CHROM 4880

CHROMATOGRAPHY OF BIOGENIC AMINE METABOLITES AND RELATED COMPOUNDS ON LIPOPHILIC SEPHADEX

II. THE CATECHOLAMINES AND THEIR 3-O-METHYLATED DERIVATIVES

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SUMMARY

N-Perfluoroacyl derivatives of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine and 3-methoxytyramine were prepared and chromatographed on Sephadex LH-20 in solvent systems of 1,2-dichloroethane and methanol. Separations of these compounds were achieved. The systems are highly efficient (about 1200 theoretical plates/m) and fast (2–4 h). The application of the method in studies on biogenic amine metabolism and purification prior to gas chromatography–mass spectrometry is described.

INTRODUCTION

Many useful methods exist for the separation of the catecholamines and their O-methylated metabolites[1,2]. Adsorption chromatography on alumina[3,4] or on thin-layer chromatography systems[5,6] have been widely used. Ion-exchange chromatography on strongly acidic columns of crosslinked polystyrene (*e.g.* Dowex 50-X4 and Amberlite CG-120) have been of great value especially in combination with quantitative fluorimetric measurements[7,8]. Weak cationic exchangers have been useful in the isolation of amines from urine. Also systems using cellulose, including paper chromatography, have been employed[9–13].

Recently SJÖVALL *et al.* have introduced the use of lipophilic Sephadex for highly efficient liquid–gel partition chromatography of steroids, fat-soluble vitamins and other compounds of biological interest[14]. In a previous communication we described the separation of acidic and neutral metabolites of several biogenic amines on Sephadex LH-20 (ref. 16). In the present study we report the preparation of derivatives of the catecholamines and the O-methylated basic metabolites suitable for liquid gel chromatography and their separation on Sephadex LH-20. Applications of the method to studies on catecholamine metabolism are described.

EXPERIMENTAL

Materials

Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. This material was refluxed three times for 2 h in methanol, dried and used to pack the columns as described below. Organic solvents were of analytical purity.

The amines were supplied by the following sources: 3-hydroxytyramine·HCl, L-noradrenaline bitartrate monohydrate and L-epinephrine bitartrate from Sigma Chemical Co., St. Louis, Mo., U.S.A., DL-metanephrine·HCl and DL-normetanephrine from Winthrop Laboratories, New York, U.S.A.; 3-methoxy-4-hydroxy-phenyl ethylamine·HCl from Calbiochem AG, Luzern, Switzerland. ^{3}H-labelled dopamine (spec. act., 2.5 Ci/mmole, generally labelled) and L-[7-^{3}H]noradrenaline (2.34 Ci/mmole) were from the Radio-chemical Centre, Amersham, Bucks., Great Britain and 3-methoxy-4-hydroxy-β-[5-^{3}H]phenylethylamine (12.6 Ci/mmole) and DL-[7-^{3}H]normetanephrine (3.8 Ci/mmole) from New England Nuclear, Boston, Mass., U.S.A.

Preparation of derivatives

Amines or their salts were dissolved in a mixture (1:1) of ethyl acetate and perfluoro acid anhydride. After 30 min at room temperature, the solvent and reagent were evaporated by a stream of nitrogen. Methanol (1–2 ml) was added and the sample left at room temperature for 1 h for the trifluoroacetyl derivatives and overnight for the pentafluoropropionyl and heptafluorobutyryl derivatives. After evaporation to dryness, the material was dissolved in a small volume of the solvent mixture of 1,2-dichloroethane–methanol and applied to the Sephadex LH-20 column.

For characterisation by gas–liquid chromatography (GLC) and mass spectrometry (MS), the N-perfluoroacyl derivatives prepared as described above were trimethylsilylated. The sample was dissolved in 10 μl of dry pyridine and 90 μl of N,O-bis-(trimethylsilyl)acetamide[15]. After 1 h at 40° the reagent was removed by a stream of nitrogen. The sample was redissolved in hexane or ethyl acetate before analysis by GLC–MS.

