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Automated on-line dialysis for sample preparation and HPLC analysis of antidepressant drugs in human plasma. Inhibition of interaction with the dialysis membrane[1]

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Abstract

Antidepressant drugs interact with the dialysis membrane and were selected as model substances to study inhibition of analyte–membrane interactions. A chemometric approach based on response surface modelling was used for screening and optimisation of dialysis recoveries. Optimal dialysis recoveries (52–65%) were obtained for the model compounds (mianserine, amitriptyline, nortriptyline, imipramine and desimipramine) when a cationic surfactant added to the donor solution of the dialyser was used to inhibit analyte–membrane interactions. Automated analysis of antidepressants in plasma was performed by connecting the ASTED™ (Automated Sequential Trace Enrichment of Dialysates) system to high-performance liquid chromatography (HPLC). The drugs were detected by ultraviolet detection and fluorescence detection after post-column photochemical reaction. Validation of the method showed linear standard curves for all the drugs in the concentration range 50–2000 nmol l^{-1}. Within- and between-day relative standard deviations ranged from 1.1 to 5.7%.

Keywords: Antidepressant drugs; Dialysis; High-performance liquid chromatography

1. Introduction

The ASTED™ (Automated Sequential Trace Enrichment of Dialysates) system processes crude biological samples in line with high-performance liquid chromatography (HPLC). Proteins and particles are removed by dialysis through a semi-permeable membrane and drug molecules collected in the dialysate are enriched on a trace enrichment column (TEC). The TEC is connected to HPLC in a column switching system. As long as the drug molecules and the proteins are not bound or associated with each other and no interaction between the analytes and the dialysis membrane takes place, rapid and efficient analyses of drugs in plasma can be performed. The system is capable of analysing more than 100 biological samples in 24 h [1–14].

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For drugs which are strongly bound to proteins, improved dialysis recoveries can be obtained by disrupting the drug–protein binding prior to dialysis. Earlier reports have described alteration of the pH in plasma and addition of displacers [7–10,13,14]. Hydrochloric acid has shown to effectively release alkaline drugs from the protein binding sites. Simultaneous addition of glycerol to the plasma sample prevented the proteins from precipitating in the plasma sample [14].

The ASTED™ system is equipped with a cellulose acetate dialysis membrane. Interactions between analytes and the cellulose acetate membrane have been reported for basic drugs such as the opiate derivative pholcodine, the benzodiazepines and the neuroleptic drug clozapine [10,11,14]. Clozapine is a hydrophobic base which may interact with the dialysis membrane by ionic and hydrophobic interactions. In a recent study, it was shown that addition of the cationic surfactant, dodecylethyldimethyl ammonium bromide, to the donor solution of the dialyser inhibited the interaction of clozapine with the dialysis membrane [14]. As most antidepressant drugs were expected to interact with the dialysis membrane in the same manner as clozapine, antidepressant drugs were selected as model substances to investigate more closely the ability of cationic surfactants to inhibit analyte–membrane interactions.

The analytical methods currently used for determination of antidepressant drugs in biological fluids are HPLC, gas chromatography (GC) and immunoassays [15–19]. Liquid–liquid extraction and solid-phase extraction methods have been reported for sample preparation prior to chromatography. Automated solid-phase extraction, both off-line and on-line to HPLC, has also been developed [19]. No automated methods based on dialysis and HPLC have been reported for determination of antidepressants in plasma.

The antidepressant drugs selected for the study were mianserine, imipramine, desimipramine, amitriptyline and nortriptyline. In this investigation, the main purpose was to study the optimisation of dialysis recoveries by addition of cationic surfactants to the donor solution in the dialyser. The effect of varying the chain length and the concentration of cationic surfactants, as well as varying the pH of the donor solution and the volume of acceptor solution, was studied by a chemometric approach applying a factorial design and response surface modelling (RSM). The optimal settings were used to develop a fully automated method for determination of the model compounds in human plasma.

2. Experimental

2.1. Chemicals and reagents

Mianserine, amitriptyline, nortriptyline, imipramine and desimipramine were gifts kindly donated from Ullevaal hospital (Oslo, Norway). Acetonitrile and methanol were supplied by Rathburn (Walkerburn, UK) Dodecyltrimethyl ammonium bromide, tetradecyltrimethyl ammonium bromide and hexadecyltrimethyl ammonium bromide were obtained from Sigma (St. Louis, MO, USA). Glycerol was obtained from Norsk Medisinal Depot (Oslo, Norway). Di-ammonium hydrogen phosphate, *ortho*-phosphoric acid and hydrochloric acid were supplied by Merck (Darmstadt, Germany). HPLC-grade water was prepared from a Milli-Q water purification system (Millipore, MA, USA).

