Quantifizierung von Angiotensin-II-Rezeptor Antagonisten in Humanplasma mittels LC-MS/MS

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Abstract

For the quantitation of angiotensin II receptor antagonist drugs (ARA-II) in human plasma, a method using liquid-chromatography (LC)-electrospray ionization tandem mass spectrometry (MS/MS) has been developed with respect to simple sample clean-up and investigation of ion suppression effects. For sample preparation, protein precipitation using zinc sulphate and methanol showed advantages in speed, recovery, and reproducibility over solid-phase extraction. A triple quadrupole mass spectrometer (Sciex API 365) with turbo ionspray source was used for detection of compounds with multireaction monitoring (MRM) of two transitions per compound. Suppression effects caused by endogenous matrix compounds were investigated by post-column infusion of analytes and LC analysis of precipitates of blank plasma samples and could be excluded. A validation was performed for the ARA-II drugs (valsartan, irbesartan, losartan and its active metabolite EXP 3174, eprosartan, candesartan, and telmisartan). The developed method showed good intra- and interday precision (12% relative standard deviation) and accuracy (11.5% bias) at different concentrations for all the studied compounds. The calculated lower limits of quantitation were between 7 and 13 ng/mL, and the compounds were stable during the analytical process. These rather expensive drugs against hypertension are prescribed with increasing numbers in Europe and the industrialized nations. Complications might arise from overdosage or metabolic disorders. However, drug monitoring is not usually performed. Because the therapeutic concentrations range from a few nanograms to hundreds of nanograms per milliliter for the different drugs, and they are not amenable to gas chromatography/MS analysis because of their high molecular weight and polarity, the LC-MS/MS method is the golden standard for therapeutic drug monitoring and for clinical and forensic toxicology of ARA-II drugs.

1. Introduction

Valsartan, irbesartan, losartan, eprosartan, candesartan and telmisartan are highly selective, non-peptide angiotensin II receptor antagonists. These antihypertensive drugs have demonstrated their ability to inhibit the angiotensin II induced vasoconstriction in preclinical species and to reduce the systolic and diastolic blood pressure at peak effect after dosing in clinical patients [1,2]. All the marketed compounds of this family are safe and effective agents for the treatment of hypertension and heart failure, either alone or together with diuretics, and they have been proposed as an alternative to the more traditional angiotensin converting enzyme (ACE) inhibitors.

Most ARA-II drugs are administered in their active form (losartan, irbesartan, valsartan, telmisartan and eprosartan), however, candesartan is administered as a prodrug (candesartan cilexetil) which is converted into the active substance after absorption. By hepatic metabolism losartan is converted into its

metabolite EXP 3174, a 40 times more potent antihypertensive compound than losartan at blocking the AT1 receptor (receptor subtype 1 for angiotensin II) [3]. The metabolites of the rest of the ARA-II drugs are compounds without antihypertensive effects.

The bioavailability of the ARA-II drugs has proven to be quite variable. It ranges from 15 % for candesartan cilexetil to 80 % for irbesartan, and in some cases, it can be decreased due to the food effect (e.g. for eprosartan, losartan, telmisartan and valsartan) [4].

All the compounds belonging to this drug family are heavily protein bound (90-99 %). The expected plasma concentration for each compound depends on the drug, the administered dose and the time after the oral intake in which the samples are collected. It can range from a few ng/mL to 1-2 µg/mL. The pharmacokinetic parameters for each ARA-II compound are summarised in Table 1.

Table 1. Pharmacokinetic parameters of the ARA-II.

Administered drug	Dose (mg/day)	Active form	C_{max} (ng/mL)*	tmax (h)	Ref.
Losartan	25-100	Losartan	296 (50)	1	[3]
		EXP 3174	249 (50)	4.1	[3]
Candesartan cilexetil	2-16	Candesartan	55 (8)	4.3	[5]
Irbesartan	150-300	Irbesartan	1900 (150)	1.5	[6]
Eprosartan	300-600	Eprosartan	1273 (400)	3	[7]
Telmisartan	40-80	Telmisartan	130 (40)	0.5-1	[8]
Valsartan	80-160	Valsartan	1640 (80)	2	[9]

* The administered dose (mg) corresponding to the C_{max} is indicated in brackets.

Several HPLC methods have been described previously for the determination of ARA-II compounds in pharmaceuticals and biological samples. Hillaert et al. [10-12] have described some electrophoretic and chromatographic screening methods for these drugs alone or in combination with diuretics in pharmaceuticals. Others [13-17] have developed methods to separate ARA-II in biological fluids such as plasma and urine but no screening methods for these compounds have been developed based on the use of liquid chromatography-tandem mass spectrometry devices. However, the mass spectrometric detection has been used with some of these drugs dealing with the search and determination of metabolites [18-21], bioavailability studies [22] and the quantitation in biological matrices [23-26].

