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Determination of the enantiomers of bunolol in human urine by high-performance liquid chromatography on a chiral AGP stationary phase and identification of their metabolites by gas chromatography–mass spectrometry

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Abstract

A high-performance liquid chromatographic method using a chiral AGP column was developed to screen and determine the enantiomers of bunolol in human urine. The recovery of (+)- and (−)-bunolol from urine was 91.79–95.23% at different concentrations. The coefficients of variation (C.V.) were less than 2.1 and 2.3% for intra- and inter-assays, respectively. Urinary metabolites were detected using GC–MS after derivatization with N-methyl(trimethylsilyl)trifluroacetamide. The influences of pH and modifier on a chiral AGP column were studied.

1. Introduction

Bunolol, 5[(*tert.*-butylamino)-2′-hydroxypropoxy]-3,4-dihydro-1-(2*H*)-naphthalenone, is a non-cardioselective β-blocker [1,2]. (−)-Bunolol is 60 times more potent than the (+)-isomer in its β-blocking activity. It is mainly used as a therapeutic agent for glaucoma and ocular hypertension [3]. Several studies have been reported on the determination of racemic bunolol using high-performance liquid chromatography (HPLC) [1] and gas chromatography–mass spectrometry (GC–MS) [4]. In this study, we developed a method for the chiral separation of bunolol with a chiral α_1-acid glycoprotein (AGP) column. Three metabolites were identified by GC–MS, two of which were found to be new minor metabolites of bunolol. Pharmacokinetic studies of stereoselectivity of bunolol were performed in human subjects using chiral HPLC.

2. Experimental

2.1. Materials and reagents

Racemic (±)-bunolol hydrochloride and (−)-bunolol hydrochloride were obtained from Allergen Canada (Markham, Ontario, Canada), (−)-moprolol from Simes (Milan, Italy), N-methyl(trimethylsilyl)trifluroacetamide (MSTFA) and trimethylchlorosilane (TMCS) from Regis (Morton Grove, IL, USA) and N,N-dimethyloctylamine (DMOA) from Aldrich. All the organic

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solvents (HPLC grade) were used as received (Caledon Labs., Georgetown, Canada). Inorganic salts were of analytical-reagent grade (J.T. Baker, Phillisburg, NJ, USA, or Caledon Labs.). β-Glucuronidase type H-1 from *Helix pomatia* was purchased from Sigma (St. Louis, MO, USA). Distilled water was further treated with a four-stage Milli-Q water purification system (Millipore, Mississauga, Ont., Canada) before used for HPLC (this is simply referred as water hereafter).

2.2. Standard solutions

Stock standard solutions of (±)-bunolol and (−)-bunolol (0.1 and 1.0 mg/ml) and (−)-moprolol used as an internal standard (I.S.) (0.1 and 1.0 mg/ml) were prepared in methanol. All the stock standard solutions and reagent solutions were sealed and stored at −20°C in the dark.

2.3. Human studies

Control urine samples were collected before the administration of a single 20-mg oral dose of racemic (±)-bunolol to two healthy male volunteers (first subject aged 34 years, weighing 63 kg, and second subject aged 57 years, weighing 63 kg). Urine samples were then collected for the next 72 h, and were frozen in the dark at −20°C immediately after collection.

2.4. Extraction procedure for chiral HPLC analysis

In a typical extraction procedure, aliquots of 5.0 ml of urine were acidified to pH 4.0 with two drops of 2 *M* HCl followed by extraction with 5 ml of hexane. The hexane phase was rejected. Then the urine samples were basified to pH 11–12 with 1.2 ml of 2 *M* potassium carbonate solution. About 1.0 g of sodium chloride was added and the resulting samples was extracted twice with 8 ml of ethyl acetate. After centrifugation, the organic layer was aspirated and dried with sodium sulfate. The organic solvent was evaporated to dryness under a stream of nitrogen at 50°C. The residue was reconstituted in 300 μl of methanol and 10 μl were injected into the liquid chromatograph. Blank and spiked urine samples were also processed using the same procedure.

2.5. High-performance liquid chromatography

An HP 1090 liquid chromatograph was equipped with a diode-array UV detector and an HP G1307A ChemStation data system (Hewlett-Packard, Palo Alto, CA, USA). The separation of (±)-bunolol and (−)-moprolol was performed on a chiral AGP column (ChromTech, Stockholm, Sweden) (150 mm × 4.0 mm I.D.) at ambient temperature. The mobile phase consisted of solvent A and B. Solvent A (0.01 *M* phosphate buffer–0.001 *M* DMOA, pH 7.0) was prepared by dissolving 1.42 g of dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) and 205 μl of DMOA in 1 l of water and adjusting the pH to 7.0 with concentrated phosphoric acid. It was filtered over a Millipore 0.45-μm HA filter and degassed with helium for 30 min before use. Solvent B was 2-propanol. The analysis was accomplished by isocratic elution with A–B (98:2) up to 20 min. The flow-rate of the mobile phase was kept at 0.9 ml/min. The UV absorbance of the column effluent was monitored at 223 nm (band width 4 nm) with a reference wavelength of 450 nm (band width 20 nm). Spectral scanning was performed from 192 to 400 nm.

2.6. Calibration

Aliquots of 5 ml of blank urine samples were spiked with (±)-bunolol (concentration 0.12–2.4 μg/ml for each enantiomer). After being equilibrated for 1 h at room temperature the urine samples were extracted as described in Section 2.4 and the extracts were analysed by chiral HPLC. For each concentration, triplicate samples were prepared and duplicate injections were made for each samples. The ratios of the peak areas of (+)- and (−)-bunolol to that of (−)-moprolol (I.S.) were measured for each analysis. The data were fitted by the linear regression equations $C_{(-)} = 0.38\ A_{(-)} + 0.090$ ($r = 0.997$)

and $C_{(+)} = 0.37\ A_{(+)} + 0.091$ ($r = 0.998$), where $C_{(-)}$ and $C_{(+)}$ are the concentrations of (−)- and (+)-bunolol in urine (μg/ml), respectively, and $A_{(-)}$ and $A_{(+)}$ are the peak-area ratios of (−)- and (+)-bunolol to that of (−)-moprolol (I.S.).

