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New Techniques for the Qualitative and Quantitative Measurement of Naturally-Ocurring Gonadotropin-Releasing Hormone Analogues by Mass Spectrometry

Tanya R. Myers

NEW TECHNIQUES FOR THE QUALITATIVE AND QUANTITATIVE MEASUREMENT OF NATURALLY-OCCURRING GONADOTROPIN-RELEASING HORMONE ANALOGUES BY MASS SPECTROMETRY

by

TANYA R. MYERS

Under the Direction of Gabor Patonay

ABSTRACT

GnRH peptides have been discovered in a wide variety of vertebrate and invertebrate organisms, and work is ongoing to characterize additional unique isoforms. This dissertation describes the investigation of reversed-phase chromatographic and mass spectrometric behavior of GnRH peptides, the development and application of an LC-MS/MS method for qualitative identification of GnRH peptides, and the comprehensive validation of an LC-MS/MS method for simultaneous, quantitative measurement of hydroxyproline^{9}GnRH (Hyp^{9}GnRH) and mammalian GnRH (mGnRH) in rat brain tissues.

Chromatographic and mass spectrometric behavior of GnRH isoforms was characterized for six GnRH model peptides. Using reversed-phase high performance liquid chromatography (HPLC), nearly complete separation of the model GnRH peptides was achieved. Evaluation of electrospray source conditions indicated that certain parameters can be adjusted to affect the abundance of selected charge states and improve response.

Using the conditions found to be optimal for GnRH peptides in general, a method was developed to facilitate characterization of novel GnRH isoforms or confirm the identity of known isoforms. Fragmentation patterns for six model GnRH isoforms were examined to determine what portion of the primary sequence could be elucidated by *de novo* sequencing, and a simple solid phase extraction protocol was developed to isolate the model GnRH compounds from tissue samples. Application of the method to rat brain samples resulted in successful isolation and structural confirmation of hydroxyproline^{9}GnRH and mammalian GnRH.

A quantitative method for the determination of concentrations of hydroxyproline^{9}GnRH and mammalian GnRH in rat brain tissue was developed and rigorously validated. Guinea pig brains were found to be a suitable substitute matrix for rat brains, and accuracy and precision were determined after four validation runs. Stability of both peptides in samples over long-term storage and under experimental conditions were evaluated, and the LC-MS/MS method was compared to an enzyme-linked immunoassay. Thirty-one brains from Sprague-Dawley rats were analyzed using the LC-MS/MS procedure and compared to published results for Hyp^{9}GnRH and mGnRH.

INDEX WORDS: GnRH, LHRH, *De novo* sequencing, Rat brain, Hydroxyproline, Mass spectrometry, Method validation, Peptide quantitation

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LIST OF ABBREVIATIONS

ACC	Accuracy
ACN	Acetonitrile
AGC	Automatic gain control
Ala (or A)	Alanine
Arg (or R)	Arginine
Asn (or N)	Asparagine
Asp (or D)	Aspartic Acid
ºC	Degrees Celcius
cDNA	Complementary deoxyribonucleic acid
CE	Capillary electrophoresis
CE/MS	Capillary electrophoresis-mass spectrometry
cGnRH-I	Chicken I GnRH
cGnRH-II	Chicken II GnRH
C.I.	Confidence interval
Cys (or C)	Cysteine
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAB	Fast atom bombardment
fmol	Femtomole
FSH	Follicle stimulating hormone
g	Centrifugal force
Gln (or Q)	Glutamine
Glu (or E)	Glutamic acid
Gly (or G)	Glycine
GnRH	Gonadotropin-releasing hormone
gpGnRH	Guinea Pig GnRH
HFBA	Heptafluorobutyric acid
His (or H)	Histidine
HPLC	High performance liquid chromatography
Hyp^9GnRH	$Hydroxyproline^9$ GnRH
irGnRH	Immunoreactive GnRH
kV	Kilovolt(s)
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
Leu (or L)	Leucine
lGnRH-I	Lamprey I GnRH
lGnRH-III	Lamprey III GnRH
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
LLOQ	Lower limit of quantitation
Lys (or K)	Lysine

Met (or M)	Methionine
mGnRH	Mammalian GnRH
mGnRH	Mammalian GnRH, free acid form
mHypGnRH	Hydroxyproline^{9} GnRH
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar
MRFA	l-methionyl-arginyl-phenylalanyl-alanine
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multiple stage mass spectrometry
m/z	Mass-to-charge ratio
MW	Molecular weight
ng	Nanogram(s)
pGlu	Pyroglutamic acid
pg	Picogram(s)
pH	Hydrogen ion concentration
Phe (or F)	Phenylalanine
pI	Isoelectric point
pmol	Picomole(s)
Pro (or P)	Proline
QC	Quality control
R^2	Coefficient of determination
RE	Relative error
REC	Recovery
RIA	Radioimmunoassay
RSD	Relative standard deviation
SEM	Standard error mean
Ser (or S)	Serine
sbGnRH	Seabream GnRH
sGnRH	Salmon GnRH
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SRM	Selected reaction monitoring
Std. Dev.	Standard deviation
TFA	Trifluoracetic acid
Thr (or T)	Threonine
Trp (or W)	Tryptophan
Tyr (or Y)	Tyrosine
µ	Micron
µL	Microliter(s)

ug	Microgram(s)
ULOQ	Upper limit of quantitation
UV	Ultraviolet
Val (or V)	Valine
V	Volt(s)
vol	Volume

CHAPTER 1: INTRODUCTION

1.1 Overview

Gonadotropin-releasing hormone (GnRH) is a critical neurohormone in the reproductive cycle of vertebrate species. In mammals, pulsatile release of the GnRH peptide from the hypothalamus stimulates the production of the gonadotropins FSH (follicle stimulating hormone) and LH (luteinizing hormone). These gonadotropins are released into systemic circulation for transport to the gonads, where they regulate gametogenesis and steroidogenesis. In addition to its critical role in reproduction, GnRH is thought to have functions beyond that of a neurotransmitter in vertebrates and has been found in tissues other than brain or pituitary (1). Fourteen GnRH isoforms have been identified thus far in vertebrates (2).

Studies of GnRH in invertebrates have thus far been limited to protochordates, in which ten distinct forms have been identified (3-5). GnRH peptides are believed to have reproductive functions in invertebrates, although their role in regards to reproduction is less well understood as compared to that in vertebrates. Several studies have found hormonal action, while others have proposed pheromonal activity (5-7). Potential unique GnRH forms have been isolated in a number of other organisms including coral, freshwater snails, marine worms and sea slugs although the structures of these forms have not been determined (8-11).

The forms of GnRH which have been identified in vertebrates and invertebrates are listed in Table 1.1. With the exception of mammalian GnRH, which was first isolated from pig and sheep and retains a more generic name, individual forms are listed by the organism in which they were first discovered. Older studies may use the name luteinizing

hormone-releasing hormone (LHRH), a more specific name originating prior to the discovery that GnRH also mediates release of FSH.

Table 1.1. Amino acid sequences of naturally-occurring GnRH forms

GnRH	Amino Acid Sequence	Ref.
Vertebrate		
Catfish	pGlu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2	(12)
Chicken I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2	(13, 14)
Chicken II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2	(15)
Dogfish	pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2	(16)
Guinea Pig	pGlu-Tyr-Trp-Ser-Tyr-Gly-Val-Arg-Pro-Gly-NH_2	(17)
Herring	pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH_2	(18)
Mammalian	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	(19, 20)
Lamprey I*	pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2	(21)
Lamprey III	pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2	(22)
Pejerrey**	pGlu-His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly-NH_2	(23)
Rana	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Trp-Pro-Gly-NH_2	(24)
Salmon	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2	(25)
Seabream	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2	(26)
Whitefish	pGlu-His-Trp-Ser-Tyr-Gly-Met-Asn-Pro-Gly-NH_2	(27)
Invertebrate		
Octopus	pGlu-Asn-Tyr-His-Phe-Ser-Asn-Gly-Trp-His-Pro-Gly-NH_2	(4)
Tunicate I	pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2	(3)
Tunicate II	pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2	(3)
Tunicate III	pGlu-His-Trp-Ser-Tyr-Glu-Phe-Met-Pro-Gly-NH_2	(5)
Tunicate IV	pGlu-His-Trp-Ser-Asn-Gln-Leu-Thr-Pro-Gly-NH_2	(5)
Tunicate V	pGlu-His-Trp-Ser-Tyr-Glu-Tyr-Met-Pro-Gly-NH_2	(5)
Tunicate VI	pGlu-His-Trp-Ser-Lys-Gly-Tyr-Ser-Pro-Gly-NH_2	(5)
Tunicate VII	pGlu-His-Trp-Ser-Tyr-Ala-Leu-Ser-Pro-Gly-NH_2	(5)
Tunicate VIII	pGlu-His-Trp-Ser-Leu-Ala-Leu-Ser-Pro-Gly-NH_2	(5)
Tunicate IX	pGlu-His-Trp-Ser-Asn-Lys-Leu-Ala-Pro-Gly-NH_2	(5)

*Lamprey II has not yet been fully characterized, and is therefore not included in the table.
**Also referred to as Medaka.

Due to the critical function of GnRH in reproductive processes, the multiplicity of forms, the presence of certain isoforms in many diverse species (some of which are so-called ancient forms), and the presence of this neuropeptide in vertebrates and invertebrates, interest in comparative studies of the GnRH family has grown considerably over the past 30 years. Such investigations of GnRH structures provide information about the evolution of the GnRH molecule and the evolution of reproductive systems.

1.2 Peptide Structure

All vertebrate forms of GnRH are decapeptides, as are all but one of the forms identified in invertebrates. Specific residues in the primary structures of the peptides are highly conserved, including the pyroglutamic acid N-terminus, the Ser^4 residue (Ser^6 in octopus GnRH), the Pro^9 residue (Pro^{11} in octopus GnRH) and the amidated glycine C-terminus. The His^2 residue and the Trp^3 residue are nearly conserved in that variations are observed in only a few organisms.

The conservation of certain residues within the GnRH structure suggests that these residues may be important for conformation and activity of the peptide. A review of the effect of amino acid substitutions on GnRH activity suggests the following generalizations: 1) residues of the C-terminus (Pro-Gly-NH_2) are important for binding to the GnRH receptor, 2) residues of the N-terminus (pGlu-His-Trp-Ser) play an important role in binding to and activation of the GnRH receptor, 3) His^2 and Trp^3 are probably involved and $pGlu^1$ may be involved in receptor activation, 4) residues in the central domain (residues 5 - 8), where more heterogeneity of amino acids is observed, are less critical although Arg^8 appears critical for high-affinity binding of mammalian GnRH to

the mammalian GnRH receptor in pituitary, and 5) Gly^6 is critical for appropriate folding of the peptide, possibly due to its lack of chirality (28).

1.3 Distribution Among Organisms

Many of the isoforms of GnRH have been identified in multiple species. The most widely distributed isoform, chicken II GnRH has been found in over 90 different organisms and is thought to be one of the most ancient isoforms found to date (29). The mammalian and chicken I isoforms also have been found in a diversity of organisms from multiple taxonomic classes, and salmon GnRH appears to be widely distributed among the Osteichthyes class of fish (bony fish) (29). Other isoforms, such as octopus, rana, and whitefish GnRH have been found in their respective species only (4, 24, 27).

Based on the reported distribution of GnRH peptides, it is believed that vertebrates express at least two isoforms. In humans, the mammalian and chicken II forms are present (30). Multiple fish species have three forms, and it has been proposed that rockfish may produce a fourth, as yet uncharacterized form (13, 31). The diversity across species has generated interest in GnRH as an indicator of the evolution of reproductive processes. In addition, the diversity within a single species has generated interest in linking particular functions to the presence and levels of the distinct forms.

1.4 Isoforms of Interest

Eight GnRH isoforms were of particular interest to the research described in this dissertation – chicken I GnRH (cGnRH-I), chicken II GnRH (cGnRH-II), guinea pig GnRH (gpGnRH), lamprey III GnRH (lGnRH-III), mammalian GnRH (mGnRH), hydroxyproline mammalian GnRH (Hyp^9GnRH), salmon GnRH (sGnRH) and seabream GnRH (sbGnRH). Six of these isoforms (cGnRH-I, cGnRH-II, lGnRH-III, mGnRH,

sGnRH and sbGnRH) were selected as model peptides for purposes of method development. cGnRH-II and mGnRH were chosen due to their importance in mammalian reproductive processes and for their reported presence in rat brain tissue; lGnRH-III and sGnRH were of interest for having been proposed also as present in rat brain tissue (32), the investigation of which is further described in Chapter 3. cGnRH-I and sbGnRH were added to the group of model isoforms for additional structural heterogeneity during methods development. Two additional isoforms, Hyp^{9}GnRH and gpGnRH, became of interest during the conduct of the experiments supporting this dissertation, as described in Chapters 3 and 4. Further details regarding distribution among organisms, localization, and functionality of these eight isoforms are described below.

Chicken I GnRH

While Chicken I GnRH (cGnRH-I) was the first isoform discovered in avian species, it has a more limited distribution among species than cGnRH-II and has been found only in avian and select reptilian species (29). This paucity suggests that cGnRH-I is a newer form than cGnRH-II. Functionally, cGnRH-I is critical to reproductive processes in birds and is localized in the hypothalamic region (33). The primary structure of the cGnRH-I isoform is shown in Figure 1.1.

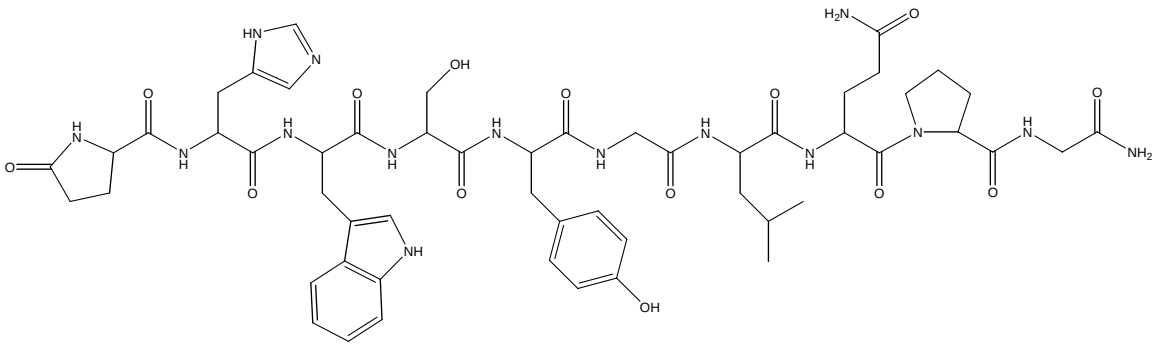

Figure 1.1. Primary structure of cGnRH-I

Chicken II GnRH

Chicken II GnRH (cGnRH-II) is the most widely distributed GnRH isoform, having been found in numerous mammals, birds, reptiles, amphibians, bony fish and cartilaginous fish (29). The breadth of distribution of this GnRH form, found in representative species of all vertebrate classes except the Agnanthans (lamprey), suggests that it is an older form than mGnRH (1). While cGnRH-II triggers FSH and LH release in mammals, it is not as effective as mGnRH (15). The locale of cGnRH-II (primarily distributed in the midbrain) differs from that of mGnRH (primarily distributed in the hypothalamus) in a number of vertebrate species, leading to speculation that the function of the two isoforms differs and that cGnRH-II may act as a neurotransmitter of unknown function (34). Similar to mGnRH, evidence of cGnRH-II has also been found in tissues outside the brain (35). The primary structure of the cGnRH-II isoform is shown in Figure 1.2.

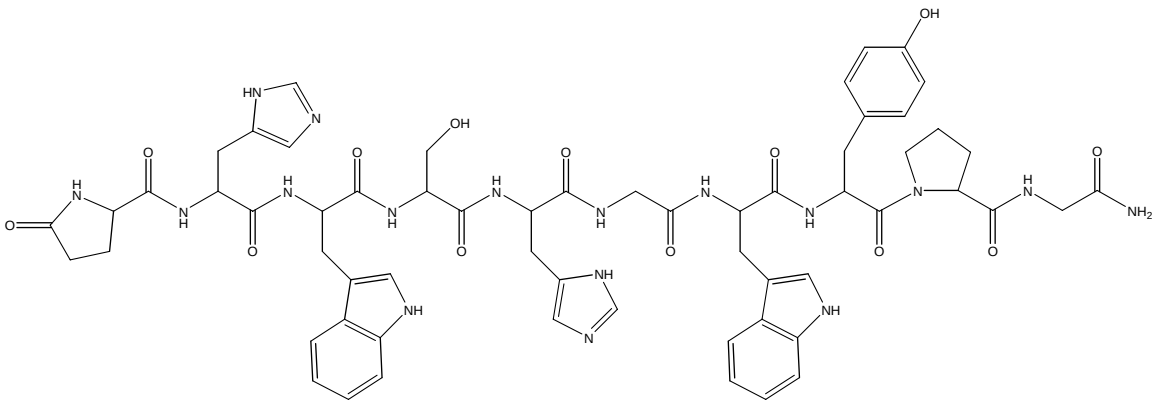

Figure 1.2. Primary structure of cGnRH-II

Guinea Pig GnRH

The occurrence of guinea pig GnRH (gpGnRH) appears to be limited to select species; to date, it has been isolated only in guinea pig and capybara (36). The primary structure of gpGnRH is shown in Figure 1.3.

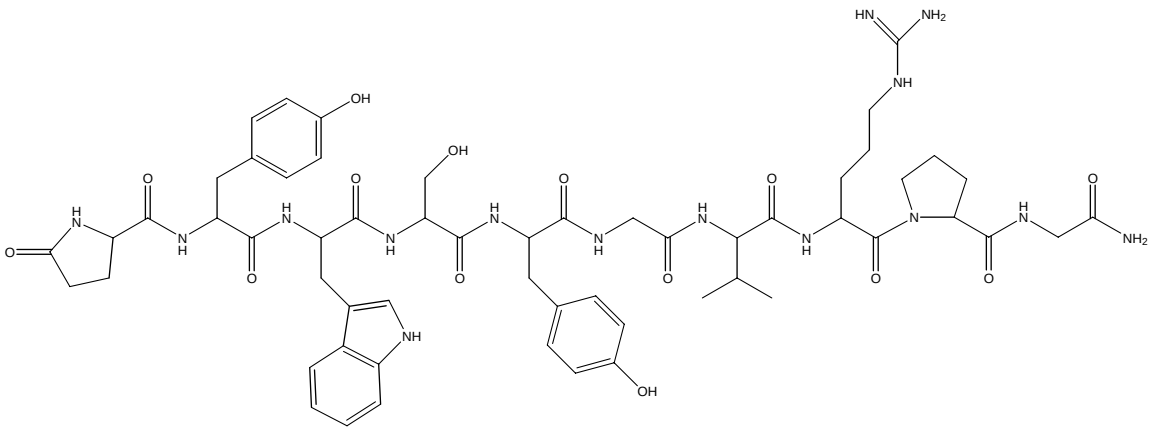

Figure 1.3. Primary structure of gpGnRH

Lamprey III GnRH

Three distinct GnRH-like peptides have been isolated in lamprey brain, and primary sequences have been proposed for two of the three, lamprey I GnRH (lGnRH-I) and lamprey III GnRH (lGnRH-III); further, both lGnRH-I and lGnRH-III have been found in three families of lamprey (21, 22, 37). While a few studies have suggested that lGnRH-I or III are expressed in rat, human and sheep and may be an FSH-releasing agent (38-41), others have found no evidence for this isoform in mammalian species (32, 42). The third GnRH-like peptide, deemed lamprey II GnRH, has never been fully characterized. The primary structure of the lGnRH-III isoform is shown in Figure 1.4.

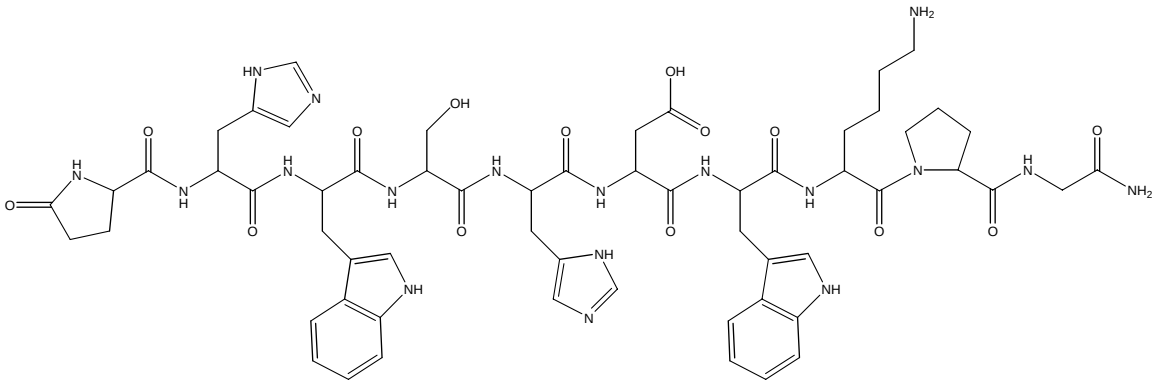

Figure 1.4. Primary structure of lGnRH-III

Mammalian GnRH and hydroxyproline mammalian GnRH

Mammalian GnRH (mGnRH) was the first LH- and FSH-releasing peptide to be isolated and characterized, initially in pig followed shortly thereafter in sheep (19, 20).

The mammalian isoform was thought to be unique until other homologous isoforms were discovered. mGnRH has subsequently been found in a wide variety of mammalian organisms (human, monkey, rodent) as well as bony fish and amphibians (29).

The mGnRH peptide is released from the hypothalamus and is transported to the pituitary, where gonadotropin receptors release FSH and LH (34). Evidence for the presence of mGnRH has also been found in extra-hypothalamic tissues, including mammary gland, prostate, pancreatic, and placental tissue (35). A post-translationally modified form of mGnRH has been isolated and identified in human, sheep, rodent and frog hypothalamus; this modified form is distinguished from mGnRH by a hydroxylated-proline in position 9 and is commonly referred to as hydroxyproline^{9} GnRH or Hyp^{9}GnRH (32, 43). The primary structures of the mGnRH and Hyp^{9}GnRH isoforms are shown in Figures 1.5 and 1.6, respectively.

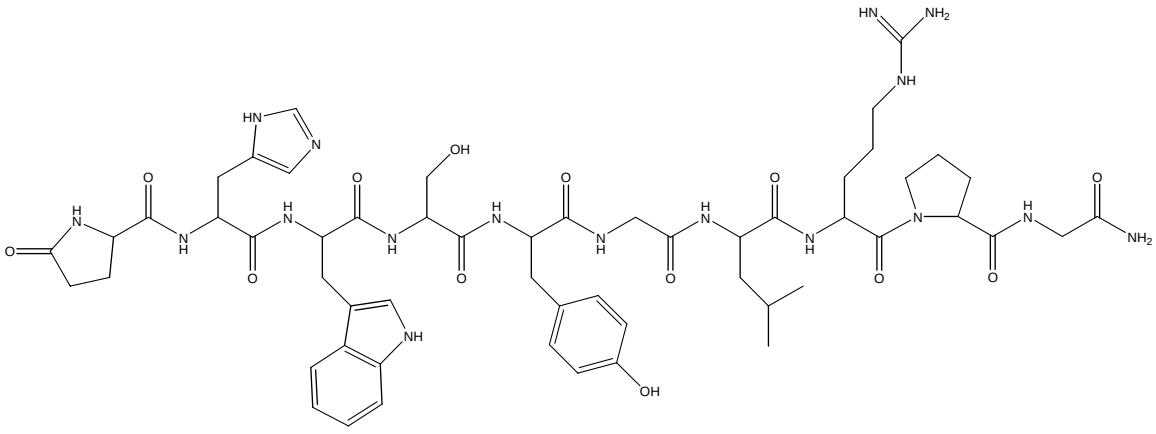

Figure 1.5. Primary structure of mGnRH

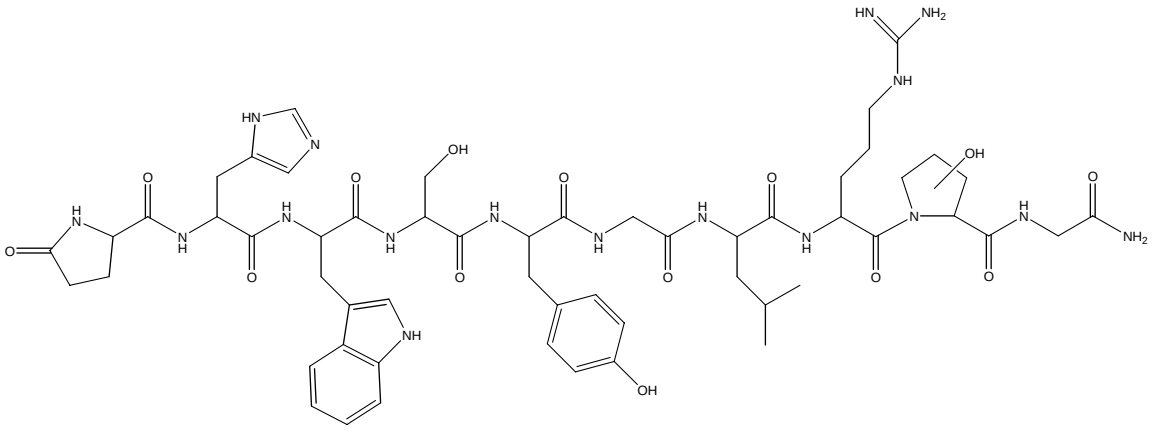

Figure 1.6. Primary structure of Hyp^{9}GnRH

Salmon GnRH

To date, salmon GnRH (sGnRH) has been found exclusively in bony fish, usually coexistent with at least one other isoform. In some species, sGnRH coordinates release of gonadotropins (analogous to mGnRH in mammals) while in other species, a different form of GnRH is responsible for this activity and the exact function of sGnRH is unclear; thus, the function of sGnRH in these fish is apparently complex and dependent upon location within the brain (29). The primary structure of the sGnRH isoform is shown in Figure 1.7.

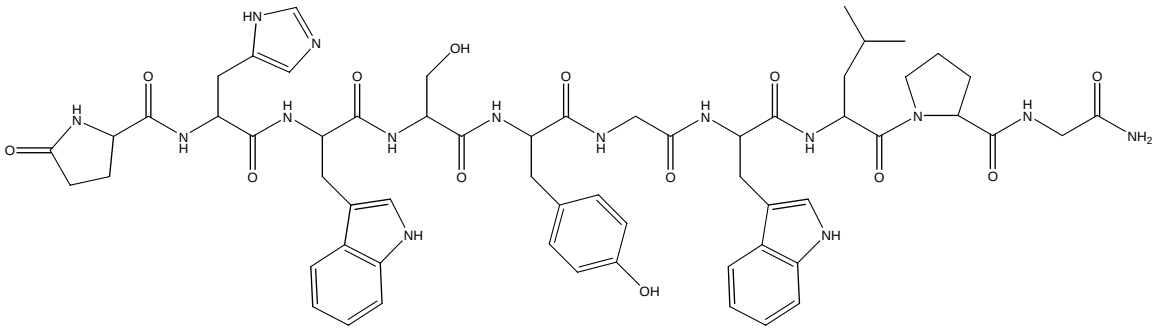

Figure 1.7. Primary structure of sGnRH

Seabream GnRH

Seabream GnRH (sbGnRH) has been isolated solely in bony fish, in combination with sGnRH and cGnRH-II (26, 44, 45). When these three isoforms are found together, sbGnRH is the form isolated in the pituitary and is presumed to be the GnRH-releasing form (45-48). The primary structure of the sbGnRH isoform is shown in Figure 1.8.

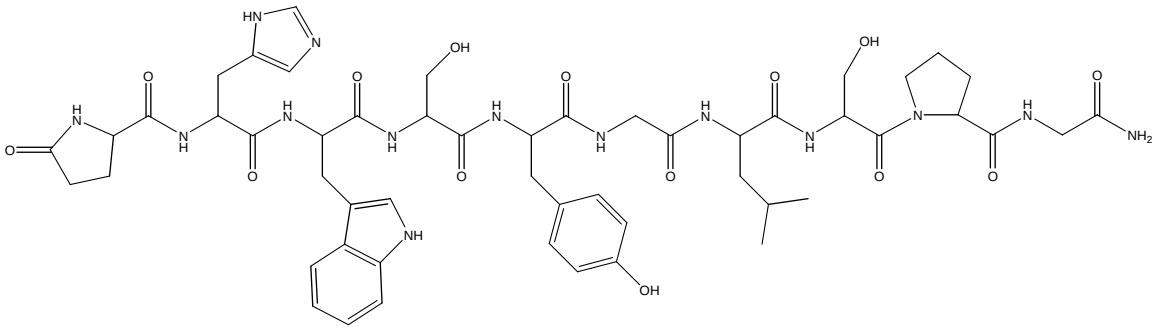

Figure 1.8. Primary structure of sbGnRH

1.5 Nomenclature

The original nomenclature applied to GnRH is somewhat ineffective in that a particular isoform can be found in many organisms. Several alternative naming strategies have been proposed in an effort to make the nomenclature more meaningful. One proposal suggests that isoforms should be referred to based on their homology to mammalian GnRH and that GnRH genes may be divided into one of three types, based on location of expression, function and molecular phylogenetic analysis (49). This scheme would redefine chicken II GnRH as {His^{5}Trp^{7}Tyr^8}GnRH, indicating which positions are substituted relative to mammalian GnRH, and would define genes as GnRH-I if hypothalamic in origin, GnRH-II if mesencephalic (midbrain), and GnRH-III if telencephalic (endbrain). A fourth type (GnRH-IV) has been suggested to include the lamprey forms of GnRH (50). The individual isoforms are sometimes referred to by type. In humans, GnRH-I is the mammalian isoform based on its hypothalamic location, and GnRH-II is the chicken II isoform based on its mesencephalic location. Another nomenclature, also based on location and function, divides GnRH isoforms into MB-GnRH (midbrain location), VF-GNRH (hypophysiotropic function located ventral forebrain), and TN-GnRH (neuromodulatory function located in terminal nerve) (51). However, confusion can be introduced when dealing with multiple organisms because the same isoform may have a different type or classification depending upon the organism (salmon GnRH is classified as GnRH-I in salmon and as GnRH-III in seabream).

Isoform names based on comparisons of homology to mammalian GnRH quickly become unwieldy when dealing with multiple isoforms. Likewise, naming the isoforms based on type can be confusing when dealing with more than one organism. In addition,

none of these naming conventions address the invertebrate isoforms of GnRH. Because this dissertation research is concerned with developing quantitative and qualitative liquid chromatography – tandem mass spectrometry (LC-MS/MS) methods that are applicable to multiple isoforms, irregardless of location or function, the original nomenclature (based on the organism in which the isoform was first discovered) will be used.

1.6 Analytical Methodologies – Limitations and Areas for Improvement

Current analytical methodologies for identification of GnRH isoforms are highly dependent upon knowledge of the isoforms that may be present in a particular sample, a requirement that is somewhat contrary to the analysis of unknown or novel forms. Additionally, current methods (both qualitative and quantitative) must be performed individually for each unique isoform, a limitation that increases the amount of sample required as well as the time required to complete analysis for all isoforms. Furthermore, these methods require lengthy preparative steps and separation procedures.

The experiments described in this dissertation demonstrate that simultaneous separation and identification of GnRH isoforms can be achieved in samples prepared using a relatively simple homogenization and clean-up procedure followed by LC-MS/MS analysis. In addition, this sample preparation procedure can be adapted to quantitative measurements of multiple isoforms in the same sample. These new strategies for qualitative and quantitative determinations of GnRH isoforms are applicable to countless forms of the peptide and provide a more efficient and faster approach to GnRH analysis than existing techniques, which are discussed in further detail below.

Current Qualitative Methods

Traditional analytical methodologies for identification of novel forms of GnRH utilize multiple HPLC fractionation steps with detection of putative GnRH peptides via immunoassay techniques, followed by structural confirmation via amino acid sequencing and molecular weight confirmation via mass spectrometry (18, 23, 27). These techniques rely on the availability of an antiserum with a broad specificity such that it will bind to the unknown form of GnRH. In the case of octopus GnRH, which has a 12 amino acid structure novel to the GnRH family of peptides, a more complicated protocol was utilized. Coarse purification of octopus GnRH was accomplished by use of an octopus heart bioassay for identification of bioreactive fractions from HPLC fractionation; subsequently, an antiserum specific to octopus GnRH was developed and used to identify specific fractions of interest for sequencing after further purification (4).

Because sequencing and mass spectrometric methods are performed only on the immunoreactive fractions, the methods described above may not detect novel forms of GnRH in cases where the antisera employed are not reactive to the unknown form. In addition, multiple fractionation procedures and associated analyses are laborious, and structural confirmation must be performed individually for each form of GnRH that is tentatively identified via immunoassay. Assays for known forms of GnRH can be accomplished using antisera with specificity for the isoforms of interest, thereby eliminating the need for sequence identification. However, the possibility for false positive results exists, and such false results may be responsible for conflicting results regarding the isoforms present in rat brains (32).

Molecular biological techniques have been applied also to identify potential new forms of GnRH. The structure of rana GnRH was determined by cloning cDNA encoding for a peptide varying from mGnRH by one amino acid; immunohistochemistry was then used to probe various tissues for expression of the new isoform (24). In addition, seven new isoforms of GnRH in tunicates were determined using in silico methods as well as cDNA cloning and immunohistochemistry (5). These methods are also laborious and should be considered indirect in that expression of the peptides is determined by immunological responses rather than by directly sequencing the peptide.

To date, no methodology has been reported that can provide simultaneous separation and sequence identification of multiple GnRH analogues. This ability to detect multiple forms would be of tremendous benefit for studies of novel GnRH isoforms as well as studies of known isoforms in previously uncharacterized organisms. Thus, one of the goals of this dissertation was to develop a broadly applicable LC-MS/MS method capable of chromatographic separation of multiple GnRH isoforms with concurrent production of mass spectra having sufficient quality to propose the identity of unknown and known GnRH isoforms; a secondary objective was that this novel methodology be more efficient and faster to employ than methods currently used to identify GnRH isoforms.

Current Quantitative Methods

The conventional method for quantitation of GnRH in biological samples involves extraction from the matrix followed by immunoassay detection. Concentrations of various isoforms of GnRH have been measured via lab-developed immunoassay methods in a variety of sample matrices including human plasma (mGnRH, detection limit 0.1

pg/mL) (52), rat plasma and brain (mGnRH, 0.7 pg/mL) (53), hagfish brain (total GnRH, 9.8 pg/mL) (54), lamprey brain (lGnRH-I and lGnRH-III, 98 pg/mL) (55), seabream pituitaries (sbGnRH, 100 pg/mL; cGnRH-II, 140 pg/mL; sGnRH, 40 pg/mL) (56), sea bass brain and pituitary (sGnRH, 40 pg/mL) (57), salmon gonad, brain and pituitary (sGnRH, 136 pg/mL) (58), perfusate from cannulized Ewes (mGnRH, 19 pg/mL) (59), and perfusate from cannulized rhesus monkeys (mGnRH, 0.7 pg/mL) (60). Commercial kits are also available. Detection limits comparable to those achieved from lab-developed immunoassay methods have been reported for measurement of mGnRH in rat plasma (0.1 pg/mL) and mGnRH in human plasma (16 pg/mL) (61, 62).

Several non-immunoassay methods have been employed for the analysis of GnRH concentration in biological samples. A liquid chromatographic assay with UV detection and lower limits of quantitation of 1.25 μg/mL has been utilized for determination of mGnRH and degradant products in intestinal extracts from possum (63). A capillary electrophoresis assay (UV detection) with lower limits of quantitation of 0.05 mM (60 ug/mL) and 0.06 mM (70 μg/mL) for mGnRH and sGnRH, respectively, has been used to assay incubations of GnRH in intestinal extracts from salmon (64).

A unique hybrid immunoassay – CE/MS method has been employed to determine mGnRH concentrations in serum and urine (65). An affinity capillary CE column selectively isolated mGnRH for quantitation by UV detection, with collection of qualitative mass spectral information. Reported detection limits were 1 ng/mL in plasma.

The popularity of immunoassay methods is most likely due to the sensitivity (picomolar concentrations) that can be achieved. However, these methods may suffer from lack of specificity depending upon the antibodies used, cross-reacting with other

GnRH isoforms or metabolic and degradation products of GnRH. Due to this concern, many methods employ lengthy cleanup and HPLC fractionation steps prior to immunoassay. In addition, comparisons across studies may be hampered by the differences in antibodies used.

A method capable of providing sensitive and selective determination of GnRH concentrations in biological samples, with broad applicability to the array of GnRH peptides, would be a useful addition to the methods currently employed for GnRH quantitative analysis. Therefore, another goal of the research described in this dissertation was to develop an LC-MS/MS-based method for quantitation of single or multiple GnRH isoforms, again with a secondary objective that this method be more efficient and faster to employ than methods currently used to determine GnRH quantities.

