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Automated on-line dialysis, trace enrichment and high-performance liquid chromatography Inhibition of interaction with the dialysis membrane and disruption of protein binding in the determination of clozapine in human plasma

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Abstract

Problems related to interaction of drugs with the dialysis membrane and to protein binding must be overcome in order to develop automated methods for drug analysis based on on-line dialysis, trace enrichment and HPLC. In order to study these problems, clozapine and its active metabolite N-desmethylclozapine were chosen as model compounds because they were found to interact with the dialysis membrane, and clozapine is highly protein bound. Addition of a cationic surfactant, dodecylethyldimethyl ammonium bromide, to the donor solution and to the plasma samples was found to inhibit interaction of the drugs with surfaces. The protein binding in plasma was disrupted prior to dialysis by lowering the pH with hydrochloric acid and the plasma proteins were solubilised with glycerol. The results obtained were used to develop a fully automated method for the determination of clozapine and N-desmethylclozapine in human plasma. More than 100 samples could be analysed within 24 h. The limit of detection in human plasma was 0.050 μmol/l for clozapine and 0.055 μmol/l for N-desmethylclozapine. Linearity was found for drug concentrations between 0.25–3 μmol/l. The relative standard deviations were between 1.2–6.7% and the method was applicable for therapeutic drug monitoring.

Keywords: Protein binding; Membrane interaction; Clozapine; N-Desmethylclozapine; Proteins

1. Introduction

Automated sequential trace enrichment of dialysate (ASTED) combined on-line with high-performance liquid chromatography (HPLC), has been successfully used to analyse a variety of drugs in biomedical samples [1–11]. The technique allows the automated removal of proteins, as only low molecular mass compounds diffuse through the pores of the semi-permeable dialysis membrane. The sample dilution caused by the dialysis step is overcome by trace enrichment of the analytes on a trace enrichment column (TEC). The use of moving acceptor and/or donor phases combined with a suitable, selective trace enrichment step results in a rapid and efficient clean-up plus enrichment. When ASTED is combined on-line with HPLC, fully automated analysis of drugs in plasma with a sample

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throughput of more than 100 samples in 24 h can be performed [12].

A problem commonly encountered in the dialysis of plasma samples is a decrease in recovery caused by drug–protein binding, as only the non-protein bound drug is able to diffuse through the dialysis membrane. Releasing the drugs from their protein binding sites is necessary in order to increase the analyte recovery. Earlier reports have described different approaches to disrupt the analyte–protein binding prior to dialysis such as addition of displacers and alteration of the pH [7–10,13].

Another potential problem is a decrease in dialysis recovery due to interaction of drugs with the dialysis membrane. Interactions between the analytes and the cellulose acetate membrane have been reported for basic drugs such as the opiate derivative pholcodine and the benzodiazepines [10,11]. Both electrostatic and hydrophobic interactions with the analytes and the dialysis membrane have been reported [10,14]. Positively charged drugs may interact electrostatically with the negatively charged surface of the cellulose acetate membrane. The negative charge on the membrane surface is probably due to incomplete acetylation of the hydroxyl groups. The interaction of drugs with the cellulose acetate membrane must be eliminated in order to develop methods with high analyte recovery, high reproducibility and high sensitivity.

Clozapine is an antipsychotic drug extensively used in the treatment of schizophrenic disorders. Several methods have been published for the determination of clozapine in plasma. Most of these methods are based on HPLC with ultraviolet [15–22] or electrochemical detection [23] after sample clean-up with liquid–liquid extraction [15–18,23] or solid-phase extraction [19–22]. Methods based on gas chromatography with nitrogen-specific detection [24,25] or mass spectrometric detection [26] and radio-immunoassay [27] have also been reported for the determination of clozapine in plasma.

