THE SEPARATION, IDENTIFICATION AND QUANTITATION OF TRICYCLIC ANTIDEPRESSANTS UTILIZING A CAPILLARY GAS CHROMATOGRAPH AND HASS SPECTROHETER

by

Robert M. Geidner

Submitted in Partial Fulfillnent of the Requirements
for the Degree of
Master of Science
in the
Chemistry
Program

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August, 1986

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THE SEPARATION, IDENTIFICATION AND QUANTITATION OF

TRYPTOPHAN METABOLITES UTILIZING [illegible]

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ABSTRACT

THE SEPARATION, IDENTIFICATION AND QUANTITATION OF TRICYCLIC ANTIDEPRESSANTS UTILIZING A CAPILLARY GAS CHROMATOGRAPH AND MASS SPECTROMETER

Robert M. Geidner

MASTER OF SCIENCE

YOUNGSTOWN STATE UNIVERSITY,1986

Many procedures have been developed to separate, identify and quantitate various tricyclic antidepressants such as amitriptyline, protriptyline, doxepin, imipramine and nortriptyline using a gas chromatograph and various detectors. Much of today's research work is done using fused silica capillary columns because of their increased resolving power due to the large number of theoretical plates. Still, much of the clinical work uoes the somewhat outdated packed columns.

A recent study commented about inaccuracies in quantitation of various tricyclic antidepresrants by many clinical laboratories (1). Many of these procedures Involve single or multi-step extractione, preparation of derivatives of these drugs and the use of various detectors in order to separate and identify them.

In the present study, state of the art equipment was ured to investigate the factors that might affect the reparation, identification and quantitation of three very

similar tricyclic antidepressants: amitriptyline, nortriptyline and protriptyline. Thir was accomplished by using the many advantages of capillary gar chromatography (GC) combined with mass rpectrometry (MS) for increared rerolution of complex mixtures and the detection of nanogram levels of the individual drugs. In order to separate and identify these three antidepressants, standards of varying concentrations of each drug and mixtures of the three drugs were run to obtain their optimal GC conditionr. In order to further increase senritivity, samples were analyzed using methane chemical ionization and selective ion monitoring. Then many of the GC/MS parameters were varied in order to see their effects on reparation and quantitation.

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I would also like to thank my family and friends, especially my wife, Mary, and my children, Christopher, Nicholas and Megan for their constant unselfish support and patience.

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- brothers

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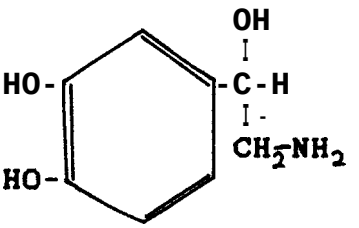
INTRODUCTION

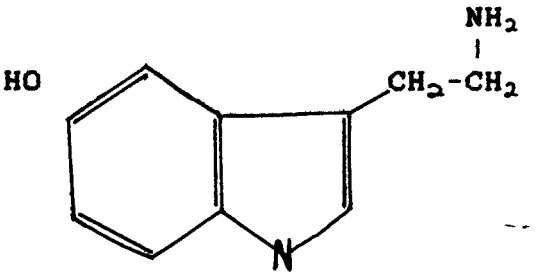
Biochemical and Pharnacoloaical Background

Tricyclic antidepressants (TCA's) are one of the most widely used clarser of psychotropic drugs for the treatment of endogenous depression. This type of depression is hypothesized to be the reoult of a genetic-biochemical abnormality affecting a person's ability to cope with stress. Depressive illness can be divided into two groups, depending on which neurotranrnitter is deficient:

1. Norepinephrine
2. Serotonin



Norepinephrine



Serotonin

Fig. 1. Chemical structure of Norepinephrine and Serotonin

CHAPTER I

INTRODUCTION

Bupropion (Wellbutrin®, Zyban®) is a novel aminoketone antidepressant with a unique pharmacological profile. Unlike most antidepressants, bupropion is a weak inhibitor of neuronal uptake of serotonin, norepinephrine and dopamine. It is also devoid of anticholinergic activity. Bupropion is a racemic mixture and the pharmacological activity has been found to reside primarily with the individual enantiomers. Hydroxybupropion, the major metabolite of bupropion, is also pharmacologically active.

1. Bupropion

2. Hydroxybupropion

3. Threohydrobupropion

Each condition can be identified by decreases in the rerpective breakdown products of metabolirm of the deficient neurotranrmitter. In carer of norepinephrine-related deprerriona, there ir a decrease in urinary levels of normetanephrine (NMET), vanillylmandelic acid (VMA), and 3-methoxy-4-hydroxyphenylglyco1 (MHPG). For those patients ruffering fror serotonin-related drpresrione, the breakdown product of serotonin, 5-hydroxyindoleacetic acid (5-HIAA) ir found in abnormally low levels in the rpinal fluid (2).

Norepinephrine and rerotonin are both products of the body's metabolism of tyrosine; the latter goes through a longer pathway via tryptophan production. There neurotransmitter. carry nerve irpulrer across the synaptic cleft to be picked up by receptors on the neuron cell membrane, at which tire the synaptic transmission is terminated. The most effective action of antidepressants to relieve depression ir to block the uptake of there amine neurotranamitters at the rynaptic cleft, thereby a1lowing longer perairtence of neurotranrmirrion.

It has been shown that amitriptyline giver better response in patients with rerotonin-related depression, while imipramine and its related tricyclic antideprerranta work better in patientr with norepinephrine defects. Since these drugs are usually given over a long period of time it is important to monitor their plasma lavele. Thie can help to

optimize therapeutic response and avoid toxic side- effects. The importance of monitoring can be realized by comparing therapeutic levels and toxic levels as seen in Table 1. It rhould also be noted that life-threatening cardiac toxicity or reizurer have been seen in cares of plasma concentrations over 1,000 ng/mL (3).

TABLE 1

THE ORAL DOSAGES, THE THERAPEUTIC PLASUA LEVELS AND THE TOXIC PLASUA LEVELS OF AUITRIPTYLINE (AMI), NORTRIPTYLINE (NOR), AND PROTRIPTYLINE (PRO) (4).

DRUG	DOSE (mg/day)	THERAPEUTIC LEVEL (ng/mL)	TOXIC LEVEL (ng/mL)
AMI	50-100	120-250	400
NOR	40-100	50-150	200
PRO	15-40	50-150	200

There are three tricyclic antideprerrants to be investigated in thir study:

1. Amitriptyline
2. Nortriptyline
3. Protriptyline

Amitriptyline is a potent antidepressant but maintains a low degree of toxicity. It also has mild side effects which include drowsiness, dizziness, nausea, headaches and hypotenrion. Nortriptyline is very similar to anitriptyline in both rtructure and function, although it does present

TABLE 1

THE ORAL DOSAGES, THE THERAPEUTIC BLOOD LEVELS, AND THE TOXIC LEVELS OF AMITRIPTYLINE (AMI), NORTRIPTYLINE (NOR), AND PROTRIPTYLINE (PRO) (*)

DRUG	DOSE (mg/day)	THERAPEUTIC LEVEL (ng/ml)	TOXIC LEVEL (ng/ml)
AMI	75-300	120-250	500
NOR	40-150	50-140	300
PRO	15-40	100-250	500

(*) Data from Gram et al. (1977).

amitriptyline is metabolized to nortriptyline. If a drug does not have its own therapeutic effects, but is converted to a drug that does, it is called a prodrug. Nortriptyline is also available for prescription.

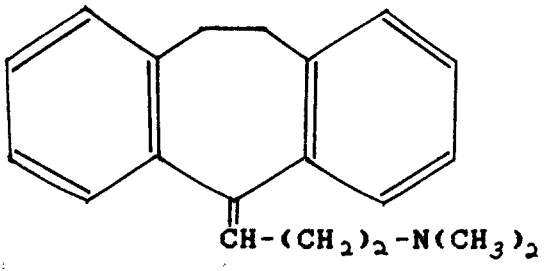
3. Protriptyline

2. Nortriptyline

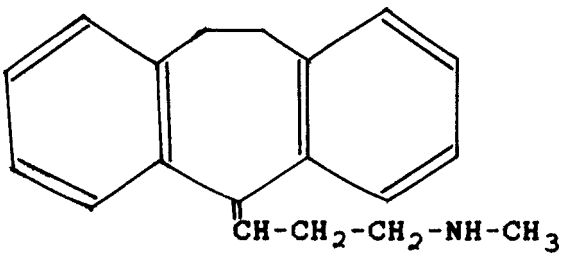
1. Amitriptyline

The tricyclic antidepressants are listed in order of potency in Table 1.

AMITRIPTYLINE(Elavil)
$C_{20}H_{23}N$
W.W. = 277



NORTRIPTYLINE(Pamelor)
$C_{19}H_{21}N$
W.W. = 263



PROTRIPTYLINE(VIVACTIL)
$C_{19}H_{21}N$
M.W. = 263

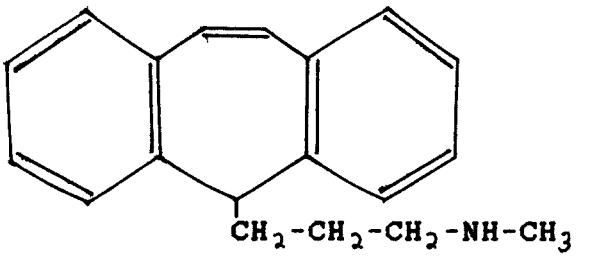


Fig. 2. The chemical structure, chemical formula, molecular weight, and trade name of Amitriptyline, Nortriptyline, and Protriptyline.

rone more serious ride-reactions in some patients that would warrant discontinuing the drug. There would include tachycardia, hypotenmion, confusion, and hallucinations. Protriptyline is a rapid acting drug without the usual tranquilizing properties of other TCA's. It is similar to nortriptylina because of the serious consequences occuring in an overdose due to cardiac involvement (5).

PROTRIPTYLINE(VIVACTIL)
$C_{19}H_{21}N$
M.W. = 263

NORTRIPTYLINE(PAMELOR)
$C_{19}H_{21}N$
M.W. = 263

AMITRIPTYLINE(ELAVIL)
$C_{20}H_{23}N$
M.W. = 277

Fig. 1. The chemical structures, chemical formula, molecular weight, and brand names of Amitriptyline, Nortriptyline, and Protriptyline.

each_word_within_this_text_block_is_reversed_to_create_the_mirror_image_effect_above

History of Analytical Methods

Since 1960 there have been nunerour methods developed for the measurement of TCA's. Most of there methods require extraction of the drug from an aqueous biological medium into an organic rolvent. This rxtract can then be analyzed by rpectrofluorometry, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC),radioimmunoassay (RIA), enzyme inmunoasrayr such as enzyme multiplied innunoarsay (EMIT), and gas chromatography (GC). The main criteria for modern toxicological methods are rpecificity, sensitivity, accuracy, reproducibility, time, and cost of equipment and analysir. The following is a review of various modern methods for the measurement of TCA's uring the above noted criteria as guidelines.

An early opectrofluoronetric procedure developed by Moody and colleague8 (6) involved a three-step extraction and acetylation before analymir. Thir method war not very rpecific because some of the parent drug's metabolites interfered with the analyrir. In 1978, Kaul and him colleagues (7) developed a method sensitive enough to measure 5 µg/L. This war a very time consuming procedure since it involved reacting amitriptyline with alpha-bromomethylacridine forming a quaternary product.

TLC procedurer have been developed by a number of researchers (8-9). It currently is a mtandard screening (qualitative) method with a sensitivity better than 1 µg/L,

Hierarchical Multiple Regression

Suppose that we have a set of predictor variables and we want to know how much variance in the criterion variable they explain. Moreover, we want to know how much variance they explain above and beyond some other predictor variables. For example, we may want to know how much variance in job performance is explained by a battery of cognitive ability tests above and beyond what is explained by a measure of conscientiousness. One way to address this question is to use hierarchical (or sequential) multiple regression. In hierarchical multiple regression, we enter predictor variables into the regression equation in a specified order. Then, we examine the R^2 change statistic to see how much variance in the criterion variable is explained by each predictor variable after controlling for the predictor variables entered before it. For example, suppose we enter a measure of conscientiousness into the regression equation first and we enter a battery of cognitive ability tests second. The R^2 change statistic tells us how much variance in the criterion variable is explained by the battery of cognitive ability tests above and beyond what is explained by conscientiousness. Another way to think about hierarchical multiple regression is that it is a way to statistically control for the predictor variables entered first (i.e., to remove their influence) when examining the relationship between the predictor variables entered second and the criterion variable. Hierarchical multiple regression is also sometimes called sequential multiple regression or blockwise multiple regression.

Hierarchical multiple regression is a powerful method that allows researchers to address many interesting research questions. However, it is rarely used in I-O psychology research (e.g., Aguinis, Pierce, & Culpepper, 2009). If researchers are not familiar with hierarchical multiple regression, they may not even realize that it is a viable option for addressing their research questions. Moreover, researchers who are familiar with hierarchical multiple regression may not use it because they are not familiar with the various decisions that must be made when using it. Thus, the purpose of this article is to provide researchers with guidelines for making these decisions. Following these guidelines should help researchers obtain more accurate results when using hierarchical multiple regression to address their research questions.

and all resultr nay be confirmed and quantitated by other methode.

The use of HPLC for the separation of TCA's was first described in 1975 by Knox and Jurand (10). The HPLC procedures are very similar in extraction method and sensitivity (approximately 1 µg/L) to GC procedures. The advantages of HPLC are its large carrying capacity and that the ranples are not destroyed as with GC. HPLC can also be used with a number of different detectorr such as mass spectrometer(MS) and visible and ultraviolet (UV) detectors.

RIA procedures for tricyclic antidepressants were introduced by Spector and colleagues in 1975 (11). Since then numerous other methods have been described with remarkable sensitivity and precision but with questionable specificity. This is due to crorr reactions with various other tricyclics and their netabolites. The RIA procedures have cone under fire in recent years because of inherent biohazard factors and are currently being replaced by enzyme labelled methods such as EUIT.

The enzyme inmunoasray procedure. have recently found wide acceptance in the area of toxicology in clinical laboratories. This is because assays such as the EMIT procedures conbine the specificity and sensitivity of immunoassay with the convenience, speed, and reproducibility of enzyme measurements.

TABLE 2

SUMMARY OF METHODS USED TO MEASURE TRICYCLIC ANTIDEPRESSANT DRUGS IN SERUM (13).