Preparation of the column

Sephadex LH-20 columns were prepared in solvent mixtures of 1,2-dichloroethane and methanol (9:1, 8:2 and 7:3, v/v) as described previously[16]. For a general discussion on the practical and theoretical aspects of liquid–gel chromatography see SJÖVALL *et al.*[14].

Sephadex LH-20 was equilibrated with an excess of the solvent mixture for 2 h, the slurry poured into the glass columns (750 × 10 mm) and allowed to settle under free flow. The bed volume was about 45 ml, the flow rate was about 0.15 ml/cm^{2}/min. Fractions of 0.8–1.0 ml were collected. A disc of porous Teflon (LKB-Produkter, Bromma, Sweden, Cat. No. 4290-04) was placed on top of the bed to protect the gel surface.

The sample was applied in 0.3–0.5 ml of solvent. After application the top of the column was connected to a reservoir which was filled with about 300 ml of the eluting solvents.

The amine derivatives were detected in the effluent by measuring the absorption at 280 nm in a spectrophotometer (Zeiss Model PMQ II). Radioactivity was

measured in a gas flow counter (Frieseke Hoepfner Model FH 51) operated in the proportional range or by liquid scintillation counting in a Packard Model 3375 or 2002 scintillation spectrometer.

The void volume (V_0) was determined to be about 33% of the bed volume by application of 10 mg of polyvinylpyrrolidone and gravimetric determination of its appearance in the eluting medium. β-Carotene (0.2–0.3 mg) was used as an internal standard. This compound which is easily observed by its orange colour appears at $1.3 \times V_0$ in the solvent system 1,2-dichloroethane–methanol (7:3).

Determination of recovery

The N-trifluoroacetamide of ^{3}H-labelled dopamine, 3-methoxytyramine, and the 7-O-methyl-N-trifluoroacetamide of ^{3}H-noradrenaline, normetanephrine, were prepared to determine the recoveries on Sephadex LH-20 columns (4×400 mm). Each compound was put on five replicate columns. The radioactivity was followed in the eluate. The fractions composing the radioactive peak were pooled and evaporated to dryness under nitrogen and counted in a liquid scintillation spectrometer.

The N-trifluoroacetamide of dopamine was also applied on the same type of column in 1 mg and 1 μg amounts. In these experiments the UV absorption at 280 nm and fluorescence at 340 (activation 270) were measured in the eluate.

Preparation of brain extract

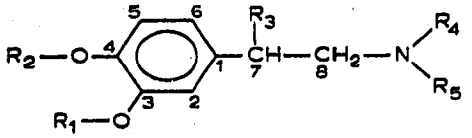
A rabbit was stunned by a blow on the head and bled to death. The brain was rapidly taken out and chilled in Krebs–Henseleit solution containing glucose 0.2%, ascorbic acid 0.02% and disodium EDTA 0.015% (ref. 17). The brain was chopped with scissors into small fragments weighing 10–20 mg each. 2 g (wet weight) of this material were incubated at 37° with 5 ml of Krebs–Henseleit solution and 2 μCi of ^{3}H-dopamine. After 60 min, 2 ml of 2 *M* HCl was added together with carrier amines (0.5 mg) and the sample was homogenised using an Ultraturrax model 45/2 (Jancke & Kunkel AG, G.F.R.) homogeniser. The sample was centrifuged and the supernatant was adjusted to pH 4 and applied to a Dowex 50-X4 column (4×60 mm). The effluent and two-ml washes were used for analysis of acids and neutral metabolites. The amines were eluted with 1 *M* HCl in methanol, and the solution was evaporated to dryness.

RESULTS AND DISCUSSION

Preparation of derivatives of amines for liquid–gel chromatography

The structures of the compounds studied are given in Fig. 1. Free amines are known to give tailing in liquid–liquid and gas–liquid chromatography systems due to hydrogen bonding effects. To protect the amino group, while keeping the hydroxyl groups free to interact during the separation, we used the reactions described by GREER *et al.*[18]. These reactions are outlined in Fig. 2 using normetanephrine as an example.

