Quantitative determination of the β-blocker labetalol in pharmaceuticals and human urine by high-performance liquid chromatography with amperometric detection

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Abstract

A rapid and simple high-performance liquid chromatographic (HPLC) method with amperometric detection has been developed for the quantitation of labetalol in urine. The chromatography was performed at 30°C using a reversed-phase column with a base deactivated silica stationary support and an alkylamide bonded phase (Supelcosil ABZ+Plus). A 5 mM acetate buffer (pH 4.5)–acetonitrile (70:30, v/v) mixture was employed as the mobile phase, pumped at a flow-rate of 1 ml/min. Sample preparation was carried out using a simple solid-phase extraction (SPE) procedure, and recoveries higher than 85% were achieved. The method was found to be accurate, precise (R.S.D lower than 8%), and sensitive enough (experimental quantitation limit of 20 ng/ml, detection limit 10 ng/ml) to be applied to doping analysis and pharmacokinetic studies in human urine. The method was applied to the determination of labetalol in pharmaceutical formulations and urine samples obtained from a healthy volunteer after the ingestion of a therapeutic dose of the drug, and the results obtained were in agreement with the pharmacokinetic data. © 1998 Elsevier Science B.V.

Keywords: Labetalol

1. Introduction

Labetalol hydrochloride, 5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]salicylamide hydrochloride, is an adrenergic β-receptor blocking agent used in the treatment of hypertension, which exhibits both α- and β-adrenoceptor blocking activity [1,2], and because of its use as doping agent in sports, this drug has been added to the list of forbidden substances issued by the International Olympic Committee (I.O.C.).

Labetalol is extensively metabolized with less than 5% of the dose being excreted unchanged in the urine [3]. Therefore it was necessary to find an analytical method sensitive enough for determining the drug in real urine samples, free from interferences caused by other metabolites and endogenous compounds.

Several HPLC methods have been reported in the literature for the determination of labetalol in biological fluids with UV [4–8], fluorimetric [9–14], and MS [15] detection, but most of them have been applied to human plasma instead of urine. There are only a few reports of HPLC–electrochemical de-

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tection (ED) of labetalol in plasma [16] and urine [17], and these have not been applied to human urine samples in a well established method (validated in terms of linearity, reproducibility, precision, quantitation limit and accuracy).

As UV detection is not sensitive enough and fluorescence and MS detection require long and tedious pretreatment of the sample, the aim of this paper is to establish a validated chromatographic method for the separation and quantitative determination of labetalol in pharmaceuticals and human urine samples.

2. Experimental

2.1. Apparatus and column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump and a Rheodyne (Pharmacia) Model 7125 injector fitted with a 20-μl injection loop.

The electrochemical detector (PAR Model 400) equipped with a glassy carbon cell (EG and G Princeton Applied Research, Madrid, Spain) was operated at +1300 mV vs. a Ag/AgCl reference electrode and a platinum auxiliary electrode in the DC mode with a 5-s low-pass filter time constant, and a current range between 20 and 100 nA. Chromatograms were recorded using an LKB Model 2221 integrator.

A Supelcosil ABZ+Plus, 25 cm×4.6 mm I.D., 5-μm (Supelco, Barcelona, Spain) HPLC column with a μ-Bondapak C_{18} precolumn module (Waters Assoc., Barcelona, Spain) were used. The column has been reported to be a silanol deactivated reversed-phase support for HPLC of clinical drugs, catecholamines and other basic compounds. This packing has a distinct advantage over C_{18} columns as it provides excellent peak symmetry and resolution without requiring the use of ion-pairing or suppressing agents, such as alkylamine salts, that can cause background problems with electrochemical detection at oxidation potentials >+0.9 V [18]. The column was kept at constant temperature using a Waters TMC temperature control system.

Solid-phase extraction was performed using Bond Elut Certify LRC columns (Varian, Barcelona, Spain) which were placed on a vacuum manifold system (Supelco).

The extracted urine samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap LV evaporator (Barcelona, Spain).

