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Determination of the enantiomers of metoprolol and its major acidic metabolite in human urine by high-performance liquid chromatography with fluorescence detection

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Abstract

Enantiomers of metoprolol and its acidic metabolite H 117/04 were determined in human urine by high-performance liquid chromatography (HPLC) with fluorometric detection after chiral derivatization. The carboxyl functional group of the major metabolite was blocked by esterification after solid-phase extraction, which helped to quantitate this compound from interfering substances. The assay method was validated. The recovery of (−)- and (+)-metoprolol from urine was 86.3–90.5%; and the recovery of the (−)- and (+)-acidic metabolite H 117/04 from urine was 74.4–83.9% at different concentrations.

1. Introduction

Metoprolol, 1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-2-propanol is a cardioselective β-adrenergic blocking agent used in the treatment of hypertension and angina [1,2]. It is therapeutically used as a racemic mixture. The differences in the pharmacokinetics of the stereoisomers were studied by Hermansson and Von Bahr [3]. Several methods for the determination of metoprolol enantiomers in biological fluids have been reported, either using chiral derivatization reagents to form diastereomers with *S*-(−)-phenylethyl isocyanate [4], chiral chloroformate [5], phosgene [1] or L-leucine [3], or using direct determination of the enantiomers of metoprolol with chiral stationary phases [6–11]. Metoprolol is mainly metabolized by oxidative deamination and O-dealkylation with subsequent oxidation and aliphatic hydroxylation [12]. The metabolites account for 85% of the dose in man [12]. Fig. 1 shows the major metabolic routes of metoprolol in mammals [13,15]. Since metoprolol is extensively metabolized in man, determination of the enantiomers of metoprolol and their major metabolites seems to be more significant.

In this paper, a method for the extraction and determination of the enantiomers of metoprolol in human urine is described. In addition, a novel method has been established for the assay of the

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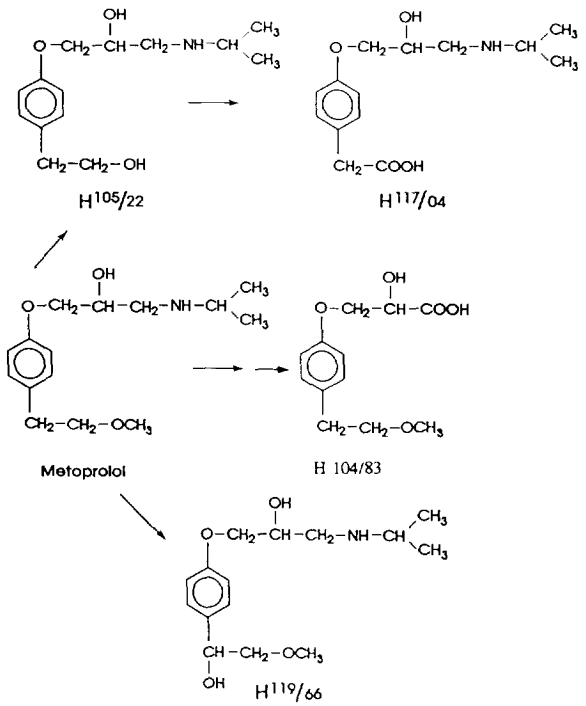

Fig. 1. Major metabolic routes of metoprolol.

enantiomers of the major acidic metabolite H 117/04 of metoprolol in urine.

2. Experimental

2.1. Materials and reagents

Racemic (±)-metoprolol tartrate (1811-01), *S*-(−)-metoprolol hydrochloride (H 18), metabolite H 105/22 benzoate, metabolite H 117/04 hydrochloride and metabolite H 119/66 benzoate were obtained from Astra Hässle AB (Mölndal, Sweden). (±)-Toliprolol (base form) was from ICI (Cheshire, UK). *S*-(−)-Menthyl chloroformate [(−)-MCF] was purchased from Aldrich (Milwaukee, WI, USA). 14% BF_3 in methanol was from Pierce (Rockford, IL, USA). Sep-Pak Plus C_{18} cartridges (containing 360 mg sorbent) were from Millipore (Milford, MA, USA). All organic 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.). Distilled water was treated with a four-stage Milli-Q water purification system (Millipore, Mississauga, Ont., Canada) before used in HPLC (this is referred as water hereafter).

