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QUANTITATION OF CHLORPROMAZINE AND ITS METABOLITES BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY AND DIRECT SCANNING MICRODENSITOMETRY*

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SUMMARY

Two-dimensional chromatography of chlorpromazine and its metabolites resulting in a resolution which permits *in situ* quantitation is described. A number of variables which affect the replication of the data has been carefully monitored and eliminated. Scanning the silica plates in two directions and averaging the counts thereby received, gives a replicable straight line within the practical limits of optical density of the instruments employed.

INTRODUCTION

For six years this laboratory has been investigating the metabolism of chlorpromazine (CPZ) and the identification and quantitative determination of each and every one of its metabolites in blood, urine, and feces of schizophrenic patients. A method has been described recently for the quantitative determination of combined phenolic and of combined non-phenolic metabolites without preliminary separation from other constituents of biological material[1]. This report will deal with the identification and quantitative determination of individual metabolites by two-dimensional microdensitometry of the thin-layer chromatographic (TLC) plate.

Identification of the metabolites of CPZ has now largely been accomplished by the work of a long list of investigators to only a few of whom we refer here [2-16]. Their work eventually has been confirmed by synthesis of a majority of these compounds.

Quantitation has, until recently, depended on separation of the metabolites, best done with TLC, and elution of spots presumed to contain all and only the individual metabolites to be determined[17,18]. Alternatively, measurement of the size of the TLC spot, and comparison with sizes of standard spots, has been recommended[19]. Although direct scanning microdensitometry has been available for at least five years no report on its use in the field of phenothiazines has appeared, a lack which we believe

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can be attributed to the variety and number of technical details which had to be resolved before a work such as ours could be reported.

METHODS AND MATERIALS

Solvent I: acetone–methanol–triethanolamine (100:30:1); solvent II: isopropyl alcohol–ethyl alcohol–ammonia (11:1:1); detection: ethanol–water–sulfuric acid–$FeCl_3 \cdot 6H_2O$ (6:1:1:0.8); thin-layer plates: Silica gel Brinkmann 254F, 250 mμ; developing chambers: Eastman-Kodak vapor chambers; sprayer: Fisher catalogue No. 5-719-5; Aminco grating monochromator No. A8401, optical bench designed to hold Aminco thin-layer scanner; American Instrument Co.: solid state microphotometer photomultiplier; Photovolt Model 43 integrator and recorder.

Standards: through the courtesy of Dr. ALBERT MANIAN, National Institute of Mental Health we received the following pure standards: CPZ, CPZSO, Nor_1CPZ, Nor_1CPZSO, Nor_2CPZ, Nor_2CPZSO, 2-Clph, CPZNO, CPZNOSO, 7-HOCPZ, 7-HOCPZSO, 7-$HONor_1$CPZ, 7-$HONor_2$CPZ, 7-$HONor_2$CPZSO, and 8-HOCPZ*. Precision Model No. 15 oven, 40°; Precision Model No. 15 oven, fitted with glass slab 1 cm above floor of oven. Temperature of glass slab was 80°; Thelco vacuum oven: Evacuated at 25 lb. and temperature 40°; scanning aperture: 1 mm (or $^1/_{25}$ in.) pinhole.

Standards in the dry state have been stored at −10° in tightly sealed desiccators. They have been stable for several years. All standards were weighed on a Cahn electrobalance and dissolved in suitable solvent (ethyl alcohol or water) so that the final solution contained 1 nmol/μl. These solutions, when stored in lightproof containers in a freezer were stable for several months. Standard mixtures were prepared from the standard solutions.

Brinkmann Silica Gel F254 thin-layer plates 250 μm (25 mm) were activated in a 100° oven for at least 4 h prior to use. Two 1 cm-tracks and a 1 cm-edge were placed on the top and left side of each plate. The non-phenolic mixture and the phenolic mixture were run on each plate in each dimension to facilitate the identification of spots. Standard mixtures were spotted 2 cm from the bottom of the plate in the center of the track. In all cases the starting zone was kept to a diameter of 3–5 mm. The sample to be chromatographed in two dimensions was spotted 2 cm from the bottom and 2 cm from the right side of the plate (Fig. 1 represents a typical mixture of polar and non-polar standards tracing after two-dimensional chromatography).

