Isocratic High Performance Liquid Chromatographic Method for Determination of Metoprolol and Its Metabolite in Human Urine

Penentuan Metoprolol dan Metabolitnya dalam Urin Manusia dengan Metode Kromatografi Cair Kinerja Tinggi secara Isokratik

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Abtract: An isocratic reversed phase high performance liquid chromatographic (HPLC) method has been developed for the determination of metoprolol (MET) and α-hydroxymetoprolol (α-OH MET) in human urine. Analytes were extracted using dichloromethane. The optimized separations were performed on a Purospher® STAR RP-18e LiChroCART® (250 x 4.6 mm, 5µm) HPLC column within 12 min by an isocratic elution with 25 mmol/L aqueous potassium dihydrogen phosphate buffer (pH 3.0) containing 15% (v/v) acetonitrile and 15% (v/v) methanol, and UV detection at 234 nm. The method was validated for linearity, accuracy, precision and specificity. The calibration curves for both analytes were linear over the range investigated (1.0-64.0 µg/mL) with correlation coefficients of 0.999. Relative standard deviation (RSD) values were below 2%. For all of the analytes, recoveries value from 98.36±1.68 to 101.32±1.36 % and the limits of detection for MET and α-OH MET were 0.22 and 0.14 µg/mL, respectively. The method developed has been demonstrated to be sensitive and reliable for the measurement of MET and α-OH MET simultaneously in human urine.

Keywords: high performance liquid chromatography, metoprolol, metabolite, human urine.

Abstrak: Metode kromatografi cair kinerja tinggi (KCKT) secara isokratik telah dikembangkan untuk penentuan metoprolol (MET) dan α-hidroksimetoprolol (α-OH MET) dalam urin manusia. Ekstraksi cair-cair menggunakan diklormetan setelah penambahan NaOH. Pemisahan dilakukan pada kolom Purospher® STAR RP-18e LiChroCART® (250 x 4,6 mm, 5µm) dengan fase gerak campuran kalium dihidrogen fosfat 25 mmol/L (pH 3,0), asetonitril dan metanol (70:15:15 v/v). Deteksi dengan detektor ultraviolet pada $\lambda_{234\ nm}$. Validasi metode meliputi linieritas, akurasi, presisi dan spesifisitas. Kurva kalibrasi linier pada rentang 1,0-64,0 µg/mL untuk kedua analit dengan koefisien korelasi 0,999. Metode menunjukkan presisi yang baik dengan nilai relative standard deviation (RSD) <2%. Perolehan kembali pada rentang 98,36±1,68 sampai 101,32±1,36 % dengan batas deteksi MET dan α-OH MET masing-masing 0,22 dan 0,14 µg/mL. Metode KCKT yang dikembangkan menunjukkan sensitivitas dan reliabilitas yang memadai untuk penentuan MET dan α-OH MET secara simultan dalam sampel urin.

Kata kunci: kromatografi cair kinerja tinggi, metoprolol, metabolit, urin.

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INTRODUCTION

METOPROLOL (MET) is a β1-selective receptor antagonist. It is widely used in the treatment of cardiovascular disorders such as coronary artery disease[1], heart failure[2], hypertension[3] and arrhythmia[4]. Metoprolol (Figure 1(a)) is extensively metabolized by phase I processes in the liver[5] by CYP2D[6] as a major catabolic enzyme[6]. Eighty-five percent of metoprolol is metabolized with α-hydroxymetoprolol (α-OH MET, Figure 1(b)) as the main metabolite. As the CYP2D6 gene is highly polymorphic, the appearance of α-OH MET varies depending on the oxidation phenotype which have different capacity to metabolize MET to α-OH MET[7]. Therefore, it is imperative to develop method for determining MET and its metabolite in biological samples either in plasma or in urine.

Several methods have been reported for the quantification of both MET and α-OH MET in human urine. These assays were based on chromatographic methods using fluorescence detector[8, 9,10], diode array detection[11]. Separation MET and its metabolite by HPLC with UV detection has been achieved with either gradient elution[11,12] or isocratic elution[13].