METHODS	SENSITIVITY(µg/L)	SPECIFICITY[a]	DERIVATIVE
Spectrometric	100	1+	YES
Fluororetric	5-30	1+	YES
TLC	5	2+	NO
EMIT	3	3+	NO
RIA	0.1-2	3+	NO
GC *[b]	10	2+	YES/NO
GC/MS	1	4+	YES/NO
HPLC /UV	2-10	2+	NO
HPLC/MS	0.5	4+	NO

[a] specificity is graded on a scale of 1-4 or poor-excellent

* denotes other detector. as noted on above.

The EMIT principle is as followrr (1) Drug-specific antibodies are added to the patient specimen. (2) Antibody-drug binding occurs. (3) The enzyme-labeled drug is added and will occupy any vacant antibody sites. (4) The free enzyme-labeled drug reacts with the substrate, resulting in coenzyme conversion and an absorbance change which is measured rpectrophotoretrically. As antibody-drug binding taker place, enzymatic activity is reduced. Therefore, only the free enzyme-labeled drug can react with the substrate and coenzyme. Ar a rrrult, the rate of enzyme

TABLE 1. COMPARISON OF METHODS USED TO MEASURE TETRACYCLINE RESIDUES IN MILK

METHOD	SENSITIVITY (ppb)	APPROXIMATE % RECOVERY	DERIVATIVE
Spectrometric	100	70	YES
Fluorometric	5-50	70	YES
TLC	5	50	NO
EMIT	3	90	NO
RIA	0.1-1	50	NO
GC**	10	50	YES/NO
GC/MS	1	40	YES/NO
HPLC (UV)	2 to 10	80	NO
HPLC/MS	0.5	40	NO

* Sensitivity may be greater if a cleanup is used before
boot-strapping.
** Derivatization is necessary to obtain adequate
sensitivity.

The EMIT procedure is as follows: (1) Dilute
antibodies are added to the test sample. (2)
Antibody-drug binding occurs. (3) The enzyme-labeled drug is
added, competing for binding sites with drug in the sample.
(4) The
resulting enzyme activity is measured spectrophotometrically. As antibody-drug
binding increases, enzyme activity decreases. The
Therefore, only the free enzyme-labeled drug can react with
the substrate and consume it as a result, the rate of enzyme

activity is directly proportional to the patient's drug concentration. In many of these procedures there ir still a lirited amount of crorr-reactivity to the drug'r own metabolite8 and other very similar drugs (13).

Gas chromatography (GC) war first described in 1952 by A. T. James and A. J. Martin with their work in reparating volatile fatty acids (14). They used a packed column in which there ir a stationary phare, urually a nonvolatile rolid or a nonvolatile liquid coating an inert rolid, and a mobile (gas) phase. Thir is a process in which volatile rubrtancer are reparated according to their vapor prerrurer. The higher the vapor prerrure of a compound, the greater is its mobile-to-rtationary phase ratio and therefore, the farter it will elute from the column (15). The solubility of the solute in the liqiud phare is also a minor factor to be considered.

There are two typer of columns presently being used: packed columnr and capillary columns. The packed columns contain a nonvolatile solid (stationary phare) and typically measure 6 ft. x 2 mm i.d. Whereas capillary columns will mearure 12-50 m x 100-500 µm i.d. There are three main typer of capillary columnr: 1) rupport coated open tubular(SCOT)-a single coating of support material in a liquid matrix; 2) porous layer open tubular(PLOT)-porous layer of support material fixed to column wall; and 3) wall coated open tubular(WCOT)-liquid phare deposited directly upon column

wall. The packed and capillary colunnr both have a wide variety of atationary pharer available.

The recent development of bonded phare capillary colunnr has rerulted in ruch more durable, reliable, and reproducible columns. In there colunnr, the stationary pharer are polymer chains, ruch am polydinethyleiloxane, that give the column a particular polarity. These polymers are covalently bonded to the rilica surface of the column and are alro crors-linked to each other. Thim bonding rerultr in a stationary phaee that will not deteriorate due to prolonged exposure to high tenperaturer or repeated injection of a wide variety of rolvents. There are alro aone columns that can tolerate multiple injections of a polar rolvent ruch as water (16).

6ar chronatography is the most widely used method for the detection of TCA. It war first used for this in 1970 by Braithwaite and Whatley (17). There have been a nunber of different types of detectors utilized much as flama-ionization, electron-capture, and alkali flame-ionization (nitrogen-sensitive) detectors. There are nunerour procedures using these detectorr and all show excellent precision and sensitivity, but the rpecificity is quertionable. Thia is because all of there detectors measure the total number of ion6 or reactive atonr reaching the detector and do not provide a means of identifying the drug being analyzed except for its retention tine.

In 1976 Hammer and colleagues ured the combined gas chromatograph/ mass rpectrometer in the datection of nortriptyline (18). The result war a very rensitiva and precise procedure with a greatly improved rpecificity. In this care, the mass spectrometer detects the total number of ions and simultaneously measures the abundance of ion fragnentr according to their mass. Thir fragmentation pattern, which ir due to electron or chemical ionization, ir unique for each drug and can be used in combination with retention times to identify a rpecific drug. Sinca Hammer's work, there have been many improvements in procedures, mass spectrometry instrumentation, and the wide use of capillary columns to give greater reparation and identification of complex mixtures of drugo. Many of the tandem (GC/MS) instruments have been interfaced to computers for improved control of instrunent parameterr and data handling.

There her been much research done on developing gas chromatography procedures for TCA's. These procedures have utilized both packed and capillary colunnn, a number of sample preparation technique., and variour detectorr. There arm many examples of techniques using packed colunnr and flame-ionization detectorr (19), mass rpectroneterr (20) and nitrogen-sensitive detectors involving ringle and multi-step extractions (21-22). In recent years, there has bean an increased amount of work uring capillary columns and nitrogen/phosphorus sensitive detectorr (23),

flame-ionization detectors (24) and mass spectrometers (25). The greatest improvement in renritivity and specificity are the result of the gar chromatograph / narr spectrometer combination.

Gas Chromatograph and Mass Spectrometer

The potential analytical power of thir combination war first realized in 1957 when Holmes and Morrell crudely coupled the two instruments (26). Mass rpectrometera have three basic function. (1) to vaporize compound8 to be analyzed, (2) to ionize neutral molecular in the gar phase, and (3) to separate and detect ions according to their mass-to-charge ratios (m/z). In the GC/MS inrtrunentr vaporization is accomplished in the oven of the gas chromatograph and maintained throughout the ryrten. Ionization can be accomplished a number of ways; the ones of interest in this rtudy are electron impact ionization and chemical ionization. In electron impact ionization, the sample is introduced to the ion source as a vapor. This vapor ir then bombarded by high energy electron8 producing a positively charged nolecular ion (M^+). The electron energy can be varied but is typically 70-80 eV. The amount of energy required for ionization of most organic compoundr ir 7-13 eV. This ionization energy is the amount of energy needed to remove an electron from the highest occupied molecular orbital. This reaction is reprerented as follows:

Il semble que l'on puisse considérer que la (12) exprime le fait que la vitesse de variation de la température dans le temps est proportionnelle à la différence de température entre le milieu et le corps.

Cette équation différentielle est linéaire du premier ordre à coefficients constants. Elle est sans second membre. Elle est homogène.

Nous allons maintenant résoudre cette équation différentielle.

Nous savons que la solution générale de cette équation différentielle est la somme de la solution générale de l'équation différentielle sans second membre et d'une solution particulière de l'équation différentielle avec second membre.

Nous allons donc commencer par résoudre l'équation différentielle sans second membre.

Nous savons que la solution générale de cette équation différentielle est de la forme $\theta = Ce^{kt}$ où C est une constante arbitraire.

Nous allons maintenant déterminer une solution particulière de l'équation différentielle avec second membre.

Nous savons que la température du milieu est constante et égale à θ_0.

Nous allons donc chercher une solution particulière de la forme $\theta = \theta_0$.

En remplaçant dans l'équation différentielle, nous obtenons $0 = k(\theta_0 - \theta_0)$, ce qui est vérifié.

Donc $\theta = \theta_0$ est une solution particulière de l'équation différentielle avec second membre.

La solution générale de l'équation différentielle avec second membre est donc $\theta = Ce^{kt} + \theta_0$.

Nous allons maintenant déterminer la constante C en utilisant la condition initiale.

Nous savons que $\theta(0) = \theta_1$.

En remplaçant dans la solution générale, nous obtenons $\theta_1 = Ce^{k \cdot 0} + \theta_0$, soit $\theta_1 = C + \theta_0$.

Donc $C = \theta_1 - \theta_0$.

La solution de l'équation différentielle est donc $\theta = (\theta_1 - \theta_0)e^{kt} + \theta_0$.

Nous allons maintenant déterminer la constante k en utilisant la condition $\theta(t_1) = \theta_2$.

En remplaçant dans la solution, nous obtenons $\theta_2 = (\theta_1 - \theta_0)e^{kt_1} + \theta_0$.

Donc $e^{kt_1} = \frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}$.

Donc $kt_1 = \ln\left(\frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}\right)$.

Donc $k = \frac{1}{t_1}\ln\left(\frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}\right)$.

La solution de l'équation différentielle est donc $\theta = (\theta_1 - \theta_0)e^{\frac{t}{t_1}\ln\left(\frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}\right)} + \theta_0$.

Nous allons maintenant déterminer la constante k en utilisant la condition $\theta(t_1) = \theta_2$.

En remplaçant dans la solution, nous obtenons $\theta_2 = (\theta_1 - \theta_0)e^{kt_1} + \theta_0$.

Donc $e^{kt_1} = \frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}$.

Donc $kt_1 = \ln\left(\frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}\right)$.

Donc $k = \frac{1}{t_1}\ln\left(\frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}\right)$.

La solution de l'équation différentielle est donc $\theta = (\theta_1 - \theta_0)e^{\frac{t}{t_1}\ln\left(\frac{\theta_2 - \theta_0}{\theta_1 - \theta_0}\right)} + \theta_0$.

$$M + e^- = M^+ + 2e^- \quad (1)$$

Upon ionization, the excess energy may caure the decomposition of M^+ to form fragmented ions ($F1^+$, $F2^+$, etc). This fragmentation pattern, or mars spectrum, can be used to identify the structure of the original molecule. The mass spectrum can be used for identification since it is like a reproducible fingerprint of the compound. It is these mass spectra and retention times that are used in identifying conponento of a conplcx mixture.

Once the compound has been identified, the relative amount of the compound can be determined and its molecular mass confirmed by the use of chemical ionization. This has been used quite often for quantitation because of its increased sensitivity. In chemical ionization (CI), the sample is vaporized in the presence of a high pressure of reagent gas such as methane or ammonia which is bombarded by electronm. Since the reagent gas is found in excess compared to the mample, it is the reagent gas which is ionized by the electrons. The ionization of the sample is the result of ion-molecule collision reaction. between the ionized reagent gas and the rample molecules. Therefore, ionization is the result of a chemical reaction rather than direct bombardment of electrons. In this study methane (CH_4) war used ar the reagent gas. The high concentration of reagent gar results in recombination reactions and further ionization. Therefore, the following reactions occur during ionization:

$$CH_4 + e^- \rightarrow CH_4^+ + 2e^- \quad (2)$$

$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2 \quad (3)$$

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3 \quad (4)$$

There is also a small amount of the $C_2H_5^+$ ions formed during ionization. It is these ionr that react with the sample nolecules (M) to form the $(M + H)^+$, $(M + CH_3)^+$, $(M + C_2H_5)^+$, and $(M + C_2H_5)^+$ ions. As a result of chemical ionization the fragmentation that is seen in electron impact ionization does not readily occur and a majority of sample ions are found in the MH^+ form.

$$W + CH_5^+ \rightarrow WH+ + CH_4 \quad (5)$$

Therefore, with a properly tuned mass rpectoneter the MH^+ ion concentration in the CI mode of operation may be ten times greater than the M^+ ion in the EI node of operation. This in effect causes a ten-fold increase in sensitivity.

Once ionization is conpleted, the mass spectromrter must separate and detect the ions according to the mass to charge ratio (m/z). There are two principal methods for separating the ions to be discussed at this tine. The first utilizes electrical and magnetic fields and the second user a quadrupole masr filter. The first magnetic-rector mass spectrometer, in which positive ions are deflected 180 degrees in a aagnetic field, was used by A. J. Denpster in 1918. The ions leaving the ion rource all have the same kinetic energy

$$KE = 1/2\ mv^2 = zeV \quad (6)$$

$$KE = \tfrac{1}{2}mv_{ion}^2 \quad \text{eqn.} \qquad (6)$$

kinetic energy.

If all three ions have the same velocity and the same mass, then the ion with the greatest kinetic energy will have the greatest momentum. Ions of the same kinetic energy but different masses can be separated by a magnetic field. A 70 eV beam of electrons is used to bombard the sample molecules in the ion source of the mass spectrometer, and the positive ions produced are accelerated by an electric field. Ions of the same kinetic energy but different masses can be separated by a magnetic field. A 70 eV beam of electrons is used to bombard the sample molecules in the ion source of the mass spectrometer, and the positive ions produced are accelerated by an electric field.

$$CH_4 + e^- \rightarrow CH_4^{+\cdot} + 2e^- \qquad (1)$$

$$CH_4^{+\cdot} + CH_4 \rightarrow C_2H_5^+ + H^\cdot \qquad (2)$$

$$CH_4^{+\cdot} + CH_4 \rightarrow CH_5^+ + CH_3^\cdot \qquad (3)$$

Reaction (2) is a radical cation fragmentation reaction. The $C_2H_5^+$ ion is a carbocation, and it is the most abundant ion in the mass spectrum of methane. Reaction (3) is a proton transfer reaction. The CH_5^+ ion is also a carbocation, but it is not as abundant as the $C_2H_5^+$ ion.

$$M + CH_4^{+\cdot} \rightarrow MH^+ + CH_3^\cdot \qquad (4)$$

The MH^+ ion is a protonated molecule, and it is the most abundant ion in the mass spectrum of many compounds. The MH^+ ion is also called the quasi-molecular ion. The MH^+ ion is the most abundant ion in the mass spectrum of many compounds because it is the most stable ion. The MH^+ ion is the most abundant ion in the mass spectrum of many compounds because it is the most stable ion. Once ionization is completed, the ions are separated according to their mass-to-charge ratio (m/z). There are two primary methods for separating ions: magnetic deflection and time-of-flight. The first method uses a magnetic field to deflect ions of different masses onto different paths. The second method measures the time it takes for ions to travel a known distance. Ions of different masses will have different velocities, with lighter ions moving faster than heavier ions. By measuring the time it takes for ions to travel a known distance, the mass-to-charge ratio can be determined.

$$M + CH_5^+ \rightarrow MH^+ + CH_4 \qquad (5)$$

Protonation of the molecule (M) to form the $(M + H)^+$, $(M + CH_5)^+$, and $(M + C_2H_5)^+$ ions. A positive ion of mass M will have a kinetic energy (KE) given by the equation:

$$KE = \tfrac{1}{2}mv^2 \qquad (6)$$

where m is the mass of the ion, v is its velocity, z is tho number of charges on an ion, e is the magnitude of the electronic charge, and V is the electrical potential. As ions enter the magnetic field they are separated according to their momentum and each m/z will have a unique radius of trajectory, R. The heavier ions have more momentum and are therefore influenced less by the magnetic field. This is seen by a flatter trajectory, whereas the lighter ions are more strongly influenced and follow a more curved trajectory. Finally, the ions must pass through a focusing slit to reach the detector. Thus, at a given magnetic field, B, only ions of a single m/z ($m/z = B^2R^2/2v$) will have the proper trajectory to reach the datector. By varying the magnetic field strength, a wide range of m/z values can be scanned to be focused at the detector slit. This type of mass spectrometer is limited to a scan rate of 0.1 s/decade for the type of magnets available. A mass decade represents a ten-fold increase in mass, for example, 50-500 anu. A time of approximately 0.2 s is needed to reset the magnet between scans. Therefore, the total scan tine is 0.3 s.

