Quantitative Analysis of Rat Spinal Cord by Acoustic Homogenization and Liquid Chromatography/Electrospray Tandem Mass Spectrometry

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INTRODUCTION

The study of neuropathic and chronic, inflammatory pain treatment involves the analysis of tissues such as spinal cord and brains. Intrathecal injection is a technique of administering the drug directly to the spinal cord with a needle or a chronically implanted catheter, bypassing the blood brain barrier.

A common method for tissue analysis involves mechanical homogenization of tissue samples by a metal blade followed by LC-MS/MS analysis of the tissue homogenate. After homogenizing a sample, the metal blade is washed with solvent(s) and used again for another sample homogenization. This process often results in cross contamination among the samples and is also time consuming and labor intensive.

In this study, we present a new method for spinal cord analysis using acoustic homogenization and LC-ESI-MS/MS. The principle of acoustic homogenization is based on computer generated acoustic shockwaves that are focused on tissue samples, leading to effective disruption and homogenization.

INSTRUMENTATION

- Sciex API5000 utilizing ESI positive mode
- Quadra 3 SPE from Tomtec Inc.
- Covaris E-series Model E-110 Acoustic Homogenizer
- Leap CTC Pal Autosampler with cooled stacker
- Shimadzu LC-20AD Pump
- Thermo Electron Corporation Hypersil Gold PFP analytical column, 150 x 2.1 mm column, 5 μm

METHOD

Rats were dosed intrathecally with gabapentin (Fig.1) into the subarachnoid space of the spinal cord. After 1 and 1.25 hours, the spinal cords were surgically removed. The spinal cord tissue samples were weighed, transferred into 4 mL glass tubes and frozen on dry ice for storage.

- 1) Samples were thawed at a later date and placed into a 4 x 6 metal rack
- 2) De-ionized water was added in a ratio of 4:1 (Water:Tissue) by weight into each of the glass tubes
- 3) The metal rack (with the spinal cord samples) was housed in a water bath maintained at a temperature of 10°C in the Covaris acoustic homogenizer system (Fig. 2), which focuses the acoustic shockwave into a single sample tube at a time
- 4) Samples were then sequentially homogenized (about 1.5 min per sample) by the Covaris system
- 5) Once the homogenization was completed, 25 μ L of the spinal cord homogenate was transferred into the Costar cluster tubes for analysis.

Spiking standards were prepared by serial dilution of the stock solution (20 $\mu g/mL$) with methanol (See Table 1).

METHOD (CONT'D)

After sample preparation (see Table 1), 20 μ L of the supernatant was injected directly into the LC/MS/MS system. The LC/MS/MS conditions are:

- Mobile Phase A: 5% MeOH in 0.1% Formic Acid
- Mobile Phase B: 95% MeOH in 0.1% Formic Acid
- Flow rate: 0.4 mL/min
- Wash 1: Acetic Acid/HPLC Water/ Isopropanol/Acetonitrile (0.1:60:20:20)
- Wash 2: Acetonitrile/MeOH (50:50) + 1% Acetic Acid

Analytical column: Thermo Electron Corporation Hypersil Gold PFP analytical column, $150 \times 2.1 \text{ mm}$ column, $5 \mu \text{m}$.

MRM: Gabapentin 172.1/154.0; Internal standard 200.1/95.1

The gradient elution program is shown in Table 2. Concentrations of gabapentin in study samples were determined using a weighted 1/x² linear regression of peak area ratios [peak area of gabapentin/peak area of internal standard] versus nominal concentrations of the calibration standards. Analytical data was captured by the Applied Biosystems program Analyst, version 1.4.1.

Peak areas were integrated by the Analyst program and the data from Analyst were imported into the Watson (Version 7.0.0.1, Thermo Electron) laboratory information software for further data processing.

