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RAPID DETERMINATION OF THE ENANTIOMERS OF METOPROLOL, OXPRENOLOL AND PROPRANOLOL IN URINE

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SUMMARY

A method is described that makes possible the rapid determination of the enantiomers of β-blocking agents. After extraction from urine samples (at pH 9.9) using toluene, the enantiomers are derivatised with *S*-(+)-benoxaprofen chloride. The chromatographic separation can be performed on thin-layer plates with toluene–acetone as mobile phase. The derivatives can be detected by measuring the fluorescence (λ_{ex} = 313 nm, λ_{em} = 365 nm).

INTRODUCTION

Most β-adrenoceptor blocking agents are used as racemic mixtures in antihypertensive therapy. Regarding β-adrenoceptor antagonist activity, the *S*-enantiomers are much more effective [1, 2], i.e the pharmacodynamic properties of the corresponding enantiomers are different. In addition, the pharmacokinetics of the two enantiomers have been shown to be different for several β-adrenoceptor blockers, such as propranolol and metoprolol. Such differences occur especially when the main route of elimination of a drug is biotransformation or if active tubular secretion is involved [3]. Therefore, it is important to investigate the enantiomer kinetics, in order to obtain information about the concentration of the effective part of the racemic mixture.

For the simultaneous determination of *R*- and *S*-enantiomers of metoprolol and propranolol in plasma, two fluorimetric methods have already been described by Hermansson and Von Bahr [4, 5]. In these two methods the enantiomers were separated by reversed-phase high-performance liquid chromatography (HPLC) after derivatisation with N-trifluoroacetyl-1-prolyl chloride and the symmetrical anhydride of *tert.*-butoxycarbonyl-L-leucine, respectively. In order to perform studies on urinary excretion of enantiomers of β-adrenoceptor blocking drugs, we

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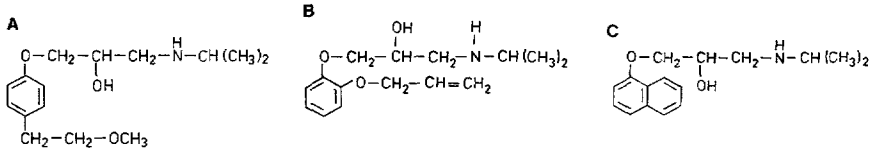

Fig. 1. Structural formulae of metoprolol (A), oxprenolol (B) and propranolol (C).

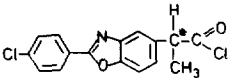

Fig. 2. Structural formula of benoxaprofen chloride.

developed a simple and rapid method for the fluorimetric determination of the enantiomers of metoprolol, oxprenolol and propranolol (Fig. 1) after derivatisation with *S*-(+)-benoxaprofen chloride [6] (Fig. 2) and thin-layer chromatographic (TLC) separation. This method allows the analysis of a large number of urine samples in a relatively short period.

EXPERIMENTAL

Instruments

A Speed Vac concentrator (Bachofer, Reutlingen, F.R.G.) was used to evaporate organic solvents to dryness. The TLC plates were scanned with a KM 3 chromatogram–spectrophotometer (Carl Zeiss, Oberkochen, F.R.G.) and a Type LS recorder (Linseis, Selb, F.R.G.). The optical purity of the reagent was determined by HPLC using a DuPont 830 liquid chromatograph and a DuPont variable-wavelength UV detector (set at 309 nm).

Materials

The chemicals, solvents and thin-layer plates (Kieselgel 60, 20×20 cm, without fluorescence indicator) were obtained from E. Merck (Darmstadt, F.R.G.). *R,S*-Metoprolol tartrate, *R*-metoprolol hydrochloride, *S*-metoprolol tartrate were kindly donated by Astra Chemicals (Wedel, F.R.G.); the available metabolites of metoprolol [H117/04 (metabolite I), H104/83 (metabolite II), H119/66 (metabolite III)] (Fig. 3) [7] by Hässle (Mölndal, Sweden); and *R,S*-propranolol hydrochloride, its enantiomers *R*- and *S*-propranolol hydrochloride and its metabolites (1-naphthoxylactic acid and 4-hydroxypropranolol hydrochloride)

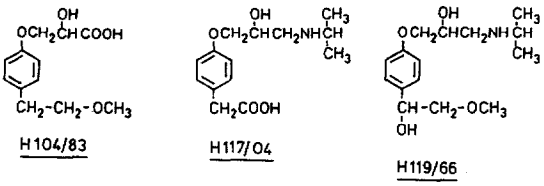

Fig. 3. Structural formulae of metoprolol metabolites.

