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Journal of Chromatography B, 657 (1994) 141–148

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Quantification of chlorprothixene, levomepromazine and promethazine in human serum using high-performance liquid chromatography with coulometric electrochemical detection☆

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(First received November 18th, 1993; revised manuscript received February 15th, 1994)

Abstract

Isocratic reversed-phase high-performance liquid chromatography with coulometric electrochemical detection was optimised to quantify the neuroleptic drugs chlorprothixene, levomepromazine, and promethazine in human serum. The method involves extraction of the neuroleptic drug in *n*-heptane–isoamylalcohol from the alkalinized serum, followed by chromatographic separation on a Nucleosil CN column with acetonitrile–pyridine–sodium acetate buffer as the mobile phase. The extraction recovery was >85% for each neuroleptic drug. The sensitivity and selectivity required for pharmacokinetic studies was obtained with a dual coulometric analytical cell operating in the oxidative screen mode. The lower limit of detection in human serum for chlorprothixene, levomepromazine, and promethazine, was 0.5, 0.2 and 0.1 ng/ml, respectively. A linear relationship ($r^2 > 0.99$) was obtained between the concentrations of each neuroleptic drug and the detector signal. The accuracy of the quality control samples was ± 7% for each neuroleptic drug with a precision within 9.5%, 8.1% and 13.5% for chlorprothixene, levomepromazine, and promethazine, respectively. The neuroleptic drugs were stable in acetonitrile and human serum for at least six months when stored at −20°C. This method is applicable to analyze a large number of serum samples for pharmacokinetic studies of the neuroleptic drugs.

1. Introduction

Chlorprothixene, levomepromazine, and promethazine are neuroleptic drugs which were developed following the introduction of chlorpromazine in the early 1950s. Although these drugs are often prescribed, information on their pharmacokinetic properties is lacking. This applies particularly to chlorprothixene. The only pharmacokinetic report on this drug is that by Raaflaub in 1975 [1] who measured chlorprothixene fluorimetrically in blood extracts after single intravenous injection or oral administration to three healthy subjects.

Solvent extraction and separation procedures that are mostly applied involve reversed-phase high-performance liquid chromatography with ultraviolet or electrochemical detection (Table 1 [2–14]). Based on previous studies [1,15–17], we

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☆ This paper is part of the dissertation of M.B., Mathematisch-Naturwissenschaftliche Fakultät der Rheinischen, Friedrich-Wilhelms Universität Bonn.

0378-4347/94/$07.00
SSDI 0378-4347(94)00161-W

Table 1
Chromatographic methods for the determination of chlorprothixene, levomepromazine, and promethazine

Ref.	Separation[a]	Detector[b]	Sample preparation	Matrix (volume used)	Sensitivity
Chlorprothixene					
2	RP-HPLC	UV Electrochemical	Solvent extraction	Plasma (1 ml)	5 ng/ml
Levomepromazine					
3	RP-HPLC	UV	Solvent extraction	Urine (20 ml) Plasma (1 ml)	0.5 ng/ml 2 ng/ml
4	RP-HPLC	UV	Solid-phase extraction	Urine (1 ml) Serum (1 ml)	2.5 ng/ml 5 ng/ml
5	RP-HPLC	Fluorescence	Solvent extraction	Plasma (2 ml)	2 ng/ml
6	GLC	FID	Solvent extraction	Plasma (6 ml)	3 ng/ml
Promethazine					
7	HPLC	UV	Solvent extraction	Blood (10 ml)	2 ng/ml
8	GC	FID	Solvent extraction	Plasma, blood, urine (up to 1 ml)	10 ng/ml
9	HPLC	Electrochemical	Solvent extraction	Plasma (2 ml)	0.1 ng/ml
9	GC	MS	Solvent extraction	Plasma (2 ml)	0.5 ng/ml
10	RP-HPLC	UV	Solvent extraction	Blood (5 ml) Saliva (5 ml)	0.2 ng/ml; 0.2 ng/ml
11	RP-HPLC	Fluorescence	Deproteination with methanol	Serum	10 ng/ml
12	HPLC	Electrochemical	Solvent extraction	Plasma (1 ml)	1 ng/ml
13	HPLC	UV	Solvent extraction	Serum (2 ml)	1 ng/ml
14	HPLC	Electrochemical	Solvent extraction	Serum (2 ml)	0.2 ng/ml

