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Optimization and validation of a SPE-HPLC-PDA-fluorescence method for the simultaneous determination of drugs used in combined cardiovascular therapy in human plasma

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ABSTRACT

This paper reports the chemometrical optimization and the validation of a quantitative high performance liquid chromatography-photodiode array-fluorescence (HPLC-PDA-Fluo) method for the simultaneous analysis, in human plasma, of drugs usually combined in cardiovascular therapy. Separation of chlorthalidone (CLTD), valsartan (VAL), valsartan-M1 (VAL-M1), fluvastatin (FLUV) and the internal standard (IS) candesartan cilexetil was performed on a dC18 Atlantis column (100 mm × 3.9 mm, 3 μm) using a gradient with a run time of 15 min. The mobile phase consisted of a mixture of acetonitrile and water containing 0.01% of formic acid and 10 mM of ammonium formate at pH 4.1. UV and fluorimetric (valsartan, its metabolite and fluvastatin) detectors were used. The sample preparation consisted of protein precipitation using acetonitrile suited to a solid-phase extraction (SPE) on a Strata-X cartridge for sample clean-up. Method validation was developed following the recommendations for bioanalytical method validation of International Conference on Harmonisation (ICH) and Food and Drug Administration (FDA) organizations. The method showed good linearity (31–3000 μg/l for chlorthalidone, 20–1000 μg/l for valsartan-M1, 10–5000 μg/l for valsartan and 14–1000 μg/l for fluvastatin), precision and accuracy. Recoveries were in the range of 78–91%. This method allowed the determination of these drugs in human plasma samples obtained from patients under cardiovascular treatment.

1. Introduction

Cardiovascular diseases are nowadays the first cause of mortality worldwide, causing around the 30% of global deaths each year. The risk of suffering from a cardiovascular disease is closely related to some factors such as hypertension, high cholesterol levels or diabetes. Due to the sedentary lifestyle and bad habits of the western society (e.g. alcohol and tobacco consume, wrong diet), these risk factors often appear together, which is known as metabolic syndrome [1,2].

When change in the lifestyle and in the diet is not enough, a medical treatment is necessary. In this way, combination of antihypertensive, hypolipemiant and antidiabetic drugs is often used in the treatment [3–5]. One of the most used combinations consists on a synergic association of a diuretic (chlorthalidone, hydrochlorothiazide, etc.) and an Angiotensin II receptor antagonist or ARA-II (valsartan, telmisartan, etc.) to control the hypertension, with a statin (fluvastatin, simvastatin, etc.) to reduce the cholesterol levels.

Valsartan (VAL) is an orally active and specific ARA-II with high bioavailability and long half-life (6–9 h) [6]. Valsartan is taken as a single dose of 80–320 mg and the highest plasmatic concentration is achieved 2–4 h after the oral intake (t_{max}). It is affected by first-pass metabolism, where the parent compound is rapidly converted (20% of initial dose) into its main metabolite valeryl-4-hydroxyvalsartan (VAL-M1), via oxidation of the C4 of the valeramide function as shown in Fig. 1 [7].

Chlorthalidone (CLTD) is a diuretic with pharmacological properties similar to the thiazides family [8]. It is absorbed slowly from the gastrointestinal tract and is excreted largely by the kidneys as unchanged drug [9]. Chlorthalidone has a very long half-life time (40–60 h) with a peak concentration 2–5 h after the oral intake, which varies from 25 to 200 mg [10].

Fluvastatin (FLUV) reduces the triglycerides and the low-density lipoprotein (LDL), known as "bad" cholesterol whereas increases

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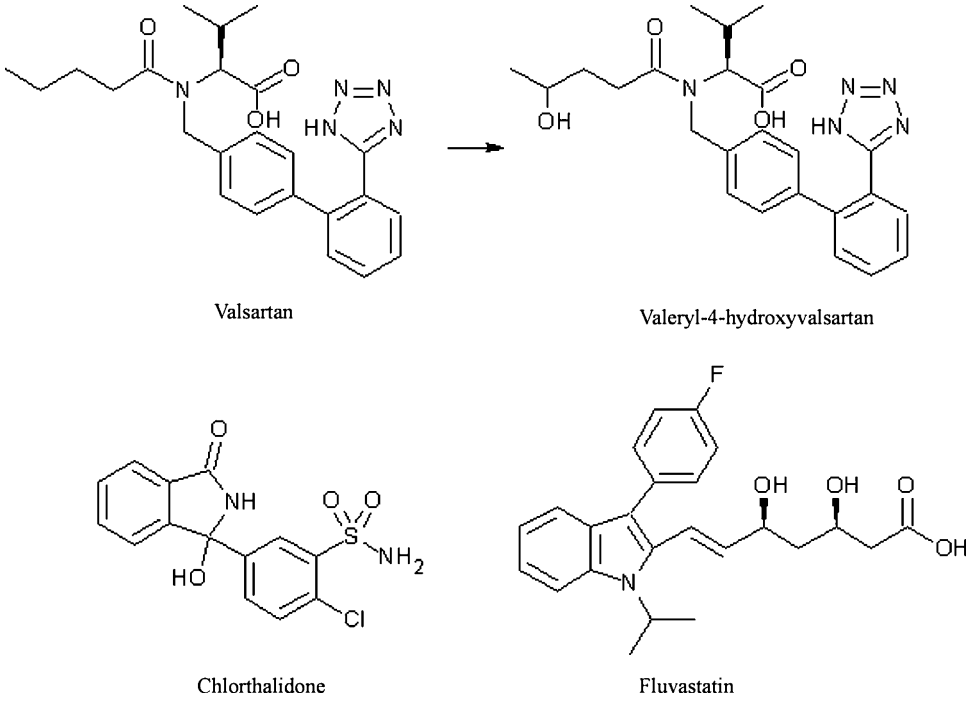

Fig. 1. Chemical structure of analyzed compounds.

the high-density lipoprotein (HDL), known as "good" cholesterol [11]. Fluvastatin absorption and metabolism are very fast, the original molecule completely disappears in 4 h due to the hepatic metabolization, having a t_{max} 0.5–1.5 h after the oral intake. The half-life time of the fluvastatin is 0.5–2.4 h [12,13] and its dose varies from 20 to 80 mg.

Determination and screening of different families of drugs used in cardiovascular therapy has been widely studied. In this way, there are several methods developed for the determination of valsartan [14–17], chlorthalidone [18–21] and fluvastatin [22–26] in biological samples (plasma and urine), most of them using liquid chromatography with UV, fluorimetric or mass spectrometry detection. Otherwise, although the metabolic syndrome is one of the most important illnesses nowadays, the different chemical (pK_a, polarity, etc.) and pharmacokinetic (t_{max}, C_{max}, etc.) properties of the drugs make more difficult the development of a unique method for their extraction and analysis. Therefore there are very few analytical methods developed for the simultaneous determination of combination of different kind of drugs used in its treatment (antihypertensive, hypolipemiant, antidiabetic, antithrombotic). These methods have been applied to pharmaceuticals [27,28] and plasma samples [29].

