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DETERMINATION OF METOPROLOL AND TWO MAJOR METABOLITES IN PLASMA AND URINE BY COLUMN LIQUID CHROMATOGRAPHY AND FLUOROMETRIC DETECTION

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SUMMARY

Metoprolol and its α-hydroxy metabolite were determined in plasma down to 2 nmol/l (S.D. 10–15%) after solvent extraction and bonded-phase liquid chromatography with fluorometric detection. The major metabolite with a carboxylic function was also measured in plasma when liquid–solid extraction on a column activated with dodecyl sulphate was applied. In urine the three components were assayed by direct injection of a diluted sample.

INTRODUCTION

Metoprolol, a β-adrenoceptor blocking drug, has been in clinical use for more than ten years. Analytical methods for metoprolol in biological samples have largely been based on gas chromatography (GC) with electron-capture detection of perfluoroacylated derivatives [1–3]. For simultaneous determination of two metabolites of metoprolol the same detection principle has been used [4], as has GC combined with mass-selective detection [5]. The major metabolite with a carboxylic function could be determined in urine after a two-step derivatization [6].

Column liquid chromatography (LC) with fluorometric detection has, in the past five years, emerged as an alternative technique for the measurement of metoprolol in plasma samples [7–10] and for the concomitant assay of metabolites in urine samples [11, 12]. In the present study we have devised a method with increased sensitivity for metoprolol (I) and the α-hydroxy metabolite (II) in

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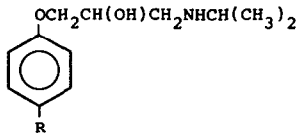

Compound	R
metoprolol (I)	$CH_2CH_2OCH_3$
α-hydroxymetoprolol (II)	$CH(OH)CH_2OCH_3$
metoprolol acid (III)	CH_2COOH
pafenolol (IV)	$CH_2CH_2NHCONHCH(CH_3)_2$

Fig. 1. Chemical structures for metoprolol (I), II, III and internal standard (pafenolol, IV).

plasma. By use of liquid–solid extraction, the carboxylic acid metabolite (III) could be included, and the same three compounds were measured in urine samples by direct injection after dilution. Fluorometric detection is employed, with bonded-phase LC in isocratic or gradient mode.

EXPERIMENTAL

Apparatus

The chromatograph comprised a Model 2150 pump and a Model 2152 gradient controller (LKB, Bromma, Sweden), a WISP Model 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.), a Brownlee CN guard column in a module (Brownlee Labs., Santa Clara, CA, U.S.A.) and a Microspher C_{18} column (3 μm, 100×4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands). A fluorescence detector LS4 (Perkin Elmer, Norwalk, CT, U.S.A.) or Model RF 530 (Shimadzu, Kyoto, Japan) was used. Chromatograms were recorded using a Model 4270 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). The vacuum manifold device (Vac Elut) and the Bond Elut C_{18} cartridges were from Analytichem (Harbor City, CA, U.S.A.).

Chemicals and reagents

Metoprolol (I), the metabolites α-hydroxymetoprolol (II) and metoprolol acid (III) and the internal standard pafenolol (IV) (Fig. 1) were synthesized at the Department of Organic Chemistry (AB Hässle, Mölndal, Sweden). Dichloromethane, methanol, 2-propanol and hexane were of HPLC-grade and acetonitrile was of specially pure HPLC S-grade (Rathburn Chemicals, Walkerburn, U.K.). Diethyl ether, analytical grade, was from May & Baker and sodium dodecyl sulphate (SDS), analytical grade, from BDH (Poole, U.K.). N,N-Dimethyloctylamine (DMOA), from ICN Pharmaceuticals (New York, U.S.A.), was redistilled before use. All other reagents and buffer substances were of analytical grade (Merck).

Chromatographic system

The mobile phase in method A (I and II in plasma) comprised acetonitrile–0.01 *M* DMOA in phosphate buffer (pH 3)–water (I=0.5) (12:10:78, v/v/v).

The aqueous mobile phase in methods B and C (I, II and III in plasma and urine) comprised 26.4% methanol, 6.6% 2-propanol and 20% 0.1 *M* SDS in phosphate buffer (pH 2) ($I=0.125$) with a gradient of 11–24% acetonitrile.

The flow-rate of the mobile phases was 1.0 ml/min for all the methods. The temperature was 22–24 °C.