[a] RP = reversed phase; HPLC = high-performance liquid chromatography; GC = gas chromatography; GLC = gas–liquid chromatography.
[b] UV = ultraviolet; MS = mass spectrometry; FID = flame-ionisation detection.

felt that a method which permits working up a large number of samples with a lower limit of detection of less than 1 ng/ml was required to carry out single dose pharmacokinetic studies in humans. In the present paper, we describe a high-performance liquid chromatographic method for the separate determination of chlorprothixene, levomepromazine, and promethazine; the method is sensitive and suitable for pharmacokinetic studies of these drugs.

2. Experimental

2.1. Chemicals

The certified reference standards of chlorprothixene [3-(2-chloro-9H-thioxanthen-9-yliden)-NN-propylamine], levomepromazine [10-(3-dimethylamino-2-methylpropyl)-2-methoxyphenothiazine], and promethazine [10-(2-dimethylaminopropyl)-phenothiazine] were provided by Troponwerke (Cologne, Germany). The structural representation of the neuroleptic drugs is given in Fig. 1. Acetonitrile (Baker, Deventer, Netherlands), water (Baker) and *n*-heptane (Rathburn, Walkerburn, UK) were high-performance liquid chromatography grade. All other chemicals were analytical grade obtained from Merck (Darmstadt, Germany).

2.2. Blood collection

In order to carry out assay validation procedures, blood was obtained from healthy subjects recruited at the Institute of Haematology of

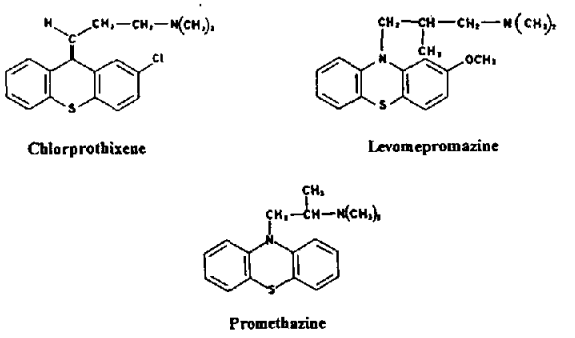

Fig. 1. Structural representation of chlorprothixene, levomepromazine, and promethazine.

the University of Bonn. Subjects taking drugs, excessively using alcohol (other than social drinking), tea or coffee, or smoking cigarettes, were excluded from the study. For pharmacokinetic studies eight to twelve healthy male volunteers received 100 mg of the neuroleptic drugs orally. In accordance with the study protocol, blood samples were collected 16–21 times for up to 72–144 h after drug administration.

2.3. Instrumentation

The high-performance liquid chromatographic system consisted of a Bischoff 2200 high-performance liquid chromatography pump (Bischoff, Leonberg, Germany) and a Waters intelligent sample processor (WISP 717) equipped with a cooling module (Millipore-Waters, Eschborn, Germany); the separation column (250 × 4.6 mm I.D.) contained Nucleosil 100 CN, 5 μm particle size. The column effluent was monitored with a Coulochem II coulometric electrochemical detector (Environmental Sciences Assoc. (ESA), Bedford, MA, USA) fitted to a high-sensitivity analytical detector cell, Model 5011 (ESA, Bedford, MA, USA). The ESA high-sensitivity analytical cell consisted of two sequential coulometric working electrodes, with reference and counter electrodes. The analyses were performed in the oxidative screen mode. The detector signals were analyzed by Hyperdata Chromsoft computer software with a Model 1605 serial chromatography signal interface (Bischoff, Leonberg, Germany).

2.4. Procedures

Blood was collected from healthy subjects into a 500-ml bottle without additives, such as EDTA, sodium citrate or heparin, and stored for 3 h at 20°C; serum was obtained by centrifugation of the blood at 2000 *g* for 15 min. The serum was combined and used immediately for the preparation of the standards for the three neuroleptic drugs. All procedures were carried out at 25°C under yellow light to protect the neuroleptic drugs from degradation. The standard curve and quality control (QC) samples were prepared with pooled serum by adding appropriate volumes of stock solution (1 mg/ml of the neuroleptic drugs in acetonitrile) to obtain concentrations in the range 0.1–1000 ng/ml. The stock solutions in acetonitrile, as well as serum standards, QC and study samples were kept at −20°C.

