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CHROMATOGRAPHY OF BIOGENIC AMINE METABOLITES AND RELATED COMPOUNDS ON LIPOPHILIC SEPHADEX

I. THE PHENOLIC ACIDS AND ALCOHOLS

ERIK ÄNGGÅRD, BIRGITTA SJÖQUIST AND ROLF SJÖSTRÖM
Department of Pharmacology, Karolinska Institutet, 104 01 Stockholm 60, and Psychiatric Research Centre, Ulleråkers Hospital, 750 17 Uppsala (Sweden)
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SUMMARY

Liquid–gel chromatography on Sephadex LH-20 in solvent systems of 1,2-dichloroethane–methanol was used to separate the aromatic acids and neutral compounds derived from catechol- and indole amines. The system is characterised by high efficiency (1600–2500 theoretical plates/m), speed (2–6 h) and high recovery in the submicrogram range. The method has been found useful in *in vivo* and *in vitro* studies on the metabolism of monoamines, as well as for isolation of metabolites prior to gas chromatographic–mass spectrometric analysis.

INTRODUCTION

Recently, new derivatives of Sephadex have extended the use of gel chromatography for lipophilic substances[1]. The methyl ethers and 2-hydroxypropyl ethers of Sephadex have been investigated by SJÖVALL *et al.*[1], who found them highly efficient for the separation of closely related substances of biological origin, such as steroids, peptides and vitamins. The separations involve liquid–gel partition and molecular sieving whereas adsorption in most cases seems to be negligible. It occurred to us that similar systems might be useful in the analysis of aromatic acids and alcohols. Some of these compounds are metabolites of the biologically important catechol- and indole amines. The present report shows that excellent separation of these substances can be achieved using Sephadex LH-20 in solvent mixtures of methanol and 1,2-dichloroethane.

EXPERIMENTAL

Materials

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. DL-3-Methoxy-4-hydroxymandelic acid (VMA); DL-3,4-dihydroxymandelic acid (DOMA); 3-methoxy-4-hydroxyphenylacetic acid (HVA); 3,4-dihydroxyphenyl-

acetic acid (DOPAC); 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG) and 3,4-dihydroxyphenyl-ethylene glycol (DOPEG) were purchased from Calbiochem AG, Luzern, Switzerland. 3,4-Dihydroxyphenylethyl alcohol (DOPET) and 3-methoxy-4-hydroxyphenylethyl alcohol (MOPET) were from Kistner Lab.tjänst, 40224 Göteborg 5, Sweden.

5-Hydroxy-3-indoleacetic acid (5-HIAA) was supplied as the dicyclohexyl ammonium salt from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The 3-indoleacetic acid was from E. Merck AG, Darmstadt, G.F.R. The 5-hydroxytryptophol was a gift from Hoffman-LaRoche AG, Basel, Switzerland. All solvents were of reagent purity as supplied by E. Merck AG. ^{3}H-labelled DL-noradrenaline (spec. act. 2.1 Ci/mmole) was from the Radiochemical Centre, Amersham, Bucks., Great Britain). The radiopurity was checked by paper chromatography and was found to be better than 95 %. The tritiated dopamine had a specific activity of 2.5 Ci/mmole. The radiopurity of this compound was checked by paper chromatography and found to be within specification (>99 %).

Preparation of the column

Sephadex was allowed to swell in methanol and was then refluxed three times for several hours with a large excess of methanol. After drying a suitable quantity was equilibrated with the solvents (1,2-dichloroethane–methanol, 9:1, 8:2 or 7:3) for a minimum of 2 h in a filtering flask. The gel was de-aerated by brief application of vacuum (*ca.* 15 mm Hg) and then immediately used for packing the chromatography column. A glass container serving as a reservoir for the solvent was fitted to the top of the column (750 × 10 mm), while the bottom of the column had a narrow stopcock. Care was taken to keep dead space to a minimum.