In this work, a HPLC method for determination of chlorthalidone, valsartan, its main metabolite and fluvastatin in human plasma samples has been developed using SPE optimized by traditional optimization and experimental design. The method was validated following Food and Drug Administration (FDA) and International Conference on Harmonisation (ICH) guidelines [30–33].

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a Waters Alliance 2695 separations module connected to a Waters 996 photodiode array detector and Waters 474 scanning fluorescence detector (Milford, MA, USA). Chromatograms were recorded by means of a computer and treated with the aid of the software Empower 5.0 from Waters.

A Waters Atlantis dC18 column (100 mm × 3.9 mm id, 3 μm, 100 Å) was used to perform the separation, with a Waters C18 Novapak, 4 μm, guard column.

Plasma samples were centrifuged in an Eppendorf model 5804R centrifuge (Hamburg, Germany) prior to the clean-up procedure. The SPE extraction was carried out in a vacuum manifold from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump from Millipore (Milford, MA, USA). After the protein precipitation procedure, the organic layer was evaporated under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain). It was also used for the total evaporation of eluted extracts prior to preconcentration.

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

2.2. Chemical and reagents

Sodium fluvastatin (7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3, 5-dihydroxy-hept-6-enoic acid), valsartan, ((*S*)-*N*-valeryl-*N*-{[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl}-valine) and its metabolite, valeryl-4-hydroxyvalsartan were kindly supplied by Novartis Pharma AG (Basel, Switzerland). Chlorthalidone (2-chloro-5-(1-hydroxy-3-oxo-1,2-dihydroisoindol-1-yl)-benzenesulfonamide) was kindly supplied by Ciba-Geigy (Barcelona, Spain) and candesartan cilexetil ((±)-1-cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-{[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl}-1H-benzimidazole-7-carboxylate), used as internal standard, by Astrazeneca (Mölndal, Sweden).

Ammonium formate, 99% purity, was purchased from Alfa Aesar (Karlsruhe, Germany) and formic acid, LC–MS quality, from Fluka (Buchs, Switzerland).

Pro-analysis quality zinc sulphate heptahydrate and 99.5% purity ammonium sulphate employed on protein precipitation

optimization step were purchased from Merck (Darmstadt, Germany).

HPLC quality methanol and acetonitrile were obtained from Scharlab (Barcelona, Spain), acetone from Merck (Darmstadt, Germany), tetrahydrofuran from Carlo-Erba (Milan, Italy), diethyl ether from Panreac (Barcelona, Spain) and propan-1-ol from Lab-Scan (Dublin, Ireland). Pro-analysis quality ethyl acetate, dichloromethane and chloroform were supplied by Carlo-Erba.

Purified water from a Milli-Q Element A10 water system (Millipore, Milford, MA, USA) was used in the preparation of buffer and reagent solutions.

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, glacial acetic acid, sodium acetate and trisodium citrate dihydrate used for preparing buffer solutions were all pro-analysis quality and obtained from Merck (Darmstadt, Germany). Pro-analysis quality phosphoric acid was purchased from Panreac (Barcelona, Spain) and pro-analysis quality disodium hydrogen citrate sesquihydrate, from Fluka (Buchs, Switzerland). Buffer solutions pH was adjusted by using volumes of 1 M HCl and KOH solutions.

2.3. Standard solutions and spiked plasma samples

Standard solutions of 1000 mg/l were prepared in 100% methanol for each drug. These solutions were diluted with methanol to obtain 100 and 20 mg/l working solutions. A 5 mg/l IS solution dissolved in methanol was also prepared.

In order to obtain representative plasma for method development and validation, a plasma pool was prepared mixing in a proportional way [34,35] six plasmas obtained from different healthy volunteers.

During SPE optimization step plasma samples were daily spiked with 1000 μg/l concentration of each analyte before the SPE procedure and IS was added with a 500 μg/l concentration after the SPE procedure, with the aim of observing the variations in the recovery of the analytes. Contrary to this, in the validation step IS was added together with the analytes before the SPE procedure.

Calibration standards were prepared by spiking a pool of drug-free human plasma with the working standard solutions. Chlorthalidone calibration curve was built from 31 to 3000 μg/l ($n = 9$), valsartan-M1 from 20 to 1000 μg/l ($n = 9$), valsartan from 10 to 5000 μg/l ($n = 9$) and fluvastatin from 14 to 1000 μg/l ($n = 9$). Quality control (QC) samples used for stability assays were prepared in a low (200 μg/l) and a high (1000 μg/l) concentration for each analyte by spiking the drug-free human plasma sample with the appropriate working standard solution volumes.

2.4. Plasma sample collection

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

Nineteen blood samples were collected from different patients under treatment with at least one of the analyzed drugs or a combination (five samples) of them between 1 and 12 h after the oral intake of the drugs. Blood samples were immediately transferred into tubes containing 18 mg of dipotassium ethylendiamine tetraacetic acid (K2EDTA) per 10 ml of blood (BD Vacutainer Systems, Plymouth, UK) and gently mixed. Then, they were centrifuged at 1.301 × *g* for 10 min at 4 °C. The plasma supernatant was carefully separated from blood cells and collected in polypropylene tubes to be frozen at −20 °C until analysis.

Table 1
Gradient elution conditions.

Time (min)	Phase A (%)	Phase B (%)	Flow rate (ml/min)
0	25	75	1.0
3	25	75	1.0
4.5	25	75	1.2
6	45	55	1.2
10	55	45	1.2
12	75	25	1.2
14	75	25	1.2
17	25	75	1.0

2.5. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile (ACN) containing 0.01% formic acid and ammonium formate (10 mM) (A) and 0.01% formic acid and ammonium formate (pH 4.1; 10 mM) (B) delivered in gradient mode (Table 1). They were prepared by means of a 1:20 dilution of a 0.2% formic acid and 200 mM ammonium formate aqueous solution. Both mobile phases were filtered through a 0.45 μm type HVLP Durapore membrane filter from Millipore. The samples were kept at 10 ± 1 °C in the autosampler and the injected volume was 10 μl. The chromatographic separation was performed at 40 ± 1 °C. The eluent was monitored with a fluorescence detector at 254 and 378 nm excitation and emission wavelengths, respectively. Photodiode array UV detector was also used. The wavelengths selected for the monitorization were: 229 nm for chlorthalidone, 254 nm for valsartan and its metabolite and 236 nm for fluvastatin. After the gradient separation, the column was re-equilibrated for 3 min.

2.6. Solid-phase extraction procedure

2.6.1. OVAT optimization

The large number of variables in the SPE procedure and the appearance of interferences would imply an extremely complicate experimental design. In order to simplify this step some variables were optimized by the traditional one-variable-at a time (OVAT) optimization before carrying out the experimental design. These variables were: protein precipitator agent, pH of conditioning and washing solutions, washing solution composition and elution solvent.

2.6.2. Chemometrical optimization

Once previous variables were fixed, optimization via experimental design was carried out. Parameters optimized were: concentration of conditioning and washing buffer solutions, drying time and activation, conditioning, washing and elution volumes. First, a screening step was carried out by a fractional factorial design (FFD) in order to study the effects of the variables [36].

