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Determination of metoprolol, and its four metabolites in dog plasma

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Abstract

Metoprolol and its metabolites α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid and deaminated metoprolol were quantified in dog plasma using an isocratic high-performance liquid chromatographic method (with a BetaBasic Cyano column) with fluorescence detection. A solid phase extraction technique (Oasis HLB, Waters) was used to extract metoprolol and its four metabolites from dog plasma with high recovery rates. The method was shown to be sensitive and reproducible and was used to determine the pharmacokinetic profile of metoprolol in dog. To the best of our knowledge, this is the only method that can extract and analyze metoprolol and its four metabolites in a single assay.
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Keyword: Metoprolol

1. Introduction

Metoprolol is a relatively selective beta(1)-adrenoceptor antagonist that has been used extensively for more than 25 years to treat such cardiovascular disorders as hypertension, arrhythmia, and heart failure. Metoprolol is metabolized to a large degree by cytochrome P-450 2D6 which is polymorphic in the human population. The appearance of metabolites α-hydroxymetoprolol, *O*-desmethylmetoprolol and metoprolol acid varies depending on oxidation phenotype [1–3]. Other metabolites, including deaminated metoprolol, are less well studied (Fig. 1).

A number of HPLC methods have been reported to analyse metoprolol and up to three of its metabolites [4–9]. To the best of our knowledge, no assay has been reported to separate metoprolol and four of its metabolites. Many of the studies of the past would have been more complete if additional metabolites had been accounted for. We describe here a single-run method for racemic metoprolol and its metabolites α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, and deaminated metoprolol in dog plasma.

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2. Experimental

2.1. Chemicals

Solvents used for extractions and for the preparation of mobile phase were HPLC grade (BDH Inc., Toronto, Ontario, Canada). Metoprolol and Alprenolol were purchased from Sigma-Aldrich Canada Ltd. (Ontario, Canada). Metoprolol metabolites were gifts from Astra Hässle (Mölndal, Sweden). All chemicals purchased were of the highest grade commercially available. Human plasma were obtained from Canadian Red Cross.

2.2. Animals

Four female beagles, 8 years of age, obtained from Marshall Farms, New York, were housed in a hutch with indoor/outdoor dog runs during the summer months (Animal Care Unit, Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada). The animals were normally fed twice, morning and afternoon (Science Diet, canine maintenance) and had fresh water available at all times.

2.3. Extraction procedure

A solid phase extraction procedure was used to extract metoprolol and four of its metabolites. Extract

