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SIMULTANEOUS DETERMINATION OF TERIFLUNOMIDE, CARBAMAZEPINE, DULOXETINE AND TAMSULOSIN IN RAT PLASMA BY LC–ESI-MS AS PRESCRIPTIVE DRUG ANALYSIS STUDY FOR MULTIPLE SCLEROSIS DISEASE

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Combination therapy or polytherapy is that uses more than one medication to treat a single disease and associated diseases. Pharmaceutical combination therapy may be achieved by prescribing separate drugs, or, where available, dosage forms that contain more than one active ingredient. A novel and accurate liquid chromatography tandem mass spectrometry method using electrospray ionization mode has been developed and validated for the simultaneous determination of Carbamazepine (CBZ), Duloxetine (DLX), Tamsulosin (TSL) and Teriflunomide (TFM) using Doxofylline (DXF) and Ibuprofen (IBP) as internal standards (IS). The separation was carried on XTerra MS C18 (100 mm x 3.9 mm, 5 μm) reversed phase column using acetonitrile and 0.01M ammonium formate as the mobile phase in gradient mode at 0.8 mL/min. The method was validated interms of linearity, accuracy and precision over the concentration range of 1–1000 ng/mL. The intra and inter-day precision and accuracy, stability and extraction recoveries of all the analytes were in the acceptable range. This method can be successfully applied to the pharmacokinetic study of CBZ, DLX, TSL and TFM when prescribed in polytherapy.

KEYWORDS : Electro spray ionization, Polytherapy, Teriflunomide, Carbamazepine, Duloxetine, Tamsulosin

INTRODUCTION

Prescribing a single drug and its administration is not sufficient in neuro diseases like multiple sclerosis. Combination therapy is growing enormously to decrease the number of medications for a single disease or their associated diseases. In clinical research estimation of concomitant drugs plays a key role to study the drug-drug interactions. The research in the current article has undertaken to provide an accurate method which can be applied to estimate the drugs which are prescribed as combination therapy.

Teriflunomide ((2Z)-2- cyano-3-hydroxy-N-[4-(trifluoro methyl) phenyl] but-2-enamide) is an immuno modulatory agent with anti-inflammatory properties that selectively and

reversibly inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHO-DH), required for de novo pyrimidine synthesis. Teriflunomide is actively being investigated for use in renal transplant recipients, not only for its immunosuppressive effects, but more important, because it has antiviral effects that assist in clearing infections common in transplant recipients, such as BK polyomavirus (BKV) and cytomegalovirus [1-3]. Carbamazepine (5*H*-dibenzo [*b*, *f*] azepine-5-carboxamide) is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder, as well as trigeminal neuralgia. Duloxetine ((+)-(*S*)-*N*-Methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine) is useful in major depressive disorder, general anxiety disorder, urinary incontinence, painful peripheral neuropathy, fibromyalgia, and chronic musculo skeletal pain associated with osteoarthritis and chronic lower back pain. Tamsulosin ((*R*)-5-(2-{[2-(2-ethoxyphenoxy) ethyl]amino} propyl)-2-methoxy benzene-1-sulfonamide) is an α_1 adrenergic receptor antagonist used in the symptomatic treatment of benign prostatic hyperplasia (BPH) and also assist the passage of kidney stones by the same mechanism of smooth muscle relaxation.

Several chromatographic techniques have been reported for DLX [4-6], CBZ [7-8], TSL [9-11] and TFM [12-15], individually and in combination with other drugs. However, so far, no single method has been reported for the simultaneous estimation of CBZ, DLX, TSL and TFM in rat plasma by LC–MS/MS. Therefore, the aim of this study was to evaluate the pharmacokinetic parameters of these four drugs when used as combination therapy. The developed bioanalytical method has been validated according to ICH guidelines [16]. This method can also be useful in estimating the plasma samples of patients receiving these drugs. The structures of analytes are presented in Fig. 1.

