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Simple and simultaneous determination for 12 phenothiazines in human serum by reversed-phase high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method has been developed for the simultaneous analysis of the 12 phenothiazines (chlorpromazine, fluphenazine, levomepromazine, perazine, perphenazine, prochlorperazine, profenamine, promethazine, propericiazine, thioproperazine, thioridazine and trifluoperazine) in human serum using HPLC/UV. The separation was achieved using a C_{18} reversed-phase column (250 mm × 4.6 mm I.D., particle size 5 μm, Inersil ODS-SP). The mobile phase, consisting of acetonitrile–methanol–30 mM NaH_2PO_4 (pH 5.6) (300:200:500, v/v/v), was delivered at a flow rate of 0.9 mL/min and UV detection was carried out at 250 nm. The recoveries of the 12 phenothiazines spiked into serum samples were 87.6–99.8%. Regression equations for the 12 phenothiazines showed excellent linearity, with detection limits of 3.2–5.5 ng/mL for serum. The inter-day and intra-day coefficients of variation for serum samples were commonly below 8.8%. The selectivity, accuracy and precision of this method are satisfactory for clinical and forensic purposes. This sensitive and selective method offers the opportunity for simultaneous screening and quantification of almost all phenothiazines available in Japan for the purposes of clinical and forensic applications.

Keywords: Phenothiazine; HPLC; Serum; Human

1. Introduction

Phenothiazines have been widely used as antipsychotic drugs for many years. These drugs are extensively metabolized by the liver and excreted in the urine in animals and humans [1]. In Japan, fetal intoxication due to these medicines is common [2] because a patient may take several drugs in combination, resulting in fetal poisoning. Therefore, there is a need for a simple and sensitive method for screening of these drugs.

Several methods have been previously described for the determination of each phenothiazine and its metabolites using high-performance liquid chromatography (HPLC), LC [3–11] and LC–mass spectrometry (MS) [12,13]. However, a simple and selective method for the simultaneous determination of these drugs using HPLC/UV has not been reported. Although LC–MS is popular, it is very expensive.

The purpose of the present study was to develop a simple, sensitive, selective and non-gradient elution HPLC/UV method for the simultaneous determination of 12 phenothiazines (chlorpromazine, fluphenazine, levomepromazine, perazine, perphenazine, prochlorperazine, profenamine, promethazine, propericiazine, thioproperazine, thioridazine and trifluoperazine) in human serum.

2. Materials and methods

2.1. Chemicals and reagents

Twelve phenothiazine derivatives were examined in this study (Fig. 1). The following is a list of the drugs considered and their therapeutic range and toxic threshold in brackets, where available. Promethazine–hydrochloride (therapeutic level: 100–400 ng/mL, toxic level: 1000–2000), profenamine–hydro-

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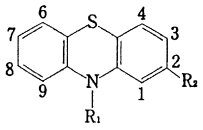

Compound	R_1	R_2
Chlorpromazine	$-CH_2CH_2CH_2N(CH_3)_2$	Cl
Promethazine	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	H
Profenamine	$-CH_2CH(CH_3)N(CH_3)_2$	H
Levomepromazine	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	OCH_3
Perazine	$-CH_2CH_2CH_2N$(piperazine)NCH_3	H
Prochlorperazine	$-CH_2CH_2CH_2N$(piperazine)NCH_3	Cl
Trifluoperazine	$-CH_2CH_2CH_2N$(piperazine)NCH_3	CF_3
Thioproperazine	$-CH_2CH_2CH_2N$(piperazine)NCH_3	$SO_2N(CH_3)_2$
Perphenazine	$-CH_2CH_2CH_2N$(piperazine)NCH_2CH_2OH	Cl
Fluphenazine	$-CH_2CH_2CH_2N$(piperazine)NCH_2CH_2OH	CF_3
Propericiazine	$-CH_2CH_2CH_2N$(piperidine)$CHOH$	CN
Thioridazine	$-CH_2CH_2$-(1-methylpiperidin-2-yl)	SCH_3

Fig. 1. Chemical structures of the 12 phenothiazines.

