ejpmr, 2016,3(7), 16-21.

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

A VALIDATED REVERSED PHASE HPLC ASSAY FOR THE DETERMINATION OF MEFENAMIC ACID IN HUMAN PLASMA

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Article Received on 25/04/2016 **Article Revised on 15/05/2016** **Article Accepted on 05/06/2016**

ABSTRACT

A simple and precise reversed-phase high performance liquid chromatographic (HPLC) assay for the measurement of mefenamic acid level in human plasma, using diclofenac as an internal standard (IS), was developed and validated. Plasma samples containing mefenamic acid were spiked with the IS then extracted with acetonitrile and reconstituted in mobile phase. The compounds of interest were efficiently separated on Atlantis dC_{18} column at room temperature and detected with photodiode array detector set at 278 nm. The mobile phase consisted of 0.025 M dibasic potassium phosphate (pH = 6.0, adjusted with phosphoric acid) and acetonitrile (65:35, v:v) and was delivered at a flow rate of 1.5 ml/min. The relationship between mefenamic acid concentration in plasma and peak area ratio of mefenamic acid/ IS was linear ($R^2 \geq 0.9987$) in the range of 0.05 – 10 µg/ml, the intra- and inter- day coefficient of variations (CV) were ≤ 5.3% and ≤ 7.2%, respectively, and the intra- and inter- day bias was ≤ 6% and ≤ 8%, respectively. Mean extraction recovery of mefenamic acid and the IS from plasma samples was 99% and 92%, respectively. The method was used to assess the stability of mefenamic acid in human plasma under various conditions encountered in the clinical laboratory. Mefenamic acid was stable for 8 weeks at -20°C (≥ 95%), 24 hours at room temperature (≥ 96%) and after 3 freeze-thaw cycles (≥ 96%) in unprocessed plasma samples, and for 48 hours at -20°C (≥ 90%) and 24 hours at room temperature (≥ 92%) in processed samples.

KEYWORDS: Mefenamic acid, Diclofenac, Human plasma, HPLC.

INTRODUCTION

Mefenamic acid, 2-[(2, 3-dimethyl-phenyl) amino] benzoic acid, is a nonsteroidal antiinflammatory, analgesic and antipyretic drug used to treat different types of pain, including menstrual pain. It inhibits the cyclooxygenase COX-1 and COX-2 and reduces the formation of prostaglandins and leukotrienes.[1] Mefenamic acid is absorbed rapidly with a peak plasma concentration of 20 µg/ml within 2-4 hours after ingestion of a single oral therapeutic dose of 250 mg.[2]

Several analytical methods for the determination of mefenamic acid in pharmaceutical preparations and biological matrixes have been reported. They include high-performance liquid chromatography (HPLC)[3-7], capillary electrophoresis (CE)[8], gas chromatographic-mass spectrometric (GC-MS)[9], ion pair system[10], and liquid chromatography-tandem mass spectrometry (LCMS/MS).[2,11] The most commonly used assay for pharmacokinetic and bioequivalence studies is HPLC. Although LCMS/MS assays have several advantages over HPLC assays, many laboratories prefer HPLC assays because of lower cost and better availability. Some of the reported HPLC methods used relatively large plasma volume[6,12] or had low recovery rate.[7,11] The most used extraction method has been liquid-liquid extraction[5,11,13], however, solid phase extraction[2,10,14] and protein precipitation[7] have been also used.

The present paper describes a precise and rapid HPLC assay that requires 0.5 ml human plasma and is based on simple liquid-liquid extraction. The method was fully validated and used to determine stability of mefenamic acid under various laboratory conditions.

MATERIAL AND METHODS

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 (Waters Associates Inc, Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat and photodiode array detector. A reversed-phase Atlantis dC_{18} column (4.6 x 150 mm, 5-µm) and a guard pak pre-column module with a Nova-pak C18 insert were used for the separation. Data were collected with a Pentium IV computer using Empower Chromatography Manager Software.

Chemical and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Mefenamic acid pure powder (99.9%)

was obtained from Riyadh Pharma Company, Riyadh, Saudi Arabia. Diclofenac sodium salt (USP reference standard) was purchased from Sigma-Aldrich Co, Steinheim, Germany. Acetonitrile, methanol, phosphoric acid (all HPLC grade), and dibasic potassium phosphate were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA).

