RESEARCH ARTICLE

High Throughput LC-MS/MS Method for the Quantitation of Mefenamic Acid in Rat Plasma by Protein Precipitation Technique Using 96 Well Plate Format

Tummuru Bhanu Prakash[1*], A Krishnamanjari Pawar[1], Beeram Geetha Madhuri[2]

[1]*College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India.*
[2]*QPS Bioserve India Pvt. Ltd., IDA Balanagar, Hyderabad, TS, India.*

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ABSTRACT

An advanced and validated high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach has been established to accurately determine the concentration of mefenamic acid in rat plasma. This method utilizes mefenamic acid D4 as the internal standard (ISTD). The analyte and ISTD underwent protein precipitation in a 96-well plate format and were then separated on a BDS Hypersil C8 column (3 µm, 100 x 4.6 mm) using a precise isocratic mobile phase of 2 mM ammonium formate by 0.1% formic acid and acetonitrile (30:70 v/v). The detection was performed on a triple quadrupole mass spectrometer utilizing positive ionization consideration. The approach demonstrated a concentration range of 20.659 to 20067.772 ng/mL, thru relative regaining reaching from 69.1 to 74.3%. The inter-batch precision was excellent, with a coefficient of variation (%CV) of ≤ 7.8%. Additionally, the inter-batch accuracy at four different quality control-points ranged from 97.0 to 100.4%, demonstrating high levels of accuracy. This method has been thoroughly validated and is extremely specific, accurate, and precise. It is perfectly suited for high-throughput investigation of mefenamic acid in rat plasma, making it ideal for routine analysis.

Keywords: Mefenamic acid, Mefenamic acid D4, Protein precipitation technique, 96 well plates.

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INTRODUCTION

Mefenamic acid, part of the anthranilic acid derivatives (fenamate) class of NSAIDs, remains employed to cure mild to moderate pain.[1,2] It remained introduced through Parke-Davis as Ponstel in the 1960s and turn into available as a generic in the 1980s, sold under various product names as Meftal. This drug remains effective for pain relief in conditions like rheumatoid arthritis, osteoarthritis, postoperative pain, acute pain, menstrual pain, and menorrhagia.[3-5] It also shows efficacy in preventing perimenstrual migraine headaches when taken two days ahead of the onset of menstruation and constant throughout the period.[2]

Mefenamic acid inhibits COX-1 and COX-2 enzymes, reducing the realization of prostaglandins involved in pain, inflammation, and fever, as well as other physiological functions.,[8,9] which contribute to the regulation of pain sensitivity, inflammation, and fever, as well as the maintenance of pregnancy, hemostasis, and the preservation of the gastric mucosa as well as kidney function.[10,11] It occurs metabolized thru liver enzyme CYP2C9 towards weakly active metabolites, which are excreted via urine and feces. The parent compound retains a half-life of approximately 2 hours (Figure 1).[4,5,7,12]

Few methods exist for quantifying mefenamic acid in rat plasma employing liquid chromatography with tandem mass spectrometry (LC-MS-MS). This study aimed to develop and validate a high-throughput LC-MS/MS technique with protein precipitation as per US Food and Drug Administration (FDA) guidelines, using mefenamic acid D4 as an internal standard.[13,14]

MATERIALS AND METHODS

Experimental

Chemicals and reagents

Mefenamic acid standard was obtained from Clearsynth, Mumbai, India, and mefenamic acid D4 from Simson

**Author for Correspondence: tummuru1@gmail.com*

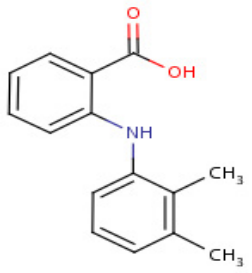

Figure 1: Structure of mefenamic acid

Pharma Limited, Mumbai, India. LC-MS grade methanol and acetonitrile were obtained from Thermo Fisher Scientific, India. GR grade ammonium formate and orthophosphoric acid stayed sourced from Merck Specialties, India. HPLC water was purified using a Milli-Q system (Millipore). Rat plasma with K2EDTA anticoagulant remained from Aptus Biosciences, Hyderabad, India. 2mL Square Well Filter Plates were bought from Phenomenex Inc, Torrance, CA, USA.

