Inter-Laboratory Comparison of Serum Lamotrigine Methods

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Abstract

Objective: To assess the inter-laboratory variability in the measurement of serum Lamotrigine (Lamictal™) by the most commonly employed methods. Lamotrigine is a newer generation antiepileptic drug and has also recently been approved for use in bipolar disorder. Currently, there is no recognized reference method for the determination of this drug in serum. Additionally, proficiency schemes are available only in the U.K. and Germany.

Methodology: Five laboratories were enrolled in this study. Two laboratories employed LC-UV techniques, one lab used GC/MS, and one lab used GC/ECD. The final laboratory used LC-UV and also performed the analyses using a new LC/MS/MS method. Labs initially validated their imprecision using three levels of quality control (Chromsystems, Munich, DE). Next they analyzed 164 unknown sample pools. Pools were composed of random patient samples, UK EQAS and INSTAND proficiency specimens, and Chromsystems QC preparations. All analyses were blind and specimens were analyzed in routine runs.

Results: Across method means were determined for each sample and the difference from the mean was determined for each methodology. Each lab's differences were then plotted against the group means. Slopes and intercepts were analyzed to assess method bias. Regression analyses were performed comparing each method's results to the LC/MS/MS results. Additionally, proficiency samples were analyzed separately to determine both assay performance and imprecision. Results from the regression analyses are shown below.

Lamotrigine Methods Compared to LC/MS/MS			
Methodology	r	Slope	Y-Intercept
LC-UV (1)	0.9220	0.9895	0.0144
LC-UV (3)	0.9274	0.9777	0.0161
LC-UV (6)	0.9644	1.0418	0.0694
GC/MS	0.9747	1.0426	0.4227
GC/ECD	0.9539	0.9316	0.4305

Conclusions: All of the methods performed satisfactorily. Proficiency samples were within acceptable ranges. Blinded inter-assay imprecision ranged from 4.1 to 16.9% depending on concentration. No systematic bias was noted for any of the methods. In general the methods were highly comparable with only occasional outliers being noted.

Introduction

Lamotrigine, 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5 diamine, (LTG) is a synthetic phenyltriazine and is a member of the newer class of antiepileptic drugs developed since the 1970s. Although structurally unrelated to other anticonvulsants, LTG was initially employed as an add-on therapy for partial seizures in epileptic patients when it was first marketed in the United States in 1994. Since then it has been found effective in the treatment of typical and atypical absence, atonic, generalized tonic-clonic, myoclonic and clonic seizures as well as drop attacks associated with Lennox-Gastaut syndrome. In 2003 it was the first antiepileptic drug approved for use in treatment of bipolar I disorder.

Though not routinely performed in the United States, LTG appears to be a good candidate for therapeutic drug management. It exhibits pronounced intra- and inter-individual variability in pharmacokinetics and a narrow therapeutic range (3 to 15 mg/L) beyond which toxic side effects have been demonstrated. LTG clearance has been shown to be influenced by concomitant drug and hormone administration. Compliance has been problematic in special populations (i.e. children, pregnancy) and a severe toxic side effect has been documented (Stevens-Johnson syndrome).

The purpose of this study was to assess the variability both within and between five laboratories routinely measuring LTG in serum using several different assay methodologies (HPLC-UV, GC-ECD, GC/MS, and LC/MS/MS). We first measured intra-laboratory precision using a commercially available QC product. Next, we measured inter-laboratory variability and total precision by assaying 164 unknown sample pools. Pools were composed of random patient samples, UK EQAS and INSTAND proficiency specimens, and QC preparations. It is hoped this data will lead to greater procedural standardization and optimization of care.

Methods

Study Design

Five laboratories participated in this study and six analytical methods were evaluated for imprecision and accuracy. Participating laboratories were first asked to evaluate their intra-assay imprecision by analyzing levels I, II, and III of Chromosystems Antiepileptic Drugs Control preparations (lots 314, 204, 463 respectively) ten times each in a single assay run.

