consent for the use of remnant material.

2.2. Patient samples

Samples obtained from patients with apparent therapy difficult-to-treat hypertension were assessed. Patients and physicians were unaware of adherence assessment. A waiver for consent was provided by the local medical ethics committee. After collection of blood, plasma samples were obtained after centrifugation and stored in −20 °C before analysis.

Long term stability was tested with plasma samples obtained by the TEMPUS study. These samples were collected between 2011 and 2012 and stored at −80 °C. The TEMPUS study was a prospective randomized open blinded endpoint cross-over trial to evaluate the effect of timing the administration of a cardiovascular polypill, a fixed-dose combination pill containing aspirin, statin, and two BP-lowering agents (Lisinopril and hydrochlorothiazide), on LDL-c and BP level. In this study drug intake was measured by the use of Medication Event Monitoring System(s) (Aardex, Zug, Switzerland). Samples with > 97% adherence according to the Medication Event Monitoring System(s) data, were used for the long term stability test. The study was conducted at the University Medical Center Utrecht, Utrecht, the Netherlands. The protocol has been approved by the Institutional Review Board. The trial is registered at clinicaltrials.gov with identification number NCT01506505 [14].

2.3. Sample preparation

100 µL plasma sample, 10 µL internal standard (caffeine C_{13}, 1000 µg/L in methanol) and 50 µL trichloroacetic acid (TCA) solution (25%) were pipetted into an 1.5 mL Eppendorf tube and vortexed for 60 s and 5 min centrifuged (24,650*g*). 60 µL was transferred into a glass vail with insert, after which they were ready for LC-MS/MS.

Table 1
Overview selected reaction monitoring transitions.

Compound Name	Quantification SRM, *m/z* (CE, v/RF, v)	Qualification SRM, *m/z* (CE, v/RF, v)
Acebutolol	337,2–218,0 (26/81)	337,2–319,1 (18/81)
Acetylsalicylic acid (–)	179.0–137,0 (10/30)	179.0–92,9 (21/30)
Aliskiren	552,4–436,3 (20/95)	552,4–418,3 (25/95)
Amlodipine	409,1–220,0 (29/57)	409,1–142,0 (42/57)
Atenolol	267,1–133,1 (32/71)	267,1–145,1 (29/71)
Barnidipine	492,2–315,1 (26/101)	492,2–91,1 (38/101)
Bisoprolol	326,2–107,1 (38/81)	326,2–56,2 (33/81)
Bumetanide	365,0–156,1 (38/81)	365,0–196,0 (36/81)
Caffeine-13C3 (IS)	198,0–140,0 (20/78)	
Canrenone	341,1–91,1 (57/78)	341,1–107,1 (30/78)
Captopril	218,1–116,0 (24/50)	218,1–47,2 (35/50)
Carvedilol	407,2–222,0 (26/88)	407,2–194,1 (38/88)
Chlorthalidone (–)	337,3–146,3 (21/75)	337,6–283,0 (19/75)
Clopidogrel	322,0–184,0 (22/77)	322,0–212,0 (16/77)
Diltiazem	415,2–178,0 (27/81)	415,2–150,0 (42/81)
Doxazosin	452,2–247,1 (41/111)	452,2–344,2 (32/111)
Enalapril	377,2–117,1 (38/71)	377,2–303,1 (20/71)
Enalaprilat	349,2–160,1 (25/79)	349,2–303,1 (17/79)
Eplerenone	415,2–163,1 (21/72)	415,2–337,1 (20/72)
Felodipine	337,9–277,9 (27/95)	337,9–305,9 (21/95)
Fosinopril	436,3–390,2 (18/84)	436,3–152,2 (31/84)
Fosinoprilat	436,2–152,2 (31/81)	436,2–390,2 (18/81)
Furosemide	329,6–205,6 (23/66)	329,6–285,6 (15/66)
Hydralazine	161,1–89,1 (26/62)	161,1–63,1 (48/62)
Hydrochlorothiazide (–)	296,6–205,6 (23/88)	296,6–269,6 (19/88)
Indapamide	366,1–132,1 (19/64)	366,1–117,3 (37/64)
Irbesartan	429,2–207,0 (26/83)	429,2–205,0 (54/83)
Labetalol	329,2–311,1 (15/65)	329,2–91,1 (34/65)
Lercanidipine	612,3–280,2 (24/114)	612,3–315,1 (32/114)
Lisinopril	406,3–246,1 (25/80)	406,3–84,1 (28/80)
Losartan	423,2–207 (24.1/76)	423,2–180 (38.1/76)
Losartan COOH	437,2–190,0 (35/70)	437,2–180,1 (34/70)
Methyldopa	212,1–103,1 (35/50)	212,1–65,1 (42/50)
Metoprolol	268,2–103,1 (41/73)	268,2–77,1 (59/73)
Minoxidil	210,1–193,0 (17/62)	210,1–164,1 (26/62)
Moxonidine	242,1–199,0 (23/75)	242,1–206,0 (20/75)
Nebivolol	406,2–151,0 (32/101)	406,2–123,1 (40/101)
Nicardipine	480,0–315,1 (25/88)	480,0–359,1 (27/88)
Nifedipine	329,0–284,1 (24/74)	329,0–268,0 (25/74)
Olmesartan	447,2–429,1 (14/74)	447,2–207,0 (26/74)
Perindopril	369,2–98,1 (33/74)	369,2–295,1 (19/74)
Perindoprilat	341,2–295,2 (18/67)	341,2–98,1 (32/67)
Propranolol	260,1–127,0 (47/69)	260,1–153,0 (36/69)
Prasugrel	374,0–149,0 (34/73)	374,0–206,0 (18/73)
Quinapril	437,8–319,7 (21/89)	437,8–347,9 (18/89)
Quinaprilat	411,2–117,1 (38/75)	411,2–91,1 (59/75)
Ramipril	417,3–117,1 (41/77)	417,3–343,1 (21/77)
Sotalol	273,1–255,0 (13/56)	273,1–133,1 (29/56)
Telmisartan	515,3–276,1 (48/139)	515,3–497,2 (34/139)
Triamterene	254,1–104,1 (40/107)	254,1–237,0 (28/107)
Ticagrelor	523,1–293,0 (31/112)	523,1–495,1 (20/112)
Valsartan	436,2–207,1 (29/60)	436,2–190,0 (39/60)
Verapamil	455,3–165,1 (29/99)	455,3–303,2 (27/99)

