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Note

High-performance liquid chromatographic determination of plasma catecholamines during α-methyldopa therapy

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α-Methyldopa [L-3-(3′,4′-dihydroxyphenyl)-2-methylalanine, Aldomet, αMD] is commonly used in the treatment of essential hypertension. In animals, the drug lowers blood pressure by reducing sympathetic activity [1]. Direct evidence for this mechanism in man is lacking since it is not possible to measure plasma noradrenaline (NE) levels, one of the most useful indices of sympathetic activity in man [2], when subjects are on αMD therapy.

Following oral administration of therapeutic doses of αMD (250—500 mg), plasma αMD concentrations may exceed those of NE by 10,000-fold [3, 4]. This high concentration of αMD competitively inhibits the enzyme catechol-O-methyltransferase, thereby greatly lowering the sensitivity of radioenzymatic assays for NE. With high-performance liquid chromatographic (HPLC) assays, analysis is limited to times at which plasma αMD concentrations are low because of the problem of resolving the high αMD concentrations from NE [3].

The aim of this study was to provide an HPLC assay for plasma NE which could also be used for patients on αMD therapy.

EXPERIMENTAL

Reagents

Noradrenaline bitartrate (NE), adrenaline bitartrate (E) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were obtained from Sigma (St. Louis, MO, U.S.A.). α-Methylnoradrenaline (αMNE) was a gift from Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). α-Methyldopa was obtained from Merck, Sharpe & Dohme (Australia) (South Granville, Australia). Bio-Rex 70, 50—100 mesh (Na^+), cation-exchange resin (Bio-Rad Labs., Richmond, CA,

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U.S.A.) was packed into polypropylene columns (4 × 1 cm, Bio-Rad Labs.) and washed three times with 1 *M* hydrochloric acid and 1 *M* sodium hydroxide, once with 1 *M* sodium acetate, pH 6.5, and once with 0.01% disodium EDTA before use. Alumina (Merck, Darmstadt, F.R.G.) was activated by the method of Anton and Sayre [5]. Water for HPLC was redistilled from alkaline potassium permanganate. All other reagents were of analytical grade.

Samples

Blood containing αMD and its metabolites was obtained from two sources: (1) supine, hypertensive subjects treated with αMD (500 mg per os); and (2) rabbits 3 h after a dose of αMD (50 mg/kg intravenously).

For precision studies, plasma was obtained from humans and rabbits under resting conditions and when sympathetic activity was reduced or increased. In humans, resting sympathetic activity was achieved by having the subjects lie in a supine position for 30 min; reduced activity was achieved pharmacologically by administering prazosin, clonidine and atropine [6], and increased activity by exercise on a bicycle ergometer [7]. In rabbits, resting, reduced and increased activity conditions were achieved respectively by the rabbit sitting quietly in its box, by intracisternal administration of αMD (600 μg/kg) [8], and by subjecting the animal to moderate noise stress.

Blood was collected into polypropylene tubes containing a solution of glutathione (30 mg/ml) and EGTA (100 mg/ml), pH 6.8—7.0, in the proportion of 20 μl per ml blood. Blood samples were immediately centrifuged at 4° C and the plasma separated and stored at —20° C.

Extraction of catecholamines

Plasma (2 ml) or distilled water (2 ml) containing catecholamine standards is added to 5 ml of 0.1% disodium EDTA, 0.5 ml of 1 *M* sodium acetate, pH 6.5, and 4 ng DHBA (internal standard). The solutions are passed through the Bio-Rex 70 columns, the resin is washed with 10 ml distilled water and both effluents are discarded. Catecholamines are eluted from the column with 1 ml of 0.7 *M* sulphuric acid and 3.5 ml of 2 *M* ammonium sulphate containing 0.1% disodium EDTA. The eluate is adjusted to pH 8.6 with 3 ml of 1 *M* Tris—HCl buffer, pH 8.6, containing 2% disodium EDTA; 100 mg activated alumina are added and the samples shaken for 3 min. After centrifugation, the supernatant is aspirated and the alumina washed three times with 10 ml distilled water. The alumina is transferred to a small filtration apparatus, excess water is removed by centrifugation and the catecholamines are eluted with 0.2 ml of 0.2 *M* perchloric acid. The eluate (50—100 μl) is assayed by HPLC.