2.7. Recovery and precision

The extraction recovery of bunolol enantiomers from urine was assessed with spiked urine samples at four different concentrations, 0.24, 0.60, 1.20 and 2.40 μg/ml, for (−)- and (+)-bunolol. Sample preparation and HPLC analysis were performed as described. The calculation of recovery was based on a comparison of the peak-area ratios of the bunolol enantiomers to that of the I.S. from two different analyses. One set of data was obtained from the analysis of the spiked urine samples and the other set from the analysis of standard solutions containing the same amount of bunolol enantiomers and I.S. The detection limit for each bunolol enantiomer was found to be 10 ng (signal-to-noise ratio = 3:1) corresponding to the absolute amount injected.

Intra- and inter-assay variabilities were de-

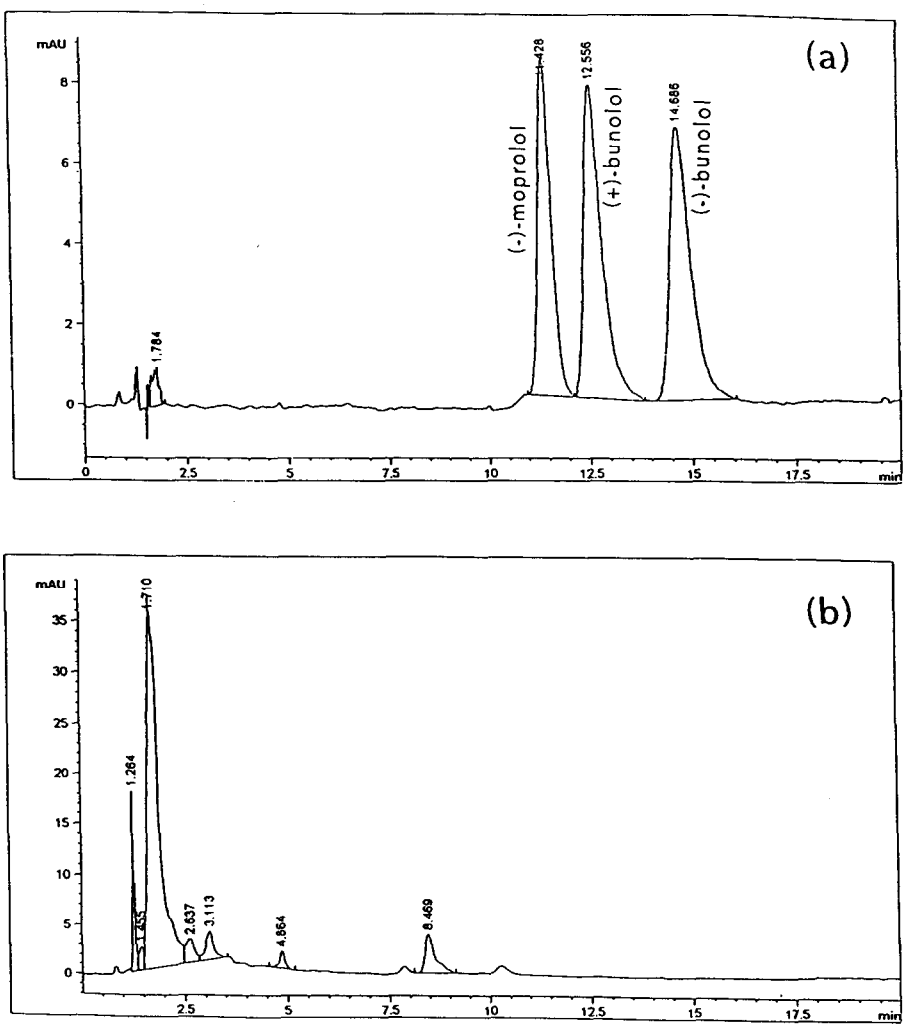


Fig. 1. HPLC of (a) standard (±)-bunolol enantiomers and (b) an extract of blank human urine sample.

