Journal of Chromatography, 583 (1992) 209–216
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6570

Determination of clozapine and its major metabolites in human serum using automated solid-phase extraction and subsequent isocratic high-performance liquid chromatography with ultraviolet detection

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(First received June 10th, 1992; revised manuscript received September 7th, 1992)

ABSTRACT

An isocratic high-performance liquid chromatographic (HPLC) method with ultraviolet detection is described for the quantification of the atypical neuroleptic clozapine and its major metabolites, N-desmethylclozapine and clozapine N-oxide, in human serum or plasma. The method included automated solid-phase extraction on C_{18} reversed-phase material. Clozapine and its metabolites were separated by HPLC on a C_{18} ODS Hypersil analytical column (5 μm particle size; 250 mm × 4.6 mm I.D.) using an acetonitrile–water (40:60, v/v) eluent buffered with 0.4% (v/v) N,N,N',N'-tetramethylethylenediamine and acetic acid to pH 6.5. Imipramine served as internal standard. After extraction of 1 ml of serum or plasma, as little as 5 ng/ml of clozapine and 10 or 20 ng/ml of the metabolites were detectable. Linearity was found for drug concentrations between 5 and 2000 ng/ml as indicated by correlation coefficients of 0.998 to 0.985. The intra- and inter-assay coefficients of variation ranged between 1 and 20%. Interferences with other psychotropic drugs such as benzodiazepines, antidepressants or neuroleptics were negligible. In all samples, collected from schizophrenic patients who had been treated with daily oral doses of 75–400 mg of clozapine, the drug and its major metabolite, N-desmethylclozapine, could be detected, while the concentrations of clozapine N-oxide were below 20 ng/ml in three of sixteen patients. Using the method described here, data regarding relations between therapeutic or toxic effects and drug blood levels or metabolism may be collected in clinical practice to improve the therapeutic efficacy of clozapine drug treatment.

INTRODUCTION

Clozapine, 8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b*,*e*]diazepine, is a member of the so-called atypical neuroleptics [1,2]. The drug does not induce the extrapyramidal side-effects [2,3] that occur frequently with standard neuroleptics [4]. Moreover, clozapine is effective in many patients who do not respond to classical neuroleptics such as chlorpromazine or haloperidol [3,5]. In spite of its high efficacy, the use of clozapine is limited by its potential haematological toxicity [3,5]. Evidence has been reported that clozapine blood levels might be useful predictors of the treatment response of schizophrenic patients [6,7], but it remains to be clarified whether high blood levels of clozapine are related to toxic side-effects, as has been shown for classical neuroleptics [8] or antidepressants [9]. A recently published study has provided evidence that toxic effects might be associated with clozapine metabolism [10]. As shown in Fig. 1 clozapine is mainly metabolized to N-desmethylclozapine and clozapine N-oxide [11]. To our knowl-

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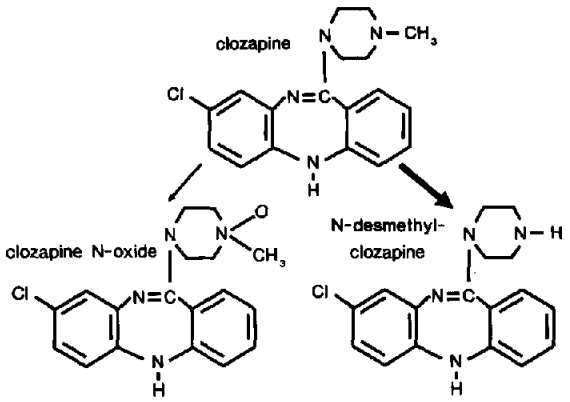

Fig. 1. Structures of clozapine and major routes of metabolism leading to the formation of N-desmethylclozapine and clozapine N-oxide.

edge the pharmacological potency of these metabolites has not been reported. In the literature, only acute toxicity in the mouse is mentioned, without giving details of the parameters tested [11]. For antidepressants it has been shown that cardiotoxic side-effects are mainly due to metabolites [12].

To obtain more information on the blood levels or metabolism of clozapine and clinical response, an appropriate assay suitable for routine determination of both clozapine and its major metabolites is necessary. Simple and robust methods have been reported in the literature [13–20], but most of them do not include the clozapine metabolites. Procedures described so far and suitable for simultaneous determination of clozapine and major metabolites, such as gas chromatography [21,22], require time-consuming extraction steps and derivatization prior to chromatographic analyses.