Chromatography

Catecholamines in the eluate are separated by HPLC using a 150 × 4.6 mm I.D., 5-μm particle size, Spherisorb ODS2 reversed-phase column (Phase Separations, Queensferry, U.K.). The mobile phase is 0.1 *M* sodium dihydrogen phosphate, 2 m*M* sodium heptanesulphonate, 0.001% disodium EDTA, pH 5.0. Flow-rate is 1.5 ml/min. Catecholamines are detected at a glassy carbon electrode (Model LC-5) using a Model LC-4A detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The potential is set at 0.50 V vs. Ag/AgCl.

Detector response is quantitated using a Model 308 computing integrator (Laboratory Data Control, Riviera Beach, FL, U.S.A.), and is expressed as the ratio of the peak height for each catecholamine relative to the internal standard, DHBA.

Analysis of α-methyldopa

Plasma αMD concentrations are measured by a method based on that of Ong et al. [9]. Plasma is deproteinated with perchloric acid and an aliquot of the diluted supernatant injected directly for HPLC. The chromatographic system is the same as above, using a potential of 0.65 V.

RESULTS AND DISCUSSION

To minimise interference of αMD in the plasma assay for NE, catecholamines were first isolated from plasma onto a weak cation-exchange resin (Bio-Rex 70). We have previously shown that αMD is not retained by this resin under conditions which successfully isolate catecholamines from urine [10]. Incorporation of this chromatographic step prior to alumina adsorption of the catecholamines and subsequent HPLC removes more than 99% of the αMD originally present in plasma. This now allows αMD (retention time 4.6 min) and NE (retention time 6.4 min) to be well resolved from each other even when the concentration differential between the two is high (Fig. 1). In the two representative chromatograms shown (Fig. 1B and C), plasma originally contained 1.04 and 4.2 μg/ml αMD whilst NE levels were 388 pg/ml and 137 pg/ml, respectively. Peak plasma αMD concentrations in humans following a therapeutic dose of αMD (500 mg per os) range from 1.4 to 5.5 μg/ml at 3 to 4 h post dose [4, 11] indicating that the assay can be used over the entire therapeutic range for αMD.

Despite the incorporation of an additional step to separate αMD from the catecholamines, overall recoveries for the catecholamines in the assay are still relatively good. Recoveries of NE, αMNE and E from plasma averaged 48, 46 and 46%, respectively. For DHBA the recovery was slightly higher averaging 55%. Recoveries of catecholamines added to distilled water or plasma were similar. Regression lines for the calibration curves for NE were $y = 0.00063x + 0.0061$ ($r^2 = 0.997$), $y = 0.00066x + 0.158$ ($r^2 = 0.995$) and $y = 0.00065x + 0.105$ ($r^2 = 0.997$) for distilled water, human and rabbit plasma, respectively, where y represents the peak height ratio of NE/DHBA and x represents the catecholamine concentration (pg/ml). The slopes of the lines are not significantly different ($P > 0.05$). The slopes of regression lines similarly obtained for αMNE and E were also not significantly different.

Because NE occurs endogenously in plasma, assay specificity for NE was validated by comparing the effect of oxidation voltage on detector response over the range 0.2—0.6 V vs. Ag/AgCl. No difference was found between NE extracted from plasma and NE standard, indicating that the chromatographic peak identified as NE in plasma extracts is due to endogenous NE. It was not possible to carry out this procedure for E because the levels of this catecholamine were always below the limit of sensitivity in our samples. No interfering peak for αMNE occurred in chromatograms, indicating that the assay is valid for measuring this metabolite of αMD.

