THERAPEUTIC DRUG MANAGEMENT (TDM) OF ANTIEPILEPTIC DRUGS - MULTIDRUG ANALYSIS USING UPLC/MS/MS

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David J Berry[1], Gareth W Hammond[2], Lisa J Calton[2] and Donald P Cooper[2]
[1] Medical Toxicology Unit, Avonley Road, London, SE14 5ER, UK. [2] Waters Corporation, Atlas Park, Simonsway, Manchester, M22 5PP, UK

ABSTRACT

INTRODUCTION: It is recognised that the desired therapeutic effect of most Anti-epileptic Drugs (AEDs) is achieved within a specific concentration range, with lower levels giving an unsatisfactory response and higher levels possibly giving undesirable side-effects. Large inter-individual differences in drug disposition occur, and many refractory patients require treatment with several AEDs. Thus, monitoring serum/plasma concentrations of AEDs has proved to be an extremely useful means of assisting in the individualisation of treatment. A large number of AEDs are available and it is necessary to quantitatively determine each compound that a patient is prescribed. Assay methods include a range of gas- and liquid- chromatographic procedures, and specific immunoassays, although the latter are not available for all AEDs. This work describes an Ultra Performance Liquid Chromatography – tandem mass spectrometry (UPLC/MS/MS) method capable of analysing a panel of AEDs, and, where appropriate, the active metabolite, simultaneously in a single procedure.
METHODOLOGY: Sample preparation consisted of a simple protein precipitation using aqueous zinc sulphate and methanol. The resulting supernatant was diluted with mobile phase to give a response in the linear range of the analyte. A reversed phase UPLC method, using a Waters ACQUITY UPLC™ BEH C18 2.1x50mm 1.7µm column was developed to meet the demands of the clinical laboratory for speed of analysis, chromatographic resolution and sensitivity. The mobile phase consisted of :- Solvent A: aqueous ammonium acetate and solvent B: methanolic ammonium acetate; A five-minute gradient elution from 2-75% organic phase, produced an effective separation. Detection was undertaken using a Waters Quattro Premier XE™ tandem quadrupole mass spectrometer operating in Multiple Reaction Monitoring (MRM) mode with fast (20ms) positive and negative ion mode switching and short (20ms) dwell times, commensurate with the UPLC peak widths.
RESULTS: The assay for Lamotrigine was linear over the analytical range 2.9 -15.3 mg/L, with a correlation coefficient (r^2) of 0.995, and a precision %RSD = 4.4% at the lower concentration (n=6). The assay for Primidone was linear over the analytical range 4.3-30.0mg/L with a r^2 of 0.998 and a precision %RDS = 3.4 at the lower concentration (n=6). The assay for Carbamazepine was linear over the analytical range 4.8-19.4mg/L with a r^2 of 0.994 and a precision %RSD = 2.9% at the lower concentration (n=6). In total, twenty four analytes have been characterised using this methodology, with linear correlation coefficients >0.99, and an average precision <10%.
CONCLUSION: An analytical protocol has been developed for the analysis of AEDs using UPLC/MS/MS. The sample preparation is straightforward, which allows a rapid turnaround time (<10 mins), with good analytical sensitivity and specificity for the individual compounds. The method is suitable for routine TDM in patients prescribed multiple drugs for treatment of their epilepsy and enables all their AED drug levels to be monitored in a single assay. There is an additional benefit of the procedure in that it has a screening capacity which allows detection of AEDs that are not suspected to be present by the requesting physician.

INTRODUCTION

- The desired therapeutic effect of most anti-epileptic drugs (AEDs) is achieved within a specific concentration range. If levels are too low convulsions may not be controlled and at high level the side-effects may be undesirable or toxic.
- There are large inter-individual differences in response to the drugs and refractory patients may be on multiple therapies. This individualization of treatment regimes can be assisted by therapeutic drug monitoring (TDM).
- The preliminary work presented here describes an Ultra Performance Liquid Chromatography tandem mass spectrometry method (UPLC/MS/MS) to quantitate a panel of AEDs in serum/plasma using a single protocol. These analyses are usually achieved by immunoassay, GC and LC procedures.

