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Bioanalytical Method Development and Validation of Metaxalone in Human Plasma by LC-MS/MS

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Subject: Analytical Chemistry

Abstract

A simple, rapid, sensitive, and selective liquid chromatography-tandem mass spectrometry (MS) method was developed and validated for the quantification of Metaxalone, a skeletal muscle relaxant, in human plasma using Metaxalone –D6 as Internal Standard (IS). Following Liquid-Liquid Extraction (LLE) , the analytes were separated using an isocratic mobile phase on a reverse phase C18 column (Chromatopak peerless basic 50×4.6mm×3.0µm) and analyzed by MS in the multiple reaction monitoring mode using the respective [M+H]+ ions, m/z 222.14 / 160.98 for Metaxalone and m/z 228.25 /167.02 for the IS. The assay exhibited a linear dynamic range of 25.19 -2521.313 ng/mL for metaxalone in human plasma. The goodness of fit was consistently greater than 0.98 during the course of validation. The range of accuracy and precision of the back-calculated concentrations of the standard curve points was from 94.1 % to 104.4 % and 0.3 % to 5.6 % for metaxalone A run time of 2.0 min for each sample and injection volume is 5 µl . The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability, or bioequivalence studies.

Keywords: *Metaxalone, Human Plasma, LC-MS-MS, Method Development, Validation.*

Introduction

Metaxalone (Skelaxin), see Figure 1, is a centrally acting skeletal muscle relaxant [1,2] .It was approved by the Food and Drug Administration (FDA) in 1964 as an adjuvant therapy to rest, physical therapy, and other measures for the relief of discomforts associated with acute, painful musculoskeletal conditions. Metaxalone has no direct relaxant effect on the "tense" skeletal muscles in humans or on the contractile mechanism of striated muscle, the motor endplate or the nerve fiber [3, 4].Although its exact mechanism of action has not been identified, it may be a general depression of the central nervous system (sedation)[3] or modification of signals conducted through polysynaptic fibers controlling passive stretch [5]. The recommended dosage for adults and children over 12 years of age is two 400-mg tablets or one 800-mg tablet three or four times a day [3].

In a clinical study, mean peak plasma concentrations (Cmax) of 865.6 ~g/L were achieved within 3.3 ± 1.2 h after dosing (Tmax)a single 400-mg metaxalone tablet under fasted condi- tions. Metaxalone concentrations declined with a mean ter- minal half-life (t1/2)of 9.2 ± 4.8 h. In the same study, following a standardized high fat meal, food statistically increased the rate (Cmax) and extent of absorption (AUC_{0-t},$AUC_{O-infinite}$) of metaxalone from tablets. Relative to the fasted treatment, the observed in- creases were 177.5%, 123.5%, and 115.4%, respectively. The mean Tmax was also increased to 4.3 ± 2.3 h, whereas the mean $t_{1/2}$ was decreased to 2.4 ± 1.2 h [3] .This decrease in half-life over that seen in the fasted subjects is felt to be because of the more complete absorption of metaxalone in the presence of a meal, resulting in a better estimate of half-life. Although a high Cmax and AUC were observed after the administration of metaxalone with a standardized high fat meal, the clinical relevance of these effects is unknown. The bioanalytical component of the pharmacokinetic study requires a drug assay with simplicity, selectivity, sensitivity, small volume requirements, and rapid turnaround time. Only a few methods have been reported for the quantification of metaxalone in biological matrices, which involves gas-liquid chromatography with flame-ionization detection (6,7) or gas chromatography-mass spectrometry (MS) [7,8]. These methods are time-consuming, and the sensitivity is not adequate for pharmacokinetic (ADME) studies. The advent of the atmospheric pressure ionization source was a breakthrough that allowed the efficient coupling of liquid chromatography (LC) and MS. The usefulness of LC-

electrospray ionization (ESI)-MS has been demonstrated for a wide range of applications in the bioanalytical, environmental, and pharmaceutical fields [9-11]. This powerful separation and detection technique is widely used for the quantification of drugs in biological fluids. The purpose of this work was to explore the high selectivity and sensitivity of a triple-quadrupole MS system operated in MS-MS mode with an ESI interface for the development and validation of a robust reversed-phase LC-MS-MS method in multiple reaction monitoring (MRM) mode for the quantification of metaxalone in human plasma. It was essential to establish an assay capable of quantifying metaxalone at concentrations down to 25 ng/mL. At the same time, it was expected that this method would be efficient in analyzing large numbers of plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies after therapeutic doses of metaxalone.