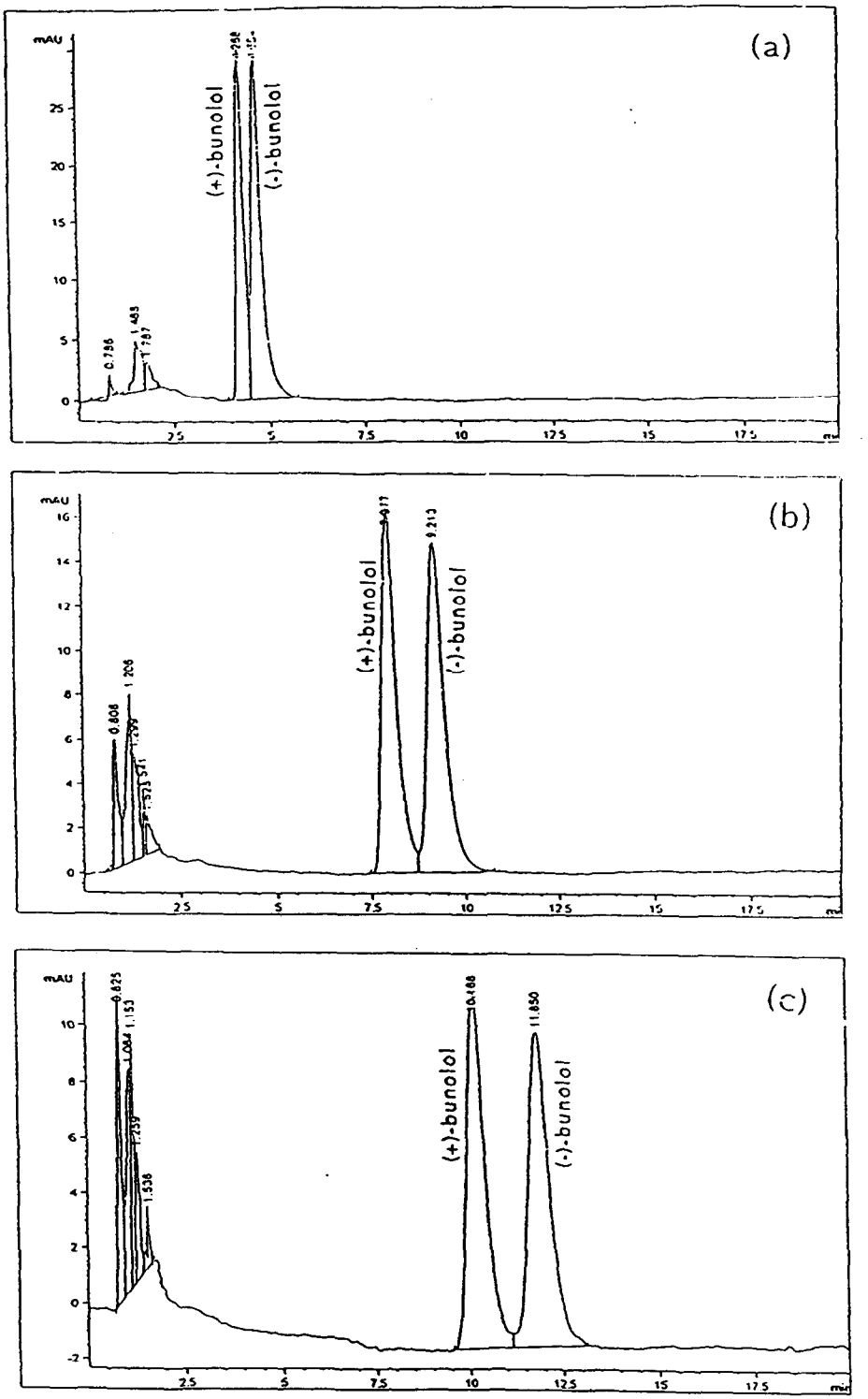


Fig. 2. HPLC of standard (±)-bunolol with a mobile phase consisting of 98% 10 m*M* phosphate buffer–1 m*M* DMOA and 2.0% 2-propanol at (a) pH 5.0 ($\alpha = 1.12$; $R_S = 0.88$), (b) pH 6.0 ($\alpha = 1.17$; $R_S = 1.40$), (c) pH 6.5 ($\alpha = 1.18$; $R_S = 1.60$) and (d) pH 7.0 ($\alpha = 1.17$; $R_S = 1.58$).

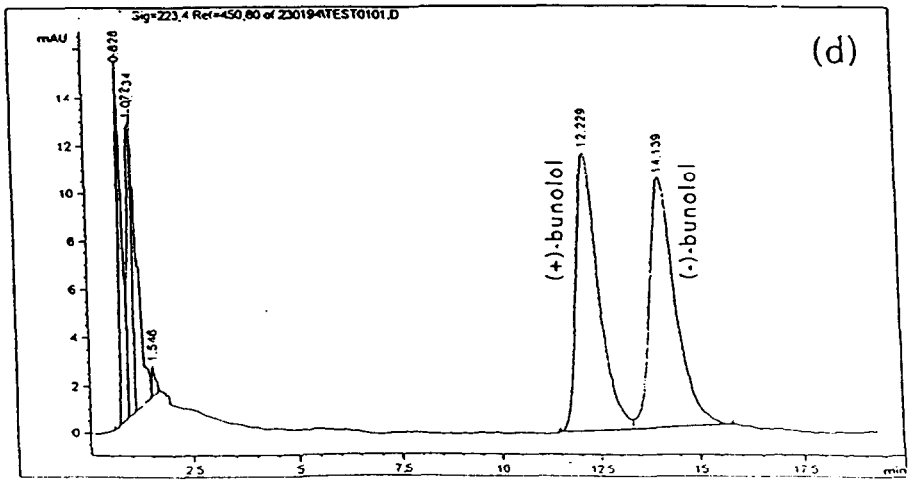


Fig. 2 (contd.)

termined by replicate analyses of (−)- and (+)-bunolol enantiomers after spiking urine samples with (±)-bunolol (at the same concentration as in the recovery experiment) on the day of preparation and on different days, respectively.

2.8. Enzymatic hydrolysis of conjugates in urine for chiral HPLC analysis

After extraction of aliquots of 5.0 ml of urine samples, as described in Section 2.4, remaining traces of ethyl acetate were removed from the aqueous phase by evaporation at 50°C under a stream of nitrogen. The pH of the aqueous phase was adjusted to pH 5.0 with 2 *M* HCl. Subsequently the samples were incubated with β-glucuronidase (3500 units) at 37°C for 18 h. The hydrolysate was basified to pH 11–12 with 1.2 ml of 2 *M* potassium carbonate and then 1.0 g of sodium chloride was added. The resulting samples were extracted twice with 8 ml of ethyl acetate. The organic layer was separated and dried with sodium sulfate. The organic solvent was evaporated under a stream of nitrogen at 50°C and the residue was reconstituted with 300 μl of methanol for HPLC analysis.

2.9. Derivatization procedure for GC–MS

The urine sample was extracted as described in Section 2.4 and 25 μl of pyridine, 25 μl of MSTFA and 2.5 μl of TMCS were added to the

Table 1
Recovery of (+)- and (−)-bunolol from human urine spiked with (±)-bunolol

Added (μg/ml)	Enantiomer	Intra-assay ($n = 3$)		Inter-assay ($n = 9$)	
		Recovery (mean ± S.D.) (%)	C.V. (%)	Recovery (mean ± S.D.) (%)	C.V. (%)
0.6	*d*	93.54 ± 1.97	2.10	91.79 ± 2.13	2.32
	l	91.94 ± 0.44	0.47	92.98 ± 1.04	1.12
1.2	*d*	93.71 ± 1.83	1.95	92.90 ± 1.42	1.53
	l	95.23 ± 1.09	1.14	93.53 ± 2.14	2.29
2.4	*d*	94.17 ± 0.73	0.77	94.19 ± 0.94	1.00
	l	94.58 ± 1.13	1.19	94.19 ± 0.92	0.98

