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Chromatographic and Mass Spectrometric Analysis of Bradykinin Related Arginyl Peptides and Other Studies

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CHROMATOGRAPHIC AND MASS SPECTROMETRIC ANALYSIS OF BRADYKININ RELATED ARGINYL PEPTIDES AND OTHER STUDIES

by

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TABLE OF CONTENTS

KEVIN DALE BALLARD. Chromatographic and mass spectrometric analysis of bradykinin related arginyl peptides and other studies. (Under the direction of DANIEL R. KNAPP.)

Dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS (DMPO-N-TFE-O-TBDMS) polyaminoalcohol derivatives of arginyl peptides were evaluated for their utility in peptide analysis by capillary gas chromatography-mass spectrometry (GC-MS). These derivatives were hydrolytically stable and sufficiently volatile for capillary GC-MS analysis of low nanomole amounts of peptides as large as RPPGF. Complete amino acid sequence information was generated under electron impact ionization conditions, including several ions diagnostic for arginine residues, with molecular weight information imparted by the $[M-15]^+$ and $[M-57]^+$ pair of ions characteristic of TBDMS derivatives. Analysis of the sequential intermediate derivatives by FAB-MS facilitated optimization of problematic derivatization steps. As little as 20 picomoles of peptide could be detected by capillary GC-MS-selected ion monitoring, but problems with the reproducibility of the diborane reduction reaction limited its utility for quantitative applications. DMPO-N,O-di-TBDMS polyaminoalcohol derivatives were found to provide similar structural information, with similar reproducibility problems at low levels.

A procedure for selectively extracting arginyl peptides using immobilized phenylboronic acid columns exhibited limited binding

with numerous co-extracted contaminants, and was judged to be impractical for biological samples. A reverse phase ion pairing liquid chromatographic procedure employing alternating ion pairing reagents successfully isolated 25-35 nanomole amounts of RPP, RPPG and RPPGF from spiked human urine. None of these peptides were detected at this level in unspiked urine.

Further studies focussed on developing sensitive and reliable means of analyzing RPPGF as the DMPO-N-trifluoroacetyl-O-methyl ester derivative by direct insertion probe MS, thus avoiding the problematic diborane reduction reaction. Under electron impact ionization conditions, this derivative generated an unusually intense molecular ion suitable for low and high resolution selected ion monitoring. Molecular specificity was obtained by employing collisionally activated dissociation linked field selected reaction monitoring mass spectrometry, monitoring the formation of a specific fragment ion from the molecular ion in the first field free region of a double focussing mass spectrometer. With low level samples, the products at each stage of the derivatization were purified by reverse or normal phase HPLC. A 12 nanomole sample of RPPGF isolated from spiked human urine was successfully analyzed and definitively identified by these methods.

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PART I

CHROMATOGRAPHIC AND MASS SPECTROMETRIC ANALYSIS OF BRADYKININ RELATED ARGINYL PEPTIDES

CHAPTER I

INTRODUCTION

The potent vasodilator peptide hormone bradykinin has a half-life in blood on the order of 16-20 seconds (1,2); furthermore, bradykinin is degraded to the extent of 80-95% by a single passage through the lungs (1-5), and to varying degrees by other tissues (1,2,6). Thus, as Kaplan has pointed out (7), "direct determination of bradykinin as a measure of disease activity is usually unproductive." Considerable insight into the activity of the kallikrein-kinin system under normal and pathologic conditions could be gained if specific end-product catabolic fragments of bradykinin were known and could be quantitated, since such fragments would reflect the total flux of bradykinin through the system.

Bradykinin is a nonapeptide of sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (single letter amino acid notation (8) RPPGFSPFR), and is well known to be degraded by two enzymes. Kininase I (EC 3.4.12.7, carboxypeptidase N) is present in plasma and cleaves the C-terminal arginine residue from bradykinin to yield des-Arg^9-bradykinin (2,7,9). Kininase II (EC 3.4.15.1, angiotensin converting enzyme) is also present in plasma (10), and is highly concentrated in the lung (2,10) and other tissues (2). This enzyme, a dipeptidyl carboxypeptidase, can cleave bradykinin in two steps, first removing the C-terminal dipeptide FR, and then removing SP, ultimately

yielding the pentapeptide RPPGF (2,7,9,10), which is not further cleaved by kininase II (7). This enzyme can also cleave des-Arg^9-bradykinin (produced by kininase I) to yield the pentapeptide RPPGF plus SPF (7,9).

While the products of the individual enzyme-substrate reactions are well characterized, the catabolic fragments of bradykinin generated in vivo are virtually unknown, although a few studies have provided some valuable information in this area. In 1968 and 1970, Ryan and co-workers (3,4) demonstrated that bradykinin is essentially quantitatively degraded by a single pass through the isolated rat lung, and gives rise to the peptide products PP, RPPG, and FR. Very little quantitative information was presented, other than to imply that PP and RPPG were generated in a 2:1 ratio.

Also in 1970, Keiser and co-workers (11) studied the metabolism of [^{14}C]-Pro^2-bradykinin after intravenous infusion in man, and attempted to identify urinary metabolites. After 24 hours, urinary radioactivity accounted for 7-13% of the administered dose, plasma radioactivity accounted for 5%, with 75-85% unaccounted for. Calculating from the data presented, 5-5.5% of the administered radioactivity emerged in urine as the dipeptide PP after 2 hours, and approximately 1.5% emerged as unidentified "basic substances" in the same time period. A weak point in this study was the use of the intravenous infusion, in light of the high efficiency of the lung for degrading bradykinin. Since venous blood obligatorily passes through the lung before reaching other organ systems, the data

generated in this study are more indicative of lung metabolism than whole body metabolism, which might be better represented after intra-aortic infusion, although this would be difficult to justify in man.

In 1985, Kaplan and co-workers (7,9) studied the degradation of bradykinin (500 ng/ml) in citrated plasma using HPLC techniques. After 15 minutes at 37°C, bradykinin, des-Arg^9-bradykinin, and the tripeptide SPF were not detectable. The degradation products identified by HPLC retention times were free phenylalanine and a peak suggestive of the tetrapeptide RPPG. These authors suggested that a plasma carboxypeptidase is capable of removing C-terminal phenylalanine residues from des-Arg^9-bradykinin, RPPGF, and SPF.

Data conflicting with the above study were generated by Marceau and co-workers in 1981 (12). These authors studied the degradation of bradykinin (100 µg/ml) in 20% heparinized plasma, and identified des-Arg^9-bradykinin as the major degradation product by HPLC and bioassay. These discrepant findings may be due to either the incredibly high concentration of bradykinin studied, the use of diluted plasma, or the use of heparin rather than citrate as an anticoagulant.

Judging from the above studies, the metabolism of bradykinin is complex and evidently involves other enzymes in addition to kininases I and II; the carboxypeptidase suggested by Kaplan (7) may be involved, as well as an aminopeptidase (kininase III) and a dipeptidyl aminopeptidase as suggested by Behal and coworkers (13,14).

In none of the above studies were C-terminal or neutral peptides longer than a dipeptide identified. The N-terminal tetrapeptide RPPG was identified in both human plasma and the isolated rat lung preparation. The unidentified "basic substances" found by Keiser's group almost certainly contained the N-terminal RP sequence. The pentapeptide RPPGF is suggested as a likely catabolic fragment from enzymatic studies, since it is generated by two-step sequential degradation by kininase II, and also by the sequential action of kininase I followed by kininase II.

Extrapolating from the piecemeal evidence in the studies described above, a working hypothesis may be formed, stating that the metabolic products of the peptide hormone bradykinin include arginine-containing peptide fragments four or more amino acid residues in length, which retain much of the original amino acid sequence and thus are highly specific metabolites of bradykinin, and which when quantitated will serve as useful indicators of the activity of the kallikrein-kinin system as a whole. That this hypothesis has not been thoroughly tested in the past is not surprising since reliable methods for isolating, identifying and quantitating small arginyl peptides at low levels have not existed.

Due to these considerations, the overall goal of the studies described here was the elucidation of some of the chemical and instrumental means which may ultimately be necessary for analyzing small arginyl peptides with high chemical specificity, high sensitivity and high reliability. These studies have focussed on the use

of the mass spectrometer as the final analytical tool in order to take advantage of the high sensitivity and specificity afforded by this instrument. Gas chromatography-mass spectrometry (GC-MS) of small peptides has long been recognized for its utility for generating amino acid sequence information (15-29), and its potential value for quantitating these compounds is beginning to become a reality (30,31). Quantitative capillary GC-MS methods using stable isotope labeled internal standards and selected ion monitoring techniques generically afford high sensitivity, high specificity, and excellent precision and accuracy (32,33). Significant advances in the quantitation of small peptides by these methods could be brought about by developing derivatives of peptides which possess good GC properties, generate intense ions at high mass in the mass spectrometer, and ensure volatilization of the highly polar guanidino group of arginine residues.

Two general methods have been developed over the past 27 years for volatilizing peptides for mass spectrometric amino acid sequence determination, namely the polyaminoalcohol derivatization procedure (15-23) and the permethylation procedure (25-29). Both of these methods have advantages, but in general the polyaminoalcohol derivatives are more volatile, produce more reliable sequence information, and are more reliably formed (19,34). With both of these procedures, arginine residues have presented a particular problem because of the high basicity and polarity of the guanidino group. The polyaminoalcohol scheme has traditionally included the conversion of

arginine residues to ornithine residues through hydrazinolysis prior to the chemical derivatization steps. However, hydrazinolysis can result in the loss of up to 60% of the original peptide through peptide bond cleavage; this type of loss would be an obvious impediment to the quantitative analysis of peptides, particularly in terms of overall sensitivity. In conjunction with the permethylation procedure, arginine residues have been successfully volatilized through derivatization with β-diketone reagents, such as 2,4-pentanedione (acetylacetone), to form substituted or unsubstituted pyrimidyl-ornithine derivatives without associated peptide bond cleavage. This type of derivatization of arginine residues has not been used in the past with the polyaminoalcohol procedure, although the two are theoretically compatible. The diborane reduction step employed in the conversion of oligopeptides to polyaminoalcohols does not reduce heterocyclic aromatic ring systems (42), as exemplified by its failure to reduce the side chain groups of tryptophan and histidine residues (43), and consequently should not reduce pyrimidyl-ornithine groups. Thus, the inclusion of this type of derivatization in the polyaminoalcohol scheme should ensure the volatilization of arginine-containing peptides without the risk of peptide bond cleavage during the derivatization reaction.

Another aspect of the polyaminoalcohol scheme which could potentially be improved is its means of volatilizing hydroxy groups. The final step in the polyaminoalcohol scheme involves the derivatization of hydroxy functions with the trimethylsilyl (TMS) group.

TMS derivatives are highly moisture sensitive and thus inherently unstable, and, while they give rise to characteristic $[M-15]^+$ ions, these are usually of relatively low intensity. Studies employing tertiary-butyldimethylsilyl (TBDMS) derivatives have demonstrated that TBDMS derivatives are much more hydrolytically stable than TMS derivatives and, through loss of both a methyl and a tertiary-butyl group, TBDMS derivatives characteristically give rise to intense ions corresponding to $[M-15]^+$ and $[M-57]^+$, which are very useful for both qualitative and quantitative work (33,44,45). The reagent N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) is potentially suitable for derivatizing polyaminoalcohols because of its high reactivity toward hydroxy groups and relatively low reactivity toward secondary amines (46,47).

The relatively new technique of fast atom bombardment mass spectrometry (FAB-MS) has proven very useful in many recent studies for ionizing and obtaining mass spectral data on nonvolatile compounds such as underivatized and partially derivatized peptides. One of the goals of the present studies was to evaluate the utility of FAB-MS for documenting the success or failure of a series of sequential derivatization reactions such as those employed with the polyaminoalcohol scheme. It was anticipated that by analyzing the sequential derivatives after each reaction, problematic steps could be readily identified and remedial measures immediately taken.

Because of the complex nature of most biological samples, it was anticipated that prepurification of the peptides of interest

would be necessary in order to reduce interference with the derivatization reactions and the GC-MS analyses. Low and high resolution reverse phase liquid chromatography of peptides using perfluorinated alkanoic acids as lipophilic ion-pairing reagents are well known and widely used (48-53). Additionally, selective extraction of arginine-containing peptides is possible (54,55). The reversible modification of arginine residues of peptides with 1,2-cyclohexanedione (CHD) to form a vicinal diol derivative (54-56), with subsequent selective extraction of the modified peptides using immobilized boronic acid columns (54,55), has been described in the literature and has been used for the selective extraction of arginine-containing peptides for GC-MS analysis in conjunction with the permethylation method (55).

The guanidino group of arginine residues reacts reversibly with CHD at pH 8-9 in borate buffers to form a vicinal diol derivative which is stable under acidic conditions and in borate buffers at pH 8-9. At pH 7-9 in the absence of borate, the derivative spontaneously regenerates CHD and the guanidino group; this reversal process is quantitative in the presence of nucleophiles such as hydroxylamine (54,56). Immobilized boronic acid groups interact selectively with vicinal diol groups at neutral or mildly alkaline pH to form covalent complexes, a process which can be reversed under acidic conditions (pH 1-2). Immobilized boronic acid columns can thus be used to selectively isolate CHD-modified arginyl peptides, with elution from the column at low pH, and with subsequent regeneration

of the arginine-containing peptide in hydroxylamine buffer at pH 7 (56). In the published work, large proteins were first modified with CHD in the presence of borate, then dialyzed against dilute acetic acid to disrupt the borate complexes and desalt the CHD-modified protein. CHD-modified peptides were then generated from the CHD-modified protein by partial hydrolysis, and the CHD-modified peptides subsequently isolated with immobilized boronic acid columns. Since the present studies dealt with small peptides rather than proteins, the dialysis step employed in the published work was not feasible, and other methods were therefore necessary to cleave the diol-borate complexes formed during the initial derivatization reaction and to desalt the sample. Toward this end, it was anticipated that acidification with a perfluorinated alkanoic acid followed by ion pairing extraction using reverse phase cartridge columns would be a suitable procedure for hydrolyzing the diol-borate complexes and desalting the CHD-modified peptides.

In summary, these studies were undertaken with a view toward ultimately permitting the testing of the hypothesis that the activity of the kallikrein-kinin system may be estimated through quantitation of putative bradykinin catabolites, a process which has been limited by the unavailability of sensitive, specific and reliable means for analyzing small arginyl peptides at low levels. Toward this end, the first specific goal was to evaluate the feasibility of incorporating the formation of volatile dimethylpyrimidylornithyl derivatives of arginyl peptides into the polyaminoalcohol

derivatization scheme for the capillary GC-MS analysis of peptides. A second related goal was to determine the feasibility of employing the TBDMS group for volatilizing the hydroxy functions of polyamino-alcohols. The third primary goal envisioned at the outset of these studies was to develop efficient and reliable means of isolating bradykinin related arginyl peptides from complex biological matrices, either by employing reverse phase ion pairing liquid chromatographic procedures alone or in combination with an arginyl peptide selective extraction procedure which was to be developed. A fourth goal was to determine the utility of employing FAB-MS as a means of verifying sequential isolation procedures and derivatization reactions. An overriding goal was to develop mass spectrometric procedures for analyzing bradykinin related peptides with high reliability at low levels. Numerous problems were encountered during the course of these studies, the nature and solutions to which will be presented in subsequent chapters.

In order to provide an overview of the detailed material which follows in subsequent chapters, the results of the present studies are summarized below. Initial studies with the simple dipeptide alanyl-alanine (AA) established the feasibility of employing the TBDMS group for derivatizing the hydroxy functions of polyamino-alcohols. Subsequent studies with bradykinin related arginyl peptides demonstrated that dimethylpyrimidylornithyl-N-trifluoro-ethyl-O-TBDMS polyaminoalcohol derivatives of these compounds may be

formed and that these derivatives are sufficiently volatile for capillary GC-MS analysis of peptides as large as the pentapeptide RPPGF. Under repetitive scanning capillary GC-MS analysis conditions, these derivatives generate complete amino acid sequence information in a fashion directly analogous to that of the well known N-trifluoroethyl-O-TMS polyaminoalcohols (17), with the additional molecular weight information provided by the easily recognized pair of ions corresponding to $[M-15]^+$ and $[M-57]^+$ which is characteristic of TBDMS derivatives. These derivatives also generate several ions diagnostic for arginine residues, and were found to be hydrolytically stable and useful for structural analysis of arginyl peptides at nanomole levels. During sensitivity studies with low level samples, as little as 20 pmoles of peptide could be detected by capillary GC-MS-SIM with good signal to noise ratio. Unfortunately, reproducibility problems with the derivatization chemistry, particularly the diborane reduction reaction, limited the utility of these derivatives for quantitative analysis of low level samples of arginyl peptides. The use of FAB-MS was particularly beneficial during these studies because it enabled the analysis of the sequential intermediate derivatives, and thus permitted ready identification of problematic derivatization steps. Some of the reaction conditions advocated in the most recently published version of the polyaminoalcohol scheme were not found to be optimal for the peptides employed in these studies. FAB-MS facilitated the identification of these problems so that corrective action could be taken.

Dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivatives of arginyl peptides were briefly explored as a possible solution to some of the reproducibility problems encountered with the N-trifluoroethyl derivatives. The di-TBDMS derivatives were found to be suitable for capillary GC-MS analysis and to provide structural information similar to their N-trifluoroethyl counterparts. However, poor reproducibility was encountered with both the diborane reduction and the silylation reactions with these derivatives even at high nanomole levels.

A general procedure was developed for the selective extraction of arginyl peptides from aqueous matrices employing immobilized phenylboronic acid columns. This procedure was found to successfully isolate standard arginyl peptides from aqueous buffers, and was demonstrated to be compatible with the derivatization procedures for capillary GC-MS analysis of these peptides. However, high performance liquid chromatographic (HPLC) analysis of the extracts showed that numerous contaminating species were present in the extracts, and that the phenylboronic acid columns employed exhibit a very limited binding capacity. This procedure was therefore judged to be impractical for the isolation of arginyl peptides from biological matrices.

A complex but highly efficient procedure was developed for the isolation of the bradykinin related peptides RPP, RPPG and RPPGF from complex biological matrices, particularly urine. This procedure was based on a combination of low and high resolution reverse

phase ion pairing liquid chromatography, with alternating ion pairing reagents. Low and high resolution FAB-MS demonstrated the successful isolation of 25-35 nmole amounts of these peptides from a spiked 20 ml human urine sample.

Further studies focussed on developing sensitive and reliable means of analyzing the pentapeptide RPPGF, which was consistently the most problematic of the peptides studied. These experiments employed the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative, which was sufficiently volatile for direct insertion probe mass spectrometric analysis. The use of this derivative avoided the problematic diborane reduction reaction. Under electron impact ionization conditions, the molecular ion of this derivative was of unusually high intensity and was suitable for direct insertion probe selected ion monitoring mass spectrometric analysis under both low and high resolution conditions. Further molecular specificity was obtained by employing direct insertion probe collisionally activated dissociation linked field selected reaction monitoring mass spectrometry, monitoring the formation of a specific fragment ion from the molecular ion in the first field free region of a double focussing mass spectrometer. Success with low level samples was obtained through HPLC isolation of the sequential derivatives, with the final derivative isolated by normal phase HPLC. A sample of RPPGF isolated from spiked human urine was successfully analyzed and definitively identified by these methods.

CHAPTER II

METHODS

A. Reagents.

Acetonitrile, chloroform, methylene chloride and methanol were glass distilled UV grade (Burdick and Jackson Laboratories, Inc., Muskegan, MI). Water used for HPLC was analytical grade deionized, while water used for derivatization reactions was distilled in glass with reflux under argon and stored under argon at 4 °C. Trifluoroacetic acid (TFA, Fisher Scientific Co., Pittsburg, PA) was redistilled with reflux under nitrogen; heptafluorobutyric acid (HFBA, Aldrich Chemical Co., Milwaukee, WI) was used without further purification. Acetylacetone (Aldrich Gold Label), triethylamine (Fisher), pyridine (Fisher) and methyl trifluoroacetate (Alfa Products, Danvers, MA) were all redistilled in glass with reflux under argon. Acetylacetone, triethylamine and pyridine were stored under argon at -80 °C and warmed to room temperature just prior to use. Tetrahydrofuran was UV grade (Burdick and Jackson) stored over 5A molecular sieves at room temperature (57). N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA, Regis Chemical Co., Morton Grove, IL) and N-methyl-bis(trifluoroacetamide) (MBTFA, Pierce Chemical Co., Rockford, IL) were stored at 4 °C and warmed to room temperature prior to use. Methanolic HCl was freshly generated prior to use by slowly adding a stoichiometric amount of acetyl

chloride (Fisher) to methanol under argon at -10 °C. Ethereal diazomethane was freshly generated prior to use from N-methyl-N-nitroso-N^1-nitroguanidine (Aldrich), 6N sodium hydroxide and diethyl ether (Fisher) using the apparatus and conditions described by Fales and co-workers (58). One molar diborane/tetrahydrofuran solution stabilized with 0.005 M sodium borohydride (Aldrich) was stored at 4 °C under nitrogen. Cyclohexanedione (Aldrich), N-ethylmorpholine (Aldrich) and glycerol (Fisher) were reagent grade.

The peptides Ala-Ala (AA, Sigma Chemical Co., St. Louis, MO), Arg-Phe (RF, Vega Biochemicals, Tucson, AZ), Arg-Pro-Pro (RPP, Serva Fine Biochemicals Inc., Westbury, NY), Arg-Pro-Pro-Gly (RPPG, Bachem Inc., Torrance, CA) and Arg-Pro-Pro-Gly-Phe (RPPGF, Serva) were stored dessicated at -20 °C. These peptides were all of high purity with the exception of RPPG, the purity of which was estimated at 30% based upon HPLC and FAB-MS analysis. The amino acid L-(+)-arginine (Aldrich) was stored at room temperature.

All other chemicals were reagent grade from commercial sources and were used without further purification.

B. Glassware.

Derivatization reactions were carried out in 1 or 2 ml Reacti-vials (Pierce) with Teflon lined caps. All glassware which came in contact with analytical samples was silanized by immersion in a 2% (v/v) solution of dimethyldichlorosilane in toluene for 20 minutes, followed by thorough rinsing with absolute methanol (59 a).

C. Evaporation techniques.

Excess derivatization reagents and solvents were removed either by evaporation under a stream of nitrogen or argon in a room temperature water bath, or by evaporation under vacuum with centrifugation on a Speed-Vac Concentrator (Savant Instruments, Inc., Hicksville, NY). The Speed-Vac was equipped with a cold trap and a Precision Scientific Model 75 vacuum pump (Precision Scientific Co., Chicago, IL).

D. High Performance Liquid Chromatography.

High performance liquid chromatographic (HPLC) analyses were performed using a Waters Associates (Milford, MA) gradient elution HPLC system consisting of two Model 510 HPLC pumps a Model 680 automated gradient controller, a Model U6K injector, and a Model 441 absorbance detector. Chromatograms were recorded with a Fisher Recordall Series 5000 recorder. Reverse phase HPLC analyses were performed using a Vydac Protein and Peptides C_{18} 5 μm column (25 cm X 4.6 mm i.d., The Sep/a/ra/tions Group, Hesperia, CA) equipped with a guard column. Normal phase HPLC analyses were performed using an Econosphere Silica 5 μm column (25 cm X 4.6 mm i.d., Alltech Associates, Inc., Deerfield, IL).

Two types of reverse phase analyses were performed during the course of these studies, depending upon whether trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA) was used as the ion pairing reagent. Water and acetonitrile were the base solvents used in both

systems, with the ion pairing reagent at a concentration of 0.1% (by volume) in each solvent. For the TFA system, the column was equilibrated with 0.1% TFA/water. At the time of injection, a linear gradient was initiated with 0.1% TFA/acetonitrile at a rate of 1% acetonitrile per minute. Similarly, for the HFBA system, the column was equilibrated with 0.1% HFBA/water, and a linear gradient with 0.1% HFBA/acetonitrile at a rate of 2% acetonitrile per minute was initiated at the time of injection. For both systems, the flow rate was 1.5 ml/min throughout, with UV detection at 214 nm and a typical sensitivity setting of 0.5 absorbance units for a full scale deflection.

Normal phase HPLC analyses were performed by equilibrating the column with chloroform, then at the time of injection initiating a linear methanol gradient to a final methanol concentration of 10% over a twenty minute time period. Detection was with UV absorbance at 254 nm, with a typical sensitivity setting of 0.05 absorbance units for a full scale pen deflection.

E. Mass Spectrometry and Gas Chromatography-Mass Spectrometry.

All mass spectrometric analyses performed during the course of these studies employed a Finnigan MAT 212 double focussing mass spectrometer. This instrument is of reverse Nier-Johnson geometry (the magnetic sector preceeds the electric sector), has a nominal accelerating voltage of 3 kV, and has a routine high resolution capability of 15-20,000. The mass spectrometer was interfaced to a

Finnigan Spectrosystem SS200 data system equipped with selected ion monitoring hardware for data acquisition during repetitive scanning and selected ion monitoring experiments. When appropriate, a Bell an Howell Model 525150 oscillographic recorder (Bell and Howell, Pasadena, CA) was used for the recording of mass spectral data.

For fast atom bombardment mass spectrometric (FAB-MS) analyses, an Ion Tech FAB11NF saddle field fast atom gun (Ion Tech Ltd., Middlesex, England) powered by an Ion Tech B50 power supply was adapted to the MAT 212. Xenon gas was introduced into the FAB gun through a Granville-Phillips Variable Leak valve (Series 203, Granville-Phillips Co., Boulder, CO). The probe used for FAB-MS was fabricated in these laboratories, employing a 316 stainless steel brush finished probe tip (angle of incidence 60°) and an insulator fabricated from Kel-F. For a typical analysis, the probe tip was first coated with a thin layer of glycerol. Then, a small amount of the solid or oily analytical sample was removed from the sample vial using a clean stainless steel wand, and was mixed with the glycerol directly on the probe tip. The FAB probe was then introduced into the ion source, the FAB gun was turned on with xenon accelerated to 6-8 kV, and several scans were recorded over the 15-1000 atomic mass unit (amu) range at a scan rate of 8 sec/decade. These techniques generally produced excellent results with very little sample consumption. Ion source potentials were optimized while monitoring the m/z 277 ion of glycerol, and a mixture of poly(ethyleneglycol)s of average molecular weight 200, 400 and 600 (Aldrich) was used for

routinely calibrating the mass spectrometer under FAB conditions. The typical ion source pressure was 5 x 10^{-6} torr, and the ion source was operated at room temperature.

A Varian 3700 gas chromatograph (Varian Associates, Inc., Palo Alto, CA) was interfaced to the MAT 212 for capillary gas chromatography-mass spectrometry (GC-MS) analyses. Columns used were fused silica DB-1 and DB-5 (0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Inc., Rancho Cordova, CA) columns 4-30 m in length, with the outlet end introduced directly into the ion source of the mass spectrometer through a heated transfer line (290-320 °C). The gas chromatograph was modified to incorporate an on-column injector fabricated in these laboratories employing glass inserts purchased from J&W Scientific. For a typical analysis, 3-5 μl of sample were slowly injected on-column in the cooled zone with the GC oven temperature 10-15 °C above the nominal boiling point of the highest-boiling solvent component in the sample. The on-column injector was then lowered and, after solvent clearance, the GC oven temperature was programmed at a rate of 5-10 °C/min to a maximum of 325 °C. Data collection was initiated at the moment of temperature program initiation. High purity helium (>99.95 %) was used as the carrier gas, with the capillary head pressure adjusted to give an average linear velocity of 45-55 cm/sec at the upper GC oven temperatures (60). The ion source of the mass spectrometer was maintained at a temperature of 250 °C. The mass spectrometer was operated in electron impact mode, with an electron energy of 70 eV and 1 mA

electron emission. For all GC-MS analyses, the ion source potentials were optimized while monitoring the m/z 207 GC bleed ion with the GC oven at or near the temperature of elution of the samples. Repetitive scanning GC-MS analyses were performed with the mass spectrometer operated at a resolution of 1,900-2,300 (50% peak height definition), scanning the 70-1000 amu range at a rate of 3 sec/decade, with an interscan time of 600 msec. For selected ion monitoring experiments, the mass spectrometer was set at a resolution af 1,000-1,200 with a flat topped peak shape. The magnet was operated in current regulation mode for field strength stability, and was manually focussed to the center of the ion of interest using a standard sample as reference material. The secondary electron multiplier was operated at 2,200 V for high level samples and at 2,800 V for low level samples. Selected ion monitoring data were collected using the SS200 data system at a rate of 5-10 intensity readings per second (each intensity reading was the average of 16 individual samples). Perfluorokerosine (PCR Inc., Gainesville, FL) was used to calibrate the mass spectrometer under electron impact conditions.

Direct insertion probe mass spectrometric (DIP-MS) analyses were performed with the FAB gun removed from the MAT 212 and replaced with a lock valve to permit the introduction of the heated DIP. For all DIP-MS experiments, 2-6 µl of dissolved sample were introduced into a 1.9 mm x 10 mm glass DIP sample vial. The DIP vial was placed into a 1 ml Reactivial, and solvents were removed

under vacuum with centrifugation on the Speed-Vac. This process was repeated if necessary to accumulate sufficient material in the DIP vial for analysis. The vial was then immediately inserted into the probe tip and introduced into the ion source of the mass spectrometer at room temperature. After instrumental stabilization, data collection and probe heating were initiated. The mass spectrometer was operated in electron impact mode for all DIP-MS experiments, with an electron energy of 70 eV, an electron emission of 1 mA, and with the ion source temperature maintained at 250 °C.

Repetitive scanning data were obtained with the instrument set at a resolution of 1,900-2,300, scanning over the 100-1000 amu range at a scan rate of 3 sec/decade. The probe was heated at a rate of 0.6-1.3 °C/sec during these experiments.

Low resolution direct insertion probe selected ion monitoring analyses were performed with the mass spectrometer set at a resolution of 1,000-1200 with a flat topped peak shape. The magnet, operated in current regulation mode, was focussed manually to the center of the ion of interest using a standard sample as reference material. The analytical sample was run immediately thereafter. The probe was heated at a rate of 1.3-2 °C/sec during these experiments.

For high resolution direct insertion probe selected ion monitoring experiments, the mass spectrometer was tuned to a resolution of 14-15,000. The magnet, operated in current regulation mode, was manually coarsely focussed to the region of the ion of interest

using a magnetic calibration table generated by the SS200 data system (CALIB routine) accurate to within 0.2 atomic mass units. Perfluorokerosene was introduced into the mass spectrometer as a reference sample, and the accelerating voltage was electrically jumped (MACQ routine using MISLRS microcode) to a perfluorokerosine ion slightly higher in mass than the ion of interest using a highly accurate electrical calibration table generated by the SS200 data system (ECAL routine). The magnet was then finely adjusted manually to focus the reference mass. Perfluorokerosine introduction was then stopped and the data system was set for selected ion monitoring of the ion of interest. The analytical sample was run immediately thereafter in order to avoid magnetic field strength drift, typically with a probe heating rate of 2 °C/sec.

Direct insertion probe collisionally activated dissociation selected reaction monitoring mass spectrometry (DIP-CAD-SRM-MS) experiments were performed with the mass spectrometer set at a resolution of 1,000-1200. Xenon gas was used for collisional activation in these experiments, and was introduced into the region of the ion source and the first field free region of the mass spectrometer through a Granville-Phillips Variable Leak valve and a 2 m segment of 0.25 mm i.d. fused silica capillary tubing. Good results were obtained with the xenon flow adjusted to give an ion source pressure of 6-8 x 10^{-6} torr. For these experiments, the linked field unit of the MAT 212 was operated in B/E mode to maintain a constant relationship between the field strengths of the magnetic

and electric sectors. The magnet and the linked field unit were adjusted to focus the precursor ion of interest using a standard sample as reference material (this process determines the constant B/E to be used by the linked field unit). The linked field unit was then switched to "measure" to maintain the ratio B/E constant, and the magnet was refocused to the center of the fragment ion of interest using the standard sample as a reference. The analytical sample was run immediately thereafter, with the data system operated in selected ion monitoring mode, and with the DIP heated at a rate of 2-3 °C/sec.

F. Condensation of arginine residues with 2,4-pentanedione (acetylacetone).

For most experiments with arginyl peptides, the procedure described by Morris and co-workers (38) was used with little modification. To the dry peptide sample (20 pmoles to 100 nmoles) were added 50 µl of water, 100 µl of absolute ethanol and 50 µl of triethylamine. The sample was capped under argon, vortexed and centrifuged. Under a flow of argon, 100 µl of acetylacetone were added. The sample was again capped under argon, vortexed and heated at 100 °C for four hours with occasional vortexing during heating. The sample was then allowed to cool and placed on the Speed-Vac to reduce the volume to less than 5 µl of yellow oil. Water (250 µl) and glacial acetic acid (25 µl) were added, and the sample was vortexed and then heated at 100 °C for twelve minutes with

occasional vortexing during heating. The sample was allowed to cool and extracted three times with 500-700 µl of diethyl ether, with centrifugation to ensure complete separation of layers. The organic layers were discarded, and the aqueous sample was placed on the Speed-Vac until the volume was reduced to dryness. The sample was suitable for FAB-MS analysis at this stage.

G. Methylation of carboxylic acid groups with diazomethane.

Methanol (250 µl) was added to the dry sample, which was then capped under nitrogen, vortexed, sonicated 15 min and cooled to 0 °C. Freshly generated ethereal diazomethane (approximately 500 µl) was added, and the sample was vortexed and immediately placed on the Speed-Vac to remove excess diazomethane and solvents. The dry sample was suitable for FAB-MS analysis.

H. Methylation of carboxylic acid groups with 3 M methanolic HCl.

To the dry sample were added approximately 500 µl of freshly generated anhydrous 3 M methanolic HCl; the sample was then sonicated for one hour with occasional vortexing. Methanol and excess HCl were removed by evaporation under a stream of nitrogen. Seven drops of methanol were added, and the sample was vortexed, centrifuged and evaporated to dryness on the Speed-Vac, after which the sample was suitable for FAB-MS analysis.

I. Trifluoroacetylation of amino groups with methyl trifluoroacetate.

Peptides were trifluoroacetylated with methyl trifluoroacetate essentially according to the published procedure (17). To the dry sample were added 250 µl of methanol, 20 µl of triethylamine and 250 µl of methyl trifluoroacetate. The sample was capped under nitrogen, vortexed and allowed to stand overnight at room temperature in the dark. The sample was then placed on the Speed-Vac to remove excess derivatization reagents and solvents. At this stage the sample was suitable for FAB-MS analysis.

J. Trifluoroacetylation of amino groups with N-methyl-bis(trifluoroacetamide) (MBTFA).

Anhydrous tetrahydrofuran (200 µl) and N-methyl-bis(trifluoroacetamide) (MBTFA, 50 µl) were added to the dry sample. The sample was then capped under nitrogen or argon, vortexed and heated at 80 °C for 30-50 minutes. The sample was then sonicated for 30 minutes to help ensure complete dissolution, and heated 30 more minutes at 80 °C, with occasional vortexing during both heating steps. The sample was then allowed to cool and evaporated to dryness on the Speed-Vac, after which the sample could be analyzed by FAB-MS.

K. Reduction of amide and ester groups with diborane.

Samples were reduced with diborane using the published procedure (17) essentially without modification. The dry sample was

capped under nitrogen and cooled to 0 °C in an ice bath. One molar diborane-tetrahydrofuran solution (200 µl) was added, and the sample was vortexed and heated at 90 °C for 30 minutes, with occasional vortexing during heating. The sample was then cooled to 0 °C in an ice bath, and excess diborane was destroyed by the careful dropwise addition of methanol, during which operation vigorous bubbling occurred. After bubbling had ceased, solvents and trimethoxyborane were removed by evaporation on the Speed-Vac. Boroamine complexes were then decomposed by the addition of 1 M methanolic HCl (approximately 500 µl), with heating at 90 °C for 30 minutes. The sample was allowed to cool, evaporated to dryness on the Speed-Vac, and the boroamine decomposition process was repeated.

In order to convert the resulting polyaminoalcohol hydrochloride salts to the free polyaminoalcohols, the dry sample was dissolved in 200 µl of 25% (w/v) aqueous potassium carbonate and extracted three times with 200 µl of methylene chloride. The sample was centrifuged to ensure complete separation of layers, and the organic layers (lower) were combined. To the aqueous layer were added 200 µl of 120% (w/v) aqueous potassium carbonate plus 50-100 mg solid potassium carbonate. The sample was then extracted three times with 400 µl of methylene chloride, and the organic layers (upper) were combined with those from the first extraction process. The combined extract was then centrifuged to separate out any residual aqueous phase, and transferred to another clean sample vial, with care taken to avoid transferring any water droplets. The

extract was then evaporated to dryness on the Speed-Vac. At this stage the sample was suitable for FAB-MS analysis.

L. Silylation of hydroxy groups with N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA).

To the dry sample were added 65 µl of pyridine and 35 µl of MTBSTFA. The sample was then vortexed and heated at 80 °C for one hour, with occasional vortexing during heating. The sample was allowed to cool and evaporated to dryness on the Speed-Vac. The dry sample could be analyzed by FAB-MS at this stage. The sample was then redissolved under nitrogen with vortexing and sonication in an appropriate volume of pyridine (typically 10-100 µl). The sample was then ready for GC-MS analysis.

M. Feasibility of N-trifluoroethyl-O-TBDMS polyaminoalcohol derivatives.

In order to explore the feasibility of forming and analyzing N-trifluoroethyl-O-TBDMS polyaminoalcohol derivatives of peptides, a series of samples of the simple dipeptide AA (0.4 - 1.2 mg) were methylated with ethereal diazomethane, trifluoroacetylated with methyl trifluoroacetate, reduced with diborane and silylated with MTBSTFA. Reactions were carried out as described above with the exception of the methylation reaction, which was performed in the following manner. The sample was first converted to the hydrochloride salt form by adding 500 µl of 1 N methanolic HCl to the dry

peptide, vortexing and immediately evaporating the sample to dryness on the Speed-Vac. The sample was then dissolved in 250 µl of methanol, and ethereal diazomethane was added until the yellow color of diazomethane persisted. The sample was then sonicated for 30 minutes and evaporated to dryness on the Speed-Vac.

Samples were anaylzed by FAB-MS after methylation, DIP-MS after trifluoroacetylation, FAB-MS after reduction, and by GC-MS after silylation.

N. Compatibility of DMPO derivatives of arginyl residues with diborane reduction.

The compatibility of the dimethylpyrimidyl ring system with the diborane reduction reaction was tested by converting arginine to dimethylpyrimidylornithine aminoalcohol at a semi-synthetic level in the following manner. Arginine (5 mg) was converted to dimethylpyrimidylornithine through derivatization with acetylacetone as described in section F above and analyzed by by FAB-MS. The sample was then sonicated in a mixture of methanol and ethereal diazomethane until all solid dissolved and the yellow color of diazomethane persisted. Solvents and excess diazomethane were removed by evaporation on the Speed-Vac, and the sample was analyzed by FAB-MS. The sample was then reduced with 800 µl of 1 M diborane-tetrahydrofuran solution as described in section K above. The isolated product was analyzed by FAB-MS.

O. Trifluoroacetylation: methyl trifluoroacetate versus MBTFA.

The efficiency of the trifluoroacetylation of the amino groups of arginyl peptides with methyl trifluoroacetate was tested using a 1.2 μmole sample of the dipeptide RF. The peptide was converted to the DMPO derivative by condensation with acetylacetone, then methylated with ethereal diazomethane. The resulting methyl ester was treated with methyl trifluoroacetate as described in section I above. After evaporation on the Speed-Vac, the sample was analyzed by FAB-MS.

Further evaluation of the trifluoroacetylation of amino groups with methyl trifluoroacetate was accomplished by carrying a series of samples of RF (0.6 - 1.6 μmoles) through the entire derivatization process (condensation with acetylacetone, methylation, trifluoroacetylation with methyl trifluoroacetate, reduction and silylation), varying the trifluoroacetylation reaction time from twelve to thirty-six hours. The fully derivatized samples were analyzed by capillary GC-MS-SIM, monitoring m/z 408.

The feasibility of employing MBTFA for trifluoroacetylating the amino groups of arginyl peptides was tested using a 300 nmole sample of RF. The dipeptide was converted to the DMPO derivative with acetylacetone and methylated with 3 M methanolic HCl. The sample was then treated with MBTFA using the procedure described in section J above. The dry sample was analyzed by FAB-MS.

P. Verification of sequential derivatization reactions by FAB-MS and capillary GC-MS.

A series of samples (170 - 600 nmoles) of RF, RPP, RPPG, and RPPGF were carried through the entire derivatization process to convert them to the corresponding DMPO-N-TFE-O-TBDMS polyaminoalcohol derivatives. Using the general procedures described above, each sample was condensed with acetylacetone, methylated with ethereal diazomethane, trifluoroacetylated with with MBTFA, reduced with diborane and silylated with MTBSTFA. At the end of each derivatization step, each sample was analyzed BY FAB-MS to determine the success or failure of the sequential reactions. The fully derivatized samples were analyzed by both FAB-MS and capillary GC-MS.

Q. Methylation - ethereal diazomethane versus methanolic HCl.

In order to evaluate the relative utility of ethereal diazomethane and methanolic HCl as methylating reagents within the overall derivatization process, the two reagents were compared in parallel experiments. Two mixtures, each containing RPP (27 nmoles), RPPG (12 nmoles), and RPPGF (17 nmoles) were carried through the entire derivatization process. One mixture was methylated with ethereal diazomethane, the other with 3 M methanolic HCl; otherwise the samples were treated identically (both were trifluoroacetylated with MBTFA). The fully derivatized samples were analyzed by capillary GC-MS-SIM, monitoring the m/z 358 ion common to the three derivatized peptides.

R. Sensitivity studies.

Overall sensitivity limits for the analysis of arginyl peptides as DMPO-N-TFE-O-TBDMS polyaminoalcohol derivatives by capillary GC-MS-SIM were evaluated using a series of sample mixtures containing from 20 pmoles to 27 nmoles of each of the peptides RPP, RPPG and RPPGF. The mixtures were treated with acetylacetone, 3 M methanolic HCl, MBTFA, diborane and MTBSTFA as described above. The mixtures were analyzed by capillary GC-MS-SIM, monitoring the m/z 358 ion common to the three derivatized peptides.

S. DMPO-N,O-di-TBDMS polyaminoalcohol derivatives of arginyl peptides.

A series of samples (50 - 250 nmoles) of the peptides RF, RPP and RPPG were condensed with acetylacetone and reduced with diborane as described above (no methylation or trifluoroacetylation). To the dry samples were added 50 µl of acetonitrile and 25 µl of MTBSTFA. The samples were capped under nitrogen and heated at 60 °C for fifteen to thirty minutes. Samples of RF were analyzed by FAB-MS after each sequential reaction. All samples were analyzed by capillary GC-MS.

T. Arginyl peptide selective extraction procedure.

The following general procedure was developed for the selective extraction of arginyl peptides from aqueous matrices. Reverse phase cartridge columns were C_{18} Sep-Paks (Waters Associates), which were

charged with 10 ml of 80% acetonitrile/water/0.1% HFBA, and washed and equilibrated with 10 ml of 0.1% HFBA/water. Immobilized phenylboronic acid (PBA) columns were Bond Elut PBA columns (Analytichem International, Harbor City, CA), which were washed with 5 ml of aqueous 1% N-ethylmorpholine buffer (NEM, pH 8.0 (55)) followed by 5 ml of 0.1% HFBA/water, then equilibrated with 5 ml of 1% NEM buffer.

The sample was first desalted by rendering it 1 to 1.5% by volume in HFBA (enough to acidify the sample to pH 1-2, and at least 1%), loading it onto a charged and washed C_{18} Sep-Pak, washing with 10 ml of 0.1% HFBA/water to effect desalting, and eluting with 2-3 ml of 80% acetonitrile/water/0.1% HFBA. The sample was then evaporated to dryness on the Speed-Vac and redissolved with sonication in 1 ml of nitrogen saturated 0.15 M 1,2-cyclohexanedione in aqueous 0.25 M sodium borate buffer (pH 8.7) (55). The reaction mixture was heated at 40 °C for three hours to form the borate-complexed dihydroxycyclohexyl derivatives of arginyl peptides. The sample was then allowed to cool and acidified to pH 1-2 with HFBA to hydrolyze the borate complexes. After 30-45 minutes at room temperature, the sample was desalted with a C_{18} Sep-Pak as described above and evaporated to dryness on the Speed-Vac. The sample was then redissolved with sonication in 2-4 ml of 1% NEM buffer and loaded onto a PBA column. The column was washed with 5 ml of 1% NEM buffer, then eluted directly onto a C_{18} Sep-Pak with 5 ml of 1% HFBA/water. The Sep-Pak was washed with 10 ml of 0.1% HFBA/water

and the sample was eluted with 2-3 ml of 80% acetonitrile/water/0.1% HFBA. The eluate was evaporated to dryness on the Speed-Vac.

Samples of RF and RPPG (250 nmoles) were extracted from phosphate buffered saline (PBS) using these methods and analyzed by FAB-MS. An RF sample so extracted was derivatized to form the DMPO-N,O-di-TBDMS polyaminoalcohol and analyzed by capillary GC-MS-SIM, monitoring m/z 176.

The arginyl peptide selective extraction procedure was further evaluated using a sample containing 175 - 250 nmoles of each of the peptides RPP, RPPG and RPPGF in PBS, and a blank sample of PBS. These two samples were carried through the extraction procedure, and the extracts were redissolved in 0.1% TFA/water and analyzed by reverse phase ion pairing HPLC using the acetonitrile/water/TFA system described above.

U. Reverse phase ion pairing chromatographic isolation of RPP, RPPG and RPPGF.

The following procedure was developed for the isolation of RPP, RPPG and RPPGF from complex biological matrices. The Sep-Pak extraction steps were perfromed using two C_{18} Sep-Paks connected in series through a short segment of silanized glass tubing to double the total binding capacity. The double Sep-Paks were charged with 10 ml of 80% acetonitrile/water/0.1% HFBA and washed with 10 ml of 0.1% HFBA/water.

The sample was rendered 1% by volume in HFBA, and the pH was checked to ensure that the buffer capacity of the sample was overcome by the HFBA. The sample was then loaded onto a charged and washed double C_{18} Sep-Pak, washed with 10 ml of 0.1% HFBA/water, and eluted with 35 ml of water saturated with diethyl ether containing 1% HFBA. After the ether was removed by rotary evaporation, the eluate was again loaded onto a double C_{18} Sep-Pak and washed with 10 ml of 0.1% HFBA/water. The double cartridge column was then eluted with 10 ml of 10% acetonitrile/water/0.1% HFBA, to yield fraction A1 (the RPP and RPPG fraction). The double column was washed with 10 ml of 18% acetonitrile/water/0.1% HFBA, and finally eluted with 10 ml of 25% acetonitrile/water/0.1% HFBA to yield fraction B1 (the RPPGF fraction).

Fractions A1 and B1 were evaporated under a stream of nitrogen to remove acetonitrile, lyophilized, redissolved with sonication in 2 ml of 0.1% TFA/water and filtered through Acrodisc 13 CR (0.45 μm, Gelman Sciences Inc., Ann Arbor, MI) filters. Each fraction was then chromatographed by reverse phase ion pairing HPLC using the acetonitrile/water/TFA system. The region of the chromatogram corresponding to RPP and RPPG was collected from fraction A1 to yield fraction A2, and the region corresponding to RPPGF was collected from fraction B1 to yield fraction B2. On a day-to-day basis, the appropriate regions of the chromatograms were located by co-injecting a small amount of the analytical samples with a known amount of the standard peptides. For sample collections, 200-650 μl

were injected per run. The individual collections from multiple chromatograms were pooled as fractions A2 and B2.

Fractions A2 and B2 were evaporated under a stream of nitrogen to remove acetonitrile, lyophilized, redissolved with sonication in 10 ml of 0.1% HFBA/water, and filtered through Acrodisc 13 CR (0.45 μm, Gelman) filters. The fractions were then rechromatographed by reverse phase ion pairing HPLC using the acetonitrile/water/HFBA system, with 0.5 to 1.8 ml injected for each run. The region of the chromatogram corresponding to RPP and RPPG was collected from fraction A2 to yield fraction A3, and the region corresponding to RPPGF was collected from fraction B2 to yield fraction B3. Again, collections from multiple chromatograms were pooled as fractions A3 and B3. Aliquots of these fractions were evaporated to dryness on the Speed-Vac for FAB-MS analysis.

Each step in this isolation procedure was carefully evaluated using mixtures of standard peptides and human urine samples, both unspiked and spiked with known amounts of the standard peptides RF, RPP, RPPG and RPPGF. For urine samples, no more than 20 ml of urine were loaded onto a double C_{18} Sep-Pak for the initial extraction step. For higher volume urine samples, multiple extractions were performed and the eluates pooled. Subsequently, proportionate aliquots of the eluate were loaded onto double Sep-Paks for the second extraction, so that the same number of extraction operations were performed during both steps.

For the final evaluation of this isolation procedure, a 100 ml sample of urine from a normal male human volunteer (25-35 year old non-smoker on no prescription or over-the-counter medications) was divided into 20 and 80 ml aliquots. To the 20 ml aliquot were added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF. The 80 ml aliquot was used as an unspiked control. Both samples were carried through the isolation procedure, and the final A3 and B3 fractions from both samples were analyzed by FAB-MS under low and high resolution conditions. The B3 fraction from the spiked sample ("RPPGF" fraction) was also derivatized and analyzed by DIP-MS as described below.

V. Derivatization of RPPGF for DIP-MS analysis.

Using standard samples (10-120 μg) of RPPGF, the following procedure was developed for generating the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of RPPGF in high yield and purity for DIP-MS analysis at fairly low levels.

To the dry peptide were added 10 μl of water, 20 μl of ethanol and 10 μl of triethylamine. The sample was capped under argon, vortexed and centrifuged. Acetylacetone (20 μl) was added under a stream of argon, and the sample was vortexed and heated at 100 °C for one hour with occasional vortexing during heating. The sample was allowed to cool and evaporated to dryness on the Speed-Vac. Schiff's bases were hydrolyzed by adding 100 μl of water and 10 μl of glacial acetic acid to the sample and heating at 100 °C for

12 minutes with occasional vortexing. The sample was then allowed to cool and extracted three times with 200 µl of diethyl ether. The aqueous sample was evaporated to dryness on the Speed-Vac, then redissolved with sonication in 250-500 µl of 0.1% TFA/water. The sample was then chromatographed by reverse phase ion pairing HPLC using the acetonitrile/water/TFA system. The region of the chromatogram corresponding to DMPO-RPPGF was collected and evaporated to dryness on the Speed-Vac. The sample was then treated with 3 M methanolic HCl as described in section H above and, after evaporation to dryness, the sample was redissolved with sonication in 1 ml of 20% methanol/water/0.1% TFA. The sample was chromatographed by reverse phase ion pairing HPLC using the acetonitrile/water/TFA system, and the region of the chromatogram corresponding to DMPO-RPPGF-O-methyl ester was collected and evaporated to dryness on the Speed-Vac. Trifluoroacetylation was accomplished by adding 250 µl of chloroform and 50 µl of MBTFA to the sample, vortexing and allowing it to stand at room temperature for one hour. The sample was evaporated to dryness on the Speed-Vac, redissolved with sonication in 250-500 µl of chloroform and chromatographed by normal phase HPLC using the chloroform/methanol gradient system. The region of the chromatogram corresponding to DMPO-RPPGF-N-TFA-O-methyl ester was collected and evaporated to dryness on the Speed-Vac. After redissolution in 50 µl of chloroform, the sample was ready for DIP-MS analysis.

W. DIP-MS analysis of standard RPPGF.

Standard samples (17-210 nmoles) of RPPGF were converted to the DMPO-N-TFA-O-ME derivative as described in section V above. After redissolution in 50 µl of chloroform, 3-12 µl aliquots were analyzed by repetitive scanning DIP-MS. Low and high resolution DIP-MS-SIM analyses were accomplished with 6-18 µl aliquots, monitoring m/z 746 (746.336 for high resolution), the molecular ion. For DIP-CAD-SRM-MS analyses, the mass spectrometer was set to monitor the reaction where an m/z 746 ion decomposes to form an m/z 429 ion in the first field-free region of the mass spectrometer, and 6-12 µl aliquots were used for these analyses.