The two levels FFD involved 16 experiments, carried out randomly by duplicate to avoid systematic errors and additional three experiments were repeated at the central point in order to estimate the experimental variance and the significance of each variable. The evaluated response was the ratio analyte area/IS area. In order to study the presence of endogenous interferences throughout the optimization process, peak symmetry and PDA spectra were checked at each experimental point, finding no trace of coeluting compounds. Blank human plasma samples were analyzed at the optimized conditions in order to cross check the absence of interfering peaks.

The data analysis of the results was performed using The Unscrambler program (CAMO, Oslo, Norway) [37]. In order to test the significance of the different variables and their interactions, analysis of variance (ANOVA) was applied. This analysis compares

the variance of the responses with the residual variance which summarizes experimental error; these ratios have a statistical distribution which is used for significance testing. The higher the ratio, the more important is the effect.

In this way, effects were declared significant (+/−) or non-significant (NS) after *p*-value analysis. The factors with *p*-values lower than 0.05 (significance level of 95%) were considered as "statistically significant". The grade of significance increased (++/− −) when *p*-value < 0.01.

Once the effects were evaluated, optimal values for the most significant parameters were found by using a central composite design (CCD) [38]. The CCD was built using the same variables as in the FFD, but excluding those which lacked significance. In this case buffer concentration, drying time and elution volume were involved in the design. Also in this case, the evaluated response was the ratio analyte area/IS area.

The CCD permits to model surface responses by fitting a second order polynomial model with a number of experiments equal to $2^k + 2k + n$ with k is the number of variables and n the number of extra points at the centre of the design. A CCD consisting of a cube samples (2^3) with star points (2×3) placed at $\pm\alpha$ from the central point of the experimental domain was applied. The axial size (α) value was 1.68 that establishes the rotatability condition. The CCD matrix consisted of 16 random experiments in which the central point value was measured twice. All the experiments were performed in triplicate.

The five-level CCD parameter variations and consequent responses allows for the fitting of a quadratic model to the data. For an experimental design with three factors, the model including linear, quadratic, and cross terms which can be expressed as (Eq. (1)):

$$Y = \beta_0 + \beta_A X_A + \beta_B X_B + \beta_C X_C + \beta_{AB} X_A X_B + \beta_{AC} X_A X_C + \beta_{BC} X_B X_C + \beta_{AA} X_A^2 + \beta_{BB} X_B^2 + \beta_{CC} X_C^2 \quad (1)$$

where Y is the response to be modelled, β is the regression coefficients and X_A, X_B and X_C represent buffer concentration (A), drying time (B) and elution volume (C), respectively.

Upon the basis of the obtained responses, The Unscrambler program directly defined a multiple linear regression model (MLR) for each response. Based in the adjustment parameters obtained after carrying out ANOVA analysis to these models, the response surface plots were built in order to select optimal conditions. However, to obtain a simple and yet a realistic model, the insignificant terms (*p*-value > 0.05) should be eliminated from the model through 'backward elimination' process. Office Excel™ 2007 (Microsoft Corporation, Redmond, Seattle, USA) software was used with this goal.

A non-significant parameter can have an influence on the response surface when the absolute value of the parameter is large (in comparison with the rest of the β values) but the *p*-value is higher than 0.05 since the standard deviation of the β is also large and thus the probability of β to be zero is also high (>0.05).

Redefined three dimensional response surface plots were used to establish the optimal condition for the SPE procedure.

2.6.3. Optimized extraction procedure

1 ml plasma was spiked with 100 μl of IS solution to achieve 500 μg/l concentration. Next, 1.5 ml of acetonitrile was added followed by vortex-mixing and centrifugation for 5 min at 10.621 × *g* and 20 °C. Supernatant was transferred to 6 ml glass tubes and it was partially dried under N_2 stream for 15 min at 60 °C in order to evaporate acetonitrile excess.

The clean-up procedure was performed using Strata-X polymeric C18 reverse phase cartridges (30 mg bed, 1 ml volume capacity) purchased from Phenomenex (Torrence, CA, USA).

The SPE cartridges were activated with 0.5 ml methanol and conditioned with 0.5 ml acetate buffer solution (pH 4; 85 mM). Plasma samples were applied to the cartridges and washed with 1 ml MeOH:acetate buffer solution (pH 4; 85 mM) (30:70, v/v). After 5 min drying at high vacuum, 1 ml of methanol was used for eluting the analytes.

The eluent was evaporated to dryness under a N_2 stream at 60 °C. The residue was reconstituted with 100 μl of methanol:aqueous mobile phase solution (70:30, v/v), vortex mixed, filtered with GHP (hydrophilic polypropylene, 0.2 μm, 13 mm diameter) filters supplied by PALL (Ann Arbor, MI, USA), transferred to autosampler vials and subsequently injected into the HPLC system for analysis.

2.7. Assay validation

In order to demonstrate the suitability of the developed analytical method, validation was carried out following FDA [32] and ICH [33] recommendations. In this way, recovery, linearity, working range, intra and inter assay accuracy and precision, limit of quantitation (LLOQ), selectivity and stability were tested for each analyte.

The method's selectivity was tested by analyzing, under optimized chromatographic conditions, blank human plasma samples from eight different sources, and by comparing them with spiked plasma samples at a concentration close to the LLOQ.

To calculate the recovery of the SPE procedure six replicates of spiked plasma samples at three different concentration levels of the four analytes were used. The obtained analyte/IS peak area ratios of samples spiked prior to SPE procedure, were compared with those obtained from samples spiked just before the evaporation step and after SPE. In all cases the IS was spiked just before the evaporation step.

Calibration curves consisting of duplicate calibration standards for each concentration were analyzed on three different days for linearity studies. The working ranges were defined considering the normal therapeutic concentration ranges [6,10,12,39]. The expected ranges were extended in order to detect potential overdoses. LLOQ was calculated by interpolating the value obtained from multiplying 10 times the signal-to-noise ratio in the calibration curve.

Three samples, corresponding to low, medium and high concentration levels, were assayed in sets of five replicates in order to evaluate the intra- and interday accuracy and precision. This procedure was repeated in three different days. The deviation of the mean from the true value, expressed as relative error (RE), served to measure the accuracy. In the same way relative standard deviation (RSD) was used to express the precision.

Stability of the four analytes was evaluated by comparing the corrected areas (analyte/IS area) of the QC samples with those obtained for samples subjected to stability tests. During long-term stability studies samples were stored frozen at −20 °C for 1, 4 and 8 weeks; during short-term stability samples were kept at room conditions for 2, 4, 6 and 24 h, and also for 24 h in the autosampler; and stability after three freeze–thaw cycles. The procedure also included a stability study of analytes in the stock solutions for 1, 7, and 30 days.

3. Results

3.1. Chromatographic separation

The pH value was a critical variable for the separation of the drugs studied. On the one hand the pH of the mobile phase was limited by the native fluorescence of valsartan and its metabolite, which disappears in the basic form ($pK_a = 3.7$). On the other

hand, spectrophotometric studies showed that fluvastatin suffered degradation in acidic conditions.

