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Efficient high-performance liquid chromatographic assay for the simultaneous determination of metoprolol and two main metabolites in human urine by solid-phase extraction and fluorescence detection

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Abstract

An improved, more efficient method for the determination of metoprolol and its two metabolites in human urine is reported. The simultaneous analysis of the zwitterionic metoprolol acidic metabolite (**III**, H117/04) with the basic metabolites α-hydroxymetoprolol (**II**, H119/66), metoprolol (**I**) and guanoxan (**IV**, internal standard) was achieved employing solid-phase extraction and isocratic reversed-phase HPLC. The analytes were extracted from urine (100 μl) using C_{18} solid-phase extraction cartridges (100 mg), and eluted with aqueous acetic acid (0.1%, v/v)–methanol mixture (40:60, v/v, 1.2 ml). The eluents were concentrated (250 μl) under vacuum, and aliquots (100 μl) were analysed by HPLC with fluorescence detection at 229 nm (excitation) and 309 nm (emission) using simple isocratic reversed-phase HPLC (Novapak C_{18} radial compression cartridge, 4 μm, 100×5 mm I.D.). Acetonitrile–methanol–TEA/phosphate buffer pH 3.0 (9:1:90, v/v) was employed as the eluent (1.4 ml/min). All components were fully resolved within 18 min, and the calibration curves for the individual analytes were linear ($r^2 \geq 0.996$) within the concentration range of 0.25–40.0 mg/ml. Recoveries for all four analytes were greater than 76% ($n=4$). The assay method was validated with intra-day and inter-day variations less than 2.5%. © 1997 Elsevier Science B.V.

Keywords: Metoprolol; α-Hydroxymetoprolol

1. Introduction

Metoprolol, 1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol (**I**), is a β_1 selective adrenoceptor antagonist used in the treatment of angina and hypertension [1]. It is mainly eliminated via hepatic metabolism. In humans, the major metabolites, α-hydroxymetoprolol (**II**, H119/66) and the carboxylic acid metabolite (**III**, H117/04), are generated by aliphatic hydroxylation, and *O*-dealkylation with subsequent oxidation, respectively. About 85% of the administered dose is excreted in the urine as metabolites with a small amount of the unchanged parent compound (**I**) [2]. The structures of the analytes and internal standard, guanoxan (**IV**) are presented in Fig. 1 (please refer to Ref. [3] for a proposed metabolic pathway).

At present metoprolol is only available as a racemic mixture. Numerous assays for the detection of the enantiomers of metoprolol and its metabolites

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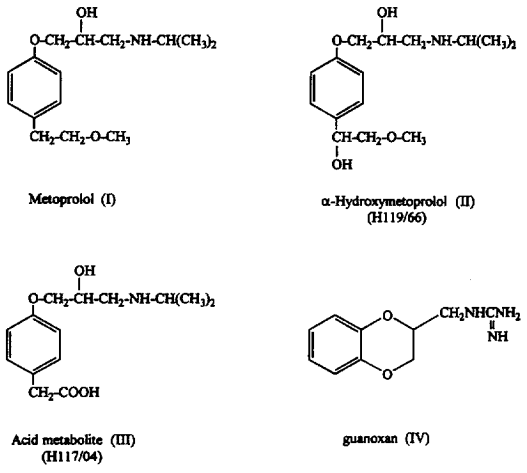

Fig. 1. Chemical structures of metoprolol (**I**), α-hydroxymetoprolol (**II**), metoprolol acid metabolite (**III**) and the internal standard, guanoxan (**IV**).

have been reported. For example, reversed-phase HPLC has been used with chiral derivatization [3], whereas chiral stationary phase HPLC has been used with underivatized samples [4]. The main objective of the current study is to compare the pharmacokinetic (PK) and pharmacodynamic (PD) differences between normal healthy Chinese and Caucasian populations. Thus, a simple and reliable assay was necessary for the determination of the total drug concentrations of metoprolol and its main metabolites. The need for a more extensive and complicated chiral assay could not be justified at the moment unless the PK/PD differences observed between the races could not be explained on the basis of total drug concentrations alone.

Previous metabolic and pharmacokinetic studies of racemic metoprolol employing HPLC methods have been largely focused on metoprolol (**I**) and its basic metabolite, α-hydroxymetoprolol (**II**) [5–7]. A GC method for the detection of three of the main metabolites has been reported but the method requires elaborate sample preparation and derivatization procedures [8]. The determination of the acidic metabolite (**III**) simultaneously with metoprolol (**I**) and α-hydroxymetoprolol (**II**) using gradient elution HPLC conditions has been reported [9]. In that report [9], plasma samples were analysed after extensive sample preparation procedure using solid-phase extraction, whereas urine samples were directly injected onto the HPLC column.