X. DIP-MS analysis of RPPGF isolated from spiked human urine.

One third of the "RPPGF" HPLC fraction (fraction B3) from the human urine sample which was spiked with standard peptides (35 nmoles of RPPGF) was derivatized to form the DMPO-N-TFA-O-ME derivative using the procedure described above. After redissolution in 50 µl of chloroform, a 6 µl aliquot was analyzed by low resolution (R=1,000-1,200) DIP-MS-SIM, monitoring m/z 746. A second 6 µl aliquot was analyzed by high resolution (R=14-15,000) DIP-MS-SIM, monitoring m/z 746.336. The sample (6 µl) was then analyzed by CAD-DIP-MS-SRM, monitoring the reaction where an m/z 746 ion decomposes to form an m/z 429 ion in the first field-free region of the mass spectrometer. Finally, a 12 µl aliquot was analyzed by repetitive scanning DIP-MS at a resolution of 1,900-2,300.

CHAPTER III

RESULTS

A. Feasibility of O-TBDMS polyaminoalcohol derivatives.

The feasibility of incorporating O-t-butyldimethylsilylation into the overall polyaminoalcohol scheme for peptides was tested with the simple dipeptide AA. Mass spectral analyses of the sequential derivatives leading to the formation of the N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of AA are presented in Figures 3.1 through 3.4.

The FAB mass spectrum of the methyl ester derivative of AA shown in Figure 3.1 demonstrates several features typical of FAB mass spectra (61). The sample has a nominal molecular weight of 174 and is ionized under FAB conditions to yield a pseudomolecular ion ($[M+H]^+$) at m/z 175. A series of glycerol clusters of the pseudomolecular ion appear at m/z 267, 359, 451, 543 and 635. Each ion in this series is incrementally 92 mass units (the molecular weight of glycerol) heavier than its predecessor, and the ions in the series are of successively decreasing intensity. Also frequently observed in FAB mass spectra is a protonated dimer of the analyte, which appears at m/z 349 in Figure 3.1. Most of the other prominent ions in this spectrum (m/z 93, 185, 277, 369, 461, 553 and 645) are background ions due to the glycerol matrix. The combination of an apparent pseudomolecular ion at m/z 175, a series of glycerol

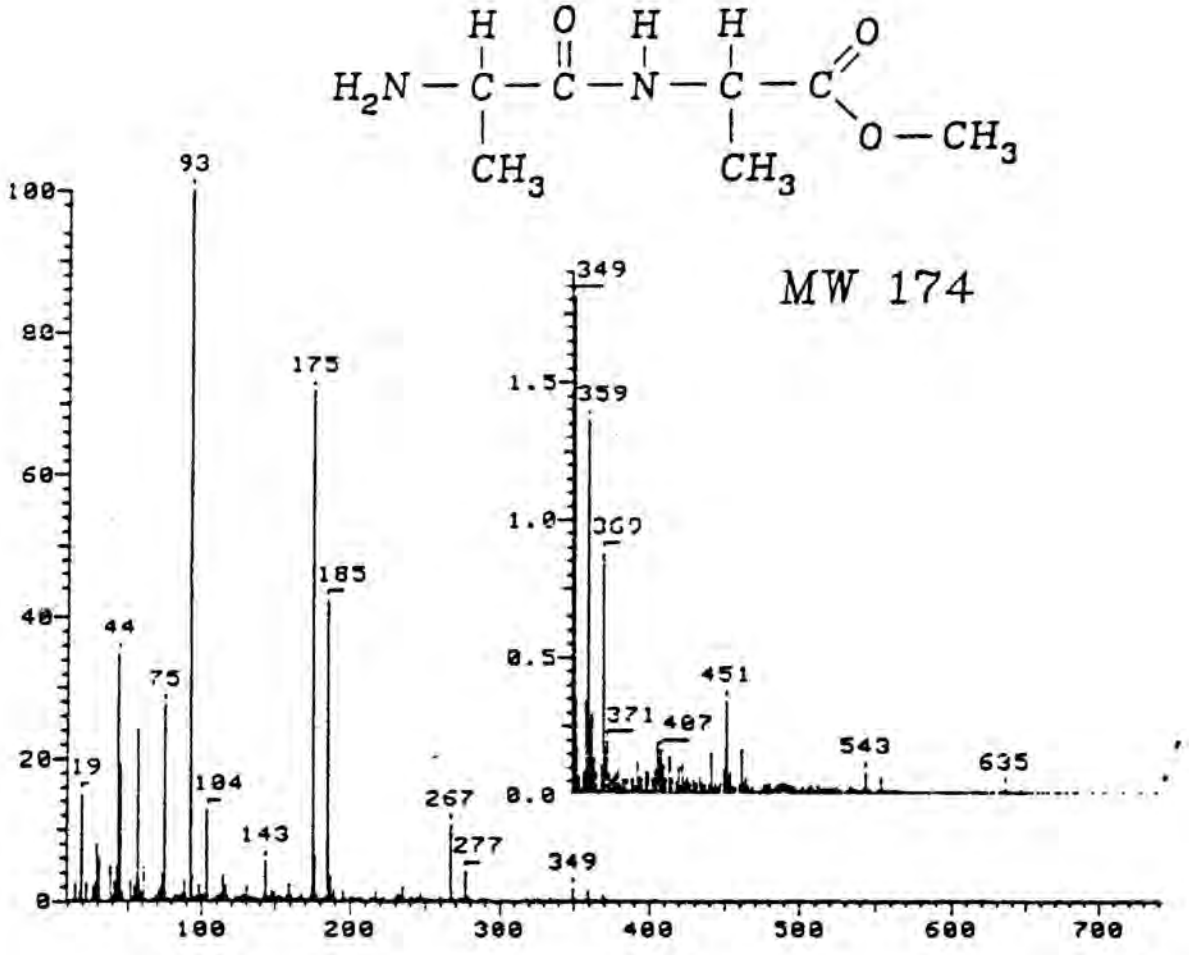


Figure 3.1. Fast atom bombardment mass spectrum of the methyl ester derivative of alanyl-alanine.

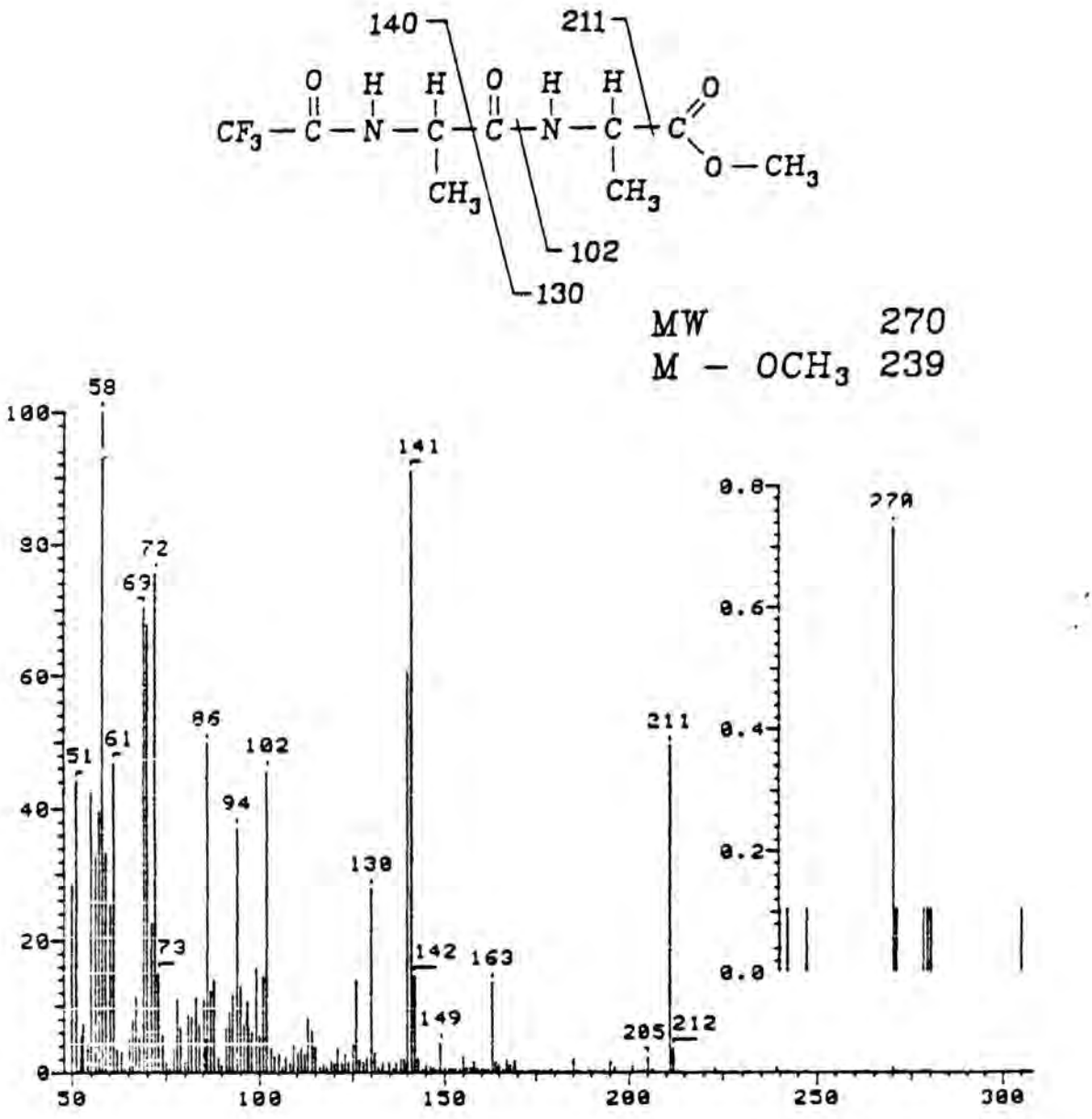
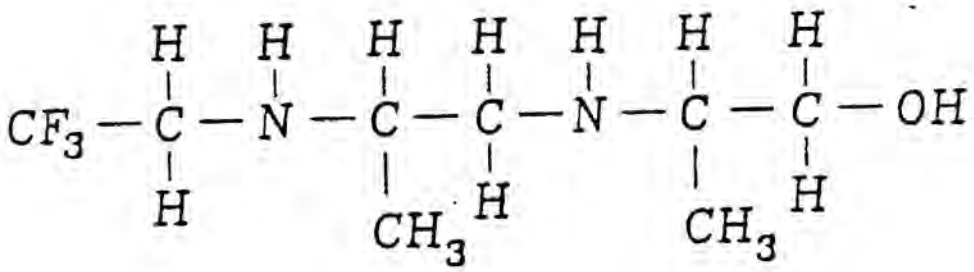


Figure 3.2. Direct insertion probe electron impact mass spectrum of the N-trifluoroacetyl-O-methyl ester derivative of alanyl-alanine.



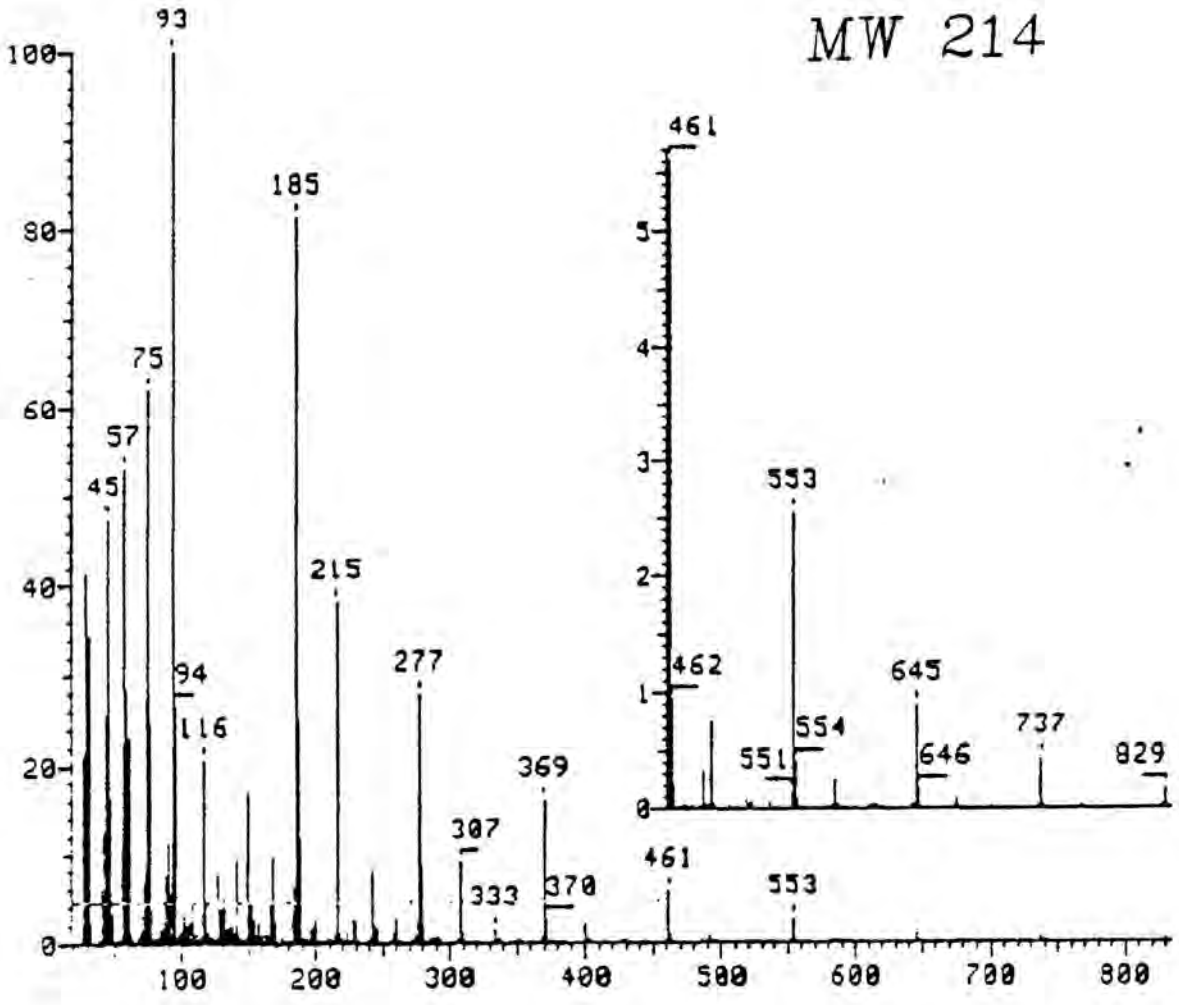


Figure 3.3. Fast atom bombardment mass spectrum of the N-trifluoroethyl polyaminoalcohol derivative of alanyl-alanine.

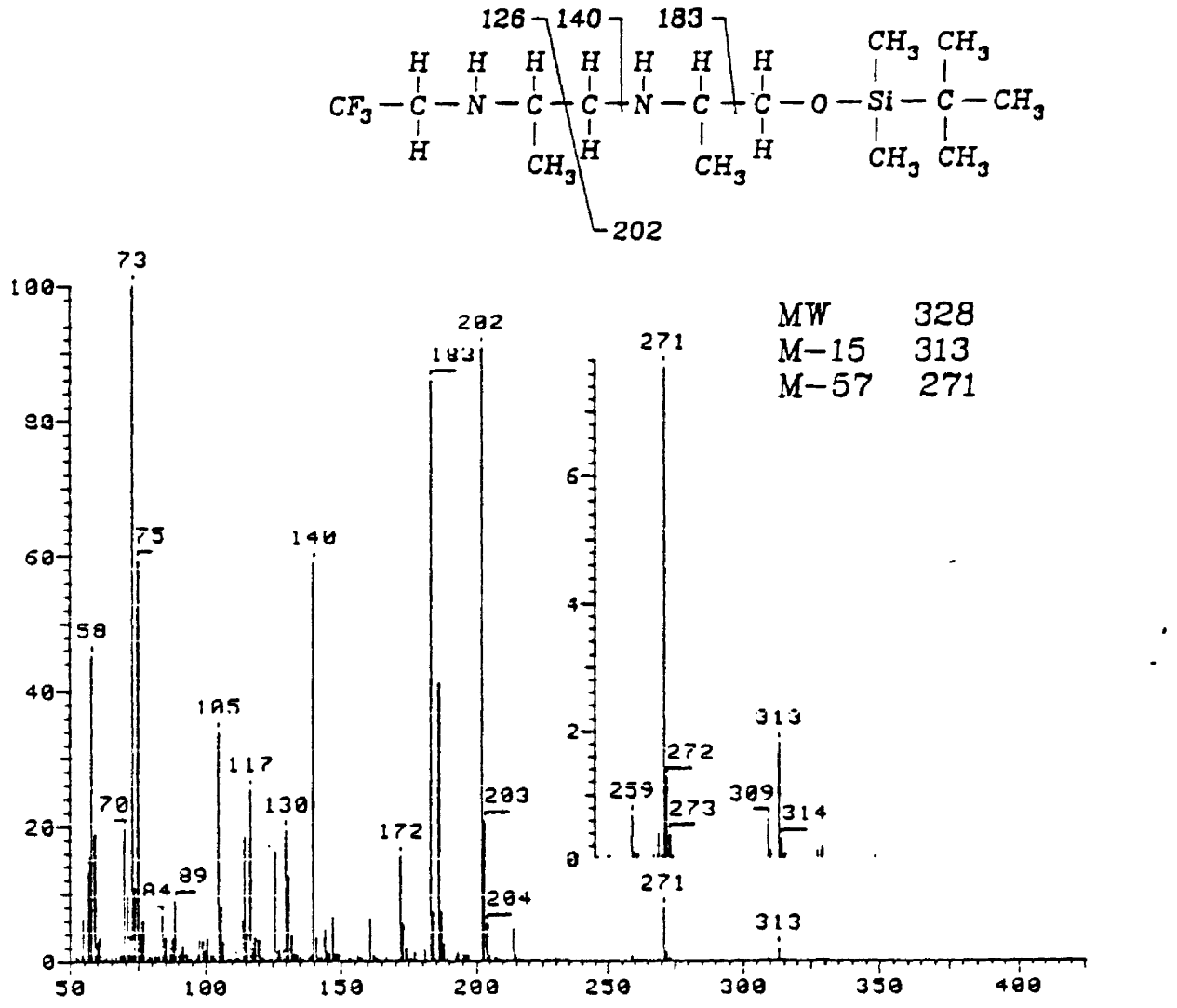


Figure 3.4. Electron impact mass spectrum of the N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of alanyl-alanine from capillary GC-MS analysis.

cluster ions starting at m/z 267 (175 + 92), and an apparent protonated dimer all form a body of evidence indicating that the analyte has a molecular weight of 174 (61). Similar features were observed in many of the FAB spectra obtained during these studies, several of which are presented below. The spectrum in Figure 3.1 provides strong evidence for the successful formation of the methyl ester of AA.

A direct insertion probe electron impact mass spectrum of the N-trifluoroacetyl-O-methyl ester derivative of AA is presented in Figure 3.2. As is typical of electron impact mass spectra, the molecular ion is of low relative intensity. The fragmentation pattern is consistent with the expected structure of the derivative. The sample was highly volatile, requiring little or no probe heating to obtain a mass spectrum.

The FAB mass spectrum of the N-trifluoroethyl polyaminoalcohol (free hydroxy group) derivative of AA (m.w. 214) is presented in Figure 3.3. The sample exhibits a prominent pseudomolecular ion at m/z 215, with the typical series of glycerol cluster ions.

An electron impact mass spectrum of the N-trifluoroethyl-O-TBDMS derivative of AA from repetitive scanning capillary GC-MS analysis is presented in Figure 3.4. The cleavage pattern evident in the mass spectrum is consistent with that of the well known N-trifluoroethyl-O-TMS polyaminoalcohols (17). Additionally, the spectrum exhibits the pair of ions corresponding to $[M-15]^+$ and $[M-57]^+$ as is characteristic of TBDMS derivatives. The nomenclature

used to indicate the ions useful for amino acid sequence determination (A_1, A_2, etc.) in this and similar spectra below is that published by Carr and co-workers (17). The derivative was highly volatile and eluted from a 30 m DB-5 capillary column at 120 °C with excellent GC properties. This sample was also analyzed by FAB-MS, and exhibited an intense pseudomolecular ion at m/z 329 (data not shown). These studies with AA indicated that volatile N-trifluoroethyl-O-TBDMS polyaminoalcohol derivatives can be formed and have potential utility for GC-MS studies of peptides.

B. Compatibility of DMPO derivatives and diborane reduction.

The next question addressed during the course of these studies was whether dimethylpyrimidylornithyl derivatives of arginyl residues are compatible with diborane reduction, i.e whether the dimethylpyrimidyl ring system is resistant to diborane reduction as expected. In order to address this question, dimethylpyrimidylornithine methyl ester was prepared at a semi-synthetic level and treated with diborane using the procedure described by Carr and co-workers (17). The FAB mass spectrum of the methyl ester of dimethylpyrimidylornithine (m.w. 252) is presented in Figure 3.5. The spectrum exhibits a very intense pseudomolecular ion at m/z 253, with a glycerol cluster ion at m/z 345 and a protonated dimer at m/z 505. The spectrum also exhibits an ion at m/z 267 which probably represents an over-methylated product which will be discussed below. Presented in Figure 3.6 is the FAB mass spectrum of the

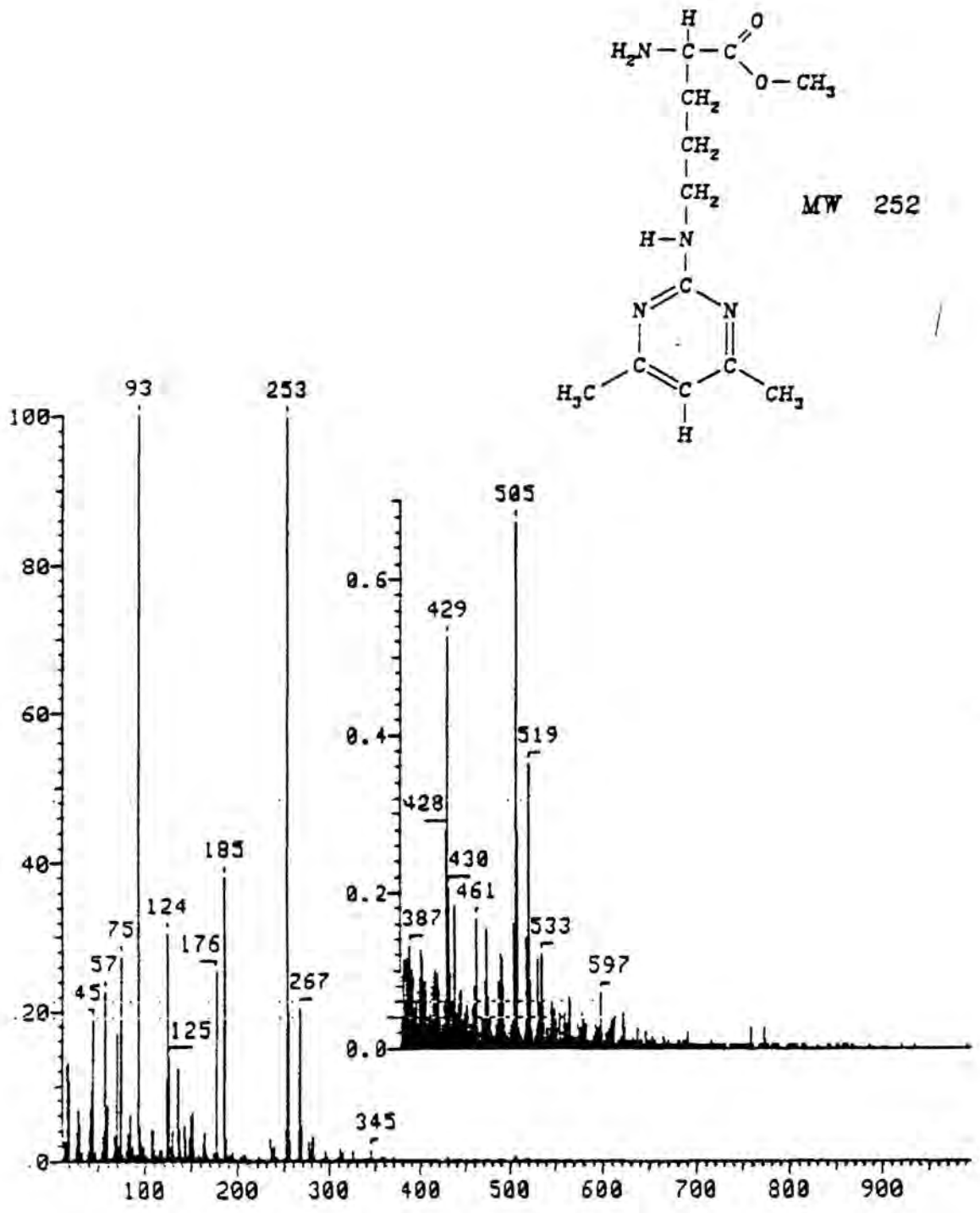


Figure 3.5. Fast atom bombardment mass spectrum of dimethylpyrimidylornithine-O-methyl ester.

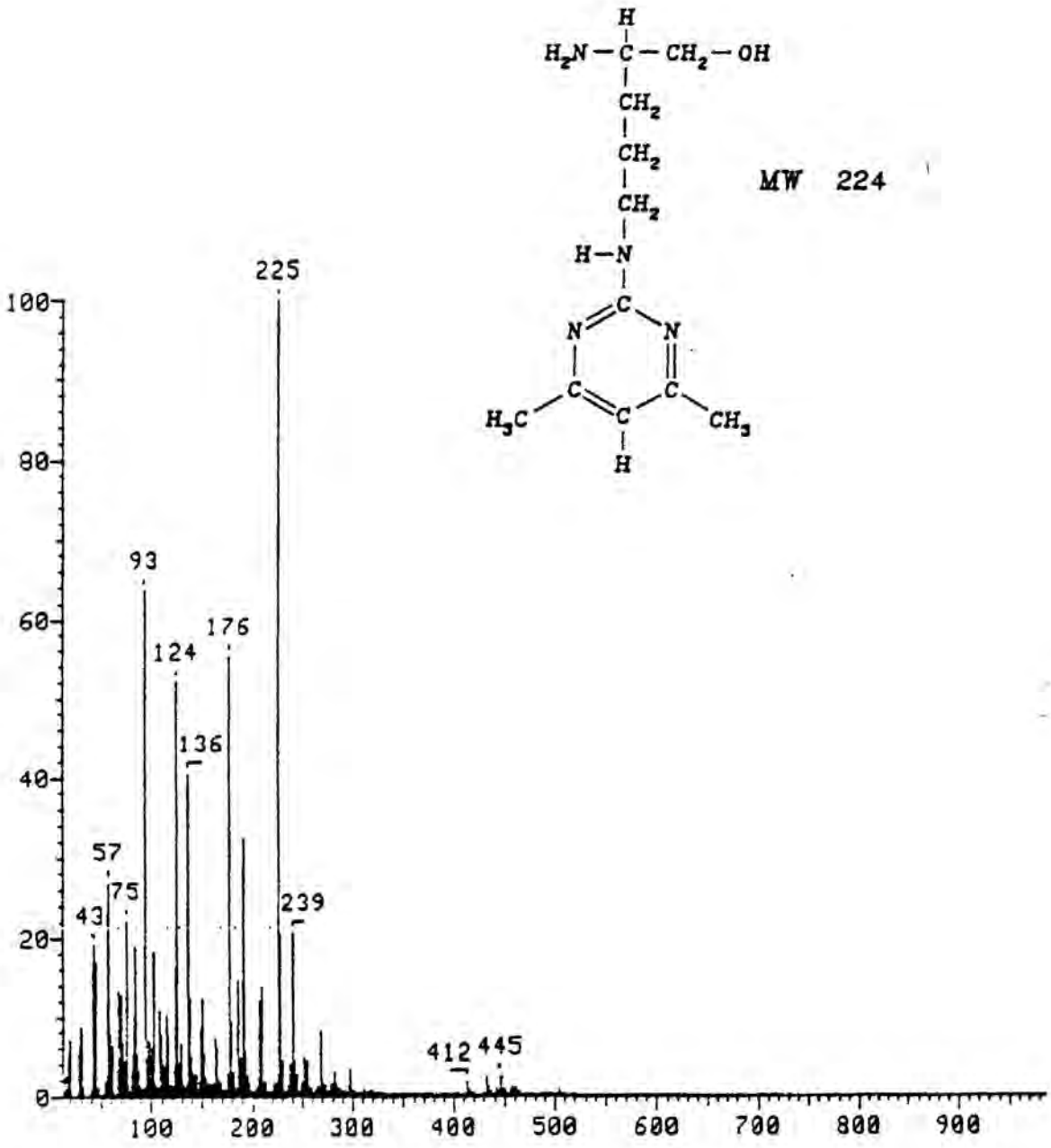


Figure 3.6. Fast atom bombardment mass spectrum of dimethylpyrimidylornithine aminoalcohol.

product isolated from the diborane reduction of dimethylpyrimidylornithine methyl ester. The expected product, with the ester group fully reduced to an alcohol group and with the dimethylpyrimidyl group unaffected, has a molecular weight of 224. The FAB mass spectrum is dominated by an extremely intense pseudomolecular ion at m/z 225, with the ionization of the glycerol matrix suppressed. Further evidence indicating that the dimethylpyrimidyl group was not reduced by diborane is the presence of the fragment ions at m/z 124, 136, 176, and 190, proposed structures for which are presented in Figure 3.7. These ions were observed in all of the electron impact mass spectra of dimethylpyrimidylornithyl derivatives of arginine and arginyl peptides as will be presented below, and in many of the FAB spectra, particularly those in which the pseudomolecular ion was very intense as in Figures 3.5 and 3.6. The presence of these ions in Figure 3.6 indicates the presence of an intact dimethylpyrimidyl group. Hence it was concluded that the dimethylpyrimidyl ring system is inherently resistant to diborane reduction under the conditions employed.

C. Trifluoroacetylation: methyl trifluoroacetate versus MBTFA.

In the most recently published version of the polyaminoalcohol derivatization scheme, methyl trifluoroacetate is used for trifluoroacetylating the amino groups of peptides (17). However, this reagent was found to be inefficient for trifluoroacetylating the amino termini of the arginyl peptides (as the

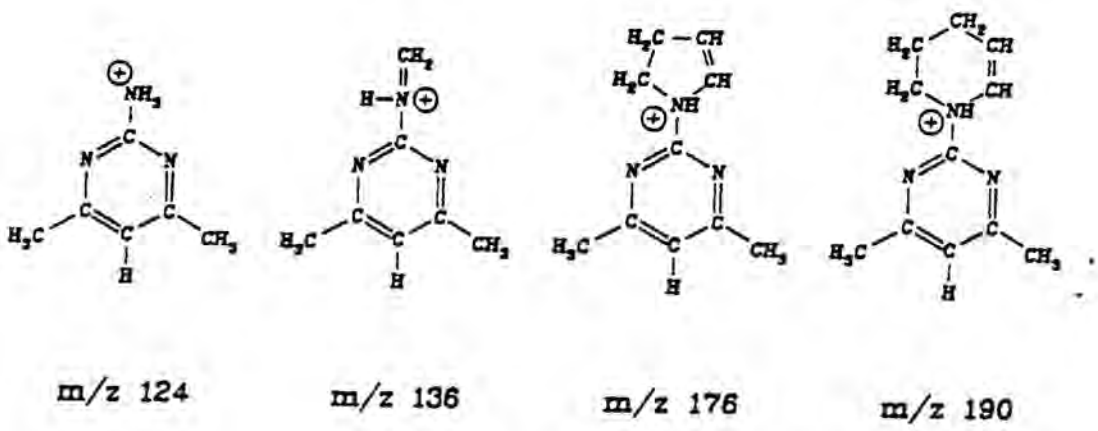


Figure 3.7. Proposed structures for the ions of m/z 124, 136, 176 and 190 derived from DMPO derivatives of arginine and arginyl peptides.

dimethylpyrimidylornithyl derivatives) employed in these studies. Evidence to this effect is presented in Figure 3.8, which is the FAB mass spectrum of the product of the trifluoroacetylation of the dimethylpyrimidylornithyl-O-methyl ester derivative of RF with methyl trifluoroacetate. The free amino precursor has a molecular weight of 399, and that of the trifluoroacetylated product is 495. Pseudomolecular ions corresponding to both the precursor and the product are present in the FAB mass spectrum, indicating the incompleteness of the reaction with methyl trifluoroacetate. Note that the relative intensities of pseudomolecular ions in FAB mass spectra are not necessarily correlated with the relative amounts present in the sample; the fact that two pseudomolecular ions are present an FAB mass spectrum simply indicates that both compounds are present in the sample. Further evidence suggesting the inefficiency of methyl trifluoroacetate for trifluoroacetylating these peptides was provided by a series of samples of RF which were carried through the entire derivatization process to yield the DMPO-N-TFE-O-TBDMS polyaminoalcohol derivative for capillary GC-MS analysis, with the trifluoroacetylation reaction time varied from twelve to thirty-six hours. Only those samples with prolonged trifluoroacetylation times ultimately yielded strong GC-MS signals (data not shown). This reaction inefficiency is not surprising in light of some work published by Steglich and Hinze in 1976 (62). These investigators studied the trifluoroacetylation of various amino acids with methyl trifluoroacetate, and found that the trifluoroacetylation reaction

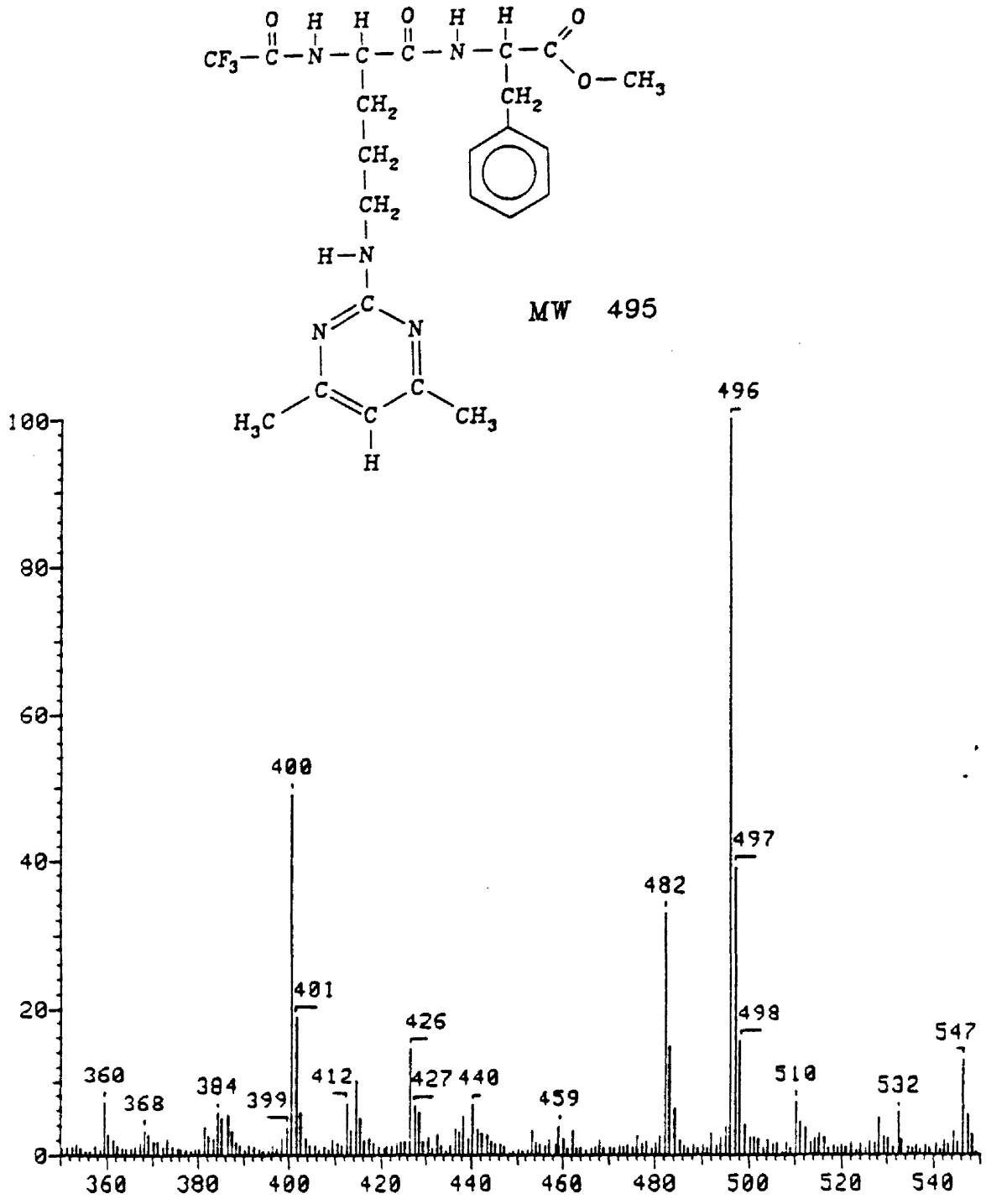


Figure 3.8. Fast atom bombardment mass spectrum of the dimethyl-pyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of arginyl-phenylalanine (RF-DMPO-N-TFA-O-ME), derivatized using methyl trifluoroacetate.

time varied from ten minutes to forty-eight hours, depending on the amino acid. That the reaction time would vary from peptide to peptide with this reagent is only to be expected.

However, in the context of an already time consuming derivatization process, a single step reaction time of 36-48 hours was clearly unacceptable. In order to circumvent this problem, a procedure was developed employing N-methyl-bis(trifluoroacetamide) (MBTFA) as a trifluoroacetylating reagent, with an overall reaction time of 90 minutes. Typical results with this procedure are presented in Figure 3.9, which is the FAB mass spectrum of the product of the trifluoroacetylation of DMPO-RF-O-methyl ester with MBTFA. The pseudomolecular ion corresponding to the trifluoroacetylated product is present and very intense at m/z 496, while that corresponding to the precursor (m/z 400) is not significant compared to the background. This reagent consistently gave good results, and the procedure was further refined during subsequent studies as described below.

D. Documentation of sequential derivatization reactions by FAB-MS.

A series of FAB mass spectra documenting the sequential derivatization reactions leading to the formation of the DMPO-N-TFE-O-TBDMS polyaminoalcohol derivative of RF are presented in Figures 3.10 through 3.14. Each spectrum in this series exhibits an intense pseudomolecular ion corresponding to the anticipated derivatization product. All of these spectra exhibit at least one

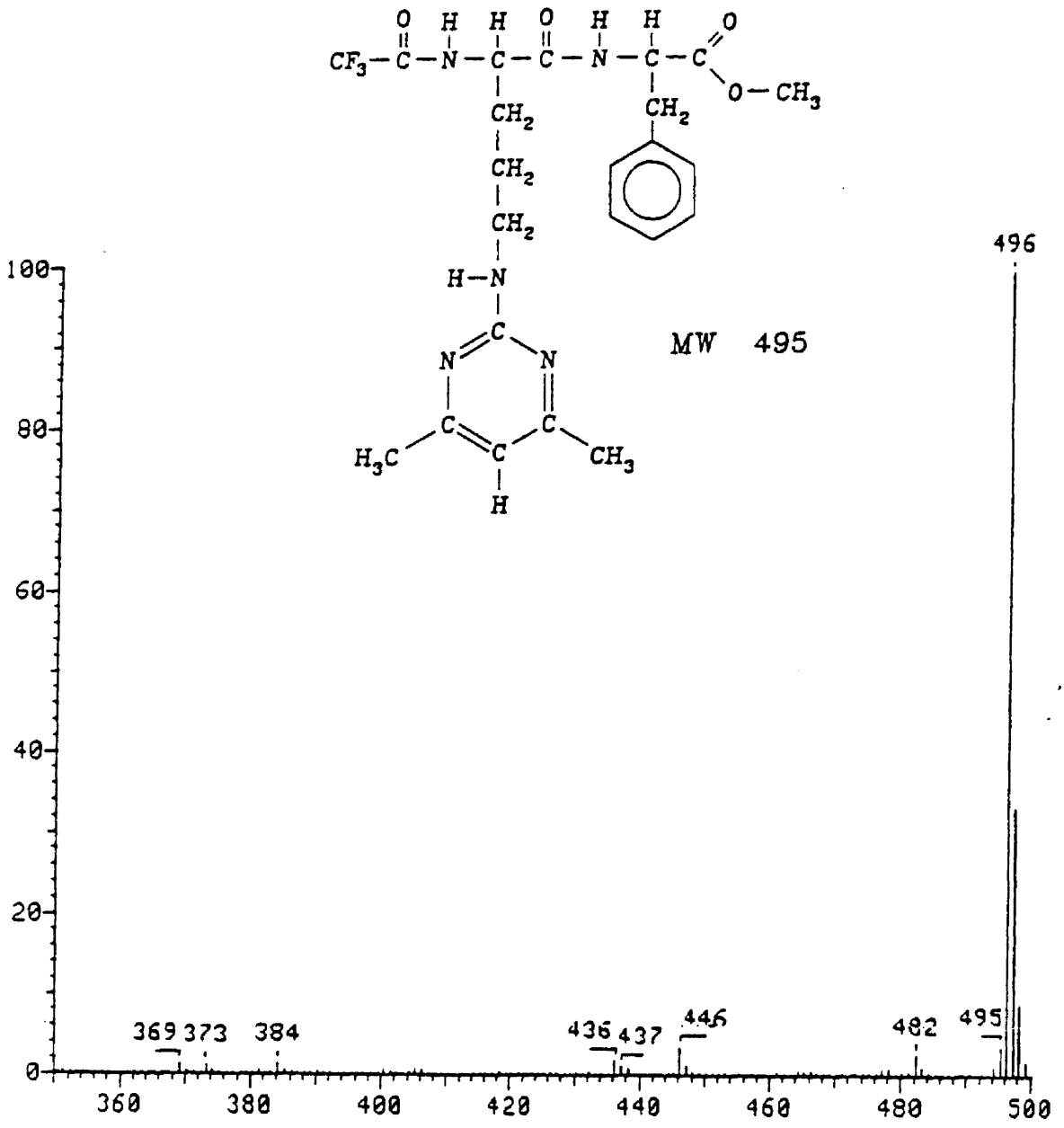


Figure 3.9. Fast atom bombardment mass spectrum of the dimethyl-pyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of arginyl-phenylalanine (RF-DMPO-N-TFA-O-ME), derivatized using MBTFA.

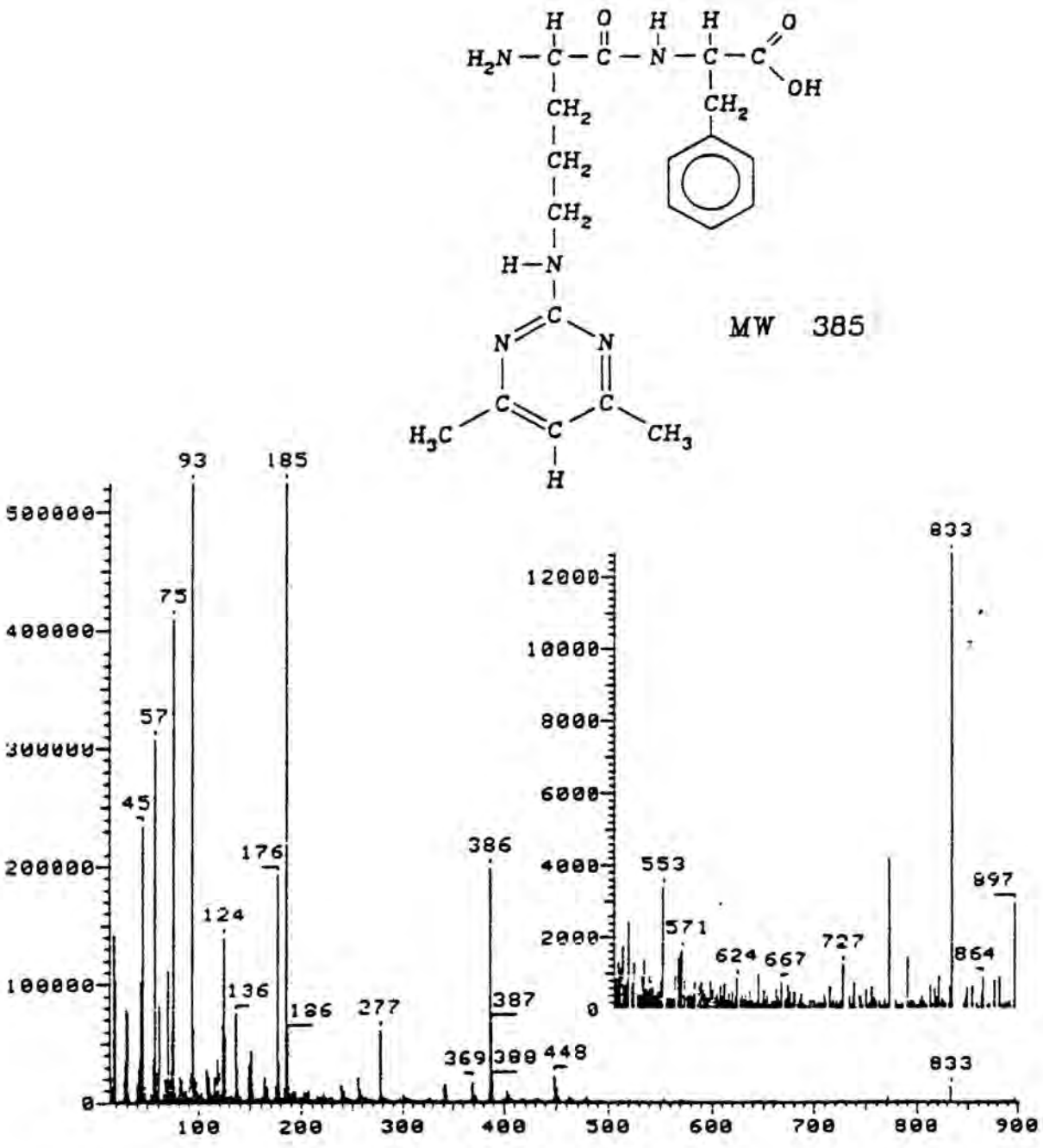


Figure 3.10. Fast atom bombardment mass spectrum of the dimethyl-pyrimidylornithyl derivative of arginyl-phenylalanine (RF-DMPO).

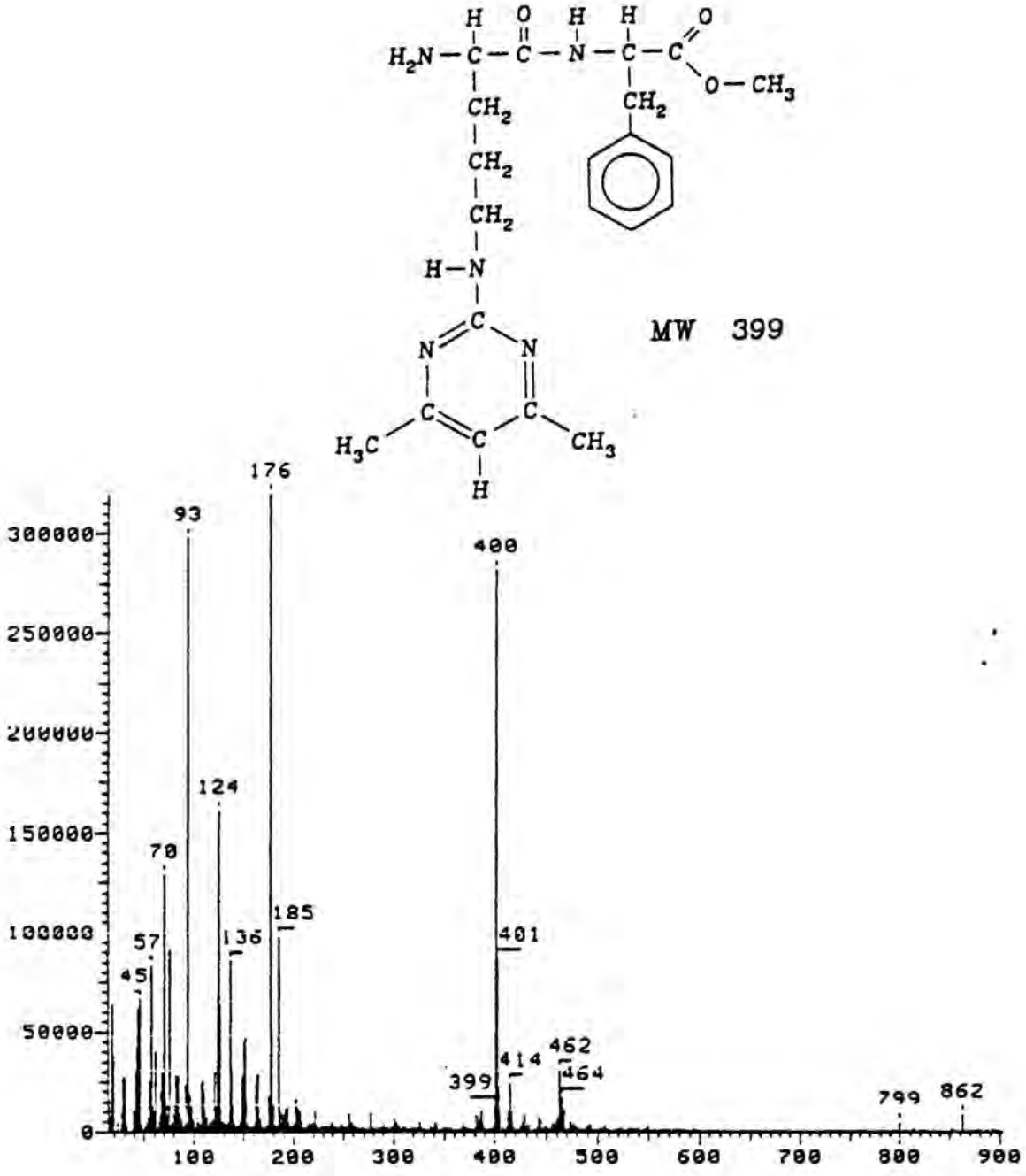


Figure 3.11. Fast atom bombardment mass spectrum of the dimethyl-pyrimidylornithyl-O-methyl ester derivative of arginyl-phenylalanine (RF-DMPO-O-ME).

