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Enantioselective determination of oxprenolol and its metabolites in human urine by cyclodextrin-modified capillary zone electrophoresis

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Abstract

A stereospecific capillary electrophoresis assay for oxprenolol enantiomers and their basic metabolites in human urine has been developed using hydroxypropyl-β-CD as a chiral selector in the mobile phase. The bioassay method has been validated and the detection limit from spiked urine samples is 0.2 μg/ml. The calibration curves are linear from 0.4 to 16 μg/ml. Extraction recovery ranged from 84.7 to 96.4% for all the compounds studied. The influence of various parameters on the chiral separation of oxprenolol and its basic metabolites have been investigated. Urinary excretion profiles of oxprenolol enantiomers and those of two metabolites have also been studied, following a single oral dose of racemic oxprenolol.

1. Introduction

Enantiomeric resolution and determination are very important in pharmaceutical analysis and quality control. Most of the synthetic chiral drugs are marketed as racemates [1], while often one of the enantiomers is more active than the other or is responsible for inadvertent side effects. In the body, biological fates of the enantiomers such as drug absorption, distribution, metabolism and elimination are in some cases very different between the enantiomers [2]. It is therefore important to develop analytical methods for monitoring stereoselective biological processes.

Oxprenolol is an important cardioprotective and noncardioselective β-adrenergic blocking agent. It has a half-life of 1.3–1.5 h for oral doses of 80 mg of the conventional tablet [3]. Oxprenolol is extensively metabolized in rat, dog and man, and various metabolites have been identified [4]. Fig. 1 shows the structures of oxprenolol, its basic metabolites and moprolol. Several methods have been reported for the determination of the enantiomers of oxprenolol in biological fluids by HPLC [5] and TLC [6] after chiral derivatization reactions.

Over the past few years, the applications of capillary electrophoresis (CE) have expanded

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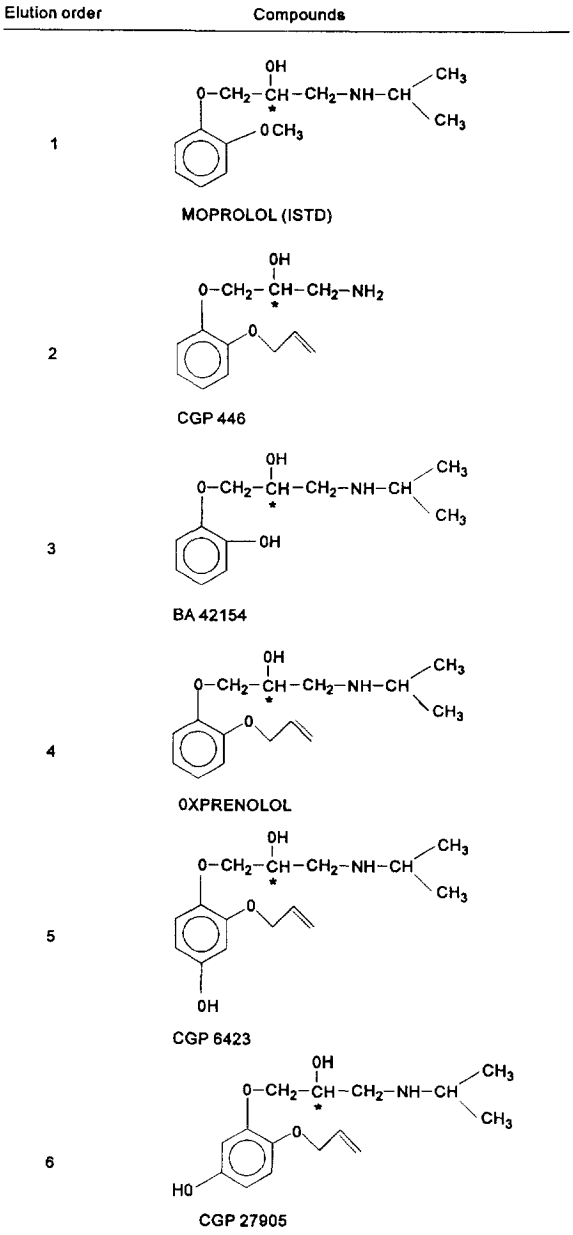

Fig. 1. Structures of oxprenolol and its basic metabolites.

rapidly, and many new applications have been introduced in the field of enantiomeric separations. Enantiomeric CE separations have been achieved using soluble [7] or immobilized [8] chiral complexing agents, such as cyclodextrins (CDs), for the separation of racemic compounds. Chiral CE separations of β-blockers have also been reported using soluble cellulase as an enantioselective protein [9] and using β-CD as a chiral recognition agent [10,11]. So far, very few applications of CE to the analysis of biological samples have been reported.