The plates were placed in Eastman vapor chambers and the chromatography begun immediately after the application of the sample spot. The use of Eastman chambers eliminated the necessity of presaturating the chromatography chamber. The samples were protected from light all through chromatography. The length of each run was exactly 12 cm from the origin. After removal from the first developing sys-

* Abbreviations: CPZ, chlorpromazine; CPZSO, chlorpromazine sulfoxide; Nor_1CPZ, monodesmethylchlorpromazine; Nor_1CPZSO, monodesmethylchlorpromazine sulfoxide; Nor_2CPZ, didesmethylchlorpromazine; Nor_2CPZSO, didesmethylchlorpromazine sulfoxide; 7-HOCPZ, 7-hydroxychlorpromazine; 7-HOCPZSO, 7-hydroxychlorpromazine sulfoxide; 7-$HONor_1$CPZ, 7-hydroxymonodesmethylchlorpromazine; 7-HONor, CPZSO, 7-hydroxymonodesmethylchlorpromazine sulfoxide; 7-$HONor_2$CPZ, 7-hydroxydidesmethylchlorpromazine; 7-$HONor_2$CPZSO, 7-hydroxydidesmethylchlorpromazine sulfoxide; 2-Clph, 2-chlorophenothiazine; CPZNO, chlorpromazine N-oxide; 8-HOCPZ, 8-hydroxychlorpromazine; CPZNOSO, chlorpromazine N-oxide sulfoxide.

