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THE ANALYSIS OF L-3-HYDROXYTYROSINE (L-DOPA) AND ITS METABOLITES

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13. ABSTRACT

A procedure for the extraction and analysis by gas-liquid chromatography of a variety of catecholamine metabolites from biological tissue was developed. An extensive investigation and evaluation of extraction techniques was undertaken. Catecholamine reactions which would result in stable, volatile derivatives suitable for separation and measurement by gas-liquid chromatography were also investigated. Since the detection by electron capture provided the greatest sensitivity in analysis for certain compounds, special consideration was given to reactions which would form derivatives suitable for this type detection. Because of their stability, volatility, and electron affinity, the pentafluoropropionyl derivatives were selected as the derivatives of choice. The rapidity of the technique and the advantage of using small sample volumes should make it applicable for the solution of a wide variety of problems in both research and clinical laboratories.

Key Words:
Analytical method
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FOREWORD

This report was prepared by members of the Chemical Hazards Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory. This work was performed in support of Project 6302, "Toxic Hazards of Propellants and Materials," Task 630203, "Identification of Toxic Materials." This study was included in a thesis submitted by the author to the Department of Biochemistry of the Pennsylvania State University in partial fulfillment of the requirements for the Doctor of Philosophy degree. The study was initiated in May 1969 and completed in June 1971.

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This technical report has been reviewed and is approved.

CLINTON L. HOLT, Colonel, USAF, MC
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GLOSSARY OF TERMS

Catecholamine metabolites: In the context of this investigation, the term catecholamine metabolite is used to refer to L-3,4 dihydroxyphenylalanine (dopa) and all of its' metabolites.

Catecholamines: All compounds which are biogenically derived from dopa and contain an amino functional group are termed catecholamines.

Biogenic amines: All compounds of biogenic origin containing an amino functional group excluding the amino acids.

Dopa or L-dopa: In this study, either term will be used to refer to the L-stereoisomer of 3, 4 dihydroxyphenylalanine only.

Alumina: Aluminum oxide (Al_2O_3) Woelm neutral activity grade 1 (Alupharm Chemicals, New Orleans, La.) which has been prepared according to the method of Anton and Sayre (1962).

Acid:methanol: Methanol which has been treated with anhydrous hydrogen chloride gas to obtain a normality of 2.0.

Schiff's base: Compound formed by the reaction of primary amines with a ketone. Nomenclature used in this study for these compounds includes the name of the ketone from which the compound is formed such as acetone Schiff's base.

TABLE OF ABBREVIATIONS

CMDMS = chloromethyl dimethyl silyl

CNS = central nervous system

CSF = cerebrospinal fluid

DC = dihydroxyphenyl acetic acid

DEG = 3, 4 dihydroxyphenylethylene glycol

DHC = 3, 4 dihydroxyhydrocinnamic acid

DM = dopamine

E = epinephrine

E.C.D. = electron capture detector

EDTA = ethylenediamine tetraacetic acid

F.I.D. = flame ionization detector

G.L.C. = gas liquid chromatography

HCl = hydrogen chloride

HFB = heptafluorobutyryl

HMDS = hexamethyldisilazane

HVA = homovanillic acid

M = metanephrine

MCA = monochloroacetyl

MD = 3 methoxytyrosine

MMH = monomethylhydrazine

MT = 3-methoxytyramine

M.U. = methylene unit

NE = norepinephrine

NM = normetanephrine

PFP = pentafluoropropionyl

TFA = trifluoroacetyl

THI = trihydroxyindole

TMCS = trimethyl chlorosilane

TMS = trimethylsilyl

I. INTRODUCTION

Survey of Pertinent Literature

The biological importance of the L-amino acid, 3, 4-dihydroxyphenylalanine (L-dopa) as the precursor of a number of biogenic amines with hormonal activity has been recognized for some time. This amino acid is first decarboxylated by dopa decarboxylase, an enzyme requiring pyridoxal 5-phosphate as a cofactor (Lovenberg et al., 1962), to the catecholamine, dopamine (DM). By hydroxylation of the ethylamine side chain of DM by the enzyme dopamine β-hydroxylase, a second catecholamine, norepinephrine (NE), is formed. This hydroxylation proceeds in the presence of ascorbic acid and oxygen (Friedmann and Kaufman, 1965). A third catecholamine, epinephrine (E) is obtained by the N-methylation of NE by the enzyme, phenylethanolamine N-methyltransferase. The methyl group donor in this reaction is S-adenosyl methionine, (Pohorecky et al., 1969). The degradation and elimination of all three of these catecholamines occurs by similar mechanisms. DM, NE, and E are all 3-0 methylated by the action of catecholamine 0-methyltransferase to 3-methoxytyramine (MT), normetanephrine (NM) and metanephrine (M), respectively. The methyl group donor of these reactions is also S-adenosyl methionine. A second pathway for the physiological inactivation of the

catecholamines is by oxidative deamination by amine oxidases. The monamine oxidases which are located in the mitochondria (Seiler, 1969) are flavoproteins containing Cu^{2+}. These enzymes convert the three catecholamines to either the respective acid or alchohol. Dihydroxyphenyl acetic acid (DC) is generally formed preferentially in the case of DM while dihydroxyphenyl ethylene glycol (DEG) is formed from both NE and E (Seiler et al., 1971). A combination of both of these pathways is also important in the degradation and elimination of these amines. All of the enzymes which have been discussed in conjunction with the biosynthesis and degradation of the catecholamines have been detected in the brain (Seiler, 1969).

The hormonal activity associated with E and NE has been recognized for some time. While these two substances are very similar structurally, and produce some effects which are similar, certain differences in action serve to distinguish these two compounds. While E increases total body oxygen consumption, glycogenolysis, blood glucose, blood lactic acid, and serum potassium, NE by contrast, is primarily a vasoconstricting agent with a prime role in the maintenance of the blood pressure (Persky, 1959). Only recently has DM been thought to have physiological activity (Blaschko, 1957). Experimental evidence is increasing which indicates that DM causes both excitatory as well as inhibitory effects at different sites of the central nervous system (Vogt, 1965). At this time, no physiological

role has been suggested for the 3-O-methylated derivatives of the catecholamines (MT, NM, M); however, MT administered in pharmacological doses has produced hyperkinesia in rats (Ernst, 1965). While L-dopa has been used extensively for the relief of Parkinsonism (Calne and Sandler, 1970) it is generally believed that the active compound is DM rather than L-dopa.

The metabolism of DM, NE and E is altered by a variety of pharmacological agents including reserpine (Carlsson, et al., 1958), chlorpromazine (Laverty and Sharman, 1965), d-amphetamine and tetrabenazine (Pletcher, et al., 1962) among others. Other compounds affecting the central nervous system (CNS) such as the hydrazines have also been shown to affect CA metabolism due to their inhibition of certain enzymes (Davison, 1957, Smith, et al., 1963). Hydrazines which have been shown to exert these effects include hydrazine, monomethylhydrazine (MMH) and unsymmetrical dimethylhydrazine (UDMH).

Since the biologically important catecholamines and their metabolites occur in very low concentrations in most tissues and body fluids (Anton and Sayre, 1962) methods for their analysis must be highly sensitive. While a variety of procedures have been used for this measurement, the most popular of these involves the measurement of the fluorescence of certain catecholamine reaction products. The two common variations involve (1) the selective oxidation of the amines at different

pH conditions to fluorescent trihydroxyindole (THI) derivatives or (2) the condensation of the amines with ethylenediamine to form the fluorophores. The former procedure is generally credited to Lund (1949) while the latter was derived from the work of Weil-Malherbe and Bone (1952). While both procedures involve the oxidation of the catecholamines to indole derivatives, the fluorophore measured has different properties including the wave length of maximum fluorescence emission spectrum in the alternate procedures (Udenfriend, 1962). Numerous oxidizing agents have been suggested under various conditions including iodine (Welch, 1969), sodium periodate (Anton and Sayre, 1964), and potassium ferricyanide (Anton and Sayre, 1962). These fluorometric methods, while offering a technique which is capable of measuring micro amounts of a number of catecholamines, as little as 0.01 ng/ml plasma according to O'Hanlan, (1970), are subject to many technical difficulties. These include rapid decomposition of the fluorescent material, suppression of the fluorescence by biological materials, extraneous fluorescence and overlap of fluorescence maxima which make specific determinations difficult. While the problem of fluorophore decomposition is alleviated by the use of ethylenediamine, the other inherent problems of overlap of fluorescence emission spectra of the various compounds have been more serious.

A variety of methods have been proposed for the concentration and separation of the catecholamines and

the removal of extraneous materials prior to fluorometric measurement. These have included solvent extraction (Shore and Olin, 1958), ion exchange chromatography (Haggendal, 1966), and adsorption on alumina (Anton and Sayre, 1962). In addition to their very complete investigation of the preparation and conditions necessary for efficiency in alumina adsorption of catecholamines, Anton and Sayre also determined optimum conditions for the formation and stabilization of the fluorophores as well as procedures for the elution of the compounds from alumina. Even with this extensive investigation of all facets of the THI-alumina extraction procedure, many variations dealing not only with the extraction and separation of catecholamines but also with the stabilization of fluorescent products (Laverty and Taylor, 1968; Valori et al., 1970) have produced a voluminous literature in this area. The large number of published variations of the fluorometric procedure is a measure of continuing attempts by many investigators to improve the method.

In addition to methods using fluorometric measurement, a variety of other procedures have been employed for catecholamine separation and measurement. A number of radiochemical procedures have been developed for the measurement of catecholamines, particularly E and NE in biological samples. Saelens (1966) described the first of these methods which was based upon the enzymatic conversion of NE to epinepherine-N-methyl-^{14}C by incubating tissues with S-adenosylmethionine-methyl-^{14}C and

a N-methyl transferase. This procedure not only had many of the same deficiencies as the fluorometric methods such as variable loss during isolation, but was also considerably less sensitive than fluorometry. To increase the precision and accuracy of radiochemical catecholamine analysis, a number of double isotope derivative procedures have been developed recently. The addition of tracer amounts of tritiated NE to samples as an internal standard by which to estimate catecholamine losses during the procedure was proposed by Engleman (1968). After addition of the internal standard, E and NE were converted to their respective 3-O-methyl derivatives with ^{14}C-methyl S-adenosyl methionine and catechol-O-methyl transferase. Other investigators have used p-toluenesulfonyl chloride-^{35}S (Aizawa and Yamada, 1969) and ^{3}H acetic anhydride (Stern, et al., 1967) as reagents for derivative formation coupled with ^{3}H or ^{14}C labelled catecholamines as internal standards respectively in other double isotope procedures. While these latter procedures have certainly aided in providing a more reproducible procedure than can be achieved by fluorometry, they lack the sensitivity of the fluorometric method in addition to being equally complex and difficult to employ. In a very recent publication, Blaedel and Anderson (1971) have described yet another isotopic method, termed by the authors as the RIDRID assay (radioisotope derivative with reverse isotope dilution assay). This

procedure involves the conversion of E and NE to iodo-aminochromes with ^{125}I, purification by column chromatography, alumina adsorption and combined spectrophotometric analysis and radioactive counting. The method was said to be sensitive to aqueous E solutions as dilute as 0.01 μM. NE, but not E, could be determined in urine by this method. While possessing sufficient sensitivity for urinary analysis, this assay is time consuming, subject to variable loss, and capable of measuring only one catecholamine in biological fluids.

While fluorometry and radiochemical procedures are certainly the most popular for catecholamine analysis, other methods have been utilized. A large number of biogenic amines were separated using thin-layer chromatography by Aures (1968) with subsequent quantitation by fluorescence measurement of the condensation products formed with paraformaldehyde. The use of certain coupling reagents such as 5-dimethylaminonapthalene-1-sulfonyl chloride (Dilbreto and DiStefano, 1969) and 4-chloro-7-nitrobenzol-(1,2,5) oxadiazole (Ghosh, 1968) prior to paper or thin layer chromatography of biological extracts has increased the sensitivity of this method to a level where urine and certain tissue determinations of most catecholamines can be achieved. In a recent publication, the use of mass spectrometry for the determination of certain amines in crude tissue extracts has been described by Boulton and Mayer (1970).

The use of dextran gel has been employed both as a method for sample purification prior to fluorometric determination (Marshall, 1963) and as a procedure for separating individual catecholamines in order to measure radioactive metabolites obtained in tissue incubation studies (Anggard and Sedvall, 1970). While all of the procedures which have been discussed possess certain attributes, lack of sensitivity and selectivity, variable loss, interference phenomenon and above all complexity and time consumption are obvious deficiencies inherent in the various procedures.

Since its inception in 1952, gas-liquid chromatography (G.L.C.) has been employed for the analysis of a wide variety of compounds of biological importance. The applications of G.L.C. have been greatly expanded in the past decade by the use of derivatives which now allow the quantitative chromatography of a wide variety of materials. By the use of appropriate derivatives, analytical procedures have been described and employed for such diverse materials as steroids (Wotiz and Clark, 1966), carbohydrates (Sweeley, 1963), nucleosides (Sasak and Hashizume, 1966), addictive drugs (Martin and Swinehart, 1966), thyroid hormones (Jaakonmaki, 1967), amino acids (Gehrke and Stalling, 1967) and even metallic cations (Taylor, Arnold and Sievers, 1968).

While the use of G.L.C. has been incorporated into analytical procedures for a wide variety of compounds

of interest in the biomedical sciences, practical application of this powerful tool in the area of biogenic amine analysis has been relatively neglected. A number of investigators have formulated procedures for the formation and chromatography of a variety of derivatives of the biogenic amines, particularly the catecholamines and their metabolites and have separated model solutions of these compounds. Sen and McGreer (1963) first used trimethyl silyl (TMS) derivatives with flame ionization detection (F.I.D.) to separate solutions of six catecholamines. A short time later, Brooks and Horning (1965) described the separation of the catecholamines and 31 other amines of the phenylalkylamine, imidazole and indole types using acetyl derivatives and argon ionization detection. M. G. Horning (1967) also used TMS derivatives to separate a group of eight catecholamines and related compounds and investigated the mechanism of formation of TMS catecholamine derivatives. Since the separation of catecholamines as their TMS ether derivatives generally requires temperature programming and rather long analysis time for acceptable separations, several investigators have evaluated the use of O-TMS catecholamine derivatives in combination with a second derivative type used to reduce the polarity of the amine group. The formation of the resulting mixed derivatives results from the condensation of the primary amine functional group of certain O-TMS catecholamines with a

ketone to form a Schiff's base suitable for G.L.C. Ketones used to form suitable Schiff's base derivatives have included acetone (Kawai and Tamura, 1967), 2-pentanone (Kawai, et al., 1966) and cyclobutanone (Capella and Horning, 1966). With the advent of the electron capture detector (E.C.D.) which is extremely sensitive to halogen containing molecules, a variety of halogenated derivatives of the biogenic amines have been investigated. Clarke, et al., (1966) investigated the chromatographic characteristics and electron-capturing ability of trifluoroacetyl (TFA), monochloroacetyl (MCA) and heptafluorobutyryl (HFB) derivatives of several amines. A variety of catecholamines were chromatographed as O-TMS, N-HFB combination derivatives by M. G. Horning et al., (1968); however, F.I.D. instead of E.C.D. was used. The excellent electron capturing ability of pentafluoropropionyl (PFP) and HFB derivatives of catecholamine metabolites as well as the conditions necessary for their chromatography has been demonstrated by Anggard and Sedvall (1969). In regard to electron capturing ability, the full heptafluorobutyryl derivative of hydroxyl-substituted amines appears to be the most sensitive (Anggard and Sedvall, 1971). Recently, however, a Schiff's base derivative obtained by the condensation of primary amines with pentafluorobenzaldehyde appears to possess superior electron capturing ability for nonsubstituted primary amines such as β-phenyl ethylamine (Moffat and Horning, 1970).

Although a variety of derivatives have been described which will impart sufficient volatility to allow G.L.C. separation and measurement of the biogenic catecholamines and their metabolites, the application of this type analysis to the problem of measurement of these compounds in biological material has been little used. Kawai and Tamura (1967) used G.L.C. of TMS ether-acetone Schiff's base derivatives to determine the E and NE content of bovine adrenals. Dopamine (DM) and its acetaldehyde condensation product, salsolinol, were measured as their TMS derivatives in brain homogenates which had been incubated with added DM and acetaldehyde (Cashaw, et al., 1971). A procedure has also been developed for the measurement of DM in normal and pathological urine by G.L.C. of TFA derivatives. Alumina adsorption and/or ion-exchange chromatography has been used in these procedures for preliminary sample clean-up. The sensitivity of these procedures is difficult to calculate; however, none appear to be capable of measuring catecholamines except where large samples (25 ml of urine) or amine-rich tissue such as the adrenals are used. While these three early attempts at a G.L.C. method for catecholamine analysis have had many deficiencies, the rapidity of analysis, separating ability and most important the extreme sensitivity of the E.C.D. to certain fluorinated catecholamine derivatives

led to this investigation and development of the procedures for the use of electron capture G.L.C. for catecholamine analysis in biological samples.