Clozapine and its major metabolite N-desmethylclozapine were chosen as model compounds in this work to study approaches to inhibit interaction between the analytes and the dialysis membrane, and additionally to disrupt drug–protein binding prior to dialysis. These analytes were selected because they were found to interact strongly with the dialysis membrane. In addition, clozapine is highly bound to plasma proteins (95%). The structures of clozapine and its metabolite N-desmethylclozapine are shown in Fig. 1. The aim of this study was to develop a fully automated method for the determination of clozapine and its metabolite N-desmethylclozapine in human plasma based on on-line dialysis, trace enrichment and HPLC.

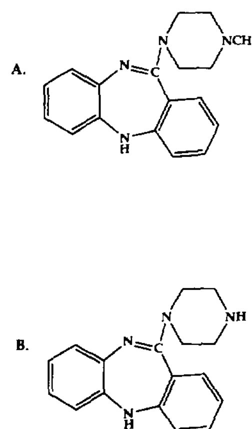


Fig. 1. Structure of clozapine (A) and N-desmethylclozapine (B).

2. Experimental

2.1. Chemicals and reagents

Clozapine and N-desmethylclozapine were supplied by Sandoz Pharma (Basel, Switzerland). Acetonitrile and methanol were supplied by Rathburn (Walkerburn, UK). Dodecylethyldimethyl ammonium bromide (DEDAB) and diethyl amine (DEA) were obtained from Fluka (Buchs, Switzerland). Tetrabutyl ammonium bromide (TBAB) and piperazine dihydrochloride (PIP) were purchased from Sigma (St. Louis, MO, USA). Glycerol was obtained from Norsk Medisinal Depot (Oslo, Norway). Acetic acid p.a., ammonium acetate p.a., hydrochloric acid and sodium hydroxide were supplied by Merck (Darmstadt, Germany). HPLC-grade

water was prepared from a Milli-Q water purification system (Millipore, MA, USA).

2.2. Equipment

The sample preparation system was an ASTED unit (Gilson, Villiers-le Bel, France) (Fig. 2) consisting of a 231 autosampling injector equipped with two 401 dilutors fitted with 1-ml syringes and an automated six-port valve (Rheodyne, Berkley, CA, USA). The dialysis cell was made of polymethylacrylate, with a donor and acceptor channel volume of 100 and 175 μl, respectively. The donor and acceptor channel were separated by a Gilson Cuprophane membrane (cellulose acetate) with a molecular mass cut-off of 15 000. For trace enrichment, a 10×2 mm I.D. stainless steel precolumn packed with 40 μm BondElut C_{18} particles (Varian, Harbor City, CA, USA) was used. The chromatographic system consisted of an LC-6A HPLC pump (Shimadzu, Kyoto, Japan) and an SPD-6A UV detector (Shimadzu) operated at 254 nm. Peak-heights were recorded on a Chromatopack C-R4A integrator (Shimadzu). The analytical column was a Brownlee cyanopropyl column (100×4.6 mm I.D., 5 μm particles) from Applied Biosystems (San Jose, CA, USA). Acetonitrile–0.05 *M* ammonium acetate buffer (pH 3.2) (22:78, v/v) was used as the HPLC mobile phase at a flow-rate of 1.0 ml/min. All assays were performed at room temperature.

2.3. Standard solutions

Stock standard solutions of clozapine and N-desmethylclozapine (2 mmol/l) were prepared in 10 m*M* methanolic solutions of DEDAB. The protein releasing reagent was an aqueous solution consisting of 1 *M* HCl, 20 m*M* DEDAB and 50% (v/v) glycerol. Citrated drug free plasma from healthy donors was obtained from The Blood Centre at Ullevaal Hospital (Oslo, Norway). Plasma samples spiked with clozapine and desmethylclozapine

