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Validation of a fast liquid chromatography–UV method for the analysis of drugs used in combined cardiovascular therapy in human plasma

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ABSTRACT

Ultra-performance liquid chromatography (UPLC) was investigated as a faster alternative to high-performance liquid chromatography (HPLC) for the simultaneous analysis of drugs usually prescribed in cardiovascular therapy. Upon a previously developed and validated solid phase extraction (SPE)–HPLC–photodiode array (PDA)–fluorescence (FLR) method, separation of chlorthalidone (CLTD; diuretic), valsartan and its metabolite (VAL and VAL-M1 respectively; angiotensin II receptor antagonist drugs) and fluvastatin (FLUV; statin) was performed in human plasma using an RP C18 column (50 mm × 2.1 mm, 1.7 μm, Waters Acquity UPLC™ (BEH)) and a tunable UV–vis (TUV) detector. After method transfer, different system variables were modulated to study the evolution of responses of the analytes and the endogenous interferences. The improved method was fully validated and the results were compared with its precursor HPLC method relating to analysis time, efficiency and sensitivity. The studied compounds were separated in less than 8 min and the method showed good linearity (20–3000 μg/L for chlorthalidone, 110–1100 μg/L for valsartan-M1, 67–1900 μg/L for valsartan and 48–1100 μg/L for fluvastatin), precision and accuracy. The proposed method was found to be reproducible (RSD < 10%), accurate (RE < 15%), robust and suitable for quantitative analysis of the studied drugs in plasma obtained from patients under combined cardiovascular treatment.

1. Introduction

The metabolic syndrome, also known as X syndrome or the insulin resistance syndrome, is a clustering of cardiovascular risk factors (low high-density lipoprotein [HDL] cholesterol and elevated triglyceride concentrations, glucose intolerance, obesity, and hypertension) that are associated with a potential risk of developing diabetes type 2 and coronary heart disease [1–4]. The presence of the metabolic syndrome predicts a two- to four-fold increase in the risk of cardiovascular disease and death [5,6], and the risk of developing diabetes type 2 is increased five- to nine-fold [7,8].

The lack of a universally established definition has complicated the epidemiologic research on the prevalence of this syndrome [9]. Nevertheless, around the 10–25% of individuals in the industrialized world are supposed to suffer from this pathology [5,10].

Risk factors of the metabolic syndrome are treated separately and there is currently no available treatment that targets all components [11]. Therefore, a combination of antihypertensive, hypolipemic and antidiabetic drugs is often used [9,12,13]. One of the most used combinations consists of a synergic association of a diuretic and an angiotensin II receptor antagonist (ARA-II) to control the hypertension, with a statin to reduce the cholesterol levels.

Due to the high prevalence of the cardiovascular diseases, several analytical methods for the determination of drug classes used in cardiovascular therapy have been already developed. In this way, determination of different classes of antihypertensive agents in urine have been carried out by CE [14–16], LC–MS [17–21], GC–MS [22–24] or HPLC–photodiode array (PDA)/fluorescence (FLR) [25–28], and in plasma or in serum by LC–MS [29–31] or by HPLC–UV/FLR [32–36].

Hypoolipemiant agents are mainly determined in plasma or serum because of their negligible renal excretion. LC–MS [37–41], GC–MS [42–44] or HPLC–UV/FLR [45,46] are the most commonly used analytical techniques.

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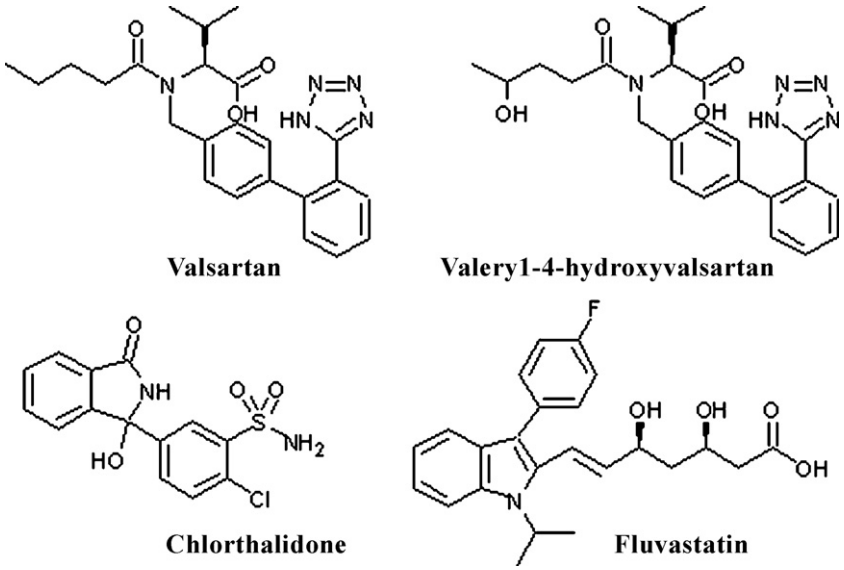

Fig. 1. Chemical structures of analyzed compounds.

