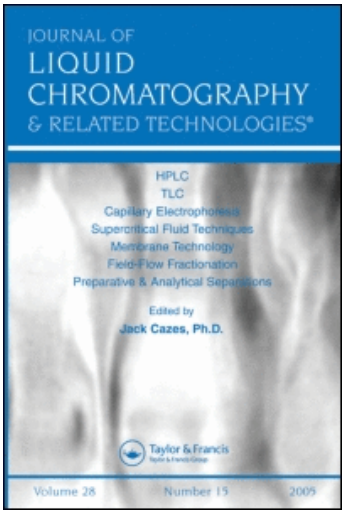

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION APPLIED TO THE DETERMINATION OF THE β-BLOCKER OXPRENOLOL IN URINE AND PHARMACEUTICALS

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ABSTRACT

A rapid and simple high performance liquid chromatographic (HPLC) method with amperometric detection has been developed for the quantitative determination of oxprenolol in human urine and pharmaceuticals. The chromatographic method was performed at (25 ± 0.2)°C on a reversed-phase column (Supelcosil ABZ + Plus) with a mobile phase of acetonitrile-water (30:70, v/v) containing 4 mM acetate buffer pH = 4.4 and with a flow rate of 1 mL/min.

The amperometric detector equipped with a glassy carbon electrode was operated at +1300 mV versus Ag/AgCl in direct current mode. A simple solid phase (SPE) extraction method was used as clean-up procedure, obtaining recoveries greater than 90% for spiked urine samples. The method was found to be accurate, precise, and sensitive enough to determine free oxprenolol in human urine samples, which would allow its application to doping analysis. The method developed allowed the analysis of urine samples obtained from a patient under treatment with oxprenolol, and to its determination in the pharmaceutical formulation Transitensin (oxprenolol 80 mg + chlortalidone 10 mg).

INTRODUCTION

Oxprenolol, 1-(2-allyloxyphenoxy)-3-isopropylaminopropan-2-ol (Figure 1), is a non-selective adrenergic β–receptor antagonist (β-blocker) widely used in the treatment of hypertension, angina pectoris, some cardiac arrhythmias, migraine, and convulsions.[1,2]

Oxprenolol as the rest of β-blockers are subjected to restrictions in sport. Their abuse was forbidden in 1987 by the International Olympic Committee (I.O.C.) in sports such as shooting, pentathlon, ski-jump, and billiard.[3] In 1994, 1% of the positive samples in doping control contained β-blockers as a forbidden substance.[4]

Oxprenolol is a lipophilic β-blocker with a rapid and complete absorption.[5] After its oral administration, a percentage from 75% to 95% is excreted in urine, but this drug is extensively metabolised, and only about 5% of the dose is excreted unchanged.[6] Therefore, it is necessary to find an analytical method sensitive enough for the determination of the drug in urine samples.

This β-blocker has mainly been determined in pharmaceutical formulations by UV-spectrophotometry[7-10] and HPLC with UV detection[11.12] methods. Several methods have been used for the quantification of this drug in plasma using HPLC[13,14] and gas chromatographic techniques.[15,16]

Gas chromatography,[17] TLC with fluorimetric detection,[18] HPLC with UV detection,[19,20] and capillary zone electrophoresis[21] have been used for the analysis of oxprenolol in urine.

HPLC with electrochemical detection (ED) has been only used by Gregg[22] for the determination of oxprenolol in plasma samples after the preanodization of the working electrode.

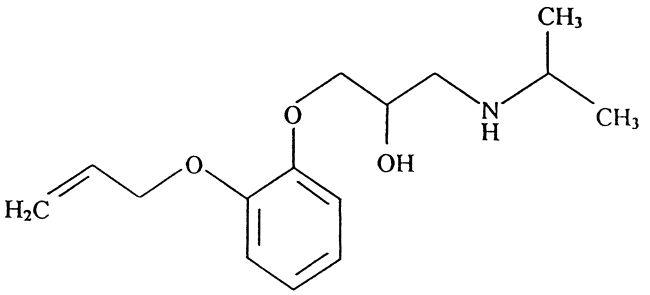

Figure 1. Chemical structure of the b-blocker oxprenolol.