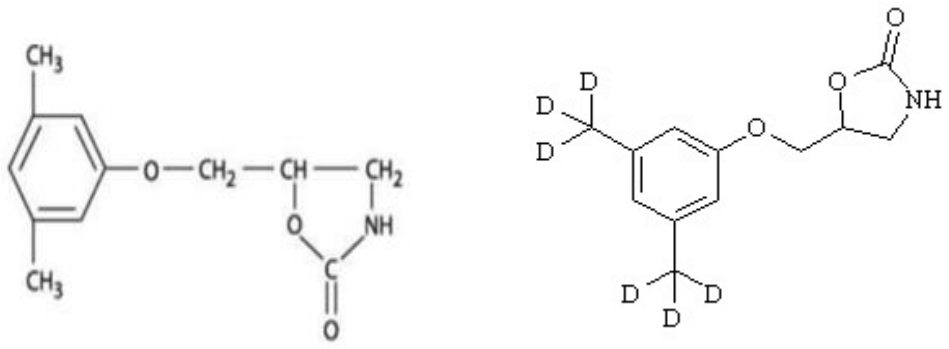

Fig.1: Chemical structures for Metaxalon & Metaxalone D6 (Internal Standard)

Experimental

Table 1:-Working Standard (WS) and Reference Standard (RS)

Sr No.	Name of Standard	Batch no.	Assay	Name of manufacturer
1.	Working Standard Metaxalone	VL/S-MT-011/c	98.84 %	VIVAN Life Sciences Pvt.Limited
2.	Metaxalone D6	VL/D-MX-230/b	98.91 %	VIVAN Life Sciences Pvt.Limited

Table 2:- Chemicals and Reagents

Sr.No	Name of chemical/Manufacturer Name	Grade
1.	Metaxalone /Metaxalone D6	Working/Reference Standard
2.	Methanol/ (J.T.Baker)	HPLC
3.	Ammonium Acetate / (Merck)	GR
4.	Methyl-tert Butyl ether-TBME/ (J.T.Baker)	HPLC
5.	Water (Rankem/Milli-Q)	HPLC
6.	Human Plasma	K_3EDTA
7.	Human Whole blood	K_3EDTA

LC-MS/MS Instrument and Conditions

LC-MS/MS Instrumentation: UPLC-MS/MS system was from Waters Acquity (USA) and consisted of a triple quadrupole Quattro premier and Quattro Premier XE mass spectrometer detector from Waters corp. (USA). Mass lynx version 4.1 software was used for data acquisition and processing.

Chromatographic Conditions: The chromatographic separation was performed on a Peerless Basic C_{18} 3µm [Chromatopack] (50×4.6 mm, Thermo Scientific, USA) at ambient temperature using an Acquity UPLC system (waters corp, Milford, MA, USA). The column temperature was maintained at 45^{o}C. The mobile phase consisted of composition of [Methanol 650 ml, Acetonitrile 150 ml and 10 mM Ammonium acetate buffer] at a flow rate of 0.700 ml/minute. The injection volume was 30 µl, and the analysis time was 2.0 min per sample.

Sample Processing

Extraction Procedure of Plasma Sample:

Take out the required number of CC/QC samples along with the subject samples from the deep freezer and allow them to thaw at room temperature. Vortex the thawed samples to ensure complete mixing of contents. Transfer the required quantit y of CC/QC samples (0.100ml) along with the subject samples and add 50µl ISTD [˜1300ng/ml] and vortex to mix it. Add 2.5ml of Methyl-Tert Butyl ether and vortex for 10 minutes. Centrifuge samples for 5 minutes at 10°C at 2000 rpm to separate the two layers. Take out organic layer in to Evaporation Test Tube. Evaporate to dryness At 40°C. Reconstitute the dried samples with 200 µl of Mobile Phase and vortex for30 seconds. Transfer into pre labeled auto sampler vials; inject 5µl of sample by using UPLC-MS/MS.

