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On-line fully automated determination of clozapine and desmethylclozapine in human serum by solid-phase extraction on exchangeable cartridges and liquid chromatography using a methanol buffer mobile phase on unmodified silica

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ABSTRACT

A fully automated method for determination of clozapine and desmethylclozapine in human serum using high-performance liquid chromatography was developed. On-line solid-phase extraction was performed on an exchangeable cyanopropyl cartridge. The analytes were eluted with a methanol–ammonium acetate buffer mobile phase, separated on a silica column, and measured by ultraviolet detection at 261 nm. The total time for one analysis was 13 min. Inter-day variation was <6% and <8% for clozapine and desmethylclozapine, respectively. Detector response was linear in the range 30–3000 ng/ml. Comparison with liquid–liquid extraction showed good agreement. The patients had clozapine serum concentrations in the range 40–1500 ng/ml. Desmethylclozapine concentrations were 25% lower and closely related.

INTRODUCTION

Clozapine is a high-dose neuroleptic drug which was developed as an antidepressant *ca.* 30 years ago [1]. Its antipsychotic effect is well documented [2,3] but the drug was temporarily withdrawn from the market due to a high incidence of drug-induced agranulocytosis [4]. However, it is estimated that presently *ca.* 10% of the schizophrenic patients in Denmark receive long-term treatment with clozapine [5]. The most prominent difference between clozapine and the low-dose neuroleptic drugs is that clozapine does not induce extrapyramidal side effects [2,3]. The most common side effects of clozapine are sedation, orthostatic hypotension and hypersalivation [2,3] but convulsions have also been reported in patients with serum concentrations above 1000 ng/ml [6].

Therapeutic drug monitoring (TDM) of neuroleptic drugs as an aid in the treatment of psychotic states is routinely used at this hospital. A therapeutic range for the minimum serum concentrations of clozapine has not yet been established although it has been shown [7] that the number of patients responding to the treatment

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was three times higher when the serum concentration was above 350 ng/ml compared to patients with serum concentrations below this level. The therapeutic threshold of *ca.* 350 ng/ml for serum concentrations of clozapine has, however, been questioned [8]. An ongoing study at this hospital aims at finding the lower limit for the therapeutically effective serum concentration of clozapine.

Most methods for routine determination of clozapine in human serum published in recent years are based on high-performance liquid chromatography (HPLC) and ultraviolet (UV) or electrochemical detection after liquid–liquid extraction [9–11]. Solid-phase extraction has also been used [12] and recently a method for automated solid-phase extraction of clozapine and metabolites was described [13]. After extraction, the eluate was evaporated to dryness and the residue dissolved in mobile phase and transferred to an autosampler.

On-line determination of the serum concentration in patients treated with low-dose neuroleptic drugs is difficult because it requires quantitation of serum concentrations below 0.5 ng/ml. The therapeutic serum levels of clozapine reported in the literature [13–15] and our own studies show that the serum concentrations are normally above 30 ng/ml which makes it possible to use on-line determination with UV detection. The main metabolites of clozapine in serum are desmethylclozapine and clozapine N-oxide [16]. Our aim was to also quantitate desmethylclozapine because this metabolite may be more toxic to bone marrow than clozapine [17].

The present paper describes a fully automated method for determination of clozapine and desmethylclozapine in human serum using on-line solid-phase extraction on exchangeable cartridges. The analytes are eluted from the cartridge with a methanol buffer mobile phase and separated on an unmodified silicagel column. Measurements were made by UV detection. The method is compared with our previously used liquid–liquid extraction method. The automated method is now in use for determination of clozapine and desmethylclozapine in serum from psychiatric patients referred to our TDM service.

EXPERIMENTAL

Chemicals

Clozapine, 8-chloro-11-(4′-methyl)piperazino-5-dibenzo[(*b*,*e*)]-1-4-diazepine, N-desmethylclozapine and clozapine N-oxide as pure substances were donated by Sandoz (Basel, Switzerland). Trifluoperazine was a gift from Rhône-Poulenc Rorer (Copenhagen, Denmark) and imipramine a gift from Ciba-Geigy (Basel, Switzerland). HPLC grade methanol, heptane and isoamylalcohol were obtained from Rathburn Chemicals (Walkerburn, UK). Sodium hydroxide, concentrated acetic acid and ammonia water were from Merck (Darmstadt, Germany). Water was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

Drug solutions

All stock solutions were prepared by dissolving pure substances in ethanol: clozapine or desmethylclozapine in a concentration of 3.268 mg/ml and 3.128 mg/ml, respectively (10 mmol/l of each) and trifluoperazine or imipramine in a concentration of 4.094 mg/ml and 2.804 mg/ml. For further dilution of stock solutions, ethanol–water (50:50, v/v) was used.

