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HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF PSYCHOPHARMACOLOGIC AGENTS IN BLOOD SERUM

D. C. FENIMORE, C. J. MEYER and C. M. DAVIS
Texas Research Institute of Mental Sciences, Texas Medical Center, Houston, Texas 77030 (U.S.A.)
and
F. HSU and A. ZLATKIS
Chemistry Department, University of Houston, Houston, Texas 77004 (U.S.A.)

SUMMARY

High-performance thin-layer chromatography affords a rapid, sensitive method for determination of psychopharmacologic agents in blood serum samples. Quantitation of the representative drugs chlorpromazine, amitriptyline, nortriptyline, imipramine, and desipramine at levels as low as 5 ng/ml is demonstrated by scanning the developed thin-layer plates with a chromatographic spectrophotometer in the ultraviolet absorption mode. Neither derivatization prior to, nor color development after chromatographic separation is required to achieve sensitivity and reproducibility of determinations.

INTRODUCTION

The quantitation of psychopharmacologic agents in blood samples is complicated by the low concentrations encountered and by interference from metabolic products which may resemble closely the parent drug. Thus, analytical procedures furnishing both sensitivity and selectivity are usually required for these determinations, and most methods applied successfully to this problem involve chromatographic separations. Chlorpromazine, for example, has been determined by electron capture gas chromatography[1–3]. Other phenothiazine and tricyclic antidepressants have been assayed using the nitrogen-sensitive flame thermionic detector[4,5]. In instances where concentrations are sufficiently high, flame ionization detection has been applied[6]. Liquid chromatographic methods have been less prominent in blood level determinations because of sensitivity limitations, but recent investigations using electrochemical detection have yielded good sensitivity for phenothiazine type drugs in human blood serum[7].

Application of either gas or liquid chromatographic procedures to routine clinical determinations is often impractical because of the excessive instrument time necessitated by sequential sampling. Thin-layer chromatography (TLC), on the other hand, permits separation of many samples simultaneously, but this technique is

hindered by relatively low sensitivity and resolution. The introduction of high-performance thin-layer chromatography (HPTLC) appeared to offer a means of overcoming these detractions[8,9]. Consequently the present study was undertaken to examine the feasibility of utilizing HPTLC in the quantitation of representative psychotropic drugs in blood serum samples at concentrations encountered in normal clinical practice.

EXPERIMENTAL

Chemicals and reagents

Psychopharmacological agents used in this study were supplied as follows: chlorpromazine, Smith Kline & French Labs. (Philadelphia, Pa., U.S.A.); loxapine Lederle Labs. (Pearl River, N.Y., U.S.A.); perphenazine, Schering Corp. (Kenilworth, N.J., U.S.A.); butaperazine, A. H. Robins Co. (Richmond, Va., U.S.A.); amitriptyline, Merck, Sharp & Dohme (West Point, Pa., U.S.A.); nortriptyline, Eli Lilly & Co. (Indianapolis, Ind., U.S.A.); imipramine, Geigy Pharmaceuticals (Summit, N.J., U.S.A.); desipramine, USV Pharmaceutical Corp. (Tuckahoe, N.Y., U.S.A.).

Solvents were purified by fractional distillation in all-glass systems and stored in bottles equipped with either PTFE-sleeved glass stoppers or with PTFE-lined screw caps.

All glassware was silylated using hexamethyldisilazane at elevated temperatures and reduced pressure[10].

Stock solutions

For chlorpromazine assays, two stock solutions were prepared using 1.5% isoamyl alcohol in heptane. The first contained chlorpromazine base at a concentration of 1 mg/ml and was used in the preparation of all calibration curves. The second contained a mixture of the internal standard, butaperazine base, at a concentration of 100 μg/ml and the "carrier", perphenazine base, at 2 mg/ml. Small amounts of the latter solution were diluted 1:100 in isoamyl alcohol–heptane (1.5:98.5) for addition to serum samples.