The aim of the presented work was to develop an LC-ESI-MS/MS screening method to quantify seven ARA-II compounds in human plasma and the validation of this method, including the optimisation of sample-clean up and investigation of possible suppression effects, to assure its application in routine analysis. After having tested solid-phase extraction procedures with RP-C18 and mixed

mode (RP-C8 and cation exchange) silica phases,- which showed lack of reproducibility especially for the drugs with biphenyltetrazole substructure - a simple sample clean-up procedure by protein precipitation was used, which finally showed good recoveries for all drugs and saved time for sample preparation.

2. Materials and Methods

Chemicals and Reagents

The ARA-II drugs were kindly supplied by the following companies: candesartan and candesartan cilexetil by Astrazeneca (Mölndal, Sweden), eprosartan by Solvay Pharma (Barcelona, Spain), losartan and its metabolite EXP 3174 by Merck (New Jersey, USA), irbesartan by Sanofi-Synthelabo (Montpellier, France), telmisartan by Boehringer Ingelheim (Rhein, Germany) and valsartan by Novartis Pharma (Basel, Switzerland). HPLC-grade methanol, acetonitrile and formic acid and zinc sulphate (analytical grade) were obtained from Merck, (Darmstadt, Germany). Ammonium formate (analytical grade) was from Sigma (Deisenhofen, Germany). Methaqualone, used as internal standard was purchased from Merck (Darmstadt, Germany). Deionised water was prepared with a cartridge-deioniser from Memtech (Moorenweis, Germany).

Blank plasma samples were obtained from the University Hospital of Freiburg (Freiburg, Germany) and the patients' samples were collected, with EDTA as anticoagulant, from patients who were treated with some of the studied drugs and with others, simultaneously.

Instrumentation

The LC-MS/MS system consisted of an API 365 triple quadrupole mass-spectrometer fitted with a turbo ionspray interface (Applied Biosystems/Sciex, Darmstadt, Germany), a Shimadzu HPLC system (three LC10AD pumps and controller unit, Shimadzu, Duisburg, Germany) and a CTC/PAL liquid autosampler (CTC PAL, Chromtech, Idstein, Germany) controlled by Analyst 1.3 software (Applied Biosystems). Analyses were performed with electrospray ionization using a turbo ionspray source in the positive mode. The ARA-II drugs were separated after protein precipitation of the plasma samples at 40 °C on a phenylhexyl reversed phase column (Luna phenyl-hexyl 50 x 2 mm I.D., 3.5 µm) with a guard column (4 x 2 mm, same packing material) (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of solvent A (0.1 % formic acid with 1 mM ammonium formate) and solvent B (acetonitrile: 0.1 % formic acid 95:5 (v/v) with 1 mM ammonium formate). The following gradient elution was used at a flow-rate of 0.25 mL/min: 0-0.2 min: 5 % B, 0.2-1 min: 5-35 % B linear; 1-8 min: 35-45 % B linear; 8-9 min: 45-70 % B linear; 9-9.5 min: 70 % B; 9.5-10.5 min: 70-5 % B linear and 10.5-12 min: 5 % B for re-equilibration.

To enhance signal intensity, acetonitrile was added with a post-column "tee" at a flow rate of 100 µl/min before the effluent enters the turbo ionspray interface. The turbo ionspray source was operated at 350 °C with an ionization voltage of 5250 V (positive mode), and nitrogen as curtain gas (11), nebulizer gas (10), turbo gas (3 L/min) and CID gas with an analyzer pressure of 2.3 x 10^{-5} torr. Analysis was performed by multi-reaction monitoring, using the precursor ions (protonated molecules) and the corresponding parameters which are shown in detail in Table 2, and unit resolution for Q1 and Q3. The dwell time was 50 ms for each transition. Chromatographic data were handled with Analyst 1.4 software (Applied Biosystems/Sciex, Darmstadt, Germany).

Preparation of calibration standards and quality control samples

All compounds were supplied as solid white powders, which were used to prepare stock solutions of 1 mg/mL of each compound in methanol. These solutions were used to prepare the working solutions as follows:
Working solution 1 contained valsartan (200 µg/mL), irbesartan (200 µg/mL), losartan (25 µg/mL), eprosartan (200 µg/mL), EXP 3174 (100 µg/mL), candesartan (25 µg/mL) and telmisartan (50 µg/mL) in methanol. It was used to prepare the highest concentration point of the calibration curve.

Working solution 2 contained valsartan (160 µg/mL), irbesartan (160 µg/mL), losartan (20 µg/mL), eprosartan (160 µg/mL), EXP 3174 (80 µg/mL), candesartan (20 µg/mL) and telmisartan (40 µg/mL) in methanol. It was used for the validation of the assay, to prepare the quality control (QC) plasma samples at a high and medium concentration level of the calibration curve.

Working solution 3 contained valsartan (1 µg/mL), irbesartan (0.7 µg/mL), losartan (1 µg/mL), eprosartan (1.3 µg/mL), EXP 3174 (0.8 µg/mL), candesartan (0.8 µg/mL) and telmisartan (1.2 µg/mL) in methanol. It was used to prepare the low concentration level (lower limit of quantitation, LLOQ) of the calibration curve and the QC plasma samples at the LLOQ.

A working solution of 1 µg/mL of each compound was prepared for spiking plasma samples for determination of LOD and LLOQ.