These methods can still be insufficient to properly resolve the analysis of the different agents involved in the combined cardiovascular therapy. Complex mixtures of lipid regulating agents (fibrates or statins), antihypertensive drugs (diuretics, ARA-II, β-blockers, inhibitors of angiotensin-converting enzyme (ACE-inhibitors) or calcium channel blockers), anticoagulants and several kinds of pharmaceuticals and personal care products have been determined by LC–MS in surface and wastewater [47], LC–MS/MS (QqQ or Q–ToF) [48–53] or recently by ultra-performance liquid chromatography (UPLC)–MS/MS (QqQ or Q–ToF) [54–57]. In this kind of multicomponent analysis, the chemical properties of the analytes are often very different. This fact leads to compromise solutions when establishing the critical parameters of the extraction process, in order to find an adequate recovery for all the analytes. The application of these methods to biological matrices is very difficult due to the presence of endogenous compounds. Kristoffersen et al. have developed an LC–MS method for the simultaneous determination of antihypertensive drugs of different families and an antiarrhythmic drug in whole blood samples from forensic autopsies. The only method focused on this type of combined therapy has been recently developed in our laboratory by using HPLC–PDA–FLR detection [58], which reports the chemometrical optimization and validation of a quantitative high-performance liquid chromatography method for the simultaneous analysis, in human plasma, of chlorthalidone (CLTD), valsartan (VAL), VAL-M1 and fluvastatin (FLUV). The quantitation limits obtained ranged from 10 μg/L for VAL to 31 μg/L for CLTD.

Most of the analytical methods reported in the literature for the determination of this kind of non-polar drugs are based on high-performance liquid chromatography coupled to mass spectrometry (LC–MS) and particularly tandem mass spectrometry (LC–MS/MS). However, despite the resolution and sensitivity provided by mass spectrometry, due to its high cost, not all laboratories can afford this kind of technology. In the present work, ultra-performance liquid chromatography, a very fast and sensitive technique, has been applied to the simultaneous determination of some antihypertensive and statin drugs, used in combined cardiovascular therapy (Fig. 1). The previous HPLC method developed for the analysis of chlorthalidone, fluvastatin, valsartan and its metabolite [58] has been successfully transferred to UPLC, saving time and resources. It has also been validated proving its suitability for the routine analysis of the studied drugs.

2. Experimental

2.1. Instrumentation

Analyses were performed on an Acquity UPLC™ system (Waters, Milford, USA), consisting of a degasser, a sample and a column manager module coupled to a UV–vis programmable detector.

The tunable UV–vis detector with 10 mm light-guided capillary flow cell and a volume of 500 nL was a two channel UV–vis absorbance detector. For all separations, the UV detector time constant was set to 0.05 s and the data sampling rate was set to 80 Hz to obtain signals of highest quality even with ultra fast separation. The wavelength was set at 220 nm in order to reach maximum absorbance for the four compounds of interest.

System control, data collection and data processing were accomplished using Waters Empower 2 chromatography data software (v.1.0, 2005).

A Waters Acquity UPLC™ BEH C18, 50 mm × 2.1 mm ID, 1.7 μm particle size (Waters, Milford, USA) column was used to perform the separation. A Waters VanGuard™ Pre-column 5 mm × 2.1 mm was placed previous to the analytical column.

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

The pH was 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 (IS), by Astrazeneca (Mölndal, Sweden).

Ammonium formate, 99% purity and pro analysi quality, was purchased from Alfa Aesar (Karlsruhe, Germany) and formic acid, 98% of LC–MS quality, from Fluka (Steinheim, Germany). They were used in the preparation of the pH 4 mobile phase.

Ammonium acetate, 99.99% purity and pro analysi quality, was purchased from Aldrich (Steinheim, Germany). Ammonium bicarbonate, pro analysi LC–MS eluent additive quality, was purchased from Fluka (Steinheim, Germany). Both ammonium acetate and acetic acid, 99% and trace analysis quality, from Fluka (Steinheim, Germany), were used in the preparation of the pH 5 and pH 6 mobile phases. In order to adjust the pH of the mobile phase to 8, diluted acetic acid was added to ammonium bicarbonate. Diluted ammonium hydroxide, pro analysi LC–MS eluent additive quality and purchased from Fluka (Steinheim, Germany), was used for adjusting the pH of the ammonium bicarbonate mobile phase to 10.

HPLC quality methanol (MeOH) and acetonitrile (ACN) were obtained from Scharlab (Barcelona, Spain). Purified water from a Milli-Q Element A10 water system (Millipore, Milford, MA, USA) was used in the preparation of buffer and reagent solutions.

Glacial acetic acid and sodium acetate used for preparing the buffer solutions used in SPE conditioning and washing steps, were both pro-analysis quality and obtained from Merck (Darmstadt, Germany). Their pH was adjusted by using different volumes of 1 M HCl and KOH solutions.

2.3. Standard solutions and spiked plasma samples

1000 mg/L standard solutions of each drug were prepared in 100% methanol. 100 and 20 mg/L multicomponent working solutions were prepared with methanol. A 5 mg/L IS solution in methanol was also prepared.

In order to obtain a representative plasma for method development and validation, a plasma pool was prepared by proportionally mixing six plasmas obtained from different healthy volunteers [59,60].

Calibration standards were prepared by spiking drug-free human plasma with the working standard solutions. CLTD calibration curve was built from 19.6 to 3000 μg/L (n = 8), VAL-M1 from 110 to 1250 μg/L (n = 8), VAL from 66.9 to 5000 μg/L (n = 8) and FLUV from 48.3 to 1250 μg/L (n = 8). In all cases the IS was added (500 μg/L) prior to the extraction procedure.