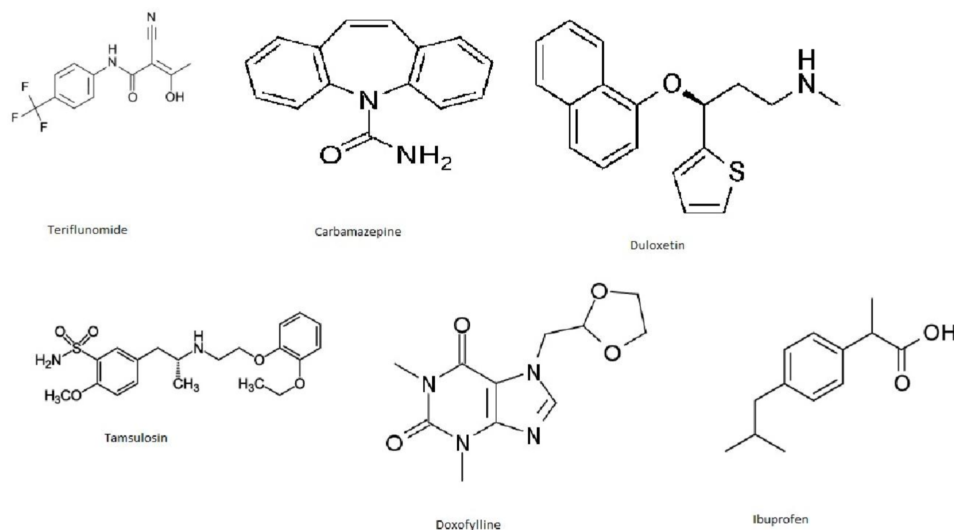

Fig. 1. Chemical structures of analytes

Materials and methods

2.1. Chemicals and apparatus

TFM was supplied by Selleckhem.com. CBZ, DLZ, TSL, IBP and DXF were supplied by Sigma-aldrich. Acetonitrile of MS grade was obtained from Merck, India. Other chemicals were all of analytical grade and purchased from Merck, India. Water used in the entire

analysis was prepared from Milli-Q water purification system from Millipore. Biological matrices were obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

2.2. Standard solutions and fortification

Standard stock solutions of CBZ, TFM, DLX, TSL, IBP, DXF were prepared by accurately weighing 10 mg of each standard on a closed electronic microbalance (Sartorius, Germany) and dissolving them separately in 10 mL of methanol. Calibration standard and quality control (QC) samples in plasma were prepared by adding corresponding working solutions with drug-free rat plasma. A volume of 10 mL of appropriate diluted stock solutions of mixture of drugs (CBZ, TFM, DLX, TSL) at different concentrations and 10 mL of ISs (IBP and DXF) at a fixed concentration were spiked into 100 mL of drug-free rat plasma to yield final concentrations of calibration samples 1, 2, 5, 10, 50, 100, 500 and 1000 ng/mL for CBZ, TFM, DLX, TSL respectively. The final concentration of ISs (IBP and DXF) was 25 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (1 ng/mL), LQC (5 ng/mL) MQC (50 ng/mL) and HQC (500 ng/mL) for CBZ, TFM, DLX and TSL.

2.2. Sample preparation

Analytes were extracted from plasma by employing the protein precipitation method. 150 µL of acetonitrile was added as a protein precipitating agent, vortexed for 1min and then centrifuged at 10,000 rpm for 10 min on refrigerated centrifuge at 4°C. The supernatant layer was separated and filtered through 0.45 µm syringe filters and 10 µL of the solution was injected for LC–MS/ MS analysis.

Table 1: Optimized LC-MS/MS conditions for TFM, CBZ, DLX, TSL, DXF and IBU

Analyte	RT (min)	ESI mode	MRM transitions	CE (ev)
TFM	5.9	Negative	269 → 150	29
IBU	5.4	Negative	205 →161	32
DLX	7.7	Positive	298 → 136	40
TSL	6.8	Positive	409 → 237	35
CBZ	5.0	Positive	237 → 136	41
DXF	4.0	Positive	267 → 181	43

2.4. Instrumentation

The LC–MS/MS analysis was carried out in electro spray ionization (ESI) positive mode for CBZ, DLX and TSL using DXF as IS and in negative ion ESI mode for TFM using IBP as IS on a mass spectrometer coupled to a Shimadzu LC system (Model : SIL-HTC) operated with Analyst 1.6.1 software. The separation of all the analytes was carried out on an XTerra MS C18 (100 mm length x 3.9 mm internal diameter and 5 µm particle size) column. Temperature was set to 25°C. The mobile phase composed acetonitrile and 0.01M ammonium formate (Gradient mode) at a flow rate of 1.0 mL/min for 15 min. The full scan MS and MS/MS spectra of each analyte were obtained by direct infusion of the respective sample solution at a concentration of 10 µg/mL solution prepared in methanol. The drugs were analyzed using multiple reactions monitoring (MRM) mode. The precursor ions, product ions, and LC–MS/MS parameters are depicted in Table 1.

