Gabapentin in Horses: Validation of an Analytical Method for Gabapentin Quantitation

A.F. Lehner[1], J. Stewart[1], A. Dafalla[2], K.J. Ely[2], A.L. Connerly[2], C.N. Jones[2], H. ElkHoly[2], K. Thompson[3], T. Tobin[4], and **L. Dirikolu[5,*]**

[1]Livestock Disease and Diagnostic Center, College of Agriculture, University of Kentucky, Lexington, Kentucky 40512-4125; [2]Department of Biomedical Sciences, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, Alabama, 36088; [3]Department of Large Animal Clinics, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, Alabama, 36088; [4]128C Maxwell Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099; and [5]Department of Veterinary Biosciences, University of Illinois, College of Veterinary Medicine, 3830 VMBSB, 2001 S. Lincoln Avenue, Urbana, Illinois 61802.

Abstract

Gabapentin [1-(aminomethyl)cyclohexaneacetic acid, Neurontin®], is a new anticonvulsant used as adjunctive therapy in the treatment of partial seizures in humans not controlled with standard antiseizure drugs, and it has also been used in veterinary medicine. In performance horses, gabapentin is listed as a class 3 performance-enhancing substance by the Association of Racing Commissioners International, and thus is considered to have the potential to influence the outcome of races. Therefore, we developed and validated a sensitive gas chromatographic–mass spectrometric (GC–MS) method for gabapentin detection. Gamma-aminobutyric acid-d_2 (GABA-d_2) was used as an internal standard during solid-phase extraction; lacking the cyclohexyl ring of gabapentin, GABA-d_2 formed a lactam structure to only a minor extent. Gabapentin, on the other hand, readily formed a lactam on thermal exposure during trimethylsilyl-derivatization and/or GC analysis; electrospray-ionization MS was employed to verify that the original compound was present as the expected 171 m.w. compound. Extraction efficiency for the assay was about 60%, and a curvilinear standard curve ranging from 50 ng/mL to 3000 ng/mL provided excellent within-run and between-run coefficients of variation and accuracies over a range of low, medium, and high values. The limit of detection, defined as the concentration calculated from the mean response at zero concentration plus two times the standard deviation, was calculated at 7.6 ng/mL; the limit of quantitation, defined as the concentration calculated from the mean of the zero responses plus five times the standard deviation, was calculated at 17 ng/mL. This method will enable accurate quantification of gabapentin in equine biological fluids for use in both pharmacokinetic and forensic studies.

Introduction

Gabapentin [1-(aminomethyl)cyclohexaneacetic acid, $C_9H_{17}NO_2$, m.w. 171.24, Neurontin®], is a relatively new anticonvulsant drug used as adjunctive therapy in the treatment of partial seizures in humans not adequately controlled with standard antiseizure drugs (9). Gabapentin has been observed to be effective in the relief of chronic neuropathic pain [diabetic neuropathy or pain following central nervous system (CNS) lesions], as an antidepressant and mood-stabilizer, and in the treatment of some forms of Parkinsonism (6,21). Gabapentin is structurally similar to gamma-aminobutyric acid (GABA), acts as an inhibitory neurotransmitter, and, therefore, has the potential to produce the following therapeutic effects in racing horses: anxiolytic, analgesic, sedative and/or anticonvulsant activities, tranquilization, or skeletal muscle relaxation.

The mode of action of gabapentin is dissimilar to that of other antiepileptic drugs. Although its structure resembles GABA, a pain inhibitor in the CNS which penetrates the blood brain barrier, gabapentin does not appear to exert its antiepileptic effects through interaction with GABA at these sites. It has been proposed that gabapentin may alter the metabolism of brain amino acids and may bind selectively to specific neuronal proteins in the brain (4,22). In addition, gabapentin may selectively inhibit voltage-operated Ca^{+2} channels in a subset of excitatory and inhibitory presynaptic terminals, thereby attenuating synaptic transmission (23).

Effective seizure control in veterinary patients is challenging, requiring careful balancing of successful seizure control with minimization of undesirable drug side effects. Gabapentin's versatile pharmacological properties encouraged veterinarians to explore the use of gabapentin as a veterinary antiepileptic, anxiolytic, analgesic, and sedative drug. However, knowledge of interspecies variations in drug disposition is required for the effective use of anticonvulsants in veterinary practice, and gabapentin appears to be no exception (3).

Medications capable of altering racehorse performance are classified by the Association of Racing Commissioners International (ARCI) based in particular on their performance-enhancing potential. Gabapentin is classified as an ARCI class 3 agent (1), and is therefore considered to have significant po-

*Author to whom correspondence should be addressed.

tential to influence the outcome of a race. As such, its administration to a horse shortly before post time would clearly contravene the rules of racing in most jurisdictions. Sensitive and specific screening and quantitative confirmation methods for gabapentin in equine biological fluids are required to enforce such rules.

Several methods have been published for the analytical detection of gabapentin in plasma (8,10,11,12,14). These methods are laborious because gabapentin has zwitterion characteristics and is difficult to extract from an aqueous matrix. Currently, high-pressure liquid chromatography (HPLC) is the most commonly used approach for the detection of gabapentin in clinical laboratories. Electrospray ionization (positive mode) mass spectrometric (ESI^+–MS) methods for detection exist (12,16), and several gas chromatography–mass spectrometry (GC–MS) approaches have been described (7,14,24). On the other hand, the chemistry of gabapentin during spectrometric analysis is confusing and has not been adequately defined. In this report, we have investigated gabapentin chemistry in order to correct current deficiencies and to develop a validated method for its measurement. The objectives of our study were therefore to optimize extraction for gabapentin (in particular by assessing various solid-phase extraction approaches), to carefully describe the chemistry of this drug on derivatization for GC–MS, and to validate a resultant method for gabapentin quantitation, the application of which to equine samples will be described in a subsequent paper (5). It will be seen that, although ESI^+–MS is still a viable alternative method for gabapentin determination, derivatization chemistry was in fact adequately explained, and a method for the much more common GC–MS instrumentation was validated.

Materials and Methods

Horse blank plasma

Mature Thoroughbred mares were used as a source of blank plasma for extraction studies. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice daily. Blood samples were collected from drug-unexposed animals in heparinized tubes that were centrifuged at 4°C 2,000 × *g* for 15 min, and the plasma was stored at –20°C until assayed.

Preparation and composition of standards

Standard solutions of gabapentin and gamma-aminobutyric acid-2,2-d_2 (GABA-d_2, as an internal standard) (Sigma Chemical Company, St. Louis, MO) and 1-tetradecanol were prepared in methanol (MeOH) and stored in a refrigerator. These standards were allowed to come to room temperature (23°C) before use. With each analytical run, serial dilutions were made from the stock gabapentin standard and added to blank plasma samples. Extraction standards were prepared by the addition of a known volume of gabapentin solution to blank plasma samples at a range of 50 to 3,000 ng/mL (0, 50, 100, 250, 500, 750, 1,500, and 3,000 ng/mL). A known volume of a GABA-d_2 (10 µL of 10 µg/mL in methanol) was added to each standard and blank plasma sample as an internal standard. 1-Tetradecanol was used in stability studies as described in the Results section.

Extraction of gabapentin

Clean 16 × 150-mm Pyrex screw-cap culture tubes with polytetrafluoroethylene cap liners were silanized with trimethylchlorosilane (TMCS) (Pierce Chemical, Rockford, IL) before use by rinsing them first with methanol, followed by a 5% solution of TMCS in hexane (Thermo Fisher Scientific, Waltham, MA), and twice more with methanol (Thermo Fisher Scientific). The silanized tubes were allowed to air-dry at room temperature.

