Development of a Candidate Reference Measurement Procedure for the Determination of Phenytoin, Phenobarbital, Lamotrigine, and Topiramate in Human Serum Using Isotope-Dilution Liquid Chromatography/Tandem Mass Spectrometry

Susan S.-C. Tai,[*] Chia-Yi Yeh,[a] and Karen W. Phinney

Analytical Chemistry Division, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8392

[*] Author for correspondence. E-mail: susan.tai@nist.gov.

[a] Permanent address: Chemical Measurement Laboratory, Industrial Technology Research Institute, Hsinchu, Taiwan, R.O.C.

Keywords: Phenytoin; PHT; Phenobarbital; PHB; Lamotrigine; LTG; Topiramate; TPM; Reference measurement procedure; RMP; Eletrospray ionization; Isotope dilution; Liquid chromatography/tandem mass spectrometry; LC-MS/MS; solid phase extraction.

ABSTRACT

Phenytoin (PHT), phenobarbital (PHB), lamotrigine (LTG), and topiramate (TPM) are some of the most widely used antiepileptic drugs (AEDs). Monitoring of their concentrations in serum is important for the treatment of epilepsy. A candidate reference measurement procedure (RMP) for the determination of PHT, PHB, LTG, and TPM in serum has been developed and critically evaluated. Isotopically labeled compounds of PHT, PHB, LTG, and TPM are used as internal standards for the four AEDs. The four drugs and their respective labeled internal standards are simultaneously extracted from serum using SPE prior to reversed-phase liquid chromatography-tandem mass (LC-MS/MS). Chromatographic separation was performed using a C_{18} column. Electrospray ionization (ESI) in the positive ion mode for PHT and LTG, and in the negative ion mode for PHB and TPM were used. The accuracy of the method was evaluated by recovery studies of measuring the four drugs in spiked samples with known drug levels. The recoveries of the added drugs ranged from 98.6 % to 102.0 %. The absolute recoveries of the four drugs with this method ranged from 97 % to 100 %. The difficulty in getting good repeatability for TPM due to the large mass difference between TPM and its internal standard (TPM-d_{12}) was overcome by shortening its retention time. The peak shape of high nitrogen containing compound of LTG was improved using ammonium acetate as an ion pairing reagent in the mobile phase. Excellent repeatability was obtained for the four drugs with between-set coefficients of variation (CVs) of < 1 %. The limit of detection at a signal-to-noise ratio of ~ 3 was 0.8 pg, 13 pg, 0.1 pg, and 0.2 pg for PHT, PHB, LTG, and TPM, respectively. This candidate LC-MS/MS RMP for the determination of PHT, PHB, LTG, and TPM in serum demonstrating good accuracy and

precision can be used to assess the accuracy of test methods used in clinical laboratories.

INTRODUCTION

Epilepsy is one of the most common dysfunctions of the nervous system, and affects almost 3 million Americans of all ages. Phenytoin (PHT), phenobarbital (PHB), lamotrigine (LTG), and topiramate (TPM) are some of the most widely used antiepileptic drugs (AEDs). These drugs may be used alone or in combination to treat epilepsy. PHT and PHB are traditional AEDs which have been used for several decades,[1] while LTG and TPM are the new generation AEDs which were approved by FDA and have been used since 1993.[2] Therapeutic drug monitoring has been playing an important role on the treatment of epilepsy to determine most effective dose for certain AEDs and to avoid toxicity. The therapeutic levels in serum are 10-20 µg/mL, 15-40 µg/mL for PHT, and PHB, respectively,[1] and 2.5-15.4 µg/mL, and 5-20 µg/mL for LTG, and TPM, respectively.[2] High levels of AEDs in serum are associated with toxicity and adverse health effects.

Approximately three decades ago, the National Institute of Standards and Technology (NIST) had issued the Standard Reference Material (SRM) 900, a lyophilized serum material containing four older AEDs (PHT, PHB, ethosuximide, and primidone) in serum for clinical laboratories to evaluate the accuracy of their methods. Recently, many patients with epilepsy are treated with newer AEDs or combination of older and newer AEDs. In response to this situation, NIST has redesigned the SRM 900 (to SRM 900a) to include newer drugs. The candidate SRM 900a is a frozen pooled serum material containing two older drugs (PHT and PHB), and two newer drugs (LTG and TPM) at two different levels. Level 1 contains approximately the therapeutic level of each drug, and

level 2 contains each drug at concentrations associated with toxicity and adverse health effects. The NIST is developing new reference measurement procedures for the new components (LTG and TPM) in SRM 900a, and the methods for PHT and PHB are also changed from liquid chromatography (LC) and gas chromatography (GC) to isotope dilution (ID) liquid chromatography/mass spectrometry (LC/MS) based methods.

Measurement of AEDs has been performed using a number of different analytical techniques. Historically, immunoassays,[3] GC,[4] and LC[1;5] have been the primary methods used for measuring older AEDs in serum. Recently, ID-MS methods coupled with GC or LC with higher specificity have been developed for newer and older AEDs.[6-15] Along with LC based ID-MS methods, conventional LC methods for newer drugs have been continuously developed[16;17] and widely used in clinical laboratories due to their capability of high throughput and simultaneously analyzing multiple AEDs in samples of patients on AED polytherapy. TPM is unable to be analyzed by conventional LC because it lacks a chromophore, and is also poorly suited for GC analysis due to its relative thermolability. However, various LC/MS based methods for TPM have been developed and used widely in clinical laboratories.[7;9;12-15] Currently, no higher-order RMPs with high precision and accuracy for the determination of the four AEDs in serum have been reported. RMPs can be used to assign the concentrations of reference materials which can be used as controls or calibrators for routine test methods. They provide means for demonstrating traceability of routine test methods and materials to higher-order reference materials. The requirements of RMPs for clinical diagnostic markers have been outlined by the International Organization for Standardization (ISO) in ISO 15193 (In vitro diagnostic medical devices - Measurement of quantities in samples of biological origin –

Requirements for content and presentation of reference measurement procedures).[18] The Joint Committee for Traceability in Laboratory Medicine (JCTLM)[19] reviews potential RMPs and compiles a list of those that meet the requirements of ISO 15193.

Recently, the NIST has developed two ID-MS methods for the determination of PHT, PHB, LTG, and TPM in serum. The first method involves solid phase extraction (SPE) to isolate the four AEDs from serum matrix and LC-MS/MS for measurement. The second method involves liquid-liquid extraction and LC/MS for measurement which will be published elsewhere. The accuracy of the LC-MS/MS method was evaluated by recovery studies of measuring the four drugs in spiked samples with known drug levels. The repeatability of the method was evaluated by repeated measurements of the four drugs in frozen serum samples in multiple days. Potential interferences from the metabolites of the four AEDs in serum were investigated. All of the requirements for an RMP recognized by the JCTLM have been met except for validation by an interlaboratory study which is currently being planned. The results from this study will complete the requirements for a RMP recognized by the JCTLM.