Chromatographic conditions

The mobile phase was composed of 0.025 M dibasic potassium phosphate (pH 6.0, adjusted with phosphoric acid) and acetonitrile (65:35, v:v). Before delivering into the system, it was filtered through 0.45 µm polyetersulfone membrane and sonicated under vacuum for 5 minutes. The analysis was carried out under isocratic conditions using a flow rate 1.5 ml/min. A photodiode array detector set at 278 nm was used for recording chromatograms.

Preparation of standard and quality control samples

Stock solutions (1.0 mg/ml) of mefenamic acid and diclofenac (internal standard, IS) were prepared in mobile phase and methanol, respectively. They were then diluted with blank human plasma or mobile phase, respectively, to produce working solutions of 10 µg/ml. Nine calibration standards in the range of 0.05 – 10 µg/ml were prepared in human plasma. Four quality control (QC) samples (0.05, 0.15, 5, and 9 µg/ml) were prepared in human plasma. QC samples were vortexed for one minute, and then 0.5 ml aliquots were transferred into Teflon-lined, screw-capped, borosilicate (13 x 100mm) glass culture tubes and stored at -20ºC until used.

Sample preparation

Aliquots of 0.5 ml of calibration curve, QC, or volunteer samples were allowed to equilibrate to room temperature. To each tube, 60 µl of the IS working solution were added and the mixture was vortexed for 10 seconds. After the addition of 5.0 ml of acetonitrile, samples were vortexed again for 10 min and then centrifuged for 10 min at 4200 rpm at ambient temperature. The organic layer was carefully collected and dried under a gentle stream of nitrogen at 40ºC and the residue was reconstituted in 250 µl mobile phase and centrifuged at 16000 rpm for 5 min. The supernatant was transferred into auto-sampler vials and 100 µl were injected into the chromatograph with a run time of 10 min.

Stability studies

Three QC samples (0.05, 0.15 and 9 µg/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, five aliquots were stored at –20°C for eight weeks before being processed and analyzed and five aliquots were processed, reconstituted and stored at room temperature for 24 hours or 48 hours at –20°C before analysis. 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.[15] The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Optimal experimental conditions consisted of a mobile phase composed of 0.025 M dibasic potassium phosphate (pH=6.0) and acetonitrile (65:35, v:v) and a flow rate of 1.5 ml/min. Mefenamic acid, IS and components of plasma exhibited a well-defined separation within a ten minutes run. The retention times of IS and mefenamic acid were around 5.6 and 8.8 minutes, respectively.

Specificity

In order to confirm method specificity, we screened six batches of blank plasma and eight frequently used medications (acetaminophen, ranitidine, nicotinic acid, ascorbic acid, caffeine, ibuprofen, omeprazole and itraconazole) for potential interference. No interference was found in plasma and none of the drugs co-eluted with mefenamic acid or the IS. **Figure 1** depicts a representative chromatogram of drug free human plasma used in preparation of standard and QC samples.

Limit of detection & quantification and linearity

The limit of quantification (LOQ) was defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤ 20%). The LOQ of mefenamic acid in human plasma was 0.05 µg/ml. The limit of detection (three times the base line noise) was 0.02 µg/ml. Linearity of mefenamic acid was evaluated by analyzing ten curves of nine standards over the range of 0.05-10 µg/ml. **Figure 2** represents an overlay of chromatograms of extracts of 0.5 ml human plasma spiked with the IS and one of nine concentrations of mefenamic acid. The peak area ratios were subjected to regression analysis. The mean regression equation was Y = 0.8025 X + 0.0110. The accuracy of the calibration curves was confirmed by back-calculating the concentration of mefenamic acid in human plasma from the calibration curves (**Table 1**). All calculated concentrations were well within the acceptable limits.

Accuracy and precision

Accuracy and precision were determined for four QC (0.05, 0.15, 5 and 9 µg/ml). The inter-day precision and accuracy of the assay were determined over three different days. The intra-day (n=10) and inter-day (n=20) imprecision was ≤ 5.3% and ≤ 7.2%, respectively. The intra-day and inter-day bias was in the range of ≤ 6% and ≤ 8%, respectively. The results are summarized in **Table 2**.

