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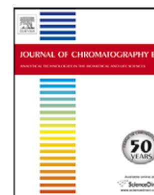

Direct analysis of valsartan or candesartan in human plasma and urines by on-line solid phase extraction coupled to electrospray tandem mass spectrometry

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ABSTRACT

AT1 receptor blockers are agents for the treatment of hypertension. Rapid and precise assay methods are needed to evaluate possible sub- or overdosage. A direct on-line solid phase extraction coupled to tandem mass spectrometry was developed and validated to determine valsartan (5–2000 ng/mL) or candesartan (1–200 ng/mL) in human plasma and urines. Both intra- and inter-assay accuracy and precision were in the range 89.2–111% and 0.9–16.9% RSD. Total run time was 4.5 min. This on-line clean-up–MS method was found to be robust for the analysis of a high number of samples with unvarying performance.
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1. Introduction

The renin–angiotensin system (RAS) is a coordinated hormonal cascade that regulates fluid, electrolyte balance, arterial pressure and plays a major role in cardiovascular, renal and adrenal function [1]. Blockade of the RAS with angiotensin I-converting enzyme (ACE) inhibitors, AT1 receptor blockers (ARBs) or a combination of these drugs [2] has become one of the most successful therapeutic approaches in medicine treating patients with broad range of cardiovascular diseases. Despite the availability of these drugs, morbidity and mortality in patients with congestive heart failure and evolution towards end-stage renal failure chronic nephropathies remain high. Multiple factors may explain the relative failure of these drugs to achieve a full cardio- and nephroprotection, including lack of compliance to treatment, genetic factors, the involvement of pathogenic pathways other than RAS, the delay in treatment start and insufficient dosing. Large between-subject variability in the pharmacokinetics of some of these drugs may also explain the variable pharmacodynamic response. Among ARBs, valsartan and candesartan cilexetil (candesartan pro-drug) which selectively blocks angiotensin II binding to the AT1 receptor (angiotensin II receptors antagonists, *i.e.* ARA-II) have been shown to have cardiovascular and renal beneficial effects. Their therapeutic concentrations range from a few nanograms to only hundreds of nanograms per milliliter of plasma. Interaction with food (50% reduction in Cmax and AUC) has already been reported for valsartan [3] but not for candesartan cilexetil [4]. In the search for sources of pharmacokinetics variability and to check for compliance, it may be necessary to measure concentrations of these drugs in biological samples, but for both technical and economical reasons these measurements are not performed. Therapeutic drug monitoring or investigational clinical studies have to benefit from fast, precise, accurate and if possible inexpensive analytical methods to reveal significant pharmacokinetic effects.

Several methods were published for the analysis of ARA-II. Single analytes in biological matrices were extracted by liquid–liquid extraction [5], solid phase extraction (SPE) [6–10] or protein precipitation [11–13]. Extracts were assayed by liquid chromatography with ultra-violet absorbance detection (LC–UV) [6,7], fluorescence detection (LC-Fluo) [5,8,13] or mass spectrometry with electrospray ionization (ESI-MS) [9–12]. Multiple analytes were analyzed in plasma or urine by on-line in tube solid phase microextraction (SPME) before LC-Fluo [14]. In pharmaceutical formulations, methodologies based on capillary zone electrophoresis [15] or micellar electrokinetic chromatography [16] were proposed.

Tandem mass spectrometry (MS/MS) with electrospray ionization coupled to separation techniques presents some obvious advantages as short time development, sensitivity, selectivity and high throughput. Despite its high selectivity and sensitivity, this