The results from this LC-MS/MS method and additional data from the LC/MS method will be used to certify the concentrations of PHT, PHB, LTG, and TPM in the candidate SRM 900a, which can be used to assess the accuracy of the test methods used in clinical laboratories.

EXPERIMENTAL SECTION

Materials. The LTG reference compound was obtained from Sigma Chemical Co. St. Louis, MO. The PHT reference compound was obtained from Parke, Davis & Co., Detroit, MI. The PHB and TPM reference compounds were obtained from the United

States Pharmacopeia, Rockville, MD. The impurities in these materials were evaluated by gas chromatography-flame ionization detection (GC-FID) and DSC. Moisture contents were determined by Karl Fischer titrations. The impurities in each material were determined to be < 0.4%, and the moisture content was < 0.04%. NO corrections were made for the impurities and moisture content in each material, instead, a type B uncertainty of 0.5% is estimated for the purity of theses compounds. An isotopically labeled internal standard for each compound was used as an internal standard. LTG-$^{13}C_2{}^{15}N_1$ and PHT-$^{13}C_1{}^{15}N_2$ were obtained from Cambridge Isotope Lab Inc., Andover, MA. PHB-d_5 was obtained from Cerilliant, Round Rock, TX. TPM-d_{12} was obtained from Toronto Research Chemicals, North York. Frozen human serum materials from individual donors were obtained from Interstate Blood Bank, Inc. (Memphis, TN). Frozen serum pooled materials at two different levels (candidate SRM 900a) were prepared by Aalto Scientific, Ltd., Carlsbad, CA. A LUNA C18 (2) column [2.0 mm (i.d.) x 15 cm, 5-µm particle diameter] was obtained from Phenomenex (Torrance, CA). C_{18} Sep-Pak SPE cartridges were obtained from Waters, Milford, MA. Solvents used for LC/MS/MS measurements were HPLC grade, and all other chemicals were reagent grade. Structures of PHT, PHB, LTG, and TPM are presented in Figure 1.

Preparation of Calibration Solutions. Three standard stock solutions for each of PHT, PHB, LTG, and TPM were gravimetrically prepared for calibration. Approximately 2 - 9 mg (6 mg for PHT, 9 mg for PHB, 2 mg for LTG, and 4 mg for TPM) for each standard stock solution was accurately weighed, and dissolved in 100 mL of anhydrous ethanol yielding the concentrations of the three stock solutions ranged from 73 µg/g to 77 µg/g for PHT, 112 µg/g to 116 µg/g for PHB, 23 µg/g to 25 µg/g for LTG, and 44 µg/g to

47 μg/g for TPM.

A solution of an isotopically labeled internal standard for each of the drugs at a concentration of approximately 79 μg/g, 28 μg/g , and 47μg/g for PHT-$^{13}C_1{}^{15}N_2$, LTG-$^{13}C_2{}^{15}N_1$, and TPM-d_{12}, respectively, was prepared in the same way as their respective unlabeled compound. For PHB-d_5, a solution of 88 μg/mL in anhydrous ethanol (with a small amount of methanol) was prepared from ampoules containing PHB-d_5 at a concentration of 1 mg/mL in methanol. The concentration of 111.44178 μg/g was calculated by dividing the combined density of methanol and ethanol (0.78965 at 20 °C).

Six calibrants were prepared for each of the four drugs. Two aliquots (145 μL - 325 μL for PHT, 135 μL - 304 μL for PHB, 163 μL - 367 μL for LTG, and 144 μL - 313 μL for TPM) from each of the three standard stock solutions for each drug were spiked with 225 μL of their respective internal standard solution, yielding six calibrants with mass ratios of unlabeled to labeled compound ranging from 0.6 to 1.4. The mixtures were dried under nitrogen at approximately 45 °C and reconstituted with 150 μL of methanol for LC-MS/MS analysis for PHB. The mixtures were further diluted 1:15 (volume fraction) with methanol for PHT and TPM analyses, and diluted 1:150 for LTG analysis.

Sample Preparation. Samples were prepared in three different sets, each set consisting of duplicate aliquots from each of two vials for each level. Each aliquot (approximately 0.8 g for level 1, and 0.4 g for level 2) was accurately weighed into a 50 mL glass centrifuge tube. Water (2.6 mL for level 1, and 5.7 mL for level 2) was added to each sample to avoid protein precipitation when samples were spiked with internal standard solutions. Each aliquot was spiked with an appropriate amount of each internal standard to obtain an approximately 1:1 ratio of analyte to internal standard, and was

adjusted to pH 2.0 ± 0.5 with 0.5 mol/L phosphoric acid (12 mL for level 1, and 13 mL for level 2). Each drug was isolated from the serum matrix using a C_{18} SPE cartridge. Each sample was loaded onto a cartridge previously conditioned by wetting with 5 mL of methanol, followed by 5 mL of water. The cartridge was then washed with 12 mL of water, followed by 5 mL of 5:95 (volume fraction) methanol:water. The drug was eluted from the cartridge with 2 mL of methanol. The eluent was dried under nitrogen at approximately 45 ^{o}C and the residues were reconstituted with methanol (150 µL for level 1, and 270 µL for level 2) for LC-MS/MS analysis for PHB. As done for the calibrants, the sample extracts were further diluted 1:15 (volume fraction) with methanol for PHT and TPM analyses, and 1:150 (volume fraction) for LTG analysis.

LC-MS/MS Analysis for AEDs. Samples and calibrants were analyzed with an Applied Biosystems API 4000 LC-MS/MS system equipped with an Agilent 1100 Series LC system. For each drug, an isocratic method was used with Phenomenex LUNA C18 (2) columns (15 cm x 2.0 mm, 5 µm particle diameter) at 22 ºC, with mobile phases of water-methanol mixtures containing 5 mmol/L ammonium acetate, operated at 0.25 mL/min. Mobile phases of water-methanol 51:49, 50:50, 65:35, 35:65 (volume fraction) for PHT, PHB, LTG, and TPM, respectively were used. After the analyte was eluted, the column was rinsed with 100% methanol for 10 min and then equilibrated at the initial condition for 12 min. The autosampler tray temperature was set at 10 ºC. The injection volume was 1 - 2 µL. Electrospray ionization (ESI) in the positive ion mode for PHT and LTG, and in the negative ion mode for PHB and TPM were used. The MS/MS parameters for each drug were optimized and summarized in Table 1.

