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Note

Clozapine plasma levels determined by high-performance liquid chromatography with ultraviolet detection

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The evaluation of the plasma concentrations of cardiac glycosides, antibiotics and antiepileptics has already become part of clinical routine. In psychiatry, however, drug monitoring has been commonly used only for lithium. Plasma levels of antidepressants are rarely measured and those of neuroleptics are hardly ever determined routinely, because the sensitivity and accuracy of available methods are insufficient and because the significance of plasma level determinations is still in doubt. But plasma level determinations for clinical applications are highly desirable for the following reasons: compliance problems caused by the adverse side-effects of neuroleptics, the wish to keep drug exposure to the minimum necessary, the existence of a therapeutic range and medicolegal problems [1].

Clozapine, 8-chloro-11-(4′-methyl)piperazino-5-dibenzo[*b,e*]-1,4-diazepine (Fig. 1A), is a neuroleptic drug finding increasing use in inpatient and outpatient management. The special feature of this substance is its antipsychotic acitivity,

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Fig. 1. Structural formulae of clozapine (A) and fluphenazine (B).

together with a lack of extrapyramidal motor side-effects, which distinguishes it from conventional high-potency neuroleptics. Determination of clozapine blood levels by chromatographic methods and radioimmunoassay [2,3] has been described, but radioimmunoassay kits are not yet commercially available, and the affinity of clozapine for dopamine receptors, which is utilized by neuroleptic radioreceptor assay, is too low to provide an advantage over UV detection [4].

In the present paper a sensitive and accurate method for the determination of clozapine plasma levels is presented. Among the available chromatographic methods high-performance liquid chromatography (HPLC) was preferred to gas chromatography (GC) and thin-layer chromatography (TLC) because of its sufficient sensitivity and its easy adaptation to routine measurements.

EXPERIMENTAL

Chemicals

For all experiments we used water from a four-stage Milli-Q system (prefilter, charcoal filter, mixed-bed ion exchange, Organex; Millipore, Milford, MA, U.S.A.), acetonitrile for chromatography (Merck, Darmstadt, F.R.G.) and *n*-hexane as organic solvent. The columns (analytical column 250 mm × 2 mm I.D., pre-column 30 mm × 2 mm I.D.) were slurry-packed with octylsilica beads (octyl Si 100 polyol; 5 μm, pore size 10 nm; Serva Feinbiochemica, Heidelberg, F.R.G.) at 400 bar using an apparatus manufactured by Ammann Technik (Kölliken, Switzerland).

Equipment

HPLC was carried out with a single-piston pump (Beckman Model 110A) and loop injector (Beckman Model 210). For absorbance measurements a UV detector (Beckman Model 160, absorbance filter 254 nm) was used. Quantification was performed with a Hewlett-Packard 3390 A integrator.

Stock solutions

Stock solutions were prepared at a concentration of 10 mg/ml in methanol and diluted to 1 μg/ml. The neuroleptics used were fluphenazine hydrochloride (Van

Heyden, Regensburg, F.R.G.) and clozapine (Sandoz, Basle, Switzerland). Plasma standards were prepared by adding an aliquot of this diluted solution; the plasma concentration of methanol did not exceed 10%. Stock solutions were stored at −20°C in the dark for not more than one month.

Extraction

The extraction procedure [5] for clozapine from plasma is set out in Table I.

Analysis

Immediately before analysis, 60 μl of eluent were added to the condensed extract and 20 μl were applied to the column. The first analysis was carried out at a sensitivity of 0.01 a.u.f.s. For isocratic chromatography the mobile phase was acetonitrile–HPLC-grade water (90:10) with 0.25 m*M* ammonium acetate [6]. The flow-rate was 0.5 ml/min. Quantification was performed by integration of peak-area and peak-height measurement; each analysis was done with duplicates of separately extracted samples. The loss during extraction was compensated by

