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SIMULTANEOUS DETERMINATION OF CHLORPROMAZINE AND LEVOMEPROMAZINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid, selective and sensitive method for the simultaneous determination of chlorpromazine and levomepromazine in human plasma and urine has been developed using high-performance liquid chromatography with electrochemical detection.

The unchanged drugs and internal standard extracted from plasma and urine were separated by reversed-phase high-performance liquid chromatography. The influence of acetonitrile concentration and of the pH of the mobile phase were investigated. The detection limits were 100 pg for chlorpromazine and for levomepromazine. In comparison with three other detection systems this was found to be the most sensitive method.

This method was successfully applied to the simultaneous determination of chlorpromazine and levomepromazine in human plasma and urine for pharmacokinetic studies.

INTRODUCTION

Antipsychotic drugs have been widely used in the treatment of psychosis.

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Also, it is well known that several drugs are simultaneously used in psychiatric practice. However, there is no convincing evidence for their efficacy [1]. To study the clinical utility of the polypharmacy from the aspect of pharmacokinetic evaluation we have developed a sensitive and selective method by reversed-phase high-performance liquid chromatography (HPLC) using electrochemical detection for the simultaneous determination of chlorpromazine (CPZ) and levomepromazine (LPZ) (Fig. 1) in biological fluids. Detection of the drugs is based on their electro-oxidizable properties.

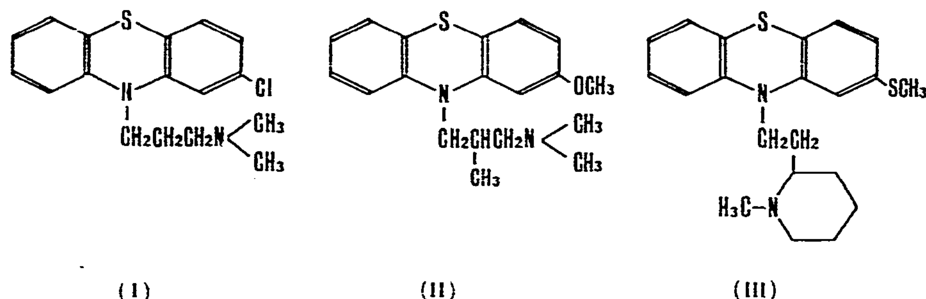

Fig. 1. Structures of chlorpromazine (I), levomepromazine (II) and thioridazine (III).

Gas—liquid chromatography (GLC) is a satisfactory and practical means for the determination of phenothiazines in biological materials. Using electron-capture detection (ECD) [2—5], CPZ and its metabolites in plasma can be determined in nanogram quantities, whereas phenothiazines (e.g. LPZ) having no halogen can not be detected. By HPLC using UV [6—11] or fluorescence [12] detection, difficulties in the determination of phenothiazines by GLC based on high polarity, low volatility and thermal instability could be eliminated. HPLC using electrochemical detection (voltammetric detection, VMD) [13, 14] has made it possible to determine the low levels of electroactive CPZ and LPZ in subnanogram quantities.

EXPERIMENTAL

Reagents

All reagents were of analytical grade. Chlorpromazine hydrochloride was kindly supplied by Yoshitomi Pharmaceutical Co. (Osaka, Japan), levomepromazine hydrochloride by Shionogi Pharmaceutical Co. (Osaka, Japan) and thioridazine (TRD) by Sandoz (Basle, Switzerland).

Stock solution

Ten milligrams each of CPZ, LPZ and TRD (internal standard) were weighed into respective 10-ml volumetric flasks and dissolved in methanol to the concentration of 1 mg/ml. Under refrigeration these solutions were stable for several weeks.

Materials

Blood and urine samples for recovery experiments were collected from several volunteers. Samples from patients who had received both CPZ and LPZ

for at least two weeks were analyzed in clinical studies. Urine samples were collected for 24 h under shaded light.

