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Enantioselective determination of metoprolol and major metabolites in human urine by capillary electrophoresis

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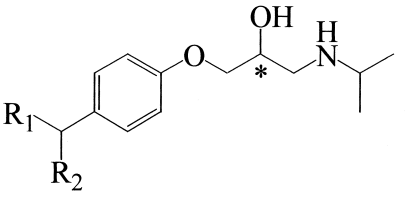
Abstract

The enantiomeric separation of metoprolol and its metabolites in human urine was undertaken using capillary electrophoresis (CE). Resolution of the enantiomers was achieved using carboxymethyl-β-cyclodextrin (CM-β-CD) as the chiral selector. A 100-m*M* acetate buffer (pH 4.0) containing 5% 2-propanol and 10 m*M* CM-β-CD resulted in the optimum separation of the metoprolol enantiomers and its acidic metabolite in human urine. Following a single metoprolol oral administration of 100 mg racemic metoprolol tartrate, stereoselective pharmacokinetic analysis showed that urinary acidic metabolite **3** of metoprolol accounted for 62.3% of the dose with an *R*/*S* ratio of 1.23 and urinary unchanged metoprolol **1** accounted for 6.3% of the dose with an *R*/*S* ratio of 0.72.

Keywords: Enantiomer separation; Metoprolol

1. Introduction

Metoprolol is a β_1 selective adrenoceptor antagonist used for the treatment of angina and hypertension (Fig. 1). The affinity of β_1 adrenergic receptor for (*S*)-metoprolol is significantly higher than for (*R*)-metoprolol [1]. Metoprolol is eliminated by the liver [2] mainly via oxidative deamination and *O*-dealkylation with further oxidation and aliphatic hydroxylation [3,4]. The enantiomers of metoprolol are, however, metabolized at different rates [5]. Since metoprolol is extensively metabolized in human, determination of the enantiomers of meto-



R_1	R_2	
CH_2OCH_3	H	Metoprolol(**1**)
CH_2OH	H	O-demethylmetoprolol(**2**)
COOH	H	Metoprolol acidic metabolite(**3**)
CH_2OCH_3	OH	α-Hydroxymetoprolol

Fig. 1. Structures of metoprolol and its main metabolites. The chiral center is marked with asterisk.

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prolol and their major metabolites is important for establishing a pharmacokinetic profile [6].

HPLC is the method most widely used for the determination of metoprolol or its metabolites, either by direct separation [7–10] or by separation after chiral derivatization [6,11]. Recently, capillary electrophoresis (CE) has developed into a powerful tool, including applications in the chiral analysis of metoprolol [12–14]. Although CE is an effective separation technique for chiral compounds, it is rarely used for the simultaneous chiral determination of metoprolol and its metabolites. For direct enantiomeric separation by CE, a large number of chiral selectors have been developed and the modified cyclodextrins (CD) are by far the most extensively used.

For the optimal resolution of chiral compounds by CE, separation parameters such as buffer composition, chiral selector and organic modifier require careful investigation. In this work, the enantioselective method for the analysis of metoprolol and its demethylated and carboxylated metabolites by CE was evaluated, and the concentration of enantiomers in human urine was determined.

2. Experimental

2.1. Apparatus

The experiments were performed on a Biofocus 3000 CE system (Bio-Rad, CA, USA) equipped with an autosampler, variable wavelength UV detector. The detection was performed at 210 and 280 nm. All separations were carried out with a 75-μm I.D. uncoated fused-silica capillary (length 47 cm, 40 cm to detector). Before use, the capillary was washed successively with 0.1 *M* NaOH for 30 s, water for 2 min and separation buffer for 2 min. The capillary was thermostated at 20°C. A computer was used for instrument control and data handling (software Biofocus integrator). Injection was performed hydrodynamically at the anodic end of the capillary with 3 p.s.i.×s. The pH of the buffer was adjusted by means of a pH meter (ATI Orion Model 370, MA, USA).

2.2. Chemicals and reagents

Carboxymethyl (CM)-, hydroxypropyl (HP)- and succinyl (SUC)-β-CD with average substitution degree of 0.5, 0.9, and 0.4, respectively, were purchased from Wacker Chemie (Munich, Germany) and dimethyl (DM)-β-CD was from Beckman (CA, USA). Racemic metoprolol tartrate was kindly supplied by Yuhan (Korea). Stereoisomers of **1**, **2** and **3** were synthesized in this laboratory by the published method [15]. The purity of synthesized chemicals was more than 98%. Other chemicals and solvents were of analytical-reagent or HPLC grade.

