Analytical Biochemistry 425 (2012) 145–150

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A liquid chromatography–tandem mass spectrometry assay for detection and quantitation of the dipeptide Gly-Gln in rat brain

Sudheer Bobba [a], Garth E. Resch [b], William G. Gutheil [a,*]

[a] Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri–Kansas City, Kansas City, MO 64108, USA
[b] School of Biological Sciences, Division of Molecular Biology and Biochemistry, University of Missouri–Kansas City, Kansas City, MO 64110, USA

ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form 8 March 2012
Accepted 14 March 2012
Available online 21 March 2012

Keywords:
Dipeptide
Brain
Marfey's reagent
LC–MS/MS
Addiction

ABSTRACT

The enzymatic cleavage products of β-endorphin (β-endorphin$_{1-27}$ 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 these effects to have an analytical method suitable for Gly-Gln detection and quantitation. Given the now widespread availability of liquid chromatography–tandem mass spectrometry (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, and 456.2/147.0 were used for detection and quantitation. This method shows good linearity from 1 to 500 pmol of Mar-Gly-Gln ($R^2 > 0.99$). The assay also demonstrated good accuracy and precision, with an average percentage standard deviation for Gly-Gln over the range of the assay of less than 5%. A combination of multiple reaction monitoring (MRM) fragment ratio normalization and chromatographic peak shifting was 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.

The opioid β-endorphin is one of many influences on voluntary ethanol consumption in rats. Two cleavage products of β-endorphin$_{1-31}$—β-endorphin$_{1-27}$ and Gly-Gln (β-endorphin$_{30-31}$)—are implicated in reduction of ethanol intake [1–6]. β-Endorphin$_{1-27}$ 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 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 [8–10]. All of 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 [1,2]. Given the widespread availability of liquid chromatography–tandem mass spectrometry (LC–MS/MS)[1] technology and its relative ease of use and lack of requirement for radiolabeled analytes, we felt that it would be beneficial to develop an LC–MS/MS assay for detection and quantitation of Gly-Gln in the brain. The ability to quantitatively determine *in vivo* levels of Gly-Gln is fundamentally important to understanding its biochemistry and physiological function and for the development of active analogs and derivatives.

Detection and quantitation of small amines in complex biological samples is a challenge and generally requires precolumn derivatization for retention and separation by reverse-phase high-performance liquid chromatography (HPLC). Ninhydrin and *o*-phthalaldehyde (OPA) have been the most widely used derivatization reagents for ultraviolet–visible (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 [13–15]. 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 current effort was directed toward expanding

* Corresponding author. Fax: +1 816 235 5779.
E-mail address: gutheilw@umkc.edu (W.G. Gutheil).

[1] *Abbreviations used:* LC–MS/MS, liquid chromatography–tandem mass spectrometry; HPLC, high-performance liquid chromatography; ESI, electrospray ionization; P, alcohol-preferring; SD, Sprague–Dawley; MS, mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation; NAc, nucleus accumbens; MRM, multiple reaction monitoring; %CV, percentage coefficient of variation; mid-LOQ, mid level of quantitation.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.03.007

the application of Marfey's derivatization and LC–MS/MS analysis to the difficult analytical problem of detecting and quantitating Gly-Gln in rat brain extracts. This assay demonstrated that *in vivo* detection and quantitation of Gly-Gln in brain extracts by LC–MS/MS is feasible, opening 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, USA), and Gly-Asn was purchased from Sigma–Aldrich (St. Louis, MO, USA). C18 silica gel was obtained from Sep-Pak Cartridges (Waters, Milford, MA, USA). Marfey's reagent (1-fluoro-2,4-dinitrophenyl-L-5-alanine amide) was purchased from Novabiochem (a division of EMD Chemicals, Gibbstown, NJ, USA). 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 software (version 1.4.2). All chromatographic separations were performed on a Nucleodur 100-3 C8 column (125 × 2.0 mm, Macherey–Nagel, Bethlehem, PA, USA).

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, USA) and male Sprague–Dawley (SD) rats (Charles River), weighing 300 to 350 g. Rats were maintained with food and water *ad libitum* under an established 12/12-h photoperiod (lights on at 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 University of Missouri–Kansas City animal care and use committee. All rats were alcohol naive. Fresh frozen pig brain was obtained from a local butcher shop (Biedermyers, Kansas City, MO, USA).

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 °C for 120 min. The derivatization reaction was quenched by acidifying with 5 μl of 1 M HCl, and the sample was 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 mass spectrometry (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 preequilibrated 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 μl/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 min, followed by a 22-min linear gradient to 75% solvent A and 25% solvent B.

Determination of response linearity, LLOD, LLOQ, possible matrix effects in pig brain samples, and carryover

For linearity, lower limit of detection (LLOD), and lower limit of quantitation (LLOQ) determination, serial dilutions of Gly-Gln were prepared in water containing 0.45 μM Gly-Asn as an internal standard. To determine whether 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 pmol).

Preparation of rat brain samples for analysis

The nucleus accumbens (NAc) samples were isolated from rat brain using the following coordinates: 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 and 400 mg, and to these were added 5 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 to 20 s on ice and centrifuged at 14,000*g* at 4 °C for 5 min, and then 200 μl of the supernatant was 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 adding 20 μl of 10 mM Marfey's reagent in acetone and 5 μl of 1 M triethylamine (dissolved in water) and incubating at 37 °C for 120 min, and then the mixture was neutralized with 5 μl of 1 M 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. Then 20 μl of this mixture was injected for LC–MS/MS analysis.

Peak shifting and MRM channel ratio normalization

Examination of rat brain extract multiple reaction monitoring (MRM) chromatograms for Mar-Gly-Gln revealed several spurious (isobar) signals in some of the MRM channels. To determine whether any possible interfering isobars were coeluting with Mar-Gly-Gln, a combination of peak shifting and MRM channel ratio normalization was performed. Peak shifting was performed