Taking into account the reported works, the aim of this work is the development of a simple HPLC method with amperometric detection for the quantitative determination of oxprenolol in urine and pharmaceuticals, which avoids the derivatization reactions necessary for the fluorometric and mass-spectrometric detection.

EXPERIMENTAL

Apparatus and Column

The HPLC system consisted of a Model 2150 LKB (Pharmacia, Barcelona, Spain) pump and a Rheodyne (Pharmacia) Model 7125 injector with a 20 μL loop. The electrochemical detector was a PAR Model 400 with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated in the d.c. mode (applying a steady direct current potential to the LC cell and measuring the resulting current versus time) at +1300 mV vs. an Ag/AgCl reference electrode, with a 5 s low-pass filter time constant, and a current range between 10 and 100 nA. Chromatograms were recorded using a LKB Model 2221 integrator. The chart speed was 0.5 cm/min, and the attenuation was 8 mV FS (8 mV for a full-scale deflection).

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 to prevent column degradation, (Waters Assoc., Barcelona, Spain) were used. The chromatographic column chosen was a C_{18} of polymeric base with silanol groups deactivated, suitable for the separation of clinical drugs, catecholamines, and other basic compounds. 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, Barcelona, Spain).

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

Voltammetric measurements were made using an Eco Chemie Autolab voltammetric analyser coupled to a Metrohm Model VA 663 three-electrode stand. The auxiliary electrode was a platinum rod, the reference electrode was a saturated Ag / AgCl / KCl 3M and the working electrode was a 3 mm i.d. Metrohm glassy carbon.

Reagents and Solutions

Oxprenolol hydrochloride was supplied by Sigma (Bilbao, Spain). Solvents were Lab-Scan HPLC grades (Dublin, Ireland). All reagents were Merck Suprapur (Bilbao, Spain). Water was obtained from Milli-RO and Milli-Q Waters systems.

A stock solution of oxprenolol (1000 μg/mL) was prepared in water and stored under refrigeration at 4°C. Working solutions were prepared by appropriate dilution in the mobile phase just before use.

Chromatographic Conditions

The separation was performed using an acetonitrile-water (30:70 v/v) mobile phase containing a 4 mM acetate buffer as supporting electrolyte. The pH was adjusted to 4.4 with acetic acid. This phase was filtered through a 0.45 μm membrane and degassed by bubbling helium through. The Supelcosil column head pressure was maintained at 94 bar at a flow-rate of 1.0 mL/min and a temperature of (25.0 ± 0.2)°C.

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 pure methanol as mobile phase at a flow-rate of 1.5 mL/min. 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.

Procedure for Tablets

The pharmaceutical formulation analysed in this work was Transitensin: oxprenolol hydrochloride 80 mg and chlorthalidone 10 mg (Ciba-Geigy S.A., Barcelona, 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 10 min, the mixture was centrifuged at 784 g for 5 min and decanted. The precipitate was washed and finally made up to a fixed volume. Aliquots of these concentrated solutions were diluted with the mobile phase and measured under calibration conditions. Different amounts of the initial solid sample were assayed in order to obtain a mean value.

Clean-up Procedure for Urine Samples

A 3.0 mL volume of human urine was made alkaline with 600 μL borate buffer (1M, pH = 9). 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 volume. The column was not allowed to dry. With the vacuum off, a 3 mL aliquot of the alkaline sample was slowly drawn through the column. Then the column was washed with 2 mL of water, 1 mL of acetate buffer (0.1 M, pH = 4) and 1 mL of methanol at a vacuum of 5 mmHg. The column was then allowed to dry under full vacuum (P > 150 mmHg) for 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 500 μL mobile phase and injected directly into the chromatographic system.

RESULTS

A study of the oxidative behaviour of oxprenolol and chlortalidone, diuretic co-administered with oxprenolol in its pharmaceutical formulation Transitensin (oxprenolol 80 mg + chlortalidone 10 mg), was carried out by differential pulse voltammetry at a glassy carbon electrode. Oxprenolol showed a single voltammetric peak in the pH range 2.4 - 8.9, with peak potential varying from 1.26 to 1.09 V versus Ag / AgCl / KCl 3M, and a maximum peak current in the pH range 4.5 - 6.3,[23] whereas chlortalidone did not show any electroactivity.

