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SYSTEMATIC IDENTIFICATION OF PSYCHOTROPIC DRUGS BY THIN LAYER CHROMATOGRAPHY. PART II

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SUMMARY

A thin-layer chromatographic technique was applied to ether extracts of blood and urine samples from patients receiving clinical doses of chlorpromazine, thioridazine, chlorprothixene, meprobamate, amitriptyline, nortriptyline, imipramine and desipramine.

Chromatography of the extracts of blood samples from patients receiving clinical doses of chlorpromazine or thioridazine were negative. Similar specimens extracted after hemolysis of the sample showed the presence of unmetabolized drug and metabolites.

In urine extracts, phenothiazine derivatives administered in daily doses lower than 75 mg were not detectable as unchanged drug; only the presence of metabolites was observed.

Urine extracts from patients on a stabilized drug dose of each of the compounds examined showed typical chromatographic patterns. The R_F values of metabolites constantly found in the samples examined are reported.

In order to demonstrate the applicability of the thin-layer chromatographic technique to the isolation and identification of psychotropic drugs and their metabolic products in biological materials, the method previously described[1] was applied to blood and urine extracts obtained from patients receiving clinical doses of some of these drugs.

The object of this study was: to determine whether the unmetabolized drug could be detected in blood and urine samples after administration of clinical doses; to establish the minimum dose that yields a detectable amount of unchanged drug; to ascertain if the metabolic products of a given drug form typical chromatographic patterns which may be used for the identification of the parent drug; and to achieve the isolation of drugs, composing a heterogeneous group of psychotropic agents, in two chromatographic systems on the basis of their different comportment in respect to the Folin–Ciocalteau reagent.

The following drugs, representing three pharmacological classes of psychotropic agents, phenothiazines, nonphenothiazine tranquilizers and stimulants of the central nervous system were investigated: chlorpromazine, thioridazine, chlorprothixene, meprobamate, amitriptyline, nortriptyline, imipramine and desipramine.

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EXPERIMENTAL

Materials

The subjects were, male and female, schizophrenic patients from Cleveland State Hospital. Blood and urine specimens were collected between one and three hours after ingestion of the drug and kept refrigerated until extracted. This was within three hours. Care was taken to ensure that the specimens were not exposed to sunlight. Urine samples provided by patients who were not receiving any psychotropic drug were used as controls. Control blood samples were made up as a pool, from specimens supplied by the Pathology Department of S. Vincent Charity Hospital.

Extraction method

25 ml of urine adjusted to pH 9–10 with 6 *N* sodium hydroxide or 5–7 ml of whole blood to which was added 1 ml of 6 *N* sodium hydroxide were extracted with two 50 ml portions of ethyl ether. The ether extracts were combined and filtered. One portion of ether (40–45 ml) was dried with 3 g of anhydrous sodium sulfate, filtered and the ether evaporated under forced air at room temperature (Extract A).

The second portion of ether was extracted with two 7 ml portions of 6 *N* hydrochloric acid. The acid solutions were combined, made alkaline (pH 9–10) with 6 *N* sodium hydroxide and re-extracted with two 50 ml portions of ether. The ether extracts were combined, dried with sodium sulfate and the ether evaporated under forced air at room temperature (Extract B). The residues from the two extractions were dissolved in a few drops of methanol. Aliquots of the two alcohol solutions were spotted on the chromatoplate.

Chromatographic method

The chromatoplates, chromatographic systems and detecting reagents were as previously described[1]. The chromatographic systems were:

System I: methanol–12 *N* ammonium hydroxide (100:1.5)
System II: cyclohexane–diethylamine–benzene (75:20:15)
System III: acetone
System IV: chloroform–methanol (90:10)
System V: benzene–ethanol–12 *N* ammonium hydroxide (95:15:5).

Plates developed in systems I and V were coated with a mixture of Silica Gel G and water. Systems II, III and IV were used with Silica Gel G plates prepared with 0.1 *N* sodium hydroxide.

