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DETERMINATION OF THE NEW β-BLOCKER BISOPROLOL AND OF METOPROLOL, ATENOLOL AND PROPRANOLOL IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatographic methods using fluorimetric detection have been developed for the determination in plasma and urine of bisoprolol, atenolol, metoprolol and propranolol. Bisoprolol, metoprolol and propranolol were extracted at alkaline pH with dichloromethane, atenolol with *n*-butanol—dichloromethane (25:75). After evaporation of the organic solvent the compounds were chromatographed on silica gel Si-60 columns (normal phase) using aqueous ammonium phosphate buffer (pH 4) containing 3—7% acetonitrile as eluent (method 1). Alternatively, the compounds were acetylated prior to chromatography on reversed-phase columns (RP-8), using acetonitrile—water mixtures as eluents (method 2). The detection limit was 1—2 ng/ml in plasma and 10 ng/ml in urine for bisoprolol and metoprolol with either method. For atenolol the detection limit was 5 ng/ml in plasma or 50 ng/ml in urine (method 1), for propranolol 1 ng/ml in plasma (method 2).

INTRODUCTION

Bisoprolol is a new β-adrenergic receptor antagonist, showing a high β_1-selectivity [1, 2]. To investigate the pharmacokinetic properties of bisoprolol and, in comparative studies, of metoprolol, atenolol and propranolol, sensitive methods for the determination of these compounds in human plasma were needed. Numerous methods for the determination of metoprolol, propranolol and atenolol using gas chromatography (GC) and high-performance liquid chromatography (HPLC) on reversed-phase columns have been described (for a review see ref. 3). Chromatographic separation of β-blocking agents on normal-phase Si-60 columns was only recently suggested by Bidlingmeyer et al. [4]. In our studies we found that chromatography on Si-60 columns with "reversed-phase" mobile phases, i.e. mobile phase containing only a few percent of

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organic modifiers, was well suited for the determination of bisoprolol, metoprolol and atenolol. In this report we describe the analytical procedures.

EXPERIMENTAL

Reagents

Bisoprolol, metoprolol, atenolol, propranolol, [4-(2-hydroxy-3-isopropylaminopropoxy)benzyloxy]acetic acid (metabolite I) and 1-[*p*-(tetrahydro-3-furanyl)phenoxy]-3-isopropylamino-2-propanol hemifumarate (II) as well as HPLC-grade acetonitrile and all other chemicals and solvents (analytical grade) were obtained from E. Merck (Darmstadt, F.R.G.). The structures of these β-blocking agents and of the main metabolite (I) of bisoprolol are given in Fig. 1.