Fig.1 Structure of Gabapentin and the Internal Standard

Gabapentin MW 171.2

Internal Standard MW 199.3

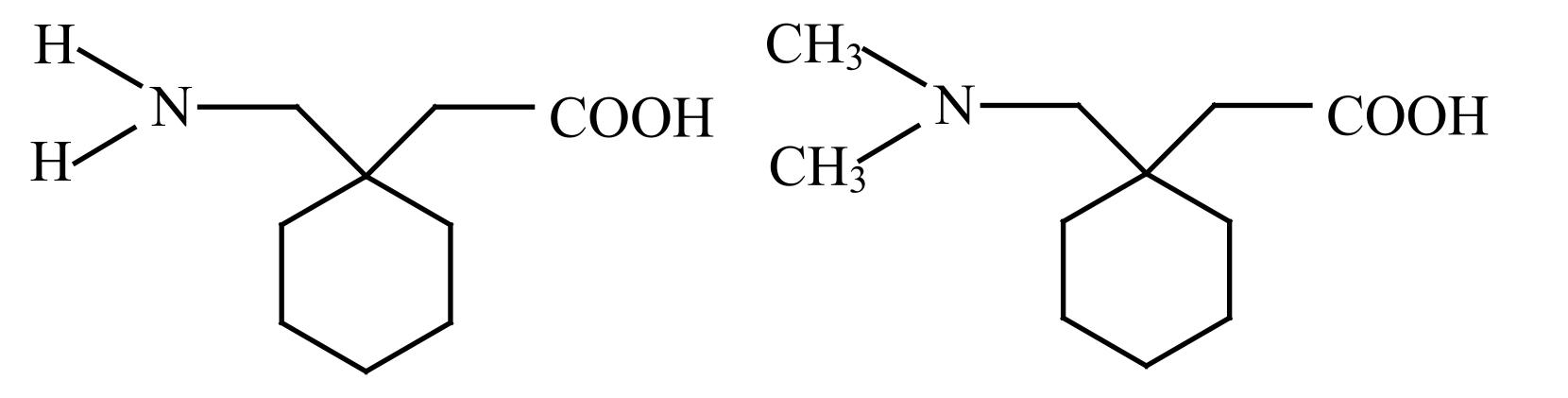
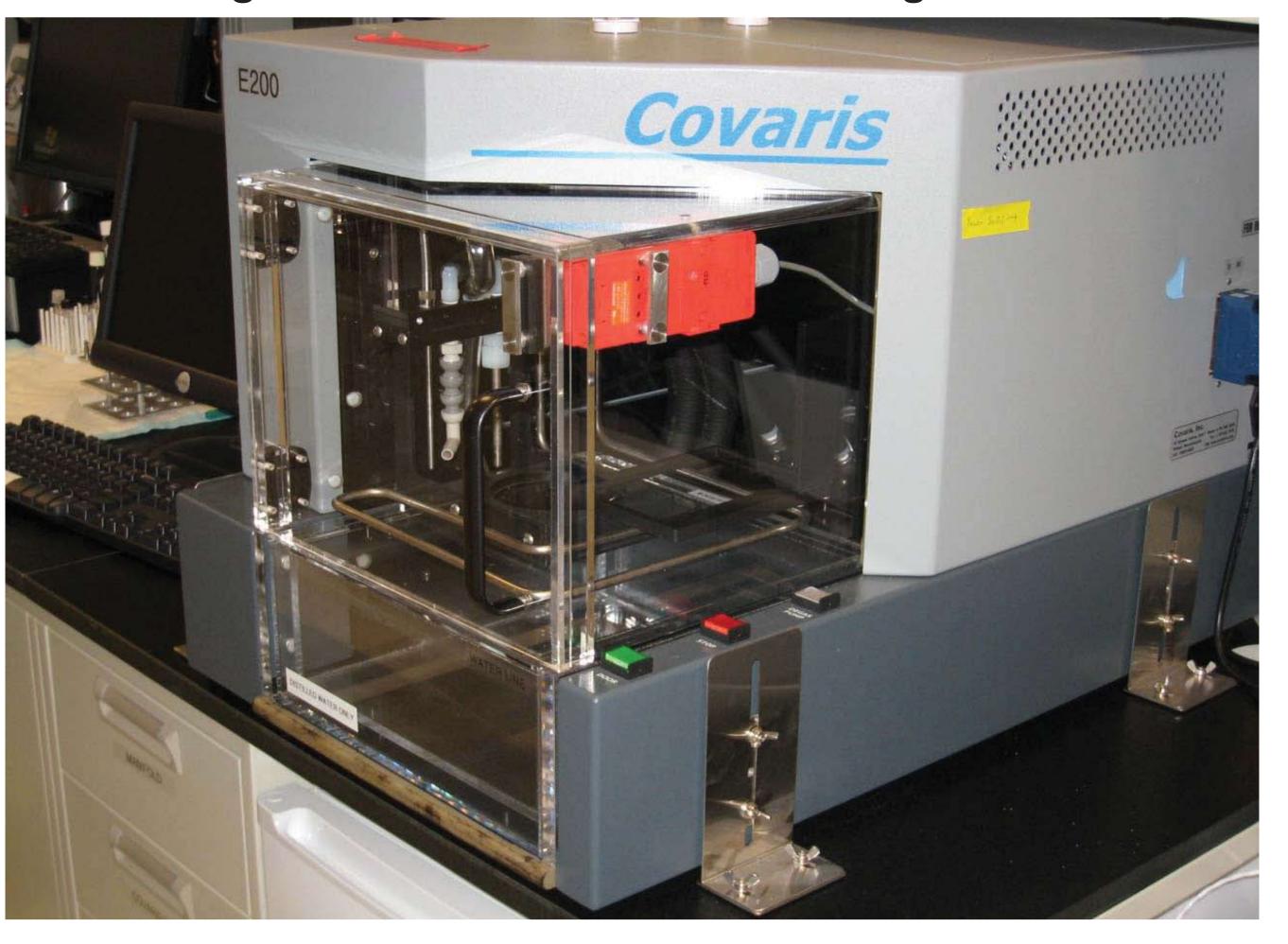