Statement of Aims

The primary aim of this study was to investigate, evaluate, and select procedures which could be integrated into a general method for the measurement of a variety of catecholamines and their metabolites in biological materials. Due to the selectivity, rapidity and sensitivity of gas-liquid chromatography (G.L.C.), special emphasis was placed upon the adaptation of this tool to this analysis. Of paramount importance in the selection of techniques were the qualities of precision, accuracy, sensitivity and simplicity of the procedure and compatibility with other parts of the general procedure. Hence, the aim of the experiment required that any developed procedures be compared with existing methods of analysis. Comparisons on these points were to be made with other methods currently in use for this type analysis.

II. PROCEDURES USED IN THE INVESTIGATION

Method Development

Preparation of catecholamine derivatives suitable for G.L.C.: Catecholamine derivatives to be investigated were prepared by various procedures depending upon the reaction or reactions involved. In all cases, however, catechol compounds used as starting materials were weighed amounts of the dry commercial preparations. Specifically these included DL-norepinephrine HCl salt, B grade; DL-normetanephrine HCl, B grade; L-epinephrine, USP; DL-metanephrine HCl, B grade; dopamine HCl, A grade; 3-methoxytyramine HCl, B grade; homovanillic acid, A grade (Calbiochem, Los Angeles, Calif.); L-3-hydroxytyrosine (dopa); L-3-methoxytyrosine (Hoffman-LaRoche Inc., Nutley, N.J.) and 3, 4-dihydroxy phenylacetic acid (K and K Chemicals, Plainview, N.J.) These compounds will be referred to in the text as catecholamines and catecholamine metabolites unless specified as a compound. All solvents used in the development and application of procedures in this investigation were "chromatoquality" reagents (Matheson, Coleman & Bell, Norwood, Ohio), unless otherwise specified.

1. Trimethylsilyl (TMS) derivatives: Hexamethyl disilazane (HMDS)--trimethylchlorosilane (TMCS) method: TMS-catecholamine derivatives were prepared according to

the method employed by Luukkainen et al., (1962) for the formation of steroid derivatives. Amounts of 100-500 μg of E, NE and DM were placed in individual 1 ml glass stoppered volumetric flasks. To each dry sample was added 0.2 ml of HMDS $(CH_3)_3SiNHSi(CH_3)_3$ (Pierce Chemical Co., Rockford, Ill.) followed by 0.2 ml of a 10% solution of TMCS $(CH_3)_3$ SiCl (Pierce Chemical Co.) in chloroform. The reaction flasks were stoppered, agitated to mix the reactants, and allowed to stand at room temperature overnight. The derivatives formed were dried under a stream of nitrogen and redissolved in 0.1-1.0 ml of either carbon disulfide for flame ionization detection (F.I.D.) or ethyl acetate for electron capture detection (E.C.D.)

N-trimethyl silylimidazole method: TMS-catecholamine derivatives were prepared by the method of Horning, et al. (1967). Approximately 500 μg of E, NE and DM were individually weighed and placed in 1 ml glass stoppered volumetric flasks. To each flask was added 0.2 ml of acetonitrile, and 0.4 ml of N-trimethyl silylimidazole $(CH_3)_3Si\overline{NCH=NCH=CH}$ (Pierce Chemical Co.) Reaction was carried out in an oil bath at 60° C for 2-3 hours. Excess reagents were removed under a stream of dry nitrogen and products dissolved in the same manner as above.

2. Chloromethyldimethyl silyl (CMDMS) derivatives: CMDMS-catecholamine derivatives were prepared by the method of Van den Heuvel (1967) for steroids. One to five hundred micrograms of NE, E, and DM were weighed into 1 ml volumetric

flasks and allowed to stand at room temperature with 0.1 ml 1,3-bis (chloromethyl dimethyl) 1, 1, 3, 3,-tetramethyl disilazane ($ClCH_2(CH_3)_2$ SiNH $Si(CH_3)_2CH_2Cl$ and 0.1 ml of a 10% solution of chloromethyl dimethyl chlorosilane $ClCH_2(CH_3)_2$ SiCl in tetrahydrofuran. After the reaction, samples were dried in the same manner as for TMS derivatives and redissolved in 1.0 ml ethylacetate for G.L.C. Both F.I.D. and E.C.D. were utilized.

3. Trifluoroacetyl (TFA) derivatives: TFA-catecholamine derivatives were prepared by a modification of the method of Clarke et al., (1966). In the initial experiments, 100-500 μg each of E, NE, M, NM and DM were placed in individual 2 ml volumetric flasks and to these were added 0.5 ml trifluoroacetic anhydride, $CF_3COOCOCF_3$ (Pierce Chemical Co.) and 1.0 ml ethyl acetate. The flask was shaken after stoppering to mix the reactants, and then heated to 50° C until all materials were dissolved (1 hour). After reaction, excess reagents and solvent were removed under a stream of dry nitrogen and the residue redissolved in 2.0 ml ethyl acetate. This solution was used without further dilution when employing F.I.D. or with a 1:10 dilution when E.C.D. was employed. In later studies, 1.0 ml of a solution of 20% trifluoroacetic anhydride in acetonitrile was employed in the derivative formation. Using this latter reagent, the reaction with any of the catecholamines was complete in

10 minutes at room temperature. Removal of excess reagents and dissolution of products was the same as described above.

4. Pentafluoropropionyl (PFP) derivatives: PFP-catecholamine derivatives were prepared in a manner similar to that used to prepare TFA derivatives. Individual 100-500 μg samples of E, NE, NM, M, DM, and MT were weighed and placed in 1 ml volumetric flasks. To each flask was added 1.0 ml of a 20% solution of pentafluoropropionic anhydride, $C_2F_5COOCOC_2F_5$, (Pierce Chemical Co.) in acetonitrile. Reactants were allowed to stand at room temperature for 10 minutes and then dried under a stream of dry nitrogen. Drying time necessary to remove all unreacted pentafluoropropionic anhydride plus the pentafluoropropionic acid formed in the reaction averaged about 30 minutes. Products were dissolved in 1.0 ml of ethyl acetate and then aliquots diluted to obtain concentrations of PFP-catecholamines of 1-10μg/ml. Samples were chromatographed using E.C.D.

5. Heptafluorobutyryl derivatives: These derivatives of the catecholamines were made in an analogous manner to PFP derivatives. A 20% solution of heptafluorobutyric anhydride $C_3F_7COOCOC_3F_7$ (Pierce Chemical Co.) in acetonitrile was used. Due to the decreased reactivity, however, reaction time at room temperature

was increased to 15 minutes. On the average, drying time was slightly longer than that necessary for PFP-catecholamine derivatives.

6. Methyl ester PFP derivatives: The presence of the carboxyl group on certain catechol compounds required that a second reaction be undertaken prior to chromatography in order to form a non polar derivative of the carboxyl group. This second procedure was necessary with L-3-hydroxytyrosine (L-dopa), homovanillic acid (HVA), 3, 4-dihydroxyphenylacetic acid (DC) and L-3-methoxy tyrosine (MD). Methyl esters were formed using a modification of the method of Stoffel et al., (1959) for fatty acid methyl ester formation. Acid methanol was prepared by bubbling anhydrous hydrogen chloride through 200 ml of methanol until sufficient HCl was dissolved to result in a net weight gain of approximately 20 grams. An aliquot of this solution was then titrated with 0.5 N potassium hydroxide in methanol to the phenolpthalein end point and the normality calculated. The remainder of the solution was diluted with sufficient methanol to obtain a 2.0 N HCl solution. Three ml of this latter solution was added to 500 μg each of any of the four catecholamine metabolites listed above contained in 10 ml glass freeze drying vials. Solutions were stirred with a glass-encased magnetic stirrer for 45 minutes at room temperature. After stirring, 1.0 ml of the solution was

transferred to a clean 10 ml glass vial and the acid-methanol removed under vacuum. The dry products were then reacted with pentafluoropropionic anhydride as in part 3 above.

7. O-TMS, N-HFB derivatives: These derivatives were formed using the method of Horning et al., (1968). One milligram of each of the catecholamines was dissolved in 0.1 ml of acetonitrile and 0.2 ml of N-TMS-imidazole was added. After heating at 60° C in a 1 ml glass-stoppered volumetric flask for 3 hours, 0.1 ml of N-heptafluorobutyryl imidazole C_3F_7-CO-$\overline{NCH=NCH-CH}$ (Pierce Chemical Co.) was added and the solution was heated for 30 minutes at 60° C. Solution volume was brought to 1.0 ml with additional acetonitrile and the solution was used directly for chromatography with F.I.D.

8. O-TMS, Schiff's Base derivatives of primary amines: Derivatives were prepared by first forming the O-TMS-derivatives of NE and DM by the HMDS-TMCS procedure discussed previously. After the TMS-catecholamine derivative had been formed and excess solvent removed, Schiff's bases were formed using the method of Kawai and Tamura (1967). To each O-TMS-catecholamine was added 1.0 ml of acetone, the 1 ml volumetric flask shaken to mix the contents and then allowed to stand for 30 minutes at room temperature. After reaction, solution

volume was reduced to 0.1 ml under a stream of dry nitrogen and sufficient ethyl acetate added to obtain 1 ml of solution for chromatography. TMS-catecholamine Schiff's bases were also formed using hexafluoroacetone, CF_3COCF_3, as the ketone. Gaseous hexafluoroacetone was condensed in a vial kept at - 70° C in a dry ice-acetone bath and added to the TMS-catecholamines which were in a 1 ml volumetric flask at the same temperature. After addition of reactants, the reaction flask was allowed to warm to room temperature to evaporate hexafluoroacetone. Products were dissolved in 1.0 ml ethyl acetate as before.

Evaluation and selection of conditions for G.L.C. of catecholamine derivatives: A Varian Aerograph model 2100 gas-liquid chromatograph was used throughout the study. This is a four column instrument with provision for operating four detectors simultaneously. Two detector types were used at various times in the investigation. The first of these was a hydrogen flame ionization detector, the second a direct current tritium electron capture detector. The E.C.D. has as its radioactive source a titanium tritide foil of 250 millicuries radioactivity. Chromatograph attenuations are given as amperage for full scale recorder deflection, i.e. 1×10^{-9} a.f.s.

Chromatographic columns utilized were either 6 or 12 feet by 2 mm I.D. glass U-tubes. Columns were

routinely silanized by filling with a 20% solution of dimethyldichlorosilane $(CH_3)_2SiCl_2$ (Pierce Chemical Co.) and allowed to stand for 2-3 hours then air dried prior to use. Direct on-column injection was used so that the samples were not in contact with metal surfaces prior to entering the detectors. All column packings were prepared by the investigator using a solvent evaporation method. An amount of liquid phase necessary to coat 10 grams of solid support was weighed in a 100 ml Erlenmeyer flask and dissolved in an appropriate solvent. This solution was quantitatively added to 10 grams of solid support in a 250 ml round bottom standard taper flask. Solvent was removed while rotating under vacuum in an all-glass rotary flash evaporator. All packings were sieved to obtain a narrow particle size range prior to using. Columns were packed by attaching one end to a vacuum line to draw air through the column while the packing material was added at the other end. Periodic vibration of the column with an electric marker resulted in a tight packing of the column.

Solid supports used during the course of the investigation were Gaschrom Q 60/80 mesh and Gaschrom Z 60/80 mesh (Applied Science Lab., State College, Pa.) and Corning GLC 110 60/80 mesh (Corning Glass Works, Corning, N.Y.). Liquid phases, their chemical form and suppliers are shown in Table I.

Table I

LIQUID PHASES USED FOR G.L.C. OF DERIVATIVES OF CATECHOLAMINE METABOLITES

Nomenclature	Type	Supplier
OV-1	non-polar methyl silicone	Supelco, Inc. Belefonte, Pa.
OV-3	non-polar 10% phenyl-methyl silicone	Supelco, Inc.
OV-7	medium polar 20% phenyl-methyl silicone	Supelco, Inc.
OV-11	medium polar 35% phenyl-methyl silicone	Supelco, Inc.
OV-17	polar 50% phenyl-methyl silicone	Supelco, Inc.
OV-25	polar 75% phenyl-methyl silicone	Supelco, Inc.
OV-210	polar fluoro silicone	Applied Sciences Lab. State College, Pa.
OV-225	polar cyanoethyl silicone	Applied Sciences
DC QF-1	polar fluoro silicone	Applied Sciences
GE SE-52	medium polar 35% phenyl-methyl silicone	Applied Sciences
EGSP-A	polar ethylene glycol succinate silicone	Applied Sciences
EGSP-X	polar ethylene glycol succinate silicone	Applied Sciences
Polyoxy ethylene diamine	very polar diamine	Applied Sciences

Two methods were utilized for the presentation of G.L.C. data. The first presents retention times in minutes measured from sample injection with column temperature, carrier gas flow rate and percentage of liquid phase specified for each set of retention times. An alternate method employs the calculation of retention indices as outlined by Horning (1967). Retention indices were obtained by chromatographing individual catecholamine derivatives under a uniform set of chromatographic conditions and recording their retention times. Under these same conditions, a series of normal hydrocarbons (C10-C18) was also chromatographed and retention times recorded. A graph was then prepared which plotted the number of carbon atoms in the n-hydrocarbon against the logarithm of its retention time. Retention indices in methylene units (M.U.) were established as being absolutely equal to the number of carbon atoms in the chain for all normal hydrocarbons, i.e. the retention index for n-decane equals 10.00 M.U. Retention times of the catecholamines were then converted to common logarithms and retention indices read directly from the graph.

Characterization of PFP-catecholamine derivatives: As the PFP-catecholamine derivatives appeared to possess sufficient volatility, stability and sensitivity to be useful in an analytical procedure, they were qualitatively

analyzed to determine the structures of the derivatives formed. Ten milligrams each of NE, E, DM, NM, M, HVA, DC and dopa catecholamines were weighed into 10 ml screw cap freeze-drying vials and PFP or methyl ester PFP derivatives formed in the same manner as outlined previously. After the derivatives were synthesized, excess reagent was removed under a stream of dry nitrogen and the products redissolved in 10 ml of benzene. This solution was transferred to a 25 ml round bottom flask and the solvent evaporated under reduced pressure in an all glass, rotary flash evaporator (Precision Scientific Co., Chicago, Ill.). This procedure was repeated and following the second flash evaporation, the derivatives were dissolved in either 1.0 ml benzene for mass spectral analysis, or 1.0 ml carbon tetrachloride for infrared spectrometry (IR) or nuclear magnetic resonance (NMR) spectrometry.

Mass spectra of the catecholamine derivatives were obtained by transferring 10 microliters of a benzene solution of the purified derivative, of a concentration equal to about 10 $\mu g/\mu l$, to a glass capillary tube which had been sealed at one end. The capillary tube was then inserted into the direct probe of a Consolidated Electronic Corp. (CEC) Model 21-491 Mass Spectrometer and the probe inserted into the ionization chamber. The pressure in the ionization chamber was reduced to $1x10^{-7}$mm Hg without heating to remove the solvent. After removal of the solvent, the probe temperature was slowly raised from ambient to 100° C and the spectrum

was scanned from 50 to 800 m/e units. Perchloro p-ditoluyl, $CCl_3\ C_6Cl_4C_6Cl_4CCl_3$, was used as a marker compound to determine mass numbers. A Varian Model A-60 NMR spectrometer was used to obtain NMR spectra. Derivatives to be examined were dissolved in a small amount of carbon tetrachloride to obtain an approximate concentration of 20 mg/ml. High concentrations were necessary to obtain acceptable spectra. Spectra were obtained at ambient temperature utilizing a sweep time of 500 sec. and a sweep width of 1000 H_z.

Infrared spectra were obtained on either a Beckman model IR5A prism IR spectrophotometer or a Perkin-Elmer model 237B grating IF spectrophotometer. When using the Beckman instrument, samples were dissolved in an appropriate solvent and deposited on sodium chloride plates using a ten microliter Hamilton syringe. Solvent was removed with a stream of dry nitrogen. From 10-20 μg of derivative was used for each analysis. When the Perkin-Elmer instrument was employed, a sodium chloride wet cell with a cell path of 0.1 mm was used. Derivatives were dissolved in sufficient carbon tetrachloride to obtain a solution containing 20-50 mg of derivative per milliliter. The IR spectrum from 2.5-8.0 microns was scanned in all cases.

