On the contrary, 0.7 mmole/kg. and 1 mmole/kg. of GGE did not increase the amount of cholesterol excreted in bile.

Other compounds such as 1 mmole/kg. of β-(4-hydroxy-2-methoxyphenoxy)lactic acid, 200 mg./kg. of GGE-MN, 300 mg./kg. of GGE, 1 mmole/kg. of nicotinic acid, 0.3 mmole/kg. of meso-inositol hexanicotinate,[3] and the equimolar mixture of GGE and nicotinic acid at 0.7- and 1-mmole/kg. doses increased the amount of cholesterol excreted in bile for some rats but did not alter that for other rats in the same group, compared with that of the control group.

On the other hand, all compounds used showed no effect on the total volume of bile excreted, compared with the control group, at 95% confidence limit.

It is interesting that (4-hydroxy-2-methoxyphenoxy)acetic acid, one of the metabolites of GGE and GGE-MN, has the effect of increasing cholesterol in bile in hypercholesteremia caused by intravenous injection of polyoxyethylene ethers. GGE-MN increased the excretion of cholesterol in bile at 1 mmole/kg., but nicotinic acid, one of the metabolites of GGE-MN, also has similar action at the same dose. Therefore, some question still remains whether the increasing effect of GGE-MN on excretion of cholesterol in bile is due to that of GGE-MN itself and/or to the metabolites of GGE-MN *in vivo*. The data concerned with mesoinositol hexanicotinate and 2,6-pyridine dimethanol bis(*N*-methylcarbamate) were determined for comparison purposes, since these compounds are now widely being used as hypocholesteremic agents.

[3] Hexanicite, Yoshitomi Pharmaceutical Co. Ltd., Japan.

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Chlorpromazine Metabolism I: Quantitative Fluorometric Method for 11 Chlorpromazine Metabolites

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Abstract □ Eleven chlorpromazine metabolites have been reacted with dimethylaminonaphthyl sulfonyl chloride to obtain fluorescent products. The reaction has been standardized and adapted to quantitative determination of nanogram amounts of the metabolites. A procedure for application to biological fluids has been developed. Addition and recovery experiments on urine and plasma indicate that the method is applicable to the study of the pharmacokinetic aspects of chlorpromazine metabolites in human subjects.

Keyphrases □ Chlorpromazine—metabolism □ Metabolites, chlorpromazine—quantitative determination □ 5-Dimethylaminonaphthalene-1-sulfonyl Cl–chlorpromazine metabolites reaction—fluorescence □ TLC—separation, identification □ Fluorometry—analysis

Despite a great deal of work done with chlorpromazine (CPZ), the significance of its biotransformation relative to its therapeutic response is not clear. The pharmacokinetics of CPZ and its metabolites in humans remain largely unexplored, possibly because of the limitations in sensitivity and precision of the analytical methods applied to the study of CPZ metabolism. Only recently (1–5) have the analytical methods been developed that offer desirable sensitivity for quantitating CPZ and its metabolites. However, their adaptation to routine determinations in clinical research is limited because these methods can quantitate only a few of the known metabolites (6).

The metabolites listed in Table I possess primary and secondary amino groups and/or hydroxy groups, all of which are capable of reacting with 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNS, dansyl) to yield fluorescent products. Dansyl has been routinely used as an end-group detector in the study of protein structure; its application for quantitation has, to date, remained limited only to amino acids (7). It has also been reported useful as a reagent for detection of some alkaloids (8).

A preliminary account of the dansylation reaction and separation of the dansylated metabolites of CPZ has already been reported (9). This paper deals with the development and standardization of the reaction between 11 CPZ metabolites and DNS. The method has been adapted to quantitative determinations of nanogram quantities of the metabolites. Addition and recovery experiments on aqueous solutions, urine, and plasma are also included.

EXPERIMENTAL

Materials and Equipment—The following were used: 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl, DNS), purity grade[1]; acetone, benzene, dichloromethane, dimethyl sulfoxide, di-

[1] Fluke-Buchs, Switzerland.