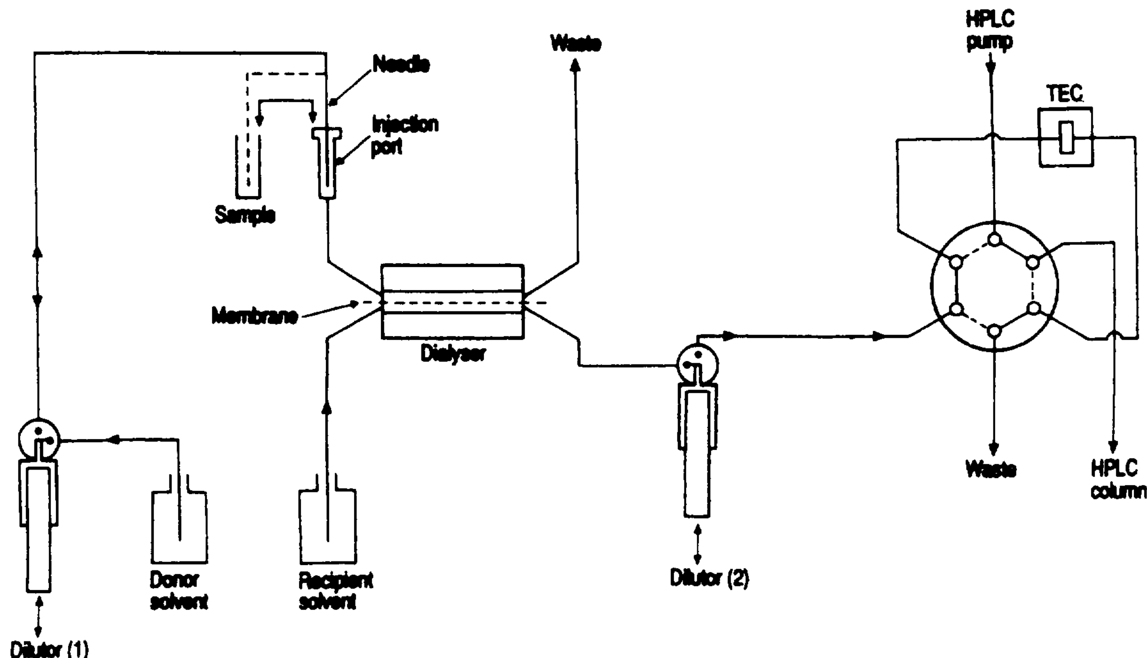


Fig. 2. Schematic representation of the on-line dialysis–HPLC system. The sample is introduced by dilutor 1 and the acceptor phase is pumped by dilutor 2.

(0.25–3 μmol/l) were prepared by mixing drug free plasma with aliquots of the stock standard solutions.

2.4. Interaction with the dialysis membrane

Working standard solutions of clozapine and desmethylclozapine (1 μmol/l) were prepared in 1 and 10 m*M* aqueous solutions of DEDAB, DEA, TBAB and PIP adjusted to pH 2.5 and 7.0 by the addition of either 0.5 *M* NaOH or 0.5 *M* HCl. All solutions were prepared in borosilicate vials and were allowed to equilibrate for 24 h. Samples (20 μl) were withdrawn from the solutions to determine the "zero" concentration values. A cellulose acetate membrane (21 cm^2) was placed in the equilibrated solutions (5 ml) and samples (20 μl) were withdrawn after 2, 8.5, 15 and 30 min. All samples were analysed by HPLC and the peak heights of clozapine and N-desmethylclozapine were recorded. The extent of interaction of clozapine and N-desmethylclozapine with the cellulose acetate membrane was determined on the basis of their peak heights compared with the corresponding "zero" values.

2.5. Automated analysis of plasma samples

2.5.1. Sample loading

An aliquot of 400 μl plasma sample was mixed with 50 μl of the protein releasing reagent and 150 μl of the mixture was injected into the donor channel of the dialysis cell by dilutor 1. The six-port valve was held in the load position.

2.5.2. Dialysis and trace enrichment

The sample was held stagnant in the donor channel while the other dilutor pulsed 4 ml of the acceptor solution (0.002 *M* ammonium acetate buffer, pH 4.0) through the acceptor channel of the dialyser and into the trace enrichment column (TEC). The acceptor solution was transported through the dialyser in pulses of 175 μl, corresponding to an average flow-rate of 0.47 ml/min. The analytes were retained on the TEC while impurities were washed out to waste.