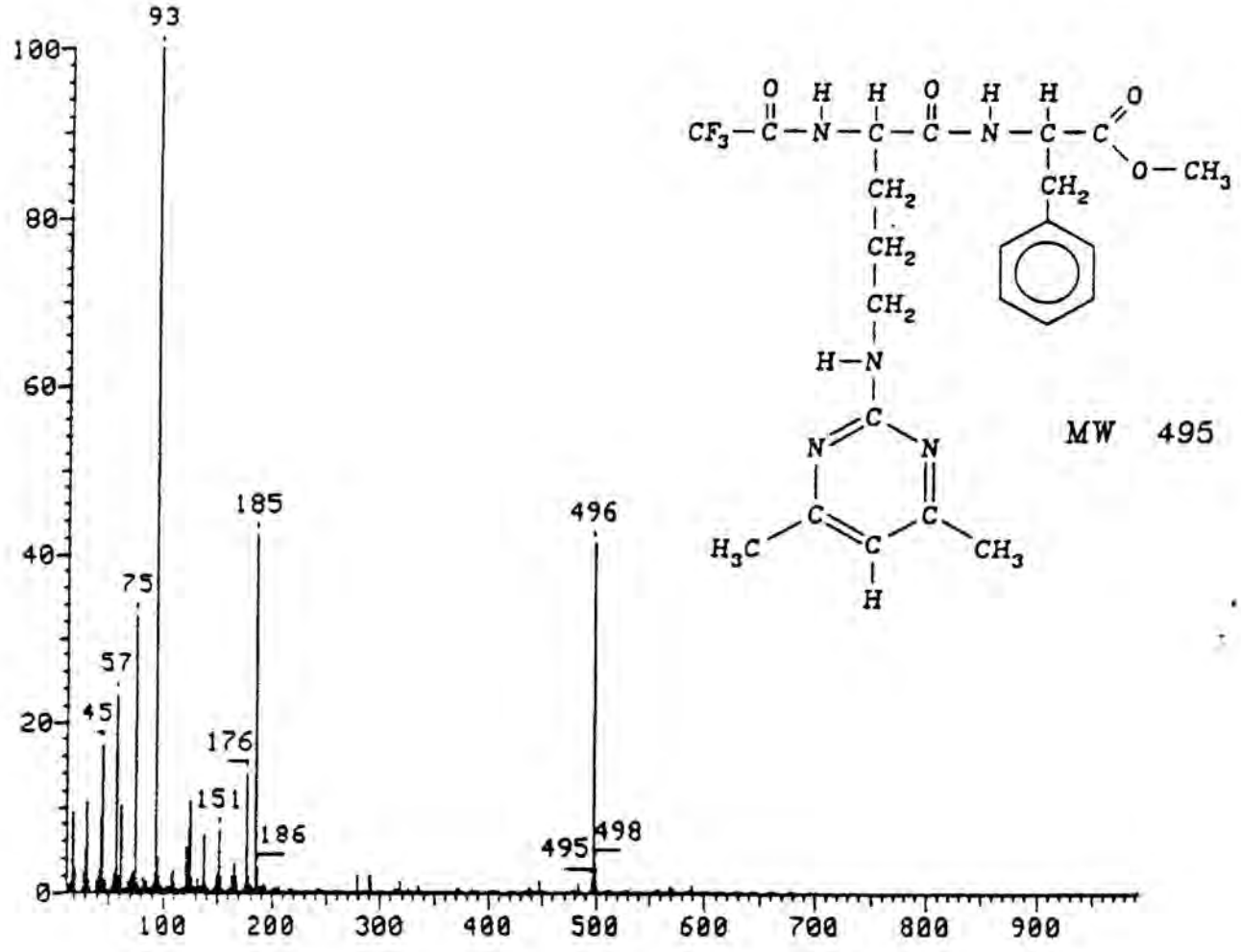


Figure 3.12. Fast atom bombardment mass spectrum of the dimethyl-pyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of arginyl-phenylalanine (RF-DMPO-N-TFA-O-ME).

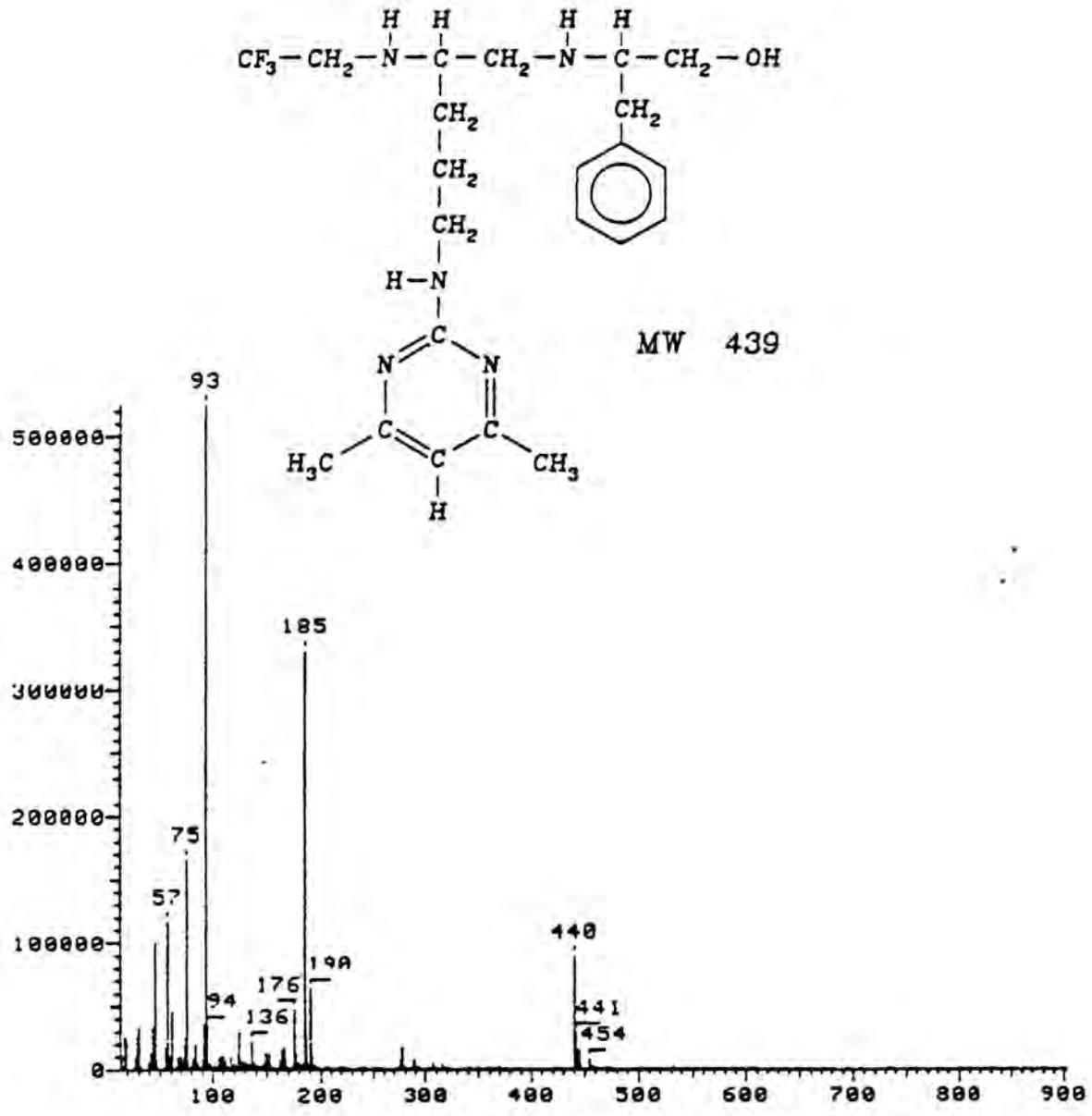


Figure 3.13. Fast atom bombardment mass spectrum of the dimethyl-pyrimidylornithyl-N-trifluoroethyl polyaminoalcohol derivative of arginyl-phenylalanine (RF-DMPO-N-TFE polyaminoalcohol).

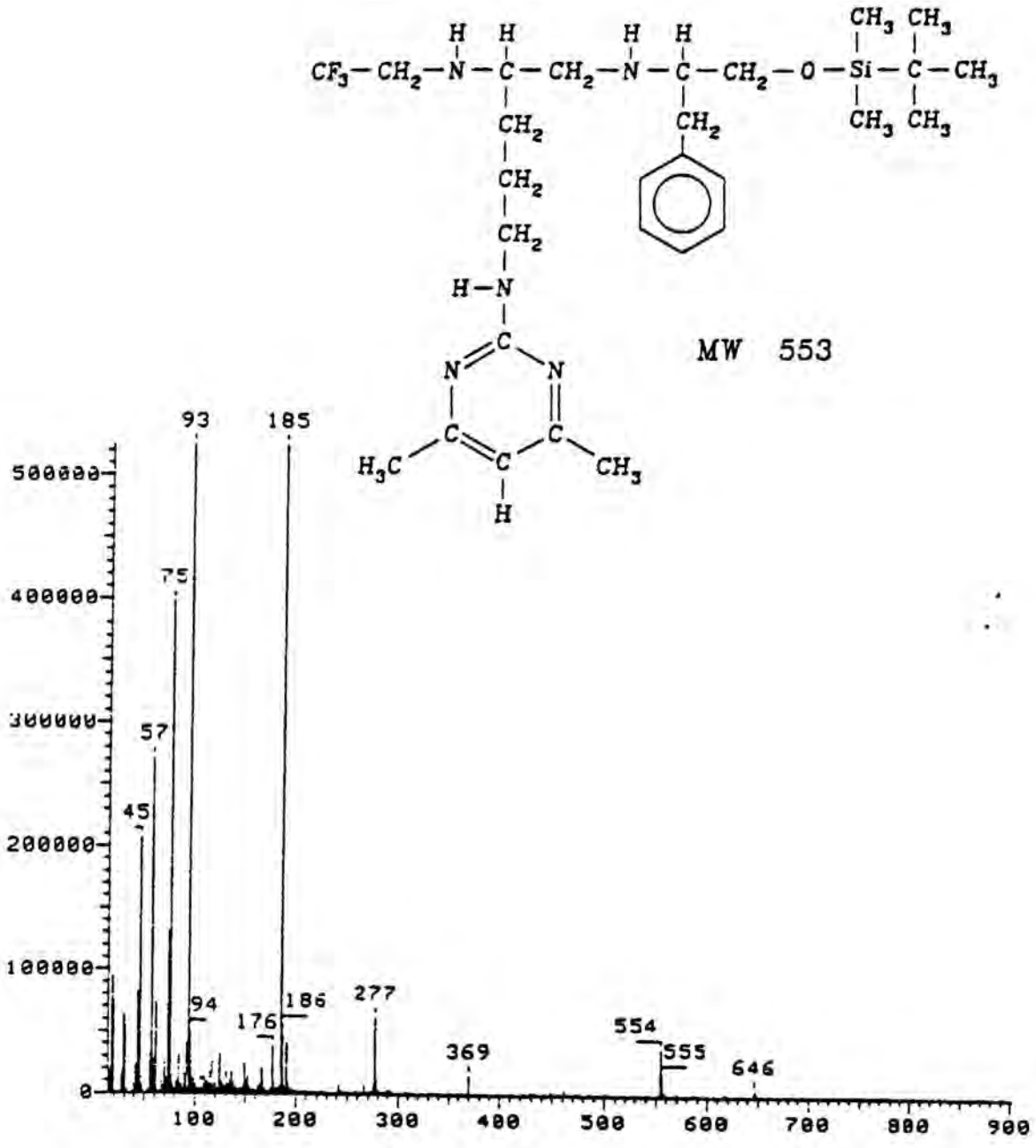


Figure 3.14. Fast atom bombardment mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of arginyl-phenylalanine (RF-DMPO-N-TFE-O-TBDMS polyaminoalcohol).

glycerol cluster ion, and two spectra (Figures 3.10 and 3.11) show a protonated dimer. Fragment ions indicative of the dimethylpyrimidylornithine side chain (m/z 124, 136 and 176) are intense in all except Figure 3.12. The m/z 190 fragment ion appears after the reduction step, lending credence to the structure proposed for this ion in Figure 3.7. If this ion were present in the FAB mass spectra of the non-reduced derivatives, this proposed structure would clearly be invalid.

It should be noted that the spectra in Figures 3.10 through 3.14 were obtained after each successive derivatization step was applied to a single 150 nmole sample of RF. This was made possible through the use of the technique where a small amount of the solid or oily sample was removed from the sample vial and mixed with glycerol directly on the FAB probe tip, leaving the majority of the sample for further chemical treatment or analysis. Serial spectra were successfully obtained using these techniques starting with as little as 15-25 nmoles of peptide sample and carrying through the entire derivatization sequence. By analyzing the sequential steps by FAB-MS in this fashion, if a particular reaction failed or was clearly inefficient, steps could immediately be taken to correct the problem (e.g. repurification of reagents, alteration of reaction conditions, etc.); otherwise, the existence of a problem would only have been learned after failure of a GC-MS analysis, with no indication as to which step was problematic.

An additional example of these analyses is presented in Figure 3.15, which shows the FAB mass spectrum of the DMPO-N-TFE polyaminoalcohol derivative of RPPGF (prior to silylation). The spectrum exhibits a prominent pseudomolecular ion corresponding to the expected derivative at m/z 649, as well as numerous higher mass ions which probably represent side reactions from the diborane reduction. Ions indicative of the dimethylpyrimidylornithine side chain are present at m/z 124, 136, 176 and 190. Additionally, two fragments from the highly favored cleavage of the polyamine backbone appear at m/z 358 and 372. Due to space considerations, spectra documenting the sequential derivatives of all of the peptides studied will not be presented. However, arginine and each of the peptides RPP, RPPG and RPPGF were successfully analyzed by FAB-MS after each step to document the sequential derivatives.

E. Capillary GC-MS of arginyl peptides as DMPO-N-TFE-O-TBDMS ployaminoalcohol derivatives.

Electron impact mass spectra from repetitive scanning capillary GC-MS analyses of arginine, RF, RPP, RPPG and RPPGF as the DMPO-N-TFE-O-TBDMS polyaminoalcohol derivatives are presented in Figures 3.16 through 3.20. All of these derivatives exhibited excellent gas chromatographic properties, with typical baseline to baseline peak widths of 6-8 seconds on DB-1 capillary columns. Temperatures of elution were 235 °C for arginine (30 m column),

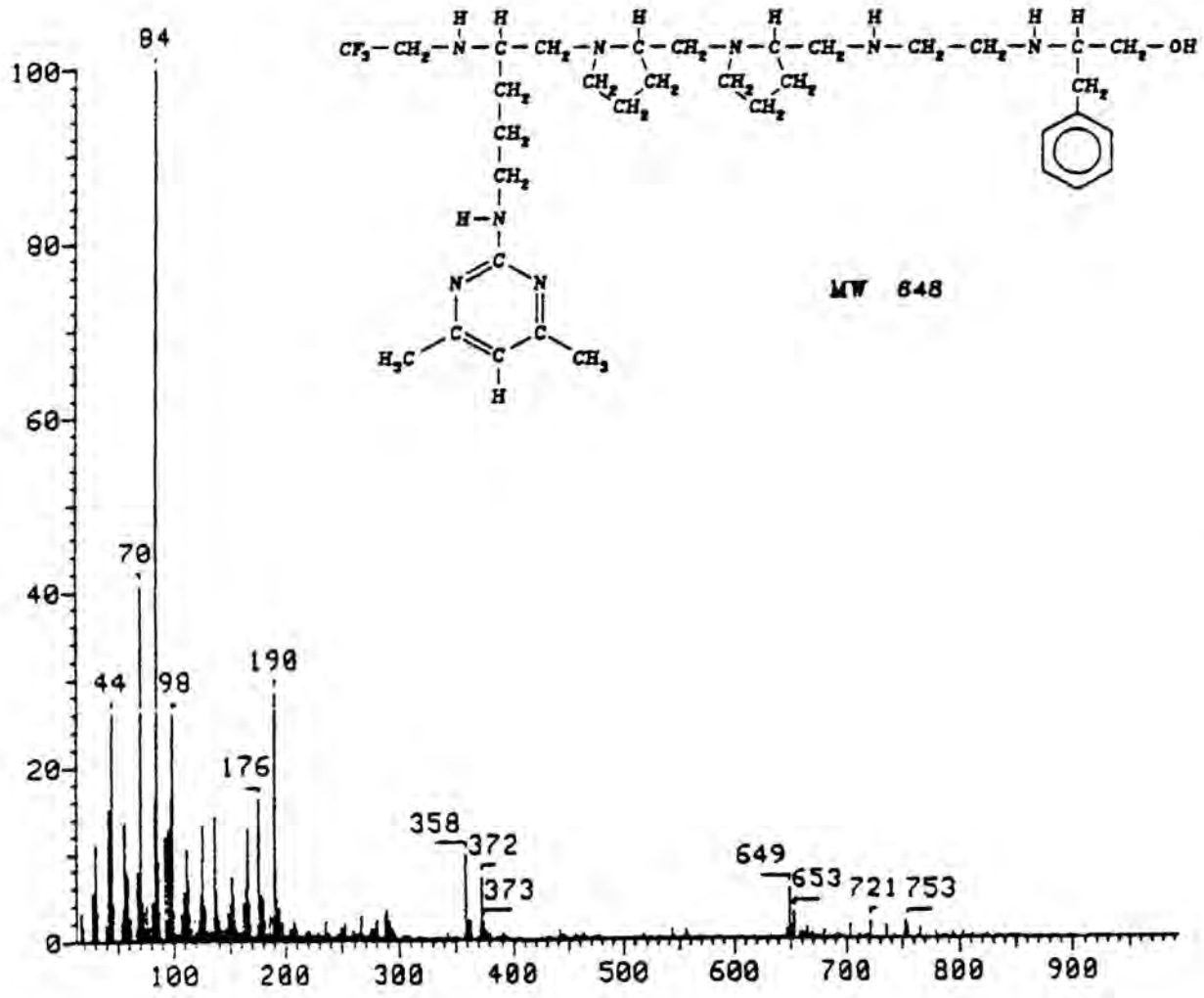


Figure 3.15. Fast atom bombardment mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of arginyl-prolyl-prolyl-glycyl-phenylalanine (RPPGF-DMPO-N-TFE-O-TBDMS polyaminoalcohol).

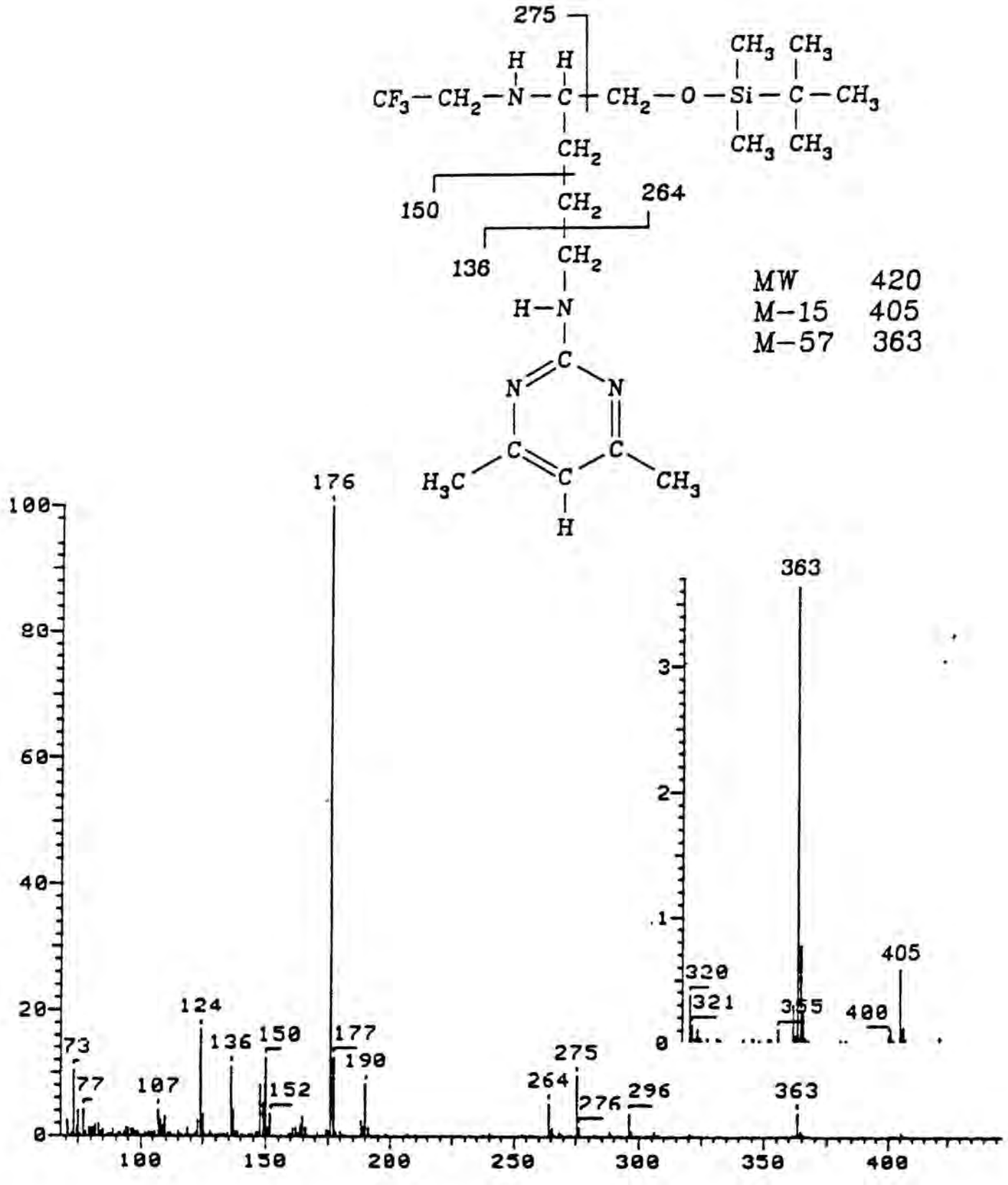
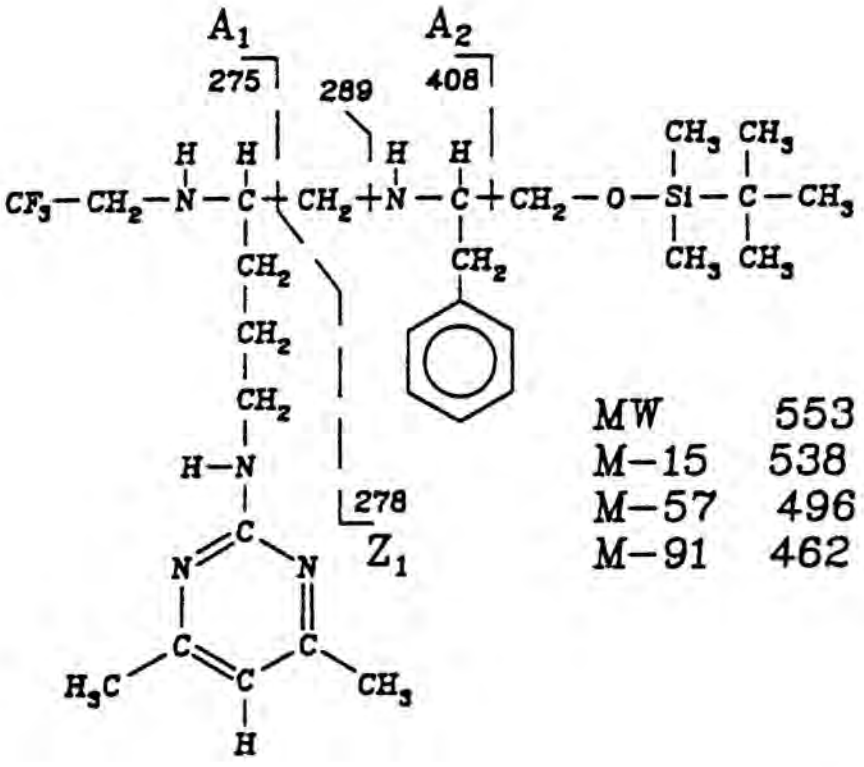


Figure 3.16. Electron impact mass spectrum of N-trifluoroethyl-O-TBDMS-dimethylpyrimidylornithine aminoalcohol from capillary GC-MS analysis.



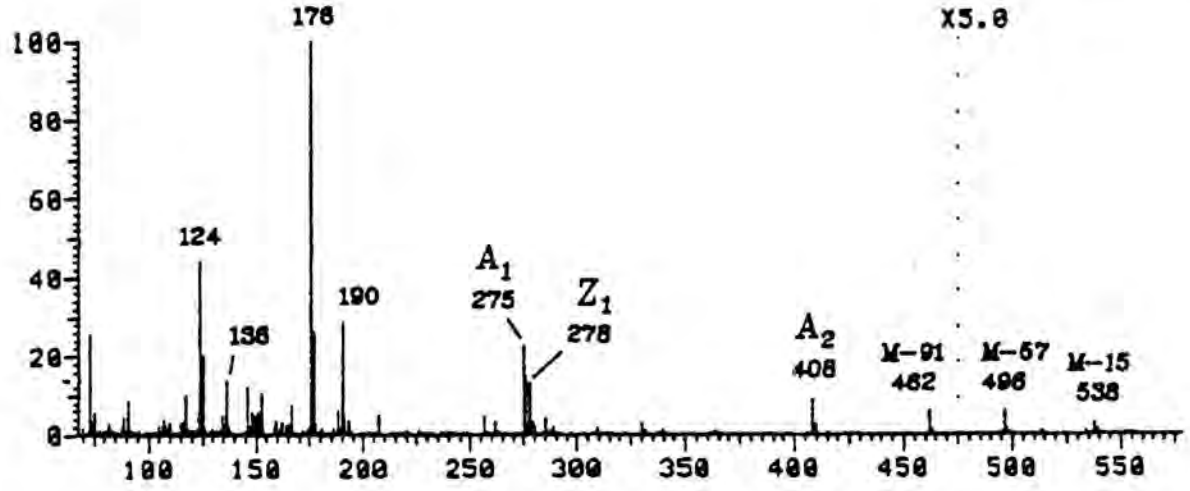
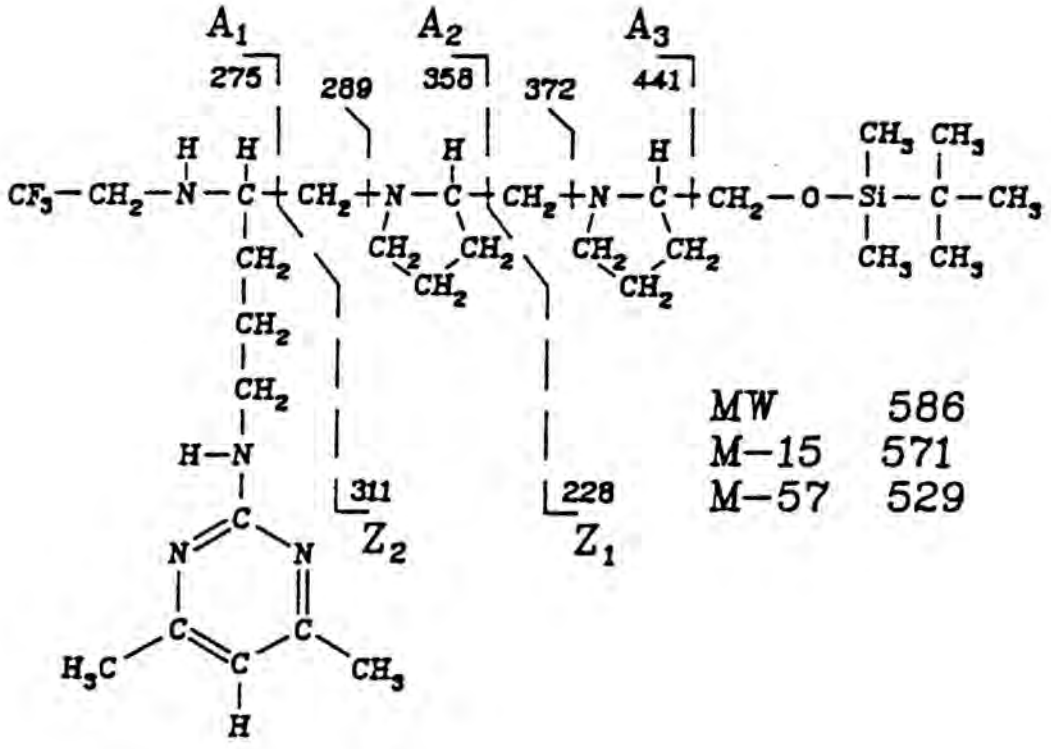


Figure 3.17. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of arginyl-phenylalanine (RF-DMPO-N-TFE-O-TBDMS polyaminoalcohol) from capillary GC-MS analysis.



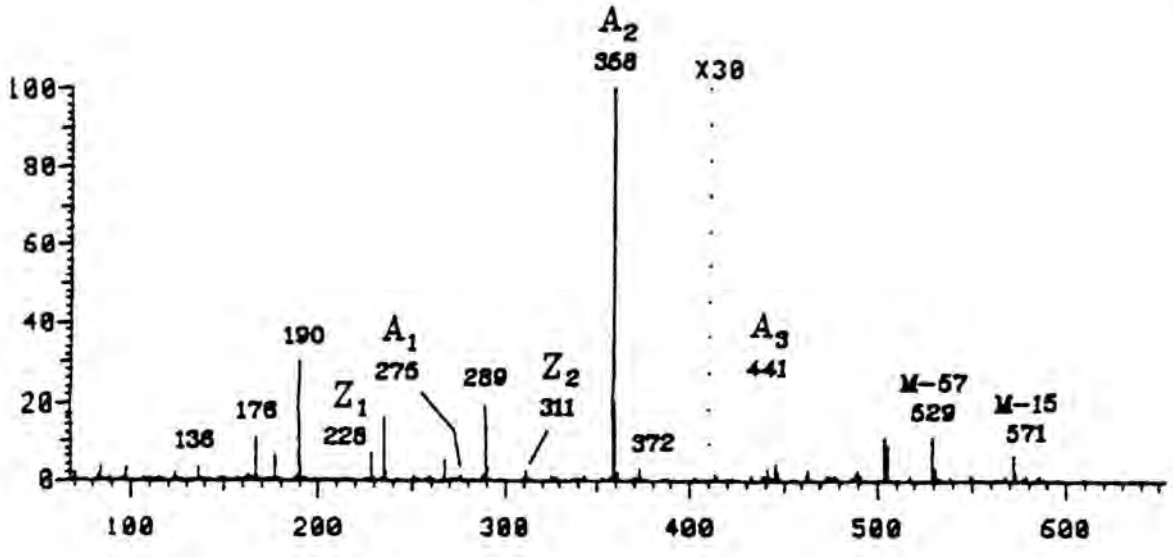


Figure 3.18. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of arginyl-prolyl-proline (RPP-DMPO-N-TFE-O-TBDMS polyaminoalcohol) from capillary GC-MS analysis.

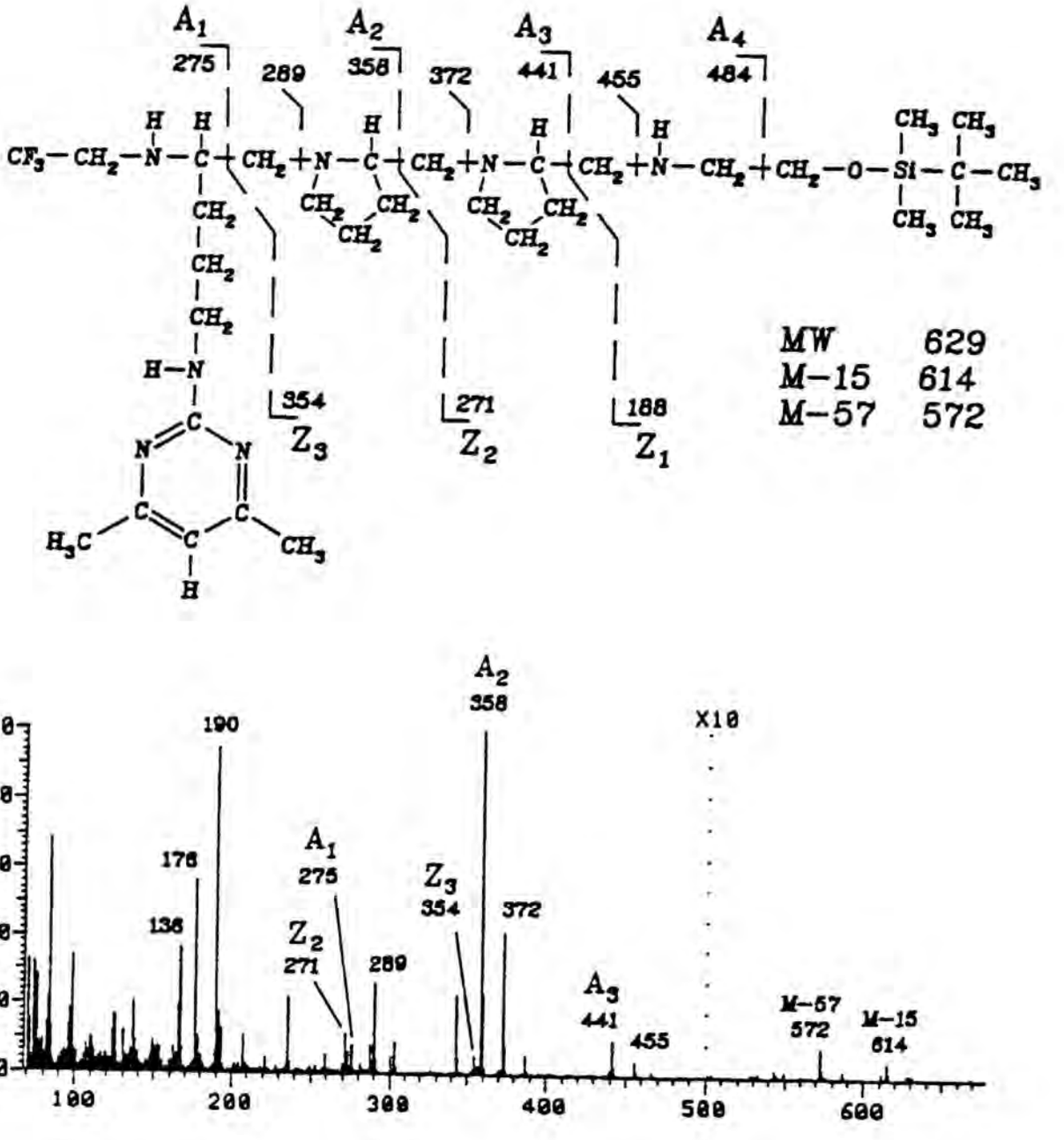
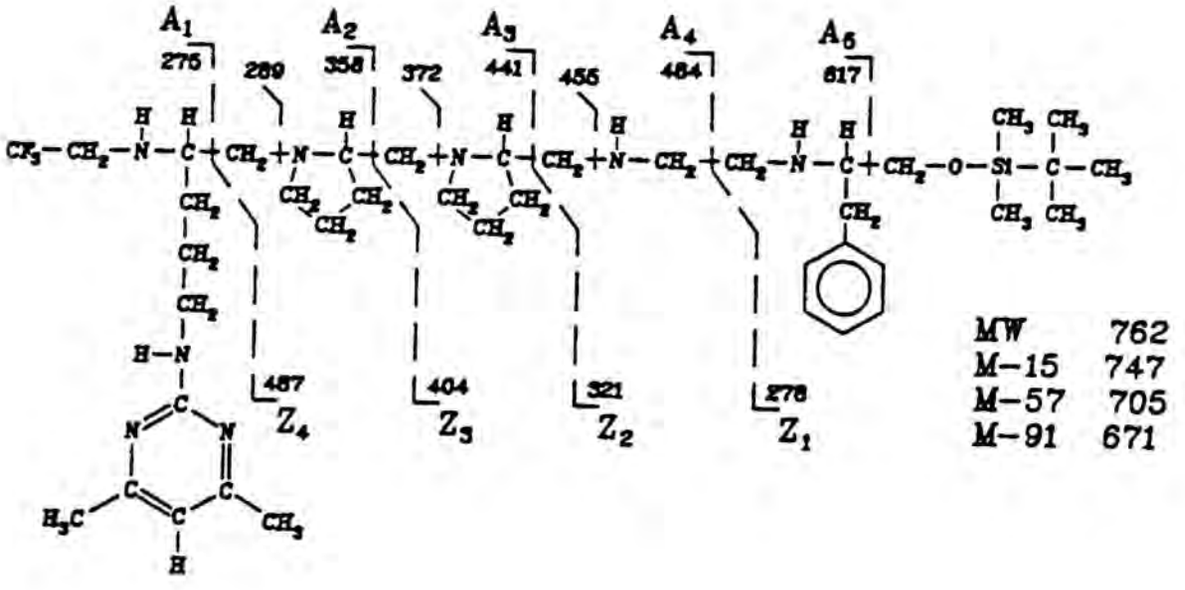


Figure 3.19. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of arginyl-prolyl-prolyl-glycine (RPPG-DMPO-N-TFE-O-TBDMS polyaminoalcohol) from capillary GC-MS analysis.



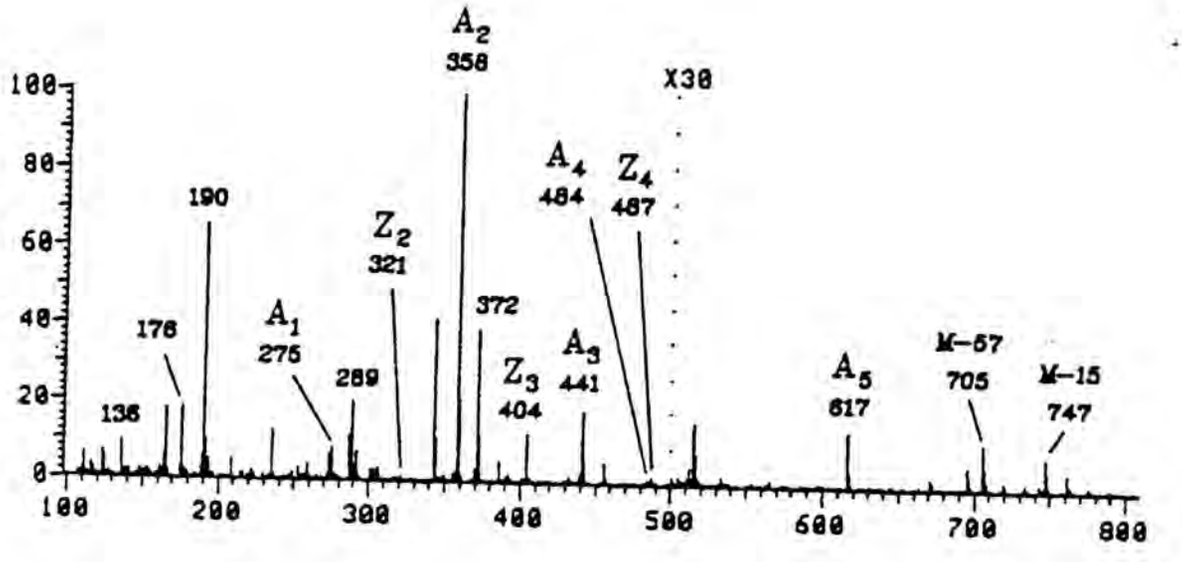


Figure 3.20. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivative of arginyl-prolyl-prolyl-glycyl-phenylalanine (RPPGF-DMPO-N-TFE-O-TBDMS polyaminoalcohol) from capillary GC-MS analysis.

295 °C for RF (30 m column), 300 °C for RPP (30 m column), 310 °C for RPPG (30 m column), and 320 °C for RPPGF (5 m column).

The electron impact spectrum of fully derivatized arginine presented in Figure 3.16 shows the series of fragment ions due to the dimethylpyrimidylornithine side chain at m/z 124, 136, 176 and 190. The related fragment ions at m/z 150 and 164, which are evident in Figure 3.16, were not consistently observed in the electron impact mass spectra of the arginyl peptides. The ion at m/z 275 represents cleavage on the carboxy side of the original α carbon, the typical cleavage pattern for derivatives of this type. Also noteworthy are the ions corresponding to $[M-15]^+$ and $[M-57]^+$ due to the loss of a methyl group and a t-butyl group from the TBDMS moiety, respectively.

The cleavage patterns evident in the electron impact mass spectra of the fully derivatized arginyl peptides in Figures 3.17 through 3.20 are directly analogous to those of the well known N-TFE-O-TMS polyaminoalcohols (17), with primary cleavages occurring on the carboxy side of the original α carbons of the polypeptide chains. Both A and Z series ions (17) are consistently observed in all of these spectra. Also consistently observed are the fragment ions at m/z 124, 136, 176, and 190, which collectively may be considered to be diagnostic for the dimethylpyrimidylornithine side chain. All of these spectra exhibit the pair of ions corresponding to $[M-15]^+$ and $[M-57]^+$ as is characteristic of TBDMS derivatives. These important ions form an easily recognizable pair which serves

as a punctuation mark for the mass spectrum, clearly denoting the molecular weight of the fully derivatized peptide. The molecular ions of all of these derivatives were detectable under selected ion monitoring conditions but were quite weak. The derivatized bradykinin related peptides RPP, RPPG and RPPGF all have m/z 358 (the A_2 ion) as the base peak in their mass spectra. This was fortuitous since it meant that all three peptides could be detected with good sensitivity and molecular specificity simply by monitoring m/z 358 under capillary GC-MS-SIM conditions.

A representative chromatogram resulting from the derivatization of a mixture of RPP, RPPG and RPPGF (17-27 nmoles of each) and analysis by capillary GC-MS-SIM is presented in Figure 3.21. The three compounds were well resolved from each other and from interfering peaks on a 5 m DB-1 capillary column, with excellent chromatographic properties. The peak corresponding to RPP is offscale (>80 V), and those corresponding to RPPG and RPPGF are of successively decreasing intensity. This can be partially accounted for by the fact that the m/z 358 ion represents an incrementally smaller percentage of the total ionization of the sample as the length of the peptide increases. However, of greater importance is the probability that the total yield of the final derivative decreases as the length of the peptide chain increases, resulting in reduced overall sensitivity for the longer peptides. As the number of peptide bonds increases, the likelihood of incomplete reduction

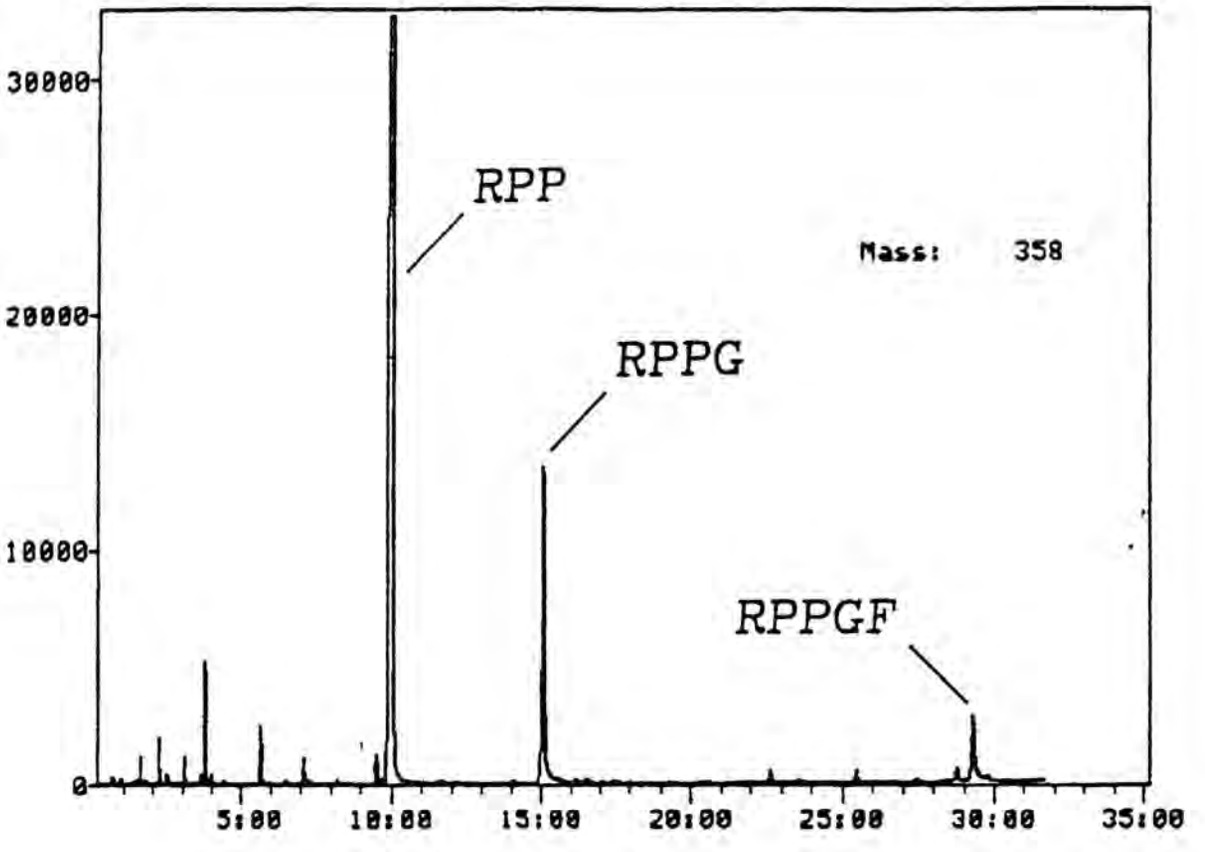


Figure 3.21. Capillary GC-MS selected ion monitoring chromatogram (monitoring m/z 358) of a mixture of RPP, RPPG and RPPGF. The amount injected represented approximately 400 ng of each peptide.

and/or incomplete decomposition of the highly stable boroamine complexes formed during reduction increases.

F. Methylation: ethereal diazomethane versus 3 M methanolic HCl.

When diazomethane was used as the methylating reagent in the derivatization process, overmethylated products were observed by FAB-MS (see Figures 3.5, 3.6, 3.8, and 3.11 through 3.14) and by capillary GC-MS. For instance, under GC-MS-SIM conditions (monitoring m/z 358), RPPGF eluted as three well resolved peaks. In light of the high reactivity of diazomethane (63), this probably represents attack on the dimethylpyrimidyl ring system, although methylation of the amino terminus or amide nitrogen have not been ruled out (overmethylated products were also observed with AA). In contrast, overmethylated products were not observed when 3 M methanolic HCl was used as the methylating reagent, as in Figure 3.21, where all three peptides eluted as single peaks. In order to evaluate the effect of the choice of methylating reagent on overall sensitivity, identical mixtures of RPP, RPPG and RPPGF (17-27 nmoles of each) were derivatized for GC-MS-SIM analysis, with one mixture methylated with diazomethane and the other with 3 M methanolic HCl. The results of GC-MS-SIM analysis of the mixtures are presented in Table 3-I. Methanolic HCL is clearly the superior reagent for these molecules, both from the standpoint of fewer side reactions, and from that of a greater overall yield of the sought derivative, with

TABLE 3-I

METHYLATION: METHANOLIC HCL VERSUS ETHEREAL DIAZOMETHANE

Peak signal intensities from capillary GC-MS-SIM analysis of mixtures of 17-27 nmoles each of RPP, RPPG and RPPGF

Mixture component	Methanolic HCl peak signal intensity (V)	Diazomethane peak signal intensity (V)
RPP	80	40
RPPG	8.8	2.2
RPPGF	1.9	not detected

attendant better overall sensitivity. Similar results were obtained with repeated experiments.

G. Sensitivity studies with DMPO-N-TFE-O-TBDMS polyaminoalcohol derivatives.

In order to evaluate the lower limits of detectability of these peptides by these methods, a series of mixtures containing from 20 pmoles to 27 nmoles of each of the peptides RPP, RPPG and RPPGF were derivatized and analyzed by capillary GC-MS-SIM, monitoring m/z 358. Some of the results of these studies are presented in Figures 3.22 and 3.23. Figure 3.22 shows the chromatogram resulting from the derivatization and analysis of 20 pmoles of each of the three peptides. The tripeptide RPP was detected with an 80:1 signal to noise ratio, while RPPG and RPPGF were not detected. The chromatogram in Figure 3.23 resulted from the derivatization and analysis of 270 pmoles of RPP, 120 pmoles of RPPG and 175 pmoles of RPPGF. The peak corresponding to RPP was offscale, RPPG was detected with a 12:1 signal to noise ratio, and RPPGF was not detected. The lowest amount of the pentapeptide RPPGF that was derivatized and definitively detected by capillary GC-MS-SIM was 1.7 nmoles (data not shown).

Unfortunately, the results depicted in Figures 3.22 and 3.23 were not typical. These results were very difficult to reproduce, and the problem was traced to two primary sources. The first of these sources of poor reproducibility was rapid deterioration of GC

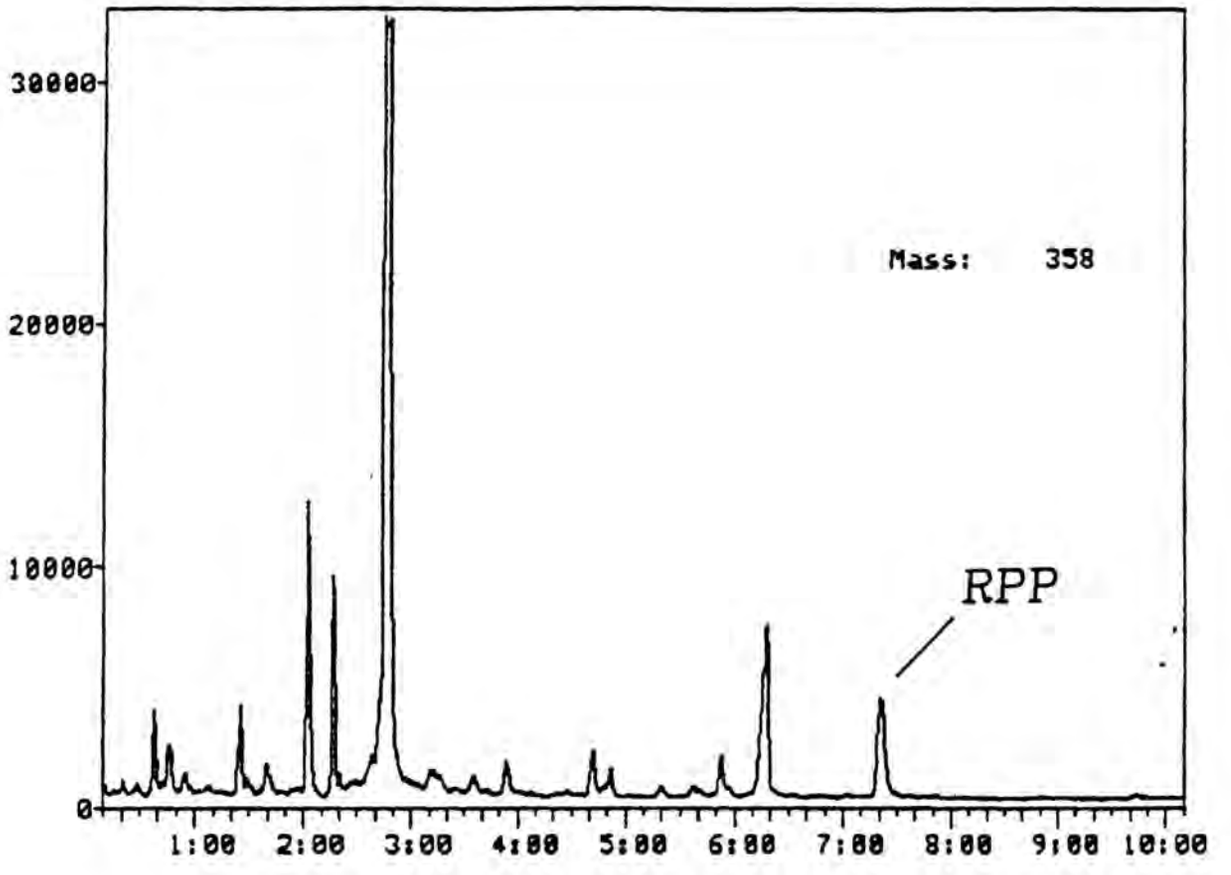


Figure 3.22. Capillary GC-MS selected ion monitoring chromatogram (monitoring m/z 358) of a mixture of 20 pmoles of each of the peptides RPP, RPPG and RPPGF. The entire sample was injected. RPPG and RPPGF were not detected.

