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Original Paper

Optimization *via* experimental design of an SPE-HPLC-UV-fluorescence method for the determination of valsartan and its metabolite in human plasma samples

A chemometric approach was applied for the optimization of the extraction and separation of the antihypertensive drug valsartan and its metabolite valeryl-4-hydroxy-valsartan from human plasma samples. Due to the high number of experimental and response variables to be studied, fractional factorial design (FFD) and central composite design (CCD) were used to optimize the HPLC-UV-fluorescence method. First, the significant variables were chosen with the help of FFD; then, a CCD was run to obtain the optimal values for the significant variables. The measured responses were the corrected areas of the two analytes and the resolution between the chromatographic peaks. Separation of valsartan, its metabolite valeryl-4-hydroxy-valsartan and candesartan M1, used as internal standard, was made using an Atlantis dC18 100 mm × 3.9 mm id, 100 Å, 3 µm chromatographic column. The mobile phase was run in gradient elution mode and consisted of ACN with 0.025% TFA and a 5 mM phosphate buffer with 0.025% TFA at pH 2.5. The initial percentage of ACN was 32% with a stepness of 4.5%/min to reach the 50%. A flow rate of 1.30 mL/min was applied throughout the chromatographic run, and the column temperature was kept to 40 ± 0.2°C. In the SPE procedure, experimental design was also used in order at achieve a maximum recovery percentage and extracts free from plasma interferences. The extraction procedure for spiked human plasma samples was carried out using C8 cartridges, phosphate buffer (pH 2, 60 mM) as conditioning agent, a washing step with methanol-phosphate buffer (40:60 v/v), a drying step of 8 min, and diethyl ether as eluent. The SPE-HPLC-UV-fluorescence method developed allowed the separation and quantitation of valsartan and its metabolite from human plasma samples with an adequate resolution and a total analysis time of 1 h.

Keywords: Experimental design / Human plasma / SPE-HPLC / Valeryl-4-hydroxy-valsartan / Valsartan

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1 Introduction

Valsartan ((*S*)-*N*-valeryl-*N*-[29-(1*H*-tetrazol-5-yl) biphenyl-4-yl)-methyl]-valine) is an orally active specific angiotensin II receptor antagonist (ARA II) used as antihypertensive drug [1 – 3]. The compound has been shown to inhibit angiotensin II induced vasoconstriction in preclinical species by reducing systolic and diastolic blood pressure at peak effect after a given dose in clinical patients [4]. ARA II are currently being administered for the treatment of hypertension and are safe and effective agents for the treatment of hypertension and heart failure, either alone, or together with diuretics [5, 6], angiotensin-converting enzyme (ACE) inhibitors [7 – 14], beta-blockers [15 – 17], or calcium channel blockers [18 – 19]. Because of this, they have been proposed as an alternative to the more traditional ACE inhibitors [20].

Valsartan is affected by first-pass metabolism. The Valsartan parent compound is rapidly converted (20% of the initial dose) in its main metabolite valeryl-4-hydroxy-val-

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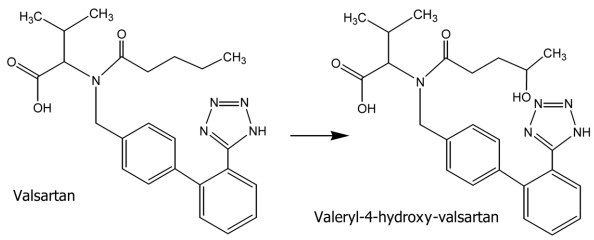
Abbreviations: ARA II, angiotensin II receptor antagonist; **CCD,** central composite design; **FFD,** fractional factorial design; **MLR,** multiple linear regression; **OVAT,** one variable at a time; **PLS-2,** partial least squares regression model



Scheme 1.

sartan *via* oxidation of the C4 of the pentamide function [21] as shown in Scheme 1.

Due to this metabolism, we will find valsartan and its metabolite in human plasma. Therefore, it was desirable to develop a simple and fast procedure that could be applied to the simultaneous determination of both compounds.

The existence of several fluorescent and chromophore functional groups in the molecular structure of the ARA II compounds such as biphenyl tetrazole, imidazole, and benzimidazole [22, 23] makes possible the development of photometric and fluorimetric methods for the determination of these compounds in biological fluids.

The determination methods reported for valsartan (alone, simultaneously with other ARA II or in combination with diuretics, usually with hydrochlorothiazide HCTZ) in biological fluids are mainly HPLC methods with photometric [24, 25] and fluorimetric [1, 26 – 35] detection. GC coupled with MS [36] and fluorimetry [37] have also been reported. No studies regarding simultaneous determination of valsartan and its metabolite have been found. Only Waldmeier *et al.* [21] have examined the pharmacokinetics and the pharmacodynamics of both compounds.

Today, it is generally accepted that the use of experimental design affords the most convenient way to deal with the optimization of processes since the traditional step-by-step approach involves a large number of independent runs. However, using chemometrical approaches, each parameter can be examined and optimized in a predefined range by constructing a series of experiments where the values for several parameters are changed at the same time [38 – 43].

Several chemometric approaches have been applied to optimize chromatographic and electrophoretic systems for the determination of drugs in biological fluids [44 – 47].

In this paper, the plasma sample treatment procedure as well as the chromatographic method for the simultaneous determination of the antihypertensive drug valsartan and its metabolite in human plasma samples have been optimized by means of experimental design.

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 were treated with the aid of the software Millenium 32 Chromatography Manager from Waters.

A Waters Atlantis dC18, 100 mm × 3.9 mm id, 100 Å, 3 µm column was used to perform the separation. It was thermostated at 40 ± 0.2°C in a column oven controlled by a Waters Temperature Control Module. Previous to the analytical column, a 10 µm Waters µBondapak C18 guard column was placed to prevent column degradation.

pH of solutions was 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 electrolyte KCl 3 M saturated AgCl.

Plasma samples were centrifuged at constant temperature (4°C) in an Eppendorf (Hamburg, Germany) model 5804R centrifuge, previous 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). Extracted plasma samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain).

The data analysis of the results (chemometric approaches, study of the regression models, *etc.*) was performed using The Unscrambler software v9.2 (Oslo, Norway; www.camo.com).

2.2 Materials and reagents

Valsartan ((*S*)-*N*-valeryl-*N*-[29-(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). 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 was provided by Astra Zeneca R&D (Möndal, Sweden).

Different HPLC-grade solvents were used: ACN and methanol were obtained from Scharlab (Barcelona, Spain); THF, chloroform, *n*-hexane, ethyl acetate, and diethyl ether were supplied by Carlo Erba (Milan, Italy); and 2-propanol was obtained from Panreac (Barcelona, Spain). Dichloromethane, analytical grade quality, was purchased from Panreac. Sodium dihydrogen phosphate,

sodium acetate, and trisodium citrate were supplied by Merck (Darmstadt, Germany) and Sigma (St. Louis, USA) and were of pro-analysis quality. Reagent grade TFA, phosphoric acid, and acetic acid were purchased from Carlo Erba.

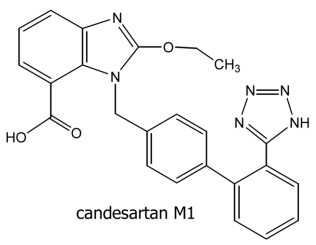
Purified water from a Milli-Q Element A10 Millipore water system was used in the preparation of the following buffer solutions: H_3PO_4/KH_2PO_4 (pH 2 and 3), CH_3COOH/CH_3COONa (pH 4 and 5), citric acid/citrate (pH = 6), KH_2PO_4/K_2HPO_4 (pH 7 and 8). The pH was adjusted to the desired value by using volumes of 1 M HCl and KOH solutions.

The clean-up procedure was performed using SPE cartridges: C2, C8, C18, CH, Ph, CN (100 mg bed packing, 1 mL volume capacity) from Varian (Harbour City, CA, USA). Extracted plasma samples were evaporated to dryness under a nitrogen stream, and their filtration was performed with polyvinylidene fluoride (PVDF) (∅ = 13 mm), hydrophobic and hydrophilic PTFE (∅ = 4, 13, and 13 mm, respectively), and nylon filters (∅ = 4 and 13 mm) obtained from Millipore; hydrophobic PTFE (∅ = 13 mm), nylon (∅ = 4 and 13 mm), and hydrophilic polypropylene (∅ = 13 mm) filters supplied by PALL (Ann Arbor, MD, USA); glass fiber filters (∅ = 13 mm) purchased from Schleicher&Schüll (Dassel, Germany); and hydrophilic cellulose filters (∅ = 4 mm) obtained from Sartorius (Goettingen, Germany).

2.3 Preparation of standard solutions and spiked plasma samples

Stock solutions (107 and 186 μg/mL) of valsartan and its metabolite valeryl-4-hydroxy-valsartan were prepared in 100% methanol by weight. A 99.26 μg/mL stock solution of candesartan M1 (Scheme 2) used as internal standard, was also prepared in 100% methanol. These solutions were diluted with methanol to produce 20 μg/mL working solutions. All solutions were stored at 8°C, protected from light.

For the chromatographic optimization, a 1 μg/mL solution of each drug was prepared. For SPE studies, blank plasma samples were daily spiked to a final concentration of 1.1 μg/mL in valsartan and in its metabolite and 1.2 μg/mL in candesartan M1.



candesartan M1

Scheme 2.

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

2.4 Chromatographic conditions

The mobile phase consisted of a mixture of ACN with 0.025% TFA and phosphate buffer (5 mM, pH = 2.5) with 0.025% TFA and was delivered in gradient mode at a flow rate of 1.30 mL/min. The gradient used is shown in 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 234 nm using a photometric detector.

2.5 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.

Venous blood samples were extracted from hypertensive patients under treatment with valsartan; they were immediately transferred into heparinized 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.

Only a single sample was obtained from each patient at a peak time interval (around 2 h after the intake, time interval which is supposed to include maximum plasma concentration levels for valsartan and a considerable amount of its metabolite).