2.3. Selection of running buffer for separation

The running buffer was prepared by adding an organic modifier (tetrahydrofuran, acetonitrile, methanol or 2-propanol) to a solution of 10 m*M* chiral selector (CM-, HP-, SUC- or DM-β-CD) in 100 m*M* phosphate buffer (pH 4.0). The resolution and migration time of enantiomers was monitored and a running buffer with suitable composition was selected. In some cases the selected buffer had to be further modified to increase the resolution and avoid some interfering compounds in human urine.

2.4. Collection and pretreatment of human urine

Blank urine was collected before the administration of a single 100-mg oral dose of racemic metoprolol tartrate to four healthy female volunteers (23 years, 45 kg; 23 years, 47 kg; 24 years, 43 kg; 27 years, 48 kg). Urine samples were collected at 1-h interval until 5 h, and 2-h interval for the next 8 h, and frozen in the dark at −70°C until analysis. For the analysis of **1** and **2**, 5 ml of urine were basified with 3 ml of 12-*M* NaOH solution and extracted twice with 5 ml of ethyl acetate. Organic layer was evaporated to dryness under vacuum. The residue was dissolved in 0.5 ml of water and injected to capillary. For the analysis of **1** and **3**, 5 ml of urine were lyophilized and dissolved in 0.5 ml water. The solution was centrifugated (Microspin, Hanil, Korea) at 15 000 *g* for 10 min and the supernatant was injected directly to the capillary.

2.5. Calibration and recovery test

Stock solutions of standards were prepared in 10 mg/ml methanol and stored at −18°C. Calibration curves were obtained by spiking blank urine with stock solutions of (*RS*)-**1**, (*RS*)-**2** and (*RS*)-**3** to the concentration of 2, 10, 20, 50 and 100 μg/ml, that is, 1, 5, 10, 25 and 50 μg/ml enantiomer. The spiked standards were treated as urine samples and analyzed accordingly. Calibration curves were obtained by plotting concentration versus peak area and analysed by linear regression analysis. Recoveries were tested by comparing the starting amount of the standards in blank urine with that in methanol.

3. Results and discussion

3.1. Effects of buffer on the resolution of enantiomers

Buffer composition and the type of chiral selector are of key importance in chiral analysis by CE, as the buffer constituents and properties can determine the migration behavior of the analytes. A preliminary experiment for the enantiomeric analysis of **1** was carried out with four different modified CDs as chiral selector. The first time the metoprolol enantiomers could not be separated with either DM-, HP-, SUC- or CM-β-CD (18 m*M* CD in 50 m*M* phosphate buffer, pH 3.0). In case of CM-β-CD, the separation was improved by changing the pH of running buffer to 4.0, so CM-β-CD was then introduced as chiral selector for separating the enantiomers of metoprolol (data not shown).

The type of organic modifier affected the separation of enantiomers in a significant and complex manner, as shown in Fig. 2. It is generally known that the organic modifier can change the zeta potential and viscosity of the buffer solution, but the relationship between resolution and physical properties of the organic modifier in this case was difficult to determine. Of the four organic modifiers tested, 2-propanol gave the best resolution, although, the resolution between (*S*)-**1** and (*R*)-**2** was still not acceptable. By changing the phosphate buffer for acetate buffer (100 m*M*, pH 4.0), the resolution was enhanced and a base line resolution could be achieved (Fig. 3).

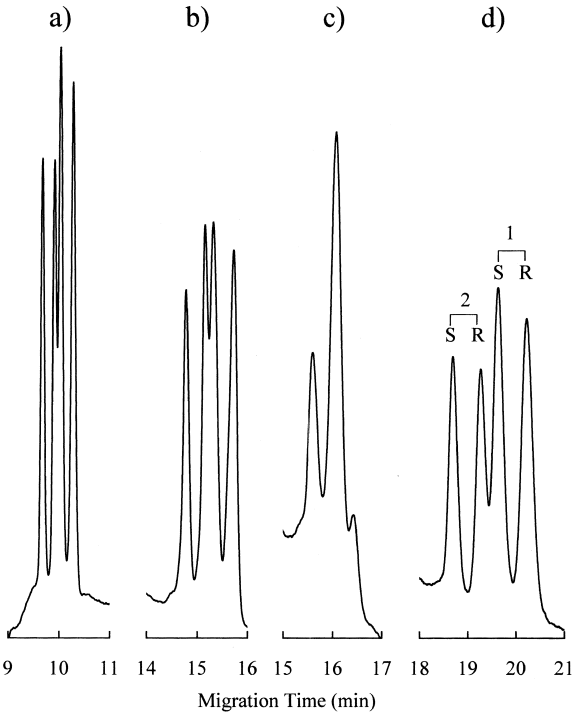

Fig. 2. Electropherograms of metoprolol (**1**) and *O*-demethylmetoprolol (**2**) with 100 m*M* phosphate buffer (pH 4.0) containing 10 m*M* CM-β-CD and 3% organic modifier. Organic modifier: (a) tetrahydrofuran, (b) acetonitrile, (c) methanol, (d) 2-propanol.