2.2. Standard solutions

The weights of all authentic standard salts were calculated in relation to their free form. Stock solutions were prepared in methanol: (±)-metoprolol (0.1 and 1 mg/ml), (−)-metoprolol (1 mg/ml), H 117/04 (0.1 mg/ml and 1 mg/ml), H 105/22 (1 mg/ml), H 119/66 (1 mg/ml), (±)-toliprolol (1 mg/ml) used as internal standard (I.S.). A solution of triethylamine (0.4%) was prepared in acetonitrile–methanol (1:1, v/v). The (−)-MCF solution (1%) was prepared every week in acetonitrile which was previously dried with anhydrous sodium sulfate. All stock solutions and reagent solutions were sealed and stored at −20°C in the dark.

2.3. Human studies

Blank urine samples were collected before administration of a single 128-mg oral dose of racemic (±)-metoprolol tartrate to two healthy male volunteers (subject 1: age 34, weighing 63 kg; subject 2: age 57, weighing 63 kg). Urine samples were then collected for the next 48 h and frozen in the dark at −20°C immediately after collection.

2.4. Sample preparation

Method A

Determination of metoprolol in urine. Aliquots of 2.0 ml of urine samples were spiked with 30 μg of (±)-toliprolol as I.S.. This mixture was basified to pH 12 with 1.0 ml of 2 *M* potassium carbonate solution. About 1.0 g of sodium chloride was added to the resulting sample, which was subsequently extracted twice with 5 ml of ethyl acetate. The organic layer was separated, dried with sodium sulfate, and evaporated to dryness under a stream of nitrogen at 50°C. To the dried extract, 100 μl of 0.4%

triethylamine solution and 100 μl of 1% (−)-MCF were added. The resulting solution was kept at room temperature for 1 h, and then evaporated to dryness under a stream of nitrogen. The sample was reconstituted with 300 μl methanol, and 10 μl were injected onto the chromatographic system.

Method B

Determination of the acidic metabolite H 117/04 in urine. A Sep-Pak C_{18} Plus cartridge was conditioned by successive washing with 5 ml of acetonitrile and 5 ml of water. A 2-ml urine sample spiked with 30 μg of (±)-toliprolol was passed through a cartridge, which was washed successively with 2 ml of water and 2 ml of dichloromethane to remove excess water in the cartridge. Metoprolol and its metabolites were eluted with 5 ml of acetonitrile containing 1% triethylamine. The solvent was evaporated to dryness under a stream of nitrogen at 50°C and the residue was derivatized with 200 μl of 14% BF_3–methanol at 100°C for 10 min to esterify the carboxyl functional group. After evaporating methanol under a stream of nitrogen at 50°C, 1 ml of water was added and the solution was acidified to pH 1–3 with 3 drops of 2 *M* HCl followed by extraction with 2 ml of diethyl ether. The diethyl ether phase was discarded. The aqueous phase was basified to pH 12 with 2 ml of 2 *M* potassium carbonate solution and the resulting sample was extracted twice with 5 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 further derivatized with 1% (−)-MCF as described above. After derivatization, the reaction solution was evaporated to dryness under a stream of nitrogen at 50°C. The residue was reconstituted with 300 μl of methanol and 10 μl were injected for HPLC analysis.

2.5. High-performance liquid chromatography

An HP 1090 liquid chromatograph was equipped with a fluorescence detector (Spectroflow 980, ABI Analytical, Kratos Division) and an HP G1307A ChemStation data system (Hewlett-Packard, Palo Alto, CA, USA). The detector was operated with an excitation wavelength of 223 nm and a cut-off emission filter of 340 nm. The separation of the (−)-MCF derivatives of (±)-metoprolol, (±)-acidic metabolite H 117/04 and (±)-toliprolol was performed on a Phenomenex Hypersil 5 C_{18} column (250 × 4.6 mm I.D.) at ambient temperature (Phenomenex, Torrance, CA, USA). A laboratory packed HP ODS Hypersil precolumn (20 × 4.0 mm I.D., 30 μm particle size) was used to protect the analytical column. The mobile phase consisted of solvents A and B. Solvent A (0.1 *M* phosphate buffer, pH 3.2) was prepared by dissolving 13.8 g of monobasic sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) and 1.59 g of propylamine hydrochloride in 1 l of water and adjusting the pH to 3.2 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 methanol. The analysis was accomplished by gradient elution starting with 75% solvent B and increasing to 85% solvent B at 15 min (held for 5 min), then increasing to 90% solvent B at 25 min (held for 3 min). The flow-rate of the mobile phase was kept at 1.0 ml/min.