In this procedure the compounds are first fully perfluoroacylated. The trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl derivatives of dopamine, noradrenaline, adrenaline, metanephrine, normetanephrine and 3-methoxytyramine



R_1 = H, CH_3
R_2 = H, CF_3CO, C_2F_5CO, C_3F_7CO, $(CH_3)_3Si$
R_3 = H, OH, CH_3O, CF_3COO, C_2F_5COO, C_3F_7COO
R_4 = H, CH_3
R_5 = H, CF_3CO, C_2F_5CO, C_3F_7CO

Fig. 1. The structure without the R_1–R_5 substituents is represented as I. The amines used in this paper are metanephrine (I, $R_1 = R_4 = CH_3$; $R_2 = R_5 = H$, $R_3 = OH$), normetanephrine (I, $R_1 = CH_3$; $R_2 = R_4 = R_5 = H$; $R_3 = OH$), 3-methoxytyramine (I, $R_1 = CH_3$; $R_2 = R_3 = R_4 = R_5 = H$), adrenaline (I, $R_1 = R_2 = R_5 = H$; $R_3 = OH$; $R_4 = CH_3$), noradrenaline (I, $R_1 = R_2 = R_4 = R_5 = H$; $R_3 = OH$) and dopamine ($R_1 = R_2 = R_3 = R_4 = R_5 = H$).

gave single peaks on GLC representing the fully acylated derivatives[19,20]. The first step in the reaction sequence outlined in Fig. 2. was fast with all three perfluoro acid anhydrides.

The second step involving methanolysis proceeded more slowly. This was more noticeable for the heptafluorobutyryl derivatives than for the trifluoroacetyl derivatives. Dopamine N-heptafluorobutyramide (I $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = C_3F_7CO$) and 3-methoxytyramine N-pentafluoropropionamide (I $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, $R_5 = C_2F_5CO$) were formed slower than the corresponding derivatives of the β-hydroxylated amines.

In methanol solution the phenolic ester group is preferentially methanolysed whereas the amide group is left intact. Compounds with a hydroxyl group α to the ring (*e.g.* noradrenaline and adrenaline) undergo a nucleophilic substitution reaction to give the methyl ether.

The nucleophilic displacement reaction could be of first (S_N1) or second (S_N2) order. Starting with an optically active compound the S_N1 mechanism gives a racemate, while the S_N2 reaction would lead to inversion of the configuration at the

