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Determination of betaxolol and its metabolites in blood and urine by high-performance liquid chromatography with fluorimetric detection

Y. W J WONG and T M LUDDEN*

Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284 (U S A)

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ABSTRACT

Analytical methods for the determination of betaxolol and two of its metabolites in blood and urine are described Betaxolol, α-hydroxybetaxolol, and the acid metabolite were extracted, with over 65% efficiency, from biological samples by liquid–liquid extraction methods Analysis was performed using reversed-phase high-performance liquid chromatography with fluorimetric detection N,N-Dimethyloctylamine (0.005 *M*) was used to improve the chromatography of betaxolol and α-hydroxybetaxolol, while acetic acid (1%) was used for the acid metabolite. An excitation wavelength of 200 nm was found to produce the best detector response. Linear standard curves were obtained for al three compounds with detection limits (signal-to-noise ratio = 3) varying between 1 and 10 ng/ml The coefficients of variation of the determination for all three compounds in blood and urine varied between 3 0 and 8 7% The metabolism of betaxolol was studied in twelve healthy male subjects. The amounts (mean ± S.D) of betaxolol, α-hydroxybetaxolol and the acid metabolite renally excreted in the first 48 h after intravenous administration of 10 mg of betaxolol hydrochloride are 17.1 ± 6 2, 0 4 ± 0.1 and 14.5 ± 3.7%, respectively, of the administered dose

INTRODUCTION

Betaxolol hydrochloride, (±)-2-propanol-1-[4-[2-(cyclopropylmethoxy)ethyl]-phenoxy]-3-[(1-methylethyl)amino] hydrochloride (I), is a cardioselective β-adrenergic antagonist effective in the treatment of hypertension. In contrast to other lipophilic β-blockers such as propranolol and metoprolol which undergo extensive first-pass metabolism in man, betaxolol exhibits excellent bioavailability and a long terminal half-life of 16–22 h [1]. Renal elimination represents a minor route of disposition of betaxolol; only about 18% of the adsorbed dose is excreted unchanged in the urine, the rest being oxidatively metabolized, presumably hepatically, yielding inactive or weakly active metabolites [2].

The metabolic pathways of betaxolol in man are shown in Fig. 1. The two major metabolic routes produce inactive metabolites by: (a) elimination of the cyclopropylmethyl group with subsequent oxidation to the amino acid (IV) and

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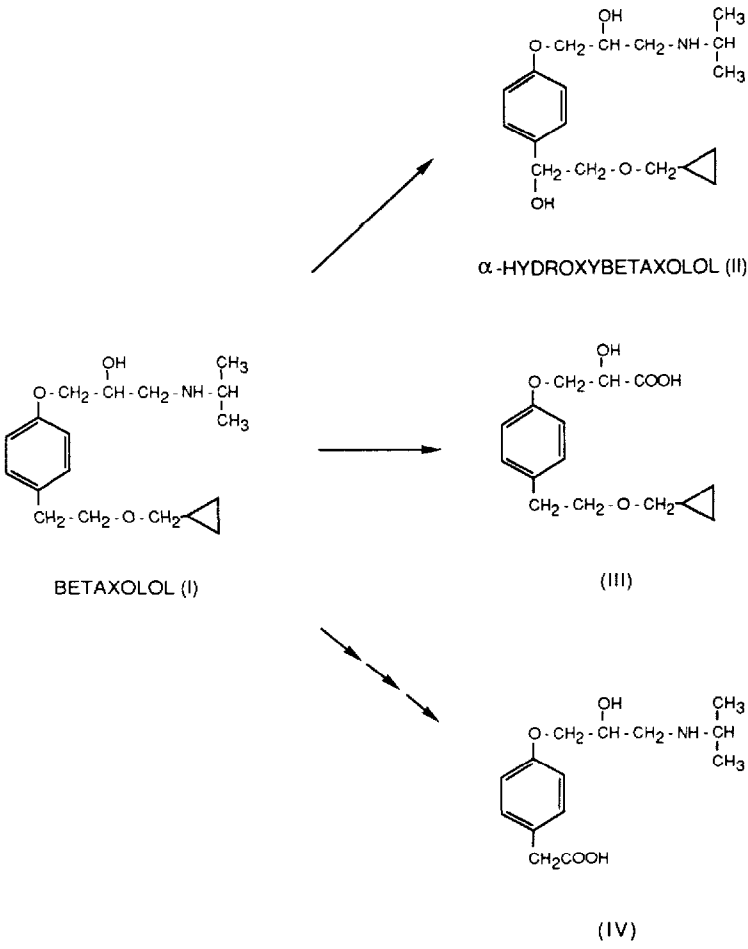