2.2. Standard solutions

Stock standard solutions of mianserine, amitriptyline, nortriptyline, desimipramine and imipramine were prepared in methanol. Citrated plasma (drug free) from healthy donors was obtained from The Blood Centre at Ullevaal Hospital (Oslo, Norway). Plasma samples with mianserine, amitriptyline, nortriptyline, desimipramine and imipramine in concentrations of 50–2000 nmol l^{-1} were prepared by spiking drug-free plasma with aliquots of the stock standard solutions. The protein releasing reagent (PRR) was an aqueous solution consisting of 1 M HCl and 25% (v/v) glycerol.

2.3. Equipment

The sample preparation system was an ASTED™ unit (Gilson, Villiers-le-Bel, France) consisting of a 231 autosampling injector equipped with two 401 dilutors fitted with 1 ml syringes and an automated six-port valve (Rheodyne, Berkeley, CA, USA). The dialysis cell was made of polymethylacrylate, with donor and acceptor channel volumes of 100 and 175 μl, respectively. The donor and acceptor channels were separated by a Cuprophane membrane (cellulose acetate) with a molecular mass cut-off of 15 kDa. The trace enrichment column (TEC) connected to the six-port valve was a 10 × 2 mm i.d. stainless steel precolumn packed with 40 μm BondElut C8 particles (Varian, Harbor City, CA, USA). The chromatographic system consisted of an LC-6A HPLC pump (Shimadzu, Kyoto, Japan). The analytical column was a Supelcosil LC-PCN cyanopropyl bonded phase column (150 × 4.6 mm i.d., 5 μm particles) from Supelco (Bellefonte, PA, USA). Acetonitrile–methanol–0.005 M ammonium phosphate buffer, pH 7.0 (70:15:15, v/v) was used as HPLC mobile phase at a flow rate of 1.5 ml min^{-1}. The analytes were detected with UV and fluorescence detection after post-column photochemical reaction. An SPD-6A UV detector (Shimadzu) operated at 254 nm was connected in series to a Beam Boost photochemical reaction unit (ICT, Frankfurt, Germany) connected to a fluorescence detector (RF-551, Shimadzu). In the photochemical reaction unit, the effluent was irradiated at 254 nm in a knitted reaction coil (10 m × 0.3 mm i.d.). The fluorescence detector was operated at 270 nm excitation wavelength and 430 nm emission wavelength. The signals were recorded on a Chromatopack C-R4A integrator (Shimadzu).

2.4. Final dialysis procedure

An aliquot of 400 μl plasma was automatically mixed with 50 μl of PRR and 150 μl of the mixture was injected into the donor channel of the dialysis cell. The six-port valve was in the load position and the sample was held static in the donor channel while 6 ml of the acceptor solution (0.001 M ammonium phosphate buffer, pH 7.0) were transported through the acceptor channel of the dialyser and into the TEC. The acceptor solution was transported through the dialyser in pulses of 175 μl at an average flow rate of 0.47 ml min^{-1}. Dialysis was performed for 12.8 min after which the six-port valve was switched to the injection position and the enriched analytes were eluted from the TEC onto the analytical column by the HPLC mobile phase. The donor side and the acceptor side of the dialyser was simultaneously washed with 5 ml of the donor solution (1 mM dodecyltrimethyl ammonium bromide in water) and acceptor solution, respectively. The precolumn was regenerated with 1 ml of the acceptor solution and the next sample was injected into donor channel of the dialysis cell.

2.5. Validation of the method

The antidepressants were determined from standard curves based on peak height measurements. For preparation of the standard curves, aliquots of 400 μl of plasma spiked with 50, 100, 250, 500, 1000, 1500 and 2000 nmol l^{-1} mianserine, amitriptyline, nortriptyline, desimipramine and imipramine were mixed with 50 μl of PRR. The plasma samples were stored at −20°C and were analysed within 1 week. Plasma samples spiked with 100, 1000 and 1500 nmol l^{-1} of the drugs were analysed for within- and between-day validation data ($n = 6$). The limit of detection was determined at a signal-to-noise ratio of 3 ($S/N = 3$).