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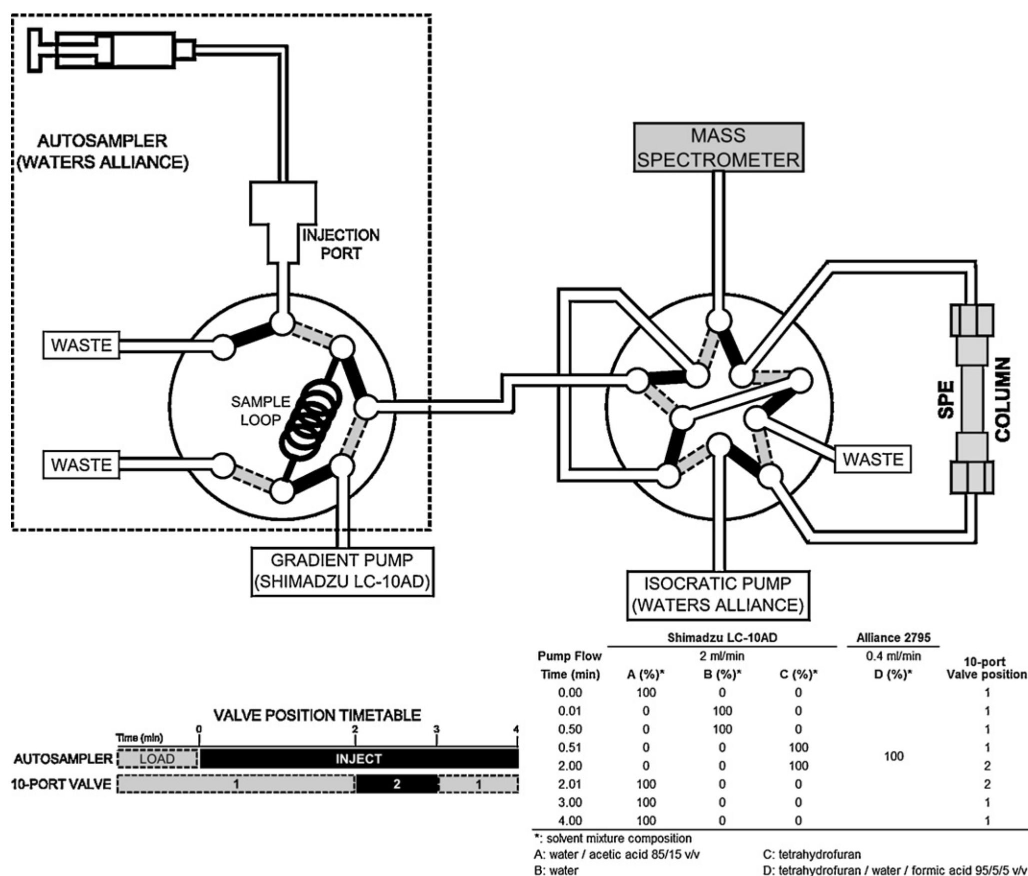

Pump Flow	Shimadzu LC-10AD 2 ml/min			Alliance 2795 0.4 ml/min	
Time (min)	A (%)*	B (%)*	C (%)*	D (%)*	10-port Valve position
0.00	100	0	0	100	1
0.01	0	100	0		1
0.50	0	100	0		1
0.51	0	0	100		1
2.00	0	0	100		2
2.01	100	0	0		2
3.00	100	0	0		1
4.00	100	0	0		1

*: solvent mixture composition
A: water / acetic acid 85/15 v/v
B: water
C: tetrahydrofuran
D: tetrahydrofuran / water / formic acid 95/5/5 v/v

Fig. 1. Scheme of the dual liquid chromatographic system connected to the mass spectrometer via the 10-port valve and time events for valve switching and solvent gradient.

technique may suffer from signal adulteration due to co-eluted endogenous compounds resulting in ionization suppression [17]. The variability of this matrix effect [18] is the main factor responsible for inaccuracy when the endogenous involved molecules have different concentrations in clinical samples because of inter- and intra-subject variability. To counteract this phenomenon, it is necessary to purify the sample as much as possible by eliminating interferences without loss of the target analytes. Extraction techniques such as liquid–liquid extraction, SPE or protein precipitation are currently employed in laboratories with varying success and recently, mixed mode sorbents for SPE were commercially introduced. These materials interact with compounds via hydrophobic and ion-exchange mechanisms and allow improved washing procedures leading to cleaner extracts [19].

On-line SPE-LC–MS/MS is a powerful alternative methodology for the analysis of xenobiotics and metabolites in biological matrices in support of pharmacokinetic or bioequivalence studies. This technique has been widely used and described [20–28] and major advantages are higher throughput, good precision, fewer stability conditions to be controlled, limited manual processing of potentially hazardous biological samples and low expense for cost and consumables. However, owing to additional time development and short lifespan related to high number of biological sample analysis leading to analytical performance decline, the use of an analytical LC column may be a limiting factor and is not absolutely necessary provided that a powerful sample cleaning is achieved.

In this report, we describe the development of an on-line SPE-MS/MS assay method for the analysis of valsartan or candesartan in human plasma and urine samples. Special emphasis was given to obtain the best precision and high accuracy in drug concentration with the aim of being able to establish even low but significant pharmacokinetic differences in future clinical studies. Here, no further separation after sample purification was necessary, *i.e.* no supplementary LC column was used to achieve the required performances of the method. Validation parameters and the application of this method to clinical samples are presented and comparison to other existing methods is discussed.

