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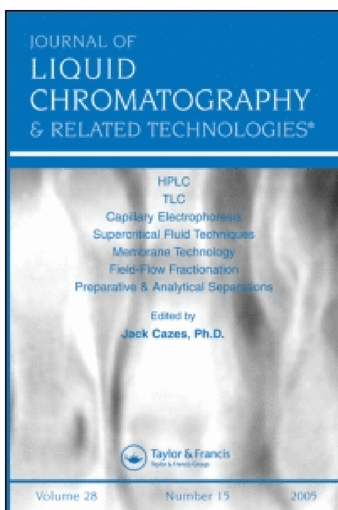

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Quantitation of the Polypeptide, Galanin, by Protein Precipitation and External Calibration LC/MS

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Abstract: The quantitation of galanin from rat plasma was conducted using protein precipitation and LC/MS. This method was externally calibrated, as no suitable internal standard was available. Galanin was detected in the positive ion mode by selected ion monitoring of the 3^{+}-charge state. The lower limit of quantitation was 10 ng/mL and calibration curves were linear over the concentration range from 10 to 1,000 ng/mL. Within and between run precision and accuracy was less than 12% at all validation points. The method was demonstrated by a limited pharmacokinetic study of galanin in rats.

Keywords: Galanin, LC-MS, Electrospray, Polypeptide, Absolute Quantitation, Plasma

INTRODUCTION

Immunoassays have commonly been used for the detection and quantitation of polypeptides since the 1960's when an immunoassay was worked out for determining insulin from human plasma.[1] These methods have been used because of their high sensitivity as well as the lack of better techniques. Immunoassays use specific antibodies that recognize and bind to certain structures of an intended analyte. One drawback to this is the very real possibility of cross-reactivity with other compounds (endogenous or other drugs) that can

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lead to erroneous test results. Even the use of monoclonal antibodies only reduces rather than eliminates cross-reactivity.[2] Quantitative techniques that further improve the confidence in a method's results are needed.

Another severe drawback to immunoassays is the long period of time and the large amount of money that is necessary for the development of new methods. If antibodies are not available for the analyte in question, it may take months to years before enough antibodies could be raised against a particular antigen.

Current advancements in the biotechnology industry may result in many new peptide based drugs for the treatment of various infections and diseases.[3–7] As is common in the pharmaceutical industry, only a small percentage of lead compounds are ever approved for retail sale. This means that there may be hundreds of potential compounds that fail toxicity and efficacy screenings. For these tests, sensitive quantitative methods are needed for each compound. Due to the high cost and time associated with the development of new immunoassays, assays that are developed more quickly and cheaply would be highly desirable to the biotechnology industry. Less severe problems associated with immunoassays include the fact that some immunoassays use radiolabels, which increase the cost of the assay and complicates sample handling and disposal.

Liquid chromatography (LC), coupled with mass spectrometry (MS), has gained ever increasing interest for the quantitation of peptides from biological matrices, as this technique can potentially meet these needs for a more reliable, inexpensive, and speedy alternative to immunoassays.[4–10] LC can effectively separate the analyte from other compounds. MS has the ability to separate and detect ionized molecules by their mass and charge. The combination of these two techniques can produce an analytical method that is both specific (minimizing the chance for false results) and sensitive to a polypeptide analyte.

Just as there are drawbacks with any analytical method, there are obstacles associated with the analysis of polypeptides by LC/MS. The first being difficulties associated with the extraction of polypeptides from a biological matrix. Biological matrices are very complex and usually contain a large amount of proteins and peptides. As a result of many peptide-protein interactions, it is not necessarily easy to remove a specific polypeptide from such a mixture. Peptides in general can be quite hydrophobic and can thereby bind to glass or plastic containers, especially when dissolved in only water. Another problem with the analysis of polypeptides is that LC methods that are used to separate peptides often use ion-pairing agents, such as trifluoroacetic acid.[11] These largely ionic compounds are often found to decrease ionization efficiency in the sources of mass spectrometers.