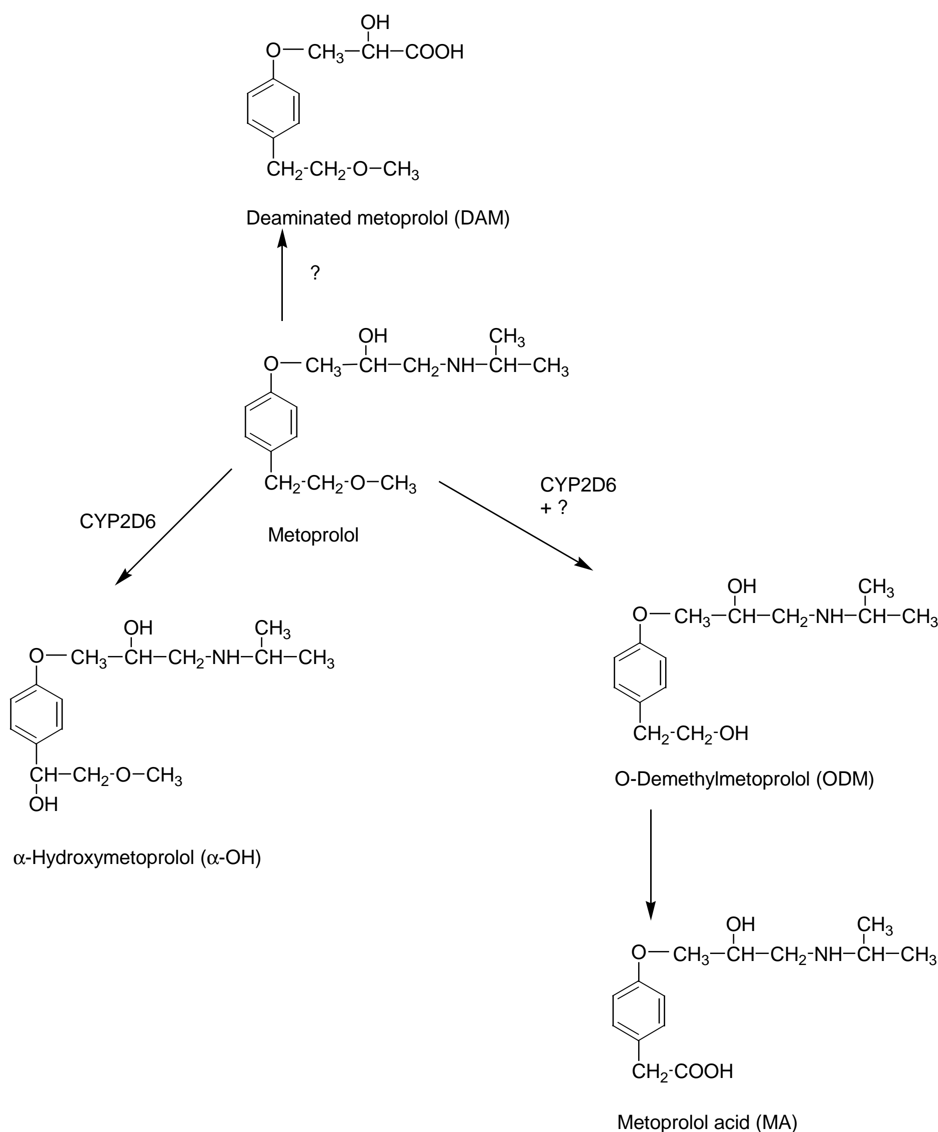

Fig. 1. Structures of metoprolol and its metabolites α-hydroxymetoprolol, *O*-demethylmetoprolol, metoprolol acid and deaminated metoprolol. Enzymes shown in the present study to be involved in each pathway are indicated.

cartridges (Oasis HLB 30 mg, Waters) were first conditioned by rinsing with methanol (1 ml), water (1 ml) and phosphate-buffered saline (200 mg/L KCl, 800 mg/L NaCl, 200 mg/L KH_2PO_4, 1150 mg/L Na_2HPO_4, pH 7.0). Plasma samples (300 μl) were mixed with 20 μl alprenolol (3.33 μg/ml in double distilled water, as internal standard) and 680 μl phosphate-buffered saline and then loaded to the column. The columns were washed with water (1 ml) and the analytes were then eluted with methanol (1 ml). The eluates were evaporated under N_2 and reconstituted in 150 μl of water.

For the standard curves, the spiked sample solutions were prepared by mixing 280 μl plasma with 20 μl of a solution (in double distilled water) containing different concentrations of metoprolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid and deaminated metoprolol. To these spiked plasma samples were then added 20 μl alprenolol (3.33 μg/ml, IS) and 680 μl phosphate-buffered saline (pH 7.0). These mixtures were then extracted as described above. All stock solutions were prepared with double distilled water.

2.4. Instrument analysis

Briefly, the system comprised a Waters 515 pump (Milford, MA, USA), a Waters 717_{plus} Autosampler. A Waters 474 Scanning fluorescence detector (excitation wavelength: 275 nm; emission: 300 nm) was used for the detection of metoprolol and its metabolites. Signals from the fluorescence detector were collected and processed by a Waters Millennium^{32} Chromatography Manager system. A BetaBasic Cyano column (5 μm, 4.6 mm × 250 mm, Keystone Scientific, Inc. PA) fitted with a SecurityGuard™ guard cartridge system (CN, Phenomenex®, Torrance, CA, USA) was

utilised. The mobile phase consisted of acetonitrile–sodium dihydrogen orthophosphate buffer (2 M)–water (5–0.5–94.5 by volume). The mixture was adjusted to pH 2.6 with phosphoric acid and the solvent was delivered at a flow rate of 0.8 ml/min. The injection volume was 20 μl.

2.5. Plasma concentration profiles of metoprolol and its metabolites in dog

The four dogs were fed 30 min before dosing with 1.37 mg/kg metoprolol (in a gelatin capsule). The bleeding schedule consisted of a pre-bleed (5 ml) before the capsule was administered, followed by blood (5 ml) being taken at the following times (after the capsule was taken): 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 h. Blood was collected from the jugular veins.

Collected blood samples were centrifuged (BECKMAN AccuspinFR) for 20 min at 2000 rpm to separate the plasma from the red blood cells. The plasma was manually pipetted to appropriately labeled Eppendorf centrifuge vials. The bulk of the samples were stored at −20 °C until all of the samples were collected.

