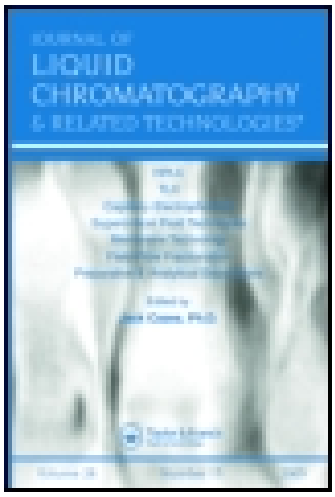

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Development of New Liquid Chromatographic Method for Mitotane and Its Metabolites Determination in Human Plasma Employing Design of Experiments Methodology

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Development of New Liquid Chromatographic Method for Mitotane and its Metabolites Determination in Human Plasma Employing Design of Experiments Methodology

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Abstract

A simple and reliable new HPLC method with UV detection has been developed and validated for simultaneous determination of mitotane and its two metabolites DDA and DDE. Method development was carried out utilizing systematical approach of the Design of Experiments (DoE) methodology. For estimation of factors influence on selected chromatographic responses and definition of the optimal chromatographic conditions, Box-Behnken experimental design was applied. The defined optimal separation conditions were: column Restek Ultra Aqua C_{18} with pre-column Restek Ultra Aqua C_{18} operating at temperature 35 °C; mixture of acetonitrile and 0.5% formic acid as mobile phase with 1.2 mL min^{-1} flow rate and detection at 230 nm. As sample preparation method, liquid-liquid extraction was chosen. Method was fully validated and LOQ and LOD were experimentally determined. Finally, method was successfully applied for determination of mitotane and its metabolites in plasma samples of patients with adrenocortical carcinoma.

KEYWORDS: mitotane, metabolites DDA and DDE, liquid chromatography, validation, experimental design

INTRODUCTION

Determination of drug and its metabolites in biological samples often presents a challenge. Knowing complexity of biological samples, assay of low levels of drug and its metabolites demands systematic approach. As many steps are included in such kind of investigation, every step should be carefully conducted in order to achieve the most suitable method. In this study, systematic approach including the Design of Experiments (DoE) methodology for a development of a new liquid chromatographic method (LC) for the analysis of mitotane and its metabolites (DDA – 1,1-(o,p'-dichlorodiphenyl) acetic acid and DDE–1,1-(o,p'-dichlorodiphenyl)-2,2 dichloroethene) in human plasma was applied.

Mitotane, chemically 1,1-(*o,p'*-dichlorodiphenyl)-2,2-dichloroethane, (*o,p'*- DDD) is used for the therapy of adrenocortical carcinoma (ACC) for the last 50 years. ACC is a rare, highly malignant neoplasm with an incidence of 2 cases per million people per year worldwide, with only 16% to 38% of patients surviving for more than 5 years after diagnosis [1-3]. Even after surgical resection of tumor, local or metastatic recurrence is frequent and 75% to 85% of patients have relapse after radical resection [4, 5]. This high recurrence rate, incompletely understood pathogenesis and poor prognosis of disease forced investigators to consider the use of adjuvant therapy - mitotane has been widely used for this purpose. Adjuvant mitotane may prolong recurrence-free survival in patients

with radically resected ACC [6]. Unfortunately, the exact mitotane adrenocorticolytic mechanism of action is unknown. After intake, mitotane undergoes biotransformation in liver and other tissues to two main metabolites – DDA and DDE catalyzed by a P-450 enzyme following α- and β- hydroxylation, respectively. β- Hydroxylation is transformed by dehydrochlorination into an acyl chloride which either covalently binds to bionucleophiles in the cancer cells or is transformed to an acetic acid derivate (DDA) for renal excretion (Figure 1). Mitotane and its metabolites are presented in Figure 2.

Careful monitoring of plasma mitotane concentrations is very important because of the narrow therapeutic window lying between 14 μg mL^{-1} and 20 μg mL^{-1}. Mitotane plasma levels above 14 μg $mL^{-1}$are required for therapeutic effects, whereas plasma concentrations higher than 20 μg mL^{-1}correlate with significant side effects, especially neurological toxicity. About 40% of patients suffer some central toxicity with dizziness, vertigo, sedation, lethargy and depression. Next to these side effects, most of the patients receiving mitotane have anorexia, nausea, vomiting and sometimes diarrhea. Due to its lipophilicity, mitotane accumulates in adipose tissue, so the plasma half-life is extremely long (18-159 days). Consequently, it can take months to reach the steady state [7]. The maximum tolerated oral dosage of mitotane range from about 2 g to 16 g (usually 6-10 g) per day [8].

