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Note

Determination of colterol in human plasma and urine by reversed-phase chromatography with amperometric detection

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Bitolterol, the di-*p*-toluate ester of colterol (Fig. 1) is an experimental bronchodilator currently undergoing clinical evaluation in both oral and aerosol formulations. Bitolterol is readily hydrolyzed in vivo to colterol, a catecholamine, which is the active moiety. Colterol is further metabolized to N-*tert.*-butylmetarterenol through the action of enzyme catechol-O-methyl transferase. Both colterol and N-*tert.*-butylmetarterenol are conjugated as the glucuronide [1] and possibly as the sulfate.

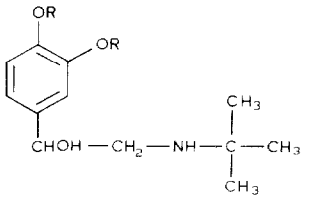

Fig. 1. Structural formulae of colterol (R=H) and bitolterol (R = —CO—C_6H_4—*p*-CH_3).

Most sample preparation techniques in catecholamine assays are based on the method of Anton and Sayre [2] in which the compound of interest is adsorbed to alumina through the catechol moiety. The use of di-(2-ethylhexyl)phosphoric acid (DEHPA) as an ion-pairing agent in the extraction of catecholamines has been reported [3, 4]. Cation-exchange resins have also been used to isolate catecholamines from biological specimens [5, 6]. Extraction of the ion-exchange eluate with a combination containing DEHPA and an organic solvent offers a convenient concentration step, with a resulting increase in sensitivity.

This report describes methods for the analysis of colterol in human plasma

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and urine by the ion-pair extraction of the catecholamine followed by high-performance liquid chromatography (HPLC) with amperometric detection.

EXPERIMENTAL

Materials

Colterol and the internal standard, the hydrochloride salt of 1-(3,4-dihydroxyphenyl)-2-cyclopentylaminoethanol, were synthesized at the Sterling-Winthrop Research Institute. Nanograde benzene (Mallinckrodt, St. Louis, MO, U.S.A.) and HPLC grade methanol were used. DEHPA was obtained from Sigma (St. Louis, MO, U.S.A.). Water was purified by the Barnstead ORGANICpure^{TM} system. All other reagents were analytical reagent grade, from commercial sources, and were used without additional purification. All glassware was silanized by treatment with 5% dimethyldichlorosilane in toluene prior to use.

Preparation of standards and samples

Stock solutions of colterol and the internal standard were prepared in 0.05 *N* sulfuric acid. Standards were prepared by adding appropriate aliquots from the stock solution to 1.0 ml of control human plasma (oxalate anticoagulant) or to 1.0 ml of control human urine. Fresh standards were prepared on the day of analysis of each set of samples.

Spiked samples were prepared for single-blind analysis in the same manner as the standards. Two sets of triplicate samples were prepared in plasma at concentrations of 0, 0.35, 0.6, 1.1 and 3.3 ng/ml. One set was analyzed upon preparation, the remaining set was stored for one week at −70°C prior to analysis. Spiked urine samples (in triplicate) were prepared in a similar manner, at concentrations of 0, 80, 180, 540 and 800 ng/ml.

Plasma assay

To 1.0 ml of plasma in a 20-ml silanized tube were added 100 μl of the internal standard solution (5 ng per 100 μl), 2.0 ml of 0.2 *M* phosphate buffer (pH 6.9), and 10 ml of 1.5% (v/v) DEHPA in benzene. The samples were mixed by shaking mechanically for 10 min, then centrifuged at 1400 *g* for 10 min. The organic phase was transferred to silanized 15-ml conical tubes, and extracted with 130 μl of 0.2 *N* sulfuric acid. The samples were shaken for 10 min followed by centrifuging at 500 *g*. The upper organic layer was carefully removed and discarded. Residual benzene vapors were evaporated under nitrogen. A 100-μl aliquot of the acid layer was injected into the HPLC system described below.

Urine assay

Cation-exchange columns were prepared by adding a slurry of resin (Bio-Rex 70, 50—100 mesh) in water to glass columns (8 mm I.D.) fitted with glass-wool plugs and PTFE stopcocks. The settled resin bed height was 4 cm. It was washed with 10 ml of water followed by 20 ml of 0.2 *M* phosphate buffer (pH 6.9).

To 1.0 ml of human urine were added 100 μl of the internal standard solu-

tion (50 ng per 100 μl) and 1.0 ml of 0.2 *M* phosphate buffer (pH 6.9). The buffered urine was transferred to the cation-exchange column and allowed to flow through the column. Following elution of the urine sample the column was rinsed with 30 ml of distilled water and drained. The catecholamines were then eluted with 4.0 ml of a 2% boric acid solution. The eluate was collected in a 20-ml silanized tube. Ten ml of a 1% DEHPA—benzene solution were added and the sample was mixed and centrifuged. The organic layer was then extracted with 1.0 ml of 0.05 *N* sulfuric acid. A 100-μl aliquot of the acid phase was chromatographed.