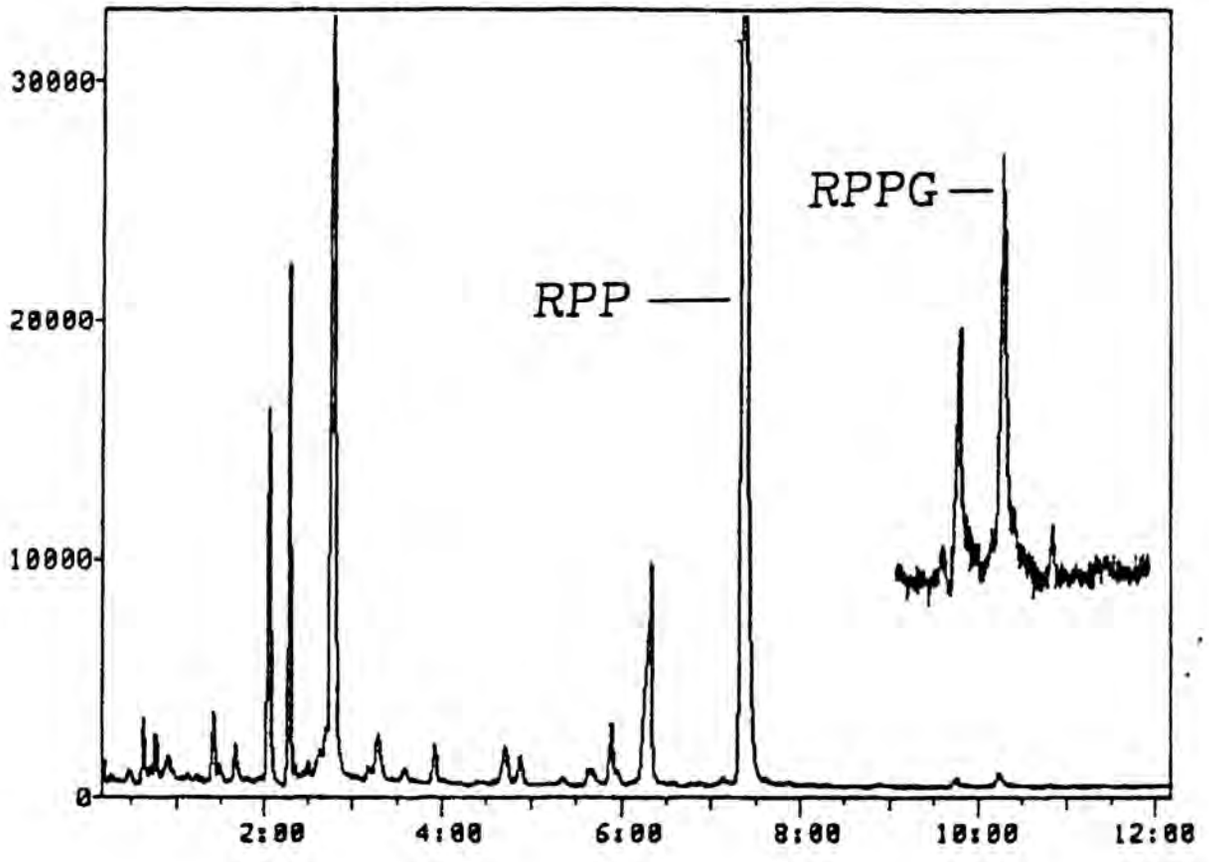


Figure 3.23. Capillary GC-MS selected ion monitoring chromatogram (monitoring m/z 358) of a mixture of 270 pmoles of RPP, 120 pmoles of RPPG and 175 pmoles of RPPGF. The entire sample was injected, and RPPGF was not detected.

column performance with repeated injection of low level samples. During the derivatization process, oily reaction by-products gradually accumulate, to the extent that the final fully derivatized sample may contain 1-2 µl of oily contaminants. Assuming that the oil has a density of 1, this means that on a mass ratio basis, a sample of 10 ng of peptide will have a contaminant to analyte ratio of 10-20,000:1. In order to detect these low level samples, the entire sample was frequently injected, meaning that 1-2 mg of unknown contaminants were co-injected with the low level analyte. Such a contaminant load could easily destroy the performance of a capillary column, particularly when heated to 320 °C, the elution temperature of the pentapeptide. Extensive washing of the columns with solvents followed by deactivation with 1,3-diphenyltetramethyldisilazane (64) did not restore column performance. Furthermore, deterioration of column performance occurred using both on-column and splitless-split injection methods, indicating that the problematic contaminants have some degree of volatility.

The second source identified for the observed reproducibility problems at low levels was the diborane reduction step of the derivatization process. The preceding three steps consisting of the condensation with acetylacetone, methylation with 3 M methanolic HCl, and trifluoroacetylation with MBTFA, all consistently yielded the sought products as demonstrated by FAB-MS. These three reactions basically worked every time at both low and high levels. The reduction step, however, was quite variable in its yield of the

sought polyaminoalcohol below a level of about 15-25 nmoles of these peptides. The irreproducibility of this reaction was most pronounced with the longer peptides. This is not surprising since the probability of incomplete reduction and unwanted side reactions increases as the number of peptide bonds increases.

The net result of these (and possibly other) sources of irreproducibility was that above a level of 15-25 nmoles, the DMPO-N-TFE-O-TBDMS polyaminoalcohol derivatives of these peptides were formed and detected with good reproducibility, although the derivative yield varied with the longer peptides even at these levels. Below the 15 nmole level, reproducibility became a significant problem.

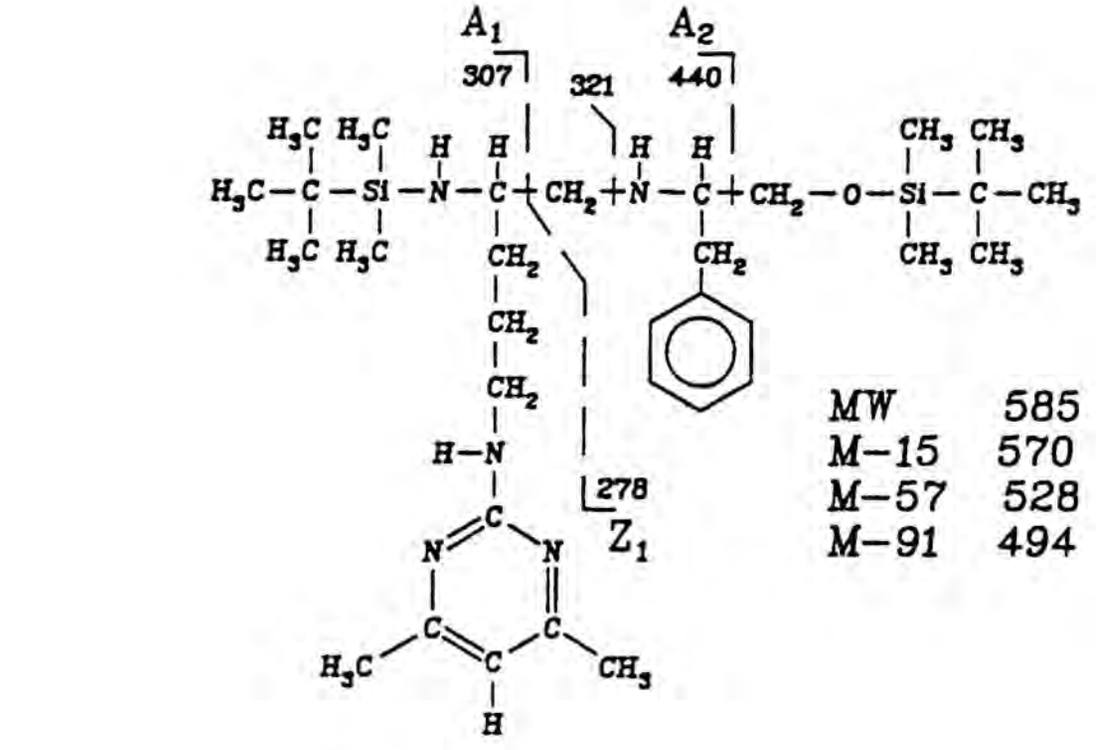
H. DMPO-N,O-di-TBDMS polyaminoalcohol derivatives.

In an effort to overcome some of the reproducibility problems at low levels with the polyaminoalcohol derivatives, particularly the problem of GC column deterioration, a brief study was undertaken to examine the feasibility of employing DMPO-N,O-di-TBDMS polyaminoalcohol derivatives for the capillary GC-MS analysis of arginyl peptides. The procedure necessary for the formation of these derivatives eliminates two steps as compared to their N-trifluoroethyl counterparts, namely the methylation and trifluoroacetylation steps. The trifluoroacetylation step could obviously be omitted, and since the methylation step was only employed to facilitate sample solubility in the triflouroacetylating reagents (17), this

step was omitted as well. Note that both ester groups and carboxylic acid groups are reduced by diborane to an alcohol function, and in fact the reaction occurs much more readily with the free carboxylic acid group than with the ester group (42).

The peptides RF, RPP and RPPG were successfully converted to the DMPO-N,O-di-TBDMS polyaminoalcohol derivatives, and Figures 3.24 through 3.26 present their electron impact mass spectra from repetitive scanning capillary GC-MS analysis. Like their N-trifluoroethyl counterparts, these derivatives yield mass spectra which contain complete amino acid sequence information including both A and Z series ions, with the pair of ions corresponding to $[M-15]^+$ and $[M-57]^+$ present in each to clearly denote the molecular weights of the derivatives. The fragments indicative of the dimethylpyrimidyl-ornithine side chain at m/z 136, 176 and 190 are present in these spectra.

These derivatives showed considerable promise from the standpoint of ultimate sensitivity. Figure 3.27 presents a chromatogram resulting from the injection of 80 pmoles of RPP as the DMPO-N,O-di-TBDMS polyaminoalcohol derivative under capillary GC-MS-SIM conditions, monitoring m/z 390, the A_2 ion. This chromatogram exhibits a 2000:1 signal to noise ratio. Similarly, an injection of 1 pmole of RF yielded an excellent signal to noise ratio as depicted in Figure 3.28. Note that these samples were derivatized at the 150-300 nmole level and diluted prior to injection.



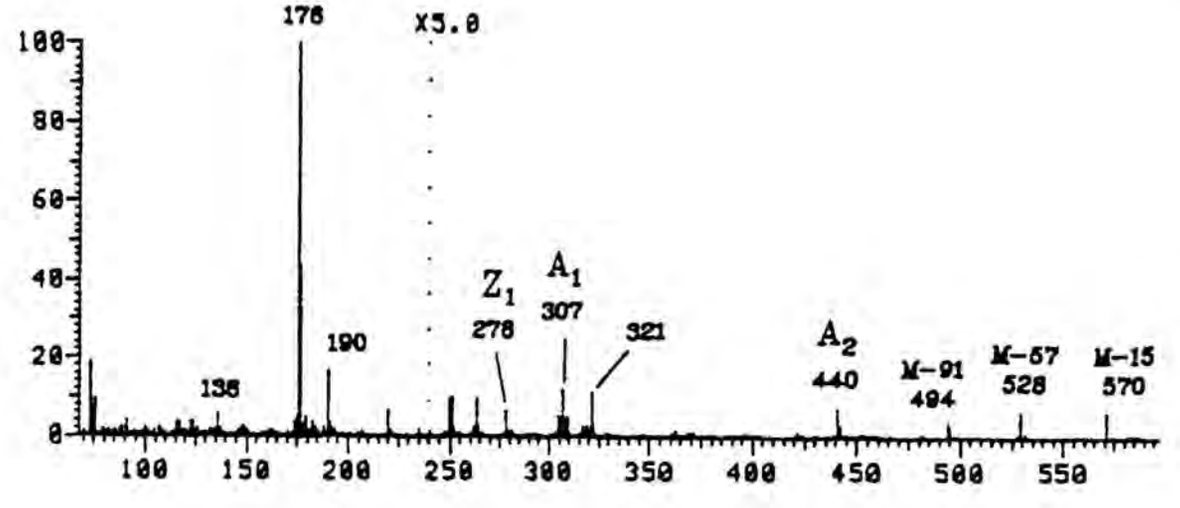
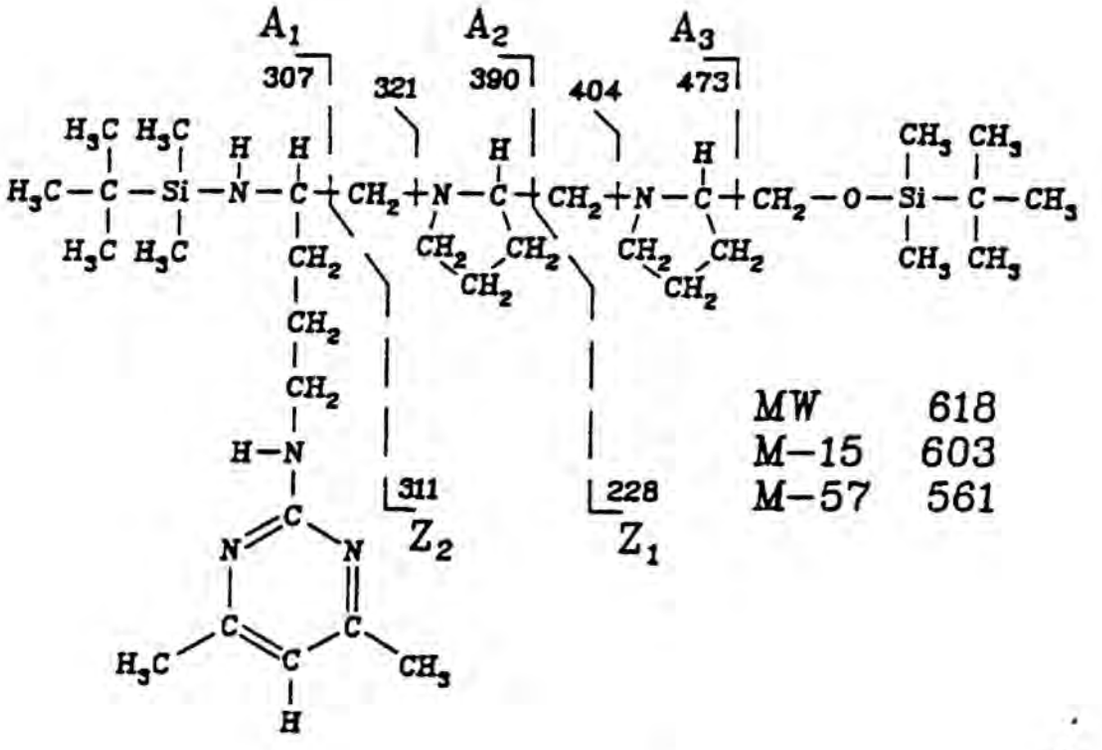


Figure 3.24. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivative of arginylphenylalanine (RF-DMPO-N,O-di-TBDMS polyaminoalcohol) from capillary GC-MS analysis.



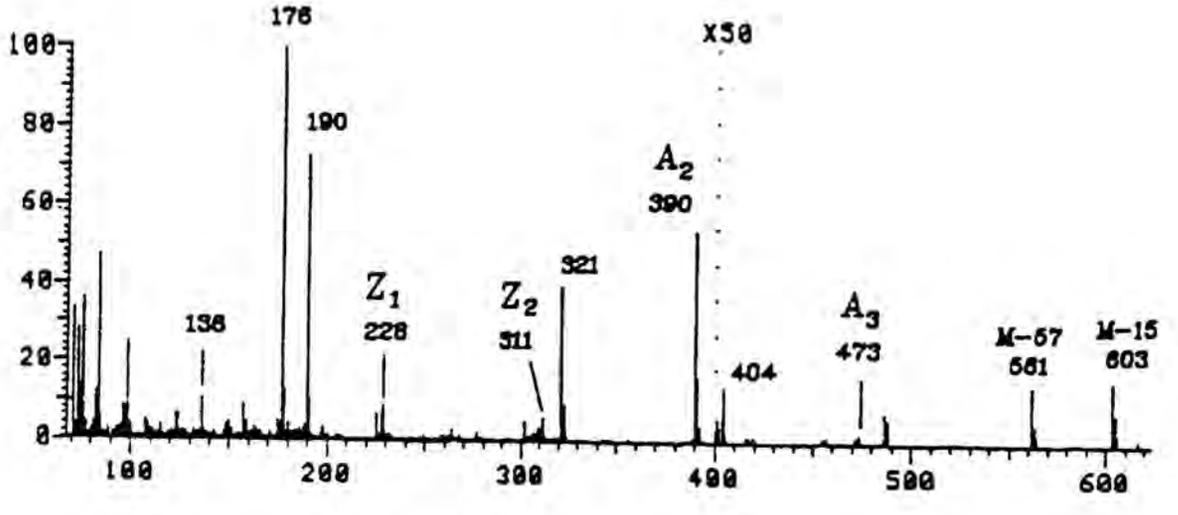
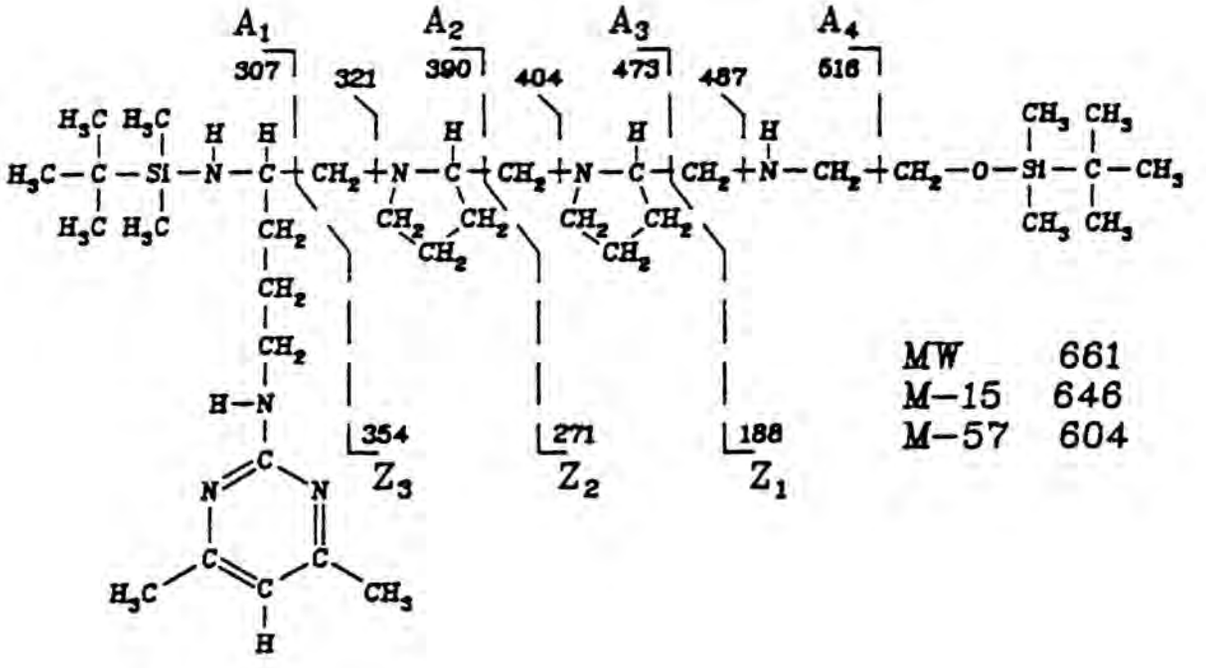


Figure 3.25. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivative of arginyl-prolyl-proline (RPP-DMPO-N,O-di-TBDMS polyaminoalcohol) from capillary GC-MS analysis.



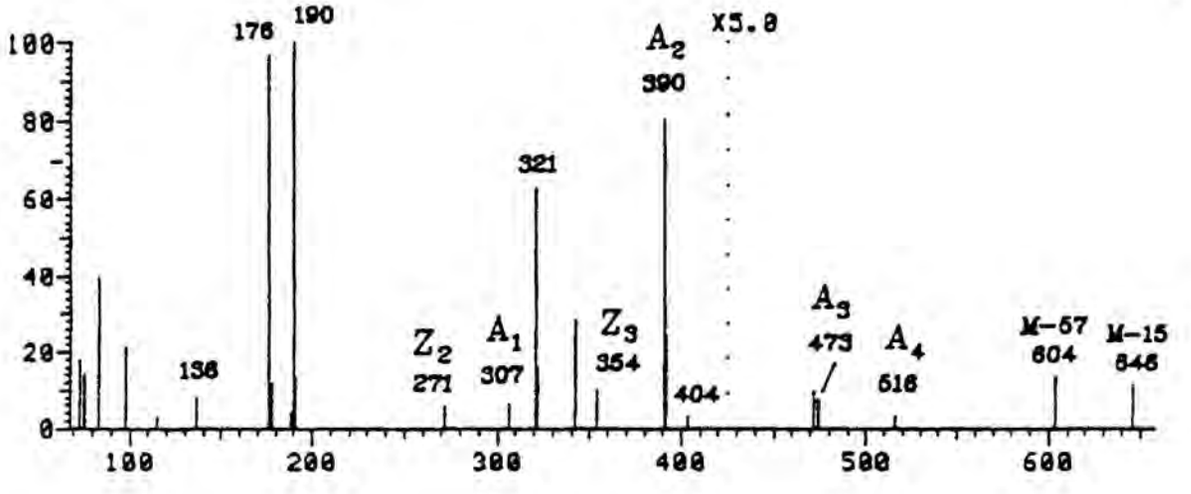


Figure 3.26. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivative of arginyl-prolyl-prolyl-glycine (RPPG-DMPO-N,O-di-TBDMS polyaminoalcohol) from capillary GC-MS analysis.

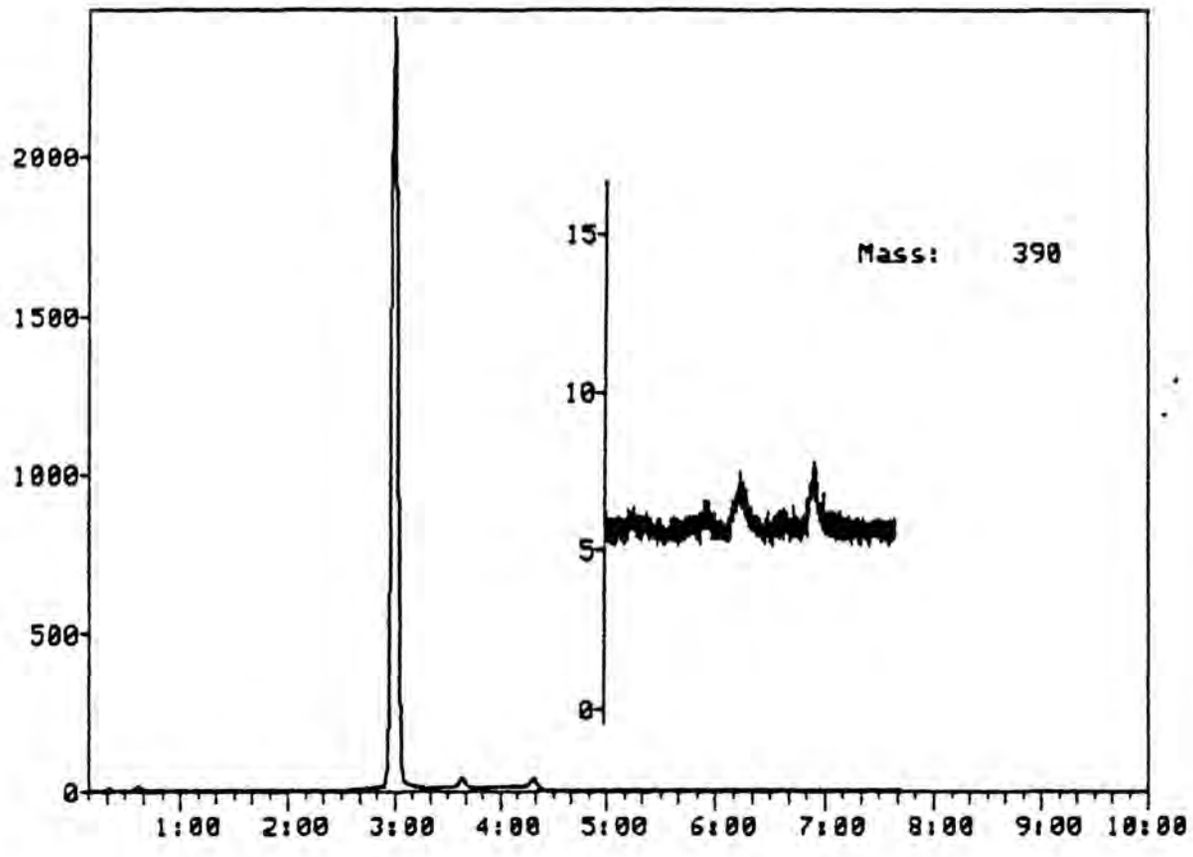


Figure 3.27. Capillary GC-MS selected ion monitoring chromatogram (monitoring m/z 390) resulting from the injection of 80 pmoles of RPP as the dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivative.

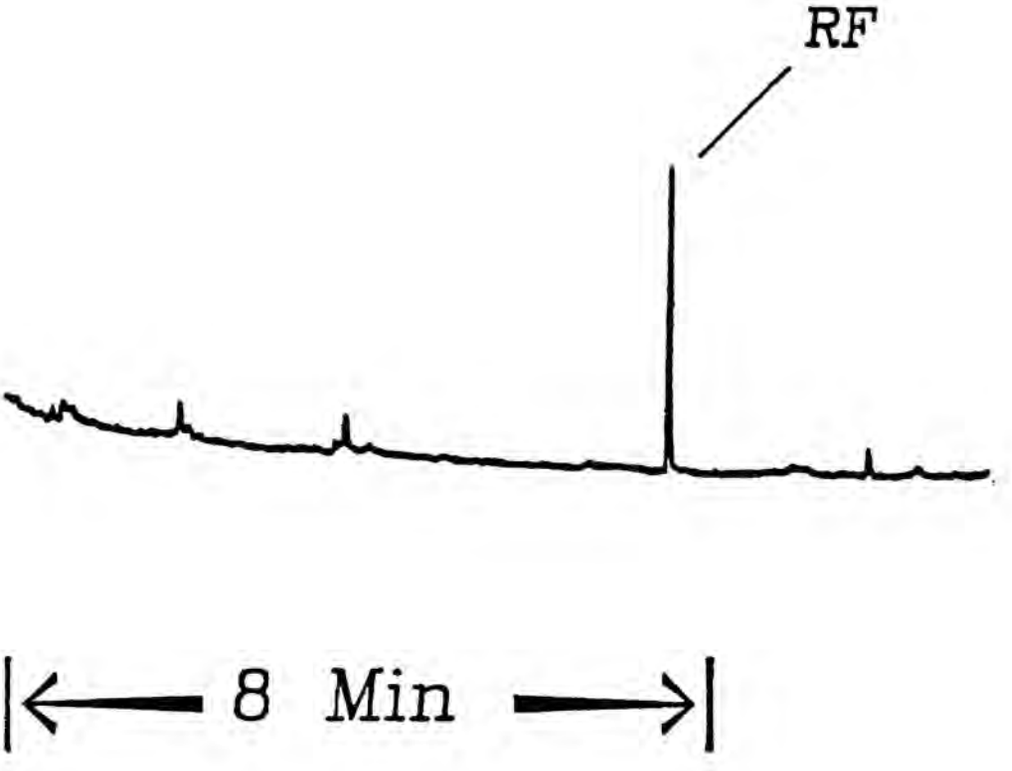


Figure 3.28. Capillary GC-MS selected ion monitoring chromatogram (monitoring m/z 176) resulting from the injection of 1 pmole of RF as the dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivative.

While these derivatives do show promise, reproducibility problems were encountered with them even at relatively high levels. The sought derivative of the pentapeptide RPPGF was not definitively identified during the course of these studies. In addition to reproducibility problems with the diborane reduction, the silylation reaction presented problems as well. With the N-trifluoroethyl derivatives, the silylation reaction could be carried out in pyridine, which supresses the reaction of MTBSTFA with secondary amines (46). Unfortunately, this element of control was not possible with the N,O-di-TBDMS derivatives, since pyridine also supressed the silylation of the primary amine functions of these molecules. Hence, the reaction was carried using acetonitrile as a solvent. What was observed on several occasions was that the sought derivative was formed and existed for a short period of time (as little as fifteen minutes), then converted to a higher mass compound which may have been over-silylated. Residual boron-containing contaminants may have also participated in this conversion to an unknown high mass product. Because of these problems, these derivatives were not pursued further during these studies.

I. Selective extraction of arginyl peptides.

In order to explore the feasibility of selectively extracting arginyl peptides from aqueous matrices, arginyl peptides were treated with 1,2-cyclohexanedione and extracted using a combination of immobilized phenylboronic acid columns and C_{18} Sep-Paks. Samples

of RF and RPPG so extracted and analyzed by FAB-MS exhibited pseudo-molecular ions corresponding to the dihydroxycyclohexyl derivatives, along with numerous contaminating species. Upon treatment with acetylacetone, the dihydroxycyclohexyl group was exchanged with acetylacetone to form the highly stable dimethylpyrimidylornithyl derivative, as was verified by FAB-MS. A sample of 300 nmoles of RF was extracted from phosphate buffered saline by these methods, then derivatized to form the DMPO-N,O-di-TBDMS polyaminoalcohol derivative and analyzed by capillary GC-MS-SIM, monitoring m/z 176. The resulting chromatogram is presented in Figure 3.29. Although this chromatogram indicates that the extraction and derivatization process are compatible, the intensity of the RF peak in Figure 3.29 is very low for the amount of sample represented. Similar results were obtained with RPP. In order to determine whether this was due to a derivatization problem or limited binding capacity in the immobilized phenylboronic acid columns, a mixture containing 175-300 nmoles of each of the peptides RPP, RPPG and RPPGF was extracted from phosphate buffered saline by these methods, and the extract was analyzed by reverse phase ion pairing HPLC. A blank sample of phosphate buffered saline was treated identically in a parallel experiment. The resulting HPLC chromatograms are presented in Figure 3.30. Two conclusions may be drawn from these virtually identical chromatograms. First, this is clearly not an interference-free system, as numerous contaminants appear in both chromatograms. Secondly, very little peptide was extracted since at this

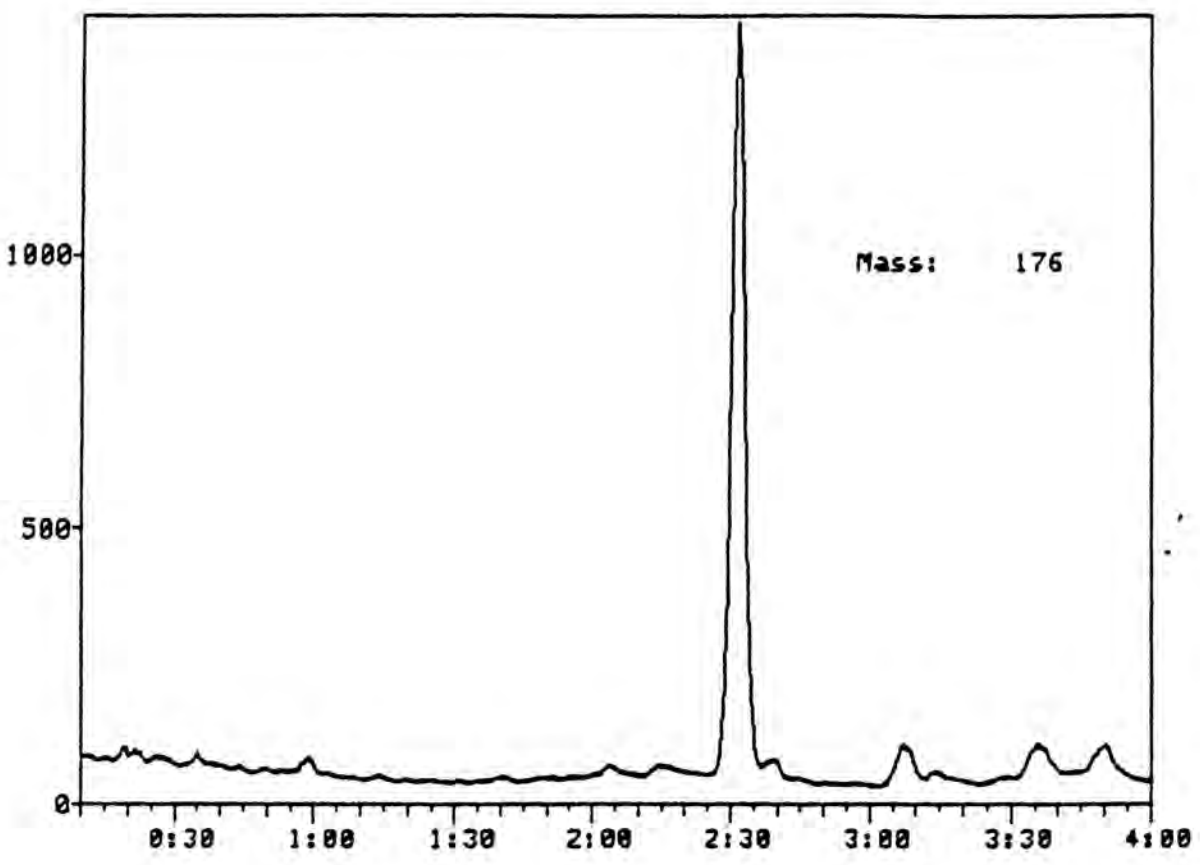


Figure 3.29. Capillary GC-MS selected ion monitoring chromatogram (monitoring m/z 176) of RF as the dimethylpyrimidylornithyl-N,O-di-TBDMS polyaminoalcohol derivative, resulting from the extraction of RF from phosphate buffered saline using the arginyl peptide selective extraction procedure.

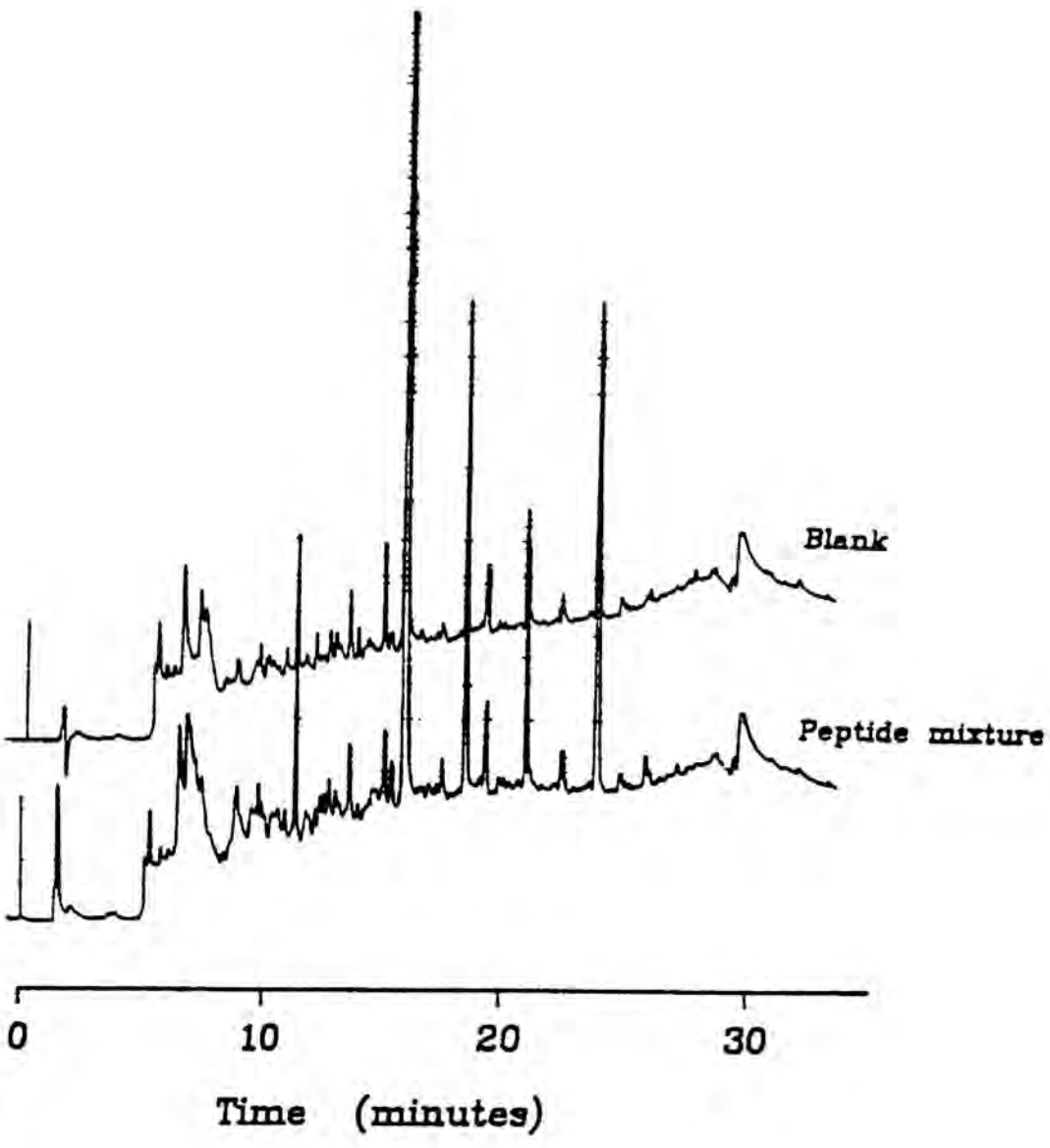


Figure 3.30. Analytical reverse phase ion pairing HPLC chromatograms of extracts of phosphate buffered saline (upper trace) and a mixture of 175-300 nmoles of each of the peptides RPP, RPPG and RPPGF in phosphate buffered saline (lower trace). The extracts were obtained using the arginyl peptide selective extraction procedure.

level of sensitivity, if the extraction were quantitative, the peptides would have yielded peaks roughly half of a full scale deflection in height, and would have been obvious as compared to the blank control sample. From these studies it was concluded that the arginyl peptide selective extraction procedure, while workable with standard samples, is probably impractical for application to complex biological samples such as urine. With the limited binding capacity of the phenylboronic acid columns, the likelihood of interfering species in biological samples effectively eliminating the binding of the peptides of interest is very high.

J. Reverse phase ion pairing liquid chromatographic isolation of RPP, RPPG and RPPGF.

A procedure based entirely on ion pairing reverse phase liquid chromatography was developed for the isolation of the bradykinin related peptides RPP, RPPG and RPPGF from complex aqueous matrices, particularly urine. The procedure employs two reverse phase ion pairing extraction steps using C_{18} Sep-Pak cartridge columns, with selective elution of the peptides from the cartridge columns at each step, and two reverse phase ion pairing HPLC isolation steps. Throughout the procedure, advantage is taken of the differing characteristics of the ion pairing reagents heptafluorobutyric acid (HFBA) and trifluoroacetic acid (TFA).

Experiments with standard samples demonstrated that the peptides RPP, RPPG and RPPGF could be extracted from aqueous matrices

and desalted using a C_{18} Sep-Pak with HFBA as an ion pairing reagent. The peptides could then be eluted from the Sep-Pak using water saturated with diethyl ether as the eluting medium, leaving strongly lipophilic materials adhering to the Sep-Pak. After removal of diethyl ether, the peptides could be re-extracted with a C_{18} Sep-Pak with HFBA as the ion pairing reagent. The more polar peptides RPP and RPPG could then be eluted using 10% acetonitrile in water, followed by a wash with 18% acetonitrile in water, and finally the more lipophilic pentapeptide RPPGF could be eluted using 25% acetonitrile in water, again leaving highly lipophilic materials adhering to the Sep-Pak. During the second extraction step, the sample is divided into two fractions: fraction A1 contains RPP and RPPG, and fraction B1 contains RPPGF.

Due to space considerations, the results of experiments with standard peptides in simple aqueous media will not be presented. Rather, the presented results will focus on experiments with urine samples, particularly a 20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF (spiked urine sample). A blank (unspiked) urine sample was run in parallel as a control.

An analytical HPLC chromatogram of the initial extract of a 20 ml urine sample, eluted from a Sep-Pak with water saturated with diethyl ether, is presented in Figure 3.31. Even with selective elution, high levels of hundreds of compounds are co-eluted with the bradykinin-related peptides. This prompted the use of the second

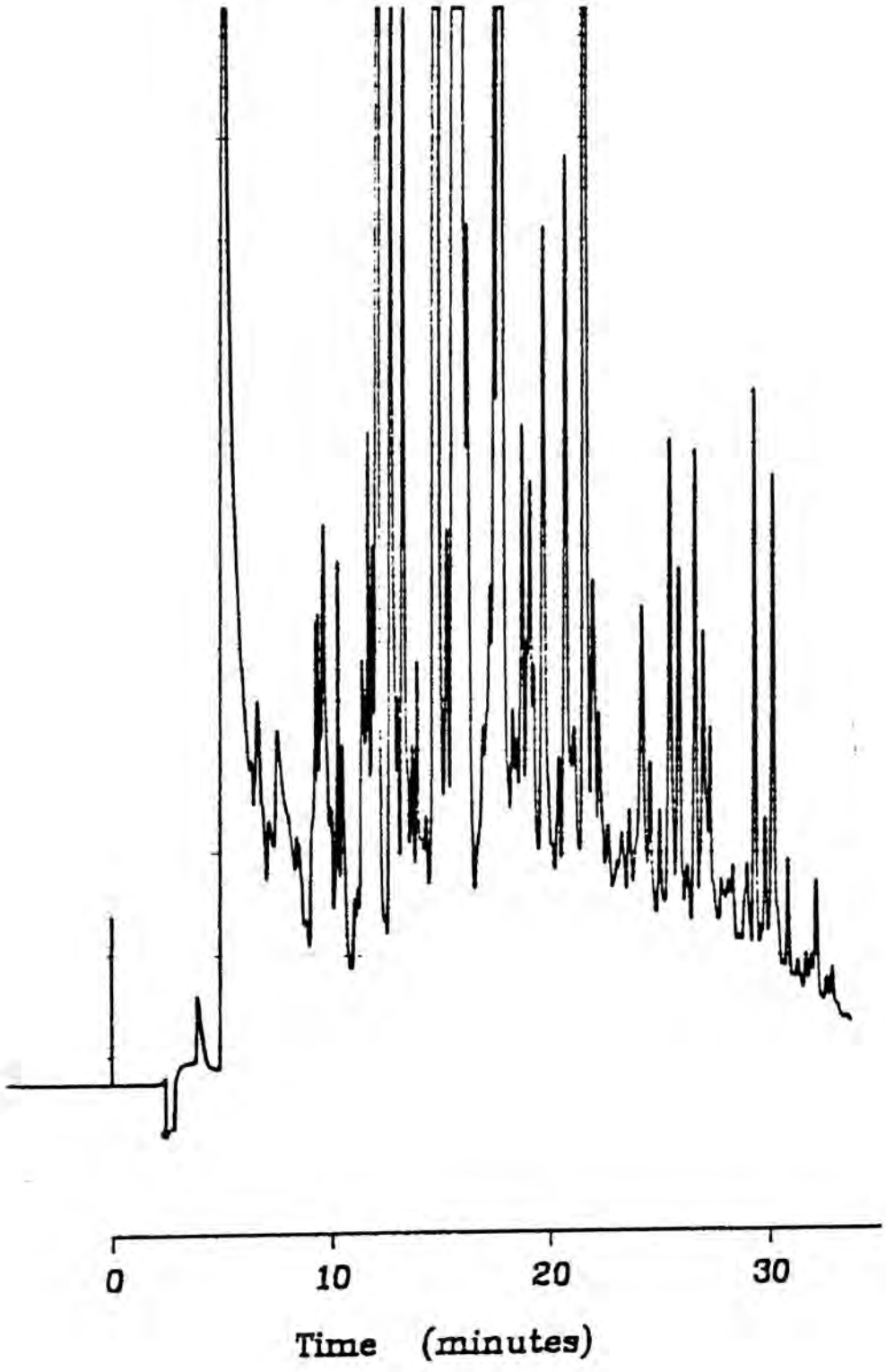


Figure 3.31. Analytical reverse phase ion pairing HPLC chromatogram of the initial extract of a 20 ml human urine sample, selectively eluted from an ODS Sep-Pak with water saturated with diethyl ether.

extraction step, with further refined selective elution using varying percentages of acetonitrile.

The next step in the isolation procedure is further purification of fractions A1 and B1 by reverse phase ion pairing HPLC, using TFA as the ion pairing reagent. Analytical HPLC chromatograms of the A1 and B1 fractions from the spiked urine sample on the TFA system are presented in Figures 3.32 and 3.33 respectively. Peptide components in the samples should be affected by the nature of the ion pairing reagent to a greater degree than non-peptide components. Since TFA is a much less lipophilic ion pairing reagent than HFBA (52), by changing the ion pairing reagent from HFBA in the extraction steps to TFA in the first HPLC step, the elution positions of the peptides of interest are shifted toward the earlier portions of the chromatograms, with non-peptide lipophilic material eluting later. The indicated regions of the chromatograms were collected under semi-preparative conditions to yield fractions A2 and B2. This required multiple injections of both samples in order to collect all of the material.

The final step in the isolation procedure is reverse phase ion pairing HPLC of fractions A2 and B2 using the HFBA system. Analytical HPLC chromatograms of these fractions from the spiked urine sample are presented in Figures 3.34 and 3.35. The large baseline shift evident in these chromatograms is an artifact due to the HFBA in the system. The peptides of interest are shifted toward the latter portions of these chromatograms relative to other components

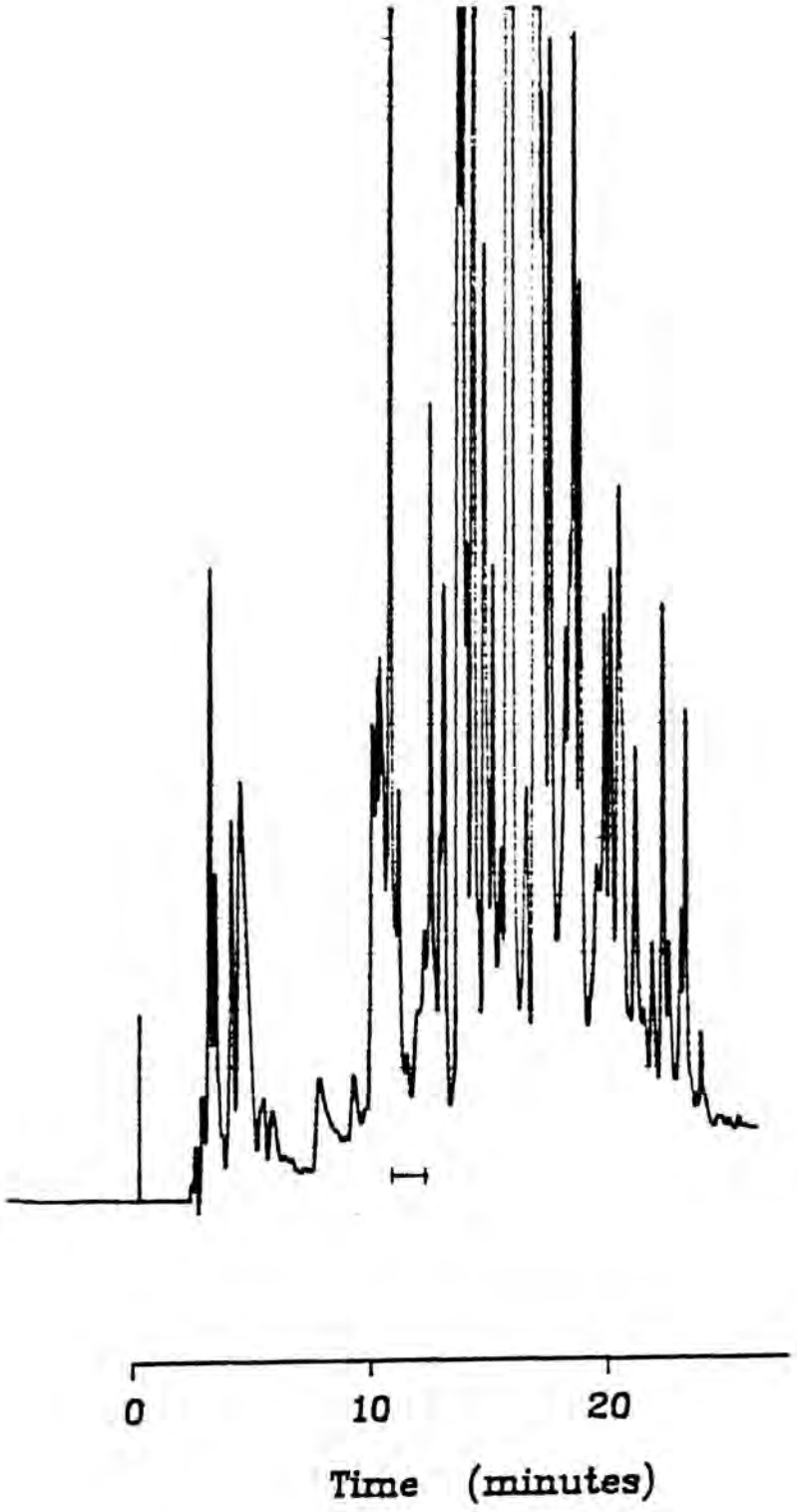


Figure 3.32. Analytical reverse phase ion pairing HPLC chromatogram (TFA system) of the A1 fraction from a spiked urine sample (20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF). The indicated region (RPP and RPPG region) was collected under semi-preparative conditions to yield fraction A2.

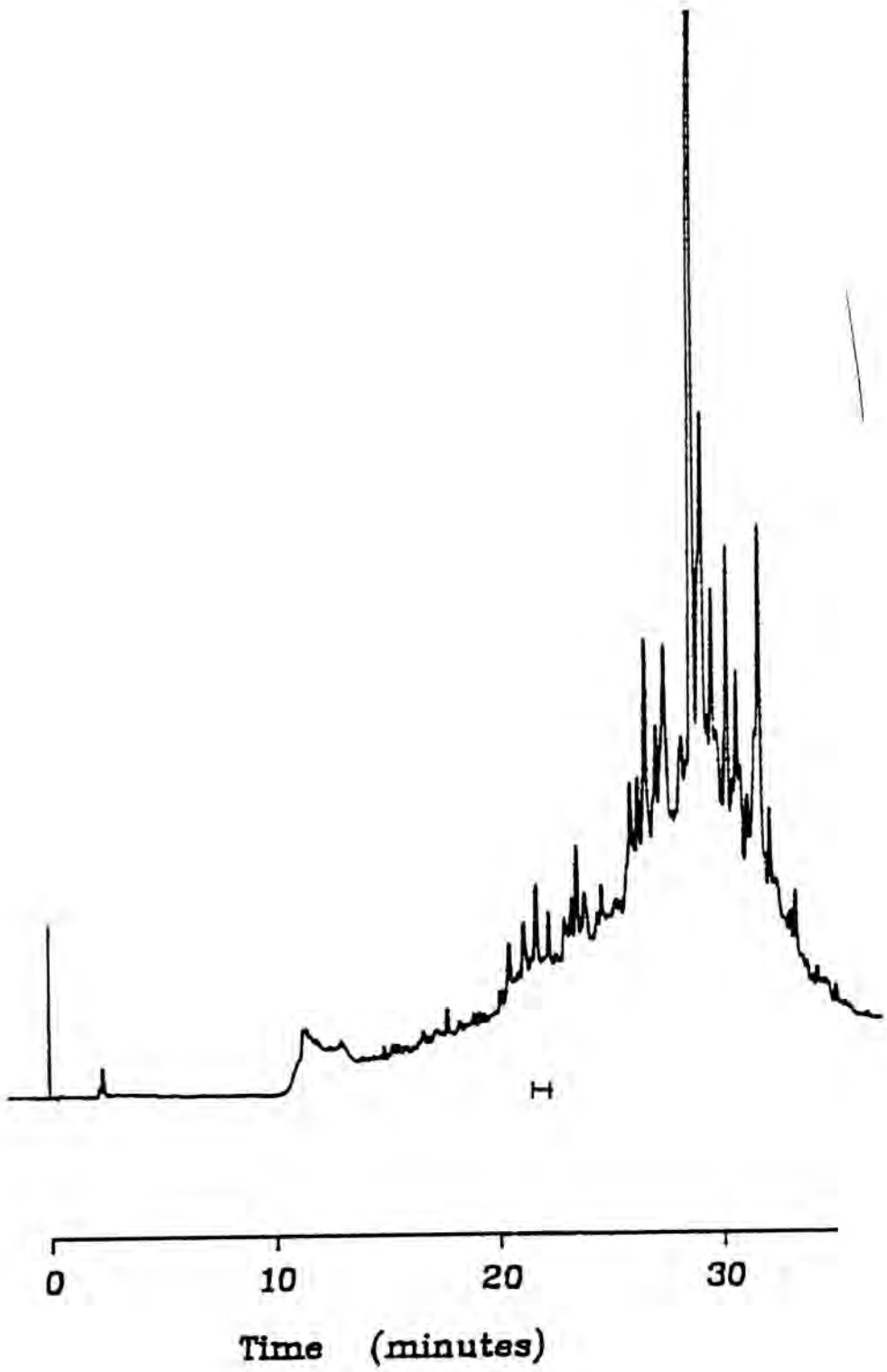


Figure 3.33. Analytical reverse phase ion pairing HPLC chromatogram (TFA system) of the B1 fraction from a spiked urine sample (20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF). The indicated region (RPPGF region) was collected under semi-preparative conditions to yield fraction B2.