Next, the laboratories assayed 164 unknown samples. The samples were integrated into their normal lamotrigine runs and the three levels of Chromsystems Q.C. were also included in any runs containing these unknowns, as an estimate of inter-assay imprecision. The unknown sample sets consisted of 111 patient samples, 25 samples from the UK EQAS (Cardiff, UK) proficiency program, 6 samples from Instand e.V (Dusseldorf, FRG) proficiency program, and the 3 Chromsystems Q.C. preparations. Sample sets were assembled from residual patient samples previously analyzed in the participating laboratories. Due to volume limitations the UK EQAS samples were placed singly in each sample set. The Instand e.V. samples were inserted six times in each samples set. All specimens were identified by a unique study number, placed in similar tubes, and stored at -80^{0}C until shipment. Samples were shipped frozen overnight to participating laboratories.

Analytical Methods

Method 1: This HPLC-UV method uses 150 µL of serum sample to which 50 µL of internal standard (oxcarbazepine) is added followed by 200 µL of methanol. The tube is mixed and centrifuged to sediment precipitated protein. 35 µL of supernatant is injected onto an Agilent 1100 HPLC system equipped with a Synergi Hydro-RP column (4µ, 50 X 4.6 mm). The separation is developed isocratically using a mobile phase of 65:20:15 (0.01M K2HPO4, pH7.0: Methanol: Acetonitrile) plus 100 µL Triethylamine. Detection is at 307 nm. Lamotrigine elutes at approximately 1.5 minutes followed by the internal standard at approximately 2.9 minutes. The analytical measurement range for the assay is from 0.5 to 60 mg/L.

Method 2: This LC-MS/MS method uses 50 µL of serum sample to which 600 µL of internal standard (UCB 17025) in methanol is added. The tube is mixed and centrifuged to sediment precipitated protein. 1 µL of supernatant is then injected onto a Waters 2795 Alliance HPLC system equipped with a Symmetry 300C18 column (5µ, 2.1 X 15.9 mm). The separation is developed isocratically using a mobile phase of 60:40 (Acetonitrile: 2mM Ammonium Acetate) plus 0.1% Formic acid. The Waters Quattro Micro API employs positive electrospray ionization in MRM mode. Lamotrigine is measured by monitoring the ammonium adduct mass transition from 256 to 211.1. A transition from 185 to 140.2 is used to monitor the internal standard. The system is also capable of measuring oxcarbazepine, carbamazepine, and their metabolites. Lamotrigine elutes at approximately 1.8 mins followed by the internal standard at approximately 2.0 minutes. The analytical measurement range for the assay is from 0.1 to 60 mg/L.

Method 3: This laboratory used the Chromsystems Antiepileptic Drugs in Serum/Plasma kit (Munich, FRG) in high resolution mode. Briefly, 150 µL of internal standard is added to 100 µL of sample and mixed. Next 50 µL of precipitation reagent is added, mixed, and centrifuged. 100 µL of supernatant is added to 100 µL of stabilization buffer and mixed. 20 µL of the mixture is then injected onto an Agilent 1100 HPLC system equipped with a proprietary HPLC column. The separation is developed isocratically using mobile phase supplied with the kit. Detection is at 204 nm. Lamotrigine elutes at approximately 6.9 minutes and the internal standard at approximately 14.5 minutes. The analytical measurement range for the assay is from 0.3 to 30 mg/L. The system is also capable of measuring oxcarbazepine, carbamazepine, their metabolites, sultiam, ethosuximide, primidone, phenobarbital, phenytoin in the same run.

Method 4: This GC/MS method uses 100 µL of serum sample to which oxazepam-d5 is added as internal standard. The preparation is the extracted using an Oasis HLB solid phase cartridge. Compounds of interested are eluted with straight methanol. The eluate is dried under nitrogen and derivatized using MTBSTFA for one hour at 85^{0}C. 1 µL of derivatized extract is then injected onto an Agilent 6890 GC system equipped with an Agilent 5963 MSD. For lamotrigine the 426.1, 428.1, and 199.1 ions are monitored and for oxazepam-d5 the 462.3, 464.2, and 519.3 ions are monitored. The analytical measurement range for the assay is from 1.0 to 40 mg/L. This group performed measurements on only half the unknown specimens.

Method 5: This GC-ECD method uses 50 µL of serum sample to which 1ml of internal standard (2-(2-chlorophenyl-4,6-diamino-1,3,5-triazine) in acetonitrile) is added. The tube is mixed and centrifuged to sediment precipitated protein. 1 µL of supernatant is then injected onto an Agilent 5890 Series II GC system equipped with a 15m, 0.53mm i.d., 1µ film DB-17 column using argon/methane for anode purge. The separation is developed isothermally using an oven temperature of 255^{0}C while carrier flow is 16.9mL/min. Detection is with an electron capture detector. The internal standard elutes at approximately 1.12 minutes followed by lamotrigine at approximately 2.87 mins. The analytical measurement range for the assay is from 0.1 to 30 mg/L.