2.2. Reagents and solutions

Labetalol hydrochloride was supplied by Sigma (Bilbao, Spain). Solvents were Lab-Scan HPLC grade (Dublin, Ireland), and all reagents were supplied by Merck Suprapur (Bilbao, Spain). The water was obtained from Milli-RO and Milli-Q Waters systems.

A stock solution of labetalol (100 μg/ml) was prepared in a water–acetonitrile mixture containing the same proportion of acetonitrile as used in the mobile phase, and stored at 4°C. Working solutions were prepared by appropiate dilution in the mobile phase just before use.

2.3. Chromatographic conditions

The separation was performed using an acetonitrile–water (30:70 v/v) mobile phase containing a 5 mM acetate buffer as the supporting electrolyte. The pH was adjusted with either acetic acid or 1 M KOH to pH 4.5. This phase was filtered through a 0.45-μm membrane and degassed by bubbling helium through.

The Supelcosil column head pressure was maintained at 89 bar at a flow-rate of 1.0 ml/min and a temperature of 30.0±0.2°C. The injection volume was 20 μl.

2.4. Electrode maintenance

The electrode was cleaned electrochemically at the end of each working day by keeping it at −600 mV for 120 s and after that at +1600 mV for 10 min. This operation was carried out using a mobile phase of pure methanol at a flow-rate of 1.5 ml/min.

Also when the baseline noise increased and the signal-to-noise ratio decreased, the glassy carbon electrode was hand-cleaned with a tissue wet with methanol to remove possible adsorbed compounds and rinsed with deionized water to dissolve precipitated salts.

2.5. Procedure for tablets

The pharmaceutical formulation analyzed in this work was 'Trandate 100 mg': labetalol hydrochloride 200 mg (Duncan Farmaceutica S.A, Madrid, Spain).

Several tablets were separately weighed and then mixed and crushed into a fine powder in a mortar. A suitable amount of this powder was weighed accurately and dissolved in deionized water. After shaking for about 20 min, the mixture was filtered through Whatman No. 41 filter-paper, washed and finally made up to a fixed volume. Aliquots of these concentrated solutions were diluted with the mobile phase and finally measured under calibration conditions.

2.6. Clean-up procedure for urine samples

A 3.75 ml volume of human urine was alkalinized with 750 μl borate buffer 1 M (pH 9.0). The mixture was centrifuged for 5 min at 734 g and decanted onto a glass tube. A Bond Elut Certify LRC SPE column was conditioned with 6 ml of methanol and washed with 6 ml of water to remove trapped methanol traces from the bed volumn. The column was not allowed to dry. With the vacuum off, a 3-ml aliquot of the alkalinized sample was slowly drawn through the column. The column was washed with 2 ml of water, 1 ml of acetate buffer (0.1 M, pH 4.0), and 2 ml of methanol at a vacuum of 5 mmHg. The column was then allowed to dry under full vacuum (P$>$150 mmHg) for about 5 min. Elution of the analyte was performed with 2 ml of a mixture of chloroform–isopropyl alcohol (60:40, v/v) $+2\%$ ammonia solution at a vacuum pressure of 2 mmHg. The eluate was evaporated to dryness at 60°C under a gentle stream of nitrogen. The remaining residue was dissolved in 1 ml of mobile phase and injected directly into the chromatographic system.

2.7. Reproducibility and extraction efficiency

The reproducibility and efficiency of the extraction procedure were determined by extracting replicate (n=5) spiked urine samples, obtained from a healthy male volunteer whose urine was collected before and after the intake of Trandate–200 mg. The samples were spiked with 0.2 and 2 μg/ml of labetalol.

The extraction efficiency was estimated by measuring the peak areas of nonextracted standard solutions in mobile phase, and comparing them with the peak areas obtained from extracting spiked urine samples of the same concentration.

The reproducibility or within-day precision of the extraction, expressed as the relative standard deviation (R.S.D.), is calculated by the formula % R.S.D.=(standard deviation/mean of the recoveries)$\times$100.

The recovery at the 2 μg/ml level was 85.57% with a % R.S.D. of 0.45, and the recovery at the 200 ng/ml level was 89.49% with a % R.S.D of 7.65 (n=5).