chloride (no data, no data), chlorpromazine–hydrochloride (therapeutic level: 50–500 ng/mL, toxic level: 1000), levomepromazine–maleate (therapeutic level: 30–150, toxic level: 500), trifluoperazine–maleate (therapeutic level: 5–50, toxic level: 100–200), perazine–maleate (therapeutic level: 25–100, toxic level: 500), prochlorperazine–maleate (therapeutic level: 10–40, toxic level: 200–300), fluphenazine–maleate (therapeutic level: 1–17, toxic level: 50–100), thioridazine–hydrochloride (therapeutic level: 200–1000, toxic level: 2000), propericiazine-free (therapeutic level: 50, toxic level: 100), perphenazine-free (therapeutic level: 4–30, toxic level: 50–100) and thioproperazine–dimethansulfonate (therapeutic level: 1–20, toxic level: 100), which were all kindly provided by Yoshitomi Pharmaceutical Ind. Co. Ltd. (Osaka, Japan) [14,15]. Blank plasma, obtained from pooled donor plasma, and diazepam (internal standard: IS) were obtained from Wako Pure Chemical Industries Ltd., Osaka. All other chemicals and reagents used were of the highest commercial quality available.

2.2. Preparation of standard solutions and calibration standards

A standard stock solution, containing the 12 drugs, was prepared at a concentration of 1 mg/mL of each compound in methanol, and it remained stable for at least 3 months at −20 °C. Serum standards were prepared at concentrations of 1, 10, 50, 100, 200 and 500 ng/mL of each compound by diluting appropriate aliquots of the stock solution with drug-free serum. The calibration curve was obtained by simple linear regression of each drug's concentration to the peak height ratio. Regression equations for the 12 phenothiazines extracted from human serum are based on the peak height ratios of drug to IS.

2.3. Extraction procedure

To 1 mL of serum was added 200 μL 1N sodium hydroxide, 3 mL *t*-butyl ethyl ether and 40 μL diazepam (10 μg/mL, IS). After vortex mixing for 3 min, the tubes were centrifuged at 1200 × *g* for 5 min. The organic phase was transferred to a clean conical tube and evaporated to dryness in a water bath at about 40 °C under a gentle stream of nitrogen. The residue was dissolved in 200 μL mobile phase and 50 μL injected into the HPLC.

2.4. Apparatus and chromatographic conditions

The HPLC equipment consisted of a pump (Model CCPS, Tosho, Tokyo, Japan) and a variable-wavelength UV detector (Model UV-8020, Tosho, Tokyo, Japan). Separation was achieved using a C_{18} reversed-phase column (250 mm × 4.6 mm I.D., particle size 5 μm, Inersil ODS-SP; GL Science, Tokyo, Japan). The mobile phase was acetonitrile–methanol–30 mM NaH_2PO_4 (pH 5.6) (300:200:500, v/v/v) and the flow rate was 0.9 mL/min. The UV absorbance of the eluate was monitored at 250 nm. All instruments were operated at ambient laboratory temperature (20–25 °C). Temperature management is important in this method.

2.5. Accuracy, recovery and linearity

The recoveries were calculated by comparing the chromatographic peak heights obtained from the extracts of the spiked serum samples with those obtained by direct LC injection of non-extracted authentic compounds dissolved in the mobile phase, and determined at two different concentrations (200 and 500 ng/mL) of each drug.

The inter-day coefficient of variation (CV) was determined by analyzing a spiked sample at two concentrations on the same day ($n = 6$). The same procedure was repeated on different days ($n = 6$) to determine the intra-day CV. The accuracy was expressed as the percentage recovery, and precision was given by the inter-day and intra-day CV values.

2.6. Limit of detection and limit of quantitation

Serum standards curves were prepared at concentrations of 2, 10, 50, 100, 200 and 500 ng/mL of each compound by diluting appropriate aliquots of the stock solutions with drug-free serum. The resulting peak heights were plotted against the concentrations.

2.7. Stability test

The stability of the 12 drugs and IS in serum was investigated. Spiked samples were prepared with drug-free serum at a single concentration level (200 ng/mL) and evaluated at room

temperature, 4 and −20 °C over 14 days. Stability tests were performed using two replicates.

2.8. Case report

Case 1: The deceased was a 40-year-old female. She had suffered from depression for about 10 years and received outpatient medication. Her eldest son found her when he came home—she was unconscious following a convulsion and vomited blood on a futon, but died in hospital. A blood specimen was submitted for toxicological examination. The autopsy findings showed no marked changes except for some organ congestion. No alcohol was detected in the blood or the urine.

