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Short communication

Simultaneous determination of the acid/base antihypertensive drugs celiprolol, bisoprolol and irbesartan in human plasma by liquid chromatography

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Abstract

A simple, rapid method for the simultaneous determination of cardiovascular drugs: celiprolol, bisoprolol and irbesartan in human plasma is described. The two main features of the proposed method deal first, with a simultaneous solid phase extraction of weakly basic beta-blockers derivatives and irbesartan which exhibit weak acidic properties; second with an absorbance monitoring using diode array detection in order to insure an improved selectivity. The separation is performed on a C_{18} Kromasil® 4.6 mm × 150 mm column using a linear gradient to achieve an entire separation of the four species in less than 20 min. The full analytical validation is performed according to guidance for industry for bioanalytical method validation. Linearity of the response was demonstrated for each drug for a range fulfilling the reported plasma levels, that is 10–500, 5–250 and 20–1000 ng l^{-1} for celiprolol, bisoprolol and irbesartan respectively. Intra- and inter-day relative standard deviations for all compounds were, in any case, lower than 11% and the method exhibits a convenient accuracy (percentage of relative error lower than 6% for each drug). In each case, the LOD were sufficient to detect post dose trough concentrations for checking patient's observance. Moreover, selectivity towards either endogenous species or co-administered drugs was demonstrated by combination of the use of the solid phase extraction process, gradient elution and diode array detection facilities, making thus, the proposed technique especially suitable for routine drug monitoring of resistant hypertensive patients.
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Keywords: Celiprolol; Bisoprolol; Irbesartan

1. Introduction

High blood pressure is quantitatively the largest single risk factor for premature death and disability due to its extremely high prevalence in industrialised countries [1]. Resistant hypertension is defined as the failure to control blood pressure to normal levels (<140/90 mmHg) using multiple antihypertensive medications. Although resistant hypertension affects a minority of treated hypertensive patients, this group continues to experience disproportionately high cardiovascular event rates [2]. Despite introduction of several new classes of antihypertensive agents over the past two decades, sub-optimal selection of drug therapy remains among the most common causes for treatment failures [3]. Drug therapy based on a treatment algorithm [3] has been reported to produce superior blood pressure control than usual care. However, some patients have still their blood pressure uncontrolled at follow-up, and a major advance would be to provide therapeutic drug monitoring in order to detect abnormalities in treatment compliance or pharmacokinetics.

The present study aimed at validating a screening method to simultaneously quantify two beta-blockers (celiprolol, bisoprolol) and one angiotensin II receptor blocker (irbesartan) by means of liquid chromatography coupled with a Diode Array Detector (DAD) detection, which would provide an efficient separation, as well as a sensitive and selective detection. International guidelines for the treatment of resistant hypertension [4] recommend that treatment include at least three antihypertensive drugs in adequate dosages, including a diuretic. The usual combinations associate to the diuretic, first, either an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker, and second, either

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a beta-blocker or a calcium channel blocker. In the present study, we arbitrarily focused on the second and third treatment steps, and selected one angiotensin receptor blocker and two beta-blockers for standardised treatment. Among angiotensin receptor blocker, we chose irbesartan because of its high bioavailability and long plasma high-life [5]. Among beta-blockers, we selected the beta-1 selective adrenoceptor antagonist bisoprolol, because of its high availability, long plasma half-life and metabolic and renal clearance characteristics that involve hepatic and renal pathway nearly equally [6]. As an alternative to bisoprolol in patients with chronic obstructive lung disease, we selected celiprolol, because of its beta-2 agonist properties [7].

Analytical methods developed for determination of these types of drugs mainly used liquid chromatography: bisoprolol [8,9], celiprolol [10–12], screening of beta-blockers [13–17], irbesartan [18,19] or screening of angiotensin II receptor antagonist [20] using UV or fluorimetic detection. To our knowledge, no simultaneous method for determination of beta-blockers and angiotensin II receptor antagonists in plasma or in other biologic fluids has been reported so far.