Procedure

A spotted plate containing the two extracts (A and B) from the unknown sample was chromatographed in system I and sprayed with the Folin–Ciocalteau reagent. The observation of the resulting color(s) limited the investigation to a group of drugs yielding the same color reaction, while the R_F value obtained for the unknown further reduced its identification within the limits of a few drugs.

A second plate was then spotted with the remaining alcoholic solutions (A and B) and with the standard solutions of those drugs that showed similar chromatographic and chromogenic comportment. The plate was developed in a second chro-

matographic system selected according to the properties shown by the unknown in system I* and then sprayed with a suitable detecting reagent.

RESULTS AND DISCUSSION

Chromatography of phenothiazines

Urine. Unchanged phenothiazines were found in both extracts A and B, or only in extract B, depending on the amount of drug ingested. Urine extracts from patients receiving small amounts of phenothiazine derivatives (from 5 to 75 mg *pro die*) did not yield any unmetabolized drug. The various metabolites present did not form any typical chromatographic pattern.

In patients on a stabilized drug dose (receiving the same medication for at least 6 weeks) the drug metabolism appeared to be qualitatively constant as shown by similar chromatographic patterns obtained from urine extractions repeated at intervals of a week on the same subjects. Variations in the excretion of the unmetabolized drug between subjects who were given the same doses of medication were noted only in a few cases.

The data collected relative to thioridazine and chlorpromazine are shown in Tables I and II, respectively. To overcome R_F value variations due to uncontrollable experimental conditions, the R_F values of the various metabolites are also reported as relative to the distance moved by the parent drug.

TABLE I

R_F VALUES OF THIORIDAZINE AND ITS METABOLITES

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Thioridazine	0.54	1.00	0.62	1.00	0.23	1.00	0.53	1.00	0.73	1.00
Spot 1*	0.37	0.68	0.53	0.85	0.04	0.17	0.39	0.73	0.65	0.89
Spot 2	0.23	0.43	0.17	0.27			0.32	0.60	0.56	0.77
Spot 3			0.06	0.10			0.18	0.34	0.39	0.53
Spot 4									0.27	0.37
Spot 5									0.14	0.19

* The spots are listed in decreasing values in respect to the position of thioridazine.

TABLE II

R_F VALUES OF CHLORPROMAZINE AND ITS METABOLITES

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Chlorpromazine	0.56	1.00	0.65	1.00	0.30	1.00	0.50	1.00	0.73	1.00
Spot 1*	0.42	0.75	0.34	0.52	0.20	0.66	0.27	0.54	0.53	0.73
Spot 2	0.38	0.68	0.22	0.34	0.06	0.20	0.18	0.36	0.30	0.41
Spot 3	0.27	0.48	0.15	0.23			0.08	0.16	0.17	0.23
Spot 4	0.17	0.30	0.05	0.08					0.07	0.10

* The spots are listed in decreasing values in respect to the position of chlorpromazine.

* See Part I, Tables V, VI and VII.

The results reported are relative to metabolites constantly found in urine extracts from patients on a stabilized drug dose varying from 75 mg to 600 mg *pro die* of thioridazine and from 100 mg to 1200 mg *pro die* of chlorpromazine.

The lower limit of detection of unmetabolized drug corresponded to a drug intake of 75 mg *pro die* of thioridazine and 75 mg *pro die* of chlorpromazine.

Fig. 1 reproduces a chromatoplate developed in system V, containing urine extract (B) from a patient receiving 100 mg of thioridazine *pro die* and from a patient receiving 150 mg of chlorpromazine *pro die*.

