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Journal of Chromatography B, 674 (1995) 85–91

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatography with amperometric detection applied to the screening of β-blockers in human urine

M.I. Maguregui, R.M. Alonso*, R.M. Jiménez

Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

First received 21 March 1995; revised manuscript received 10 July 1995; accepted 11 July 1995

Abstract

A high-performance liquid chromatographic method with electrochemical detection has been developed for the determination of six β-blockers: atenolol, nadolol, timolol, metoprolol, oxprenolol, and alprenolol.

The chromatographic separation was performed using a μBondapack C_{18} column, a mobile phase of acetonitrile–water (40:60), containing 5 m*M* KH_2PO_4/K_2HPO_4 proved to be optimal at a 1.3 ml/min flow-rate, and a pH of 6.5. The temperature was optimized at 30 ± 0.2°C. The amperometric detector, equipped with a glassy carbon electrode, was operated at 1300 mV versus Ag/AgCl in the direct current mode. The method was applied to the determination of these compounds at two concentration levels: ppm and ppb (ng/ml), obtaining relative standard deviations lower than 5% at ppm levels and lower than 10% at ppb levels, and quantitation limits ranging from 15 ppb to 500 ppb.

The method was applied to the screening of β-blockers in spiked urine samples, with a total elution time lower than 12 min, obtaining the best recoveries for timolol and metoprolol (never greater than 93%). These recoveries together with the low limits of quantitation achieved, allows its application to doping analysis in human urine.

1. Introduction

Since the discovery of the blocker properties of dichloroisoproterenol by Powell and Slater in 1958 [1], β-adrenergic receptor antagonists have found wide applications in the treatment of hypertension, cardiac arrhythmias, and other cardiovascular disorders.

These drugs block the β-receptors in the heart, resulting in a decreased cardiac rhythm, and this fact can be useful in sports where aiming is important [2]. Due to their sedative effect, in January 1987 the use of β-blockers was forbidden in sports such as pentathlon, shooting and billiards.

The screening analysis of β-blocking drugs in doping control has been preferably done by gas chromatography–mass spectrometry after derivatization of the bifunctional polar groups of their aminopropanol side-chain, using N-methyl-N-trimethylsilyltrifluoroacetamide/N-methyl-bis-(trifluoroacetamide) (MSTFA/MBTFA), following their extraction from urine [3]. However the poor gas chromatographic properties of trimethylsilyl/trifluoroacetamide (TMS/TFA) derivatives of several polar β-blockers have limited the use of GC–MS for doping analysis of these compounds [4].

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00303-7

Current methods for the determination and identification of β-blockers usually involve high performance liquid chromatography (HPLC) combined with UV or fluorimetric detection [5–16].

There are only a few reports on HPLC with electrochemical detection (ED) applied to the determination of β-blockers. These reports have dealt mainly with coulometric detection, and have only been applied to a few β-blockers, generally in single determinations and mostly in plasma: timolol in plasma [17,18], labetalol in plasma and urine [19–21], and oxprenolol in plasma [22].

The aim of this paper is the application of an HPLC–electrochemical detection (ED) method for the separation and determination of six β-blockers in urine samples: 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy] benzeneacetamide (atenolol); 5-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy] - 1,2,3,4 - tetrahydro - 2,3 - naphthalenediol, (nadolol); 1-[(1,1-dimethylethyl)-amino] - 3 - [[4 - morpholinyl - 1,2,5 - thiadiazol - 3 - yl]-oxy 2-propanol(timolol); 1-[4(2-methoxyethyl)-phenoxy]-3-[(1- methylethyl)-amino]-2-propanol-(metoprolol); 1-[(1-methylethyl)-amino]-3-[2-(2-propenyloxy)phenoxy]-2-propanol (oxprenolol); and 1-[(1-methylethyl) amino]-3-[2-(2-propenyl)phenoxy]-2-propanol (alprenolol).

2. Experimental

2.1. Apparatus and column

The HPLC system consisted of a Waters Model 510 (Waters, Assoc., Barcelona, Spain) HPLC pump, and a Rheodyne Model 7125 (Pharmacia, Barcelona, Spain) injector with a loop of 20 μl.