The gel was poured into the column on top of a tightly packed wad of glass wool and allowed to settle under gravity. A small circular plug of porous teflon was placed on the top of the gel surface to prevent disturbances to the fragile gel surface. Since the gel had a lower specific gravity than some of the solvents the plug also kept the gel from floating. The columns were conditioned by running the solvent mixture through the column for 6–12 h. The sample was applied in the smallest possible volume of the solvent mixture and allowed to sink into the gel. The sample was rinsed from the teflon disc by several 0.5 ml volumes of solvent mixture. The column and top reservoir were filled with about 300 ml of solvent mixture. The total bed volume of the columns was about 45 ml. The flow rate under the stated conditions was about 0.15 ml/cm^2/min. Fractions of about 0.8 ml were collected.

Aromatic amine metabolites were detected in the effluent by measuring the absorption at 280 nm in a Zeiss spectrophotometer Model PMQ II. Radioactivity was measured in a gas flow counter (Frieseke Hoepfner Model FH 51) after drying an aliquot on aluminium planchets. The void volume (V_0) was found to be about 33 % of the bed volume. This was determined by application of 10 mg of polyvinylpyrrolidone and gravimetric determination of its appearance in the eluting medium. β-Carotene, which appears at 1.3 × V_0 in the solvent system 1,2-dichloroethane–methanol (7:3) was used as an internal standard.

Preparation of derivatives

Aromatic acids or acidic extracts were esterified by the use of freshly prepared

diazomethane. The sample was dissolved in a small amount of ethyl acetate and sufficient methanol to act as a catalyst added. After addition of an excess of diazomethane in cold ether (−20°), the reagent was immediately removed by a stream of nitrogen. Esterification under these conditions is instantaneous. Longer exposure to diazomethane, when methanol is used as solvent, leads to the methylation of phenolic groups[2–4]. This tendency is especially noticeable with compounds having a catechol structure. The structures of all derivatives were verified by gas chromatography–mass spectrometry[4].

Preparation of urine and tissue extracts

An amount of 10 ml of rat urine was subjected to enzymatic hydrolysis for 16 h at room temperature, at pH 5.5, using 0.05 volumes of Glusulase (Endo Lab., New York, U.S.A.). The urine was acidified to pH 2–3 and extracted 5 times with 25 ml of ethyl acetate. The combined organic phases were washed repeatedly with small volumes of distilled water and then evaporated *in vacuo* to dryness. The dried extract, containing the acidic and neutral amine metabolites, was treated with diazomethane as described above and subjected to chromatography on a Sephadex LH-20 column.

Rabbits were used for the study of the brain metabolism of ^{3}H-dopamine. The animals were stunned by a blow on the neck and exsanguinated from the large vessels of the neck. The whole brain was taken out, immersed in ice cold Krebs Henseleit solution containing glucose 0.2 %, ascorbic acid 0.02 % and disodium EDTA 0.015 % (ref. 5). The whole brain was rapidly chopped with scissors into smaller fragments weighing about 10 mg each. 2 g (wet weight) of chopped rabbit brain was combined with 5 ml of the Krebs Henseleit solution and 2 μCi of ^{3}H-dopamine. Incubation was carried out at 37° with shaking. After 60 min, 2 ml of 2 *M* HCl was added together with carrier metabolites (about 0.5 mg each) and the sample was homogenised using an Ultraturrax model 45/2 homogeniser. The sample was centrifuged and the supernatant was adjusted to pH 4 with 1 *M* NaOH and applied on a short Dowex 50 4X (H) 200–400 mesh column. The effluent and two 10 ml washes were combined; after adjusting the pH to 2 with 1 *M* HCl, it was extracted twice with 50 ml of ethyl acetate and twice with 50 ml of ether. The combined organic phases were washed with small volumes of water until neutral and evaporated to dryness *in vacuo*. The residue was dissolved in methanol and transferred to a small test tube and reduced to dryness under nitrogen. After conversion of the acid to the methyl esters by diazomethane as described above, the sample was dissolved 0.15 ml methanol and 0.35 ml of 1,2-dichloroethane and applied on the Sephadex LH-20 column.