A methanolic solution of methaqualone was prepared and diluted to a working solution with a concentration of 50 µg/mL of methaqualone in methanol.

All solutions were stored at 4 °C and protected from light using brown glass vials. Calibrators were prepared in 1 mL aliquots of human plasma by spiking drug free control plasma using the stock and working solutions.

Protein precipitation

For protein precipitation a solution containing zinc sulphate 0.1 M and methanol (1:4, v/v) was used. This solution was stored at 4 ºC. 0.1 mL of the calibration standards, control blanks and QC samples was transferred to an 1.5 mL

plastic tube (Eppendorf cup) and spiked with 10 µL of the working solution of methaqualone. After vortexing, 0.2 mL of the protein precipitation reagent solution was added by slow dropping to avoid the precipitation of the proteins as clusters which would retain the analytes inside. Then this solution was vortex mixed for 1 minute and centrifuged at 13200 r.p.m. for 10 min using a micro centrifuge (model 5415 D centrifuge, Eppendorf, Hamburg, Germany). 170 µL of the supernatant was transferred to a 2 ml autosampler vial with glass micro insert and 20 µL was injected into the HPLC.

Setting up an MRM method

Full-scan mass spectra for the determination of protonated molecules and product ion mass spectra were acquired using our general procedure for setting up a MS/MS spectra library of drugs [27]. MRM transitions were selected from the product ion spectra and mass spectrometric parameters were optimised by infusion of single compound solutions at 10 µg/ml concentrations using the tune function of the Analyst software. Optimised parameters for ionisation and collision are shown in Table 2.

Table 2. Tandem mass spectrometry parameters and retention times of ARA-II drugs and internal standard methaqualone (IS).

Drug and retention time t_R (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	FP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
Valsartan, 7.29	436	291	15	190	10	15	24	25
	436	235	20	185	10	14	25	22
Irbesartan, 4.84	429	207	30	200	10	15	35	17
	429	195	30	210	9	13	30	22
Losartan, 4.96	423	377	16	182	10	13	20	24
	423	207	16	172	10	12	31	19
Eprosartan, 2.42	425	207	40	225	8	15	33	19
	425	135	30	210	9	18	43	14
EXP 3174, 6.73	437	235	17	190	8	15	23	22
	437	207	17	190	8	15	30	20
Candesartan, 5.04	441	423	12	178	10	14	16	29
	441	263	12	178	10	14	17	23
Telmisartan, 3.88	515	305	30	220	9	25	50	25
	515	276	50	260	8	20	60	20
IS, 4.45	251	132	20	230	10	7	35	15

DP: defragmentation potential, FP: focusing potential, EP: entrance potential, CEP: collision cell entrance potential, CE: collision energy, CXP: collision cell exit potential.

Validation procedures and method characteristics

Recovery

To calculate the absolute recovery of the protein precipitation procedure, three replicates of spiked plasma samples at different concentration levels (low, medium and high, see Table 4) of valsartan, irbesartan, losartan and its active metabolite EXP 3174, eprosartan, candesartan and telmisartan were used. These

samples were compared with methanolic solutions of the drugs at the studied concentrations which have been processed following the same sample preparation procedure as the plasma samples. The obtained recovery values for each concentration level and drug are summarised in Table 3.

Table 3. Absolute recovery values for valsartan, irbesartan, losartan and its active metabolite EXP 3174, eprosartan, candesartan and telmisartan at the studied concentration levels. (n=3)

	Concentration level		
	low	medium	high
Drug	Absolute recovery (%)		
Valsartan	87.9	99.6	112
Irbesartan	89.3	102	123
Losartan	99.3	103	119
Eprosartan	74.1	89	106
EXP 3174	90.8	94.3	104
Candesartan	97.7	89.4	94
Telmisartan	93	102	105

Determination of linearity and working range

The linearity of an analytical method is its ability to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample within a defined range [28]. Blank plasma was spiked with the appropriate volume of working solution 1 to achieve the following concentrations: valsartan (2000 ng/mL), irbesartan (2000 ng/mL), losartan (250 ng/mL), eprosartan (2000 ng/mL), EXP 3174 (1000 ng/mL), candesartan (250 ng/mL) and telmisartan (500 ng/mL). The other calibration levels were prepared by subsequent dilutions of this sample, covering the whole working range. The calibration standard corresponding to the LLOQ was prepared with the appropriate volume of the working solution 3.

The working range was defined considering the normal therapeutic doses of each drug and the maximum plasma concentrations. The expected range was extended in order to detect potential overdoses. In the case of irbesartan, the response was not linear over the whole selected range and therefore the calibration curve was divided in two linear fractions: from LLOQ to 250 ng/mL and from 250 to 2000 ng/mL.

Three series of plasma samples were prepared and analysed on three different days. The correlation coefficients and the slope values (parameters shown in Table 4) were reproducible for the three curves. Correlation coefficients ranged from 0.9940 to 0.9998. The relative standard deviations of slope values for each compound were less than 10 %.

As no calibration sample exceeded the acceptable limit of relative error (< 15 %) for the interpolated concentration with regard to nominal concentration, the

proposed linear model was accepted for the studied compounds in the working ranges described in Table 4.

