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Research Article

Bioanalytical Method Development and Validation of Valproate Semisodium in Human Plasma by LC–MS/MS

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ABSTRACT

An LC–MS method for the determination of Valproate semisodium in human plasma was developed and validated. Sample preparation involved the protein precipitation method. Chromatographic separation was performed on Sunfire C18 5µm (150 X 4.6) mm column with the mobile phase consisting of Acetonitrile: 10 mM ammonium acetate buffer ::80:20 v/v. The interface used with the API 3000 LC-MS/MS was a turbo ion spray in which negetive ions were measured in MRM mode. The method was validated over the concentration range of 1.012 µg/mL. to 120.399 µg/mL. and the Lower limit of quantitation (LLOQ) was 1.012 µg/mL. The intra- and inter-day precision of the method at four concentrations was 2.26-10.67% and 3.58-10.49% .Stability of compounds was established in a series of stability experiments. The method can be used for the simultaneous determination of Valproate semisodium in human plasma.

Key words: Divalproex, Valproic acid, Valproic acid D4, Valproate semisodium, LC-MS/MS, Bioanalysis, Bioequivalence, Protein Precipitation

INTRODUCTION

Valproate semisodium consists of a compound of sodium Valproate and ValproicAcid in a 1:1 molar relationship in an enteric coated form. Valproate semisodium is used as mood stabilizer in bipolar disorders. It is used as an antiepileptic agent (1,2) and to treat episodes of mania. This medicine is also reported to treat migraine.

Valproate semisodium also known as Divalproex Sodium converts in to Valproic acid in the stomach (3) hence the active product is Valproic acid. The Valproic acid that circulates in blood is 85% to 90% protein bound under normal circumstances. (4)

This drug is reported to increase levels of GABA (Gamma aminobutyric acid) which plays important role in regulation neuronal excitability throughout the nervous system.

(5)

Valproate was first approved in the united state in 1978 as an immediate release formulations (Depakene) for the treatment of seizure. In 1983 divalproex sodium (Depakote) was introduced. Which is an enteric coated stable co-ordination complex of Valproic acid and valproate.

Along with the useful effects this medicine do have side effects so it is highly recommended to evaluate its plasma concentration through effective method development and validation process.(6)

Various derivitazition method were developed to analyse the drug (7)(8). This method is time consuming and affect the cost and the recovery of the analyte. Analysis of Valproate semisodium is certainly a challenge due to molecule sensitivity and volatile nature hence optimization of analytical and instrumental parameters are important while analysis.

The aim of this work was to explore the quantification of Valproate semisodium (Divalproex) in human plasma using a highly selective, sensitive validated LC-MS/MS method in consideration of molecule sensitivity, volatile nature and cost effectiveness.

EXPERIMENTAL

Chemicals and materials: Divalproex sodium (mol.wt. 310.40; 99.24% by HPLC), and Valproicacid D4 (mol.wt. 148.24, 100.00 % by HPLC) were obtained from Clearsynth Labs (P) Ltd.

Control buffered (K_2EDTA) human plasma was procured from Laxmi Sai Clinical lab., India. All other reagents/chemicals were of AR grade.

LC–MS/MS instrumentation and settings: A high performance liquid chromatography system (Shimadzu Co., Kyoto, Japan) with Sunfire C18,5µm 4.6 mm x 150 mm column was used in this project. The column oven and autosampler temperature were maintained at 35± 2 °C and 5±1ºC respectively. The flow rate was set at 0.800 mL/min with addition of spliter and divertor, Divertor activates till 0 to 2 min to divert the LC flow to the waste. Spitter ratio was be 70:30 (70% flow to waste)

Ionization and detection of analyte and IS was performed on a triple quadruple mass spectrometer, API 3000 LC-MS/MS equipped with Turbo Ion spray®, from MDS SCIEX (Toronto, Canada) operated in the negative ion mode.