Mobile phases in different formic acid/formate proportion were tested in order to establish the range where fluvastatin was stable and ARA-II analytes kept their fluorescence. 0.01% formic acid/10 mM ammonium formate (pH 4.1) was chosen as appropriate buffer for both organic and aqueous phases (see Section 2.5).

In order to achieve a suitable separation of analytes from endogenous compounds of plasma matrix, gradient elution mode showed in Table 1 was chosen.

3.2. Solid-phase extraction procedure

3.2.1. OVAT optimization

Different protein precipitants were tested according to the studies carried out by several authors [40,41]: methanol, acetonitrile, phosphoric acid (0.5 M), zinc sulphate (10%, w/v):NaOH (0.5 M) (1:1) and saturated ammonium sulphate. All of them were added in precipitant:plasma (2:1) proportion, except ammonium sulphate which was used in (3:1) proportion. After precipitant agent addition, samples were vortex mixed and centrifuged for 5 min at 10.621 × *g* and 20 °C (4 °C in the case of the phosphoric acid).

Despite the good recoveries obtained with phosphoric acid for the majority of analytes, fluvastatin degradation was observed in acidic conditions. Therefore acidic precipitants were avoided. The highest recoveries were obtained with organic solvents. No significant differences between acetonitrile and methanol were found, but the first one offered a more compact precipitate minimizing the risk of cartridge obstruction. Due to this fact acetonitrile was used for protein precipitation.

Conditioning and washing solutions at different pH values (from 2 to 7) were tested, keeping the same pH for conditioning and washing steps. According to obtained recoveries acetate buffer solution at pH 4 was chosen as optimal solution for cartridge conditioning and washing.

In order to get the optimal washing solution composition, washing profiles were constructed using two organic modifiers with different eluent strength: methanol and acetonitrile. For this aim, different washing solutions, covering the range from 0% to 90% organic modifier proportion, were assayed using previously chosen acetic acid/acetate buffer solution as aqueous phase (Fig. 2). According to analytes' recoveries and eluted interferences, MeOH:acetic acid/acetate buffer solution (30:70, v/v) was chosen for the washing step.

Solvents with different polarities (methanol, acetonitrile, acetone, chloroform, propan-1-ol, tetrahydrofuran, ethyl acetate, dichloromethane and diethyl ether) were tested for the elution step. Although the lower number of interferences was obtained with non-polar eluents, the recoveries reached were not satisfactory. Therefore, methanol, which offered the highest recoveries with the lowest number of interferences, was chosen as elution solvent.

When the proportion of acetonitrile in reconstitution solution was higher than that in the initial gradient conditions of the mobile phase, band broadening was observed (especially in the chromatographic peaks with shorter retention times). In order to avoid this, methanol was used instead of acetonitrile as solvent for the reconstitution solution.

3.2.2. Chemometrical optimization

Once reduced the number of the variables affecting the SPE procedure, concentration of conditioning and washing buffer solutions (*A*), washing volume (*B*), drying time (*C*), elution volume (*D*), activation volume (*E*) and conditioning volume (*F*) were optimized by using experimental design.

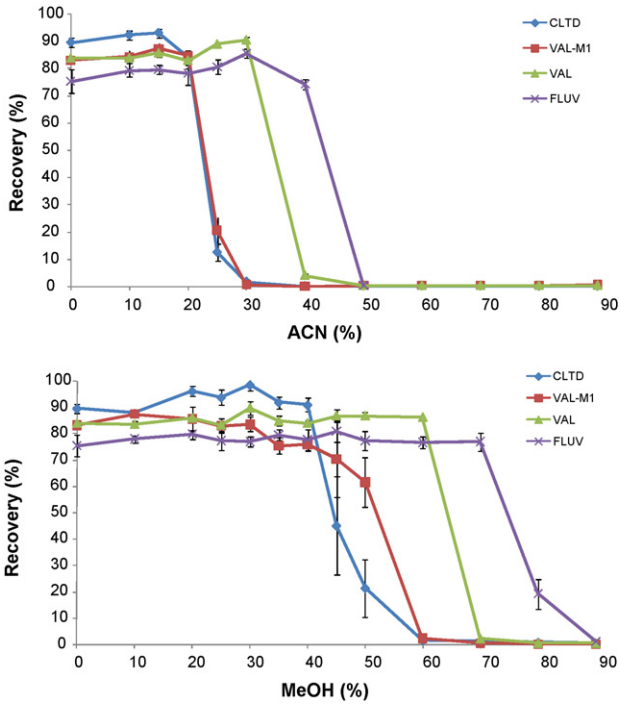

Fig. 2. Washing profiles, acetonitrile: acetate buffer (pH 4; 50 mM) (above) and methanol:acetate buffer (pH 4; 50 mM) (below) for human plasma sample spiked with 1000 μg/l of CLTD, VAL-M1, VAL and FLUV.

3.2.2.1. Screening phase: FFD. A FFD was used for the screening step. In order to perform a low number of experiments (2^{6-2}, resolution IV), variables *E* and *F* were combined with the others in a balanced way (*E* = *ABC* and *F* = *BCD*). The high, medium and low levels for each variable are shown in Table 2.

Obtained data (ratio analyte area/IS area) were fitted to a mathematical model using a multiple regression algorithm, based on ordinary least squares regression. These regression equations (one per analyte) were statistically evaluated by ANOVA at the 5% significance level, in order to estimate and determine effects and interactions. Model suitability was checked regarding the obtained R^2 (percentage of variance explained) for each response model and studying residuals distribution. The R^2 found showed good fit in all cases (Table 3). Both variables' and studied interactions' residuals did not diverge significantly from the normal distribution.

Effects of different variables and their interactions were studied using data obtained from the ANOVA. As it can be seen in Table 3, buffer concentration (*A*), drying time (*B*) and elution volume (*D*) had a significant effect (p-value < 0.05).

Since activation and conditioning volume (*E* and *F*) effect on analytes recoveries were negligible, the minimum value (0.5 ml) was chosen for both variables. The effect of washing volume on

Table 2
Variables studied in the SPE optimization procedure with a fractioned factorial experimental design (FFD, 2^{6-2} experiments, resolution IV) at low (−), medium (0) and high (+) levels.

Variable	Level		
	−	0	+
Buffer concentration, *A* (mM)	50	75	100
Washing volume, *B* (ml)	0.5	1.25	2
Drying time, *C* (min)	2	6	10
Elution volume, *D* (ml)	0.5	1.25	2
Activation volume, *E* (ml)	0.5	1.25	2
Conditioning volume, *F* (ml)	0.5	1.25	2

Table 3
Significance of variables and their interactions studied in the SPE optimization procedure over the ratio analyte area/IS area obtained after FFD (2^{6-2} experiments, resolution IV) in the screening phase.

