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Original Paper

# Biovalidation of an SPE-HPLC-UV-fluorescence method for the determination of Valsartan and its metabolite valeryl-4-hydroxy-valsartan in human plasma

A simple and fast method for the simultaneous determination of the antihypertensive drug Valsartan and its metabolite in human plasma has been validated. The proposed method deals with SPE, followed by an HPLC separation coupled with fluorimetric and photometric detection. The optimization of the SPE-HPLC method was achieved by an experimental design. The separation was performed on an RP C18 Atlantis 100 mm × 3.9 mm column. The mobile phase consisted of a mixture of ACN 0.025% TFA and phosphate buffer (5 mM, pH = 2.5) 0.025% TFA and was delivered in gradient mode at a flow rate of 1.30 mL/min. The eluent was monitored with a fluorescence detector at 234 and 378 nm excitation and emission wavelengths, respectively, and at 254 nm using a photometric detector. The full analytical validation was performed according to the Food and Drug Administration (FDA) 'guidance for industry: bioanalytical method validation' and the recoveries obtained for Valsartan and its metabolite ranged from 94.6 to 108.8%. The validated method was successfully applied to 12 plasma samples obtained from patients under antihypertensive treatment with Valsartan.

**Keywords:** Biovalidation / HPLC / Human plasma / Valsartan / Valeryl-4-hydroxy-valsartan

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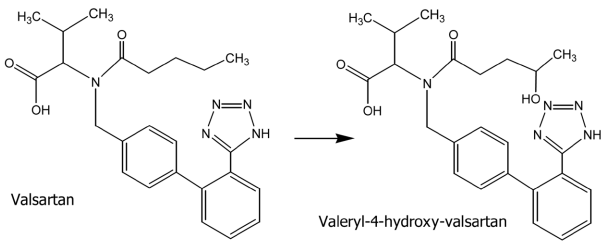
## 1 Introduction

The present study is focused on the therapy based on the use of a single antihypertensive agent: Valsartan, ((*S*)-*N*-valeryl-*N*-[29-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]-valine), an orally active specific Angiotensin II (ARA-II) with high bioavailability and log half-life [1 – 3], which induces vasoconstriction in preclinical species by reducing systolic and diastolic blood pressure at peak effect after dosing [4].

Valsartan is affected by first-pass metabolism. The Valsartan parent compound is rapidly converted (20% of initial dose) into its main metabolite valeryl-4-hydroxy-valsartan, *via* oxidation of the C4 of the pentamide function [5] as shown in the following structural scheme:




**Scheme 1.**

Due to this metabolism, Valsartan and its metabolite can be found in human plasma. The correct separation and quantitation of Valsartan and its metabolite in human plasma samples is crucial to monitor the plasma concentration levels, which will inform us if the therapeutic levels are kept during the interdose range. Therefore, the development of a simple and fast procedure for the simultaneous determination of both compounds is desirable.

The determination methods developed for Valsartan (alone, simultaneously with other ARA-II or in combination with diuretics usually with hydrochlorothiazide, HCTZ) are mainly HPLC methods with photometric [6 –

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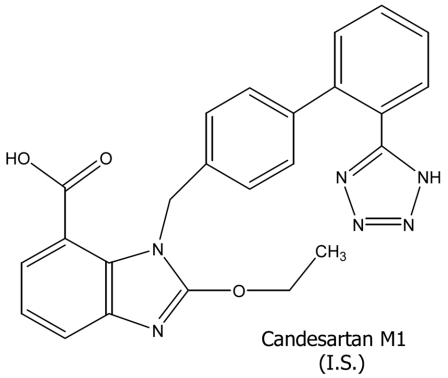
**Abbreviations: LLOQ,** lower limit of quantitation; **QC,** quality control

14] and fluorimetric [15 – 19] detection. GC coupled with mass spectrometry [20], micellar EKC-UV [21] and CE-UV [22] as well as the first derivative UV-spectrophotometry [23, 24] methods have also been reported. No studies regarding simultaneous determination of Valsartan and its metabolite have been found. Only Waldmeier *et al*. [5] have examined the pharmacokinetics and the pharmacodynamics of both compounds.

The aim of the present study is to validate an SPE-HPLC-UV-fluorescence method previously optimized in our laboratory for the simultaneous quantitation of Valsartan and its metabolite in plasma samples obtained from patients under cardiovascular treatment [25].




**Scheme 2.**

## 2 Experimental

### 2.1 Instrumentation

The chromatographic system consisted of two Waters Model 510 HPLC pumps, a Waters Model 717 Plus Autosampler, a Waters 490E programmable multiwavelength detector and a Waters 474 scanning fluorescence detector (Milford, MA, USA). Chromatograms were recorded by means of a computer and treated with the aid of the software Millenium 32 Chromatography Manager from Waters.

A Waters Atlantis dC18, 100 mm × 3.9 mm id, 3 µm, 100 Å column was used to perform the separation. It was thermostated in a column oven controlled by a Waters Temperature Control Module. Previous to the analytical column, a Waters µBondapak C18 guard column 10 µm was placed to prevent column degradation.

Plasma samples were centrifuged at constant temperature (4°C) in an Eppendorf model 5804R centrifuge (Hamburg, Germany), prior to the clean-up procedure. The SPE cartridges were placed in a vacuum manifold from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump from Millipore (Bedford, MA, USA) to perform the SPE step. Plasma extracted samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain).