The monitoring of plasma levels of mitotane and its metabolite is required in order to balance the good therapeutic efficacy with the acceptable toxicity. Literature survey shows that there are only few papers dealing with determination of mitotane and its

metabolites in plasma [9–11]. In papers [9, 10] gradient elution mode was employed while in [11] isocratic mode was used. In all three published methods UV detection was utilized [9–11]. In previously published studies, a simple trial and error approach was used in method development. In this paper, a strategy which included DoE methodology was employed in the development of new LC method for the analysis of mitotane and its metabolites in plasma samples. The application of DoE methodology in LC method development provides a detailed view of the analyzed system and enables a mathematical description of the system behavior, after performing a minimal number of well-planned experiments. Following this strategy, a improvement of LC method for mitotane and its metabolite analysis was achieved and, consequently, optimal chromatographic conditions were successfully defined. Finally, the method was fully validated and applied for the determination of mitotane and its metabolites in patients' plasma samples.

EXPERIMENTAL

Reagents and Standard Substances

Mitotane, *p,p'*-DDA, *p,p'*-DDE and DDT – dichlorodiphenyltrichloroethane (internal standard) and formic acid were purchased from Sigma-Aldrich, Germany. Acetonitrile was obtained from J. T. Baker, The Netherlands and HPLC grade ethanol from Fischer Scientific, U. K.

Stock Solutions

All stock standard solutions of the studied compounds and internal standard were prepared in ethanol HPLC grade at the concentration of 0.5 mg mL^{-1}.

Chromatographic Conditions

The chromatographic system Spectra System consisting of Spectra P1000 HPLC Pump, Spectra AS3000 autosampler, Spectra UV1000 UV absorbance detector and Chromeleon Client Program (Software Windows XP) was used for data collection. Separations were performed on a Restek Ultra Aqua C_{18}, (30 mm × 4.6 mm, 3 μm particle size) with pre-column Restek Ultra Aqua C_{18} (10 mm ×4.6 mm, 3 μm particle size), temperature at 35° C with UV detection at 230 nm and mobile phase flow rate 1.2 mL min^{-1}. Injection volume was 50 μL.

Mobile phases were prepared by mixing water phase (water and formic acid) with acetonitrile. Final content of mobile phase as well as its final column temperature were defined during the method optimization.

METHODS OPTIMIZATION

Solutions for Method Optimization

For method optimization the stock solutions of mitotane, DDA and DDE were used to prepare a mixture with concentration of 60 μg mL^{-1} for mitotane, DDA and DDE in ethanol HPLC grade.

Experimental Procedure

Experiments were done according to the matrix of experiments created by Box-Behnken design. All factors were investigated in three levels (−1, 0 and +1). Factors, their levels in real and coded values are given in Table 1.

Software

Experimental design and data analysis were performed using Design−Expert® 7.0.0. (Stat−Ease Inc., Minneapolis).

SAMPLE PREPARATION

400 µL thawed plasma samples were spiked with 200 µL of the internal standard DDT (0.05 mg mL^{-1} in ethanol) in microcentrifuge tubes and vortexed well. Plasma standards and patient samples were treated in the same way. Proteins were precipitated by adding 500 µL of cold HPLC quality ethanol, followed by vortex mixing and centrifugation at 14000 *x g* at 4 °C for 10 minutes in the Universal 32R (Hettich-Zentrifugen). After transferring supernatant into a new microcentrifuge tube, 100 µL of 0.5% formic acid was added, vortexed and centrifuged again at 14000 *x g* at 4 °C for 10 minutes. The final supernatant was transferred into autosampler tubes of HPLC and 50 µL was injected. In case when measured peaks were out of calibration curve, appropriate dilutions were made.