Preparation of labelled DOPAC, HVA and VMA

^{3}H-labelled DOPAC was prepared by incubating 230 μCi^{3}H-dopamine with liver mitocondria in 40 ml 0.15 *M* potassium phosphate buffer, pH 7. This solution also contained 0.5 m*M* ascorbic acid, 5 m*M* pyrogallol, 0.05 m*M* ethanethiol and 0.5 m*M* nicotinamide-adenine dinucleotide (NAD^+). The incubation (37°) was interrupted after 60 min by adding 360 ml of chloroform–ethanol (1:1). The protein precipitate was removed by filtration. The filtrate was evaporated *in vacuo*. A small amount of water, pH 3, was added and the mixture was extracted with ethyl acetate three times. The combined ethyl acetate extracts were washed repeatedly with small amounts of water and evaporated *in vacuo*. ^{3}H-labelled DOPAC was isolated by preparative

thin-layer chromatography (toluene–ethyl acetate–water (70:36:1.5). Yield 20 μCi.

The preparation of ^{3}H-labelled HVA was performed in the same manner as for DOPAC, but in this case 2 mg S-adenosyl-L-methione chloride was added and the supernatant from centrifugation at 15 000 g was used as solvent.

For preparation of ^{3}H-VMA, [^{3}H]noradrenaline was incubated with chopped pieces of whole rabbit brain essentially as described above for preparation of ^{3}H-HVA and DOPAC.

Recovery

To calculate the recoveries in the submicrogram range the methyl esters of the ^{3}H-labelled acids, prepared as described above, were put on Sephadex LH-20 columns (4 × 400 mm). Each compound was put on four replicate columns. The radioactivity in the eluate was followed. The fractions in the peak corresponding to the methyl ester of the radioactive compound were pooled and evaporated to dryness unter nitrogen and counted in a liquid scintillation spectrometer. The recovery of 3–5 μg of 5-HIAA-Me was determined on columns (4 × 400 mm) of LH-20 using fluorometry (Aminco-Bowman Spectrofluorometer).

RESULTS

Metabolites of catecholamines

Three solvent systems, using different concentrations (10, 20 and 30 vol. %) of methanol in 1,2-dichloroethane, were used to separate the amine metabolites. The elution volumes relative to β-carotene are given in Table I. The shape of the peaks for these compounds was narrow and symmetrical indicating high efficiency of the separations. A chromatogram showing the separation of the methyl esters of HVA, VMA, DOPAC and DOMA as well as the glycols MOPEG and DOPEG is shown in Fig. 1. The number of theoretical plates/m was 1700 for HVA-Me, 2100 for VMA-Me and 2300 for MOPEG.

It proved difficult to analyse these compounds as the free acids because the

TABLE I

ELUTION VOLUMES RELATIVE TO β-CAROTENE

Compound[a]	*1,2-Dichloroethane–methanol*		
	9:1	*8:2*	*7:3*
3-Methoxy-4-hydroxyphenylacetic acid (HVA)	1.7	1.7	1.7
3,4-Dihydroxyphenylacetic acid (DOPAC)	—	2.5	2.3
3-Methoxy-4-hydroxymandelic acid (VMA)	2.6	2.1	2.1
3,4-Dihydroxymandelic acid (DOMA)	—	3.5	2.9
3-Methoxy-4-hydroxyphenylethylene glycol (MOPEG)	5.8	3.3	2.7
3,4-Dihydroxyphenylethylene glycol (DOPEG)	—	5.4	4.5
3-Methoxy-4-hydroxyphenylethanol (MOPET)	—	2.4	2.1
3,4-Dihydroxyphenylethanol (DOPET)	—	4.7	3.6
5-Hydroxy-3-indole acetic acid (5-HIAA)	—	—	2.3
3-Indoleacetic acid (IAA)	—	—	1.8
5-Hydroxytryptophol	—	—	3.3

[a] All acids were run as the methyl esters.