The analytical method used was as follows: plasma samples (1 mL) were pipetted into 15-mL screw-cap tubes. Ten microliters of 10 µg/mL internal standard was added to each tube, followed by 1.5 mL of acetonitrile. The tubes were then shaken on a reciprocating shaker for 5 min, and then centrifuged at 3000 rpm for 5 min to precipitate proteins. The supernatant liquid was transferred into clean tubes and reconstituted with 1.5 mL 0.1M HCl and applied to solid-phase extraction (SPE) columns. We evaluated various SPE columns, the types and manufacturers of which are described in the Results section. The reconstituted residues were applied to SPE columns previously conditioned with 1.5 mL methanol and 1 mL of 0.1M HCl. The columns were washed with 1 mL of MeOH, dried for 2 min, and the extracted compounds were eluted with 2 mL of 2% NH_4OH (conc.) in MeOH. The solvent was decanted into silanized test tubes and evaporated to dryness under a stream of N_2 at < 40°C in a water bath, and the residue was reconstituted with 20 µL of *N*,*N*-dimethylformamide (DMF) (Sigma Chemical) and 80 µL of *N*-methyl-*N*-trimethlysilyltrifluoroacetamide (MSTFA) (Pierce Chemicals, Rockford, IL), vortex-mixed briefly, incubated at 70°C for 1 h, and then transferred to an autosampler vial equipped with a 200 µL spring-loaded insert. Other reagents examined for derivatization of gabapentin included *N*-methyl-*N*-[tert-butyldimethylsilyl]trifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA +1% TBDMCS), *N,O*-bis-trimethylsilyltrifluoroacetamide with 1% trimethyl-chlorosilane (BSTFA + 1% TMCS), and trimethylanilinium hydroxide (TMAH) (all from Pierce Chemicals).

Preliminary mass spectrometric analyses (University of Kentucky)

Gabapentin (Sigma standard) was studied at the University of Kentucky campus as follows: Gabapentin dissolved in 65 µL BSTFA + 1% TMCS and 50 µL DMF was derivatized by reaction at 75°C for 30 min. Derivatized compounds were analyzed by GC–MS on a Hewlett-Packard 5890 GC equipped with a Zebron ZB-5MS column (30 m × 0.25 mm × 0.25 µm film thickness) (Phenomenex, Torrance, CA) with a He flow rate of 1 mL/min. Standard operating conditions were as follows. The injector temperature was 250°C; the GC oven was programmed from 70°C (held 1 min) to 280°C at 20°C/min, and the final temperature held for 18 min. The effluent was carried through a 280°C transfer line to the mass spectrometric detector. Electron impact mass spectra were recorded in the *m/z* 50–550

mass range and analyzed by HP Chemstation software. Resulting chromatograms and spectra were compared to reference material or to spectra within the National Institute of Standards and Testing Spectral Database (NIST02, ChemSW, Fairfield, CA). Where necessary, some spectra were interpreted with the assistance of Mass Spec Calculator Pro, Version 4.03 (Quadtech Associates, ChemSW).

ESI^+–MS was performed on the Micromass Quattro II HPLC-tandem quadrupole MS (LC–MS–MS) (Beverly, MA). Conditions were as follows: Full scan ESI mass spectra were obtained on analytical standards at 10 µg/mL in 50:50 (v/v) acetonitrile–0.1% formic acid (aq), by infusion at 1.2 mL/h with a Harvard syringe pump (Holliston, MA) into the electrospray probe set in positive ion (+) mode. All spectra were optimized by combination of 1–2 min of uniformly acquired data, background subtraction, and peak smoothing. Interpretation of mass spectra was assisted by Mass Spec Calculator Pro, v. 4.03 (Scientific Instrument Services, Inc., Ringoes, NJ). The mass spectrometer was tuned by direct injection of 10 ng/µL gabapentin in 50:50 (v/v) acetonitrile–0.1% formic acid (aq). The peak shape and intensity of the monoprotonated gabapentin *m/z* 172 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Skimmer lens offset was left at 5 V. Collision gas (argon) and collision energy were adjusted for collision-induced dissociation (CID) in the central hexapole by optimizing settings for the *m/z* 154 and 137 daughter ions from 172 in MS^2. Generally, the collision gas was set to 2.5×10^{-3} mbar. Increasing the photomultiplier setting to 750–800 V sufficiently increased the sensitivity. In general, the source cone voltage was set at +25 V, the collision energy was set between –15 and –18 V, the capillary of the ESI probe was set at +3.70 kV and the HV lens was set at +0.87 kV. Source temperature was set between 120–140°C.

AutoSystem XL GC and TurboMass MS analysis

Development and validation of the extraction method were done at the Tuskegee campus with MS parameters worked out by consultation with the University of Kentucky campus. The method for GC–MS was performed at Tuskegee for subsequent validation and use in later pharmacology studies as follows. Samples were analyzed using Perkin Elmer AutoSystem XL Gas Chromatography and TurboMass Mass Spectrometer (Perkin Elmer, Norwalk, CT) set in positive ion mode. The GC conditions used for identification of the corresponding gabapentin peak were as follows: column, HP-5ms [(5% phenyl)-95% methylpolysiloxane)] 30 m, 0.50-µm film thickness, 0.25-mm internal diameter (Agilent Technologies, Wilmington, DE) (helium flow rate: 1.5 mL/min); injector, 250°C; transfer line, 250°C; the oven was temperature programmed from 70°C (2 min at initial temperature) with a rate of increase of 20°C/min to 280°C (holding temperature, held for 10 min). The MS was set to acquire from *m/z* 50 to 650 at 1 scan/s. In quantitative experiments, selected ion monitoring (SIM) was performed for ions *m/z* 210, 211, and 225 and *m/z* 102 for internal standard. The *m/z* 102 of bis–TMS-GABA-d_2 derivative is the major fragmentation product and was monitored as the internal standard ion. For the TMS–gabapentin derivative, quantitation was based on the most abundant ion, *m/z* 210. For chromatographic and mass spectrometric identification of gabapentin and GABA-d_2, AORC guidelines (2) were followed.

A standard curve was constructed by plotting standard gabapentin concentration versus the ratio of gabapentin–internal standard peak areas. Standard curves were generated with Sigma Plot for Windows (Aspire Software International, Leesburg, VA). The areas of peaks corresponding to gabapentin and the internal standard were recorded, and the internal standard values were thereby used to normalize the gabapentin areas. Integrated peak values were entered into QuattroPro for Windows (Borland Software Corporation, Scotts Valley, CA) for statistical analysis of standards and for interpretation of unknown amounts of gabapentin in test animals (5). An estimate of gabapentin concentration in unknown samples was obtained by comparing unknown:internal standard area ratio obtained from the unknown sample and interpolated on the standard curve.

Validation of the assay

The GC–MS method for the quantitation of gabapentin was validated by using the most recent Standard Operating Procedure for Validation available to us from Dr. Rick Sams, formerly of The Ohio State University (personal communication). The quantitative method of gabapentin was validated by examining the measurement of consistency of results (within-run and between-run), correlation (coefficient of determination of the standard curve), and extraction efficiency of the assay. The within-run precision was calculated from similar responses from six repeats of three control samples (60, 500, 2400 ng/mL) in one run. The between-run precision was determined by comparing the calculated response (in ng/mL backfit of the standard curve) of the low (60 ng/mL), medium (500 ng/mL), and high (2400 ng/mL) control samples over three consecutive daily runs (total of six runs). The assay accuracy for within-run and between-run was established by determining the ratio of calculated response to expected response for low (60 ng/mL), medium (500 ng/mL), and high (2400 ng/mL) control samples over six runs.

Standard curve correlation was measured by the mean coefficient of determination (r^2) for six consecutive daily runs. The extraction efficiency was determined by comparing the response (in area) of low (100 ng/mL), medium (500 ng/mL), and high (1500 ng/mL) standards, and the internal standard (100 ng/mL) spiked into blank plasma eluent before evaporation to the equivalent extracted standards. The lower limit of detection (LOD) was calculated from six consecutive runs. The concentration calculated from the mean of the responses at zero concentration (y-intercepts) was determined. The LOD was defined as the concentration calculated from the mean response at zero concentration plus two times the standard deviations (the upper 95% confidence limit for zero). In addition to this determination of the LOD, an alternate calculation was performed utilizing the analyte's peak height compared to the baseline noise in the *m/z* 210 fragmentation chromatogram. By this method, the LOD was defined as the lowest concentration of analyte producing a peak greater than or equal to three times the baseline noise of the ion chromatogram. The

lower limit of quantitation (LOQ) was defined as the concentration calculated from the mean of the zero responses plus five times the standard deviation.