Figure 1. System configuration of a Waters Acquity UPLC™ and Quattro Premier™ XE.

METHODS

Mass Spectrometry

A Quattro Premier™ XE tandem mass spectrometer coupled to an Acquity UPLC™ (Waters Corporation, Manchester, UK) was used for all analyses (Figure 1). The instrument was operated in electrospray ionization mode utilizing short (20ms) positive negative polarity switching. All data acquisition was performed using MassLynx v4.1 software with auto data processing using the QuanLynx™ Application Manager.

The ionization mode and compound dependent cone voltage was optimized for each analyte to produce either a $[M+H]^+$ or $[M-H]^-$ precursor ion. Collision induced dissociation of the precursor ion was facilitated by argon and collision energy to produce characteristic product ions. Using this information a specific Multiple Reaction Monitoring experiment was created as shown in Table 1.

Compound	MRM Function	Dwell (ms)	Cone (V)	Collision Energy (eV)	Retention Time (min)
Vigabatrin	130.1 > 71.2	15	16	13	0.31
Pregabalin	160.2 > 97.2	15	20	14	1.08
Gabapentin	172.2 > 137.2	15	24	16	1.13
Levetiracetam	171.1 > 126.1	15	14	15	1.36
Ethosuximide	140.1 > 42.3	35	35	18	1.69
Zonisamide	213.1 > 132.1	15	26	15	1.77
Primidone	219.2 > 162.2	25	25	12	2.45
Felbamate	239.2 > 178.2	25	14	7	2.59
Lamotrigine	256.1 > 211.1	25	48	26	2.83
Phenobarbitone	231.2 > 188.0	35	28	10	3.03
Desmethylmethsuximide	190.1 > 120.1	25	26	14	3.18
Valproic acid/Na valproate	143.1 > 143.1	75	30	7	3.59
Topiramate	340.2 > 264.2	26	26	8	3.70
10-hydroxycarbamazepine	255.2 > 194.2	26	16	20	3.87
Carbamazepine epoxide	253.2 > 180.2	26	20	25	4.05
Phenytoin	253.2 > 182.2	26	28	18	4.76
Carbamazepine	237.1 > 194.2	15	32	20	5.06
Clonazepam	316.1 > 270.2	15	40	25	5.16
Desmethylclobazam	287.2 > 210.2	15	35	30	5.20
Clobazam	301.2 > 259.2	15	35	20	5.50
Tiagabine	376.2 > 149.1	15	35	28	5.87
Desmethyldiazepam	271.2 > 140.1	15	45	28	6.05
Diazepam	285.2 > 154.1	15	45	26	6.24

Table 1. The tuning parameters used when monitoring 24 AEDs in MRM mode.

MS conditions

Polarity	ES+	ES-
Capillary (kV)	2.0	2.0
Source Temperature (°C)	120	120
Desolvation Temperature (°C)	400	400
Cone Gas Flow (L/Hour)	25	25
Desolvation Gas Flow (L/Hour)	800	800
Gas Cell Pressure (mbar)	$4.0e^{-3}$	$4.0e^{-3}$

UPLC Conditions

Column:	UPLC BEH 1.7µm, 2.1 x 50mm
Mobile Phase A:	10mM Ammonium Acetate
Mobile phase B:	Methanol
Column Temperature:	50°C
Injection Volume:	20µL

Gradient Table

Time(mins)	Flow Rate (mL/min)	%A	%B	Curve
Initial	0.6	98	2	1
1.5	0.6	80	20	6
2.0	0.6	80	20	11
3.5	0.6	70	30	6
7.5	0.6	25	75	6
8.0	0.6	5	95	6
8.5	0.6	5	95	11
8.6	0.6	98	2	11

Calibrators and QCs

Mixed analyte calibration curves were prepared in water from individual stock solutions (1ng/mL, methanol). The calibration series was 0, 1, 5, 10, 50, 100, 500, 1000, 5000 and 10000ng/mL and matrix controls containing AEDs were obtained from Chromsystems, Munich, Germany.