In this paper, the chiral CE separation and quantitation of oxprenolol and its metabolites in human urine samples using hydroxypropyl-β-CD is described. In addition, important parameters concerning the selectivity and resolution of the enantiomers are discussed.

2. Experimental

2.1. Materials and reagents

Racemic (±)-oxprenolol hydrochloride, (+)-oxprenolol (BA 42155), (−)-oxprenolol (BA 42244), and the metabolites BA 42154, CGP 6423, CGP 446 and CGP 27905 were obtained from Ciba-Geigy (Basel, Switzerland), and (−)-moprolol from Simes (Milan, Italy). β-Cyclodextrin (β-CD) and heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) were purchased from Sigma (St. Louis, MO, USA) and hydroxypropyl-β-cyclodextrin (HP-β-CD, degree of substitution DS = 4.3) was donated by American Maize-Products (Hammond, IN, USA). Polyethylene glycol (PEG, M_r 5000–6000) was obtained from J.T. Baker, Phillipsburg, NJ, USA and tetrabutylammonium dihydrogen phosphate (TBA) from Aldrich (Milwaukee, WI, USA). Fused-silica capillaries (50 μm I.D. × 365 μm O.D., 78 cm long) were purchased from Chromabec (Montreal, Canada). Organic solvents (HPLC grade) were used as received (Caledon Labs., Georgetown, Canada). Inorganic salts were of analytical reagent grade (J.T. Baker or Fisher Scientific, NJ, USA). β-Glucuronidase (type H-1) was obtained from Sigma. Distilled water was further treated with a four-stage Milli-Q water purification system (Millipore, Mississauga, Canada), and this was used throughout.

2.2. Capillary electrophoresis

An Applied Biosystems (Toronto, Canada) Model 270A Capillary Electrophoresis system

equipped with a variable-wavelength UV detector was used with an untreated fused-silica capillary of 58 cm effective length. The oven temperature was set at 27°C. The separation voltage was 26 kV. Samples were introduced onto the capillary by hydrodynamic injection for 2 s. On-column detection was carried out by UV absorbance measurements at 200 nm, with a detector rise time of 0.5 s. The electropherograms were recorded using a Model HP 3392A integrator (Hewlett-Packard, Palo Alto, CA, USA). A 100 m*M* NaH_2PO_4 solution was prepared in water, adjusted to pH 2.5 with concentrated phosphoric acid, and filtered through a Millipore 0.45-μm HA filter. Electrode buffers were prepared by degassing the above buffer in an ultrasonic bath for about 2 min before use. The run buffer contained 50 m*M* HP-β-CD, 0.05% (w/v) PEG and 0.03 m*M* TBA in the above buffer, and 2 ml of run buffer was filtered through a cellulose acetate 0.2-μm frit (provided by Chromabec, Montreal, Canada) and degassed in an ultrasonic bath for about 2 min before use. Initially, the capillary was washed with 1 *M* NaOH for 20 min, and then washed with water for 20 min. Before each analysis the capillary was washed with 0.01 *M* NaOH for 15 min and then with run buffer for 15 min. After each working day, the capillary was washed with 0.01 *M* NaOH for 60 min and later with water for 60 min.

2.3. Standard solutions

Stock solutions of the following compounds were prepared in methanol (1.0 mg/ml): (±)-oxprenolol, (−)-oxprenolol, (+)-oxprenolol, (±)-CGP 6423, (±)-BA 42154, (±)-CGP 27905, (±)-CGP 446 and (−)-moprolol used as an internal standard (I.S.). All the stock standard solutions were sealed and stored at −20°C in the dark. They were diluted with 5 m*M* phosphate buffer (pH 2.5) to appropriate concentrations before use.

2.4. Human studies

A blank urine sample was collected from a healthy male volunteer (age 35 years, weight 64 kg) before the administration of a single 100-mg oral dose of (±)-oxprenolol (calculated as the base form). Urine samples were then collected for 28 h, and were frozen in the dark at −20°C immediately after collection.