2. Experimental

2.1. Reagents and chemicals

Valsartan and candesartan, in their free acid forms, were purchased from Manus Aktteva (Ahmedabad, India). Reagent grade ammonia solution, formic acid and acetic acid were obtained from Merck (VWR, Fontenay sous Bois, France). Methanol was supplied by Merck, acetonitrile and tetrahydrofuran (THF) by Sigma (St Quentin-Fallavier, France), all in LC gradient grade. Ultrapure water (18.2 MΩ) was produced by a Maxima II system (Elga Labwater, Le Plessis Robinson, France). The SPE column (20 mm × 2.1 mm) packed with OASIS MAX 30 μm particles was purchased from Waters (St Quentin en Yvelines, France). Drug free human plasma from whole blood collected on EDTA K3 was obtained from Biopredic (Rennes, France). Drug free human urines were collected from the laboratory staff. For both biological matrices, six individual samples and a pool of four donors were used from selectivity and other validation experiments, respectively. Candesartan and valsartan were used as internal standard (IS) during the assay of the other one.

2.2. Instrumentation

The analytical system was formed by coupling an Alliance 2795 HT chromatographic system (Waters) with a quaternary low

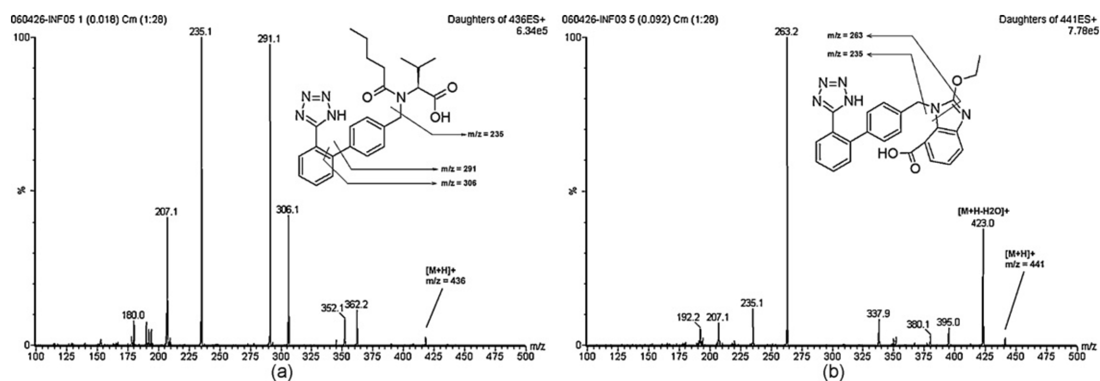

Fig. 2. Ion product MS/MS spectra of valsartan and candesartan.

pressure gradient pump LC-10AD (Shimadzu, Champs sur Marne, France) through a 10-port two positions switching valve LabMX Pro (Rheodyne, Alsbach, Germany). The Alliance system was modified by connecting the LC-10AD pump outlet to the autosampler injection valve. The Alliance pumping line was connected directly to the switching valve (Fig. 1).Detection was achieved with a Quattro micro-triple quadrupole mass spectrometer from Waters operating in positive electrospray ionization mode and controlled by Masslynx version 4.0 (Waters).

2.3. Standards and quality controls (QCs) preparation

Valsartan and candesartan stock solutions, at a concentration of 4 and 1 mg/mL, respectively, were prepared in methanol with 0.5% NH_4OH and were stored at −20 °C in amber glass vials. Independent stock solutions were prepared for calibration and quality controls. Working solutions were made by dilution in methanol. Matrices were then spiked with working solutions with a final amount of 0.4% of organic solvent to obtain the calibration standards and QCs. Calibration curves consisted of eight levels ranging from 5 to 2000 ng/mL for valsartan and from 1 to 200 ng/mL for candesartan. QCs concentrations were chosen to be equivalent to the lower limit of quantification (LLOQ), three times the LLOQ, the middle of the range and about 90% of the upper limit of quantification. LLOQ samples were also prepared in six individual matrices to evaluate the selectivity of the method. Aliquots were stored at −20 °C until analysis.

2.4. Sample preparation

After thawing at room temperature, samples were centrifuged at 2400 × *g* for 5 min. Fifty microliters of sample were deposited in a 96-well polypropylene plate (Waters). After addition of 50 μL of aqueous acetic acid (15%, v/v) and 10 μL of IS solution, the plate was sealed with a polypropylene cap and vortexed for 10 s at 1000 rpm. Samples were kept at +4 °C in the autosampler until injection.

2.5. Chromatographic and mass spectrometric conditions

The SPE column was connected to the 10-port switching valve. The sample (40 μL) was flushed from the sample loop at 2 mL/min with the mobile phase A (water/acetic acid 85/15) and loaded on the SPE column kept at 45 °C. After 0.01 min, the solvent was changed to a mobile phase B (water) to remove the acetic acid and followed by mobile phase C (THF) at 0.5 min until the valve switching. During this step, all unretained interferences were flushed from the column and directed to waste. After 2 min, the valve was switched to its second position in order to back-flush the column with the mobile phase D (THF/water/formic acid 95/5/5, v/v) and to elute the compounds towards the mass spectrometer. The valve was switched in its original position at 3 min to allow the column reconditioning (Fig. 1). In these conditions the two compounds were eluted at around 2.33 min.