Method 6: This HPLC-UV method uses 200 µL of serum sample to which 200 µL of 0.8M Tris buffer (pH 10.9), 50 µL of internal standard (methoxycarbamazepine), and 7 ml of dichloromethane is added. The tube is mixed and allowed to stand until the layers separate. The aqueous phase is aspirated to waste. The organic layer is filtered (Whatman no.1 paper) and evaporated to dryness under a stream of air in a warm water bath. The residue is then reconstituted in 100 µL of mobile phase. 40 µL extract is injected onto the HPLC system equipped with a Jasco PU 880 series pump, UVIDEC-100V detector, 851-AS autosampler, an HP3395 integrator, and a Spherisorb ODS-2 column (5µ, 150 X 4.0 mm). The separation is developed isocratically using a mobile phase of 54:36:11(0.1M Acetic Acid / NAOH buffer, pH7.5: Acetonitrile: Methanol). Detection is at 325 nm. Lamotrigine elutes at approximately 2.3 minutes followed by the internal standard at approximately 5.3 minutes. The analytical measurement range for the assay is from 0.5 to 20 mg/L.

Data Analysis

Intra-method and inter-method imprecision were evaluated by calculating the coefficients of variation (C.V.) for the 3 levels of Chromosystems Q.C. product sent to each laboratory prior to the analysis of unknowns and run with the unknowns.

To assess inter-laboratory variability the average result from each of the six methods for each unknown sample was calculated. The difference between each method's result and the average of the six methods was then calculated. The differences for each method were then plotted against the six method means and a regression analysis was performed. Total imprecision was evaluated as above using the Instand e.V. proficiency samples included in the unknown set.

As a test of accuracy , a similar approach was used to evaluate results for the UK EQAS samples included in the unknown group compared to the proficiency organization's consensus mean. In another test for accuracy, each method's results were compared to those of the LC/MS/MS method by linear regression and residual analysis.

Results

1. Method Imprecision

Intra-Assay (n=10 replicates)

Determined using the Chromsystems Q.C. products .

	I		II		III	
Method	Mean	%C.V.	Mean	%C.V.	Mean	%C.V.
#1 (HPLC-UV)	1.31	7.6	7.83	1.1	13.57	2.5
#2 (LC-MS/MS)	2.61	5.3	9.12	3.9	14.02	2.6
#3 (HPLC-UV)	2.58	11.1	8.72	8.3	14.55	9.5
#4 (GC-MS)	ND	ND	ND	ND	ND	ND
#5 (GC-ECD)	3.40	20.0	8.59	6.0	14.40	16.9
#6 (HPLC-UV)	2.92	2.6	8.68	2.8	14.46	8.9

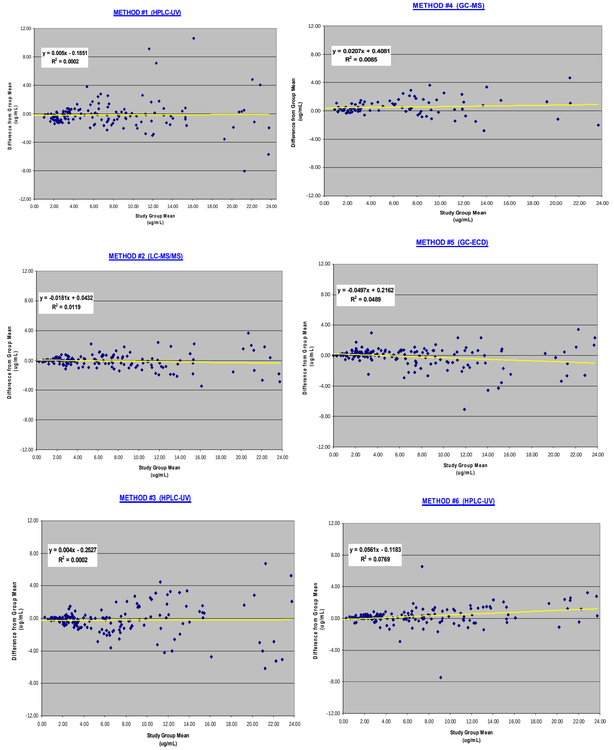
Inter-Assay

Inter-assay variation for each method was determined using the same Q.C. product. The number of runs varied for each laboratory and ranged from 8 to 14.