Acid hydrolysis

To 1.0 ml of urine, 0.5 ml of 1 *N* hydrochloric acid was added. The sample was placed in a 90°C water bath for about 30 min. The mixture was neutralized with 1 *N* sodium hydroxide (approx. 0.5 ml) before the addition of 1.0 ml of 0.2 *M* phosphate buffer (pH 6.9). The hydrolyzed sample was processed as described above.

Chromatography

Chromatographic separation was performed at ambient temperature on a modular system composed of a Dupont Model 870 pump, a Rheodyne Model 7125 sample injector, and an Altex Ultrasphere ODS reversed-phase 5-μm column (25 cm × 4.6 mm). A Bioanalytical Systems Model LC-4 amperometric detector with a TL-5 glassy carbon electrode containing a 0.002-in. spacer was used. The applied potential was +0.60 V vs. Ag/AgCl (3 *M* sodium chloride). The flow-rate was 1.0 ml/min at 125 bar.

Mobile phase

The chromatographic mobile phase consisted of 88 parts (v/v) of 0.1 *M* sodium sulfate (containing 100 mg of disodium ethylenediaminetetraacetate (EDTA) per liter adjusted to pH 2.8 with phosphoric acid, and then brought to a final pH of 3.0 with 1 *N* sodium hydroxide) and 12 parts of methanol. The mobile phase was filtered through 0.45-μm filters, and purged with helium before use.

Animal study

To demonstrate the biological applicability of the method, rats were medicated orally with 25 mg of bitolterol. Blood was drawn from the aorta and placed in tubes containing oxalate as the anticoagulant, 60 min after medication. Glass syringes were used when drawing blood samples. (A large peak which interfered with the chromatography of colterol was observed when plastic syringes were used.) Plasma was processed as described above. Urine was collected from a similarly dosed rat over a 0—6 h period and treated as described above; an aliquot of the urine was acid-hydrolyzed before analysis.

Calculations

A least-squares regression analysis of the peak height ratios (colterol:internal standard) obtained for the standards was performed. Linearity of response was evaluated on the basis of visual examination of the plot, examination of the

residuals, and an F-test for lack of fit. From the slope and y-intercept of the regression line, the concentrations of colterol in the samples were determined. The minimum quantifiable limit of the assay, defined as the concentration whose lower 80% confidence limit just encompasses zero, was estimated from the least-squares regression analysis [7].

The assayed levels for the validation samples were expressed as percent differences from the nominal values and analyzed by a two-way analysis of variance with replication to test for a concentration effect, a time effect, and a concentration-times-time effect (fresh vs. frozen). The accuracy of the assay was estimated from the mean percent differences at each concentration. The precision of the assay was estimated from the derived standard deviation of the percent differences.

RESULTS AND DISCUSSION

Chromatograms of extracted plasma and urine samples from rats medicated orally with bitolterol are shown in Fig. 2. Retention times for colterol and the internal standard are 8.5 min and 13.5 min, respectively. Regression analysis on each set of standards demonstrated a linear relationship between peak height ratio and concentration over the range of 0 and 0.25 to 5.0 ng/ml for plasma and 0 and 25 to 1000 ng/ml for urine. The concentration of each of the prepared plasma and urine specimens was estimated by inverse prediction [8] from the appropriate equation which was generated from the linear regression analysis; the results of these calculations for plasma and urine samples are summarized in Tables I and II, respectively.

A two-way analysis of variance of the nominal values for the plasma samples indicated no significant sources (either concentration, time, or concentration-times-time) of variation at $p \leqslant 0.01$. An overall estimate of the precision of the plasma method, based on the variance of the replicate determinations at each

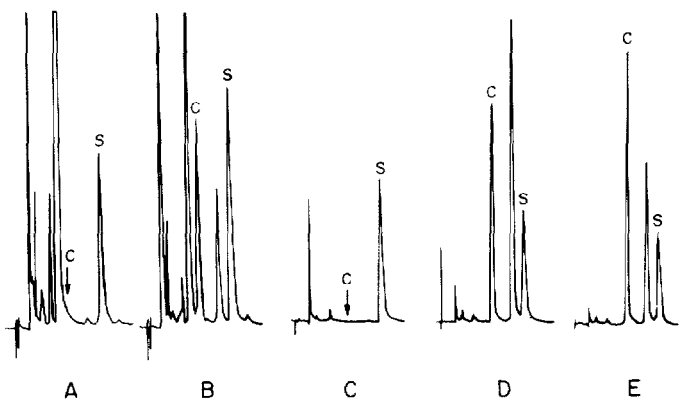

Fig. 2. Chromatograms of (A) processed rat plasma containing only the internal standard (s); (B) processed rat plasma sample taken 1.0 h after oral bitolterol dosing at 25 mg, showing colterol (c); (C) processed rat urine containing only the internal standard (s); (D) processed rat urine sample collected 0—6 h after bitolterol dosing at 25 mg; (E) same as (D), subjected to acid hydrolysis before processing. Attenuation settings: plasma samples 5 nA; urine samples 50 nA.