This paper describes an improved method for determination of clozapine, N-desmethylclozapine and clozapine N-oxide in human serum or plasma using automated solid-phase extraction and subsequent isocratic high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The determination fulfils the requirements of a method to be used for therapeutic drug monitoring.

EXPERIMENTAL

Chemicals

Clozapine, 8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b*,*e*]diazepine, and its major metabolites, clozapine N-oxide and N-desmethylclozapine (Fig. 1), were kindly donated by Sandoz (Basle, Switzerland). Amitriptyline, nortriptyline and protriptyline were obtained from Merck, Sharp and Dohme (Rahway, NJ, USA), caffeine and theophylline from Sigma (Munich, Germany), carbamazepine, carbamazepine 10,11-epoxide, clomipramine, N-desmethylclomipramine, 8-hydroxyclomipramine, 8-hydroxy-N-desmethylclomipramine, imipramine and maprotiline from Ciba Geigy (Basle, Switzerland), benperidol from Tropon (Cologne, Germany), haloperidol from Janssen (Beerse, Belgium), perazine from Promonta (Hamburg, Germany), doxepin from Pfizer (Karlsruhe, Germany), mianserine from Organon (Munich, Germany), lorazepam from Wyeth (Münster, Germany) and flunitrazepam, nordiazepam and diazepam from Hoffmann La-Roche (Basle, Switzerland). Methanol (LiChrosolv, Merck, Darmstadt, Germany) and acetonitrile (LiChrosolv, Merck) were used without further purification. N,N,N′,N′-Tetramethylethylenediamine (TEMED) was obtained from Sigma and concentrated acetic acid from Merck. Water was deionized and purified by a Milli-Q water-processing system (Millipore, Eschborn, Germany).

Drug solutions

Drug solutions were prepared by dissolving pure substances in methanol. Clozapine, N-desmethylclozapine and clozapine N-oxide were dissolved at a concentration of 1 mg of free base per ml. They were diluted to final concentrations using HPLC eluent. Stock solutions could be stored in the dark at −20°C for several months without measurable decomposition, and no instabilities were observed for serum or plasma samples either spiked with drug or obtained from patients treated with clozapine.

Serum or plasma samples containing known amounts of drug were spiked with standard solu-

tions to obtain final concentrations of 10–2000 ng/ml clozapine, 10–500 ng/ml N-desmethylclozapine and 20–400 ng/ml clozapine N-oxide.

Prior to extraction 100 μl of a solution containing 1 μg of imipramine were added to 1 ml of serum or plasma containing unknown amounts of drugs.

Serum or plasma samples

Blood was withdrawn from the antecubital vein of drug-free healthy volunteers or schizophrenic patients who had been treated daily with clozapine for seven days. Patients' blood was collected in the morning, 12 h after the last drug administration. Serum or plasma was prepared by centrifugation of blood samples at 3000 *g* for 10 min and stored at −20°C until assayed. After thawing the samples were recentrifuged before analysis.

Solid-phase extraction

Unless otherwise indicated, the solid-phase extraction of clozapine and its major metabolites included application of 1 ml of serum or plasma samples on C_{18} reversed-phase cartridges (Bond-Elut, ict Handels GmbH, Frankfurt, Germany), which were mounted on a sample processor (ASPEC from Gilson, Villiers-le-Bel, France), performing all extraction steps automatically. Prior to application of the sample, the clean-up column was conditioned by consecutively rinsing 1 ml of methanol and 1 ml of water through the columns. Serum or plasma was mixed with 100 μl of internal standard plus 1 ml of 0.2 *M* potassium chloride adjusted to pH 12.0 using 0.2 *M* sodium hydroxide and applied onto the cartridges. After washing with 50% methanol in water (v/v) the drugs were eluted with 0.01 *M* acetic acid in methanol. The final eluate was evaporated to dryness and the residue dissolved in 1 ml of HPLC eluent for subsequent HPLC analysis.

