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Note

Determination of α-methyldopa and methyldopate in human breast milk and plasma by ion-exchange chromatography using electrochemical detection

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Hypertension during pregnancy is not uncommon and may require drug therapy. α-Methyldopa and, if an injected form of the drug is required, methyldopate (methyldopa hydrochloride ethyl ester), are often prescribed. Since any drug regimen at this time may be dangerous the clinical need must outweigh the risk. The active treatment of hypertension during pregnancy however, has been shown to be associated with a significantly improved foetal outcome [1]. A disadvantage is that in the neo-natal period the child will carry a load of the drug transferred in utero [2, 3] and in addition may receive further doses from its mother's milk [2, 4]. At birth the child may have a sufficiently high plasma level of methyldopa to produce a clinically measurable effect [5]. Although the few results published so far [2, 4] suggest that there is little clinical significance in the small additional amounts transferred in the breastmilk, insufficient work has been done to confirm this and establish the amount excreted in the milk and its relation, both temporal and absolute, to methyldopa dosage.

Many assays for methyldopa in plasma and a few for methyldopate have been published. Most recently the methods are by high-performance liquid chromatography (HPLC) using a variety of strategies. Although some early work was reported using ion-exchange methods [6, 7], recent workers have favoured a reversed-phase column and an ion-pair technique [8—10]. This is a good method for the related catecholamines but less so for methyldopa. The use of 3,4-dihydroxybenzylamine (DHBA) [11] as an internal standard is becoming accepted for these assays as well as for those of the catecholamines. Detection by UV [7, 12] and also by fluorescence [2, 13] have been reported. However UV detection is not quite sensitive enough for the lowest level of drug found in clinical samples and, although more sensitive, fluorescence methods require a comparison with blank samples which may not always be available. Electrochemical detection is emerging as the method of choice [8—10]. This

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paper describes an assay using a strong cation-exchange material coupled with the sensitivity and specificity of electrochemical detection.

MATERIALS AND METHODS

Chemicals and reagents

2-Methyl-3-(3,4-dihydroxyphenyl)-L-alanine (L-α-methyldopa) and 3,4,-dihydroxybenzylamine were supplied by Sigma (St. Louis, MO, U.S.A.). α-Methyldopate was a gift from Merck Sharp and Dohme Research Laboratories (Hoddesdon, Great Britain). The internal standard (DHBA) was dissolved in the running solvent: 5 μl (equivalent to 2.5 μg DHBA) of a stock solution containing 500 μg/ml was added to each assay. The sulphatase preparation used was Helix pomatia juice (Industrie Biologique Française, Paris, France). All reagents and solvents were Analar grade.

High-performance liquid chromatography

Isocratic elution was performed with a Waters Assoc. (Milford, MA, U.S.A.) 6000A pump. Samples were applied through a Rheodyne 7125 injection valve with a 20-μl loop and detection made with a TL-8A transducer (glassy carbon electrodes) connected to an LC-4 electronic controller (Bioanalytical Systems, Lafayette, IN, U.S.A.). The column (24 cm × 4.6 mm I.D.) was packed with Partisil-10 SCX strong cation exchanger, particle size 10 μm (Whatman, Maidstone, Great Britain). The mobile phase was 33.8 m*M* perchloric acid (0.2% v/v), 50 m*M* sodium perchlorate and 0.2 m*M* EDTA in 20% (v/v) methanol in water at a flow-rate of 1.5 ml/min. Before starting all the stainless-steel surfaces to the top of the column were passivated with 2 *M* nitric acid. The column was then pre-washed with a solution of 250 m*M* sodium perchlorate in aqueous 0.2% (v/v) perchloric acid, followed by water (25 ml), then methanol (25 ml) before equilibrating with the eluting solvent. The column was run at ambient temperature. A hydrodynamic voltammogram for α-methyldopa with glassy carbon electrodes at a current of 100 nA was constant above an applied voltage of 0.75 V; the applied voltage used in these analyses was 0.80 V.

Extraction procedure and sample preparation

Methyldopa is present free and conjugated as the sulphate in both milk and plasma. Total methyldopa in milk was assayed after deconjugation of the sulphate with sulphatase. Milk (1 ml) containing DHBA (2.5 μg) was adjusted to pH 4.8 with 0.2 *M* acetate buffer, sulphatase (0.01 ml) added and the mixture incubated at 37°C for 48 h. Methyldopa was then assayed as below. Total methyldopa in plasma was assayed after acid hydrolysis, according to the method of Saavedra et al. [13]. The analytical method for the analysis of methyldopa in both milk and plasma was similar except that plasma samples were not submitted to a defatting step.