The pH values of solutions were measured with a Crison GLP 22 pH-meter (Barcelona, Spain) using a Crison glass-combined electrode model 5209 with a reference system Ag/AgCl and KCl 3 M saturated in AgCl as electrolyte.

### 2.2 Chemicals and reagents

Valsartan ((*S*)-*N*-valeryl-*N*-[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)-methyl]-valine) and its metabolite, valeryl-4-hydroxy-valsartan (*N*-(4-hydroxy-1-oxopentyl)-*N*-[[2′-(1*H*-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]methyl]-L-valine), were kindly supplied by Novartis Pharma AG (Basel, Switzerland) (Scheme 1). Candesartan M1 (2-Ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid), used as internal standard (Scheme 2), was kindly supplied by AstraZeneca R&D (Möndal, Sweden).

Reagent grade TFA and phosphoric acid were purchased from Carlo Erba (Milan, Italy). Sodium monohydrogen and dihydrogen phosphate were supplied by Merck (Darmstadt, Germany) and were of proanalysis quality. HPLC grade ACN, methanol and diethyl ether were obtained from Scharlab (Barcelona, Spain) and Carlo Erba.

Purified water from a Milli-Q Element A10 water system (Millipore) was used in the preparation of buffer solutions and reagent solutions. The pH value was adjusted by using volumes of 1 M HCl and KOH solutions.

The cleanup procedure was performed using C8 SPE cartridges (100 mg bed packing, 1 mL volume capacity), purchased from Varian (Harbour City, CA, USA). Prior to the chromatographic separation of extracted samples, they were filtered with hydrophilic polypropylene (∅ = 13 mm) filters supplied by PALL (Ann Arbor, MI, USA).

### 2.3 Standards, calibration curves and quality control samples

Stock solutions of 217 and 114 µg/mL of Valsartan and its metabolite valeryl-4-hydroxy-valsartan, respectively, were prepared in 100% methanol by weight. A 201 µg/mL stock solution of candesartan M1, used as internal standard, was also prepared in 100% methanol.

Calibration solutions were obtained by serial dilutions of stock solutions with methanol, covering the ranges 5 – 1250 and 5 – 4000 ng/mL for valeryl-4-hydroxy-valsartan and Valsartan, respectively, to encompass the expected concentrations in real samples. The internal standard concentration was 650 ng/mL in all samples. All solutions were stored at 4°C and protected from light.

For the validation of the assay, three quality control (QC) plasma samples were prepared containing low

(100 ng/mL of both analytes), medium (1000 and 450 ng/mL of Valsartan and its metabolite) and high (3000 and 1000 ng/mL of each compound) concentration levels. These QC samples were stored at −20°C to study the stability after thaw–freeze cycles and also long-term stability.

### 2.4 Plasma sample collection

Drug-free control human plasma was purchased from the Blood Bank of Galdakao Hospital (Bizkaia, Basque Country) and collected in polypropylene tubes to be frozen at −20°C until analysis.

Plasma samples were also collected from 12 hypertensive patients under treatment with different Valsartan-containing pharmaceutical formulations: DIOVAN-80®, VALS-80, Co-VALS-80, DIOVAN-160, VALS-160, Co-VALS-160 and different cardiovascular drugs, such as diuretics (chlortalidone) and statins (atorvastatine).

A single blood sample was collected from each patient at a peak time interval, between 2 and 4 h after the intake of an oral dose, where the maximum plasma concentrations for Valsartan are expected, and it was immediately transferred into tubes containing tripotassium EDTA and gently mixed. Blood samples were centrifuged at 3500 rpm for 10 min under controlled temperature (4°C) to avoid either decomposition or biological activity. The plasma supernatant was carefully separated from blood cells and collected in polypropylene tubes to be frozen at −20°C until analysis.

### 2.5 Extraction procedure

Once thawed and vortex-mixed, calibration and validation (QC) aliquots of 1 mL of blank human plasma samples were spiked with an appropriate quantity of Valsartan and its metabolite, as well as with the working solution of candesartan M1 to produce an internal standard concentration of 650 ng/mL. Samples extracted from patients under antihypertensive treatment were only spiked with the IS. Afterwards, 1 mL of 0.5 M phosphoric acid was pipetted in all samples in order to precipitate proteins and clarify the plasma sample by eliminating fibrinous material, followed by a brief vortex mixing and centrifugation for 5 min in a high speed centrifuge (10 000 rpm) refrigerated at 4°C.

The C8 SPE cartridges were conditioned with 2 mL of methanol, followed by 1 mL of phosphate buffer (60 mM, pH 2). The plasma samples (1 mL) were manually applied to the cartridges and washed with 1 mL of methanol–phosphate buffer solution (40:60 v/v), followed by an 8 min drying period at high vacuum. Before elution, 0.1 mL of 10% v/v ethyleneglycol solution in methanol was added to the test tubes in order to prevent adsorption processes of the ARA-II [26, 27]. Finally, the analytes of interest were eluted with 0.5 mL of diethyl ether.

