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DETECTION OF CHLORPROMAZINE AND THIORIDAZINE METABOLITES IN HUMAN ERYTHROCYTES

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SUMMARY

Chlorpromazine and thioridazine metabolites were detected in bound and unbound forms in the red blood cells of patients receiving these drugs.

The metabolites were separated by thin-layer chromatography and identified, by comparison to reference compounds, as: unmetabolized chlorpromazine (CPZ), CPZ mono-demethyl, CPZ di-demethyl, CPZ sulfoxide, mono-demethyl CPZ sulfoxide, di-demethyl CPZ sulfoxide; unmetabolized thioridazine (TH), TH ring sulfoxide, TH side-chain sulfoxide, demethyl TH side-chain sulfoxide.

By visual comparison of the size and color intensity of the spots with those of standards at various concentrations, the content of free metabolites in 10 cc of packed red cells was, in most cases, equivalent to their concentration in 4 ml of plasma. In blood samples from patients on long-term medication, free chlorpromazine metabolites were present in the entire red cell fraction of the sample at a higher concentration than in the corresponding plasma fraction.

The chromatographic patterns formed by the plasma extracts showed large daily variations in the concentration of the free metabolites as well as in the ratio unbound/bound forms. More reproducible patterns were obtained from the extraction of the free metabolites present in red cells.

INTRODUCTION

Although the literature on psychoactive phenothiazine derivatives is particularly extensive, little is known relative to the metabolic picture and to the distribution of these drugs in blood.

Studies on the pharmacological properties of the phenothiazines and their metabolites in animals[1,2] and in humans[3–7] indicate that the therapeutic effects of these compounds are related to the metabolic pathways and to the rate of biotransformation which the drug undergoes. Inter-patient and intra-patient variations in response to phenothiazine therapy have been studied mainly in relation to excretion patterns and

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correlated to phenolic and conjugated metabolites[8]. Analyses of serum or plasma[9–16], or whole blood[17], have been limited to the determination of total drug metabolites detectable by the adopted analytical procedure, or to the separation and identification of some metabolites by thin-layer chromatography[18–20]. Technical difficulties in the detection of the submicrogram amounts of these compounds present in blood have been overcome by gas chromatography[21–23]. Recently CURRY AND MARSHALL[24] and CURRY[25] reported on the determination by gas chromatography of plasma levels of chlorpromazine, the sulfoxide and the mono- and di-demethyl derivatives in a group of patients receiving various dosages of the drug. The results showed a large variation in the plasma concentration of the drug in patients receiving similar dosages. Only the unbound forms of the four compounds were determined.

It has been observed that plasma levels of unbound metabolites, at a given time, do not necessarily reflect the concentration of the active compound(s) at the site of action and consequently may not be directly related to the therapeutical effect of the drug[26]. The concentration in plasma of the active unbounds forms of the drug, available for diffusion into the sites of action, is influenced by many factors: absorption of the compound, binding to plasma and tissue proteins and degree of reversibility of the complexes formed, transport across membranes, degree of interaction at level of the sites of action and biotransformation of the drug into metabolites with specific pharmacological activity[27].

Perhaps a better understanding of individual variations in response to phenothiazine therapy could be achieved by studying the complete picture of the distribution of the drug and its metabolites in bound and unbound forms, in both plasma and blood cells.

SALZMAN AND BRODIE[10] postulated that chlorpromazine is bound to serum proteins to a great extent. Differences in the degree of plasma protein binding of chlorpromazine and two metabolites have been observed *in vitro*: chlorpromazine and the mono-demethyl derivative showed a considerable protein binding with respect to the sulfoxide[28]. When chlorpromazine and chlorpromazine sulfoxide were incubated for 1 h with whole blood[29], different concentrations were found in the plasma, red cell and platelet fractions. These variations were related to the relative degree of protein plasma binding of the two compounds. Further experiments, *in vitro*, confirmed the uptake of some phenothiazines by red cells and platelets[30,31].

Organic bases of high lipid solubility are known to penetrate the human erythrocyte at rates depending on their lipid-to-water partition[32]. Studies on the distribution of drugs in blood have led to the observation that red cell concentration of sulfanilamide exceeds its plasma level[33]; acetazolamide accumulates and persists in the erythrocytes independently of its concentration in plasma[34]. No reference has been found in the literature concerning the presence of phenothiazine derivatives in human blood cells.

This study, prompted by previous observations[35], was designed as a complement to a project currently in progress in this laboratory on the distribution of psychoactive phenothiazine compounds in blood. It is limited to the detection of phenothiazine derivatives in the red cells of patients receiving various dosages of chlorpromazine or thioridazine and to the identification of some of their metabolites.

EXPERIMENTAL

Material

Blood samples were obtained from patients hospitalized in Cleveland State Hospital and receiving 800–1200 mg of chlorpromazine or 600–800 mg of thioridazine *pro die*.

