BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF TOLPERISONE HYDROCHLORIDE IN HUMAN PLASMA BY HPLC

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ABSTRACT

A simple, rapid, selective, sensitive, accurate and precise High Performance Liquid Chromatography (HPLC) with UV detection method has been developed and validated for determination of Tolperisone hydrochloride in human plasma. Tolperisone hydrochloride is a piperidine derivative, centrally-acting muscle relaxant used in the treatment of acute muscle spasms in back pain. Eperisone hydrochloride is used as internal standard. The chromatographic separation of Tolperisone hydrochloride and internal standard were achieved with Chromasil C18 column using a mobile phase Acetonitrile: water pH adjusted to 3.0 with O- Phosphoric acid (60:40 v/v) at flow rate 1.0 ml/min. The measurements were made with injection volume 20 μl and UV detection at 260.0 nm. Plasma samples were processed using acetonitrile as precipitating agent to extract drug. The linearity for Tolperisone hydrochloride was found to be 50-2000 ng/ml with regression coefficient (r^2) 0.9990. The limit of quantification in plasma for Tolperisone hydrochloride was found to be 50 ng/ml. The mean recovery was obtained at 74.22 %. The method has been used to perform pharmacokinetic and bioequivalence studies in human plasma.

Key Words : Tolperisone hydrochloride, Eperisone hydrochloride, Human plasma, Method validation, HPLC-UV detection.

INTRODUCTION

Tolperisone hydrochloride (TOL) is a piperidine derivative, is a centrally-acting muscle relaxant used in the treatment of acute muscle spasms in back pain and spasticity in neurological diseases.[1,2] Chemically it is 2-methyl-1-(4-methylphenyl)-3-(1-piperidyl) propan-1-one[1,2] (Fig.1). Tolperisone has the unique property of mediating muscle relaxation without concomitant sedation and it does not cause weakness and mental confusion in contrast to other muscle relaxants. Its molecular formula and molecular weight are $C_{16}H_{23}NO.HCl$ and 281.83 respectively. Tolperisone hydrochloride is extremely water soluble. It is more stable in acidic medium. Tolperisone hydrochloride is official in Martindale, The Extra Pharmacopoeia[1] and The Merck Index[2]. Literature survey reveals that many analytical methods such as UV spectrophotometric[3-6], HPLC methods[7-10], HPTLC[11,12] and TLC[13] methods are reported for determination of Tolperisone hydrochloride individually from pharmaceutical dosage form and UV spectrophotometric[14-16], HPLC[17,18] methods are reported for determination of TOL with other drugs in combined dosage form.

MATERIALS AND METHODS

Chemicals and reagents

Pure sample of Tolperisone (HPLC grade) was procured from Emcure Pharmaceuticals Ltd., Pune. Methanol (HPLC grade) and Acetonitrile (HPLC grade) were obtained from Qualigen Laboratories Pvt. Ltd., Mumbai.

Instrumentation and chromatographic condition

The analysis of Tolperisone hydrochloride was carried out on an Agilent 1120 series, which comprised a degasser, binary gradient pump and UV detector. The system was controlled through Ezchrome software using Chromasil C18 (4.6 x 250 mm, 5 μm; Advanced Chromatography Systems, Johns Island, SC) column maintained at 30°C temperature and flow rate of mobile phase 1.0 ml/min. The mobile phase was composed of acetonitrile: water pH adjusted to 3.0 with O- Phosphoric acid (60:40 v/v). The mobile phase was kept in ultrasonicator for 30 min. and filtered through a 0.22 μm nylon membrane filter. The measurements were made with injection volume 20 μL and UV detection at 260.0 nm.

Preparation of standard stock solutions

A Standard stock solution of Tolperisone hydrochloride was prepared by dissolving 10 mg drug in HPLC grade methanol in 100 ml volumetric flask to get concentration 100 μg/ml. To

prepare working standard solution of TOL was diluted to 0.5, 1, 2, 4, 8, 12, 16 and 20 µg/ml with methanol.

Preparation of Internal standard stock solution

Accurately about 10 mg Eperisone hydrochloride (Internal standard Fig. 2) was dissolved in HPLC grade methanol in 100 ml volumetric flask to get concentration 100 µg/ml. Using a calibrated pipette, 2.0 ml of Internal standard stock solution (100 µg/ml) was pipetted into a 10.0 ml volumetric flask and made up the volume with the methanol to get concentration of 20 µg/ml.