The following measurement protocol was used for LC-MS/MS analysis. The six

calibrants were analyzed along with the samples. The calibrants were analyzed first, followed by the samples, and then the samples and calibrants were analyzed in reverse order. Instrumental response was determined from a linear regression fit of the calibration data using a y = mx + b regression model.

Method Validation for the Four AEDs. A commercially available drugs free frozen human serum material from a single donor was used for the absolute recovery study of AEDs from serum. The approaches described in previously published papers for steroid hormones[20;21] were used to determine the absolute recovery of AEDs from serum with this extraction method. The recoveries of AEDs were evaluated with the serum samples that were spiked with AEDs at 15.3 μg/g, 21.6 μg/g, 5.3 μg/g, and 9.1 μg/g, for PHT, PHB, LTG, and TPM, respectively. For each of the four AEDs, two groups of samples were prepared. For the first group, serum was spiked with each drug before extraction and the respective labeled internal standard after extraction. For the second group, both the drug and the respective labeled internal standard were spiked before extraction. The samples were processed according to the procedure described above in the sample preparation for LC-MS/MS measurement. The absolute recovery of AEDs from serum was calculated from comparison of the results from the two groups.

The accuracy of the method was evaluated by measuring the four drugs in spiked samples with known drug levels. The previously mentioned serum material for the study of absolute recovery was used for this study. The serum samples were subdivided into twelve aliquots, and spiked with the four AEDs at three concentrations (four aliquots for each of the three concentrations at approximately 4 μg/g, 14 μg/g, and 33 μg/g for PTH, at approximately 7 μg/g, 21 μg/g, and 63 μg/g for PHB, at approximately 1 μg/g, 6 μg/g,

and 15 μg/g for LTG, and at approximately 2 μg/g, 9 μg/g, and 25 μg/g for TPM). Appropriate amounts of the four internal standards were added to all aliquots, and the aliquots were processed using the procedure described in the experiment section for LC-MS/MS measurement.

The candidate SRM 900a at two different levels was used to evaluate the repeatability of the method. Samples were prepared in three different sets (each set on a different day), each set consisting of four aliquots from each level. Repeatability (within-set precision) and intermediate precision (between-set precision) were evaluated for the LC-MS/MS method.

Uncertainty Evaluation. NIST guidelines[22] which conform to the ISO Guide to the Expression of Uncertainty in Measurement[23] were followed for statistical treatment of the data. Potential sources of uncertainty were evaluated, and those factors that could contribute significantly were used to calculate the standard uncertainty. For measurement imprecision uncertainty (type A component), an analysis of variance calculation was performed on the measurement data to determine if set-to-set differences were statistically significant. This analysis determined the number of independent measurements, n, used for calculating the measurement standard deviation of the mean. Other uncertainty components (type B) were based on the uncertainties in the purity of the reference compounds, in the weighing of the reference compounds, and unknown systematic errors in the sample preparation and undetected interferences in the LC-MS/MS analysis. Type A and type B uncertainty components were combined quadratically to determine the standard uncertainty, u_c, which was multiplied by a coverage factor, k, to calculate the expanded uncertainty, U.

RESULTS AND DISCUSSION

Method Validation. Most AEDs are bound to protein in the circulation.[1;24;25] PHT is highly protein bound (90% to 95%). PHB and LTG are approximately 50% bound to serum proteins, while the binding of TPM to serum proteins is low. Prior to C_{18} SPE isolation of the four AEDs from serum matrix, the serum samples were adjusted to pH 2 to liberate the AEDs from their binding proteins.[20;26] The SPE extraction procedure produced a clean extract with no interferences detected at ions monitored for the four AEDs by LC-MS/MS. The absolute recovery of the four AEDs from the serum with this extraction method averaged 97% (3.0% CV, n=4), 100% (1.5 % CV, n=4), 99% (0.2% CV, n=4), and 99% (1% CV, n=4), for PHT, PHB, LTG, and TPM, respectively. Absolute recoveries of less than 100% should not influence method accuracy, because quantitation is based on the ratio of unlabeled to labeled AEDs when ID-MS was used.

The recoveries of the four AEDs added to the serum (accuracy test) are listed in Tables 2A, 2B, 2C, and 2D, for PHT, PHB, LTG, and TPM, respectively. The drug free serum material was spiked with AEDs at three different concentrations (concentration 2 is within the therapeutic level for each AED, concentration 1 is below the therapeutic level and concentration 3 is above the therapeutic level). The amounts recovered and added were in very good agreement at all three concentrations for the four AEDs; the mean recoveries ranged from 98.6 % to 102.0 %.

Repeatability (within-set precision) and intermediate precision (between-set precision) were evaluated for this LC-MS/MS method. The method was applied to the candidate SRM 900a at two levels. Three sets of samples were analyzed, each set

consisting of four samples for each of the two levels. The results are summarized in Tables 3A-3D. Within-set coefficients of variations (CVs) and between-set CV for all the four AEDs at the two levels were all within 1%. Selected ion chromatograms from level 1 samples of the candidate SRM 900a are shown in Figures 2 to 5 for PHT, PHB, LTG, and TPM, respectively.

The only commercially available isotopically labeled internal standard for TPM is TPM-d_{12}. The large mass difference between TPM and TPM-d_{12} makes their LC retention times significantly different. In the initial attempt of TPM analysis, much larger CVs were obtained. Shortening their retention times to approximately 3 min resulted in co-elution of the two peaks and minimized the instrument drift, thus improving the repeatability of the measurements to approximately 1%.

LTG is a high nitrogen containing compound. Getting a good peak shape is a measurement challenge for LTG. Use of ammonium acetate as an ion pairing agent in the mobile phase significantly improves the peak shape of LTG, thus reduces the measurement error caused by improper peak integration.

To satisfy the requirements of ISO 15193, an RMP must be thoroughly tested for sources of bias. The blank pooled serum material that was used to prepare the candidate SRM 900a was analyzed for these AEDs by this method. No interferences were detected at ions monitored for the four AEDs. The metabolites of the four AEDs in serum were investigated from literature for potential interferences. PHT and PHB are primarily metabolized to hydroxylated derivatives of the drugs,[1;27] LTG to its methyl, oxide, and glucuronide of LTG,[6] and TPM to its hydroxyl and diol of TPM.[7] Due to large differences in the molecular masses, the metabolites of these AEDs should not interfere

with the LC-MS/MS method.