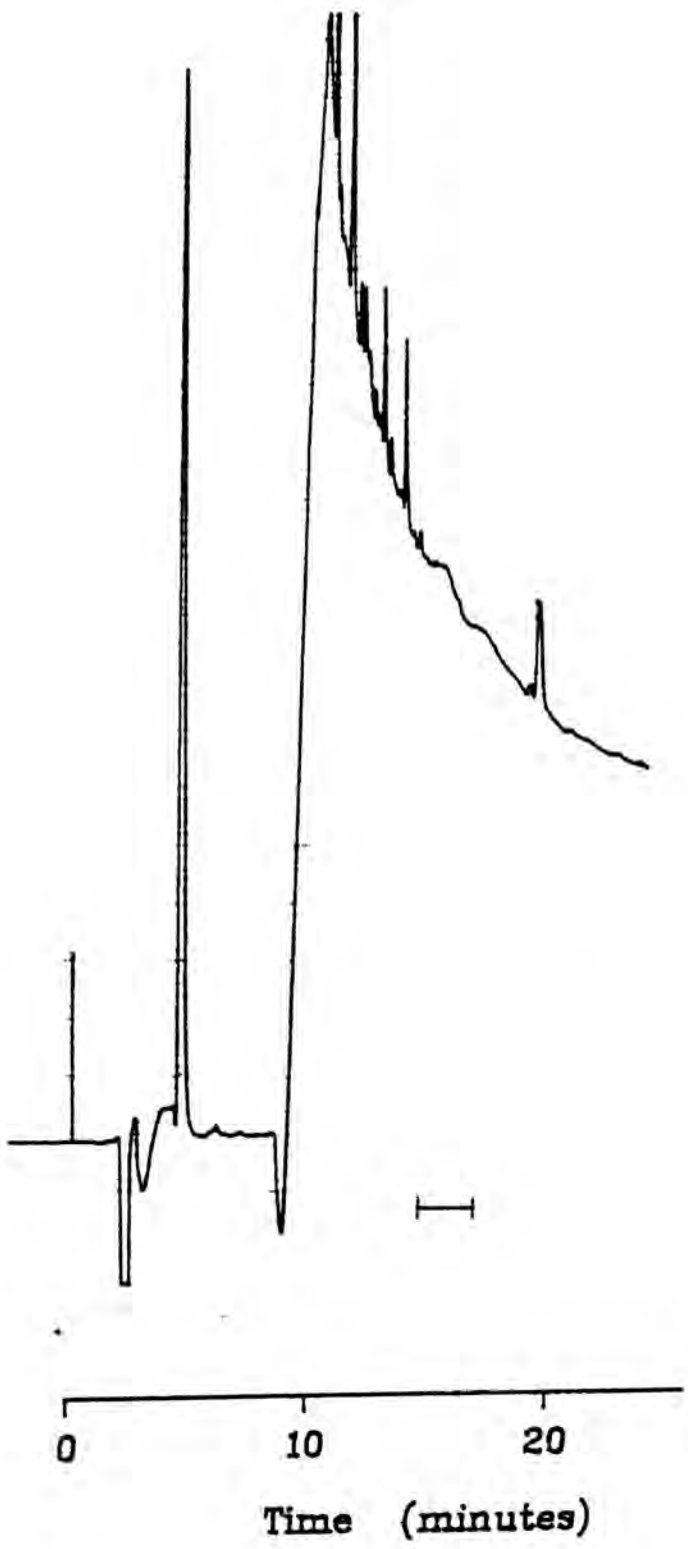


Figure 3.34. Analytical reverse phase ion pairing HPLC chromatogram (HFBA system) of the A2 fraction from a spiked urine sample (20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF). The indicated region (RPP and RPPG region) was collected under semi-preparative conditions to yield fraction A3.

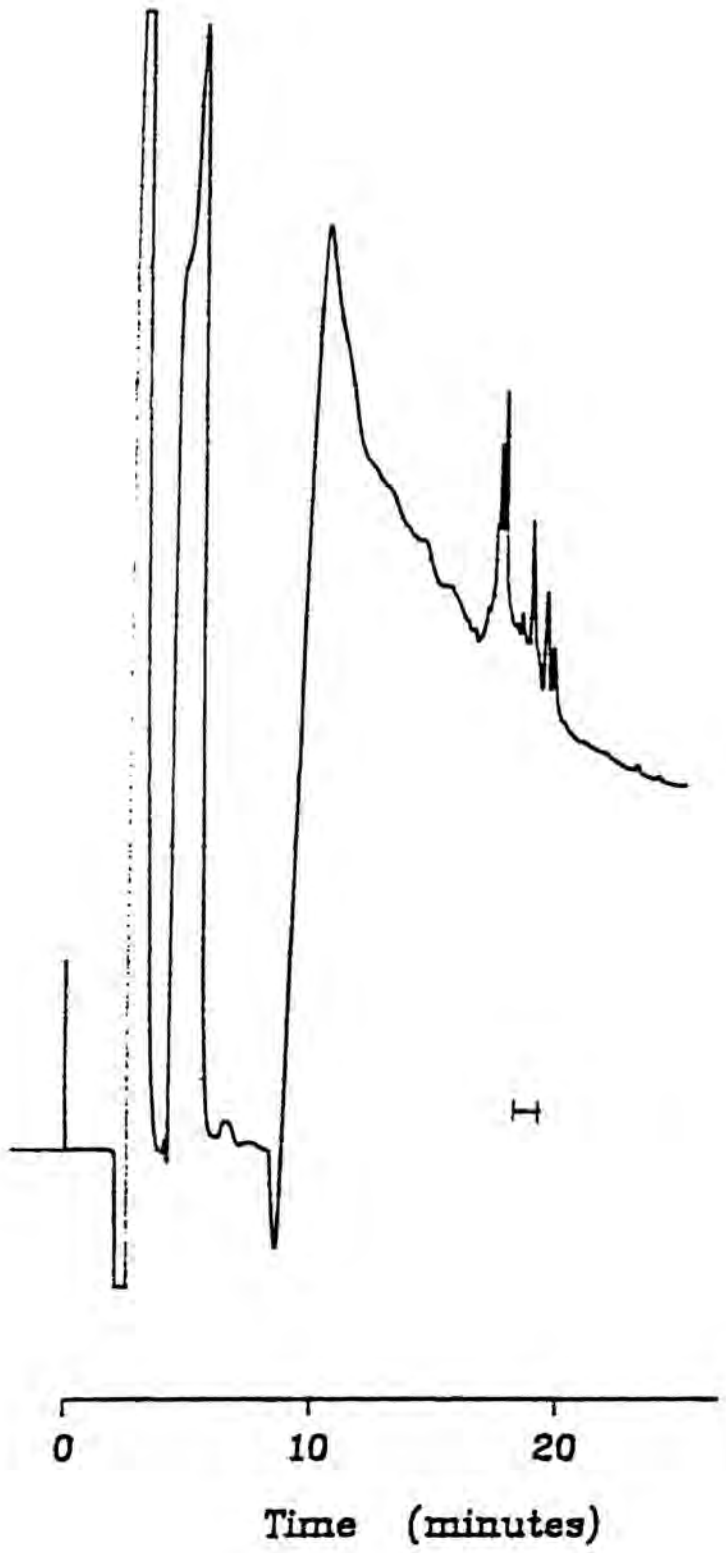


Figure 3.35. Analytical reverse phase ion pairing HPLC chromatogram (HFBA system) of the B2 fraction from a spiked urine sample (20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF). The indicated region (RPPGF region) was collected under semi-preparative conditions to yield fraction A3.

in the samples due to the use of HFBA as the ion pairing reagent. The indicated regions were collected from multiple chromatograms to yield fractions A3 and B3.

K. FAB-MS analysis of RPP, RPPG and RPPGF isolated from spiked human urine.

The FAB mass spectrum of fraction A3 isolated from the spiked urine sample is presented in Figure 3.36. For the sake of clarity, only the portion of the spectrum encompassing the ions of interest is displayed. The molecular weights of RPP and RPPG are 368 and 425, respectively, and pseudomolecular ions corresponding to each of these peptides are present in the spectrum at m/z 369 and 426. Fortuitously, glycerol ionization was supressed with this sample, which enabled the visualization of the pseudomolecular ion corresponding to RPP without interference from the m/z 369 background ion due to the glycerol matrix. Despite four successive steps of purification, numerous other species are present in this sample. Similar contaminant ions were in the blank urine sample as well, and ions at m/z 369 and 426 were not significant compared to background in the blank urine sample. While the ions corresponding to RPP and RPPG were not the most intense in this region of the spectrum in Figure 3.36, they were nevertheless reasonably intense for the amount of material represented in the spectrum. In fact, these ions were sufficiently intense to permit their visualization under FAB conditions at a resolution of 6-7,000. In order to better

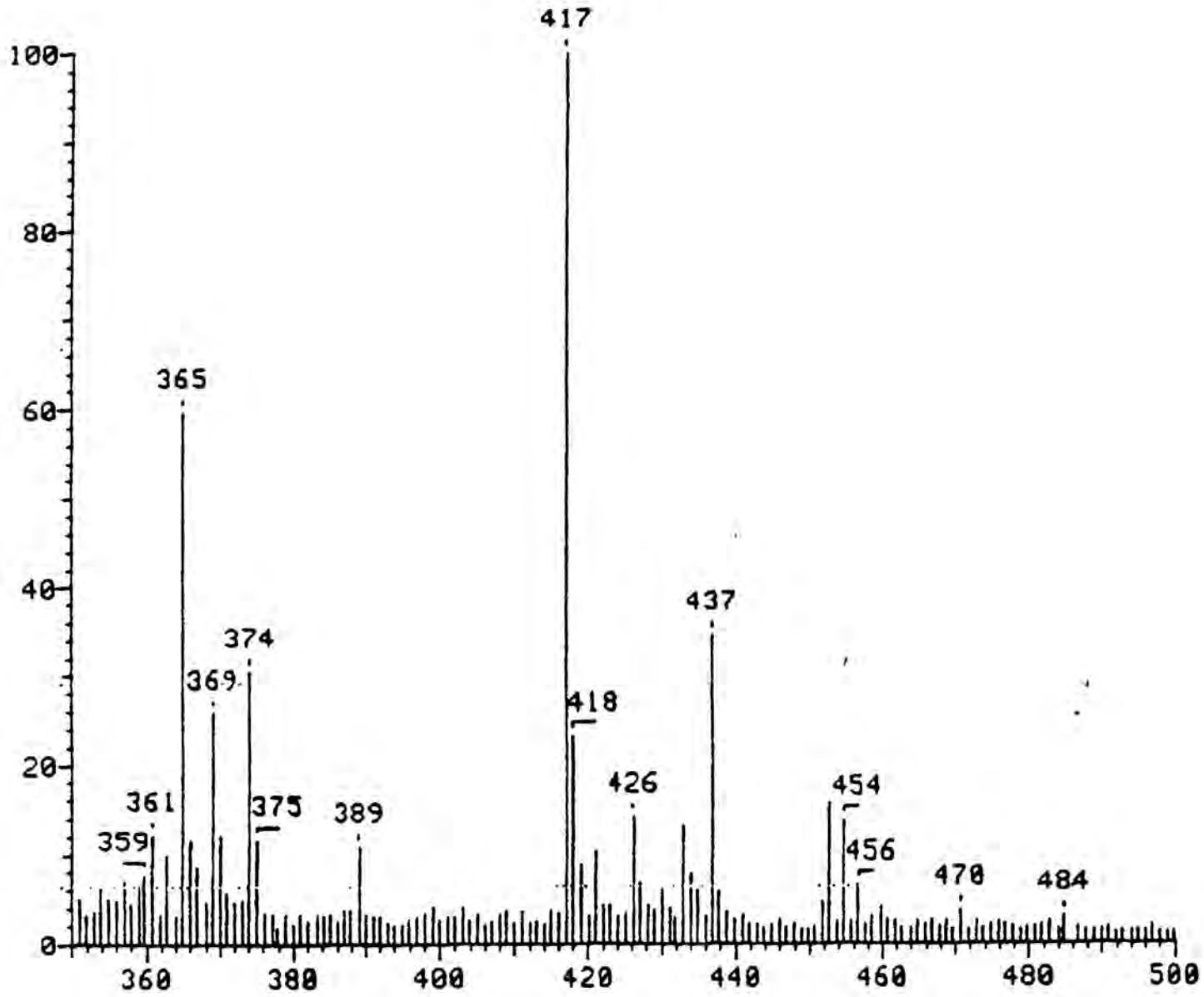


Figure 3.36. Fast atom bombardment mass spectrum of the A3 fraction (RPP and RPPG fraction) from a spiked urine sample (20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF).

characterize the nature of the m/z 369 and 426 ions in this sample, both ions were scanned at a resolution of 6-7,000 using the oscillographic recorder in order to visualize the peak profiles. Then, the authentic peptides were added to the glycerol-solvated sample, and these ions were re-scanned. The results of these scans are presented in Figure 3.37. In both instances, the ion of interest increased in intensity while maintaining the peak profile of a single peak, rather than appearing as a doublet in the presence of the authentic peptides. These results indicate that the isolated materials have elemental compositions consistent with RPP and RPPG.

Figure 3.38 presents the FAB mass spectrum of the B3 fraction isolated from the spiked urine sample. The pseudomolecular ion corresponding to RPPGF is quite intense at m/z 573. Again, numerous unknown species are present in the sample despite four steps of purification. This sample was not analyzed by FAB-MS under high resolution conditions. Rather, this sample was derivatized and analyzed by DIP-MS methods as described below.

L. Derivatization and DIP-MS analysis of standard RPPGF.

Since the pentapeptide RPPGF was consistently the most difficult to analyze of the peptides employed in these studies, efforts were directed toward developing sensitive, specific and reliable methodology for analyzing this peptide at reasonably low levels. Toward this end, DIP-MS methods were developed employing the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester (DMPO-N-TFA-

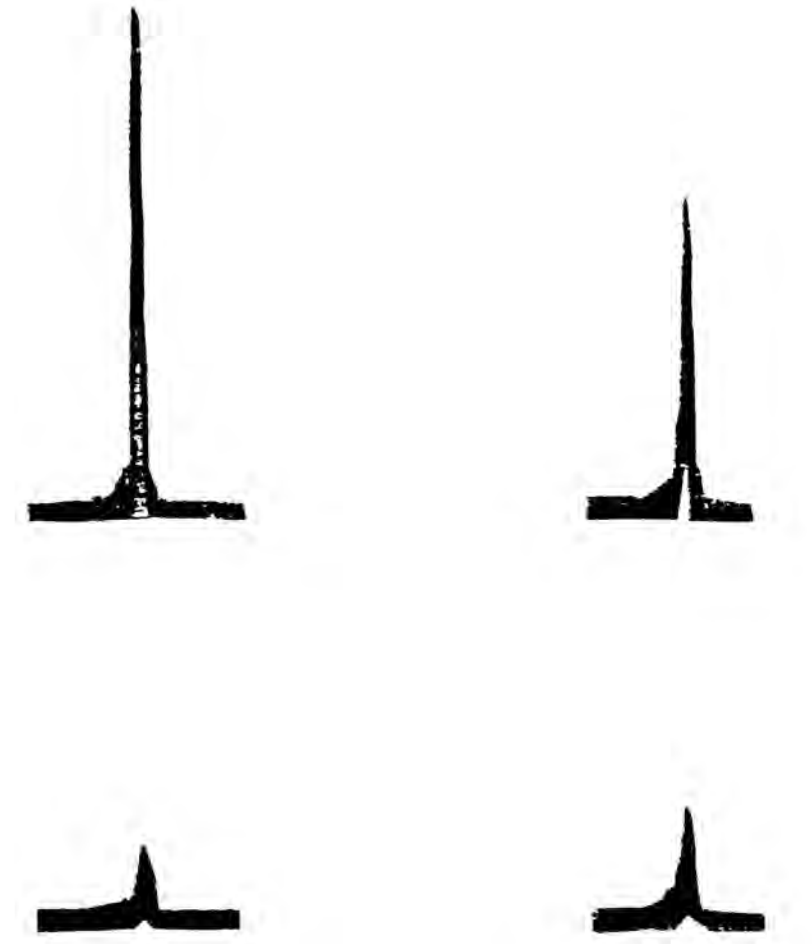


Figure 3.37. High resolution (R=6-7,000) fast atom bombardment mass spectral analysis of the pseudomolecular ion regions corresponding to RPP (m/z 369) and RPPG (m/z 426) of fraction A3 from the skiked urine sample. The traces show the pseudomolecular ion regions before (lower) and after (upper) the addition of authentic RPP and RPPG to FAB sample.

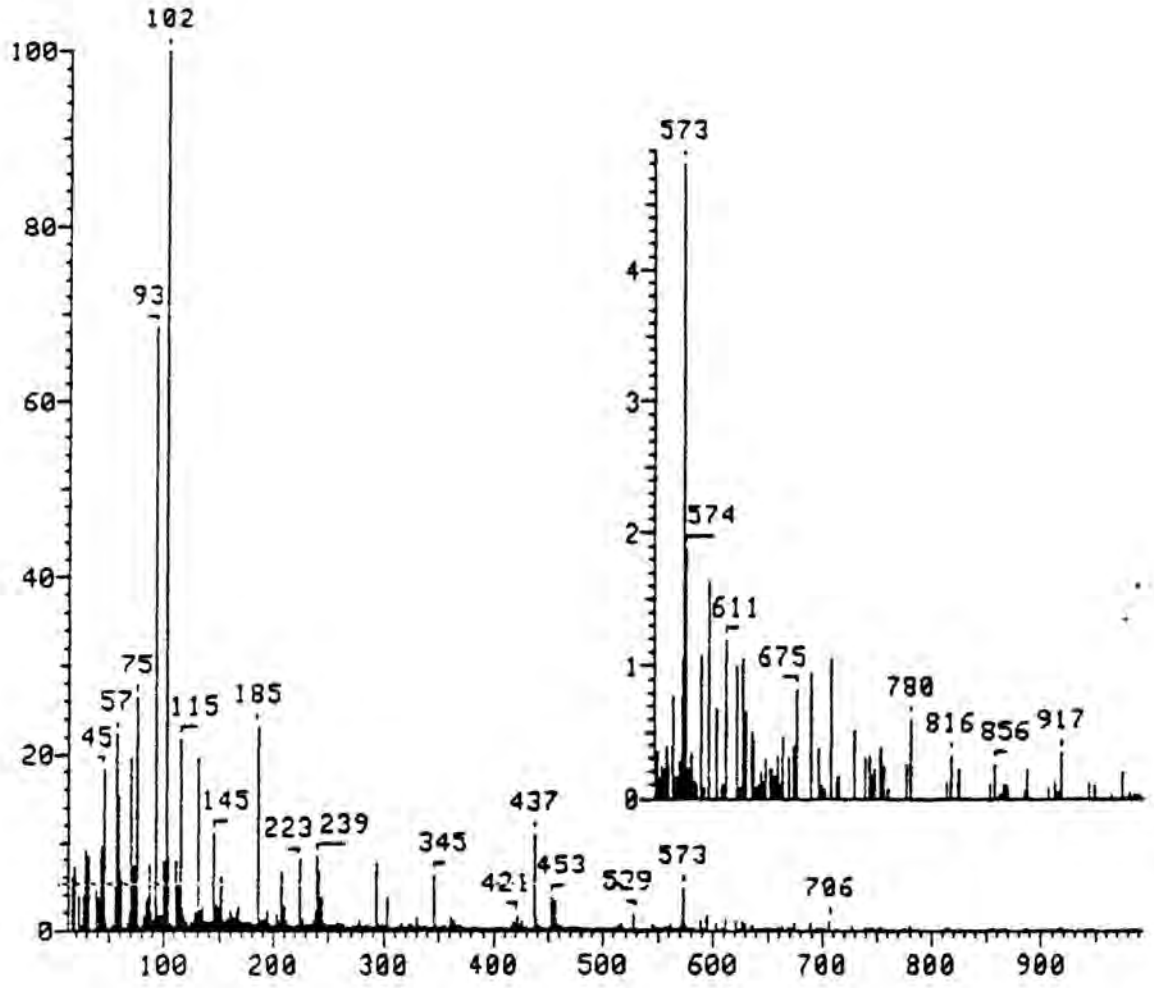


Figure 3.38. Fast atom bombardment mass spectrum of the B3 fraction (RPPGF fraction) from a spiked urine sample (20 ml sample of human urine to which was added 54 nmoles of RPP, 24 nmoles of RPPG and 35 nmoles of RPPGF).

O-ME) derivative of RPPGF. Unfortunately, this derivative possesses insufficient volatility for capillary GC-MS analysis, but it is sufficiently volatile for DIP-MS, giving peak signal intensity with the direct insertion probe tip at a temperature of approximately 250 °C, with the ion source pressure maintained at approximately 10^{-7}-10^{-6} torr. Figure 3.39 presents an electron impact mass spectrum of RPPGF as the DMPO-N-TFA-O-ME derivative from repetitive scanning DIP-MS analysis. The spectrum exhibits fragment ions consistent with the expected structure of the derivative, including the series of ions diagnostic for the dimethylpyrimidylornithine side chain, and ions representing cleavage on both sides of the carbonyl groups of all four peptide bonds. Most notably, the spectrum exhibits a surprisingly intense molecular ion at m/z 746, roughly 20% as intense as the base peak in the spectrum, which is quite unusual for an electron impact mass spectrum. This intense molecular ion was suitable for direct insertion probe selected ion monitoring mass spectrometric analysis under both low (R=1,900-2,300) and high (R=14-15,000) resolution conditions as demonstrated in Figures 3.40 and 3.41, respectively. Unfortunately, DIP-MS methods suffer a loss of molecular specificity as compared to capillary GC-MS methods. In order to compensate for this loss of specificity, methods were developed employing direct insertion probe collisionally activated dissociation selected reaction monitoring mass spectrometry (DIP-CAD-SRM-MS) for the assay. The reaction wherein an m/z 746 ion decomposes to form an m/z 429 ion in the

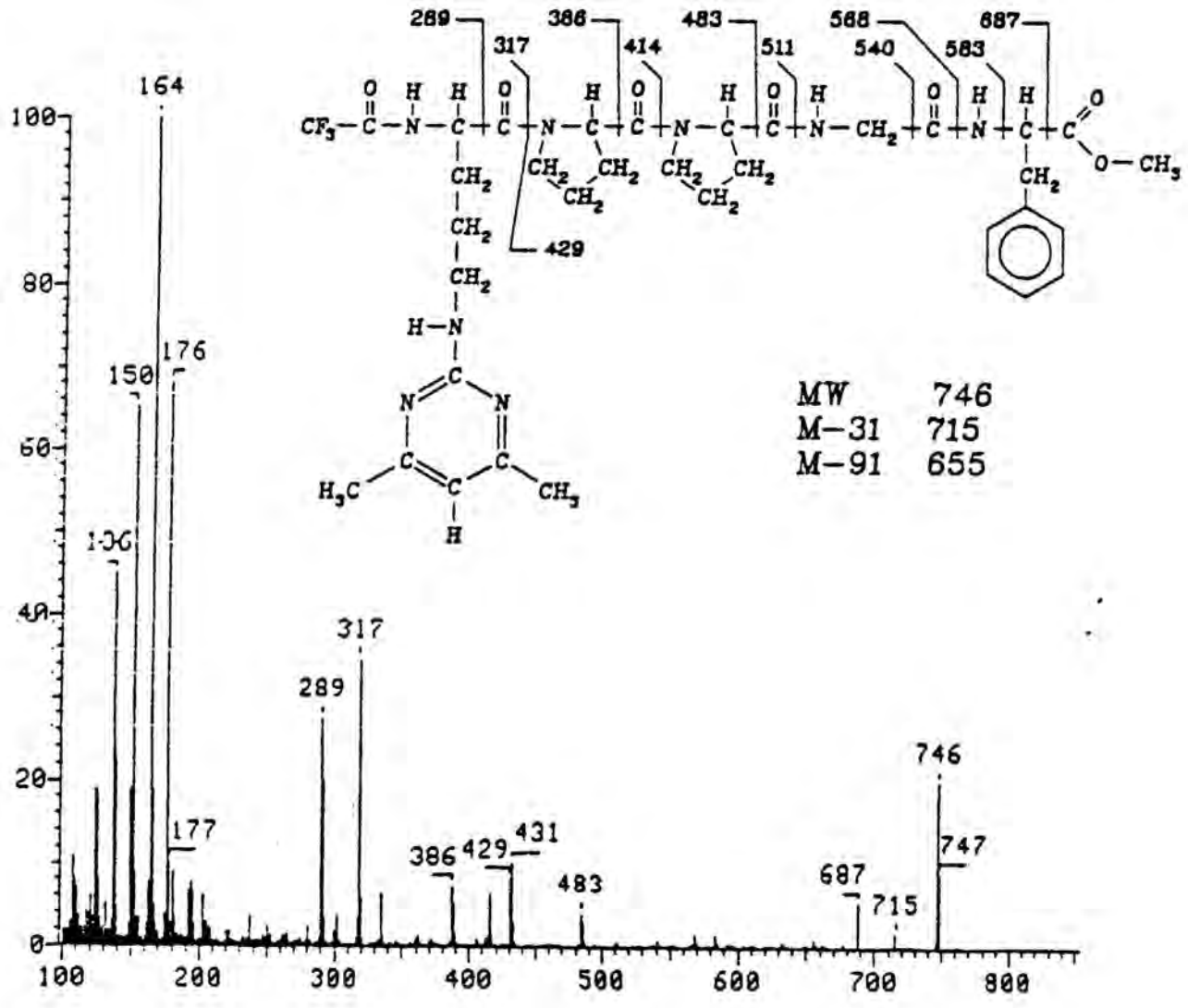


Figure 3.39. Electron impact mass spectrum of RPPGF as the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative (RPPGF-DMPO-N-TFA-O-ME) from repetitive scanning DIP-MS analysis.

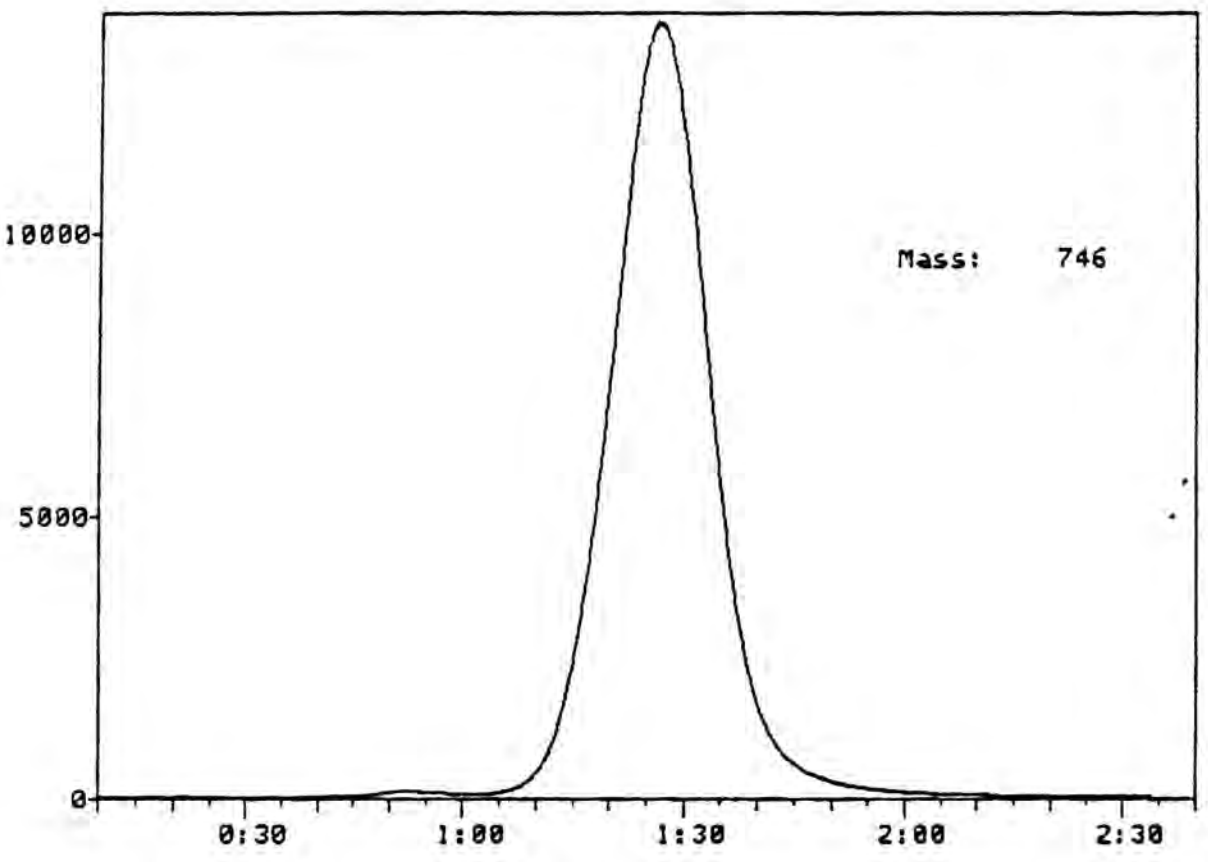


Figure 3.40. Low resolution (R=1,900-2,300) direct insertion probe selected ion monitoring trace (monitoring m/z 746) of approximately 1 nmole of RPPGF as the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative (RPPGF-DMPO-N-TFA-O-ME).

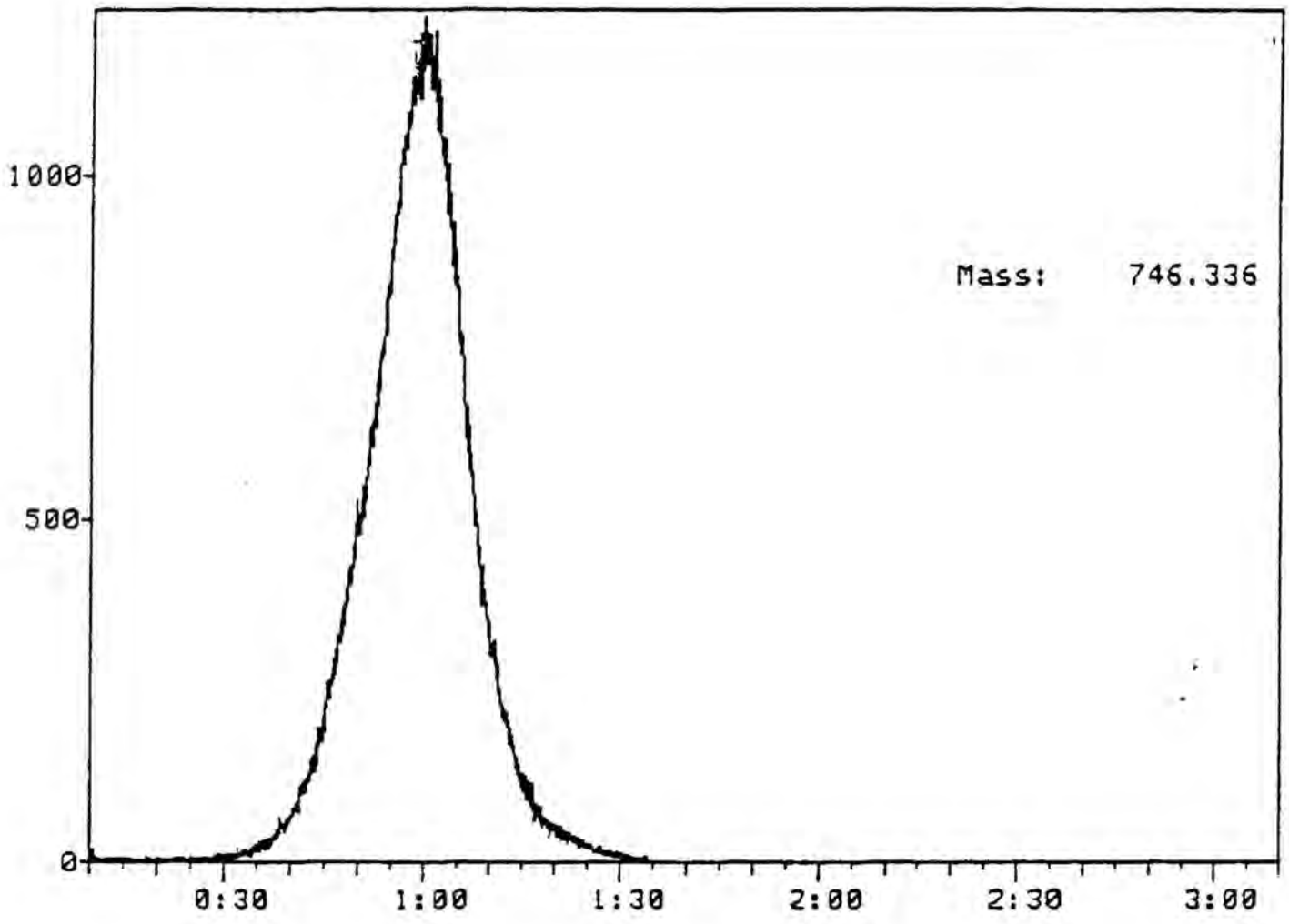


Figure 3.41. High resolution (R=14-15,000) direct insertion probe selected ion monitoring trace (monitoring m/z 746) of approximately 900 pmoles of RPPGF as the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative (RPPGF-DMPO-N-TFA-O-ME).

first field free region of the mass spectrometer was arbitrarily chosen for these analyses (see Figure 3.39). Figure 3.42 presents an exemplary DIP-CAD-SRM-MS analysis of standard RPPGF.

The results presented in Figures 3.40 through 3.42 were obtained using samples derivatized at the 175 nmole level, with small aliquots removed for analysis. Initial efforts to reproduce these results results using lower level samples (e.g. 10-20 nmoles) failed, with little or no signal for the molecular ion of the derivative detected by DIP-MS-SIM. However, after the attempted DIP-MS analyses, relatively large amounts of brown, obviously decomposed material were observed in the DIP sample vials. From this, coupled with the fact that the presence of the sought derivative in the samples could be demonstrated by FAB-MS, it was concluded that accumulated reaction by-products from the serial derivatization steps were probably reacting with the derivative under the heating conditions of DIP-MS, thus preventing the detection of the derivative.

The obvious solution to this problem was separation of the sought derivative from the problematic contaminants. Toward this end, HPLC isolation of the successive derivatives was incorporated into the overall process, with the final derivative isolated by normal phase HPLC. Experiments with 17 nmole samples of RPPGF demonstrated that the DMPO-N-TFA-O-ME could be formed, isolated and successfully analyzed by DIP-MS using these methods. Due to space considerations, these experiments with lower levels of standard

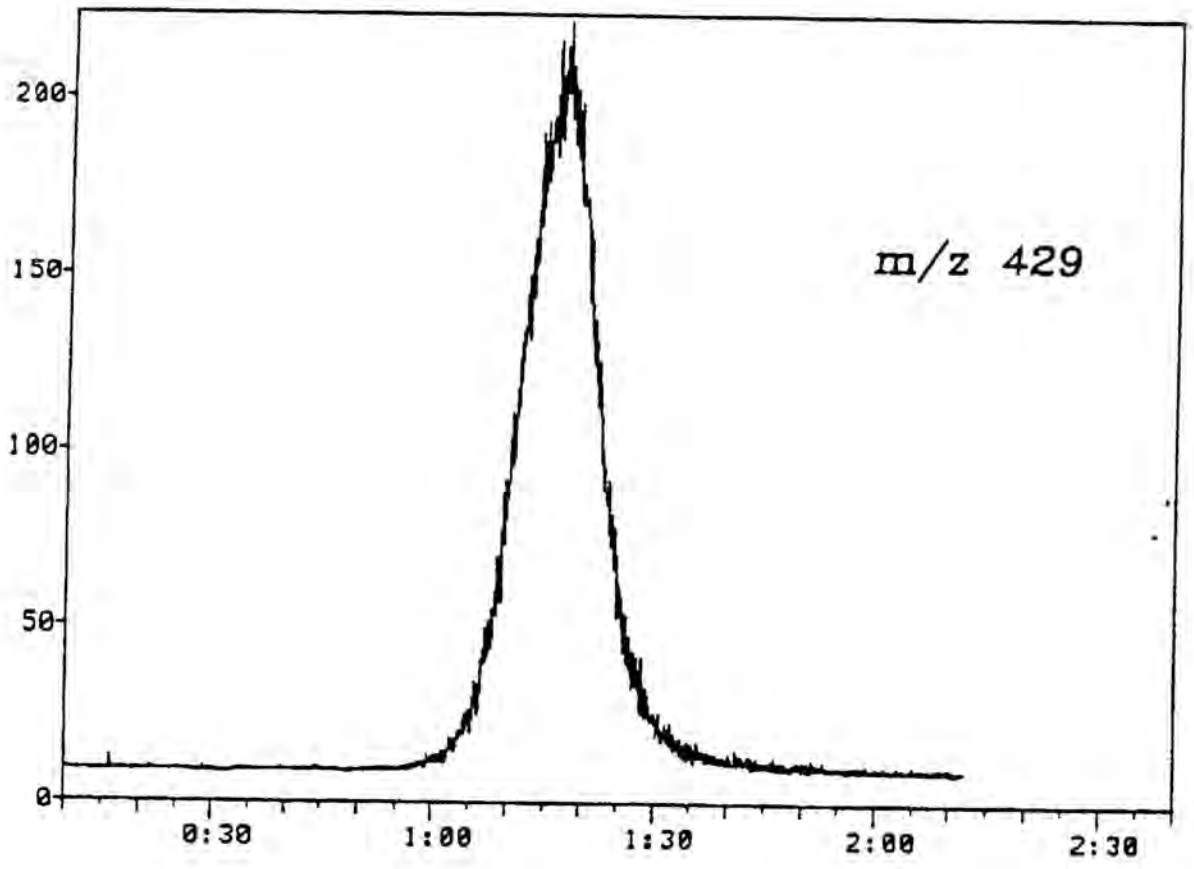


Figure 3.42. Direct insertion probe collisionally activated dissociation linked field selected reaction monitoring mass spectrometric analysis of approximately 200 pmoles of RPPGF as the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative (RPPGF-DMPO-N-TFA-O-ME). The formation of an m/z 429 fragment ion from an m/z 746 precursor ion was monitored.

RPPGF will not be presented. The results with the B3 ("RPPGF") fraction isolated from spiked human urine are presented below.

An additional benefit of incorporating HPLC into the derivatization process was that it permitted visualization and quantitative estimation of the precursor, product, and some of the reaction by-products of each of the three derivatization steps. With the aid of HPLC, the methods used in the acetylacetone condensation and trifluoroacetylation steps were further optimized to maximize the yield of the sought derivatives. The methylation reaction consistently yielded 90% or more of the sought methyl ester and was not further opitmized.

M. Derivatization and DIP-MS analysis of RPPGF isolated from spiked human urine.

One third of fraction B3 from the spiked human urine sample (representing approximately 12 nmoles of RPPGF) was derivatized to form the DMPO-N-TFA-O-ME derivative for DIP-MS analysis, with HPLC isolation of the sequential derivatives. The reverse phase ion pairing HPLC chromatogram for the isolation of DMPO-RPPGF is presented in Figure 3.43. The unreacted peptide eluted from this gradient system at 25% acetonitrile concentration, and the DMPO derivative eluted at 28% acetonitrile (these concentrations and those below are corrected for the void volume of the system). The broad peak eluting at 41% acetonitrile represents a peptide-related reaction by-product. The formation of the DMPO derivative was

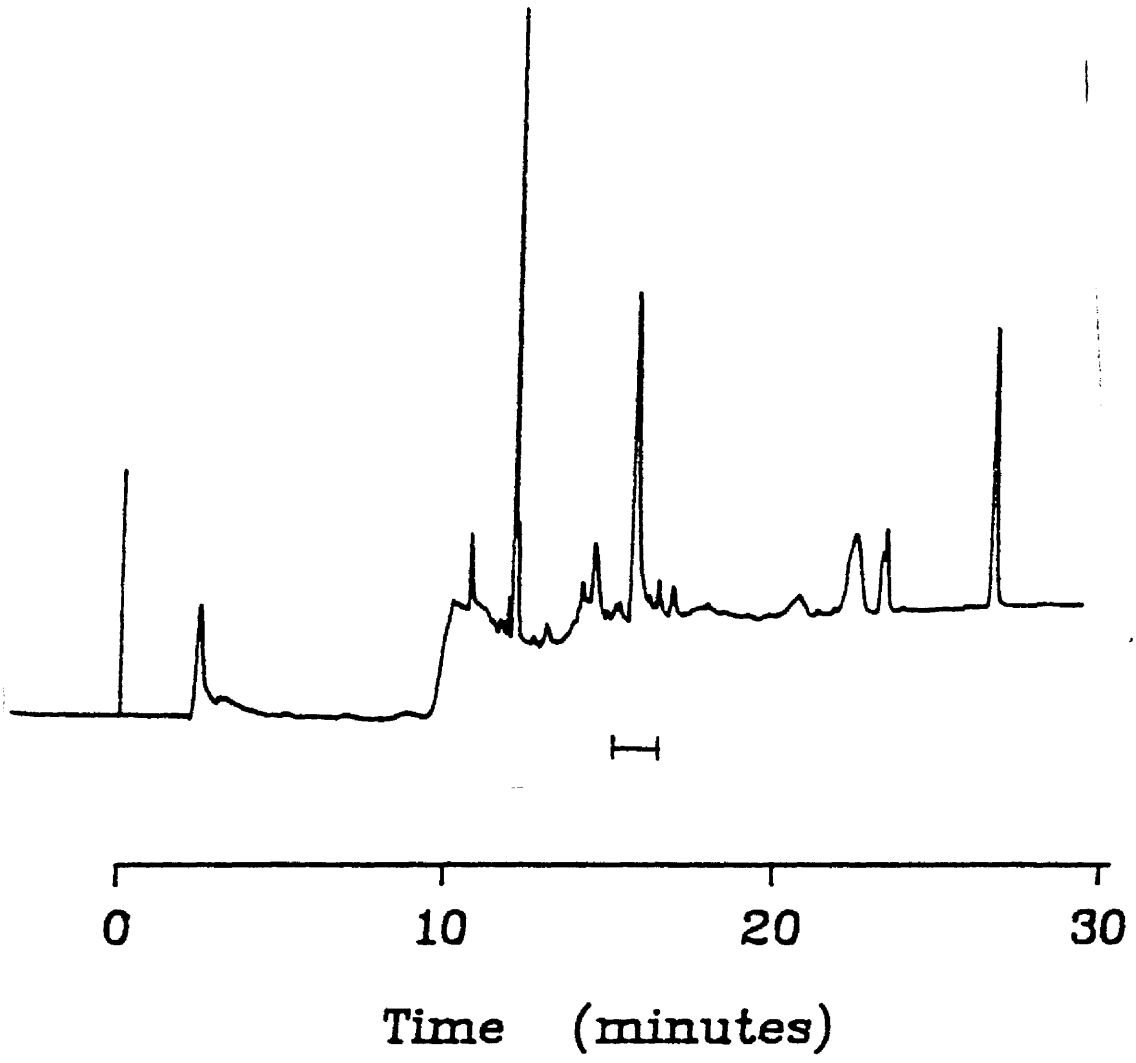


Figure 3.43. Reverse phase ion pairing HPLC chromatogram (TFA system) of the DMPO-derivatized B3 fraction from the spiked urine sample. The indicated region of the chromatogram, corresponding to RPPGF-DMPO, was collected.

maximized and that of the by-product was minimized using the modified acetylacetone condensation procedure. The indicated region of the chromatogram was collected, dried, treated with 3 M methanolic HCl and chromatographed by reverse phase ion pairing HPLC. Figure 3.44 presents the resulting chromatogram. RPPGF-DMPO-O-ME eluted at 31% acetonitrile on this HPLC system, and the indicated region of the chromatogram was collected and dried. After trifluoroacetylation, the sample was chromatographed by normal phase HPLC using a chloroform-methanol gradient system. The resulting chromatogram is presented in Figure 3.45. The downward baseline drift evident in this chromatogram is due to the increased UV transparancy of the solvent system as the methanol concentration increases. On this system, RPPGF-DMPO-N-TFA-O-ME eluted at 15% methanol in chloroform, and the indicated region of the chromatogram was collected.

The isolated derivative of fraction B3 from the spiked human urine sample was analyzed by DIP-MS-SIM under low (R=1,900-2,300) and high (R=15,000) resolution conditions, monitoring m/z 746, the molecular ion of the sought derivative of RPPGF. The resultant DIP-MS-SIM profiles are presented in Figures 3.46 and 3.47. Both of these analyses exhibit a single peak consistent with the presence of the RPPGF derivative. The sample was next analyzed by the more molecularly specific technique of DIP-CAD-SRM-MS, with the instrument set to monitor the formation of an m/z 429 ion from an m/z 746 precursor ion in the first field free region of the mass

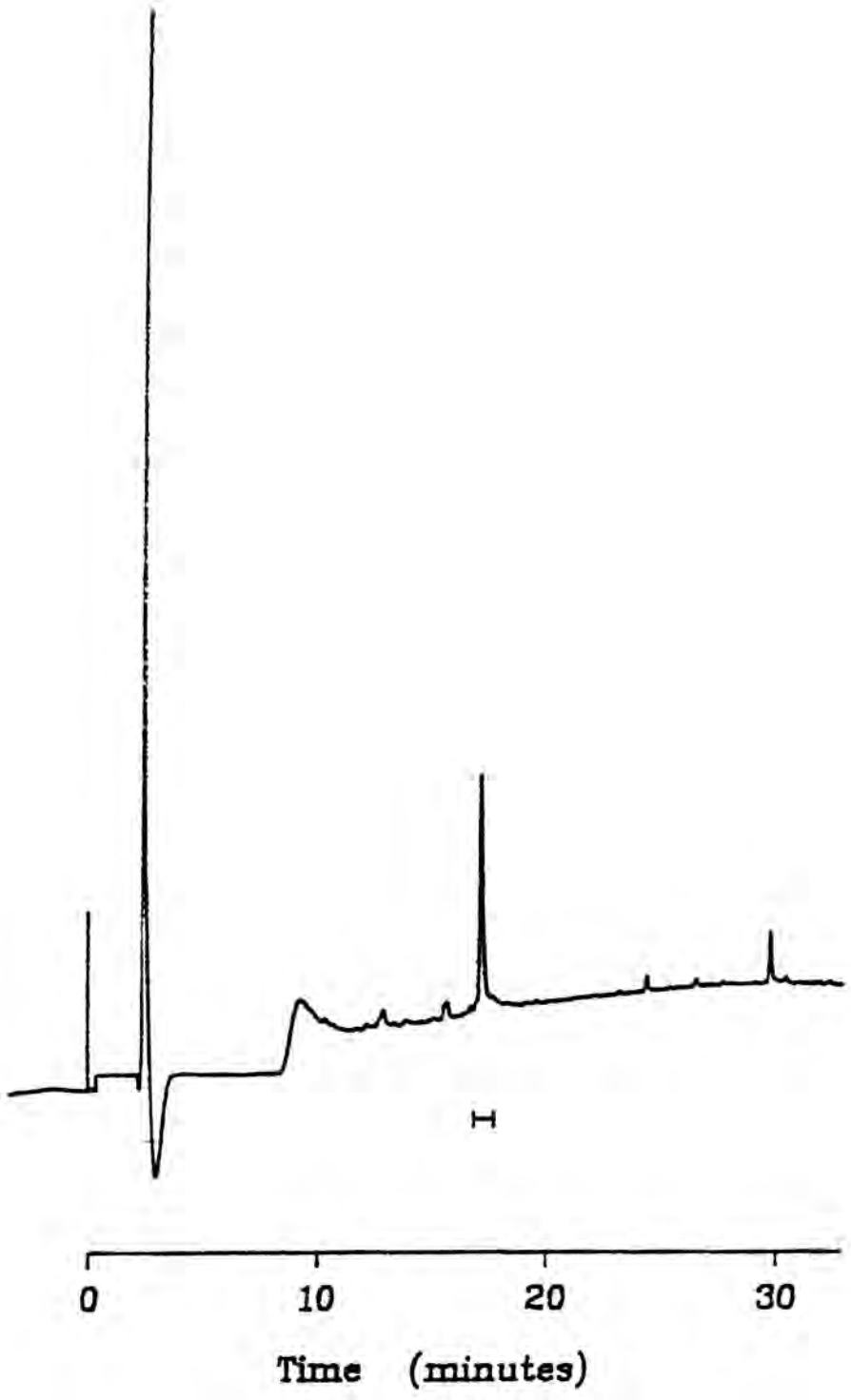


Figure 3.44. Reverse phase ion pairing HPLC chromatogram (TFA system) of the DMPO-derivatized and methyl-esterified B3 fraction from the spiked urine sample. The indicated region of the chromatogram, corresponding to RPPGF-DMPO-O-ME, was collected.

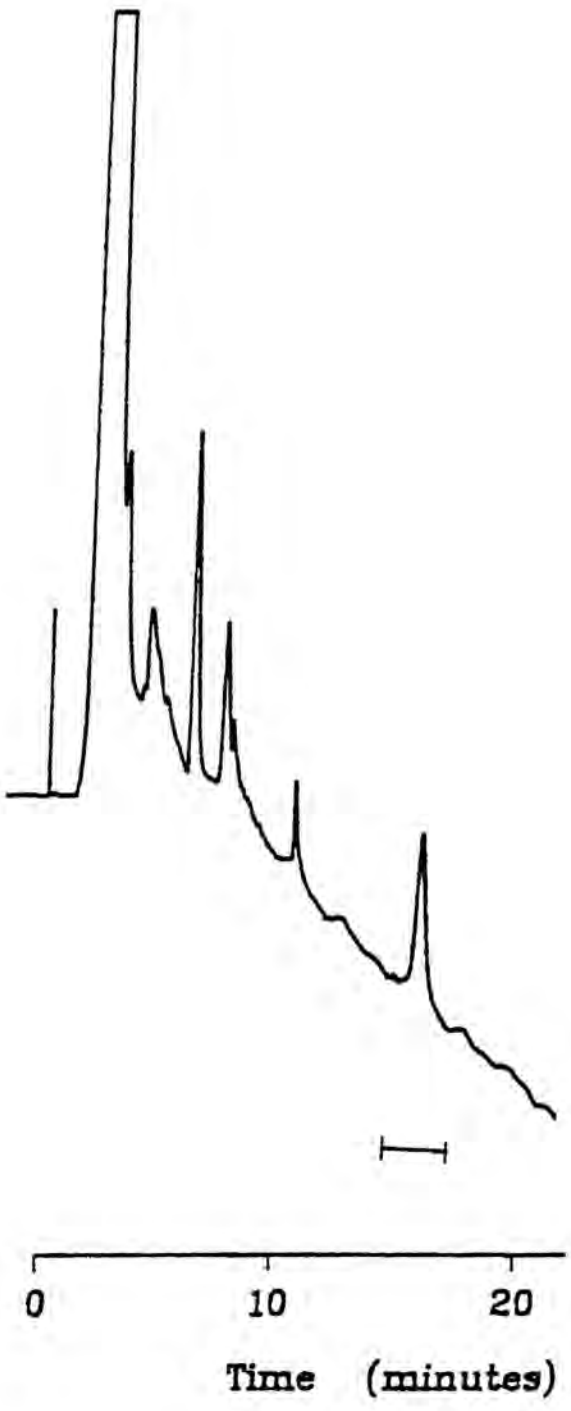


Figure 3.45. Normal phase HPLC chromatogram of the DMPO-derivatized, methyl-esterified and trifluoroacetylated B3 fraction from the spiked urine sample. The indicated region of the chromatogram, corresponding to RPPGF-DMPO-N-TFA-O-ME, was collected.

