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Determination of doxazosin and verapamil in human serum by fast LC–MS/MS: Application to document non-compliance of patients

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ABSTRACT

A rapid and sensitive method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for simultaneous determination of doxazosin and verapamil in human serum has been developed. Trimipramine-d_3 as an isotopic labelled internal standard was used for quantification. Serum samples were prepared by simple liquid–liquid extraction with mixture of *tert* butyl methyl ether and ethyl acetate (1:1, v:v). The analytes and internal standard were separated on C18 column using an isocratic elution with 5 mM ammonium formate with 0.02% formic acid and 0.02% formic acid in acetonitrile (55:45, v:v) at a flow rate of 1.1 mL/min. Positive TurboIonSpray mass spectrometry was used with multiple reaction monitoring of the transitions at: m/z 455.3 → 165.2 and 150.2 for verapamil, m/z 452.2 → 344.4 and 247.4 for doxazosin, m/z 298.2 → 103.1 for trimipramine-d_3. Linearity was achieved between 1 and 500 ng/mL ($R^2 \geq 0.997$) for both analytes. An extensive pre-study method validation was carried out in accordance with FDA guidelines. This assay was successfully applied to determine the serum concentrations of doxazosin and verapamil in suspect non-compliance patients.

1. Introduction

Essential or primary hypertension refers to high blood pressure with no identifiable cause and remains a major modifiable risk factor for cardiovascular disease. Essential hypertension is nowadays one of the most common disorders of western civilisation. The estimates predict further increase in number of adults suffering from hypertension by about 60% till 2025, when 1.56 billion (1.54–1.58 billion) of people are predicted to have hypertension [1]. If lifestyle and diet modifications are not satisfactory, medical treatment with antihypertensive drugs is necessary. Resistant hypertension is defined as a state, when the treatment with at least 3 antihypertensive drugs (including a thiazide diuretic) is not able to normalize the blood pressure. This is a relatively frequent, but often omitted problem. Exclusion of secondary hypertension, including endocrine hypertension (mainly primary aldosteronism, but also pheochromocytoma and hypercortisolism) is recommended in these patients. Therefore the patients are switched to a drug combination of alpha 1 adrenoreceptor antagonists (A1AAs) [2,3] and/or calcium channel blockers (CCBs) [4–6], which does not affect plasma concentration of the endogenous hormones connected to secondary hypertension. Doxazosin and verapamil (Fig. 1) are used as the substitutive antihypertensive drug combination [7–10].

Doxazosin mesylate [(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(1,4-benzodioxan-2-yl-carbonyl) piperazine monomethansulphonate], a quinazoline derivate, is a long-acting postsynaptic A1AA [11] displaying similar efficacy to that of other A1AAs, beta adrenoreceptor antagonists, diuretics, CCB and angiotensin-converting enzyme inhibitors [12]. Like structurally similar prazosin, doxazosin exerts its antihypertensive effect by reducing total peripheral resistance [13]. Verapamil, (±)-(alpha)[3-[[2-(3,4-di-dimethoxyphenyl)ethyl] methylamino]propyl]-3,4-dimethoxy-(alpha)-(1-methylethyl) monohydrochloride, is a selective CCB effective in the treatment of hypertension, arrhythmia and angina pectoris [10,14,15].

Due to wide use of doxazosin and verapamil, several analytical methods for the determination of verapamil and doxazosin individually have been described in literature. Verapamil was preferably determined in human plasma or in other biological specimens by liquid chromatographic methods coupled with ultra-violet detector (HPLC-UV) [16–20], fluorimetric detection (HPLC-FLR) [21–25] and mass spectrometric detection (LC–MS) [26–28]. Methods