Fig 1 Major pathways of betaxolol metabolism in man

(b) oxidative deamination to form the acid metabolite (III). An active metabolite, α-hydroxybetaxolol (II), is also produced in small amount by hydroxylation of the carbon α to the benzene ring.

Analytical methods for betaxolol in biological samples have largely been based on gas chromatography (GC) with electron-capture detection (ECD) of the heptafluorobutyrated derivative [3,4]. Several high-performance liquid chromatographic (HPLC) methods [5–7] and a gas chromatographic–mass spectrometric (GC–MS) assay have also been published [8]. However, sensitive methods for the determination of certain of its metabolites are not available. Methods for the analysis of the zwitterionic metabolite (IV), which is also the major metabolite of metoprolol, has been described by Lennard [9] and Balmér *et al.* [10]. Although the acid metabolite (III) may be assayed by GC–MS, the method involves a

laborious double-derivatization procedure and has been reported to produce unpredictable results due to ring opening of the cyclopropyl group during derivatization resulting in the production of double peaks [8].

In this paper, sensitive methods are described for the analysis of betaxolol (I), α-hydroxybetaxolol (II) and the acid metabolite (III) in blood and urine using HPLC with direct fluorimetric detection.

EXPERIMENTAL

Reagents and chemicals

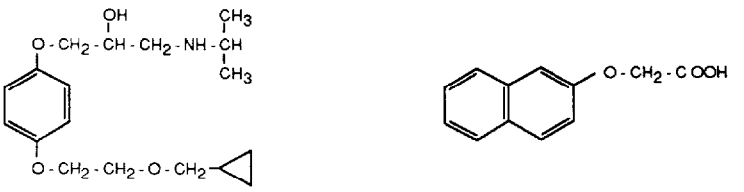
Betaxolol hydrochloride (I), α-hydroxybetaxolol (II), acid metabolite (III) and the internal standard, cicloprolol hydrochloride (V, Fig. 2), were provided by Lorex Pharmaceuticals (Skokie, IL, U.S.A.). (2-Naphthoxy)acetic acid (VI, Fig 2) and N,N-dimethyloctylamine (DMOA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile for HPLC was HPLC grade (OmniSolv, MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.). Methylene chloride (HPLC grade) was from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Diethyl ether was freshly distilled from copper turnings and potassium hydroxide pellets before use. All other reagents were of analytical-grade purity. 0.05 *M* DMOA (pH 3.0) was prepared by suspending 4.14 g of DMOA in 450 ml of distilled water with stirring. Phosphoric acid (85%) was added to the mixture dropwise until the pH was 3.0. The resulting solution was made up to 500 ml with distilled water. 0.005 *M* DMOA was prepared by mixing 1 ml of 0.05 *M* DMOA (pH 3.0) with 9 ml of water.

Standard solutions

Stock solutions (1 mg/ml) of betaxolol (I), α-hydroxybetaxolol (II), betaxolol acid (III) and the internal standards, cicloprolol hydrochloride (V) and (2-naphthoxy)acetic acid (VI), were prepared in methanol. These solutions were stored at 4°C until use.

HPLC instrumentation

The chromatograph comprised a Waters Model 600A pump (Milford, MA,



CICLOPROLOL (V) (2-NAPHTHOXY)ACETIC ACID (VI)

Fig 2 Structures of the internal standards cicloprolol (V) and (2-naphthoxy)acetic acid (VI).

