Bioorganic & Medicinal Chemistry Letters, Vol 1, No 8, pp. 415-420, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

ANALYSIS OF RAT AMYLIN AMIDE FROM COMMERCIAL SOURCES: IDENTIFICATION OF A MERCURY COMPLEX.

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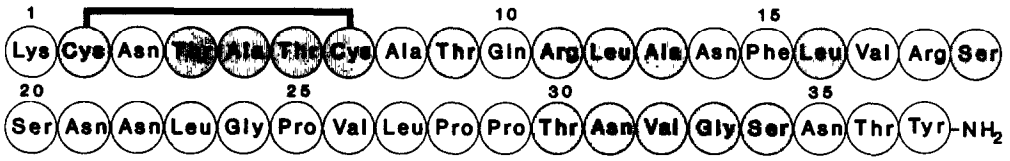
(*Received* 10 *July* 1991)

Abstract: Analysis of four commercial samples of rat amylin amide by HPLC, CZE and biological activity showed one sample (A) was different from the other three (B, C and D). Additional structural analysis of sample A (Edman sequencing, amino acid analysis, ^{1}H NMR and FAB-MS) suggested it was indeed rat amylin amide. However, electrospray ionization mass spectrometry and scanning electron microscopy showed sample A contained mercury covalently bound to the side chains of the cysteines in positions 2 and 7.

Amylin (Diabetes-Associated Peptide), initially isolated from pancreatic islet amyloid deposits and present in 90% of patients suffering from non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes mellitus),[1-4] is a 37 amino acid peptide containing one disulfide bridge. Amylin has been reported to inhibit insulin-stimulated glycogen synthesis in skeletal muscle, but not affect insulin-stimulated carbohydrate metabolism in adipose tissue, suggesting that amylin may act as a regulator of insulin secretion under normal conditions.[5-7] In the anesthetized rat, infusion of amylin enhances in vivo hepatic glucose output in the presence of elevated insulin levels. The amylins (rat and human) are homologous (> 40%) with both α- and ß-calcitonin gene-related peptide (α- and ß-CGRP, Figure 1) and show limited homology to the A chain of insulin.[2] The CGRP's impart similar effects on skeletal cells in vitro and hepatic glucose output in vivo.[5,9] These and additional observations led to the speculation that overproduction of amylin and/or CGRP may play a role in NIDDM pathogenesis.[8-12] Structure-activity analyses have shown that an intact disulfide bridge and a C-terminal amide are critical for high biological activity.[11]

Figure 1: Primary Sequence of Rat Amylin Amide (Shaded residues are conserved in the CGRP's).



Recently, the role of amylin in insulin regulation and the development of type 2 diabetes mellitus has been reevaluated.[13-15] In one study, amylin specific receptors could not be found in isolated rat hepatocytes but were shown to be localized to the nonparenchymal cells and therefore may not be directly involved in glucose metabolism.[15] Likewise, specific amylin receptors could not be identified in L6 myocytes (rat skeletal cells), rat liver or brain and it was suggested that amylin may be acting through CGRP receptors.[16] In addition,

we observed that the pharmacology of amylin may differ depending upon its source. In order to determine the homogeneity and structural integrity of commercial rat amylin amide preparations with respect to its biological activity, we have characterized samples from four different suppliers.

Experimental:

Rat amylin amide was purchased from four independent commercial sources (samples A, B, C and D) and was used without further purification or modification. The relative biological activity was determined for samples A and B only, by their ability to enhance in vitro cAMP accumulation in cultured L6 myocytes.[16]

The purity of samples A, B, C and D was assessed by high performance liquid chromatography (HPLC) on a Waters system with a Vydac 218TP54 column (0.46 X 25.0 cm, 214 and 280 nm) The mobile phase was a linear gradient from 10% to 50% acetonitrile in water over 60 min (1.5 mL/min) with a constant trifluoroacetic acid (TFA) concentration of 0.10%. Electrophoretic purity was determined on an Applied Biosystems (ABI) 270A capillary zone electrophoresis system (CZE) equipped with a 72 cm capillary (30°C), operating with an applied field of +12 kV (20 mM aqueous sodium citrate, pH 2.5, 210 nm). Automated pulsed-liquid phase Edman sequencing and amino acid analysis (following acid hydrolysis) of samples A and B was performed on ABI 473A and ABI 420H instruments, respectively, using standard methods.