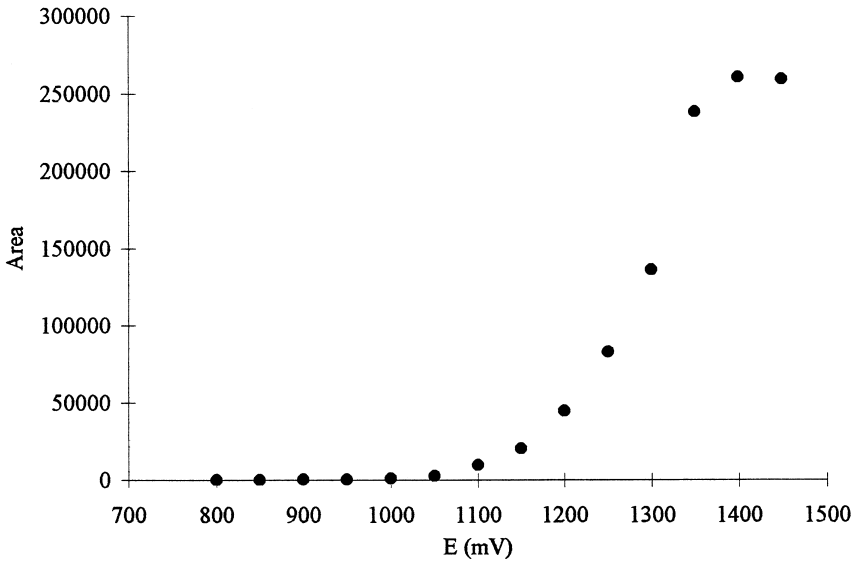

Figure 2. Hydrodynamic voltammogram of oxprenolol. Chromatographic conditions: 5 mM acetate buffer-acetonitrile (70:30 v/v), pH=5, flow rate = 1mL/min, T = (25 ± 0.2)°C. Amount of oxprenolol hydrochloride injected: 100 ng.

Based on this oxidative behaviour, a chromatographic system with amperometric detection was developed for the quantitative determination of the drug in the pharmaceutical formulation Transitensin, and in urine samples obtained from a hypertensive patient.

Optimisation of the Chromatographic System

The similar chromatographic behaviour shown for oxprenolol and labetalol at pH lower than 6 in the screening method for β-blockers, previously developed,[24] led us to use initially the separation conditions reported by Ceniceros et al.[25] for labetalol analysis.

A hydrodynamic voltammogram of the compound (Figure 2) was carried out in order to choose the optimum potential value for the determination of oxprenolol. An oxidative potential of +1300 mV was chosen as the working potential, although it was not the one at which the current was maximum, but induced a lower baseline noise (therefore a higher signal-to-noise ratio). This potential also provided a higher reproducibility and selectivity because lower amounts of endogenous urine compound were oxidised.

The study of the effect of the pH value (4-6.5) of the mobile phase on the chromatographic behaviour showed that retention time and baseline noise increased with the increment of pH. A value of 4.4 was considered as optimum.

The influence of the electrolyte concentration on the signal-to-noise ratio was also studied. Concentrations ranging from 2 mM to 10 mM were assayed. An increase in the concentration caused a reduction of k´ and an increase in the background signal. So the optimal electrolyte concentration was set at 4 mM.

Different proportions of methanol-water and acetonitrile-water containing 4 mM acetate buffer were tested as the mobile phase. Methanol showed to provide less sensitivity than acetonitrile in the same conditions, so acetonitrile was chosen as organic modifier. The 70:30 (water-acetonitrile) ratio was used, since a good resolution, as well as a low retention time was achieved ($t_r = 5.45 \pm 0.04$ min). These conditions allowed the separation of oxprenolol from the interfering endogenous compounds of urine, keeping a low retention time.