**Table 1.** Gradient elution conditions

| Time (min) | ACN 0.025% TFA | Phosphate buffer 0.025% TFA | Flow rate (mL/min) |
|---|---|---|---|
| 0 | 32 | 68 | 1.30 |
| 4 | 50 | 50 | 1.30 |
| 9 | 50 | 50 | 1.30 |
| 9.5 | 32 | 68 | 1.30 |
| 14 | 32 | 68 | 1.30 |

The eluent was evaporated to dryness under a nitrogen stream at 60°C. The remaining residue was reconstituted with 100 μL of mobile phase, vortex mixed, filtered with a PALL GH Polypro (GHP) Acrodisc minispike outlet syringe filter (∅ = 13 mm, 0.45 μm, GHP hydrophilic polypropylene membrane) and transferred to autosampler vials. Aliquots of 20 μL were injected into the HPLC system for analysis.

### 2.6 Chromatographic conditions

The mobile phase consisted of a mixture of ACN 0.025% TFA and phosphate buffer (5 mM, pH = 2.5) 0.025% TFA and was delivered in gradient mode at a flow rate of 1.30 mL/min (Table 1). Before use, the mobile phase was filtered through a 0.45 μm type HVLP Durapore membrane filter from Millipore and degassed in an ultrasonic bath. The sample volume injected into the chromatographic system was 20 μL and the chromatographic separation was performed at 40 ± 0.2°C. The eluent was monitored with a fluorescence detector at 234 and 378 nm excitation and emission wavelengths, respectively, and at 254 nm using a photometric detector [25]. After elution of the analytes, the column was re-equilibrated for 4.5 min before injecting the next sample.

### 2.7 Measurements and calculations

The quantitation of the ARA-II drug concentration levels in human plasma was based on the chromatographic peak area ratios (drug to internal standard candesartan M1). The standard calibration curves were fitted to a linear regression equation of the peak area ratios *versus* analyte concentrations. ARA-II drug concentrations in the clinical samples were then determined from the regression equation obtained from spiked human plasma samples.

### 2.8 Assay validation

In order to carry out sample analysis in a Good Laboratory Practice (GLP)-compliant manner, the developed

**Table 2.** Mean recovery percentages obtained at three concentration levels for valeryl-4-hydroxy-valsartan and Valsartan after the mentioned SPE-HPLC procedures (Sections 2.5 and 2.6)

| | Valeryl-4-hydroxy-valsartan | | | Valsartan | | |
|---|---|---|---|---|---|---|
| Nominal concentration (ng/mL) | 100 | 450 | 1000 | 100 | 1000 | 3000 |
| Recovery(%) | 94.6 | 107.3 | 108.8 | 98.0 | 96.6 | 101.2 |
| RSD(%) | 3.1 | 1.4 | 1.2 | 1.6 | 0.7 | 0.8 |

The extracted plasma samples were spiked with 650 ng/mL of IS after the SPE ($n$ = 6 replicates).

method was validated according to the currently accepted FDA bioanalytical method validation guidance [28] and to the summary report of the conference on 'Analytical Method Validation: Bioavailabilty, Bioequivalence and Pharmacokinetics Studies', which has provided guidelines for pharmacokinetics studies in humans and animals [29].

## 3 Results and discussion

The Atlantis C18 analytical column with a flow rate of 1.3 mL/min provided acceptable retention times allowing relatively short chromatograms (less than 9 min). Valeryl-4-hydroxy-valsartan eluted at 4.5 ± 0.5 min, IS at 6.3 ± 0.4 min and Valsartan at 8.3 ± 0.5 min. The system needed no more than 4.5 min for re-equilibration between injections.

### 3.1 Assay validation

#### 3.1.1 Selectivity

The method's specificity was tested by analysing, under optimized chromatographic conditions, blank human plasma samples from six different sources (or donors) and by comparing them with spiked plasma samples at a concentration close to the lower limit of quantitation (LLOQ).

Blank samples did not show any significant differences among them, neither area values higher than 20% of the LOQ's areas at the analytes retention times nor higher than 5% of the IS's area at its corresponding retention time. Representative chromatograms obtained from control human plasma and plasma spiked with 200 ng/mL and 250 ng/mL for MV and its precursor respectively, and 650 ng/mL of candesartan M1 (IS) are shown in Fig. 1.

Although method selectivity was proved in this way, due to the high interindividual variability and the simultaneous intake of other drugs, it would be advisable to analyse a 'before dose' sample in order to evaluate the suitability of the method in each patient.

**Table 3.** Parameters corresponding to linear regressions obtained from the calibration curves ($n$ = 10) for Valsartan and its metabolite

| | Slope (estimated ± SD) | Intercept (estimated ± SD) | Correlation coefficient ($R^2$) | Linear range (ng/mL) |
|---|---|---|---|---|
| Valeryl-4-hydroxy-valsartan | | | | |
| Curve 1 | 1.31 ± 0.01 | 0.013 ± 0.009 | 0.999 | 5 – 1250 |
| Curve 2 | 1.34 ± 0.03 | −0.002 ± 0.017 | 0.997 | 5 – 1250 |
| Curve 3 | 1.18 ± 0.01 | 0.011 ± 0.006 | 0.999 | 5 – 1250 |
| Valsartan | | | | |
| Curve 1 | 1.38 ± 0.02 | 0.06 ± 0.02 | 0.999 | 5 – 4000 |
| Curve 2 | 1.43 ± 0.02 | 0.03 ± 0.03 | 0.999 | 5 – 4000 |
| Curve 3 | 1.39 ± 0.01 | 0.01 ± 0.02 | 0.999 | 5 – 4000 |

#### 3.1.2 Recovery and reproducibility of the extraction procedure

The recoveries obtained for Valsartan and its metabolite ranged from 94.6 to 108.8% (Table 2). The recoveries did not appear to be concentration dependent.

