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Note

Assay and stability of α-methyldopa in man using high-performance liquid chromatography with electrochemical detection

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Due to the important intersubject [1] and intrasubject [2] variation of the bioavailability of α-methyldopa, plasma monitoring of this antihypertensive drug is considered necessary. Previous measurements of α-methyldopa in biological fluids have been carried out by fluorimetric [1, 3] and gas—liquid chromatographic procedures [4]. More recently, two methods by high-performance liquid chromatography (HPLC) with electrochemical detection were proposed [5, 6]. All methods, with the exception of that of Cooper et al. [6], employ a sample clean-up step involving adsorption on alumina, a difficult and time-consuming procedure. In the latter method, however, the acid supernatant from the deproteinization with perchloric acid is chromatographed directly. Our preliminary experiments using this method (unpublished observation) did not give satisfactory results, possibly due to the instability of the drug and its metabolites: inconsistent results were obtained on the concentration profiles of this drug in plasma from 3—8 h following a single 250-mg dose administered to volunteers, when an apparent increase of its concentration was observed.

The addition of the antioxidant, sodium metabisulfite, to the evacuated blood collection tubes has been described by Kim and Koda [3] in connection with their fluorimetric procedure. However, most workers use heparinized tubes without further additives.

Perchloric acid has been used by Kwan et al. [1] for the release of α-methyldopa from its conjugated metabolites, but the rate of hydrolysis at room temperature (during the deproteinization step) has never been reported. The present study was undertaken to optimize conditions for the rapid analysis of α-methyldopa in biological specimens.

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EXPERIMENTAL

Reagents and standards

Perchloric acid, sodium bisulfite, disodium EDTA, citric acid and disodium hydrogen phosphate were reagent grade. Water was deionized and glass double distilled. The mobile phase was passed through a 0.2-μm filter (Millipore, Bedford, MA, U.S.A., FGLP 025 00) before introduction to the system. α-Methyldopa (Aldrich, Milwaukee, WI, U.S.A.) and sodium octanesulfonate (Eastman Chemicals, Rochester, NY, U.S.A.) were used without further purification. The stock solution of α-methyldopa (1 mg/ml) in perchloric acid (0.05 *M*) containing sodium bisulfite (0.1%) and disodium EDTA (0.005–0.05%) was freshly prepared every week. The working standards were prepared fresh each day by dilution of the stock solution with blank plasma (outdated plasma, Canadian Red Cross, Ottawa, Canada) or fresh whole blood from human volunteers. The protein precipitant solution was perchloric acid (1 *M*) containing sodium bisulfite (0.2 m*M*).

For blood collection commercially available evacuated containers with EDTA as anticoagulant (Vacutainers, 10 ml, Lavender stopper, B-D, Canlab, Ottawa, Canada) were used, to which 0.1 ml of 1 *M* sodium metabisulfite solution was added. The addition was done with a 26-gauge needle to prevent the loss of the vacuum.

Equipment

The chromatographic system consisted of a pump (Waters Assoc., Milford, MA, U.S.A., Model 6000A), an injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7125) with a 10-μl loop and a reversed-phase column (Waters Assoc., μBondapak alkyl phenyl column, mean particle size 10-μm, 30 × 0.39 cm). The detection system consisted of a thin-layer flow-through electrochemical cell with glassy carbon as the working electrode, and a silver–silver chloride reference electrode (Bioanalytical Systems, W. Lafayette, IN, U.S.A., Models TL5 and LC2A) and a recorder (Perkin-Elmer, Norwalk, CT, U.S.A., Model 56). The glassy carbon electrode was repolished once a week, or as required, using the polishing kit supplied with the detector.

Chromatographic conditions

The mobile phase consisted of a 2:1 mixture of 0.02 *M* citric acid and 0.02 *M* Na_2HPO_4 containing sodium octanesulfonate (0.1%) and sodium EDTA (0.055 *M*) adjusted to pH 3.05 with phosphoric acid. After warming to slightly above room temperature, the mobile phase was sparged with helium and pumped at a flow-rate of 2 ml/min. The effluent was passed through the detector cell and monitored at a potential of + 0.75 V versus the silver–silver chloride reference electrode.