Apparatus

Analyses were performed on a ALC/GPC 204 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Waters Model U6K injector and a Yanaco electrochemical detector Model VMD-101 (Yanagimoto Manufacturing Co., Kyoto, Japan). The column (150 mm × 4 mm I.D.) was stainless steel and packed with Nucleosil C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) of 5 μm particle size. The detector consisted of a low-volume thin-layer cell housing a three-electrode detection system, i.e. a glassy carbon working electrode, a stainless tube auxiliary electrode and an Ag/AgCl reference electrode (Fig. 2), and an electrochemical control unit. The detector output was connected to a linear potentiometric recorder.

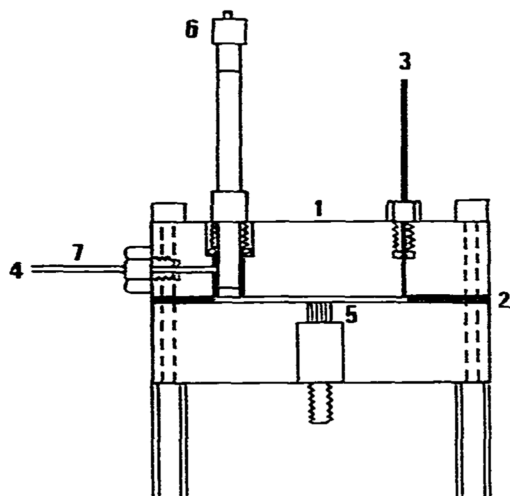

Fig. 2. Cross-section of the low-volume thin-layer cell for HPLC. 1 = cell body (polychlorotrifluoroethylene); 2 = spacer (polytetrafluoroethylene film, 50 μm); 3 = solute inlet; 4 = solute outlet; 5 = glassy carbon working electrode; 6 = Ag/AgCl reference electrode; 7 = stainless tube auxiliary electrode.

In GLC analyses a Yanagimoto GC-1800 gas chromatograph equipped with a flame ionization detector (FID) (Yanagimoto Manufactoring Co.) was used. The glass column (2.0 m × 2 mm I.D.) was packed with 4% PEG 20M on Chromosorb W AW HMDS (80—100 mesh).

Chromatographic conditions

Conditions for HPLC. The mobile phase consisted of pyridine—tetrahydrofuran—acetonitrile—0.1 *M* acetate buffer (pH 3.5) (0.1:1.0:68.9:30.0, v/v) containing 20 mmol/l of $NaClO_4$. The solvent mixture was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed before use under negative pressure. The flow-rate was adjusted to 0.7 ml/min, generating a pressure of 1200—1300 p.s.i. (ca. 8.2—9.0 MPa).

Conditions for GLC. The operating conditions were as follows: oven tem-

perature 246°C; detector temperature 290°C; inlet temperature 290°C; carrier gas (nitrogen) flow-rate 25 ml/min; hydrogen flow-rate 20 ml/min; air flow-rate 750 ml/ min.

All operations in both HPLC and GLC were carried out at room temperature.

Extraction procedures

Plasma. To 1 ml of plasma were added 4 ml of distilled water, 1 ml of internal standard solution (TRD, 160 ng/ml) and 0.8 ml of 1 *N* sodium hydroxide. The mixture was extracted three times with 15 ml of *n*-heptane containing 1.5% isoamyl alcohol by shaking for 10 min. After centrifugation at 700 *g*, the combined organic layer was evaporated to dryness. The residue was dissolved in 10 ml of 0.05 *N* hydrochloric acid, and 20 ml of diethyl ether were added subsequently. After shaking for 3 min, the ether was removed by aspiration. The acidic aqueous layer was alkalinized with the addition of 1 ml of 5 *N* sodium hydroxide and extracted again with 10 ml of *n*-heptane—isoamyl alcohol mixture. After shaking and centrifugation, the organic layer was evaporated to dryness. The residue was dissolved in 1 ml of acetonitrile and 50 µl were injected into the chromatograph.

Urine. To 20 ml of urine were added 3 ml of 1 *N* hydrochloric acid and 1 ml of internal standard solution (TRD, 1 µg/ml). The procedure is summarized as follows. Washing with diethyl ether, alkalinization of the acidic aqueous layer and extraction with *n*-heptane—isoamyl alcohol mixture are successively carried out as described above. Finally, the residue is dissolved in 1 ml of acetonitrile and 10 µl are injected into the chromatograph.