2.6 Extraction procedure for plasma samples

One milliliter aliquots of blank plasma samples were spiked with the working solutions of valsartan, valeryl-4-hydroxy-valsartan, and IS to achieve 1.1 μg/mL concentration for both analytes and 1.2 μg/mL for the IS; 1 mL of 0.5 M phosphoric acid was added to all samples, followed by brief vortex mixing and centrifuged for 5 min at

10 000 rpm in a high speed centrifuge 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 were manually applied to the cartridges and washed with 1 mL methanol–phosphate buffer solution (40:60 v/v), followed by an 8 min drying period at high vacuum. Before elution, 0.2 mL of 10% v/v ethyleneglycol solution in methanol was added to the test tubes to prevent adsorption processes of these ARA II [28, 48]. Finally, the compounds were eluted with 0.5 mL of diethyl ether.

The eluent was evaporated to dryness under a nitrogen stream at 60°C. The 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. Twenty microliter aliquots were injected onto the HPLC system for analysis.

2.7 Efficiency and repeatability of the extraction procedure

The repeatability and efficiency of the extraction procedure were determined by extracting replicates of spiked plasma samples (n = 6).

The extraction efficiency, expressed in terms of recovery percentage, was estimated by comparing the chromatographic peak areas obtained for the compounds in spiked plasma samples with those of the reference samples. To prepare these reference samples, blank plasma samples were extracted and previous to the evaporation step, spiked with the same drug concentration levels. Corrected peak areas were considered for all calculations (peak area ratios of valsartan and its metabolite against internal standard).

The repeatability of the extraction was expressed as percentage of RSD.

2.8 Selectivity of the extraction procedure

Selectivity of the assay was established with six independent sources of blank plasma samples by comparing the chromatograms obtained from these plasma samples without and with the addition of low concentrations of the studied compounds (150 ng/mL for valsartan and its metabolite and 650 ng/mL for the IS). The chromatograms were visually inspected for possible interfering chromatographic peaks from plasma endogenous substances.

3 Results and discussion

3.1 Optimization of the chromatographic separation

The large number of variables to be considered in the HPLC separation would imply an extremely complicated experimental design. Therefore, in order to reduce the number of experiments, some of these variables were studied by means of traditional methodology "one variable at a time" (OVAT) and were fixed prior to the use of experimental design. These variables were the analytical column, the organic modifier in the mobile phase, the elution mode, the internal standard, and the detection wavelengths (excitation and emission).

In a second step, two different experimental designs were applied: a fractional factorial design (FFD) to evaluate which of the considered variables were significant factors, and a central composite design (CCD) to optimize these factors in the previously selected experimental domain. To define this domain, the experiments using OVAT methodology were taken into account.

For all the experiments carried out during the chromatographic optimization, a 1 μg/mL standard solution was prepared, containing valsartan, its metabolite and candesartan M1 (IS).

3.1.1 OVAT methodology

In order to choose the optimal detection wavelengths, several excitation and emission spectra were collected for valsartan, its metabolite and the internal standard at different pH values. All compounds were fluorescent only at pH values lower than 5.0, thus below their pK_a values: 4.90 ± 0.09 for valsartan and 3.9 ± 0.1 for candesartan M1 [49]. Excitation and emission at 254 and 378 nm, respectively, were chosen as wavelengths since they provided the maximum relative fluorescent intensity; 254 nm was also chosen for the photometric detection.

The performance of seven analytical columns was checked by running different mobile phases, containing methanol, ACN, or mixtures of both organic modifiers in isocratic mode. The analytical columns tested were: μBondapak C18 300 mm × 3.9 mm id, 125 Å, 10 μm; Supelcolsil ABZ+Plus 250 mm × 4.6 mm id, 100 Å, 5 μm; Nova-Pak Silica 150 mm × 3.9 mm id, 60 Å, 4 μm; Nova-Pak CN HP 150 mm × 3.9 mm id, 60 Å, 4 μm; Atlantis dC18 100 mm × 3.9 mm id, 100 Å, 3 μm; Nova-Pak C18 150 mm × 3.9 mm id, 60 Å, 4 μm and Nova-Pak C8 150 mm × 3.9 mm id, 60 Å, 4 μm. The organic modifier percentages ranged from 20 to 50% and the pH from 2 to 4. The flow rate and the temperature of the column were fixed at 1 mL/min and 30°C.

ACN was chosen as organic modifier since it provided sharper and higher chromatographic peaks than the ones obtained by using methanol or mixtures of both. By using ACN, we obtained better resolution. The analytical column which offered the best chromatographic peak shapes and resolution, using ACN as organic modifier, was Atlantis dC18.

Since the metabolite exhibited a slight peak tail in all chromatographic conditions, a TFA percentage was added to the mobile phase in order to optimize the peak shapes [21, 50].

The use of an isocratic elution mode did not allow the optimum resolution between the studied drugs: IS and plasma endogenous compounds. Due to this fact, a gradient elution mode was used. Nevertheless, the isocratic conditions tested were of great help in setting the gradient elution conditions.

The last step in the OVAT methodology was the selection of the internal standard. For quantitative determination of any drug belonging to the ARA II family, any other compound from the same family is normally used as the internal standard. In this case, candesartan M1 was chosen as internal standard since the rest of the compounds tested either gave rise to chromatographic peaks which overlapped with the metabolite or eluted at too high retention times.

3.1.2 Screening phase: FFD

The experiments needed to complete a whole factorial design were reduced by introducing a confounding and running the so-called FFD. The variables considered in the FFD for valsartan and its metabolite were the flow rate (A), the temperature of the column (B), the pH of the mobile phase (C), the percentage of TFA in the mobile phase (D), the initial composition of mobile phase (E), and the stepness of ACN during the gradient elution mode (F). The defined response variables were the corrected area (analyte area/internal standard area) and the resolution between the chromatographic peaks (R_s). A minimum resolution of 1 was considered taking into account the quantitative aim of this work. Two responses were defined as $R_{s(IS-M1)}$, $R_{s(Vals-IS)}$ as:

$$R_2 = 2\frac{(t_2 - t_1)}{(w_2 + w_1)} \quad (1)$$

where t_1 and t_2 represent retention times of peaks 1 and 2 and w_1 and w_2 represent widths of peaks 1 and 2, respectively.

The FFD is able to identify the influence of each parameter as well as first-order interactions between factors. FFD involves 2^{k-p} experiments, where k is the number of factors studied and p accounts for the degree of fractionality of the FFD ($p < k$) [41].

In this case, in order to perform a lower number of experiments (2^{6-2}), two factors were confused with the other factors as follows:

ACN% at the beginning of the gradient
(E) = flow (A) × temperature (B) × pH (C)

Stepness (F) = temperature (B) × pH (C) × TFA%
in the mobile phase (D)

A two level FFD involving 16 runs was carried out. All experiments were performed in random order to avoid systematic errors. Furthermore, two replicates of the central point were run corresponding to the center of the experimental domain to test the model linearity and to obtain an estimation of experimental variance. Matrix for the experimental domain in which every factor was evaluated in the FFD is shown in Table 2.

Limit values for the flow rate of the mobile phase were set to the usual working parameters. In order to avoid damage to the column, the highest value for the temperature of the column was fixed at 50°C. Based on the fluorescent properties of valsartan, its metabolite and the internal standard, pH values were fixed below 5 and above 2 in order to prevent column degradation. TFA percentages added to organic and aqueous phases were similar to those found in [21, 50].

As mentioned before, the use of gradient elution mode was necessary to perform the optimization. In order to simplify the number of variables corresponding to the gradient, the duration of gradient stepness was fixed to 4 min. The initial composition of organic modifier percentage, one of the theoretically most influential factors, was varied in order to get stronger or weaker mobile phases. Some experiments previously carried out aided to fix the interval between 20 and 40% of ACN. The linear stepness was varied from 1 to 6 ACN%/min.

The data analysis of the results was performed using The Unscrambler program. This program defines the responses as a function of the considered variables (x_i) by using analysis of variance. The treatment of these data and the analysis of variance gave rise to the results shown in Table 3, in which the significant variables and interactions are qualitatively collected, considering a significance level of 95% (if p value <0.05, the effect is regarded to be significant).

According to the results obtained from this model, only the initial percentage of the organic modifier in the mobile phase and the interaction between the ACN percentage and the gradient stepness were significant and affected the valsartan's corrected area, while no variable affected the metabolite's corrected area. Resolution between chromatographic peaks was significantly affected by most of the considered variables.

Table 2. Variables (A, B, C, D, E, and F) and responses (corrected areas and resolutions) from the 2^{6-2} proposed FFD for the optimization of the chromatographic system HPLC-UV-fluorescence for the separation of valsartan, valeryl-4-hydroxy-valsartan and the internal standard candesartan M1 in standard solutions

Experiment	Flow (mL/min) (A)	T (°C) (B)	pH (C)	%TFA (D)	%ACN (E)	Stepness (during 4 min) (F)	Corrected area Vals-M1	Corrected area Vals	$R_{s(IS\text{-}Vals.M1)}$	$R_{s(Vals\text{-}IS)}$
1	0.8	30	2.00	0	20	1	1.24	8.71×10^{-3}	8.00	12.00
2	1.3	30	2.00	0	40	1	1.26	1.69	1.20	4.11
3	0.8	50	2.00	0	40	6	1.07	1.48	1.15	3.12
4	1.3	50	2.00	0	20	6	1.16	1.56	1.22	2.94
5	0.8	30	4.00	0	40	6	1.31	1.68	1.06	2.84
6	1.3	30	4.00	0	20	6	1.29	1.47	1.56	2.02
7	0.8	50	4.00	0	20	1	1.91	2.25	2.92	6.39
8	1.3	50	4.00	0	40	1	1.33	1.69	0.96	2.57
9	0.8	30	2.00	0.050	20	6	1.08	1.43	1.09	3.62
10	1.3	30	2.00	0.050	40	6	1.20	1.60	1.07	3.39
11	0.8	50	2.00	0.050	40	1	1.22	1.62	1.09	3.97
12	1.3	50	2.00	0.050	20	1	1.28	2.79×10^{-2}	6.00	10.00
13	0.8	30	4.00	0.050	40	1	1.13	1.49	1.84	3.68
14	1.3	30	4.00	0.050	20	1	1.27	1.80×10^{-2}	7.00	11.00
15	0.8	50	4.00	0.050	20	6	0.99	1.32	1.69	2.57
16	1.3	50	4.00	0.050	40	6	1.10	1.45	1.62	2.78
17	1.05	40	3.00	0.025	30	3.5	1.10	1.44	2.17	3.21
18	1.05	40	3.00	0.025	30	3.5	1.18	1.54	2.09	3.27

Table 3. Significance of the variables over the defined responses by using analysis of variance (considering a significance level of 95%) in the 2^{6-2} proposed FFD for the optimization of the chromatographic system HPLC-UV-fluorescence to separate valsartan, valeryl-4-hydroxy-valsartan, and IS in stock solutions

Effects overview significance testing method: Center Variable	Corrected area Vals-M1	Corrected area Valsartan	$R_{s(IS\text{-}Vals\text{-}M1)}$	$R_{s(Vals\text{-}IS)}$
Flow (A)	NS	NS	NS	NS
Temperature (B)	NS	NS	−	−
pH (C)	NS	NS	NS	−
%TFA (D)	NS	NS	+	+
%ACN (E)	NS	+	−	−
Gradient stepness (F)	NS	NS	−	−
AB = CE	NS	NS	+	+
AC = BE	NS	NS	+	+
AD = EF	NS	−	++	++
AE = BC = DF	NS	NS	NS	NS
AF = DE	NS	NS	NS	-
BD = CF	NS	NS	+	+
BF = CD	NS	NS	+	+

(NS): nonsignificant; (+): positive influence; (−): negative influence; (++/−): grade of influence.

