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ANODIC COULOMETRIC DETECTION WITH A GLASSY CARBON ELECTRODE IN COMBINATION WITH REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

DETERMINATION OF BLOOD LEVELS OF PERPHENAZINE AND FLUPHENAZINE*

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SUMMARY

A detector for liquid chromatography, based on anodic electrochemical oxidation at a glassy carbon electrode, is used in combination with reversed-phase liquid chromatography for the determination of the long-acting tricyclic psychotropic drugs fluphenazine and perphenazine in blood.

Completely porous microparticulate silica material, modified so as to give a methyl silica, was used as the stationary phase and buffered mixtures of methanol and water as the mobile phase. The low detection limit and the selectivity of the coulometric detector made possible the determination of concentrations of 1–20 ppb of the compounds of interest.

An extraction procedure was in order to obtain sufficient selectivity.

Data for the precision, detection limit and yield of the extraction procedure are given. A number of serum levels of perphenazine after oral administration and of fluphenazine after i.m. doses of fluphenazine decanoate are stated.

INTRODUCTION

Most phenothiazines used in medical practice are neuroleptics, which are usually given orally once or several times a day. One of the main problems of prescribing oral medication to psychiatric out-patients is that about 50% of the patients fail to take the prescribed medication after a few months[1]. From this group of drug-withdrawn patients, about 60% relapse within several months[2]. Most of these drug-withdrawn patients recover when the same dosage of the original neuroleptic is reinstituted.

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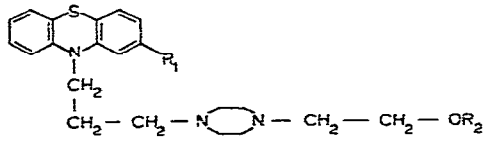
TABLE I
STRUCTURES OF FLUPHENAZINE AND PERPHENAZINE



Compound	*Abbreviation*	R_1	R_2
Fluphenazine	FPZ	CF_3	H
Perphenazine	PPZ	Cl	H
Perphenazine enanthate	—	Cl	$-CO-(CH_2)_5-CH_3$
Perphenazine decanoate	—	Cl	$-CO-(CH_2)_8-CH_3$

From phenothiazines that contain an alcoholic group such as fluphenazine (FPZ) and perphenazine (PPZ), esters can be synthesized, which are more lipophilic than the original compounds. The structures are given in Table I. The decanoic ester of fluphenazine or perphenazine is dissolved in sesame oil and given by intramuscular injection. Contrary to oral medication, this injection has to be given only once or twice a month. The prolonged action of these depot formulations must be attributed to the slow release of the ester from the oily depot. Once in the blood, the phenothiazine esters are hydrolyzed rapidly by esterases into the psycho-active phenothiazines. In dog blood, only trace amounts of the esters could be detected 1 h after i.v. injection of the ester, more than 95% having been hydrolyzed[3].

The percentage of psychiatric out-patients relapsing within 1 year has been reduced to about 20% by the introduction of long-acting phenothiazines. It is possible that a part of the remaining relapses have pharmacological reasons. Therefore, it is important to be able to determine phenothiazines and their metabolites in body fluids, giving the psychiatrist a new parameter for the institution of calculated doses and dose intervals.

TABLE II
PLASMA LEVELS (ng/ml) OF PERPHENAZINE AFTER DIFFERENT DOSAGE FORMS IN MAN

All determinations were carried out by GLC using electron-capture detection. A: Oral administration of 8 mg of PPZ, 3 patients[4]. B: i.m. administration of 5 mg of PPZ, 3 patients[4]. C: i.m. administration of 100 mg per 2 weeks of PPZ enanthate, 3 patients[4]. D: i.m. administration of 100 mg per 2 weeks of PPZ enanthate, 1 patient[5].

