Development and Validation of RP-HPLC Method with Diode Array Detection for Estimation of Metaxalone in Rat Plasma

Iffath Rizwana[1,2], Karanam Vanitha Prakash[3*] and Ganta Krishna Mohan[4]

[1]*Research Scholar, R & D, Jawaharlal Nehru Technological University Kakinada, Kakinada, A. P. India.*

[2]*Department of Pharmaceutical Analysis, Deccan School of Pharmacy, Hyderabad, A. P. India.*

[3]*Department of Pharmaceutical Analysis, SSJ College of Pharmacy, Gandipet, Hyderabad, A.P. India.*

[4]*Centre for Pharmaceutical Sciences, IST, JNTU Hyderabad, A.P. India.*

ABSTRACT

Purpose: To develop a simple, highly sensitive, precise and accurate high-performance liquid chromatographic method with photodiode array detection and validated for the rapid quantification of metaxalone in rat plasma samples. **Method:** Following Liquid-Liquid Extraction (LLE), metaxalone and the internal standard Phenytoin (PHY) were extracted from an aliquot of 200 mL of plasma. Chromatographic separation was carried out using Phenomenex Luna C_8 column (250 mmμ 4.6 mmμ 5 mm) with mobile phase composed of phosphate buffer, pH 7 and acetonitrile in 35:65, v/v ratio. The analyte was monitored with UV detector at 219 nm. The developed method was validated with respect to linearity, accuracy, precision, specificity and stability. The peak area ratio of MET to that of internal standard, PHY was used for the quantification of samples. **Results:** The retention time of MET and PHY were found to be 2.30 and 3.02 min respectively. The calibration curve was linear (r^2> or=0.99) ranging from 1.505-538.254 ng/ml and the lower limit of quantification was 1.505 ng/mL. Interday and Intraday precision were lower than 5% (CV) and accuracy ranged from 95 to 105% in terms of percent accuracy. Mean extraction recovery was found to be above 94%. **Conclusion:** A simple, alternative, reproducible and sensitive HPLC-DAD method was developed for MET that can be used in preclinical pharmacokinetics.

Key words: Ammonium formate buffer, Acetonitrile, Metaxalone, Phenytoin, Preclinical pharmacokinetics.

INTRODUCTION

Metaxalone (Figure 1) has the molecular formula $C_{12}H_{15}NO_3$ and chemical name 5-[(3, 5-dimethylphenoxy) methyl]-2-oxazolidinone) with a molecular mass of 221.26 g/mol and absorption maxima around 345 nm. Metaxalone belong to the BCS class II of centrally acting skeletal muscle relaxant drug with antispasmodic effect.[1] Metaxalone belongs to non-benzodiazepine antispasmodics with a structure similar to mephenaxalone nucleus.[2] Metaxalone (skelaxin) got FDA approval in 1962 by King Pharmaceuticals mainly for the treatment of acute, painful and musculoskeletal conditions like fractures, dislocations, and trauma to tendons and ligaments and other measures for the relief of discomforts.[3] The mode of action of the metaxalone is clearly unknown but hypothesized as CNS depressant drug which causes skeletal muscle relaxation and sedation.[4] It acts through inhibiting interneuronal activity and blocking polysynaptic reflex pathways at spinal cord and at descending reticular formation in brain but leaving monosynaptic pathways intact like other similar class of skeletal muscle relaxants.[5,6]

***Corresponding address:**
Dr. Karanam Vanitha Prakash
Department of Pharmaceutical Analysis
Professor and Principal,
SSJ College of Pharmacy, Gandipet,
Ranga Hyderabad, A.P. India.
E-mail: vanithaprakashssj@gmail.com