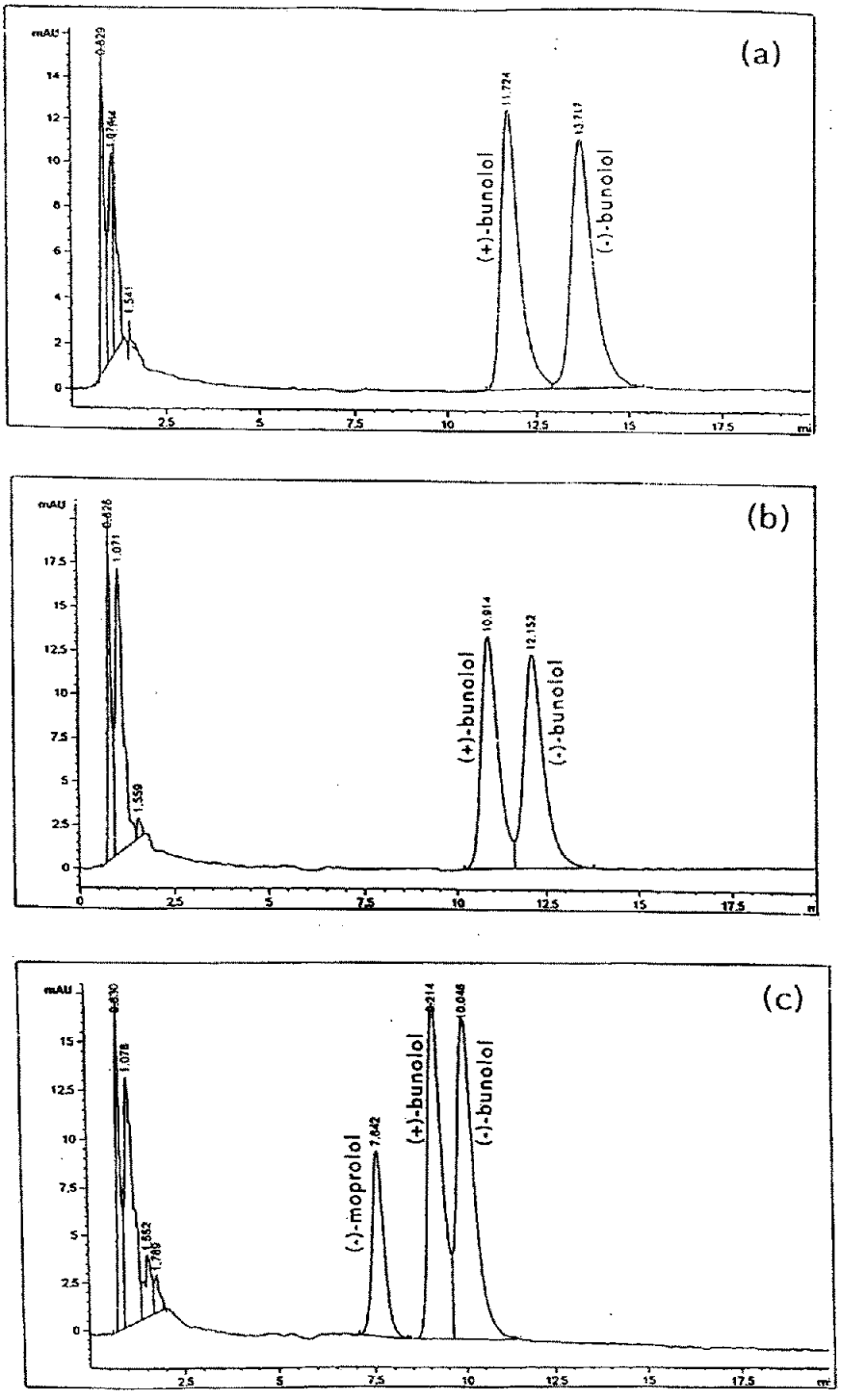


Fig. 3. HPLC of standard (±)-bunolol with a mobile phase consisting of 10 m*M* phosphate buffer (pH 7.0) and 2-propanol (98:2) with DMOA at concentrations of (a) 1, (b) 2 and (c) 3 m*M*.

dried extract. The solution was kept at 70°C for 30 min and then evaporated to dryness. The residue was reconstituted with 100 μl of hexane and 1 μl was injected for GC–MS analysis.

2.10. Gas chromatography–mass spectrometry

Identification of bunolol and its metabolites was carried out using an HP 5970 mass-selective detector (Hewlett-Packard) equipped with an HP 5890 gas chromatograph fitted with an HP-5 fused-silica capillary column (25 m × 0.2 mm I.D.; 0.33-μm film thickness). The injections were made in the splitless mode using helium as the carrier gas at a flow-rate of 0.8 ml/min. The oven temperature was programmed from 100°C to 15°C/min to 250°C (held for 1 min) and then at 1.5°C/min to 280°C (maintained for 5 min). The injector and the detector temperatures were 250 and 300°C, respectively. The mass spectrometer was operated in the full-scan mode for bunolol and its metabolites. Mass spectra of the MSTFA derivatives of standard bunolol and its metabolites of interest were recorded at a scanning speed of 1 s per decade over the mass range 50–550 u.

3. Results and discussion

3.1. Chiral separation of bunolol enantiomers

The separation of bunolol enantiomers was achieved by using a chiral α-AGP column. The standard racemic (±)-bunolol gave two peaks at retention times of 12.6 and 14.7 min when the pH of the mobile phase was 7.0 (Fig. 1a). The peaks of standard (+)-bunolol and (−)-bunolol appeared at 12.6 and 14.7 min, respectively. The retention time of (−)-moprolol was 11.4 min. No interfering peaks were visible in blank urine samples at the retention times of (±)-bunolol and the I.S. (−)-moprolol. Fig. 1b shows the chromatogram from an extract of blank human urine sample.

The chiral separation of enantiomers was very dependent on the pH of the mobile phase. Mobile phase pH values in the range 5.0–7.0 and a DMOA concentration of 1 m*M* gave different values of the separation (α) and resolution (R_S) factors (Fig. 2). Changes in the concentration of charged modifier DMOA (1–3 m*M*) in mobile phase can greatly influence the enantioselectivity and the retention time (Fig. 3).

With increasing number of analysed urine samples the retention time was shifted and the enantioselectivity decreased. This phenomenon has also been observed by other workers [5].