Serum standards and controls containing known amounts of clozapine and desmethylclozapine were prepared by spiking serum from healthy drug-free blood donors to obtain final concentrations of clozapine and desmethylclozapine in the range 32.7–3268 ng/ml.

Prior to analysis, 300 μl of water containing trifluoperazine and imipramine (internal standards) was mixed with 600 μl of serum standard and control or serum containing unknown amounts of drugs.

Blood samples

Blood samples for determination of clozapine and desmethylclozapine were drawn by venepuncture in the morning *ca.* 12 h after the last dose and before the morning dose was given. The patients should be in steady state, *i.e.* treated with a constant dose for at least five days. After centrifugation at 1500 *g* for 10 min, serum was

kept frozen at −18° if not analyzed within two days.

On-line extraction

The apparatus consisted of a solvent delivery unit (SDU) with a purge pump and a six-port solvent selection valve, an autosampler (Marathon) and a programmable on-line solid-phase extraction unit (Prospekt), all three units from Spark-Holland (Emmen, Netherlands). A detailed description of the function of the programmable extraction unit with exchangeable cartridge (Prospekt) has been published previously [18]. The unit was optionally equipped with a Model 7125 manual injector (Rheodyne, Cotali, CA, USA), which allows direct injection without cartridge. The internal diameter and length of the cartridge were 2 mm and 10 mm, respectively, and it was filled with 40 μm particles of cyanopropyl (CN) bonded phase (J.T. Baker Chemicals, Deventer, Nederlands). To 600 μl of serum was added 300 μl of water containing the internal standards, and the vials were placed in the autosampler. The sampler had a loop volume of 200 μl. It was programmed to use 100 μl of the sample as flush volume and then twice the loop volume for filling. Thus, a fixed volume of 200 μl corresponding to 133 μl of serum was applied to a cartridge. Used in this way the carry-over (memory effect) was less than or equal to 1% for the compounds of interest.

The purge pump flow-rate from the SDU was initially programmed on Prospekt to deliver 1.5 ml/min. Before application of the serum sample, the cartridge was flushed for 1 min with methanol; the flow was then increased to 4.5 ml/min and the cartridge was flushed for 2 min with water in order to wash out the possibly remaining fine particles from the CN material. The cartridge was then flushed for 2 min with 0.1 *M* ammonia acetate buffer at pH 8.0 at a flow-rate of 1.5 ml/min; subsequently, the flow was directed through the loop of the autosampler and serum transported to the cartridge. The cartridge was rinsed for 0.5 min with the buffer and then for 1 min with 9% methanol in water, maintaining the flow-rate of 1.5 ml/min. The analytes were now eluted with mobile phase for 1 min at a flow-rate of 1.4 ml/min. The Prospekt cannot be programmed to use a cartridge more than once in a sequenced but in order to be able to reuse it in a new series, it was purged for 1 min with water and 1 min with methanol. The cartridge was automatically changed for a new one, and a new cycle started.

Liquid–liquid extraction

In 12-ml centrifuge tubes, 1 ml of serum was mixed with 1000 μl of 0.1 *M* NaOH and 50 μl of 50% ethanol containing 4098 and 2801 ng/ml of the internal standards trifluoperazine and imipramine, respectively. Five ml of heptane–isoamylalcohol (99:1, v/v) was added and the mixture was shaken for 5 min at 250 shakings/min on a HS 500 (Janke & Kunkel, Staufen, Germany) shaking apparatus. After centrifugation at 1500 *g* for 10 min, the aqueous layer was frozen by immersing the tubes into a cooling bath consisting of dry ice and ethanol. The heptan layer was decanted into centrifuge tubes and evaporated to dryness at 50° under a gentle stream of nitrogen. The residue was dissolved in 75 μl of mobile phase and 65 μl was injected into the chromatograph.

