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A simple HPLC method for plasma level monitoring of mitotane and its two main metabolites in adrenocortical cancer patients

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ABSTRACT

Mitotane (o,p′-DDD or (1,1-dichloro-2-[o-chlorophenyl]-2-[p-chlorophenyl]ethane, DDD) is the drug of choice for non-resectable and metastatic adrenocortical carcinomas (ACC). Measurement of mitotane and metabolites, o,p′-DDE (1,1-dichloro-2-[p-chlorophenyl]-2-[o-chlorophenyl]ethene, DDE) and o,p′-DDA (1,1-[o,p′-dichlorodiphenyl] acetic acid, DDA) provides a better understanding of mitotane pharmacokinetics and pharmacodynamics. We have developed a simple, robust and efficient high performance liquid chromatography (HPLC) method to measure mitotane and its two main metabolites, DDE and DDA. The method involves a single ethanol extraction of mitotane, DDE, DDA, and an internal standard (int std) p,p′-DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) with an extraction efficiency of 77–88%. All compounds are measured simultaneously using a reversed-phase phenyl HPLC column with an isocratic elution of mobile phase at a flow rate of 0.6 ml/min followed by UV detection at λ 226 nm. Inter and intra-day validation demonstrates good reproducibility and accuracy. Limits of quantitation are 0.2 μg/ml for mitotane and DDE, and 0.5 μg/ml for DDA. The method has been evaluated in plasma from 23 patients on mitotane therapy, revealing DDA concentrations 1–18 times higher than the parent compound.

1. Introduction

Mitotane (o,p′-DDD, DDD), an analogue of the insecticide dichloro-diphenyl-dichloroethane, (DDT) was originally developed in 1960 and has been used alone in the treatment of adrenocortical carcinoma (ACC) or in combination with other agents [1,2]. Mitotane is the drug of choice for non-resectable, metastatic or recurrent ACC. Because of high local or distant recurrence rates, mitotane has also been suggested as adjuvant therapy after tumor resection in some studies [3–6].

The antitumor effect of mitotane is related to the maintenance of adequate plasma drug levels [7–10]. It is suggested that patient plasma mitotane levels ≥14 μg/ml lead to a 55–60% response rate, whereas those with lower levels have a low chance of response. In addition, significant side effects have generally only been observed in patients with plasma levels greater than 20 μg/ml [7,10]. Plasma level monitoring of mitotane during treatment allows the more rapid attainment of a therapeutic plasma level (14–20 μg/ml), as well as minimization of adverse effects due to an excessively high plasma level [9,11].

Intraadrenal metabolic transformation is essential for the therapeutic effect of mitotane, which is metabolised to DDE and DDA (Fig. 1) by α- and β-hydroxylation, respectively [12], with DDA levels increasing rapidly to high levels [13–18] while DDE levels rise slowly. The importance of measuring plasma mitotane and its metabolites DDE and DDA has been documented in a number of studies [9,13–17,19]. A recent long term follow up study [17] suggests that the plasma concentrations of DDE were more closely related to clinical improvement or remission than the mitotane levels. Thus pharmacokinetic monitoring of mitotane and its metabolites could provide a better understanding of mitotane metabolism and guide effective management.

Measurement of plasma mitotane and its metabolites has been undertaken using gas chromatography (GC) with electron capture detection and GC/MS methods [15,16,18] which involve laborious sample preparation or specialized equipment such as mass spectrometry. HPLC methods constitute a valid alternative to GC methods [13,14,20] but some do not use internal standard in the analysis [13] while other methods [20] do not assess all compounds simultaneously. One recent method did separate all compounds simultaneously, however, extra solid phase extraction steps were needed, differing HPLC gradient flow conditions were utilised to

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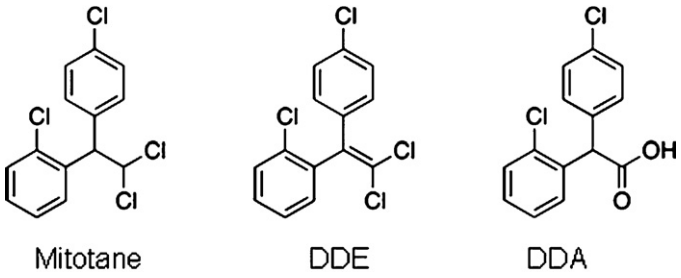

Fig. 1. Chemical structures of mitotane and its metabolites DDE and DDA.

assess DDA along with mitotane, DDE and the internal standard and resolution between some peaks was poor [14]. We have developed a simple, robust, efficient and sensitive single-run HPLC method with UV detection to measure all four compounds simultaneously: mitotane, its two principal metabolites DDE, DDA and internal standard p,p′-DDD. This method overcomes the limitations of previous methods [13,14,20] and is ideal for clinical pharmacokinetic analysis with good performance parameters.