Patient Blood Sample Preparation

Patients were informed about the purpose of the study and provided written informed consent before the enrolment. Mitotane dosage regimens were in the range from 3 to 12 g, three times per day. Patient blood samples were collected in a 5 mL potassium -

ethylenediamine tetraacetate (K-EDTA) tubes 8 h after the consumption of the last mitotane daily dose. The plasma was separated using the centrifugation and the samples were stored at −80 °C until assayed.

METHOD′S VALIDATION

Solutions for Specificity Testing

In order to prove specificity of the method 400 μL of blank plasma was spiked with 200 μL of mixture containing 60 μg mL^{-1} of mitotane, DDA, DDE and 0.05 mg mL^{-1} of DDT followed by the above described extraction procedure. Obtained chromatogram was compared to chromatogram of the blank plasma.

Solutions for Linearity Testing

For the linearity testing of mitotane, DDA and DDE a stock standard solution of 0.5 mg mL^{-1} of each compound (prepared in HPLC grade ethanol) was used. The first nine working standard solutions with following concentrations 2.5, 5, 15, 30, 45, 60, 90, 120 and 150 μg mL^{-1} of each substance were prepared. Additionally, standard solution of 5 mg mL^{-1} of DDA was used to prepare additional five standard solutions at concentrations of 300, 450, 600, 900 and 1200 μg mL^{-1}. Aliquots of 200 μL of analyte standard solutions were added to 400 μL of blank plasma, followed by above described extraction procedure. Internal standard (DDT) was added in all standard solutions in the concentration of 0.05 mg mL^{-1}. Calibration curves and equations were obtained using least-squares linear regression analysis of the peak area (AUC) *vs* analyte concentrations.

Extraction Efficiency Testing

Extraction efficiency of mitotane and its metabolites was determined by adding 200 μL of working standard solutions at four different concentrations (5, 45, 120 and 600 μg mL^{-1} – QC samples) – DDA and three different concentration (5, 45 and 120 μg mL^{-1} – QC samples) – for mitotane and DDE to the 400 μL of blank plasma, followed by described extraction and repeating the whole procedure three times. The HPLC – derived peak areas obtained were compared with those obtained from standard solutions at the same four concentrations, six times diluted and injected directly into the liquid chromatographic apparatus. The percentage of the extraction yield was calculated and presented as Recovery value.

Accuracy Testing

The accuracy of the method was determined in four different concentrations (5, 45, 120 and 600 μg mL^{-1} – QC samples) for DDA and three different concentrations (5, 45 and 120 μg mL^{-1} – QC samples) for mitotane and DDE. Aliquots of 200 μL of working standard solutions in these mentioned concentrations were added to 400 μL of blank plasma and extracted as described. The procedure was repeated three times at each concentration level and the accuracy of the measurements was expressed in terms of Recovery.

Precision Testing

The precision experiments for mitotane, DDA and DDE were performed by spiking 400 μL of blank plasma with 200 μL of working standard solution of the QC samples,

followed by extraction. The procedure was repeated six times within the same day to obtain intra-day precision and the inter-day precision was assessed by replicate analysis on the second and the third day. The results of the precision measurements were expressed as relative standard deviation (RSD) values.

Freeze and Thaw Stability Testing

Six thawed blank plasma samples were spiked with 200 μL of working standard solution of the QC samples for mitotane, DDA and DDE, vortexed well and frozen at −80°C for 30 days in order to test stability of studied substances. Three sets of these samples were prepared. After 30 days all sets of samples were thawed at the room temperature and first set of samples were extracted and injected in HPLC system, while the other two sets are returned and stored again at −80 °C. The freeze/thaw cycle followed by extraction procedure was repeated for remaining two sets of samples after another 30 (60) days.

RESULTS AND DISCUSSION

Method Development

In order to achieve the most suitable performance in chromatographic method it is necessary to apply the systematic approach. In the beginning, this approach demands understanding of nature and chemical characteristics of investigated substance in order to establish relationship between its properties and chromatographic conditions. This is important in order to define desirable characteristics of chromatogram at the start.