DOI: 10.5530/phm.2014.2.4

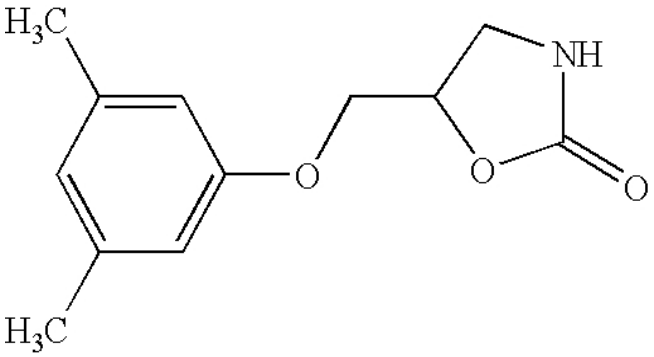

Figure 1: Chemical structure of Metaxalone

The pharmacokinetic data for metaxalone are interesting. As compared to fasted conditions, the presence of a high fat meal at the time of drug administration increased metaxalone C_{max} by 177.5% and AUC (AUC0–t, AUC0–∞) by 123.5% and 115.4% respectively. Time-to-peak concentration (T_{max}) was also delayed (4.3 h versus 3.3 h) and terminal half-life was decreased (2.4 h versus 9.0 h) under fed conditions compared to fasted condition.[7] The drug is not recommended for patients with significant renal, hepatic disease and drug induced anaemias.[8]

Literature survey has revealed that there only few methods were reported for the determination of metaxalone in plasma by liquid chromatography. Methods reported in the literature for the estimation of metaxalone in bulk and biological fluids include soft ionization interfaces like electrospray ionization (ESI) for determining metaxalone (LC-MS/MS),[10] ultraviolet spectroscopy with LC Chromatography method (HPLC-UV)[9], gas chromatography with flame ionization detection,[10,11] gas chromatography with mass detection.[12,13] The quantification of drugs, metabolites and poisonous chemicals from biological fluids using mass spectrometry has gained importance in forensic toxicology, clinical and pharmaceutical sector for wide range of application.[14]

From the literature survey, reported methods are mainly designed for human biological samples typically above 0.2 mL of human plasma reveals the usage of high quantity of sample in terms of volume, high solvent consumption and tedious sample processing includes control of the factors like pH, extraction solvent, evaporating temperature which is highly time consuming and laborious analysis. Consequently, in rodents very little plasma volume would be available from each animal for processing. Under the scope of this view, aim of our research work is to develop a highly specific, reliable and sensitive method for metaxalone determination in rat plasma that proves to be of immense importance for conducting preclinical studies efficiently in terms of less sample volume, short run time, less tedious processing and sensitive analysis. Hence, a highly sensitive isocratic HPLC-DAD method was developed and validated according to the international guidelines for quantifying metaxalone in rat plasma at a concentration range (0.98 ng/mL). Furthermore, a pharmacokinetics study of metaxalone in rat model was conducted, in order to validate the method in study samples.

EXPERIMENTAL

Reagent and materials

Metaxalone sample was obtained from Orchid Pharma, Chennai and Internal standard (IS), Phenytoin, was obtained from Cipla Ltd, Mumbai. Analytical grade reagent such as ammonium formate was purchased from Merck specialties private limited, Mumbai. HPLC grade reagents such as methanol and acetonitrile were purchased from Merck specialties private limited, Mumbai. Millipore water (0.22 mm membrane filtered) was produced in the laboratory by Millipore system (Model: Direct-Q^L3 water purification system) Millipore Corporation, Billerica, MA, U.S.A.

Chromatographic instrumentation

The analysis was carried out on a Shimadzu LCe2010CHT (Shimadzu Corporation, Kyoto, Japan) equipped with low pressure quaternary gradient pump along with dual wavelength UV detector, column oven, auto sampler and LC solution 1.24SP1 software. Phenomenex Luna C 8 column (250 mm x 4.6 mm x 5 mm) was used for drug separation. The analyte was monitored with UV detector at 219 nm. A glass vacuum-filtration apparatus (fitted with 0.22 m filter) were used to filter mobile phase. Ultrasonic bath was used to remove dissolved gases and entrapped air in mobile phase. A model Genie-2 Spinix vortex mixer, a cold centrifugation (Sigma, Germany) were employed during sample processing and TurboVap LV Evaporator (Caliper Life Sciences, Hopkinton, MA, USA) for evaporation purpose after extraction.

Preparation of standard solutions

All standard stock solution and working standard solution were prepared in amber colored volumetric flask. An accurately weighted sample of Metaxalone was dissolved in methanol to give standard stock solution of 100 mg mL^{-1}. Phenytoin was used as an Internal Standard (IS). The working standard solutions were prepared by further serial dilution from the stock solutions with mobile phase mixture. Calibration standards (20, 50, 100, 200, 500,

1000, 2000 ng mL^{-1}) in control rabbit plasma samples were prepared by spiking with 20 mL of working stock solutions of MKS. Three Quality Control (QC) solutions containing low (75 ng mL^{-1}; LQC), medium (750 ng mL^{-1}; MQC) and high (1750 ng mL^{-1}; HQC) were prepared in a similar way. The IS working solutions were prepared providing finally a plasma concentration of 5000 ng mL^{-1}.