A study of the influence of the temperature on the chromatographic separation was carried out. An increase in the temperature caused a reduction in the retention time decreasing the selectivity of the process without having an effect on sensitivity. A temperature of (25 ± 0.2)°C was used throughout the work.

A value of flow rate of the mobile phase of 1 mL/min was used. Once the optimum chromatographic conditions had been established (Table 1), a quantitative method for the determination of oxprenolol was developed, at two concentration levels: ng/mL and μg/mL.

Reproducibility and Extraction Efficiency

The reproducibility and efficiency of the extraction procedure were determined by extracting replicates (n = 5) spiked urine samples obtained from a healthy volunteer. Urine samples were treated following the clean-up procedure described in the experimental section. The samples were spiked with 0.2 and 2 μg/mL of oxprenolol hydrochloride.

The extraction efficiency was estimated by measuring the peak areas of non-extracted 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. = (standard deviation/mean of the recoveries) × 100), and the recovery obtained are shown in Table 2.

Table 1

Chromatographic Conditions for the Determination of Oxprenolol by HPLC-ED

Mobile phase:	Water: acetonitrile (70:30 v/v)
Electrolyte:	Acetate buffer 4 mM pH - 4.4
Applied potential:	+ 1300 mV
Flow rate:	1 mL/min
Column temperature:	(25 ± 0.2)°C
Pre-column:	μ-Bondapack C18
Column:	Supelcosil ABZ + Plus 5μm, 25 cm x 4.6 mm I.D.
Injection volume:	20 μL
Detection mode:	Amperometric Working electrode: glassy carbon Reference electrode: Ag/AgCl/KCl 3M Auxiliary electrode: platinum

Table 2

Reproducibility and Efficiency of the Extraction Procedure in Spiked Urine Samples*

Concentration	% Recovery	% R.S.D.
200 ng/mL	93.09 ± 3.20[a]	2.16
2 μg/mL	93.11 ± 3.20[a]	2.16

* For chromatographic conditions see Table 1. [a] Amount ± t s/√n (n = 5), where t is the student parameter (95% level of confidence).

Linearity, Reproducibility and Accuracy

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

Linearity and accuracy of the method were determined by spiking human blank urine samples obtained from a healthy volunteer, with different concentrations of oxprenolol and treating and processing them as samples. Good linearity in terms of peak area response as a function of analyte concentration is demonstrated by the high correlation coefficients observed for the regression lines.

Table 3

Analytical Parameters for the Determination of Oxprenolol in Urine*

Retention time ± S.D. (min):	5.45 ± 0.04	
Quantitation limit (ng/mL):	50	
Linear range μg/mL:	0.1 - 1	1 - 4
Slope ± t s (area /concentration):	80728 ± 10517	35876 ± 6920
Intercept ± t s:	3304 ± 2308	27837 ± 12599
Correlation coefficient (r^2):	0.996	0.995
Within-day reproducibility (% RSD):	4.32[a]	1.15[b]
Interday reproducibility (% RSD):	5.40[a]	2.90[b]

* For chromatographic conditions see Table 1. [a] 10 determinations at 200 ng/mL. [b] 10 determinations at 1 μg/mL.

The within-day and interday reproducibilities, expressed as relative standard deviation (%R.S.D.), were determined by injecting replicate samples (n=10) of the β-blocker at two concentration levels, Table 3. The accuracy of the method was determined by the analysis of control urine samples (n=5) that were spiked with 100 ng/mL of oxprenolol. A good accuracy of (100.05 ± 2.58)% was achieved in the assay. Accuracy was expressed by the formula: (found concentration / added concentration) x 100 ± t s.

The experimental quantitation limit, defined as the lowest concentration of oxprenolol in a spiked urine sample, which gives rise to a signal able to be quantified by the integrator with an %RSD ≤ 5 was found to be 50 ng/mL.

Analytical Applications

Firstly, the method developed was applied to the determination of oxprenolol in pharmaceutical formulations. In Figure 3, chromatograms of a dilute solution of a tablet of Transitensin (oxprenolol hydrochloride + chlortalidone) and of a standard solution of oxprenolol are shown. The obtained values (79.94 ± 0.98 mg) were in concordance with the certified (80 mg) by the pharmaceutical company, with a relative error of 0.075%.