For the assay of the tricyclic antidepressants, 1 mg/ml solutions of each base (imipramine, desipramine, amitriptyline and nortriptyline) were made using the 1.5% isoamyl alcohol–heptane solvent. A mixture of the internal standard, loxapine base, at a concentration of 100 μg/ml and the perphenazine carrier at 2 mg/ml was made in the 1.5% isoamyl alcohol–heptane solvent to be later diluted 1:100 and added to serum samples.

Sample preparation

A 100-μl portion of the diluted stock solution containing the appropriate internal standard and carrier mixture was added to a 15-ml silylated screw-top test tube and dried under reduced pressure. To this was added 1 ml of the serum to be tested together with 1 ml of 1 *N* NaOH. This basic serum was then extracted with a single 10-ml portion of the 1.5% isoamyl alcohol–heptane solvent. A tube rocker having gentle action (Labindustries, Berkeley, Calif., U.S.A.) was used in this initial extraction from serum for a period of 30 min, followed by separation in a bench-top

centrifuge for 5 min. The aqueous serum layer was frozen using an acetone–dry ice bath, and the organic layer was transferred to another 15-ml tube. This supernatant was then made acidic by the addition of 1 ml of 0.05 *N* HCl, and vortexed. After separating by centrifugation, the organic layer was removed by aspiration, and the remaining aqueous phase alkalinized to pH 10 by the addition of 0.2 ml of 1 *N* NH_4OH. This was extracted with 2 ml of the 1.5% isoamyl alcohol–heptane solvent by mixing and centrifuging. The aqueous layer was then frozen in a dry ice–acetone bath to enable easy, complete transfer of the organic solvent to a silylated Reactivial (Pierce, Rockford, Ill., U.S.A.). This final extract was taken to dryness under reduced pressure and later reconstituted in 15 μl of the 1.5% isoamyl alcohol–heptane solvent for HPTLC.

High-performance thin-layer chromatography

All HPTLC separations were carried out using 10 × 10 cm pre-coated HPTLC silica gel 60 plates (E. Merck, Darmstadt, G.F.R.). In order to assure low background, ultraviolet absorbing materials were moved to the solvent front by performing two pre-development washes with hexane–acetone–diethylamine (80:20:3). The plates were then dried in a vacuum oven for 1 h and stored in a glass desiccator.

Extracts obtained from the sample preparation were applied to the HPTLC plates by means of platinum–iridium capillary pipettes obtained from R. E. Kaiser (Antech, Bad Dürkheim, G.F.R.). The pipette was placed in a counter-balanced applicator (EVA-Chrom; W & W, Basel, Switzerland) and the total volume of serum extract was spotted in small portions on the plate. A glass tube connected to vacuum was held besides the spot during the application procedure to remove the solvent rapidly thus preventing excessive enlargement of the spot. The spot diameter was usually held to less than 2 mm diameter, and each spot was separated by 7 mm. After sample application the plate was held in a vacuum desiccator for 10 min to insure removal of residual isoamyl alcohol. After this drying step the plates were allowed to stand in room atmosphere for at least 5 min before placing them in the developing tank. This step appeared to be necessary to obtain a perfectly straight solvent front, and may be related to equilibration of the moisture content of the plate. Plates were developed in twin-trough chambers (Camag, Muttenz, Switzerland) with one side lined with Whatman No. 3 chromatography paper to provide a well-saturated atmosphere in the tank. 20–25 ml of solvent were required for use in this particular chamber.

After equilibration in the laboratory atmosphere, the plate was placed in the chamber for 5 min of solvent pre-loading before tilting to allow the development solvent to flow over the dividing ridge to reach the plate.

Development for chlorpromazine samples required about 5 min for 6 cm solvent front travel. Double development was employed with the tricyclics in which the solvent front traveled 6.5 cm. The plate was dried for about 2 min in a stream of cool air, and the plate was re-developed after another 5 min solvent vapor pre-loading. After chromatographic development, the plates with the resolved drugs were dried briefly under vacuum and were then ready for scanning without further processing.

