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Note

Sensitive high-performance liquid chromatographic method for procaterol in human urine

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Procaterol hydrochloride, 8-hydroxy-5-[1-hydroxy-2-[(1-methylethyl)-amino]butyl]-2(1H)-quinolinone, has been reported to exhibit β2-adrenergic stimulation, producing selective bronchodilation. Because of its potency and selectivity, the therapeutic potential of procaterol for treating asthma is encouraging. Procaterol has more potent bronchodilating activity than isoproterenol and is more selective than salbutamol for β2-adrenergic receptors [1–3].

Procaterol was synthesized by Yoshizaki et al. [1] and has the chemical structure given in Fig. 1. Apparent pK_a values for procaterol are 7.55 and 9.46 for the phenolic and side-chain amino group, respectively. Solubility varied from 36.4 mg/ml in 0.1 *M* hydrochloric acid to 53.4 mg/ml in water. Apparent partition coefficients (log *K*, *n*-octanol/water) ranged from −1.06 for 0.1 *M* hydrochloric acid to −0.05 for water. Procaterol was stable for three days at room temperature in aqueous solutions at pH 1, 4 and 7, and stable for 24 h at 37°C.

Pharmacokinetics and metabolism of procaterol have been examined in animal studies [2,3]. For these studies plasma and urine concentrations were determined by gas chromatography–mass spectrometry (GC–MS) procedures requiring extensive sample preparation The pharmacokinetics of procaterol in rat, rabbit, and dog were examined by Ishigami et al. [2] using liquid–liquid extraction and a complex derivatization sequence. In vivo and in vitro metabolism of procaterol in rats were studied by Shimizu et al. [3]; several solid-phase isolation steps were used including Amberlite XAD-2, preparative thin-layer chromatography, and Sephadex LH-20.

The complexity and sophisticated instrumentation of these techniques neces-

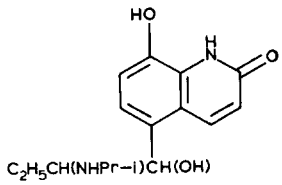

Fig. 1. Chemical structure of procaterol.

sitated development of an alternative method for routine analysis of procaterol. This report describes the development and validation of a high-performance liquid chromatographic (HPLC) assay for procaterol in urine. Analysis of procaterol in urine was a difficult analytical problem, since procaterol is a highly polar compound and is administered at low doses (100–200 μg per day). Analyte isolation and HPLC systems were extensively surveyed to identify a sensitive and selective method. A carboxylic acid solid-phase isolation system was most useful as it allowed isolation of procaterol from a relatively large urine volume. Several HPLC columns were examined with conventional mobile phases; the C_{18} in an ion-pairing mode was the most selective and time efficient.

EXPERIMENTAL

Reagents

Procaterol hydrochloride hemihydrate, desisopropylprocaterol hydrochloride monohydrate, and 5-formyl-8-hydroxy-2(1H)-quinolinone were synthesized at Otsuka Pharmaceutical in Japan. Anhydrous dibasic sodium phosphate (MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.), monohydrated monobasic sodium phosphate (MCB), acetone (Fisher, Pittsburgh, PA, U.S.A.), 1-octane sulfonic acid sodium salt (Aldrich, Milwaukee, WI, U.S.A.), glacial acetic acid (J.T. Baker, Phillipsburg, WI, U.S.A.), hydrochloric acid (MCB), and nitric acid (Fisher) were reagent grade and used as received. Acetonitrile (Fisher) was HPLC grade and used as received.

Standard solutions

Procaterol hydrochloride (145 mg) was weighed into a 100-ml flask and diluted to volume with Milli-Q-purified water. An aliquot of this solution was added to a 50-ml volumetric flask and diluted to volume with water to yield a concentration of 8.7 μg/ml (free base equivalents). Aliquots were diluted with water to produce eight working solutions: 87, 174, 434, 868, 1300, 1740, 2170 and 2600 ng/ml.

Calibration standards were prepared daily with drug-free urine at procaterol concentrations of 4.3, 8.7, 21.7, 43.4, 65.1, 86.6, 108, and 130 ng/ml. Standards were prepared by taking 1.25-ml aliquots from the respective eight aqueous working solutions and diluting to 25 ml with urine. Three urine procaterol control pools at 25, 65.1, and 108 ng/ml were prepared by diluting aliquots of the 8.7 μg/ml working solution with pooled drug-free urine to 200 ml. Each control pool was subdivided into 8-ml volumes and stored frozen in glass vials.