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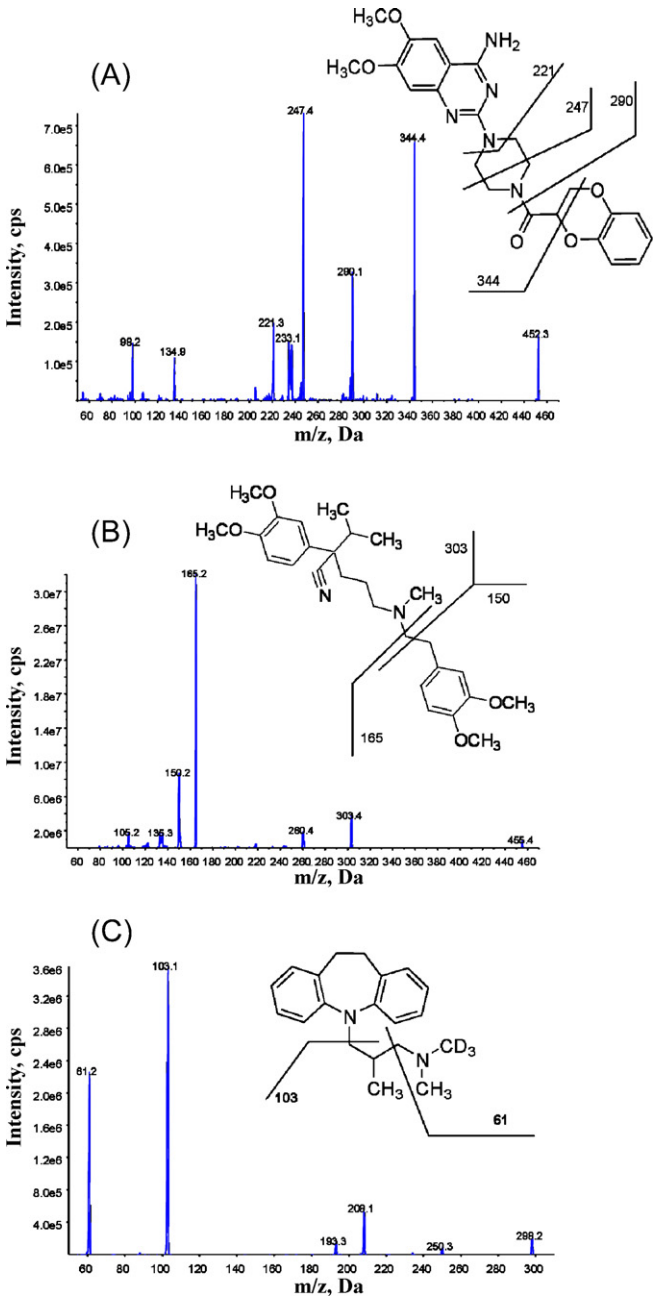

Fig. 1. Chemical structures and product ion spectra of $[M+H]^+$ of doxazosin (A), verapamil (B) and trimipramine-d_3 (C).

involving gas chromatography (GC) were also published [29–32]. Determination of doxazosin in pharmaceutical formulations and biological matrices was performed using HPLC-UV [33], HPLC-FLR [34–40] and LC–MS [41–43]. Doxazosin was also analysed using electrochemical techniques [44,45]. A method for simultaneous determination of both analytes was not published so far.

Determination of both doxazosin and verapamil in biological matrices is routinely used in pharmacokinetic studies. However, structurally related antihypertensive agents are used as an internal standard (IS), which prevents possible application of such methods into real clinical settings because there is a high risk of interferences by co-medications. Structures like tetrazosin [42], prazosin [34,35,37,39,41], cisapride [43], propranolol [24,36,38], metoprolol [26], dextrometorphan [20] or gallopamil [25] have been used as an IS in methods for applications in controlled pharmacokinetic studies.

We describe a fast, selective and accurate LC–MS/MS method for the simultaneous quantification of verapamil and doxazosin using trimipramine-d_3 as an IS in this paper. This method was specially developed to confirm suspect non-compliance of patients treated with verapamil and (or) doxazosin, since non-compliance has been previously reported as a prevalent cause of pseudo-resistant hypertension [46,47]. This method is easy to provide and is applicable for routine determination of both medicaments in clinical practice. The time non-consuming liquid–liquid extraction (LLE) was used for sample pre-treatment. Additionally, we used high resolution mass spectrometry for the interpretation of unknown fragment ion spectra of doxazosin.