It was decided to run a more complex experimental design to exhaustively set the influence of these factors on experimental response (CCD). For this purpose, it was necessary to reduce the number of factors to be considered [38]. Since the column temperature and the TFA% showed a negligible effect on the shape of chromatographic peaks, we decided to fix these two factors in the 0 level used in the FFD (T = 40°C, TFA% = 0.025%), thus simplifying the experimental design.

3.1.3 Optimization phase: CCD

A CCD was built using the remaining four variables: flow rate of the mobile phase (A), pH value (B), initial ACN% (C), and gradient stepness (D).

A CCD consists of a full factorial design plus an orthogonal star design. This CCD involved 24 runs plus three replicates of the central point. The design matrix for the experiments is given in Table 4. The high number of runs together with the time needed for experimental procedure made it impossible to carry out a whole sequence in the same day, so it was decided to divide the experiments into 3 days.

Fortunately, the CCD consists of two main sets of experiments: cube and star samples. These two groups have the mathematical property of contributing to the estimation of a quadratic model independently from each other. As a consequence, if some of the experimental conditions vary slightly between the first group and the second one, it will not change the computed effects [38]. As a result of the long sequence, cube experiments were performed on the first day; star experiments on the second day; and on the third day, those experiments that by previous tests were anticipated incoherent or too long. All experiments were performed in random order, and every day a replicate of the central point was carried out.

Table 4. Variables (A, B, C, and D) and responses (corrected areas, resolutions, and retention times) obtained from the CCD used for optimization of the chromatographic system HPLC-UV-fluorescence to separate valsartan, valeryl-4-hydroxy-valsartan, and IS in standard solutions

Experiment	Flow (mL/min) (A)	pH (B)	%ACN (C)	Stepness (during 4 min) (D)	Corrected area Vals-M1	Corrected area Vals	$R_{s(IS\text{-}Vals.M1)}$	$R_{s(Vals\text{-}IS)}$	$t_{r(MIN)}$ (min)	$t_{r(MAX)}$ (min)
1.*L:A-a	0.675	3.00	30.0	3.750	1.02	1.63	2.37	3.46	9.08	15.72
2.*H:A-a	1.425	3.00	30.0	3.750	1.05	1.68	2.74	3.20	4.80	8.86
3.*L:B-a	1.050	1.50	30.0	3.750	0.47	1.43	0.31	6.08	6.23	11.23
4.*H:B-a	1.050	4.50	30.0	3.750	0.86	1.64	3.44	3.67	5.01	10.79
5.*L:C-a	1.050	3.00	15.0	3.750	1.12	1.72	4.65	7.94	13.52	45.99
6.*H:C-a	1.050	3.00	45.0	3.750	0.97	1.62	1.48	2.54	2.14	5.26
7.*L:D-a	1.050	3.00	30.0	$2.9802 \cdot 10^{-7} \approx 0$	1.14	1.79	4.24	7.62	6.93	39.23
8.*H:D-a	1.050	3.00	30.0	7.500	1.02	1.61	2.17	2.14	5.92	8.43
9. Cube001a	0.800	2.00	20.0	1.250	1.48	1.95	4.33	14.19	25.67	161.00
10.Cube002a	1.300	2.00	20.0	1.250	1.44	1.88	4.01	14.07	16.61	97.25
11.Cube003a	0.800	4.00	20.0	1.250	1.28	1.77	8.12	11.68	23.49	140.65
12.Cube004a	1.300	4.00	20.0	1.250	1.25	1.67	7.28	11.67	14.94	86.90
13.Cube005a	0.800	2.00	40.0	1.250	1.20	1.89	1.42	5.41	3.54	10.06
14.Cube006a	1.300	2.00	40.0	1.250	1.16	1.86	1.30	4.82	2.24	6.58
15.Cube007a	0.800	4.00	40.0	1.250	1.06	1.64	2.20	4.22	3.57	10.15
16.Cube008a	1.300	4.00	40.0	1.250	1.04	1.65	2.05	3.98	2.24	6.58
17.Cube009a	0.800	2.00	20.0	6.250	1.13	1.82	1.39	4.17	10.21	15.10
18.Cube010a	1.300	2.00	20.0	6.250	1.15	1.84	1.35	3.65	7.21	10.37
19.Cube011a	0.800	4.00	20.0	6.250	1.01	1.62	1.94	2.86	10.05	14.89
20.Cube012a	1.300	4.00	20.0	6.250	1.04	1.67	2.09	2.88	7.17	10.35
21.Cube013a	0.800	2.00	40.0	6.250	1.16	1.84	1.32	4.06	3.54	8.28
22.Cube014a	1.300	2.00	40.0	6.250	1.17	1.89	1.41	4.29	2.28	5.65
23.Cube015a	0.800	4.00	40.0	6.250	1.04	1.62	2.41	2.90	3.59	8.36
24.Cube016a	1.300	4.00	40.0	6.250	1.06	1.69	2.77	3.95	2.23	5.60
25.Cent-a	1.050	3.00	30.0	3.750	1.03	1.63	2.57	3.44	6.20	11.05
26.Cent-b	1.050	3.00	30.0	3.750	1.03	1.64	2.56	3.34	6.29	11.17
27.Cent-c	1.050	3.00	30.0	3.750	1.03	1.64	2.60	3.45	6.28	11.13

With the aim to delimit the analysis time in which the separation should be achieved, it was advisable to add two other variables to the previously defined response variables (corrected areas and resolution between the chromatographic peaks): minimum and maximum analysis time. Thus, in order to get the equations for these two new responses, the retention times from all the first and last chromatographic peaks of the experiments run in the CCD were taken into account.

Upon the basis of the obtained responses, The Unscrambler defined a multiple linear regression MLR model for each response. The analysis of variance applied to these MLR models showed lack of fit of experimental data, and therefore a new model was built. Due to the complexity of the system (four variables and six responses), the multivariate data analysis computer program defined a partial least squares regression model (PLS-2).

As a result, the fit to the regression (percentage of variance explained) improved with PLS-2, as shown in Table 5.

In order to study the influence of the variables on the answers in PLS-2, the regression coefficients were studied. If a regression coefficient for a variable is larger than 0.2 in absolute value, the effect of the variable is most probably significant [38]. The regression coefficients which affected the two corrected areas were originated by pH (B), ACN% (C), and stepness (D) as variables and CD, BB, CC, and DD as interactions.

Thus, taking these influences into account, the response surface of each corrected area was analyzed (Fig. 1). Different maxima values were obtained and although all of them could be considered valid, only those providing retention time maxima below 10 min were taken into account.

In that way, satisfactory results were obtained for the chromatographic separation when the composition of the mobile phase was A : B (A: ACN + 0.025% TFA; B: 5 mM phosphate buffer pH = 2.5 + 0.025% TFA), and the gradient elution mode started at an initial percentage of ACN of 32% and reached 50% in 4 min. Then, this mobile phase was kept constant for 5 min. The flow rate was 1.30 mL/min during all the chromatographic runs. In these conditions (collected in Table 1), valeryl-4-hydroxy-valsartan, candesartan M1 and valsartan chromatographic peaks eluted in the isocratic elution zone, as it can be seen in Fig. 2.

Table 5. Treatment of the responses obtained from the CCD by two different multivariate calibration methods: MLR and PLS-2. Both models collect for each response the obtained regression coefficient and multiple correlation coefficients (MLR) as well as the error of model (root mean square of calibration, RMSEC)

Response	MLR		PLS-2 Y-Explained[a]:%71(1PC),%19 (2PC) (PC = 3)	
	Regression coefficient (R^2)	Multiple correlation coefficient	Regression coefficient (R^2)	RMSEC
Corrected area Vals.–M1	0.707	0.841	0.952	0.041
Corrected area Vals	0.635	0.797	0.950	0.033
$R_{s(IS\text{-}Vals.M1)}$	0.955	0.977	0.962	0.466
$R_{s(Vals\text{-}IS)}$	0.994	0.988	0.986	0.577
$t_{r(MIN)}$	0.950	0.975	0.971	1.486
$t_{r(MAX)}$	0.931	0.965	0.960	11.713

a) Results obtained after recalculation of the model. 9 and 11 experiments were not taken into account, since they were recognized as outliers.

The developed method has proved to be rugged under light variations in the composition of the mobile phase such as buffer concentration, pH, and percentage of TFA. Slight variations in the chromatographic conditions have not shown significant variances in the chromatograms obtained.

3.2 Extraction procedure: Experimental design

The most used extraction techniques for the determination of ARA II compounds in biological samples are liquid–liquid extraction (LLE) [51, 52] and SPE [28, 37, 52–55].

The optimization of the extraction procedure was carried out following the same experimental design methodology as the one used for the optimization of the chromatographic separation.

The variables studied were as follows: pretreatment of plasma samples, the extraction cartridge, the convenience of using surfactant, the pH of sample application, the elution liquid composition, the washing liquid, and the type of membrane used to filter the plasma extracts.

Taking into account that the SPE procedure is influenced by many parameters, but generally, the measured response is always one (the corrected area or the recovery), the OVAT optimization methodology together with the use of experimental design was decided. A four factor considering FFD enabled the selection of critical factors on response. Response surface modeling from the three-factor considering CCD design allowed the selection of the optimal analysis conditions for the clean-up procedure for valsartan and its metabolite in human plasma samples.