The quadrupole mass filter consists of four rods carrying variable combinations of both radio frequency (RF) and direct current (DC) voltages. Ions interact with the electrostatic forces created by the rods aftrr they are accelerated towards the detector. By controlling the DC-to-RF ratio, an electrical field can be established so

that ions of only one specific m/z value can pass through the mass analyzer, whereae almost all other m/z value ions collide with the rods and are neutralized. If the RF and DC amplitudes are simultaneously varied, a wide range of m/z value ions can be analyzed by the detector. The mass scanning range of the quadrupole mass filter is approximately 10-800 anu. A maximum scan rate of 780 amu/s can be reached before there is a significant decrease in resolution, peak shape, and intensity. This type of instrumentation is especially useful when a capillary column GC technique is performed. Capillary columnr with up to 10^6 theoretical plates can resolve on the order of ten peaks/s, especially early in the chronatogram. Therefore, in order to get reasonable reconstructed ion chromatograms, RIC, (graph of total ion current versus time) under these conditions, a scan rate of 100 scans/s is necessary (27-28). The conditions noted above are extremes and are beyond the capabilities of the instrumentation used in this study. The specifications of the Finnigan Model 1020B and capillary column used in this study are noted in the materialo-and apparatus section of this study.

dependent upon the specific application. Typical values are 50 to 100 ohms for RG-58/U, 75 ohms for RG-59/U, and 93 ohms for RG-62/U. The characteristic impedance of the Ethernet Model 10Mbit and cable is 50 ohms. Maximum cable length is 500 meters (1640 feet) for thick cable and 185 meters (607 feet) for thin cable. Maximum number of transceivers per segment is 100. Minimum distance between transceivers is 2.5 meters (8 feet). Transceiver cable length is 50 meters (164 feet) maximum. The transceiver cable contains four twisted pairs of 24 AWG wire. Two pairs are used for data in and data out, one pair is used for collision detect, and one pair is used to provide power to the transceiver from the computer. The maximum length of the transceiver cable for the thick cable is 50 meters (164 feet). The maximum length of the transceiver cable for the thin cable is 4 meters (13 feet). The minimum length of the transceiver cable is 0.5 meters (1.5 feet). The transceiver cable connects to the computer's Ethernet interface board through a 15-pin D-shell connector. The maximum number of transceivers per segment is 100. The minimum distance between transceivers is 2.5 meters (8 feet). The transceiver cable length is 50 meters (164 feet) maximum. The transceiver cable contains four twisted pairs of 24 AWG wire. Two pairs are used for data in and data out, one pair is used for collision detect, and one pair is used to provide power to the transceiver from the computer. The maximum length of the transceiver cable for the thick cable is 50 meters (164 feet). The maximum length of the transceiver cable for the thin cable is 4 meters (13 feet). The minimum length of the transceiver cable is 0.5 meters (1.5 feet). The transceiver cable connects to the computer's Ethernet interface board through a 15-pin D-shell connector. The maximum number of transceivers per segment is 100. The minimum distance between transceivers is 2.5 meters (8 feet). 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CHAPTER II

PURPOSE OF STUDY

It hae been noted in a previous study that many clinical laboratories doing work in toxicology have great difficulty in the detection and quantitation of nunerous drugs (1). It is the purpose of this study to develop the optimum operating criteria for the separation, identification and quantitation of three very similar tricyclic antidepressants (AMI, NOR, and PRO) utilizing the Finnigan Model 1020 GC/MS with a capillary column. I have chosen to do this work using standards and not to deal with drug extraction procedures themselves because of the vast amount of work done in this area and due to the extra equipment and tine necessary. It is also my intention to use available equipment such as a capillary column and an injector liner with as little pretreatment as possible to make clinical adaptation easier and less time consuming.

I will also attempt to thoroughly investigate as many variables or adjustable instrument parameters as possible to see their effects on the procedure. Therefore, the scope of this study is two-fold, to investigate the problem of developing an analytical procedure and to understand the capabilities of a state-of-art GC/MS instrument.

EL CAPITAN

VISTA OF TOWER

CHAPTER III

UATERIALS AND APPARATUS

All reagents were analytical grade including methanol supplied by Fisher Scientific Co. (Orangeburg, N. J. 10962) Methane, the reagent gas, and Helium, the carrier gas, were ultra-high purity grade supplied by Airco Products. The tricyclic antidepressants were supplied by Youngstown Osteopathic Hospital and purchased from the following companies: amitriptyline HCl (Elavil) and protriptyline HCl (Vivactil) from Merck Sharp & Dohme (West Point, PA 19486); and nortriptyline HCl (Pamelor) from Sandoz Pharnaceutical Div. (East Hanover, N. J. 07936).

All analyses were performed on a Finnigan Model 10208 Automated GC/MS which is computer interfaced for instrument parameter control and data processing. In this study the chemical ionization (CI) ion source war used, as opposed to the electron ionization (EI) ion source, in order to give optimum results during chemical ionization -- procedures while allowing the flexibility to perform electron ionization without a large loss in sensitivity. The ion source is the actual enclosed compartment where ionization occurs. The column is a fused silica capillary column measuring 15 m X 0.25 mm I.D. with a polymer stationary phase of polyvinyl- phenylnethylsiloxane (SE-54)

with a film thickness of 0.25 μm. It was purchased from J & W Scientific, Inc. (Rancho Cordova, CA. 95670).

CHAPTER IV

EXPERIXENTAL

The drugs were ured in their hydrochloride salt forms and were dissolved in methanol to obtain stock rtandards of a concentration of 1 µg/µL for both anitriptyline (AMI) and nortriptyline (NOR) and 0.5 µg/µL for protriptyline (PRO). This was done so that approximately 1 µg of sample could be injected onto the colunn in a rrall solvent volume without overloading the column. The Finnigan Model 10208 GC/MS war zeroed and calibrated daily according to the manufacturer's specifications. A nanual tune was also carried out daily in order to adjust the instrument to obtain optimum resolution and intensity of the ions of interest in the range of 40-350 atomic mass units (amu). An example of the Finnigan computer syrten's typica1 calibration report and the nanual tune settings are seen in Figures 3 and 4, respectively.

The standards and dilutions of the standards were ured to run analyrer in the electron ionization and chemical ionization modes to obtain reconstructed ion chromatogram and mass spectra of each drug standard. The mass spectrum of each drug was confirmed by comparing it to the electron ionization work by T. Mills et al (29)

CHAPTER IV

EXPERIMENTAL

CALIBRATION REPORT:

18 = LOWEST PEAK IN REFERENCE TABLE FOUND

614 = HIGHEST PEAK IN REFERENCE TABLE FOUND

21 OF 21 REFERENCE PEAKS WERE FOUND

1% OF PEAK WIDTH = RUS FIT ERROR
(RUS FIT ERROR <10% ACCEPTABLE)

Figure 3. An example of a calibration report

1.	ELECTRON HULTIPLIER SWITCH	ON
2.	ELECTRON UULTIPLIER VOLTAGE	-2200.00
3.	HIGH RESOLUTION	133.00
4.	LOW RESOLUTION	125.00
5.	ION ENERGY	2.51
6.	FILAMENT SWITCH	ON
7.	ION PROGRAM	3.22
8.	LENS VOLTAGE	-166.00
9.	EXTRACTOR	2.00
10.	ELECTROHETER RANGE	7.00
11.	ELECTROHETER ZERO	2.05

Figure 4. Example of the typical manual tune settings

There mass rpectra are seen below in Figures 5-7. A mixture of the three drug etandards was then run in order to assure that separation was possible wth the SE-54 capillary column. These standards were analyzed using tho ieothorma1

and programmed temperature nodes to determine the optimum analysis conditions. Later many of the parameters (those denoted by an * in Figure 8) were altered in order to determine their effects on the reparation and quantitation of AMI, NOR, and PRO.

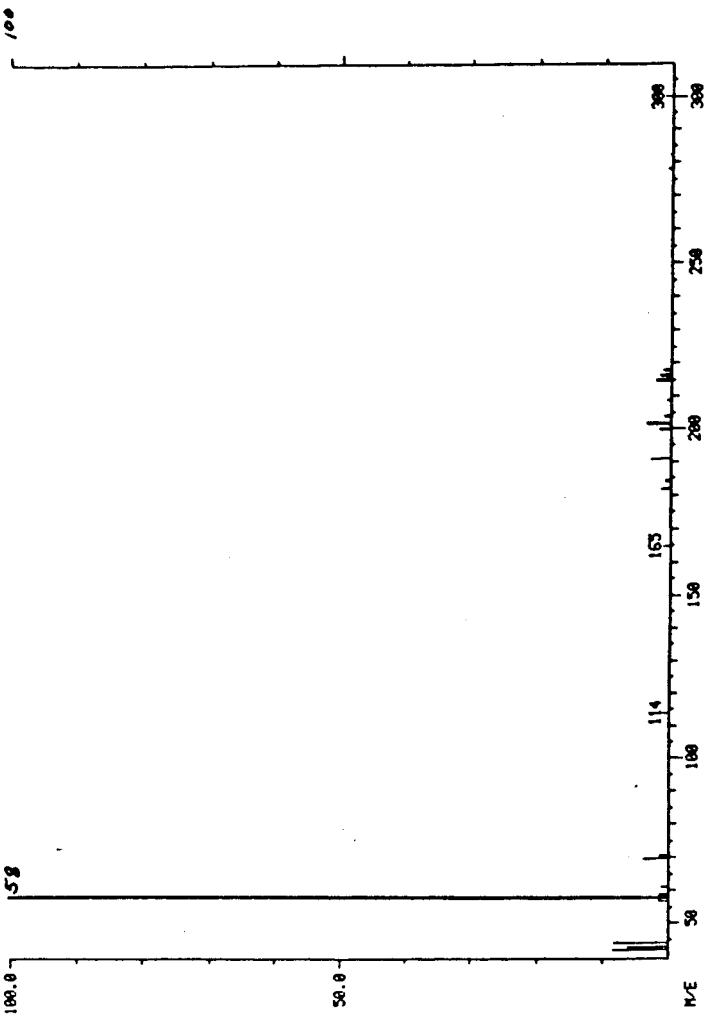

Figure 5. Reference EI Mass Spectrum of Amitriptyline from the work by T. Mills III.

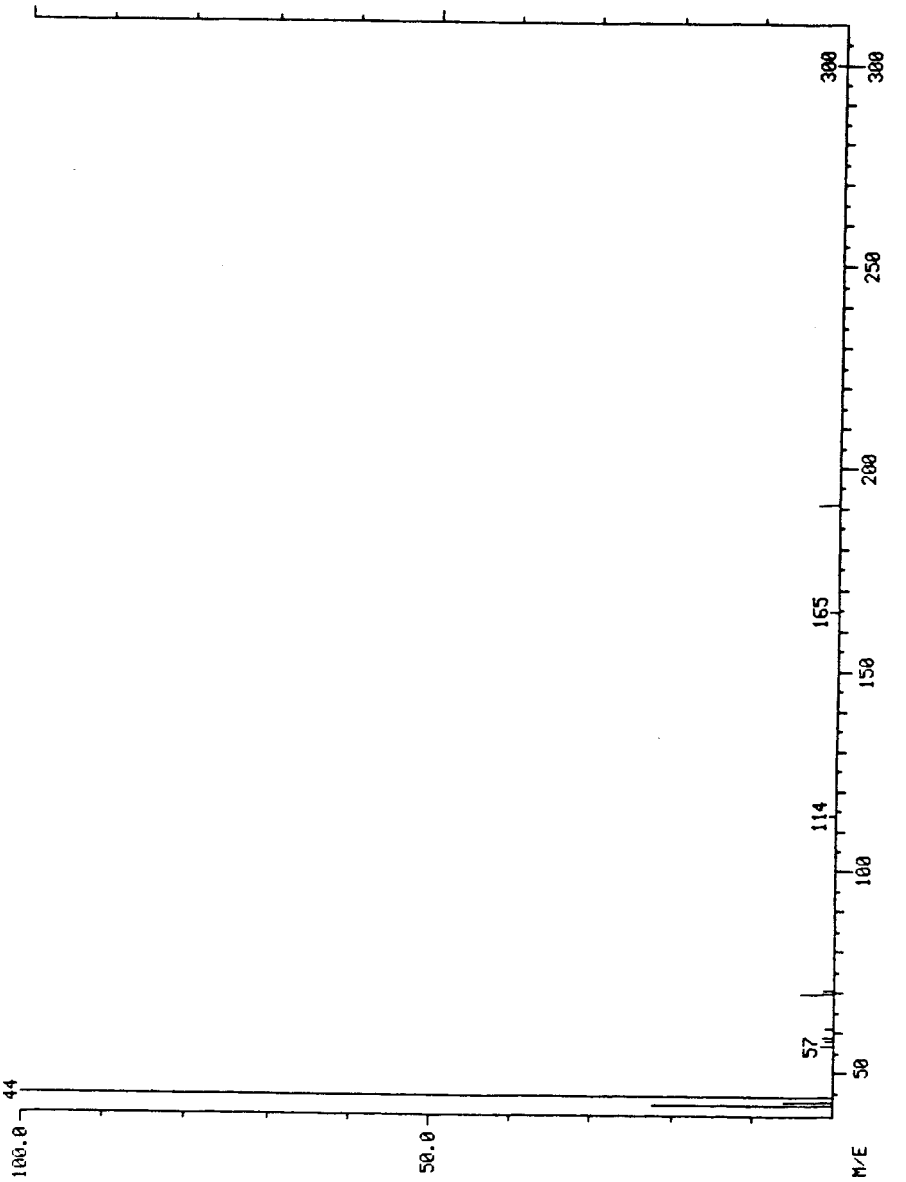

Figure 6. Reference EI Mass Spectrum of Mortriptyline from the work by T. Mills III.

