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USE OF 2,6-DINITRO-4-TRIFLUOROMETHYLBENZENESULFONIC ACID AS A NOVEL DERIVATIZING REAGENT FOR THE ANALYSIS OF CATECHOLAMINES, HISTAMINES AND RELATED AMINES BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

PRAVIN S. DOSHI and DAVID J. EDWARDS*

Department of Psychiatry, Western Psychiatric Institute and Clinic, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15261 (U.S.A.)

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SUMMARY

We have found that 2,6-dinitro-4-trifluoromethylbenzenesulfonic acid reacts rapidly and specifically with primary amines at room temperature. We have used this reagent for derivatizing phenylethylamines, including catecholamines, and histamine and 1-methylhistamine. After the reaction, hydroxyl groups were derivatized to form the corresponding trimethylsilyl ethers, and the final derivatives were analyzed by gas chromatography with electron-capture detection. These derivatives are stable, possess excellent gas chromatographic properties and are detected with high sensitivity. We have applied this method to the analysis of histamine and 1-methylhistamine in human urine.

INTRODUCTION

Amines, such as the catecholamines, dopamine and norepinephrine, and histamine, have key physiological roles in organisms from lower invertebrates to man, even though they are present in very low concentration. Other amines, such as phenylethylamine and octopamine, are usually present in even lower concentration, and have been referred to as "trace amines"[1]. Attempts to elucidate the possible physiological function of these compounds have required the use of extremely sensitive and specific analytical techniques. One approach has been to convert the amines into volatile derivatives which can be separated by gas chromatography (GC) and detected by electron-capture detection (ECD). Several derivatives, including trifluoroacetyl (TFA)[2–6], pentafluoropropionyl (PFP)[6–8], heptafluorobutyryl (HFB)[8–10], O-trimethylsilyl (TMS) ether N-pentafluorobenzylimine[11], and dinitrophenyl (DNP)[12,13], derivatives have been found to be suitable for the analysis of amines by GC–ECD.

The DNP derivatives are particularly advantageous for the analysis of amines in tissues, since they are stable to solvent extraction and/or chromatographic proce-

* To whom correspondence should be addressed. Present address: Department of Pharmacology-Physiology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pa. 15261, U.S.A.

dures which can be used to isolate them from the bulk of the tissue components. Day *et al.*[12] used 2,4-dinitrofluorobenzene (DNFB) to dinitrophenylate several aliphatic amines and to separate them by GC. However, since DNFB reacts with hydroxyl groups in addition to amino groups, compounds which contain both functional groups would incorporate two or more DNP groups and would therefore be too non-volatile to be separated by GC. In order to avoid this problem, Edwards and Blau[13] used 2,4-dinitrobenzenesulfonic acid (DNBS), which reacts relatively specifically with primary amino groups, and to subsequently derivatize the hydroxyl groups with a trimethylsilylating reagent. These procedures have been successfully used for the analysis of several phenylethylamines in tissues by GC combined with either ECD[13] or chemical-ionization mass spectrometry (CI-MS)[14].

However, we have found that the DNP derivatives of histamine and related compounds are unsuitable for GC analysis, since they exhibited excessive tailing. In an attempt to avoid this problem, we have studied the chromatographic properties of the corresponding 2,6-dinitro-4-trifluoromethyl (DNT) derivatives. Crosby and Bowers[15] originally used α,α,α-trifluoro-3,5-dinitro-4-chlorotoluene to form the DNT derivatives of amines released by the hydrolysis of carbamate pesticides. These derivatives could be detected by ECD in amounts as low as 50 pg. We also observed[13] that the DNT derivatives were more volatile, exhibited less tailing, and could be detected in lower amounts than could the corresponding DNP-phenylethylamines by GC–ECD. However, the reagent which Crosby and Bowers[15] used suffers from the same lack of specificity as DNFB in that it reacts not only with primary amino groups but also with secondary amino groups and hydroxyl groups. In the present report, we have used 2,6-dinitro-4-trifluoromethylbenzenesulfonic acid (DNTS) to derivatize various amines. This reagent has the advantages of introducing the DNT-group, but, as expected, it had the specificity towards primary amino groups as did DNBS. The DNT derivatives of histamine and 1-methylhistamine, unlike the DNP derivatives, had good GC properties and could be used for the analysis of these compounds as well as of other amines in biological fluids. An additional advantage of DNTS is that it has a much greater reactivity than does DNBS and completely derivatizes primary amines in 10 min at room temperature. As a result, catecholamines, which are destroyed under the conditions needed to react with DNBS[13], may be reacted with DNTS to form stable derivatives.

