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Gas Chromatography and Electron Capture Detection of Tetrahydroisoquinolines and Related Catecholamines

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GAS CHROMATOGRAPHY AND ELECTRON CAPTURE DETECTION OF TETRAHYDROISOQUINOLINES AND RELATED CATECHOLAMINES

BY

MOSTAFA G. BIGDELI

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirement for the Degree of Master of Science

June, 1972

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CHAPTER ONE

INTRODUCTION

A. PURPOSE

In order to examine critically the tetrahydroisoquinoline (THIQ)* biosynthesis hypothesis in alcoholism, a remarkably sensitive and selective assay method is needed. The objective of the work in this thesis is to develop such an analytical method for the isoquinolines and their parent catechoamine, using electron capture gas chromatography.

B. BACKGROUND

THE THEORY OF FORMATION OF TETRAHYDROISOQUINOLINE

a) Tetrahydroisoquinoline Formation in Animal Tissue during Alcohol Consumption

The role of epinephrine and norepinephrine:

In 1970, Collins and Cohen reported (1) that THIQ biosynthesis takes place in rat adrenal glands during $^{14}$C-methyl alcohol metabolism. Radioactive materials, epinephrine (E), and norepinephrine (NE), were extracted by an alumina absorption method from the animal received six i. p. injections of $^{14}$C-methanol over thirty hours period. They observed the presence of two

*A complete list of abbreviations used is given in the Appendix.
radioactive THIQs on thin layer chromatography plates, while E and NE were not radioactive.

The presumed reaction sequence of THIQ formation is presented in Fig. 1.

\[ R'\text{CHO} + \text{CATECHOLAMINE} \rightarrow \text{ALDEHYDE} \]

\[ R^* = \text{CH}_3 = \text{Epinephrine} \]
\[ R^* = \text{H} = \text{Norepinephrine} \]

Fig. 1. The reaction sequence for the formation of tetrahydroisoquinoline from catecholamine and alcohol metabolites.
They had previously identified (2) the THIQs in the medulla of cow adrenal glands following perfusion with dilute solutions of aldehyde in isotonic phosphate buffer (pH = 7.0) at 37.0°C. Further evidence for THIQ synthesis in vivo, was obtained by Cohen and Barrett (3), who reported that THIQs were detected by fluorescence microscopy in freeze-dried slices of adrenal tissue from rats chronically intoxicated with non-radioactive methanol.

Indoleamines (e.g., tryptamine, 5-OH-tryptamine) and catecholamines (CA) (e.g., dopamine, E, NE) condense with certain aldehydes like formaldehyde (4, 5), acetaldehyde (6, 7, 8, 9), 3, 4-dihydroxyphenylacetaldehyde (10, 11), pyridoxal phosphate (12), and glyoxalic acid (13) in vitro to form tetrahydrocarboline alkaloids and THIQ alkaloids, respectively. Robbins (14) suggested that some of these naturally occurring amines might form alkaloids, especially when the latter occur in abnormally high concentrations, e.g. acetaldehyde after ingestion of ethanol.

Based on the tissue perfusions, Cohen and Collins (2) proposed that THIQ alkaloids may be forming in the adrenal and neural cells (rich sources of CAs) of human alcoholics during chronic ethanol intoxication and methanol poisoning. It was suggested (2) that the alkaloids might play a role in some of the behavior of alcohol-withdrawn individuals, because there are structural similarities of these THIQs to a group of synthetic and naturally occurring alkaloids which have a variety of pharmacological actions (to be discussed in the section on toxigenicity of THIQ).
A similar condensation reaction involving an indoleamine rather than CA, was observed in vivo by McIsaac (15). He detected 5-methoxy-tetrahydroxy-β-carboline in the urine of rats treated with ethanol and 5-methoxy-tryptamine along with iproniazid (a mono-amine oxidase inhibitor) and disulfiram (an aldehyde oxidase inhibitor).

The role of dopamine

Davis and Walsh (11) hypothesized and supported by in vitro data that biotransformation of ethanol to its active metabolite (acetaldehyde) induces alteration in metabolism of dopamine (DA) to 3, 4-dihydroxyphenylacetaldehyde. This latter metabolite, in tissue such as brain with comparatively low aldehyde oxidizing activity, condenses with the second molecule of DA, and a benzyl-THIQ alkaloid, tetrahydopapaveroline (THP) is formed (Fig. 2).

Since THP is intermediate compound in the biosynthesis of morphine in the opium poppy, alcoholic addiction was suggested to be associated with the formation of this THIQ alkaloid.

b) THIQ alkaloids in plants

Peyote from Caectaceae family contains at least fifteen different THIQs such as anhalanine, carnegine, salsoline, and -phenethylamine (mescaline, normescaline) (16). The biosynthesis of these alkaloids in plants have been studied by Agurell (17). Using gas chromatography-mass spectrometry, he detected tyramine, dopamine, 3, 4, 5-trihydroxy phenethylamine and also dopa in plant extract, indicating them as the precursors of THIQs formed in Peyot
Fig. 2. A representation of a hypothesis depicting the relation of alcohol-induced alteration of the metabolic disposition of the biogenic amine, dopamine with the postulated resultant formation of morphine-like alkaloids as a biochemical basis for the addiction liability of alcohol.
Toxigenicity of THIQs