3. Results

3.1. Chromatography

Fig. 2A is a chromatogram of blank dog plasma spiked with a mixture of standards of α-hydroxymetoprolol, *O*-desmethyl-metoprolol, metoprolol acid, metoprolol, deaminated metoprolol and the internal standard, alprenolol. The peaks are well separated. Fig. 2B is a chromatogram of extracted dog plasma obtained 0.5 h after administering metorpolol. The compounds are well resolved and there is no interference from biological impurities. A chromatogram of blank human plasma is shown in Fig. 2C.

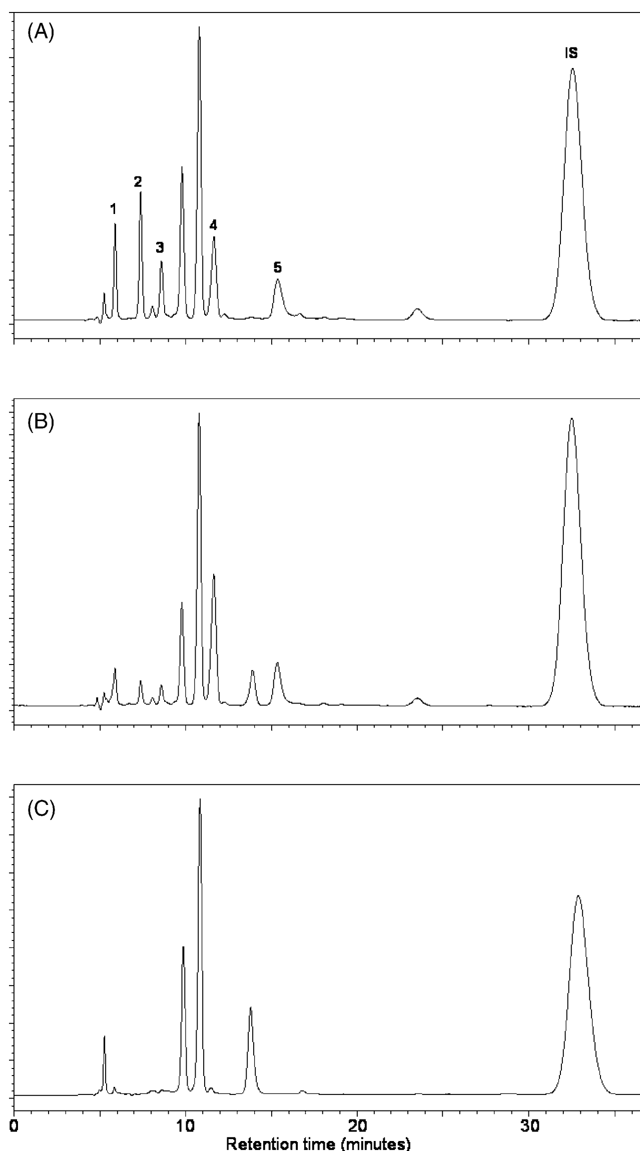

Fig. 2. HPLC chromatograms of metoprolol and its metabolites in dog plasma. (A) Extracted dog plasma spiked with metoprolol and its metabolites; (B) extracted dog plasma 0.5 h after administering 1.37 mg/kg metoprolol; (C) extracted blank human plasma. Peak 1: α-hydroxymetoprolol; peak 2: *O*-demethylmetoprolol; peak 3: metoprolol acid; peak 4: metoprolol; peak 5: deaminated metoprolol; and peak IS: internal standard.

3.2. Recovery and linearity

Plasma samples containing α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, metoprolol, deaminated metoprolol and the internal standard (alprenolol) were extracted according to the procedure described above. The percent recovery for each drug was determined by comparing peak heights of each drug in the extracted samples with those obtained from direct injection of the non-extracted (standard) compounds. The average percent recovery was calculated for each compound (see Table 1). The recoveries of metoprolol and its four metabolites and alprenolol were around 80%.

A standard calibration curve ($n = 6$), ranging from 0.047 to 1.5 μg/ml, was constructed. The peak height of metoprolol and each of its metabolites were divided by the peak height of alprenolol (internal standard) to attain a peak height ratio. The data from the standard curves were analyzed using regression analysis to obtain the slopes, the intercepts and correlation coefficients. The correlation coefficients for metoprolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid and deaminated metoprolol were typically 0.99.

3.3. Reproducibility and accuracy

Data taken from samples prepared on three different days was used to determine inter-day reproducibility for metoprolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, deaminated metoprolol (0.0079–0.25 μg/ml).