2.5. Extraction of urine samples

Aliquots (5 ml) of urine samples were adjusted to approximately pH 10–11 by the addition of 1 ml of 2 *M* potassium carbonate solution after spiking with 10 μg of (−)-moprolol as I.S. The samples were equilibrated for 1 h at room temperature. Sodium chloride (1.0 g) was added and the resulting samples were extracted twice with 8 ml of ethyl acetate. After centrifugation, the organic layer was aspirated, dried with sodium sulfate, and evaporated under a stream of nitrogen at 50°C. The residue was reconstituted in 200 μl of 5 m*M* phosphate buffer for CE analysis. Blank and spiked urine samples were processed by the same procedure.

2.6. Enzymatic hydrolysis

Aliquots (5 ml) of urine samples were spiked with 10 μg of I.S. and incubated with β-glucuronidase (3500 units) at 50°C for 2 h. The hydrolysates were adjusted to pH 10–11 with 2 *M* potassium carbonate. Extraction was carried out by the procedure described above.

2.7. Calibration curves

Standards were prepared by the addition of known amounts (2, 4, 10, 20, 40 and 80 μg, respectively) of racemic oxprenolol, CGP 6423, BA 42154, CGP 27905, CGP 446 and 10 μg of (−)-moprolol (I.S.) to 5 ml of blank urine samples. After equilibration for 1 h at room temperature, the urine samples were extracted as described above, and the extracts were analyzed by CE. Calibration curves for each enantiomer were calculated by linear regression of the peak-area ratios of (−)- and (+)-standard compounds to the area of (−)-moprolol (I.S.) against the concentration.

2.8. Recovery and assay precision from urine

Recovery studies were performed at two different concentrations (0.8 and 4.0 μg/ml) for each compound and the internal standard was added at the end of the extraction procedure. The calculation of recovery was based on a comparison of the peak-area ratio of each enantiomeric compound to I.S. from two different analyses. One set of data was obtained from the analysis of the spiked samples, while the other was from the analysis of standard solutions containing the same quantities of the compounds and the I.S.

Intra- and inter-assay variabilities were determined by replicate analyses of the (−)- and (+)-standard enantiomers after spiking the urine samples with racemic standard compounds (with the same concentrations as in the recovery experiment). CE was carried out on the day of preparation and on different days, to determine intra- and inter-assay reproducibilities, respectively.

3. Results and discussion

3.1. Chiral separation of oxprenolol and metabolites

When chiral complexing agents are included as soluble CE buffer components, the resolution of enantiomers is described by Eq. 1:

$$R_s = (0.177\Delta\mu)[V/D(\mu_{avg} + \mu_{eo})]^{1/2} \quad (1)$$

where $\Delta\mu$ is the difference in the electrophoretic mobilities of the enantiomers, μ_{avg} is the average electrophoretic mobility, μ_{eo} is the electroosmotic mobility, D is the diffusion coefficient and V is the applied voltage [12]. From this expression, it can be seen that an increase in $\Delta\mu$, or a decrease in the sum of $\mu_{avg} + \mu_{eo}$, will lead to improved enantiomeric resolution. Decreasing, and even reversing the direction of electroosmotic flow has been accomplished by lowering the buffer pH to around 2.5, to protonate silanol groups on the capillary wall, by adding neutral polymers such as poly(vinyl alcohol) or poly(ethylene glycol) that adsorb to silica and alter the zeta potential at the capillary/buffer interface, and by adding cationic surfactants such as hexadecyltrimethylammonium bromide or tetrabutylammonium phosphate, that neutralize or even change the polarity of the capillary wall, again by adsorption [13].

Chiral complexing agents, such as cyclodextrins, are used to generate a non-zero value of $\Delta\mu$. The electrophoretic mobility of an enantiomer capable of a dynamic equilibrium between free and CD-bound forms is described as the weighted average of the mobilities of the two forms, given by Eq. 2:

$$\mu = (\mu_F + K_{CD}[CD]\mu_C)/(1 + K_{CD}[CD]) \quad (2)$$

where μ_F and μ_C are the electrophoretic mobilities of the free and CD-bound forms of the enantiomer, [CD] is the cyclodextrin concentration in the CE buffer, and K_{CD} is the association constant for the enantiomer with CD [14]. Clearly, any significant difference in electrophoretic mobilities between enantiomers will result from different K_{CD} values, and a three-point interaction model [13] has been used to account for observed differences. Both the kind of cyclodextrin and its concentration affect the observed differences in the electrophoretic mobilities of enantiomers.