2.6. Calibration curves

Calibration curves were constructed by spiking urine with known amounts of metoprolol, its acidic metabolite H 117/04 and the internal standard toliprolol. Aliquots of 2 ml of blank urine samples were spiked with (±)-metoprolol (concentration: 0.75, 1.5, 4.5, 12, 18 and 22.5 μg/ml) and 30 μg of I.S.. The other half of the 2-ml aliquots of blank urine samples were spiked with (±)-acidic metabolite H 117/04 (concentration: 1.5, 3.0, 9.0, 18, 30 and 45 μg/ml) and 30 μg of I.S.. The urine samples were equilibrated for 1 h at room temperature. Subsequently they were extracted as described in section 2.4., and the samples were analyzed by HPLC. For each concentration, triplicate samples were prepared. The peak-area ratio of (−)- and (+)-metoprolol or (−)- and (+)-acidic metabolite H 117/04 to the (−)- and (+)-toliprolol (I.S.) were

measured for each analysis. The data were fitted by linear regression equations, for metoprolol: $C_{(-)} = 4.99A_{(-)} - 0.24$ ($r = 0.998$) and $C_{(+)} = 4.97A_{(+)} - 0.52$ ($r = 0.997$), and for the acidic metabolite H 117/04: $C_{(-)} = 14.09A_{(-)} + 0.41$ ($r = 0.998$) and $C_{(+)} = 14.38A_{(+)} + 0.98$ ($r = 0.998$), where $C_{(-)}$ and $C_{(+)}$ are the concentration of (−)- and (+)-metoprolol or (−)- and (+)-acidic metabolite H 117/04 in urine (μg/ml), respectively; $A_{(-)}$ and $A_{(+)}$ are the peak-area ratios of the (−)-MCF derivatives of (−)- and (+)-metoprolol or (−)- and (+)-acidic metabolite H 117/04 to that of (−)- and (+)-toliprolol (I.S.).

2.7. Recovery and precision

The extraction recovery of metoprolol and its acidic metabolite H 117/04 enantiomers from urine was measured with spiked urine samples at four different concentrations, 0.75, 2.25, 4.50 and 7.50 μg/ml for (−)- and (+)-metoprolol or (−)- and (+)-acidic metabolite H 117/04. Sample preparation and HPLC analysis were performed as mentioned previously. For the recovery study, 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 the (−)-MCF derivatives of metoprolol and its acidic metabolite H 117/04 enantiomers to that of I.S. enantiomers from two different analysis. One set of data was obtained from the analysis of the spiked samples while the other set of data was from the analysis of standard solutions containing the same amount of metoprolol, acidic metabolite H 117/04 and toliprolol enantiomers.

Intra- and inter-assay variabilities were determined by replicate analysis of (−)- and (+)-metoprolol or (−)- and (+)-acidic metabolite H 117/04 enantiomers after spiking urine samples with (±)-metoprolol and (±)-acidic metabolite H 117/04 (with the same concentration as in the recovery experiment) on the day of preparation and on different days, respectively (Tables 1 and 2).

3. Results and discussion

3.1. Extraction procedure

Since the acidic metabolite H 117/04 has both amino and carboxylic acid functions, it is difficult to extract and quantitate from aqueous media by liquid–liquid extraction. A method has been reported to determine the acidic metabolite by

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

Amount added (μg/ml)	Intra-assay ($n = 3$)		Inter-assay ($n = 9$)	
	Recovery (mean ± S.D.) (%)	C.V. (%)	Recovery (mean ± S.D.) (%)	C.V. (%)
0.75	(−) 89.7 ± 4.3	4.8	(−) 86.9 ± 6.9	7.9
	(+) 87.1 ± 4.6	5.3	(+) 86.3 ± 8.4	9.7
2.25	(−) 87.0 ± 4.4	5.0	(−) 88.7 ± 4.6	5.2
	(+) 88.5 ± 3.1	3.5	(+) 90.5 ± 3.6	4.0
4.50	(−) 86.6 ± 4.6	5.3	(−) 87.5 ± 4.5	5.2
	(+) 88.5 ± 5.5	6.3	(+) 89.5 ± 5.6	6.3
7.50	(−) 86.8 ± 3.0	3.4	(−) 86.6 ± 2.3	2.6
	(+) 87.7 ± 3.6	4.2	(−) 87.8 ± 2.6	2.9