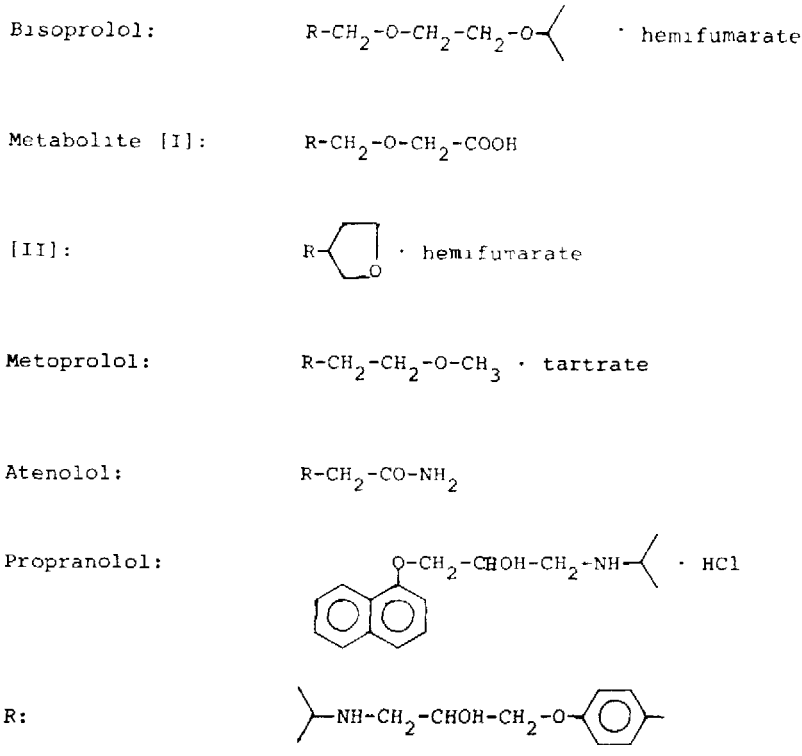

Fig. 1. Structure of the β-blockers investigated, of the internal standard (II) and the main metabolite (I) of bisoprolol.

Internal standard and reference solutions

As internal standard we used II for the determination of bisoprolol, bisoprolol for the determination of metoprolol and propranolol, and metoprolol for the determination of atenolol. Stock solutions of these compounds in distilled water (1 mg/ml) were stable at −20°C for at least two months. Appropriate dilutions were made when needed.

Sample preparation

Blood samples were taken by venipuncture into lithium heparin tubes and plasma was separated by centrifugation. Plasma and urine samples were stored at −20°C until analysis. Aliquots (1 ml) of plasma (0.5 ml in the case of atenolol and urine) were placed in glass tubes which could be closed with PTFE-lined screw caps. Prior to sample work-up the glass tubes were washed with dichloromethane. These samples were shaken for 15 min after addition of an appropriate amount of internal standard, dissolved in 20 μl of distilled water, 0.1 ml of 1 *M* sodium hydroxide and 5 ml of dichloromethane (dichloromethane—butanol, 75:25, in the case of atenolol). After centrifugation the organic phase was transferred and evaporated to dryness in a stream of nitrogen. Lipophilic contaminants were removed as follows: The residue was dissolved in 0.2 ml of 1 *M* acetic acid, 5 ml of hexane were added and the sample was shaken for 5 min. The organic phase was discarded and the water phase evaporated to dryness in a stream of nitrogen. In the case of propranolol, which is considerably more lipophilic than the other compounds studied, this washing step was omitted, since even at acidic pH small amounts of propranolol were extracted with hexane. For chromatography on normal-phase Si-60 columns the residue was taken up in 30—500 μl of the appropriate mobile phase. For derivatization with acetic anhydride the residue was dissolved in 0.2 ml of acetic acid—acetic anhydride (1:2) and heated to 60°C for 2 h. Excess reagent was evaporated with a stream of nitrogen and the residue dissolved in 30—500 μl of the appropriate mobile phase.

Chromatography

The following equipment was used: 655-A11 HPLC pump, F1000 fluorescence detector, 833 A integrator (all from Merck) and a six-port injection valve (Rheodyne), equipped with a 20-μl sample loop.

For the determination of bisoprolol, metoprolol and atenolol the fluorescence detector was operated at an excitation wavelength of 225 nm and an emission wavelength of 300 nm. For the determination of propranolol an emission wavelength of 320 nm was used. It should be noted that the emission maximum of propranolol is 340 nm. Since no suitable internal standard was available at the time of these investigations, bisoprolol was used and an emission wavelength chosen at which both compounds showed sufficient fluorescence.

For the chromatographic separation of underivatized bisoprolol, metoprolol and atenolol, LiChrosorb Si-60 columns (7 μm, 125 × 4 mm I.D., Merck) and the following mobile phases were used: (A) for detection of bisoprolol and metoprolol, acetonitrile—distilled water—1 *M* ammonium phosphate buffer (pH 4) (7:88:5); (B) for determination of atenolol, acetonitrile—distilled water—1 *M* ammonium phosphate buffer (pH 4) (6:89:5 for plasma samples and 3:92:5 for urine samples).

Prior to use, the Si-60 columns were conditioned by pumping ca. 50 column volumes of methanol and 50 column volumes of the appropriate mobile phase through the column.

For separation of the acetylated derivatives LiChrosorb RP-8 columns (7 μm, 125 × 4 mm I.D., Merck) and the following mobile phases were used:

(A) for determination of bisoprolol and metoprolol, acetonitrile—distilled water (50:50); (B) for determination of propranolol, acetonitrile—distilled water (60:40).