Time	*A*	*B*	*C*	*D*
Peak level after a single dose	1–3 (after 1–4 h)	2–3 (after ½–3 h)	2–7 (after ½–3½ days)	2 (after 3–7 days)
Time after first dosage:				
1 day	<0.2	<0.2	1.6–6.6	1.4
3 days	—	—	1.6–5.8	2.0
9 days	—	—	0.7–1.0	0.8
14 days	—	—	0 –0.5	0.6
Time after second dosage:				
3 days	—	—	—	4.5
9 days	—	—	—	2.7

For this purpose, concentrations of the order of 1–10 ng/ml must be measurable in blood, etc. Only a limited number of reports have described a successful approach to this problem.

Hansen and Larsen[4] and Larsen and Naestoft[5] described the use of gas chromatography, in combination with derivatization and electron-capture detection. Some of their results are given in Table II. The derivatization step necessary in these procedures is tedious, and risky at these low concentration levels.

The tracer methods used by Schreiber and Grozier[6] and McIsaac[7] can only be used in research work. The results obtained with these methods are reviewed in Table III.

TABLE III

PLASMA LEVELS (ng/ml) OF FLUPHENAZINE AFTER DIFFERENT DOSAGE FORMS IN MAN

In all determinations, radioactively labelled compounds were applied. No separation was carried out before determination. A: oral administration of 10 mg of FPZ, 3 patients[6]. B: i.m. administration of 25 mg of FPZ enanthate, 4 patients[6]. C: i.m. administration of 25 mg of FPZ enanthate, 5 patients[7]. D: i.m. administration of 25 mg of FPZ decanoate, 4 patients[6]. E: i.m. administration of 25 mg of FPZ decanoate, 5 patients[7].

Time after first dosage	*A*	*B*	*C*	*D*	*E*
2–24 h			0.30–0.50		0.03–0.21
3 days	0.006–0.011		0.72–0.94		0.16–0.72
6 days		0.57–1.15	1.07–1.18		0.12–0.21
12 days			0.66		0.13–0.35
25 days				0.04–0.29	

The fluorimetric method described by Smulevitch *et al.*[8] and the phosphorimetric–chromatographic methods described by Gifford and co-workers[9,10] are very unlikely to be suitable for analyses at the nanogram level. Smulevitch *et al.* reported plasma levels, but these are substantially higher.

During the last few years, a number of papers dealing with the electrochemical measurement of drugs in biological fluids have been published[11–16]. The applicability of electroanalytical techniques for the determination of drugs without a preceding separation is limited, because of the lack of selectivity.

Riggen *et al.*[17] showed the powerful combination of high-performance liquid chromatography and electrochemical detection for the determination of drugs in body fluids.

The determination of phenothiazines in blood at very low concentrations (PPZ, 0–20 ng/ml; FPZ, 0–1.5 ng/ml) is possible only when the chromatographic system, the detection system and the sample pre-treatment are optimized with respect to this particular problem.

This paper describes the use of a reversed-phase system with large selectivities towards phenothiazines[18], combined with the highly sensitive coulometric detector[11] for the determination of PPZ and FPZ in serum.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from custom-made and commercially available parts and consisted of a glass thermostated eluent reservoir, a high-pressure pump (Orlita, Giesen, G.F.R.; Type DMP 1515), a flow-through Bourdon-type manometer, serving as a damping device, a high-pressure sampling valve (Valco; Type CV-6-UHPa-C20) and a thermostated stainless-steel column of I.D. 2.8 mm. In order to prevent contamination of the separation column, a pre-column (300 × 7.5 mm) was installed. The coulometric detector was custom-made and has been described elsewhere[11]. The chromatograms were recorded on a linear potentiometric recorder (Goertz, Austria; Servogor RE 511). An integrator (Spectra Physics Autolab System I) was used in the quantitative experiments. A homogenizer (Sorvall) was used in the extraction experiments.

Chemicals and materials

In all experiments, doubly distilled water and organic solvents of analytical grade (Merck, Darmstadt, G.F.R.) were used. *n*-Hexane, used as extraction solvent, was freshly distilled. The methyl silica used as the stationary phase was prepared from narrow-sized silica (Merck; LiChrosorb SI 60) treated with dichlorodimethylsilane[18]. The pre-column was filled with silanized silica, 63–200 μm (Merck).