The limit of detection at a signal-to-noise ratio of ~ 3 was 0.8 pg, 13 pg, 0.1 pg, and 0.2 pg on column for PHT, PHB, LTG, and TPM, respectively. LTG does not fragment well in collision induced dissociation (CID) in Q2. The strongest ion found in Q3 is m/z 256, thus the transition of *m/z* 256→256 was monitored for LTG.[28;29] Strong product ions were obtained in the positive mode for PHT, and in the negative mode for TPM. Product ions of PHB were not very strong.[11] The sensitivity of PHB is one to two orders of magnitude worse than those for the other three drugs (undiluted extract was used for PHB analysis, while the extract was further diluted 15 or 150 times for the other three drugs). However, the therapeutic level of PHB in serum is at μg/mL level, the sensitivity of this method for PHB is far more than sufficient. As shown in Tables 2B and 3B, good accuracy and precision were obtained with the recovery of PHB added to serum ranging from 100.5% to 102%, and the overall CVs for PHB within 1%.

Statistical Analysis of Results. The summaries of the statistical analyses for the results are shown in Tables 4A, 4B, 4C, and 4D for PHT, PHB, LTG, and TPM, respectively. For each level of each of the four drugs, three sets of four samples each were analyzed. To determine if set-to-set differences were significant for measurement imprecision uncertainty (type A component), each sample mean was normalized by dividing by the overall mean of that level. For PHT and PHB, an analysis of variance found that the p-values were less than 0.05 for all the normalized sample means, indicating that set-to-set differences were statistically significant, effectively reducing the number of independent observations to the number of sets. Thus, the standard deviation of the mean for each level was calculated by dividing the standard deviation of the set

means for that level by the square root of n, where n=3. For LTG and TPM, an analysis of variance found that the p-values were greater than 0.05 for all the normalized sample means, indicating that set-to-set differences were not statistically significant. Thus, the standard deviation of the mean for each level was calculated by dividing the standard deviation of the sample means for that level by the square root of n, where n = 12.

To calculate the standard uncertainty, u_c, the standard deviation of the mean for the measurements was combined quadratically with the type B factors, which include uncertainties related to the purity of the four unlabeled reference compounds, the weighing of the reference compounds, and unknown systematic errors in the sample preparation and in the LC-MS/MS system. The uncertainties in the purity of the reference compound were estimated to correspond to a relative standard deviation of 0.5% for the four drugs. The uncertainty in the weighing was estimated to be 0.1%. Finally, the uncertainty of other unknown sources in the sample preparation was estimated to be 1 %. Because the type B components contribute most of the uncertainty and have very large degrees of freedom, the effective degrees of freedom for each level are large, resulting in a coverage factor, k, near 2. A value of 2 was used for multiplying the standard uncertainty for each level to calculate the expanded uncertainty, which is intended to represent a 95% confidence interval. The relative expanded uncertainties are 2.3% for all four drugs at both levels.

CONCLUSIONS

An LC-MS/MS RMP for AEDs in serum has been developed. This method demonstrates good accuracy and precision and low susceptibility to interferences. The NIST is going to use the results from this LC-MS/MS method with additional data from a

second method to certify PHT, PHB, LTG, and TPM in candidate SRM 900a which can be used to validate the accuracy of the test methods used in clinical laboratories.

ACKNOWLEDGMENTS

We thank Michele Schantz (NIST) for GC-FID analysis, Barbara Porter (NIST) for DSC analysis and Brian Lang (NIST) for Karl Fischer analysis for the purity assessment of the PHT, PHB, LTG, and TPM reference compounds.

DISCLAIMER

Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

Table 1. The MS/MS Parameters for Analyses of PHT, PHB, LTG, and TPM in Serum

Paramenters	**PHT**	**PHB**	**LTG**	**TPM**
Ion mode	Positive	Negative	Positive	Negative
MRM transition, analyte	253→182	231→188	256→256	338→78
MRM transition, internal standard	256→183	236→193	259→259	350→78
Curtain gas, psi	30	45	30	40
Collision gas, psi	7	7	3	7
Ion source gas 1, psi	75	50	70	40
Ion source gas 2, psi	75	75	75	65
Electrospray voltage, V	5500	-4500	5000	-4500
Turbo gas temperature, ℃	700	700	700	750
Declustering potential, V	61	-48	99	-85
Entrance potential, V	10	-10	10	-10
Collision energy, V	25	-15	7	-52
Collision exit potential, V	12	-3	18	-5

Table 2A. Recovery of PHT[a] Added to Serum

Conc	Added ng/g	Detected ng/g	Recovery %	Mean Recovery %	CV[b] n=3
1	3.85	3.78	98.1	98.6	0.6
	3.87	3.81	98.3		
	3.89	3.86	99.3		
	3.87	3.83	98.9		
2	13.48	13.60	100.9	100.1	0.5
	13.48	13.45	99.7		
	13.47	13.45	99.9		
	13.64	13.61	99.8		
3	33.45	33.32	99.6	100.0	0.4
	33.28	33.36	100.2		
	33.66	33.61	99.8		
	33.17	33.32	100.5		

[a] Based on known additions to a serum sample.
[b] CV, coefficient of variation.

Table 2B. Recovery of PHB[a] Added to Serum

Conc	Added ng/g	Detected ng/g	Recovery %	Mean Recovery %	CV[b] n=3
1	6.85	7.02	102.5	102.0	1.0
	6.84	6.89	100.8		
	6.86	7.07	103.2		
	6.88	6.99	101.7		
2	21.36	21.56	101.0	101.7	0.5
	21.32	21.76	102.1		
	21.28	21.67	101.8		
	21.53	21.92	101.8		
3	62.84	62.68	99.8	100.5	0.5
	62.98	63.30	100.5		
	63.86	64.46	100.9		
	62.84	63.23	100.6		

[a] Based on known additions to a serum sample.
[b] CV, coefficient of variation.

Table 2C. Recovery of LTG[a] Added to Serum

Conc	Added ng/g	Detected ng/g	Recovery %	Mean Recovery %	CV[b] n=3
1	1.47	1.47	100.1	100.4	0.3
	1.47	1.48	100.6		
	1.46	1.47	100.7		
	1.47	1.47	100.4		
2	5.85	5.87	100.3	100.7	0.5
	5.83	5.86	100.6		
	5.86	5.95	101.5		
	5.91	5.94	100.5		
3	15.15	15.19	100.2	100.4	0.2
	15.37	15.46	100.6		
	15.21	15.32	100.7		
	15.09	15.13	100.3		

[a] Based on known additions to a serum sample.
[b] CV, coefficient of variation.

Table 2D. Recovery of TPM[a] Added to Serum

Conc	Added ng/g	Detected ng/g	Recovery %	Mean Recovery %	CV[b] n=3
1	2.45	2.44	99.5	99.5	0.2
	2.46	2.45	99.3		
	2.45	2.44	99.7		
	2.45	2.44	99.7		
2	8.64	8.65	100.2	100.1	0.2
	8.62	8.64	100.3		
	8.60	8.59	99.8		
	8.66	8.67	100.1		
3	25.42	25.31	99.6	99.0	0.6
	25.67	25.20	98.2		
	25.55	25.31	99.1		
	25.31	25.13	99.3		

[a] Based on known additions to a serum sample.
[b] CV, coefficient of variation.