The patients received the same medication for at least four weeks prior to sampling. No other drugs which could interfere with the analytical procedure adopted were administered. Blood specimens were collected in the morning, 90 min after ingestion of the drug. Patients from the same hospital receiving no phenothiazine derivatives served as control subjects.

Reagents and reference solutions

All reagents were of analytical grade purity. The diethyl ether contained 0.0001 % peroxides.

The Folin–Ciocalteu reagent (Fisher Scientific Co.) was diluted with an equal part of distilled water prior to use.

Reference solutions were prepared from the following chlorpromazine and thioridazine metabolites: chlorpromazine, CPZ mono-demethyl, CPZ di-demethyl, CPZ sulfoxide, mono-demethyl CPZ sulfoxide, di-demethyl CPZ sulfoxide, 7-hydroxy-chlorpromazine, 8-hydroxy-chlorpromazine; thioridazine, TH demethyl, TH ring sulfoxide, TH side-chain sulfoxide, TH disulfoxide, TH disulfone and demethyl TH side-chain sulfoxide.

Stock solutions were prepared by dissolving 10 mg of each substance in 10 ml of 95 % ethyl alcohol. The solutions were stable for ten weeks when stored in the dark at 4°. Aliquots of the stock solutions were used to prepare working standard solutions at various concentrations which ranged from 0.25 to 1 mg %. The alcoholic solvent was evaporated under nitrogen; the residue, dissolved in 0.001 *N* hydrochloric acid, was extracted according to the procedure adopted for the biological samples.

Extraction method

Free metabolites. Chlorpromazine, thioridazine, and their metabolites, were extracted from the biological samples into diethyl ether. The sample, made alkaline by addition of 1 *N* sodium hydroxide (pH 9–10), was shaken twice with 2 vol. of ether for 2 min, and twice with 5 vol. of ether for 10 min. Each ether phase, as soon as it was separated from the aqueous phase, was transferred to a flask containing 2–5 ml portions of 1 *N* hydrochloric acid and shaken for 10 min. The aqueous acidic phases were collected, made alkaline (pH 12) and re-extracted with two 25-ml portions of methylene chloride. The organic phases were combined and evaporated to dryness under a stream of nitrogen.

Bound metabolites. 5 *N* sodium hydroxide was added to the aqueous residue after extraction of the free metabolites. The final concentration of sodium hydroxide was 3 % (w/v). The flask containing the sample was placed for a few minutes in hot water and gently shaken in order to remove traces of ether remaining in the residue. It was then heated in a boiling water bath for 1 h. After cooling, the extraction was continued as outlined above for the free metabolites.

Recovery of chlorpromazine and thioridazine metabolites. In order to validate the

extraction method adopted, aliquots of the reference solutions corresponding to a content of 7 and 15 μg of substance were transferred to a test tube. The ethanol was evaporated under nitrogen. Three milliliters of 0.001 *N* hydrochloric acid were added to the test tube to dissolve the residue. The absorption spectrum of this solution was determined over the range of 200 to 400 mμ on a Beckman DB-G recording spectrophotometer. The solution was then neutralized and transferred to a flask containing 10 ml of phenothiazine free plasma or 20 ml of red cell hemolysate. The extraction was continued as for the biological samples. After evaporation of the methylene chloride, the residue was dissolved in 3 ml of 0.001 *N* hydrochloric acid. The absorbance of this solution was determined again, *versus* a blank sample prepared from the same plasma or hemolysate under identical experimental conditions. The recovery of the added substance was calculated from the difference between the initial and the final readings of the acidic solution at the wavelength of maximum absorption of the compound analyzed. Correction was made for the loss occurring during the various steps of the extraction procedure. Decomposition of the compounds was checked by thin-layer chromatography.

Chromatographic method

Merck Silica Gel G plates without fluorescence indicator, 20 × 20 cm, 0.25 mm, used during the experiments, were obtained from Brinkmann Instruments.

The plates were re-activated for 30 min at 100° and used the same day. Two parallel lines were marked 13 and 14 cm from the line of the application points which was 3 cm from the base of the plate. The silica gel between the two parallel lines was scraped off. A series of vertical parallel strips 2 cm wide was also scribed on each plate. The chromatographic tank was previously saturated for 1 h with the solvent. Two plates containing biological extracts and reference solutions were developed simultaneously in each tank. The chromatographic mixture was used for only one development. The chromatographic systems used were:

System I: acetone–12 *N* ammonium hydroxide (100:7);
System II: 95 % ethyl alcohol–ethyl acetate–glacial acetic acid–water (15:60:15:10);
System III: chloroform–absolute methanol–glacial acetic acid (90:10:10).

The development was allowed to proceed until the solvent front had reached 13 cm from the line of the application points. Then the plates were overrun for 10 min, removed from the tank, air-dried, sprayed lightly with the Folin–Ciocalteu reagent and observed for color development. A subsequent spray of 50 % sulfuric acid (v/v) was applied after 5 min and the color development observed again at room temperature as well as after heating the plate at 100° for a few minutes.