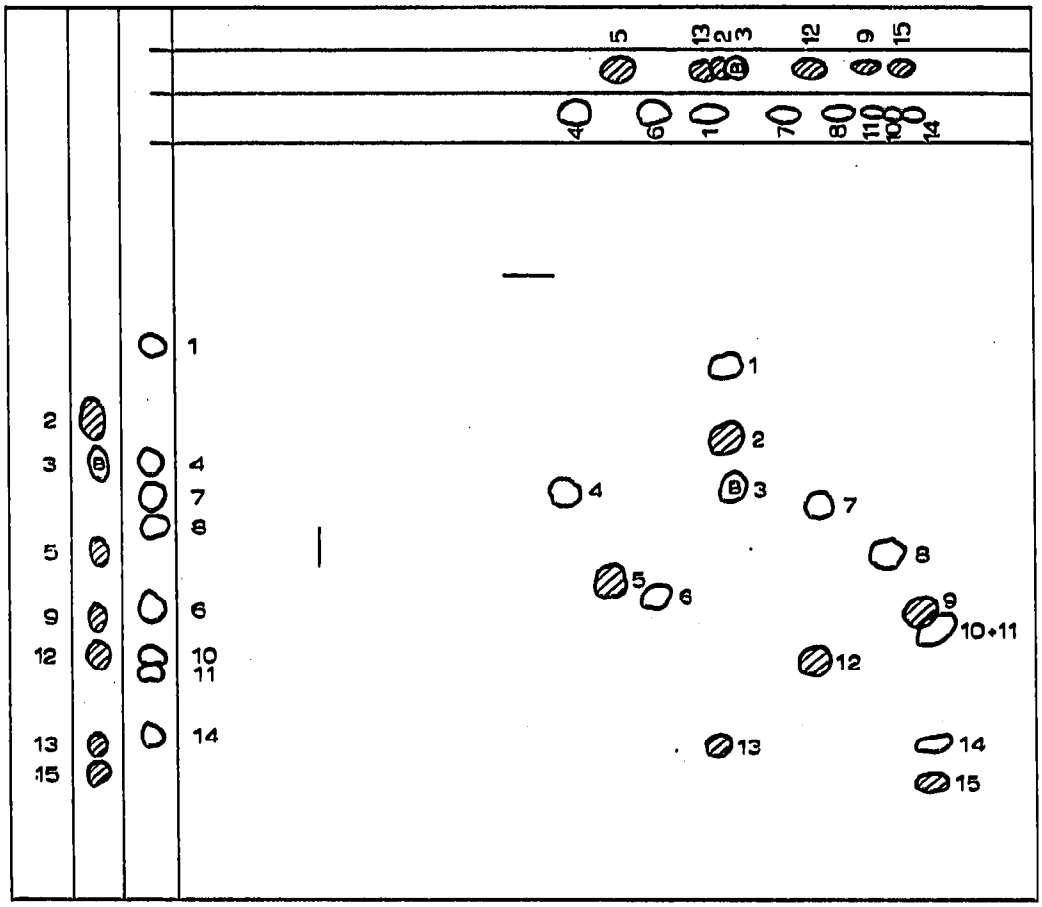

Fig. 1. Tracing from a mixture of polar (phenolic) and non-polar (non-phenolic) standards after two-dimensional development on silica gel plates sprayed and heated (see text). ○, pink; ◉, purple and B, blue. 1 = CPZ, 2 = 7-HOCPZ, 3 = 8-HOCPZ, 4 = Nor_2CPZ, 5 = 7-HONor_2CPZ, 6 = Nor_2CPZSO, 7 = CPZSO, 8 = Nor_1CPZ, 9 = 7-HONor_1CPZ, 10 = CPZNO, 11 = Nor_1 CPZSO, 12 = 7-HOCPZSO, 13 = 7-HONor_2CPZSO, 14 = CPZNOSO, 15 = 7-HONor_1CPZSO.

tem the plates were allowed to air-dry briefly and then were placed in a 42° drying oven for 10 min, after which time the second run was begun (solvent II). After development to 12 cm from origin the plates again were allowed to air-dry briefly, and placed in a 42° drying oven for a minimum of 10 min or until such time as they were sprayed for color development. At this step the plates could be stored for at least 1 week in a 40° vacuum oven without deterioration. The spray was delivered as finely as possible to avoid dripping. The thin-layer plate was placed glass side down on the glass slab of the Precision oven for 3 min by which time the plate was entirely dried and the background color from the chromogenic spray had entirely faded. A desiccant was kept in the thin-layer scanner while the plates were being scanned to retard absorption of moisture by the plates.

The phenolic and non-phenolic spots were scanned at 530 nm. While the maximum absorbance of the 7-HOCPZ metabolites is 565 nm and the isobestic point for the phenolic and non-phenolic metabolites is 545 nm, we have found 530 nm to be most practical with the plates used by us. The loss of sensitivity for the hydroxy compounds is approximately 60% but at the isobestic point the efficiency is not much greater while the loss for non-phenolic compounds amounts to 50%. 8-HOCPZ, a