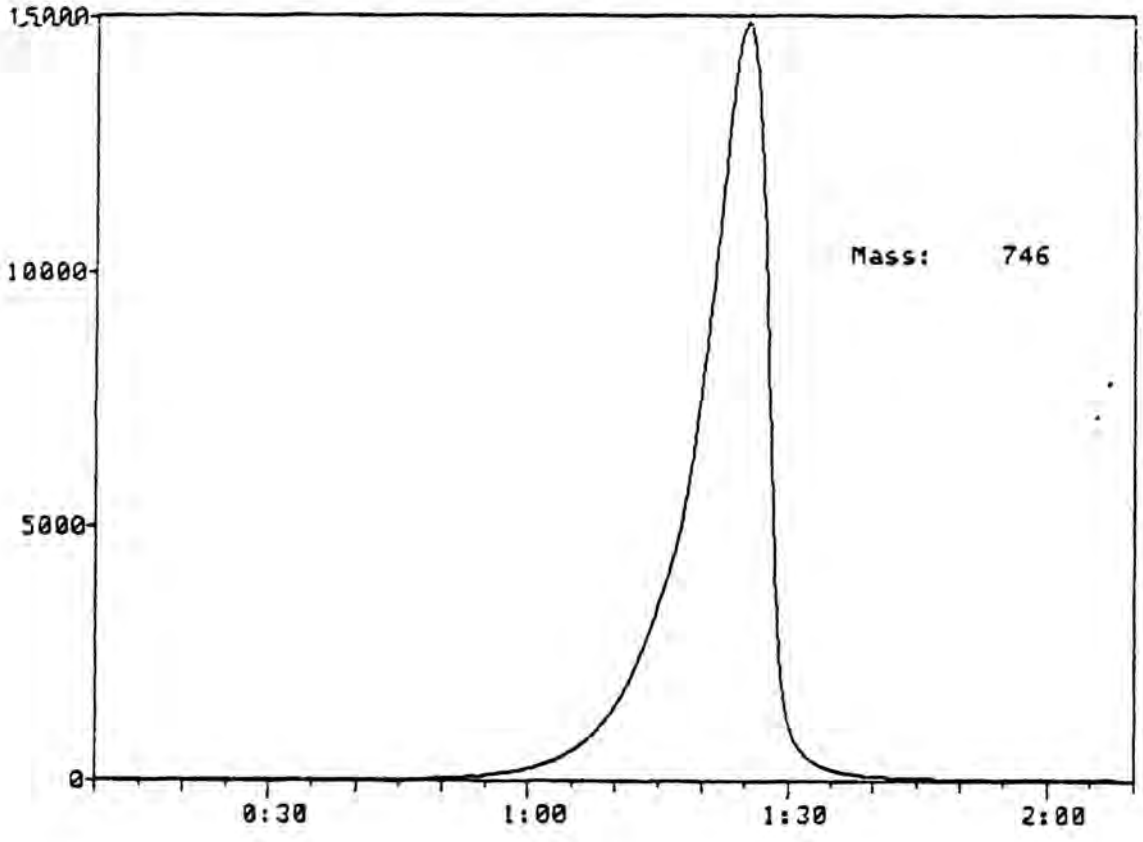


Figure 3.46. Low resolution (R=1,900-2,300) direct insertion probe selected ion monitoring trace (monitoring m/z 746) of the dimethyl-pyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of the B3 fraction isolated from spiked human urine.

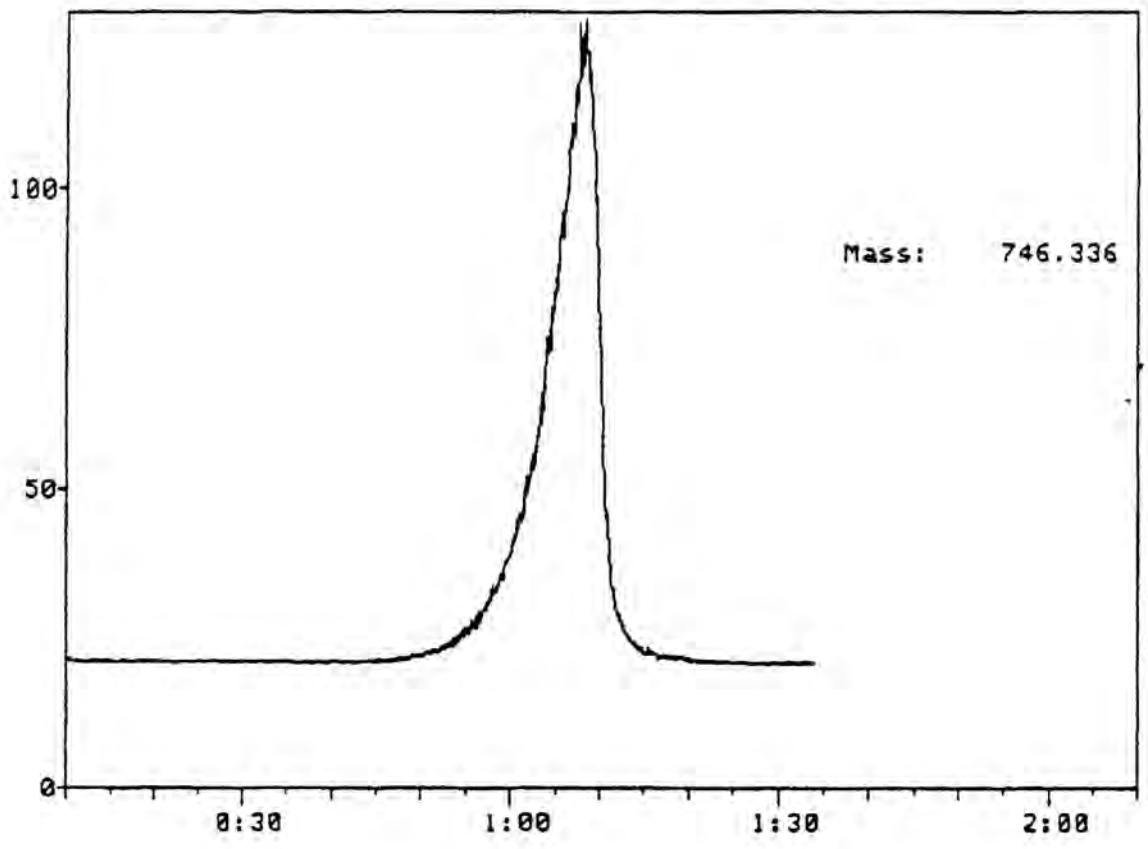


Figure 3.47. High resolution (R=14-15,000) direct insertion probe selected ion monitoring trace (monitoring m/z 746) of the dimethyl-pyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of the B3 fraction isolated from spiked human urine.

spectrometer. Figure 3.48 presents the resultant profile. Again, a single peak consistent with RPPGF is observed. The sample was then analyzed by repetitive scanning DIP-MS, yielding the electron impact mass spectrum presented in Figure 3.49, which is virtually identical to that of the standard material in Figure 3.39. As a final analysis, roughly equal quantities of the derivatized B3 fraction and derivatized standard peptide were mixed in a DIP sample vial and analyzed by DIP-MS under high resolution (R=15,000) conditions, scanning the molecular ion region of the mass spectrum in raw data acquisition mode. The resultant peak profiles are presented in Figure 3.50. The molecular ion and the higher mass isotope peaks appear as singlets, indicating that the elemental composition of the derivatized standard peptide and the derivatized B3 fraction are indistinguishable at this resolution. Collectively, the data presented in Figures 3.46 through 3.50 unambiguously identify the presence of the pentapeptide RPPGF in the B3 fraction from spiked human urine.

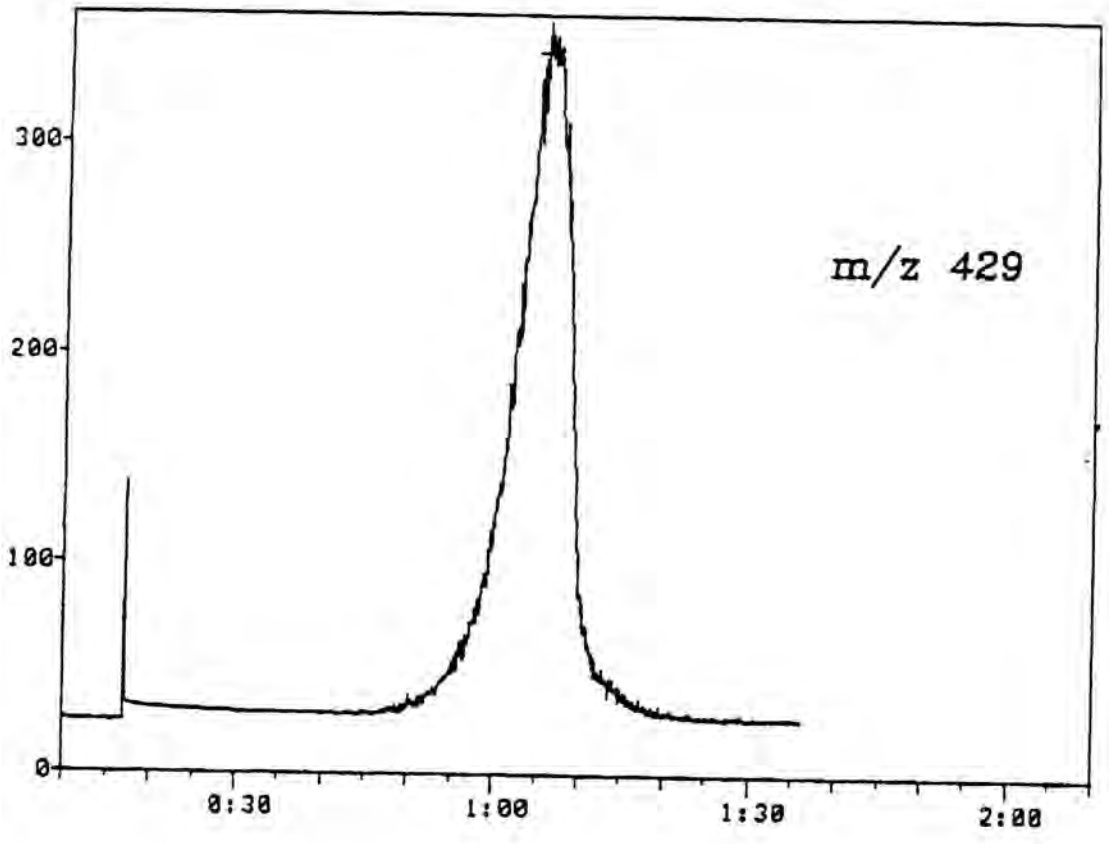


Figure 3.48. Direct insertion probe collisionally activated dissociation linked field selected reaction monitoring mass spectrometric analysis of the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of the B3 fraction isolated from spiked human urine.

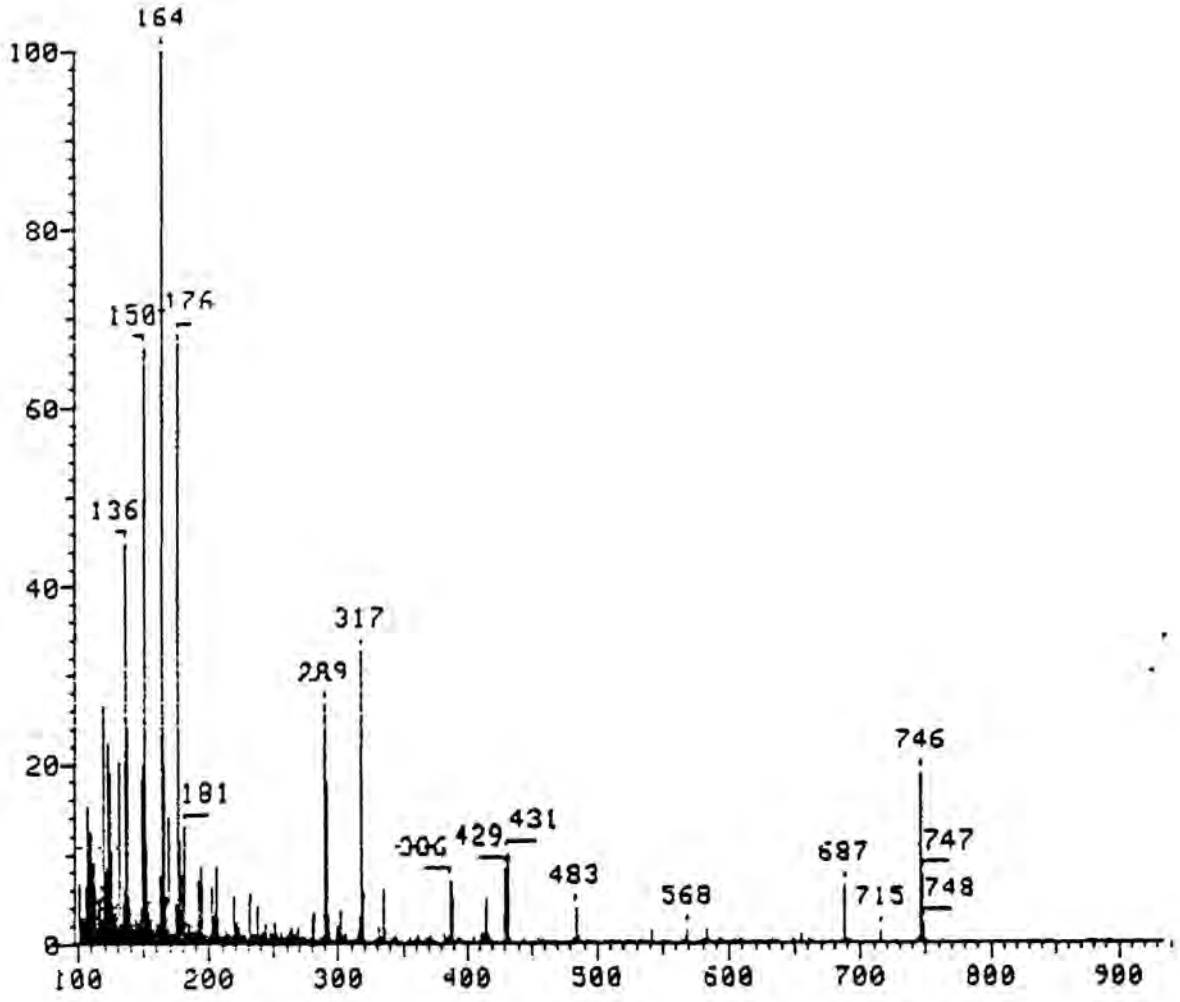


Figure 3.49. Electron impact mass spectrum of the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of the B3 fraction isolated from spiked human urine, from repetitive scanning DIP-MS analysis.

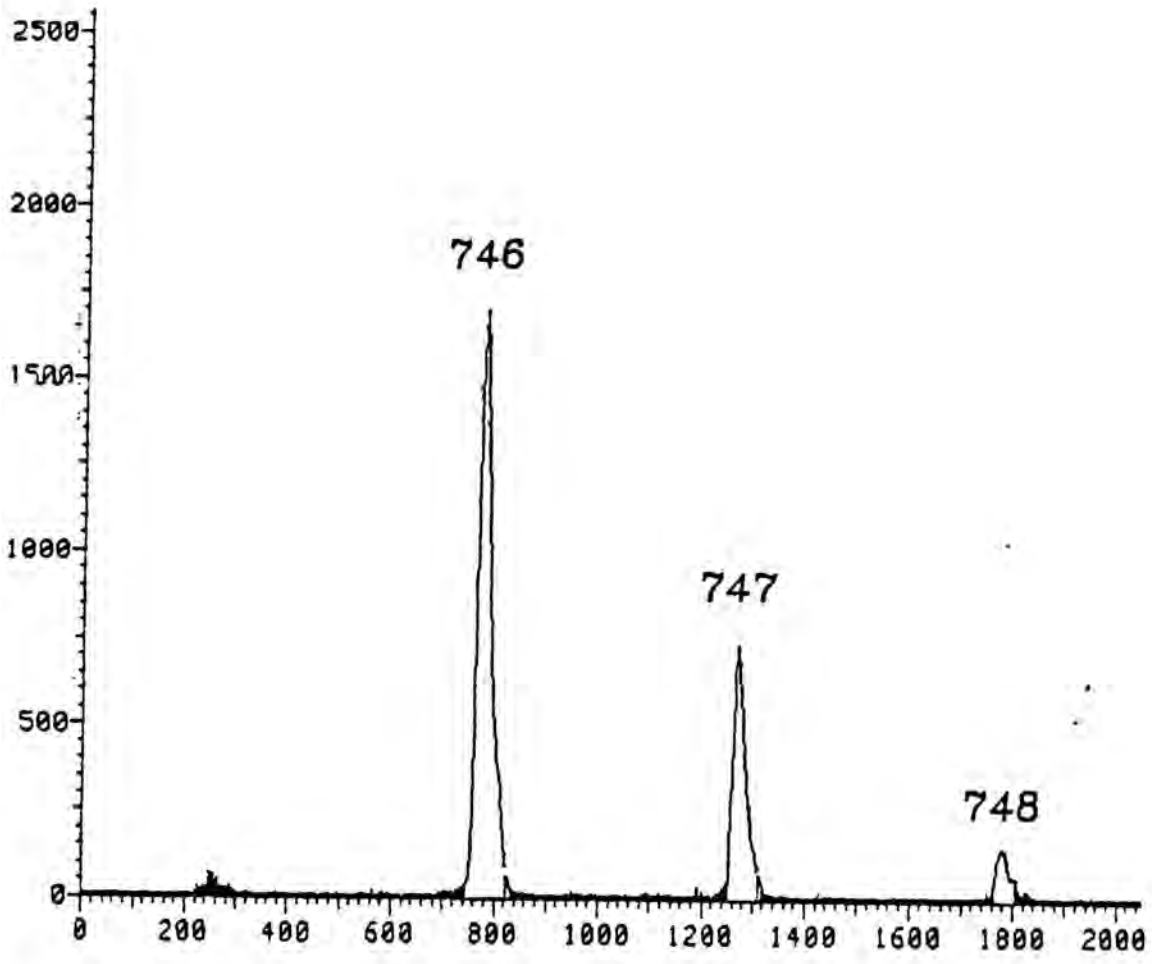


Figure 3.50. High resolution (R=14-15,000) scan of the region of the mass spectrum corresponding to the molecular ion of the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of RPPGF. The sample was a mixture of roughly equal quantities of the derivatized B3 fraction and derivatized authentic RPPGF.

CHAPTER IV

DISCUSSION

A. Polyaminoalcohol derivatives.

During the past thirty years, various forms of polyaminoalcohol derivatives have been used for determining the amino acid sequence of small peptides by gas chromatography-mass spectrometry. These derivatives have generally been used for the qualitative analysis of high nanomole to micromole amounts of peptides, with very few claims of successful qualitative or quantitative applications to lower level peptide samples (30,65). The application of these derivatives to low level samples has not been restricted due to mass spectrometric sensitivity limitations; rather, the problem has been inherent to the complex derivatization chemistry necessary for the formation of these derivatives. Detection limits have gradually been improved over the years, but the ultimate sensitivity which could theoretically be obtained with these derivatives has not yet been approached in practical terms. The present studies have pointed out some of the likely reasons for these persistent problems. These studies probably represent the first time that the sequential derivatives generated by the successive derivatization steps have been analyzed by direct chemical means, namely FAB-MS and, to some extent, HPLC. Fast atom bombardment mass spectrometry has only recently become generally available, and many advances in HPLC technology have been

made during the past decade. These factors rendered these sequential analyses possible, thus helping to identify some of the problems inherent in the most recently published version of the polyaminoalcohol derivatization scheme (17).

According to Carr and co-workers (17), ethereal diazomethane and methanolic HCl may be used virtually interchangeably for methylating the carboxylic acid functions of peptides. However, the present studies demonstrated that methanolic HCl is the superior reagent for this purpose. Diazomethane is an extremely reactive reagent (63), capable of reacting with with numerous functions likely to be present in a peptide by a variety of mechanisms; thus the use of diazomethane carries considerable risk of unwanted side reactions. Furthermore, peptides are generally poorly soluble in diethyl ether, thus necessitating prolonged reaction times which in turn increases the risk of side reactions. Methanolic HCl, on the other hand, is much less reactive than diazomethane, yet is a highly efficient reagent for methylating carboxylic acid groups. Furthermore, peptides are generally highly soluble in this reagent, thus increasing the likelihood of a high overall yield of the sought methyl ester.

Also in the paper published by Carr and co-workers, a procedure employing methyl trifluoroacetate was presented as an optimized means of trifluoroacetylating the amino functions of peptides. This procedure was found to be inefficient for the arginyl peptides employed in these studies. Steglich and Hinze (62) found that

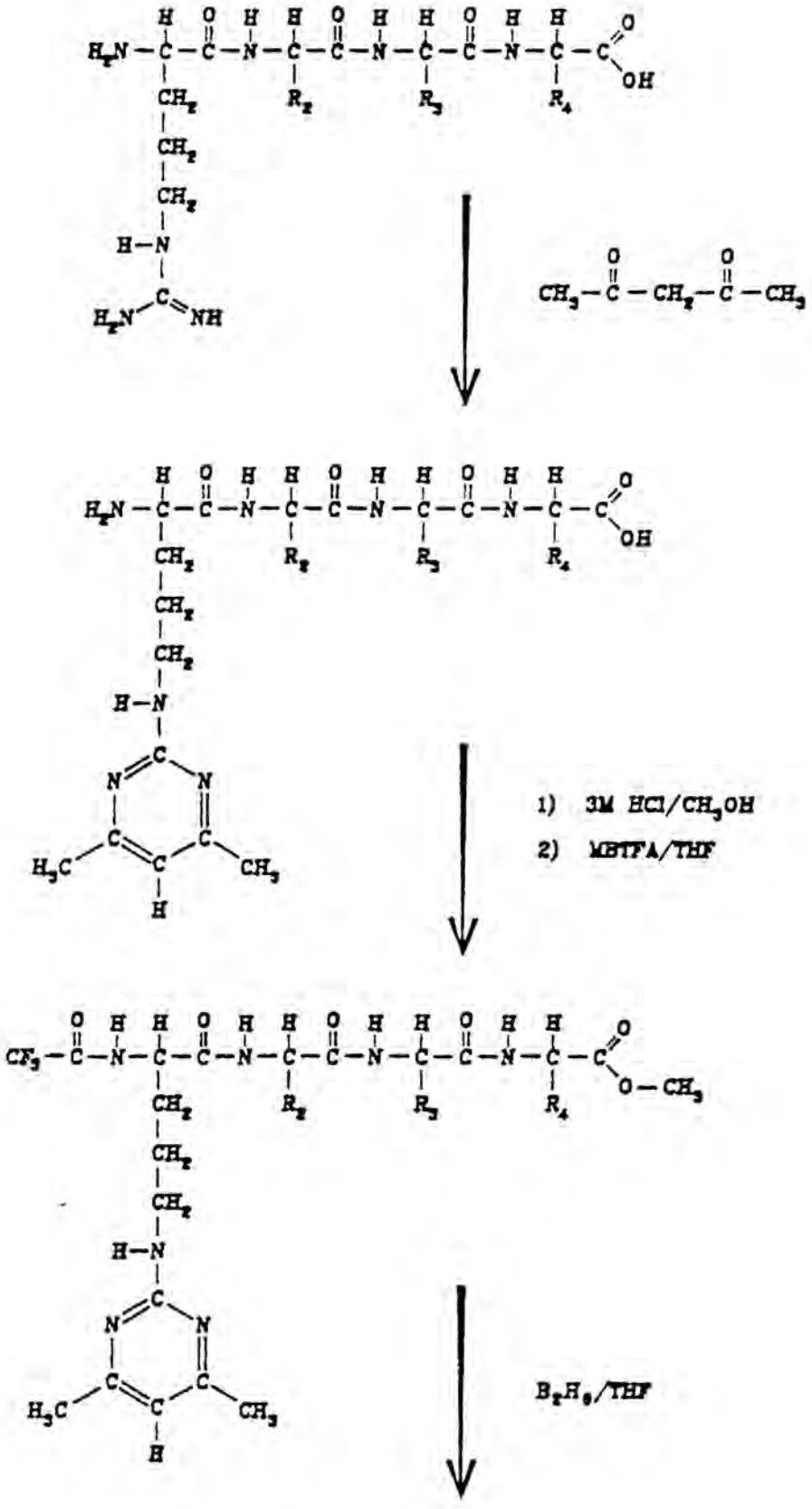
methyl trifluoroacetate reacts with different amino acids at varying rates, requiring from ten minutes to forty-eight hours for complete reaction, depending on the amino acid. Similar variability of reaction efficiency with different peptides is thus hardly surprising. The procedures developed during the present studies employing MBTFA as a trifluoroacetylating reagent proved to be highly efficient for the peptides studied, although whether these procedures will be generally applicable to other peptides remains to be determined.

The present studies demonstrated that employing the TBDMS group for derivatizing the hydroxy functions of polyaminoalcohols offers some significant advantages over the TMS group which has traditionally been used for this purpose. The TBDMS derivatives exhibit hydrolytic stability, which is not the case with TMS derivatives; one sample of the DMPO-N-TFE-O-TBDMS polyaminoalcohol derivative of RF, stored in pyridine solution at room temperature without special precautions, was stable for at least five weeks without apparent decomposition. Under electron impact ionization conditions, the TBDMS derivatives give rise to a pair of ions corresponding to $[M-15]^+$ and $[M-57]^+$; this easily recognized pair clearly denotes the molecular weight of the derivative. This information is frequently not obvious from the $[M-15]^+$ ions of TMS derivatives of polyaminoalcohols, which are typically of low intensity and may be easily confused with chemical noise. The potential disadvantage of slightly reduced volatility of the TBDMS group as compared to TMS was

found not to be prohibitive, since the TBDMS derivatives were sufficiently volatile for capillary GC-MS analysis of molecules as large as the pentapeptide RPPGF. The O-TBDMS polyaminoalcohol derivatives may thus represent a new class of derivatives useful for the amino acid sequencing of small peptides by GC-MS methods.

The present studies have shown that the condensation of the guanidino groups of arginyl peptides with acetylacetone can be successfully incorporated into the polyaminoalcohol scheme, thus ensuring volatilization of the guanidino groups without the risk of peptide bond cleavage. This represents an improvement over the hydrazinolysis method traditionally employed with the polyaminoalcohol scheme. The reaction sequence employed for forming the DMPO-N-TFE-O-TBDMS polyaminoalcohol derivatives is presented in Figure 4.1. These derivatives exhibit good volatility and excellent capillary gas chromatographic properties for arginyl peptides as large as RPPGF. Under both electron impact and fast atom bombardment ionization conditions, these derivatives generate several ions diagnostic for arginyl residues. The overall cleavage patterns observed with these derivatives were typical of the polyaminoalcohols, and thus the new derivatives generate complete amino acid sequence information in a fashion directly analogous to their well known O-TMS counterparts.

One of the goals of the present studies was to develop sensitive and reliable means of analyzing low level samples of arginyl peptides. Even with the improved derivatization methodologies



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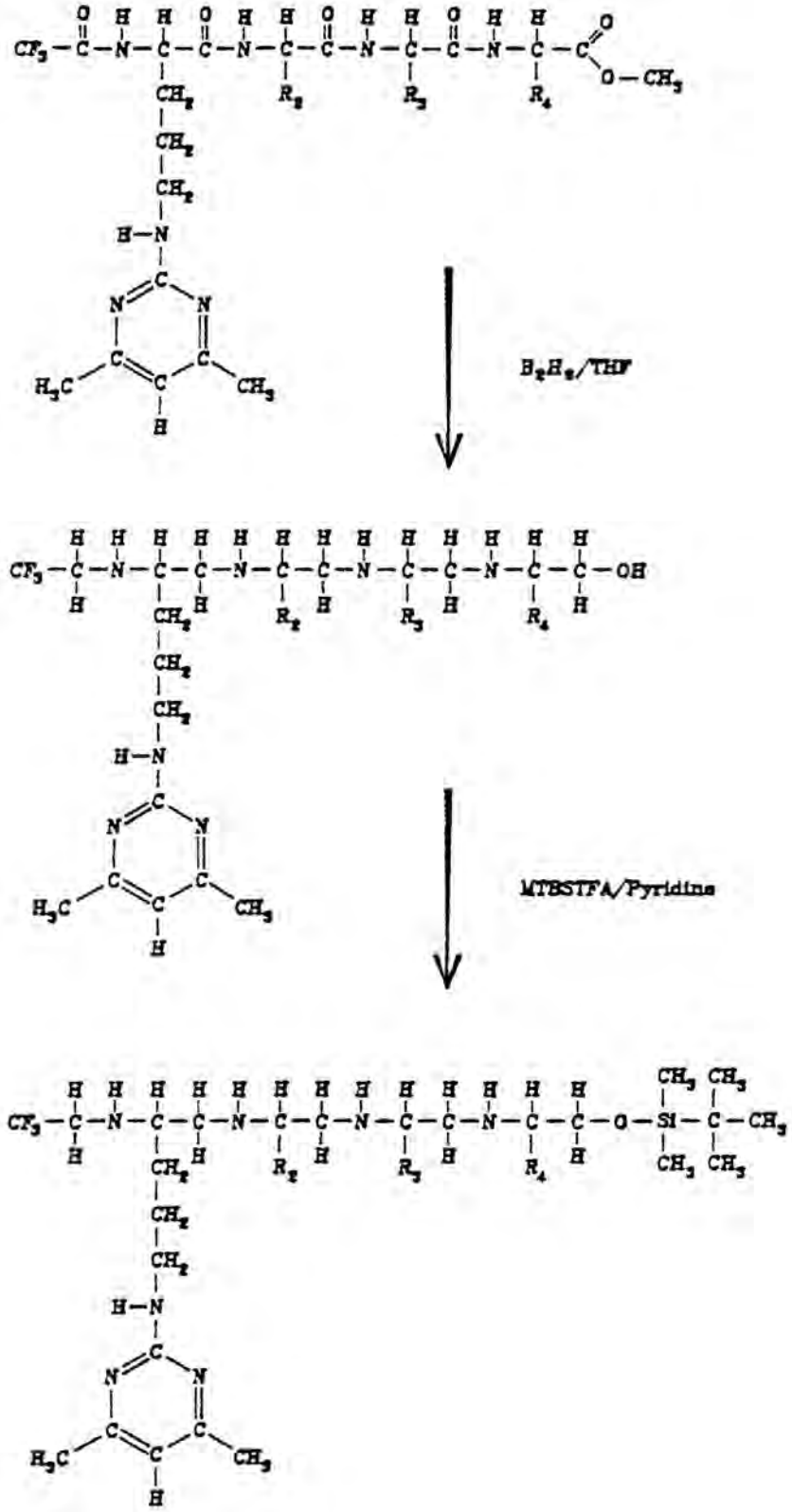

Figure 4.1. Reaction sequence for forming dimethylpyrimidylornithyl-N-trifluoroethyl-O-TBDMS polyaminoalcohol derivatives of arginyl peptides.

developed during the course of these studies, the polyaminoalcohol derivatives were not successfully utilized for this purpose. Problems arose when attempting to extend the sensitivity limits of these derivatives for quantitative purposes. For qualitative applications, e.g. repetitive scanning analyses, a threshold was identified at around 10-20 nanomoles of peptide above which sufficient derivative was generated to permit consistent qualitative identification of the peptides. Below this threshold derivative yield was quite variable, so that detection of the derivatives even by the highly sensitive method of capillary GC-MS-SIM was only sporadically successful. The criterion for high reliability in a quantitative method was thus not satisfied with the polyaminoalcohol derivatives. This judgement of poor reproducibility at low levels was based upon many repeated experiments, during which as little as 20 picomoles of RPP were successfully analyzed, and during which as much as 20 nanomoles of the pentapeptide were not successfully analyzed. Sporadic results such as these were clearly unacceptable for a general analytical method.

Two major areas were identified as the probable sources for the observed poor reproducibility with low level samples. The first of these was the diborane reduction reaction employed for converting the polyamide peptide backbone to the more volatile polyamine structure. For many years, lithium aluminum hydride was the reagent employed for this purpose. The use of diborane for the reduction, originally developed by Frank and Desiderio (16) and later improved

by Carr and co-workers, represented a significant advance for the polyaminoalcohol derivatives in that it permitted the derivatization and detection of lower level peptide samples than was possible with lithium aluminum hydride. However, the use of diborane still poses problems, to the extent that two identical low level samples treated identically by a single researcher can produce widely differing results. That the diborane reduction step was the problematic point in the overall sequence was demonstrable by FAB-MS. However, the specific nature of the problem, whether it is incomplete reduction of the polyamide backbone or ester groups, incomplete decomposition of the highly stable boroamine complexes, or a problem with the extraction process still remains unclear. Throughout the long history of the use of diborane for synthesizing secondary and tertiary amines from substituted amides, the nature of the series of reactions involved has never been rigorously elucidated. Further research on the nature of these reaction mechanisms could be of considerable benefit to future efforts to extend the sensitivity limits of the polyaminoalcohols.

The second source identified as a possible cause of the observed reproducibility problems with low level samples was residual reagents and reaction by-products which accumulate during the conversion of peptides to the fully derivatized polyaminoalcohols. The derivatization of an arginyl peptide to form the DMPO-N-TFE-O-TBDMS polyaminoalcohol involves treating the sample with ten successive reagents and reagent mixtures which consist of a total of

sixteen different chemical species. It is thus likely that the observed oily contaminants consisted of mixtures of trace levels of some of the derivatization reagents as well as by-products from the individual reactions. Accumulated contaminants from one reaction sequence could certainly have interfered with a subsequent derivatization step. Furthermore, with low level samples, the total amount of the accumulated contaminants may have been several orders of magnitude higher than the analyte, so that when nanogram quantities of the fully derivatized peptides were injected onto a capillary column, hundreds of micrograms of contaminants were co-injected. Repeated injections of low level samples was observed to cause rapid and irreversible deterioration of capillary column performance, particularly with the larger peptides. This was less problematic with the higher level samples for qualitative analysis, e.g. 20-50 nanomoles. However, the observed deterioration of column performance further reduced the reliability of the overall methods for quantitative applications with low level samples. The use of HPLC for the isolation of the derivatives in high purity might obviate this problem; however, the ultimate value of such a procedure would be questionable in light of the reproducibility problems associated with the diborane reduction reaction.

The dimethylpyrimidylornithyl-N,O-di-TBDMS derivatives of arginyl peptides which were briefly explored showed considerable promise in terms of the amino acid sequence information generated and the sensitivity for capillary GC-MS-SIM applications which might

ultimately be obtained. The reaction sequence employed for forming these derivatives is presented in Figure 4.2. One obvious advantage offerred by these derivatives was the elimination of two major derivatization steps and their attendant reaction by-products. A second potential advantage was the elimination of a possible unfavorable interaction due to the presence of both silicon and fluorine in the fully derivatized N-trifluoroethyl derivatives. During a study of propranolol metabolism (66), Walle and co-workers found that trifluoroacetyl derivatives partially decompose on gas chromatographic systems previously used with TMS derivatives, to the extent that entirely separate GC systems had to be used for the two derivative types (67). Similar results have since been obtained from capillary GC-MS analysis of silylated and trifluoroacetylated derivatives of adenosine (unpublished results from these laboratories). The silicon-flourine bond is one of the strongest known covalent bonds (68), and thus it was speculated that the observed deterioration of capillary GC column performance might be related to an unknown but highly favored anamolous interaction between silicon- or flourine-containing species in the accumulated contaminants and the derivatized peptides. Unfortunately, the reproducibility problems encountered with these derivatives prevented a complete assessment of this possibility. If reliable methodology for forming these derivatives can be developed in the future, these derivatives may prove to be very useful for both qualitative and quantitative applications.

B. Isolation procedures.

The arginyl peptide selective extraction procedure which was developed during the course of these studies was a modified version of a procedure developed by Rose and co-workers (55). While this procedure was found to be workable with standard samples of arginyl peptides in simple aqueous buffers, it was found not to provide the interference-free, high yield extracts which were anticipated. The high levels of contaminating species in the extracts which were observed by HPLC were probably residual reagents and reaction by-products generated during the derivatization and extraction process. These contaminants likely contributed to the surprisingly low binding observed with the phenylboronic acid columns employed. Because of the high levels of interfering species and the low binding capacity, this procedure was judged to be inappropriate for application to complex biological samples such as urine.

In order to develop efficient and reliable means for isolating bradykinin related arginyl peptides from complex matrices, advantage was taken of the favorable properties of silica based reverse phase matrices. Both low and high resolution reverse phase columns exhibit a high binding capacity with little risk of loss of low level analytes. Further advantage was taken of the shift in the elution position of peptides relative to non-peptide species which can be obtained through alternating the nature of the ion pairing reagent employed in successive chromatographic procedures. It was serendipitously discovered that water saturated with diethyl ether

containing heptafluorobutyric acid could elute all of the peptides of interest from reverse phase cartridge columns, and this formed the basis of one stage of selective elution. While the overall procedures developed were complex and time consuming, they nevertheless proved to be efficient means for isolating bradykinin related arginyl peptides from human urine, which is an especially complex matrix, and from which peptides are particularly difficult to isolate. It was reasoned that if these procedures could be successfully employed with the urinary matrix, they or variants of them could be used with practically any biological matrix, most of which are less complex than urine. Indeed, for simple systems such as cell culture supernatants, a single extraction followed by a single HPLC isolation step, with alternating ion pairing reagents, would probably prove sufficient for isolating these peptides in high purity for further analysis. It is anticipated that the isolation methods developed during these studies will be beneficial for future studies of bradykinin metabolism.

C. Derivatization and DIP-MS analysis of RPPGF.

Because the pentapeptide RPPGF was consistently the most difficult of the arginyl peptides studied to analyze by mass spectrometric means other than the relatively insensitive method of FAB-MS, and because this peptide is one of the more likely metabolites of bradykinin, efforts were directed toward developing sensitive, specific and reliable means of analyzing this compound.

The dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of RPPGF was employed for these studies. The scheme employed for forming this derivative is presented in Figure 4.3. This derivative offered a significant advantage over the polyamino-alcohol approach in the simpler derivatization chemistry necessary for its formation. In particular, the use of this derivative avoided the diborane reduction step, which was identified as a major source of unreliability at low levels with the polyaminoalcohols. Unfortunately, this derivative of the pentapeptide was insufficient-ly volatile for GC-MS analysis, and thus sufferred the disadvantage of a loss of the molecular specificity afforded by capillary GC-MS methods. However, this derivative had the unexpected advantage of a surprisingly intense molecular ion under electron impact ionization conditions; this intense molecular ion proved suitable for both selected ion monitoring and selected reaction monitoring, and thus allowed a high degree of molecular specificity in the assay to be regained. A similarly intense molecular ion was also observed with the dipeptide RF as the DMPO-N-TFA-O-ME derivative, which was sufficiently volatile for GC-MS analysis (data not shown). Thus, this may be a general phenomenon with this type of derivative of small arginyl peptides.

Once again, accumulated contaminants from successive deriva-tization reactions threatened the reliability of the overall method with low level samples by preventing the volatilization of the derivative in the mass spectrometer. With the pentapeptide, this

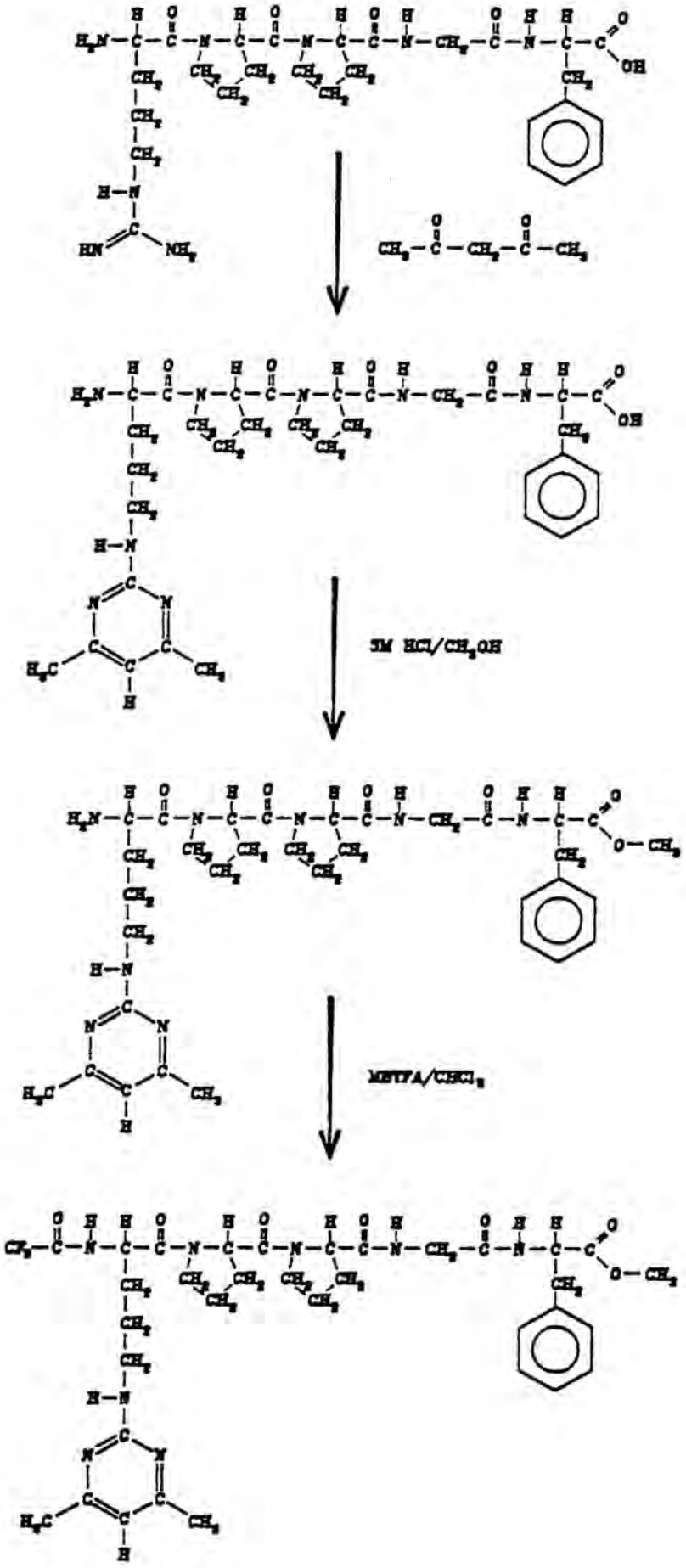

Figure 4.3. Reaction scheme for forming the dimethylpyrimidylornithyl-N-trifluoroacetyl-O-methyl ester derivative of RPPGF.

problem was obviated through HPLC isolation of the three sequential derivatives. Normal phase HPLC permitted the isolation of the final derivative in highly pure form, so that very intense signals were consistently obtained from DIP-MS analysis of low level samples under low resolution, high resolution and selected reaction monitoring conditions. Furthermore, the inclusion of three HPLC isolation steps in the derivatization process added an additional degree of molecular specificity.

The use of HPLC also permitted further optimization of the individual derivatization reactions. During the acetylacetone condensation reaction, a peptide related reaction by-product was observed, the formation of which increased with the prolonged heating conditions recommended in the literature (38). The optimized procedure developed during these studies permitted complete consumption of the underivatized peptide with minimal formation of this by-product. The trifluoroacetylation reaction was also optimized to permit maximal yield of the fully derivatized peptide. The overall yield of the derivative using the new procedures was estimated at 50-60%, as compared with around 20% with the non-optimized procedures, based upon HPLC analysis.

These high-yield, high-purity derivatization methods, in combination with the highly sensitive techniques of DIP-MS, permitted the unequivocal qualitative identification of RPPGF isolated from spiked human urine at the 12 nanomole level (one third of a 35 nanomole sample). These overall methods should be readily

applicable to quantitative analyses with stable isotope labeled internal standards.

D. Conclusions.

The analysis of bradykinin metabolites could ultimately prove to be a powerful means of evaluating the activity of the kallikrein-kinin system. From enzymatic and other studies, N-terminal fragments of bradykinin are likely to be significant metabolites, and may be useful for quantitating the flux of bradykinin through the kallikrein-kinin-degradation system. The studies described here have focussed on developing means for isolating and analyzing the N-terminal tri- through pentapeptide fragments of bradykinin with high sensitivity, molecular specificity and reliability. Efficient but time consuming means for isolating all three of these compounds have been developed. The developed mass spectrometric analytical methodology was most highly refined with the pentapeptide RPPGF during these studies, but the new methods should be readily extendable to the shorter N-terminal peptides. A relatively low level (spiked) sample of RPPGF was successfully isolated and unequivocally identified from the exceedingly complex biological matrix of human urine. It is hoped that the isolation and analytical techniques described here will facilitate the elucidation of bradykinin metabolism in biological systems.

PART II

OTHER STUDIES

Chapter V

USE OF O-(−)-MENTHYL-N,N'-DIISOPROPYLISOUREA FOR THE PREPARATION OF DIASTEREOMERIC MENTHYL ESTERS FOR THE CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERIC CARBOXYLIC ACIDS

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INTRODUCTION

One of the techniques commonly employed in the separation of enantiomers involves the preparation of diastereomeric derivatives using an optically pure chiral derivatizing reagent. The diastereomers are then separated by various chromatographic means, and the resulting elution patterns reflect the nature of the underivatized material. The purpose of the study presented here was to examine the usefulness of O-(−)-menthyl-N,N'-diisopropylisourea as a reagent for preparing (−)-menthyl esters of enantiomeric carboxylic acids.

Previously used methods for the preparation of menthyl esters include acid catalyzed esterification using (−)-menthol (69). This method requires the use of dry hydrogen chloride gas and a very large excess of (−)-menthol. Menthyl esters have also been prepared by first converting the acid to the acid chloride by refluxing with thionyl chloride (70) or oxalyl chloride (71), followed by treatment of the acid chloride with (−)-menthol. While all of these methods produce the desired end-product, namely diastereomeric (−)-menthyl esters of enantiomeric acids, the derivatization procedures themselves are somewhat involved and require considerable care in the maintenance of anhydrous conditions. Among the proposed advantages of O-(−)-menthylN,N'-diisopropylisourea ((−)-MDI) as a chiral derivatizing reagent are the simple techniques involved with its use, the completeness of its reaction with carboxylic acids, and the lack of potential racemization during derivatization.

(−)-MDI is an adduct of (−)-menthol and N,N'-diisopropylcarbodiimide, as shown in Figure 5.1. This reaction is catalyzed by monovalent copper. The derivatization reaction, depicted in Figure 5.2, produces the two diastereomeric esters and N,N'-diisopropylurea. Tertiary amines catalyze the derivatization; triethylamine was used as the catalyst in these studies.

Naphthoxylactic acid (2-hydroxy-3-(1-naphthoxy)propanoic acid; NLA), a major urinary metabolite of the β-adrenergic antagonist propranolol, and mandelic acid were used as test samples for the (−)-MDI reagent. The structures of these compounds are shown in Figure 5.3. Both of these acids have one chiral center. The diastereomeric derivatives were analyzed by capillary gas chromatography (GC) and capillary gas chromatography-mass spectrometry (GC-MS).

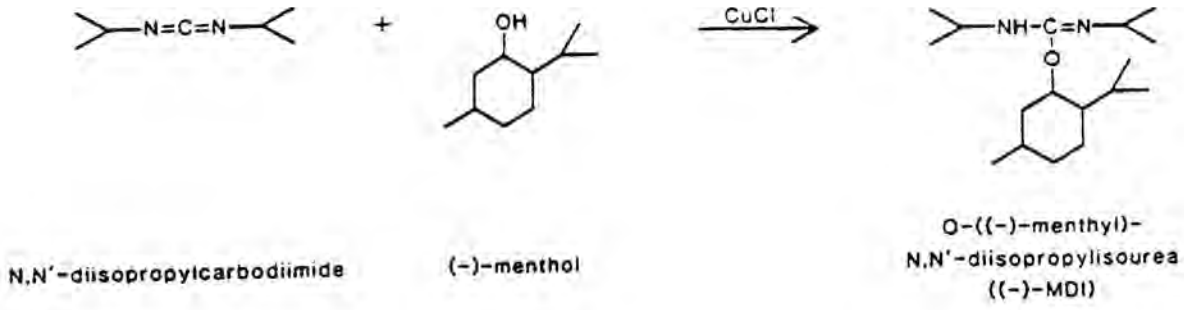

Figure 5.1. Synthesis of O-(−)-menthyl-N,N'-diisopropylisourea.

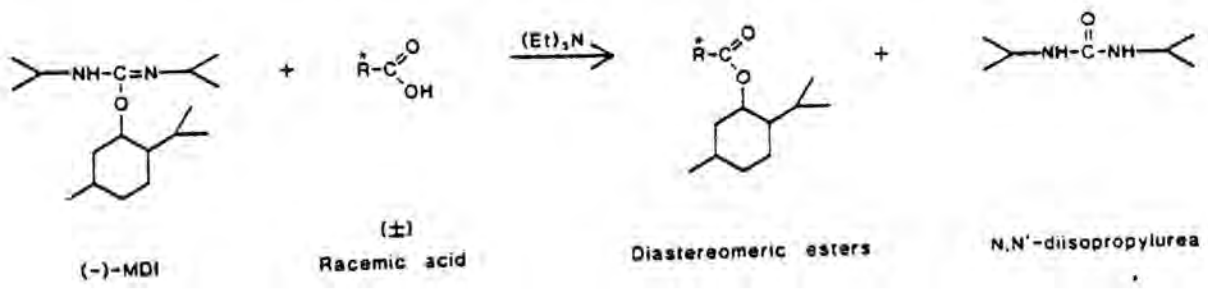

Figure 5.2. Derivatization of the carboxylic acid group with O-(−)-menthyl-N,N'-diisopropylisourea.

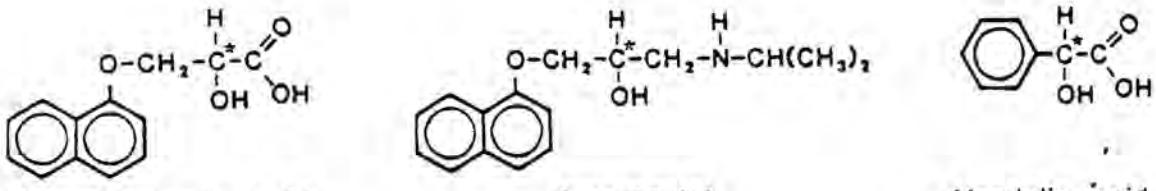

Figure 5.3. Structures of naphthoxylactic acid, propranolol and mandelic acid. The asterisks (*) indicate chiral centers.

MATERIALS AND METHODS

Synthesis of O-(−)-menthyl-N,N'-diisopropylisourea.

(−)-Menthol (7.8 g, 0.050 mol; Mallinkrodt, New York, NY, USA) and N,N'-diisopropylcarbodiimide (6.3 g, 0.050 mol; Aldrich, Milwaukee, WI, USA) were dissolved in 25 ml tetrahydrofuran (Aldrich). Copper(I) chloride (10 mg; Fisher, Fair Lawn, NJ, USA) was added, and the mixture was stirred at room temperature overnight. The mixture was then evaporated in vacuo to 8 ml and chromatographed on a 250 g Al_2O_3 (W200 Basic, Woelm, Eschwege, GFR) column eluting with 250 ml tetrahydrofuran. The tetrahydrofuran was removed by evaporation in vacuo, yielding 5.86 g of (−)-MDI (41.6% of theoretical) as a pale yellow liquid. No appreciable decomposition of (−)-MDI was detected four years after synthesis.

Other reagents.