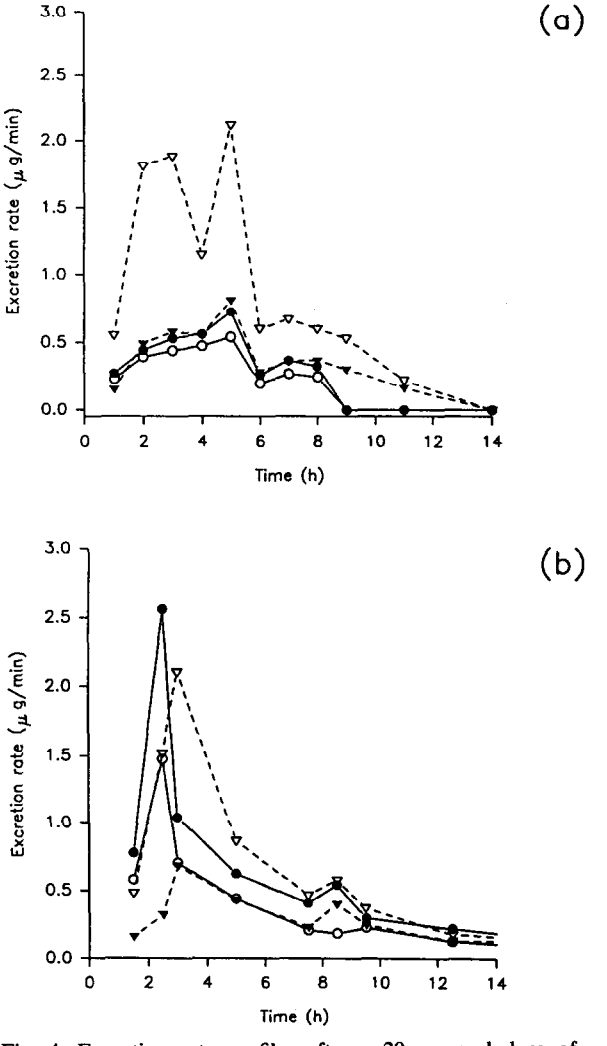


Fig. 4. Excretion rate profiles after a 20 mg oral dose of (±)-bunolol to (a) an older volunteer (57 years) and (b) a young volunteer (34 years). ○ = Unconjugated (+); ● = unconjugated (−); ▽ = conjugated (+); ▼ = conjugated (−).

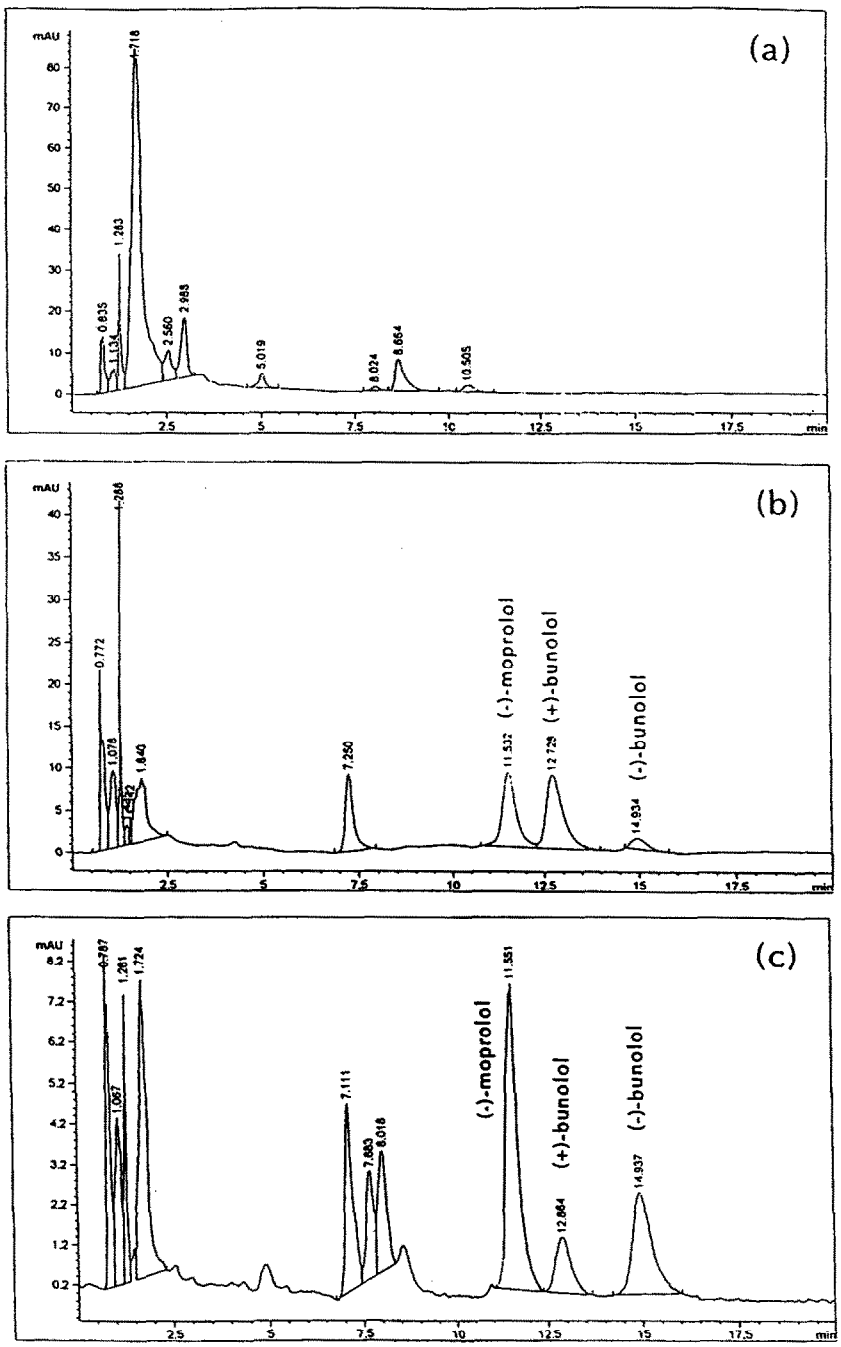


Fig. 5. HPLC of (a) blank, (b) conjugated bunolol and (c) unconjugated bunolol, 3 h after oral administration of (±)-bunolol to a young volunteer (34 years).

