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MultiSimplex and experimental design as chemometric tools to optimize a SPE-HPLC-UV method for the determination of eprosartan in human plasma samples

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Abstract

A chemometric approach was applied for the optimization of the extraction and separation of the antihypertensive drug eprosartan from human plasma samples. MultiSimplex program was used to optimize the HPLC-UV method due to the number of experimental and response variables to be studied. The measured responses were the corrected area, the separation of eprosartan chromatographic peak from plasma interferences peaks and the retention time of the analyte.

The use of an Atlantis dC18, 100 mm × 3.9 mm i.d. chromatographic column with a 0.026% trifluoroacetic acid (TFA) in the organic phase and 0.031% TFA in the aqueous phase, an initial composition of 80% aqueous phase in the mobile phase, a stepness of acetonitrile of 3% during the gradient elution mode with a flow rate of 1.25 mL/min and a column temperature of 35 ± 0.2 °C allowed the separation of eprosartan and irbesartan used as internal standard from plasma endogenous compounds. In the solid phase extraction procedure, experimental design was used in order to achieve a maximum recovery percentage. Firstly, the significant variables were chosen by way of fractional factorial design; then, a central composite design was run to obtain the more adequate values of the significant variables. Thus, the extraction procedure for spiked human plasma samples was carried out using C8 cartridges, phosphate buffer pH 2 as conditioning agent, a drying step of 10 min, a washing step with methanol–phosphate buffer (20:80, v/v) and methanol as eluent liquid. The SPE-HPLC-UV developed method allowed the separation and quantitation of eprosartan from human plasma samples with an adequate resolution and a total analysis time of 1 h.

Keywords: Eprosartan; Human plasma; HPLC; SPE; MultiSimplex; Experimental design

1. Introduction

Eprosartan is a highly selective, non-peptide angiotensin-II antagonist. The compound has been shown to inhibit angiotensin-II induced vasoconstriction in preclinical species and cause reductions in systolic and diastolic blood pressure at peak effect after dosing in clinical patients [1]. It is currently being developed for the treatment of hypertension as other compounds of the angiotensin-II receptor antagonists (ARA-II) family. These are safe and effective agents for the treatment of hypertension and heart failure, either alone, or together with diuretics. Because of this, they have been proposed as an alternative to the more traditional angiotensin-converting enzyme (ACE) inhibitors.

The pharmacokinetic properties of eprosartan after oral administration have been determined primarily in healthy adults. Oral bioavailability is approximately 13% and is not significantly affected by food. Time to achieve maximum plasma drug concentration (C_{max}) ranges from 1 to 3 h after oral dose. The expected C_{max} depends on the administered dose and varies from 702 to 1857 μg/mL in case of 200 and 800 mg dose, respectively. The drug has a distribution volume of approximately 13 L and it is highly bound to plasma proteins (98%). Approximately two-thirds of eprosartan reaching the systemic circulation is eliminated unchanged by biliary excretion, while the remaining one-third is eliminated in the urine primarily as unchanged drug. Approximately 20% of renal elimination of eprosartan is as the acyl glucuronide. The drug has an elimination half-life of

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approximately 4.5–9 h [2]. The development of analytical methods for the determination of eprosartan is crucial for the study of the antihypertensive efficacy of this single dose drug during posological range, since they would allow the monitorization of its plasmatic concentration levels.

There are some works dealing with the determination of ARA-II in pharmaceuticals and biological samples. Hillaert et al. [3–5] have described some electrophoretic and chromatographic screening methods for eprosartan and other ARA-II alone or in combination with diuretics in pharmaceuticals. Other authors [6–9] have developed methods to separate ARA-II in biological fluids such as plasma and urine, but these do not include eprosartan. This fact could be due to the high polarity of this drug, which makes difficult the separation of eprosartan from some plasmatic interferences in human samples and its quantitation. Only Lundberg et al. [10] report the analysis of eprosartan in biological samples by HPLC-UV. This method achieves the separation of eprosartan and an internal standard from clinical plasma samples in 14 min with maximum recoveries of 71.8%.

Many methodologies have been developed to optimize the parameters of interest in chromatography and related techniques (electrochromatography and electrophoresis) [11–14]. In chemometric approaches, experimental measurements are performed in such a way that all factors vary together. An objective function is utilized in which the analyst introduces the desired criteria (selectivity, resolution, time of analysis, . . .).

The aim of actual optimization strategies is to obtain the largest quality information while carrying out a limited number of experiments. The great number of chromatographic parameters and the relationship between them rules out the possibility of empirical optimization by trial and error or intentional variation of one or two parameters. These intentional variations fails to take interactions between two or more parameters into account. But, although it suffers from severe limitations such as the success in the optimization procedure mainly relays on the starting conditions and the researcher's experience, one variable-at-a-time (OVAT) optimization method is still nowadays very popular and extremely used in analytical applications. This traditional methodology may become erratic and, which is more important, possible sinergetic effects among variables are not considered at all. Use of experimental design combined with analysis of variance, and Simplex or MultiSimplex methods are much more confident and avoid the limitations mentioned above.

A variety of systematic methods has been developed to optimize the parameters of interest. In this paper, MultiSimplex program has been used to optimize the chromatographic conditions to separate the antihypertensive drug eprosartan from human plasma interferences. On the other hand, a fractional factorial design (FFD) and a central composite design (CCD) have been chosen for the optimization of the plasma sample treatment procedure. The different methodologies have been chosen taking into account the number of response variables. So, in the chromatographic separation three responses were evaluated while in the extraction procedure, the measured response was only one.

Upon the basis of a chromatographic method developed in our laboratory for the screening of ARA-II family drugs in human plasma samples, and due to the different polarity of eprosartan related to other members of its family, the aim of this work is to develop a SPE-HPLC-UV method for the future monitorization of eprosartan, which could be applied to other polar drugs in elderly patients who are under cardiovascular treatment.