2.4. Instrumentation

All samples were analyzed with an Ultimate 3000 UHPLC coupled to a triple quadrupole TSQ Quantiva, both Thermo Fisher (Waltham, MA). The method was validated with the following settings: Samples (10 µL) were injected onto a Waters acquity UPLC BEH C18 (2.1 ∗ 150 mm, 1.7 µm particle size) analytical column (column temperature 50 °C), Thermo Scientific (Milford, MA). Water with 10 mM ammonium formate and 0.1% formic acid (eluent A) and methanol with 0.1% formic acid (eluent B) was used as eluents. With eluents profile: 0–0.5 min isocratic 5% B, 0.5–12 min linear gradient from 5 to 95% B, 12–13 min isocratic gradient 95% B, 13–13.1 min linear gradient from 95 to 5%, and 13.1–15 min isocratic gradient 5% B, used flow rate was 0.3 ml/min. Compounds were analyzed by selected reaction monitoring (SRM) (see Table 1 for mass transitions). Analytes were analyzed in positive

mode (3500 V) or in negative mode (3200 V). Ion transfer tube temperature was 325 °C and vaporize temperature was 300 °C.

2.5. Analytical validation

Validation for the lower limit of quantification (LLOQ) and linearity was performed in accordance with European Medicines Agency (EMA) guidelines [15]. The LLOQ was determined for the individual compounds by analyzing standards made in blank calf plasma in 5-fold in a range from 0.001 μg/L to 20 μg/L (0.005 μg/L, 0.01 μg/L, 0.05 μg/L, 0.1 μg/L, 0.5 μg/L, 1 μg/L, 5 μg/L, 10 μg/L and 20 μg/L) and analyzed for 3 days. For the compounds with less sensitivity an additional calibration curve was made in a range from 1 μg/L to 2000 μg/L (10 μg/L 20 μg/L 50 μg/L 100 μg/L 500 μg/L 1000 μg/L and 2000 μg/L) and analyzed in 5-fold for 3 days. After validation of LLOQ and linearity, a LLOQ standard and 4 levels from low to high within the linearity of each compound was made. Due to potential compound instability [16], these standard mixtures were made in MeOH, stored at −20 °C and spiked to blank calf plasma before sample preparation. These standard mixtures were used for the validation of stability, matrix effect and for routine analysis. For the detection of matrix effects, the recovery of the analyte through the analytical method was used, as described by Hewavitharana et al. [17]. Whereby, the concentration of analyte recovered ((Sample + spike) − Sample) was divided by the analyte added known concentration ∗ 100. In total, 20 different samples were spiked with level 4 standard and the concentration of the spiked and unspiked plasma samples were calculated with matrix matched standards. Autosampler stability was tested by reanalyzing three high level calibration standards after 12 h and 24 h. For room temperature stability, three high level standards were stored for 3 h and 20 h at room temperature and analyzed.

