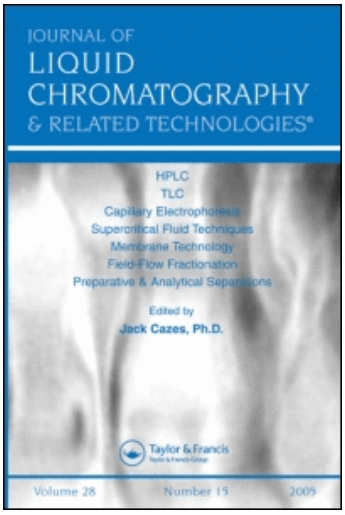

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Characterization of Tryptic Peptides of a Potent Growth Hormone Releasing Hormone Analog by Reversed Phase High Performance Liquid Chromatography-Ionspray Mass Spectrometry

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CHARACTERIZATION OF TRYPTIC PEPTIDES OF A POTENT GROWTH HORMONE RELEASING HORMONE ANALOG BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-IONSPRAY MASS SPECTROMETRY

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ABSTRACT

A highly reproducible tryptic digestion procedure was developed for the characterization of a potent growth hormone releasing hormone (GHRH) analog, para-methylhippuroyl derivative of the N-terminus of a precursor porcine-GHRH (2-76) (pMHpGHRH) derived by recombinant DNA technology. Tryptic digestion can be completed within one hour at room temperature. All tryptic peptides can be separated by reversed phase high performance liquid chromatography(RPHPLC) on a Vydac C18 protein column with trifluoroacetic acid(TFA)-acetonitrile(ACN) gradient elution. Eleven single fragments obtained in tryptic peptide mapping have been identified by coupled Ionspray mass spectrometry. Combined fragments T2-3 and T4-5 were also observed and

verified by Ionspray mass spectrometry and microsequencing analysis. A stability study indicated that the digestion solution will remain stable for 9 days at 5 °C. The retention time variations for the tryptic peptides found to be 1.9% relative standard deviation(RSD).

INTRODUCTION

Growth hormone releasing hormones (GHRH) are a series of hypothalamic peptides that stimulate the synthesis and release of growth hormone (1). Native GHRH and its analogs have been reported to have long term effects on increasing growth rate in growth hormone deficient children (2) and lactation in dairy cows (3). In practical use, however, stable analogs were required in the use of long term processes. Several hybrid GHRH produced by recombinant DNA technology have been reported (4). A para-methylhippuroyl derivative of the N-terminus of a recombinant precusor porcine-GHRH(2-76) (pMHpGHRH) has been developed(5). This new modified recombinant GHRH which is composed of 76 amino acids, results in an increase in production of growth hormone in mammals. pMHpGHRH may be used to increase lean muscle mass, to foster wound healing and to counter the effects of aging (5).

Peptide mapping is a powerful technique to characterize the primary structure of proteins. Reversed-phase high performance liquid chromatography (RPHPLC) is commonly used for the separation of enzymatic digest peptides. In a previous paper, a free solution capillary electrophoresis (FSCE) method and RPHPLC have been used for the characterization of tryptic peptides of pGHRH 2-76, the parent peptide of pMHpGHRH (6). However, the recognition of digest peptide fragments was carried out by adding synthesized peptide fragments in the tryptic digestion solution. The weakness of this procedure is the lack of selectivity since two or more different peptide fragments can have identical retention times.

The combination of high performance liquid chromatography and mass spectrometry (HPLC-MS) offers one of the most powerful techniques in modern time for the characterization of biomolecules. Several ionization interfaces such as thermospray (7) and particle beam (8) have been used in LC-MS. These interfaces, however, have been less successful for the analysis of biopolymers with relatively large molecular weights. A technique using the combination of high-performance displacement chromatography with continuous flow fast atom bombardment (FAB)-mass spectrometry has been used for the characterization of tryptic peptides of recombinant human growth hormone (9),but this method has had only limited success in the use of routine analysis of proteins. Ionspray (IS) is a very gentle ionization process and operates without the input of heat into the spray-ionization step. As a consequence, sample molecules are ionized without thermal degradation (10, 11) and generally little or no fragmentation for proteins and peptides. A particularly significant aspect of this technique is the multiple charging observed for large biomolecules, such as proteins, peptides and nucleic acids, that can be used for highly accurate molecular weight determination of biomolecules.

In this paper, based upon the studies of effects of digestion temperature and incubation time on peptide separation, a highly reproducible tryptic digestion procedure was developed for the characterization of pMHpGHRH. The combination of HPLC-ionspray MS was employed to identify tryptic fragments of pMHpGHRH.