Fig. 2 Covaris Acoustic Homogenizer



RESULTS

Table 1: Preparation of Spiking Standards (2.5–2500 ng/g)

Stock Concentration	Volume (μL)	MeOH (μL)	Final Concentration (ng/g)
20 μg/mL	20	380	5000.00
5.00 μg/g	240	360	2000.00
2.00 μg/g	280	280	1000.00
1.00 µg/g	280	280	500.00
0.50 μg/g	250	250	250.00
0.25 μg/g	220	330	100.00
0.10 μg/g	100	300	25.00
0.025 μg/g	110	440	5.00
0.005 μg/g	250	250	2.50

- 1) For calibration standards, aliquot 25 μ L of spiking standards and 25 μ L control blank spinal cord homogenate to the Costar tubes. For unknown samples, aliquot 25 μ L MeOH and 25 μ L control blank spinal cord homogenate to the Costar tubes.
- 2) Add 150 μ L of internal standard (25 ng/mL in MeOH), with an eight channel pipette, except for the double blank (sample #1) which receives 150 μ L MeOH only.
- 3) Cover with a clear web-seal mat, vortex on medium, and centrifuge @ 3000 rpm for 5 min.
- 4) Transfer 150 μ L from the 96 well block and dispense into a 96 well plate by running the TomTec program.
- 5) Seal the 96 well plate with webseal mat (Chromocol WSM-2).

Table 2: Gradient Elution Program

Minute	% A	% B	μ L/min
0.01	90	10	400
1.50	90	10	400
2.00	70	30	400
3.70	70	30	400
3.71	90	10	400
4.49	90	10	400
4.50	Controller	Stop	

Fig. 3 Spinal Cord Sample Before and After Homogenization

Spinal Cord Sample Before Homogenization



Spinal Cord Sample After

Homogenization

Fig. 4 Chromatogram of Gabapentin at 2.5 ng/g (LLOQ) in Rat Spinal Cord Homogenate