Quantitation was done using MRM mode to monitor protonated precursor product ion transition of m/z 143.10

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Table 1 Back calculated concentration of calibration curve standards (N=3)

		STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H
	Nominal con. (µg/mL.)	1.012	2.024	15.050	30.100	60.199	78.296	102.671	120.399
PA BATCH 1	CC 1	1.083	1.847	14.860	31.990	58.297	78.989	97.726	124.961
PA BATCH 2	CC 2	1.029	1.882	15.08	33.041	59.615	75.228	100.058	123.82
PA BATCH 3	CC 3	0.978	2.029	14.96	29.149	64.537	81.767	96.786	119.925
	N	3	3	3	3	3	3	3	3
	Mean	1.030	1.919	14.967	31.393	60.816	78.661	98.190	122.902
	SD	0.0525	0.0966	0.1105	2.0134	3.2889	3.2818	1.6846	2.6405
	% CV	5.10	5.03	0.74	6.41	5.41	4.17	1.72	2.15
	Mean % Nominal	101.78	94.83	99.45	104.30	101.03	100.47	95.64	102.08

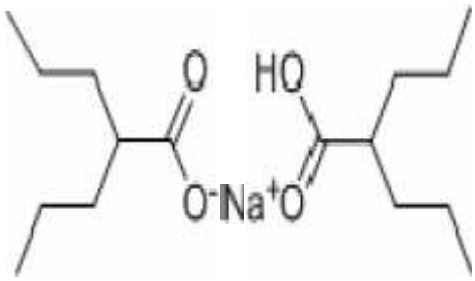

Fig1 (A): Divalproex sodium

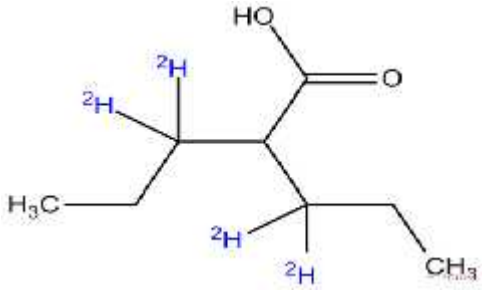

Fig1 (B): Valproic acid D4

→143.10 amu, and 147.20 →147.20 Divalproex and Valproicacid D4 respectively. Pseudo MRM was performed *i.e.* parent and product ion kept same as this molecule was not given any significant, stable fragment during product ion scanning. All the parameters of HPLC and MS were controlled by Analyst software version 1.4.2. The mass spectrometer was tuned to its optimum sensitivity. Mass spectrometry conditions were optimized using a syringe pump infusion of 5 µL/min with the fresh dilution of 20 µg/mL.

The source dependent parameters maintained for analyte and IS were Nebulizer Gas (NEB): 10 psi, Curtain Gas (CUR): 15.00 psi, Collision Activation Dissociation (CAD): 0.00 psi, Turbo Heater Temperature (TEM): 400.00 ºC, Ion Spray Voltage (IS): -3500 V. The compound dependent parameters for analyte and internal standard were Declustering Potential (DP): -42 V (for Divalproex) & -31 V (for IS), Focusing Potential (FP): -113.00V(for Divalproex) & -93.00V (for IS) ,Collision Energy(CE):-130 V ,Collision Cell Exit Potential(CXP): -6V, Entrance Potential(EP): -10 V.

Preparation of standard stock and plasma samples: The standard stock solution of Divalproex (10mg/mL), and Valproic Acid D4 (1mg/10mL) were prepared by dissolving the standard in methanol. Further dilutions from the stock solutions were prepared using diluent solution (Methanol: Milli-Q Water: 50:50, v/v) for spiking in plasma to obtain calibration curve (CC) standards and quality control (QC) samples. CC standards consisting of a set of eight non-zero concentrations were prepared by spiking 2% of Divalproex aqueous mixture to the plasma to obtain calibration standards and quality control samples. Calibration standards ranges 1.012 µg/mL, 2.024 µg/mL, 15.050 µg/mL, 30.100 µg/mL, 60.199 µg/mL, 78.296 µg/mL, 102.671 µg/mL and 120.399 µg/mL for Divalproex.