2. Experimental

2.1. Chemicals and reagents

Standard of doxazosin mesylate (99.4%) was kindly supplied by Zentiva (Prague, Czech Republic). Verapamil hydrochloride (99.0%) was purchased from Sigma Aldrich (Steinheim, Germany) and trimipramine-d_3 (99.0%) was obtained from Alltech (Prague, Czech Republic). Formic acid (p.a.) and ammonium formate (p.a.) were purchased from Sigma Aldrich (Steinheim, Germany). Acetonitrile (HPLC grade) and extra pure solvents *tert*-butyl methylether (TBME) and ethyl acetate were obtained from Merck (Darmstadt, Germany). Deionised water was produced in-house using a Milli-Q-System from Millipore (Bedford, MA, USA).

2.2. Instrumentation

The chromatographic separation was performed on a 1200 RRLC (Agilent, Waldbronn, Germany), consisting of a degasser, binary pump, autosampler and thermostatted column compartment. The MS/MS analysis was performed using a 3200 Q-trap triple quadrupole/linear ion trap mass spectrometer with a TurboIonSpray source (MDS Sciex, Ontario, Canada). For data analysis was used Analyst software version 1.5.1.

2.3. LC–MS/MS

Chromatographic separation was achieved with an Agilent Zorbax Eclipse XBD-C18 column (1.8 μm, 50 × 4.6 mm I.D.), protected by a C18 security guard cartridge (4 × 2 mm I.D.). Isocratic elution occurred with (A) 5 mM ammonium formate with 0.02% formic acid and (B) 0.02% formic acid in acetonitrile (55:45, v:v) at a flow rate of 1.1 mL/min. The mobile phase was thermostatted at 40 ± 0.5 °C. The mass spectrometer operated in positive TurboIonSpray mode and selected reaction monitoring (SRM) was used for data acquisition of both analytes and IS. The following transitions were monitored: *m/z* 455.3 → 165.2 and 150.2 for verapamil, *m/z* 344.4 → 165.2 and 247.4 for doxazosin, *m/z* 298.2 → 103.1 for IS. The underlined transitions were used for quantification. The MS parameters for the analysis were as follows: ion source temperature 550 °C; ion-spray voltage 5000 V; nebulizer gas 45 psi; auxiliary gas 50 psi; curtain gas 10 psi and medium collision gas. Conditions of mass spectrometric detection were optimized by direct infusion of standard solutions into the MS. The final parameters settings are shown in Table 1.

2.4. High resolution mass spectrometry

High resolution exact mass MS/MS spectra for doxazosin were obtained with an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The spectrometer was operated in positive

Table 1
Parameters of MS detection for doxazosin, verapamil and trimipramine-d_3.

	Q1 mass (amu)	Q3 mass (amu)	Dwell time (ms)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision entrance potential (V)	Cell exit potential (V)	Ion ratio, SRM2/SRM1
Doxazosin	452.2	344.4	75	70	7	43	15	4	0.58
		247.4	75	70	7	57	15	4	
Verapamil	455.3	165.2	75	50	4.5	35	20	3	0.29
		150.2	75	50	4.5	63	20	3	
Trimipramine-d_3	298.2	103.1	75	30	4.5	25	15	3	–

ion mode with a mass resolving power of 60.000. The MS/MS experiments were performed in CID and HCD mode with normalized collision energies 30–40%. Nitrogen was used as sheath/auxiliary gas (15 a.u./5 a.u.) and helium served as the collision gas. The mobile phase was delivered using Rheos ALLEGRO UHPLC pump (Flux Instruments AG, Switzerland) and consisted of methanol/water (1:1), flow rate 50 μL/min. The sample was diluted with the mobile phase and injected using a 2 μL loop. Spray voltage, capillary voltage, tube lens voltage and capillary temperature were 4.3 kV, 40 V, 155 V and 275 °C, respectively. The mass spectra were internally calibrated using protonated caffeine (m/z 195.08765) to provide high-accuracy mass measurements within 2.0 ppm.