Blood samples from volunteers participating in the pharmacokinetic study were drawn from the antecubital vein into 10-ml venoject blood collecting tubes (Terumo, Leuven, Belgium) with brown stoppers without additives. After 30 min the serum was obtained by centrifugation at 1800 *g* for 10 min at 4°C.

The extraction of the neuroleptic drugs was carried out as follows: 2.0 ml of water and 2.0 ml of 2 *M* sodium hydroxide were added to 1.0 ml serum standard, QC or study sample in 15-ml screw-capped borosilicate glass tubes, and the mixture was vortex-mixed for 10 s. The mixture was extracted with 5.0 ml water-saturated *n*-heptane–isoamylalcohol (99:1, v/v) by shaking gently for 20 min. After centrifugation at 2800 *g* at 4°C the top layer was aspirated; this procedure was repeated once and the two extracts were combined. The solvent was evaporated to dryness under vacuum and the residue was dissolved in 500 μl acetonitrile; 100 μl of this solution was used for the analysis and the remainder was stored at −20°C for possible repetition of the analysis.

2.5. Chromatography

The mobile phase (acetonitrile–pyridine–0.14 *M* sodium acetate, pH 3.1 (698:2:300, v/v)) was filtered through a 0.22-μm Nylon filter (Millipore, Eschborn, Germany) and degassed with helium immediately prior to the chromatographic procedure. The reconstituted solution was kept in the autosampler at 5°C for chromatography; 30 μl was injected onto the chromatographic system. The mobile phase was continuously recirculated at a flow-rate of 0.9 ml/min; the chromatography for any of the three neuroleptic drugs was completed within 20 min. The cell potentials of the screen electrode were +0.5, +0.35 and +0.25 V, and those of the sample electrode +0.85, +0.65 and +0.5 V for chlorprothixene, levomepromazine, and promethazine, respectively. The signals of the sample electrode were used for quantification. The reconstituted solutions with concentrations of more than 50 ng/ml (which exceeded the detector range) were diluted with acetonitrile prior to the analysis.

The mobile phase was recycled for 7 days without adversely affecting the shape of the peaks or the sensitivity of the assay. A problem encountered during the analysis of samples was coating of the analytical cell. This was attributed to the polymerisation of the analyte and to impurities on the electrode surface. To reduce the passivation of the analytical cell, it was removed from the chromatographic system and treated with 3 ml of 6 *M* nitric acid and rinsed with water. This treatment was necessary after processing approx. 500–700 samples in order to maintain constant sensitivity.

2.6. Calculation

The calibration was performed by regression of the peak area of each neuroleptic drug *vs.* the appropriate standard concentration. The best fit was obtained by the least square linear regression procedure with a weighing factor of $1/\text{concentration}^2$ [18–20] and the concentrations of the QC and unknown samples were calculated accordingly.

In each set of experiments the QC samples were analyzed along with calibration standards and unknown samples to check the reliability of the assay. Each sample (QC, calibration and unknown samples) was analyzed in duplicate. The accuracy and precision of the assay procedure were monitored for each analytical run. The recovery of the extraction procedure for each neuroleptic drug was calculated by comparing the slope of the regression line of the extracted standards to that of the directly injected standards.

2.7. Acceptance criteria

The quality criteria to fulfil the requirements for pharmacokinetic investigations were chosen as follows: In general, the accuracy should be within ± 10%. The concentration of the standard samples in human serum *vs.* peak area should yield a coefficient of determination (r^2) of > 0.99. The intra- and inter-assay coefficient of variation should be $\leq 10\%$; but, close to the lower limit of detection the value may be higher than 10% but not more than 20%.

