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Note

Determination of free and total metaproterenol in human plasma by high-performance liquid chromatography with fluorimetric detection

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One of the most common and widespread diseases of modern times is bronchial asthma. The antiasthmatic drugs used to combat this illness can be divided into several groups, one of which are β-adrenergic stimulants. Metaproterenol is one of the β-adrenergic stimulants along with others such as albuterol, terbutaline, isoproterenol and epinephrine. Without question, the most popular drug in the group is albuterol, for which several analytical methods have been developed [1–6] and for which the physical properties, metabolism and excretion are well known [7]. On the other hand, such data on metaproterenol is scant. Even the 1985 review [7] quoted no methods suitable to measure therapeutic levels of metaproterenol.

Over the ensuing period of time, several papers have been published which provide more information concerning metabolic fate, therapeutic levels, pharmacokinetic data and analytical methods for metaproterenol. Metaproterenol has recently replaced isoproterenol because it causes less tachycardia and hypertension. After oral administration, metaproterenol is metabolized in the gut wall to a 3-O-sulfate conjugate [8].

As metaproterenol possesses a resorcinol ring structure, it is electroactive and can be detected by an electrochemical detector. This is the basis for a high-performance liquid chromatographic (HPLC) method achieving a detection

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limit of 0.5 ng/ml metaproterenol in plasma [9]. In this case, solid-phase extraction was used.

By far the most relevant is the paper by Hatch et al. [10] which described a gas chromatographic–mass spectrometric (GC–MS) method using a stable isotope. The assay was linear over the range 0.5–8.0 ng/ml for metaproterenol in plasma. They reported a peak plasma concentration after a 10-mg dose of metaproterenol sulfate varying between 2.2 and 17.3 ng/ml; time to peak, 0.75–3.0 h; mean half-life, 2.1 h. The other finding was of a great inter- and intra-subject variability of pharmacokinetic parameters which might make a cross-over study on bioavailability difficult or impossible.

The goal of this research was two-fold. First to develop a robust and simple HPLC method using fluorescence detection to assay free metaproterenol in plasma which could be used in pharmacokinetic studies. Secondly, to develop a similar method to measure total concentration of metaproterenol after releasing it from its 3-O-sulfate, in expectation that such an approach would make a crossover bioavailability study feasible.

EXPERIMENTAL

Methods

Metaproterenol sulfate, USP reference standard, was obtained from American Chemicals (Montreal, Canada) while terbutaline sulfate, also USP reference standard, was from Nucro Technics (Scarborough, Canada). Acetonitrile, methanol, dichloromethane (all HPLC grade) and *n*-butanol (glass-distilled) were supplied by Caledon Labs. (Georgetown, Canada). Sodium phosphate monobasic, potassium bisulphite, sodium carbonate and bicarbonate, hydrochloric acid (all ACS certified) and orthophosphoric acid (HPLC grade) were supplied by Fisher (Montreal, Canada). Trichloroacetic acid and sulfatase/β-glucuronidase (*Helix pomatia*) were purchased from Sigma (St. Louis, MO, U.S.A.). Disposable C_{18} (3 ml) Bond-Elut extraction columns were supplied by Rayonics (Downsview, Canada).

Chromatographic conditions

The chromatographic system consisted of a Waters Model 590 programmable solvent delivery module, a Waters WISP 710B autosampler (Milford, MA, U.S.A.), a Spectraflow 980 fluorescence detector (Kratos Analytical, Ramsey, NJ, U.S.A.), an analytical column, 25 cm $\times$ 4.9 mm I.D., in-house packed with C_8 Spherisorb, 5 μm particle size (Phase Sep, Norfolk, CO, U.S.A.), and an SP4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.). Data was collected on a Hewlett Packard 3357 laboratory automation system. The excitation wavelength was 200 nm (slit width 5 nm), while emission wavelength was controlled by a 300-nm cut-off filter. The flow-rate was 1.8 ml/min with a

resulting back-pressure of 20 MPa. Total run time was 18 min. The same system was used for both free and total metaproterenol and kept at 22 ± 3 °C.

Mobile phase

Mobile phase was prepared by adding 80 ml of acetonitrile to a 2-l volumetric flask and 30 ml of 1 *M* sodium phosphate adjusted to pH 3.0 with 85% (w/w) orthophosphoric acid. The flask was then brought to volume with water, stirred and filtered through a 0.45-μm nylon filter. The same mobile phase was used for both free and total metaproterenol.