EXPERIMENTAL

Materials

pMHpGHRH samples were obtained from Eli Lilly & Co (Indianapolis, IN, USA). TPCK-treated trypsin was purchased from Fluka Chemie AG (Switzerland). HPLC grade tri-

fluoroacetic acid (TFA) (Fischer, NJ, USA), Trizma base (Sigma, St. Louis, MO, USA), HPLC-grade acetonitrile (ACN) (Mallinckrodt, St. Louis, MO, USA), all analytical grade reagent and Millipore Milli Q water were used in this work.

Methods

Tryptic digestion. Tryptic digestions of pMHpGHRH were performed at room temperature in a Tris-acetate buffer system. One milligram of the pMHpGHRH sample was dissolved in 0.5 mL of water and mixed with 0.5 mL of 100 mM Tris-acetate buffer(pH 8.5). In this case, the solution became cloudy because of the poor solubility of pMHpGHRH at this pH condition but digestion results were not affected. A 40 µL aliquot of TPCK treated trypsin solution (1 mg/mL in 100 mM Tris-acetate buffer, pH 8.5) was added and vortexed for 2 minutes. The digest mixture was incubated for 1 hour at room temperature. After digestion, the mixture was maintained under refrigeration throughout the chromatographic analysis. Aliquots of the digest mixture were stored frozen at - 20 ° C for use at a later time.

Reversed-phase HPLC. Reversed-phase chromatographic analyses of tryptic digest peptides were performed on a Waters 625 LC-system equipped with a Waters 991+ photodiode array detector (Waters, Milford, MA, USA) and a Vydac protein and peptide C18 column (4.6 x 250 mm) (Vydac, Hesperia, CA, USA). Mobile phase consisted of 0.1% aqueous TFA(Solution A) and (v/v) 0.1% TFA in 50% ACN/H2O (Solution B). The flow rate was 1.0 mL/min. A 50 µL aliguot of the tryptic digest mixture was injected onto the column at room temperature. A linear gradient from 0% to 100% solution B over 50 min was carried out upon injection. Peptides were detected at 214 nm. A Beckman System-Gold instrument consisting of a model 126 programmable solvent delivery system, a Model 168 photodiode array detector and a Beckman manual injector with a 100µL sample loop was used for the LC-MS experiments.

Mass Spectrometry . Ionspray mass spectrometry(ISMS) was conducted using a Sciex API III triple quadrupole mass spectrometer equipped with a pneumatically assisted ionspray interface. The mass calibration of the instrument was performed according to manufacturer's instuctions using a mixture of polypropylene glycols. A post-column flow split tee was used to maintain a split flow from HPLC eluant in a ratio of 50:1. A second tee was set up downstream of the flow split tee into which a 10 μL/min post column addition of a solution of 1% (v/v) acetic acid in acetonitrile was introduced using a Harvard syringe pump (model #22) for increasing the sensitivity in Ionspray MS. The instrument was operated in the positive ion detection mode with an inlet orifice potential of 50V (+20 volts relative to RO). Spectra were collected over a range of 150-2400 amu at 0.33 amu intervals with a dwell time of 0.7 msec. per interval. The duration of a single scan was 5.10 sec.

N-terminal peptide sequencing. Sequencing of pMHpGHRH tryptic peptides was performed using the Edman degradation technique on an Applied Biosystems Model 477A Protein Sequencer and analyzed using an Applied biosystems Model 120A Analyzer.

RESULTS AND DISCUSSION

The structure of pMHpGHRH is shown in Fig. 1. Complete tryptic digestion of pMHpGHRH should generate 12 tryptic peptide fragments. Table 1 lists the amino acid sequences and masses of the peptide fragments expected from the digestion mapping and two combined peptide fragments. A chromatogram of the tryptic digestion mixture of pMHpGHRH under linear gradient elution conditions is shown in Fig. 2. The tryptic peptide mapping of pMHpGHRH is very similar to that of pGHRH 2-76 previously reported (7) except the T1 peak of pMHpGHRH appears at a later retention time in the chromatographic profile because of the more hydrophobicity of this peptide.