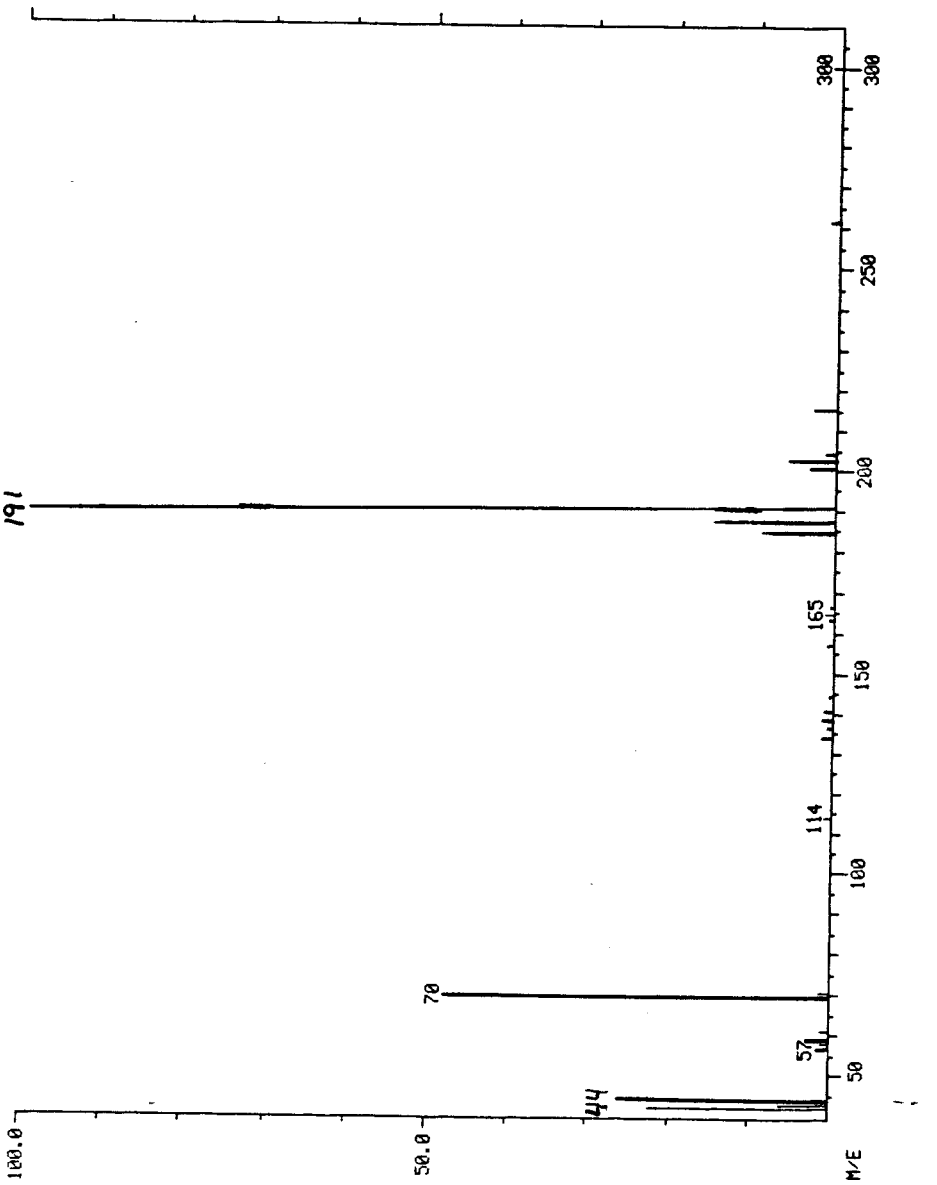

Figure 7. Reference EI Mass Spectrum of Protriptyline from the work by T. Mills III.

GC PARAMETERS:

250 deg. = Injector Temp. *

150 deg, = Initial Temp. *

220 deg. = Final Temp. *

1 min. = Initial Time *

5.0 D/H = Ramp Rate (deg/min) optional *

10 nin. = Final Time *

250 deg. = Separator Temp.

80 deg. = MS Manifold Temp.

INJECTION MODE: CAPILLARY

60 sec. = Split/Sweep Valve Time

90 sec. = Filament/Multiplier Off Time

SCAN FROU 40 AMU TO 350 AMU IN 1.0 sec. *

*The separator is the area where the GC connects with the MS. A temperature higher than the final tamp. is needed to prevent condensation.
*The manifold is the structure which houses the ion source, quadrupole analyzer, and ion detector.

Figure 8. An example of the typical GC/MS conditions

CHAPTER V

RESULTS AND DISCUSSION

Electron Ionization

The problem presented here is the separation of three tricyclic antidepressants, very similar in both structure and molecular weight. Uany procedures involve derivatization techniques to increase resolving power and sensitivity (30-31) but these problems should be eliminated by using capillary GC/MS. So, as in the work of Chinn *et al* (32) and Vinet (33) underivatized TCA's were used. First the etandards were analyzed in a programmed temperature node as seen in Figure 8. The data from these individual runs can be seen in Table 3. Thia phase of the analysis resulted in small but rather wide peaks in the reconstructed ion chromatograms (RIC) of all the drugs. The computer enhancement showed well-defined peaks with a slight amount of tailing resulting in excellent sensitivity for AMI and NOR and slightly decreased sensitivity for PRO. The sensitivity was determined by comparing peak height to baseline and background noise. The mass spectra for AMI, NOR and PRO can be seen in Figures 9-11 and all of these spectra correspond to the results seen by T. Mills III *et al* (30).

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Introduction

The results of the experimental work carried out in the laboratory are presented and discussed in this chapter. The results are presented in the following order: Section 5.2 presents the results of the preliminary experiments carried out to establish the optimum conditions for the subsequent experiments. Section 5.3 presents the results of the experiments carried out to investigate the effect of the concentration of the reactants on the rate of the reaction. Section 5.4 presents the results of the experiments carried out to investigate the effect of temperature on the rate of the reaction. Section 5.5 presents the results of the experiments carried out to investigate the effect of the ionic strength of the solution on the rate of the reaction. Section 5.6 presents the results of the experiments carried out to investigate the effect of the dielectric constant of the solution on the rate of the reaction. Section 5.7 presents the results of the experiments carried out to investigate the effect of the solvent on the rate of the reaction. Section 5.8 presents the results of the experiments carried out to investigate the effect of the catalyst on the rate of the reaction. Section 5.9 presents the results of the experiments carried out to investigate the effect of the inhibitor on the rate of the reaction. Section 5.10 presents the results of the experiments carried out to investigate the effect of the initiator on the rate of the reaction. Section 5.11 presents the results of the experiments carried out to investigate the effect of the temperature on the rate of the reaction. Section 5.12 presents the results of the experiments carried out to investigate the effect of the pressure on the rate of the reaction. Section 5.13 presents the results of the experiments carried out to investigate the effect of the volume on the rate of the reaction. Section 5.14 presents the results of the experiments carried out to investigate the effect of the surface area on the rate of the reaction. Section 5.15 presents the results of the experiments carried out to investigate the effect of the particle size on the rate of the reaction. Section 5.16 presents the results of the experiments carried out to investigate the effect of the shape on the rate of the reaction. Section 5.17 presents the results of the experiments carried out to investigate the effect of the orientation on the rate of the reaction. Section 5.18 presents the results of the experiments carried out to investigate the effect of the collision frequency on the rate of the reaction. Section 5.19 presents the results of the experiments carried out to investigate the effect of the activation energy on the rate of the reaction. Section 5.20 presents the results of the experiments carried out to investigate the effect of the entropy on the rate of the reaction. Section 5.21 presents the results of the experiments carried out to investigate the effect of the enthalpy on the rate of the reaction. Section 5.22 presents the results of the experiments carried out to investigate the effect of the free energy on the rate of the reaction. Section 5.23 presents the results of the experiments carried out to investigate the effect of the equilibrium constant on the rate of the reaction. Section 5.24 presents the results of the experiments carried out to investigate the effect of the rate constant on the rate of the reaction. Section 5.25 presents the results of the experiments carried out to investigate the effect of the order of the reaction on the rate of the reaction. Section 5.26 presents the results of the experiments carried out to investigate the effect of the molecularity of the reaction on the rate of the reaction. Section 5.27 presents the results of the experiments carried out to investigate the effect of the mechanism of the reaction on the rate of the reaction. Section 5.28 presents the results of the experiments carried out to investigate the effect of the transition state on the rate of the reaction. Section 5.29 presents the results of the experiments carried out to investigate the effect of the activated complex on the rate of the reaction. Section 5.30 presents the results of the experiments carried out to investigate the effect of the intermediate on the rate of the reaction. Section 5.31 presents the results of the experiments carried out to investigate the effect of the product on the rate of the reaction. Section 5.32 presents the results of the experiments carried out to investigate the effect of the reactant on the rate of the reaction. Section 5.33 presents the results of the experiments carried out to investigate the effect of the solvent on the rate of the reaction. Section 5.34 presents the results of the experiments carried out to investigate the effect of the catalyst on the rate of the reaction. Section 5.35 presents the results of the experiments carried out to investigate the effect of the inhibitor on the rate of the reaction. Section 5.36 presents the results of the experiments carried out to investigate the effect of the initiator on the rate of the reaction. Section 5.37 presents the results of the experiments carried out to investigate the effect of the temperature on the rate of the reaction. Section 5.38 presents the results of the experiments carried out to investigate the effect of the pressure on the rate of the reaction. Section 5.39 presents the results of the experiments carried out to investigate the effect of the volume on the rate of the reaction. Section 5.40 presents the results of the experiments carried out to investigate the effect of the surface area on the rate of the reaction. Section 5.41 presents the results of the experiments carried out to investigate the effect of the particle size on the rate of the reaction. Section 5.42 presents the results of the experiments carried out to investigate the effect of the shape on the rate of the reaction. Section 5.43 presents the results of the experiments carried out to investigate the effect of the orientation on the rate of the reaction. Section 5.44 presents the results of the experiments carried out to investigate the effect of the collision frequency on the rate of the reaction. Section 5.45 presents the results of the experiments carried out to investigate the effect of the activation energy on the rate of the reaction. Section 5.46 presents the results of the experiments carried out to investigate the effect of the entropy on the rate of the reaction. Section 5.47 presents the results of the experiments carried out to investigate the effect of the enthalpy on the rate of the reaction. Section 5.48 presents the results of the experiments carried out to investigate the effect of the free energy on the rate of the reaction. Section 5.49 presents the results of the experiments carried out to investigate the effect of the equilibrium constant on the rate of the reaction. Section 5.50 presents the results of the experiments carried out to investigate the effect of the rate constant on the rate of the reaction. Section 5.51 presents the results of the experiments carried out to investigate the effect of the order of the reaction on the rate of the reaction. Section 5.52 presents the results of the experiments carried out to investigate the effect of the molecularity of the reaction on the rate of the reaction. Section 5.53 presents the results of the experiments carried out to investigate the effect of the mechanism of the reaction on the rate of the reaction. Section 5.54 presents the results of the experiments carried out to investigate the effect of the transition state on the rate of the reaction. Section 5.55 presents the results of the experiments carried out to investigate the effect of the activated complex on the rate of the reaction. Section 5.56 presents the results of the experiments carried out to investigate the effect of the intermediate on the rate of the reaction. Section 5.57 presents the results of the experiments carried out to investigate the effect of the product on the rate of the reaction. Section 5.58 presents the results of the experiments carried out to investigate the effect of the reactant on the rate of the reaction. Section 5.59 presents the results of the experiments carried out to investigate the effect of the solvent on the rate of the reaction. Section 5.60 presents the results of the experiments carried out to investigate the effect of the catalyst on the rate of the reaction. Section 5.61 presents the results of the experiments carried out to investigate the effect of the inhibitor on the rate of the reaction. Section 5.62 presents the results of the experiments carried out to investigate the effect of the initiator on the rate of the reaction. Section 5.63 presents the results of the experiments carried out to investigate the effect of the temperature on the rate of the reaction. Section 5.64 presents the results of the experiments carried out to investigate the effect of the pressure on the rate of the reaction. Section 5.65 presents the results of the experiments carried out to investigate the effect of the volume on the rate of the reaction. Section 5.66 presents the results of the experiments carried out to investigate the effect of the surface area on the rate of the reaction. Section 5.67 presents the results of the experiments carried out to investigate the effect of the particle size on the rate of the reaction. Section 5.68 presents the results of the experiments carried out to investigate the effect of the shape on the rate of the reaction. Section 5.69 presents the results of the experiments carried out to investigate the effect of the orientation on the rate of the reaction. Section 5.70 presents the results of the experiments carried out to investigate the effect of the collision frequency on the rate of the reaction. Section 5.71 presents the results of the experiments carried out to investigate the effect of the activation energy on the rate of the reaction. Section 5.72 presents the results of the experiments carried out to investigate the effect of the entropy on the rate of the reaction. Section 5.73 presents the results of the experiments carried out to investigate the effect of the enthalpy on the rate of the reaction. Section 5.74 presents the results of the experiments carried out to investigate the effect of the free energy on the rate of the reaction. Section 5.75 presents the results of the experiments carried out to investigate the effect of the equilibrium constant on the rate of the reaction. Section 5.76 presents the results of the experiments carried out to investigate the effect of the rate constant on the rate of the reaction. Section 5.77 presents the results of the experiments carried out to investigate the effect of the order of the reaction on the rate of the reaction. Section 5.78 presents the results of the experiments carried out to investigate the effect of the molecularity of the reaction on the rate of the reaction. Section 5.79 presents the results of the experiments carried out to investigate the effect of the mechanism of the reaction on the rate of the reaction. Section 5.80 presents the results of the experiments carried out to investigate the effect of the transition state on the rate of the reaction. Section 5.81 presents the results of the experiments carried out to investigate the effect of the activated complex on the rate of the reaction. Section 5.82 presents the results of the experiments carried out to investigate the effect of the intermediate on the rate of the reaction. Section 5.83 presents the results of the experiments carried out to investigate the effect of the product on the rate of the reaction. Section 5.84 presents the results of the experiments carried out to investigate the effect of the reactant on the rate of the reaction. Section 5.85 presents the results of the experiments carried out to investigate the effect of the solvent on the rate of the reaction. Section 5.86 presents the results of the experiments carried out to investigate the effect of the catalyst on the rate of the reaction. Section 5.87 presents the results of the experiments carried out to investigate the effect of the inhibitor on the rate of the reaction. Section 5.88 presents the results of the experiments carried out to investigate the effect of the initiator on the rate of the reaction. Section 5.89 presents the results of the experiments carried out to investigate the effect of the temperature on the rate of the reaction. Section 5.90 presents the results of the experiments carried out to investigate the effect of the pressure on the rate of the reaction. Section 5.91 presents the results of the experiments carried out to investigate the effect of the volume on the rate of the reaction. Section 5.92 presents the results of the experiments carried out to investigate the effect of the surface area on the rate of the reaction. Section 5.93 presents the results of the experiments carried out to investigate the effect of the particle size on the rate of the reaction. Section 5.94 presents the results of the experiments carried out to investigate the effect of the shape on the rate of the reaction. Section 5.95 presents the results of the experiments carried out to investigate the effect of the orientation on the rate of the reaction. Section 5.96 presents the results of the experiments carried out to investigate the effect of the collision frequency on the rate of the reaction. Section 5.97 presents the results of the experiments carried out to investigate the effect of the activation energy on the rate of the reaction. Section 5.98 presents the results of the experiments carried out to investigate the effect of the entropy on the rate of the reaction. Section 5.99 presents the results of the experiments carried out to investigate the effect of the enthalpy on the rate of the reaction. Section 5.100 presents the results of the experiments carried out to investigate the effect of the free energy on the rate of the reaction.