Protein Precipitation

The blank plasma sample was prepared by adding 1ml of plasma and 1ml of Acetonitrile and vortex for 1.0 min. Then the solution was centrifuged at 3000 RPM for 10 min. The supernatant liquid was taken and transferred to HPLC vials. The blank sample was injected to HPLC (Fig.3). The blank plasma sample containing internal standard was prepared by adding 0.95 ml of spiked plasma, 1ml of acetonitrile and 50 µl of Eperisone hydrochloride (20 µg/ml) and vortex for 1.0 min. Then the solution was centrifuged at 3000 RPM for 10 min. The supernatant liquid was taken and transferred to HPLC vials.

Preparation of calibration curve (cc) standards and quality control samples

The working standard solutions of TOL with concentrations of 0.5, 1, 2, 4, 8, 12, 16 and 20 µg/ml was prepared by diluting with methanol. Aliquots of 0.85 ml of blank human plasma were spiked with 100 µl of working standard solutions of 0.5, 1, 2, 4, 8, 12, 16 and 20 µg/ml to get CC standards containing 50, 100, 200, 400, 800, 1200, 1600 and 2000 ng/ml of Tolperisone hydrochloride. A volume of 50 µl of an internal standard (EPE) solution (20 µg/ml) was added in each CC standards separately. Similarly the QC samples were prepared to contain three concentrations [150 ng/ml low quality control (LQC), 1000 ng/ml middle quality control (MQC) and 1500 ng/ml high quality control (HQC)].

Preparation of plasma sample solution

To 0.85 ml of plasma, 50 µl of an Eperisone hydrochloride (Internal standard) solution (20 µg/ml), 100 µl of Tolperisone hydrochloride standard solutions of 0.5, 1, 2, 4, 8, 12, 16 and 20 µg/ml, 1.0 ml acetonitrile were added to a glass tubes. Each sample was vortex mixed for 1.0 min and centrifuged (3000 rpm for 10 min). After centrifugation 50 µl aliquots of supernatant of each concentration were injected into the HPLC system (Fig. 4 and Fig. 5).

METHOD VALIDATION[19-21]

The method was validated in accordance with FDA guidelines

Calibration Plot

The calibration plot for the HPLC method was constructed by analysis of six solutions containing different concentrations of Tolperisone hydrochloride (50, 100, 200, 400, 800, 1200, 1600 and 2000 ng/ml). In the range 50- 2000 ng/ml the data were best fitted by a linear equation y = mx + b, the coefficient of determination (R^2) was 0.9990.

Evaluation of Selectivity

This parameter was evaluated at the Lower Limit of Quantification (LLOQ) i.e. 50 ng/ml per ml of plasma. The LLOQ sample was mixed with 100 μl of methanol, analyzed and the drug peak area was noted.

Similarly, 1 ml blank plasma was mixed with 100 μl of methanol, analyzed and the detector response for the blank was noted at the retention time of the drug. The blank response was compared with the peak area of the LLOQ sample.

Precision and accuracy

The intra- and inter-day variation of the assay were determined by replicate analysis (n = 6) of three concentrations of TOL [150 ng/ml low quality control (LQC), 1000 ng/ml middle quality control (MQC) and 1500 ng/ml high quality control (HQC)] on the same day and at different days, respectively, using the same stock solutions and plasma batches. The percent of relative standard deviations of results of the assay were determined.

Repeatability

The repeatability (intraday precision) of the method was evaluated in triplicate on the same day for three different concentrations performing of Tolperisone hydrochloride.

Recovery

Recovery from human plasma samples was evaluated in triplicate for each of three concentrations of Tolperisone hydrochloride. The response for each level being compared with that from the corresponding standard solution.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The parameters LOD and LOQ were determined using the signal-to-noise ratio by comparing results of the test of samples with known concentrations of analyte to blank samples. The

analyte concentration that produced a signal-to noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision and variability.

Stability

Short term stock stability

A Stock solution of Tolperisone hydrochloride and Eperisone hydrochloride (EPR) was kept at room temperature for 6 hours.

