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An LC-MS/MS Assay for Detection and Quantification of the Dipeptide Gly-Gln in Rat Brain

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Abstract

The enzymatic cleavage products of β -endorphin (β -endorphin₁₋₂₇ and Gly-Gln) reduce voluntary alcohol consumption in alcohol preferring P rats. Gly-Gln also inhibits the reward benefiting effects of morphine and nicotine. It would be useful for the investigation of this effect to have an analytical method suitable for Gly-Gln detection and quantitation. Given the now widespread availability of LC-MS/MS instruments, the development of an LC-MS/MS-based approach seemed a viable option. An LC-MS/MS method for Gly-Gln quantitation was developed based on derivatization with Marfey's reagent. The Marfey's adduct of Gly-Gln (Mar-Gly-Gln) was chromatographically resolved and readily detected and quantitated by LC-MS/MS. Precursor/product positive ions of 456.2/366.2, 456.2/237.2, 456.2/147.0 were used for detection and quantitation. This method shows good linearity from 1 to 500 pmole of Mar-Gly-Gln ($R^2 > 0.99$). The assay also demonstrated good accuracy and precision, with an average percent standard deviation for Gly-Gln over the range of the assay of <5%. A combination of MRM fragment ratio normalization and chromatographic peak shifting were used to ensure that the LC-MS/MS peak for Mar-Gly-Gln was free from possible isobar interferences. This assay was then demonstrated for the determination of *in vivo* Gly-Gln levels in P and Sprague-Dawley rat cortex and nucleus accumbens samples.

Keywords

Dipeptide; brain; Marfey's reagent; LC-MS/MS; addiction

The opioid β -endorphin is one of many influences on voluntary ethanol consumption in rats. Two cleavage products of β -endorphin₁₋₃₁ – β -endorphin₁₋₂₇ and Gly-Gln (β -endorphin₃₀₋₃₁) – are implicated in reduction of ethanol intake [1; 2; 3; 4; 5; 6]. β -endorphin₁₋₂₇ and Gly-Gln act in different ways. The 27 residue peptide and classical opioid antagonists act by blocking mu and delta receptors, whereas Gly-Gln does not significantly bind to opioid receptors, does not displace naltrexone bound to mu or delta receptors, and does not bind to other known neurotransmitter receptors or transporters [7]. It apparently acts by a unique

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and as yet undefined mechanism [5]. Along with inhibition of voluntary alcohol consumption activity, Gly-Gln has also been shown to inhibit the reward benefiting effects of morphine and nicotine [8; 9; 10]. All these findings corroborate the fact that Gly-Gln has an interesting and potentially useful pharmacological activity. Gly-Gln has previously been quantitated in sheep and pig pituitary gland using radioimmunoassay (RIA) [1; 2]. Given the widespread availability LC-MS/MS technology, and its relative ease of use and lack of requirement for radiolabeled analytes, we felt it would be beneficial to develop an LC-MS/MS assay for detection and quantification of Gly-Gln in the brain. The ability to quantitatively determine *in vivo* levels of Gly-Gln is fundamentally important to understanding its biochemistry, physiological function, and for the development of active analogs and derivatives.

Detection and quantification of small amines in complex biological samples is a challenge, and generally requires precolumn derivatization for retention and separation by reverse-phase HPLC. Ninhydrin and α -phthalaldehyde (OPA) have been the most widely used derivatization reagents for UV-vis detected analyses, however the instability of their adducts is a significant limitation [11; 12]. Marfey's reagent is a chiral analog of Sanger's reagent, which is used to derivatize chiral amino acid mixtures prior to separation on achiral media to determine chiral purity [13; 14; 15]. In a recent study we reported the use of LC-MS/MS detection and quantitation for Marfey's derivatives of the racemic amino acids L-Ala and D-Ala, and the dipeptide D-Ala-D-Ala [16]. LC-MS/MS detection of Marfey's derivatives of low molecular mass hydrophilic amines appears to be a potentially general approach to the stereospecific detection and quantitation of this otherwise challenging class of biomolecules. Given our interest in Gly-Gln, the present effort was directed towards expanding the application of Marfey's derivatization and LC-MS/MS analysis to the difficult analytical problem of detecting and quantitating of Gly-Gln in rat brain extracts. This assay demonstrated that *in vivo* detection and quantification of Gly-Gln in brain extracts by LC-MS/MS is feasible, which opens the door for an improved understanding of how Gly-Gln works to reduce addictive behavior, and for the development and characterization new anti-addictive Gly-Gln analogs and derivatives in a similar fashion.