Oxprenolol and its metabolites (Fig. 1) are small molecules with a single positive charge at acidic pH, and migrate in the same direction as electroosmotic flow. For example, in 100 m*M* sodium phosphate buffer at pH 4.5, with 80 m*M* hydroxypropyl-β-CD, oxprenolol eluted as a racemic mixture after 13.9 min. While in 100 m*M* sodium phosphate buffer with 80 m*M* hydroxypropyl-β-CD, the pH was lowered to 2.5, slight separation of the enantiomers was observed, and migration times were much longer at 30.8 and 31.1 min. At pH 2.5, electroosmotic flow is greatly reduced [15], and the slight separation of the enantiomers results from the greater relative importance of their electrophoretic mobilities, which depend on cyclodextrin binding.

Three cyclodextrins were examined as chiral

selectors in a 100 m*M* phosphate buffer (pH 2.5) mobile phase: di-O-methyl-β-CD, β-CD and hydroxypropyl-β-CD, over concentration ranges of 10–80 m*M*. Chiral recognition via CD inclusion complexation has been described by a three-point interaction involving hydrogen bonding between aliphatic hydroxyl and amine groups on the alkanolamine side chains of the β-blockers with secondary CD hydroxyl groups, and inclusion of the phenyl ring in the CD cavity [13]. Clearly, the di-O-methyl-β-CD derivative would be expected to have reduced hydrogen bonding capabilities, due to methylation of half of the secondary hydroxyls. The different resolutions obtained with β-CD and its primary hydroxypropyl derivative are probably related to their different solubilities rather than intrinsic differences in complexing abilities, since similar concentrations of these cyclodextrins yielded similar resolutions (0.64 and 0.66 for 40 m*M* β-CD and hydroxypropyl-β-CD, respectively). The best resolution of oxprenolol enantiomers ($R_s = 0.71$) was achieved with 40–60 m*M* hydroxypropyl-β-CD and 48 cm effective length of capillary, and all further experiments employed 50 m*M* hydroxypropyl-β-CD as the chiral selector.

Attempts to improve enantiomeric resolution by the addition of 10–20% methanol as an organic modifier, as suggested by Fanali [11], were unsuccessful. However, further improvements in resolution were achieved by adding 0.05% poly(ethylene glycol) and 30 μ*M* tetrabutylammonium phosphate to the mobile phase. Under these conditions the resolution of the oxprenolol enantiomers increased to $R_s = 1.20$, with the same 48 cm capillary. Increasing the effective length of the capillary to 58 cm afforded baseline resolution ($R_s = 1.73$), as shown in Fig. 2. The identities of the enantiomers were confirmed by CE of a solution of racemic oxprenolol to which (−)-oxprenolol had been added.

Fig. 3 shows chiral separations of oxprenolol and four metabolites present in urine samples under these optimized conditions, with a moprolol internal standard. Complete chiral separation was achieved for BA 42154, oxprenolol, CGP 6423 and CGP 27905, whereas CGP 446 was partly resolved. The identities of the metabolites were confirmed by comparison with electropherograms obtained for the standard compounds in separately spiked blank urine samples. Since the six compounds are quite similar in terms of molecular weight, structure and charge, differences in migration times must be strongly influenced by association constants with hydroxypropyl-β-CD. Thus, moprolol binding is weakest and the binding of the metabolite CGP 27905 is strongest with hydroxypropyl-β-CD. The elution order suggests that both the substituents on the phenyl ring and the isopropylamine group are important for strong CD binding.