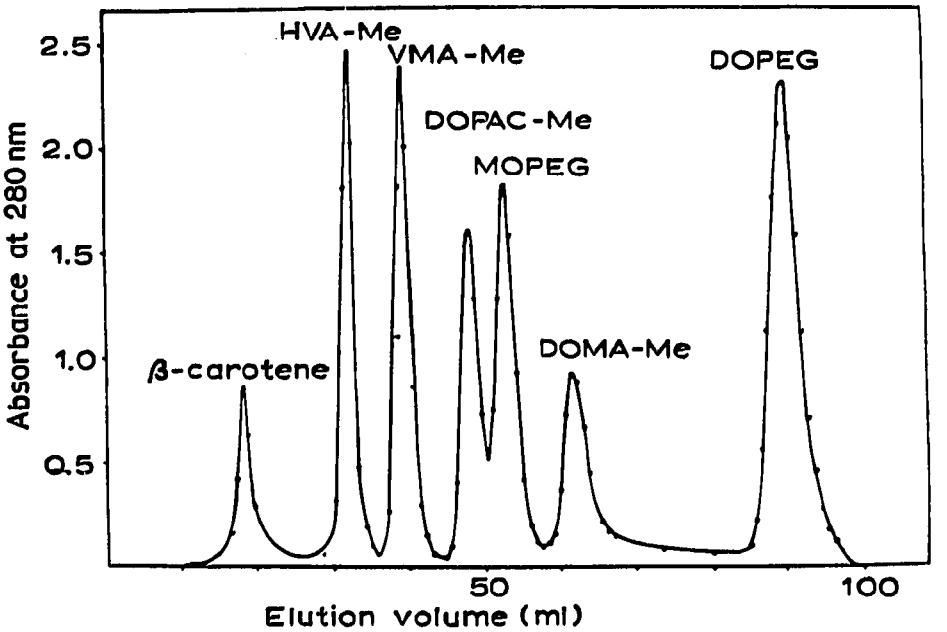

Fig. 1. Separation of 3-methoxy-4-hydroxyphenylacetic acid methyl ester (HVA-Me); 3-methoxy-4-hydroxymandelic acid methyl ester (VMA-Me); 3,4-dihydroxymandelic acid methyl ester (DOMA-Me); 3,4-dihydroxyphenylacetic acid methyl ester (DOPAC-Me); 3-methoxy-4-hydroxy phenylethylene glycol (MOPEG); and 3,4-dihydroxyphenylethylene glycol (DOPEG) on Sephadex LH-20. Solvent systems: 1,2-dichloroethane–methanol (7:3).

peaks were broad and showed pronounced tailing. This was not altered by the addition of 1 % formic acid to the elution medium.

For various analytical purposes it could be desirable to hydrolyse the methyl esters to the free acids. Acidic hydrolysis (2 *M* HCl, 60° for 2 h) gave quantitative conversion of the methyl esters of HVA, DOPAC, VMA, but not of DOMA, to the acids. The glycols are probably dehydrated under these conditions. Mild alkaline hydrolysis (0.1 *M* NaOH in EtOH, 60° for 2 h) was efficient for HVA-Me but the catecholic and β-hydroxylated structures were labile in alkali, as expected.

The recoveries for the methyl esters of labelled HVA and VMA from four determinations were 99.1 ± 0.6 % (S.D.) and, 97.5 ± 5.8 % (S.D.), respectively, while the ^{3}H-DOPAC-methyl ester in these small amounts gave lower and more variable figures. The recovery of 1 mg of DOPAC methyl ester was found to be 92 %, while 5 HIAA-Me in the microgram range from six determinations gave 97.0 ± 6.2 % (S.D.).

The practical use of the chromatography system was demonstrated by two experiments. A rat was given an intravenous injection of ^{3}H-labelled noradrenaline and the urinary acids and alcohols were extracted as described under EXPERIMENTAL and chromatographed on Sephadex LH-20 in 1,2-dichloroethane–methanol (8:2), after the extract had been treated with diazomethane. The chromatogram is shown in Fig. 2. Two major radioactive peaks corresponding to VMA-Me and MOPEG, respectively, were observed. A great deal of material absorbing at 280 nm was also present in various parts of the chromatogram indicating other acids and neutral compounds present in the urine.