Recovery

The absolute recovery of mefenamic acid was assessed by direct comparison of absolute peak areas of plasma and mobile phase samples, using five replicates of 4 QCs (0.05, 0.15, 5 and 9 µg/ml). Similarly, the recovery of the IS was determined by comparing the peak area of the IS in five aliquots of human plasma spiked with 0.6 µg of IS with the peak areas of equivalent samples prepared in mobile phase. The results are presented in **Table 3.**

Stability

Mefenamic acid and IS stabilities in processed and unprocessed plasma samples were investigated **(Table 4)**. No significant change in chromatographic behavior of mefenamic acid or the IS were observed. Mefenamic acid in processed samples (0.05, 0.15 and 9 µg/ml) was stable for at least 24 hours at room temperature (≥ 92%) and 48 hours at -20°C (≥90%). Mefenamic acid in unprocessed plasma samples was stable for at least eight weeks at -20°C (≥ 95%), for at least 24 hours at room temperature(≥ 96%), and after three freeze-and thaw cycles (≥96%). Further, mefenamic acid and IS stock solutions were stable for at least 48 hours at room temperature (100%) and for at least 8 weeks at –20 C (98%). Mefenamic acid and IS working solutions were stable for at least 2 weeks at –20 C (101% and 91%) respectively.

Figure Captions

Fig. 1 Representative chromatogram of a drug-free human plasma. The arrows indicate the retention times of mefenamic acid (8.8 min) and the internal standard (IS), diclofenac (5.6 min).

Fig. 2 Overlay of chromatograms of extracts of 0.5 ml blank human plasma and human plasma spiked with one of nine concentrations of mefenamic acid, 0.05, 0.1, 0.2, 0.3, 0.6, 3, 4, 8 and 10 µg/ml and with the internal standard (IS).

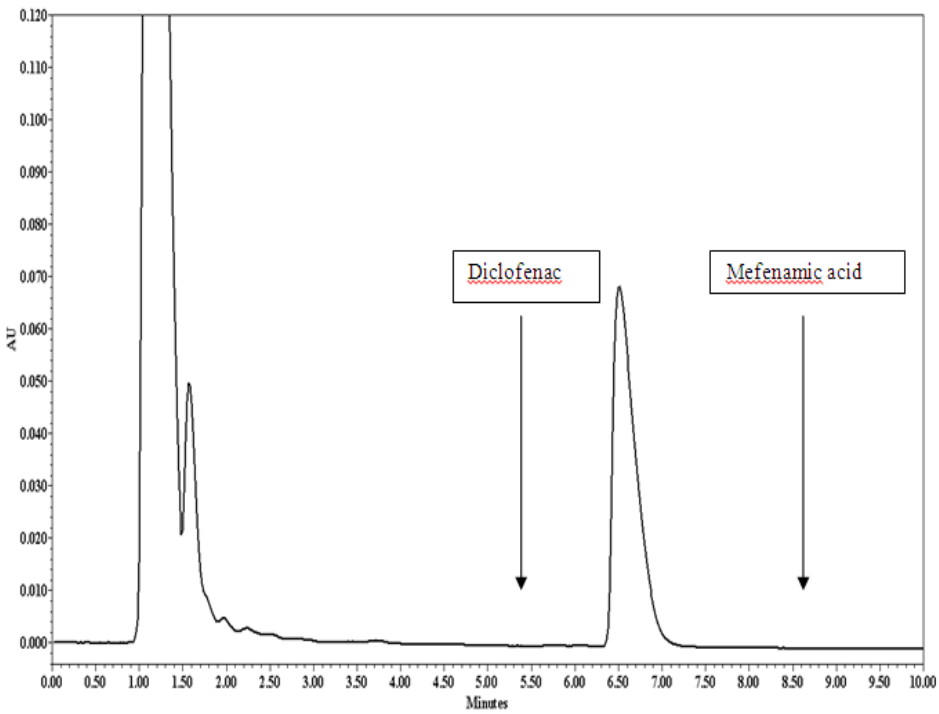

Fig. 1:

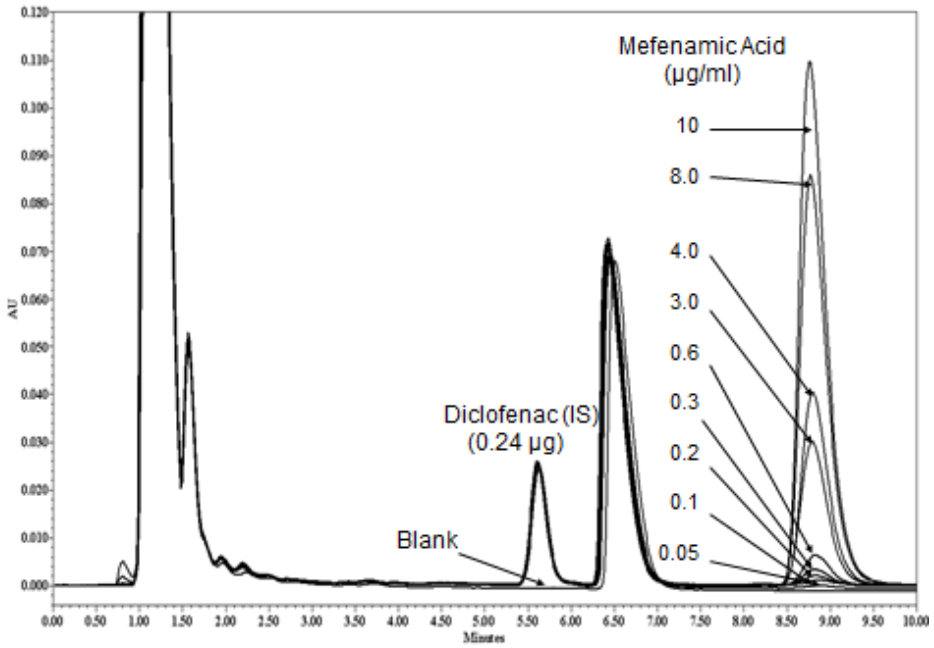

Fig. 2:

Table 1: Back-calculated mefenamic acid concentrations from ten calibration curves

Nominal Level (µg/ml)	Calculated level (µg/ml)		CV (%)	Accuracy (%)
		Mean (SD)		
0.05		0.0508 (0.0030)	5.9	102
0.1		0.0992 (0.0067)	6.7	99
0.2		0.1997 (0.0107)	5.4	100
0.3		0.3114 (0.0184)	5.9	104
0.6		0.6042 (0.0218)	3.6	101
3.0		2.9223 (0.0976)	3.3	97
4.0		4.0102 (0.0845)	2.1	100
8.0		8.0869 (0.1547)	1.9	101
10.0		9.9426 (0.1089)	1.1	99

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100
Accuracy, measured level divided by nominal level x 100.

Table 2: Intra and inter-day precision and accuracy of mefenamic acid assay

Nominal Level (µg/ml)	Measured level Mean (SD) (µg/ml)		CV (%)	Accuracy (%)
Intra-day (n=10)				
0.05	0.0509	(0.0020)	3.9	102
0.15	0.1539	(0.0081)	5.3	103
5	5.3093	(0.0563)	1.1	106
9	9.4398	(0.0804)	0.9	105
Inter-day (n=20)				
0.05	0.0541	(0.0039)	7.2	108
0.15	0.1535	(0.0088)	5.8	102
5	5.2221	(0.1002)	1.9	104
9	9.3726	(0.0985)	1.1	104

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100
Accuracy, measured level divided by nominal level x 100.

Table 3: Recovery of mefenamic acid and the internal standard from 0.5 ml of human plasma

Concentration (µg/ml)	Human plasma*	Mobile phase*	Recovery** (%)
Mefenamic acid			
0.05	12611 (1079)	12791 (397)	99
0.15	35922 (905)	36740 (739)	98
5	1198716 (23198)	1194514 (2219)	100
9	2223906 (47956)	2278439 (28235)	98
Internal standard			
0.6	302994 (2858)	330774 (845)	92

* Mean peak area (SD), n = 5. ****** Recovery is ratio of mean peak area in human plasma divided by mean peak area in mobile phase x 100

Table 4: Stability of mefenamic acid in human plasma

Stability (%)							
Nominal level	**Unprocessed**		**Processed**		**Freeze-Thaw**		
(µg/ml)	**24 hrs RT**	**8 wks -20°C**	**24 hrs RT**	**48 hrs -20°C**	**Cycle**		
					1	**2**	**3**
0.05	103	97	100	99	112	94	108
0.15	98	95	96	90	107	95	98
9	96	100	92	92		97	96

Stability (%) = 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 24 hours at room temperature (24 hrs RT), after freezing at –20ºC for 8 weeks (8 wks. –20ºC), or after 1-3 cycles of freezing at -20ºC and thawing at room temperature; or processed and then analyzed after storing for 24 hours at room temperature (24 hrs RT) or 48 hours at –20ºC (48 hrs -20ºC).

CONCLUSION

The described HPLC assay is precise and rapid. It requires only 0.5 ml plasma and utilizes a simple and convenient method for sample preparation. The assay was applied to monitor stability of mefenamic acid under various conditions generally encountered in the clinical laboratories.

ACKNOWLEDGEMENT

Authors wish to thanks administration of research center in king faisal specialist hospital and research center for their support and cooperation.

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