Quantitation of HPTLC spots

In situ quantitation of the developed HPTLC plates was accomplished utilizing

a Zeiss Model KM-3 chromatogram spectrophotometer (Zeiss, New York, N.Y., U.S.A.). Measurements were made in the reflectance mode for UV absorption. A deuterium light source was used with the monochromator set for 254 nm for chlorpromazine, 240 nm for amitriptyline and nortriptyline, and 275 nm for imipramine and desipramine. The slit width was 1.0 mm and slit length was 3.5 mm. Scanning speed was 30 mm/min.

Calibration curves

The concentrations of psychotropic drugs in serum were predicted using known peak area ratios (drug/internal standard) displayed as calibration curves. These calibration curves were scrutinized for linearity in the form of a linear regression analysis, which was used as the basis of general prediction. To avoid extraction variability, calibration data was generated by adding known amounts of the drug to be determined to fresh, pooled, drug-free serum and extracting as described above. Thus, values are based on the entire analytical procedure.

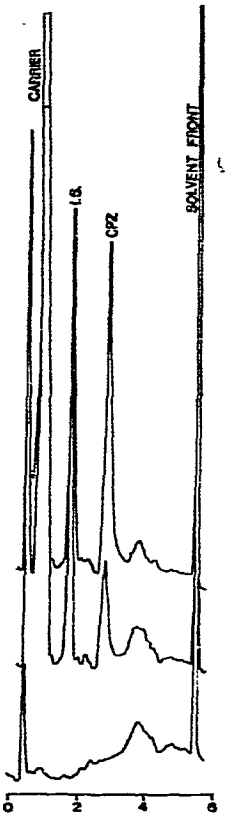

Fig. 1. Scan of HPTLC separation of blood serum extracts containing 100 ng/ml (upper trace) and 25 ng/ml (middle trace) chlorpromazine (CPZ) standard with butaperazine as internal standard I.S.). Abscissa indicates distance (cm) from origin of components on HPTLC plate.

RESULTS AND DISCUSSION

The chromatographic spectrophotometer used for quantitation of the spots separated on the HPTLC plates is capable of several modes of operation including UV and visible absorption by reflectance or transmission, fluorescence, and fluorescence quenching (if the plates contain a fluorescent indicator). Most of these methods are applicable to the quantitation of psychopharmacologic agents either directly, as with UV absorption, or after development of color or fluorescence with suitable reagents. Investigation of the various options indicated that UV absorption by reflectance yielded the most reliable determinations for the compounds studied when recovered from a biological sample such as blood serum.

Fig. 1 shows the UV absorption trace of chromatograms of varying concentrations of chlorpromazine recovered from 1-ml serum samples. The plate was developed to a little less than 6 cm and scanned in the direction of solvent flow. As can be seen from the blank serum sample, little or no interference is present at the position of the chlorpromazine or the butaperazine internal standard.

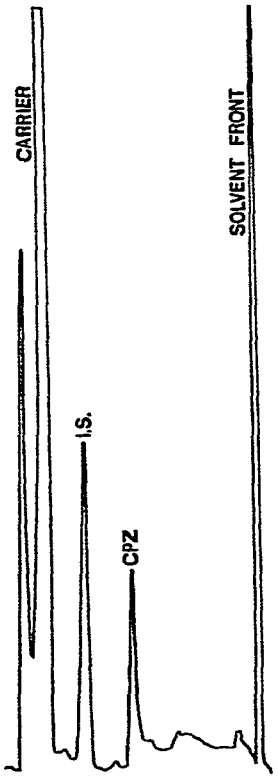

Fig. 2. High-performance thin-layer chromatogram of patient's blood serum containing 45 ng/ml chlorpromazine.