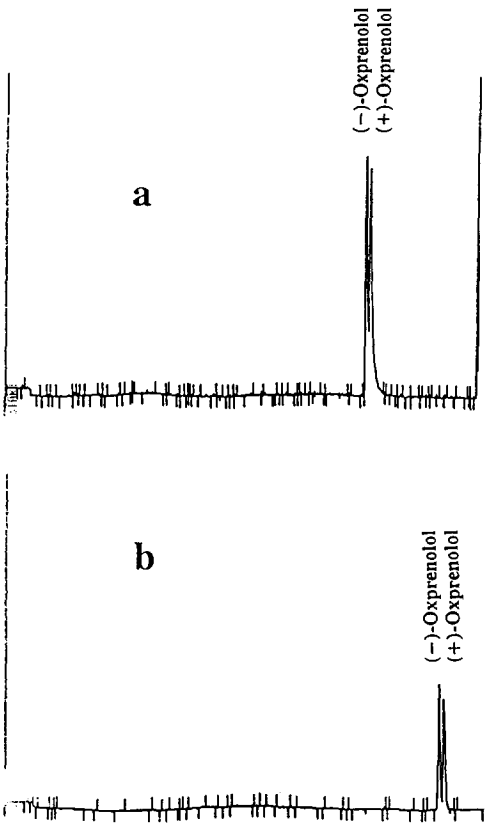

Fig. 2. Effect of capillary length on chiral separation of racemic oxprenolol using 100 m*M* phosphate buffer, pH 2.5, 50 m*M* HP-β-CD, 0.05% PEG, 0.03 m*M* TBA: (a) 48 cm effective length; (b) 58 cm effective length.

Fig. 3c shows results obtained with a real urine sample, where the excretion of oxprenolol and its metabolites was monitored 4 h after the ingestion

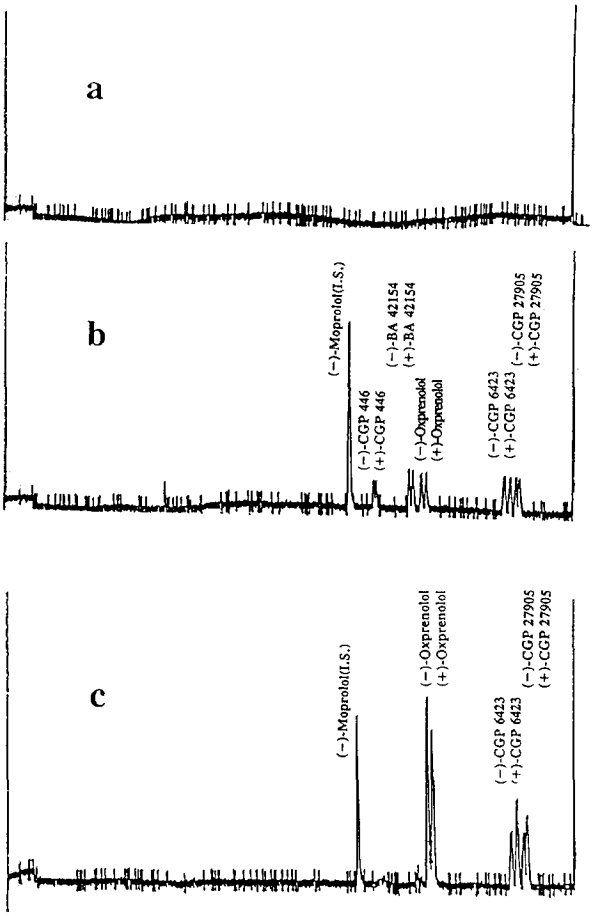

Fig. 3. Electropherograms of oxprenolol and its basic metabolites using 100 m*M* phosphate buffer, pH 2.5, 50 m*M* HP-β-CD, 0.05% PEG, 0.03 m*M* TBA. 26 kV, 17 μA. (a) Blank urine; (b) blank urine spiked with standard compounds; (c) real urine sample, collected 4 h after a 100 mg oral ingestion of racemic oxprenolol.

of a 100-mg oral dose of racemic oxprenolol. It can be seen that the (+)-oxprenolol enantiomer is metabolized more rapidly than its antipode, and that two metabolites, CGP 6423 and CGP 27905, are excreted in significant quantities, with their (+)-enantiomers being more abundant.

3.2. Calibration curves

Calibration data were obtained using blank human urine spiked with oxprenolol and its metabolites. All eleven species, including the moprolol internal standard and the enantiomers of CGP 446, BA 42154, oxprenolol, CGP 6423 and CGP 27905, showed intra- and inter-assay migration time reproducibilities of 1.0% R.S.D. or better. For example, inter-assay ($n = 6$) migration time reproducibilities ranged from 0.85% to 1.0% for the eleven compounds.

Linear regression of the analyte:moprolol peak-area ratios against concentration (in μg/ml) over the 0.4–16 μg/ml range yielded correlation coefficients in excess of 0.993, as shown in Table 1. Detection limits ($S/N = 3$) of 0.2 μg/ml were obtained for the enantiomers of oxprenolol and its two major metabolites, CGP 6423 and CGP 27905. For these species, quantitation in spiked urine samples (Table 2) showed intra-assay precisions ($n = 3$) better than 3.4% R.S.D., and inter-assay precision ($n = 6$) was better than 5.7% R.S.D.