Instrumentation

A binary pump was utilized for the delivery of the solvent in order to carry out the analysis, which was carried out with an Agilent 1200 Series system. An API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) installed with a turbo ion spray interface was utilised for the purpose of carrying out the detection technique. The quantitative analysis was carried out in MRM mode with the assistance of Analyst software version 1.4.2 (SCIEX) for the purpose of controlling the hardware and managing databases.

Chromatographic environment

At a flow rate of 0.8 mL/min, the separation occurred *via* a BDS Hypersil C8 column thru a particle size of 3 µm and a dimension of 100 x 4.6 mm. The mobile phase consisted of an isocratic mixture of 2 mM ammonium formate (0.1% formic acid) and acetonitrile on a 30:70 v/v ratio. The autosampler remained insisted at 5 ± 2°C with a 2 µL injection volume. The column oven temperature stayed set at 40.0 ± 2.0°C. The retention times stayed 2.28 minutes for mefenamic acid and 2.29 minutes for mefenamic acid D4, through a total run time of 3.2 minutes.

Mass spectrometric conditions

The LC-MS/MS stood accomplished using positive ionization mode with optimized parameters, including curtain gas (CUR), collision energy (CE), and other settings, as detailed in Table 1. The isocratic mobile phase allowed effective elution of the analyte and ISTD with good sensitivity at low concentrations (Table 2).

Table 1: Optimized mass spectrometry parameters

Parameters	*Q1 (amu)*	*Q3 (amu)*	*Dwell Time (msec)*	*DP (volts)*	*CE (volts)*	*CXP (volts)*	*EP (volts)*
Mefenamic Acid	240.1	196.1	200	-53	-24	-11	-10
Mefenamic Acid D4	244.3	200.1	200	-53	-24	-11	-10
Parameters	CUR (psi)	GS1 (psi)	GS2 (psi)	IS (Volts)	CAD (psi)	TEMP (°C)	
Source/Gas	35	60	40	-4500	6	500	
Ionization mode	Negative ionization						
Resolution	Q1 Unit; Q3 Unit						

Table 2: Optimized chromatography parameters

Parameter	*Condition*
HPLC system	Agilent 1200 (Make: Agilent, USA)
Mobile phase	2 mM ammonium formate in a 30:70 v/v solution of acetonitrile and 0.1% formic acid
Column	BDS Hypersil C8 3 µm, 100 X 4.6 mm
Flow rate	0.8 mL/min.
Inj. vol.	2 µL
Column oven temp.	40 ± 2°C
Autosampler temp.	5 ± 2°C
Retention time	Mefenamic Acid: 2.28 minutes; Mefenamic Acid-D4: 2.29 minutes (ISTD)
Run time	3.2 minutes

Preparation of Calibration Standards and QC Samples

Stock solutions of mefenamic acid and mefenamic acid D4 stood organized in methanol to achieve a final concentration of 1000 mg/mL'. Working solutions stayed made thru diluting stock solutions in 50% methanol and stored at 2 to 8°C. Internal standard working solutions (1000 ng/mL) were prepared daily. Blank plasma was spiked with working mixtures (5% of total volume) from separate stock weightings to provide calibration standards and QC trials, stored below 2 to 8°C, and conveyed to room temperature earlier practice. Calibration standards ranged from 413.180 to 401355.430 ng/mL, and QC samples remained made at specified concentrations 413.180 (LLoQ QC), 1220.760 (LQC), 34779.500 (MQC-I), 194765.200 (MQC-II) and 295625.750 (HQC) ng/mL).