(±)-Naphthoxylactic acid was prepared in these laboratories by a procedure analogous to that reported by Nelson and Bartols (72). Racemic mandelic acid was obtained from Chem Service (Westchester, PA, USA); (−)-mandelic acid and (+)- mandelic acid were obtained from Aldrich, triethylamine from Eastman Kodak (Rochester, NY, USA), and ethyl acetate from Fisher. All solvents were ACS reagent grade or better. Ethereal diazomethane was generated from Diazald (Aldrich).

Equipment.

Capillary GC analyses were performed using a Varian 3700 gas chromatograph equipped with a split injector and a flame ionization detector. The column was a Grade A 60 m x 0.25 mm I.D. SP-2100 wall coated open tubular (WCOT) glass capillary obtained from J&W Scientific (Orangeville, CA, USA). The straightened capillary ends were deactivated with Carbowax 20M.

Capillary GC-MS analyses were performed using the same gas chromatograph and column interfaced through an open split interface to a Finnigan MAT 212 mass spectrometer with a Spectrosystem SS200 data system.

Derivatization of enantiomeric acids.

A 1 mg amount of each sample acid was dissolved in 400 µl of a solution of tetrahydrofuran-triethylamine (9:1; v/v); 10 µl of (−)-MDI were added, and the reaction mixture was capped tightly in a vial and heated at 100 °C for 16 hours. The completeness of reaction was monitored by forming the methyl ester of any unreacted acid by derivatization with diazomethane in diethyl ether followed by capillary GC analysis. No methyl ester peaks were observed after 16 h heating with triethylamine as a catalyst.

Capillary GC-MS resolution of diastereomers.

The diastereomeric menthyl naphthoxylactates were resolved under the following conditions: injector temperature 260 °C; split

ratio 1:350; column temperature programmed from 225 °C to 240 °C at a rate of 0.2 °C/min; average linear velocity 21-22 cm/sec; open split interface temperature 260 °C; ionization energy 70 eV. The conditions for the resolution of the diastereomeric menthyl mandelates were identical except that the injector temperature was 200 °C and the column temperature was programmed from 165 °C to 180 °C at 0.3 °C/min. Both analyses were complete before the final column temperature was reached. During both analyses the mass spectrometer was scanned from 45 to 400 amu at a rate of 3 sec/decade with an interscan time of 1 sec.

Determination of elution order.

Menthyl mandelates. R-(−)-Mandelic acid and S-(+)-mandelic acid were separately derivatized. (In the Cahn-Ingold-Prelog system of chiral notation, used here for the purposes of consistency, D-(−)-mandelic acid has the R configuration.) Aliquots of the two solutions were mixed in a 4:1 (v/v) ratio (S:R and R:S) and analyzed by capillary GC. Elution order was determined based upon the retention times of the separately derivatized R and S standards and the relative peak intensities of the two mixtures.

Menthyl naphthoxylactates. Because of the unavailability of optically pure R- and S- naphthoxylactic acid, and because NLA is a major urinary metabolite of propranolol, R-naphthoxylactic acid was extracted from the urine of a rat dosed with S-(−)-propranolol. The sample was a 24-hour urine collection from a male Sprague-Dawley rat

injected intraperitoneally with 10 mg/kg of S-(−)-propranolol hydrochloride (Ayerst Labs., New York, NY, USA). The total urine volume was 14 ml. A 2 ml aliquot was adjusted to pH 1.5 with 6 N hydrochloric acid and extracted with 2 x 5 ml of ethyl acetate. The combined organic phases were evaporated to dryness under a stream of nitrogen; the residue was derivatized using 90 µl of tetrahydrofuran-triethylamine (9:1) and 10 µl of (−)-MDI. A 6 µl aliquot of this solution was mixed with 6 µl of previously derivatized racemic NLA of comparable concentration. The resulting mixture was analyzed by selected ion monitoring (m/z 370, the molecular ion) capillary GC-MS. The elution order was determined by comparing the relative peak intensities of derivatized racemic NLA with and without the added derivatized urinary R-NLA.

RESULTS

(−)-MDI was found to react to completion with carboxylic acids to form menthyl esters after 16 h heating in tetrahydrofuran with triethylamine as a catalyst.

The retention behavior of the diastereomeric menthyl naphthoxylactates and menthyl mandelates from capillary GC-MS analysis is presented in Table 5-I. The retention times presented are adjusted for the void volume of the system. Exemplary chromatograms from the two analyses are presented in Figures 5.4 and 5.5. Figure 5.4 is a retrospective single ion plot of the molecular ion (m/z 370) of menthyl naphthoxylactate. No molecular ion was observed for the menthyl mandelates; the plot presented in Figure 5.5 is a retrospective plot of m/z 107, corresponding to the $[(C_6H_5)C(OH)H]^+$ fragment of menthyl mandelate. Both sets of diastereomers were resolvable on an SP2100 WCOT capillary column with slight peak overlap. Baseline separation was obtainable under isothermal column conditions (225 °C for the menthyl naphthoxylactates and 170 °C for the menthyl mandelates) with some sacrifice in analysis time and peak broadening.

The elution order of the menthyl mandelates was determined by comparing the retention times of the separately derivatized optically pure mandelic acid enantiomers, and by comparing the relative peak intensities of 4:1 (v/v) mixtures of the separately derivatized enantiomers. Under the conditions of the analyses, (−)-menthyl-R-(−)-mandelate eluted with a peak maximum at 40.5 min (adjusted

TABLE 5-I

RETENTION BEHAVIOR OF THE DIASTEREOMERIC MENTHYL NAPHTHOXYLACTATES AND MENTHYL MANDELATES ON AN SP2100 WCOT CAPILLARY COLUMN

Analysis	Column temperature	Adjusted retention time* (min, ± 0.1)	Carboxylic acid enantiomer	
Menthyl naphthoxy-lactates	225-240 °C at 0.2 °C/min	51.2 53.7	R-(−) S-(+)	**
Menthyl mandelates	165-180 °C at 0.3 °C/min	40.5 42.6	R-(−) S-(+)	***

* The elution time of a non-retained compound (butane) was subtracted from the absolute retention times to obtain the adjusted retention times.

** Determined from the data presented in Table 5-II.

*** Determined from chromatograms of derivatized optically pure mandelic acid standards.

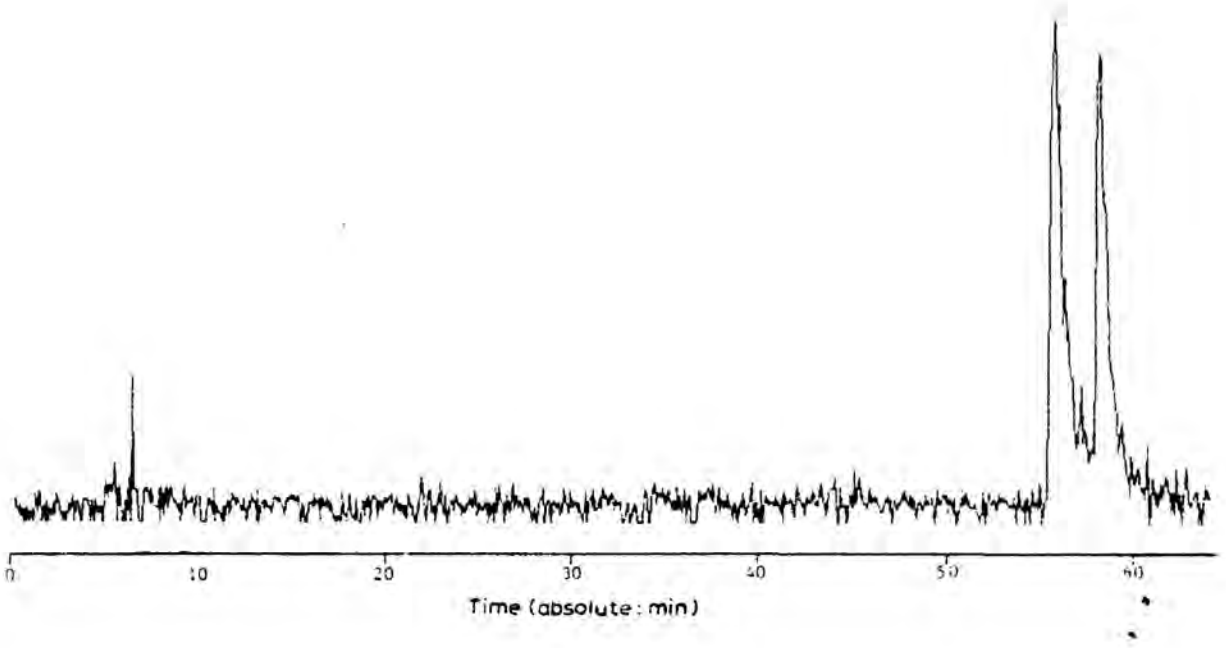

Figure 5.4. Capillary GC-MS chromatogram of the diastereomeric menthyl naphthoxylactates. A 3 µl sample was injected (7-8 µg) at a split ratio of 1:350. The chromatogram is a retrospective single ion plot of the molecular ion (m/z 370).

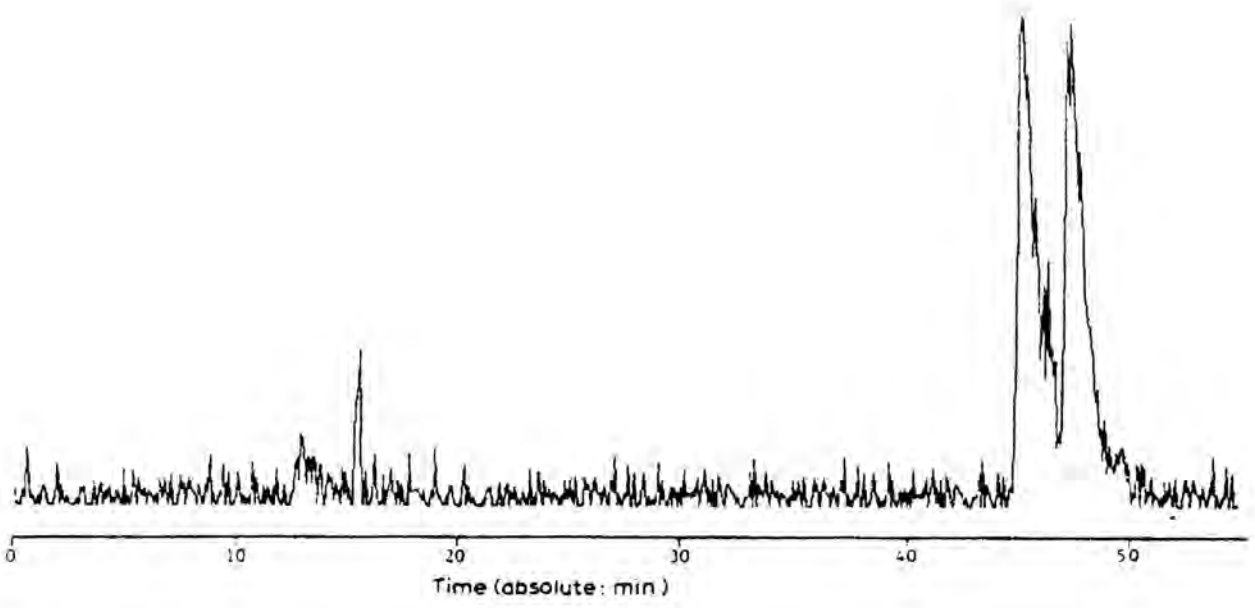

Figure 5.5. Capillary GC-MS chromatogram of the diastereomeric menthyl mandelates, as a retrospective single ion plot of the [(C6H5)C(OH)H]+ fragment (m/z 107) of menthyl mandelate. The sample size was 3 µl (7-8 µg) injected with a split ratio of 1:350.

retention time), and (−)-menthyl-S-(+)-mandelate eluted at 42.6 min. These retention times were reproducible to within ± 0.1 min. The relative peak intensities of the two 4:1 mixtures were consistent with the above elution order.

In order to determine the order of elution of the menthyl naphthoxylactates, R-naphthoxylactic acid (R-NLA) was extracted from the urine of a rat dosed with S-(−)-propranolol. The shift in the notation of the absolute configuration derives from the shift in priorities in the Cahn-Ingold-Prelog system of notation. It is assumed that the chiral center of the β-blocking side chain of propranolol remains unaltered during metabolism to NLA. The extracted R-NLA was derivatized and mixed with derivatized racemic NLA. Table 5-II presents the relative peak intensities of this mixture and those of derivatized racemic NLA alone (from selected ion monitoring capillary GC-MS, monitoring m/z 370). From the results of the work of Nelson and Bartols (72), R-NLA is the negative rotating isomer. (−)-Menthyl-R-(−)-naphthoxylactate was found to elute before (−)-menthyl-S-(+)-naphthoxylactate on an SP2100 column.

TABLE 5-II

DETERMINATION OF THE ORDER OF ELUTION OF THE DIASTEREOMERIC MENTHYL NAPHTHOXYLACTATES FROM SELECTED ION MONITORING (m/z 370) CAPILLARY GC-MS

Derivatized sample	Adjusted retention time (min, ± 0.1)	Relative peak intensity
Racemic NLA	51.2	0.95
	53.7	1.00
Urinary R-NLA and recemic NLA	51.2	1.50
	53.7	1.00

DISCUSSION

O-(−)-menthyl-N,N'-diisopropylisourea has been synthesized and found to be useful for the preparation of diastereomeric (−)-menthyl esters of enantiomeric acids. (−)-Menthyl esters (from (−)-menthol) have been used in the past in the diastereomeric separation of lactic and glyceric acids (69), acyclic isoprenoid acids (70), phytanic (73) and pristanic (74) acids, and chrysanthemic acids (75). The techniques involved with the use of (−)-MDI are relatively simple, with the minor disadvantage of the requirement of overnight heating in order to effect completeness of reaction. While this study has demonstrated the applicability of (−)-MDI to analytical samples, it could be useful on a preparative scale as well. For instance, a large quantity of a racemic acid could be treated with (−)-MDI, and the resulting diastereomers resolved by preparative high performance liquid chromatography. The separate esters could then be hydrolyzed and the optically pure enantiomers isolated. Such a procedure may offer advantages over repeated fractional crystallization in terms of yield, purity, or both.

The determination of the order of elution of the menthyl mandelates was straightforward due to the availability of optically pure standards and the extremely reproducible retention times obtainable from capillary GC techniques. While the determination of the elution order of the menthyl naphthoxylactates was indirect, it afforded a further demonstration of the usefulness of (−)-MDI in its

application to the concentration levels of samples obtained from biological fluids. (−)-MDI thus has a wide range of potential applications as an analytical tool, and could be useful for preparative work as well.

CHAPTER VI

RESOLUTION OF THE SEVEN ISOMERIC RING-HYDROXYLATED PROPRANOLOLS AS *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES BY CAPILLARY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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INTRODUCTION

Three previously unknown ring-hydroxylated metabolites of the β-adrenergic antagonist drug propranolol were recently identified by Walle and co-workers (66). In rats and in man, 2-hydroxypropranolol (2-HO-P), 5-HO-P, and 7-HO-P were found, as well as the previously known 4-HO-P (76-80), which is the major monohydroxylated urinary metabolite of propranolol in both species, and the only hydroxylation product identified in the dog. Briefly, these compounds were identified in the following fashion. Urine samples were treated enzymatically to hydrolyze glucuronide conjugates; then the monohydroxypropranolols were extracted and analyzed as trimethylsilyl (TMS) and trifluoroacetyl (TFA) derivatives by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) using packed column techniques (3% OV-1). Identification of the isomers was based upon comparison of retention times and mass spectra with those of derivatized synthetic monohydroxypropranolols (66,81). Two derivatization techniques were employed because, under the conditions of the analyses, 2-HO-P and 8-HO-P were unresolved as TMS derivatives, and 4-HO-P and 5-HO-P were unresolved as TFA derivatives. Another complication encountered was that as TFA derivatives the propranolol peak overlapped with the 2-HO-P peak, necessitating the use of selected ion monitoring to distinguish these two compounds. Furthermore, since TFA derivatives partially decompose on columns previously used with TMS derivatives, separate columns had to be used for the two types of derivatives.

The purpose of the work presented here was to develop a simpler technique for separating and identifying the seven isomeric ring-hydroxylated propranolols in order to facilitate future studies with these compounds. Such a technique has been developed, using a relatively new silylating reagent, N-methyl-N-(tert.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), which produces volatile tert.-butyldimethylsilyl (TBDMS) derivatives of aliphatic and aromatic hydroxy groups (46), yielding mono-derivatized propranolol (TBDMS-P) and di-derivatized hydroxypropranolols (TBDMS-HO-P), the structures of which are presented in Figure 6.1.

When pyridine is used as a solvent for the derivatization, secondary amines are derivatized very slowly by MTBSTFA (46); and, with the TBDMS group attached to the aliphatic oxygen which occurs far more rapidly in pyridine (46), derivatization of the secondary amine group is further sterically hindered. No tri-derivatized hydroxypropranolols were observed during the course of this study. (It may be possible to produce the tri-TBDMS derivatives with acetonitrile as the solvent (45,46,59b) but these derivatives would be expected to be less volatile than the di-TBDMS derivatives.) Separations were carried out using glass capillary GC-MS. The applicibility of the method was demonstrated by analysis of a urine sample from a rat dosed with (±)-propranolool and identification of the ring-hydroxylated metabolites.

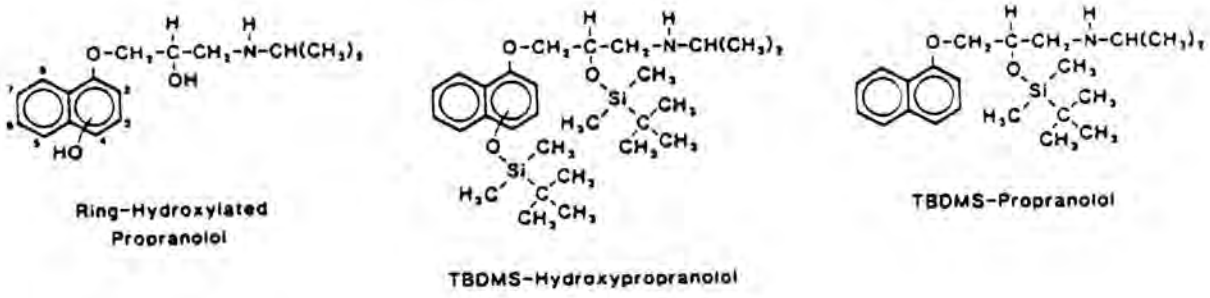


Figure 6.1. Structures of ring-hydroxylated propranolol, TBDMS-hydroxypropranolol and TBDMS-propranolol.

MATERIALS AND METHODS

Chemicals.

(±)-Propranolol hydrochloride was obtained from Sigma (St. Louis, MO). 2-HO-P and 4- through 8-HO-P were synthesized as their hydrochloride salts as previously described by Oatis and co-workers (81); 3-HO-P (the least stable isomer) was freshly synthesized as the free base according to the previously described procedure (81). N-methyl-N-(tert.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% tert.-butyldimethylsilylchlorosilane was obtained from Regis Chemical (Morton Grove, IL), and pyridine from Fisher (Fair Lawn, NJ).

Equipment.

Capillary GC-MS analyses were performed using a Varian 3700 gas chromatograph equipped with a split injector. The column, a Grade A, 60 m x 0.25 mm I.D. wall coated open tubular (WCOT) glass capillary obtained from J&W Scientific (Orangeville, CA), was interfaced through an open split interface to a Finnigan MAT 212 mass spectrometer equipped with a Spectrosystem SS200 data system. A fisher Recordall Series 5000 stripchart recorder was used during selected ion monitoring in analoque mode.

Preparation of TBDMS derivatives of propranolol and monohydroxypropranolols.

A 1-1.5 mg sample of each compound was dissolved in 200 µl of pyridine; 20 µl of MTBSTFA were added, and the reaction mixture was tightly capped and heated at 100 °C for 2 h. Derivatization of 8-HO-P is apparently sterically hindered, and requires 1-2 h heating to drive it to the di-derivatized product. The other compounds were more readily derivatized, but all of the individual samples were treated identically. A mixture of propranolol and the seven monohydroxypropranolols (1-1.5 mg of each) was similarly derivatized using 100 µl of pyridine and 120 µl of MTBSTFA; this mixture required heating for 16 h at 100 °C to complete the derivatization of 8-HO-P. The derivatives were stable in solution for at least two weeks.

Biological sample.

The hydroxypropranolol metabolites from a 2 ml aliquot of a 24 hour urine collection (total urine volume was 26 ml) from a male Sprague-Dawley rat injected intraperitoneally with 10 mg/kg (±)-propranolol were enzymatically hydrolyzed and extracted according to the method described by Walle and co-workers (66). The extracted metabolites were derivatized using 100 µl of pyridine and 20 µl of MTBSTFA with 16 h heating at 100 °C.

Capillary GC-MS resolution of propranolol and the monohydroxypropranolols.

The TBDMS derivatives of propranolol and the seven isomeric monohydroxypropranolols were resolved under the following conditions: injector temperature 240 °C; split ratio 1:350; column temperature programmed from 240 to 255 °C at a rate of 0.2 °C/min; helium carrier gas average linear velicity 23-25 cm/sec; open split interface temperature 260 °C; line-of-sight temperature 250 °C; ion source temperature 240 °C; ionization energy 70 eV. During the analyses of synthetic samples the mass spectrometer was scanned repetitively from 30 to 550 amu at a rate of 3 sec/decade with an interscan time of 1 sec. Selected ion monitoring was used as the detection system during the analysis of the biological sample because of the enhanced sensitivity of this technique. The mass spectrometer was set to monitor m/z 217 using a direct probe sample of TBDMS-derivatized 2-HO-P for calibration. All of the compounds eluted before the final column temperature was reached.

RESULTS

The TBDMS derivatives of propranolol and the hydroxypropranolols (with the exception of 8-HO-P) were readily prepared using MTBSTFA with pyridine as a solvent. 8-HO-P formed the di-derivatized product less rapidly than the other hydroxypropranolols, but was driven to completion with heating at 100 °C for up to 16 h. With insufficient heating, two peaks were observed corresponding to mono- and di-derivatized 8-HO-P, with the mono-derivatized product eluting first. With sufficient heating, only the later eluting peak was observed.

Table 6-I presents the elution order and the retention times of the separately derivatized synthetic compounds; the retention times presented have been adjusted for the viod volume of the system. A representative chromatogram of the results obtained when all eight compounds were derivatized as a mixture is presented in Figure 6.2, as a mass chromatogram of the sum of m/z 72, 217, and 274. Proposed structures of these ionic species are depicted in Figure 6.3. A profile essentially identical to that in Figure 6.2 was obtained for m/z 72 alone, and the same profile minus the propranolol peak was obtained for m/z 217 or 274 alone. All three of these ions are abundant in the mass spectra of the hydroxypropranolols, and each is potentially suited for selected ion monitoring. The m/z 217 and 274 ions are more specific for the hydroxypropranolols, as the m/z 72 ion is subject to interference from smaller molecular weight species likely to be present in a biological sample. Alternatively, the

TABLE 6-I

ELUTION ORDER OF THE TBDMS DERIVATIVES OF PROPRANOLOL AND THE MONOHYDROXYPROPRANOLOLS ON AN SP2100 WCOT CAPILLARY COLUMN*

Derivatized compound	Adjusted retention time** (min, ± 0.1)	Relative retention time*** (± 0.01)
propranolol	14.7	1.00
8-HO-P	34.4	2.34
2-HO-P	36.1	2.46
3-HO-P	46.1	3.14
7-HO-P	49.0	3.33
4-HO-P	54.1	3.68
6-HO-P	59.4	4.04
5-HO-P	60.0	4.08

* The column temperature was programmed from 240 to 255 °C at a rate of 0.2 °C/min.

** The elution time of a non-retained compound (butane) was subtracted from the absolute retention times to obtain the adjusted retention times.

*** Relative to TBDMS-propranolol.

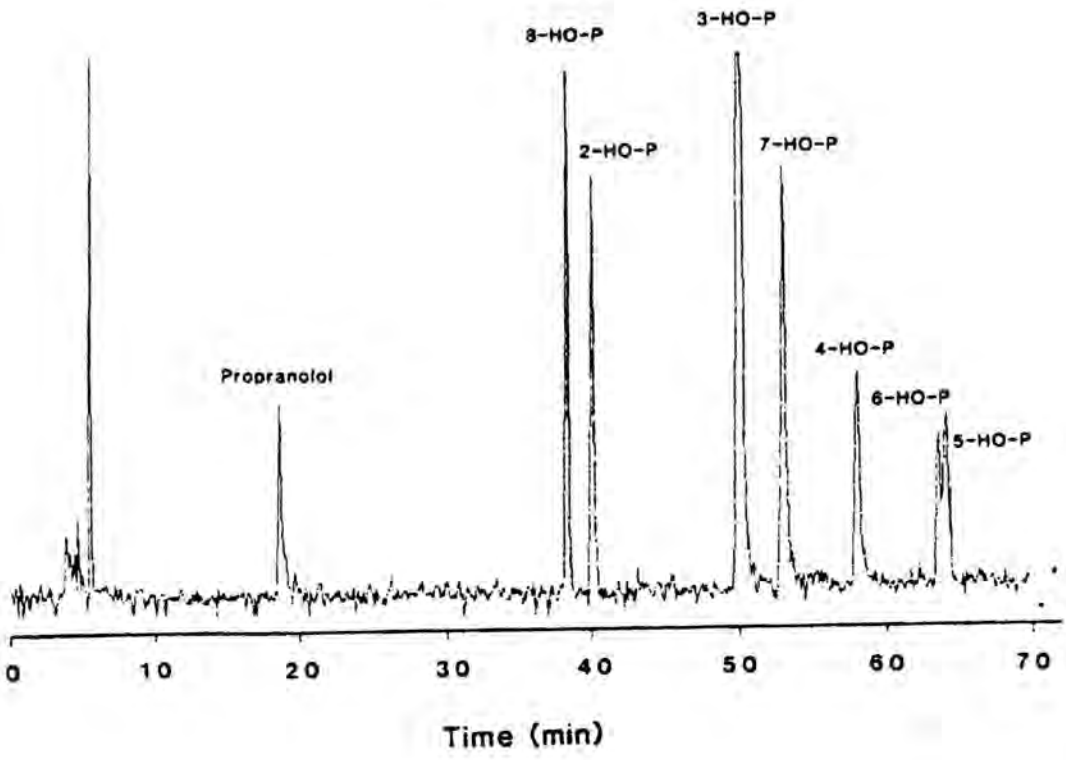
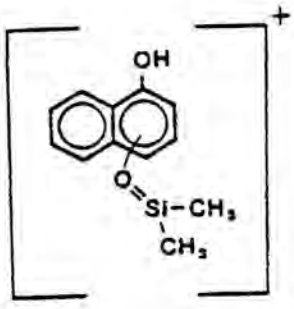


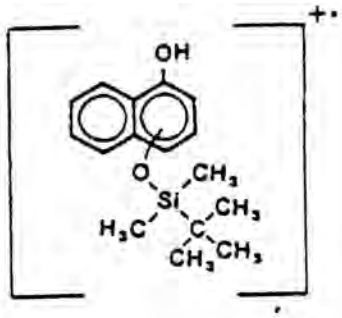
Figure 6.2. Capillary GC-MS chromatogram of the TBDMS derivatives of propranolol and ring-hydroxylated propranolols, as a retrospective combined plot of three ionic species, m/z 72, 217 and 274 (see Figure 6.3). The sample size was 3 µl (100 µg), injected with a split ratio of 1:350.



M/z 217

$[H_2C{=}NH{-}CH(CH_3)_2]^+$

M/z 72



M/z 274

Figure 6.3. Proposed structures of the ionic species traced in Figure 6.2.

molecular ion (m/z 503) could be monitored to enhance specificity for the hydroxypropranolols, but with a sacrifice in sensitivity.

The TBDMS derivatives of propranolol and the hydroxypropranolols were well resolved on an SP2100 WCOT capillary column, with the exception of 5- and 6-HO-P, which were separated by only 0.5-0.6 min with an overall retention time of about 1 h. This degree of resolution was obtained only when care was taken to ensure that the initial average linear velocity was only slightly above the optimal average linear velocity for the carrier gas (helium), so that the system would approach maximum efficiency at the higher elution temperatures. Under a given set of flow conditions, capillary GC-MS retention times were reproducible to within ± 0.1 min.

A selected ion monitoring (m/z 217) chromatogram of the TBDMS derivatives of the ring-hydroxylated propranolol metabolites extracted from the urine of a rat dosed with (±)-propranolol is presented in Figure 6.4. The retention times of the peaks observed from this sample were consistent with the observed retention times of derivatized synthetic 2-, 7-, 4-, and 5-hydroxypropranolol run on the same day. The species identified by these techniques, and their relative proportions, are consistent with the results previously obtained in rats by Walle and co-workers (66).

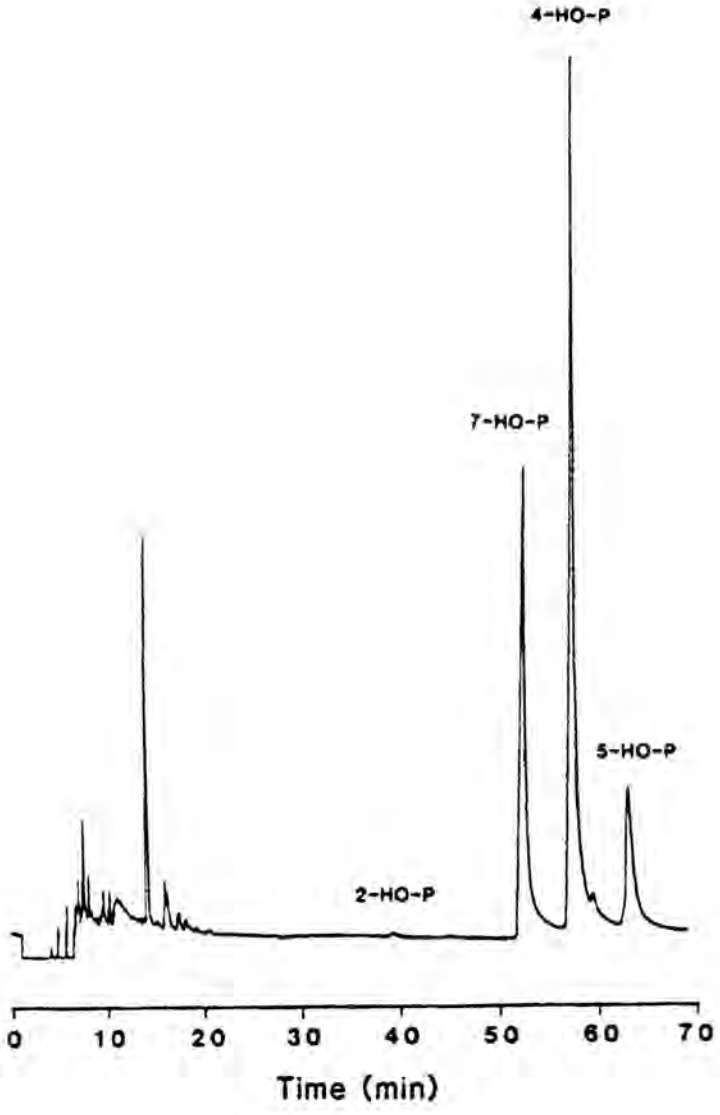


Figure 6.4. Selected ion monitoring (m/z 217) capillary GC-MS chromatogram of the ring-hydroxylated metabolites of (±)-propranolol extracted from rat urine. The sample size was 5 µl (approximately 10 µg) injected with a split ratio of 1:350. Peaks are labeled in accordance with the retention times of derivatized synthetic compounds chromatographed on the same day.

DISCUSSION

Propranolol and the seven ring-hydroxylated propranolols can be separated and readily identified as TBDMS derivatives using capillary GC-MS techniques. The identification methodology presented here affords significant advantages over the techniques used in the past (66). The TBDMS derivatives are well suited for several detection systems, including flame ionization (data not shown), repetitive scanning mass spectrometry, ans selected ion monotoring MS. Only one derivatization technique is employed, eliminating the necessity for using two separate GC columns. TBDMS-propranolol is well separated from the other seven compounds, so that special techniques for distinguishing the parent compound from one of its metabolites are not necessary.

The only major problem encountered using the techniques presented here was the moderate peak overlap between the TBDMS derivatives of 5- and 6-HO-P. In order to ensure reliable identification of these two compounds, care must be taken to ensure that the capillary GC system is operating at near-maximal efficiency. Problems concerning the identity of either or both of these two compounds in a mixture may be overcome by adding a small quantity of derivatized synthetic 5- or 6-HO-P and comparing relative peak heights with and without the added compound. The method presented here should facilitate further studies of ring oxidation metabolic pathways for propranolol, and should also be applicable to isomeric metabolites of related compounds.

CHAPTER VII.

QUANTITATIVE ANALYSIS OF ADENOSINE: STATISTICAL COMPARISON OF RADIOIMMUNOASSAY AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY-SELECTED ION MONITORING METHODS

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ABSTRACT

A new method for quantitating adenosine concentration by capillary gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) has been developed and used as a reference method for evaluating a newly developed radioimmunoassay (RIA) for adenosine. Details of the GC-MS-SIM method are presented, along with the comparative results and uncertainties of both methods. General considerations in the statistical analysis of method comparison data are discussed with particular reference to studies using quantitative mass spectrometry as the standard method; the adenosine methods are used as specific examples in this discussion. Simultaneous estimation of the y-intercept and slope of the least squares regression line relating the results of the two methods using the 95% joint confidence ellipse demonstrated the absence of either constant or proportional error between the two methods. The relatively small uncertainty in the GC-MS-SIM measurements had no significant effect on the linear regression. Random error between the two methods was detected, and was estimated by the coefficient of variation in the RIA data as ten percent of the RIA value.

INTRODUCTION

In method comparison studies, analytical techniques involving quantitative mass spectrometry are being used with increasing frequency as reference methods for determining the validity of other quantitative analytical methods. In a 1981 review article, Garland and Powell (32) cited thirty-six such studies, and at least twenty-four similar studies have appeared since that time (83-106). The theoretical basis for using quantitative mass spectrometric methods as reference or "standard" methods in such studies stems from the high specificity and sensitivity for the compounds of interest afforded by the mass spectrometer, particularly when combined with the specificity of high resolution chromatographic techniques such as capillary gas chromatography. We have developed a new capillary gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) method for quantitating adenosine, and have used this method to evaluate a newly developed radioimmunoassay (RIA) for adenosine. The GC-MS-SIM method employs 5',5'-dideuteroadenosine as a stable isotope labeled internal standard, and uses the tetra-t-butyldimethylsilyl (tetra-TBDMS) derivatives of adenosine and its dideuterated analog, with selected ion monitoring of the M-57 ions characteristic of TBDMS derivatives under electron impact ionization conditions (44,45). This chapter discusses details of the GC-MS-SIM method and the comparison of the results of this method with those obtained with the radioimmunoassay.

In evaluating one analytical method using another "standard" method one is faced with the problem of how to compare the data in a statistically valid manner and how to arrive at a quantitative measure of the nature and magnitude of errors in the method under examination. The use, and misuse, of statistical techniques in comparisons of analytical methods have been discussed previously (107-111). In this paper we discuss the use of statistical techniques in method comparison studies using quantitative GC-MS-SIM as the reference method, with application to the adenosine methods as a specific example.

MATERIALS AND METHODS

All glassware used in these experiments was silanized.

Chemicals.

5',5'-[$^2H_2$]-adenosine (112) (isotopic purity estimated at greater than 98%) was a generous gift from Karl H. Schram (University of Arizona, Tucson, AZ). Unlabeled adenosine and n-dodecane were reagent grade from Aldrich Chemical Co. (Milwaukee, WI). Glass-distilled water was generated in these laboratories, and Distilled-In-Glass grade methanol and acetonitrile were obtained from Burdick and Jackson Laboratories, Inc. (Muskegan, MI). N-Methyl-N-t-butyl-dimethylsilyltrifluoroacetamide (MTBSTFA) was obtained from Regis Chemical Co. (Morton Grove, IL). All other chemicals were reagent grade from commercial sources and were used without further purification.

Unknown samples.

Thirty-nine unknown samples were used for comparative measurements of adenosine concentration by RIA and GC-MS-SIM. Thirty-six of these were coronary effluent samples from Langendorf style perfused guinea pig hearts. The perfusing medium was a modified Krebs-Henseleit solution (113) (KHS), equilibrated with 95% oxygen-5% carbon dioxide, and containing NaCl (127.2 mM) KCl (4.7 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.1 mM), $NaHCO_3$ (24.9 mM), sodium pyruvate (2.0 mM), and glucose (5.5 mM). Samples were taken under control conditions and after various pharmacological manipulations including infusion of adenosine and bolus injections of

isoproterenol. All coronary effluent samples were collected for 30 seconds and immediately immersed in boiling water for 5 minutes in order to denature any degradative enzymes released from the heart.

The three remaining unknown samples were left ventricular tissue extracts. Portions of left ventricle (approximately 100 mg) were quickly frozen between two copper blocks precooled with dry ice. The frozen tissue samples were homogenized with a Polytron^{R} homogenizer in 1 ml of ice cold 10 mM Tris HCl, pH 7.2. The homogenate was acidified with 1 ml of 14% trichloroacetic acid, then centrifuged. The supernatant was decanted and saved. The pellet was resuspended in 2 ml of 7% trichloroacetic acid, centrifuged, and the supernatant combined with the first. The combined supernatant was neutralized with sodium hydroxide prior to analysis by RIA and GC-MS-SIM.

The results of the biological experiments have been published elsewhere (114).

Preparation of standard samples and internal standard solution for GC-MS-SIM analysis.

Standard samples ranging in concentration from 1 to 100 ng/ml in adenosine concentration were prepared by serial dilution in KHS. The initial solution from which serial dilutions were made was freshly prepared to four significant figures at the start of each analysis. An internal standard solution containing approximately 40 ng/100 µl dideuteroadenosine in KHS was also freshly prepared for each analysis.

Addition of internal standard.

An aliquot (1 ml) of each standard and unknown sample was pipetted into a polypropylene tube (17 x 100 mm, Elkay Products). For unknown samples out of the range of the standard curve according to previous analysis, smaller aliquots were diluted with KHS to a final volume of 1 ml to bring them into range. The internal standard solution (100 µl, 40 ng) was then added to each sample. Control samples included a blank sample of KHS (1 ml), to which the internal standard was added, and an additional 1 ml aliquot of the 100 ng/ml adenosine standard solution, to which no internal standard was added. All samples were vortexed and allowed to equilibrate for 30 minutes.

Extraction of adenosine with reverse-phase cartridge columns.

Because buffer salts can interfere with silylation reactions, all samples were desalted using reverse-phase cartridge columns. A separate C18 Sep-Pak^{R} was used for each sample. The cartridge columns were charged with 10 ml of methanol, and washed with 10 ml of water. The samples were then loaded, washed with 10 ml of water to effect desalting, and finally eluted with 2.5 ml of 40% (v/v) aqueous methanol. The samples were eluted directly into 10 ml sealable ampules (Wheaton). Eluting solvent was removed by evaporation under a stream of nitrogen in a boiling water bath.

Formation of the tetra-TBDMS derivatives of adenosine and dideutero-adenosine.

To each dry sample were added 100 µl of MTBSTFA and 400 µl of aceto-
nitrile. The ampules were sealed and heated for five hours in a wax bath maintained at 150 °C. The ampules were then opened, and the contents transferred to 1 ml Reacti-Vials^{R} (Pierce). Excess derivatization reagent
and solvent were removed by evaporation under a stream of nitrogen in a 40 °C
water bath. The derivatized samples were redissolved with sonication in 20 µl
n-dodecane.

GC-MS-SIM.

Quantitative GC-MS analyses were performed using a Varian 3700 gas chromatograph interfaced to a Finnigan MAT 212 mass spectrometer equipped with a Spectrosystem SS200 data system with selected ion monitoring hardware. The GC column was a 30 m DB-5 (0.259 mm i.d., 0.25 µm film thickness, J & W Scientific) fused silica capillary column, the outlet end of which was introduced directly into the ion source of the mass spectrometer through a heated transfer line maintained at 290 °C. The gas chromatograph was equipped with an on-column injector and utilized helium carrier gas with a capillary head pressure of 35 psi (average linear velocity 40-45 cm/sec at the upper GC oven temperatures). A sample, typically 3 µl, was slowly

injected on-column with the GC oven at 225 °C; after the solvent had passed through the column, the oven temperature was rapidly ramped to 280 °C, then programmed to 300 °C at 3 °C/minute.

The mass spectrometer was operated in electron-impact mode (70 eV ionization energy, 1 mA emission) with the ion source at 220 °C, and a typical source pressure of 1×10^{-5} torr. Mass spectral resolution was set at 700 with a flat-topped peak shape. Ion source potentials were optimized while monitoring the m/z 207 column bleed peak with the GC oven at 300 °C.

The magnet was operated in current regulation mode for stability. The magnetic field was manually set to focus m/z 666.4 at the nominal accelerating voltage of 3 kV; magnetic focusing was checked immediately prior to each sample injection in order to correct for any drift in magnetic field strength.

The ion intensities at m/z 666.4 and 668.4 were monitored by alternating the accelerating voltage only (no magnetic jumping was employed). The intensity corresponding to each ion was recorded 10 times per second, with each recorded measurement being the average of 16 individual measurements. This high rate of sampling was used in order to maximize the precision of the ion intensity area ratio measurements from narrow capillary GC peaks. The areas under the GC ion intensity profiles were measured using the standard software with the SS200 data system.

Data reduction

The 666/668 ion intensity ratio measurements were converted to idealized mole ratios ($\underline{x}/\underline{y}$, sample to internal standard) using equation (1) (115):

$$\frac{x}{y} = \frac{(Ry - Rm)(Rx + 1)}{(Rm - Rx)(Ry + 1)} \qquad (1)$$

where Rx is the 666/668 ion intensity ratio for pure unlabeled sample, Ry is the corresponding ratio from the pure internal standard, and Rm is the ratio for each mixture. The utility of this equation has been discussed thoroughly in the literature (115,116). Standard curves were generated by plotting $\underline{x}/\underline{y}$ calculated from the known samples versus the adenosine concentration. The $\underline{x}/\underline{y}$ ratio was calculated for each unknown sample, and the adenosine concentration calculated from the standard curve; any necessary corrections for sample dilution were then applied.

Radioimmunoassay.

A detailed description of the new RIA for adenosine is beyond the scope of this manuscript. Briefly, the antiserum was raised in rabbits using an immunogen of 2',3'-O-disuccinyl adenosine coupled to bovine serum albumin with isobutyl chloroformate. Free and bound adenosine were separated using dextran-charcoal, with iodinated tyramine bound to disuccinyl adenosine used as a tracer. The detection limit was established at approximately 280 pg/ml. Specificity studies employing a series of related compounds showed maximum cross reactivities of 1.7% for deoxyadenosine and 0.06% for

adenosine monophosphate. The intraassay coefficient of variation averaged 3.2%, and the interassay coefficient of variation was 3-5%.

RIA linear transformation

The standard linear transformation for radioimmunoassay data was used, where the logit of B/B_m (binding over maximal binding) is plotted against the logarithm of adenosine concentration (117).

Statistics

The lines of best fit for the linearly transformed standard curves from both GC-MS-SIM and RIA analyses were calculated using classical least squares linear regression techniques (118). For both standard curves, 95% confidence limits on X values calculated from measured Y values were determined using the 95% inverse tolerance limits for the entire regression line as described by Williams (118).

Several statistical calculations were employed in comparing the RIA and GC-MS-SIM quantitative results. The y-intercept and the slope of the line relating $[A]_{RIA}$ (Y) and $[A]_{GCMS}$ (X) were calculated using classical least squares procedures (118), as were the independent tests for the significance of the y-intercept and the slope. The 95% joint confidence ellipse for testing the hypothesis that the y-intercept and slope are simultaneously 0 and 1 respectively was generated as described by Neter and Wasserman (119). Deming's method for determining a linear equation for data involving errors in both ordinate and abscissa was employed in the manner described by Cornbleet and Gochman (111).

RESULTS AND DISCUSSION

Mass spectra of the tetra-TBDMS derivatives of adenosine and 5',5'-dideuteroadenosine are presented in Figure 7.1. The fragmentation patterns in these spectra are very similar to those described for trimethylsilyl derivatives of nucleosides by Pang and co-workers (120). The molecular ion regions of both spectra are of very low intensity, while the M-57 ions, characteristic of TBDMS derivatives (44,45), are prominent in both. The molecular ions and fragment ions involving the ribose (sugar) moiety (S) differ by two m/z units between the two spectra, while fragments involving the base (B) but not the ribose are identical. The M-57+2 ions are also prominent in these spectra; since m/z 666 and 668 were the ions monitored for quantitative GC-MS analysis, the presence of an intense m/z 668 peak in the unlabeled spectrum presents an ion overlap problem (121), as will be discussed below. The base peak in these spectra, m/z 73, was identified as the rearrangement ion $[(CH_3)_3Si]^+$ by measuring its exact mass by high resolution (R=10,000) direct probe mass spectrometry with peak matching against the m/z 69 ion of perfluorokerosene (theoretical exact mass 73.0474 amu; experimental 73.0470 amu).

Representative GC-MS-SIM traces at m/z 666.4 and 668.4 from a perfusate sample are presented in Figure 7.2. The tetra-TBDMS derivative of adenosine has excellent chromatographic properties on a fused silica DB-5 capillary GC column, and retention times were reproducible to within ± 3 seconds. The SIM traces were entirely free of interference in this region of the chromatogram.

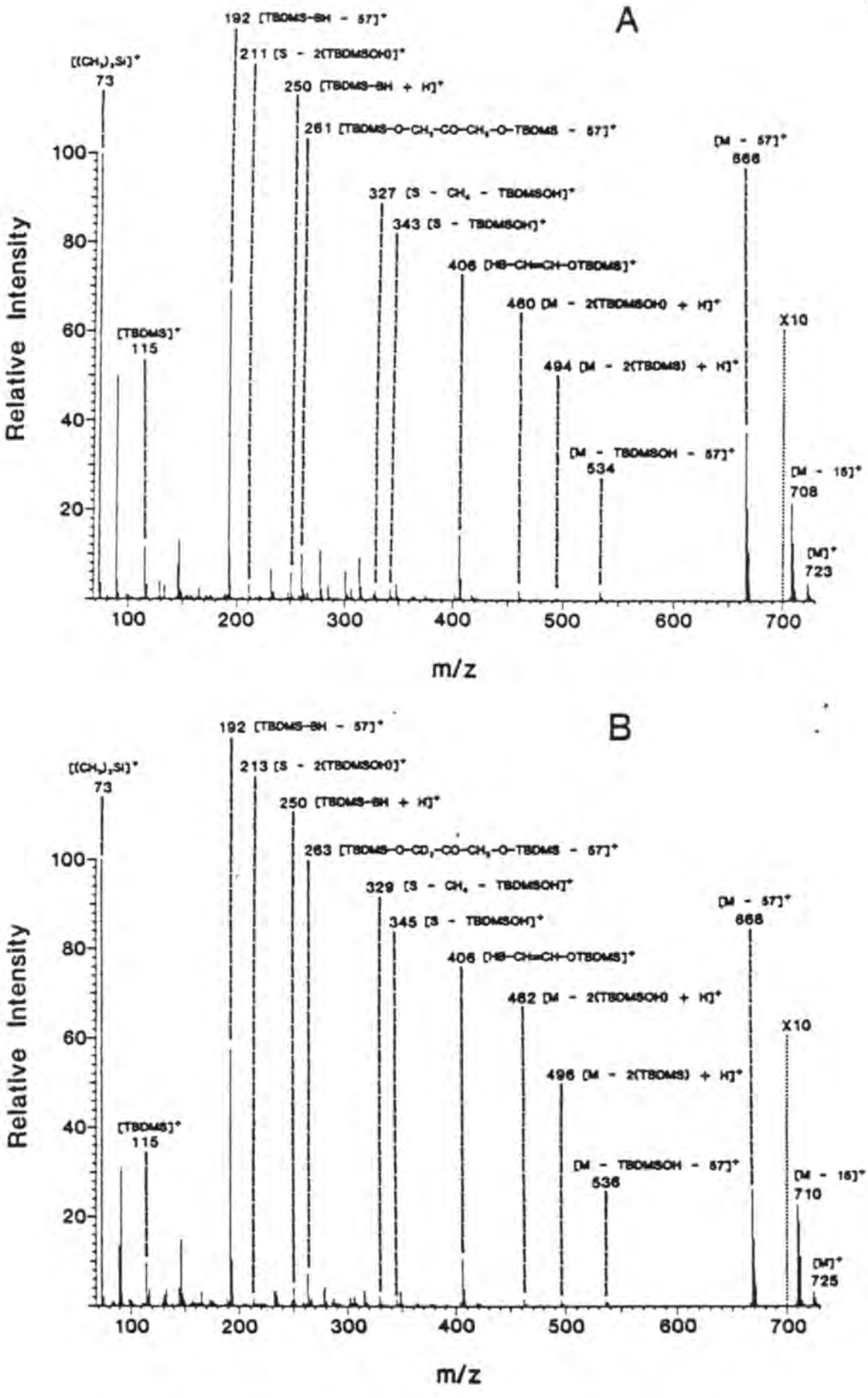

Figure 7.1. Averaged, background subtracted repetitive scanning mass spectra of the tetra-TBDMS derivatives of adenosine (A) and 5',5'-dideuteroadenosine (B).

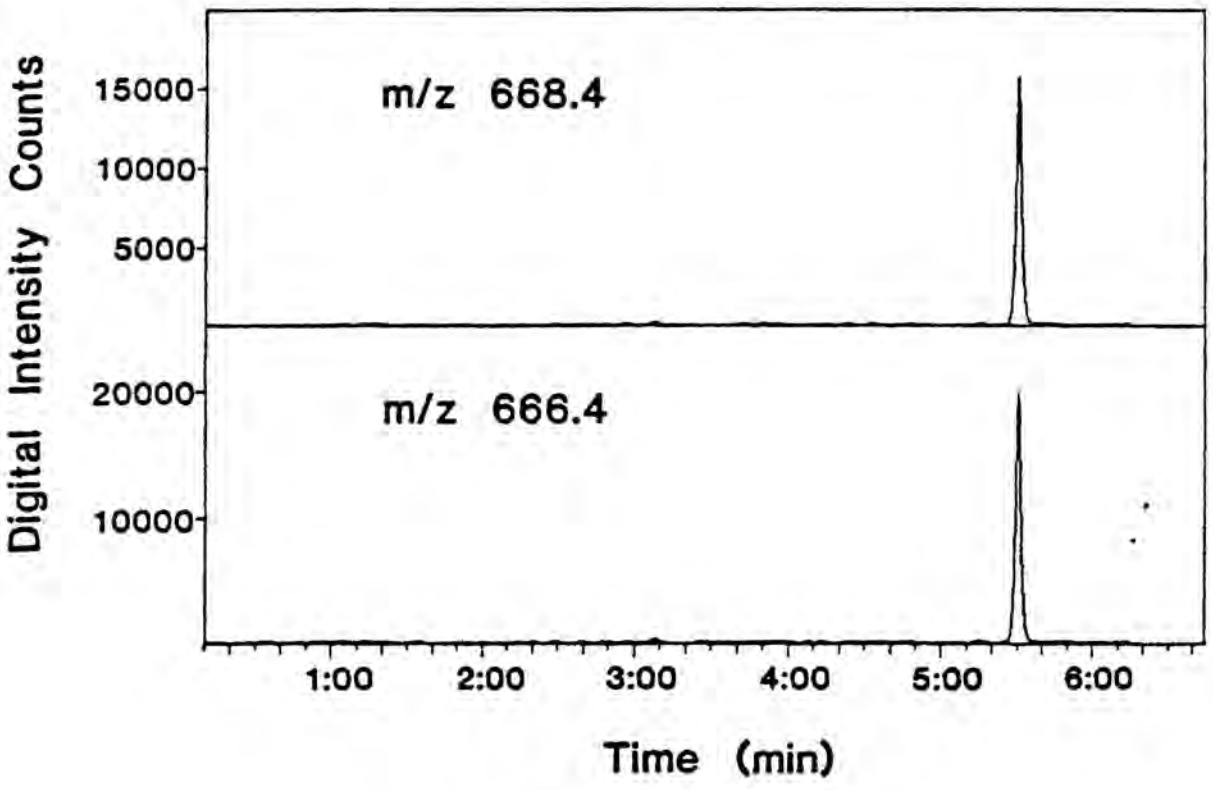

Figure 7.2. Capillary GC-MS-SIM traces of m/z 666.4 and 668.4 from a perfusate sample (76.4 ± 1.9 ng/ml, 6-8 ng injected on-column).