The quality control samples were prepared worth concentration of Divalproex at LLOQ QC 1.016 µg/mL, LQC 2.846 µg/mL, MQC 58.403 µg/mL and HQC 100.543 µg/mL.

After bulk spiking, 250 µL of spiked plasma samples were pipetted out in pre-labeled polypropylene tubes. The calibration curve standards and quality control samples were logged in Ultra Low temperature deep freezer (Temp range: -55ºC to -75°C) except 30 samples each of LQC and HQC which were transferred for storage in Cell Frost deep freezer (Temp range: -17ºC to -27ºC) for the generation of Long Term Stability at – 22ºC ± 5ºC. These samples were used for performing the method validation.

Preparation of Mobile phase and Extraction method: A Buffer solution was prepared by dissolving approximately 770.0 mg of ammonium acetate in 1000 mL of milli-Q water and 0.2 mL of formic acid added in the buffer solution. Mobile phase was prepared as the mixture of acetonitrile: buffer solution in the ratio 80:20, v/v. For bioanalysis Protein precipitation method was performed. A set of calibration curve standards, quality control samples and blank samples were withdrawn from the deep freezer and allowed to thaw at room temperature. 50 µL of ValproicAcid D4 as internal standard dilution (5.000 µg/mL) was added into labeled eppendorf tubes except in blank and blank QC samples. 150 µL of plasma