One-dimensional 500 MHz proton NMR (^{1}H NMR) spectra of samples A and B dissolved in 0.5 mL of 90% H_2O/10% D_2O (292 K), were recorded on a Bruker AMX spectrometer (< 1.0 mg) The spectra were the Fourier transformed average of 1024 scans with presaturation of the H_2O signal. Electrospray ionization mass spectrometry (ES-MS) data were acquired on a Finnigan TSQ-70 instrument equipped with an Analytica of Branford electrospray ionization source. Samples were introduced from a capillary HPLC column (0 075 mm x 10 cm, slurry packed with LiChrosorb Si-60 C-18 silica gel). The mobile phase was a linear gradient from 0% to 40% acetonitrile in water over 40 min (200 µL/min) with a constant TFA concentration of 0.10%. Liquid secondary ion mass spectrometry (liquid SIMS) analyses were performed on a VG Analytical ZAB 2-SE high field mass spectrometer operating at an accelerating voltage of 8 kV, a resolving power of ~1500 and using a cesium ion gun (M-Scan, Inc., West Chester, PA). Scanning electron microscopy (SEM) analyses of samples A and B were performed on a JEOL JSM 820I scanning electron microscope with energy dispersive X-ray detection on a Kevex system 8000 with light element detection (Oneida Research Services, Inc., Whitesboro, NY). The samples were mounted on aluminum SEM stubs with carbon paint, and coated with a thin film (~30 nm) of gold/palladium as needed. Spectra were collected with accelerating voltages of 15 and 30 kV.

Results:

Samples A and B vary substantially in the ability to stimulate cAMP accumulation in rat L6 myocytes (Figure 2). Sample B caused significant accumulation of cAMP at concentrations of less than 100 nM, but sample A showed only minimal effects at concentrations of up to 1.0 µM.

All samples were reported by the suppliers to be greater than 98% homogenous, but our analysis found the major component of sample A to be only 93% of the total peak area. Upon coinjection on HPLC, the major component of samples B, C and D (>98%, in all cases) coeluted, but the major component of sample A eluted approximately 15 seconds earlier (Figure 3A). Capillary zone electrophoresis supported the HPLC results.

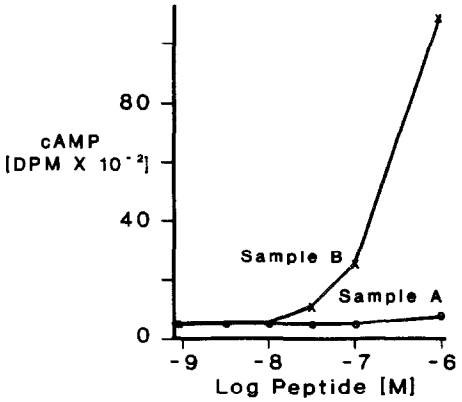


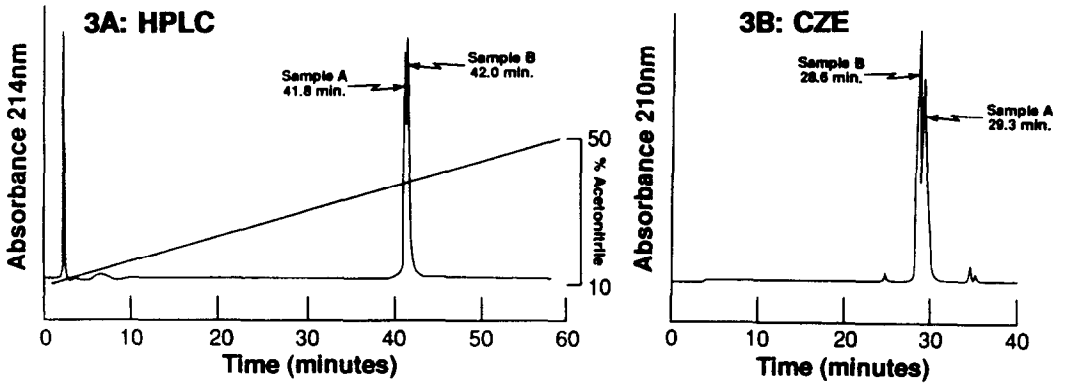
Figure 2: Stimulation of cAMP Production in Rat L6 Myocytes by Rat Amylin Amide Obtained from Two Different Commercial Sources (Samples A and B, n = 2).

DPM = Disintegrations Per Minute

Impurities in sample A were responsible for the additional peaks observed in the CZE trace (Figure 3B), and it was also shown that the major components of samples A and B did not comigrate. Independently, sample B was shown to comigrate with samples C and D.