According to Schultes (16), THIQs found in peyote are hallucinogenic. Fasset and coworkers extensively studied the physiological action and toxigenicity of different THIQs. They observed (18) in general, that compounds with substituents at position 6 are the most toxic and the compound with substituents at position 5 and 6 are the least toxic in mice. They also presented the evidence that location of these actions is on the central nervous system of frogs. They later reported the action of a number of these compounds on blood pressure, pulse rate, respiration and smooth muscles (19). It was found that the physiological action of these compounds is related to their chemical substitution. This same group did research (20) on the antianesthetic action of THIQs in mice treated with urea. The result was quite varied from actual prolongation of sleeping time in one THIQ to remarkable shortening in another. The other report indicates that some THIQs have reverse action on epinephrine vaso-motor activity. Simple THIQ alkaloids possessing hydroxyl groups at the 4-position, with one possible exception—a 1, 2-dimethyl-4-hydroxy-6, 7-dimethoxy THIQ (21), are not known to occur naturally and no physiological studies seem to have been done on these compounds produced from condensation of E and NE with aldehydes.

2. GENERAL ASPECTS OF GAS CHROMATOGRAPHY

The gas chromatograph (GC) volatilizes and separates very
similar and even isomeric compounds. The sample, after injection into a headspace, is carried by an inert gas (carrier gas) like nitrogen or helium through the column. The compounds are partitioned between the carrier gas and the non-volatile solvent (liquid phase). The solvent selectively retards the sample components according to their distribution coefficients, until they form separate bands in the carrier gas. These component bands leave the column in the gas stream and are recorded as a function of time.

Since the initial experiments with gas chromatography, over twenty different kinds of detectors have been developed, but only six of them are generally used. The selection of an appropriate detector to demonstrate the material flowing from the column is dependent on a number of intrinsic properties of the GC system. The thermal conductivity (TC) detector responds according to differences in heat generated between a carrier gas and gas solute mixture. Detectors of this type perform most satisfactorily with large sample loads and are used for collecting the sample for further analysis. The ionization detector detects the partially ionized compound which was burned with hydrogen. The two universally used detectors of this type are hydrogen and argon flame ionization (FI) detectors. Such detectors are more sensitive than TC detectors.

Extremely sensitive results can be obtained with the electron capture (EC) detector. In this detector the cathode consists of a metal foil impregnated with a β-emitting isotope. Commonly employed radioisotopes are tritium ($^{3}\text{H}$) and nickel ($^{63}\text{Ni}$). Under
the influence of an applied potential, electrons emitted from carrier gas by the cathode will migrate to the anode, thereby establishing a standing current. This current will decrease if a compound possessing electron affinity is vaporized into the cell (22, 23), thus providing a signal output. Although the exact mechanism of capture is not completely understood, the two following equations are the most widely accepted (24, 25).

\[
\begin{align*}
(1) \quad & AB + e^- \rightarrow AB^- \\
(2) \quad & AB + e^- \rightarrow A^- + B^-
\end{align*}
\]

In both instances, negative ions are generated. The mobility of these negative ions, however, is much less than electrons. Thus, the current decreases following the electron capture. As for the particular reaction, Samelson (26) notes that reaction (1) takes place with compounds having low energy vacant atomic or molecular orbitals, either nonbonding (e.g. lead alkyls) or antibonding (e.g. polycyclic aromatic hydrocarbons), or substances with highly dipolar functional groups (α-dicarbonyl compounds). Reaction (2) on the other hand, is directly dissociative. It occurs primarily with compounds containing high electronegative groups as well as with halogenated substances.

Since relatively few compounds show significant electron affinity, the detector is remarkably selective.
Derivative formation

Not all compounds are suitable to direct GC analysis as "free" compounds. Different functional groups make the compound very polar and generally reduce its volatility. Such a polyfunctional compound will exhibit a long retention time, or may not be eluted from the column at all. A decrease in polarity is generally accomplished by derivative formation with one or all the functional groups; subsequently volatility is increased and the compound can be eluted from a GC column in a reasonable time.

Functional parts in THIQs and CAs are aromatic and aliphatic hydroxyl groups and amine groups. Reaction of these groups can be represented in Fig. 3.

\[
\begin{align*}
- \text{OH} & + \text{RX} \quad \rightarrow \quad - \text{OR} + \text{XH} \\
- \text{NH} & + \text{RX} \quad \rightarrow \quad - \text{NR} + \text{XH}
\end{align*}
\]

Figure 3

The replacing agent (R) can be acyl, fluoroacetyl or methyl-silyl and donating agent (X) usually is imidazole or anhydride.

The compounds generally used in preparation of silyl derivatives are:

- N-trimethylsilyl acetamide (TMSiA)

\[
\begin{align*}
\text{CH}_3 - \overset{\text{NH}}{\text{C}} - \overset{\text{Si}}{\text{CH}_3} & \quad \text{CH}_3
\end{align*}
\]
Fluorinated acyl derivatives of CAs have been formed using the following reagents.