Procedures used for the extraction of catecholamines from biological material: Catecholamines were

extracted from a variety of biological materials including whole blood, plasma, cerebrospinal fluid (CSF), urine and tissue. A number of different procedures were used during the investigation to remove the compounds of interest from other biological molecules. Derivatives for chromatography were formed from dry extracts since all reactions for derivative formation required anhydrous conditions.

Samples used in all extraction experiments were prepared in a like manner. Plasma samples were prepared by adding 5 mg sodium metabisulfate, $Na_2S_2O_5$, as an antioxidant to 10.0 ml of human plasma. Urine samples were prepared in a like manner. E and NE standard solutions were prepared by dissolving weighed amounts of the catecholamines in 0.01 N HCl. A 0.1 ml aliquot of these standards was then diluted 1:10 with the biological sample. Lower concentrations were prepared by diluting this initial sample with additional biological fluid. Tissue samples were prepared by homogenizing 500 mg of tissue in 2 ml cold 0.4N perchloric acid in a Tembroeck homogenizer. The homogenizer was washed twice with additional cold 0.4N perchloric acid to obtain a final volume of 4.5 ml of homogenate. One half milliliter of a solution containing 50 mg/ml sodium metabisulfate was added and the homogenate centrifuged at 2,400 rpm (1570 x g) for 30 minutes in an International model PR-2 refrigerated centrifuge equipped with a number 269 rotar. The supernatant liquid was then used as the sample.

Three methods were investigated for extraction and these are described below:

1. Molecular sieve method: The method described by Marshall (1963) was employed for the removal of E and NE from urine and plasma to which amounts of these catecholamines had been added for determination of precision and accuracy of recovery. Ten grams of dry Sephadex G-10 (Pharmacia Chemicals, Inc., Piscataway, N.J.) was allowed to swell overnight in distilled water and then poured into all glass liquid chromatography columns to prepare columns of 1 x 15 cm dimensions. After pouring, columns were stabilized by passing through 100 ml of 0.01 M sodium acetate at a flow rate of 40 ml per hour. Void volume for the column was calculated to be 4.0 ml.

One ml of sample was carefully pipetted on the top of the prepared Sephadex columns, washed in with two 1 ml volumes of 0.01 M sodium acetate, and allowed to flow through the column at a constant rate of 0.5 ml/min. Additional 0.01 M sodium acetate was used as the eluent. Two ml fractions were collected. Proteins were detected in the eluted fractions by adding 20% trichloroacetic acid to the fractions and observing the white precipitate. Catecholamines were detected by removing the water from the fractions by freeze-drying, forming the PFP derivatives using the method discussed previously with measurement by G.L.C.

Once elution volumes for the catecholamines had been established, those fractions containing the catecholamines were combined, freeze-dried and derivatives formed as before.

2. Alumina adsorption procedure: A modification of the extraction procedure of Anton and Sayre (1962) was followed. Aluminum Oxide (Woelm neutral activity grade 1) was cleaned and activated by stirring with hot 2.0N HCl, washing repeatedly with double distilled water, and drying at a high temperature prior to use. About 100 g of alumina were placed in a 1 liter beaker and heated for 1 hour at 90° C while stirring with 500 ml 2.0N HCl. The acid was decanted after allowing the alumina particles to settle. This procedure was repeated twice with additional 250 ml portions of 2.0N HCl for one-half hour periods. Sufficient double distilled water was added to cover the alumina and after slurrying, the material was transferred to a clean 500 ml beaker where it was washed repeatedly with 200 ml portions of double distilled water. Washing and decanting were continued until the pH of the wash water had risen to pH 3.4. After the final wash, the alumina was filtered through glass fiber filter paper (Reeve Angel) and dried for 1 hour at 120° C. After drying, the alumina was activated at 200° C for 3 hours in a high temperature drying oven. This activated alumina was stored in an incubator at 40° C until used.

For catecholamine extraction, 200 mg of the activated alumina was placed in a 10 ml freeze-drying vial. One to four milliliters of sample (plasma, urine, CSF, or tissue homogenate supernatant) was added to the alumina along with 1 ml of a solution containing 100 mg/ml disodium ethylenediamine tetraacetic acid (EDTA). The pH of the mixture was quickly adjusted to 8.6 with 0.5 or 0.05N sodium hydroxide depending upon the original pH of the solution and maintained at this pH for 5 minutes while stirring with a magnetic stirrer. The pH was measured using a Corning model 12 pH meter equipped with a Beckman 39013 probe combination pH electrode. The alumina was allowed to settle by gravity and the supernatant liquid removed with a pipette and discarded. This procedure was repeated four times using 3 ml aliquots of double distilled water. A 0.2 ml volume of 0.1 N HCl was added to the damp alumina, the mixture frozen in a dry ice-acetone bath and all water removed by lyophylizing with a Virtis model 10-010BA freeze-dryer. The tops of the individual drying vials were covered with a circle of Whatman #1 filter paper to prevent loss of alumina during feeze-drying.

Following drying, two methods were used to remove the catecholamines from the alumina. The first or direct method involved the addition of 1 ml of a 20% solution of pentafluoropropionic acid anhydride in acetonitrile

directly to the dry alumina sample. This mixture was allowed to stand at room temperature for ten minutes, a 0.8 ml aliquot of the liquid removed and the solvent and excess reagent evaporated under a stream of dry nitrogen. The second or indirect method involved the addition of 3.0 ml of a 2N solution of anhydrous HCl in methanol to the dry alumina sample. These samples were stirred for 30 minutes with a magnetic stirrer, the contents transferred to a 10 ml centrifuge tube and centrifuged in a Lourdes model AAC clinical centrifuge at 7,000 rpm for 15 minutes to precipitate the alumina. A 2 ml aliquot of the methanol solution was withdrawn and transferred to another 10 ml freeze-drying vial and the methanol removed under vacuum. Derivatives for chromatography were prepared in the same manner as in the direct method. An indirect method was required when an analysis for L-dopa or other acidic metabolic product was desired.

3. Trifluoroacetic acid method: This method was used with whole blood, urine or plasma samples. Two milliliters of whole blood, plasma or urine was combined with 2.0 ml of a 20% aqueous solution of trifluoroacetic acid in a graduated conical centrifuge tube and mixed with a vortex mixer to precipitate proteinaceous materials in the sample. The mixture was centrifuged in an International refrigerated centrifuge at 3,000 rpm for 30

minutes. The supernatant was transferred to a 10 ml freeze-drying vial and the solution lyophylized to remove water and trifluoroacetic acid. Following drying, the residue containing the catecholamines was reacted for derivative formation with a 20% solution of pentafluoro-propionic anhydride.

III. EXPERIMENTAL RESULTS

Development of the Analytical Method

Formation and G.L.C. of various catecholamine derivatives: The group of biogenic amines commonly called "catecholamines" due to the presence of the 3,4-dioxyphenyl (catechol) structure in all members have such low vapor pressures that G.L.C. of the free compounds is not feasible. These compounds, however, all contain from two to five labile hydrogen atoms within their structure which can be replaced with other groups to increase their volatility and make them suitable for chromatography. This portion of the investigation was concerned with the study of a variety of chemical reactions which occur with all catecholamines and which result in the formation of volatile products suitable for G.L.C. The criteria used for the evaluation of reaction products were:

a. Ease, specificity and completeness of the derivative formation.

b. Volatility and stability of the derivatives produced.

c. Sensitivity of G.L.C. detectors to the derivatives.

d. Symmetry of chromatographic peaks, and separation efficiency with various commercial gas

chromatographic liquid phases on specific solid supports.

All derivatives were prepared for evaluation and ultimate selection of the most suitable for G.L.C. analysis.

The first group of reactions investigated were those which resulted in the conversion of hydroxyl and amino groups on the catecholamines to N-silyl and/or O-silyl groups. Also considered were two step reactions whereby the hydroxyl groups were converted to silyl ethers while the amino function was converted to either a fluorinated alkyl amide or Schiff's base. The O-TMS derivatives, N,O-TMS derivatives, and N,O-CMDMS derivatives of DM, NE and E as well as the O-TMS, N-HFB and O-TMS Schiff's bases of NE and DM were prepared as outlined in Section II. Derivatives prepared were chromatographed on 6 ft x 2 mm glass columns packed with 5% SE-52 on 60/80 mesh Gaschrom Z, 3% OV-25 on 60/80 mesh Gaschrom Q, or 1% EGSP-Z on 60/80 mesh Gaschrom Q. Injection port and detector temperatures were maintained at 155° C and 200° C respectively. Chromatographic data for silyl derivatives are shown in Table II. It should be noted that the acetone or hexafluoroacetone Schiff's base could be formed only with NE and DM. Secondary amines such as E and M will not form these compounds. Apparently the HMDS-TMCS method and the TMS-imidazole method for TMS derivative synthesis form different derivatives as evidenced by the differences in

Table II

CHROMATOGRAPHIC RETENTION DATA FOR CATECHOLAMINE SILYL DERIVATIVES

Catecholamine	Derivative Type	Column Temp (°C)	Carrier Flow (cc/min)	Retention Time (cm)		
				OV-25	SE 52	EGSP-Z
Norepinephrine	N, O-TMS*	140°	60	4.5	18.9	5.7
Epinephrine	N, O-TMS*	140°	60	4.3	19.5	4.9
Dopamine	N, O-TMS*	140°	60	3.9	19.1	4.8
Norepinephrine	O-TMS**	140°	60	4.7	19.0	7.9
Epinephrine	O-TMS**	140°	60	4.4	19.8	6.9
Dopamine	O-TMS**	140°	60	4.4	19.1	6.7
Norepinephrine	N-HFB, O-TMS	140°	60	4.7	21.0	5.1
Epinephrine	N-HFB, O-TMS	140°	60	4.6	22.1	4.6
Dopamine	N-HFB, O-TMS	140°	60	4.4	20.4	5.3
Norepinephrine	O-TMS Acetone Schiff's Base	150°	30	7.9	26.2	-
Dopamine	O-TMS Acetone Schiff's Base	150°	30	7.0	25.1	-

Table II (continued)

Catecholamine	Derivative Type	Column Temp (°C)	Carrier Flow (cc/min)	Retention Time (cm)		
				OV-25	SE 52	EGSP-Z
Norepinephrine	O-TMS, Hexa-fluoroacetone Schiff's Base	150°	30	2.0	14.9	-
Dopamine	O-TMS, Hexa-fluoroacetone Schiff's Base	150°	30	2.0	14.5	-
Epinephrine	N, O, CMDMS	150°	60	18.2	32.0	14.0
Norepinephrine	N, O, CMDMS	150°	60	14.6	25.9	13.8
Dopamine	N, O, CMDMS	150°	60	13.0	27.0	13.8

*Prepared using HMDS-TMCS method.

**Prepared using TMS-imidazole method.

retention times of the derivatives formed by the alternate methods. Comparison of the chromatographic behavior on columns of varying polarity suggests that TMS-imidazole produces O-TMS ether derivatives in all cases while HMDS-TMCS produces a N, O-TMS derivatives in the case of secondary amines and a N, N, O-TMS derivative in the case of primary amines. This conclusion is supported by the work of Horning et al. (1967).

Catecholamine derivatives of the silyl type varied greatly in ease of formation, minimum detectable amount, and symmetry of chromatographic peaks. This data is displayed in Table III. All derivatives could be prepared using moderate conditions; however, long time periods were required for quantitative formation in some cases. For instance, two days were required to prepare fully trimethylsilylated derivatives using the HMDS-TMCS method, and quantitative formation of CMDMS derivatives was never achieved. Single peaks were obtained in all cases. All derivatives with the exception of the Schiff's bases formed with hexafluoroacetone were sufficientlv stable for G.L.C. This type derivative (HFA - Schiffs base) gave a widely varying chromatographic response with repeated injections of the same solution, indicating their chromatographic instability. Also, solutions of these derivatives could not be stored without decomposition, even at 0° C.

Table III

DETECTOR RESPONSE, STABILITY AND PEAK SYMMETRY OF CATECHOLAMINE SILYL DERIVATIVES

Derivative Type	Minimum Amount Detectable (μg)		Peak Symmetry	Reaction Time	Stability*
	F.I.D.	E.C.D.			
N, O-TMS	0.01	0.5	Symmetrical	24 hrs	4 weeks
O-TMS	0.01	0.5	Slight-trailing	2-3 hrs	4 weeks
N, O-CMDMS	0.03	0.001	Moderate trailing	24 hrs	2-3 weeks
N-HFB, O-TMS	0.01	0.0005	Symmetrical	3-4 hrs	1 week
O-TMS, acetone Schiff's Base	0.01	0.5	Symmetrical	1 hr	2-3 days
O-TMS, hexafluoro-acetone Schiff's Base	0.05	0.0002	Asymmetrical	1 hr	1 day

*Stability defined in terms of time required for 50% of the derivative to decompose in solution at room temperature.

In addition to the formation of silyl derivatives, a second group of reactions was investigated. These reactions were of the general type

$$R\text{-}OH + R'COOCOR' \rightarrow R\text{-}O\text{-}R' + R'COOH$$

$$R\text{-}NH_2 + R'COOCOR' \rightarrow R\text{-}NH\text{-}R' + R'COOH$$

where R = any aromatic or aliphatic hydrocarbon and

$R' = CF_3$, C_2F_5 or C_3F_7

The trifluoroacetyl (TFA), pentafluoropropionyl (PFP) and heptafluorobutyryl (HFB) derivatives of E, NE, DM, M, NM and MT were prepared as described in Section II. All derivatives could be prepared using the appropriate fluoroacyl anhydride; however, the speed and completeness of the reaction in many cases was governed by the choice of solvents. For instance, the PFP, TFA and HFB derivatives of E could be quantitatively formed in 15 minutes at room temperature using methylene chloride, chloroform, ethyl acetate or acetonitrile as the reaction solvent while NE derivatives could only be formed quantitatively using acetonitrile or pyridine as solvents regardless of the reaction conditions. Derivatives of all of the catecholamines could be rapidly and completely synthesized using acetonitrile as a solvent, and since this solvent was obtainable commercially without interfering electron capturing impurities, it is the solvent of choice for this reaction.

While both the flame ionization detector (F.I.D.) and the electron capture detector (E.C.D.) can be used for the detection of fluorinated acyl catecholamine derivatives, the E.C.D. is at least three orders of magnitude more sensitive to these derivatives than the F.I.D. Except where it was desirable to use a F.I.D. so that the retention times of these derivatives could be compared under identical conditions to normal hydrocarbons, E.C.D. was used exclusively in this study. The E.C.D. response of the various derivatives prepared varied considerably both as a function of the catecholamine and the fluoroacyl group. This response is tabulated on the basis of peak area per nanogram of catecholamine in Table IV. As can be seen from the table, with the majority of the compounds the magnitude of the E.C.D. response increases in the order TFA < PFP < HFB.

While the three types of fluoroacyl derivatives did not vary appreciably in regard to E.C.D. response, the stability of the compounds was markedly different. The solvent used to prepare the derivative solutions also significantly affected this aspect of stability. All compounds were readily soluble in all organic solvents surveyed; however, as would be expected, polar solvents such as methanol and ethanol immediately resulted in hydrolysis. Stability in other solvents increased in the following order: carbon disulfide < heptane < hexane < acetonitrile < ethyl acetate < benzene. As can be seen

Table IV

STABILITY AND E.C.D. RESPONSE OF
CATECHOLAMINE FLUOROACYL DERIVATIVES

Compound	Derivative	Stability*	Detector Response** cm^2/ng
Epinephrine	PFP	14 days	0.961
	TFA	2	0.271
	HFB	12	0.912
Norepinephrine	PFP	12	0.831
	TFA	1	0.169
	HFB	10	0.878
Dopamine	PFP	14	1.423
	TFA	4	0.508
	HFB	14	1.755
Metanephrine	PFP	14	1.681
	TFA	9	0.887
	HFB	14	1.838
Normetanephrine	PFP	14	1.679
	TFA	4	1.116
	HFB	14	1.883
3-Methoxytyramine	PFP	14	1.010
	TFA	6	0.768
	HFB	14	1.197

*Time required for 20% sample loss in benzene solution at room temperature.

