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Journal of Chromatography A, 949 (2002) 91–97

JOURNAL OF CHROMATOGRAPHY A

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# Quantitative determination of oxprenolol and timolol in urine by capillary zone electrophoresis

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**Abstract**

A simple capillary zone electrophoretic method with UV detection has been developed for the quantitative determination of the β-adrenoreceptor antagonists (β-blockers) oxprenolol and timolol in human urine, preceded by a solid-phase extraction step. The electrophoretic separation was performed on a 78 cm×75 μm I.D. fused-silica capillary (effective capillary length: 70 cm). The electrolyte consisted of a $Na_2B_4O_7$–$H_3BO_3$ (50 m*M*), pH 9. The introduction of the sample was made hydrostatically for 20 s and the running voltage 25 kV at the injector end of the capillary. Photometric detection was used at a wavelength of 229 nm for oxprenolol and 280 nm for timolol. Under these conditions oxprenolol migrated at 4.76±0.05 min and timolol at 4.97±0.05 min. The solid-phase extraction methods were optimised for each β-blocker and provided recoveries of 72.8% for timolol and 94.52% for oxprenolol. Good resolution from the endogenous compounds present in the urine matrix were achieved for both compounds. The method was applied to the determination of both β-blockers in pharmaceutical formulations and urine samples obtained from hypertensive patients after the ingestion of a therapeutic dose (in a 24-h time interval after the ingestion). The quantitative results were compared with results previously obtained at our laboratories by HPLC and were found to be in good agreement. Good reproducibility, linearity, accuracy and quantitation limits (in urine) of 0.19 μg/ml for timolol and 0.20 μg/ml for oxprenolol were obtained, allowing the method to be applied to pharmacokinetic studies of these compounds. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Oxprenolol; Timolol; β-Blockers

## 1. Introduction

Oxprenolol and timolol are antihypertensive drugs that belong to the family of nonselective β-adrenoreceptor antagonists (β-blockers) widely used in the treatment of hypertension, angina pectoris and cardiac arrhythmias [1,2]. Due to its sedative effect, in January 1987 the use of these compounds was forbidden in sports such as pentathlon and billiards [3].

Timolol has an oral bioavailability of about 75% and undergoes oxidative biotransformation. About 20% of the dose is excreted unchanged [4] and the mean elimination half-life is 4 h.

Oxprenolol is a lipophilic β-blocker with a rapid and almost complete absorption. It is extensively metabolised and only about 5% of the dose is excreted unchanged in the urine [4]. Oxprenolol is also commercialised in combination with the diuretic chlortalidone in the pharmaceutical formulation Trasitensin (oxprenolol 80 mg+chlortalidone 10 mg).

Determination of β-blockers in biological fluids is required in many areas, including doping control, forensic analysis, toxicology and pharmacokinetic

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PII: S0021-9673(01)01320-6

studies. Several methods have been reported for the determination of timolol and oxprenolol in plasma and urine, including mainly high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [5–7], HPLC with electrochemical detection [8–10], HPLC with fluorimetric detection [11] and gas chromatography with mass spectrometric detection [12–14]. Micellar electrokinetic chromatography (MEKC) [15–17] and capillary zone electrophoresis (CZE) methods [18,19] have also been reported for the qualitative analysis of these β-blockers. We have not found however any report dealing with the quantitative determination of oxprenolol or timolol in human urine by CZE.

The aim of this work was to develop a simple and reliable CZE method for the quantitative determination of timolol and oxprenolol in pharmaceutical formulations and urine samples obtained from hypertensive patients under treatment with the corresponding pharmaceutical formulations.

## 2. Experimental

### 2.1. Reagents and solutions

Timolol maleate and oxprenolol hydrochloride were 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 Millipore Milli-RO and Milli-Q Waters systems.

Stock solutions of oxprenolol and timolol (100 μg/ml) were prepared in water and stored at 4°C. Working solutions were prepared by appropriate dilution in electrolyte just before use.

Bond-Elut Certify LRC solid-phase extraction (SPE) columns (10 ml/130 mg) were supplied by Varian (Barcelona, Spain).