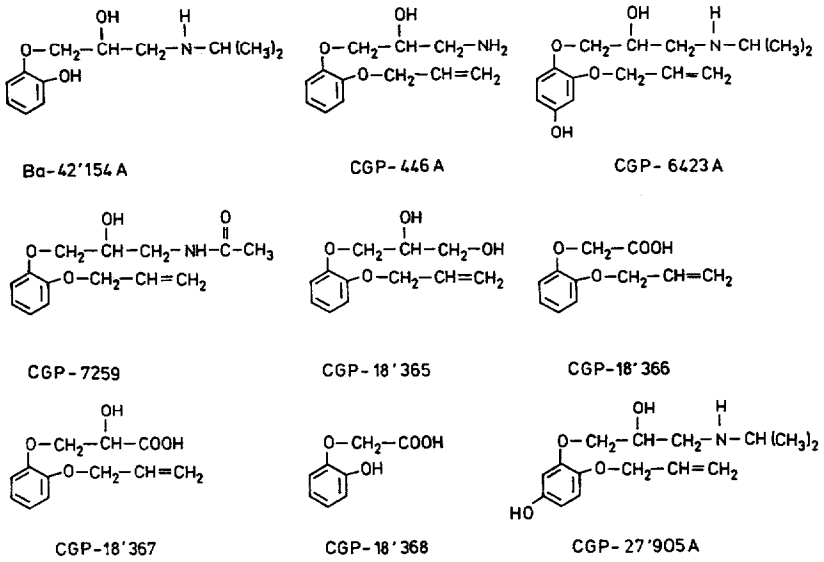

Fig. 4. Structural formulae of oxprenolol metabolites.

by ICI-Pharma (Plankstadt, F.R.G.). *R,S*-Oxprenolol hydrochloride, *R*-oxprenolol hydrochloride, *S*-oxprenolol hydrochloride and its metabolites CGP-6423A, CGP-27'905A, Ba-42'154A, CGP-446A, CGP-18'367, CGP-18'366, CGP18'368, CGP-18'365 and CGP-7259 [8] were obtained from Ciba-Geigy (Basel, Switzerland). The enantiomers of benoxaprofen were isolated by preparative HPLC as described previously [9]. A solution of 3 mg of *S*-benoxaprofen chloride (BOP-Cl, optical purity 96.5%) in toluene (analytical grade) was used as reagent solution.

Synthesis of reference compounds (e.g. propranolol)

A sample of 0.9 mmol of propranolol (as base) was dissolved in methylene chloride (20 ml), and 1.2 mmol of sodium carbonate were added. Then 1 mmol of benoxaprofen chloride [6, 9] in 20 ml of methylene chloride was added slowly at room temperature and allowed to react for 24 h. The organic phase was twice washed with 1 *M* hydrochloric acid and water. The propranolol derivative was crystallised from methanol, yielding a white powder. The elementary analysis for propranolol showed: C 70.49 (theoretical 70.77); H 5.67 (theoretical 5.75); N 5.02 (theoretical 5.16).

Fluorescence properties of the derivatives

The chromophoric properties of the reference compound were measured on the TLC plate after chromatography using the Zeiss chromatogram-spectrophotometer with a mercury medium-pressure lamp (ST 41) and compared with those of the metoprolol and oxprenolol derivatives.

Extraction

To 1.0 ml of urine in a stoppered test-tube, 1.0 ml of 0.5 *M* carbonate buffer (pH 9.9) was added, and the mixture was shaken with 6.0 ml of toluene for 10

min. After centrifugation (15 min), 4.0 ml of organic phase were transferred into another tube and evaporated to dryness in vacuum.