**Trifluoroacetic anhydride (TFAA)**

\[
\text{CF}_3 - \text{C} - \text{O} - \text{C} - \text{CF}_3
\]

**Perfluoropropionic anhydride (PFPA)**

\[
\text{CF}_3 - \text{CF}_2 - \text{C} - \text{O} - \text{C} - \text{CF}_2 - \text{CF}_3
\]

**Heptafluorobutyric anhydride (HFBA)**

\[
\text{CF}_3 - \text{CF}_2 - \text{CF}_2 - \text{C} - \text{O} - \text{C} - \text{CF}_2 - \text{CF}_2 - \text{CF}_3
\]
Heptfluorobutyryl imidazole (HFBI)

\[
\text{CF}_3 - \text{CF}_2 - \text{CF}_2 - \text{C} - \text{N}^\circ \\
\quad \text{HC} = \text{N} \\
\quad \text{HC} = \text{CH}
\]

The choice of reagent and solvent varies with the type of compound and purpose. The most obvious route to prepare derivatives is acylation of both hydroxyl and amine groups. In Fig. 3 the donating agent (X) has been mostly anhydride for application in EC detector. However, recently heptfluorobutyryl imidazole has been used (27) in preparation of fluorinated amides of CAs, following the trimethylsilylation of hydroxyl groups. A selection of an appropriate solvent media is of great importance in the production of a single derivative. Multiple products of CAs are often obtained under relatively mild conditions of derivatization. Different solvent media have been employed in different cases of CA derivatization, in order to obtain single derivatives in good yield. These include ethyl acetate (28, 29), tetrahydrofuran (30), pyridine (27), and acetonitrile (31). In some cases the procedure has been conducted in the absence of solvent (39).

Detection and quantitation of CAs

Gas chromatography of CAs and their metabolites have been studied by a number of groups using trimethylsilyl derivatives. Horning et al. published a method (31) of O, N-trimethylsilyl formation of CAs with TMSiI and Bis-trimethylsilyl actamide (ESA). Recently, this laboratory reported a method of derivatiza-
tion with combination of TMSiI and HFBI which could be useful for EC detection (27). According to their findings, TMSiI react with hydroxyl groups to form trimethylsilyl ethers, and acyl groups could be donated by HFBI to primary and secondary amines. Fig. 4 shows the reaction sequences.

Wilk and Gitlow (33) were the first to report some biological amine studies with the EC detector. Kawai and Tamura (34) achieved sensitivity in the nanogram (10^-9 gram) range using TFAA as the derivatizing agent for CAs. Anggard and Sedvall (28) compared the EC responses of phenolic metabolites of CAs. After reaction with different fluoroacylating agents, they found that sensitivity increased in the order of TFAA PPFA HFBA. Cattabeni and Costa (35) recently reported the mass fragment assay of femt mole (10^-15 mole) concentrations of CAs in rat brain tissue, following the formation of derivatives with PFPA. In recent work, Moffat and Horning (36) prepared a new derivative by Schiff base formation when primary CAs were heated in with pentafluorobenzaldehyde, after with hydroxyl groups were converted to trimethylsilyl ethers with BSA.

Measurement of THIQ alkaloids with HFI detector

The GC characteristics of THIQs have not been extensively studied. The HFI detector has been used by several investigators, but there have been no reports of EC determinations of these or similar alkaloids. Lundstrom and Agurell (37), with XE-60 columns, separated and measured THIQs in Peyote cacti. Using
Fig. 4. Derivatization of catecholamines with trimethylsilyl imidazole and heptafluorobutyryl-imidazole.

SE-30 as the liquid phase THIQ separation was not as well resolved. The same deficiency has been observed by Kapadia and Rao (38). Agurell, in HFI detector gas chromatography determinations of plant alkaloids, detected these compounds at microgram levels. Also in HFI detector studies, Davis and Walsh (39) were able to quantify fifty nanograms and detected one nanogram of THIQ with the preparation of TMSi-derivatives.

Since THIQs can be viewed as cyclic CAs, the method for CAs studies might be adaptable for THIQs.
Extraction of THIQs

Cohen and Collins worked out the extraction of CA-derived THIQs from tissue homogenate (2). This procedure was a modified method of Goldenberg (40) employed for CA extraction. Aluminum hydroxide freshly prepared from aluminum sulfate (the pH of which adjusted to 8.0), was used. Many investigators have employed activated aluminum oxide for extraction of CAs. Shellenberger (41) has been able to get 75-95% recoveries using 0.05 N perchloric acid as the eluant and fluorescence assay. Kawai and Tamura (34) eluted CAs from alumina with 0.2 N acetic acid and reported 80% recovery. Different factors affecting the aluminum oxide procedure for the analysis of CAs have been studied by Anton and Sayre (42). Organic solvents like ethyl acetate (35) and secondary butanol (43) have been utilized by some investigators for the extraction of CAs.
CHAPTER TWO

MATERIALS AND METHODS

EXPERIMENTAL

The analyses were performed on a Varian model 2100 gas chromatograph equipped with both HFI and EC (Ni-63) detectors. 6' U-shaped glass columns (1/8 I. D.) packed with 3% or 5% OV-17 or 5% SE-30 on 80/100 mesh Gas Chrom Q were obtained from Applied Science Laboratories or were packed in this laboratory according to standard procedures (44). All were conditioned at 270° C (30° C less than the maximum temperature stability of liquid phase), with the column end unattached to the detector. Zero grade nitrogen from "Matheson Gas Products" was used as a carrier gas.

Materials

Derivatizing agents TFA, HFBA, HFBI, TMSiI and solvents (pyridine, acetonitrile, ethyl acetate) were purchased from Pierce Chemical Company. All of these reagents were kept over drying agent (Drierite) at -20° C. Ethyl acetate was batch distilled before use. L (-)-epinephrine and L (-)-norepinephrine were obtained from Regis Chemical Company. Dopamine-HCl, Metanephrine-HCl, and normetanephrine-HCl were purchased from Winthrop Labora-
Hydrochloride salts of tetrahydroisoquinolines were synthesized in our laboratories by Dr. Frank Kernozek and Dr. Michael Collins. The structure and purity of these compounds were confirmed by Infra-red Spectrometry (IRS), Nuclear Magnetic Resonance (NMR), melting point determinations and thin layer chromatography (TLC). The structure of some of these are shown in Fig. 5.