TABLE 3

DATA FOR EI OF INDIVIDUAL DRUG STANDARDS

DRUGS	Retention tine (min:sec)	Intensity[a] (units)	Sensitivity (ng)
AMI	2:34	8608	20
NOR	2:35	19680	30
PRO	2:45	7200[b]	50

[a] Intensity is measured by arbitrary units to compare the anounto of iono reaching the MS detector.
[b] PRO's intensity value was doubled for comparison purposes because ita concentration was half of AMI's and NOR's.

Figures 9-11 also show how there fragmentation patterns in the mars spectra occur as a result of electron ionization. Note that the molecular ion has a very low abundance in each case. It rhould also be noted that some of the primary ion fragment8 are further fragmented and are not seen in the mass rpectra.

TABLE 5

DAY-AM FOR EI OF INDIVIDUAL DUAL TASKERS

SETTING	Information Flow (min:sec)	Intensity (cm)	Complexity (%)
AMI	5:34	Above	50
NOE	5:32	Level	30
PPO	5:42	Below	20

a Intensity to proceed in straight ahead flight

Comparable info amounts of road knowledge and MC assessed

= PPO.E Information Noise Level for

coordination tribesse pedagogy the a normalization see point of

AMI.E and NOE.E

Figures 8-11 also show how the three training methods

the mean absolute error is a measure of overall performance

Note that the majority of pilots had fewer than 10 hours

ceed. It should also be noted that the criteria for

training was not met by all pilots

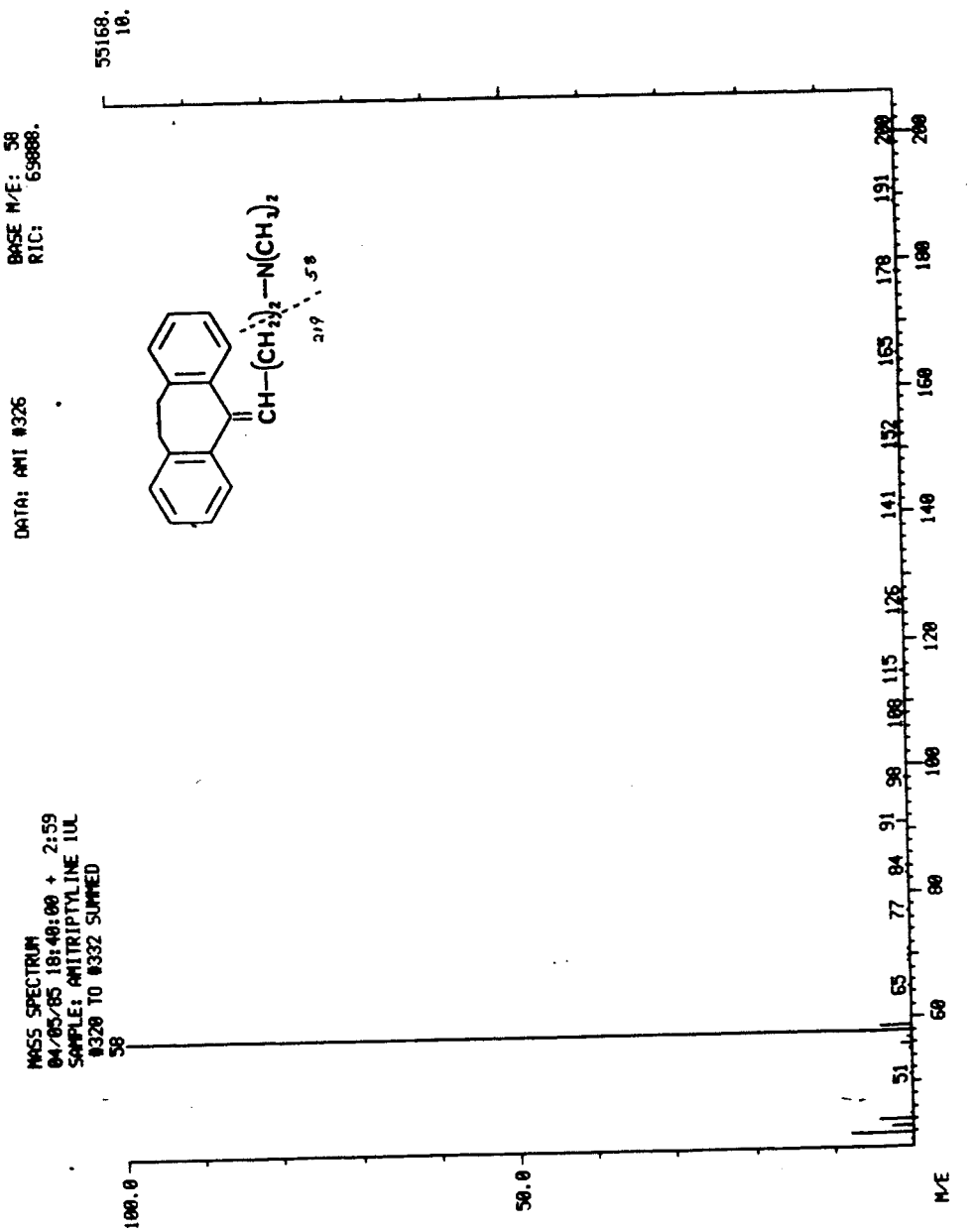

Figure 9. EI Mass Spectrum and Molecular Fragmentation of AMI.

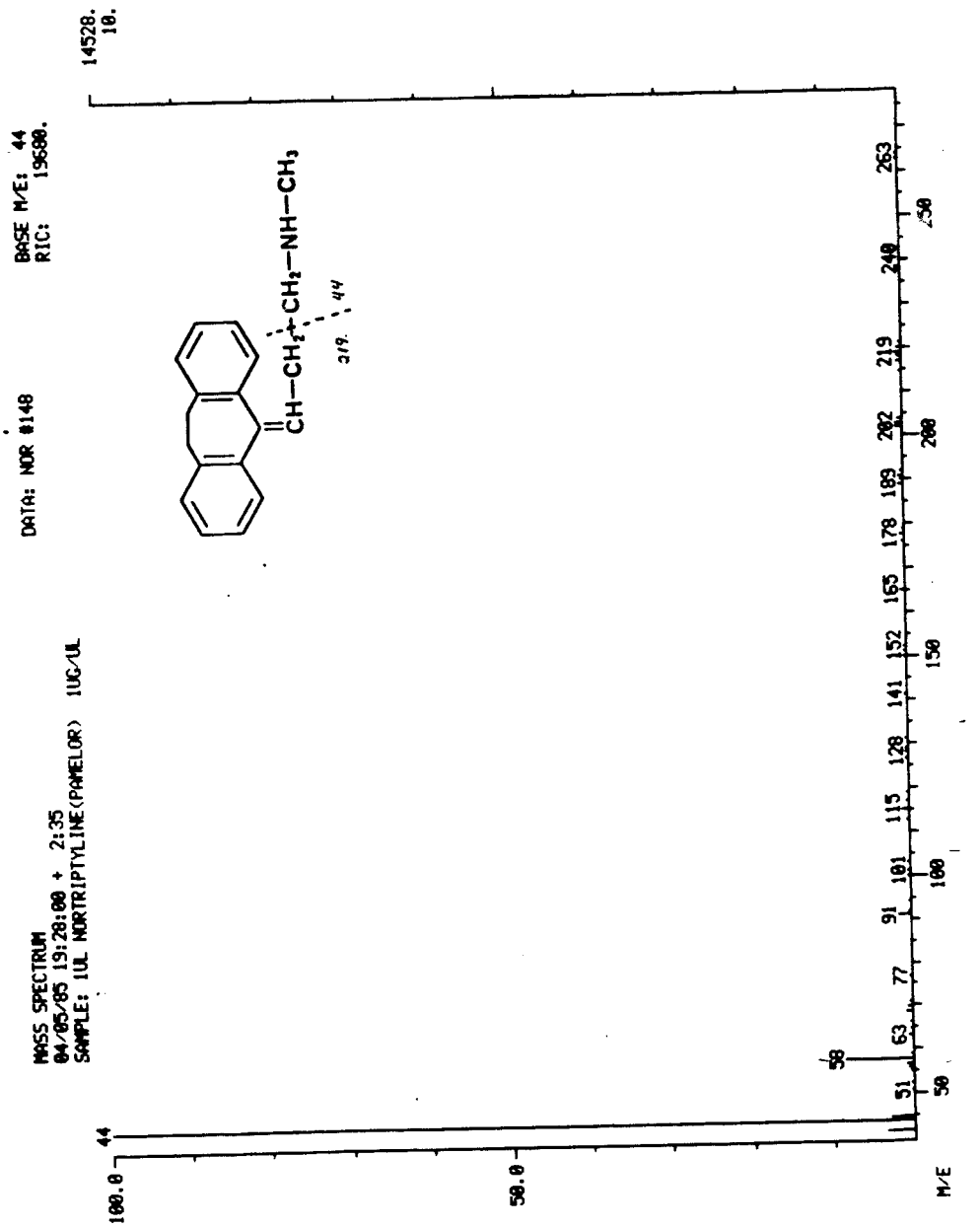

Figure 10. EI Mass Spectrum and Holecular Fragmentation of NOR.

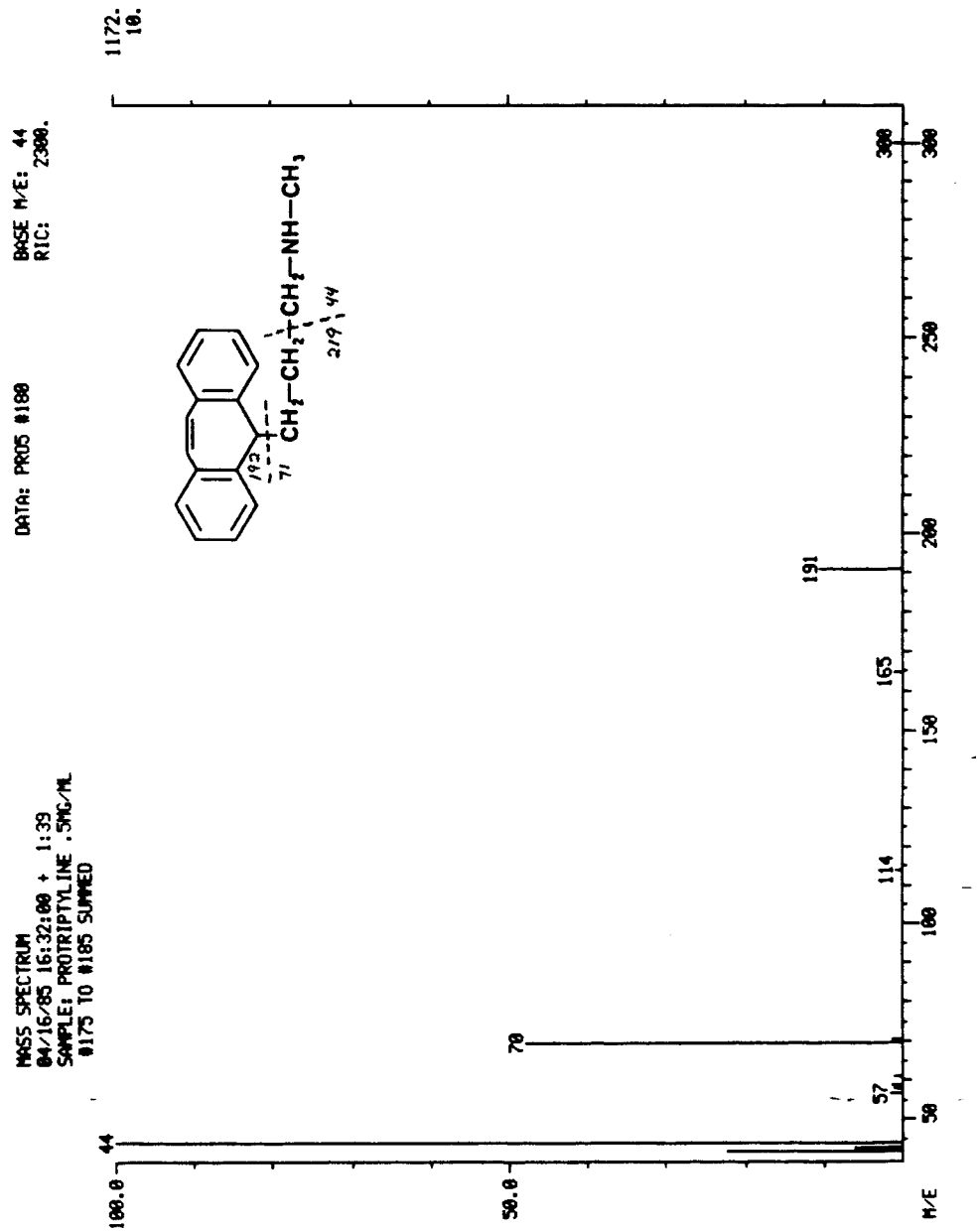

Figure 11. EI Mass Spectrum and Molecular Fragmentation of PRO.