We describe here a simple, robust and more efficient isocratic HPLC assay for the simultaneous determination of metoprolol and its two main metabolites (**II**) and (**III**) in urine using solid-phase extraction (SPE) procedure and fluorescence detection. This method has been successfully employed on a routine basis for our study on the comparative metabolism and pharmacokinetics of metoprolol in healthy Chinese and Caucasian volunteers.

2. Experimental

2.1. Materials

Metoprolol tartrate (**I**) and guanoxan sulphate (**IV**) were kindly provided by Professor G.T. Tucker, Department of Medicine and Pharmacology, University of Sheffield, UK. The metoprolol acidic metabolite (**III**, H117/04) and α-hydroxymetoprolol *p*-hydroxybenzoate (**II**, H119/66), were kindly provided by Astra Hassle (Mölndal, Sweden, via Mr. Henry Li in their Hong Kong Office). HPLC grade acetonitrile and methanol were purchased from Mallinckrodt Chemical (Chesterfield, MO, USA). Analytical grade triethylamine (TEA) was purchased from Riedel-de Häen (Seelze, Germany). Analytical grade phosphoric acid, sodium dihydrogen phosphate, sodium hydroxide and sulphuric acid were purchased from BDH (Poole, UK). All reagents were used without further purification. Distilled and deionized water was used throughout the study. Aqueous stock solutions of metoprolol (**I**), α-hydroxymetoprolol (**II**), the acid metabolite (**III**) and the internal standard, guanoxan sulphate (**IV**), were prepared at 200 μg/ml and stored at 4°C. The working solutions for spiking blank urine samples were prepared fresh daily. Isolute C_{18} solid-phase extraction (SPE) cartridges (100 mg) were purchased from International Sorbent Technology (Mid Glamorgan, UK).

2.2. Chromatography

A Hewlett-Packard series 1050 HPLC system equipped with an HP 1046A programmable fluores-

cence detector and ChemStation software package (Hewlett-Packard Company, Wilmington, DE, USA) was employed. Separation was achieved using a Waters (Milford, MA, USA) Novapak C_{18} radial compression cartridge column (4 μm, 100×5 mm I.D.). The HPLC mobile phase was composed of acetonitrile–methanol–TEA/phosphate buffer (0.5% TEA adjusted to pH 3.0 with phosphoric acid) (9:1:90, v/v) at a flow-rate of 1.4 ml/min. The eluent was monitored by fluorescence detection at 229 nm (excitation) and 309 nm (emission).

2.3. Sample preparation

Each urine sample (100 μl) was mixed with water (100 μl), internal standard (100 μl, 10 μg/ml guanoxan sulphate) and sodium phosphate buffer (0.7 ml, pH 7, 50 m*M*). The mixture was applied onto a C_{18} solid-phase extraction cartridge previously pre-conditioned by washing sequentially with methanol (1 ml), water (1 ml) and sodium phosphate buffer (0.3 ml). The sample solution was allowed to run through by gravity and the cartridge was washed with water (1 ml). The analytes were eluted from the cartridge with aqueous acetic acid–methanol mixture (1.2 ml, methanol–0.1% aqueous acetic acid, 60:40, v/v). The eluent was collected in a glass centrifuge tube and concentrated in vacuo to about 250 μl (Centrivap vacuum concentrator, Labcono, MO, USA). An aliquot (100 μl) was analysed by HPLC.

The standard samples for the individual calibration curves were prepared by spiking blank urine samples (100 μl) with the appropriate standard solutions (100 μl) to give equivalent concentrations of metoprolol (**I**) and α-hydroxymetoprolol (**II**) at 0.25, 0.50, 1.0, 2.0 and 4.0 μg/ml, and the acid metabolite (**III**) at 2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml. The samples were then spiked with the internal standard (**IV**), diluted with buffer, extracted using the SPE procedure and analysed in an identical manner to that of the test samples. The results from three calibration determinations carried out on three separate days were combined for the calculation of the standard curve.

The extraction recoveries of the analytes and internal standard were determined by performing HPLC runs of SPE prepared blank urine samples spiked with known amounts of the analytes, and reference samples containing the equivalent amounts of the analytes in water without any sample preparation procedures. The percentage recoveries of the individual compounds were calculated by comparing the respective analyte peak areas of the extracted samples relative to those of the untreated reference samples.