Quality control (QC) samples used for stability assays were prepared at a low (200 μg/L) and a high (1000 μg/L) concentration for each analyte by spiking the drug-free human plasma 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 stored in polypropylene tubes at −20 °C until analysis.

Blood samples were collected from 19 different patients under treatment with at least one of the analyzed drugs or a combination of them (five samples) 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 (K_2EDTA) per 10 mL of blood (BD Vacutainer Systems, Plymouth, UK) and gently mixed. Then, they were centrifuged at 1.301 × g (3500 rpm) for 10 min at 4 °C. The plasma supernatant was carefully separated from blood cells and transferred to polypropylene tubes which were stored at −20 °C until analysis.

Table 1
Gradient elution conditions.

Time (min)	Phase A_1 (%)	Phase B_1 (%)	Flow rate (mL/min)
0	90	10	0.5
2.6	78	22	0.5
3.9	76	24	0.5
4.7	60	40	0.5
5.9	50	50	0.5
6.5	45	55	0.5
7.8	35	65	0.5
8.0	35	65	0.5
8.1	90	10	0.5
9.5	90	10	0.5

2.5. Chromatographic conditions

The mobile phase consisted of a mixture of an aqueous solution of ammonium formate (pH 4.1, 10 mM) and 0.01% formic acid (A_1), and an organic solution of acetonitrile (ACN) containing ammonium formate (10 mM) and 0.01% formic acid (B_1), delivered in gradient mode at a flow rate of 0.5 mL/min (Table 1). Under these conditions, the backpressure in the system was about 9.700 psi. Mobile phases were filtered through a 0.2 μm type GH Polypro hydrophilic polypropylene membrane filter from PALL (Port Washington, NY, USA). The samples were kept at 10 ± 1 °C in the autosampler and the injected volume was 2.5 μL in a partial loop with needle overfill mode. Amber glass vials were used throughout the study and the window of the autosampler was also covered with an opaque film, to protect FLUV from the light exposure.

The chromatographic separation was performed at 30 ± 1 °C. The eluent was monitored with a tunable UV detector at 220 nm absorption wavelength. After the gradient separation, the column was re-equilibrated and conditioned with 90 volumes of A_1 and 10 volumes of B_1 for 2 min.

200 μL strong (35:65 A_1:B_1) and 600 μL weak (90:10 A_1:B_1) needle wash solutions were injected after sample injection in order to avoid carry over effects. A seal wash solution (90:10 H_2O:ACN) was also programmed every 5 min. All the solutions mentioned above were filtered through 0.2 μm membranes under vacuum before their usage. The total run time of analysis was 10 min. Conical vials were employed to maximize the number of injections per sample.

2.6. Solid phase extraction procedure

The solid phase extraction procedure applied to plasma samples was the one previously optimized in our laboratory by Gonzalez et al. [58]. The reconstituted sample was filtered directly into 100 μL polypropylene inserts (PN 5182-0549, Agilent, Santa Clara, CA, USA). The maximum recovery volume was obtained by placing manually the injector's needle 1.5 mm from the bottom.

2.7. Assay validation

In order to demonstrate the suitability of the transferred and improved analytical method, validation was carried out following the guidelines given by the Food and Drug Administration (FDA) [61] and the International Conference on Harmonization (ICH) [62]. In this way, recovery, linearity, working range, intra- and inter-assay accuracy and precision, lower limit of quantitation (LLOQ), selectivity and stability studies were tested for each analyte.

The method's selectivity was tested by analyzing blank human plasma samples from eight different sources, under optimized

chromatographic conditions and by comparing them with spiked plasma samples at a concentration near 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, were compared with those obtained from samples spiked just after SPE and before the evaporation step. In all cases the IS was added just before the evaporation step.

Calibration curves consisting of duplicate calibration standards for each concentration were analyzed on 3 different days for linearity studies. The working ranges were defined considering the normal therapeutic concentration ranges [63–66]. 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.

Four samples, corresponding to LLOQ, low, medium and high concentration levels were assayed in sets of five replicates in order to evaluate the intra- and inter-day accuracy and precision. This procedure was repeated on 3 different days. The deviation of the mean from the true value, expressed as relative error (RE), was used to measure the accuracy. Precision was calculated in terms of relative standard deviation (RSD).

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, spiked plasma samples were stored frozen at −20 °C for 1, 4 and 8 weeks; during short-term stability, samples were kept at room conditions (25 °C and light exposure) for 2, 4, 6 and 24 h. Furthermore, stability of spiked plasma samples after freeze–thaw cycles was studied. In order to test stability in the autosampler, processed plasma samples were kept in the autosampler for 24 h (termostated at 10 °C) and then were again analyzed. Stability of analytes in working solutions for 1, 7, and 30 days was also investigated.

3. Results and discussion

3.1. Chromatographic separation

When column dimensions and particle size are reduced for transferring methods from HPLC to UPLC, an improvement in efficiency, resolution, peak capacity and benefit of reducing analysis time are obtained. Care must be taken to ensure operating flow rate, gradient profiles and injection volumes are scaled appropriately to obtain an equivalent or superior separation.