The columns were operated at ambient temperature and at a flow-rate of 1.8 ml/min (50—60 bar).

Calibration

Calibration standards were prepared by adding 20 μl of calibration solution containing known amounts of the respective β-blocker and internal standard in distilled water to 1 ml of plasma. The equation of the regression lines was calculated as $y = Ax + B$ or as $\log y = A \log x + B$, where x is the peak height ratio multiplied by the amount of internal standard (ng/ml) and y the concentration of the respective β-blocker. Using the logarithms of y and x has the advantage that the relative rather than the absolute deviation from the theoretical value is considered.

RESULTS AND DISCUSSION

Stability of bisoprolol in plasma and urine

Plasma samples containing 50 ng of bisoprolol were stored at −20°C and analysed after various time intervals. No decrease of bisoprolol concentration was observed during storage of up to one year. Also after incubation of plasma samples for 24 h at 37°C no degradation of bisoprolol was observed.

Chromatography

The chromatographic properties of bisoprolol, metoprolol, atenolol and propranolol were studied on normal-phase Si-60 columns and after acetylation on reversed-phase RP-8 columns. At the start of the development of bisoprolol we studied the chromatographic properties of this compound without derivatization on RP-8 columns. However, these were found to be rather poor. Addition of an ion-pairing agent (dodecane-1-sulphonic acid) considerably improved the peak symmetry, but the stability of the column was not optimal, and late-eluting contaminants from plasma made long analysis times necessary.

Derivatization with acetic anhydride results in the acetylation of the secondary amine as well as the secondary hydroxy function, according to the mass spectra of the acetylated compounds. These acetyl derivatives showed good chromatographic properties, and the stability of the column using acetonitrile—distilled water as the mobile phase was excellent. At 60°C the derivatization is complete after 1 h and at longer incubation times (8 h) no degradation of bisoprolol or the internal standard was observed. At 80°C the reaction of bisoprolol proceeds considerably faster, but small amounts of degradation products were observed after only 2 h and the amount of these products increased with time. An incubation time of 2 h at 60°C was therefore considered optimal. Under these chromatographic conditions, samples extracted with dichloromethane were essentially free from interfering contaminants. In the case of atenolol, which was extracted with butanol—dichloromethane because of its rather polar nature, a satisfying separation from co-extracted plasma constituents could not be achieved.

Separation of β-blocking agents on normal-phase Si-60 columns with "reversed-phase" mobile phases containing only a few percent of organic modifiers was, to our knowledge, first suggested by Bidlingmeyer et al. [4]. We found this method well suited for the determination of bisoprolol, metoprolol and atenolol. The compounds showed acceptable peak symmetry, and the background due to plasma and urine constituents and common contaminants of organic solvents used for extraction was rather low (see Figs. 2—4). Column stability was excellent: after chromatography of more than 500 samples no degradation of the column was observed. The exact mechanism of retention is not known at present. It has been suggested that silica gel at pH values lower than the pK of silicic acid shows a similar retention mechanisms as reversed phases, i.e. lipophilic compounds exhibit longer retention times than hydrophilic ones.

This is true for atenolol, metoprolol and bisoprolol, i.e. these compounds were eluted in the order of increasing lipophilicity. Propranolol, however, although being much more lipophilic than any of the other three, is eluted between atenolol and metoprolol.

