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RESEARCH ARTICLE

LC–MS/MS Determination of Prednisone, A Drug in Phase 4 of Multiple Sclerosis Therapy along with Teriflunomide and Pioglitazone in Rat Plasma

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ABSTRACT

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 randomized efficacy phase 4 study is going on for prednisone in the treatment of multiple sclerosis, which is a neurodegenerative disease associated with many other disorders. A novel and accurate liquid chromatography tandem mass spectrometry method using electrospray ionization mode has been developed and validated for the simultaneous determination of prednisone (PDN), pioglitazone (PGZ) and teriflunomide (TFM) using imipramine (IMP) 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 1.0 mL/min. The method was validated in terms of specificity, linearity, accuracy and precision over the concentration range of 1–500 ng/mL. The intra and inter-day precision and accuracy, stability and extraction recoveries of all the analytes were found in the range of 97.2-102.2%. The lower limit of quantitation was 1.0 ng/mL for all the 3 analytes and the extraction recovery values were more than 65%. The method proved highly reproducible and sensitive and was successfully applied to pharmacokinetic study after single dose oral administration of PDN, PGZ and TFM to the rats.

Keywords: Polytherapy; Teriflunomide; Prednisone; Pioglitazone; Phase 4; Multiple Sclerosis; Pharmacokinetics

INTRODUCTION

Prescribing a single drug and its administration is not sufficient in neurological 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 of the drug-drug interactions. Recently clinical trials.gov has updated the information regarding the phase 4 studies of prednisone (PDN) in the treatment of multiple sclerosis disease. The research in the current study has undertaken to provide an accurate method which can be applied to estimate prednisone concomitantly along with drugs like teriflunomide (TFM) and pioglitazone (PGZ) which are prescribed as combination therapy.

PDN is a synthetic corticosteroid drug that is particularly effective as an immunosuppressant drug. It is used to treat certain inflammatory diseases (such as moderate allergic reactions) and (at higher doses) some types of cancer, but has significant adverse effects. TFM ((2Z)-2- cyano-3-hydroxy-N-[4-(trifluoromethyl)phenyl]but-2-enamide) is an immunomodulatory agent with anti-inflammatory properties that selectively and reversibly inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), required for de novo pyrimidine synthesis. TFM is actively being investigated for use in renal transplant recipients, not only for its immunosuppressive effects, but more importantly, because it has antiviral effects that assist in clearing infections common in transplant recipients, such as BK polyomavirus (BKV) and cytomegalovirus.[1–3] Pioglitazone hydrochloride, (±)-5-{4-[2-(5-ethyl-2-pyridyl) ethoxy] benzyl}-2,4-thiazolidinedione hydrochloride salt, is an oral anti-hyperglycemic agent used in the treatment

of type 2 diabetes.[4,5] PGZ exerts its glucose-lowering effects by binding to peroxisome proliferator activated receptors gamma (PPARγ) and increasing the receptor sensitivity to insulin.[6,7] PGZ is rapidly absorbed and extensively metabolized by hydroxylation and oxidation to active and inactive metabolites in the liver.[8] Two high-performance liquid chromatography (HPLC) methods have been described for the determination of PGZ and its metabolites in human serum.[9,10] The solid-phase extraction method was used in both methods and also the second method involves additional liquid extraction and gradient HPLC, which is a time consuming process. The run time of these methods is more than 20 min. Another HPLC method was also reported for the determination of PGZ in rat plasma by Sripalakit et al.[11] using solid-phase extraction sample preparation method. Liquid chromatography with tandem mass spectroscopy (LC–MS–MS) was also reported for determination of PGZ with a low limit of quantitation.[12–14] The reported methods have applied different extraction procedures and different sensitivity ranges in different biological fluids. In the current method a single step protein precipitation is used for all the three analytes in rat plasma which is a very useful technique for biological samples. Several chromatographic techniques have been reported for TFM[15–18], PGZ[13,19,20] and PDN[21–25] individually and in combination with other drugs. However, so far, no single method has been reported for the simultaneous estimation of PGZ, PDN and TFM in rat plasma by LC–MS/MS. Therefore, the aim of this study was to simultaneously estimate TFM, PDN & PGZ in pharmacokinetic study in rats as combination therapy. The developed bioanalytical method has been validated according to USFDA and ICH (International Conference on Harmonization) guidelines.[26] This method may also be useful in estimating the plasma samples of patients receiving these drugs in clinical trials. The structural formulae of analytes are presented in Figure 1.