Sample Preparation

Calibration standards and QCs were processed using a Phenomenex Vacuum Manifold with 100 µL of plasma. Detailed steps included spiking plasma with working solutions, adding the internal standard, and transferring the mixture to a 96-well plate containing 0.1% FA in acetonitrile. The plate was vortexed, and the filtrate was collected under a vacuum, evaporated, and altered in mobile phase for LC-MS/MS analysis.

Method Validation

In compliance with the recommendations of the EMEA and the USFDA, a way for the detection of mefenamic acid in rat plasma remained supported. As part of the process, modified standards, blank samples, and QC samples occurred utilized. During the first stages of the validation process, the standard samples were tested, and quality control samples were distributed across the process.

Six sets of rodent plasma trials remained extracted and analyzed using LC-MS/MS without an internal standard to ascertain selectivity. In order to assess selectivity, the responses of the lowest limit of quantification (LLoQ) plasma samples (n = 6) were compared to those of the blanks. Four calibration curves were generated over a three-day period to evaluate linearity. Each curve contained ten concentration points that were distributed throughout the dynamic range. Data was analysed using linear least squares regression, thru the weighting factor (1/X2) being the reciprocal of drug concentration. The analyte concentrations were directly correlated with the peak area ratios of plasma-spiked calibration standards.[15-18]

At five different concentrations, precision and accuracy stood estimated. These concentrations were as follows: LLoQ, LQC, MQC, HQC, and ULoQ. The evaluation was conducted within a single batch (intra-batch) and across multiple batches (inter-batch). In order to evaluate precision and accuracy, %CV and relative error were computed. The peak topics of extracted trials were compared to those of un-extracted samples at LQC, MQC, and HQC ranks to determine the extraction recovery of mefenamic acid.

The matrix effect remained investigated using six plasma samples, one of which had hemolysis and the other had lipemia, at together low and high QC heights. The matrix factor ensued and was regulated through assessing the peak responses of post-extraction trials to those of aqueous trials equipped in the mobile phase. The matrix factor of the internal standard was compared to that of the analyte to derive the internal typical normalised matrix factor.[19]

The analyte's stability was evaluated in biological matrices and aqueous solutions under a variety of conditions. Plasma stability was assessed under frozen-thaw, long-term, bench-top, and extracted sample conditions. After seven cycles of chilling at -70ºC and thawing at room temperature, the freeze-thaw stability occurred assessed. The stability of the bench-top remained evaluated at ambient temperature, and the long-term stability stood measured at -20 and -70ºC. The stability of reconstituted samples remained verified through storage at 10°C, while the stability of whole blood stood assessed at room temperature. Freshly prepared samples were compared to stability samples at LQC and HQC levels.

In comparison to fresh solutions, the stability of stock and working solutions was evaluated under various temperature conditions, such as ambient temperature and 2 to 8ºC. The system's suitability was confirmed by injecting six MQC sample repeats prior to each analytical run, thereby guaranteeing a coefficient of variation for reaction ratios below 5.0%.

RESULTS AND DISCUSSION

Method Development

In order to ensure the reliable and consistent inference of analytes, it became essential to adjust the extraction technique, chromatographic, and mass spectrometric circumstances. Electrospray ionization was used to calibrate the analyte and ISTD in positive polarity mode. Q1 and MS/MS measurements were conducted in infusion mode, and additional factors stood optimized for flow injection analysis. [M-H] peaks detected at m/z 240.1 and 244.3 for mefenamic acid and mefenamic acid D4, correspondingly. The utmost profuse product ions existed identified at m/z of 196.3 and 200.1 for both mefenamic acid and mefenamic acid D4 with collision-activated dissociation gas and energy. Intensity increased above 475ºC. No effect on signal intensity from a 5% vary in ionspray voltage and gas restrictions.[20,21]

Isocratic mode was chosen for chromatographic optimization due to no crosstalk among analytes and ISTD. Acetonitrile in the mobile phase enhances signal intensities compared to methanol. Substituting milli-Q water per 2 mM ammonium formate buffer in the mobile phase and adding 0.02% formic acid improved chromatographic peak shapes. Higher buffer concentration caused a loss of reply. Flow rate of 0.8 mL/min stayed worked towards a lower run duration.