Materials and methods

General

Gly-Gln was purchased from Bachem (Torrance, CA) and Gly-Asn from Sigma-Aldrich (St. Louis, MO). C18-silica gel was obtained from Sep-Pak Cartridges (Waters Co, Milford MA). Marfey's reagent (1-fluoro-2,4-dinitrophenyl-L-5-alanine amide) was purchased from Novabiochem (a division of EMD Chemicals, Gibbstown, NJ). LC-MS/MS was performed on an Applied Biosystems Sciex 3200 QTrap LC-MS/MS mass spectrometer equipped with a Shimadzu UFLC HPLC system using an electrospray ionization (ESI) source in positive mode, and run using Analyst® v. 1.4.2. All chromatographic separations were performed on Nucleodur 100-3 C8 125 × 2.0 mm column (Macherey-Nagel, Bethlehem PA).

Animals

Two strains of rats were used in this work. Male alcohol preferring (P) rats (Center for Alcohol Studies, Indiana University Medical Center, Indianapolis, IN) and male Sprague-Dawley rats (SD) (Charles River), weighing 300–350 g. Rats were maintained with food and water *ad libitum* under an established 12/12 h photoperiod (lights on: 0700 h). Each rat was handled daily and maintained in individual cages in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication No. 80-23, revised 1996), with all experimental protocols approved by the UMKC Animal Care and Use

Committee. All rats were alcohol naive. Fresh frozen pig brain was obtained from a local butcher shop (Biedermeyers, Kansas City, MO).

Marfey's Gly-Gln derivatization reaction

A standard solution of the Marfey's adduct of Gly-Gln (Mar-Gly-Gln) was prepared by diluting 1.5 μL of 5 mM Gly-Gln to 150 μL with H_2O to give a 50 μM sample. A 20 μL aliquot of this sample (1 nmol) was added to 20 μL of 10 mM Marfey's reagent in acetone, and then 5 μL of 1 M triethylamine was added to this mixture to initiate the reaction. The contents were mixed well and kept in an incubator at 37 $^{\circ}\text{C}$ for 120 minutes. The derivatization reaction was quenched by acidifying with 5 μL 1 M HCl, and the sample diluted with 150 μL of 70% H_2O /30% acetonitrile/0.1 % formic acid (final Mar-Gly-Gln concentration of 5 μM). This provided a standard solution of Mar-Gly-Gln suitable for use in initial experiments to optimize separation and quantitation parameters.

MS and MS/MS optimization

Salts interfere with MS detection of Marfey's derivatives. To remove salts prior to infusion for MS and MS/MS optimization, a 100 μL sample of the Mar-Gly-Gln standard solution was purified on 200 mg of C18-silica packed in a 1 mL syringe. C18-silica was prepared by first washing with MeOH (1 mL), and then pre-equilibrated with 1 mL of H_2O /0.1% formic acid. After adding the Marfey's derivatized sample, the C18-silica was washed with 100% H_2O /0.1% formic acid. Marfey's adducts were then eluted with 75% MeCN/25% H_2O /0.1% formic acid to provide a sample free of salts. MS, MS/MS and flow (LC-MS/MS) parameter optimization were performed using the desalted Marfey's adduct sample and the automated quantitative optimization routine in Analyst®. This optimization process resulted in the MS/MS settings given in Table 1.

Standard chromatographic conditions

Chromatographic elution was performed at a flow rate of 300 $\mu\text{L}/\text{min}$, with a gradient of 85% solvent A (100% H_2O /0.1% formic acid) and 15% solvent B (70% MeCN/30% H_2O /0.1% formic acid) for 1 minute, followed by a 22 minute linear gradient to 75% solvent A and 25% solvent B.

Determination of response linearity, lower limit of detection (LLOD), lower limit of quantitation (LLOQ), possible matrix effects in pig brain samples, and carryover

For linearity, LLOD, and LLOQ determination, serial dilutions of Gly-Gln were prepared in water containing 0.45 μM Gly-Asn as an internal standard. To determine if matrix (brain extract) would interfere with analyte detection, a pig brain extract was prepared (using the same procedure as described below for rat brain samples), and an identical serial dilution was prepared in this extract with 0.45 μM Gly-Asn added to all samples as an internal standard. These serially diluted samples were derivatized with Marfey's reagent as described above, and LC-MS/MS analysis was performed using the MS/MS settings given in Table 1. Carryover was analyzed by alternately analyzing blank samples and Gly-Gln standard at upper limit of quantitation (ULOQ, 500 pmoles).