The six replicates of the plasma samples spiked at three concentration levels (50, 1000 and 3000 ng/mL for Valsartan and 50, 450 and 1000 ng/mL for its metabolite) used to calculate the relative recoveries were also used for calculating the reproducibility of the extraction procedure. The reproducibility obtained in terms of RSD% did not exceed 10% in any case.

#### 3.1.3 Linearity, quantitation limits (LLOQ) and working range

To generate a standard calibration curve for each analyte, blank plasma samples were spiked with appropriate volumes of Valsartan and its metabolite stock solutions in order to achieve the following concentrations: 5, 30, 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000 ng/mL for Valsartan and 5, 30, 50, 100, 200, 275, 350, 450, 650, 800, 1000, 1250 ng/mL for Valsartan's metabolite. In all the cases, the IS (650 ng/mL) was added, prior to the SPE procedure. Three replicates of each calibration curve were carried out. The working range was defined considering the normal therapeutic dosage (80 – 320 mg/day) and the time needed to achieve the maximum plasma levels (1 – 3 h after the intake of the therapeutic dose,

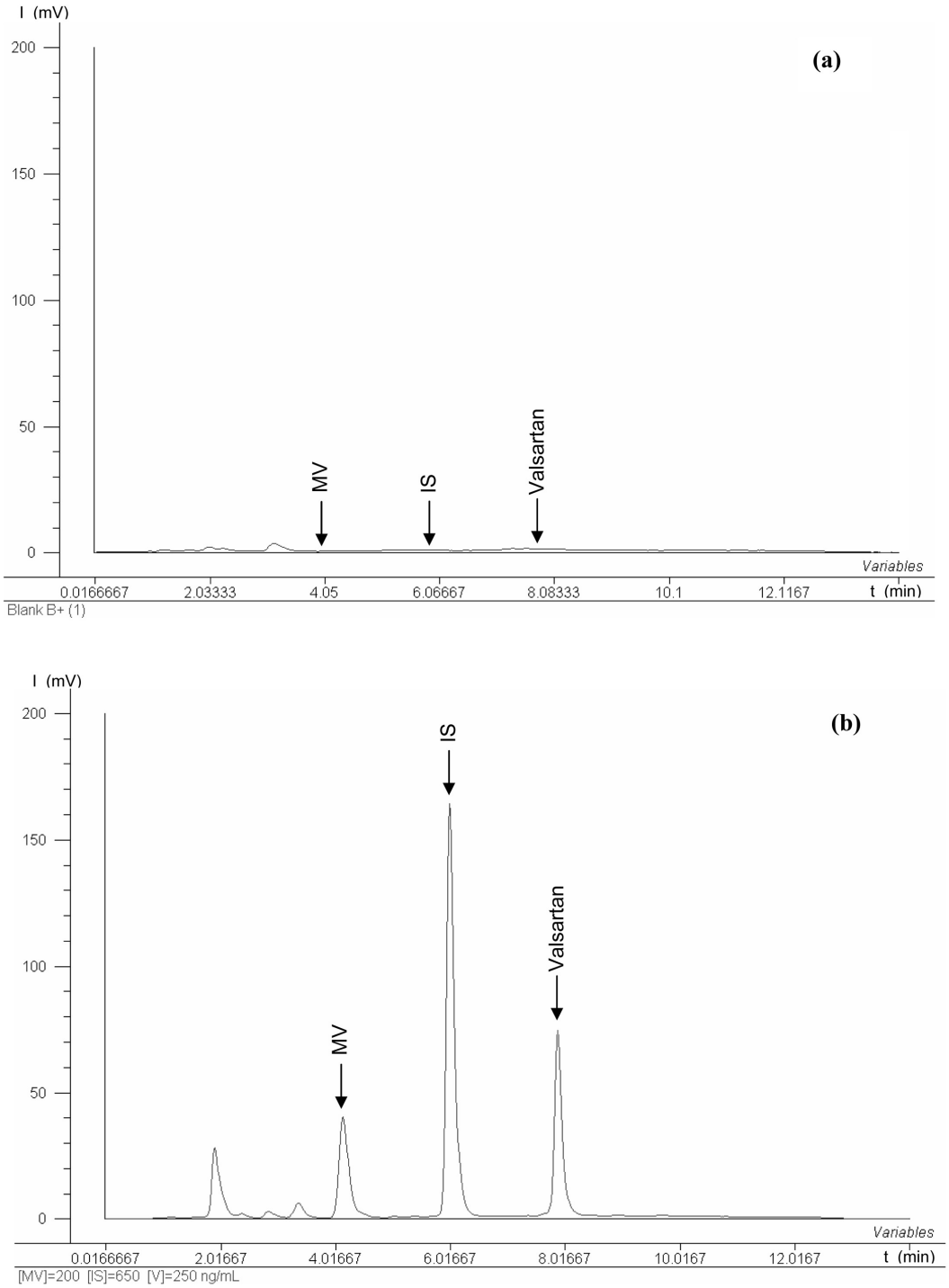



**Figure 1.** Chromatograms obtained with fluorescence detection corresponding to extracts of: a blank plasma extracted sample (a) and a spiked plasma sample with valeryl-4-hydroxy-valsartan (200 ng/mL), Valsartan (250 ng/mL) and candesartan M1 (IS, 650 ng/mL) (b) SPE and chromatographic conditions as described in Sections 2.5 and 2.6.

