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LC–MS/MS method for the determination of several drugs used in combined cardiovascular therapy in human plasma

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ABSTRACT

A simple, fast and validated method is reported for the simultaneous analysis, in human plasma, of several drugs usually combined in cardiovascular therapy (atenolol, bisoprolol, hydrochlorothiazide, chlorthalidone, salicylic acid, enalapril and its active metabolite enalaprilat, valsartan and fluvastatin) using high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI), working in multiple reaction monitoring mode (MRM). Separation of analytes and internal standard (pravastatin) was performed on a Luna C18(2) (150 mm × 4.6 mm, 3 μm) column using a gradient elution mode with a run time of 15 min. The mobile phase consisted of a mixture of acetonitrile and water containing 0.01% formic acid and 10 mM ammonium formate at pH 4.1. Sample treatment consisted of a simple protein precipitation with acetonitrile, enabling a fast analysis. The method showed good linearity, precision (RSD% values between 0.7% and 12.7%) and accuracy (relative error values between 0.9% and 14.0%). Recoveries were within 68–106% range and the ion-suppression was not higher than 22% for any analyte. The method was successfully applied to plasma samples obtained from patients under combined cardiovascular treatment.

1. Introduction

Cardiovascular diseases are the first cause of mortality worldwide, causing around the 30% of the global deaths, a number which will significantly increase in the following years according to the World Health Organization (WHO). These illnesses have been traditionally associated to the western society, but in fact 80% of the deaths take place in low- and middle-income countries due to the troubles to access medicines and also due to their unhealthier diet [1,2].

Actually, inappropriate diet and other bad habits like alcohol and tobacco consumption are some of the factors closely related to the risk of suffering from a cardiovascular illness. Other important risk factors are hypertension, dyslipidemia and diabetes. The suffering from some of these pathologies simultaneously is known as metabolic syndrome [3,4].

Due to the factors involved, different drugs must be used to fight the metabolic syndrome, thus a combined cardiovascular therapy is necessary. This therapy usually involves different antihypertensive (diuretics, angiotensin II receptor antagonists (ARA-II) and β-blockers), lipid lowering drugs (statins, ezetimibe), antiplatelet (salicylic acid, clopidogrel) and antidiabetic (metfomin, glibenclamide) drugs [5,6].

The monitoring of the plasmatic concentrations of cardiovascular drugs is crucial for understanding their pharmacokinetics and pharmacodynamics. Moreover, it provides valuable information about possible interactions. Therefore the simultaneous determination of these analytes turns interesting. This is a complex task since analytes from different families have different physicochemical properties. The complexity of the biological matrices and the low expected concentrations of some analytes require the development of sensitive and selective determination methods. In this context, one of the most suitable techniques to achieve this goal is the liquid chromatography–tandem mass spectrometry (LC–MS/MS).

On the other hand, although quantitative analysis for different drug families have been widely developed, very few analytical methods have been focused on the simultaneous analysis of drugs from different families [7–9]. In this work, a simple and fast method for the determination of drugs used in combined cardiovascular therapy has been developed, considering the most prescribed drugs in our geographical area: atenolol, bisoprolol (β-

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Table 1
Chemical structures and normal therapeutic concentration ranges for analyzed compounds.

