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Development of an LC–MS/MS method for the quantitation of 55 compounds prescribed in combined cardiovascular therapy

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ABSTRACT

This paper reports an LC–MS/MS method with positive electrospray ionization for the screening of commonly prescribed cardiovascular drugs in human plasma, including compounds with antihypertensive (57), antidiabetic (12), hypolipemiant (5), anticoagulant (2) and platelet anti-aggregation (2) effects. Sample treatment consisted of a simple protein precipitation with MeOH/0.1 M $ZnSO_4$ (4:1, v/v) solution after the addition of internal standard, followed by evaporation and reconstitution. Analytes separation was performed on a Polar-RP column (150 mm × 2 mm, 4 μm) using a gradient elution of 15 min. The MS system was operated in MRM mode, monitoring one quantitation and one confirmation transition for each analyte. The recovery of the protein precipitation step ranged from 50 to 70% for most of the compounds, while some were considerably affected by matrix effects. Since several analytes fulfilled the linearity, accuracy and precision values required by the ICH guidelines, the method proved to be suitable for their quantitative analysis. The limits of quantitation varied from 0.38 to 9.1 μg/L and the limits of detection from 0.12 to 5.34 μg/L. The method showed to be suitable for the detection of plasma samples of patients under cardiovascular treatment with the studied drugs, and for 55 compounds reliable quantitative results could be obtained.

1. Introduction

The probability of suffering from cardiovascular diseases is closely related to several risk factors such as high blood pressure, obesity, high blood levels of cholesterol and triglycerides and insulin resistance. The combination of these medical disorders is known as metabolic syndrome [1–3] and it is the first cause of mortality worldwide, with more than 17 million deaths each year [4]. Therefore, the prevention and treatment of the disorders associated to the metabolic syndrome is one of the main tasks of the pharmacological therapy. The pathology of the disorders implies a combined cardiovascular therapy [5–7] with drugs which have different targets and mechanisms of action, to regulate each factor separately: reducing the high blood pressure with antihypertensive compounds, decreasing the triglyceride and cholesterol levels (lipid lowering drugs), lowering the sugar concentration in blood (antidiabetics) and increasing the fluidity of blood to avoid thrombus formation (anti-platelets, anticoagulants).

Monitoring of blood concentrations of drugs is always crucial to understand their pharmacokinetics and pharmacodynamics. But in the case of several compounds used in combined cardiovascular therapy, it is also important to detect misuse of these drugs, i.e. for doping, or to reveal accidental or intentional intoxications [8–10] or to check the compliance of patients. Moreover, it provides very valuable information about possible interactions and secondary effects derived from the co-administration of several drugs which share metabolic and/or excretion pathways [11–13].

The analysis of multiple substances with different physicochemical properties and physiological behaviour is always a challenge for the analytical chemist, due to the different expected concentrations in biological fluids (from several micrograms to few picograms) and due to the difficulty of developing an extraction process adequate for all the analytes but selective enough to reduce as much as possible the matrix effects. These factors are responsible for the small

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number of publications aimed on the simultaneous target screening of a high number of drugs belonging to different compound classes [14–16]. However, several analytical methods have been reported for the simultaneous determination of the frequently prescribed associations [17–21].

Therefore, the aim of this work is the development of an analytical method for the simultaneous screening of the most commonly drugs prescribed in combined cardiovascular therapy in human plasma; including 57 antihypertensive (8 angiotensin converting enzyme inhibitors (ACEI) of which 6 are prodrugs and 2 are active compounds, 6 angiotensin-II-receptor antagonists (ARA-II), 29 β-blockers, 2 diuretics and 12 calcium antagonists), 12 antidiabetic (9 belonging to sulfonylurea class, 1 biguanide, 1 meglitinide, 1 thiazolidinedione), 2 anticoagulant (acenocoumarol and warfarin), 5 hypolipemiant (statins) and 2 anti-platelet (ticlopidine, clopidogrel) compounds. Concurrently, a sample preparation procedure has been completed.

Due to the required sensitivity and selectivity, and the convenient sample preparation without derivatization procedures, liquid chromatography–tandem mass spectrometry (LC–MS/MS) [22] was the selected technique to fulfil the objective of this research work.

2. Experimental

2.1. Chemicals and reagents

Fluvastatin was kindly supplied by Novartis Pharma AG (Basel, Switzerland). All the other compounds were generously provided by the Institute of Legal Medicine, Humboldt University (Berlin, Germany).

HPLC-grade methanol was obtained from J.T. Baker Mallinckrodt (Deventer, The Netherlands). Deionized water was prepared with a cartridge deionizer from Memtech (Moorenweis, Germany). Formic acid was purchased from Carl Roth GmbH (Karlsruhe, Germany) and ammonium formate and zinc sulfate from Sigma–Aldrich (Steinheim, Germany). Blank serum samples were obtained from the University Medical Centre of Freiburg (Freiburg, Germany), and were tested by GC/MS for the absence of drugs prior to use.

2.2. Instrumentation

The LC–MS/MS system consisted of a 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIonSpray interface (AB Sciex, Darmstadt, Germany) and a Shimadzu Prominence HPLC system: two LC-20ADsp isocratic pumps, a CTO-20AC column oven, an SIL-20AC autosampler, a DGU-20A3 degasser, and a CBM-20A controller (Shimadzu, Duisburg, Germany). A Polar RP 150 mm × 2 mm, 4 μm column (Phenomenex, Aschaffenburg, Germany) with a guard column of the same material (4 mm × 2 mm) was used for the chromatographic separation.

Eppendorf centrifuge 5415D (Hamburg, Germany) was used for plasma samples centrifugation after protein precipitation.

2.3. Standard solutions and plasma samples

Standard stock solutions of 1000 mg/L were prepared in methanol for each analyte separately. With those solutions a working solution containing the adequate concentrations of all the analytes to spike the plasma samples covering the linearity range (0.12–30 mg/L) was prepared in methanol. A 2.5 mg/L solution containing the three IS (d_3-doxepin, d_5-diazepam and methaqualone) was also prepared in methanol.

Calibration standards were prepared by spiking a pool of drug-free human plasma with the working standard solution and diluting it covering the full studied concentration range. The calibration curves were built from 25 to 5000 μg/L for carbutamide, eprosartan, glibornuride, gliclazide, metformin, tolazamide, tolbutamide, torasemide, valsartan, and warfarin; from 10 to 2000 μg/L for candesartan, glipizide, gliquidone and irbesartan; from 4 to 800 μg/L for atenolol, candesartan cilexetil (cand. cilex.), fluvastatin, glibenclamide, metoprolol, quinaprilat and ticlopidine; from 3 to 600 μg/L for glimepiride; from 2 to 400 μg/L for acenocoumarol, enalapril, enalaprilat, fendiline, nadolol, nicardipine, nifedipine, rosiglitazone, sotalol, telmisartan, tertalolol and toliprolol; from 1 to 200 μg/L for alprenolol, amiloride, betaxolol, carteolol, celiprolol, cilazapril, gallopamil, imidapril, lisinopril, lovastatin, nimodipine, nisoldipine, nitrendipine, oxprenolol, penbutolol, perindopril, pindolol, propranolol, repaglinide, simvastatin, talinolol, timolol and verapamil; from 0.5 to 100 μg/L for acebutolol, amlodipine, atorvastatin, befunolol, bisoprolol, bopindolol, bunitrolol, bupranolol, carazolol, carvedilol, cerivastatin, esmolol, felodipine, isradipine, labetalol, lercanidipine, levobunolol, mepindolol, practolol and ramipril and from 0.5 to 20 μg/L for clopidogrel. All calibration curves consisted of seven calibration points.