### 2.2. Apparatus and electrophoretic conditions

This work was performed in a 78 cm×75 μm I.D. fused-silica capillary (Composite Metal Services, UK). The effective separation distance was 70 cm. The capillary electrophoresis system was a Waters Quanta 4000, and data were collected with the help of a personal computer and treated with the software MILLENNIUM 2010 (Waters Chromatography Division, Barcelona, Spain). The wavelengths used for the photometric detection were 229 nm for oxprenolol and 280 nm for timolol.

Both compounds showed similar electrophoretic behaviour [17]. The study of the influence of the pH and composition of the electrolyte gave an optimum electrolyte consisting of $Na_2B_4O_7$ (50 m*M*)–$H_3BO_3$ (50 m*M*) mixed to give a pH of 9, inducing a 70 μA current across the capillary when a 25 kV voltage was applied. This buffer induced a relatively low current across the capillary providing good reproducibility and resolution from the endogenous compounds present in the urine matrix.

The capillary was conditioned every day with an initial wash cycle consisting of 1 *M* NaOH, 20 min and deionized water, 20 min.

Wash cycles were also carried out before each injection in order to reduce fouling: 0.1 *M* NaOH and running buffer, 3 min each.

Daily wash cycles after finishing experiments consisted of 1 *M* NaOH, 5 min and deionized water, 5 min.

### 2.3. Procedure for tablets

The pharmaceutical formulations analysed in this work were Trasitensin (oxprenolol hydrochloride 80 mg+chlortalidone 10 mg) and Blocadren (timolol maleate 10 mg).

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 electrolyte and finally measured under calibration conditions.

### 2.4. Clean-up procedure for urine samples

The pH of the urine samples (sample volume=5 ml) was adjusted to 9 with borate buffer (1 ml, 1 *M*), vortex mixed for 5 s, centrifuged for 5 min at 734 *g* and filtered through a 45-μm membrane filter.

Bond Elut Certify LRC SPE columns were inserted into a vacuum manifold and conditioned with 6 ml of methanol and washed with 6 ml of water to remove trapped methanol traces. The columns were prevented from drying. With the vacuum off, a 2.5-ml aliquot of alkalinized sample was slowly drawn through the column. The column was washed with 2 ml of water, 1 ml of 0.1 *M* acetate buffer, pH 4.0, and 2 ml of methanol at a vacuum of 5 mmHg (1 mmHg=133.322 Pa). The column was then allowed to dry under full vacuum ($P>150$ mmHg) for about 10 min. Elution of the analyte was performed with 2 ml of chloroform–isopropyl alcohol (60:40, v/v)+ 2% ammonia solution at a vacuum pressure of 2 mmHg for oxprenolol and (80:20, v/v) for oxprenolol. The eluate was evaporated to dryness at 60°C under a gentle stream of nitrogen, using a Zymark Turbo Vap LV evaporator (Barcelona, Spain). The remaining residue was dissolved in 50 μl of electrolyte and measured under calibration conditions.

### 2.5. Reproducibility and extraction efficiency

The reproducibility and efficiency of the extraction procedure was determined by extracting replicate ($n=5$) spiked urine samples, doped with 20 μg/ml. Each of the extracts was injected and its concentration determined three times. This procedure was repeated on 3 different days.

The extraction efficiency was estimated by measuring the peak areas of nonextracted standard solutions in electrolyte and comparing them with the peak areas obtained from extracted urine samples of the same concentration.

The reproducibility was expressed as the relative standard deviation (RSD) and is calculated by the formula:

$$\%\text{RSD} = \frac{\text{standard deviation}}{\text{mean of the recoveries}} \cdot 100$$

The recoveries obtained for oxprenolol were (93.2±2.5)% at the 2 μg/ml level and (94.5±2.8)% at the 200 ng/ml level. The recovery for timolol was only determined at the 400 ng/ml level since this was the expected concentration range in the urine: (72.8±2.7)%.

## 3. Results and discussion

Oxprenolol and timolol exhibit similar electrophoretic behaviours. They both have p$K_a$ values around 9.5 [20]. At pH values below their p$K_a$, the molecules will have an overall positive charge while at pH>p$K_a$, they will be neutral and migrate with the electroosmotic flow.

Based on this acid–base behaviour a validated capillary electrophoresis method for the quantitation of the β-blockers oxprenolol and timolol in human urine samples was optimized.