Results

We sought to develop a GC–MS analytical procedure for gabapentin, but reports to date have left a somewhat confusing picture of gabapentin behavior with or without derivatization. In order to develop a sensitive GC–MS method for quantitation of gabapentin, we therefore first studied its chemical behavior. Gabapentin in methanol was found to generate a 153 m.w. species on electron-impact GC–MS, corresponding to loss of 18 amu, presumably a neutral water molecule, from the expected 171 m.w. compound (Figure 1A). We believe that the *m/z* 153 corresponds to the M^+ molecular ion because, firstly, ion chromatography disclosed no convincing higher mass alternatives; secondly, isotope analysis revealed a reasonable correspondence between expected (100%, 10.7%, 0.59%) and found (100%, 10.0%, 0.50%) M, M+1, and M+2 isotope ratios for a $C_9H_{15}NO$ compound's *m/z* 153, 154, and 155 ions, respectively. We noted that a considerable M–1 was also present at *m/z* 152, supporting later speculations of gas phase intermolecular proton transfer.

Generation of the gabapentin lactam appears to occur in a quantitative (i.e., dependably complete) manner for the following reasons: (*i*) repeated injection of 0.1 µL 100 ng/µL gabapentin in methanol reproducibly provided the *m/z* 153 peak on ion chromatography, with integration of its chro-

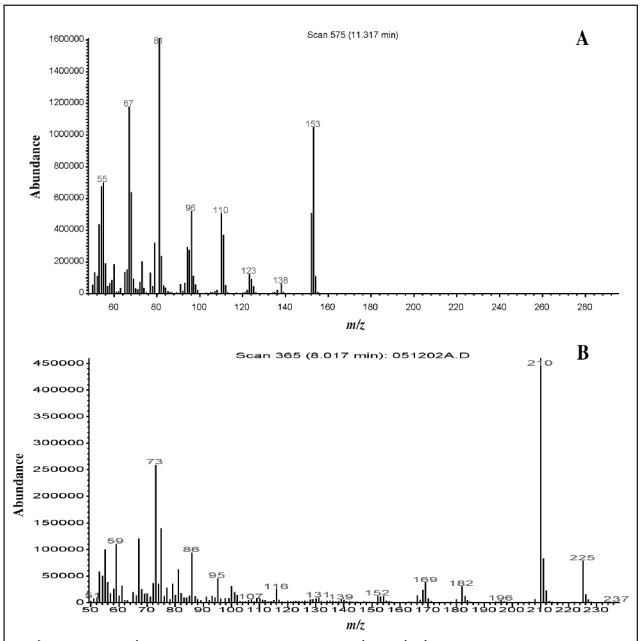

Figure 1. Gabapentin GC–MS. Principal peak from injection as a 10 µg/mL solution in methanol (A); principal peak from injection of an equivalent amount following BSTFA/1%TMCS derivatization (30 min at 70°C) (B). GC conditions: 0.3 µL injection, GC: injector, 250°C; oven, 70°C (2 min) → 280°C at 20°C/min; transfer line, 280°C.

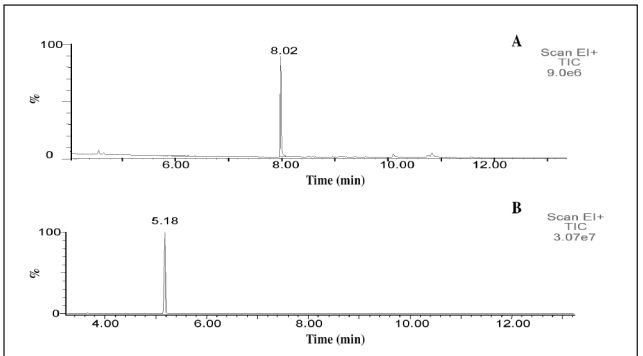

Figure 2. Total ion chromatograms for gabapentin (A) and GABA-d_2 (B) derivatized at 500 ng sample (A) and 100 µg sample (B) in 10 µL DMF and 90 µL MSTFA, incubated at 70°C for 1 h (5 ng/µL injection).

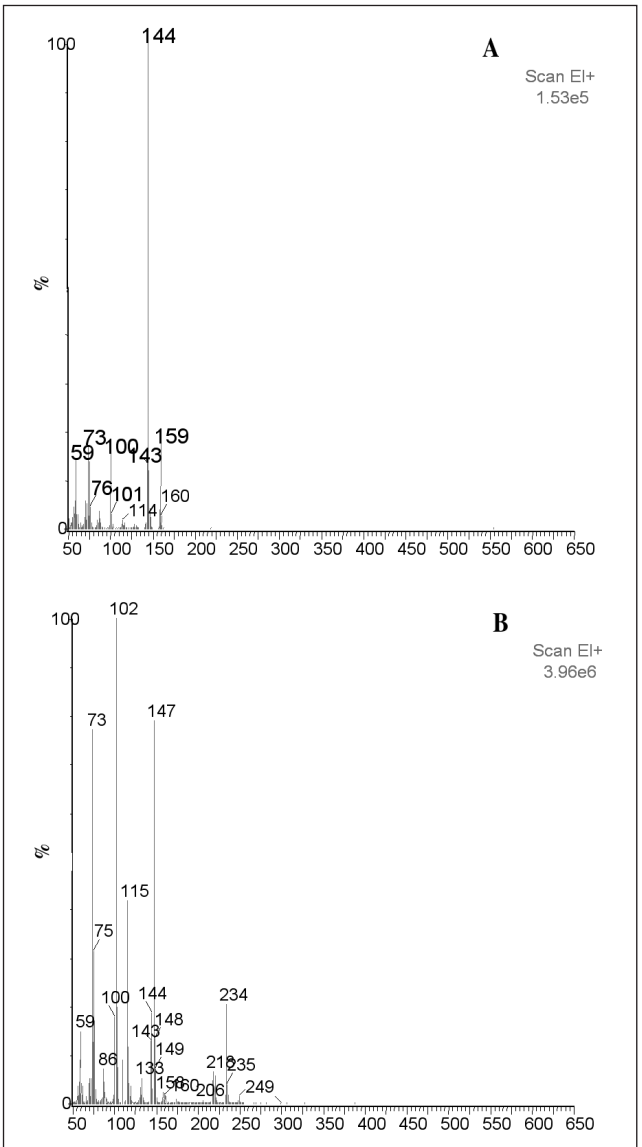

Figure 3. Significant mass spectra from MSTFA derivatization of GABA-d_2, Figure 2B, showing GABA-d_2 spectra occurring at 3.6 min [minor species (A): 159 m.w.] and 5.18 min [principal species (B): 249 m.w.]. During similar chromatography, the principal gabapentin peak occurred at 7.97 min and provided a 225 m.w. compound with spectrum identical to that in Figure 1B.

matographic peak uncorrected by internal standard as 1.62 × 10^6 area counts ± 6.5% relative standard deviation (RSD); (*ii*) this generally agreed with the precision of the autosampler used in this manner and assessed separately with ion chromatography of the *m/z* 97 peak of the stable compound 1-tetradecanol as 3.3% RSD; (*iii*) injection of TMS-derivatized gabapentin standard at 100 ng/µL in BSTFA/1%TMCS provided > 96% gabapentin lactam-TMS with traces of underivatized lactam (< 3%) and lactam bis-TMS (< 1%) and no other visible species, such as uncyclized gabapentin-mono, -bis, or -tris-TMS.

