Determination of Chlorpromazine in the Blood and Brain of Mice by High Performance Liquid Chromatography Combined with Electrochemical Detection

Shinji SHIBANOKI, Yukinobu GOTOH and Koichi ISHIKAWA

Department of Pharmacology, Nihon University School of Medicine,
Itabashi, Tokyo 173, Japan

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Abstract—A simple and sensitive procedure was developed for determining the concentration of chlorpromazine (CPZ) in the blood and brain of mice with promethazine as the internal standard. The procedure involves (1) primary extraction by a mixture of heptane and isoamyl alcohol (99:1) in an alkaline condition and (2) determination by means of high performance liquid chromatography with electrochemical detection. The detection limit of CPZ was 0.5 ng for one chromatographic injection. The drug was detectable in a 100 μ l sample of the blood by 6 hr after an intravenous injection of 0.25 mg/kg. The concentration of CPZ was about 40 times higher in the brain compared with that in the blood. The biological half life was estimated to be 87 and 65 min, in the blood and brain, respectively. The proposed method is also applicable for the determination of other phenothiazine derivatives and effective for pharmacokinetic study of the compounds in clinics and laboratories.

Electrochemical detection has been confirmed to be a successful procedure for the determination of substances having phenolic hydroxy radicals. Many reports demonstrated the application of the detector in combination with high performance liquid chromatography for assaying monoamines in the brain (1–4), blood (5) and urine (6). Other applications of electrochemical detection have appeared for drugs which possess phenolic OH in their molecular structure (7–9). Heterocyclic compounds seem to react electrochemically and have been reported to be determined by the electrochemical detector (10, 11).

Chlorpromazine (CPZ) is a drug used in the treatment of psychotic disorders. CPZ is a strong dopamine receptor blockade and a useful drug for determining dopaminergic function in the central nervous system. Thus the intracerebral concentration of the drug gives significant information about its effects on the dopaminergic system. The conventional procedure for assaying sensitively the concentration of CPZ is gas chromatography with an electron capture

detector (12), for which complex derivatization is required because of the non-volatile property of this drug. CPZ, a phenothiazine derivative, is a heterocyclic compound and is considered to be electrochemically reactive. A report recently demonstrated the application of HPLC-ECD for determining the concentration of promethazine and other phenothiazine derivatives including CPZ in human plasma (13, 14). The present study shows a simplified procedure for the assay of CPZ in the brain and blood of mice.

Materials and Methods

Apparatus and reagents: A liquid chromatographic system (Yanagimoto L-2000, Kyoto, Japan) was used with a six port injector (Rheodyne 7120, Berkeley, CA, U.S.A.) and a glassy carbon electrochemical detector (Yanagimoto VMD-101). The analytical column was an Ultrasphere-ODS reversed-phase column (average particle size, 5 μ m; 250 $\times$ 4.6 mm i.d.; Altex Scientific, Berkeley, CA, U.S.A.). To protect the analytical column,

a short column packed with the ODS resin (10×4.5 mm i.d.) was equipped. The detector potentials were set at 700 mV versus the Ag/AgCl reference electrode.

Cyclic voltammetry was performed using CV-1A (Bioanalytical Systems, West Lafayette, Indiana, U.S.A.). The working electrode was of glassy carbon and the reference electrode, Ag/AgCl. The scan rate was 100 mV/sec.

CPZ hydrochloride was obtained from Shionogi Pharmaceutical Co. (Osaka, Japan). The drug was dissolved in saline immediately prior to injection. Promethazine, internal standard for the chromatography, was also purchased from Shionogi Pharmaceutical Co. Reagent grade chemicals for extraction and chromatography were obtained from a single source (Wako, Osaka, Japan) and used without further purification. The chromatographic mobile phase was a mixture of 0.1 M acetate buffer (pH 3.5)-acetonitrile-tetrahydrofuran-pyridine (100:200:3:0.3).

Animals: ICR mice, each weighing about 30 g, were used. All animals were kept in a room in which the temperature and humidity were controlled at $23 \pm 0.5^\circ\text{C}$ and $55 \pm 5\%$, respectively, prior to drug administration. The animals were injected intravenously with CPZ at dose levels of 0.1, 0.5, 1, 2.5, 5, 7.5, 10, 15 and 20 mg/kg and sacrificed by decapitation 1 hr after the administration. In another series of experiments, 10 mg/kg of the drug was intravenously injected into mice which were sacrificed 5, 15 or 30 min or 1, 2, 3, 4 or 6 hr after the administration.