In a first approach to transfer the previously developed HPLC method for the analysis of these five compounds [58], Acquity UPLC Columns Calculator software (v.1.0, Waters, Milford, USA) was used. An initial adjustment of the system, employing a UPLC linear velocity–geometrically scaled gradient method, gave very good results for the stock solutions. The studied drugs were eluted in a very short time interval (~2 min), with an excellent resolution (Fig. 2). However, when plasma extracts were injected, several endogenous interferences were observed due to the fact that the detection mode employed in the UPLC method (UV vs. PDA and FLR) forced us to use a less selective wave length. Therefore a further optimization of the method was required.

Several mobile phases were tested at different pH values (4, 5, 6, 8 and 10), always higher than pH 3 to avoid FLUV degradation [58]. At the established wavelength compromise (220 nm), mobile phases of pH 6, 8 and 10 were rejected due to the coelution of endogenous interferences of plasma. The absorbance of interfering compounds was significantly lower in the case of pH 4 and 5. A pH value of 4 was chosen because it induced narrower chromatographic peaks, providing a higher sensitivity. The percentage

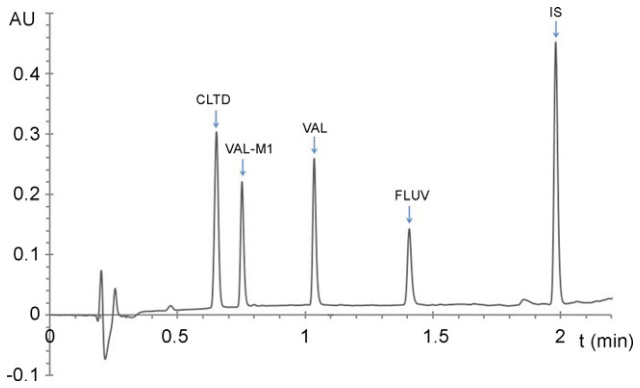

Fig. 2. Chromatogram corresponding to a methanolic stock solution of the studied compounds (10 μg/mL) employing an improved UPLC linear velocity–geometrically scaled gradient. Oven temperature and flow velocity were fixed at 45 °C and 0.6 mL/min respectively.

of formic acid was fixed to 0.01% and ammonium formate to 10 mM. [58].

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

This experimental work evidenced the strong interdependence between the pH of the reconstitution solution (A_1:B_1), the percentage and nature of the organic modifier (ACN or MeOH) in this solution (A_1:B_1) and the injected volume on the system.

Firstly, an attempt was made respecting the composition of the mobile phase (pH and B_1) in the reconstitution solution. Proportions higher than 50% of B_1 gave rise to chromatographic signals which suffered major frontings. Below 50% B_1, fluvastatin and IS were not correctly dissolved. Chromatographic peaks obtained at pH 4 (A_1) were narrower than those observed at pH 5 (A_1). Peak broadening was minimized using lower injection volume, but in order to keep the sensitivity, it was decided not to reduce the injected sample volume under values of 1 μL.

The same tests were repeated with MeOH, due to its lower eluotropic strength. The fronting effect appeared at higher percentages (70%), which allowed the dissolution of all the analytes. This organic modifier allowed to work with larger injection volumes resulting in increase of sensitivity.

Thus, the validation of the method was performed by injecting 2.5 μL of sample reconstituted with a solution of, A_1 (pH 4):MeOH (B_1) (30:70; v/v).

3.2. Assay validation

3.2.1. Selectivity

Selectivity was studied by analyzing eight plasma samples from different healthy volunteers. As the ICH guideline requires [61], no interfering peaks were observed in blank plasma, indicating the high selectivity of the method. Representative chromatograms obtained from blank human plasma and plasma spiked with 200 μg/L of each analyte, and 500 μg/L of candesartan cilexetil (IS) are shown in Fig. 3.

3.2.2. Recovery

The recoveries were calculated for each analyte in low, medium and high concentrations (n = 6) and were found between 77% and 91% as shown in Table 2. As was expected, these values are according to those obtained by the previous HPLC method (78–91%).

3.2.3. Linearity, LLOQ and working range

Calibration curves were obtained by plotting the normalized area (ratio analyte area/IS area) for each concentration level versus

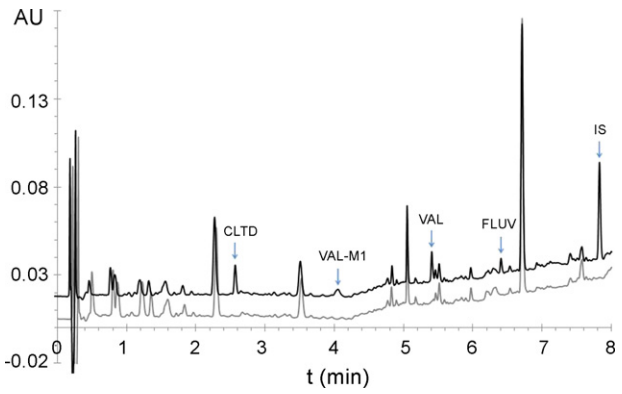

Fig. 3. Chromatograms obtained for blank plasma sample (grey) and a 200 μg/L spiked plasma sample with 500 μg/L IS (black).

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 squares method. The statistical significance of regression was evaluated using the ANOVA and checking the adjustment of the linear model, validity of the regression and efficiency of the regression tests. Eight concentration levels were used in all calibration curves. The correlation coefficients, slope and intercept values are given in Table 3.