1.7 Scope and Goals of the Research Project

The focus of the dissertation research is the application of mass spectrometric methods to analysis of naturally-occurring GnRH peptides in biological samples. The work is directed toward three areas: exploration of the chromatographic and mass spectrometric behavior of the peptides, development of a qualitative method simultaneously applicable to multiple GnRH analogues, and development of a quantitative strategy that can be applied to multiple GnRH analogues.

These methods offer significant improvements to current techniques for multiple reasons. Qualitative determinations of multiple GnRH isoforms by LC-MS/MS methods can be made in one analysis without prior knowledge of which isoforms may be in the sample, and without the need to develop or purchase antibody or cDNA products pertinent to unknown forms. Since multiple isoforms can be assayed in a single run, the

sample burden posed by multi-step procedures is reduced. The qualitative LC-MS/MS methods described in this dissertation also provide immediate insight into post-translational modifications of expressed peptides, as spectral information is readily available to compare to that of the unmodified isoform. Finally, the LC-MS/MS-based approach allows quantitative measurements of multiple GnRH isoforms in one analysis and without concerns regarding cross-reactivity of antibody products. Subsequent chapters of this dissertation describe the preliminary experiments that were used to inform development of the qualitative and quantitative methods, followed by discussion of the qualitative and quantitative methods and application to brain tissue samples obtained from rat.

The initial experiments conducted to define chromatographic and mass spectrometric behavior are discussed in Chapter 2. Factors affecting chromatographic separation, including mobile phase composition and additives, reversed-phase column composition, and gradient profiles were investigated using cGnRH-I, cGnRH-II, lGnRH-III, mGnRH, sGnRH and sbGnRH. The effect of solution composition and electrospray parameters on peptide charge states during the electrospray process was evaluated as well. Knowledge gained from this work was used to develop the instrumental methods applied in the qualitative and quantitative portions of the dissertation research.

Chapter 3 describes the development of a qualitative method with broad applicability to multiple GnRH isoforms. MS/MS fragmentation patterns for six GnRH isoforms were examined in detail to determine whether common fragments or patterns are formed that may be helpful in identifying putative GnRH peptide spectra. The six model GnRH isoforms were used to prepared spike samples for development of an

appropriate clean-up method and application of the method to isolation and identification of mGnRH and Hyp^{9}GnRH in rat brain is described.

In chapter 4, development of LC-MS/MS strategies for quantification of mGnRH and Hyp^{9}GnRH in rat brains is described. Selection of an appropriate calibration preparation strategy, selection of the regression model, recovery of mGnRH and Hyp^{9}GnRH after sample preparation, and stability of the analytes in samples under storage and experimental conditions are discussed. Figures of merit, such as accuracy and precision, are evaluated in addition to assay specificity and sensitivity. Comparison of sample analysis via the new LC-MS/MS method versus an existing immunoassay technique is included, and concentrations of mGnRH and Hyp^{9}GnRH in male and female Sprague-Dawley rat brains, as determined by the LC-MS/MS assay, are presented.

The dissertation concludes with a discussion of the advantages and limitations of the new LC-MS/MS techniques in Chapter 5.

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CHAPTER 2: CHROMATOGRAPHIC AND MASS SPECTROMETRIC BEHAVIOR OF GnRH PEPTIDES

2.1 Introduction

With the realized success of genome-sequencing projects, interest has rapidly grown toward identifying the protein and peptide profiles in organisms and how these profiles vary with age, stress, disease-state, and other conditions. Broad-spectrum mass spectrometric methods are frequently applied for proteomics and peptidomics studies in which the goal is the identification of the entire array of proteins or peptides in a sample. However, these studies require generic approaches designed to prevent discrimination, as much as possible, against any of a myriad of proteins and are not well suited to experiments for more specific targets.

Analytical methods developed for a particular protein/peptide or family of proteins/peptides can be optimized to enhance specificity and sensitivity, unlike methods intended for generalized applications. In this dissertation, reversed-phase HPLC and quadrupole ion trap mass spectrometry methods are explored for qualitative and quantitative determinations of GnRH peptides. Clearly, selection of stationary phase and choice of mobile phase components affect chromatographic resolution (1). In addition, substantial enhancements in electrospray response have been observed for a variety of compounds when methanol was compared to acetonitrile as the organic component for reversed-phase chromatography (2, 3). Further, mass spectrometric settings have been shown to impact response dramatically in a mixture of five proteins with varying physicochemical properties (4).

No systematic evaluation of the parameters affecting chromatographic resolution and mass spectrometric response of GnRH isoforms has been reported. Thus, it is expected that an investigation of the chromatographic and mass spectrometric characteristics of GnRH peptides would be useful in optimization of qualitative LC-MS/MS methods for GnRH isoforms and quantitative LC-MS/MS methods for specific GnRH isoforms. To this end, a variety of parameters were explored, including mobile phase composition, reversed-phase column composition, and effects of solution composition and electrospray parameters on signal intensity and peptide charge states. Six GnRH peptides (cGnRH-I, cGnRH-II, lGnRH-III, mGnRH, sGnRH and sbGnRH) were chosen to investigate the impact of structural differences. These preliminary experiments will be used to inform development of HPLC-MS/MS methods for the qualitative and quantitative determination of GnRH isoforms in biological samples.

2.2 Structure of the Peptides

While the GnRH isoforms described in Table 1.1 have significant homology, amino acid variations amongst the isoforms result in differences in hydrophobicity and ionization that affect chromatographic retention and charge state. These differences in amino acid groups enable chromatographic separation of the peptide isoforms and affect the distribution of charge states (both in solution and in the gaseous phase). Ionizable residues and their respective acid dissociation constants (presented as a range expected within the local environment of peptide or protein) for the six representative isoforms are shown in Figure 2.1 (5).

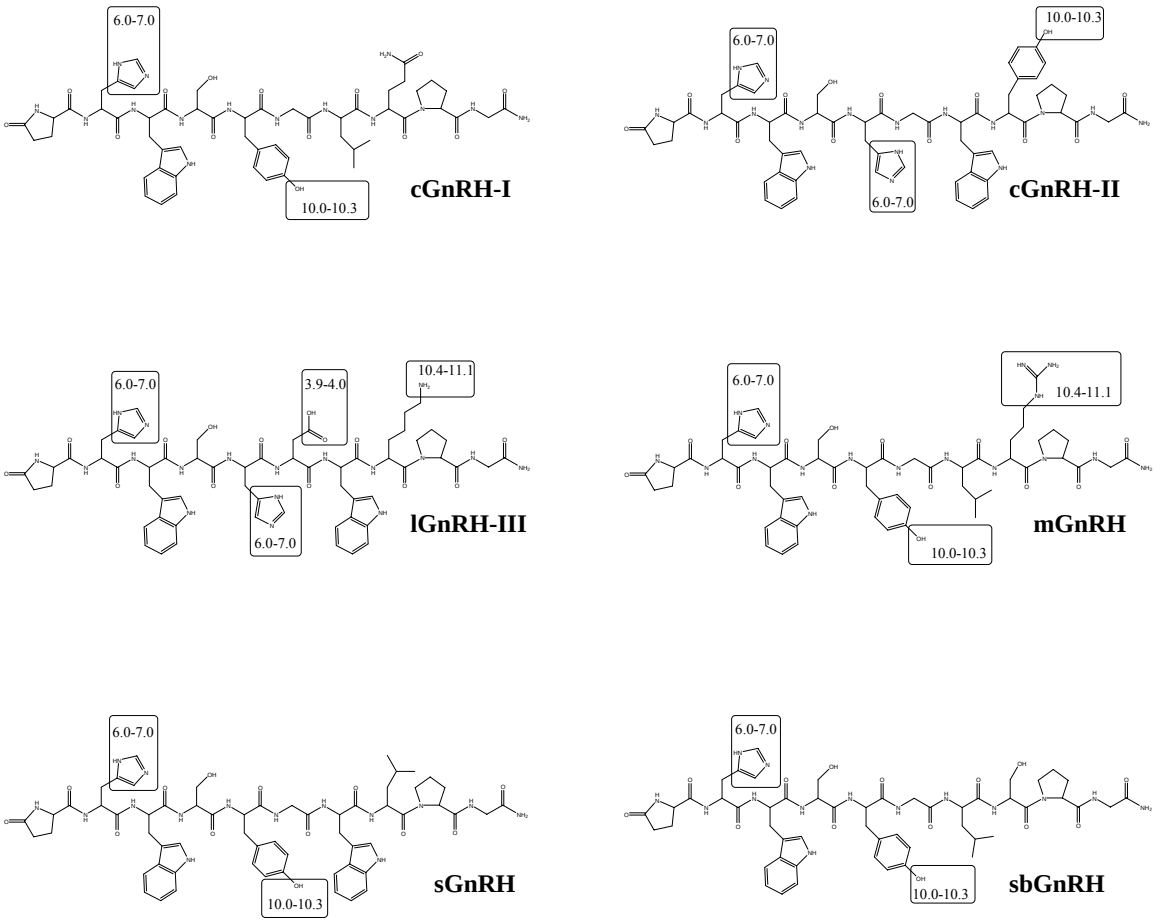

Figure 2.1. GnRH isoforms and their ionizable residues

Of the 24 GnRH isoforms thus far identified (see Table 1.1), twelve have one basic amino acid residue, eleven have two, and one has three. Five of the isoforms have one acidic residue also. Because the terminal ends of the GnRH isoforms are consistent and are not expected to be significantly charged as a consequence of their modified structures, the acidic and basic side chains of the amino acids will determine the total charge state of each peptide at a given pH. Our selection of three isoforms with one basic residue (cGnRH-I, sGnRH, and sbGnRH), two isoforms with two basic residues (cGnRH-II and mGnRH) and one isoform containing three basic residues and one acidic

residue (lGnRH-III) was expected to be representative of the family of GnRH isoforms such that conditions amenable to these six peptides should be applicable to most GnRH isoforms. Four of these six isoforms (cGnRH-II, lGnRH-III, mGnRH and sGnRH) were also of specific interest to the characterization of GnRH isoforms in rat brains (6).

Our goal was to find a set of experimental conditions that would provide optimal chromatographic resolution and mass spectrometric response for cGnRH-I, cGnRH-II, lGnRH-III, mGnRH, sGnRH and sbGnRH. Chromatographic separation of the isoforms was desirable, especially for data dependent experiments in which the most abundant signals trigger MS/MS experiments as compounds elute from the chromatographic column. Because some of the peptides differ in structure only by a single amino acid, full chromatographic resolution may be challenging to achieve. Thus, it was important to investigate the effect of mobile phase components and column composition on chromatographic resolution. Also, optimization of the mass spectrometric response was critical because the concentrations of GnRH in extracted samples were expected to be low (fmol/brain). The electrospray parameters required for optimal response were expected to differ somewhat due to heterologous structures in this peptide family, and the differing number of ionizable amino acid residues was expected to present some challenges in terms of promoting a single charge state (rather than splitting response across multiple charge states). Finally, differing conditions may be optimal in regards to chromatographic resolution versus mass spectrometric response and require that some compromises be made to achieve a generic LC-MS/MS method suitable for as many GnRH isoforms as possible.

2.3 Experimental

Chemicals

Purified cGnRH-I, cGnRH-II, mGnRH, lGnRH-III, sGnRH and sbGnRH were obtained from BAChem (Torrance, CA, USA). Glacial acetic acid, formic acid (88%), trifluoracetic acid (TFA), ammonium acetate, ammonium formate, and ammonium hydroxide (28-30%) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Heptafluorobutyric acid (HFBA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA), and water was purchased from the same or supplied by an in-house Vantage reverse-osmosis system (US Filter, Colorado Springs, CO, USA).

Infusion experiments

Effect of varying pH

Stock solutions were prepared by diluting the purified peptide in deoxygenated water to 100 ug/mL, after which solutions were frozen at -70°C until use. Aqueous solutions were prepared across a range of acidic pH values typical for reversed-phase chromatography and at one basic pH value (see Table 2.1 for preparation schemes). Solution pH was measured in the aqueous solutions (prior to addition of organic solvent) using a calibrated pH meter (Orion 420A, Boston, Massachusetts, USA). A dilution of each peptide to 1 ug/mL was prepared from stocks in 95:5 pH-adjusted solution:acetonitrile; this small percentage of organic (calculated as volume:volume) was necessary to obtain reasonable signal intensity from the electrospray source.

Table 2.1. Preparation of pH-adjusted solutions

pH	Buffer Component	Acid/Base used for pH Adjustment
2.7	None	Formic Acid
3.8	10 mM Ammonium Formate	Formic Acid
4.8	10 mM Ammonium Acetate	Acetic Acid
5.3	10 mM Ammonium Acetate	Acetic Acid
5.7	10 mM Ammonium Acetate	Acetic Acid
9.1	10 mM Ammonium Acetate	Ammonium Hydroxide

Effect of varying organic concentration

Solutions of each peptide were prepared from stocks diluted to a final concentration of 1 μg/mL with varying percentages of acetonitrile or methanol, two organic solvents commonly used for reversed-phase chromatographic separations of proteins and peptides. Because acidic conditions are frequently used for HPLC-MS/MS analysis of proteins and peptides, the experiments were repeated at three acidic pH values (2.7, 3.8 and 4.8).

Effect of varying source conditions

The effect of varying source conditions was explored using the 1 ug/mL solutions of each peptide prepared in 0.1% formic acid (pH=2.7):acetonitrile (75:25, volume:volume). The following parameters were varied: capillary heater (175-350ºC, 25º increments), capillary voltage (-16 to 132 V, 10V increments), tube lens voltage (-10 to 140V, 10V increments), sheath gas (50 – 100 units, 10 unit increments), and source voltage (0 – 8.0kV, 1kV increments).

Chromatographic experiments

Preliminary experiments indicated that nearly complete separation (cGnRH-I and sbGnRH coeluted) of the six GnRH isoforms could be achieved using a Phenomenex Luna C8(2) 50 x 1.0 mm column, 5 µ particle size (Torrance, CA, USA) and acidified mobile phase. To further characterize the chromatographic behavior of the six isoforms on this column, several commonly used mobile phase modifiers were investigated for differences in chromatographic separation (if any) and response. Formic and acetic acid solutions were prepared at 0.01%, 0.05%, 0.1% and 0.5% (prepared as % volume) in water and paired with the same concentration of acid in acetonitrile for gradient chromatography; in addition, 0.01% TFA, 0.005% HFBA and the combinations of 0.01% acetic acid with 0.001% HFBA and 0.01% acetic acid with 0.005% HFBA were explored. A gradient program increasing from 5% organic to 60% organic over 55 min was used to achieve separation, and 10 µL of a 100 pg/µL mix of the six GnRH peptides was injected on-column. Phenomenex Luna C18(2) and Luna phenylhexyl columns were also evaluated, both 50 x 1.0 mm, 5 µ particle size (Torrance, CA, USA). A Phenomenex Jupiter Proteo (C12) column, designed specifically for peptide separations, was included in these experiments as well; the 50 x 1.0 mm, 4 µ particle size dimensions were chosen to be as comparable as possible with the conventional C18 and phenylhexyl phases.

Mass spectrometry

All experiments were performed on a Finnigan LCQ Deca quadrupole ion trap mass spectrometer (San Jose, CA, USA) operated in positive electrospray mode. The electrospray source was fitted with a stainless steel needle.

For the infusion experiments, solutions were infused into the electrospray source from the on-board syringe pump via capillary tubing. The infusion rate was 20 µL/min for the experiments exploring the effect of varying pH and organic content and 100 µL/min for the experiments exploring varying source conditions. Ten to twenty MS scans were captured and averaged, with the scanned mass range selected based on the anticipated charge states for each peptide. Unless otherwise noted above, electrospray source conditions were as follows: source voltage – 3.0 kV, sheath gas flow (nitrogen) – 50 units, auxiliary gas flow – 0 units, capillary voltage – 36 to 46 V, and capillary temperature – 175°C.

For the chromatography experiments, column effluent at 100 µL/min was directed into the electrospray source without splitting. The following source conditions were selected: source voltage – 3.0 kV, sheath gas flow (nitrogen) – 100 units, auxiliary gas flow – 0 units, capillary voltage – 46 V, and capillary temperature - 200°C.

2.4 Results and Discussion

Effect of pH on charge state and absolute signal intensity

Results from the infusion of GnRH peptide solutions prepared in solutions of different pH are shown in Figure 2.3 and 2.4, depicting relative intensity and absolute intensity of each charge state, respectively.

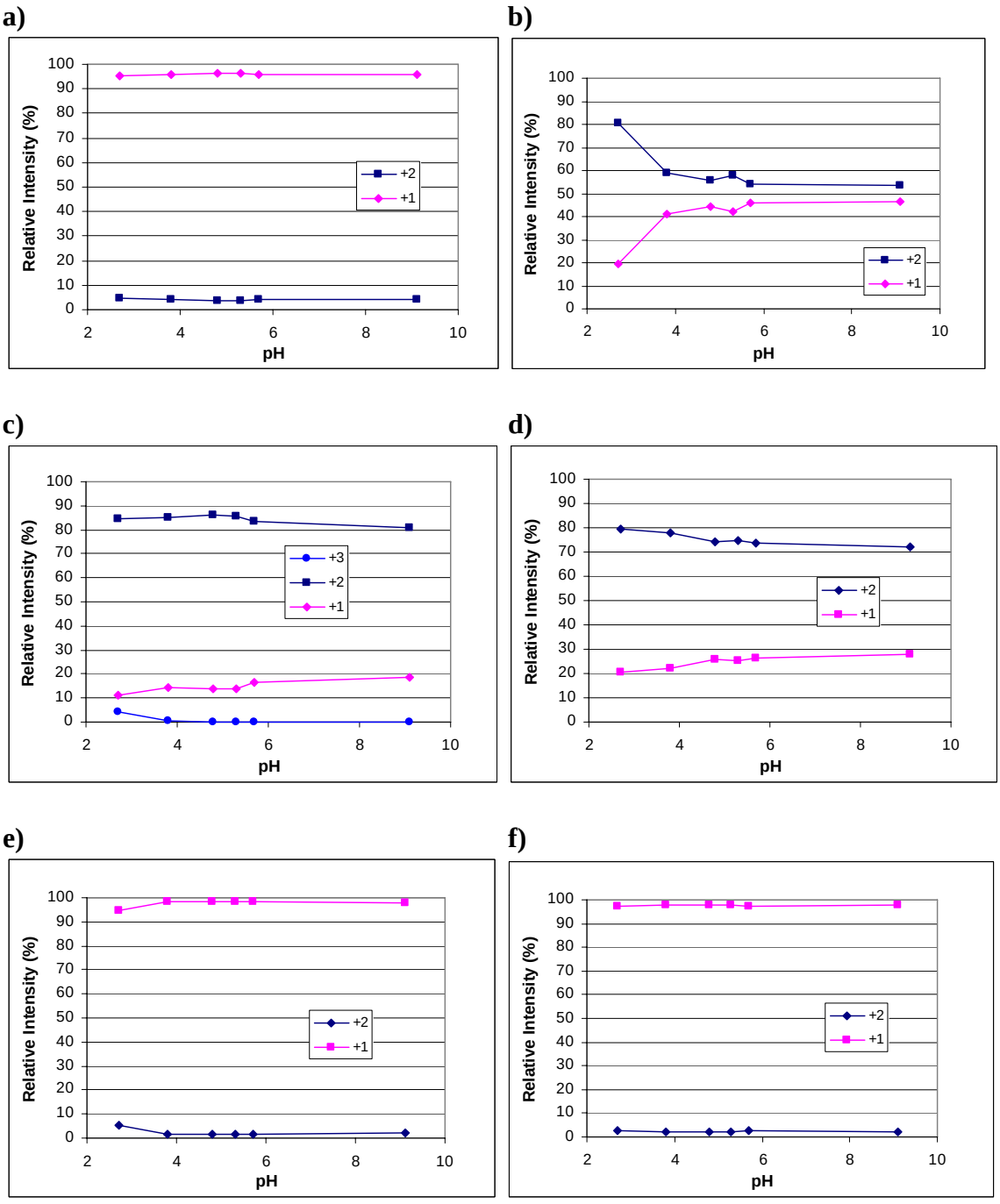

Figure 2.2. Infusion of GnRH isoforms, 1 µg/mL, at 20 uL/min, with varying pH. Data derived from average of 20 MS scans. Relative intensity for MH^{+}, MH_2^{+2} and MH_3^{+3} (lamprey only) is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^{+} is designated +1 and MH_2^{+2} is designated +2.

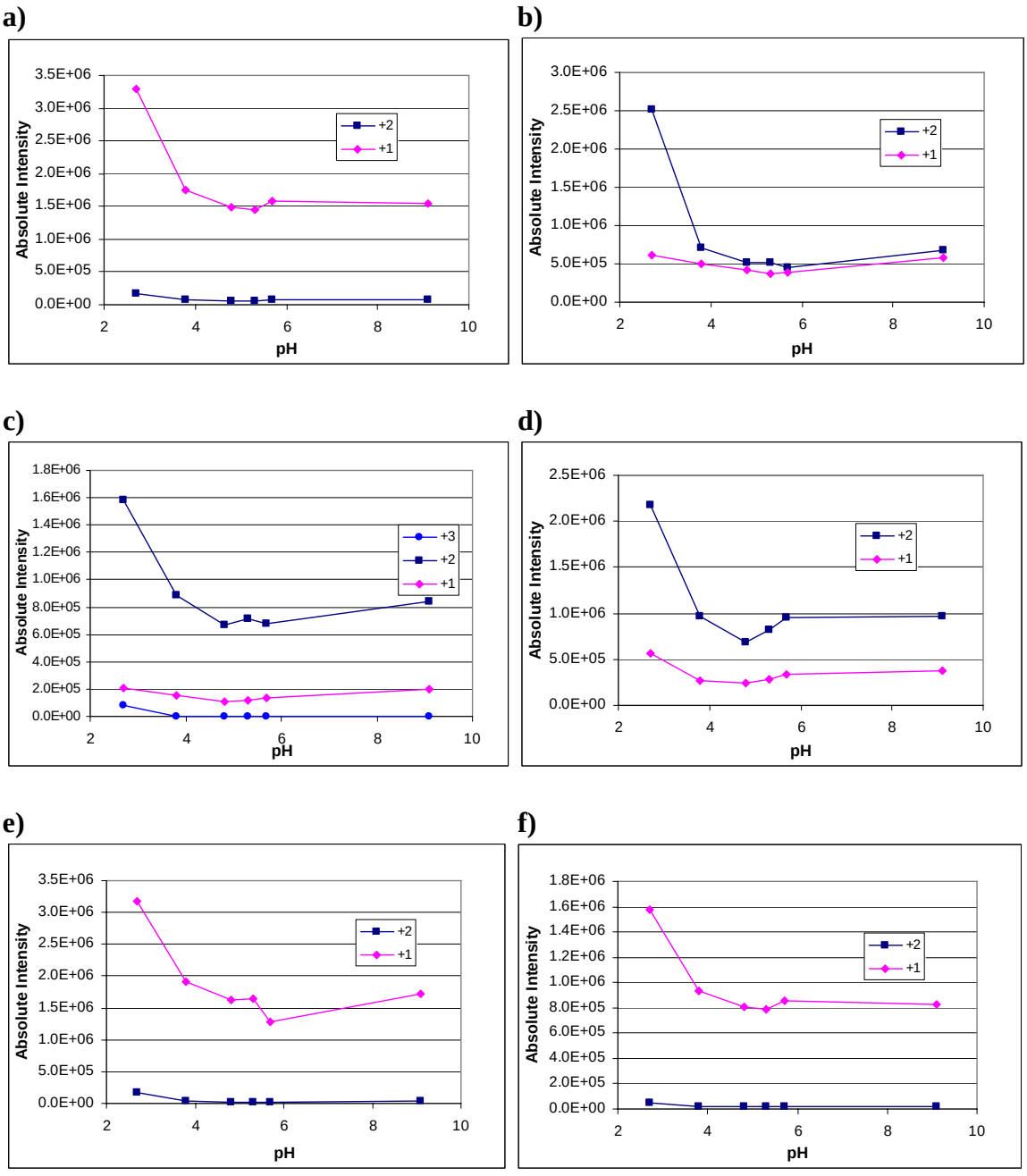

Figure 2.3. Infusion of GnRH isoforms, 1 µg/mL, at 20 uL/min, with varying pH. Data derived from average of 20 MS scans. Absolute intensity for MH^+, MH_2^{+2} and MH_3^{+3} (lamprey only) is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1 and MH_2^{+2} is designated +2.

The single and double charge states were observed for all isoforms, and for lGnRH-III, the triply-charged form was observed as well. For cGnRH-I, sGnRH and sbGnRH, the singly-charged state was the predominant state (greater than 90% of total signal for MH^+ and MH_2^{+2}), irregardless of solution pH; the similar behavior of these peptides is logical in that all three of these GnRH isoforms have a basic histidine residue in position 2 and an acidic tyrosine residue in position 5. For cGnRH-II and mGnRH, the doubly-charged state was the more abundant state, influenced somewhat by pH; these GnRH isoforms both have two basic residues in addition to an acidic tyrosine residue. The doubly-charged state was the most abundant charge state for lGnRH-III as well, ranging from 81 – 86% of the total signal for MH^+, MH_2^{+2}, and MH_3^{+3}. The triply-charged form of lGnRH-III was observed predominantly at the most acidic pH with signal intensity for this form dropping to less than 0.5% of the total signal at pH=3.7 and above. The singly-charged state of lGnRH-III ranged from 11% at pH=2.7 to 19% at pH=9.1. The primary structure for lGnRH-III includes four ionizable residues: two basic histidine residues at positions 2 and 5, an aspartic acid residue at position 6, and a basic lysine residue at position 8.

The maximum charge observed for each peptide under the electrospray conditions employed in this experiment correlates to the maximum charge state expected based on solution chemistry (under moderately acidic conditions, +1 for cGnRH-I, sGnRH and sbGnRH; +2 for cGnRH-II and mGnRH; +3 for lGnRH-III). However, the maximum charge state observed did not always trend with pH as might be expected based on solution chemistry; this was especially apparent at pH = 9.1. These observations suggest that the electrospray process and reactions after transfer of the ions to gas phase are also

important determinants of charge state for the GnRH peptides, a phenomenon that has been observed for other peptides and proteins (7, 8). The absolute signal intensity for all of the charge states was greatest at pH=2.7, probably due to acid-enhanced charge separation during the electrospray process (9).

Effect of organic composition on charge state and absolute signal intensity

Results from the infusion of GnRH peptide solutions prepared at varying concentrations of acetonitrile or methanol are shown in Figures 2.4 and 2.5. In most cases, increasing percentages of organic solvent resulted in increases in signal intensity irrespective of charge state. This result is consistent with previous observations that increasing percentages of organic solvent can stabilize the electrospray current and enhance response, probably due to reduced droplet surface tension and enhanced desolvation at higher concentrations of organic (9). Notable exceptions to this observation were cGnRH-I, sGnRH and sbGnRH, for which the MH^+ signal diminished at higher percentages of acetonitrile at pH=2.7. Irregardless of whether the solutions were prepared with acetonitrile or methanol, electrospray response was enhanced at lower pH.

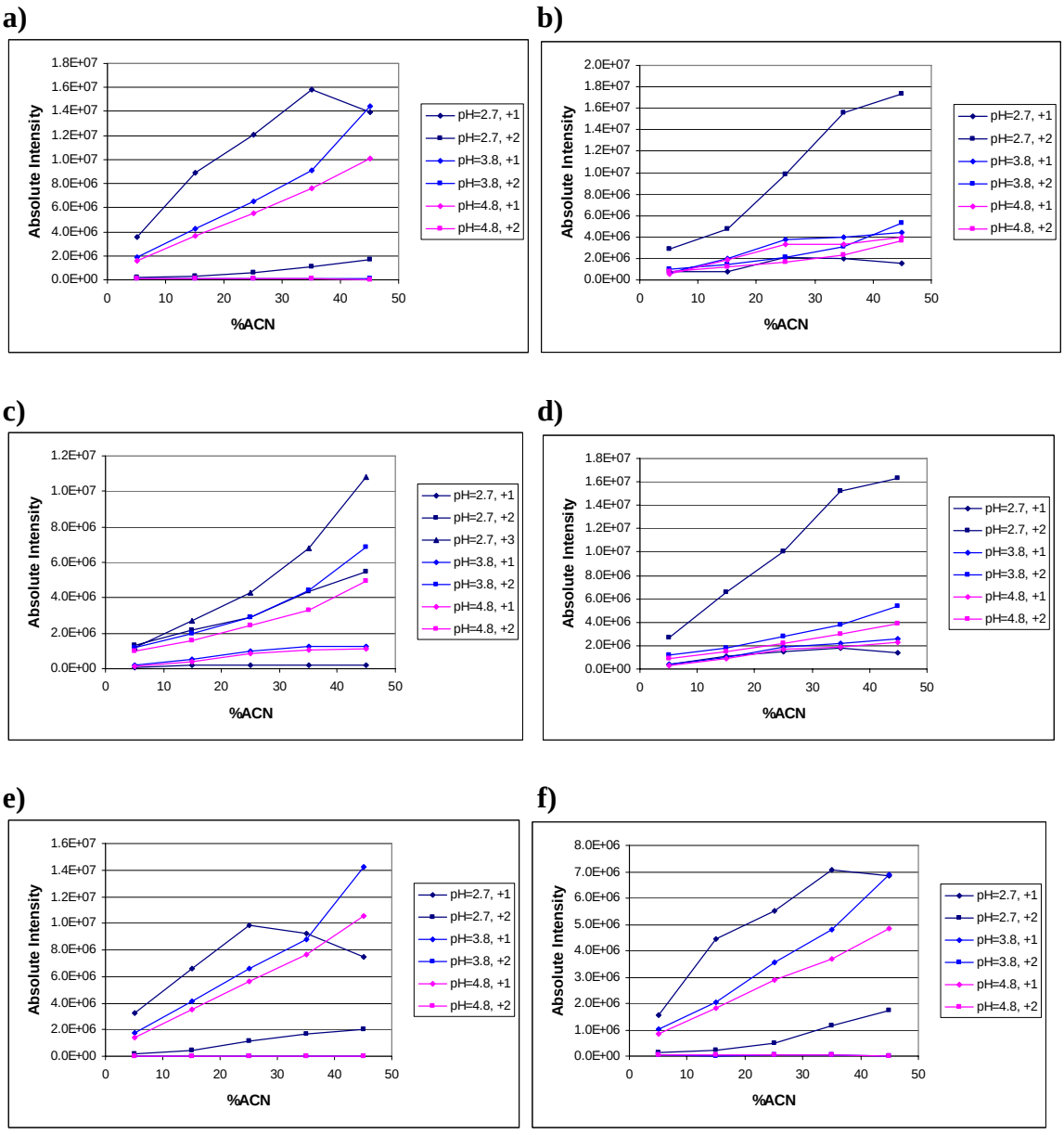

Figure 2.4. Infusion of GnRH isoforms, 1 µg/mL, at 20 uL/min, with varying concentrations of acetonitrile. Data derived from average of 20 MS scans. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

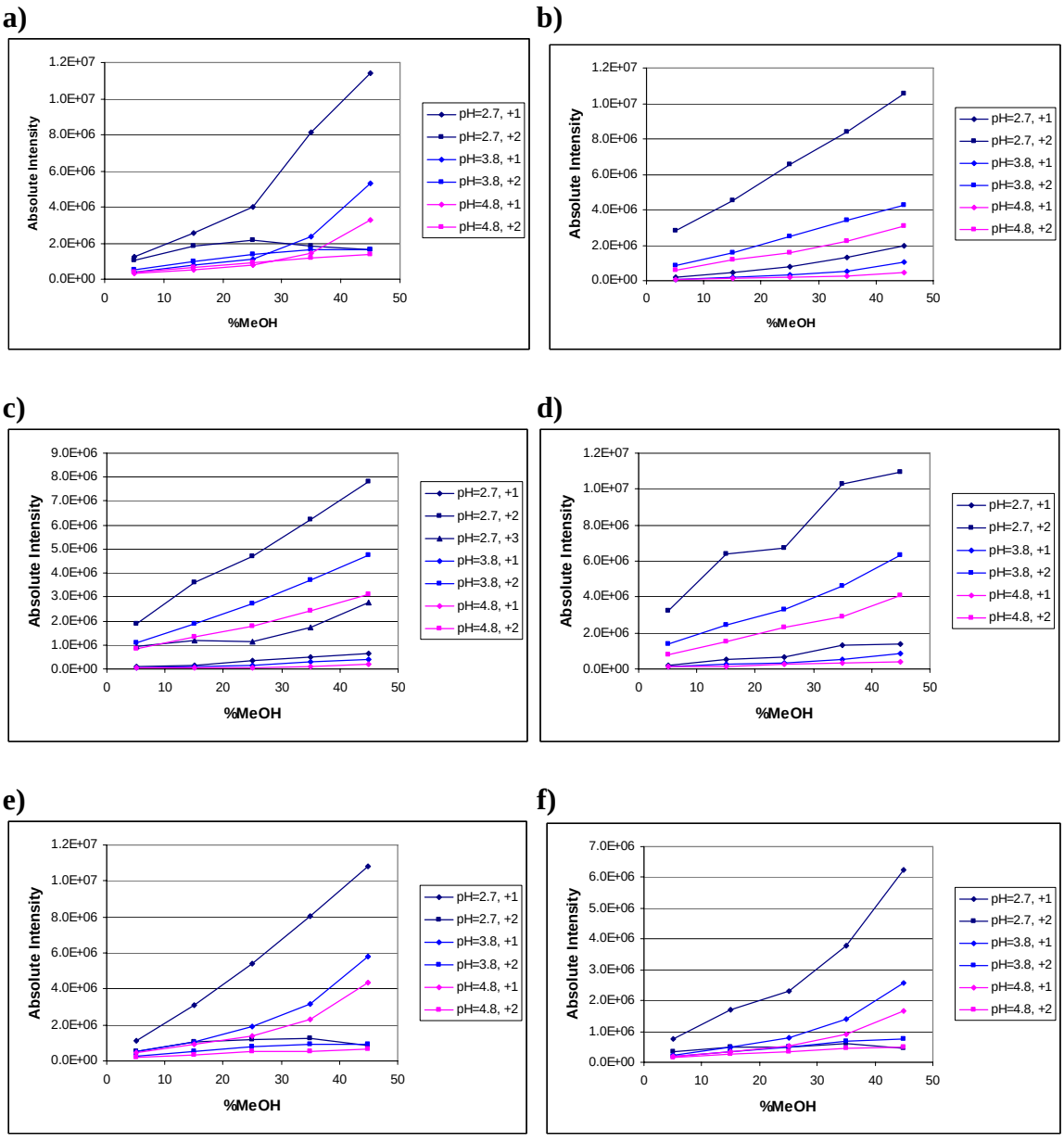

Figure 2.5. Infusion of GnRH isoforms, 1 µg/mL, at 20 uL/min, with varying concentrations of methanol. Data derived from average of 20 MS scans. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

In general, acetonitrile enhanced signal intensity for the primary charge states of all six GnRH isoforms compared to methanol. This finding is the converse of that obtained during analysis of a non-natural mGnRH analogue, goserelin, for which response was enhanced four to eight-fold with methanol as the organic component of the mobile phase as compared to acetonitrile (3). The chemical structures of mGnRH and goserelin are similar (the glycine residue in position 6 and the amidated glycine in position 10 are replaced with a tert-butylserine reside and a carbazamide terminus, respectively), so it is not clear whether structural differences in goserelin lead to enhanced electrospray response from solutions containing methanol. It may be that differences in electrospray sources influence which organic modifer is optimal (the goserelin study was conducted using a Micromass Platform 2 single quadrupole mass spectrometer).

2.5 Effect of electrospray source conditions on charge state

Results from varying capillary heater temperature while infusing 1 μg/mL GnRH peptide solutions prepared in 0.1% formic acid, pH=2.7:acetonitrile (75:25, volume:volume) are shown in Figures 2.6. Likewise, results from varying capillary voltage, tube lens voltage, sheath gas flow, and source voltage are shown in Figures 2.7, 2.8 2.9, and 2.10, respectively.