Table 1
Recovery rate of metoprolol and its metabolites α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, metoprolol, deaminated metoprolol and the internal standard alprenolol from dog or human plasma

	Dog (recovery %)			Human (recovery %)		
	Experiment 1	Experiment 2	Mean	Experiment 1	Experiment 2	Mean
α-Hydroxymetoprolol	77.1	86.1	81.6	77.2	86.7	82
O-Desmethylmetoprolol	77.4	86.1	81.7	80.0	89.3	84.7
Metoprolol acid	81.5	86.1	83.8	85.5	89.0	87.3
Metoprolol	78.9	88.4	83.6	80.2	88.7	84.5
Deaminated metoprolol	74.1	80.4	77.3	74.4	82.7	78.5
Alprenolol	84.2	98.2	91.2	79.1	91.1	85.1

The concentrations are 0.5 μg/ml for all analytes.

Table 2
Inter-day variation of metoprolol and its metabolites α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, metoprolol, and deaminated metoprolol

Concentration (μg/ml)	S.D. (%)				
	α-OH	ODM	MA	Metoprolol	DAM
0.00785	15.5	11.9	5.2	8.6	4.9
0.0157	8.1	3	13.9	5.1	8.6
0.0313	5	6.1	12.1	5.9	7.8
0.0625	11.3	7.4	12.7	17.3	9.6
0.125	4.3	5.9	10.1	3.6	2.5
0.25	6.3	3	13.4	0.9	4.6

Data are from experiments from three days.

Inter-day variation coefficiency varied between 0.9 and 17.3% with an average value of 7.8%. The measured value of the quality controls are 103.94, 101.85, 102.31, 105.17, 98.38% of the actual value for metoprolol (0.108 μg/ml), α-hydroxymetoprolol (0.108 μg/ml), *O*-desmethylmetoprolol (0.108 μg/ml), metoprolol acid (0.542 μg/ml), and deaminated metoprolol (0.108 μg/ml), respectively (Tables 2 and 3).

3.4. Lower limit of quantitation

The lower limits of quantitation for α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, metoprolol and deaminated metoprolol were 0.0078, 0.0039, 0.0157, 0.0157 and 0.0039 μg/ml, respectively. These limits were determined based on their respective inter-day variation (S.D.% < 20%).

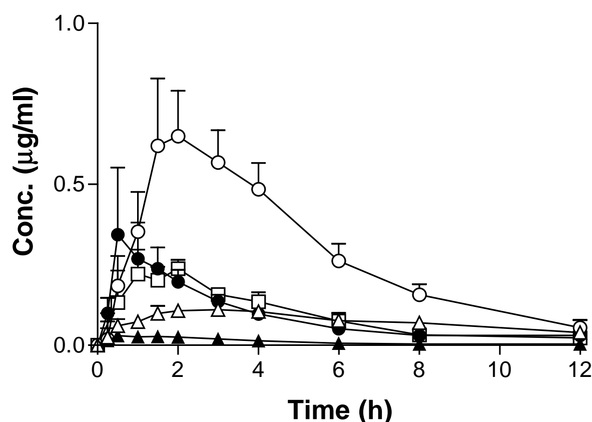

Fig. 3. Plasma concentration profile of metoprolol and its metabolites following an oral dose of metoprolol (1.37 mg/kg) in dog. Closed circles: metoprolol; open triangles: α-hydroxymetoprolol; triangles: *O*-demethylmetoprolol; open circles: metoprolol acid and open squares: deaminated metoprolol. Data are presented as mean ± S.E.M. from four dogs. Each plasma sample was assayed in duplicate.

3.5. Pharmacokinetics of a single dose of metoprolol

The HPLC method was used to determine the concentration profile of metoprolol and its four metabolites in dog following administration of 1.37 mg/kg metoprolol. Duplicate samples from each time point were analyzed and the average concentration was used to plot the graph (see Fig. 3). Minimal interference from endogenous plasma matrix substances was observed in the four dogs used. AUC values (0–48 h) for α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, metoprolol, and deaminated metoprolol were calculated to be 2.05, 0.34,

Table 3
Slopes, intercepts and correlation coefficients from the calibrations curves of metoprolol and its metabolites

	Slopes	Intercepts	Correlation coefficients
α-Hydroxymetoprolol	6.91 ± 0.43	0.03 ± 0.01	0.999 ± 0.001
O-Desmethylmetoprolol	13.72 ± 1.29	0.01 ± 0.01	0.999 ± 0.001
Metoprolol acid	1.14 ± 0.09	0.02 ± 0.01	0.997 ± 0.003
Metoprolol	8.39 ± 0.73	0.15 ± 0.05	0.997 ± 0.003
Deaminated metoprolol	3.53 ± 0.33	0.01 ± 0.01	0.999 ± 0.001

Results are presented as mean ± S.E.M. ($n = 4$).