Despite the increased interest in quantitation of peptides by LC/MS, there are relatively few published methods to quantify polypeptides larger than 3000 Daltons.[4–10,12] To illustrate the ability of LC/MS methods to quantify large polypeptides, we present a method for the quantitation of the

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polypeptide galanin from rat plasma using protein precipitation sample preparation followed by LC separation and MS detection. Galanin is a 30 amino acid polypeptide with a molecular weight of 3157 Daltons.[13] As a peptide neurotransmitter, galanin is a critical part of the cholinergic system, and as such, it is involved in insulin release, spinal reflex, growth, depression, learning, and memory, among others.[14–16] As related to memory, galanin is associated with Alzheimer's disease.[14–16]

EXPERIMENTAL

Materials

Synthetic human galanin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetic acid was purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). Blank rat plasma was purchased from Harlan Laboratories (Indianapolis, IN, USA).

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LC/MS Assay

An Agilent 1100 series LC system (Palo Alto, CA, USA), consisting of a solvent degassing module, quaternary gradient pump, autosampler, and thermostated column compartment, was coupled with a Micromass Quattro II mass spectrometer fitted with a Z-spray ion source (Manchester, England, UK). Galanin was separated on a Biobasic C_{18} (2.0 x 50 mm) with a 5 μm particle size and a 300 Å pore size with a Biobasic C_{18} cartridge guard column (Keystone Scientific, Bellefonte, PA, USA). The column was maintained at 60°C. The mobile phase consisted of (A) water and (B) acetonitrile, each with 0.2% acetic acid and 0.02% trifluoroacetic acid (TFA). The organic gradient used for separation was linear from 19 to 35% B over eight minutes. Prior to column reequilibration, the column was washed with 95% B (see Table 1). The total run time was 24 minutes at a flow rate of 0.2 mL/min. The LC flow was directed into the mass spectrometer source without splitting. Prior to the injection of a sample set, at least 10 blank extractions were run in order to stabilize the retention time of the analyte.

The mass spectrometer was operated in positive ion mode with a capillary voltage of 4.0 kV and a cone voltage of 43 V. The source block was heated to 100°C, the desolvation gas was heated to 350°C at a flow rate of approximately 250 L/hour, and the nebulizing gas was set at its maximum flow rate. Selected ion monitoring (SIM) was used to monitor for galanin in its 3^+-charge state at m/z (mass to charge ratio) 1053.4. Galanin did not produce an abundant product ion upon collisionally-induced dissociation.

Table 1. Gradient used for separation, (A) water and (B) acetonitrile, each with 0.2% acetic acid and 0.02% trifluoroacetic acid. The total run time was 24 minutes

Time	A (%)	B (%)
0	81	19
8	65	35
9	5	95
13	5	95
15	81	19
24	81	19

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The instruments were controlled and the data processed by Micromass Masslynx v. 3.1 software.

Preparation of Stock and Standard Solutions

A stock solution of galanin was made by dissolving the lyophilized powder in blank rat plasma to give a concentration of 100 μg/mL and was stored at −20°C until use. Serial dilutions were made with blank plasma to give the following concentrations: 30, 24, 18, 12, 9, 6, 3, 2.25, 1.5, 0.75, and 0.30 μg/mL. All standards were made fresh each day. Of each of these standards, 5 μL were added to 145 μL aliquots of rat plasma. This yielded final concentrations of 1000, 800, 600, 400, 300, 200, 100, 75, 50, 25, and 10 ng/mL and a total volume of 150 μL. Both calibration and validation samples were then processed as described below.