The Perkin Elmer LS4 detector was set at 228 nm (excitation) and 306 nm (emission) and the Shimadzu RF 530 detector at 272 nm and 306 nm.

Standard solutions

Stock solutions of the compounds were prepared in 0.01 *M* hydrochloric acid containing 5% methanol, and were found to be stable for at least three months at 4 °C. When required, 100 µl were added to 1 ml of plasma or urine, giving a concentration of 200 nmol/l for I and II and 500 nmol/l for III and IV in plasma, and 50 µmol/l for I, II and IV and 200 µmol/l for III in urine.

Storage and preparation of samples

After collection, the plasma and urine samples were stored at −20 °C and found to be stable for at least four months [3]. The samples were allowed to thaw at room temperature, mixed on a whirl-mixer for 30 s and centrifuged at 1500 *g* for 5 min before aliquots were removed.

Analytical procedure

Method A: metoprolol (I) and α-hydroxymetoprolol (II) in plasma. Plasma (1.0 ml) was combined with 100 µl of internal standard (IV) solution, 100 µl of 1 *M* sodium hydroxide and 0.5 g of sodium chloride and was shaken with 5.0 ml of dichloromethane–diethyl ether (1:4) for 10 min. After centrifugation (1500 *g*, 5 min) the aqueous phase was frozen in a bath of dry-ice–ethanol, and the organic layer was transferred to a conical test-tube. After back-extraction to 250 µl of a solution of 0.001 *M* DMOA in phosphate buffer (pH 3) ($I=0.05$) by shaking for 5 min, centrifugation (1500 *g*, 2 min) and freezing, the organic phase was discarded. The aqueous phase was washed by shaking for 2 min with 1 ml of hexane, which was discarded after centrifugation and freezing. An aliquot (10–100 µl) of the aqueous phase was injected onto the chromatograph.

Method B: analytical procedure for I, II and III in plasma. A Bond Elut C_{18} cartridge was activated and conditioned by successive washing with 1 ml of methanol, 1 ml of distilled water and 1 ml of a solution containing 0.001 *M* SDS in 0.01 *M* phosphoric acid. A mixture of 1.0 ml of plasma, 1 ml of 0.2 *M* phosphoric acid and 100 µl of internal standard (IV) was loaded onto the Bond Elut C_{18} cartridge. The plasma sample was passed at low pressure through the cartridge in 1–5 min. The cartridge was washed with 0.2 ml of 0.001 *M* SDS in 0.01 *M* phosphoric acid. The substances were eluted from the cartridge by passing 2 ml of 25% acetonitrile in dichloromethane followed by 0.2 ml of acetonitrile. The total volume was transferred to a conical test-tube containing 0.5 ml of 5% 2-propanol in 0.01 *M* phosphoric acid, and was combined with 2.5 ml of hexane used to wash the collection tube. The eluate was evaporated at 37 °C under a gentle stream of nitrogen to 0.5 ml, and the residue was washed with 1 ml of

hexane by shaking for 2 min. After centrifugation (1500 *g*, 2 min), the aqueous phase was frozen in a bath of dry-ice–ethanol, and the organic layer was discarded. An aliquot (50–200 μl) of the aqueous solution was injected onto the chromatograph.

Method C: analytical procedure for I, II and III in urine. Urine sample (1.0 ml) and 100 μl of the internal standard solution (IV) were diluted to 10.0 ml with a solution of 0.02 *M* SDS in phosphate buffer (pH 2) ($I=0.025$), and 100–150 μl of the solution was injected onto the chromatograph.

RESULTS AND DISCUSSION

Extraction

The liquid–liquid extraction of I and II from plasma into diethyl ether–dichloromethane (4:1) gives a distinct phase separation. After back-extraction into a small volume of aqueous phase and washing with hexane, chromatograms without interfering peaks are obtained by fluorometric detection. Absolute extraction recoveries of I and II were ca. 100% compared with injection of the substances in the back-extraction solution.

If only metoprolol is to be assayed, addition of sodium chloride to the plasma may be omitted and the recovery is still 94%. (The recovery of II will then be ca. 65%.) This omission facilitates freezing of the aqueous phase.

Compound III, which has both amino and carboxylic acid functions, cannot easily be isolated from plasma by liquid–liquid extraction. Liquid–solid extraction on a Bond-Elut C_{18} column had then to be applied to enable measurement of III besides from I and II. After the sample is applied to the C_{18} cartridge, proteins and some endogenous components in plasma are washed off. Compounds I, II and III and the internal standard are retained as ion pairs with SDS and are then eluted with an organic solvent mixture, acetonitrile–dichloromethane. This eluent was found to be more efficient than methanol, pure acetonitrile and other solvents tested.