Preparation of Aqueous Sample Processing:
Take 1480µl of Mobile Phase + 500µl of internal standard + 20µl of respective Spiking solution, vortex it for proper mixing

Table 3: Mass Tune Parameter

Sr.No	Parameters	Drug (Metaxalone)	ISTD (Metaxalone D6)
1.	Capillary (kV)	3.00	3.00
2.	Source Temperature	100°C	100°C
3.	Desolvation Temperature	250°C	250°C
4.	Cone (V)	25	25
5.	LM Resolution 1	16.5	16.5
6.	HM Resolution 1	16.5	16.5
7.	Collision Energy (eV)	15	15
8.	Entrance	2	2
9.	Exit	2	2
10.	LM Resolution 2	15.0	15.0
11.	HM Resolution 2	15.0	15.0
12.	Detection	~ 224.14>160.98	~228.25>167.02
13.	Dwell time	0.200 sec	0.200 sec
14.	Desolvation Gas Flow	750	750
15.	Cone Gas Flow	20	20
16.	Ion Energy 1	0.5	0.5
17.	Ion Energy 2	1.5	1.5

Scanning profile of Drug and IS

Table 4:- MRM Parameters

Function type	MRM of 2 channels			
Chan Reaction	**Dwell (Secs)**	**Cone Volt**	**Col.Energy**	**Energy Delay (Secs)**
1:222.14>160.98`	0.200	25.0	15.0	NA
228.25>167.02	0.200	25.0	15.0	NA

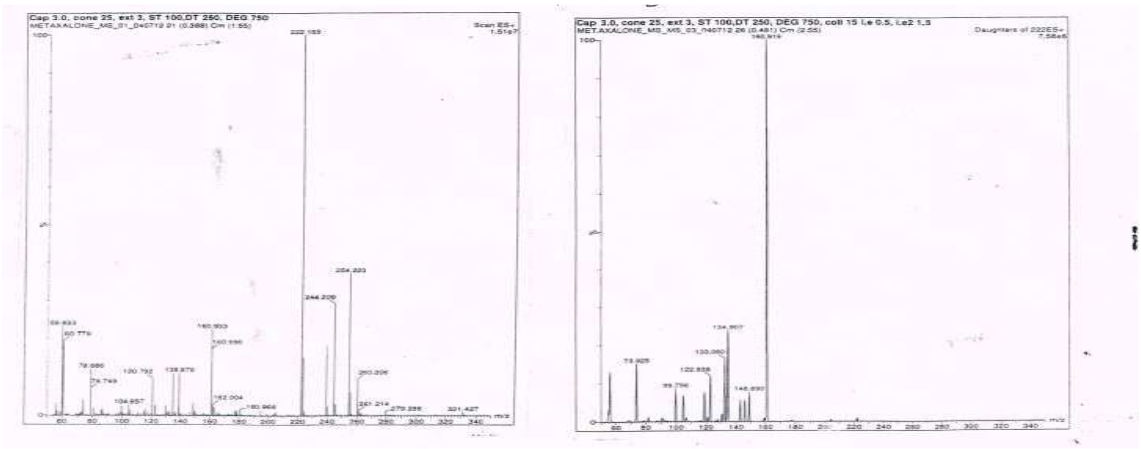

Figure 2:- Metaxalone Parent and Daughter Ion Scanning

Conclusion: - According to this time parameters and parents daughter mass, we generate MRM file as under

Table 5: MRM File of Metaxalone Parent and Daughter Ion Scanning

Parents (m/z)	Daughter (m/z)	Dwell (secs)	Cone volt	Cell energy
222.14	160.98	0.200	25.0	15.0