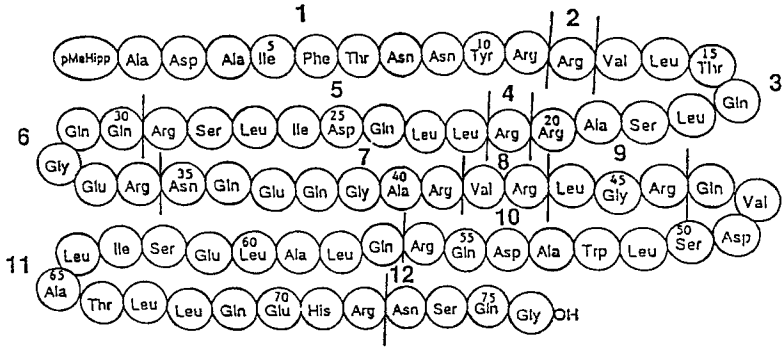

FIG. 1
Sequence structure of pMHpGHRH and predicted tryptic cleavage sites.

TABLE 1. TRYPTIC PEPTIDE FRAGMENTS OF pMHpGHRH

Num.	Fragment	MH+(mass)	Sequence
T1	1 - 11	1359.6	pMH-ADAIFTNNYR
T2	12 - 12	175.1	R
T2-3	12 - 20	1043.6	RVLTQLSAR
T3	13 - 20	887.5	VLTQLSAR
T4	21 - 21	175.1	R
T4-5	21 - 29	1113.7	RLLQDILSR
T5	22 - 29	957.6	LLQDILSR
T6	30 - 34	617.3	QQGER
T7	35 - 41	802.4	NQEQGAR
T8	42 - 43	274.2	VR
T9	44 - 46	345.2	LGR
T10	47 - 56	1217.6	QVDSLWADQR
T11	57 - 72	1835.0	QLALESILATLLQEHR
T12	73 - 76	405.2	NSQG

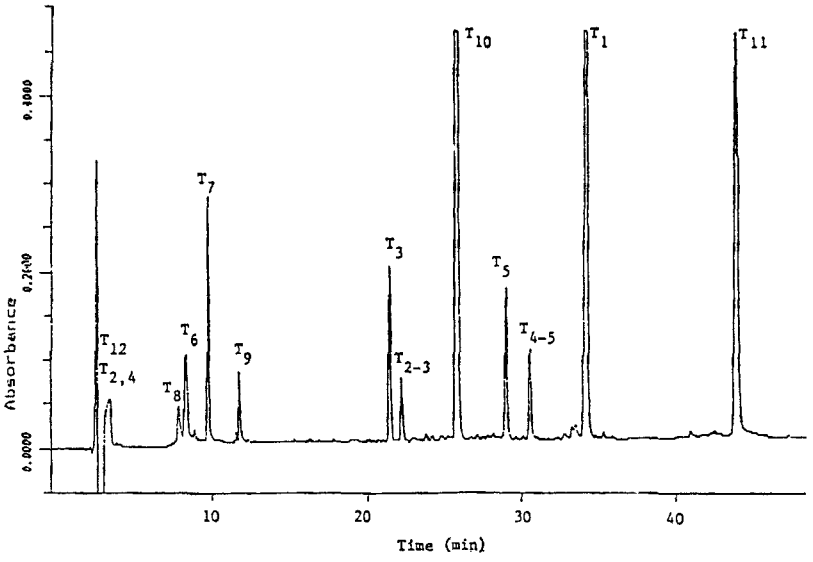

Fig. 2
RPHPLC tryptic mapping obtained from the digest mixture of pMHpGHRH. Tryptic fragments are numbered sequencially from N-terminus of the peptide and given in Table 1.

Peak identification

Peak identification of the tryptic peptide fragments of pMHpGHRH was performed on a HPLC system interfaced with an ionspray triple quadrupole mass spectrometer (Fig. 3). All tryptic peptides found in the digest mixture have been identified and are listed in Table 2. The sequence assignment of fragments was made by comparison of the observed mass with that calculated for the most abundant isotopes of each peptide. It can be seen that two tryptic peptides comprising T2(or T4), arginine, and T12, a tetrapeptide were found in the void peak by plotting selected ion chromatogram (SIC). Fig. 4a and 4b show the reconstructed SIC of fragment T2(or T4) and T12 which are dominated by ions corresponding to a $[M + H]^+$ of 175.3 amu for T2(or T4) and 405.4 amu for T12. The