3.2. Method validation

The method was validated in the concentration range of 1–50 μg/ml enantiomer in urine. Calibration curves obtained by plotting concentration versus peak area showed linearity. The results of recovery test are presented in Table 1. Both accuracy and precision indicated that this method is suitable and applicable for the determination of enantiomers of metoprolol and metabolites in human urine. The detection limit of enantiomers at a signal-to-noise ratio of 3 was ~0.5 μg/ml urine.

Sometimes EOF instability was observed and some difficulties occurred in determining the enantiomer concentration in standard solutions as well

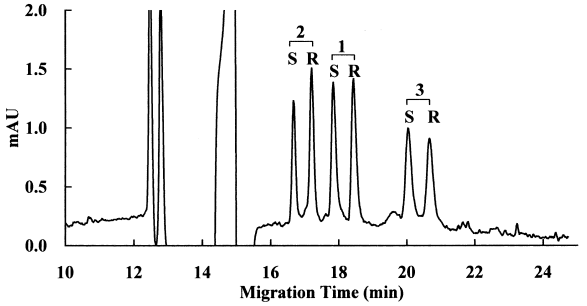

Fig. 3. Electropherogram of mixed standard solution of metoprolol and metabolites at the concentration of 50 μg/ml for each enantiomer. Running buffer: 100 m*M* acetate buffer (pH 4.0) containing 3% 2-propanol and 10 m*M* CM-β-CD. CE condition: capillary, fused-silica, 75 μm I.D.×47 cm (40 cm to detector); temperature of capillary, 20°C; applied voltage, 10 kV; detection, UV 210 nm. Peaks: (1) metoprolol, (2) *O*-demethylmetoprolol, (3) metoprolol acidic metabolite.

as urine samples. This may be caused by the incomplete reaction of siloxane to silanol groups during the preconditioning process. This phenomenon could be reduced by longer preconditioning times (more than 1 h) with 1 *M* NaOH.

3.3. Analysis of the enantiomers of metoprolol and metabolites

The CE separation method was applied to urine samples taken from four healthy volunteers following a single oral dose of racemic metoprolol tartrate (100 mg). The initial sample preparation was carried out by liquid–liquid extraction using ethyl acetate to reduce the effects of the endogenous matrix in the urine. More than 90% of **1** and metabolite **2** was recovered, but metabolite **3** was not extracted by ethyl acetate. Consequently, direct injection was performed with preconcentrated urine samples by the lyophilization method. Due to presence of interfering substances in human urine that were apparent in the lyophilized samples, the buffer composition was further modified to avoid the effects of interference. When the concentration of 2-propanol in the running buffer was increased to 5%, **1** and **3** in the lyophilized samples could be analyzed without any interferences, but **2** still suffered from interferences in the urine (Fig. 4b). Although metabolite **2** could be analyzed by liquid–liquid extraction, its concentration in urine was too low to be significant in the calculation of excretion (Fig. 4a). From the low concentration of **2**, it seems that the formation of **2** is a rate-determining step in the metabolism of **1** to **3** through **2**.

Stereoselectivity data on the excretion of **1** and formation of **3** at different collection intervals are summarized in Table 2. In human urine, stereoselectivity was observed in both **1** and **3**. The *R*/*S* ratio of the formation of **3** was 1.23 across the interval of 0–11 h, favoring the metabolism of (*R*)-enantiomer. Stereoselectivity was high in the early stage of metabolism and decreased by the late collection intervals; for example, the *R*/*S* ratios of **3** were 1.25 and 1.10 in the intervals of 0–5 and 5–11 h, respectively. Approximately 6.3% of the dose with an *R*/*S* ratio of 0.72 was excreted in urine as unchanged **1**. The metabolite **3** accounted for 62.3% of the dose and this amount is comparable to that

Table 1
Recovery of enantiomers of metoprolol and metabolites from human urine spiked with racemic standards (mean(%)±standard deviation of four experiments)