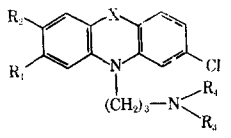

Table I—Various Chlorpromazine Metabolites Reactive with Dansyl[a]

Metabolite Compounds	Abbreviation	R_1	R_2	R_3	R_4	X
7-Hydroxychlorpromazine	7-OH CPZ	H	OH	CH_3	CH_3	S
8-Hydroxychlorpromazine	8-OH CPZ	OH	H	CH_3	CH_3	S
Monodesmethylchlorpromazine	Nor_1 CPZ	H	H	H	CH_3	S
Didesmethylchlorpromazine	Nor_2 CPZ	H	H	H	H	S
7-Hydroxychlorpromazine sulfoxide	7-OH CPZ-SO	H	OH	CH_3	CH_3	S → O
Monodesmethylchlorpromazine sulfoxide	Nor_1 CPZ-SO	H	H	H	CH_3	S → O
Didesmethylchlorpromazine sulfoxide	Nor_2 CPZ-SO	H	H	H	H	S → O
7-Hydroxymonodesmethylchlorpromazine	7-OH Nor_1 CPZ	H	OH	H	CH_3	S
7-Hydroxydidesmethylchlorpromazine	7-OH Nor_2 CPZ	H	OH	H	H	S
7-Hydroxymonodesmethylchlorpromazine sulfoxide	7-OH Nor_1 CPZ-SO	H	OH	H	CH_3	S → O
7-Hydroxydidesmethylchlorpromazine sulfoxide	7-OH Nor_2 CPZ-SO	H	OH	H	H	S → O

[a] The test samples of CPZ metabolites were supplied by the National Institute of Mental Health, Bethesda, Md.

oxane, ethanol, and pyridine, all nanograde; various phosphate and carbonate buffer salts, A.R. grade; Eastman 100-μ polyethylene terephthalate TLC plates coated with silica gel without fluorescent indicator; Eastman TLC chambers; and Vortex shakers. A recording Aminco-Bowman spectrofluorometer was used to record the fluorescence spectra, while a Turner model 110 filter fluorometer, fitted with a high sensitivity door, was used to quantitate the solutions. A Turner model 330 spectrophotometer was used for predetermination of the metabolite concentration range in the biological fluids prior to quantitation *via* the dansylation procedure.

Analytical Method—To 0.003–0.3 μmole of CPZ metabolites in acetone in a small screw-capped graduated glass centrifuge tube were added 0.015 μmole of DNS in acetone and 0.05–5 μmoles of phosphate buffer at pH 12, the total reaction volume being made up with acetone to 1 ml. The tube was capped and incubated at 45° for 2 hr. After cooling to room temperature, 10 μl. of the reaction mixture was spotted on a TLC plate and the chromatogram was developed in the appropriate solvent system (Table II). The individual DNS–metabolite spot was cut out, transferred to a 50-ml. centrifuge tube, and eluted with 10 ml. of ethanol by shaking for 1 min. on a Vortex shaker. The tube was centrifuged at 2000 r.p.m. for 2 min., and the supernatant was decanted for subsequent fluorescence analysis.

Table II—Solvent Systems for TLC Fractionation of DNS–CPZ Metabolite Products and the Corresponding R_f Values

System	Products	R_f Values
Benzene–acetone (1:1)	DNS-8-OH CPZ	0.67
	DNS-7-OH CPZ	0.50
	DNS-7-OH CPZ-SO	0.39
	DNS-Cl	0.95
	DNS-NH_2	0.84
	DNS-OH	0.07
Benzene–acetone (9:1)	DNS-Nor_1 CPZ	0.84
	DNS-Nor_1 CPZ-SO	0.37
	DNS-Nor_2 CPZ	0.74
	DNS-Nor_2 CPZ-SO	0.25
	DNS-Cl	0.89
	DNS-NH_2	0.46
	DNS-OH	0.00
Benzene–acetone (12:1)	DNS-7-OH Nor_1 CPZ	
	N-reacted	0.73
	OH-reacted	0.47
	DNS-7-OH Nor_2 CPZ	
	N-reacted	0.59
	OH-reacted	0.27
	DNS-7-OH Nor_1 CPZ-SO	
	N-reacted	0.18
	OH-reacted	0.02
	DNS-7-OH Nor_2 CPZ-SO	
	N-reacted	0.10
	OH-reacted	0.01
	DNS-Cl	0.92
	DNS-NH_2	0.26
	DNS-OH	0.00

Proof of Dansylation—A brilliantly fluorescent spot appeared on the developed chromatogram, with a R_f value different from that of the DNS and those of the reaction controls. On spraying the chromatogram with the Forrest reagent (10), the DNS–metabolite spot became purple, confirming the presence of a phenothiazine nucleus in the product. Figure 1 shows a typical chromatogram for a few of the DNS–metabolite reaction products.