$C_{max}$ = 2∼4 μg/mL [30 – 33]. The expected range was extended in order to detect potential overdoses.

Three calibration curves were obtained representing the corrected areas (analyte /IS area ratio) *versus* the nominal concentration levels corresponding to each standard sample.

The correlation coefficient, the slope and intercept values were reproducible, as it can be seen in Table 3. No cal-

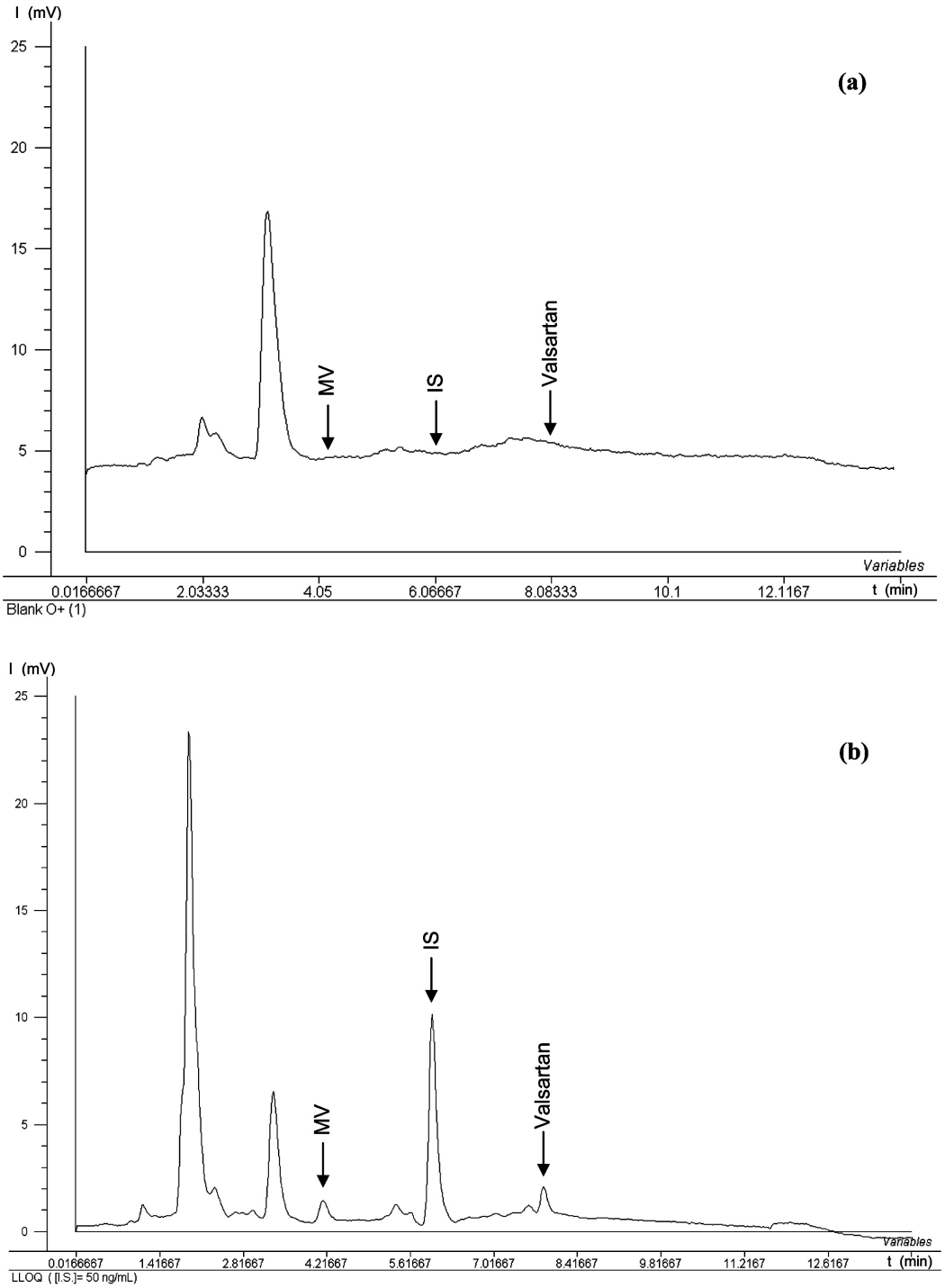



**Figure 2.** HPLC-fluorescence chromatograms corresponding to extracts of: (a) blank plasma and (b) a spiked human plasma sample with valeryl-4-hydroxy-valsartan and Valsartan at a final concentration of 5 ng/mL (LLOQ), and the IS candesartan M1 (IS, 50 ng/mL).

ibration sample exceeded the limit value (RE% >15%) for the interpolated concentration with regard to nominal concentration, so the proposed models were accepted for the studied linear ranges.