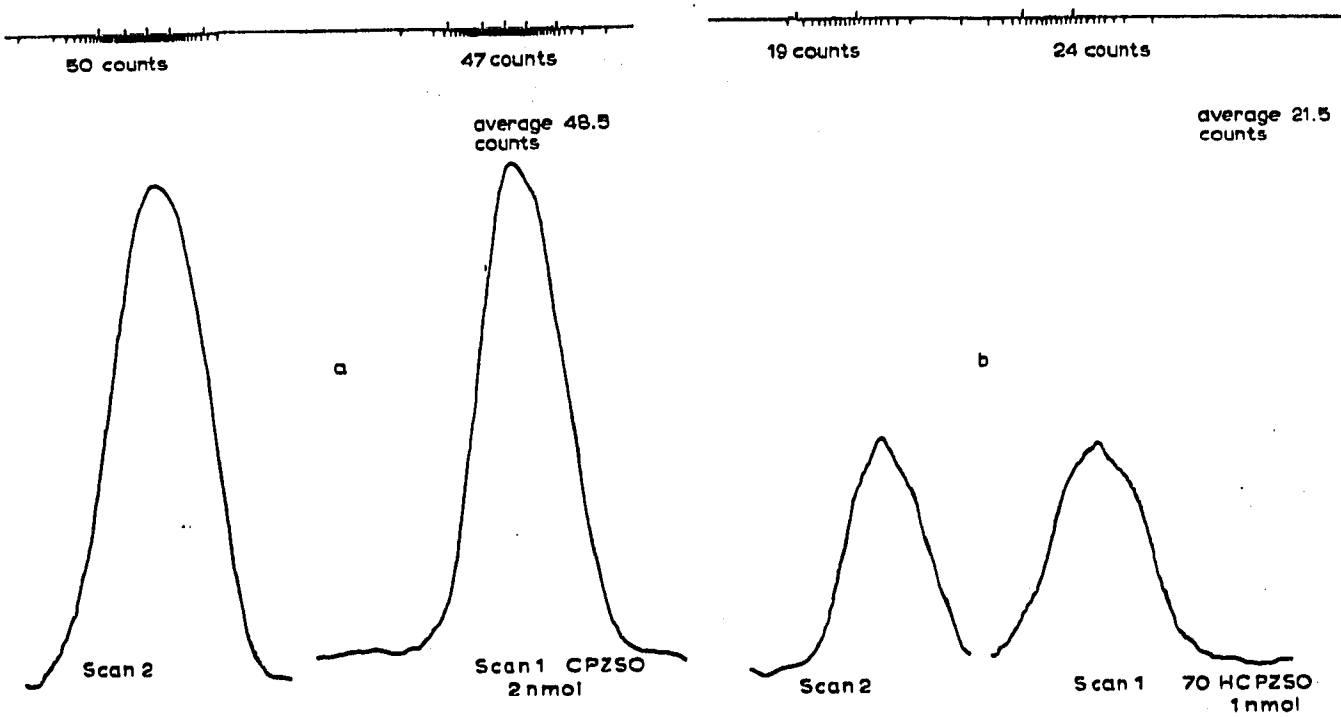

Fig. 2. Segment of integrator chart obtained on scan of one phenolic and one non-phenolic CPZ metabolite. (a) in direction opposite flow of solvent, (b) 90° to (a).