U S.A.), a Rheodyne 7120 injector (Berkeley, CA, U.S.A.), fitted with a 200-μl loop, and a Schoeffel Model FS 970 fluorimetric detector (Kratos, Westwood, NJ, U.S.A.). Chromatographic peaks were monitored at an excitation wavelength of 200 nm with a No. 280 emission filter and recorded on a Hewlett-Packard Model 3390A integrator (Houston, TX, U S.A.).

Column packings and mobile phases

Method A: betaxolol (I) and α-hydroxybetaxolol (II). The mobile phase comprised acetonitrile–0.05 *M* DMOA (pH 3.0)–water (8.10:82). A Beckman Ultrasphere C_{18} (3 μm, 7.5 cm × 0.45 cm I.D.) column was used as the analytical column for blood samples. For urine samples, a Beckman Ultrasphere C_{18} (5 μm, 15 cm × 0.45 cm I.D.) was used. The flow-rate of the mobile phase for each chromatographic system was 1.0 ml/min.

Methods B and C: acid metabolite (III). The mobile phase was acetonitrile–glacial acetic acid–distilled water (29:1:70) eluting the HPLC column at a flow-rate of 0.9 ml/min. A Waters Nova-Pak C_{18} (5 μm, 15 cm × 0.39 cm I.D.) was used as the analytical column for both blood and urine samples.

Sample preparation

All blood and urine samples were stored at −20°C. Each sample was allowed to thaw at room temperature and mixed on a whirl-mixer for 30 s before an aliquot was removed.

Method A: betaxolol (I) and α-hydroxybetaxolol (II) in blood and urine. The blood sample (1.0 ml) was mixed with the internal standard [cicloprolol hydrochloride (V), 20 ng in 100 μl distilled water], distilled water (1.0 ml) and 1.0 *M* sodium hydroxide (200 μl) in a screw-capped silanized culture tube by vortex-mixing. The mixture was then extracted with diethyl ether (10 ml) by mixing for 30 min on a mechanical shaker After separation of phases by centrifugation (1400 *g* for 15 min), the organic layer was transferred to another tube and then back-extracted with 0.05 *M* hydrochloric acid (2.0 ml) by mixing for 10 min. The organic phase was discarded after phase separation. The aqueous phase was mixed with 1.0 *M* sodium hydroxide (200 μl) and then extracted with diethyl ether (10 ml) by mixing for 10 min. The organic layer was transferred to another culture tube and evaporated to total dryness under dry nitrogen at 40–50°C. The residue was reconstituted with 0.005 *M* DMOA (100 μl), and 90 μl were injected into the chromatograph. Calibration plots were constructed with blood samples supplemented with known amounts of betaxolol (5–60 ng/ml) and α-hydroxybetaxolol (3–20 ng/ml).

The same extraction protocol was used for urine samples except that 90 ng of cicloprolol hydrochloride (in 90 μl of distilled water) was used as the internal standard. The residue was reconstituted with 0.005 *M* DMOA (100 μl) and 20–90 μl were injected into the chromatograph. Calibration plots were constructed with urine samples supplemented with betaxolol (50–200 ng/ml) and α-hydroxybetaxolol (10–50 ng/ml).

Method B: acid metabolite (III) in blood. The blood sample (1.0 ml) was mixed with the internal standard [(2-naphthoxy)acetic acid, 10 ng in 100 μl of 0.01 *M* sodium hydroxide], distilled water (2.0 ml) and 5.0 *M* hydrochloric acid (150 μl) in a screw-capped silanized culture tube by vortex-mixing. The mixture was extracted with diethyl ether (10 ml) by mixing for 15 min on a mechanical shaker. After phase separation, the organic layer was transferred to another tube containing 0.1 *M* sodium phosphate buffer (2.0 ml, pH 7.0). The mixture was shaken for 30 min on a mechanical shaker. After separation of phases by centrifugation (1400 *g*, 10 min), the organic layer was discarded. The aqueous phase was acidified with 1 0 *M* phosphoric acid (1.0 ml) and then extracted with methylene chloride (10 ml) by mixing for 15 min. The aqueous phase was discarded and the organic layer was washed with 0.1 *M* phosphoric acid (2.0 ml). The organic layer was then transferred to a clean screw-capped culture tube and evaporated to total dryness under nitrogen at 40–50°C. The residue was reconstituted with 15% acetonitrile in 1% acetic acid (100 μl), and 90 μl were injected into the chromatograph. Calibration plot was constructed with blood samples supplemented with the metabolite (10–50 ng/ml).