The triple quadrupole mass spectrometer was operating in positive electrospray ionization. Nitrogen was used as desolvatation, nebulization and cone gas and argon was used as collision gas. Compounds were infused in mobile phase D for tuning the MS/MS conditions and were analyzed in the multiple reaction monitoring mode using the following transitions using the $[M+H]^+$ ion as precursor: valsartan *m*/*z* 436.2 → 291.1 (dwell time 0.3 s, cone voltage 20 V, collision energy 20 eV) and candesartan *m*/*z* 441.2 → 263.2 (dwell time 0.3 s, cone voltage 20 V, collision energy 15 eV). Half-unit resolution (full with at half maximum) was applied for both quadrupoles in all experiments. Source and desolvatation temperatures were set at 100 °C and 350 °C, respectively. Capillary voltage was set at 5 kV. The desolvatation flow was set at 500 L/h and the collision gas pressure was set at 2×10^{-3} mbar.

2.6. Assay validation

Validation parameters were assessed according to the international guidelines [29].

The selectivity of the method was assessed by analyzing six samples from different donors as blanks and spiked at the LLOQ level.

In LC–MS bioanalysis, extraction and ionization recoveries are parameters classically evaluated. On the contrary to the off-line methods, with on-line SPE these parameters cannot be evaluated separately. Recovery measurements were made by injecting QCs at the three levels in biological matrix and in pure water at the same concentration and comparing the peak areas, allowing the measurement of a total recovery (*i.e.* extraction + ionization). However, in case of poor recovery, post-column infusions were performed to determine whether this effect could be attributed to ionization or extraction. Blank matrix samples (water, plasma and urine) were injected in the system while the compounds dissolved in mobile phase D were infused via a tee between the switching valve and the mass spectrometer source. The concentrations of the compounds were calculated to be equivalent to the peak concentration of a middle QC level. Total ion recordings were normalized versus the one of water and compared to this latter.

Intra-assay precision and accuracy were evaluated by measuring five times each QC level except for the LLOQ for which six samples prepared in six different donor matrix were analyzed in order to assess the selectivity of the method. The inter-assay precision and accuracy were determined by measuring single QC sample at each level during 5 days.

Table 1
Validation results for valsartan and candesartan in human plasma and urines.

	Valsartan		Candesartan	
	Plasma	Urines	Plasma	Urines
Calibration curve ($n=5$)	$Y=aX+b$; weighting $1/X^2$		$Y=aX+b$; weighting $1/X$	
Mean slope (SD)	6.1×10^{-4} (1.5×10^{-4})	4.4×10^{-4} (0.4×10^{-4})	12.54×10^{-3} (2.97×10^{-3})	16.83×10^{-3} (1.25×10^{-3})
Mean intercept (SD)	10.1×10^{-4} (5.7×10^{-4})	7.3×10^{-4} (2.3×10^{-4})	5.32×10^{-3} (2.62×10^{-3})	7.61×10^{-3} (3.56×10^{-3})
Mean R^2 (SD)	0.9968 (0.0018)	0.9969 (0.0013)	0.9957 (0.0055)	0.9987 (0.0005)

	Valsartan								Candesartan							
	Plasma				Urines				Plasma				Urines			
	QC level (ng/mL)															
	5 (LLOQ)	15	1000	1800	5 (LLOQ)	15	1000	1800	1 (LLOQ)	3	100	180	1 (LLOQ)	3	100	180
Total recovery ($n=3$)																
Accuracy (%)	–	104	108	101	–	41.7	45.0	46.4	–	88.4	87.9	96.5	–	54.3	49.8	56.3
Intra-assay ($n=5$)[a]																
Precision (CV%)	8.5	5.9	4.5	2.0	3.8	9.3	3.4	0.6	10.6	6.7	1.5	2.4	9.1	7.7	0.9	5.6
Accuracy (%)	105	96.0	101	102	100	95.6	100	100	89.2	94.8	104	99.2	108	105	105	103
Inter-assay ($n=5$)																
Precision (CV%)	16.9	10.4	2.1	2.8	11.5	5.0	4.0	2.7	10.0	5.4	5.1	3.6	7.4	8.9	2.0	2.8
Accuracy (%)	94.9	99.8	98.9	97.2	92.1	99.0	93.7	98.1	100	102	104	101	111	98.2	101	96.4
Stability ($n=3$)—accuracy (%)																
Three freeze/thaw cycles	–	104	–	99.2	–	93.5	–	99.2	–	106	–	101	–	100	–	102
Autosampler 72 h + 4 °C	–	109	–	98.8	–	95.3	–	99.2	–	93.4	–	98.8	–	101	–	101
Storage 6 months −20 °C	–	110	–	99.5	–	119	–	110	–	94.0	–	107	–	88.2	–	91.5

[a] For LLOQ, $n=6$ independent matrices.