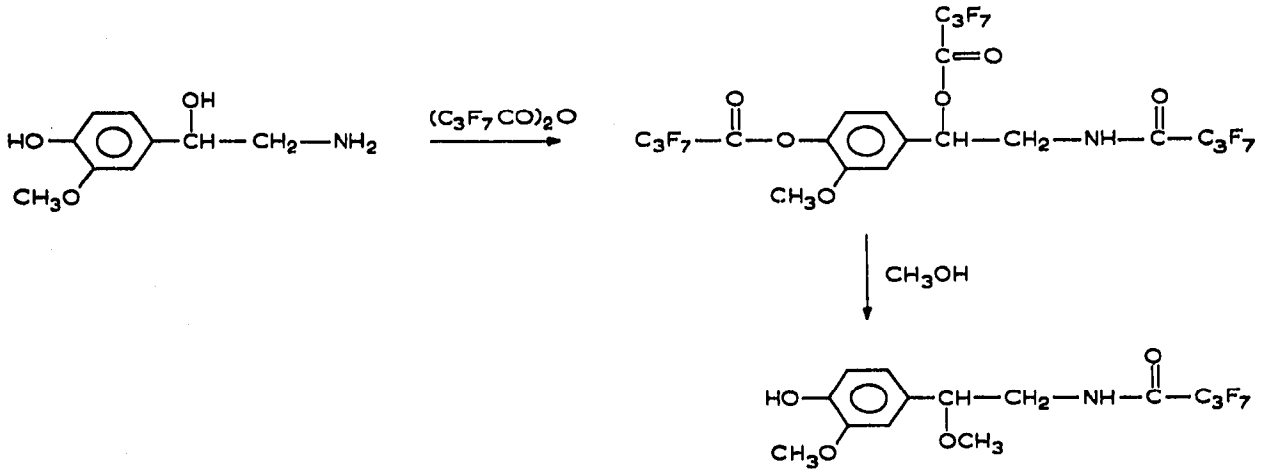
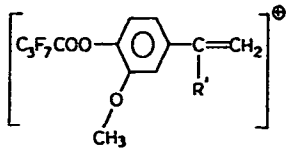
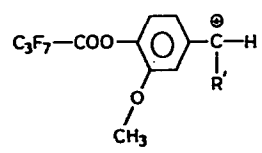
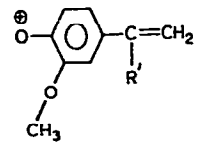
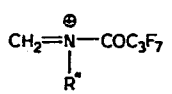
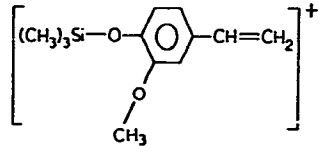
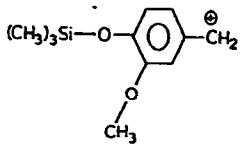
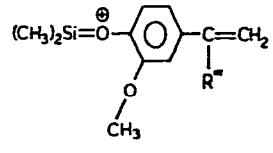
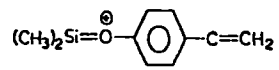


Fig. 2. Normetanephrine is O,O,N-acylated with heptafluorobutyric anhydride (a fast reaction). This derivative dissolved in methanol forms slowly (overnight) a compound with the structure I, $R_1 = CH_3$; $R_2 = R_4 = H$; $R_3 = CH_3O$; $R_5 = C_3F_7CO$.

TABLE I

MASS SPECTROMETRIC DATA FOR DERIVATIVES OF 3-METHOXYTYRAMINE (3-MT), NORMETANEPHRINE (NM) AND METANEPHRINE (M)
M^+ = molecular ion.

4-O,N-Diheptafluorobutyryl derivatives	M^+				
3-MT; R′ = H, R″ = H	559 (12%)	346 (100%)	333 (12%)	149 (29%)	226 (3%)
NM; R′ = C_3F_7COO, R″ = H	771 (7%)	558 (100%)	545 (3%)	361 (14%)	226 (11%)
M; R′ = C_3F_7COO, R″ = CH_3	785 (1%)	558 (25%)	545 (3%)	361 (2%)	240 (100%)

7-O-Methyl-4-O,N-diheptafluorobutyryl derivatives	$M^{\oplus}$	$M^{\oplus}$-15				
3-MT	435 (45%)	420 (17%)	222 (50%)	209 (100%)	207 (13%)	
NM; R‴ = OCH_3	465 (14%)	450 (18%)			237 (100%)	206 (53%)
M; R‴ = OCH_3	479 (12%)	464 (50%)			237 (100%)	206 (100%)

asymmetric centre, giving an optically active product. To investigate this mechanism L-noradrenaline was heptafluorobutyrylated and then methanolysed. The product was found to have opposite sign of the optical rotation as compared to the parent compound. Thus the reaction seems to be of the S_N2 type.

The UV spectrum of the O-methyl-N-heptafluorobutyryl derivative of normetanephrine showed a $\lambda_{max.}$ at 278 nm in methanol. In 0.01 *M* NaOH in methanol the absorption maximum underwent a bathochromic shift to a $\lambda_{max.}$ of 288 nm, indicating the presence of a phenolic hydroxyl group.

The structures of the derivatives were confirmed by their mass spectra. Table I presents the *m/e* values and probable structures of the main fragments of O,N-heptafluorobutyryl and 7-O-methyl-O,N-heptafluorobutyryl derivatives of 3-methoxytyramine, normetanephrine and metanephrine. The fragmentation α to the nitrogen atom is more frequent than the β cleavage. In the trimethylsilyl derivatives the molecular ion with a loss of a methyl group (M^+-15) is also prominent.