Aluminum sulfate (analytical reagent) and sodium hydroxide were obtained from Mallinckrodt Chemical Works. Aluminum oxide (Woelm Neutral Activity Grade I) was prepared according to the method of Anton and Sayre (42) and was kept in a desiccator over drying agent (Drierite).

**ANALYTICAL PROCEDURES**

Determination of detector responses and calibration of the instrument

HFI and EC detector responses of THIQ and CA derivatives was determined relative to lindane (hexachlorobenzene). Solutions of lindane and derivatives (1.0 mg/ml) were prepared in distilled ethyl acetate and used for evaluation of HFI responses. The same solution was diluted by a factor of $10^5$ (10 pg/ul) with ethyl acetate and was used to evaluate the EC responses. Peaks area was calculated by planimetry method or by peak height times width at half the height (44).
Fig. 5. Crystalline THIQs used in study
Steps involved in derivative formation from crystalline THIQs and CAs

a) THSi-HFB derivatives
1. One milligram of CA or THIQ was weighed into a 4 ml screw-capped vial.
2. 0.1 ml acetonitrile (AN) and 0.2 ml TMSiI was added with dry syringes.
3. Vials were covered with aluminum foil, capped tightly, and heated for three hours at 60° C.
4. 0.1 ml of HFBI was added and heated for thirty minutes at 60° C.

The solution was used directly for GC analysis.

b) HFB-derivatives
1. One milligram of either CA or THIQ was weighed in separate screw-capped vials which were dried in the oven before use.
2. 0.1 ml AN and 0.2 ml HFBA was added with dry syringes and left at room temperature.
3. After 45 minutes, the solution was dried with a stream of nitrogen, tightly capped, and stored at -20° C until used.
4. The dried compound obtained in this way was redissolved in distilled ethyl acetate to the appropriate concentration prior to GC analysis.
Derivative Formation following Extraction from Aqueous Solution

A. Ethyl acetate extraction

1. One milligram of either CA or THIQ was dissolved in 0.5 ml of 1 N perchloric acid (HClO₄) which contained 5 mMol/l of sodium metabisulfite (Na₂S₂O₅).

2. The solution was mixed with 5.0 ml of saturated solution of sodium chloride in distilled water.

3. 5.0 ml of ethyl acetate was added and the two phase mixture was shaken for three minutes.

4. The organic layer was allowed to separate, then was transferred to a 50 ml round bottom flask.

5. Steps 3 and 4 was repeated four times and the organic layers were combined.

6. The combined organic layers were evaporated on a rotary evaporator at 40°C, and the residue was derivatized with HFBA in acetonitrile (method b, above).

B. Formation of HFBA-derivatives of CAs and THIQs bound to freshly prepared aluminum hydroxide

Reagents:

1 N perchloric acid containing 0.5 mMol/l sodium metabisulfite (Na₂S₂O₅).

Aluminum hydroxide: 10.0 ml of a 20% solution of crystalline aluminum sulfate (Al₂(SO₄)₃ · 18 H₂O) was diluted with 10.0 ml distilled water. The pH was adjusted to 8.2 - 8.4 with
Methods

1. One milligram of the hydrochloride salt of 4, 6, 7-trihydroxy THIQ or L-epinephrine was dissolved in 0.5 cc of 1 N perchloric acid in a 15 ml centrifuge tube.

2. 10.0 ml freshly prepared aluminum hydroxide was added (pH of final solution was carefully adjusted to 8.0 - 8.2 with 0.2 N sodium hydroxide). The tube was tightly stoppered and was shaken on a mechanical shaker.

3. After 20 minutes the aluminum hydroxide material was separated by centrifuging for 5 minutes at 2000 r.p.m. The supernatant was discarded and the precipitate was washed three times with 10.0 ml portions of distilled water.

4. A portion (usually one-third) of precipitate was dried under vacuum (0.5 mm Hg) for three hours at room temperature and the alumina-bound CA or THIQ was derivatized with 0.2 ml HFBA in acetonitrile. After 75 minutes, the rea-
gent and solvent are evaporated under vacuum (0.5 mm Hg). The derivatized materials are eluted from alumina with 4 portions of 0.5 ml ethyl acetate, each time shaking for 5 minutes. Combined eluants were dried under stream of nitrogen and redissolved in ethyl acetate to the desired concentration for GC analysis.

5. 0.5 ml of 4 N hydrochloric acid was added to the remaining two-thirds portion of alumina material to elute the catechol compounds.

6. After two hours at 4.0°C temperature, all the precipitate was dissolved. 0.5 ml of 1 M K₂HPO₄ was added and the pH of mixture was adjusted to 3.5 - 3.8 (glass electrode, Beckman pH meter) by dropwise addition of 1.0 N sodium hydroxide. In this stage, all aluminum ions were precipitated and the mixture became quite viscous. The volume of this mixture was estimated. Four volumes of 50:50 (v/v) ethanol-acetone solution were added. The mixture was chilled at 4.0°C for at least two hours.