Preparation of rat brain samples for analysis

The nucleus accumbens (NAc) samples were isolated from rat brain using the following coordinates. NAc: anterior-posterior +2 to -4.8 mm from bregma; -5 mm from dorsal surface to base of brain; +/- 1.5 mm lateral to the midline. The cortex sample was taken from above the NAc from above the corpus callosum [17]. Samples weighed between 200-400 mg, and to these were added five volumes (v/w) of ice cold acetone/water (80/20) containing internal standard (Gly-Asn) at 0.45 μM . The mixture was homogenized for 10-20

seconds on ice, centrifuged at 14,000×g at 4°C for 5 minutes, and 200 µL of the supernatant taken and dried under vacuum. Dried samples were reconstituted with 100 µL of 70% water/30% acetonitrile/0.1% formic acid. This was followed by addition of 20 µL of 10 mM Marfey's reagent in acetone, 5 µL of 1M triethylamine (dissolved in water), incubated at 37°C for 120 min and then the mixture was neutralized with 5 µL 1M HCl. This was again dried down under high vacuum and made up to a final volume of 60 µL with 70% water/30% acetonitrile/0.1% formic acid. 20 µL of this mixture was injected for LC-MS/MS analysis.

Peak shifting and MRM channel ratio normalization

Examination of rat brain extract MRM chromatograms for Mar-Gly-Gln revealed several spurious (isobar) signals in some of the MRM channels. To determine if any possible interfering isobars were co-eluting with Mar-Gly-Gln, a combination of peak shifting and MRM channel ratio normalization was performed. Peak shifting was performed using the standard chromatographic conditions described above with a constant admixture of 0-20 % of channel D (25% methanol) in place of that percentage of solvent A. This had the effect of reducing the retention time of eluted peaks. Normalization was performed by using the MRM peak ratios from a Mar-Gly-Gln standard as a reference. The normalized peak ratios were calculated relative to the most intense peak (456.2/336.2). The other peak ratios for the Mar-Gly-Gln standard for 456.2/237.2 and 466.2/147.0 were 0.41 and 0.21 respectively (1:0.41:0.21). Normalizing the three channels for sample MRM chromatograms based on this ratio had the effect of making all three MRM channels of equal intensity for pure Mar-Gly-Gln.

Results and Discussion

Method development

A desalted sample of Mar-Gly-Gln was prepared and infused into the MS instrument, and a good signal for the precursor $[M+H]^+$ ion was observed. Analyst® software quantitative optimization algorithms were used to optimize for LC-MS/MS-based quantitation on the three most intense parent ion fragment peaks (Fig.1). This process gave the parameters listed in Table 1 for optimum sensitivity for detection of Mar-Gly-Gln. The same procedure was followed to develop a method for quantification of Mar-Gly-Asn for use as an internal standard.

The next step was to determine if this method could be used to detect Gly-Gln in rat brain extracts. A portion of the rat brain containing the nucleus accumbens (NAc) was isolated, homogenized, derivatized with Marfey's reagent, and analyzed by LC-MS/MS. A signal for Mar-Gly-Gln was observed, but was weak. The extraction procedure was altered to concentrate the analyte before injection, revealing a readily detectible peak for Mar-Gly-Gln (Fig. 2).

Assessment of analyte recovery and stability

To assess analyte recovery using our extraction procedure, rat brain samples were processed as described above to obtain extracts. The resulting pellets were re-extracted by re-suspending in five volumes of acetone/water with internal standard, incubated on ice for 10 min, and the supernatant recovered. The analyte recovered from the second extraction averaged 15% of the amount of the analyte recovered in the first extraction, demonstrating an 85% recovery in the one step extraction process used in this study.

Gly-Asn was chosen as internal standard as it is structurally similar to Gly-Gln, and initial results did not show any detectable levels of Gly-Asn nor interfering isobars in rat or pig

brain samples. Marfey's adducts were stable for at least 48 h at room temperature and for at least 3 months at -20°C and are stable in an auto sampler at room temperature during analysis or in a freezer for long term storage. To assess the recovery of internal standard, Gly-Asn was added to a concentration of $0.45\ \mu\text{M}$ to a fresh crude homogenate of rat brain in five volumes of acetone/water (80/20) and $0.45\ \mu\text{M}$ of Gly-Asn was similarly diluted. The recovery of Gly-Asn in the brain extract sample was 95% relative to the water extract sample.