Statistical analyses of within-run coefficient of variation (CV), between-run CV and total CV were performed for LLOQ for each compound, by using one-way analysis of variance (ANOVA).

2.6. Clinical validation

The assay was clinically tested using 91 samples drawn from patients with apparent therapy difficult-to-treat hypertension, including a total of 37 different prescribed medications. 16 of these compounds were prescribed to five or more patients. Adherence to blood pressure lowering medication was expressed as the concentration ratio (CR), as described by Hugen et al. [18], defined as the ratio of the detected concentration by LC-MS/MS and the literature-based population trough concentration (PTC). Whereby a CR ≥ 1, means a concentration found by LC-MS/MS was higher compared by the PTC, contrary, a CR < 1 means a concentration found by LC-MSMS was lower compared the PTC. For concentrations below the LLOQ with a signal to noise ratio (s/n) of at least 10, the corresponding CR was used instead of a negative report. For patients adherence classification (Table 2) different CR-cut-off for the prescribed medication in a range from 0.1 to 1 values (with an interval of 0.1) were used.

3. Results and discussion

3.1. Method development

For sample clean-up, sample preparations such as protein precipitation by acetonitrile, TCA and use of solid phase extraction, were tested (data not shown). Due to the large number of compounds with a wide range of physiochemical properties, selection of a sample preparation optimal for all compounds was challenging. Preliminary data showed that protein precipitation by acetonitrile did not improve sensitivity for most of the compounds regards to TCA. However, protein precipitation with use of TCA was more efficient (plasma:TCA ratio is 1:0,2) compared to ACN (1:2) and/or MeOH (1:3). TCA proved to be a fast and effective method for protein precipitation as described by Polson et al. [19], and was therefore used: 43 of the antihypertensive agents were detected at levels ≤ 1 μg/L. To increase sensitivity, an optimum was found by using a highly concentrated TCA solution of 25% preventing further sample dilution.

3.2. LLOQ and linearity

For all compounds, the LLOQ was validated for accuracy and precision by the coefficient of variation (CV) for within run accuracy, between run accuracy and total CV with a maximal acceptable error of 20% (Table 3). A full assay validation according to EMA guidelines would also require a low, medium and high quality control however, the assay was not feasible for pk studies and TDM purposes because the linear dynamic range (Table 3, linear range) various to their therapeutic ranges (Table 3, Cmin–Cmax). Linearity (r^2) for all compounds was ≥0.995, except for acetylsalicylic acid (r^2 0.991). For the detection of non-adherence an LLOQ of each compound lower than the PTC was predefined, this was succeeded for 50 of the 52 (96%) compounds, in which the LLOQ was at least equal to or lower than the theoretical PTC and for 65% of the compounds the LLOQ was > 10 times lower than the PTC. For nebivolol and lercandipine the assay lacked sensitivity based on the theoretical PTC, however, for lercandipine (total prescribed n = 6) and nebivolol (total prescribed n = 3) the concentrations were found above the LLOQ. The theoretical PTC for fosinopril and prasugrel were, to the best of our knowledge, not reported in literature. For enalapril, fosinopril, losartan, perindopril and quinapril, the (active) metabolite was included in the assay, since the active metabolites have a substantially lower LLOQ compared to the PTC and/or longer half-life.

3.3. Stability

Short term stability testing at room temperature (3 h) and freeze/thaw stability testing showed that captopril, hydralazine, nifedipine, methyldopa and prasugrel where unstable. All of these compounds have a short half-life. Long term stability testing at room temperature (20 h), showed further degradation of these compounds. After 20 h also acetylsalicylic acid, amlodipine, barnidipine, lercanidipine, nicardipine and telmisartan started to degrade > 20% (Table 3). In total 10 of the 52 compounds started to degrade > 20% after 20 h. Fosinoprilat showed an increase in recovery, this was related to a broad peak shape of

Table 2
Adherence classification for patients.