Table 3A. Repeatability of LC-MS/MS Measurements of PHT in Serum

Level	Set	Mean ng/g	Within-set CV, %	Overall Mean, ng/g	Between-set CV, %
1	1	11.62	0.2	11.65	0.3
	2	11.65	0.1		
	3	11.69	0.2		
2	1	46.11	0.3	46.10	0.2
	2	46.02	0.2		
	3	46.16	0.3		

Table 3B. Repeatability of LC-MS/MS Measurements of PHB in Serum

Level	Set	Mean ng/g	Within-set CV, %	Overall Mean, ng/g	Between-set CV, %
1	1	16.90	0.4	16.92	0.5
	2	16.85	0.2		
	3	17.01	0.3		
2	1	69.45	0.1	69.13	0.5
	2	68.78	0.2		
	3	69.17	0.3		

Table 3C. Repeatability of LC-MS/MS Measurements of LTG in Serum

Level	Set	Mean ng/g	Within-set CV, %	Overall Mean, ng/g	Between-set CV, %
1	1	3.95	0.2	3.95	0.3
	2	3.94	0.4		
	3	3.95	0.2		
2	1	14.59	0.2	14.62	0.3
	2	14.62	0.3		
	3	14.64	0.4		

Table 3D. Repeatability of LC-MS/MS Measurements of TPM in Serum

Level	Set	Mean ng/g	Within-set CV, %	Overall Mean, ng/g	Between-set CV, %
1	1	6.85	0.4	6.89	0.8
	2	6.95	0.2		
	3	6.86	0.6		
2	1	19.17	0.3	19.08	0.7
	2	19.02	1.0		
	3	19.03	0.3		

Table 4A. Estimation of Expanded Uncertainties for LC-MS/MS Measurements of PHT in Serum

	Level 1 (μg/g)	Level 2 (μg/g)
Mean	11.651	46.099
Type A		
Standard deviation	0.036	0.069
Standard deviation of mean	0.021	0.040
Type B		
1 % Uncertainty of systemic error	0.117	0.461
0.5 % Uncertainty of purity of reference compound	0.058	0.230
0.1 % Uncertainty of weighing	0.012	0.046
Combined standard uncertainty (u_c)	0.132	0.519
Coverage factor (k)	2	2
Expanded uncertainty (U) [a]	0.265	1.038
Relative expanded uncertainty, %	2.3	2.3

[a] 95 % confidence interval.

Table 4B. Estimation of Expanded Uncertainties for LC-MS/MS Measurements of PHB in Serum

	Level 1 (μg/g)	Level 2 (μg/g)
Mean	16.920	69.135
Type A		
Standard deviation	0.084	0.337
Standard deviation of mean	0.048	0.194
Type B		
1 % Uncertainty of systemic error	0.169	0.691
0.5 % Uncertainty of purity of reference compound	0.085	0.346
0.1 % Uncertainty of weighing	0.017	0.069
Combined standard uncertainty (u_c)	0.196	0.800
Coverage factor (k)	2	2
Expanded uncertainty (U) [a]	0.392	1.600
Relative expanded uncertainty, %	2.3	2.3

[a] 95 % confidence interval.

Table 4C. Estimation of Expanded Uncertainties for LC-MS/MS Measurements of LTG in Serum

	Level 1 (μg/g)	Level 2 (μg/g)
Mean	3.948	14.618
Type A		
Standard deviation	0.011	0.044
Standard deviation of mean	0.003	0.013
Type B		
1 % Uncertainty of systemic error	0.039	0.146
0.5 % Uncertainty of purity of reference compound	0.020	0.073
0.1 % Uncertainty of weighing	0.004	0.015
Combined standard uncertainty (u_c)	0.044	0.165
Coverage factor (k)	2	2
Expanded uncertainty (U) [a]	0.089	0.329
Relative expanded uncertainty, %	2.3	2.3

[a] 95 % confidence interval.

Table 4D. Estimation of Expanded Uncertainties for LC-MS/MS Measurements of TPM in Serum

	Level 1 (µg/g)	Level 2 (µg/g)
Mean	6.888	19.077
Type A		
Standard deviation	0.055	0.130
Standard deviation of mean	0.016	0.038
Type B		
1 % Uncertainty of systemic error	0.069	0.191
0.5 % Uncertainty of purity of reference compound	0.034	0.095
0.1 % Uncertainty of weighing	0.007	0.019
Combined standard uncertainty (u_c)	0.079	0.218
Coverage factor (k)	2	2
Expanded uncertainty (U) [a]	0.159	0.437
Relative expanded uncertainty, %	2.3	2.3

[a] 95 % confidence interval.

Figure 1. Structures of PHT, PHB, LTG, and TPM

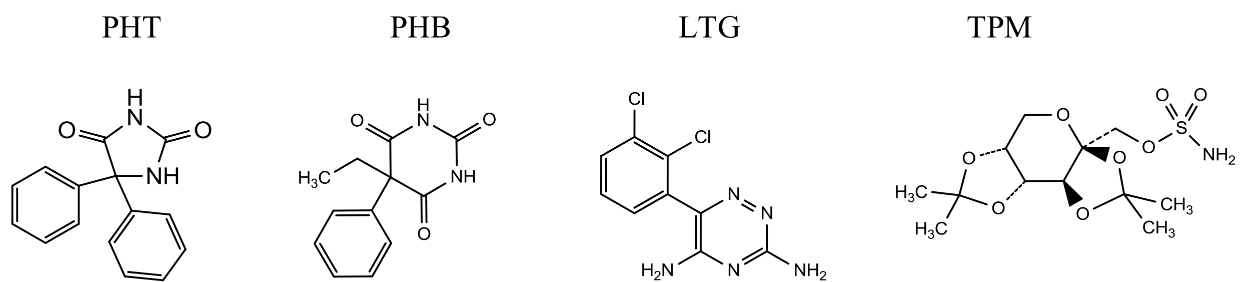


Figure 2. Selected ion chromatograms for PHT and PHT-$^{13}C_1{}^{15}N$ in serum at a concentration of 12 µg/g obtained by LC-MS/MS.