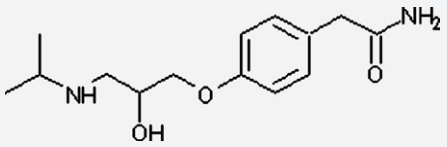
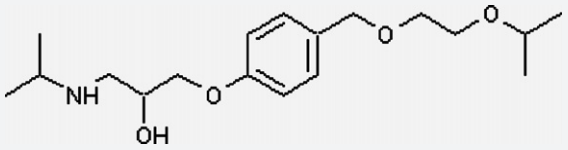
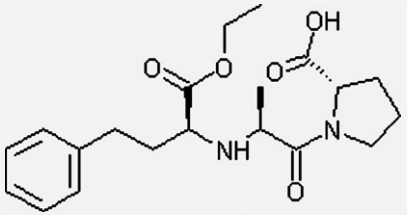
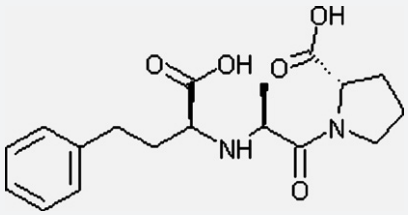
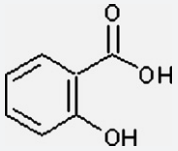
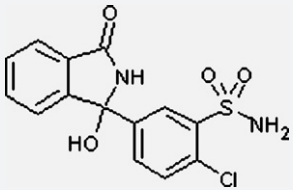
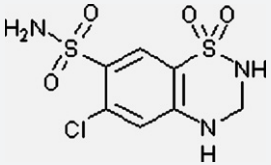
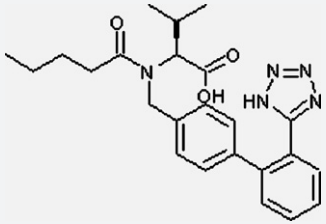
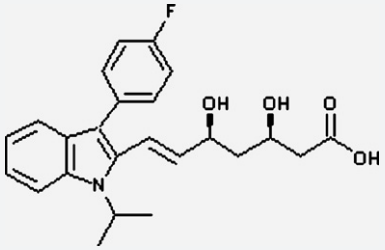
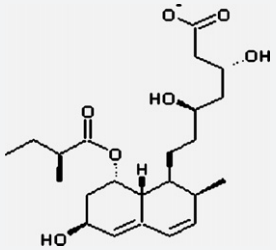
Name and type	Structures	Therapeutic Range (μg/L)	Name and type	Structures	Therapeutic Range (μg/L)
Atenolol (β-blocker)		100–1000	Bisoprolol (β-blocker)		10–100
Enalapril (IECA)		20–350	Enalaprilat (IECA, metabolite)		20–100
Salicylic acid (antiaggregant)		1500–30,000	Chlorthalidone (diuretic)		10–600
Hydrochloro thiazide (diuretic)		50–400	Valsartan (ARA-II)		700–4000
Fluvastatin (statin)		40–320	Pravastatin (statin) internal standard		–

Table 2
Optimized gradient for LC–MS/MS analysis.

Time (min)	Organic phase (%)	Aqueous phase (%)
0	5	95
2	28	72
6	40	60
10	90	10
15	90	10
16	5	95
20	5	95

blockers), hydrochlorothiazide, chlorthalidone (diuretics), salicylic acid (active metabolite of aspirin, antiplatelet), enalapril (ACEI) and its active metabolite enalaprilat, valsartan (ARA-II), and fluvastatin (statin).

Although most of these drugs have been individually analyzed in plasma by LC coupled to mass spectrometry techniques [10–14], in this work, a simple and fast LC–MS/MS method has been developed for their simultaneous analysis in human plasma. The suitability of the method has been demonstrated by validation, carried out following the guidelines proposed by Food and Drug Administration (FDA) [15] and International Conference on Harmonisation (ICH) [16]. The method has also been successfully applied to samples obtained from patients under cardiovascular treatment.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted on a Waters Alliance 2695 separation module connected to a Waters 996 photodiode array detector (PDA) and to a Micromass Quatro Micro tandem quadrupole mass spectrometer operated in electrospray ionization mode (Milford, MA, USA). Chromatograms were recorded by means of a computer and treated with the aid of the software MassLynx 4.0 from Waters.

A Phenomenex Luna C18(2), 150 mm × 4.6 mm I.D., 3 μm, 100 Å column was used to perform the separation. A Phenomenex C18, 4 mm × 3 mm, Security guard cartridge was placed prior to the analytical column in order to prevent its degradation.

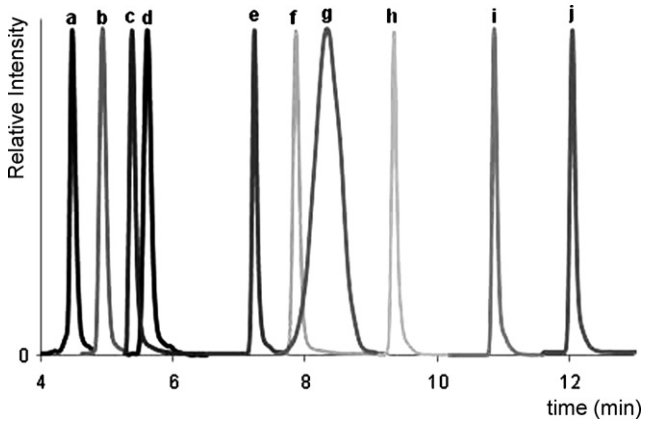


Fig. 1. LC–MS/MS chromatogram corresponding to a calibration standard solution (normalized heights): atenolol (a), enalaprilat (b), salicylic acid (c), hydrochlorothiazide (d), chlorthalidone (e), bisoprolol (f), enalapril (g), pravastatin (I.S) (h), valsartan (i) and fluvastatin (j). LC and MS conditions described in Section 3.1.