Case 2: The deceased was a 19-year-old female. She had suffered from depression for a few years and received outpatient medication. Her mother found her when she went to her room following no reaction, but she died in hospital. A blood specimen was submitted for toxicological examination. The physical findings showed no appreciable injury or abnormality in the head, limbs, or neck, apart from a mild dilatation of the left external carotid artery.

Case 3: The deceased was a 35-year-old female. She had suffered from depression for about 10 years and received outpatient medication. Her father found her when he came home—she was sleeping in the same position as in the morning and exhibited no reactions; she later died in hospital. A blood specimen was submitted for toxicological examination. The autopsy findings showed no marked changes other than organ congestion. No alcohol was detected.

3. Results and discussion

3.1. Selectivity and chromatography

Fig. 2 shows the chromatograms of the 12 phenothiazines. These drugs and the IS were well separated. The retention times (RT) of propericiazine, promethazine, profenamine, levomepromazine, thioproperazine, perazine, chlorpromazine, IS, perphenazine, thioridazine, fluphenazine, prochlorperazine and trifluoperazine were 11.3, 15.3, 16.2, 17.8, 19.3, 21.1, 22.3, 25.7, 27.5, 31.7, 39.6, 43.2 and 62.0 min, respectively. Under the described optimized chromatographic conditions, they all eluted within 65 min.

No interfering peaks appeared when the following drugs were added to serum lofepramine (RT: 4.2 min), theophylline (4.5 min), caffeine (5 min), thiamyral (7.1 min), phenobarbital (7.6 min), carbamazepine (10.7 min), desipramine (12.2 min), estazolam (12.6 min), nitrazepam (13 min), oxazolam (13.1 min), dosulepin (14.3 min), imipramine (14.7 min), triazolam (14.8 min), flunitrazepam (15.6 min), etizolam (17.4 min), deorodone (20.1 min), midazolam (25 min) and haloxazolam (28 min).

3.2. Limit of quantification

The limit of quantification is the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. The lower practical limit of quantification of propericiazine, promethazine, profenamine, levomepromazine, thioproperazine, perazine, chlorpromazine, perphenazine, thioridazine, fluphenazine, prochlorperazine and trifluoperazine was 3.7, 3.2, 3.5, 4.5, 4.1, 4.6, 3.6, 4.0, 4.1, 5.5, 4.9 and 5.2 ng/mL, respectively. All of these quantification limits are adequate for clinical and forensic analyses. This method offered an approximately 10-fold higher sensitivity compared with the previous methods that have been reported [11–13].

Table 1
Recoveries of the 12 phenothiazines added to human drug-free serum

Drug	Amount added (ng/mL)	Recovery (%)	CV (%)
Chlorpromazine	100	96.6	2.6
	200	93.0	3.6
Fluphenazine	100	97.4	1.8
	200	97.8	3.4
Levomepromazine	100	97.3	1.5
	200	98.8	2.6
Perazine	100	96.0	2.3
	200	98.0	4.1
Perphenazine	100	95.6	3.6
	200	92.4	4.9
Prochlorperazine	100	93.9	3.8
	200	87.6	4.7
Profenamine	100	98.9	1.8
	200	98.7	3.1
Promethazine	100	97.5	2.2
	200	98.5	3.6
Propericiazine	100	99.8	2.1
	200	99.3	2.9
Thioproperazine	100	98.9	3.3
	200	99.5	3.6
Thioridazine	100	91.8	2.2
	200	94.7	4.3
Trifluoperazine	100	95.5	2.2
	200	95.7	3.1

Data for each drug were determined by measuring two concentrations in six samples. CV is the coefficient of variation.

3.3. Recovery

Three liquid–liquid extraction solvents were investigated, namely *t*-butyl ethyl ether, diethyl ether and hexane. *t*-Butyl ethyl ether was chosen as the extraction solvent because it provided >85% absolute recoveries for the 12 drugs. Actually, the recovery rate of these drugs was >85% and the CVs ranged from 1.5 to 4.9% for all drugs (Table 1). The mean recovery of the IS was 98%.