2. Materials and methods

2.1. Chemicals and reagents

Mitotane, DDE, p,p′-DDD (int std) and p,p′-DDA (1,1-(p,p′-dichlorodiphenyl) acetic acid) were purchased from Supelco (Sigma–Aldrich, NSW, Australia) while o,p′-DDA (DDA) was kindly provided by Prof. Andres Andersen (Norwegian Radium Hospital, Oslo, Norway) on request. p,p′-DDA, an isomer of DDA was used as a reference standard because DDA is not available commercially, its suitability as a reference standard is detailed in Section 3.1. Ethanol and methanol (MeOH) of HPLC grade were purchased from Crown Scientific Australia (Sydney, Australia). All other chemicals used were of best commercially available grades. Water was purified by passage through a Nanopure II systemB (Sybron/Barnstead, Boston, MA, USA) and was further filtered through a 0.45 μm membrane filter.

2.2. Solutions

All stock standard solutions of compounds at a concentration of 1 mg/ml were prepared in ethanol and stored at −20 °C. These solutions were diluted in heparinised plasma to prepare a series of plasma standard and quality control (QC) samples.

2.3. Plasma samples: calibration standards and patient samples

Drug-free pooled human plasma was purchased from Australian Red Cross Blood Service (ARCBS) Sydney, Australia. Plasma standard solutions for calibration and quality control (QC) samples were prepared by spiking the control plasma with stock standard solutions to achieve concentrations of 50, 40, 25, 20, 10, 5, 2.5, 2, 1, 0.5 and 0.2 μg/ml for mitotane and DDE and 250, 200, 125, 100, 50, 25, 10, 5, 2.5, and 1.0 μg/ml for p,p′-DDA. A stock solution of 5 mg/ml of each compound was used to prepare the first four plasma standard concentrations. Serial dilutions of these were then used to prepare the remaining plasma standards.

The method has been evaluated using samples from 23 patients with adrenal cancer undergoing treatment with mitotane doses ranging from 1.0 g/day to12 g/day in one or two daily doses. Trough plasma samples were taken at least 4–8 h after dosing. Informed consent for the mitotane treatment and for plasma mitotane levels determination was obtained. Blood (5–6 ml) was collected in K3 ethylenediamine tetraacetate tube and kept on ice until centrifuged at 1712 × *g* for15 min at −4 °C. Plasma was transferred to 5 ml polypropylene tubes and shipped on dry ice with storage at −20 °C until analysis.

2.4. Sample preparation

0.2 ml thawed plasma samples were aliquoted into microcentrifuge tubes in duplicate and spiked with 25 μl of internal standard p,p′-DDD (25 μg/ml in ethanol) and vortexed well. Plasma standards and controls and patients samples were treated the same way. Protein was precipitated by the addition of 1.5 volumes (300 μl) of cold HPLC grade ethanol followed by immediate vortex mixing. Precipitate was pelleted by centrifugation at 11,337 × *g* at 4 °C for 6 min in a Heraeus Fresco microcentrifuge (K.I. Scientific Pty. Ltd., NSW, Australia). Supernatant was transferred into a fresh microcentrifuge tube, and 50 μl of 50 mM potassium phosphate (KH_2PO_4) buffer, pH 2.5 was added, vortex mixed and centrifuged at 11,337 × *g* at 4 °C for 5 min again. The supernatant was transferred to glass autosampler tubes and 10 μl was injected onto the HPLC.

2.5. Chromatographic conditions

All chromatographic equipment was manufactured by Shimadzu (Toyko, Japan). The solvent delivery system consisted of a DGU-14A online degasser coupled to a LC-10AD dual piston pump. Column temperature was maintained at 57 °C using a CTO-10AS VP column oven. Samples were injected with a SIL-10A autoinjector and detected by a SPD-10A variable wavelength UV detector. Data acquisition and integration were performed using Class-VP 7 computer-based integration system to determine peak areas.