Variable	CLTD	VAL-M1	VAL	FLUV
Buffer concentration (*A*)	**0.0047 (++)**	0.1486 (NS)	**0.0446 (+)**	**0.0156 (+)**
Washing volume (*B*)	0.0972 (NS)	0.0975 (NS)	0.1807 (NS)	0.0591 (NS)
Drying time (*C*)	**0.0024 (− −)**	**0.0194 (−)**	**0.0285 (−)**	**0.0337 (−)**
Elution volume (*D*)	**0.0034 (++)**	**0.0333 (+)**	**0.0430 (+)**	**0.0438 (+)**
Activation volume (*E*)	0.6287 (NS)	0.5933 (NS)	0.6058 (NS)	0.4279 (NS)
Conditioning volume (*F*)	0.5534 (NS)	0.1651 (NS)	0.1840 (NS)	0.1884 (NS)
AB = *CE*	**0.0130 (+)**	**0.0453 (+)**	**0.0297 (+)**	0.1224 (NS)
AC = *BE*	**0.0047 (++)**	**0.0404 (+)**	**0.0353 (+)**	0.3293 (NS)
AD = *EF*	**0.0116 (−)**	0.1729 (NS)	0.1991 (NS)	0.1385 (NS)
AE = *BC* = *DF*	**0.0452 (+)**	0.1038 (NS)	0.0831 (NS)	0.4270 (NS)
AF = *DE*	0.7471 (NS)	0.4764 (NS)	0.3743 (NS)	0.3592 (NS)
BD = *CF*	0.1023 (NS)	0.9307 (NS)	0.2200 (NS)	0.1033 (NS)
BF = *CD*	**0.0060 (++)**	0.1434 (NS)	**0.0350 (+)**	0.9456 (NS)
R^2	0.915	0.898	0.893	0.965

The significant values ($p < 0.05$) are in bold, and the effect in parenthesis.

Table 4
Variables studied in the SPE optimization procedure with a central composite design (CCD) at cube (±1), star (±α) and center (0) levels.

Variable	Level				
	−α	−1	0	+1	+α
Buffer concentration, *A* (mM)	6.2	30.0	65.0	100.0	123.8
Drying time, *B* (min)	0.6	3.0	6.5	10.0	12.4
Elution volume, *C* (ml)	0.2	0.5	1.0	1.5	2.0

the recovery of the SPE was insignificant too, but it was observed that the endogenous compounds coeluting with the analytes decreased with increasing the washing volume, obtaining cleaner extracts. Nevertheless, the use of volumes greater than 1 ml did not improve the cleaning step. In this way, 1 ml was chosen as optimal value.

3.2.2.2. Optimization phase: CCD. Optimization design was built in order to find the optimal values for significant variables: buffer concentration (*A*), drying time (*B*) and elution volume (*C*).

Since the optimization procedure was too long to be completed in one day, CCD experiments were divided in two days. Fortunately, this kind of design consists on two main sets of experiments: cube and star samples. Each sample set contributes independently to the quadratic model, so sets can be performed in different days. In this case, the first day cube experiments (8) and the central sample were performed by triplicate (8 × 3; 1 × 3). The next day, star samples (6) and the central sample analysis were carried out by triplicate (6 × 3; 1 × 3) (Table 4).

Using The Unscrambler software, obtained results were statistically treated by means of MLR in order to build the response

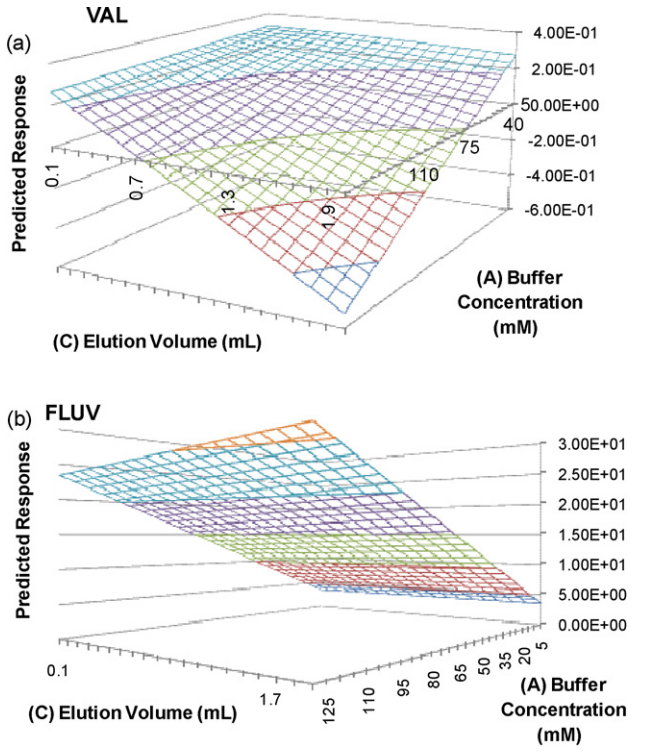

Fig. 3. Responses surfaces obtained after MLR regression in the SPE optimization design (CCD) corresponding to: (a) VAL; (b) FLUV. In (a), *B* variable does not affect. In (b), *B* variable has been fixed in 5 min. (a) presents the same tendency as CLTD and VAL-M1 (responses not shown).

Table 5
β-Coefficients and significance of variables and their interactions studied in the SPE optimization procedure over the ratio analyte area/IS area obtained after CCD.

Affecting variables/interactions	CLTD	VAL-M1	VAL	FLUV
A (buffer concentration)	$\mathbf{-1.33 \times 10^{-4}}$ (−)	$\mathbf{-1.49 \times 10^{-4}}$ (− −)	$\mathbf{-1.46 \times 10^{-4}}$ (− −)	$\mathbf{1.97 \times 10^{-4}}$ (++)
B (drying time)	$\mathbf{-2.93 \times 10^{-3}}$ (− −)	$\mathbf{-1.76 \times 10^{-3}}$ (− −)	$\mathbf{-2.18 \times 10^{-3}}$ (− −)	$\mathbf{-9.19 \times 10^{-4}}$ (−)
C (elution volume)	$\mathbf{+1.06 \times 10^{-2}}$ (+)	$\mathbf{+6.66 \times 10^{-3}}$ (+)	$\mathbf{+5.68 \times 10^{-3}}$ (+)	$\mathbf{1.21 \times 10^{-2}}$ (++)
$A \times B$	$+1.91 \times 10^{-3}$ (NS)	$+2.38 \times 10^{-3}$ (NS)	$+2.16 \times 10^{-3}$ (NS)	$\mathbf{-3.55 \times 10^{-3}}$ (−)
$A \times C$	$+4.19 \times 10^{-4}$ (NS)	-1.93×10^{-3} (NS)	$\mathbf{-3.15 \times 10^{-3}}$ (−)	$\mathbf{3.91 \times 10^{-3}}$ (+)
$B \times C$	$+3.16 \times 10^{-3}$ (NS)	$+1.63 \times 10^{-3}$ (NS)	$+1.49 \times 10^{-3}$ (NS)	$+1.19 \times 10^{-3}$ (NS)
A^2	-1.15×10^{-3} (NS)	-1.29×10^{-3} (NS)	-1.58×10^{-3} (NS)	-2.60×10^{-3} (NS)
B^2	$\mathbf{-1.38 \times 10^{-2}}$ (− −)	$\mathbf{-8.17 \times 10^{-3}}$ (− −)	$\mathbf{-8.19 \times 10^{-3}}$ (− −)	-2.56×10^{-3} (NS)
C^2	$\mathbf{-9.82 \times 10^{-3}}$ (− −)	$\mathbf{-5.13 \times 10^{-3}}$ (− −)	$\mathbf{-6.98 \times 10^{-3}}$ (− −)	$+9.88 \times 10^{-4}$ (NS)
R^2	0.751	0.791	0.819	0.730

The significant values ($p < 0.05$) are in bold, and the effect in parenthesis.