Sample Preparation

The initial sample volume of 150 μL plasma was placed in 1.5 mL polypropylene centrifuge tubes. Acetonitrile was added to the plasma over 20 seconds with an infusion pump while vortexing to precipitate the plasma proteins. The sample was then centrifuged at 16,000 xg for five minutes. The supernatant was transferred to clean tubes and evaporated to dryness under vacuum. Dried samples were then reconstituted in 50 μL of a mixture of water, ACN, acetic acid, and TFA (85:15:0.2:0.02). Reconstituted samples were then centrifuged at 60,000 g for 10 minutes in order to remove insoluble particulates. Of the reconstituted sample, 40 μL was injected into the LC system for analysis.

Method Validation

Calibration curves for galanin were generated as spiked plasma samples. The calibration curves were constructed over the range from 10 to 1000 ng/mL using weighted (1/y) least-squares linear regression analysis of galanin peak areas. The precision and accuracy of the method was determined using five replicates of each of five concentrations of galanin; 10, 25, 75, 300, and 1000 ng/mL. Precision is indicated by the percent relative standard deviation within a single concentration in a validation set, and accuracy is expressed as the average percent error of the calculated concentration to the nominal concentration of each validation sample. Within-run precision and accuracy is calculated from all of the replicates of each of the five concentrations run in one set. Between-run precision and accuracy is calculated from all replicates of the same concentrations over three separately prepared sets.

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Animal Treatment

Galanin was dissolved in a buffer containing 0.01 M phosphate and 0.15 M sodium and chloride. The solution was then intravenously administered as a single dose of 0.5 mg/kg to male Sprague-Dawley rats. Each rat weighed approximately 250 to 300 grams. Blood was collected by jugular canula (c.a. 500 40 μL) and placed in heparinized 1.5 mL centrifuge tubes. Collection time points were 0, 0.5, 1, 2, 3, 4, 5, 7, 9, and 12 minutes after administration. Freshly collected samples were stored on ice no longer than 30 minutes before processing. The blood samples were centrifuged at 9,500 × g for five minutes to separate the plasma from the red blood cells. Plasma aliquots, 150 μL, were placed in clean tubes and immediately processed as described above.

RESULTS AND DISCUSSION

LC/MS Assay

The chromatographic gradient was optimized to provide good separation between galanin and endogenous compounds in the ion chromatograms. Initial organic composition was optimized with the gradient to produce a minimal analyte peak width. Some TFA (trifluoroacetic acid) was necessary to obtain a satisfactory peak shape. No significant ion suppression was observed with the low levels of TFA used. The addition of acetic acid was to lower the pH of the mobile phase for chromatographic separation, as well as to aid analyte ionization in the source of the mass spectrometer. The resulting chromatography provided baseline resolution of galanin from endogenous compounds, see Figure 1.

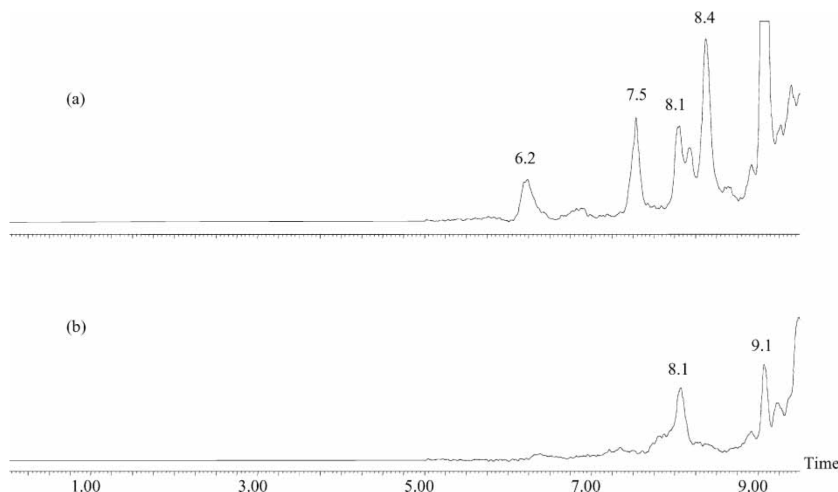

Figure 1. Typical ion chromatograms of m/z 1053.4 for (a) galanin (8.4 min) at the LLOQ (10 ng/mL), and (b) blank extracted plasma.