Patient classification	Description
Fully non-adherent	0% match prescribed, all prescribed medication was below the defined CR ratio
Partially adherent	1–80% match prescribed, 1–80% of the prescribed medication was below the CR ratio
Adherent	80%–100% match prescribed, all of the prescribed medication was above the defined CR ratio

Table 3
Overview of the validation results for 52 compounds.

	Cmin[a]–Cmax (µg/L)	Half-life $T_{0,5}$ (h) [51]	Linear range[b] (µg/L)	LLOQ for within run		Freeze/thaw	Room temperature stability		Autosampler stability		Matrix effect
				CV, %/between run CV, %/overall CV, %	Overall bias, %	Stability Avg, %/CV, %	After 3 h Avg, %/CV, %	After 20 h Avg, %/CV, %	After 12 h Avg, %/CV, %	After 24 h Avg, %/CV, %	Avg, %/CV, %
Acebutolol	200–2000 [11]	3–4	0.05–100	7/5/8	9.5	105/1	100/4	98/3	97/8	90/2	91/7
Acetylsalicylic acid	10–400	3–4	10–500	4/17/17	0.2	113/12	92/8	44/7	94/3	94/24	142/5
Aliskiren	5–500 [24]	34–41	0.1–100	10/13/17	1.7	100/3	101/2	96/2	105/8	97/1	104/4
Amlodipine	15–30 [25]	6–12	0.5–1000	17/0/17	10.5	91/2	90/19	56/62	102/8	92/4	105/42
Atenolol	100–1000 [11]	6–9	0.05–100	8/0/8	3.3	96/1	102/6	105/3	92/6	89/1	99/4
Barnidipine	0.1–5 [26]	20	0.1–2000	16/0/16	−6.7	108/3	88/2	57/2	112/5	101/1	111/11
Bisoprolol	10–100 [11]	10–12	0.5–500	5/3/6	−11.0	98/2	101/4	105/1	89/7	76/0	89/2
Bumetanide	1–200 [27]	0.6–2.5	0.5–1000	14/4/15	5.3	97/4	97/2	92/1	107/13	101/5	133/7
Canrenone	100–500 [12]	10–35	20–1000	8/6/10	2.4	118/4	105/7	85/4	89/10	67/3	106/8
Captopril	50–1000 [12]	2	1–2000	8/0/8	1.4	60/6	4/40	2/64	116/14	69/6	30/30
Carvedilol	50–500 [11]	6–10	0.5–2000	8/11/13	−6.7	106/3	97/4	84/2	104/5	96/6	102/11
Chlortalidone	10–1000 [28]	4–8	1–2000	12/0/12	2.9	87/4	101/5	91/1	103/10	103/6	124/4
Clopidogrel	1–15 [29]	6	0.1–500	11/0/11	−6.8	100/8	98/5	90/4	100/1	104/15	111/4
Diltiazem	50–400 [12]	4–8	0.005–500	12/3/13	−4.5	96/3	98/5	94/3	96/7	78/1	101/5
Doxazosin	10–50 [30]	16–30	0.5–2000	8/10/12	4.1	100/3	98/3	82/2	112/6	101/2	100/7
Enalapril	1–250 [31]	–	0.05–500	16/3/17	6.4	99/1	99/3	96/1	106/9	99/3	99/3
Enalaprilat	1–50 [31]	11	1–500	6/9/11	5.3	97/1	102/4	99/1	102/1	118/13	108/2
Eplerenone	20–2000 [32]	3–6	0.1–2000	10/0/10	10.2	99/1	100/2	95/0	108/3	103/2	96/4
Felodipine	1–10 [33]	25	1–1000	7/7/10	1.5	97/6	92/17	102/7	89/8	83/7	136/52
Fosinopril	–	11.5–14	7.5–500	9/7/11	1.7	90/7	98/3	73/6	52/22	16/7	112/18
Fosinoprilat	10–6000 [34]	–	5–1000	10/6/11	1.5	152/10	216/41	175/28	102/7	104/8	85/61
Furosemide	50–500 [35]	0.5–1	5–2000	9/5/10	6.5	99/4	97/4	91/7	99/4	82/6	124/8