Both samples A and B were reduced with dithiothreitol (37°C, 1 h, pH > 8). HPLC analysis showed that the major component of these solutions coeluted. Reoxidation with excess potassium ferricyanide (37°C, 1 h, pH > 8), resulted in products which also coeluted with each other and untreated sample B.

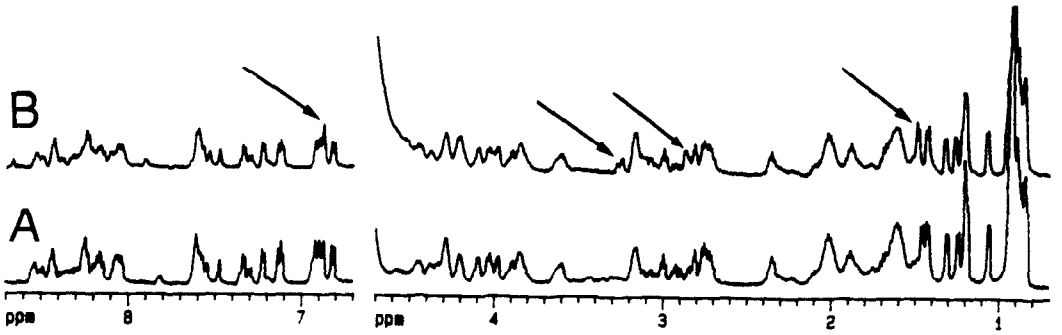
Figure 3: Analytical HPLC and CZE Traces for the Coinjection of Samples A and B.



Automated Edman sequencing of samples A and B showed that the primary sequence of both samples was consistent with rat amylin amide. Cysteine had not been derivatized and therefore was not directly observed, but a peak resulting from its decomposition to dehydroalanine was present. Amino acid analysis following acid hydrolysis supported the sequencing results and indicated the presence of cysteine.

Only relatively minor chemical shift differences were apparent in the one-dimensional 500 MHz proton NMR spectra of samples A and B (Figure 4). These differing resonances were assigned to the primary amide protons of an asparagine or glutamine (6.9 ppm), the methyl protons of an alanine (1.5 ppm) and the beta protons of an amino acid with an AMX-type spin system (2.9 and 3.2 ppm). From the primary sequence of

Figure 4: One-Dimensional 500 MHz ^{1}H NMR Spectra of Samples A and B.



rat amylin amide, these resonances must correspond to the beta protons of either a cysteine, tyrosine or phenylalanine residue.[17]

Capillary HPLC with ES-MS detection revealed that the actual molecular weight of the major component of sample A was actually 4120 and at least two other minor components were present with molecular weights of approximately 4087 and 3923. The results from liquid-SIMS analyses of both samples in a nonreducing matrix (2-nitrobenzyl alcohol) were consistent with those obtained by electrospray ionization (Figure 5). Scanning electron microscopy coupled with energy dispersive X-ray analysis specifically indicated the *presence of mercury in sample A, but not in sample B.*

Discussion:

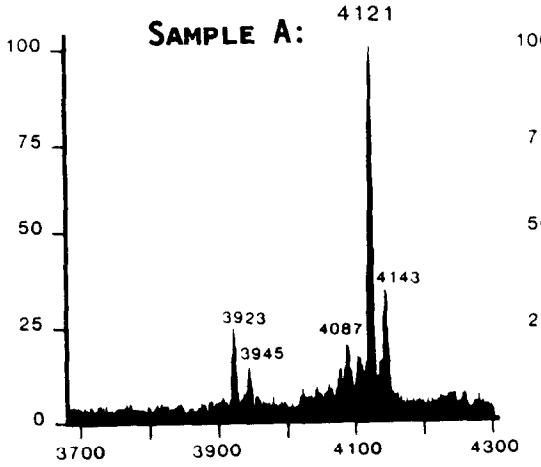
Three of the four commercial samples (B, C and D) were homogenous and structurally equivalent to each other based upon HPLC coelution and CZE comigration, but sample A was clearly different by both techniques. However, additional analyses of sample A by Edman sequencing, amino acid analysis, ^{1}H-NMR and a limited mass spectral analysis was consistent with the primary structure of rat amylin amide. The possible hydrolysis of the C-terminal or a side chain amide of an asparagine or glutamine residue could not *be readily determined from the first mass spectral analysis tried, due to the lack of sufficient resolution in this* range. However, attempted derivatizations of both samples A and B with methanolic HCl did not provide any evidence of methyl ester formation to suggest that amide hydrolysis had not occurred.