2.5.3. Elution and purging

Upon switching the six-port valve to the injection position, the enriched analytes were eluted from the TEC by the HPLC mobile phase and into the analytical column for separation. The donor side of the dialyser was washed with 5 ml of donor solution (1 m*M* DEDAB). The acceptor side was simultaneously washed with 5 ml of acceptor solution (0.002 *M* ammonium acetate buffer, pH 4.0).

2.5.4. Regeneration

The six-port valve was switched back to the load position to connect the TEC with the recipient channel of the dialyser. The trace enrichment column was regenerated with 1 ml of acceptor solution and the next sample was injected into the dialyser.

2.6. Validation of the method

For preparation of the standard curves, an aliquot of 400 μl of plasma spiked with 0.25, 0.5, 1, 2 and 3 μmol/l of clozapine and its metabolite were mixed with 50 μl of the protein releasing reagent. The plasma samples were stored at −20°C prior to analysis and were analysed within one week. The standard curves for clozapine and N-desmethylclozapine were based on peak-height measurements. Plasma samples spiked with 0.25, 1 and 2 μmol/l of clozapine and N-desmethylclozapine were analysed ($n=6$) for within- and between-day validation data. The limit of detection was determined at a signal-to-noise ratio of 3 ($S/N=3$).

3. Results and discussion

Clozapine is a hydrophobic base and it is partly positively charged in plasma ($pK_{a1}=3.7$, $pK_{a2}=7.6$). It may interact with surfaces by ionic and/or hydrophobic forces. One approach to overcome interaction is the protonation of the negative surface charges by adjustment of the pH. The limited pH stability of cellulose acetate (pH 2–8) is a disadvantage in this respect. Another approach is to inhibit interaction by addition of displacers, such as positively charged surface active substances. Positively charged surface active substances may inhibit both ionic and hydrophobic interactions between the analytes and the membrane. Both approaches were investigated in this study.

In order to reduce the protein binding of clozapine

and its metabolite prior to dialysis, lowering the pH in plasma was investigated as well as methods to solubilise plasma proteins after alteration of the pH.

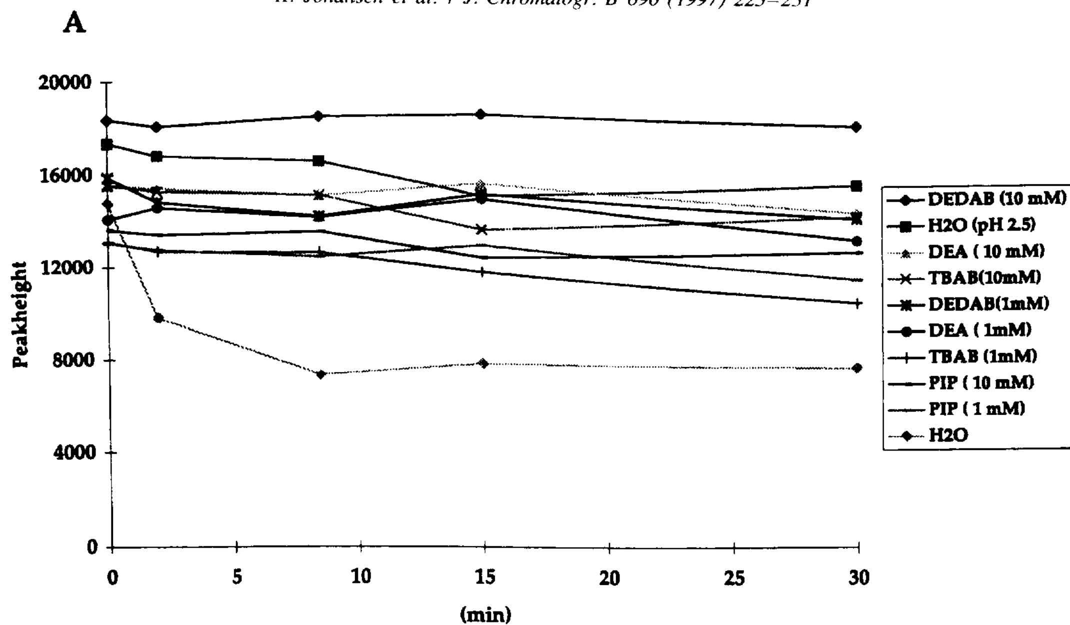
3.1. Interaction with the dialysis membrane

