OPEN ACCESS
Saudi Journal of Medical and Pharmaceutical Sciences
Abbreviated Key Title: Saudi J Med Pharm Sci
ISSN 2413-4929 (Print) |ISSN 2413-4910 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com/journal/sjmps/home

Original Research Article

Rapid Determination of Tolperisone in Human Plasma by Reversed Phase High Performance Liquid Chromatography

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| **Received:** 11.04.2019 | **Accepted:** 25.04.2019 | **Published:** 30.04.2019
DOI: 10.36348/sjmps.2019.v05i04.008

Abstract

A simple and rapid reversed-phase high performance liquid chromatography (HPLC) assay for determination of tolperisone concentration in human plasma was developed and validated. Using prednisolone as an internal standard (IS), 1.0 ml plasma samples were extracted with a mixture of methyl tert. butyl ether and dichloromethane (70:30, v:v). Analysis was performed using Atlantis dC18 column with a mobile phase composed of 0.05 M (mono basic) potassium phosphate (pH=3.0) and acetonitrile (70:30, v:v). The eluent was monitored spectrophotometrically at 262 nm. No interference with tolperisone or IS peaks by extracted blank plasma components or commonly used drugs was observed. The relationship of tolperisone concentration and peak area ratio of tolperisone / IS was linear ($R^2 \geq 0.9961$) in the range of 10 – 800 ng/ml, the intra and inter-day coefficient of variations were ≤ 4.1% and ≤5.7%, respectively with a corresponding bias of ±11.4% and ±8.4%, respectively. Mean extraction recovery of tolperisone and the IS were 95% and 83%, respectively. The method was applied to assess the stability of tolperisone under various conditions encountered in the clinical laboratory. Tolperisone stability in processed samples stored at room temperature for 24 hours or at -20 °C for 48 hours, in unprocessed samples stored for 24 hours at room temperature or for 8 weeks at -20 °C, and after 3 freeze and thaw cycles was ≥ 91%.

Keywords: Tolperisone, Prednisolone, Human plasma, HPLC.

INTRODUCTION

Tolperisone (CAS: 728-88-1), chemically known as 2-methyl-1-(4-methylphenyl)-3-(1-piperidyl) propan-1-one, is a centrally-acting muscle relaxant used in the treatment of acute muscle spasms and spasticity [1, 2]. Its mean (SD) oral bioavailability is about 22.3 (6.3) % with a peak plasma concentration of 784.9 ng/ml within 1.2 hours after ingestion of a 450 mg therapeutic dosage [3, 4]. Figure-1 depicts the chemical structures of tolperisone and the internal standard (IS) used in this study, prednisolone.

Several analytical methods have been reported for the determination of tolperisone concentration in pharmaceutical formulations that used various techniques such as potentiometry [5], spectrometry [6], high performance thin layer chromatography [7], and high performance liquid chromatography (HPLC) [8, 9]. However, only few assays were reported for biological sample analysis. Tolperisone level in human serum/plasma has been often determined by HPLC with UV detector [10] or liquid chromatography-tandem mass spectrometry (LC-MS/MS) [11]. The reported HPLC assay involved liquid-liquid extraction using 100% dichloromethane. Dichloromethane is considered carcinogenic solvent, and according to international safety guidelines, its exposure must be reduced [12].

In the present study, we developed a simple and rapid tolperisone HPLC assay using reduced amount of dichloromethane in sample preparation. The method was fully validated and successfully applied to assess the stability of tolperisone under various laboratory conditions.

MATERIAL AND METHODS

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 system (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. We used a reversed-phase Atlantis dC18 (4.6 x 150 mm, 5 µm) column protected by guard column Symmetry C18 (3.9 x 20 mm, 5 µm). Data were collected with a Pentium IV computer, using Empower Chromatography Software.

Chemical and Reagents

All reagents were of analytical-reagent grade unless stated otherwise. Tolperisone and prednisolone were obtained from Sigma-Aldrich MO, USA. Acetonitrile, methanol (both HPLC grade), methyl tert. butyl ether, dichloromethane, and (mono basic) potassium phosphate were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia.

Chromatographic Conditions

The mobile phase was composed of 0.05 M (mono basic) potassium phosphate (pH=3.0±0.1, adjusted with phosphoric acid) and acetonitrile (70:30, v:v) and was delivered at a flow rate of 1.0 ml/minute at ambient temperature, with a run time of 8.0 minutes. A photodiode array detector set at 262 nm was used.

Preparation of Standard and Quality Control Samples

Stock solution of tolperisone and prednisolone (1.0 mg/ml) were prepared in methanol. They were further diluted with methanol to produce working solutions of 8.0 and 10 µg/ml. Nine calibration standards in the range of 10 – 800 ng/ml and four quality control (QC) samples (10, 30, 400, and 750 ng/ml) were prepared in human plasma. Calibration standards and QC samples were vortexed for one minute and 1.0 ml aliquots were transferred into 7 ml culture tubes with flat top cap and stored at –20 °C until used.

