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A SENSITIVE AND SPECIFIC THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE IDENTIFICATION OF 3,4-DIMETHOXYPHENETHYLAMINE IN URINE SAMPLES

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SUMMARY

Procedures for the separation and quantitative determination of 3,4-dimethoxyphenethylamine as the isothiocyanate derivative are given and the thin-layer chromatographic properties of 3,4-dimethoxyphenethylamine isothiocyanate are described. The sensitivity of this method for qualitative identification on thin-layer chromatography is 0.003 μg and for quantitative determination 0.01 μg. Recovery experiments were carried out with varying quantities of 3,4-dimethoxyphenethylamine added to 80% of a 24-h urine collection. Levels as low as 5 μg of 3,4-dimethoxyphenethylamine added to a urine sample yielded a distinct 3,4-dimethoxyphenethylamine isothiocyanate spot on thin-layer chromatography when 10% of an aliquot of the final concentrate was spotted.

INTRODUCTION

3,4-Dimethoxyphenethylamine (DMPEA) was first implicated as an abnormal metabolite in urines of schizophrenic patients by FRIEDHOFF AND VAN WINKLE[1], in 1962, who identified the amine by paper chromatograms of chloroform extracts of urine samples at pH 9, which were sprayed first with ninhydrin (acetone–pyridine) and then with modified Ehrlich's reagent. During the past decade this compound, popularly referred to as "pink spot" has been the subject of much controversy. The current status of the pink spot in schizophrenia has been reviewed recently[2–4]. A pink spot is reported to have been found in cases of thyrotoxicosis[5] and the authors comment that "it is safe to assume the area that has been labelled as pink spot is not homogeneous and is composed of a number of chemical components". However, it should be noted that the mass spectrometric evidence for the presence of this compound was provided through its dansyl derivative[6]. In a recent study DMPEA was found both in normal controls and in schizophrenics and was shown to have been due to an exogenous dietary source: tea[7]. Therefore it is clear that before a definite conclusion in regard to an association between this catecholamine derivative and

schizophrenia can be drawn, more detailed studies of urinary samples under rigorously controlled conditions are necessary.

From a survey of the literature it seemed obvious to us that a more sensitive and specific test was needed and that the methods used for the extraction of the material should be modified so that there would be a preferential concentration of the component to be tested.

We recently reported on the use of carbon disulfide (CS_2) for the separation of the primary and secondary amines from the tertiary amines[8]. In a subsequent communication[9] the structure of the reaction product of primary amines and CS_2 was shown to be the isothiocyanate (NCS), and the gas–liquid chromatographic (GLC) and gas chromatographic–mass spectrometric (GC–MS) characteristics of the NCS derivatives of several biologically active primary amines were described. Because the reaction between the amines and CS_2 to form the NCS derivatives was stoichiometric and was completed in a short time (30 min) and also because of its higher volatility for GLC and GC–MS identification, we also examined the thin-layer chromatographic (TLC) properties of the NCS derivatives of DMPEA and of other normal urinary metabolites such as tryptamine, *p*-tyramine, etc.

EXPERIMENTAL

Materials and methods

The free amines in ethyl acetate solution (1 mg/ml) were shaken with CS_2 (10% by volume) for 30 min and set aside for 2 h. The completion of the reaction was indicated by monitoring the reaction mixture by TLC and GLC. The solution was evaporated to dryness under vacuum and then reconstituted with ethyl acetate to contain 1 μg/μl. This solution, which corresponds to 1 μg/μl of the original base, was used for quantitative TLC and GLC.

For TLC, glass plates coated with Silica Gel G (E. Merck), 250 mμ thickness, were used, and the solvent systems were chloroform; chloroform–acetic acid (50:1); benzene; benzene–acetic acid (50:1); and benzene–methanol (10:1). Since the spot was not visible in visible or UV light, methanolic sulfuric acid (1:1) was used as a spray reagent. A yellow fluorescent spot developed under UV, and the fluorescence became more intense on heating at 100° for 10 min.

Recovery, reproducibility and sensitivity

The quantitative nature and the reproducibility of the reaction were then compared with a standard sample of DMPEA. A solution containing 1 mg/ml was made by dissolving 30 mg of DMPEA hydrochloride in 25 ml of water; 1-ml aliquots were taken in triplicate, the pH was adjusted to 12 or 10 with 4 *N* sodium hydroxide and the base was extracted with 5 ml ethyl acetate by shaking for 15 min. This solution was then centrifuged and the aqueous layer drawn off. The ethyl acetate extract was shaken with CS_2 (0.5 ml) for 30 min, then set aside for 2 h and evaporated to dryness under vacuum. The residue was redissolved in 1 ml of ethyl acetate (1 μg/μl of DMPEA). Aliquots of this solution in the range of 0.5–1.5 μl were used for GLC, and the area under the peak/μl of the sample was calculated. Similarly 1 μl of the sample was spotted (2 spots for each sample) on a Silica Gel G TLC plate, developed with chloroform, sprayed with methanolic sulfuric acid (1:1) and, after being heated

at 100° for 10 min, eluted with methanol (1.5 ml) and read on an Aminco Bowman Spectrofluorometer at the fluorescence maximum ($F_{max.}$) 465 nm, activation 365 nm. The sensitivity of this method was determined by spotting serially diluted samples ranging from 0.001–1.0 μg of DMPEA on the TLC plate and spraying with sulfuric acid.