3. Experimental design and optimisation

The chain length of surfactant added to the donor solution (C12, dodecyltrimethyl ammonium bromide; C14, tetradecyltrimethyl ammonium bromide; and C16, hexadecyltrimethyl ammonium bromide), the concentration of surfactant (0.02, 1.01 and 2 mM), the pH of the donor solution (2.5, 4.75 and 7.0) and the volume of the acceptor solution (2, 6 and 10 ml) were included as variables in the screening design. A fractional factorial design with centre-points (central composite face centred) was used to examine the

Table 1
The two variable experimental design used for the response surface optimisation experiment

Variable	Low (−1)	Medium (0)	High (+1)
1. Concentration of surfactant (mM)	0.5	1.0	1.5
2. Volume of acceptor (ml)	4	6	8

Exp No.	Variable	
	1	2
1	−1	−1
2	+1	−1
3	−1	+1
4	+1	+1
5	−1	0
6	+1	0
7	0	−1
8	0	+1
9	0	0
10	0	0
11	0	0

influence of these variables on the dialysis recovery after performing 23 experiments.

The most significant factors (volume of the acceptor solution and the concentration of the surfactant in the donor solution) from the screening were selected for further optimisation. The factor levels which showed to be optimal in the screening experiments were set as centre-point values in the optimisation experiment based on response surface modelling (RSM). The RSM design with levels of the factors is listed in Table 1. The experiment was performed with one replicate. Multiple linear regression analysis was used to determine if linear, quadratic or interaction terms of the factors were significant for the effect. Both the screening and the optimisation experiment were carried out in a randomised order.

The dialysis recovery for the model compounds was used as the response variable in all experiments. For each of the drugs, a second-order regression model (Eq. (1)) was developed by means of multiple linear regression:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_1^2 + b_4x_2^2 + b_5x_1x_2 \qquad (1)$$

where Y is the dialysis recovery, $b_0 \ldots b_5$ represents the regression coefficients, and x_1 and x_2 are the coded levels for the concentration of the surfactant and the volume of acceptor in Table 1. Non-significant regression coefficients ($\alpha = 0.05$) were excluded from the models. The programme MODDE version 3.0 from Umetri AB (Umeå, Sweden) was used for design and evaluation of the chemometric studies.

4. Results and discussion

4.1. Preliminary screening of dialysis conditions

The two major problems which must be solved in order to develop methods based on dialysis for determination of the model compounds in plasma are inhibition of protein binding and analyte–membrane interactions. Protein releasing reagent (PRR) was added to the plasma samples prior to dialysis. The pH of the plasma–PRR mixture was 2.5. When the plasma–PRR mixtures were analysed by the ASTED™ system, the dialysis recoveries of the analytes were the same as those obtained when aqueous solutions of the analytes were dialysed. This experiment showed that drug–protein interactions were completely inhib-

ited in the plasma–PRR mixture. However, the dialysis recoveries were only 11–28%. Without interaction with the membrane, dialysis recoveries in the range 50–60% were expected for these drugs. This clearly demonstrates the need for finding a common approach for inhibition of interactions with hydrophobic basic drugs and the dialysis membrane.

Both electrostatic and hydrophobic interactions with basic hydrophobic drugs and the membrane have been reported [10,11,14]. The pK_a values of the amino groups on the antidepressant drugs range from 9 to 10, implying that they are positively charged in plasma. The electrostatic interaction with negatively charged groups on the membrane is probably caused by incomplete acetylation of the hydroxyl groups. In this study, the pH of the sample was 2.5. At this pH value, acidic groups on the membrane are protonated and ionic analyte–membrane interactions are less important. However, both electrostatic and hydrophobic interactions can be inhibited by cationic surfactants added to the donor solution. Cationic surfactants interact with negative and hydrophobic binding sites on the membrane. By equilibrating the dialysis membrane with a donor solution containing a sufficiently high concentration of surfactant before a sample is dialysed, analyte–membane interactions may be inhibited. In this investigation, the membrane was washed and equilibrated between each sample with 5 ml of a donor solution to which a cationic surfactant was added.