2.4. Assay validation

Multiple spiked blank urine samples (n=4) at 1.0, 1.0 and 10.0 μg/ml of metoprolol (**I**), α-hydroxymetoprolol (**II**) and the acid metabolite (**III**), respectively were analysed over three separate days using the procedures described in Sections 2.2 and 2.3. The accuracy of the assay was expressed as the mean percentage deviation from the expected values (%bias). The intra-day and inter-day variabilities were expressed as coefficient of variation (%C.V.).

3. Results and discussion

3.1. Sample preparation

Due to the zwitterionic nature of the acid metabolite (**III**), a simple solvent extraction procedure is not feasible for the concurrent analysis of this metabolite with the basic compounds, metoprolol (**I**) and α-hydroxymetoprolol (**II**). Two procedures involving solid-phase extraction (of plasma) and direct HPLC injection (of urine) have been reported by Balmer et al. [9]. Interference from other urine components was the major problem in the method using direct injection of untreated urine. In addition, this practice would also shorten the life span of the HPLC column. The solid-phase extraction method reported by Balmer et al. [9] for the analysis of plasma samples required the use of sodium dodecyl sulphate (SDS) as an ion-pairing reagent to improve the adsorption of the analytes onto the C_{18} matrix. Subsequent elution was carried out with acetonitrile–dichloromethane mixture, and finally a back extraction into aqueous acid was required. This multi-step sample preparation procedure was highly cumbersome, and not suitable for processing the large number of samples generated from our study.

In the current study using the solid-phase ex-

traction procedure, all three analytes were found to be efficiently extracted by the SPE cartridges (recovery>76%) when diluted urine samples buffered at neutral pH were loaded. After washing with water, the analytes were eluted using aqueous acetic acid (0.1%, v/v)–methanol mixture (40:60, v/v). The eluents were concentrated to remove excess methanol before HPLC analysis as the retention times and resolution of the polar components (**II**) and (**III**) were influenced by the presence of organic modifiers in the sample. The resulting extracts were free from endogenous interference peaks when analysed by HPLC (cf., Fig. 2b after SPE and Fig. 2c direct injection of untreated urine sample).

The extraction recoveries of the analytes and internal standard were determined by comparing the respective peak areas of each compounds from the chromatograms of samples prepared with SPE procedure relative to the untreated reference sample containing an equivalent amount of the compounds. The recoveries of the basic compounds, metoprolol (**I**), α-hydroxymetoprolol (**II**) and the internal standard, guanoxan (**IV**), over the concentration range of the assay were uniformly high at 85%. The recovery of the acidic metabolite (**III**) over the concentration range of the calibration curve was 76%.

It was observed that for the extremely polar acidic metabolite (**III**) of metoprolol, the extraction efficiency using SPE method is highly dependent on the extraction conditions, and a moderate rate of sample application and elution is desirable. Thus, the urine sample was allowed to percolate through the SPE cartridge by gravity. The time required to process a single sample is around 20 min, and around 30 min for a batch of ten samples processed in sequence.

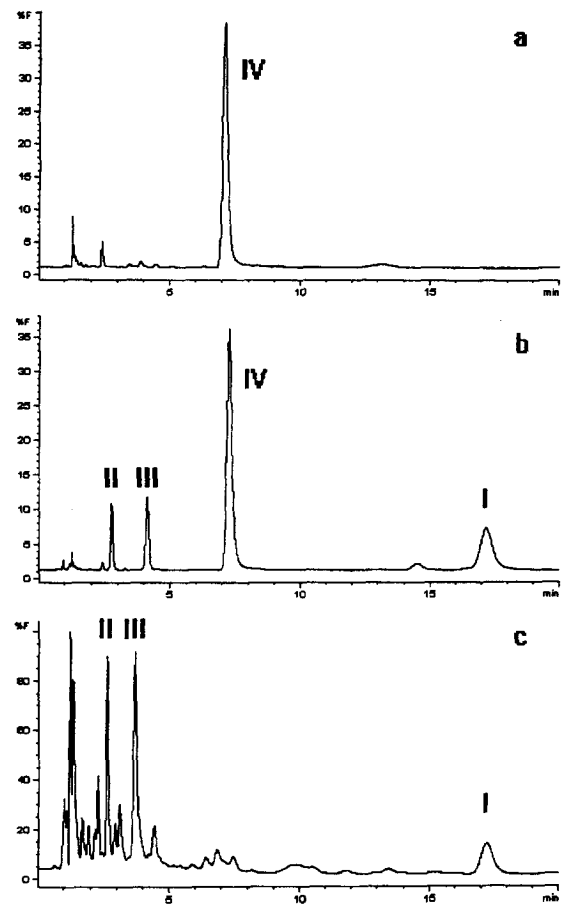

Fig. 2. Chromatograms of authentic human urine samples after a 100 mg oral dose of metoprolol tartrate: (a) pre-dose urine after SPE extraction; (b) urine sample (6–10 h) with SPE sample preparation; (c) urine sample (6–10 h) injected directly for HPLC analysis (50 μl). The authentic urine sample (6–10 h after dosing) containing metoprolol (**I**) (0.637 μg/ml), α-hydroxymetoprolol (**II**) (0.617 μg/ml) and the acid metabolite (**III**) (4.88 μg/ml). Peak (IV) is the internal standard, guanoxan (**IV**).