3.4. Precision and accuracy

The inter-day and intra-day CVs are shown in Table 2. The inter-day reproducibility was assessed using six samples at two different concentrations (100 and 200 ng/mL) in three samples that were analyzed on the same day. The CVs ranged from 1.2 to 6.3%; the accuracy was found to be in the range of 95.7–102.1%. The intra-day repro-

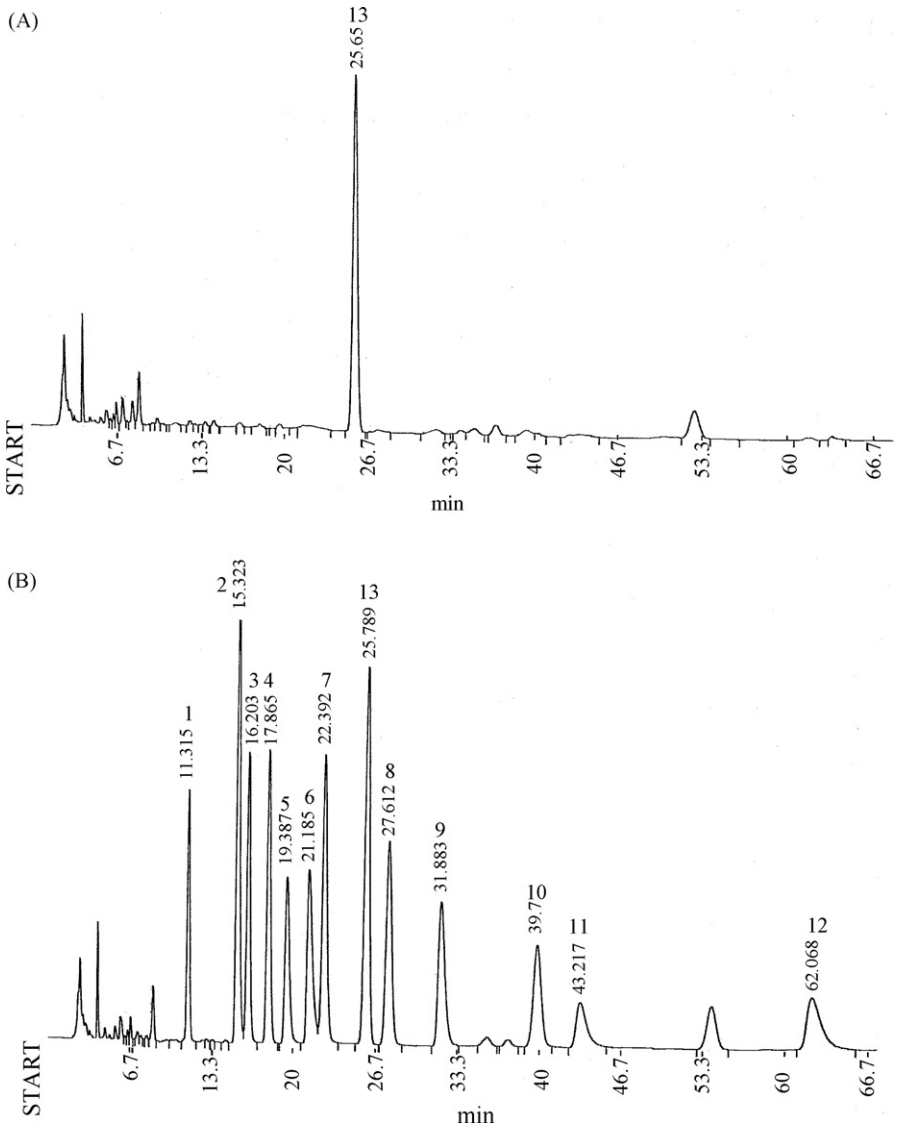

Fig. 2. Chromatograms of the 12 phenothiazines in human serum. Conditions: column, 250 × 4.6 mm I.D., particle size 5 μm, Inersil ODS-SP; mobile phase, acetonitrile–methanol–30 mM NaH_2PO_4 (pH 5.6) (300:200:500, v/v/v); flow rate, 0.9 mL/min; detection wavelength, 250 nm. (A) Pooled blank serum. (B) Drugs added to the drug-free serum. The concentration of the 12 compounds is 50 ng/mL. 1 = propericiazine, 2 = promethazine, 3 = profenamine, 4 = levomepromazine, 5 = thioproperazine, 6 = perazine, 7 = chlorpromazine, 8 = perphenazine, 9 = thioridazine, 10 = fluphenazine, 11 = prochlorperazine, 12 = trifluoperazine, 13 = diazepam (internal standard, 40 ng/mL).

ducibility was determined using two different quality control samples over a 2-week period. The CVs ranged from 2.7 to 6.3%; the accuracy was found to be in the range of 95.5–102.1%.