Sample preparation

Aliquots of 1 ml of calibration standards or QC samples in culture tubes were allowed to equilibrate to room temperature. To each tube, 100 µl of 10 µg/ml IS working solution was added and the mixture was vortexed for 10 seconds. After the addition of 5 ml of a mixture of methyl tert. butyl ether and dichloromethane (70:30, v:v), the samples were vortexed again for 5 minutes and centrifuged for 15 minutes at 4700 rpm at room temperature. The supernatant organic layer was carefully collected into a clean tube, evaporated under gentle steam of nitrogen, reconstituted in 300 µl of the mobile phase, and 100 µl were injected into the HPLC system.

Stability Studies

Two of QC samples (30 and 750 ng/ml) were used for stability studies. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at –20 °C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed and stored at room temperature for 24 hours, or in –20 °C for 48 hours before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at –20 °C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20 °C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Method Validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [13]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

The mobile phase was composed of 0.05 M (mono basic) potassium phosphate (pH=3.0±0.1, adjusted with phosphoric acid) and acetonitrile (70:30, v:v) and was delivered at a flow rate of 1.0 ml/minute at ambient temperature, with a run time of 8.0 minutes. A photodiode array detector set at 262 nm was used. The retention times of tolperisone and the IS were around 4.6 and 6.6 minutes, respectively.

Specificity

Figure-2 depicts a representative chromatogram of drug free human plasma used in preparation of calibration standards and QC samples. No endogenous component from six batches of extracted human plasma co-eluted with tolperisone or the IS. Further, none of eight commonly used drugs (aspirin, acetaminophen, ranitidine, nicotinic acid, ascorbic acid, caffeine, ibuprofen, and diclofenac) co-eluted with tolperisone or the IS.

Linearity, Accuracy and Precision

Linearity of tolperisone assay was evaluated by analyzing nine curves of nine calibration standards over the range (10-800 ng/ml) prepared in human plasma. Figure-3 represents an overlay of chromatograms of extracts of 1.0 ml human plasma spiked with the IS with or without nine concentrations of tolperisone. The peak height ratios were subjected to regression analysis. The suitability of the calibration curves was confirmed by back-calculating tolperisone concentration from the calibration curves (Table-1). All back-calculated concentrations were well within the acceptable limits (11.5%). Precision and bias were also determined for four QC samples (10, 30, 400, and 750 ng/ml). The intra-day (n=10) and inter-day (n=20, over three consecutive days) precision was ≤ 4.1% and ≤ 5.7%, respectively. The intra-day and inter-day bias was in the range of -3.4 to +11.4% and of -0.7 to

+8.4%, respectively. The results are summarized in Table-2.

Recovery

Tolperisone recovery was assessed by direct comparison of its peak height in plasma and in methanol samples, using five replicates for each of four QC samples (10, 30, 400, and 750 ng/ml). Similarly, the recovery of the IS was determined by comparing its peak height (at 10.0 µg/ml) in plasma and in methanol samples. The results are presented in Table-3. Recovery of tolperisone and the IS was in on average 95% and 83%, respectively.

Stability

As shown in Table-4, tolperisone stability in processed and unprocessed QC samples (10, 30 and 750 ng/ml) was investigated. Tolperisone was stable in processed samples for at least 24 hours at room temperature (≥ 96%) or 48 hours at -20 °C (≥ 92%). Tolperisone in unprocessed samples was stable for at least 24 hours at room temperature (≥ 94%), eight weeks at -20 °C (≥ 91%), or after three freeze-and thaw cycles (≥ 92%).

Table-1: Back-calculated tolperisone concentrations from nine calibration curves

Nominal concentration (ng/ml)	Calculated level (ng/ml)		CV (%)*	Bias** (%)
	Mean	SD		
10	11.15	0.27	2.4	11.5
20	21.64	0.89	4.1	8.2
50	53.01	2.98	5.6	6.0
100	98.18	4.63	4.7	-1.8
200	193.54	9.11	4.7	-3.2
300	299.43	13.73	4.6	-0.2
600	588.90	19.96	3.4	-1.9
700	711.83	21.27	3.0	1.7
800	800.66	14.29	1.8	0.1

*Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100. **Bias = measured level – nominal level divided by nominal level x 100.

Table-2: Intra- and inter-day precision and bias of tolperisone assay

Nominal concentration (ng/ml)	Measured level (ng/ml)		CV (%)*	Bias (%)**
	Mean	SD		
Intra-day (n=10)				
10	11.14	0.45	4.1	11.4
30	32.46	0.63	1.9	8.2
400	389.10	6.76	1.7	-2.7
750	724.05	22.21	3.1	-3.4
Inter-day (n=20)				
10	10.84	0.41	3.8	8.4
30	32.39	1.84	5.7	8.0
400	397.15	11.17	2.8	-0.7
750	748.16	30.04	4.0	-0.2

*CV, coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x100. **Bias = measured level - nominal level divided by nominal level x 100.

