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Simultaneous determination of olanzapine, clozapine and demethylated metabolites in serum by on-line column-switching high-performance liquid chromatography

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Abstract

An automated method for simultaneous routine quantification of the antipsychotic drugs clozapine, olanzapine and their demethylated metabolites is described. The method included adsorption on a cyanopropyl (CPS) coated clean-up column (10 μm; 10×2.0 mm I.D.), washing off interfering serum constituents to waste, and separation on C18 ODS Hypersil reversed phase material (5 μm; 250×4.6 mm I.D.) using acetonitrile–water–tetramethylethylenediamine (37:62.6:0.4, v/v/v) adjusted to pH 6.5 with concentrated acetic acid. UV-detection was performed at 254 nm. The limit of quantification was 10–20 ng/ml. Relative day to day standard variations ranged between 4.5 and 13.5%. The method is suitable for routine monitoring of olanzapine and clozapine including their demethylated metabolites.

Keywords: Olanzapine; Clozapine

1. Introduction

Clozapine and olanzapine are structurally related psychotropic drugs belonging to the class of atypical antipsychotics [1,2]. In spite of its high efficacy, the use of clozapine is limited by its potential risk to cause agranulocytosis [3]. The recently introduced antipsychotic olanzapine is an effective alternative to clozapine [4,5], lacking the need for weekly controls of white blood cells.

The advantageous therapeutic profiles of the two drugs have led to an increasing use of both clozapine and olanzapine in treatment of schizophrenic patients. Therapeutic drug monitoring improves the use of these neuroleptics, because adequate clozapine [6–14] or olanzapine [10] serum concentrations are reported to enhance the treatment response and reduce side effects due to overdosing.

The major metabolite of clozapine in serum is desmethylclozapine. The metabolite is pharmacologically active [15], but passes the blood–brain barrier only to a minor extent [16]. For olanzapine *N*-desmethylolanzapine seems to be the major metabolite besides a 10-*N*-glucuronide [17]. Though olan-

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zapine's metabolites are likely to be pharmacologically inactive [18], determination of concentrations of demethylated metabolites in serum are informative for compliance and individual metabolic activity.

To obtain more information about metabolism, side effects, clinical response, and their correlation to serum concentrations of clozapine, desmethylclozapine, olanzapine and desmethylolanzapine an appropriate assay suitable for routine determination of these compounds is necessary.

Simple and robust methods with high-performance liquid chromatography (HPLC) have been reported for clozapine [19–36] and olanzapine [37–41]. They all require more or less laborious and time consuming off-line sample pretreatment for extraction of drugs before HPLC analysis.

Here we describe an HPLC method for simultaneous determination of clozapine, desmethylclozapine, olanzapine and desmethylolanzapine in human serum. It enables fully automated and rapid (results within 1 h) analysis of clozapine, desmethylclozapine, olanzapine and desmethylolanzapine in serum or plasma by using on-line column-switching and HPLC with ultraviolet (UV) detection under clinical routine conditions

2. Experimental

2.1. Chemicals

Clozapine, 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzodiazepine, and its metabolite desmethylclozapine as well as fluperlapine and thioridazine were kindly donated by Sandoz (Basel, Switzerland). Olanzapine (2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3][1,5]benzodiazepine) was obtained from Lilly (Bad Homburg, Germany). Drugs to be tested for interference with the analytes were kindly donated by the manufacturers or received from commercial sources. Methanol (LiChrosolve, Merck, Darmstadt, Germany) and acetonitrile (LiChrosolve, Merck) were used without further purification. *N*,*N*,*N′*,*N′*-Tetramethylethylenediamine (TEMED) was obtained from Sigma (München, Germany) and concentrated acetic acid from Merck. Water was deionized and purified by a Milli-Q water processing system (Millipore, Eschborn, Germany).

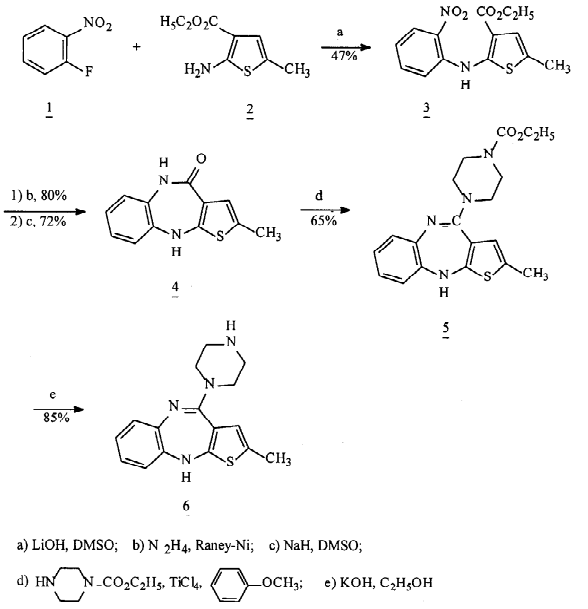

Fig. 1. Synthesis of desmethylolanzapine.