Procedure

Blood specimens collected in EDTA tubes were centrifuged for 10 min at 2500 r.p.m. The plasma and the buffy coat were removed and the red cells washed three times with 100 ml of 0.9 % sodium chloride solution each time. The third washing solution was subjected to extraction. The washed red cells were hemolyzed by adding an equal volume of distilled water and by shaking the test tube for a few minutes. Six extracts were prepared from each blood sample analyzed:

Extract A: hemolysate obtained from 10 cc of packed red cells;
Extract B: 10 ml of plasma;
Extract C: 4 ml of plasma;
Extract D: third washing solution (see above);
Extract E: hydrolysate from the residue after extraction A;
Extract F: hydrolysate from the residue after extraction B.

After evaporation of the methylene chloride, each residue was dissolved in acetone and quantitatively spotted on the chromatographic plate. Aliquots of the reference solutions mentioned above were extracted with the same procedure and chromatographed simultaneously with the biological extracts. Samples of phenothiazine-free plasma and hemolysate were also included in the analysis.

RESULTS AND DISCUSSION

Extraction procedure and recovery studies

Analytical methods previously used for the extraction of the phenothiazine derivatives from biological material involved the use of *n*-heptane[10,16], ethylene dichloride[36], carbon tetrachloride[37], chloroform[19,38] or methylene chloride[8,22]. When these solvents were used for recovery studies of the pure substances from aqueous alkaline solutions, the figures obtained showed considerable discrepancies with respect to the relative amounts of sulfides and sulfoxides recovered. Furthermore, they proved to be unsuitable for the extraction of the red blood cell hemolysates because of the tendency for gel formation which caused a considerable percentage of organic solvent to be lost during the extraction operations. The use of a single large volume of solvent to extract successively small portions of the sample resulted in an extract containing considerable amounts of normally occurring substances which interfered with the chromatography of the phenothiazine derivatives. Re-extraction of chlorpromazine and thioridazine from chloroform or methylene chloride in hydrochloric acid at various concentrations proved to be unsuitable, as the partition between the two phases is in favor of the organic solvent. Re-extraction in dilute sulfuric acid yielded poor recoveries of the sulfoxide forms. Unidentified green spots were observed in the chromatograms of thioridazine-containing samples when the concentration of the sulfuric acid was increased.

Extraction with diethyl ether[6,11] was preferred despite the content of peroxides present in the commercially available product. Attempts to remove the peroxides were only partially successful: the recoveries of the sulfide forms of the two drugs after a single 10 min-extraction were inconsistent and the oxidation to the corresponding sulfoxides was still considerable. By reducing the contact time between sample and ether to 2 min and by re-extracting each ether phase with the acid solution as soon as it was separated from the aqueous phase, no oxidation of the sulfide forms to the corresponding sulfoxides was noted.

Tables I and II show respectively the recoveries of 7 and 15 μg of chlorpromazine and thioridazine metabolites added to 10 ml of phenothiazine free plasma or to 20 ml of hemolysate, when extracted according to the procedure described.

Hydrolysis of the bound metabolites

During preliminary experiments, acid or alkaline hydrolysis was applied to por-

TABLE I

RECOVERY OF KNOWN AMOUNTS OF CHLORPROMAZINE METABOLITES FROM DRUG-FREE PLASMA AND HEMOLYSATE[a]

Compound	*7 μg added*		*15 μg added*	
	Plasma	*Hemolysate*	*Plasma*	*Hemolysate*
Chlorpromazine	68 ± 3.4	60 ± 2.2	79 ± 5.6	65 ± 4.2
CPZ mono-demethyl	76 ± 6.5	72 ± 5.6	86 ± 3.7	79 ± 5.1
CPZ di-demethyl	77 ± 3.1	69 ± 5.2	82 ± 6.1	76 ± 3.8
CPZ sulfoxide	97 ± 1.5	93 ± 4.4	98 ± 1.1	91 ± 1.9
Mono-demethyl CPZ sulfoxide	96 ± 2.3	91 ± 3.1	101 ± 2.1	96 ± 4.4
Di-demethyl CPZ sulfoxide	89 ± 4.8	88 ± 3.2	99 ± 1.3	94 ± 2.5
7-Hydroxy-chlorpromazine	62 ± 5.6	61 ± 4.6	74 ± 3.8	71 ± 3.6
8-Hydroxy-chlorpromazine	75 ± 3.6	77 ± 4.8	81 ± 1.4	79 ± 3.8

[a] Each figure is the average of four determinations.