Plasma samples were centrifuged in an Eppendorf model 5804R centrifuge (Hamburg, Germany) after protein precipitation procedure (PPT). The supernatant was evaporated under a nitrogen stream using a Zymark Turbovap evaporator LV (Barcelona, Spain).

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 and valsartan were kindly supplied by Novartis Pharma AG (Basel, Switzerland) and bisoprolol fumarate by Merck (Darmstadt, Germany). Chlorthalidone was kindly supplied by Ciba-Geigy (Barcelona, Spain). Enalapril maleate, atenolol, sodium pravastatin, salicylic acid and hydrochlorothiazide were supplied by Sigma–Aldirch (St. Louis, MO, USA). Enalaprilat was synthesized in our laboratories and characterized by ^{1}H NMR, ^{13}C NMR and elemental analysis. Chemical structures of these compounds are shown in Table 1.

Table 3
Optimal MS/MS conditions for analyzed compounds and their quantitation limits. First row corresponds to quantitation transition and the second one to the confirmation transition.

Analyte	ESI mode	Precursor ion	Fragment ion	Cone voltage (V)	Collision energy (eV)	LOQ (μg/L)
Atenolol	+	267.0 267.0	144.9 132.9	30 30	30 30	2.0
Enalaprilat	+	349.0 349.0	206.0 117.0	15 15	15 30	2.5
Salicylic acid	−	136.8 136.8	92.7 64.5	15 15	15 30	75.0
Hydrochlorothiazide	−	295.8 295.8	268.8 204.8	30 30	15 15	20.0
Chlorthalidone	+	338.8 338.8	321.8 242.9	15 15	15 30	5.0
Enalapril	+	376.9 376.9	234.0 129.9	15 15	15 30	3.5
Bisoprolol	+	326.0 326.0	115.8 73.6	30 30	15 30	1.5
Valsartan	+	436.0 436.0	234.9 207.0	15 15	15 30	2.0
Fluvastatin	+	411.9 411.9	224.0 266.0	15 15	30 15	1.0
Pravastatin (I.S.)	−	422.9	321.0	30	15	–