Method C: acid metabolite (III) in urine. The urine sample (0.5 ml) was mixed with the internal standard [(2-naphthoxy)acetic acid, 60 ng in 60 μl of 0.01 *M* sodium hydroxide] and 0.1 *M* phosphoric acid (2.0 ml) in a screw-capped silanized culture tube by vortex-mixing. The mixture was extracted with methylene chloride (10 ml) by mixing on a mechanical shaker for 15 min. The aqueous layer was discarded after phase separation by centrifugation for 5 min (1400 *g*). The organic layer was further washed with 0.1 *M* phosphoric acid (2.0 ml) by mixing for 5 min. The organic layer was transferred to another tube and evaporated under nitrogen to total dryness at 40–50°C. The residue was reconstituted with the mobile phase (50 μl), and 10 μl were injected in the chromatograph. Calibration plot was constructed with urine samples supplemented with metabolite (100.0–1000.0 ng/ml).

Metabolism of betaxolol in man

The metabolism of betaxolol in man was studied in twelve healthy adult male subjects [1]. Each subject received intravenously 10 mg of betaxolol hydrochloride over a period of 30 min. Urine was collected over the following intervals: 0–3, 3–6, 6–12, 12–24 and 24–48 h. The urine samples for an individual subject were pooled in proportion to each original voided volume so as to provide a single sample for each subject.

RESULTS AND DISCUSSION

Extraction procedures

Betaxolol (I) and α-hydroxybetaxolol (II) were efficiently extracted with diethyl ether from alkalinized blood and urine. After back-extraction into

hydrochloric acid (0.05 *M*) and further extraction into diethyl ether, both compounds were analyzed by HPLC. Although our experience with the GC–ECD assay suggests the advantage of an additional clean-up step with Chem Elut tube for the analysis of blood samples, this was found to be unnecessary for the present assay.

Metabolite III, a carboxylic acid, was extracted from acidified urine with methylene chloride prior to HPLC analysis. For the analysis of blood samples, an additional back-extraction step with pH 7.0 phosphate buffer (0.1 *M*) was necessary to produce chromatograms free from interference.

The absolute recoveries of betaxolol (I), α-hydroxybetaxolol (II) and metabolite III from blood, measured by comparison with direct injection of the compounds in mobile phase, were 80, 74 and 68%, respectively, within the calibration range.

Chromatographic procedures

Due to the presence of residual basic and acidic silanol groups on silica-based bonded-phase packings such as the C_{18} phase, a major problem in the analysis of basic and acidic compounds by HPLC is peak tailing [11] The result is a severe reduction in sensitivity in the analysis of these compounds. Most efforts to suppress peak tailing are aimed at minimizing these silanol interactions. While deactivated columns have been made available in recent years, the most commonly employed method is to pre-saturate these free silanol groups with suitable modifiers in the mobile phase.

Triethylamine has been used previously as a mobile phase additive for the chromatography of betaxolol and other β-blockers [6]. Our investigations showed DMOA to be superior for betaxolol (I) and α-hydroxybetaxolol (II). The application of DMOA for the analysis of β-adrenergic drugs have been recently reviewed [12]. Similarly, the chromatography of the acid metabolite (III), which contains both a carboxyl and a hydroxyl group, was substantially improved with 1% acetic acid in the mobile phase.