Separation of amine derivatives on Sephadex LH-20

The 7-O-methyl-N-trifluoroacetyl and 7-O-methyl-N-heptafluorobutyryl derivatives of metanephrine, normetanephrine and noradrenaline were prepared. The N-trifluoroacetyl and the N-heptafluorobytyramides of dopamine and 3-methoxytyramine were produced. The 7-O-methyl-N-trifluoroacetyl derivative of adrenaline was readily prepared while the preparation of the 7-O-methyl-N-heptafluorobutyryl derivative unexpectedly was not successful. The compounds were chromatographed on Sephadex LH-20 in solvent systems of 1,2-dichloroethane–methanol (8:1 or 7:3). The elution volumes relative to β-carotene are given in Table II.

The above 7-O-methyl-N-trifluoroacetyl derivatives of metanephrine, normetanephrine and 3-methoxytyramine separated well in systems of 1,2-dichloroethane–methanol (9:1 and 8:2). This is illustrated in Fig. 3. The 7-O-methyl-N-trifluoroacetyl derivatives of the corresponding catecholamines (adrenaline, noradrenaline, dopamine) required the more polar solvent mixture (7:3) for elution within a reasonable volume.

TABLE II

SEPARATION OF CATECHOLAMINE DERIVATIVES ON A SEPHADEX LH-20 COLUMN

Parent compound	*Structure (I)*[a]					*$ClCH_2$-CH_2Cl–CH_3OH (8:2)*	*$ClCH_2$-CH_2Cl–CH_3OH (7:3)*
	R_1	R_2	R_3	R_4	R_5		
Metanephrine	CH_3	H	CH_3O	CH_3	CF_3CO	1.55	1.44
Normetanephrine	CH_3	H	CH_3O	H	CF_3CO	1.90	1.74
3-Methoxytyramine	CH_3	H	H	H	CF_3CO	2.20	1.93
Adrenaline	H	H	CH_3O	CH_3	CF_3CO	—	1.92
Noradrenaline	H	H	CH_3O	H	CF_3CO	—	2.35
Dopamine	H	H	H	H	CF_3CO	—	2.65
Metanephrine	CH_3	H	CH_3O	CH_3	C_3F_7CO	1.53	1.40
Normetanephrine	CH_3	H	CH_3O	H	C_3F_7CO	1.77	1.65
3-Methoxytyramine	CH_3	H	H	H	C_3F_7CO	2.10	1.88
Adrenaline	H	H	CH_3O	CH_3	C_3F_7CO	—	—
Noradrenaline	H	H	CH_3O	H	C_3F_7CO	2.85	2.18
Dopamine	H	H	CH_3	H	C_3F_7CO	3.40	2.61

[a] See Fig. 1.