Chromatography

The HPLC system consisted of the following components: SP8700 XR pump (Spectra-Physics, San Jose, CA, U.S.A.), SP8780 XR autosampler, Kratos (Ramsey, NJ, U.S.A.) Spectroflow 783 programmable absorbance detector coupled to an SP4270 integrator system. The column was a Hypersil ODS (3 μm, 60×4.6 mm; Hewlett-Packard, Palo Alto, CA, U.S.A.). The guard column (20×2 mm; Upchurch Scientific, Oak Harbor, WA, U.S.A.) was dry-packed with Whatman pellicular ODS (37–53 μm; Whatman, Clifton, NJ, U.S.A.). A Rainin column heater (Rainin, Woburn, MA, U.S.A.) was used to thermostat the guard and analytical columns at 30°C. The ion-pairing mobile phase consisted of aqueous 50 m*M* 1-octane sulfonic acid–8% glacial acetic acid (v/v, with water)–acetonitrile (49:38:13). The flow-rate was 1 ml/min. The column effluent was monitored at 260 nm, and the procaterol peak height was measured from a tangent skim baseline.

Sample preparation

Procaterol was isolated from 5 ml of human urine using a Bond Elut® carboxylic acid (CBA) solid-phase extraction column (1 g sorbent mass per 6 ml column volume, Lot No. 152818, Analytichem, Harbor City, CA, U.S.A.). Sep-Pak® reservoirs (Waters Assoc., Milford, MA, U.S.A.) were coupled to the CBA columns to enable large volume processing. A Vac Elut® vacuum manifold system (Analytichem) was used to regulate fluid flow through the CBA column.

The CBA column was conditioned with two column volumes of both methanol and water. Afterwards, 20 ml of 0.01 *M* phosphate buffer (pH 7.0) were added to the column followed by 5 ml of urine standard, control, or blank. Approximately 1 min was allowed for thermal mixing of phosphate buffer and urine before applying vacuum (330–380 mmHg) to the column. A series of three washes was used to remove interferences: 15 ml water (vacuum: 330–380 mmHg); 1.0 ml methanol (low vacuum was applied until a yellowish band migrated approximately halfway down the column, afterwards a higher vacuum was applied); 0.5 ml acetone. Procaterol was eluted from the column with 2.0 ml of methanolic 0.005 *M* hydrochloric acid. The eluent was collected in a test tube and evaporated to dryness (40°C) by a gentle stream of nitrogen. The residue was reconstituted with 0.4 ml aqueous 0.01 *M* nitric acid. The contents were vortexed, centrifuged, and transferred to a glass injection vial; 110 μl of the reconstituted volume were injected into the ion-pairing HPLC system.

Calibration

The method was calibrated for each run by regressing procaterol peak heights against procaterol concentration (C) of the calibration standards. The best-fit line was determined by the linear least-squares method using a weighting factor of $1/C$. The procaterol concentration in unknown samples was calculated using the regression equation.

TABLE I

RECOVERY AND INTRA-ASSAY PRECISION OF PROCATEROL ($n=6$)

Concentration (ng/ml)	Recovery (mean ± S.D.) (%)	Relative standard deviation (%)
25	75.5 ± 7.6	10.1
65.1	67.9 ± 1.6	2.4
108	66.7 ± 2.8	4.2

RESULTS AND DISCUSSION

The method was evaluated over a procaterol concentration range of 4.3–130 ng/ml in human urine. Isolation efficiency, selectivity, suitability, linearity, precision, and accuracy were evaluated to establish the validity of the assay.

Isolation efficiency

Several types of solid-phase extraction columns (non-polar, polar, and ion-exchange) were investigated; the most useful was a carboxylic acid (CBA) type since it provided optimal recovery and selectivity. This column is a weak cation exchanger ($pK_a=4.8$). Given a mean human urine pH of 5.8 [4] 10 m*M* phosphate buffer (pH 7.0) was added to 5 ml of urine to adjust the pH in the range 6–6.5. In this range the CBA column is negatively charged and procaterol is positively charged. The 15-ml water wash was used to remove salts and other hydrophyllic constituents from the column. The methanol and acetone washes were necessary to remove the more hydrophobic interferences. Finally, the elution solvent (0.005 *M* hydrochloric acid in methanol) was a weak solvent which left strongly retained interferences behind, yet eluted procaterol. The relatively low boiling point of the solvent also enabled an evaporation step for preconcentration. Mean isolation efficiencies of procaterol from 5 ml of urine (Table I) ranged from 67 to 76% over a concentration range of 25–108 ng/ml. Relative standard deviations (R.S.D.) were less than 10% for six replicates at each of three urine concentrations.

Selectivity

Representative chromatograms which demonstrate assay selectivity are given in Fig. 2: (A) an aqueous standard containing 65 ng/ml procaterol; (B) drug-free pooled human urine; (C) human urine calibration standard containing 65 ng/ml procaterol; (D) human urine sample containing 53.5 ng/ml procaterol. The retention time for procaterol was approximately 17 min. Although urine samples contained components which eluted near procaterol, their peak heights were typically small and caused no interference. Resolution of procaterol from the nearest peak (Fig. 2D) was 1.6.

