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Note

Determination of methyldopa and metabolites in human serum by high-performance liquid chromatography with electrochemical detection*

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Methyldopa [(S)-3-(3,4-dihyroxyphenyl)-2-methylalanine] is an effective antihypertensive agent which has been used extensively for the treatment of hypertension. Previous studies [1—5] have shown that the absorption, biotransformation and incidence of side effects produced by methyldopa vary in individual patients and may be significantly altered in patients with impaired renal function [6]. Thus, correlating serum levels of methyldopa and its metabolites with therapeutic response may provide a more effective means of using this valuable agent.

The quantitative analysis of methyldopa in biological specimens has most commonly been carried out by fluorometric procedures which are protracted and rather non-spe[illegible] [1—5]. Other methods utilizing [^{14}C]methyldopa in conjunction with pa[illegible] and thin-layer radiochromatography [7] or gas chromatography requiring derivatization procedures prior to analysis, have also been reported [8, 9]. Recently, a technique employing high-performance liquid chromatography (HPLC) with ultraviolet detection has been described for assay of methyldopa in serum and urine [10, 11].

This report describes a highly sensitive and selective method for the determination of methyldopa and its principal biotransformation products in serum, utilizing HPLC with electrochemical detection. This recently developed detection system [12] has proved to be extremely sensitive and specific for catecholamines, and compounds of similar chemical structure present in biological

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material [13—16]. The technique presented requires a minimum sample work-up time, making this method attractive for routine analyses.

EXPERIMENTAL

All chemicals employed were of reagent grade. Water used in the preparation of buffers was glass-distilled over ethylene diamine tetraacetic acid (EDTA).

High-performance liquid chromatography

The apparatus consisted of a pumping system (Waters Assoc., Model 6000) and an electrochemical detector (Bioanalytical Systems, Model LC-2A). A glass column, 500 mm × 2 mm I.D. (Altex Scientific) was dry-packed with Vydac SCX cation-exchange resin, particle size 30—44 μm (Altex Scientific). The eluent was 20 m*M* ammonium dihydrogen phosphate (pH 2.55) containing 0.1 m*M* EDTA pumped at a flow-rate of 0.4 ml/min with a column pressure of 300—350 p.s.i. The inclusion of EDTA in elution buffers of pH values less than 4.0 was shown to be necessary to suppress erratic fluctuations in the background current, presumably due to electroactive materials present on the resin or in the pump itself. The working electrode potential was set at +0.54 V against an Ag/AgCl reference electrode, and the detector operated at a sensitivity of 2—20 nA f.s.d. depending on the concentration of methyldopa in the biological specimen.

Procedure

To 1.0 ml serum, 50 μl of concentrated (70%) perchloric acid were added. The sample was immediately agitated on a Vortex mixer for 30 sec and kept on ice for 15 min. After centrifugation for 15 min at 1000 *g* (4°), an aliquot of the acid-extracted supernatant was filtered through a Centriflo membrane cone (Amicon) by re-centrifugation at 1000 *g*. An appropriate volume (usually 10 μl) of the ultrafiltrate was injected onto the chromatographic column and the value obtained represented the amount of unconjugated methyldopa present. In order to determine total drug levels, the remainder of the sample was kept in a boiling water bath for 20 min. The hydrolyzed sample was then processed in the same manner as the non-hydrolyzed one. Working standards of methyldopa or its various metabolites containing 0.1—10.0 μg/ml were prepared from outdated normal human serum and treated identically as above.

RESULTS AND DISCUSSION

A representative chromatogram demonstrating the resolution of methyldopa, its metabolite methyldopamine and the endogenous catecholamine neurotransmitters, norepinephrine and dopamine, is shown in Fig. 1A. In addition, 3,4-dihydroxybenzylamine, an exogenous catechol used routinely as an internal standard for tissue catecholamine determinations, has also been included. Under these conditions, serum concentrations of methyldopa in the range of 50—100 ng/ml ($2—5 \cdot 10^{-7}$ *M*) were routinely detected. A chromatogram of a patient's serum prior to and following hydrolysis at 90° is shown in Fig. 1B. Since only the unconjugated species of methyldopa can be detected directly,