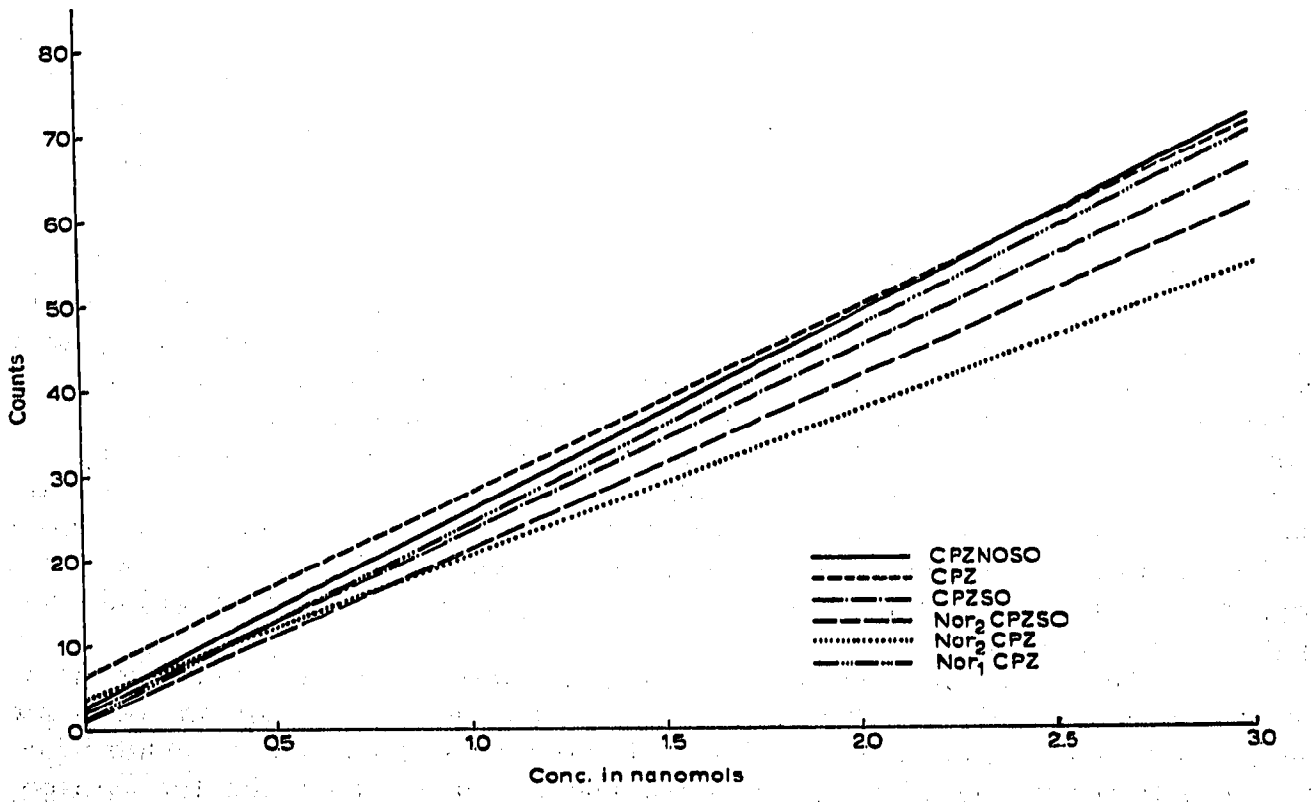

Fig. 3. Relationship between integrator pip counts and quantity of non-phenolic CPZ metabolites per TLC spot. Lines drawn from regression equations (see text).

minor component giving a blue spot, can be quantitated reasonably well at 530 nm. The integrator was adjusted for 95%-transmission in the blank areas preceeding each spot. In this manner we eliminated as far as possible any background deviation either inherent in the plate or due to solvent gradation.

The integrator was adjusted so that a deviation of 1–2% from the baseline initiated the counting. The plates were then scanned in the direction opposite the flow of the solvents. For each individual spot, the average of the counts from Scan I and II was calculated and used as a reference.

Mixtures of standards were chromatographed repeatedly in quantities varying from 0.5 to 3.5 nmols. The practical limits of optical density was reached at 3.0 to 3.5 nmols.

RESULTS

Fig. 2 is a segment of integrator chart representing the scan of a spot in each direction. Figs. 3 and 4 are the graphs of integrator pip counts against quantity per spot for non-phenolic and phenolic, respectively. The lines drawn for each compound are derived from the regression equation, except at the upper limits where the line curves.

Table I gives the means and the standard deviations of determination of each of fifteen metabolites at quantities from 0.5 to 3.5 nmols. Nor_1CPZSO, CPZNO, and 7-HONor_1CPZ resolve very closely in the standard mixtures. For this reason these