Chromatography

The chromatographic system comprised an LKB 2153 autosampler (Pharmacia, Freiburg, Germany) equipped with a 7010 Rheodyne switching valve and a 100-μl sample loop, a Constametric III HPLC pump (LDC Analytical, Gelnhausen, Germany) and a pulse dampener (Bischoff, Leonberg, Germany). For chromatographic separation an analytical column (250 mm × 4.6 mm I.D.) filled with C_{18} ODS Hypersil of 5 μm particle size (VDS Optilab, Berlin, Germany) was used. The analytical column was protected by a C_{18} ODS Hypersil precolumn (20 mm × 4.6 mm I.D.) obtained from Chemie- und Werkstofftechnik GmbH Idstein (Idstein, Germany). For detection a UV spectromonitor III (LDC Analytical) of variable wavelength was set at 254 nm. Chromatograms were recorded and integrated by an MP 3000E integration system (LDC Analytical).

Unless otherwise indicated, the HPLC eluent consisted of acetonitrile–water (40:60, v/v) containing 0.4% (v/v) TEMED and adjusted to pH 6.5 with concentrated acetic acid. Prior to use the HPLC eluent was degassed by sonication. Residual oxygen was removed with helium gas. The isocratic separation was performed at 1.5 ml/min flow-rate at ambient temperature.

Calculations

From recorded peak heights the ratios of drug to internal standard were calculated. The results obtained from spiked plasma samples containing known amounts of drug were subjected to linear regression analysis for the calculation of correlation coefficients, slopes and intercepts. Drug concentrations were computed on the basis of the regression lines. For determination of recoveries plasma samples were supplemented with standard solutions dissolved in HPLC eluent and subjected to HPLC analysis including solid-phase extraction. The results of these measurements were related to results obtained from standard solutions and analysed without solid-phase extraction to calculate the recovery rates.

RESULTS

Chromatography

Using an analytical column filled with C_{18} reversed-phase material, clozapine and its two major metabolites could be well separated in a mo-

bile phase containing 40% acetonitrile in water buffered with TEMED acetate to pH 6.5. Acetonitrile was found to be superior to methanol as organic modifier. At pH 5.5, which has been considered optimal by others [20], a 30–35% acetonitrile eluent enabled baseline separation of all compounds of interest within 20 min (data not shown). The peak shape of the metabolites, howevere, exhibited marked asymmetry. At concentrations of 100 ng/ml a clozapine N-oxide peak could hardly be distinguished from the baseline. Peak symmetry was improved by increasing the acetonitrile content of the eluent to 40% (Fig. 2, left panel). Under these conditions, however, the resolution became poorer. When increasing the pH of the mobile phase from 5.5 to 6.5 the retention times changed from 6.2 to 7.4 min for clozapine, from 5.8 to 5.6 min for N-desmethylclozapine and from 4.8 to 3.9 min for clozapine N-oxide, thus allowing baseline separation of all three substances (Fig. 2, right panel) within less than 10 min. Changes in the pH below 5.5 or above 6.5 impaired the chromatographic resolution.

When testing standard solutions containing various other psychotropic drugs that may be applied in combination with clozapine, minimal interferences with clozapine or clozapine metabolites were found for any drug or metabolite that had been tested (Table I).

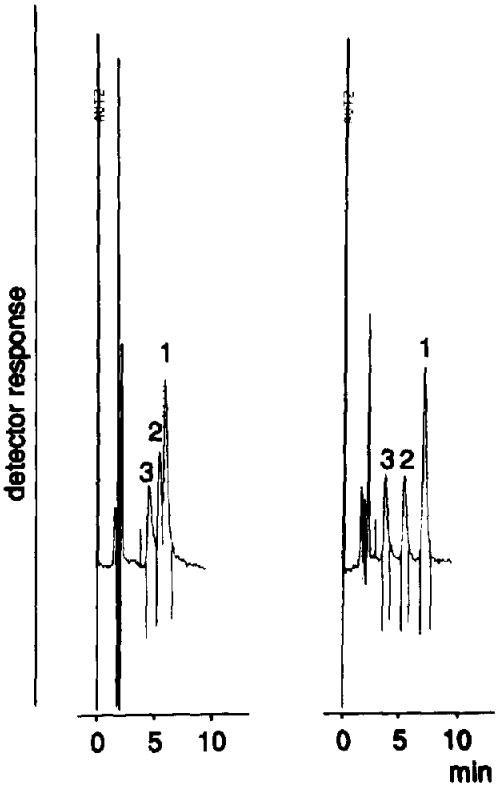

Fig. 2. Chromatographic separation of standard solutions containing clozapine (1), N-desmethylclozapine (2) and clozapine N-oxide (3) at concentrations of 330 ng/ml (clozapine) or 100 ng/ml at pH 5.5 (left panel) or 6.5 (right panel). Standard solutions were dissolved in the HPLC eluent, which consisted of acetonitrile–water (40:60, v/v) containing 0.4% TEMED. The pH was adjusted with acetic acid.