Procedures

Chromatography. The separation columns were packed using a pressurized balanced slurry technique, as described elsewhere[19]. The pre-column was packed by means of a dry packing technique.

The phase systems used consisted of mixtures of methanol and 0.05 *M* phosphate buffer in water, in combination with the methyl silica. To all mobile phases, 7 g/l of potassium chloride was added in order to obtain stable potentials of the reference electrode in the detector. The capacity ratios were calculated from the retention times of the phenothiazines and an unretarded compound (sodium sulphite). The selectivity coefficients were calculated as the ratios of capacity ratios.

The samples were dissolved in the eluent and injected by means of the sampling valve with a loop of 104 μl.

Extraction. The procedure used for the extraction of the phenothiazines from serum is outlined in Table IV. In order to allow for a rapid and nearly quantitative separation of the phases after equilibration, the extraction mixtures were centrifuged and frozen, after which the organic phase can be decanted easily. In order to prevent emulsification during mixing of alkaline serum and *n*-hexane in the first step, a homogenizer was used instead of a whirl-mixer.

RESULTS AND DISCUSSION

In order to determine phenothiazines at very low concentrations in blood, the parameters of the chromatographic process and the detection system should be chosen so that both the resolution and the detection limit are adequate[20]. For the de-

TABLE IV

SCHEME FOR EXTRACTION OF PHENOTHIAZINES FROM PLASMA

Stage	*Operation*
3 ml serum	(1) Add 0.3 ml of 7 *N* NaOH and 12 ml of *n*-hexane (2) Homogenize for 120 sec (4000 r.p.m.) (3) Pour in glass tube, centrifuge for 120 sec (4) Freeze and decant the *n*-hexane phase
Hexane phase	(1) Add 2 ml of 0.1 *N* H_2SO_4 (2) Mix for 60 sec (whirl-mixer) (3) Decant the *n*-hexane phase
Aqueous phase	(1) Add 0.2 ml of 7 *N* NaOH and 6 ml of *n*-hexane (2) Mix for 60 sec (whirl-mixer) (3) Centrifuge for 120 sec (2500 r.p.m.) (4) Freeze and decant the *n*-hexane phase
Hexane phase	(1) Add 10 μl of isopropylamine (2) Evaporate the solvent (40°, nitrogen stream)
Residue	(1) Dissolve in 150 μl of eluent (2) Ultrasonicate (3) Analyse aliquots of 104 μl by LC

tection limit, the absolute amount rather than the concentration in the sample is relevant, as only a few millilitres of blood are usually available.

The fundamental chromatographic equation giving the resolution, R_{ji}, of a pair of compounds is

$$R_{ji} = (r_{ji} - 1)\frac{\kappa_i}{1 + \kappa_i}\sqrt{\frac{L}{H_i}} \tag{1}$$

where r_{ji} is the selectivity coefficient of the components i and j, κ_i is the capacity ratio of component i, L is the length of the column and H_i is the theoretical plate height for component i. The first factor describes the influence of the selectivity of the phase system, the second that of the retardation and the third that of the dispersion in the column.

The relationship between the maximum concentration of the solute in the mobile phase at the end of the column, $\langle c_j^m \rangle_{max}$, and the amount injected, Q_j, is expressed by the equation

$$\langle c_j^m \rangle_{max} = \frac{Q_j}{\sqrt{2\pi}\,\varepsilon_m A (1 + \kappa_j)(H_j L)^{\frac{1}{2}}} \tag{2}$$

where ε_m is the porosity of the mobile phase and A is the cross-sectional area of the column. From eqns. 1 and 2, it can be concluded that large selectivity coefficients, small column diameters and low theoretical plate heights are favourable and that large capacity ratios must be avoided in order to achieve low detection limits and adequate resolution.