Calculation

The calculations of the concentrations of CPZ and LPZ in plasma and urine were always made by the internal standard method using the peak-height ratio technique.

RESULTS AND DISCUSSION

Chromatographic conditions

In order to achieve the best chromatographic conditons, the capacity factor (k') was measured as a function of acetonitrile content and pH of the mobile phase. When the acetonitrile concentration exceeded 80%, the resolution became poorer and the retention time decreased. By lowering the acetonitrile content the resolution was improved but the retention time was more than 15 min (Fig. 3). As the pH was increased above 4 the k' and the retention time gradually increased. At a pH of less than 3.5 excellent resolutions were attained in a short time (Fig. 4). In order to achieve good resolution it is necessary that the acetonitrile content and the pH of the mobile phase are fixed at 68% and 3.5, respectively.

The addition of pyridine to the mobile phase caused the oxidation potential to shift to a more negative potential [15]. Sodium perchlorate was added as a supporting electrolyte to improve conductivity. TRD was chosen as an internal standard because it is a structural analogue to CPZ and was not co-administered to patients in these experiments.

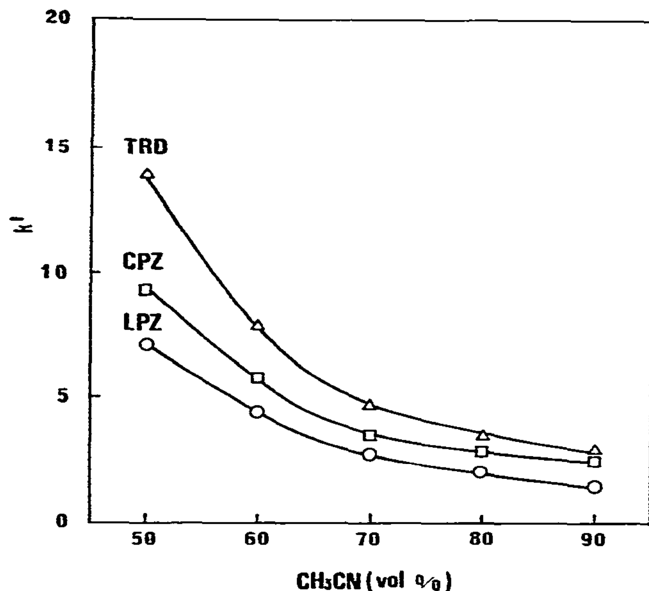

Fig. 3. Effect of the solvent acetonitrile content at pH 3.5 on capacity factors (k') of CPZ (□), LPZ (○) and internal standard (△). A 5-µl volume of the standard mixture (5 ng of CPZ and LPZ, and 7 ng of internal standard) was injected. Other conditions are described in the text.

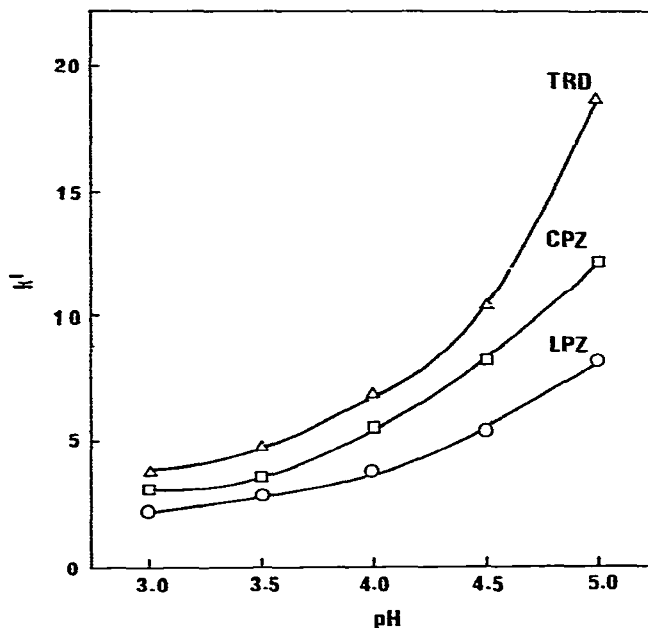

Fig. 4. Effect of the solvent pH on capacity factors (k') of CPZ (□), LPZ (○) and internal standard (△). Other conditions are described in Fig. 3.