Sample preparation

200 mL aliquot of a rat plasma sample was spiked with 10 mL internal standard solution. Subsequently, sample was extracted with 1.5 ml of tertiary-butyl methyl ether (TBME). The mixture was vortexed for 15 min and centrifuged at 10,000 rpm for 10 min. After centrifugation, the organic phase was transferred to glass tubes and evaporated to dryness using TurboVap LV Evaporator (Caliper Life Sciences, Hopkinton, MA, USA) at 50°C under stream of nitrogen for 5 min. The residue remaining after drying was reconstituted with 150 mL of mobile phase mixture and vortexed for 1 min then a volume of 50 mL was injected into the HPLC system.

Validation of method

Validation of the developed method was carried out as per US FDA guidelines for accuracy, precision, linearity, selectivity, sensitivity, reproducibility, and stability.[15]

Selectivity

The selectivity of method was proved by processing and analyzing blanks prepared from six independent lots of control plasma along with six extracted LOQ-QC samples. The method is selective if there is no interfering peak present at the retention time of the drug or IS. If there is any interfering peak present at the retention time of drug then its response should be less than 20% of mean response of six extracted LOQ-QC samples.

Linearity (calibration curve)

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. The linearity of developed method was evaluated with a total of three calibration curves over the concentration range 20e 2000 ng mL^{-1} by plotting the peak response (area) ratio of MET versus concentration of MET in plasma.

Detection and quantification limit

The limit of detection (LOD) is defined as the lowest quantity of substance which gives a peak area of three times the baseline noise. The equation 2H/h was used to calculate the ratio of signal size to that of noise. Where, H is the height of the peak in a chromatogram obtained with the prescribed reference solution and h is the noise in blank chromatogram. The limit of quantification (LLOQ) was defined as the lowest concentration with ratio of signal-to-noise more than 5 with accuracy of 80 to120% and precision of 20% to its nominal value.

Accuracy and precision

The different concentrations including lower and upper limits of each QC sample (LOQ-QC, LQC, MQC and HQC) in six replicates were analyzed on the same day and on three different days in order to determine the intra-day inter-day accuracy and precision. The accuracy (%bias) was calculated as follows

Accuracy (%bias) = (Concentration found/Nominal concentration) X 100

The percent coefficient of variation, %CV was calculated as follows

%CV = (Standard deviation/Mean) X _ 100

The accuracy determined at each concentration level must be within 15% except at LOQ-QC where it must not exceed 20% of the respective nominal value. The precision around the mean value must not exceed 15% except for LOQ-QC where it must be within 20% of the %CV.

Recovery

Recovery is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of standard. It is accessed by comparing the mean peak areas of extracted LQC, MQC and HQC samples to the one obtained after the direct injection of a solution with corresponding concentration (unextracted).

Recovery = (Mean peak area response of extracted samples at LQC; MQC; HQC)/ (Mean peak area response of un extracted samples at LQC; MQC; HQC).

Stability studies

Stock solution stability

The drug stability is a function of the storage conditions and the chemical properties of the drug. Stock solution stability was performed at room temperature for 8.0 h and at 2 to 8°C

for 30 days. Stock solution stability was assessed by comparing freshly prepared samples of MET and IS with that of stability samples at MQC level by performing five injections of each. Mean percentage change was calculated for both MET and IS. Stock solution of MET and IS is deemed stable if mean percentage change of IS and MKS was within _10%.

Bench top stability

Six replicates of LQC and HQC in biological matrix were withdrawn and thawed unassisted at room temperature and kept unprocessed for 8 h (stability samples). After 8 h fresh calibration was prepared with one set of low and high QC samples (comparison samples).

Freeze thaw stability

Freeze thaw stability in plasma was assessed by analyzing six replicates of LQC and HQC samples after three freeze and thaw cycles. Samples were kept at _70°C and frozen for 24 h and thawed unassisted at room temperature. The freeze-thaw cycle was repeated two more times; samples were then analyzed after the third cycle.

Long term stability

Six replicates of LQC and HQC in biological matrix were withdrawn from deep freezer (_70°C) after 30 days and thawed at room temperature (stability samples). Fresh calibration was prepared with six replicates of low and high QC samples (comparison samples). MET was deemed stable in matrix if mean percentage change in concentration was within _15%.