Underivatized gabapentin was somewhat subject to tailing on GC, and therefore functional group derivatization was considered a viable approach to curtailing this problem. In addition, silyl derivatives were roughly an order of magnitude more sensitive than the underivatized compound. Various derivatizations were attempted including MTBSTFA, MSTFA, BSTFA, TMAH, etc. with the intention of generating stable tert-butyldimethylsilyl (*tBu*DMS), bis(TMS), methyl, etc. derivatives of gabapentin, respectively. Some of these studies included catalysts, such as BSTFA + 1% TMCS, MSTFA + 1% TMCS, and MTBSTFA + 1% TBDMCS. The TMS derivatives were found to be the most dependable in terms of ease of reaction, reproducibility of reaction outcome, and the presence of a single chromatographic peak and corresponding linearity of response. MSTFA provided the lowest background with respectable yield of product and was chosen as the basis of the derivatization reaction in the validated procedure.

However, as can be seen from the representative spectrum in Figure 1B, the TMS derivative was 225 m.w., not 315 m.w. as expected for a bis(TMS) derivative. No ions above *m/z* 225 could be convincingly demonstrated to be associated with the TMS derivative of gabapentin by ion chromatography, and once again isotope analysis revealed a reasonable correspondence between expected (100%, 19.2%, 5.1%, 0.52%) and found (100%,

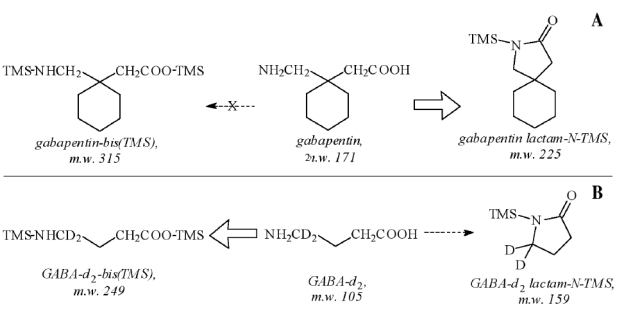

Figure 4. Illustration of gabapentin (A) and GABA-d_2 (B) reactions during derivatization and/or GC. A gabapentin-bis(TMS) product (A, left) was never identified, whereas derivatized gabapentin gave the lactam-N-TMS (A, right). GABA-d_2, and presumably GABA, has the opposite pattern of favoring conversion to the bis(TMS) derivative (B, right) yet still affording a minimum conversion to the corresponding lactam, here labeled as GABA-d2 lactam-N-TMS, but equivalent to pyrrolidone-d_2-*N*-TMS.

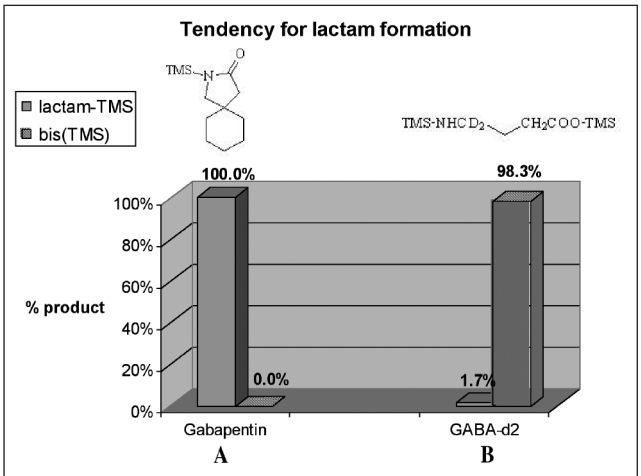

Figure 5. Illustration of the stark differences between gabapentin (A) and GABA-d_2 (B) in their respective abilities to generate lactam structures following derivatization and/or during conditions of GC–MS. Assigned structures of principal products are shown.

Table I. Comparison of Fragmentations of Gabapentin Lactam and its TMS Derivative as Examined by EI–GC–MS

Fragment	Gabapentin lactam	Gabapentin lactam-TMS
Molecular ion, $M^{+\cdot}$	153	225
$M^{+\cdot} - CH_3$	–	210
$M^{+\cdot} - CH_2{=}CHCH_3$	–	182
$M^{+\cdot} - CH_2CH_2CHCH_3$	–	169
$M^{+\cdot}$ – TMS	–	152
$M^{+\cdot} - C_6H_{10}(CH_2)_2$	–	116
$M^{+\cdot} - CH_2{=}NH_2$	123	–
$M^{+\cdot}$ – NH-C=O	110	–
$M^{+\cdot}$ – NH-[C=O]-CH_2 [with or without N-TMS]	96	95
$M^{+\cdot} - CH_2NH[C{=}O]CH_3$ [with or without N-TMS]	81	81
TMS	–	73
$M^{+\cdot} - CH_2{=}CHCH_3$ and –NH-C=O [with or without N-TMS]	67	67

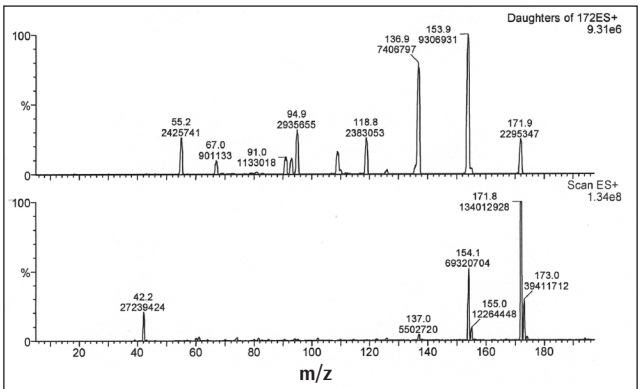

Figure 6. Gabapentin standard at 10 µg/mL run by Direct Infusion-ESI+–MS. Scan (B) demonstrates strong tendency of native gabapentin+H+ to lose water, as *m/z* 172 minus water (18) = *m/z* 154. Daughter ions (A) indicate the same tendency, with the significant m/z 137, also seen in the full scan. Technical info: Sigma gabapentin at 10 µg/mL in 0.1% formic acid–acetonitrile, 1:1 was infused at 2.2 mL/h, with settings: cone volts = 25, collision energy = 15.

18.7%, 5.6%, 0.6%) M, M+1, M+2, and M+3 isotope ratios for a $C_{12}H_{23}NOSi$ compound's *m/z* 225, 226, 227, and 228 ions, respectively, supporting the assignment of *m/z* 225 as the molecular ion. The m.w. 225 would then correspond to a mono-TMS derivative of the 153 m.w. species seen without derivatization.

Figure 2A illustrates a single gabapentin-TMS peak at 8.02 min retention time. The intended structurally-related internal standard, GABA-d_2, gave a single principal peak at 5.18 min retention time under the same conditions of derivatization and chromatography (Figure 2B). Closer examination of the GABA-d_2 chromatogram revealed a minor species at 3.6 min retention time, with the principal peak at 5.18 min. Figure 3 shows the EI-mass spectra of these GABA-d_2 components, revealing that they correspond to 159 and 249 m.w. species, respectively. These in turn correspond in size to loss of water plus mono-TMS derivatization (a picture similar to that of gabapentin) and bis(TMS) derivatization for GABA-d_2, respectively.

Figure 4 illustrates the most succinct interpretation of these findings. During derivatization and/or chromatography, gabapentin forms a cyclic dehydrate, in this case a lactam, by splitting out water between the carboxylic acid and amine functions of the gamma-amino acid. The amido N-H group is the most likely target for TMS derivatization, although enolization of the amide oxygen and generation of an O-TMS derivative is not formally excluded. In contrast, for reasons of higher activation energy leading to the lactam, or greater reactivity with MSTFA, or both, GABA-d_2 is much more capable of forming the bis(TMS) derivative of the otherwise unaltered parent. Figure 5 depicts the relative tendencies to form lactams for gabapentin and GABA-d_2, respectively. This reasoning allowed us to make structural assignments to principal peaks of the gabapentin EI-mass spectra of Figure 1, and these assignments for lactam and lactam-TMS are summarized in Table I. The results of this analysis provided reasonable assignments, in turn supporting the structural interpretations of Figure 4.