Table 3: Recovery of tolperisone and internal standard (IS) from 1.0 ml human plasma

Nominal concentration (ng/ml)	Mean peak height (SD)		Recovery* (%)
	Human plasma (n=5)	Mobile phase (n=5)	
Tolperisone 10	1026 (15)	1059 (53)	97
30	2670 (24)	2952 (208)	90
400	32862 (883)	35493 (199)	93
750	62388 (320)	63581 (1460)	98
Internal standard 1000	20418 (15)	24674 (462)	83

* Recovery = peak height in human plasma divided by peak height in mobile phase x 100.

Table-4: Stability of tolperisone under various clinical laboratory conditions

Nominal concentration	Unprocessed		Processed		Freeze-Thaw Cycle		
(ng/ml)	24 hrs RT	8 wks -20 °C	24 hrs RT	48 hrs -20 °C	1	2	3
10	94	91	96	97	92	96	96
30	101	98	109	109	105	96	100
750	102	100	98	92	96	98	98

Data represent stability (%) calculated as mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after storing for 24 hours at room temperature (24 hrs RT), freezing at –20 ºC for 8 weeks (8 wks., –20 ºC), or after 1 to 3 cycles of freezing at –20 ºC and thawing at room temperature (freeze-thaw); or were processed and then analyzed after storing for 24 hours at room temperature (24 hrs., RT) or 48 hours at –20 ºC (48 hrs., -20 ºC).

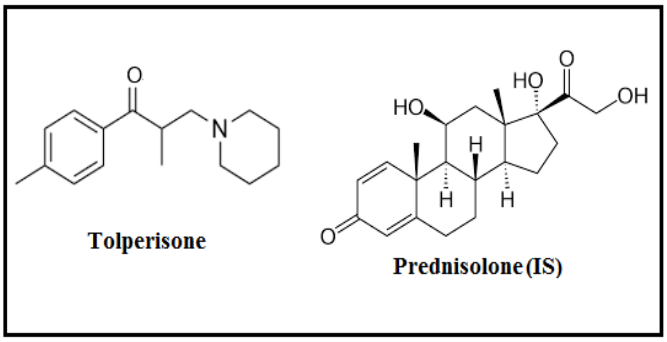

Fig-1: Chemical structures of tolperisone and prednisolone (IS)

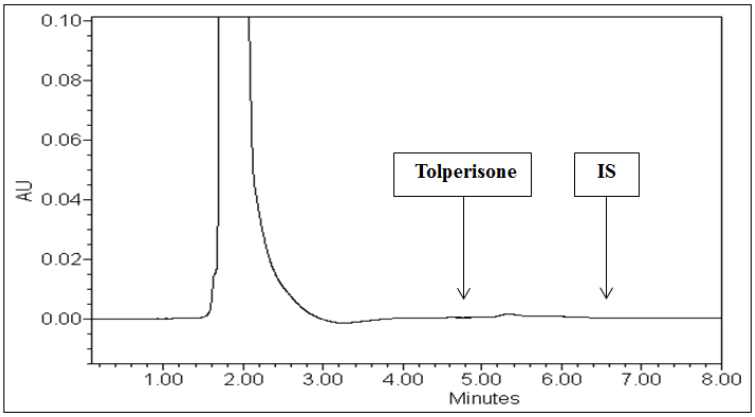

Fig-2: Representative chromatogram of blank human plasma

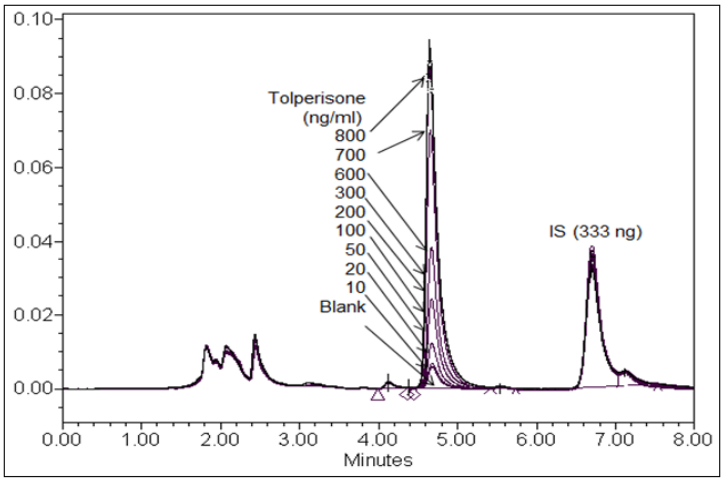

Fig-3: Overlay of chromatograms of extracts of 1.0 ml human plasma spiked with the internal standard (IS) alone or with tolperisone at one of nine concentrations

CONCLUSION

The described HPLC assay is accurate, precise, and rapid. It requires only 1.0 ml plasma and utilizes a simple and convenient liquid-liquid extraction procedure. Further, it requires the use of dichloromethane at a reduced concentration of 30% (v:v). The assay was successfully applied to monitor stability of tolperisone under various conditions generally encountered in the clinical laboratories.

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