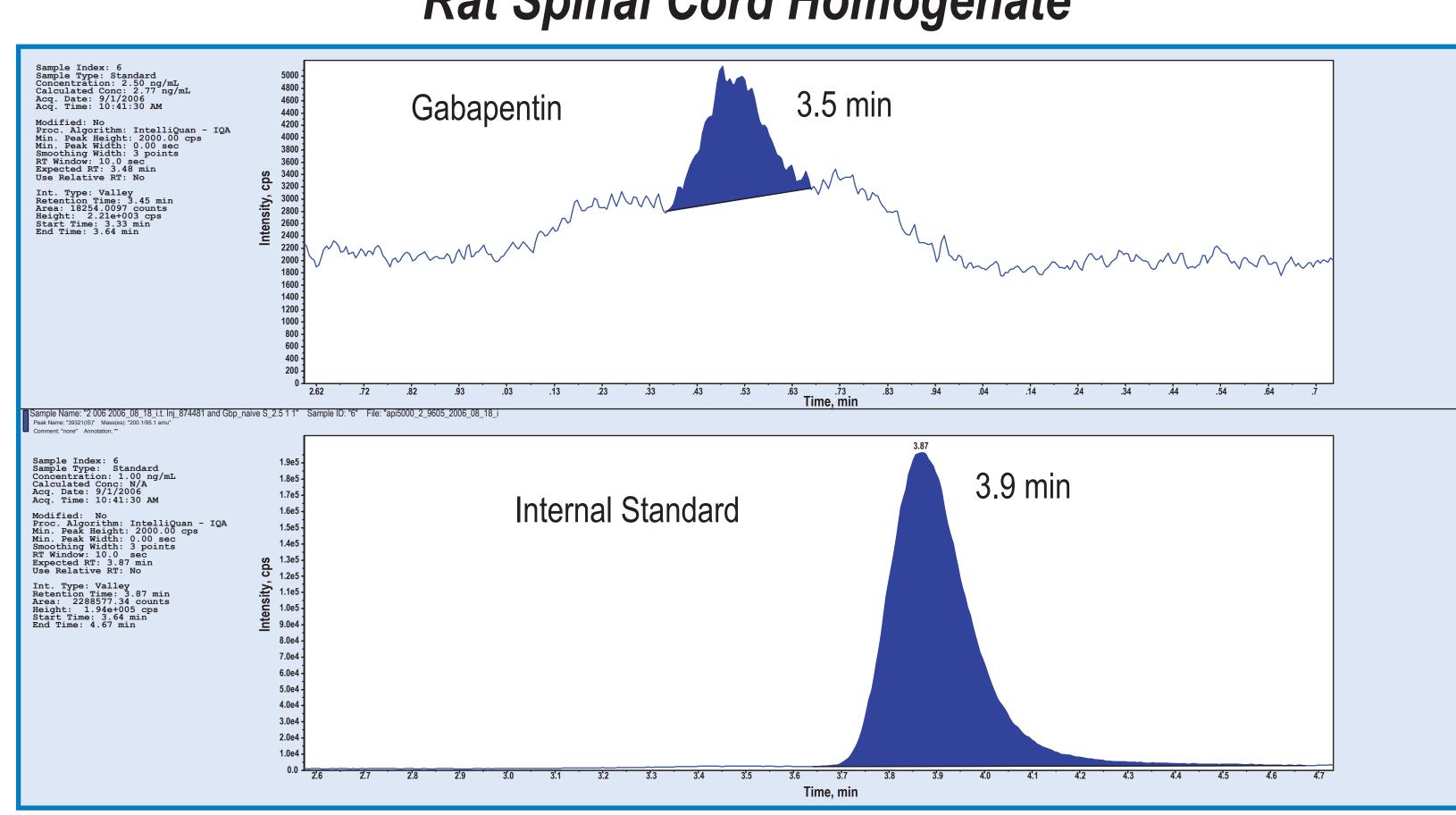


Fig. 5 Chromatogram of Gabapentin at 2000 ng/g (ULOQ) in Rat Spinal Cord Homogenate