Characteristics of the Dansylated CPZ Metabolites—Table II includes the solvent systems used to fractionate various groups of the dansylated metabolites and also their corresponding R_f values. The spectrofluorescent characteristics are given in Table III. Figure 2 represents the excitation and fluorescence spectra of a few representative metabolite products.

Optimum Conditions of Reaction—The optimum conditions were determined by studying only a few metabolites representing a primary amine, a secondary amine, and a phenol. The dansylation reactions were carried out under different conditions, varying one parameter at a time.

Effect of Temperature and Time—Nor_2 CPZ was reacted in the presence of phosphate buffer at pH 12 with an excess of DNS in acetone at 0, 25, and 45°. The reaction product formed was assayed at varying time intervals. Figure 3 shows the time course of the reaction at the different temperatures. Most of the reaction occurred within the first 30 min. Two hours at 45° or 6 hr. at room tempera-

Table III—Fluorescence Characteristics of the Dansylated Chlorpromazine Metabolites in Ethanol

CPZ Metabolites	Wavelength Maxima, mμ[a] Excitation	Emission
Nor_1 CPZ	345	520
Nor_1 CPZ-SO	345 (300)[b]	528
Nor_2 CPZ	340	510
Nor_2 CPZ-SO	340 (305)	515
7-OH Nor_1 CPZ		
N-reacted	344	520
OH-reacted	344	516
7-OH Nor_1 CPZ-SO		
N-reacted	344	520
OH-reacted	356 (288, 320)	520
7-OH Nor_2 CPZ		
N-reacted	342	512
OH-reacted	340	510
7-OH Nor_2 CPZ-SO		
N-reacted	346	510
OH-reacted	358 (282, 316)	512
7-OH CPZ	350	530
7-OH CPZ-SO	348 (302)	535
8-OH CPZ	346	530
DNS-Cl	340	520
DNS-OH	320	460
DNS-NH_2	340	500

[a] From uncorrected spectra recorded on Aminco-Bowman spectrofluorometer. [b] Numbers in parentheses represent secondary peaks.

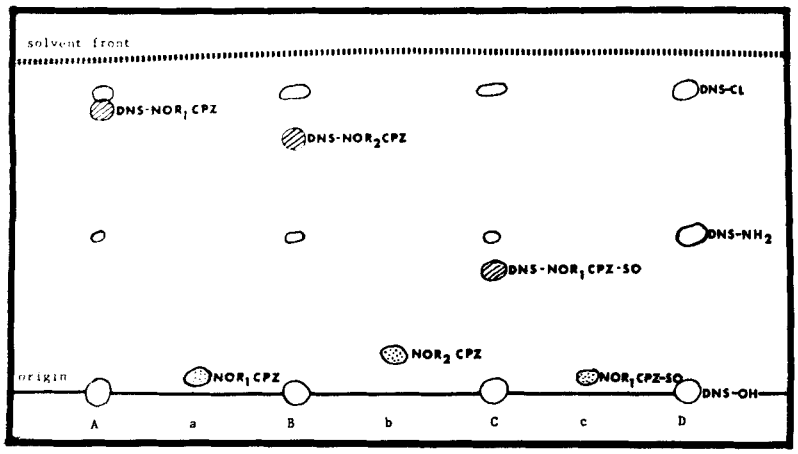

Figure 1—*TLC on a 100-μ Eastman plate developed with benzene-acetone (9,1), showing separation of some of the DNS-reacted CPZ metabolites. Dansylation reaction mixtures of the metabolites were spotted at A, B, and C, whereas at a, b, and c only the metabolites were spotted. D is the DNS-Cl reagent blank without any metabolite. Key: ○, fluorescent, nonreactive to Forrest reagent (F.R.); ◍, fluorescent, becoming purple when sprayed with F.R.; and ⊛, nonfluorescent but purple when sprayed with F.R.*

ture seems to be the practical optimum time–temperature combinations.