The LLOQ was calculated from a relationship S/N equal to 10. The concentrations obtained were 20, 110, 67 and 48 μg/L for CLTD, VAL-M1, VAL and FLUV, respectively.

LLOQ values obtained for CLTD and FLUV were lower than those obtained with the HPLC method (20 and 48 μg/L instead of 31 and 85 μg/L), while higher values were obtained for VAL and its metabolite (67 and 110 μg/L instead of 44 and 41 μg/L). However, the LLOQ values are low enough to use the UPLC method for therapeutic drug monitoring of patients under treatment with these drugs.

Calibration standards did not exceed the limit value (RE > 15%) for the interpolated concentration with regard to nominal concentration. 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: 20–2900 μg/L for CLTD, 110–1100 μg/L for VAL-M1, 67–2900 μg/L for VAL and 48–1100 μg/L for FLUV.

3.2.4. 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 inter-day accuracy (RE) and precision (RSD) are summarized in Table 4. Intra-day precision varied between 3.2% and 8.0%, and inter-day precision between 4.0% and 9.9%. Intra-day accuracy varied from 0.2% to 14.7% and inter-day accuracy from 0.2% to 14.9%. Obtained values are in accordance with the FDA and ICH recommendations.

3.2.5. Stability

The analyzed drugs, except FLUV, were found to be stable under the studied stability conditions. The chromatographic signal of FLUV changed with the time in those plasma samples which were exposed to light at room conditions, surely due to the photodegradation of the molecule reported by Mielcarek et al. [67]. As result of this degradation two photoproducts appeared, which can be identified with the two new chromatographic peaks shown in Fig. 4. This degradation was not significant for analysis times below 24 h, using amber vials in order to avoid light exposure

3.3. Application to real samples

Plasma samples obtained from patients under cardiovascular treatment with CLTD, VAL, FLUV or a combination of these were both determined by UPLC and HPLC. These patients were also treated with other co-administered drugs: β-blockers, ARA-II, diuretics or statins.

Patients' samples were preferably taken at their highest plasmatic concentration (t_{max}) (1 h for FLUV, 2–3 h for VAL and CLTD)

Table 2
Mean recoveries obtained with the UPLC system with TUV (220 nm) detection mode ($n = 6$).

	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
Recovery (%)	87.4	91.8	88.3	77.8	83.7	81.5	85.2	85.2	83.0	80.9	80.2	78.3
RSD (%)	1.4	1.8	3.8	4.6	0.8	1.5	2.6	1.7	2.1	3.3	3.6	4.1

Table 3
Parameters corresponding to linear regression obtained from the calibration curves ($n = 8$) using UPLC–TUV (220 nm) detection mode at 3 different days.

CLTD	Slope (estimated ± SD)	Intercept (estimated ± SD)	Correlation coefficient	Linear range (μg/L)
Curve 1	1.94 ± 0.02	−0.03 ± 0.02	0.996	
Curve 2	2.04 ± 0.01	−0.03 ± 0.02	0.997	20–2900
Curve 3	1.95 ± 0.03	−0.03 ± 0.04	0.994	
VAL-M1	Slope (estimated ± SD)	Intercept (estimated ± SD)	Correlation coefficient	Linear range (μg/L)
Curve 1	1.07 ± 0.01	−0.08 ± 0.01	0.996	
Curve 2	1.07 ± 0.01	−0.05 ± 0.01	0.996	110–1100
Curve 3	1.05 ± 0.03	−0.07 ± 0.04	0.995	
VAL	Slope (estimated ± SD)	Intercept (estimated ± SD)	Correlation coefficient	Linear range (μg/L)
Curve 1	1.34 ± 0.01	0.01 ± 0.01	0.996	
Curve 2	1.38 ± 0.01	−0.01 ± 0.01	0.997	67–2900
Curve 3	1.31 ± 0.02	−0.004 ± 0.02	0.995	
FLUV	Slope (estimated ± SD)	Intercept (estimated ± SD)	Correlation coefficient	Linear range (μg/L)
Curve 1	0.814 ± 0.005	−0.015 ± 0.007	0.999	
Curve 2	0.844 ± 0.004	−0.011 ± 0.005	0.999	48–1100
Curve 3	0.78 ± 0.01	−0.014 ± 0.018	0.995	

Table 4
Intra-day and inter-day precision and accuracy in the assay ($n = 5$) at the lower limit of quantitation (LLOQ), low (200 μg/L), medium (600 μg/L) and high (1100 μg/L for VAL-M1 and FLUV; 2900 μg/L for CLTD and VAL) concentration levels, defined within the range of expected concentrations.

	LLOQ (μg/L)		Low (μg/L)		Medium (μg/L)		High (μg/L)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
RSD (%)								
CLTD	4.2	5.3	4.5	6.1	4.9	6.3	7.2	7.9
VAL-M1	3.2	4.0	4.6	5.9	4.8	5.8	7.0	7.1
VAL	8.0	9.9	5.0	6.1	4.9	7.1	7.4	7.9
FLUV	6.3	6.8	4.8	6.1	3.6	4.1	5.2	6.0
RE (%)								
CLTD	14.7	14.9	0.4	1.7	5.6	5.8	1.4	1.9
VAL-M1	11.8	12.8	1.6	0.4	5.3	5.4	0.2	2.7
VAL	13.6	14.9	0.5	1.8	2.9	4.4	1.2	2.2
FLUV	7.2	9.2	3.5	1.9	0.4	3.0	2.2	0.2

[63–66]. 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 them had a combination of the studied drugs.