Hydralazine	100–500 [12]	2–4	0.5–2000	6/10/12	4.9	66/18	64/13	67/9	103/6	94/7	85/14
Hydrochlorothiazide	10–250 [25]	9.5–13	5–1000	13/10/17	−6.9	93/9	97/14	90/10	112/10	95/7	116/10
Indapamide	30–100 [36]	14–24	0.5–500	3/5/6	−14.9	104/14	96/9	101/3	108/4	104/1	96/13
Irbesartan	20–5000 [37]	11–15	0.1–2000	10/12/16	7.0	106/3	100/5	86/3	104/6	98/3	109/6
Labetalol	80–650 [11]	4	0.5–2000	5/10/12	−0.5	95/4	98/3	91/2	114/6	115/6	104/3
Lercanidipine	0.1–2 [38]	8–10	0.5–1000	11/15/19	0.0	120/6	92/7	57/7	94/4	95/8	109/9
Lisinopril	1–100 [12]	12.6	0.5–2000	7/14/15	6.3	92/3	101/4	98/1	102/4	96/4	96/6
Losartan	1–500 [39]	2	0.05–100	13/1/13	4.9	104/2	100/4	93/2	114/7	106/2	105/5
Losartan COOH	1–1000 [39]	6–9	1–1000	14/0/14	−5.1	102/1	102/4	88/5	84/7	74/3	126/7
Methyldopa	100–5000 [12]	2	100–2000	5/10/11	0.3	92/9	79/10	38/23	99/6	75/14	79/33
Metoprolol	35–500 [11]	3.5	0.5–100	3/6/6	−0.7	101/2	101/3	100/1	108/9	102/3	96/2
Minoxidil	1–10 [40]	1	0.05–100	5/15/16	−0.8	91/2	97/3	104/2	100/7	89/2	97/6
Moxonidine	1–10 [41]	1	0.05–100	3/4/5	−6.5	96/1	99/2	99/0	93/7	83/1	95/4
Nebivolol	0.05–0.1 [42]	8–27	0.1–2000	16/6/17	−4.9	128/8	101/5	89/2	112/8	103/4	105/8
Nifedipine	5–150 [43]	2–3	5–150	14/37/40	22.7	41/22	26/126	-/-	68/8	64/9	19/72
Nicardipine	10–100 [44]	7–9	0.05–2000	13/5/14	−10.9	110/3	95/4	74/3	105/4	94/3	109/10
Olmesartan	20–500 [45]	10–15	0.5–2000	7/6/9	−6.0	105/3	102/4	97/3	98/5	93/1	119/5
Perindopril	4–14 [46]	1	0.1–2000	11/0/11	−2.2	97/0	100/3	97/0	112/7	103/2	98/2
Perindoprilat	3–20 [46]	10–12	0.5–2000	11/15/18	3.7	99/1	102/4	100/1	103/29	96/15	103/2
Prasugrel	- - -	2–15	0.1–100	11/15/19	−7.2	25/75	118/122	NF	66/0	4/0	7/44
Propranolol	20–300 [11]	3–6	0.1–100	15/10/18	5.8	103/3	99/4	92/0	93/10	77/5	92/5
Quinapril	10–1500 [47]	1	10–2000	11/3/11	−7.5	95/8	101/6	92/7	114/12	100/19	132/5
Quinaprilat	30–1500 [47]	3	0.5–1000	9/18/20	1.8	102/1	101/4	92/3	108/2	106/4	111/4
Ramipril	1–50 [48]	13–17	0.1–500	10/13/17	3.6	104/1	100/4	95/1	114/8	103/1	112/5
Sotalol	500–3000 [11]	10–20	0.05–50	6/6/8	−4.3	100/0	102/5	101/1	106/7	104/3	90/10
Telmisartan	30–1200 [49]	24	0.5–1000	6/0/6	−2.2	116/4	99/5	79/3	102/4	93/1	98/7
Ticagrelor	25–800 [50]	7	1–500	12/5/13	2.3	100/2	106/7	93/7	103/4	80/8	126/9
Triamterene	16–45 [12]	2–4	0.05–100	5/4/6	−4.6	102/1	98/3	92/2	102/6	98/2	94/3
Valsartan	200–6000 [21]	6	0.5–1000	11/0/11	15.8	100/3	104/7	90/7	109/9	111/3	124/7
Verapamil	30–500 [43]	3–7	0.05–2000	10/13/17	−3.5	102/3	101/4	90/2	111/9	99/3	90/31