The brain was obtained as quickly as possible after sacrifice. The sample was weighed and then wrapped in aluminium foil and stored at -80°C until the assay. The blood sample was collected from the carotid artery when the animal was decapitated. The whole blood was also stored at -80°C .

Extraction procedure: The blood sample (100 μl) was transferred to a glass-stoppered tube and agitated with 100 μl of distilled water containing internal standard (promethazine, 100 ng) and 200 μl of 1 N NaOH. Five ml of an organic mixture of heptane: isoamyl alcohol (99:1) was added to the tube. The tube was vortexed for 1 min and centrifuged for 3 min to separate the organic

layer of which 4 ml was transferred to another tube. The tube was added with 100 μl of 0.1 N HCl and then vortexed for 1 min. After a brief centrifugation to separate the layers, a portion of the HCl layer was used as the chromatographic sample.

The brain sample (about 400 mg) was homogenized in distilled water containing internal standard (2,000 ng). A portion (500 μl , in general) of homogenate was transferred to a glass-stoppered tube containing 500 μl of 1 N NaOH. From this point, the same procedure as for the blood sample was applied. The final HCl layer was injected into the HPLC system.

Results

Static experiments: Hydrodynamic voltammograms of CPZ and promethazine are shown in Fig. 1A. CPZ initiated the electrochemical response at 475 mV. The internal standard, promethazine, also showed the initial response at 475 mV. Both responses increased steadily to about 700 mV and then decreased. The maximum response was higher in the internal standard than CPZ. These results agreed with those obtained by cyclic voltammetry, in which the oxidation (anodic) current appeared at about 500 mV (Fig. 1B). The cyclic voltammograms showed a reduction (cathodic) current of about 1/3 of the oxidation phase, indicating reversible reactions of the substances.

The detector response varied directly with the amount of CPZ injected. The ratio of CPZ to the internal standard showed linear responding ($r=0.998$, $N=11$, $P<0.0001$) in a wide range of dosages between 500 pg and 1 μg (Fig. 2). This means that a simple comparison of the peak height is adequate for the calibration of concentrations of the substance.

The recovery rate of CPZ was over 90% after adjusting for solvent loss, and the ratio of the substance to promethazine was constant in a dose range between 500 pg and 1 μg .

Chromatogram: In electrochemical detection, the mobile phase must be aqueous because the reaction does not occur in the organic phase. However, acetonitrile, an organic solvent, has been reported to be

used in the buffer solution for the electrochemical detector. The solvent has been utilized for shortening the retention time of hydrophobic substances in the reverse phase column. Thus the present study successfully used acetonitril for shortening and differentiating the retention times of CPZ and

promethazine. Typical chromatograms for the substances are illustrated in Fig. 3. Biogenic substances did not interfere with the quan-

Fig. 1. Electrochemical reactivity of the substances used in the present study. A, Hydrodynamic voltammograms of CPZ (●) and promethazine (○). Detector, VMD 101 (Yanagimoto); mobile phase, 0.1 M acetate buffer (pH 3.5)-acetonitril-tetrahydrofuran-pyridine (100:200:3:0.3). B, Cyclic voltammograms of CPZ (—) and promethazine (···). Note the similarity of both voltammograms, demonstrating that the cyclic voltammogram is useful for selecting the applied voltage for the electrochemical detector.

Fig. 2. Linearity of the ratio of CPZ and promethazine (internal standard).

Fig. 3. Typical chromatograms of blood and brain samples. Chromatograms were obtained after a saline (A) or CPZ (B) injection. For details of chromatographic conditions, see the Methods section. Peak identities: 1, promethazine (internal standard); 2, CPZ. Note that no biogenic substances interfered with the determination of CPZ. One run for a single assay needed about 8 min.

titations of CPZ and the internal standard in both the blood and brain. CPZ had a retention time of 5.7 min. Assays of over 80 samples were completed in a regular 8 hr routine workday. It may be possible to assay the drug in the blood even when it is injected intravenously at a dose level of 0.1 mg/kg.

Concentrations of CPZ in the blood and brain: When the determination was performed 30 min after the intracerebral administration

of CPZ at a dose level of 0.25 mg/kg, the blood concentration of the drug was estimated as 13.1 ± 3.2 ng/ml (mean $\pm$ S.D. of 5 determinations). In this case, the intracerebral concentration was 557.3 ± 4.5 ng/g wet tissue, being about 40 times higher than that in the blood. This value was maintained when CPZ doses were increased by 20 mg/kg (Fig. 4). The concentrations in the blood and brain correlated significantly ($r=0.975$, $P<0.001$).