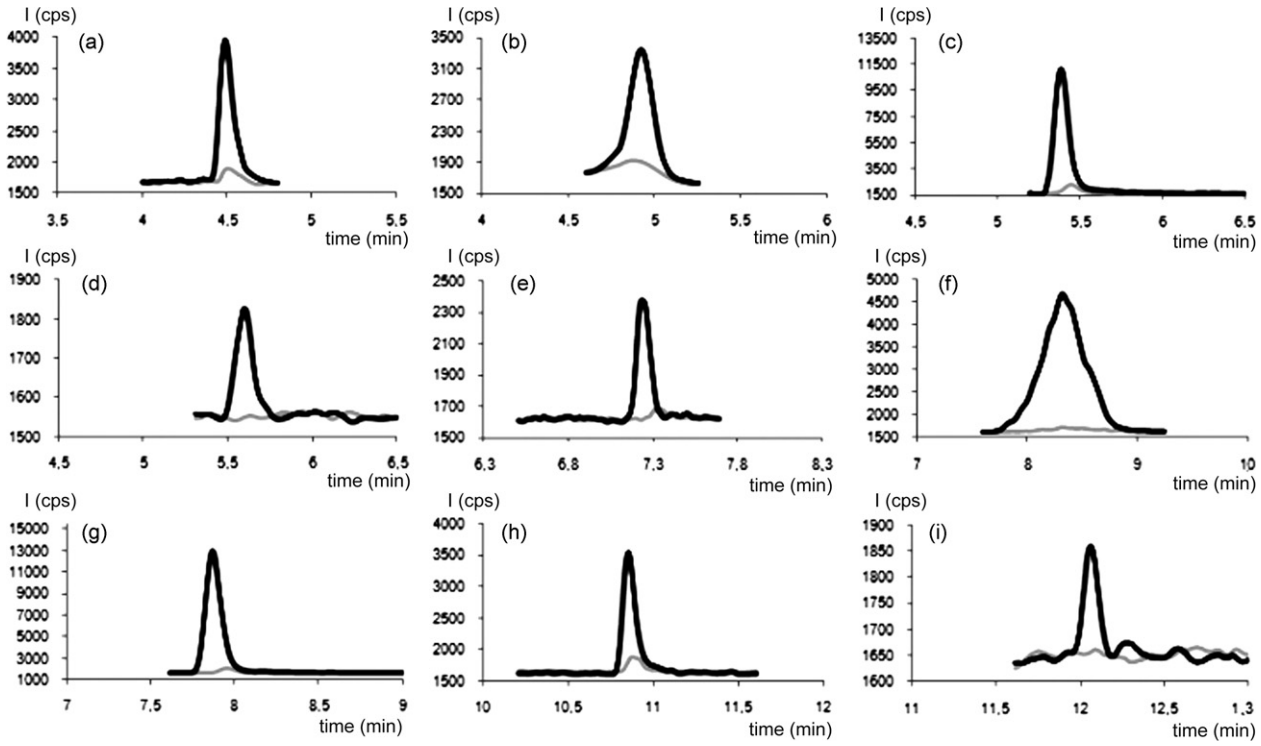


Fig. 2. MRM windows' chromatographic signals corresponding to a plasma sample spiked with quantitation limit concentrations: (a) atenolol (2 μg/L), (b) enalaprilat (2.5 μg/L), (c) salicylic acid (75 μg/L), (d) hydrochlorothiazide (20 μg/L), (e) chlorthalidone (5 μg/L), (f) enalapril (3.5 μg/L), (g) bisoprolol (1.5 μg/L), (h) valsartan (2 μg/L), and (i) fluvastatin (1 μg/L) with their blank signal (gray lines). LC and MS conditions described in Section 3.1.

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

HPLC quality methanol and acetonitrile were obtained from VWR (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.

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

2.3. Standard solutions and spiked plasma samples

Standard solutions between 500 and 10000 mg/L were prepared in 100% acetonitrile for valsartan and salicylic acid, and in acetonitrile:water (90:10) for the other drugs. These solutions were diluted with acetonitrile:water to obtain the necessary multicomponent working solutions for spiking the plasma samples. A 6 mg/L pravastatin solution in acetonitrile was also prepared to use as internal standard (IS).

In order to obtain representative plasma for method development, a plasma pool was prepared by mixing in a proportional way eight plasma samples obtained from different healthy volunteers.

During the optimization step of the extraction procedure, plasma samples were spiked with 950 μg/L of each analyte.

Calibration standards were prepared by spiking a pool plasma with the working solutions. Calibration curve for atenolol was built from 2 to 1000 μg/L (n = 8), enalaprilat from 2.5 to 250 μg/L (n = 7), salicylic acid from 187.5 to 7500 μg/L (n = 7), hydrochlorothiazide from 20 to 2000 μg/L, chlorthalidone from 5 to 500 μg/L (n = 7), enalapril from 3.5 to 350 μg/L (n = 7), bisoprolol from 1.5 to 150 μg/L (n = 7), valsartan from 10 to 5000 μg/L (n = 9) and fluvastatin from 1 to 500 μg/L (n = 8). In all the cases the IS was added (1000 μg/L) prior to the extraction procedure.

Quality control (QC) samples used for stability assays were prepared by spiking a drug-free plasma with all the analytes, in low and high concentrations, using the appropriate working standard solution volumes, taking into account their working ranges.

2.4. Chromatographic conditions

The mobile phase consisted of solvent A (acetonitrile with 0.01% formic acid and 10 mM ammonium formate) and solvent B (0.01% formic acid with 10 mM ammonium formate (pH 4.1)) delivered in gradient mode (Table 2) at a flow rate of 0.8 mL/min. After the PDA the flow was split so that only 0.28 mL/min reached the mass spectrometer. The mobile phases were prepared by a 1:20 dilution of a 0.2% formic acid and 200 mM ammonium formate water solution. Before the chromatographic use, the mobile phases were filtered through a 0.45 μm type HVLP Durapore membrane filter from Millipore.

During the chromatographic analysis, samples were kept at 10 ± 1 °C in the autosampler and the injected volume was 30 μL. The chromatographic separation was performed at 40 ± 1 °C. The column was re-equilibrated for 6 min after the gradient separation.

2.5. MS conditions

The optimal precursor and fragment ions chosen for quantitation and confirmation are shown in Table 3, together with their optimal cone voltage, collision energy and ionization mode. The dwell time for the analysis was set at 0.2 s and the inter-scan delay at 0.1 s. The following parameters were fixed according to the manufacturer's recommendations: capillary voltage: 3.2 kV (for ESI+) or 2.6 (for ESI−); desolvation gas (N_2) flow: 450 L/h, source temperature: 120 °C and desolvation temperature: 300 °C. The scan time for each analyte was set at 1.0 s for mass and daughters scans.