Chemical Ionization

In an attempt to improve the sensitivity of the individual drugs, they were analyzed by chemical ionization (CI) utilizing methane as the reagent gas as was done by Jenkins and Friedel (34). To determine the methane pressure to be used, mass spectra were obtained for the methane alone. This was done by varying the methane pressure by 0.1 torr increments from 0.0 torr to 1.0 torr (instrument manufacturer's recommended reagent gar range), and the results can be seen in Figure 12. It was by thie means that

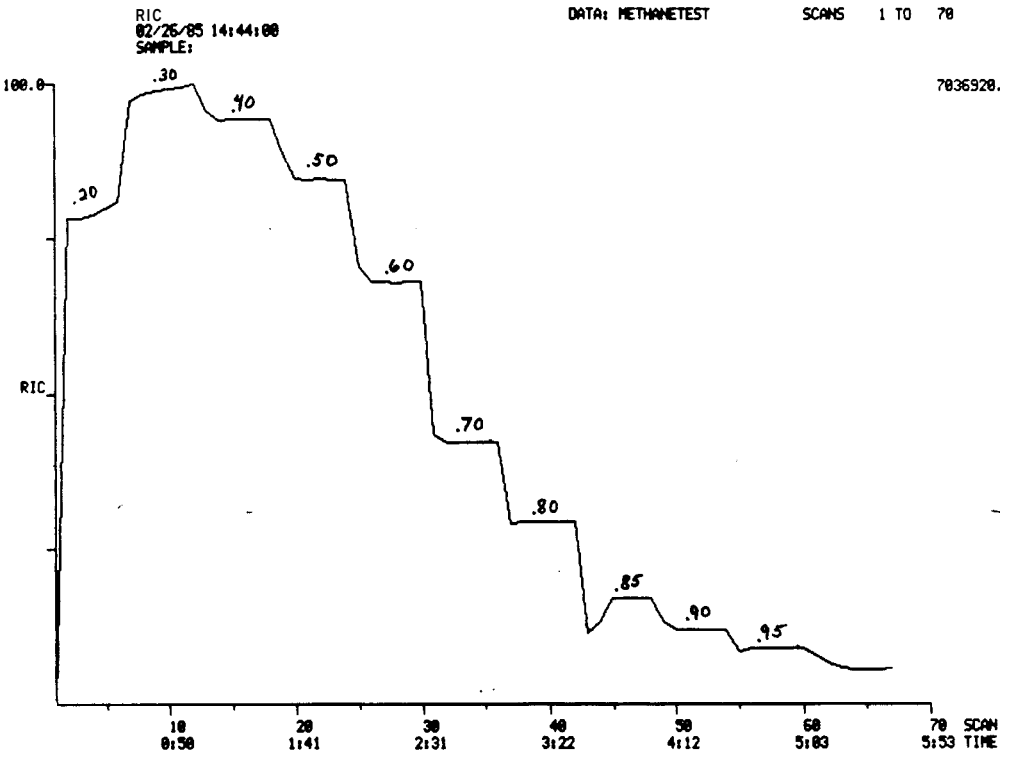

Figure 12. The total ion intensity of methane (the reagent gar). The pressure in torr is indicated at each level.

it war determined that the maximum concentration of the reactive CH_5^+ ions occurs at 0.4 torr and, therefore, this is the optimum methane operating pressure. The following changer in the GC parameter. were made in order to get the desired sensitivity and peak sharpness.

1. INITIAL TEMP. = 150 deg.
2. RAMP RATE = 10.0 deg/min.
3. SPLIT/SWEEP VALVE = 60 sec.
4. FIL./MULT. OFF TIME = 90 rec.
5. SCAN FROM 150 TO 350 AMU IN 1.0 sec.

The split/sweep valve tine is the amount of time that the valve is initially closed to allow all of the sample to get onto the column. The results are noted below in Table 4 and ar can be seen there war a vast improvement in the relative intensitier and sensitivity of all three drugr. The

TABLE 4

DATA FOR CI OF INDIVIDUAL DRUG STANDARDS

DRUGS	Retention time (min:sec)	Intenrity (units)	Sensitivity (ng)
AMI	5:44	14944	10
NOR	5:53	11264	20
PRO	6:19	16480	30

[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

DATA FOR ID OR INDIVIDUAL PT. [illegible]

TABLE I

[illegible]

intensities for NOR and PRO, because of the broadness of their peaks, reflect the areas under the peaks. It must be remembered that the intensity referred to here is a relative term and will vary depending on the manual tune settings and other inrtrument conditions. The chemical ionizaton mass mpectra of AMI, NOR and PRO are seen below in Figuree 13-15. It should be noted that the ratio of MH^+ to M^+ is approximately 10 to 1. This agrees with statements made by the nanufacturer on CI results. The $(M + C_2H_5)^+$ ions and $(M + C_2H_5)^+$ ions are seen in the mass spectra, but $(M + CH_5)^+$ ions are not detected by this MS technique. This is because nearly all of the CH_5^+ that reacts with M forms MH+ and CH_4 as in reaction 5. It is this MH^+ ion that remultr in the base peak in each of the cases. At this methane pressure some fragmentation of the drug molecule occure. This results in the detection of many characteristic mass peaks that can be umed to confirm the identification of the analyzed drugs. So under these conditions, there are three criteria for identifying a ruspected drug (1) retention tine, (2) the rolecular weight of the compound am obtained from the mass of the MH^+ ion, and (3) the nolecular fragmentation pattern.

A standard drug mixture of AMI, NOR and PRO, each with a concentration of 250 ng/µL, was analyzed using the same GC parameters am noted above. Since the split/sweep valve off-time is 60 sec the injection is splitlers and the vast majority of the sample reaches the column.

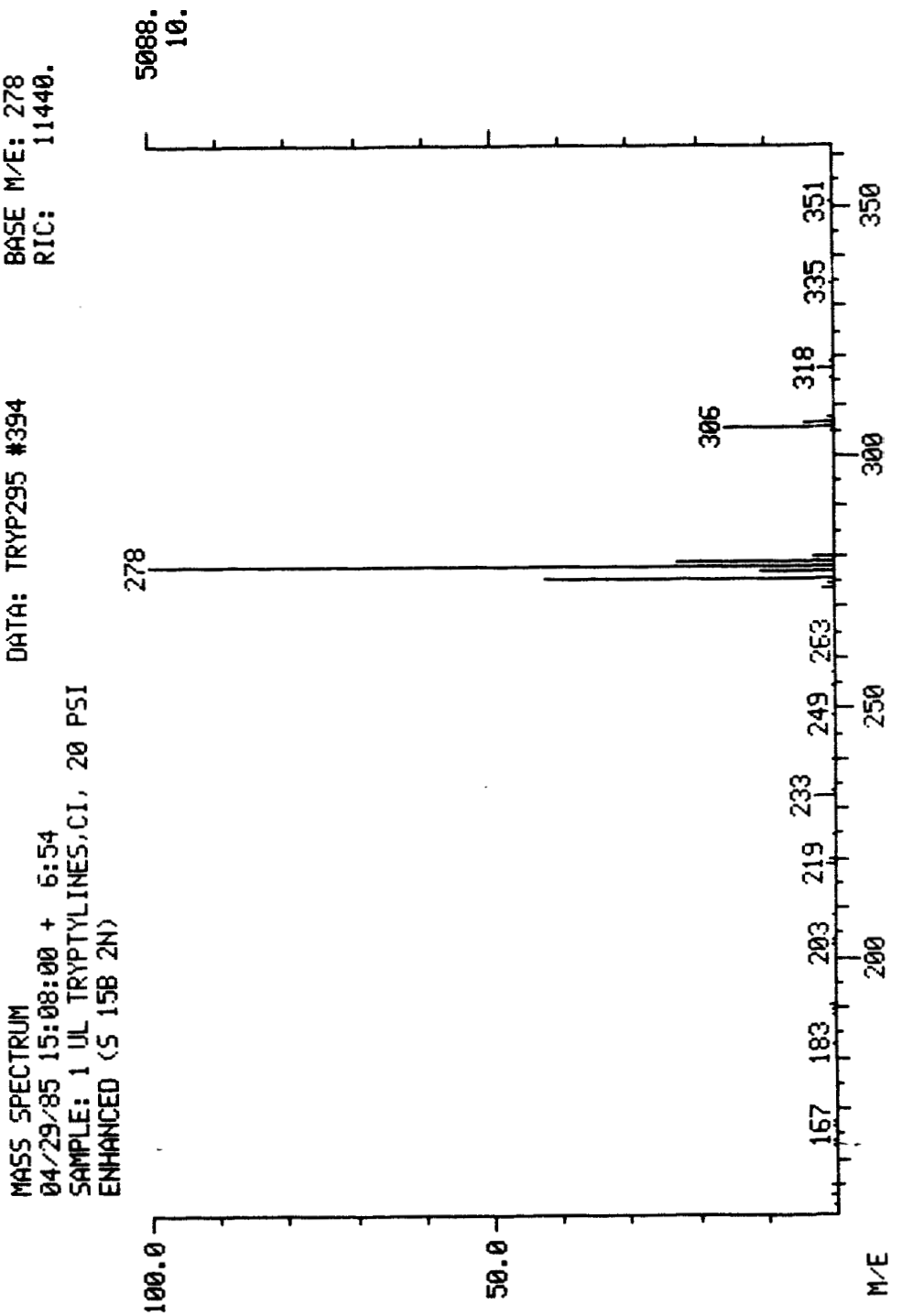

Figure 13. Chemical Ionization Mass Spectrum of AMI.

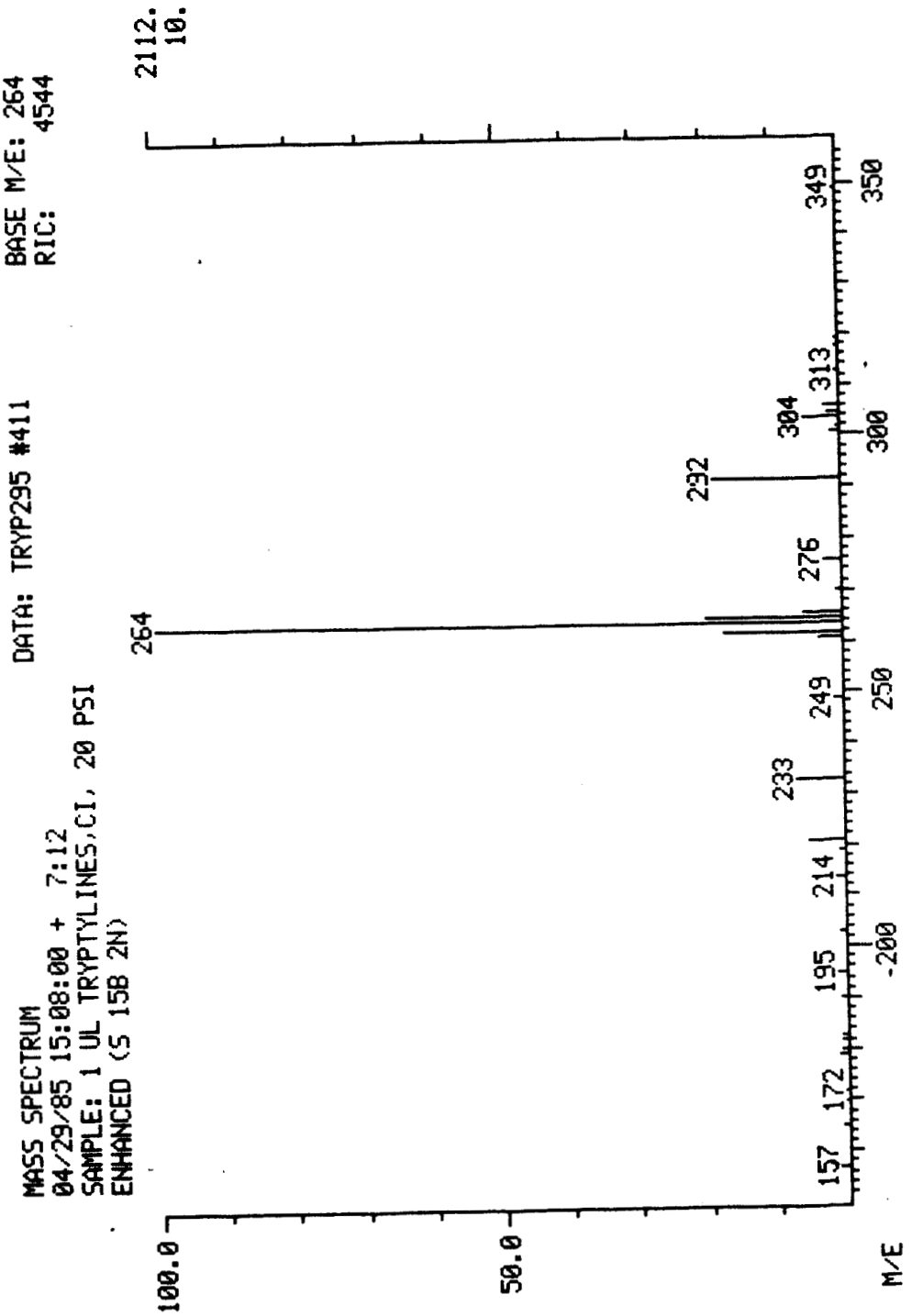

Figure 14. Chemical Ionization Mass Spectrum of NOR.