TABLE I

EXTRACTION OF CLOZAPINE FROM HUMAN PLASMA

Stage	Procedure
Blood samples	Draw samples of 20 ml of blood, add 10 drops of Heparin-Novo, 5000 I.E./ml, shake, centrifuge at 3000 *g* for 10 min, use the supernatant for extraction.
Internal standard	Pipette every 2 ml of plasma into a Corex tube (30 ml), add 100 μl of fluphenazine hydrochloride stock solution (2.5 μg fluphenazine per ml methanol), analyse this stock solution separately for calculation of recovery.
Pre-extraction	(1) Add 100 μl of 1 *M* hydrochloric acid. (2) Pipette 5 ml of *n*-hexane into every sample under continuous vortex motion. (3) Shake for 15 s. (4) Centrifuge at 12 000 *g* (35°C) for 10 min. (5) Freeze phases at −80°C (10 min) in the bio-freezer, discard the supernatant (*n*-hexane).
First analytical extraction	(1) Thaw plasma in hot water-bath. (2) Add 100 μl of 7 *M* sodium hydroxide. (3) Pipette 5 ml of *n*-hexane into every sample. (4) Shake for 30 s. (5) Centrifuge at 12 000 *g* (35°C) for 5 min. (6) Liquefy the gelatinous supernatant with a Pasteur pipette and centrifuge at 12 000 *g* (35°C) for 5 min. (7) Freeze phases at −80°C in the bio-freezer for 10 min. (8) Evaporate the supernatant (*n*-hexane with clozapine) in a disposable 10 cm × 1.2 cm I.D. glass tube at 40°C under a nitrogen stream.
Second analytical extraction	(1) Thaw plasma in hot water-bath (2) Continue with steps 3–7 as in first extraction. (3) Evaporate the supernatant in the same tube.

using fluphenazine as an internal standard. The total recovery was determined by calculating the amount of fluphenazine (Fig. 1B) found by HPLC.

The calculated recoveries were checked by extraction of radioactive fluphenazine (internal standard): 100 μl of radioactive [1,2-^{3}H]fluphenazine (NET-805; New England Nuclear, Boston, MA, U.S.A.) were added to 2 ml of plasma and mixed, and an aliquot of 100 μl was counted in the liquid scintillation counter. Extraction was carried out according to Table I. After every step, aliquots of the supernatant and the sediment were counted in the scintillation counter.

RESULTS AND DISCUSSION

For highest sensitivity of measurement of clozapine in plasma a three-step extraction procedure was developed (Table I). This procedure involved extraction of the acidified plasma to eliminate acidic and uncharged organic contaminants.