Multiple linear regression analysis of the screening experiment showed significant terms for the concentration of the surfactant and the volume of the acceptor. This was valid for all the model compounds. The statistical analysis of a model containing the volume of acceptor, concentration of surfactant, the quadratic term of volume of acceptor and the interaction term volume of acceptor/concentration of surfactant, gave R^2 values above 0.9 for all the compounds. The models were significant for all the experiments ($P < 0.001$).

The results from the screening concluded that neither the chain length of the surfactant added to the donor solution, nor the pH of the acceptor solution, had a significant effect on the dialysis recovery. Cationic surfactants with chain lengths C12–C16 can therefore be added to the donor solution to inhibit analyte–membrane interactions. Since the pH of the donor did not influence the dialysis recovery, the surfactant was dissolved in water in the final method.

Increasing the concentration of the surfactant from 0.02 to 2 mM was found to be highly significant ($P < 0.001$) for the dialysis recoveries. Under the conditions used, the effect showed a quadratic term implying that there will be an optimum surfactant concentration. High concentrations of surfactant give breakthrough of the analytes on the TEC. The surfactant diffuses through the dialysis membrane into the acceptor solution and the TEC will be overloaded.

Varying the volume of acceptor solution from 2 to 10 ml was also significant for the dialysis recovery. This effect was investigated by keeping the flow rate of the acceptor solution (0.47 ml min^{-1}) and the dialysis time constant. The volume of the acceptor solution affects the concentration gradient over the membrane. Increasing the acceptor solution volume ensures a high concentration gradient of the analytes during dialysis. The terms were both linear and quadratic, indicating that there must be an optimal acceptor solution volume which can be explained by breakthrough of the analytes at high volumes of acceptor solution.

4.2. Optimisation using response surface modelling (RSM)

The acceptor volume and the concentration of the surfactant were selected for further optimisation by RSM. The values which were shown to be optimal in the screening experiment were set as centre-points. A central composite face-centred design was chosen for the RSM experiments in Table 1 [20]. The surfactant concentration varied from 0.5 to 1.5 mM and the volume of the acceptor solution from 4 to 8 ml. A summary of the statistical evaluation is shown in Table 2. The interaction term was found to be non-significant according to analysis of variance (ANOVA) and was eliminated from the models. The most impor-