In another experiment, ^{3}H-labelled dopamine was incubated with chopped pieces of whole rabbit brain. After termination of the incubation, carrier metabolites

were added and the acidic and neutral metabolites were extracted as described under EXPERIMENTAL. After treatment of the extract with diazomethane as described above, the extracted material was chromatographed on Sephadex LH-20 in 1,2-dichloroethane–methanol (7:3). The chromatogram is shown in Fig. 3. Two peaks of radioactivity are visible, the major one coinciding with the carrier DOPAC-Me and the minor peak with the carrier HVA-Me.

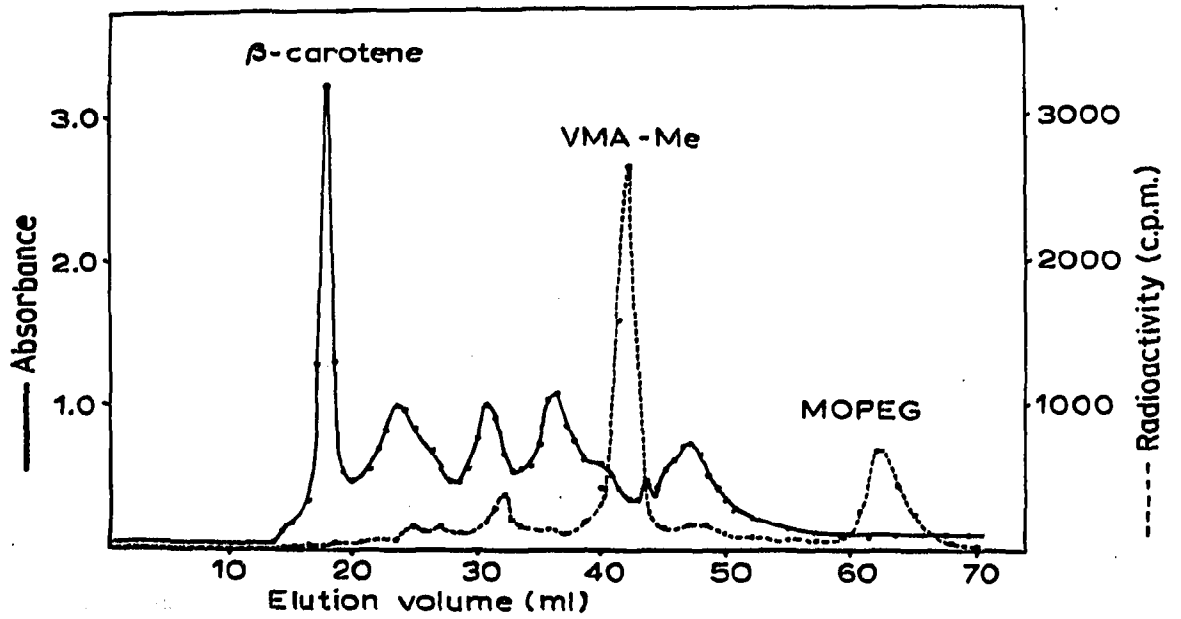

Fig. 2. Chromatography of urinary acidic and neutral metabolites from a rat given [^{3}H]noradrenaline. The solid line represents absorption at 280 nm and the broken line represents radioactivity. Sephadex LH-20, solvent system 1,2-dichloroethane–methanol (4:1).