The standard deviation of the baseline noise measured during the same period of time as corresponds to the peak integral corresponds to a concentration of $1.6 \cdot 10^{-10}$

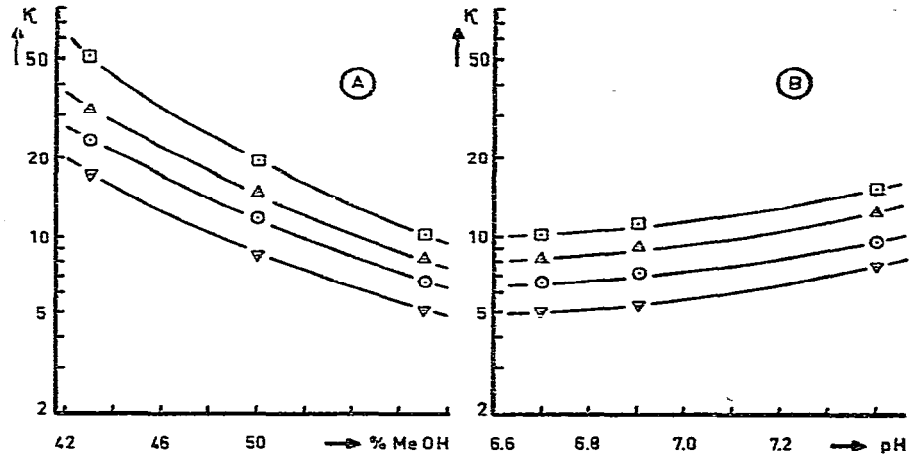


Fig. 1. Dependence of the capacity ratio of some neuroleptics on the methanol content and the pH of the buffer with methyl silica as the stationary phase. A, methanol–phosphate buffer (0.05 *M*, pH 6.70); B, methanol–phosphate buffer (0.05 *M*) (57:43). ⊡, Flupenthixol; △, fluphenazine; ○, clopenthixol; ▽, perphenazine.

mole/l (ref. 11). If a signal-to-noise ratio of 3 is chosen, it can be calculated from eqn. 2 that a detection limit of 30 pg is set by the baseline noise of the detector.

Selectivity of the phase system

In Fig. 1A, log κ is plotted *versus* the methanol content of the mobile phase for some neuroleptics. It can be seen that an increase in the methanol content leads

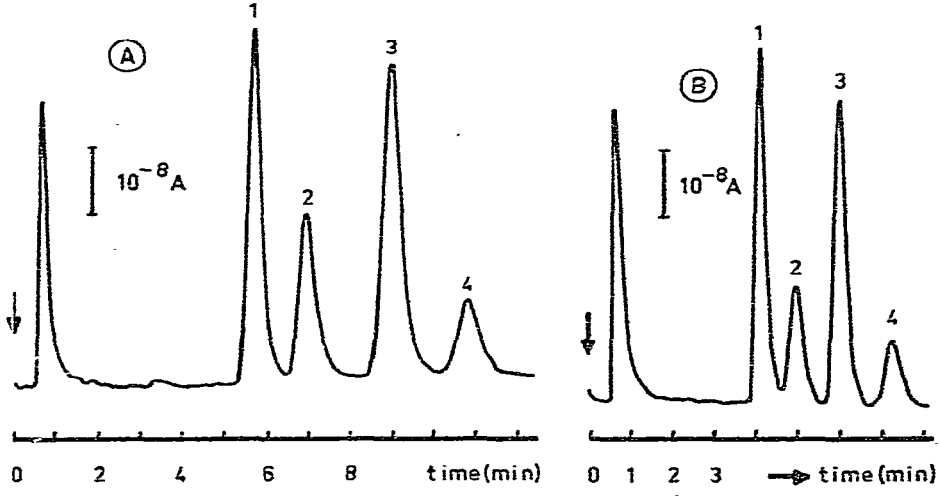


Fig. 2. High-speed separation of a test mixture of four neuroleptics. Column, 100 × 2.8 mm; packing, methyl-bonded silica (SI 60, 6–7 μm). Eluent: A, methanol–phosphate buffer (0.05 *M*, pH 7.40) (57:43, v/v); B, methanol–phosphate buffer (0.05 *M*, pH 6.90) (53:47, v/v). Peaks: 1 = perphenazine; 2 = clopenthixol; 3 = fluphenazine; 4 = flupenthixol (amount of each compound injected, 7 ng).

to a decrease in both the capacity ratios and the selectivity coefficients. Fig. 1B shows the relationship between the pH of the buffer used and the capacity ratios. It appears that the pH has a strong influence. The ability of the phase system is demonstrated in Fig. 2, which shows the separation of four neuroleptics using two different phase systems.