2.5. Method Validation

The bio analytical method was validated according to the FDA guidelines (US Food and Drug Administration, May 2001). The method was validated interms of selectivity, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision,

recovery, matrix effect and stability. The selectivity and specificity were assessed by comparing the chromatograms of six different sources of blank rat plasma with those of the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and LC–MS/MS conditions to ensure no interference of TFM, CBZ, TSL, DLX and ISs from blank plasma. The linearity of the assay was evaluated by constructing calibration curves with different concentrations ranging from 1 to 1000 ng/mL for all the analytes. The calibration curves were constructed by plotting each respective peak area ratios of TFM to IBU (IS) and CBZ, DLX, TSL to DXF (IS) against the concentrations of each analyte, using the weighting factor of $1/x^2$. LODs of the drugs were determined based on signal intensity three times more than base line noise (S/N = 3) and LOQs of the drugs were determined based on intensity of signal which was ten times more than the noise (S/N=10). QC samples were prepared in blank plasma at the concentrations of 1 (LLOQ), 5 (LQC), 50 (MQC) and 500 (HQC) ng/mL for all the four analytes in six replicates (n = 6) for assessing the accuracy, intra-and inter-day precisions (reproducibility) of the method. All QC samples were prepared freshly on three consecutive days and analyzed in each analytical batch along with the unknown samples. The matrix effect and recoveries of analytes were quantitatively measured by comparing the signal intensities and the peak area ratios (analyte/IS) obtained from post extraction spiking (A) (extracting 50 µL of rat plasma with 150 µL of acetonitrile. The residues, after evaporation of solvent by nitrogen purging, were reconstituted with 10 µL of standard solution containing TFM, CBZ, TSL and DLX at concentrations of 5, 50 and 500 ng/mL and internal standards IBU and DXF at 25 ng/mL) and standard solutions (B) (samples prepared in methanol) at the same concentrations in six replicates (n = 6). The ratio (A/Bx100%) was used to evaluate the matrix effect. The stabilities of all four analytes in plasma at different storage conditions were evaluated and the results were expressed as mean percentage accuracies. The short-term stability was determined by keeping QC samples in six replicates (n=6) at room temperature for 24h. The autosampler stability was evaluated by keeping the QC samples at 4°C for 24h in autosampler before analysis. Freeze–thaw stability of QC samples was analyzed after four freeze–thaw cycles by freezing at -70°C for 24h and thawing at room temperature for 24h.

RESULTS AND DISCUSSION

3.1. Mass spectrometric and chromatographic conditions

To optimize peak shape with appropriate retention time various combinations of mobile phases were investigated. Separation of these drugs was attempted using various combinations of acetonitrile and water with different percentage of modifiers and buffers. The best separation was achieved with acetonitrile and 0.01M ammonium formate for estimating TFM, CBZ, TSL and DLX along with ISs (DXF and IBU). The reversed-phase XTerra MS C18 column (100 mm x 3.9 mm, 5 µm) was used with a flow rate of 0.8 mL/min. The retention times of DXF, IBU, TFM, CBZ, TSL and DLX were found to be 4.6, 5.6, 5.9, 6.3, 6.8 and 7.6 min, respectively. The total chromatographic run time was 15 min in estimating all the analytes. The standard solutions of 10 µg/mL with respect to TFM, CBZ, TSL, DLX in methanol were in fused directly into the mass spectrometer. The observed full scan mass spectra in positive mode showed prominent protonated molecular ions $[M+H]^+$ of m/z 237, 298, and 409 for CBZ, DLX and TSL respectively in positive ion mode, and prominent deprotonated molecular ions $[M-H]^-$ of m/z 269 and 205 for TFM and IBU respectively, in negative ion mode. The $[M+H]^+$ ions and $[M-H]^-$ ions of respective analytes were subjected to collision-induced dissociation (CID) at average collision energy of 30%. The collision

energies were optimized for each analyte to obtain the most intense fragment ions. The molecules underwent fragmentation to yield the following fragment ions of m/z 160, 237, 136 and 136 for TFM, TSL, DLX, and CBZ respectively. The MS/MS spectra of four analytes are presented in Fig. 2. Based on their mass spectra and tandem mass spectra, the following MRM transitions: m/z 269→160, m/z 409→237, m/z 298→136, m/z 237→136, m/z were selected for analysis of TFM, TSL, DLX, and CBZ respectively.