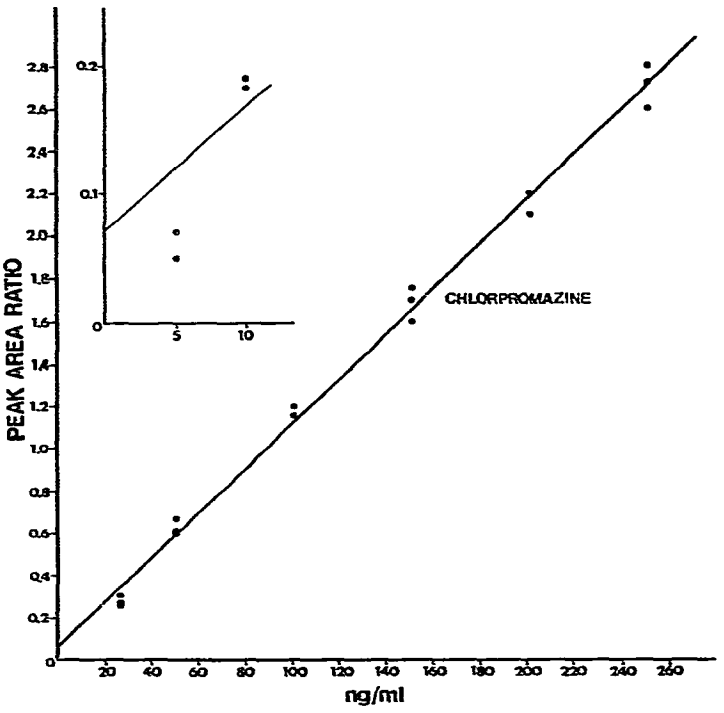

Fig. 3. Calibration graph of chlorpromazine standards extracted from blood serum. Response is peak area ratio of chlorpromazine to butaperazine internal standard.

Interference from the metabolites of chlorpromazine is a possibility to be considered in samples obtained from patients receiving the drug[11]. Fig. 2, a chromatogram of a blood sample obtained from a patient receiving chlorpromazine (CPZ), shows that the CPZ is well isolated, and the chromatogram is very similar to the previous calibration samples. Estimates of the amount of chlorpromazine present in blood serum during therapy range from about 10 ng/ml to about 300 ng/ml[1,2,12,13]. A calibration curve for CPZ in shown in Fig. 3 based on replicate determinations of known amounts added to drug-free serum. Some departure from linearity is apparent at 5 ng/ml, but the overall linear correlation coefficient is 0.998. Calibration curves were very reproducible from plate to plate which reflects favorably on both the quality of the plates and the analytical procedure.

UV absorption scans of a chromatogram of amitriptyline, imipramine, nortriptyline, and desipramine shown in Fig. 4 is illustrative of the effect of wavelength on the response to the individual drugs. Separation of these drugs by HPTLC is excellent; but even if this were not the case, some selectivity of detection can be achieved by choosing the appropriate wavelength for UV absorption.

