A new simple HPLC method for measuring mitotane and its two principal metabolites
Tests in animals and mitotane-treated patients

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Abstract

A new C18 reversed-phase column and UV HPLC method for the detection of mitotane, its principal metabolites, dichlorodiphenylacetate and dichlrodiphenylethene, and its precursor DDT is described. In this article mitotane, dichlorodiphenylacetate, and dichlrodiphenylethene concentrations in organs of rats fed on a mitotane diet, and the effects of erythromycin and grapefruit juice as cytochrome P450 common inhibitors are presented. Tissue accumulation of mitotane and dichlrodiphenylethene, the acquired ability to eliminate dichlorodiphenylacetate, and inhibition of β-hydroxylation by both inhibitors are illustrated here. Blood samples from mitotane-treated patients revealed two correlations: plasma mitotane/dichlrodiphenylethene and plasma mitotane/red cell mitotane.
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1. Introduction

Common dichlorodiphenyltrichloroethane (DDT) preparations are mixtures of p,p′ (predominant), o,p′, o,o′ isomers which are metabolized by liver into mitotane (dichlorodifenyldichloroethane, DDD), dichlorodiphenylacetate (DDA), and dichlorodiphenylethene (DDE). DDD and DDE accumulate in fat tissue, whereas DDA is eliminated in urine and bile [1]. Oestrogen and androgen activity of these compounds has been described previously. DDD is the o,p′ derivative of DDT and an efficacious palliative in inoperable adrenocortical carcinoma [2–6]. Maximum doses range from 2 to 16 g (usually 6–10 g) per day. Plasma concentrations of 14–20 µg/ml are considered therapeutic, though the best way to reach this levels is

still not clear [7,8]. Adverse reactions are common mainly to the gastrointestinal, central nervous system, and derma, but non-tolerable toxic events are rare when the plasma concentration is less than 15 µg/ml. Adrenocortical glands become necrotic and 17-hydroxycorticosteroid secretion is inhibited [9,10]. DDD's exact antitumour molecular mechanism is unknown and it is still unclear whether it is induced by DDD itself or one of its metabolites such as DDE or DDA following α- and β-hydroxylation, respectively (Fig. 1) [11]. Monitoring plasma DDD and its metabolite levels is thus required to combine good therapeutic efficacy with acceptable toxicity. HPLC methods constitute a valid alternative to gas chromatography [12,13] in monitoring plasma DDD levels, however they do not always asses DDD, DDA, DDE, and DDT [14,15]. A new HPLC method that measures all four compounds, with some changes in order to ameliorate DDA revealing, is described in this paper. This method has been used to reveal DDD, DDA, and DDE in rat blood, rat tissues and the blood of patients treated with DDD,
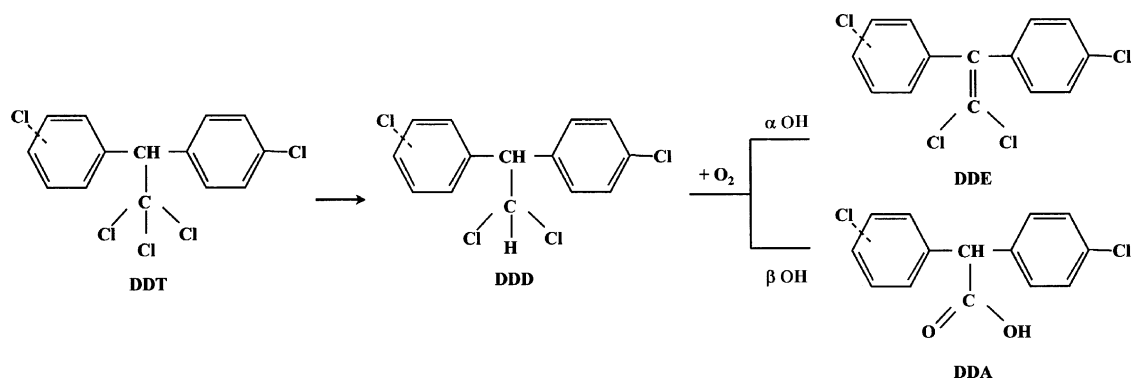
Fig. 1. DDT metabolic steps.

whose red cell concentrations were used as evidence of organ accumulation. Since erythromycin and grapefruit juice (GFJ) are known to be cytochrome P450 inhibitors, their influence on DDD and its metabolite levels in experimental animals was also assessed in order to understand the role of this enzyme system and its modulation on DDD pharmacokinetic [16–18].