Isocratic HPLC with acetonitrile: acetate buffer (pH 3.5) mixture as mobile phase gives a long analysis time[13]. The purpose of the present study was to develop a simple isocratic HPLC method with UV detection for MET and α-OH MET assay in human urine and to validate it according to International

Figure 1. Chemical structure of MET(a) and α-OH MET (b).

Conference on Harmonisation (ICH) guidelines[14].

MATERIALS AND METHOD

MATERIALS. Metoprolol tartrate and Caffeine as an internal standard (IS) were purchased from Sigma–Aldrich (St.Louis, MO, USA). While α-hydroxymetoprolol was purchased from Toronto Research Chemical. HPLC-grade acetonitrile and methanol were purchased from Merck. Potassium dihydrogen phosphate, *ortho*-phosphoric acid, sodium hydroxide and dichlormethane were obtained from Merck. All other chemicals were HPLC or AR-grade and were used as received.

METHODS. **Chromatographic Conditions**. The HPLC system used was LC-10 AT VP (Shimadzu) consisted of manual injection facility with 20 µL fixed loop, Shimadzu System Controller SCL-10A and UV-Vis SPD 10A and software provided by the manufacturer. Chromatographic separation was performed isocratically on a Purospher® STAR RP-18e LiChroCART® (250 x 4.6 mm, 5µm) HPLC column. The mobile phase consisted of 15% (v/v) acetonitrile, 15% (v/v) methanol and 70% (v/v) 25 mM potassium dihydrogen phosphate buffer (pH 3). The UV detector was set at 234 nm. The flow rate was 1 mL/min with the injection volume of 20 µL.

Validation Method. The method was validated as recommended by ICH for the parameters like specificity, precision, linearity, limit of detection, limit of quantitation and accuracy. The specificity of the method was determined by comparing the chromatograms obtained from the samples containing MET, α-OH MET and IS with those obtained from blank samples. Intra-day and inter-day precision studies were carried out by estimating the corresponding responses 6 times on the same day and on 2 different days at the concentrations of 2; 8; and 32 µg/mL of MET and OH-MET. Linearity was studied by preparing standard MET and OH-MET solutions in blank urine at different concentrations from 1.0 to 64.0 µg/mL. Curves were constructed by plotting concentrations against peak area ratio (peak area compound/peak area internal standard(IS)). Linearity was determine by least-squares regression for both the analytes. Three individually prepared replicates at each concentration were analyzed. Accuracy was evaluated in triplicate, at three different concentrations (2; 8 and 32 µg/mL) by adding a known amount of MET and α-OH MET standard to a pre-analyzed sample. The accuracy was determined by calculating the recovery of MET and α-OH MET, RSD and % recovery for each concentration.

System suitability tests are an integral part of chromatographic methods. The tests are used to verify reproducibility of the chromatographic system. A system suitability test was performed by injecting six injections of the reference solution at the concentration of 5 µg/mL for MET and α-OH MET, to check the reproducibility of the system. The limits of detection (LOD) and quantitation (LOQ) were calculated from

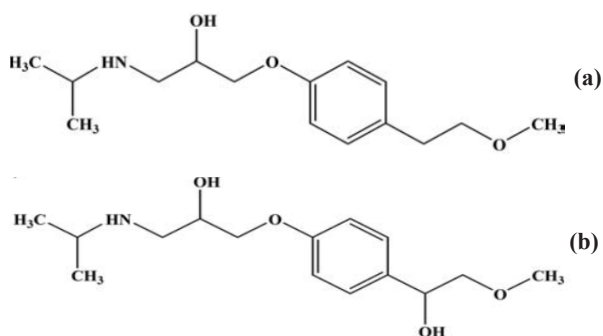a separate calibration curve at low level concentration.

Preparation of Standard Solution. The standard stock solutions of MET and α-OH MET were prepared by dissolving of accurately weighed 10 mg of MET and α-OH MET reference in a 10 mL of volumetric flask and made up to volume with the mobile phase to obtain a concentration of 1000 µg/mL.