In spite of its inability to undergo lactam formation, GABA-d_2 was viewed as a suitable internal standard for our research in the absence of a deuterated gabapentin standard for the following reasons: (*i*) GABA has a pKa_1 of 4.2 and pKa_2 of 10.43 with free solubility in water, comparable to gabapentin's pKa_1 of 3.68 and pKa_2 of 10.70, respectively, and excellent solubility in water, making it an excellent choice for a standard with nearly equivalent extractability; (*ii*) GABA-d_2 provided dependable chromatography, with its derivatized peak well-separated from that of gabapentin; (*iii*) GABA-d_2 avoided any potential interference from native GABA-d0; (*iv*) as will be seen, GABA-d_2 provided a rugged and dependable standard curve with excellent coefficient of correlation on data refit.

We were concerned as to whether the gabapentin standard was itself present as a lactam [which is itself a pharmacological agent with its own range of properties (15,26)] or whether the lactam was simply a byproduct of our analyses following dissolution in methanol. Suspension in the aprotic solvent ethyl acetate disclosed no dependence of lactam formation on methanol's weak capacity to protonate, generating the same spectrum as Figure 1A. Application of heat (2 h at 75°C.) did, however, disclose that, in contrast to ethyl acetate, methanol caused gabapentin breakdown, but the products were not identifiable and offered no further clues as to mechanisms of gabapentin rearrangements.

ESI+–MS was considered an appropriate alternative analyt-

Table II. Peak Assignments for ESI–MS–MS Daughter Ions of Gabapentin M+H+, *m/z* 172

m/z	Assignment
172	Molecular ion, M+H+
154	Lactam formation; see Figure 7
137	Deamination and further rearrangement of M+H+ to a cyclic ether; see Figure 7
119	Possible loss of H_2O from *m/z* 137 by unknown mechanism
109	*m/z* 154 minus neutral formamide
95	*m/z* 137 minus $H_2C{=}C{=}O$
93	Unidentified
91	Possible loss of C_2H_4 from *m/z* 119
67	*m/z* 137 minus $(CH_2)_5$
55	*m/z* 137 minus $(CH_2)_5C$

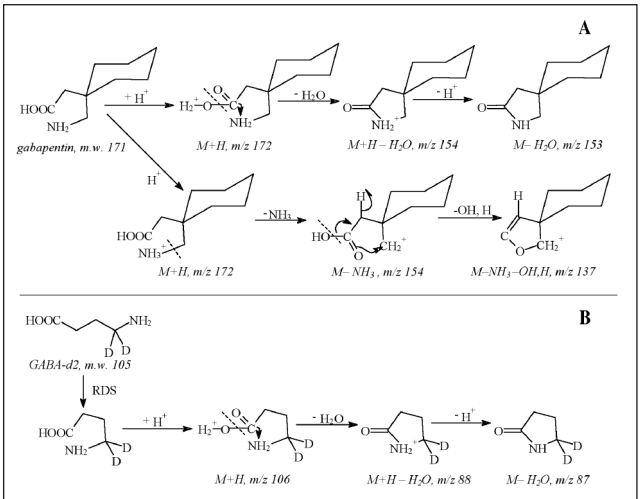

Figure 7. Putative mechanisms of gabapentin and GABA-d_2 lactam formation and fragmentation. Formation of gabapentin-lactam and the minor ESI+–MS *m/z* 137 species (A) are shown originating from gabapentin. The next section (B) illustrates lactam formation from GABA-d_2, limited by a rate determining step (RDS).

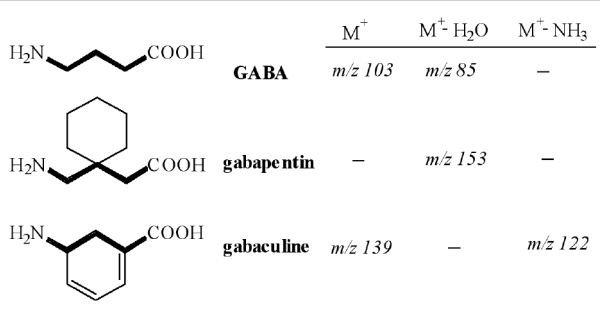

Figure 8. Loss of NH_3 posited in Figure 7 is generally a relatively minor mechanism (17), but may be of greater significance with certain amino acids. GABA and gabapentin Wiley library entries showed no significant M-17, whereas the more rigid analog gabaculine does show such a loss, demonstrating its feasibility.

ical method capable of sorting out gabapentin solution chemistry. ESI+–MS applied to gabapentin in an acetonitrile–formic acid solution revealed the following: (*i*) the gabapentin standard must have been present as a 171 m.w. gamma-amino acid, because the molecular ion + proton (M+H) pseudomolecular ion of *m/z* 171.8 could be readily seen (Figure 6B); (*ii*) gabapentin exposed to ESI source temperatures of 200°C generated a considerable loss of water to the presumed lactam +H at *m/z* 154, both in source (Figure 6B) or on examination of *m/z* 172 daughter ions (Figure 6A); (*iii*) peak assignments could be made for gabapentin M+H daughter ions consistent with gabapentin structure (Table II); (*iv*) both full scan and daughter ion analyses indicated a further capacity to generate a loss of NH_3 to yield an *m/z* 137 species under these conditions.

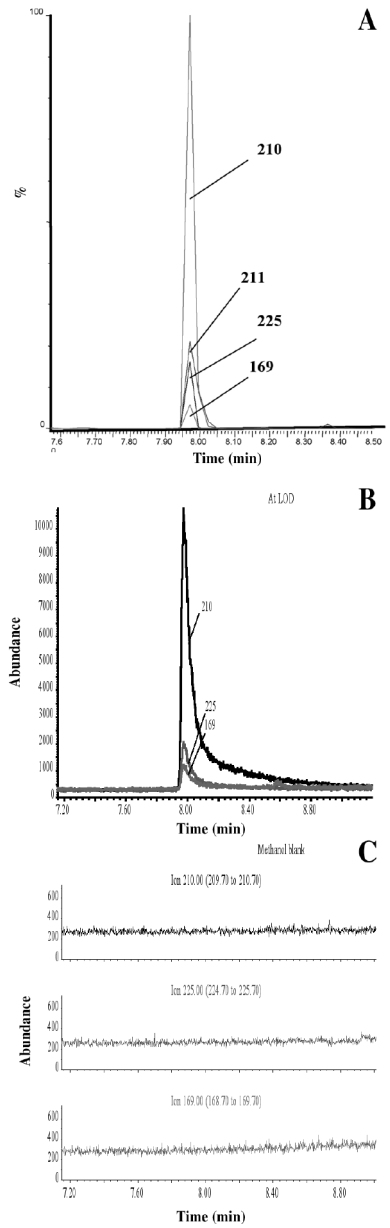

Figure 9. Selected ion chromatogram of the TMS derivative of gabapentin-lactam extracted from plasma at 500 ng/mL gabapentin with the standard peak at 7.96 min. The overlap of ions in the order of *m/z* 210, 211, 225, and 169 is displayed (A). Figure 9B illustrates unextracted gabapentin-lactam-TMS (0.1 µL of 2 ng/µL) approximately at the method LOD, and Figure 9C illustrates a methanol blank run under the same conditions; in both cases, ion chromatograms for *m/z* 169, 210, and 225 are labeled.

Figure 7 shows likely routes to the gabapentin lactam and the minor *m/z* 137 species (A). During ESI–MS, gabapentin has a route to fragmentation involving (left-to-right) protonation to an *m/z* 172 pseudomolecular ion, followed by dehydration to an *m/z* 154 dehydrate, equivalent to the *m/z* 154 fragments seen in Figure 6. Loss of a proton accounts formally for the dehydration product seen in Figure 1 at *m/z* 153 by EI–MS. Gabapentin can also fragment first by loss of NH_3 to a hypothetical variant *m/z* 154 fragment, which undergoes concerted rearrangement with losses of OH and H to give an *m/z* 137 fragment. In contrast, GABA-d_2 has a Rate Determining Step involving conformational rearrangement, after which dehydration and proton loss would proceed similarly to gabapentin to give an *m/z* 87 dehydrate, which, upon TMS derivatization, would give the 159 m.w. lactam-TMS compound seen in Figure 3A.