The interaction of clozapine and its metabolite with the dialysis membrane was studied in pure water and in aqueous solutions adjusted to pH 2.5 and 7, to which were added different concentrations of positively charged surface active substances. The positively charged substances investigated were the cationic surfactant, dodecylethyldimethyl ammonium bromide (DEDAB), the quarternary ammonium compound tetrabutyl ammonium bromide (TBAB), the secondary amine diethyl amine (DEA) and the heterocyclic amine piperazine dihydrochloride (PIP). The peak-heights of clozapine and its metabolite plotted vs. time, after placing the dialysis membrane in the solutions, are shown in Fig. 3. A 55% reduction in peak-heights was observed after 30 min when the dialysis membrane was placed in pure aqueous solutions of clozapine and its metabolite, showing the strong interaction of these substances with the membrane. A 10% reduction in peak heights was found after 30 min of storage when the membrane was placed in acidic solution at pH 2.5. Ionic interactions are reduced at pH 2.5 because acidic groups on the membrane are protonated. However, clozapine and its metabolite may also interact with the membrane by hydrophobic forces. Hydrophobic interactions have been reported for another group of hydrophobic bases, the benzodiazepines [10]. The addition of cationic substances to aqueous solutions at pH 7 reduced the adsorption, as shown in Fig. 3. The most effective substance was DEDAB. No reduction in peak-height was observed for clozapine or N-desmethylclozapine when the membrane was placed in standard solutions to which was added 10 m*M* DEDAB. Although both TBAB and DEDAB are quaternary ammonium salts, the substantially higher recoveries obtained by use of the markedly more hydrophobic DEDAB imply that hydrophobic forces may prevail in the interaction, in addition to ionic forces. The concentration of DEDAB was important. The addition of DEDAB in a concentration of 1 m*M* did not completely eliminate the interaction. Addition of DEDAB in a concentration exceeding its critical micelle concentration (cmc=14 m*M*) decreased its effect to inhibit the interaction.

The different peak heights observed prior to placing the membrane in the solutions (zero on the *x*-axis in Fig. 3) indicate that both clozapine and N-desmethylclozapine interacted with the surface of the glass vials. The studies on interaction with the dialysis membrane were therefore carried out in solutions which had been equilibrated in glass vials for 24 h. A 20% reduction in peak-height was observed when pure aqueous solutions of clozapine and N-desmethylclozapine were stored in glass vials for 24 h, as compared to standard solutions to which was added 10 m*M* DEDAB. Therefore, 10 m*M* DEDAB was added to stock standard solutions of clozapine and N-desmethylclozapine to inhibit interaction with the glass surfaces.

3.2. Dialysis of aqueous standards.

To inhibit interaction between clozapine and its metabolite with the dialysis membrane in automated on-line dialysis, DEDAB was added to the donor and the acceptor solutions. Dialysis was performed in the static-pulsed mode i.e. the sample solution was held static in the donor channel while the acceptor solution was pumped in pulses of 175 μl through the acceptor channel. DEDAB added to the acceptor solution in a concentration of 10 m*M* caused breakthrough of the analytes from the TEC. As DEDAB is able to diffuse through the dialysis membrane and into the acceptor solution, a high concentration of DEDAB (10 m*M*) in the donor solution also caused breakthrough of the analytes from the TEC. No breakthrough from the TEC was observed after addition of 1 m*M* DEDAB to the donor solution. The dialysis recoveries were 45–50% for clozapine and N-desmethylclozapine when 4 ml of acceptor solution (0.002 *M* ammonium acetate buffer, pH 4.0) was pumped through the acceptor channel with a total dialysis time of 8.5 min. No significant increase in dialysis recovery was observed when the acceptor volume was increased from 4 to 10 ml. Similar recoveries have been obtained for other drugs which have been analysed by similar dialysis procedures [12]. In the final set-up, dialysis was performed with a donor solution consisting of 1 m*M* DEDAB in water. The membrane was washed and equilibrated