Mean percentage change = [(Calculated concentration of stability samples/Calculated concentration of comparison samples)-1] X100

Application to preclinical pharmacokinetics

To assess the applicability of the method, it was used to evaluate the pharmacokinetic of MET in rats. The study was conducted after approval by the Institutional Animal Ethics Committee (IAEC), Deccan Medical College (IAEC/DMC/14/2012-2013). Albino wister rats weighing 150-200 gm were housed with free access to food and water. Rabbits in group (n=3) were dosed orally by gavages with developed tablet formulation (800 mg/tablet). After a single oral administration; 0.6 ml of blood samples were collected from the retro orbital at control and 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 18 and 24 h after the administration into tubes containing EDTA. The plasma was separated immediately using cold centrifugation (Sigma, Germany) at 10,000 rpm for 10 min and stored at -70°C until analysis.

RESULT AND DISCUSSION

Optimization of LC–MS parameters

Phenomenex Luna 5 µ, C_8,100×4.6 mm was selected after trying other different columns since it provided good peak shape and high intensity with greater signal to noise ratio (S/N) when Phosphate buffer pH 7 was used in the combination of acetonitrile was used as organic component in mobile phase. Analyte and IS responses in presence of ammonium formate were optimum and stable at pH 7 (adjusted with sodium hydroxide). The mobile phase consisting of phosphate buffer pH 7 (adjusted with sodium hydroxide) and Acetonitrile (35:65% V/V) was used to complete each run within 4.0 min.

Protein precipitation technique was extensively investigated for extraction of MET from biological matrix in previously published reports. It provided with comparatively less sensitive detector with limited amount of processing volume (which is usually the case in preclinical studies), protein precipitation technique despite of having good recovery faces the problem of sample dilution during extraction, which affects the sensitivity of method adversely. For this reason liquid-liquid extraction was investigated as a sample extraction technique. Due to high logP (9.890) value of MET, various non-polar extraction solvents like diethyl ether, n-hexane, tertiary-butyl methyl ether (TBME) and chloroform were tried. Amongst all, TBME showed good

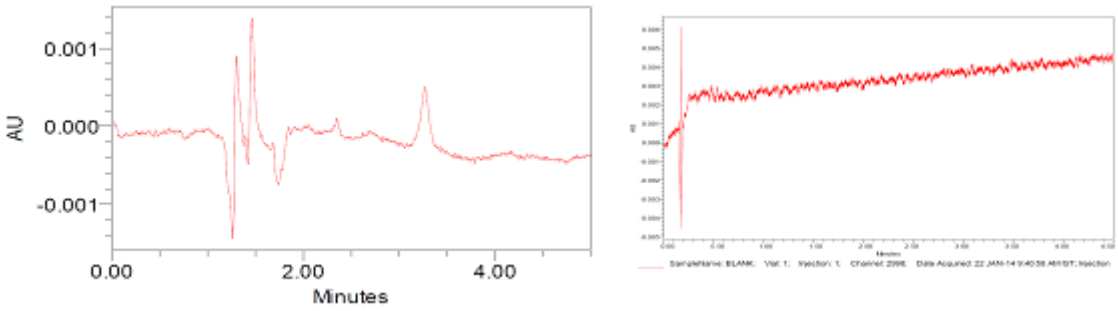

Figure 2: Representative chromatogram of drug (MET) and IS (PHY) free blank rat plasma

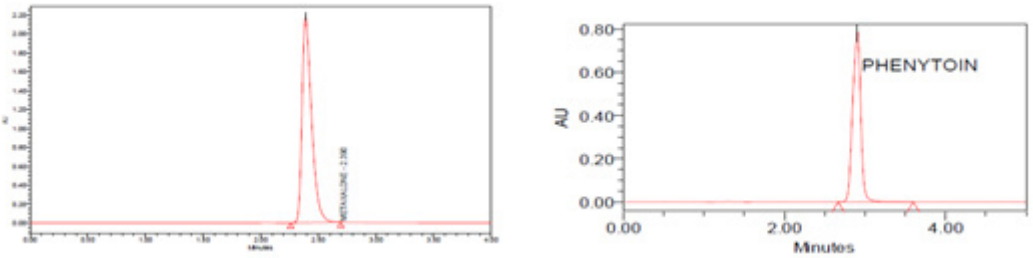

Figure 3: Representative chromatogram of spiked rat plasma at LQC of concentration 4.492 ng/mL.