2.2. Synthesis of desmethylolanzapine

The synthesis of desmethylolanzapine is as outlined in Fig. 1. The amino thiophene derivative (2) was prepared under the well-established Gewald conditions [42]. Reaction of the above thiophene with commercially available fluoro nitrobenzene, based on a reported method [43] with minor modifications, led to (3). Reduction of the nitro group was achieved using hydrazine/Raney Ni instead of Pd/C. Ring closure to diazepinone (4) followed the method of Chakrabarti which was converted into the corresponding amidine (5) by reaction with *N*-ethoxycarbonyl piperazine in the presence of $TiCl_4$ and anisole [44]. Saponification of (5) gave rise to desmethylolanzapine (6). All the compounds were characterized by physical methods using IR and NMR, the microanalyses were within $+0.4\%$ of the calculated values.

2.3. Drug solutions

Drug solutions were prepared by dissolving pure substance in methanol. Clozapine, desmethylcloza-

pine, olanzapine and desmethylolanzapine as well as the internal standard fluperlapine were dissolved to a concentration of 1 mg per ml. Stock solutions in methanol could be stored in the dark at −20°C for several months without measurable decomposition. Instabilities were not observed for serum or plasma samples either spiked with drug or obtained from patients treated with clozapine or olanzapine.

Blank plasma samples were spiked with standard solutions, to obtain final concentrations of 10–800 ng/ml clozapine, 20–450 ng/ml desmethylclozapine, 10–180 ng/ml olanzapine and 10–180 ng/ml desmethylolanzapine.

Fluperlapine, the internal standard, was added to serum or plasma samples prior to analysis by pipetting 10 μl of an aqueous solution into 500 μl of sample. The final concentration of fluperlapine was 250 ng/ml.

2.4. Serum or plasma samples

Blood of schizophrenic patients who had been treated with a fixed dose of clozapine or olanzapine for at least 7 days, were collected in the morning, 12 h after the last drug administration. Blood was withdrawn from the antecubital vein. Serum or plasma were prepared by centrifugation of blood samples at 3000 *g* for 10 min and, if not assayed on the same day, stored at −20°C. After thawing, the samples were centrifuged at 15 000 *g* before analysis.

2.5. Equipment

The chromatographic system comprised an autosampler 231 XL (Gilson, Villiers le Bel, France) equipped with a 7010 Rheodyne injection valve and a 100 μl sample loop, two Bischoff HPLC pumps model 2250 (Bischoff, Leonberg, Germany), and an automated switching valve with a 7000 Rheodyne (Besta, Wilhelmsfeld, Germany). The UV detector was a Shimadzu SPD-10A (Shimadzu, Duisburg, Germany) with variable wavelength set at 254 nm. The chromatograms were recorded and integrated by a Kontron integration pack 3.9 (Kontron, Milano, Italy). Moving the switching valves and starting the integration system was managed by a time program of the autosampler.

2.6. Sample preparation and chromatography

One hundred μl of serum or plasma were injected automatically onto the clean-up column (10×2,0 mm) filled with 10 μm particles of cyanopropyl (CPS) bonded material, Hypersil CPS (ict, Frankfurt, Germany). Proteins, lipids and other interfering compounds were washed to waste with deionized water, buffered to pH 4.6, at a flow rate of 1.5 ml/min for 2 min. The HPLC eluent consisted of acetonitrile–water–tetramethylethylenediamine (37: 62,6: 0.4, v/v/v) adjusted to pH 6.5 with concentrated acetic acid. Prior to use, the HPLC eluent was degassed by sonication. After the six-port valve had been switched, the drugs to be determined were eluted from the clean-up column at a flow rate of 1.5 ml/min by the HPLC eluent and separated on the analytical column (250×4.6 mm) filled with ODS Hypersil C18 of 5-μm particle size (MZ-Analysentechnik, Mainz, Germany). Sample clean-up and chromatographic separation were performed at room temperature. If serum concentrations of clozapine, olanzapine or its metabolites exceeded the detection range, samples were dissolved with blank serum to reach quantifiable concentrations.