MATERIALS AND METHODS

Reagents and solvents

All standard compounds were obtained in the highest purity available from commercial sources and used without further purification. The following amines were obtained as the hydrochloride salts: benzylamine and phenylethylamine (K & K Labs., Plainview, N.Y., U.S.A.), phenylethanolamine, DL-normetanephrine and 4-hydroxy-3-methoxyphenylethylamine (3-methoxytyramine) (Regis, Chicago, Ill., U.S.A.), *m*-octopamine (norphenylephrine), *p*-octopamine, *p*-tyramine, phenylpropanolamine (norephedrine) and piperidine (Aldrich, Milwaukee, Wisc., U.S.A.), and *m*-tyramine (Vega-Fox, Tucson, Ariz., U.S.A.). *o*-Tyramine hydrogen bromide was synthesized by Dr B. L. Goodwin (Queen Charlotte's Maternity Hospital, London, Great Britain). L-Epinephrine and the hydrochloride salts of L-norepinephrine, do-

pamine and tryptamine were obtained from Sigma (St. Louis, Mo., U.S.A.). Histamine dihydrochloride, 1-methylhistamine dihydrochloride and 3-methylhistamine dihydrochloride were purchased from Calbiochem (San Diego, Calif., U.S.A.). D-Amphetamine sulfate was purchased from Sigma, L-metaraminol-D-bitartrate from Regis, and serotonin creatinine sulfate monohydrate from Aldrich. The hydrochloride salt of *p*-chlorophenylethylamine was prepared from the free base (Aldrich). Putrescine, cadaverine, 1,7-diaminoheptane, spermidine and spermine were obtained from Aldrich. N,O-bis-(trimethylsilyl)acetamine (BSA) was obtained from Pierce (Rockford, Ill., U.S.A.). Nanograde benzene and ethyl acetate were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). Reagent grade 1-butanol and chloroform were obtained from Fisher (Pittsburgh, Pa., U.S.A.). 4-Chloro-3,5-dinitrobenzotrifluoride was obtained from K & K Labs.

Derivative formation

The sodium salt of DNTS was synthesized by the nucleophilic substitution reaction in which the sulfite ion acting as a nucleophile displaces the halide on the activated aromatic ring of 4-chloro-3,5-dinitrobenzotrifluoride[16].

Stock solutions of each amine were prepared in 0.001 *N* HCl (except 0.1 *N* HCl for epinephrine) at a concentration of 1 mg/ml of the free base. The standard solutions were stored at —20°. A 10-μl amine solution was reacted with 100 μl of 0.11 *M* DNTS (in 50% saturated sodium borate) for 10 min at room temperature. The reaction mixture immediately turned yellow on addition of DNTS. The DNT-amines were extracted twice with 400 μl of benzene (or ethyl acetate in the case of the dopamine, norepinephrine, diamine and polyamine derivatives). After centrifugation 750 *g* for 5 min), the organic layers were transferred to acid-washed, silanized 0.3-ml Reacti-Vials (Pierce) and evaporated to dryness under nitrogen. Hydroxylated amine derivatives were converted into the corresponding TMS-ethers by addition of 10 μl of BSA and 40 μl of benzene (or ethyl acetate) and heating to 60° for 15 min. N-DNT, O-TMS derivatives were evaporated to dryness under nitrogen. Appropriate dilutions of the derivatives were made before injection into the gas chromatograph.