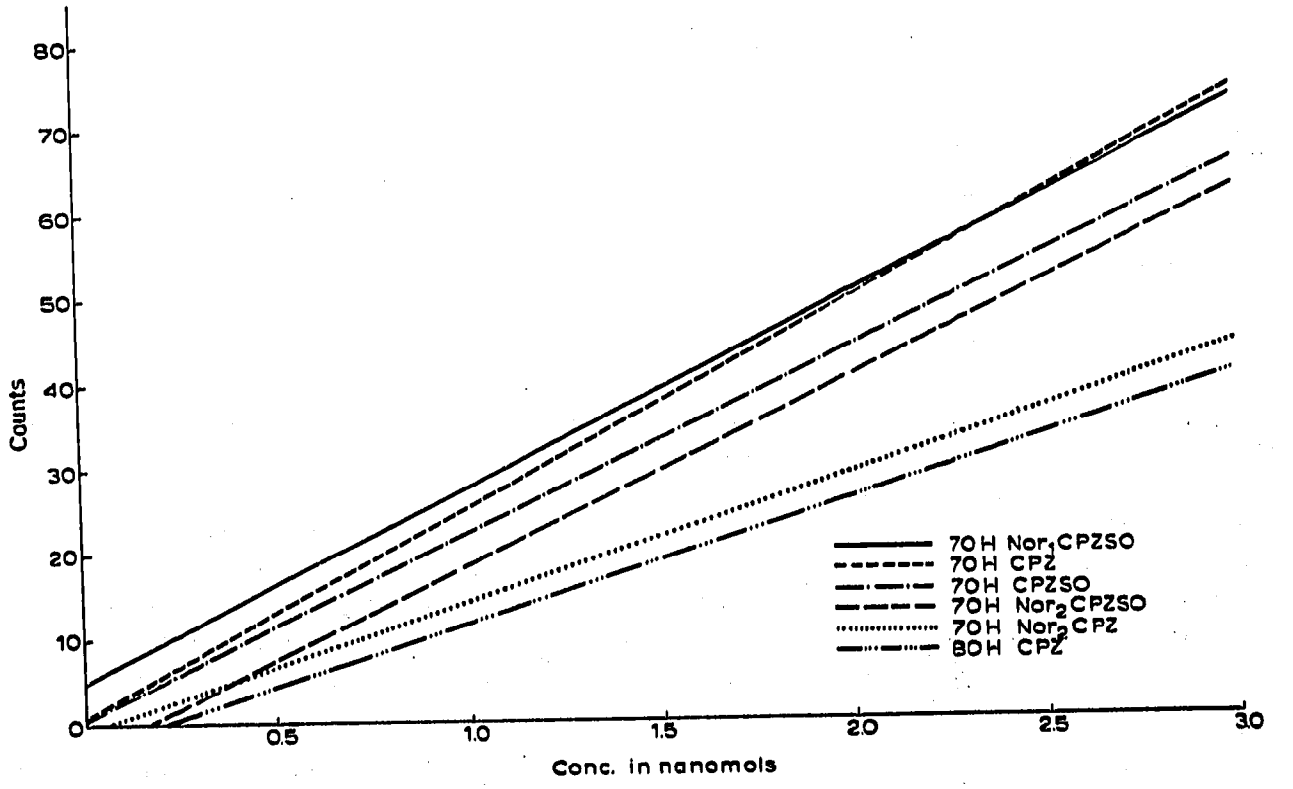

Fig. 4. Relationship between integrator pip counts and quantity of phenolic CPZ metabolite per TLC spot. Lines drawn from regression equations (see text).

TABLE I

MEAN, NUMBER AND STANDARD DEVIATION OF VARYING QUANTITIES OF CHLORPROMAZINE METABOLITES AFTER TWO-DIMENSIONAL CHROMATOGRAPHY

	0.5 nm			*1.0 nm*			*1.5 nm*			*2.0 nm*			*2.5 nm*			*3.0 nm*			*3.5 nm*		
	M	*N*	*SD*	*M*	*N*	*SD*	*M*	*N*	*SD*	*M*	*N*	*SD*	*M*	*N*	*SD*	*M*	*N*	*SD*	*M*	*N*	*SD*
CPZ	17.8	10	1.07	27	13	2.9	40	13	3	50	15	2.9	62.8	12	3.8	69	6	3.4	77.3	4	2.0
CPZSO	12.5	10	1.1	23.6	10	1.7	35	7	2.2	44.6	12	1.9	55.6	10	2.1	65.7	7	2.7	77.9	4	3.6
CPZNOSO	14	6	1.7	25	9	1.2	38.1	11	3.1	49.9	12	2.6	61.6	11	2.1	70.4	6	2.1	75.4	5	2.0
NOR_1CPZ	13.5	11	1.8	24.2	12	2.1	35	10	2.2	46.7	12	1.2	57.7	8	2.9	71.2	5	2.2	78	4	2.1
NOR_2CPZ	10.6	8	2.1	21.5	7	1.5	29.4	11	2.9	39.6	10	1.3	47.9	7	3.6	51.4	4	3.5	65.5	4	2.4
NOR_2CPZSO	12	6	1.09	21.5	1	1.8	31.5	8	0.9	41	11	3.3	52.5	8	2.6	62.1	5	3.4	73.8	3	0.25
CPZNO	18.5			30.3	6	2.8	39.3			54.4	4	1.5				61.5	3	0.4			
NOR_1CPZSO	15.3			28.3	8	2.5	34.5			53.6	6	2.6				66.8	6	2.6			
$7\text{-}HONOR_1CPZ$	14.3						44.5			57.9						81					
$7\text{-}HONOR_1CPZSO$	14.2	10	1.3	27.6	9	1.0	41	14	3.3	52.5	7	1.6	65.2	7	3.5	67.7	6	1.3	72.1	5	4.8
$7\text{-}HONOR_2CPZ$	5.5	5	1.4	13.6	9	1.6	23	11	1.7	30.6	4	1.6	38.8	7	2.3	41.2	8	4.1	56.1	4	3.4
$7\text{-}HONOR_2CPZSO$	11.3	6	1.5	14.3	6	1.3	27	7	1.7	40.1	11	1.6	52.4	9	3.1	63.3	5	2.9	79.3	4	3.7
7-HOCPZSO	11.8	9	2.5	21.8	9	2.3	32.2	7	2.4	45.2	9	2.2	57.3	6	3	64	4	2.4	75.8	4	2.1
7-HOCPZ	10.2	7	1.8	22.9	8	1.3	36	9	1.7	46.8	8	3.1	59	7	3.7	68.6	5	4.2	70	4	1.2
8-HOCPZ	5.7	10	1.1	11.1	11	1.6	18.5	12	1.3	24.6	8	1.8	33.1	7	3.1	41.5	5	2.6	47	4	1.0

