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Note

Rapid and sensitive determination of clozapine in human plasma using high-performance liquid chromatography and amperometric detection

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Clozapine (Leponex®) is an atypical neuroleptic with novel properties, including a high antipsychotic activity and the lack of extrapyramidal motor side-effects. Determination of clozapine plasma levels by gas chromatography [1], thin-layer chromatography [2] and radioimmunoassay [3–5] has been described, but a radioimmunoassay kit for clinical routine is not commercially available. A method using high-performance liquid chromatography (HPLC) and UV detection has been developed in our laboratory by Haring et al. [6]. This method, however, requires at least 2 ml of plasma for a single determination and a rather time-consuming extraction. The neuroleptic drugs fluphenazine and perphenazine, which share structural similarities with clozapine, are electrochemically active [7]. The aim of the present study was to develop an HPLC method with amperometric detection after a simple extraction step to reduce the analysis time and to increase the sensitivity so that lower sample volumes would be required.

EXPERIMENTAL

Chemicals

For all experiments water from a Milli-Q system (Millipore, Milford, MA, U.S.A.), acetonitrile for chromatography (Merck, Darmstadt, F.R.G.) and analytical *n*-hexane for extraction were used. Clozapine, 8-chloro-11-(4′-

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methyl)piperazino-5-dibenzo[*b,e*]-1,4-diazepine was a gift from Sandoz (Basel, Switzerland) and dibenzepine a gift from Biochemie Kundl (Kundl, Austria).

Equipment

HPLC was carried out with a double-piston pump (LKB Model 2150), a 100-μl loop injector (Valco) and an amperometric detector (Bioanalytical Systems LC4B; range, 20 nA full scale; potential +0.7 V vs. Ag/AgCl reference electrode; filter, 0.1 Hz). Quantification was performed with a Shimadzu C-R1A integrator.

Extraction

In order to simplify the extraction of clozapine for easier routine measurements, the three-step extraction method described by Haring et al. [6] was modified as follows: 5 μl of 7 *M* sodium hydroxide, 5 μl of internal standard (1 mg of dibenzepine per 1 ml of methanol) and 0.5 ml of *n*-hexane were added to 100 μl of plasma in disposable 10 cm × 1.2 cm I.D. glass tubes. The samples were shaken for 10 s, then centrifuged for 5 min at 3000 *g* (30°C). The phases were cooled to −70°C in a freezer, and the supernatant was decanted and evaporated under a nitrogen stream at 45°C. The residue was dissolved in 130 μl of mobile phase and injected into the HPLC system.

Analysis

Reversed-phase HPLC was performed with a Beckman, 3 μm particle size column (Ultrasphere XL-Octyl; 70 mm × 4.6 mm I.D.) and a pre-column (5 mm × 4.6 mm I.D.) of the same material. For isocratic chromatography the following eluent was used as mobile phase: 90% (v/v) acetonitrile, 10% (v/v) HPLC-grade water, 0.25 m*M* ammonium acetate. The flow-rate was 0.6 ml/min. For quantification the peak height was used and compared with that of dibenzepine as an internal standard. Extraction and recovery of dibenzepine are similar to those of clozapine (unpublished data). The total recovery was determined by calculating the amount of dibenzepine. The method was compared with the HPLC method described by Haring et al. [6], carried out with UV detection and a plasma volume of 2 ml.

Patient samples

Blood samples were taken from inpatient schizophrenics (five male and one female), treated with 50–600 mg of clozapine (Leponex), 4 h after the last administration. The blood (1 ml from the vein or 0.4 ml from the finger tip) was collected in polypropylene vials containing 10 μl of Heparin-Novo® (5000 I.U./ml). After immediate centrifugation for 10 min at 3000 *g* and 4°C, plasma was stored at −20°C and analysed within 24 h. A blood sample of 10 ml was taken from the vein of those patients where clozapine was determined by UV detection and electrochemical detection (ED) and treated as above. From the

obtained plasma, 100 μl were used for ED. The remaining plasma was determined as previously described [6] using UV detection. One sample from the vein and one from the finger tip from the same patient were analysed simultaneously.

RESULTS AND DISCUSSION

Clozapine was found to be electrochemically active, reaching the maximum signal at an oxidation potential of +0.7 V vs. Ag/AgCl (Fig. 1). Under these conditions the retention times were 4.7 min for clozapine and 7.1 min for dibenzepine. A standard curve ($r=0.9928, p=0.9999, n=26$) was established by extraction of different amounts of clozapine (25–400 ng/ml) from the stripped plasma, which was purified from organic substances by charcoal treatment (Fig. 2). The intra-assay variation was found to be 8.8%, obtained by repeated measurement ($n=26$) of the same sample. The mean deviation of all data points from the common regression line was 13.2%, indicating the inter-assay variation. The average (±S.E.M.) extraction recovery was calculated as 26.9±0.9% ($n=21$). The detection limit for clozapine was 500 pg at a signal-to-noise ratio of 5:1 (Fig. 2A). This limit allows the measurement of as little as 20 ng/ml from a sample of 100 μl of plasma. Therefore it is sufficient for routine measurements, because the therapeutic range of clozapine in plasma is between 50 and 600 ng/ml [8–10].