3. Results

The average recoveries (mean ± S.D.) of the extraction over the entire calibration range were 93.0 ± 6.2%, 84.6 ± 11.5% and 88.5 ± 7.1% for chlorprothixene, levomepromazine, and promethazine, respectively. The specificity of the assay was monitored by checking the chromatograms for interfering peaks from endogenous components in drug-free human serum. From the chromatograms of blank serum it may be inferred, that there were no interfering substances at the respective retention times of the neuroleptic drugs (Fig. 2.). This also holds for the chromatograms of volunteer serum samples obtained 5 h after oral administration of 100 mg chlorprothixene, levomepromazine, or promethazine (Fig. 3.).

Using 1.0 ml serum the lower limits of sen-

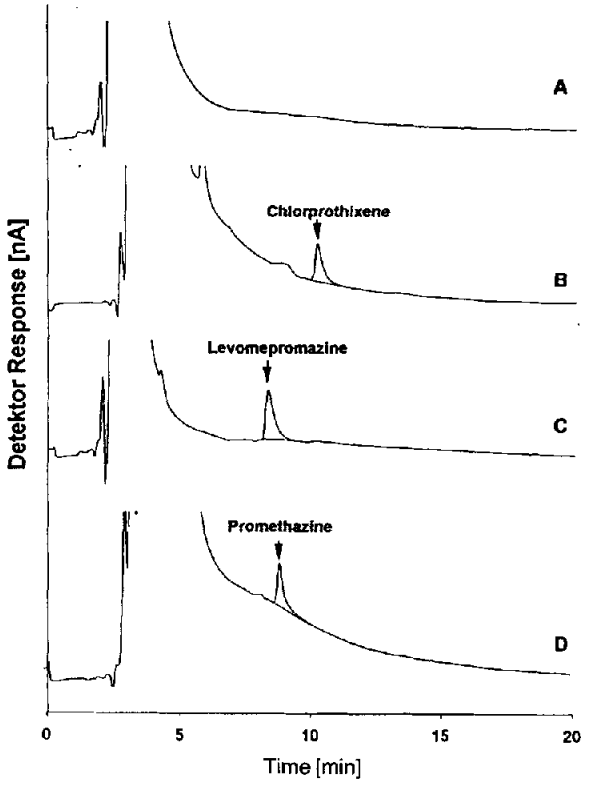

Fig. 2. Chromatograms of extracts from: (A) drug-free human serum, and human sera containing 10 ng/ml of each (B) chlorprothixene, (C) levomepromazine, and (D) promethazine. The retention times of the neuroleptics are indicated by the arrows and were 10.2, 8.3, and 8.9 min for chlorprothixene, levomepromazine, and promethazine, respectively.

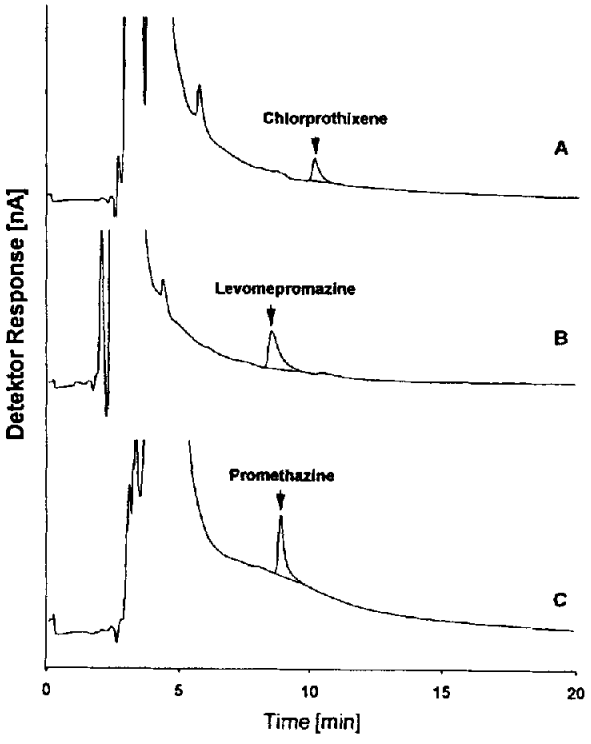

Fig. 3. Chromatograms of extracts from sera of human volunteers 5 h after receiving orally 100 mg (A) chlorprothixene, (B) levomepromazine, and (C) promethazine. The retention times were the same as in Fig. 1.