Sample Preparation

Samples (50µL) were pipetted into a microfuge tube, methanol (200µL) added, vortex mixed (20s) and centrifuged (5mins @ 13,000rpm) to precipitate and remove proteins. The supernatant (50µL) was diluted with water (950µL) before injecting 20µL onto the UPLC/MS/MS system.

Ion Suppression Studies

Blank matrix or water were processed and the extract analyzed with post-column addition of the AEDs each at 10µg/mL and a flow rate of 10µL/min using the integral syringe pump.

Experiments

Preliminary investigations into the linearity, limit of detection and reproducibility of the analytes in solvent and serum matrix have been performed.

RESULTS

- An example of a solvent chromatogram of the 24 AEDs using UPLC is shown in Figure 2.
- A chromatogram of the lowest extracted matrix control (Figure 3) is annotated with the compound name and the signal-to-noise ratio (SNR) for the analyte. All responses are well above the limit of detection (SNR 8:1)
- Example ion suppression chromatograms shown in Figure 4 demonstrate that the one area of significant ion suppression, at 0.25mins, is coincident with vigabatrin only.
- Linear calibration curves have been obtained in both solvent and matrix (Figures 5 and 6). All calibration curves in solvent were linear (r^2>0.99) and average precision <10% RSDs. The linearity and precision summary (Table 2) is for Chromsystems controls treated as matrix calibrators.

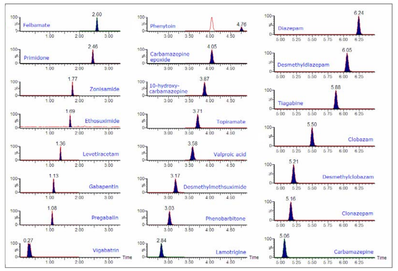

Figure 2. Chromatography of a solvent standard showing MRM transitions of individual analytes and their retention times. Data shown is a single acquisition using time windows.

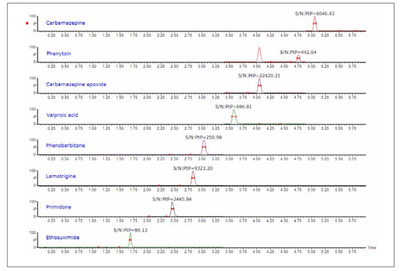

Figure 3. Chromatogam showing the lowest serum control and the calculated signal-to-noise ratio for each AED.

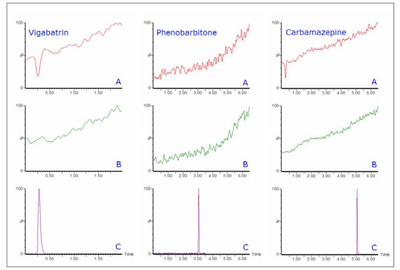

Figure 4. Example chromatograms showing post-column infusion of AEDs. A is injected matrix, B is injected water. C demonstrates the retention of the AED.

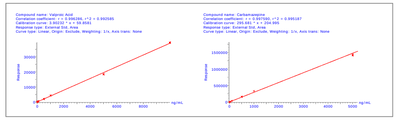

Figure 5. Example calibration curves for valproic acid and carbamazepine prepared in solvent.

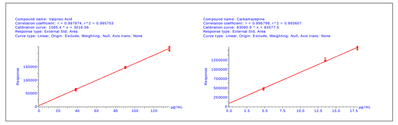

Figure 6. Example calibration curves for valproic acid and carbamazepine extracted from serum matrix.