2. Experimental

2.1. Chemicals and sample preparation procedures

DDD, DDA, DDE, DDT, dieldrin, methanol, acetonitrile and acetone were purchased from Sigma (USA). All solutions were in methanol and stored at $4\,°C$. Blood samples were prepared after separation of plasma from red cells by centrifugation at $1500 \times g$ at $4\,°C$. Red cells were then lysed by suspension in an equal volume of water. Solid organs were homogenated in liquid nitrogen by a dismembrator (mikro-dismembrator II Braun, Germany) and suspended in water or in mineral oil for fat [19] in the ratio of $400\,\mu l/100\,mg$ tissue to obtain a homogenous suspension. Since in DDD therapy DDT is not concerned, $100\,\mu l$ of $100\,\mu g/ml$ DDT solution was proposed as internal standard for asses DDA, DDD, and DDE in therapy monitoring. In DDT evaluation internal standard was constituted by $100\,\mu g/ml$ dieldrin solution. DDD, DDE, and DDT were extracted by vortex mixing of $500\,\mu l$ samples (plasma, red cell or solid tissue suspensions) with $100\,\mu l$ internal standard and $750\,\mu l$ acetone, centrifugation at $2500 \times g$ for 5 min, transfer of $500\,\mu l$ organic layer to a HPLC vial mixed with $500\,\mu l$ recovering phase (water–methanol–acetonitrile, 40:10:50, v/v/v). For DDA, after the first extraction, $500\,\mu l$ organic phase were transferred onto C18 SPE column (Bioanwers, Italy), eluted twice with methanol and suspended in $150\,\mu l$ recovering phase after evaporation to dryness [15]. Each drug was added to blank plasma or blank tissue suspension at concentration of 0.625, 1.25, 2.5, 5, $10\,\mu g/ml$ in order to perform calibration curves.

2.2. Chromatographic system

HPLC was performed with a Merck–Hitachi system (Lachrom7000) equipped with autosampler, spectrophotometer, and heated column compartment. System management and data acquisition were performed with the HSM software on a PC.

Fifty microlitres of the sample mixture were injected. Separation was achieved with a C18 reversed-phase column (Lichrocart 250-4 Lichrospher 100 RP-18, 5 μm, VWR, Germany) preceded by a guard column (VWR). The mobile phase for DDD, DDE, and DDT (standard method) consisted of a gradient of water, methanol, acetonitrile (0–6.5 min: 10:10:80, v/v/v; 6.6–9.7 min: 5:5:90, v/v/v; 9.7–15 min: 10:10:80, v/v/v) at the constant flow rate of 1 ml/min at $35\,°C$ and the eluate was monitored at 218 nm. For DDA, the flow rate was 0.8 ml/min with a different gradient: 0–3.5 min: 20:80 (methanol–acetonitrile); 3.6–5 min: 10:10:80 (water–methanol–acetonitrile); 6.6–9.6 min: 5:5:90 (water–methanol–acetonitrile); and 9.7–11 min: 10:10:80 (water–methanol–acetonitrile). These variations were adopted to eliminate interference by plasma and delay its retention time.

