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RAPID MICROMETHOD FOR THE ANALYSIS OF MITOTANE AND ITS METABOLITE IN PLASMA BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A rapid micromethod for 50-μl samples is described for the analysis in plasma of mitotane (*o,p'*-DDD) and its metabolite (*o,p'*-DDE), using *p,p'*-DDD as internal standard. The compounds are extracted with heptane without sample pretreatment, and an aliquot is directly analysed by gas chromatography with electron-capture detection. Absolute recoveries for all three compounds were ca. 87% and coefficients of variation were less than 5%. The method is being used successfully for monitoring patients with Cushing's syndrome who receive chronic oral mitotane therapy.

INTRODUCTION

Mitotane, 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane, is commonly used as an oal chemotherapeutic agent in the treatment of inoperable adrenocortical carcinoma [1–4]. Other authors have reported positive effects in the treatment of Cushing's syndrome [5–18]. The compound is also known as *o,p'*-DDD (I, Fig. 1) and is marketed for therapeutic use as Lysodren® (Calbio Pharmaceuticals, La Jolla, CA, U.S.A.). In plasma, the only metabolite of mitotane found is *o,p'*-DDE [19] (II, Fig. 1). Mitotane appears to be heavily metabolized and is not excreted as such in urine. It does occur in faeces after oral

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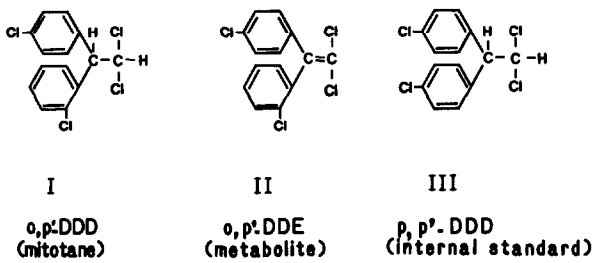

Fig. 1. Structures of *o,p'*-DDD, *o,p'*-DDE and *p,p'*-DDD.

application, but this may reflect non-absorbed material. Besides DDE, the principal metabolites are dichlorodiphenylacetic acid (DDA) derivatives [20].

In order to monitor effectively mitotane therapy in patients, it is essential to follow both the parent drug and the metabolite, *o,p'*-DDE. Though not likely to be active itself [21], *o,p'*-DDE may be an indicator of how the metabolic ratio changes with time. Gas chromatography (GC) with electron-capture detection (ECD) has previously been used to monitor the two substances [13, 22]. However, the methods include a protein precipitation step as sample pretreatment, either with formic acid or with acetone. Unfortunately this not only decreases the precision, but also induces big, tailing solvent peaks and other interferences in vital areas of the chromatogram. These phenomena are particularly troublesome at lower plasma concentrations.

In this paper we describe a rapid, simple and selective GC micromethod for mitotane and its metabolite without sample pretreatment and evaporation steps. The method is suitable for monitoring oral mitotane therapy and for pharmacokinetic studies.

EXPERIMENTAL

Reagents and solvents

o,p'-DDD, *o,p'*-DDE and the internal standard (I.S.), 2-bis(4-chlorophenyl)-1,1-dichloroethane, *p,p'*-DDD (III, Fig. 1), were obtained from Serva Feinbiochemica (Heidelberg, F.R.G.). Ethanol (puriss. p.a.) was obtained from Laborchemie (Apolda, G.D.R.). Heptane (p.a.) from Berlin-Chemie (Berlin, G.D.R.) was refluxed for 8 h over potassium hydroxide pellets and then distilled using a 120 cm long column. Anhydrous sodium sulphate was extracted in a Soxhlet apparatus with hexane for 5 h and then activated at 200°C for 2 h.

Standards

o,p'-DDD, *o,p'*-DDE and *p,p'*-DDD (100 mg each) were dissolved in 100 ml of ethanol to yield stock standard solutions (1 mg/ml). A series of working standard solutions containing 4, 8, 20, 40, 60, 80, 100 and 120 μg/ml *o,p'*-DDD and *o,p'*-DDE and a constant amount of 40 μg/ml *p,p'*-DDD in each solution was prepared by serial dilution of the stock solutions with ethanol.

Plasma standards were freshly prepared each day by spiking 50 μl of blank

plasma with 5 μl of the working standard solutions to yield *o,p'*-DDD and *o,p'*-DDE concentrations ranging from 0.4 to 12 μg/ml in plasma, the concentration of the I.S. then being 4 μg/ml.