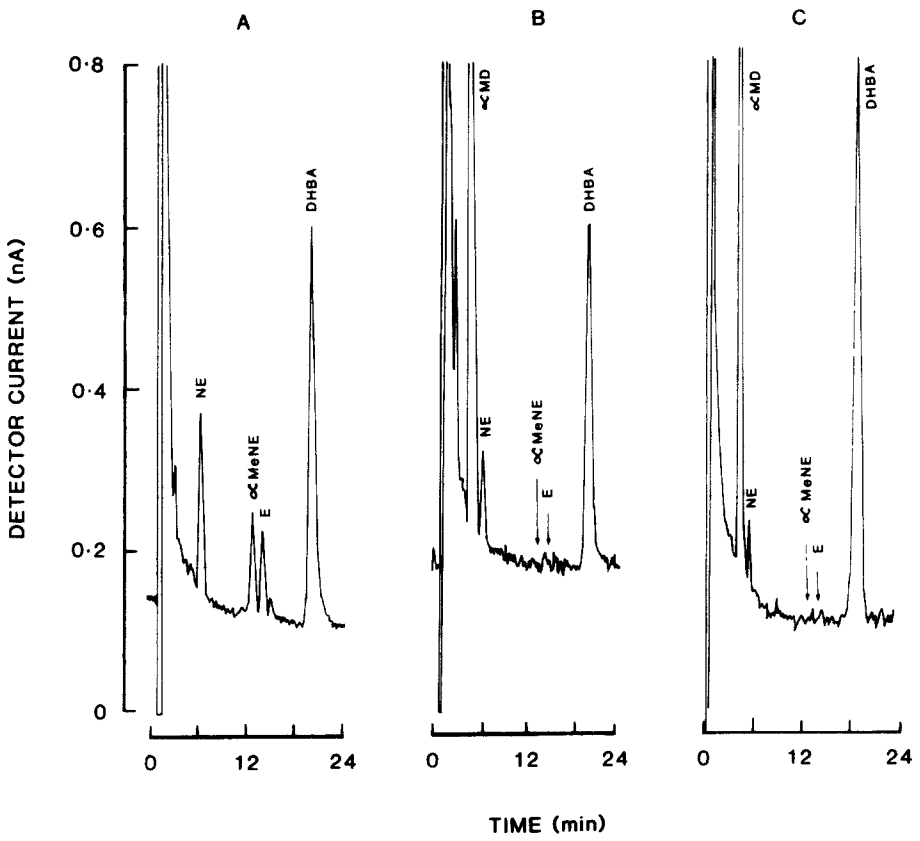

Fig. 1. Chromatographic traces of: (A) standard catecholamines, NE, αMNE and E (500 pg/ml), and internal standard, DHBA (4 ng), extracted from distilled water; (B) extract of human plasma 4 h following a dose of αMD (500 mg, per os), containing 388 pg/ml NE; and (C) extract of rabbit plasma 3 h following αMD administration (50 mg/kg, intravenously), containing 137 pg/ml NE.

TABLE I

PRECISION OF THE ASSAY

Sample	Relative sympathetic activity	Plasma NE		
		Mean ± S.D. (pg/ml)	*n*	C.V. (%)
Human	Low	171 ± 13.5	5	7.9
	Normal	224 ± 8.9	6	4.0
	High	1252 ± 38.7	6	3.1
Rabbit	Low	92.8 ± 6.3	6	6.8
	Normal	231 ± 6.9	6	3.0
	High	570 ± 20.7	6	3.6

The limits of sensitivity for NE, αMNE and E are 30, 50 and 70 pg/ml respectively when 2 ml plasma are assayed. Assay sensitivity may be further increased by using 4 ml plasma without modifying the method.

Assay precision for NE was established by replicate analysis of human and rabbit plasma samples over the concentration range 90—1200 pg/ml. This represents the entire range of catecholamine levels normally encountered. Coefficients of variation were 7—8% for levels below 200 pg/ml and 3—4% for higher NE levels (Table I). These values compare favourably with other HPLC or radioenzymatic methods [12—15].

Following αMD administration, no αMNE could be detected in plasma from either humans or rabbits. This finding is in agreement with the work of Jenner et al. [3] in which αMNE was only detected during severe exercise. It would appear that αMNE is not released in significantly large amounts from sympathetic nerves under resting conditions. This finding is consistent with αMNE reducing sympathetic activity by activating central noradrenergic neurones [1] as well as inhibiting NE release from sympathetic nerves by activating peripheral presynaptic α_2 adrenoceptors [16].

In summary, we have developed an assay for plasma catecholamines which is applicable to samples taken over the entire therapeutic range of αMD. The method can also be used in human and animal studies elucidating the mechanism of action of αMD.

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