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Note

Determination of α-methyldopa, α-methylnoradrenaline, noradrenaline and adrenaline in plasma using high-performance liquid chromatography with electrochemical detection

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α-Methyldopa is widely used in the treatment of hypertension. The mechanisms underlying its hypotensive effect are still unclear. Although the most important action of α-methyldopa is thought to be a centrally mediated reduction in sympathetic activity [1], this cannot be reliably assessed by measurement of plasma catecholamines if there is also appreciable release of the α-methyldopa metabolite, α-methylnoradrenaline. Thus it is of interest to correlate changes in plasma concentrations of noradrenaline, adrenaline and α-methylnoradrenaline with changing levels of α-methyldopa.

Of the available methods, high-performance liquid chromatography (HPLC) with electrochemical detection was most suitable because its separation power permits determination of closely related catecholamine compounds with a high degree of specificity [2, 3]. Radioenzymatic assays which have been used for the determination of noradrenaline and adrenaline in human plasma [4, 5] do not allow discrimination between adrenaline and α-methylnoradrenaline.

The approach has been used previously for the detection of α-methyldopa in biological fluids [6—8]. It has also been shown to be capable of providing sufficient sensitivity to allow determination of concentrations of endogenous catecholamines in the plasma of normal subjects [9, 10]. The present paper describes a method for the simultaneous determination of noradrenaline, adrenaline, α-methyldopa and α-methylnoradrenaline in plasma. It is an extension of a method that has been used to measure noradrenaline and adrenaline in

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a wide range of patients and normal subjects. It involves a simple extraction procedure and a single, isocratic, chromatographic run.

EXPERIMENTAL

Reagents

Noradrenaline (NA), adrenaline (A) dihydroxybenzylamine (DHBA) and α-methyldopa (αMD) were obtained from Sigma (London, Great Britain). α-Methylnoradrenaline (αMNA) was a gift from Hoechst (U.K.) (Hounslow, Great Britain). Sodium octanesulphonate was obtained in solid form from Fisons Scientific Apparatus (Loughborough, Great Britain). All other chemicals used were of reagent grade and solutions were prepared using glass-distilled water.

Stock solutions of catecholamines and αMD, 1 mg/ml in 0.1 *M* hydrochloric acid were prepared and stored at 4°C. Working standards were prepared by making suitable dilutions of the stock solutions in 0.01 *M* hydrochloric acid.

Sample collection

At the beginning of each study, subjects (untreated hypertensives or patients receiving long-term αMD therapy) had indwelling catheters inserted into forearm veins. They then rested. Three samples were taken at 5-min intervals to establish basal plasma catechol concentrations.

Further blood samples were taken immediately after 5 min of sub-maximal exercise on a bicycle ergometer.

On each occasion, 10 ml blood was collected in a chilled lithium heparin tube and immediately centrifuged at 4°C. The plasma was collected and stored at −80°C. Prior to analysis, it was thawed and centrifuged for 5 min at 4°C.

Extraction of catechols

Plasma (1—4 ml) and 2 ng of internal standard (DHBA, in 20 μl of 0.01 *M* hydrochloric acid) were added to a chilled tube containing 75 mg alumina and 1 ml of 1 m*M* hydrochloric acid containing 0.1 m*M* EDTA. A 1-ml aliquot of 3 *M* Tris, pH 8.5 was added and the tube quickly stoppered and mixed for 10 min on a roller/shaker mixer.

The liquid was then aspirated and the alumina washed three times with 12 ml water. Elution of the catechol compounds was achieved by mixing the alumina with 200 μl of 0.091 *M* orthosphosphoric acid for 1 min.

Chromatography

The chromatograph comprised an Altex 100A pump (Altex Scientific, Berkeley, CA, U.S.A.), Altex 210 injection valve, Altex Ultrasphere Octyl (5 μm, C_8-bonded silica) column (150 × 4.6 mm I.D.) and precolumn (45 × 4.6 mm I.D.), B.A.S. TL5 glassy-carbon electrode and LC4 amperometric controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A sheet-aluminium Faraday cage enclosing column, electrode and controller were used to decrease electrical noise. The chromatograms were recorded on a Servoscribe 1S potentiometric recorder.