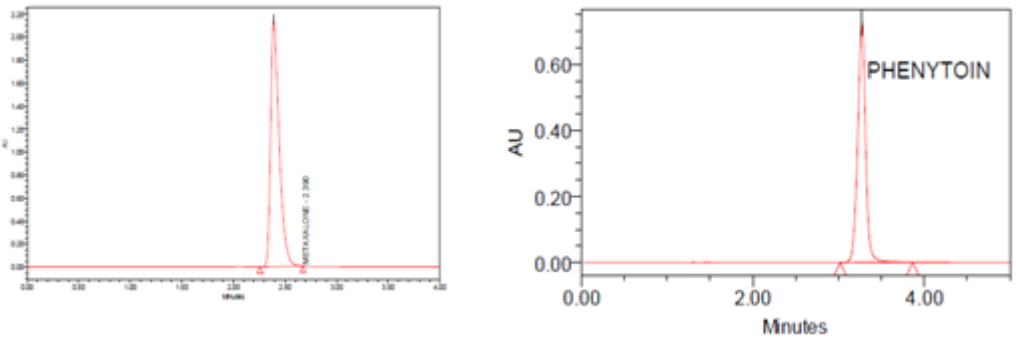

Figure 4: Representative chromatogram of spiked rat plasma at HQC of concentration 417.819 ng/mL

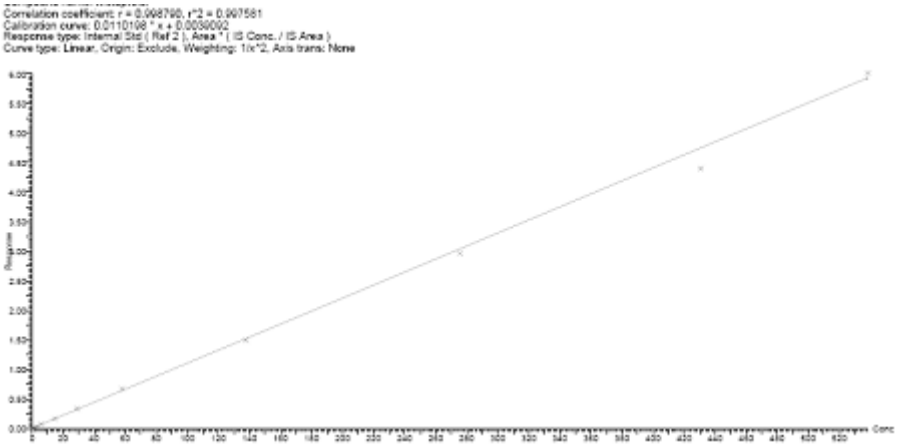

Figure 5: Linearity graph of MET

recovery and no interference was observed either at drug or IS retention time. 200 ml processing volume of rat plasma was used which finally concentrated by reconstituting to 100 ml volume increasing drug's response.

Method validation

Selectivity

The developed method was found selective for both MET and IS, as no interference was detected at the respective retention times. The representative chromatograms of blank extracted rat plasma and spiked rat plasma at LQC and HQC concentration are shown in the Figure 2, 3, and 4 respectively.

Linearity (calibration curve)

The peak area ratio of standard (MET) to that of internal standard was used for the quantification of MET in plasma samples. Calibration curves were linear in the concentration range of 1.505-538.254 ng mL^{-1} with correlation coefficient (r^2) of 0.9981. The mean regression equation was: y=0.0110198x + 0.0039092, Where y is peak area ratio and x is the plasma concentration of MET. The linearity graph was shown in Figure 5.

Detection and quantification limit

In alignment with the criteria for clinical and preclinical bioequivalence studies, this demand LLOQ of bioassay to

Table 1: Inter-day accuracy and precision data for Metaxalone at five concentration level (n=6)