Firstly, plasma extracts obtained from the clean-up procedure were injected into the HPLC. 36 h later, these samples were injected into the UPLC system. Plasmatic concentrations obtained for these samples both by UPLC or by HPLC are collected in Tables 5 and 6.

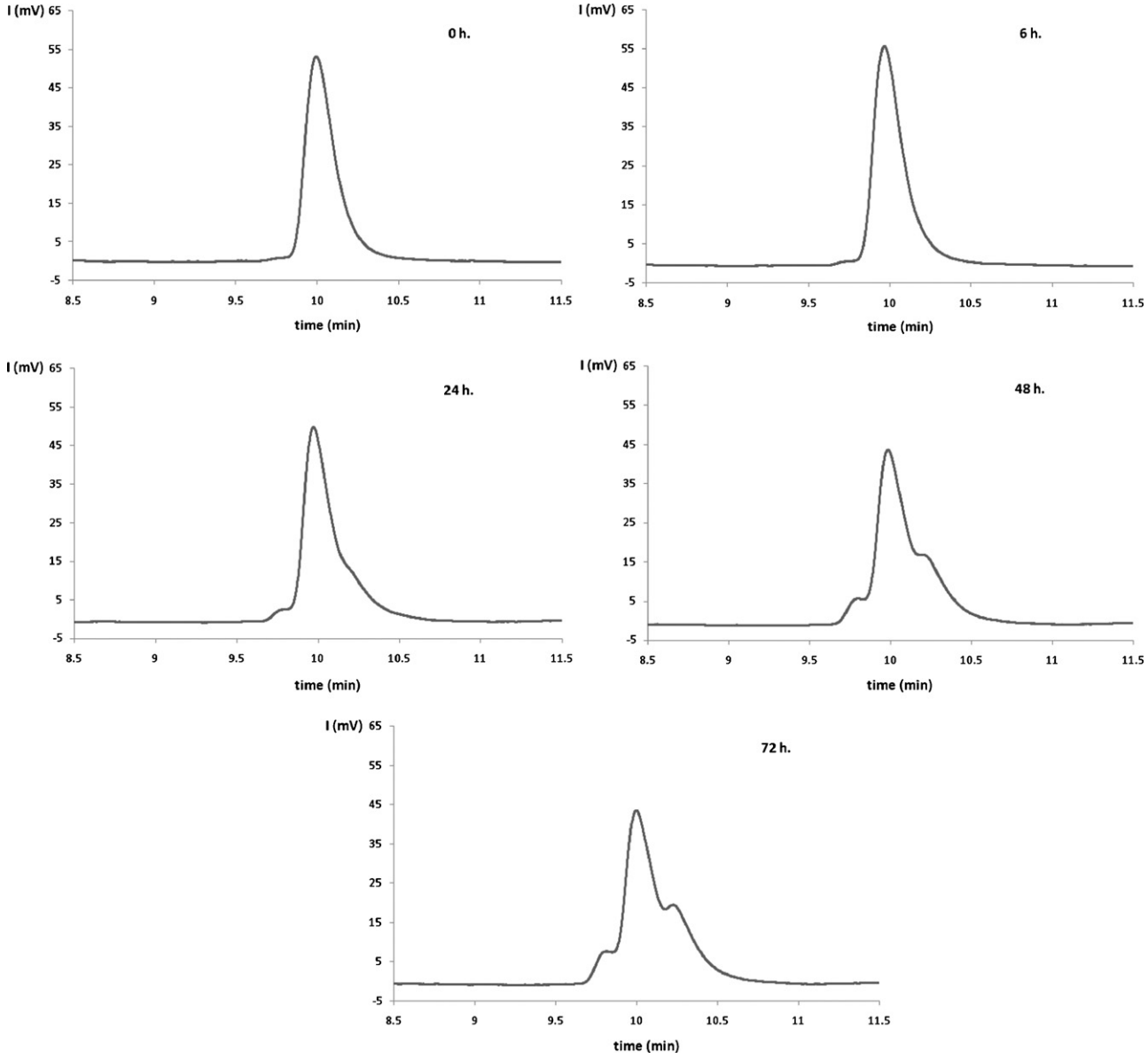

Fig. 4. Chromatograms of a methanolic solution of fluvastatin (10 mg/L) at different times of light exposure. HPLC–PDA–FLR chromatographic conditions are described by Gonzalez et al. [58].

Table 5
Concentration values obtained for studied drugs after SPE extraction of plasma samples obtained from patients under one of the drugs used in the combined cardiovascular treatment (results given as mean results).

Patient number	Ingested amount (mg)	*t* after ingestion (h)	Concentration (μg/L) CLTD		
			UPLC–TUV	HPLC–PDA	HPLC–FLR
1	Higrotona 50 mg (CLTD 50 mg)	2 h 50 min	346	320	–
2	Higrotona 50 mg (CLTD 50 mg)	2 h 45 min	254	246	–
3	Higrotona 50 mg (CLTD 50 mg)	1 h 45 min	607	582	–
4	1/1 Higrotona 50 mg (CLTD 25 mg)	2 h 30 min	233	260	–
5	Blokium-Diu 100 mg (CLTD 25 mg)	2 h 10 min	152	152	–
			VAL		
			UPLC–TUV	HPLC–PDA	HPLC–FLR
11	Vals-160 mg (VAL 160 mg)	3 h 15 min	2690	2649	2527
12	Vals-160 mg (VAL 160 mg)	3 h 15 min	3146	3082	3049
13	Diovan-80 mg (VAL 80 mg)	2 h 50 min	1048	1010	976
14	Co-Diovan-160 mg (VAL 160 mg)	2 h	6363	6286	6076

Table 6
Concentration values obtained by UPLC and HPLC for studied drugs after SPE extraction of plasma samples obtained from four patients under combined cardiovascular treatment (results given as mean results).