TABLE II

RECOVERY OF KNOWN AMOUNTS OF THIORIDAZINE METABOLITES FROM DRUG-FREE PLASMA AND HEMOLYSATE[a]

Compound	*7 μg added*		*15 μg added*	
	Plasma	*Hemolysate*	*Plasma*	*Hemolysate*
Thioridazine	73 ± 2.1	69 ± 5.1	76 ± 4.3	78 ± 2.4
TH demethyl	85 ± 4.2	82 ± 3.3	83 ± 1.5	80 ± 3.7
TH ring sulfoxide	97 ± 3.3	98 ± 2.3	102 ± 2.4	99 ± 2.1
TH side-chain sulfoxide	95 ± 3.5	92 ± 2.1	99 ± 2.1	101 ± 2.7
TH disulfoxide	61 ± 3.8	59 ± 4.6	65 ± 4.5	67 ± 3.5
TH disulfone	101 ± 3.6	97 ± 2.2	96 ± 3.1	98 ± 3.7
Demethyl TH side chain sulfoxide	81 ± 2.3	79 ± 3.7	89 ± 4.7	86 ± 3.5

[a] Each figure is the average of four determinations.

tions of the aqueous residues after extraction of the free metabolites from the plasma samples. The use of hydrochloric acid or sulfuric acid at various concentrations led to higher quantities of unmetabolized chlorpromazine and thioridazine than the alkaline hydrolysis, and to lower quantities of the corresponding sulfoxides. Recovery studies on the available reference compounds added to phenothiazine-free plasma, showed that the sulfoxides are, by the action of the strong acid, partially converted to the corresponding sulfides, depending on the concentration of the acid and on the time of hydrolysis. Blank samples to which thioridazine metabolites were added and plasma samples from patients receiving thioridazine, when hydrolyzed with sulfuric acid or hydrochloric acid, yielded green unindentified derivatives[39].

Recoveries of the pure substances, added to phenothiazine-free samples hydrolyzed with 3 % sodium hydroxide, were similar to those shown in Tables I and II.

Chromatography of the extracts

Reference compounds. The R_F values of chlorpromazine and thioridazine metabolites in the three chromatographic systems used are tabulated respectively in

Tables III and IV together with the color reactions formed with the Folin–Ciocalteu reagent and the subsequent spray with the sulfuric acid solution, at room temperature and after heating the plate at 100°.

TABLE III

R_F VALUES (× 100) AND COLOR REACTIONS OF CHLORPROMAZINE METABOLITES

Compound	*System No.*			*Reagents*	
	I	*II*	*III*	*Folin*	H_2SO_4
CPZ di-demethyl	90	72	43	Fuchsia	
Chlorpromazine	85	50	40	Fuchsia	
Di-demethyl CPZ sulfoxide	79	42	11	—	Pink
7-Hydroxy-chlorpromazine	73	66	23	Violet	
CPZ mono-demethyl	60	61	52	Fuchsia	
CPZ sulfoxide	51	13	15	—	Pink
8-Hydroxy-chlorpromazine	41	66	25	Blue-violet	
Mono-demethyl CPZ sulfoxide	25	33	23	—	Pink

TABLE IV

R_F VALUES (× 100) AND COLOR REACTIONS OF THIORIDAZINE METABOLITES

Compound	*System No.*			*Reagents*		
	I	*II*	*III*	*Folin*	H_2SO_4	100°
Thioridazine	83	66	48	Blue-green		
TH demethyl	75	86	56	Blue-green		
TH disulfone[a]	70	40	27	—	—	—
TH ring sulfoxide	54	23	17	—	Blue violet	
TH side-chain sulfoxide	45	20	10	Pink		
Demethyl TH side-chain sulfoxide	33	45	21	Pink		
TH disulfoxide	29	6	4	—	—	Pink

[a] For the localization of the spot, see text.

The Folin reagent was useful for the identification of the sulfide forms of the two drugs. The subsequent spray with sulfuric acid resulted in color reactions with the sulfoxide forms and in an intensification of the colors produced by the Folin reagent. Under the chromatographic conditions described, the sulfoxide derivatives of the two drugs (0.5–1 μg) formed colored spots at room temperature, with the exception of thioridazine disulfoxide, which develops a pink color only after heating the plate. Thioridazine disulfone did not give any color formation and was localized as a blue fluorescent spot when the untreated plate was observed in UV light.

Blood extracts. The results obtained by the analyses of the bound and unbound metabolites in the red cell and plasma fractions from the blood samples of 50 patients in chlorpromazine and 24 patients in thioridazine therapy are tabulated respectively in Tables V and VI, and in Tables VII and VIII. The extraction of each sample was repeated twice at intervals of two days. The resultant extracts were chromatographed in two different systems. System I was used for the chromatography of the extracts from the first sample, independently of its content in chlorpromazine or thioridazine. The extracts from the second sample of blood were chromatographed in System II if

TABLE V

CHROMATOGRAPHIC PATTERNS OF UNBOUND AND BOUND CHLORPROMAZINE METABOLITES IN THE RED CELL AND PLASMA FRACTIONS OF FIFTY BLOOD SAMPLES (EXTRACTS A, B, E AND F)