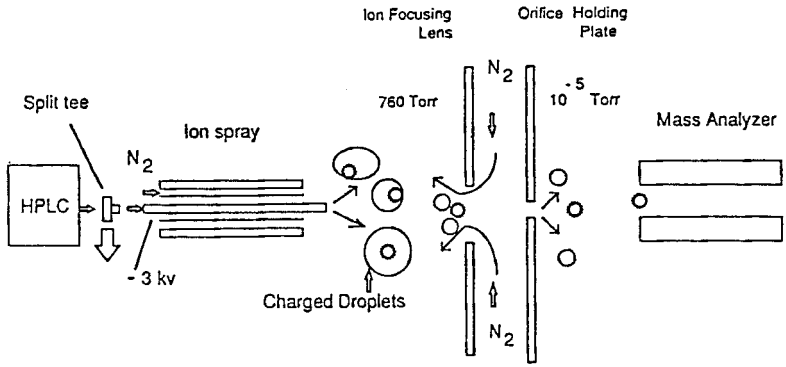

Fig. 3
Schematic diagram of the HPLC-ISMS.

TABLE 2. MOLECULAR IONS OBSERVED IN RPHPLC - ISMS

Fragment	Observed[M+H]			Calculated[M+H]		
	Z=1	Z=2	Z=3	Z=1	Z=2	Z=3
T1		680.8		1359.6	680.8	
T2,T4	175.3			175.1		
T3		444.4		887.5	444.8	
T2+T3		522.6		1043.6	522.8	
T4+T5		557.6		1113.7	557.9	
T5		479.7		957.6	479.8	
T6	617.5	309.2		617.3	309.7	
T7		402.1		802.4	402.2	
T8	274.2			274.2		
T9	345.2	173.0		346.2	173.6	
T10		609.5		1217.6	610.3	
T11		918.6	612.5	1836.0	918.5	612.7
T12	405.4			406.2		

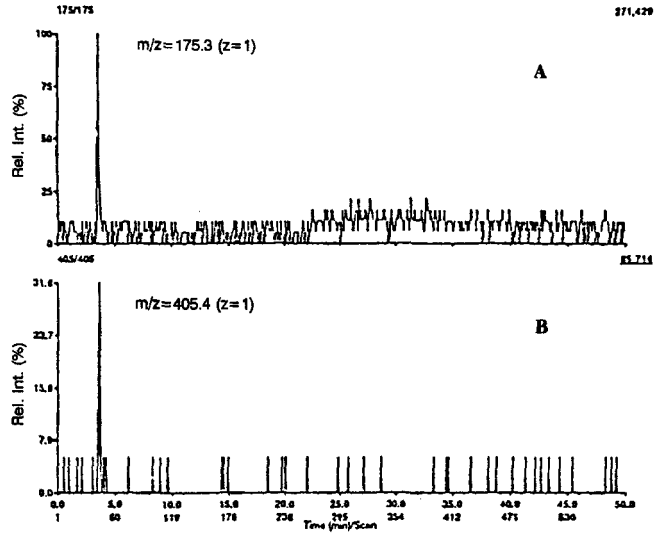

Fig. 4
Reconstructed single ion current chromatograms.
A: $[M + H]^+$ of 175.3 amu.(T2 or T4)
B.: $[M + H]^+$ = 405.4.(T12)

observed masses are identical with the predicted masses. In fact, a slightly longer retention time for T12 peak was observed in SIC that is due to the slightly stronger hydrophobicity of T12 than that of T2(or T4).

Incomplete digestion was also observed at two sites as indicated in Table 2. The mass spectral analysis assigned the observed molecular weight of these peptides to the sequences comprising residues T2-3 or T3-4 ($[M + 2H]^{2+}$ = 522.8)(Fig. 5a) and T4-5 ($[M + 2H]^{2+}$ = 557.9)(Fig. 5b) in which additional arginine is retained at the N-terminus bound of the fragments. It is not possible to identify the sequence of the combined fragment, that is T2-3 or T3-4, by MS because both fragments have the same molecular weight. In order to