Patient number	Ingested amount (mg)	*t* after ingestion (h)	Concentration (μg/L)		Concentration (μg/L)		
			CLTD		VAL		
			UPLC–TUV	HPLC–PDA	UPLC–TUV	HPLC–PDA	HPLC–FLR
16	Higrotona 50 mg (CLTD 50 mg) + Diovan 160 mg (VAL 160 mg)	1 h 50 min (both)	301	303	4150	4164	3750
17	Higrotona 50 mg (CLTD 50 mg) + Diovan 80 mg (VAL 80 mg)	12 h (CLTD) 2 h (VAL)	447	385	2783	2779	2564
18	Higrotona 50 mg (CLTD 50 mg) + Diovan 160 mg (VAL 160 mg)	2 h 40 min (both)	109	124	1287	1216	1187
19	Higrotona 50 mg (CLTD 50 mg) + Vals-160 mg (VAL 160 mg)	12 h (CLTD) 1 h 55 min (VAL)	619	533	2446	2474	2515

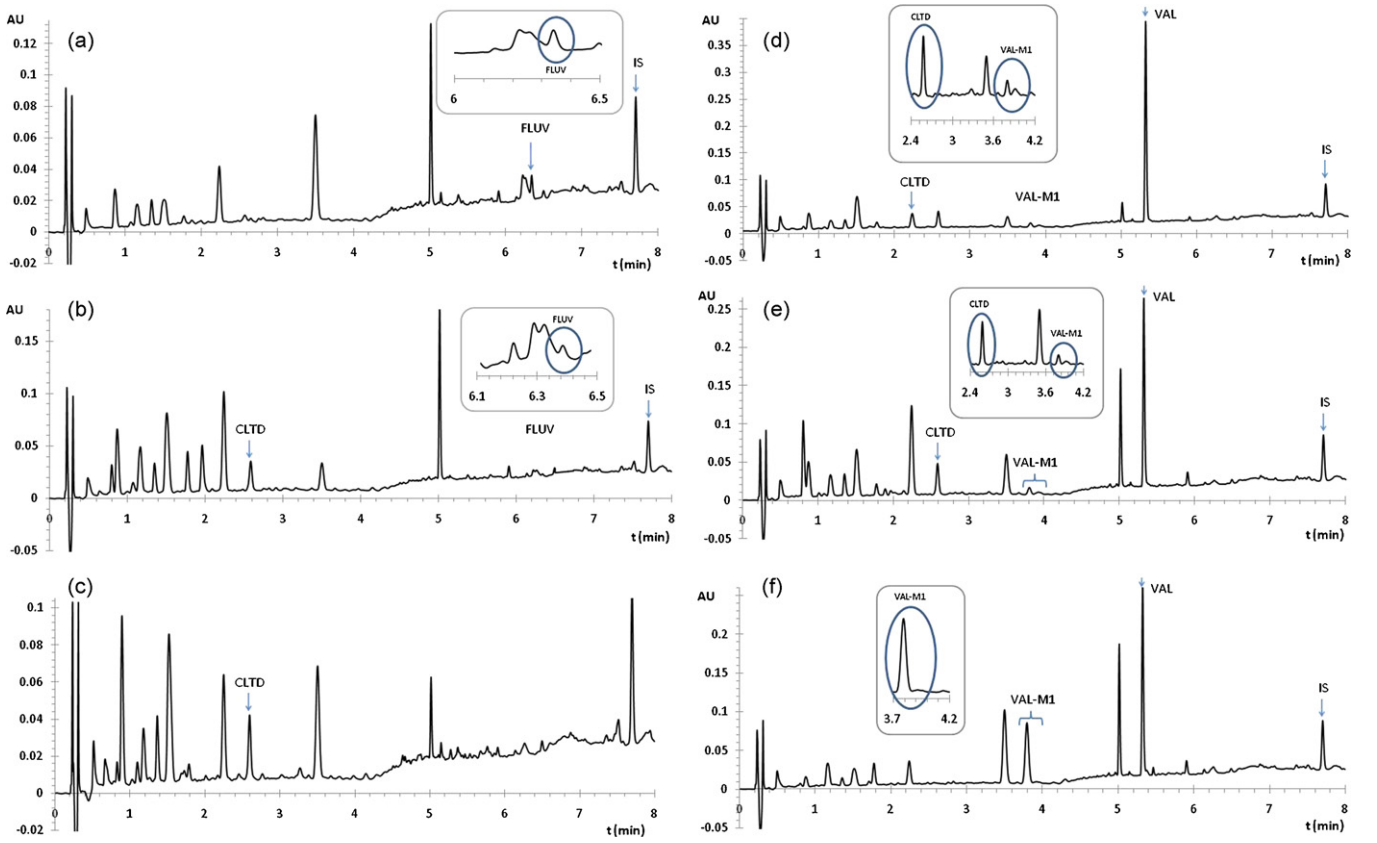

Fig. 5. UPLC–TUV (220 nm) chromatograms obtained for plasma samples collected from different patients under cardiovascular treatment with: (a) FLUV (40 mg) 1 h after the oral intake (patient 7); (b) FLUV 1 h 10 min and CLTD (80 mg and 25 mg) 11 h after the oral intake (patient 15); (c) CLTD (50 mg) 2 h 45 min after the oral intake (patient 2); (d) CLTD and VAL (50 mg and 160 mg) both 1 h 50 min after the oral intake (patient 16); (e) CLTD 12 h and VAL (50 mg and 80 mg) 2 h after the oral intake (patient 17); and, (f) VAL (160 mg) 3 h 15 min after the oral intake (patient 11). IS: candesartan cilexetil 500 μg/L.

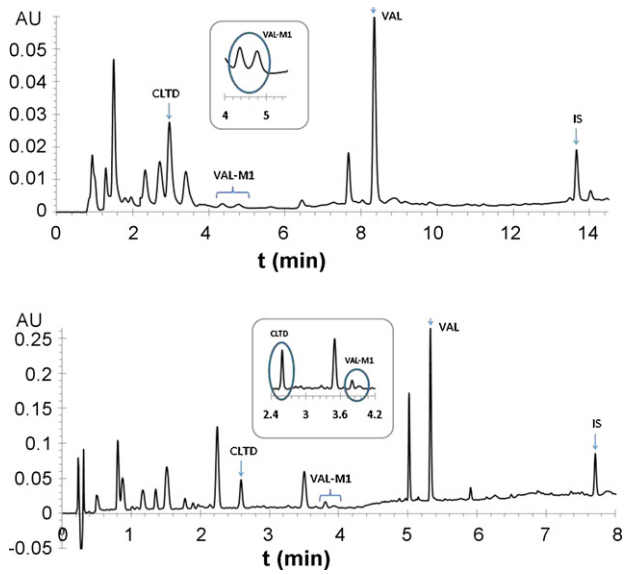

Fig. 6. Chromatograms corresponding to plasma extracts of the patient 17 (under cardiovascular treatment with CLTD-50 mg and VAL-80 mg) obtained with: (above) HPL–PDA (CLTD at 229 nm; VAL-M1 and VAL at 254 nm); (below) UPL–TUV (220 nm). IS: candesartan cilexetil 500 μg/L. Time transcurred after the oral intake: 1 h 50 min for VAL and 12 h for CLTD.

The results obtained using UPLC and HPLC (UV or FLR) methods showed a good concordance, with the exception of FLUV ones. FLUV containing samples could not be properly quantified due to instability problems, probably due to the long time elapsed between the extraction of analytes and the UPLC injection.

Chromatograms corresponding to plasma samples of patients under treatment with CLTD and VAL or CLTD and FLUV are shown in Figs. 5 and 6. No interferences between analytes and co-administered drugs were observed.

UPLC quantitation of VAL-M1 in samples from valsartan-treated patients was not possible due to splitting of the chromatographic peak of this compound. Not interference appeared at this retention time when analyzing samples from patients not treated with valsartan. This chromatographic double peak was not observed when spiked plasma samples were analyzed. Due to these facts and in accordance to results previously obtained with HPLC, we suggest that this double peak results from a metabolic transformation of valsartan or its metabolite. In order to confirm this hypothesis, LC–MS studies should be carried out.