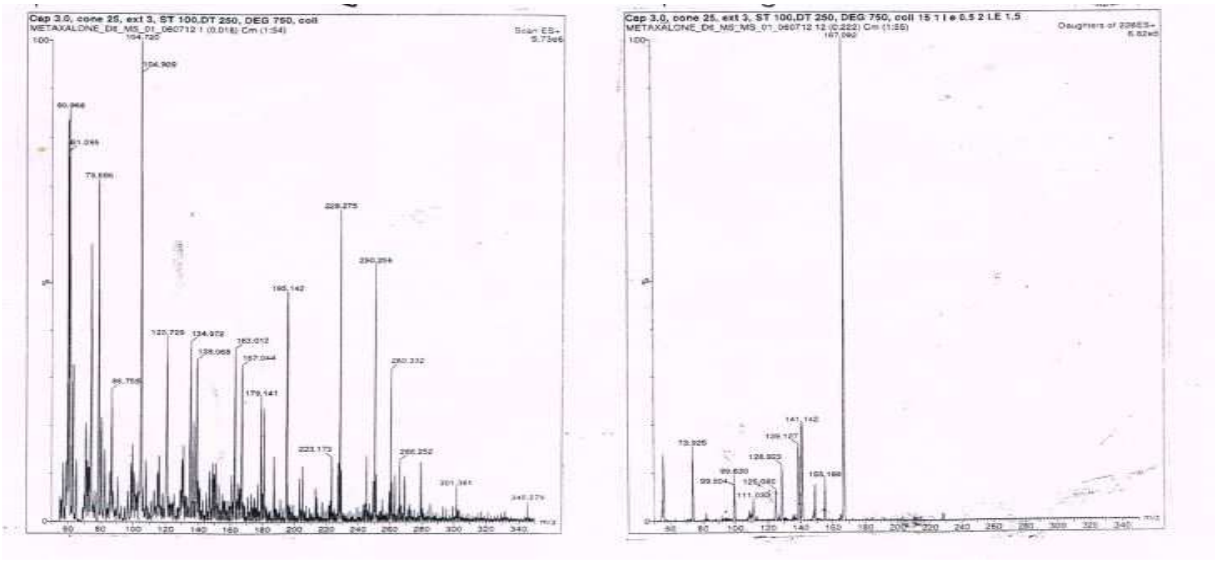

Figure 3:- Metaxalone D6 Parent and Daughter Ion Scanning

Conclusion: we have added trance of ISTD which we found from scanning and Generated MRM file which content following parameters of parent & daughters

Table 6: MRM File of Metaxalone D6 Parent and Daughter Ion Scanning

Parents (m/z)	Daughter (m/z)	Dwell (secs)	Cone volt	Cell energy
228.25	167.02	0.200	250	15.0

Table 7: Chromatographic condition

HPLC	Waters Acquity UPLC
Column	Chromatopak ,Peerless Basic C18 (50 *4.6 mm) 3.0 µm
Column oven	$40^0C \pm 5^0C$
Mobile Phase	Methanol :5mM Ammonium Acetate (85 :15 v/v)
Needle Wash	Strong Wash:-Methanol Weak Wash:-Mobile Phase
Flow rate	0.500mL/minute
Detection	Q3 [Quattro premier XE,Waters ,USA]
Ion Mode	Positive
Injection volume	5µl (Partial Loop With Needle overfill)
Sample Cooler temp	$5^0C \pm 5^0C$
Retention Time	Metaxalone around 1.51 min,Metaxalone D6 around 1.49 min
Run Time	2.2 min
Injection volume	5µl (Partial Loop With Needle overfill)

Bioanalytical Method Development [13-18]

The process by which a specific bioanalytical method is developed,validated and used in routine sample analysis can be divided into 1. Reference standard preparation 2.

Bioanalytical method development and establishment of assay procedure 3 Application of validated bioanalytical method to routine drug

Method Development and Trials

- Optimization of extraction procedure
- Optimization of mobile phase
- Optimization of pH

- Optimization of column
- Optimization of flow rate of mobile phase
- Optimization of column oven temperature
- Optimization of Mass Detector parameters

Bioanalytical Method Validation Parameter (BMVP) : Preparation of Calibration Curve Standards: Preparation of Spiking Solution of Metaxalone:-Calibration curve range is prepared from ~2521 ng/ml to 25 ng/ml. Prepare the following spiking solution with diluent as per example given below:

Table 8: Spiking Solution with Diluents

Stock Conc. (ng/ml)	Stock Volume (ml)	Total Volume after addition of Diluent. (ml)	Spiking Conc. (ng/ml)	Batch No.
750390	4.90	25.00	126065	STD 1 SS
126065	9.00	10.00	113459	STD 2 SS
113459	8.33	10.00	94511	STD 3 SS
94511	6.66	10.00	63039	STD 4 SS
63039	3.00	10.00	18911	STD 5 SS
18911	3.33	10.00	6297	STD 6 SS
6297	4.00	10.00	2519	STD 7 SS
2519	5.00	10.00	1259	STD 8 SS

Spiking of Metaxalone in Human Plasma:
Spike above spiking solution in Human Plasma as per example given in below:

Table 9: Spiking of Meatxalone

Spiking conc. (ng/ml)	Volume of Spiking solutions	Total Volume after addition of Human Plasma (ml)	Final Conc.In Human Plasma (ng/ml)	Batch No.
126065	0.02	1.00	2521	STD 1 SM
113459	0.02	1.00	2269	STD 2 SM
94511	0.02	1.00	1890	STD 3 SM
63039	0.02	1.00	1260	STD 4 SM
18911	0.02	1.00	378	STD 5 SM
6297	0.02	1.00	125	STD 6 SM
2519	0.02	1.00	50	STD 7 SM
1259	0.02	1.00	25	STD 8 SM

Quality Control Samples: Prepare Quality Control samples from different stock solution. Use highest standard for preparing Quality Control samples. Prepare QC samples according to Calibration Curve Standards and Quality Control Samples Specification and Acceptance Criteria).

Storage Details for Dilutions and Aliquots: All spiking solutions and dilutions are stored at approximately below 8°C. Aliquot 200µl of each spiked calibration standards and Quality control samples into different polypropylene-capped tubes and store at 20°C until analysis.
Preparation of Quality Control Standards:

Table 10:- Preparation of spiking solution of Metaxalone

Stock conc.(ng/ml)	Stock volume(ml)	Total volume after addition of Diluent (ml)	Spiking Conc. (ng/ml)	Batch No.
750539	4.90	25.00	126090	STD 1SS
126090	8.00	10.00	100872	HQC SS
100872	6.20	10.00	62540	MQC-1 SS
62540	4.85	10.00	30332	MQC-2 SS
30332	1.24	10.00	3730	LQC SS
3730	3.40	10.00	1268	LLOQ QC SS

Table 11: Spiking of Metaxalone in Human Plasma: Spike above spiking solution in human plasma as given in below table 11: Spiking of QC Sample in Human Plasma

SpikingConc. (ng/ml)	Volume of spiking solutions (ml)	Total volume after addition of human plasma (ml)	Final conc. In human plasma (ng/ml)	Batch No.
126090	0.02	1.00	2521	STD 1 SS
100872	0.02	1.00	2017	HQC SS
62540	0.02	1.00	1250	MQC-1 SS
30332	0.02	1.00	606	MQC-2 SS
3730	0.02	1.00	74	LQC SS
1268	0.02	1.00	25	LLOQ QC SS

Calibration Curve:

Linearity and Regression: **Linearity and Goodness-of-fit**

Assay performance and validation The eight-point calibration curve was linear over the concentration range 2521.313 ng/ml to 25.19 ng/ml..The calibration model was selected based on the analysis of the data by linear regression with or without intercepts and weighting factors (l/x, 1/x 2, and A linear equation was judged to produce the best fit for the concentration/response relationship. The regression type was 1/ (concentration) 2 and peak area ratio for 8-point calibration curve of human Plasma was found to be linear from 2521.313 ng/ml to 25.19 ng/ml for Metaxalone. The goodness of fit was consistently greater than 0.98 during the course of validation. The range of accuracy and precision of the back-calculated concentrations of the standard curve points was from 94.1% to 104.4 % and 0.3 % to 5.6% for Metaxalone.

Weighting factor :

To determine weighting factor for Metaxalone calibration standards accuracy results were calculated for three P&A batches with linear regression using weighting factors as 1/X, $1/X^2$ and no weighting. The percent relative error(%RE) were calculated for each standards level as percent difference between the observed value of all %RE are summed for each weighting factor in three calibration standards. The weighting factor that gives the smallest values of the sum of %RE is taken as concentration response relationship for Metaxalone. The sum of %RE for weighting factor none , 1/X and $1/X^2$ For Metaxalone is 156.1 % ,56.0 % and 53.1 % Respectively

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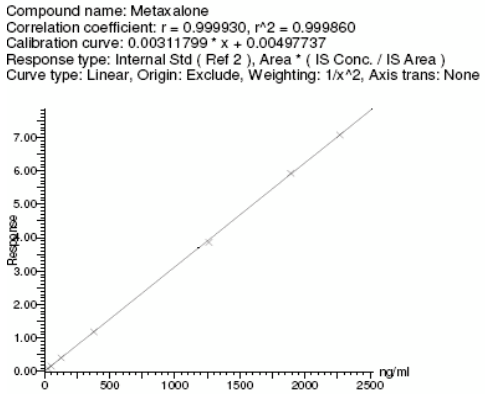

Fig.4: A Representative Regression Analysis of a Calibration curve

Validation parameters at the LLOQ:

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision and was found to be 25.19 ng/ml in human plasma..According to global data % accuracy of LQC is 97.5 % and LLOQ was 98.0%, and LQC Intraday accuracy was 98.5 % and LLOQ QC the accuracy was 99.1% for metaxalone.