### 3.1. Optimization of the electrophoretic system

The optimisation process was carried out by running real urine samples (obtained after the ingestion of a therapeutic dose) in parallel with standard solutions, urine blank samples and spiked samples. This way we take into account all possible interferences and metabolites excreted at different time intervals. An optimisation process using only standard solutions and spiked samples has proven not to be a realistic approach since many interferences are not taken into account.

The fact that oxprenolol is habitually administered simultaneously with the diuretic chlortalidone in the pharmaceutical formulation Trasitensin was also taken into account during the optimisation process (including chlortalidone in the optimisation of the electrophoretic conditions). A complete resolution between oxprenolol and chlortalidone was achieved under the optimised conditions, using spiked urine samples (chlortalidone migrated in 6.5 min). This way we could be sure that possible interferences arising from chlortalidone would have no influence in the determination of the β-blocker. Such interferences would however not need to be taken into account during a 24-h study because the amount of chlortalidone expected in the urine during this time interval is negligible and far below our detection limit. The half-life of chortalidone is 44 h and Trasitensin contains only 10 mg of chlortalidone, but could acquire greater significance in longer studies.

Based on previous studies carried out in our laboratories for the determination of other β-blockers [21], samples were injected hydrostatically for 20 s (injection end was raised to 10 cm) and the running

voltage was +25 kV. Temperature was kept constant at 25°C to ensure reproducible results. The length and diameter of the capillary were also optimised. Capillaries shorter than 70 cm did not provide enough resolution between the β-blockers and the endogenous substances present in the urine. Therefore an optimum effective length of 70 cm was chosen. Internal diameters smaller than 75 μm did not provide enough sensitivity due to limited detector pathlength.

Phosphate and borate buffers with different composition and pH values ranging from 5 to 11 were assayed as running electrolyte. The +25 kV running voltage induced currents between 30 and 70 μA across the capillary.

The study of the influence of the pH and composition of the electrolyte gave an optimum value of pH 9 and a composition consisting of $Na_2B_4O_7$–$H_3BO_3$ (50 m*M*), inducing a 70 μA current.

The wavelengths assayed for the photometric detection were 214, 229 and 280 nm since these were the wavelengths closest to the absorption maxima of the compounds; 229 nm was chosen for the detection of oxprenolol and 280 nm for timolol since these wavelengths provided the highest signal-to-noise ratios in each case.

Once the optimum electrophoretic conditions had been established, a quantitative method for the determination of oxprenolol and timolol in urine samples was developed (Table 1). The RSD of the migration times were close to 1%, thus indicating high stability for the system. Linearity and accuracy were determined by spiking human blank urine samples obtained from five different healthy volunteers. The linearity of the calibration curve was calculated by linear regression and a correlation coefficient higher than 0.99 was obtained from the quantitation limit to 100 μg/ml. The within- and inter-day reproducibilities were determined by extracting replicate spiked urine samples ($n=5$) at 20 μg/ml concentration level.

The experimental quantitation limit, defined as the lowest concentration of β-blocker in a spiked urine sample which gives rise to a signal able to be quantified with a % RSD below 5% ($n=5$), was 0.19 μg/ml for timolol and 0.2 μg/ml for oxprenolol.

### 3.2. Analytical applications

The method developed was applied to the determination of oxprenolol, chlortalidone and timolol in pharmaceutical formulations (Fig. 1). The obtained values were in agreement with those certified by the pharmaceutical companies, with relative errors lower than 1% (Table 2).

The electrophoretic method was also applied to the quantitative determination of oxprenolol and timolol in urine samples obtained from hypertensive patients under treatment with the pharmaceutical formulations Trasitensin and Blocadren.

Urine was collected at different time intervals after the ingestion of the therapeutical dose.