Evaluation of precision and accuracy

The method precision (relative standard deviation, RSD), was determined on one day (intraday precision) and on three different days (intermediate precision or repeatability). A calibration was performed on each day. To cover the whole working range, three concentration levels were chosen: low level (LLOQ), intermediate level and high level, which was 80 % of the highest value of the calibration curve. These concentrations levels, which were different for each compound depending on the linear range, are shown in Table 4. Precision was measured using six spiked plasma samples for each concentration level and each day.

Intra- and inter-day accuracy (expressed as bias) was determined by measuring six replicates for each concentration level of each drug (on three different days). Precision and accuracy, summarised in Table 5, were in accordance with the validation guidelines of the Food and Drug Administration (FDA) (RSD < 15 % and bias < 15 %) [29].

Table 4. Linear range of the calibration curves and quality controls for the studied drugs, including the correlation coefficients and slope media values (n=3) corresponding to the linear regressions of those curves. All the concentration values are in ng/mL.

Drug	LOQ	LOD	QC low	QC medium	QC high	Linear range	R^2	Slope
Valsartan	10	1.7	10	800	600	10-2000	0.9994	4.36E-05
Irbesartan*	7	2.6	7	100	200	7-250	0.9992	4.84E-04
				800	1600	250-2000	0.9998	2.21E-04
Losartan	10	1.7	10	100	200	10-250	0.9940	6.43E-05
Eprosartan	13	3.3	13	800	1600	13-2000	0.9992	2.00E-05
EXP 3174	8	3.6	8	400	800	8-1000	0.9995	3.65E-05
Candesartan	8	1.7	8	100	200	8-250	0.9993	1.00E-04
Telmisartan	12	4.1	12	200	400	12-500	0.9986	1.38E-04

* Irbesartan presents two linearity ranges which were separately studied.

Evaluation of selectivity

Selectivity has been studied by analysing independent blank plasma samples from six different sources. The chromatograms of the blank samples did not show significant differences when compared to each other. Signals from matrix compounds at the retention times corresponding to the analytes were all below 20% of the signal obtained for the analytes at the LLOQs and below 5% of the area of the internal standard.

Limit of quantitation and limit of detection

To determine the limit of quantitation (LLOQ) and the limit of detection (LOD) the software B.E.N. version 2.0 (Arvecon, Waldorf, Germany) was used. It calculates the LLOQ by a linear regression of a calibration curve with equidistant points (blank included) using a significance level of 99 % for the LLOQ (alpha-error 1 %). The more abundant transition of each compound was used for determination of the LLOQ. For calculation of the limit of detection, the less abundant transition ("qualifier ion") was used with a significance level of 90 % (alpha-error 10 %) – which is in accordance to forensic guidelines of the GTFCh for GC/MS analysis of drugs of abuse [30]. To construct this calibration curve, six human plasma samples were spiked with the appropriate volume of the working solution containing 1 µg/mL of each drug to achieve the following concentration levels: 0, 5, 10, 15, 20 and 25 ng/ml.

Table 5. Precision and accuracy.

Drug	Conc. (ng/mL)	Intra-day (n=6)		Inter-day (n=6)	
		RSD (%)	Bias (%)	RSD (%)	Bias (%)
Valsartan	10	6.71	3.85	11.76	5.83
	800	6.92	1.49	7.62	7.10
	1600	4.34	8.07	4.7	8.60
Irbesartan	7	5.91	8.54	7.53	11.07
	250	2.76	1.04	3.52	3.70
	800	4.07	1.34	5.97	5.90
	1600	3.43	3.68	3.80	5.43
Losartan	10	5.33	2.50	7.74	4.84
	100	6.77	1.73	10.16	3.84
	200	5.05	2.11	8.08	3.89
Eprosartan	13	10.33	4.56	11.29	5.68
	800	5.82	1.22	7.02	2.86
	1600	5.18	4.01	9.07	6.29
EXP 3174	8	6.68	2.42	9.01	7.40
	400	6.16	1.94	8.48	4.74
	800	6.05	4.31	7.02	4.86
Candesartan	8	2.62	3.37	6.54	6.76
	100	9.07	2.28	9.21	3.36
	200	5.53	2.37	7.22	2.45
Telmisartan	12	3.55	1.56	5.28	7.55
	200	4.13	3.04	6.18	4.60
	400	3.59	5.65	3.92	7.77

The values of the calculated LLOQ and the regression coefficients for each compound and the transition used for this calculation are collected in Table 6. The chromatograms corresponding to the calculated LLOQ for each compound and those corresponding to blank plasma samples are shown in Figure 1a and 1b respectively.