Standard and quality control preparation

The standard samples in plasma were prepared by adding appropriate volumes of aqueous metaproterenol sulfate to human plasma containing EDTA as an anti-coagulant and potassium bisulphite (1.5 mg/ml) as an anti-oxidant. The volume added was always less than or equal to 2% of total volume of the sample so that the integrity of plasma was maintained. Quality control samples were prepared in the same way using separately weighed stock solutions. After aliquoting, 1-ml samples were stored at −80 °C until required.

Clinical pharmacokinetic study

Five healthy male volunteers, 18–45 years of age, weighing at least 60 kg and not receiving any medication for the seven days preceding the study, participated in the pilot project after giving a written informed consent. A single dose of metaproterenol sulfate (20 mg) was administered orally with 250 ml of water, and blood samples were collected at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 6, 8 and 12 h. After centrifugation, potassium bisulphite was added to plasma as anti-oxidant. Samples were mixed and stored at −80 °C.

Excess of plasma at peak time (1–3.5 h) was pooled to provide hydrolysis control samples to be run on each analytical day to monitor the completeness of hydrolysis.

Ethical aspects of this study were considered and approved by the institutional review board.

Extraction of free metaproterenol

To a standard, quality control plasma or clinical sample (1.0 ml each), the internal standard was added (100 μl of 2.0 μg/ml terbutaline sulfate) and also 0.02 *M* sodium phosphate dibasic solution, pH 9.0 (1.0 ml). After mixing, the solution was transferred to a C_{18} Bond-Elut column, prewashed with acetonitrile and water, where the compounds of interest were absorbed. The extraction column was washed with 5 column volumes of water, and the eluates were discarded. Next, the column was dried for 5 min and washed with 3% (v/v) *n*-butanol in dichloromethane (3 ml). The drug and internal standard were eluted with 0.09% (w/v) hydrochloric acid in acetonitrile (2 × 1 ml), the fractions

collected in a tube, and the acetonitrile was evaporated under a nitrogen stream at 37°C. The residue was dissolved in mobile phase (300 μl) and an aliquot (200 μl) injected into the chromatographic system.

Hydrolysis of 3-O-metaproterenol sulfate

To a standard, quality control, hydrolysis control or clinical sample (1.0 ml each), the internal standard was added (200 μl of 2.0 μg/ml terbutaline sulfate) and the solution mixed. Proteins were precipitated with trichloroacetic acid (6%, w/v, 1.0 ml), the tubes were then mixed on a vortex and centrifuged at 20°C for 15 min at 1000 *g*. The supernatant was poured into another tube, where 200 μl of 2 *M* hydrochloric acid were added. The sample was incubated for 90 min at 65°C. After cooling down, the pH of the sample was adjusted to 10 by the addition of 400 μl of 2 *M* carbonate buffer. Extraction of liberated metaproterenol was performed in the same way as described above.

RESULTS AND DISCUSSION

Precision and accuracy

Free metaproterenol. A set of six calibration standards, a zero and a drug-free blank were analyzed with every batch of clinical samples. The inter-assay precision and accuracy were assessed by the repeated analysis of quality control samples containing different concentrations of metaproterenol. The results are shown in Table I. Linear response of metaproterenol and internal standard peak-height ratio were observed over the concentration range 0.5–20.0 ng/ml. A linear regression analysis using a least-squares fit was performed with the reciprocal of the drug concentration as weight. The correlation coefficients, an indication of linearity, were equal to or better than 0.9986 over five curves.

Total metaproterenol. A set of seven calibration standards, a zero, a blank and a hydrolysis control were analyzed with every batch of clinical samples.

TABLE I

METAPROTERENOL INTER-ASSAY PRECISION AND ACCURACY

Compound	Nominal concentration (ng/ml)	*n*	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Percentage of nominal concentration
Free metaproterenol	1.50	9	1.427 ± 0.1146	8.0	91.5
	6.00	10	5.776 ± 0.2222	3.8	96.3
	18.0	10	16.48 ± 0.986	6.0	91.6
Total metaproterenol	6.00	10	6.009 ± 0.5689	9.5	100.2
	70.0	10	69.24 ± 2.594	3.7	98.9
	135	10	134.7 ± 5.91	4.4	99.8

The inter-assay precision and accuracy are shown in Table I. The intra-assay precision was evaluated by running, in multiplicate, plasma obtained from a subject 2 h after administration of 20 mg metaproterenol sulfate. The results were as follows: concentration found (mean ± S.D.), 53.5 ± 2.50 ng/ml; coefficient of variation (C.V.), 4.7%; $n=5$. Linear response was observed over the concentration range 1.0–150.0 ng/ml. The correlation coefficients were equal to or better than 0.9981 over five curves. Hydrolysis control samples, which monitor the completeness of the acidic hydrolysis of 3-O-metaproterenol sulfate, provided a C.V. of 8.6% ($n=4$), suggesting a good reproducibility of the process.