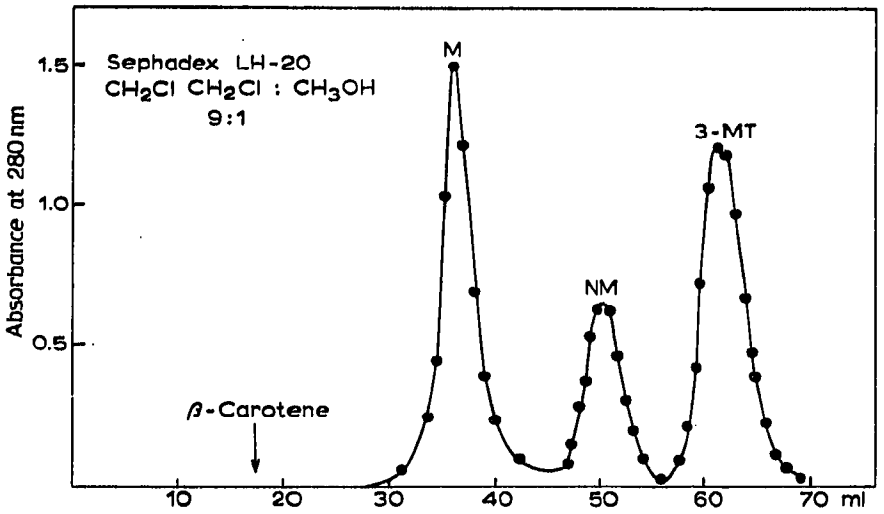


Fig. 3. Separation of 7-O-methyl-N-trifluoroacetyl derivative of metanephrine (= M), normetanephrine (= NM) and 3-methoxytyramine (= 3MT).

In this solvent system the catecholamines were separated from each other (Fig. 4) and also from the corresponding O-methylated metabolites, except for the 3-methoxytyramine and adrenaline derivatives which were not resolved under these conditions.

The separation between the N-trifluoroacetyl derivatives of the amines was better than that of the corresponding N-heptafluorobutyryl compounds (Table II). The peak shapes for the metanephrine, normetanephrine and 3-methoxytyramine derivatives were symmetrical, indicating that adsorption plays a minor role in the

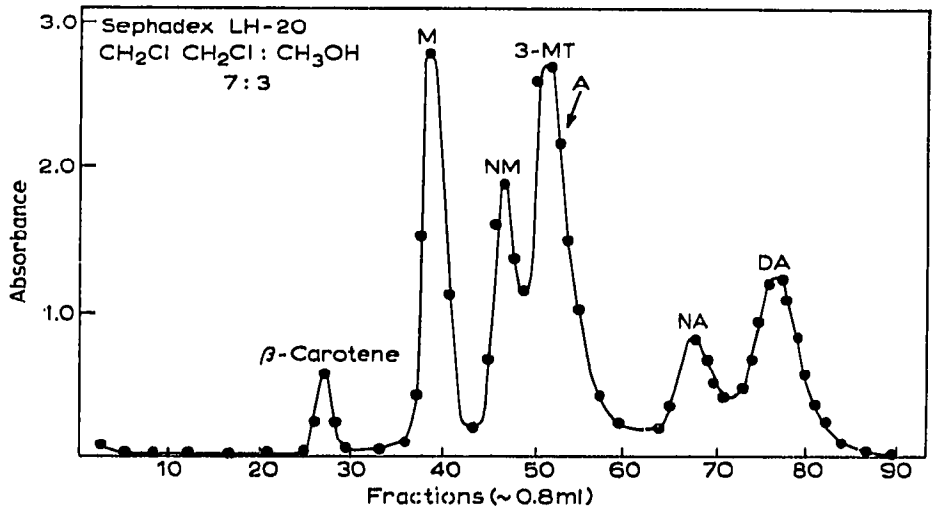


Fig. 4. Separation of metanephrine-O-methyl-N-trifluoroacetamide (M) (I, $R_1 = R_4 = CH_3$; $R_2 = H$; $R_3 = CH_3O$; $R_5 = CF_3CO$), normetanephrine 7-O-methyl-N-trifluoroacetamide (NM) (I, $R_1 = CH_3$; $R_2 = R_4 = H$; $R_3 = CH_3O$; $R_5 = CF_3CO$), 3 MT = 3-methoxytyramine-N-trifluoroacetamide (3-MT) (I, $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$; $R_5 = CF_3CO$) not resolved from A = adrenaline 7-O-methyl-N-trifluoroacetamide (I, $R_1 = R_2$ H; $R_3 = CH_3O$; $R_4 = CH_3$; $R_5 = CF_3CO$), NA = noradrenaline 7-O-methyl-N-trifluoroacetamide (NA) (I, $R_1 = R_2 = R_4 = H$; $R_3 = CH_3O$; $R_5 = CF_3CO$), DA = dopamine N-trifluoroacetamide (DA) (I, $R_1 = R_2 = R_3 = R_4 = H$; $R_5 = CF_3CO$).