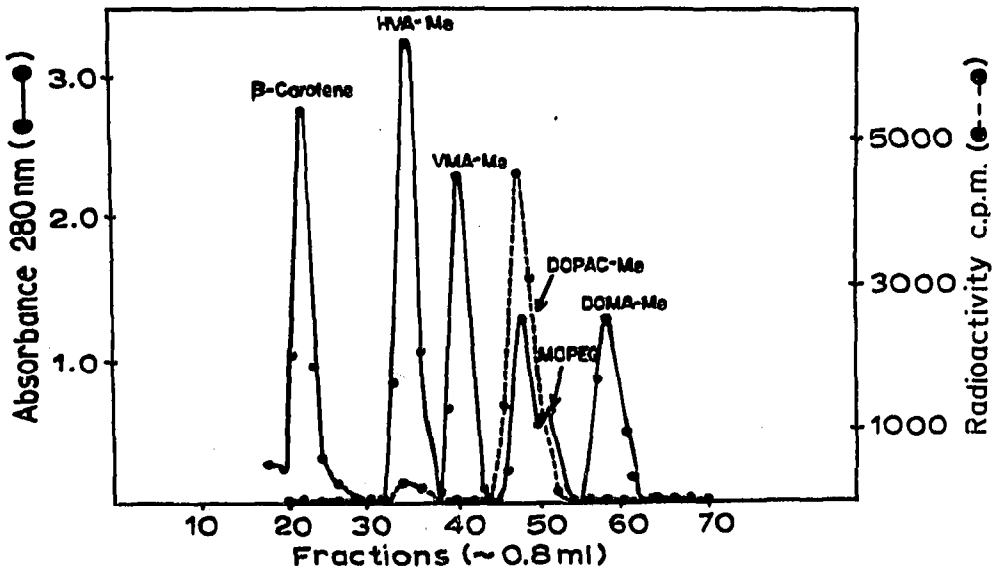

Fig. 3. Chromatography of acidic metabolites of [^{3}H]dopamine after incubation with chopped rabbit brain. The solid line represents absorption at 280 nm due to carrier metabolites and the broken line represents radioactivity. Sephadex LH-20, solvent system: 1,2-dichloroethane–methanol (7:3).

Metabolites of indoleamines

The columns also proved suitable for the separation of indoleacetic acid (IAA), 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophol (5-HIOL). The acids were quantitatively converted to the methyl ester using diazomethane as described under EXPERIMENTAL.

The elution volumes of the methyl esters and of 5-HIOL are given in Table I. Excellent separation was obtained with 1,2-dichloroethane–methanol (7:3) (Fig. 4). The peaks were symmetrical. The number of theoretical plates/column was 900 for IAA methyl ester, 1400 for 5-HIAA methyl ester and 1600 for 5-HIOL.

The methyl ester of 5-HIAA could not be hydrolysed in acid or alkali without degradation.