Of the electrospray source parameters manipulated in these experiments, capillary heater temperature and source voltage had the most significant effect on response of the six GnRH isoforms. For isoforms in which the +2 charge state was the more prominent form at pH=2.7 (cGnRH-II, lGnRH-III, and mGnRH), increasing capillary temperatures

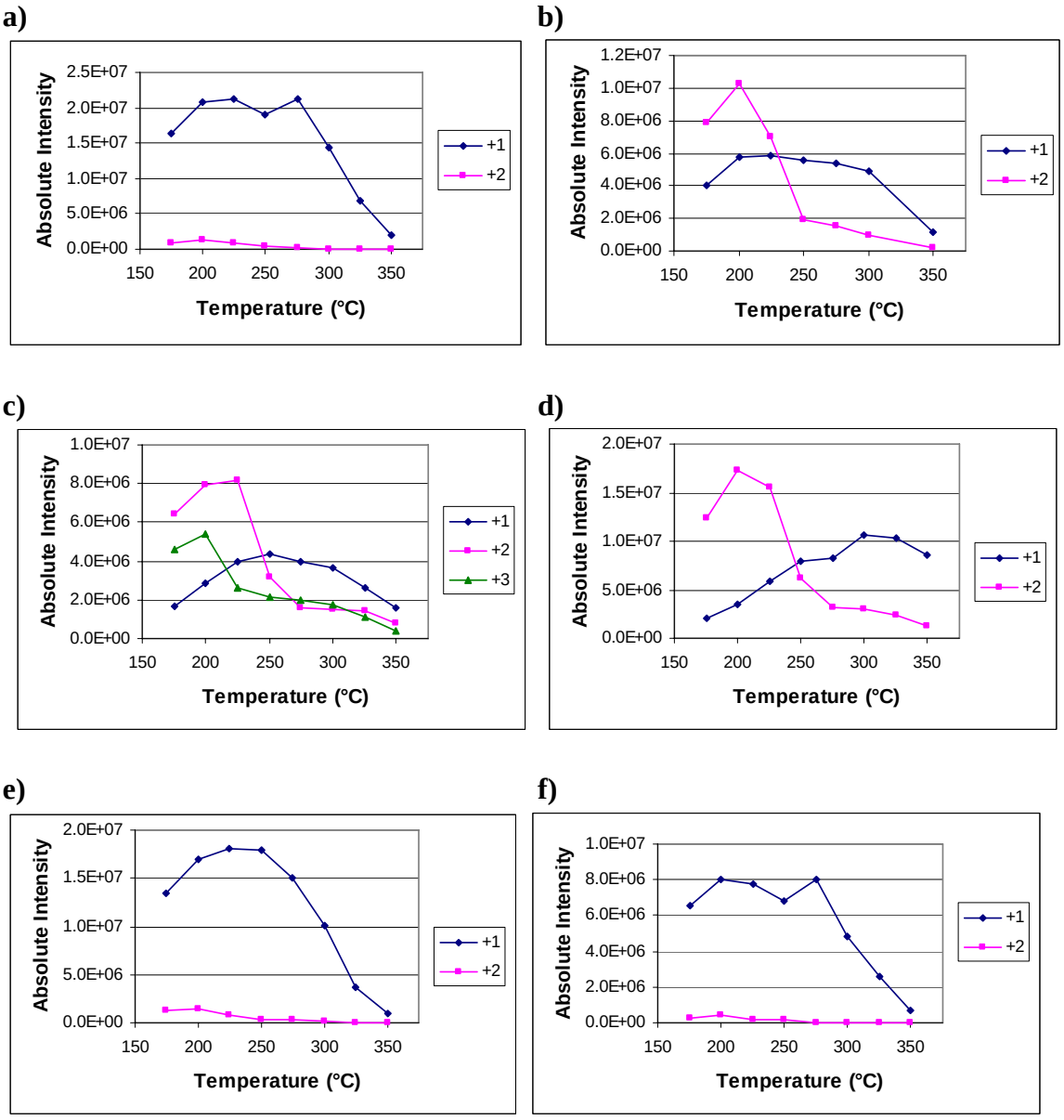

Figure 2.6. Effect of capillary heater temperature upon response. Infusion of 1 µg/mL GnRH isoforms, in 75:25 1% formic acid:acetonitrile, at 100 uL/min. Data derived from average of 10 MS scans. Sheath gas flow was set to 50 units, source voltage was set to 3 kV, capillary voltage was set to 46 V, and the tube lens voltage was set to 5V. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

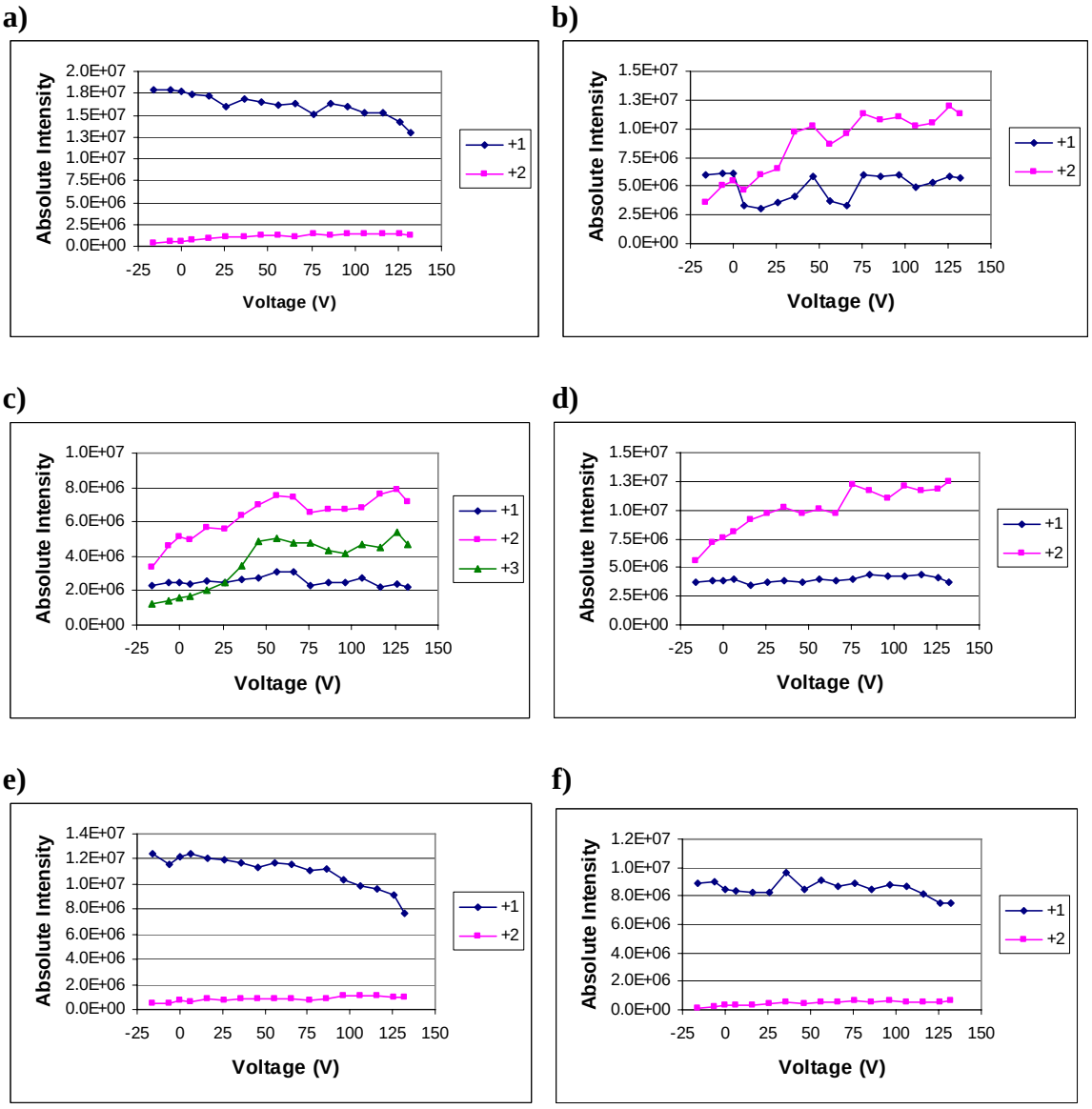

Figure 2.7. Effect of capillary voltage upon response. Infusion of 1 µg/mL GnRH isoforms, in 75:25 1% formic acid:acetonitrile, at 100 uL/min. Data derived from average of 10 MS scans. Sheath gas flow was set to 50 units, source voltage was set to 3 kV, capillary heater was set to 200 V, and the tube lens voltage was set to 5V. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

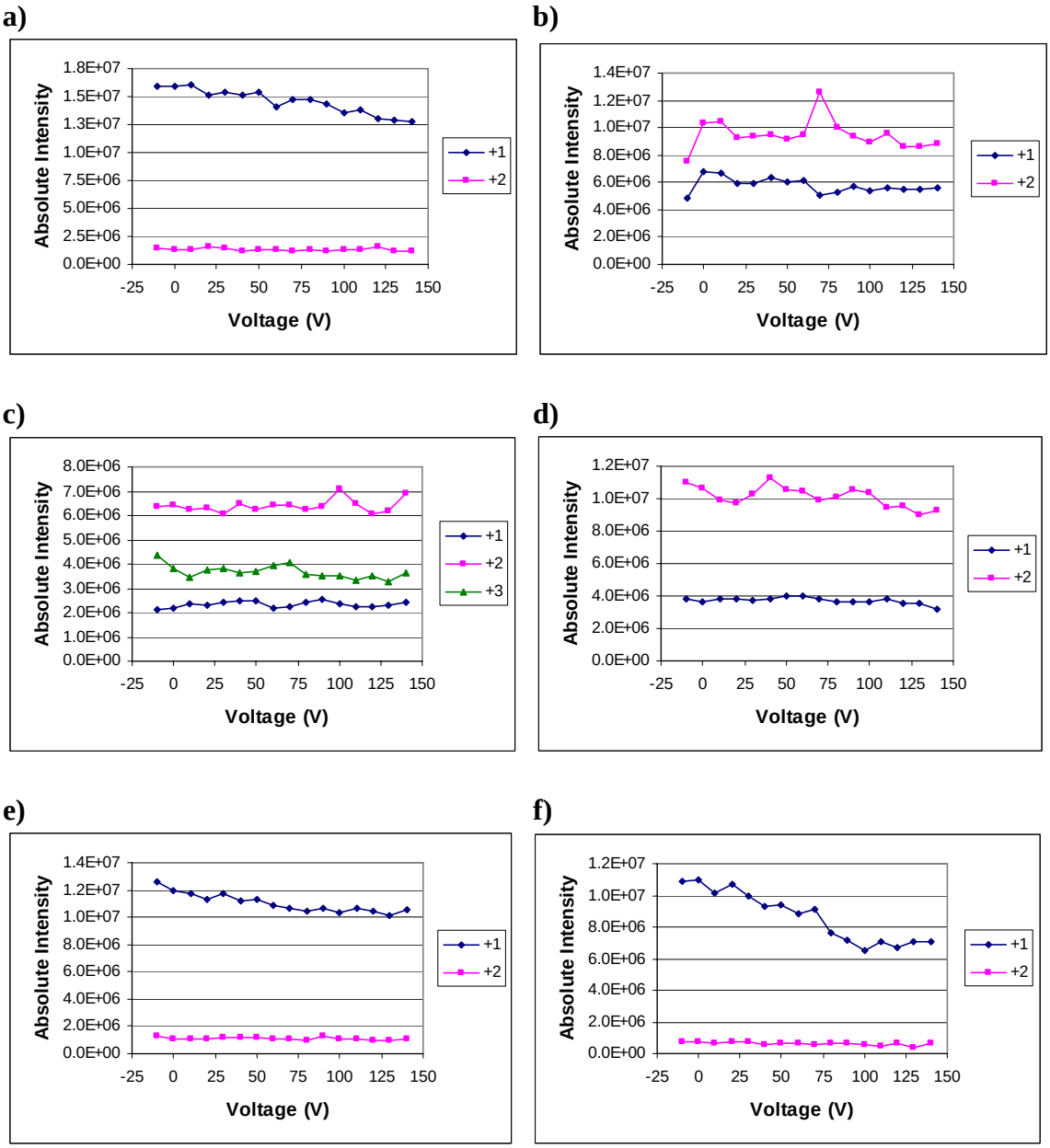

Figure 2.8. Effect of tube lens voltage upon response. Infusion of 1 µg/mL GnRH isoforms, in 75:25 1% formic acid:acetonitrile, at 100 uL/min. Data derived from average of 10 MS scans. Sheath gas flow was set to 50 units, source voltage was set to 3 kV, capillary heater was set to 200 V, and capillary voltage was set to 46V. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

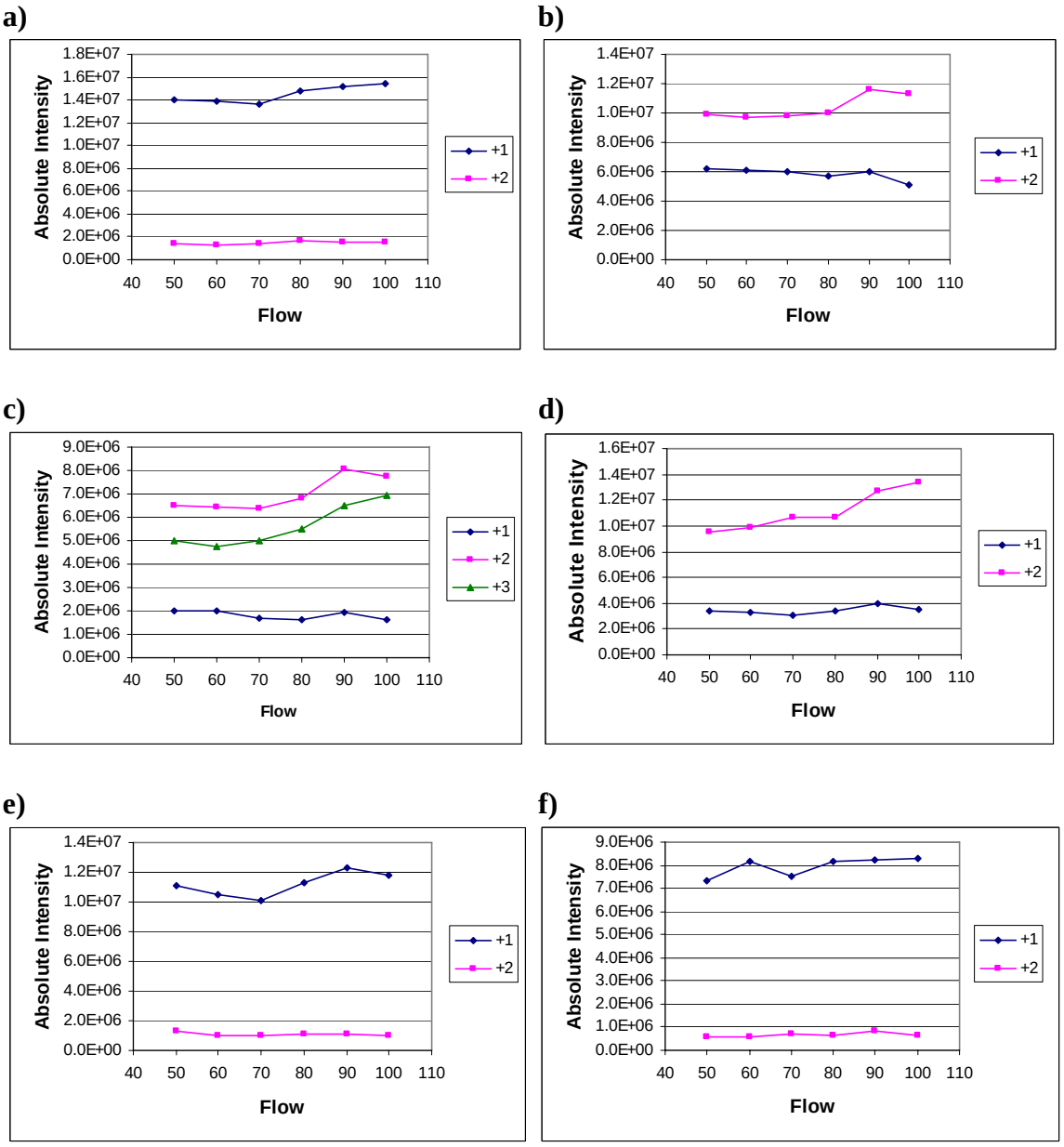

Figure 2.9. Effect of sheath gas flow upon response (flow setting to arbitrary units). Infusion of 1 µg/mL GnRH isoforms, in 75:25 1% formic acid:acetonitrile, at 100 uL/min. Data derived from average of 10 MS scans. Tube lens voltage was set to 5V, source voltage was set to 3 kV, capillary heater was set to 200 V, and capillary voltage was set to 46V. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

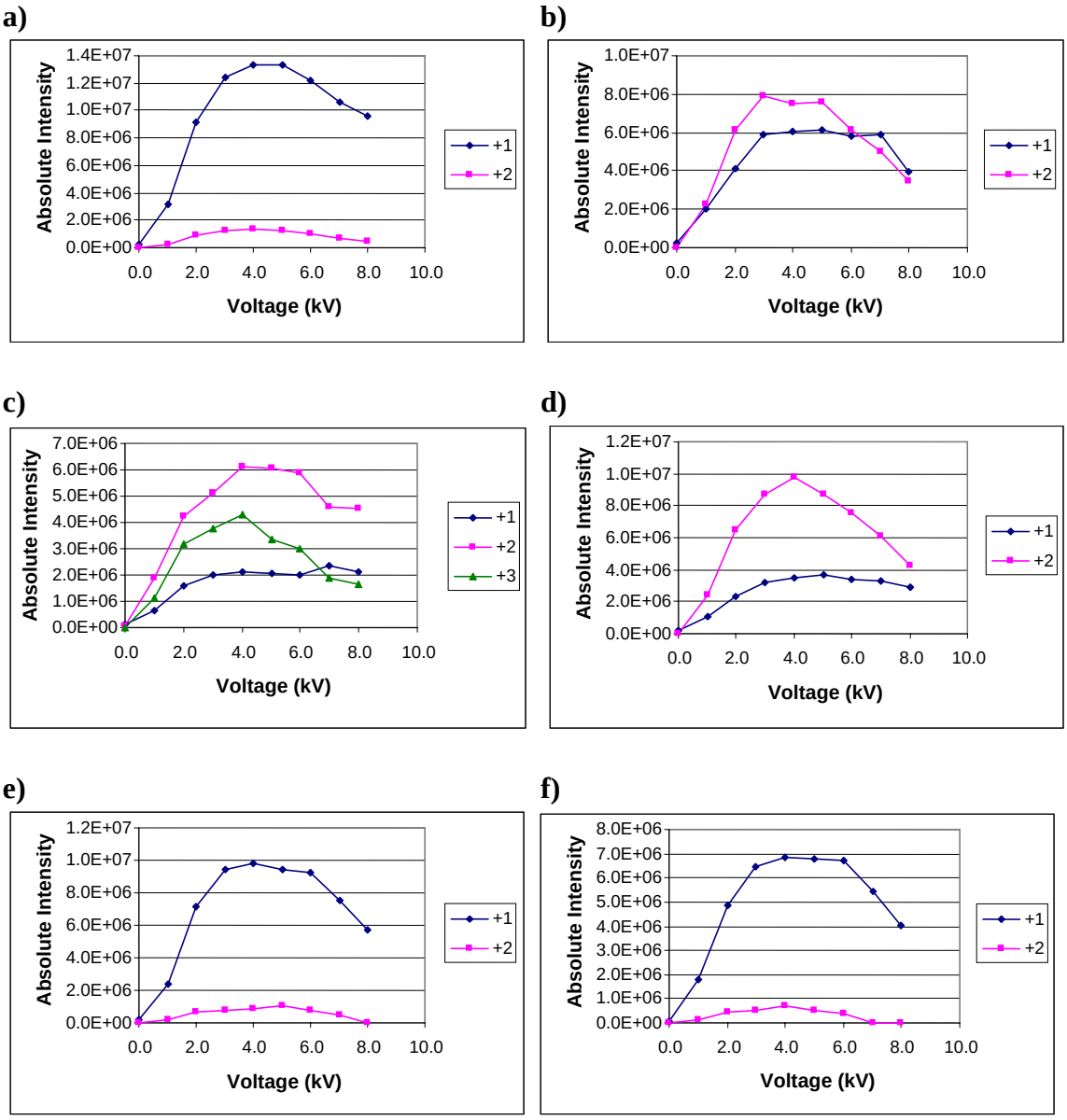

Figure 2.10. Effect of source voltage upon response. Infusion of 1 µg/mL GnRH isoforms, in 75:25 1% formic acid:acetonitrile, at 100 uL/min. Data derived from average of 10 MS scans. Sheath gas flow was set to 50 units, tube lens voltage was set to 5V, capillary heater was set to 200 V, and capillary voltage was set to 46V. Absolute intensity for MH^+, MH_2^{+2}, and MH_3^{+3} is shown for a) cGnRH-I, b) cGnRH-II, c) lGnRH-III, d) mGnRH, e) sGnRH and f) sbGnRH. MH^+ is designated +1, MH_2^{+2} is designated +2, and MH_3^{+3} is designated +3.

resulted in a diminished response for MH_2^{+2} and an enhancement in MH^{+1} response. At even higher capillary temperatures, MH^{+1} response was diminished also. Similarly, the MH^{+1} response of cGnRH-I, sGnRH and sbGnRh, for which the +1 charge state was the more prominent form at pH=2.7, was diminished at the higher capillary temperatures.

The effect of source voltage variation on response was similar for all charge states and all isoforms in that there seemed to be an optimal range of settings for GnRH peptides centered at approximately 4 V; lower and higher settings resulted in significantly diminished response. Capillary voltage, tube lens voltage, and sheath gas flow changes affected response to a lesser extent, depending upon the charge state and isoform.

The effect of higher capillary temperatures may be explained by an apparent conversion of higher charge states to lower charge states and has been dubbed “charge stripping” by the manufacturer of the LCQ Deca instrument used to gather these data (10, 11). This effect may be unique to the design of the electrospray source and particularly, the heated capillary tube used to transfer ions from the atmospheric pressure region to the vacuum region. Interestingly, the manufacturer also reports that this phenomenon can occur at higher settings of tube lens voltage as well. However, increasing the tube lens voltage during infusion of the six GnRH isoforms did not affect the response in a uniform manner that would be suggestive of charge stripping. These results highlight the importance of characterization of peptide behavior in a particular mass spectrometric system, particularly if optimal response for a particular charge state is desired.

2.6 Effect of acid modifiers on chromatography

Results from the reversed-phase gradient separation of cGnRH-I, cGnRH-II, lGnRH-III, mGnRH, sbGnRH, and sGnRH on a Luna C8(2), 50 x 1.0 mm column with

varying concentrations of acetic acid and formic acid are shown in Figures 2.11 and 2.12, respectively. Results obtained using TFA and HFBA as mobile phase modifiers are shown in Figure 2.13.

The elution order of the six GnRH isoforms was similar for the mobile phases modified with acetic acid, formic acid, and TFA, with complete resolution of all the isoforms except cGnRH-I and sbGnRH. Retention of the GnRH isoforms on the C8 stationary phase was enhanced in the formic acid-modified mobile phase as compared to that modified with acetic acid. This increased retention was somewhat surprising in that while both acids have weak ion pairing capacities, the uncharged portion of the acetic acid group (CH_3CO-) would be expected to interact slightly more effectively than the uncharged portion of the formic acid group (HCO-) with the non-polar stationary phase.

Of the acid modifiers explored, the best electrospray response was observed in mobile phases prepared with acetic acid. Increasing concentrations of the acid modifiers lowered electrospray response. As would be expected, response was lowered also in mobile phases prepared with the strong ion-pairing reagents TFA and HFBA.

Peak broadening for lGnRH-III, mGnRH and cGnRH-II was observed in mobile phase modified with 0.01% acetic acid and for lGnRH-III in mobile phases modified with 0.01% formic acid or with 0.01% acetic acid and 0.001% HFBA. This peak broadening is likely due to interactions of the peptides with active silanol groups which can be ionized under moderately acidic conditions ($pH \geq 3.5$) and become available for secondary chromatographic interactions (12, 13). The lGnRH-III, mGnRH and cGnRH-II isoforms are predominantly in a +2 or +3 charge state at acidic pH, thereby increasing the potential

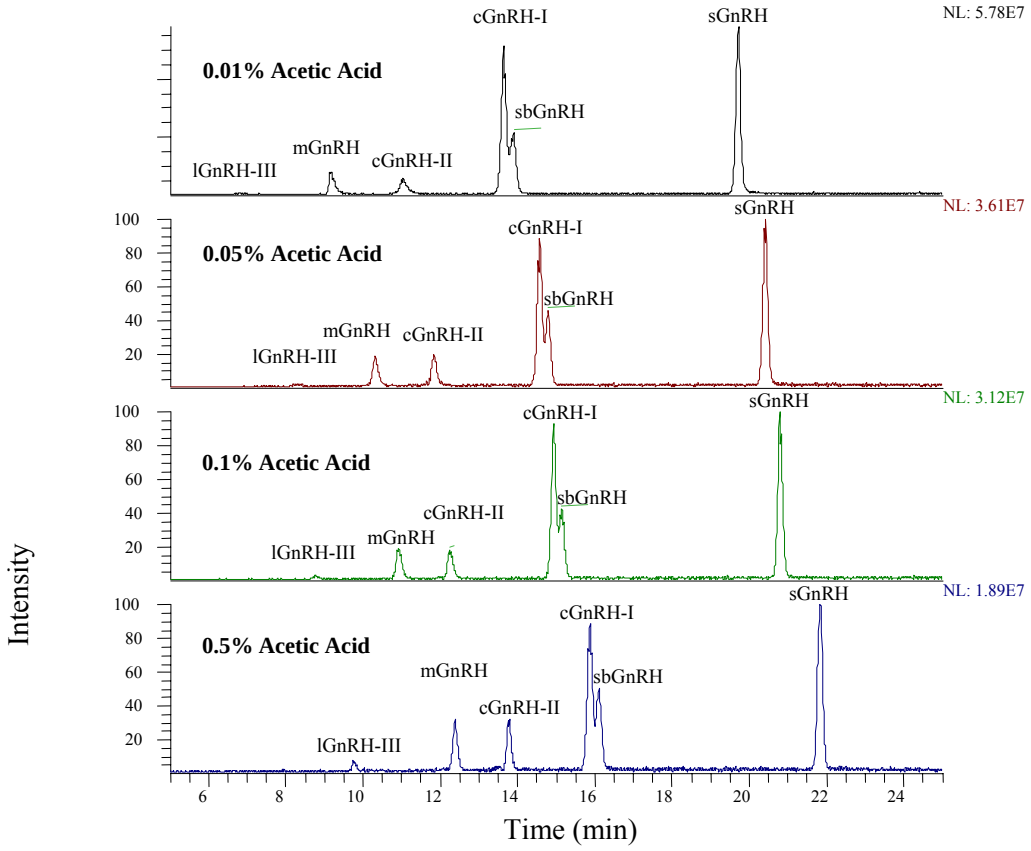

Figure 2.11. Effect of varying concentrations of acetic acid in mobile phase, reversed-phase chromatography on Luna C8(2). Chromatograms are normalized to the most intense peak, with maximum intensity displayed in the right-hand corner.

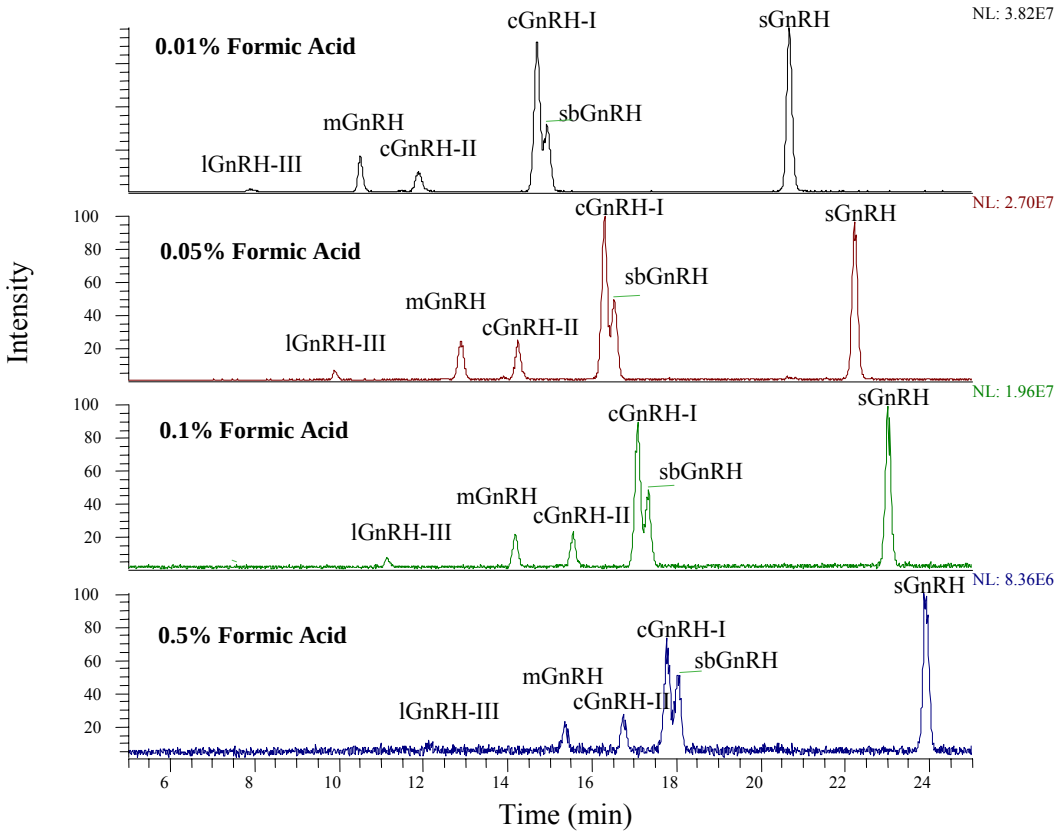

Figure 2.12. Effect of varying concentrations of formic acid in mobile phase, reversed-phase chromatography on Luna C8(2). Chromatograms are normalized to the most intense peak, with maximum intensity displayed in the right-hand corner.

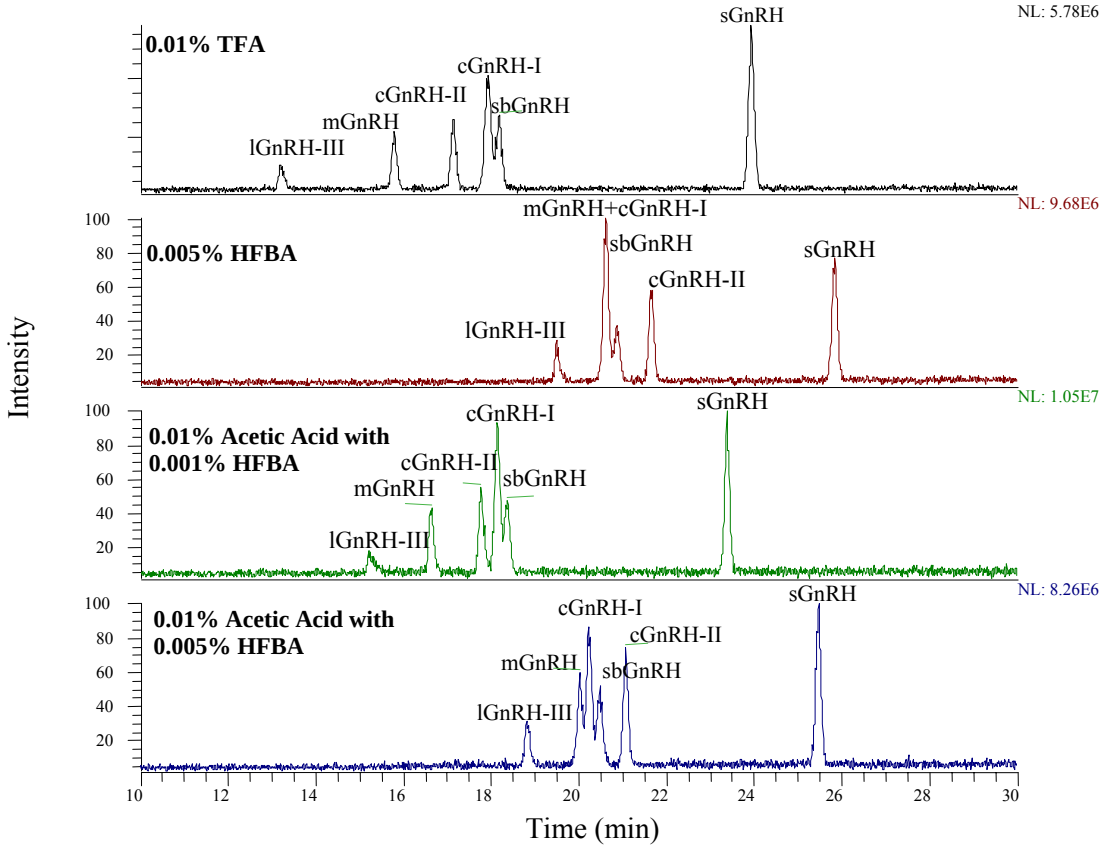

Figure 2.13. Effect of varying concentrations of TFA and HFBA in mobile phase, reversed-phase chromatography on Luna C8(2). Chromatograms are normalized to the most intense peak, with maximum intensity displayed in the right-hand corner.

ionized sites on these peptides for interaction with active silanol groups. Mobile phases modified with either HFBA alone or acetic acid-HFBA combinations displayed significantly different elution orders for the isoforms and shifted the retention times of lGnRH-III, mGnRH and cGnRH-II later relative to the times for cGnRH-I, sbGnRH and sGnRH. This result may be explained by the greater hydrophobicity of HFBA as an ion-pairing reagent, with an increased impact on higher net-charge peptides that have multiple sites available for ion-pairing (13).

2.7 Effect of column composition and organic modifier on chromatography

Conventional C18 and phenylhexyl-based stationary phases as well as a C12 column designed especially for peptide separations were evaluated to determine whether chromatographic separation achieved on the C8 column could be improved. Each column was evaluated with 0.01% acetic acid, 0.5% acetic acid, 0.01% formic acid and 0.01% acetic acid with 0.001% HFBA as the mobile phase modifiers. In all cases, mGnRH and cGnRH-II eluted in broad peaks and lGnRH-III was either broadened to the extent that it became indistinguishable from baseline or was not retained by the column. Comparison of separations using the C8, C18, phenylhexyl and Proteo (C12) column are shown in Figure 2.14, using 0.5% acetic acid. Conversations with the manufacturer of these reversed-phase chromatography columns indicated that the C18 and phenylhexyl columns can have enhanced silanol activity relative to the C8 column due to less successful endcapping of active silanols in the presence of bulkier ligand groups (C18 and phenylhexyl) (14). None of the columns investigated offered an improvement in resolution of cGnRH-I and sbGnRH over that achieved on the C8 column. Given that nearly full resolution of the six GnRH isoforms was achieved on the C8 column and that

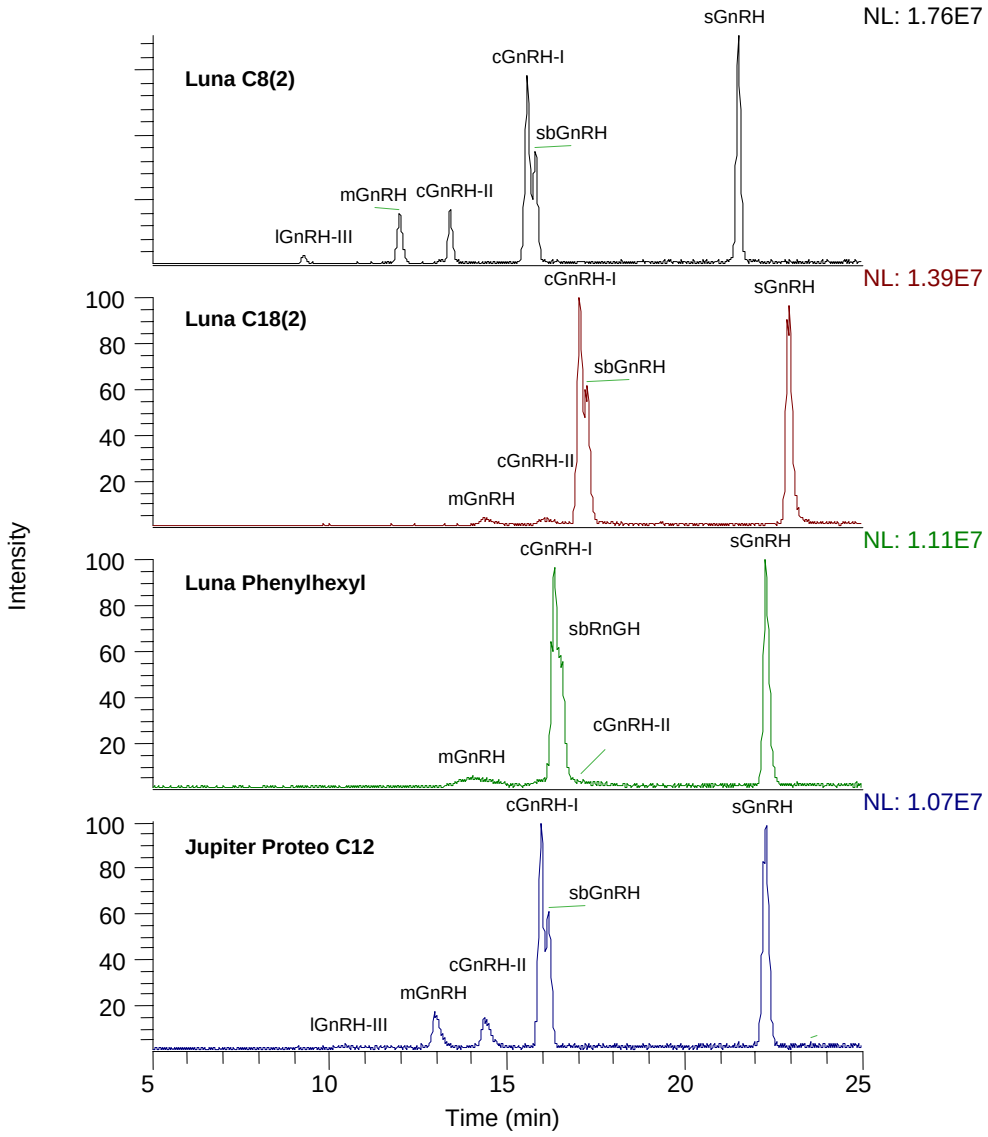

Figure 2.14. Effect of varying column composition on separation of GnRH isoforms. Chromatograms are normalized to the most intense peak, with maximum intensity displayed in the right-hand corner.

evaluation of additional columns would be cost-prohibitive, it was determined that further evaluation of column compositions would not be pursued.