Procedure

The standard curve was prepared by adding α-methyldopa, 0, 0.05, 0.1, 0.5 and 1.0 μg/ml, to commercial blank plasma or whole blood samples; four aliquots at each concentration were assayed as described below, and the recoveries were estimated by comparing the peak heights obtained to those observed

after direct chromatography of the stock solution. A calibration curve with working standards in the expected concentration range was prepared daily.

For analysis of plasma or whole blood samples a 100-μl aliquot was mixed with 200 μl of the perchloric acid precipitant, the sample was immediately agitated on a Vortex mixer for 30 sec and cooled in an ice bath for 5 10 min. After centrifugation at 2000 *g* at 4°C for 20 min, aliquots (10 μl) of the supernatant were chromatographed. The samples were kept on ice until the injection which was done within 30 min after the addition of perchloric acid.

Method validation

One healthy male volunteer (86 kg, 50 years old) was given an oral dose of 250 mg α-methyldopa with 100 ml water after an overnight fast. Two 10-ml blood samples were collected from the antecubital vein at 0, 0.5, 1, 2, 3, 5 and 8 h after the dose using the evacuated containers described above. One of each of the tubes was centrifuged immediately (600 *g*, 5 min) and, after noting the hematocrit, the plasma was removed. Both the plasma and the whole blood samples were divided into several aliquots, a third of which were analyzed immediately, another third after one week, and the rest after four weeks of storage at −18°C. The acid supernatants of the 3- and 5-h plasma samples were rechromatographed after incubation for various times at room temperature and 4°C.

Quantitation

All measurements were done by peak heights. Recovery was estimated by comparing the peak heights of the standard curve with that of the stock α-methyldopa solution. The slope and intercept of the standard curve were obtained by linear regression of peak height on concentration ($Y = AX + B$).

RESULTS AND DISCUSSION

Chromatography

The chromatographic conditions presented here permit the resolution of α-methyldopa from the endogenous catecholamine neurotransmitters (Fig. 1A). The plasma of a human volunteer at 0 h, as well as outdated plasma obtained commercially were found to give essentially clean blanks (Fig. 1B). Fig. 1C shows a chromatogram of plasma extract of the volunteer 5 h after a 250-mg oral dose of α-methyldopa (estimated concentration: 437 ng/ml), and Fig. 1E of blank plasma to which 100 ng α-methyldopa were added.

After several weeks of repeated use of the same column there was a marked deterioration in the resolution between an endogenous substance and the drug; however, by increasing the octanesulfonate concentration in the mobile phase from 0.10 to 0.11% the original resolution was re-established (Fig. 1D and E).

Standard curve

Using the method described above standard curves were constructed for both plasma and whole blood at the concentrations of 0, 0.05, 0.1, 0.5 and 1 μg/ml. The linearity for the standard curve for plasma, with four determinations at each concentration, was excellent ($r^2 > 0.99$, $n = 16$) and a least-squares linear