2.3. Validation

In validation procedure each drug was added to blank plasma and extracted using the protocol described above. Linearity,
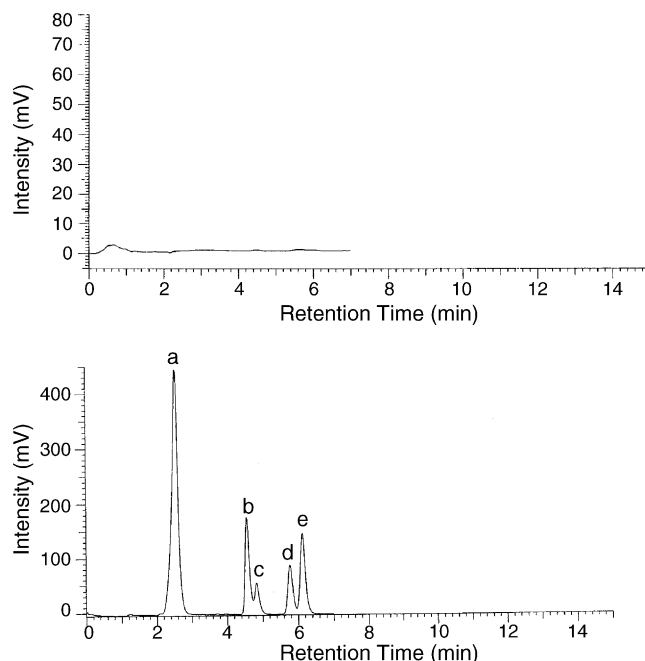

Fig. 2. Base line and HPLC separation of DDA (a), DDD (b), dieldrin (c), DDT (d), DDE (e).

Table 1
Intra- and inter-day variability and repetitive error measurements

Drug	Actual concentration	Detected concentration (μg/ml) (mean $\pm$ SD)	Variability (RSD) %	Repetitive error: detected concentration (mean $\pm$ SD) − RSD%
DDT	Intra-day ($n = 3$)			
	2.500	3.00 ± 0.08	2.71	
	5.000	4.85 ± 0.11	2.22	
	10.000	9.58 ± 0.42	4.41	$10.70 \pm 0.28 - 2.62$
	20.000	21.13 ± 1.67	7.91	
	Inter-day ($n = 6$)			
	2.500	3.05 ± 0.13	4.25	
	5.000	5.27 ± 0.47	8.84	
	10.000	10.35 ± 1.12	10.86	
	20.000	20.02 ± 2.49	12.42	
DDD	Intra-day ($n = 3$)			
	2.500	2.64 ± 0.13	4.84	
	5.000	5.22 ± 0.08	1.61	
	10.000	9.78 ± 0.29	2.94	$9.91 \pm 0.60 - 6.05$
	20.000	19.18 ± 0.17	0.91	
	Inter-day ($n = 6$)			
	2.500	2.59 ± 0.17	6.50	
	5.000	5.31 ± 0.16	3.04	
	10.000	10.19 ± 0.75	7.39	
	20.000	19.45 ± 0.44	2.27	
DDE	Intra-day ($n = 3$)			
	2.500	2.45 ± 0.09	3.84	
	5.000	4.76 ± 0.13	2.70	
	10.000	9.32 ± 0.18	1.98	$12.09 \pm 0.29 - 2.41$
	20.000	21.17 ± 1.96	9.25	
	Inter-day ($n = 6$)			
	2.500	2.48 ± 0.13	5.08	
	5.000	5.36 ± 0.59	11.07	
	10.000	10.46 ± 1.31	12.50	
	20.000	20.51 ± 3.18	15.49	
DDA	Intra-day ($n = 3$)			
	2.500	2.37 ± 0.12	5.08	
	5.000	8.58 ± 0.82	9.58	
	10.000	9.51 ± 0.49	5.17	$11.36 \pm 0.66 - 5.81$
	20.000	21.14 ± 2.23	10.57	
	Inter-day ($n = 6$)			
	2.500	2.42 ± 0.16	6.58	
	5.000	8.76 ± 0.75	8.61	
	10.000	10.51 ± 1.49	14.15	
	20.000	21.75 ± 2.55	11.75	

intra- and inter-day variability, repetitive error and recovery were measured over 3 days. Standard calibration curves were built with the spike height ratios of each drug at concentration of 0.0781, 0.1562, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 μg/ml and the internal standard, and fitted using linear regression. Intra-day variability was defined as relative standard deviation (RSD) calculated from the values measured from three samples at concentration of 2.5, 5, 10, 20 μg/ml, respectively. Inter-day variability was calculated using the values measured from six different samples (two samples for each day) at concentration of 2.5, 5, 10, 20 μg/ml, respectively. Repetitive error was defined as RSD calculated from the values measured from ten samples at concentration of 10 μg/ml. Percent recovery was obtained

from the spike height ratio between extracted sample and drug methanol solution at equal concentration.