Long term stock stability

A Stock solution of Tolperisone hydrochloride and Eperisone hydrochloride (EPR) was kept at room temperature for 15 days.

Freeze thaw stability

The stability of low and high quality control samples were determined after three freeze thaw cycles.

RESULTS AND DISCUSSION

The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability.

Linearity

The linearity was found to be 50-2000 ng/ml.

Sensitivity

In the plasma, limit of quantification was found to be 50 ng/ml for Tolperisone hydrochloride**.**

Precision and Accuracy

The accuracy, precision and intraday precision were carried out by preparing 6 individual samples of HQC, MQC and LQC and the percentage of mean and C.V. was calculated (Table 1).

Recovery

The percentage recovery of the drug was found to be 97.88%. The results of recovery study are given in Table 2.

Stability

Stability of method was carried out by performing short term and long-term stock stability and freeze thaw stability. The results of recovery study are given in Table 3.

Table 1: Intraday, Interday precision and accuracy of Tolperisone hydrochloride in human plasma QC samples

Concentration of QC samples (ng/ml)	Observed Concentration* (mean ng/ml)	Precision (% RSD)	Accuracy* (% mean)
Intraday Precision			
150	145.37±0.9530	0.6555	96.91
1000	973.37±2.545	0.2614	97.33
1500	1454.19±7.599	0.5225	96.94
Interday Precision			
150	142.85±1.069	0.7483	95.23
1000	953.94±4.091	0.4288	95.39
1500	1437.75±5.958	0.4143	95.84

 *Average of six reading

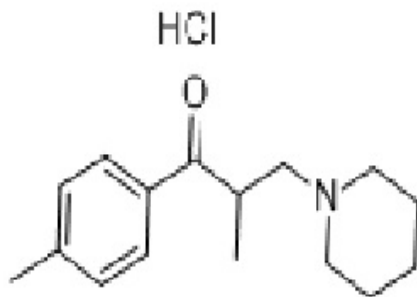**Table 2: Recovery of Tolperisone hydrochloride QC samples in human plasma**

| Concentration of QC samples (ng/ml)	Observed Concentration* (mean ng/ml)	Recovery* (% mean)	% RSD
150	105.73	70.49	0.6152
1000	766.20	76.62	0.7814
1500	1133.40	75.56	0.5347
Mean		74.22	

 *Average of six readings

Table 3: Stability of Tolperisone hydrochloride QC samples in human plasma

| Stability	Concentration of QCsamples (ng/ml)	Concentration found* (mean ng/ml)	% RSD	% Mean*
Short term stability	150	145.94±1.286	0.8811	97.29
	1500	1450.04±24.275	1.674	96.66
Long term stability	150	145.33±0.9008	0.6198	96.88
	1500	1446.18±3.780	0.2613	96.41
Freeze thaw stability	150	144.85±0.7517	0.5189	96.56
	1500	1437.32±15.857	0.9931	95.82

