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RELIABLE ROUTINE METHOD FOR DETERMINATION OF PERAZINE IN SERUM BY THIN-LAYER CHROMATOGRAPHY WITH AN INTERNAL STANDARD

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SUMMARY

The use of a high-performance thin-layer chromatography linear chamber and of thioridazine as internal standard increases the performance of thin-layer chromatography (TLC) with direct densitometric scanning, and allows the rapid determination of serum levels of the neuroleptic drug perazine under usual therapeutic conditions. TLC is superior to gas—liquid chromatography in so far as the main metabolite desmethylperazine can be easily separated and detected by the same procedure.

INTRODUCTION

In 1976 two methods for the quantitative determination of perazine (P) in human sera were published, which greatly advanced the search for a practical and precise analytical procedure at reasonable costs. One of the methods used was gas—liquid chromatography (GLC) [1], the other thin-layer chromatography (TLC) [2].

As the GLC method was not fully optimized and not sensitive enough for clinical conditions, an improved method was developed in our laboratory [3,4]. The latter procedure proved to be very satisfactory with regard to specificity and sensitivity, but had the disadvantage that the main metabolite desmethylperazine could not be assessed together with perazine in human sera. To overcome this difficulty and to have an independent method for an internal comparative control in our laboratory, we adopted the TLC method by Breyer and Villumsen [2]. This method was easily reproducible but was too time-consuming and sophisticated for clinical routine procedures.

However, it turned out that a faster and simpler modification of this TLC method could be developed, which is described in this paper.

MATERIALS AND METHODS

Reference compounds and reagents

Reference compounds were gifts from the following companies: perazine base from Chem. Fabrik Promonta (Hamburg, G.F.R.; thioridazine from Ciba (Basle, Switzerland). Desmethylperazine (DMP) was prepared by reacting phenothiazine with 1-bromo-3-chloropropane followed by reaction with piperazine in methanolic solution, and perazine sulphoxide (PSO) was prepared according to the method of Breyer [5].

Reagents and solvents were Merck p.a. quality (Merck, Darmstadt, G.F.R.). Sodiumdioxycholate was obtained from Serva (Heidelberg, G.F.R.).

Stock solutions

Stock solutions of perazine (P), desmethylperazine (DMP), and thioridazine (THIO) were prepared by dissolving 10 mg of each compound in 10 ml of ethanol. These three solutions were mixed and further diluted with ethanol to obtain standard solutions of 5—50 mg/l of P and DMP, and 30 mg/l of THIO, respectively. The solutions were stored at 4°C. They were found to be stable for at least one month.

Plasma samples

Patients. Blood samples from perazine-treated patients were obtained within the context of a double-blind study described elsewhere [6].

Blank plasma. For recovery experiments we used plasma from blood donors. Blood (400 ml) was collected into a glass bottle containing 100 ml of ACD (acid—citrate—dextrose) solution as anticoagulant and preservative. The blood was centrifuged at 1930 *g* for 10 min, and the plasma stored in 10-ml portions at −20°C. The plasma was used for extraction within a period of two months.

Both the patients' and the blank plasma were thawed shortly before processing.

Extraction procedure

To 5 ml of blank plasma were added 50 μl of one of the standard solutions. To patients' plasma or serum (generally 5 ml) 1500 ng THIO as internal standard were added. Extraction was carried out according to Breyer's method [2] with minor modifications: *n*-heptane was used instead of benzene as solvent; THIO was added as the internal standard; after evaporation of the *n*-heptane phase, two portions of ethanol (100 μl) were used to transfer the residue into a polyethylene microtube (Eppendorf 3810 Reaktionsgefässe) taking care to rinse the sides of the glass vessels, and stirring carefully with a spatula to ensure complete dissolution of the residue. After evaporating the ethanol phase, the residue was dissolved in 50 μl of ethanol. A 10-μl aliquot of this solution was spotted onto the TLC plates.

Thin-layer chromatography

Plates. Precoated silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (10 × 20 cm) with a layer thickness of 0.25 mm without fluorescent indicator were used (Merck).

Chromatography was performed in a HPTLC linear chamber 28520 (Camag, Muttenz, Switzerland) using the horizontal technique at room temperature with protection from daylight.

Treatment of the HPTLC plate. Plates were prerun twice with methanol—ammonia (18:1) and stored in a closed box. Prior to use, they were activated at 100°C for 10 min.