A final concern was the stability of the analyte in the crude rat brain extract in acetone/water. To address this concern we performed a stability time course by incubating an acetone/water rat brain homogenate spiked with Gly-Gln on ice (i.e., as generated and handled as described during the extraction process), taking samples periodically over 4 hours for immediate derivatization with Marfey's reagent. No significant decrease in measured Gly-Gln levels (e.g. $<3\%$ decrease) were observed over the 4 hr incubation period. This demonstrates that the ice cold acetone extraction process effectively stops metabolism of Gly-Gln.

Assessment of linearity and matrix effects

In order to determine the linearity and sensitivity of this assay for Gly-Gln, serially diluted samples were prepared, derivatized with Marfey's reagent, and analyzed with this method. To assess the possible matrix effect of brain extract on analyte sensitivity, serially diluted samples were prepared both in water and pig brain extract for comparison. Analysis of these samples demonstrated good linearity, with $R^2 > 0.99$ (Fig. 3). Samples diluted in water gave a small but significantly higher signal than samples diluted in matrix (pig brain extract), with matrix giving 90% of the signal observed in water. The %CV at a given analyte level ranged from 4% at the ULOQ to 15% at the LLOQ. Error bars are not shown in Figure 3 due to crowding. The response curve therefore has good dynamic range and good quantitative (linear response) characteristics.

Sensitivity and carryover

Using this assay the lower limit of detection (LLOD) was determined to be 1 pmole ($1.5\ \text{pmoles/mg}$ of brain) (by the criteria of signal/noise (SD) ratio of 3:1) and the lower limit of quantitation (LLOQ) was 4 pmoles ($5.8\ \text{pmoles/mg}$ of brain) (by the criteria of signal/noise ratio of 7:1). There was no detectable carryover observed between samples even at the highest concentration (500 pmoles) of Mar-Gly-Gln.

Selectivity

Isobar peaks were observed in the MRM chromatogram of rat brain extract. To exclude the possibility of isobar interference in the detection and quantitation of Mar-Gly-Gln, a combination of peak shifting and MRM channel ratio normalization were used (Fig. 4). Signals from the three MRM channels were overlaid after normalizing their intensity to the MRM peak intensities observed for a Mar-Gly-Gln standard run under identical conditions. Such a normalized MRM chromatogram will show equal MRM channel peak heights for Mar-Gly-Gln, but isobars will be unlikely to show identical MRM peak ratios. Peak shifting was performed with a fixed level of Channel D (25% methanol) added to the gradient. In general, different compounds will be shifted in the chromatogram to different degrees. This combined MRM peak ratio normalization and chromatographic peak shifting analysis demonstrated that Mar-Gly-Gln is well resolved and free from possible isobar interference (Fig. 4). As a further test of selectivity, a blank and LLOQ spiked pig brain extract were also analyzed (Fig. 5). (Pig brain showed undetectable levels of Gly-Gln (Fig. 5), in contrast to rat brain which showed readily detectable levels (Fig. 4). This demonstrates that this analytical method has a high degree of selectivity for Mar-Gly-Gln.

Accuracy and precision

The accuracy and precision were determined by analyzing Mar-Gly-Gln samples (n=6) at concentrations at LLOQ, Mid LOQ and ULOQ. Samples and standards were run intraday (same day) and interday (two weeks) (Table 2). The average percent coefficient of variation (%CV) for Mar-Gly-Gln at the ULOQ and Mid LOQ was 4%, which is a measure of the precision of the assay. The average deviation of the %nominal determined Mar-Gly-Gln for ULOQ and Mid LOQ intraday samples was 2.5%, which is a measure of the accuracy of the assay.

Quantitation of Gly-Gln in rat brain samples

This method was then applied to the *in vivo* detection and quantification of Gly-Gln in different rat strains (SD and P) and in different parts of the rat brain (cortex and nucleus accumbens). All samples were analyzed in quadruplicate (Fig. 6), and the quantity of Gly-Gln measured was standardized to the amount of rat brain initially extracted (pmoles Gly-Gln/mg of brain). The measured levels of Gly-Gln in SD and P rats were statistically the same, whereas the levels in different parts of rat brain were higher in cortex compared to nucleus accumbens in both SD and P rats ($P < 0.05$, t-test).