Derivatisation

The residues were dissolved in 1.0 ml of methylene chloride. Then 100 mg of sodium carbonate (without crystal water) and 100 µl of the reagent solution were added. The samples were allowed to react overnight at room temperature. Following the addition of 50 µl of methanol (to stop the reaction) the solution is again evaporated to dryness in vacuum.

Chromatographic conditions

The residue was redissolved in 500 µl of cyclohexane by shaking vigorously, and 20 µl of the solution were applied onto a thin-layer plate (width of strip 5 mm) with a Linomat III (Camag, Muttenz, Switzerland). The plate was developed in a glass tank (Desaga) in the freshly prepared mobile phase [toluene–acetone (100:10, v/v), ammonia atmosphere, produced by two open 50-ml beakers filled with ammonia (33%) and standing in the glass tank] at room temperature. After development (for metoprolol and oxprenolol 19.5 cm; for propranolol 18.5 cm) the plate is air-dried. The R_F values for the derivatives with *S*-(+)-BOP-Cl are as follows: metoprolol, 0.24 (*R*)/0.28 (*S*); oxprenolol, 0.32 (*R*)/0.38 (*S*); propranolol, 0.32 (*R*)/0.39 (*S*).

Measurements

Excitation was measured with the 313-nm line of an ST 41 mercury medium pressure lamp, and emission with an M 365 monochromatic filter; the slit measured 1.0×6 mm. Unknown concentrations were determined by calculating the peak areas of the urine standards and the samples.

Standard curves and reproducibility

Standards were prepared by adding known amounts of racemic metoprolol tartrate (0.5–40 µg), oxprenolol hydrochloride (0.2–30 µg) and propranolol hydrochloride (0.1–30 µg) to 1.0 ml of drug-free urine, which was then treated like the urine samples. Standard curves were calculated by linear regression analysis using peak areas. Reproducibility studies were performed at two different concentrations (1.0 and 10.0 µg/ml) for each β-blocking drug. Eight urine samples of each concentration were prepared and analysed according to the described procedure, and coefficients of variation were calculated.

Interference with metabolites

The available metabolites of metoprolol, oxprenolol and propranolol were tested for interference with the corresponding enantiomers of the β-blocking agents. For this purpose, 1.0 ml of blank urine was spiked with the drug itself and the corresponding metabolites (0.5 and 10 µg/ml), respectively, and treated like samples using the described procedure.

Quantitation of R-benoxaprofen chloride in the reagent

The optical purity of *S*-benoxaprofen chloride was determined using a method published by Wainer and Doyle [10]. *S*-Benoxaprofen (as acid) was converted into the acid chloride by treating it with thionyl chloride and afterwards derivatised with 1-naphthalenemethylamine. The resulting compound was directly resolved as enantiomeric derivatives on an HPLC chiral stationary phase consisting of covalently bound (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine (Bakerbond®; column length 250 mm; Baker, Phillipsburg, NJ, U.S.A.). A mixture of *n*-hexane–propan-2-ol (90:10, v/v) was used as mobile phase. Chromatographic separation was obtained at 25 °C at a pressure of 4 MPa. Retention times of the enantiomers were 37 and 44 min.

Racemisation test

To confirm that racemisation does not occur during extraction, urine samples containing just one of the enantiomers of metoprolol, oxprenolol or propranolol, i.e. either *R* or *S*, were treated as described above and compared with the pure enantiomers.

Furthermore, the enantiomeric stability of the compounds in biological material during storage was investigated. Urine standards containing 5 μg of *R*- or *S*-enantiomer per ml, respectively, were kept frozen at −20 °C for three months. Then the enantiomer concentrations were determined and the enantiomeric ratios compared with the pure reference compounds.

Correction of the results

The results were corrected by the method of Hermansson and Von Bahr [4]. The following equations were used:

$$x = x_{\text{chrom}} + Ax - Ay \tag{1}$$

$$y = y_{\text{chrom}} + Ay - Ax \tag{2}$$

where x and y are the concentrations of the enantiomers, x_{chrom} and y_{chrom} are the calculated concentrations of the enantiomers after TLC analysis and A is the chiral impurity of the reagent in percent. By combination of the eqns. 1 and 2, eqns. 3 and 4 result:

$$x = x_{\text{chrom}} \frac{1-A}{1-2A} - y_{\text{chrom}} \frac{A}{1-2A} \tag{3}$$

$$y = y_{\text{chrom}} \frac{1-A}{1-2A} - x_{\text{chrom}} \frac{A}{1-2A} \tag{4}$$

where $\frac{1-A}{1-2A} = K_1$ and $\frac{A}{1-2A} = K_2$.