sitivity were 0.5, 0.2 and 0.1 ng/ml for chlorprothixene, levomepromazine, and promethazine, with inter-assay coefficients of variation of 12.2, 3.3 and 5.3%, respectively. The calibration curves were linear for chlorprothixene (range: 0.5 to 500 ng/ml; $r^2 = 0.9973 \pm 0.0028$), levomepromazine (range: 0.2 to 500 ng/ml; $r^2 = 0.9994 \pm 0.0005$) and promethazine (range: 0.1 to 50 ng/ml; $r^2 = 0.9993 \pm 0.0004$). The intra-assay coefficients of variation were established at concentrations of 10 ng/ml of chlorprothixene, 5 and 20 ng/ml of levomepromazine, and 0.5 and 20 ng/ml of promethazine ($n = 10$ for each concentration), resulting in a precision estimate of 2.5% for chlorprothixene, 5.6 and 0.9% for levomepromazine, and 5.3 and 0.9% for promethazine. The inter-assay coefficients of variation for the entire standard concentration range and QC samples were within 12.2%, 9.3% and 13.5% for chlorprothixene, levomepromazine, and promethazine, respectively (Table 2). The accuracy of the standards and QC samples deviated by ± 7% at the various concentrations investigated for each neuroleptic drug.

The neuroleptic drugs were stable in acetonitrile and serum samples for at least six months when stored at −20°C (Table 3).

The individual serum concentrations *vs.* time profiles (Fig. 4.) indicated that the concentrations of the neuroleptic drugs are measurable up to 144 h following oral administration of 100 mg of chlorprothixene, levomepromazine, and promethazine to healthy volunteers.

Table 2
Accuracy and precision of the determination of serum neuroleptic drug levels by HPLC

Concentration (ng/ml)	Chlorprothixene		Levomepromazine		Promethazine	
	Accuracy[a] (%)	C.V.[b] (%)	Accuracy[a] (%)	C.V.[b] (%)	Accuracy[a] (%)	C.V.[b] (%)
Calibration standards						
0.1	n.m.[c]	n.m.	n.m.	n.m.	103.4	5.3
0.2	n.m.	n.m.	103.6	3.3	101.1	5.9
0.5	108.0	12.2	102.0	4.0	98.7	7.1
1.0	104.3	10.9	96.4	4.9	96.4	5.7
2.0	107.5	12.1	98.0	7.3	98.0	4.3
5.0	103.5	8.2	103.3	3.8	103.4	3.3
10	101.0	4.8	97.6	2.9	101.6	3.3
20	99.3	3.3	103.0	3.2	105.4	6.0
50	99.9	3.9	106.7	3.4	102.4	4.0
100	101.2	3.2	98.5	4.1	n.m.	n.m.
200	102.1	3.0	102.4	1.2	n.m.	n.m.
500	104.1	2.1	100.3	0.9	n.m.	n.m.
Quality control samples						
0.4	n.m.	n.m.	100.1	8.1	93.3	13.5
4.0	104.6	9.5	n.m.	n.m.	n.m.	n.m.
40	n.m.	n.m.	97.2	6.3	93.4	7.4
400	106.7	6.2	99.5	3.1	n.m.	n.m.

[a]The added concentration was taken as 100%.
[b]Inter-assay coefficient of variation.
[c]n.m. = not measured.

Table 3
Stability data for chlorprothixene, levomepromazine, and promethazine in acetonitrile and human serum

Matrix	Concentration taken (ng/ml)	Concentration measured (ng/ml)		
		After 1 month	After 2 months	After 6 months
Chlorprothixene				
Acetonitrile	5	4.2	4.3	4.7
	50	54.5	50.1	50.2
Human serum	4.0	4.4	4.05	4.2
	49.9	49.1	44.5	47.8
Levomepromazine				
Acetonitrile	20	n.m.[a]	21.5	19.2
Human serum	36.8	n.m.[a]	34.7	34.1
Promethazine				
Acetonitrile	20	n.m.[a]	18.2	22.8
Human serum	43.2	n.m.[a]	46.2	38.1

[a] n.m. = not measured.