	I		II		III	
Method	Mean	%C.V.	Mean	%C.V.	Mean	%C.V.
#1 (HPLC-UV)	1.63	34.8	8.18	8.2	14.32	12.5
#2 (LC-MS/MS)	2.47	3.8	8.62	6.8	14.73	5.0
#3 (HPLC-UV)	2.72	14.4	8.87	12.1	14.20	13.5
#4 (GC-MS)	2.47	12.2	8.52	8.9	12.35	6.1
#5 (GC-ECD)	2.64	13.1	8.94	13.9	14.04	7.5
#6 (HPLC-UV)	2.78	10.1	9.00	5.0	15.22	5.2

2. Inter-Laboratory Variability

Assessed using total sample set of 164 unknowns



3. Total Assay Imprecision

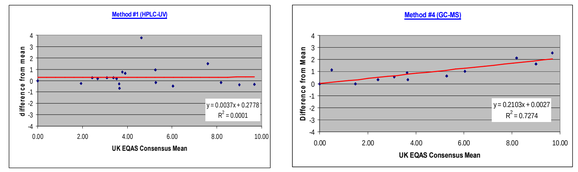
Total inter-assay imprecision was also determined using the proficiency materials provided by the Instand e.V. program. Four proficiency samples were included in the unknown sample sets, six times each.

	314-I		314-II		154-I		154-II	
Method	Mean	%C.V.	Mean	%C.V.	Mean	%C.V.	Mean	%C.V.
#1 (HPLC-UV)	6.1	5.7	20.0	16.8	1.3	8.9	9.4	11.5
#2 (LC-MS/MS)	7.2	5.6	22.5	7.9	2.4	18.6	10.2	6.0
#3 (HPLC-UV)	5.8	32.5	20.1	26.8	2.9	16.3	12.9	14.1
#4 (GC-MS)	8.2	20.0	24.2	10.2	2.9	19.2	12.2	7.6
#5 (GC-ECD)	6.9	18.5	21.6	16.9	2.7	23.7	10.2	8.3
#6 (HPLC-UV)	6.9	12.2	23.4	2.9	2.7	4.1	9.4	40.6

4. Accuracy

Comparison to UK EQAS Consensus Means

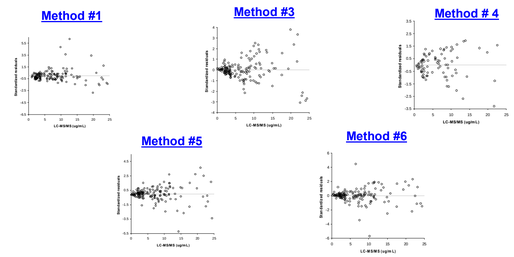
25 UK EQAS proficiency specimens were placed randomly throughout the unknown specimen set. Results for each method were then compared to the UK EQAS consensus means



5. Comparison To LC-MS/MS Method

Values from each method compared to the single LC-MS/MS method

Comparison to LC-MS/MS			
Methodology	r	Slope	Y-Intercept
#1 (HPLC-UV)	0.9220	0.9895	0.0144
#3 (HPLC-UV)	0.9274	0.9777	0.0161
#6 (HPLC-UV)	0.9644	1.0418	0.0694
#4 (GC/MS)	0.9747	1.0426	0.4227
#5 (GC/ECD)	0.9539	0.9316	0.4305



Conclusions

1. **All of the methods performed satisfactorily with various degrees of imprecision on the known controls (Intra-assay C.V.s ranged from 1.1 to 20%, Interassay C.V.s ranged from 5.0 to 34.8%)**
2. **Total assay imprecision ranged from 2.9 to 32.5% as determined on blinded specimens.**
3. **No systematic bias was noted for any of the methods when compared to the group means.**
4. **Methods #3 (HPLC-UV), #4 (GC-MS), and #5 (GC-ECD) did exhibit a proportional bias when compared to the UK EQAS consensus means.**
5. **In general all of the methods compared well with the LC-MS/MS method, with only a small constant bias in the two GC methods and occasional outliers being noted.**
6. **This work highlights the continued need for proficiency testing when doing therapeutic drug determinations.**

Acknowledgement

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