2.4. Animals and patients

Male Sprague–Dawley rats (C. River, Italy) weighing about 200 g were fed with a standard diet containing 1% DDD and sacrificed after 3, 40, and 80 days to obtain blood and other organs. Tail blood was withdrawn at the same times from control and other animals receiving 0.075% dietary erythromycin (Abbott, Italy) or 30% commercial GFJ.

Blood was also obtained from six adrenocortical carcinoma patients chronically treated with varying doses of DDD.

Table 2
Cytochrome P450 inhibitor influence in rat blood (plasma and red cells) DDD and principal metabolites

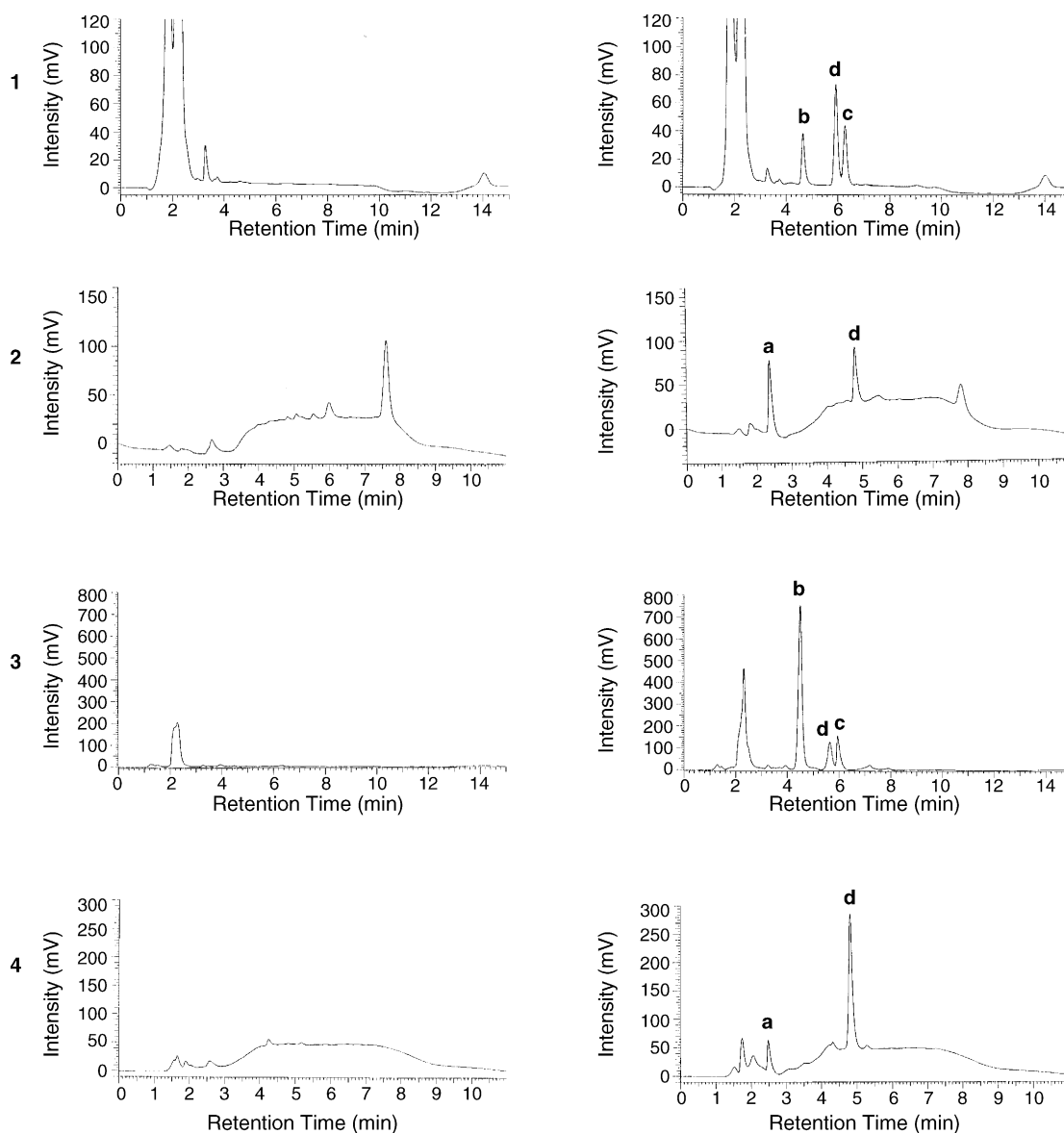|	Control at different times	Grapefruit (time (days))			Erythromycin (time (days))		
		3	40	80	3	40	80
DDD, plasma (%)	100	347	79	39	170	108	131
DDD, red cells (%)	100	111	209	95	85	175	119
DDE, plasma (%)	100	692	36	0	47	141	0
DDE, red cells (%)	100	76	99	0	34	99	0
DDA, plasma (%)	100	–	–	64	–	–	187
DDA, red cells (%)	100	–	–	21	–	–	44