Throughout the SPE optimization, before the extraction process, all plasma samples were spiked with 1.1 μg/mL of valsartan metabolite and 1.1 μg/mL of valsartan. In order to avoid variances coming from the chromatographic method, once extracted all samples were spiked with 1.2 μg/mL of IS, previous to the evaporation process.

3.2.1 OVAT methodology

In order to define the SPE procedure, different RP cartridges (CH, C2, C8, C18, CN, and Ph) were tested. The highest recoveries were obtained by using CH, C18, and C8 cartridges. CH and C18 cartridges despite providing the highest recoveries did not give extracts sufficiently free of endogenous compounds, therefore C8 cartridges were used.

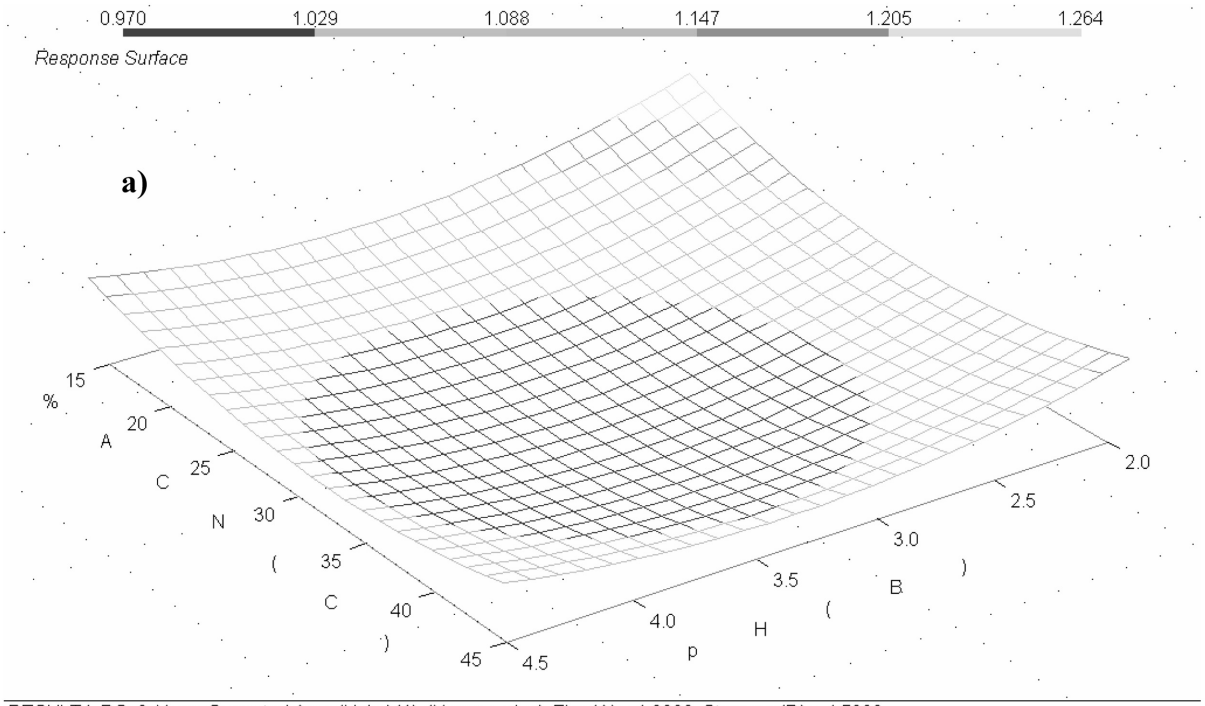
The effect of sample pH was clearly stated with all the solvents and packings. Samples were applied at different pH values, ranging from 2 to 5. In all cases, stronger ionization of the analytes at pH >5 induced important losses (recovery <50%) due to their weak interactions with no polar cartridges. In addition to this, these pH values were simultaneously tested in the protein precipitation as shown in Table 6. The recoveries were higher when proteins were precipitated with 0.5 M phosphoric acid.

Despite the slightly higher recoveries obtained when sample application was made at pH 4, pH 2 was used throughout the extraction procedure since cleaner plasma extracts were obtained together with acceptable recovery percentages (higher than 85%).

ACN provided the best recoveries of all the assayed elution solvents: methanol, ACN, chloroform, 2-propanol, THF, ethyl acetate, dichloromethane, diethyl ether, and *n*-hexane. Nevertheless, selectivity was preferred to extraction efficiency and diethyl ether showed a higher selectivity giving cleaner extracts.

In order to improve the recoveries obtained with diethyl ether, addition of low methanol percentages on the elution liquid was tested.

Different mixtures of methanol–phosphate buffer solutions (50 mM, pH 2) were tested as washing solutions



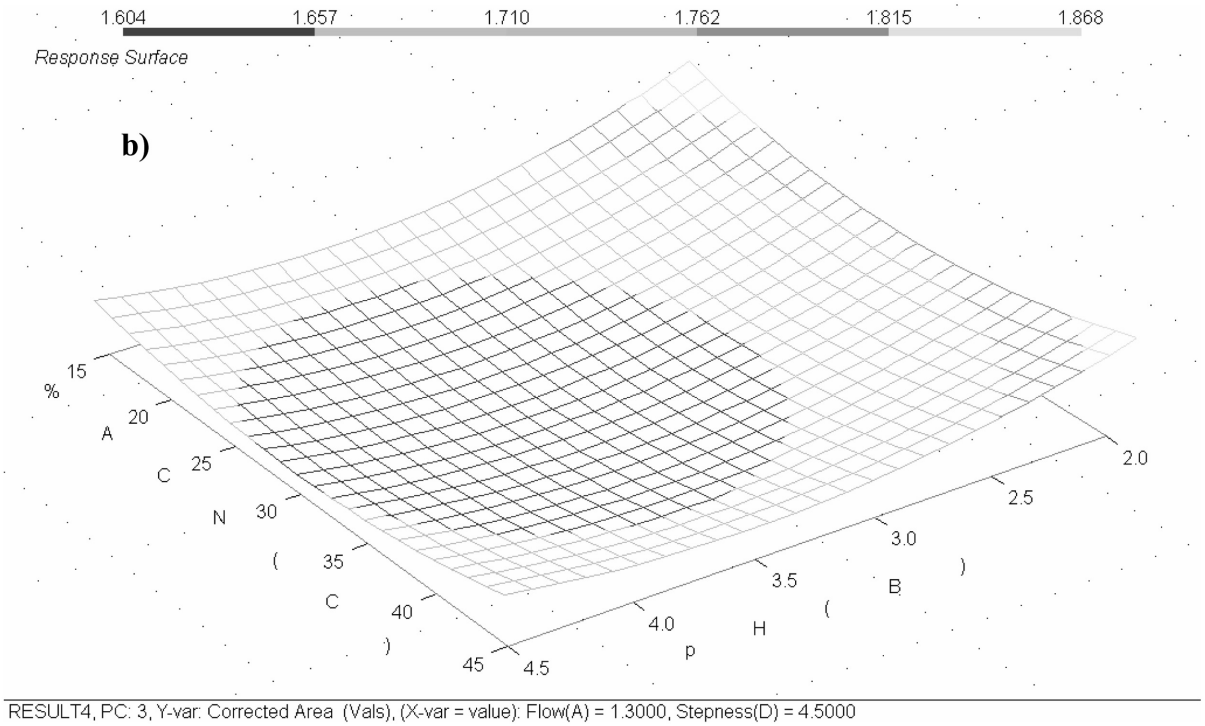
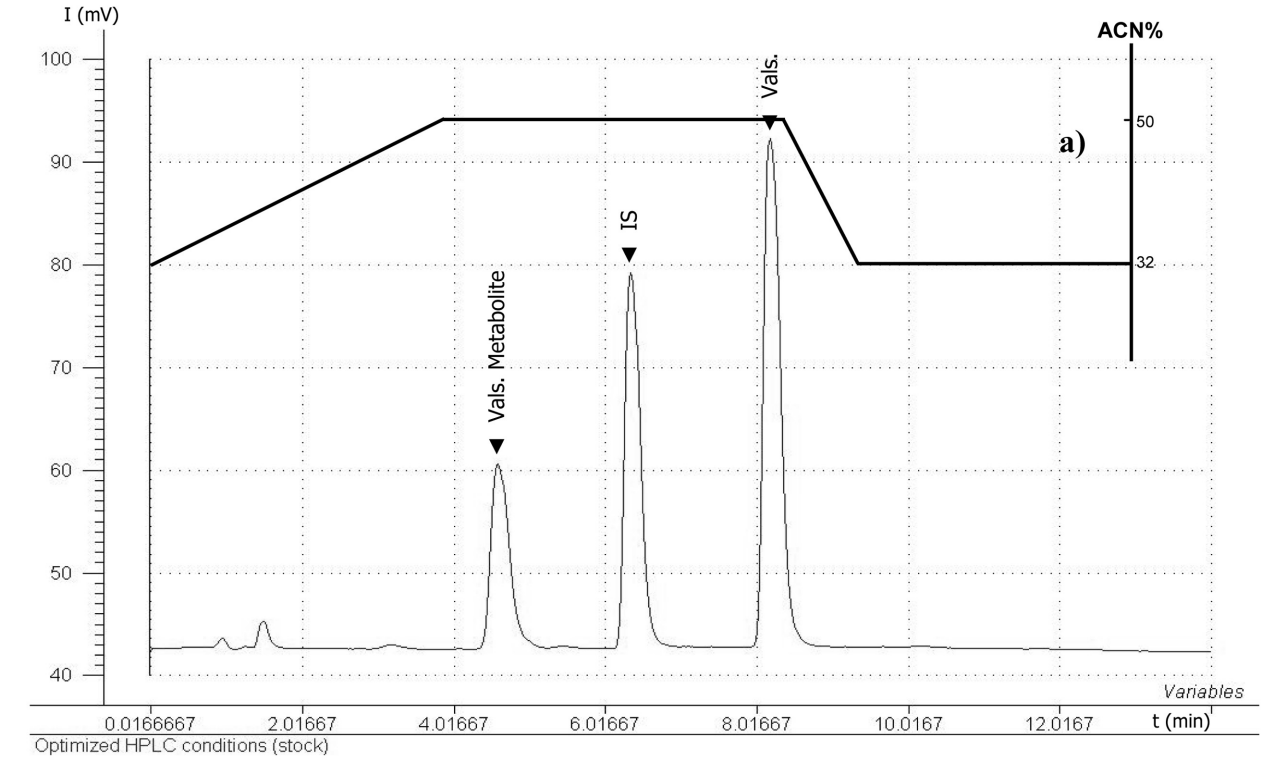


Figure 1. Response surfaces for the most important functions, obtained from CCD: (a) corrected area of valeryl-4-hydroxy-valsartan and (b) corrected area of valsartan. These two response surfaces are defined by the pH (B) and the initial composition of ACN (C), while the flow (A) and the gradient stepness (D) are fixed at 1.3 and 4.5 mL/min, respectively.