In order to achieve low detection limits, it was decided to use a mixture of methanol and phosphate buffer (0.05 *M*, pH 6.90) in the volume ratio 53:47.

Development of the procedure

As a first approach, direct injection of the serum on the column was tried because it was thought that a combination of reversed-phase liquid chromatography and selective electrochemical detection might have sufficient selectivity. Experiments with the injection of 0.25 ml of serum, partly deproteinized with an equal amount of methanol and to which appropriate amounts of buffer and chloride were added, were carried out. The detection limit of this method for FPZ was estimated to be *ca.* 25 ng/ml, owing to the large amount of oxidizable material in the serum.

Further work on the selectivity of the measurement via anodic electrochemical oxidation, especially for biological samples, is necessary.

On the basis of this poor result, it was decided to use an extraction procedure, which would have the additional advantage that an increase in concentration (by a factor of about 20) would be obtained, with the result that it would no longer be necessary to work at the limit of the capability of the detector.

The extraction procedure is straightforward for these basic drugs. Iso-propylamine is added in the last stage in order to diminish adsorption on glass.

Precision of the method

The precision of the quantitative determination of phenothiazines was investigated by injection of a constant volume (104 μl) of a solution of PPZ and FPZ in different concentrations (8–200 ng/ml). Fig. 3 shows the relationship between peak area and amount injected. The broken lines show the confidence limits for $\pm$ 3 times the standard deviation. For PPZ, the standard deviation was about 0.5% at 200 ng/ml and 7% at 8 ng/ml, and for FPZ it was 0.8% and 16%, respectively, at these concentrations. The standard deviations at low concentrations are higher than can be expected from the baseline noise figures stated for the detector[11]. As no higher noise levels were observed, other contribution to the variance must be present, *e.g.*, in the chromatographic and sampling processes.

Recoveries for PPZ and FPZ were determined by extraction of known amounts added to serum. In the extraction of 10 ng of PPZ and 10 ng of FPZ added to 3 ml of serum, the recovery of each compound was 50% with a standard deviation of 5%.

Determination of PPZ and FPZ in serum

The method described was applied to the determination of FPZ and PPZ in serum samples from psychiatric patients receiving neuroleptics. The samples were treated as described before.

Fig. 4 shows the chromatogram of a serum extract, FPZ being added as an internal standard. Fig. 5 shows that in co-medication, the determination of PPZ is still possible, although the precision is lower because of the presence of overlapping