2.4. Chromatographic and MS conditions

The mobile phase consisted of solvent A (0.1% formic acid (v/v) with 1 mmol/L ammonium formate) and solvent B (methanol: 0.1% formic acid (v/v) with 1 mmol/L ammonium formate). The gradient applied was the following: 0–1 min, 0% B; 1–5 min, 0–65% B; 5–8 min, 65–95% B, 8–11 min 95% B, 11–12.5 min 95–0% B, 12.5–15.5 min 0% B. Flow started at 0.2 mL/min and linearly increased to 0.4 mL/min during the first minute, then it decreased again to 0.2 mL/min from minute 11 to 12.5. During the chromatographic analysis the column was thermostated at 40 °C and samples were kept at 10 ± 1 °C in the autosampler.

The TurboIonSpray source was operated in positive mode at 500 °C with an ionization voltage of 5500 V. Nitrogen was used as curtain gas (10 psi), gas 1 (80 psi), gas 2 (70 psi) and collision gas (6 psi). Analysis was performed by multiple reaction monitoring mode (MRM), using the precursor ions and the corresponding product ions. Two transitions were monitored for each analyte, one for quantitation and the other one for confirmation (Table 1). In order to obtain a minimum of 10 data points for each chromatographic peak scheduled MRM was used. The cycle time and the retention time window were fixed at 1.8 s and 90 s, respectively, which determines a minimum dwell time of 17 ms.

2.5. Sample treatment

100 μL of human plasma sample were transferred to a 1.5 mL Eppendorf cup and spiked with 10 μL of a 2.5 mg/L IS solution (d_5-diazepam, d_3-doxepin and methaqualone). Protein precipitation was carried out using 200 μL of MeOH/0.1 M $ZnSO_4$ (4:1, v/v) solution. After vortex mixing and centrifugation for 5 min at 16,100 × *g*, the supernatant was transferred to a 2 mL glass vial and evaporated to dryness under N_2 stream at 60 °C. The residue was then reconstituted with 100 μL of mobile phase (*A*/*B*, 60:40 (v/v)) and vortex mixed. 30 μL of aliquots were injected into the LC system for analysis.

2.6. Method validation

Recovery and matrix effect for each analyte were determined at three different concentrations (low, middle and high) following the strategies reported by Matuszewski et al. [23]. Three sets of samples were used for this aim: standards prepared in neat solvent (*A*, *n* = 5), plasma samples spiked after the protein precipitation (PPT) but before the evaporation step (*B*, *n* = 5) and plasma samples spiked before PPT (*C*, *n* = 5). Recovery was calculated by compar-

Table 1
MRM analysis conditions.

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Acebutolol	337.2	319.2	53	5	11	22	3.5	
Acebutolol	**337.2**	**116.1**	**53**	**5**	**11**	**32**	**2.0**	**6.07**
Acenocoumarol	354.1	296.1	33	5	16	25	3.5	
Acenocoumarol	**354.1**	**163.1**	**33**	**5**	**16**	**24**	**2.5**	**8.75**
Alprenolol	**250.2**	**116.1**	**47**	**4**	**9**	**24**	**2.0**	
Alprenolol	250.2	91.1	47	4	9	56	2.0	**6.88**
Amiloride	**230.1**	**171.1**	**41**	**5**	**9**	**24**	**2.5**	
Amiloride	230.1	161.1	41	5	9	35	2.5	**4.92**
Amlodipine	**409.2**	**294.1**	**28**	**4**	**16**	**17**	**3.5**	
Amlodipine	409.2	238.1	28	4	16	19	3.5	**7.61**
Atenolol	267.2	190.2	43	5	10	26	3.0	
Atenolol	**267.2**	**145.1**	**43**	**5**	**10**	**36**	**2.5**	**4.63**
Atorvastatin	**559.3**	**440.2**	**53**	**5**	**17**	**32**	**4.0**	
Atorvastatin	559.3	250.2	53	5	17	61	3.0	**8.74**
Befunolol	**292.2**	**250.2**	**50**	**5**	**11**	**24**	**3.0**	
Befunolol	292.2	203.2	50	5	11	30	3.0	**6.50**
Betaxolol	308.2	133.1	40	5	10	37	2.0	
Betaxolol	**308.2**	**116.1**	**40**	**5**	**10**	**28**	**2.0**	**7.02**
Bisoprolol	326.2	222.2	53	4	10	19	3.0	
Bisoprolol	**326.2**	**116.1**	**53**	**4**	**10**	**26**	**2.0**	**6.72**
Bopindolol	**381.2**	**186.1**	**51**	**5**	**13**	**31**	**2.5**	
Bopindolol	381.2	158.1	51	5	13	49	2.5	**7.62**
Bunitrolol	**249.2**	**193.2**	**31**	**5**	**9**	**20**	**2.5**	
Bunitrolol	249.2	175.2	31	5	9	24	2.5	**6.20**
Bupranolol	**272.1**	**216.1**	**41**	**5**	**9**	**20**	**3.0**	
Bupranolol	272.1	155.1	41	5	9	34	2.5	**7.02**
Candesartan	441.2	423.2	37	5	15	18	4.0	
Candesartan	**441.2**	**263.2**	**37**	**5**	**15**	**18**	**3.0**	**8.34**
Cand. Cilex.	**611.3**	**423.2**	**39**	**5**	**22**	**18**	**4.0**	
Cand. Cilex.	611.3	207.2	39	5	58	18	2.5	**10.12**
Carazolol	299.2	222.2	43	5	10	27	3.0	
Carazolol	**299.2**	**116.2**	**43**	**5**	**10**	**29**	**2.5**	**6.80**
Carbutamide	**272.2**	**156.1**	**32**	**5**	**11**	**22**	**2.5**	
Carbutamide	272.2	92.1	32	5	11	42	1.5	**6.59**
Carteolol	**293.2**	**237.2**	**36**	**5**	**10**	**21**	**3.0**	
Carteolol	293.2	202.2	36	5	10	30	2.5	**5.60**
Carvedilol	407.2	222.2	60	5	13	35	3.0	
Carvedilol	**407.2**	**100.1**	**60**	**5**	**13**	**44**	**2.0**	**7.70**
Celiprolol	380.2	307.2	50	5	12	25	3.5	
Celiprolol	**380.2**	**251.2**	**50**	**5**	**12**	**31**	**3.0**	**6.49**
Cerivastatin	**460.2**	**356.2**	**78**	**5**	**15**	**50**	**3.5**	
Cerivastatin	460.2	324.2	78	5	15	67	3.5	**8.54**
Cilazapril	**418.2**	**211.2**	**34**	**5**	**15**	**28**	**3.0**	
Cilazapril	418.2	70	34	5	15	70	1.5	**7.70**
Clopidogrel	**322.1**	**212.1**	**33**	**5**	**12**	**23**	**3.0**	
Clopidogrel	322.1	184.1	33	5	12	30	3.0	**8.97**
Enalapril	**377.2**	**234.2**	**45**	**5**	**12**	**27**	**3.5**	
Enalapril	377.2	117.1	45	5	12	58	2.5	**7.08**
Enalaprilat	**349.2**	**206.2**	**46**	**5**	**12**	**26**	**3.0**	
Enalaprilat	349.2	117.1	46	5	12	49	2.0	**6.10**
Eprosartan	425.2	207.2	65	5	16	33	3.0	
Eprosartan	**425.2**	**107**	**65**	**5**	**16**	**75**	**2.0**	**7.17**
Esmolol	296.2	219.2	53	5	10	27	3.0	
Esmolol	**296.2**	**145.1**	**53**	**5**	**10**	**38**	**2.0**	**6.50**
Etacrynic acid	303	303	30	4	10	5	4.0	
Etacrynic acid	**303**	**257**	**30**	**4**	**10**	**13**	**3.0**	**8.14**
Felodipine	384.1	352.1	25	4	12	17	3.5	
Felodipine	**384.1**	**338.1**	**25**	**4**	**12**	**17**	**3.5**	**8.89**
Fendiline	316.2	212.2	40	5	13	19	3.0	
Fendiline	**316.2**	**105.1**	**40**	**5**	**13**	**40**	**2.0**	**8.09**
Fluvastatin	**412.2**	**266.2**	**46**	**5**	**13**	**26**	**3.5**	
Fluvastatin	412.2	224.2	46	5	13	44	3.0	**8.74**
Gallopamil	**485.3**	**165.1**	**68**	**5**	**17**	**42**	**2.5**	
Gallopamil	485.3	150.1	68	5	17	63	2.5	**7.60**
Glibenclamide	**494.2**	**369.1**	**25**	**5**	**19**	**21**	**3.5**	
Glibenclamide	494.2	169.1	25	5	19	50	2.5	**8.92**
Glibornuride	367.2	170.2	43	5	13	27	3.0	
Glibornuride	**367.2**	**152.2**	**43**	**5**	**13**	**27**	**2.5**	**8.20**
Gliclazide	**324.2**	**127.1**	**47**	**5**	**13**	**27**	**2.5**	
Gliclazide	324.2	110.1	47	5	13	42	2.0	**8.15**
Glimepiride	**491.2**	**352.2**	**38**	**5**	**15**	**20**	**4.0**	
Glimepiride	491.2	126.1	38	5	15	47	2.5	**9.19**
Glipizide	446.2	347.2	34	5	15	20	3.5	
Glipizide	**446.2**	**321.1**	**34**	**5**	**15**	**20**	**3.5**	**8.21**
Gliquidone	**528.2**	**403.2**	**40**	**5**	**16**	**18**	**3.5**	
Gliquidone	528.2	386.2	40	5	16	30	3.5	**9.57**