Urine samples were treated following the clean-up procedure described in Section 2.4 and measured under calibration conditions (Fig. 2). Oxprenolol samples collected between 8 and 24 h after the ingestion of the dose contained concentrations below the quantitation limit of the method and therefore a

Table 1
Analytical parameters for the determination of oxprenolol and timolol in urine

| | Oxprenolol | Timolol |
|---|---|---|
| Migration time±SD (min) | 4.76±0.05 | 4.97±0.06 |
| Calibration range (μg/ml) [b] | 10–100 | 20–100 |
| Slope$\pm ts^c/\sqrt{n}$ (confidence interval 95% and $n=6$) | 96.73±2.44 | 89.97±1.96 |
| Intercept$\pm ts^c/\sqrt{n}$ (confidence interval 95% and $n=6$) | −437.37±60.14 | −398.28±203.13 |
| Regression coefficient ($r^2$) | 0.993 | 0.995 |
| Within-day reproducibility (RSD, %)[a] | 0.86 | 1.07 |
| Inter-day reproducibility (RSD, %)[a] | 3.43 | 4.22 |
| Detection limit ($S/N=3$) (μg/ml)[b] | 4.75 | 13.70 |

[a] Ten determinations at the 20 μg/ml level.
[b] Final concentrations after extraction and reconstitution of the sample (preconcentration factor=50 for oxprenol and 100 for timolol).
[c] *t*, statistical value used for the determination of the confidence limits at a 95% confidence level; *s*, standard deviation.

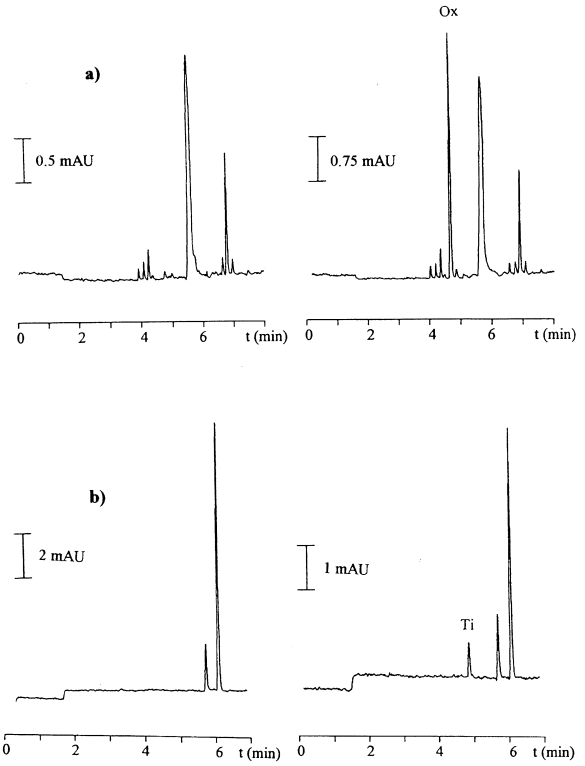


Fig. 1. Electropherograms obtained from the extracts of (a) blank urine sample and urine sample spiked with 4 μg/ml of oxprenolol; (b) blank urine sample and urine sample spiked with 400 ng/ml of timolol. Electrophoretic conditions: the separation was performed on a fused-silica capillary with a 70 cm effective capillary length×75 μm I.D. The wavelengths used for the photometric detection were 229 nm for oxprenolol and 280 nm for timolol. The electrolyte used consisted of a 50 m*M* $Na_2B_4O_7$–50 m*M* $H_3BO_3$ buffer mixed to give a pH of 9. A 25-kV voltage was applied across the capillary. Hydrostatical introduction of the sample for 20 s (10 cm).

higher preconcentration of the samples was required: 5 ml of urine were extracted and the extract was reconstituted to a final 50-μl volume.

Table 2
Determination of oxprenolol, chlortalidone and timolol in pharmaceutical formulations

| Formulation | Nominal (mg) | Found (mg)[a] |
|---|---|---|
| Trasitensin | Oxprenolol 80 mg | 79.78±0.01 |
| | Chlortalidone 10 mg | 9.94±0.02 |
| Blocadren | Timolol 10 mg | 9.96±0.02 |

[a] $x \pm ts/\sqrt{n}$ for a 95% confidence interval, $n=3$ and three replicates of each sample.