Chromatography

The chromatographic system consisted of an isochratic LC Model 250 pump (Perkin-Elmer, Norwalk, CT, USA). The analytical column (150 mm × 4.6 mm I.D.) was filled with Spherisorb S5W, 5 μ particle size (Phase Separation Ltd., Queensferry, UK). For UV detection, a SpectroMonitor 4100 (LDC Analytical, Riviera Beach, CA, USA) was set at 261 nm. Chromatograms were recorded on a D-2000 Chromato-Integrator (Merck). The mobile phase was methanol–ammonia acetate buffer 50 m*M*, pH 9.9 (85:15, v/v). The flow-rate was 1.4 ml/min.

Calculations

From recorded peak heights, the ratios of drug to internal standard were calculated. The results obtained from serum standards spiked with three different known amounts of clozapine and its metabolite were used to calculate the factor for multiplying the ratios between the heights of the

unknown and internal standard peaks (three points calibration).

RESULTS

Chromatography

A chromatogram of pure standards dissolved in mobile phase and injected manually without solid-phase extraction is shown in Fig. 1A. By changing the mobile phase with respect to molarity and pH of the buffer as well as the methanol concentration, a composition was found which allowed baseline separation of the compounds of interest in less than 9.0 min. Fig. 1B shows a chromatogram of a serum blank from a healthy drug-free blood donor. Only one small interfering peak (IP) was consistently found at a retention time eluting *ca.* 3 s earlier than clozapine. This peak was present both when serum blank and water were extracted and when the cartridge was filled with other brands of solid phase. A chromatogram of an extracted serum standard containing 327 ng/ml of clozapine and 313 ng/ml of desmethylclozapine (1000 nmol/l of each), and the internal standards is shown in Fig. 1C. The mean retention times for clozapine, trifluoperazine, desmethylclozapine and imipramine were 2.4, 3.3, 4.8, and 7.0 min, respectively. The chromatogram shown in Fig. 1D originates from a patient receiving 600 mg clozapine per day. In addition to clozapinesulphoxide, an unidentified asymmetric peak (UM), presumably a metabolite, was seen at *ca.* 6.4 min in all serum samples from clozapine-treated patients.

Recovery and linearity

The peak heights of clozapine, desmethylclozapine and the internal standards were measured in chromatograms obtained after manual injection of the four compounds dissolved in mobile phase without extraction (Fig. 1A). The peak heights were compared with those obtained when blank serum spiked with known amounts of the four compounds was analysed (Fig. 1C). In this way, the recovery of clozapine and imipramine was found to be more than 90% and that of desmethylclozapine and trifluoperazine *ca.* 80% relative to the unextracted compounds.