3. Results and discussion

Under static conditions labetalol, just like some other β-blockers (metoprolol and atenolol) is hardly oxidized at a glassy carbon electrode. Labetalol shows a well defined voltammetric peak only at neutral and slightly alkaline pH values, and its oxidation peak potentials shifts to less positive potential values as the pH increased [19,20].

Based on this oxidative behaviour, a chromatographic system with amperometric detection was developed for the quantitative determination of labetalol in human urine.

The proposed method presents some advantages over other previously reported methods: J. Wang et al. [17] reported an HPLC–ED method, but did not apply it to urine samples obtained after the intake of labetalol. It was only applied to spiked urine, spiked with very high concentrations of the drug, and no clean-up method was proposed. They also obtained higher limits of detection (30 ng/ml in standard solutions).

A paper written by M.T. Saarinen et al. [8] reported an HPLC screening method for β-blockers in urine, but they did not quantify labetalol or any other β-blocker.

3.1. Optimization of the chromatographic system

A hydrodynamic voltammogram of the compound was carried out in order to choose the optimum potential (Fig. 1). An oxidative potential of $+1300$
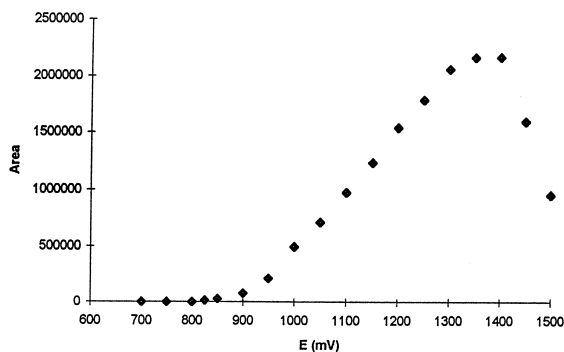
Fig. 1. Hydrodynamic voltammogram of labetalol. Amount of drug injected: 100 ng. Mobile phase: 5 m*M* acetate buffer–acetonitrile (70:30, v/v), pH 4.5.

mV was chosen as the working potential, and although this potential was not the optimal in terms of sensitivity, the +1300 mV potential induced a lower baseline noise (and therefore a higher signal-to-noise ratio). This potential also provided a higher reproducibility and selectivity because a lower amount of endogenous urine compounds were oxidized. Potential values higher than +1400 mV induced a poor reproducibility and poor sensitivity, probably due to an adsorptive process at these high potential values.

A correlation of retention time with pH (3–6.5) of the mobile phase showed that retention time, and baseline noise, increased with increasing pH. The optimum pH was found to be 4.50.

The acetate buffer was chosen because of its high capacity at pH 4.50. Assays were also made using phosphate buffers at different pH values and different organic modifier proportions, but the *k'* of labetalol was always too low, and no sufficient resolution from the urine matrix was obtained (the retention time was about 2 min shorter than using acetate buffer at the same pH value). It is possible that the acetate buffer acts as an ion-pairing agent, retarding the elution of labetalol.

The effect of the electrolyte concentration on the signal-to-noise ratio was also studied. Concentrations ranging from 2.5 m*M* to 20 m*M* were assayed and an increase in the background signal was observed when the electrolyte concentration exceeded 5 m*M*. Optimal electrolyte concentration was set at 5 m*M*.

Different proportions of methanol–water and ace-

tonitrile–water containing 5 m*M* acetate buffer were tested as the mobile phase. The 70:30 (water–acetonitrile) ratio was chosen as the most adequate, since a good resolution, as well as a low retention time, was achieved ($t_r = 5.95 \pm 0.02$ min)

A study of the influence of the flow-rate on the chromatographic separation was carried out. As expected, the peak area decreased with increasing flow-rate whithout affecting the resolution. A flow-rate of 1 ml/min was used.

An increase in the temperature caused a reduction in the retention time decreasing the selectivity of the process without affecting the sentisivity. A temperature of $30 \pm 0.2°C$ was used throughout the work.

When optimum chromatographic conditions had been established, a quantitative method for the determination of labetalol was developed, at two concentration levels: ng/ml and μg/ml (Table 1).