2.8 Discussion

Development of combined reversed-phase HPLC and mass spectrometric methods requires balancing the best parameters for resolution and for electrospray response, as the conditions producing the desired chromatographic resolution may not produce the optimal electrospray response. Furthermore, the conditions best for one compound may not be best for another. For qualitative method development, resolution of the test GnRH compounds was desired (to facilitate data-dependent experiments during which the mass spectrometer triggers MS/MS experiments based on MS response as compounds elute from the HPLC system). Because biological samples are expected to contain low concentrations of GnRH peptides, it was important also to identify general conditions that would produce optimal electrospray response for the GnRH peptide family, using the six isoforms as model compounds. For development of a quantitative method for a particular isoform or isoform(s), chromatographic resolution may be modified from the conditions employed for qualitative analysis and electrospray parameters can be selected to fully optimize conditions to those particular isoforms.

The results obtained from infusions of solutions containing cGnRH-I, cGnRH-II, lGnRH-III, mGnRH, sGnRH and sbGnRH at different pH indicate that increased electrospray response (irregardless of charge state) was obtained at the lowest pH evaluated, 2.7. This pH is fully compatible with reversed-phase chromatography of peptides, as interactions with non-endcapped silanol groups are minimized at pH below 3. Experiments with varying capillary heater temperature and source voltage settings

revealed optimal ranges for qualitative methods; however, other electrospray parameters had less consistent effects on response, although these could be optimized for a particular isoforms if desired. Infusions of the GnRH isoforms in solutions of varying organic content demonstrated that, in general, response was enhanced with higher concentrations of organic and at lower pH. While response in solutions containing acetonitrile tended to be somewhat higher than those containing methanol, the difference was not completely consistent among isoforms and charge states, and either solvent would be suitable as the organic modifier for mobile phase.

To achieve optimal sensitivity, it would also be desirable to select conditions whereby one of the charge states is dominant so that the signal for a particular peptide is not distributed between several *m/z* ratios. For cGnRH-I, sGnRH, and sbGnRH, over 90% of the absolute signal existed in the +1 charge state, irregardless of pH. However, for cGnRH-II, lGnRH-III and mGnRH, the signal was split between several charge states at all pH evaluated. While the electrospray response for the +2 charge states of these peptides was higher, the actual sensitivity achieved in practice with biological samples may be better at the higher m/z associated with the +1 charge states. The capillary temperature setting appears to offer a mechanism by which the charge states produced during the electrospray process may be shifted toward the +1 charge state, especially for lGnRH-III and mGnRH.

Some initial starting conditions for chromatographic separation had been identified via experimentation with a 50 x 1.0 mm C8(2) Luna reversed-phase chromatography column prior to the work described in this chapter; in general, these initial conditions proved to be a good starting point. Nearly complete resolution of the six

model isoforms was achieved on this column with mobile phases prepared using formic and acetic acid. While formic acid and acetic acid produced similar results in terms of chromatographic separation, the electrospray response was somewhat enhanced in separations using acetic acid. Changing to a peptide specialty (C12), C18, or phenylhexyl column composition produced degraded chromatographic resolution or resulted in broadened peaks as compared to the C8 column. Mobile phases prepared with TFA or HFBA, two ion-pairing reagents that are frequently used for peptide/protein reversed-phase separations, resulted in an expected loss of sensitivity and offered no advantage in terms of the chromatographic separation.

2.9 Conclusions

We sought to systematically evaluate the LC-MS/MS conditions that would affect chromatographic resolution and mass spectrometric response in our laboratory. Results demonstrated that nearly complete separation of the six model GnRH isoforms could be achieved within a reasonable time frame (30 minutes) when using mobile phases modified with either acetic or formic acid on the C8(2) 50 x 1.0 mm column. While the electrospray parameters were optimized for and may be peculiar to an LCQ Deca quadrupole ion trap mass spectrometer, the results indicate that certain source conditions can significantly affect charge state as well as response. In particular, we found that capillary heater temperature and source voltage settings might be useful to select for a particular charge state or optimizing response.

These findings were used to develop the qualitative method described in Chapter 3, which presents a novel LC-MS/MS approach to GnRH characterization that is more efficient and consumes less tissue than traditional approaches and eliminates the need for

specific antibodies. This method can be used to confirm the presence of any of the twenty-four known isoforms or to search for unknown isoforms in samples which have not been fully characterized for their GnRH content. The chromatographic separation developed in this chapter was important for the data-dependent experiments described in Chapter 3, and the investigation of source conditions allowed the selection of settings for optimal response of the model isoforms (and presumably the family of GnRH peptides). Because the LC-MS/MS method relies on *de novo* sequencing techniques to provide strong evidence of primary structure, further characterization of the model isoforms in regards to MS/MS fragmentation is described in Chapter 3.

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CHAPTER 3: A NEW ELECTROSPRAY IONIZATION-QUADRUPOLE ION TRAP MASS SPECTROMETRY-BASED STRATEGY FOR THE QUALITATIVE DETERMINATION OF GnRH PEPTIDES

(The experiments described in this chapter have been published in Myers, T.R. and Patonay, G., A new strategy utilizing electrospray ionization-quadrupole ion trap mass spectrometry for the qualitative determination of GnRH peptides. *Journal of Mass Spectrometry* **2006**, 41, 950-959.

3.1 Introduction

Gonadotropin-releasing hormone (GnRH) is a critical neurotransmitter in vertebrate species, acting to stimulate and release gonadotropins for regulation of reproductive activities. GnRH is now thought to have functions beyond that of a neurotransmitter in vertebrates and has been found in tissues other than brain or pituitary, including those from mammary gland tumors, pancreas, and placenta (1). While several forms of GnRH have been found in invertebrates, functionality in these species is less understood.

Twenty-four naturally-occurring forms of GnRH have been identified (2). All forms identified from vertebrates are decapeptides, as are all but one of the forms identified in invertebrates. The primary structures of the peptides are highly conserved, and all have an N-terminal pyroglutamic acid and a C-terminal amidated glycine (see Table 3.1). Most isoforms have been found in multiple species. In addition, most vertebrates have been found to express at least two isoforms. This diversity across species has generated interest in GnRH as an indicator of the evolution of reproductive processes. In addition, the presence of multiple forms within a single species has generated interest in linking particular functions to the presence and levels of the distinct forms.

Table 3.1. Primary structure and expected $(M+H)^+$ for known GnRH isoforms

GnRH Isoform	Amino Acid Sequence	$(M+H)^+$
Catfish	pGlu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2	1114.5
Chicken I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2	1154.5
Chicken II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2	1236.5
Dogfish	pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2	1186.6
Guinea Pig	pGlu-Tyr-Trp-Ser-Tyr-Gly-Val-Arg-Pro-Gly-NH_2	1194.6
Herring	pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH_2	1087.5
Lamprey I	pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2	1226.6
Lamprey III	pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2	1259.6
Mammalian	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	1182.6
Octopus	pGlu-Asn-Tyr-His-Phe-Ser-Asn-Gly-Trp-His-Pro-Gly-NH_2	1425.6
Pejerrey	pGlu-His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly-NH_2	1097.5
Rana	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Trp-Pro-Gly-NH_2	1212.6
Salmon	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2	1212.6
Seabream	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2	1113.5
Tunicate I	pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2	1246.6
Tunicate II	pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2	1117.5
Tunicate III	pGlu-His-Trp-Ser-Tyr-Glu-Phe-Met-Pro-Gly-NH_2	1263.5
Tunicate IV	pGlu-His-Trp-Ser-Asn-Gln-Leu-Thr-Pro-Gly-NH_2	1149.5
Tunicate V	pGlu-His-Trp-Ser-Tyr-Glu-Tyr-Met-Pro-Gly-NH_2	1279.5
Tunicate VI	pGlu-His-Trp-Ser-Lys-Gly-Tyr-Ser-Pro-Gly-NH_2	1128.5
Tunicate VII	pGlu-His-Trp-Ser-Tyr-Ala-Leu-Ser-Pro-Gly-NH_2	1127.5
Tunicate VIII	pGlu-His-Trp-Ser-Leu-Ala-Leu-Ser-Pro-Gly-NH_2	1077.5
Tunicate IX	pGlu-His-Trp-Ser-Asn-Lys-Leu-Ala-Pro-Gly-NH_2	1119.6
Whitefish	pGlu-His-Trp-Ser-Tyr-Gly-Met-Asn-Pro-Gly-NH_2	1158.5

Traditional analytical methodologies for identification of GnRH typically involve multiple HPLC fractionation steps followed by detection via immunoassay techniques, with confirmation of molecular weight by mass spectrometry and of primary structure by amino acid sequencing (3-5). Because only immunoreactive fractions are investigated, traditional methods may not detect GnRH in cases where the antisera employed are not reactive to the unknown form. In addition, multiple fractionation procedures and associated analyses are laborious, and structural confirmation must be performed individually for each form of GnRH that is tentatively identified via immunoassay. More recently, molecular biological techniques (in silico methods or cloning of cDNA) have been used to identify novel forms of GnRH (6-8). These techniques are also laborious, and expression of a particular GnRH form as well as any post-translational modifications must be confirmed individually.

In this study, we demonstrate that *de novo* sequencing from ESI-MS/MS spectra of intact GnRH peptides provides adequate sequence coverage for proposing or confirming primary structure. Using simple sample preparation techniques, we were able to isolate and identify the chicken I, chicken II, lamprey III, mammalian, salmon and seabream isoforms in a spiked control sample. In addition, we were able to isolate and identify the mammalian GnRH (mGnRH) isoform as well as a modified version, hydroxyproline^{9} mammalian GnRH (Hyp^{9}GnRH), in Sprague-Dawley rat brains. To our knowledge, no methodology has been shown to provide simultaneous separation and identification of multiple GnRH analogues.

3.2 EXPERIMENTAL

Chemicals and Reagents

Glacial acetic acid, trifluoroacetic acid (TFA) and formic acid (88%) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA). Water was either purchased from Burdick & Jackson (Muskegon, MI, USA) or supplied by an in-house Vantage reverse-osmosis system (US Filter, Colorado Springs, CO, USA). Bond Elut C18 solid-phase extraction cartridges (200 mg) were purchased from Varian (Palo Alto, CA, USA).

Ultramark 1621 and l-methionyl-arginyl-phenylalanyl-alanine (MRFA) were purchased from ThermoFinnigan (San Jose, CA, USA). Caffeine was purchased in powder form from Sigma-Aldrich (St. Louis, MO, USA). Peptide standards (chicken I, chicken II, lamprey III, mammalian, salmon and seabream GnRH) were purchased from BAChem (Torrance, CA, USA). Hydroxyproline^{9} GnRH was purchased from California Peptide Research (Napa, CA, USA).

Standard preparation

Stock standards of each GnRH peptide were prepared at 100 μg mL^{-1} in deoxygenated H_2O. Subsequent dilutions were made into 1% formic acid to minimize adsorption to tube and vial walls.

Mass spectrometry

HPLC separations were performed using a Hewlett-Packard 1100 series binary pump and autosampler with vacuum degasser (Palo Alto, CA, USA) connected to a Phenomenex Luna C8(2) 50 x 1.0 mm column, 5μ particle size (Torrance, CA, USA). Mobile phase A consisted of 0.5% (vol/vol) acetic acid in water and mobile phase B

consisted of 0.5% (vol/vol) acetic acid in acetonitrile. A gradient program increasing from 5% B to 60% B over 55 minutes was used to achieve separation of the GnRH peptides. Column effluent, at 100 μL min^{-1}, was directed into the electrospray source of the mass spectrometer without splitting.

All experiments were performed using a Finnigan LCQ Deca quadrupole ion trap mass spectrometer (San Jose, CA, USA) operated in positive electrospray mode. The electrospray source was fitted with a stainless steel needle. Calibration of the mass spectrometer was performed using a mixture of caffeine, MRFA, and Ultramark 1621 as recommended by the manufacturer. Optimization of source conditions was performed while infusing sample from a syringe pump into mobile phase flow at 100 μL min^{-1}. Evaluation of MS/MS fragmentation patterns was performed while infusing 1 μg mL^{-1} sample (in 25:75 acetonitrile:0.1% formic acid) into the source from a syringe pump at 10 μL min^{-1}. MS/MS spectra were acquired over a series of collision energies to determine optimum conditions for *de novo* sequencing.

The following source settings were selected as optimal for GnRH peptides: sheath flow (Nitrogen) - 100 units, auxiliary flow - off, source voltage – 4.5 kV, capillary temperature – 250°C, and capillary voltage – 22.0 V. Automatic gain control (AGC) was set to on, with a full AGC target of 5000000. Microscans were set to 3, with an injection time of 50 msec. Data-dependent experiments were designed such that MS scans (*m/z* 1000-1275) reaching a threshold of 50 x 10^4 counts triggered an MS^2 scan (default setting *m/z* 100-1275) on the most intense ion from the MS scan. A default isolation width of *m/z* 2.8 was selected, collision energy was set to either 30 or 35%, and the activation Q was set to 0.250 with an activation time of 30 msec. No inclusion or exclusion criteria were

set for the data-dependent experiments. Targeted product-ion analysis experiments were conducted on specific GnRH precursor ions, with collision energy set to 35%, a default isolation width of 2.5, an activation Q of 0.250, and an activation time of 30 msec. The volume of sample extract consumed was 50 μL for data-dependent experiments, and 25-50 μL for product-ion analysis and SIM experiments.

Extraction procedure

Rat brains were homogenized in polypropylene test tubes with 2-3 mL acetonitrile using a Tissue-Tearor tissue homogenizer (Biospec Products, Bartlesville, OK, USA). A control was prepared by homogenizing a single rat brain, to which was added 50 ng each chicken I and II, lamprey III, mammalian, salmon and seabream GnRH. Acetonitrile was added to bring the total volume of solvent to 5 mL, after which tubes were vortexed and centrifuged at 2500 g, 4ºC for 30 minutes. The supernatant was evaporated under nitrogen at 60ºC and reconstituted in 3 mL of the loading solution, which consisted of water:acetic acid:TFA (96:3:1, volume/volume).

Solid phase extraction (SPE) was used to isolate GnRH from the brain homogenate samples. The SPE cartridges were preconditioned with 3 mL methanol followed by 3 mL of the loading solution. The reconstituted supernatant was transferred to the cartridges, which were subsequently washed with 3 mL loading solution followed by 3 mL 70:30 loading solution:methanol. Samples were eluted with 3 mL 30:70 3% acetic acid:methanol, which was collected and evaporated under nitrogen at 60ºC. For experiments where brain extracts were combined, extracts were pooled into one extract as they neared dryness, with the final combined extract taken fully to dryness. Samples were

reconstituted in 200 µL 95:5 3% acetic acid:methanol, vortexed for 30 seconds and transferred to glass autosampler vials for analysis.

Peptide confirmation with synthetic GnRH standards

Aliquots of 50 µL were removed from the total reconstitution volume (200 µL) of a single brain extract and transferred to separate glass autosampler vials. A standard solution of 5 pg/uL Hyp^{9}GnRH and 20 pg/uL mGnRH was prepared in 95:5 3% acetic acid:methanol, and 10 µL of this standard was transferred to one of the aliquots while 10 µL of 95:5 3% acetic acid:methanol was added to the second aliquot. The total volume of both aliquots was analyzed in product-ion analysis experiments for *m/z* 1182 and 1198.

3.3 RESULTS AND DISCUSSION

Mass spectral data/fragmentation patterns

Known GnRH peptides have certain similarities in primary structure. The proline residue, at one position removed from the C-terminal amidated glycine (see Table 3.1), is such a constant residue that is expected in new forms of GnRH. Proline residues can have significant implications for MS/MS fragmentation by promoting intense fragmentation on the N-terminal side of the proline residue (9-11). Selective production of a single prominent y or b ion could obscure the series of ions necessary to perform *de novo* sequencing of novel GnRH forms. To determine whether an adequate series of ions could be observed from fragmentation in the quadrupole ion trap, MS/MS fragmentation patterns were examined for chicken I and II, lamprey III, mammalian, salmon and seabream GnRH**.**

Optimal % collision energies were selected as those which optimized fragmentation without a severe reduction in overall signal intensity. Fragmentation data

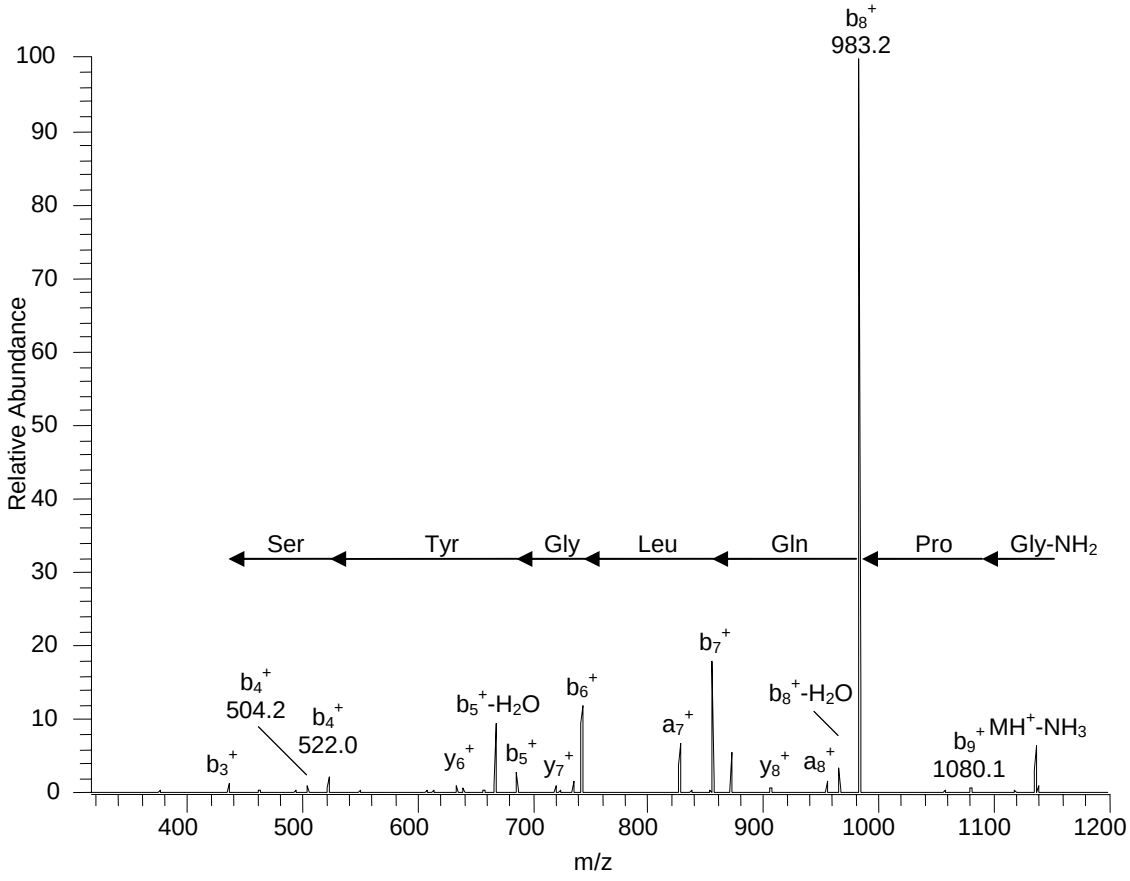

Figure 3.1. ESI-MS/MS spectrum of singly-charged cGnRH-I, precursor ion at *m/z* 1154.4, average of 10 scans.

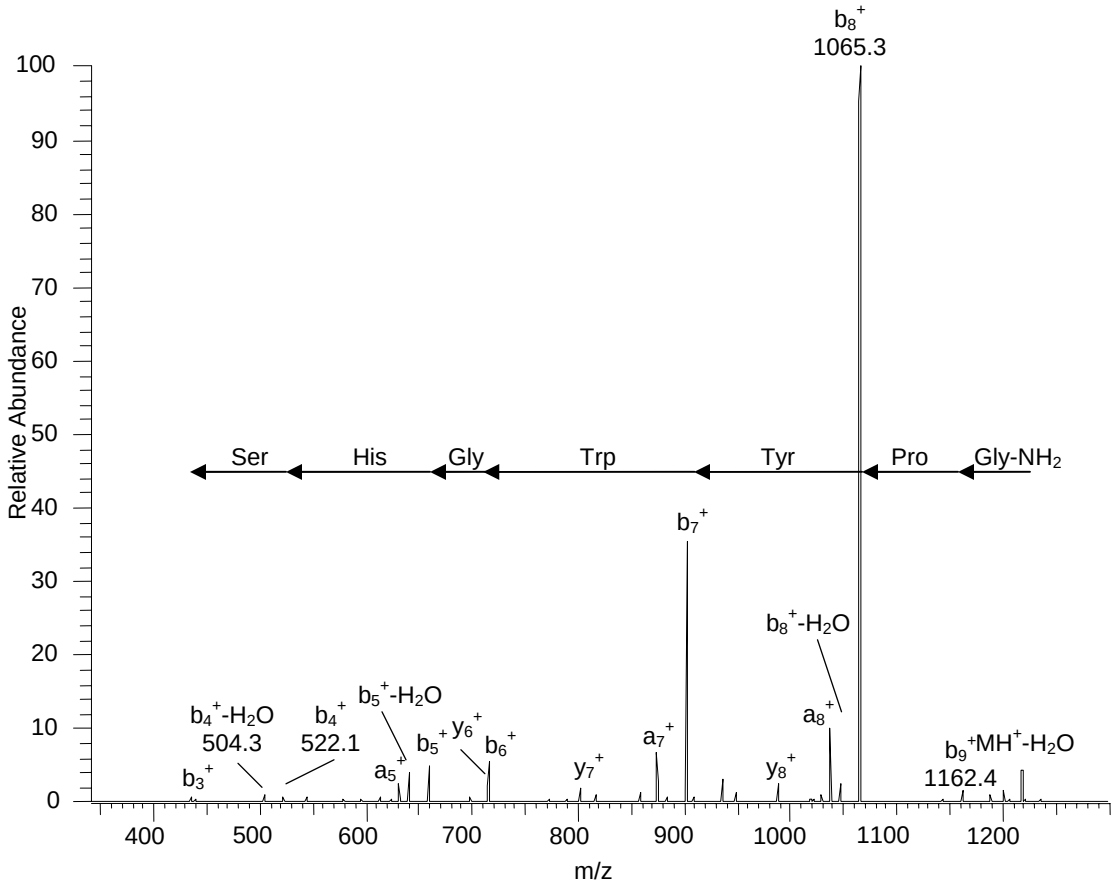

Figure 3.2. ESI-MS/MS spectrum of singly-charged cGnRH-II, precursor ion at *m/z* 1236.4, average of 10 scans.

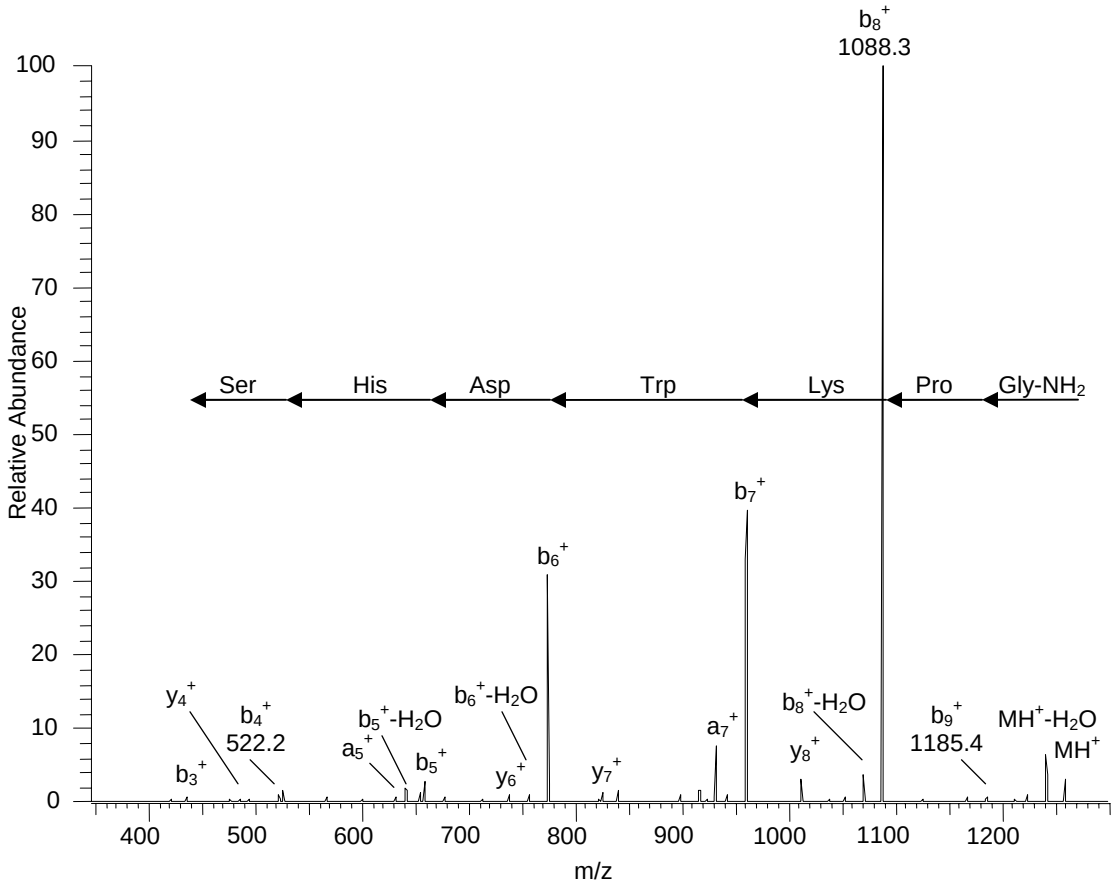

Figure 3.3. ESI-MS/MS spectrum of singly-charged lGnRH-III, precursor ion at *m/z* 1259.5, average of 10 scans.

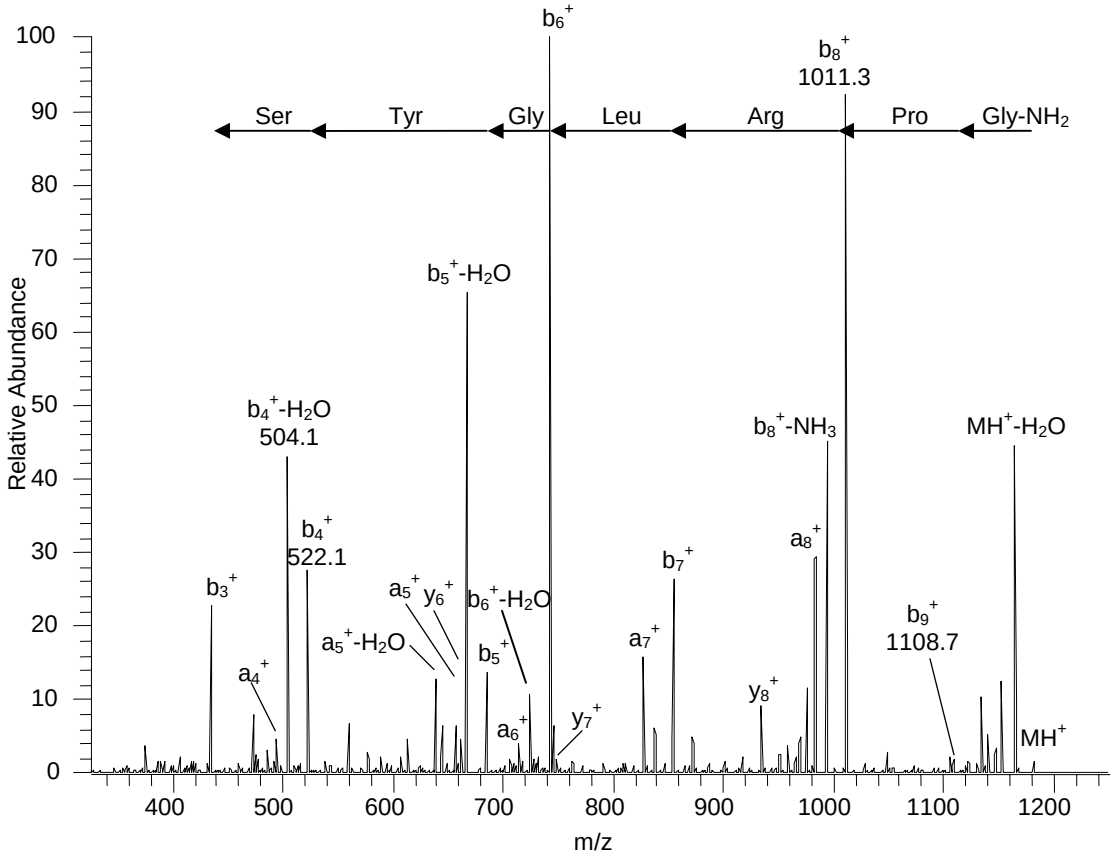

Figure 3.4. ESI-MS/MS spectrum of singly-charged mGnRH, precursor ion at *m/z* 1182.6, average of 10 scans.

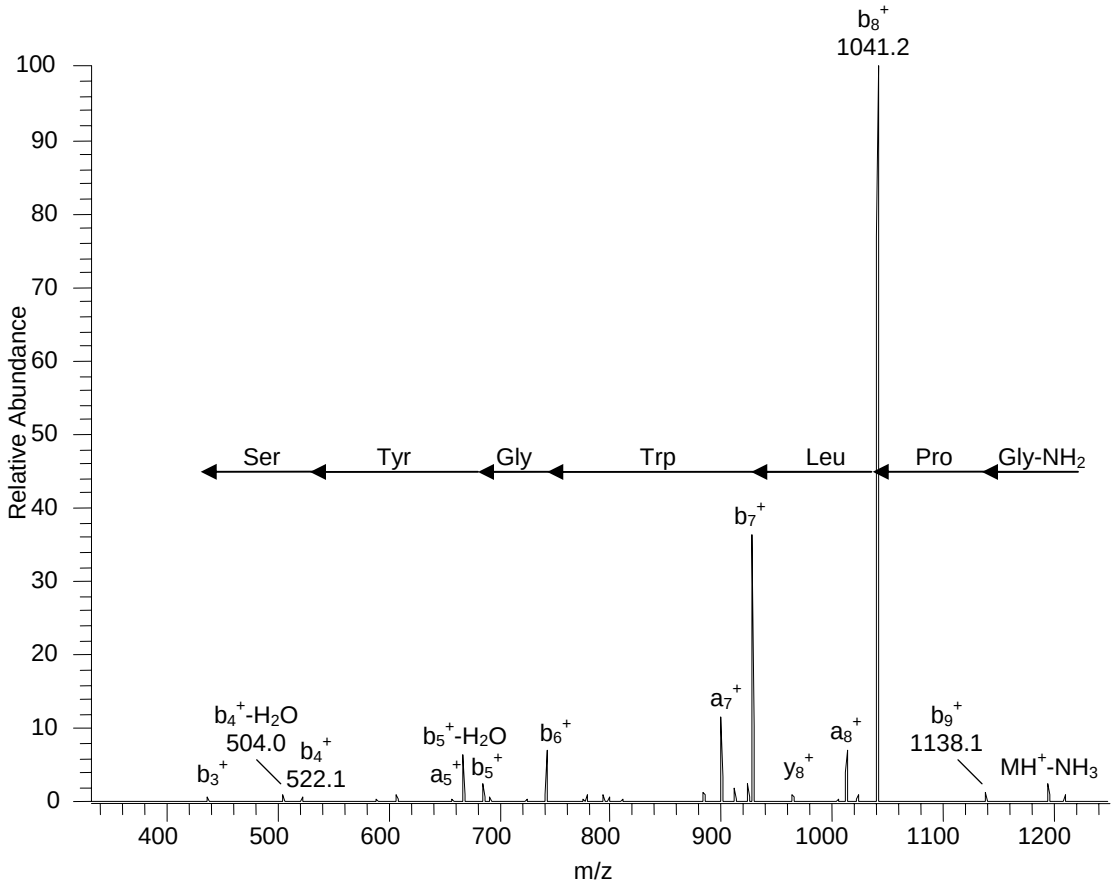

Figure 3.5. ESI-MS/MS spectrum of singly-charged sGnRH, precursor ion at *m/z* 1212.4, average of 10 scans.

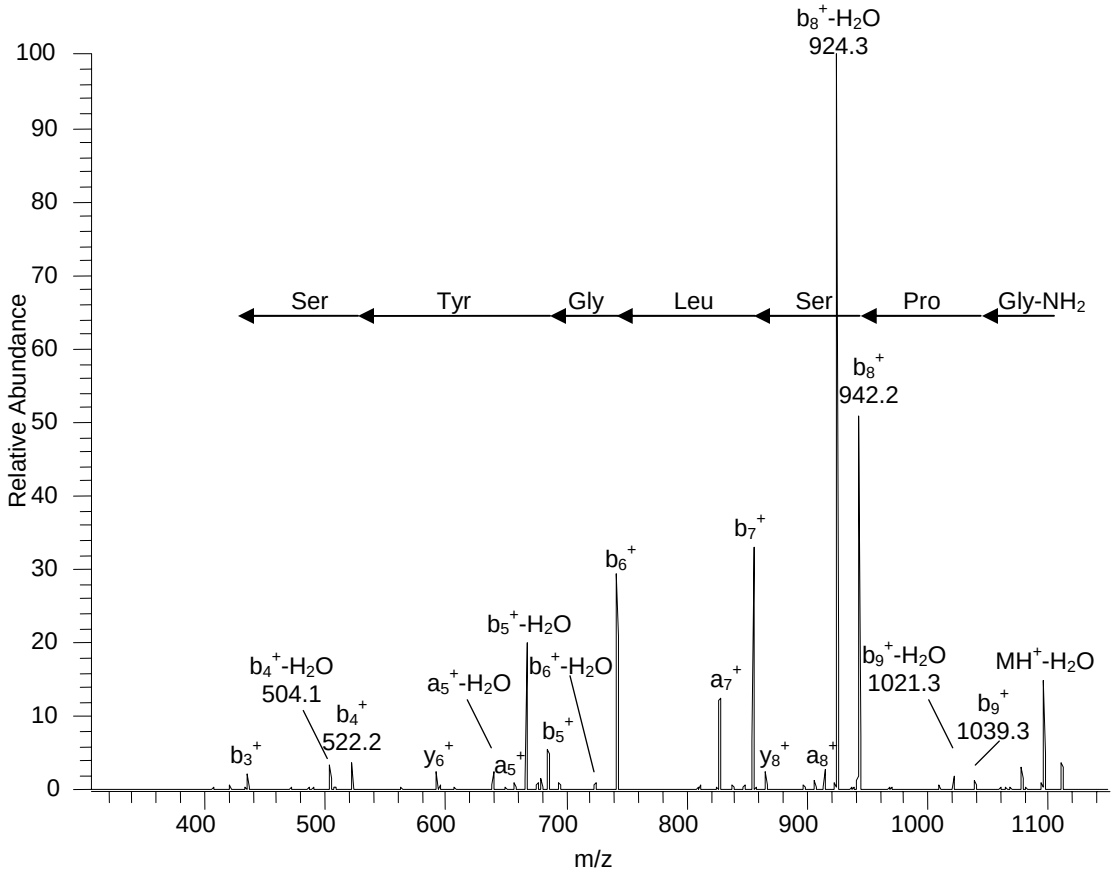

Figure 3.6. ESI-MS/MS spectrum of singly-charged sbGnRH, precursor ion at *m/z* 1113.4, average of 10 scans.

were collected for all charge states observed for the peptides. The MS/MS spectra for the singly-charged states are shown in Figures 3.1 through 3.6, and the fragment ions observed for each charge state are shown in Figure 3.7. Only a, b, and y fragment ions are indicated in Figures 3.1 through 3.7 as these are the most useful for *de novo* sequencing. The observed *m/z* is also indicated for fragments of special interest in identifying spectra from GnRH peptides.