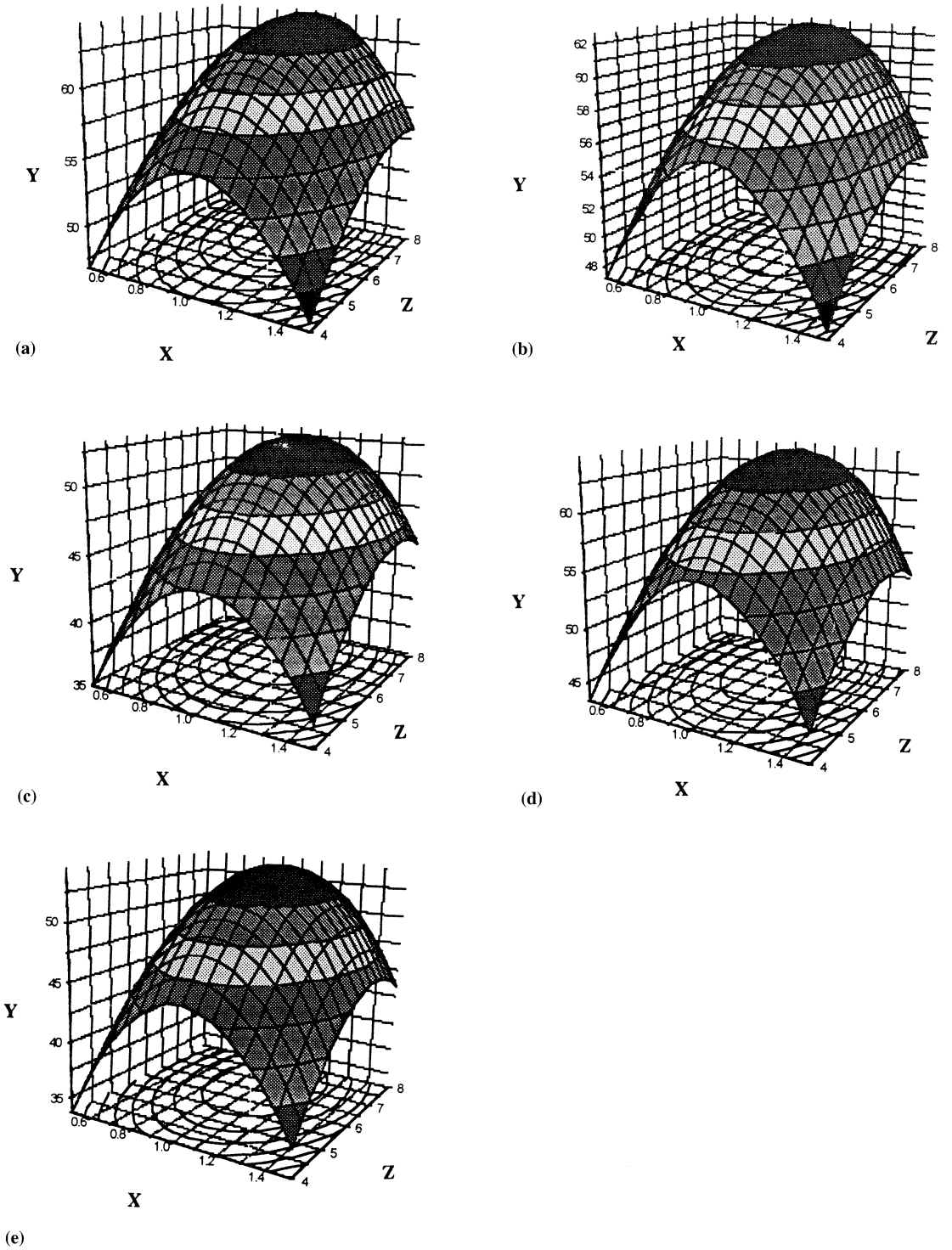

Fig. 1. Response surfaces according to the central composite face-centred design showing *Y*, the dialysis recovery (%), as a function of *X*, the concentration of surfactant in the donor solution (mM), and *Z*, the volume of the acceptor solution (ml). Three-dimensional contour plots. Experimental conditions: see Table 1. A, Mianserine; B, imipramine; C, desimipramine; D, amitriptyline; E, nortriptyline.

Table 2
Predicting equations for the dialysis recovery of the model drugs based on coded influence variables

Drug	Equation	R^2	Q^2	P_{reg}	$P_{lack\ of\ fit}$
Mianserine	$62.8+0.2x_1+4.3x_2-8.6x_1^2-3.0x_2^2$	0.996	0.988	<0.001	0.833
Amitriptyline	$63.9+1.2x_1+3.7x_2-11.5x_1^2-4.3x_2^2$	0.992	0.975	<0.001	0.639
Nortriptyline	$53.6+1.1x_1+3.8x_2-10.3x_1^2-4.8x_2^2$	0.960	0.856	<0.001	0.517
Imipramine	$61.8-0.2x_1+3.0x_2-8.7x_1^2-2.8x_2^2$	0.944	0.807	<0.001	0.425
Desimipramine	$52.1-0.3x_1+4.0x_2-8.4x_1^2-4.1x_2^2$	0.954	0.867	<0.001	0.768

The fraction of variation of the response explained by the model (R^2), the fraction of variation of the response predicted by the model (Q^2) and the P-values for regression and lack of fit obtained in the ANOVA for the second-order models are also shown. All models were significant ($\alpha = 0.05$). x_1, Concentration of the surfactant; x_2, volume of acceptor solution.

tant terms for all the models were the linear and quadratic terms of the volume of acceptor solution and the quadratic term of the concentration of the surfactant in the donor. The linear term of the concentration of the surfactant was not significant. However, it was kept in the model in order to enforce hierarchy of the model terms, since a higher order term of this factor was still in the model. The multiple linear regression analysis gave an acceptable summary of fit $R^2 > 0.944$ and $Q^2 > 0.807$. All models were highly significant ($P < 0.001$).

The three-dimensional contour plots constructed for the dialysis recoveries of the model compounds are shown in Fig. 1. These plots show the predicted values for the dialysis recoveries at various combinations of variables. From the response surface plots, it can be concluded that an optimum dialysis recovery of 52–65% is obtained for all model compounds when dialysis is performed at intermediate levels of the two factors. In the final method for determination of the model compounds in plasma, dialysis was performed with an acceptor solution of 6 ml. Between analysis, the dialysis membrane was washed and equilibrated with 5 ml donor solution containing a cationic surfactant at a concentration of 1 mM for inhibition of the interaction with the analytes and the dialysis membrane.