Recovery

Recovery was calculated by comparing quality control samples, extracted according to the respective procedure for the free or total metaproterenol, with an additionally prepared calibration curve, which represented 100% recovery, correcting for all the losses in volume due to sample transfer. The recovery of free metaproterenol was 108.0% (at 1.50 ng/ml, $n=8$, C.V. = 4.7%) and 88.9% (at 18.00 ng/ml, $n=8$, C.V. = 1.6%). The recovery of the drug, while using the procedure for total metaproterenol, was 98.0% (at 6.00 ng/ml, $n=8$, C.V. = 3.0%) and 110.8% (at 135.0 ng/ml, $n=6$, C.V. = 5.3%).

The recovery of terbutaline ranged from 77.3 to 91.9% over the range 80.0–325 ng per sample. Both procedures provided similar recoveries of the internal standard.

Chromatography

Chromatograms obtained while using the procedure for free metaproterenol are shown in Fig. 1. Panel A shows a drug-free plasma, panel B shows subject plasma 1.5 h after the drug administration and panel C represents a plasma spiked at 4.0 ng/ml.

Fig. 2 shows chromatograms resulting from the use of the procedure for total metaproterenol. Panel A represents a drug-free plasma, panel B is a subject plasma 1.5 h after the drug administration and panel C is a standard at 50 ng/ml.

Plasma was collected from ten healthy donors and screened for interference at the retention times of metaproterenol and internal standard. No significant interference was observed in drug-free plasma samples.

Pharmacokinetic data

Plots of logarithm of plasma concentration versus time were constructed for free and total metaproterenol. The slope of the log-linear terminal phase was determined by least-squares regression analysis. The apparent elimination half-life ($t_{1/2}$) was calculated directly from the elimination constant K_{el} [11]. The trapezoidal method was used to calculate the area under the curve (AUC) until

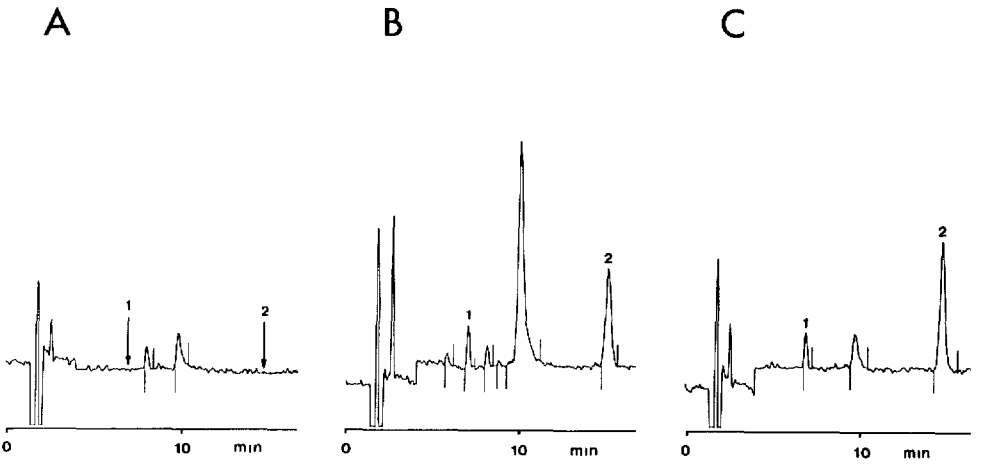

Fig. 1. Representative chromatograms of free metaproterenol extracted from human plasma. (A) Drug-free plasma; (B) sample from a subject, 1.5 h after dosing (6.42 ng/ml); (C) quality control sample (4.00 ng/ml). Conditions: excitation wavelength, 200 nm; emission filter, 300 nm; PMT signal, 0.1; range, 0.01. Peaks: 1 = metaproterenol (retention time 6.9 min); 2 = terbutaline (internal standard) (retention time 14.8 min). The pronounced peak at approximately 10 min is of endogenous origin and has not been identified. Vertical bars represent integration marks.