The mobile phase was composed of 70 m*M* KH_2PO_4, 1 m*M* EDTA, 6 m*M* sodium octanesulphonate and 14% methanol, pH 6.4 (using 5 *M* sodium hydroxide). Before use, the solution was filtered and then degassed by a flow of helium. Mobile phase flow-rate was 1.5 ml/min. Working electrode potential was +0.5 V.

The orthophosphoric acid eluate (100 μl) was mixed with 30 μl of 0.27 *M* tripotassium citrate solution containing sodium octanesulphonate, EDTA and methanol in amounts calculated to equalize concentrations between the mobile phase and the injected material. This has the effect of buffering the orthophosphoric acid eluate to approximately pH 5.5. A 100-μl aliquot of the buffered eluate was injected for chromatography.

Calculation of catechol concentrations

The concentrations of αMD, NA, A and αMNA in each sample were determined by calculating the ratios of heights of each catechol peak with that of the DHBA peak in that particular chromatogram. These ratios were then compared with equivalent ratios obtained from a chromatogram of known quantities of a standard mixture, extracted and separated using a similar procedure.

RESULTS

Sample chromatogram

Fig. 1 represents a chromatogram obtained from plasma from a patient on

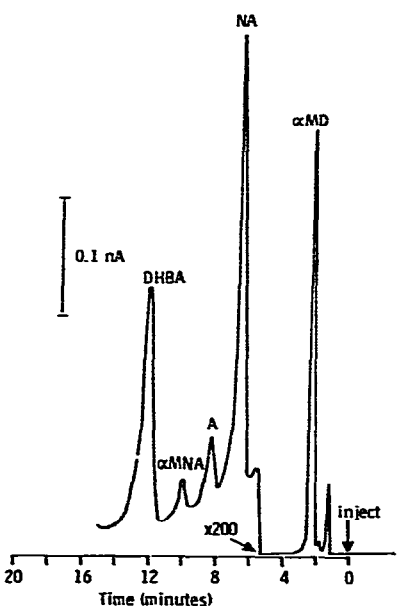

Fig. 1. Chromatogram of a plasma sample obtained from a patient on α-methyldopa therapy attending a regular hypertension clinic. Chromatographic conditions as described in Experimental. Determined concentrations: αMD, 0.275 μg/ml; NA, 0.770 ng/ml; A, 91 pg/ml; αMNA, 79 pg/ml; added internal standard (DHBA) concentration, 0.5 ng/ml.

αMD therapy, showing peaks corresponding to NA, A, the parent drug αMD, its metabolite αMNA and internal standard DHBA.

Standard curves

By comparing determined and added concentrations after the addition of various amounts of catechol standards to aliquots of a plasma pool, the method was shown to be linear in the range 20 pg/ml—20 ng/ml for NA and A, 40 pg/ml—20 ng/ml for αMNA and 10 ng/ml—2.5 μg/ml for αMD.

Precision

Repeated determinations of endogenous A and NA and added αMD and αMNA (in aliquots of pooled plasma) gave the following coefficients of variation (C.V.): A, C.V. = 4.7% at a mean calculated concentration of 51 pg/ml; NA, C.V. = 4.0% at 188 pg/ml; αMD, C.V. = 4.1% at 97.2 ng/ml; αMD, C.V. = 3.9% at 97 pg/ml.

Application in experimental subjects

Table I shows determined plasma catechol concentrations in four patients receiving αMD therapy, before and after exercise on a bicycle ergometer.

TABLE I

PLASMA CATECHOL CONCENTRATIONS IN αMD PATIENTS BEFORE AND AFTER EXERCISE

Limits of detection: αMD, 10 ng/ml; NA, 20 pg/ml; A, 20 pg/ml; αMNA, 40 pg/ml. N.D. = not detectable.