2.7. Calculations

From the recorded peak heights, the ratios of drug to internal standard were calculated. The results obtained from spiked serum or plasma samples, containing known amounts of drugs, were subjected to linear regression analysis for the calculation of correlation coefficients, slopes and intercepts. Drug concentrations in samples containing unknown amounts of drug were calculated on the basis of the computed regression lines.

The lower limit of quantification was defined as ten standard deviations above the mean blank value, and the lower limit of detection as three standard deviations above the mean blank value according to the IUPAC definitions. The calculated limit of quantification was accepted, when precision was better than 25%.

3. Results

3.1. Pre-purification

Clozapine, desmethylclozapine, olanzapine and desmethylolanzapine as well as the internal standard fluperlapine were retained by CPS bonded silica material in the clean-up column, while proteins, lipids and various other serum constituents were eluted to waste by deionized water. After switching to the HPLC eluent, the analytes were removed from the clean-up column into the analytical column. Using other stationary phases for clean-up, such as C8 or C18 bonded silica, the analysis was impaired for all constituents of interest in comparison to CPS material (data not shown). After injection of 30–40 serum samples the clean-up column had to be replaced, because recovery of all analytes began to decrease.

3.2. Chromatographic separation

Optimal chromatographic separation with base line separation of clozapine, desmethylclozapine, olanzapine, desmethylolanzapine and fluperlapine was achieved on ODS Hypersil C18 within 15 min (Fig. 2B).

3.3. Interferences

When the experimental conditions that were considered optimal for sample pre-purification and drug retention were used for assaying blank serum samples obtained from five nonmedicated healthy volunteers, no significant interferences from endogenous constituents were observed (Fig. 2A).

Testing standard solutions containing other psychotropic drugs that may be applied in combination with clozapine, interferences were revealed for sertraline, 9-OH risperidone, omeprazol, carbamazepine, pipamperone and melperone with olanzapine or desmethylolanzapine, as well as doxepine with clozapine and paroxetine with the internal standard fluperlapine (Table 1). However, only carbamazepine, pipamperone and melperone reached disturbing UV-absorptions in the analysis of patients serum under clinical routine conditions. In the case of carbamazepine, pipamperone and melperone the determination of olanzapine or desmethylolanzapine was possible by a slight modification. Adding water to the HPLC eluent to reduce the acetonitrile concentration to 35% (v) enabled baseline separation of carbamazepine, pipamperone, melperone, desmethylolanzapine and olanzapine with slightly longer retention times.

3.4. Linearity and sensitivity

When analyzing blank plasma samples spiked with 10–800 ng/ml of clozapine, 20–450 ng/ml of desmethylclozapine or 10–180 ng/ml of olanzapine and desmethylolanzapine, the detector responses were linear for all substances. Linear regression analyses revealed coefficients of correlation between 0.993 and 0.994 for all analytes. The curve intercepts were close to 0 (5 ng/ml for clozapine, 16 ng/ml for desmethylclozapine, −2 ng/ml for olanzapine and 0.5 ng/ml for desmethylolanzapine). This was within the range of the limit of quantification, which was considered to be about 10 ng/ml for clozapine, 20 ng/ml for desmethylclozapine, 10 ng/ml for olanzapine and 10 ng/ml for desmethylolanzapine.

3.5. Precision and accuracy

The day-to-day variations calculated from spiked plasma samples containing clozapine (50–500 ng/ml), desmethylclozapine (50–300 ng/ml), olanzapine (25–75 ng/ml) or desmethylolanzapine (25–75 ng/ml) were 6.3–7.5% for clozapine, 4.5–11.5% for desmethylclozapine, 4.5–13.5% for olanzapine and 5.8–12.2% for desmethylolanzapine, respectively. When each concentration was analyzed once on five different days to calculate the coefficients of variation, the resulting accuracy ranged between −10 and 4% for clozapine, −8.5 and 7.5% for desmethylclozapine, −5.9 and −2% for olanzapine and −12.4 and −4.7% for desmethylolanzapine.