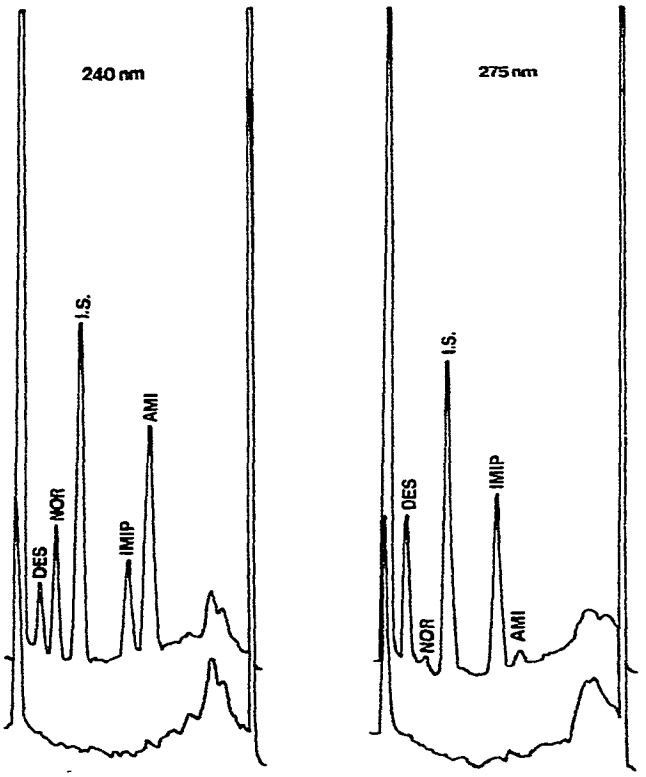

Fig. 4. Scans of tricyclic antidepressants at wavelengths selected for maximum absorption. Amitriptyline (AMI) and nortriptyline (NOR), 240 nm; imipramine (IMIP) and desipramine (DES), 275 nm. Internal standard (I.S.) is loxapine.

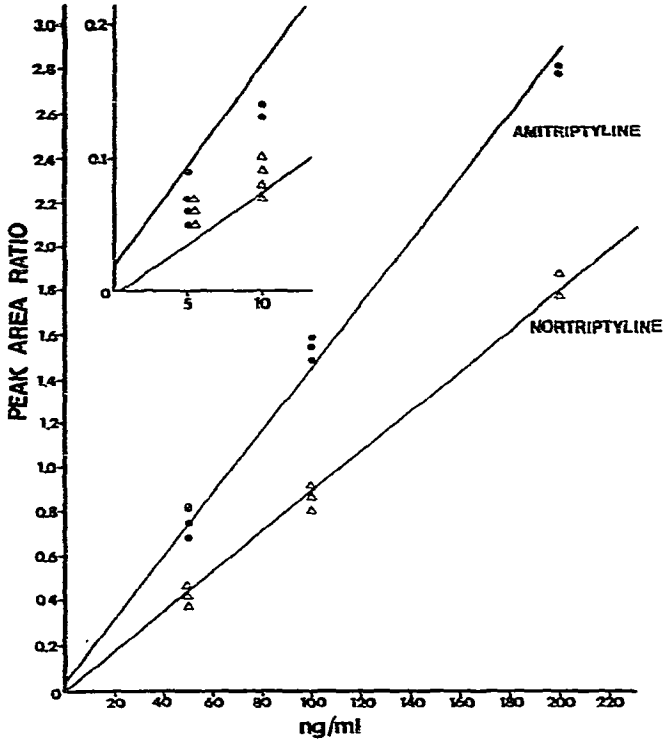

Fig. 5. Calibration graphs of amitriptyline and nortriptyline standards extracted from blood serum. Response is peak area ratio of drugs to loxapine internal standard.

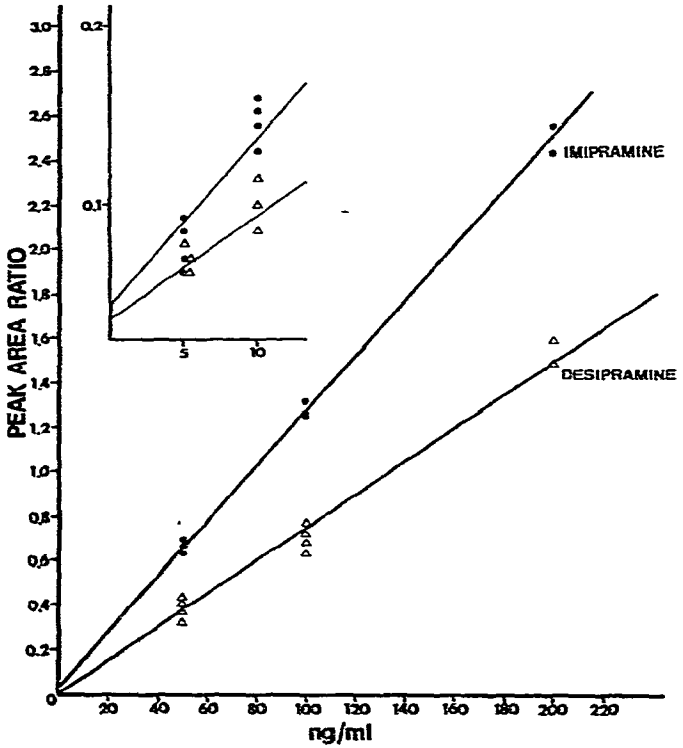

Fig. 6. Calibration graphs of imipramine and desipramine standards extracted from blood serum. Response is peak area ratio of drugs to loxapine internal standard.