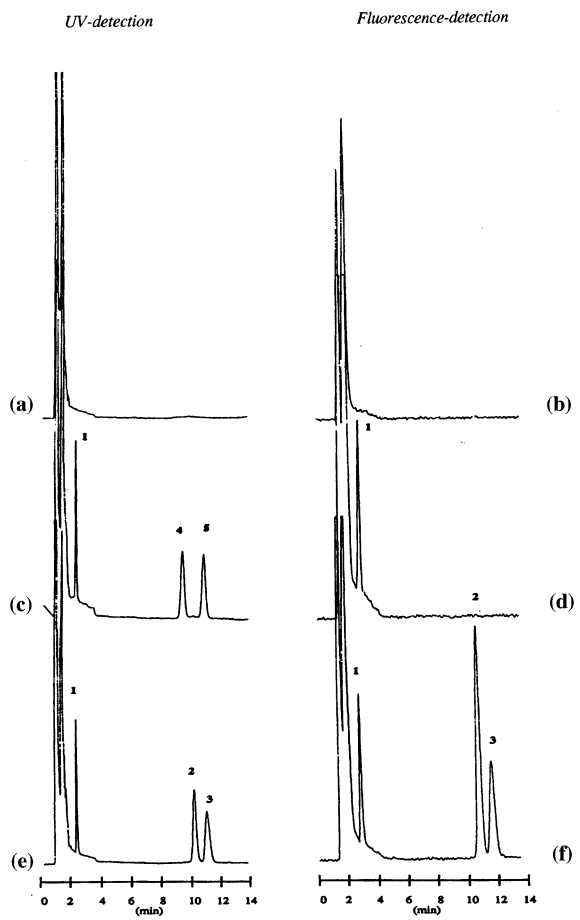

Fig. 2. Chromatograms of a drug-free human plasma sample ((A) and (B)), a plasma sample spiked with 1000 nmol l^{-1} mianserine, amitriptyline and nortriptyline ((C) and (D)), and a plasma sample spiked with 1000 nmol l^{-1} of mianserine, imipramine and desimipramine ((E) and (F)). UV detection: (A), (C) and (E). Fluorescence detection after post-column photochemical derivatisation: (B), (D) and (F). For experimental conditions, see text. Peaks: 1, mianserine; 2, imipramine; 3, desimipramine; 4, amitriptyline; 5, nortriptyline.

Table 3
Within-day validation data for the determination of mianserine, desimipramine, imipramine, amitriptyline and nortriptyline in human plasma determined with ASTED dialysis and HPLC

Added conc. (nmol l^{-1})	Measured conc. (mean ± S.D., $n = 6$) (nmol l^{-1})	R.S.D (%)	Accuracy (%)
Mianserine			
UV detection			
100	102 ± 4	4.4	102.1
1000	1001 ± 36	3.6	100.1
1500	1466 ± 27	1.8	97.7
Fluorescent detection			
100	101 ± 4	3.6	100.6
1000	991 ± 26	2.6	99.1
1500	1540 ± 34	2.2	102.6
Imipramine			
UV detection			
100	101 ± 5	5	100.8
1000	1017 ± 35	3.4	101.7
1500	1480 ± 44	2.9	98.7
Fluorescent detection			
100	102 ± 6	5.7	101.7
1000	1010 ± 14	1.4	101.0
1500	1455 ± 26	1.8	97.0
Desimipramine			
UV detection			
100	104 ± 2	1.8	104.1
1000	1001 ± 34	3.4	100.1
1500	1483 ± 42	2.8	98.8
Fluorescent detection			
100	101 ± 2	2.4	101.4
1000	1010 ± 37	3.7	101.0
1500	1474 ± 49	3.3	98.3
Amitriptyline			
UV detection			
100	102 ± 3	3.3	101.8
1000	999 ± 30	3.0	99.9
1500	1516 ± 34	2.2	101.1
Nortriptyline			
UV detection			
100	106 ± 3	2.9	105.8
1000	992 ± 32	3.2	99.1
1500	1490 ± 39	2.6	99.3

S.D., standard deviation; R.S.D., relative standard deviation.