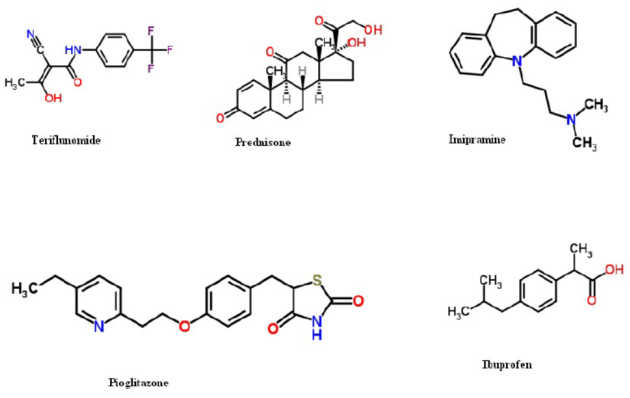

Fig. 1: Structural formulae of the analytes

EXPERIMENTAL

Chemicals and apparatus

TFM was supplied by Selleckhem.com. PDN and PGZ were supplied by Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). Acetonitrile of MS grade was obtained from Merck (Mumbai, India). Other chemicals were all of analytical grade and purchased from Merck (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA). Biological matrices were obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until used.

Standard solutions and fortification

Standard stock solutions of TFM, PDN, PGZ 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 (TFM, PDN, PGZ) at different concentrations and 10 mL of ISs (IBP and IMP) at a fixed concentration were spiked into 100 mL of drug-free rat plasma to yield final concentrations of calibration samples 1,5,10,50,100,200,400 and 500 ng/mL for TFM, PDN, PGZ respectively. The final concentration of ISs (IBP and IMP) was 25 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (1 ng/mL), LQC (5 ng/mL) MQC (100 ng/mL) and HQC (500 ng/mL) for TFM, PDN and PGZ.

Sample preparation

Analytes were extracted from plasma by employing the protein precipitation method. 150 μL of acetonitrile was added as a protein precipitating agent; to 50 μL plasma, 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.

Instrumentation

The LC–MS/MS analysis was carried out in electrospray ionization (ESI) positive mode for PDN and PGZ using IMP as IS and in negative ion mode, for TFM using IBP as IS on a mass spectrometer coupled to a Shimadzu LC system (Make: API 3000, 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 x 3.9 mm id and 5 μm) column. Temperature was set at 30°C. The mobile phase composed of 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.

Table 1: Optimized LC-MS/MS conditions for TFM, PDN, PGZ, IMP and IBU

Analyte	RT (min)	ESI mode	MRM transitions	CE (ev)
TFM	5.9	Negative	269 → 160	29
IBU	5.6	Negative	205 →161	32
PDN	6.5	Positive	359 → 341	25
PGZ	6.9	Positive	357 → 122	35
IMP	7.3	Positive	237 → 136	41

Method Validation

The bio analytical method was validated according to ICH and FDA guidelines (US Food and Drug Administration, May 2001). The method was validated in terms 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 (50 μL) was tested using the proposed extraction procedure and LC–MS/MS conditions to ensure no interference of TFM, PDN, PGZ and ISs from blank plasma. The linearity of the assay was evaluated by constructing calibration curves with different concentrations ranging from 1 to 500 ng/mL for all the analytes. The calibration curves were constructed by plotting each respective peak area ratios of TFM to IBU (IS) and PDN and PGZ to IMP (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), 100 (MQC) and 500 (HQC) ng/mL for all the three 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 neat standard solutions (B) (samples prepared in methanol) at the same concentrations in six replicates (n=6) of all the 3 drugs were prepared. The stabilities of all three analytes in plasma at different storage conditions were evaluated and the results are 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 auto sampler stability was evaluated by keeping the QC samples at 4°C for 24h in auto sampler 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.