2. Experimental

2.1. Chemicals

Celiprolol hydrochloride (+/−), Bisoprolol fumarate (+/−) and irbesartan were kindly provided by Aventis pharma (Dagenham, UK), Merck (Damstadt, Germany) and Sanofi (Aramon, France), respectively. Their chemical structures are represented in Fig. 1. Internal Standard (I.S.): propranolol (+/−) was purchased from Sigma–Aldrich (Saint-Quentin, France).

LC grade acetonitrile were purchased from Sigma–Aldrich (Seelze, Germany), and LC grade methylic alcohol from Prolabo (Briave-le-Canal, France).

Potassium dihydrogenophosphate, 25% ammonia solution and 37% hydrochloric acid were purchased from Merck (Damstadt, Germany). All reagents were of analytical grade and used without further purification.

2.2. Standard solutions

Individual 1000 μg/ml methanolic stock solutions of celiprolol, bisoprolol (as free base), irbesartan and internal standard were first prepared. Each of these stock solutions was used for preparing an intermediate solution containing all the compounds of interest (25, 12.5 and 50 μg/ml for celiprolol, bisoprolol and irbesartan, respectively). Final working solutions were obtained by appropriate dilution of the intermediate solutions with deionised water. Quality control (QC) and calibration standard samples were prepared by adding appropriate volumes of these solutions to drug-free plasma. Six calibration concentrations were used to establish the standard curves and these concentrations were defined in accordance with reported plasma trough concentrations in the literature for bisoprolol [21], celiprolol [22] and irbesartan [19,23,24]. Stock and working solutions were stored at −22 °C for 3 months.

2.3. Equipment

The clean-up procedure consisted of solid-phase extraction and was performed using Oasis MCX® (Mixed-mode Cation eXchanger) columns ($1\,cm^3$/30 mg) obtained from Waters (Milford, MA, USA). These columns were adapted

Compound	pKa
Bisoprolol	9.5
Celiprolol	9.7
Irbesartan	4.7

Fig. 1. Chemical structures of celiprolol, bisoprolol and irbesartan and their respective pK_a.

onto in a Baker® SPE-24G vacuum system (Deventer, Holland).

Chromatography was performed using Waters Associates chromatographic equipment (Milford, MA, USA) consisting of a model 600 solvent delivery pump used with a pump control module, a model 717 plus auto sampler (used at room temperature), a model 996 Photodiode Array Detector (DAD). Data monitoring was performed with a Millennium® (version 3.05.01) software. A Kromasil C18 column (5 μm, 4.6 mm × 150 mm) from Interchim (Montluçon, France) was used for all separations.

2.4. Mobile phase

Solvent A consisted of phosphate buffer 0.1 M adjusted to pH 3.4 ± 0.1 with hydrochloric acid. Phosphate buffer was filtered using a vacuum filter system equipped with a Hydro-Aqueous Teflon Filter (HATF®) 0.45 μm filter (Millipore, Bedford, MA). Solvent B was pure acetonitrile. The starting mobile phase (S) consisted of 76% solvent A and 24% solvent B (v/v).

2.5. Sample treatment

Blood was collected from consulting patients into Heparin-Vacutainer® tubes and gently mixed. Samples were immediately centrifuged 10 min at 3000 rpm (1880 × g) at +4 °C. The plasma obtained was transferred to 5 ml tubes and stored at −22 °C until use.

The extraction columns were preconditioned by passing 1 ml of methanol followed by 1 ml of water. Plasma samples (1 ml) spiked with 50 μl of I.S solution and 40 μl hydrochloric acid 4.5N were passed through the SPE column. Then the loaded columns were washed with 2 × 1 ml of phosphate buffer (0.1 M, pH 3.4) and then with 1 ml of methanol. Celiprolol, bisoprolol, irbesartan and I.S. were eluted from the column using 2 × 1 ml of methanol–ammonia solution (95:5, v/v). All elution processes including washing steps were performed under a −20 kPa negative pressure. The solvent extracts were evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 200 μl of S, sonicated for 30 s and 100 μl of the solution was injected.