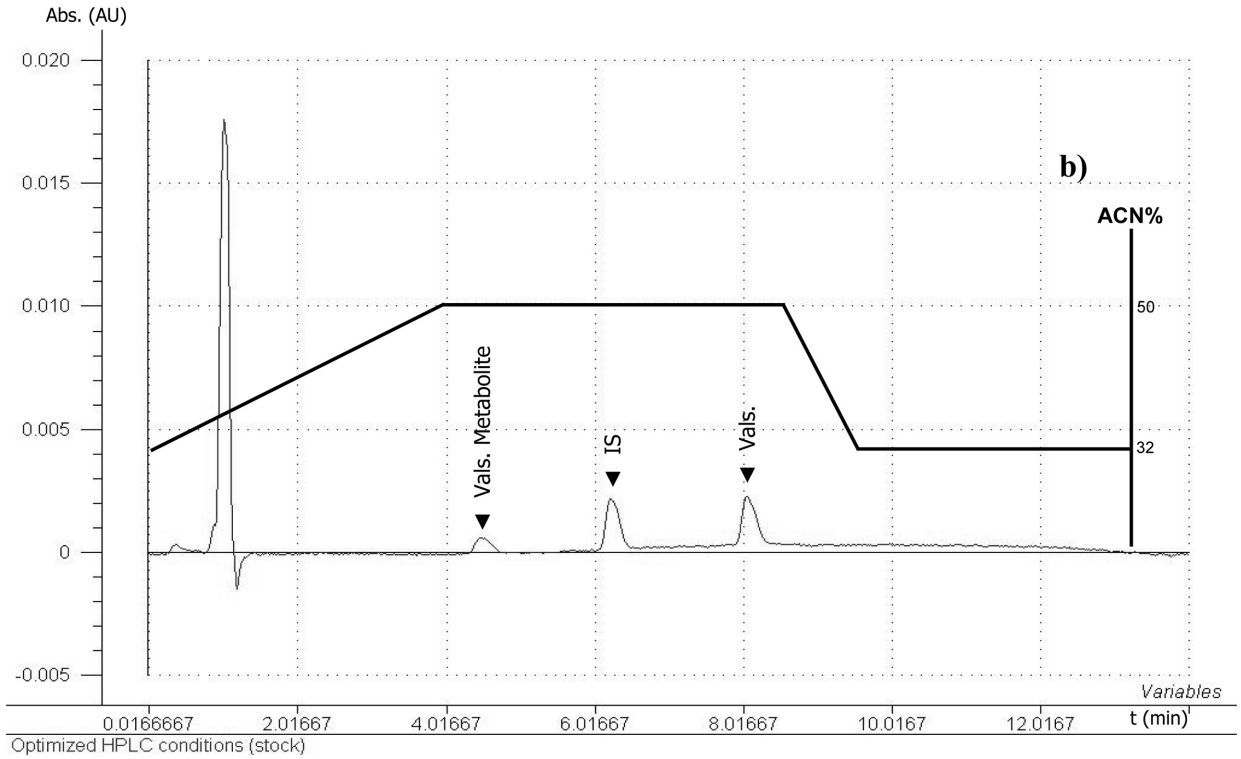


Figure 2. Chromatograms corresponding to a mixture of valeryl-4-hydroxy-valsartan (450 ng/mL), valsartan (1000 ng/mL), and candesartan M1 (IS, 650 ng/mL) prepared in methanol. (a) Fluorescence and (b) UV. Chromatographic conditions as described in Section 2.4.

Table 6. Effect of sample pH and protein precipitant pH on the recovery percentage of SPE

Protein precipitant pH	Cartridge conditioning pH	Recovery (%) Valsartan-M1	% RSD	Recovery (%) Valsartan	% RSD
1.2	2	86.27	3.12	90.40	1.36
2	2	55.52	1.02	40.25	7.56
1.2	3	88.33	11.81	85.69	11.15
3	3	58.30	0.80	40.00	0.02
1.2	4	96.92	0.37	93.20	2.87
4	4	53.19	7.23	27.92	13.35
1.2	5	8.17	26.30	79.56	3.20
5	5	1.01	21.23	9.08	9.02

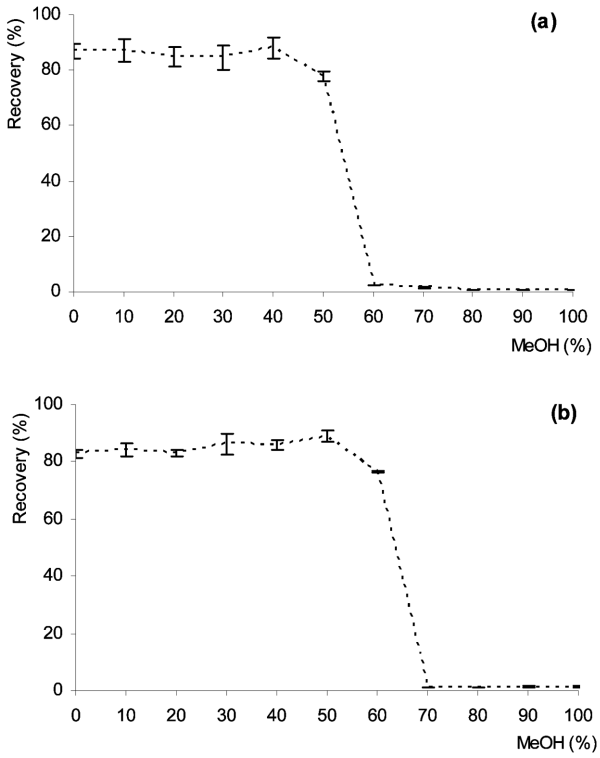


Figure 3. Elution profiles (methanol–phosphate buffer, 50 mM pH 2) for human plasma samples spiked with both analytes: (a) valeryl-4-hydroxy-valsartan and (b) valsartan.

(Fig. 3). The washing solution methanol–50 mM phosphate buffer solution, pH 2, 40:60 v/v, was chosen and it was valid for both analytes, yielding a chromatogram free of interferences.

The use of ethyleneglycol as surfactant to prevent adsorption of ARA II compounds (mainly candesartan cilexetyl) on the tube walls has been reported by several authors [28, 48]. In this work, the convenience of using 0.2 mL of 10% v/v ethyleneglycol in methanol as surfactant was studied. This surfactant induced slightly higher recoveries for the internal standard as well as for valsartan.

Filtering the sample constituted the last step in the sample treatment. An exhaustive study for the evaluation of the filter type to be used previous to injection on the chromatographic system was carried out. Different types of membranes (PVDF, hydrophobic and hydrophilic PTFE, nylon, hydrophilic cellulose, hydrophilic polypropylene, and glass fiber) with several diameters (∅ = 4 and 13 mm) were assayed obtaining the recoveries shown in Table 7.

Filters of diameter 4 mm were discarded due to saturation problems and sample losses. Hydrophobic and hydrophilic PTFE, hydrophilic polypropylene and nylon membranes provided acceptable recoveries. Universal hydrophilic polypropylene membrane was chosen based on the most favorable recovery/price relation.

3.2.2 Screening phase: FFD

The experimental variables considered in the FFD for the extraction of valsartan, its metabolite and the internal standard candesartan M1 from human plasma samples were as follows: buffer solution concentration (A), drying time (B), elution liquid volume, (C) and methanol percentage added to the diethyl ether elution solution (D). In this case, the main effect estimated for the D factor (methanol percentage added to the elution solution) was confused with the interaction effect for the other three variables as shown in the following relation:

MeOH% added to the elution liquid (D) =
C_{Buffer}(A) × drying time (B) × volume of elution liquid (C)

Since it was predictable that the D factor would not significantly affect the other variables. A two level FFD involving eight runs and two replicates of the central point was carried out. All experiments were performed in random order to avoid systematic errors. The proposed experiments for FFD are shown in Table 8.

In order to obtain an adequate extraction procedure, and considering that the chromatographic method had been previously optimized, the response variables measured were the corrected areas for both analytes.

The buffer solution concentration ranged from 5 to 50 mM according to the most commonly used values. The drying time was varied between 0 and 10 min, to avoid too long extraction times. The elution solvent volume varied between 0.5 and 2 mL. The methanol percentage added to the elution solvent varied from 0 to 40%.