Patient No.	*Unknown*[a]	*CPZ di-demethyl*	*CPZ mono-demethyl*	*Chlorpromazine*	*Di-demethyl CPZ sulfoxide*	*Mono-demethyl CPZ sulfoxide*	*Unknown*[a]	*CPZ sulfoxide*
1		A B F		A B F	B F			A B I
2		A B F		A B F				
3		B F			F			I
4		A B		A B F				A B I
5		A B		A B F				
6	B	A B E F	A B F	A B E F	A B F	B F		A B I
7		A B		A B F				
8		B						B
9		A B F		A B F				
10		B			F			I
11		A B		A B F				
12	E	A B F		A B F	B F			A B I
13		A B		A B F	B			A B I
14		A B F	F	A B F				
15		A B F		A B F				
16	E	A B F		A B F	B F			A B I
17		A B		A B F				
18	E	A B F		A B F	B F			A B I
19	E	A B F		A B F	B F			A B I
20		A B		A B F				
21	E	A B F		A B F	B F			A B I
22		A B F		A B F				
23		B			F			B I
24	B E	A B E F	B F	A B E F	A B F	B	B F	A B I
25		A B F		A B F				
26		B						B
27		A B F		A B F				
28		A B F		A B F				
29		A B		A B F				
30	E	A B F		A B F	B F			A B I
31		A B F		A B F				
32	E	A B F		A B F	B F			A B I
33		A B		A B F				
34		A B F		A B F				
35	E	A B		A B F	B F			A B I
36		B			F			I
37		A B		A B				
38		A B F		A B F	B F			A B I
39	E	A B F		A B F	B F			A B I
40		A B		A B				
41		A B		A B				
42		A B F		A B F				
43		A B F		A B F	B F			A B I
44		A B F		A B F	B F			A B I
45		A B F		A B F				
46		A B		A B				
47	E	A B E F	A B E F	A B E F		A F	B F	A B I
48		A B		A B F				
49	E	A B		A B F	B F			A B I
50		B						B

[a] See test.

TABLE VI

CHLORPROMAZINE METABOLITES DETECTED IN EXTRACTS A, B, E AND F AND OCCURRENCE OF THE SPOTS (R_F VALUES IN SYSTEM II)

Groups and % of cases	*R_F values (× 100) of the spots*	*Reference compounds*	*% appearance of the spots within each group*[a]			
			Unbound metabolites		*Bound metabolites*	
			Extract A	*Extract B*	*Extract E*	*Extract F*
Group A	72	CPZ di-demethyl	—	100	—	14
14 % (7 patients)	42	Di-demethyl CPZ sulfoxide	—	—	—	57
	13	CPZ sulfoxide	—	57	—	57
Group B	72	CPZ di-demethyl	100	100	—	50
48 % (24 patients)	61	CPZ mono-demethyl	—	—	—	4
	50	Chlorpromazine	100	100	—	83
Group C	81	Unknown[b]	—	—	62	—
32 % (16 patients)	72	CPZ di-demethyl	100	100	—	75
	50	Chlorpromazine	100	100	—	100
	42	Di-demethyl CPZ sulfoxide	—	94	—	87
	13	CPZ sulfoxide	100	100	—	100
Group D	81	Unknown[b]	—	67	67	—
6 % (3 patients)	72	CPZ di-demethyl	100	100	100	100
	61	CPZ mono-demethyl	67	100	33	100
	50	Chlorpromazine	100	100	100	100
	42	Di-demethyl CPZ sulfoxide	67	67	—	67
	33	Mono-demethyl CPZ sulfoxide	33	67	—	67
	22	Unknown[c]	—	67	—	67
	13	CPZ sulfoxide	100	100	—	100

[a] Figurs were rounded for simplicity.
[b] See test.
[c] See test.

TABLE VII

CHROMATOGRAPHIC PATTERNS OF UNBOUND AND BOUND THIORIDAZINE METABOLITES IN THE RED CELL AND PLASMA FRACTIONS OF TWENTY-FOUR BLOOD SAMPLES (EXTRACTS A, B, E AND F)

Patient No.	*Thioridazine*	*Unknown*[a]	*TH ring sulfoxide*	*TH side-chain sulfoxide*	*Demethyl TH side-chain sulfoxide*
1			A B F	A B F	
2	A B		A B E F	A B E F	
3	A B F	B F	A B E F	A B E F	A B F
4			A B F	A B	
5	A B		A B E F	A B F	
6			B	B	
7	A B		A B F	A B F	
8	A B E F	B F	A B E F	A B E F	A B F
9	A B		A B E F	A B E F	A B F
10	A B		A B F	A B	
11	A B		A B E F	A B E F	A B F
12			A B	A B	
13	A B		A B E F	A B	
14			B F	B F	
15	A B E F	B F	A B E F	A B E F	A B F
16			B		
17	A B		A B E F	A B E F	A B F
18	A B		A B F	A B	
19	A B		A B E F	A B E F	A B F
20			A B F	A B F	
21	A B		A B F	A B	
22	A B E F	B F	A B E F	A B E F	A B F
23			A B	A B F	
24	A B		A B E F	A B F	

[a] The spot is pink after Folin reagent. Its R_F value in System III was 39.

chlorpromazine had to be analyzed and in System III if the sample contained thioridazine. The use of different chromatographic systems for the second development was necessary because of the close R_F values of chlorpromazine and chlorpromazine di-demethyl in System III; System II did not give satisfactory separation between thioridazine ring sulfoxide and thioridazine side-chain sulfoxide.