These routes are most likely favored by (*i*) the relatively small range of conformations available to the gabapentin carboxy methylene and amino methylene functions, in contrast to those available to GABA-d_2 (Figure 7B), which must presumably pass through a significant RDS to enter a conformation favorable to lactam formation; and (*ii*) presence of acid in the solvent and/or intermolecular proton transfer to provide a catalyst either in the gas phase during chromatography and/or in solution during derivatization. Note that loss of NH_3 (minus 17 amu) is a relatively minor mass spectrometric fragmentation (17), and examination of a Wiley EI–MS library entry for GABA (m.w. 103) showed no such loss, whereas it was not possible to see such a loss in gabapentin owing to its rapid conversion to a lactam during GC–MS. However, the related compound gaba-

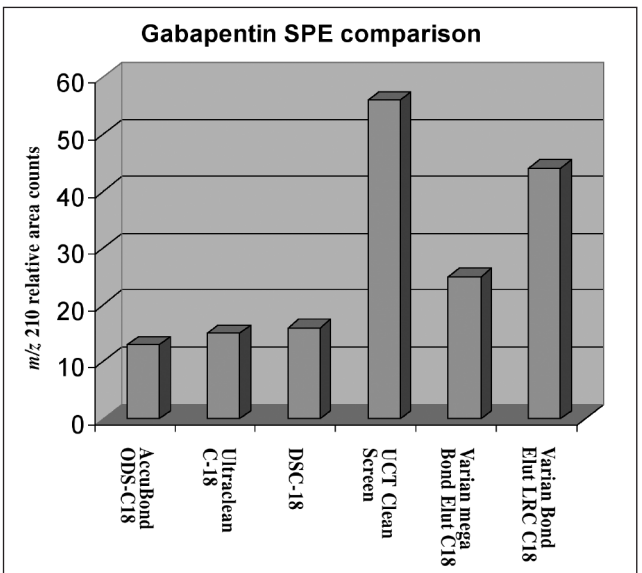

Figure 10. Comparison of the yields of the gabapentin TMS derivatization product of MSTFA on SPE cartridges from various manufacturers. Data were obtained from full scan GC–MS runs with plotting of the 8-min *m/z* 210 integrated area counts.

culine, m.w. 139, a *Streptomyces* natural product capable of potent irreversible inhibition of GABA-transaminases (20), did reveal a significant M–17, demonstrating at least the feasibility of participation of such a loss in generation of gabapentin's *m/z* 137 fragment seen by ESI^+–MS; these fragmentations are summarized in Figure 8.

The selected ion chromatogram of TMS derivative of gabapentin-lactam extracted from plasma spiked with 500 ng/mL of gabapentin standard is shown in Figure 9. The *m/z* 210 base peak suggested itself as an appropriate quantitative ion, particularly because it was reasonably above the *m/z* 50–150 region. The *m/z* 211 peak was consistent at 20% of the area counts of *m/z* 210, demonstrating its reliable origin as an isotopic M+1 ion of *m/z* 210. An equally good qualifier ion was *m/z* 225, which fulfilled the AORC criteria for monitoring the molecular ion when present above 10% (2).

We evaluated various SPE columns, such as Supelco Discovery (DSC-18) columns (Sigma Chemical, Bellefonte, PA), Ultra Clean C-18 columns (Alltech Company, Deerfield, IL), Accu Bond II ODS C-18 columns (Agilent Technologies, Wilmington, DE), Clean Screen columns (United Chemical Technologies, Bristol, PA), and Varian Mega Bond Elut C-18 and Bond Elut LRC C-18 columns (Varian Analytichem, Walnut Creek, CA) for the analytical detection of gabapentin in the biological samples of horses. The columns are based on similar matrices comprising hydrophobic resins in some cases with mixed bed anionic resins and gave relatively similar results; Figure 10 shows a comparison of relative yields on the extraction of gabapentin from horse plasma. Relative yields were assessed by extraction of 150 ng gabapentin spiked into 1 mL plasma samples without internal standard. The absolute area of the *m/z* 210 peak following MSTFA derivatization and GC–MS analysis provided sufficient comparability between columns. We chose Clean Screen columns owing to long-standing experience with the performance characteristics and proven reliability of these columns and their high yield. Other good choices would be the Varian products, particularly the Mega Bond Elut which gave the lowest gas chromatographic background overall.

For quantitation, the intense ion at *m/z* 102 was chosen for SIM of GABA-d_2 TMS derivative (internal standard, data not shown) and the intense ion at *m/z* 210 was chosen for SIM of gabapentin-lactam TMS derivative. The resultant standard curve was curvilinear from 50 ng/mL to 3000 ng/mL, with the r^2 value of the assay (n = 6) being 0.994 ± 0.0026 (SD). Figure 11 presents a typical standard curve for gabapentin. Standard curve correlation was measured by the mean coefficient of determination (r^2) for six consecutive daily runs. The extraction efficiency was determined by comparing the response (in area) of low (100 ng/mL), medium (500 ng/mL), and high (1500 ng/mL) standards, and the internal standard (100 ng/mL) was spiked into blank plasma eluent before evaporation to the equivalent extracted standards. The LOD was calculated from six consecutive runs. The concentration calculated from the mean of the responses at zero concentration (y-intercepts) was determined. The LOD was defined as the concentration calculated from the mean response at zero concentration plus two times the standard deviation (the upper 95% confidence limit for zero). In addition to this determination of the LOD, an alternate calculation was performed utilizing the analyte's peak height compared to the baseline noise in the *m/z* 210 fragmentation chromatogram. By this method, the LOD was defined as the lowest concentration of analyte producing a peak greater than or equal to three times the baseline noise of the ion chromatogram. The LOQ was defined as the concentration calculated from the mean of the zero responses plus five times the standard deviation.

The LOD calculated by the method of standard deviations described earlier was 7.6 ng/mL. The LOD estimated by consideration of the baseline noise of the ion chromatogram was 0.1 ng on column (10 ng/100 µL, data not shown), roughly equivalent to 10 ng/mL in a 1 mL plasma sample. It was reassuring that the estimate was close to the calculated value, particularly because both values fell below our lowest non-zero calibrator. The LOQ also fell below the lowest non-zero calibrator and was calculated as 17 ng/mL.

For within-run precision, the coefficient of variation (CV) for 60 ng/mL (n = 6) was 7.47%, for 500 ng/mL (n = 6) was 4.66%, and for 2400 ng/mL (n = 6) was 6.26% with the mean CV of 6.13% (Table III), less than the 15% CV value acceptable for the

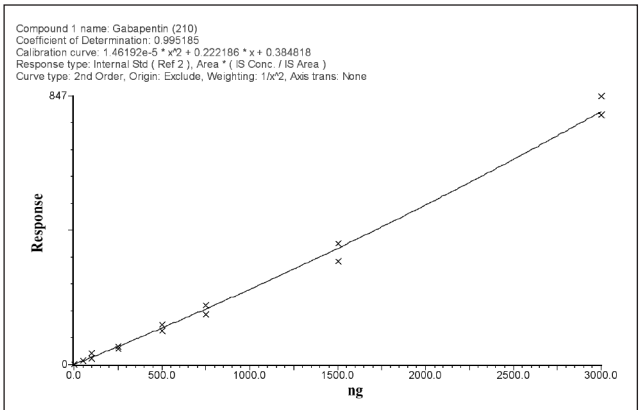

Figure 11. Typical calibration curve for gabapentin extracted from equine plasma samples, indicating a coefficient of determination of 0.995185.