As mentioned above, the analysis of the experimental results was accomplished by means of The Unscrambler program. Among other features, this program allows the linear regression analysis of data, including two factor

Table 7. Mean recoveries obtained with different types of membrane syringe filters (∅ = 13 mm)

Filter ∅ = 13 mm	Filtration area and pore size	Hold-up volume	Type of membrane	Recovery (%)			
				Vals-M1:	%RSD	Vals:	%RSD
Millipore: Ref: SLHV 013 NL	0.65 cm^2 0.45 μm	<25 μL	PVDF	64.64	7.38	48.80	8.27
Millipore: Ref: SLCR 013 NL	0.65 cm^2 0.45 μm	<25 μL	Hydrophilic PTFE	91.50	4.95	79.02	8.32
Millipore: Ref: SLFH R04 NL	0.65 cm^2 0.45μm	<25 μL	Hydrophobic PTFE	82.82	1.46	66.90	1.22
PALL: Ref: S4553.	1.00 cm^2 0.45 μm	<14 μL	Hydrophobic PTFE	86.35	2.84	66.30	8.43
Millipore: Ref: SLHN 013 NL	0.65 cm^2 0.45 μm	<25 μL	Nylon	79.63	9.07	69.72	2.06
PALL: Ref: S4551.	1.00 cm^2 0.45 μm	<14 μL	Nylon	86.71	8.35	46.85	8.12
Schleicher&Schuell: Ref: 463030	cm^2 0.45μm	<10 μL	Glass fiber	78.10	6.70	62.30	15.26
PALL: Ref: S4556.	1.00 cm^2 0.45 μm	<14 μL	Hydrophilic polypropylene	85.60	1.87	72.02	4.27

Table 8. Variables (A, B, C, and D) and responses (corrected areas) from the 2^{4-1} proposed FFD for the optimization of the SPE procedure of valsartan and valeryl-4-hydroxy-valsartan from human plasma

Exp	Buffer conc. (mM) (A)	Drying time (min) (B)	Elution sol. volume (mL) (C)	MeOH% added (D)	Corrected area Vals.M1	Corrected area Vals
1	5	0	0.5	0	0.80	0.63
2	50	0	0.5	40	0.85	0.66
3	5	10	0.5	40	0.92	0.76
4	50	10	0.5	0	0.84	0.73
5	5	0	2	40	0.82	0.62
6	50	0	2	0	0.88	0.70
7	5	10	2	0	0.90	0.74
8	50	10	2	40	0.90	0.74
9	27.5	5	1.25	20	0.89	0.75
10	27.5	5	1.25	20	0.91	0.74

Table 9. Significance of the variables over the defined responses by using analysis of variance (considering a significance level of 95%) in the 2^{4-2} proposed FFD for the optimization of the SPE procedure of valsartan, valeryl-4-hydroxy-valsartan from human plasma

Effects overview Significance testing method: center Variable	Corrected area Vals-M1	Corrected area Valsartan
Buffer conc. (A)	NS	+
Drying time (B)	NS	++
Elution sol. volume (C)	NS	NS
MeOH% added (D)	NS	NS
AB = CD	NS	−
AC = BD	NS	+
AD = BC	NS	NS

(NS): Nonsignificant; (+): positive influence; (−): negative influence; (++/−): grade of influence.

interactions, and gives the ANOVA of the regression model and the response surface. The treatment of these data and the analysis of variance gaves rise to the results collected in Table 9. The significant variables and interactions are qualitatively given considering a significance level of 95% (if p value <0.05, the effect is regarded to be significant).

These results allowed us to conclude that, apparently, no variable influenced the recovery of the extraction of valeryl-4-hydroxy-valsartan. Nevertheless, it was observed that buffer concentration, drying time, as well as certain interactions between variables affected the recovery of valsartan.

With the purpose of studying the influence of these factors more exhaustively, it was decided to run a CCD. In order to reduce the number of factors to consider in this design, it was decided to add no methanol to the elution solution, since it was observed that even small percentages of methanol induced the coelution of several interferent endogenous compounds.

3.2.3 Optimization phase: CCD

The information obtained from the FFD was used to build a CCD. The experimental variables considered in the CCD were as follows: buffer solution concentration (A), drying time (B), and elution liquid volume (C).

This CCD involved 14 runs plus four replicates of the central point, all of them randomized and carried out on the same day. The design matrix for the experiments is given in Table 10. The response variables were measured by the corrected areas for both analytes.

Table 10. Variables (A, B, and C) and responses (corrected areas) obtained for the CCD for the optimization of an SPE procedure of valsartan and valeryl-4-hydroxy-valsartan from human plasma

Experiment	Buffer conc. (mM) (A)	Drying time (min) (B)	Elution sol. volume (mL) (C)	Corrected area Vals.M1	Corrected area Vals
1.*L:A-a	4.98	11.25	1.45	0.81	0.80
2.*H:A-a	61.42	11.25	1.45	0.83	0.81
3. *L:B-a	33.20	11.25	0.53	0.73	0.70
4.*H:B-a	33.20	11.25	2.37	0.81	0.75
5.*L:C-a	33.20	4.95	1.45	0.82	0.78
6.*H:C-a	33.20	17.55	1.45	0.84	0.81
7.*L:D-a	16.40	7.50	0.90	0.85	0.80
8.*H:D-a	50.00	7.50	0.90	0.87	0.80
9.Cube001a	16.40	7.50	2.00	0.71	0.70
10.Cube002a	50.00	7.50	2.00	0.75	0.72
11.Cube003a	16.40	15.00	0.90	0.86	0.82
12.Cube004a	50.00	15.00	0.90	0.76	0.75
13.Cube005a	16.40	15.00	2.00	0.80	0.75
14.Cube006a	50.00	15.00	2.00	0.78	0.73
15.Cent-a	33.20	11.25	1.450	0.88	0.83
16.Cent-a	33.20	11.25	1.450	0.80	0.76
17.Cent-b	33.20	11.25	1.450	0.80	0.75
18.Cent-c	33.20	11.25	1.450	0.77	0.74

As it was done in the previous design, The Unscrambler program was used to study the regression models, including all the square and interaction terms in order to get the response surfaces for the two analytes.

On the basis of the obtained responses, The Unscrambler defined an MLR for both responses. According to this model, neither the variable nor the interaction was significant (p level <0.05, for a significance level of 95%). In contrast, the analysis of the regression models showed a significant lack of fit for the compounds (0.3085 and 0.4919 for the p values of lack of fit for valsartan's metabolite and valsartan). This meant that the models were not adequate to explain the experimental variance (lack of fit <0.05). The multivariate data analysis computer program defined a PLS-2 to improve the fit of results (Table 11).

In order to study the influence of the variables on the responses in PLS-2, the coefficients of regression were studied ($|0.2|<$ regression coefficient). The variable and interactions which affected the responses were as follows: drying time (B) and AC, BC, AA, and BB interactions.

The parameters which had influence on the response of each corrected area defined the response surfaces shown in Fig. 4. These response surfaces had two zones in which the response was maximum (Table 12). For similar retention times, in the first zone the response values rose when the maximum concentration (60 mM) and the minimum elution volume (0.5 mL) correlated. In the second one, the minimum concentration (5 mM) and the maximum elution volume (2 mL) correlated. Predictions and experimental responses for both zones and responses were similar. In this way, in order to reduce the analysis time and the amount of reagents used, the optimum conditions for the SPE were fixed as follows: buffer concentration of 60 mM, a washing solution volume of 0.5 mL, and 8 min drying time, avoiding a too long analysis time.

The recovery percentages for valeryl-4-hydroxy-valsartan and valsartan obtained after this optimized plasma sample clean-up procedure were calculated at three concentration levels low, medium, and high levels, according to the pharmacokinetics of each compound [1, 21, 27, 56, 57], and they are collected in Table 13. In Fig. 5, a chromatogram of a spiked plasma sample at a concentration of 650 ng/mL for both compounds in the optimized chromatographic and extraction conditions is shown.

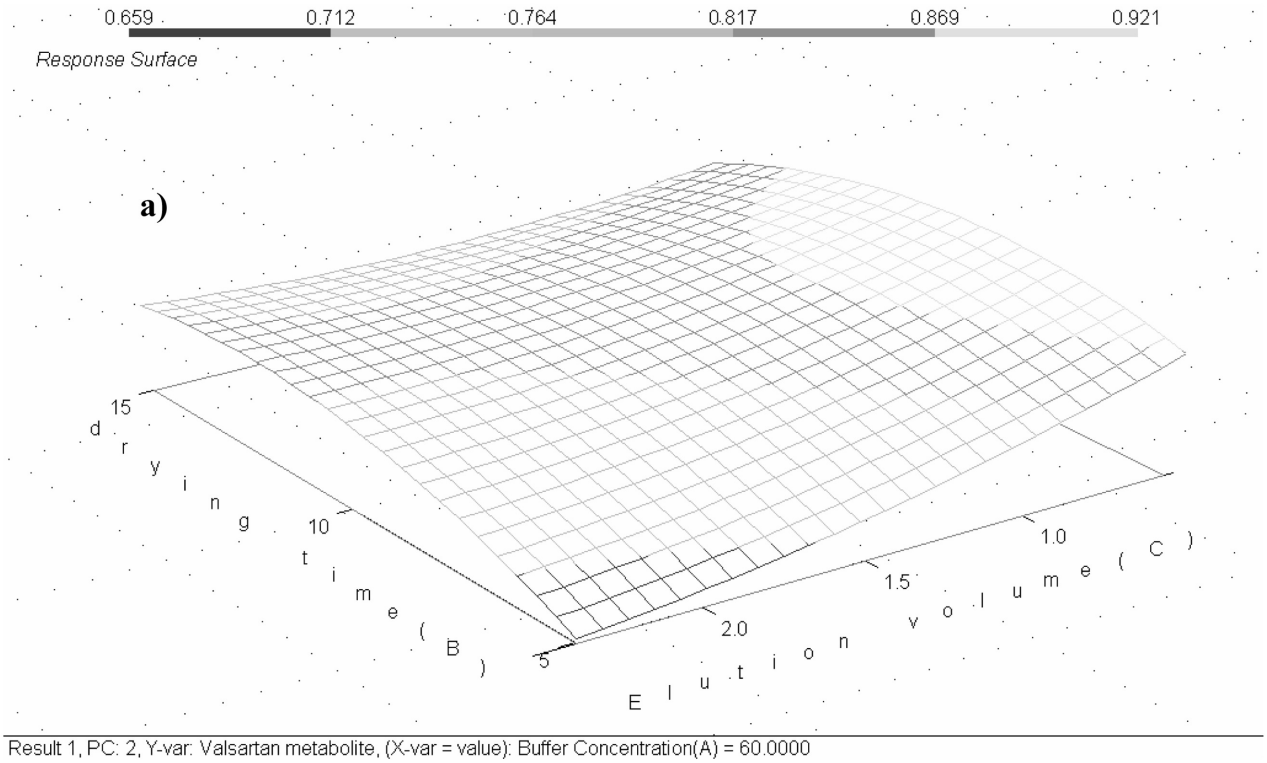
3.3 Analytical application

The SPE-HPLC-UV-fluorescence analytical method for the determination of valsartan and its metabolite valeryl-4-hydroxy-valsartan was optimized in spiked plasma samples. This method was applied to the plasma samples obtained from hypertensive patients. Figures 6, 7 show the chromatograms of a plasma sample obtained from patients treated with *Diovan*-80 mg and *Vals*-160 mg, respectively, after the addition of 650 ng/mL of internal

Table 11. Treatment of the responses obtained from the CCD by two different multivariate calibration methods: MLR and PLS-2. Both models collect for each response the obtained regression coefficient and multiple correlation coefficients (MLR) as well as the error of model (root mean square of calibration, RMSEC)

Response	MLR		PLS-2 Y-Explained[a]:%75 (1PC),%3 (2PC) (PC = 2)	
	Regression coefficient (R^2)	Multiple correlation coefficient	Regression coefficient (R^2)	RMSEC
Corrected area Vals.M1	0.507	0.712	0.877	0.022
Corrected area Vals	0.520	0.721	0.891	0.018

[a] Results obtained after recalculation of the model. 6 experiment was not taken into account, since it was recognized as outlier.