Recovery experiments with urine samples. Levels in the range of 25–50 μg of DMPEA/24-h urine collection are reported in the literature[2]. Therefore, recovery experiments were carried out with varying quantities of DMPEA—30, 15, 10 and 5 μg—added to 80% of a 24-h collection.

The urine with added DMPEA was first concentrated to 10% of the original volume and then extracted with ethyl acetate at pH 2 to remove the acidic material. The aqueous layer was adjusted to pH 12 or 10 with 4 *N* sodium hydroxide, and extracted twice with equal volumes of ethyl acetate by shaking for 15 min. After centrifuging, the ethyl acetate extracts were pooled and concentrated to 10 ml under vacuum. The extract was then dried (with sodium sulfate), shaken with CS_2 (1.0 ml) for 30 min and set aside for 2 h. The solution was then extracted with 0.5 *N* HCl (3 ml) to remove all basic components. The ethyl acetate solution, now containing the NCS derivatives, was evaporated to dryness under vacuum. The residue was dissolved in ethyl acetate (100 μl), and 10 μl of the solution were spotted on a Silica Gel G TLC plate and developed two-dimensionally with chloroform and benzene–acetic acid (50:1) as solvent systems. Standard DMPEA-NCS was used for reference spots.

In another experiment the urine sample (24-h collection) was divided into two equal parts; to one part 5 μg of DMPEA was added and both the parts were processed as described above.

Modified method

The extraction step and the reaction with CS_2 are combined in one step by adjusting the pH of the urine sample to 12 or 10 and extracting into a mixture of ethyl acetate and CS_2 (95:5). The extract is then concentrated to 10 ml, washed with 0.5 *N* HCl (3 ml) and the final ethyl acetate extract evaporated to dryness.

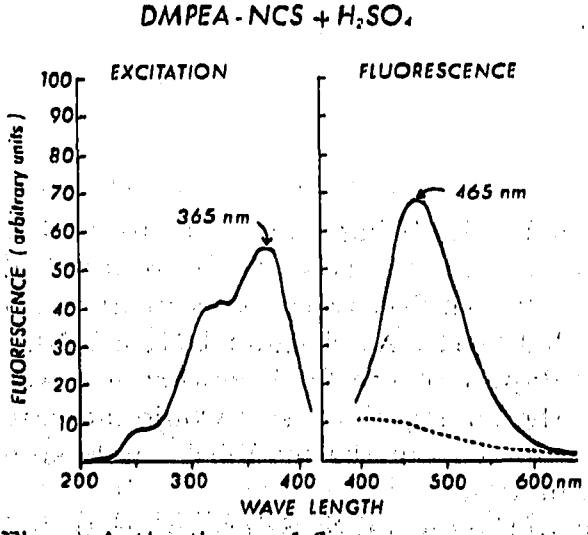

Fig. 1. Activation and fluorescence spectra of DMPEA-NCS after sulfuric acid spray, heating at 100°, and elution with methanol.

RESULTS AND DISCUSSION

The reaction between the primary amines and CS_2 to yield NCS derivatives was established by a GC–MS technique[9]. Our present study relates to the use of these derivatives for TLC. Spraying with sulfuric acid produced an intense bluish fluorescence under UV light, which was further intensified by heating at 100°. The fluorescence characteristics of this spot when eluted with methanol are shown in Fig. 1. When 1 μl of DMPEA-NCS solution containing 1 μg was heated with 0.1 ml of methanolic sulfuric acid (1:1) for 10 min and the solution made up with methanol (1.5 ml), the fluorescence was identical. Sensitivity tests showed that the fluorescence was distinctly seen with levels as low as 0.003 μg.

The quantitative reproducibility of the reaction was determined both by GLC and spectrofluorometric methods by replicate analyses involving all the steps of extraction, reaction with CS_2 and GLC or TLC analysis. The results are shown in Table I. The GLC data are reported as the area of the GLC peak/μg of DMPEA and the spectrofluorometric data as the fluorescence units of the reading of a 1-μg spot on TLC.

While pH 10 or 12 did not materially alter the extraction efficiency, pH 12 was preferred because it eliminated the free phenolic compounds, and thus increased the specificity for fully methylated compounds.