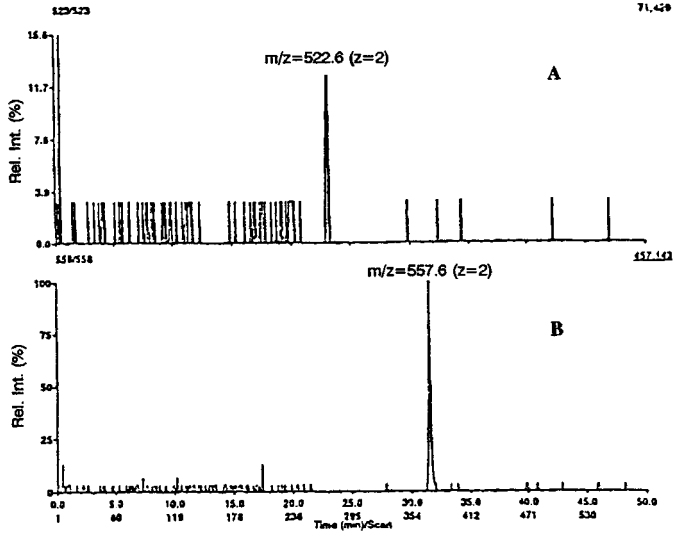

Fig.5
Reconstructed single ion current chromatogram for the combined peptides.
A: $[M + H]^+ = 522.6$ (T2-T3)
B: $[M + H]^+ = 557.6$ (T4-T5)

identify the sequence of this fragment, the collected fraction of this fragment was examined by micro-sequence analysis. The result obtained indicated that the combined fragment is T2-3.

RPHPLC of tryptic peptide mapping

In RPHPLC, the retention order depends on the molecular size and hydrophobicity of the peptide. In general, the larger the more hydrophobic peptides the stronger the retention. In Fig.2, we can see that the retention order basically follows the molecular size of the peptides. For example, T2, and T4, monopeptide, eluted at void volume and T8, dipeptide, T6, pentapeptide, and T7, heptapeptide eluted

successively afterwards. In some cases, however, hydrophobicity of the peptide strongly effects the retention order. The tripeptide T9 exhibits a stronger hydrophobicity because of its two hydrophobic amino acid, glycine and isoleucine. Consequently, T9 elutes at a retention time later than that of T6. Peptide T12 eluted at the void volume owing to three strong polar amino acids in this tetrapeptide. In addition, T5 containing four hydrophobic amino acids exhibits stronger retention although the molecular size of this peptide is smaller than T10. The largest peptide T11 presents the strongest retention in the peptide mapping.

Validation of the tryptic digestion of pMHpGHRH

The tryptic digestion procedure has been validated for temperature effects, incubation time, stability, and reproducibility.

The temperature effect on the tryptic digestion of pMHpGHRH is shown in Fig.6. The maps obtained from the peptide mixtures digested at 22°C and 37°C demonstrate identical chromatographic profiles. The slight variation in retention time for some peaks is due to slight fluctuation in the gradient elution profile.

The effect of incubation time on tryptic digestion has been examined. Chromatograms in Fig.7 demonstrated that when the incubation time varied from 1 to 7 hours identical peptide maps were obtained. This result indicated the tryptic digestion of pMHpGHRH can be completed within one hour.

In the stability study, the same profiles of digest peptide mapping were obtained after the peptide digestion solutions were stored in a refrigerator at 5 °C for 9 days.

Reproducibility of tryptic mapping has also been determined by using 4 different lots with duplicate or

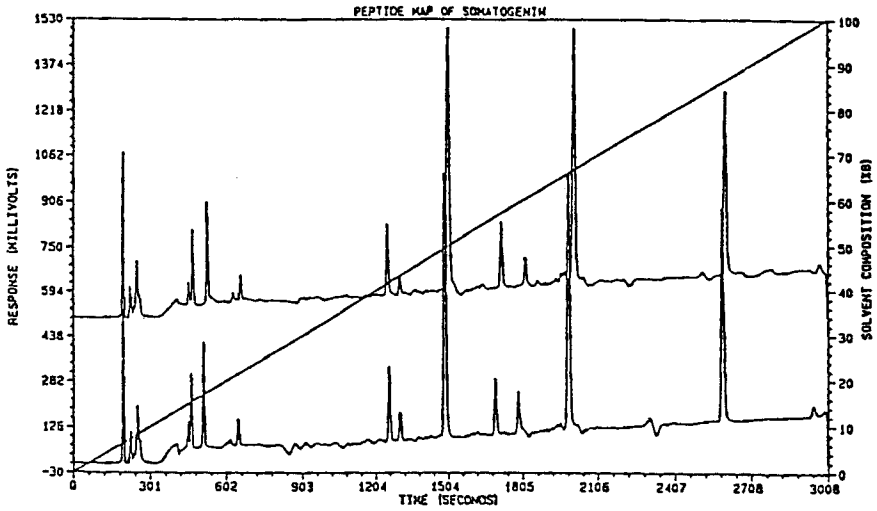

Fig. 6
Effect of digestion temperature on the tryptic peptide map of pMHpGHRH. A: 22°C. B: 37°C.