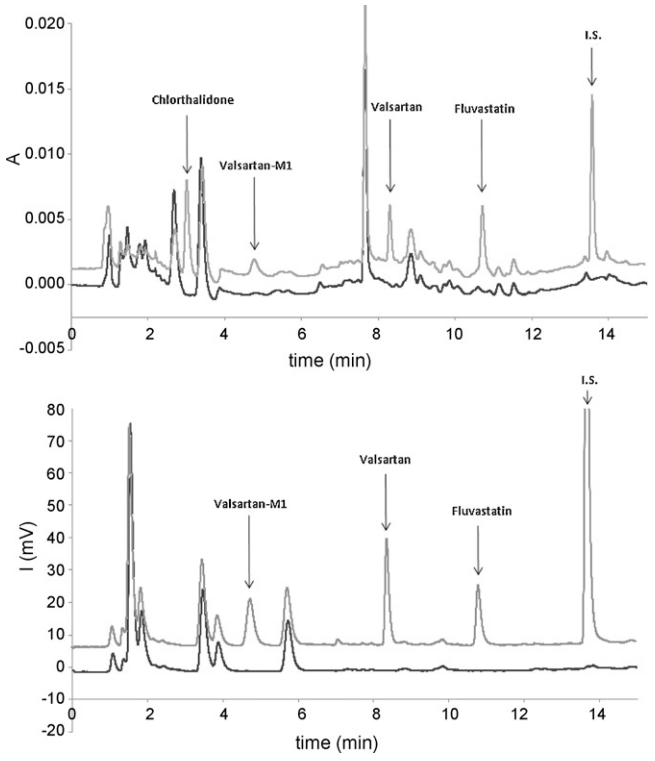

Fig. 4. Chromatograms obtained for blank plasma sample (black) and a 200 μg/l spiked plasma sample with 500 μg/l IS (grey). Photometric (above) and fluorimetric detection (below).

surfaces. Classical statistical tools, as ANOVA and residual analysis, were employed to validate each model.

At first, the obtained models (one for each analyte) showed the lack of fit of experimental data. Experiment corresponding to the star point for the *C* variable (0.16 ml of elution volume), found to be an outlier. The model obtained after rejecting the values for this experiment, showed an adequate distribution of the residuals. The error of each model, that is, what each model fails to explain, in all cases was less than 4.0×10^{-3}. The R^2 values were in the range of 0.730–0.819 (see Table 5). Models' suitability was acceptable since the obtained R^2 for each response model were within an adequate order and distributions of residuals were random.

Once the model's suitability was checked, optimal values were found according to the response surfaces. However, in order to build the response surfaces and since The Unscrambler considers all adjustment parameter, no matter if they are significant or not, the Office Excel™ 2007 software was used and the best conditions were fixed in accordance to these surfaces.

In the case of CLTD and VAL-M1, no interaction between variables was found. Two dimensions response plots were enough to evaluate the affecting sense of the system. In the case of VAL, $A \times C$ interaction was observed and the corresponding response surface was plotted in three-dimensional space. As in the case of CLTD and VAL-M1, the response increased as the values of *A* and *C* decreased. Value of *B* was predicted as well as for previous analytes: response decreased with the drying time.

The prediction for FLUV was more complex, due to $A \times B$ and $A \times C$ interactions. As it can be shown in Fig. 3, trends found were totally opposed to the three previous analytes.

As analytes with different chemical properties were simultaneously studied, the obtained optimal conditions for all analytes were also different. Therefore compromise decisions had to be taken in order to obtain the maximum common recovery. On this way 85 mM buffer concentration, 5 min drying time and 1 ml elution volume were chosen.

3.3. Assay validation

3.3.1. Selectivity

In the present study, selectivity has been studied by analyzing eight plasma samples from different healthy volunteers. As the ICH guideline requires [33], the studied blanks showed neither area values higher than 20% of the LLOQ's areas at the analytes retention times nor higher than 5% of the IS area at its corresponding retention time. Representative chromatograms obtained from control human plasma and plasma spiked with 200 μg/l of each analyte, and 500 μg/l of candesartan cilexetil (IS) are shown in Fig. 4.

3.3.2. Recovery

The recoveries were calculated for each analyte in low, medium and high concentrations ($n = 6$) and were found between 78% and 91% as shown in Table 6. As it was expected, the recoveries obtained with both detectors are comparable, except at the low concentration for fluvastatin, probably due to the worse sensitivity of the UV compared to fluorimetric detection.

3.3.3. Linearity, LLOQ and working range

Calibration curves were obtained plotting the corrected area (ratio analyte area/IS area) for each concentration level versus the nominal concentration levels corresponding to each standard solution. The calibration curves generated were fitted to a regression line by applying the lineal regression model based on the least square method. At least, seven concentration levels were used in all calibration curves. The correlation coefficients ranged from 0.993 to 0.999 for all the compounds and slope and intercept values showed good reproducibility between days.

The limit of quantitation (LLOQ) was calculated from a relationship S/N equal to 10. The concentrations obtained by UV detection were 31, 41, 44 and 85 μg/l for chlorthalidone, valsartan-M1, valsartan and fluvastatin, respectively. Limits of quantitation obtained by fluorimetric detection were 20, 10 and 14 μg/l for valsartan-M1, valsartan and fluvastatin, respectively.

Table 6
Recoveries obtained with photometric and fluorimetric (Fluo.) detection modes ($n = 6$).

	Concentration CLTD (μg/l)			Concentration VAL-M1 (μg/l)			Concentration VAL (μg/l)			Concentration FLUV (μg/l)		
	200	1000	2500	200	500	1250	200	1000	2500	200	500	1250
UV												
Recovery (%)	91.1	90.1	87.0	80.8	82.4	80.9	82.5	79.8	78.9	91.2	78.1	79.6
RSD (%)	0.6	2.0	1.2	2.3	2.4	1.3	1.5	2.6	1.0	2.0	3.8	3.1
Fluo												
Recovery (%)				82.4	82.2	80.4	82.3	79.3	78.3	82.0	80.1	80.7
RSD (%)				0.7	2.8	1.3	1.1	2.8	1.4	2.1	3.4	3.4

Table 7
Precision and accuracy obtained for three different analyte concentration levels ($n = 5$) using UV and fluorimetric (Fluo.) detection modes.