The spectra collected from singly-charged peptides contained predominantly a and b fragment ions with some y and internal cleavage fragment ions. The b_8 ion was prominent in all cases, indicating that the proline residue does promote selective fragmentation N-terminal to position 9. While a full series of b ions was not observed, b_3-b_9 were detected in all cases; this series can be used to deduce the amino acid residues in positions 4-9. The amino acid residue in position 3 could be deduced by identification of the y_8 fragment ion (also observed for all GnRH forms) and a priori knowledge of the amidated glycine at the C-terminus. Based on these experiments and assuming that the structural similarities on each terminus are consistent, most known forms of GnRH could be identified using MS/MS fragmentation experiments on a quadrupole ion trap. Octopus GnRH (with an unusual 12 residue length) was not investigated, but it seems likely that similar fragmentation patterns would be observed, yielding useful sequencing information and possibly sufficient data to propose a complete sequence.

MS/MS spectra of the doubly-charged mammalian and lamprey III GnRH peptides contained useful b and y fragment series that could be used for sequencing as noted above for the singly-charged peptides. The spectra obtained from the doubly-charged forms of chicken I, chicken II and seabream GnRH and the triply-charged

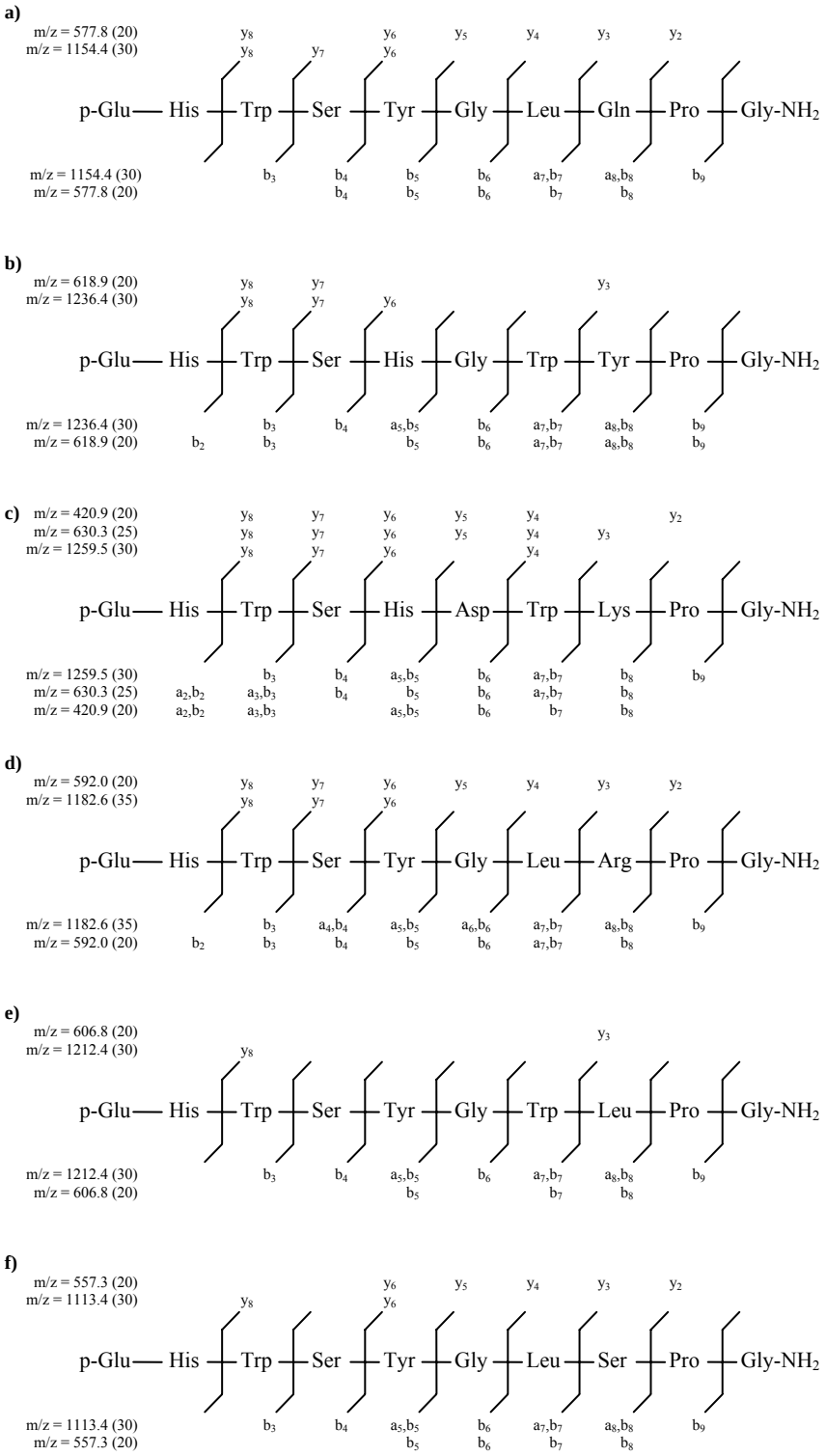

Figure 3.7. a, b, and y fragment ions identified in ESI-MS/MS spectra for GnRH peptides, with optimal collision energy shown in parenthesis. (a) cGnRH-I, (b) cGnRH-II, (c) lGnRH-III, (d) mGnRH, (e) sGnRH and (f) sbGnRH.

lamprey GnRH contained shorter series of b and y fragment ions. In cases where insufficient data were obtained from the multiply-charged forms to deduce the complete GnRH structure, complementary information was observed that could be useful to support a proposed *de novo* sequence obtained from the singly-charged peptide.

GnRH isolation and identification from Sprague-Dawley rat brains

In data-dependent MS/MS scanning experiments, manual review of data to identify the peptide spectra of interest can be laborious without a strategy for selection of relevant data. The product-ion analysis data from the six model peptides and the analogous terminal structures of the known forms of GnRH can be used to predict features that are expected in MS/MS spectra of these peptides. A common pattern arises from the structural similarity observed through the fourth amino acid position on the N-terminus for nearly all known forms. This similarity leads to a characteristic b_4 and b_4-H_2O fragment ions at *m/z* 522 and 504, respectively, which can be used as keystones to identify potential GnRH spectra. The structures of lamprey I and guinea pig GnRH suggest that additional forms may be discovered that contain substitutions in positions 2 and 3, and in these cases, marker ions at m/z 522 and 504 are not expected. An additional indicator based on the separation of the b_8 and b_9 fragment ions by 97 *m/z* units may be useful and should be easily identifiable since the b_8 fragment is one of the most predominant ions in the spectrum. This pattern would be expected for all forms of GnRH, except the unique octopus isoform for which the b_{10} and b_{11} fragment ions would be separated by 97 *m/z* units.

Data-dependent scanning experiments were performed to examine a control sample which was spiked with 50 ng each chicken I and II, lamprey III, mammalian,

salmon and seabream GnRH. The mass range (*m/z* 1000-1275) was selected to encompass singly-charged peptides of known vertebrate GnRH isoforms; this range was chosen after exploratory experiments indicated that higher background levels at lower mass ranges would preclude sufficient sensitivity for detection of doubly- and triply-charged GnRH peptides in data-dependent experiments. Strategies described above were used to review the spectra obtained, and each of the isoforms added to the control sample were detected by data-dependent scanning. The spectra obtained for each isoform are shown in Figures 3.8 through 3.13. Chromatographic separation of the GnRH isoforms in the control sample is shown in Figure 3.14. This proof-of-concept experiment confirmed the viability of the method at a level (approximately 40 pmoL) that would be expected from a small pool of rat brains, based on results reported for mGnRH in mature rat brains (12).

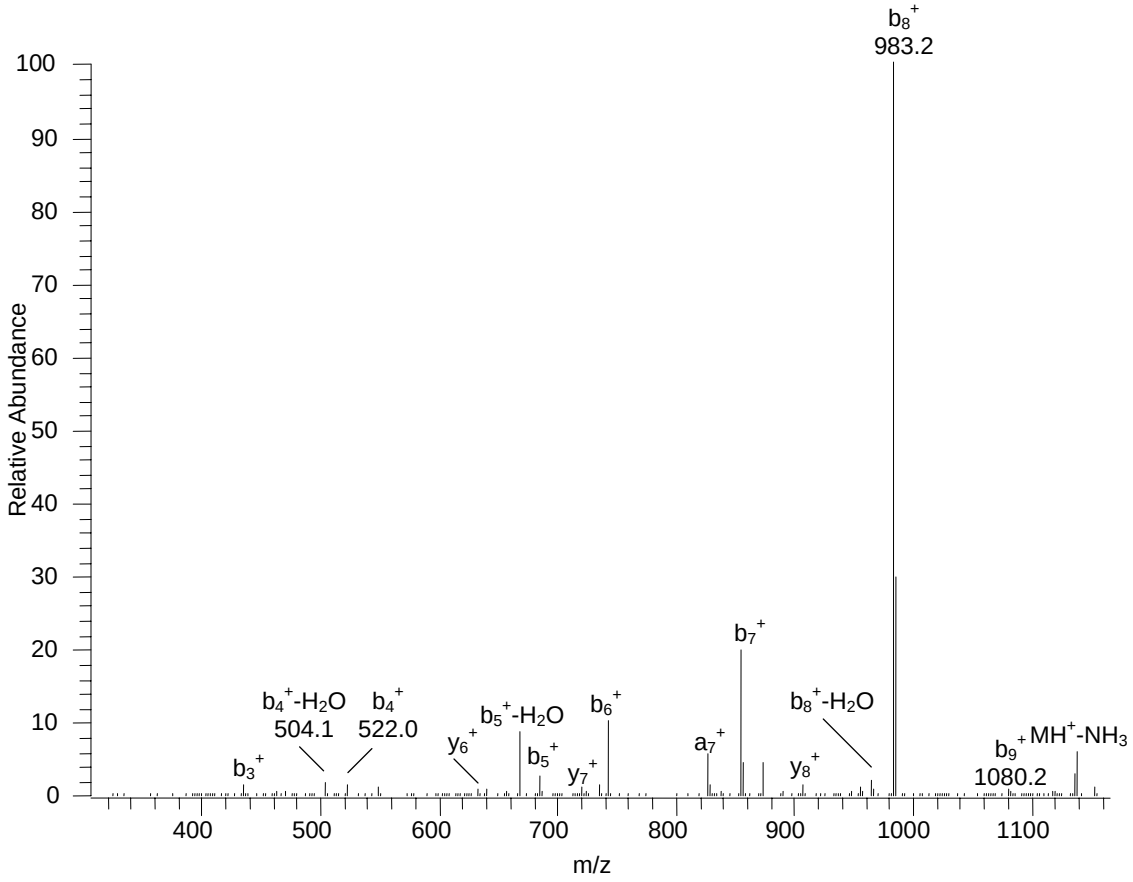

Figure 3.8. ESI-MS/MS spectra of cGnRH-I extracted from Sprague-Dawley rat brain control samples, precursor ion at *m/z* 1154.4. Data acquired in data-dependent scan mode with %CE=30. Spectra are averaged across the peak of interest.

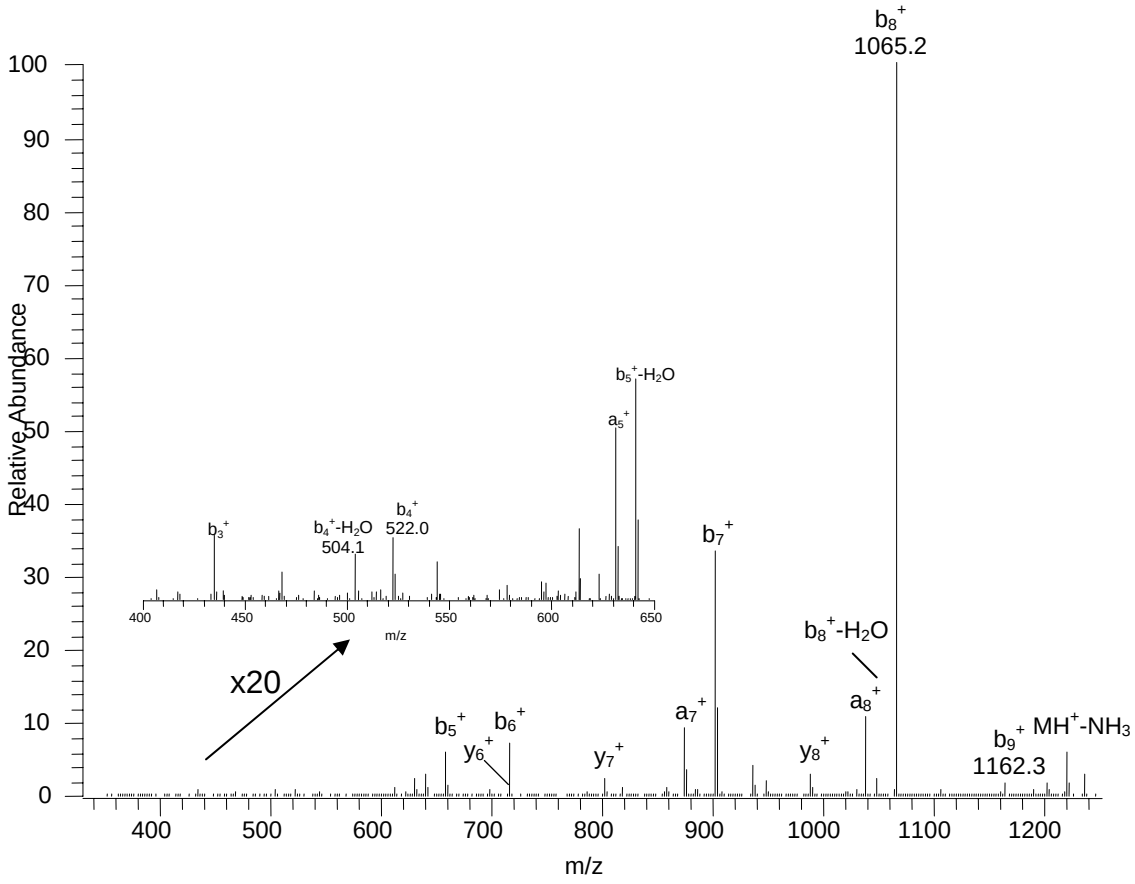

Figure 3.9. ESI-MS/MS spectra of cGnRH-II extracted from Sprague-Dawley rat brain control samples, precursor ion at m/z 1236.4. Data acquired in data-dependent scan mode with %CE=30. Spectra are averaged across the peak of interest.

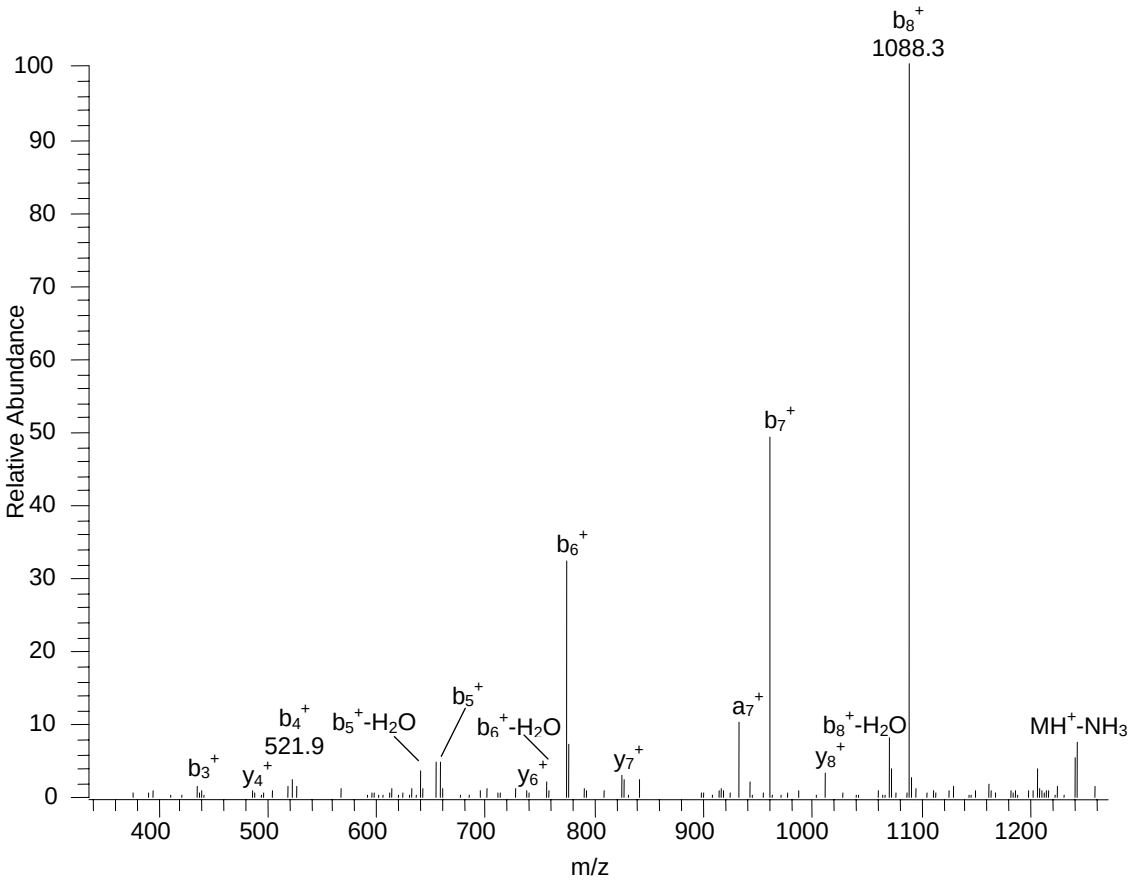

Figure 3.10. ESI-MS/MS spectra of lGnRH-III, extracted from Sprague-Dawley rat brain control samples, precursor ion at m/z 1259.5. Data acquired in data-dependent scan mode with %CE=30. Spectra are averaged across the peak of interest.

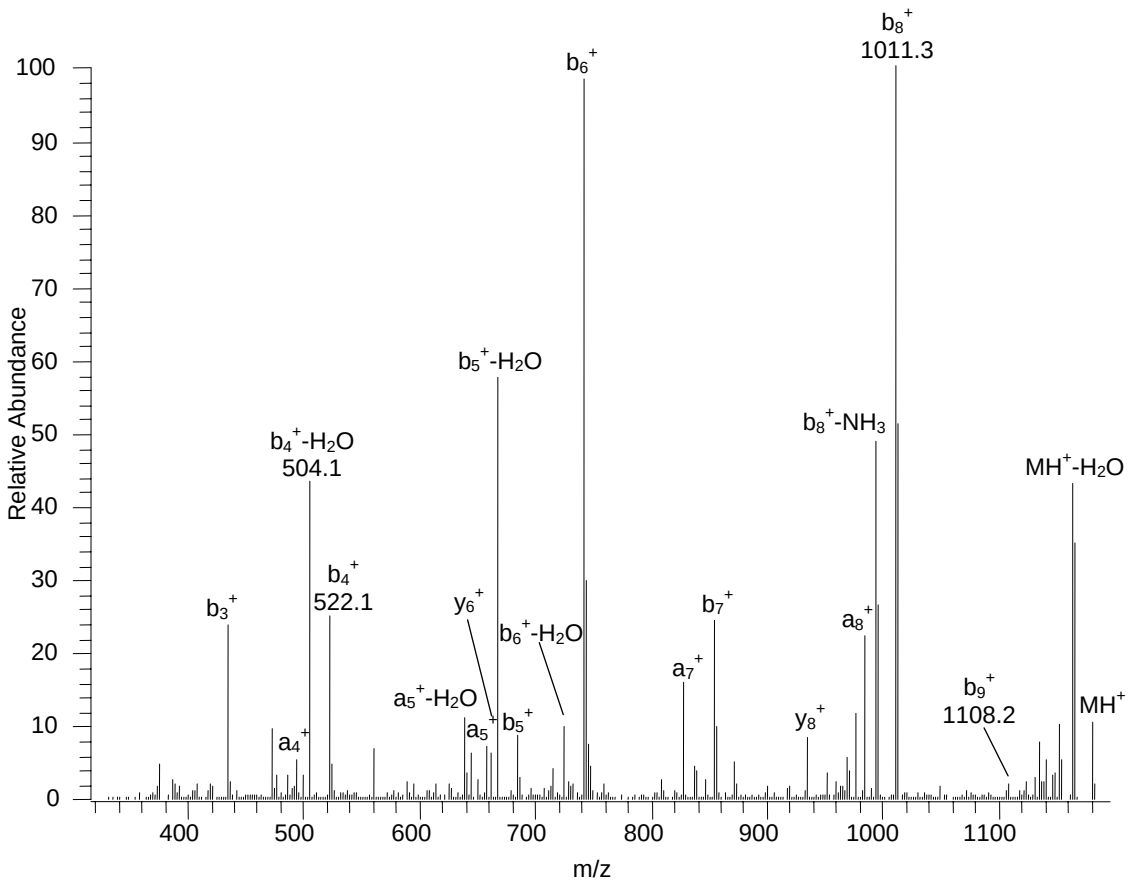

Figure 3.11. ESI-MS/MS spectra of mGnRH extracted from Sprague-Dawley rat brain control samples, precursor ion at m/z 1182.4. Data acquired in data-dependent scan mode with %CE=35. Spectra are averaged across the peak of interest.

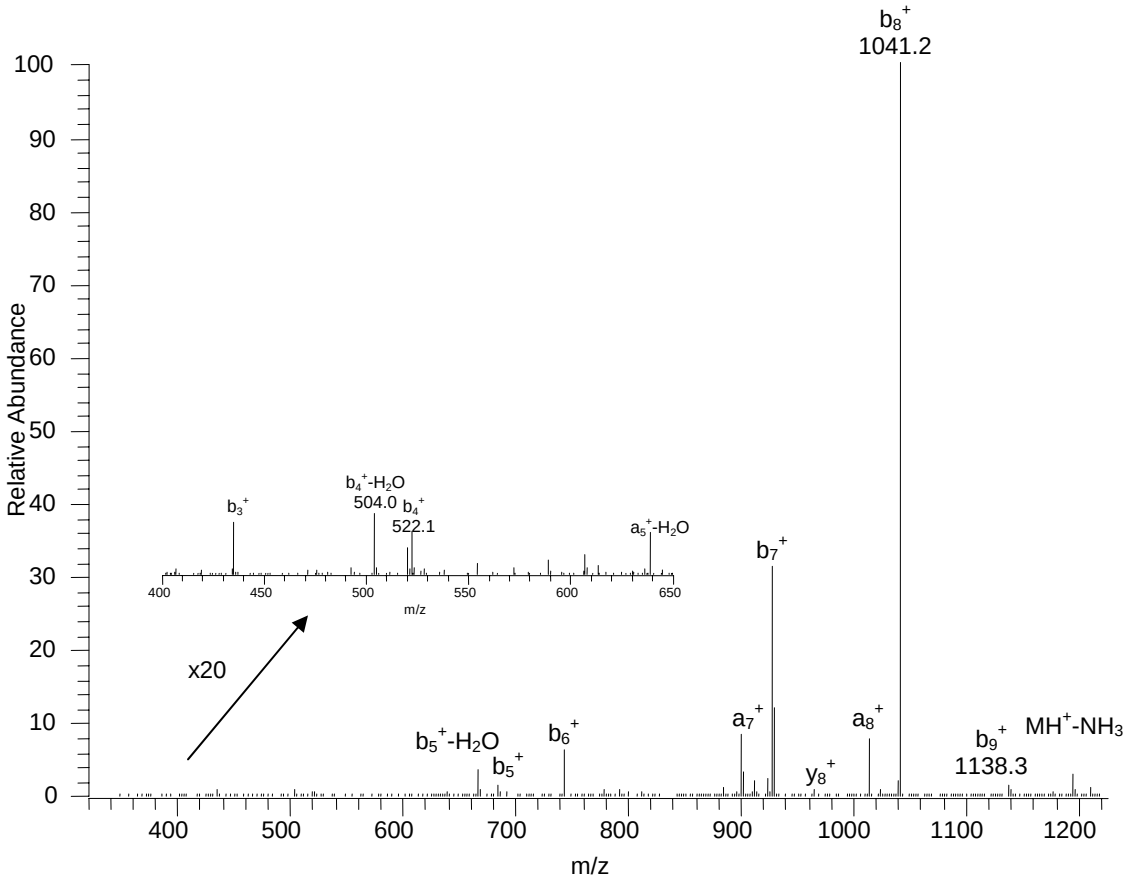

Figure 3.12. ESI-MS/MS spectra of sGnRH extracted from Sprague-Dawley rat brain control samples, precursor ion at m/z 1212.6. Data acquired in data-dependent scan mode with %CE=30. Spectra are averaged across the peak of interest.

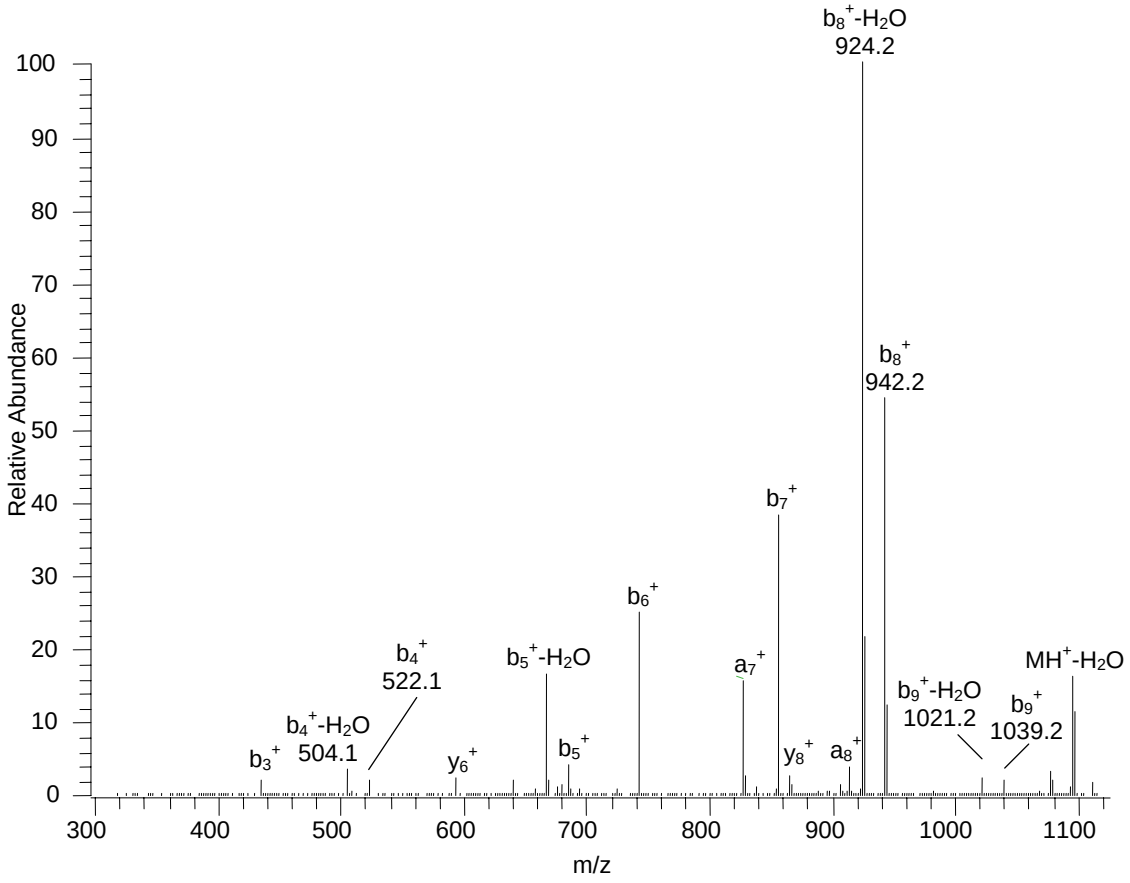

Figure 3.13. ESI-MS/MS spectra of sbGnRH extracted from Sprague-Dawley rat brain control samples, precursor ion at m/z 1113.4. Data acquired in data-dependent scan mode with %CE=30. Spectra are averaged across the peak of interest.

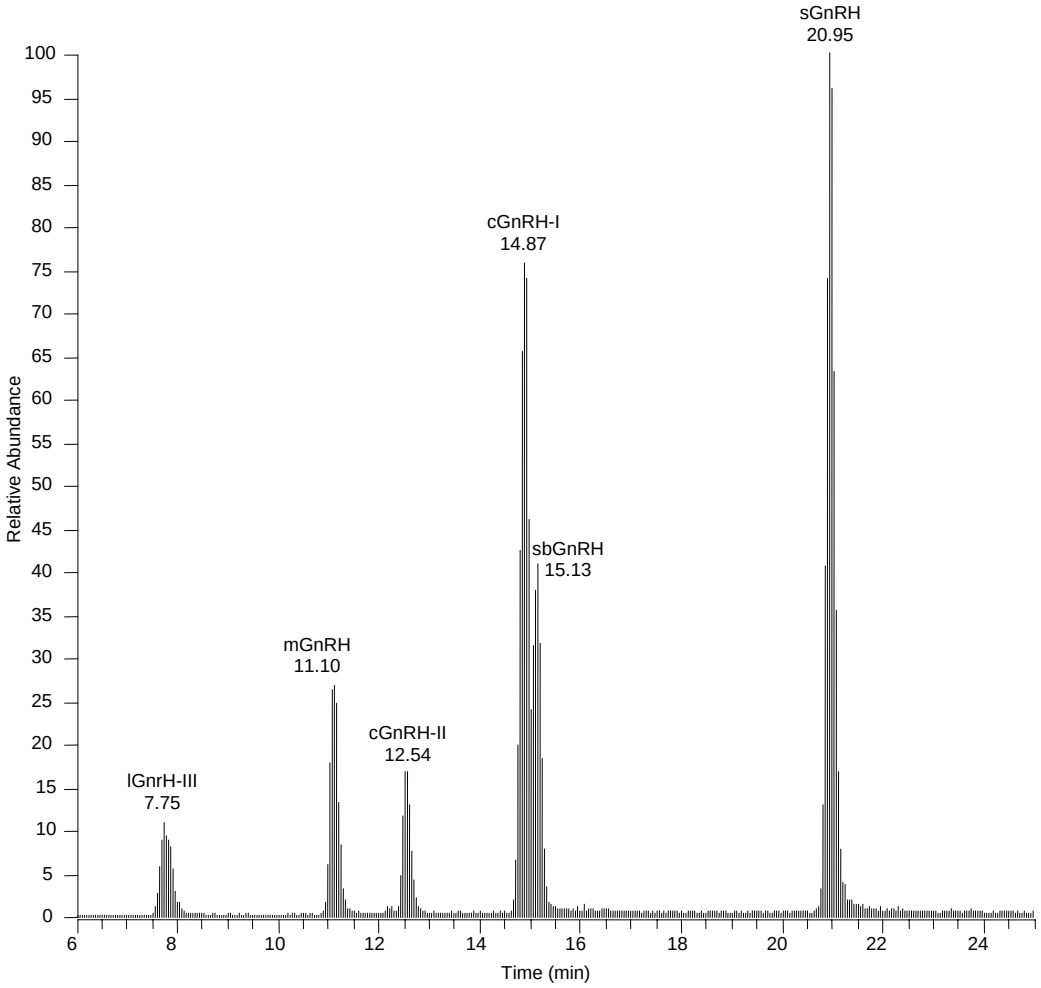

Figure 3.14. Reconstructed ion chromatogram of GnRH peptides extracted from Sprague-Dawley rat brain control samples.

When a pooled extract from 10 Sprague-Dawley rat brains was examined in data-dependent experiments, putative peptides of MH^+ at m/z 1182 and 1198 were detected. Spectra for both peptides are shown in Figure 3.15. No spectra were observed that would be indicative of chicken II, lamprey III, or salmon GnRH or any other novel forms of GnRH. The same experiment conducted on the extract from a single brain also produced spectra for a putative peptide at *m/z* 1182 but was unable to detect the putative peptide at *m/z* 1198.

The averaged spectrum obtained for the putative peptide at *m/z* 1182 was consistent with spectra obtained for mGnRH in the infusion experiment and in the control sample. The b_4-H_2O (*m/z* 504) and b_4 ions (*m/z* 522), useful for identifying spectra from potential GnRH isoforms, were readily apparent. The b_8 ion was the most prominent ion, but no b_9 ion was observed above baseline noise level. The retention time for the putative peptide at *m/z* 1182 was consistent with that obtained for mGnRH in the control sample.

Comparison of the spectra for the two putative peptides at *m/z* 1182 and 1198 suggested strong homology. An identical series of b_3-b_8 ions were observed, with the b_8 ion a prominent feature in the fragmentation pattern. An addition of +16 to the y_8 ion was also observed, indicating structural modification to one of the two amino acids on the carboxy-terminus. The retention time for this peptide under reversed phase conditions was somewhat earlier than mGnRH, suggesting that the structure was slightly more polar than mGnRH. Taken together, these patterns suggest the Hyp^9GnRH isoform; however, based on these results alone we could not eliminate the possibility of other structures, especially since hydroxyproline and leucine/isoleucine are isobaric amino acids.

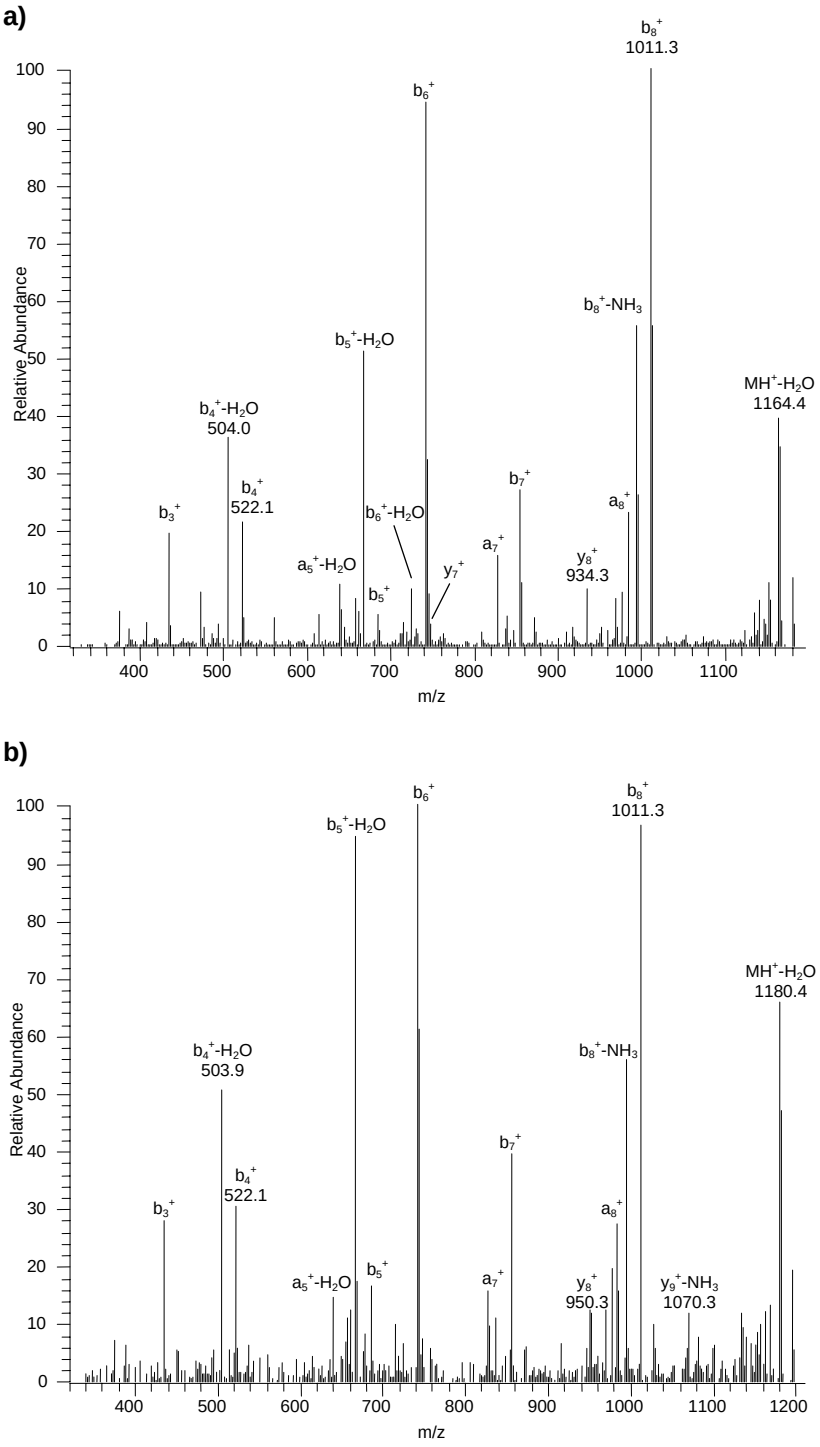

Figure 3.15. Combined extracts from 10 Sprague-Dawley rat brains, (a) putative mGnRH peptide, ESI-MS/MS at *m/z* 1182.5, %CE=35, average of 11 scans and (b) putative hydroxyproline GnRH peptide, ESI-MS/MS at *m/z* 1198.7, %CE=35, average of 3 scans.