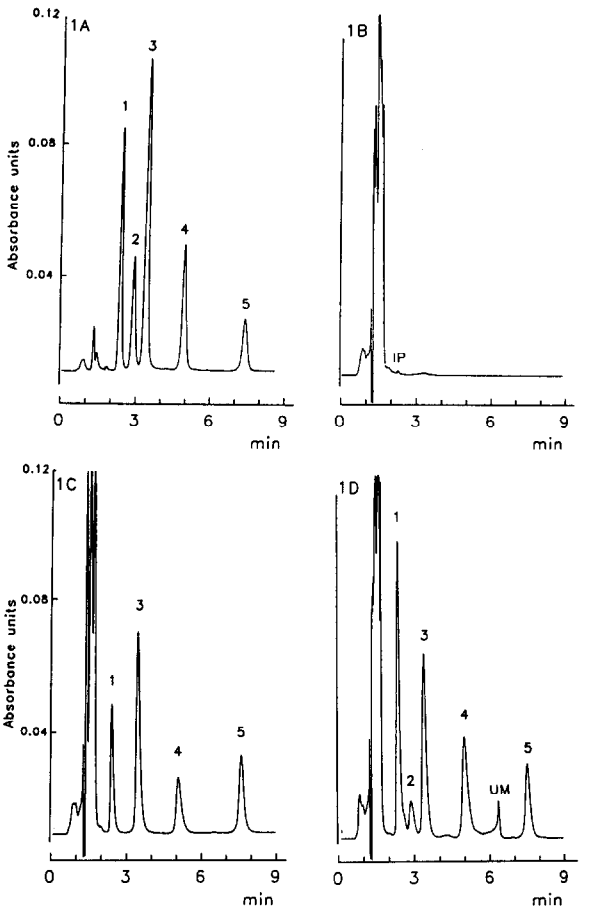

Fig. 1. Chromatograms obtained under the following conditions; Mobile phase: methanol–ammonium acetate 50 m*M*, pH 9.9 (85:15, v/v); column: silica S5W, 5 μm (150 × 4.6 mm I.D.); flow: 1.4 ml/min; detection: UV at 261 nm. (A) Separation of clozapine (1), clozapinesulphoxide (2), trifluoperazine (I.S.) (3), desmethylclozapine (4), and imipramine (I.S.) (5). The compounds were dissolved in mobile phase and injected manually without extraction. (B) Serum blank extracted on-line as described in the Experimental section. I.P. indicates the position of an unknown interfering peak not originating from endogenous material. (C) Serum blank spiked with 326.8 ng/ml of 1 and 312.5 ng/ml of 4. After dilution with water, the concentrations of the internal standards 3 and 5 were 437 and 374 ng/ml, respectively. (D) Serum from patient receiving 600 mg clozapine/day. The results of the analysis were 831 ng/ml of clozapine and 607 ng/ml of desmethylclozapine.

When analysing blank serum spiked with 0–3268 ng/ml of clozapine and 0–3128 ng/ml of desmethylclozapine the detector response was linear for both substances (Fig. 2) and the intercept of the regression line close to zero.

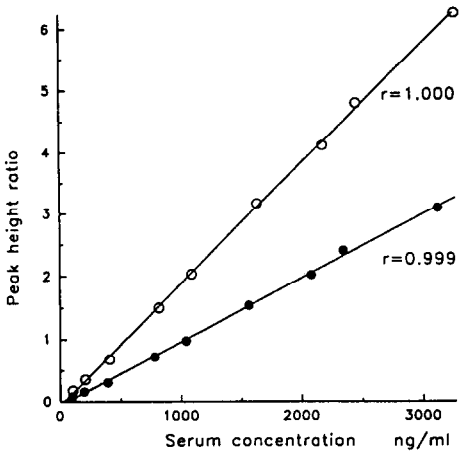

Fig. 2. Clozapine/internal standard (○) and desmethylclozapine/internal standard (●) ratios as a function of the amounts of the two compounds added to blank serum. On-line analysis was performed as described in the Experimental section.

Precision and accuracy

The within-day and day-to-day precision and accuracy were evaluated by analysing blank serum spiked with different amounts of clozapine and desmethylclozapine. The results are given in Table I which shows that in the range investigated both the intra-day and the inter-day variations for clozapine were less than 6%; for desmethylclozapine the corresponding variations were less than 8%.

The lower limit of detection was *ca.* 15 ng/ml and that of accurate quantitation *ca.* 30 ng/ml for both substances. The latter was examined by analysing five blank serum samples spiked with 32.7 ng/ml of clozapine and 31.3 ng/ml of desmethylclozapine. The accuracy for clozapine determination was 108% and the C.V. was 3.5%. The corresponding figures for desmethylclozapine were 98% and 5.0%, respectively.

Comparison with liquid–liquid extraction

In serum from 25 patients treated with different doses of clozapine the concentration of clozapine was determined using on-line extraction and liquid–liquid extraction, respectively. The results obtained by the two methods were highly correlated (Fig. 3) and the equation for the linear regression line was $y = 1.00x + 10$.

Analysis for interference

Serum from patients receiving antipsychotic drugs commonly used in combination with clozapine was analysed for interference with clozapine or desmethylclozapine determination.

TABLE I

VARIATION AND ACCURACY OF THE DETERMINATION OF CLOZAPINE AND DESMETHYLCLOZAPINE IN SPIKED SERUM

Concentration (ng/ml)	*n*	Intra-day		Inter-day	
		C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)
Clozapine					
65.4	10	4.9	110.4		
163.4	15			4.7	101.7
326.8	10	2.2	106.1		
490.2	15			5.1	96.4
980.4	10	5.8	102.3		
Desmethylclozapine					
62.5	10	4.5	106.6		
156.3	15			7.7	101.4
312.5	10	2.7	102.8		
468.8	15			5.8	96.3
937.5	10	6.7	99.5		