Table 1 (*Continued*)

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Imidapril	406.2	332.2	51	5	14	26	3.5	
Imidapril	**406.2**	**234.2**	**51**	**5**	**14**	**28**	**3.0**	**7.17**
Irbesartan	429.2	207.1	54	5	17	34	3.0	
Irbesartan	**429.2**	**180.1**	**54**	**5**	**17**	**60**	**2.5**	**8.37**
Isradipine	372.2	340.2	21	4	13	17	3.5	
Isradipine	**372.2**	**312.2**	**21**	**4**	**13**	**17**	**3.0**	**8.45**
Labetalol	**329.2**	**311.3**	**30**	**5**	**13**	**18**	**3.5**	
Labetalol	329.2	91.1	30	5	13	65	2.0	**6.65**
Lercanidipine	**612.3**	**280.2**	**50**	**10**	**22**	**32**	**3.5**	
Lercanidipine	612.3	100.1	50	10	22	52	2.5	**8.85**
Levobunolol	**292.2**	**236.2**	**45**	**5**	**11**	**22**	**3.5**	
Levobunolol	292.2	201.2	45	5	11	28	3.0	**6.43**
Lisinopril	406.2	246.2	50	5	15	34	3.0	
Lisinopril	**406.2**	**84.1**	**50**	**5**	**15**	**59**	**2.0**	**5.39**
Lovastatin	**405.3**	**285.3**	**28**	**4**	**12**	**16**	**3.0**	
Lovastatin	405.3	199.2	28	4	12	27	2.5	**9.34**
Mepindolol	263.2	186.2	39	5	10	24	2.5	
Mepindolol	**263.2**	**116.1**	**39**	**5**	**10**	**27**	**2.0**	**6.05**
Metformin	**130.1**	**71**	**33**	**4**	**7**	**32**	**2.0**	
Metformin	130.1	60	33	4	7	19	2.0	**1.58**
Metipranolol	310.2	233.2	55	5	12	25	2.5	
Metipranolol	**310.2**	**191.2**	**55**	**5**	**12**	**31**	**2.5**	**6.72**
Metoprolol	268.2	191.2	50	5	10	25	3.0	
Metoprolol	**268.2**	**133.1**	**50**	**5**	**10**	**36**	**2.5**	**6.20**
Nadolol	310.2	254.2	48	5	10	24	3.0	
Nadolol	**310.2**	**201.2**	**48**	**5**	**10**	**32**	**3.0**	**5.53**
Nifedipine	**347.1**	**315.1**	**15**	**4.5**	**13**	**12**	**3.5**	
Nifedipine	347.1	254.2	15	4.5	13	27	3.5	**7.69**
Nimodipine	**419.2**	**343.2**	**24**	**4**	**15**	**18**	**3.5**	
Nimodipine	419.2	301.1	24	4	15	32	3.5	**8.06**
Nicardipine	**480.2**	**315.2**	**52**	**5**	**17**	**33**	**3.5**	
Nicardipine	480.2	166.2	52	5	17	25	2.5	**8.73**
Nisoldipine	**389.2**	**357.2**	**30**	**4**	**14**	**14**	**3.5**	
Nisoldipine	389.2	315.2	30	4	14	14	3.5	**8.72**
Nitrendipine	361.1	329.2	29	4	13	18	3.5	
Nitrendipine	**361.1**	**315.1**	**29**	**4**	**13**	**18**	**3.5**	**8.59**
Oxprenolol	266.2	225.2	33	5	13	18	3.0	
Oxprenolol	**266.2**	**116.1**	**33**	**5**	**13**	**24**	**2.5**	**6.65**
Penbutolol	**292.2**	**236.2**	**47**	**5**	**10**	**23**	**3.0**	
Penbutolol	292.2	133.1	47	5	10	34	2.5	**7.69**
Perindopril	369.2	295.2	49	5	11	24	3.0	
Perindopril	**369.2**	**172.2**	**49**	**5**	**11**	**28**	**2.5**	**7.17**
Pindolol	249.2	172.2	40	5	12	23	2.5	
Pindolol	**249.2**	**116.1**	**40**	**5**	**12**	**24**	**2.0**	**5.67**
Practolol	267.2	190.2	40	5	10	25	3.0	
Practolol	**267.2**	**148.2**	**40**	**5**	**10**	**31**	**2.5**	**4.84**
Propranolol	**260.2**	**183.2**	**49**	**4.5**	**10**	**26**	**3.0**	
Propranolol	260.2	116	49	4.5	10	26	2.5	**6.90**
Quinaprilat	**411.2**	**206.2**	**37**	**5**	**15**	**28**	**2.5**	
Quinaprilat	411.2	117.1	37	5	15	55	2.0	**7.20**
Ramipril	**417.2**	**234.2**	**52**	**4.5**	**15**	**30**	**3.0**	
Ramipril	417.2	117.1	52	4.5	15	57	2.5	**7.67**
Repaglinide	**453.2**	**230.2**	**54**	**5**	**18**	**39**	**3.0**	
Repaglinide	453.2	162.2	54	5	18	30	2.5	**8.33**
Rosiglitazone	**358.1**	**135.1**	**60**	**4**	**13**	**38**	**2.5**	
Rosiglitazone	358.1	107.1	60	4	13	55	2.0	**6.79**
Simvastatin	**419.3**	**285.3**	**36**	**5**	**14**	**18**	**3.0**	
Simvastatin	419.3	199.2	36	5	14	28	2.5	**9.41**
Sotalol	**273.2**	**255.2**	**28**	**4**	**10**	**16**	**3.0**	
Sotalol	273.2	133.1	28	4	10	38	2.5	**4.43**
Talinolol	364.2	308.2	54	5	12	25	3.5	
Talinolol	**364.2**	**100.1**	**54**	**5**	**12**	**33**	**2.0**	6.79
Telmisartan	515.2	497.3	83	5	16	47	4.0	
Telmisartan	**515.2**	**276.2**	**83**	**5**	**16**	**65**	**3.0**	**8.96**
Tertalolol	296.2	240.2	45	5	11	23	3.0	
Tertalolol	**296.2**	**222.2**	**45**	**5**	**11**	**27**	**3.0**	**7.18**
Ticlopidine	**264.1**	**154.1**	**41**	**5**	**12**	**24**	**2.5**	
Ticlopidine	264.1	125.0	41	5	12	44	2.5	**6.81**
Timolol	**317.2**	**261.2**	**45**	**5**	**11**	**23**	**3.5**	
Timolol	317.2	244.2	45	5	11	30	3.5	**6.20**
Tolazemide	**312.1**	**115.1**	**43**	**5**	**12**	**27**	**2.0**	
Tolazemide	312.1	91.1	43	5	12	57	2.0	**7.90**
Tolbutamide	**271.1**	**155.1**	**35**	**5**	**9**	**24**	**2.5**	
Tolbutamide	271.1	91.1	35	5	9	49	2.0	**7.53**
Toliprolol	**224.2**	**147.1**	**45**	**5**	**9**	**24**	**2.5**	
Toliprolol	224.2	119.1	45	5	9	31	2.0	**6.21**