The electrochemical detector was a PAR Model 400 with a glassy carbon cell (EG&G Princeton Applied Research, Instrumatic, Madrid, Spain). It was operated in the d.c. mode at +1300 mV versus an Ag/AgCl electrode, with a 5-s low-pass filter time constant, and a current range between 50 and 200 nA. Chromatograms were recorded with the help of a computer and were treated with the software Millenium 2010 Chromatography Manger from Waters.

The column used was a 30 cm × 3.9 mm I.D., 10-μm, 125-Å μBondapack C_{18} column (Waters Assoc.). A μBondapack C_{18} guard column (Waters Assoc.) was used to prevent column degradation. The column was kept at constant temperature using a Waters TMC temperature control system.

The organic layer obtained in the clean-up procedure, was evaporated to dryness under a nitrogen stream, using the Turvo Vap LV Evaporator from Zymark Corporation (Varian, Barcelona, Spain).

2.2. Reagents and solutions

The β-blockers studied were alprenolol hydrochloride, atenolol, (±)-metoprolol (+)-tartrate, oxprenolol hydrochloride, timolol maleate and nadolol, all from Sigma (Bilbao, Spain). Potassium dihydrogenphosphate, and dipotassium hydrogenphosphate were Merck Suprapur (Bilbao, Spain). All solvents were Lab-Scan HPLC grade, and the water used was obtained by Milli-RO and Milli-Q Millipore systems.

Stock solutions of pure β-blockers (100 μg/ml) were prepared in water–acetonitrile mixtures containing the same proportion of acetonitrile as used in the mobile phase, and stored at 4°C. Working solutions were obtained by appropriate dilution, just before use.

2.3. Chromatographic conditions

Acetonitrile–water (40:60) containing 5 m*M* potassium dihydrogenphosphate/dipotassium-hydrogenphosphate was used as the mobile phase. The salts served as the supporting electrolyte. The pH was adjusted with either phosphoric acid or 1 *M* KOH. The mobile phase was filtered with a 0.45-μm membrane, and the air was removed from the phase by bubbling helium through.

The μBondapack column head pressure was 1500 p.s.i. at a flow-rate of 1.3 ml/min. The

injection volume was 20 μl. The column was kept constant at 30 ± 0.2°C.

2.4. Sample clean-up procedure

β-Blockers in general can be considered as lipophilic compounds, and as such can be extracted by various organic liquids. The lipophilicity of these drugs is bound to their non-ionized forms, therefore the adjustment of the pH to a value higher than their pK_a values (pK_a values of around 9) by ca. 2 units is the first step of each extraction.

The procedure used was as follows: 0.5 ml of NaOH (5 *M*), 2 ml of diethyl ether and 1 g of Na_2SO_4 were mixed with 3 ml of urine. The mixture was shaken mechanically for 15 min and centrifuged for 5 min at 734 *g*. The diethyl ether layer was separated and evaporated to dryness at 60°C under a gentle stream of nitrogen. The residue was dissolved in 10 ml of mobile phase and was measured under calibration conditions.

2.5. Reproducibility and extraction efficiency

The reproducibility and efficiency of the extraction procedure was determined by extracting replicate ($n = 3$) spiked urine samples. The samples were spiked with 2 ppm of each β-blocker.

The recoveries of the β-blockers studied were estimated by measuring the peak areas of non-extracted standard solutions in the appropriate solvent, and comparing them with the peak areas obtained from extracting spiked urine samples of the same concentration. Quantitative recoveries calculated from urine samples spiked with 2μg/ml of each β-blocker are collected in Table 1.

2.6. Electrode maintenance

The electrode was cleaned at the end of each working day by keeping it at −600 mV for 60 s and after that at +1500 mV for 10 min. This operation was repeated three times, using a mobile phase of pure methanol at a flow-rate of 1.3 ml/min. Also when the signal-to-noise ratio decreased, the glassy carbon electrode was cleaned with a tissue wetted with water to remove any precipitated salt, and then with a tissue soaked with methanol.