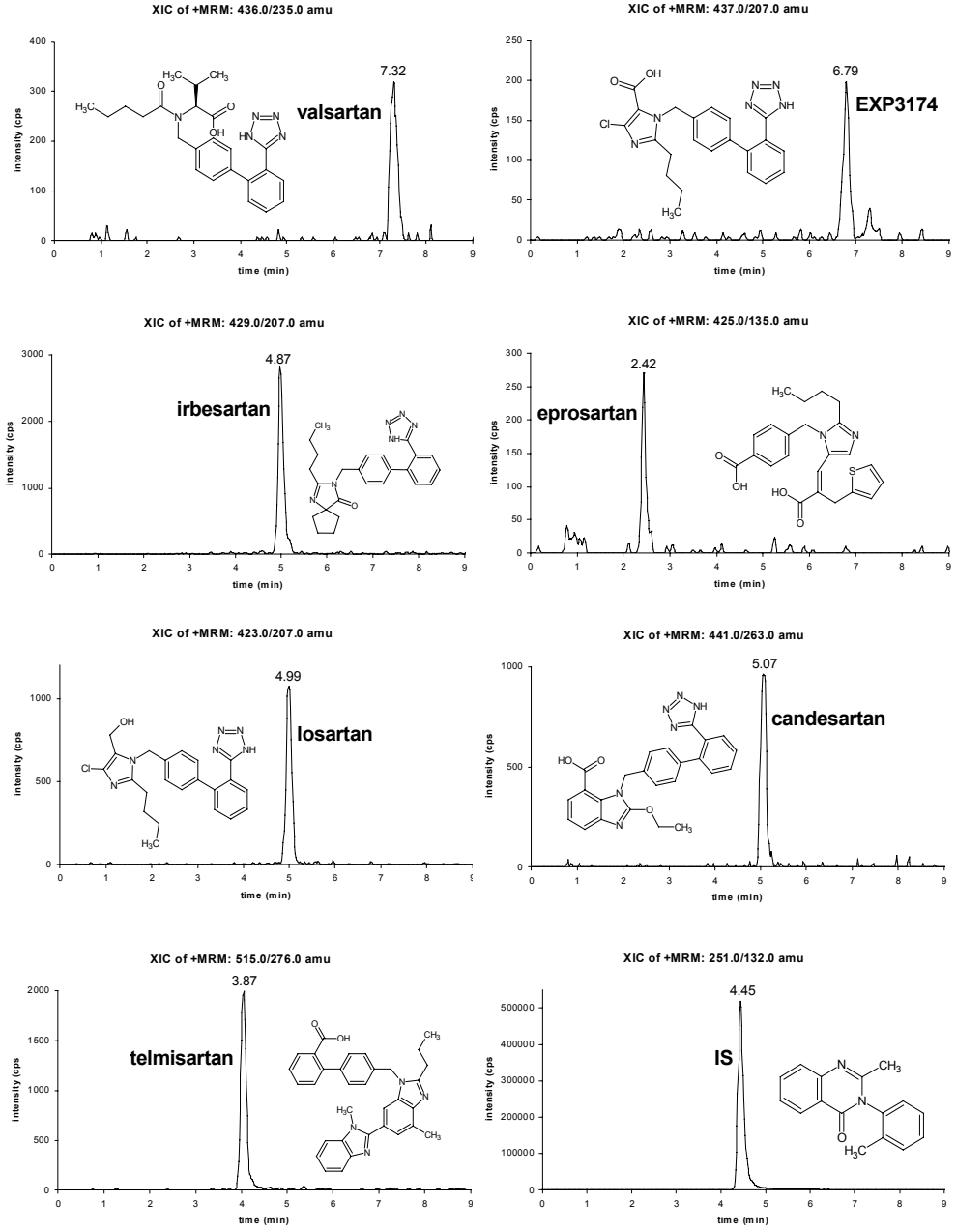

Figure 1 (A). Extracted ion chromatograms of human plasma sample spiked with a concentration of each compound corresponding to the calculated lower limit of quantitation. For comparison to blank plasma sample see Fig. 1 (B).