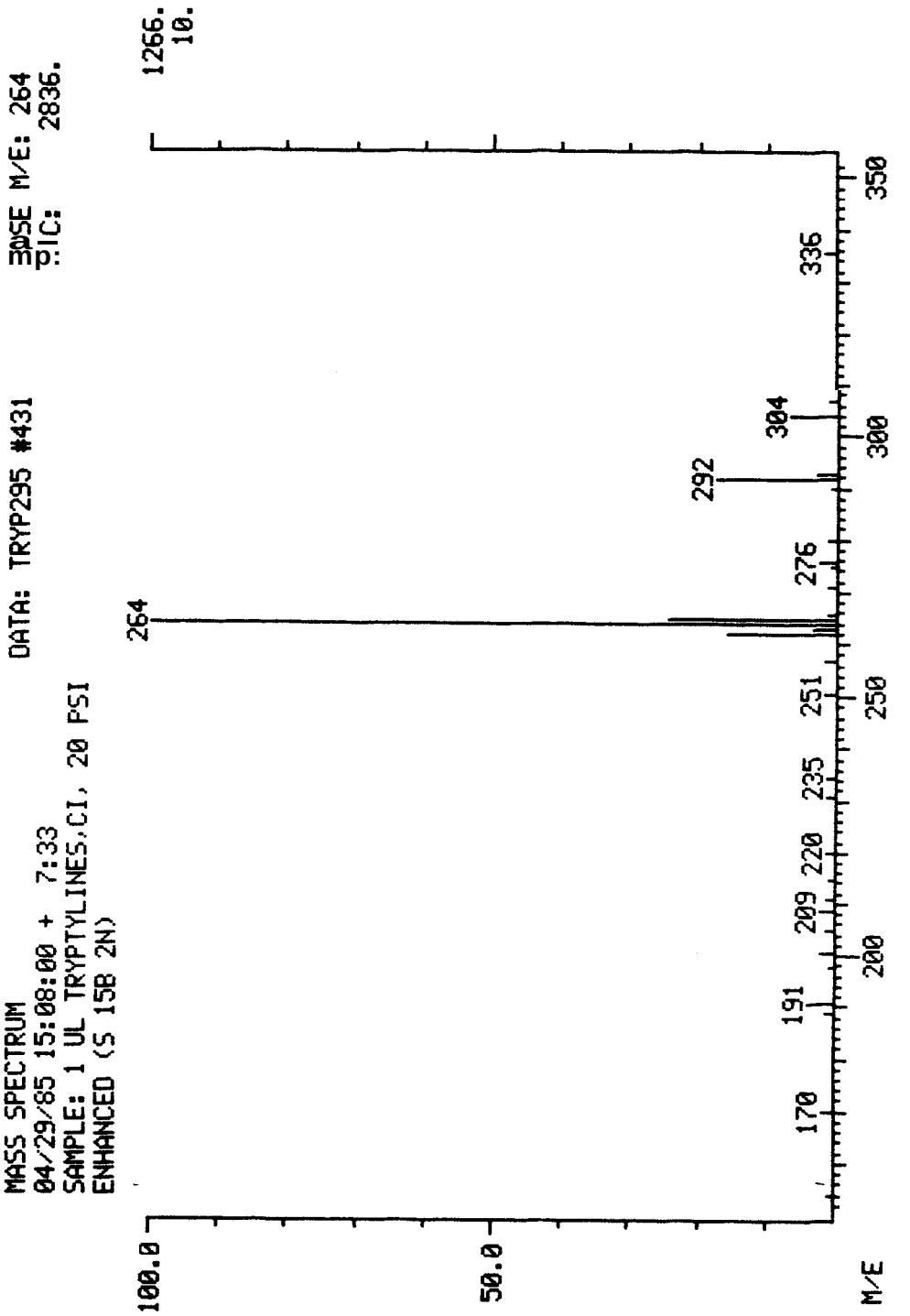

Figure 15. Chemical Ionization Mass Spectrum of PRO.

[illegible]

The RIC for one of the samplings is seen in Figure 16, and it shows excellent resolution, intensity, and sensitivity. The statistical information for this analysis is seen in Table 5. It should also be noted that the intensities in

TABLE 5

Data for Chemical Ionization of Drug Mixture

Drug	RT	Intensity(Ht/A)	Rerponse Factor*
AMI	6:56	44032/176496	0.85
NOR	7:13	23872/151524	1.00
PRO	7:36	18784/ 57925	2.60

* The peak aroa intensity value was urad to calculated tho response factor

Table 5 are expressed in terms of peak height (Ht) and peak area (A). The response factors (Rf) seen in Table 5 are used in the quantitation of the drugs in an actual analysis. The response factor value is determined by comparing the instrument's ability to detect various substances of equal or known concentrations (as in this study). The response factor is calculated from the intensity readings of the various drugs compared to a reference. In this study, I used NOR as the drug of reference to calculate the response factors.

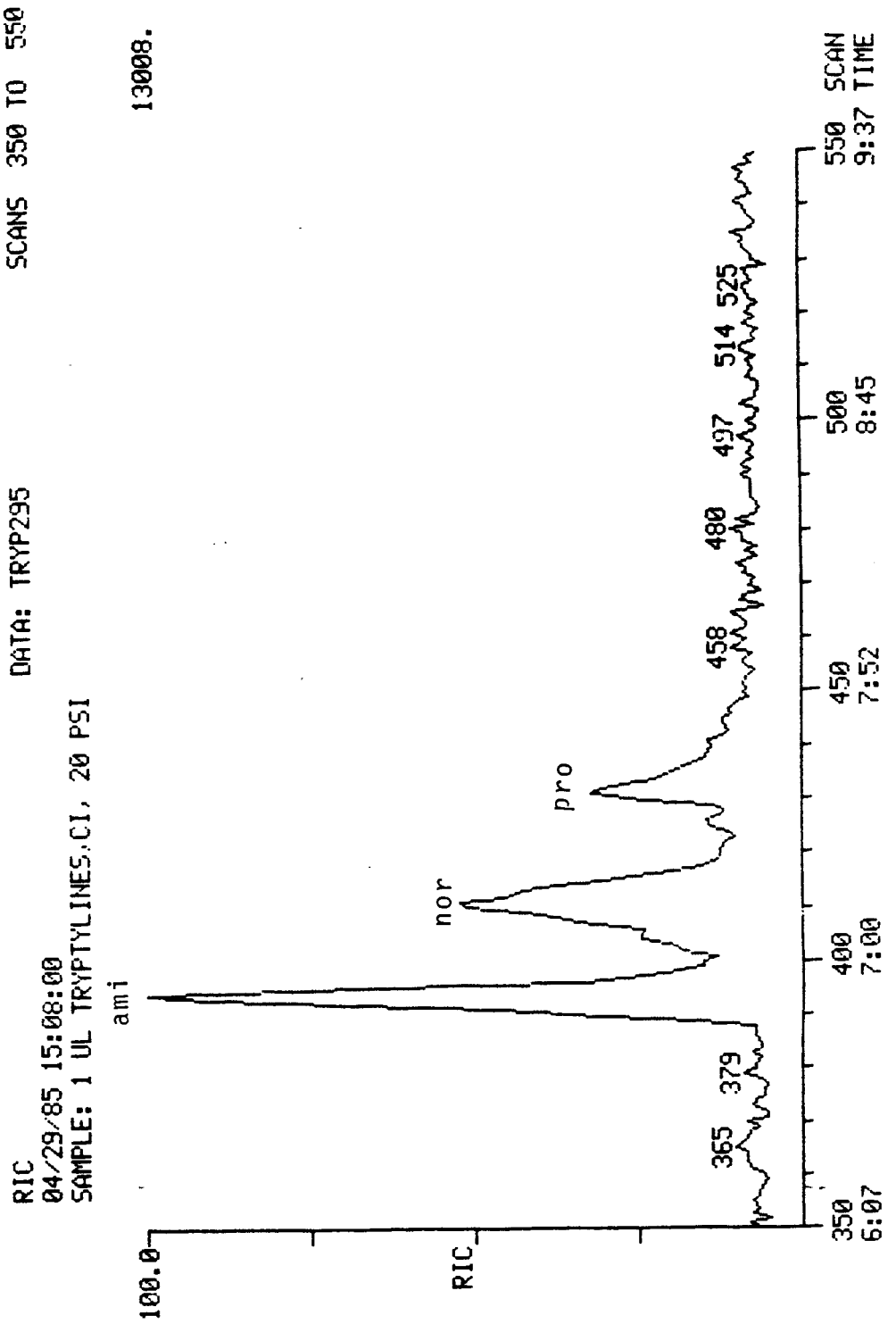

Figure 16. The RIC of 250 ng of each of the three drug standards analyzed in the programmed temperature mode.-

NOR I/ AMI I = AMI Rf (7)

151524/ 176496 = 0.89 (8)

A response factor would have to be calculated for all rubstances to be analyzed. The concentration of unknown drugs would be determined using the following equation

$(I_{unk} \times Rf \times Conc.I_{std}) / I_{std} = Conc._{unk}$ (9)

As was noted above, intensity is a relative determination and can vary considerably, but this variation had little effect on calculating the response factors as seen in Table 5. Because of the broadness of the peaks and the large difference in peak height and peak area, I felt a more accurate response factor would be obtained by using peak area rather than peak height.

Isothermal Conditions

If a procedure is to find clinical application, it should be as simple end easy to perform as possible. The programmed temperature mode is vary useful in analyzing complex mixtures and resulted in more than adequate separation of the drugs. But in clinical laboratorieg, a majority of their work involves therapeutic drug monitoring. In this case, we are not working with a complex mixture. Therefore, I have explored the possibilities of an isothermal procedure. The isothermal procedures are simpler and faster since the column does not need to reequilibrate its temperature (cool down to the initial temperature)

before the next analysis. During this portion of the study only the initial and final temperature settings were varied but kept equal to each other. The same standard drug mixture war run through a series of CI analyses to find the optimum isothermal operating temperature. The RIC and the optimum temperature and other GC conditions are seen in Figures 17 and 18, respectively. The RIC shows good resolution and sensitivity for the relatively short retention times in comparison to the programmed mode. All three drugs ware injected onto a 200°C column and due to similarities in molecular weight and rtructure the rerult is three rather uniform peaks. Thie caused a substantial change in the response factors compared to earlier programmed temperature data. A tabular listing of the results of this isothermal analysis is seen in Table 6. The response factors in thie care were calculated using the peak height because of a difficulty in determining peak area due to peak overlap, although all peaks did appear uniform.

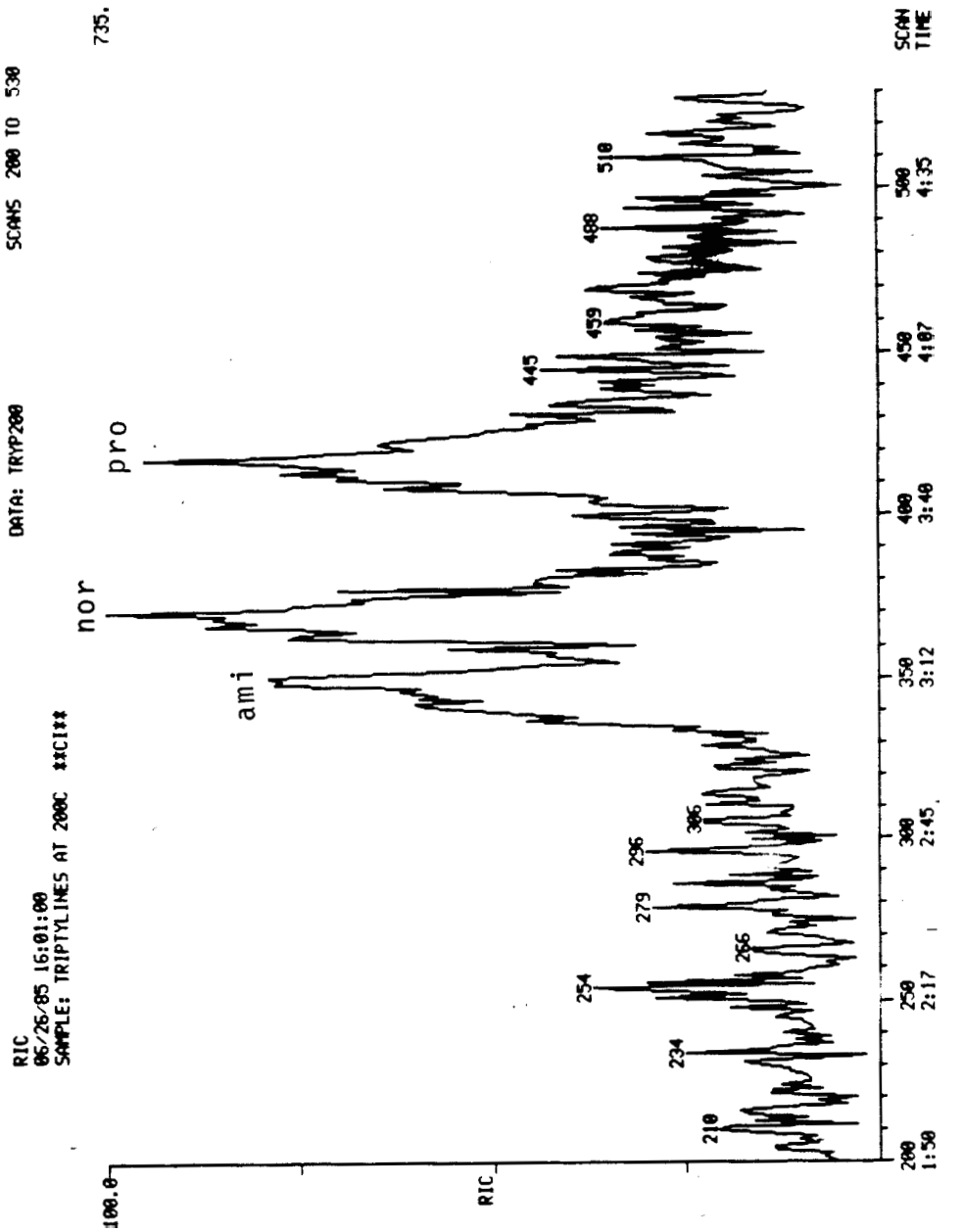

Figure 17. The RIC for the isothermal CI of the drug mixture analyzed at 200°C.

GC PARAMETERS:

250 deg. = Injector Temp.

200 deg. = Initial Tenp.

200 deg. = Final Tenp.

1 min. = Initial Time

0.0 D/H = Ramp Rate (deg/min)

10 nin. = Final time

250 deg. = Separator Tenp.

80 deg. = Hanifold Temp.

INJECTOR MODE: CAPILARY

60 sec. = Split/Sweep Valve Closed Time

90 sec. = Filament/Multiplier Off Time

SCAN FROM 150 AMU TO 350 AMU IN 0.5 sec.

UETHANE PRESSURE: 0.40 torr (used in CI)

Figure 18. Typical computer printout of GC conditions for isothernal analysis.

TABLE 6

Data for 200°C Ioothernal Analysis of the Drug Mixture[a]

Drug	RT	Intensity	RF
AMI	4:29	771	1.13
NOR	4:43	870	1.00
PRO	5:23	631	1.38

[a]The GC/MS conditions for isothermal analysis are eean in figure 18.