Fluorimetric detection

Betaxolol, like other β-blocking agents such as metoprolol, produces an intense fluorescence at pH 3.0. The optimum wavelength of excitation appears to be detector-dependent. Several excitation wavelengths, including 198, 220 and 275 nm, have been used for betaxolol and metoprolol [3,4,9,10]. Fig. 3 shows that the detector used for the present assay produced the best detector response for betaxolol when an excitation wavelength of 200 nm was used.

Analysis of betaxolol and its metabolites in biological fluids

In method A (blood) the retention times for α-hydroxybetaxolol (II), ciclopro-lol (V) and betaxolol (I) were 2.3, 6.9 and 9.9 min, respectively. Fig. 4 shows a chromatogram of a blood sample obtained from an individual 24 h after the last

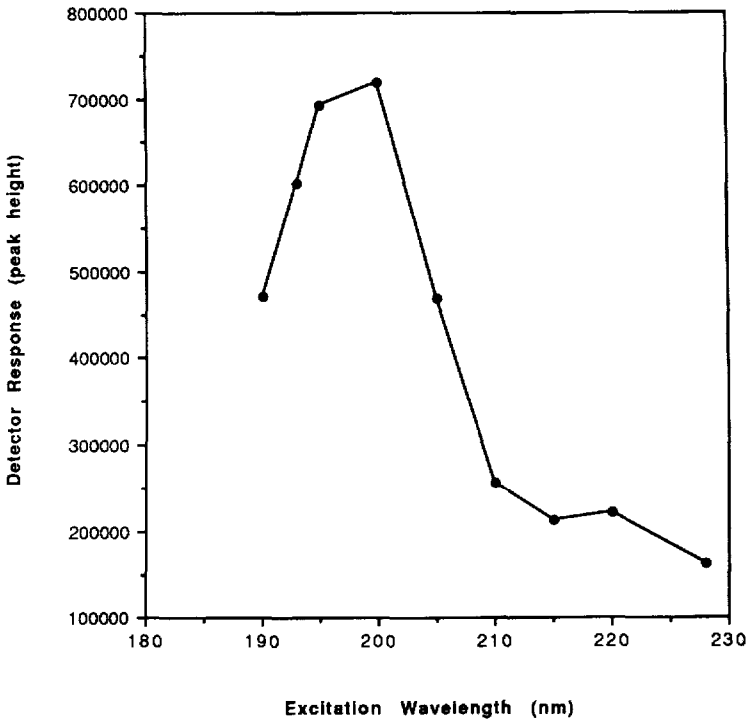


Fig 3. Detector response at different excitation wavelengths. The detector response was taken as the peak height as recorded on the integrator The results were obtained by direct injection of 10 ng of betaxolol hydrochloride into the HPLC system

dose of an oral regimen of 20 mg of betaxolol per day for seven days. Although the lowest calibration points for I and II in blood were 5 and 3 ng/ml, respectively, the limits of detection were about 1 ng/ml for both compounds for a signal-to-noise ratio of 3. A longer column was employed for urine samples to avoid interfering peaks observed occasionally. This resulted in an anticipated increase in retention time and a reduction in assay sensitivity. A typical chromatogram is shown in Fig. 5. Limits of detection for I and II in urine were about 10 and 5 ng/ml, respectively.

Figs. 6 and 7 show chromatograms resulting from the analysis of the acid metabolite (III) in blood and urine (methods B and C). The retention times were 7.4 and 12.0 min for III and VI (internal standard), respectively. The sensitivity of the assay for III was 5 ng/ml in blood and 20 ng/ml of urine. Assay precision was examined with standard blood and urine samples supplemented with known amounts of betaxolol and its metabolites. The results are shown in Table I.