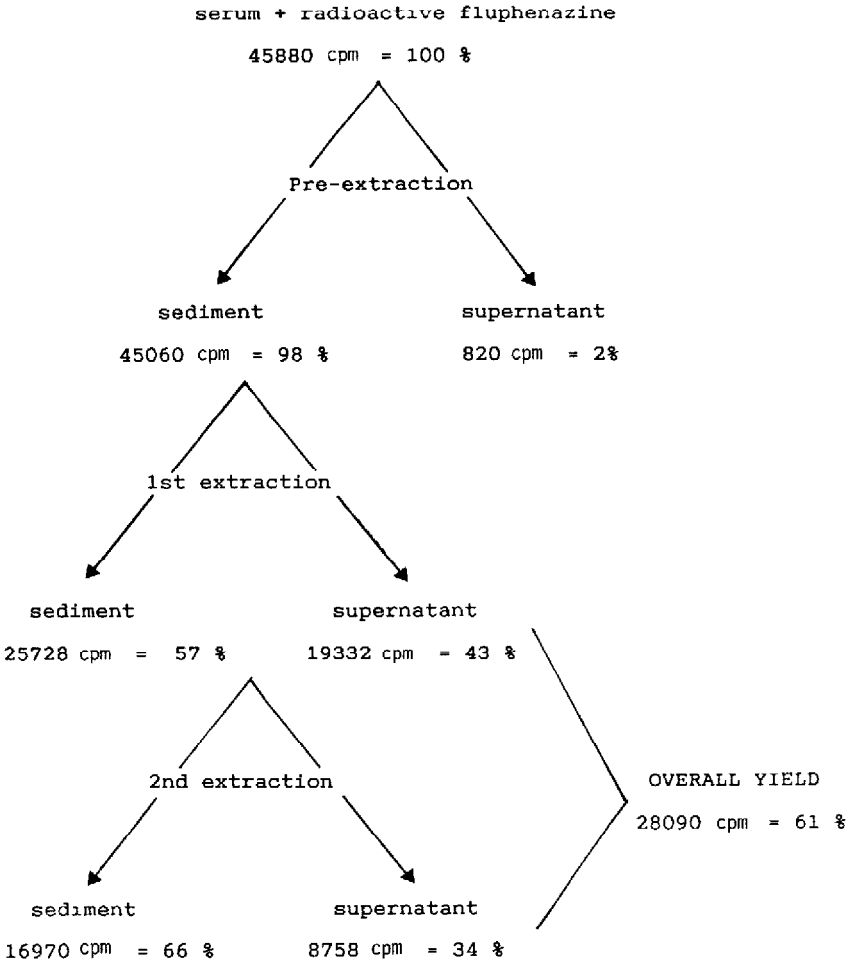

Fig. 2. Analysis of the extraction with radioactive fluphenazine.

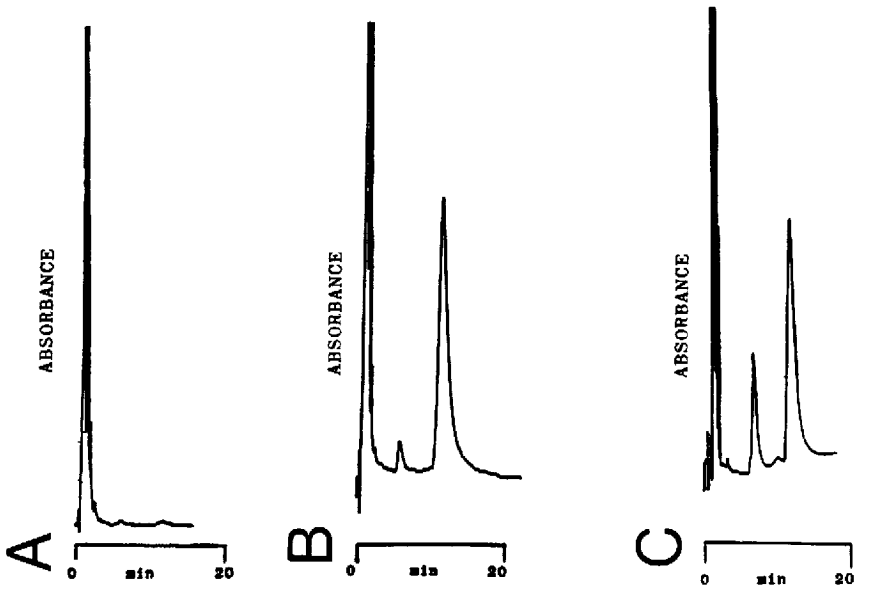

Fig. 3. (A) Extraction of pure plasma. (B) Separation of a clozapine standard (50 ng/ml) with fluphenazine (2.5 μg/ml in methanol) as internal standard. (C) Analysis of plasma from a patient. The daily dose of Leponex® was 400 mg; a plasma concentration of 208.9 ng/ml was measured (internal standard fluphenazine, 2.5 μg/ml). Retention times: clozapine, 5.9 min; fluphenazine, 12.5 min. Measurement at 0.01 a.u.f.s. (254 nm); eluent, acetonitrile–HPLC-grade water (90:10) with 0.25 m*M* ammonium acetate; column, RP-8; flow-rate, 0.5 ml/min.

Clozapine was extracted by the following two treatments of the alkaline plasma with *n*-hexane. The average recovery rate of clozapine from plasma was $61.7 \pm 6.4\%$ ($n=3$). To compensate for irreproducible losses during the complicated extraction procedure we used fluphenazine (Fig. 1B) as internal standard. Recovery of fluphenazine was similar to that of clozapine ($56.1 \pm 14.5\%$, $n=19$). These results were confirmed by extraction with radioactive fluphenazine. Fig. 2 shows the activities (counts per minute, cpm) for every step of the extraction as measured in the scintillation counter.

The activity after the first analytical extraction was 19 332 cpm, and after the second analytical extraction it was 8758 cpm. The total activity counted was 28 090, so 61% of the 45 880 cpm were extracted (extraction recovery). This corresponds well with the recovery found by the internal standard method. The loss of 2% by pre-extraction is negligible.

The retention times of clozapine and fluphenazine were determined. Under the conditions mentioned, with a flow-rate of 0.5 ml/min, the retention times were 5.9 min for clozapine and 12.5 min for fluphenazine, which provided for clear separation.