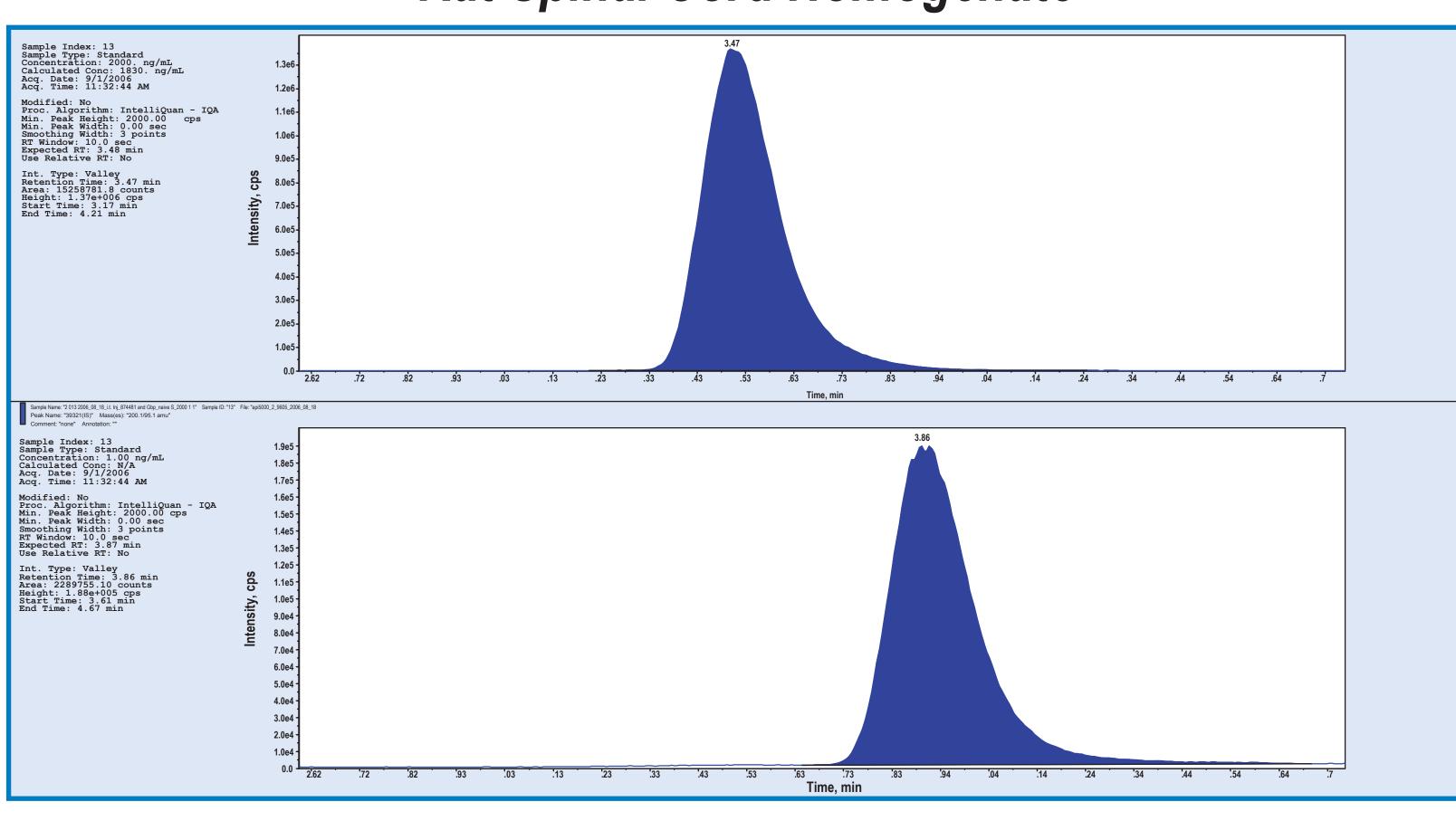


Fig. 6 Chromatogram of a Control Blank Spinal Cord Homogenate

