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Short communication

Determination of clozapine and its *N*-desmethyl metabolite by high-performance liquid chromatography with ultraviolet detection

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Abstract

A rapid high-performance liquid chromatographic method has been developed for the simultaneous determination of the atypical antipsychotic drug clozapine and its principal metabolite, *N*-desmethyl clozapine in human plasma. After liquid–liquid extraction the compounds were separated in a reversed-phase column and measured by ultraviolet absorption at 230 nm. For both compounds inter-day variations were <3.8%, and, based on a plasma sample volume of 2 ml, the limits of quantification were 25 ng/ml. Analytical interference from coadministered psychoactive drugs and their metabolites was also studied, and no interference was found from the most commonly used antidepressants and antipsychotic drugs. The assay is sufficiently sensitive and easy to use for the analysis of plasma samples in human clinical trials and therapeutic drug monitoring.

Keywords: Clozapine; *N*-desmethyl clozapine

1. Introduction

Clozapine is an atypical antipsychotic agent with a dibenzodiazepine structure (Fig. 1). Unlike conventional neuroleptics, clozapine is less likely to cause extrapyramidal side-effects [1]. The use of clozapine has a substantial impact on the management of psychotic disorders: over 30% of patients who are otherwise non-responsive or intolerant of standard neuroleptic therapy respond to this drug, which is therefore of major importance as a second-choice drug in the treatment of psychosis [2]. However, despite these advantages, its use has been severely limited by the occurrence of agranulocytosis in 1–2% of the patient population [1,3].

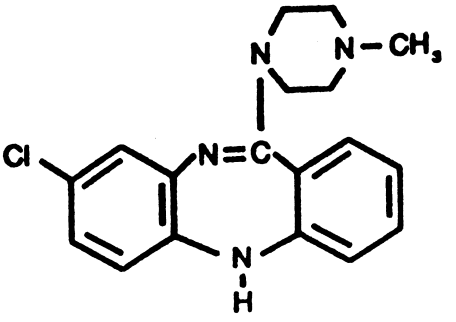

Fig. 1. Chemical structure of clozapine.

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In the human body, hepatic microsomal enzymes metabolize clozapine extensively: only a trace amount of the unchanged drug is recovered in the urine. The main metabolites are *N*-desmethyl clozapine and clozapine *N*-oxide. The pharmacological activity of these two metabolites appears to be much lower than the parent compound, and their plasma concentrations range approximately between 75 and 90% for *N*-desmethyl clozapine and 10–35% for clozapine *N*-oxide of the clozapine concentrations [4].

Genetic and environmental factors influence the drug's metabolism, which may result in variability of clozapine plasma levels. Clozapine is mainly (~70%) metabolized by CYP1A2, and to a lesser extent by CYP3A4 [5,6]. However, it appears it may also be metabolized by CYP2D6 [7].

Some studies have investigated the relationship between clinical response and plasma clozapine concentrations in the range of 100–800 ng/ml [8,9]. Significantly higher plasma clozapine concentrations were found in patients under 35 years of age than in those over 45 years of age. Plasma clozapine concentrations in men were only ~69.3% of those in women. The average plasma concentrations of clozapine were found to be 20% lower in smokers than in non-smokers [10]. In view of these effects, routine therapeutic drug monitoring could therefore be useful in clinical practice. In spite of the relationship between dose and plasma concentration, the measurement of plasma levels of clozapine and its metabolites might be useful in the study of possible interactions [11] with drugs metabolized by the cytochromes (CYP1A2, CYP3A4) implicated in clozapine metabolism, such us benzodiazepines, arrhythmic drugs, antidepressants or caffeine [12]. Measurement of clozapine plasma levels can also be a useful tool for compliance monitoring, a very important issue in psychiatric patients.

In the literature, high-performance liquid chromatographic methods have been described using liquid–liquid and solid-phase extraction procedures to determine the plasma levels of clozapine and its metabolites [13–21]. However many of them need special equipment or are expensive.

The aim of the present study was to develop a simple, easy-to-handle, accurate and inexpensive method to assay clozapine and its main metabolite *N*-desmethyl-clozapine in plasma using liquid–liquid extraction and reversed-phase high-performance liquid chromatography with ultraviolet detection. The conditions fulfil the requirements of a method to be used for routine therapeutic drug monitoring.