Abbreviations: avg = average, CV = coefficient of variation.
[a] C_{min} is equal to PTC.
[b] All values r^2 were above 0.995 except for acetylsalicylic acid (r^2 0.991).

fosinoprilat. Autosampler stability tests (12 h and 24 h), showed instability for fosinopril, nifedipine and prasugrel. The instability for nifedipine was related to light sensitivity as described by Jakobsen et al. [16]. For captopril and the active metabolite of prasugrel (R-138727), derivatization was needed in able to analyze the stable molecule, as described by Vancea et al. [20] and Kakarla et al. [21] respectively. However, this was not performed due to unpractical and infeasible for this multi-component method. For the determination of LLOQ and linearity of canrenone, a second metabolite or possible spironolactone itself was noticed during analysis [22]. This was reduced by spiking the blanc calf's plasma before sample preparation.

3.4. Matrix effects

Matrix effects were monitored by spiking 20 patient samples. With use of matrix matched standards, the matrix effects for most of the compounds were within an acceptable range. 69% of the compounds showed a minimal matrix effect (recovery between 85% and 115%),

Table 4
Results of long term stability. n = total number of prescribed medications, values expressed as CR. S = sample number. Positive gives the percentage of positive samples according to qualitative analysis.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	Total prescribed, n	Positive
Acetylsalicylic acid	2,6	neg		13	6,7	0,4	0,4	2,5	neg	neg	neg	8,9	4,8	70	21		neg				15	67%
Amlodipine	0,7	40	0,2	0,7	0,2	neg	0,7	0,3	0,5		0,2	0,5	0,4	0,6	0,4		neg		neg		16	81%
Atenolol						neg			0,4					0,9		3,3					4	75%
Bisoprolol																		0,9			1	100%
Clopidogrel										2,3				7,1							2	100%
Enalapril							15														1	100%
Enalaprilat							60														1	100%
Hydrochlorothiazide	4,8	71	0,8	12	neg		5,1	5,5	7,1	2,1				1,1		18	11	12	11	2,2	15	93%
Lercanidipine										3,5											1	100%
Lisinopril	72	533	38	69	neg	8,4			13	77				11		14	75	24	75	20	14	93%
Losartan											12										1	100%
Losartan COOH											119										1	100%
Metoprolol		35			3,7		0,9					0,6	2,8							1,2	6	100%
Perindopril													1,1								1	100%
Perindoprilat													2,1								1	100%
Sotalol										0,9											1	100%
Telmisartan															0,9						1	100%
Triamterene	2,9								10												2	100%
Ticagrelor	0,01																				1	100%

21% had a recovery between 70% and 130%. For prasugrel, nifedipine and captopril the recovery was < 50%, which can be explained by compound instability (see Section 3.3).

To control matrix effects it is recommended to use stable isotope labelled standards in the bioanalysis of drugs. However, the addition of 52 stable isotope labelled standards per sample run is very costly and, above all, not commercial available for all compounds. An alternative method to control matrix effects is standard addition. Unfortunately, the concentrations of the compounds in our samples were unknown and could be anywhere within the therapeutic range or in case of non-compliance far below the PTC. Therefore, standard addition for the correction of matrix effects with an optimum spike-analyte concentration [23] was also considered unfeasible.

3.5. Long term stability

Long term stability was tested by analyzing blood samples from 20 different patients from the TEMPUS study. Patients in this study took their medication under controlled conditions (as described in Section 2.6) using Medication Event Monitoring System(s) for monitoring and controlling patients administration adherence. In total, 68% of the compounds had a recovery of 100% for long term stability (Table 4). Acetylsalicylic acid (n = 15) had a low recovery, which was related to instability as shown by the validation results. The degradation of amlodipine (positive found 81%) for 20 h at room temperature could be an indication of long term instability. Atenolol and clopidogrel did not show instability during validation. For these compounds the low recovery could be related to non-adherence or long term instability. Hydrochlorothiazide and lisinopril both had a recovery of 93% and where found negative in the same patient.