So, we began primarily with some general consideration. Taking into account that mitotane and its metabolites are highly lipophilic non-polar compounds, the reversed phase chromatography mode should be the best choice. High dose of mitotane used in therapy and previously published studies led to conclusion that UV detector has quite enough sensitivity for this analysis. In line with the reference [11], wavelength of 230 nm was chosen. Further, according to previously published papers, where mitotane and its metabolites were investigated chromatographically [9–11], early eluting substance was DDA because of the presence of carboxylic group while the last eluted substance was DDE. De Francia et al. (9) in the paper, applied two separate gradient methods in order to solve this challenge. In the remaining papers [10, 11] isocratic elution was applied, but DDA was eluted very near to the mobile phase peak. According to these findings as well as characteristics of the sample, as the first desired characteristic of chromatogram in present study, the retention factor for DDA was defined to be not less than 3.0. The other two substances are more lipophilic and, in order to avoid their long elution, short octadecyl column was selected. Based on these considerations, the second important characteristic of chromatogram is the retention time of the last eluting substance acting as an indicator of the overall run time. Finally, as a last parameter of the chromatogram´s quality, the resolution between last eluted substances was set at 1.5. After the analysis of already existing data and defining the quality of desirable chromatogram, preliminary experiments were conducted.

Based on the chemical structure of investigated substances, especially acid character of DDA, mobile phase with acidic pH was selected. As an organic modifier in mobile phase,

acetonitrile was chosen. Taking into account previously discussed considerations, scouting experiments on short C_{18} columns (Restek Ultra Aqua C_{18} (30 mm × 4.6 mm, 3 μm particle size) and XTerra® C_{18} (20 mm × 4.6 mm, 3.5 μm particle size column)) were done. In these experiments the content of acetonitrile varied from 40% to 80%. Simultaneously, different water phase was used – various additives were used for pH adjustment (glacial acetic acid, phosphoric acid and formic acid). In line with the previously defined desirable characteristics of chromatogram and based on the scouting experiments, Restek Ultra Aqua C_{18} (30 mm × 4.6 mm, 3 μm particle size) was chosen. Further on, about 50% of acetonitrile was selected as acceptable, with formic acid in water phase. Flow rate was varied from 1.0 mL min^{-1} to 1.5 mL min^{-1}, where 1.2 mL min^{-1} was set as final flow rate.

Based on the preliminary experiments, some frameworks of chromatographic conditions were defined (e. g. column type, flow rate and organic modifier) but other factors (acetonitrile content, content of formic acid and column temperature) were to be optimized. In order to choose optimal chromatographic conditions and investigate behavior of substances in certain experimental space, DoE methodology was chosen. Successful application of this methodology in LC method optimization was published in many papers such as [12–17].

In this study, from preliminary experiments three factors were selected to be changed during the optimization: content of acetonitrile in mobile phase, content of formic acid in water phase and column temperature. Factors and their levels are presented in Table 1.

Other chromatographic factors (flow rate and wavelength kept on the constant level during optimization).

For the optimization, Box-Behnken design (BBD) as a kind of response surface design was chosen. BBD is based on three–level incomplete factorial designs where two factors are arranged in a full two–level design, while the level of the third factor is set at zero [12]. If three factors are to be examined, twelve experiments plus central point replications are required. Also, BBD is nearly rotatable and especially useful when the points on one or more corners of the cube represent factor–level combinations unacceptably expensive or impossible to carry out due to physical constraints of the experimentation [18].

As the responses, previously defined important characteristics of chromatogram (retention factor of DDA, retention time of DDE and resolution between DDE and mitotane) were followed and the obtained results are given in Table 1.
Further, the results were statistically evaluated. The obtained values for coefficients and important statistical data are given in Table 2.

Table 3 presents coefficients for coded factor levels with corresponding *p*–values. Also, coefficients of determination (R^2) and adjusted values of the coefficients of determination (adj. R^2) are given. All the obtained models had high values of R^2 (> 0.98) and adj. R^2 (> 0.95), which revealed that experimental data fitted well in the second–order polynomial equations. Also, values for predicted R^2 is higher than 0.80 which confirmed that

obtained models have good predictive ability. For all tested models lack of fit value was not significant. Next to that, values for *p* were analyzed and values greater than 0.1000 indicate that model terms are significant. Acetonitrile is a factor with the strongest influence on all followed responses (value *p* is lower than 0.1000). In addition, in all three created models the coefficients corresponding to acetonitrile (b_1) had minus sign, which means that higher values of acetonitrile influence the decrease of all three analyzed responses. Additionally, *p* value for coefficient b_{23} (interaction of formic acid content and temperature) for all followed responses was considered significant. The third followed response (R) is under the strong influence of acetonitrile, column temperature and the interactions acetonitrile – formic acid content (b_{12}) and content of formic acid – temperature (b_{23}). Besides these significant influences, resolution factor in all experiments had adequate values leading to conclusion that in all investigated experimental space proper separation was achieved.