Effect of DNS Concentration—Figure 4 shows the formation of the dansylated product of Nor_2 CPZ under varying concentrations of DNS at three different reaction times. It appears that the optimal concentration of DNS on a molar basis is at least 5 times the concentration of the metabolite. In the actual procedure, the amount of DNS used was usually in excess of 6–8 times the molar concentration of the CPZ metabolite(s).

Effect of Hydrogen Ion, Buffer Salt, and Water Concentrations—The compounds Nor_1 CPZ, Nor_2 CPZ, and 7-OH CPZ, selected as representatives of three reactive sites in various groups of CPZ metabolites, were reacted with 6–8 times their concentration of DNS at room temperature for 6 hr. but in the presence of varying hydrogen-ion concentrations. Figure 5 shows the pH-dependent profile of the reaction products. In the optimal reaction procedure, pH 12 was used for the dansylation reaction. Figure 6 represents the influence of the buffer concentration on the dansylation, indicating that at least 0.05 μmole is required for the reaction and that higher concentrations are without any significant effect. In the absence of any buffer, the reaction is extremely slow. Figure 7 shows that the presence of water in the reaction mixture is critical.

Effect of Organic Solvents—Of several organic solvents used, the reaction proceeded only in relatively polar solvents. Of these, acetone seemed to show an optimum effect on the reaction (Fig. 8). Ethanol and methanol, when used as the reaction medium, gave their own dansylated products which interfered in the TLC fractionation. Alcohols were, therefore, avoided in all procedural steps until after the dansylation reaction. Dimethyl sulfoxide also yielded several additional fluorescent spots.

Effects of Atmosphere and Light—Dansylation of Nor_2 CPZ was carried out in nitrogen, oxygen, and air atmospheres, but no effect was observed on the formation of the reaction products. Reactions run in the dark, in ordinary fluorescent room light, and in UV light also did not show any difference in the product concentration.

Effect of Metabolite Concentration—Figure 9 shows the linear correlation of the fluorescence with the metabolite concentration. The linearity holds over a wide range of concentration.

Stability of Reaction Product—DNS–metabolite products appear to be stable with time for at least 24 hr. in solution. The atmosphere and light conditions do not seem to affect the amine-dansylated metabolites on the TLC, but the phenol-dansylated metabolites appear to be somewhat photosensitive.

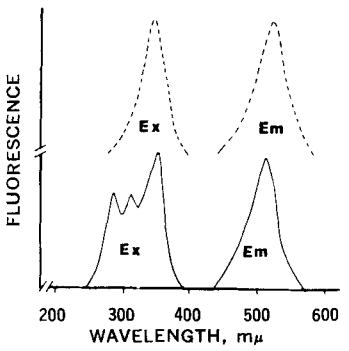

Figure 2—*Excitation (Ex) and emission (Em) curves of DNS–metabolite products. Key: - -, Nor_1 CPZ; and ——, 7-OH Nor_1 CPZ-SO (N-reacted).*

Adaptation to Studies on CPZ Metabolism—To apply the dansylation assay to the CPZ metabolites in the biological fluids of humans, it was necessary to develop an isolation and purification procedure for the metabolites prior to the dansylation reaction. For fractionation purposes, most of the CPZ metabolites may be classified into unconjugated and conjugated groups. The existing procedures (11–13) for isolation of these metabolite groups were extensively studied, modified, and adapted to quantitative determination *via* dansylation.

Procedure for Biological Materials—One-tenth to one milliliter of the biological fluid in a screw-capped centrifuge tube was extracted at pH 10 with 3 ml. of dichloromethane (DCM) for 3 min. and centrifuged. A 2-ml. aliquot of the organic layer was trans-