Separation was achieved by isocratic elution of the mobile phase, methanol: 50 mM KH_2PO_4 buffer pH 2.5 (71:29, v/v) at a flow rate of 0.6 ml/min through a Waters Nova-Pak Phenyl column (150 mm × 3.9 mm I.D., 4 μm particle size) (Waters Australia Pty. Ltd., Rydalmere, NSW, Australia) kept at 57 °C preceded by Waters Nova-Pak Phenyl (3.9 mm × 20 mm, 4 μm) guard column. The eluent was monitored at 226 nm. The total analysis time for each run was 21 min.

In the course of development of this assay, different columns and mobile phases from other reported methods [7,13,14] were studied. Problems encountered with these methods included interference from endogenous plasma constituents, low resolution between peaks, and baseline instability. This plasma extraction and HPLC method is an adaptation of the method described by Andersen et al. [13] and is designed to be more applicable for routine clinical laboratory practice, using internal standard, without compromising sensitivity and precision. Initially we used a column temperature of 50 °C as detailed by Andersen et al. [13]. Under our assay conditions this produced tailing peaks with low resolution between some peaks. As a result, the column temperature was increased stepwise until better quality peaks were attained. The optimum conditions included a column temperature of 57 °C with a low mobile phase flow rate of 0.6 ml/min and a run time of 21 min.

2.6. Analytical method validation

2.6.1. Selectivity and specificity

The extraction and HPLC assay resulted in symmetrical peak shape and good baseline resolution of DDA (metabolite), p,p′-DDA (isomer of DDA), mitotane, p,p′-DDD (internal standard) and DDE (metabolite) with retention times of 5.6 min, 6.7 min, 15.5 min, 17.0 min and 18.0 min, respectively. Endogenous plasma compounds did not interfere with the analysis except for a small peak

eluting at about 6.3 min just after the DDA peak, which separated well from p,p′-DDA peak in plasma standards and QC samples.

2.6.2. Precision and accuracy

The limit of quantification (LOQ) was defined as the minimum concentration that could be detected and quantified with ≤10% standard deviation from the actual concentration. To determine LOQ, concentrations of 0.1, 0.2, 0.5, and 1 μg/ml of mitotane and DDE and concentration of 0.5, 1.0, 2.5 and 5 μg/ml of p,p′-DDA and DDA were run in triplicate. The concentrations which could be reliably measured and quantified with ≤10% standard deviation from the actual concentration were 0.2 μg/ml for mitotane and DDE, and 0.5 μg/ml for p,p′-DDA and DDA, which is comparable or better that previous published methods [14].

Linearity of detector response was assessed for extracted plasma standards over the range of 1.0–50 μg/ml for mitotane and DDE and 5–250 μg/ml for p,p′-DDA. Data were added serially from low to high concentrations. The limit of linearity was taken as the highest concentration that maintained an $r^2 > 0.998$. For 0.2 ml plasma standards the assay was linear up to 40 μg/ml for mitotane and DDE and up to 200 μg/ml for p,p′-DDA. Calibration curves for mitotane (1.0–20 μg/ml), DDE (1.0–20 μg/ml), and p,p′-DDA (5.0–100 μg/ml) were produced during each patient analysis. The mean ± SD ($n = 5$) for the slope and intercept for mitotane was 0.327 ± 0.009 and 0.005 ± 0.045, respectively for DDE was 0.280 ± 0.008 and −0.053 ± 0.029, respectively and for p,p′-DDA was 0.537 ± 0.024 and 0.300 ± 0.212, respectively. Peak purity was tested by comparing water standards of pure compounds with extracted plasma standards. This comparison showed no variation in retention times or peak shape of the standards, and no interfering plasma peaks.

Intra-day variability was defined as relative standard deviation (RSD) calculated from the values measured from three samples at concentrations of 2.5 and 20 μg/ml for mitotane and DDE and 12.5 and 100 μg/ml for p,p′-DDA. Inter-day variability was calculated using the measured concentration from these samples over 3 days. Intra and inter-day variability is shown in Table 1.