TABLE I

QUANTITATIVE REPRODUCIBILITY OF DMPEA–CS_2 REACTION: GLC AND TLC DATA

GLC peak (area/μg DMPEA)		*Fluorescence (units/μg DMPEA[a])*	
Experiment		*Experiment*	
I	*II*	*I*	*II*
3.7	3.0	1.37	2.24
3.3	3.8	1.35	2.11
3.6	3.8	1.38	1.69
3.0	4.0	1.32	1.73
3.0	4.0	1.67	2.48
3.8	4.2	1.61	2.41
4.0	4.4		

[a] Reading of a 1-μg spot on TLC.

Recoveries varied with the concentration levels, being higher at the higher concentrations (60–70% at 20–30 μg). For this reason it is advisable to use internal standards at appropriate levels for quantitative studies. Recovery experiments with urine samples showed that even with 5 μg of added DMPEA a distinct DMPEA-NCS spot could be seen on TLC when 10% of an aliquot of the final concentrate was spotted. So it is safe to assume that with this method a negative result would indicate a level below 5 μg/24-h urine collection. The results from the modified single step method are similar to those from the two step method.

In regard to the specificity of this reaction, of the several NCS derivatives tested only three compounds, DMPEA, 3,4-dimethoxyphenylisopropylamine (DMPIA) and

TABLE II

TLC, GLC AND GC–MS DATA ON DMPEA-NCS

TLC		*GLC*			*GC–MS*
Solvent system	R_F	*Column*	RR_T[a]	*MU*	
Chloroform	0.66	1% OV-101 170° iso	4.0		m/e 223 (27)[b]
Benzene	0.12	3% OV-17 190° iso	4.0		m/3 151 (100)[b]
Chloroform–acetic acid (50:1)	0.65	3% SE-30		18.68	
Benzene–acetic acid (50:1)	0.28	1% OV-101		18.43	
Benzene–methanol (10:1)	0.70	2.5% OV-225		26.76	
		3% OV-17		22.04	

[a] DMPEA 1.0.
[b] Relative abundance of the ions of the indicated mass.

3-methoxytyramine (3-MT) yielded a similar fluorescence. Of these DMPEA and DMPIA were very much higher in intensity and gave identical fluorescence spectra. 3-MT gave only a weakly fluorescent spot and did not show any $F_{max.}$ at 365 nm activation. Furthermore 3-MT could be separated from DMPEA on TLC and GLC. DMPIA is not a natural metabolite and it also could be separated from DMPEA on GLC (ref. 9). Therefore it seems that fluorescence tests coupled with $F_{max.}$ are quite specific for DMPEA-NCS derivatives and can be used for the qualitative identification and quantitation of DMPEA in urine samples. There is an added advantage in that the same derivative can also be used for GLC and GC–MS studies. The MS characteristics of the NCS derivatives have recently been reported[9] and the major fragments are *m/e* 223 (M^+) and *m/e* 151 $(M\text{-}72)^+$. The present method is superior to previously reported methods in sensitivity and specificity both by fractionation at the extraction step and specific fluorescence reaction. The TLC, GLC, MS and fluorescence characteristics of DMPEA-NCS are summarized in Table II and Fig. 1.

We have used both the TLC and GLC methods with the NCS derivatives in screening DMPEA in urine samples of normal and schizophrenic patients. The results of our investigations will be reported elsewhere. In some cases we obtained evidence for the presence of DMPEA with GLC, which was not substantiated by TLC. The mass spectrum of such a peak was negative for DMPEA. These findings reemphasize and further support our insistence on the need for more than one parameter in the identification of biological metabolites.

REFERENCES

1 A. J. FRIEDHOFF AND E. VAN WINKLE, *Nature*, 194 (1962) 897.
2 A. J. FRIEDHOFF, in A. J. MANDELL AND M. P. MANDELL (Editors), *Psychochemical Research in Man: Methods, Strategy and Theory*, Academic Press, New York, 1969, p. 161–174.
3 J. A. G. WATT, G. W. ASHCROFT, R. J. DALY AND J. R. SMYTHIES, *Acta Psychiat. Scand.*, 46 (1970) 374.
4 A. A. BOULTON, *Nature*, 231 (1971) 22.
5 M. WIDELITZ AND W. FELDMAN, *Biol. Psychiat.*, 3 (1971) 181.
6 C. R. CREVELING AND J. W. DALY, *Nature*, 216 (1967) 190.
7 J. R. STABENAU, C. R. CREVELING AND J. DALY, *Amer. J. Psychiat.*, 127 (1970) 611.
8 N. NARASIMHACHARI, J. M. PLAUT AND K. Y. LEINER, *Biochem. Med.*, 5 (1971) 304.
9 N. NARASIMHACHARI AND P. VOUROS, *Anal. Biochem.*, in press.