Table 3 shows the results of recovery experiments, where human urine samples spiked with known quantities of oxprenolol and its two major metabolites were extracted and analyzed by CE. Recoveries ranged from 84.7% to 96.4%, with intra-assay ($n = 3$) and inter-assay ($n = 6$) precisions better than 8.9% and 10.2% R.S.D., respectively.

3.3. Excretion of oxprenolol and metabolites in urine

The urinary excretion profiles of the enantiomers of oxprenolol and its two major metabolites were determined using the developed CE meth-

Table 1
Regression of peak-area ratio against concentration, with (−)-moprolol internal standard[a]

Compound	*R*	Slope	*Y*-Intercept
Oxprenolol	(−) 0.998	(−) 0.380	(−) −0.026
	(+) 0.998	(+) 0.389	(+) −0.167
CGP 6423	(−) 0.998	(−) 0.397	(−) −0.116
	(+) 0.997	(+) 0.412	(+) −0.133
CGP 27905	(−) 0.997	(−) 0.389	(−) −0.127
	(+) 0.993	(+) 0.490	(+) −0.274

[a] For concentrations in μg/ml, from three replicate injections at each of six concentrations covering the 0.4–16 μg/ml range.

Table 2
Precision for the quantitation of oxprenolol and its metabolites in spiked human urine samples

Compound (4 μg/ml)	Intra-assay response ($n = 3$) Area ratio of compound to I.S.		Inter-assay response ($n = 6$) Area ratio of compound to I.S.	
	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
Oxprenolol	(−)1.1988 ± 0.0145	1.21	(−)1.1488 ± 0.0526	4.58
	(+)1.2983 ± 0.0442	3.40	(+)1.2443 ± 0.0637	5.12
CGP 6423	(−)1.3573 ± 0.0108	0.79	(−)1.3072 ± 0.0566	4.33
	(+)1.4105 ± 0.0094	0.67	(+)1.3481 ± 0.0686	5.09
CGP 27905	(−)1.3546 ± 0.0044	0.32	(−)1.3095 ± 0.0514	3.93
	(+)1.4676 ± 0.0295	2.01	(+)1.3987 ± 0.0798	5.71

od, following the ingestion of a single 100 mg oral dose of racemic oxprenolol by a healthy male volunteer. Quantitation was performed on samples before and after treatment with the enzyme β-glucuronidase, which hydrolyzes the glucuronide derivatives to release free oxprenolol and metabolites. The results, shown in Fig. 4, indicate that the majority of the species excreted between 2 and 14 h after ingestion are in the glucuronide forms, and that the maximum excretion rates for all species occurred between 2 and 4 h after ingestion. The free drugs can be detected up to 7 h after ingestion, while total (free + glucuronide) species are detectable up to 14 h after ingestion. The results shown for oxprenolol in Fig. 4a are consistent with previous reports conducted with urine [6] and plasma [5] samples, in that the excretion of both free and conjugated (+)-oxprenolol is greater than that of its (−)-antipode.

Fig. 4b, the excretion profiles for metabolite CGP 6423, shows that the (−)-enantiomer is excreted at a higher rate than the (+)-enantiomer when the free form of the metabolite is extracted, but the reverse is true if the extraction and CE quantitation are performed after enzymatic hydrolysis of the glucuronides. Most of the (+)-enantiomer of this metabolite therefore exists as the glucuronide derivative.

Quantitation of the metabolite CGP 27905 resulted in the excretion profiles shown in Fig. 4c. These profiles are similar to those obtained