Table III. Accuracies, Within-Run, and Between-Run Precisions of the GC–MS Assay used to Quantify Gabapentin in Horse Plasma Samples

Theoretic concentration (ng/mL)	Measured concentration (ng/mL; mean ± SD) (n = 6)	Accuracy (%) (mean ± SD)	Coefficient of variation (%)
Within-run			
60	62.33 ± 4.63	103.67 ± 7.74	7.47
500	498 ± 24.16	101.17 ± 4.71	4.66
2400	2398.5 ± 145.33	100 ± 6.26	6.26
Mean		101.61	6.13
Between-run			
60	58.33 ± 5.89	97.3 ± 9.6	9.87
500	518.33 ± 56.46	103.83 ± 10.96	10.56
2400	2521.67 ± 49.27	105.33 ± 2.25	2.14
Mean		102.15	7.52

assay validation. In addition, the within-run accuracies for this method (n = 6 for each value) were 103.67% ± 7.74 (SD), 101.17% ± 4.71 (SD), and 100% ± 6.26 (SD) for 60, 500, and 2400 ng/mL standard solutions, respectively (Table III); the accuracy requirements for validation of the assay are between 85% and 115%.

For between-run precision, the CV for 60 ng/mL (n = 6) was 9.87%, for 500 ng/mL (n = 6) was 10.56%, and for 2400 ng/mL was 2.14% with the mean CV of 7.52% (Table III). In addition, the between-run accuracies for this method (n = 6 for each value) were 97.3% ± 9.6 (SD), 103.83% ± 10.96 (SD), and 105.33% ± 2.25 (SD) for 60, 500, and 2400 ng/mL standard solutions, respectively (Table III). The extraction efficiency was determined in three different concentrations (n = 6 for each value): low (100 ng/mL) = 64.9% ± 6.58 (SD), medium (500 ng/mL) = 63.2% ± 9.96 (SD), and high (1500 ng/mL) = 52.67% ± 6.65 (Table IV). Note that extraction efficiencies ranging from 53–65% generally agreed with the initial estimate of recovery described in Figure 10 for UCT Clean Screen. The measurement uncertainty and expanded uncertainty were determined in three different concentrations (n = 6 for each value): low (100 ng/mL) = 2.69% and 6.73%, medium (500 ng/mL) = 4.07% and 10.18%, and high (1500 ng/mL) = 2.72% and 6.8%, respectively (Table IV).

Long term stability of standards of gabapentin stored at 4°C has already been demonstrated by Wolf et al. (25); these authors also showed 6 month stability of serum gabapentin stored at –15°C. This is in agreement with the earlier work of Hengy & Kölle (10), who showed 6 month stability of gabapentin in plasma. Equine samples were generally analyzed within 1–2 weeks following collection in our study, so stability was not considered a likely problem.

GC of the gabapentin-lactam-TMS derivative must proceed promptly after derivatization. Stability of derivatives awaiting injection by autosamplers was studied by repeated injection of 0.1 µL of individual 10 ng/µL or 2 ng/µL gabapentin samples in BSTFA/1% TMCS in the absence of internal standard and monitoring the *m/z* 210 area on GC–MS over a 10-h period. The derivative absolute area was found to degrade by 3% over a 100-min period, or 0.03%/min for the 10 ng/µL standard. Results for the 2 ng/µL standard (roughly at the LOD) degraded at 0.08%/min in comparison, possibly accounting to some extent for the slight curvilinearity of calibration curves. These effects would be ameliorated by taking into account the internal standard, particularly if a deuterated gabapentin standard were to become available.

Table IV. Extraction Efficiencies, Measured, and Expanded Uncertainties of the GC–MS Assay Used to Quantify Gabapentin in Horse Plasma Samples*

Theoretical Concentration (ng/mL)	Extraction Efficiency (%) (mean ± SEM) (n = 6)	Measured Uncertainty (%)	Expanded Uncertainty (%)
100	64.9 ± 6.58	2.69	6.73
500	63.2 ± 9.96	4.07	10.18
1500	52.67 ± 6.65	2.72	6.8
Mean	60.26	3.16	7.9

* Uncertainty was determined using the method of A2LA (2002, personal communication from Dr. Cornelius Uboh, Equine Toxicology and Research Laboratory, University of Pennsylvania, School of Veterinary Medicine, Kennet Square, PA). Mean ± expanded uncertainty = 95% confidence range.

Discussion

Gabapentin is a relatively new antiepileptic drug listed as a Class 3 drug by the ARCI. As such, it requires definitive understanding of its side-reaction, derivatization and fragmentation chemistries in the quest for a validated method. Our spectrum of underivatized gabapentin (Figure 1A) with a significant *m/z* 153 suggested correspondence to the work of Van Lente and Gatautis (24), whose GC–MS method for underivatized gabapentin focused on the principal *m/z* 153. In addition, our ESI^+–MS spectra of gabapentin M+H (Figure 6) corresponded well to spectra reported by Ifa et al. (12) and Matar and Abdel-Hamid (16). However, comparisons were not so straightforward regarding the TMS derivative of the cyclized gabapentin lactam, and this represented a principal source of confusion during the elaboration of a gabapentin-TMS method on GC–MS, and we compare our work to one such report next.

Gambelunghe et al. (7) offered an EI-mass spectrum for gabapentin-TMS with a putative molecular ion at *m/z* 226. There is no resemblance to our gabapentin-lactam-TMS (Figure 1B), and the principal peaks (*m/z* 154, 110, 81, 67) have much more in common with the underivatized gabapentin lactam (Figure 1A), particularly *m/z* 110, 81, and 67. The *m/z* 226 peak of the Gambelunghe et al. (7) EI-spectrum has no associated isotopic peaks of which the M+1 of a presumed $C_{12}H_{24}NOSi$ compound should have a rather significant 19.2% intensity. The authors offer no structural analysis, although they do provide the EI daughter ion spectrum of *m/z* 226, with principal peaks at *m/z* 207, 191, 177, 153, 144, 133, 119, and 111. In the Experimental Section, the authors label the product of MSTFA derivatization as the bis(trimethylsilyl) ether (*sic*) of gabapentin; a bis(TMS) derivative should have a molecular ion at *m/z* 315, which differs by 89 amu from their putative molecular ion at *m/z* 226. One simple possibility is that their conditions favored bis(TMS) formation and subsequent loss of O-TMS (89 amu) to provide a *m/z* 226 fragment; unfortunately, this possibility does not offer straightforward interpretation of the *m/z* 226 daughter ions. Other serious complications: there is no straightforward mechanism to produce a loss of 19 amu to explain *m/z* 207, and the EI-mass spectrum lacks an *m/z* 73 typical of TMS derivatives. Because we found it difficult to rationalize a possible bis(TMS) derivative in light of the derivatization behavior we have observed (Figure 5), the more likely explanation of the results of Gambelunghe et al. is (*i*) mono(TMS) derivatization of a gabapentin lactam; (*ii*) space charging in their Varian 2000 ion trap to give a high population of M+1 molecular ions (*m/z* 226); (*iii*) although *m/z* 111, 144, and 153 daughter ions become explicable in this scenario, the possibility exists that the spectra are plagued by artifactual

spectrometry induced by space charging effects, particularly in the absence of any exercise of dilute-and-rerun for more accurate spectra or similar verification procedures.

Extraction efficiency was adequate to the method, considering that zwitterionic species are generally more difficult to extract by SPE. Considering that only 1 mL plasma was used, the LOD and LOQ were excellent and likely to conform well to the needs of racing labs. A parallel paper on the equine pharmacology of gabapentin (5) will demonstrate that plasma concentrations were well above the LOQ during the first 24 h following a reasonable 5 mg/kg dose.

The method validated in this study offered the following advantages: (*i*) it utilizes the more prevalent GC–MS instrumentation likely to be present in racing laboratories; (*ii*) it avoids exclusive dependence on the thermally-derived lactam by locking it in as an O-TMS derivative; (*iii*) TMS derivatization also avoids issues of tailing on GC; (*iv*) it provides a clear-cut explanation for the derivatization chemistry and mass spectral fragmentation of the lactam-TMS derivative.