Subject		αMD (μg/ml)	NA (ng/ml)	A (ng/ml)	αMNA (ng/ml)
1	basal	1.36	0.107	N.D.	N.D.
	post-exercise	1.44	1.317	0.228	0.233
2	basal	0.22	0.248	N.D.	N.D.
	post-exercise	0.22	1.770	0.110	0.354
3	basal	0.74	0.151	N.D.	N.D.
	post-exercise	0.77	0.374	0.077	0.090
4	basal	1.06	0.063	N.D.	N.D.
	post-exercise	1.03	0.998	0.085	0.301

DISCUSSION

Interference from other substances (unidentified, but thought to include acidic catechol compounds co-extracted on alumina) was decreased by using a relatively high mobile phase pH (6.4) and sodium octanesulphonate concentration (6 m*M*). Increasing pH in the range 3.0—7.0 has the effect of selectively decreasing protonation of the acidic species, the fully protonated state of the basic species e.g. catecholamines, being largely unaffected. In the pH range 6.0—7.0, the latter are still capable of a high degree of ion-pair bonding with the octanesulphonate and thus have relatively long retention

times. Catecholamine retention times can effectively be further selectively increased by increasing the concentration of the ion-pair reagent [11].

An additional benefit obtained by using a mobile phase with high pH is improved detector response [11]. It is of note that new columns could not be used with pH 6.4 mobile phase immediately. It took some days before auto-oxidation of catecholamines (catalysed by new metallic surfaces) was reduced to a negligible level.

An operating potential of +0.5 V (relative to the B.A.S. RE1 Ag/AgCl reference electrode) proved to be optimal for achieving the maximal signal from the oxidation of catecholamines without oxidation of any other compounds with similar retention times. (Using potentials greater than +0.5 V, there is little further gain in signal from a fixed catecholamine concentration [12].)

The previously adopted system for the measurement of NA and A only, utilised mobile phase containing 5 m*M* sodium octanesulphonate and 12% methanol. 6 m*M* sodium octanesulphonate and 14% methanol were found to give better resolution of A and αMNA and were thus adopted for the present application, maintaining other factors constant. Resolution of these two substances was also found to be better using a C_8 column than a C_{18} column.

Using the present system, αMD has a very short retention time (see Fig. 1) running close to the solvent front where it may, in fact, co-chromatograph with other unidentified peaks. Although these components have not been investigated in any detail, they generally have associated peak heights which are negligible compared with those associated with αMD in the samples analysed (see e.g. Fig. 2 which shows a chromatogram obtained from plasma from a

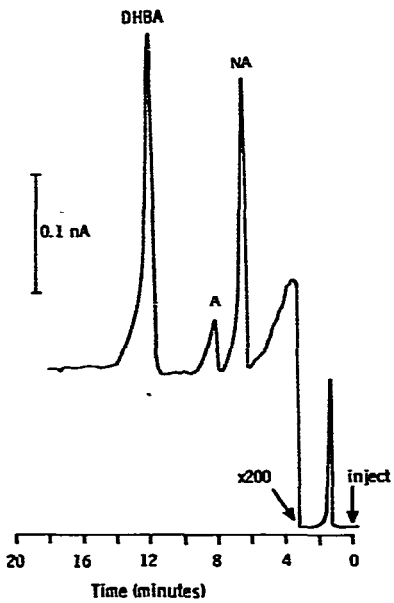

Fig. 2. Chromatogram of a plasma sample obtained from a normal subject. Chromatographic conditions as described in Experimental. Determined concentrations: NA, 0.349 ng/ml; A, 66 pg/ml; added internal standard (DHBA) concentration, 0.5 ng/ml.

normal subject). In patients on αMD therapy, determined plasma αMD concentrations were in the range 0.2—2.0 μg/ml. Comparable levels have been reported by other workers [8, 13]. These concentrations are three to four orders of magnitude higher than concentrations of endogenous catecholamines. Chromatographic problems associated with peak overlapping have been noted by Moyer et al. [14], who used a similar system, but by using an efficient column and ensuring sufficient difference in retention times between αMD and later eluting peaks, this problem is diminished. Using the present system, αMD peak tailing only becomes a problem at high plasma levels, > 2 μg/ml. In order to measure αMD, NA, A, αMNA and internal standard DHBA in the same chromatogram, it is necessary to employ large sensitivity changes in mid-run as illustrated in Fig. 1. These sensitivity changes do not lead to problems in quantitation, high plasma αMD concentrations are still within the linear range of the system.

The method described was shown to be capable of detection of plasma concentrations of NA in resting subjects and of NA and A in subjects after exercise. Although detection limits for αMNA were similar, it is of interest that the substance was not found in plasmas from resting αMD patients. However, its appearance on exercise demonstrates that it is clearly present in the sympathetic nerve endings of these patients.

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