The time course of CPZ disappearance was examined after an intravenous injection of the drug at a dose level of 10 mg/kg (Fig. 5). The disappearance curves were linear both in the blood and brain with correlation coefficients of -0.994 and -0.995 , respectively ($P<0.001$). The biological half lives of CPZ were estimated as 87 and 65 min for the blood and brain, respectively.

Discussion

The present study demonstrated a simple procedure for the determination of CPZ, a neuroleptic drug. Previous reports have demonstrated the application of gas chromatography (12) or liquid chromatography combined with ultraviolet spectrometry (15, 16) for determination of the drug. The electrochemical reaction sufficiently occurred for the determination of CPZ with promethazine as the internal standard, and the proposed HPLC-ECD method was more sensitive than those conventional approaches. The heterocyclic compound has a long retention time on a reverse phase column, such as ODS in the present study, because of its physicochemical properties. If the mobile phase used for routine assay of monoamines, a citrate buffer containing tetrahydrofuran (4), was applied, the retention time was about 4 hr for CPZ. This means that the mobile phase must include a stronger organic component. A high concentration of organic solvents is not, in general, applicable to the electrochemical detector because the reaction occurs in only aqueous states. Acetonitril is an organic solvent which is utilized as a medium for promoting reactions involving ionization. A previous report utilized, indeed, successfully acetonitril in electrochemical detection (13). This fact prompted us to

Fig. 4. Correlation of the concentration of CPZ in the blood and brain. The correlation coefficient was calculated to be 0.975. Note that the concentrations were about 40 times higher in the brain than in the blood for all dosages tested.

Fig. 5. Disappearance curves of CPZ from the blood (●) and brain (○). The biological half life was estimated to be 87 and 65 min in the blood and brain, respectively. The regression coefficients for linearity were calculated to be -0.994 and -0.995 , respectively.

choose the solvent in the chromatographic system in the present study. Finally, 66% of acetonitril was found to be the most efficient for separating CPZ and promethazine, the internal standard, with a citrate buffer (pH 3.5). No biogenic substances interfered with the determination of both CPZ and internal standard under the chromatographic conditions cited in the Methods section.

Electrode responses depend upon the applied voltage. Although increases in the applied voltages result in more responses, the increases are accompanied by increases in background and noise currents which interfere with suitable determination of the substances (1, 6). This means that the voltage has to be selected for CPZ and the internal standard. Both substances showed similar electrochemical behavior with the maximum response occurring at about 700 mV. The electrochemical reaction reportedly occurs at the hydroxy group on the benzene ring (phenolic OH). However, the present substances showed the reaction, even though they have no such moiety. The electrochemical reaction is, therefore, considered to be induced not only on phenolic OH but also on another site in a molecular structure. This fact extends the application of electrochemical detection. Cyclic voltammetry is the simple approach to demonstrate whether a substance is detectable by HPLC-ECD or not and to decide the suitable applied voltage for the determination.

Both CPZ and promethazine showed linear responses in a wide range of doses between 500 pg and 1 μ g. This resulted in a linearity of the ratio of the drug to internal standard. All quantitations were therefore based on peak heights of the resulting chromatogram. Ratios of the peak heights for the substances and the internal standard were compared for samples and standards taken through the entire extraction procedure, as described previously (4). The procedure also provided a treatment to include the recovery rate in the estimation of tissue concentration. The recovery rate was also constant for both substances in the wide range tested, showing values over 90% after adjusting for solvent loss. The overall precision of the present procedure was found

to be less than $\pm 3.7\%$ standard deviation for the drug.

The drug was detectable in the blood after an intravenous injection of 0.25 mg/kg. While a recent report demonstrated the application of HPLC-ECD for the determination of phenothiazines in human plasma (14), the present modification yielded a higher sensitivity. The dose administered in the present study was about 10 to 40 times less than routine doses administered in clinics. Furthermore, the sample volume required for the assay was 100 μ l. This implies that the present procedure can be applied for clinical laboratories as well as for pharmacological research.

The intracerebral concentration of CPZ was 40 times higher than that in the blood. The ratio was very high in comparison with that of, for example, morphine, of which the concentration was 10 times less in the brain than in the blood (9). This means that the drug penetrates into the brain easily from the blood. When CPZ was injected at a dose level of 10 mg/kg, it was possible to monitor its concentration in the blood with a 100 μ l sample by 24 hr after the administration. This fact is useful for pharmacokinetic experiments with small laboratory animals.

In conclusion, HPLC-ECD is a sensitive procedure for determining the pharmacokinetics of CPZ, a phenothiazine derivative, in both clinical and laboratory experiments. The method may also be applicable for other heterocyclic compounds as well as for phenothiazines.

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