Application of the method to pharmacokinetic study

All animal experiments were approved (No. 1722/RO/ERe/S/13/CPCSEA) by Local Animal Ethics Committee (Albino Research Lab, Miyapur, Hyderabad, Andhra Pradesh, India). In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed LC-MS/MS method was successfully applied to a pharmacokinetic study by administration of TFM, PDN, PGZ as single solution to six male Wistar rats by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 3 mg/kg body weight. Approximately, 0.2 mL of blood samples from each anesthetized (isoflurane) rat at pre-determined time intervals was collected using a capillary tube into pre-labeled eppendorf tubes containing 10% of K_2EDTA anticoagulant (20 μL). The time intervals for the sample collection were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12 and 24 h (post dose). The total blood volume collected from each rat was approximately 1.7 to 1.9 mL which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a 200 g body weight rat). Plasma was obtained by centrifuging blood samples at 4,000 rpm for 10 min. The obtained plasma samples were transferred into pre-labeled micro centrifuge tubes and stored at −70°C.

RESULTS

Mass spectrometric and chromatographic conditions

To optimize peak shape with appropriate retention time, various combinations of mobile phases were investigated. The chromatographic separations were initiated to achieve a short runtime, symmetric peak shapes, minimum matrix interference and solvent consumption. Previous studies for teriflunomide[15–18], pioglitazone[19,20] and prednisone 21-25] have reported different columns with 5μm particle size, 3–4 mm inner diameter and columns lengths (125–150 mm) with runtimes ≥15 min. Thus, in the present work chromatographic separation was tried on XBridge C18 (150 mm×4.6 mm, 5 μm), Waters X-Terra MS C18 (100 mm×3.9 mm, 5 μm) and Inertsil ODS-3 C18 (150 mm×4.6 mm, 5 μm) columns. To find the best eluting solvent system, various combinations of methanol/acetonitrile with additives like ammonium acetate and ammonium formate in different concentration and volume ratios were tested. Best results were obtained in terms of higher sensitivity,

superior retention and better peak shapes on X-Terra MS C18 column. The best separation was achieved with 0.01M ammonium formate (A) and acetonitrile (B) for estimating TFM, PDN and PGZ along with ISs (IBU and IMP). Initial gradient conditions were set at 95% A and 5% B. From 2 to 8 min, %B increased to 95% and the same maintained till 10 min. Again %B was decreased linearly to 5% upto 12 min and held at the same till 15 min (total runtime is 15 min). Reproducibility and recovery data for all the analytes and IS supported protein precipitation to be used as the preferred extraction technique. All the chromatographic separations are achieved with single step protein precipitation technique using acetonitrile without any interference from one another. The reversed-phase XTerra MS C18 column (100 mm x 3.9 mm, 5 μm) was used with a flow rate of 1.0 mL/min. The retention times of IBU, TFM, PDN, PGZ, and IMP were found to be 5.6, 5.9, 6.5, 6.9 and 7.6 min, respectively. LC chromatogram showing proper separation of all the analytes is represented in Figure 2.

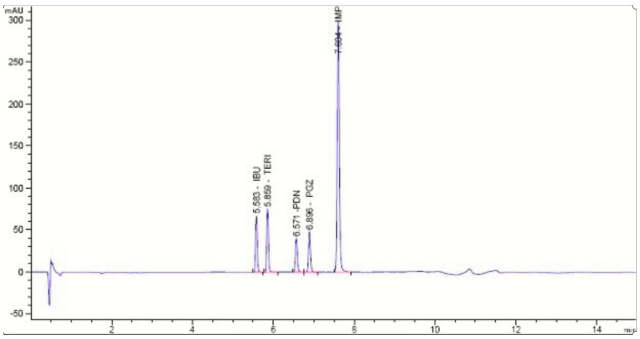

Fig. 2: LC Chromatogram showing the proper separation of drugs

The standard solutions of 10 μg/mL with respect to TFM, PDN, PGZ in methanol were infused directly into the mass spectrometer. The electrospray ionization (ESI) of TFM was conducted in negative ionization mode as it has high electron affinity due to the presence of trifluoromethyl group. Internal standard, IBP has given higher response in negative mode. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response for all the analytes. The recovery in other solvent systems was between 50% and 80%, but was inconsistent with some ion suppression(greater than 15% CV).