The stock solution of *p,p'*-DDD was separately diluted with ethanol to give a concentration of 40 μg/ml, and 5 μl of this I.S. solution were used to spike samples from patients.

All standard solutions were stored at 4°C and were stable for at least one year.

Gas chromatography

A gas chromatograph GC HF 18.3 equipped with a ^{3}H electron-capture detector (Chromatron, G.D.R.) operated in a constant-current mode [23] was used. The glass column (2 m×3 mm I.D.) was packed with 1.5% QF-1 plus 1.95% OV-17 on 80–100 mesh Chromosorb W HP (RFR, Hope, RI, U.S.A.).

The GC conditions were as follows: carrier gas, nitrogen; flow-rate, 70–80 ml/min; column temperature, 180°C; injector temperature, 240°C; detector temperature, 180°C.

Sample preparation

To 50 μl of drug-free plasma in a 5-ml glass-stoppered centrifuge tube were added 5 μl of working standard solution containing the required amounts of *o,p'*-DDD, *o,p'*-DDE and *p,p'*-DDD. The mixture was shaken mechanically for 30 min. After the addition of 2 ml of heptane, the drugs were extracted by mechanical shaking for 5 min, which was adequate. Then, 0.5 g of anhydrous sodium sulphate were added, and the mixture was centrifuged for 2 min at 3000 *g*. The clear and dry heptane layer was transferred to another glass-stoppered tube and 1 μl was injected into the chromatograph.

Plasma samples (50 μl) from patients treated with Lysodren were spiked with 5 μl of 40 μg/ml I.S. solution, and then treated in the same way.

Absolute recovery

The completeness of the exctraction of *o,p'*-DDD, *o,p'*-DDE and *p,p'*-DDD from plasma into heptane was examined. Plasma samples containing 0.8, 4, 8 and 12 μg/ml each of *o,p'*-DDD and *o,p'*-DDE and 4 μg/ml *p,p'*-DDD were extracted and analysed as described. The individual peak heights were compared with those resulting from direct injection of standard solutions of the corresponding substances at the same concentrations.

Plasma standard curve

Plasma samples (50 μl) were spiked with 5 μl of the required working standard solutions, extracted and chromatographed as indicated above. They gave plasma standard solutions with 0.4, 0.8, 2, 4, 6, 8, 10 and 12 μg/ml *o,p'*-DDD and *o,p'*-DDE, respectively, as well as 4 μg/ml *p,p'*-DDD in plasma. Peak-height ratios (drug/I.S.) were plotted against the plasma concentrations of *o,p'*-DDD and *o,p'*-DDE. The data were subjected to linear regression analysis to give the appropriate calibration factors. Each concentration was analysed ten times.

Accuracy and precision

Spiked plasma samples (0.4, 0.8, 2, 4, 6, 8, 10 and 12 μg/ml each of *o,p'*-DDD and *o,p'*-DDE) were extracted and analysed as described above. The calibration factors obtained on the day of analysis were used to calculate the concentrations of *o,p'*-DDD and *o,p'*-DDE. This was repeated on ten different days, and the means and standard deviations were calculated.

Analysis of patient plasma samples

Paediatric patients with hypothalamic-pituitary dependent Cushing's syndrome were treated chronically with low doses of Lysodren for periods up to ten years. Determination of plasma levels of *o,p'*-DDD and *o,p'*-DDE was necessary for therapy control and optimum dose adjustment. After each blood sample had been taken, the plasma was immediately separated and frozen (−20°C) until analysed.

RESULTS AND DISCUSSION

Under the GC conditions described, *o,p'*-DDD, *o,p'*-DDE and *p,p'*-DDD gave well resolved and well defined peaks. Fig. 2A shows a typical chromatogram for an extract from plasma sample from a patient.

A number of drug-free plasma samples from different human subjects and aliquots of pooled plasma were tested for the presence of interfering endogenous compounds using the outlined extraction procedure. Fig. 2B, a blank plasma, shows that no interferences were observed. Thus, no further clean-up of the plasma extracts is necessary.

Incomplete extraction of organochlorine compounds from plasma and serum because of their binding to proteins appeared to be a main problem in the development of analytical procedures [22, 24, 25]. According to Moolenaar et al. [22], recoveries of *o,p'*-DDD and *o,p'*-DDE by heptane extraction were 25–68% and 19–31%, respectively. Protein precipitation with acetone apparently released the analyte and increased these recoveries to over 93% [22].