In order to define optimal region overlay plot was constructed. Desirable values for investigated responses was set, for k_1 maximal values, for t_3 values lower than 16 minutes while value for R was set in range presented in Table 2. Column temperature was set at nominal value (35°C) and obtained overlay plot is presented in Figure 3.

Yellow part of graph presented range of factors whose gives desirable responses. From this region we choose 53% of acetonitrile in mobile phase and 0.5% of formic acid as optimal values. Under chosen experimental conditions, all previously defined goals of the

method optimization were achieved with optimal run times, optimal resolution and retention of DDA.

The presented DoE methodology enables analysis of the chromatographic system in defined range of investigated factors. It makes easier to realize the regions with optimal as well as desirable responses (Figure 3). In this case, where DoE was applied in the development of LC method for mitotane and metabolites determination, all the previously defined aims were accomplished. Finally, some significant advantages were achieved in comparison to previously published methods. In comparison to paper [9], where gradient elution was applied, this investigation brings the isocratic method. Furthermore, the run time in [10] paper was 20 minutes, while in this study the separation is achieved in less than 15 minutes. Also, in the presented study, a significantly lower content of acetonitrile in the mobile phase was used compared to the previous publications [10, 11], which is considered as a very important environmental toxicity aspect. Generally, the employment of DoE strategy enables the improved understanding of the given LC system, thus providing an efficient LC method for the analysis of mitotane and its metabolites.

Next, under the optimal chromatographic conditions internal standard was chosen. Considering mitotane as a derivate of DDT and taking into account their similar chemical structures, DDT was injected under the obtained chromatographic conditions. It was confirmed that appropriate separation from other compounds in the mixture was achieved. Under the optimal chromatographic conditions chromatograms of the blank plasma and plasma spiked with working standards were recorded and presented in Figure 4.

For the sample preparation, liquid-liquid extraction was performed. Guideline for sample preparation was found in paper published [10]. Procedure was tested in our laboratory and feasibility was confirmed. Considering different water phase, final dilution was made with water phase consisting of 0.5% formic acid. Details for sample preparation are given in Experimental part.

Method′S Validation

Firstly, method specificity was investigated. Under the optimal conditions the solutions of the blank plasma and plasma spiked with standards of DDA, DDT, mitotane and DDE were injected into HPLC system and chromatograms were recorded. Obtained chromatograms are presented in Figure 4. There is no endogenous interference from drug-free plasma and the method is selective for determination of above mentioned drug and its metabolites in plasma.

As a next important parameter of method validation the linearity of the method was tested. Linearity for mitotane and DDE were tested in range from 0.417 μg mL^{-1} to 25.0 μg mL^{-1} and for DDA in range from 0.417 μg mL^{-1} to 200.0 μg mL^{-1}. The calibration curve was linear over the studied concentration range (R^2 >0.9968) for all investigated compounds. The obtained results for linearity testing are presented in Table 3.

LOQ and LOD were experimentally determined and obtained values for LOQ were 0.4 μg mL^{-1} for mitotane, DDA and DDE and LOD was 0.2 μg mL^{-1} for mitotane

and DDA and μg mL^{-1} for DDE. Obtained values for LOD and LOQ were in agreement with the previously published confirming the adequate sensitivity of method for the analysis of mitotane, DDA and DDE metabolites.

Further, the extraction efficiency was evaluated according to the procedure given in Experimental part. Range of the obtained Recovery values for investigated substances were 96.09% – 101.32%, 99.37% – 102.08% and 98.02%–99.75% for DDA, mitotane and DDE, respectively. It should be pointed out that adequate extraction efficiency was achieved.

Accuracy of the presented chromatographic method was determined in four different levels for DDA and in three different levels for mitotane and DDE (see Experimental part for procedure). Results are shown in Table 3. Obtained results for Recovery values confirmed good accuracy of the method.