The problem involved in the determination of neuroleptics from plasma is the fact that a clear separation of the neuroleptic from plasma components is essential for optimal quantitation. This is why we made a preliminary extraction and

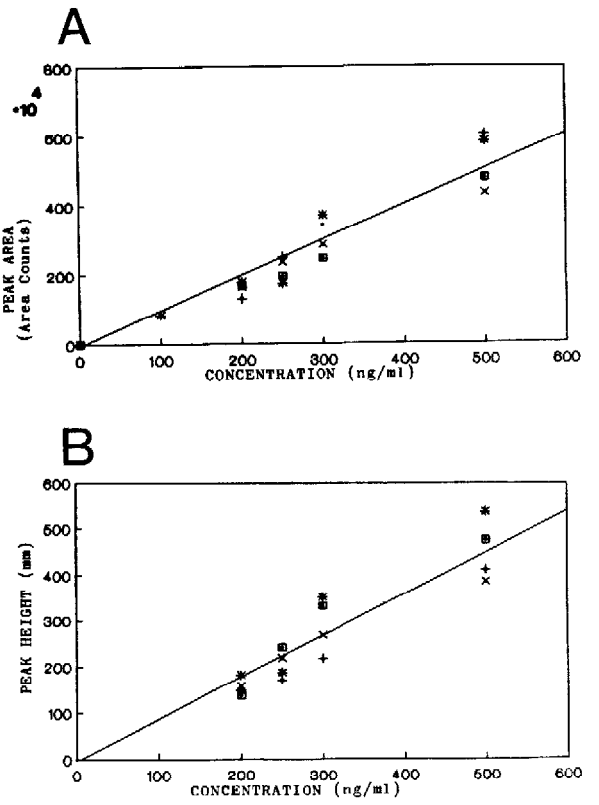

Fig. 4. Extraction of different clozapine concentrations from plasma. Quantification by integration of peak area (A) and measurement of peak height (B). The units of both ordinates were corrected for recovery. The four different symbols represent four separate standard curves obtained on different days of analysis.

analysis of blank samples of plasma. Fig. 3A and B demonstrates that the plasma components pass through the column during the first 4 min and are well separated from clozapine. The principal metabolite of clozapine in humans is the corresponding N-oxide [7]. Since this metabolite is not commercially available, it could not be tested in the present study. However, there was no indication that any other peak overlapped the clozapine peak. The unknown peak between the clozapine and fluphenazine peak in the plasma sample (Fig. 3C) may be due to this metabolite.

Standard curves were established by extraction of different amounts of clozapine (10–500 ng/ml) from the stripped plasma, which was previously purified from organic substances by charcoal treatment. Concentrations were calculated using both the integrated peak area and peak height. The mean percentage deviation of single standard concentrations from the regression lines of four standard curves (Fig. 4) was found to be between 5.6 and 14.9% when peak area was calculated and between 4.8 and 10.4% when peak height was calculated (intra-assay variation). The mean percentage deviation of all data points in Fig. 4 from the common regression line was 12.8% (peak area) and 13.7% (peak height), respectively, indicating inter-assay variation. Inter- and intra-assay variations

were not significantly different as calculated by variance analysis. Correlation coefficients of the standard curves shown in Fig. 4A ranged from 0.973 to 0.996 and those of the standard curves shown in Fig. 4B from 0.972 to 0.994.

The chosen eluent and the flow-rate of 0.5 ml/min provided for optimal separation from plasma. Although the detector was not used at full capacity, the lower detection limit was 5 ng/ml, demonstrating the high sensitivity of this method. The plasma levels measured in a number of patients were found to be between 100 and 400 ng/ml (to be published). This indicates that the sensitivity of this method comfortably exceeds the demands placed on it. Petruch et al. [8] used a TLC method with a minimum level of detectability of ca. 10 ng/ml and an accuracy of $\pm 10\%$. Heipertz et al. [9] described a GC method, but the lower detection limit was only 100 ng/ml. A drawback of our HPLC method is the rather time-consuming extraction.

A current topic of much debate is the question of the correlation between dosage and plasma levels [9–12]. The accuracy of our method, however, gives rise to the hope of further clarification of inter- and intra-individual variations in plasma levels.

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