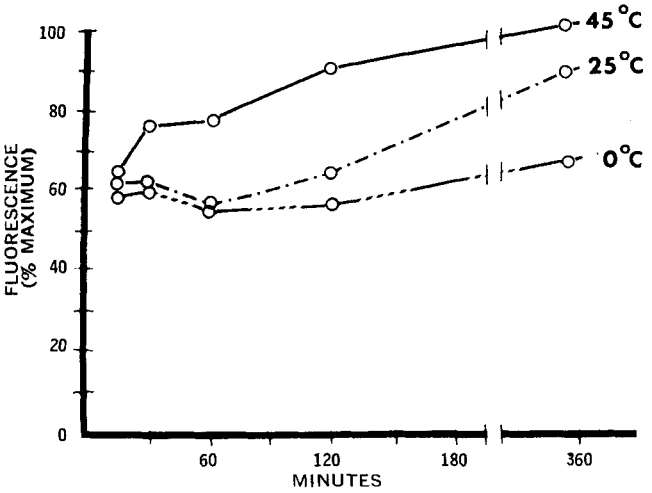

Figure 3—*Time course of the Nor_2 CPZ dansylation at various temperatures.*

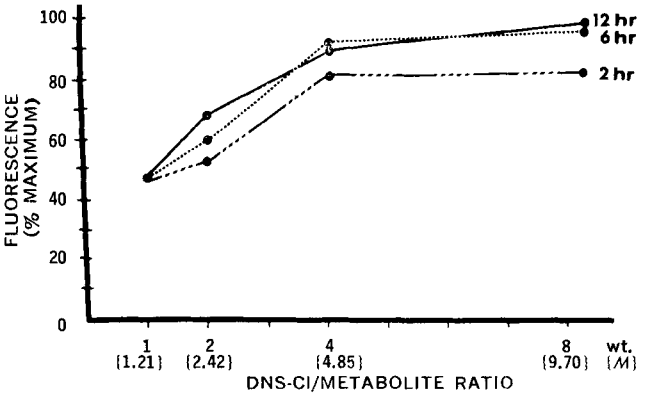

Figure 4—*Influence of DNS concentration on the dansylation of Nor_2 CPZ at varying time intervals.*

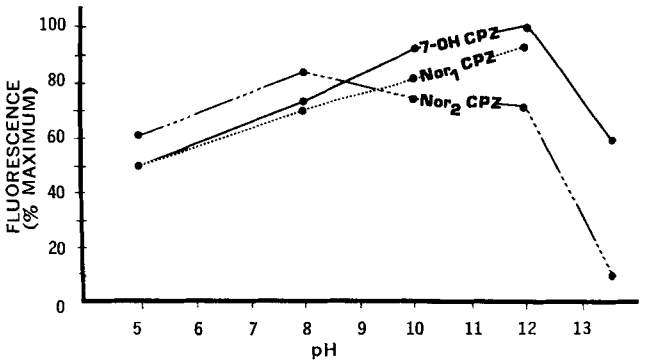

Figure 5—*Effect of pH on the course of dansylation reaction of various CPZ metabolites at room temperature in 6 hr.*

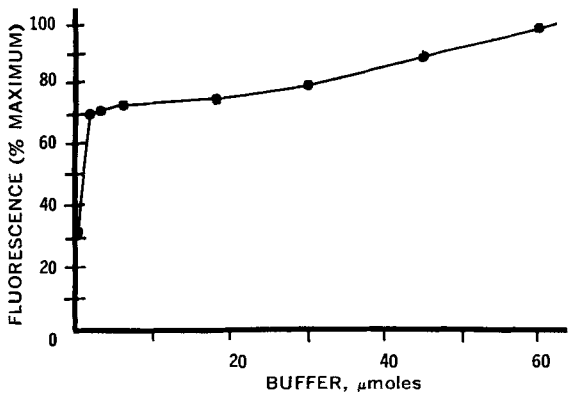

Figure 6—*Influence of pH 12 phosphate buffer concentration on the formation of dansylated Nor₂ CPZ at 1 hr.*

ferred into another centrifuge tube containing 5 ml. of modified Forrest reagent (5% ferric chloride in 50% sulfuric acid). The mixture was shaken for 1 min. and centrifuged. The lower acid layer was read on a spectrophotometer at 528 mμ, and the concentration of unconjugated metabolites extractable at pH 10 was computed from a predetermined standard curve based on the estimations of known CPZ concentrations by the same method. Although this represents only a few of the determinable metabolites, in any individual subject there should be a definite ratio between these and other CPZ metabolites. It should, therefore, serve as an index of the gross concentration of metabolites in the biological material.