Table 1 (*Continued*)

Analyte	Q1	Q3	DP	EP	CEP	CE	CXP	t_r
Torasemide	**349.1**	**264.1**	**38**	**5**	**11**	**25**	**3.0**	**7.11**
Torasemide	349.1	168.1	38	5	11	66	2.5	
Valsartan	**436.2**	**235.2**	**37**	**5**	**15**	**27**	**3.0**	**8.30**
Valsartan	436.2	207.1	37	5	15	40	3.0	
Verapamil	**455.3**	**165.1**	**60**	**5**	**16**	**40**	**3.0**	**7.54**
Verapamil	455.3	150.1	60	5	16	59	2.5	
Warfarin	309.1	251.1	27	5	12	26	3.0	**8.35**
Warfarin	**309.1**	**163.1**	**27**	**5**	**12**	**22**	**2.5**	
d_3-doxepin	283.1	107.1	40	10	18	35	4	7.19
d_5-diazepam	290.1	154.1	40	10	18	35	4	8.75
Methaqualone	251.1	91.1	40	10	21	50	4	8.07

Q1: precursor ion (m/z); Q3: fragment ion (m/z); DP: declustering potential (V); EP: entrance potential (V); CEP: cell entrance potential (V); CE: collision energy (V); CXP: cell exit potential (V); t_r: retention time (min). Quantitation transitions are shown in bold.

ing the areas of *B* and *C* samples (Rec (%) = $C_{area}/B_{area} \times 100$) and matrix effect by comparing the areas of *A* and *B* samples (M.E. (%) = $B_{area}/A_{area} \times 100$). A matrix effect value higher than 100% indicates enhancement, whereas a lower one indicates suppression effects.

The matrix effect was also qualitatively studied using the post-column infusion technique reported by Bonfiglio et al. [24]. For this purpose, a methanolic solution of the studied compounds (120 μg/L) was infused post-column via a mixing tee at a flow rate of 25 μL/min while the analysis of a pretreated blank plasma sample was carried out.

The selectivity of the method was tested by analyzing blank human plasma samples from 6 different sources under optimized chromatographic conditions, and by comparing them with spiked plasma samples at a concentration close to the lower limit of quantitation (LLOQ).

LLOQ was calculated according to criteria of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [25]: a specific calibration curve containing the analytes in the range of the LLOQ was studied and the value of the LLOQ was determined based on the standard deviation of the regression curve. Signal to noise ratio corresponding to the LLOQ was required to be higher than 10. Limit of detection (LOD) was calculated following the same approach using the confirmation transition with a signal to noise ratio of 3.

Considering the large concentration ranges for several analytes, an $1/x^2$ statistical weight was applied in order to obtain the most reliable calibration curves [26]. Calibration curves ($n = 7$) were built by plotting the corrected areas (analyte area/IS area) for each concentration level versus the nominal concentration of each calibration standard.

Three samples, corresponding to low, medium and high concentration levels, were assessed in sets of five replicates in order to evaluate the intra- and interday accuracy and precision. This procedure was repeated at 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.

2.7. Application to real samples

Blood samples were collected from 19 different patients under treatment with a combination of the studied drugs 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 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.

3. Results

3.1. Matrix effect and recovery

The chromatographic separation obtained for a spiked sample of 50 μg/L is shown in Fig. 1. The blue line corresponds to the total ion current as the sum of all MRM signals of the analytes during a post-column infusion of a 120 μg/L solution containing all the studied analytes. Considerable ion suppression at the elution peak retention time (1–2 min) and around the last part of the gradient elution (9–10 min) can be observed. The ion suppression at the beginning of the chromatographic separation is attributed to the elution of polar compounds present in plasma, whereas the suppression at the final part is associated with the elution of non-polar phospholipids [27,28]. Consequently, the analytes eluting in those ranges of retention time suffer from high signal suppression as it can be observed in Table 2. Metformin, coeluting with the injection peak, suffers a suppression of more than ten times in its intensity and lovastatin, simvastatin and gliquidone, which all co-elute with phospholipids also show significant signal suppression. Matrix effects were studied quantitatively for all the analytes and it showed to be comparable at the three different studied concentrations ($n = 5$). In Table 2, the average values obtained for the three concentrations are shown.

Recovery of the analytes, also gathered in Table 2, ranges from 29.9% to 74.5%. For most of the analytes, the recovery value was higher than 50%. Only candesartan cilexetil, enalaprilat, eprosartan, gliclazide, lisinopril, quinaprilat and tolazemide did not fulfil this criterion.

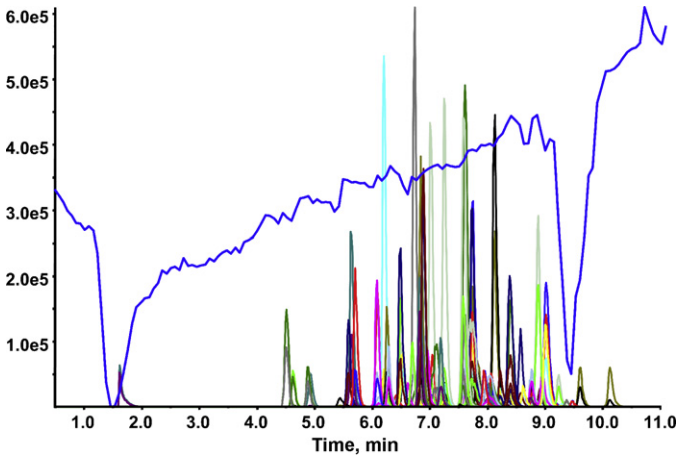

Fig. 1. Chromatographic separation obtained for a plasma sample spiked with all the studied analytes (50 μg/L) together with the TIC corresponding to a post-column infused 120 μg/mL solution (blue line).

Table 2
Average matrix effect (M.E.) and recovery (Rec.) values obtained from three different concentrations (values showed as average ± standard deviation, $n = 5$).