We investigated the phenomenon of incomplete extraction in detail and found that the introduction of the right amount of I.S. at the correct time (i.e. to the undiluted plasma sample in as small a volume as possible), followed by adequate equilibration of the I.S. with the plasma (30 min), were key factors in obtaining good absolute recoveries as well as good precision. This is shown in Table I, in which data obtained with our method are compared with the procedure suggested by Moolenaar et al. [22]. As can be seen, there were no significant differences in recovery and precision between the two methods. However, the use of acetone resulted in big, tailing solvent peaks, which tended to interfere with the accurate measurement of the I.S. peak (see standard deviations in Table I). Moreover, acetone also proved to be a source of small interfering peaks in the chromatogram. Formic acid as a precipitating reagent [13, 24, 25] produced similar results.

The use of *p,p'*-DDD as I.S proved to be very suitable, because its extraction properties are very similar to those of the two analytes. This resulted in relative recoveries of the drugs of essentially 100%.

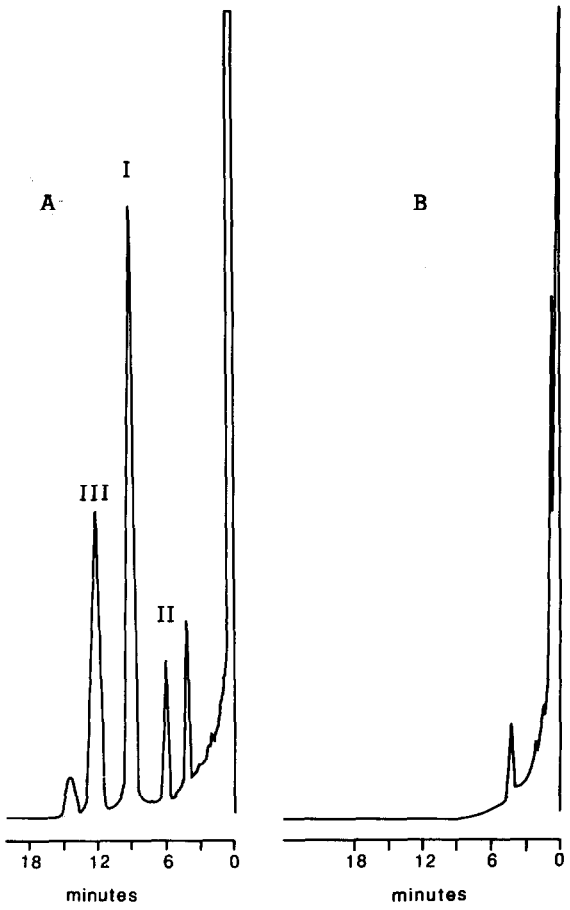

Fig. 2. (A) Chromatogram of a plasma sample of a patient receiving Lysodren therapy. Concentrations were determined as 9.02 μg/ml *o,p'*-DDD (I) and 1.28 μg/ml *o,p'*-DDE (II). I.S. concentration is 4.0 μg/ml (III). (B) Chromatogram of a drug-free plasma sample.

The analytical procedure was found to be accurate, precise and linear over the range 0.8–12 μg/ml for *o,p'*-DDD and 0.4–12 μg/ml for *o,p'*-DDE. Calibration line equations were as follows. For *o,p'*-DDD: $y=0.03312+1.1117x$, $r=0.9996$, $n=10$; for *o,p'*-DDE: $y=0.0283+2.0005x$, $r=0.9999$, $n=10$.

The accuracy was 98.5% (range 88.8–103.4%) for *o,p'*-DDD and 101.2% (range 92.5–104.3%) for *o,p'*-DDE. The precision expressed as coefficient of variation (C.V.) was 2.38% (range 1.4–4.2%) and 2.55% (range 1.9–3.7%) for *o,p'*-DDD and *o,p'*-DDE, respectively (see Table II for some values within and outside these ranges).

If a maximum C.V. of 5% is accepted, the assay limits for the present procedure are 0.8 μg/ml *o,p'*-DDD and 0.4 μg/ml *o,p'*-DDE. Limits of detection at a signal-to-noise ratio of 10 were 0.05 μg/ml *o,p'*-DDD and 0.03 μg/ml *o,p'*-DDE. This would indicate that assay limits may be lowered by using smaller amounts of I.S. (now 4 μg/ml).