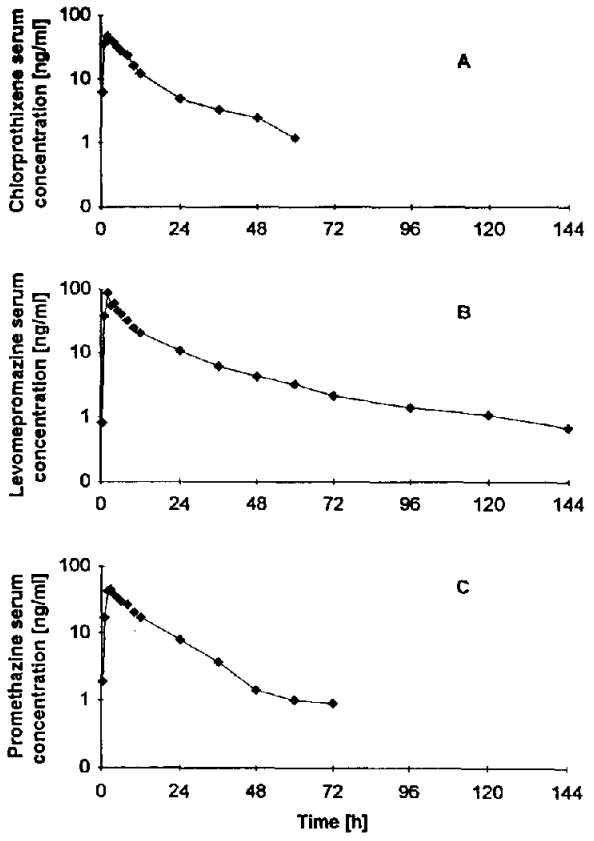

Fig. 4. Individual serum concentration *versus* time profiles (semi-logarithmic plot) measured following oral administration of 100 mg chlorprothixene (A), levomepromazine (B), and promethazine (C) to human volunteers.

4. Discussion

In the present paper, we describe a sensitive, selective and accurate assay for the analysis of chlorprothixene, levomepromazine, and promethazine in human serum that can be applied for single-dose pharmacokinetic studies. This method includes a rapid and simple one-step solvent extraction with water-saturated *n*-heptane–isoamylalcohol that allows the processing of a large number of samples within a short time. The extraction efficiency of the procedure was close to 90% for these three neuroleptic drugs and was in good agreement with previous results [2,3,7–10,13,14]. Using the present method, the extracts were less contaminated than those obtained by extraction with dichloromethane or ethyl acetate, thereby resulting in cleaner chromatography with no interferences from serum constituents. The method was optimised to carry out the assay for each neuroleptic drug without using an internal standard to avoid the coating of the analytical cell with additional sample constituents. Furthermore, to prolong the life of the analytical cell, samples containing concentrations of more than 50 ng/ml were diluted prior to the injection.

The neuroleptic drugs were oxidized coulometrically on a porous graphite surface. Compared to the commonly used amperometric detection where the current is monitored with an efficiency of 1–4% as a function of elution time, the yield of the coulometric detector in the present method is close to 100% and by Faraday's law the peak area is directly proportional to the quantity of the analyte. Thus, the quantification of the neuroleptic concentrations by operating the two coulometric electrode cells in the screen mode, resulted in a higher sensitivity compared with previous methods. Although, Leelavanthi *et al.* [9] achieved a sensitivity of 0.1 ng/ml for the determination of promethazine, their method required 2.0 ml of plasma.

Apart from the adjustment for optimal detector response, necessary for each neuroleptic drug, the present method can be used for the determination of chlorprothixene, levomepromazine or promethazine without any further modification.

Preliminary results from our pharmacokinetic studies indicated that the sensitivity, accuracy and precision are adequate to monitor serum levels after an intravenous or oral administration of the neuroleptic drug over a period of up to 144 h. The method is adequate to work up a total of approx. 250 serum samples including QC and standard samples per week.

5. Acknowledgements

The financial support and provision of reference standards of chlorprothixene, levomepromazine, and promethazine by Troponwerke (Cologne, Germany) are gratefully acknowl-

edged. We thank Dr. Langer, Troponwerke, for his valuable suggestions and discussion and Drs. P. Hanfland and R.E. Scharf, Institut für Experimentelle Haematologie und Transfussionsmedizin der Universität Bonn, for supplying drug-free serum from healthy subjects.

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