2.5. Standard working solutions, calibration samples and quality control samples

Standard solutions (200 μg/mL) of each substance were prepared in methanol. 20, 2 and 0.2 μg/mL two-component working solutions were prepared by diluting with mixture of methanol and water (50:50, v:v). The IS working solution was prepared with the same mixture of methanol and water at the final concentration of 2 μg/mL.

Working solutions were used for preparing the calibration samples by diluting them with pooled human serum creating final concentration from 1 to 500 ng/mL of both analysed compounds. Quality control samples (QC) used for stability assays at concentrations of 10, 80 and 300 ng/mL were prepared by spiking working solutions to pooled human serum.

2.6. Liquid–liquid extraction

All calibration standards, QC samples and patient samples were prepared by liquid–liquid extraction. A 10 μL aliquot of trimipramine-d_3 solution (2 μg/mL) was added to 250 μL of serum sample and briefly mixed. After pH adjustment with 0.5 mL of phosphate buffer (pH 6.0), the mixture was extracted with 1.5 mL of a mixture of TBME–ethyl acetate (1:1, v:v). After centrifugation (4000 *g* for 8 min), the organic phase was separated and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was dissolved in 500 μL of LC mobile phase and a 5 μL aliquot was injected into the chromatographic system.

2.7. Assay validation

The following parameters were evaluated for the validation of the LC–MS/MS method for the simultaneous determination of verapamil and doxazosin in human serum: selectivity, sensitivity (limits of detection (LOD) and quantification (LOQ)), linearity, precision, accuracy, extraction recovery, matrix effect, carryover effect and stability.

The method selectivity was assessed by analyzing six different lots of pooled blank human serum and by comparing them with spiked serum samples at concentration near LLOQ. Additionally, serum samples from patients receiving other medicaments commonly used for therapy of cardiovascular diseases (amlodipine, betaxolol, bisoprolol, metoprolol, hydrochlorothiazide, prazosin, losartan and telmisartan) were tested to exclude possible interferences.

LLOQ refers to the lowest concentration of each compounds in human serum, that can be analysed quantitatively by the LC–MS/MS method with precision less than or equal to 20% and accuracy within 80–120% ($n = 6$). LOD is the lowest concentration with a signal to noise ratio higher than 3:1. Both parameters were empirically evaluated for qualifier transition by analyzing samples with low concentrations of analytes.

Peak-area ratio of verapamil and doxazosin/IS was measured and plotted against the theoretical concentration of the spiked standards. Six-point calibration curves were constructed over the whole concentration range (1–500 ng/mL) with a weighting factor of $1/x$ and least-square linear regression analysis was performed to determine slopes, intercepts and correlation coefficients (R^2), required being ≥0.99. The calibration ranges were defined considering the normal therapeutic concentration ranges of doxazosin (10–150 ng/mL) and verapamil (10–400 ng/mL) [48]. To detect potentially overdosed patients were expected ranges extended.

Accuracy, intra- and inter-day precision for verapamil and doxazosin were evaluated according to the requirements of FDA guideline on bioanalytical method validation [49]. Intra-day variation was assessed by six replicate determinations of three concentrations (low, medium and high) over the tested range. Intra-day accuracies were expressed as the mean of the assays relative to the true value. The intra-day precision of the method was calculated as the relative standard deviation (RSD) of the assay made for intra-day accuracy. Inter-day variation was determined by analysing replicates of QC samples with the same concentrations on three days. Accuracies were calculated as the mean of the assays relative to the nominal value. The inter-day precision of this method was expresses as the RSD of the assays made for inter-day accuracy.

