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Thin-Layer and Gas-Liquid Chromatographic Methods for the Identification and Estimation of Indoleamines in Urine Samples

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Several methods for the separation of amines from urine samples have been published. Chief among these are ion-exchange chromatography (1), solvent extraction (2–4), and acetone treatment of the butanol concentrate (4). However, because these methods lack the ability to fractionate different amines, their use results in a multiplicity of possibly overlapping spots in thin-layer chromatography (TLC) or a multiplicity of peaks in gas-liquid chromatography (GLC). Gross and Franzen (5,6) in determining bufotenin and *N,N*-dimethyltryptamine in blood and urine used a carbon disulfide reaction to remove the primary and secondary amines but did not confirm the efficacy of the method by the concomitant use of alternate methods such as paper chromatography or TLC. The nonspecificity of their method has been stressed in several critical reports and will be discussed in the latter part of this paper. Fisher and Spatz (7) used the reaction with acetic anhydride to acetylate the primary and secondary amines. Since this reaction is not specific for the primary and secondary amino groups only, hydroxy and phenolic compounds from the tertiary amines may also be acetylated and result in more than one spot by TLC.

In our studies on the determination of dimethylated indoleamines we reevaluated the carbon disulfide method of Gross and Franzen (5) by monitoring the reaction with two-dimensional TLC and GLC techniques.

METHODS

A mixture containing either 100 or 10 μg of each of the following amines: tryptamine, 5-methoxytryptamine (5-MT), serotonin (5-HT), *N*-methyltryptamine (NMT), *N*-methylserotonin (NMS), *N,N*-dimethyltryptamine (DMT), 5-methoxy-*N,N*-dimethyltryptamine (5-MeO DMT).

and bufotenin (5-OH DMT) was dissolved in 10 ml of ethyl acetate and shaken with 1 ml of carbon disulfide for 15 minutes. The ethyl acetate solution was washed with 0.1 N borate buffer (pH 10.1) (3 ml). The ethyl acetate layer was then extracted with 0.5 N HCl (2 ml) and the acid extract made alkaline (pH 10+) with ammonia, and reextracted into ethyl acetate after adding sodium chloride (1 gm). The ethyl acetate extract was dried over sodium sulfate and concentrated under vacuum. With either mixture TLCs of the final fraction showed only three spots corresponding to the three tertiary amines DMT, 5-MeO DMT, and bufotenin. GLC of this fraction also showed three peaks corresponding to the standards. The TLC and GLC data showed a recovery of 80–90% of the added tertiary amines after separation.

The same results were obtained when amounts as low as 5 μg of the dimethylated amines DMT, 5-MeO DMT, and bufotenin were added to urine samples and the same separation technique was used. When 5 μg of each of the standards (DMT, 5-MeO DMT, and bufotenin) was added to a 24-hr urine collection and subjected to the different steps of extraction and separation, an aliquot corresponding to one-fifth of the final concentrate showed distinct spots of the added standards on TLC. This indicates that levels as low as 5 μg per 24-hour collection of urine can be identified by this extraction procedure and TLC. These results were also confirmed by GLC. The recoveries in a series of experiments done by different technicians varied between 70 and 80%.

Modified Method. A slight modification of the above method was used to combine the extraction of the free amines from an aqueous solution and the reaction with carbon disulfide. The aqueous solution (5 ml) containing 10 μg each of the eight amines listed above in 0.2 N HCl was adjusted to pH 10 with ammonia and shaken with ethyl acetate (10 ml) containing 1% (v/v) of carbon disulfide for 15 minutes. The aqueous layer turned yellow during this procedure. After centrifuging, the ethyl acetate layer was separated and extracted with 2 ml of 0.5 N HCl. The acid extract was reextracted into ethyl acetate at pH 10.0. This fraction showed only the three tertiary amines on GLC and TLC.

Ion-Exchange Chromatography. The method used was similar to that described by Kakimoto and Armstrong (1). After the urine was extracted with ethyl acetate to remove the acidic and neutral compounds, and the pH adjusted to 5.5 with ammonia, it was passed through a Dowex 50 (H^+ Form) column. The effluent was discarded. The column was washed with water and with ethanol and was then eluted with ethanolic ammonium hydroxide (3.5% in 60% ethanol) and the eluate was collected in three fractions, pH 5–7, 7–9, and >9. The fractions D-1, D-2, and D-3 were then concentrated under reduced pressure, frozen, and lyophilized.