3.6. Adherence per cardiovascular agent

After bioanalytical validation, measurements of prescribed cardiovascular medications were performed in samples obtained from patients with apparent therapeutic hypertension for routine diagnostics. As shown in Table 5, a total of 37 different compounds were prescribed to 91 individual patients. A proportion of 25%–99% undetected medication was found for 7 compounds and for 10 compounds the undetected proportion was 1–24%. In total, 20 compounds were detected in all patients (0% undetected). Nifedipine, a compound which critically suffered from instability issues like freeze taw, room temperature and matrix effects, was still good detectable in these patients. For compounds suffering from 20 h room temperature instability, only amlodipine (n = 35) was in patients undetected (n = 6). When comparing our data with the PTC, for five compounds the minimum values were > 10 times lower than their PTC's and for four of these compounds the median was under the PTC. In contrast, for 11 compounds no concentrations below their PTC were found.

The prevalence of adherence differed per agents and per CR cut-off value (Table 6). While interpreting the results the following should be taken into account: 1) the difference in between-drug pharmacokinetics including the within- and between-population variation, 2) the fact that samples were drawn randomly over the day, and 3) the fact that the assay was not fully validated according to FDA/EMA guidelines. For example, the median concentration found for perindopril, a compound with a very short half-life, was lower than the PTC, while perindoprilat, its active metabolite with a half-life around 10–12 h, showed a median within the population range. Further investigation and refinement of PTC values by means of pharmacokinetic studies should be performed, since both quantity and quality of available pharmacokinetic data for some agents were limited.

3.7. Patient adherence for different cut-off values

Patients were divided into three groups: adherent (81%–100% match prescribed), partially adherent (1–80% match prescribed) and completely non-adherent (0% match prescribed), according to different CR cut-off values (0.1 to 1). An increase in non-adherence (2.2%) was noticed between qualitative results and at CR 0.1. The proportion of patients classified as completely non-adherent increased from 8.7% for CR 0.1 to 19.7% for CR 0.5 and 27% for a CR cut-off value of 1. The proportion of partially adherent patient increased from 15.4% for qualitative analysis, to 18.6% at CR 0.1, 27.4% for CR 0.5 and 38.4% for CR 1 cut-off value. As a result, qualitative analysis would classify 78% of the patients as fully adherent, while using CR cut-off of 0.1, 0.5 or 1 would classify 72.5%, 52.7% and 31.9% respectively as adherent (Fig. 1). Concerning the use of CR instead of qualitative analysis, the literature based PTC is a population reference and was used to determine if the concentration of a compound is within the effective therapeutic range. Nevertheless, due to the individual variation and as mentioned in Section 3.6, using PTC to discriminate adherence from nonadherence has his limitations. However, the alternative is classification of adherence only based on interpretation on positive/negative. In this case bioanalytical differences in compound sensitivity can lead to misclassification depending to LOD in relation to PTC. Qualitative

Table 5
Cardiovascular medications prescribed and analyzed in 91 individual patients.

	Total		Concentration range			PTC
	Prescribed	Undetected	Minimum, µg/L	Maximum, µg/L	Median, µg/L	
Aliskiren	4	1	9,5	100	34	5
Amlodipine	35	6	0,9	62	9	15
Atenolol	3	2	40	40	–	100
Barnidipine	12	0	0,1	1	0	0,1
Bisoprolol	1	0	< 0,5	0	0	10
Bumetanide	2	0	0,5	32	16	1
Canrenone	12	4	1	1100	30	100
Carvedilol	1	0	22	22	–	50
Chlortalidone	6	1	39	710	160	10
Doxazosin	11	3	< 0,5	60	18	10
Enalapril	2	0	17	208	113	1
Eplerenone	8	2	0,4	56	4	1
Fosinopril	1	1	–	–	–	–
Fosinoprilat	1	0	290	290	–	10
Furosemide	8	4	15,0	900	129	50
Hydrochlorothiazide	28	5	< 5	190	47	10
Indapamide	1	0	19	19	–	30
Irbesartan	3	0	78	2300	82	20
Labetalol	3	1	15	29	22	80
Lercanidipine	6	0	< 0,5	7	2	0,1
Lisinopril	10	0	14	183	62	1
Losartan	7	0	0,2	190	5	1
Methyldopa	1	0	> 2000	> 2000	> 2000	100
Metoprolol	32	3	0,7	420	15	35
Nebivolol	3	0	0,1	1	0	0
Nifedipine	9	1	–	–	–	5
Olmesartan	10	5	17,6	640	26	20
Perindopril	12	1	0,1	56	0,2	4
Perindoprilat	12	1	0,9	21	6	3
Propranolol	1	0	44	44	–	20
Quinapril	1	0	34	34	–	10
Quinaprilat	1	0	1020	1020	–	30
Ramipril	1	0	0,3	0,3	–	1
Sotalol	1	0	520	520	–	500
Telmisartan	3	0	8,7	234	156	30
Triamterene	3	0	0,6	7	1	16
Valsartan	13	1	2,0	7200	440	200