The milk—sulphatase—buffer mixture after deconjugation (see above), or for free methyldopa milk (1 ml) containing DHBA (2.5 μg), was cooled in ice and defatted by extraction with diethyl ether (3 × 1 ml). Separation of the layers by centrifugation was only found necessary for the final extraction volume. It is important at this stage to keep the mixture cold and avoid too vigorous

shaking, e.g. with the Whirlimixer, which produces an intractable gel. Residual ether was removed by bubbling nitrogen through the cooled sample. The defatted milk was deproteinised by first adding 4 *M* perchloric acid (0.1 ml per ml sample), Whirlimix for 15 sec, standing for 5 min then heating in a water bath at 90°C for 5 min. After cooling the mixture was centrifuged (at 2000 *g* for 10 min), the supernatant removed and the residue washed with water (1 ml), recentrifuged and the supernatants combined. Filtration through a 1.2-μm filter gave the sample for analysis.

Calibration curves and extraction yields

The validity of the method was established by adding known amounts of the compounds to drug-free samples of milk and plasma and assaying the mixture by the same extraction procedure described above; peak heights were plotted against the concentrations of the compound. Calibration curves were linear throughout the range studied as shown in Fig. 1 which also illustrates the greater response of the detector for the amine compared to the amino acid. Because of its particular clinical use methyldopate was only assayed in plasma.

The relative response of the detector to the analytes was determined using standard solutions of the compounds in the running solvent. Work-up of milk and plasma samples containing added known amounts of one compound with addition of another as standard at the end of the procedure enabled the efficiency of the method to be determined. Methyldopa and methyldopate are carried through the work-up of both milk and plasma without loss (95—106% recovered), however some DHBA is lost during the procedure (70—79% recovered).

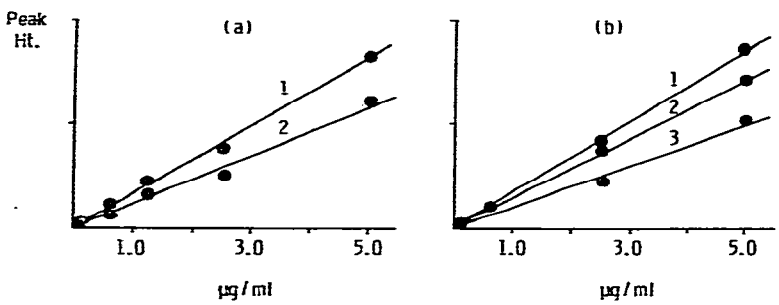

Fig. 1. Calibration curves of standard mixtures extracted from (a) milk and (b) plasma. 1, DHBA; 2, methyldopa; 3, methyldopate. The dots encompass the S.E.M. from replicate assays.

RESULTS AND DISCUSSION

Milk is well recognised by the analyst as an awkward matrix to work with, human milk presenting more problems than cow milk. An assay as described herein for non-lipid-soluble drugs requires the removal and discarding of the fat as a preliminary step. The lipid concentration of milk varies throughout a feed increasing to the end. For the assay of non-lipid-soluble materials it is preferable therefore to obtain a sample by expression from a full breast thus obtaining a sample containing less lipid. Mechanical removal of the fat after centrifu-

gation followed by cooling in ice [14] was not satisfactory in our hands due to low mechanical strength of the fat pellet in our samples. Extraction with diethyl ether proved superior if care was taken not to agitate the system excessively.

The removal of proteins from the sample is not a simple matter as human milk contains the soluble, or whey, proteins lactalbumin and lactoglobulin. Whey proteins are not coagulated by acid but they are coagulated by heat. Thus precipitation of casein and similar proteins by perchloric acid was followed by heating to precipitate the remaining soluble protein material. This work-up resulted in no loss of the analytes methyldopa and methyldopate but a consistent loss of some of the internal standard both from milk and plasma. Also, under these conditions no observable hydrolysis of the conjugated drug occurred.