Hexane is added prior to evaporation in order to wash the collection tube and to get an upper organic phase. The eluate is not evaporated to dryness, as this seems to cause degradation of II. The final washing with hexane appears to improve column stability.

The extraction efficiency for metoprolol and the two metabolites using the Bond Elut C_{18} cartridge was measured by comparison with direct injection of the compounds in 0.1 *M* phosphoric acid. The results are shown in Table I. Solid-phase extraction had been used previously for metoprolol, when atenolol was also assayed [13].

The sample preparation for the assay of metoprolol and the metabolites II and III in urine includes only addition of internal standard and a ten-fold dilution with 0.02 *M* SDS in phosphate buffer (pH 2).

Separation and quantification

The separation of I, II and the internal standard, IV (method A) on a C_{18} column employed a mobile phase of 12% acetonitrile and 0.001 *M* DMOA in phos-

TABLE I

ABSOLUTE RECOVERY AND REPEATABILITY FOR I, II AND III IN PLASMA (METHOD B)

Substance	Concentration (nmol/l)	Recovery (%)	Repeatability ($n=3$) (%S.D.)
I	120	78	7.9
	1200	85	3.1
	3000	89	2.3
II	60	81	5.6
	600	86	4.2
	1500	82	2.6
III	200	80	7.8
	2000	88	3.5
	5000	86	1.6

phate buffer (pH 3) ($I=0.05$) An earlier study showed that DMOA improves the column efficiency [14]. The retention times were 2.1, 4.7 and 8.7 min for II, IV and I, respectively. Fig. 2 shows a chromatogram of an authentic plasma sample 60 min after administration of 100 mg of metoprolol.

If II is not to be measured, a retention time of ca. 3 min for metoprolol can be achieved if the proportion of acetonitrile in the mobile phase is increased to 20%.

The injection volume may be varied from 10 to 150 μl without any change in column efficiency, as the injection solution does not contain any acetonitrile. With an injection volume of 100 μl the limit of determination (S.D. $\leqslant$ 10–15%) is 2 nmol/l (0.5 ng/ml) for I and II.

The ion-pair chromatographic system with SDS in phosphate buffer (pH 2) enables a baseline separation of II and III when the mobile phase contains methanol, 2-propanol and acetonitrile in the proportion 26.4:6.6:11. The retention times are 8 and 9 min, respectively. The fluorescence response for III is only one quarter of that of II, probably because of the electron-withdrawing group COOH in III. The acetonitrile concentration is increased by the gradient controller, and metoprolol is eluted in the same system, giving almost the same limit of determination as for II.

A chromatogram of a plasma sample 75 min after administration of 100 mg of metoprolol (method B), is shown in Fig. 3. The limit of determination for I, II and III is 15, 10 and 20 nmol/l plasma, respectively if 200 μl of the extract is injected. Increasing the acetonitrile content in the mobile phase too rapidly when running the gradient will give rise to an increased background, deleterious for low-concentration plasma samples. A slow gradient of acetonitrile may prevent such interferences.

In method C (urine) it is possible to increase the acetonitrile concentration faster than in method B (plasma). The sensitivity of the detector does not have