Conclusion

Gly-Gln is a known inhibitor of voluntary ethanol drinking in rats acting within the nucleus accumbens, and has also been shown to inhibit the reward benefiting effects of morphine and nicotine [3; 4; 5; 6; 7; 8; 9]. An assay for Gly-Gln would be of great utility for further investigation of this effect and in efforts to exploit this effect for the development of novel anti-addictive agents. The hydrophilic nature and low molecular weight of Gly-Gln precludes direct analysis by LC-MS/MS. To address this analytical problem a Marfey's derivatization based strategy was explored, which we have previously demonstrated for quantitation of L-Ala, D-Ala, and D-Ala-D-Ala from bacterial extracts [16]. Good linearity between 1 and 500 pmol was observed, with a LLOQ of 4 pmol. MRM peak ratio normalization and chromatographic peak shifting were used to demonstrate this specificity of this method, and finally demonstrated for detection of Gly-Gln in rat brain samples.

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Figure 1. MS/MS spectrum of Mar-Gly-Gln with the possible fragment ions (M of $[M+H]^+$).

Figure 2. LC-MS/MS chromatogram of Marfey's derivatized rat brain nucleus accumbens extract detected in positive mode at m/z 456.2/366.2 (Precursor ion/Product ion).

Figure 3. Standard curve of Gly-Gln serially diluted in pig brain extract, derivatized with Marfey's reagent, and quantitated by LC-MS/MS using (Precursor ion/Product ion)= 456.2/366.2.

Figure 4.

Results from MRM channel normalization and chromatographic peak shifting for Mar-Gly-Gln in rat brain nucleus accumbens extract. Raw data (collected at 3.6 sec time intervals sequentially in each channel) were smoothed by seven point moving average. Then the data were then normalized to the MRM peak ratios obtained from a pure standard sample of Mar-Gly-Gln under identical conditions. Top: normalized MRM chromatogram with the standard chromatographic (0% methanol) solvent system. Bottom: normalized MRM chromatogram peak shifted with 10% channel D (25% methanol) admixture into the standard chromatographic solvent system. MRM channels: Blue - 456.2/336.2; Green - 456.2/237.2; and Red-147.0/456.2.

Figure 5.

Results from analysis of pig brain extract with (red line) and without (blue line) the addition of Gly-Gln at LLOQ. Pig brain extract has undetectable levels of Gly-Gln present, in contrast to rat brain which has readily detectable levels (Fig. 4).

Figure 6.

Measured levels of Gly-Gln in rat brain extracts. Each individual analyte represented the Gly-Gln extracted from 2.8 mg of brain. Error bars represent the standard error ($n = 4$) for each measurement. SD(NAC), Sprague-Dawley (nucleus accumbens); SD(C), Sprague-Dawley (cortex); P(NAC), Alcohol preferring (nucleus accumbens); P(C), Alcohol preferring (cortex).

Table 1

Summary of optimized parameters for MS/MS detection of Marfey's adducts of Gly-Gln and Gly-Asn.

	Mar-Gly-Gln		Mar-Gly-Asn (IS)	
	m/z	Parent ion [M+H] ⁺ /Fragment ion	m/z	Parent ion [M+H] ⁺ /Fragment ion
<i>MS parameters</i>	456.2/ 366.2	456.2/ 237.2	456.2/ 147.0	442.2/ 352.5
DP (V)	—	51 —	—	51 —
EP (V)	—	5.5 —	—	5.5 —
CEP (V)	—	22 —	—	22 —
<i>MS/MS parameters</i>				
CE (V)	20	40	57	23
CXP (V)	6	6	4	6
CAD (Arb)	—	5 —	—	5 —
<i>ESI parameters</i>				
TEM (°C)	—	400 —	—	400 —
CUR (Arb)	—	26 —	—	26 —
GS1 (Arb)	—	60 —	—	60 —
GS2 (Arb)	—	60 —	—	60 —

Arb, arbitrary instrument-based setting; CAD, collisionally activated dissociation gas level; CE, collision energy; CEP, collision cell exit potential; CUR, curtain gas setting; CXP, collision cell exit potential; EP, entrance potential; DP, declustering potential; GS1 and GS2, gas flow 1 and 2 settingsd, respectively; IS, internal standard; TEM, ion spray temperature.

Table 2

Intra- and inter-day (2 weeks) precision and accuracy of Mar- Gly-Gln samples (n=6) in pig brain homogenate.

	ULOQ	Mid LOQ	LLOQ
Quantity (pmoles)	500	62.5	3.9
Intraday mean	490	60	4.3
Intraday SD	20	4	1.5
Intraday %CV	3.8	6.1	15.2
Intraday %nominal	99	96	110
Interday mean	500	58.5	4.1
Interday SD	20	0.5	2.0
Interday %CV	2.9	0.9	14.2
Interday %nominal	100	94	106

Intraday values from first day samples and standards, and interday values from the same samples and standards run after two weeks.