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Analysis of clozapine and norclozapine by high-performance liquid chromatography

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Abstract

A simple and reliable method for analyzing the concentrations of clozapine and its biologically active metabolite, norclozapine, in human serum or plasma has been developed. This method is based on reversed-phase high-performance liquid chromatography (HPLC) with automated solid-phase extraction (SPE). For HPLC analysis, samples and standards are prepared with an ASPEC automatic sample preparator using 100 mg Bond-Elut C_{18} SPE columns. The HPLC assay is an isocratic method with a mobile phase of acetonitrile–methanol–10 m*M* dipotassium hydrogenphosphate, pH 3.7 (30:2:100, v/v/v) at a flow-rate of 1.5 ml/min with a C_8 reversed-phase column. Detection is performed with a diode array detector set at 220 nm and with peak purity analyses at 210–365 nm. The absolute recovery varied from 85 and 95%. The intra-assay coefficients of variation (C.V.s) were from 4.2 to 8.0% and the inter-assay C.V.s were from 1.1 to 9.3% at therapeutic drug concentrations. The detection limit is 15 nmol/l. The method has been developed for use in a clinical laboratory for therapeutic drug monitoring. © 1997 Elsevier Science B.V.

Keywords: Clozapine; Norclozapine

1. Introduction

Clozapine, 8-chloro-11-(methyl-1-piperazinyl)-5H-dibenzo[*b*,*e*]diazepine, is classified structurally as an atypical neuroleptic. It is mainly used in patients who do not respond to classical neuroleptics (e.g. phenothiazines) [1]. Clozapine has high anti-psychotic activity, but it does not induce extrapyramidal side-effects and, in this respect, it differs from other neuroleptics. However, due to its hematological side-effects (agranulocytosis), it was withdrawn from markets in all countries in 1975 [2]. However, in recent years, clozapine has been re-introduced and, because of its high efficacy in certain schizophrenic patients, it is increasingly used [3,4], with cautious follow-up of the safety parameters (e.g. blood granulocyte count).

Clozapine is metabolized to N-desmethylclozapine and clozapine-N-oxide [1]. The biologically active metabolite, N-desmethylclozapine, has weaker anti-psychotic effects and the concentration in serum is usually lower when compared to that of clozapine. The other metabolite, clozapine-N-oxide, is present in patients' serum only in minor amounts. Several reports have shown that the dose and effect of clozapine are not linearly related, but there is a clear correlation between response and clozapine serum or plasma concentration [5–9]. The metabolites of clozapine seem to influence the metabolism of the parent drug, leading to lower elimination. Some

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reports have shown that other drugs may affect clozapine metabolism and could lead to changes in its serum concentrations [10–14]. The hematological side-effect is not related to serum concentration, but many other side-effects are predominantly related to high clozapine serum concentrations [8,15].

A number of methods have been published for analyzing serum concentrations of clozapine and norclozapine [16–24], some of which are based on gas chromatography and others on liquid chromatography. An extensive sample preparation method is always needed in all chromatographic methods. In some methods, sample purification is based on liquid–liquid extraction. The preferred technique to isolate and purify drugs from a serum sample is solid-phase extraction [24,25].

In this study, a sensitive and automated solid-phase extraction (SPE) system-based purification method was combined with a specific isocratic high-performance liquid chromatography (HPLC) method for analyzing clozapine and norclozapine concentrations in human serum samples. Furthermore, patients' data from routine therapeutic drug monitoring (TDM) is included, in which the clozapine serum concentration is compared to the clozapine daily dose and also to norclozapine serum concentrations.