2. Theory

Simplex-based optimizations of instrumental parameters and experimental conditions have been already used [15–18] and they have been demonstrated to be a good alternative to optimize many experimental procedures [19]. The MultiSimplex program [20] is designed as a true multivariate non-linear optimization tool that combines the modified Simplex method [21] with the fuzzy set theory [22] by means of the membership functions or the point response measure called the "aggregated value of membership". The most outstanding feature of MultiSimplex is that it allows simultaneous optimization of several response signals. MultiSimplex optimization is very easy to follow and it has been already used in some analytical applications [23,24]. First of all, the variables, the range of each variable and the responses that are going to be followed are defined. Then, MultiSimplex suggests a $k+1$ number of experiments, where k is the number of variables to be studied. Once the experiments are carried out, the result of the experiments are introduced and MultiSimplex suggests one new experiment. And the process goes on until the optimum conditions are reached. In order to measure the closeness to the optimum, MultiSimplex makes use of the "membership value" [21]. This value ranges from 0 to 1 and takes into account the results of all responses considered in the optimization. Optimized conditions are achieved when the membership value is close to 1. The optimization procedure includes a re-evaluation rule that means that, for every certain number of experiments, a previous trial is repeated experimentally [20].

When applying experimental design methodologies, it is advisable to keep the number of variables as low as possible in order to avoid very complex response models and large variability [25]. Thus, in this case, the 2^4 experiments needed to complete a whole factorial design for the extraction procedure's optimization, were reduced by introducing a confusion and running the so-called fractional factorial design. This design confounds some main effects with interactions or interactions among themselves, resulting in a smaller set of experiments and nevertheless, it 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 studies and p accounts for the degree of fractionality of the FFD ($p<k$) [25].

From the results of the fractional factorial design, a central composite design is built using the same variables as in the FFD but excluding those which present lack of significance. A central composite design, proposed by Box and Wilson [26], consists of a full factorial design plus an orthogonal star design. In this way, each factor is studied at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$). To maintain the highest symmetry as possible, $-\alpha$ and $+\alpha$ levels

were located at $-(2^{1/2})$ and $+2^{1/2}$ respectively. In order to estimate the pure experimental uncertainty of CCD, it is important to measure repeatedly the response function at the conditions determined by the central point [27].

3. Experimental

3.1. Materials and reagents

Eprosartan {(E)-3-[2-butyl-1-[(4-carboxy-phenyl)methyl]-1H-imidazol-5-yl]-2-[(2-thienyl)-methyl]propenoic acid}, provided as mesylate salt, and the internal standard irbesartan {2-butyl-3-[[2′-(1H-tetrazole-5-yl)(1,1′-biphenyl)-4-yl]methyl]-1,3-diazaspiro[4,4]non-1-en-4-one} were kindly supplied by Solvay Pharma (Barcelona, Spain) and Sanofi-Synthelabo (Montpellier, France), respectively. Both drugs are shown in Fig. 1.

Reagent grade trifluoroacetic acid, phosphoric acid, sodium dihydrogen phosphate, acetic acid, sodium acetate, boric acid and sodium borate were obtained from Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germany). HPLC grade acetonitrile and methanol were obtained from Scharlab (Barcelona, Spain) and chloroform, acetone, ethyl acetate and diethyl ether from Carlo Erba (Milan, Italy). Purified water from a Milli-Q Element A10 water system (Millipore, Bedford, MA, USA) was used in the preparation of buffer and reagent solutions. Solid-phase extraction (SPE) cartridges: C2, C8, C18, CH, PH, CN, 2OH, SCX, MCX, SAX, MAX, SI (100 mg bed packing, 1 mL volume capacity) were purchased from Varian (Harbour City, CA, USA) and Waters (Milford, MA, USA). Drug-free control human plasma was purchased from Blood Bank of Galdakao Hospital (Bizkaia, Spain).

3.2. Apparatus

The chromatographic system consisted of two Waters Model 510 HPLC pumps, a Waters Model 717 Plus Autosampler, and a Waters 490E programmable multiwavelength detector. 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^{TM} dC18, 100 mm × 3.9 mm i.d., 3 μm, 100 Å column was used to perform the separation. It was thermostated at 35 ± 0.2 °C in a column oven controlled by a Waters Temperature Control Mode. Previous to the analytical column, a Waters μBondapak C18 guard column 10 μm was placed to prevent column degradation.

The clean-up procedure consisted of a solid-phase extraction and was performed using a vacuum manifold system from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump from Millipore (Bedford, MA, USA).

Plasma extracted samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain).

Plasma samples were centrifuged in a 5904 R Eppendorf refrigerated centrifuge (Hamburg, Germany).

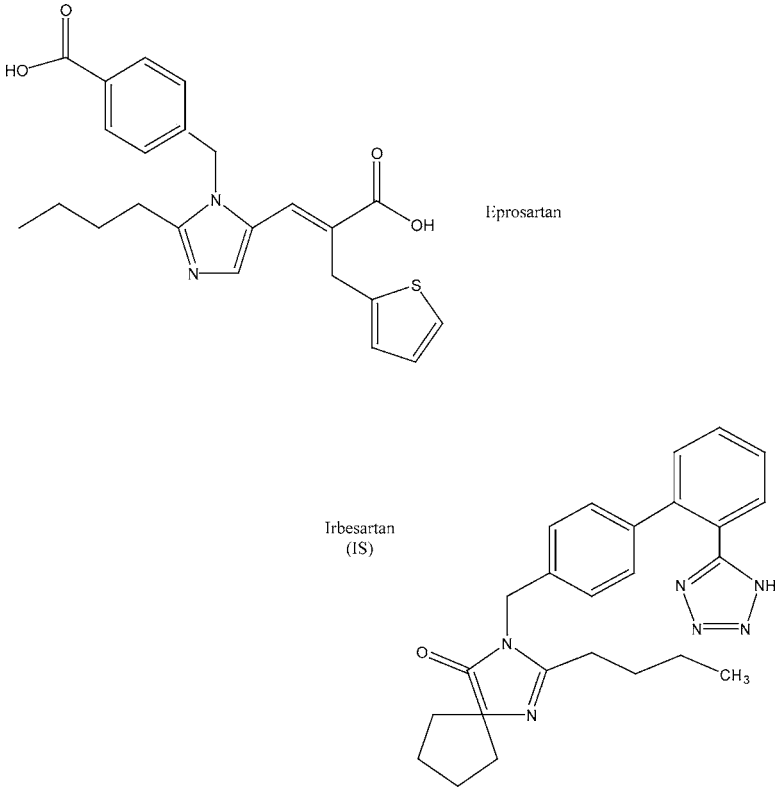


Fig. 1. Chemical structures for eprosartan and the internal standard irbesartan.