4.39, 1.89, and 1.11 μg ml^{-1} h, respectively. The apparent half-life of metoprolol was estimated to be 2 h.

4. Discussion

This report describes a fast, sensitive and simple HPLC method for the determination of racemic metoprolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid and deaminated metoprolol in dog plasma.

To the best of our knowledge, this is the only HPLC assay which can separate metoprolol and four of its metabolites. Wang and Semple [9] analyzed metoprolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol, and metoprolol acid with a C18 column. Lennard [8] separated metoprolol, α-hydroxymetoprolol, metoprolol acid and deaminated metoprolol. Metoprolol, α-hydroxymetoprolol and *O*-desmethylmetoprolol were analyzed by a number of research groups [4,7]. Metoprolol, α-hydroxymetoprolol and metoprolol acid were analyzed by Chiu et al. [5] and Godbillon and Duval [6].

This solid phase extraction method is also the most comprehensive method which can extract metoprolol and its four metabolites in a single extraction procedure. The difficulty in extracting these compounds with a single extraction procedure is apparent because these compounds vary widely in their chemical properties. Among these compounds, α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol and alprenolol are bases, metoprolol acid is amphoteric and deaminated metoprolol is an acid. High recovery rates were achieved through the use of a Waters Oasis HLB Extract cartridges (30 mm), and careful choice of loading buffer, rinsing and eluting solvents. While our solid extraction method resulted in excellent recoveries for metoprolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid and deaminated metoprolol, the previously reported extraction procedures resulted in recoveries of only three of these compounds. Metoprolol, α-hydroxymetoprolol, and deaminated metoprolol were extracted with a liquid-liquid extraction procedure [8]. Metoprolol, α-hydroxymetoprolol, and *O*-desmethylmetoprolol were extracted with another liquid–liquid extraction method [7]. Metoprolol, α-hydroxymetoprolol, and metoprolol acid were extracted with a solid extraction procedure [5].

Cyano columns have been successfully applied to analyze a number of drugs and their metabolites in this laboratory [10–14]. Generally speaking, phase I metabolites are considerably more polar than their parent compounds. When lipophilic columns are used, the retention time of the parent compounds are often too long, which necessitates the use of gradient systems. A polar column such as a cyano column reduces the differences in retention time for the polar and lipophilic compounds and enable analysis with an isocratic system. The lack of interfering peaks at any of the retention times of the analytes in the human blank plasma chromatogram in Fig. 2C is evidence that the method can be extended to human plasma. Neverthless, cyano columns are usually less selective than C18 columns, interferences from co-administrered drugs should be ruled out before this method can be used to analyze samples of human taking co-medications.

A pharmacokinetic study of metoprolol was conducted in dog (Fig. 3). AUC values were highest for metoprolol acid and the lowest for *O*-desmethylmetoprolol. This indicates that *O*-desmethylmetoprolol was rapidly converted to metoprolol acid in vivo (Fig. 1). This is consistent with Yin and Zhang [15] who observed two compartment model with the pharmacokinetic of metoprolol in dog. Half-life for the α and β phase were reported to be around 0.5 and 2.5 h, respectively. In our experiment, solid dosage forms were administered which could significantly delayed the absorption of metoprolol. Thus, the α phase probably overlapped with the absorption phase of metoprolol. However, the apparent terminal half-life was found to be similar (2 h).

Metoprolol undergoes extensive first-pass hepatic metabolism, and it has been used as a model drug in many studies exploring various aspects of its pharmacokinetics and metabolism over the past two decades. Some studies explored the high degree of pharmacokinetic variability caused by the first-pass effect [16–18]. When metoprolol is administered with a high protein meal, its bioavailability is increased due to a reduction of hepatic metabolism. The mechanism of this "food effect" has been of considerable interest, but it remains to be completely elucidated [9,19]. Because of its variable kinetics and relatively short half-life, metoprolol has been used as a model drug for the development of sustained-release dosage forms that would permit once daily dosing [20]. Like other beta blockers, metoprolol has a chiral centre. While it is administered as the racemate, its pharmacological activity is stereospecific, with the (−) (S) isomer exhibiting more activity, and its pharmacokinetics are also stereospecific [21–24]. Since the HPLC method is not stereoselective, it would be most useful in pharmacokinetic stdies where stereoisomers of metoprolol were to be administered.

In conclusion, this is the first method to extract and quantify metoprolol, and its metabolites α-hydroxymetoprolol, *O*-desmethylmetoprolol, metoprolol acid, deaminated metoprolol in a single assay. It would be useful in therapeutic drug monitoring and/or pharmacokinetics studies of metoprolol.

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