Protein precipitation, liquid-liquid extraction, and solid phasa extraction practices were used for extraction method selection. Protein precipitation stayed done in 1.5 ml Eppendorf centrifuge containers. The methodology was converted to a 96-well plate format. The ultimate optimized restrictions described, and the bearing of altered solutions and their concentrations continuously analyte revival was monitored. Emphasis was placed on enhancing sensitivity and recovery

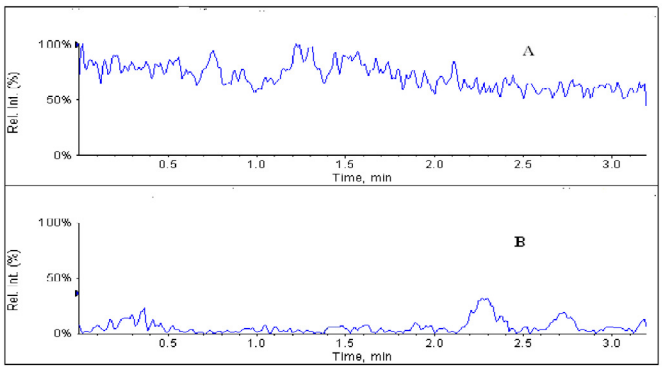

Figure 2: Chromatogram of blank plasma (A-Analyte; B-ISTD)

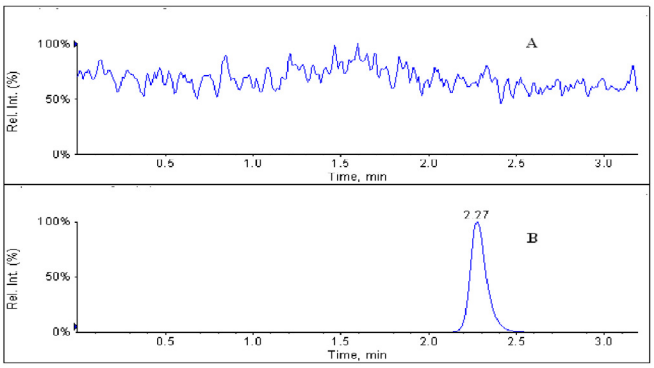

Figure 3: Chromatogram of zero standard (A-Analyte; B-ISTD)

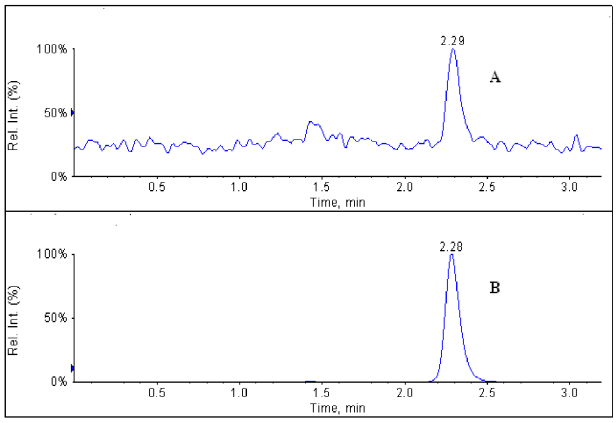

Figure 4: Chromatogram of LLoQ (A-Analyte; B-ISTD)

Table 3: Calibration standard overview

Analyte	*Nominal (ng/mL)*	*Mean[a] (ng/mL)*	*%CV*	*%Nominal*
Mefenamic acid	20.659	19.686	3.5	95.3
	41.318	45.581	8.9	110.3
	103.295	104.283	2.5	101.0
	278.236	272.141	2.9	97.8
	626.031	638.497	2.0	102.0
	1391.180	1450.105	1.3	104.2
	2851.919	2647.087	3.9	92.8
	6955.900	6408.212	1.6	92.1
	15650.775	14171.690	2.9	90.5
	20067.772	19404.432	4.5	96.7

throughout the optimization of chromatographic circumstances and extraction practice. No matrix effects are perceived with projected chromatographic and extraction circumstances.[22]