2. Experimental

2.1. Chemicals

Clozapine (CZP), *N*-desmethyl clozapine (NCZ) and protriptyline (PRO) were from Research Biochemicals (Natick, MA, USA). Analytical grade potassium dihydrogenphosphate, perchloric acid, phosphoric acid, and high-performance liquid chromatography (HPLC) grade acetonitrile were purchased from Merck (Darmstadt, Germany). Water was de-ionized and purified by a Milli-Q Water processing system (Millipore, Molsheim, France).

2.2. Instrumentation

The liquid chromatographic system consisted of a Beckman model 110B pump, and a Beckman 166 programmable detector module coupled to a 386 PC with Beckman Gold software V.3.2 (Beckman Instruments, Fullertone, CA, USA). The mobile phase was a mixture of 5.0 g potassium dihydrogenphosphate, 320 ml acetonitrile, 190 μl perchloric acid, and 100 μl phosphoric acid completed to 1 l with water. Before analysis the mobile phase was filtered through a 0.22-μm filter (Millipore, Ireland). Separation was carried out at room temperature using an ODS Beckman Partisil (5 μ; 150×4.6 mm I.D.) column. The flow-rate was set at 1 ml/min and detection wavelength at 230 nm.

The ratios of drug and metabolite to PRO were calculated from the recorded peak heights. The results obtained from plasma standards spiked with different known amounts of CZP and NCZ were used to calculate the factor for multiplying the ratios between heights of unknown and PRO peaks.

2.3. Drug solutions

Stock solutions of CZP, NCZ and internal standard (PRO) were prepared by dissolving the pure sub-

stance in methanol–water (1:1). CZP was dissolved to a concentration of 0.5 mg/ml. NCZ and PRO were dissolved to a concentration of 1.0 mg/ml. The working concentrations for the compounds were 0.01 mg/ml for CZP and NCZ, and 0.005 mg/ml for PRO.

Blank plasma samples were spiked with standard solutions to obtain final concentrations of 50–500 ng/ml of CZP and NCZ. PRO was added to the plasma before analysis by pipetting 100 μl into 2 ml of plasma.

2.4. Extraction

Sample preparation was carried out by liquid–liquid extraction. Briefly, 2 ml of human plasma was pipetted into a 10-ml polypropylene tube, and 10–100 μl of 0.01 mg/ml CZP and NCZ, 100 μl of 0.005 mg/ml PRO and 200 μl of 2 *M* sodium hydroxide were added. The plasma was mixed with a hand vortexer, and then 5 ml of hexane:isoamyl alcohol (98.5:1.5, v/v) was added to the tubes, which were then capped and placed in a shaker for 20 min. The tubes were then centrifuged at 3600 rpm for 5 min. The organic phase was drawn off and put into a 10-ml polypropylene tube, to which 120 μl of 0.1 *M* hydrochloric acid was added. This tube was vortexed and centrifuged at 3600 rpm for 5 min. The organic layer was drawn off and evaporated at 40°C in an N_2 atmosphere for 2 h. The residue was redissolved in 110 μl of 0.1 *M* hydrochloric acid and an aliquot of 100 μl of this solution was injected into the HPLC system for analysis.

3. Results

3.1. Chromatography

The retention times were 3.9, 5.1 and 12.4 min, for NCZ, CZP and PRO, respectively. Under the isocratic conditions, all compounds eluted within 15 min. Fig. 2A shows a chromatogram of extracted plasma from a healthy drug-free blood donor spiked with internal standard. Fig. 2B shows an example of the clinical use of the present method in a 30-year-old male schizophrenic patient treated with 175 mg/day of clozapine: plasma levels are 78 ng/ml NCZ and 100 ng/ml CZP. The optimal wavelength was 230 nm under the present conditions.

3.2. Extraction efficiency

The absolute recoveries of CZP and NCZ were determined by comparing the peak heights of extracted standards with the peak heights of standards injected directly into the HPLC system. Recovery of the analytes (0–500 ng/ml) was 92% for CZP and 37% for NCZ.