Analyte	M.E. %	Rec. %	Compound name	M.E. %	Rec. %
Acebutolol	90.3 ± 2.5	62.8 ± 0.5	Isradipine	65.8 ± 4.3	65.4 ± 5.0
Acenocoumarol	64.9 ± 5.4	67.9 ± 3.4	Labetalol	116.8 ± 16.1	57.9 ± 2.8
Alprenolol	94.8 ± 3.9	65.3 ± 4.0	Lercanidipine	68.6 ± 4.6	56.1 ± 3.4
Amiloride	78.2 ± 1.9	52.3 ± 3.0	Levobunolol	105.2 ± 7.2	68.0 ± 4.1
Amlodipine	77.7 ± 3.5	56.8 ± 2.2	Lisinopril	178.7 ± 14.4	23.0 ± 1.0
Atenolol	66.9 ± 6.0	65.8 ± 3.5	Lovastatin	16.6 ± 2.1	59.1 ± 1.9
Atorvastatin	75.0 ± 1.0	62.6 ± 2.7	Mepindolol	82.0 ± 6.2	64.1 ± 2.6
Befunolol	104.1 ± 5.8	64.2 ± 4.2	Metformin	8.0 ± 1.5	65.1 ± 4.6
Betaxolol	96.9 ± 2.0	65.0 ± 3.4	Metoprolol	96.1 ± 3.3	66.5 ± 3.5
Bisoprolol	103.5 ± 4.6	67.3 ± 3.8	Nadolol	109.1 ± 6.1	65.0 ± 2.4
Bopindolol	90.1 ± 4.8	57.8 ± 0.3	Nicardipine	90.6 ± 1.3	64.0 ± 3.0
Bunitrolol	84.8 ± 0.8	64.4 ± 3.8	Nifedipine	73.5 ± 3.2	68.5 ± 2.9
Bupranolol	88.1 ± 1.0	63.1 ± 3.5	Nimodipine	71.7 ± 5.4	65.9 ± 2.2
Candesartan	100.3 ± 6.5	51.9 ± 3.2	Nisoldipine	55.1 ± 2.0	68.4 ± 2.1
Cand Cilex	104.6 ± 6.1	48.7 ± 2.3	Nitrendipine	59.9 ± 3.5	67.2 ± 1.9
Carazolol	101.1 ± 4.5	62.5 ± 6.2	Oxprenolol	95.1 ± 1.4	67.0 ± 2.8
Carbutamide	103.1 ± 14.9	66.6 ± 1.3	Penbutolol	88.7 ± 4.4	64.7 ± 4.2
Carteolol	90.4 ± 5.2	64.9 ± 3.5	Perindopril	113.3 ± 10.9	67.2 ± 3.3
Carvedilol	96.3 ± 1.2	55.5 ± 3.2	Pindolol	91.6 ± 4.1	63.0 ± 2.1
Celiprolol	104.7 ± 6.6	65.2 ± 1.4	Practolol	84.4 ± 4.2	65.0 ± 3.0
Cerivastatin	78.8 ± 5.9	61.2 ± 1.8	Propranolol	94.8 ± 3.6	63.2 ± 3.9
Cilazapril	142.0 ± 8.6	65.5 ± 3.4	Quinaprilat	112.3 ± 13.5	29.9 ± 0.8
Clopidogrel	58.0 ± 4.0	60.5 ± 4.9	Ramipril	117.3 ± 6.4	68.5 ± 3.2
Enalapril	129.3 ± 11.6	64.6 ± 2.7	Repaglinide	98.2 ± 11.7	58.7 ± 3.1
Enalaprilat	104 ± 9.6	42.6 ± 2.9	Rosiglitazone	91.6 ± 4.2	57.5 ± 6.0
Eprosartan	103.3 ± 7.1	44.9 ± 3.0	Simvastatin	23.3 ± 0.3	59.2 ± 3.2
Esmolol	103.1 ± 7.5	65.8 ± 2.2	Sotalol	73.1 ± 8.6	66.5 ± 3.0
Felodipine	81.4 ± 5.5	63.5 ± 3.1	Talinolol	114.6 ± 5.9	65.0 ± 3.3
Fendiline	74.9 ± 3.5	66.9 ± 2.4	Telmisartan	86.6 ± 7.8	56.1 ± 3.1
Fluvastatin	112.9 ± 13.0	55.6 ± 1.8	Tertatolol	93.2 ± 2.6	66.2 ± 3.5
Gallopamil	91.6 ± 1.9	65.1 ± 1.8	Ticlopidine	81.6 ± 2.0	51.4 ± 4.2
Glibenclamide	115.2 ± 8.3	62.0 ± 2.1	Timolol	99.9 ± 5.7	67.6 ± 3.8
Glibornuride	89.9 ± 5.3	63.6 ± 6.4	Tolazamide	101.3 ± 6.5	47.3 ± 2.6
Gliclazide	98.2 ± 8.6	39.0 ± 6.2	Tolbutamide	92.4 ± 4.7	65.8 ± 3.2
Glimepiride	63.9 ± 3.2	61.8 ± 1.8	Toliprolol	90.1 ± 4.6	65.6 ± 3.3
Glipizide	97.6 ± 9.7	74.5 ± 9.9	Torasemide	107.5 ± 14.5	58.9 ± 4.9
Gliquidone	48.2 ± 4.5	56.1 ± 2.0	Valsartan	103.9 ± 14.2	53.3 ± 3.5
Imidapril	111.2 ± 8.7	61.9 ± 4.0	Verapamil	90.7 ± 1.3	64.5 ± 1.9
Irbesartan	98.4 ± 7.1	64.5 ± 3.6	Warfarin	83.5 ± 6.8	70.7 ± 8.0

3.2. Selectivity

Selectivity has been studied by analyzing 6 blank plasma samples from different healthy volunteers. The chromatograms did not show interfering signals within the retention time windows of the chromatographic peaks of the analytes and the internal standards, which could be misinterpreted as the target compounds or could affect the bias of the method.

The selectivity of the isobaric transitions has also been tested. From the 156 transitions only atenolol and practolol shared an isobaric transition (267.2 → 190.2). In the case of atenolol the transition (267.2 → 145.1) was used for quantitation, because it was the most intensive; whereas for practolol the transition (267.2 → 148.2) was used, in order to avoid possible interferences between the analytes. Nevertheless, as it can be seen in Fig. 2, both analytes are separated to baseline, which confirms the suitability of the isobaric transition for confirmation or quantitation purposes.

3.3. LOD, LLOQ and linearity

LOD and LLOQ, calculated following the ICH criteria, are shown in Table 3.

For calibration purposes, the most suitable IS was chosen by studying the relative error values of the calibration standard concentrations and the linearity of the calibration curves. For some analytes (atorvastatin, candesartan cilexetil, cerivastatin, enalaprilat, fluvastatin, glimepiride, impidapril, lisinopril, lovastatin, quinaprilat and simvastatin) better results were obtained with an external calibration compared to a calibration using the reported IS. The applicability of external calibration using electrospray as ionization source is debatable due to its associated matrix effects. Moreover, only the use of isotopic marked IS can correct the matrix effects caused by the ionization technique, which is not feasible with these types of screening methods. Therefore, for the above mentioned substances, other IS should be tested for quantitation purposes [29].

Some calcium channel blockers (isradipine, nifedipine, nimodipine, nisoldipine, and nitrendipine) did not fit conve-

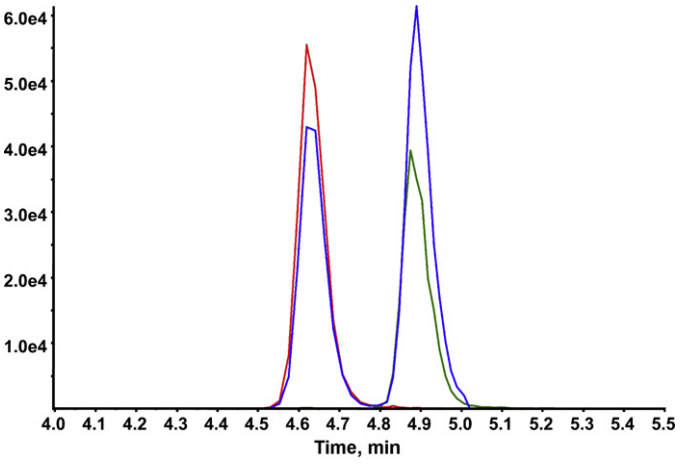

Fig. 2. Chromatogram with the isobaric analytes atenolol (left) and practolol (right).