three standards were calculated separately. The counts listed in Table I are therefore slightly higher than they would appear if chromatographed together.

The variability of color development for equimolar quantities of the members of a class of compounds, *e.g.*, non-phenolic sulfoxides, is quite low. It might seem adequate therefore, in the quantitation of an unknown, to employ the average absorbance of the class. A better way, however, is to use the absorbance of that identifiable member of the class closest on the thin-layer plate. As indicated by the data in Table I the number of counts for equimolar concentrations of the metabolites within a class varies inversely with the R_F values. The exception to this general rule is CPZ for the non-phenolics and 8-HOCPZ for the phenolics.

DISCUSSION

The effects of variation in one or more steps of TLC have been discussed in many papers[20,21]. PATAKI[22] listed seven factors which affect reproducibility: drying of the chromatogram and the time-delay between chromatography and actual measurement; positioning of the scanner; loading volume and size of origin; developing distance; influence of neighboring spots; accurate spotting of samples; layer thickness.

STAHL AND YORK[23] emphasized the necessity of keeping the starting zone constant, as variations in this area will affect the area under the curve later. DE ZEEUW[24] stated that considerable changes appear in the R_F values with varying amounts of water vapor in the ambient atmosphere. The R_F rises at first with an increase in humidity, then drops. He suggested the use of a constant humidity room. We monitored the humidity of the laboratory for several weeks with respect to the resolution of the chromatograms and found no variation great enough to affect reproducibility, provided the plates were placed quickly in the ovens and prevented from absorbing moisture.

Scraping plates in the blind has many possible sources of error. Almost everyone has had the experience of having had bowing or overlapping in TLC. Further, the shifting of R_F and tailing in mixtures, particularly in those of biological origin are well-known. This tailing is greater with solvent mixtures[25–27].

Although quantitative analysis by light absorption of paper chromatography goes back many years[28], surprisingly little has been published on direct microdensitometry of TLC since HAMMAN AND MARTIN described their work with the Aminco instrument[29,30]. GRAHAM *et al.*[31] claimed that the direct densitometry was equivalent in accuracy to absorptiometric methods and had the added advantages of speed and increased efficiency in reading spots difficult to remove from the silica. Other considerations, reviewed by GUNNER[32] and by GOLDMAN AND GOODALL[33] beyond those inherent in TLC as noted above, have been choice of slit dimensions, reproducibility of spot size and color intensity, choice of wavelength of light, and rate of passage of plate over the light source. When these have been appropriately chosen, the standard deviations of measurements have been quite good. Our own data in this regard are well in accord with those of GOLDMAN AND GOODALL.