The method was selective for procaterol in human urine as no interfering peaks were observed during the analysis of more than 200 samples. Caffeine, uric acid, and xanthine were examined for possible interference with procaterol. All three had retention times less than 5 min, thus posing no interference problems.

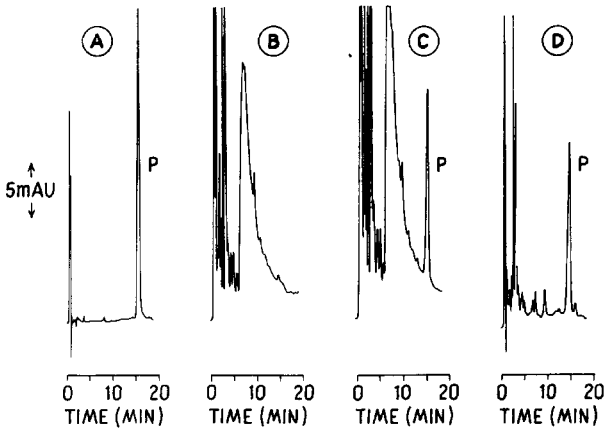

Fig. 2. Representative chromatograms of (A) an aqueous standard containing 65 ng/ml procaterol, (B) drug-free pooled human urine, (C) human urine calibration standard containing 65 ng/ml procaterol, and (D) human urine sample containing 53.5 ng/ml procaterol. Peak P = procaterol.

System suitability

An external standard method was used. The external standard approach gave a shorter analysis time and acceptable precision, when compared to an internal standard method developed in our laboratory. The most favorable internal standard (8-ethoxyprocaterol) of many evaluated, required gradient elution which resulted in a 52-min chromatographic run time per sample.

Solid-phase extraction of procaterol from urine was performed in batches of ten with a processing time of approximately 30 min. The time required for evaporation and reconstitution of two batches was approximately 30 min. The chromatographic run time per sample was 20 min. The separation factors (α) for desisopropylprocaterol and 5-formyl-8-hydroxy-2(1H)-quinolinone (major metabolites) with respect to procaterol were 2.6 and 11.2, respectively. The capacity factors for procaterol and the two major metabolites were 13.4, 5.1 and 1.2, respectively. The number of theoretical plates per meter was 35 000. Precision of the procaterol retention time was typically 0.7% R.S.D.

System reproducibility was excellent. Six replicate injections of solid-phase-extracted human urine standards at 65 and 108 ng/ml procaterol gave R.S.D. values of 5.3 and 5.0%, respectively, using peak heights.

Linearity

Peak-height responses were proportional to the concentration of procaterol in human urine over the range of 4.3–130 ng/ml. The best-fit straight line was determined by a least-squares method using a weighting factor of $1/C$. Results from a typical regression analysis were: peak height = 157 × procaterol concentration (ng/ml) + 671; $r = 0.997$. The minimum quantitation limit was 4.3 ng/ml.

Precision and accuracy

Eight urine procaterol calibration standards and three urine procaterol controls were analyzed in triplicate on three separate days. Precision (R.S.D.) for

TABLE II

INTER-ASSAY PRECISION AND ACCURACY OF PROCATEROL OVER A THREE-DAY PERIOD

Calibration standards and seeded controls were analyzed in triplicate on three separate days ($n=9$).

Added (ng/ml)	Found (ng/ml)	Relative standard deviation (%)	Relative error (%)
Calibration standards			
4.3	4.8	20.8	11.6
8.7	9.0	17.8	3.4
21.7	18.9	9.5	−12.9
43.4*	44.5	7.0	2.5
65.1	69.8	7.6	7.2
86.8	89.4	6.9	3.0
108	109	7.2	0.9
130	127	8.0	−2.3
Seeded controls			
25	27.6	9.8	10.4
65.1	71.0	8.5	9.1
108	108	9.1	0.0

*Calibration standard was analyzed in triplicate on two separate days ($n=6$).

the back-calculated concentrations of the calibration standards using the regression parameters ranged from 20.8% for the lowest standard to 6.9% (Table II). Relative error values ranged from −12.9 to 11.6% (Table II). Assay precision and accuracy were evaluated using the procaterol urine controls. Precision (R.S.D.) of the concentration found in urine controls ranged from 8.5 to 9.8%, relative error was less than 10.4% (Table II).

In summary, an accurate, selective, and precise HPLC assay has been developed and validated to monitor trace levels of procaterol in urine using UV spectrophotometric detection. The method is suitable for routine analysis of as little as 4.3 ng/ml procaterol in clinical samples. A major advantage of the HPLC assay when compared to currently available GC–MS methods is that sophisticated instrumentation and lengthy derivitization schemes are not required.

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