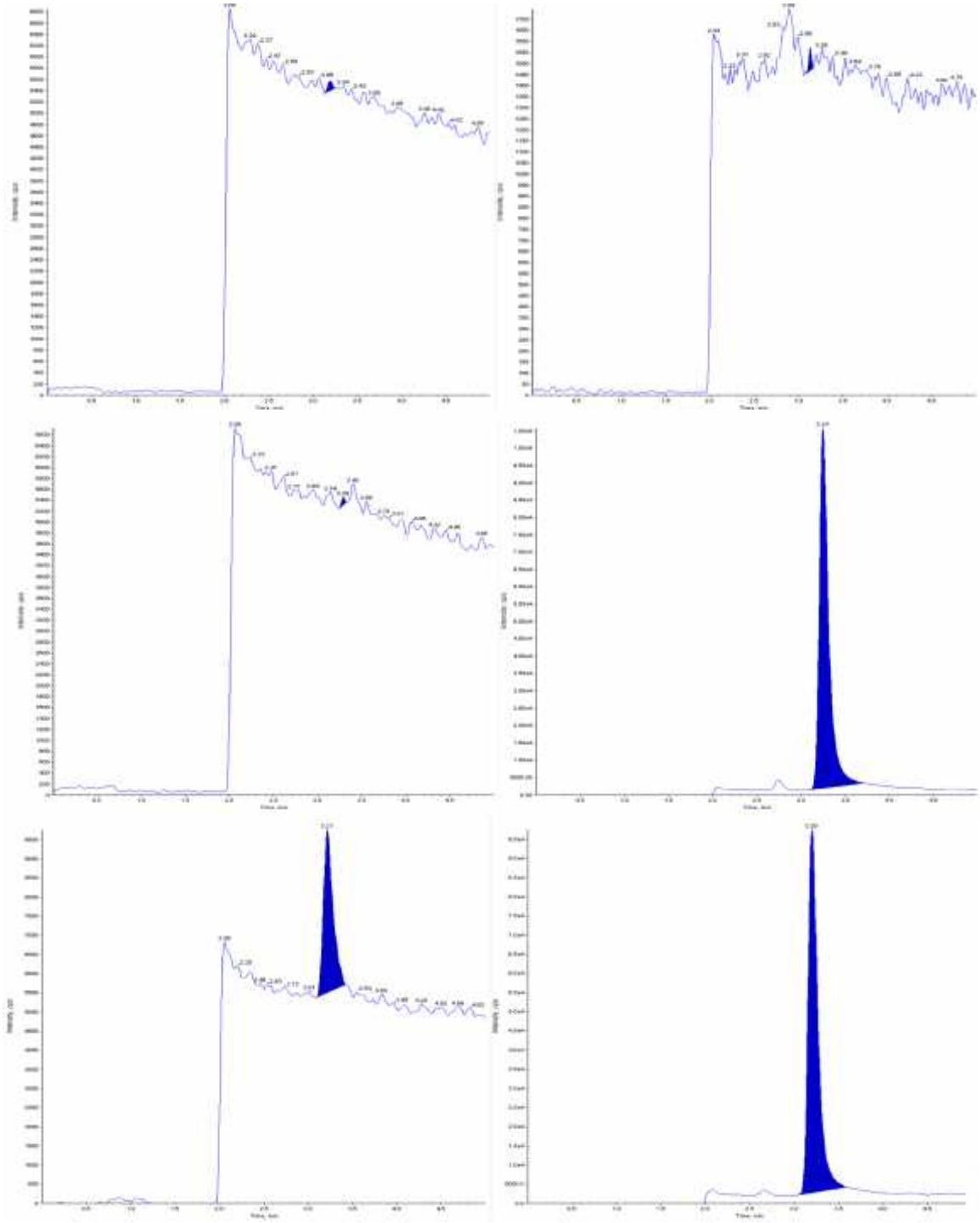

Fig 2: Plasma Blank ,Zero Blank and LLOQ of Divalproex and Valproic acid D4, Diverter diverts the flow to waste till 2 min. than the eluent passes through the instrument source to avoid any contamination after protein precipitation method.

was aliquoted to the pre-labeled eppendorf tubes, vortexed and added 50 µL of sample buffer Solution (10% Acetic acid solution in water) followed by vortexing for approximately 2 min. Added 600 µL of Acetonitrile and allowed the samples to vortexed in Heidolf multireax for about 10 minutes for 1800 RPM. Centrifuged the samples at 15000 rpm for about 10 min at 4 ºC. Pipetted out 0.500 mL of extract and transferred into HPLC vials for analysis.

Method validation: The method has been validated for selectivity, sensitivity, linearity, matrix effect, calibration curve standards and quality control samples, precision and accuracy batches. The results of various stabilities i.e. (stock dilution stability at refrigerator temperature and room temperature, standard stock solution stability in refrigerator temperature and room temperature, auto sampler stability, re-injection reproducibility, freeze thaw stability, long term stability at - 65ºC ± 10°C and at - 22ºC ± 5ºC, reagent stability, bench top stability, dry ice

stability, extended bench top stability, wet extract stability in refrigerator, Lipemic and Haemolysed plasma stability), blood stability, effect of potentially interfering drugs, dilution integrity, recovery, ion suppression through infusion, ruggedness, robustness and extended batch verification meeting the acceptance criteria as per the USFDA guidelines (FDA, 2001). Selectivity was performed in six lots of Normal, three lots of Lipemic and three lots of Haemolysed Plasma containing K2EDTA (Potassium salt of Ethylene di-amine Tetra Acetic Acid) as an Anticoagulant. Sensitivity of the method was determined on six LLOQ samples. For matrix effect, 12 blank samples were processed from 6 normal plasma lots (Two aliquots prepared from each plasma lot) and six blank samples were processed from 3 Lipemic plasma lots and six blank samples were processed from 3 Haemolysed plasma lots respectively.