After this predetermination, the biological material was diluted to contain 0.2–20 mcg./ml. of the index group metabolites. A 5-ml. sample in a 50-ml. centrifuge tube was adjusted to pH 10 with an excess addition of solid sodium bicarbonate and a dropwise addition of 6 *N* sodium hydroxide. It was extracted twice with 10 ml. of DCM for 3 min. each, 9 ml. of the organic phase being removed each time and combined. The combined organic extracts were evaporated to dryness under reduced pressure at low temperature on a rotary vacuum evaporator. The residue containing unconjugated metabolites was dansylated and assayed as described under *Analytical Method.*

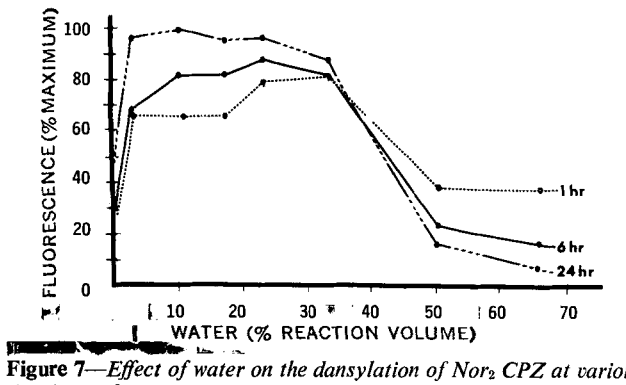

Figure 7—*Effect of water on the dansylation of Nor₂ CPZ at various time intervals.*

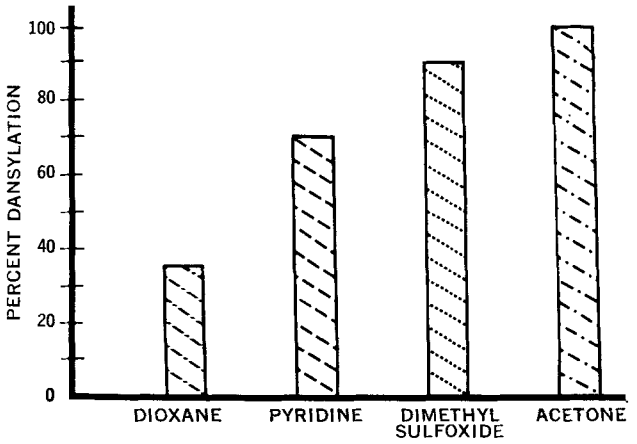

Figure 8—*Effect of solvents present in the reaction mixture at 45° on the dansylation of Nor₂ CPZ in 2 hr.*

The aqueous phase containing the polar conjugated metabolites was quantitatively transferred to a 50-ml. round-bottom flask. Sodium hydroxide solution was added to make the contents 2.5 *N* with respect to the base. The mixture was refluxed for 1 hr., cooled, and neutralized with HCl to pH 9. It was quantitatively transferred to a 50-ml. centrifuge tube and extracted for 2 min. with 20 ml. of DCM. After centrifugation, an 18-ml. aliquot of the organic phase was evaporated to dryness under reduced pressure and low temperature and was subjected to the dansylation assay.

Addition and Recovery—Known amounts of various representative metabolites of CPZ were added to water and to fresh urine and plasma samples. Aliquots of 5 ml. were subjected to fractionation, described under *Procedure for Biological Materials*, and then assayed by the *Analytical Method.* The addition and recovery data are included in Table IV.

Precision, Blanks, and Specificity—Replicate analyses varied less than ±5% in relative fluorescence whereas the precision of the Turner fluorometer was ±3–5%. A general 10% blank was contributed largely by the TLC plates, but this can be avoided by using self-made plates instead of commercial ones. The urine blanks contributed about ±10%, whereas plasma blanks varied from 0–10%. The blanks depend on the position of the DNS–metabolite on the chromatogram, since biological blank controls, particularly of urine, fractionate into several dull spots, some of which may occur at the R_f value of some of the DNS–metabolites. However, the high precision of the method, as revealed by negligible variation between the replicates, makes it feasible to subtract the blanks for concentra-