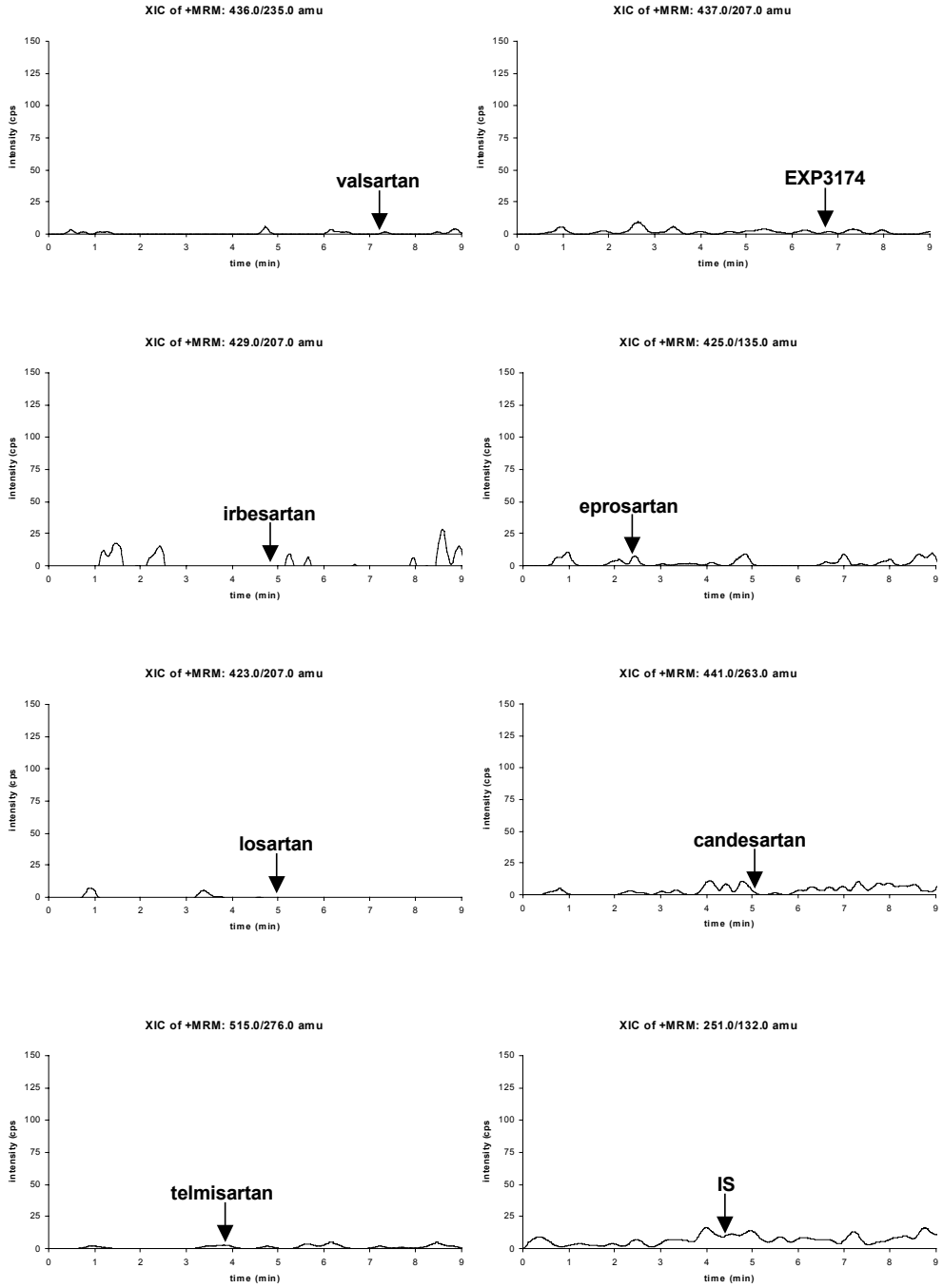

Figure 1 (continued) (B) Extracted ion chromatograms corresponding to blank human plasma sample. The arrows point to the expected retention times. For comparison to spiked sample see Fig. 1 (A)

Stability during the analytical processing of samples

The stability of all analytes was demonstrated by the mean and standard deviation of the ratios between the repeated and the initial measurements that were carried out at two different concentration levels using the following conditions:

After three thaw-freeze cycles, at room temperature for 4 hours and during 24 hours in the autosampler. The obtained values are shown in Table 7.

To study the long term stability (three months) at –20 ºC, a calibration curve was constructed the same day the samples were measured. The concentrations calculated by using this curve, were compared with the initial spiked concentrations for each drug in the samples. The stability of all the ARA-II substances in methanolic solutions at refrigerator temperature was also found to be acceptable for six months.

Table 6. Values of LLOQ and the regression coefficient for each compound (n=6).

Drug	Transition m/z	LLOQ (ng/mL)	R^2
Valsartan	436 → 235	10	0.996
Irbesartan	429 → 207	7	0.998
Losartan	423 → 207	10	0.996
Eprosartan	425 → 135	13	0.993
EXP 3174	437 → 207	8	0.998
Candesartan	441 → 263	8	0.997
Telmisartan	515 → 276	12	0.994

Table 7. Concentration ratios (measured concentration after freeze thaw cycle divided by spiked concentration): mean values and standard deviations of n=3 measurements at two different concentration levels for studying the stability.

Drug	Conc. (ng/mL)	Stability (ratio ± SD)			
		Room temp. t=4 h	After freeze-thaw cycles		
			Cycle 1	Cycle 2	Cycle 3
Valsartan	50	1.05±0.04	0.93±0.02	1.10±0.10	1.1±0.17
	1600	1.00±0.05	0.95±0.02	0.99±0.05	0.94±0.05
Irbesartan	35	0.96±0.01	1.00±0.03	0.99±0.03	0.93±0.02
	250	1.02±0.03	1.04±0.02	1.01±0.07	0.91±0.04
	1600	0.97±0.01	0.92±0.02	0.95±0.05	0.94±0.06
Losartan	50	0.93±0.06	0.93±0.07	1.04±0.18	0.91±0.03
	200	0.97±0.04	0.94±0.02	1.00±0.07	0.96±0.09
Eprosartan	65	1.07±0.01	0.93±0.03	0.93±0.04	0.95±0.08
	1600	1.09±0.03	1.08±0.02	1.03±0.12	1.06±0.07
EXP 3174	40	1.00±0.07	1.05±0.04	1.04±0.06	0.95±0.01
	800	1.03±0.04	1.02±0.02	1.04±0.04	1.00±0.07
Candesarta	40	0.99±0.07	1.02±0.07	1.09±0.06	0.97±0.07
n	200	1.06±0.04	1.00±0.03	0.99±0.08	1.02±0.08
Telmisarta	60	1.00±0.05	1.02±0.03	1.03±0.05	0.96±0.02