4.3. HPLC analysis and detection

The model compounds were separated in less than 13 min on a cyano-column. The mobile phase was an acetonitrile–methanol–phosphate buffer mixture with 85% organic modifier. Initially, a modified method with acetonitrile–phosphate buffer was used as mobile phase [21]. However, serious baseline disturbances were observed when the ASTED™ was connected to the HPLC system. Addition of methanol to the mobile phase eliminated these problems. In this system, amitryptiline/imipramine and nortryptiline/desimipramine coeluted. These analytes were,

Table 4
Between-day validation data for the determination of mianserine, desimipramine, imipramine, amitriptyline and nortriptyline in human plasma determined with ASTED dialysis and HPLC

Added conc. (nmol l^{-1})	Measured conc. (mean ± S.D., $n = 6$) (nmol l^{-1})	R.S.D (%)	Accuracy (%)
Mianserine			
UV detection			
100	99 ± 4	3.7	98.7
1000	1040 ± 12	1.1	104.0
1500	1478 ± 31	2.1	98.6
Fluorescent detection			
100	98 ± 3	2.6	97.9
1000	1026 ± 11	1.1	102.6
1500	1477 ± 28	1.9	98.5
Imipramine			
UV detection			
100	97 ± 5	4.7	97.4
1000	1052 ± 31	2.9	105.2
1500	1478 ± 31	2.1	98.6
Fluorescent detection			
100	103 ± 4	2.8	102.8
1000	1020 ± 18	1.7	102.0
1500	1484 ± 37	2.5	98.9
Desimipramine			
UV detection			
100	103 ± 5	5.5	103.3
1000	1044 ± 25	2.4	104.4
1500	1483 ± 32	2.2	98.9
Fluorescent detection			
100	99 ± 4	3.1	98.6
1000	1053 ± 27	2.6	105.3
1500	1564 ± 26	1.7	104.3
Amitriptyline			
UV detection			
100	103 ± 4	4.1	103.1
1000	1002 ± 42	4.8	101.5
1500	1567 ± 63	4.0	104.4
Nortriptyline			
UV detection			
100	104 ± 6	5.6	103.9
1000	992 ± 37	3.7	99.2
1500	1516 ± 69	4.6	101.1

S.D., standard deviation; R.S.D., relative standard deviation.

however, easily distinguished by the dual detection system employed. The analytes were simultaneously detected with UV and fluorescence detection after post-column photochemical derivatisation. It is known that irradiation with UV light converts weakly or non-fluorescent analytes into reaction products which are highly fluorescent [22–28]. In this study, fluorescent products of mianserine, imipramine and desimipramine were formed upon UV irradiation, which enabled specific detection of these compounds by fluorescence detection as shown in Fig. 2. The irradiation time was optimised by varying the length of the reaction coil in the Beam Boost reactor between 5 and 20 m. Optimal fluorescence was obtained in a 10 m reactor coil. These conditions were used in the final method.

4.4. Validation of the method

Validation of the method was carried out with two mixtures of the test compounds added to drug-free plasma. One mixture contained mianserine, imipramine and desimipramine and the other mixture contained mianserine, amitriptyline and nortriptyline. The standard curves for mianserine, imipramine, desimipramine, amitriptyline and nortriptyline were linear in the concentration range 50–2000 nmol l^{-1} with correlation coefficients in the range 0.997–0.999. The within- and between-day validation data of the procedure are shown in Tables 3 and 4. The relative standard deviations were 1.1–5.7%. The limit of detection in human plasma with UV detection was 17, 29, 40, 29 and 39 nmol l^{-1} for mianserine, amitriptyline, nortriptyline, imipramine and desimipramine, respectively. Correspondingly, the limit of detection for mianserine, imipramine and desimipramine with the fluorescence detection were 6, 4 and 17 nmol l^{-1}, respectively.

5. Conclusion

Response surface modelling was successfully used to optimise dialysis recoveries of antidepressant drugs. The optimal conditions for dialysis of antidepressants were found when a donor solution containing a cationic surfactant at a concentration of 1 mM was used to wash and equilibrate the dialysis membrane between analysis. Dialysis of plasma samples were performed with an acceptor solution volume of 6 ml. Varying the pH of the donor solution from 2.5 to 7.0, and the chain length of the surfactant from C12 to C16, had no significant effect on the dialysis recovery. Automated on-line dialysis, trace enrichment and HPLC was shown to be an accurate and reproducible method for the determination of the antidepressant drugs in human plasma.

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