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The source conditions for the mass spectrometer were optimized by flow injection of a solution of galanin (10 μg/mL). For optimization, the mobile phase composition needed to be as close as possible to conditions of analyte elution. To do this, a makeup flow of 150 μL/min from the LC system was added to the flow from the infusion pump (50 μL/min). This resulted in a total flow rate of 200 μL/min entering the source of the mass spectrometer. The total flow had an organic and pH composition estimated to be the same as at the time of elution from the column. By altering the capillary and cone voltages, we were able to selectively maximize the 3^+-charge state of galanin. This particular charge state was chosen because it had the largest signal when optimized, as compared to optimizing for other charge states. The optimized source conditions produced the spectrum seen in Figure 2. Using collision induced dissociation, it was not possible to generate an abundant product ion for use in a multiple reaction monitoring (MRM) transition.

There was no commercially available stable isotope labeled galanin, so other peptides were tested as possible internal standards. Several peptides were tested including angiotensin I, II, and III, β-endorphin, motilin, and glucagon. In every case, galanin and the possible internal standard did not extract in the same way. Galanin always produced a better calibration curve with less variability in replicate samples when no internal standard was used. For this reason, we did not use an internal standard. If stable isotope labeled galanin was used as an internal standard, the LLOQ could likely be reduced to less than 5 ng/mL.

During the first several injections of a sample set, variability in the retention time of galanin was observed. The time increased by approximately

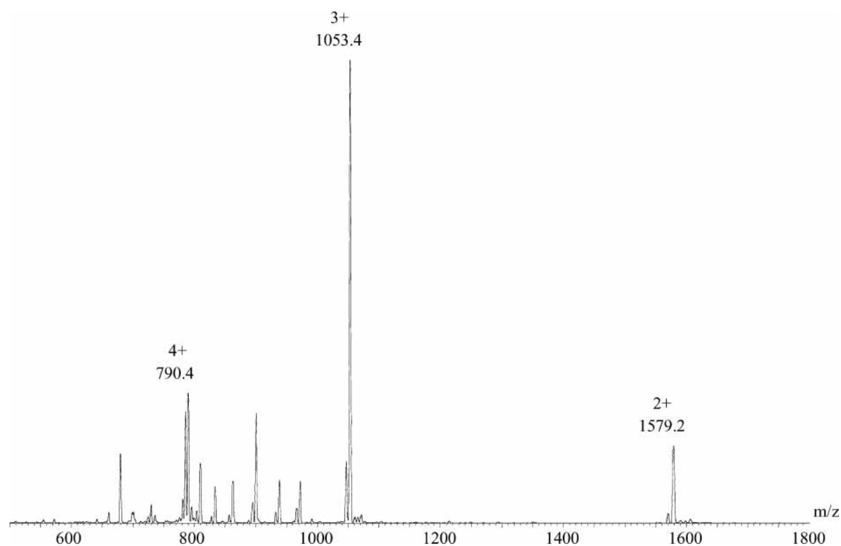

Figure 2. Spectrum of galanin showing its multiple charge states.

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one minute over the first eight injections of extracted plasma. We attribute this to an accumulation of peptides and proteins on the analytical column. They appear to have a strong affinity for the column as the organic wash does not remove them, although they do eventually run off of the column. We believe that these compounds provide an additional partitioning interaction with galanin resulting in an increased retention time. Allowing mobile phase alone to run through the column for several hours returned the elution back to its original retention time.