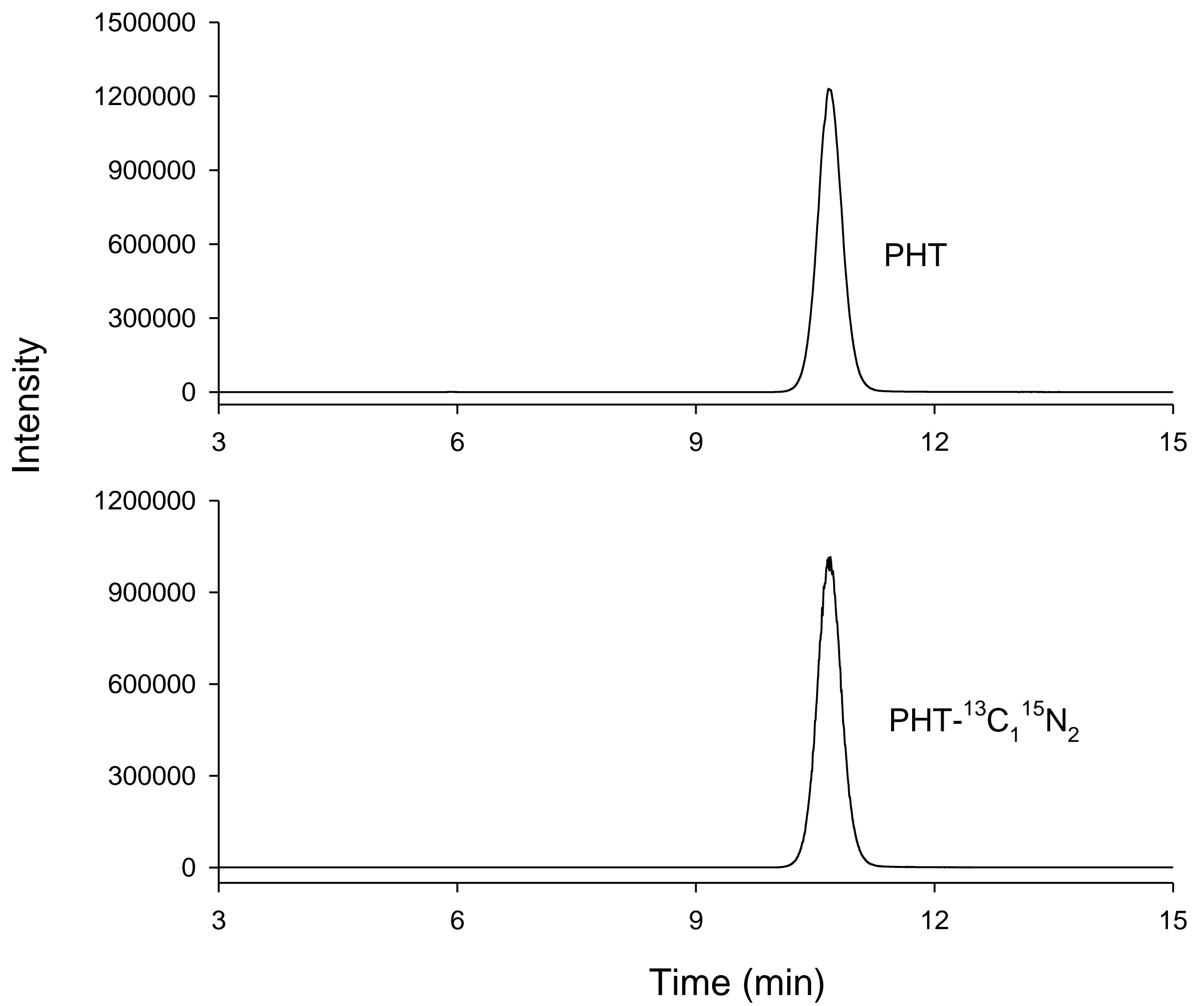
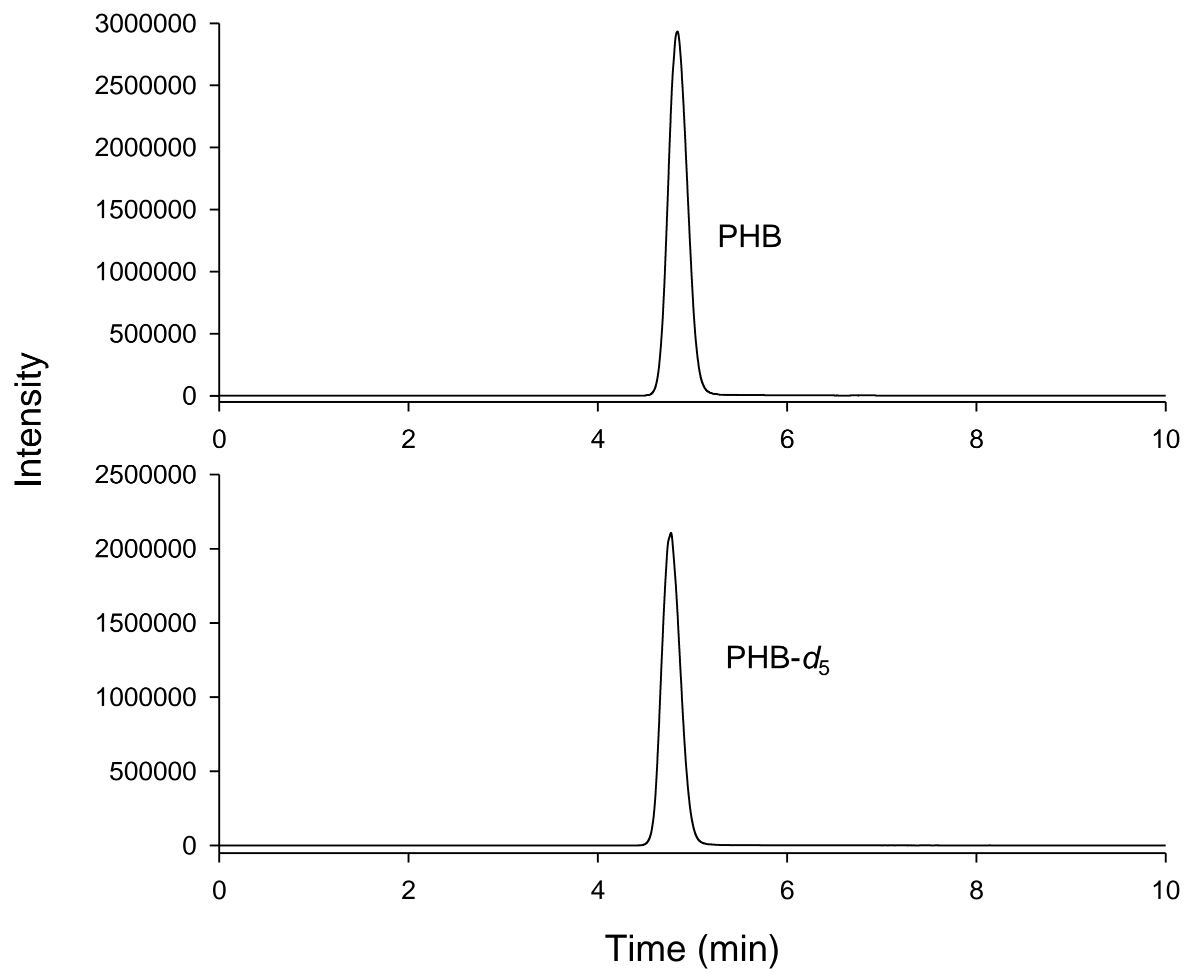
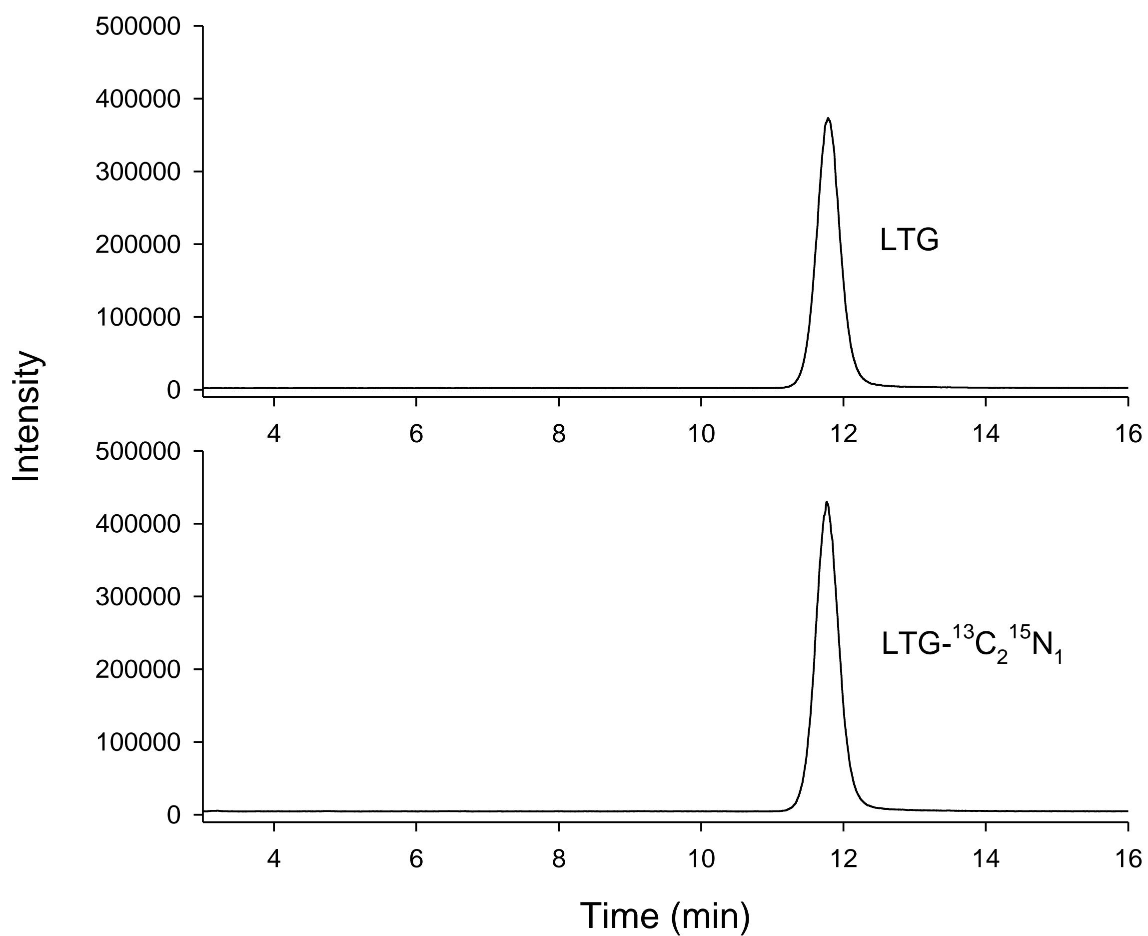