separation. The height equivalent to a theoretical plate (HETP) in 1,2-dichloroethane–methanol (7:3) was for the 7-O-methyl-N-trifluoroacetyl derivative of metanephrine (I $R_1 = R_4 = CH_3$, $R_2 = H$, $R_3 = CH_3O$, $R_5 = CF_3CO$) 0.72 mm, for normetanephrine (I $R_1 = CH_3$, $R_2 = R_4 = H$, $R_3 = CH_3O$, $R_5 = CF_3CO$) 0.79 mm and for the N-trifluoroacetyl derivative of 3-methoxytyramine (I $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, $R_5 = CF_3CO$) 0.61 mm. The derivatives of the catecholamines adrenaline, noradrenaline and dopamine gave peaks which were broader and sometimes showed a tendency to tailing (Fig. 4).

Recovery

The recoveries in the submicrogram range of the derivatives of ^3H-labelled 3-methoxytyramine, noradrenaline and normetanephrine were 94.0 ± 15% (SD), 89.8 ± 2.4% (SD) and 105.5 ± 6% (SD), respectively.

The recovery of 1 mg of dopamine was 92.0 ± 5.6% (SD) (four columns). In the microgram range the recovery of dopamine was of the same order, while in the submicrogram range the yield was lower and more variable.

Application in studies on catecholamine metabolism

Chopped rabbit brain was incubated with ^3H-dopamine as described under EXPERIMENTAL. Carrier normetanephrine, 3-methoxytyramine, dopamine and

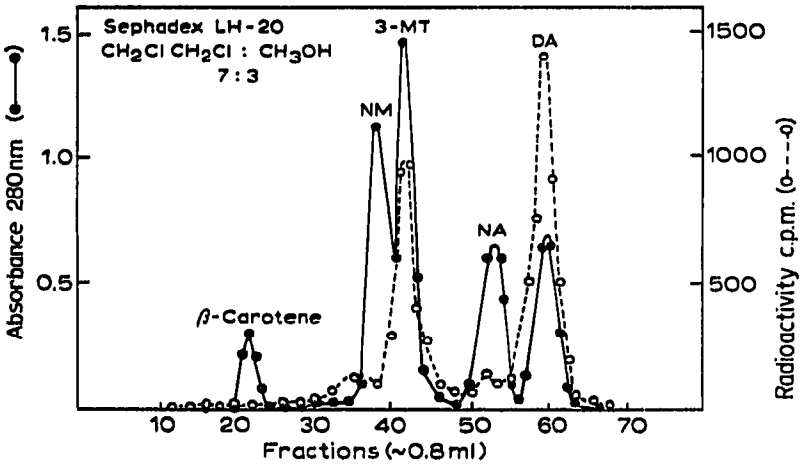


Fig. 5. Separation of brain basic metabolites of [^3H]dopamine after incubation with chopped rabbit brain. The solid line represents absorption at 280 nm due to 7-O-methyl-trifluoroacetyl amides of carrier metabolites and the broken line represents the corresponding radioactive derivatives.

noradrenaline were added and the basic metabolites of dopamine isolated. After preparation of trifluoroacetyl derivatives and methanolysis, the material was subjected to chromatography on Sephadex LH-20 in 1,2-dichloroethane–methanol (7:3). The chromatography is shown in Fig. 5. Two major radioactive peaks appear corresponding to the retention volume of the 3-methoxytyramine and dopamine derivative, respectively. A smaller peak at the position of the noradrenaline derivative was also observed.

Use of derivatives in gas chromatography with electron capture detection

GREER *et al.*[18] used trifluoroacetic anhydride to acylate metanephrine, normetanephrine and 3-methoxytyramine. According to HORNING *et al.*[21], the trimethylsilyl ethers of the N-heptafluorobutyryl derivatives of catecholamines were proposed to have high electron capture responses.