7. The mixture was centrifuged for 20 minutes at 2000 r.p.m. and the supernatant was carefully decanted into a 50 ml round bottom flask.

8. The precipitate was dispersed in 20 ml of ethanol-acetone solution and recentrifuged. The supernatant was combined with the first supernatant and vacuum-evaporated at 40°C.
9. The residue was dissolved in 1.0 ml of ethyl acetate and transferred to a small screw-capped vial.

10. The ethyl acetate was dried under a stream of nitrogen and the residue was derivatized with HFBA in acetonitrile as described above (method b).

C. Formation of HFB-derivatives of Woelm aluminum oxide bound CAs or THIQs

1. One milligram of E or 4, 6, 7-trihydroxy-THIQ-HCl was dissolved in 1.0 ml of 1 M perchloric acid.

2. The pH was adjusted to 8.2 carefully with 0.2 N sodium hydroxide.

3. 2.0 gr. activated aluminum oxide was added into the test tube, was stoppered and shaken on a mechanical shaker.

4. After 20 minutes, the aluminum oxide material was separated by centrifuging at 2000 r.p.m. in a clinical centrifuge and was washed three times with 5 ml portions of distilled water.

5. The same as step 4 in experiment B.

6. 10.0 ml of 0.2 N acetic acid was added to the remaining two-third portion and was shaken for 5 minutes, to elute the catechols in to the acid portion.
7. The eluate was divided into two parts. The first part was dried, using a vacuum pump at room temperature and the second part was freeze-dried with the Vertis apparatus.

8. To the both residues was added HFBA in acetonitrile, and derivatization continued as described in method b, above.

D. Formation of HFB-derivatives from CAs and THIQs extracted from tissue homogenate

1. A fresh pair of rat adrenal glands was homogenized in 2 ml of 1.0 N perchloric acid.

2. The homogenate was centrifuged at 2000 r.p.m. for 20 minutes with a clinical centrifuge.

3. The precipitate was discarded and 0.05 mg of 4, 6, 7-trihydroxy-THIQ,HCl in 0.5 ml of 1.0 N HClO₄ was added to the supernatant. The pH was adjusted to 8.2 with 0.2 N sodium hydroxide.

4. 100 mg activated aluminum oxide (Woelm) was added to the solution and it was shaken for 20 minutes.

5. Steps 4 and 5 from method (C) were followed.
CHAPTER THREE

RESULTS AND DISCUSSION

I. Derivative Formation

A. TMSi-derivatives with TMSiI

This method in use to prepare CAs for HFI detection, was successful in our hands for formation of single derivatives from CA-derived THIQs. In Fig. 6, the distinctly different retention times of the TMSi-THIQs and TMSi-CAs along with their relative sensitivity to the HFI detector can be observed. The sensitivity of our results, (1 ng) in some cases, was higher than those obtained by Davis and Walsh (10 ng) (38) from salsolinol derivatives prepared by N, O-Bis trimethylsilyl acetamide.

B. TMSi-HFB-derivatives

For the detection of very small amounts of THIQ alkaloids possibly present in biological samples, the formation of fluorinated compounds applicable to EC detector is required. Our next approach was the formation of the TMSi-HFB derivatives, the usefulness of which was proposed by Horning et al. (27). The results were erratic for all THIQs named in Fig. 5. Multiple products were formed. Also the shape and size of peaks on the chromatogram were not consistent for each injec-
Fig. 6. Relative sensitivities and retention times of TMSi-derivatives of E, NE, DA, and THIQs, 4, 6, 7-trihydroxy (I), 6, 7-dihydroxy (II), 1-methyl-6, 7-dihydroxy (III) and 4-hydroxy-6, 7-methylenedioxy (IV).

Conditions: 5% OV-17 on 80/100 mesh Gas Chrom Q; Column T = 160 C; flow rate = 30 ml/min; the range of HFI detector = 8 x 10^{-10}
tion over the first 3-4 hours, possibly because of instability of those particular derivatives. Changing the reaction conditions of time and temperature did not lead to the formation of single derivatives. Also, large tailing interfered with the separation and EC or HFI detection of the multiple derivatives. On the other hand, E, NE, and DA used as controls in the procedure, produced a single peak in agreement with the literature (27). The retention time of each obtained by HFI detection is demonstrated in Fig. 7. The results from this procedure are summarized in Table I.

The EC detector for studying these derivatives produced no advantage of sensitivity over CAs studies by HFI detector. Moreover, the compounds were highly obscured by tailing solvent.

As mentioned previously, the silylation of the hydroxyl groups and the fluoro-amidation of the amine group takes place during TMSi-HFB derivatization to give a single derivative (Fig. 4). In the derivatization of THIQ, multiple products were formed probably due to the structural environment of the aliphatic hydroxy and amine groups.

C. HFB-derivatives

Different groups of investigators have reported a very sensitive method for gas chromatographic studies of CAs by formation of completely fluoroacylated (amidated) derivatives. To prepare such derivatives from THIQs, we attempted various derivatizing procedures utilizing different fluoroacylating
Fig. 7. Separation of the TMSi-HFB derivatives of DA, NE, and E (HFI detector).