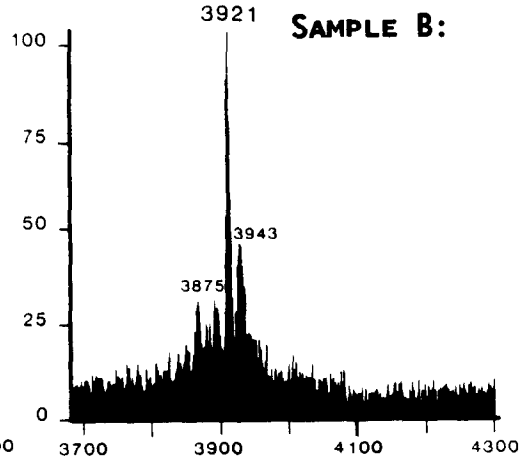
The results of the reduction/reoxidation experiment suggests that the difference between samples A and B is localized to the disulfide bond and that the pharmacologically inactive sample A is not rat amylin amide. The relatively small differences in HPLC elution and CZE mobility did not suggest that the peptide had dimerized or oligomerized during the oxidation of the cysteines to cystine or that the two samples differed in *overall net charge. Further evidence to implicate the disulfide bond was obtained from the one-dimensional* ^{1}H NMR spectra. All proton chemical shift differences between the two samples could be attributed to residues in the 2-7 cystine bridged sequence.

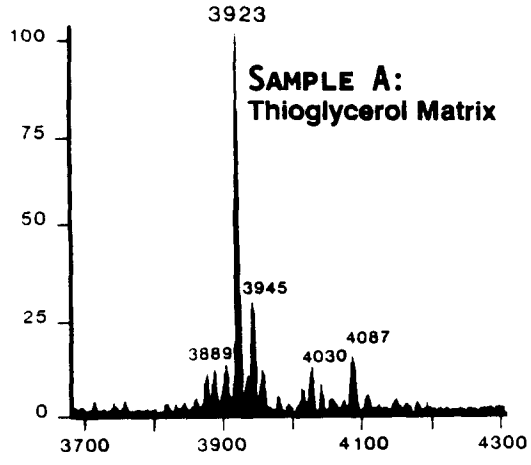
Electrospray ionization mass spectrometry showed that the actual molecular weight of sample A was approximately 200 mass units greater than that expected for rat amylin amide (3920.5). Since ^{1}H NMR evidence does not suggest the presence of residual protecting groups or other obvious modifications,

contamination with heavy metals was considered. The data for sample A was consistent with the covalently bound mercury complex of rat amylin amide, probably complexed to the sulfurs of cysteine 2 and 7. Mercury may have been introduced during the deprotection and oxidation of the cysteines to cystine since mercuric acetate is routinely utilized for removal of the acetamidomethyl (Acm) protecting group.[16] In fact a peak which may correspond to sodium cationized bis-Acm protected rat amylin amide (m/z 4087) was observed in both the ES-MS and liquid SIMS analyses (Figure 5). It should be noted that previous FAB-MS analyses failed to

Figure 5: Liquid SIMS Mass Spectral Data for Samples A and B in a 3-Nitrobenzyl Alcohol Matrix and Sample A following the Addition of Thioglycerol to the Matrix.







ANALYSIS OF MASS SPECTRAL DATA FOR RAT AMYLIN AMIDE[a] (RAA)

M/Z	IDENTIFICATION
3921	$[M + H]^+$, RAA
3923	$[M + H]^+$, reduced RAA
3943	$[M + Na]^+$, RAA
4087	$[M + Na]^+$, bis-Acm protected RAA
4121	$[M + H]^+$, Hg-RAA[b]
4143	$[M + Na]^+$, Hg-RAA[b]

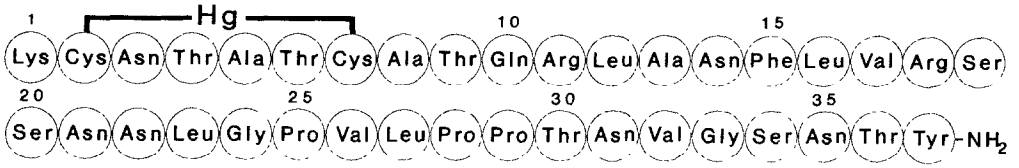
[a]Average mass of RAA = 3920.5
[b]Mercury complex of RAA

detect the mercury containing peptide, due in part to the upper range limitations of the instrumentation (m/z 4000) and the choice of thioglycerol for the matrix. Ultimately, the presence of mercury was verified by scanning electron microscopy with energy dispersive X-ray detection.