3.3. Preparation of standard solutions and spiked plasma samples

A 232 μg/mL stock solution of eprosartan was prepared in 100% methanol by weight of eprosartan mesylate. A 174 μg/mL stock solution of irbesartan, used as internal standard, was also prepared in 100% methanol. These solutions were diluted to produce 20 μg/mL working solutions with methanol. All solutions were stored at 8 °C and protected from light. For the chromatographic optimization, blank plasma samples were daily spiked to achieve drug concentrations of 1 μg/mL each drug. For SPE studies, the plasma samples were daily spiked to be up to 1 μg/mL eprosartan and 300 ng/mL irbesartan.

3.4. Extraction procedure of plasma samples

The 1 mL aliquots of blank plasma samples were spiked with the working solutions of eprosartan and irbesartan to achieve a concentration of 1000 and 300 ng/mL, respectively. An aliquot (1 mL) of phosphoric acid 1 M was added to all samples, which was followed by brief vortex mixing and centrifugation for 5 min in a high-speed centrifuge (10,000 rpm) refrigerated at 4 °C.

The SPE cartridges were conditioned with 2 mL of methanol, followed by 2 mL of phosphate buffer (50 mM, pH 2). The plasma samples were applied to the cartridges manually and washed with 1 mL methanol–phosphate buffer solution (20:80, v/v), followed by a 10 min drying period at high vacuum. The cartridges were then eluted with 2 mL of methanol. The eluent was evaporated to dryness under nitrogen at 60 °C. The residue was reconstituted with 100 μL of acetonitrile, vortex mixed, filtered with a PALL GHP Acrodisc 13 mm syringe filter with 0.45 μm GHP membrane and transferred to autosampler vials. The 20 μL aliquots were injected onto the HPLC system for analysis.

3.5. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile 0.026% TFA and water 0.031% TFA and was delivered in gradient mode at a flow rate of 1.25 mL/min. The gradient is shown in Table 1. Before using, the mobile phase was passed through a 0.45 μm membrane filter from Millipore (Bedford, MA, USA) and degassed in an ultrasonic bath. The chromatographic separation was performed at 35 ± 0.2 °C and the analyte and internal standard were monitorized photometrically at 232 nm.

Table 1
Gradient elution conditions

Time (min)	ACN (0.026% TFA)	H_2O (0.031% TFA)	Flow rate (mL/min)
0	20	80	1.25
5	45	55	1.25
10	45	55	1.25
14	20	80	1.25

3.6. Recovery and repeatability of the extraction procedure

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

The extraction efficiency, expressed in terms of percentage of recovery, was estimated by comparing the peak area of the compound in spiked plasma samples with that from the blank plasma samples to which the compound was added previous the evaporation step. Data were collected as peak areas and peak ratio of eprosartan against IS was considered for all the calculations.

The repeatibility of the extraction was expressed as percentage of relative standard deviation (% R.S.D.).

3.7. Specificity of the extraction procedure

Specificity of the assay was established with six independent sources of blank plasma samples, and comparing these with control plasma spiked with the studied compound (150 ng/mL). The chromatograms were visually inspected for interfering chromatographic peaks from plasma endogenous substances.

4. Results and discussion

4.1. Factors affecting the chromatographic separation: MultiSimplex

Optimization of the chromatographic separation method was made on spiked plasma samples by means of MultiSimplex program. As plasma samples needed a clean-up procedure before introducing them onto HPLC system, a SPE procedure was developed. This was optimized after the chromatographic separation but, in order to use plasma samples for the chromatographic optimization, a basic SPE procedure was used. This was based in previous experiences of the research group on a SPE methodology applied to the separation of 1,4-dihydropyridines drugs [28]. It consisted in a conditioning step of C8 cartridges with methanol and phosphate buffer (100 mM, pH 2.5) and the application of 2 mL of the spiked plasma sample which had been treated with phosphoric acid as protein precipitant agent (1:1), and had been centrifuged. Then, the cartridges were washed with a solution of methanol–phosphate buffer (20:80, v/v), dried at 20 mmHg for 5 min and eluted with 2 mL of methanol. These extracts were evaporated under nitrogen at 60 °C.

Due to the large number of variables in the HPLC operating conditions, some of them were studied by means of traditional methodology one variable-at-a-time and were fixed previously to the use of experimental design. In that way, the ability of six analytical columns: μBondapak C18 300 mm × 3.9 mm i.d., 125 Å, 10 μm; Supelcolsil ABZ + Plus 250 mm × 4.6 mm i.d., 100 Å, 5 μm; Nova-Pak Silica 150 mm × 3.9 mm i.d., 60 Å, 4 μm; Atlantis dC18 100 mm × 3.9 mm i.d., 100 Å, 3 μm; Nova-Pak C18 150 mm × 3.9 mm i.d., 60 Å, 4 μm and Nova-Pak C8 150 mm × 3.9 mm i.d., 60 Å, 4 μm were checked. The organic modifier in the mobile phase, the elution mode, the internal standard and the detection wavelength were chosen upon the basis of different previous experiments.

The use of an isocratic elution mode did not allow the optimum resolution of the studied drug, probably due to the similar polarity of eprosartan and plasma endogenous compounds. Due to this fact, a gradient elution mode was used. Moreover, it was observed that using acetonitrile as organic modifier the chromatographic peaks had a higher area than using methanol or a mixture of both solvents. To improve chromatographic peaks shape and avoid tails a little percentage of TFA was added to the mobile phase. The gradient established by the organic modifier and the organic acid allowed to avoid employing buffer solution in the chromatographic method and the precipitation of the buffer salt in the instrument. The detection wavelenght was fixed in 232 nm since eprosartan presents an absorption maximum at this wavelength. The use of this low wavelength determined also the suitability of using a solvent which low absortivity at this wavelength, as acetonitrile, instead of methanol or tetrahydrofurane.