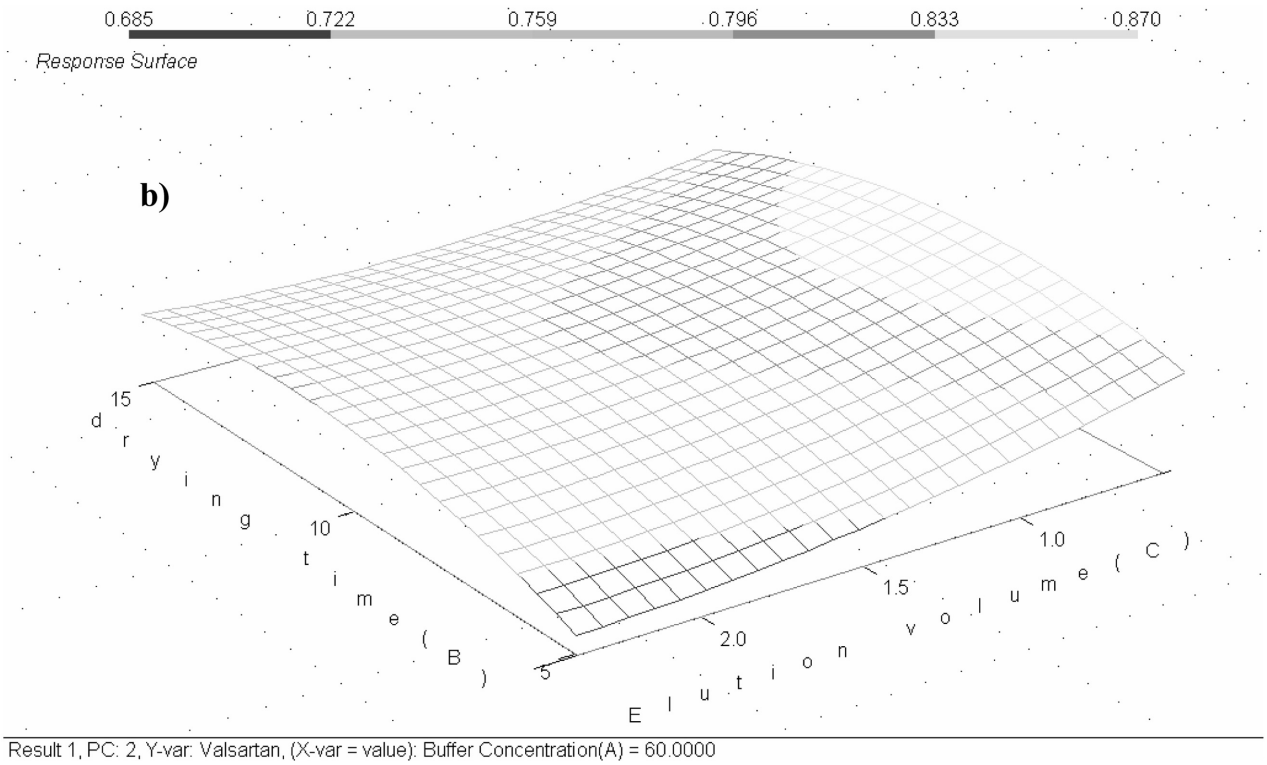


Figure 4. Response surfaces for the most important functions, obtained from CCD: (a) corrected area of valeryl-4-hydroxy-valsartan and (b) corrected area of valsartan. These two response surfaces are defined by the drying time (B) and the elution volume (C), while the buffer concentration (A) is fixed at 60 mM.

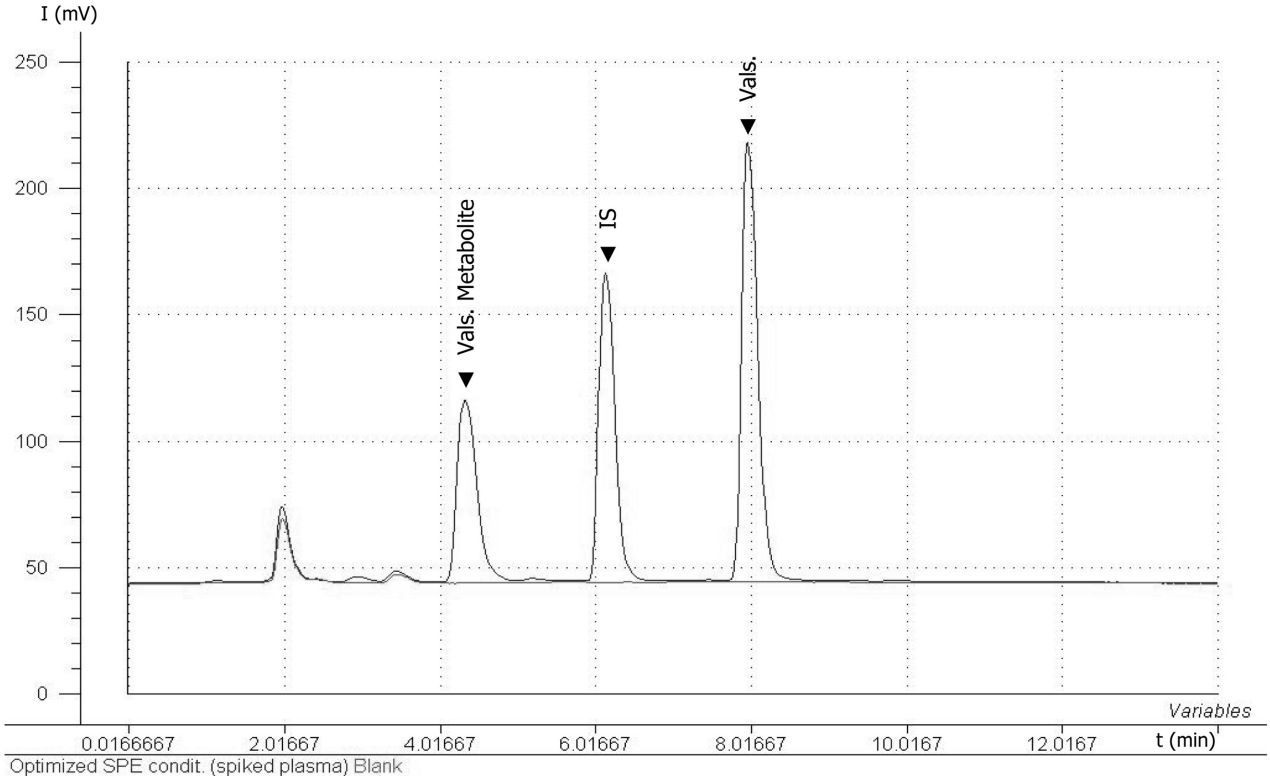


Figure 5. Chromatograms obtained with fluorescence detection corresponding to a blank plasma extracted sample and a spiked plasma sample with valeryl-4-hydroxy-valsartan (450 ng/mL), valsartan (1000 ng/mL), and candesartan M1 (IS, 650 ng/mL) in the optimized SPE and chromatographic conditions as described in Sections 2.4 and 2.6.

Table 12. Predictions and experimental responses for both zones of maximum response proposed by PLS-2 multivariate regression model for the optimization of an SPE procedure of valsartan and valeryl-4-hydroxy-valsartan from human plasma

Theoretical values (variables)			Theoretical responses	
Buffer conc. (mM) (A)	Drying time (min) (B)	Elution sol. volume (mL) (C)	Corrected areas (Vals-M1)	Corrected areas (Vals)
60	8.30	0.52	0.940	0.864
4.97	9.97	2.37	0.942	0.896
Experimental values (variables)			Experimental responses	
60	8	0.5	0.016	1.749
			0.010	1.291
5	10	2	0.045	5.053
			0.039	4.819

Table 13. Recovery percentages obtained at three concentration levels for valeryl-4-hydroxy-valsartan and valsartan after the optimized SPE procedure. The extracted plasma samples were spiked with 650 ng/mL of IS after the SPE (n = 6 replicates)

Valsartan[a)] Concentration (μg/L)	Recovery (%)	Standard deviation	%RSD
100	98.0	1.6	1.6
1000	109.1	0.7	0.6
3000	106.7	0.3	0.3
Valsartan-M1[a)] Concentration (μg/L)	Recovery (%)	Standard deviation	%RSD
100	94.6	2.9	3.1
450	108.9	1.5	1.4
1000	108.8	1.3	1.2

[a)] IS concentration = 650 μg/L.

standard. It can be observed that the extract of plasma sample was free of interferences from endogenous compounds.