Table 2
Recovery of (−)- and (+)-acidic metabolite H 117/04 from human urine spiked with (±)-acidic metabolite H 117/04

Amount added (μg/ml)	Intra-assay ($n = 3$)		Inter-assay ($n = 9$)	
	Recovery (mean ± S.D.) (%)	C.V. (%)	Recovery (mean ± S.D.) (%)	C.V. (%)
0.75	(−) 80.0 ± 7.5	9.3	(−) 80.3 ± 7.0	8.7
	(+) 75.4 ± 4.6	6.1	(+) 76.3 ± 7.8	10.2
2.25	(−) 76.1 ± 8.8	11.5	(−) 74.4 ± 9.2	12.4
	(+) 74.1 ± 4.5	6.1	(+) 77.4 ± 6.2	8.0
4.50	(−) 81.9 ± 5.5	6.7	(−) 75.5 ± 7.5	10.0
	(+) 79.5 ± 8.8	11.0	(+) 79.3 ± 7.8	9.8
7.50	(−) 83.0 ± 6.1	7.3	(−) 82.4 ± 10.9	13.3
	(+) 79.5 ± 4.4	5.5	(+) 83.9 ± 8.5	10.1

direct injection of a diluted urine sample [14]. Direct injection of the urine sample onto a reversed-phase column can shorten lifetime of the column and also lacks selectivity. Gyllenhaal and Hoffmann [15] reported another method for the quantitation of basic and acidic metabolites: the functional groups in the aminopropanol side-chain were blocked by oxazolidineone formation and neutral or acidic derivatives were isolated by extraction into an organic phase at low pH. In the present paper, metoprolol and its acidic metabolite H 117/04 were extracted with solid-phase extraction using C_{18} reversed-phase cartridges, in order to remove interfering substances from urine extracts. The extracted and eluted residue was reacted with the esterification reagent BF_3–methanol to block the carboxylic acid group. Further purification and isolation was carried out by liquid–liquid extraction. Due to the poor recovery of metoprolol with extraction method B, method A was applied for the extraction of metoprolol to improve the recovery.

3.2. Chiral separation of metoprolol and its acidic metabolite

The (−)-MCF derivatives of enantiomers of metoprolol and acidic metabolite H 117/04 were separated from each other and from all interfering substances in urine by this chromatographic method. Comparison of the (−)-MCF derivatives of a real urine sample with those of standard compounds (±)-H 119/66 and (±)-H 105/22, shows that the diastereomers of the metabolite H 119/66 and those of metabolite H 105/22 have very close retention times. The peaks of (−)-H 119/66 and (−)-H 105/22 (retention time of about 13.9 min) and those of (+)-H 119/66 and (+)-H 105/22 (retention time of about 14.6 min) overlap each other. These overlapping peaks do not interfere in the analysis of (±)-metoprolol and (±)-acidic metabolite H 117/04 (Fig. 2c). However, (±)-H 119/66 and (±)-H 105/22 cannot be analyzed with this method. The chromatograms of the diastereomers of (+)- and (−)-metoprolol and those of the (+)- and (−)-acidic metabolite H 117/04 after extraction from urine are shown in Figs. 2 and 3, respectively. Baseline separation of the diastereomers of metoprolol and the acidic metabolite H 117/04 was achieved in this experiment. By comparing the (−)-MCF derivatives of standard (±)-metoprolol with those of standard (−)-metoprolol in the chromatogram, we found that the diastereomer derived from (−)-metoprolol has a shorter retention time than that derived from (+)-metoprolol.