The analytical column which offered the best chromatographic peak shape and sensitivity was Atlantis dC18, using acetonitrile as organic modifier. This column presents a minor particle size (3 μm) than the other assayed columns. The Atlantis silica-based difunctionally bonded reversed phase C18 column, recently marketed, has been included in this work since it is recommended for the separation of polar compounds and provides full LC–MS compatibility, superior peak shapes and excellent column to column reproducibility [29]. It also allowed to cut down the retention times of the analytes due to its shorter length, last trend in chromatographic separations.

The internal standard, irbesartan, was chosen taking into account that it was a compound belonging to the same antihypertensive family of drugs, it has an absorption maximum at 230 nm, very close to that of eprosartan (232 nm) and it is not co-administered with the studied drug. Once these chromatographic variables were fixed, MultiSimplex software was used to optimize the following parameters: percentage of TFA in each component of the mobile phase, the temperature of the column, the initial composition of mobile phase, the stepness of acetonitrile during the gradient elution mode and the flow rate. The defined response variables were the corrected area (analyte's area/internal standard's area), the separation of eprosartan chromatographic peak against interference chromatographic peaks and the retention time of the analyte and the internal standard. All the variables are shown in Table 2. The range of each variable was based in the experiments carried out previously.

The experiments proposed by the MultiSimplex program and the membership value of each of the experiments (which is drawn in Fig. 2) decided to stop the optimization after the 30th run. As it can be seen in Fig. 2, 5 of the 30 experiments were considered as impossible due to the chromatographic conditions proposed by MultiSimplex. After running the rest 25 experiments, it was managed to observe that for the last five experiments, the membership function values were similar. So, agreement conditions were established upon the basis of the experiment 30. To avoid working with an excess of control variables, it was decided to use a gradient elution mode depending on the composition of the mobile phase which started in an aqueous percentage determinated by MultiSimplex till a 50%, and a recovery time of 5 min. To achieve a reliable chromatographic peaks quantitation, from the optimized conditions, the variables were slighty adapted to obtain the chromatographic peaks in an isocratic region of the gradient. In that way, satisfactory results were obtained when the gradient elution mode started at an initial percentage of water of 80% and reached the 55% in 5 min. Then, this mobile phase was kept constant for 5 min. In these conditions (collected in Table 1), eprosartan and irbesartan chromatographic peaks appeared in the isocratic elution zone, as it can be seen in Fig. 3.

Table 2
Variables and levels considered for the optimization of the chromatographic system HPLC-UV to separate eprosartan and irbesartan in human plasma samples by using MultiSimplex

Control variables	TFA in water (%)	TFA in ACN (%)	Column T_a (°C)
Initial range	0.02	0.02	10
Reference value	0.02	0.02	35
Minimum	0.01	0.01	25
Maximum	0.03	0.03	45

Control variables	H_2O initial mobile phase (%)	ACN stepness (% ACN/min)	Flow rate (mL/min)
Initial range	20	2	0.8
Reference value	80	3	1.4
Minimum	70	2	1.0
Maximum	90	4	1.8

Response variables	Corrected area	Interference separation (min)	Retention time (min)
Weight	1	1	0.67
Minimum	0	0	4
Maximum	0.8	0.7	10

The developed method has demonstrated to be rugged under light variations in the composition of the percentage of TFA in the mobile phase. The variations in the instrumental conditions have not shown significant changes in the obtained chromatograms.

4.2. Extraction procedure: experimental design

Experimental design has been applied to the optimization of different clean-up procedures [30–32]. In this work, the optimization of the extraction procedure was made by means

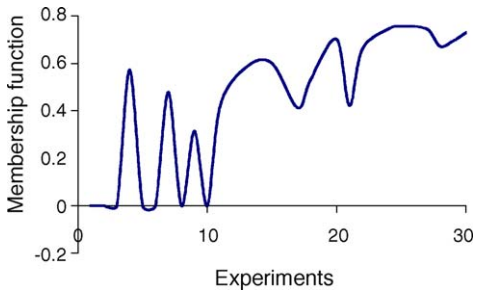


Fig. 2. Evolution of the membership function value depending on the experiment during the optimization of the chromatographic system.