Stability of the compounds in biological matrix was investigated by analyzing three samples of the QC at low and high level. The stability conditions comprised three freeze/thaw cycles, short-term storage on autosampler during 72 h at +4 °C and long-term storage during 6 months at −20 °C.

Impact of the dilution for out-of-range samples was evaluated by spiking matrix at a level equal to two times and five times the highest calibration standard for plasma and urines, respectively. These samples were diluted in blank matrix to reach the middle range of the calibration curve.

3. Results

3.1. Mass spectrometry detection

Ion product spectra of valsartan and candesartan as well as corresponding structure proposal are presented in Fig. 2.

3.2. Selectivity

The blank samples did not exhibit any detectable peak at the compound retention times (Fig. 3). Accuracy and precision measurements for the LLOQ samples performed with different source of plasma and urine were in the regulatory limits for each compound in both matrices (Table 1).

3.3. Linearity

The ratio of compound area/IS area versus concentration was fitted by weighted linear least-squares regression. A $1/X$ and $1/X^2$ weight factors were used for candesartan and valsartan, respectively. For valsartan and candesartan, calibration curves parameters were presented in Table 1.

3.4. Precision and accuracy

For intra- and inter-assay precision (Table 1), the variability of the results was below the regulatory limits of 15% and 20% RSD for the LLOQ. Accuracy ranged from 89.2% to 111%, allowing the accurate assay of these compounds in both matrices.

3.5. Stability

The compounds stability after three freeze/thaw cycles was satisfactory in plasma and urines. With the relatively low volume of sample needed (50 μl), it was possible to assay samples several times to confirm ambiguous results. The sample stability on autosampler, half diluted in mobile phase A and kept at +4 °C, and in storage conditions at −20 °C for 6 months were within the regulatory limits (±15% of the nominal value).

3.6. Sample dilution

This test was made to check whether it was possible to dilute out-of-range samples in blank matrix without compromising accuracy of the results. The accuracy obtained were of 93.9% and 102% for valsartan, and of 115% and 104% for candesartan, in plasma and urines ($n=3$), respectively.

3.7. Clinical application

This method was applied to 896 plasma and 448 urines samples in 64 healthy volunteers after administration of a single oral dose of either Tareg® 160 mg (valsartan, $n=32$) or Atacand® 8 mg (candesartan cilexetil, $n=32$). This study was approved by the Comité de Protection des Personnes Paris-Cochin and was compliant with the ethical principles defined in the Helsinki Declaration. Analytical parameters obtained within this study fulfilled all the validation performance criteria (Table 2) and typical chromatograms are presented (Fig. 3). The pharmacokinetic parameters found in the present study were in accordance with previously results (M. Azizi, et al., unpublished results) and typical plasma pharmacokinetic profiles of valsartan ($n=4$) and candesartan ($n=4$) obtained in healthy volunteers were presented (Fig. 4).