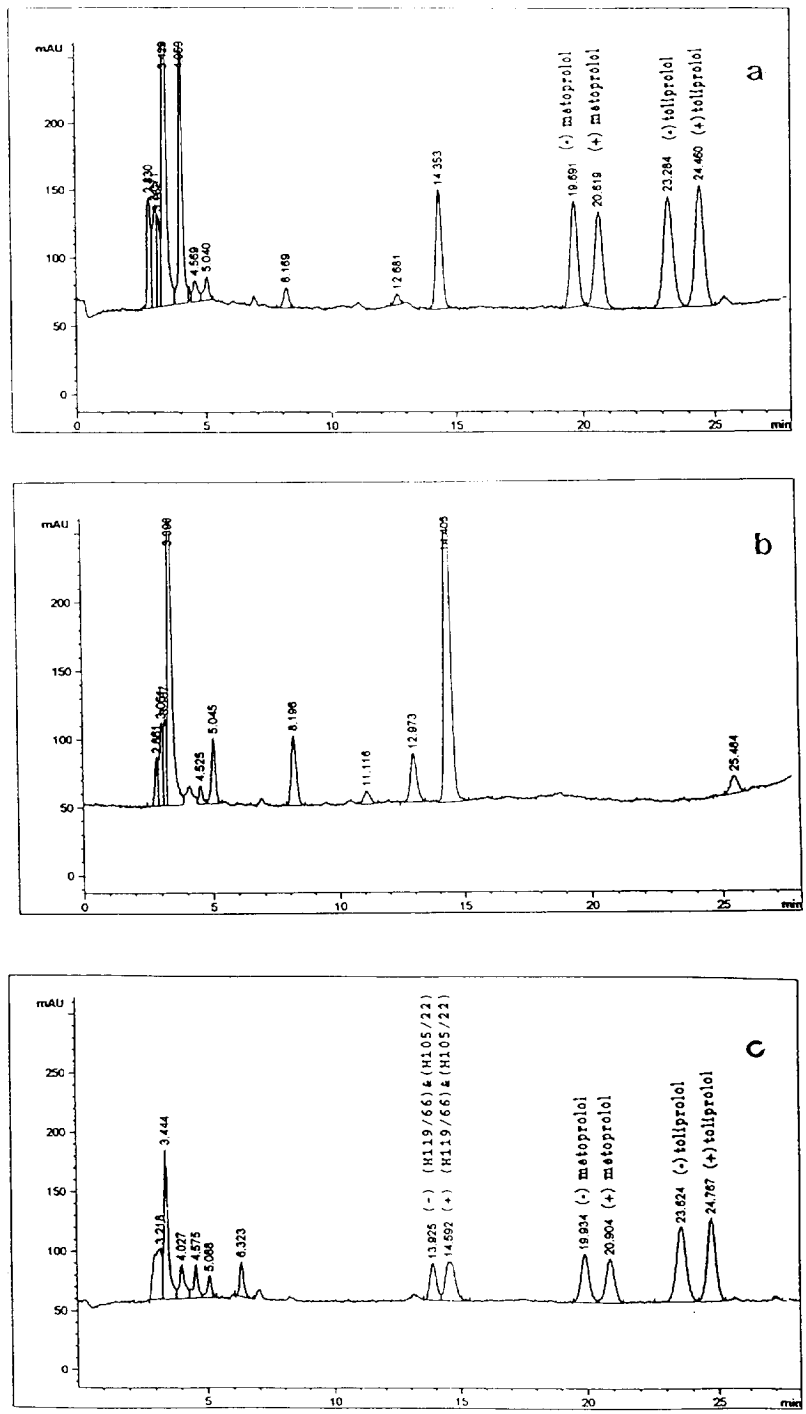

Fig. 2. Chromatograms of metoprolol enantiomers obtained after extraction and derivatization with (−)-MCF using method A. For conditions see Experimental section. (a) Spiked urine; (b) Blank urine; (c) 4-h urine sample.