**Chromatograph electrometer attenuation = 16×10^{-9} a.f.s.

from Table IV, most TFA derivatives were quite labile, even in benzene solution. Chromatographic stability also varied as a function of the catecholamine and derivative type as well as differing dependent upon the liquid phase used for G.L.C. Once again, the TFA derivatives appeared to be much more susceptible to on-column degradation than were the corresponding PFP or HFB derivatives. Certain TFA-catecholamines could not be chromatographed on very polar liquid phases such as OV-225 cyanoethylsilicone. A significant degradation of all TFA derivatives and, in some cases, PFP and HFB derivatives on all liquid phases more polar than OV-7 was noted; however, this deficiency could be overcome in the case of HFB and PFP derivatives by initial injections of rather large amounts (~ 0.5 μg) of either ethyl pentafluoropropionate or ethyl heptafluorobutyrate.

The fluoroacyl catecholamines are all volatile and sufficiently stable to permit G.L.C. on a variety of different liquid phases. Most catecholamine derivatives could be chromatographed on thinly coated supports (1-5%) using any of the liquid phases listed in Table I. Since many of these liquid phases possess very similar separation characteristics, only five representative types will be discussed here. Table V shows the retention data for the derivatives on two polar liquid phases, OV-210 fluorosilicone oil and EGSP-Z ethylene glycol succinate silicone gum. Since some of the derivatives were retained

Table V

CHROMATOGRAPHIC RETENTION DATA FOR CATECHOLAMINE FLUOROACYL DERIVATIVES ON POLAR LIQUID PHASES

Compound	Derivative	Retention Index on 1% EGSPZ	Retention Index on 1% OV-210	Retention Index on 1:1 mixture
Epinephrine	TFA	19.50	23.69	21.67
	PFP	17.97	25.41	21.78
	HFB	18.92	25.25	22.99
Norepinephrine	TFA	23.80	23.20	22.77
	PFP	19.52	21.24	22.16
	HFB	20.05	24.00	23.36
Dopamine	TFA	23.92	21.00	22.48
	PFP	20.22	23.12	21.88
	HFB	20.30	24.68	22.82
3-Methoxytyramine	TFA	25.46	20.50	22.98
	PFP	22.15	22.05	22.10
	HFB	21.82	23.42	22.62
Metanephrine	TFA	21.25	25.84	22.56
	PFP	19.32	23.87	21.95
	HFB	19.40	24.58	22.62
Normetanephrine	TFA	25.32	25.62	23.47
	PFP	21.15	23.51	22.33
	HFB	20.73	21.63	23.18

for an excessive length of time on polar phases of relatively high loading, 1% coating was employed with these phases. Gaschrom Q, 60/80 mesh was used as the solid support in 6 ft x 2 mm I.D. columns. As a general rule, retention times increased in the order E < M < NE < DM < NM < MT and HFB < PFP < TFA. The separation characteristics of these polar phases were very good for the above six amines regardless of the derivative type. A combination of the two phases provided even better separations.

Retention data for two phases of moderate polarity, OV-7 and SE-52, are presented in Table VI. A 3% coating of the liquid phase on 60/80 Gaschrom Z in 6 ft x 2 mm columns was used. With the decreased polarity on the stationary phase, the order of elution was significantly changed. With these phases retention times generally increased in the order: NE < E < DM < NM < M < MT and PFP < TFA < HFB. Separation characteristics of phases of this type were less desirable than with more polar types; however, less on-column sample degradation was noted.

Columns prepared with a nonpolar stationary phase, OV-1 and a combination of this phase with a moderately polar phase, SE-52, were used to obtain the retention data presented in Table VII. Five percent coatings of the phases on Gaschrom Q, 60/80 mesh in 12 ft x 2 mm columns were used. The order of elution on OV-1 was

Table VI

CHROMATOGRAPHIC RETENTION DATA FOR CATECHOLAMINE FLUOROACYL DERIVATIVES ON MODERATELY POLAR LIQUID PHASES

Compound	Derivative	Retention Index on 3% OV-7	Retention Index on 3% SE-52
Epinephrine	TFA	15.82	15.64
	PFP	15.31	15.80
	HFB	16.08	16.80
Norepinephrine	TFA	15.78	15.56
	PFP	15.24	15.60
	HFB	15.96	16.60
Dopamine	TFA	16.60	15.96
	PFP	16.37	15.80
	HFB	17.01	16.80
3-Methoxytyramine	TFA	17.58	16.66
	PFP	17.34	16.68
	HFB	17.82	17.40
Normetanephrine	TFA	17.08	16.42
	PFP	16.56	16.44
	HFB	17.24	17.38
Metanephrine	TFA	17.34	16.68
	PFP	17.31	16.86
	HFB	17.61	17.76

Table VII

CHROMATOGRAPHIC RETENTION DATA FOR CATECHOLAMINE FLUOROACYL DERIVATIVES ON NON-POLAR LIQUID PHASES

Compound	Derivative	Retention Index on 5% OV-1	Retention Index on 5% OV-1: SE-52 1:1 w/w
Epinephrine	TFA	15.62	15.66
	PFP	15.92	15.88
	HFB	16.88	16.76
Norepinephrine	TFA	15.38	15.50
	PFP	15.62	15.66
	HFB	16.84	16.58
Dopamine	TFA	16.08	15.76
	PFP	15.96	16.12
	HFB	17.02	16.74
3-Methoxytyramine	TFA	16.48	16.30
	PFP	16.54	16.36
	HFB	17.26	16.92
Normetanephrine	TFA	16.22	16.14
	PFP	16.40	16.48
	HFB	17.36	16.98
Metanephrine	TFA	16.60	16.44
	PFP	16.86	16.86
	HFB	17.82	17.32

essentially the same as with the moderately polar phases discussed previously. With the increased column length and separation efficiency of OV-1, five of the six catecholamines mentioned initially could be separated as their PFP or HFB derivatives on this phase alone. By preparing a column containing equal amounts of OV-1 and SE-52 at 3% loading on Gaschrom Q, all six catecholamines could be efficiently separated as their PFP or TFA derivatives using a 12 foot column length. Figure 1 illustrates the separation of E, NE, DM, NM and M as both TFA and PFP derivatives on this column. With these liquid phases, preconditioning with ethyl pentafluoropropionate was not necessary to prevent on-column degradation even when TFA derivatives were chromatographed.

Certain acidic catecholamine metabolites require the formation of a derivative of the carboxylic acid functional group in addition to fluoroacylation of the other functional groups prior to gas-liquid chromatography. The methyl esters of L-dopa, dihydroxy phenylacetic acid (DC), homovanillic acid (HVA) and 3-methoxy tyrosine (MD) were prepared as described previously (Section II). After esterification and removal of excess reagents, fluoroacyl derivatives were prepared in an identical manner to that used with the catecholamines. Derivatives were chromatographed on 12 ft x 2 mm glass columns packed with 5% OV-1:SE-52 1:1 w/w on 60/80 mesh

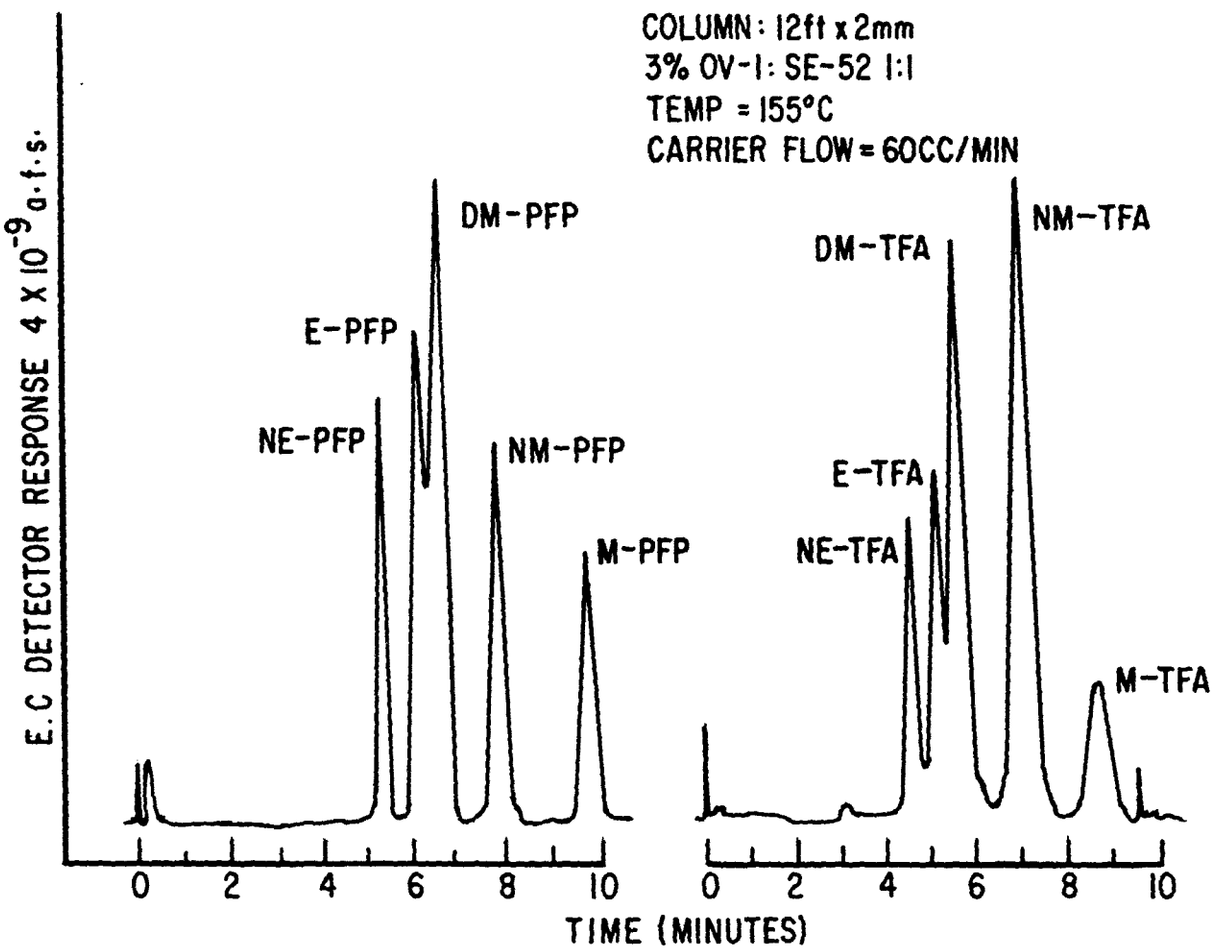

FIGURE 1. Chromatograms Illustrating the Separation of Trifluoroacetyl and Pentafluoropropionyl Derivatives of Five Catecholamines.

Gaschrom Q. The retention indices and E.C.D. response for the methyl ester-fluoroacyl derivatives of these compounds are listed in Table VIII. In addition, the fluoroacyl derivatives of β-methoxy dopamine and N-methyl β-methoxy dopamine were also chromatographed under the same conditions and are included in this table. These two compounds are quantitatively formed when norepinephrine and epinephrine are treated in the same manner used to form methyl esters of the acidic catecholamine metabolites. Chromatographic characteristics, stability, and E.C.D. response for these compounds did not differ appreciably from other catecholamine fluoroacyl derivatives. The TFA derivatives were, in all cases, the least stable of the three derivative types and had the lowest retention indices for each respective compound. The TFA derivatives also elicited the lowest E.C.D. response. The E.C.D. response elicited by all three derivatives of dopa was considerably less than for other catecholamine metabolites investigated. All compounds were sufficiently stable as their methyl-PFP or methyl-HFB derivatives to store in benzene solution for several weeks without significant decomposition. Peak shapes were symmetrical for all compounds regardless of derivative type.

Table VIII

CHROMATOGRAPHIC RETENTION DATA AND E.C.D. RESPONSE FOR FLUOROACYL DERIVATIVES OF ACIDIC CATECHOLAMINE METABOLITES

Compound	Derivative	Retention Index* (M.U.)	E.C.D. Response* cm^2/ng
L-dopa	TFA	16.56	0.210
	PFP	16.86	0.446
	HFB	17.89	0.475
Dihydroxy phenyl acetic acid (DC)	TFA	14.28	0.942
	PFP	14.78	1.460
	HFB	15.66	1.692
Homovanillic acid (HVA)	TFA	15.21	.876
	PFP	15.52	1.218
	HFB	16.42	1.392
3-methoxytyrosine	TFA	17.00	4.468
	PFP	17.20	0.892
	HFB	18.62	0.900
β-methoxy dopamine	TFA	16.00	0.585
	PFP	16.30	1.309
	HFB	17.30	1.348
N-methyl, β-methoxy dopamine	TFA	16.10	.585
	PFP	16.56	1.406
	HFB	17.92	1.487

Characterization of the pentafluoropropionyl derivatives of catecholamine metabolites: The PFP derivatives of E, NE, M, NM, DM, DC, HVA and L-dopa were examined by mass spectrometry to elucidate the structures of these derivatives. In the case of the acidic compounds, spectra of the methyl esters were obtained rather than of the free acids since esterification was necessary for G.L.C. Originally, DC was not considered in the investigation; however, during subsequent analyses, the compound appeared as an unknown chromatographic peak in urinary extracts. Mass spectral data was obtained for HVA to provide a reference aid in the identification of the purified unknown.

Mass spectra of the derivatives above are shown in Figures 2 through 5. As can be seen from the spectra, four of the amines (E, NE, M, NM) fragment in a similar manner. None of the four provide significant molecular (M^+) ion peaks. Major peaks in the spectrum for the primary amines NE and NM represent the $M^+ - H_2NCOC_2F_5$ and $M^+ - CH_2HNCOC_2F_5$ fragments with a smaller peak representing the $CH_2NHCOC_2F_5$ fragment alone. In the case of the secondary amines, E and M, the largest peak was the $CH_2CH_3NHCOC_2F_5$ fragment with smaller peaks evident at $M^+ - CH_2CH_3NHCOC_2F_5$ and $M^+ - CH_3NHCOC_2F_5$. The spectrum from DM was slightly different. In this case, the largest peak represented the $M^+ - NHCOC_2F_5$ fragment while the $M^+ - CH_2NHCOC_2F_5$ fragment

is much less abundant. In this spectrum a large peak is also in evidence at 176 m/e as was the case with the other primary amines. Derivatives of the amino acid, L-dopa, produced a more complex but similar spectrum. The major fragment for this derivative once again represented the $M^+ - NHCOC_2F_5$ ion; however, another large peak (415) represented cleavage between the α and β carbon atoms of the aliphatic chain. The molecular ion was present but a much more significant feature of the spectrum was the peak representing the loss of the ester methyl group from the parent ion. Other peaks apparent in the spectrum are due to rearrangements which occur in the mass spectrometer.

The catechol acids, HVA and DC, produced the most abundant molecular ion of all the compounds analyzed by mass spectrometry. In addition to the molecular ion, peaks representing $M^+ - COOCH_3$ and $M^+ - COC_2F_5$ were significant features of the spectra. The unknown material isolated from urine and DC produced identical mass spectra, thus confirming the identification of this compound.