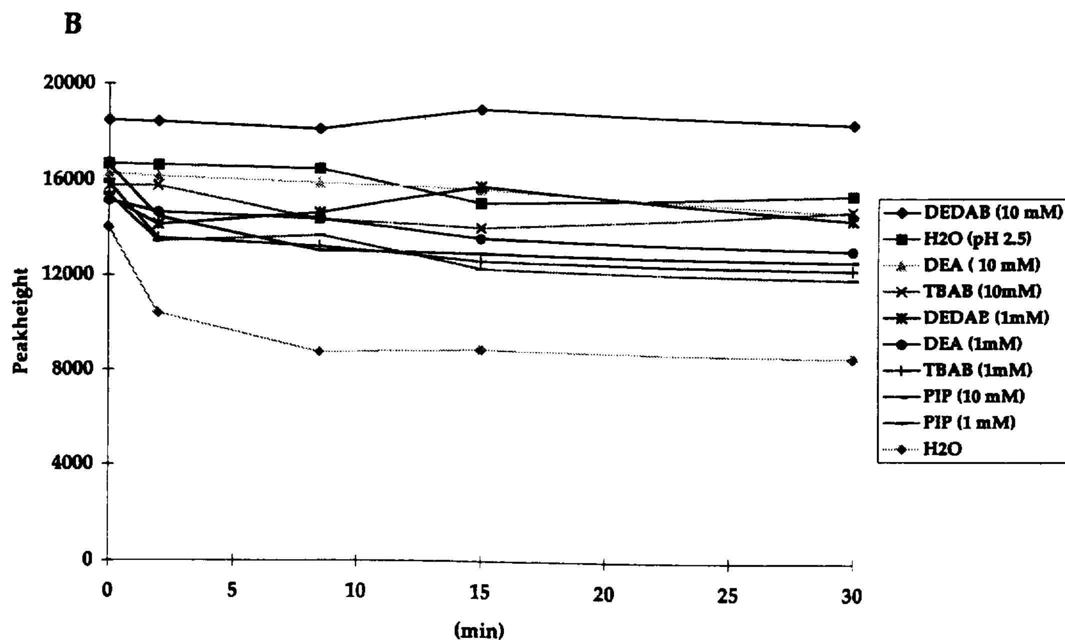


Fig. 3. Peak-heights of N-desmethylclozapine (A) and clozapine (B) plotted vs. time after placing a dialysis membrane into the solutions.

with 5 ml of this solution between the analyses. No memory effects between the analyses were observed.

3.3. Dialysis of plasma samples

Chromatograms from the analysis of clozapine and N-desmethylclozapine in spiked plasma, analysed using different procedures to inhibit interaction with the dialysis membrane and reduction of protein binding, are shown in Fig. 4. Chromatogram A shows dialysis of a plasma sample with water as donor and acceptor solution. The dialysis recoveries of clozapine and N-desmethylclozapine were low (6–8%) as no measures were taken to reduce either interaction with the dialysis membrane or drug–protein binding. Chromatogram B shows dialysis of a plasma sample when 1 m*M* DEDAB was used as donor solution and water as acceptor solution. The dialysis recovery increased to 20%. DEDAB was able to inhibit interaction of the drugs to the the membrane, but had little or no effect on the protein binding of clozapine and N-desmethylclozapine. Decreasing the pH of the acceptor solution may reduce the protein binding of basic drugs in plasma [11]. Decreasing the pH to 4.0 in the acceptor solution reduced the protein binding of clozapine and N-desmethylclozapine, and the dialysis recoveries increased as demonstrated by the higher peak heights observed in chromatogram C as compared to chromatogram B. When the pH value in plasma was decreased to 2.4 by addition of HCl prior to dialysis, the drugs were released from the proteins. However,