To calculate recoveries of analysed compounds were compared peak areas obtained from QC serum samples and those found by direct injection of mobile phase solutions at the same concentration levels. The matrix effect was calculated according to the method by Matuszewski et al. [50] as peak areas of the samples spiked after extraction procedure divided by the corresponding areas of the standard solutions dissolved in serum before extraction. Recovery and matrix effect experiments were performed for five different lots of human serum on three concentration levels (QC) of both analytes and on a middle concentration level of IS. Acceptable carryover was defined as no quantifiable SRM transition peaks in a blank serum sample containing IS immediately injected after a sample containing two times greater amount of analytes than upper LOQ.

Stability was evaluated with human serum fortified with both analytes at the three QC concentrations ($n = 6$). Short-term temperature stability was tested during the whole working day at room temperature, at 4 °C for 10 days and at −20 °C for 1 month. QC samples were kept at different storage conditions and calculated

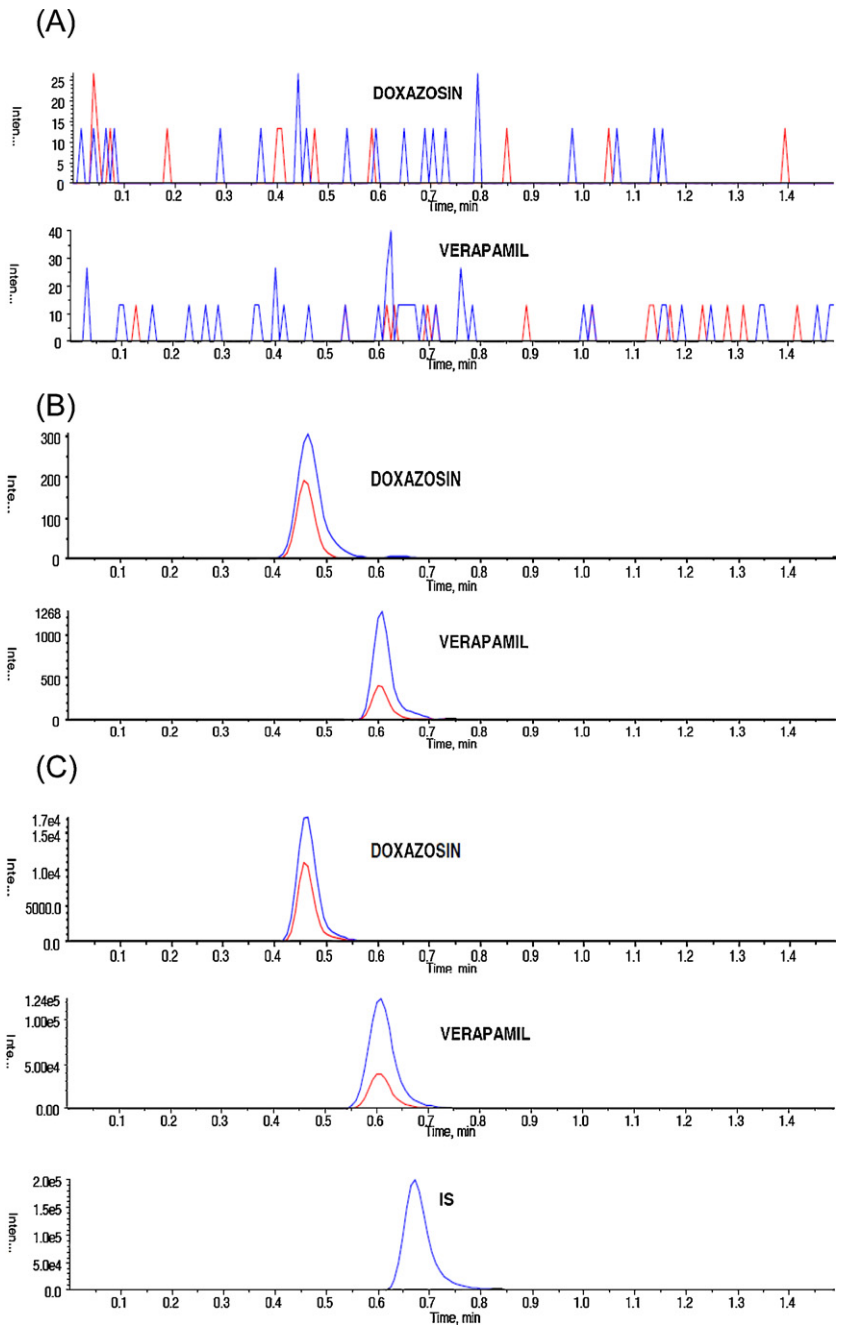

Fig. 2. SRM chromatograms obtained after analysis of blank human serum (A), serum spiked with 1 ng of doxazosin and verapamil (B) and serum sample containing 64.2 ng/mL of doxazosin and 95.2 ng/mL of verapamil (C).

concentrations of stability specimens were compared to QC samples prepared freshly on the day of analysis.

2.8. Application of the method

To apply the method a single blood sample was analysed from 93 different patients treated with one of the analysed drugs (40) or their combination (53). Blood samples were collected between 1 and 12 h after the oral intake of the prescribed drugs. Samples were transferred into venous blood collection tubes (4 mL, BD Vacutainer, Heidelberg, Germany) containing no additives or gels for serum separations. Samples were centrifuged at 4000 *g* for 5 min. The serum supernatant was carefully separated from blood cells and transferred to Eppendorf vials which were stored at −20 °C until analysis.

Samples with S/N ratio lower than 3 were evaluated as negative, while samples with concentrations below the lower limit of therapeutic window and higher than LLOQ were evaluated as samples out of therapeutic range. Negative samples were not included in the computations of descriptive statistics. Statistical evaluation of the obtained data was done using χ^2 test with significance level at $p < 0.05$.

3. Results and discussion