In an attempt to obtain additional structural information from the pooled extract, product-ion analysis experiments were conducted for the putative peptides. These experiments were conducted for singly- and doubly-charged forms of the peptides. Spectra obtained from singly-charged peptides (*m/z* 1182 and 1198) did not contain any additional structural information beyond that obtained during data-dependent experiments. Spectra obtained from doubly-charged peptides (m/z 592 and 600) demonstrated a series of y-type ions from y^2-y^8, all of which showed an increase of +16 for the proposed hydroxyproline[9] isoform. MS^3 scans on the y^2 ion were dominated by y^2-NH_3 and an immonium ion for the amino acid in position 9 (*m/z* 70 for mGnRH and *m/z* 86 for the proposed hydroxyproline[9] isoform). While this information did not identify the amino acid in position 9, it did allow assignment of the +16 shift to that position. MS^2 and MS^3 spectra for both peptides are shown in Figure 3.16.

A Hyp[9]GnRH peptide standard was obtained for confirmatory experiments. Fragmentation spectra of the precursor ion at *m/z* 1198 were evaluated for the standard (1 ng injected on-column) using the same HPLC and product-ion analysis conditions as for the pooled extracts, with results indicating a similar retention time and fragmentation pattern as the putative peptide observed at *m/z* 1198. Comparison of two aliquots from a single rat brain extract, one fortified with 10 µL reconstitution solution and one fortified with 10 µL reconstitution solution containing 50 pg Hyp[9]GnRH and 200 pg mGnRH confirmed that the synthetic Hyp[9]GnRH and mGnRH standards coeluted with the putative peptides. Reconstructed ion chromatograms and spectra from this experiment are displayed in Figure 3.17. The spectra observed in the sample containing the synthetic peptides are consistent with those observed in the unadulterated sample. This experiment

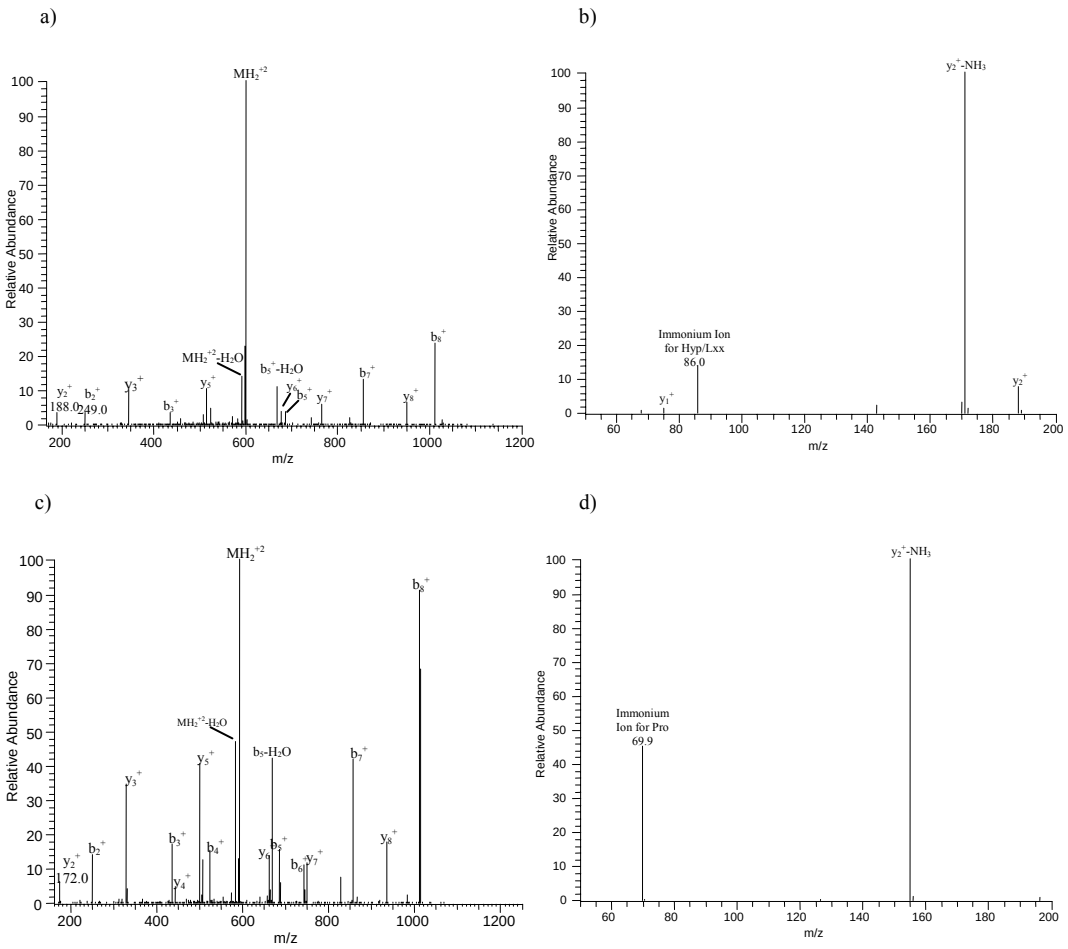

Figure 3.16. Combined extracts from 10 Sprague-Dawley rat brains, (a) product-ion spectra of *m/z* 599.7, %CE=20, average of 10 scans, (b) product-ion spectra of *m/z* 599.7 →m/z 188.0, %CE=25, average of 22 scans, (c) product-ion spectra of *m/z* 591.7, %CE=20, average of 11 scans and (d) product-ion spectra of *m/z* 591.7 → *m/z* 171.9, %CE=25, average of 29 scans.

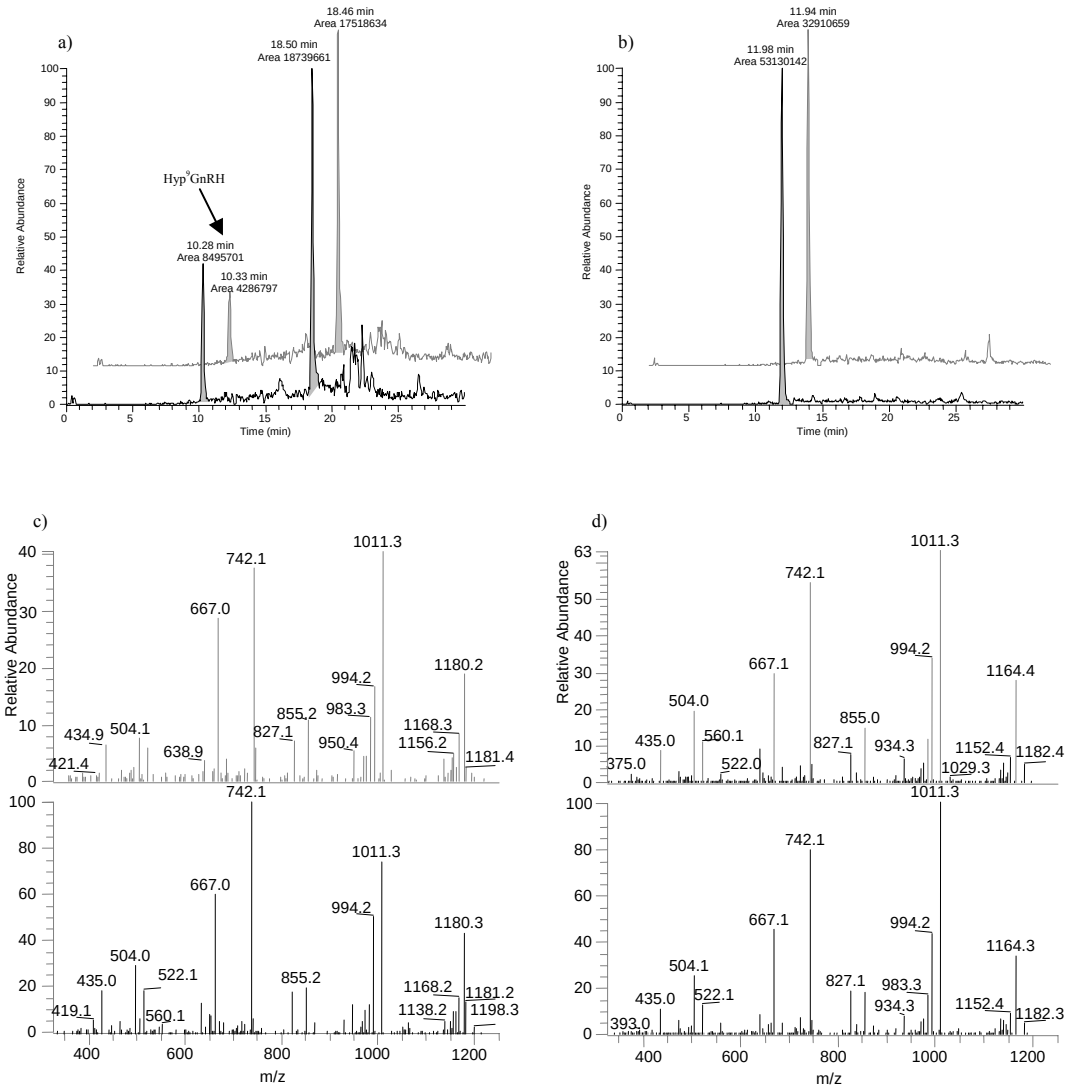

Figure 3.17. Comparison between single Sprague-Dawley rat brain extract fortified with 10 µL reconstitution solution (shown in gray) or 10 µL same containing 50 pg Hyp^9 GnRH and 200 pg mGnRH (shown in black). (a) reconstructed ion chromatogram of *m/z* 1198.4, (b) reconstructed ion chromatogram of *m/z* 1182.5, (c) product-ion spectrum of *m/z* 1198.4 and (d) product ion spectra of *m/z* 1182.5.

also allowed an approximation of Hyp^{9}GnRH and mGnRH quantities in this rat brain tissue of 200 pg (200 fmol) and 1000 pg (1 pmol), respectively.

Previous work has suggested the presence of chicken II (13), lamprey III (14), and salmon GnRH (15) isoforms in addition to Hyp^{9}GnRH and mGnRH in the brains of rats (12, 16). To investigate whether these additional isoforms may have been missed by the data-dependent algorithm, a targeted SIM experiment for the expected precursor masses of these isoforms was conducted on the pooled extract. No peaks were observed at the expected retention times for chicken II, lamprey III, and salmon GnRH in the rat brains. The aforementioned studies utilized detection via antisera to the respective GnRH forms. Because these studies relied on the specificity of the antisera employed and did not include any structural identification of the peptides, it is possible that false positive responses were obtained for chicken II, lamprey III, and salmon GnRH.

The techniques described here provide a methodology by which GnRH isoforms can be separated and identified by *de novo* sequencing, avoiding the specificity problems associated with immunoassay-related methods. Targeted product-ion analysis experiments can be easily designed for known forms of GnRH, while data-dependent experiments provide a means to detect novel forms of GnRH. Although we were successful using manual interpretation of the data, recognition of GnRH-related spectral features, such as the characteristic ions observed for b_4 and b_4-H_2O and the difference in m/z between b_8 and b_9, could be facilitated by use of software algorithms (17, 18). The presence of structural isomers (leucine/isoleucine) or isobaric amino acids (glutamine and lysine, hydroxyproline and leucine/isoleucine) in the proposed peptide sequence may require the use of additional techniques. In this study, addition of a synthetic Hyp^{9}GnRH

standard to an extracted sample was required to provide confirmation of the proposed peptide sequence.

One of the main advantages of this methodology is that it can identify putative GnRH peptide structures from a smaller tissue sample than other reported methods. Ten brains were required to produce pooled extract with sufficient signal for data-dependent experiments, and a single rat brain was sufficient to conduct multiple experiments on known or suspected GnRH precursor ions. In contrast, 100-200 rat brains were reported consumed in an effort to fully characterize GnRH forms (12); while the total tissue consumed was not devoted solely to qualitative determinations, this work still required a substantial quantity of animals. Of course, the quantity of tissue (or other matrix) required will vary depending upon the organism under study.

The detection limit for this method will vary depending upon whether product-ion analysis experiments are sufficient to characterize a sample for known GnRH peptides or whether data-dependent experiments are required to elucidate unknown GnRH variants. For product-ion analysis experiments, a practical limit of detection of 10 pg (10 fmol) for both Hyp^{9}GnRH and mGnRH was estimated by determining the smallest volume of extract (single brain) capable of generating GnRH peaks with a signal-to-noise ratio greater than 3 and with reasonable mass spectral quality. The practical limit of detection for data-dependent experiments will vary considerably depending upon experimental setup and sample quality; in this case, 300-500 pg was sufficient to trigger MS/MS scans useful for sequence determination. While the detection limits of immunoassay methods for GnRH are typically on the order of pg/sample, this HPLC-MS/MS methodology

allows simultaneous detection and identification of multiple GnRH isoforms while collecting valuable structural information.

3.4 CONCLUSIONS

The experiments described in this chapter were focused on developing a generalized approach to the separation and identification of GnRH isoforms. The method utilized a simple solid-phase extraction followed by LC-MS/MS analysis to acquire sequence information about the peptides. The method also provides simultaneous detection of multiple isoforms, and does not require the lengthy multi-chromatographic fractionation typically used prior to traditional immunoassay measurements. Because immunoreactive detection is not used, the possibility that an isoform will be missed if it does not cross-react with the antibodies employed during purification is eliminated. Also, the LC-MS/MS approach does not require any prior knowledge of the expected isoforms aside from the expected homology in the GnRH family, although experiments can be tailored to detect specific isoforms.

Given the strong interest in identifying the multiple forms of GnRH in a variety of organisms, less laborious qualitative approaches would be welcome. These results demonstrate that ESI-MS/MS techniques provide a simple approach to GnRH characterization that is rich in qualitative information. The fragmentation patterns obtained from product-ion analysis experiments with six model GnRH peptides contained sufficient sequencing information, when used in conjunction with knowledge about the conserved amino acid residues in GnRH peptides, to define the intact peptide structure. Data-dependent experiments followed by targeted product-ion analysis experiments were conducted on extracts from Sprague-Dawley rat brains, and mammalian GnRH and

hydroxyproline^{9} GnRH, a post-translationally modified form of mammalian GnRH, were identified. These results confirm the ability of a relatively simple extraction and ESI-MS/MS experiment to isolate and identify GnRH peptides in biological samples. Using this methodology, we were able to determine GnRH identities using a minimal quantity of tissue. In addition, the technique should also be applicable as an indicator of the presence of novel GnRH forms and may assist in the identification of such forms.

Targeted product-ion analysis experiments were conducted in the extract from a pool of rat brains to search specifically for GnRH isoforms that have been previously reported – chicken II GnRH, lamprey III GnRH, and salmon GnRH. No evidence supporting the expression of any of these isoforms was found. These results support the hypothesis that mGnRH and its post-translationally modified form, Hyp^{9}GnRH, are the primary, if not only, forms of GnRH expressed in rat brains.

Previous applications of mass spectrometry to GnRH identification have been primarily limited to confirmation of a molecular weight (MW), and comparison to the corresponding MW for the amino acid sequence obtained from chemical sequence analysis of a putative GnRH peptide isolated after HPLC fractionation. A number of studies have utilized matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry to confirm MW, but did not report the amount of sample required (3, 5, 16, 19, 20). Other studies indicate that MW determinations by a variety of mass spectrometry techniques, including MALDI-TOF MS, fast atom bombardment (FAB) MS, and liquid secondary ion (LSI) MS, required ng – ug quantities of purified GnRH (21-24). Several studies have explored partial sequence determination by mass spectrometry in addition to MW confirmation. After proposal of a primary sequence by

chemical sequence analysis, the MW as well as terminal structural features and a portion of the internal sequence of the octopus GnRH peptide were confirmed using nano-ESI-TOF mass spectrometry; however, the amount of sample required was not reported (4). Similarly, the MW and partial sequence for Lamprey I was determined by FAB-MS using approximately 0.8-3 ng peptide for each MS experiment (25). While the absolute amounts of peptide reported for chemical sequencing methods was not reported for the above studies, techniques such as Edman degradation typically require ng quantities of purified peptide. All of these methods required pre-purification of the GnRH isoforms prior to chemical sequencing and mass spectrometric analysis, with initial isolation of the putative peptides from immunoreactive HPLC fractions. The LC-MS/MS method reported here required approximately 10 pg for detection of a known isoform and 300-500 pg for unknown isoforms via data-dependent experiments. Thus, not only is this LC-MS/MS method more efficient but it also allows for identification of GnRH isoforms from less extracted material than previously reported methods.

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CHAPTER 4: A NEW METHOD FOR QUANTITATIVE MEASUREMENT OF MAMMALIAN GnRH ISOFORMS IN RAT BRAIN TISSUE

4.1 Introduction

Mammalian GnRH (mGnRH) was the first LH- and FSH-releasing peptide to be isolated and characterized (1, 2). The mammalian isoform was thought to be unique until other homologous isoforms were discovered, and mGnRH has subsequently been found in a wide variety of mammalian organisms (human, monkey, rodent) as well as bony fish and amphibians (3). The mGnRH peptide is released from the hypothalamus and is transported to the pituitary, where gonadotropin receptors facilitate release of FSH and LH (4). Evidence for the presence of mGnRH has also been found in extra-hypothalamic tissues, including mammary gland, prostate, pancreatic, and placental tissue (5).

A modified form of mGnRH has been isolated and identified in human, sheep, rodent, frog and toad hypothalamus; this modified form is distinguished from mGnRH by a hydroxylated-proline in position 9 and is commonly referred to as hydroxyproline^{9} GnRH or Hyp^{9}GnRH (6-8). The modification to the proline residue is post-translational, achieved through the action of prolyl-hydroxylase enzymes; alteration at the proline residue confers greater resistance to enzymatic degradation compared to mGnRH (6). Relative distribution of Hyp^{9}GnRH as compared to mGnRH in brain tissue is higher in tissues from fetal and young rats than in those from adults (6, 7), and *in vitro* experiments have demonstrated that release of Hyp^{9}GnRH may differ from that of mGnRH during physiological development (9). Additionally, Hyp^{9}GnRH has been demonstrated to induce release of LH and FSH in-vitro and in-vivo, and is thought to have a role in regulation of these gonadotropins (10).

Accurate measurements of concentrations in tissue or other biological matrices are essential for further study of differential occurrence of Hyp^{9}GnRH and mGnRH not only in rats but in other mammalian species as well. Current methods rely on lengthy chromatographic procedures to assure adequate separation of potential GnRH isoforms prior to radioimmunoassay (RIA) analysis; these methods typically measure Hyp^{9}GnRH and mGnRH collected in fractions from the chromatographic assay, utilizing multiple antibodies with differing cross-reactivities to enhance selectivity (6, 7, 9). Sample throughput would be greatly enhanced if the multiple chromatographic separation steps and detection steps could be combined into one method, using a technique such as LC-MS/MS. An LC-MS/MS method would also enable simultaneous measurement of Hyp^{9}GnRH and mGnRH concentrations in one sample, since these isoforms would be chromatographically separated and detected based on their distinct molecular weights.

Development of an optimal LC-MS/MS method for accurate and precise quantitation of peptides relies on the use of a calibration curve and an appropriate internal standard. The internal standard should be a stable-isotope labeled analogue of the analyte or an analogue having a very similar chemical structure, and hence, similar chemical behavior to the analyte over the course of the experiment (11, 12). An additional challenge for assays of endogenous peptides is the identification of an appropriate substitute matrix for standard preparation; this was especially problematic for this project, in that all rat brains (irrespective of age or gender) were expected to have measurable levels of Hyp^{9}GnRH and mGnRH.

Assessment of accuracy and precision of an LC-MS/MS method as well as evaluation of critical parameters such as linearity of the calibration curve, selectivity of

the method, analyte and internal standard recovery, in-process stability, and storage stability are best accomplished through validation of the proposed method (13, 14). While validation of bioanalytical methods is carried out most rigorously in the fields of clinical chemistry and pharmaceutical analysis, where specific regulatory criteria must be met, the elements of validation described above are applicable to cases such as this research where no specific criteria were defined a priori.

We were able to develop and validate an LC-MS/MS method capable of accurate and precise measurement of Hyp^{9}GnRH and mGnRH in brain tissues collected from Sprague-Dawley rats. This is the first report of simultaneous quantitative measurements of Hyp^{9}GnRH and mGnRH in any biological matrix, and to our knowledge, the first report of a rigorously validated method supporting the measurement of endogenous GnRH in tissue samples.

Previously reported quantitative methods for Hyp^{9}GnRH and mGnRH in tissue samples require two to three chromatographic steps, with fractions collected from each chromatographic separation evaluated for immunoreactive-GnRH by RIA prior to application in the next chromatographic experiment (6, 7, 9). Our method eliminates the need for antibody-related detection of GnRH isoforms by utilizing mass spectrometric detection; not only does this allow for simultaneous detection of Hyp^{9}GnRH and mGnRH during a single chromatographic experiment based on their differing masses, but also it improves selectivity of the method regarding potential peptide fragments or post-translationally modified isoforms. Additionally, the method is self-correcting for recovery due to the use of an internal standard and calibration standards prepared in tissue samples, unlike RIA-based methods which require correction by a pre-determined

factor assumed to be constant from experiment to experiment. Experimental and long-term stability were demonstrated during validation, parameters which have not been previously reported for Hyp^{9}GnRH and mGnRH in tissue samples. Finally, the LC-MS/MS method greatly improves upon efficiency for Hyp^{9}GnRH and mGnRH quantitation by eliminating lengthy multi-day RIA incubation steps.

Potential applications of the new methodology include continued research regarding differential expression of Hyp^{9}GnRH relative to mGnRH as the distinct functions of these peptides are further investigated. In addition, the assay may have clinical relevance for monitoring the up- or down-regulation of Hyp^{9}GnRH and mGnRH release after dosing with GnRH agonist- or antagonist-compounds in animal models. Finally, this methodology can be adapted to measurement of other GnRH isoforms, requiring only that synthetic reference standard material be available and appropriate blank matrices can be identified.

4.2 EXPERIMENTAL

Chemicals and Reagents

Glacial acetic acid and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA). Water was either purchased from Burdick & Jackson (Muskegon, MI, USA) or supplied by an in-house Vantage reverse-osmosis system (US Filter, Colorado Springs, CO, USA). Bond Elut C18 solid-phase extraction cartridges (200 mg) were purchased from Varian (Palo Alto, CA, USA).

Ultramark 1621 and l-methionyl-arginyl-phenylalanyl-alanine (MRFA) were purchased from ThermoFinnigan (San Jose, CA, USA). Caffeine was purchased in

powder form from Sigma-Aldrich (St. Louis, MO, USA). mGnRH (native and free acid forms) and chicken II GnRH standards (cGnRH-II) were purchased from BAChem (Torrance, CA, USA). Hyp^{9}GnRH was purchased from California Peptide Research (Napa, CA, USA).

Sprague-Dawley rat brains and guinea pig brains were obtained frozen over dry ice from BioChemed Services (Winchester, VA, USA) and were stored at -70°C prior to analysis. LHRH ELISA kits were purchased from BAChem, Peninsula Labs division (San Carlos, CA).

Standard preparation

Stock standards of each GnRH peptide were prepared at 100 μg mL^{-1} in deoxygenated H_2O. Subsequent dilutions were made into 50/50 acetonitrile/ H_2O to minimize adsorption to tube and vial walls. Calibration standard spiking solutions were prepared at 4, 10, 20, 40, 100, 200 and 400 pg μL^{-1} such that 50 μL added to brain homogenate produced calibration standards ranging from 200 – 20000 pg total per tissue extracted. Two internal standard candidates were evaluated, mGnRH-free acid (mGnRH-fa) and chicken II GnRH (cGnRH-II); a stock solution containing 100 pg μL^{-1} mGnRH-fa and cGnRH-II was prepared such that 20 μL added to brain homogenates produced 2000 pg total per tissue extracted. Quality control samples were prepared by adding 30 μL of the 20 pg μL^{-1} calibration spiking solution into homogenate to produce the LOW QC at 600 pg, by adding 20 μL of the 100 pg μL^{-1} calibration spiking solution into homogenate to produce the MID QC at 2000 pg and by adding 37.5 μL of the 400 pg μL^{-1} calibration spiking solution into homogenate to produce the HIGH QC at 15000 pg.

Extraction procedure

Rat and guinea pig brains were homogenized in polypropylene test tubes with 3 mL acetonitrile using a Tissue-Tearor tissue homogenizer (Biospec Products, Bartlesville, OK, USA), with an additional 2 mL acetonitrile used to rinse the homogenizer probe and combined with the homogenate. After addition of calibration and internal standard spiking solutions to the samples, homogenates were vortexed and then centrifuged at 2500 g, 4ºC for 30 minutes. The supernatant was evaporated under nitrogen at 65ºC and reconstituted in 3 mL of the loading solution, which consisted of water:acetic acid:TFA (96:3:1, volume/volume).

Solid phase extraction (SPE) was used to isolate GnRH from the brain homogenate samples. The SPE cartridges were preconditioned with 3 mL methanol followed by 3 mL of the loading solution. The reconstituted supernatant was transferred to the cartridges, which were subsequently washed with 3 mL loading solution followed by 3 mL loading solution:methanol (70:30, volume/volume). Samples were eluted with 3 mL 3% acetic acid:methanol (30:70, volume/volume), which was collected and evaporated under nitrogen at 70ºC. Samples were reconstituted in 100 µL 3% acetic acid:methanol (95:5, volume/volume), vortexed for 30 seconds and transferred to glass autosampler vials for analysis.

HPLC-MS/MS

HPLC separations were performed using a Hewlett-Packard 1100 series binary pump and autosampler with vacuum degasser (Palo Alto, CA, USA) connected to a Phenomenex Luna C8(2) 50 x 1.0 mm column, 5µ particle size (Torrance, CA, USA). Mobile phase A consisted of 0.5% (volume/volume) acetic acid in water and mobile

phase B consisted of 0.5% (vol/vol) acetic acid in acetonitrile. A gradient program increasing from 5% B to 20% B over 15 minutes was used to achieve separation of the GnRH peptides. The volume of sample extract injected was 50 μL, and column effluent, at 100 μL min^{-1}, was directed into the electrospray source of the mass spectrometer without splitting.

All experiments were performed using a Finnigan LCQ Deca quadrupole ion trap mass spectrometer (San Jose, CA, USA) operated in positive electrospray mode. The electrospray source was fitted with a stainless steel needle. Calibration of the mass spectrometer was performed using a mixture of caffeine, MRFA, and Ultramark 1621 as recommended by the manufacturer (15).

Optimization of source conditions was performed while infusing sample from a syringe pump into mobile phase flow at 100 μL min^{-1}. The following source settings were selected as optimal: sheath flow (Nitrogen) - 100 units, auxiliary flow - off, source voltage – 5.0 kV, capillary temperature – 275°C, and capillary voltage – 7.0 V. Automatic gain control (AGC) was set to on, with a full AGC target of 5000000.

The MS-MS acquisition was divided into three segments, with data acquired over the course of the chromatographic run. The scan events in each segment, as well as mass spectrometry settings for the scan events, are shown in Table 4.1. The default isolation width was set to 3.0 and the activation Q was set to 0.250 with an activation time of 30 msec for all peptides.

Table 4.1. Scan events and settings for MS-MS quantitation

GnRH Peptide	Precursor Ion (m/z)	Event Time (min)	Collision Energy (%)	Fragment Mass Range Scanned (m/z)
Hyp^{9}GnRH	1198.5	8.00-10.50	35	325-1200
mGnRH	1182.5	10.50-12.25	35	325-1200
mGnRH-fa	1183.5	12.25-15.00	35	325-1200
cGnRH-II	1236.5	12.25-15.00	30	340-1250

Validation Experiments

Validation of method performance was conducted to verify that the extraction and instrumental methods provided accurate and reproducible results over multiple instances of use. Key parameters of interest for the intended application in this study were accuracy and precision, extraction recovery, stability during sample processing, and long-term storage stability.

Calibration standards and quality control (QC) samples were prepared with each validation run. Because the GnRH peptides of interest are endogenous in rat brain tissue, guinea pig brains were evaluated and found to be a suitable blank matrix. To provide a tissue matrix of comparable mass to the rat brain samples, guinea pig brains were halved laterally and then spiked with the appropriate calibration and internal standard solutions for calibration and QC sample preparation.

Each validation batch included seven calibration standards containing Hyp^{9}GnRH and mGnRH ranging from 20 to 20000 pg/tissue; calibration standards were prepared in singlicate and were distributed throughout the analytical run sequence on the instrument. QC samples were prepared in duplicate at three levels for each run, excepting one validation run in which five replicates at each level were prepared and analyzed to assess accuracy and precision. A blank matrix sample, prepared from guinea pig brain, was also included with each validation batch to monitor possible cross-contamination and instrument carryover. Reconstructed ion chromatograms were prepared for Hyp^{9}GnRH, mGnRH, mGnRH-fa and cGnRH-II from which peak areas were integrated; ratios of peak area analyte to peak area internal standard were used to prepare calibration curves. After determining a suitable calibration model, least-squares linear-regression analysis with $1/x^2$ weighting was applied to each validation run, from which back-calculated concentrations for the calibration standards, QCs and samples were derived. All regressions were performed using XCalibur version 1.2 (Thermo, San Jose, CA, USA).

Extraction recovery was assessed by comparison of samples prepared from rat brains spiked with 20 ng Hyp^{9}GnRH and mGnRH and 2 ng mGnRH-fa to samples prepared from rat brain extracts spiked with the same amounts post-extraction and cleanup. The recovery experiments were designed to compare pre- and post-extraction spiked samples such that any influence of sample matrix components on ionization within the electrospray source of the mass spectrometer would be normalized (16, 17).

To assess in-process stability, rat brains subjected to room temperature for 2 hours prior to processing and rat brains subjected to 3 freeze-thaw cycles prior to processing were compared to rat brains thawed and processed immediately. Stability of Hyp^{9}GnRH

and mGnRH after long-term freezer storage was explored by comparing brains stored for 21 days prior to extraction with brains stored for 112 days prior to extraction. Stability of extracted samples stored at room temperature was assessed by reinjecting a set of calibration standards and quality control samples 27 hours after the initial analysis start time. Injection volume was changed to 40 µL for the second injection, as insufficient volume remained to allow for a 50 µL injection.

Comparison to ELISA

A comparison of mGnRH values determined via the new LC-MS/MS method to values determined using a commercially available mGnRH immunoassay was performed. To create equivalent samples for analysis, five male and five female rat brains were homogenized with the resulting supernatant divided equally. The aliquot reserved for LC-MS/MS analysis was spiked with internal standard solution (mGnRH-fa). No internal standard was added to the aliquot reserved for the immunoassay to eliminate any response due to potential cross-reactivity with mGnRH-fa. All samples were extracted by the solid phase extraction method described above, with final reconstitution in assay buffer for the ELISA procedure or in 95:5 3% acetic acid:methanol for the LC-MS/MS method. Attempts to assess control samples prepared from guinea pig brains by the ELISA method were hampered by suspected cross-reactivity of guinea pig GnRH with the anti-mGnRH antibody.

The 96-well plates provided with the ELISA kit were utilized per kit instructions for competitive enzyme immunoassay and subsequently transferred to a Spectramax250 plate reader (Molecular Devices, Sunnyvale, CA, USA) for determination of absorbance at 450 nm. Data from the Spectramax was transferred to SigmaPlot 10.0, where sigmoidal

(3-parameter) regression analysis was performed with results corrected for recovery losses during extraction. Data provided by the manufacturer specified that the antibody had crossreactivity of 0.1% or less with lamprey III GnRH, salmon GnRH, and several synthetic analogues of mGnRH; no data was provided for cross-reactivity with Hyp^9GnRH, mGnRH-fa or gpGnRH (18).

Quantitative Analysis of Hyp^9GnRH and mGNRH in Rat Brain

Concentrations of Hyp^9GnRH and mGnRH in 16 male and 15 female rat brains (Sprague-Dawley) were determined using the validated method and are reported below along with a comparison to previous reports in the literature. To our knowledge, this is the first method reported to provide simultaneous quantification of multiple GnRH isoforms.

4.3 RESULTS AND DISCUSSION

Selection of blank matrix

Selection of an appropriate matrix for preparation of calibration standards and quality control samples for exogenous compounds is straightforward in that blank tissue or fluid can be obtained, which is then spiked with known amounts of the analyte. Thus, for accurate quantification of endogenous GnRH isoforms, a different strategy was required. Following unsuccessful attempts to reduce GnRH concentrations to undetectable levels via degradation at extended room temperature storage or gross removal of hypothalamic tissues, it became clear that a matrix other than rat brain tissue would be needed for preparation of calibration standards and quality control samples.

While standards of known concentration can be prepared and extracted from neat solutions, there is no compensation for the influence of matrix on recovery or instrument

signal. Standard addition methods can achieve accurate quantitative results with endogenous compounds. However, because standards are prepared from aliquots of the actual sample, standard addition requires a generous amount of material for each sample tested. Recently, "surrogate analytes" have been proposed as a strategy for accurate quantitation of endogenous compounds (19); this technique utilizes a surrogate (usually an isotopically-labeled compound) that closely mimics the behavior of the endogenous analyte for calibration standards.

Given that the sample material for this project was limited to single brain tissues and that isotopically-labeled forms of Hyp^{9}GnRH and mGnRH were not readily available, we chose to investigate whether guinea pig might provide a suitable brain tissue matrix for preparation of Hyp^{9}GnRH and mGnRH standards. There is some discrepancy in the literature regarding whether mGnRH is expressed in guinea pig; while the initial work proposing the structure of gpGnRH failed to isolate mRNA specific to mGnRH, a later study suggested coexpression of gpGnRH with mGnRH (20, 21). In this context, it seemed prudent to establish the presence or absence of a detectable amount of mGnRH in guinea pig brain before proceeding with use of this tissue for preparation of calibration standards and quality control samples.

To determine whether mGnRH was detectable, a single intact guinea pig brain was extracted and subjected to a targeted product ion analysis experiment by LC-MS/MS as described for single rat brains in Chapter 3. Neither Hyp^{9}GnRH nor mGnRH was detected. However, a strong signal for gpGnRH was observed with a fragmentation pattern suggestive of the sequence predicted for gpGnRH based on mRNA (20),

indicating that extraction of GnRH from the brain was successful and mGnRH, if present, was undetectable.

Assay range and calibration model

During initial method development, a reasonable lower limit of quantitation (LLOQ) for extracted samples was determined to be 200 pg Hyp^{9}GnRH and mGnRH. The upper limit of quantitation (ULOQ) for the calibration curve was set at 20000 pg Hyp^{9}GnRH and mGnRH. Chromatograms for unspiked (blank) guinea pig brain and for guinea pig brains spiked with 200 and 20000 pg Hyp^{9}GnRH and mGnRH are shown in Figure 4.1. The signal to noise (S/N) ratios at the LLOQ concentration were 15 and 21, respectively, for Hyp^{9}GnRH and mGnRH. The effect of fragment ion selection for quantitation on S/N ratios is discussed further in below sections.