QC ID	HQC	MQC1	MQC2	LQC	LLOQ QC
Nominal Concentration (ng/mL)	417.819	233.978	112.310	4.492	1.572
	355.146-480.492	198.881-269.075	95.464-129.157	3.818-5.166	1.258-1.886
P&A ID		Day 1: Calculated Concentration (ng/mL)			
P&A I	397.460	237.428	104.937	4.380	1.632
	394.112	228.384	116.452	4.643	1.452
	343.854	216.232	111.877	4.643	1.451
	399.045	232.060	110.733	4.507	1.556
	387.911	227.668	114.956	4.360	1.592
	393.530	233.282	117.146	4.654	1.415
Mean	385.9853	229.1757	112.6835	4.5312	1.5163
SD	20.9950	7.2625	4.5551	0.1363	0.0887
%CV	5.44	3.17	4.04	3.01	5.85
%Mean Accuracy	92.38	97.95	100.33	100.87	96.46
N	6	6	6	6	6
P&A II		Day 2: Calculated Concentration (ng/mL)			
	358.122	213.875	114.804	4.643	1.589
	380.730	223.638	111.822	4.390	1.490
	334.387	195.705	98.656	3.832	1.404
	336.247	204.902	98.335	4.315	1.453
	362.590	209.083	110.367	4.182	1.535
	363.169	216.604	103.261	4.138	1.444
Mean	355.8742	210.6345	106.2075	4.2500	1.4858
SD	17.7094	9.7302	7.0768	0.2721	0.0672
%CV	4.98	4.62	6.66	6.40	4.52
%Mean Accuracy	85.17	90.02	94.57	94.61	94.52
N	6	6	6	6	6
		Day 3: Calculated Concentration (ng/mL)			
	388.429	217.064	112.127	4.600	1.300
	378.886	220.547	112.314	4.241	1.394
P&A III	382.086	227.048	112.336	4.320	1.326
	378.252	222.227	110.336	4.248	1.541
	390.265	229.275	110.157	4.360	1.421
	394.997	233.301	115.538	4.599	1.184
Mean	385.4858	224.9103	112.1347	4.3947	1.3610
SD	6.7736	6.0304	1.9392	0.1648	0.1211
%CV	1.76	2.68	1.73	3.75	8.90
%Mean Accuracy	92.26	96.12	99.84	97.83	86.58
N	6	6	6	6	6
		Day 4: Calculated Concentration (ng/mL)			
	397.334	224.904	108.747	4.499	1.592
	378.861	232.063	110.035	4.241	1.552
P&A IV	389.779	226.804	96.948	4.293	1.371
	381.369	225.106	112.195	4.574	1.414
	385.163	221.856	106.040	4.565	1.594
	394.466	218.449	109.480	4.585	1.446
Mean	387.8287	224.8637	107.2408	4.4595	1.4948
SD	7.3138	4.6011	5.4225	0.1530	0.0967
%CV	1.89	2.05	5.06	3.43	6.47
%Mean Accuracy	92.82	96.10	95.49	99.28	95.09
N	6	6	6	6	6
Between Batch Precision and Accuracy					
Mean	378.7935	222.3960	109.5666	4.4088	1.4645
SD	19.2123	9.7914	5.5872	0.2057	0.1085
%CV	5.07	4.40	5.10	4.67	7.41
%Mean	90.66	95.05	97.56	98.15	93.16
N	24	24	24	24	24

Table 2: Recovery results of MET at four concentration level (n=6)

Replicate No.	HQC		MQC1		MQC2		LQC	
	Aqueous Response	Extracted Response	Aqueous Response	Extracted Response	Aqueous Response	Extracted Response	Aqueous Response	Extracted Response
1	153154	105918	85932	63990	49343	34780	2039	1615
2	153545	106782	91014	64022	50893	35003	2164	1484
3	136415	108238	92305	65090	52332	34086	2252	1486
4	162929	106021	95595	64745	54490	32098	2318	1470
5	169275	107696	98500	65467	57669	32771	1943	1515
6	142295	109434	91391	66501	51126	34199	1894	1608
Mean	152935.5	107348.2	92456.2	64969.2	52642.2	33822.8	2101.7	1529.7
SD	12275.3	1370.5	4293.8	950.3	3000.8	1149.0	170.6	65.1
% CV	8.03	1.28	4.64	1.46	5.70	3.40	8.12	4.26
% Mean Recovery	70.19		70.27		64.25		72.78	
Overall % Mean Recovery	69.37							
Overall SD	3.62							
Overall % CV	5.22							

be at least 10% of the C_{max} or five $T_{1/2}$ of the drug under consideration, whichever is smallest, the LLOQ of present method was found out to be 1.505 ng/mL for the MET. The LOD considering the signal-to noise ratio of 3:1, was estimated to be 417.819.

Accuracy and precision

The intra-day and inter-day accuracy and precision values of the MET in rat plasma shown in Table 1, are well within the acceptable limits. The intra-day and inter-day precision (%CV) values for MET were below 6.06% and 8.43%. Intra-day and inter-day accuracies were within 95.81% and 110.90%, respectively, as acceptable per guidelines.