2. Experimental

2.1. Reagents

Clozapine and norclozapine were purchased from Sandoz (Basel, Switzerland). Protriptyline was from Ciba-Geigy (Basel, Switzerland). HPLC-grade acetonitrile and methanol as well as other reagents of analytical grade were obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation

The samples and standards were prepared with an ASPEC automatic sample preparator (Gilson Medical Electronics, Villiers-le-Bel, France) using 100 mg Bond-Elut C_{18} solid-phase extraction columns (Varian, Sunnyvale, CA, USA). A Perkin-Elmer liquid chromatography system (ISS-200 autosampler, Binary LC 250 pump, 235C diode-array detector), controlled by Turbochrom chromatography workstation (Perkin-Elmer, Norwalk, CT, USA), was used. Chromatographic separations were achieved using a Select-B 125×4 mm 5 μm C_8 analytical column (Merck) at room temperature. The elution was isocratic with a mobile phase of acetonitrile–methanol–10 m*M* dipotassium hydrogenphosphate, pH 3.7 (30:2:100) at a flow-rate of 1.5 ml/min. The drugs were detected at 220 nm and peak purity analyses were performed at 210–365 nm.

2.3. Preparation of standard and control solutions

The stock solutions of clozapine and norclozapine were made by dissolving 10 mg of clozapine in 10 ml of methanol and by dissolving 10 mg of norclozapine in 10 ml methanol. Finally, the standards and controls were dispensed in a 50 g/l albumin solution (ionic strength, 0.15 mol/l with NaCl) as follows. The appropriate volumes of stock solutions were evaporated to dryness at 50°C with a gentle stream of air and the residue was dissolved in 100 ml of albumin solution by mixing it gently using a magnetic stirrer at room temperature for 30 min. The stock solution of internal standard was made by dissolving 10 mg of protriptyline in 10 ml of methanol. Before analysis, the internal standard solution was diluted to its final concentration (660 nmol/l) with a 5% solution of methanol in water.

2.4. Sample collection

Blood samples to be used for determination of clozapine and norclozapine concentrations were collected as steady-state concentration samples. After blood coagulation, the samples were centrifuged 1500 *g* for 10 min, the serum was separated and stored at +4°C until analysis, which occurred within one week.

2.5. Sample preparation

The ASPEC system was programmed first to add and mix 200 μl of internal standard solution to a 1.0-ml sample (and then to prepare each sample separately). The extraction column was activated with 2.0 ml of methanol and then washed with 2.0

Table 1
Absolute recovery, inter- and intra-assay precision for compounds present at different concentrations

Compound	Concentration added (nmol/l)	Absolute recovery (%)	Intra-assay C.V. (n=10) (%)	Inter-assay C.V. (n=30) (%)
Norclozapine	250	95	9.3	6.4
	875	88	2.3	5.8
	4500	86	1.1	6.2
Clozapine	250	89	6.9	8.0
	875	85	1.7	4.2
	4500	85	1.7	5.3

ml of HPLC-grade water. The whole sample was then added to the column, followed by two washing steps with 2.0 ml of HPLC-grade water. Next, the pH of the column was made acidic using 1.0 ml of a 10% methanol solution in 0.25 *M* hydrochloric acid. Before the elution step, the column was washed with 500 μl of acetonitrile. The analytes were eluted with two 500 μl portions of 10 m*M* acetic acid, 5 m*M* diethylamine in methanol. Finally, the eluates were evaporated to dryness with a Techne sample concentrator (Techne, Cambridge, UK) at 37°C with a gentle stream of air. The residue was reconstituted in 100 μl of mobile phase. The injection volume was 40 μl.

3. Results

3.1. Recovery and precision

To test the efficiency of the extraction procedure, absolute recoveries from spiked serum samples were analyzed at three concentrations with triplicate measurements (Table 1). The recovery ranged from 85 to 95%. Table 1 shows the precision of the method. With the automated sample preparation method, the C.V.s (%) are low. The intra-assay C.V. ranged from 1.1–2.3% with therapeutic and toxic concentrations and, even with low concentrations, it was below 10%. The inter-assay concentration varied from 4.2 to 8.0% at all tested concentrations. The relative recovery tested with spiked serum samples at five concentrations ranged from 84 to 105%.

3.2. Linearity

The linearity of the assay was determined at five concentrations, with duplicate measurements. The correlation coefficients were better than 0.999. The linearity of the assay is presented in Table 2. The detection limit is 15 nmol/l. Detection limit calculations were based on a signal-to-noise ratio of 5:1.