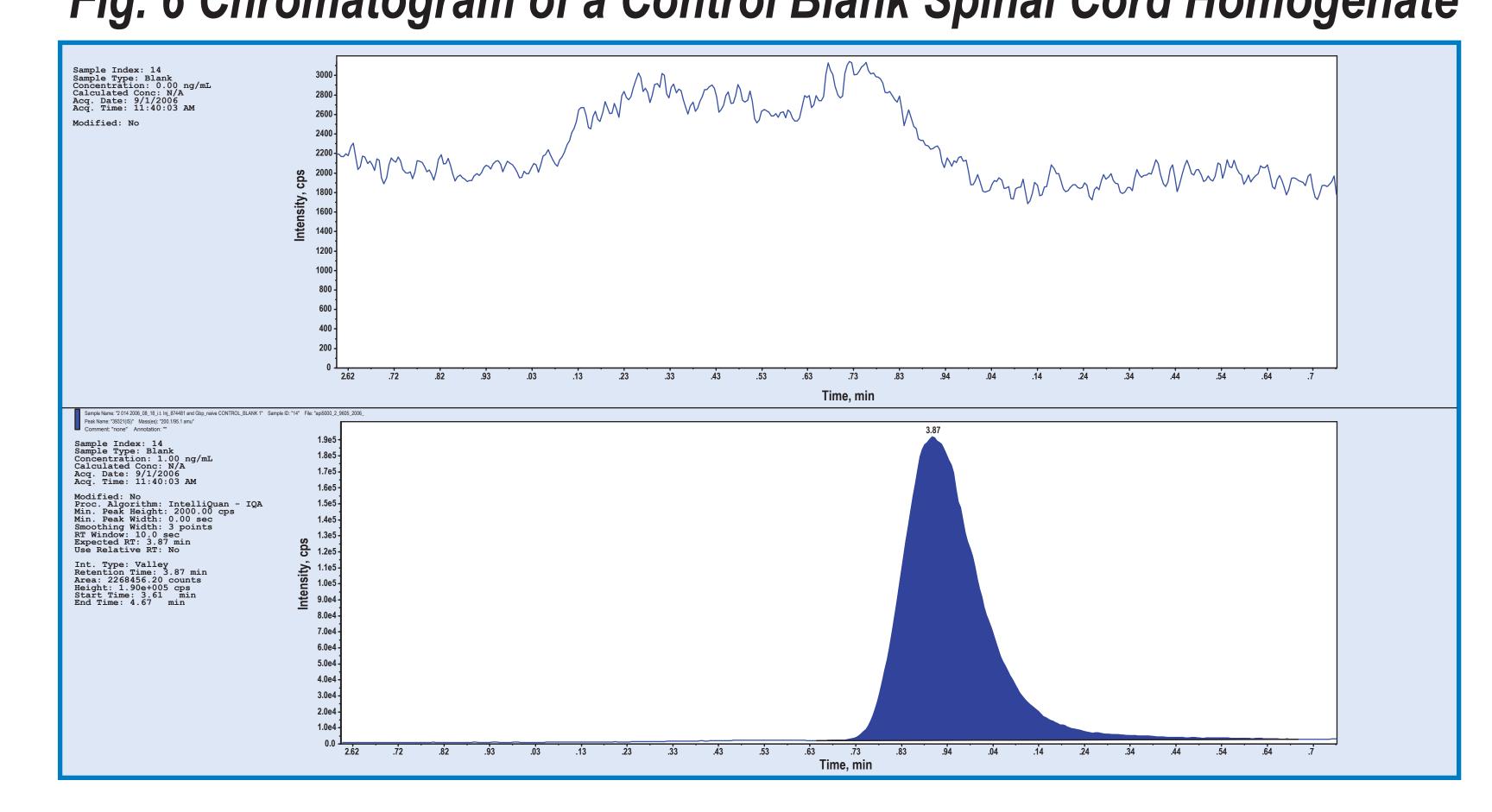


Fig. 7 Chromatogram of a Spinal Cord Homogenate Study Sample After a 100 Fold Dilution