These curves defined the LLOQ as 5 ng/mL for both analytes. The analysis of six spiked plasma samples showed that the precision (lower than 20%) and accuracy (80 – 120%) of the LLOQ are acceptable in both cases (RSD%

**Table 4.** Intraday and interday precision and accuracy in the assay ($n = 6$) at the LLOQ, low, medium and high concentration levels, defined within the range of expected concentrations

| | Concentration level | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Valeryl-4-hydroxy-valsartan | LLOQ | | 100 ng/mL | | 450 ng/mL | | 1000 ng/mL | |
| Precision (RSD%) | Intra | Inter | Intra | Inter | Intra | Inter | Intra | Inter |
| | 7.2 | 9.5 | 1.2 | 5.7 | 1.8 | 4.0 | 1.6 | 3.3 |
| Accuracy (ER%) | 1.6 | 4.4 | 3.3 | 4.0 | 2.4 | 3.7 | 7.3 | 9.1 |
| Valsartan | LLOQ | | 100 ng/mL | | 1000 ng/mL | | 3000 ng/mL | |
| Precision (RSD%) | Intra | Inter | Intra | Inter | Intra | Inter | Intra | Inter |
| | 1.6 | 8.0 | 3.2 | 7.0 | 0.8 | 6.0 | 0.3 | 3.3 |
| Accuracy (ER%) | 8.2 | 9.1 | 5.7 | 6.3 | 6.6 | 0.3 | 1.9 | 3.9 |

Inter, interday; intra, intraday.

**Table 5.** Means and SDs of the ratios between the initial conditions (control samples, QC) and repeated measurements ($n = 3$) carried out at three different concentration levels to study the stability for Valsartan and its metabolite

| Concentration level (ng/mL) | Valeryl-4-hydroxy-valsartan | | | Valsartan | | |
|---|---|---|---|---|---|---|
| | 100 | 450 | 1000 | 100 | 1000 | 3000 |
| QC samples | 0.124 ± 0.002 | 0.617 ± 0.011 | 1.499 ± 0.032 | 0.155 ± 0.006 | 1.404 ± 0.023 | 4.377 ± 0.027 |
| Freeze and thaw stability | | | | | | |
| 1 Cycle (24 h) | 0.122 ± 0.002 | 0.610 ± 0.008 | 1.592 ± 0.101 | 0.149 ± 0.002 | 1.364 ± 0.016 | 4.477 ± 0.084 |
| 2 Cycle (48 h) | 0.116 ± 0.001 | 0.577 ± 0.004 | 1.441 ± 0.054 | 0.157 ± 0.003 | 1.377 ± 0.008 | 4.364 ± 0.031 |
| 3 Cycle (96 h) | 0.128 ± 0.004 | 0.609 ± 0.001 | 1.515 ± 0.034 | 0.150 ± 0.001 | 1.325 ± 0.011 | 4.188 ± 0.038 |
| Long-term stability | | | | | | |
| 1 month | 0.112 ± 0.007 | 0.601 ± 0.021 | 1.471 ± 0.016 | 0.142 ± 0.015 | 1.320 ± 0.032 | 4.129 ± 0.021 |
| 2 months | 0.105 ± 0.013 | 0.609 ± 0.035 | 1.429 ± 0.054 | 0.136 ± 0.011 | 1.308 ± 0.056 | 4.067 ± 0.42 |
| Postpreparative stability (24 h in the auto sampler) | | | | | | |
| | 0.128 ± 0.003 | 0.611 ± 0.012 | 1.522 ± 0.022 | 0.158 ± 0.007 | 1.408 ± 0.034 | 4.462 ± 0.037 |
| Short-term stability (at room temperature) | | | | | | |
| QC samples | 0.138 ± 0.003 | 0.672 ± 0.023 | 1.522 ± 0.037 | 0.155 ± 0.004 | 1.577 ± 0.035 | 5.842 ± 0.097 |
| 8 h | 0.140 ± 0.003 | 0.670 ± 0.054 | 1.521 ± 0.029 | 0.154 ± 0.002 | 1.596 ± 0.028 | 5.777 ± 0.014 |
| 22 h | 0.143 ± 0.004 | 0.672 ± 0.008 | 1.503 ± 0.051 | 0.155 ± 0.002 | 1.594 ± 0.004 | 5.823 ± 0.034 |
| Stock solution stability | | | | | | |
| QC stock-solutions | 0.103 ± 0.001 | 0.492 ± 0.001 | 1.145 ± 0.001 | 0.150 ± 0.001 | 1.385 ± 0.002 | 4.535 ± 0.006 |
| 8 h | 0.103 ± 0.001 | 0.488 ± 0.002 | 1.154 ± 0.001 | 0.0152 ± 0.001 | 1.383 ± 0.003 | 4.561 ± 0.008 |
| 22 h | 0.104 ± 0.001 | 0.479 ± 0.003 | 1.143 ± 0.008 | 0.0149 ± 0.003 | 1.355 ± 0.004 | 4.502 ± 0.033 |

and RE%, respectively). A chromatogram corresponding to LLOQ is shown in Fig. 2.

### 3.1.4 Precision and accuracy

As it can be seen in Table 4, the obtained values for precision (RSD% <15%) and accuracy (RE% <15%) fulfilled the FDA proposed validation rules [28].

In conclusion, statistical evaluation of the results established good accuracy and precision of the method, whatever the concentration tested according to the validation guides mentioned [28, 34, 35].

### 3.1.5 Stability

For evaluating the short-term stability, QC plasma aliquots were thawed and kept at room temperature for 4–22 h, based on the expected duration of sample processing and analytical procedure at our laboratories.

For checking the long-term stability, QC spiked plasma aliquots were stored in the freezer at −20°C for 5–11 wk and then thawed (room temperature). Stability was evaluated over three freeze–thaw cycles (24–48–96 h).