Conditions: 3% SE-30 on 80/100 mesh Gas Chrom Q; temperature programming at 2°C/min from 140°C; flow rate = 30 ml of nitrogen per min; HFI detector with range of 8 x 10^{-10}. 
### TABLE I

Amount of each Derivative Yielding Peak Area of 100 mm$^2$ on Electron Capture and Flame Ionization Detectors

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Sensitivity* of detectors to different derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HFI$^+$</td>
</tr>
<tr>
<td></td>
<td>TMSi-</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>0.5</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.5</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.2</td>
</tr>
<tr>
<td>4,6,7-trihydroxy-THIQ (# 1)</td>
<td>2.0</td>
</tr>
<tr>
<td>6,7-dihydroxy-THIQ (# 2)</td>
<td>5.0</td>
</tr>
<tr>
<td>I-methyl-6,7-dihydroxy-THIQ (# 3)</td>
<td>10</td>
</tr>
</tbody>
</table>

* The sensitivity of HFI detector is in nanogram and of EC detector is in picogram range.

+ Condition for HFI detector: temperature for 3% OV-17 column = 160°C; flow rate of nitrogen = 30 ml/min; range of detector = $2 \times 10^{-12}$

++ Condition for EC detector: temperature for 3% OV-17 column = 160°C; flow rate of nitrogen = 18 ml/min; range of detector = $8 \times 10^{-10}$

a The detector was contaminated in this experiment. Greater sensitivity can be obtained with a clean detector.
agents, donating agents, and reaction conditions. The results are summarized in Table II.

Using HFBA in acetonitrile, single products with distinct retention times were obtained for each CA and each THIQ named in Fig. 5. The retention times for E and NE were very similar; for better separation of these two compounds in the future, GC conditions should be changed. Fig. 8 demonstrates the retention times of model compounds.

Quantitative reproducibility of this method was excellent (8 ± 5%). The presence of α-hydroxyl in the CA and similarly the hydroxyl group at 4-position in THIQ did not interfere with our results, although it has been reported that sometimes, they produce extra derivatives (29).

With the HFI detector, the peak areas were linearly related to the quantity of derivatives injected. In the case of EC detection, linearity exists up to the 100 picograms of 4, 6, 7-trihydroxy-THIQ. The relative sensitivity of HFI and EC detectors to HFB-derivatives as compared to lindane are shown in Table III.

The standard curve (Fig. 9) which can be used for quantitative determinations, was prepared with the following conditions: 3% OV-17 column, heated to 160° C; flow rate was adjusted on 30 ml/min and HFI detector range = 2 x 10⁻¹¹. Each point on the calibration curve is the average of five injections from each of five sets of HFB-derivatives. The standard deviation of these derivatives falls in the range of ± 5 - 8%.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Solvent</th>
<th>Condition (RX. Time, Temp.)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. TFAA (trifluoroacetic</td>
<td>ETOAc</td>
<td>1 Hr., 30° and 60° C.</td>
<td>Single CA Derivatives Usually: Multiple THIQ Derivatives</td>
</tr>
<tr>
<td>anhydride)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. TFAA</td>
<td>CH₃CN</td>
<td>1 Hr., 30° C.</td>
<td>Similar to 1.</td>
</tr>
<tr>
<td>3. TMSi-Imidazole/</td>
<td>CH₃CN</td>
<td>TMSiI, 3 Hrs.; HFBI, 30°;</td>
<td>Similar to 1; but poor EC Response</td>
</tr>
<tr>
<td>HFB-Imidazole</td>
<td></td>
<td>60° C</td>
<td></td>
</tr>
<tr>
<td>4. HFBI</td>
<td>ETOAc</td>
<td>30° to 24 Hrs.; 30° C</td>
<td>Multiple Derivatives in all Cases; Tailing</td>
</tr>
<tr>
<td>5. HFBA (heptafluorobutyric</td>
<td>ETOAc</td>
<td>10° to 24 Hrs.; 30° C</td>
<td>Similar to 1</td>
</tr>
<tr>
<td>anhydride)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. HFBA</td>
<td>CH₃CN</td>
<td>10° to 24 Hrs.; 30° C</td>
<td>Single THIQ and CA Derivatives at 30° - 60° RX. Times</td>
</tr>
</tbody>
</table>
Fig. 8. Separation of HFB-derivatives of 0.2 ng. each of the norepinephrine, dopamine, 6, 7-dihydroxy-THIQ and 4, 6, 7-trihydroxy-THIQ.

Condition: Column, 5% OV-17 on 80/100Gas Chrom Q. 
T = 145° C, N2 = 20 ml/min, Ni-63 detector, 4 x 10^{-10}
Fig. 9. Calibration curve* for:

- □ 6,7-dihydroxy-THIQ
- △ Dopamine
- ▲ Epinephrine
- ■ 4,6,7-trihydroxy-THIQ
- ○ Norepinephrine

*HFI detector was employed
TABLE III
The Relative Response* of HFI and EC Detectors to HFB-derivatives (HFBA-Acetonitrile Method)

<table>
<thead>
<tr>
<th>Compounds (HFB-derivatives except lindane)</th>
<th>HFI</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lindane</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>2.50</td>
<td>0.60</td>
</tr>
<tr>
<td>Dopamine</td>
<td>1.50</td>
<td>0.38</td>
</tr>
<tr>
<td>4,6,7-trihydroxy-THIQ</td>
<td>2.10</td>
<td>0.45</td>
</tr>
<tr>
<td>6,7-dihydroxy-THIQ</td>
<td>1.20</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Lindane being the base = 1.00

The use of standard curve is valid as long as the detector sensitivity remains constant. This may be checked by injection of any suitable standard on each day's use of the GC. The maximum response of the EC detector to HFB-derivatives of THIQs and CAs is shown in Table I.