Si-60 columns were also very suitable for the separation of urinary metabolites of bisoprolol. These metabolites included conjugates of bisoprolol and

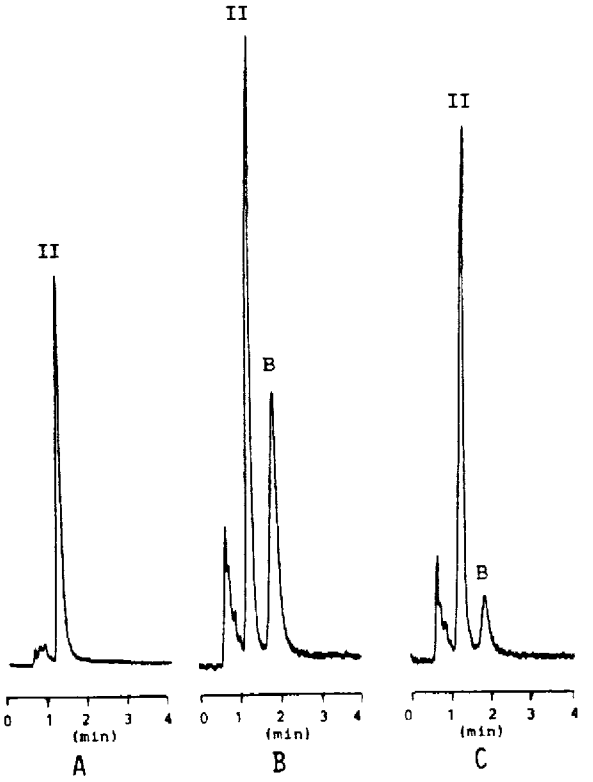

Fig. 2. Chromatograms (method 1) of plasma samples of a subject before oral administration (A) and 3 h (B) and 24 h (C) after oral administration of 20 mg of bisoprolol (peak B). In each case 100 ng of internal standard (peak II) per ml of plasma were added. The amount of bisoprolol was found to be 58 ng/ml (B) and 14 ng/ml (C).

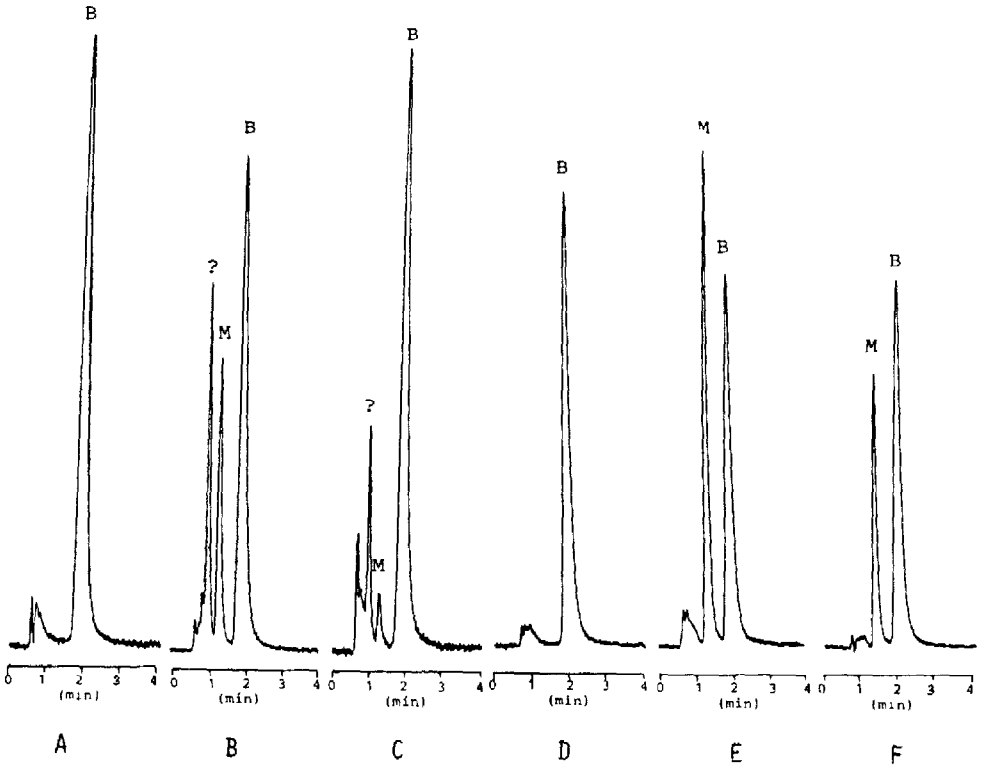

Fig. 3. Chromatograms (method 1) of plasma samples of two subjects before and after oral administration of 50 mg of metoprolol (M). Subject 1: (A) before administration, (B) 3 h after and (C) 12 h after administration. Subject 2: (D) before administration; (E) 3 h after and (F) 12 h after administration. Internal standard was 200 ng of bisoprolol (peak B) per ml of plasma. The amount of metoprolol was found to be 86 ng/ml (B), 12 ng/ml (C), 220 ng/ml (E) and 110 ng/ml (F).

products formed by N- and O-dealkylation and subsequent oxidation to the corresponding carboxylic acids [5].

