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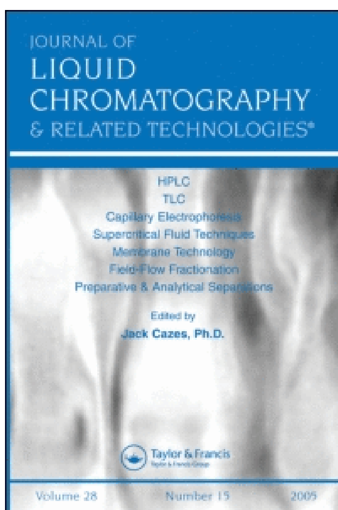

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A RAPID QUANTITATIVE ANALYSIS OF THE β-BLOCKER TIMOLOL IN HUMAN URINE BY HPLC-ELECTROCHEMICAL DETECTION

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ABSTRACT

A High Performance Liquid Chromatographic method with amperometric detection has been developed for the determination of the β-blocker 1-[(1, 1-dimethylethyl) amino]-3-[[4-(4-morpholinyl)-1, 2, 5-thiadiazol-3-yl] oxy], timolol.

The chromatographic separation was performed using a μ-Bondapak C_{18} column and a mobile phase of acetonitrile:water (30:70), containing 5mM KH_2PO_4/K_2HPO_4, pH 6.5 pumped at a flow rate of 1.3 mL/min. The amperometric detector equipped with a glassy carbon electrode was operated at +1000 mV. The method was applied to the determination of timolol in urine samples obtained from a hypertensive patient under medical treatment with the pharmaceutical formulation Blocadren 10 mg (timolol 10 mg). Using a simple liquid-liquid extraction procedure, a good recovery (93.25±2.5) and separation from the urine matrix is achieved. A good reproducibility, linearity and

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accuracy are obtained, and the quantitation limit of 10 ng/mL, allows the method to be applied to doping analysis in human urine, and pharmacokinetic studies.

INTRODUCTION

Timolol maleate is a β-adrenergic blocker used for the treatment of hypertension, angina pectoris, and glaucoma. Due to its sedative effect, in January 1987 the use of this compound and other β-blockers was forbidden in sports such as pentathlon, shooting and billiards.[1]

This β-blocker has an oral bioavailability of about 75 % and undergoes oxidative biotransformation. About 20 % of the dose is excreted unchanged,[2-3] and the mean elimination half-life is 4 hours.

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Methods for the detection and identification of timolol have been described using mainly gas chromatography (GC) with either electron capture detection (ECD),[4] flame ionization detection (FID) and mass spectrometry (MS) achieving detection limits as low as 0.5 ng/mL.[5-6] All these methods need the use of tedious extraction and back extraction procedures to minimize interferences from endogenous components of the matrixes (mainly plasma and serum).

There are several articles describing HPLC methods with different detection techniques such as UV detection[7] and atmospheric pressure chemical ionization mass spectrometry.[8] These determinations have been mainly done in plasma and serum matrixes and some of them in pharmaceutical formulations.[9-10] There is only one report dealing with urine matrixes: horse urine.[6]

With respect to the use of HPLC-electrochemical detection (ED) applied to the determination of timolol, Gregg et al.[11] describe a method for the determination of timolol in plasma and breast milk. The limits of detection given in this work are based only on signal-to-noise measurements (2 ng/mL) and should not be confused with the lower quantifiable limits.

On the other hand the extraction methods proposed are long and complicated. He et al.[12] report a determination of timolol in plasma monitored by coulometric detection, including also a complicated liquid-liquid extraction procedure.

Upon the basis of a recently developed method for the screening of several β-blockers using HPLC-ED,[13] and based on the good results obtained we are now developing methods for the quantitative determination of each β-blockers in human urine.

The aim of this paper is the application of a simple HPLC system with amperometric detection to the quantitation of timolol in human urine, preceded by a simple and fast liquid-liquid extraction procedure.

MATERIALS AND METHODS

Reagents, Chemicals and Standard Solutions

Timolol Maleate was supplied by Sigma (Bilbao, Spain). Potassium dihydrogenphosphate, and dipotassium hydrogenphosphate were Merck Suprapur (Bilbao, Spain).

HPLC grade solvents were purchased from Lab-Scan (Dublin, Ireland), and the water used was obtained from the Milli-RO and Milli-Q Millipore systems.

A stock solution of timolol (100μg/mL) was prepared in aqueous-acetonitrile mixture 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.