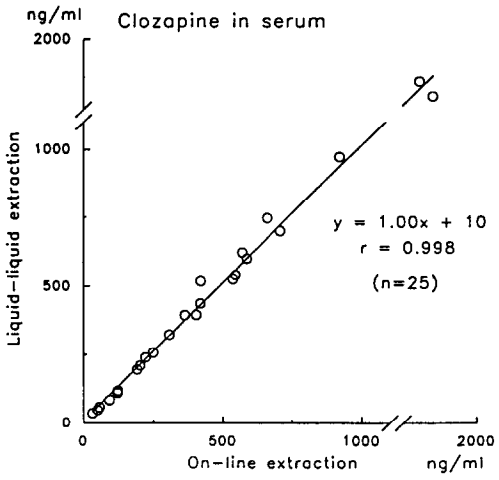

Fig. 3. Comparison between the concentrations of clozapine in serum determined by the present on-line extraction method and the liquid–liquid extraction method described in the Experimental section. The serum samples were from 25 different patients treated with clozapine.

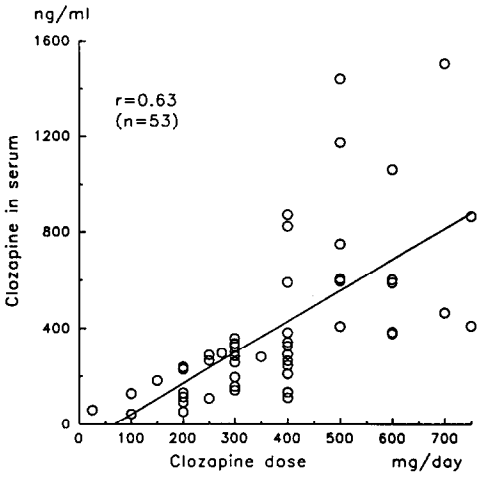

Fig. 4. Clozapine in serum determined by the present on-line method as a function of the clozapine dose given in mg per day. The serum samples were from 53 patients treated with clozapine.

Analysis of serum from patients receiving the tricyclic antidepressant drugs amitriptyline, clomipramine, imipramine, and nortriptyline showed that both the parent drugs and their metabolites had retention times in the range 6–12 min. The high-dose neuroleptic drugs levomepromazine and chlorprothixene and their metabolites were eluted between clozapine and desmethylclozapine while their metabolites had retention times in the range 8–12 min. Carbamazepine had a retention time of 10 min.

The low-dose neuroleptic drugs perphenazine and zuclopenthixol did, in fact, interfere with the determination of clozapine but their concentration in serum seldom exceeds 15 ng/ml. Therefore, the error in the quantitative analysis of clozapine caused by these drugs was accepted in our routine TDM. Mianserine could not be separated from clozapine by the present method and the concentration of this drug in serum was of the same magnitude as that of clozapine.

Serum concentrations in patients

The serum concentration of clozapine and desmethylclozapine was determined by the present on-line method in 53 patients referred to our TDM service. Fig. 4 shows the clozapine concentration as a function of the dose in milligrams per day. There was a significant correlation between dose and the concentration of the drug in serum but it appears from the figure that the inter-individual variation of the serum concentrations obtained at equal doses was considerable.

Fig. 5 shows a close relationship between the clozapine and the desmethylclozapine concentration in the same 53 serum samples. The mean ratio clozapine/desmethylclozapine was 0.74 and

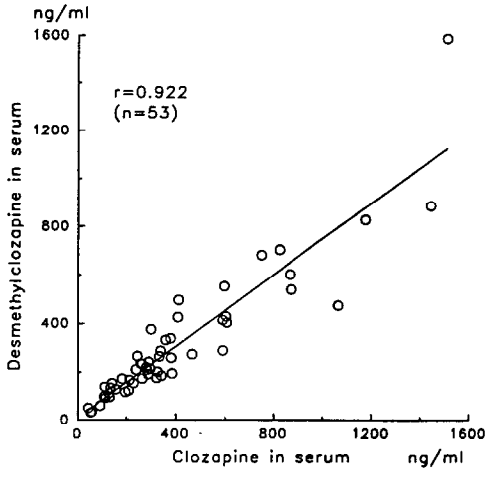

Fig. 5. Desmethylclozapine concentration in serum as a function of the clozapine concentration in serum from 53 patients treated with clozapine. Both concentrations were determined by the present on-line method.

the lowest concentrations of the two compounds found in clozapine-treated patients were 43 and 31 ng/ml, respectively.