3.3. Precision, linearity and detection limit

The assay validation was performed over 3 days with spiked plasma samples at concentrations of CZP and NCZ of 50–500 ng/ml containing 100 μl of internal standard. The inter-assay precision was determined by measuring the spiked plasma concentrations for each calibration sample from its corresponding calibration curve. Samples were analyzed three times at each concentration level. The inter-assay precision was calculated as the coefficient of variation (% C.V.). The % C.V. was less than 3.8% for both CZP and NZC at all calibration points. The intra-assay precision was determined by analyzing four spiked plasma samples at each concentration on the same day: the % C.V. was less than 1.0% for both substances.

Blank plasma samples were spiked with CZP and NCZ in the range 50–500 ng/ml. At each concentration level ($n=5$), the spiked samples were analyzed in duplicate. The standard curves for CZP and NCZ were linear in each case with correlation coefficients of 0.9956 and 0.9938, respectively. The limit of detection, defined as a signal-to-noise ratio of 4, was 1.8 ng/ml for CLZ and 7.6 ng/ml for NCZ. The limit of quantification determined in triplicate was 25 ng/ml for both CLZ and NCZ.

3.4. Analysis of interference from other drugs

A total of 49 drugs that could be used in combination with clozapine — including 11 antipsychotics, 10 antidepressants, 16 benzodiazepines, and 12 others — were tested for possible chromatographic