Table 4
Recovery percentages (*R*%) and matrix effect (ME%) obtained for all the cardiovascular drugs studied at three different concentration levels. Expressed as mean value ± standard deviation (s).

Compound	Low concentration		Middle concentration		High concentration	
	R% (±s)	ME% (±s)	*R*% (±s)	ME% (±s)	*R*% (±s)	ME% (±s)
Atenolol	79 ± 3	78 ± 3	90 ± 3	80 ± 2	89 ± 3	93 ± 5
Enalaprilat	76 ± 5	88 ± 5	68 ± 9	82 ± 3	76 ± 2	82 ± 4
Salicylic acid	85 ± 6	85 ± 7	85 ± 2	84 ± 4	85 ± 1	91 ± 3
Hydrochlorothiazide	100 ± 7	98 ± 7	97 ± 11	97 ± 5	97 ± 7	97 ± 6
Chlorthalidone	91 ± 6	84 ± 8	87 ± 8	82 ± 3	93 ± 4	84 ± 3
Enalapril	91 ± 3	92 ± 5	93 ± 9	85 ± 2	90 ± 2	87 ± 3
Bisoprolol	98 ± 4	93 ± 4	96 ± 4	89 ± 1	93 ± 3	92 ± 2
Valsartan	88 ± 7	97 ± 3	92 ± 11	90 ± 6	88 ± 12	88 ± 2
Fluvastatin	96 ± 8	107 ± 6	106 ± 8	102 ± 13	97 ± 10	119 ± 16

2.6. Extraction procedure

2.6.1. Stability of the compounds

Since extraction procedure involves sample evaporation under a N_2 stream, stability of the analytes at different temperatures was studied prior to the optimization process.

For this purpose 800 μg/L concentration samples in acetonitrile:water (60:40) were prepared, simulating the conditions required for protein precipitation (PPT). These samples were kept in the turbovap at 60, 70 or 80 °C (n = 3) for a time slightly longer than they needed for a total evaporation (40, 30 and 20 min, respectively). Then, they were reconstituted with 100 μL methanol:aqueous phase (60:40) and injected in the LC system. The stability of the compounds was tested by comparing the obtained chromatographic responses with those obtained for the standard solutions.

2.6.2. Optimized extraction procedure

Different parameters involved in the extraction procedure were studied by experimental design, using *The Unscrambler* program [17]: precipitant agent (methanol and acetonitrile), precipitant agent's temperature (room and fridge) and centrifugation temperature (20 and 4 °C).

The extraction procedure was carried out as follows: A 0.5 mL plasma aliquot was spiked with 100 μL IS (pravastatin) to achieve a concentration of 1000 μg/L. Next, 0.65 mL of acetonitrile, at room temperature, was added followed by vortex-mixing and centrifugation for 5 min at 10,000 rpm and 20 °C. Supernatant was transferred to 6 mL glass tube and it was evaporated to dryness under N_2 stream at 80 °C for 15 min. The extract was then reconstituted with 100 μL methanol:aqueous phase (60:40) and vortex mixed in order to help dissolving the sample. Then, it was filtered and transferred to autosampler vials. 30 μL aliquots were injected into the LC system for analysis.

Table 5
Intra- and inter-day accuracy and precision values obtained for the analytes at three different concentrations.

Compound	Concentration (μg/L)	RSD (%)		RE (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Atenolol	2 (LOQ)	9.0	3.1	9.8	12.9
	100	5.4	2.7	7.8	8.4
	1000	2.4	3.8	11.4	9.0
Enalaprilat	2.5 (LOQ)	10.0	0.7	1.9	1.8
	25	5.1	4.6	3.8	1.9
	250	1.8	2.4	2.0	0.6
Salicylic acid	187.5	1.8	5.9	2.1	4.3
	750	4.0	0.7	10.8	11.4
	7500	3.4	2.1	8.5	9.7
Hydrochlorothiazide	20 (LOQ)	9.7	12.7	7.2	6.0
	200	8.2	6.3	6.6	6.1
	2000	3.2	9.0	5.5	3.6
Chlorthalidone	5 (LOQ)	6.6	3.3	2.3	1.9
	50	3.8	4.5	3.0	0.9
	500	2.9	1.9	5.6	3.7
Enalapril	3.5 (LOQ)	4.4	1.0	1.4	1.6
	35	3.9	3.4	3.4	2.2
	350	2.9	1.6	2.2	2.2
Bisoprolol	1.5 (LOQ)	6.4	2.3	13.1	14.0
	15	3.9	3.7	12.5	10.8
	150	4.0	2.4	9.4	10.0
Valsartan	10	6.2	2.9	3.1	3.5
	100	3.7	1.2	5.1	5.4
	5000	2.7	1.2	11.5	10.3
Fluvastatin	1 (LOQ)	5.0	11.4	3.8	8.0
	50	3.3	7.0	4.7	4.3
	500	4.2	7.3	7.6	8.3

Table 6
Plasmatic concentrations of the studied drugs obtained for patients under combined cardiovascular therapy (SA: salicylic acid).