Respective intra-day variations were 2.9–8.9% for clozapine, 1.6–5.1% for desmethylclozapine 1.2–5.6% for olanzapine and 1.5–8.5% for desmethylolanzapine. Accuracy ranged between −2.3% and 3.7% for clozapine, −4.6 and 5.9% for desmethyl-

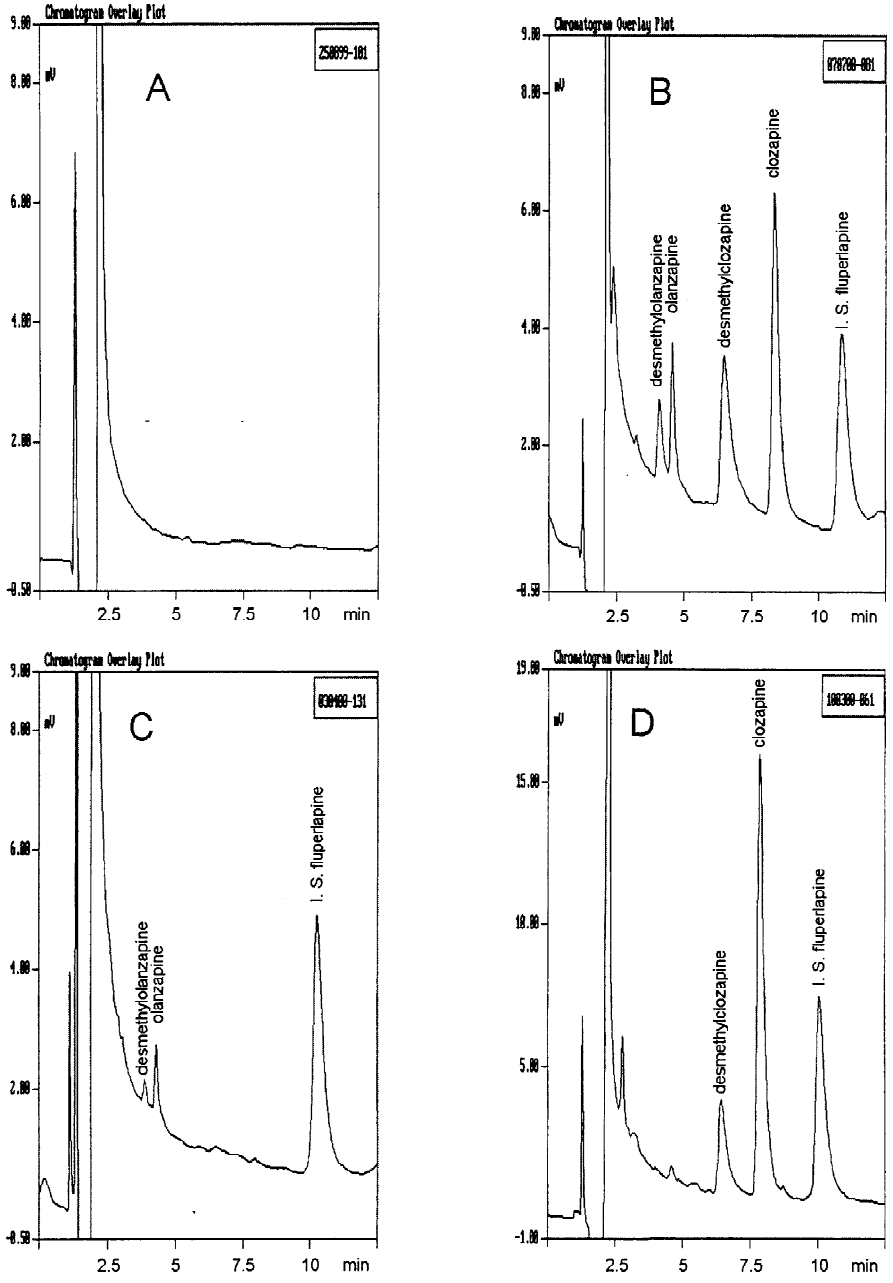

Fig. 2. Representative chromatograms of serum samples analyzed by the described method for clozapine, olanzapine and its demethylated metabolites: (A) Serum of a non-treated healthy subject. (B) Blank serum sample spiked with desmethylolanzapine 60 ng/ml, olanzapine 60 ng/ml, desmethylclozapine 175 ng/ml, clozapine 300 ng/ml and the internal standard fluperlapine 250 ng/ml. (C) Serum obtained from a patient receiving 20 mg/d olanzapine orally for 2 weeks. In the patient's sample the serum concentrations of olanzapine and its demethylated metabolite were 19 ng/ml for desmethylolanzapine and 32 ng/ml for olanzapine. Fluperlapine was used as internal standard in a final concentration of 250 ng/ml. (D) Serum obtained from a patient receiving 125 mg/d clozapine orally for 2 weeks combined with fluvoxamine 100 mg/d and amisulpride 100 mg/d. In the patient's sample the serum concentrations of clozapine and its demethylated metabolite were 156 ng/ml for desmethylclozapine, and 483 ng/ml for clozapine. Fluperlapine was used as internal standard in a final concentration of 500 ng/ml.