4. Conclusions

The optimized UPLC method has proved to be sensitive, selective and rapid for the simultaneous determination of CLTD and VAL in human plasma samples.

The UPLC technology has widely improved the method optimization process (method development) since lower re-equilibration times (2 min vs. 5–8 min in HPLC) and shorter chromatograms allow a greater number of experimental testing conditions than with a conventional HPLC. The sample volume required is also much lower than in HPLC (2.5 μL vs. 10 μL), enabling larger number of injections per sample. Shorter analysis times together with slower flow rates reduce the organic solvent consumption. Taking into account the shortage of acetonitrile worldwide, the UPLC achieves a remarkable 73% acetonitrile saving in comparison with the previous HPLC method.

Shorter analysis times require higher data-acquisition rates (80 Hz vs. 1 Hz), resulting in sharper and higher chromatographic peaks and thus, improving the peak capacity. In terms of efficiency UPLC has notorious advantages in comparison with conventional HPLC. Nevertheless, less tedious extraction methods should be developed to couple with the fastness of UPLC.

The sensitivity has found to be analyte dependent, since that improvement has not been proportional for all the analytes. Even so, this fact does not affect the viability of the method, since the concentrations ranges expected for the four analytes are widely covered.

The analysis of the FLUV should be carried out immediately after the clean-up process, since a significant loss in concentration has been observed between the HPLC analysis and the UPLC analysis.

As reported for HPLC, samples obtained from patients under treatment with valsartan showed the splitting of the VAL-M1 chromatographic peak. Even if different gradient conditions were assayed, the complete resolution of both peaks by UPLC–tunable UV–vis (TUV) resulted impossible. This fact could be explained on the assumption that both peaks matched two metabolites of valsartan presenting major structural similarities. This hypothesis should be validated with a further mass spectrometry (MS) study of both peaks.

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