Spotting the plates. Ethanolic solution of extracts or standards were applied with disposable micro-pipettes (10 μl) (Brand, Wertheim, G.F.R.) in lines 10 mm long. Extracts were applied twice, four on each plate. Together with six standards, 14 spots were applied per plate.

TLC developing solvent. The solvent was freshly prepared before use and consisted of isopropanol—ethyl acetate—chloroform—methanol—25% ammonia (25:10:6:2.4). The plates were removed from the chamber 10 min after the two solvent fronts had met in the middle of the plate (45 mm). Only 6 ml of solvent per run were required.

Photometry. The Chromatogramm-Spektralphotometer KM3 (Carl Zeiss, Oberkochen, G.F.R.) was used in remission mode at the UV absorption maximum of perazine (250 nm). Peak areas were evaluated by means of a Summagraphics digitizer (HW-TT) attached to a HP 9825 desk computer.

Recovery experiments. For recovery experiments, standards and extracts were applied in alternating spots (10 μl each, in ethanolic solution). Using this method, a comparison between the recovery and the internal standard method was possible on the same plates.

Preparation of glass vessels. All glass tubes were cleaned with dichromate—sulfuric acid. Silanization was omitted because it produced no better results.

RESULTS

Calibration curves with ethanolic standard solutions

Calibration curves were calculated and plotted with a linear regression program (Hewlett-Packard) on an HP 9825 calculator. Calibration curves with reference compounds were linear over a range of 50—500 ng. There was no loss

TABLE I

PLATE-TO-PLATE VARIABILITY OF LINEAR REGRESSION PARAMETERS: ETHANOLIC STANDARD SOLUTIONS ON SIX DIFFERENT HPTLC PLATES

Data are given as means ± S.D. I = Area (mm^2) as a function of amount of substance (ng). II = (P/THIO or DMP/THIO) × 100 as a function of amount of substance (ng). A = Intercept; B = slope; *r* = correlation coefficient; C.V. = coefficient of variation.

Function	A	C.V.	B	C.V.	*r*	C.V.
Perazine						
I	83.5 ± 14.2	17	1.40 ± 0.05	4	0.99 ± 0.00	0.0
II	−12.9 ± 3.0	24	0.60 ± 0.04	7	1.00 ± 0.00	0.0
Desmethylperazine						
I	77.1 ± 8.1	11	1.30 ± 0.04	3	0.98 ± 0.01	1.0
II	−9.6 ± 1.3	13	0.50 ± 0.00	0.0	1.00 ± 0.00	0.0

of linearity when the ratio DMP/THIO or P/THIO was used instead of area (Table I).

Effect of the time interval between development and scanning of the plates on UV absorption of DMP and THIO

Time intervals between development and scanning considerably influence the absorption of P, DMP, and THIO. This is illustrated by the peak-height ratios of P and DMP to THIO, determined in 10- and 20-min intervals over a total period of 220 min. Table II records some selected ratios. The decreases in peak-height ratios were due to a faster reduction of absorption of P and DMP than of THIO, which might be caused by oxidative decomposition of P and DMP. The interval between development and scanning must, therefore, be kept constant, e.g. 30 min.

TABLE II

EFFECT OF THE TIME INTERVAL BETWEEN PLATE DEVELOPMENT AND SCANNING ON THE ABSORPTION RATE AT 250 mm

Time (min)	P/THIO	DMP/THIO
30	1.5	1.4
40	1.4	1.4
50	1.4	1.2
60	1.2	1.1
80	1.2	0.9
100	1.1	0.9
160	1.1	0.9
220	1.0	0.8

Separation

No significant differences between normal and HPTLC plates regarding the R_F values of DMP, P, PSO, and THIO could be observed. (One normal plate was cut into two pieces of 20 × 10 cm and processed in the same way as a HPTLC plate.)

To improve separation, Breyer's solvent [2] had to be modified. R_F values were kept low to avoid interference from endogeneous material. With this solvent it was not possible, however, to separate PSO from DMP within the short migration distance (45 mm) in the HPTLC chamber. For complete separation of PSO runs of at least 80 mm on both types of plates are necessary. Examples of chromatogram scans are shown in Fig. 1.

Identification and specificity

R_F values for DMP, P, THIC, and other psychoactive drugs are listed in Table III. Due to the short run of only 45 mm, there is considerable interference from other drugs, particularly with THIO.