The observed full scan mass spectra in positive mode showed prominent protonated molecules $[M+H]^+$ of m/z 359 and 357 for PDN and PGZ respectively in positive ion mode, and prominent deprotonated molecules $[M-H]^-$ 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 spectra were acquired using the following conditions: ion spray voltage, 5000 V; turbo gas temperature, 250°C; nebulizer gas (compressed air), 55 psi; curtain gas (N_2), 20 psi; declustering potential, 80 eV; focusing potential, 200 eV; entrance potential, 10 eV. The optimum values for compound dependent parameters like declustering potential (DP), collision energy (CE), entrance potential (EP), focusing potential (FP) and cell exit potential (CXP) set were −46, −30, −10, −283 and −13V in negative mode and 40, 25, 12, 230 and 13V in positive mode respectively. The molecules underwent fragmentation to yield the following fragment ions of m/z 160, 237 and 122 for TFM, PDN and PGZ respectively. The MS spectra of three analytes are presented in Figure 3.

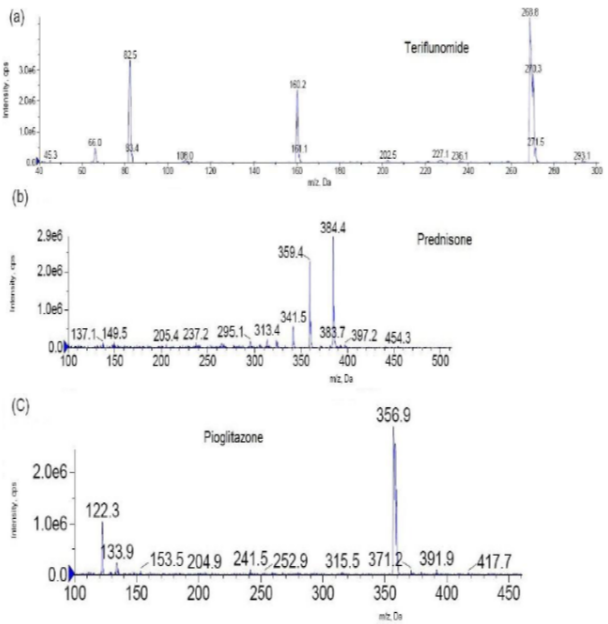

Fig. 3: Product ion mass spectra of (a) Teriflunomide (b) Prednisone (c)Pioglitazone

Based on their mass spectra and tandem mass spectra, the following MRM transitions: m/z 269→160, m/z 359→237, m/z 357→122 were selected for analysis of TFM, PDN, and PGZ respectively. The extracted MRM ion chromatograms of teriflunomide, prednisone, pioglitazone, ibuprofen and imipramine are represented in Figure 4.

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. PDN, PGZ and IMP (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

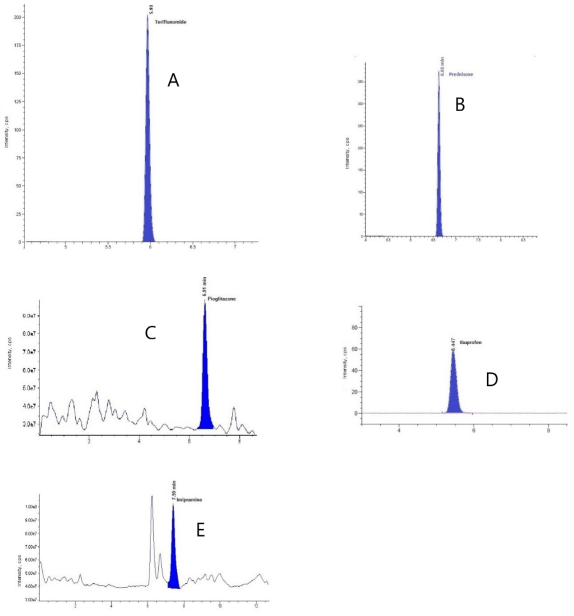

Fig. 4: 4. MRM-ion chromatograms of (A)Teriflunomide (TFM), (B) Prednisone (PDN), (C) Pioglitazone (PGZ), (D) Ibuprofen (IBU) and (E) Imipramine (IMP)

linearity over the studied concentration range of 1–500 ng/mL for TFM, PDN and PGZ with correlation coefficients (r^2) 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, PDN and PGZ were less than 8.2%. The obtained intra-day accuracies were in the range of 97.2–102.2% and inter-day accuracies were in the range of 97.3–101.8%. The validation parameters are depicted in Table 2.