The problems arising from the assay of the O-sulphate have not previously been discussed fully. It has been stated that deconjugation with sulphatase results "in substantial degradation of methyldopa" [15]. We have found that loss of methyldopa and the internal standard occurs upon incubation, with and without the enzyme present, in plasma samples. There is no similar loss in milk samples; it is not unreasonable to propose that the fat layer of the milk is protective possibly by the exclusion of oxygen. We explored the alternative acid hydrolysis for the plasma samples. Generally losses occurred or interfering compounds developed. Most previous assays have relied upon fluorescence measurements: loss of methyldopa, as measured by HPLC with electrochemical detection, may not be loss of a fluophore. It is almost certain that plasma levels of the O-sulphate conjugate have no pharmacological significance and therefore we have not exhaustively explored the possibilities of assaying plasma for this compound. Milk, like urine, represents an end compartment and the concentrations of conjugate therein are the result of active or passive transport from the blood. The pharmacological significance of the conjugate in the milk relies upon gut absorption and the sulphatase activity in those compartments of the infant where the drug conjugate appears.

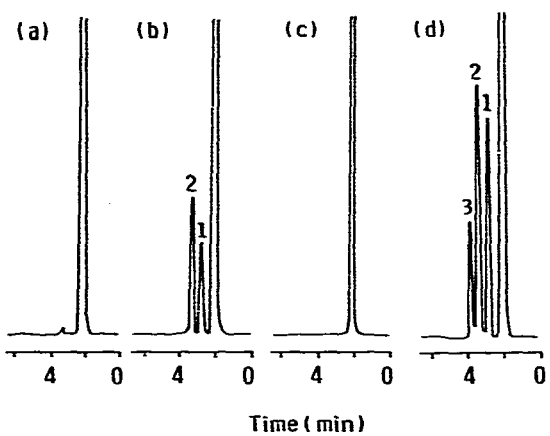

Fig. 2. Chromatograms showing the separation of (1) methyldopa, (2) DHBA and (3) methyldopate extracted from (b) milk and (d) plasma (2.5 μg/ml; 20 nA) compared to drug-free extracts of (a) milk and (c) plasma at the same sensitivity.

The chromatograms in Fig. 2 show the adequate separation and good peak symmetry obtained with this method. No significant background interferes with the assay over the concentration range studied but it was noted that an extension of the assay, looking for the drugs in erythrocytes, would be frustrated in the case of methyldopate by the presence of a background peak. The sulphatase preparation used did not introduce any interfering compound. Neither was there interference from other drugs prescribed: anti-anxiolytics, diuretics and anti-bacterials.

The use of an ion exchanger rather than an ion-pairing technique apparently gives superior results and eliminates the many problems associated with the latter method. No degradation of the column performance was noted over several months. When not in use the column and detector were stored with 20% methanol in water, or methanol when the period was a week or more, and reequilibration with the running solvent was quickly established.

The test of any assay method is its use for real samples. Unfortunately throughout the period of this study only samples containing methyldopa were received. Methyldopate is however hydrolysed in vivo to methyldopa [7, 13] and we have shown that the assay works for the mixture of the two compounds that is found clinically when this drug is prescribed [7, 13]. The results are shown in Table I. The concentration of methyldopa, free and conjugated, is less in milk than in plasma for samples collected at the same time as expected for a weakly acidic material. Milk (average pH 7.0) is more acid than plasma (average pH 7.4) and the distribution of a drug between the two, in the absence of an active transport system, may be calculated by the method of Rasmussen [16], using a modification of the Henderson—Hasselbach equation:

$$\frac{[\text{milk}]}{[\text{plasma}]} = \frac{1 + 10^{\text{pH}_{\text{milk}} - \text{p}K_a}}{1 + 10^{\text{pH}_{\text{plasma}} - \text{p}K_a}}$$

The pK (COOH) of methyldopa is 2.2, thus for plasma at pH 7.4 and milk pH 7.0 the distribution of the drug [milk]/[plasma] = 0.40. This compares well

TABLE I

PATIENTS TAKING METHYLDOPA (n = 8), 0.25—1.5 g PER DAY DIVIDED DOSE, WHILE BREASTFEEDING

As time of dose is different and variable from time of sampling there is no correlation between dose and drug levels.

Sample			Concentration of drug ± S.D. (μg/ml)
Milk	Free	(n = 14)*	0.17 ± 0.08
	Conjugated	(n = 17)*	0.26 ± 0.16
Plasma**	Free	(n = 8)	0.37 ± 0.26

*When only trace quantities (< 0.05 μg/ml) were detected these are not included.

**Consistent results for conjugated drug in plasma were not obtained. Inconsistencies were reproducible, differed between patients but not between samples from the same patient; a probable reason is polypharmacy.

with the figure of 0.46 calculated from the total number of samples examined and 0.41 when paired milk and plasma samples only are included.

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