Several of the PFP derivatives of the catecholamine metabolites were further examined by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy. Figure 6 illustrates the NMR spectrum of PFP-DM. The three areas of adsorption in the spectrum were located at 2.48, 3.17 and 6.67 ppm (δ). Ratios of the areas of the peaks

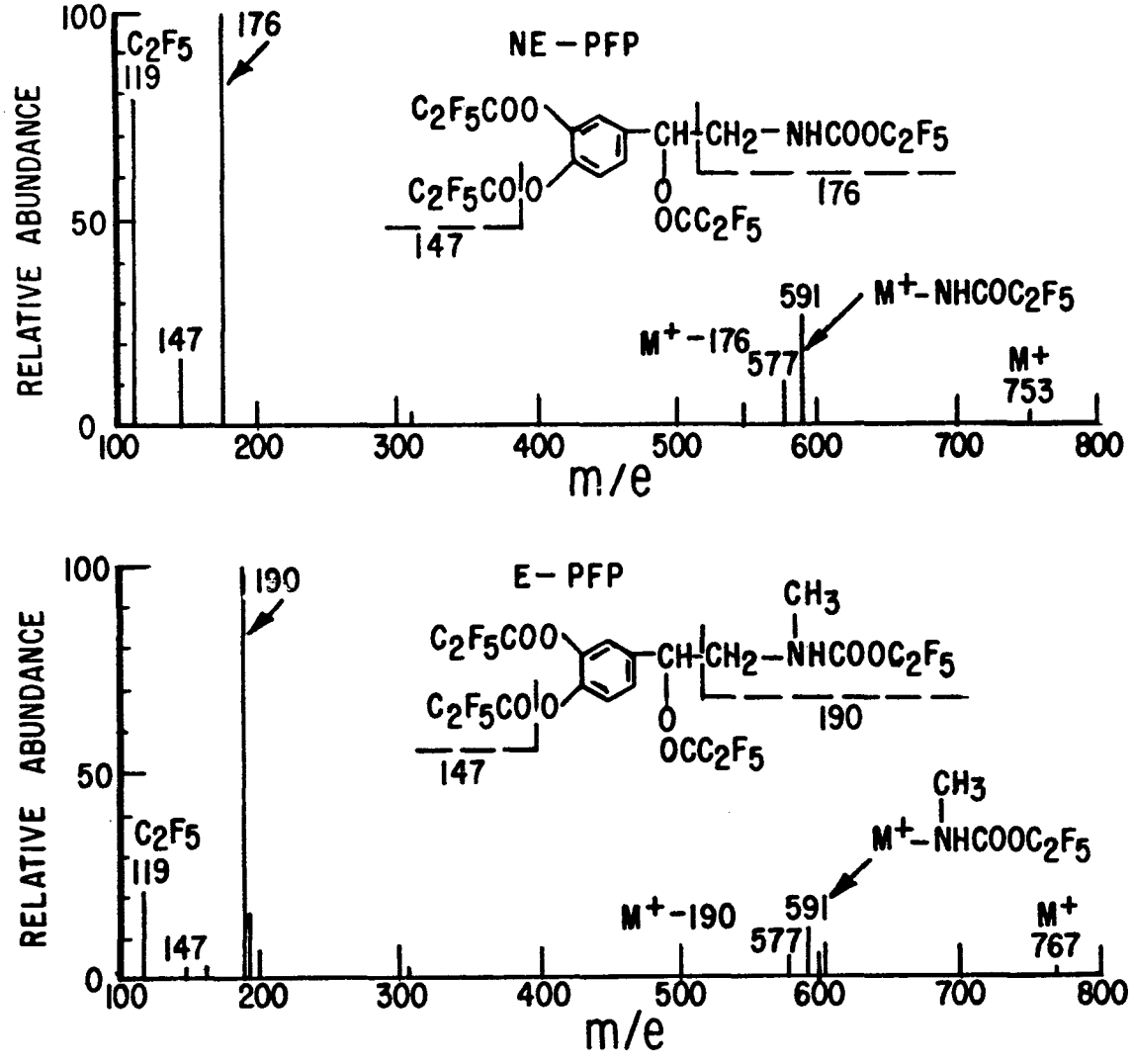

FIGURE 2: Mass Spectra of Epinephrine and Norepinephrine Pentafluoropropionyl Derivatives.

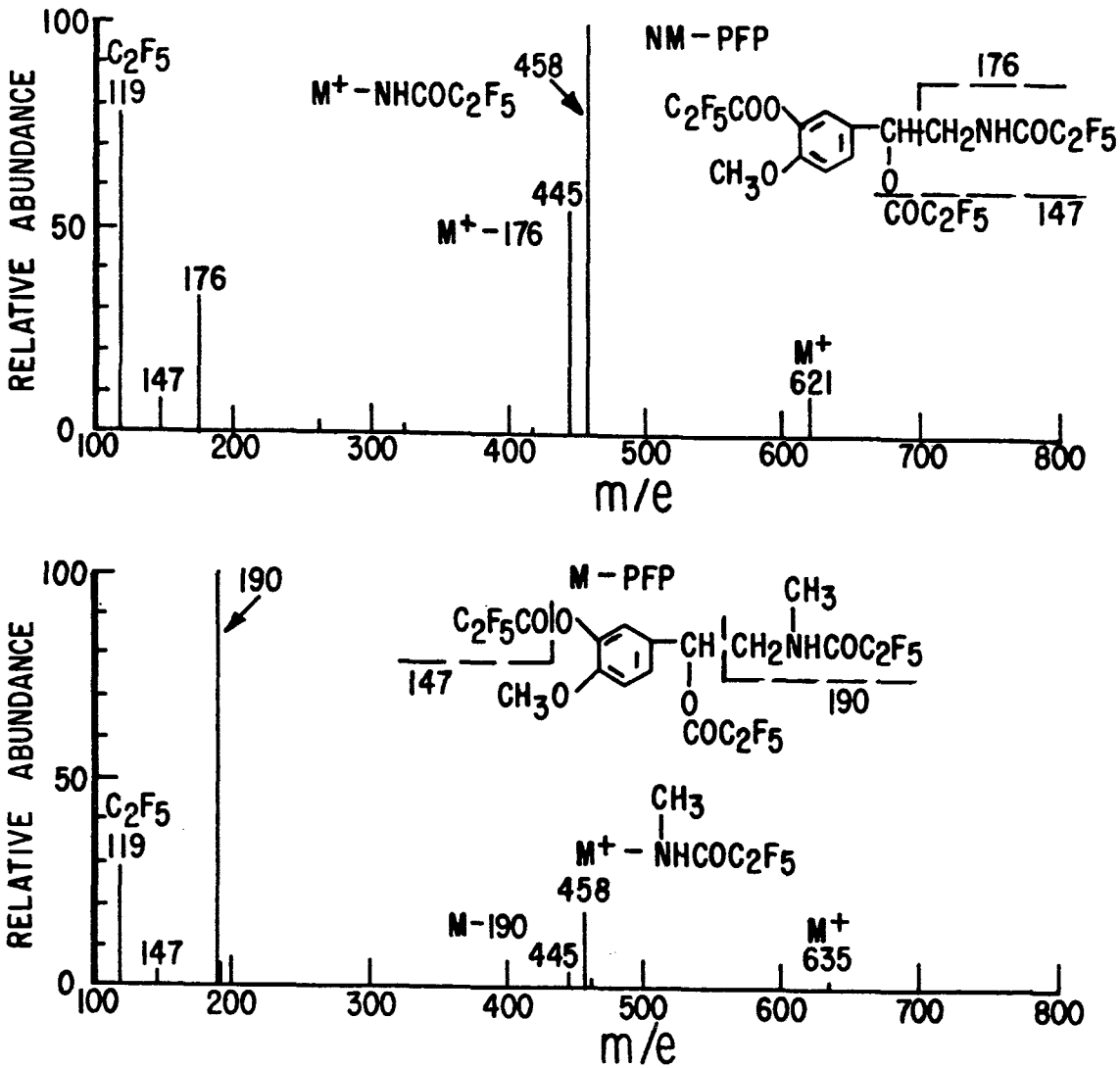

FIGURE 3. Mass Spectra of Metanephrine and Normetanephrine Pentafluoropropionyl Derivatives.

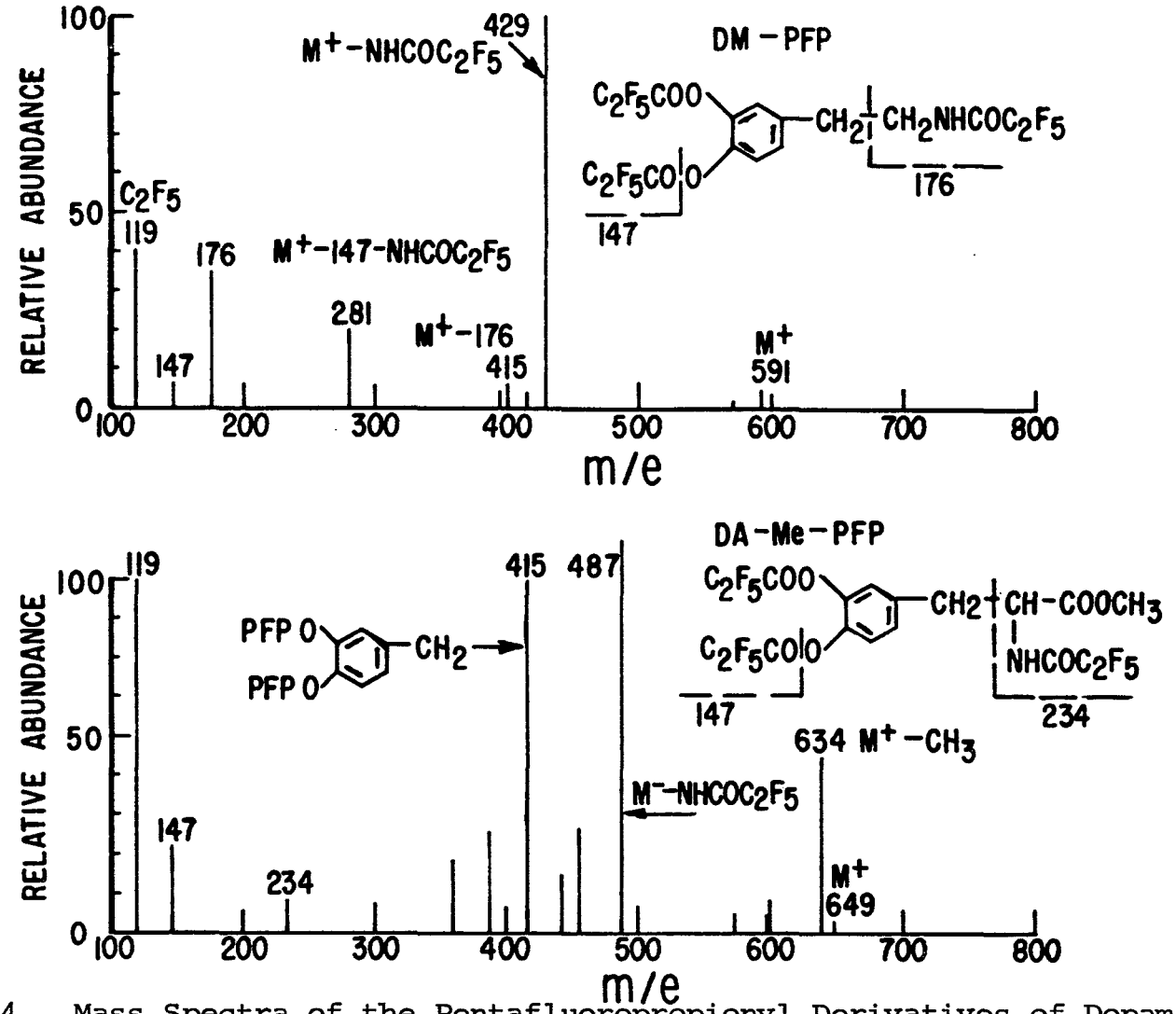

FIGURE 4. Mass Spectra of the Pentafluoropropionyl Derivatives of Dopamine and the Methyl Ester of L-Dopa.

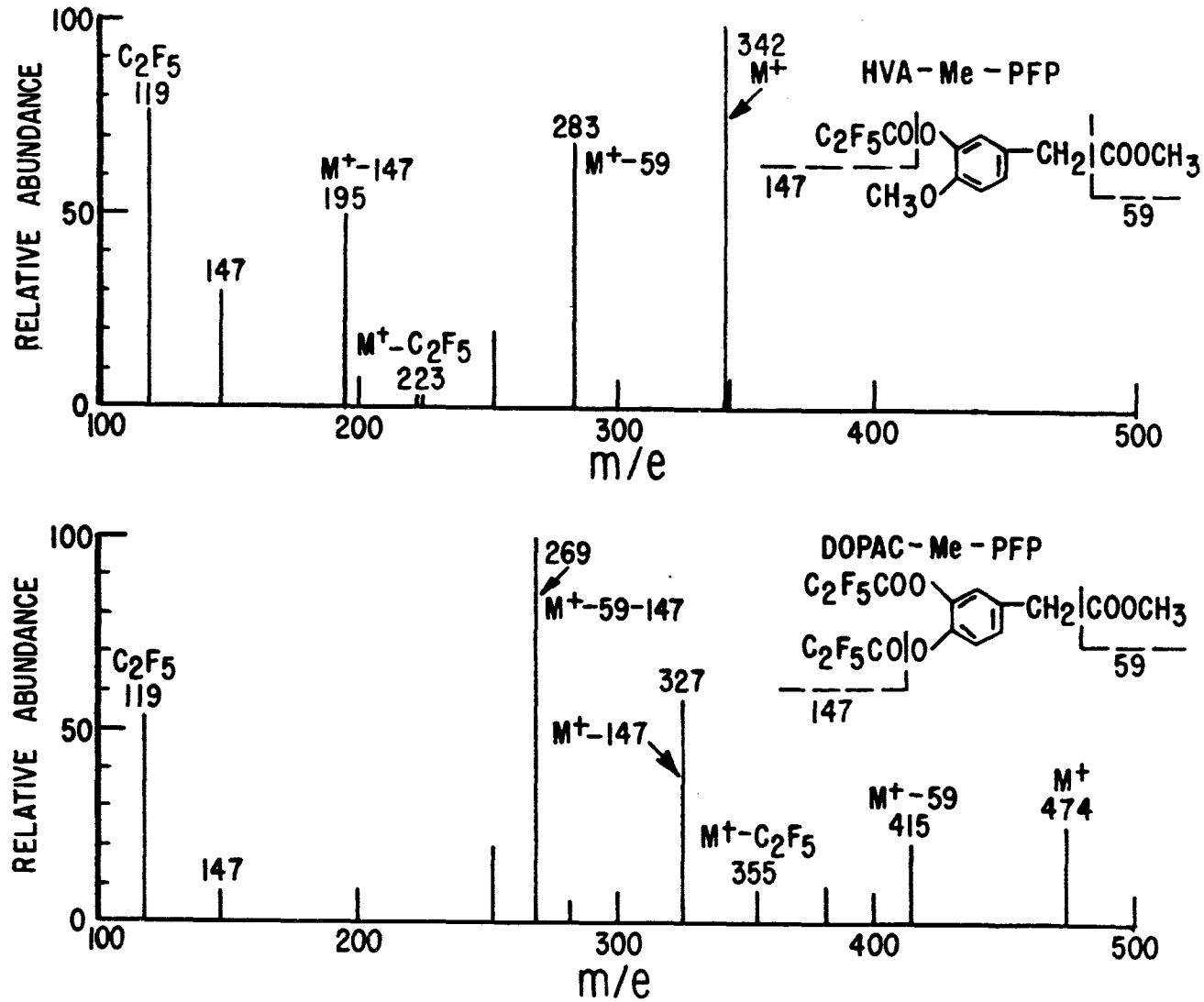

FIGURE 5. Mass Spectra of the Pentfluoropropionyl Derivatives of the Methyl Esters of Homovanillic Acid and Dihydroxyphenylacetic Acid.

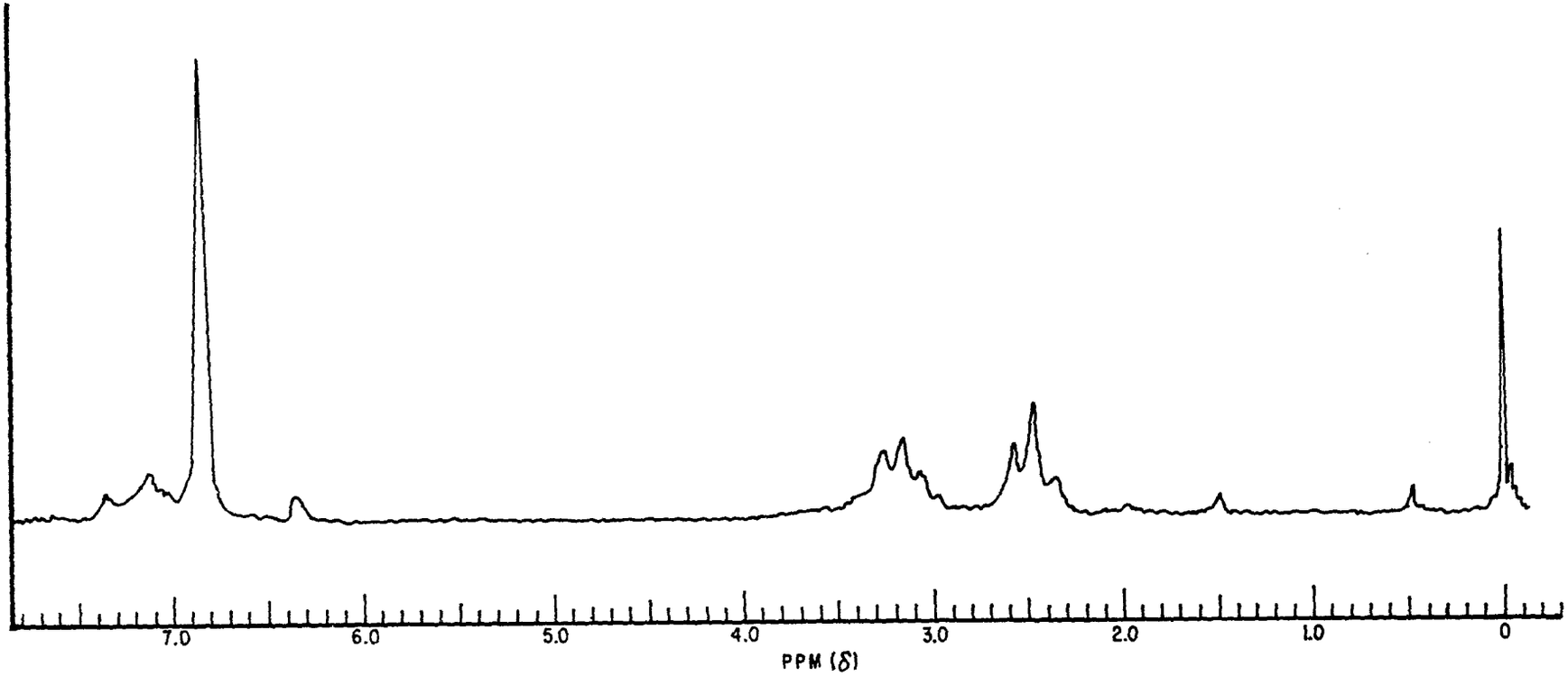

FIGURE 6. Nuclear Magnetic Resonance Spectrum of Pentafluoropropionyl-Dopamine.

obtained by integration was 2:2:4. The two proton triplet at 2.48 ppm apparently results from the protons of the methylene group adjacent to the aromatic ring, the two proton quartet at 3.17 ppm represents the methylene protons adjacent to the amide nitrogen while the singlet at 6.67 ppm is due to the overlapping of the three ring protons and the amine proton. NMR spectra of other catecholamine PFP derivatives were similar and slightly more complex due to additional methyl group protons which appear at lower ppm values and a general shifting to slightly higher ppm values for the other protons. The absence of any absorptions due to hydroxyl protons provided further evidence to support the existence of a single fully fluoroacylated derivative for each of the individual catecholamines.