3.3. Chromatography and selectivity

Fig. 1 shows chromatograms from a standard and from a patient's sample. The separation of the analyzed compounds is excellent, even with very high concentrations. Retention indices of the analyzed compounds and of 30 other drugs that are also extracted with this method are shown in Table 3. Of the compounds analyzed, only desmethylcitalopram could cause a problem, if it were present at very high concentrations. Many antiepileptic drugs (10-hy-

Table 2
Non-weighted linear regression equations for the assayed drugs

Compound	Concentration range added (nmol/l)	Equation of the non-weighted linear regression curve ($y=ax+b$)	Correlation coefficient (r)
Norclozapine	36–13500	$y=0.017x+1.838$	0.9992
Clozapine	36–13500	$y=0.013x+1.876$	0.9996

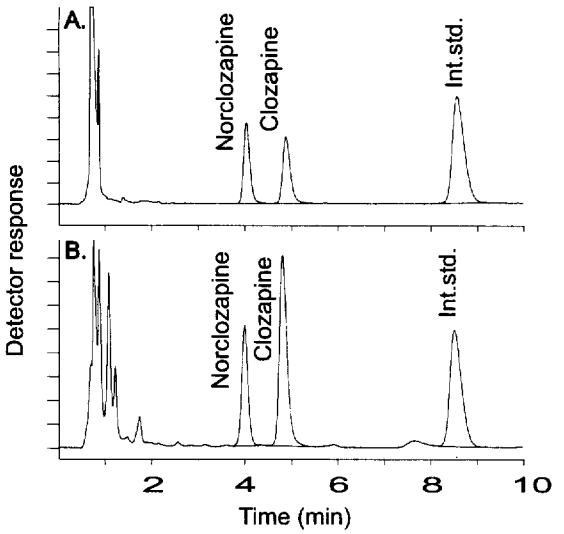

Fig. 1. Chromatograms obtained on analysis of an extracted standard sample (A) containing clozapine (1050 nmol/l) and norclozapine (1080 nmol/l) and of a patient's sample (B) containing clozapine (2890 nmol/l) and norclozapine (1420 nmol/l).

droxy-carbazepine, carbamazepine, carbamazepine-10,11-epoxide, ethosuximide, oxcarbazepine, pentobarbital, phenobarbital, primidone) and benzodiazepines (clobazam, flunitrazepam, norclobazam, oxazepam, temazepam) are excluded with our extraction method.

3.4. Serum concentrations in patients

The data of patients TDM serum concentrations of clozapine and norclozapine, determined with the present method over a six-month period, were combined. Fig. 2 shows the norclozapine concentration as a function of clozapine concentration in 333 samples from patients. The correlation coefficient (r) is 0.873. The mean clozapine concentration in serum was 1591 nmol/l and the mean norclozapine concentration in serum was 1075 nmol/l. Fig. 3 clarifies the results by showing the large individual variation in the norclozapine concentration when compared to the same concentration of clozapine. In some patients, the concentration of norclozapine could be over two times higher than the clozapine concentration. In most of the patients, the norclozapine concentration was somewhat lower than the clozapine concentration. Fig. 4 shows the correlation between clozapine and norclozapine concentrations in serum following daily doses of clozapine. A correlation between dose and concentration is clearly seen in the figure, but it is also possible to see the extensive inter-individual variation within the dose groups.

Table 3
Specificity of the assay

Compound	Retention index compared to internal standard (protriptyline)
Medazepam	0.14
Thioridazine	0.36
Trazodone	0.41
Nordoxepin	0.43
Midazolam	0.43
Norclozapine	0.49
Mianserin	0.56
Desmethylcitalopram	0.58
Clozapine	0.59
Citalopram	0.66
Thiotixene	0.69
Doxepin	0.71
Nitrazepam	0.74
Normaprotiline	0.92
Desipramine	0.94
Protriptyline	1.00
Haloperidol	1.02
Imipramine	1.08
Nortriptyline	1.11
Maprotiline	1.19
Amitriptyline	1.29
Levomepromazine	1.34
Norfluoxetine	1.41
Nortrimipramine	1.44
Fluoxetine	1.69
Norclomipramine	1.83
Trimipramine	1.85
Diazepam	1.90
Chlorprotixene	2.05
Chlorpromazine	2.07
Clomipramine	2.13