For the chiral reagent *S*-benoxaprofen chloride, an optical impurity of 3.5% was determined using this method. The constants K_1 and K_2 were calculated to be 1.04 and 0.04. The concentrations resulting from peak-area calculation were

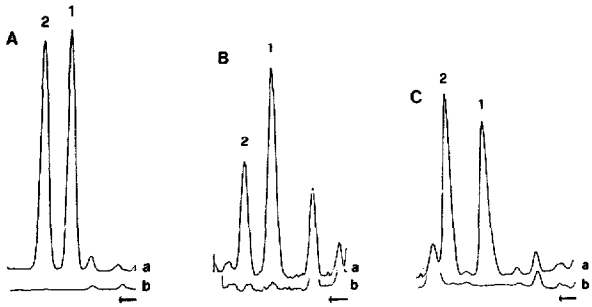

Fig. 5. Thin-layer chromatograms of metoprolol (A; 9.54 μg/ml), oxprenolol (B; 1.81 μg/ml) and propranolol (C; 1.67 μg/ml) after extraction from urine (2 h after oral administration of a commercial preparation) and derivatisation with *S*-(+)-BOP-Cl. Peaks: 1 = *R*-(+)-enantiomer; 2 = *S*-(−)-enantiomer. Traces: a = 0–2 h sample; b = blank urine. The concentrations given for A, B and C are total concentrations (*R*+*S*).

corrected by applying eqns. 3 and 4 on the basis of the K_1 and K_2 values.

Application of the method

Two healthy volunteers received 100 mg of *R/S*-metoprolol tartrate, 80 mg of *R/S*-oxprenolol hydrochloride and 80 mg of *R/S*-propranolol hydrochloride orally in a randomised cross-over. Urine was collected at the following time intervals: 0–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–24 and 24–32 h. The cumulative urinary excretion was calculated until infinity using a monoexponential function, including a lag-time [11].

RESULTS AND DISCUSSION

Fluorescence properties of the derivatives

Using the described procedure it was possible to obtain the derivatisation product of propranolol in a sufficient amount. The investigation of the fluorescence properties of the derivatives resulted in the following excitation and emission maxima, respectively: metoprolol, 313 and 360 nm; oxprenolol, 313 and 364 nm; propranolol, 313 and 359 nm.

Analytical procedure

The enantiomers (as diastereomeric derivatives) were separated from each other and from all interfering substances in urine by TLC. The chromatograms of all investigated compounds after extraction from urine and derivatisation with *S*-(+)-BOP-Cl are shown in Fig. 5. Baseline separation of the enantiomers was achieved in each case. Under the described conditions the derivatisation is almost complete (95%). However, for standardisation it is advantageous to stop the reaction in all samples simultaneously by adding an excess of methanol. Methanol (or other alcohols) converts the excess benoxaprofen chloride into the corresponding ester. The detection limit for all compounds is ca. 100 ng/ml (1.6 ng per peak) after extraction from urine and derivatisation.

TABLE I

COEFFICIENTS OF VARIATION FOR URINE STANDARD SOLUTIONS

Compound	Enantiomer	Coefficient of variation (%)	
		1 μg/ml	10 μg/ml
Metoprolol	*R*-(+)	5.6	3.3
	S-(−)	5.3	3.1
Oxprenolol	*R*-(+)	6.8	5.6
	S-(−)	7.0	5.9
Propranolol	*R*-(+)	6.3	4.8
	S-(−)	6.6	5.0

In the case of propranolol the yield after extraction from a sample and derivatisation could be determined, because the pure reference compound (derivatisation product) was available. The absolute recovery after extraction and derivatisation is 78% for propranolol. The detection limit obtained with the pure reference compound of propranolol is 0.5 ng of enantiomer per spot.