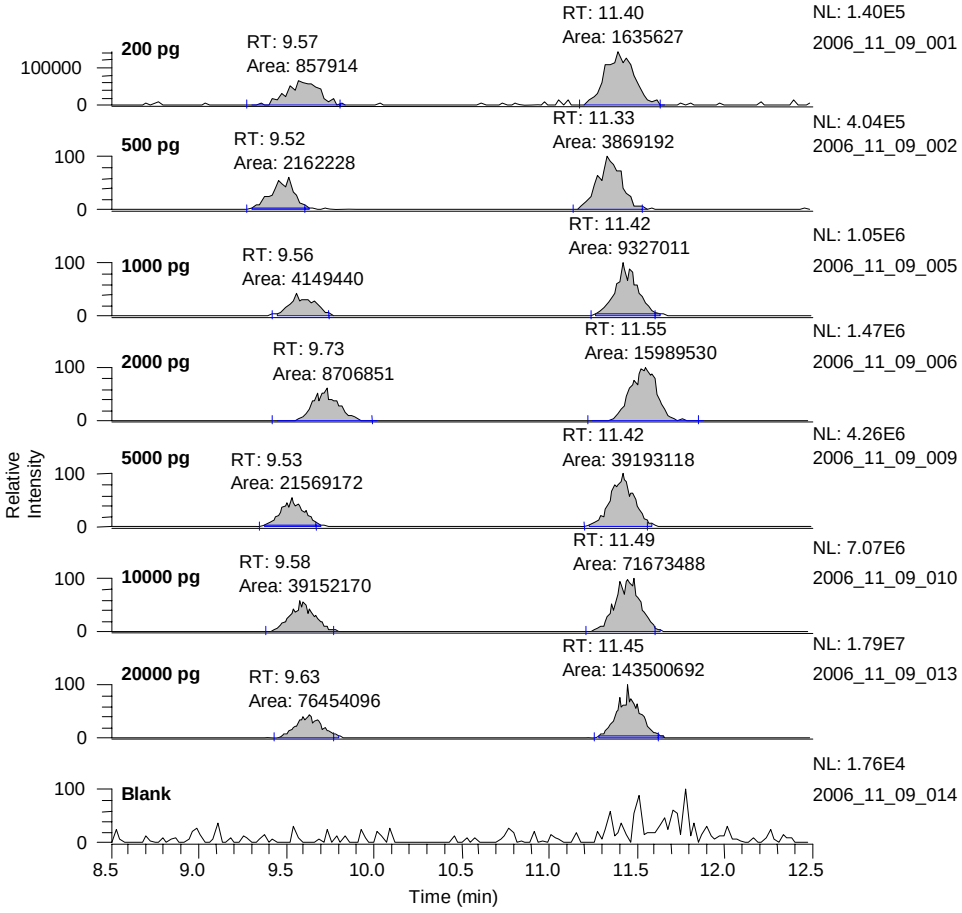
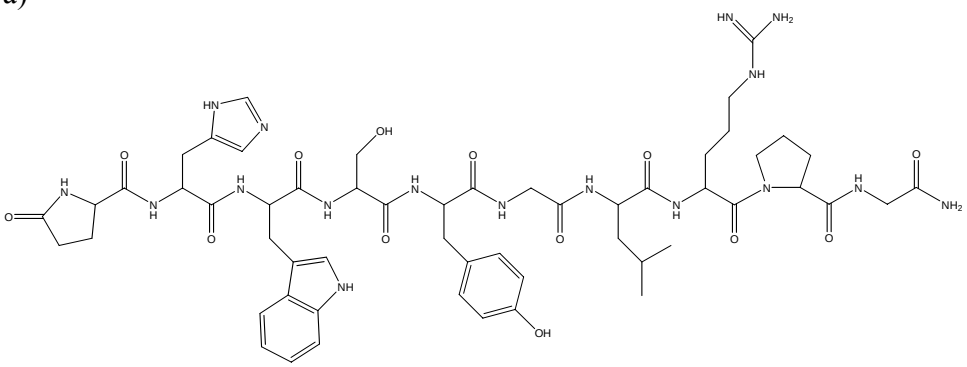


Figure 4.1. Reconstructed ion chromatograms of *m/z* 1198.5 (precursor ion for Hyp^{9}GnRH) and *m/z* 1182.5 (precursor ion for mGnRH) for extracted calibration standards at 200, 500, 1000, 2000, 5000, 10000, and 20000 pg/tissue and an extracted blank sample.

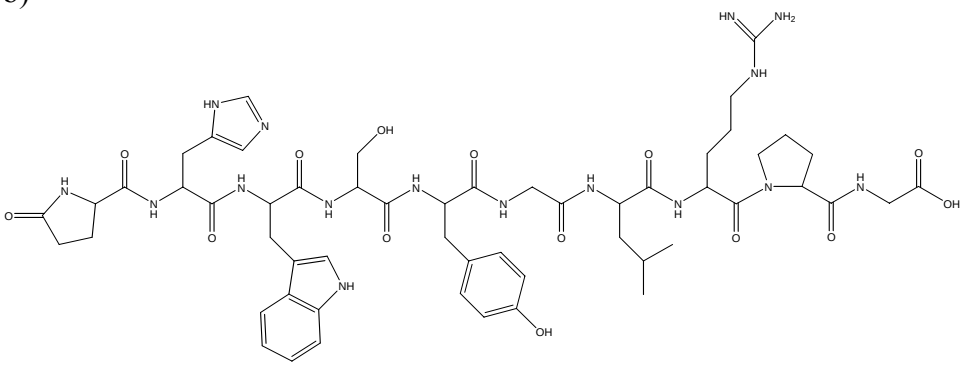
Two internal standards were chosen for evaluation during method validation, mGnRH in free acid form (mGNRH-fa) and chicken II GnRH (cGnRH-II). mGnRH-fa was chosen for structural similarity and similar fragmentation behavior as the analytes. cGnRH-II was selected because it eluted most closely to mGnRH under the HPLC conditions employed as compared to other GnRH isoforms available in our laboratory (including chicken I GnRH, lamprey III GnRH, salmon GnRH, and seabream GnRH). The structures of mGnRH, mGNRH-fa, Hyp^{9}GnRH and cGnRH-II are shown in Figure 4.2 and ESI-MS/MS spectra for each isoform are shown in Figure 4.3. mGnRH-fa was ultimately selected as the more appropriate internal standard for both Hyp^{9}GnRH and mGnRH, as accuracies were improved over those obtained with cGnRH-II as the internal standard. This result may be because the MS/MS fragmentation pattern of mGnRH-fa was more similar to that of Hyp^{9}GnRH and mGnRH than that of cGnRH-II, making mGNRH-fa a better analogue internal standard.

A characteristic of quadrupole ion trap mass spectrometers is that product ion scans over a wide mass range can be detected in a shorter scan time than would be possible with triple quadrupole mass spectrometers. While triple quadrupole mass spectrometers are commonly used and perform well for quantitative applications, analysis is typically by selected reaction monitoring (SRM) whereby a single fragment mass is monitored. The capability of the ion trap to provide MS/MS scan data in a time frame compatible with chromatographic elution is a distinct advantage for analyses involving compounds such as peptides which undergo fragmentation into many ions because the signal from multiple fragment ions can be summed to produce a more abundant signal than could be achieved from a single fragment ion (12, 22).

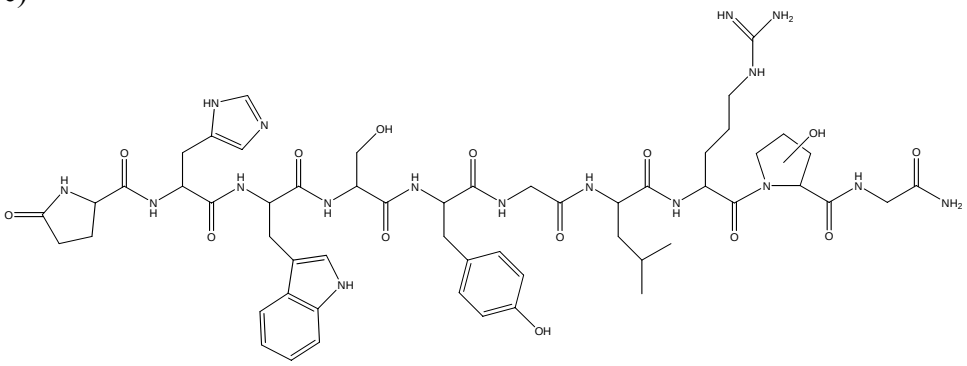
a)



b)



c)



d)

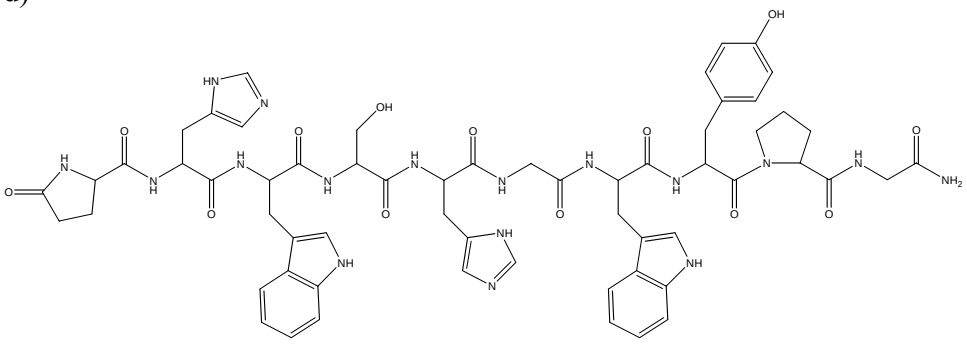


Figure 4.2. Chemical structures of a) mGnRH, b) mGnRH-fa, c) Hyp^{9}GnRH and d) cGnRH-II.

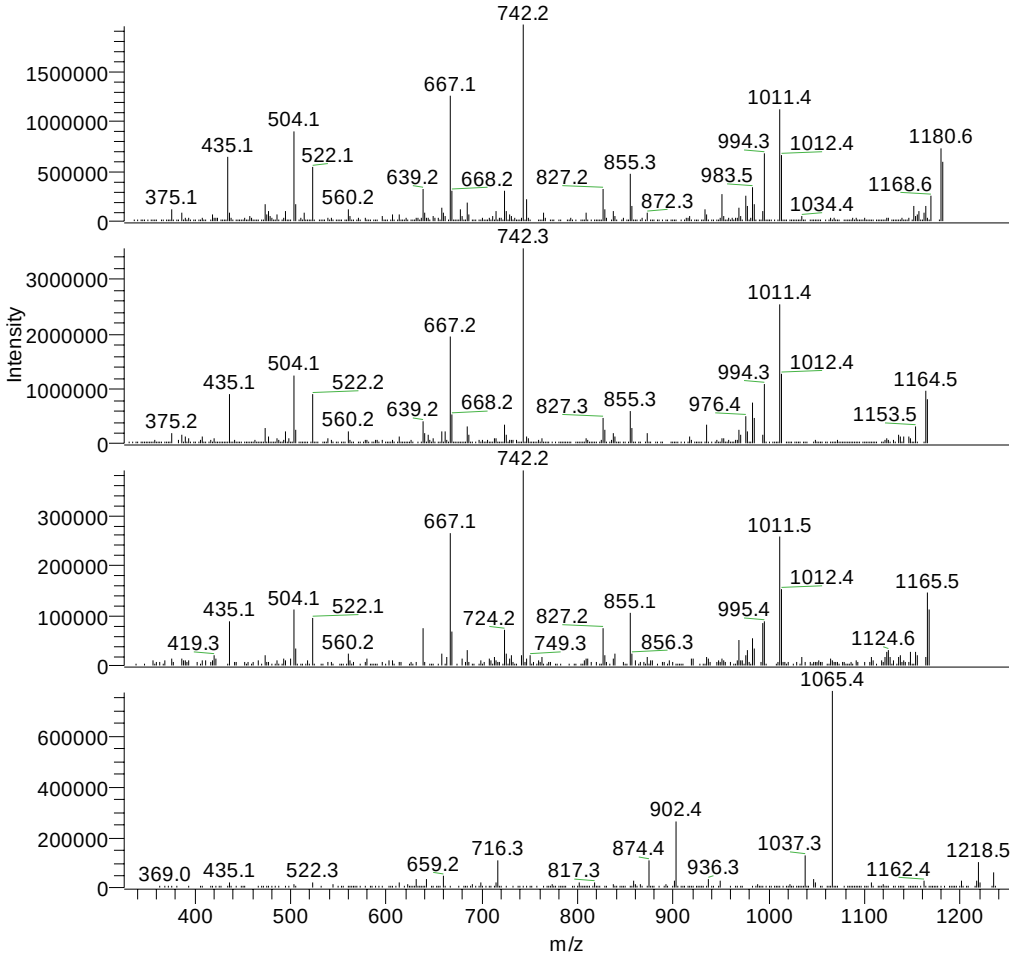


Figure 4.3. Product ion spectra of a) Hyp^{9}GnRH, *m/z* 1198.5, b) mGnRH, *m/z* 1182.5, c) mGnRH-fa, *m/z* 1183.5, and d) cGnRH-II, *m/z* 1236.5.

To determine an optimal quantification strategy given the multiple fragment ions produced, results from the first validation run were evaluated after quantification based on the single most abundant fragment ion, the sum of the two most abundant fragment ions, and so forth up to a total of five fragment ions for each analyte and internal standard compound. The transitions evaluated are shown in Table 4.2. Due to the homologous amino acid sequences of the GnRH peptides, four of the five ions evaluated in this exercise were common fragments to Hyp^{9}GnRH, mGnRH and mGNRH-fa. The fifth ion was the consequence of dehydration of the precursor ion, and the *m/z* associated with this fragment ion varied accordingly.

Table 4.2. Mass transitions selected for quantitative comparison

Fragment Ions Listed in Order of Descending Abundance (fragment ion type)	**GnRH Peptide (precursor *m/z*)**		
	Hyp^{9}GnRH (1198.5)	**mGnRH (1182.5)**	**mGnRH-fa (1183.5)**
1 (b_6^+)	742.2	742.2	742.2
2 (b_8^+)	1011.4	1011.4	1011.4
3 (b_5^+-H_2O)	667.2	667.2	667.2
4 (b_4^+-H_2O)	504.1	504.1	504.1
5 (MH^+-H_2O)	1180.6	1164.6	1165.5

To evaluate results obtained from the quantitative comparison, relative error (residuals) for each calibration standard and QC in the first validation experiment as well as the mean of the absolute relative error for each iteration of the run are shown in Table 4.3 for Hyp^{9}GnRH and Table 4.4 for mGnRH. A linear regression with $1/x^2$-weighting was used for this comparison. Results can be seen to improve as additional ions are added until five ions are included in the summed signal, when a slight increase in the mean residual is observed.

Table 4.3. Relative Errors for Hyp^{9}GnRH, Validation Run #1

	% Relative Error (# Ions included in quantitative analysis)				
Stds & QCs	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**
C1 (200 pg)	-4.0	-0.1	0.7	0.1	0.6
C2 (500 pg)	8.3	-1.9	-3.4	-2.8	-2.5
C3 (1000 pg)	9.3	6.8	5.4	6.4	3.1
C4 (2000 pg)	-14.7	-7.9	-8.2	-5.6	-6.5
C5 (5000 pg)	8.2	7.9	12.2	7.9	11.6
C6 (10000 pg)	6.4	1.9	2.3	-0.1	0.8
C7 (20000 pg)	-13.5	-6.7	-8.9	-6.0	-7.2
LOW QC (600 pg)	-3.4	-0.1	2.8	5.5	7.5
LOW QC (600 pg)	-14.1	2.2	6.3	5.8	6.6
MID QC (2000 pg)	21.9	24.7	21.3	12.1	13.0
MID QC (2000 pg)	6.5	5.7	-0.8	1.2	-0.3
HIGH QC (15000 pg)	-4.1	2.2	0.8	-3.8	-4.1
HIGH QC (15000 pg)	-6.2	-2.4	1.3	-1.4	1.2
Mean |relative error|	9.3	5.4	5.7	4.5	5.0

Table 4.4. Relative Errors for mGnRH, Validation Run #1

	% Relative Error (# Ions included in quantitative analysis)				
Stds & QCs	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**
C1 (200 pg)	0.6	0.9	-0.6	0.3	0.8
C2 (500 pg)	-10.2	-12.0	-7.7	-10.8	-11.2
C3 (1000 pg)	23.8	23.6	22.9	23.2	22.4
C4 (2000 pg)	-13.7	-8.0	-10.1	-7.3	-10.4
C5 (5000 pg)	1.3	-0.3	5.2	3.9	7.8
C6 (10000 pg)	4.8	0.1	-0.3	-2.4	-2.8
C7 (20000 pg)	-6.6	-4.3	-9.5	-7.1	-6.7
LOW QC (600 pg)	-8.9	-1.1	-1.4	1.9	5.9
LOW QC (600 pg)	-25.2	-10.2	-9.4	-8.6	-11.8
MID QC (2000 pg)	19.3	21.3	19.2	9.4	8.6
MID QC (2000 pg)	13.2	8.1	-1.6	1.3	-1.6
HIGH QC (15000 pg)	-10.0	-2.0	-2.7	-3.9	-1.0
HIGH QC (15000 pg)	-6.2	-6.7	-4.2	-7.5	-5.0
Mean |relative error|	11.1	7.6	7.3	6.7	7.4

Reconstructed ion chromatograms of the 200 pg calibration standard for each iteration of this exercise are shown in Figure 4.4 for Hyp^{9}GnRH and Figure 4.5 for mGnRH. While signal intensity increases with the addition of each successive ion, a potential interference peak immediately prior to mGnRH is observed when five ions are included in the reconstructed ion chromatogram. Additionally, the signal to noise ratio is

decreased to less than 10 upon addition of the MH^+-H_2O fragment ion in the reconstructed ion chromatogram. Given that the smallest mean relative error was obtained from the summed signal of four ions, that a potential interference to mGnRH was observed upon the inclusion of a fifth ion, and that signal to noise ratios were lessened by addition of the fifth ion, quantification for both Hyp^9GnRH and mGnRH was based on the four most abundant ions in Table 4.2 and the MH^+-H_2O ion fragment was dropped from the quantitative analysis program.

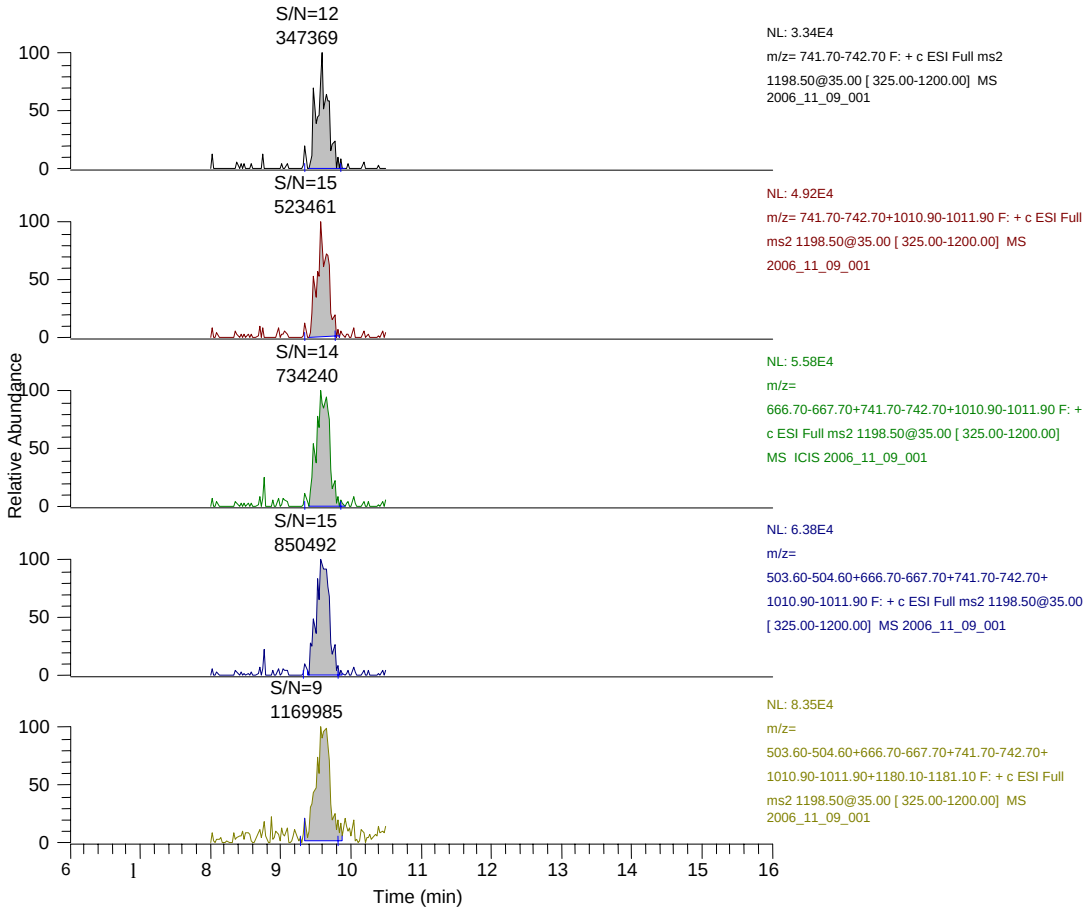


Figure 4.4. Reconstructed ion chromatograms of *m/z* 1198.5 (precursor ion for Hyp^{9}GnRH) for an extracted 200 pg calibration standard, shown in increasing order of ions included in reconstruction. S/N and integrated areas are shown above each peak.

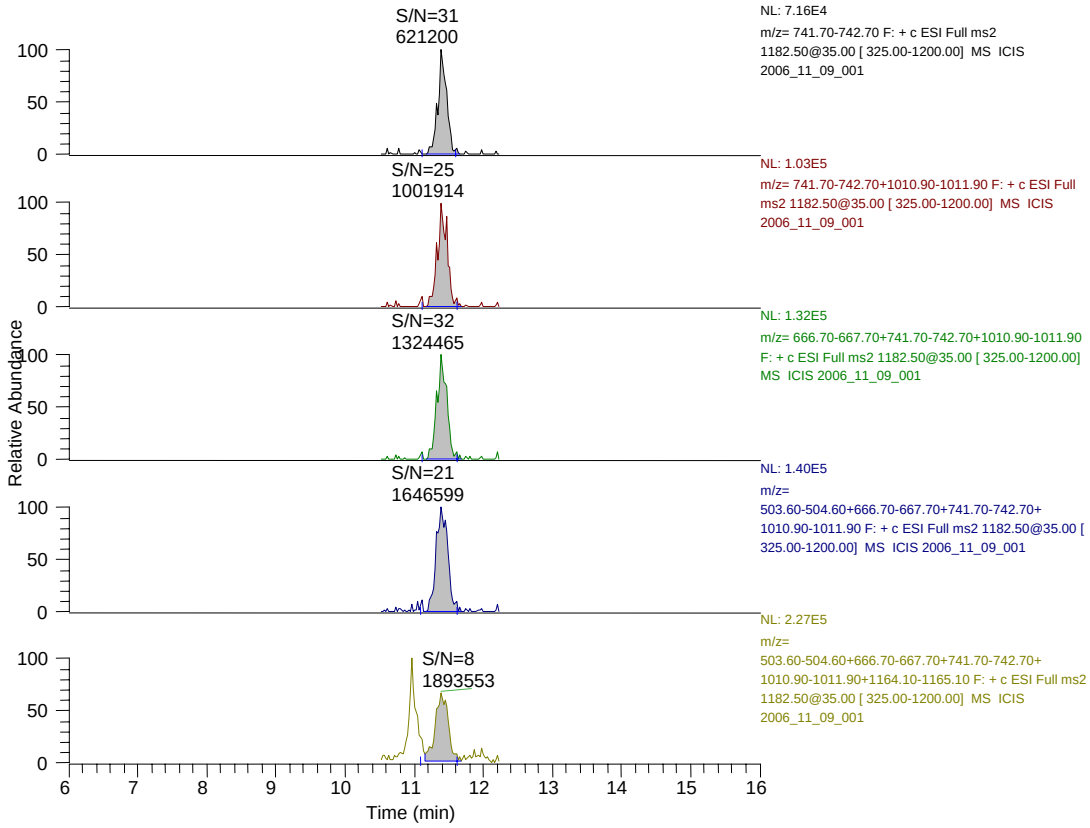


Figure 4.5. Reconstructed ion chromatograms of *m/z* 1182.5 (precursor ion for mGnRH) for an extracted 200 pg calibration standard, shown in increasing order of ions included in reconstruction. S/N and integrated areas are shown above each peak.

Selection of a suitable regression model was performed by evaluating data from four analytical batches, each containing calibration standards prepared at seven concentration levels between 200-20000 pg/tissue and QC samples prepared at three concentration levels within the calibration range. Relative errors were compared for the following regression models: linear with no weighting, linear with 1/x weighting and linear with $1/x^2$ weighting. Plots of relative errors by concentration (calibration standards and QC samples) are shown for Hyp^9GnRH in Figure 4.4 and mGnRH in Figure 4.6, and the mean absolute relative error for each validation run is shown in Table 4.7 for Hyp^9GnRH and mGnRH. Coefficients of determination (R^2) for both analytes calculated using the different weighting schemes are shown in Table 4.6. The application of weighting greatly improved the fit at lower concentrations and reduced the mean relative error. For both Hyp^9GnRH and mGnRH, R^2 decreased in most instances as 1/x and $1/x^2$ weighting were applied; however, R^2 was greater than 0.98 for all runs. Therefore, a linear calibration model with $1/x^2$ weighting was chosen.

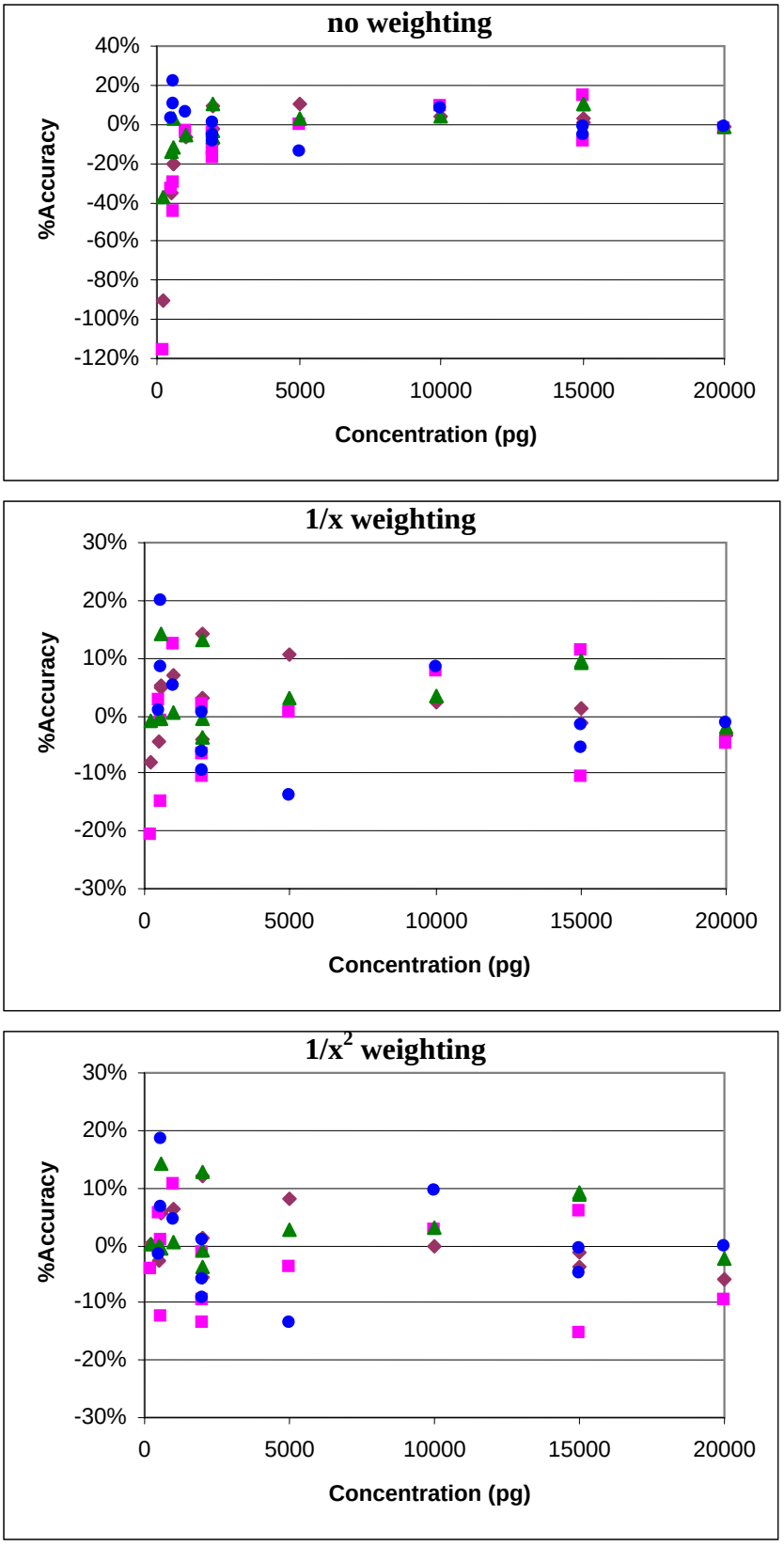


Figure 4.6. Relative error plots from regression models evaluated for Hyp^{9}GnRH

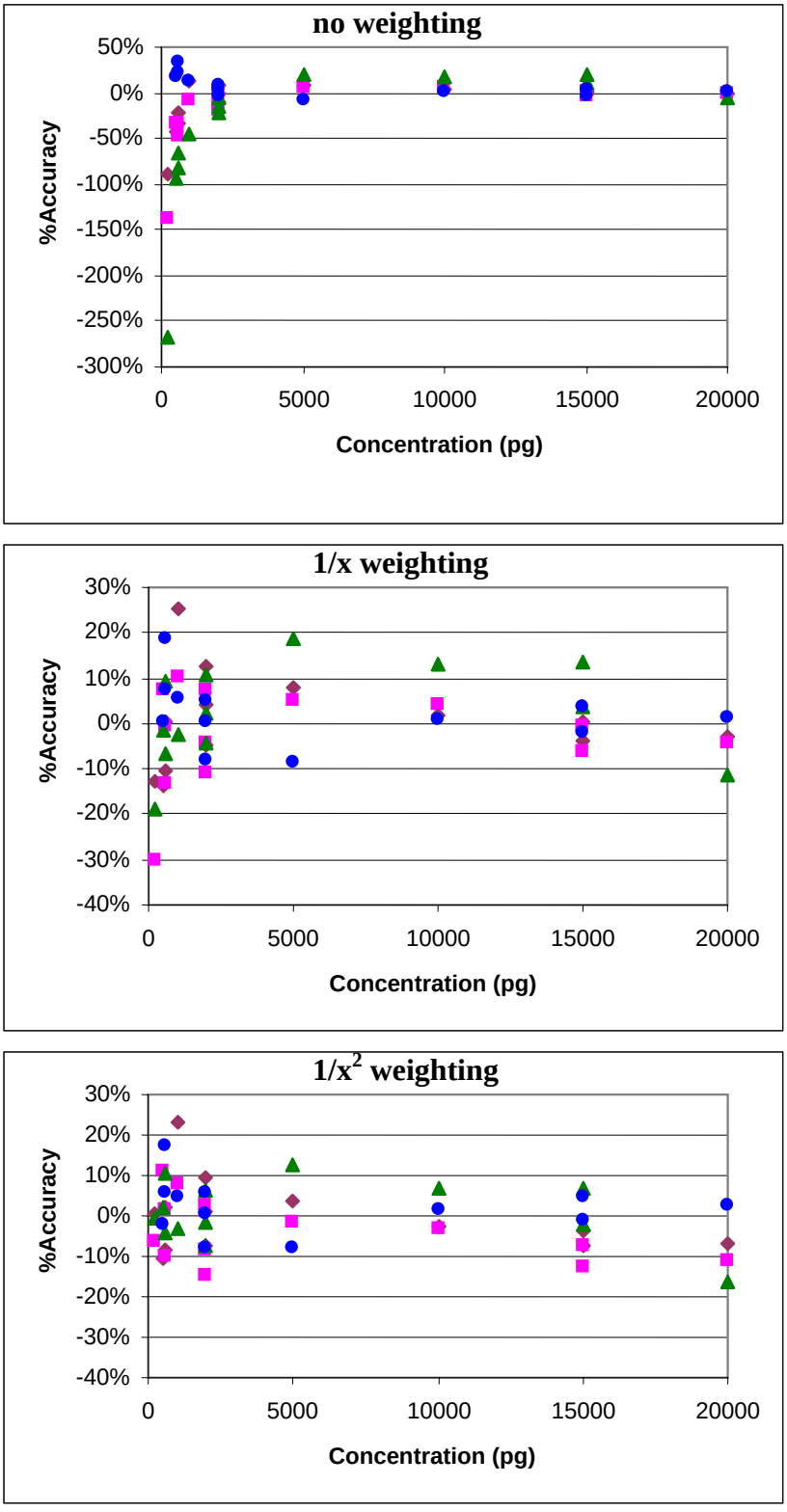


Figure 4.7. Relative error plots from regression models evaluated for mGnRH

Table 4.5. Mean |% Relative Error|

	Mean |%RE| Hyp^{9}GnRH			Mean |%RE| mGnRH		
Run#	**none**	**1/x**	**1/x^2**	**none**	**1/x**	**1/x^2**
1	16.4	5.34	4.53	18.2	7.75	6.73
2	22.7	8.16	7.28	23.7	8.01	7.51
3	9.27	4.67	4.54	51.3	8.96	6.23
4	7.39	6.79	6.34	9.86	5.23	5.19
Mean	13.9	6.24	5.67	25.8	7.49	6.42

Table 4.6. Coefficients of Determination (R^2)

	Hyp^{9}GnRH			mGnRH		
Run#	**none**	**1/x**	**1/x^2**	**none**	**1/x**	**1/x^2**
1	0.9981	0.9971	0.9960	0.9987	0.9960	0.9826
2	0.9960	0.9960	0.9938	0.9979	0.9966	0.9918
3	0.9992	0.9992	0.9991	0.9802	0.9820	0.9891
4	0.9955	0.9940	0.9909	0.9993	0.9985	0.9969
Mean	0.9972	0.9966	0.9950	0.9940	0.9933	0.9901

Linearity of calibration curve

The calibration curve was constructed to measure 200-20000 pg/tissue Hyp^{9}GnRH and mGnRH. None of the seven calibration standards were excluded from any of the regression analyses, which were performed using a $1/x^2$-weighted linear regression. In validation run 4, a loss of instrument sensitivity prevented detection of Hyp^{9}GnRH and mGnRH in the 200 pg/tissue calibration standard; this problem was rectified by cleaning of the instrument and has not recurred. Slope, intercept and R^2 values for each regression analysis during validation are presented in Table 4.7. Calibration curves constructed from the largest batch, validation run 4, for Hyp^{9}GnRH and mGnRH are presented in Figures 4.8 and 4.9, respectively.