Table 1
Retention times of drugs analyzed by the described method established for the determination of clozapine, olanzapine and metabolites. The drug concentrations were 100 and 1000 ng/ml[a]

Drug	Retention time, min
Metoprolol	2.19
Carbamazepine-10-11-epoxide	2.57
9-OH Risperidone	3.13
Desmethylolanzapin	*3.68*
Sertralin	3.97
Risperidone	4,10
Omeprazol	4.13
Carbamazepine	4.21
Dipiperon	4.21
Olanzapin	*4.46*
Melperon	4.64
Mirtazapin	4.65
Benperidol	4.67
Zolpidem	4.83
Lorazepam	5.42
Desmethylclozapine	*6.36*
Flunitrazepam	7.21
Nordiazepam	7.22
Clozapine	*8.25*
Doxepin	8.83
Haloperidol	9.24
Paroxetine	10.75
Fluperlapine (Internal Standard)	*11.18*
Diazepam	13.47
Desipramine	13.48
Imipramine	14.84
Fluvoxamine	15.04
Perazine	18.89
Amitriptyline	20.56
Ethosuximide	nd
Fluoxetine	nd
Furosemid	nd
Maprotiline	nd
Phenobarbital	nd
Thioridazine	nd

[a] nd = not detectable.

clozapine, −2.3 and 2.9% for olanzapine and –6.1 and 6.6% for desmethylolanzapine.

3.6. Recovery

At serum concentrations of 100–400 ng/ml for clozapine, 50–200 ng/ml for desmethylclozapine, 10–50 ng/ml for olanzapine and 10–50 ng/ml of desmethylolanzapine, recovery of analytes ranged from 77 to 86% for clozapine, 67 to 80% for desmethylclozapine, 73 to 95% for olanzapine and 86 to 96% for desmethylolanzapine. Recovery of the internal standard fluperlapine at a concentration of 250 ng/ml averaged 93%.

3.7. Application to patients' serum or plasma

In serum from patients, who had been treated with clozapine or olanzapine, the drugs and their *N*-demethylated metabolites could be well detected (Fig. 2C and D). The method could be used with similar precision, linearity and sensitivity for either serum or plasma samples. In 22 patients, who had received fixed daily oral doses of clozapine for at least 7 days (steady state conditions), the clozapine serum concentrations ranged between 57 and 702 ng/ml (Table 2). The metabolite desmethylclozapine was found in all serum samples. Its concentrations ranged between 39 and 466 ng/ml. In 24 patients, who had received fixed daily oral doses of olanzapine for at least 7 days (steady state conditions),

Table 2
Steady state concentrations of clozapine and its major metabolite in serum of schizophrenic patients[a]

Patient no.	Daily doses (mg)	Serum concentrations (ng/ml)	
	Clozapine	Clozapine	Desmethylclozapine
1	25	57	39
2	75	110	75
3	100	123	100
4	100	234	198
5	150	170	175
6	200	327	104
7	200	283	143
8	200	134	170
9	225	302	189
10	225	354	276
11	250	152	150
12	275	167	125
13	300	414	348
14	300	444	314
15	300	283	225
16	300	702	292
17	400	359	261
18	400	277	371
19	500	437	384
20	600	248	290
21	600	556	466
22	700	421	380

[a] All patients had been treated for at least 7 days with fixed doses of clozapine.

Table 3
Steady state concentrations of olanzapine and its major metabolite in serum of schizophrenic patients. All patients had been treated for at least 7 days with fixed doses of olanzapine[a]

Patient no.	Daily doses (mg) Olanzapine	Serum concentrations (ng/ml)	
		Olanzapine	Desmethylolanzapine
1	7.5	32	<10
2	7.5	49	<10
3	10	15	<10
4	10	14	nd
5	10	21	nd
6	10	42	nd
7	10	51	10
8	10	39	nd
9	10	37	nd
10	15	<10	nd
11	15	49	13
12	15	27	<10
13	15	15	nd
14	20	68	13
15	20	21	15
16	20	28	<10
17	20	30	<10
18	20	20	nd
19	20	12	<10
20	20	<10	nd
21	20	75	12
22	20	54	<10
23	20	53	20
24	40	27	<10

[a] nd = not detectable.

the olanzapine serum concentrations ranged between <10 and 75 ng/ml (Table 3). The metabolite desmethylolanzapine could be quantified in 6 (<10 ng/ml), detected in 9 and not detected in another 9 patients.