After processing, 550 µL supernatant solution was withdrawn from blank samples and added 80 µL of drug and internal standard mixture. The drug and internal standard mixture was prepared by adding 270 µL of acetonitrile, 30 µL of Divalproex (LQC or HQC), 500 µL of internal standard dilution mixture. Matrix effect was calculated as per the following formula:

Matrix factor = (Peak response in the presence of matrix ions)/ (Peak response in the absence of matrix ions)

%Matrix effect = (1-Mean of matrix factor) X 100

The precision of the assay was calculated as percent coefficient of variation over the concentration range of LLOQ QC, LQC, MQC and HQC samples, respectively. The accuracy of the assay was calculated as the ratio of the calculated mean values of the LLOQ QC, LQC, MQC and HQC samples to their respective nominal values. The data of three precision & accuracy batches were subjected for goodness of fit analysis. The back-calculated concentrations of Calibration Curve standards using 1/x and 1/x2 weighing were considered for finding the best fit for regression. Linearity was calculated using a regression equation with a weighting factor of 1/x2 for drug to IS concentration to produce the best fit for the concentration-detector response relationship for Divalproex. Stock solution and stock dilution stability in refrigerator for Divalproex and Valproic Acid D4 (IS) was carried out for 7 days while Stock solution and stock dilution stability at room temperature was carried out for 48 hours. For all the aqueous related stability studies two aqueous mixtures were prepared, one from the stability standard stock solution and the other from fresh standard stock solution (comparison stock). Six replicates of aqueous mixture from each, stability stock and comparison stock were injected. The response of stability sample was corrected using a correction factor.

Correction factor= (Conc.of fresh standard sol.)/(Conc.of stability standard sol.)

Corrected response=Stability Stock Response X Correction Factor

% Change= ((Mean Response of Comparison Samples - Mean Corrected Response of Stability Samples))/(Mean response of comparison samples) X 100

Aqueous recovery comparison samples (LQC, MQC and HQC) were prepared by adding 0.012 mL aqueous dilution of each Divalproex quality control (LQC, MQC and HQC), 0.200 mL of internal standard dilution (approximately 5.000 µg/mL), 0.200 mL of sample buffer and 1.988 mL of Acetonitrile (representing 100 % extraction). The aqueous samples (LQC, MQC and HQC) of Divalproex were compared against 6 sets of LQC, MQC and HQC. Recovery of internal standard was compared at LQC, MQC and HQC level.

The aqueous samples (LQC, MQC and HQC) of Divalproex were compared against 6 sets of extracted LQC, MQC and HQC. Recovery of internal standard was compared at LQC, MQC and HQC level.

Recovery of internal standard was compared at LQC, MQC and HQC level.

% Recovery = (Mean Peak Area Response of Extracted sample) / (Corrected Mean Peak Area Response of Unextracted sample) X 100

The effect of Potentially interfering drugs (PIDs) i.e. Ibuprofen, Caffeine, Acetaminophen and Acetyl salicylic acid on Divalproex analysis was performed by spiking PID's at their approximately Cmax concentration in the