The infrared spectrum of PFP-DM is shown in Figure 7. Once again all of the PFP catecholamine derivatives produced very similar spectra. Major areas of absorption can be seen in the amide (5.9μ) and ester (5.6μ) carbonyl regions of the spectrum. Stretching vibrations of the N-H group are visible at 3.0 microns. Strong absorption is also present in the region from 7.2 to 9.0 microns as well as the sharp peaks at 6.25, 6.42, 6.65 and 6.9 μ all of which are due to the aromatic nature of the compounds. After the above mentioned analyses were

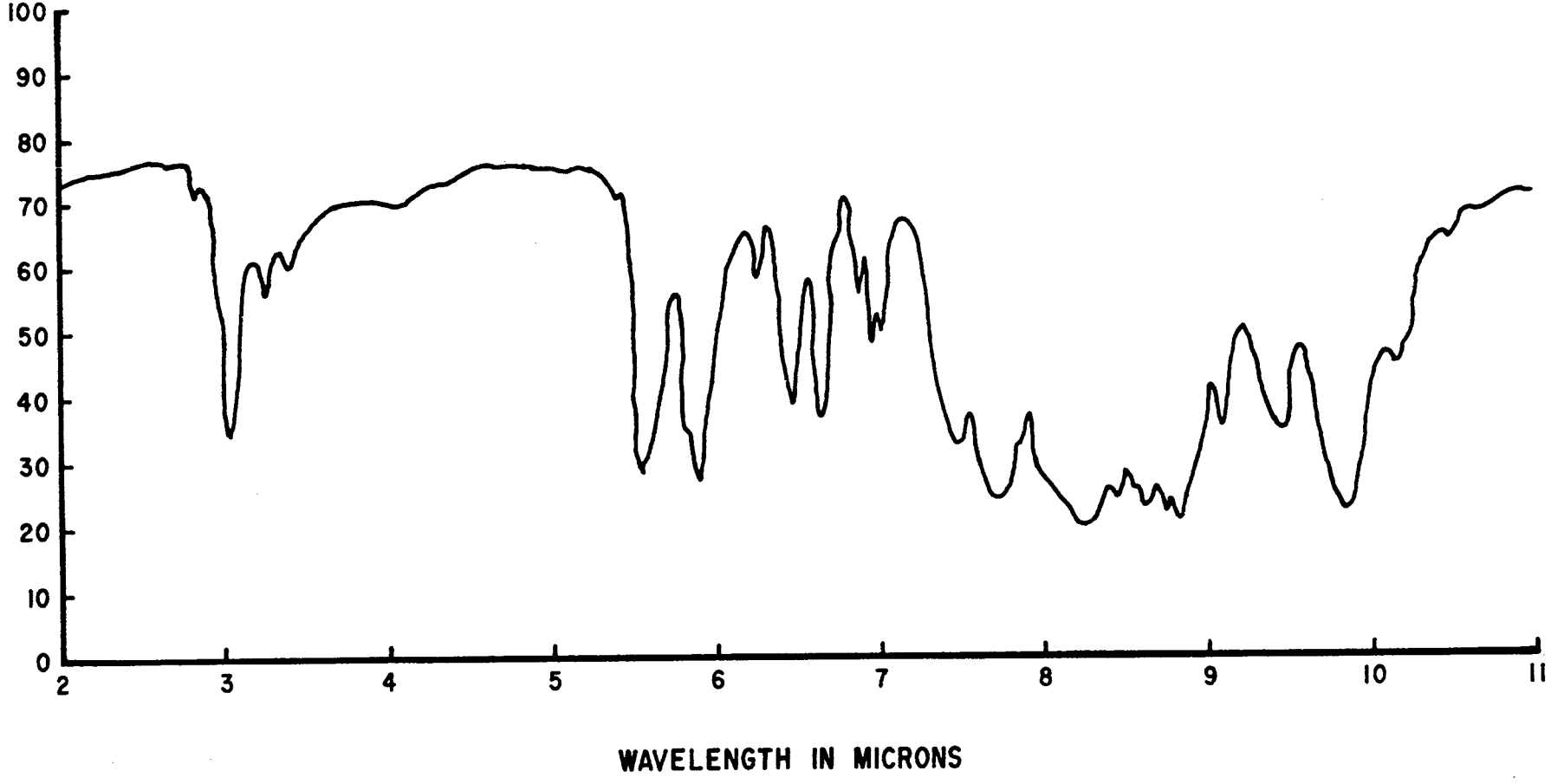

FIGURE 7. Infrared Spectrum of Pentafluoropropionyl-Dopamine.

performed, the PFP-DM was recrystallized from benzene to obtain a white crystalline material with melting point of 72 ± 1° C.

Evaluation of methods used for the extraction of catecholamine metabolites from biological materials: Three different procedures for the separation of catecholamines and their metabolites from other compounds present in biological materials were evaluated. Specifically these procedures included: 1. separation by precipitation of proteinaceous materials; 2. separation by gel filtration and, 3. separation by selective adsorption. The final step in all of these procedures was the removal of water from the sample by lyophylization to provide anhydrous conditions for subsequent derivative synthesis and G.L.C.

The criteria used for evaluation of these procedures were: 1. efficiency of the separation; 2. number of catecholamines recovered; 3. time requirement; and 4. accuracy and precision of recovery. Separation efficiency and recovery were measured by adding small volumes of a standard solution of the catecholamines of interest to the biological material, extracting with one of the above procedures, followed by derivative formation and G.L.C. The variety of catecholamines recovered was determined in a like manner except that a solution of a single catecholamine was added to the biological material

rather than adding a solution containing several catecholamines. Time required for each procedure was measured from the time of addition of the catecholamine solution to the time when a dry sample suitable for derivative formation was obtained.

The simple procedure of derivative synthesis and G.L.C. of the catecholamines contained in a dry protein-free filtrate allowed the extraction of the greatest variety of catecholamines from a single sample in addition to being the least time consuming. Most common protein precipitating agents such as trichloroacetic acid or perchloric acid were not useful for this purpose due to their introduction of impurities detectable by E.C.D. Trifluoroacetic acid in 20% aqueous solution did not present this drawback. Its high vapor pressure allowed its removal with the water during dehydration of the sample. As can be seen in Table IX recoveries of added catecholamines from whole blood were very good at high catecholamine concentrations; however, they became much less with decreasing concentration. Chromatographic backgrounds were sufficiently low to permit chromatography with concentrated samples, but were unacceptable due to trailing peaks when high sensitivity detection was required. Chromatographic peaks were considerably less symmetrical than those obtained when derived from standard aqueous solutions. It is proposed that the asymmetrical nature of the peaks was due to the

Table IX

RECOVERY OF ADDED CATECHOLAMINES FROM WHOLE BLOOD AFTER PROTEIN PRECIPITATION WITH TRIFLUOROACETIC ACID

Compound Added	Amount Added mg/ml	Average* Amount Recovered mg/ml	Percentage Recovery
Epinephrine	1.0	0.92	92
Norepinephrine	1.0	0.94	94
Dopamine	1.0	0.86	86
Epinephrine	0.5	0.43	86
Norepinephrine	0.5	0.41	82
Dopamine	0.5	0.45	90
Epinephrine	0.1	0.084	84
Norepinephrine	0.1	0.079	79
Dopamine	0.1	0.079	79
Epinephrine	0.01	0.0056	56
Norepinephrine	0.01	0.0044	44
Dopamine	0.01	0.0047	47

*Average of five samples analyzed at each concentration.

formation of trifluoroacetate salts during the precipitation which prevents fluoroacylamide derivatives from forming. These salts can be formed with various amines such as β-phenyl ethylamine and have sufficient volatility to be chromatographed with resulting asymmetric peaks.

The use of gel filtration on Sephadex G-10 was effective in separating many catecholamines from other biological compounds. Figure 8 illustrates the elution pattern obtained by the gel filtration of one milliliter of plasma containing 10 μg NE and 5.1 μg E by the procedure outlined in Section II. The proteinaceous material from the plasma eluted rapidly from the column and no high molecular weight materials were present after 2.5 void volumes (11.5 ml) had passed through the column. After 2.5 void volumes, E was detected in the column effluent, and continued through 5.5 void volumes while NE was detected at 3.0 void volumes and continued through 6.0 void volumes. The entire catecholamine fraction was contained in a total of 16.1 ml. Other catecholamines had elution volumes similar to E and NE with the primary amines being retained slightly longer than the corresponding secondary amine. All of the catecholamines were sufficiently separated from other interfering materials to permit chromatography at high detector sensitivity. Recovery of E and NE from plasma samples to which these amines had been added averaged 89.9% and 85.5% respectively

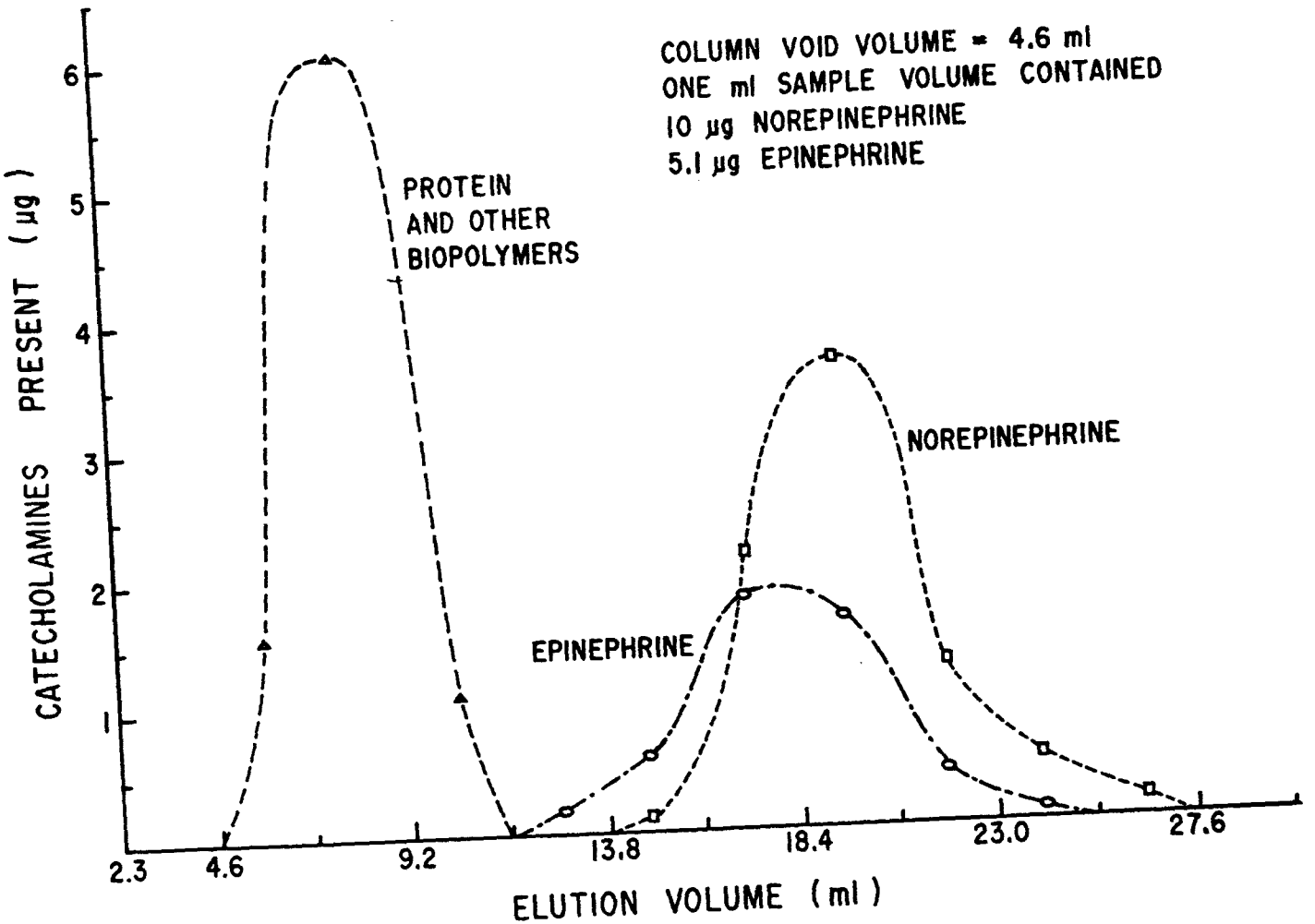

FIGURE 8. Elution Diagram of the Separation of Norepinephrine and Epinephrine from Plasma Using Gel-Filtration.

on samples containing from 0.05 to 10.0 μg/ml of each catecholamine. As can be seen in Table X, recoveries decreased slightly with decreasing concentration. This may be due to losses experienced during the lyophylization of the very dilute solutions obtained after filtration. While the use of gel filtration has the advantages of good recovery and separation efficiency, the time required was excessively long, averaging in excess of 8 hours per sample.

Two variations of a third procedure for catecholamine extraction were pursued. This method employed a selective adsorption of certain catecholamines on activated alumina by a method similar to that employed by Anton and Sayre (1962). Following the adsorption step and subsequent dehydration by freeze-drying, the adsorbed catecholamines were eluted from the alumina and derivatives were formed simultaneously. All non acidic catecholamines could be converted to fluoroacyl derivatives and removed from the alumina by using the appropriate fluoroacyl acid anhydride in acetonitrile solution, which was employed directly with the dry catecholamines on alumina. When analysis for any of the acidic catecholamine metabolites was desired, methyl esters could be formed with simultaneous elution by using 2N HCl in anhydrous methanol. Fluoroacyl derivatives could subsequently be formed from these methyl esters after removal of the acid-methanol under vacuum. Only amines or

Table X

RECOVERY OF CATECHOLAMINES ADDED TO BLOOD PLASMA AFTER GEL-FILTRATION ON SEPHADEX G-10

Compound Added	Amount Added µg/ml	Average Amount Recovered µg/ml	Percentage Recovery
Norepinephrine	5.11	4.41	86.3
Epinephrine	9.89	9.12	92.2
Norepinephrine	1.10	0.98	89.1
Epinephrine	0.98	0.92	93.9
Norepinephrine	0.470	0.424	90.2
Epinephrine	0.536	0.487	90.8
Norepinephrine	0.121	0.102	84.3
Epinephrine	0.110	0.097	88.2
Norepinephrine	0.054	0.042	77.8
Epinephrine	0.052	0.044	84.6

*Average of five samples analyzed at each concentration.

acids containing the dihydroxy phenyl structure could be extracted by this method due to the selectivity of the alumina. All methoxy metabolites such as M, NM and MT were lost during the washings of the alumina.