Selectivity

About six lots of rat K2 EDTA plasma matrix, one lot of lipemic serum, and one lot of hemolytic serum stood used to evaluate the selectivity of the procedure. At the analyte retention time and ISTD, there was very little interference when comparing the response of injected LLoQ to the peak responses in blank lots. The selectivity of the procedure is demonstrated in Figures 2-4 by analyzing chromatograms of the LLoQ sample, blank plasma with and without ISTD.

Linearity and sensitivity

An evaluation of the linearity of the calibration curve occurred performed *via* mapping the peak area ratio (y) of analytes to ISTD vs. the nominal concentration (x). The linearity of the calibration curves was more than 0.9995, and they ranged from 20.659 to 20067.772 ng/mL. The weighted linear regression method was utilized in order to determine the r values, slopes, & intercepts of 3 different calibration curves. The mean concentrations that were back-calculated are presented in Table 3, including both precision (%CV) and correctness (%Nominal). The LLoQ stayed obtained remained 20.659 ng/mL. The LLoQ accuracy stood 95.2%, and the CV was 3.5%.

Precision and accuracy

Three intra and inter-day precision and accuracy trials stood used to assess the quality control sample's precision and accuracy. Every batch contained 6 replicates at 5 concentration points. Intra-batch precision ranged from 1.4 to 3.5%, with a

Table 4: Intra, inter batch precession & accuracy

QC level	*Nominal conc. (ng/mL)*	*Intra Batch [a]*			*Inter Batch [b]*		
		Mean Conc Found (ng/mL)	*% CV*	*% Nominal*	*Mean Conc Found (ng/mL)*	*% CV*	*% Nominal*
LLOQQC	20.659	19.668	3.5	95.2	20.042	7.8	97.0
LQC	61.038	57.661	2.5	94.5	60.480	6.2	99.1
MQC-I	1738.975	1712.436	2.5	98.5	1714.606	3.0	98.6
MQC	9738.260	9852.096	1.4	101.2	9744.327	3.6	100.1
HQC	14781.288	15186.517	3.4	102.7	14840.416	4.6	100.4

Table 5: Matrix impact

	LQC			*HQC*		
Lot #	*MF of Analyte*	*MF of ISTD*	*ISTD Normalized Factor*	*MF of Analyte*	*MF of ISTD*	*ISTD Normalized Factor*
1	0.874	0.839	1.042	0.832	0.927	0.898
2	0.839	0.927	0.905	0.926	0.972	0.953
3	0.892	0.973	0.917	0.873	0.829	1.053
4	0.923	0.936	0.986	0.839	0.889	0.944
5	0.828	0.962	0.861	0.883	0.873	1.011
6	0.839	0.923	0.909	0.894	0.898	0.996
Mean	-		0.9366	-		0.9757
SD	0.07			0.06		
% CV	7.0			5.7		
N	6			6		

Table 6: Recovery

Analyte		*A*	*B*	*%Recovery*	*Mean recovery*	*% CV*
Mefenamic acid	LQC	9283.8	12699.9	73.1	71.8	3.2
	MQC-I	264494.5	355917.2	74.3		
	MQC	2011405.5	2843718.7	70.7		
	HQC	3053026.2	4416018.4	69.1		
Mefenamic acid d6	-	221325.2	301418.1	73.4	-	-