N	Sex	Administered drugs	Time after oral intake	Concentration μg/L (±s)
1	F	Atenolol 50 mg	2 h	225 ± 2
		Aspirin 250 mg (as SA)	20 h	1837 ± 44
2	F	Bisoprolol 10 mg	2 h	18.0 ± 0.3
		Valsartan 160 mg	2 h	152 ± 3
3	M	Enalapril 20 mg	1 h	183 ± 2
		Enalaprilat	–	49.2 ± 0.3
		Chlorthalidone	1 h	364 ± 3
		Aspirin 100 mg (as SA)	20 h	362 ± 10
4	F	Hydrochlorothiazide 12.5 mg	2 h	43 ± 3
		Valsartan 80 mg	2 h	355 ± 6
5	F	Enalapril 50 mg	Unknown	32.1 ± 0.2
		Enalaprilat		62.7 ± 0.4
		Aspirin 100 mg (as SA)	20 h	79 ± 8
6	F	Atenolol 50 mg	Unknown	75.1 ± 0.8
		Enalapril 20 mg	Unknown	121 ± 1
		Enalaprilat		35.5 ± 0.2
7	M	Hydrochlorothiazide 12.5 mg	1 h	94 ± 3
		Bisoprolol 10 mg	1 h	53 ± 1
8	F	Hydrochlorothiazide 25 mg	2 h	174 ± 5
		Valsartan 160 mg	2 h	581 ± 10
		Fluvastatin 80 mg	12 h	15.6 ± 0.2
9	F	Enalapril 20 mg	3 h	66.5 ± 0.5
		Enalaprilat		18.4 ± 0.1
		Chlorthalidone 50 mg	3 h	209 ± 2
10	M	Atenolol 100 mg	2 h	391 ± 4
		Enalapril (5 mg)	>24 h	<LOQ
		Enalaprilat		2.6 ± 0.1
		Chlorthalidone	2 h	148 ± 1
11	F	Hydrochlorothiazide 25 mg	1 h	195 ± 6
		Fluvastatin 80 mg	1 h	46.4 ± 0.5
12	F	Bisoprolol 5 mg	3 h	37.6 ± 0.7
		Valsartan 160 mg	3 h	2516 ± 43
13	F	Hydrochlorothiazide 25 mg	8 h	446 ± 13
		Valsartan 160 mg	8 h	4019 ± 44
		Fluvastatin 80 mg	2 h	6.7 ± 0.1
14	M	Atenolol 100 mg	12 h	310 ± 3
		Chlorthalidone 25 mg	11 h	228 ± 2
		Fluvastatin 80 mg	1 h	28.7 ± 0.3

2.7. Validation

In order to demonstrate the suitability of the developed analytical method, validation was carried out following FDA [15] and ICH [16] recommendations: linearity, working range, intra and inter-assay accuracy and precision, limit of quantitation (LOQ), selectivity and stability were tested for each analyte. Recovery and matrix effect were also studied.

Recovery and matrix effect were determined at three different concentrations following the strategies reported by Matuszewski et al. [18]. Three sets of samples were used for this purpose: standards prepared in the reconstitution solution (A, $n = 3$), plasma samples spiked after the PPT and before the evaporation step (B, $n = 5$) and plasma samples spiked before PPT (C, $n = 5$). Recovery was calculated by comparing the areas of B and C samples (Rec (%) = C area/B area × 100) and matrix effect by comparing the areas of A and B samples (ME (%) = B area/A area × 100). A matrix effect value higher than 100% indicates enhancement, whereas a lower one indicates suppression effects.

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

Taking into account the wide concentration ranges, $1/x^2$ statistical weight was applied in order to obtain the most reliable calibration curves [19]. Calibration curves consisting of triplicate calibration standards for each concentration were analyzed on three different days for linearity studies. They were built by plotting the corrected areas for each concentration level versus the nominal concentration of each calibration standard, taking into account the selected weighting factor.