In our experience[22] and according to CLARKE *et al.*[19] the heptafluorobutyryl amides of phenylalkyl amines give considerably lower EC-responses than the corresponding esters. To investigate this matter more closely we prepared the O-trimethylsilyl-N-heptafluorobutyryl and O,N-heptafluorobutyryl derivatives of 7-O-methylmetanephrine and 3-methoxytyramine and compared the EC-responses of these derivatives. The results are shown in Table III. It appeared that the O-trimethylsilyl-N-heptafluorobutyryl derivatives gave 5% or less of the EC-response of the O,N-heptafluorobutyryl compounds. It seems therefore that O-trimethylsilyl-N-heptafluorobutyryl derivatives have only limited use in conjunction with EC-detection. Our results therefore do not support the proposal made by HORNING *et al.*[21] that these derivatives would be suitable for use with EC-detectors. The pentafluoropropionyl or heptafluorobutyryl esters seem to be the best derivatives so far [22].

TABLE III

ELECTRON CAPTURE RESPONSES FOR N-HEPTAFLUOROBUTYRYL AND 7-O,N-DIHEPTAFLUOROBUTYRYL DERIVATIVES OF O-METHYLATED CATECHOLAMINES

Parent compound	*Derivative (I)*[a]					*Electron capture response*[b] *(dieldrin = 100)*
	R_1	R_2	R_3	R_4	R_5	
Metanephrine	CH_3	$(CH_3)_3Si$	CH_3O	CH_3	C_3F_7CO	2.9
Metanephrine	CH_3	C_3F_7CO	CH_3O	CH_3	C_3F_7CO	55
3-Methoxytyramine	CH_3	$(CH_3)_3Si$	H	H	C_3F_7CO	0.7
3-Methoxytyramine	CH_3	C_3F_7CO	H	H	C_3F_7CO	65

[a] See Fig. 1.
[b] Area measurement by triangulation.

The mechanisms of separation in liquid–gel chromatography on lipophilic Sephadex have been discussed in detail by SJÖVALL *et al.*[14]. Depending on the solvent system and the dextran derivative used, "straight-phase" or "reversed-phase" partition effects are achieved in addition to the molecular-sieve mechanism.

Both straight-phase liquid–gel partition and gel filtration mechanisms seem to operate in the separations described here. The compounds with the relatively polar catechol structure were as a group retained as compared to their O-methylated analogues. Within each of these groups there are differences in polarity. The tertiary amides are less polar than the secondary. There are also small differences in molecular size: adrenaline > noradrenaline > dopamine and metanephrine > normetanephrine > 3-methoxytyramine. Both these factors seem to be important in the separation of these closely related compounds. Accordingly, the order of elution is adrenaline, noradrenaline and dopamine derivatives in the catecholic group and metanephrine, normetanephrine and 3-methoxytyramine derivatives among the 3-O-methylated compounds (Table II, Fig. 3). A gel filtration effect is also supported by the finding

that the N-heptafluorobutyryl derivatives were much less well separated from each other as compared to the N-trifluoroacetyl compounds (Table II).

The chromatography system was developed with the aim to produce an efficient and convenient purification step prior to quantitative analysis by gas chromatography. Since the N-heptafluorobutyryl derivatives seem to have no advantages in terms of high EC-response, it would be preferable to use the N-trifluoroacetyl derivatives as these compounds separate better on the Sephadex LH-20 column. To obtain a derivative suitable for analysis with EC-detector, the free phenolic group may be heptafluorobutyrylated (Table III).

In conclusion a simple two-step reaction sequence is used to prepare derivatives of the catecholamines and their O-methylated metabolites for column chromatography on Sephadex LH-20. The separation of these closely related compounds was achieved, probably by a combination of a liquid–gel partition and a molecular-sieve mechanism in an organic solvent mixture.

These chromatography systems incorporate many attractive features such as high efficiency, speed and high recovery[14] in the submicrogram range. The columns can be used repeatedly without repacking. The separated compounds appear in a small volume of volatile organic solvent that is easily removed. This and the fact that the amine group already is protected by an acyl group should make the procedure suitable for use in connection with GLC and MS.

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