In summary, it has been demonstrated that at least one commercial source of rat amylin amide is contaminated with mercury, which is probably covalently bound to the sulfurs of cysteine 2 and 7 (Figure 6).

This observation may account for some of the apparently contradictory reports on the pharmacology of rat amylin amide. This study shows that heavy metal contamination must be considered as a possible cause when discrepancies in the biological properties of peptides are observed.[19,20]

Figure 6: Proposed Structure of the Rat Amylin Amide Mercury Complex (Peptide A).



Acknowledgement: The authors wish to thank Dr. Philip C. Andrews (University of Michigan) for the peptide sequencing data, Drs. Donald Hunt and Jeffrey Shabanowitz (University of Virginia), Mark Rodgers (M-Scan, Inc.), Bruce Wegter and Ms. Karen Thomann (Oneida Research Services, Inc.) for spectral data and Mr. David Oliver (DRDA Graphic Services) for preparation of the illustrations.

References and Notes:

1. Westermark, P.; Wernstedt, C ; O'Brien, T.D., Hayden, D.W ; Johnson, K H. *Am. J. Pathol.* **1987,** *127,* 414.
2. Copper, G.J S., Willis, A.C ; Clark, A, Turner, R C , Sim R.B.; Reid, K B.M. *Proc. Natl Acad. Sci. USA* **1987,** *84,* 8628
3 Lukinius, A.; Wilander E.; Westermark, G T.; Engstrom, U , Westermark, P *Diabetologia* **1989,** *32,* 240
4 Kahn, S E ; D'Alessio, D A ; Schwartz, M.W , Fujimoto, W.Y., Ensinck, G W. Jr , Porte, D Jr. *Diabetes* **1990,** *39,* 634.
5. Leighton, B., Cooper, G.J.S *Nature (Lond.)* **1988,** *335,* 632.
6 Cooper, G J.S ; Leighton, B; Dimitriadis, G D., Parry-Billings, M.; Kowalchuk, J M., Howland, K , Rothbard, J B ; Willis, A C ; Reid, K B.M. *Proc. Natl. Acad. Sci. USA* **1988,** *85,* 7763.
7. Leighton, B ; Foot, E *Biochem. J.* **1990,** *269,* 19
8. Leighton, B.; Cooper, G J S *Trends Pharmacol Sci.* **1990,** *15,* 295.
9. Molina, J.M ; Cooper, G.J S , Leighton, B.; Olefsky, J.M. *Diabetes* **1990,** *39,* 260
10. Kreutter, D.; Orena, S J.; Andrews, K M. *Biochem. Biophys. Res. Commun* **1989,** *164,* 461
11 Roberts, A.N.; Leighton, B., Todd, J A.; Cockburn, D , Schofield, P.N , Sutton, R; Holt, S., Boyd, Y , Day, A J , Foot, E A.; Willis, A C., Reid, K.B.M ; Cooper, G.J.S *Proc Natl Acad. Sci. USA* **1989,** *86,* 9662.
12. Nishi, M., Sanke, T., Nagamatsu, S.; Bell, G.I , Steiner, D F *J. Biol. Chem* **1990,** *265,* 4173.
13 Steiner, D.F ; Ohagi, S ; Nagamatsu, S ; Bell, G I., Nishi, M. *Diabetes* **1991,** *40,* 305
14. Johnson, K.H., O'Brien, T D., Westermark, P. *Diabetes* **1991,** *40,* 310
15 Stephens, T W ; Heath, W.F , Hermeling, R.N. *Diabetes* **1991,** *40,* 395.
16. Zhu, G.; Dudley, D.T , Saltiel, A.R *Biochem. Biophys Res. Commun* **1991,** *177,* 771
17 Wüthrich, K *NMR in Biological Research. Peptides and Proteins,* North-Holland Publishing Co. Inc.: Amsterdam, 1976, pp 42-55
18. Veber, D F.; Milkowski, J D., Vargas, S L.; Denkewalter, R G., Hirschmann, R. *J. Amer. Chem. Soc.* **1972,** *94,* 5456
19 Heavner, G.A., Cody, W L., Crowther, J ; Duhl-Emswiler, B , May, K., Salomons, P ; Pascone, J *Int. J Peptide Protein Res.* **1990,** *36,* 188.
20. Upon completion of this study a similar observation was reported using ES-MS and atomic absorption; Berman, J D., Kassel, D B , Cobb, J E.; Anderegg, R.J Poster No. 585, *at The 12th American Peptide Symposium,* Cambridge, MS, **1991.**