Gas–liquid chromatography

A Model 7400 Packard gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. Glass columns, either 6 ft. or 3 ft. in length and 2 mm I.D. were silanized and respectively packed with 3% OV-1 or 3% SP-2250 on 80–100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S A.).

GC–ECD analysis of histamine and 1-methylhistamine in human urine

The extraction of histamine and 1-methylhistamine from the urine of a human subject was carried out following essentially the method of Fram and Green[17]. A 4-ml urine sample (in duplicate) was placed in a 15-ml conical centrifuge tube and its pH adjusted to 13 with 10 *N* NaOH. The sample was saturated with NaCl and shaken for 15 min with 4 ml of a mixture of 1-butanol and chloroform (3:2, v/v). After centrifugation at 1650 *g* for 15 min, the organic layer was transferred to a 15-ml centrifuge tube containing 6 ml of *n*-heptane and 0.5 ml of 0.2 *N* HCl. The mixture was shaken for 15 min and then centrifuged at 750 *g* for 15 min. After the upper phase had been aspirated off, the aqueous phase was transferred to a 5-ml conical centrifuge

tube and lyophilized. The residue was then derivatized using 1 ml of 0.11 *M* DNTS as described above. The DNT derivatives were extracted four times with 400 μl of benzene. After centrifugation, the combined organic phase was evaporated to dryness. The residue was taken up in benzene and injected into the gas chromatograph.

Gas chromatography–mass spectrometry

The chemical-ionization mass spectra of the derivatives were obtained on a Finnigan Model 3200F combined gas chromatograph–mass spectrometer. The gas chromatograph was equipped with a 3 ft. × 2 mm I.D. silanized U-column packed with 3% SP-2250 on 80–100 mesh on Supelcoport. The GC–CI-MS conditions were: column and injection port temperature, 230°; transfer line temperature, 265°; analyzer, 70°; electron energy, 120 eV; emission current, 0.49 mA; electron multiplier voltage, 1800 V; ion source pressure, 0.9 Torr (methane); methane flow-rate, 12 ml/min.

TABLE I

CHROMATOGRAPHIC RETENTION DATA OF N-DNT, O-TMS AMINES

n.d. = not determined; t_R = retention time (min); Rel. t_R = relative retention time, phenylethylamine = 1.0. No peaks were observed for the secondary amines, piperidine and epinephrine. At 230°, 3-methylhistamine eluted with a t_R = 6.69 min, using 3% SP-2250.

Amine	*Column*					
	*3% OV-1**		*3% SP-2250***			
	250°		*220°*		*230°*	
	t_R	*Rel.* t_R	t_R	*Rel.* t_R	t_R	*Rel.* t_R
Benzylamine	0.89	0.75	1.38	0.70	0.98	0.71
Amphetamine	0.98	0.83	1.18	0.60	0.89	0.64
Phenylethylamine	1.18	1.00	1.97	1.00	1.38	1.00
Phenylpropanolamine	1.38	1.17	1.38	0.70	0.98	0.71
Phenylethanolamine	1.58	1.34	1.97	1.00	1.38	1.00
1-Methylhistamine	1.77	1.50	4.92	2.47	3.35	2.43
o-Tyramine	1.87	1.58	2.76	1.40	1.77	1.28
p-Chlorophenylethylamine	2.17	1.84	4.13	2.10	2.76	2.00
m-Tyramine	2.36	2.00	3.94	2.00	2.56	1.86
Histamine	2.56	2.17	9.15	4.64	5.91	4.28
p-Tyramine	2.56	2.17	4.63	2.35	2.95	2.14
Metaraminol	2.56	2.17	2.46	1.25	1.77	1.28
m-Octopamine	2.76	2.34	3.64	1.85	2.36	1.71
p-Octopamine	3.25	2.75	4.33	2.12	2.76	2.00
3-Methoxytyramine	3.94	3.34	7.28	3.70	4.92	3.57
Dopamine	4.53	3.84	6.99	3.55	4.53	3.28
Normetanephrine	4.53	3.84	6.00	3.05	3.94	2.85
Norepinephrine	5.12	4.33	5.91	3.00	3.74	2.71
Tryptamine	5.71	4.84	n.d.	n.d.	n.d.	n.d.
Serotonin	10.83	9.18	n.d.	n.d.	n.d.	n.d.