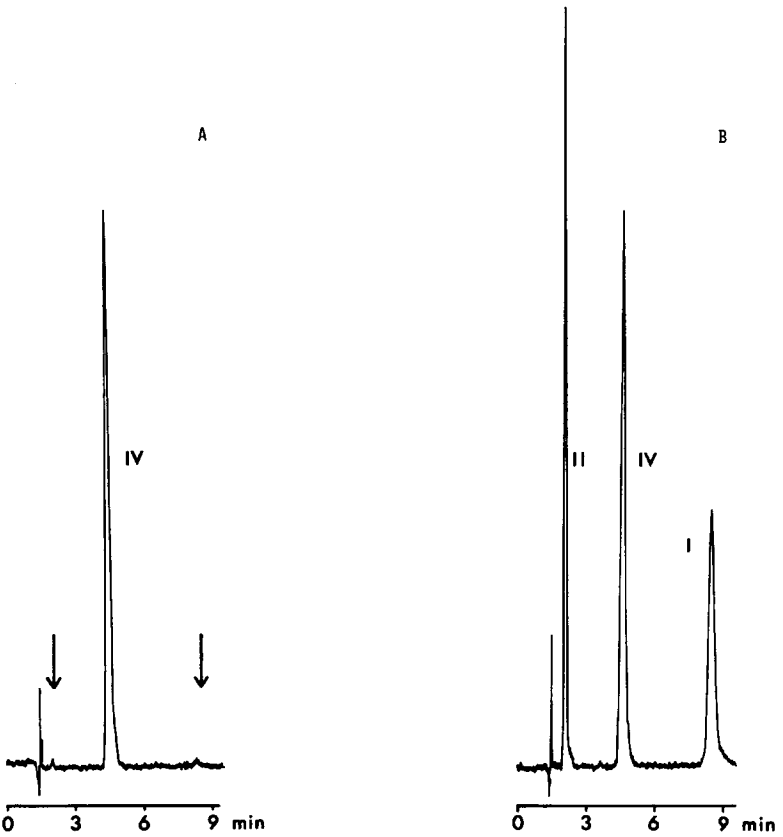

Fig. 2. Metoprolol and II in authentic plasma samples (method A). Plasma sample (A) before and (B) 60 min after administration of 100 mg of metoprolol containing 91 nmol/l metoprolol and 97 nmol/l II. Injection volumes, 50 μl; detector, Perkin Elmer LS4, slit ex 10/em 10.

to be in a highly amplified mode in order to determine the current concentrations of I, II and III present in urine. The limit of determination for I, II and III is 0.5, 0.5 and 2 μmol/l urine, respectively, when injecting 150 μl of the urine sample diluted with mobile phase without modifier.

A chromatogram of a urine sample (method C) from a volunteer 0–24 h after administration of 100 mg of metoprolol is shown in Fig. 4.

Precision and repeatability

In method A the repeatability measured as percentage S.D. was 2.8–3.8% ($n=10$) for the concentration range 15–400 nmol/l for both I and II. The modified method A (no addition of sodium chloride) has been compared with a GC assay [3] in which only metoprolol was measured. The ratio of the results from the two methods was 1.01 (S.D. 9.3%) in the concentration range 100–1000 nmol/l ($n=67$). The repeatability of the modified LC method for plasma samples was S.D. 2.3% ($n=8$) at a concentration level of 300 nmol/l.

The liquid–solid extraction of I, II and III on a Bond Elut C_{18} column (method