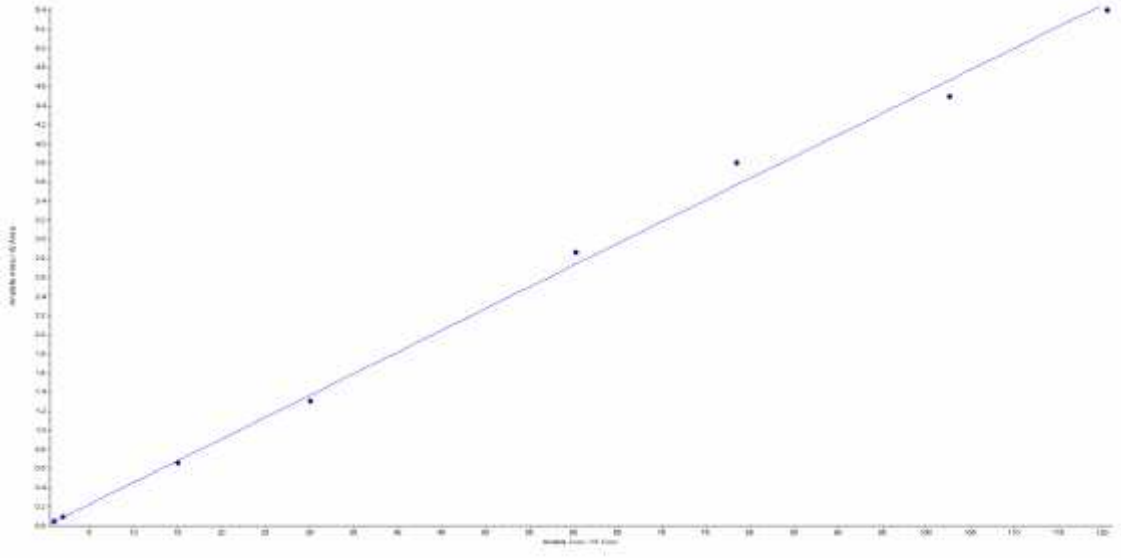

Fig 3: Calibration Curve of Divalproex

Table 2 Inter day and intraday precision and accuracy of the method

PA BATCH 1	LLOQ QC		LQC		MQC		HQC	
NOMINAL CONC.	10.202	µg/mL.	30.58	µg/mL.	2590.836	µg/mL.	4343.21	µg/mL.
QC ID	Observed Conc	% Nominal	Observed Conc	% Nominal	Observed Conc	% Nominal	Observed Conc	% Nominal
1	1.160	114.173	2.875	101.019	58.845	100.757	104.774	104.208
2	1.129	111.122	2.926	102.811	61.113	104.640	110.917	110.318
3	1.194	117.520	2.907	102.143	59.955	102.657	110.871	110.272
4	1.101	108.366	2.988	104.989	55.518	95.060	99.124	98.589
5	1.010	99.409	2.944	103.443	58.254	99.745	114.771	114.151
6	0.936	92.126	3.296	115.812	58.220	99.687	108.306	107.721
N	6	6	6	6	6	6	6	6
Mean	1.088	107.12	2.989	105.04	58.651	100.42	108.127	107.54
SD	0.0974		0.1549		1.8952		5.5097	
% CV	8.95		5.18		3.23		5.10	
Mean % Nominal	10.67		9.78		2.26		2.49	
PA BATCH 2	LLOQ QC		LQC		MQC		HQC	
7	0.881	86.713	2.527	88.791	59.101	101.195	111.323	110.722
8	1.005	98.917	2.657	93.359	62.193	106.489	111.444	110.842
9	0.886	87.205	2.797	98.278	61.148	104.700	106.398	105.823
10	1.014	99.803	2.873	100.949	56.543	96.815	103.559	103.000
11	0.850	83.661	2.952	103.725	56.590	96.896	110.148	109.553
12	0.938	92.323	2.768	97.259	59.129	101.243	110.544	109.947
N	6	6	6	6	6	6	6	6
Mean	0.929	91.44	2.762	97.06	59.117	101.22	108.903	108.31
SD	0.0685		0.1523		2.3064		3.2059	
% CV	7.38		5.51		3.90		2.94	
Mean % Nominal	91.44		97.06		101.22		108.31	
PA BATCH 3	LLOQ QC		LQC		MQC		HQC	
13	1.077	106.004	2.897	101.792	54.384	93.119	104.036	103.474
14	0.970	95.472	2.831	99.473	56.413	96.593	100.847	100.302
15	0.879	86.516	2.939	103.268	56.362	96.505	101.443	100.895
16	1.018	100.197	2.666	93.675	57.817	98.997	98.675	98.142
17	0.881	86.713	2.964	104.146	56.903	97.432	101.897	101.347
18	0.963	94.783	2.842	99.859	57.980	99.276	99.692	99.154
N	6	6	6	6	6	6	6	6
Mean	0.965	94.95	2.857	100.37	56.643	96.99	101.098	100.55
SD	0.0772		0.1069		1.3014		1.8601	
% CV	8.01		3.74		2.30		1.84	
Mean % Nominal	94.95		100.37		96.99		100.55	
Global calculation								
	LLOQ QC		LQC		MQC		HQC	
N	18	18	18	18	18	18	18	18
Mean	0.994	97.83	2.869	100.82	58.137	99.54	106.043	105.47
SD	0.1042		0.1625		2.0831		5.1008	
% CV	10.49		5.66		3.58		4.81	
Mean % Nominal	97.83		100.82		99.54		105.47	