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Procedure for Urine Samples

The clean-up procedure for urine samples was a simple liquid-liquid extraction : 0.5mL of NaOH (5M), 4mL of diethyl ether and 1g of Na_2SO_4 were mixed with 4mL of urine. The mixture was shaken mechanically for 15min and centrifuged for 5min at 734g.

The diethyl layer was separated and evaporated to dryness at 60°C under a gentle stream of nitrogen, using a Zymark TurboVap LV evaporator (Barcelona, Spain). The residue was dissolved in 2 mL of mobile phase and was measured under calibration conditions.

The reproducibility and efficiency of the extraction procedure was determined by extracting replicate spiked urine samples, doped with 2ppm of timolol. A quantitative recovery of (mean value±R.S.D%) 93.25±2.50% was achieved.

Chromatographic Conditions

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

The electrochemical detector (PAR Model 400) equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1000mV vs a Ag/AgCl electrode, in the DC mode with a 5-s low pass-filter time constant, and a current range between 2 and 100nA. Chromatograms were recorded with the help of a computer and treated with the software Millenium 2010 Chromatography Manager from Waters.

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The column used was a 30cm×3.9mm 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 mobile phase was a mixture acetonitrile:water (30:70) containing 5mM potassium dihydrogenphosphate/dipotassium hydrogenphosphate. The pH was adjusted to 6.5 and the buffer served as supporting electrolyte. The μ Bondapack column head pressure was 1500PSI at a flow rate of 1.3mL/min. The injection volume was 20μL. The column was kept constant at 30±0.2°C.

RESULTS AND DISCUSSION

Hydrodynamic voltammetry of the compound was carried out in order to choose the optimum potential value for the amperometric detection of this β-blocker. An oxidative potential of 1000mV was chosen as the working potential, since it was the potential which provided the maximum sensitivity for timolol.

The study of the influence of pH and composition of the mobile phase gave an optimum value of pH 6.5 and a water:acetonitrile ratio of 70:30, which allowed the separation of timolol from the interfering endogenous compounds of the urine, keeping a low retention time.

Table 1

Determination of Timolol at Two Concentration Levels: μg/mL and ng/mL

	β-Blocker: Timolol	
Retention Time ± S.D. (min)	6.47 ± 0.03	
Linear Range	10 - 1000 ng/mL	0.5 - μg/mL
Slope of Calibration graph[a]	157412.1	15887.5
r^2	0.9996	0.9994
Repeatability Intraday R.S.D. (%)	6.16[b]	3.53[c]
Repeatability Interday R.S.D. (%)	---	3.37[d]
Experimental Quantitation Limit (ng/mL)	10	---

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

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The buffer potassium dihydrogenphosphate/dipotassium hydrogenphosphate was used as supporting electrolyte since it gave the best signal to noise ratio at a concentration of 5mM.

An increase in the temperature caused a reduction of k′, without affecting the sensitivity. A temperature of 30±0.2°C was used throughout the work. A value of flow rate of 1.3mL/min was chosen as the optimum.

Once the optimum chromatographic conditions had been established, a quantitative method for the determination of timolol in urine samples was developed at two concentration levels: ppm and ppb (Table 1).

The relative standard deviation of the retention times is less than 1%, thus indicating high stability for the system.

Linearity ocurred at least from the limits of quantitation to 10ppm, with a good correlation.

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) at a 4 ppm and a 400 ppb level, and it is expressed as the relative standard deviation (R.S.D.) and listed in Table 1. The R.S.D is calculated by the formula R.S.D.=(standard deviation/mean of peak area)×100%.

The accuracy of the method was determined by the analysis of 5 control urine samples spiked with 2ppm of timolol. Acceptable accuracy, defined as mean (found concentration/actual concentration)×100, was achieved: 100.05 ± 8.6%.

The experimental quantitation limit, defined as the minimum concentration of timolol which gives rise to a signal able to be quantified by the computer program used, was 10 ng/mL, in spiked urine samples.

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Analytical Applications

The method developed was applied to the determination of timolol in urine samples obtained from a patient suffering from hypertension and under medical treatment with the pharmaceutical formulation Blocadren 10mg (timolol maleate 10mg).

Urine was collected at different time intervals for the quantitative determination of the β-blocker: 0-2 hours, 2-4 hours and 4-12 hours.