Preparation of Stock and Standard Solutions

All stock and standard solutions were prepared in blank plasma. This was done because plasma samples spiked with water standards were found to have more variability in resulting peak areas. When stocks were made in plasma, improvement was seen in response variability, sensitivity, and the linearity of calibration curves. This is likely a result of galanin adhering to sample vials when dissolved in water. The addition of plasma may add additional peptides and proteins that compete or saturate vial wall binding sites. It was found through stability experiments that galanin concentrations of standard solutions dropped very quickly when they were in water alone. By dissolving galanin in plasma, standard solution stability was greatly improved. Standard solutions of galanin dissolved in plasma still lost approximately 10% of the peptide within 20 minutes, but this allowed enough time to prepare and

process all samples before galanin concentrations diminished to a great extent. Yet because of the eventual loss of galanin in the standard solutions, fresh standard solutions were made daily, just prior to use. The stock solution (1 mg/mL) was kept frozen at -20°C to maintain stability. No significant loss of galanin was observed in the stock solution, as indicated by similar peak areas from the same concentration samples prepared on different days. Neither borosilicate glass nor deactivated glass tubes had improved analyte adhesion characteristics.

Sample Preparation

One of the most important steps for minimizing variability in the sample preparation was found in the precipitation of plasma proteins. It was found that the addition of acetonitrile for precipitation must be done slowly. Rapid precipitation yielded more variability in the responses and reduced sensitivity. This may be a result of clumping of the precipitate, possibly trapping some of the analyte. Slower precipitation while vortexing produced a much finer precipitate, and it also improved the sensitivity, as well as the variability in galanin peak areas.

It was also found that extracted galanin samples could not be filtered. Galanin bound so strongly to the filtration membranes that when samples were filtered, nearly 100% of the galanin was lost. In order to remove insoluble particulates prior to LC injection, all extracted samples were centrifuged at 60,000 g.

Due to galanin's tendency to adhere to sample vials and filtration membranes, there was concern over the possibility of galanin not being stable in the autosampler during the entire time needed for analysis for a sample set. In order to test this, eight galanin samples were extracted. After they were reconstituted and centrifuged, they were mixed and then redistributed into eight autosampler tubes and placed in the autosampler. The samples were mixed to remove any variability between samples as a result of preparation. The samples remained in the autosampler, and one was injected every three hours. No decrease in galanin peak areas was observed. The standard deviation of the peak areas was 5.9%, indicating that the samples were stable for at least 21 hours. This was more than the time needed for the analysis of a whole sample set.

Method Validation

With an initial sample volume of 150 μL plasma, this method for the quantitation of galanin was validated over the range from 10 to 1000 ng/mL. Accuracy and precision of the method were determined over three sample sets using five validation points at 10, 25, 75, 300, and 1,000 ng/mL with

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five replicates of each point on each day. Calibration points were at 10, 25, 100, 250, and 1000 ng/mL. Calibrations curves were generated by plotting these known galanin concentrations vs. the corresponding peak area with a 1/y weighting scheme. Weighting of 1/y puts additional emphasis on lower concentrations during linear regression. This weighting scheme was chosen over 1/x, $1/y^2$, and $1/x^2$ weightings because it gave the lowest sum of the percent residuals for the calibration curve. Percent residuals are calculated by dividing the residual for each point (from the linear regression) by the corresponding peak area and multiplying by 100%. This allowed the optimization of the calibration curve to give the most accurate calculated concentration, both at the top and at the bottom of the calibration range. All calibration curves had a minimum R^2 value of 0.99. In order to eliminate any bias based on injection order, all samples in a set were randomized prior to injection.

The LLOQ (lower limit of quantitation) for the method was set at 10 ng/mL. A typical chromatogram for galanin at the LLOQ is shown in Figure 1. Due to variation associated with protein precipitation, differing levels of endogenous compounds appear from sample to sample, as seen in Figure 1, peaks at 6.2, 7.5, 8.1, and 9.1 minutes. Even so, the extraction of galanin (8.4 minutes) remains consistent. With an appropriately labeled galanin internal standard, one may be able to decrease the LLOQ.