LLOQ sample in triplicate.

Bench top stability was determined for 12 hours using six sets each of LQC and HQC samples while Extended Bench Top Stability was determined in spiked samples to assess the stability of Divalproex at each step of extraction. The freeze and thaw stability was determined for five freeze thaw cycles. Six sets of LQC and HOC samples were analyzed after five freeze-thaw cycles. Long Term Stability (at - 65 ºC ± 10ºC and -22 ºC±5ºC) was carried out in plasma for 32 days by using six sets of LQC and HQC. Dry Extract stability was carried out by processing six sets of LQC and HQC, stored at -22 ºC±5ºC without reconstitution while Wet extract stability was carried out by processing the six sets of LQC and HQC, stored at 2-8 ºC after reconstitution. The samples of wet extract stability was analyzed after 55 hrs storage. All stability QC's were analyzed against the freshly spiked calibration curve standards and six sets of freshly spiked LQC and HQC (prepared from the fresh stock solution) to calculate the % change between the stability QC's and Comparison QC's.

For Robustness six sets of LQC and HQC were analysed against a calibration curve standards at different chromatographic conditions i.e. Robustness experiment was performed at different column temperatures (33ºC and 42ºC), at different flow rates (0.780 mL/min and 0.820 mL/min) and at different mobile phase compositions Acetonitrile: 10 mm ammonium acetate at 75:25 v/v and 85:15 v/v. To evaluate ruggedness, precision and accuracy batch was processed against calibration curve standards and analysed by a different analyst using different column and different sets of solutions.

RESULTS AND DISCUSSION

LC–MS/MS settings: Internal standards Valproic acid D4 was expected show nearly similar chromatographic behavior as of analyte because they are differing only in terms of possessing different isotopic atoms. Retention time (RT) of Divalproex and Valproic acid D4 were found to be 3.24 ± 0.3 min and 3.22 ± 0.3 min respectively. Furthermore, as expected IS produces similar recovery as of analyte in the negative ion mode. Electro Spray Ionization (ESI) provided high ionization efficiencies for both analyte and IS in negetive ion mode which resulted in admirable sensitivity of the method.

Sample preparation: Protein precipitation method was used for sample preparation because of high affinity towards plasma proteins of the analyte. Liquid liquid extraction and Solid phase extraction procedures were also tried during the method development but protein precipitation and direct injection was found more suitable method interms of reproducibility and recovery. As in Liquid liquid method after drying the sample due to volatile nature of the drug sensitivity and reproducibility of the method was impacted. Further in solid phase extraction the results were not satisfactory interms of sensitivity, reproducibility and recovery. Thus Protein precipitation procedure was found to be most effective for extraction of Divalproex and Valproic acid D4 with minimal interference.