The compound was easily detected at 0-2 and 2-4 hours intervals and the concentrations determinated, collected in Table 2, were in agreement with the pharmacokinetic data .[14] Timolol was not detectable at 4-12 hours interval. Urine samples were treated following the clean-up procedure described in the experimental section. Fig. 1 shows the chromatograms corresponding to a

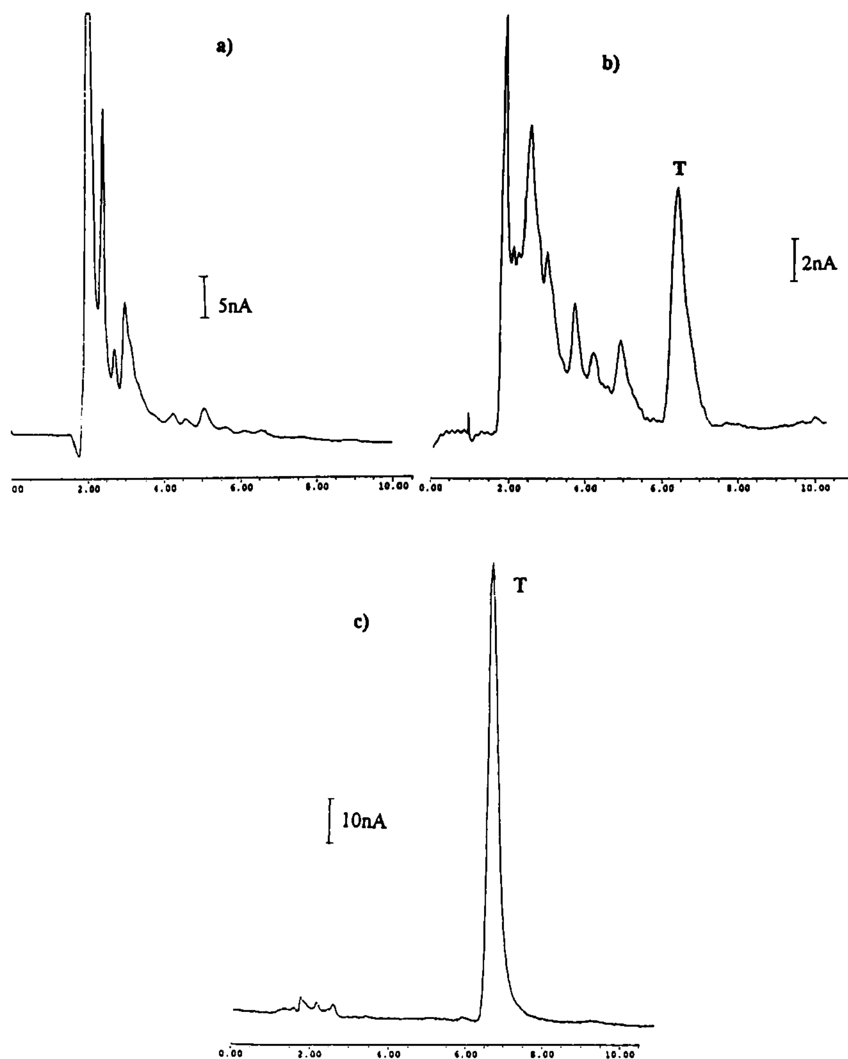

Figure 1. Chromatograms obtained from an extract of: a) blank urine sample, b) urine sample 0-2 hours after the oral administration of 1 tablet of timolol 10mg (Blocadren 10mg) to a hypertensive patient and c) a diluted solution of a tablet of Blocadren 10mg, containing 10 mg of timolol. For chromatographic conditions see experimental section.

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Table 2

Determination of Timolol in Urine

Urine Samples			
Time Interval	0-2 hours	2-4 hours	4-12 hours
Timolol conc. (μg/mL)	0.96	0.415	---

(Urine obtained from a hypertensive patient under treatment with Blocadren 10 mg).

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blank urine sample, a diluted solution of a tablet of Blocadren 10mg and a urine sample 0-2 hours after the administration of a dose of Blocadren.

Discussion

Liquid chromatography with amperometric detection has been shown to be a fast and powerful method for the identification and determination of timolol in urine samples. The clean-up procedure is very simple with recoveries of 93.25 ± 2.50%, and the chromatographic separation is made in less than 7 minutes.

The relatively low potential used in the detection +1000mV induces a low background signal and provides high stability to the system. This fact constitute an advantage with respect to the work done by Gregg et al.[11] who used +1200mV as optimal potential for plasma and breast milk samples.

The limit of quantitation of 10ng/mL has proved to be sensitive enough for the determination of the free timolol in urine samples of hypertensive patients in the 0-2 and 2-4 hours intervals.

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