It should be noted that for complete separation on a HPTLC plate a difference in peak distance of at least 4 mm is needed, i.e. the difference in R_F × 100 values must be at least 8. Endogenous material (e.g. lipids) moved to the upper third of the migration distance without interfering with the drugs. Thus, there

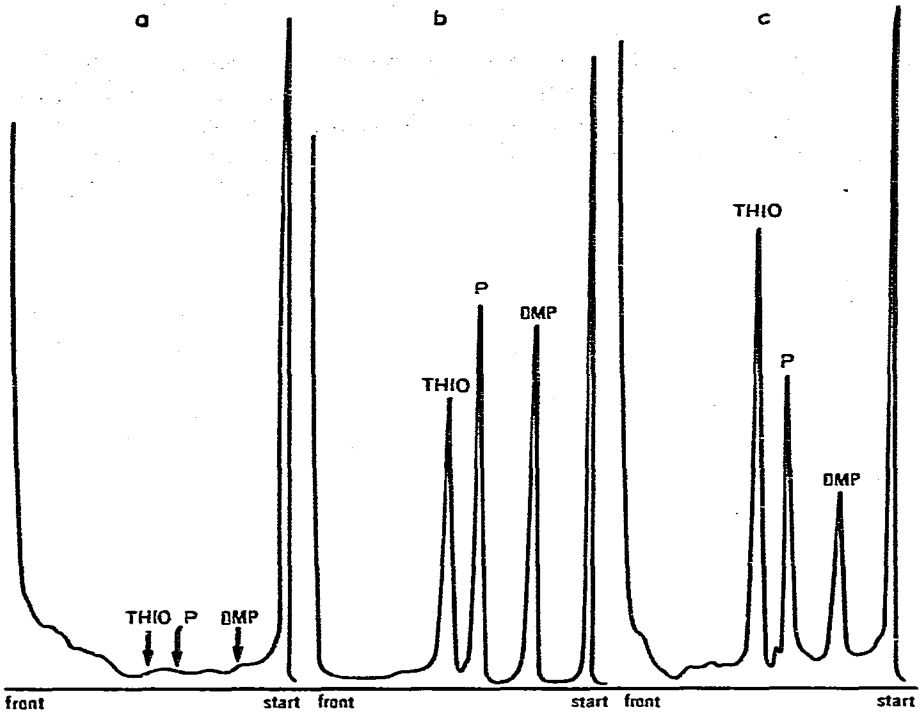

Fig. 1. Examples of chromatogram scans. (a) Blank patient serum; (b) ethanolic standard solutions: THIO, 300 ng; P, 100 ng; DMP, 100 ng; (c) serum from a patient treated with perazine (200 mg t.i.d., orally).

TABLE III

R_F VALUES OF SOME DRUGS ON HPTLC PLATES

Compound	R_F
Desmethylperazine	0.13
Desipramine	0.22
Perazine	0.30
Nortriptyline	0.36
Imipramine	0.40
Thioridazine	0.44
Trifluoperazine	0.44
Perphenazine	0.47
Amitriptyline	0.49
Prochlorperazine	0.53
Biperidene	0.85

was no need for running the plates with chloroform—isopropanol (20:2) before development, as recommended by Breyer and Villumsen [2]. Besides, considerably less interferences from endogenous material occurred due to extraction with *n*-heptane [3]. The homogeneity of the spots was proved in some samples by two-dimensional chromatography [2].

Sensitivity

Amounts of at least 20 ng/ml of P and DMP were detectable on the TLC plates with moderate gain of the amplifiers. As to sera spiked with perazine, the sensitivity was slightly lower (approximately 30 ng/ml) due to an increased variability of the recoveries with smaller amounts of the compounds (Table IV). Sensitivity can be increased up to 10 ng/ml by applying 90 or 100 µl of the extract per spot.

Recovery and reproducibility

Recovery rates are listed in Table IV. Every extract was chromatographed in duplicate, and the mean area was transformed into (ng/spot) on the basis of the corresponding standard curve obtained from the same plate.

Table V presents a comparison of the linear regressions of areas obtained with standard solutions vs. areas obtained with spiked sera, and of the corresponding peak-area ratios when the internal standard is used.

TABLE IV

RECOVERY OF P AND DMP FROM SPIKED HUMAN SERA

Recovered amounts are given as ng/ml (mean ± S.D.); *N* = number of extractions; *R* = recovery; C.V.$_1$ = Coefficient of variation and C.V.$_2$ = Coefficient of variation of the ratio DMP/THIO or P/THIO.