3.1. LC–MS/MS optimization

Separation of doxazosin, verapamil and IS was performed under acidic conditions. Chromatographic behaviour of both analytes and IS on a C18 reversed phase packing material was proper.

Table 2
Parameters of the calibration curves.

$n=6$	a Doxazosin	b	R^2	a Verapamil	b	R^2
Mean	0.178	0.0027	0.9985	0.499	0.0039	0.9979
SD	0.011	0.0015	0.0012	0.014	0.0010	0.0011
SE	0.005	0.0006		0.006	0.0004	
RSD (%)	6.46			2.76		

a: slope; b: intercept; R^2: correlation coefficient; SD: standard deviation; SE: standard error.

No extensive tailing was observed during the chromatographic method development. Different ratios of acetonitrile and formic acid, temperature and flow rate were tested for obtaining suitable chromatographic separation. Higher content of acetonitrile (≥80%) resulted in faster chromatographic run, but without satisfactory analytes separation and stability of retention times. Higher content of water (≥70%) gave rise to substantially longer chromatographic run with an insufficient response of analytical system. Finally, using mobile phase with 45% fraction of acetonitrile the best separation and good response of MS detector was obtained. The optimal flow rate of mobile phase was found to be 1.1 mL/min. Column temperature (20–50 °C) did not substantially affect the time of analysis and response of MS detector. Stability of the chromatographic method was evaluated by calculating retention time variability. RSD for retention times was lower than 0.19% for all analytes over 20 consecutive injections. Analytes were eluted within 0.9 min with a total chromatographic run time of 1.5 min (Fig. 2).

Conditions of MS detection were optimized for maximum fragment ion formation by direct infusion of single compound of interest. Fragment spectra of pseudomolecular ions $[M+H]^+$ of detected compounds are displayed in Fig. 1. For both analytes two SRM were set up, one for quantification and one as qualifier using one precursor ion and two fragment ions per compound as shown in Table 1. The ratios of SRM1 and SRM2 and retention time deviations were used for analytes identification in analysed unknown samples. The RSD of SRM ratios were lower than 5.56% for all analytes over the whole working range. The fragmentation of protonated doxazosin was studied using high-resolution mass spectrometry. The same procedure was previously used for elucidation of fragmentation pathways of verapamil [51]. The structures of trimipramine-d_3 fragments were derived from the fragmentation mechanism of trimipramine, which was also previously published [52,53].