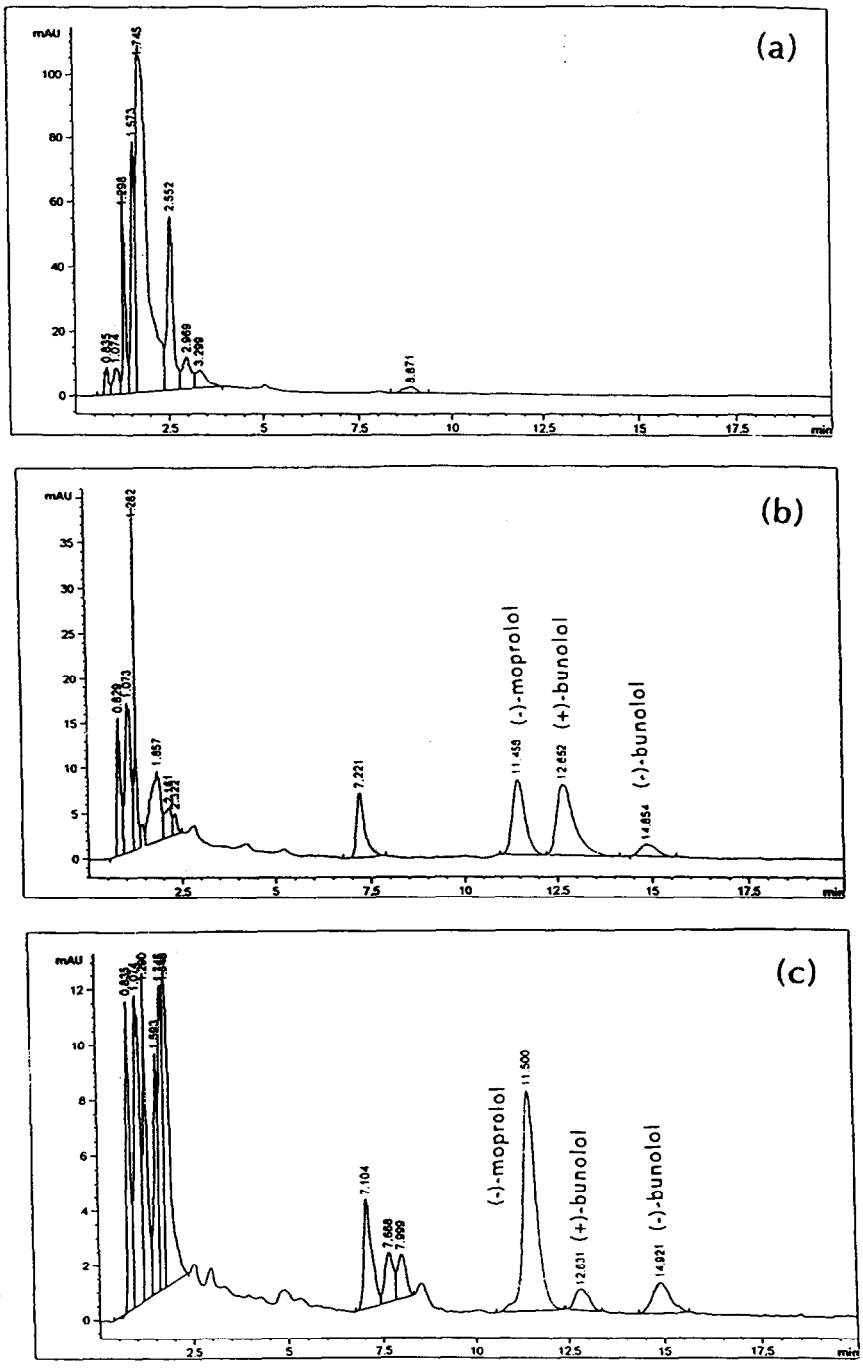


Fig. 6. HPLC of (a) blank (b) conjugated bunolol and (c) unconjugated bunolol, 3 h after oral administration of (±)-bunolol to an older volunteer (57 years).

3.2. Recovery and precision

The method was validated by determining the recoveries of bunolol enantiomers on spiking these compounds at different concentrations in blank urine samples, which were further processed as described in Section 2.4. The results for the reproducibility and recovery of (+)- and (−)-bunolol are given in Table 1. The overall coefficient of variation (C.V.) was below 5%. The calibration graphs were linear over the range 0.12–2.4 μg/ml for each enantiomer.

3.3. Stereoselective urinary excretion

Both conjugated and unconjugated excretion profiles were studied in two volunteers (aged 34 and 57 years). Bunolol enantiomers were first detected 1 h after oral administration of 20 mg of the racemic drug. The urinary excretion rate profiles of two volunteers are shown in Fig. 4.

In the older volunteer (Fig. 4a), a large amount of (+)-bunolol was excreted in the conjugated form. The conjugated (+)-bunolol had a much higher excretion rate than the (−)-antipode. There was no significant difference in the excretion rate between (+)- and (−)-enantiomers of unconjugated bunolol.

In the younger volunteer (Fig. 4b), conjugated (+)-bunolol had a much higher excretion rate than the conjugated (−)-antipode as in the older subject. In contrast to the excretion profiles of unconjugated (+)- and (−)-bunolol enantiomers in the older subject, the excretion rate of these antipodes was much higher in the younger volunteer.