Table 2: Intra-day and Inter-day variation for TFM, CBZ, DLX and TSL in six replicates (n=6) at each concentration

Analyte	Nominal concentration (ng/mL)	Intra-day		Inter-day	
		% Recovery	% RSD	% Recovery	% RSD
TFM	1	104.609	6.923	106.394	2.905
	5	101.021	1.593	100.884	0.606
	50	100.266	2.010	103.240	2.626
	100	100.330	1.182	100.819	0.304
CBZ	1	106.536	4.442	103.468	5.684
	5	101.098	1.663	100.667	0.766
	50	101.519	2.066	99.880	2.933
	100	100.242	0.570	100.078	0.307
DLX	1	104.289	6.454	104.324	2.248
	5	100.961	1.678	100.312	1.303
	50	100.119	2.093	100.268	1.219
	100	100.079	1.312	99.729	0.678
TSL	1	98.873	4.441	98.873	1.153
	5	99.545	1.605	99.918	0.837
	50	97.103	2.702	97.535	1.050
	100	98.234	1.131	98.481	0.435

3.3. Method Validation

The specificity of this method was confirmed by comparing chromatograms of blank plasma, spiked plasma with analytes at a concentration of 1 ng/mL. CBZ, TSL, DLX and DXF (IS) in positive ESI experiment and TFM and IBU (IS) in negative ESI experiment were well separated under the described chromatographic conditions. No interfering endogenous peaks were observed around their retention times. The calibration curves of the analytes showed a good linearity over the studied concentration range of 1–1000 ng/mL for TFM, CBZ, DLX, and TSL with correlation coefficients (r2) 0.999. The LODs for all studied analytes were found to be 0.5 ng/mL. The LLOQs for all analytes were 1 ng/mL with acceptable precision and accuracy. The intra- and inter-day precisions for TFM, CBZ, DLX and TSL were less than 8.5%. The obtained intra-day accuracies were in the range of 96.6–106.5% and inter-day accuracies were in the range of 97.5–106.4%. The validation parameters are depicted in Table 2. The extraction recoveries of all drugs from rat plasma were in the range of 95.1–103.1%

with relative standard deviations less than 3.6%, which indicates the sample preparation technique is suitable for extracting the studied drugs from rat plasma. The recovery results are displayed in Table 3. The stability studies of these drugs were performed at three QC concentration (low, medium and high) levels in six replicates (n = 6). The predicted concentrations for each analyte deviated within ±2.0% of nominal concentrations after storage of plasma samples at room temperature for 24 h, four freeze–thaw cycles and in autosampler for 12 h at 4°C. The mean accuracies were found to be more than 95% with relative standard deviations less than 5.7%, which are summarized in Table 4.

Table 3. Recovery Values of TFM, CBZ, DLX and TSL (n = 6)

Analyte	Nominal concentration (ng/mL)	Mean Recovery	% RSD
TFM	5	97.505	3.617
	50	100.788	1.495
	500	99.282	1.285
CBZ	5	100.122	2.804
	50	100.635	1.599
	500	98.511	0.482
DLX	5	95.054	2.044
	50	100.028	0.816
	500	99.979	1.003
	5	103.148	3.324
TSL	50	100.598	2.220
	500	98.993	2.565

Table 4. Stability studies of TFM, CBZ, DLX and TSL in rat plasma at two QC levels (*n* = 6)

Analyte	Mean accuracy ±RSD		
	Short term stability (24h RT)	Freeze-thaw stability (4 cycles)	Autosampler stability
TFM	106.220 ± 7.525	99.277 ± 2.592	102.243 ± 4.689
	99.677 ± 1.298	100.512 ± 0.854	98.615 ± 3.858
	99.997 ± 6.464	99.460 ± 1.684	98.217 ± 3.103
CBZ	99.509 ± 2.879	100.626 ± 1.649	101.740 ± 3.456
	98.630 ± 6.010	99.577 ± 3.470	100.654 ± 4.613
DLX	99.540 ± 3.007	100.250 ± 2.763	95.043 ± 3.454
	101.887 ± 2.980	97.994 ± 2.744	99.949 ± 3.326
TSL	99.311 ± 1.971	99.327 ± 2.532	98.792 ± 3.917