3.2. Accurate mass measurement of the fragment ions of doxazosin

The protonated molecule of doxazosin provided several fragments identified by high resolution mass spectrometry as follows: The ion m/z 344 was found to be a fragment $C_{17}H_{22}N_5O_3^+$ (344.1714, −0.9 ppm) formed by elimination of $C_6H_4O_2$ from benzodioxane part of the molecule. Loss of ketene $C_9H_6O_3$ gave m/z 290 ($C_{14}H_{20}N_5O_2^+$; 290.1608, −1.1 ppm). The cleavage of piperazine cycle provided m/z 247, 233 and 221 identified as $C_{12}H_{15}N_4O_2^+$ (247.1186, −1.2 ppm), $C_{11}H_{13}N_4O_2^+$ (233.1030, −1.4 ppm), and $C_{10}H_{13}N_4O_2^+$ (221.1029, −1.6 ppm), respectively. The ions m/z 135 and 98 were cations $C_8H_7O_2^+$ (135.0438, −2.0 ppm) and $C_5H_8O_2N^+$ (98.0600, −0.8 ppm), respectively. The schematic fragmentation of doxazosin is shown in Fig. 1A.

3.3. Extraction method development

Different extraction techniques were tested during a method development. Consistent parameters of calibration curves, good repeatability and high recoveries were obtained using a simple protein precipitation with acetonitrile. The disadvantage of this route was an unacceptable extent of ion suppression for analytes (doxazosin > 33.6%, verapamil > 16.1%, IS 11.1%). The SPE cartridges (BondElut Certify, Varian), usually used in our laboratory [54,55], were also examined for extraction of analytes. Regrettably, the correlation coefficients of the linear regression were out of acceptable criterion (<0.99) and any modifications of SPE procedure did not improve them. Finally, the LLE procedure was investigated for optimization of serum sample clean up. Diverse buffer for pH (6.0–9.1) adjustment and different solvents were tested during optimization. The high extraction recovery, minimal matrix effect and suitable values of precision and accuracy data were obtained using phosphate buffer (pH 6.0) for pH adjustment and a mixture of TBME and ethyl acetate (1:1, v:v) as an extraction solvent.

3.4. Assay validation

The selectivity of the method was tested by comparing the chromatograms of six different lots of blank human serum. All blank serum samples were found to be free of interferences with respect to all SRM transitions. Additionally, no interfering peaks to verapamil and doxazosin were found with potentially interfering compounds.

The assay was found to be linear over the whole calibration span. The parameters of calibration curves equations are described in Table 2. Criteria for LLOQ were fulfilled by the lowest point of the calibration curve (1 ng/mL for both substances). Chromatograms of spiked serum sample containing 1 ng/mL of doxazosin and verapamil and chromatograms of blank serum samples are presented on Fig. 2.

Intra-day accuracies ranged between 94.7% and 107.3% throughout the three concentrations of both analytes. Inter-day accuracies

Table 3
Inter- and intra-day variation data from the determination of doxazosin and verapamil in human serum.

Nominal concentration (ng/mL)	LOQ		10		80		300	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Intra-day variation								
Doxazosin	92.7	7.5	104.8	7.8	107.3	4.3	100.4	3.6
Verapamil	93.1	10.6	94.7	2.5	105.1	5.1	98.0	7.8
Inter-day variation								
Doxazosin	103.5	9.1	101.3	6.7	94.0	5.2	102.8	6.1
Verapamil	94.1	10.0	97.4	4.2	101.3	2.9	105.2	5.2

Table 4
Recovery and matrix effect for doxazosin, verapamil and trimipramine-d_3 extracted from human serum.