The stock solution stability was evaluated at room temperature for 8–22 h. For the postpreparative stability, extracted samples were kept for 24 h in the auto sampler. In all the evaluated stability tests, the mean values

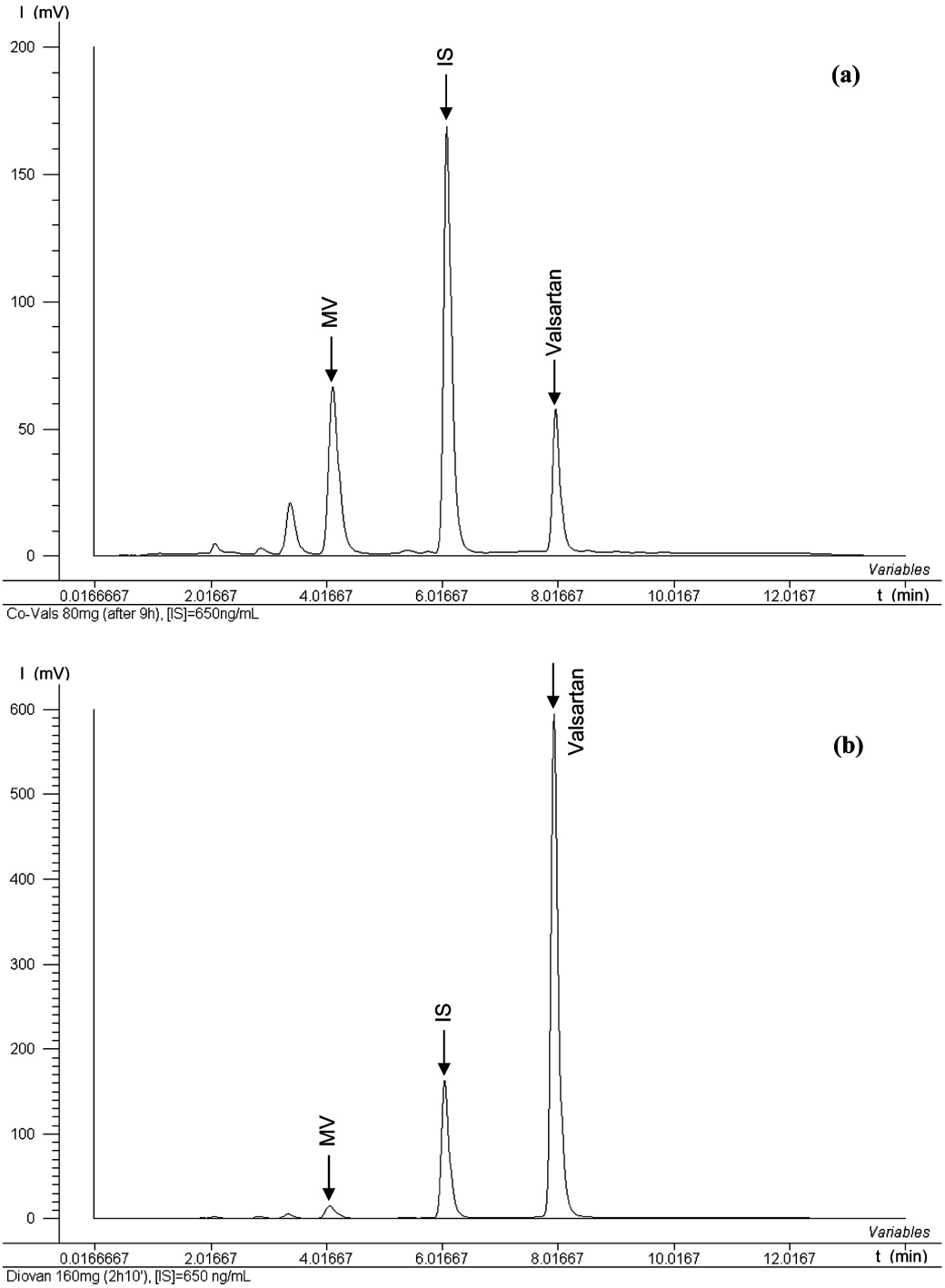



**Figure 3.** Chromatograms for extracts obtained from plasma samples collected from two different hypertensive patients at a different time interval after the intake of their cardiovascular medication: (a) Co-Vals-80 after 9 h; (b) Diovan-160 after 2 h 10 min. IS: candesartan M1 650 ng/mL.

obtained were compared with the initial ones, which were obtained immediately after the preparation of the stability control plasma samples (QC values).

The stability of valeryl-4-hydroxy-valsartan and its precursor was demonstrated by comparing the means and SDs of the measurements carried out at three different concentration levels at the initial conditions with those measurements carried out for the situations mentioned above.