LOQ were calculated by interpolating in the calibration curve, the value obtained from multiplying 10 times the average signal of 6 different drug free plasma samples. These LOQs were validated in terms of relative standard deviation (RSD) and RE < 20% and signals at least 10 times higher than the blank's response. The working ranges were defined considering the LOQ, the normal therapeutic dosage and the time needed to achieve the maximum plasmatic levels [7,10–14,20–24].

Three samples, corresponding to 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 in three different days. The deviation of the mean from the true value, expressed as RE, served to measure the accuracy. In the same way RSD was used to express the precision.

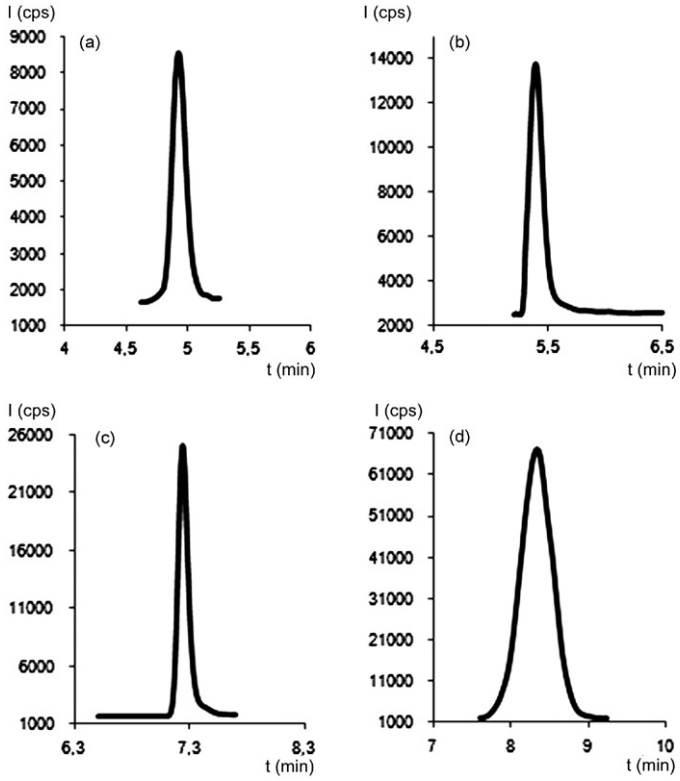


Fig. 3. MRM windows' chromatographic signals corresponding to a plasma extract from patient number 3: enalaprilat (a), salicylic acid (b), chlorthalidone (c), and enalapril (d). LC and MS conditions described in Section 3.1.

Short-term stability (bench top, room temperature), long-term stability (frozen at the intended storage temperature for 1 month), stability after three freeze–thaw cycles and stability in the autosampler were tested at low and high concentrations. The procedure also included an evaluation of analytes stability in the stock solutions.

2.8. Plasma sample collection

14 blood samples were collected from different patients under treatment with combinations of the studied drugs. Samples were taken between 1 and 20 h after the oral intake of the drugs. Blood samples were immediately transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) and gently mixed. Then, they were centrifuged at 3500 rpm 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. This procedure underwent the revision of the Ethical Committee of the Basque Country University.

3. Results

3.1. LC and MS conditions

Chromatographic separation was optimized based on a previously developed method for chlorthalidone, fluvastatin and valsartan [7]. The gradient chosen for the analysis is shown in Table 2.

In Fig. 1 a chromatogram obtained for a calibration standard under optimized MS conditions can be observed.

3.2. Extraction procedure

The relative errors obtained by comparing the chromatographic signals from samples evaporated at 80 °C for 20 min with those obtained from standard solutions were lower than 3.5%. Therefore, no degradation of the analytes was observed and 80 °C was chosen as the temperature for the evaporation in order to reduce the extraction procedure time.

In the sample treatment optimization step none of the parameters studied by experimental design showed a significant effect over the analytes' recoveries within the studied ranges at 95% confidence level. Thus, the parameters chosen were the ones that provided the simplest and fastest treatment method.

3.3. Validation

3.3.1. Recovery and matrix effect

The recoveries and matrix effect obtained for each analyte at three different concentration levels are shown in Table 4. Recoveries ranged between 68% and 106% and matrix effect between 78% and 119%. These values showed no significant differences at different concentration levels, for most of the analytes. Matrix effect values suggest ion suppression for all the analytes except for fluvastatin. On the other hand, higher RSD values for this analyte could be attributed to its photodegradation [25,26].

3.3.2. Selectivity

In the present study, selectivity has been studied by analyzing 6 plasma samples from different healthy volunteers. As the ICH guideline requires [16], the studied blanks did not show area values higher than 20% of the LOQ's areas at the analyte's retention times, neither higher than 5% of the IS area at its corresponding retention time. Representative chromatograms obtained from drug-free plasma and plasma sample spiked with a concentration equivalent to the LOQ are shown for each analyte in Fig. 2.