n	400	0.97±0.05	0.96±0.02	0.99±0.05	0.96±0.07

Test of ion suppression

A methanolic solution of the studied compounds and the IS, was infused post-column via an additional mixing tee (before passing the post-column tee for acetonitrile addition just in front of the ionspray source) at a concentration of 1000 ng/mL and 500 ng/mL respectively and at 25 µl/min. Ion suppression resulting from matrix components in the eluent was monitored during gradient elution by continuously monitoring the MRM-transitions for all compounds over a 12 min period. Control plasma blanks were prepared by using the protein precipitation procedure described above and injected into the LC–MS/MS system (see Fig. 2 and "Results").

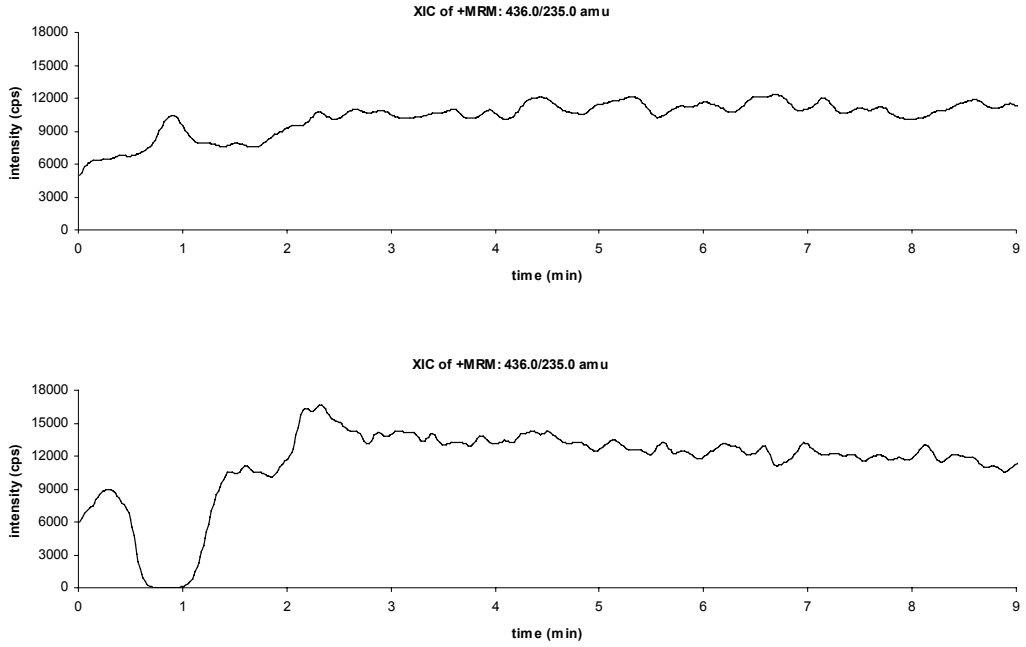

Figure 2. Extracted ion chromatograms for valsartan during post-column infusion and subsequent injection of acetonitrile (above) and a control blank plasma sample (below).

3. Results and discussion

The results of method validation are summarised in Tables 2 to Table 7. The evaluation of ion suppression – which should be required at least for procedures, where no isotope-labelled homologues of the analytes are used [31] - was performed as suggested by Bonfiglio et al. [32] and this method has been applied in our laboratory before for the evaluation of ion suppression with different sample preparation procedures [33]. A decrease or increase in the intensity of the monitored ion trace was considered to be an effect of suppression or enhancement of ionisation. As an example, the extracted ion chromatogram

corresponding to valsartan is shown in Figure 2.The first major ion suppression region, most likely caused by the elution of polar compounds (salts, peptides, organic acids and bases, amino acids and carbohydrates etc.) occurs near the void volume of the column; i.e., between 0.7 and 1 min. The second major ion suppression region, occurring around 10 min after injection, is caused by lipophilic compounds (e.g. phospholipids or others) [34]. All the studied drugs elute between 2.4 and 7.3 min, where no ion suppression phenomena have been observed. The retention time for each compound is shown in Table 2.

Figures 1a and 1b show the ion-chromatograms of the analytes at their lower limit of detection (1a) and a blank extract (1b).

The fast sample preparation and the LC-MS/MS analysis have been shown to be adequate for the quantitation of seven ARA-II drugs in therapeutic concentration ranges in human plasma samples and can be applied for drug monitoring in clinical and – due to the high identification power of LC-MS/MS by use of two MRM transitions per compound- also in forensic cases.

Application to patients' blood samples

Plasma samples obtained from patients under cardiovascular treatment with one of the studied ARA-II compounds and co-administered drugs, such as β-blockers, 1,4-dihydropiridines, statins, ACE inhibitors, diuretics or non-steroidal anti-inflammatory drugs have been analysed.