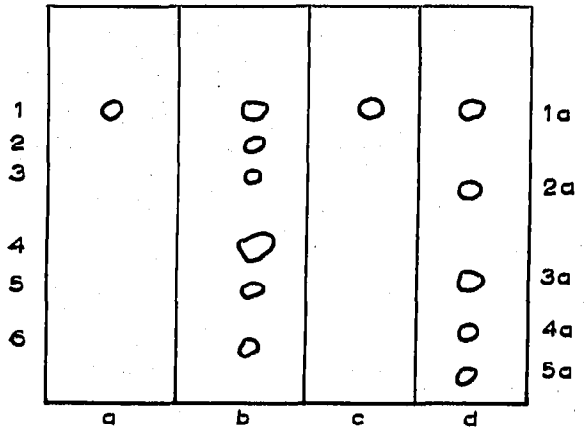

Fig. 1. Thin-layer chromatography of phenothiazines from urine extracts. (a) Thioridazine standard solution; (b) urine extract B from a patient receiving 100 mg *pro die* of thioridazine; (c) chlorpromazine standard solution; (d) urine extract B from a patient receiving 150 mg *pro die* of chlorpromazine. Chromatographic system: benzene–ethanol–12 *N* ammonium hydroxide (95:15:5). Detecting reagent: Folin–Ciocalteau reagent. Colors of the spots: spots 1 and 2 blue-green, 3 and 6 pink, 4 and 5 fuchsia, 1a and 2a fuchsia, 3a and 4a violet, 5a pink.

Blood. With a drug intake of 600 mg of thioridazine or 800 mg of chlorpromazine *pro die*, both the extracts A and B from 5–7 ml of whole blood were negative. Urine specimens collected simultaneously from the same patients and examined using the same procedure confirmed medication intake as shown by the chromatographic patterns obtained. Plates spotted with blood extract B from a patient receiving only chlorpromazine (1200 mg *pro die*) and developed in system IV showed, sporadically, when sprayed with the Folin–Ciocalteau reagent, a reddish-pink spot with an R_F value corresponding to 0.39–0.41. Negative results were also obtained when a pool of 15 ml of whole blood from patients who were given 600 mg of thioridazine *pro die* was extracted according to the procedure described and also on using different extraction procedures and chromatographic methods reported in literature[2,3].

No attempt was made to verify the eventual presence of unmetabolized drug by spectrophotometric studies after elution of the substance from the silica gel, as the object of this investigation was to prove the validity of the thin-layer chromatographic technique and its limits.

Surprisingly positive were, on the other hand, the results obtained when 5 ml of whole blood from patients receiving daily doses of 200–600 mg of thioridazine or 200–800 mg of chlorpromazine were hemolyzed by shaking the sample with 20 ml of distilled water, extracted and the extracts chromatographed according to the procedure described. Unchanged drug was found in 8 of the 10 samples examined in extract B, while the presence of metabolites was constantly observed on all the plates.

The few experiments conducted on these lines did not permit any interpretation of the nature of the reactions involved. However, the possibility of detecting phenothiazine blood levels after hemolysis of the sample appears fairly promising. No reference was found in the literature on this particular subject which is still under investigation in this laboratory.

Chromatography of nonphenothiazine tranquilizers

Chlorprothixene. This drug reacts with the Folin–Ciocalteau reagent at room temperature yielding a bluish color. In the group of psychotropic agents previously investigated, other compounds (clopenthixol, etryptamine, iproniazid, isocarboxazid, nialamide, phenelzine and rescinnamine) have similar chromogenic behavior. Their R_F values in system I are clearly distinct from the R_F value of chlorprothixene, with the exception of clopenthixol and nialamide. The R_F values of these three drugs in system I are: chlorprothixene 0.61; clopenthixol 0.62; nialamide 0.58. To differentiate chlorprothixene from clopenthixol and nialamide system II or system V were used as a second chromatographic system. A good resolution of the three compounds was achieved (Table III).

TABLE III

R_F VALUES OF CHLORPROTHIXENE, CLOPENTHIXOL AND NIALAMIDE IN SYSTEMS II AND V

	Chlorprothixene	*Clopenthixol*	*Nialamide*
System II	0.66	0.15	0
System V	0.75	0.36	0.07

The plate developed in the second system was sprayed with Mandelin reagent. Chlorprothixene and its metabolites show characteristic brilliant orange or green fluorescences. Table IV shows the R_F values of chlorprothixene and its metabolites in the five chromatographic systems used. Fig. 2 illustrates the chromatographic