Amount added (racemate, μg/ml)	Metoprolol	*O*-demethyl -metoprolol	Acidic metabolite
2	(*S*) 92.1±6.3	(*S*) 91.3±6.2	(*S*) 89.2±7.2
	(*R*) 91.4±5.8	(*R*) 92.5±5.7	(*R*) 90.1±8.8
10	(*S*) 91.8±4.2	(*S*) 91.3±5.5	(*S*) 87.5±6.7
	(*R*) 91.4±5.1	(*R*) 93.6±5.3	(*R*) 89.7±5.4
20	(*S*) 90.7±4.6	(*S*) 92.1±4.2	(*S*) 90.1±4.7
	(*R*) 92.3±3.5	(*R*) 91.2±3.2	(*R*) 87.9±4.9
50	(*S*) 90.6±4.1	(*S*) 90.5±3.7	(*S*) 91.2±4.8
	(*R*) 92.5±3.3	(*R*) 91.2±4.4	(*R*) 90.3±5.5

Metoprolol and acidic metabolite were analyzed by lyophilization method and *O*-demethylmetoprolol was by extraction with ethyl acetate.

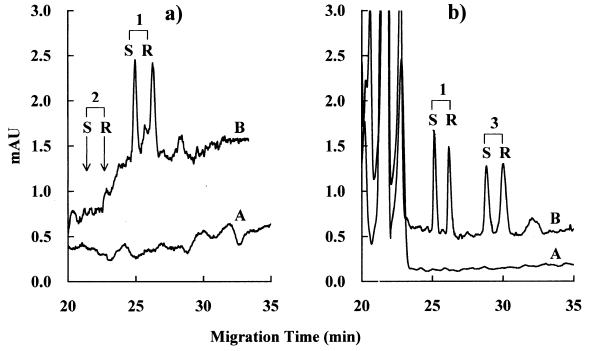

Fig. 4. Representative electropherogram of metoprolol and metabolites assayed in human urine collected to 1 h after the administration of 100-mg oral dose of racemic metoprolol tartrate (B) and blank urine (A). Human urine was pretreated by liquid–liquid extraction with ethyl acetate (a) or lyophilization (b). Running buffer: 100 m*M* acetate buffer (pH 4.0) containing 5% 2-propanol and 10 m*M* CM-β-CD. CE condition: capillary, fused-silica, 75 μm I.D.×47 cm (40 cm to detector); temperature of capillary, 20°C; applied voltage, 10 kV; injection (pressure), 3 p.s.i.×s; detection, UV 210 nm. Peaks: (1) metoprolol, (2) *O*-demethylmetoprolol, (3) metoprolol acidic metabolite.

observed by Murthy et al. [5] (52.7% in 0–24-h interval) and by Lennard [16] (57% in 0–12 h).

4. Conclusions

CE is a useful method in pharmacokinetics because the parent drug and metabolites can be analyzed simultaneously. This study investigated a number of the running parameters for the CE of metoprolol and its metabolites, including the type of buffer, chiral selector and organic modifier. The CE assay method developed in this work is convenient and simple when compared with HPLC. This method was applied successfully to the stereoselective determination of metoprolol and its metabolites in human urine. This pharmacokinetic study of metoprolol following oral administration showed that the acidic metabolite was predominant. The *R*/*S* ratio of 1.23 for the formation of this metabolite favored the metabolism of (*R*)-enantiomer.

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Table 2
The amount of excreted enantiomers of metoprolol and acidic metabolite in human urine after single administration of 100 mg racemic metoprolol tartrate in different collection intervals

Interval (h)	Metoprolol tartrate			Metoprolol acidic metabolite		
	(*S*)	(*R*)	(*R*)/(*S*)	(*S*)	(*R*)	(*R*)/(*S*)
0–1	0.45±0.13	0.34±0.09	0.76±0.03	1.24±0.27	1.55±0.35	1.25±0.01
1–2	0.97±0.22	0.67±0.14	0.69±0.05	4.85±1.30	6.08±1.61	1.26±0.02
2–3	0.99±0.19	0.64±0.12	0.64±0.04	4.90±1.42	6.15±1.75	1.26±0.01
3–4	0.63±0.29	0.45±0.19	0.72±0.06	3.54±0.78	4.44±0.94	1.25±0.01
4–5	0.25±0.14	0.22±0.11	0.90±0.08	2.18±0.48	2.69±0.57	1.24±0.03
5–7	0.32±0.05	0.32±0.09	0.98±0.30	2.79±0.38	3.34±0.49	1.19±0.08
7–9	n.d.	n.d.	n.d.	1.93±0.34	1.91±0.38	0.99±0.19
9–11	n.d.	n.d	n.d.	0.59±0.14	0.56±0.15	0.94±0.21
0–11	3.62±0.97	2.64±0.66	0.72±0.05	22.02±3.42	26.73±3.89	1.23±0.02

Values represent the mean±S.D. of four volunteers. The excreted amount (mg) was calculated as mean concentration×volume of total urine in the interval. n.d., not determined.

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