**Table 6.** Quantitative values obtained for valeryl-4-hydroxy-valsartan and its precursor after SPE extraction of plasma samples obtained from 12 patients under antihypertensive treatment

| Patient | Age | Sex | Coadministrated drugs | Antihypertensive treatment (mg) | $t$ (h) | $C_{MV}$ (μg/mL) | $C_{vals}$ (μg/mL) |
|---|---|---|---|---|---|---|---|
| 1 | 79 | F | Simvastatin, ASA, Staticum, Hidrosaluretil, Glucobay 50 mg, Carduran Neo | Diovan 160 | 0.75 | 0.093 ± 0.004 | 0.261 ± 0.004 |
| 2 | 75 | F | Higrotona 1/2 | Diovan 80 | 0.5 | 0.132 ± 0.005 | 1.434 ± 0,029 |
| 3 | 74 | F | Pantecta 40 mg, Daonils, Zyloric | Co-Vals 160 | 24 | 0.228 ± 0.010 | 0.112 ± 0,004 |
| 4 | – | – | – | Vals 80 | 2.5 | 0.025 ± 0.001 | 0.129 ± 0,003 |
| 5 | – | – | – | Diovan 80 | 2.25 | 0.149 ± 0.002 | 1.092 ± 0,005 |
| 6 | – | – | – | Vals 160 | 2 | 0.031 ± 0.002 | 0.575 ± 0,007 |
| 7 | 75 | F | Pantecta 40 mg, Daonil 5 mg, Dianben 850 mg, Voltaren Retard 100 mg, Zyloric 300 mg | Co-Vals 80 | 9 | 0.110 ± 0.002 | 0.043 ± 0,001 |
| 8 | 67 | F | Prevencor 10 mg, Oculotect 50 mg/mL, Zomig Flas 2.5 mg, Blister, Terbasmin-Turbuhaler 0.5 mg | Co-Vals 80 | 9 | 0.380 ± 0.001 | 0.195 ± 0,002 |
| 9 | 60 | F | Higrotona 50 mg | Diovan 80 | 2.5 | 0.176 ± 0.004 | 0.459 ± 0,002 |
| 10 | 64 | F | Ideos 1250 mg, Evista 60 mg, Artedil | Diovan 160 | 2.2 | 0.085 ± 0.001 | 2.469 ± 0,009 |
| 11 | 71 | M | Beglan 0.025 mg | Vals 160 | 2.5 | 0.089 ± 0.002 | 0.560 ± 0,014 |
| 12 | 75 | F | Prevencor 20 mg, Emconor Cor 2.5 mg, Airtal 100 mg | Co-Vals 160 | 1.2 | 0.288 ± 0.003 | 4.744 ± 0,009 |

Calibration curves ($n$ = 10) range: 5 – 1250 and 5 – 4000 ng/mL respectively; IS concentration: 650 ng/mL.

The results are shown in Table 5. The concentrations did not change significantly, indicating no significant substance degradation in any of the tested conditions.

### 3.1.6 Application to real samples

The developed method was applied to the determination of Valsartan and its metabolite in plasma samples obtained from 12 different hypertensive patients under cardiovascular treatment with Valsartan and other coadministered drugs, such as diuretics, β-blockers, dihydropiridines, inhibitors of the angiotensin converting enzyme, statins and nonsteroid anti-inflammatories. Figure 3 shows the chromatograms corresponding to plasma samples collected from different hypertensive patients after the oral intake of ARA-II formulations and treated as reported in Section 2. Plasma concentration values (expressed as mean ± SD in (g/mL) found for clinical samples were obtained by interpolation from the daily calibration curves. Patients were under treatment with DIOVAN-80®, VALS-80®, Co-VALS-80®, DIOVAN-160®, VALS-160® and Co-VALS-160®. Concentration values are presented in Table 6. No interferences were observed at the analytes or ISs corresponding retention times.

As it can be observed from the data collected in Table 6, the metabolism of Valsartan presents a high interindividual variation; even though, the calculated values for its concentrations are in accordance with the reported pharmacokinetic data ($C_{max\,(Valsartan)}$ ~2 μg/mL (from 80 mg) at $t_{max}$ ~2 – 3 h) [30 – 33]. In spite of this, it would be advisable to apply this method to a wider range of hypertensive population to verify these data and carefully control sample collection time to measure the real peak plasma concentration.

## 4 Concluding remarks

The proposed method, based on the SPE-HPLC-fluorimetric-UV – Vis detection [25] is adequate for the quantitation of Valsartan and its metabolite in human plasma samples at different concentration levels, using candesartan M1 as an internal standard. In spite of the complexity of the plasma matrix, the validation of the assay is adequate in terms of the generally accepted guidelines for linearity, LOQ, stability, selectivity, precision and accuracy in the bioanalytical laboratory.

The SPE procedure, previously optimized in our laboratory [25], is very simple and effective providing extracts free from interferences arising from endogenous components. Thus, the method proved to be selective.

The method also proved to be sufficiently sensitive for the determination of these ARA-II compounds in plasma samples obtained from hypertensive patients under treatment with Valsartan, given that the obtained LOQ was 5 ng/mL for both Valsartan and valeryl-4-hydroxy-valsartan.

Since the extraction analysis time, including extraction step for 12 samples, can be carried out in 1 h, separation is accomplished in less than 10 min.

The use of this method can save efforts when monitoring patients under treatment with several drugs, which is very common in elderly patients. The selectivity of the fluorescence detector avoids interferences arising from

those compounds, which do not have native fluorescence. The obtained chromatographic peak shape and width were comparable with the peaks obtained for the standard solutions thus indicating the absence of interferences. However, to ensure the absence of interferences the use of LC-MS or GC-MS would be preferable.

The validity, LOQ and the linearity range of this method makes it an acceptable method for drug and metabolite monitorizing during 24 h after dose intake. This is necessary to ensure that antihypertensive plasma levels are kept in the therapeutic level during all the interdose range, in order to decrease the incidence of cardiovascular events.

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