The chromatographic method was also applied to the analysis of oxprenolol in urine samples obtained from a hypertensive patient under treatment with the pharmaceutical formulation Transitensin. Urine was collected at different time intervals for the quantitative determination of the

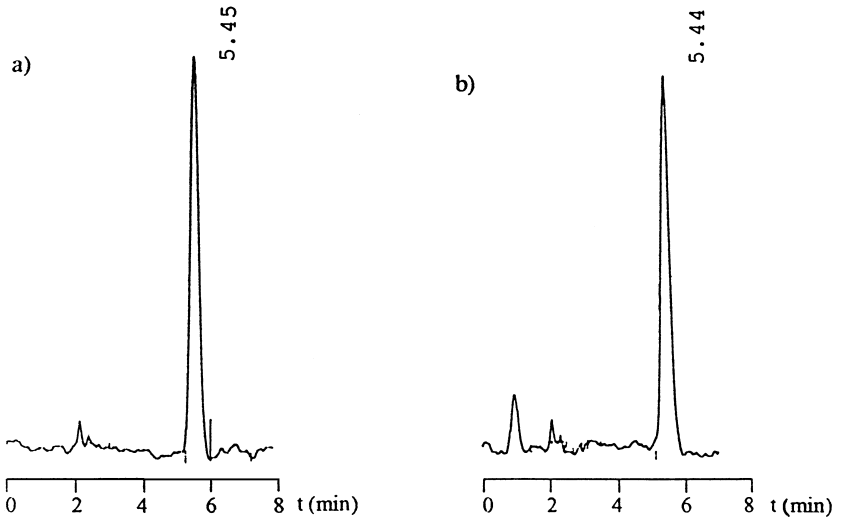

Figure 3. Chromatograms of (a) a standard solution of oxprenolol hydrochloride 1 mg/mL, and (b) a diluted solution of transitensin (oxprenolol hydrochloride 80 mg + chlortalidone 10 mg). Full scale: 50 nA. For chromatographic conditions see Table 1.

β-blocker: 0 - 4 h, 4 - 8 h, 8 - 12 h, and 12 - 24 h. The urine samples were treated following the clean-up procedure already described. The samples collected in the interval time 0 - 4 h and 4 - 8 h were measured using a calibration curve ranging from 1 - 4 μg/mL, whereas a calibration curve in the range 0.1 - 1 μg/mL was used for the measurement of the ones collected in the interval time 8 - 12 h and 12 - 24 h, due to the low concentration of oxprenolol which is excreted as unchanged drug in the urine in these time intervals.

The method was applied to three replicates of each sample. Figure 4 shows the chromatograms of extracted urine samples, free of interferences from the endogenous compounds of the matrix. The results obtained are listed in Table 4.

DISCUSSION

High-performance liquid chromatographic method with amperometric detection has shown to be adequate in terms of reproducibility, linearity and accuracy for the quantitative determination of oxprenolol. Chlorthalidone, diuretic co-administered with oxprenolol, does not interfere in its determination.