DISCUSSION

The method described allows unattended on-line determination of clozapine and its major metabolite desmethylclozapine in human serum. The manual (off-line) sample preparation was restricted to diluting serum with water containing the internal standards. Less than 15 min is needed to analyse a sample and theoretically the apparatus can function during 24 hours a day, performing more than 100 determinations per day.

In the 53 clozapine-treated patients, the serum concentration (mean ± S.D.) was 402 ± 333 ng/ml; within this range, the day-to-day variation of the method was 5–6% which is acceptable for routine TDM and equally good as the precision reported by others using liquid–liquid [9–11] or solid-phase [12,13] extraction. A total amount of 600 μl serum was used for one analysis (133 μl for extraction). Our previously used liquid–liquid extraction method was not suitable for determination of desmethylclozapine due to a low extraction yield. This problem was solved by using the present method.

The main difference between the present and the other HPLC on-line methods is the use of exchangeable cartridges for sample concentration and clean-up. On-line methods for the determination of tricyclic antidepressant drugs in human serum make use of different types of column-switching techniques [19,20]. These techniques imply that the drugs are concentrated on a pre-column; after washing the pre-column is automatically back-flushed with the mobile phase to transfer the analytes to the analytical column. The problem connected with repeated use of the pre-column is that it may deteriorate due to precipitation of protein and the risk of blocking [21]; also, there is a risk that late eluting compounds will interfere with a succeeding chromatogram. The exchangeable cartridge (pre-column) used in the present study contained a cyanopropyl bonded phase with a particle size of 40 μm sealed with 25 μm stainless steel sieves instead of frits. Due to the relatively large particle size and the wide bore of the sieves there was no increase of the back-pressure when the sample was loaded on a cartridge. Furthermore, no clogging has been observed. The short length of the cartridge (10 mm) allows a forward-flush mode for elution of the analytes with mobile phase and at the same time the cartridge acts as a protective filter [18]. The wide bore of the sieves necessitates a high uniformity of the particle size of the packing material in order to avoid fine particles from being eluted from the material and thereby causing blockage of the column inlet frit. When we developed the present method for clozapine determination, a steady increase of the column back-pressure was observed. This problem was solved by flushing the cartridge with water at a flow-rate of 4.5 ml/min for 2 min after the methanol activation.

Our own results with solid-phase extraction of tricyclic antidepressant drugs from serum (unpublished data) showed a decreased recovery when the water used to wash the cartridge contained more than 10% of methanol. Since no interfering peaks from endogenous material were found, no further experiments were performed and a methanol content of 9% was used. No obvious advantage was obtained by diluting the serum with buffer instead of water before extraction and since the recovery of the compounds of interest was acceptable and comparable with other methods [13], no attempts were made to improve this.

The original suggestion to use unmodified silica as column material and a mixture of methanol and aqueous buffer as mobile phase for separation of basic drugs was made by Jane in 1975 [22] and modifications of the method have been used in collaborative studies on inter-laboratory reproducibility [23,24]. A mobile phase with 80% methanol and 5 m*M* ammonia hydroxide has been used for on-line determination of tricyclic antidepressant drugs [25]. In accordance with these authors [23–25] we found very reproducible inter-column retention times and we also found that the silica columns were surprisingly stable at basic pH – a column can be used for more than 600 determinations before significant peak broadening occurs.

Patients given clozapine are often simultaneously treated with other drugs. In our choice of a mobile phase we had to assure that other commonly used antipsychotic drugs on the one hand should not interfere directly, and on the other hand, should elute within a reasonable time in order not to disturb succeeding chromatograms. As a compromise, a 50 m*M* buffer with pH 9.9 was chosen and in order to establish baseline separation of clozapine and clozapinesulphoxide, a methanol content of 85% was found to be the optimal choice.

In the introduction of new methods we should show consideration for both environment and economy. Liquid–liquid extraction is a tedious and time consuming procedure and it implies an almost unavoidable exposure to organic vapor. The present on-line method requires a $30 000 investment to cover the solvent delivery unit, the autosampler and the programmable cartridge exchange unit. The exchangeable cartridges can be used twice which reduces this part of the expenses to *ca.* $2 per analysis. Until now, we have made no systematic attempts to use the cartridges three or more times and thereby reduce the expenses even further.

We have now used the present on-line method for more than six months in our routine TDM service. Our aim is to relate the results of the clozapine and desmethylclozapine serum concentration determinations to the antipsychotic effect and side effects of the drug.

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