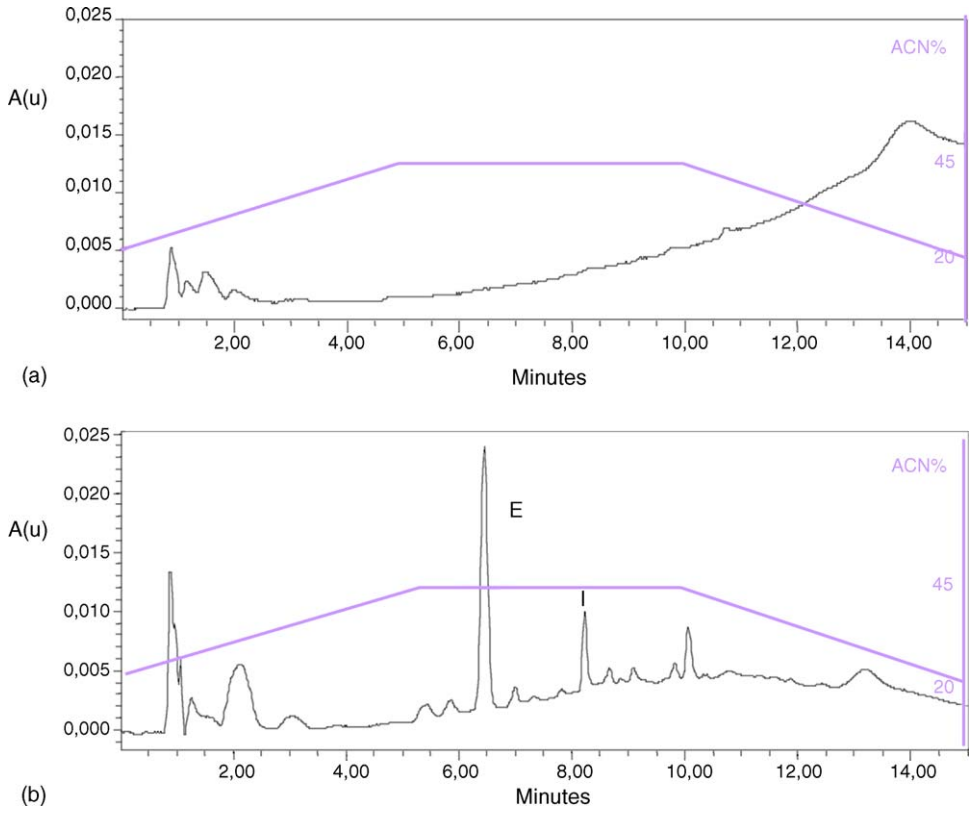


Fig. 3. Chromatograms of: (a) human plasma blank extract and (b) human plasma extract spiked with eprosartan 3.65 μg/mL (E) and irbesartan 0.59 μg/mL (I) in the optimized chromatographic conditions collected in Section 4.1.

of two different experimental designs: a fractional factorial design to evaluate which of the considered variables were significant factors, and a central composite design to optimize these factors in the previously selected experimental domain. To define this domain, some experiments were carried out. In these, some variables were optimized each one at a time to avoid extending the number of variables included in the experimental domain and the interpretation of the results. These variables were: the pretreatment of plasma samples (using phosphoric acid as protein precipitant agent), the extraction cartridge (the best recovery percentage was obtained with C8 cartridges), the pH of sample application (pH 2), the elution liquid composition (methanol–phosphate buffer solution (50 mM, pH 2) 20:80, v/v) and the washing liquid (100% methanol).

The SPE procedure is also influenced by a lot of parameters, but generally, the measured response is only one (the higher corrected area or the recovery). Thus, together with one variable-at-a-time optimization methodology, the use of experimental design was decided. A four-factor considering FFD enabled the selection of critical factors on response. Response surface modelling from the three-factor considering CCD design allowed the selection of the optimal analysis conditions for the clean-up procedure for eprosartan in human plasma samples.

4.2.1. Protein precipitant agent

Phosphoric acid and acetonitrile were tested as protein precipitant agents. The organic solvent induced the formation of clusters containing the drug, so the precipitant agent chosen was phosphoric acid.

4.2.2. OVAT methodology

Eprosartan has two different pK_a values around 3.6 and 6.8 (calculated by using spectrophotometric methods) [33]. Effect of sample pH was clearly stated with all the solvents and packings.

In order to define the solid phase extraction procedure different mechanisms were tested: reversed phase, normal phase and ionic exchange. The highest recoveries were obtained by using reversed phase C8 cartridges (see Fig. 4a).

Plasma samples buffered at pH 2 and 5 gave the highest recoveries in the extraction procedure. In all the cases, stronger ionization of eprosartan at pH > 7 produced important losses of analyte (recovery < 50%) due to its weak interactions with apolar cartridges; pH 2 was used in the extraction procedure since the best protein precipitant agent was phosphoric acid and in this way, a pH change was not necessary.

The choice of the methanol as elution liquid provided the best recoveries between all the assayed eluting solvents: methanol, acetonitrile, acetone, chloroform, ethyl acetate, dichloromethane, diethyl ether and hexane (see Fig. 4b). Extraction efficiency was initially preferred to selectivity and pure methanol was selected as eluting solvent. In order to choose an adequate washing solvent to enhance selectivity, elution of eprosartan was examined as a function of the composition of mixtures methanol–phosphate buffer solution (0.1 M, pH 2), as it is shown in Fig. 4c. The elution profiles obtained from plots

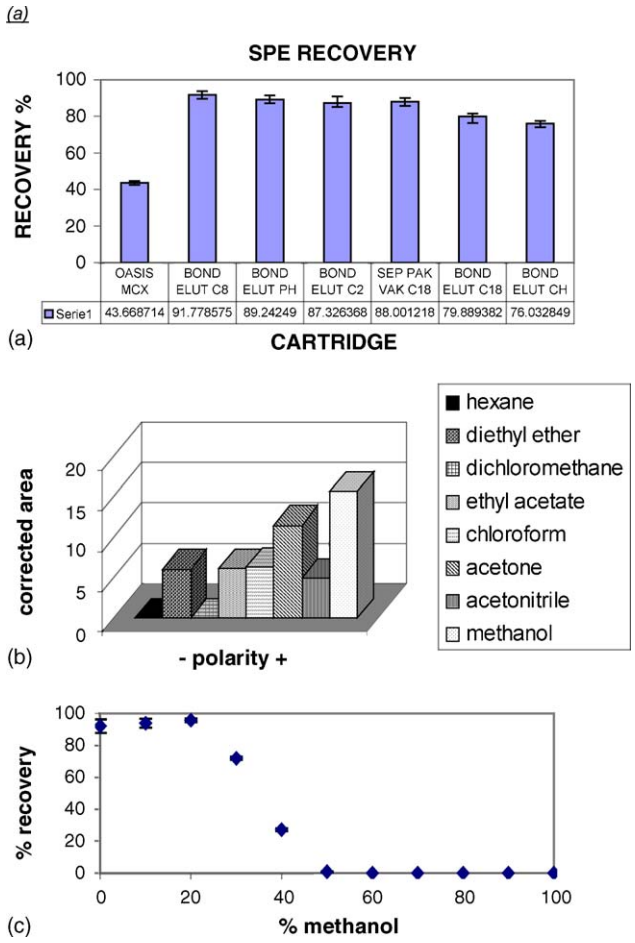


Fig. 4. Effect of cartridge characteristics on the recovery percentage of SPE (a), elution solvent on chromatographic peak area (b) and elution profile methanol–phosphate buffer 0.1 M pH 2 (c) for human plasma samples spiked with eprosartan.

of recovery percentage against percentage of methanol in the mixture gave information about the best composition for the washing solvent (that containing the largest amount of methanol without eluting the drug) and also the best elution solvent (the lowest amount of methanol affording complete recovery of the drug). The washing solution chosen (methanol–phosphate buffer solution (0.1 M, pH 2) 20:80, v/v) eliminated the endogenous compounds from the matrix without eluting the analytes, yielding a chromatogram free of interferences. Although a solution containing a percentage of 50% of methanol was enough for totally recovery of the drug, pure methanol was chosen because the evaporation of hydromethanolic solutions spends more time and resources.