2.6. Chromatographic conditions

Chromatography was performed at room temperature. The flow-rate used was set at 1 ml/min and gradient profile was linear. Initial condition was S and the gradient run over 15 min up to 40% A, 60% B then held constant for 6 min. One min was necessary to return to S and held constant 10 min. Optimisation has show that after 17 min of chromatographic run, 4 min were necessary to the column from impurities and 10 min to re-equilibrate the system. The DAD detector was used to monitor chromatographic data using a spectral range from 200–280 nm. Quantification of celiprolol, bisoprolol and irbesartan were carried out at 225 nm. Each collected ultraviolet spectrum was compared with that of corresponding reference standard compound. Peak purity calculation facilities were used to ensure that the analyte peak of interest was free from co-eluting impurities with different spectral properties.

2.7. Validation

For the validation of the proposed method, the parameters of accuracy, precision, limit of quantification (LOQ), linearity, recovery, drugs stability in plasma and selectivity were investigated. The validation was performed in accordance to the guidance of industry for bioanalytical method validation [25].

3. Results and discussion

3.1. Sample treatment optimisation

One of the main characteristics of the proposed method comes from the simultaneous extraction procedure involved. That is an extraction of basic species (i.e., beta-blockers) with acidic one (i.e., irbesartan). In fact, the respective pK_a of the secondary amines of celiprolol and bisoprolol ranging from 9.5 to 9.7, respectively [26] as well as the acidic functionality of tetrazolic moiety of irbesartan with a pK_a of 4.7 [27] showed that there is no pH value for which the two types of compounds can be simultaneously uncharged.

Consequently, when classical liquid-liquid extraction procedure, largely used for extraction of beta-blockers [8–12,14] and irbesartan [18], was envisaged; two successive steps: acidic (for irbesartan), then alkaline (for beta blockers) pH extraction range has to be implemented. Our experiments demonstrated the tedious time consuming character, as well as the poor reproducibility of such a procedure. A second approach consisted of simple protein precipitation using acetonitrile without any pH adjustment. Unfortunately, although some convenient yields (with an average recovery of about 90% for each compound) and a relative satisfying reproducibility (CV% $<$ 15% for each compound), the chromatograms exhibited a large number of numerous endogenous peaks illustrating the limited selectivity of the sample pre-treatment. This is why a SPE sample treatment was finally envisaged with the aim of increasing the throughput of the sample treatment step for further routine analysis. After numerous attempts and many tested extraction support, a convenient solution was found thanks to the use of MCX® phase acting both by cationic exchange as well as a reverse stationary phase with a partition mechanism [28]. The optimisation of the procedure showed that 1 ml plasma sample acidified with hydrochloric acid 4.5N, 40 μl (pH 2) led to the more efficient retention and to the highest selectivity onto the SPE column. This clearly demonstrated the cationic exchange mechanism involved at

Table 1
Intra-day and inter-day precision (expressed as R.S.D. (%)) and accuracy (expressed as relative error: R.E. (%)) for celiprolol, bisoprolol and irbesartan in human plasma

Compound	Concentration added (ng/ml)	Intra-day ($n = 6$)			Inter-day ($n = 6$)		
		Concentration measured (ng/ml)	R.S.D. (%)	R.E. (%)	Concentration measured (ng/ml)	R.S.D. (%)	R.E. (%)
Celiprolol	30	29.0	7.1	−3.3	28.4	6.6	−5.4
	100	96.9	2.6	−3.1	98.8	3.1	−1.2
	400	397.5	1.8	−0.6	387.6	2.4	−3.1
Bisoprolol	15	15.1	6.8	0.7	14.6	10.6	−2.8
	50	49.5	5.8	−0.9	47.0	8.9	−6.0
	200	196.2	5.3	−1.9	192.2	5.8	−3.9
Irbesartan	60	60.5	2.4	0.9	57.1	7.2	−4.8
	200	201.1	1.4	0.6	197.8	3.0	−1.1
	800	814.4	2.4	1.8	773.1	1.8	−3.8

R.S.D. (%): relative standard deviation; R.E. (%): deviation from nominal value.

this pH with the protonated beta-blockers species, whereas the more lipophilic and uncharged irbesartan is retained mainly with hydrophobic interactions. Elution of both types of compounds was obtained with 1 ml methanol/ammonia (95:5, v/v) eluent after making the basic derivatives uncharged and due to the high solubility of non-protonated irbesartan in methanol. In such a way, a relatively clean chromatogram profile permitting the further determination of beta-blockers other than celiprolol and bisoprolol and the determination of angiotensin II receptor antagonist other than irbesartan was obtained.