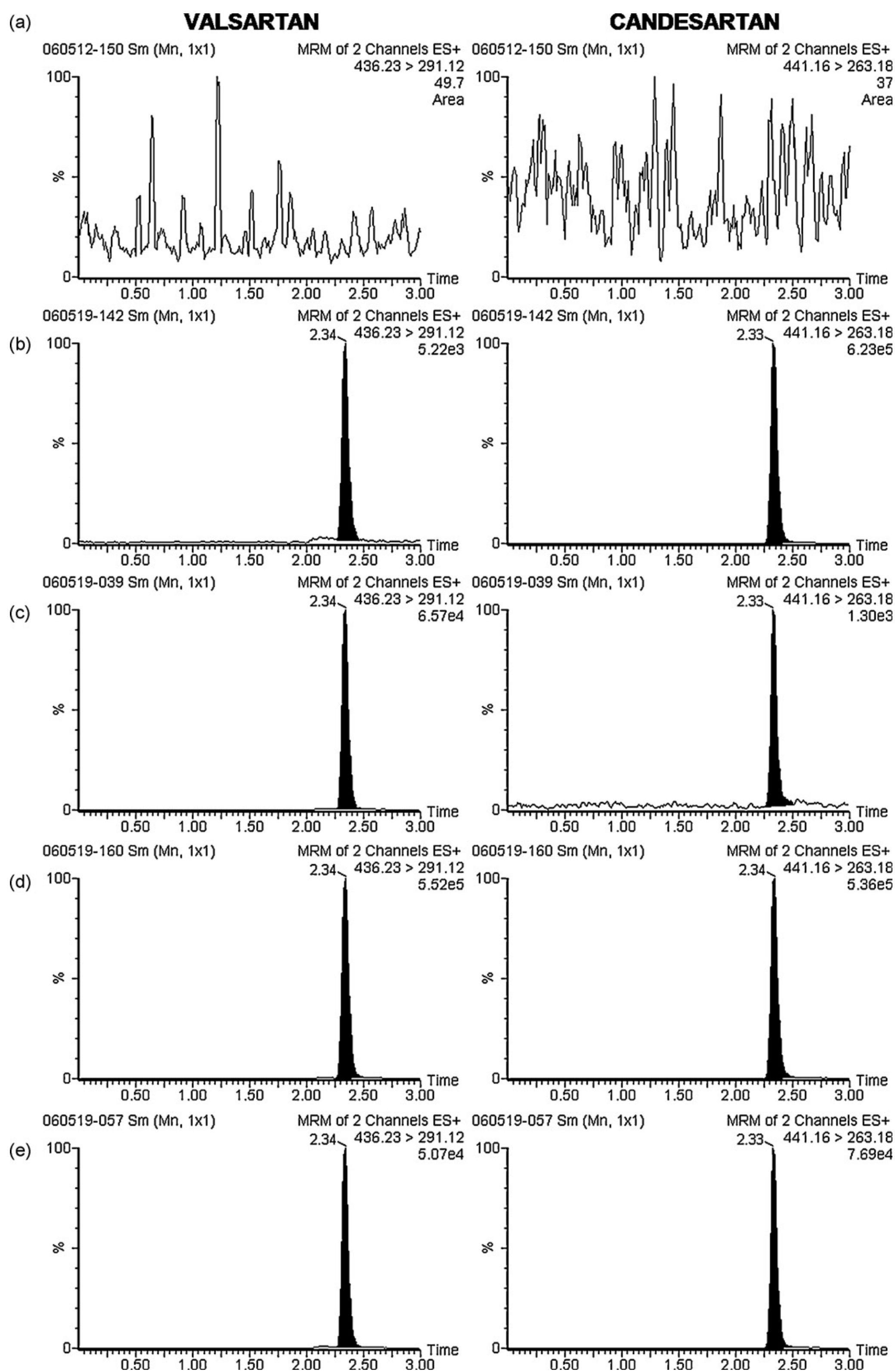

Fig. 3. Typical chromatograms obtained after analysis of valsartan or candesartan in human plasma. (a) Blank plasma, (b) LLOQ of valsartan, (c) LLOQ for candesartan, (d) plasma sample (T1h, 1103 ng/mL) of healthy volunteer after administration of valsartan 160 mg and (e) plasma sample (T4h, 104.4 ng/mL) of healthy volunteer after administration of candesartan cilexetil 8 mg.

Table 2
Valsartan and candesartan assay performance in plasma and urine from healthy volunteers.

	Valsartan		Candesartan	
	Plasma	Urines	Plasma	Urines
Calibration curve				
Plasma ($n=4$)	$Y=aX+b$; weighting $1/X^2$		$Y=aX+b$; weighting $1/X$	
Urines ($n=3$)				
Mean slope (SD)	5.0×10^{-4} (0.3×10^{-4})	3.4×10^{-4} (0.2×10^{-4})	10.09×10^{-3} (0.48×10^{-3})	15.89×10^{-3} (1.04×10^{-3})
Mean intercept (SD)	18.5×10^{-4} (1.2×10^{-4})	7.7×10^{-4} (3.1×10^{-4})	1.90×10^{-3} (1.63×10^{-3})	6.81×10^{-3} (1.66×10^{-3})
Mean R^2 (SD)	0.9978 (0.0009)	0.9931 (0.0033)	0.9938 (0.00012)	0.9973(0.0018)
Standard bias				
Mean precision (%) range	1.7–10.2	3.3–7.4	1.5–9.9	3.7–7.6
Mean accuracy (%) range	92.2–112	86.3–107.8	86.6–108	91.3–105
QC bias				
Mean precision (%) range	4.8–7.9	6.1–10.8	9.0–15.2	9.0–12.0
Mean accuracy (%) range	90.5–112	97.3–111	96.2–100	87.8–108