The lyophilized residues were then extracted into ethyl acetate (10 ml). The ethyl acetate extracts containing the free amines were fractionated into the tertiary amines by the carbon disulfide method described earlier. In experiments with added standards (5 μg each) DMT, 5-MeO DMT, and bufotenin were found in the D-2 and D-3 fractions. Ethyl acetate-insoluble residues in these fractions were found to be mainly glucuronides of amines since on glucuronidase or HCl hydrolysis these gave the free amines on TLC and GLC. With added standards (5 μg/24-hour sample) recoveries in the range of 75–85% were obtained.

Thin-Layer Chromatography (TLC): Spray Reagents. The final concentrate containing the tertiary amine fraction was dissolved in ethyl acetate (50 μliters). A 10-μliter aliquot as well as a reference mixture were applied on each silica gel G (Merck) thin-layer plate. The two solvent systems used were $CHCl_3$, MeOH, NH_3 (12:7:1) and isopropanol, NH_3, H_2O (85:15:5). A solution of *p*-dimethylaminocinnamaldehyde (DACA) in ethanolic HCl is the spray reagent of choice for indoleamines. However, in view of the controversial findings in the detection of bufotenin we felt it necessary to confirm the identification of this substance by a second spray reagent. Diazotized *o*-tolidine was used and found to be even more sensitive than DACA. O-Phthalaldehyde (OPT), also reported to be a sensitive reagent for 5-hydroxyindoles, has been used in the quantitation of low concentrations of 5-HT and 5-HIAA in biological material. 5-MT also gives an intense fluorescence under UV when sprayed with OPT. There has been no report on the use of this reagent for dimethylated indoleamines such as bufotenin and 5-MeO DMT. We evaluated this reagent for these two compounds (8). After spraying with the reagent (10 mg OPT, 100 mg L-cysteine in 5 ml methanol and 5 ml concd. HCl) and heating the TLC plate for 15 minutes at 100° an intense yellowish fluorescent spot was seen under UV for bufotenin and a blue fluorescent spot for 5-MeO DMT. The silica gel when scraped from the plate and extracted with 6 N HCl (2 ml) showed the characteristic fluorescent maxima at 490 mμ (ex. 360 mμ). This is the most sensitive of the three spray reagents, the lower limit of identification being 0.05 μg.

Gas-Liquid Chromatography (GLC). The GLC separation of the three dimethylated tryptamines (DMT, 5-MeO DMT, and bufotenin) is reported in the literature (9). In our studies we used a 1 or 3% SE-30 6-ft column. The retention times of the three compounds are in the ratio of 1:2.3:3.26.

When the standard mixture of the three amines (1 μliter) was injected along with 3 μliters of Regisil TM-TMCS (bistrimethylsilyltrifluoroacetamide plus 1% trimethylchorosilane), DMT and 5-MeO DMT were