3.2. Chromatography

One other important aspect of the present work was to perform an assay with a relatively short analysis time, convenient for the routine analysis of three compounds of interest and I.S. One of the difficulties to be solved was the large difference in polarity between the two types of compounds (beta-blockers and irbesartan). To circumvent this problem, we implemented a polarity gradient based on acidic pH eluent, in order to increase the polarity of the beta-blockers by protonating their basic functionalities, combined with a limited acetonitrile content. This was demonstrated as sufficient enough to obtain a rapid elution in less than 20 min with a convenient resolution between compounds of interest. This, associated with the efficacy of the sample purification described above, makes easy the transposition to determinate of beta-blockers other than celiprolol and bisoprolol or angiotensin II receptor antagonists other than irbesartan.

3.3. Analytical figures of merits

3.3.1. Accuracy and precision

The accuracy and precision of the assay were determined by analysing three levels (low, medium, high) of plasma samples corresponding to the three QC concentrations, six times within day and at six different days. The QC concentration levels were 30, 100, 400 ng/ml for celiprolol; 15, 50, 200 ng/ml for bisoprolol and 60,200 and 800 ng/ml for irbesartan, respectively. Precision was calculated as relative standard deviation (R.S.D., %), and accuracy as the relative error (R.E., %). The intra- and the inter-day precision data are summarised in Table 1. R.S.D. values remain lower than 11% whatever the compound and the concentration tested.

In addition, the method could be considered as conveniently accurate since derivation from the theoretical value was, in any case less than 6% (Table 1).

3.3.2. Linearity and limit of quantification

Calibration curves were prepared in the range of 10–500 ng/ml for celiprolol, 5–250 ng/ml for bisoprolol and 20–1000 ng/ml for irbesartan. No weighting was applied. The parameters of the calibration curves and their correlation coefficients show a good linearity in the aforesaid concentration ranges (Table 2) and cover concentration ranges for the active substances accordingly to the literature.

Table 2
Assay validation of celiprolol, bisoprolol and irbesartan by HPLC with DAD detection in human plasma

Compound	Linear range (ng/ml)	Slope ± S.D. ($\times 10^{-3}$)	Intercept ± S.D. ($\times 10^{-3}$)	Linear regression coefficient (r)
Celiprolol	10–500	9.03 ± 0.49	−33.4 ± 17.2	0.9996
Bisoprolol	5–250	5.59 ± 0.40	11.2 ± 42.7	0.9990
Irbesartan	20–1000	9.65 ± 0.55	−8.64 ± 38.0	0.9994

The limits of quantification (LOQ) were determined as the lowest concentration used in the construction of the corresponding standard curve and defined as 10, 5 and 20 ng/ml for celiprolol, bisoprolol and irbesartan, respectively.

3.3.3. Recovery

Three QC concentrations were used to calculate recovery. Recovery was determined from the percentage ratio of the mean peak area of the extracted samples ($n = 3$) to that of the unextracted samples ($n = 3$). Recoveries calculated