4. Discussion

In this study, a TDM application for the simultaneous analysis of clozapine and its biologically active metabolite, norclozapine, with HPLC–diode array detection (DAD) is reported. This HPLC method provides an excellent option for analyzing

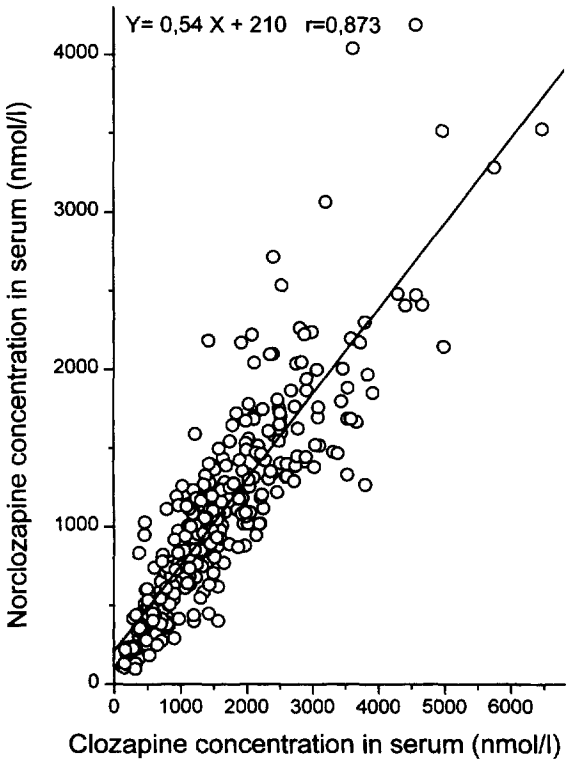

Fig. 2. Correlation between clozapine and norclozapine concentrations in 333 samples from patients. The concentrations of both compounds were determined using the presented method. (y=0.544x+210; r=0.873).

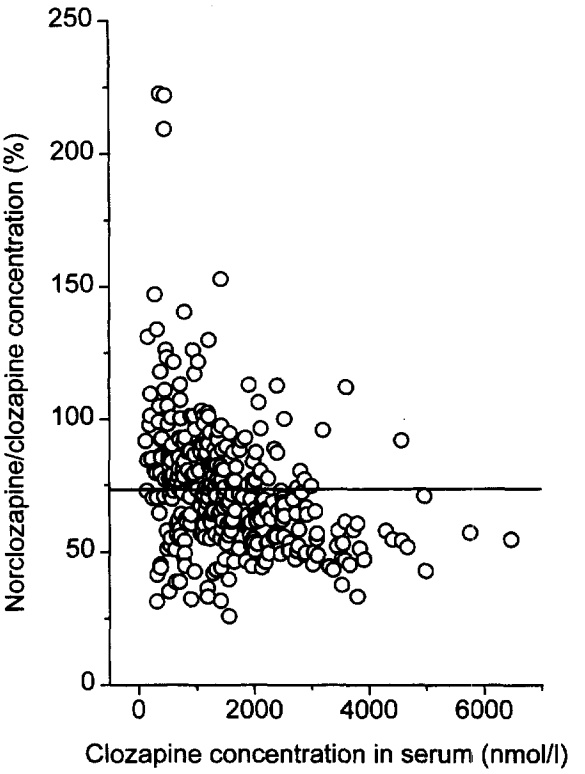

Fig. 3. Correlation of clozapine concentration in serum to the ratio of norclozapine/clozapine concentrations.