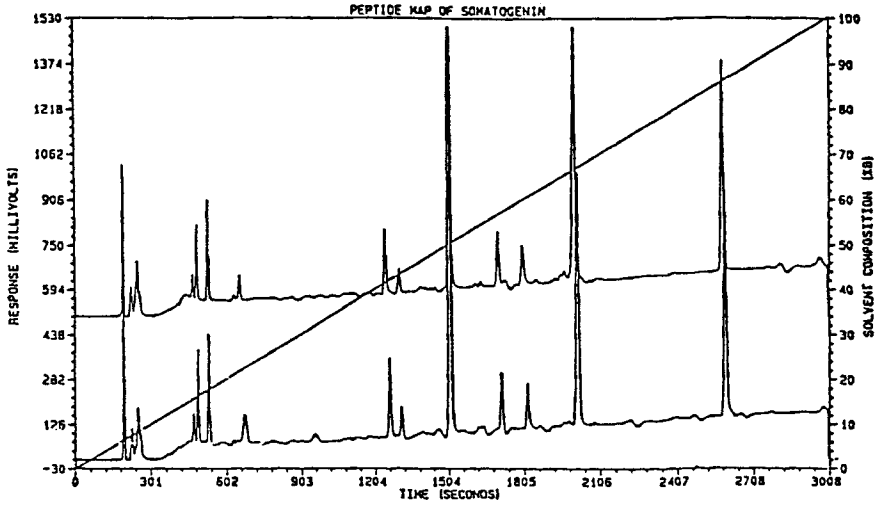

Fig.7
Effect of digestion time on the tryptic peptide map of pMHpGHRH.
A: 1 hour incubation. B: 7 hours incubation.

TABLE 3. REPRODUCIBILITY OF THE RETENTION TIME OF FRAGMENT PEAKS

Fragment	Sample No.	Run No.	tr	RSD%
T6	4	10	7.78	4.32
T7	4	10	8.95	3.49
T9	4	10	11.04	3.90
T3	4	10	21.04	2.02
T2-3	4	10	21.94	2.11
T10	4	10	25.33	1.11
T5	4	10	28.56	0.86
T4-5	4	10	30.18	0.57
T1	4	10	33.57	0.39
T11	4	10	42.63	0.49

triplicate analyses. Results listed in Table 3 indicated that the variability in the retention time of the tryptic fragments appears to be quite small. Peaks with less retention have larger relative standard deviation (RSD).

CONCLUSION

RPHPLC-ionspray MS is a fast, sensitive method for the identification of tryptic digest peptides. Analysis of tryptic peptides of pMHpGHRH by this method demonstrates the presence of all the expected peptide fragments. High-quality mass spectra were obtained for the tryptic peptides which co-eluted at the void volume. Two combined peptides were also observed in the analysis. Those have been comfirmed to be T2-3 and T4-5.

The tryptic digestion procedure developed in this paper is a simple and highly reproducible method for the characterization of pMHpGHRH.

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REFERENCES

1. L. A. Frohman and J. O. Jansson, Endocrine Rev. 7: 223(1986)

2. P. E. Rochiccioli, M. T. Tauber, F. X. Coude, M. Arnone, M. Moore, F. Uboldi and C. Barbeau, J. Clin. Endocrinol. Metab. 85: 268(1987).

3. W. J. Enright, L. T. Chapin, W. M. Moseley and H. A. Tucker, J. Dairy Sci., 71: 99(1988).

4. D. S. Smith, M. L. Heiman, J. F. Wagner, R. L. Jackson, R. A. Bimm and H. M. Hsiung, Biotechnology 10: 315(1992).

5. R. D. Dimarchi, M. L. Heiman, J. E. Shields, D. L. Smiley, European Patent Application 511003 (1992)

6. J. P. Chang, D. L. Smiley and M. R. Coleman, M.R. J. Liquid Chromatog., In press.

7. K. Stachowiak, C. Wilder, M. L. Vestal, D. F. Dyckes, J. Am. Chem. Soc. 110: 1758(1988).

8. Winkler, P.C.; Perkins, D.D.; Williams, D.K.; Browner, R. F. Anal. Chem. 60: 489(1988).

9. J. Frenz, J. Bourell, W. S. Hancock, J. Chromatogr. 512: 299(1990).

10. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, Science, 246: 64(1989).

11. S. A. McLuckey, G. J. Van Berkel, G. L. Glish, E. C. Huand, and J. D. Henion, Anal. Chem. 1991, 63: 375(1991).

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