The urinary excretion rates of bunolol enantiomers were maximum between 2 and 3 h. Renal clearance of bunolol enantiomers indicates stereospecificity. Both the younger and older volunteers demonstrated similar excretion patterns as far as the stereoselective conjugation of (+)- and (−)-bunolol is concerned. On the other hand, the urinary concentration of unconjugated (−)-bunolol was significantly higher in the younger volunteer, 3 h post-administration of the

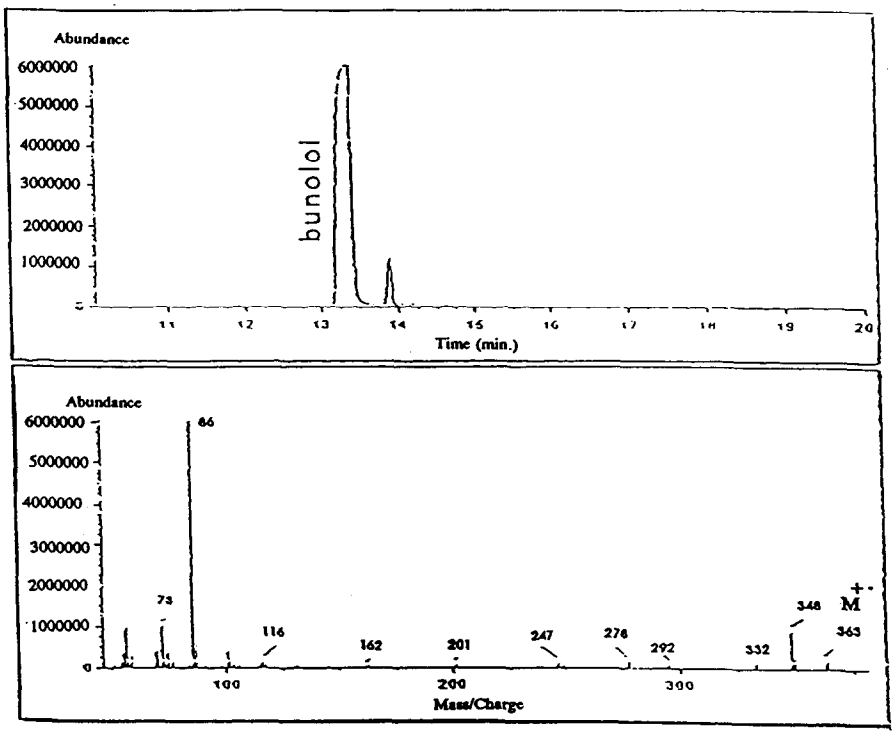


Fig. 7. GC–MS of standard (±)-bunolol derivatized with MSTFA.

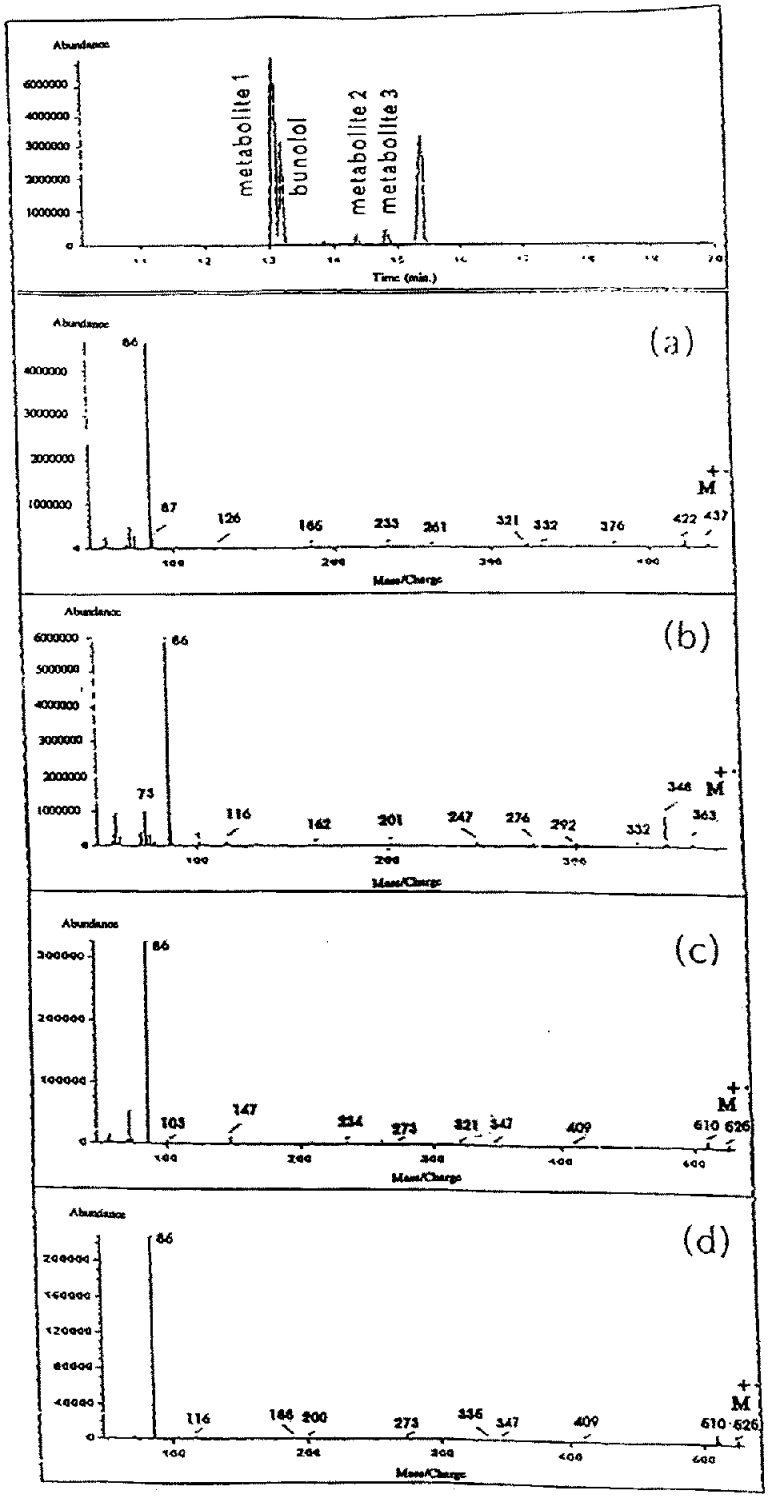


Fig. 8. GC–MS of a urine sample collected 5 h after oral administration of the drug. (a) metabolite 1; (b) bunolol (parent drug); (c) metabolite 2; (d) metabolite 3.

racemic parent drug (Figs. 5 and 6). The modification in the stereoselectivity of the urinary excretion pattern might be due to either inter-individual or age difference between the two subjects.