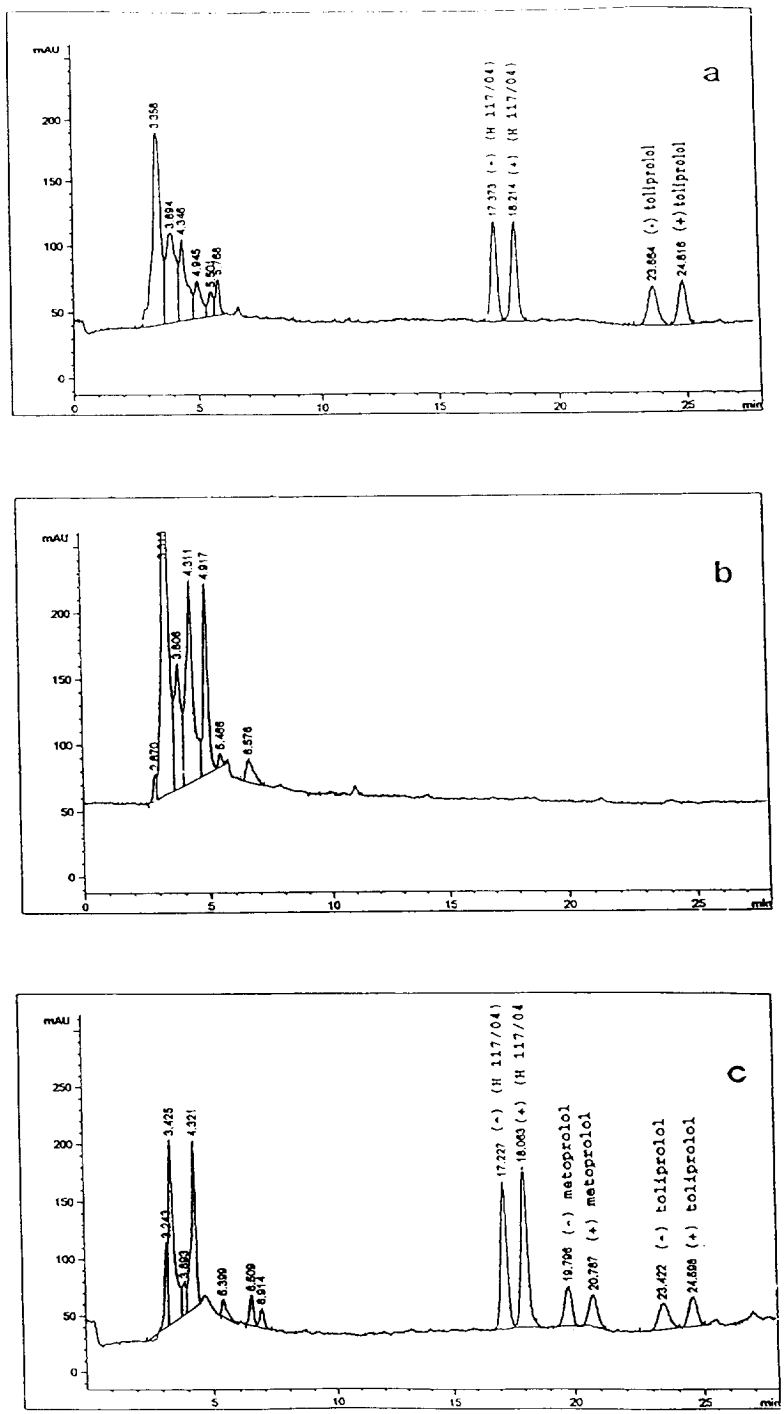

Fig. 3. Chromatograms of acidic metabolite H 117/04 enantiomers after extraction, esterification and derivatization with (−)-MCF using method B. (a) Spiked urine; (b) blank urine; (c) 4-h urine sample.

3.3. Excretion rate and stereoselective metabolism

Racemic metoprolol and its major metabolites in human urine were previously analyzed by Gyllenhaal and Hoffmann [15] and by Regardh et al. [16]. After oral administration of (±)-metoprolol, the parent compound is mainly eliminated from the body by biotransformation: about 95% of the oral dose was excreted in urine over a period of 72 h, mainly in metabolized form. The recovery of metoprolol in the urine