Table 3
Recovery and precision of oxprenolol and its major metabolites

Compound	Added (μg/ml)	Intra-assay ($n = 3$) recovery (%)		Inter-assay ($n = 6$) recovery (%)	
		Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
Oxprenolol	0.8	(−) 86.1 ± 4.6	5.3	(−) 85.0 ± 6.6	7.7
		(+) 84.7 ± 1.5	1.8	(+) 86.9 ± 5.5	6.3
	4.0	(−) 94.3 ± 2.6	2.7	(−) 93.0 ± 4.1	4.4
		(+) 93.6 ± 1.6	1.7	(+) 94.4 ± 3.8	4.0
CGP 6423	0.8	(−) 95.7 ± 8.5	8.9	(−) 94.5 ± 6.4	6.7
		(+) 89.9 ± 1.8	2.0	(+) 89.7 ± 7.5	8.4
	4.0	(−) 94.6 ± 3.7	4.0	(−) 95.2 ± 5.9	6.2
		(+) 95.0 ± 2.7	2.9	(+) 96.0 ± 5.3	5.5
CGP 27905	0.8	(−) 86.4 ± 1.1	1.3	(−) 91.5 ± 9.3	10.2
		(+) 95.3 ± 3.9	4.1	(+) 96.4 ± 8.2	8.5
	4.0	(−) 92.9 ± 2.9	3.1	(−) 94.5 ± 3.0	3.2
		(+) 94.1 ± 1.9	2.0	(+) 96.1 ± 3.8	4.0

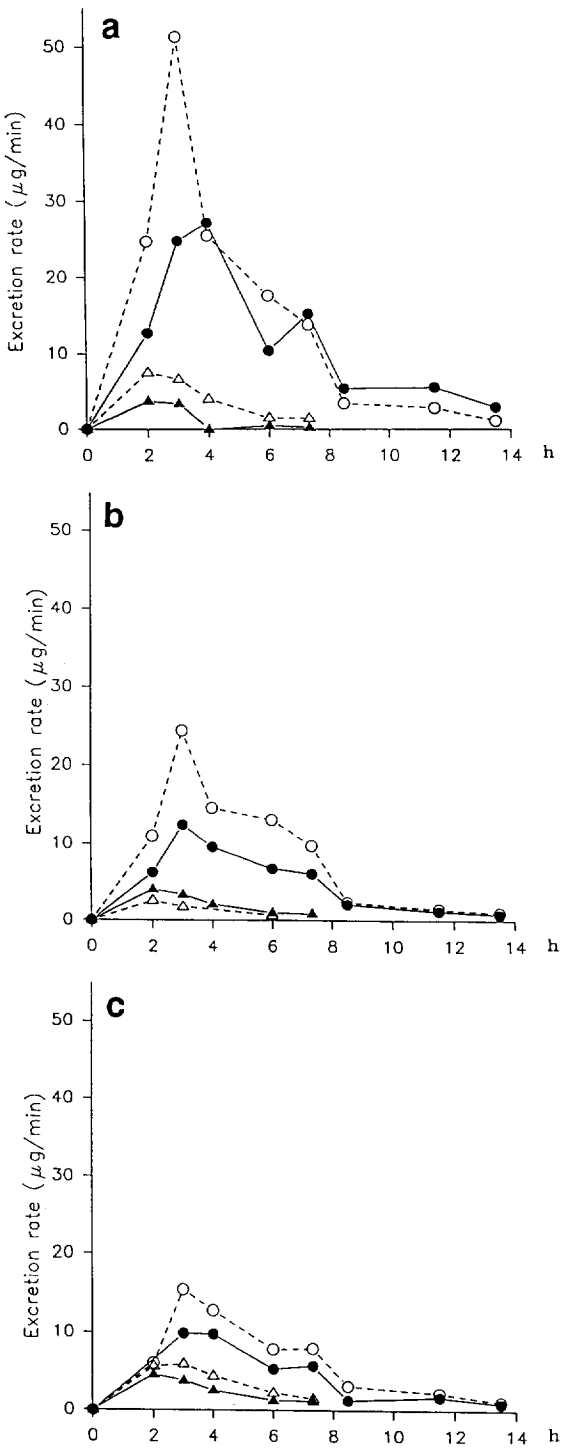

Fig. 4. Urinary excretion profiles of (a) oxprenolol, (b) CGP 6423, and (c) CGP 27905. Before enzyme hydrolysis ▲ = (−) and △ = (+); after enzyme hydrolysis ● = (−) and ○ = (+).

for oxprenolol, with the (+)-enantiomer having a higher excretion rate in both the free and glucuronide forms.

These results demonstrate that the mechanism of oxprenolol metabolism is stereoselective in humans. With all three species, oxprenolol and its metabolites CGP 6423 and CGP 27905, elimination of the (+)-enantiomers occurs at a higher rate than that of the (−)-enantiomers. The biological activities and fates of the selectively retained (−)-enantiomers would be an interesting subject for further investigation.

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