Application of Proposed Method to Urine Samples. To an aliquot of 500 µL urine samples from healthy subjects who had been given an oral tablet of 100 mg metoprolol was added 150 µL sodium hydroxide solution (2M), 20 µL internal standard (100µg/mL), and 1000 µL dichlormethane. The samples were vortex-mixed and centrifugated at 8000 rpm for 10 min at room temperature. Then, the organic phase was transferred into a vial and evaporated to dryness with a stream of nitrogen. The residue was dissolved in the mobile phase (1000 µL) and 20 µL of the solution was injected into the HPLC. The concentration of MET and α-OH MET in urine sample was calculated from the peak area ratio using calibration curves for each analytes.

Data Analysis and Statistics. Data were given as arithmetic mean. The precision was expressed as the percentage relative standard deviation (RSD), while the accuracy was expressed as percentage of the MET and α-OH MET concentration measured in each sampel referring to the known amount of MET and α-OH MET added.

RESULTS AND DISCUSSION

The mobile phase consisting of KH_2PO_4 25 mmol/L pH 3 adjusted with ortho-phosphoric acid, acetonitrile and methanol (70:15:15, v/v) with flow rate 1 mL/min was found to be suitable for the chromatographic separation of MET, α-OH MET and IS. The optimized mobile phase gave three sharp peaks with good asymmetry factor for MET, α-OH MET and IS (Figure 2). The representative chromatograms obtained in the absence and presence of MET and α-OH MET are depicted in figure 2 which shows that under presented chromatographic conditions, the retention times for α-OH MET, IS and MET were 3.9; 5.3 and 10.9 min, resepectively. As also shown in figure 2, MET, α-OH MET and IS are well separated from the matrix components with no endogenous interfering peaks at the retention times of the compounds of interest. The effective separation of α-OH MET and MET was
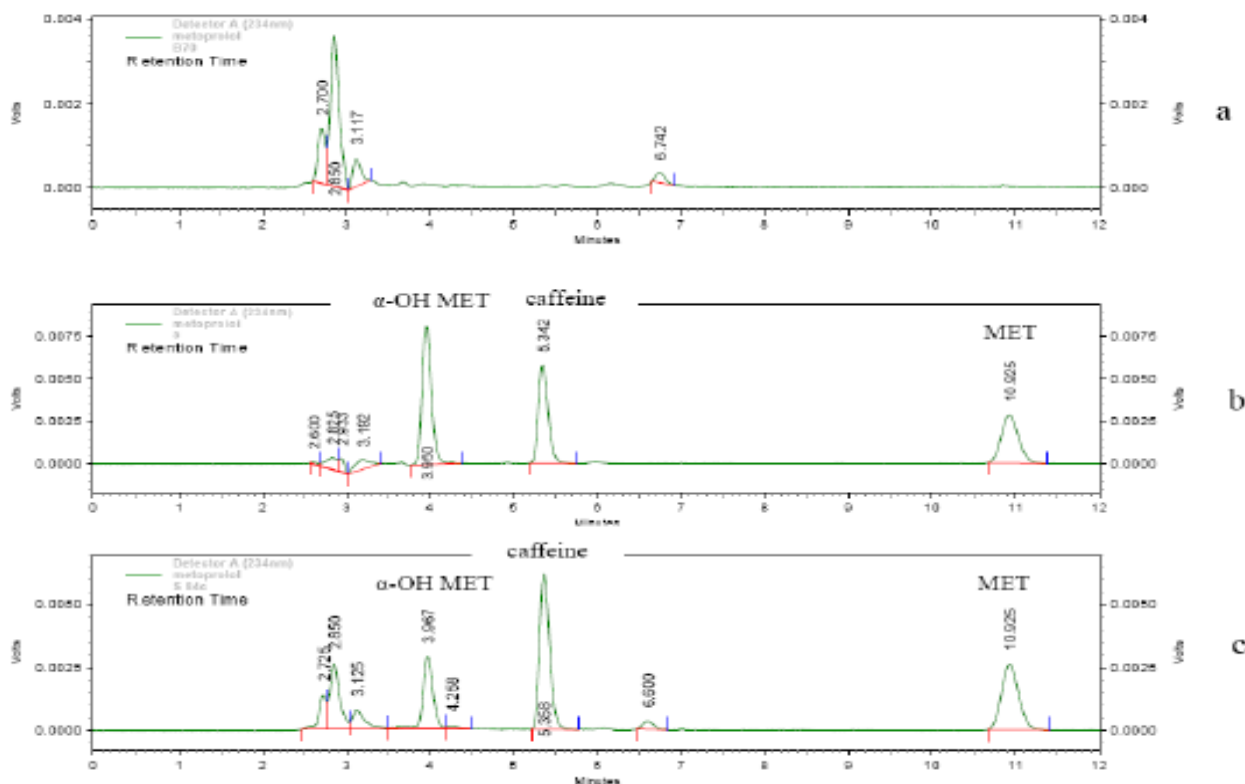
Figure 2. Representative chromatograms obtained during quantification of metoprolol tartrate (tR=10.9 min) and α-hydroxymetoprolol (tR=3.9 min) in human urine with caffeine (tR=5.3 min) as Internal Standard (IS). (a) Blank human urine, (b) standard solution containing MET, α-OH MET and IS, (c) urine sample collected 8 hours after oral administration of a 100 mg MET tablet.