Conclusions

(*i*) We have visualized gabapentin as the gabapentin lactam in every case, particularly during GC, either as free compound or as TMS derivative. (*ii*) Solvent choices may be important, as protic solvents such as methanol may lead to degradation of gabapentin and its lactam into multiple products in the presence of heat. (*iii*) Figure 12 indicates our interpretation that the gabapentin lactam, once formed, most likely derivatizes at the amido N-atom, which basically agrees with findings summarized by Pierce (18) for similar compounds; however, the formal possibility exists of occasional O-TMS formation via keto-enol tautomerism or amido-iminol tautomerism. Appearance of peaks *m/z* 116 and 95 in the TMS derivative suggest that the TMS is located on the N-atom, because cleavage of the enol double bond is less likely than cleavage of C-N bonds. (*iv*) According to ESI$^+$–MS results, the gabapentin standard is in fact gabapentin, and the lactam is solely a derivatization and/or chromatographic artifact. (*v*) The method should be more than adequate for accurate quantitation of gabapentin following administration of this drug; it does not, however, enable differentiation between the administered drug and any *in vivo* conversion to gabapentin lactam. Even though the anticonvulsant efficacy of gabapentin is lost by lactam formation in humans (19) and this information may be of significance in horses or other animals, this should be only a minor difficulty because conversion estimates in humans administered gabapentin are relatively small (13).

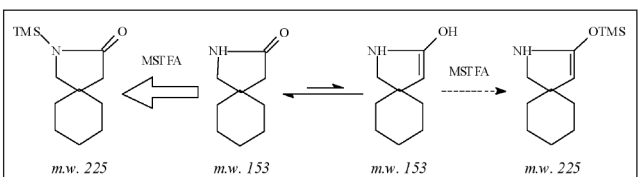

Figure 12. The most likely route to a gabapentin lactam-TMS derivative is trimethylsilylation of the amido N-atom, converting the 153 m.w. lactam to its 225 m.w. derivative. However, the formal possibility exists that keto-enol conversion to the lactam enol isomer (or to the lactam iminol isomer with double bond between C and N [not shown]) may provide targets for *O*-trimethylsilylation.

Acknowledgements

This research was supported by grants from the Birmingham Racing Commission Breeding and Development Fund, Birmingham, AL, and Tuskegee University Research and Graduate Studies Development Fund, Tuskegee, AL. The authors thank Pharsight Corporation (Cary, NC) for providing WinNonlin, version 5.1, for pharmacokinetics analysis.

References

1. Association of Racing Commissioners International. *Uniform Classification Guidelines for Foreign Substances and Recommended Penalties and Model Rule*. Lexington, KY, 1998.
2. AORC. *Minimum criteria for identification by chromatography and mass spectrometry.* Association of Official Racing Chemist Proposed Guidelines, Lexington, KY, 2001.
3. D.M. Boothe. Anticonvulsant therapy in small animals. *Veterinary Clinical Practice of North America: Small Animal Practice* **28:** 410–448 (1998).
4. D. Chadwick. Drug profile: gabapentin. *Lancet* **343:** 89–91 (1994).
5. L. Dirikolu, A. Dafalla, K.J. Ely, A.L. Connerly, C.N. Jones, H. ElkHoly, A.F. Lehner, K. Thompson, and T. Tobin. Gabapentin in horses: pharmacokinetics and clinical pharmacology. *Journal of Veterinary Pharmacology and Therapeutics*, in press.
6. N.B. Finnerup, H. Gottrup, and T.S. Jensen. Anticonvulsants in central pain. *Expert Opinion on Pharmacotheraphy* **3:** 1411–1420 (2002).
7. C. Gambelunghe, G. Mariucci, M. Tantucci, and M.V. Ambrosini. Gas chromatography-tandem mass spectrometry analysis of gabapentin in serum. *Biomed Chromatogr.* **19:** 63–67 (2005).
8. D. Gauthiera and R. Gupta. Determination of gabapentin in plasma by liquid chromatography with fluorescence detection after solid-phase extraction with a C18 column. *Clin. Chem.* **48:** 2259–2261 (2002).
9. K.L. Goa and E.M. Sorkin. Gabapentin, a review of its pharmacological properties and clinical potential in epilepsy. *Drugs* **46:** 409–427 (1993).
10. H. Hengy and E.U. Kölle. Determination of gabapentin in plasma and urine by high-performance liquid chromatography and precolumn labeling for ultraviolet detection. *J. Chromatogr. B* **341:** 473–478 (1985).
11. W.D. Hooper, M.C. Kavanagh, and R.G. Dickinson. Determination of gabapentin in plasma and urine by capillary column gas chromatography. *J. Chromatogr. B* **529:** 167–174 (1990).
12. D.R. Ifa, M. Falci, M.E. Moraes, F.A.F. Bezerra, M.O. Moraes, and G. de Nucci. Gabapentin quantification in human plasma by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry. Application to bioequivalence study. *J. Mass Spectrom.* **36:** 188–194 (2001).
13. R. Knorle, T.J. Feuerstein, and A. Schulze-Bonhage. Determination of gabapentin-lactam in serum of patients under gabapentin therapy. *Arzneimittelforschung* **54:** 139–142 (2004).
14. M.M. Kushnir, J. Crossett, P.I. Brown, and F.M. Urry. Analysis of

gabapentin in serum and plasma by solid-phase extraction and gas chromatography–mass spectrometry for therapeutic drug monitoring. *J. Anal. Toxicol.* **23:** 1–6 (1999).
15. W.A. Lagreze, R. Muller-Velten, and T.J. Feuerstein. The neuroprotective properties of gabapentin-lactam. *Graefes Arch Clin Exp Ophthalmol* **239:** 845–849 (2001).
16. K.M. Matar and M.E. Abdel-Hamid. Rapid tandem mass spectrometric method for determination of gabapentin in human plasma. *Chromatographia* **61:** 499–504 (2005).
17. F.W. McLafferty and F. Turecek. *Interpretation of Mass Spectra*, 4th Ed. University Science Books, Sausalito, CA, 1993.
18. A.E. Pierce. *Silylation of Organic Compounds*. Pierce Chemical Co., Rockford, IL, 1982.
19. H. Potschka, T.J. Feuerstein, and W. Loscher. Gabapentin-lactam, a close analogue of the anticonvulsant gabapentin, exerts convulsant activity in amygdala kindled rats. *Naunyn Schmiedebergs Arch Pharmacol.* **361:** 200–205 (2000).
20. R.R. Rando and F.W. Bangerter. The irreversible inhibition of mouse brain gamma-aminobutyric acid (GABA)-alpha-ketoglutaric acid transaminase by gabaculine. *J. Amer. Chem. Soc.* **98:** 6762–6764 (1976).
21. H. Rosner, L. Rubin, and A. Kestenbaum. Gabapentin adjunctive therapy in neuropathic pain states. *Clinical Journal of Pain* **12:** 56–58 (1996).
22. C. Taylor. Emerging perspectives on the mechanism of action of gabapentin. *Neurology* **44:** S10–16 (1994).
23. J.A. van Hooft, D. Endeman, R.A. Nichols, and W.J. Wadman. Gabapentin inhibits presynaptic Ca^{+2} influx and synaptic transmission in rat hippocampus and neocortex. *Eur. J. Pharmacol.* **449:** 221–228 (2003).
24. F. Van Lente and V. Gatautis. Cost-efficient use of gas chromatography-mass spectrometry: a "piggyback" method for analysis of gabapentin. *Clin. Chem.* **44:** 2044–2045 (1998)
25. C.E. Wolf II, J.J. Saady, and A. Poklis. Determination of gabapentin in serum using solid-phase extraction and gas-liquid chromatography. *J. Anal. Toxicol.* **20:** 498–501 (1996).
26. B. Zucker, D.E. Ludin, T.A. Gerds, C.H. Lucking, G.B. Landwehrmeyer, and T.J. Feuerstein. Gabapentin-lactam, but not gabapentin, reduces protein aggregates and improves motor performance in a transgenic mouse model of Huntington's disease. *Naunyn Schmiedebergs Arch Pharmacol.* **370:** 131–139 (2004).

Manuscript received July 4, 2007
Revision received April 6, 2007