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 and accuracy were determined by spiking human blank urine samples obtained from the healthy male volunteer used throughout the work, with different concentrations of labetalol and treating and processing them as samples. The linearity of the calibration curve was calculated by linear regression and a correlation coefficient higher than 0.999 was obtained from the quantitation limit to 10 μg/ml.

The within-day and inter-day repeatabilities were determined by injecting replicate samples ($n=10$ for the within-day, and $n=9$ for the inter-day repeatability) at 0.2 μg/ml and 2 μg/ml levels. The R.S.D is presented in Table 1.

The accuracy of the method was obtained analys-

Table 1

Recoveries and within-day precissions of labetalol in human urine samples, spiked at the ng/ml and μg/ml levels ($n=5$)

Concentration	% Recovery	% R.S.D.
2 μg/ml	85.57	0.45
200 ng/ml	89.49	7.65

Chromatographic and clean-up conditions as described in the Section 2.

ing control urine samples spiked with 400 ng/ml and 1.10 µg/ml labetalol solutions. Acceptable accuracy was achieved in the assay: 101.64% at 400 ng/ml and 100.61% at 1.10 µg/ml.

The experimental quantitation limit, defined as the lowest concentration of labetalol in a spiked urine sample which gives rise to a signal able to be quantified by the integrator (signal-to-noise ratio=5), was found to be 20 ng/ml. The detection limit was 10 ng/ml for spiked urine samples and 5 ng/ml for standard solutions (signal-to-noise ratio=3) (Table 2).

3.3. Analytical applications

The method developed was applied to the determination of labetalol in pharmaceutical formulations. The obtained values were in accordance with those certified by the pharmaceutical company (relative error lower than 1%, see Table 3). In Fig. 2, chromatograms of a dilute solution of a tablet of Trandate-100 mg (labetalol hydrochloride) and of a standard solution of labetalol are shown.

The chromatographic method was applied to the analysis of labetalol in urine samples obtained from a healthy volunteer after oral administration of a single dose of the pharmaceutical formulation Trandate 200
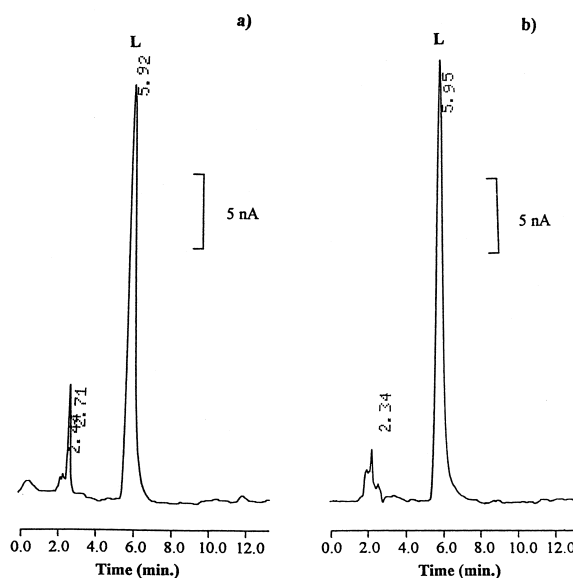

Fig. 2. Chromatograms of (a) a standard solution of labetalol 2 µg/ml, and (b) diluted solution of Trandate–200 mg (labetalol 200 mg), 2 µg/ml. Full current scale: 50 nA.

mg. Urine was collected at different time intervals for the quantitative determination of the β-blocker: 0–2 h, 4–6 h, 8–12 h, and 12–20 h. Urine samples were treated following the clean-up procedure al-

Table 2
Determination of labetalol at two concentration levels: ng/ml and µg/ml

Retention time±S.D (min)	Linear range	Slope[a]	r^2	Within-day repeatability (% R.S.D.)	Inter-day repeatability (% R.S.D.)	Experimental quantitation limit (ng/ml)
5.95±0.02	20–1000 ng/ml	304.7	0.9996	7.84[b]	6.99[e]	20
	1–10 µg/ml	306093.1	0.9995	2.05[c]	7.38[d]	

For chromatographic conditions see the Section 2.
[a] Area/concentration; [b] 10 determinations at the 200 ng/ml level; [c] 10 determinations at the 2 µg/ml level; [d] 9 determinations at the 2 µg/ml level; [e] 7 determinations at the 200 ng/ml level.