For the determination of bisoprolol and metoprolol both methods were equally suitable but we preferred chromatographic separation on Si-60 columns (method 1), since the derivatization with acetic anhydride could be omitted. For the determination of atenolol method 2 was inferior owing to insufficient separation of acetylated atenolol from plasma constituents.

In the case of propranolol method 2 was used. When propranolol was chromatographed on Si-60 columns the peak height of this compound showed large variations [coefficient of variation (C.V.) up to 20%] even when pure standard solutions were used. The reason for this is not yet known. We assume it is due to poor solubility of this rather lipophilic compound in the mobile phase and, in consequence, to irreproducible absorption by the equipment used.

The chromatograms of some plasma samples showed peaks with very long retention times, making long analysis times necessary especially at low propranolol concentrations. The broad peaks seen in chromatogram B in Fig. 5 are due to these substances and originated from a plasma sample injected ca.

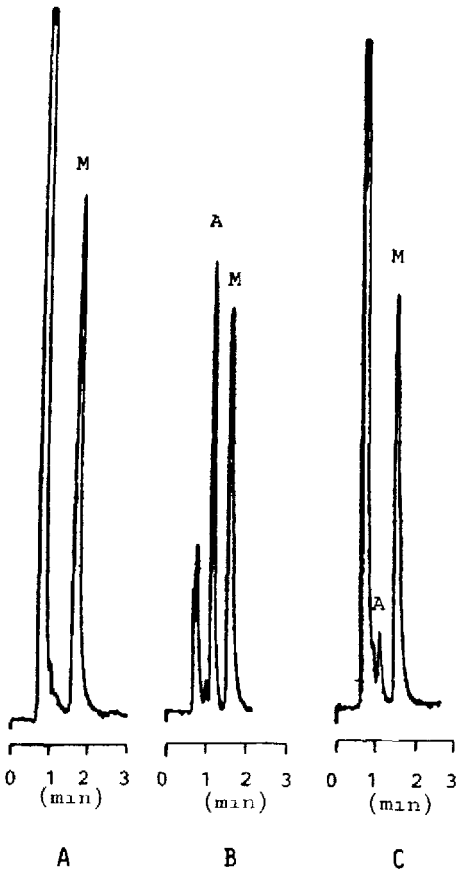

Fig. 4. Chromatograms (method 1) of 1-ml plasma samples of a subject before oral administration (A) and 3 h (B) and 48 h (C) after oral administration of 100 mg of atenolol (A). Internal standard was 100 ng of metoprolol (M) with samples A and C and 1 μg of metoprolol with sample B. The amount of atenolol was found to be 650 ng/ml (B) and 8 ng/ml (C).

6 min earlier. These compounds could be removed by washing the samples at acid pH with hexane prior to acetylation but, as already mentioned, propranolol was also extracted to a slight extent under these conditions.

Precision and recovery

The standard curves are linear in the concentration range studied, with a regression coefficient of close to 1. The quantitation limit, defined as three times the baseline noise, in plasma was 1—2 ng/ml for bisoprolol, metoprolol (methods 1 and 2), and propranolol (method 2) and 5 ng/ml for atenolol (method 1). The coefficient of variation was in the range 3—5%. Overall recovery of metoprolol, bisoprolol and propranolol was between 95 and 100% and between 80 and 90% in the case of atenolol (see Table I). When the proportion of butanol was increased to ca. 75% for extraction of atenolol, the recovery was close to 100%, but the background from interfering plasma constituent was also considerably increased.