Table 3
LOD, LLOQ, precision (as average inter and intra-RSD for three different concentrations) and accuracy (as average inter an intra RE for three different concentrations) for each analyte with the corresponding IS (d_5-diaz: d_5-diazepam; d_3-dox: d_3-doxepin; Meth: methaqualone; Ext: external calibration; Screen: only for qualitative purposes).

Analyte	I.S.	LOD (μg/L)	LLOQ (μg/L)	RSD Inter	RSD Intra	RE Inter	RE Intra
Acebutolol	d_5-diaz	0.18	0.50	5.6 ± 3.3	6.7 ± 0.5	5.1 ± 3.0	5.7 ± 2.0
Acenocoumarol	d_5-diaz	0.65	1.02	4.1 ± 1.8	6.2 ± 2.7	2.7 ± 3.3	3.8 ± 1.9
Alprenolol	d_3-dox	0.16	0.60	3.6 ± 0.6	4.8 ± 1.6	7.1 ± 5.6	7.1 ± 4.7
Amiloride	d_5-diaz	0.24	0.60	5.6 ± 1.6	9.1 ± 2.3	5.3 ± 5.3	6.8 ± 2.8
Amlodipine	d_3-dox	0.17	0.50	7.9 ± 6.1	8.1 ± 0.9	4.9 ± 4.1	7.3 ± 0.5
Atenolol	d_3-dox	0.22	0.80	5.5 ± 4.8	6.0 ± 0.6	4.7 ± 4.4	6.5 ± 3.5
Befunolol	d_3-dox	0.16	0.50	4.7 ± 1.5	5.7 ± 2.4	5.0 ± 1.1	4.8 ± 1.0
Betaxolol	d_3-dox	0.40	0.47	5.0 ± 1.7	5.0 ± 1.5	8.4 ± 1.2	7.6 ± 1.5
Bopindolol	d_5-diaz	0.27	0.50	6.5 ± 2.3	6.5 ± 1.0	2.1 ± 1.4	4.3 ± 1.8
Bunitrolol	d_3-dox	0.17	0.50	3.0 ± 0.4	5.0 ± 1.6	7.2 ± 3.3	6.3 ± 3.0
Bupranolol	d_3-dox	0.16	0.48	2.7 ± 0.7	4.6 ± 1.6	5.4 ± 5.7	5.5 ± 4.0
Candesartan	d_3-dox	0.34	1.12	11.7 ± 2.6	9.3 ± 1.7	1.3 ± 0.9	8.6 ± 1.6
Carazolol	d_3-dox	0.17	0.43	3.1 ± 0.6	5.0 ± 2.1	8.4 ± 4.6	8.3 ± 3.6
Carteolol	d_3-dox	0.48	0.81	2.8 ± 1.4	4.7 ± 0.9	8.2 ± 7.0	8.1 ± 6.1
Carvedilol	d_3-dox	0.16	0.50	7.2 ± 0.9	6.9 ± 1.7	8.0 ± 0.9	8.0 ± 1.3
Celiprolol	d_3-dox	0.14	0.52	6.8 ± 1.3	5.8 ± 0.2	4.0 ± 4.8	6.1 ± 2.1
Cilazapril	d_3-dox	0.17	0.53	5.5 ± 2.0	6.3 ± 1.2	4.6 ± 3.0	5.6 ± 3.0
Clopidogrel	d_5-diaz	0.13	0.49	7.6 ± 4.1	5.1 ± 2.0	9.1 ± 3.6	8.7 ± 4.3
Enalapril	d_5-diaz	0.18	0.55	7.2 ± 3.7	8.4 ± 2.2	7.0 ± 5.8	8.7 ± 3.1
Eprosartan	d_3-dox	5.34	9.10	5.5 ± 6.0	8.0 ± 2.5	6.9 ± 3.7	8.6 ± 3.7
Esmolol	d_3-dox	0.21	0.50	6.6 ± 1.1	5.4 ± 2.2	2.7 ± 1.5	5.2 ± 1.6
Felodipine	d_5-diaz	0.43	0.50	8.1 ± 0.5	8.7 ± 2.8	4.1 ± 0.6	6.1 ± 1.0
Fendiline	d_3-dox	0.32	0.61	5.7 ± 2.9	7.9 ± 1.7	6.7 ± 4.0	6.9 ± 3.7
Gallopamil	d_3-dox	0.17	0.48	6.5 ± 0.9	6.3 ± 1.5	8.4 ± 2.7	8.2 ± 2.2
Glibenclamide	d_5-diaz	0.18	0.62	5.1 ± 1.1	7.7 ± 3.1	4.4 ± 4.5	5.5 ± 2.1
Glibornuride	Meth	0.25	0.48	10.5 ± 3.2	6.7 ± 2.0	2.0 ± 2.3	7.8 ± 2.4
Gliclazide	d_3-dox	0.61	1.46	2.9 ± 0.3	7.4 ± 2.0	9.9 ± 1.8	9.1 ± 1.6
Glipizide	d_3-dox	0.24	0.69	7.5 ± 4.0	6.1 ± 3.5	2.5 ± 3.1	5.7 ± 2.2
Gliquidone	d_5-diaz	0.39	0.61	6.1 ± 5.8	6.9 ± 0.6	3.9 ± 2.4	6.2 ± 4.3
Irbesartan	d_3-dox	0.37	1.25	3.7 ± 2.6	5.6 ± 1.5	8.5 ± 4.0	7.9 ± 3.3
Lercanidipine	d_3-dox	0.19	0.50	4.4 ± 1.6	5.1 ± 1.8	3.6 ± 2.6	3.8 ± 1.4
Levobunolol	d_3-dox	0.12	0.42	1.8 ± 0.8	4.7 ± 1.8	8.8 ± 7.0	8.7 ± 6.0
Mepindolol	d_5-diaz	0.50	0.79	9.2 ± 2.7	8.4 ± 1.7	2.5 ± 3.6	7.1 ± 2.9
Metformin	d_5-diaz	0.76	0.84	5.1 ± 2.3	7.9 ± 2.5	1.4 ± 1.6	4.5 ± 2.0
Nadolol	d_3-dox	0.30	1.00	5.0 ± 2.6	5.5 ± 1.8	7.6 ± 3.6	7.5 ± 3.4
Nicardipine	d_3-dox	0.15	0.51	4.5 ± 0.9	4.1 ± 1.2	7.7 ± 3.2	6.8 ± 3.5
Penbutolol	d_3-dox	0.20	0.42	9.9 ± 0.8	5.5 ± 2.0	1.8 ± 0.6	7.4 ± 1.0
Perindopril	d_3-dox	0.61	1.14	7.0 ± 1.5	5.9 ± 3.3	4.4 ± 1.1	6.2 ± 1.7
Pindolol	d_3-dox	0.26	0.74	3.0 ± 0.4	5.4 ± 0.8	5.1 ± 1.1	4.5 ± 1.0
Practolol	d_5-diaz	0.22	0.50	4.7 ± 3.4	10.2 ± 2.1	2.2 ± 0.8	3.3 ± 1.9
Propranolol	d_3-dox	0.13	0.38	2.4 ± 1.9	4.1 ± 1.0	7.4 ± 5.1	7.5 ± 4.0
Ramipril	d_3-dox	0.20	0.50	3.3 ± 2.6	5.6 ± 1.8	6.9 ± 6.1	7.1 ± 4.7
Repaglinide	d_5-diaz	0.20	0.73	4.1 ± 1.6	6.2 ± 0.2	3.3 ± 2.9	3.7 ± 1.9
Rosiglitazone	d_3-dox	0.20	0.77	3.1 ± 1.5	5.2 ± 1.4	8.4 ± 6.3	8.6 ± 4.8
Sotalol	d_3-dox	0.20	0.60	5.4 ± 0.8	6.2 ± 0.6	3.7 ± 1.6	4.2 ± 0.9
Talinolol	d_3-dox	0.13	0.43	8.6 ± 5.0	5.2 ± 2.3	2.5 ± 1.3	6.8 ± 5.1
Telmisartan	d_5-diaz	0.14	0.62	8.4 ± 2.9	4.6 ± 1.5	4.0 ± 4.4	7.5 ± 1.4
Tertatolol	d_3-dox	0.15	0.58	3.2 ± 2.2	5.0 ± 2.0	4.7 ± 4.8	5.6 ± 2.8
Ticlopidine	d_3-dox	0.20	0.68	5.1 ± 5.2	5.3 ± 0.5	7.6 ± 2.6	8.4 ± 1.6
Tolbutamide	d_3-dox	0.57	2.52	8.0 ± 4.3	6.4 ± 1.6	2.9 ± 2.4	6.8 ± 4.4
Toliprolol	d_3-dox	0.52	0.52	3.6 ± 1.1	4.6 ± 1.0	8.6 ± 5.6	8.5 ± 4.7
Torasemide	d_3-dox	0.19	0.77	7.4 ± 5.2	6.8 ± 1.4	5.5 ± 4.6	7.9 ± 1.5
Valsartan	d_3-dox	0.30	1.00	8.2 ± 4.3	7.3 ± 2.5	3.8 ± 5.6	7.7 ± 1.3
Verapamil	d_3-dox	0.20	0.69	6.1 ± 5.0	5.1 ± 1.7	5.6 ± 4.8	6.8 ± 3.6
Warfarin	d_3-dox	0.31	1.52	1.6 ± 1.4	7.3 ± 2.5	8.4 ± 4.5	8.3 ± 3.7
Bisoprolol[a]	d_3-dox	0.15	0.51	3.1 ± 3.6	4.5 ± 2.0	11.4 ± 3.1	11.4 ± 2.2
Carbutamide[a]	d_3-dox	0.32	1.13	3.2 ± 1.0	5.7 ± 3.1	6.3 ± 7.0	7.3 ± 4.8
Labetalol[a]	d_3-dox	0.50	0.50	2.5 ± 1.1	4.9 ± 3.7	14.3 ± 0.6	14.2 ± 0.3
Metoprolol[a]	d_3-dox	0.18	0.66	3.6 ± 0.8	5.5 ± 3.3	7.9 ± 6.8	7.9 ± 5.8
Oxprenolol[a]	d_3-dox	0.17	0.65	1.4 ± 0.6	3.9 ± 1.1	10.2 ± 6.2	9.5 ± 6.2
Timolol[a]	d_3-dox	0.18	0.54	4.2 ± 2.5	5.3 ± 1.9	6.0 ± 7.2	7.3 ± 4.4
Tolazamide[a]	d_3-dox	0.47	1.20	3.0 ± 1.5	8.0 ± 2.3	7.2 ± 7.6	7.7 ± 5.9
Atorvastatin	Ext	0.24	0.48	9.0 ± 1.4	4.9 ± 3.2	4.8 ± 1.8	7.9 ± 1.6
Cand Cilex	Ext	0.47	1.10	12.1 ± 4.6	7.1 ± 3.7	2.7 ± 1.0	9.1 ± 3.6
Cerivastatin	Ext	0.20	0.42	10.6 ± 1.2	8.0 ± 4.6	2.8 ± 2.3	8.1 ± 1.9
Enalaprilat	Ext	0.25	0.78	9.6 ± 3.4	4.2 ± 1.9	3.5 ± 0.8	6.7 ± 3.2
Fluvastatin	Ext	0.18	1.42	6.8 ± 1.0	5.0 ± 1.4	1.6 ± 1.0	5.3 ± 1.1
Glimepiride	Ext	0.20	0.60	8.0 ± 1.5	6.1 ± 3.7	5.6 ± 0.8	7.4 ± 0.4
Imidapril	Ext	0.30	0.81	10.1 ± 6.6	4.3 ± 1.6	1.7 ± 1.7	7.4 ± 5.1
Lisinopril	Ext	0.34	1.00	7.5 ± 2.5	5.0 ± 1.4	5.8 ± 2.9	6.8 ± 3.0
Lovastatin	Ext	1.00	1.50	5.3 ± 3.0	7.9 ± 3.3	5.7 ± 1.9	7.0 ± 1.3
Quinaprilat	Ext	0.79	4.45	11.0 ± 4.5	4.0 ± 0.2	3.6 ± 2.8	8.9 ± 3.2