The stability of HFB derivatives in the dried state is good. They can be kept at -20°C for several days. However, after being dissolved in ethyl acetate at room temperature, 50% decomposition takes place in the first six hours and after 24 hours more than 80% would be decomposed. At -20°C, the decomposition of ethyl acetate solutions of derivatives during the first six hours is unnoticeable. Fig. 10 shows
Fig. 10. Decomposition curve of HFI-derivatives of CASs and THIQs in ETOAc at -20° C.

- ▲ Epinephrine
- ○ Norepinephrine
- ■ Dopamine
- △ 4,6,7-trihydroxy-THIQ
- □ 6,7-dihydroxy-THIQ
the decomposition of HFB-derivatives in ethyl acetate stored at \(-20^\circ C\) over seven days.

In the process of derivatization of CAs with fluoroacylating agents ester and amide formation, respectively, with hydroxyl and amine groups exists a well-established practice (27, 33, 34) (Fig. 11). More studies should be done to confirm structural conformations. However, it is not unreasonable to expect that similar chemical substitution happens during HFB-derivatization of tetrahydroisoquinolines.

Fig. 11. Derivatization of catecholamines with fluoroacyl reagent.
II. Formation of HFB-Derivatives of CAs and THIQs after Extraction from Aqueous Solution

A. Ethyl acetate extraction

Cattabeni and Costa (35) recently reported a successful extraction of CAs from rat brain homogenate with ethylacetate. However, the formation of HFB-derivatives of model compounds (epinephrine and 4, 6, 7-trihydroxy-THIQ) following the same method of extraction was relatively unsuccessful in our hands. Table IV (method 1) shows that multiple products were obtained. The results of four attempts were identical, and the area under the peaks, when compared to chromatograms obtained from crystalline HFB-derivatives, was low, indicating loss of compound during the procedure.

B. Freshly prepared aluminum hydroxide extraction

1. Elution with acid and evaporation prior to derivatization

The extraction of CAs with aluminum hydroxide has been a relatively common method for bioassay (40) or thin layer chromatographic studies (2). Our results of attempts to derivatize CAs and THIQs after elution by hydrochloric acid from aluminum hydroxide followed by evaporation on a rotary evaporator were not encouraging. The recovery of compounds was low, about 5-10% as it is pointed out in Table IV (method 2).
<table>
<thead>
<tr>
<th>Extraction methods</th>
<th># of experiments</th>
<th># of steps involved</th>
<th>Minimum time required (hours)</th>
<th># of GC products</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ethyl acetate fractionation</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2 Freshly prepared aluminum hydroxide followed by HCl elution and evaporation</td>
<td>4</td>
<td>10</td>
<td>16</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>3 Direct derivative formation on freshly prepared aluminum hydroxide</td>
<td>6</td>
<td>5</td>
<td>16</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4 Activated aluminum oxide (Woelm) followed by acid elution and evaporation</td>
<td>8</td>
<td>10</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5 Method #4 freeze-dried</td>
<td>4</td>
<td>10</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6 Direct derivative formation on active aluminum oxide</td>
<td>12</td>
<td>5</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7 Aluminum oxide column</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
2. Direct derivative formation in the aluminum hydroxide bound state

Destruction of CAs after elution from alumina is believed to be due to the traces of alumina in the residue following evaporation (29). To avoid the acid concentration step, the compounds directly derivatized in the aluminum hydroxide bound state, were produced. In reference to the data listed on Table IV, it appears that this modification, when used with freshly prepared aluminum hydroxide, did not improve the recovery (5-10%).

C. Activated aluminum oxide (Woelm) extraction

1. Elution with acid and vacuum evaporation prior to derivatization

Another form of alumina, aluminum oxide (commercially available—Woelm) has been used for extraction of CAs and their metabolites for fluorometric and gas chromatographic studies (29, 30, 41). This procedure led to the formation of single derivatives from E and 4, 6, 7-trihydroxy-THIQ with the retention times identical to the derivatives of crystalline materials. The use of 0.2 N acetic acid as the eluant produced higher yields than strong acids like 2 N HCl. Shellenberger (41) obtained 80-90% recoveries in fluorometric studies of tissue CAs extracted with aluminum oxide prior of elution with 0.05 N HClO₄. We checked the effect of perchloric acid on our recover-
ies. This result was also lower than the one with 0.2 N acetic acid. Our results did not exceed 40%, although Kawai and Tamura (34) reported 80% recovery of CAs from urine and tissue derivatized with TPAA in tetrahydrofuran. The average value reported in the literature is between 40-50% (27). In an attempt to increase the yield, we employed the Virtis apparatus to freeze-dry the acid-eluted materials, usually vacuum evaporated at room temperature. The result from Table IV (method 5) demonstrates that this modification was not successful.

2. Direct derivative formation in the aluminum oxide-bound state:

To avoid the acid concentration step and cut down on some unnecessary steps, we again attempted to derivatize the model compounds directly in the alumina-bound state. Single HFB-derivatives were formed from THIQ and E with retention time identical to those obtained from crystalline derivatives. This method is simple and rapid (Table IV). Reproducibility was checked by twelve sets of experiments and the standard deviation was ± 3.2%. About a 75% decrease in deviation and a large improvement of the percent recovery as compared to the other methods in Table IV, resulted from this modification.