Accuracy and precision are represented as percent error and percent relative standard deviation (% RSD), respectively. Within run accuracy and precision at the LLOQ was determined to be 11.4% error and 10.3% RSD. The within run precision and accuracy was found to be less than 11.6% error and less than 6.5% RSD for all other points. Between run accuracy and precision at the LLOQ was 17.4% error and 10.9% RSD, respectively. All other validation points were found to have an error of less than 8.0% and less than 6.8% RSD for between run accuracy and precision. Detailed accuracy and precision data can be found in Table 2.

Recovery could not be calculated for the extraction because all galanin stock and standard solutions were made in plasma rather than water. Again, this was to slow analyte loss due to sample tube wall binding. As a result of galanin loss, water solutions were found to have much lower peak areas than equal concentration samples extracted from plasma.

Method Application

We demonstrated the utility of this method by determining the half-life of galanin in rats. We analyzed plasma from rats that were given a single IV bolus dose of galanin (0.5 mg/kg). Due to the expected short half-life of galanin, blood was drawn within the first 30 seconds after dosing. After collection, all samples were processed by the described method within 30 minutes.

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Table 2. Accuracy (average % error) and precision (% standard deviation) at five concentrations of galanin extracted from plasma for within run (n = 5) and between run (n = 15) validation

Conc (ng/mL)	Avg. error (%)	Std. dev. (%)
Within run		
10	11.4	10.3
25	5.8	6.4
75	2.5	2.9
300	11.5	3.4
1000	5.1	6.2
Between run		
10	17.4	10.9
25	5.7	6.7
75	3.9	3.1
300	7.9	3.7
1000	6.0	6.4

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A concentration vs. time plot of galanin is shown in Figure 3. Pharmacokinetic analysis shows that human galanin has a half-life of 1.0 minutes in rats. The calculated half-life is reasonable considering the half-life of galanin in humans is approximately 3.5 to 4.0 minutes.[17,18]

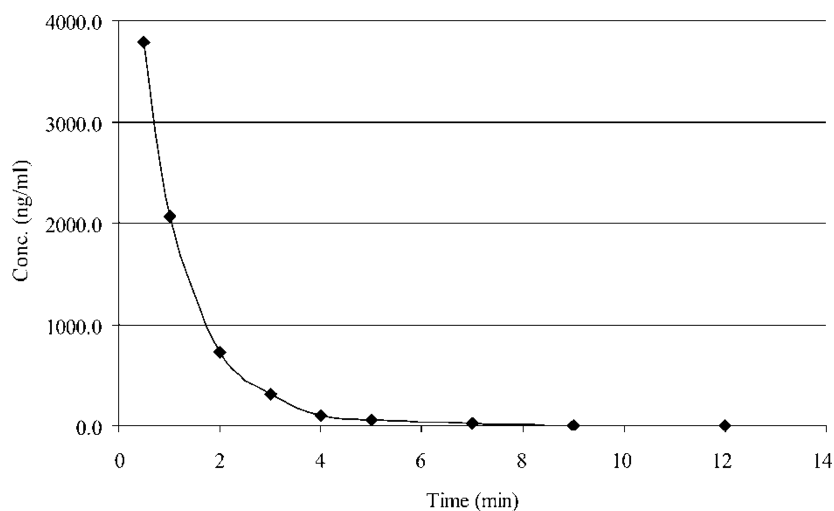

Figure 3. Concentration vs time plot for galanin administered to a male Sprague-Dawley rat with a dose of 0.5 mg/kg. The half-life was determined to be 1.0 minutes.

CONCLUSION

We have described a new LC/MS method for the quantitation of the polypeptide galanin from rat plasma. We were able to validate this method from 10 to 1000 ng/mL without the use of an internal standard. The LLOQ may be reduced, with an appropriate internal standard, to a level as low as 1 ng/mL. The method is sensitive, accurate, and specific to galanin. The method has proven to be useful and well suited for the determination of pharmacokinetic parameters.

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