		Concentration chlortalidone (μg/l)			Concentration valsartan-M1 (μg/l)			Concentration valsartan (μg/l)			Concentration fluvastatin (μg/l)		
		40	600	2900	40	600	1100	40	600	2900	40	600	1100
UV													
RSD (%)	Intraday	4.5	3.6	3.0	4.4	3.7	4.7	5.7	4.0	3.6	6.4	3.6	2.9
	Interday	7.0	5.7	3.1	7.8	6.3	5.3	9.6	5.8	4.4	13.0	3.9	3.2
RE (%)	Intraday	8.8	4.2	0.6	12.1	4.8	1.5	5.6	4.2	2.6	11.7	2.9	3.5
	Interday	5.8	1.5	0.9	11.9	1.0	1.1	3.7	0.6	3.0	3.8	1.3	3.2
Fluo.													
RSD (%)	Intraday				4.5	4.2	6.3	5.8	4.8	3.7	4.8	2.9	4.5
	Interday				6.8	7.2	7.6	7.9	7.7	4.2	8.6	4.2	5.6
RE (%)	Intraday				2.3	5.3	2.8	5.0	6.8	3.4	8.0	2.7	3.8
	Interday				9.4	1.5	2.2	11.3	2.8	3.8	1.8	2.1	1.8

Calibration standards did not exceed the limit value (RE > 15%) for the interpolated concentration with regard to nominal concentration. The precision and accuracy of the LLOQ were acceptable since the RSD and RE values were lower than 20%. Therefore, the calibration curves were accepted for the linear ranges established: 31–3000 μg/l for chlorthalidone, 20–1000 μg/l for valsartan-M1, 10–5000 μg/l for valsartan and 14–1000 μg/l for fluvastatin.

3.3.4. Precision and accuracy

Plasma samples spiked with low, medium and high concentrations of drugs were prepared and their concentrations were obtained from interpolation of their respective calibration curves. The intra- and interday accuracy (RE) and precision (RSD) is summarized in Table 7. As it can be seen, intraday precision varied between 2.9% and 6.4%, and interday precision between 3.1% and 13.0%. Intraday accuracy varied from 0.6% to 12.1% and interday accuracy from 0.6% to 11.9%. Obtained values agree with the FDA and ICH recommendations.

3.3.5. Stability

The ARA-II drugs were found to be stable under the studied stability conditions. The obtained responses did not change significantly thus indicating no substance loss during repeated thawing and freezing as well as long- and short-term stability tests.

The stability of chlorthalidone, fluvastatin, valsartan and its metabolite in methanolic solutions at refrigerator temperature of 4 °C for two months was also found to be acceptable with deviations from the QC concentrations always below 5%.

When stability at room conditions (25 °C and light exposure) was studied, chlorthalidone, valsartan and its metabolite were sta-

Table 8
Concentration values obtained for studied drugs after SPE extraction of plasma samples obtained from five patients under combined cardiovascular treatment.

Patient	Co-administered drugs		Concentration (μg/l)			
			CLTD	VAL	FLUV	VAL-M1
1	Alendronic acid					
		Ingested amount (mg)	Higrotona 25 mg	Diovan 160 mg	–	–
		t after ingestion (h)	1 h 50 min	1 h 50 min		1 h 50 min
		UV detection	303.0 ± 21.8	4164 ± 254	–	286.1 ± 31.8
		Fluorescence detection	–	3750 ± 232	–	283.7 ± 13.9
2	Alendronic acid					
		Ingested amount (mg)	Higrotona 25 mg	Diovan 80 mg	–	–
		t after ingestion (h)	1 h 30 min	1 h 30 min		1 h 30 min
		UV detection	385 ± 22	2779 ± 230	–	320.2 ± 32.4
		Fluorescence detection	–	2564 ± 215	–	314.7 ± 14.0
3	Morphine sulphate, amitriptiline chloridrate, estriol					
		Ingested amount (mg)	Higrotona 25 mg	Diovan 160 mg	–	–
		t after ingestion (h)	2 h 40 min	2 h 40 min		2 h 40 min
		UV detection	123.6 ± 22.6	1216 ± 222	–	66.3 ± 31.2
		Fluorescence detection	–	1187 ± 211	–	52.5 ± 14.4
4	Atenolol, alopurinol					
		Ingested amount (mg)	Tenoretic 25 mg	–	Vaditon prolib 80 mg	–
		t after ingestion (h)	12 h 10 min		1 h 10 min	
		UV detection	331.7 ± 21.8	–	114.1 ± 20.1	–
		Fluorescence detection	–	–	72.6 ± 13.0	–
5	Lormetazepam, alopurinol, ascorbic acid, potassium bicarbonate					
		Ingested amount (mg)	Higrotona 50 mg	Vals 160 mg	–	–
		t after ingestion (h)	11 h 35 min	1 h 55 min		1 h 55 min
		UV detection	533.0 ± 23.3	2474 ± 229	–	175.4 ± 31.3
		Fluorescence detection	–	2515 ± 215	–	158.7 ± 13.8

ble. On the other hand, the chromatographic signal of fluvastatin changed with the time, surely due to the photodegradation of the molecule reported by Mielcarek et al. [42]. As result of this degradation two new chromatographic peaks appeared (results not shown). This degradation was not significant during required analysis time, but in order to avoid it, samples' light exposure was minimized and amber vials were used.

3.4. Application to real samples

The developed method has been applied to plasma samples obtained from patients under cardiovascular treatment with chlorthalidone, valsartan, fluvastatin or a combination of them. These patients were also treated with other co-administered drugs: β-blockers, ARA-II, diuretics or statins.

Real samples were preferably taken at t_{max} (1 h for fluvastatin, 2–3 h for valsartan and chlorthalidone [6,10,12,39]). Plasma concentration values (expressed as mean ± SD (μg/l)) found for clinical samples were obtained by interpolation from the daily calibration curves. A total of 19 samples were analyzed, 5 of those had a combination of studied drugs. Obtained plasmatic concentrations for these five samples are collected in Table 8.

Chromatograms corresponding to plasma samples of patients under treatment with chlorthalidone and valsartan or chlorthalidone and fluvastatin are shown in Figs. 5 and 6. In all cases, no interferences between analytes and co-administered drugs were observed. However, there is a double peak in the signal corresponding to valsartan-M1. It is worth noting that this double peak only appears in real samples and not in the spiked ones. Furthermore, there is a double peak both with UV and fluorimetric detection and no interference appeared at that retention time on samples without valsartan. Due to these facts, this double peak could be probably due to a metabolic transformation. In order to confirm this hypothesis, LC–MS studies should be carried out.