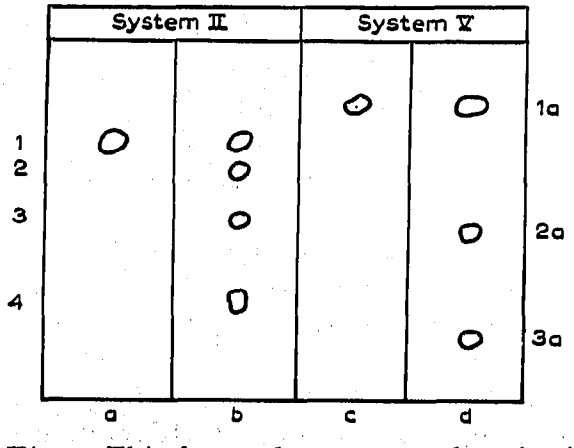

Fig. 2. Thin-layer chromatography of urine extract containing chlorprothixene. (a) and (c) chlorprothixene standard solution. (b) and (d) urine extracts A. System II: cyclohexane–diethylamine–benzene (75:20:15). System V: benzene–ethanol–12 *N* ammonium hydroxide (95:15:5). Detecting reagent: Mandelin reagent. Colors of the spots: spots 1 and 1a orange with brilliant green (orange) fluorescence; spots 2, 3, 4 and 3a orange with brilliant orange fluorescence; spot 2a orange with brilliant green fluorescence. In parentheses the outside fluorescence of the spot.

TABLE IV

R_F VALUES OF CHLORPROTHIXENE AND ITS METABOLITES

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Chlorprothixene	0.61	1.00	0.66	1.00	0.37	1.00	0.60	1.00	0.75	1.00
Spot 1	0.31	0.51	0.59	0.89	0.21	0.57	0.47	0.78	0.44	0.59
Spot 2			0.47	0.71	0.06	0.16	0.31	0.52	0.17	0.23
Spot 3			0.26	0.39			0.20	0.33		

pattern obtained from a urine extract (A) from a patient receiving 150 mg *pro die* of the drug. The plates were developed in systems II and V. Colors and fluorescences reported are those formed by means of the Mandelin reagent. Urine and blood extracts from patients receiving daily doses lower than 50 mg showed only the presence of metabolites.

Meprobamate. Blood and urine samples from patients receiving daily doses of 400–1200 mg of the drug were extracted according to the procedure described. The specimens, after extraction, were re-extracted with two 50 ml portions of ethyl acetate. The ethyl acetate layers were separated, collected, filtered and the ethyl acetate evaporated under forced air at room temperature. The residue was taken up in methanol. The ethyl acetate extracts were found to contain the hydroxy-meprobamate[4], which is not extracted by the ether.

In system I, the drug and its metabolite show very close R_F values, 0.71 and 0.68 respectively, corresponding to the R_F values of the relative standard solutions. The best separation between the hydroxy-meprobamate and the parent drug was obtained in system IV. This system also differentiates the meprobamate from drugs showing the same color reaction with the Folin–Ciocalteau reagent and having similar R_F values in system I: chlordiazepoxide (0.66), benactizine (0.68), diazepam (0.72), tybamate (0.74), buclizine (0.76). In system IV the R_F values of these compounds are clearly distinct in respect to the R_F value of meprobamate, with the exception of chlordiazepoxide (meprobamate 0.42; chlordiazepoxide 0.49). To identify the meprobamate, the plate developed in system IV was sprayed with furfural reagent[5]. Chlordiazepoxide showed a bluish color, while meprobamate and hydroxy-meprobamate form violet colors with characteristic brilliant red fluorescences.

Table V reports the data collected for hydroxy-meprobamate and the parent drug. No specimens were available from patients receiving doses of meprobamate lower than 400 mg *pro die*.