clozapine and norclozapine concentrations in serum. Those patients receiving clozapine have invariably many other drugs in their therapy and this could pose a problem for the analytical method. In this particular method, all the antidepressants and neuroleptics tested were eluted within 20 min of injection. Only very high concentrations of desmethylcitalopram may interfere with the assay. Also, many benzodiazepines and antiepileptic drugs are excluded by the SPE method presented. It also minimizes the hands-on time by making use of an automated sample preparation method. The method could even be converted to an on-line method by using the sample processor as an autosampler. If we used the method to analyze the other antidepressant drugs mentioned in Table 3, we had to concentrate the final eluates ten-fold before HPLC analysis. The sensitivity of the assay was satisfactory, even with low concentrations. The detection limit was as low as 15 nmol/l and the limit of quantitation was about 50 nmol/l, where the total variation exceeds 20%.

The mean ratio of norclozapine to clozapine was 0.73, which is almost the same as that reported by Olesen and Poulsen [24] and Olesen et al. [8], who had smaller groups of patients, and it also very similar to the values described by other groups [15,21]. Many reports show that a steady-state plasma concentration of about 1065–1130 nmol/l (350–370 ng/ml) distinguishes responders from non-responders [1,6,7,26,27]. As can be seen from Fig. 4, the mean clozapine concentration in our patients' serum is commonly above this concentration limit in most of the daily doses. The high inter-individual variation in the serum levels of clozapine and norclozapine in all daily-dose groups could be due to the high variation in clozapine bioavailability [27,28]. The clozapine and norclozapine concentrations in serum are closely corre-

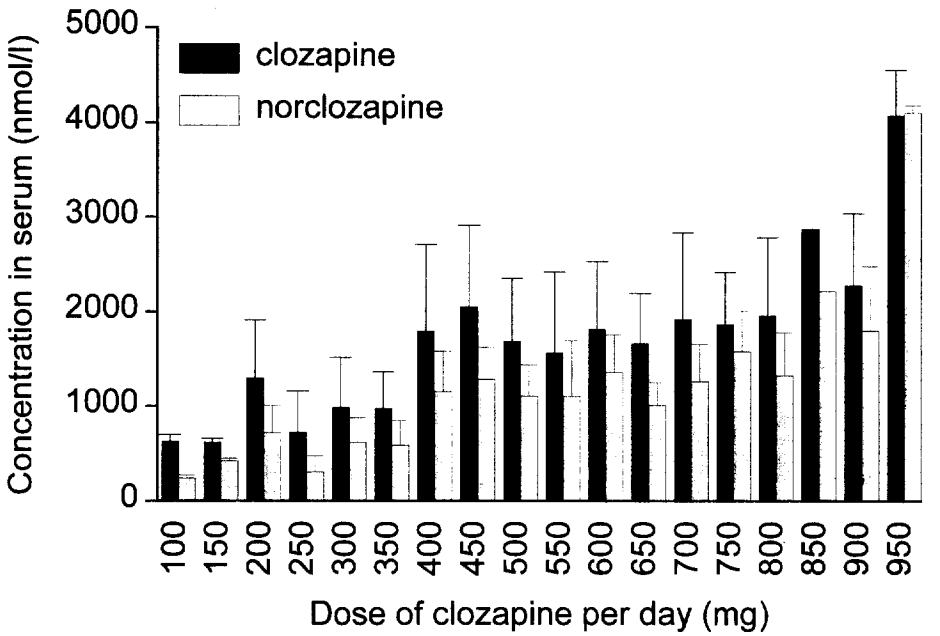

Fig. 4. Correlation between the clozapine and norclozapine concentrations in serum to the daily dose of clozapine administered (in mg per day). The serum samples were from 228 patients treated with clozapine.

lated to each other, and one might conclude that it is not necessary to analyze norclozapine serum concentrations. However, as can be seen from Fig. 3, the variation in the norclozapine concentration is large with respect to the corresponding clozapine concentration.

In conclusion, this reversed-phase HPLC–DAD method, with automated sample preparation, provides an excellent, inexpensive, reliable and time-saving assay system in TDM purposes for a clinical laboratory. The assay is also an excellent choice for clinical trials, since all validation parameters meet the recently recommended criteria [29]. The method is applied to the routine TDM service in our laboratory.

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