Under the chromatographic conditions described above, the retention times of CPZ, LPZ and internal standard were 8.0, 7.0 and 9.5 min, respectively. Fig. 5 shows a typical chromatogram of a standard mixture. No interfering peaks in

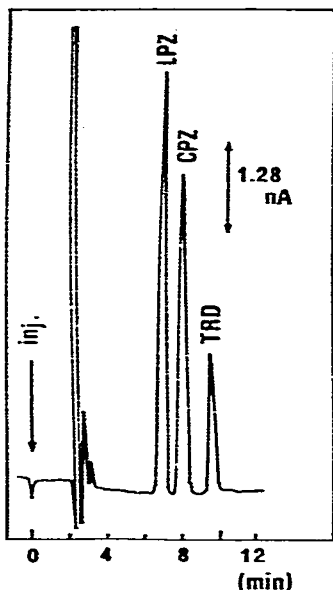

Fig. 5. Chromatogram of standard mixture. A 5-μl volume of the standard mixture (5 ng of CPZ and LPZ, and 7 ng of internal standard) was injected. Chromatographic conditions are described in the text.

the region of the standards have been observed from the blank plasma and urine samples analyzed.

Detection and sensitivity

Electrochemical detection is extremely selective because the sensitivity for each compound can be changed by altering the potential applied on the working electrode. We obtained three hydrodynamic voltammograms from the chromatographic peak currents of the respective drugs at each potential (Fig. 6). The limiting currents reached a plateau at 0.85, 0.80 and 0.75 V for CPZ,

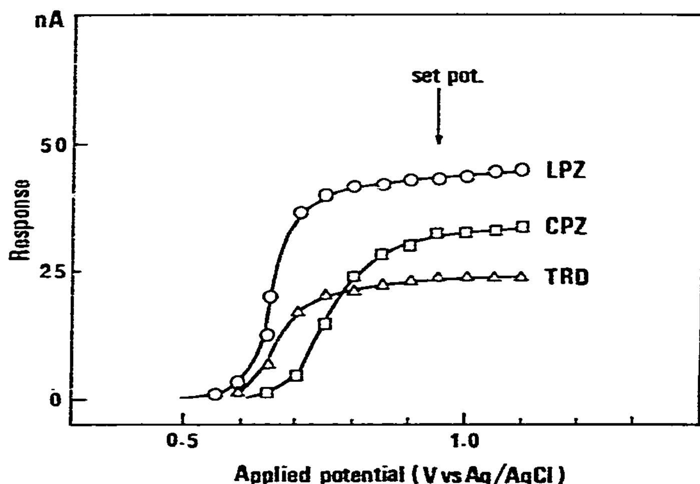

Fig. 6. Current—potential curves for CPZ, LPZ and internal standard. A 32-ng amount of each compound was injected. Chromatographic conditions are described in the text.

LPZ and TRD, respectively. When the applied potential was set at more than 0.85 V against the Ag/AgCl electrode, each drug could be quite sensitively and selectively oxidized in the VMD cell. In these experiments the potential was fixed at 0.95 V vs. Ag/AgCl.

The sensitivity of the electrochemical detector gradually declined in the continuous analysis. But it was restored by cleaning the electrode surface with a soft cloth loaded with hexane once a month, and by setting the voltammetric detector at −0.5 V for a few minutes before the analysis.

Table I shows comparable results obtained using four detection systems, minimum detectable amounts for CPZ and LPZ and the detection limits in plasma and urine. VMD and UV detection revealed a more sensitive response to the drugs than FID-GLC. ECD-GLC could sensitively respond to CPZ, but not to LPZ. With FID-GLC these drugs were simultaneously detected with poorer sensitivity. For simultaneous determination VMD is found to be the most sensitive method.