Linearity and reproducibility of the analytical method

The calibration graphs were linear up to 40 μg of metoprolol, 30 μg of oxprenolol and 30 μg of propranolol per ml of urine. The correlation coefficient was above 0.998 in all cases. The standard deviations for the compounds at different concentrations are given in Table I.

Quantitation of R-(−)-BOP chloride in the reagent

The optical impurity of the chiral reagent caused by *R*-(−)-BOP chloride was confirmed to be 3.5% by chromatographic separation of the enantiomers on a chiral phase, using the method of Wainer and Doyle [10].

Racemisation test

Before routine analyses were started, the method was checked for racemisation during the analytical procedure and storage. The chromatograms obtained for the *R*- and *S*-enantiomers of propranolol in the racemisation test are shown in Fig. 6. The second peak could be determined as the sum of the optical impurity of the chiral reagent (3.5%) and of the enantiomers for all β-adrenoceptor blocking agents. For propranolol the area of the additional peak was 6.5% of the total area (sum of both peaks). Taking into account that the optical purity of the BOP enantiomer and the drug enantiomer was 3.5 and 2.5%, respectively, the area of the additional peak was in the expected range. Therefore, it can be concluded that the enantiomers – of both the β-adrenoceptor blocking agents and the reagent – are almost entirely stable during the whole procedure. The peak ratios did not change when the spiked urine samples were stored for three months, i.e. the enantiomers were stable in biological material as well.

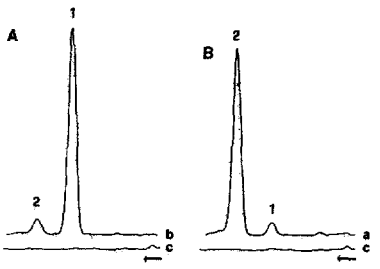

Fig. 6. TLC scans of urine samples containing *R*-(+)-propranolol (A) and *S*-(−)-propranolol (B) after extraction and derivatisation. The urine standards were stored for three months at −20°C. Peaks: 1 = *R*-(+)-propranolol; 2 = *S*-(−)-propranolol. Traces: a and b = urine standards containing propranolol enantiomers; c = blank urine.

Interference of metabolites

All metabolites that were available were investigated for interference. These metabolites do not interfere with any of the enantiomers of metoprolol or oxprenolol or propranolol.

Application of the method

The cumulative urinary excretion of the enantiomers after a single dose of 100 mg of *R/S*-metoprolol tartrate, 80 mg of *R/S*-oxprenolol hydrochloride and 80 mg of *R/S*-propranolol hydrochloride are given in Table II.

For the two investigated volunteers, mean enantiomeric ratios (*R/S*) of 0.86 for metoprolol, 1.6 for oxprenolol and 0.85 for propranolol were found. The application of the method proved it to be reliable and easy to perform.

The procedure can also be transferred to HPLC. For metoprolol, a possible

TABLE II

URINARY EXCRETION OF *R*- AND *S*-ENANTIOMERS OF METOPROLOL, OXPRENOLOL AND PROPRANOLOL AFTER ORAL ADMINISTRATION TO TWO HEALTHY VOLUNTEERS

Commercial tablets, 100 mg of *R/S*-(±)-metoprolol tartrate, 80 mg of *R/S*-(+)-oxprenolol hydrochloride and 80 mg of *R/S*-(±)-propranolol hydrochloride.

Compound	Enantiomer	Urinary excretion (%)	
		Volunteer 1	Volunteer 2
Metoprolol	*R*-(+)	2.79	0.92
	S-(−)	2.99	1.17
	Ratio *R/S*	0.93	0.79
Oxprenolol	*R*-(+)	0.68	0.87
	S-(−)	0.40	0.58
	Ratio *R/S*	1.7	1.5
Propranolol	*R*-(+)	0.17	–
	S-(−)	0.20	–
	Ratio *R/S*	0.85	–

mobile phase is *n*-hexane–methylene chloride–propan-2-ol (380:20:8, v/v/v) on a CN stationary phase (25 cm, 6 μm).

In summary, it can be stated that activated *S*-(+)-benoxaprofen, which was recently described as chiral fluorescence reagent by our group, is suitable for the determination of the urinary excretion of the enantiomers of β-adrenoceptor blocking agents.

ACKNOWLEDGEMENTS

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