TABLE V

R_F VALUES OF MEPROBAMATE AND HYDROXY-MEPROBAMATE

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Meprobamate	0.71	1.00	0.03	1.00	0.76	1.00	0.42	1.00	0.08	1.00
Hydroxy-meprobamate	0.69	0.97	0	0	0.65	0.85	0.17	0.40	0	0

TABLE VI

R_F VALUES OF AMITRIPTYLINE, NORTRIPTYLINE AND THEIR METABOLITES

	System I				*System II*				*System IV*				*System V*			
	Amitriptyline		*Nortriptyline*		*Amitriptyline*		*Nortriptyline*		*Amitriptyline*		*Nortriptyline*		*Amitriptyline*		*Nortriptyline*	
	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F
Unchanged drug	0.55	1.00	0.31	1.00	0.71	1.00	0.50	1.00	0.46	1.00	0.19	1.00	0.76	1.00	0.51	1.00
Spot 1	0.31	0.56			0.50	0.70	0.28	0.56	0.19	0.41	0.08	0.42	0.51	0.67	0.25	0.49
Spot 2					0.28	0.39			0.08	0.17			0.25	0.33		

TABLE VII

R_F VALUES OF IMIPRAMINE, DESIPRAMINE AND THEIR METABOLITES

	System I				*System II*				*System IV*				*System V*			
	Imipramine		*Desipramine*		*Imipramine*		*Desipramine*		*Imipramine*		*Desipramine*		*Imipramine*		*Desipramine*	
	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F	R_F	*Rel.*R_F
Unchanged drug	0.49	1.00	0.23	1.00	0.65	1.00	0.42	1.00	0.38	1.00	0.12	1.00	0.65	1.00	0.34	1.00
Spot 1	0.41	0.84			0.48	0.74	0.14	0.33	0.12	0.32	0.05	0.42	0.34	0.52	0.09	0.26
Spot 2	0.31	0.63			0.42	0.65	0.06	0.14	0.05	0.13			0.09	0.14		
Spot 3	0.23	0.47			0.14	0.21										
Spot 4					0.06	0.09										

Chromatography of stimulants

Parallel experiments were conducted on two pairs of commonly used antidepressant agents: amitriptyline and its monomethyl analog nortriptyline, and imipramine and its monomethyl analog desipramine.

It appeared advisable to ascertain whether the drug and its monomethyl analog form similar chromatographic patterns and if demethylation reactions take place during the metabolism of the dimethyl compound, yielding metabolites corresponding chromatographically to the respective monomethyl analog.

Amitriptyline–nortriptyline. According to HUCKER[6], demethylation and hydroxylation are the major metabolic reactions of amitriptyline. In a study concerning the excretion of nortriptyline, AMUNDSON AND MANTHEY[7] attempted to identify one of the spots, obtained after chromatographic separation of urine extracts, as 10-hydroxynortriptyline. On the bases of these observations, urine and blood extracts from patients receiving daily doses of 25–75 mg of amitriptyline or nortriptyline were spotted on the same plate and chromatographed in the five systems used.

The results of the experiments are shown in Table VI. In systems I, II, IV and V the major metabolite of amitriptyline corresponded to the position of unmetabolized nortriptyline and relative standard solution. Only the presence of the unchanged drugs was observed in system III for the two compounds (amitriptyline R_F 0.26; nortriptyline R_F 0.06). In systems II, IV and V a second metabolite was found to be common to the two drugs.

The spots were localized by means of the Folin–Ciocalteau reagent and by heating the plate at 100° for 10 min. The metabolites and the parent drugs form bluish colored spots. The lower limit of detection of unmetabolized drug corresponded to a daily administration of 25 mg of amitriptyline or nortriptyline.

Imipramine–desipramine. The chromatographic behavior of these two drugs was found to be similar to that of the two cycloheptadiene derivatives described. In system III only the unchanged drugs were detected (imipramine R_F 0.19; desipramine R_F 0.03). In this system a spot corresponding to the position of desipramine was observed irregularly in urine extracts of imipramine. Metabolites of imipramine were found in systems I, II, IV, V. The major spot corresponded consistently to the position of desipramine.

Metabolites of desipramine, found in systems II, IV and V corresponded to analogous spots separated from urine extracts of the dimethyl compound.

Table VII reports the R_F values of imipramine and desipramine and their metabolites. Extracts from specimens corresponding to daily doses lower than 25 mg of the two dibenz-azepine derivatives showed only the presence of metabolites.

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