These studies of CAs and THIQs derivatized in the alumina-bound state represent the first report of this novel adaptation for GC analysis. Meanwhile, Franklin
and Mayer (45), interested in double isotope analysis of CAs in the urine, acetylated urine CAs, along with C-14 labeled CAs added in the same urine, with H-3 acetic anhydride directly in an alumina bound state. Since their determination of endogenous amine was based on ratio of H-3/C-14 rather than absolute amount, they were not concerned with high recoveries.

Less than five nanomole of the catechol compounds were detected with this method. Also this method was successfully employed for extraction and detection of endogenous CAs from the rat adrenal glands. In the next experiment THIQ was added to the homogenate of the rat adrenal and brain tissue which was then detected and separated from endogenous CAs with this method. The retention times of both CAs and THIQs was identical to the crystalline materials.

The optimum pH in adsorption steps and the amount of alumina required for maximum results are shown in Fig. 12.

D. Aluminum oxide (Woelm) column

We attempted to use an aluminum oxide column and allowed HFBA to react with epinephrine and 4, 6, 7-trihydroxytetrahydroisoquinoline bound to alumina and followed by elution with ethyl acetate. Low yields of HFB-derivatives were obtained. It was thought that the problem was in the elution process. Usually three minutes shaking with each portion of ethyl acetate (4-5 times) is required (step 4 from
Fig. 12. The effect of pH (———) and the amount of aluminum oxide (-------) on per cent recovery of 4, 6, 7-THIQ from extract.
method B or C of derivative formation).
CHAPTER FOUR

SUMMARY AND CONCLUSIONS

A successful method for the preparation of fluoro-acylated tetrahydroisoquinolines (THIQs) and catecholamines (CAs), detectable with electron capture detector, by addition of heptafluorobutyric anhydride in acetonitrile to the compounds at room temperature was established. Among the different derivatization methods examined, this procedure was the only one which provided single derivatives from both THIQs and CAs. The reproducibility of this method was excellent. The presence of ω-hydroxy of CAs and similar 4-hydroxy-groups of THIQs did not interfere with the results. Because of great sensitivity and reproducibility, this method can be ideally substituted for the previous methods used for gas chromatographic studies. The advantage of this method is that CAs can be detected and separated from related THIQs.

THIQs in the presence of CAs were extracted from 1.0 N perchloric acid solution by aluminum oxide (Woelm) at pH 8.0 - 8.2 and were successfully derivatized in the alumina-bound state. This novel method of extraction followed by derivatization of catechol compounds without elution from absorbant proved to be much easier and faster than the resulted derivatization procedure on acid-eluted materials. This method is much more dependable than the classic one involving acid elution and lyophilization.
The deviation of the latter method is 75% more than this new adaptation. The average recovery of 42% agrees with those reported in the literature.

Endogenous CAs from a pair of rat adrenal glands were detected by new method of alumina extraction plus direct derivative formation with heptafluorobutyric anhydride and acetonitrile. By applying this method we have been able to extract $2 \times 10^{-7}$ grams of epinephrine or 4, 6, 7-trihydroxy-THIQ from perchloric acid solution and detect them with electron capture gas chromatography. The percentage of recovery of 4, 6, 7-trihydroxy-THIQ after addition to the brain and adrenal tissue homogenate, was the same as by extraction of the compound from an aqueous mixture.

With this method the tissues of ethanol and methanol intoxicated animals will be examined for evidence of in vivo alkaloid biosynthesis.
# APPENDIX

<table>
<thead>
<tr>
<th>Term</th>
<th>Short Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>AN</td>
</tr>
<tr>
<td>N, O-Bis trimethylsilyl</td>
<td>BSA</td>
</tr>
<tr>
<td>Catecholamine</td>
<td>CA</td>
</tr>
<tr>
<td>Dopamine</td>
<td>DA</td>
</tr>
<tr>
<td>Electron capture</td>
<td>EC</td>
</tr>
<tr>
<td>Epinephrine</td>
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<tr>
<td>Gas chromatography</td>
<td>GC</td>
</tr>
<tr>
<td>Heptafluorobutyric</td>
<td>HFB</td>
</tr>
<tr>
<td>Heptafluorobutyric anhydride</td>
<td>HFBA</td>
</tr>
<tr>
<td>Heptafluorobutyric imidazole</td>
<td>HFBI</td>
</tr>
<tr>
<td>Hydrogen flame ionization</td>
<td>HFI</td>
</tr>
<tr>
<td>Infrared spectrometry</td>
<td>IRS</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>NE</td>
</tr>
<tr>
<td>Nuclear magnetic resonance</td>
<td>NMR</td>
</tr>
<tr>
<td>Perfluorobutyric anhydride</td>
<td>PFBA</td>
</tr>
<tr>
<td>Tetrahydroisoquinoline</td>
<td>THIQ</td>
</tr>
<tr>
<td>Tetrahydropapaveroline</td>
<td>THP</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>TC</td>
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<tr>
<td>Thin layer chromatography</td>
<td>TLC</td>
</tr>
<tr>
<td>Trifluoroacetic anhydride</td>
<td>TFAA</td>
</tr>
<tr>
<td>Trimethylsilyl</td>
<td>TMSi</td>
</tr>
<tr>
<td>Trimethylsilyl anhydride</td>
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</tr>
<tr>
<td>Trimethylsilyl imidazole</td>
<td>TMSiI</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY

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The thesis submitted by Mostafa G. Bigdeli has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

[Signature]

[Date]