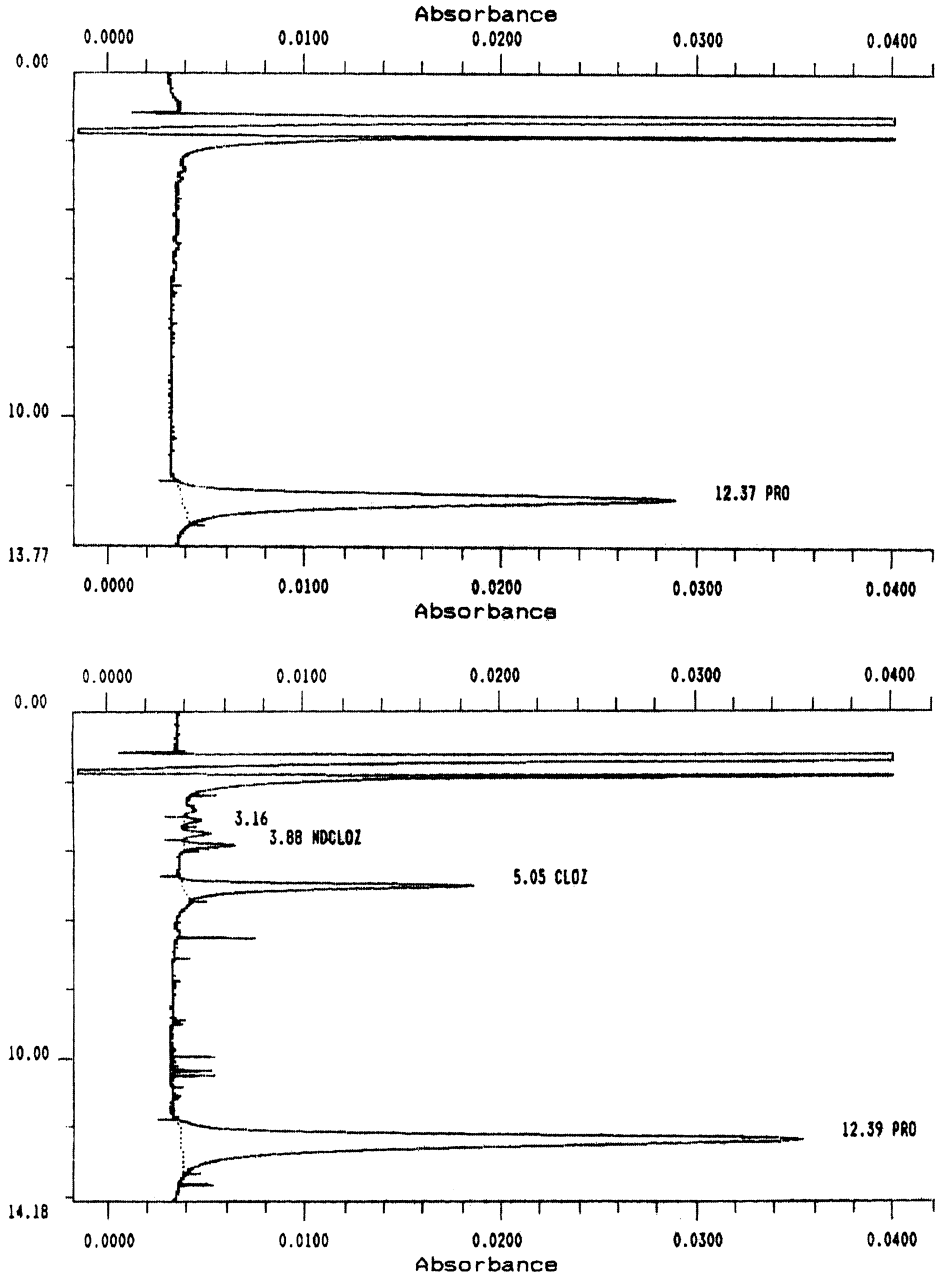

Fig. 2. Chromatograms: (A) human plasma from healthy drug-free blood donor spiked with internal standard (protriptyline); (B) patient plasma sample receiving 175 mg/day clozapine. Peaks are: NDCLOZ: *N*-desmethyl clozapine; CLOZ: clozapine; and PRO: protriptyline.

interference. Testing standard solutions containing these drugs revealed no interference in any of the cases. The retention times for the most important psychotropic drugs are given in Table 1.

4. Discussion

The aim of the study was to establish a simple HPLC method suitable for simultaneous determi-

Table 1
Relative retention times (t_R) from internal standard (protriptyline 12.4) of some important psychotropic drugs that could be used in combination with clozapine, out of 49 tested[a]

Drug	t_R (min)	Drug	t_R (min)
Clozapine	−7.2	Chlorpromazine	−4.5
N-Desmethyl clozapine	−8.5	Haloperidol	−2.4
Protriptyline (I.S.)	0	Perphenazine	n.d.
Trimipramine	−3.2	Prometazine	−6.8
Amitriptyline	−3.5	Alprazolam	1.2
Imipramine	1.5	Diazepam	−7.7
Maprotiline	2.6	Lorazepam	−1.3
Fluoxetine	n.d.	Pinazepam	−6.5
Fluvoxamine	n.d.	Bentazepam	−8.3

[a] n.d., not detectable.

nation of clozapine and its major metabolite, *N*-desmethyl clozapine, in human plasma samples, and which can be used with easy-to-handle equipment for the routine monitoring of plasma levels in a general psychiatric department or unit. Therefore the method was designed to be rapid, simple, specific, easy to perform, and inexpensive. Protriptyline was taken to be an acceptable internal standard because it exhibits similar linear extraction properties, i.e. hydrophobicity, basic pK_a, and chromatographs close to the two analyte peaks.

Baseline HPLC separation was obtained for all assayed compounds within less than 15 min. The procedure enabled the determination of both CZP and NCZ using a liquid–liquid extraction method and HPLC with ultraviolet detection. Lovdahl et al. [14] report extraction recoveries of 84% for CZP and 28% for NCZ with a similar extraction method. The present method proved to be more efficient, with extraction recoveries of 92% for CZP and 37% for NCZ. The intra-day and inter-day variations of CZP and NCZ were found to be better than in most other published methods: <1 versus 4% for intra-day and <3.8 versus 6% for inter-day variations [13–21].

CZP and its metabolite were simultaneously quantified at concentrations as low as 25 ng/ml. Plasma levels of these drugs at therapeutically effective doses (100–800 ng/ml) are far above the detection limit of this method [22]. Since the method was aimed at routine plasma level measurement of clozapine, the range of reliable response was designed to cover the usual plasma concentration levels of patients taking clozapine.

With the HPLC system described here, no other important psychiatric drug showed any interference with CZP, its metabolite or the internal standard. The most significant advantage of the present method is that the chromatographic conditions are simple to adapt for the analysis of the most commonly used psychotropic drugs (e.g. haloperidol [23]). This method is therefore suitable for use in clinical practice for therapeutic drug monitoring.

Acknowledgements

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