Percent of control values.

Fig. 3. HPLC separation of plasma and fat extracts. Left: blanks and right: drug extracts; (1) plasma (standard method); (2) plasma (DDA method); (3) fat (standard method); and (4) fat (DDA method). DDA (a) DDD (b), DDE (c), DDT (d).
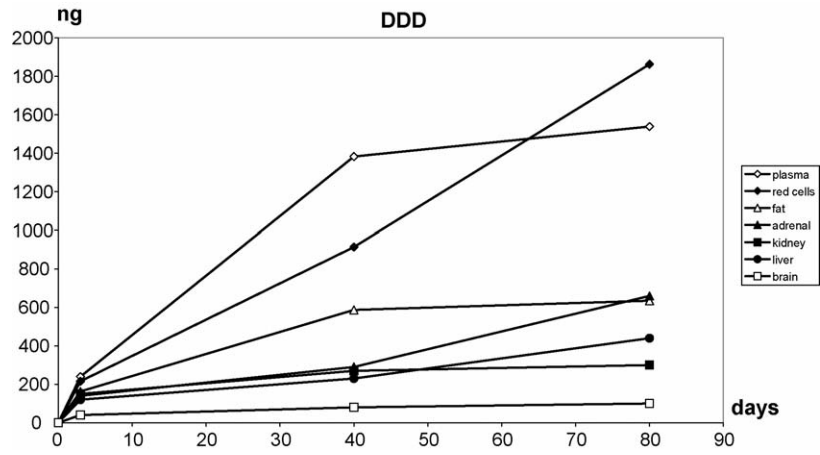
Fig. 4. Time course of DDD concentrations in rat blood and organs.
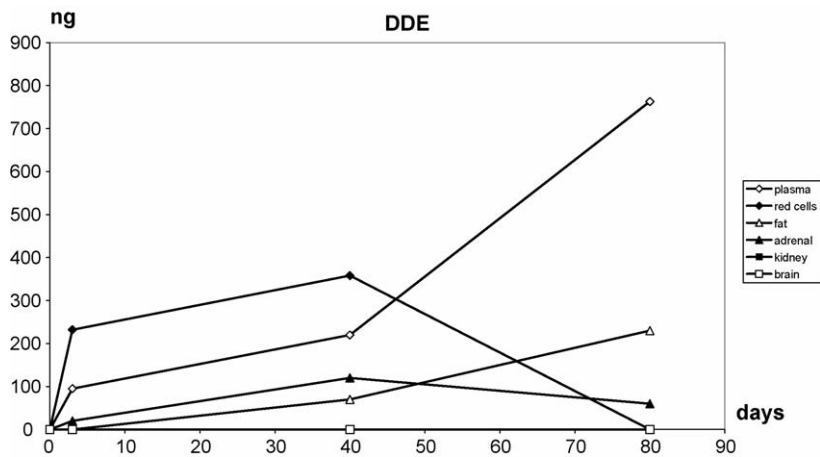
Fig. 5. Time course of DDE concentrations in rat blood and organs.