Only metabolites detected in plates developed in two different systems were included in the results. Analyses of the third-washing solutions were always negative.

Chlorpromazine metabolites. The chromatographic patterns resulting from the analysis of blood samples from patients receiving chlorpromazine were grouped according to the absence of any free chlorpromazine metabolites in the red cell fraction (Group A), or to the presence, in this fraction, of unmetabolized chlorpromazine together with: chlorpromazine di-demethyl (Group B), or chlorpromazine di-demethyl and chlorpromazine sulfoxide (Group C), or chlorpromazine di-demethyl and chlorpromazine sulfoxide in addition to other metabolites (Group D). Table VI shows the percentage of appearance of the metabolites detected in Extracts A, B, E and F, within each of these groups.

Patients in Groups A, B, and C were receiving different dosages of chlorpromazine, varying between 800 and 1200 mg *pro die*. No considerable differences were noted in these patients with respect to the terms of medication. The three patients in Group D were in chlorpromazine therapy for longer periods of time. Two of them, at the time

TABLE VIII

THIORIDAZINE METABOLITES DETECTED IN EXTRACTS A, B, E AND F, AND OCCURRENCE OF THE SPOTS (R_F VALUES IN SYSTEM I)

Groups and % of cases	*R_F values (× 100) of the spots*	*Reference compounds*	*% appearance of the spots within each group*[a]			
			Unbound metabolites		*Bound metabolites*	
			Extract A	*Extract B*	*Extract E*	*Extract F*
Group A	54	TH ring sulfoxide	—	100	—	33
12% (3 patients)	45	TH side-chain sulfoxide	—	66	—	33
Group B	54	TH ring sulfoxide	100	100	—	60
21% (5 patients)	45	TH side-chain sulfoxide	100	100	—	60
Group C	83	Thioridazine	100	100	19	25
67% (16 patients)	60	Unknown[b]	—	25	—	25
	54	TH ring sulfoxide	100	100	75	100
	45	TH side-chain sulfoxide	100	100	56	75
	33	Demethyl TH side-chain sulfoxide	50	50	—	50

[a] Figures were rounded for simiplicity.
[b] The spot is pink after Folin reagent. Its R_F value in System III was 39.

of sampling, were receiving 800 mg, and the third 1000 mg of the drug *pro die*.

Unbound and bound chlorpromazine metabolites were absent in the red cell fractions from the seven patients in Group A. Analysis of 4 ml of plasma was negative. The concentration of free and bound forms in 10 ml of plasma was at the limits of detection. The sulfoxide form of chlorpromazine di-demethyl appeared in Extracts F in four samples, together with chlorpromazine sulfoxide. Unmetabolized chlorpromazine was absent in all the plasma extracts or its concentration was below the limit of detection. In these patients, chlorpromazine di-demethyl was constantly the major metabolite present in free form in plasma.

The chromatographic patterns obtained from the 24 patients in Group B showed, as a common characteristic, the presence in the red cell fraction of free unmetabolized chlorpromazine and the di-demethyl derivative. The two metabolites were also present in free form in the extracts from the corresponding plasma (Extracts B) in all the samples, and simultaneously in bound form in twelve samples of plasma. Extracts E were negative. No sulfoxides were detected in any of the extracts analyzed in this group. As estimated by visual comparison of the size and color intensity of the spots, unmetabolized chlorpromazine appeared to be the major metabolite present in free form in red cells as well as in plasma.

Free chlorpromazine sulfoxide was present together with unmetabolized chlorpromazine and the di-demethyl derivative in the red cell fraction of all the samples in Group C. Chlorpromazine and its sulfoxide were constantly detected in free and bound form in the plasma extracts. The total free chlorpromazine content in red cells appeared to be equivalent to 4 ml of plasma. The analysis of the bound forms in red cells was negative with respect to the metabolites identified in the other fractions; an unidentified derivative was detected in ten of the sixteen samples analyzed in this group. It formed a pink-violet spot when the sprayed plate was heated at 100°. Its R_F values were: 97 in System I, 81 in System II, and 63 in System III. The spot was absent in chromatograms of similar extracts from phenothiazine-free samples.

The same unknown metabolite was present in Extracts B and/or E from the patients in Group D. It was detected in free form in plasma and in bound form in red cells in the blood samples of these patients over a period of five months. Unmetabolized chlorpromazine and the di-demethyl derivative were present in free and bound form in the red cells and plasma of all the three patients in this group. Chlorpromazine mono-demethyl appeared in the two plasma fractions (Extracts B and F) in all samples, in free form in the red cells of two patients, and in bound form only in one of these samples. Chlorpromazine sulfoxide was also constantly detected in Extracts A, B and F. It represented the major metabolite within the sulfoxide derivatives detected. The sulfoxide forms of the two demethyl derivatives appeared randomly in Extracts A or B or F. Hydrolysis of the red cells yielded only sulfide forms. A metabolite, constantly detected in the two plasma extracts of two patients in this group, remained unidentified. It formed a violet spot at room temperature with the Folin reagent. Its R_F values were: 10 in System I, 22 in System II and 24 in System III.