3.3.3. Linearity, LOQ and working range

LOQ calculated from a relationship S/N equal to 10 are shown in Table 3. Salicylic acid showed the highest quantitation limit, probably due to its poor ionization and to the fact that the fragment ion is quite small facilitating the appearance of interferences. Anyway, concentration of salicylic acid is usually quite higher than this LOQ and this would not be a problem when analyzing samples of patients under treatment with aspirin.

Calibration standards did not exceed the limit values established by FDA and ICH neither for the accuracy or the precision. Therefore the models were accepted for the linear ranges established: from 2 to 1000 μg/L for atenolol, from 2.5 to 250 μg/L for enalaprilat, from 187.5 to 7500 μg/L for salicylic acid, from 20 to 2000 μg/L for hydrochlorothiazide, from 5 to 500 μg/L for chlorthalidone, from 3.5 to 3500 μg/L for enalapril, from 1.5 to 150 μg/L for bisoprolol, from 10 to 5000 μg/L for valsartan and from 1 to 500 μg/L for fluvastatin.

3.3.4. Accuracy and precision

Plasma samples spiked with low, medium and high concentrations of drugs were prepared and their concentrations were obtained from the interpolation on their respective calibration curves. The intra and inter-day accuracy (RE) and precision (RSD) is summarized in Table 5. As it can be seen intra-day precision varied between 1.8% and 10.0%, and inter-day precision between 0.7% and 12.7%. Intra-day accuracy varied from 1.4% to 13.0% and inter-day accuracy from 0.9% to 14.0%. Therefore, obtained values agree with the FDA and ICH recommendations.

3.3.5. Stability

Stability was studied by comparing the corrected area of QC samples with those samples subjected to stability tests. No significant changes in corrected areas were noticed after three freeze–thaw cycles, long term storage or after 24 h in autosampler storage. Therefore, all analytes seem to be stable in those conditions, both at high concentration as well as at low concentration. Working solutions used in sample preparation were also stable for at least 1 month.

When stability in room conditions (bench top, room temperature) was studied degradation of fluvastatin was observed, surely due to the photodegradation of the molecule reported by Mielcarek et al. [25]. 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 14 patients under cardiovascular treatment with different combinations of atenolol, enalapril, hydrochlorothiazide, aspirin, chlorthalidone, bisoprolol, valsartan or fluvastatin. These patients were also co-administered with other β-blockers (metoprolol), statins (atorvastatin, simvastatin), ARA-II (olmesartan), calcium channel blockers (amlodipine, felodipine), antidiabetic drugs (metformin, glibenclamide) and other drugs not involved in cardiovascular therapy (omeprazole, allopurinol, salmeterol, etc.).

Real samples were collected early in the morning, 1–3 h after the oral intake (around the time of maximum concentration) for most of the analytes. Statins are usually prescribed to be taken late in the evening, so not to change the habits of the patients samples were collected in the morning, 12 h after of the oral intake instead of taking them at the time of maximum concentration. The same criterion was applied to the salicylic acid which was administered 20 h before the blood extraction.

A total of 14 samples were analyzed with the developed method, the plasmatic concentrations obtained are gathered in Table 6 and the signals obtained for one of them is shown as example in Fig. 3 (patient number 3).

4. Conclusions

Even if several methods have been reported for the quantitation of drugs prescribed against hypertension, high cholesterol level or diabetes, very few have been developed for the simultaneous determination of drugs used in combined cardiovascular therapy. In this way, this paper reports a method for the simultaneous determination of two diuretics (chlorthalidone and hydrochlorothiazide), two β-blockers (atenolol and bisoprolol) two ACEI (enalapril and its active metabolite enalaprilat), one statin (fluvastatin), one antiplatelet agent (salicylic acid) and one ARA-II (valsartan).

The proposed LC–MS was fully validated and showed an appropriate specificity, linearity, sensitivity and precision for all the analytes studied. It was successfully applied to the determination of these drugs in plasma samples obtained from patients under combined cardiovascular therapy.

The calibration curve of salicylic acid covers a shorter range than other methods previously reported [12,22], but taking into account that the purpose of this analytical method is the determination of samples from patients under cardiovascular treatment, where salicylic acid is used in lower concentration levels, this range is adequate for the expected plasmatic concentrations.

The developed method appears to be the first direct method for the simultaneous analysis of the studied drugs.

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