achieved in less than 12 min. The retention time of MET (10.9 min) and α-OH MET (3.9 min) were quite shorter than that studied by Godbillon & Duval[13] and Baranowska & Wilczek[11]

System Suitability. System suitability testing is an integral part of analytical procedures[15]. The performance qualification of HPLC was determined using the system suitability to verify system performance under actual running conditions. The quality of the chromatographic method was monitored by applying the following system for the suitability tests : capacity factor, asymmetry factor and theoretical plates (Table 1). The peak asymmetry factors for MET and α-OH MET were 1.10 and 1.19, respectively. Chromatographic conditions in this study resulted in the peak asymmetry factor and theoretical plates that meets the requirements[15]. The HPLC system also produces good separation results which showed with resolution > 3.

Table 1. System suitability.

Characteristics	α-OH MET	MET
Capacity factor	0.40	2.88
Asymmetry factor	1.19	1.10
Theoretical plates	24171.04	50350.70
Resolution	3.12	18.25

Linearity. Calibration standard of various concentrations of α-OH MET and MET in human blank urine were assayed. The linearity of the calibration curves for MET and α-OH MET in human blank urine at concentration 1-64 µg/mL were excellent (r = 0.999) over the range investigated (Table 2). The linear regression data for the calibration curves (Figure 3) indicate that the response is linear over the

concentration range studied with correlation coefficient (r) value of 0.999. The range of concentrations used in this study is wider than that used in previous studies[11].

Precision. The precision of the assay method was studied with respect to both intra-day and inter-day precision. The method indicated very good precision. Intra-day and inter-day precisions for MET and α-OH MET are summarized in Table 3. The intra-day and inter-day precision were found to be within 0.978 to 1.831% for MET and 0.983 to 1.871 % for α-OH MET, respectively. Intra-day and inter-day precision of the present study were comparable to previously reported method [13].

Table 3. Precision data.

Conc. (µg/mL)	Intra-day precision[a] (%RSD)		Inter-day precision[b] (%RSD)	
	α-OH MET	MET	α-OH MET	MET
2	1.33	1.87	1.64	1.83
8	0.98	1.69	0.98	1.69
32	1.10	1.80	1.23	1.58

Note: a: n = 6; b: n = 12.