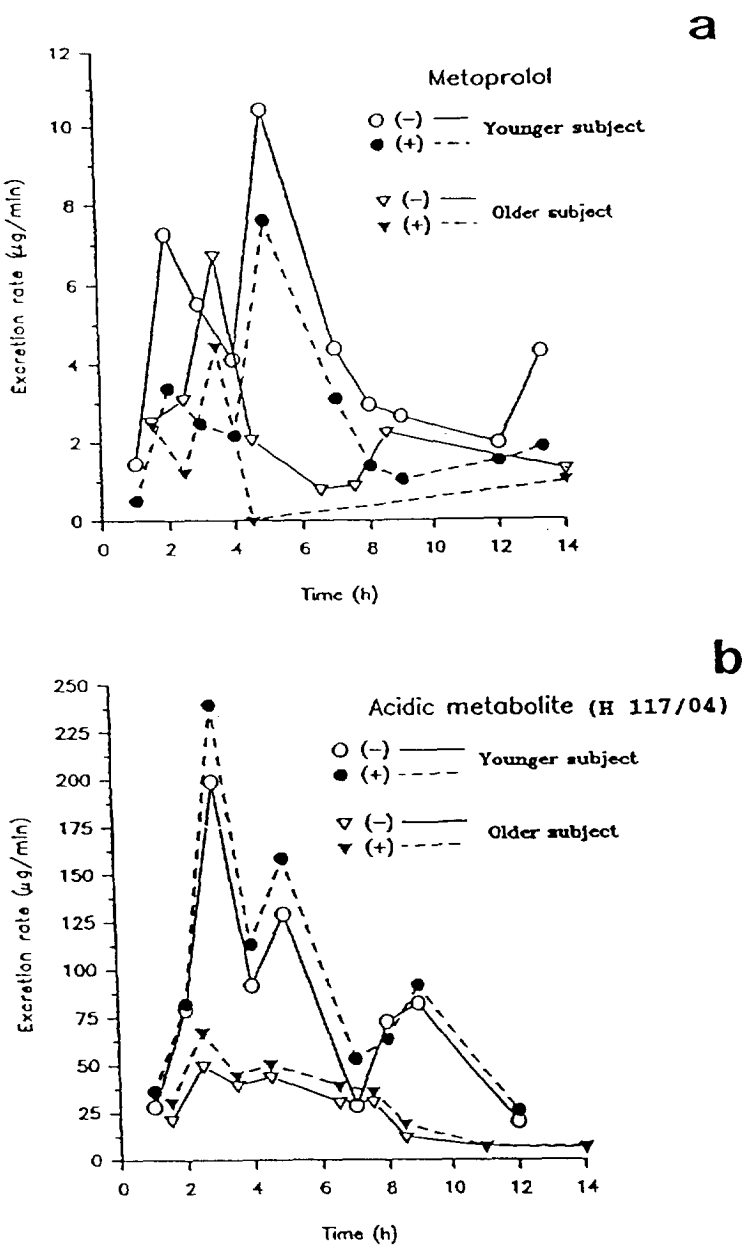

Fig. 4. Excretion rate profiles in younger and older subjects receiving 128 mg of racemic metoprolol tartrate for (a) metoprolol, (b) acidic metabolite H 117/04.

cannot be used for evaluation of the bioavailability of the oral dose [16]. The stereoselective metabolism of metoprolol was studied in the present paper. The data on urinary excretion rate indicated that the acidic metabolite H 117/04 is the major urinary excretion product in man after an oral dose of metoprolol (Fig. 4a,b). The (−)-metoprolol has a slightly higher excretion rate than (+)-metoprolol, while the (−)-acidic metabolite H 117/04 has a slightly lower excretion rate than (+)-acidic metabolite H 117/04 for both younger and older subjects (Fig. 4a,b). Our results for enantiomeric metoprolol excretion rates in human urine are similar to the findings previously reported in human plasma [4,10,11].

3.4. Detection limit and recovery studies

The detection limit of the assay determined from extracted spiked urine standard was 5 ng for each enantiomer of metoprolol and 25 ng for each enantiomer of acidic metabolite H 117/04 (corresponding to absolute amount injected). The detection limit was defined at a signal-to-noise ratio of 3:1. The reproducibility of the results and the recovery of the enantiomers of metoprolol and acidic metabolite H 117/04 are given in Tables 1 and 2, respectively. The maximum coefficient of variation (C.V.) is 9.7% for metoprolol and 13.3% for the acidic metabolite H 117/04. The calibration curves were linear up to 22.5 μg/ml for (−)- and (+)-metoprolol, and up to 45 μg/ml for (−)- and (+)-acidic metabolite H 117/04.

3.5. Esterification after solid-phase extraction

The carboxylic group was protected by esterification with BF_3 in methanol and the excess reagent was decomposed by adjusting the pH to 12 during the extraction procedure. Diazomethane was also tried to protect the carboxylic group. Due to the variation in the absorbance of diazomethane in diethyl ether, this reagent could not be used for the quantitative determination of the acidic metabolite H 117/04 in urine in our studies.

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