Concentration (ng/ml)	*N*	(ng/ml)	*R* (%)	C.V.$_1$ (%)	C.V.$_2$ (%)
Perazine					
50	8	49.0 ± 8.5	98.0	17	7
100	17	84.5 ± 8.7	84.5	8	8
200	8	167.8 ± 13.6	84.0	8	6
500	4	366.0 ± 22.3	73.0	6	2
Desmethylperazine					
50	8	42.2 ± 5.4	84.0	13	30
100	17	82.2 ± 6.8	82.2	8	7
200	8	150.0 ± 17.9	75.0	12	7
500	4	337.0 ± 62.4	67.0	19	3

TABLE V

LINEAR REGRESSION RESULTS

Obtained by correlating: I, areas calculated for spiked sera with areas for standard solutions and II, ratios of (P and DMP/THIO) × 100 for spiked sera with ratios × 100 calculated for standard solutions. A = Intercept; B = slope; *r* = correlation coefficient. Data from 4 plates.

Function	A	B	*r*
Perazine			
I	−3.9	0.86	0.99
II	22.8	0.84	0.97
Desmethylperazine			
I	39.7	0.72	0.99
II	27.6	0.75	0.99

DISCUSSION

The reason for modifying Breyer's method [2] was to render it more suitable to the demands of the clinical laboratory, i.e. to achieve a facilitated and faster performance without any loss of specificity and sensitivity.

The introduction of an internal standard offers the following advantages: (i) the performance can be greatly augmented since there is no need for running standards together with the extracts; (ii) it appears to be much more suitable to handle relative rather than absolute data, e.g. in our laboratory we worked with two different recorders and it was not possible to compare areas recorded on the one with areas recorded on the other — using ratios instead of absolute areas solved the problem; (iii) a simple and fast daily recovery control is achieved by running two standards per plate [7,8]; if the then computable recovery for THIO is within an acceptable range, one can be quite sure that the recovery for P and DMP is under control too.

The use of an internal standard presents many problems known from GLC [7—10]: (i) the chemical structure of the internal standard must not be too different from the compound to be determined so that analogous protein-binding and extraction properties can be assumed; (ii) it must be different enough from the compound to be determined so that it can be separated easily from it; (iii) it should have an absorbance maximum close to the one of the drug to be determined (THIO, 265 nm; P and DMP, 250 nm).

Considering these aspects, THIO appears to be a satisfactory compound. The correlation of quotients with the amount of P and DMP per spot is as good as when absolute areas are computed (Table I).

As to serum extracts, the variability of the peak area ratios is equal or even less than that of absolute areas (Table IV).

In Table V is shown to what extent the absolute areas and the ratios of standards are modified by the extraction procedure: the correlation coefficient for both substances and functions was excellent; the slope coincides well with the mean recovery for both substances (Table IV) (P: ϕR = 84.9, slopes = 0.86 and 0.84; and DMP: ϕR = 77.2, slopes = 0.72 and 0.75); and the intercept can most likely be interpreted as interference from endogeneous material. This interference seems to influence mainly DMP and frequently led to a broadening of the extraction peaks, which might be due to the low R_F value of DMP.

The usefulness of thioridazine as internal standard and the preciseness of the method was also proven by the analysis of 30 plasma samples from perazine-treated patients. These 30 plasma samples were each analysed twice, by a GLC method [4] and by the TLC method described above, resulting in an excellent correlation between both methods [12].

As thioridazine is a very common drug in psychiatry, it is easily available without the need of any synthetic procedures. On the other hand, as it can be separated from perazine, and can be extracted by the same procedure, it can be assayed with perazine as internal standard using the same method.

The use of the Camag HPTLC chamber reduced the time required for the performance of Breyer and Villumsen's [2] original method by 1 h because only 30 min were required to obtain values for 14 extracts. Another 30 min were saved by the use of *n*-heptane as the solvent for extraction, because predevelopment of the plates proved to be unnecessary. Besides, *n*-heptane is less toxic than benzene. A drawback to using *n*-heptane is the decreased recovery of DMP. The low solubility of DMP in *n*-heptane may lead to variable and erroneous results when small volumes of *n*-heptane are used for dissolution of the final residue; ethanol was used instead, therefore.

In contrast, perazine sulphoxide can only be extracted from serum with *n*-heptane if concentrations greater than 1000 ng/ml are present. Thus, PSO in patients' sera will not interfere with the determination of DMP (from which it cannot be separated satisfactorily).

Due to the short migration distance of only 45 mm interferences from other drugs cannot be excluded. If such interferences are to be expected from the therapeutic strategy in an individual patient, then the use of alternate methods such as GLC might become necessary to overcome this difficulty.

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