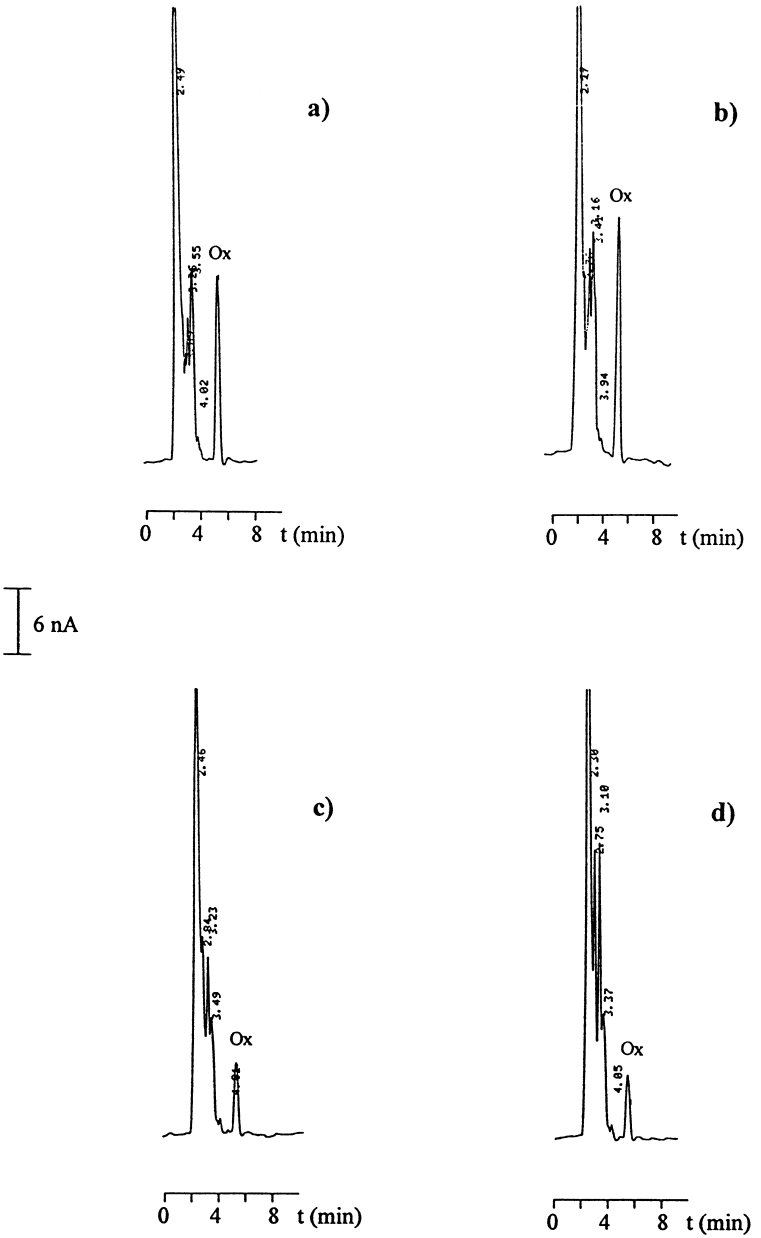

Figure 4. Chromatograms of urine extracts obtained from a hypertensive patient under treatment with Transitensin at different interval times: a) 0-4 h, b) 4-8 h, c) 8-12 h, and d) 12-24 h. For chromatographic conditions see Table1.

Table 4

Quantitative Determination of Oxprenolol in Human Urine Obtained from a Patient Under Treatment with the Pharmaceutical Formulation "Transitensin" Collected at Different Time Intervals*

Interval Time (h)	Urine Volume (mL)	Amount of Oxprenolol (mg)[a]
0 - 4	1250	0.58 ± 0.11
4 - 8	1700	1.09 ± 0.02
8 - 12	1500	0.22 ± 0.01
12 - 24	570	0.05 ± 0.01

* For chromatographic conditions see Table 1. [a] Amount ± $t s/\sqrt{n}$ ($n = 3$), where t is the student parameter (95% level of confidence).

The solid-liquid extraction used as clean-up procedure is very simple and effective. A chromatogram free of interferences from the endogenous compounds of the urine matrix is achieved in less than 6 min. The clean-up procedure used gives rise to percentages of recovery greater than 90%.

The chromatographic method has proven to be useful for the identification and determination of the β-blocker oxprenolol in human urine samples obtained from a hypertensive patient after the administration of the pharmaceutical association Transitensin.

Amperometric detection has demonstrated to be sensitive enough for the determination of free oxprenolol in urine samples at all time intervals studied. The amount of oxprenolol found in urine at different time intervals is in agreement with that expected from the pharmacokinetic data, less than 5% of the dose is excreted in 24 h.[5] Electrochemistry provides a sensitive detection for the drug and it constitutes a good alternative to the photometric detection used in some works reported in literature.

The chromatographic method is easily used for the determination of the β-blocker in the pharmaceutical formulations for quality control purposes.

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