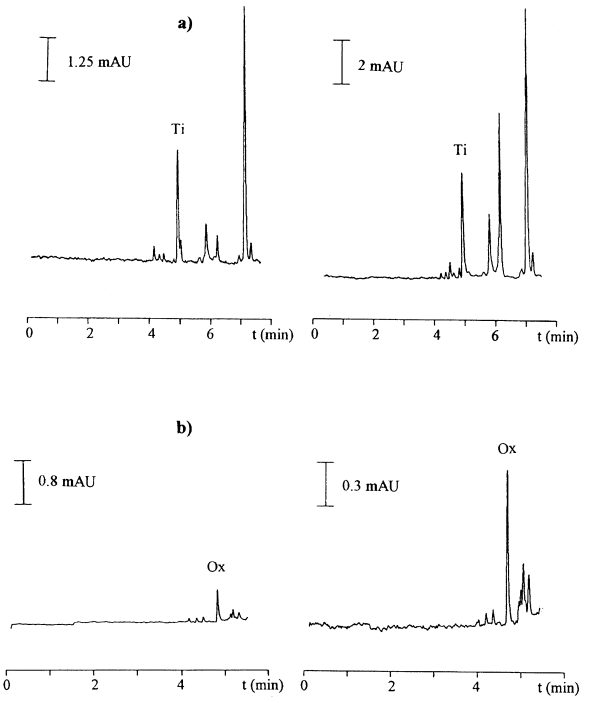


Fig. 2. Electropherograms of urine samples obtained from hypertensive patients. Samples were collected at different time intervals after the intake of a therapeutic dose of Blocadren (timolol 10 mg) and Trasitensin (oxprenolol 8 mg+chlortalidone 10 mg): (a) samples collected at 0–2 h and 2–4 h time intervals after the intake of Blocadren. (b) Samples collected at 0–4 h and 4–8 h time intervals after the intake of Trasitensin. Same CE conditions as in Fig. 1.

Analyses were carried out in triplicate (three different urine aliquots were extracted and analysed for each time interval and three replicate injections per aliquot were carried out). The different oxprenolol and timolol amounts found are reported in Table 3. These results were compared to those previously obtained at our laboratories by HPLC with electrochemical detection (ED) and no significant differences were found [10].

## 4. Conclusions

The described electrophoretic method has proved to be a fast, simple and useful method for the determination of oxprenolol and timolol in real human urine samples. Chlortalidone, diuretic coad-

Table 3
Determination of oxprenolol and timolol in human urine samples collected at different time intervals after the ingestion of a therapeutic dose of (a) Trasitensin and (b) blocadren

| Pharmaceutical formulation | Time interval (h) | Volume collected (ml) | Oxprenolol (mg)[a] | Timolol (mg)[a] |
|---|---|---|---|---|
| Trasitensin | 0–4 | 1250 | 0.56±0.05 | |
| (oxprenolol 80 mg + | 4–8 | 1700 | 1.08±0.09 | |
| Chlortalidone 10 mg) | 8–12 | 1500 | 0.17±0.02 | |
| | 12–24 | 570 | 0.040±0.003 | |
| Blocadren 10 mg | 0–2 | 250 | | 0.36±0.03 |
| Timolol 10 mg | 2–4 | 250 | | 0.17±0.01 |
| | 4–12 | 250 | | 0.56±0.05 |

[a] $x \pm ts/\sqrt{n}$ for a 95% confidence interval, $n=3$ and 2 replicates of each sample.

ministered with oxprenolol, does not interfere with its determination.

In spite of the matrix interference, acceptable RSD values were obtained for peak areas and migration times when wash cycles were carried out between runs. If these wash cycles are not carried out, endogenous compounds from the urine matrix could be adsorbed on the capillary surface, affecting the electroosmotic flow and inducing irreproducibilities between runs.

The sample clean-up procedure is simple and effective, providing extracts that are sufficiently free from interference to allow the preconcentration of the samples.

The electrophoretic elution is achieved in $<5$ min and the analysis takes $<20$ min, including the clean-up step.

Quantitation limits are good enough to determine oxprenolol and timolol in a 24-h time interval. The quantitation limits could be improved if a larger urine volume would be extracted or the residue would be dissolved in a smaller final volume.

The results obtained from the quantitation at different time intervals after the ingestion of the drugs are in good agreement with those expected from the pharmacokinetic data [4]. Moreover, these results were also in good agreement with previous results obtained at our laboratories by HPLC–ED [10].

## Acknowledgements

The authors thank the University of the Basque Country UPV-EHU (project UPV171.310-EB 149/99) for financial support.

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