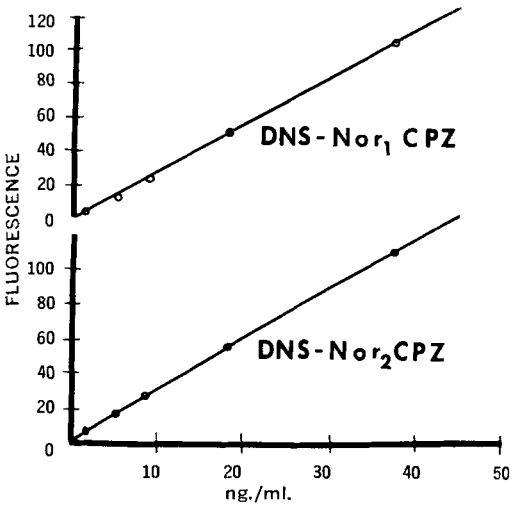

Figure 9—*Plots showing a linear correlation between the concentration of metabolites and fluorescence of their corresponding dansylated products in ethanol.*

Table IV—Addition of Various CPZ Metabolites to and Their Recovery from Biological Fluids

Metabolite	μmoles Added	Percent Recovery[a] Theoretical[b]	Water	Plasma	Urine
Nor_1 CPZ	0.318	100.00	85.81	75.88	61.70
		±1.78	±4.34	±0.71	±1.78
		Cor.[c]	100.00	88.42	71.90
Nor_2 CPZ	0.284	100.00	92.06	78.58	66.66
		±0.91	±7.77	±5.99	±1.52
		Cor.	100.00	84.34	72.41
Nor_2 CPZ-SO	0.277	100.00	78.16	82.39	76.40
		±0.91	±0.70	±0.40	±4.44
		Cor.	100.00	105.40	97.74
7-OH CPZ	0.808	100.00	83.61	77.96	116.94
		±4.26	±1.95	±3.14	±7.02
		Cor.	100.00	93.24	139.96

[a] Average of four samples ± *SE*. [b] Calculated from fluorescence values when processed directly without going through the *Procedure for Biological Materials*. [c] Corrected, basing recoveries from water as 100%

tion computation purposes. The selective extraction processes and chromatographic fractionations make the dansylation method specific to the CPZ metabolites studied.

DISCUSSION

The reaction between dansyl and the primary or secondary amine metabolites of CPZ may be regarded as a nucleophilic substitution of the S_N2 type, wherein the electron-rich amine nitrogen attacks the partially positive sulfur atom of dansyl chloride, the chloride ion departing as the nitrogen-sulfur bond is being formed. A hydrogen attached to the nitrogen may subsequently come off as a proton to form hydrochloric acid.

The dansylation reaction in absence of any buffer proceeds very slowly. This is understandable, since the acid produced during the reaction, if not buffered, will tend to protonate the amine reactant and thus reduce the nucleophile concentration in the reaction mixture. Buffer concentrations above a certain threshold level did not seem to affect the dansylation rate.

Water appears to be necessary for the optimum reaction rate, possibly because it serves as a medium for the buffer to operate. However, its concentration is critical. In quantities greater than 30% of the reaction mixture volume, water tends to hydrolyze the formed dansylated product. Also, excess water would compete with the nucleophilic metabolite for DNS molecules, decreasing the probability for DNS–metabolite formation.

As a solvent for the reaction medium, acetone appears to be the most suitable among those tested. Alcohols, in general, react with dansyl chloride, yielding highly fluorescent products which interfere in the subsequent chromatographic separation of the reaction mixture. Any alcohol should, therefore, be avoided during the dansylation process.

The dansylation assay method shows a high precision in replicates. The various blanks are within the acceptable range. It is encouraging that plasma gave negligible blanks, and the recoveries from it were better than 80%. A linear correlation graph between the concentration of the metabolites and their dansylated fluorescent products extends over a fairly large concentration range. By decreasing the dansylation reaction volume and by increasing the volume spotted on the TLC plates, the sensitivity of the method can be increased 10–50-fold. This should not only make it possible to quantitate minute amounts of the metabolites but also make feasible the detection of yet unknown trace metabolites of CPZ if any exist. The method is currently being applied to studying the pharmacokinetic aspects of CPZ metabolites in schizophrenic patients.

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