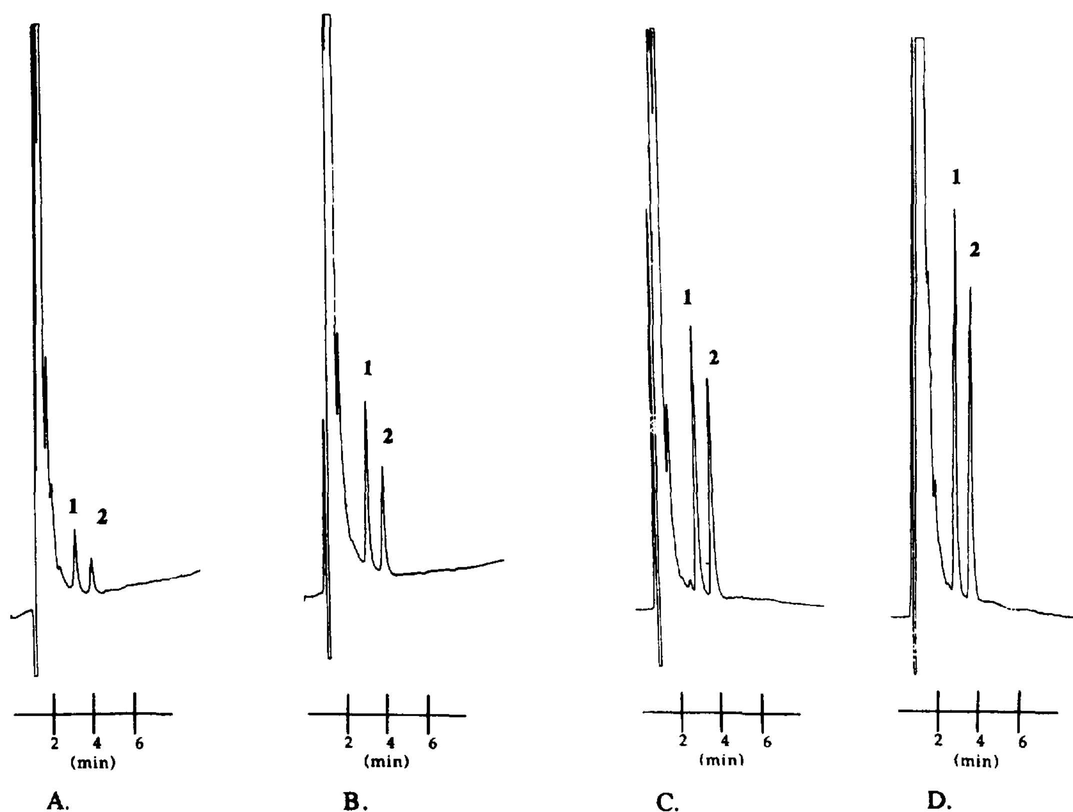


Fig. 4. Chromatograms of plasma samples spiked with 1 μmol/l of clozapine and N-desmethylclozapine after dialysis with water as donor and acceptor solution (A), dialysis with 1 m*M* DEDAB as donor solution and water as acceptor solution (B), dialysis with 1 m*M* DEDAB as donor solution and 0.002 *M* ammonium acetate (pH 4.0) as acceptor solution (C), dialysis conditions as in (C). An aliquot of 400 μl plasma was mixed with 50 μl of protein releasing reagent prior to dialysis (D). The donor channel volume (sample volume) of the dialysis cell was 100 μl. Peaks: 1=N-desmethylclozapine, 2=clozapine.

protein precipitation occurred in the sample vials. Glycerol was able to solubilise the plasma proteins. The analysis of a plasma sample after mixing the plasma sample with the protein releasing reagent consisting of HCl, glycerol and DEDAB is shown in chromatogram D. The dialysis recovery increased to 50%, which was equivalent to the recovery obtained after dialysis of aqueous solutions of clozapine and desmethylclozapine i.e. the biological matrix did not affect the dialysis recovery.