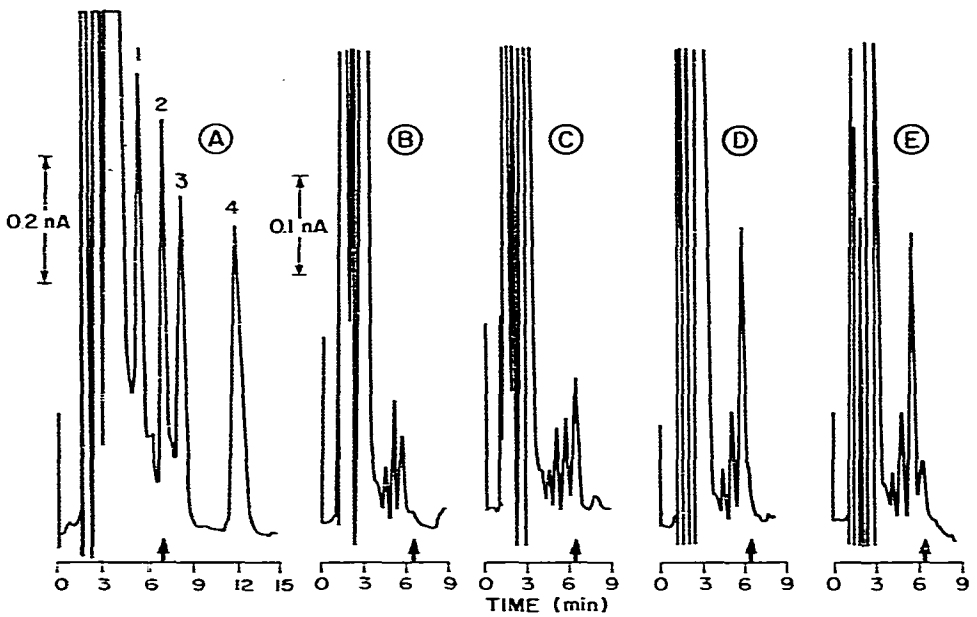

Fig. 1. Chromatography of α-methyldopa and catecholamines. (A) Chromatograms of aqueous standards. Peaks: 1, norepinephrine; 2, α-methyldopa (arrow); 3, epinephrine; 4, dopamine. (B) Plasma blank (volunteer, 0 h). (C) Volunteer 5-h plasma sample following ingestion of 250 mg α-methyldopa (estimated concentration 0.437 μg/ml). (D) Blank plasma spiked with 0.1 μg/ml drug (old column). (E) Same as (D) but with 0.11% sodium octanesulfonate in the mobile phase. Conditions: alkyl phenyl column; mobile phase, 0.02 *M* citrate phosphate buffer, pH 3.05, containing 0.055 *M* disodium EDTA and 0.1% (A—D) or 0.11% (E) sodium octanesulfonate; flow-rate, 2 ml/min; detector, + 0.75 V vs. the Ag/AgCl reference electrode.

regression of peak height on concentration gave a slope (± 95% confidence interval) of 0.429 (± 0.015) nA/ng, a negligible intercept of 0.006 (± 0.008) nA and a mean coefficient of variation (C.V.) of 6% (n = 4 at each concentration). The mean recovery was 93.7% (n = 16). Because of good recovery and the use of constant-volume injection loop the use of an internal standard, as recommended by Cooper et al. [6] was found unnecessary.

Results on whole blood appeared to be much more variable, with a mean C.V. of 17%, possibly due to interference from some components of the erythrocytes. However, as shown below (cf. Fig. 2), α-methyldopa appears to be at least partially excluded from the erythrocytes, reducing the need for an assay method for this drug in whole blood.

Stability

Preliminary experiments using the blood and plasma of three human volunteers to whom α-methyldopa had been administered and whose blood was collected in the presence of heparin only (Vacutainers, Green Stoppers, Becton-Dickinson, Canlab, Ottawa, Canada) gave variable and unreproducible results (personal observations): fresh plasma from volunteers given α-methyldopa, precipitated and kept at room temperature until chromatography, appeared to give bimodal plasma concentration vs. time profiles; repeat analyses of plasma

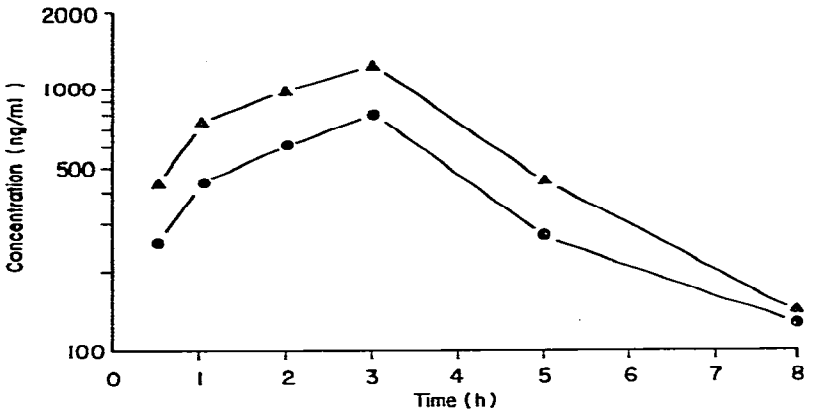

Fig. 2. α-Methyldopa concentrations in the plasma (▲) and whole blood (●) of a volunteer after 250 mg of the drug taken orally. Assay as described in the text using aliquots stored at −18°C for 1 week; for chromatographic conditions, see Fig. 1.

samples which had been kept frozen for several weeks showed a sharp decline in concentrations.