Analyte	Recovery (%, $n=5$)			Matrix effect (%, $n=5$, RSD)		
	10*	80*	300*	10*	80*	300*
Doxazosin	82.1	75.0	71.3	94.3 (7%)	96.6 (6%)	92.6 (8%)
Verapamil	85.3	91.4	81.2	93.1 (5%)	102.2 (5%)	92.7 (6%)
Trimipramine-d_3		90.0			100.5 (9%)	

* Nominal concentration (ng/mL).

Table 5
Results obtained after analysis of serum samples obtained from patients under cardiovascular treatment.

	Negative samples (%)	Below therapeutic range (%)	Mean ± SD
Group 1 ($n=6$)	1 (16.7)	1 (16.7)	148.5 ± 126.06
Group 2 ($n=34$)	16 (47.1)	1 (2.9)	27.7 ± 16.2
Group 3 ($n=53$)	DOX: 4 (7.5)	DOX: 2 (3.8)	DOX: 46.2 ± 38.9
	VER: 3 (5.6)	VER: 4 (7.5)	VER: 139.5 ± 111.6
	VER + DOX: 6 (11.3)		
Total ($n=93$)	30 (32.3)	8 (8.6)	

DOX: Doxazosin; VER: Verapamil.

ranged between 94.0% and 105.2% throughout the three concentrations of both analytes. Precision was 7.8% or less in both analyses. The results are listed in Table 3.

Mean extraction recoveries of doxazosin and verapamil at three QC concentrations were higher than 71.3 and 81.2, respectively. The mean extraction recovery of IS at a middle concentration level was 90.0%. The average matrix effect values for both analytes at three concentration levels ranged between 92.6% and 102.2%. Matrix effect for the IS at middle concentration was 100.5%. These results (Table 4.) indicate that no co-eluting substance significantly influenced the ionization of analysed compounds and IS. Blank serum samples injected after the sample containing two times the upper LOQ did not show carryover.

The stability experiments were conducted to simulate worst case scenario of sample shipping or handling in clinical praxis. Both substances were stable for 24 h at room temperature in the short-term stability testing and long-term stability for 1 month has been shown at −20 °C. The calculated deviations during short-term stability were less than −7.2% for doxazosin and −3.1% for verapamil. The calculated deviations were better than −3.1% for doxazosin and −2.6% for verapamil in long-term stability testing. The stability of analytes in serum at 4 °C was investigated for 10 day because of largely variable time of handling and shipping in naturalistic conditions of clinical praxis. Both analytes were stable under these conditions with deviations less than 7.7%. Thus, storage of serum samples under above mentioned laboratory conditions was not critical for this method.

3.5. Application of the method

Six patients were treated only with verapamil (group 1), 34 patients only with doxazosin (group 2) and 53 of them with a combination of both drugs (group 3) (Table 5). The worst compliance was observed in patients treated by doxazosin alone (47.1%), while the best adherence to therapy was noted in patients under combined medication (5.6–11.3%). Total ratio of non-compliant patients was higher than 32%. Forty-one serum samples from women were analysed with 21 (51.2%) of them classified as negative, whilst there were significantly less frequent negative samples 9 of 52 (17%) in the male subgroup ($\chi^2 = 12.06$; $p = 0.0005$). Two (28.6%) non-compliant subjects were classified in age subgroup below 40 years ($n = 7$) and similar frequency of negative samples 22.8% was noted in age group above 60 years. The highest proportion of negative samples 39.2% was found in the group of patients aged between 40 and 60 years ($n = 51$).

4. Conclusion

In this paper, we describe the first method for simultaneous determination of doxazosin and verapamil in human serum using LC–MS/MS in positive turbo ion-spray source with SRM mode. A simple LLE method was used for the isolation of drugs from the serum samples in this method. The method has a short run time and provides good accuracy and precision. The assay was successfully applied to a measurement of both drugs in clinical practice and the results were also presented. Trimipramine-d_3 was used for quantification to avoid potential interferences from co-medicated structurally related compounds, which were used for quantification in previously published papers.

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