4.2.3. *Fractional factorial design*

The experimental variables considered in the FFD for the separation of eprosartan and the internal standard irbesartan from human plasma samples were: buffer solution concentration (x_1), washing liquid volume (x_2), drying time (x_3) and elution liquid volume (x_4).

In this case, the main effect estimated for factor buffer solution concentration was confused with the interaction effect for the other three variables as it is shown in Eq. (1). A two-level FFD involving eight runs and two replicates of the central point was carried out. The proposed experiments for FFD are shown in Table 3:

Table 3
Corrected areas obtained from the 2^{4-1} proposed fractional factorial design for the optimization of a solid phase extraction procedure of eprosartan from human plasma

Experiment	Washing solution volume (mL)	Drying time (min)	Elution solution volume (mL)	Buffer concentration (mM)	Corrected area
1	0.5	5	1	10	1.78
2	2	5	1	50	2.57
3	0.5	15	1	50	2.27
4	2	15	1	10	2.08
5	0.5	5	3	50	1.99
6	2	5	3	10	1.71
7	0.5	15	3	10	2.13
8	2	15	3	50	2.90
9	1.25	10	2	30	2.34
10	1.25	10	2	30	2.30

$$\text{Buffer solution concentration} = \text{volume of washing liquid} \times \text{drying time} \times \text{volume of elution liquid} \quad (1)$$

In order to obtain an adequate extraction procedure, and considering that the separation method has been previously optimized, the response variable was measured by the corrected area. Level codification was established taking into account the domain where experiments could be easily interpreted. The washing solution volume had to be enough to eliminate the more interferences as possible but not too much to avoid enlarging the extraction procedure (each cartridge volume is 1 mL) and losing the analyte. It ranged from 0.5 to 2 mL. In the same way, an excess of elution liquid volume would involve a great waste of resources and would enlarge the evaporation step, and the increase of drying time would give rise to enhance the analysis time. Thus, the drying time was varied between 5 and 10 min, the elution solvent volume, between 1 and 3 mL and the buffer solution concentration, between 10 and 50 mM.

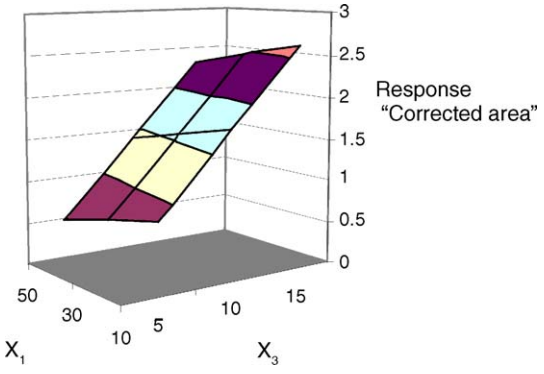


Fig. 5. Response surface defined by the variables x_1 (buffer concentration) and x_3 (drying time) in the FFD.

The results of the analysis for eprosartan and irbesartan were treated using The Unscrambler program [34]. This program defines the response as a function of the considered variables (x_i) by using analysis of variance. Table 3 shows the FFD matrix along with the response value for each run. The treatment of these data and the analysis of variance allowed us to conclude that the elution solution volume did not influence on the recovery of the extraction considering a significance level of 95%. Taking into account this level, the only significant variable would be the buffer solution concentration. However, if the significance level falls until 90%, it is also necessary include as significant variables the washing solution volume, the drying time and the interaction washing solution volume–buffer solution concentration. As the obtained p-value was close to 0.05, these three variables were used in the CCD. Fig. 5 shows the response surface defined by the variables x_1 (buffer solution concentration) and x_3 (drying time). The elution solution volume was fixed in 2 mL, to ensure the total recovery of the retained analytes from the cartridges.

4.2.4. Central composite design

From the four proposed variables for the FFD, the volume of elution solution has shown lack of significance, so it has been fixed (2 mL). Thus, the CCD was a $2^3 + (3 \times 2) + k$ design, k being the number of replicates of the centre point ($k = 2$).

Table 4 shows the three studied factors and the obtained responses. The randomized experiments were run in the order collected in Table 4, which was obtained from The Unscrambler program.

Upon the basis of the obtained responses, The Unscrambler defined a regression model with R^2 value of 0.844. In this case, it was observed that the interaction between the buffer solution concentration and the washing solution volume was not significant (for a significance level of 95%). The parameters which had influence on the response were the concentration of buffer solution and the drying time. These two variables defined the response surface that is shown in Fig. 6. The response rises when the two variables are increased, until reaching a plateau, where the response is practically constant in spite of the increase of the two significant variables. In that way, the optimum conditions for the solid phase extraction were a concentration of buffer of 50 mM to avoid saturating the cartridges, and a drying time of 10 min to avoid the increase of the whole analysis time. The volume of washing solution was fixed in 1 mL.

Table 4
Variables and responses obtained for the central composite design for the optimization of a solid phase extraction procedure of eprosartan from human plasma

Experiment	Washing solution volume (mL)	Drying time (min)	Buffer concentration (mM)	Corrected area
1	0.60	5.0	50.0	1.87
2	1.30	10.0	60.2	2.51
3	**1.30**	**10.0**	**35.0**	2.18
4	0.12	10.0	35.0	1.88
5	0.60	15.0	50.0	2.17
6	2.00	5.0	20.0	1.26
7	**1.30**	**10.0**	**35.0**	2.22
8	2.50	10.0	35.0	2.56
9	2.00	15.0	50.0	2.58
10	1.30	1.6	35.0	1.81
11	2.00	15.0	20.0	1.52
12	2.00	5.0	50.0	1.97
13	1.30	18.4	35.0	2.75
14	0.60	5.0	20.0	1.30
15	0.60	15.0	20.0	2.07
16	1.30	10.0	9.8	1.34

The replicates of the central point are in bold.