The samples were taken between 1 and 24 hours after the oral administration of the substances. In all cases, no interferences from the co-administered substances were observed (Figure 3). Plasma concentration levels (expressed as mean ± SD in ng/mL) were obtained by interpolation from the calibration curve and are summarised in Table 8.

Table 8. Measured concentration of the ARA-II in plasma samples of patients under cardiovascular treatment (mean ± SD (n=3)).

Drug and dose (mg/day)	Time after dose (h)	Calculated conc. (ng/mL)	Coadministered drugs
Irbesartan (150)	1.5	1969.7±12.7	metformin, insulin, atorvastatin
Telmisartan (80)	24	16.2±1.5	glibenclamide, cinarizine+dihidroergocristine, rosiglitazone, atorvastatin, doxazosin, ASA
Telmisartan (80)	1	483.9±10.2	chlortalidon, lacidipine, allopurinol, atorvastatin
Candesartan (16)	9	25.0±0.5	---
Valsartan (160)	3.5	505.5±9.5	ASA, atorvastatin
Valsartan (160)	2	674.0±10.7	---
Losartan (not reported)	Not reported	<LLOQ losartan 8.3±0.7 EXP 3174	benzodiazepines
Eprosartan (600)	2	195.5±4.1	atorvastatin

ASA: Acetylsalicylic acid

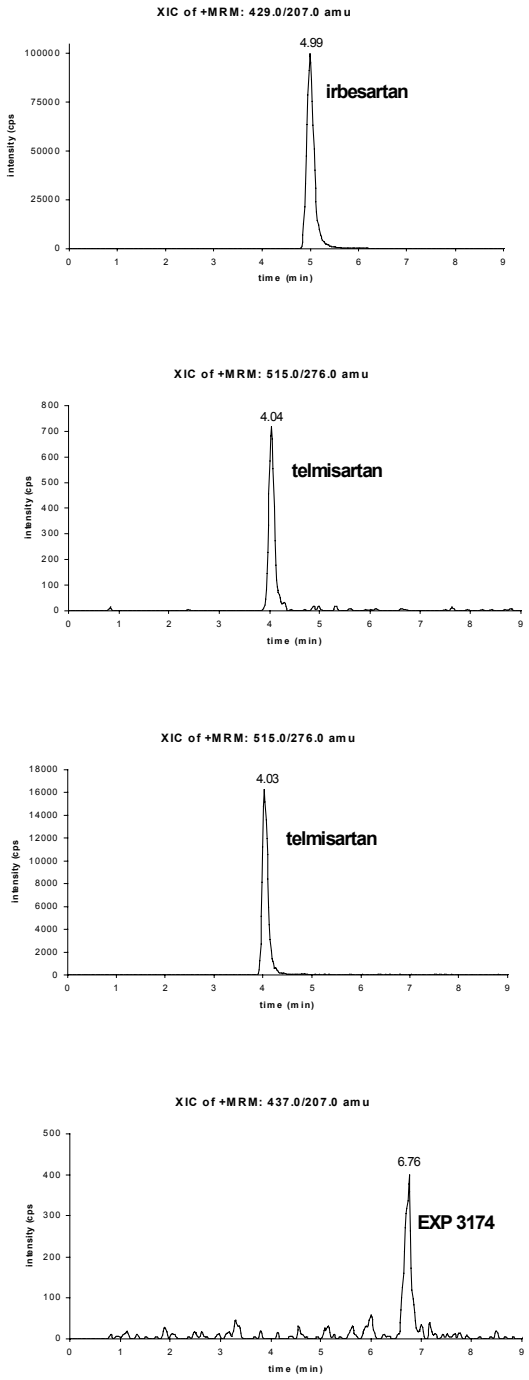

Figure 3. Extracted ion chromatograms corresponding to plasma samples containing irbesartan (1969.7 ng/mL), telmisartan (16.2 ng/mL), telmisartan (483.9 ng/mL), and EXP 3174 (8.3 ng/mL).

4. Conclusions

This new LC-MS/MS procedure has been developed for the simultaneous determination of valsartan, irbesartan, losartan and its metabolite EXP 3174, eprosartan, candesartan and telmisartan in one single analysis, to cover their whole therapeutic ranges in plasma samples obtained from patients under cardiovascular treatment. The method proved to be sensitive, accurate, precise and reproducible and sample preparation showed high recovery for the quantitative determination of the ARA-II compounds in human plasma. Suppression tests – which are required for each quantitative LC-MS/MS procedure using ESI or APCI - have been performed and suppression effects could be excluded by testing different plasma sample batches. The simultaneous detection and quantitation of the ARA-II drugs by LC-MS/MS is advantageous compared to other methods – most of them using fluorescence detection - especially when patients take several different drugs or analysis for different drugs is required. Protein-precipitation instead of solid-phase extraction saves sample pre-treatment time, and the high specificity of LC-MS/MS reduces analysis time. In addition, eprosartan, which does not show native fluorescence has been included in the LC-MS/MS procedure [15,35].

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