Table 3 (*Continued*)

Analyte	I.S.	LOD (μg/L)	LLOQ (μg/L)	RSD Inter	RSD Intra	RE Inter	RE Intra
Simvastatin	Ext	0.25	1.25	10.5 ± 1.6	5.1 ± 1.6	2.6 ± 2.6	7.2 ± 0.9
Isradipine	Screen	0.39	1.35	–	–	–	–
Nifedipine	Screen	0.56	2.00	–	–	–	–
Nimodipine	Screen	0.21	0.68	–	–	–	–
Nisoldipine	Screen	0.44	1.37	–	–	–	–
Nitrendipine	Screen	0.17	1.21	–	–	–	–

[a] RSD and RE values of the high concentrations of these analytes were not taken into account for the calculation of the average RSD and RE values due to the lack of linearity of the last calibration points.

niently to a calibration curve with any IS and showed high imprecision among their replicas, which is probably due to the fact that these analytes degrade under light exposure [30]. Thus, even the method is not acceptable for the quantitative analysis of these drugs; it can be used to detect the presence of these calcium channel blockers in plasma samples.

Seven analytes (bisoprolol, carbutamide, labetalol, metoprolol, oxprenolol, timolol and tolazemide) lost their linearity in the higher range of the calibration curve, which may indicate a saturation of the detector due to the high intensity of the response. Problems of ion suppression were rejected since the quantitative values obtained for matrix effect showed no difference between high and low concentrations. In order to evaluate the suitable calibration range the highest calibration standard was removed from the calibration curves of metoprolol and oxprenolol, which improved linearity of both compounds. For the other five analytes the last two calibration standards were rejected to obtain satisfying calibration curves. Therefore the calibration range for the quantitative analysis of these compounds was fixed as follows: from 0.5 to 38 μg/L for bisoprolol and labetalol; from 1 to 75 μg/L for timolol; from 1 to 125 μg/L for oxprenolol; from 4 to 500 μg/L for metoprolol and from 25 to 1880 μg/L for carbutamide and tolazemide. Dilution of plasma samples might be necessary in cases the concentrations of the analytes exceed the calibration range.

3.4. Precision and accuracy

Plasma samples spiked with low, medium and high concentrations of drugs were processed and analyzed. Their concentrations were obtained from the interpolation of the corrected area of each analyte on its corresponding calibration curve. Results obtained for the intra and inter day accuracy (RE) and precision (RSD) are summarized in Table 3 as the average values and their corresponding standard deviation obtained for the three different concentration levels. Among the studied compounds 65 fulfil the precision (RSD < 15%) and accuracy (RE < 15%) criteria, including the compounds that fit better to an external calibration. Isradipine, nifedipine, nimodipine, nisoldipine and nitrendipine showed high RSD values and the calibration curves were not adequate, thus, the method is only suitable for their qualitative analysis. Considering the lack of linearity of bisoprolol, carbutamide, labetalol, metoprolol, oxprenolol timolol and tolazemide calibration curves, RE and RSD values corresponding to the high concentration of these analytes were not determined.