Although the GC–ECD assay has been the preferred method for betaxolol in biological fluids, preliminary investigations revealed that it is not suitable for α-hydroxybetaxolol (II) because of the production of multiple chromatographic

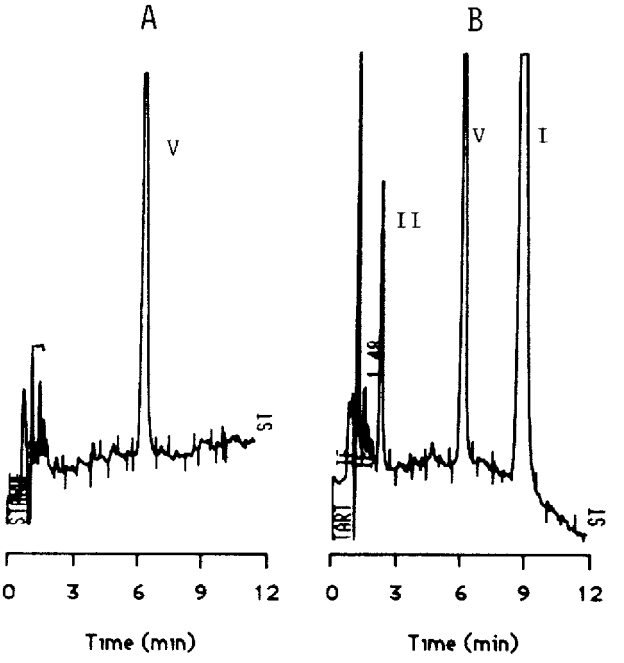


Fig. 4. Chromatograms of (A) 1 ml of blank blood and (B) 1 ml of blood containing 6.6 ng of α-hydroxybetaxolol (II) and 45.4 ng of betaxolol (I) The sample was obtained from an individual 24 h after the last dose of an oral regimen of 20 mg of betaxolol hydrochloride per day V = cicloprolol (internal standard) The chromatograms were generated using method A (see Experimental).

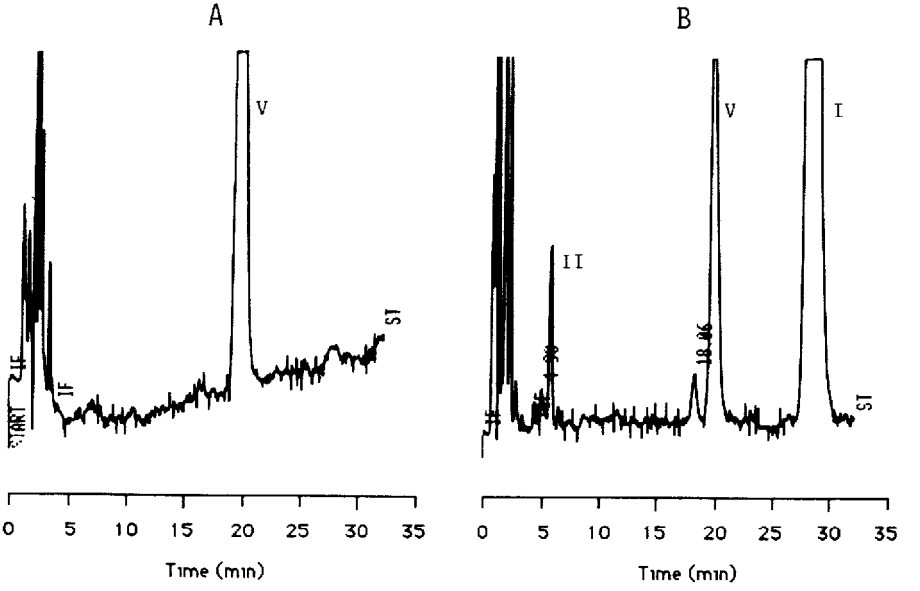


Fig 5 Chromatograms of (A) 1 ml of blank urine and (B) 1 ml of urine containing 14.8 ng of α-hydroxybetaxolol (II) and 514.4 ng of betaxolol (I). This sample was obtained by pooling all urine samples collected from an individual within a study period of 48 h after receiving 10 mg of betaxolol hydrochloride intravenously. V = cicloprolol (internal standard). The chromatograms were generated using method A (see Experimental) The injected volumes were 80 μl for (A) and 25 μl for (B)