The results of the precision experiments were expressed as the relative standard deviation (RSD) and the obtained values were 1.77%, 3.38% and 0.85% for mitotane, DDA and DDE, respectively. At the same concentration levels freeze and thaw stability was checked (see Experimental part for procedure) and obtained results for RSD (the highest value was 0.6%) confirm adequate stability of the samples.

Patient Samples

The optimized and validated LC method was used to determine plasma levels of mitotane and its metabolites in plasma samples of 6 patients with mitotane doses ranging from 3 to 12 g per day, taken in three daily doses. Plasma samples were retrieved in the morning, at least 6-8 h after administration. Mitotane levels ranged from 6.6 μg mL^{-1} to 43.6 μg mL^{-1} and DDA levels from 11.28 μg mL^{-1} to 151.47 μg mL^{-1}. Also, the ratios of DDA/mitotane were followed and the obtained values ranged from 1.53-10.59. In the analyzed samples DDE level was lower than LOQ. As it is previously mentioned, pharmacokinetic characteristics of mitotane are complex because of its lipophilicity and consequently very long elimination half-time. In addition, the measurement of patients' mitotane and its metabolites plasma levels show significant variability in metabolism between patients indicating how more regular therapeutic drug monitoring may contribute to the main goal in therapy – good therapeutic efficacy with acceptable toxicity. Finally, the chromatogram of one patient sample is given in Figure 5.

CONCLUSION

In this study, the development of the new LC method for determination of mitotane and its two metabolites in human plasma employing DoE methodology is presented. In order to achieve defined aim in method optimization (acceptable retention factor of DDA, optimal run time and adequate separation) Box-Behnken design was used. Statistical and graphical interpretation of the obtained results enables setting of optimal chromatographic conditions where significant improvement of the method was achieved. Thereafter, the method was validated and successfully applied for the analysis of plasma samples obtained from patients treated with different daily doses of mitotane.

Concentration levels of mitotane and metabolite DDA were quantified while level for DDE was below limit of quantification.

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Table 1. Plan of experiments for Box-Benhken design and results obtained for responses

A	B	C	k_{DDA}	t_{DDE}	$R_{DDE/mitotan}$
51 (−1)*	0.3 (−1)	35 (0)	4.768	18.868	4.944
55 (+1)	0.3 (−1)	35 (0)	3.561	12.110	4.300
51 (−1)	0.7 (+1)	35 (0)	4.563	18.600	5.100
55 (+1)	0.7 (+1)	35 (0)	3.182	11.528	4.079
51 (−1)	0.5 (0)	30 (−1)	4.861	20.203	5.130
55 (+1)	0.5 (0)	30 (−1)	3.350	12.228	4.204
51 (−1)	0.5 (0)	40 (+1)	4.587	18.839	4.950
55 (+1)	0.5 (0)	40 (+1)	3.048	11.82	3.969
53 (0)	0.3 (−1)	30 (−1)	4.05	15.052	4.562
53 (0)	0.7 (+1)	30 (−1)	4.352	16.755	4.831
53 (0)	0.3 (−1)	40 (+1)	4.287	16.911	4.677
53 (0)	0.7 (+1)	40 (+1)	3.77	15.039	4.414
53 (0)	0.5 (0)	35 (0)	3.95	15.705	4.662
53 (0)	0.5 (0)	35 (0)	4.086	15.465	4.686
53 (0)	0.5 (0)	35 (0)	4.108	15.836	4.512

A – concentration of acetonitrile (%); B – concentration of formic acid; C – column temperature (°C);

k_{DDA} – retention factor of DDA; t_{DDE} – retention time for DDE; R – resolution between DDE and mitotane