GC/MS Variable Parameters

Many researchers in reporting clinical procedureo merely state the conditions at which the analysis is performed. At this tire I will show the effect of varying many of the GC/MS parameters. This is done in order to give a better understanding of the overall GC/MS operation. This will show that adjusting many of the variables will result in a considerable change in the RIC. The first variable to be considered is the column temperature. It can be varied from 25°C to 300°C. The compromise is between the analysis tine, intensity, and resolution. As temperature is increased the analysis tine and the rasolution will decrease. The sensitivity will increase with temperature and will plateau at the optimum temperature. As this temperature is exceeded, sensitivity and resolution will decrease as peaks begin to overlap. Table 7 shows the retention tire (RT) and the intensity (I) for the three drug rtandards at column temperatures from 190°C to 220°C while all other parameters were held constant. Table 8 shows the relative retention time (RRT) and the intensity (I) of the three rtandardr at varying column temperatures. This was accomplished by using the information from Table 7. The RRT is a means of standardizing retention tines from numerous chronatograms. The RRT is determined by establishing one of the drugs as the standard or reference drug and its RRT is 1.00. All of the other drugs RRT are determined by

TABLE 7

The Retention Times (RT) and Intenritier (I) of the Drug Standards at Various Isothermal Temperature8

COLUMN TEMP.	AMI(RT/I)	NOR(RT/I)	PRO(RT/I)
190	4:55/1830	5:15/347	5:57/223
200	3:11/1898	3:23/2037	3:49/3561
210	2:17/1898	2:26/4244	2:44/1868
220	1:58/1180	2:02/1500	2:12/1650

TABLE 8

The Relative Retention Timer (RRT) and Intensities (I) of the Drug Standardr at Various Isothermal Temperatures

COLUMN TEHP.	AMI(RRT/I)	NOR(RRT/I)	PRO(RRT/I)
190	1.00/1830	1.13/347	1.22/223
200	1.00/1898	1.04/2037	1.12/3561
210	1.00/1898	1.04/4244	1.12/1868
220	1.00/1180	1.27/1500	1.34/1650

establishing a ratio between then and the reference drug. Analyres at temperatures less than 190°C reeulted in extremely long RT and decreased intensity and sensitivty due to peak broadening. Analyses at temperatures greater than 220°C resulted in a complete loss of resolution because all three drugs appear as one large peak with an extremely

short retention time. The drugs could be distinguished only through conputer enhancement by scanning for individual characterietic masses.

This same effect is seen in the programmed temperature analyses when the column temperatures (initial and final) and the ramp rate are varied. It therefore appears that the degree of sensitivity and the resolution attained is directly related to the retention time which is controlled by the column temperature. Table 9 showe a comparison of the retention timer (RT) and the intensities (I) of the drug standards to the operating ramp rate in degrees per minute (D/M).

TABLE 9

The Retention tines and Intensities of the Drug Standards at Variour Ramp Rates[a]

Ramp Rate(D/M)	AMI(RT/I)	NOR(RT/I)	PRO(RT/)
5	9:33/21180	9:49/9316	9:57/7252
10	6:54/57228	7:12/49799	7:33/22466
15	5:36/43846	5:43/32458	6:00/10334
20	4:58/8768	---/---[b]	---/---[b]

[a] All analyses were performed according to the conditions seen in Figure 8, except for ramp rate.
[b] NOR and PRO appear as one peak at 5:02

The next parameter requiring investigation was the helium head pressure. Thir pressure can typically vary from 5 to 30 psi (pounds per square inch). The more common terminology for carrier gas (helium) neasurements is flow rate. Thie was mearured at preeeures of 5 and 10 psi and rerulted in helium flow rates of 1.0 and 2.0 mL/min. Table 10 shows the effect of helium pressure on the drug's retention tine while all other parameters remain constant. The RICs of this series of analyrer showed again that there is a direct correlation between intensity and retention tine. The lower pressures resulted in long retention times and poor intensity due to very broad peaks that at times were nearly lost in the background noise, whereas the higher preesures resulted in rhorter retention tines, greater intensities and excellent sensitivity. It must be

TABLE 10

The Retention Times of Drug Standards at Variour Helium Pressures (psi)[a]

HELIUM PRESSURE (psi)	RETENTION TIME (min:sec)		
	AMI	NOR	PRO
10	11:02	11:30	12:05
20	9:33	9:49	9:57
30	8:33	8:45	9:25

[a]All analyrar were at 5 deg/min ramp rate.

remembered that varying this and other parameters that affect retention times nay reault in loss of resolution due to overlapping of RIC peaks.

As noted earlier the MS has the ability to scan for ions with a aasr-to-charge ratio from 1 to 800. In this study scanning war typically from 150 to 350 amu in 0.5 to 1.0 sec. The Finnigan model 1020B can also scan for a number of selected ion masses in a tine frame of 0.030 to 1.000 eec for each mass or mase range while the total scan nay take 0.50 to 4.00 rec. The instrument will then reset itself and begin scanning again. This concept is termed selected ion nonitoring (SIM). The advantage of this is two-fold; the background is decreased because fewer masses are being rcanned so fewer interfering ions are detected, and the selected masses are scanned for a longer period of tine than in previouely noted ranger resulting in more ions of a specific mass detected and higher intensity readings. These advantages result in an overall increase in detection sensitivity.

The effective use of CI and SIM are dependent upon the ability of the reagent gas to react with the parent molecules to be studied. This ability is reflected in the conversion rate of M^+ to MH^+ and the effectiveness is measured by the MH^+ to M^+ ratio. As noted earlier in Chapter V, methane is the reagent gar in this study and its optimum pressure was determined through independent methane

studies. The experimental proof of that optimum pressure war demonrtrated through a series of analyses of the drug standards at various methane pressures. This was experimentally demonstrated through analyses of AMI in which the methane presaure was varied at 0.1 torr increments while monitoring the relative percentage of MH^+ formed compared to the total amount of ions detected. Table 11 shows the results of this series of analyses.

TABLE 11

Comparison of Methane pressures and the Relative amount of the UH+ formed during AMI analyses

Methane Pressure (torr)	Rel. % of MH^+
0.20	11.93
0.30	18.10
0.40	12.00
0.50	9.71

This pressure ir measured in the ion source of the MS and it varies depending on the type of rolvent and the sample size, but mainly on the flow rate of reagent gas entering the ion source.

evidence. The antibacterial potency of this preparation was
checked against a series of bacteria by the cup plate
method. Bacteriostatic activity was also found to be present
in the culture medium from which the bacteria had been
removed by centrifugation. The following table shows the
results of the tests carried out:

TABLE II

Comparison of Warfarex prepared by the Polyclar method
and the 1MA cultured bacterial preparation
(Zone of inhibition in mm.)

Warfarex Preparation (1000)	Bact. v. Coli
0.50	11.25
0.25	18.75
0.12	14.00
0.06	12.75

Clinical Comparison of Study

In thie study it was determined that sensitivity of the developed procedures ranged from 10 to 30 ng. It must be remembered that this is the actual amount of the drug injected onto the column. In many of the clinical procedures the following steps are followed

1. 1-3 mL of serum from patient is required.
2. 100-200 ng of internal standard is added.
3. A basic buffer is added to attain pH 9-11.
4. Extracting solution (such as hexane/alcohol) is added.
5. 70-99% of drug is extracted into organic phare.
6. Organic phase is drawn off and evaporated to dryness.
7. 5-100 µL of pure hexane is added to reconstitute residue.
8. 1-3 µL concentrated drug in hexane solution is injected.

The critical steps in concentrating the drug samples are steps 6 and 7. The above noted method is similar to the procedure developed by Rovei *et al* (30). Thus, the serum AMI concentration of 100 ng/mL would result in the injection of 30-40 ng of AMI being injected into the column. This is well within the sensitivity range of the above developed GC/MS procedure. The mathematical explanation of this is as follows:

$$100\ ng/mL \times 2mL = 200\ ng \qquad (10)$$

Claims: Method of Making Same

1. A method for making a polymeric bead useful in chromatography comprising the steps of:

   a. providing a first aqueous phase comprising a water-soluble polymer having a plurality of pendant hydroxyl groups;

   b. providing a second phase comprising a water-immiscible organic solvent;

   c. dispersing said first phase in said second phase to form droplets;

   d. reacting said droplets with a polyfunctional crosslinking agent to form crosslinked polymeric beads;

   e. recovering said beads; and

   f. sizing said beads to a predetermined particle size distribution.

2. The method of claim 1 wherein said water-soluble polymer is selected from the group consisting of dextran, starch, cellulose, polyvinyl alcohol, polyhydroxyethylacrylate, polyhydroxyethylmethacrylate, polyacrylamide, polyhydroxypropylacrylate, polyhydroxypropylmethacrylate, hydroxyethylcellulose, hydroxypropylcellulose, hydroxybutylcellulose, and copolymers of hydroxyethylmethacrylate and styrene.

3. The method of claim 2 wherein said water-soluble polymer is dextran.

4. The method of claim 3 wherein said dextran has a molecular weight of about 4 x 10^6 daltons.

5. The method of claim 1 wherein said second phase is selected from the group consisting of cyclohexane, chloroform, carbon tetrachloride, chlorobenzene, dichloroethylene, trichloroethylene, toluene, and xylene.

6. The method of claim 5 wherein said second phase is cyclohexane.

7. The method of claim 1 wherein said polyfunctional crosslinking agent is selected from the group consisting of epichlorohydrin, bischloromethyloxirane, diglycidylether, polyglycidylether, dialdehyde, diisocyanate, divinylsulfone, and 2,3-dibromopropanol.

8. The method of claim 7 wherein said polyfunctional crosslinking agent is epichlorohydrin.

9. The method of claim 1 wherein said droplets are formed by adding said first phase to said second phase.

10. The method of claim 1 wherein said droplets are formed by dispersing said first phase in said second phase.

11. The method of claim 1 wherein said droplets are formed by dispersing said first phase in said second phase with a stirring rate sufficient to produce droplets having a diameter of about 10 to about 1000 microns.

12. The method of claim 1 wherein said droplets are formed by dispersing said first phase in said second phase with a stirring rate sufficient to produce droplets having a diameter of about 50 to about 500 microns.

13. The method of claim 1 wherein said droplets are formed by dispersing said first phase in said second phase with a stirring rate sufficient to produce droplets having a diameter of about 100 to about 300 microns.

200 ng/ 20 µL = 10 ng/ µL (11)

Equation 10 shows that 2 mL of serum from this patient would contain 200 ng of AMI. The drug is completely extracted into an organic solvent and then this solvent is evaporated at 60°C under a stream of nitrogen gas. According to this procedure the residua is reconstituted with 20 µL of hexane. Therefore, as shown by equation 11 the 200 ng of AMI is now found in 20 µL or 10 ng/µL of hexane. Then 3-4 µL of this mixture or 30-40 ng of AMI is injected into the GC.

Some of the newer procedures such as that developed by Chinn et al (32) have eliminated the drying and concentrating steps. In this procedure the drug is extracted into 200 µL of a toluene/hexane/alcohol mixture and then 6 µL is injected onto column. According to this extraction procedure a rerun AMI concentration of 120-250 ng/mL would result in the injection of 7.2 to 15.0 ng of AMI onto the column.

CHAPTER VI

CONCLUSION

As noted earlier, there have been numerous procedures developed for the separation, identification and quantitation of numerous tricyclic antidepressants including amitriptyline, nortriptyline and protriptylina. These procedures have included spectrometric methods, radioimmunoassays, thin-layer chromatography, enzyme-labelled immunoarsays, and most recently high prersure liquid chromatography (HPLC) and gas chromatography (GC). The latter method, GC, has become the most widely accepted methods for drug studies. Through the years many types of detectors have been used with gar chromatographs such as ultraviolet detectors, nitrogen-phosphorous sensitive detectors and mass spectrometers. It is the combination of gas chromatograph/ mass spectrometer (GC/MS) that has resulted in a highly senritive and highly specific instrument capable of detecting and identifying picogram quantities of drugs.

In spite of all the research accomplished, there was a limited amount of work done using fused silica capillary columns combined with GC/MS. This was especially true for capillary columns with the SE-54 stationary phase, since no research work was found that used this particular stationary

phase with GC/MS. There is also a very small amount of work involved with studying the capabilitier and the limitations of capillary GC/MS. In this study, procedures and GC/MS parameters for both programmed or ramping temperature and isothermal nodes were investigated. The programmed temperature procedure could rupply excellent separation of a complex drug mixture and could be very useful in identifying an unknown drug. The isothermal procedure would provide more than adequate separation and more reliable response factors for the quantitation of drugs as in the case of therapeutic drug monitoring. In this case the use of electron ionization appeared less effective due to a lower sensitivity and specificity, whereas chemical ionization utilizing methane as the reagent gas resulted in significant increases in sensitivity and specificity. The specificity increased because the molecular weight of the parent molecule can easily be determined and the presence of additional fragmentation resulted in identifying many characteristic mass peaks.

It was the purpose of this study not only to develop a procedure of optimum conditions but also to report the effect of varying the conditions. The following is a list of the optimum GC/MS conditions for the programmed temperature node and the isothermal rode. The isothermal mode conditions are in parentheses if they are different than the programmed mode.

GC PARAMETERS:

250 deg. = Injector Temp.

150 deg. = Initial Temp. (200 deg.)

220 deg. = Final Temp. (200 deg.)

1 min. = Initial Time

10.0 D/M = Ramp Rate deg/min (0.0 D/M)

10 min. = Final tine

260 deg. = Separator Temp.

80 deg. = Manifold Temp.

INJECTOR MODE: CAPILLARY

60 rec. = Split/Sweep Valve Closed Time

90 sec. = Filament/Multiplier Off Time

SCAN FROM 150 AMU TO 350 AMU IN 0.5 sec.

METHANE PRESSURE: 0.40 torr (used in CI)

HELIUM PRESSURE: 20 psi

Figure 19. The typical computer printout of the optimum GC/MS programmed temperature mode conditions. The optimum irothermal conditions, if different, are noted parenthetically.

In invertigating many of the adjustable parameters the key factor in reparating a drug mixture appears to be the retention time. This is affected by several variables such as column temperature (isothermal or programmed modes), carrier gas flow rate and column diameter and length. It must be remembered that an adjustment of any parameter may

result in a compromise in resolution, retention time, or sensitivity of a procedure's results.

One of the factors that was not investigated and could have a definite effect on the procedure is the stationary phase coating of the capillary column. The SE-54 coating used in this study is slightly polar and nay tend to cause the tailing and broadened peaks evident in some of the RICs, whereas a less polar column coating, such as SE-30 or OV-101 that contain polydimathylsiloxane polymers, may be more effective due to the slightly polar characteristics of these drugs. There are also some columns available with slightly larger inside diameters or film thickness. These various column characteristice may result in sharper peaks without the tailing but some of the resolving power of the SE-54 coating would be lost.

Another factor that could be investigated is the reagent gas. Methane was used in this study, but as suggested by Chinn *et al* (32), by using a methane-ammonia mixture as the reagent gas a larger percentage of the MH^+ ions were produced. This is because a more stable MH^+ ion is formed, less fragmentation of the parent molecule occure, and fewer secondary reactions occur. This could result in better sensitivity when analyses are performed by chemical-ionization selected-ion monitoring.

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