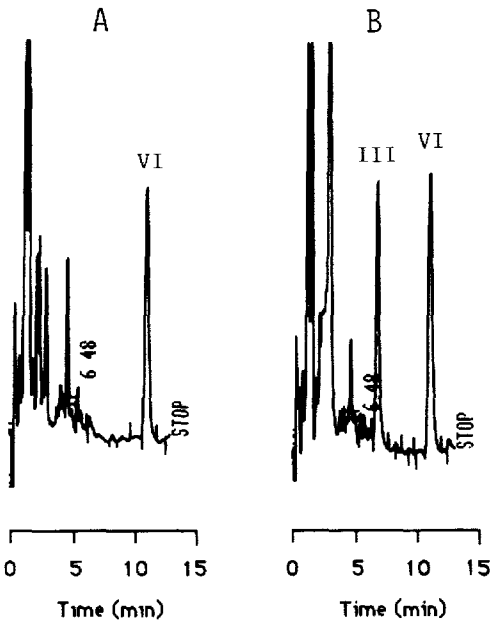


Fig. 6 Chromatograms of (A) 1 ml of blank blood and (B) 1 ml of blood containing 24.6 ng of acid metabolite (III) The sample was obtained from an individual 4 h after the last dose of an oral regimen of 20 mg of betaxolol hydrochloride per day for seven days VI = (2-naphthoxy)acetic acid (internal standard). The chromatograms were generated using method B (see Experimental)

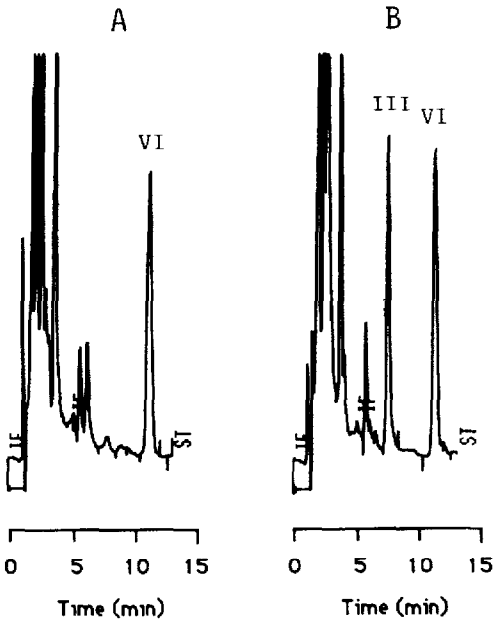


Fig 7 Chromatograms of (A) 0.5 ml of blank urine and (B) 0.5 ml of urine containing 176 5 ng of acid metabolite (III). This sample was obtained by pooling all urine samples collected from an individual within a study period of 48 h after receiving 10 mg of betaxolol hydrochloride intravenously VI = (2-naphthoxy)-acetic acid (internal standard) The chromatograms were generated using method C (see Experimental)

TABLE I

CALIBRATION AND PRECISION DATA FOR THE MEASUREMENT OF BETAXOLOL (I), α-HYDROXYBETAXOLOL (II) AND ACID METABOLITE (III) IN BLOOD AND URINE

Drug/ metabolite	Calibration range (ng/ml)	Linearity (r^2)	Coefficient of variation (%)	n	Concentration[a] (ng/ml)	Limit of detection[b] (ng/ml)
Blood						
I	5.0–60.0	> 0.99	3.7	12	12.0	1.0
			4.0	12	40.0	
II	3.0–20.0	> 0.99	6.9	12	7.0	1.0
			5.7	12	18.0	
III	10.0–50.0	> 0.99	8.7	10	25.0	5.0
Urine						
I	50.0–200.0	> 0.99	4.0	8	175.0	10.0
			3.8	8	60.0	
II	10.0–50.0	> 0.99	3.2	8	45.0	5.0
			6.3	8	15.0	
III	100.0–1000.0	> 0.99	3.0	10	800.0	10.0
			3.9	10	200.0	

[a] Concentration at which the coefficient of variation was performed

[b] Taken at signal-to-noise ratio of 3.