screening does not take therapeutic exposure in account in contrast to quantitative measurement. Quantitation enables a more detailed information of drug adherence in individual patients by a combination of the measured drug level, the PTC, half-life, and time between last drug intake and sampling. Furthermore, the assay was designed for alleged therapy-resistant patients, who were treated for a long time without/limited effect and were prescribed > 1 drug. The latter facilitates an integrated pharmacological advice using the PTC's of multiple agents from one analytical run.

4. Conclusions

Screening and semi-quantification of 52 cardiovascular medications and their metabolites in plasma assay using LC-MS/MS was successfully developed fulfilling predetermined qualification and quantification validation requirements for LLOQ, linearity, stability and matrix effects. Instability of some compounds necessitates a high through put under controlled cool temperature condition with a fast sample preparation and using amber glass to protect against UV. A sensitive method was

Table 6
Percentage adherence per cardiovascular agent, only those compounds prescribed at least 5 times are presented.

	CR[a] 0,1	CR[a] 0,2	CR[a] 0,3	CR[a] 0,4	CR[a] 0,5	CR[a] 0,6	CR[a] 0,7	CR[a] 0,8	CR[a] 0,9	CR[a] 1
Lercanidipine	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Lisinopril	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Barnidipine	100%	100%	100%	100%	100%	100%	92%	92%	83%	83%
Chloortalidon	83%	83%	83%	83%	83%	83%	83%	83%	83%	83%
Hydrochloorthiazide	82%	79%	79%	79%	75%	75%	75%	71%	71%	68%
Losartan	100%	100%	86%	71%	71%	71%	71%	71%	71%	57%
Eplerenone	75%	75%	75%	75%	63%	63%	63%	63%	63%	63%
Perindoprilaat	92%	92%	83%	75%	67%	67%	67%	67%	67%	58%
Valsartan	85%	85%	85%	69%	69%	69%	62%	54%	54%	54%
Doxazosine	64%	64%	64%	64%	64%	64%	55%	55%	45%	45%
Furosemide	50%	50%	38%	38%	38%	38%	25%	25%	25%	25%
Amlodipine	77%	74%	69%	60%	57%	43%	37%	34%	31%	23%
Metoprolol	81%	66%	56%	47%	41%	38%	34%	28%	28%	19%

[a] CR = analyzed with LCMSMS / Population though concentration.

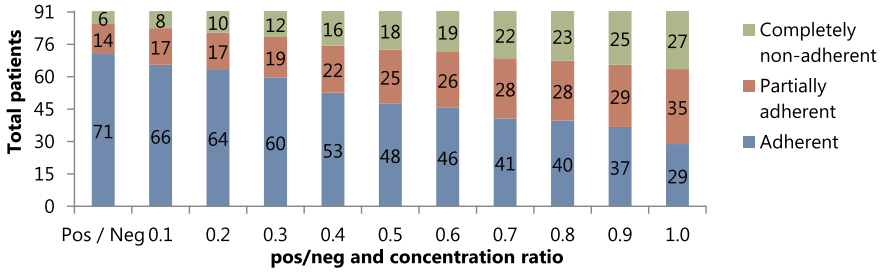

Fig. 1. Medication adherence according to qualitative (pos/neg) and semi-quantitative analysis (different cut-off values of the concentration ratio (CR)) in 91 patients with apparent difficult-to-treat hypertension.

required to detect compounds with LLOQ values below their PTC's. Furthermore, the introduction of CR cut offs had a significant and relevant effect on patient adherence identification and classification. We conclude, that plasma screening and subsequent quantification of cardiovascular agents with LC-MS/MS is a valuable tool for assessment of medication adherence in patients with apparent difficult-to-treat hypertension.

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