Solid-phase extraction

Minimal losses and maximal recoveries of clozapine and metabolites were found when 1 ml of 50% methanol was used for washing and 1 ml of methanol containing 0.01 *M* acetic acid was used for drug elution. After applying a 2.1-ml sample consisting of 1 ml of serum, 1 ml of water and 0.1 ml of internal standard on C_{18} cartridges, most samples prepared from blood of non-treated healthy volunteers, HPLC analyses revealed no or minimal interferences. Some serum or plasma samples contained impurities of unknown chemical identity interfering with the analytes of interest. They could be easily removed by the 1 ml methanol–water (50%, v/v) wash. At lower methanol concentrations, washing was less effective (Fig. 3). When the methanol content exceeded 50%, clozapine N-oxide was lost (Fig. 3). Increasing the volumes of the washing solutions did not improve the purification (data not shown).

Under the above-mentioned optimized extraction conditions the mean recovery rates tested for 500 ng/ml were 85% (clozapine), 78% (N-desmethylclozapine) and 77% (clozapine N-oxide).

Internal standard

Of the various drugs tested for interference, imipramine was selected as internal standard. The tricylic antidepressant was well separated from clozapine and its metabolites (Table I). Moreover, after the extraction procedure of a sample containing 1 μg/ml imipramine, 95% was recovered (Fig. 3).

Linearity

When analysing 1-ml blank serum samples spiked with 0–2000 ng of clozapine, 0–500 ng of N-desmethylclozapine or 0–400 ng of clozapine N-oxide the detector responses were linear for all substances. The correlation coefficients calculated by linear regression analyses were 0.998 ± 0.001 for clozapine, 0.995 ± 0.001 for N-desmethylclozapine and 0.985 ± 0.002 for clozapine N-oxide. The curve intercepts were close to 0, ranging between −0.077 and 0.006. This was within the range of the detection limit, which was considered to be about 5 ng/ml for clozapine, 10 ng/ml for N-desmethylclozapine and 20 ng/ml for the N-oxide.

TABLE I

RETENTION TIMES OF PSYCHOTROPIC DRUGS AND SOME RELATED METABOLITES ANALYSED FOR INTERFERENCES

Solutions contained 100 ng/ml except caffeine, theophylline, doxepin and mianserine (1 μg/ml each). The analyses did not include solid-phase extraction prior to HPLC.

Drug	Retention time (min)
Caffeine	<2
Theophylline	<2
Carbamazepine 10,11-epoxide	3.49
Clozapine N-oxide	3.80
Benperidol	4.97
Carbamazepine	5.06
8-Hydroxy-N-desmethylclomipramine	5.35
Lorazepam	6.10
N-Desmethylclozapine	6.44
8-Hydroxyclomipramine	6.96
Doxepin	7.31
Clozapine	8.00
Flunitrazepam	8.38
Nordiazepam	9.15
Haloperidol	9.68
Fluvoxamine	10.18
Maprotiline	11.95
Mianserine	11.96
Nortriptyline	11.96
Protriptyline	12.98
Imipramine	13.47
Diazepam	14.80
Perazine	15.18
Amitryptyline	17.76
Desmethylclomipramine	21.48
Clomipramine	23.71