3.5. Linearity

The calibration curves (the peak height ratio of the concentration of each drug) was linear over the concentration range of 2–500 ng/mL serum. The coefficients of determination (r^2) from regression analyses of the 12 drugs were between 0.996 and 0.998.

The range of linearity of most phenothiazines was satisfactory, with respect to the therapeutic range for forensic and clinical purposes.

3.6. Stability test

A stability study was conducted to determine the best storage temperature for serum samples. The results demonstrated that these drugs and the IS were stable up to 8 h at room temperature. Furthermore, these drugs were stable up to 2 weeks when stored at 4 and −20 °C. Therefore, all extracted samples were stored refrigerated at 4 °C for same-day analysis, whereas serum samples were frozen at −20 °C until analysis by HPLC.

3.7. Case report

The serum level in each case was as follows: for Case 1: propericiazine 130 ng/mL, levomepromazine 36 ng/mL,

Table 2
Precision and accuracy data for the 12 phenothiazines added to human drug-free serum

Drug	Amount added (ng/mL)	Accuracy (%)	Inter-day CV (%)	Accuracy (%)	Intra-day CV (%)
Chlorpromazine	100	98.2	4.3	98.8	4.4
	200	96.5	5.3	101.2	6.3
Fluphenazine	100	96.5	3.1	95.5	3.2
	200	98.1	4.2	97.8	4.4
Levomepromazine	100	97.7	3.3	97.7	3.7
	200	96.2	4.5	96.6	4.7
Perazine	100	98.2	1.2	101.8	2.6
	200	97.8	3.5	98.8	3.1
Perphenazine	100	101.2	2.7	98.8	8.8
	200	97.6	3.6	99.1	4.3
Prochlorperazine	100	102.1	1.9	99.7	2.6
	200	98.6	3.1	97.3	5.1
Profenamine	100	97.8	4.1	98.8	5.1
	200	97.7	4.3	98.5	5.2
Promethazine	100	99.1	3.3	99.6	3.8
	200	101.3	3.6	102.1	3.9
Propericiazine	100	98.8	4.2	98.9	4.2
	200	97.6	4.7	98.8	5.2
Thioproperazine	100	98.6	2.4	101.1	2.7
	200	95.7	3.1	99.7	4.3
Thioridazine	100	99.4	2.2	98.6	2.2
	200	97.3	3.8	98.5	3.9
Trifluoperazine	100	98.1	2.6	99.2	3.1
	200	97.9	2.9	98.7	4.4

Data for each drug was determined by measuring two concentrations in six samples. CV is the coefficient of variation.

chlorpromazine 72 ng/mL; Case 2: propericiazine 312 ng/mL, promethazine 34 ng/mL, levomepromazine 1119 ng/mL, chlorpromazine 687 ng/mL, other drugs nortriptyrine 2034 ng/mL, imipramine 1842 ng/mL, mianserin 4518 ng/mL; Case 3: promethazine 765 ng/mL, levomepromazine 772 ng/mL, chlorpromazine 178 ng/mL, other drugs carbamazepine 8 ng/mL, phenobarbital 23 ng/mL, flunitrazepam 34 ng/mL.

4. Conclusion

The suitability of the HPLC method for the determination of the 12 phenothiazines has been studied. The LC–MS method reported by Mizuno et al. [13] involve a complicated extraction, but this method is simple. Finally, this HPLC method has sensitivity, precision and accuracy and may be useful for the determination of the blood levels of the 12 phenothiazines in clinical and forensic investigations. Both low and high doses of the 12 phenothiazines can be measured simultaneously, with low detection limits, low limits of quantification and satisfactory validation characteristics.

In conclusion, the present method offers the opportunity for screening and quantification of the 12 phenothiazines, which are available in Japan and are commonly found in clinical and forensic cases.

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