4 Concluding remarks

The optimization using experimental design strategies allowed an efficient development of the extraction method and the chromatographic separation, showing that a correct use of an appropriate experimental design is of considerable benefit in setting up the experimental conditions. Furthermore, the chemometric approach allowed us to reduce the number of experiments needed

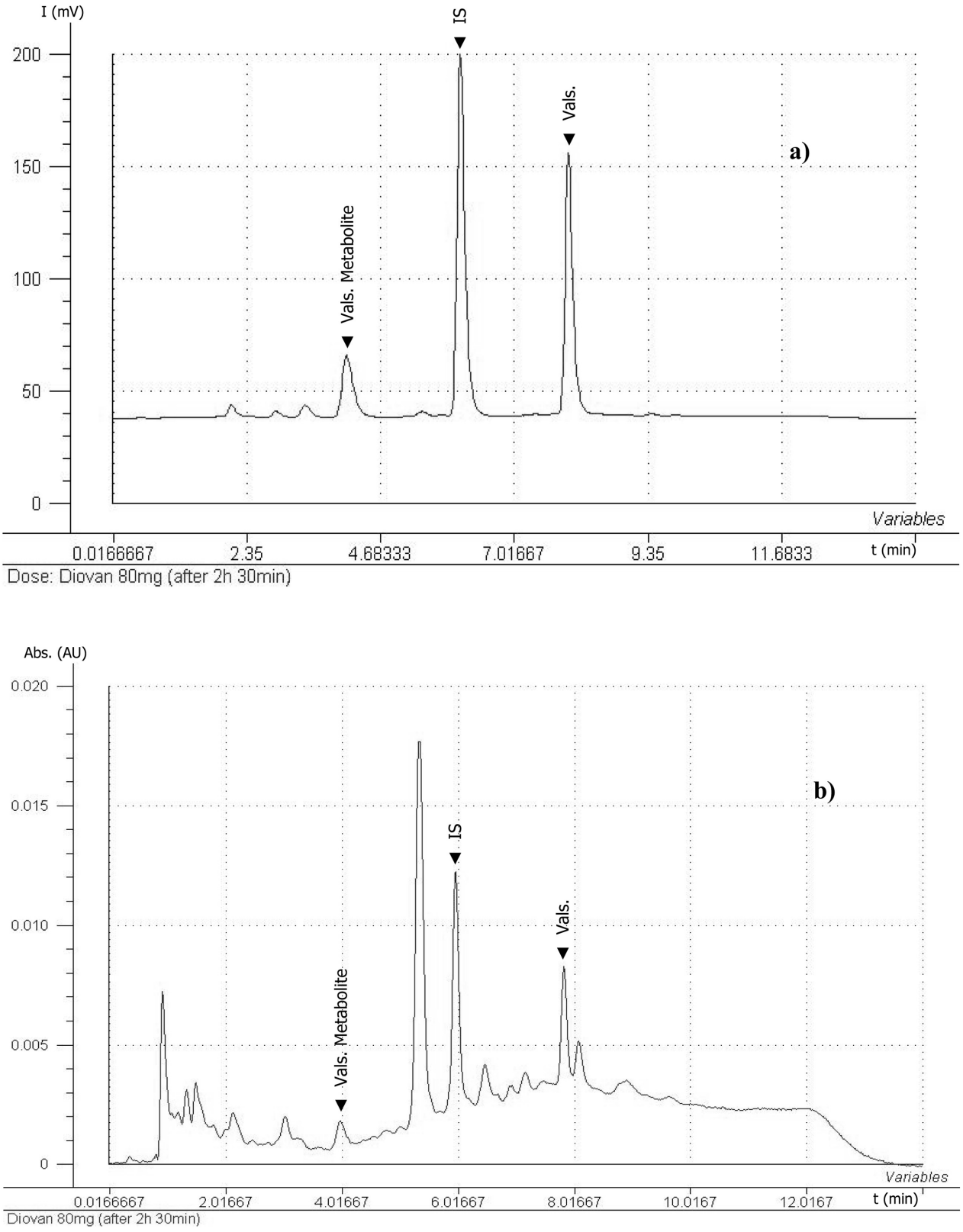
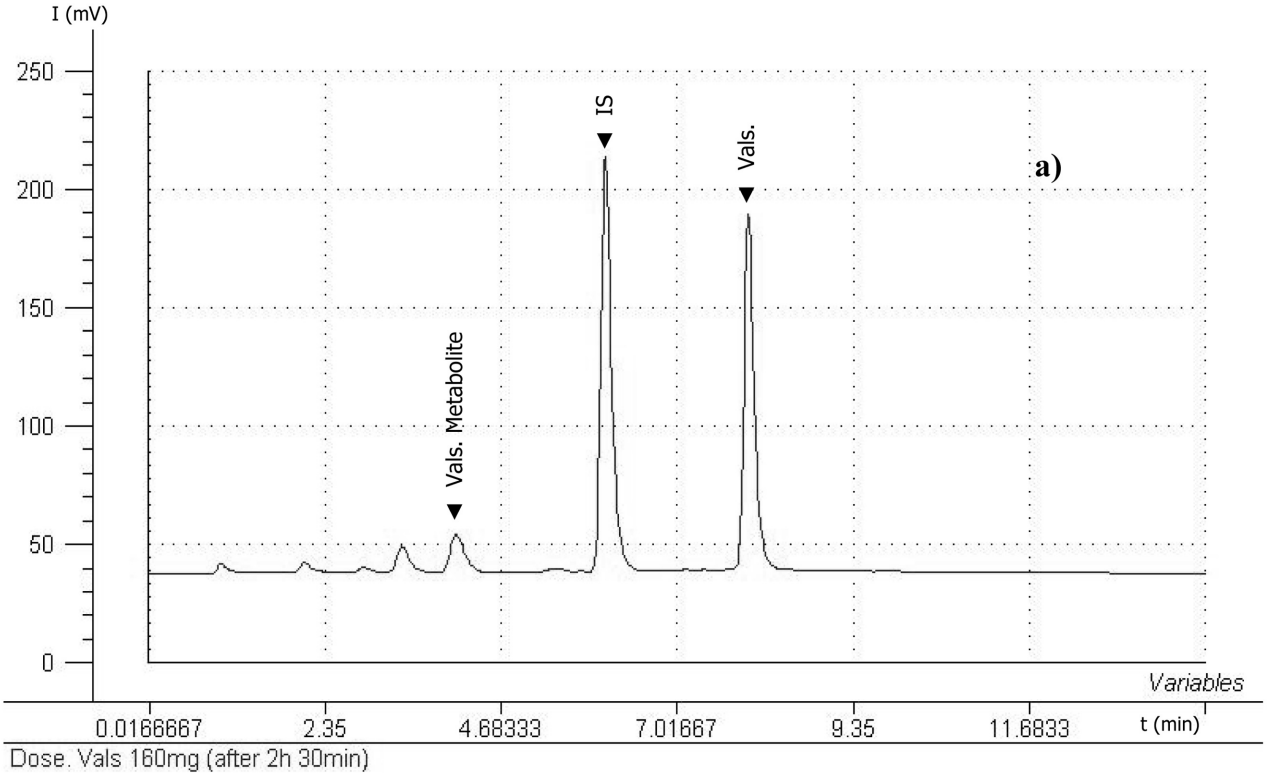


Figure 6. Chromatograms of plasma sample extracts obtained from a hypertensive patient 2 h 30 min after the intake of Diovan-80 mg in the optimized SPE and chromatographic conditions as described in Sections 2.4 and 2.6. Internal standard: candesartan M1 650 ng/mL. (a) Fluorescence and (b) UV.



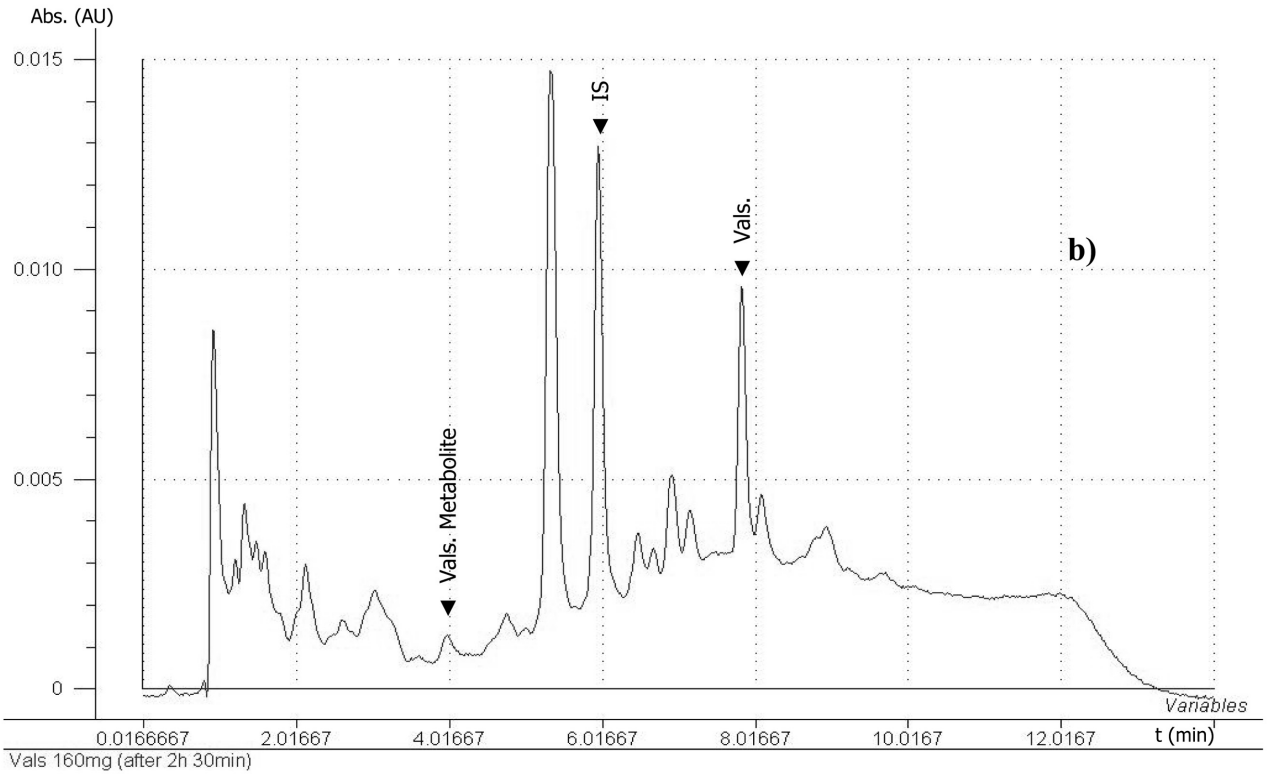


Figure 7. Chromatograms of plasma sample extracts obtained from a hypertensive patient 2 h 30 min after the intake of Vals-160 mg in the optimized SPE and chromatographic conditions as described in Sections 2.4 and 2.6. Internal standard: candesartan M1 650 ng/mL. (a) Fluorescence and (b) UV.

to optimize this analytical method, saving time as well as reagents and solvents.

Due to an intense metabolic transformation of valsartan in the liver, plasma concentrations for valsartan and mainly for its metabolite become low (μg/L) once the interval of maximum plasma concentration (t_{max}) was surpassed and until the ingestion of the next dose. Therefore, in the development of bioanalytical methods for their determination not only a sensitive analytical method has to be performed, but also an extraction procedure which yields high recovery percentages is required.

The optimized SPE method provides good recoveries and low values of RSD which makes it reliable for the quantification of both compounds in plasma samples.

The proposed analytical method SPE-HPLC-UV-fluorescence has been shown to be adequate for the separation and determination of valsartan and its metabolite in plasma samples obtained from patients under cardiovascular treatment.

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