* A 6 ft. × 2 mm I.D. glass column was used with nitrogen flow-rate of 32 ml/min. Injection port and detector: 275°.

** A 3 ft. × 2 mm I.D. glass column was used with a nitrogen flow-rate of 44 ml/min. Injection port and detector: 245°.

RESULTS

The GC characteristics of the derivatives of 20 amines on 3% OV-1 and 3% SP-2250 are shown in Table I. Although neither stationary phase is able to separate all of the amine derivatives, those not separated on one phase may be separated on the other. Thus, phenylethylamine and phenylethanolamine have identical retention times on 3% SP-2250, but they are well separated on 3% OV-1. On the other hand, histamine, *p*-tyramine, and metaraminol elute with retention times which are identical on 3% OV-1 but which greatly differ on 3% SP-2250. In cases where the identity of a peak is ambiguous, the use of two different columns may aid in an unequivocal identification. Alternatively, the sample may be injected with and without trimethylsilylation, since DNT-amines which contain hydroxyl groups (*e.g.* phenylethanolamine and *p*-tyramine) will elute from the column only when the hydroxyl groups have been derivatized. Peaks corresponding to non-hydroxylated DNT-amines (*e.g.*, phenylethylamine and histamine) will be unaltered by trimethylsilylation.

Fig. 1 illustrates the separation of phenylethylamine, phenylethanolamine, the *ortho*, *meta*, and *para* isomers of tyramine, dopamine, norepinephrine and tryptamine on 3% OV-1. A mixture containing 1-methylhistamine and histamine was separated on 3% SP-2250 (Fig. 2). The DNT derivatives of diamines, except for putrescine (*e.g.*, cadaverine and 1,7-diaminoheptane) and polyamines (*e.g.*, spermidine and spermine) exhibited poor GC properties and low sensitivity towards the ECD. Putrescine gave a sharp peak which appeared before phenylethylamine on both stationary phases.

We have obtained preliminary results for the determination of histamine and 1-methylhistamine in human urine (Fig. 3). The excretion of these two compounds was estimated to be 55 and 285 μg/24 h, respectively.