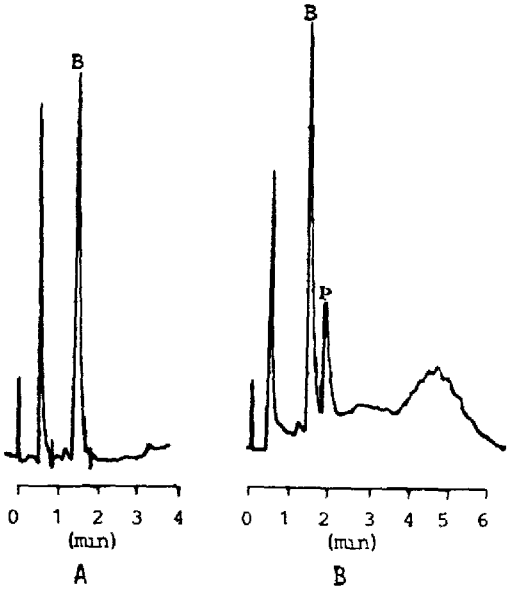

Fig. 5. Chromatograms (method 2) of 1-ml plasma samples containing (A) 200 ng of standard (bisoprolol, peak B) and (B) 200 ng of standard and 10 ng of propranolol (peak P).

TABLE I

PRECISION AND ACCURACY OF THE DETERMINATION OF BISOPROLOL, ATENOLOL, METOPROLOL AND PROPRANOLOL IN HUMAN PLASMA

Regression lines: bisoprolol: $y = 1.20x + 0.24$ ($r = 0.9997$); atenolol: $y = 0.51x + 4.83$ ($r = 0.9988$); [$\log y = 0.97 \log x - 0.19$; ($r = 0.9998$)]; metoprolol: $y = 0.63x + 0.12$ ($r = 0.9998$); propranolol. $y = 0.14x + 0.33$ ($r = 0.9998$). x = peak-height ratio multiplied by the standard concentration (ng/ml); y = concentration (ng/ml) of the compound determined.

Added (ng/ml)	Found (ng/ml)							
	Bisoprolol*		Atenolol*		Metoprolol*		Propranolol**	
	Mean (n=3)	C.V. %	Mean (n=5)	C.V. %	Mean (n=3)	C.V. (%)	Mean (n=3)	C.V. (%)
2	2.0	3.0			1.9	10.5	1.9	2.6
5	5.0	3.0	4.1	1.5	4.8	6.3	4.9	2.9
10	9.8	3.6	8.3	3.6	9.4	5.0	9.3	1.1
20	20.1	3.3	18	3.4	19.5	5.6	22	0
50	51	1.1	43	2.8	49	1.6	55	1.0
100	102	2.1	86	2.8	98	0.6	108	0.5
200			182	3.1				
500			468	5.8				

*Method 1 was used.
**Method 2 was used

Interference of other drugs and metabolites

Interference of other drugs with the determination of bisoprolol (method 1) was studied for trichlormethiazide, hydrochlorothiazide, nifedipine and rifam-

picin. These compounds were found to produce no interfering peaks. The same was true for the known metabolites of bisoprolol. Of the metabolites of bisoprolol only two can be extracted with dichloromethane. These metabolites were found in rats and dogs but not in humans and elute before the internal standard (II) [5]. After administration of metoprolol the chromatograms (method 1) showed a large peak with a retention time of ca. 1 min (Fig. 3). This peak was not present in blank plasma and therefore we assume that it is due to α-hydroxymetoprolol, a metabolite which can be quantitatively extracted with dichloromethane [6]. These results suggest that method 1 may be useful for the simultaneous determination of metoprolol and this metabolite, but since no reference compound was available at the time of this investigation, we did not study its quantitative recovery and fluorescence yield. Interestingly this metabolite was not present in plasma samples of a subject which showed elevated plasma concentrations and a rather long plasma half-life of ca. 8 h (Fig. 3). It is known that the formation of α-hydroxymetoprolol is impaired in poor metabolisers of the polymorphically oxidized drug, debrisoquine [6—8].