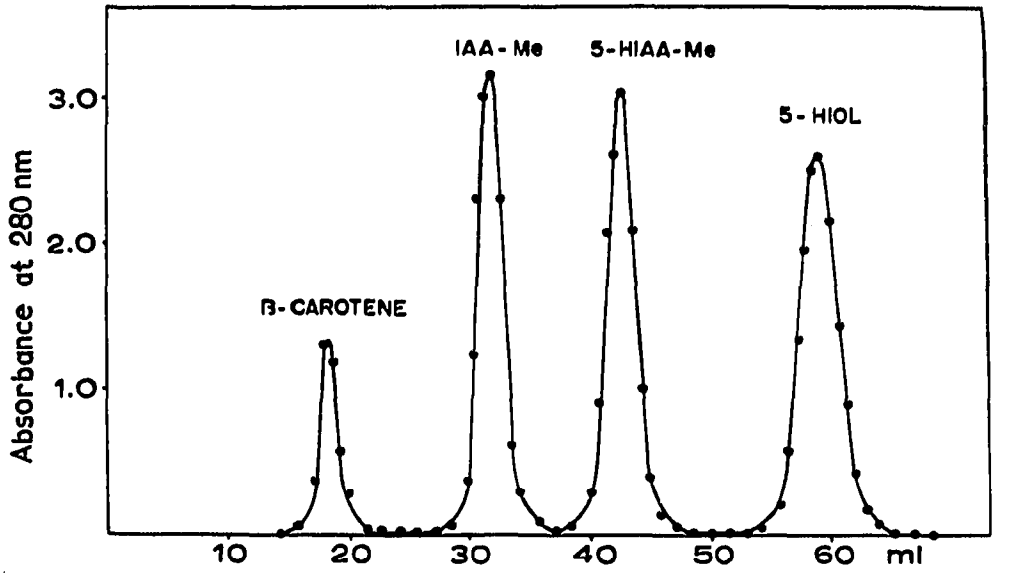

Fig. 4. Chromatography of 5-hydroxytryptophol (5-HIOL) and the methyl esters of 5-hydroxy-3-indoleacetic acid (5-HIAA) and indoleacetic acid (IAA) on Sephadex LH-20. Solvent system: 1,2-dichloroethane–methanol (7:3).

DISCUSSION

The separation and analysis of aromatic acids and alcohols by nondestructive methods generally include the principles of ion exchange, adsorption and electrophoresis[6,7]. None of these methods have the efficiency inherent in liquid–liquid and gas–liquid partition systems. In the present report, chromatography on Sephadex LH-20 was found to give excellent separations of the above-mentioned phenolic acids and alcohols in solvent systems of 1,2-dichloroethane and methanol.

Sephadex LH-20 is prepared from Sephadex G-25 in bead form by hydroxypropylation of the hydroxyl groups in the dextran chain. In a mixed solvent system the beads swell, possibly incorporating one of the solvent components preferentially in the gel phase. The molecules solvating and surrounding the substituted dextran will therefore have a different composition from those of the mobile phase. Solutes

may therefore be separated according to their distribution between the mobile phase and the stationary gel phase. They may also be distributed according to their size by molecular sieving mechanisms. These separations have been designated liquid–gel chromatography and liquid–gel filtration chromatography respectively by SJÖVALL *et al.*[1]. For a thorough discussion of the principles and practice of liquid–gel chromatography in organic solvents the reader is referred to the review by these authors.

The compounds separated in the present investigation are roughly of the same size. Therefore liquid–gel chromatography rather than liquid–gel filtration seems to be the separating mechanism. Since the peaks are symmetrical adsorption seems to be minimal. The compounds appear in order of increasing polarity. The partition chromatography is therefore of "straight-phase" type. Since more lipophilic Sephadex gels are now available[8–10], it appears possible to construct "reversed-phase" liquid–gel chromatographic systems for the aromatic acid and alcohols as has been done for cholesterol esters, bile acids and other lipids[9,10].

Liquid–gel chromatography of the aromatic acids and alcohols possesses advantages over other methods based on adsorption, ion-exchange and paper chromatography. In many cases, the efficiency of the separation approaches those of the analytical gas–liquid chromatography systems. The recovery is high and for the O-methylated compounds practically quantitative. The number of theoretical plates/m was around 2000 for most of the compounds studied. Thus the compounds appear separated from each other in small volumes of organic solvent, which are easily removed prior to analysis by other methods. As in gas chromatography, the column is used repeatedly without any alteration of the composition of the mobile phase although possibilities for programmed variations exist. Furthermore, the elution volume of a given compound seems very reproducible. It is therefore possible in routine analyses to discard most of the effluent while collecting only those fractions containing the desired compounds, thus rendering expensive fraction collectors unnecessary.

With the compounds studied under the conditions described a separation takes 2–6 h. The chromatogram shown in Fig. 1 was run in about 6 h. The virtual absence of column bleed from a well washed and conditioned column, and the absence of adsorption effects, is of value in isolation of submicrogram amounts of monoamine metabolites.

The application of this liquid–gel chromatography system to *in vivo* and *in vitro* studies on the metabolism of catecholamines was demonstrated. Another obvious use of the method — and the use for which it was designed — is for isolation of individual metabolites prior to analysis by gas chromatography–mass spectrometry. Here the conversion of the acids to the methyl esters represents no problem since these or similar derivatives have to be prepared anyway. For fluorimetric measurement some of the methylesters may be quantitatively hydrolysed.

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