Recoveries of certain catecholamines from plasma, urine, spinal fluid and deproteinated tissue homogenates are shown in Tables XI and XII. Results shown in Table XI were obtained using plasma and urine to which E and NE had been added and elution accomplished with 20% pentafluoropropionic anhydride in acetonitrile. Table XII shows the recoveries obtained using acid:methanol elution with deproteinated brain tissue homogenates with added L-dopa, DM and NE. Average recoveries of added catecholamines by both variations of this procedure over the concentration range of 0.01 to 1.0 μg per ml plasma, urine or spinal fluid and from 0.05 to 5.0 μg per gram of brain tissue were in excess of 80%. Erratic variation in recoveries is due to irreproducible losses experienced during washing and freeze-drying of the alumina. It should be noted that when NE and E are eluted from alumina with 2.0N HCl: methanol, they are quantitatively converted to β-methoxy, 3, 4-dihydroxyphenyl ethylamine and β-methoxy, N-methyl 3, 4-dihydroxyphenyl ethylamine respectively. These compounds, as their tri-PFP derivatives, can be separated from each other and other catecholamines extracted by this method on 12 ft x 2 mm I.D. columns packed with 3% OV-1:SE-52 1:1 w/w on 60/80 mesh Gaschrom Q (Figure 9).

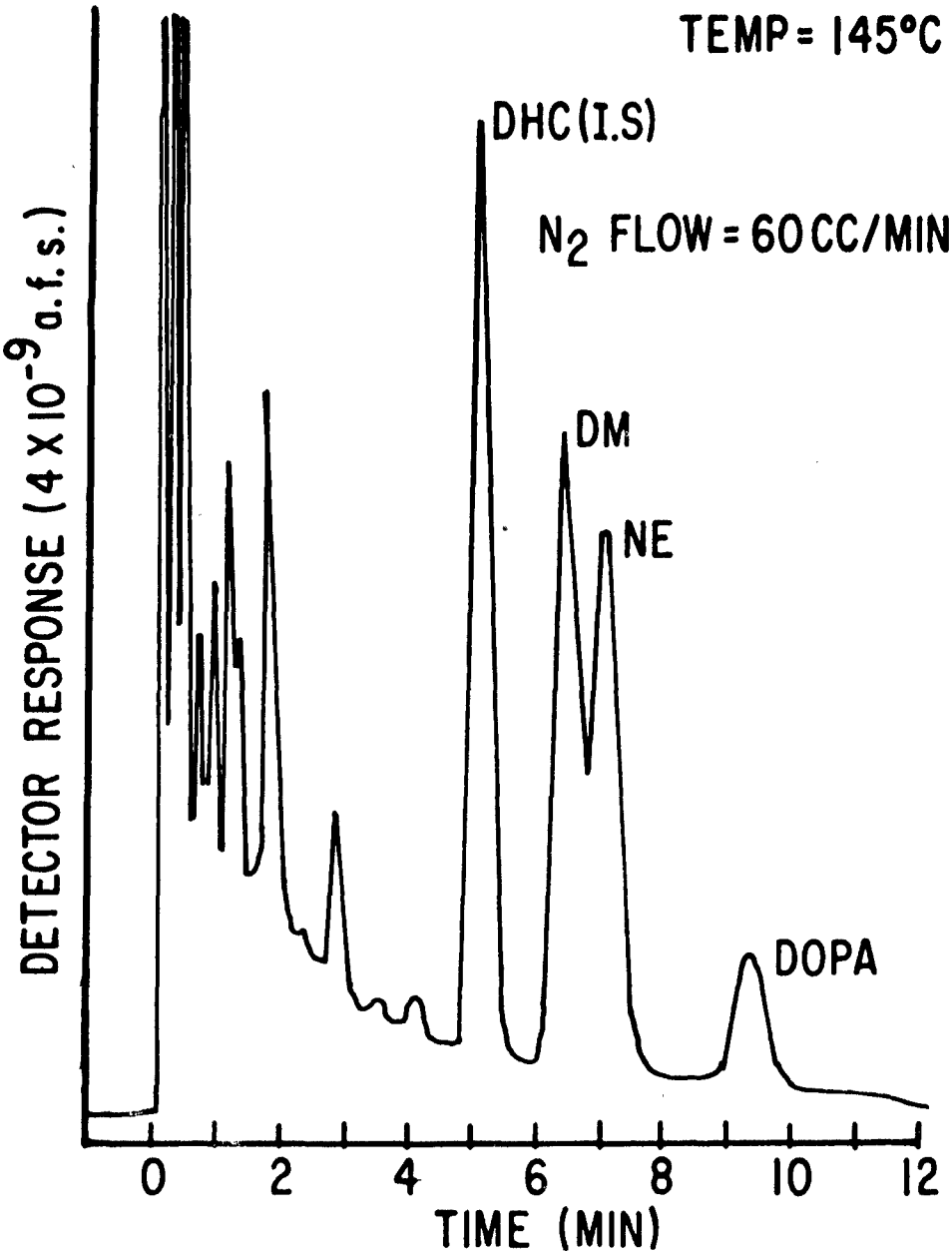

Figure 9: Chromatogram of a Brain Tissue Homogenate Sample Containing Added Dopamine, Norepinephrine and Dopa.

Table XI

RECOVERY OF CATECHOLAMINES ADDED TO BLOOD PLASMA OR URINE AFTER ALUMINA ADSORPTION AND DIRECT DERIVATIVE FORMATION

Compound	Sample	Amount Added to 1 ml Sample (μg)	Average Amount Recovered* (μg)	Recovery Percentage
Epinephrine	Plasma	1.104	1.063 ± .12	96.3
Norepinephrine	Plasma	1.316	1.171 ± .13	89.0
Epinephrine	Urine	0.964	0.845 ± .12	87.7
Norepinephrine	Urine	1.041	0.884 ± .13	84.9
Epinephrine	Plasma	0.241	0.215 ± .03	89.2
Norepinephrine	Plasma	0.207	0.191 ± .04	92.3
Epinephrine	Urine	0.198	0.161 ± .02	81.3
Norepinephrine	Urine	0.226	0.173 ± .03	76.6
Epinephrine	Plasma	0.047	0.041 ± .009	87.2
Norepinephrine	Plasma	0.062	0.052 ± .007	83.8
Epinephrine	Plasma	0.012	0.011 ± .004	92.0
Norepinephrine	Plasma	0.018	0.016 ± .007	89.0

*Average of five samples at each concentration ± maximum deviation from the mean.

Table XII

RECOVERIES OF ADDED DOPA, DOPAMINE AND NOREPINEPHRINE FROM BRAIN HOMOGENATES AFTER ALUMINA ADSORPTION AND INDIRECT DERIVATIVE FORMATION

Compound	Amount Added (μg/g Tissue)	Average Amount Recovered* (μg/g Tissue)	Percentage Recovery
Dopamine	13.625	13.407 ± .402	98.4
Norepinephrine	13.450	12.939 ± .464	96.2
Dopa	12.000	10.920 ± .458	91.0
Dopamine	5.452	5.250 ± .100	96.3
Norepinephrine	5.380	5.186 ± .109	96.4
Dopa	4.810	4.315 ± .137	89.7
Dopamine	1.090	1.064 ± .022	97.6
Norepinephrine	1.078	1.019 ± .030	94.5
Dopa	0.960	0.893 ± .040	93.0
Dopamine	0.545	0.514 ± .028	94.3
Norepinephrine	0.538	0.522 ± .017	97.0
Dopa	0.481	0.433 ± .022	90.0
Dopamine	0.218	0.212 ± .012	97.2
Norepinephrine	0.216	0.215 ± .020	99.5
Dopa	0.192	0.168 ± .007	87.5
Dopamine**	5.452	4.961 ± .476	91.0
Norepinephrine**	5.380	5.025 ± .431	93.4
Dopa**	4.810	4.161 ± .482	86.5

Table XII (continued)

Compound	Amount Added (μg/g Tissue)	Average Amount Recovered* (μg/g Tissue)	Percentage Recovery
Dopamine**	0.545	0.483 ± .053	88.6
Norepinephrine**	0.538	0.584 ± .062	90.1
Dopa**	0.481	0.404 ± .059	84.0

*Average amount recovered calculated on the basis of four determinations for each data point ± maximum deviation from the mean.

**Recoveries calculated using external standard. Internal standard employed in all other determinations.

The sensitivity of the extraction procedure is enhanced when the acid: methanol variation is employed. Apparently a number of by-products are formed when pentafluoropropionic anhydride contacts alumina and these products cause large chromatographic peaks which trail into the catecholamine region of the chromatograms when low attenuations are employed. Since the acid anhydride does not directly contact the alumina when acid: methanol elution is employed, derivatives can be dissolved in micro amounts of solvent (50 to 100 microliters) thus concentrating the catecholamines without increasing interferences to the point that they become unacceptable. Average time required for the extraction of the catecholamines from four samples is 3 hours or 45 minutes per sample for the second variation and 2 hours for four samples with the first variation.

Since the loss of alumina after adsorption of catecholamines results in somewhat erratic recoveries, an internal standard which could be added to the samples prior to adsorption and carried through the entire procedure was advisable. To be useful as an internal standard, a compound must be 1. quantitatively adsorbed on alumina, 2. form derivatives similar to those formed by the catecholamines, 3. be easily separable from the compounds of interest by G.L.C. and 4. not be normally found in the biological samples to be analyzed. Compounds

with extremely short or long retention indices (< 10 M.U. or > 20 M.U.) are also undesirable. Compounds with structures similar to that of certain catecholamines were evaluated. Table XIII compares a number of these compounds in regard to their adsorption and chromatographic characteristics. Retention indices for those compounds of interest which were extractable with alumina and acid: methanol are listed at the bottom of the table. A difference in retention indices of 0.18 M.U. is required for complete separation with the chromatographic conditions used. As can be seen from the table, 3, 4-dihydroxyhydrocinnamic acid $\left[(HO)_2C_6H_3CH_2CH_2COOH\right]$ (DHC) meets all of the requirements for an internal standard mentioned above. When added to plasma samples along with DM and processed through the alumina; acid-methanol method, identical recoveries of added materials were obtained. This indicates that both compounds behave alike in this method and that less than 100% recoveries experienced are due to similar losses during the procedure. Sensitivity of the E.C.D. to the PFP-DHC derivative is 4.6 times less than to PFP-DM. The internal standard (DHC) was added to the samples as an aqueous solution of a concentration of 5 μg/ml. This solution was kept refrigerated when not in use where it could be stored for at least five days without detectable loss.

Table XIII

A COMPARISON OF THE CHARACTERISTICS OF VARIOUS COMPOUNDS THAT AFFECT THEIR USEFULNESS AS INTERNAL STANDARDS

Compound	Retention Index*	Adsorbs on Alumina	Derivative Formed	Naturally Occurring
α-Methyl dopa (Aldomet)	16.64	yes	Methyl PFP	no
α-Propyl dopamine	15.87	yes	PFP	no
Isoproterenol (Isuprel)	16.25	yes	PFP	yes
3-Methoxy tyrosine	17.20	no	Methyl PFP	yes
Bromo dopamine	17.92	yes	PFP	no
Dibromo dopamine	18.58	yes	PFP	no
Tribromodopamine	19.16	yes	PFP	no
Dihydroxycinnamic acid	16.40	yes	Methyl PFP	no
Dihydroxyhydrocinnamic acid	15.62	yes	Methyl PFP	no
Tyrosine	16.11	no	Methyl PFP	yes
Benzedrine	13.78	no	PFP	no
α-Methyl dopamine (epinine)	16.21	yes	PFP	yes
6-Hydroxy dopamine	15.58	yes	PFP	no

Table XIII (continued)

Compound	Retention Index*	Adsorbs on Alumina	Derivative Formed	Naturally Occurring
3,4-Dihydroxyphenyl acetic acid	14.78	yes	Methyl PFP	yes
L-dopa	16.86	yes	Methyl PFP	yes
Dopamine	16.12	yes	PFP	yes
Epinephrine	16.56	yes	β-O-Methyl PFP	yes
Norepinephrine	16.30	yes	β-O-Methyl PFP	yes

*Determined on 12 ft x 2 mm I.D. column packed with 5% OV-1:SE-52; 1:1 on 60/80 mesh Gaschrom Q.

IV. DISCUSSION

The development in this study of a G.L.C. method for the measurement of several catecholamines and their metabolites in biological materials involved the investigation and evaluation of many procedures and ultimately the incorporation of the most effective of those procedures into a total analytical method. Specifically, procedures were evaluated for 1. the collection and preservation of the biological samples; 2. the removal of the compounds of interest from other biological compounds in the sample; 3. the preparation of the extracted compounds for G.L.C. and 4. the selection of effective G.L.C. conditions.

Since catecholamine metabolites are known to be present in varying quantities in the blood, urine, CSF and many tissues of most higher animals, effective methods for their measurement had to be compatible with any of these materials. Regardless of the sample type, precautions were taken to avoid alteration of the catecholamines by both oxidative and enzymatic degradation. In liquid samples such as blood, urine and CSF the use of the reducing agent, sodium metabisulfite $Na_2S_2O_5$ proved effective in preventing oxidative changes in certain catecholamines

due to the presence of dissolved oxygen and other oxidizing agents in the sample. Other antioxidants such as ascorbic acid and sodium thiosulfate were used at various times in this investigation; however, neither were as effective as $Na_2S_2O_5$. In the case of whole blood samples, $Na_2S_2O_5$ was added as the crystalline solid to raise the osmolality of the solution thus preventing hemolysis of the red cells which occurs upon addition of aqueous solutions of low salt concentration to blood. Either crystals or aqueous solutions were acceptable for addition to CSF, tissue or urine samples. Changes in the catecholamines due to enzymes contained in the biological fluids can be significantly retarded by acidification with HCl. An equal volume of 0.1 N HCl was sufficient to prevent the metabolism of the most susceptible catecholamines contained in the biological fluid. The use of HCl has the added advantage of converting all of the amines of interest to their hydrochloride salts which are considerably more stable than the free amines or conjugates. Where storage of the samples is required, enzymatic activity can be reduced to an insigificant level for periods in excess of one week by acidification to pH 2 combined with refrigeration at 0° C. Samples from the various tissues required different procedures to prevent degradation of the compounds of interest. The rapid freezing of the organs by immersion in liquid nitrogen was a convenient and effective way to

prevent all degradation. After sectioning of the frozen organ, the sections were kept frozen until homogenization in an acid media. The use of cold perchloric acid (0.4N) as a homogenization medium was effective. Enzymes contained in the tissue were inactivated rapidly by this acid preventing metabolic changes in the compounds. The low pH ($\sim$1.5) obtained during homogenization was sufficient to retard or eliminate oxidation by inorganic oxidizing agents such as dissolved O_2.

The use of an extraction procedure similar to that of Anton and Sayre (1962) provided the most efficient removal of E, NE, dopa, DM and DC from all biological samples. This procedure has several advantages over other procedures tested in this study. Most significant of these was the employment of alumina as a selective adsorbant. Only those compounds containing adjacent hydroxyl groups on an aromatic ring (i.e. catechol structure) in conjunction with an aliphatic chain containing either a carboxyl or amine (or both) at the end of the chain opposite the aromatic structure were adsorbed. This selectivity proved advantageous both from the standpoint of freedom of G.L.C. samples from interfering materials as well as providing an effective means of concentrating the catecholamines in relatively large volumes of fluid. While samples of urine, plasma and spinal fluid used in this investigation seldom exceeded 1 ml, volumes of up to

10 ml could easily have been extracted on the same amount of alumina (200 mg). The selectivity of the alumina used for extraction has, however, the disadvantage that all of the catecholamine metabolites with 3-0 methyl groups, such as M and NM, are lost during the extraction step. In order to analyze for these metabolites, an additional extraction of the alumina-extracted sample would have been necessary. Since the major aim of the toxicological portion of this study was the measurement of dopa, DM and NE, no effort was necessary to develop an analytical schedule for metabolites with methoxy groups at position 3.

For the small sample volumes employed in this study (< 2 ml), 200 mg of alumina was an optimum amount. The use of smaller amounts resulted in depressed recoveries of catecholamines due to the higher percentage loss of alumina particles and concomitantly higher losses of the adsorbed compounds. The use of larger amounts of alumina offered no significant advantages. The pH of 8.6 ± 0.1, recommended by Anton and Sayre (1962), resulted in the highest recoveries of added compounds. While the use of disodium ethylenediamine tetra acetic acid (Na_2EDTA) did not appear to significantly affect the percentage recoveries as suggested by the above authors, 1 ml of a solution containing 100 mg of this acid was added to all samples during adsorption due to its excellent buffering properties. A stable pH of 8.6 was difficult to attain without the

addition of Na_2EDTA. The alumina samples could be washed with distilled water as many as six to eight times without significant desorption of the catechols. Since three or four washes were sufficient to remove all contaminants which would interfere with subsequent analysis, additional washings were not considered necessary. It was necessary, however, to add a small volume of 0.1N HCl (0.2 ml) to protect the adsorbed catecholamines from oxidation during subsequent lyophylization. The alumina extraction procedure was employed with plasma, urine, CSF and brain tissue filtrates as the sample with similar separation efficiencies and recoveries regardless of sample type.