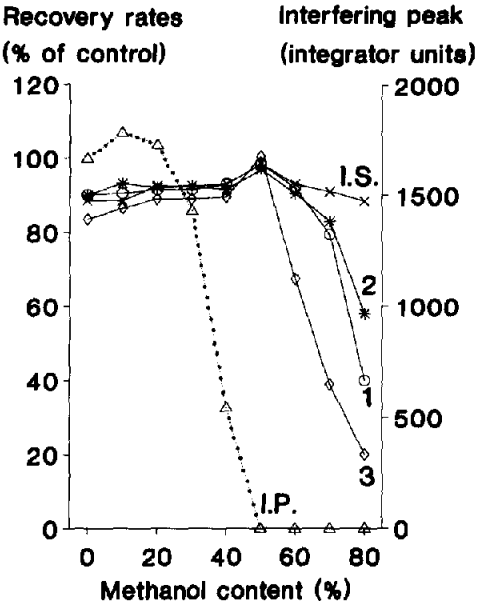

Fig. 3. Recoveries of clozapine (1), N-desmethylclozapine (2), clozapine N-oxide (3), the internal standard imipramine (I.S.) and an interfering peak (I.P.) in a blank serum sample using variable methanol contents in the washing solutions (1 ml) for solid-phase extraction. The concentrations of clozapine and metabolites were 500 ng/ml, that of imipramine 1 μg/ml. The drugs were eluted by 1 ml of methanol containing 0.01 *M* acetic acid and the eluates submitted to HPLC analysis.

Precision

The day-to-day variations calculated from spiked serum samples containing clozapine (100–1000 ng/ml), N-desmethylclozapine (25–250 ng/ml) or clozapine N-oxide (20–200 ng/ml) were 1.7–6.3, 5.4–16.7 and 6.3–17.5%, respectively.

Application to patient serum or plasma

When applying the steps considered as optimal for sample extraction and chromatographic separation to serum or plasma samples of twelve healthy control subjects all blank samples tested revealed no or negligible interferences with endogenous substances (Fig. 4, left panel). In serum from patients who had received daily oral doses of clozapine, the drug and the two metabolites could be well detected (Fig. 4, right panel). Using either serum or plasma samples identical results were obtained, indicating that the method could be applied to both specimens. In sixteen patients who had been treated with doses of clozapine

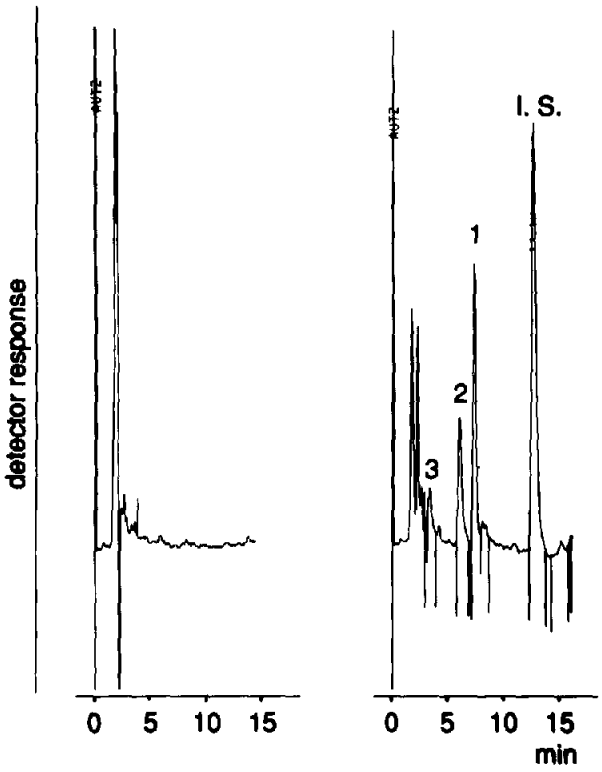

Fig. 4. Representative chromatograms obtained from the analyses of a blank serum sample of a non-treated healthy subject and of a serum sample from a patient who had received daily oral doses of 200 mg of clozapine over seven days. For the analysis of patient serum imipramine was used as internal standard (I.S.). The serum concentrations were 270 ng/ml for clozapine (1), 205 ng/ml for N-desmethylclozapine (2) and 83 ng/ml for clozapine N-oxide (3).

varying from 75 to 300 mg per day the clozapine plasma concentrations ranged between 75 and 524 ng/ml. The metabolite N-desmethylclozapine was found in all serum samples, while clozapine N-oxide was not detectable (<20 ng/ml) in three of the sixteen patients (Table II).