TABLE I

EFFECT OF CARRIER (PERPHENAZINE) ON RECOVERY OF PSYCHOTROPIC DRUGS FROM SERUM

Mean: mean of six determinations expressed as percent of recovery. S.D.: standard deviation. C.V.: coefficient of variation expressed as percent of the mean.

Conc. of sample (ng/ml)	*Parameter*	*Chlorpromazine*		*Nortriptyline*		*Amitriptyline*		*Desipramine*		*Imipramine*	
		With	*Without*	*With*	*Without*	*With*	*Without*	*With*	*Without*	*With*	*Without*
10	Mean	80.3	54.5	83.5	61.9	95.3	71.5	81.9	71.6	86.2	77.4
	S.D.	2.4	21.0	3.1	18.1	16.4	10.2	13.7	23.8	15.2	10.4
	C.V.	3.0	38.6	3.7	29.2	17.3	14.3	16.9	33.2	17.7	13.4
100	Mean	95.7	87.0	77.5	72.8	99.6	86.7	69.7	61.9	91.1	85.7
	S.D.	3.2	3.4	10.2	23.1	2.3	6.4	7.2	7.1	5.2	7.3
	C.V.	3.4	3.9	13.2	31.8	2.3	7.3	10.3	11.4	5.8	8.5

TABLE II

EFFECT OF CARRIER (PERPHENAZINE) ON REPRODUCIBILITY OF DETERMINATION OF PSYCHOTROPIC DRUGS IN SERUM

Mean: mean of six determinations expressed as peak area ratio of drug/internal standard. S.D.: standard deviation. C.V.: coefficient of variation expressed as percent of the mean.

Conc. of sample (ng/ml)	*Parameter*	*Chlorpromazine*		*Nortriptyline*		*Amitriptyline*		*Desipramine*		*Imipramine*	
		With	*Without*	*With*	*Without*	*With*	*Without*	*With*	*Without*	*With*	*Without*
10	Mean	0.088	0.059	0.083	0.064	0.160	0.121	0.079	0.062	0.180	0.158
	S.D.	0.007	0.020	0.007	0.012	0.013	0.014	0.009	0.011	0.008	0.018
	C.V.	8.0	34.2	8.5	18.5	7.8	11.3	10.7	17.7	4.2	11.3
100	Mean	1.25	1.14	0.647	0.555	1.444	1.257	0.584	0.486	1.229	1.151
	S.D.	0.04	0.03	0.020	0.069	0.037	0.100	0.035	0.066	0.049	0.090
	C.V.	3.3	2.6	3.1	12.4	2.6	7.9	6.0	13.5	4.0	7.9

Calibration curves of the antidepressants are shown in Figs. 5 and 6. Again replicate determinations of the ratio of peak areas of the drugs to the peak area of the internal standard exhibit some scatter especially at low levels, but the linearity of response in each case is quite good with correlation coefficients as follows: amitriptyline, 0.997; nortriptyline, 0.998; imipramine, 0.999; and desipramine, 0.994.

Factors affecting reproducibility and recovery

In a previous study of a GC procedure for determination of CPZ in blood serum[3] it was observed that adding relatively large amounts of a compound similar to CPZ to the serum sample improved both the reproducibility of the assay and the recovery of CPZ and the internal standard through the extraction and chromatographic processes. Addition of a "carrier" compound was also found to be advantageous in the HPTLC procedure. Perphenazine was selected for this purpose, because it had low R_F values in the described solvent systems and therefore offered no interferences to the compounds being measured. Table I shows the difference in total recovery of the various drugs from serum samples in the presence and absence of carrier. The effect was particularly profound at low sample concentrations, which is to be expected, but even at 100 ng/ml concentrations there was a marked difference in recovery.