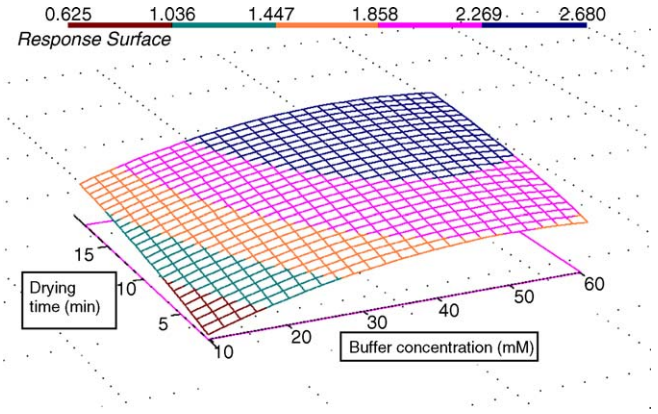
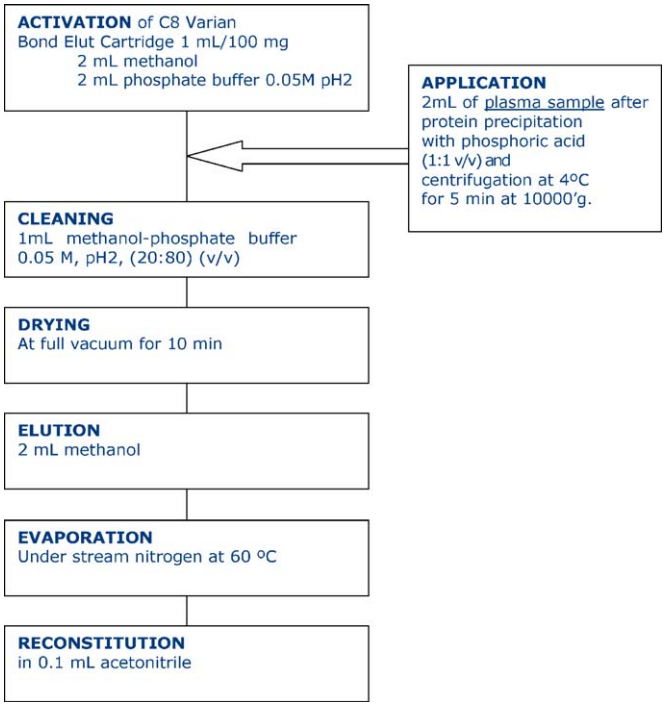


Fig. 6. Respone surface, obtained from CCD, defined by the buffer solution concentration and the drying time.

In Scheme 1, the optimized plasma samples clean-up procedure is given. In these conditions, the recovery percentage for eprosartan was 97 ± 5, 103 ± 5 and 99 ± 5, calculated at three concentration levels 150, 600 and 4000 ng/mL, respectively. In Fig. 7, a chromatogram of a spiked plasma sample at a concentration of 150 ng/mL in the optimized chromatographic and extraction conditions is shown.



Scheme 1. Optimized SPE procedure for plasma samples containing eprosartan.

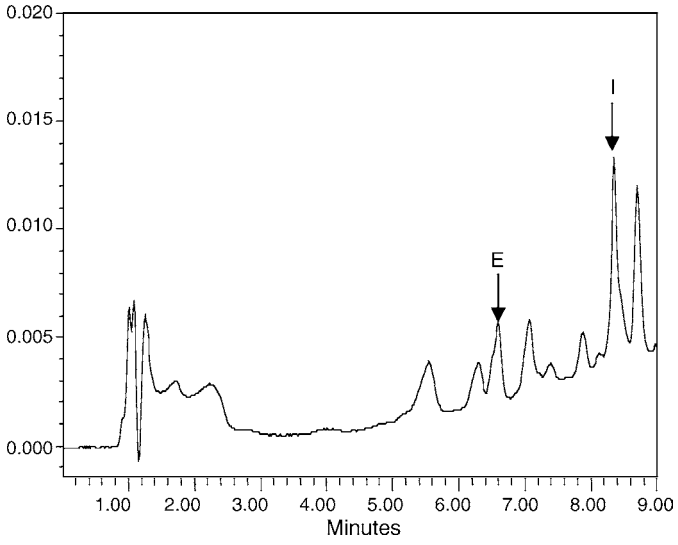


Fig. 7. Chromatogram corresponding to a spiked plasma sample with eprosartan (E) 150 ng/mL and irbesartan (I) 300 ng/mL in the optimized chromatographic and SPE conditions.

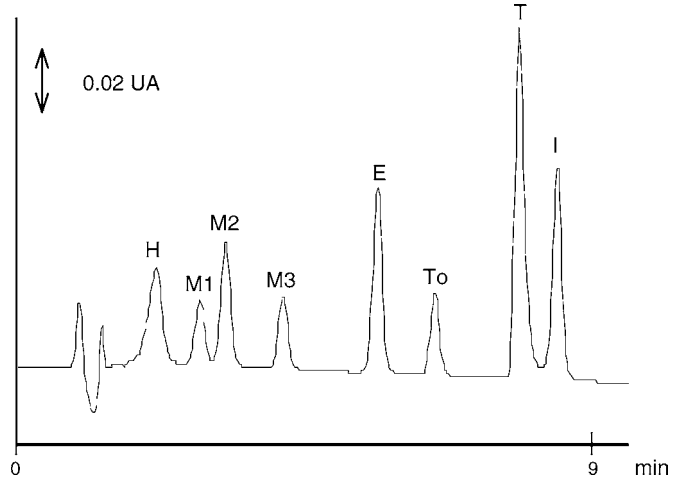


Fig. 8. Chromatogram corresponding to a spiked plasma sample with hydrochlorotiazide (H), torasemide (To) and three of its metabolites (M1–M3), eprosartan (E), telmisartan (T) and irbesartan (I) in the chromatographic conditions of the optimized HPLC-UV method.