TABLE I

DETECTION LIMIT OF CPZ AND LPZ IN FOUR DETECTION SYSTEMS

The final residue from the extraction procedure for GLC was dissolved in 0.3 ml of acetone and 5 μl were injected. Other conditions were as described in the text.

Method	Detection method	Minimum detectable amount (ng)		Detection limit (ng/ml)			
				Plasma		Urine	
		CPZ	LPZ	CPZ	LPZ	CPZ	LPZ
HPLC	UV	1	1	20	20	5	5
	VMD	0.1	0.1	2	2	0.5	0.5
GLC	FID	10	10	600	600	30	30
	ECD*	0.2	—	12	—	0.6	—

*Values cited from ref. 16.

The precision and linearity of this method were determined by injecting 10- or 50-μl aliquots of the standard mixture, in which the concentration of CPZ or LPZ was between 0.002 and 6.4 μg/ml, and that of TRD 0.16 or 1.0 μg/ml. Linear regression analyses were carried out by plotting the ratios of the peak height of CPZ or LPZ to that of internal standard as a function of the ratios of the weight of each drug to that of the standard. Both calibration graphs gave excellent linearity with a correlation coefficient (r) of 0.999 (n = 8). The linear regression equations calculated by the least-squares method were $y = 1.335x + 0.048$ for CPZ and $y = 1.952x - 0.028$ for LPZ when the concentration of TRD was 1.0 μg/ml. The reproducibility of the method was studied by the repeated injection (n = 10) of 1 or 10 ng of each drug. The standard deviations of peak currents were 1.8% and 1.6% for CPZ and LPZ, respectively.

Recovery

The recovery and reproducibility of the extraction were determined by spiking blank plasma and urine samples with known amounts of CPZ, LPZ and internal standard (plasma 20, 50 and 100 ng/ml; urine 0.1 and 1.0 μg per 20 ml) and extracting as described under procedures. The average recovery from plasma samples was 89.8 ± 1.38% (S.D.) for CPZ, 93.2 ± 2.07% for LPZ and 82.1 ± 2.34% for TRD; from urine the average recoveries were 84.0 ± 3.00% (S.D.) for CPZ, 98.0 ± 3.20% for LPZ and 80.5 ± 1.35% for TRD. The coefficients of variation in plasma samples were calculated to be 1.54%, 2.22%, and 2.85% for CPZ, LPZ and TRD, respectively, while those in urine samples were 3.62%, 3.27% and 1.61% for CPZ, LPZ and TRD, respectively.

Clinical studies

The HPLC—VMD system was applied to the analysis of plasma and urine samples from patients receiving daily 200-mg doses of CPZ and LPZ. Fig. 7a shows typical chromatograms of extracts of blank plasma and of plasma of patients receiving the drugs. Fig. 7b also shows the chromatograms of extracts from urine samples. Fig. 8 shows two types of patterns (I and II) of CPZ and LPZ levels in the plasma of patients who received the drugs on a divided-dose schedule. Urinary excretion of the drugs in the patients for 24 h is given in Table II.