4. Discussion

It was the aim of the study to establish an HPLC method suitable for determination of clozapine, olanzapine and its major metabolites in serum or plasma of patients undergoing antipsychotic treatment. The method described here was found to be rapid, simple, specific, accurate and robust in application.

Column-switching techniques are most useful in the analysis of drugs in complex matrices [36,45–53]. In addition to a conventional isocratic HPLC system, only a pre-column for sample clean-up, a second HPLC pump and a six-port switching valve are needed.

Serum or plasma samples were injected directly into the system. Baseline separation could be obtained by HPLC for all compounds to be determined within less than 15 min. At least 30 serum samples could be processed per clean-up column before replacement was required. We consider it as a major advantage of the method that urgent results, for example in case of suspicion on intoxication, can be obtained within less than 1 h. In daily clinical routine, results of up to 20 samples, requested by a large department of psychiatry, were easily available after an overnight run in the next morning.

Day-to-day variations of 4–13% for clozapine, desmethylclozapine, olanzapine and desmethylolanzapine were in the range of those found by others [19–29,31,33,34,38–41]. This precision is consid-

ered acceptable for monitoring of drugs such as antiepileptics which underlie regular quantitative assessments legally required in many countries [54].

All analytes showed highly variable concentrations in serum of treated patients. Serum levels of clozapine and its *N*-demethylated metabolite at therapeutically effective doses exceeded by far their quantification limits of 10 and 20 ng/ml, respectively (Table 2), while serum levels of olanzapine and desmethylolanzapine observed in clinical routine were sometimes under the limit of quantification (Table 3). Serum levels of olanzapine of up to 75 ng/ml under treatment with 20 mg of olanzapine could be observed. In two of 24 patients treated with 15 or 20 mg of olanzapine respectively serum levels of the drug could not be quantified ($<$10 ng/ml). This is in good agreement with results shown by others [10,37,39,41].

Serum concentrations of desmethylolanzapine showed an interindividual variability like olanzapine itself (Table 2). In only 6 out of 24 patients treated with oral doses of olanzapine between 7.5 and 40 mg, the major metabolite desmethylolanzapine could be quantified. In addition results from animal trials suggest that desmethylolanzapine seems to be pharmacologically inactive [18]. However, drug–drug interactions can lead to higher serum concentrations of metabolites like it was shown for the interaction between clozapine and fluvoxamine [55]. Thus information about the metabolite concentrations can be useful to elucidate the mechanisms of interaction. Beyond that, for use of the method in clinical routine, it was necessary to control if the metabolite, does not interfere with the detection of olanzapine. Using the described method, desmethylolanzapine was well separated from olanzapine (Fig. 2B).

Three clinically relevant disturbing interferences with the detection of olanzapine could be observed: carbamazepine, pipamperone and melperone, drugs often used in clinical routine. Nevertheless, the separation of these compounds from olanzapine can easily be attained by increasing the water content of the analytical eluent.

Fluperlapine was found to be a suitable internal standard. It has a structure similar to clozapine and olanzapine. The retention time was well reproducible about 3 min longer than for clozapine and 7 min longer than for olanzapine, allowing base line separation (Fig. 2B).

To our knowledge this is the first report describing a method which allows the separation of both clozapine and olanzapine and their demethylated metabolites using the same automated isocratic HPLC system. Each sample can be analysed separately in combining sample pretreatment directly to the HPLC. The on-line pretreatment allows the complete analysis of a sample within 1 h, because it is not necessary to collect a batch of samples before the start of the extraction process. In addition, this is the first method enabling the detection of the demethylated metabolite of olanzapine. To our knowledge all methods described in literature so far need time consuming off-line sample preparation and in part electrochemical detection, which is not practical for clinical routine measurements.

In conclusion, the fully automated column-switching HPLC-method described here seems advantageous over other methods reported so far in literature. The described method has sufficient accuracy and precision and is thus suitable for therapeutic drug monitoring. It is a most rapid method which is of importance, not only in cases of intoxications, but also for immediate medical decisions in clinical routine.

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