TABLE I

CONCENTRATION OF COLTEROL (FREE BASE) FOUND IN SPIKED PLASMA SAMPLES

Nominal concentration (ng/ml)	Mean concentration found (n = 3) (ng/ml)		
		Non-frozen	Frozen
0		<MQL*	<MQL**
0.35	Mean	0.32	0.37
	% S.E.M.***	1.03	5.43
	Mean % difference§	−7.62	6.67
	% C.V.§§	1.79	9.41
0.60	Mean	0.59	0.61
	% S.E.M.	2.48	4.64
	Mean % difference	−2.22	2.22
	% C.V.	4.29	8.04
1.1	Mean	1.12	1.06
	% S.E.M.	2.39	3.64
	Mean % difference	1.52	−3.94
	% C.V.	4.14	6.30
3.3	Mean	3.25	3.19
	% S.E.M.	2.65	0.69
	Mean % difference	−1.52	−3.43
	% C.V.	4.59	1.19

*Minimum quantifiable limit = 0.09 ng/ml.
**Minimum quantifiable limit = 0.14 ng/ml.
***% S.E.M. = S.E.M./mean × 100.
§Mean % difference = assayed mean/(nominal level − 1) × 100.
§§% Coefficient of variation (C.V.) = S.D./mean.

concentration level, was 5.8%. The accuracy of the analysis, defined by the ranges of the mean percent differences from the nominal concentrations, varied from −7.6% to +5.7%. The mean (±S.E.) minimum quantifiable level of the plasma assay was 0.11 (±0.02) ng/ml, n = 2.

An overall estimate of the precision of the urine assay, based on the variance of the repeat determinations, was 3.8%. The accuracy of the urine method, defined by the mean percent difference of the assayed value from the nominal value, ranged from −0.2% to +2.6%. The minimum quantifiable level was 5 ng/ml. Omission of the step involving isolation on the ion-exchange resin reduced the sensitivity to 19 ng/ml; since the ion-exchange procedure removes much endogenous material, the background noise level is reduced.

The extraction efficiencies of colterol and the internal standard were based on a comparison of peak heights of extracted versus direct standards and were independent of the concentration. From plasma the mean efficiency was 61% for colterol and 59% for the internal standard; from urine, the percent recovery was 75% for both colterol and the internal standard.

The concentration of colterol in rat plasma 1 h after a 25-mg oral dose of bitolterol was 36 ng/ml. The concentration of colterol in unhydrolyzed urine was 240 ng/ml; hydrolysis increased the concentration to 930 ng/ml.

In conclusion, an accurate, sensitive and reproducible HPLC assay with

TABLE II

CONCENTRATION OF COLTEROL (FREE BASE) FOUND IN SPIKED URINE SAMPLES

Nominal concentration (μg/ml)		Mean concentration found, n = 3 (μg/ml)
0		<MQL*
0.08	Mean	0.081
	S.E.M. (%)	2.14
	Mean percent difference	1.25
	C.V. (%)	3.70
0.18	Mean	0.18
	S.E.M. (%)	0.18
	Mean percent difference	0.19
	C.V. (%)	0.32
0.54	Mean	0.55
	S.E.M. (%)	2.20
	Mean percent difference	0.99
	C.V. (%)	3.80
0.80	Mean	0.82
	S.E.M. (%)	3.00
	Mean percent difference	2.63
	C.V. (%)	5.20

*Less than the minimum quantifiable level, 5 ng/ml.

amperometric detection has been developed for the determination of colterol in both plasma and urine; its utility in biological specimens has been demonstrated.

REFERENCES

1 T. Aimoto, O. Ito, R. Kimura and T. Murata, Xenobiotica, 9 (1979) 173.
2 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.
3 B.-A. Persson and P.-O. Lagerström, J. Chromatogr., 122 (1976) 305.
4 G.B. Park, R.F. Koss, J. Utter, B.A. Mayes and J. Edelson, J. Pharm. Sci., 71 (1982) 932.
5 G.M. Tyce, N.S. Sharpless and C.A. Owen, Biochem. Pharmacol., 21, (1972) 2409.
6 G.P. Jackman, Clin. Chem., 27 (1981) 1202.
7 R.W. Ross and H. Stander, Some Statistical Problems in Drug Metabolism, presented at the Princeton Conference on Applied Statistics, December 1975.
8 R.R. Sokal and F.J. Rohlf, Biometry, W.H. Freeman, San Francisco, CA, 1969.