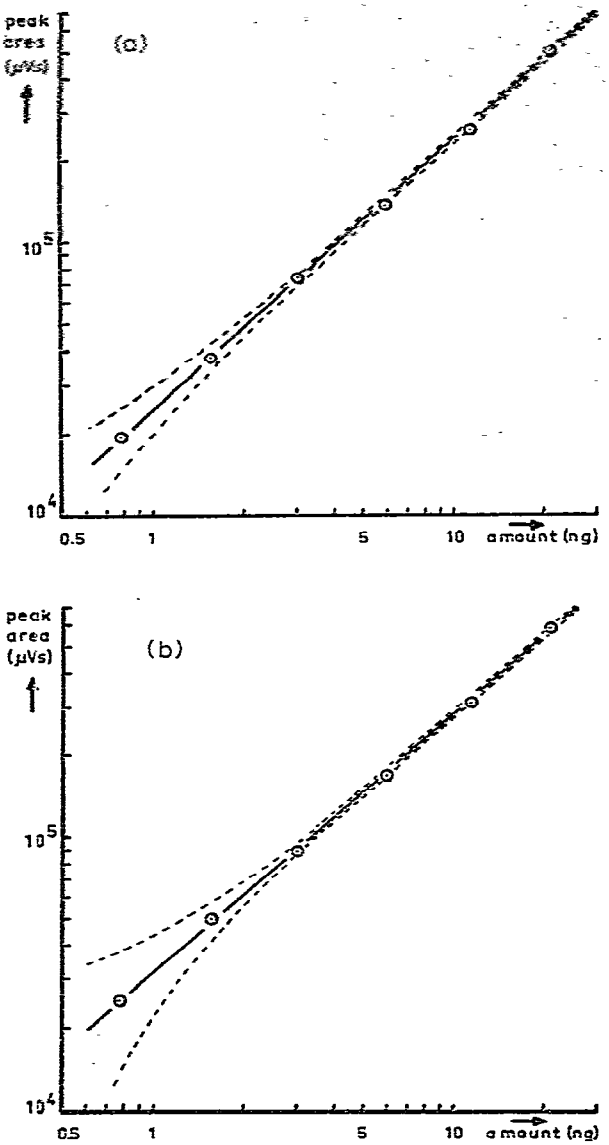


Fig. 3. Peak area *versus* amount injected for (a) perphenazine ($\kappa = 5.2$) and (b) fluphenazine ($\kappa = 8.8$). The broken lines show ± 3 times the standard deviation. Conditions as in Fig. 2 (eluent B).

peaks in the chromatogram. Table V gives the results of the determinations of serum levels of PPZ after oral doses. The difference between these results and those in Table II should be attributed to the accumulation of the drug, when given as a maintenance dose instead of as a single dose.

The determination of FPZ is more critical because of the lower concentrations occurring in blood. Fig. 6 represents the chromatogram of an extract of serum, taken from a subject 4 days after the first dose of the long-acting formulation (125 mg of

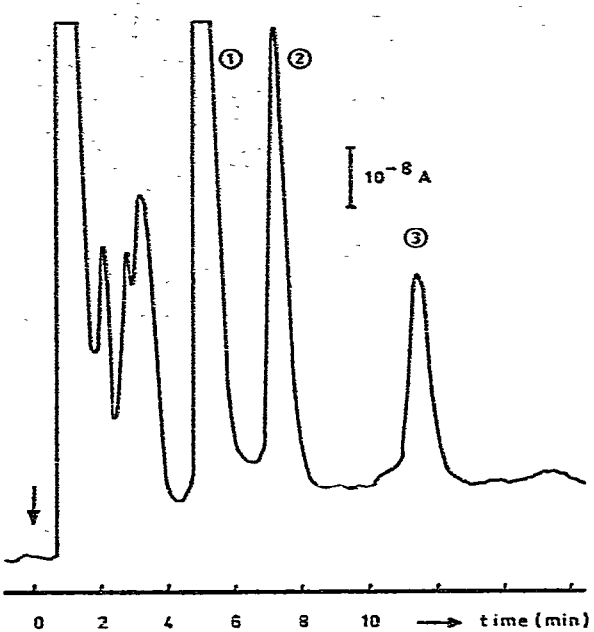


Fig. 4. Chromatogram of an extract from serum of subject S.L.W. with a maintenance dose of 3 × 8 mg of perphenazine and 3 × 50 mg of orphenadrine daily. Blood sample was taken before the morning dose. Fluphenazine was added as internal standard. Column, 100 × 2.8 mm; packing, methyl silica (SI 60, 6–7 μm); eluent, methanol–phosphate buffer (0.05 *M*, pH 6.90) (53:47, v/v). Peaks: 1 = orphenadrine; 2 = perphenazine; 3 = fluphenazine.