1500000
1200000
900000
600000
300000
0
PHT
3
6
9
12
15
Intensity
1200000
900000
600000
300000
0
PHT-13C1 15N2
3
6
9
12
15
Time (min)

Figure 3. Selected ion chromatograms for PHB and PHB-d_5 in serum at a concentration of 17 μg/g obtained by LC-MS/MS.



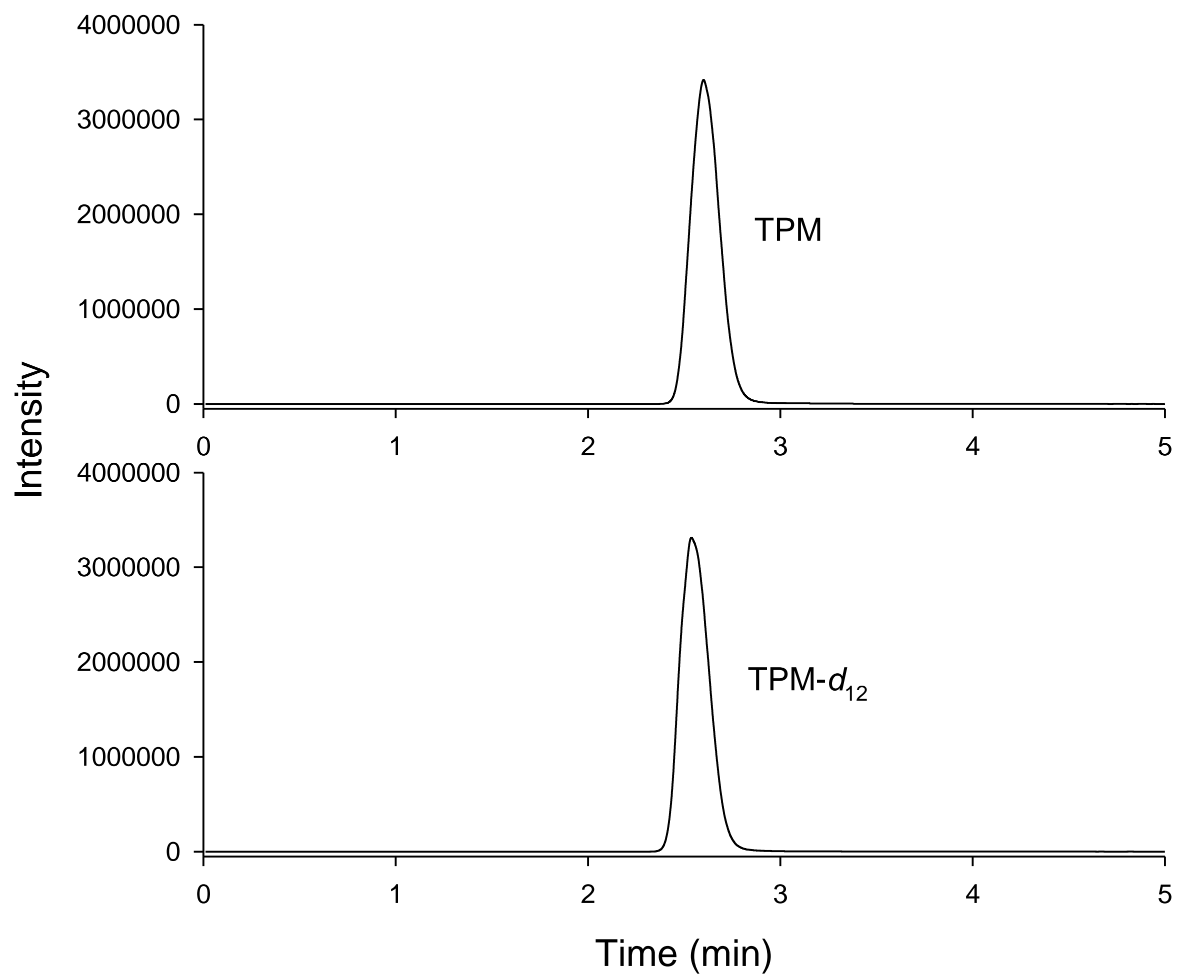
3000000
2500000
2000000
1500000
1000000
500000
0
PHB
0
2
4
6
8
10
Intensity
2500000
2000000
1500000
1000000
500000
0
PHB-d_5
0
2
4
6
8
10
Time (min)