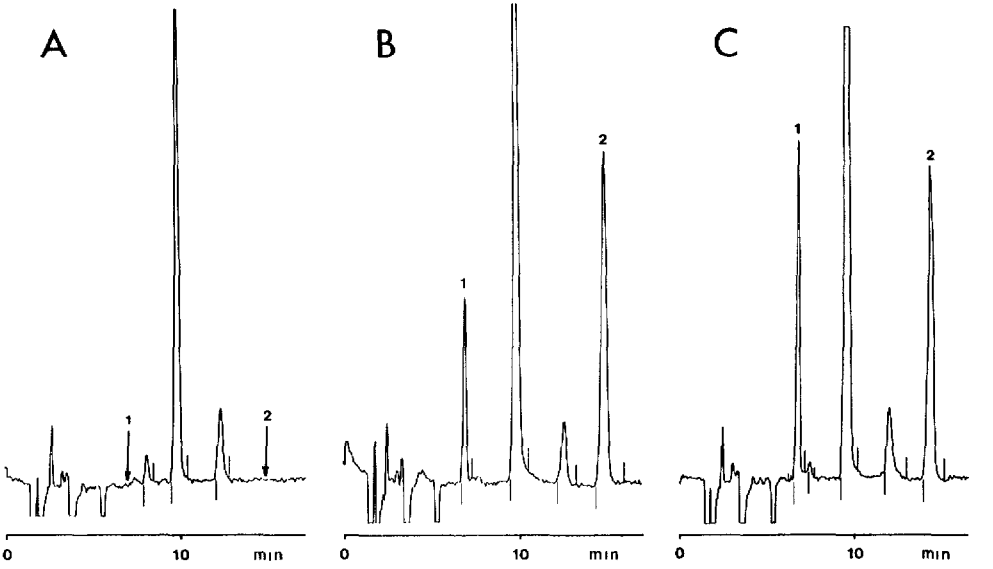

Fig. 2. Representative chromatograms obtained by the procedure for total metaproterenol. (A) Drug-free plasma; (B) sample from a subject, 1.5 h after dosing (23.7 ng/ml); (C) standard (50.0 ng/ml). Conditions and peak identification as in Fig. 1.

the final detectable plasma concentration. To this area, the residual area, extrapolated to infinity, was added, calculated by the dividing of the final concentration by K_{el} [11].

The main pharmacokinetic data are shown in Table II. The limit of quantification of 0.5 ng/ml is necessary to obtain meaningful pharmacokinetic data for the free metaproterenol after a 20-mg dose administration, yet for the total metaproterenol, 5 ng/ml would be sufficient, which is very easily achieved. At a concentration of 0.5 ng/ml, the signal-to-noise ratio was 3.5. The profiles are shown in Fig. 3. Generally, a large inter-subject variability of metaproterenol concentration was observed with the C.V. ranging from 56 to 103% for free metaproterenol and 43 to 63% for the total.

The pharmacokinetic data for the free metaproterenol obtained by this

TABLE II

PHARMACOKINETIC DATA OF METAPROTERENOL FOLLOWING SINGLE ORAL 20-mg DOSE

Values are means ± S.D. ($n=5$).

Parameter	Free metaproterenol	Total metaproterenol
Elimination constant (K_{el})	0.278 ± 0.119	0.271 ± 0.081
Elimination half-life ($t_{1/2}$)	2.49	2.56
Area under the curve (AUC_{0-12}) (h ng/ml)	23.02 ± 10.38	583.4 ± 211.7
Maximum plasma concentration (C_{max}) (ng/ml)	5.52 ± 3.089	120.3 ± 60.84
Time of C_{max} (t_{max}) (h)	1.45 ± 0.798	1.60 ± 0.418

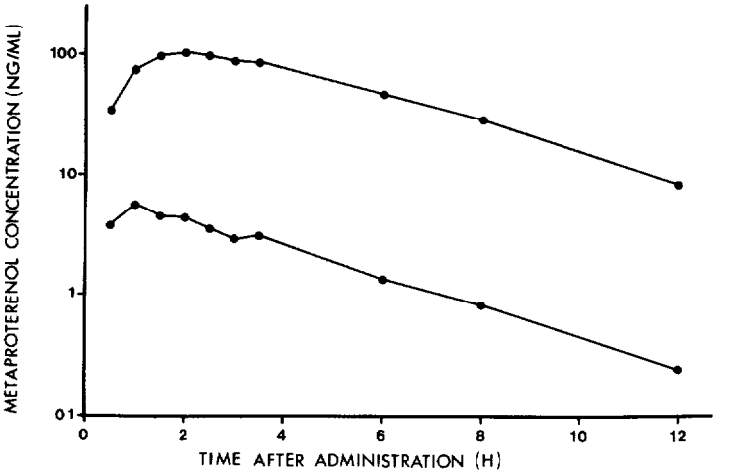

Fig. 3. Plasma metaproterenol profile after administration of 20 mg metaproterenol sulfate (mean values, $n=5$). The upper track represents total metaproterenol; the lower track represents free metaproterenol.

method seem to correspond with those reported by Hatch et al. [10], with a half-life of 2.49 h agreeing well with the value of to 2.1 h in the quoted study. All the other parameters seem to fall in line, considering oral doses of 20 mg (our study) and 10 mg (Hatch et al. [10]).