3. Results and discussion

3.1. p,p′-DDA as a reference standard for DDA

Because DDA is not commercially available, we tested p,p′-DDA for suitability as a reference standard. Standard solutions of DDA and p,p′-DDA (5.0 μg/ml in ethanol) were injected into the HPLC system and detected at a range of UV wavelengths (218–236 nm) under our assay conditions, and peak areas of DDA and p,p′-DDA peaks were compared (Fig. 2). A detection wavelength of 226 nm was chosen as these two compounds have almost identical absorbance at this wavelength and other compounds were also well detected (Fig. 2). Therefore, we concluded that p,p′-DDA may be used in this assay at a wavelength of 226 nm as reference standard for DDA. At this wavelength, all four compounds could be detected simultaneously in one run.

3.2. Chromatograms

The extraction and HPLC method we have developed resulted in symmetrical peak shape and good baseline resolution of DDA (metabolite), p,p′-DDA (isomer of DDA), mitotane, p,p′-DDD (int std) and DDE (metabolite) with retention times of 5.6 min, 6.7 min, 15.5 min, 17.0 min and 18.0 min, respectively (Fig. 3). Plasma matrix components did not interfere with the analysis except for a small peak eluting at about 6.3 min just after the DDA peak, which had to be manually integrated.

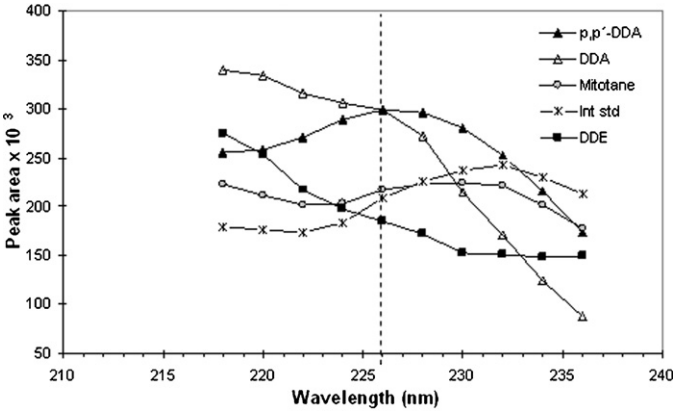

Fig. 2. Plot of Peak areas at different wavelengths for DDA, p,p′-DDA (isomer of DDA), mitotane, p,p′-DDD (int std) and metabolite DDE.

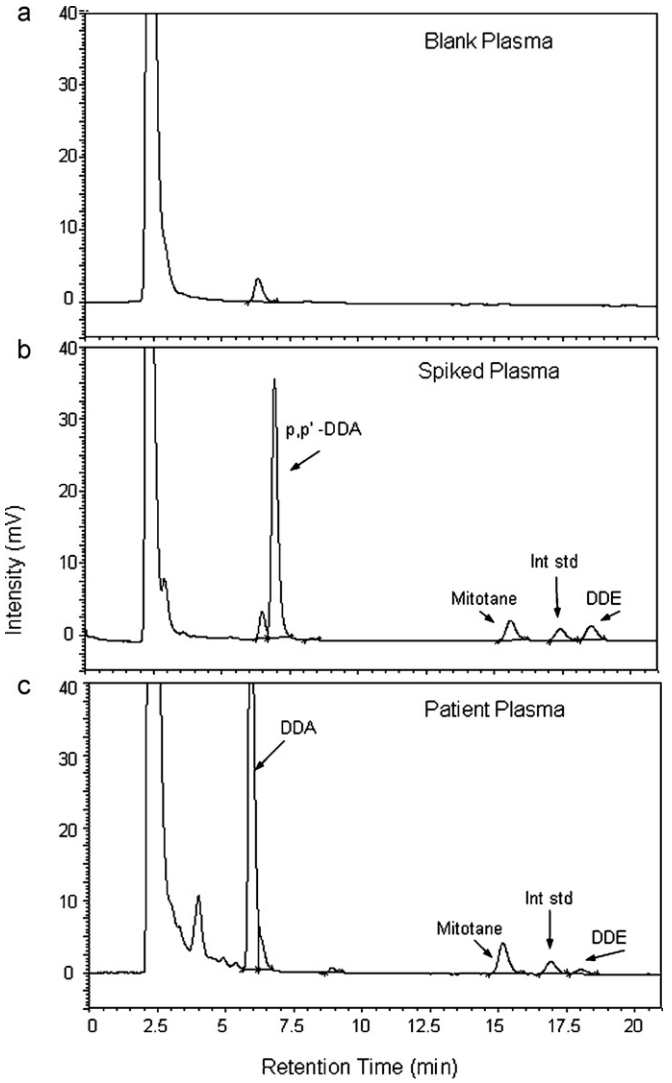

Fig. 3. Chromatograms of plasma extracts obtained from (a) a blank plasma sample, (b) a plasma sample spiked with 25.0 μg/ml p,p′-DDA and 5.0 μg/ml mitotane and DDE and 3.125 μg/ml internal standard p,p′-DDD and (c) a patient sample containing 38.51 μg/ml DDA, 7.37 μg/ml mitotane, 3.125 μg/ml int std and 1.56 μg/ml DDE.