The effect of carrier on reproducibility is shown in Table II. Replicate determinations of known amounts of drugs added to serum samples were calculated as ratio of the peak area to the area of a fixed amount of internal standard added to the extract just prior to application of the sample to the HPTLC plate. Again the variance of the values is significantly less in the samples to which carrier was added.

In order to test the accuracy of the analytical procedure, samples of serum with known amounts of the various drugs were prepared by one of the investigators and given to another for determination. The results of this single blind study are shown in Table III. Agreement of these values were within the range usually experienced in blood serum assays, and should be of acceptable accuracy and precision for clinical use.

TABLE III

SINGLE BLIND DETERMINATIONS OF PSYCHOTROPIC DRUGS FROM SPIKED SERUM SAMPLES

Ami = amitryptiline; nor = nortryptiline; imi = imipramine; des = desipramine.

Chlorpromazine			*Tricyclic antidepressants*					
Sample	*Known conc. (ng/ml)*	*Observed conc. (ng/ml)*	*Sample*	*Known conc. (ng/ml)*	*Observed conc. (ng/ml)*			
					Ami	*Nor*	*Imi*	*Des*
1	50.0*	51.0	1	100.0	95.3	98.9	93.0	94.7
		53.0			91.4	97.3	97.1	94.6
2	100.0	105.0	2	10.0	11.4	7.5	7.8	13.7
		108.3			9.7	8.2	7.5	13.3
3	10.0	8.2	3	50.0	47.6	43.6	45.7	47.5
		7.5			48.0	48.0	41.7	48.5

* ng/μl.

Ideally the carrier and the internal standard should be compounds which would not be encountered in the blood samples because of prior administration to the patient. While polypharmacy in the treatment of mental illness has been encountered frequently in the past, this practice has now declined to the point that interference from this source should no longer be significant. Table IV lists the R_F values for a number of psychotropic drugs, drug metabolites, and other compounds which might be present in patient blood samples. As can be seen from these values, the possibility of interference is actually quite low because of the excellent selectivity of the HPTLC systems.

While this study has dealt in detail with only five of the numerous drugs currently used in the treatment of mental disorders, there is reason to believe that HPTLC analysis could be applied to most of these therapeutic agents as recovered from biological samples. This method may not necessarily supplant other procedures for determination of drug levels in pharmacokinetic and bioavailability research; but the speed of analysis, superior separating power, elimination of derivatization, and excellent sensitivity all contribute to the practicality of this procedure when applied to routine clinical determinations.

TABLE IV

R_F VALUES OF 26 COMMON BASIC DRUGS SCREENED FOR INTERFERENCE

Solvent system 1, hexane–benzene–diethylamine (96:12:3); solvent system 2, hexane–acetone–diethylamine (80:20:3).

Drug	R_F	
	Solvent system 1	*Solvent system 2*
Acetophenazine	0.00	0.03
Amitriptyline	0.60	0.47
Butaperazine	0.22	0.22
Caffeine	0.04	0.09
Chlordiazepoxide	0.00	0.03
Chlorpromazine	0.54	0.45
Chlorpromazine 7-hydroxy	0.00	0.04
Chlorpromazine sulfoxide	0.02	0.10
Chlorprothixine	0.53	0.45
Clozapine	0.00	0.11
Desipramine	0.09	0.23
Diazepam	0.15	0.27
Fluphenazine	0.00	0.07
Haloperidol	0.03	0.17
Imipramine	0.45	0.48
Loxapine	0.24	0.35
Nicotine	0.35	0.40
Nortriptyline	0.18	0.18
Penfluridol	0.05	0.24
Perphenazine	0.00	0.09
Phenothiazine	0.07	0.29
Prochlorperazine	0.19	0.26
Promazine	0.26	0.40
Thioridazine	0.24	0.40
Thiothixine	0.03	0.08
Trifluroperazine	0.20	0.29

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