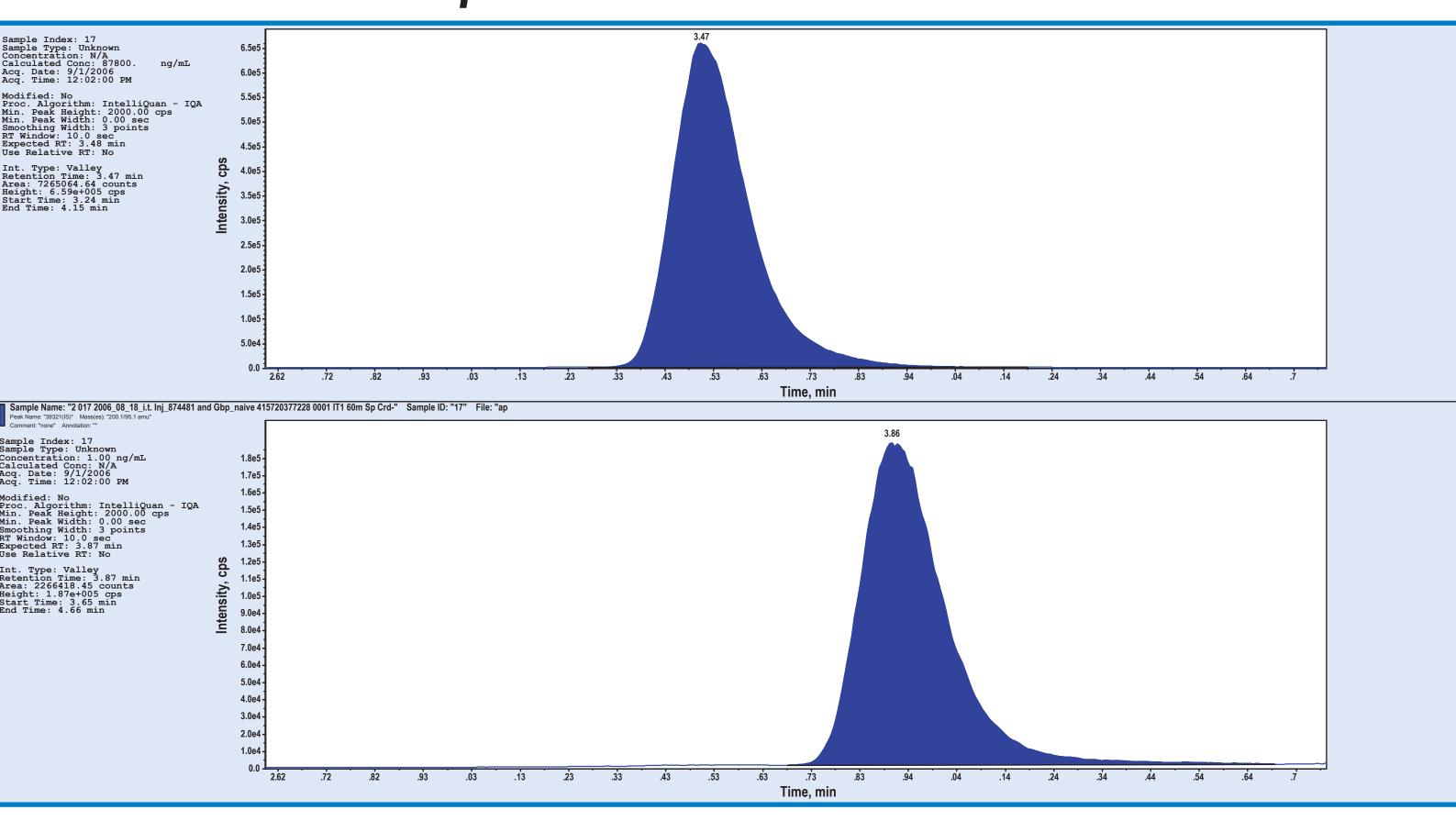


Fig. 8 A Linear Calibration Curve (2.5–2000 ng/g)