A plot of the 666/668 ion intensity ratio versus the adenosine concentration from the standard curve data was hyperbolic, asymptotically approaching the ratio for pure derivatized unlabeled adenosine as the relative concentration of internal standard decreased. This phenomenon occurred because of the presence of the m/z 668 peak in the mass spectrum of the unlabeled material (Figure 7.1A), and necessitated the use of a linear transformation on the standard curve data, which was performed using equation (1) as recommended by Colby and McCaman (115). The standard curves of the transformed data (Figure 7.3) were linear over the 1-100 ng/ml adenosine concentration range, with a typical correlation coefficient of 0.9998. At higher levels of adenosine concentration (e.g. 500 ng/ml), propagated errors reached an unacceptable level (115), thus limiting the upper end of the standard curve to 100 ng/ml with 40 ng/ml internal standard. The lower end of the range was dictated by the anticipated lowest concentration of the samples to be analyzed (5-10 ng/ml), and was not due to sensitivity limitations. The GC-MS-SIM methodology described here should be useful in the picogram per milliliter range, with an appropriate reduction in the amount of added internal standard.

The derivatization reaction was not complete under the conditions employed, with approximately 30-35% of the adenosine driven to the tetra-derivatized form. However, variations in extraction and derivatization efficiency were well accounted for through the use of

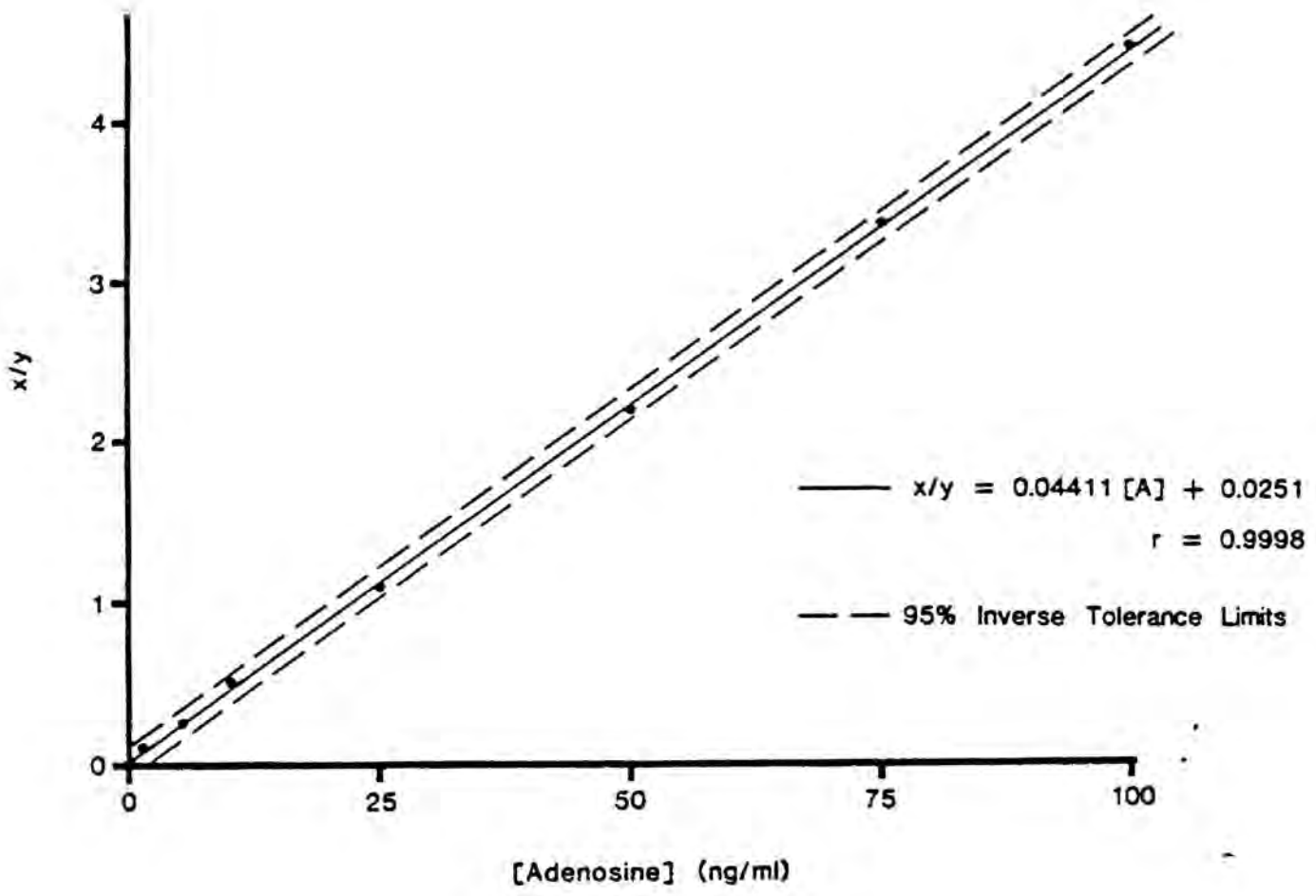

Figure 7.3. Linearly transformed standard curve from capillary GC-MS-SIM analysis. The ratio x/y is the transformed 666/668 ion intensity ratio. The solid line represents the estimated least squares regression line, and the dashed lines represent the 95% inverse tolerance limits for the estimated regression line.

the stable isotope labeled internal standard, as evidenced by the excellent linearity of the transformed standard curve.

Also shown in Figure 7.3 are the 95% inverse tolerance limits for the GC-MS-SIM standard curve regression line. These were used to place confidence intervals on calculated X values (adenosine concentration) from measured, transformed Y values ($\underline{x}/\underline{y}$, the transformed 666/668 ion intensity ratio). While actually hyperbolic, these two lines are approximately parallel to the regression line, giving a 95% confidence band of ± 2 ng/ml across the entire range of the standard curve. This establishes the lower limit of quantitation with this standard curve at around 5 ng/ml, since at the lower standard concentration, 1 ng/ml, the uncertainty is larger than the known value. For concentrations below 5 ng/ml, a new standard curve covering a lower range of concentrations would be needed. The lowest level of adenosine concentration in an unknown sample measured in these analyses was 5.0 ± 2.1 ng/ml.

Figure 7.4, a typical linearly transformed standard curve from RIA analysis, shows the 95% inverse tolerance limits on the logarithm of adenosine concentration. By definition, these tolerance limits are hyperbolic, and thus automatically incorporate the heteroscedacity (nonconstant variance) inherent in RIA measurements. However, both the RIA and GC-MS standard curves exhibited excellent linearity, and the hyperbolic nature of the tolerance limits, although present, is not visually apparent in either standard curve. For the RIA analyses, application of the inverse tolerance limits

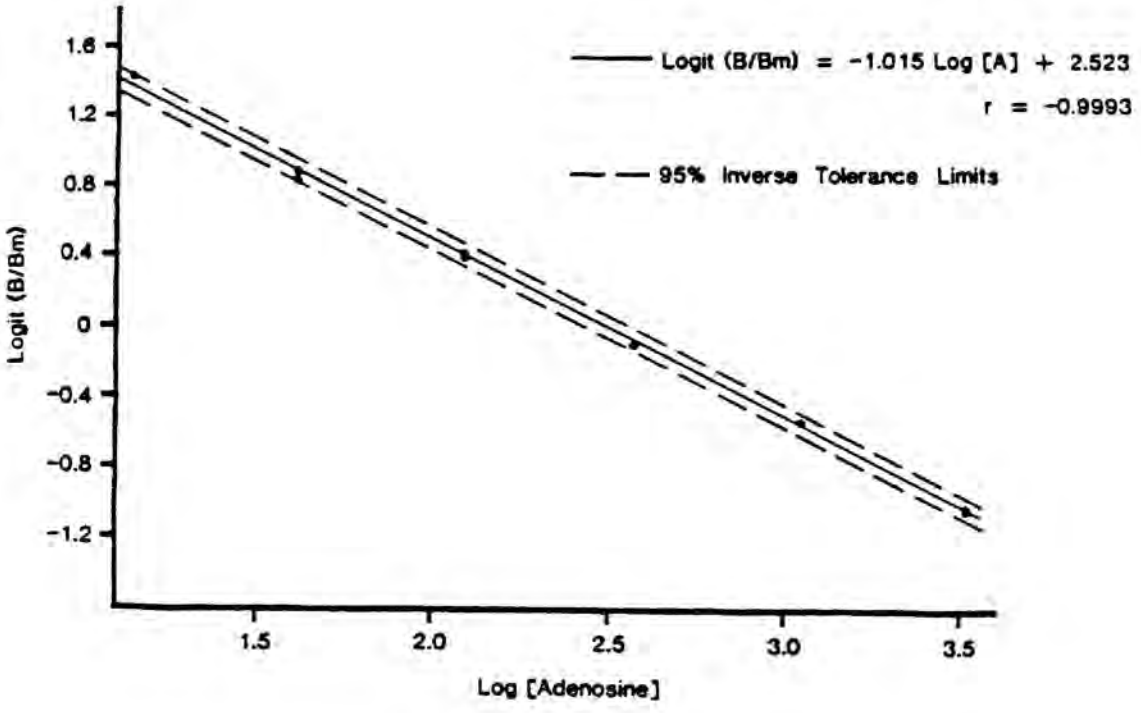

Figure 7.4. Linearly transformed standard curve for the radioimmunoassay for adenosine; B/Bm is the ratio of binding (B) to maximal binding (Bm). The solid line represents the estimated least squares regression line, and the dashed lines represent the 95% inverse tolerance limits for the estimated regression line.

equation to an unknown sample yielded 95% confidence limits for the logarithm of adenosine concentration, which, when exponentiated, generated a confidence interval for adenosine concentration. This confidence interval is asymmetric due to the logarithmic nature of the standard curve.

The comparative results between RIA and GC-MS-SIM analyses are plotted in Figure 7.5, with Figure 7.5A covering the entire range of observations (5 to 3000 ng/ml), and Figure 7.5B expanded to show the cluster of values in the 5-300 ng/ml range. (The higher concentrations were measured by GC-MS by diluting the sample into the 1-100 ng/ml range.) The vertical bars indicate the 95% confidence limits on [A] for each sample from RIA analysis, and the horizontal bars show the corresponding 95% confidence limits for [A] from GC-MS-SIM analysis. The estimated regression line relating $[A]_{RIA}$ (Y) and $[A]_{GCMS}$ (X) obtained by classical least squares analysis is shown as the solid line. The ideal relationship between the two methods occurs when identical results are obtained with both methods, and is represented mathematically by the functional relationship Y=X or $[A]_{RIA} = [A]_{GCMS}$. This relationship occurs when the regression line relating $[A]_{RIA}$ and $[A]_{GCMS}$ has simulatneously a y-intercept and slope of zero and one, respectively. This ideal line of identity is represented by the dashed line in Figure 7.5. In order to investigate the agreement of the results of the two methods, it is necessary to test the hypothesis that the true (population) y-intercept and slope of the estimated regression line relating $[A]_{RIA}$ and

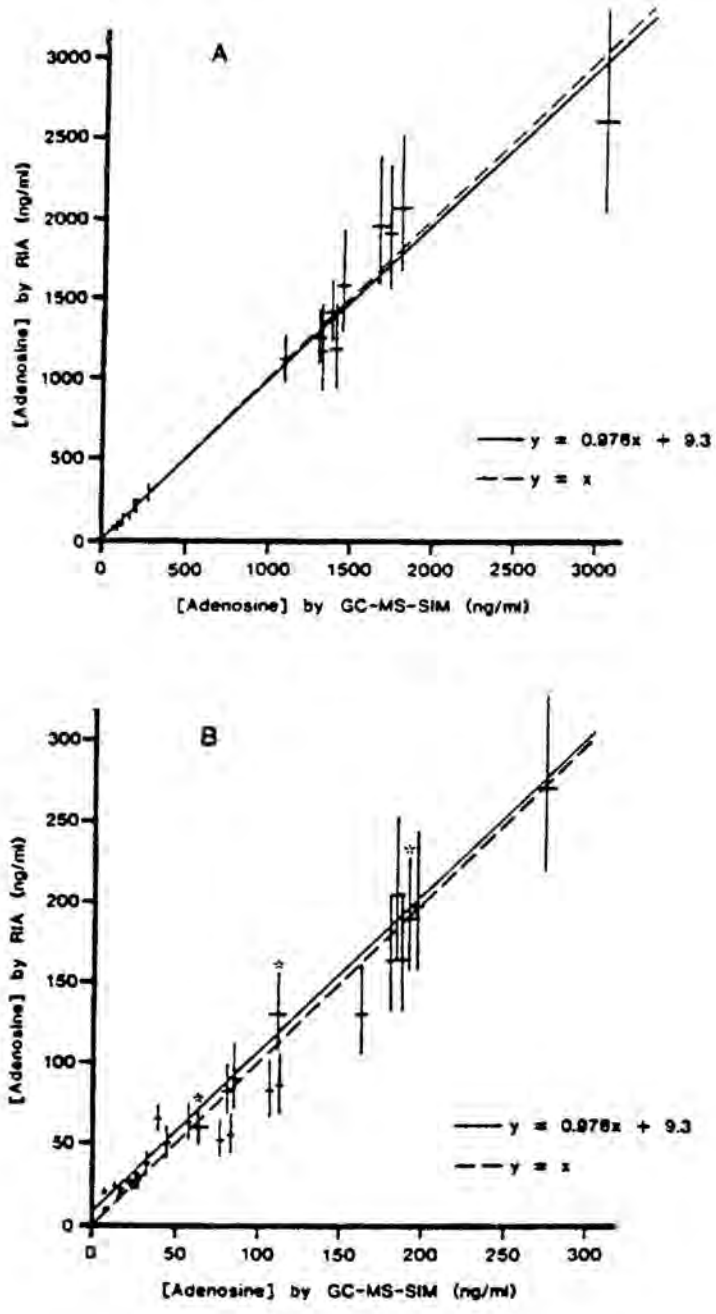

Figure 7.5. Plot of the quantitative measurements of adenosine concentration obtained by radioimmunoassay versus those obtained by capillary GC-MS-SIM. Vertical and horizontal bars represent 95% confidence limits for individual samples from RIA and GC-MS-SIM respectively. The concentration ranges displayed are 5-3000 ng/ml (A) and 5-300 ng/ml (B). The solid line represents the estimated least squares regression line (correlation coefficient r = 0.9888), and the dashed line represents the line of identity (Y=X). The points indicated with an asterisk were ventricular tissue extracts.

$[A]_{GCMS}$ do not deviate significantly, in the statistical sense, from zero and one, respectively. It is common practice to test this hypothesis by breaking it into two hypotheses and testing them separately and independently, i.e. testing the hypothesis that the y-intercept is equal to zero, and then testing the hypothesis that the slope is equal to one. These tests are performed by calculating confidence intervals (e.g. 95%) for each of the two parameters and determining whether or not each confidence interval overlaps with its ideal value. For the regression line presented in Figure 7.5, the 95% confidence interval for the y-intercept is 9.3 ± 42.7, and that for the slope is 0.976 ± 0.049. Both of these confidence intervals overlap with the ideal values, and one is tempted to conclude at this point that the ideal relationship holds, i.e. that the results obtained with the RIA are not significantly different from those obtained by GC-MS-SIM.

However, separate hypothesis tests for the y-intercept and slope do not provide assurance at the given level of significance (e.g. 95%) that conclusions for <u>both</u> the intercept and slope are <u>simultaneously</u> correct. In order to test the two hypotheses simultaneously, a joint confidence ellipse may be constructed which incorporates all simultaneous values for y-intercept and slope supported by the data at the chosen significance level (119, 122). Figure 7.6 shows the 95% joint confidence ellipse for y-intercept and slope constructed using the data presented in Figure 7.5. The ellipse is centered at the least squares regression estimates of the

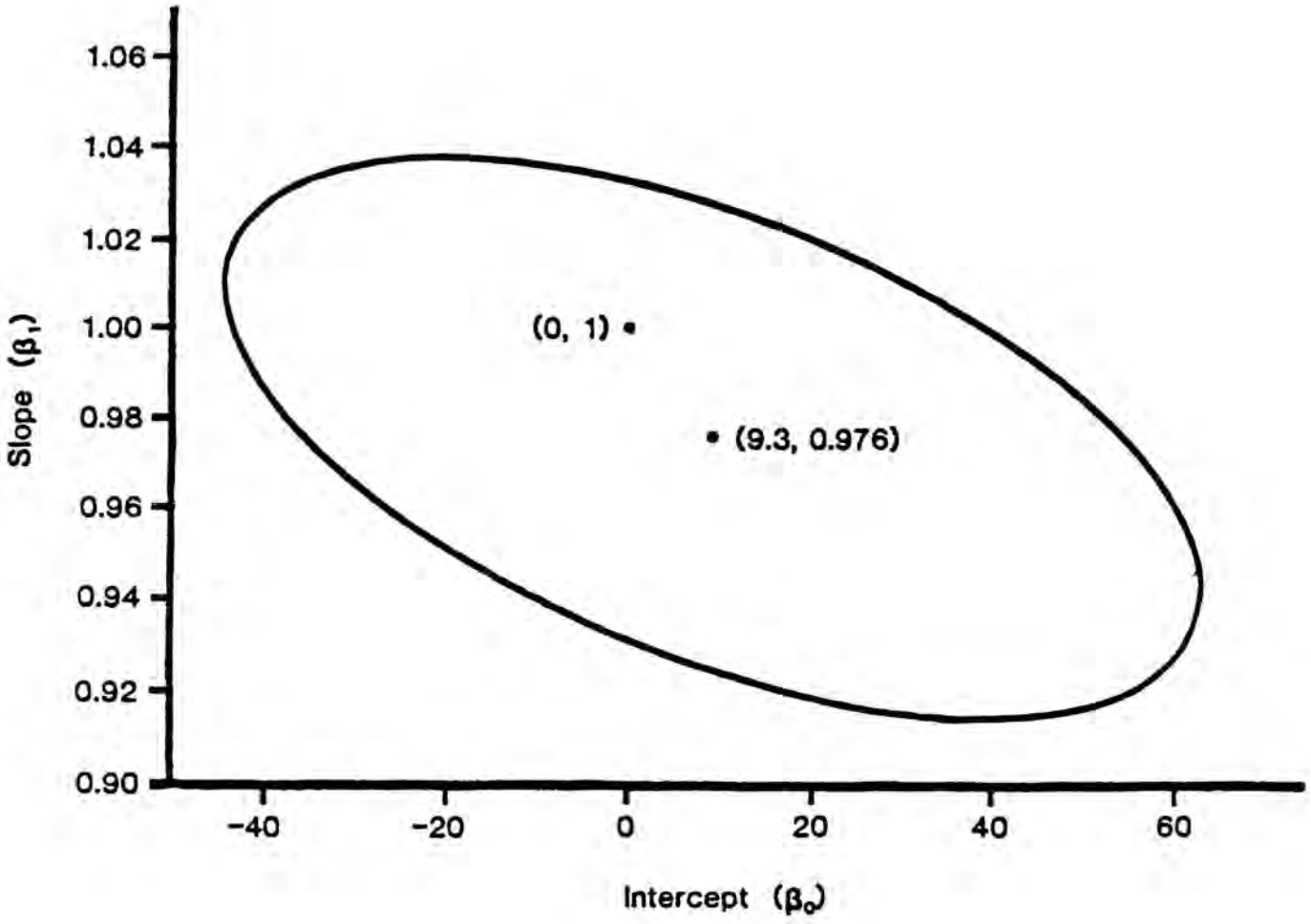

Figure 7.6. Elliptical 95% joint confidence region for simultaneous estimation of the y-intercept and the slope for the data presented in Figure 7.5.

y-intercept and slope, shown as the point (9.3, 0.976). The conclusion that the y-intercept and slope are simultaneously not significantly different from zero and one, respectively, is supported by the data if the point (0,1) is included within the boundaries of the ellipse, as it is in Figure 7.6. This suggests that that observed deviations of the y-intercept and slope from their hypothesized values of zero and one are likely to be due to random chance. If the point (0,1) had fallen outside the boundaries of the 95% joint confidence ellipse, then it would be very unlikely that the observed deviations of the y-intercept and slope from their ideal values were due to random chance, and it would be concluded that either the y-intercept or slope (or both) differs significantly from the ideal relationship. Note that it is possible for the values calculated from a least squares regression analysis to pass both independent tests while not passing the simultaneous test. The data presented in Figure 7.5 pass all of these.

The statistical analysis described above is based upon the techniques of classical least squares regression. In order for such an analysis to be strictly valid, the data analyzed should meet certain criteria. First, there should be no uncertainty in the values of the independent (X) variable, and secondly, the uncertainty in the dependent (Y) variable should be constant for all values of X (constant variance). Unfortunately, these criteria are seldom met in method comparison studies. Both of the methods for measuring adenosine concentration used in the present study incorporate

inherent uncertainty, as shown by the error bars in Figure 7.5. The uncertainty in the GC-MS-SIM measurements is small compared to that in the RIA measurements, which lends justification to the use of GC-MS-SIM as the reference method. However, the presence of a small uncertainty in the reference method should not be completely ignored. Other authors (110,111) have recommended the use of Deming's method for determining the y-intercept and slope in regression analyses involving errors in both X and Y. For the data in this study, Deming's method generates a y-intercept of 9.1 and a slope of 0.976; the close similarity between these values and those obtained through classical least squares regression procedures suggests that the relatively small uncertainty in the GC-MS-SIM measurements has a negligible effect on the linear regression.

The criterion for constant variance in the Y (RIA) data is also not met by the data in this study. As demonstrated by the vertical bars in Figure 7.5, the magnitude of the uncertainty in the RIA data increases as the RIA value increases. On the average, this uncertainty is a constant percentage of the RIA value, and the Y data in Figure 7.5 can thus be described as having a constant coefficient of variation, defined as the standard deviation of repeated measurements of a single Y value divided by the mean of those repeated measurements. Although such repetitive measurements were not performed in this study, the coefficient of variation may be estimated as one fourth of the magnitude of the RIA 95% confidence interval for a given point divided by the RIA value of the point

(note: the 95% confidence interval is approximately four standard deviations). When this calculation is performed for all of the points in Figure 7.5 and averaged, the coefficient of variation in the RIA data is estimated to be 0.1, or 10% of the RIA value. Data involving a constant coefficient of variation may be analyzed through weighted least squares regression analysis; however, Cornbleet and Gochman (111) have demonstrated that the classical procedure calculates the correct regression line even with a coefficient of variation as high as 20%, so that weighted least squares analysis is not required. While this description of the RIA data as incorporating a constant coefficient of variation must be regarded as an approximation, it is a reasonable one in this case due to the virtually parallel nature of the 95% inverse tolerance limits associated with the logarithmic RIA standard curve (Figure 7.4).

Three major types of errors may occur between the results of two analytical methods. These are constant, proportional, and random errors. Westgard and Hunt (109) have demonstrated the utility of statistical analysis, particularly least squares regression, in determining the nature and estimating the magnitude of such errors in method comparison studies. From least squares linear regression analysis, a constant discrepancy between two methods is reflected in the value of the y-intercept, and a proportional error is measured as the deviation of the slope from the ideal value of one. Neither constant nor proportional error has been detected between the RIA and GC-MS-SIM methods in this study, since the

y-intercept and slope have been shown to be simultaneously not significantly different from zero and one, respectively.

The major error detected statistically between these two analytical methods is of the random type. Random errors may be measured from least squares regression analysis as the standard deviation of the residual error of regression (Syx). For the data in Figure 7.5, Syx is calculated to be 110 ng/ml by the classical procedure and 108 ng/ml by Deming's procedure. Westgard and Hunt (109) have shown that Syx is a good estimator of random error when the Y data have constant variance. However, Cornbleet and Gochman (111) point out that when the Y data have a constant coefficient of variation, rather than constant variance, Syx is not constant, but increases as X and Y increase. Thus, a more reasonable estimate of the random error between these two analytical methods is the coefficient of variation, or ten percent of the RIA value.

As has been pointed out (109) statistics are not a substitute for judgements upon the acceptability of an analytical method, but they can provide a basis upon which judgements can be made. The radioimmunoassay evaluated in this study has the advantages of simplicity, speed, and a broad range of quantitation, and is thus well suited for use with large numbers of samples. In contrast, the GC-MS-SIM method is labor intensive, time consuming, and has a restricted range of quantitation. However, the RIA measurements incorporate a greater uncertainty than the GC-MS-SIM measurements, and the utility of the RIA may be limited by this uncertainty.

Based upon the statistical analysis described in this study, the radioimmunoassay for adenosine will be useful in experiments where a random error of ten percent of the "true" value can be tolerated, since neither constant nor proportional errors were detected.

REFERENCES

1. Ferreira S.H. and J.R. Vane. "The disappearance of bradykinin and eledoisin in the circulation and vascular beds of the cat." Br. J. Pharmac. Chemother. 30; 417-424, 1967.

2. Regoli D. and J. Barabé. "Pharmacology of bradykinin and related kinins." Pharmacol. Rev. 32; 1-46, 1980.

3. Ryan, J.W., J. Roblero and J.M. Stewart. "Inactivation of bradykinin in the pulmonary circulation." Biochem. J. 110; 795-797, 1968.

4. Ryan, J.W., J. Roblero and J.M. Stewart. "Inactivation of bradykinin in rat lung." Advan. Exp. Med. Biol. 8; 263-271, 1970.

5. Ferreira S.H. and Y.S. Bakhle. "Inactivation of bradykinin and related peptides in the lung." In: Metabolic Functions of the Lung, edited by Y.S. Bakhle and J.R. Vane. New York: Marcel Dekker, 1977, 4:33-53.

6. Camargo, A.C.M., M.A. Cicilini and K.M. Carvalho. "Breakdown of biologically active peptides by brain endo-oligopeptidases (kininase A and B)." Advan. Exp. Med. Biol. 156B; 855-862, 1983.

7. Kaplan, A.P., I. Sheikh and M.H. Frendscho. "Assessment of histamine release and kinin formation in man: identification of kinin degradation products and characterization of a lymphocyte-dependent histamine releasing factor." Int. Archs. Allergy appl. Immun. 77; 64-68, 1985.

8. Cohn, W.E. "Nomenclature and symbolism of α-amino acids." Meth Enzymol. 106; 3-17, 1984.

9. Sheikh, I. and A.P. Kaplan. "Assessment of lysyl bradykinin (LBK), bradykinin (BK) and bradykinin degradation products by high-performance liquid chromatography (HPLC)." Fed. Proc. 44; 1242, 1985.

10. Ryan, J.W. "Processing of endogenous polypeptides by the lungs." Ann. Rev. Physiol. 44; 241-255, 1982.

11. Keiser, H.R., M.P. Peyton, J.J. Pisano and A. Sjoerdsma. "Metabolism of ^{14}C-bradykinin in man." In: Bradykinin and Related Kinins: Cardiovascular, Biochemical, and Neural Actions, edited by F. Sicuteri, M. Roche de Silva and N. Back. New York: Plenum Press, 1970, pp. 315-319.

12. Marceau, F., M. Gendreau, J. Barabé, S. St-Pierre and D. Regoli. "The degradation of bradykinin (BK) and of des-Arg^9-BK in plasma." Can. J. Physiol. Pharmacol. 59; 131-138, 1981.

13. Behal, F.J., W. Sidorowicz, J. Heins and A. Barth. "Human lung kinin catabolism." Fed. Proc. 42; 1021, 1983.

14. Szechinski, J. and F.J. Behal. "Human lung kininase." Fed. Proc. 41; 1452, 1982.

15. Biemann, K., F. Gapp and J. Seibl. "Application of mass spectrometry to structure problems. I. Amino acid sequence in peptides." J. Amer. Chem. Soc. 81; 2274-2275, 1959.

16. Frank, H. and D.M. Desiderio. "Reduction of oligopeptides to amino alcohols with borane." Anal. Biochem. 90; 413-419, 1978.

17. Carr, S.A., W.C. Herlihy and K. Biemann. "Advances in gas chromatographic mass spectrometric protein sequencing." Biomed. Mass Spectrom. 8; 51-61, 1981.

18. Biemann, K. "Amino acid sequence in oligopeptides." In: Biochemical Applications of Mass Spectrometry, edited by G.R. Waller. New York: John Wiley, 1972, pp. 405-428.

19. Biemann, K. "Amino acid sequence in oligopeptides and proteins." In: Biochemical Applications of Mass Spectrometry, First Suppl. Vol., edited by G.R. Waller and O.C. Dermer. New York: John Wiley, 1980, pp. 469-525.

20. Nau, H. "Sequence analysis of polypeptides and proteins by combined gas chromatography-mass spectrometry." Angew. Chem. Int. Ed. Eng. 15; 75-86, 1976.

21. Shackleton, C.H.L. and W. Chai, "Mass spectrometry: application to steroid and peptide research." Endocrine Rev. 6; 441-486, 1985.

22. Carr, S.A. and K. Biemann. "Identification of posttranslationally modified amino acids in proteins by mass spectrometry." Meth. Enzymol. 106; 29-58, 1984.

23. Larson, B.R. "A model system for mass spectrometric sequencing of orthopthalaldehyde peptide derivatives." Life Sci. 30; 1003-1012, 1982.

24. Priddle, J.D., K. Rose and R.E. Offord. "The separation and sequencing of permethylated peptides by mass spectrometry directly coupled to gas-liquid chromatography." Biochem J. 157; 777-780, 1976.

25. Priddle, J.D., K. Rose and R.E. Offord. "Amino acid sequence determination by direct e.i./c.i. mass spectrometry of N-acyl;N,O-permethylated oligopeptides separated by gas chromatography." Adv. Mass Spectrom. 7B; 1502-1505, 1978.

26. Rose, K., A. Bairoch and R.E. Offord. "Amino acid sequence determination by gas chromatography-mass spectrometry of permethylated peptides." J. Chromatogr. 268; 197-206, 1983.

27. Rose, K., M.G. Simona and R.E. Offord. "Amino acid sequence determination by g.l.c.-mass spectrometry of permethylated peptides." Biochem J. 215; 261-272, 1983.

28. Rose, K., H.P. Kocher, B.M. Blumberg and D. Kolakofsky. "An improved procedure, involving mass spectrometry, for N-terminal amino acid sequence determination of proteins which are N^{α}-blocked." Biochem. J. 217; 253-257, 1984.

29. Mallet, A.I., K. Rose and J.D. Priddle. "Development of a gas chromatography mass spectrometry assay for the two eosinophil chemotactic factors of anaphylaxis." Biomed. Mass Spectrom. 10; 120-125, 1983.

30. Peralta, E., H.-Y.T. Yang, J. Hong and E. Costa. "Combination of enzymatic and mass fragmentographic assays for the identification and measurement of [met^5]-enkephalin." J. Chromatogr. 190; 43-51, 1980.

31. Hartman, N.R. and I. Jardine. "Mass spectrometric analysis of cyclosporine metabolites." Biomed. Environ. Mass Spectrom. 13; 361-372, 1986.

32. Garland, W.A. and M.L. Powell. "Quantitative selected ion monitoring (QSIM) of drugs and/or drug metabolites in biological matrices." J. Chromatogr. Sci. 19; 392-434, 1981.

33. K.D., Ballard, R.G. Knapp, T.D. Eller, J.G. Webb, W.H. Newman and D.R. Knapp. "Quantitative analysis of adenosine: statistical comparison of radioimmunoassay and gas chromatography-mass spectrometry-selected ion monitoring methods." Biomed. Environ. Mass Spectrom. 13; 667-675, 1986.

34. Pedersen, C.T. and P. Roepstorff. "Comparison of different derivatives for GC/MS analysis of peptides." Int. J. Mass Spectrom. Ion Phys. 48; 193-196, 1983.

35. Shemyakin, M.M., Yu. A. Ovchinnikov, E.I. Vinogradova, M.Yu. Feigina, A.A. Kiryushkin, N.A. Aldanova, Yu.B. Alakhov, V.M. Lipkin and B.V. Rosinov. "Mass spectrometric determination of the amino acid sequence in arginine-containing peptides." Experientia 23; 428-430, 1967.

36. King, T.P. "Selective chemical modification of arginyl residues." Biochemistry 5; 3454-3459, 1966.

37. Leclercq, P.A., L.C. Smith and D.M. Desiderio. "Modification, permethylation and mass spectrometry of arginine-containing oligopeptides." Biochem. Biophys. Res. Commun. 45; 937-944, 1971.

38. Morris, H.R., R.J. Dickinson and D.H. Williams. "Studies towards the complete sequence determination of proteins by mass spectrometry: derivatisation of methionine, cysteine and arginine containing peptides." Biochem. Biophys. Res. Commun. 51; 247-255, 1973.

39. Mori, A., T. Ichimura and H. Matsumoto. "Gas chromatography-mass spectrometry of guanidino compounds in brain." Anal. Biochem. 89; 393-399, 1978.

40. Erdtmansky, P. and T.J. Goehl. "Gas-liquid chromatographic electron capture determination of some monosubstituted guanidino-containing drugs." Anal. Chem. 47; 750-752, 1975.

41. Malcolm, S.L. and T.R. Marten. "Determination of debrisoquin and its 4-hydroxy metabolite in plasma by gas chromatography/mass spectrometry. Anal. Chem. 48; 807-809, 1976.

42. Brown, H.C., P. Heim and N.M. Yoon. "Selective reductions. XV. Reaction of diborane in tetrahydrofuran with selected organic compounds containing representative functional groups." J. Amer. Chem. Soc. 92; 1637-1646, 1970.

43. Frank, H., H.J.C. Das Neves and E. Bayer. "Use of borane as reducing agent in sequence analysis of peptides by gas chromatography-mass spectrometry." J. Chromatogr. 152; 357-362, 1978.

44. Bazan, A.C. and D.R. Knapp. "Improved derivative of 6-keto-prostaglandin $F_{1\alpha}$ for gas chromatographic-mass spectrometric analysis." J. Chromatogr. 236; 201-207, 1982.

45. Mawhinney, T.P. and M.A. Madsen. "N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide and related N-tert-butyldimethylsilyl amides as protective silyl donors." J. Org. Chem. 47; 3336-3339, 1982.

46. Kutschinski, J.L. "Silylation: use of the t-BuMe_2Si- group." Ph.D. Thesis, University of California 1974. Diss. Abstr. Int., B 35; 4838, 1975.

47. K.D., Ballard, D.R. Knapp, J.E. Oatis and T. Walle, "Resolution of the seven isomeric ring-hydroxylated propranolols as tert.-butyldimethylsilyl derivatives by capillary gas chromatography-mass spectrometry." J. Chromatogr. 277; 333, 1983.

48. Böhlen, P., F. Castillo, N. Ling and R. Guillemin. "Purification of peptides: an efficient procedure for the separation of peptides from amino acids and salt." Int. J. Peptide Protein Res. 16; 306-310, 1980.

49. Bennett, H.P.J., A.M. Hudson, C. McMartin and G.E. Pardon. "Use of octadecylsilyl-silica for the extraction and purification of peptides in biological samples." Biochem. J. 168; 9-13, 1977.

50. Schöneshöfer M. and A. Fenner. "Hydrophilic ion-pair reversed-phase chromatography of biogenic peptides prior to immunoassay." J. Chromatogr. 224; 472-476, 1981.

51. Browne, C.A., H.P.J. Bennett and S. Solomon. "The isolation of peptides by high-performance liquid chromatography using predicted elution positions." In: High Performance Liquid Chromatography of Proteins and Peptides, edited by M.T.W. Hearn, F.E. Regnier and C.T. Wehr. New York: Academic Press, 1983, pp. 65-72.

52. Schaaper, W.M.M., D. Voskamp and C. Olieman. "Perfluoroalkanoic acids as lipophilic ion-pairing reagents in reversed-phase liquid chromatography of peptides including secretin." J. Chromatogr. 195; 181-186, 1980.

53. Voskamp, D., C. Olieman and H.C. Beyerman. "The use of trifluoroacetic acid in the reverse-phase liquid chromatography of peptides including secretin." Recl. Trav. Chim. Pays-Bas 99; 105-108, 1980.

54. Patthy, L., A. Varadi, J. Thész and K. Kovacs. "Identification of the C-1-phosphate-binding arginine residue of rabbit-muscle aldolase." Eur J. Biochem. 99; 309-313, 1979.

55. Rose, K., J.D. Priddle and R.E. Offord. "Affinity technique for the isolation of polypeptides containing arginine modified with cyclohexane-1,2-dione, and their analysis by combined gas-liquid chromatography-mass spectrometry." J. Chromatogr. 210; 301-309, 1981.

56. Patthy, L. and E.L. Smith. "Reversible modification of arginine residues." J. Biol. Chem. 250; 557-564, 1975.

57. Feiser, L.F. and M. Feiser. "Reagents for Organic Synthesis." New York: Wiley, 1967, p. 705

58. Fales, H.M., T.M. Jaouni and J.F. Babashak. "Simple device for preparing ethereal diazomethane without resorting to codistillation." Anal. Chem. 45; 2302-2303, 2973.

59. Knapp, D.R. Handbook of Analytical Derivatization Reactions. New York: Wiley, 1979, pp. 20-21 (a) and 87 (b).

60. Cramers, C.A., G.J. Scherpenzeel and P.A. Leclercq. "Increased speed of analysis in directly coupled gas chromatography-mass spectrometry systems. Capillary columns at sub-atmospheric outlet pressures." J. Chromatogr. 203; 207-216, 1981.

61. Martin, S.A., C.E. Costello and K. Biemann. "Optimization of experimental procedures for fast atom bombardment mass spectrometry." Anal Chem. 54; 2362-2368, 1982.

62. Steglich, W. und S. Hinze. "Eine rationelle synthese von N-trifluoroacetylaminosäuren." Synthesis 1976; 399-401, 1976.

63. Pizey, J.S. Synthetic Reagents Volume II. New York: Wiley, 1974, pp. 65-143.

64. Grob, K. Jr. and G. Grob. "Capillary columns with immobilized stationary phases. Part 5. Determination of column bleeding; re-silylation." J. H. Res. Chrom. and Chrom. Commun. 5; 349-354, 1982.

65. Steiner, W. and A. Niederwieser. "Peptide analysis as amino alcohols by gas chromatography-mass spectrometry. Application to hyperoligopeptiduria. Detection of Gly-3Hyp-4Hyp and Gly-Pro-4Hyp-Gly." Clin. Chem. A. 92; 431-441, 1979.

66. Walle, T., J.E. Oatis, Jr., U.K. Walle and D.R. Knapp. "New ring-hydroxylated metabolites of propranolol. Species differences and stereospecific 7-hydroxylation." Drug Metab. Disp. 10; 122-127, 1982.

67. Walle, T. Personal communication, 1982.

68. Weast, R.C., editor. Handbook of Chemistry and Physics. Cleveland: The Chemical Rubber Company, 1972, pp. F183-F187.

69. Kamerling, J.P., G.J. Gerwig, J.F.G. Vliegenhart, M. Duran, D. Keting and S.K. Wadman. "Determination of the configuration of lactic and glyceric acids from human serum and urine by capillary gas-liquid chromatography." J. Chromatogr. 143; 117-123, 1977.

70. Ackman, R.G., R.E. Cox, G. Eglinton, S.N. Hooper and J.R. Maxwell. "Stereochemical studies of acyclic isoprenoid compounds I - gas chromatographic analysis of stereoisomers of a series of standard isoprenoid acids." J. Chromatogr. Sci. 10; 392-401, 1972.

71. Guette, J.P. and A. Horeau. "Determination of the optical purity and the value of the rotary power of racemic constituents by gas phase chromatography." Tetrahedron Lett. 1965; 3049-3056, 1965.

72. Nelson, W.L. and M.J. Bartols. "Absolute configuration of glycerol derivatives. 8. 2-Hydroxy-3-(1-naphthoxy)propionic acid, a major oxidative metabolite of the β-adrenergic antagonist propranolol." J. Org. Chem. 47; 1574-1576, 1982.

73. Ackman, R.G., S.N. Hooper, M. Kates, A.K. Sen Gupta, G. Eglinton and I. Maclean. "Phytanic acid 1-menthyl esters. Gas-liquid chromatographic separation of diastereomers." J. Chromatogr. 44; 256-261, 1969.

74. Ackman, R.G., M. Kates and R.P. Hansen. "Diastereomeric composition of pristanic acids of marine and terrestrial origin." Biochem. Biophys. Acta. 176; 673-675, 1969.

75. Murano, A. "Analytical studies on pesticides and related compounds XIII. Gas chromatographic separation and determination of optical isomers of chrysanthemic acid." Agr. Biol. Chem. 36; 917-923, 1972.

76. Bond, P.A. "Metabolism of propranolol (Inderal), a potent, specific β-adrenergic receptor blocking agent." Nature, London. 2; 721, 1967.

77. Hayes, A. and R.G. Cooper. "Studies on the absorption, distribution and excretion of propranolol in rat, dog and monkey." J. Pharmacol. Exp. Ther. 176; 302-311, 1971.

78. Patterson, J.W., M.E. Conolly, C.T. Dollery, A. Hayes and R.G. Cooper. Pharmacol. Clin. 2; 127, 1970.

79. Walle, T. and T.E. Gaffney. "Propranolol metabolism in man and dog: mass spectrometric identification of six new metabolites." J. Pharmacol. Exp. Ther. 182; 83-92, 1972.

80. Tindell, G.L., T. Walle and T.E. Gaffney. "Rat liver microsomal metabolism of propranolol: identification of seven metabolites by gas chromatography-mass spectrometry." Life Sci., Part II, 11; 1029-1036, 1972.

81. Oatis, J.E. Jr., M.P. Russell, D.R. Knapp and T. Walle. "Ring hydroxylated propranolol: synthesis and β-receptor antagonist and vasodilating activities of the seven isomers." J. Med. Chem. 24; 309-314, 1981.

82. Garland, W.A., and M.L. Powell. J. Chromatogr. Sci. 19; 392, 1981.

83. Hensby, C.N., M. Jogee, M.G. Elder and L. Myatt. "A comparison of the quantitative analysis of 6-oxo-$PGF_{1\alpha}$ in biological fluids by gas chromatography mass spectrometry and radioimmunoassay." Biomed. Mass Spectrom. 8; 111-117, 1981.

84. Bjorkhem, I., A. Bergman, O. Falk, A. Kallner, O. Lantto, L. Svensson, E. Akerlof and R. Blomstrand. "Accuracy of some routine methods used in clinical chemistry as judged by isotope dilution-mass spectrometry." Clin. Chem. 27; 733-735, 1981.

85. Trafford, D.J.H., S.A. Seamark, H. Turnbull and H.L.J. Makin. "High performance liquid chromatography of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in human plasma. Use of isotachysterols and a comparison with gas chromatography-mass spectrometry." J. Chromatogr. 226; 351-360, 1981.

86. Hensby, C.N., M. Jogee and L. Myatt. "Comparison of GC-MS and RIA methods for 6-oxo-$PGF_{1\alpha}$." Prog. Lip. Res. 20; 779-782, 1981.

87. Holdiness, M.R. "A comparison of measurement of homovanillic acid in brain tissue by gas chromatography mass spectrometry and liquid chromatography with electrochemical detection." Anal. Lett. B15(13); 1099-1109, 1982.

88. Lindberg, C., S. Jonsson, P. Hedner and A. Gustafsson. "Radioimmunoassay and chemical ionization mass spectrometry compared for plasma cortisol determination." Clin. Chem. 28; 174-177, 1982.

89. Smith, G.H. and R.H. Levy. "Serum quinidine determination: comparison of mass spectrometric and extraction-fluorescence methods." Drug Intell. Clin. Pharm. 16; 693-695, 1982.

90. McKay, G., K. Hall, J.K. Cooper, E.M. Hawes and K.K. Midha. "Gas chromatographic-mass spectrometric procedure for the quantitation of chlorpromazine in plasma and its comparison with a new high-performance liquid chromatographic assay with electrochemical detection." J. Chromatogr. 232; 275-282, 1982.

91. Lambert, W.E., J.P.M. De Slypere, J.A. Jonckheere, A. Vermeulen and A.P. De Leenheer. "Improved liquid chromatographic determination of serum cortisol with double internal standardization compared to radioimmunoassay and fluorometry, and evaluated by isotope dilution mass spectrometry." Anal. Biochem. 134; 216-223, 1983.

92. Heumann, K.G., W. Schrodl and H. Weiss. "Mass spectrometric and electrochemical bromide trace determination in geochemical stansard reference materials." Z. Anal. Chem. 315; 213, 1983.

93. Bukhave, K., K. Green and J. Rask-Madsen. "Comparison of radioimmunological determinations with gas chromatography mass spectrometry dosage. A study of PGE_2 and $PGF_{2\alpha}$ in gastrointestinal fluids." Biomed. Mass Spectrom. 10; 265-268, 1983.

94. Fraser, S., P. Cowen and M. Franklin. "Direct radioimmunoassay and gas chromatography-mass spectrometry compared for determination of melatonin in plasma." Clin. Chem. 29; 1703-1704, 1983.

95. Anderson, G.M., M.B. Bowers, R.H. Roth, J.G. Young, C.C. Hrbek and D.J. Cohen. "Comparison of high performance liquid chromatographic, gas chromatographic-mass spectrometric, and fluorometric methods for the determination of homovanillic acid and 5-hydroxyindoleacetic acid in human cerebrospinal fluid." J. Chromatogr. 277; 282-286, 1983.

96. Welch, M.J., A. Cohen, H.S. Hertz, F.C. Ruegg, R. Schaffer, L.T. Sniegoski and E. White V. "Determination of serum urea by isotope dilution mass spectrometry as a candidate definitive method." Anal. Chem. 56; 713-719, 1984.

97. Patterson, D.G., M.B. Patterson, P.H. Culbreth, D.M. Fast, J.S. Holler, E.J. Sampson and D.D. Bayse. "Determination of steroid hormones in a human-serum reference material by isotope dilution-mass spectrometry: a candidate definitive method for cortisol." Clin. Chem. 30; 619-626, 1984.

98. Tusell, J.M., F. Artigas, C. Sunol, E. Martines and E. Gelpi. "Comparison of high-performance liquid chromatography and gas chromatography-mass spectrometry for the analysis of indole-3-acetic acid in brain tissue." J. Chromatogr. 306; 338-344, 1984.

99. Chiabrando, C., M.N. Castagnoli, A. Noseda and R. Fanelli. "Comparison of radioimmunoassay and high-resolution gas chromatography mass spectrometry for the quantitative determination of serum thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ after pharmacological blockade of thromboxane synthetase." Prostaglandins, Leukotrienes Med. 16; 79-88, 1984.

100. Garrigues, P., G. Bourgeois, A. Veyres, J. Rima, M. Lamotte and M. Ewald. "Comparison of methylphenanthrene isomer detection in petroleum extracts by gas chromatography/mass spectrometry and Shpol'skii luminescence spectrometry." Anal. Chem. 57; 1068-1070, 1985.

101. Min, B.H., W.A. Garland, T.M. Lewinson and B.P. Mehta. "Comparison of gas chromatographic/mass spectrometric and microbiological assays for 5-fluorouracil in plasma." Biomed. Mass Spectrom. 12; 238-240, 1985.

102. Ziegler, J.M., O. Houot, M. Hernot and G. Siest. "Plasma uric acid: mass spectrometric method for control and comparison of intra- and interlaboratory studies." Clin. Chem. Acta 147; 135-143, 1985.

103. Herold, D.A., R.M. Ross, J.R. Shipe Jr., G.T. Redpath, J.H. Bowden III and A.F. Arlinghaus. "Comparison of enzymatic and mass spectrometric determination of urinary oxalate." Clin. Chem. 31; 993, 1985.

104. Tada, M., H. Matsuda, T. Kuzuya, M. Inui and H. Abe. "Development of a quantitative analysis of plasma thromboxane B_2 by gas chromatography-mass spectrometry." Jpn. Circ. J. 49; 276-283, 1985.

105. Roberts, L.J. II, K.A. Aulsebrook and J.A. Oates. "Comparative evaluation of the radioenzymatic method for the determination of urinary histamine with a mass spectrometric assay. J. Chromatogr. 338; 41-49, 1985.

106. Zipser, R.D., A. Morrison, G. Laffi and R. Duke. "Assay methods for 6-keto-prostaglandin $F_{1\alpha}$ in human urine. Comparison of chromatographic techniques with radioimmunoassay and gas chromatography-negative ion chemical-ionization mass spectrometry." J. Chromatogr. 339; 1-9, 1985.

107. Barnett, R.N. "A scheme for the comparison of quantitative methods." Amer. J. Clin. Path. 43; 562-569, 1965.

108. Barnett, R.N. and W.J. Youden. "A revised scheme for the comparison of quantitative methods." Amer. J. Clin. Path. 54; 454-462, 1970.

109. Westgard, J.O. and M.R. Hunt. "Use and interpretation of common statistical tests in method-comparison studies." Clin. Chem. 19; 49-57, 1973.

110. Wakkers, P.J.M., H.B.A. Hellendoorn, G.J. Op De Weegh, and W. Heerspink. "Applications of statistics in clinical chemistry. A critical evaluation of regression lines." Clin. Chem. Acta 64; 173-184, 1975.

111. Cornbleet, P.J. and N. Gochman. "Incorrect least-squares regression coefficients in method-comparison analysis." Clin. Chem. 25; 432-438, 1979.

112. Schubert, E.M., and K.H. Schram, presented at the International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Kansas City, Missouri, June 6-11, 1982.

113. Bunger, R., F.J. Haddy, A. Querengasser and E. Gerlach. "An isolated guinea pig heart preparation with in vivo like features." Pflugers Arch. 353; 317-326, 1975.

114. Newman, W.H., J.T. Lee, T.D. Eller and J.G. Webb. "Persistent desensitization of the heart to the inotropic action of isoproterenol by adenosine." Circulation 72; III-315, 1985.

115. Colby, B.N. and M.W. McCaman. "A comparison of calculation procedures for isotope dilution determinations using gas chromatography mass spectrometry." Biomed. Mass Spectrom. 6; 225-230, 1979.

116. Pickup, J.F. and K. McPherson. "Theoretical considerations in stable isotope dilution mass spectrometry for organic analysis." Anal. Chem. 48; 1885-1890, 1976.

117. Robard, D. and J.E. Lewald. "Computer analysis of radiological assay and radioimmunoassay data." Acta Endocrinol., Copenhagen, Suppl. 147; 79-92, 1970.

118. Williams, E.J. Regression Analysis. New York: Wiley, 1959, pp. 212-214.

119. Neter, J. and W. Wasserman. Applied Linear Statistical Models; Regression, Analysis of Variance and Experimental Designs. Homewood, Illinois: Irwin, 1974, pp. 140-145.

120. Pang, H., K.H. Schram, D.L. Smith, S.P. Gupta, L.B. Townsend and J.A. McClosky. "Mass spectrometry of nucleic acid constituents. Trimethylsilyl derivatives of nucleosides." J. Org. Chem. 47; 3923-3932, 1982.

121. Bush, E.D. and W.F. Trager. "Analysis of linear approaches to quantitative stable isotope methodology in mass spectrometry." Biomed. Mass Spectrom. 8; 211-218, 1981.

122. Munson, P.J. and D. Rodbard. "Statistical comparison of two assay methods (weighted linear regression with errors in x and y)." In: Quality Control in Clinical Endocrinology. Proceedings of the Eighth Tenovus Workshop, edited by D.W. Wilson, S.J. Gaskell and K.W. Kemp. London: Alpha Omega Publishing Limited, 1979, pp. 89-98.