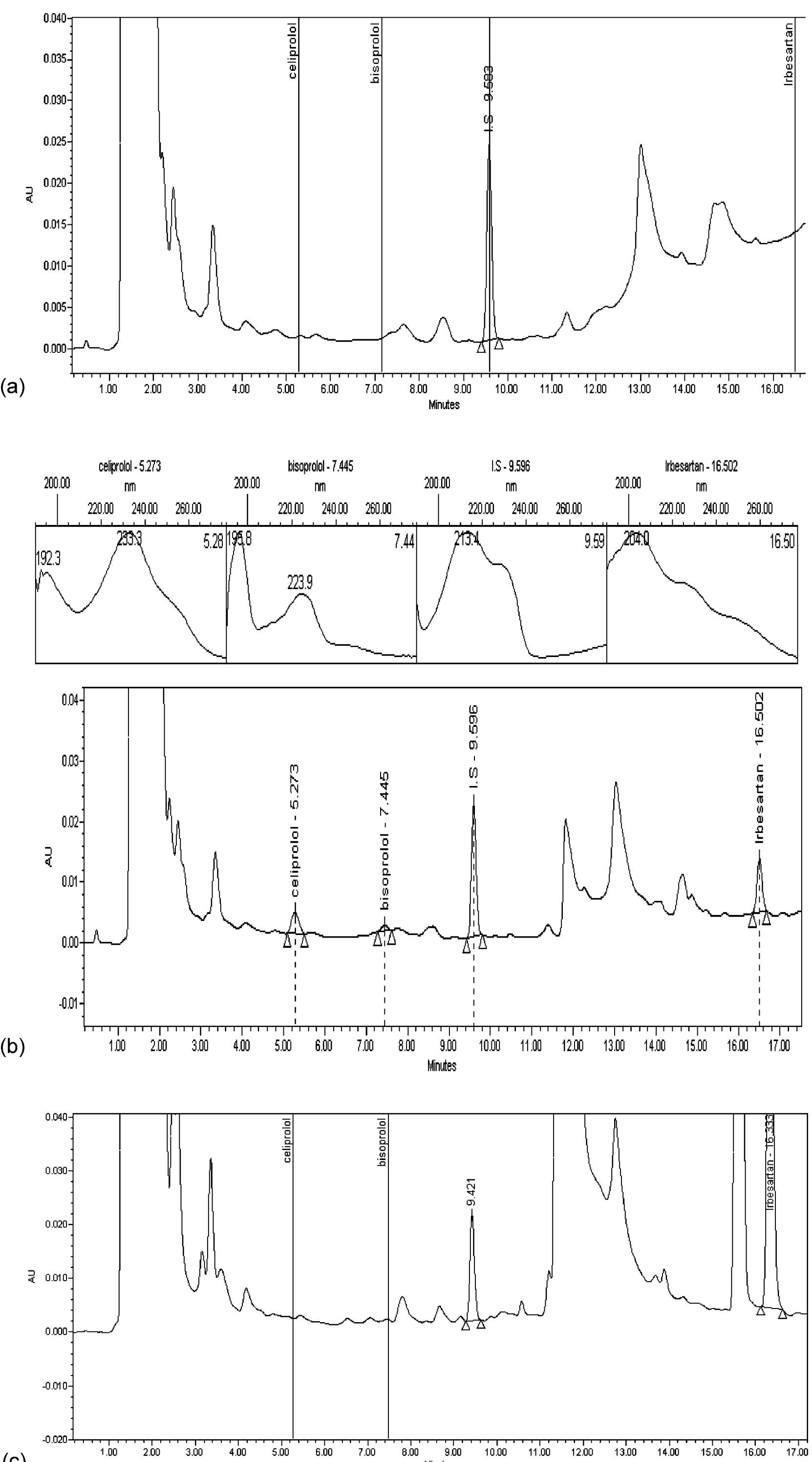

Fig. 2. Typical chromatogram of a control plasma sample (a), LOQ level plasma sample spiked with 10 ng/ml celiprolol; 5 ng/ml bisoprolol and 20 ng/ml irbesartan (b), treated irbesartan patient plasma sample (c). For experimental conditions, see text.

Table 3
Celiprolol, bisoprolol and irbesartan recoveries from human plasma at quality control (QC) concentrations.

Compound	Recovery (%) ± S.D.		
	Low concentration level ($n = 3$)	Medium concentration level ($n = 3$)	High concentration level ($n = 3$)
Celiprolol	90.3 ± 3.8	87.5 ± 1.3	84.5 ± 2.1
Bisoprolol	84.3 ± 2.4	79.1 ± 7.0	82.1 ± 0.7
Irbesartan	87.1 ± 2.5	90.6 ± 1.7	95.1 ± 3.2

are reported in Table 3. The mean absolute recoveries were demonstrated to be independent of the concentration level and were at least 79% for all drugs. The recovery of I.S. was 90% for a 50 ng/ml plasma concentration.