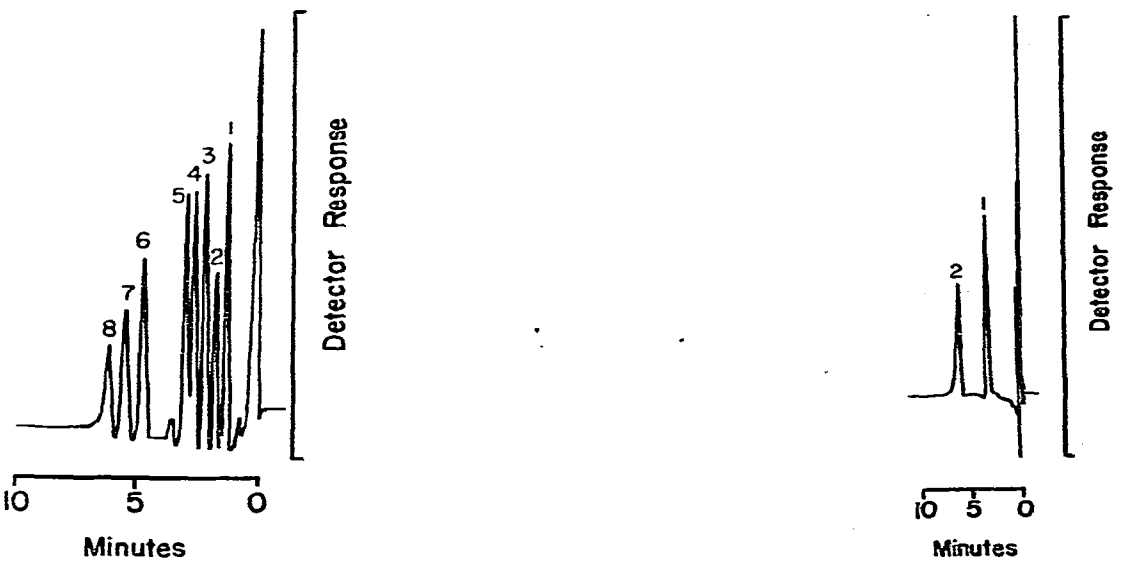


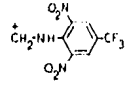
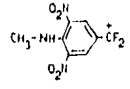
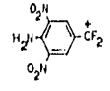
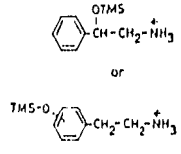
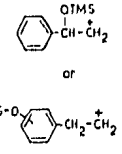
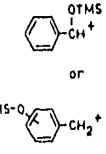
Fig. 1. Gas chromatographic separation of N-DNT, O-TMS derivatives of 0.2 ng each of (1) phenylethylamine, (2) phenylethanolamine, (3) *o*-tyramine, (4) *m*-tyramine, (5) *p*-tyramine, (6) dopamine, (7) norepinephrine, and (8) tryptamine. Conditions: 6 ft. × 2 mm I.D. glass column packed with 3% OV-1 on Supelcoport (80–100 mesh); column, 250°; injection port and detector, 275°; flow-rate, 30 ml/min.

Fig. 2. Gas chromatographic separation of N-DNT derivatives of 1-methylhistamine (5 ng) and histamine (4 ng). Conditions: 3 ft. × 2 mm I.D. glass column packed with 3% SP-2250 on Supelcoport (80–100 mesh); column 230°; injection port and detector, 245°; flow-rate, 44 ml/min. Peaks: (1) 1-methylhistamine, (2) histamine.

TABLE II

PARTIAL METHANE CI MASS SPECTRA OF REPRESENTATIVE N-DNT, O-TMS AMINES

Data are *m/e* (relative abundance). Probable structures of the ion fragments are shown.

*Amine**	MH^+	$MH^+ - HF$	$MH^+ -$ *TMSOH*						
Bz	342 (11)	322 (100)	–	264 (19)	–	–	–	–	–
PE	356 (63)	336 (100)	–	264 (21)	246 (11)	232 (16)	–	–	–
PEOH	444 (3)	424 (23)	354 (100)	264 (6)	246 (22)	232 (13)	210 (6)	193 (18)	179 (66)
m-Tyr	444 (17)	424 (23)	–	264 (5)	246 (3)	232 (12)	210 (23)	193 (100)	179 (27)
p-Tyr	444 (2)	424 (6)	–	264 (4)	246 (2)	232 (8)	210 (25)	193 (100)	179 (33)
p-Oct	–	512 (2)	442 (28)	264 (57)	246 (50)	232 (21)	210 (9)	193 (100)	179 (14)
HA	346 (100)	326 (13)	–	–	–	232 (44)	–	–	–
1-MHA	360 (100)	340 (33)	–	–	–	232 (19)	–	–	–

* Bz, benzylamine; PE, phenylethylamine; PEOH, phenylethanolamine; *m*-Tyr, *m*-tyramine; *p*-Tyr, *p*-tyramine; *p*-Oct, *p*-octopamine; HA, histamine; 1-MHA, 1-methylhistamine.