In all the three patients the level of total free chlorpromazine material in 10 cc of red cells was equivalent or higher than in 10 ml of plasma. The bound forms in plasma appeared to be also at a higher concentration than the free forms.

As mentioned above, the daily dosages of these three patients were below the maximum reached in some of the cases studied: at the time of sampling, they had been

in chlorpromazine therapy at various dosages for longer periods of time ranging from six to eight months.

A characteristic pattern resulted from the analysis of the blood sample from one patient in this group. During the experiments it had been observed that each free metabolite detected in the red cell fraction was constantly present also in the extract from the corresponding 10 ml of plasma. In the case mentioned, mono-demethyl chlorpromazine sulfoxide was detected in free form in the red cells (Extract A) and in bound form in plasma (Extract F), but it was absent as a free metabolite in the plasma extract B (Fig. 1). Chlorpromazine mono-demethyl appeared in all the four fractions and distinctly at a higher concentration in free form in red cells. Unmetabolized chlorpromazine and the di-demethyl derivative were also present in Extracts A, B, E and F.

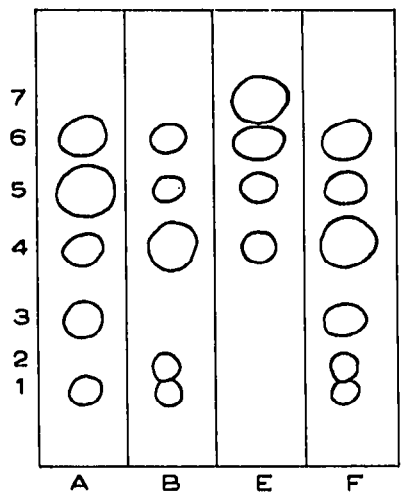

Fig. 1. Chromatographic patterns of Extracts A, B, E and F from a blood sample of a patient in chlorpromazine therapy (1000 mg/day).

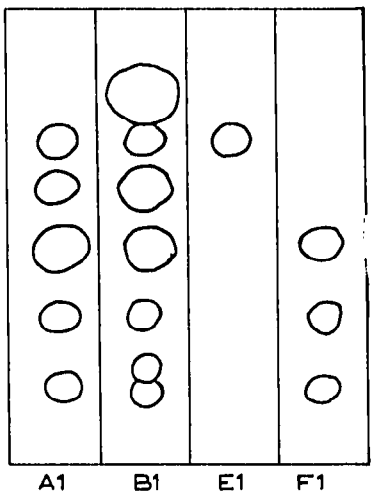

Fig. 2. Chromatographic patterns of Extracts A, B, E and F (reported in the test and in the figure as: A1, B1, E1 and F1) from a blood sample from the same patient, five days later.

The analyses of the two samples were performed on 10.6 cc of packed red cells and on 12.1 ml of plasma, representing the entire content of the samples. The plates were developed in System II. The spots, in order of increasing R_F values, correspond to: CPZ sulfoxide (1), unknown (2), mono-demethyl CPZ sulfoxide (3), chlorpromazine (4), CPZ mono-demethyl (5), CPZ di-demethyl (6), unknown (7).

The predominant spot in the two plasma fractions (B and F) corresponded to unmetabolized chlorpromazine; in the red cell fraction, the major free metabolite was the mono-demethyl derivative.

The analysis repeated on a second blood sample after two days, according to the procedure adopted, yielded similar patterns. The analysis was performed again five days later and then at intervals of weeks for five months.

In Fig. 2 are shown, for comparison purposes, the chromatographic patterns resulting from the extraction of the sample obtained from this patient five days after the analysis represented in Fig. 1.

Mono-demethyl chlorpromazine sulfoxide appeared as a free metabolite in plasma (Extract B1), and nearly at the same concentration as in red cells (Extract A1).

The level of chlorpromazine mono-demethyl in plasma (B1) increased with respect to Extract B, while its concentration in the red cell fraction (A1) appeared to be lower than in Extract A. Correspondingly, the bound form of this metabolite disappeared from plasma and from red cells. Unmetabolized chlorpromazine and the di-demethyl derivative were the only bound forms of sulfides present respectively in plasma and in red cells.

In this sample, free unmetabolized chlorpromazine was the major metabolite detected in the red cell fraction. The major spot in the plasma extract (B1) showed the same chromatographic properties as the unknown detected in Extract E (Fig. 1).