In the final method, as described in the experimental section, the stability of the sample was greatly enhanced by collecting the blood in the presence of EDTA and sodium metabisulfite (to prevent oxidation of the catechol) and reducing the time of contact with the acid precipitant to 30 min at 0°C (to prevent hydrolysis of the conjugated metabolites). Fig. 2 illustrates the plasma and whole blood concentrations in a volunteer during the 8 h following a single oral dose of 250 mg α-methyldopa. From the plasma values it appears that the drug is readily absorbed, absorption half-life being less than 1 h, and rapidly eliminated, with an approximate elimination half-life of less than 2 h. Exact pharmacokinetic modeling was not possible because of the paucity of values, especially between 4 and 8 h. The values for whole blood were consistently lower, indicating a partial, though not complete, exclusion from the red cells.

Plasma α-methyldopa concentrations following 1 and 4 weeks of storage at −18°C are shown in Table I. There was a slight decline in plasma concentra-

TABLE I

THE EFFECT OF STORAGE ON THE CONCENTRATION OF α-METHYLDOPA IN THE PLASMA

The 1- and 8-h samples were not available for immediate analysis.

Time (h) after dose	Concentration (ng/ml) assayed immediately	Percentage remaining after storage at −18°C for	
		1 week	4 weeks
0	0	—	—
0.5	440	98.9	92.3
2	1016	95.6	95.9
3	1193	102.6	97.6
5	475	92.0	82.7
Mean	—	97.3	92.1

tions after 1 week, as compared to the aliquots assayed immediately after blood collection. The mean loss amounted to 2.7%. A somewhat greater loss (mean 7.9%) was observed after 4 weeks at −18°C.

The effect of perchloric acid on the precision of the assay, attributable to possible hydrolysis of the conjugated metabolites, was investigated by chromatographing the acid supernatant of the 3-h and 5-h plasma samples after incubating for 2, 6 and 24 h at room temperature or 24 h at 4°C. The results in Fig. 3 show that the apparent concentration of α-methyldopa increases steadily, this increase being greater at room temperature, and especially with the 5-h sample (more than double by 24 h), presumably due to the greater proportion of metabolites.

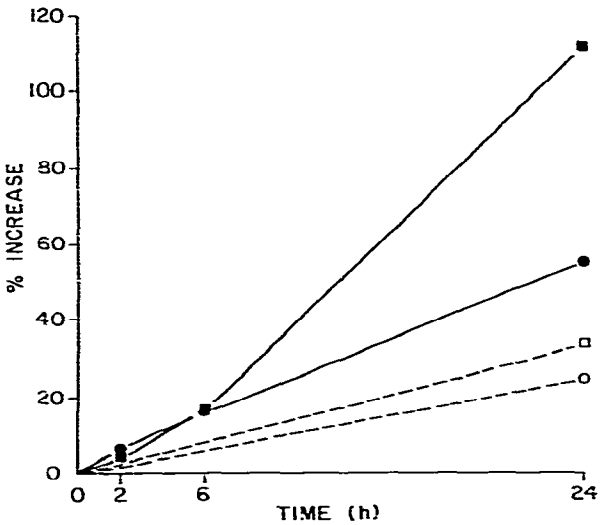

Fig. 3. Release of α-methyldopa from its conjugates in the presence of perchloric acid. Plasma taken 3 h after 250-mg oral dose, incubated at 4°C (○) and at room temperature (●); plasma samples 5 h after the dose, incubated at 4°C (□) and at room temperature (■).

In conclusion, difficulties due to instability associated with blood collection, plasma storage and sample work-up have been reduced to manageable proportions. By using an inhibitor in the collection tubes, avoiding prolonged storage of the plasma samples and keeping the time of contact between the acid precipitant and supernatant to a minimum, a rapid, simple and sensitive assay for this drug in the plasma has been developed which can be used for clinical analyses, as well as for pharmacokinetic studies. Similar precautions may well prove necessary for other, naturally occurring catechols, because of their similar redox and metabolic behavior.

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