peaks. The presence of an additional hydroxyl group on the molecule might have led to incomplete derivatization with heptafluorobutyric anhydride. Other published HPLC assays for betaxolol have not been applied for the analysis of its metabolites [5–7]. In addition, two such methods were not designed for betaxolol concentrations below 10 ng/ml [5,6]. The method of Darmon and Thenot [7] requires derivatization of betaxolol with a fluorescent agent to achieve the required sensitivity. The present HPLC methods make use of the native fluorescence of betaxolol and its metabolite molecules. Comparison of this and the GC–ECD method for betaxolol in the blood concentration range 5–60 ng/ml indicated excellent correlation (Fig. 8). Although the HPLC method provides a simpler approach to the analysis of betaxolol, our experience with this assay suggested that it is less selective than the GC–ECD method; analysis of blood samples from patients receiving other drugs revealed that interfering peaks were more likely to occur with the HPLC assay than with the GC–ECD method.

Metabolism of betaxolol in man

Studies performed in healthy volunteers with [^{14}C]betaxolol have shown that 16% of a 20-mg oral dose is eliminated unchanged in urine in seven days. The two

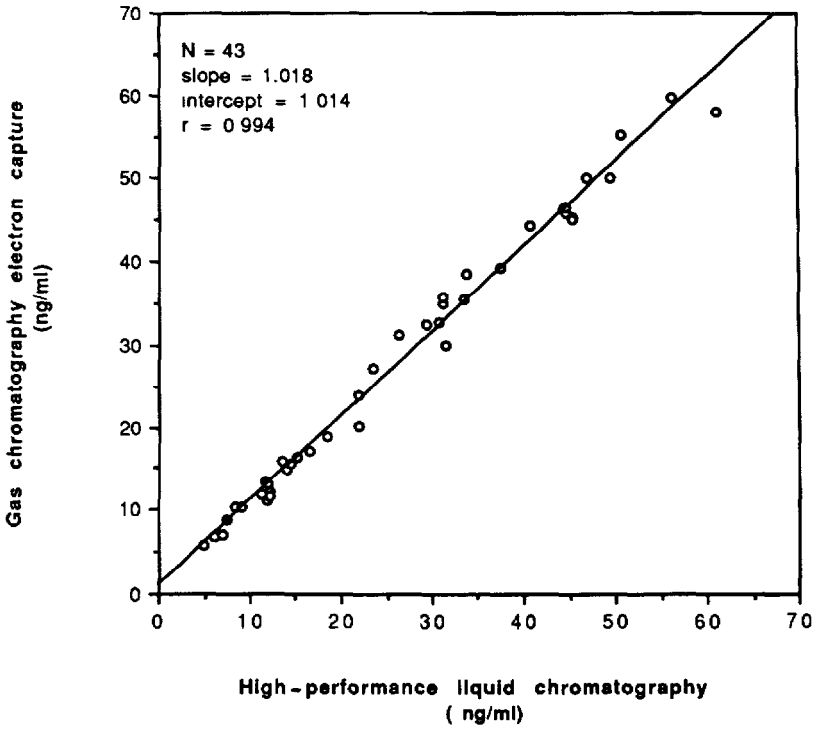


Fig 8. Correlation curve between the concentrations of betaxolol in subject blood samples measured by GC–ECD and those measured by HPLC

metabolites, α-hydroxybetaxolol (II) and the acid metabolite (III), account for about 1 and 35% of the administered dose, respectively [2].

In the present study, the metabolism of betaxolol was investigated in twelve healthy male subjects. The amounts (mean ± S.D.) of betaxolol (I), α-hydroxybetaxolol (II) and the acid metabolite (III) renally excreted in the first 48 h after intravenous administration of 10 mg of betaxolol hydrochloride were 17.1 ± 6.2, 0.4 ± 0.1 and 14.5 ± 3.7%, respectively, of the administered dose. The discrepancy in the results for III obtained in the two studies might be attributable to the route of administration, the sampling duration or the analytical methods used.

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