Comparing the present results determined in 100 μl of plasma with the results previously obtained with UV detection in 2 ml of plasma [6] by Student's t-test, there was no significant difference ($t=0.503$; $p=0.623$; $n=34$; Pearsons's correlation coefficient =0.9911; Fig. 3).

In six different patients, clozapine plasma levels were determined from the vein as well as from the finger tip. Plasma levels determined in samples from either the vein or the finger tip were not significantly different (levels divided by dose and compared with paired t-test; $p=0.173$; $n=6$), although the mean

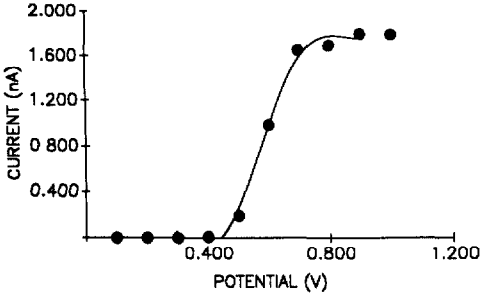

Fig. 1. Analysis of 10 ng of clozapine at different oxidation potentials. Analytical conditions as described in Experimental.

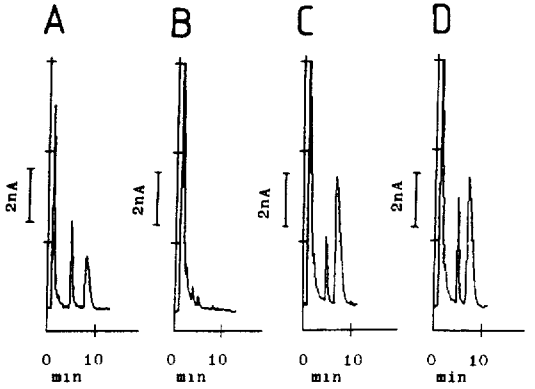

Fig. 2. (A) Chromatogram of a standard solution of clozapine (3.3 ng on column) and dibenzepine (666.6 ng on column). (B) Chromatogram of an extract of charcoal-treated 'pure' plasma. (C) Chromatogram of an extract of a charcoal-stripped plasma (50 ng/ml clozapine); dibenzepine was used as an internal standard. (D) Chromatogram of a venous plasma from a patient. The patient received a daily dose of clozapine (Leponex) of 100 mg; a plasma concentration of 85.3 ng/ml was measured. Retention times were 4.7 min for clozapine and 7.1 min for dibenzepine. Analytical conditions as described in Experimental.

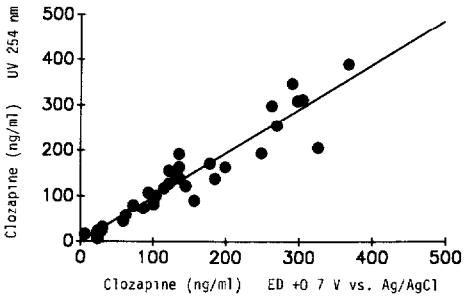

Fig. 3. Clozapine plasma levels in patients receiving different doses, determined simultaneously by either UV (254 nm) or amperometric (+0.7 V vs. Ag/AgCl) detection. Pearson's correlation coefficient = 0.9911 ($n = 34$). Analytical conditions as described in Experimental.

value of the concentration measured in plasma obtained from the finger tip was somewhat higher (Table I).

The principal metabolites of clozapine in humans (the corresponding N-oxide and N-desmethyl derivatives [11]) are not commercially available and were not tested in the present study. There was, however, no indication that any other peak in plasma from untreated patients overlapped the clozapine peak. The following drugs frequently used in psychiatry do not interfere with clozapine: haloperidol, fluphenazine, amitryptiline, nortryptiline, biperiden, imipramine, desipramine and chlorpromazine. In summary, the present study allows the quantitative determination of clozapine, after a single extraction

TABLE I

CLOZAPINE PLASMA LEVELS IN PATIENTS

Values measured from 100 μl of plasma.

Patient	Dose (mg)	Plasma level (ng/ml)	
		Finger tip	Vein
1	50	157	108
2	50	45	45
3	75	94	45
4	100	87	101
5	500	291	277
6	600	319	207

step, in low volumes of blood with a sensitivity comparable with radioimmunoassay and gas chromatography.

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