*in the brackets coded values for factor levels are given

Table 2. Coefficients of quadratic model $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$

	k_1		t_3		R	
	coefficient	*p*-value	coefficient	*p*-value	coefficient	*p*-value
b_0	4.04		15.65		4.62	
b_1	−0.71	<0.0001*	−3.60	<0.0001*	−0.45	<0.0001*
b_2	−0.100	0.0545	−0.13	0.3928	−0.0074	0.7886
b_3	−0.11	0.0359*	−0.20	0.1953	−0.090	0.0186*
b_{12}	−0.043	0.4801	−0.078	0.7006	−0.094	0.0516*
b_{13}	−0.0004	0.9477	+0.24	0.2699	−0.014	0.7234
b_{23}	−0.20	0.0151*	−0.89	0.0056*	−0.13	0.0158*
b_{11}	−0.089	0.1897	−0.27	0.2334	−0.036	0.3917
b_{22}	+0.069	0.2947	−0.11	0.6228	−0.022	0.5932
b_{33}	+0,0069	0.9109	+0.39	0.1088	−0.021	0.6126
Lack of fit	0.2670		0.1125		0.7930	
R^2	0.9857		0.9932		0.9848	
adj. R^2	0.9600		0.9810		0.9575	
Pred. R^2	0.8083		0.8986		0.8928	

k_1 – retention factor of DDA, t_3 – retention time of DDE; R – resolution between DDE and mitotane

Table 3. Results from linearity and accuracy testing

Substance	Linearity			Accuracy		
	Concentration range ($\mu g\ mL^{-1}$)	Equation	Correlation coefficient	Spiked concentration ($\mu g\ mL^{-1}$)	Recovery (%)	RSD (%)
DDA	0.417 – 200.0	y=0.1746x+0.00836	0.9968	0.833	92.7	5.99
				7.5	88.67	0.89
				20	86.89	0.61
				100	97.2	1.36
Mitotane	0.417 – 25.0	y=0.1457x−0.06112	0.9979	0.833	102.78	6.12
				7.5	91.67	1.12
				20	91.72	0.45
DDE	0.417 – 25.0	y=0.165x−0.016	0.9990	0.833	92.97	1.73
				7.5	100.18	0.51

				20	99.27	0.74

Figure 1. Structures of mitotane, its two metabolites (DDA and DDE) and internal standard (DDT)