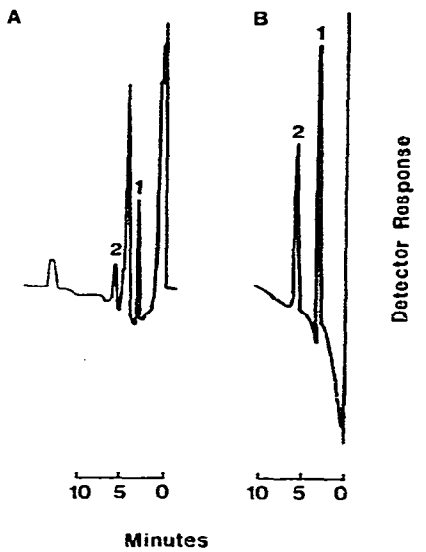


Fig. 3. GC–ECD analysis of histamine and 1-methylhistamine in human urine. (B) Standard: 10 μg of each amine in 4 ml of water and extracted as described in the text. (A) Sample: 4 ml of a urine specimen carried through the extraction procedure. Peaks: (1) 1-methylhistamine, (2) histamine. Conditions were the same as in Fig. 2.

The partial methane CI mass spectra of the N-DNT, O-TMS derivatives of some representative amines are given in Table II. All of the amines except octopamine have an M + 1 peak. However, this represents the base peak in only the spectra of the histamine derivatives. The derivatives of phenylethylamine and benzylamine have base peaks resulting from the loss of HF, while the derivatives of hydroxylated phenylethylamines have base peaks (*m/e* 193) corresponding to the O-TMS phenylethyl fragment.

DISCUSSION

Our results suggest that DNTS is an important new reagent which may effectively replace DNBS in derivatizing amines for their analysis by GC–ECD. The DNT derivatives are more volatile, exhibit improved GC properties and are more sensitive to ECD than are the corresponding DNP-amines. An important advantage of DNTS is that it is extremely reactive compared with DNBS and can form the DNT-amines under very mild conditions (*i.e.* 10 min at room temperature). This is apparently due to the fact that the electron-withdrawing trifluoromethyl group of DNTS facilitates the removal of the sulfonic acid group during the reaction with an amine. Consequently, whereas catecholamines and serotonin are destroyed during their reaction with DNBS[13], they can be easily derivatized with DNTS to form stable derivatives.

In spite of the high reactivity of DNTS, it nevertheless has a specificity towards primary amino groups that is comparable to that of DNBS. None of the secondary amines tested produced any color change in the reaction mixture nor did they result in any peaks in the gas chromatograms. That hydroxyl groups did not react with DNTS is shown by the fact that the DNT derivatives of hydroxylated amines failed to produce a peak unless they were trimethylsilylated. Moreover, the mass spectra (Table II) indicated that all of the derivatized compounds had the structures expected

The utility of DNTS is illustrated by the fact that it forms derivatives with histamine and 1-methylhistamine which have excellent GC properties. Presumably owing to the polar nature of the imidazole ring, previous attempts have largely been unsuccessful in analyzing these amines by GC. For example, Cancalon and Klingman[18] failed to observe any GC peaks for either the TFA or TMS derivatives of histamine. Although Navert[19] reported that the TFA, HFB and TMS derivatives of histidine, histamine and various N-methylhistamines could be analyzed by GC with flame ionization detection, we could not detect either the TFA or the HFB derivatives of histamine and 1-methylhistamine by GC–ECD.

GC–ECD analysis of the DNT derivatives formed from human urine extracts gave values of histamine and 1-methylhistamine excretion of 55 and 285 μg/day, respectively. These values are in good agreement with earlier results of 16–53 μg/day of histamine and 140–480 μg/day of 1-methylhistamine reported by Fram and Green[17], using a spectrophotometric technique.

Our results also suggest that DNTderivatives could be used to determine concentrations of catecholamines and other related amines in biological samples by GC–ECD. In addition, the high molar extinction coefficient of these derivatives makes them amenable to analysis by high performance liquid chromatography. Finally, it should be possible to analyze the DNT derivatives by GC–MS by procedures similar to those used for the analysis of DNP derivatives[14].

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