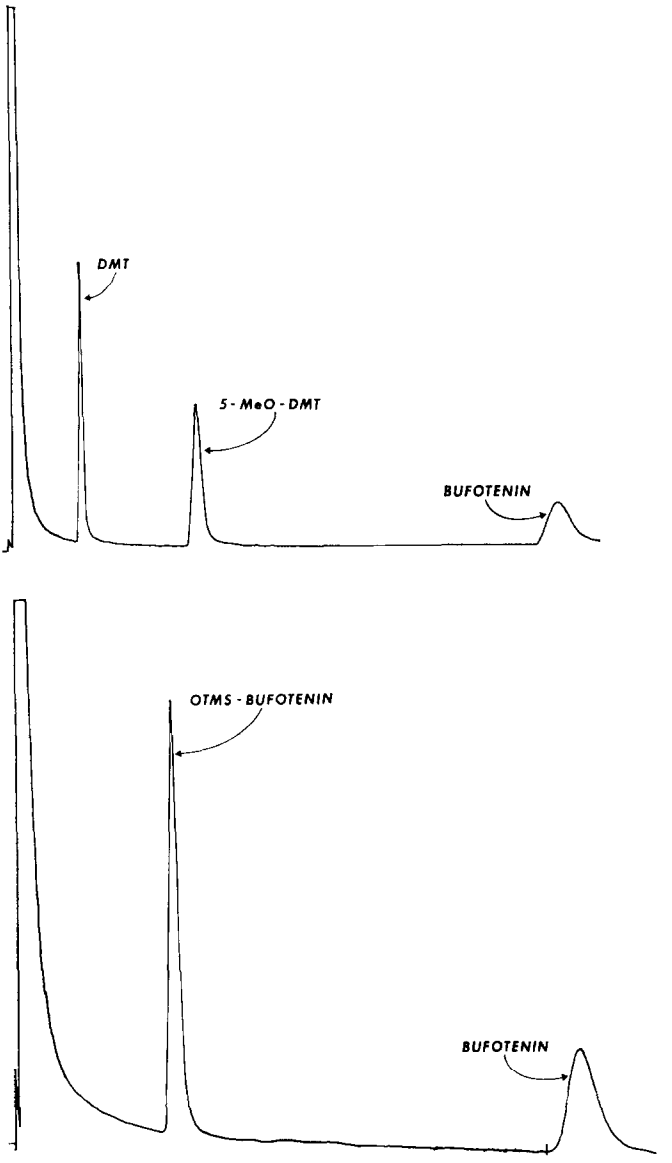

FIG. 1. GLC of dimethylated tryptamines on OV 225.

unaffected and bufotenin gave a sharp peak with a retention time 0.9 of that of the parent compound. This would be the *O*-trimethylsilyl (TMS) derivative of bufotenin. This reaction on the GLC column could be used for the identification of specific peaks.

The retention times of these amines were increased when a more polar column packing such as OV-225 (2.5%) was used. The chromatogram of the standards on a 4-ft 2.5% OV-225 column is shown in Fig. 1. The relative retention times were 1:2.7:7.7. In this column the TMS derivative of bufotenin had a retention time 0.28 of that of the hydroxy compound.

Quantitative Analysis for Dimethylated Indoleamines. For a quantitative evaluation of the dimethyltryptamines it is necessary to subject the tertiary amine fraction to preliminary preparative thin-layer chromatography (PTLC). Two aliquots of urine, one of which contains added standards, are extracted by the procedure outlined earlier, and the tertiary amine fractions of the two aliquots are applied as two separate bands and developed in one of the two solvent systems simultaneously with a spot of standards on the same plate. The bands corresponding to the reference standards are eluted with HCl and determined spectrofluorometrically.

DISCUSSION

In the past, methods used for the quantitation of *N,N*-dimethylated indoleamines (DMT and bufotenin) have lacked specificity for the components they were claimed to determine. The method of Gross and Franzen (5) is based on spectrofluorometry after chemical separation. Our experiments reported earlier support the claim of separation of primary and secondary amines from the tertiary amines. However, our recovery experiments fully confirm the criticism of Siegel (10) in regard to the nonspecificity of the fluorescence and the absence of maxima even when standards are added to the urine. We have found Gross and Franzen's method to be superior to that of Fisher and Spatz (7), who used acetic anhydride for the separation of primary and secondary amines from tertiary amines and reported that they obtained only one spot positive to diazotized *o*-tolidine in the urines of both normals and schizophrenics. They also clamied that the colors produced by some other phenolic amines were different.

In our experiments with 31 urine samples of normals, chronic schizophrenics, and acute schizophrenics we obtained on an average 4.6 spots (minimum 2, maximum 8) positive to *o*-tolidine.

From the results obtained in the experiments decribed above using solvent extraction and the carbon disulfide method of separation it is

evident that concentrations of the three dimethylated indoleamines as low as 5 μg of these amines in 24-hour urine collections are in the detectable range and therefore samples reported negative are below this range.

Supportive evidence of the identification is provided by GLC of the free amine fraction and of the TMS derivatives. A point worthy of mention is the incomplete reaction with the silylating agent. While with the SE-30 column the reaction was always complete to give a single peak, with the OV-225 column there were always two peaks (Fig. 1), one of the silyl derivative and one of the parent compound. It appears therefore the nature of the column plays a part in such column reactions.

Additional evidence for the presence of bufotenin can be provided by using three different spray reagents: (1) DACA for indoleamines, (2) diazotized *o*-tolidine for phenolic amines, and (3) OPT for 5-hydroxyindoles.

Since both the ion-exchange and the solvent extraction methods gave similar results and recoveries of the same order, the latter easier method was chosen as a routine procedure in our investigations. The methods described here have been in use in our laboratories for the past 2 years and have supplied the data presented in recent studies of dimethylated indoleamines in samples of urine and blood (11, 12).

The application of a combination of TLC and GLC methods and the use of more than one spray reagent in the identification of bufotenin as well as the use of *O*-phthalaldehyde in its quantitation are discussed in detail in a separate communication (13).

SUMMARY

The efficacy of the carbon disulfide reaction in separating the tertiary amines from the primary and secondary amines was reevaluated by monitoring the separation by GLC and TLC techniques. The need of more than one method for the identification of dimethylated indoleamines is emphasized. *O*-Phthalaldehyde is shown to be the most sensitive reagent for the qualitative identification of bufotenin and 5-MeO DMT on TLC.

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