Table 7: Dilution integrity

Dilution factor[a]	*%Nominal*	*%CV*
1/5	98.9	3.4
1/10	95.1	4.1
1/20	93.1	4.2

Table 8: Stability results

Stability	*QC Level*	*A*	*%CV*	*B*	*%CV*	*%Change*
Stability of Bench-tops at room Temp. (8 hours.)	LQC	60.025	12.5	63.477	4.9	-5.4
	HQC	15008.190	10.4	14334.745	3.3	4.7
Freeze-thaw (After 6 cycle)	LQC	59.747	4.7	64.284	3.9	-7.1
	HQC	13710.972	6.7	14334.745	3.3	-4.4
Auto sampler stability (48 hours 24 minutes)	LQC	58.198	9.6	63.477	4.9	-8.3
	HQC	14664.412	9.4	14334.745	3.3	2.3
Long term stability for 30 days (Below-20ºC)	LQC	63.933	6.4	64.284	3.9	-0.5
	HQC	13320.923	7.1	14790.901	6.7	-9.9
Long term stability for 30 days (Below -50ºC)	LQC	63.720	15.1	64.284	3.9	-0.9
	HQC	13070.478	10.4	14790.901	6.7	-11.6

nominal percentage of 94.5 to 102.7%. The nominal percentage ranged from 97.0 to 100, while the inter-batch precision was between 3.0 and 7.8%. Table 4 shows precision and accuracy results.

Matrix effect

The ionisation is able to be subdued or enriched by co-eluting matrix elements, but the selectivity of the MS detection may prevent a noticeable rejoinder in matrix blanks. Nevertheless, they are able to influence the precision and accuracy of the assay.[23] Consequently, the IS normalized matrix factor was used to assess the possibility for mutable matrix-related ion restraint in six independent resources of rat plasma, each of which contained one hemolytic and one lipemic lot. The mean IS normalized matrix factor occurred at 0.9366, by a %CV of 5.7 to 7.0, as illustrated in Table 5.

Extraction recovery and dilution integrity

The peak reactions of plasma samples (n = 6) pointed earlier abstraction were compared to those pointed afterward extraction to ascertain the extraction recapture of analytes after EDTA plasma.[24] The recovery rates were 73.1, 74.3, 70.7, and 69.1% at the LQC-I, MQC, and HQC points, correspondingly. According to Table 6, the mean recovery was 71.8% with a %CV of 3.2%. The recovery rate for the internal standard was determined to be 73.4%.

The experiment to determine dilution integrity was conducted at a concentration that was three times the ULoQ. The indicated back intended concentration for dilution QC sections stood inside 85 to 115% of the supposed significance after 1/5, 1/10, and 1/20 dilution, as demonstrated in Table 7. The %CV was ≤8.1.

Stability

Assessments were conducted on matrix-based and aqueous samples. The stock solutions remain constant for 26 days at 2 to 8ºC and for 8 hours at room temp. Stock dilution in 50% methanol stayed constant for 8 hours at ambient temp. Stability evaluations were conducted using freshly produced QC trials alongside freshly spiked calibration principles. The analyte remained stable for 4 hours and 50 minutes at room temp. and through 6 freeze-thaw cycles. The tests remained stable for 48 hours and 24 minutes in the autosampler at 5°C. The injection's reproducibility is assessed for 48 hours and 13 minutes. Matrix stability was assessed for 30 days at -20 and -50ºC. No degradation of analytes occurred during the stability period. The outcomes in Table 8 occurred 85 to 115%.

CONCLUSION

An approach was developed to influence mefenamic acid in rat plasma employing liquid chromatography with electrospray ionization tandem mass spectrometry. The run time is 3.2

minutes. The method has a LoD of 20.659 ng/mL, ensuring high selectivity. The extraction method consistently recovers analyte and ISTD by minimal plasma interference & matrix effect despite using a low sample volume of 100 µL. The validated method can analyze samples from pharmacokinetic and toxicology investigations. Mefenamic acid D4, the deuterated compound of the analyte, is a reliable ISTD that does not affect analytical accuracy. The high throughput approach can lower handling time and analyse over 80 samples at once.

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