Figure 4. Selected ion chromatograms for LTG and LTG-$^{13}C_2{}^{15}N_1$ in serum at a concentration of 4 μg/g obtained by LC-MS/MS.



500000
400000
300000
200000
100000
0
LTG
4
6
8
10
12
14
16
Intensity
500000
400000
300000
200000
100000
0
LTG-13C2 15N1
4
6
8
10
12
14
16
Time (min)

Figure 5. Selected ion chromatograms for TPM and TPM-d_{12} in serum at a concentration of 7 μg/g obtained by LC-MS/MS.


4000000
3000000
2000000
1000000
0
TPM
0
1
2
3
4
5
Intensity
4000000
3000000
2000000
1000000
0
TPM-d_{12}
0
1
2
3
4
5
Time (min)

Reference List

1. Tietz, N. W. Tietz Textbook of Clinical Chemistry, 2 ed.; Saunders: Philadelphia, 1994.
2. Johannessen, S. I.; Battino, D.; Berry, D. J.; Bialer, M.; Kramer, G.; Tomson, T.; Patsalos, P. N. *Ther.Drug Monit.* **2003,** *25*, 347-63.
3. Tracy, R. P.; Ebnet, L. E.; Moyer, T. P. *Clin Chem* **1979,** *25*, 1868-69.
4. Foerster, E. H.; Dempsey, J.; Garriott, J. C. *J.Anal.Toxicol* **1979,** *3*, 87-91.
5. Gerson, B.; Anhalt, J. P. High Pressure Liquid Chromatography and Therapeeutic Drug Monitoring. ASCP Press, Chicago, 1980.
6. Beck, O.; Ohman, I.; Nordgren, H. K. *Ther.Drug Monit.* **2006,** *28*, 603-07.
7. Britzi, M.; Soback, S.; Isoherranen, N.; Levy, R. H.; Perucca, E.; Doose, D. R.; Maryanoff, B. E.; Bialer, M. *Ther.Drug Monit.* **2003,** *25*, 314-22.
8. Zhang, Y.; Mehrotra, N.; Budha, N. R.; Christensen, M. L.; and Meibohm, B. *Clin.Chim.Acta.* **2008,** *398*, 105-12.
9. Subramanian M.; Birnbaum, A. K.; Remmel, R. P. *The Drug Monit.* **2008,** *30*, 347-56.
10. Speed, D. J.; Dickson, S. J.; Cairns, E. R.; Kim, N. D. *J.Anal.Toxicol* **2000,** *24*, 685-90.
11. Spell, J. C.; Srinivasan, K.; Stewart, J. T.; Bartleett, M. G. *Rapid Communication in Mass spectrometry* **1998,** *12*, 890-94.
12. Chen, S.; Carvey, P. M. *Rapid Communication in Mass spectrometry* **1999,** *13*, 1980-84.
13. Matar, K. M. *Clin Chim.Acta* **2010,** *411*, 729-34.
14. Park, J.; Park, Y.; Lee, M.; Rhim, S.; Song, J.; Lee, S.; Kim, J.; Shaw, L. M.; Kang, J. *Biomed.Chromatography* **2008,** *22*, 822-29.
15. Christensen, J.; Hojskov, C. S.; Poulsen, J. H. *The Drug Monit.* **2002,** *24*, 659-64.
16. Greiner-Sosanko, E.; Lower, D. R.; Virji, M. A.; Krasowski, M. D. *Biomed.Chromatography* **2007,** *21*, 225-28.
17. Vermeij, T. A. C.; Edelbroek, P. M. *J Chromatogr.B* **2007,** *857*, 40-46.
18. ISO 15193 *www.iso.ch/iso/en/CatalogueListPage.CatalogueList*. **2003**.
19. JCTLM *http://www.bipm.org/en/committees/jc/jctlm/jctlm-db* **2005**.
20. Tai, S. S.; Xu, B.; Welch, M. J.; Phinney, K. W. *Anal.Bioanal.Chem.* **2007,** *388*, 1087-94.

21. Tai, S. S.; Xu, B.; Welch, M. J. *Anal Chem* **2006,** *78*, 6628-33.

22. Taylor, B. N.; Kuyatt, C. E. *NIST Technical Note 1297(available at http://physics.nist.gov/Pubs/guidelines/contents.html)*. **1994**.

23. ISO *Guide to the Expression of Uncertainty in Measurement (International Organization for Standardization, Geneva, Switzerland)* **1993**.

24. Bourgeois, B. F. *Drugs Today (BARC)* **1999,** *35*, 43-48.

25. Tsiropoulos, I.; Kristensen, O.; Klitgaard, N. A. *The Drug Monit.* **2000,** *22*, 517-21.

26. Tai, S. S.; Welch, M. J. *Anal.Chem.* **2004,** *76*, 1008-14.

27. Ohnmacht, C. M.; Chen, S.; Tong, Z.; Hage, D. S. *J Chromatogr.B Analyt.Technol.Biomed.Life Sci.* **2006,** *836*, 83-91.

28. Carrier, D. J.; Eckers, C.; Wolff, J. C. *J Pharm.Biomed.Anal* **2008,** *47*, 731-37.

29. Matsuura, H.; Ohmori, T.; Nakamura, M.; Itoh, Y.; Hirano, K. *Biomed.Chromatography* **2008,** *22*, 387-93.