3. Results and discussion

3.1. Method validation

The lowest DDD, DDA, DDE, and DDT level whose peak height was three times the base line noise (limit of detection) was 300 ng/ml and the limit of quantification (five times the base line noise) was 500 ng/ml. Over the plasma concentration range from 0.0781 to 40 μg/ml regression analysis indicated that there was an acceptable linearity between UV absorption and drug concentration (DDT: $r > 0.996$, DDD: $r > 0.998$, DDE: $r > 0.993$, DDA: $r > 0.974$). The mean ± standard devia-

Fig. 6. Time course of DDA concentrations in rat blood and organs.

Fig. 7. Mitotane, DDA, and DDE concentrations in rat organs: control and animals fed with cytochrome P450 inhibitors (erythromycin and grapefruit juice).

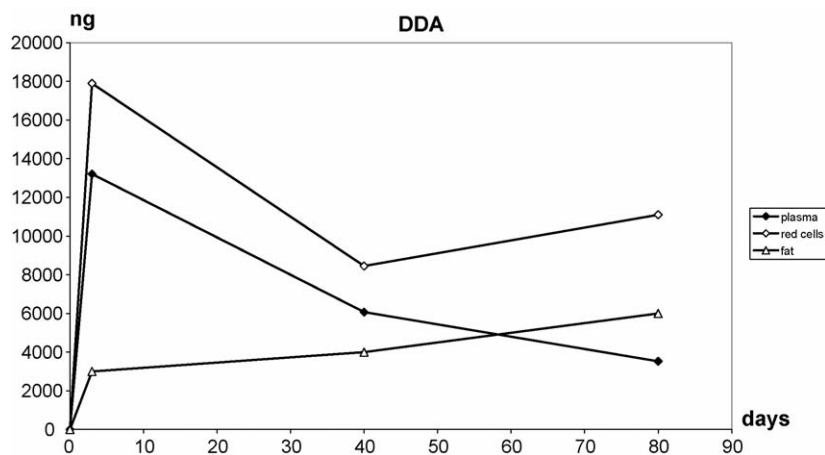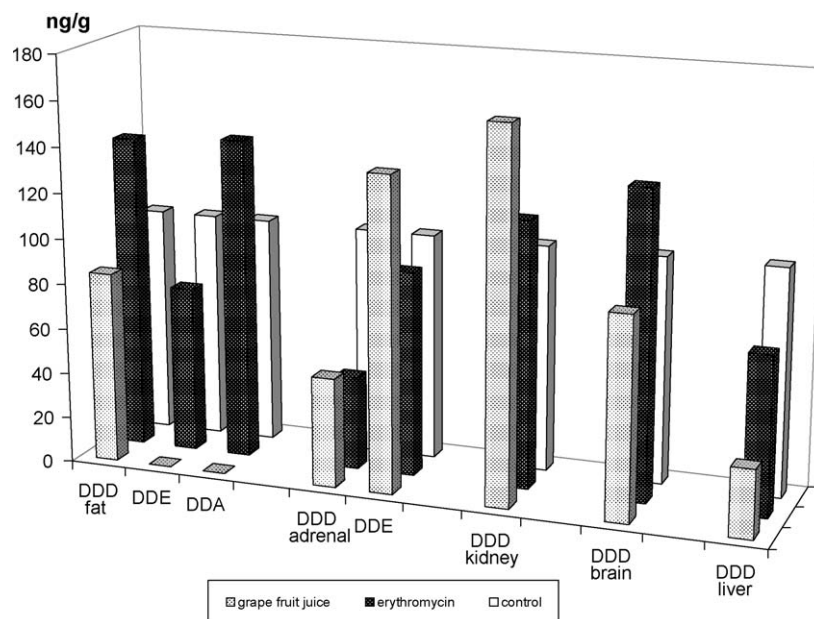tion for the slope and intercept were for DDT 0.106 ± 0.012 and -0.012 ± 0.021, for DDD 0.060 ± 0.001 and 0.015 ± 0.002, for DDE 0.160 ± 0.019 and 0.009 ± 0.035, for DDA 0.081 ± 0.018 and 0.235 ± 0.161, respectively. Intra- and inter-day variability and repetitive error measurements are summarized in Table 1. Plasma/red cell and organ recovery were for DDD 95% and 65%, for DDE 99% and 67%, for DDA 62% and 55%, for DDT 85% and 60%, respectively. Fig. 2 shows chromatograms of both base line (mobile phase injection) and mixture of DDD, DDA, DDE, DDT, and dieldrin (standard method). Fig. 3 shows on the left blanks of both human plasma and rat fat and on the right both human plasma and rat fat DDD, DDE, DDT and DDA extracts. In spite of the low wavelength employed no significant interference could be observed in base line and blank chromatograms. The sensitivity of this new method is sufficient to evaluate DDD, DDA, and DDE in plasma, red cells, and principal organs of experimental animals as well as in DDD-treated patient blood. Our chromatograms showed DDT as perfect internal standard in monitoring adrenocortical carcinoma. Our method could also be applied to evaluate DDT and its metabolites as pollutants, but in this case, dieldrin should be utilized as internal standard.