5. Discussion and conclusions

5.1. Discussion

With the aim of spread the applicability of the developed method to the screening of several members of ARA-II family and also to diuretics, frequently co-administered with ARA-II drugs, it was applied to a sample containing diuretics: hydrochlorotiazide and torasemide and three of its metabolites, and ARA-II compounds: eprosartan, telmisartan and irbesartan. The HPLC-UV method allowed the separation of all of them with a good resolution as it can be seen in Fig. 8. This fact confirmed the application of this method to determine polar compounds, which are frequently co-administered to elderly patients under cardiovascular treatment.

5.2. Conclusions

Chemometric approach allowed us to reduce the number of experiments needed for optimization of extraction procedure and chromatographic separation, as well as the attainment of a true optimum set of conditions.

The HPLC-UV developed method by using gradient elution mode, achieved the separation of eprosartan and the internal standard irbesartan in human plasma samples in 9 min, by using an Atlantis dC18 column 100 mm × 3.9 mm i.d., 100 Å, 3 μm, and a mobile phase of water 0.031% TFA and ACN 0.026% TFA.

The developed SPE procedure was very simple and effective (97% recovery percentage). The whole analytical procedure (extraction and separation of plasma samples) can be carried out in 1 h.

The SPE-HPLC-UV method achieved the separation of eprosartan in a shorter analysis time (9 min) by using a short-length column with a smaller particle size and acetonitrile as organic modifier, compared with that obtained by Lundberg et al. (14 min) utilized a BDS-Hypersil C18 150 mm × 2 mm and a mobile phase consisted of 0.05 M citrate buffer solution (pH 3.5) tetrahydrofuran (34:16, v/v). On the other hand, the recovery percentages were higher (97 ± 5%) than those described in bibliography (79.1%).

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References

[1] K.J. McClellan, J.A. Balfour, Drugs 55 (5) (1998) 713.
[2] G.L. Plosker, R.H. Foster, Drugs 60 (1) (2000) 177.
[3] S. Hillaert, W. Van den Bossche, J. Chromatogr. A 979 (1–2) (2002) 323.
[4] S. Hillaert, W. Van den Bossche, J. Pharm. Biomed. Anal. 31 (2) (2003) 329.
[5] S. Hillaert, T.R.M. De Beer, J.O. De Beer, W. Van den Bossche, J. Chromatogr. A 984 (1) (2003) 135.
[6] L. Gonzalez, R.M. Alonso, R.M. Jimenez, Chromatographia 52 (11/12) (2000) 735.
[7] E. Cagigal, L. Gonzalez, R.M. Alonso, R.M. Jimenez, Talanta 54 (6) (2001) 1121.
[8] L. Gonzalez, J.A. Lopez, R.M. Alonso, R.M. Jimenez, J. Chromatogr. A 949 (1–2) (2002) 49.
[9] N. Torrealday, L. Gonzalez, R.M. Alonso, R.M. Jimenez, E. Ortiz Lastra, J. Pharm. Biomed. Anal. 32 (4–5) (2003) 847.
[10] D.E. Lundberg, R.C. Person, S. Knox, M.J. Cyronak, J. Chromatogr. B 707 (1 and 2) (1998) 328.
[11] A.M. Siouffi, R. Phan-Tan-Luu, J. Chromatogr. A 892 (2000) 75.
[12] E. Marengo, V. Gianotti, S. Angioi, M.C. Gennaro, J. Chromatogr. A 1029 (1–2) (2004) 57.
[13] Y.P. Zhang, X.J. Li, Z.B. Yuan, Microchem. J. 73 (2002) 307.

[14] L. González, U. Akesolo, R.M. Jiménez, R.M. Alonso, Electrophoresis 23 (2002) 223.
[15] F. Walters, L.R. Parker Jr., S.L. Morgan, S.N. Deming, Sequential Simplex Optimization, CRC Press, Boca Raton, FL, 1991.
[16] O. Zuloaga, N. Etxebarria, L.A. Fernández, J.M. Madariaga, Anal. Chim. Acta 416 (2000) 43.
[17] A. Fernández-Gónzalez, R. Badía, M.E. Díaz-García, J. Pharm. Biomed. Anal. 29 (2002) 669.
[18] A. Numan, B.A. Musial, N.D. Danielson, J. Pharm. Biomed. Anal. 30 (2002) 761.
[19] S.N. Deming, S.L. Morgan, Experimental Design: A Chemometrical Approach, Elsevier, Ámsterdam, 1993.
[20] J. Bergström, T. Öberg, MultiSimplex 98, Karlskrona, Sweeden, 1998.
[21] J.A. Nelder, R. Mead, Comput. J. 5 (1965) 308.
[22] A. Otto, Chemom. Intell. Lab. Syst. 4 (1988) 101.
[23] J. Sanz, A. de Diego, J.C. Raposo, J.M. Madariaga, Anal. Chim. Acta 486 (2003) 255.
[24] E. Cortazar, O. Zuloaga, J. Sanz, J.C. Raposo, N. Etxebarria, L.A. Fernández, J. Chromatogr. A 978 (2002) 165.
[25] A.C. Atkinson, A.N. Donev, Optimum Experimental Designs, Oxford Science, Oxford, 1992.
[26] G.E.P. Box, K.B. Wilson, J. R. Stat. Soc. Ser. B Stat. Methodol. 13 (1951) 1.
[27] E.D. Morgan, Acol Analytical Chemistry by Open Learning, Chichester, Wiley, 1991.
[28] A.B. Baranda, C.A. Mueller, R.M. Alonso, R.M. Jiménez, W. Weinmann, Ther. Drug Monit. 27 (2005) 44.
[29] www.waters.com.
[30] J. Wieling, H. Dijkstra, C.K. Mensink, J.H.G. Jonkman, J. Chromatogr. 629 (2) (1993) 181.
[31] H. Svennberg, S. Bergh, H. Stenhoff, J. Chromatogr. B 787 (2) (2003) 231.
[32] C. Prado, J. Garrido, J.F. Periago, J. Chromatogr. B 804 (2) (2004) 255.
[33] N. Ferreirós, M.Sc. Thesis, University of Basque Country/EHU, Vitoria (Spain), 2004.
[34] www.camo.no.