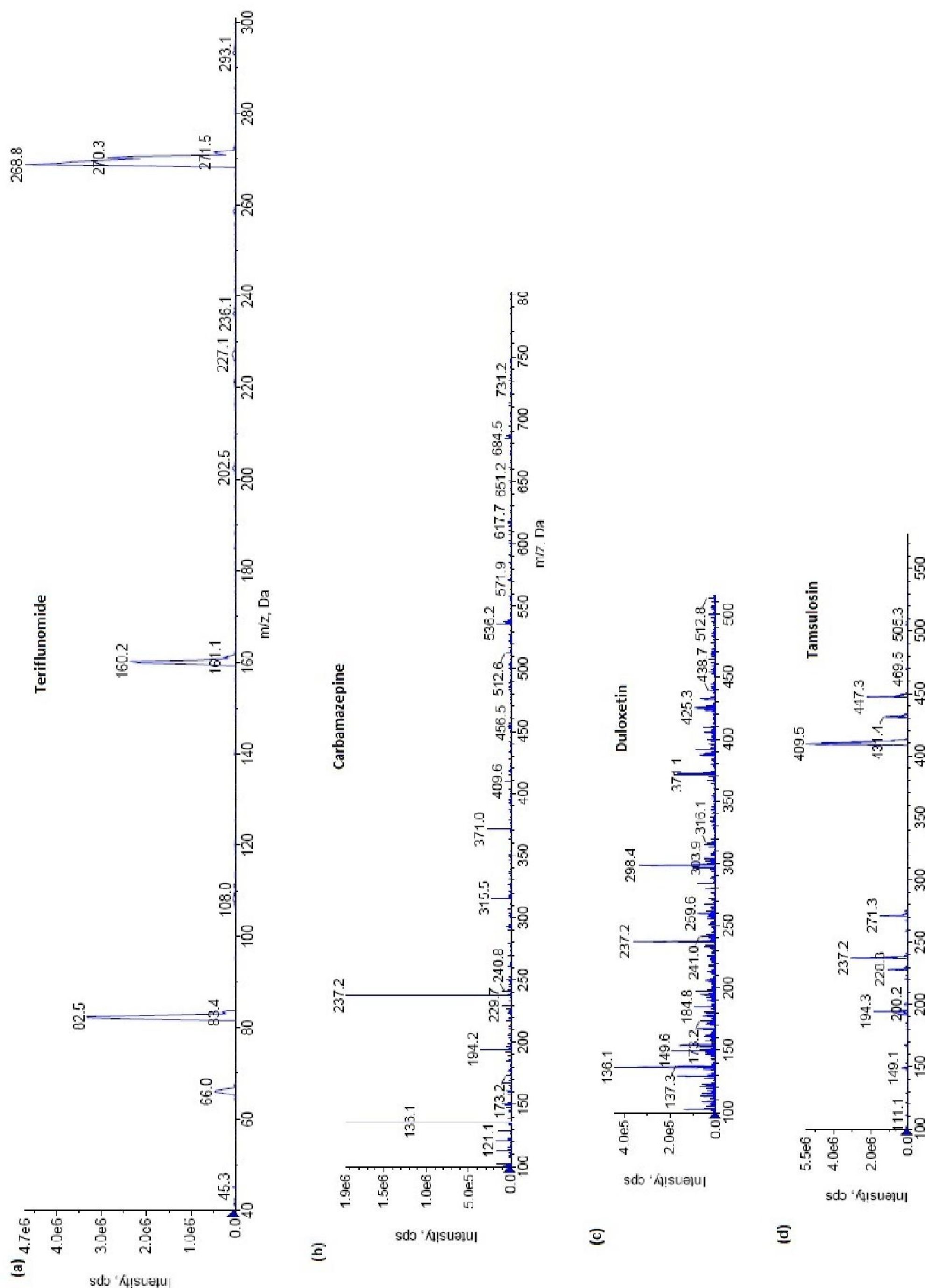

Fig. 2. Product ion spectra of (a) Teriflunomide (b) Carbamazepine (c) Duloxetine (d)Tamsulosin

Conclusion

We have developed and validated a highly sensitive, specific, reproducible and high throughput LC–MS/MS assay to quantify TFM, CBZ, DLX and TSL simultaneously in rat plasma. Simple and single step protein precipitation was used to extract analytes from rat plasma. The major advantages of the assay are simple sample preparation with equal sensitivity for all the four analytes. The obtained LODs and LOQs of all the drugs were adequate and may useful to perform the pharmacokinetic study in rat plasma. Based on the results, we can conclude that the present method is suitable for quantification of multiple analytes simultaneously without any interference and matrix effects. The concomitant drug analysis along with the target analyte is more advantageous than single compound analysis and also useful in drug interaction and toxicology studies.

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