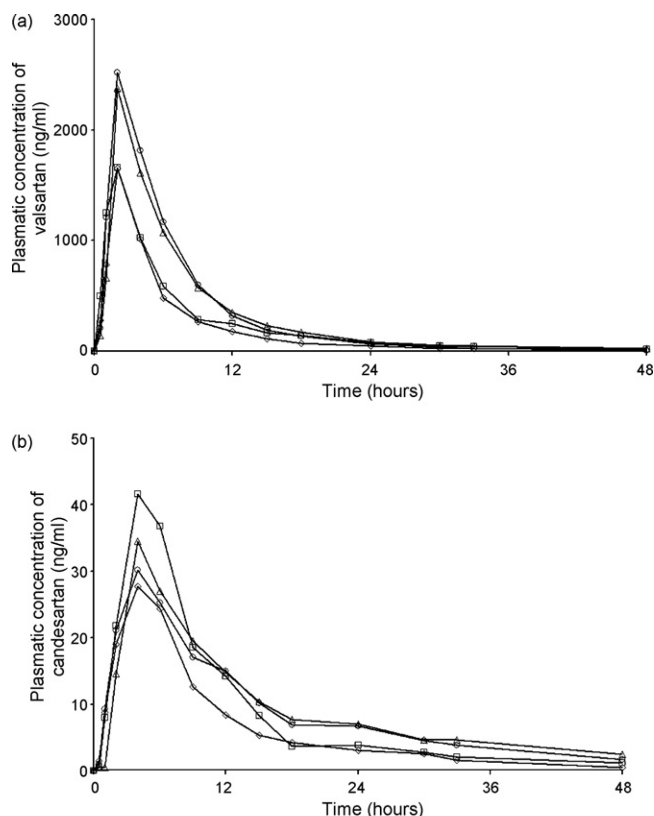

Fig. 4. Plasma pharmacokinetic profile of valsartan (a) and candesartan (b) obtained after administration in healthy volunteers of Tareg® 160 mg ($n=4$) and Atacand® 8 mg ($n=4$), respectively.

4. Discussion

4.1. Choice of the mobile phase

Valsartan and candesartan are relatively highly lipophilic drugs with a log $P_{o/w}$ around 5. In addition, their structure comprises two acidic functions such as a tetrazole ring and a carboxylic acid. Consequently the use of a mixed mode sorbent for SPE seemed to be the key factor to achieve efficient sample cleaning prior to analysis. This allowed to wash the SPE column with full organic phases to remove the hydrophobic interferences with minimal loss of target compound which are bounded to the sorbent via anion exchange.

Many different endogenous components must be removed from the sample. Proteins may precipitate in the analytical system when using highly organic mobile phases or adsorb onto the sorbent, shortening its lifetime. Lipids, and especially phospholipids, are known to reduce signal in electrospray when they are co-eluted with analytes. Zeng et al. [30] proposed the use of aqueous acetic acid solutions (15%) to enhance the solubility of proteins and so eluting them from the column during the sample loading step. They also showed that the use of THF between two injections, permitted to eliminate lipids adsorbed on the column. We choose to use aqueous acetic acid (15%) as sample loading mobile phase and THF as washing mobile phase. However, a washing step made of pure water was necessary between these two phases to prevent their mixing in the column and thus the loss of the compound of interest.

For accurate and reproducible chromatographic peak integration and sensitivity, it is useful to elute the compounds in the sharpest possible band. For this purpose it was necessary to use a mobile phase that creates “on–off” retention behavior for both compounds on the mixed mode sorbent. THF mixed with water and formic acid (95/5/5, v/v) was found to be the most efficient mobile phase. Acetonitrile was tested too, but the peaks were much wider. To compensate peak dispersion, the SPE column was back-flushed with this solvent mixture. The use of THF in ESI-MS has not been described very often [31,32]. The compound response in product ion scan mode was compared by infusion in THF and acetonitrile with the same amount of water and formic acid. The signal was slightly less intense in THF for candesartan and twofold more intense for valsartan. Consequently the use of THF did not compromise the sensitivity and was useable as mobile phase component. Of note, the analytical system was set-up to support high THF concentration, thus stainless steel tubing and adapted piston seals were used.

4.2. Internal standard selection

While stable-isotope-labeled compounds were available, authors have chosen to use each compound as IS of the other one to simplify this assay. This decision was evident during method development because both compounds had a very similar behavior during extraction. This was attributed to the fact that both molecules present the two acidic functions, *i.e.* carboxylic acid and a tetrazole ring. For example when bearing only one of these acidic functions, other ARA-II compounds, such as losartan (no carboxylic acid) or telmisartan (no tetrazole ring), were not retained on SPE column and then were not detected in MS. Moreover, candesartan and valsartan are never co-administered. As noted and as it was expected in this kind of elution system, the two compounds had the same retention time. At the beginning of the method development, it had been checked in the MS spectra that neither

valsartan nor candesartan gave a signal corresponding to the other compound in order to avoid inaccuracy in their quantitation. A same retention time for the two compounds was also an advantage in the control of a potential matrix effect as it would be the case with a stable-isotope-labeled molecule used as IS.