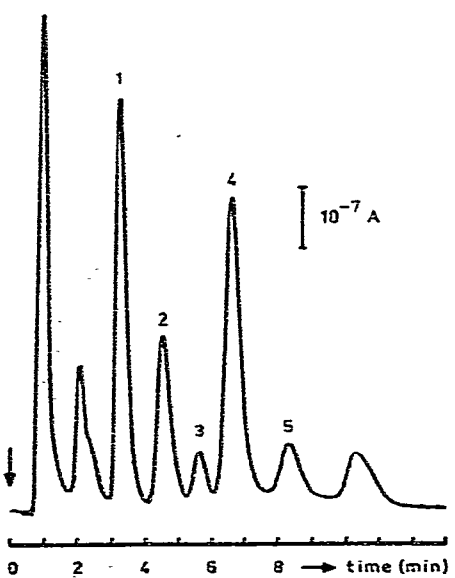


Fig. 5. Determination of perphenazine in a serum extract. Subject, L.G.R. Maintenance dose, 3 × 16 mg of perphenazine, 3 × 2 mg of biperiden, 3 × 100 mg of orphenadrine, 3 × 50 mg of phenobarbital, 3 × 50 mg of promethazine, 3 × 50 mg of chlorpromazine. Conditions as in Fig. 4. Peaks: 1 = biperiden; 2 = orphenadrine; 3 = perphenazine; 4 = chlorpromazine; 5 = fluphenazine (internal standard).

TABLE V

SERUM LEVELS OF PERPHENAZINE AFTER ORAL DOSES

Blood samples were taken before the morning dose. All subjects received the stated doses for several months.

Subject	*Dose (mg/day)*	*Concentration (ng/ml)*
L.G.R.	3 × 16	~10
S.L.W.	3 × 8	17
H.Z.M.	3 × 16	20

FPZ decanoate). PPZ was added as an internal standard. In Table VI, data are given for assays of samples taken from a number of different subjects. The results are comparable to those obtained by McIsaac[7] (Table III), assuming a linear relationship between dose and blood level.

From duplicate measurements, a standard deviation of 0.2 ng/ml can be estimated for the overall procedure ($n = 3$).

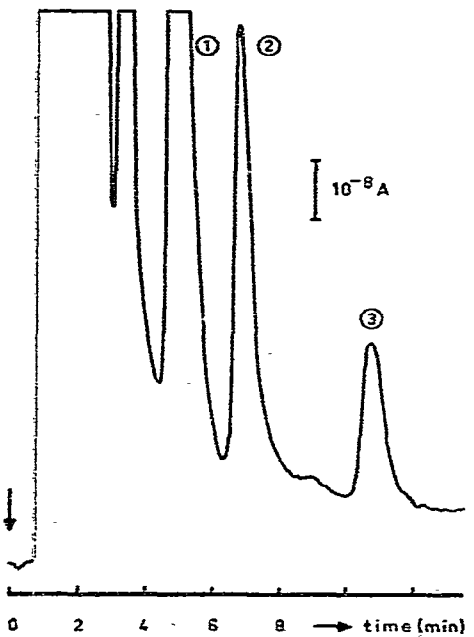


Fig. 6. Determination of fluphenazine in a serum extract. Subject, S.C.H. Medication, 125 mg of fluphenazine decanoate (first intramuscular dose). Blood sample was taken 4 days after dosage. Perphenazine was added as an internal standard. Conditions as in Fig. 4. Peaks: 1 = orphenadrine; 2 = perphenazine; 3 = fluphenazine.

CONCLUSIONS

The method described for the determination of parts per billion levels of psychotropic drugs in blood is based on the combination of a highly efficient column

TABLE VI

SERUM LEVELS OF FLUPHENAZINE AFTER INTRAMUSCULAR DOSES OF FLUPHENAZINE DECANOATE

Subject	*Dose*	*Dose cycle*	*Days after dosage*	*Concentration (ng/ml)*
V.A.D.	19 mg/3 weeks	10th	20	0.8
			23	0.7
			28	Trace
V.V.Z.	25 mg	1st	5	0.7
			8	2.3
S.T.R.	75 mg/3 weeks	30th	8	1.4
D.B.R.	75 mg/3 weeks	50th	8	1.3
O.T.S.	125 mg	1st	3	2.1
			6	6.1
S.C.H.	125 mg	1st	4	1.4
			7	1.3
			11	1.2
G.R.N.	125 mg	1st	12	4.0
			14	3.4
			16	3.3

packing material with suitable selectivity towards the various compounds of interest and electrochemical detection with a very low detection limit and with a selective response towards the group of compounds at hand[11]. For the determination of parts per billion levels in plasma, such a method, combining the selectivity of extraction, of chromatography and of detection is required.

Future work will be devoted to the determination of metabolites of phenothiazines in blood.

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