3.4. Identification of bunolol and its metabolites by GC–MS

Typical mass spectra obtained from GC–MS of the TMS derivatives of standard (±)-bunolol and urine specimens collected 5 h after administration of the racemic drug are shown in Figs. 7 and 8, respectively. The mass spectrum of standard bunolol indicates that the ion at m/z 363 is the molecular ion of the TMS derivative of bunolol. Other diagnostic ions at m/z 348 and 278 arise from β-cleavages. The base peak at m/z 86 is the typical ion of β-blockers which have a tertiary butylamine group (Fig. 9).

Metabolite 1

This compound was the most abundant metabolite detected in urine. By comparing the mass spectrum of its TMS derivative with that of standard bunolol (Fig. 8), the presence of the molecular ion at m/z 437 and other structurally diagnostic ions at m/z 422 and 86 were indicative of the existence of two hydroxyl groups. During the metabolism, the keto group was bio-

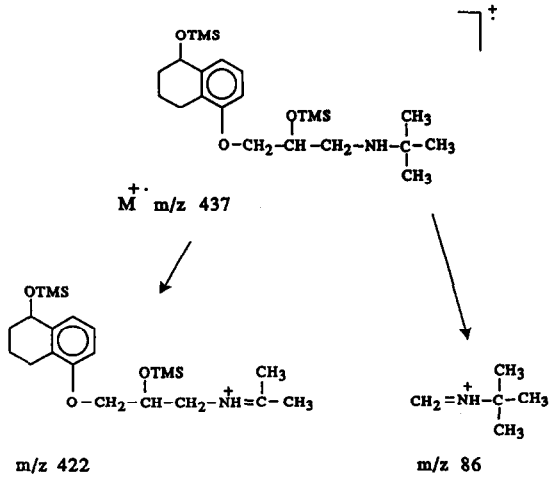


Fig. 10. Proposed fragmentation routes of metabolite 1.

transformed to a hydroxyl group (Fig. 10). Levobunolol was reported to be extensively metabolized in ocular tissues of rabbits via reduction of the cyclohexanone functional group to dihydrolevobunolol [6]. Hence dihydrobunolol, which results from the reduction of the keto group, is the major metabolite. The molecular ion at m/z 437 and ion fragments at m/z 422 and 86 are consistent with the proposed structure.

The possibility of stereospecificity of the biological reduction to dihydro-(−)-bunolol in vivo after administration of (−)-bunolol could not be

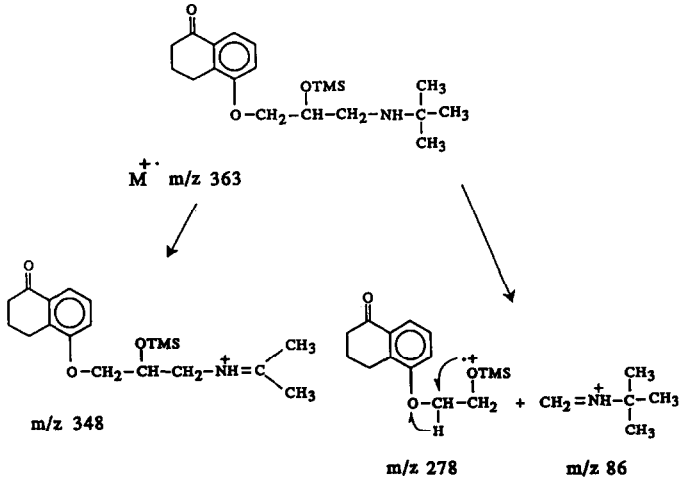


Fig. 9. Proposed fragmentation routes of standard bunolol.

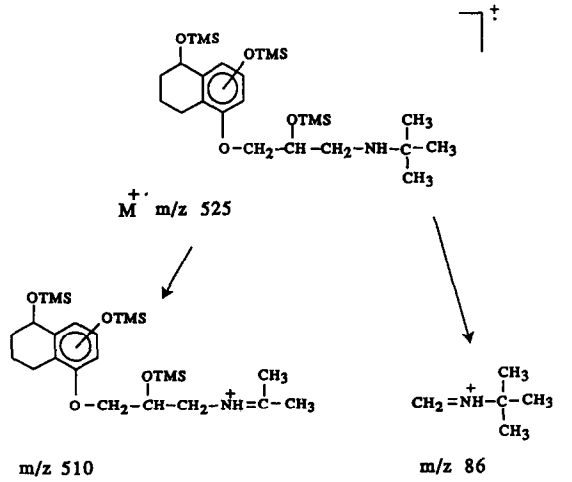


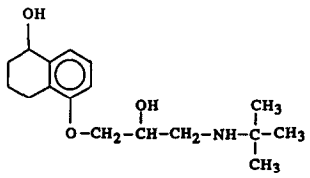
Fig. 11. Proposed fragmentation routes of metabolites 2 and 3.

established in this work, as reported by Watson and Midgley [4], because the authentic enantiomers of this metabolite were not available.

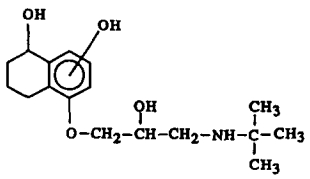
Metabolites 2 and 3

The mass spectra of metabolites 2 and 3 resemble each other, but they show different retention times (Fig. 8). The TMS derivatives of these minor metabolites showed identical molecular ions at *m/z* 525; the fragment ion at *m/z* 510 is the OTMS analogue of the ion at *m/z* 422 in the mass spectrum of bunolol metabolite 1. The molecular ion and fragment ions were shifted by 88 u to *m/z* 525 (*m/z* 437) and *m/z* 510 (*m/z* 422), respectively, with respect to those of metabolite 1 (Fig. 11). These data suggest that metabolites 2 and 3 have one hydroxyl group on the aromatic ring and are positioned isomers bearing hydroxyl groups at different positions (Fig. 12).

The metabolic studies of different xenobiotics have shown that an aromatic ring is more susceptible to hydroxylation than a saturated ring [7]. Further work will be done by isolating these unknown metabolites from urine samples by preparative HPLC and confirming their chemical structures by NMR and GC–MS analysis.



metabolite 1



metabolites 2 and 3

Fig. 12. Suggested structures of metabolites 1, 2 and 3.

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