4.3. Recovery measurements

The results showed a lower recovery in urines than in plasma for both compounds. Total ion current recording obtained with post-infusion experiment showed a decrease in signal intensity at the retention time of the compounds for urine samples but not for plasma samples. These experiments demonstrate that the lowest recovery measured in urine samples should be mainly attributed to ion suppression. To control this effect, clinical samples free from candesartan and valsartan were spiked at the low level QC to check the signal response versus calibration standards. No significant difference was measured, allowing the accurate assay of the clinical samples (data not shown). Another way to circumvent this lower ionization recovery would have been to dilute urine samples in plasma or water before analysis. As shown by the results of the clinical study, the valsartan urinary concentrations were high enough what would allow us to dilute urine samples with at least a dilution factor 5.

4.4. Selectivity and matrix effect

Regarding possible interferences due to the presence of metabolites associated with low chromatographic conditions and electrospray ionization, one major source of interference comes from the potential presence of conjugated compounds since they may be present in great and variable quantity and may be so responsible for a major matrix effect. They also may fragment in the ionization source providing the parent compound and resulting in a false quantitative result. As explained before, compounds not bearing the two acidic functions (*i.e.* carboxylic acid and tetrazole ring) were not retained on the SPE cartridge of this method. Consequently this ruled out the possibility for conjugated metabolites to be retained and analyzed in MS source since one of these acidic functions was masked to the sorbent by the simple fact of the conjugation. Furthermore, as it is recommended for selectivity, matrix effect evaluation should be evaluated throughout application of a method to study samples. For example with this method, valsartan was quantified in 448 plasma samples from 32 different patients and covering 14 different time points from 0 to 48 h after dosing. We examined the IS (candesartan) peak area variability which is the contribution of all variability (volume of sample dilution with acetic acid, volume of IS added to the sample, extraction on SPE, injection volume, ionization process and potential matrix effect). Precision of IS peak area was 17.1%RSD (mean, SD, n; 14,339, 2452, 74) for calibration and QC samples (same pool of plasma) and 16.7%RSD (15,773, 2643, 448) for the quantified samples. This resulted in an overall precision of 17.1%RSD (15,569, 2662, 522). This demonstrates that IS behavior was not different between pool of plasma used for both calibrators and QCs and plasma of healthy volunteer samples, thus showing the absence of relative matrix effect. A similar observation was made for urines where the precision of IS peak area was 10.3%RSD (mean, SD, n; 7697, 791, 54) for calibration and QC samples (same pool of urines) and 13.8%RSD (8884, 1226, 224) for the quantified samples of the study, resulting in an overall 14.4%RSD (8652, 1246, 278). As we can observe for plasma and urines, respectively, IS peak intensity and precision were identical in matrices used for calibration standards and QCs and those of many healthy volunteers, thus demonstrating the absence of a relative matrix effect.

4.5. System ruggedness

One aim of the development process was to obtain a reliable and accurate method with keeping in mind some of the today's constraints for bioanalysis and clinical laboratory, *i.e.* high sample throughput and low consumables waste. The cycle time from injection to injection was of 4.5 min. This allowed to assay about 190 samples per day comprising calibration standards and QCs. Furthermore, the on-line sample clean-up was effective enough to lengthen the SPE column lifetime up to 4500 injections without loss of performance and with plasma and urines samples assayed the same day during validation. Moreover, for the pharmacokinetic study of valsartan and candesartan, 4 and 3 analytical runs were performed for plasma and urine, respectively, for each compound without excluding any analytical series.

In conclusion, by evaluating some key points of this on-line SPE-MS/MS method in comparison with previous methods developed for the quantitation of ARA-II in biological matrices and more particularly valsartan and candesartan [5,7–9,11–14], we found that with the very low volume of biological sample used, it is among the most sensitive described methods. Moreover, to our knowledge it presents also the shortest analysis run time including chiefly the sample clean-up. Furthermore, owing to the high number of samples analyzed with only one SPE column, this method presents an asserted character of ruggedness and so the advantage of a moderate cost.

This method, exhibited the required performance in term of selectivity, precision, accuracy, for its application in pharmacokinetic or interaction studies. The use of on-line cleaning reduced the manual processing of biological samples and showed satisfactory result dispersion with high throughput. This methodology, with minor modifications could be applied to other drugs bearing same chemical functions for pharmacokinetic studies or drug monitoring.

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