Table 1
Intra and inter-day variability.

	Actual conc. (μg/ml)	Intra-day (n = 3)			Inter-day (n = 3)		
		Detected conc. (μg/ml) (mean ± SD)	RSD (%)	Accuracy (%)	Detected conc. (μg/ml) (mean ± SD)	RSD (%)	Accuracy (%)
Mitotane	2.5	2.53 ± 0.05	1.97	101.4	2.48 ± 0.09	3.79	99.4
	20.0	20.11 ± 0.83	4.10	100.6	19.99 ± 0.10	0.52	99.9
DDE	2.5	2.60 ± 0.03	1.31	104.2	2.56 ± 0.06	2.41	102.7
	20.0	20.50 ± 0.86	4.21	102.5	20.02 ± 0.10	0.48	100.1
p,p′-DDA	12.5	12.23 ± 0.19	1.57	97.9	11.63 ± 0.40	3.42	93.0
	100.0	100.65 ± 2.73	2.71	100.6	99.25 ± 0.14	0.14	99.2

3.3. Extraction efficiency

Extraction efficiency of mitotane, its metabolites and the internal standard were determined by spiking 0.2 ml aliquots of plasma and mobile phase simultaneously with stock solutions, extracting the plasma samples as described above, analysing both sets and comparing the HPLC-derived peak areas. Extraction efficiency for mitotane, DDE, DDA and int std p,p′-DDD at the concentration of 5.0 μg/ml (n = 4) was 83.0 ± 7.4, 84.8 ± 2.4, 85.6 ± 11.5 and 77.4 ± 5.5, respectively and for p,p′-DDA at the concentration of 25 μg/ml (n = 4) was 87.6 ± 3.8.

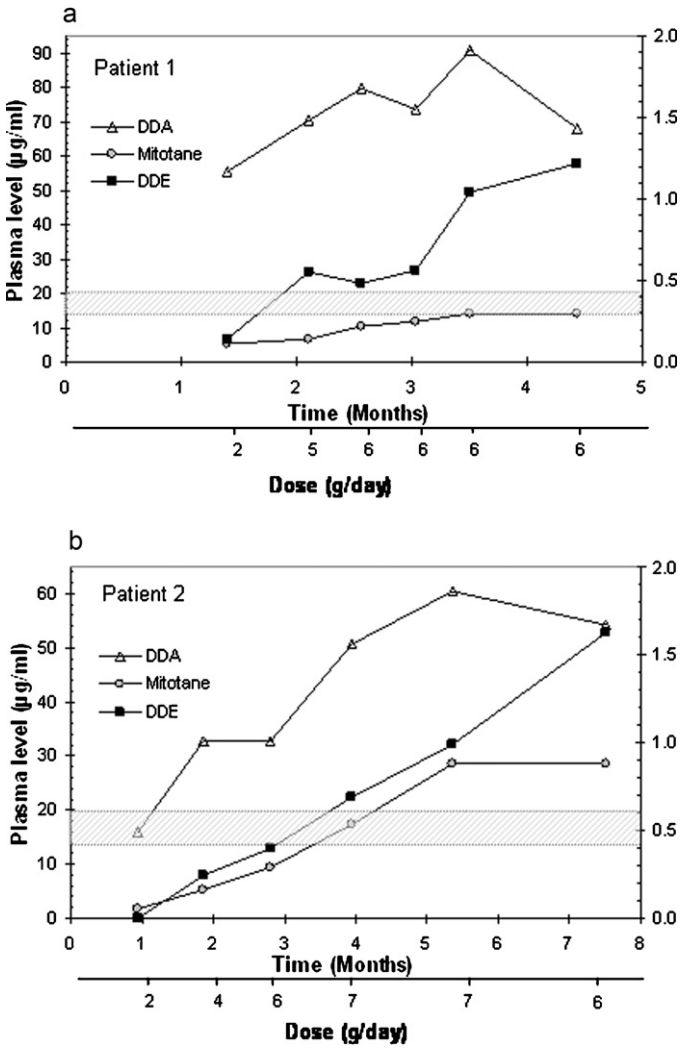

Fig. 4. Patient plasma level curves for mitotane (left axis), DDA (left axis) DDE (right axis) vs time (months) on mitotane therapy and vs dose of mitotane (g/day) for patient 1 (a) and patient 2 (b). Hatched zone represents therapeutic range (14–20 μg/ml) for mitotane level.