Table 1
Recoveries of β-blocking drugs from 4 ml human urine spiked with 2 ppm of each compound ($n = 3$)

Compound	Recovery (%)	R.S.D (%)
Atenolol	—	—
Nadolol	32.85	5.48
Timolol	93.25	2.50
Metoprolol	87.09	2.04
Oxprenolol	92.99	14.96
Alprenolol	81.82	2.39

3. Results and discussion

In static conditions some β-blockers such as metoprolol and atenolol are hardly oxidized at a glassy carbon electrode, others like oxprenolol and timolol proved to be electroactive in the whole pH range studied (1–12), while alprenolol and nadolol only showed well defined voltammetric peaks in alkaline medium, undergoing a unique oxidation wave. The oxidation peak potentials shift to less positive potentials as the pH increases [23].

Upon the basis of this oxidative behaviour, a chromatographic system with amperometric detection can be developed for the determination of these compounds.

3.1. Optimization of the chromatographic system

Hydrodynamic voltammograms of each compound were carried out to choose the optimum potential (Fig. 1). An oxidative potential of 1.3 V was chosen as the working potential, although this potential is not the optimal for the oxidation of atenolol (1600 mV), oxprenolol (1400 mV) and metoprolol (1400 mV), induced a lower baseline noise, still providing a good sensitivity and selectivity.

The pH of the mobile phase was considered as one of the main parameters for the optimization of the retention times and peak symmetry. These

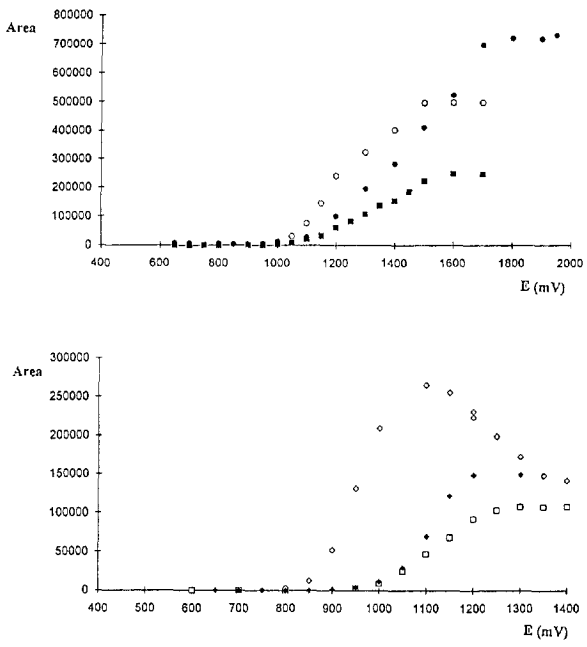

Fig. 1. Hydrodynamic voltammograms of (●) atenolol, (□) nadolol, (◇) timolol, (■) metoprolol, (○) oxprenolol and (◆) alprenolol. Amount of drug injected: 200 ng. The chromatographic conditions can be found in the experimental section.

drugs can be classified in different groups with regard to their distribution coefficients. Nadolol and atenolol are lipophobic, while metoprolol, oxprenolol and alprenolol are intermediates in the lipophilicity range. The highly lipophilic drugs have distribution coefficients up to 2000 times greater than those of the least lipid soluble drugs. It has been shown that small changes in pH result in large changes in distribution coefficients; an increase of the pH from 7.0 to 7.4 generally causes up to three fold difference in the distribution coefficient [24].

Buffers in the pH range 3–7 were prepared and tested for the chromatographic separation of these analytes. All of the drugs chromatographed in this study showed an increase in retention time with an increase in the pH value. A pH of 6.5 was chosen as optimal for the separation and resolution of the six compounds.

The supporting electrolyte used, which is necessary for the amperometric detection, was the buffer KH_2PO_4/K_2HPO_4. The effect of the electrolyte concentration on the signal-to-noise ratio was studied. Concentrations ranging from 1 to 8 m*M* were assayed and an increase of the background signal was observed when the electrolyte concentration increased over 5 m*M*. A concentration of 5 m*M* was chosen as optimum.

Different ratios of methanol–water and acetonitrile–water containing 5 m*M* KH_2PO_4/K_2HPO_4 were used as the mobile phase. The ratio 60:40 (water–acetonitrile) was chosen as the optimum, since a good resolution in terms of k' (atenolol = 0.36, nadolol = 0.56, timolol = 1.42, metoprolol = 1.73, oxprenolol = 2.81, alprenolol = 4.71) as well as a good elution time, lower than 12 min, was achieved for the six β-blockers studied (Fig. 2).