3.2. Animals

Figs. 4–6 show DDD, DDA, and DDE concentrations in rat tissues. DDE could not be detected in liver because of an interference peak in blank chromatogram at the DDE time and was virtually absent in brain. DDA was detected in blood and in fat only. Table 2 shows the effect of erythromycin and GFJ on blood DDD. Fig. 7 indicates final solid organ concentrations of DDD, DDA, and DDE. The animal data indicate that DDD tends to accumulate in all tissues and is also present, although in low concentrations, in brain; plasma and red cell DDD levels run in the same direction as those of fat and other organs; DDE, at

a lower level, displays the same tendency as DDD, though it is virtually absent at 80 days in red cells. Initially high DDA blood and fat levels point to a high β-hydroxylation capability, whereas their subsequent decline indicates higher elimination activity acquired by the animal during drug exposure. After 80 days, both erythromycin and GFJ lower DDE concentration in blood, while DDD accumulation increases in the fat and the kidney of the erythromycin-treated and GFJ-treated animals, respectively, and is less represented in the latter's organs. Moreover erythromycin enhances DDA in both plasma and fat. α-hydroxylation is inhibited by both erythromycin and GFJ. Even so β-hydroxylation remains active and allows elimination of DDD (GFJ) or accumulation of DDA (erythromycin). Attention should be paid to medical and dietary consumption of substances influencing cytochrome P450 activity.

3.3. Patients

Table 3 summarises the patient blood data. Positive correlations between the patient parameters were found only for DDD in plasma and red cells ($r = 0.85452$, $p < 0.01$, $y = 6.2867x + 341.8$) and for DDD and DDE in plasma ($r = 0.69735$, $p < 0.01$, $y = 0.1635x + 101.18$).

Table 3
Patient blood (plasma and red cells) measurements of DDD and its principal metabolites in six adenocortical carcinoma patients treated chronically with varying doses of DDD at different times

Molecule (material)	No. of dosages (patients)	Mean (µg/ml)	SD
DDD (plasma)	16 (6)	3.17	2.98
DDD (red cells)	16 (6)	0.56	0.40
DDE (plasma)	15 (6)	0.56	0.54
DDE (red cells)	15 (6)	0.02	0.06
DDA (plasma)	16 (6)	18.06	14.86
DDA (red cells)	16 (6)	1.47	1.37

The human blood data show that DDA is the most represented and that all three compounds are more concentrated in plasma than in red cells. DDA is a metabolite ready for elimination but also the end product of a process in which highly reactant intermediate acyl-chloride is formed as has been suggested in bovine studies [11]. Plasma and red cell DDD are correlated and hence red cell DDD concentrations can be deduced from the plasma data and the plasma DDE concentration can be deduced from the DDD concentration. By contrast, there are no correlations for DDA. Since the relative weights of these three compounds in determining the efficacy of DDD are not known, both DDD and DDA should be monitored, the latter both in plasma and red cells. Further studies are necessary to assess the predictive effectiveness of blood monitoring of each compound.

Acknowledgements

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