Table 4.7. Calibration Curve Parameters

	Hyp^{9}GnRH			mGnRH		
Run#	**Slope**	**Intercept**	**R^2**	**Slope**	**Intercept**	**R^2**
1	0.000328	0.000305	0.9960	0.000605	0.00894	0.9826
2	0.000365	0.00613	0.9938	0.000671	0.00437	0.9918
3	0.000325	0.00388	0.9991	0.000576	0.0134	0.9891
4	0.000353	-0.00167	0.9909	0.000669	0.00211	0.9969
Mean (%RSD)	0.000343 (5.67)	0.00216 (162)	0.9950 (0.348)	0.000630 (7.52)	0.00721 (69.6)	0.9901 (0.601)

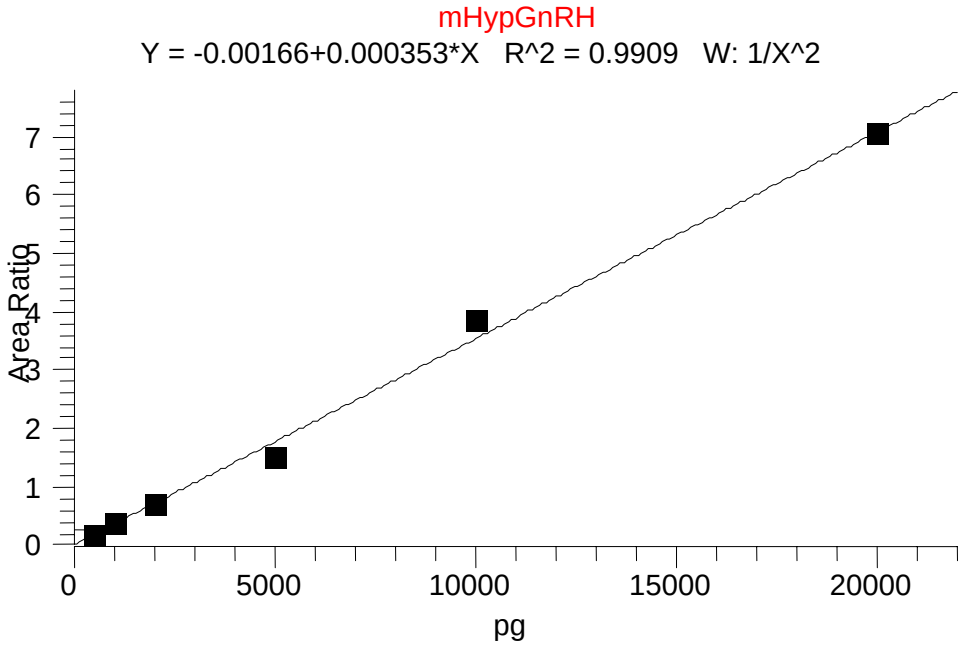


Figure 4.8. Calibration curve for Hyp^{9}GnRH, validation run 4

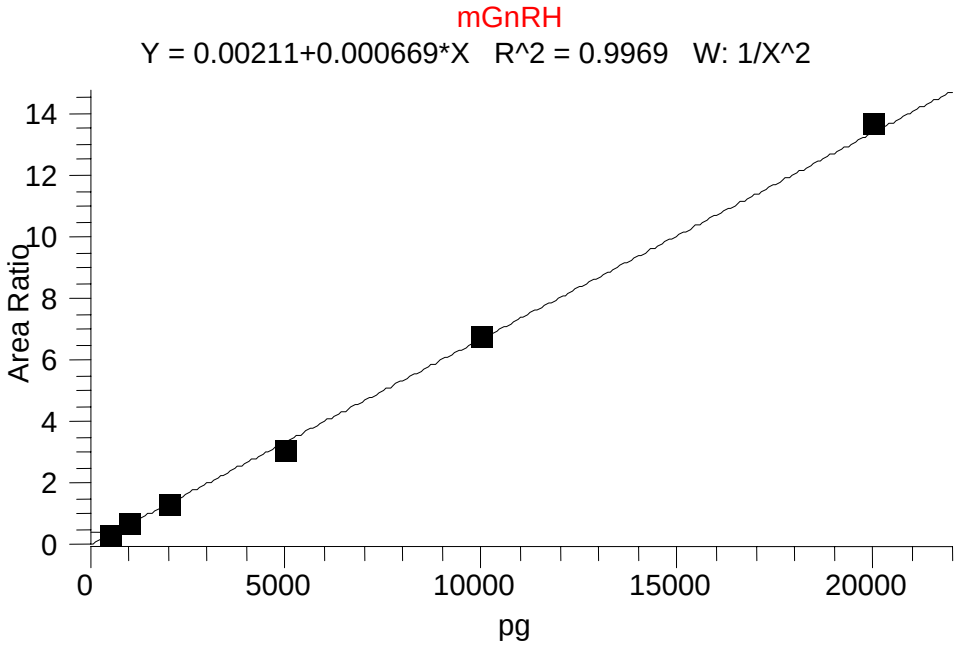


Figure 4.9. Calibration curve for mGnRH, validation run 4

Back-calculated concentrations for Hyp^{9}GnRH and mGnRH in calibration standards are presented in Tables 4.8 and 4.9, respectively. Inter-run accuracy ranged from 95.5 to 106% for Hyp^{9}GnRH and 92.1 to 108% for mGnRH. Inter-run precision, reported as relative standard deviation (%RSD), ranged from 2.34 to 9.40% for Hyp^{9}GnRH and 3.57 to 10.2% for mGnRH. These data further suggest that linear-regression with $1/x^2$-weighting provided a suitable model for fitting the data obtained from these experiments.

Table 4.8. Back-calculated values for Hyp^{9}GnRH calibration standards

	Nominal Concentration (pg/tissue)						
Run#	**200**	**500**	**1000**	**2000**	**5000**	**10000**	**20000**
1	200	486	1060	1890	5400	9990	18800
2	192	527	1110	1970	4800	10300	18100
3	200	499	1010	1920	5140	10300	19500
4	*	492	1050	2020	4330	11000	20000
Mean	197	501	1060	1950	4920	10400	19100
%Accuracy	98.7	100	106	97.5	98.4	104	95.5
%RSD	2.34	3.62	3.89	2.93	9.40	4.11	4.34

*not detected

Table 4.9. Back-calculated values for mGnRH calibration standards

	Nominal Concentration (pg/tissue)						
Run#	**200**	**500**	**1000**	**2000**	**5000**	**10000**	**20000**
1	201	446	1230	1850	5200	9760	18600
2	188	555	1080	2050	4930	9710	17800
3	199	509	966	1970	5630	10700	16800
4	*	490	1050	2010	4610	10200	20500
Mean	196	500	1080	1970	5090	10100	18400
%Accuracy	98.0	100	108	98.5	102	101	92.1
%RSD	3.57	9.03	10.2	4.39	8.48	4.57	8.51

*not detected

Accuracy and precision

Accuracy and precision of the method were assessed by including quality control samples at 600 pg/tissue (LOW QC), 2000 pg/tissue (MID QC) and 15000 pg/tissue (HIGH QC) in each run. Validation runs 1-3 included duplicate QC samples at each level for assessment of inter-run accuracy and precision, while validation run 4 included five replicates at each level for assessment of intra-run accuracy and precision. QC results for Hyp^{9}GnRH and mGnRH are presented in Tables 4.10 and 4.11, respectively. Intra-run accuracy ranged from 94.0 – 110% for Hyp^{9}GnRH and 98.4 – 111% for mGnRH, with intra-run precision ranging from 3.24 – 8.84% for Hyp^{9}GnRH and 4.43 – 8.16% for mGnRH. Inter-run accuracy ranged from 100 - 102% for Hyp^{9}GnRH and 95.6 – 98.7% for mGnRH, with inter-run precision (%RSD) ranging from 8.68 – 10.8% for Hyp^{9}GnRH and 6.85 – 9.65% for mGnRH.

Table 4.10. Back-calculated values for Hyp^{9}GnRH quality control samples

LOW QC (600 pg/tissue)			
Run 1	**Run 2**	**Run 3**	**Run 4**
633	525	597	640
635	604	685	711
			568
			703
			677

Inter-day (n=6)		Intra-day (n=5)	
Mean	613	Mean	660
%ACC	102	%ACC	110
%RSD	8.68	%RSD	8.84

MID QC (2000 pg/tissue)			
Run 1	**Run 2**	**Run 3**	**Run 4**
2240	1810	1990	1880
2020	1730	2260	1810
			1810
			1990
			1910

Inter-day (n=6)		Intra-day (n=5)	
Mean	2010	Mean	1880
%ACC	100	%ACC	94.0
%RSD	10.8	%RSD	4.02

HIGH QC (15000 pg/tissue)			
Run 1	**Run 2**	**Run 3**	**Run 4**
14400	12700	16300	14900
14800	15900	16400	14300
			13600
			14300
			14200

Inter-day (n=6)		Intra-day (n=5)	
Mean	15100	Mean	14300
%ACC	101	%ACC	95.1
%RSD	9.43	%RSD	3.24

Table 4.11. Back-calculated values for mGnRH quality control samples

LOW QC (600 pg/tissue)			
Run 1	**Run 2**	**Run 3**	**Run 4**
611	541	575	636
549	611	665	704
			614
			679
			696

Inter-day (n=6)		Intra-day (n=5)	
Mean	592	Mean	666
%ACC	98.7	%ACC	111
%RSD	7.85	%RSD	5.87

MID QC (2000 pg/tissue)			
Run 1	**Run 2**	**Run 3**	**Run 4**
2190	1830	1850	2110
2030	1710	2130	1850
			2160
			1940
			1790

Inter-day (n=6)		Intra-day (n=5)	
Mean	1960	Mean	1970
%ACC	97.8	%ACC	98.5
%RSD	9.65	%RSD	8.16

HIGH QC (15000 pg/tissue)			
Run 1	**Run 2**	**Run 3**	**Run 4**
14400	13100	16000	15700
13900	13900	14700	14800
			14900
			13900
			14500

Inter-day (n=6)		Intra-day (n=5)	
Mean	14300	Mean	14800
%ACC	95.6	%ACC	98.4
%RSD	6.85	%RSD	4.43

Recovery

Results obtained from samples prepared to assess recovery are shown in Table 4.12. The five samples spiked prior to extraction were designated as "extracted", while the five samples spiked post-extraction were designated "non-extracted". Mean areas derived from the reconstructed ion chromatograms were compared for Hyp^{9}GnRH, mGnRH and mGnRH-fa. %Recovery (%REC) was calculated as a ratio of the mean area from the extracted samples to the mean area from the non-extracted samples. Results were similar, with 44.8% Hyp^{9}GnRH recovered, 51.7% mGnRH recovered, and 46.3% mGnRH-fa recovered.

Table 4.12. Recovery of Hyp^{9}GnRH, mGnRH and mGnRH-fa

	Hyp^{9}GnRH		mGnRH		mGnRH-fa	
	Area Extracted	**Area Nonextracted**	**Area Extracted**	**Area Nonextracted**	**Area Extracted**	**Area Nonextracted**
	84142541	171958909	147682906	281973711	12328887	22122647
	85734167	189529217	141409126	273353435	12305379	24862010
	75164616	192535276	129912990	267259826	10599715	28376566
	83114284	195848822	142498625	265558625	11623932	26344277
	84749569	170972721	134345940	257636670	12381540	26134739
Mean	82581035	184168989	139169917	269156453	11847891	25568048
%RSD	5.15	6.42	5.05	3.38	6.45	9.00
%REC		44.8		51.7		46.3

Stability of Hyp^{9}GnRH and mGnRH in rat brains at room temperature

To assess the impact of room temperature storage on Hyp^{9}GnRH and mGnRH in rat brains, five rat brains were extracted immediately after thawing and were compared to five rat brains extracted after two hours at room temperature. Results from these experiments are shown in Table 4.13. Means were compared using a t-test (95% confidence interval) and were not found to be significantly different.

Table 4.13. Evaluation of room temperature storage on concentrations of Hyp^{9}GnRH and mGnRH

	Hyp^{9}GnRH		**mGnRH**	
	pg/tissue 0 hr RT	**pg/tissue 2 hrs RT**	**pg/tissue 0 hr RT**	**pg/tissue 2 hrs RT**
	243	199	900	797
	230	407	1050	1480
	277	410	1370	1860
	348	312	1680	1590
	277	260	1240	1250
Mean	275	318	1250	1400
Std. Dev.	46	92	301	400
t-value (95% C.I.) t_{crit}=2.31		0.92		0.65

Stability of Hyp^{9}GnRH and mGnRH in rat brains after freeze/thaw cycling

To assess the impact of multiple freeze/thaw cycles on Hyp^{9}GnRH and mGnRH in rat brains, five rat brains were extracted immediately after thawing and were compared to five rat brains extracted after enduring three freeze/thaw cycles. Results from these experiments are shown in Table 4.14. Means were compared using a t-test (95% confidence interval) and were not found to be significantly different.

Table 4.14. Evaluation of 3 freeze/thaw cycles on concentrations of Hyp^{9}GnRH and mGnRH

	Hyp^{9}GnRH		mGnRH	
	pg/tissue 1 F/T cycle	**pg/tissue 3 F/T cycles**	**pg/tissue 1 F/T cycle**	**pg/tissue 3 F/T cycles**
	243	175	900	731
	230	379	1050	1890
	277	267	1370	1070
	348	246	1680	1200
	277	164	1240	634
Mean	275	246	1250	1110
Std. Dev.	46	86	301	497
t-value (95% C.I.) t_{crit}=2.31		0.67		0.56

Stability of Hyp^{9}GnRH and mGnRH under long-term storage conditions

Assessment of the long-term stability of Hyp^{9}GnRH and mGnRH in Sprague-Dawley rat brains stored at -70ºC was performed by comparing concentrations obtained from five brains stored for a relatively short period of time (21 days) to five brains stored for over 3 months (112 days). Results from these experiments are shown in Table 4.15. Means were compared using a t-test (95% confidence interval) and were not found to be significantly different. While limited supplies prevented analysis of a larger sample set, evaluation of these samples does demonstrate that gross losses do not occur during long-term storage of up to 3.5 months. Evaluation of long-term storage effects on endogenous GnRH in brain tissues has not been previously reported in the literature.

Table 4.15. Evaluation of effect of long-term storage at -70°C on concentrations of Hyp^{9}GnRH and mGnRH in rat brain

	Hyp^{9}GnRH		**mGnRH**	
	pg/tissue 21 days	**pg/tissue 112 days**	**pg/tissue 21 days**	**pg/tissue 112 days**
male	198	231	865	996
male	515	489	2480	2580
male	328	348	1290	1840
female	231	255	1290	1560
female	341	163	1900	798
Mean	323	297	1570	1550
Std. Dev.	124	126	631	710
t-value (95% C.I.) t_{crit}=2.31		0.32		0.02

Stability Hyp^{9}GnRH and mGnRH in sample extracts

The stability of Hyp^{9}GnRH and mGnRH in sample extracts after completion of processing was assessed by re-injecting a set of calibration standards and quality control samples after 24 hours at room temperature. Results for calibration standards and quality control samples were compared by evaluating a linear regression of the back-calculated concentrations obtained from the reinjection assay against the back-calculated concentrations obtained from the initial assay. The resulting slope, intercept, and coefficient of determination (R^2) are shown in Table 4.16. Slope and intercept are presented with 95% confidence limits. A perfect correlation would yield a slope = 1 and an intercept = 0. For both Hyp^{9}GnRH and mGnRH, the ideal slope and intercept fall within the 95% confidence limits. Based on these results, samples stored at room temperature and subsequently analyzed up to 24-hours post-extraction should provide results comparable to those obtained from analysis immediately post-extraction.

Table 4.16. Regression results for evaluation of re-injection stability

	Hyp^{9}GnRH	**mGnRH**
Slope	1.01 ± 0.08	0.961 ± 0.046
Intercept	-58 ± 716	80 ± 398
R^2	0.987	0.995

Comparison to ELISA assay

To create equivalent samples for measurement by both ELISA and LC-MS/MS, five male rat brains and five female rat brains were homogenized and extracted using the validated method, with the samples split into equal aliquots. After the final evaporation step, one portion was allocated to LC-MS/MS analysis and reconstituted with 95:5 3% acetic acid:methanol while the other portion was allocated to ELISA and reconstituted with the ELISA buffer solution.

Recovery of the internal standard in the comparison samples differed from recovery in the calibration standards (as evaluated by comparison of mGnRH-fa area counts for samples versus calibration standards); therefore, external standard calibration ($1/x^2$-weighted linear regression*)* was used to determine mGnRH concentrations. The accuracy of back-calculated concentrations for calibration standards ranged from 94.2 to 106%, while the accuracies of quality control standards ranged from 85.1 to 106%; thus, use of an external standard regression for LC-MS/MS quantitation was deemed valid for this experiment.

The calibration range for the ELISA method was 0.04 – 25 ng/mL, with a reported detection limit of 1-2 pg/well and a practical linear range in our laboratory of 0.16 – 2.5 ng/mL. Brain tissue extracts were reconstituted in 500 µL assay buffer to generate concentrations on the linear portion of the ELISA calibration curve, and aliquots of 50 uL were assayed in duplicate with the mean results reported in Table 4.17. %CV for the duplicate measurements was ≤30%. Using this ELISA kit, mGnRH might be reliably measured in tissue samples with as little as 32 pg/tissue (accounting for the practical linear range, and duplicate measurements as is customary for ELISA procedures).

Results obtained for these comparative samples are presented in Table 4.17. The results are shown in Figure 4.10 and were well correlated, with an R^2=0.968. The values obtained from the LC-MS/MS data were generally higher than those obtained from the ELISA assay, as manifested in the regression results as a slope > 1. Without a comparative sample in which the actual (nominal) concentration is known, it is difficult to make judgements regarding which of these datasets is more accurate. Nonetheless, reasonable agreement is demonstrated between the two methods.

Table 4.17. Comparison of ELISA and MS

Gender	ELISA pg/tissue	LC-MS/MS pg/tissue	|%Difference| (calculated relative to mean results)
male	307	368	18.1
male	545	575	5.36
male	535	602	11.8
male	211	279	27.8
male	295	269	9.22
female	1040	1310	23.0
female	285	423	39.0
female	262	340	25.9
female	214	303	34.4
female	225	243	7.69
Mean	392	471	
Std. Dev.	258	320	

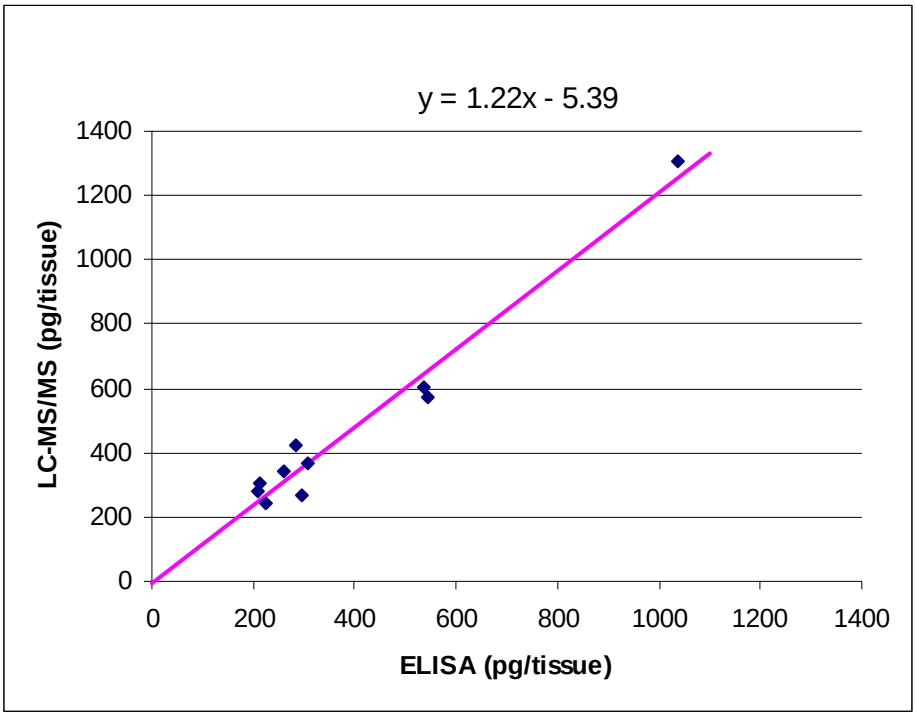


Figure 4.10. Comparison of mGnRH measured by ELISA and LC-MS/MS

Sprague-Dawley Rat Brains

Brains from sixteen male and fifteen female Sprague-Dawley rats were processed and analyzed with the validated LC-MS/MS method. Male rats ranged from 8 to 10 weeks in age, and female rats from 20 to 22 and 26 to 30 weeks. Hyp^{9}GnRH and mGnRH were detected in all tissues, as shown in Table 4.18, with consistently lower concentrations of Hyp^{9}GnRH than mGnRH.

Table 4.18. Concentrations of Hyp^{9}GnRH and mGnRH in male and female Sprague-Dawley rat brains

male				female			
Age (weeks)	**Hyp^{9}GnRH pg/tissue**	**mGnRH pg/tissue**	**Ratio Hyp^9/m**	**Age (weeks)**	**Hyp^{9}GnRH pg/tissue**	**mGnRH pg/tissue**	**Ratio Hyp^9/m**
8-10	271	1320	0.21	26-30	*159*	1150	0.14
8-10	*168*	1100	0.15	26-30	*177*	1350	0.13
8-10	333	1820	0.18	26-30	*129*	692	0.19
8-10	260	1070	0.24	26-30	*74.5*	509	0.15
8-10	291	1280	0.23	26-30	291	1820	0.16
8-10	244	900	0.27	26-30	*169*	966	0.18
8-10	230	1050	0.22	26-30	*121*	607	0.20
8-10	277	1370	0.20	26-30	*67.2*	524	0.13
8-10	348	1680	0.21	26-30	294	1730	0.17
8-10	277	1240	0.22	26-30	298	1860	0.16
8	231	996	0.23	26-30	222	1510	0.15
8	489	2580	0.19	20-22	255	1560	0.16
8	348	1840	0.19	20-22	*163*	798	0.20
8	*198*	865	0.23	20-22	231	1290	0.18
8	515	2480	0.21	20-22	341	1900	0.18
8	328	1290	0.25				
Mean ± SEM	301 ± 24	1430 ± 130	0.21	**Mean ± SEM**	199 ± 22	1220 ± 131	0.16
Range	168-515	865-2580		**Range**	67.2-341	509-1900	

Italicized text indicates value lower than the lower limit of quantitation

Mean levels of 301 pg/tissue Hyp^{9}GnRH and 1430 pg/tissue mGnRH were measured in brains from male rats, and 199 pg/tissue Hyp^{9}GnRH and 1220 pg/tissue mGnRH were measured in brains from female rats. Concentrations of Hyp^{9}GnRH were

below the lower limit of quantitation in two of the male rat brains and in eight of the female rat brains. While accuracy and precision of the assay below 200 pg Hyp^9GnRH/tissue have not been demonstrated, the ratio of Hyp^9GnRH to mGnRH in these samples was consistent with the ratios observed for samples where Hyp^9GnRH values were within the calibration range. All mGnRH determinations were within the calibration range of 200-20000 pg/tissue. The mean Hyp^9GnRH/mGnRH ratio was 0.21 in male rats and 0.16 in female rats.

There are few reports of Hyp^9GnRH and mGnRH quantitative measurement in individual brain tissues to which these results may be compared. Hypothalamic tissue is expected to contain greater than 95% of the Hyp^9GnRH and mGnRH present in whole brain tissue (7); thus, we expected our measurements to be comparable to those previously reported for hypothalamus in the literature. Gautron and colleagues reported concentrations in extracts from hypothalami of 500 pg/tissue Hyp^9GnRH and 5010 pg/tissue mGnRH in intact male rats and 251 pg/tissue Hyp^9GnRH and 1830 pg/tissue mGnRH in intact female rats (age unreported); castration resulted in lower levels for both genders, with reported concentrations of 165 pg/tissue Hyp^9GnRH and 1380 pg/tissue mGnRH in castrated male rats and 113 pg/tissue Hyp^9GnRH and 1040 pg/tissue mGnRH in female rats (23). Rochdi and colleagues measured mean concentrations of 739 pg/tissue Hyp^9GnRH and 4495 pg/tissue mGnRH in hypothalamic tissues from Sprague-Dawley rats (age not reported) (9), and found that Hyp^9GnRH and mGnRH concentrations in hypothalamic tissues increase with age from 2 to 45 days post-natal, with the Hyp^9GnRH/mGnRH ratio decreasing over that period to approximately 0.15 at 45 days irrespective of gender (24). All methods measured Hyp^9GnRH and mGnRH by

separation based on multiple chromatographic methods with fractions collected for separate RIA measurements of Hyp^{9}GnRH and mGnRH.

Mean concentrations of Hyp^{9}GnRH and mGnRH measured in brains from Sprague-Dawley rats by LC-MS/MS methodology were somewhat lower than those observed in previous studies for intact rats and were similar to those observed for castrated rats. The ratio of Hyp^{9}GnRH and mGnRH was consistent with that derived from previous observations. GnRH release is known to be pulsatile in nature and inhibited by conditions of stress (25-27). Also, the age of animals in our study may differ significantly from those utilized in previous studies. These factors make direct comparisons between our study and previous work difficult. Nonetheless, our method has been demonstrated to have good accuracy and precision, and the values obtained after measurement of 31 male and female rat brains are reasonably close to those reported previously in the literature. Thus, these results further validate use of the LC-MS/MS method for quantification of Hyp^{9}GnRH and mGnRH in samples derived from brain tissue. Strategies to lower the calibration range for Hyp^{9}GnRH for future work would be desirable; to this end, improved recovery of the GnRH isoforms during extraction and lower detection capabilities via nanoflow liquid chromatography could be pursued.

4.4 CONCLUSIONS

We have developed and validated an LC-MS/MS method for the simultaneous measurement of two GnRH isoforms, Hyp^{9}GnRH and mGnRH, in individual rat brains. The method utilizes SPE to isolate GnRH isoforms from the sample followed by a single high performance liquid chromatography method interfaced directly with a quadrupole

ion trap mass spectrometer. The validated concentration range is 200-20000 pg/tissue for both Hyp^{9}GnRH and mGnRH.

Calibration standards and quality control samples were prepared in halved guinea pig brains, which were found to be a suitable substitute blank matrix. Results from the validation experiments demonstrated that the method is accurate and precise, with suitable stability to allow for analysis of samples up to 24 hours after extraction. Furthermore, there was no evidence that Hyp^{9}GnRH or mGnRH were significantly degraded in rat brain tissue stored for 112 days at -70ºC. Results from the analysis of 31 brains from Sprague-Dawley rats using the LC-MS/MS method compare favorably with previous studies. To our knowledge, this is the first report of a rigorously validated method for measurement of GnRH isoforms in a tissue matrix and the first report of simultaneous quantitative measurement of two GnRH isoforms in any biological matrix. In addition, this is the first report to assess experimental conditions and long-term storage on stability of Hyp^{9}GnRH and mGnRH.

Replacement of antibody-related detection with LC-MS/MS-based detection of Hyp^{9}GnRH and mGnRH has multiple benefits, including improvements in selectivity, efficiency, sample throughput, and more precise correction for recovery. Selectivity is greatly enhanced because the LC-MS/MS method combines chromatographic separation with detection based on a mass/charge ratio rather than on an epitope of the peptide structure. The potential for interference from peptide fragments or metabolic products is greatly reduced, and additionally, full-scan spectra obtained during the quantitative experiment can be assessed for the characteristic fragmentation pattern associated with GnRH peptides without consuming additional material in a separate experiment (28).

Sample throughput and efficiency are also improved by replacing antibody-based detection with the LC-MS/MS method for several reasons. First, because multiple isoforms can be measured simultaneously rather than performing immunoassay analysis separately for each isoform, only one analysis step is required. Secondly, the experimental time with the LC-MS/MS method is greatly reduced compared to immunoassay techniques. For the LC-MS/MS analysis, extraction and a complete instrument run can be completed within 24 hours; in contrast, RIA procedures alone for GnRH analysis can require multi-day incubation steps (7). Furthermore, the LC-MS/MS method proved capable of measuring Hyp^{9}GnRH and mGnRH in single rat brain tissues, whereas previous studies have utilized large pools of tissue, ranging from tens to thousands of hypothalamus and brains (6-8).

With the LC-MS/MS method, recovery is accounted for within each experiment and for every sample due to the use of calibration standards and a structurally-analogous internal standard. With the previously-utilized RIA methods, a general recovery factor was applied to each individual experiment, requiring the assumption of constant recovery from experiment to experiment and sample to sample. While the accuracy and precision of RIA methods used for tissue analyses of Hyp^{9}GnRH and mGnRH have not been reported, making it difficult to assess how much of an improvement the LC-MS/MS method offers over previously-utilized methods, use of internal standard quantitation would be expected to improve upon these parameters.

In terms of comparison to other similar methods, the data collected for intra- and inter-day accuracy and precision during validation of the LC-MS/MS method were within the ranges expected for bioanalytical methods of exogenous compounds and compare

favorably to reports in the literature for peptide analysis by LC-MS/MS. An LC-MS/MS method for EPI-nHE4, a 56-amino acid protein, in plasma samples has been validated with reported accuracies of 90.6-106.7%, precision (%RSD) ≤ 13.6, and an LLOQ of 5 ng/mL (250 pg on-column) (29). A multi-analyte LC-MS/MS assay for tifuvirtide, efuvirtide, and a metabolite of efuvirtide in plasma samples has been validated with reported accuracies of 87.7-106.9%, precision (%RSD) ≤ 13.4%, and an LLOQ of 20 ng/mL each peptide (100 pg on-column) (30). The penta-peptide Gluten Exorphin A5 has been measured in cerebrospinal fluid by LC-MS with reported accuracies of 99.6-103%, precision (%RSD) ≤ 4.99, and an LLOQ of 0.96 ng/mL (160 pg on-column) (31). Kahalalide F, a depsipeptide containing 13 amino acids, has been measured in plasma by LC-MS/MS with reported accuracies of 84.9-97.3%, precision (%RSD) ≤ 9.91, and an LLOQ of 1 ng/mL (12 pg on-column) (32). Finally, an LC-MS method has been validated for Cetrorelix, a decapeptide synthesized as a GnRH antagonist and having limited structural similarity to mGnRH (50% homology); accuracies of 96.0-114%, precision (%RSD) ≤ 13.5, and an LLOQ of 1 ng/mL (400 pg on-column) were reported for plasma samples, and accuracies of 86.6-103%, precision (%RSD) ≤ 17.1, and an LLOQ of 2 ng/mL (1.7 ng on-column) were reported for urine samples (33). Our LC-MS/MS method for quantitative measurement of Hyp^9GnRH and mGnRH achieved accuracies ranging from 94.0-111%, precision (%RSD) ≤ 10.8%, and an LLOQ of 200 pg/tissue (~50 pg on-column); these measures compare favorably with the data reported for bioanalysis of other peptides as described above.

The calibration range as validated, with a lower limit of quantitation of 200 pg/tissue, did not encompass the lowest concentrations of Hyp^9GnRH measured in rat

brain samples; approximately a third of the brains sampled in this study contained concentrations of Hyp^{9}GnRH lower than 200 pg/tissue. These results suggest that a slightly lower limit of quantitation for Hyp^{9}GnRH should be pursued as a method improvement for assay of rat brain samples. Sample reconstitution volumes could be halved while maintaining the HPLC injection volume; theoretically, this method change could be used to lower the lower limit of quantitation by a factor of 2, to 100 pg/tissue. Improved recoveries of GnRH from extracted tissue would also assist in decreasing the LLOQ. In combination, these method changes would be expected to reduce the LLOQ such that the lower concentrations of Hyp^{9}GnRH in rat brain would be within the calibration curve range. For samples where substantially lower concentrations of GnRH are expected, conversion of the HPLC method from a microbore column (in this case, 1.0 mm diameter column) to capillary chromatography (<0.5 mm diameter column) or transfer of the method to a newer mass spectrometry platform such as the linear ion trap would provide a means to substantially decrease the LLOQ of the method.

The LC-MS/MS method described here could be extended to other species than rat, and the results presented here suggest that guinea pig brain may be a suitable tissue matrix for many mammalian species. Adaptation of the LC-MS/MS method to other GnRH isoforms should be straightforward, so long as synthetic peptides for use in calibration standards and for analogous internal standards are available. The ease with which peptides are routinely synthesized means that this methodology can be adapted to virtually any GnRH isoform. Extension of the method to other matrices such as blood and serum should be feasible as well.

We have demonstrated that simultaneous quantitation of two GnRH isoforms with good accuracy and precision can be achieved via a new LC-MS/MS method. The new method offers substantial improvements over previously utilized immunoassay-based methods, including increased specificity, efficiency, and recovery-corrected values. Accuracy and precision were difficult to compare to previous GnRH methods, as metrics for these parameters have not been reported; however, the new LC-MS/MS method compared favorably with other LC-MS and LC-MS/MS methods validated for peptide quantitation with regards to accuracy, precision, and limits of quantitation. Future applications of the method include continued exploration of the precise function of Hyp^9GnRH and mGnRH in the rat animal model or investigations of up- or down-regulation of Hyp^9GnRH and mGnRH release after administration of GnRH agonist- or antagonist-compounds. For both of these applications, accurate and precise methods of quantitation of Hyp^9GnRH and mGnRH will be essential. Additionally, it is expected that this method could be used as a guide to validate LC-MS/MS methods for quantitative measurement of Hyp^9GnRH or mGnRH in species other than rat or extended to measurement of other GnRH isoforms with relative ease.

4.5 References

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CHAPTER 5: SUMMARY

Gonadotropin-releasing hormones (GnRH) have been found in a wide diversity of species. Twenty four isoforms have been characterized thus far in vertebrate and invertebrate species, and it is likely that additional isoforms will be discovered. This dissertation describes the investigation of reversed-phase chromatographic and mass spectrometric behavior of GnRH peptides, the development and application of an LC-MS/MS method for qualitative identification of GnRH peptides, and the comprehensive validation of an LC-MS/MS method for simultaneous, quantitative measurement of hydroxyproline^{9}GnRH (Hyp^{9}GnRH) and mammalian GnRH (mGnRH) in rat brain tissues. The LC-MS/MS-based qualitative and quantitative methods described in this dissertation provide alternatives to the antibody-based detection techniques that have been the predominant techniques for GnRH characterization and measurement, with the advantages of these new LC-MS/MS methods described below.

Chapter 2 describes the chromatographic and mass spectrometric behavior of a model set of six GnRH peptides, chicken I GnRH, chicken II GnRH, lamprey III GnRH, mGnRH, salmon GnRH and seabream GnRH. Mobile phase composition and reversed-phase column composition were explored to optimize chromatography, and effects of solution composition and electrospray parameters on signal intensity and peptide charge states were investigated. Nearly complete separation of the six model peptides was achieved using gradient reversed phase chromatography with a 50 x 1.0 mm C8 chromatography column, and mobile phases prepared with acetic acid were found to provide optimized response. The capillary temperature setting for the electrospray source

was found to affect the charge state distribution for GnRH peptides having more than one main charge state, offering a mechanism to "tune" the electrospray settings for a particular charge state. The electrospray source voltage setting was found to have an optimal range for the six GnRH peptides. These results were used to develop the qualitative LC-MS/MS method presented in Chapter 3, which was developed as a generalized approach to facilitate identification of any isoform in the GnRH peptide group, and the quantitative LC-MS/MS method described in Chapter 4.

In Chapter 3, the development and application of an LC-MS/MS method capable of simultaneous separation and identification of multiple GnRH isoforms is described. MS/MS fragmentation patterns observed from the model set of six GnRH isoforms were used to define characteristic transitions and marker ions for the family of peptides and to ensure that the proline in position 9 would not dominate fragmentation and prevent obtaining an adequate series of ions for *de novo* sequencing. A simple solid phase extraction procedure was developed to prepare rat brain homogenates for LC-MS/MS analysis. To investigate feasibility, six model GnRH peptides were used to prepare a control sample from Sprague-Dawley rat brain for data-dependent experiments; results indicated that sufficient sequencing information could be obtained to identify all six GnRH peptide structures by *de novo* sequencing. In an unspiked pool of ten Sprague-Dawley rat brains, two putative GnRH peptides were detected with molecular weights and fragmentation patterns consistent with what would be expected from Hyp^{9}GnRH and mGnRH. An extract containing these natural peptides was augmented with synthetic Hyp^{9}GnRH and mGnRH peptide standards, demonstrating coelution of the synthetic and natural peptides and similarity of MS/MS fragmentation patterns; these results confirmed

the identify of Hyp9GnRH and mGnRH in extracts from Sprague-Dawley rat brains. The LC-MS/MS method described in this chapter was able to provide qualitative information with as little as 10 pg peptide for detection of GnRH peptides of known sequence or 300-500 pg peptide for detection and sequencing of novel GnRH isoforms. Targeted product-ion analysis experiments were conducted in pooled extract from rat brains to search for GnRH isoforms that have been previously reported – chicken II GnRH, lamprey III GnRH, and salmon GnRH. No evidence supporting the expression of any of these isoforms was found. These results support the hypothesis that mGnRH and its post-translationally modified form, Hyp^9GnRH, are the primary, if not only, forms of GnRH expressed in rat brains.

Chapter 4 describes the comprehensive validation of an LC-MS/MS method for simultaneous quantitative measurement of Hyp^9GnRH and mGnRH in rat brain samples. Calibration standards and quality control samples were prepared in guinea pig brains, which were found to be a suitable blank matrix for the assay. A simple solid phase extraction procedure was employed prior to LC-MS/MS analysis. Internal standard quantitation was performed with an analogue to mGnRH, and the validated concentration range was 200 - 20000 pg for each peptide. For both Hyp^9GnRH and mGnRH, the intra- and inter-run accuracy and precision measures were good. Sufficient stability at room temperature, during freeze/thaw cycles, after extraction and under long-term storage conditions were found to accommodate typical laboratory procedures. Comparison of mGnRH quantitation using the LC-MS/MS assay to a commercially available ELISA kit produced reasonable agreement between the two methods. Thirty-one brains from Sprague-Dawley rats were analyzed using the LC-MS/MS procedure and compared to

published results for Hyp^{9}GnRH and mGnRH. The validated LC-MS/MS method offers numerous improvements to preceding methods for Hyp^{9}GnRH and mGnRH measurement in tissue samples. Replacement of antibody-based detection with detection based on a mass/charge ratio improved selectivity, efficiency and sample throughput as compared to previously reported methods; also, the inclusion of extracted calibration standards and internal standard quantitation allowed for a more precise accounting of recovery within each experiment and for each sample. The method should be easily adaptable to the measurement of other GnRH isoforms. To our knowledge, this is the first report of a rigorously validated method for measurement of GnRH isoforms in a tissue matrix and the first report of simultaneous quantitative measurement of two GnRH isoforms in any biological matrix. In addition, these experiments provide the first reported assessment of experimental conditions and long-term storage on stability of Hyp^{9}GnRH and mGnRH.