3.2. Chromatography

An established assay employing isocratic HPLC elution conditions and guanoxan as internal standard for the analysis of the plasma level of metoprolol and its metabolites has been routinely used in our laboratory [10]. It was found in the present study that the assay is also adequate for the complete separation of the analytes in urine samples; interference from other urine components was not observed. Metoprolol (**I**), α-hydroxymetoprolol (**II**), the acid metabolite (**III**) and the internal standard, guanoxan (**IV**), were eluted at 17.1, 2.8, 4.1 and 7.2 min, respectively. Ion-pairing reagents and gradient elution were not required. Fig. 2 shows the chromatograms of blank and authentic urine samples after SPE treatment, and authentic urine sample injected directly. These chromatograms demonstrated that the internal standard, guanoxan (**IV**), is well resolved from other analytes, and it also has good fluorescence intensity and extractability. However, other suitable compounds with adequate resolution and fluorescence property,

if identified, can be used as alternative internal standards.

3.3. Assay linearity

Quantitative analysis was performed using the peak area ratio method with respect to the internal standard, guanoxan. The calibration curves of the analytes obtained over three independent runs were linear within the calibration range. The regression equations describing the calibration runs were: metoprolol (**I**) (0.25 to 4.00 μg/ml), $y=5.037x+0.203$ ($r^2=0.9981$); α-hydroxymetoprolol (**II**) (0.25 to 4.00 μg/ml), $y=5.518x+0.399$ ($r^2=0.9999$); metoprolol acidic metabolite (**III**) (2.50 to 40.0 μg/ml), $y=0.956x+0.937$ ($r^2=0.9963$).

3.4. Assay validation

The accuracy and precision of the assay were estimated by performing analysis on replicates ($n=4$) of spiked urine samples at 1.0, 1.0 and 10.0 μg/ml of metoprolol (**I**), α-hydroxymetoprolol (**II**) and the acid metabolite (**III**), respectively over three separate days. The accuracy (%bias) was within 7% for all three analytes, and the intra-day and inter-day variations of the three analytes were all less than 2.5% (%C.V.) (Table 1).

3.5. Calibration ranges and detection limits

The observed urinary concentrations of metoprolol and its metabolites after a 100 mg dose of metoprolol tartrate over a 24 h duration were relatively high compared to the plasma concentrations. In the current study, the calibration ranges of the analytes were chosen to cover the expected range of urinary concentrations. Moreover, under the current chromatographic conditions, the detection limits were 1 ng/ml for metoprolol (**I**) and α-hydroxymetoprolol (**II**) and 10 ng/ml for the acid metabolite (**III**) at a signal-to-noise ratio of 5:1.

4. Conclusions

The assay procedure described in this report is simple, robust and highly efficient. It requires minimal sample preparation and simple isocratic HPLC conditions when metoprolol (**I**) and its two main metabolites, (**II**) and (**III**), can be simultaneously determined. This procedure was successfully applied in the study of the urinary elimination of a single 100 mg oral dose of metoprolol in healthy Chinese subjects ($n=6$) over 24 h. Metoprolol (**I**) was excreted (expressed as the % administered dose) mainly as the acid metabolite (**III**) (51.4±7.6%) with a small amount of α-hydroxymetoprolol (**II**) (7.0±2.0%) and the unchanged compound (8.3±2.8%).

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Table 1
Validation and extraction recovery data of metoprolol, α-hydroxymetoprolol, metoprolol acid metabolite and guanoxan

	Spiked concentration (μg/ml)	Estimated concentration (μg/ml)	%C.V.	%Bias	Recovery (%)
Metoprolol (**I**)	1.0	1.065	0.8	+6.5	85.3
α-Hydroxymetoprolol (**II**)	1.0	1.020	1.3	+2.0	84.8
Metoprolol acidic metabolite (**III**)	10.0	10.71	2.1	+7.1	77.3
Guanoxan (**IV**)	10.0	–	–	–	86.8

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