Method validation

Selectivity and matrix effect: Figure 2(A and B) shows typical MRM chromatograms of a blank plasma sample, a plasma sample spiked with Divalproex at the LLOQ (1.012 µg/mL.). Base line was relatively high due to pseudo MRM which certainly not impacting method sensitivity and signal to noise ratio. No significant interferences was observed from endogenous substances at the retention times of the analyte and internal standard in normal, haemolysed or lipemic Plasma. The variability of matrix factor (reported as %CV of matrix factor) was 2.14 % (LQC) and 5.16 % (HQC) for Divalproex, and 1.39 % (LQC) and 2.58 % (HQC) for Valproic acid D4 and the variability of IS-Normalized Matrix Factor on normal plasma (reported as %CV of matrix factor) was -0.46 % (LQC) and 0.37 % (HQC) for Divalproex, 1.78 % (LQC) and 2.76 % (HQC) Valproic acid D4. The results were within the acceptance criteria and indicate no ion suppression or enhancement due to the plasma matrix was consistent and would not interfere with the quantitation of analytes.

Linearity and sensitivity: The correlation coefficients (R^2) were greater than 0.99 over the range of 1.012 µg/mL. and 120.399 µg/mL. . Typical equations of calibration curves are as follows:

$y = 0.0455 x + (0.00078)$, $R^2=0.9990$

Where y represents the analyte\ I.S. peak area ratio and x represents the plasma concentration of the analyte. The sensitivity experiment was performed and precision and accuracy at LLOQ was 5.36 % and 104.25 % respectively revealing a prodigious sensitivity of the method.

Precision and accuracy: Table 1 summarizes back calculated concentrations of calibration curve standards Divalproex whereas Table 2 represents the intraday and inter days precision and accuracy data. The inter-day accuracy was 97.83%-105.47% and precision was 3.58%-10.49%.The results showed that method is fairly precise and accurate within the acceptable limits.

Recovery: The mean % recovery of Divalproex was 75.318 % with a precision of 3.76 % and Valproic acid D4 was 74.038 % with a precision of 0.58 % .The data show that the Protien Precipitation procedure efficiently extracts analyte as well as IS from human plasma.

Stability and other parameters: All the stabilities are been successfully established as per future PK study requirements. The outcomes of other parameters like Ruggedness, Reinjection reproducibility, Effect of Potentially Interfering Drugs (PID), Dilution Integrity, Extended Batch Verification and Robustness were found to be within the acceptance criteria as per USFDA guidelines (Anonymous, 2001).

CONCLUSION

A highly sensitive and selective method for the quantitative determination of Divalproex in human plasma was developed using HPLC–MS/MS with turbo-ion spray in negetive ion mode. The method was validated as per USFDA guidelines and found to be well

suited for the PK study. This method is effective allows for a much higher sample throughput due to the simple extraction procedure shorter chromatographic run time (5.0 min) .The method has been successfully used in a PK study of orally administered Divalproex Sodium extended release tablets.

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REFERENCES

1. Simom,D,Penry JK. Sodium di-N-propylacetate(DPA) in the treatment of epilepsy,A review.Epilepsia,16(4)(1975)549-573
2. Richard,H.Mattson,Joyce A.Cramer,Peter D. Williamson,Robert A.Novelly>Valproic acid in Epilepsy:Clinical and Pharmacological effects.Annals of Neurology 3(1) (1978) 20-25
3. Sandeep Dutta, Ronald C. Reed Disclosures Clin Drug Invest. 2004;24(9)
4. Willmore,L.Divalproex and epilepsy J. Psychopharmacol.Bull.37(2)(2003) 43-53.
5. Laeng P1, Pitts RL, Lemire AL, Drabik CE, Weiner A, Tang H, Thyagarajan R, Mallon BS, Altar CA J Neurochem. 2004 Oct;91(1):238-51
6. Dasgupta A, Volk A: Displacement of valproic acid and carbamazepine from protein binding in normal and uremic sera by tolmetin, ibuprofen, and naproxen: presence of inhibitor in uremic serum that blocks valproic acid-naproxen interactions. Ther Drug Monit 1996;18:284-287
7. H.Cheng et.al./J.Chromatogr.B 850(2007)206-212
8. B.Mohammadi et.al./J.Chromatography.B 880(2012)12-18
9. Anonymous. Guidance for Industry, Bionanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001