Molecular sieving on Sephadex G-10 provided a method of separation of catecholamines from other biological material; however, this method had serious deficiencies when compared to sample separation by adsorption on alumina. While plasma, urine and CSF samples could be extracted by sieving, protein-free filtrates of tissue samples could not be prepared due to reaction between the gel and the perchloric acid in the filtrate. In addition, time required to achieve separation was considerably longer than the alumina adsorption procedure previously discussed. At least 55 minutes was required to filter a single sample while alumina adsorption required only 18 minutes. Since the catecholamines extracted from the samples must be made anhydrous prior to derivative formation

for G.L.C., the time and effort required for removal of water was another deficiency in the molecular sieving procedure. Lyophylization was the method of choice for the removal of water since it provided low temperatures and in vacuuo conditions which significantly reduced oxidation of the catecholamine metabolites. Sephadex G-10 columns of sufficiently large dimensions to obtain acceptable separations resulted in a volume of solution containing the eluted catecholamines of from 16-20 ml. Even when 100 ml flasks were used for freeze-drying, at least three hours was required for the complete removal of this volume of water. This was contrasted with a maximum of 45 minutes required to completely dry the 200 mg of alumina used in the adsorption procedure. Recoveries of added catecholamines by the gel filtration procedure were comparable to those attained using alumina adsorption when relatively large concentrations were present. Recoveries averaging in excess of 87% of added E and NE were obtained when sample concentrations were in excess of 0.05 μg of each catecholamine per milliliter (Table X). Recovery values at lower catecholamine concentrations were not determined for the gel filtration procedure due to the disadvantages inherent in the procedure outline above. One advantage of the method, however, is its usefulness if analysis of O-methylated catecholamine metabolites is desirable. Metanephrine and normetanephrine were eluted

from the Sephadex column in the same fraction as contained epinephrine and norepinephrine.

The use of a protein precipitation procedure for the removal of extraneous materials was also evaluated. This method was totally unacceptable due to low recoveries of added catecholamines (average ~50% at concentrations of 10 μg/ml) and inefficient separations (Table IX).

A large number of different derivatives of the catecholamines and their metabolites can be formed which are sufficiently volatile for G.L.C. Of the derivative types evaluated in this study, many had characteristics which limited their usefulness in a microanalytical method. Trimethyl silyl (TMS) derivatives possess excellent properties for flame-ionization detection; however, they could not be detected by E.C.D. except when large quantities were present. The use of CMDMS derivatives resulted in improved electron capture sensitivity; however, the decreased volatility of these derivatives lengthened retention times excessively unless high column temperatures were used. Since chromatography with E.C.D. is limited by the maximum temperature limit of the detector (225° C), high column temperatures are not advisable. CMDMS derivatives of E, NE and DM could be effectively separated using columns packed with OV-25 as the liquid phase; however, acceptable separations of the TMS derivatives were not achieved on any of the stationary phases tested. Reaction

times necessary for quantitative formation of either of these derivative types was either excessively long or required elevated temperatures. The combination of an O-TMS ether derivatives with a fluoroacetone Schiff's base condensation product resulted in products which elicited very good E.C.D. response; however, this type derivative was not sufficiently stable to permit accurate quantitative G.L.C. Only primary amines yielded this type derivative.

The fluoroacyl derivatives had many significant advantages over other types studied in this investigation. Conditions required for their quantitative formation were mild (room temperature) and formation was rapid (10 minutes). The method of choice for fluoroacylation employed the reaction of the catecholamines with the appropriate fluoroacyl anhydride using acetonitrile as the solvent. Acetonitrile apparently acts as a catalyst in this reaction since in most cases its elimination or replacement resulted in incomplete or slow reactions.

The chromatographic behavior of the catecholamine fluoroacyl derivatives was excellent. All of the catecholamines examined in this study produced symmetrical peaks on all of the liquid phases employed. Retention indices varied from 15.24 for the PFP derivative of NE on the moderately polar liquid phase OV-7 to 25.84 for the TFA derivative of M on the polar liquid phase OV-210. At a

column temperature of 155° C and carrier gas flow rate of 40 cc/min, maximum retention time for any of the eighteen different catecholamine-derivative combinations was fifteen minutes. By the correct selection of fluoroacyl derivative and liquid phase, the six catecholamines; E, NE, M, NM, DM and MT, could be separated in a single run. For instance, these six amines are effectively separated as their PFP derivatives on a 12 ft x 2 mm I.D. column packed with 3% OV-1:SE-52 1:1 w/w on 60/80 mesh Gaschrom Q employing a column temperature of 145° C and carrier gas flow of 40 cc/min. While separation efficiency was a primary consideration in column selection, the stability of the derivatives on a particular liquid phase was also of concern. In general, the fluoroacyl catecholamines were all much more stable on the less polar phases studied. While the degree of separation that could be achieved between different amine derivatives varied considerably as a function of column polarity, overall separation efficiency when all six amines were considered did not change significantly regardless of the liquid phase used. It is interesting to note that a large amount of structural information can be obtained about individual catecholamines by G.L.C. of the amines as fluoroacyl derivatives on liquid phases of different polarity. For example, primary amines had shorter retention times than the corresponding secondary amines on non-polar phases (OV-1) while the reverse is true

on polar phases (EGSP-Z). An increase in the number of functional groups which can be fluoroacylated tended to increase the retention time regardless of the liquid phase. Separation achieved between the catecholamines E, NE and DM and their respective 3-O-methyl metabolites increased with increasing liquid phase polarity.

Of the three types of fluoroacyl derivatives which can be formed with the catecholamines, the pentafluoropropionyl (PFP) derivatives have several characteristics which make them the most useful for incorporation into an analytical method. Most important of these characteristics is the excellent stability of the PFP derivatives. The PFP derivatives of the six catecholamines could be stored at room temperature in benzene solution for periods in excess of two weeks while the TFA derivatives showed significant hydrolysis as early as an hour after the solution was prepared. Heptafluorobutyryl (HFB) derivatives were much more stable than the TFA derivatives but not as stable as the PFP. Chromatographic stabilities followed the same pattern. Separation efficiency on the columns employed was generally higher for the PFP derivatives although this factor was not particularly significant. While all three types elicited excellent electron capture response, the E.C.D. was from 5-10 times more sensitive to the PFP or HFB derivatives than to the TFA derivative of any particular amine (Table IV). Minimum

detectable amount of the six catecholamines as their PFP or HFB derivatives was from 5 to 12 picograms depending upon the amine. The time required to form the TFA or PFP derivatives was slightly less than for the HFB derivative and the time required to remove excess reagents was considerably less. The fluoroacyl derivatives of the methyl esters of acidic catecholamine metabolites displayed G.L.C. characteristics similar to the other compounds discussed previously. These derivatives of dopa, DC, HVA and MD were all volatile and sufficiently stable for use in quantitative G.L.C. analysis. Because of their stability, ease of formation, and excellent E.C.D. response, the PFP derivatives of these compounds were the most desirable. While the E.C.D. response of the PFP derivatives of HVA and DC was similar to that of the PFP derivatives of the other catecholamine metabolites, PFP dopa methyl ester and PFP-MD methyl ester elicited a reduced E.C.D. response (Table VIII).

In order to elucidate the structure of the catecholamine PFP derivatives, a number of these compounds were prepared, purified and subjected to mass spectral analysis. This analysis provided conclusive evidence that pentafluoropropionic anhydride reacts with all hydroxyl and amine groups to form a completely fluoroacylated molecule. For instance, in the case of E and NE, the tetrapentafluoropropionyl compound is formed.

Evidence for this structure is the presence of a molecular ion peak of correct mass and large peaks corresponding to a fragmenting of the fully fluoroacylated derivative between the α and β carbon atoms in the mass spectra of all amines examined. No peaks of the correct mass to correspond to partial derivative formation were found in any of the spectra. Mass spectra of the methyl esters of acidic catecholamine metabolites also presented evidence for full derivative formation in these compounds. In addition to mass spectrometry, infrared and nuclear magnetic resonance spectroscopy provided evidence for the existence of a fully fluoroacylated species in all derivatives examined. These latter analyses were accomplished on DC, DM, NM, NE and dopa.

The formation of PFP esters and amides requires anhydrous conditions to prevent hydrolysis of both the anhydride and the products formed. Anhydrous conditions were obtained by freeze-drying the solutions of catecholamine metabolites which were obtained by extraction of biological fluids. Where alumina adsorption was used to extract these compounds from the biological material, the alumina was freeze-dried prior to the elution of adsorbed materials. Alumina powder can be lyophilized easily in any commercial freeze-dryer, as long as care is taken to prevent powder loss from the drying vials into the vacuum system. A disc of Whatman #42 filter paper

placed over the mouth of the drying vial was effective for this purpose. When either the gel-filtration or protein precipitation procedure was employed, the dry extract could be reacted to form derivatives directly, excess reagent removed with a nitrogen stream and chromatographed after dissolving the product in an appropriate solvent. The alumina extraction procedure required the elution of the compounds from the dry alumina to prepare them for chromatography. Two routes are possible for this step. The alumina can either be exposed to the reaction solution which forms the derivatives and elutes them simultaneously or the catecholamine metabolites can first be eluted with acid:methanol, followed by drying of the eluate under vacuum and then reaction with the appropriate anhydride. The latter procedure has the advantage that acidic catecholamine metabolites react with the methanol to form methyl esters suitable for chromatography after subsequent fluoroacylation. By the former procedure, only E, NE and DM can be measured in a single run while the latter procedure allows measurement of dopa and dihydroxyphenylacetic acid in addition to the other three catecholamines. In addition to the inclusion of two more compounds into the general analytical procedure, the use of acid:methanol also has several other advantages. Most important of these is the relative freedom of the samples prepared in this manner from

interfering substances. While recoveries obtained by either elution procedure were nearly identical, a large number of electron-capturing materials were eluted from the G.L.C. column when the former procedure was used. Certain early eluting materials tended to trail excessively and cause the chromatogram baseline to rise to unacceptable levels. Another material, identified by mass spectrometry as aluminum tripentafluoropropionate, had an erratic retention time and tended to condense on the tritium foil of the detector. Due to this contamination, the maximum sensitivity of the overall method of NE in plasma was only 15 μg/L when the alumina was eluted with PFP anhydride:acetonitrile, while a sensitivity greater than 1 μg/L could be realized with acid:methanol as the eluent. It should be noted that when catecholamines containing hydroxyl groups are eluted from alumina using acid:methanol, a reaction occurs which results in the conversion of this hydroxyl to a methoxyl group. These compounds react with PFP anhydride in the same manner as the parent compounds to form tripentafluoropropionyl derivatives which can be chromatographed and separated from each other and other compounds of interest using 5% OV-1:SE-52 1:1 as the liquid phase. Retention times of the respective PFP derivatives were slightly longer than those of the parent compounds. Stability and E.C.D. sensitivity of the PFP derivatives of these latter compounds were the same or

slightly better than the parent amines. Structures of both compounds were elucidated by mass spectrometry. The accuracy and precision of the method was greatly enhanced by the use of 3, 4-dihydroxyhydrocinnamic acid as an internal standard which was added to the samples prior to adsorption on alumina. This compound is quantitatively adsorbed on alumina and undergoes all other reactions discussed previously for catecholamine metabolites. By the use of this compound as an internal standard, the deleterious effects of alumina loss and volumetric errors on the accuracy and precision of the method were greatly minimized.

After evaluation of all of the different procedural steps, the following general method provided the most effective means of analyzing small volumes of biological fluids for the catecholamine metabolites epinephrine, norepinephrine, dopamine, dopa and dihydroxy phenylacetic acid:

Step 1: Catecholamine metabolites are extracted from the preserved biological fluids using activated alumina at pH 8.6, the alumina washed with distilled water, and freeze-dried. The internal standard (DHC) is added to the sample prior to extraction with alumina.

Step 2: The catechols are eluted from the dry alumina with 2.0N HCl in methanol with the simultaneous formation of methyl esters of all carboxylic acids.

Step 3: The methanolic solution is dried under vacuum followed by subsequent reaction of the residue with pentafluoropropionic anhydride using acetonitrile as the solvent.

Step 4: Excess reagents and solvents are removed under a stream of nitrogen, the products redissolved in benzene and chromatographed on 12 ft x 2 mm glass columns packed with 3% OV-1:SE-52 1:1 w/w on Gaschrom Q and measured with an E.C.D.

The method outlined above is effective in measuring dopa, DM, E, NE and DC in 1 ml samples of plasma, urine, and CSF and 500 mg samples of tissue. Minimum concentration detectable by this method is less than 1 μg/L of each compound in fluids and less than .05 μg/g of each compound in tissue. Average recovery of added catecholamines from tissue homogenates varied from 90% for dopa to 96% for dopamine when the internal standard was used for the calculation.

V. SUMMARY AND CONCLUSIONS

A procedure for the extraction and analysis by gas-liquid chromatography of a variety of catecholamine metabolites from biological tissue was developed. An extensive investigation and evaluation of extraction techniques was undertaken. Catecholamine reactions which would result in stable, volatile derivatives suitable for separation and measurement by gas-liquid chromatography were also investigated. Since the detection by electron capture provided the greatest sensitivity in analysis for certain compounds, special consideration was given to reactions which would form derivatives suitable for this type detection. Because of their stability, volatility, and electron affinity, the pentafluoropropionyl derivatives were selected as the derivatives of choice. This derivative type could be formed quantitatively by a simple reaction from all catecholamines and their metabolites. The structures proposed for these derivatives was verified by quantitative analysis using infrared spectrometry, nuclear magnetic resonance spectroscopy and mass spectrometry. Optimum conditions for the chromatography and measurement of these compounds were determined experimentally. Three procedures for the removal of catecholamines from biological samples were evaluated. Specific

techniques which were experimentally appraised included gel filtration, alumina adsorption, and selective precipitation. Gel filtration and alumina adsorption both had significant advantages; however, because of the simplicity, efficiency and compatibility of the alumina adsorption procedures with gas-liquid chromatography and electron capture detection, this procedure was selected over the more versatile gel filtration procedure. By the use of the alumina adsorption procedure, dopa, dopamine, norepinephrine, epinephrine and dihydroxyphenyl acetic acid were extracted into a single sample. If these compounds were eluted from the alumina by a solution of anhydrous hydrogen chloride in methanol, methyl esters of the acidic compounds were formed which were suitable for fluoroacylation and subsequent chromatography. This allowed measurement of all five compounds in a single biological sample. A non-biogenic compound, dihydroxyhydrocinnamic acid was chosen as an internal standard for the analytical procedure. Dihyroxyhydrocinnamic acid was found to exhibit properties similar to the compounds of interest and therefore was carried through the entire analytical procedure. The use of this internal standard was shown to increase the accuracy and precision of analysis by 5-10% and reproducibility by at least 12%. Techniques for the handling of biological samples (i.e., blood, urine, cerebrospinal fluid and tissue) were investigated

and those which resulted in the least alteration of the compound of interest were incorporated into the general procedure.

As a result of this experimentation, a general procedure was developed for the measurement of norepinephrine, epinepherine, dopamine, dopa and dihydroxyphenyl acetic acid in a single sample of blood, urine, CSF, or tissue. The steps in this procedure involved: 1. extraction of the compounds from the sample with activated alumina, 2. removal of water by lyophilization of the alumina, 3. elution of the compounds with acid:methanol and simultaneous esterification of acidic metabolites 4. conversion of the eluted compounds to volatile pentafluoropropionyl derivatives by reaction with pentafluoropropionic anhydride and 5. the gas-liquid chromatographic separation and measurement of these derivatives with electron capture detection. This general analytical procedure is capable of measuring dopamine levels of less than 1.0 μg/L in biological fluids and 0.05 μg/g in brain tissue when either 2.0 ml fluid or 500 mg tissue samples are employed. Minimum detectable levels for the other compounds measured were similar. When an internal standard was used, precision of the determinations averaged ± 2.4% and recoveries of added compound from brain tissue extracts were in excess of 90%.

The analytical procedure developed in this study is capable of providing accurate and reproducible measurements of certain catechol containg compounds in biological matrices. Precision, accuracy and sensitivity of the procedure is comparable to other published analytical techniques. The rapidity of the technique and the advantage of using small sample volumes should make it applicable for the solution of a wide variety of problems in both research and clinical laboratories.

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