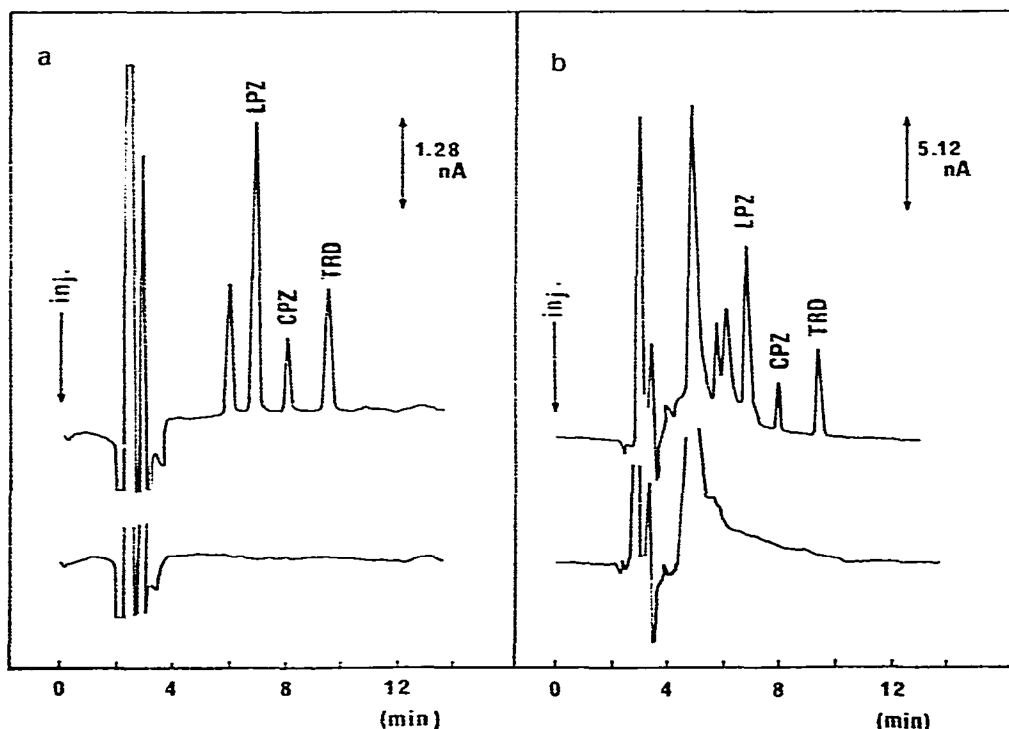

Fig. 7. (a) Chromatograms of extracts of blank plasma and of plasma of patients receiving daily 200 mg of CPZ and 200 mg of LPZ. (b) Chromatograms of extracts of blank urine and of urine of patients receiving the drugs as in part (a). These were determined at a different VMD sensitivity from the measurements in (a).

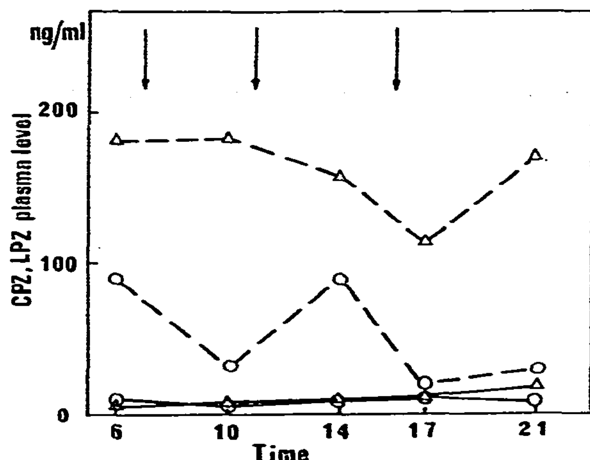

Fig. 8. The patterns (type I, — — —; and type II, ——) of CPZ (○) and LPZ (△) levels in plasma of patients receiving the drugs on a divided-dose schedule. Arrows show the times the patients received the drugs (200 mg per day of each drug).

TABLE II

URINARY EXCRETION OF CPZ AND LPZ IN PATIENTS

Patient	Drug administration			Urine volume for 24 h (ml)	Urinary excretion (mg/day)	
	Dose (mg/day)		Duration (months)		CPZ	LPZ
	CPZ	LPZ				
A	200	350	3	1210	1.343	0.762
B	200	100	17	1090	0.458	0.425
C	200	250	1	1100	0.319	0.112
D	200	150	20	1500	1.395	0.315
E	300	150	3	1650	1.419	0.611

Our preliminary results of CPZ and LPZ levels in plasma and urine show as follows. In type I pattern the drugs maintain relatively high concentrations in plasma all day, and in type II lower concentrations. LPZ is always at a higher concentration than CPZ in both types. More CPZ than LPZ tends to be excreted in the urine. These results might suggest that CPZ would inhibit the metabolism of LPZ or LPZ would activate that of CPZ in patients. Our subsequent paper will present a further detailed report about the interaction between phenothiazines in psychotic patients.

The system has proved to be useful for pharmacokinetic studies in humans.

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