Recovery

The extraction recovery of MET at LQC, MQC and HQC is 72.78%, 70.27%, 70.19% respectively. Results of recovery studies was shown in Table 2. The extraction recovery was found to be efficient and consistent.

Stability studies

The stock solution of MET was stable at least for 8 h at room temperature and 30 days when stored at 4°C, since %change was found to be less than 10%. Bench top stability of MET in plasma was investigated at the concentrations of 75 and 1750 ng/ml and the results revealed that the MET in plasma was stable for at least 8 h at room temperature. The repeated freezing and thawing (three cycles) of plasma samples spiked with MET at two levels 75 and 1750 ng/ml, showed mean percentage change of-4.94 and-2.73% respectively. Long term stability of the MET in plasma at -70°C was also performed after 30 days of storage at two (75 and 1750 ng/ml) levels, which showed mean percentage change of -5.23 and -3.47%, respectively. The results of the stability studies indicated that the MET was stable in the studied conditions.

CONCLUSION

A simple, alternative, reproducible and sensitive HPLC-DAD method was developed for MET in rat plasma. The method was validated over concentration range 1.505-538.254 ng/ml (r^2=0.997) and was found to offer good accuracy and precision for monitoring the full pharmacokinetic profile of MET in individual rodent, like rats. The exclusive advantage of the method is the small processing volume used for extraction without jeopardizing the sensitivity. This method can be used to estimate the concentration of MET in rat and human plasma after performing partial method validation.

REFERENCES

1. See S, Giuzburg R. Choosing a skeletal muscle relaxant. Am Fam Physician. 2008; 78(3): 365-70.
2. Beebe FA, Barkin RL, Barkin SA. A clinical and pharmacologic review of skeletal muscle relaxants for musculoskeletal conditions. Am J Ther. 2005; 12(2): 151-71.
3. Robert AS, Menelaos K, Rahul R, Myint M. Pharmacological options for low back pain. Semin Pain Med. 2004; 2(3): 175-85.
4. Elenbass JK. Centrally acting oral skeletal muscle relaxants. Am J Hosp Pharm. 1980; 37(10): 1313-23.
5. Karla AM, Barry L, David F. A fatality involving metaxalone. Forensic Science International 2005; 149(1): 249-51.
6. Bruce RB, Turnbull L, Newman J, Pitts J. Metabolism of Metaxalone. J Med Chem. 1966; 9(3): 286-8.
7. Dipanjan Goswami, Arabinda Saha, Sanjay Gurule, Arshad Khuroo, Tausif Monif, Poonam Vats. Metaxalone estimation in biological matrix using high-throughput LC–MS/MS bioanalytical method. Journal of Chromatography B. 2012; 902: 132-6.
8. Gruszecki AC, Kloda S, Simmons GT, Daly TM, Hardy RW, *et al*. Polydrug fatality involving metaxalone. J Forensic Sci. 2003; 48(2): 432-4.
9. Ramakrishna VSN, Vishwottam NK, Manoj S, Koteshwara M, Wishu S, *et al* . Quantification of Metaxalone in Human Plasma by Liquid Chromatography Coupled to Tandem Mass Spectrometry. J Anal Toxicol. 2006; 30(4): 245-51 .
10. Janice C, Reilly EE, Anton A. Comparison of the dissolution of metaxalone tablets (skelaxin) using USP apparatus 2 and 3. AAPS Pharm Sci Tech. 2004; 5(1): E6.
11. Jemal M. High-throughput quantitative bioanalysis by LC–MS/MS. Biomed Chromatogr. 2000; 14(6): 422-9.
12. Nowatzke W, Woolf E. Best practices during bioanalytical method validation for the characterization of assay reagents and the evaluation of analyte stability in assay standards, quality controls, and study samples . AAPSJ. 2007; 9(2): E117-22.
13. Bansal S, DeStefano A. Key elements of bioanalytical method validation for small molecules. AAPS J. 2007; 9(1): E109-14.
14. Causon R. Validation of chromatographic methods in biomedical analysis viewpoint and discussion . J Chromatogr B Biomed Sci Appl. 1997; 689(1): 175-80.
15. FDAUS. Guidance for Industry. Bioanalytical Method Validation. Rockville, MD, USA: US Department of Health and Human Services. US FDA/Center for Drug Evaluation and Research; 2001.