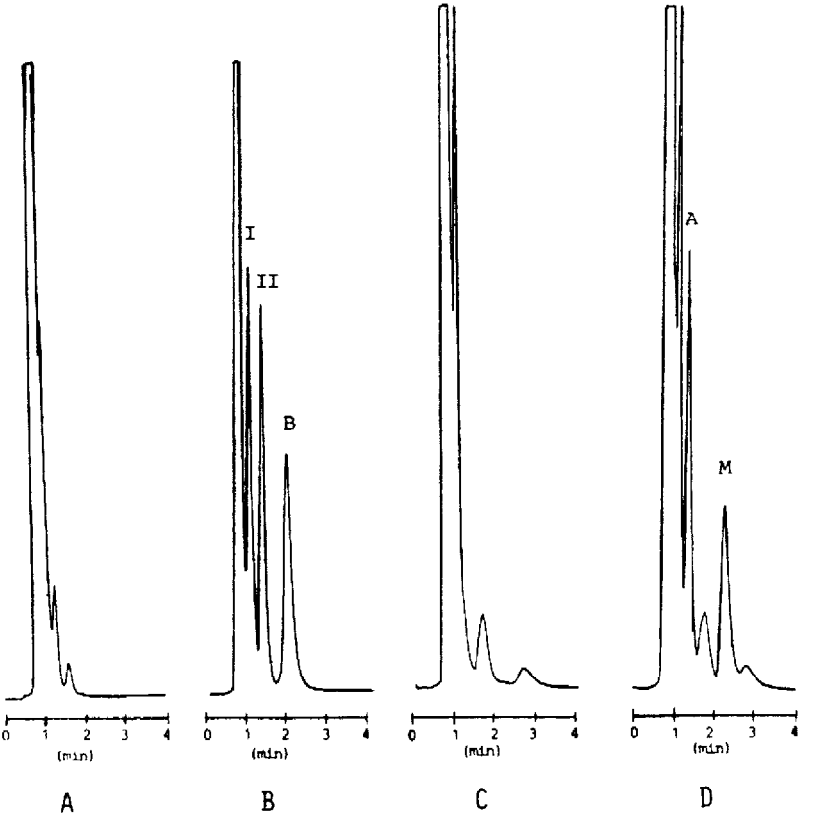

Fig. 6. Chromatograms (method 1) of urine samples. (A) and (C) urine blank. (B) Urine (0—12 h collection) of a subject after oral administration of 20 mg of bisoprolol (peak B); 10 μg of II per ml were added as internal standard The amount of bisoprolol in this sample was found to be 8 9 μg/ml. (D) Urine sample containing 5 μg/ml atenolol and 5 μg/ml metoprolol. Mobile phase acetonitrile—distilled water—1 *M* ammonium phosphate buffer (pH 4) (7 88:5 for A and B; 3:92:5 for C and D).

Application to pharmacokinetic studies

The methods described in this paper were sufficiently sensitive to follow the plasma concentration in humans for at least three half-lives at the usual therapeutic doses (5—20 mg of bisoprolol [9], 50—100 mg of metoprolol, 50—100 mg of atenolol and 40—240 mg of propranolol). Representative chromatograms of plasma samples before and after administration of the drugs are given in Figs. 2—5.

Determination of bisoprolol and atenolol in urine

Bisoprolol and atenolol were also determined in urine (method 1). The detection limit of these compounds was 10 ng/ml in the case of bisoprolol and ca. 50 ng/ml in the case of atenolol. At a dose of 10 mg of bisoprolol and 50 mg of atenolol, urine concentrations (0—24 h collection interval) are high enough to allow determination of these compounds with good accuracy without prior extraction (see Fig. 6). For this purpose, urine samples containing the appropriate amount of internal standard were diluted prior to chromatography with three volumes of the respective mobile phase. The peak designated as I in Fig. 6B is due to the main metabolite (I) of bisoprolol. To achieve a better separation of this compound from urine constituents, a slightly modified mobile phase was used [5].

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