In the final procedure all plasma samples were treated in the same manner by mixing of the plasma sample with the protein releasing reagent. The protein releasing reagent consisted of HCl to release the drugs from the protein binding sites, glycerol to solubilise the plasma proteins and DEDAB to inhibit interaction of the analytes with surfaces.

The ASTED method was found to be highly reliable when applied to routine monitoring of clozapine and its metabolite in patient plasma. More than 100 samples could be automatically analysed in 24 h. The membrane was replaced after 1000 analyses and the TEC was replaced after 250 analyses. The effectiveness of on-line dialysis combined with trace enrichment for sample clean-up is demonstrated by the narrow solvent front and the clean baseline shown in chromatograms of a drug-free plasma sample and a patient plasma sample (Fig. 5).

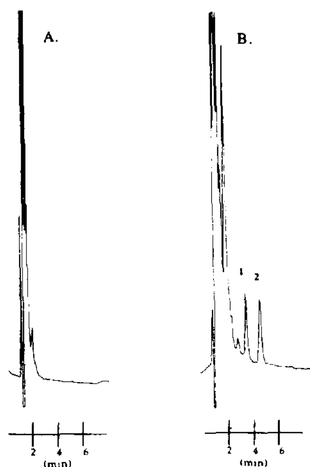


Fig. 5. Chromatograms of a drug-free human plasma (A) and a patient plasma containing 0.32 μmol/l N-desmethylclozapine (1) and 0.42 μmol/l clozapine (B). The donor channel volume (sample volume) of the dialysis cell was 100 μl. Peaks: 1=N-desmethylclozapine, 2=clozapine.

Table 1
Within- and between-day validation data for clozapine and desmethylclozapine in human plasma determined with ASTED dialysis and HPLC

Added concentration (μmol/l)	Measured concentration (mean±S.D., $n=6$) (μmol/l)	R.S.D. (%)	Accuracy (%)
Within-day variation			
Clozapine			
0.25	0.24±0.01	2.6	95.0
1.00	0.98±0.04	3.7	97.6
2.00	2.07±0.04	1.6	103.6
Desmethylclozapine			
0.25	0.25±0.01	2.8	97.8
1.00	0.98±0.03	2.6	97.5
2.00	2.07±0.04	2.2	103.4
Between-day variation			
Clozapine			
0.25	0.26±0.02	6.7	102.4
1.00	1.01±0.03	3.3	100.1
2.00	2.07±0.02	1.2	103.5
Desmethylclozapine			
0.25	0.26±0.02	6.3	105.6
1.00	1.01±0.03	3.2	100.1
2.00	2.06±0.04	1.7	103.4

S.D.=Standard deviation. R.S.D.=Relative standard deviation.

3.4. Validation of the procedure

The calibration graphs for clozapine and N-desmethylclozapine were linear in the concentration range 0.25–3 μmol/l with correlation coefficients ranging from $r=0.994$–0.998. The within- and between-day validation data of the procedure are shown in Table 1. The relative standard deviations were between 1.2–6.7% ($n=6$). The limit of detection in human plasma ($S/N=3$) was found to be 0.050 and 0.055 μmol/l for clozapine and N-desmethylclozapine, respectively. The limit of detection is dependent upon the sample size (donor channel volume). If necessary, the limit of detection can be reduced by using a dialysis cell with a larger acceptor volume or by connecting two dialysis cells in series.

4. Conclusions

This work demonstrates that automated on-line dialysis, trace enrichment and HPLC could be suc-

cessfully used to analyse drugs (clozapine and its metabolite N-desmethylclozapine) which interact strongly with the dialysis membrane and which are highly protein bound. To the donor solution was added dodecylethyldimethyl ammonium bromide (DEDAB) to inhibit interaction with the dialysis membrane. The plasma sample was mixed with a protein releasing reagent prior to dialysis. The protein releasing reagent contained HCl to release the drugs from protein binding sites, glycerol to solubilize plasma proteins and DEDAB to inhibit interaction of the analytes with surfaces. The method was found to be highly reproducible and has been successfully used in therapeutic drug monitoring of plasma samples from patients.

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