The extraction recoveries of all drugs from rat plasma were in the range of 65.5–85.3% with relative standard deviations less than 8.1%, which indicates the sample preparation technique is suitable for extracting the studied drugs from rat plasma. The results are displayed in Table **??**.

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 ±5.6% of nominal concentrations after storage of plasma samples at room temperature for 24 h, four freeze–thaw cycles at -20°C. The mean accuracies were found to be more than 95% with relative standard deviations less than 4.3%, which are summarized in Table 4. Long-term stability of all the samples after Day-30 were found to be within 4.5% of actual concentrations.

Pharmacokinetic study results

All the samples were analyzed by the developed method and the mean plasma concentrations vs time profile of prednisone, pioglitazone and teriflunomide are shown in

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

Analyte	Nominal concentration (ng/mL)	Intra-day		Inter-day	
		% Recovery	% RSD	% Recovery	% RSD
PDN	1	100.6	6.811	100.8	1.692
	5	98.6	3.331	98.8	1.383
	100	99.5	3.272	99.3	0.579
	400	99.3	1.800	99.3	0.328
PGZ	1	97.3	8.123	101.8	5.111
	5	100.3	3.832	97.3	2.433
	100	102.2	6.263	100.5	1.611
	400	102.2	7.172	99.6	1.066
TFM	1	97.9	6.963	97.7	1.127
	5	99.6	4.621	100.0	1.147
	100	98.3	5.888	99.1	1.362
	400	97.2	4.718	97.7	0.926
	stability (24h RT)	eeze-thaw stability (4 cycles)		Longterm stability (Day-30)	

Table 3: Recovery Values of TFM, PDN and PGZ (n=6)

Analyte	Nominal concentration (ng/mL)	Mean Recovery	% RSD
PDN	5	66.0	9.868
	100	65.9	2.986
	400	80.8	4.687
PGZ	5	66.6	7.330
	100	65.2	3.162
	400	82.3	7.833
TFM	5	77.7	10.589
	100	80.9	6.153
	400	78.5	5.315

Table 4: Stability studies of TFM, PDN and PGZ in rat plasma at Low & High QC levels (n=6)

Analyte	Mean accuracy ± RSD		
	Short term stability (24h RT)	Freeze-thaw stability (4 cycles)	Freeze-thaw stability (4 cycles)
PDN	97.5 ± 2.6	99.3 ± 2.3	99.9 ± 1.9
	99.9 ± 3.2	98.4 ± 4.3	97.6 ± 3.8
PGZ	99.0 ± 3.2	100.6 ± 1.9	100.0 ± 2.0
	101.4 ± 5.6	99.4 ± 3.2	97.4 ± 4.5
TFM	101.7 ± 4.6	99.8 ± 4.7	99.6 ± 2.2
	98.1 ± 2.8	99.3 ± 3.1	98.3 ± 3.8

Figure 3.

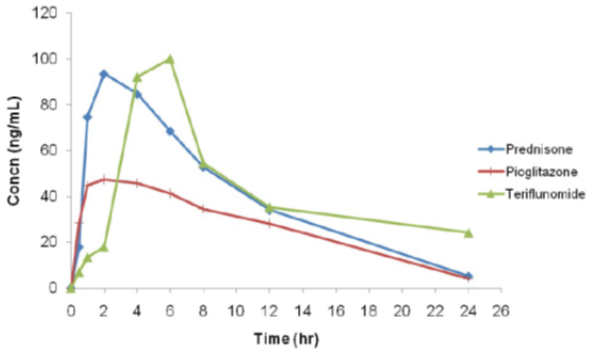

Fig. 5: Mean plasma concentration–time profiles of PDN, PGZ and TFM in rat plasma

The plasma concentration–time profile of all the three analytes was analyzed by the non–compartmental method using WinNonlin Version 5.1. The estimated pharmacokinetic parameters are shown in Table 5.