Compound	Linearity (r^2)	Control	Expected (µg/mL)	%CV
Ethosuximide	0.98	Control 1	43.9	12.9
		Control 2	88.6	5.3
		Control 3	120.0	2.6
Primidone	0.99	Control 1	4.3	5.8
		Control 2	17.8	2.6
		Control 3	26.7	3.7
Lamotrigine	0.99	Control 1	2.9	4.9
		Control 2	9.8	2.0
		Control 3	13.9	2.7
Phenobarbitone	0.99	Control 1	9.5	10.0
		Control 2	34.6	5.8
		Control 3	43.2	5.6
Valproic acid	0.996	Control 1	38.4	4.9
		Control 2	90.1	1.5
		Control 3	136.0	3.1
Carbamazepine epoxide	0.99	Control 1	2.0	3.7
		Control 2	4.9	1.7
		Control 3	7.0	2.4
Phenytoin	0.99	Control 1	4.8	9.7
		Control 2	17.9	5.0
		Control 3	26.7	5.2
Carbamazepine	0.994	Control 1	4.8	6.0
		Control 2	13.5	2.9
		Control 3	18.0	2.4

Table 2: Summary of the linearity for the 8 AEDs contained in the serum controls and the precision (n=5) at each level.

DISCUSSION

- Using ES+ and ES– modes it was possible to ionize 24 AEDs. However, valproic acid and sodium valproate dissolve in an aqueous solution to the same acidic moiety and only one MRM transition is need.
- AEDs are a diverse group of compounds including acids (e.g. valproic acid), weak bases (e.g. carbamazepine) and zwitterions (e.g. vigabatrin). Some compounds, e.g. clonazepam and diazepam ionize readily whilst ethosuximide ionizes poorly. The therapeutic ranges of the AEDs are generally at the µg/mL level and are well above the limit of detection for each analyte, even though there is a large spread of ionization efficiencies.
- Different mobile phase modifiers were considered whilst developing the chromatographic assay. These involved lowering and raising the pH as well as looking at the concentration of buffers. Given the range of compounds the best compromise was to use ammonium acetate with no other modifiers
- Vigabatrin was unretained chromatographically and eluted in the column void volume. The solvent calibration curve was linear but the robustness of assay in serum may be compromised by ion suppression and patient variability.
- Carbamazepine and phenytoin are isobaric and have the same precursor ion at *m/z*253. When carbamazepine is fragmented in the collision cell the major product ion is at *m/z*180 and there is also a minor product ion at *m/z*182. The major product ion for phenytoin is at *m/z*182. When phenytoin elutes from column the product ion at *m/z*182 gives a response at the detector. However, when carbamazepine elutes a response will be seen both in the MRM channel for carbamazepine and phenytoin because of the common product ion at *m/z*182. Therefore, it is necessary to resolve these compounds chromatographically.
- The calibration curves prepared in solvent were used to demonstrate that the AEDs could be detected with adequate sensitivity,the response was linear and reproducible.
- The material to produce mixed serum calibrators was unavailable so the serum controls were used to determine that the AEDs contained were detectable, linear and precise using the extraction procedure.

CONCLUSIONS

- A protocol for the determination of 24 AEDs in serum/plasma has been developed.
- The method is short with a sample to sample injection time of 10 minutes.
- Calibration curves for the 24 AEDs prepared in water and analysed on the UPLC/MS/MS system produced quantitative data with good limits of detection, linearity and reproducibility in a single acquisition. This is facilitated by the speed and chromatographic resolution of UPLC and the scanning capabilities (minimum 5ms dwell and 20ms polarity switching) of the Quattro Premier XE.
- The 8 AEDs contained in the matrix controls when extracted using the simple sample extraction and dilution process were adequately detected above the limit of detection for each analyte. The calibration curves obtained were linear and the results reproducible.
- After further validation the method may be suitable to check compliance and indicate a patient has taken an AED without the knowledge of the prescribing physician.

References

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