Sensitivity. The sensitivity was evaluated by the limits of detection (LOD) and quantitation (LOQ). LOD and LOQ were calculated from calibration curve using the formula LOD=3.3 σ/S and LOQ=10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve[15]. The value of LOD for MET and α-OH MET found were 0.22 and 0.14 µg/mL, respectively, while the limit of quantitation (LOQ) for MET and α-OH MET were 0.67 and 0.42 µg/mL, respectively. The present method provides the LOD and LOQ values for α-OH MET lower than that from previously reported method [11,13], while the LOD and LOQ values for MET

Table 2. Linearity of α-OH MET and MET by HPLC.

Characteristic	Range (µg/mL)	Linear regression	r	LOD (µg/mL)	LOQ (µg/ml
α-OH MET	1.0-64.0	y = 0.22 x + 0.09	0.999	0.14	0.42
MET	1.0-64.0	y = 0.15x - 0.04	0.999	0.30	0.90

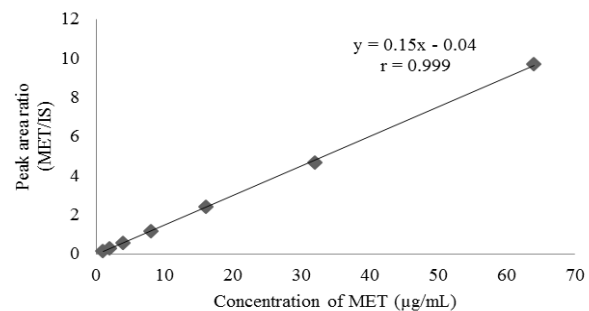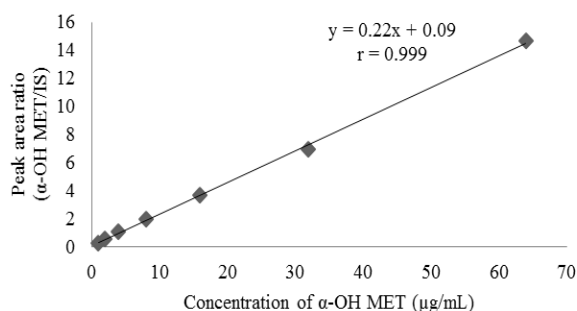**Figure 3. Calibration curves α-OH MET and MET in human urine.**

were lower than that from Godbillon & Duval[13] and comparable to Baranowska & Wilczek[11].

Accuracy. The accuracy of the method was indicated by the values of recovery. The studies were performed at three different concentration levels. The recovery studies were carried out using a spiking method of known amount of α-OH MET and MET, and found to be in the range from 98.36 to 101.32 % as listed in Table 4. RSD was always <2%, which indicates that the method has a high accuration level. The mean recoveries are better than those of the studied reported by Godbillon & Duval[13], Albers et al.[16] and Fang et al.[4]. Precision and accuracy were adequate for analysis of MET and its metabolite in human urine.

Table 4. Accuracy of the MPLC method.

Compounds	Concentration (µg/mL)		Recovery (%)	Precision of recovery (%RSD)
	Spiked	Found		
α-OH MET	2.02	2.02	99.99±1.29	1.30
	8.09	7.96	99.69±1.53	1.53
	32.35	32.35	99.91±1.76	1.77
MET	2.02	1.99	98.36±1.68	1.71
	8.07	8.02	99.36±1.80	1.82
	32.29	32.72	101.32±1.36	1.35

Assay. The validated HPLC method was used for simultaneous determination of MET and α-OH MET in human urine. The method has been applied successfully to analyse MET and α-OH MET in urine samples collected 8 hours after oral administration of a 100 mg metoprolol tablet from 90 healthy volunteers. The concentration ranges of MET and α-OH MET in urine samples were 1.44 to 167.8 µg/mL for MET and 0.32 to 68.79 µg/mL for α-OH MET. The proposed method provides an isocratic HPLC method with adequate precision and accuracy with standard HPLC equipment to measure MET and α-OH MET in urine samples for studying oxidation capacity of CYP2D6 in healthy volunteers.

CONCLUSIONS

The chromatographic method developed has been demonstrated to be sensitive and reliable for the measurements of MET and α-OH MET simultaneously in human urine.

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