3.5. Application to real samples

Plasma samples obtained from patients under treatment with acenocoumarol, amlodipine, atenolol, atorvastatin, bisoprolol, enalapril, enalaprilat, felodipine, fluvastatin, lisinopril, metformin, metoprolol, quinaprilat, repaglinide, simvastatin, valsartan or a combination of them were analyzed using the described methodology. Besides studying quantitation and confirmation transitions, their ratio was measured and compared with the ratio obtained for a standard solution. All of these drugs were detected in the plasma samples except simvastatin, which was only detected in three plasma samples of the five samples of patients treated with this drug. Only in one sample simvastatin could be quantified.

Probable reasons for the lack of detection are its low therapeutic concentration range and the high ion suppression it suffers. In Table 4 the plasmatic concentration levels for 13 plasma samples obtained from patients under treatment with a combination of the studied compounds are reported. The chromatogram corresponding to sample 6 containing atorvastatin, bisoprolol and valsartan and spiked with the three different IS is shown in Fig. 3.

4. Discussion and conclusions

We present a comprehensive approach for simultaneous analysis of many compounds usually prescribed in cardiovascular combined therapy. The proposed LC–MS/MS method is adequate for the screening of 78 drugs and showed and appropriate specificity, precision and accuracy for the quantitative determination of 55 compounds, using the internal standard approach. It was successfully applied to the detection and quantitation of several of the studied analytes in plasma samples obtained from patients under treatment with these drugs.

Some of the compounds of interest fulfilled the precision and accuracy requirements only for external calibration. This kind of calibration is not suitable for the analysis of biological matrices with electrospray ionization, thus, different IS should be assessed to find a suitable one. If it is possible, the authors recommend the use of isotopic marked internal standards. A standard addition method could be an adequate alternative as well, since matrix effects are

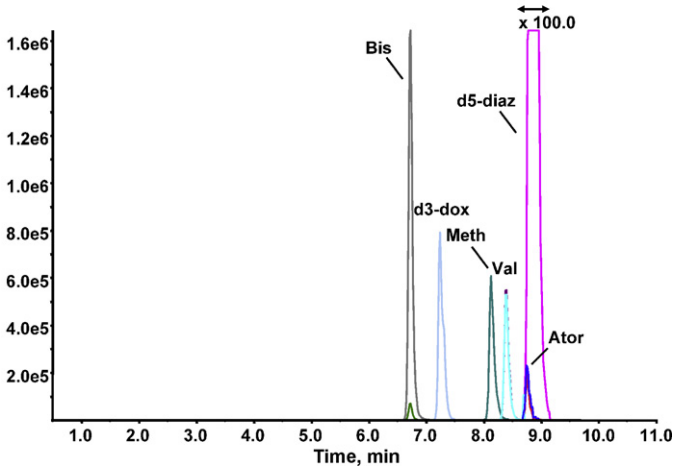

Fig. 3. Chromatogram corresponding to a plasma sample of a patient (no. 6) under treatment with atorvastatin (Ator), bisoprolol (Bis) and valsartan (Vals) with the internal standards: d_5-diazepam (d_5-diaz), d_3-doxepin (d_3-dox) and methaqualone (meth).

Table 4
Concentration values for plasma samples obtained from patients under cardiovascular treatment with a combination of the studied drugs (results reported with a 95% confidence level).

Patient	Administered drug	Time after oral intake (h)	Dose (mg)	Concentration (μg/L) ± ts
1	Amlodipine	Unknown	Unknown	126.5 ± 22.0
	Fluvastatin	12	40	4.7 ± 0.3
2	Atorvastatin	12	5	1.0 ± 0.1
	Bisoprolol	3	160	2.4 ± 0.6
	Valsartan	3	10	732.9 ± 42.7
3	Amlodipine	12	10	128.4 ± 22.4
	Bisoprolol	12	10	2.8 ± 0.7
	Quinaprilat	1	12.5	363.9 ± 33.0
	Simvastatin	12	5	<LLOQ
4	Felodipine	12	5	1.4 ± 0.2
	Fluvastatin	12	80	20.0 ± 1.3
	Metoprolol	12	50	16.2 ± 2.2
	Valsartan	3	160	94.7 ± 5.5
5	Atorvastatin	12	8	0.9 ± 0.1
	Bisoprolol	2	10	<LLOQ
6	Atenolol	2	50	53.8 ± 5.2
	Enalapril	2	20	71.0 ± 6.2
	Enalaprilat	–	–	25.0 ± 2.5
	Simvastatin	12	40	<LLOQ
7	Atorvastatin	12	20	6.7 ± 0.5
	Enalapril	3	5	16.0 ± 1.4
	Enalaprilat	–	–	26.5 ± 2.6
	Metformin	3	850	755.5 ± 13.6
	Repaglinide	3	1	10.4 ± 1.0
8	Amlodipine	12	5	1.5 ± 0.2
	Atorvastatin	12	40	9.7 ± 0.7
	Metformin	18	425	57.3 ± 1.0
	Quinaprilat	2	20	6.1 ± 0.56
9	Felodipine	12	5	1.5 ± 0.2
	Fluvastatin	12	80	7.5 ± 0.5
	Metoprolol	12	50	14.4 ± 1.9
	Valsartan	3	160	1039.9 ± 60.6
10	Atorvastatin	12	5	0.9 ± 0.1
	Valsartan	2	180	68.36 ± 4.0
11	Enalapril	3	20	103.3 ± 9.0
	Enalaprilat	–	20	15.1 ± 1.5
	Simvastatin	12	20	<LOD
12	Amlodipine	12	5	60.8 ± 10.6
	Atenolol	2	50	88.7 ± 8.6
	Lisinopril	2	20	43.5 ± 6.5
	Simvastatin	22	10	1.9 ± 0.2
13	Valsartan	3	160	87.9 ± 5.12
	Simvastatin	12	10	<LOD

constant within the same serum sample, but this method is very time consuming and a higher volume of sample is needed.

Isradipine, nifedipine, nimodipine, nisoldipine, and nitrendipine showed a very high variability probably due to their photodegradation. In order to avoid the degradation of these compounds light exposure of the sample should be minimized and amber vials used.

The recovery range is very wide (29.9–74.5%), probably due to the different chemical properties of the compounds, and for some analytes is lower than 50%. However, the sensitivity of the method allows the analysis of those compounds in their therapeutic range, although some compounds showed a high matrix effect. Since protein precipitation does not remove endogenous plasma compounds (such as phospholipids), they cause high ion suppression to some of the studied analytes. This was the case for simvastatin, for which detection and quantitation limits were too high to cover the therapeutic range, as it was observed in the analysis of this drug in plasma samples. The same problem is expected to occur to lovastatin, taking into account the similar therapeutic range and quantitation limit. Alternative sample treatments such as solid phase extraction might reduce the matrix effects and improve the sensitivity, but the sample preparation process would be more time consuming and acceptable recoveries for all analytes included into this method would probably not be feasible with one extraction method.

The simple and non-selective pre-treatment procedure allows adding further analytes in case of addition of new compounds of the studied substance class, new drug families and new associations to maintain the applicability of the method for pharmacological therapies of the metabolic syndrome.

However, the suitability of using the method for quantitation purposes must be studied for each single compound, assuring the usefulness of the respective internal standard and including a validation for each analyte.

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