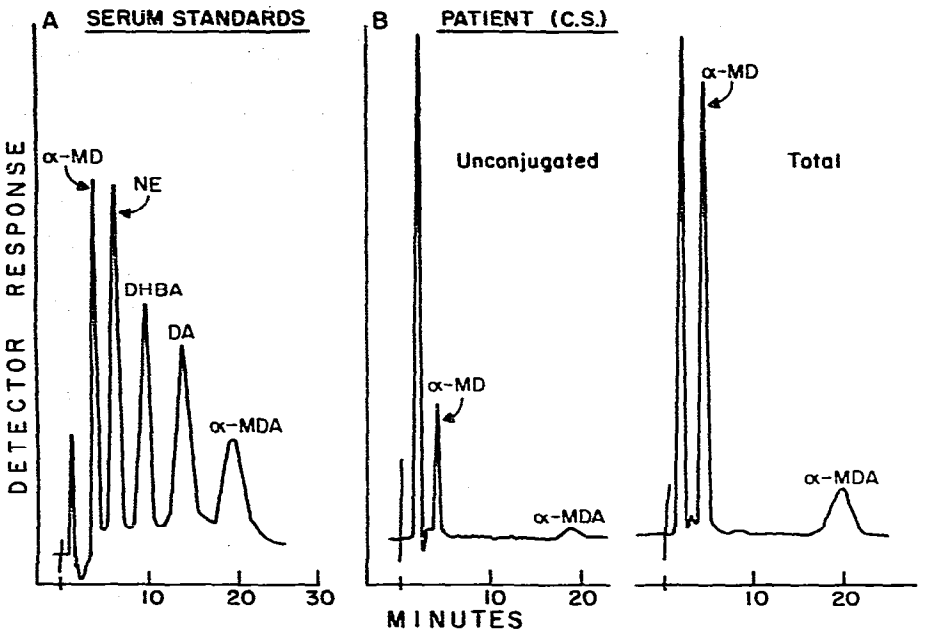

Fig. 1. (A) High-performance liquid chromatogram of reference compounds in human serum. Each peak represents 10 ng of standard compound: α-MD = methyldopa, NE = norepinephrine, DHBA = dihydroxybenzylamine, DA = dopamine, α-MDA = methyldopamine. (B) Elution profile of patient serum before (left) and after (right) hydrolysis at 90°. Unconjugated α-MD, 0.8 μg/ml; total α-MD, 3.4 μg/ml; unconjugated α-MDA, 0.2 μg/ml; total α-MDA, 1.0 μg/ml. Conditions: 500 × 2 mm I.D. Vydac SCX column, with 20 m*M* ammonium dihydrogen phosphate and 0.1 m*M* EDTA as eluent; flow-rate, 0.4 ml/min; electrochemical detection at +0.54 V.

the absolute quantity of sulfated metabolite present in a given specimen is represented by the difference between the total (determined after hydrolysis) and unconjugated (determined prior to hydrolysis) compounds. The decarboxylated metabolite of methyldopa, methyldopamine, can also be quantitatively determined in patient serum by this method, as demonstrated in Fig. 1B. Like the parent compound, methyldopamine also appears to be present both as an unconjugated and sulfate-conjugated species.

Standard curves for methyldopa in human serum were linear over the range 0.05—5.0 μg/ml. Therefore, serial dilutions of sera were routinely performed to insure that measurements fell on the linear portion of the standard curve. The average recovery of methyldopa added to serum when concentrations between 0.05 and 10.0 μg/ml were studied was 89.2 ± 4.1% (mean ± S.D., $n = 35$).

In a preliminary series of investigations, this method has been applied to the quantitation of methyldopa in sera obtained from hypertensive pediatric patients with normal renal function (serum creatinine <1.3 mg/dl). The mean serum concentration of unconjugated methyldopa 8 h after a morning dosage (7.5 mg/kg) was 0.63 ± 0.23 μg/ml, which represents 58% of the total methyldopa present in serum. This value is essentially identical to those recently reported by Myrhe et al. [3]; however, the absolute values reported in this

study were slightly lower. This discrepancy most likely results from the longer interval between dosage and sampling time (8 vs. 4 h), or possibly because the fluorescence method used by Myrhe and coworkers [3, 4] cannot distinguish between methyldopa and methyldopamine, resulting thereby in falsely elevated values.

Serum volumes of 1 ml were used in our investigation, but when sample size is limited, as in pediatric applications, the whole procedure can be successfully performed with serum volumes as small as 100 μl [6]. In addition, this method for the determination of methyldopa and its metabolites in human serum has several important advantages over those previously described. It is more rapid, sensitive and specific than fluorometric techniques and the ultrafiltration purification eliminates the alumina adsorption step [11]. Furthermore, sample derivatization is not needed as for gas chromatographic analysis [8, 9]. The use of electrochemical detection also provides a greatly enhanced sensitivity compared to ultraviolet absorption [10, 11]; this factor also enables the analysis of much smaller volumes. In the procedure of Walson et al. [10] the lower limit of detection was 1 μg/ml, using 1-ml serum samples. Mell and Gustafson [11] processed 5—30-ml aliquots of urine for each analysis, and, moreover, the entire procedure was more time consuming since an alumina adsorption step was used. Both these systems analyzed only parent drug; the current procedure enables analysis of metabolites as well. This technique should facilitate the quantitation of methyldopa and its metabolites in biological specimens acquired from patients on antihypertensive drug regimens.

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