A comparsion of the chromatographic patterns of each fraction in Fig. 1 and in Fig. 2 showed also evident variations in the relative concentrations of total chlorpromazine derivatives, free and bound, in plasma as well as in red cells. These differences were more considerable with respect to the distribution and to the concentrations of the various metabolites between the four fractions, rather than with respect to the level of total chlorpromazine material present in each of the two different samples of blood. Extract B1 in Fig. 2 showed an evident increase in the plasma concentration of total free metabolites with respect to the analogous Extract B in Fig. 1; concomitantly the level of the bound forms in plasma (Extract F1) decreased with respect to Extract F in Fig. 1. Lower levels were also noted in the content of the bound forms in red cells. A more reproducible pattern appeared to be obtained from the extraction of the free metabolites present in red cells.

Similar variations were noted in many samples when the analyses were repeated at intervals of weeks under identical experimental conditions. The more apparent discrepancies observed were related to the concentration of the various metabolites free in plasma and to the ratio unbound/bound forms in this fraction.

Thioridazine metabolites. The results from the analysis of blood samples from patients in thioridazine therapy were grouped according to the absence of any free thioridazine metabolites in the red cell fraction (Group A), or to the presence, in this fraction, of thioridazine ring sulfoxide and thioridazine side-chain sulfoxide (Group B), or to the presence of the two sulfoxides together with unmetabolized thioridazine (Group C) (Table VIII).

The extracts from the patients in Group A formed poor patterns. Free thioridazine ring sulfoxide was present in the plasma extracts (10 ml) in all three cases. The side-chain sulfoxide was detected in this fraction in two samples. Extracts A, C and E were negative.

Contrary to the patterns formed by chlorpromazine, the two thioridazine sulfoxides appeared in free form in red cells before the unmetabolized drug and the demethyl derivative could be detected. Ring and side-chain sulfoxides were present simultaneously as free metabolites in the red cells and in the corresponding 10 ml of plasma, in the five patients in Group B. No bound metabolites were detected in Extracts E.

The chromatographic patterns of the red cell fractions from the sixteen patients in Group C showed the presence of unmetabolized thioridazine; in all the samples it was detected constantly together with the two sulfoxides. The corresponding plasma extracts showed the same free forms. In eight samples demethyl thioridazine side-chain sulfoxide was present, in free form, in red cells and in plasma, and simultaneously in bound form in the plasma fraction. Extracts F contained thioridazine ring sulfoxide in all the cases.

Contrary to the patterns formed by the bound chlorpromazine metabolites in red cells, the two sulfoxide derivatives of thioridazine were detected in the fractions E of some samples. In no case was the content of free metabolites in red cells higher than in 4 ml of plasma. The concentration of bound forms in plasma appeared to be lower than the level of the free metabolites.

In all the blood samples analyzed, thioridazine ring sulfoxide represented apparently the major metabolite; nevertheless, the higher sensitivity of this metabolite to the color developers used must be taken into consideration, when compared with the other thioridazine reference compounds studied.

CONCLUSIONS

From the results obtained during the experiments, it appeared that the concentration of unbound forms of chlorpromazine and thioridazine metabolites in red cells reflects the level of the total drug present in plasma.

Plasma levels appeared not to be a reflection of the administered dosage, but rather to be depending on the terms of medication. Plasma protein binding did not limit the diffusion of the drug derivatives into red cells, as shown by comparison of the chromatographic patterns obtained from these two fractions.

In patients receiving various dosages of the drug for periods of time ranging between four and nine weeks, the concentration of chlorpromazine and thioridazine metabolites in 10 cc of packed red cells was estimated, by visual comparison of the size and color intensity of the spots, to be equivalent to the drug content in 4 ml of plasma.

Samples from patients in longer-term medication (chlorpromazine) yielded more intense patterns, regardless of the administered dosage. In some of these samples, free forms of chlorpromazine were detected in the red cell fraction at a higher concentration than in 10 ml of plasma; correspondingly the ratio unbound/bound forms in plasma was exceptionally reversed, the bound metabolites appearing at a higher concentration than the free forms.

In the 96% of the total samples analyzed (74 patients) the distribution of chlorpromazine and thioridazine metabolites in the four fractions (unbound and bound forms in red cells, and unbound and bound forms in plasma) appeared to be as follows: plasma free forms > plasma bound forms > red cell free forms > red cell bound forms. In patients in long-term chlorpromazine therapy, and depending on the day of sampling, this relation was: plasma bound forms > red cell free forms > plasma free forms > red cell bound forms (Fig. 1), or it was: plasma free forms > red cell free forms > plasma bound forms > red cell bound forms (Fig. 2).

The variation in the relative concentration of the various metabolites in each of the four fractions did not appear to affect the total drug content in the whole sample.

The most apparent daily variations in the chromatographic patterns formed by the two drugs studied in each of the four fractions analyzed were related to the level of the free metabolites in plasma. As these variations appeared to be inconsistently depending on the day of sampling, any approach made in order to establish a correlation between plasma level of the drug and patient response to the phenothiazine therapy should be reviewed. Clearly, such a correlation cannot rely exclusively on the determination of the plasma level of unbound metabolites present in any given sample.

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