A study of the influence of the flow-rate on the chromatographic separation was carried out. As

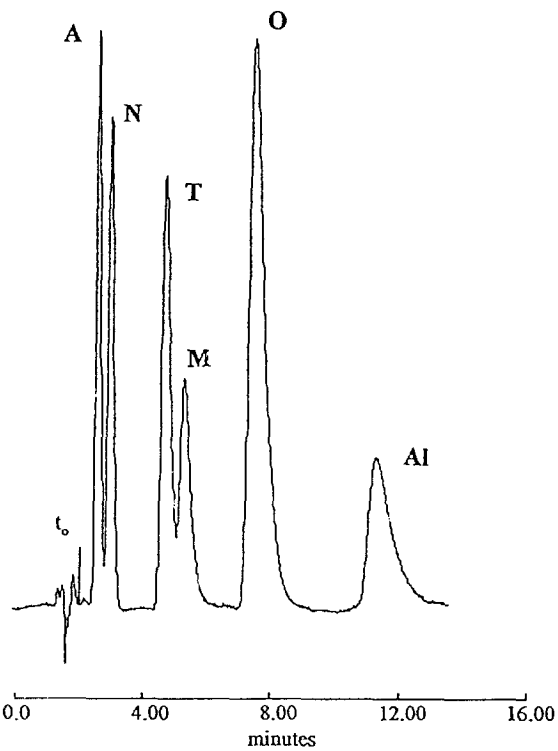

Fig. 2. Separation of a 10 ppm standard solution of atenolol (A), nadolol (N), timolol (T), metoprolol (M), oxprenolol (O) and alprenolol (Al). Eluent, phosphate buffer 5 m*M*–acetonitrile (60:40), pH = 6.5. Electrochemical detection at a working potential of 1300 mV vs. Ag/AgCl. Full current scale: 0.1 μA.

expected, the peak area decreased with the increase of the flow-rate, while the effect on the k' was practically negligible. A value of 1.3 ml min^{-1} was chosen as the optimum flow-rate.

As expected [25], increased temperature caused a reduction of k', which decreased the selectivity of the process without affecting the sensitivity. A temperature of 30 ± 0.2°C was used throughout the work.

When optimum chromatographic conditions had been established, a quantitative method for the simultaneous determination of atenolol, nadolol, timolol, metoprolol, oxprenolol and alprenolol was developed at two concentration levels: ppm and ppb (Table 2).

3.2. Linearity, repeatability and accuracy

The relative standard deviation of the retention times was less than 1%, thus indicating high stability for the system. Linearity occurred at least from the limits of quantitation to 10 ppm, and the correlation was good (Table 2).

The intra-day and inter-day repeatabilities were determined by injecting replicate samples ($n = 10$ for the intra-day, and $n = 6$ for the inter-day repeatability) of each β-blocker at a 4 ppm and a 400 ppb level, and they are expressed as the relative standard deviation (R.S.D) and listed in Table 2. The R.S.D. is calculated by the formula R.S.D = (standard deviation/mean of the peak areas) · 100%.

The accuracy of the method was determined by the analysis of 5 control urine samples spiked with 4 ppm of each β-blocker. Acceptable accuracy, defined as mean (found concentration/actual concentration) × 100%, was demonstrated in the assay: nadolol 100.882%, timolol 100.046%, metoprolol 102.292%, oxprenolol 103.158%, and alprenolol 100.933%.

3.3. Analytical applications

We found a sufficient clean-up of human urine but relatively low recoveries for polar β blockers (nadolol), using the conventional liquid–liquid extraction described before. Such low recoveries will not seriously affect their urine analysis, since the experimental limits of determination proved to be low enough for the relatively high urine

Table 2
Determination of atenolol, nadolol, timolol, metoprolol, oxprenolol and alprenolol at two concentration levels: ppm and ppb