Validation parameters at the middle and upper concentrations: The middle and upper quantification levels of metaxalone ranged from 500 to 2500 ng/mL in human plasma. For the intra day experiments the % CV ranged from 1.1 % to 2.5 % and the accuracy from 95.2 % to 101.6% For the inter day (Global data) experiments the precision and accuracy for the analyte met the acceptance criteria (= < + 15%).Recoveries of the analyte and IS were high, and it was consistent, precise, and reproducible. Therefore, the assay has proved to be robust in high throughput bioanalysis

Table 12: Stability Studies

Stability Studies	Metaxalone	ISTD
Short Term Stock Solution Stability (STD-1,HQC,LQC & ISTD) (09:03Hrs for Drug and ISTD)	111.53 %, 99.41 %, 101.05 %	95.85 %
Long Term Stock Stability (20 ±5°C for drug & 2-8°C for ISTD) (STD-1, HQC, LQC & ISTD) (21 Days for Drug and 19 Days for ISTD)	99.43 %, 100.25 %, 98.38 %	91.77 %
Auto Sampler Stability (5 ± 5°C) (Low & High QC) (48: 59Hrs)	98.53 %, 101.66 %	NA
Wet Extract Stability (2 - 8°C) (Low & High QC) (44:35Hrs)	99.48 %, 100.25 %	NA
Freeze & Thaw Stability (-20 ± 5°C) After 3 Cycles (Low & High QC)	99.42 %, 98.40 %	NA
Freeze & Thaw Stability (-20 ± 5°C) After 4 Cycles (Low & High QC)	99.90 %, 98.13 %	NA
Bench Top Stability (at Room Temperature) (Low & High QC) (06:08Hrs)	103.35 %, 98.85 %	NA
Stability in whole blood (01: 52 Hrs Room Temperature)	99.65 %	NA
Stability in whole blood (01: 40 Hrs at 2 - 8°C)	94.0 %	A
Dry Extract Stability (-20 ± 5°C) (Low & High QC) (28:28 Hrs)	98.26%, 100.82 %	NA
Dilution Stability (Std-1, HQC,LQC) (21 Days for Drug)	109.74 %, 100.28 %, 98.36 %	NA
Stability of Internal Standard Working Solution (08 Days)	NA	100.32 %
Freeze & Thaw Stability (-20 ± 5°C) After 4 Cycles (Low & High QC)	99.90 %, 98.13 %	NA

Application [19]**:-**The validated method has been successfully used to quantify metaxalone concentrations in human plasma samples after the administration of an 800-mg oral dose of metaxalone under both fasted and fed conditions. Mean plasma concentration versus time profiles for six subjects, each receiving a single dose, is presented in **below figure 5**

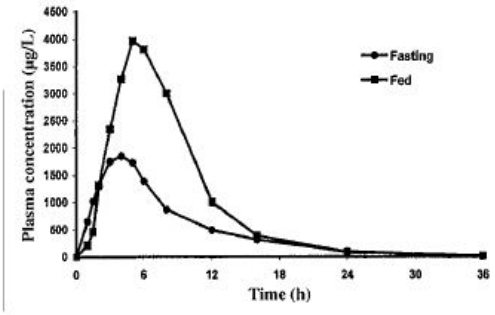

Figure 5: Mean plasma concentration –time profiles of six healthy subjects after the administration of a single oral dose of 800 mg of metaxalone under both fasted and fed conditions,

Conclusions:

In summary, a method is described for the quantification of metaxalone from human plasma by LC-MS-MS in positive ionization mode using MRM. The current method has shown acceptable precision and adequate sensitivity for the quantification of metaxalone in human plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies. Furthermore, it was utilized for the analysis of hundreds of subject's samples. The method described is simple, rapid, sensitive, selective, and fully validated according to commonly accepted criteria [13] .The simplicity of the assay and rapid LLE and sample turnover rate of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of metaxalone. The validated method allows quantification of metaxalone in the 2521.313 ng/ml to 25.19 ng/ml range.

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