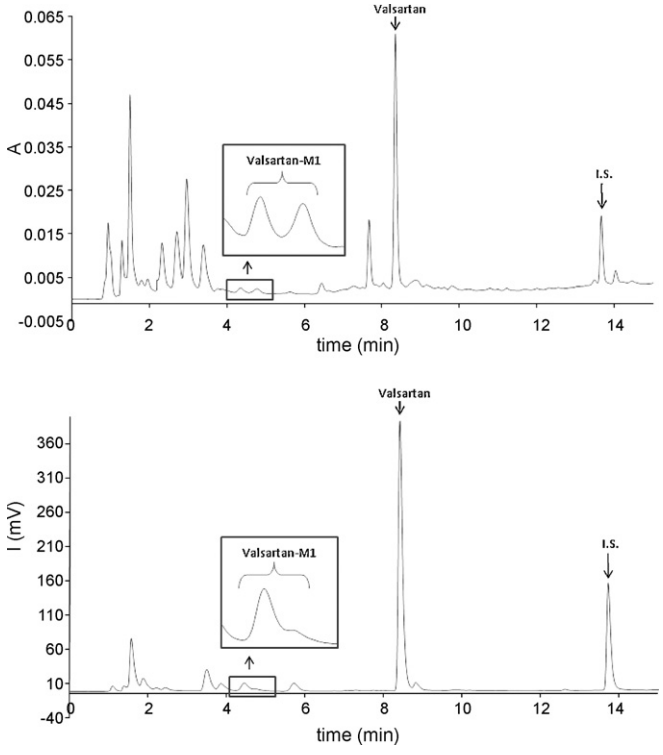

Fig. 5. Chromatograms obtained for a plasma sample collected from a patient under cardiovascular treatment with VAL (160 mg) and CLTD (25 mg) 2 h and 12 h after the oral intake of VAL and CLTD, respectively. IS: candesartan cilexetil 500 μg/l. Photometric (above) and fluorimetric detection (below) (Patient-5).

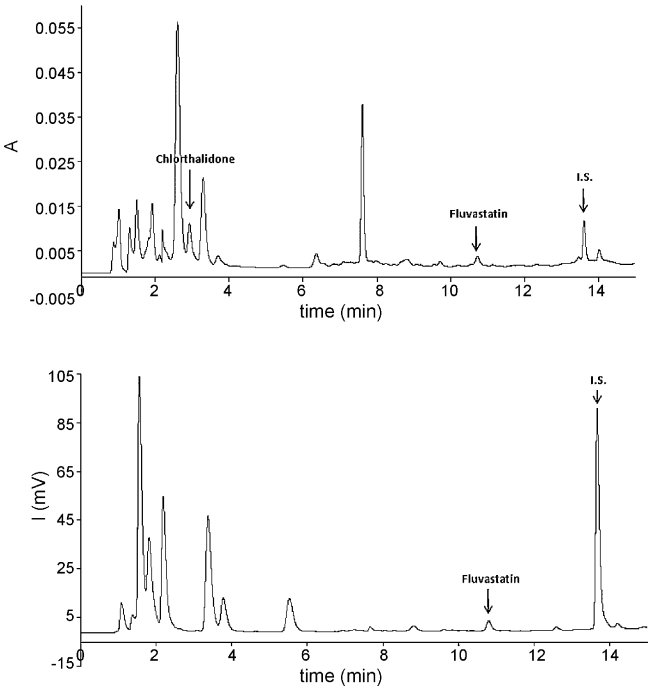

Fig. 6. Chromatograms obtained from a plasma sample collected from a patient under cardiovascular treatment with FLUV (80 mg) and CLTD (25 mg) 1 h and 12 h after the oral intake of FLUV and CLTD, respectively. IS: candesartan cilexetil 500 μg/l. Photometric (above) and fluorimetric detection (below) (Patient-4).

4. Discussion

Several HPLC methods have been developed for the quantitation of agents closely related to cardiovascular risk factors as hypertension, high cholesterol level or diabetes. But so far, no one has developed a full analytical method to quantify drugs used in a combined cardiovascular treatment, to face the metabolic syndrome as a whole. Only the method developed by Kristoffersen et al. [29] by SPE-LC–MS in post-mortem whole blood samples could be considered, although it is more focused on antihypertensive drugs. In this case, as they faced a 14 drugs analysis they were obliged to reach compromise decisions achieving recoveries under 50% for some analytes (including valsartan, with recovery percentage of 9–18%). In this way, our laboratory has developed a novel analytical method which provides the control of one of the most used treatment consisting of a synergic combination of a diuretic (chlorthalidone) and an ARA-II (valsartan) to control the hypertension, with a statin (fluvastatin) to reduce the high cholesterol levels.

The proposed method, based on the HPLC-PDA-fluorimetric detection is adequate for the quantitation of chlorthalidone, valsartan and fluvastatin in human plasma samples, using candesartan cilexetil as internal standard. The method involved a protein precipitation prior to SPE, with recoveries greater than 78%. It also provides superior sensitivity and selectivity with the fluorimetric detector for fluvastatin, valsartan and its metabolite. Chlorthalidone demanded UV detection due to its lack of native fluorescence. The use of any of the proposed detection types allows the detection of lower quantities of the analytes than those expected for the therapeutic ranges.

The chemometrical approach carried out in this study, reduced the number of experiments necessary to achieve the optimal conditions for the SPE of the four drugs in plasma samples. Nei-

ther endogenous compounds nor other co-administrated drugs in patients showed interferences in terms of selectivity. Thus, the method proved to be selective. The use of this method can save efforts when monitoring patients under treatment with several drugs.

The developed methodology showed appropriate selectivity, linearity, sensitivity and precision, which allowed clinical studies of patients under mentioned therapeutic conditions. The method was rugged and was successfully applied to the determination of plasma samples obtained from patients under combined cardiovascular therapy. It can be concluded that this procedure is an acceptable method for drug monitorization during 24 h after dose intake. This is necessary to ensure that drug plasma levels are kept in the therapeutic level during all the time between drug intakes, in order to maintain the pharmaceutical protection and decrease the incidence of cardiovascular events.

Simultaneously, stability of these four analytes was investigated. Chlorthalidone, valsartan and its metabolite's standard stock solutions and plasma samples remained stable during pretreatment at room temperature, and after storage in refrigerating or freezing conditions. In the case of fluvastatin, as the literature reported [42,43], a slight lack of stability was observed. Despite of the observed slight instability for fluvastantin in the stock solutions and to a lesser extent in spiked plasma samples, stability for this compound was assured for at least 2–3 h, period required in the pretreatment step (under light exposure). At the same time, it was observed the impossibility to work under acidic conditions, because the fluvastatin degraded rapidly. This fact was decisive to carry out the optimization of the extraction procedure, since the possibility of using acidic agents as precipitating proteins agents was rejected. The use of organic solvents needed an evaporation step previous the injection of analytes into the SPE cartridge, so that the extraction time of plasma treatment was lengthening.

Once the validation was successfully completed, processing of real samples showed that when analyzing valsartan-M1, a double peak appears in all the samples taken from patients under treatment with valsartan. This double was not observed when spiked plasma samples were analyzed, as it could be seen by the fact of all the parameters required for validation were successfully overcome. This peak splitting can be attributed to the presence of a new metabolite of valsartan. The absence of interferences at the retention time of valsartan-M1, both in fluorimetric detection and photometric detection, in samples obtained from patients who were not under treatment with valsartan corroborate this hypothesis. This splitting conditions the application of the method to the determination of the metabolite. A further MS study of the splitting occurred for the valsartan-M1 peak in plasma samples obtained from patients under treatment with valsartan should be carried out.

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