Total metaproterenol seemed to mirror the profile of the free metaproterenol with a half-life of 2.56 h and concentrations of total metaproterenol 22 times higher than the free compound.

Hydrolysis of 3-O-metaproterenol sulfate

Hydrolysis of a conjugate is often the second best solution, as conjugates are notoriously difficult to obtain, either through synthesis (e.g. ref. 12) or separation from biological fluids like urine [8].

Two methods of hydrolysis are to be considered: enzymatic or acidic. Enzymatic hydrolysis was used successfully by MacGregor et al. [8] in urine, salbutamol-O-sulfate (a similar compound) was also hydrolyzed down to the parent drug by Morgan et al. [13] in a lengthy 120 h long incubation. Our early experiments, similar to those described in ref. 13, showed that enzymatic hydrolysis of 3-O-metaproterenol sulfate in plasma is difficult to complete. In the experiment, 1 ml of a subject plasma was adjusted to pH 5.0 with acetate buffer, and 600 U of sulfatase enzyme were added to the sample. The sample was then incubated for 120 h at 37°C. Every 24 h a sample was taken and extracted. No plateau value was obtained even after 120 h, while samples were increasingly difficult to process due to their viscosity and changing integrity. Such an approach, including lengthy incubation, is very impractical, even with moderate volumes of samples being involved. Morgan et al. [13] also described an acidic hydrolysis which was modified for our purposes. In fact, while our procedure has been developed independently, it is very similar to the one published a few years earlier by Causon and co-workers [14, 15] for hydrolysis of isoproterenol sulfate.

The acidic hydrolysis of 3-O-metaproterenol sulfate was tested by hydrolyzing the control samples as described in one of the above paragraphs. Samples were incubated at 65°C for 0, 30, 60, 90, 120 and 150 min. Incubation for 90 min was sufficient to complete hydrolysis as the metaproterenol concentration reaches a plateau and remains constant for at least 30 min. After incubation of 150 min the concentration of metaproterenol started to decrease, probably due to drug oxidation, since a faint pinkish color was observed. The addition of potassium bisulfite as an anti-oxidant was hence essential.

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REFERENCES

1 P.V. Colthup, F.A.A. Dallas, D.A. Saynor, P.F. Carey, L.F. Skidmore, L.E. Martin and K. Wilson, J. Chromatogr., 345 (1985) 111.
2 B. Oosterhuis and C.J. van Boxtel, J. Chromatogr., 232 (1982) 327.
3 J.G. Leferink, J. Danhers and R.A.A. Maes, J. Chromatogr., 229 (1982) 217.
4 Y.K. Tan and S.J. Soldin, J. Chromatogr., 311 (1984) 311.
5 M.J. Hutchings, J.D. Paull and D.J. Morgan, J. Chromatogr., 277 (1983) 423.
6 M. Weisberger, J.E. Patrick and M.L. Powell, Biomed. Mass Spectrom., 10 (1983) 556.
7 N. Kucharczyk and F.H. Segelman, J. Chromatogr., 340 (1985) 243.
8 T.R. MacGregor, L. Nastasi, P.R. Farina and J.J. Keirns, Drug Metab. Dispos., 11 (1983) 568.
9 W.J. Cooke and L. Moore, Clin. Chem., 31 (1985) 942.
10 F. Hatch, K. McKellop, G. Hansen and T. MacGregor, J. Pharm. Sci., 75 (1986) 886.
11 M. Rowland and T.N. Tozer, Clinical Pharmacokinetics, Lea and Febiger, Philadelphia, PA, London, 1989.
12 E. Kienzl and K. Eichinger, J. Chromatogr., 430 (1988) 263.
13 D.J. Morgan, J.D. Paull, B.H. Richmond, E. Wilson-Evered and S.P. Ziccone, Br. J. Clin. Pharmacol., 22 (1986) 587.
14 R.C. Causon, R. Desjardins, M.J. Brown and D.S. Davies, J. Chromatogr., 306 (1984) 257.
15 R.C. Causon, M.J. Brown and D.S. Davies, J. Chromatogr., 337 (1985) 311.