Table 3
Determination of labetalol in pharmaceutical formulations

Formulation	Nominal (mg)	Found (mg)[a]	% Relative error
Trandate	100	99.08±2.23	0.91

[a] Amount±$ts/\sqrt{n}$, where t is the student parameter (95% confidence), s is the standard deviation and n is the number of replicates; $n=3$ different samples and 3 replicates of each sample
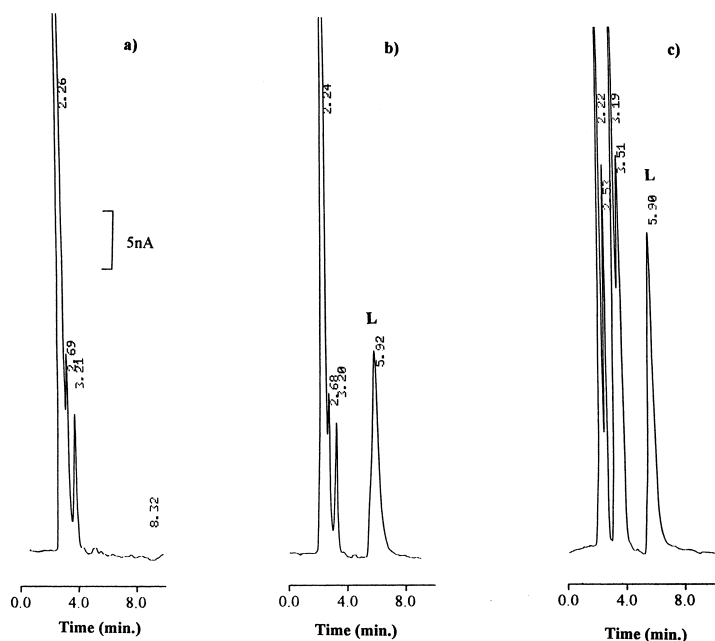
Fig. 3. Chromatograms obtained from an extract of (a) blank urine sample, (b) urine sample spiked with 2 μg/ml of labetalol, and (c) urine sample 2–4 h after the oral administration of 200 mg of labetalol (Trandate-200 mg) to a healthy volunteer. Full current scale: 50 nA.

ready described, and measured under calibration conditions (Fig. 3). Labetalol was easily detected at the different time intervals, and the concentrations are collected in Table 4.

4. Conclusions

The described chromatographic method has shown to be a useful method for the identification and determination of the β-blocker labetalol in real human urine samples. The clean-up procedure is simple and effective, and the separation from the urine matrix is achieved in less than 6 min.

The chromatographic method with amperometric detection presents some advantages over other reported methods: better limit of detection (5 ng/ml in standard solutions and 10 ng/ml in spiked urine samples), good accuracy, precision and enough sensitity for the quantitation of free labetalol in urine at all time intervals studied.

The results obtained from the quantitation of

Table 4
Determination of labetalol in human urine samples obtained from a healthy volunteer, collected at different time intervals after the ingestion of a single dose of Trandate (labetalol chlorhidrate 200 mg)

Time interval (h)	Urine volume (ml)	Labetalol concentration (μg/ml)[a]	Excreted labetalol amount (mg)[a]	Cumulative excreted labetalol %[a]
0–2	200	0.36±0.01	0.072±0.002	0.036±0.001
2–4	430	1.14±0.04	0.49±0.02	0.28±0.01
4–6	300	0.53±0.02	0.158±0.007	0.36±0.01
6–8	215	0.51±0.06	0.11±0.01	0.41±0.01
8–12	180	0.86±0.03	0.154±0.006	0.49±0.01
12–20	410	0.45±0.03	0.19±0.01	0.58±0.01
		Total excreted amount (mg): 1.17±0.03		

[a] Amount$\pm ts/\sqrt{n}$, $n=3$ different samples and 3 replicates of each sample.

labetalol at different time intervals after the ingestion of the drug are in good agreement with those expected from the pharmacokinetic data, with the peak concentration found at the time interval 0–4 h and less than 5% of the dose being excreted in 24 h.

Acknowledgement

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