3.4. Patient data

This method has been used to determine plasma levels of mitotane and its metabolites DDE and DDA as therapeutic drug monitoring for 23 patients with mitotane doses ranging from 1.0 to 12 g/day in one or two daily doses. Trough plasma samples were taken at least 4–8 h after dosing. Mitotane levels ranged from 0.8 to 38.6 μg/ml, DDA levels from 4.07 to 118.6 μg/ml and DDE levels from 0.0 to 4.42 μg/ml (data not shown). DDA is the most represented metabolite, giving ratios of DDA/mitotane from 1.2 to 18.1 while ratios of DDE/mitotane ranged from 0.0 to 0.32. Two patients on mitotane therapy were monitored for several months (Fig. 4a and b). This data indicates that for patient 1, at 6 g/day there was some accumulation of DDE, but not of mitotane or DDA, whereas for patient 2, at 6–7 g/day there was accumulation of mitotane and DDE, but not of DDA. This finding suggests significant unexplained variability in metabolism between patients, and indicates potential value in therapeutic drug monitoring including metabolites.

3.5. Stability

Previous studies have evaluated stability of processed and unprocessed plasma standards to determine their optimal requirements for storage and processing during analysis. Mitotane, DDE and DDA are stable in solutions for long periods of time [13,16,19], although p,p′-DDA has been reported to decompose on exposure to light [21]. Therefore we recommend that plasma standards should be processed and analysed quickly to minimise exposure to light, and refrigerated between processing periods. All plasma standards and controls prepared in this study were stored as 0.2 ml aliquots at −20 °C. Patient plasma samples were shipped frozen and stored at −20 °C until analysis and 0.2 ml was aliquoted in duplicate at analysis time. Plasma standards and patient samples did not go through freeze–thaw cycles.

4. Conclusions

There is considerable interpatient variability in the dose–plasma concentration relationship for mitotane. Therefore plasma level monitoring during treatment allows more rapid attainment of a therapeutic plasma level (14–20 μg/ml), as well as minimization of adverse effects due to an excessively high plasma level. Measurement of its metabolites DDE and DDA along with mitotane could provide a better understanding of mitotane metabolism and may give a closer correlation to clinical parameters (toxicity and anticancer effects) than merely measuring the parent drug alone.

We have developed and validated a simple, robust, efficient and sensitive HPLC method with UV detection to simultaneously measure mitotane, and its principal metabolites DDE and DDA in

patients plasma samples along with an internal standard, which involves only one ethanol extraction step and isocratic elution of mobile phase at a low flow rate. All compounds are well separated from each other and from other interfering peaks. Though the run time is longer than some published methods [13,14], the mobile phase flow rate was kept slower at 0.6 ml/min to achieve the best resolution between the four compounds. The assay is reproducible with good performance parameters and is therefore ideal for clinical pharmacokinetic analysis. Overall the method is more efficient and comprehensive than previous methods [13,14,20] and is ideally suited for therapeutic drug monitoring, with limits of quantitation (LOQ) as 0.2 μg/ml for mitotane and DDE and 0.5 μg/ml for DDA and p,p′-DDA.

We tested the suitability of p,p′-DDA as a reference standard for DDA as DDA is not available commercially. We found that the two compounds had almost identical absorbance at 226 nm wavelength, with no material difference in extraction efficiency or any other parameter, indicating that p,p′-DDA can be used to standardize DDA. In patients plasma DDA is the most represented, with ratios of 1.2–18 relative to parent compound, suggesting highly variable biotransformation in patients, confirming the results of others [17].

The method has been evaluated using samples from 23 adrenocortical cancer patients on mitotane therapy, and found to be consistent in its behaviour, allowing accurate therapeutic drug monitoring and appropriate dose modification to maximize the therapeutic index of mitotane in this disease. Ongoing studies will permit evaluation of the interpatient variability in metabolism of mitotane.

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