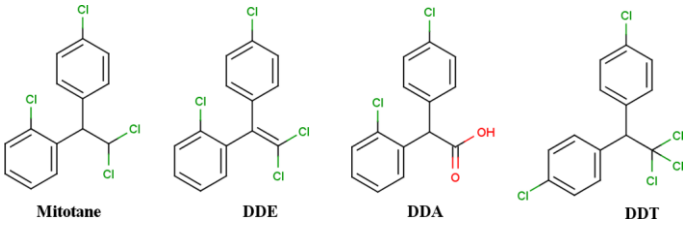

Figure 2. Metabolic pathway of mitotane

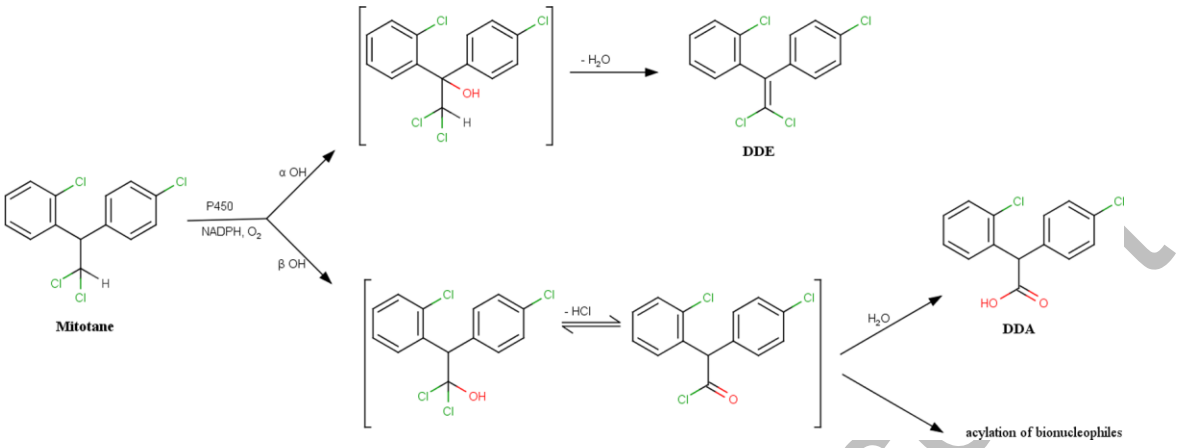

Figure 3. Overlay plot

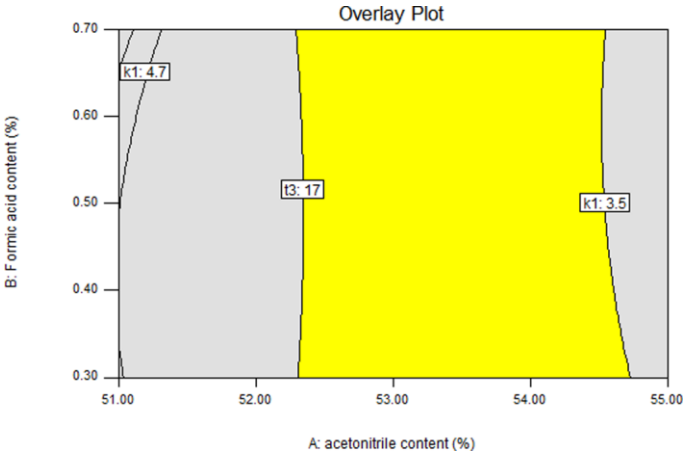

Figure 4. Chromatograms for A. blank plasma, B. plasma spiked with standards of DDA, DDT, mitotane and DDE; Chromatographic condition: Column Restek Ultra Aqua C18 (30 mm × 4.6 mm, 3 μm particle size) with pre-column Restek Ultra Aqua C_{18} (10 mm ×4.6 mm, 3 μm particle size), with mobile phase consisted of acetonitrile and 0.5% formic acid in ratio 53:47 V/V, column temperature 35 °C, flow rate 1.2 mL min^{-1}, wavelength 230 nm and injection volume of 50 μL.

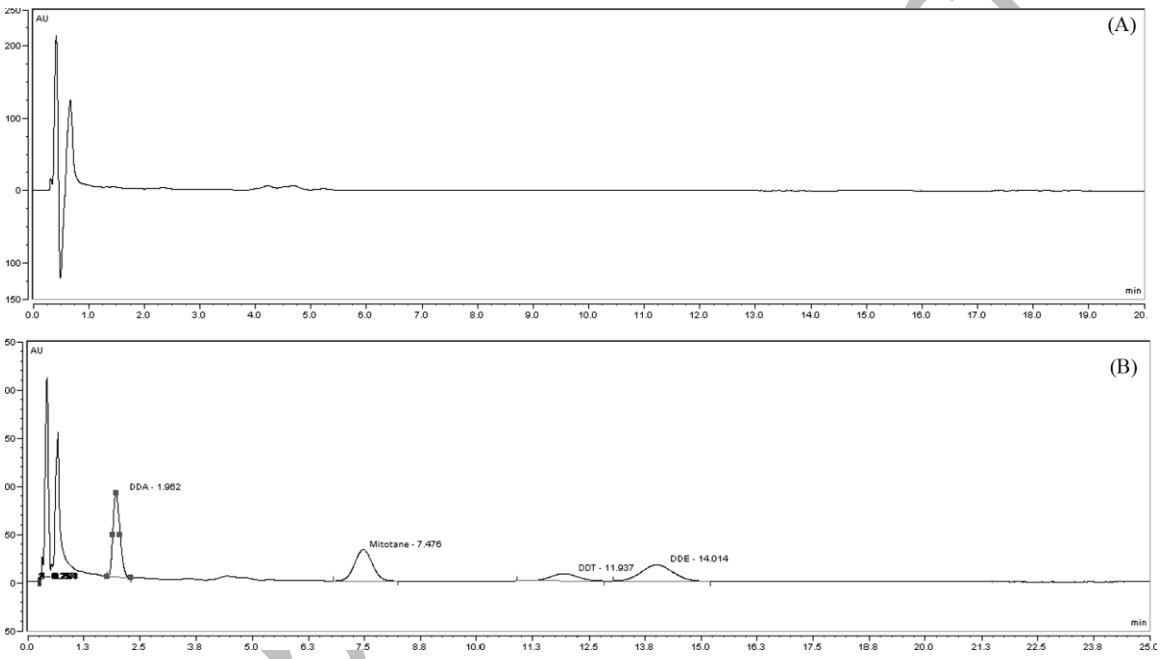

Figure 5. Patient sample chromatogram