β-Blocker	Retention time ± S.D (min)	Linear range	Slope[a]	r^2	Repeatability intra-day R.S.D (%)	Repeatability inter-day R.S.D (%)[e]	Experimental quantitation limit (ppb)
Atenolol	2.70 ± 0.01	50–1000 ppb	10814.5	0.9978	5.15[b]	15.28	50
		0.5–10 ppm	26571.2	0.9981	1.36[c]		
Nadolol	3.08 ± 0.02	100–1000 ppb	9424.1	0.9993	7.83[b]	9.57	100
		0.5–10 ppm	9493.6	0.9992	1.04[c]		
Timolol	4.88 ± 0.03	15–1000 ppb	157412.1	0.9996	6.16[b]	3.37	15
		0.5–10 ppm	15887.5	0.9994	3.53[c]		
Metoprolol	5.42 ± 0.02	400–1000 ppb	15425.0	0.9987	5.11[b]	8.09	400
		0.5–10 ppm	27830.0	0.9989	2.43[c]		
Oxprenolol	7.21 ± 0.02	120–1000 ppb	30243.0	0.9971	9.01[b]	5.24	120
		0.5–10 ppm	42384.4	0.9993	9.81[c]		
Alprenolol	11.42 ± 0.02	500–1000 ppb	22122.0	0.9985	7.10[d]	6.01	500
		0.5–10 ppm	25378.0	0.9997	2.07[c]		

For chromatographic conditions see the Experimental section.
[a] Area/concentration.
[b] Ten determinations at the 400 ppb level.
[c] Ten determinations at the 4 ppm level.
[d] Ten determinations at the 600 ppb level.
[e] Six determinations at the 4 ppm level.

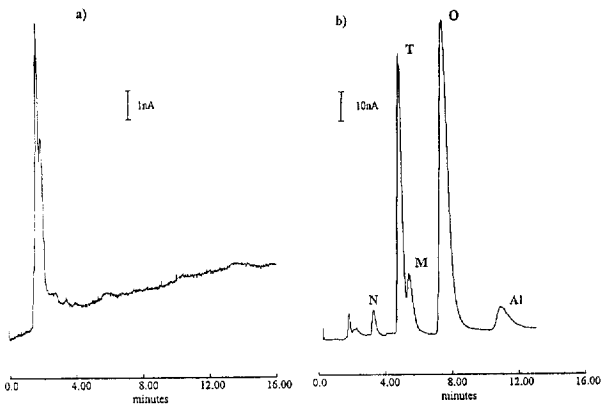

Fig. 3. Chromatograms obtained from an extract of (a) blank urine sample and (b) urine sample spiked with 6 ppm of nadolol, timolol, metoprolol, oxprenolol and alprenolol. A C_{18} column, and a mobile phase consisting of phosphate buffer 5 mM–acetonitrile (60:40), pH = 6.5 was used. Flow-rate: 1.3 ml/min. Detection potential: 1300 mV. Full current scale: 0.1 μA.

concentrations of these compounds. Atenolol was not extracted at all due to its very lipophobic character; new clean-up methods are being assayed for the most polar β-blockers in order to improve their recoveries.

The low limits of quantitation achieved, defined as the minimum concentration which gives a quantifiable chromatographic peak, allow the method to be applied to the quantitation, in urine, of each β-blocker studied (except for atenolol), as well as to a fast screening of the afore-mentioned β-blockers. Fig. 3 shows chromatograms for blank urine and urine samples spiked with 6 ppm of nadolol, timolol, metoprolol, oxprenolol and alprenolol after clean-up with the method described before.

4. Conclusion

Reversed-phase chromatography with electrochemical detection has proved to be adequate for the screening and the determination of the β-blockers studied in urine samples, although the clean-up method assayed should be improved for the most polar compound, atenolol. The assay limits ranged from 15 to 500 ppb, improving the determination limits given by other authors in earlier works, carried out with other detection techniques [16], and extending the applications of the ED detection to the simultaneous determinations in urine. The assay validation was adequate in terms of reproducibility, linearity and accuracy (Table 2). Therefore, this method could be applied to doping analysis.

New reversed-phase methods with electrochemical detection are being developed in our laboratories, for the determination of these β-blockers in real urine samples obtained from hypertensive patients after the ingestion of the pharmaceutical dosage forms of each β-blocker.

Acknowledgements

The authors thank the Basque Government for financial support (Project PGV 92/24, and Project PGV 94/118). M.I. Maguregui thanks the Ministry of Education and Science for an FPI grant.

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