 *Average of six readings

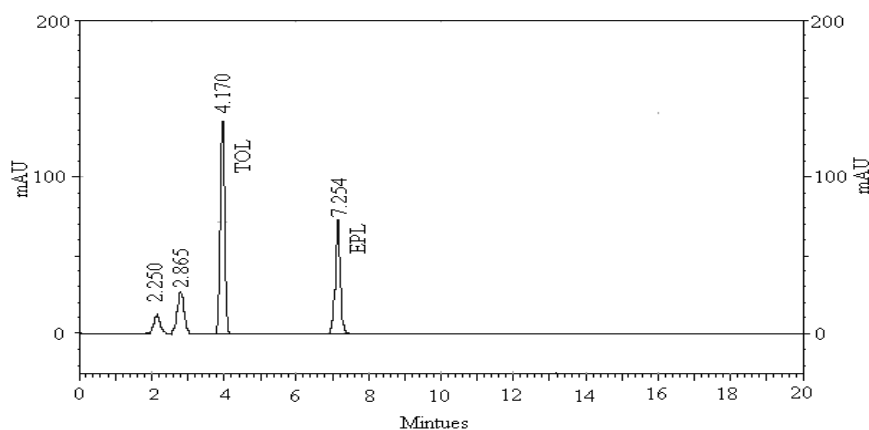**Fig.1: Chemical structure of Tolperisone hydrochloride**
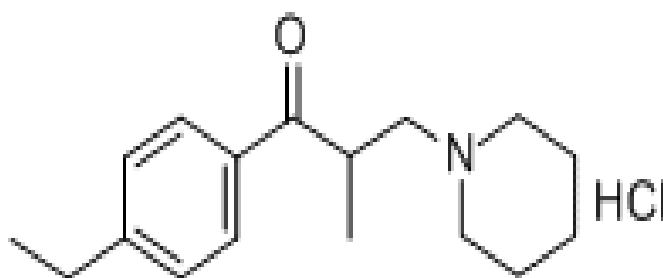
Fig. 2: Chemical structure of Eperisone hydrochloride
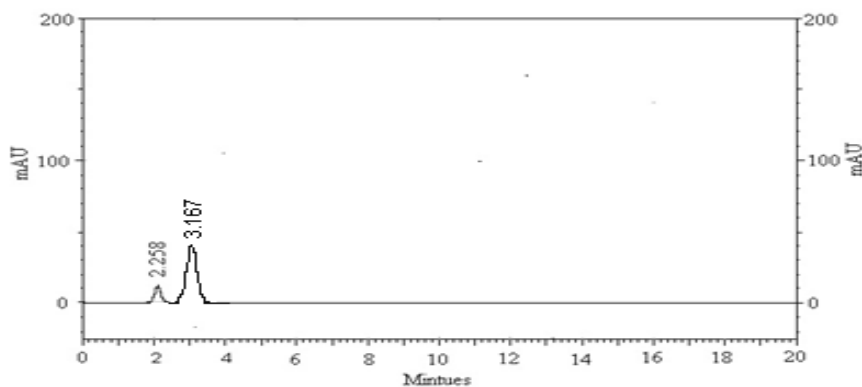
Fig.3: Typical chromatogram of Blank Human Plasma
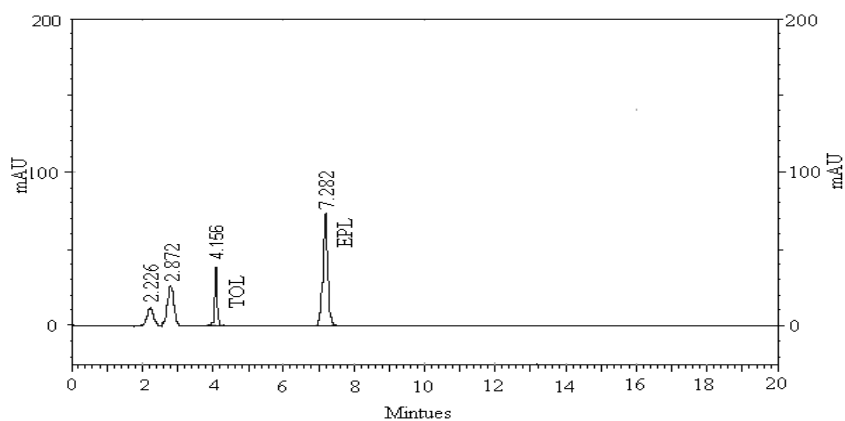
Fig. 4 : Typical chromatogram of LQC sample of TOL in human   plasma

Fig.5: Typical chromatogram of HQC sample of TOL in human   plasma

Table legends

Table 1. Intraday, Interday precision and accuracy of Tolperisone hydrochloride in human
plasma QC samples

Table 2. Recovery of Tolperisone hydrochloride QC samples in human plasma

Table 3: Stability of Tolperisone hydrochloride QC samples in human plasma

Figure legends

Fig.1: Chemical structure of Tolperisone hydrochloride

Fig. 2: Chemical structure of Eperisone hydrochloride

Fig.3: Typical chromatogram of Blank Human Plasma

Fig.4: Typical chromatogram of LQC sample of TOL in Human Plasma

Fig.5: Typical chromatogram of HQC sample of TOL in Human Plasma

CONCLUSION

This HPLC method for quantification of Tolperisone hydrochloride in human plasma is simple, accurate, precise, rapid, reproducible and selective. It is a simple, rapid, practical and economical alternative for studies of the bioavailability, bioequivalence and pharmacokinetics of this drug in human plasma.

ACKNOWLEDGMENTS

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