The mean ratios of the concentration of N-desmethylclozapine or clozapine N-oxide to the concentration of clozapine, indices of N-demethylation or N-oxidation, were 0.70 ± 0.16 and 0.22 ± 0.16 (mean ± S.D.), respectively, without obvious dependence on the clozapine dose. Patient 5, however, who had received 150 mg of clozapine, behaved differently. The concentrations of the metabolites (396 ng/ml) exceeded by 267% those of clozapine (108 ng/ml).

DISCUSSION

It was the aim of this study to establish an HPLC method suitable for simultaneous determination of clozapine and its major metabolites in the serum or plasma of patients undergoing clozapine therapy. The method had to be rapid, simple, specific, accurate and robust in application.

Baseline separation could be obtained by HPLC for all compounds to be determined within less than 15 min. Increasing the acetonitrile content of the eluent decreased the retention times for all three substances and the internal standard, probably by reducing the lipophilic interactions of the drugs with the column matrix. After having changed the pH of the mobile phase from 5.5 to 6.5, the elution of clozapine was delayed while the elution of the N-oxide was accelerated. These opposite effects were probably due to differences in the dissociation of the basic groups of the two substances, a tertiary amine function in the clozapine molecule and a less basic N-oxide function in that of the metabolite.

The extraction of serum or plasma on a solid phase could be performed automatically, which is a great advantage in comparison with liquid extractions used by others [13–18,20]. The finding that the serum or plasma samples could be purified sufficiently by a 50% methanol wash before elution indicated that this procedure may be further developed in the future to a column-switching system which enables on-line purification prior to HPLC analysis [23].

In comparison with HPLC assays reported so far for clozapine in the literature [13–20], our method seems advantageous in several respects. The procedure enabled the determination of both clozapine and its major metabolites within a single run using automated solid-phase extraction and subsequent isocratic HPLC with UV detection. Recovery rates of 77–85% and day-to-day variations of 1–20% were in the range of those found by others [13–20]. Such precision is considered acceptable for monitoring of drugs such as antiepileptics which underlie regular quantitative assessments, legally required in many countries [24,25].

TABLE II

CONCENTRATION OF CLOZAPINE AND MAJOR METABOLITES IN THE SERUM OF SCHIZOPHRENIC PATIENTS TREATED FOR SEVEN DAYS WITH INCREASING DOSES OF CLOZAPINE

Patient No.	Final dose (mg of clozapine per day)	Plasma concentrations (ng/ml)		
		Clozapine	N-Desmethyl clozapine	Clozapine N-oxide
1	75	237	110	N.D.[a]
2	125	211	120	21
3	150	275	203	27
4	150	236	149	23
5	150	108	203	193
6	175	280	196	25
7	200	74	80	N.D.
8	200	270	205	82
9	250	141	96	59
10	300	170	167	98
11	300	524	303	63
12	300	313	168	38
13	300	182	138	N.D.
14	300	228	132	61
15	300	144	94	22
16	400	429	337	87

[a] N.D. = Not detectable.

For the procedure described here, 2 h were necessary to analyse a sample, and during a 24-h day it was possible to perform 36 determinations within a single run. Thus it is possible that results may be reported within 24 h after blood withdrawal.

The sensitivity of the assay was satisfactory, since plasma levels of clozapine and its N-demethylated metabolite were far above their detection limits of 5 and 10 ng/ml, respectively. Only in three of the sixteen patients analysed so far was the N-oxide of clozapine not detectable in the serum. Since the N-oxide seems to be the least pharmacologically effective of the three substances [1], the detection limit of our method may be considered acceptable for routine determination.

The range and the high inter-individual variations in the serum levels of clozapine found by us were in agreement with data reported so far in the literature [6,7,26,27]. Currently, we do not know whether the patient exhibiting metabolite concentrations exceeding those of clozapine had excessive metabolizing activities in liver, lung [28] or other organs. Moreover, information is lacking as to whether the metabolic deviation was related to any abnormality in the clinical response to the drug. The finding, however, showed that additional data on clozapine blood levels, its metabolism and clinical outcome must be collected for psychiatric patients undergoing clozapine therapy. The method described here provides a useful tool to obtain such data.

ACKNOWLEDGEMENTS

The authors thank the nursing staff and the physicians of the Department of Psychiatry, University of Mainz, for helping us with blood sampling. The generous gifts of pure drug substances by the pharmaceutical companies are gratefully acknowledged. We thank Mrs. Michaela Jahnke for typing the manuscript.

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