The method was found to be sufficiently sensitive to follow plasma levels of

TABLE I

ABSOLUTE RECOVERIES FROM PLASMA BY HEPTANE EXTRACTION ($n=5$)

Compound	Added (μg/ml)	Recovery (mean ± S.D.) (%)	
		Without acetone	With acetone*
o,p'-DDD	0.8	84.1 ± 1.14	85.3 ± 2.49
	4.0	89.5 ± 2.05	90.6 ± 2.51
	8.0	88.6 ± 1.37	95.1 ± 1.60
	12.0	89.8 ± 1.77	94.6 ± 1.62
Mean		88.0 ± 1.58	91.4 ± 2.06
o,p'-DDE	0.8	80.0 ± 4.24	82.0 ± 3.90
	4.0	84.5 ± 0.98	95.4 ± 1.15
	8.0	89.3 ± 2.28	90.6 ± 2.33
Mean		84.6 ± 2.50	89.3 ± 2.46
p,p'-DDD (I.S.)	4.0	87.8 ± 1.55	91.1 ± 2.68

*Method of ref. 22.

TABLE II

PRECISION AND ACCURACY OF THE METHOD ($n=10$)

Compound	Added (μg/ml)	Found (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (found %)
o,p'-DDD	0.4	0.23 ± 0.03	13.04	57.5
	0.8	0.71 ± 0.03	4.23	88.8
	4.0	3.90 ± 0.14	3.59	97.5
o,p'-DDE	0.2	0.13 ± 0.02	15.38	65.0
	0.4	0.37 ± 0.01	2.70	92.5
	4.0	4.00 ± 0.10	2.50	100.0

o,p'-DDD and *o,p'*-DDE in patients put on long-term Lysodren therapy. This is demonstrated in Fig. 3 for a paediatric patient with hypothalamic-pituitary Cushing's disease. In our opinion, monitoring *o,p'*-DDD and *o,p'*-DDE in plasma is essential to assure achievement of therapeutic, yet non-toxic concentrations [26]. In addition, the present micromethod provides opportunities for pharmacokinetic investigations, especially in those subjects in whom sampling of large blood volumes would be deleterious (infants, neonates).

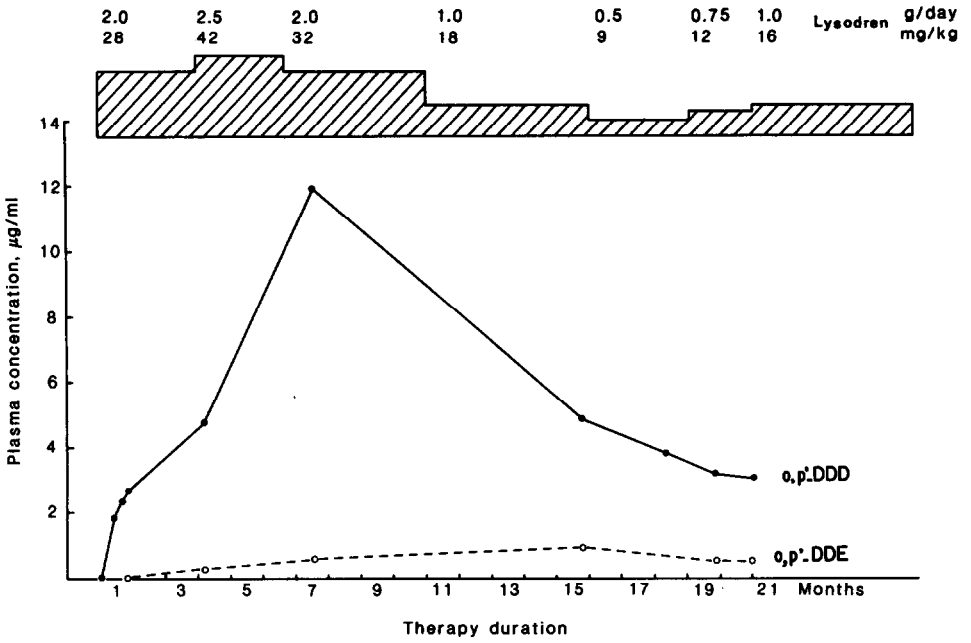

Fig. 3. Plasma concentration-time profiles for *o,p'*-DDD and *o,p'*-DDE of a patient with Cushing's syndrome receiving Lysodren therapy. The dose regimen is given in the block at the top.

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