Table 5: Pharmacokinetic parameters of TFM, PDN and PGZ in rat plasma (n=6, Mean ± SD)

Parameter	TFM	PDN	PGZ
Cmax (ng/mL)	100.1 ± 13.6	93.6 ± 17.0	47.4 ± 9.7
Tmax (h)	5.0 ± 1.4	2.3 ± 1.5	1.5 ± 0.7
t1/2 (h)	11.7 ± 4.6	4.8 ± 4.7	4.6 ± 2.2
Kel (h-1)	0.059 ± 0.098	0.144 ± 0.081	0.114 ± 0.021

Cmax: maximum plasma concentration.
Tmax: time point of maximum plasma concentration.
$t_{1/2}$: half life of drug elimination during the terminal phase.
K_{el}: elimination rate constant

DISCUSSION

The present study was undertaken to develop a rapid and simple method to determine the prednisone which is in Phase-4 clinical trials for multiple sclerosis disease. Along with teriflunomide and pioglitazone this method has been developed and validated which can be highly useful in drug interaction studies with prednisone and also other possible random combinations. The current method is proved for its ruggedness with sufficient recovery and low matrix effect in a simple protein precipitation method with acetonitrile. This method may be applicable to other preclinical samples as well as clinical sample analysis of these three drugs given to the patients suffering with multiple sclerosis disease. In drug interaction studies, the concomitant drugs can be analyzed in a single run with current developed methods. Separation of ibuprofen, imipramine and teriflunomide is based on their affinity and interaction with the reverse phase C-18 column and the mobile phase. In the present study, based on sensitivity, matrix effect and reproducibility requirements extraction techniques of LLE (Liquid-liquid extraction) and SPE (Solid phase extraction) were tried during method development for the combinations associating teriflunomide. Thus, three analytes were distinctly resolved from one another by the present LC method. It should be noted that interfering peaks from endogenous compounds were not observed. Selectivity of the method in rat K_2EDTA plasma was evaluated in twelve individual matrix lots along with one lipemic and one hemolytic lot. Peak responses in blank lots were compared against the response of spiked LLOQ and negligible interference was observed at the retention time of analytes and internal standards. Calibration for the quantification was performed using the ratio of peak area of the analyte to that of IS. The results of intra- and inter- day accuracy and precision listed in Table 2 indicated that the method was accurate with excellent accuracy range of 97.2–102.2%, and the CV was within 10%. Matrix effect is a special phenomenon associated with LC–MS determination of drugs from biological fluids such as plasma and other matrices. Endogenous components extracted from plasma may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes. It is for this reason that matrix effect was evaluated under the experimental conditions used in this study. The average ion suppression or enhancement at low QC and high QC levels was < 11%, suggesting that matrix effect on the analysis was negligible. The stability results (Table 4) showed that the mixture of three analytes spiked into rat plasma was stable for 24 h at ambient temperature, for 30 days at 4°C, and four freeze–thaw cycles at -70°C. Stability of the analyte in the sample is of crucial importance to the validity of the split sample program. Thus, proper storage of all samples is very important to obtaining reliable results and their interpretation in analysis. Application of the current validated method has resulted positive results for pharmacokinetic study in rats which shall be highly useful information in clinical trials. Fig-5 shows the plasma-time concentration profiles of in vivo study conducted in albino rats for PDN, TFM, and PGZ. The extraction recovery of analytes from K_2EDTA plasma was determined by comparing the peak responses of plasma samples (n = 6) spiked before extraction with that of plasma samples spiked after extraction. The mean recovery of PDN, TFM and PGZ was found to be 70.9%, 79.0% and 71.4% respectively with %CV across the three levels ranging between 3.0 and 10.6%, as shown in Table 3. Dilution integrity experiment was carried out at 2 times the ULOQ concentration for all the three analytes. After 1/2 and 1/4 dilution the mean back calculated concentration for dilution QC samples was within 85–115% of nominal value with a %CV of ≤ 8.1.

CONCLUSIONS

We have developed and validated a highly sensitive, specific, reproducible and high throughput LC–MS/MS assay to quantify TFM, PDN and PGZ 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 three analytes. The obtained LODs and LOQs of all the drugs were adequate and proved successful 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. This method may be useful for clinical sample analysis of prednisone which is currently in Phase 4 trials of multiple sclerosis disease along with coadministered drugs.

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