3.3.4. Selectivity

Interferences of endogenous matrix components, metabolites, decomposition products or other exogenous xenobiotics were evaluated first, by injections of six lots of plasma from male and female volunteers; second by direct injection of solutions prepared in the solvent S of various cardiovascular drugs or other drugs frequently co-administered in hypertension treatments.

After evaluation of six plasma, no interfering compounds were detected. Typical chromatograms obtained from control human plasma; plasma spiked with 10 ng/ml of celiprolol, 5 ng/ml of bisoprolol and 20 ng/ml of irbesartan (corresponding of the limit of detection) and a patient plasma with irbesartan are show in Fig. 2.

Among approximately 20 evaluated drugs were selected for their possible co-prescription (Table 4), some of them were detected but their retention time was found, in all cases, different from the drug or I.S. retention time (i.e., Rs > 1.5).

Moreover, the DAD facility was used to confirm the quality of the selectivity using a home made spectrum library obtained by direct injection of drugs. Peaks of compounds of interest were identified and confirmed by comparison with the standard spectra library. At last, comparison of the spectrum taken at the peak apex to the spectra corresponding to the ascending and decreasing part of the peak confirmed that celiprolol, bisoprolol, irbesartan and I.S. peaks were free from co-eluting substances.

Table 4
Drugs tested as possible interferents in the determination of celiprolol, bisoprolol and irbesartan in plasma

Drugs	Retention time (min) ± S.D.	Drugs	Retention time (min) ± S.D.
Celiprolol	5.19 ± 0.07	I.S.: propranolol	9.46 ± 0.06
Bisoprolol	7.31 ± 0.08	Irbesartan	16.32 ± 0.11
Acebutolol	3.50	Losartan	14.76
Aspirin	ND	Metoprolol	4.04
Atenolol	2.25	Nifedipine	ND
Cafeine	2.56	Paracetamol	2.46
Candesartan	ND	Perindopril	ND
Captopril	ND	Piretanide	15.39
Carvediol	13.03	Ramipril	ND
Enalapril	ND	Simvastatine	ND
Hydrochlorothiazide	3.73	Sotalol	2.27
Indapamide	14.05	Telmisartan	17.07
Labetolol	8.11	Valsartan	17.20
Lisinopril	ND		

ND: not detected.

3.3.5. Drugs stability in plasma

The stability of celiprolol, bisoprolol and irbesartan in plasma was investigated by assaying three concentration levels of plasma samples corresponding to the QC concentrations and stored at −22 °C, +4 °C or at room temperature (protected from light) for 1 month. Results showed the absence of degradation products at −22 °C for this period. One month stability at +4 °C was demonstrated too, but in this case, some additional peaks were observed. At last, at room temperature, irbesartan degradation was detected and occurrence of interfering peak at bisoprolol retention time was detected. In summary, plasma can be kept frozen at −22 °C at least for one month before to be analysed.

The stability of these three compounds after three freeze (−22 °C) and thaw cycles was also investigated at the three QC concentration levels. These three drugs were demonstrated to be stable upon three freeze-thaw cycles.

4. Conclusion

This work permits for the first time, the simultaneous determination of various beta-blockers in the presence of irbesartan, making thus possible a partial therapeutic drug monitoring of patient treated with an antihypertensive polytherapy. The proposed method on one hand, appeared as convenient for a better monitoring of the patient observance; and on the other hand, for further pharmacokinetic studies. As already point-out, the selectivity study had reasonably demonstrated that other treatments schedules using beta-blockers and angiotensin receptor blockers different from the presently studied drugs should be easily monitored. The following step of the preliminary study, presently in progress, will be the determination of the whole prescribed drug regimen, i.e. beta-blockers, angiotensin receptor blockers and thiazidic diuretic and among them: especially hydrocholorothiazide.

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