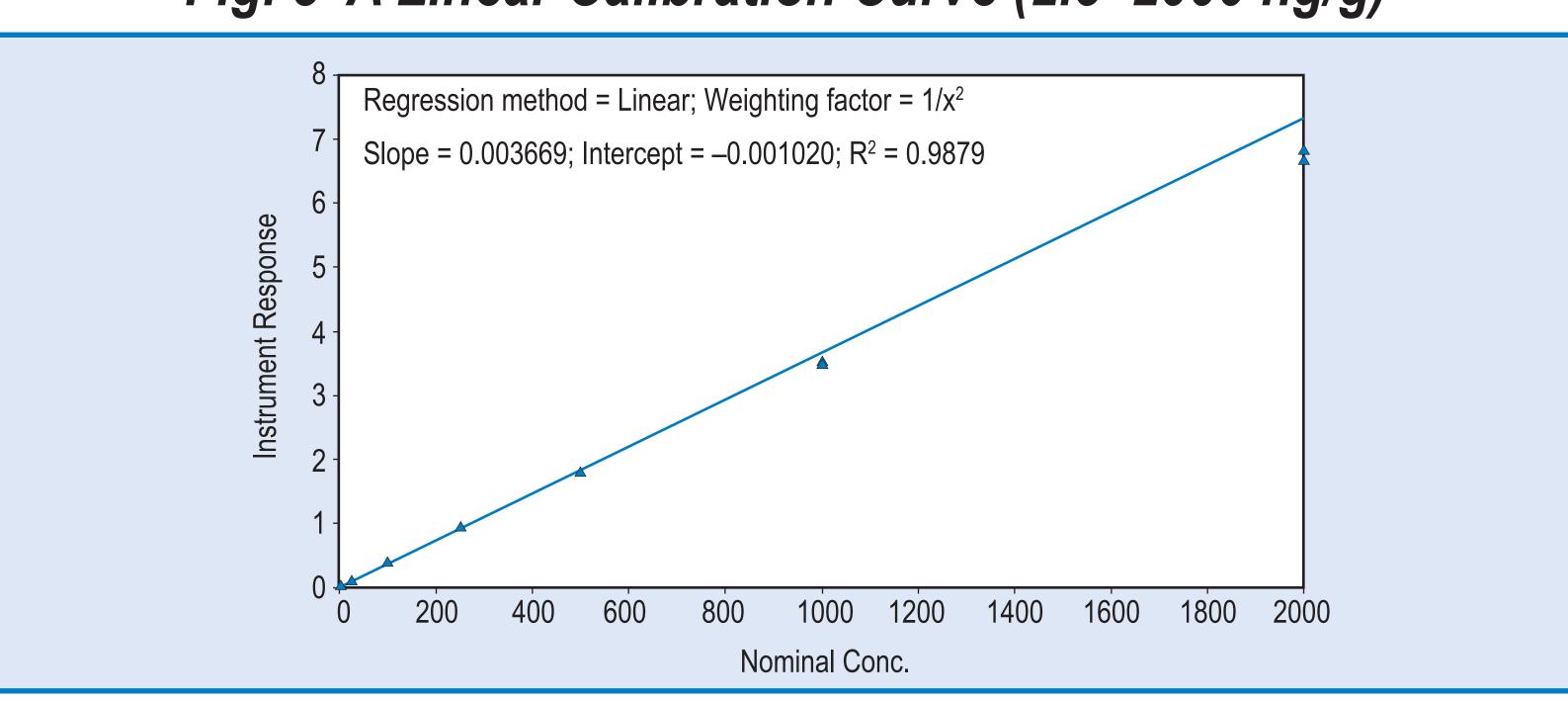


Table 3: Results for Gabapentin Spinal Cord Samples

Study #	Amount Dosed/Rat	Results (ng/g)	Average (ng/g)	Time (hours)
1	200 μg in Lidocaine	140,000	N/A	1.25
2	200 μg in saline	87,400 99,500 134,000 73,700	98,650	1.00

CONCLUSION

- A LC-MS/MS method has been developed for the determination of gabapentin in rat spinal cord homogenate.
- The Covaris acoustic homogenizer is shown to be effective in homogenizing spinal cord samples.
- Advantages:
- absence of cross contamination
- reduced labor
- high throughput sample analysis compared to mechanical homogenization.

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