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REFERENCES

1 W. J. TURNER, P. A. TURANO AND J. E. MARCH, *Clin. Chem.*, 16 (1970) 916.
2 C. L. HUANG AND A. A. KURLAND, *Amer. J. Psychiat.*, 118 (1961) 428.
3 M. B. WECHSLER, R. V. WHARTON, E. TANAKA AND S. MALITZ, *J. Psychiat. Res.*, 5 (1967) 327.
4 I. S. FORREST, D. E. GREEN AND B. P. UDALE, *Proc. W. Pharm. Soc.*, 7 (1964) 35.
5 J. J. ROSS, JR., T. L. FLANAGAN AND A. R. MAASS, *J. Med. Pharm. Chem.*, 5 (1962) 1035.
6 R. M. ROSE, A. DIMASCIO AND G. L. KLERMAN, *J. Phychiat. Res.*, 2 (1964) 299.
7 I. S. FORREST, A. G. BOLT AND R. C. ABER, *Agressologie*, 9 (1968) 259.
8 V. FISHMAN AND H. GOLDENBERG, *Proc. Soc. Exp. Biol. Med.*, 112 (1963) 501.
9 A. H. BECKETT, M. A. BEAVEN AND A. E. ROBINSON, *Biochem. Pharmacol.*, 12 (1963) 779.
10 N. P. SALZMAN, N. C. MORAN AND B. B. BRODIE, *Nature*, 176 (1955) 1122.
11 V. FISHMAN, A. HEATON AND H. GOLDENBERG, *Proc. Soc. Exp. Biol. Med.*, 109 (1962) 548.
12 C. F. RODRIQUEZ AND D. E. JOHNSON, *Life Sci.*, 5 (1966) 1283.
13 P. F. COCCIA AND W. W. WESTERFELD, *J. Pharmacol. Exp. Ther.*, 157 (1967) 446.
14 V. FISHMAN AND H. GOLDENBERG, *J. Pharmacol. Exp. Ther.*, 150 (1965) 122.
15 A. H. BECKETT AND D. S. HEWICK, *J. Pharm. Pharmacol.*, 19 (1967) 134.
16 T. H. LIN, L. W. REYNOLDS, I. M. RONDISH AND E. J. VAN LOON, *Proc. Soc. Exp. Biol. Med.*, 102 (1959) 602.
17 ANONYMOUS, *Psychopharmacol. Bull.*, 6 (1970) 44.
18 N. H. CHOULIS AND C. E. CAREY, *J. Pharm. Sci.*, 57 (1968) 1048.
19 J. J. BIEZINSKI, W. POMERANCE AND J. GOODMAN, *J. Chromatogr.*, 38 (1968) 148.
20 M. LEDERER, K. MACEK AND I. M. HAIS (Editors,) *Reproducibility in Paper and Thin-Layer Chromatography*, Vol. 33, No. 1, 1968.
21 M. LEDERER, K. MACEK AND I. M. HAIS (Editors), *Reproducibility in Paper and Thin-Layer Chromatography*, Vol. 33, No. 2, 1968.
22 G. PATAKI, *Chromatographia*, 1 (1968) 492.
23 E. STAHL AND H. JORK, *Zeiss Inform.*, 68 (1968).
24 R. A. DE ZEEUW, *J. Chromatogr.*, 33 (1968) 227.
25 S. TURINA, L. HORVATH AND V. MARGANOVIC, *J. Chromatogr.*, 37 (1968) 234.
26 F. C. HAER, *An Introduction to Chromatography on Impregnated Glass Fiber*, Ann Arbor Science Publishers.
27 F. GEISS AND S. SANDRONI, *J. Chromatogr.*, 33 (1968) 201.
28 T. D. PRICE, P. B. HUDSON AND D. F. ASHMAN, *Nature*, 175 (1955) 45.
29 B. L. HAMMAN AND M. M. MARTIN, *Anal. Biochem.*, 20 (1967) 423.
30 B. L. HAMMAN AND M. M. MARTIN, *Anal. Biochem.*, 15 (1967) 305.
31 R. J. T. GRAHAM, L. B. BARK AND D. A. TENSLEY, *J. Chromatogr.*, 39 (1969) 218.
32 S. W. GUNNER, *J. Chromatogr.*, 40 (1969) 85.
33 J. GOLDMAN AND R. R. GOODALL, *J. Chromatogr.*, 40 (1969) 345.