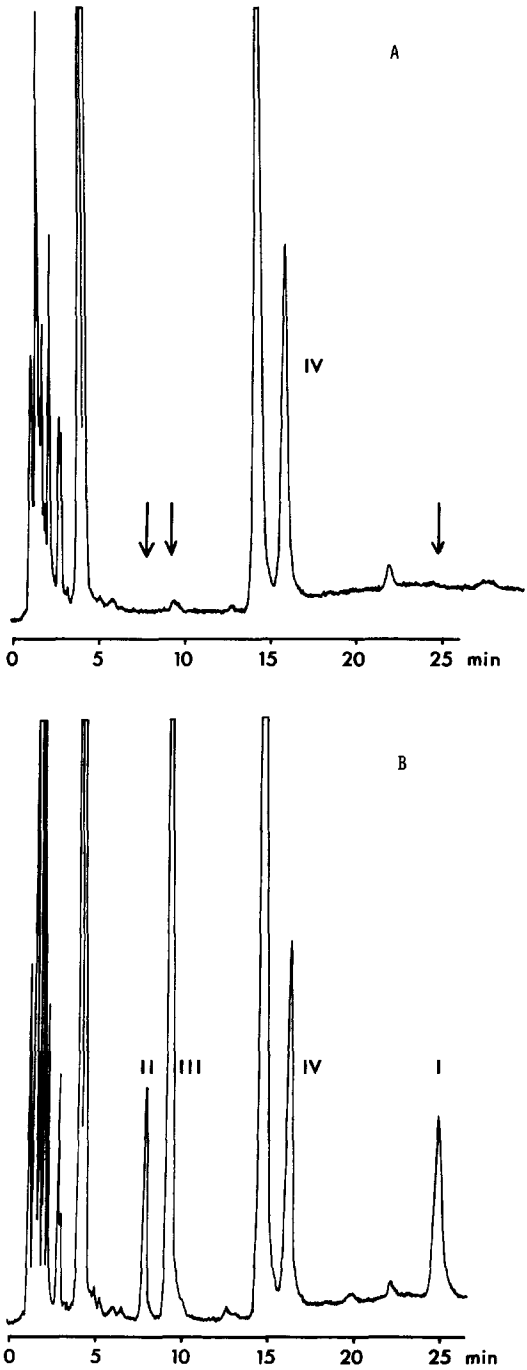

Fig. 3. Metoprolol, II and III in authentic plasma samples (method B). Plasma sample (A) before and (B) 75 min after administration of 100 mg of metoprolol containing 380 nmol/l metoprolol, 250 nmol/l II and 4150 nmol/l III. Injection volume, 100 μl; gradient mode, 0–10 min 11% acetonitrile, 10–20 min 11–17% acetonitrile, and 20–25 min 17% acetonitrile; detector, Perkin Elmer LS4, slit ex 10/em 2.5.

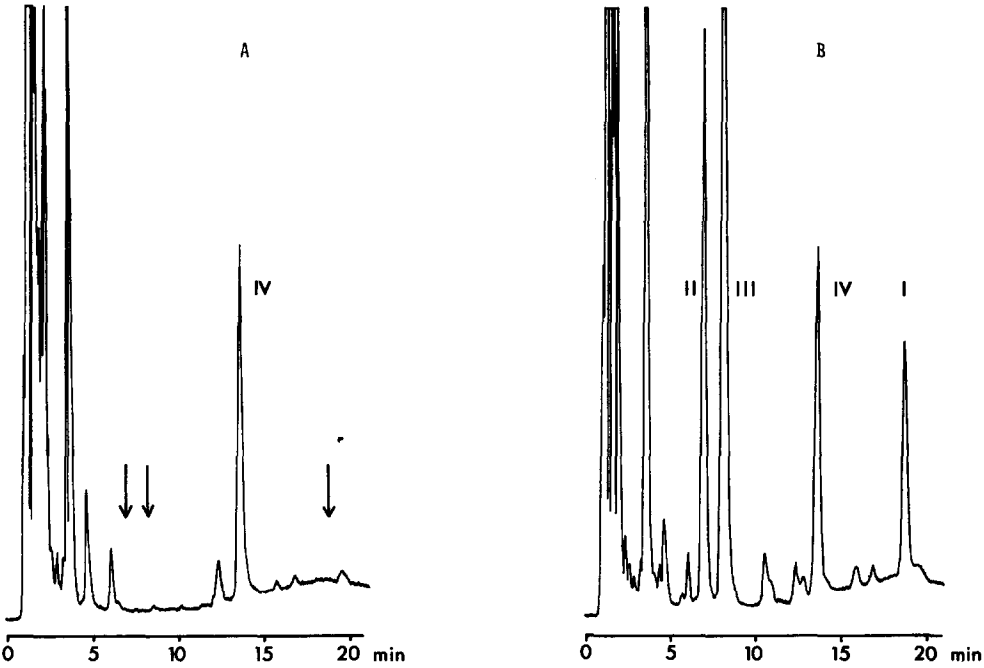

Fig. 4. Metoprolol, II and III in authentic urine samples (method C). Urine sample (A) before and (B) 0–24 h after administration of 100 mg of metoprolol containing 14 μmol/l metoprolol, 32 μmol/l II and 209 μmol/l III. Injection volume, 100 μl; gradient mode, 0–8 min 11% acetonitrile, 8–15 min 11–24% acetonitrile, 15–22 min 24% acetonitrile; detector, Perkin Elmer LS4, slit ex 10/em 2.5.

B) of a plasma sample with concentrations of 90, 70 and 260 nmol/l, respectively, gave an S.D. of 6.3, 4.0 and 2.5% ($n=8$) when 200 μl of the extract were injected.

The absolute recoveries of I, II and III and the repeatability at three concentration levels in the liquid–solid extraction procedure (method B) are shown in Table I.

The repeatability of method C, by repeated injections of 150 μl ($n=10$) of the same diluted urine sample, was 1.9–2.2% S.D. for I, II and III at concentrations of 20, 30 and 300 μmol/l, respectively.

Seventeen authentic urine samples were analysed in duplicate by this method. The mean differences of each pair of samples were 3.1, 2.5 and 2.5% for I, II and III in the concentration ranges 1.5–80, 4–40 and 15–350 μmol/l, respectively.

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