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A Validated Reversed-phase HPLC Assay for the Determination of Meloxicam in Human Plasma

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ABSTRACT

A simple and precise reversed-phase high performance liquid chromatography (HPLC) method for the determination of meloxicam in human plasma was developed and validated. Using piroxicam as an internal standard (IS), separation was achieved on Symmetry shield RP-18 column. 0.5 ml plasma samples were prepared by protein precipitation using trifluoroacetic acid and acetonitrile. The mobile phase consisted of 0.025 M dibasic potassium phosphate (pH=6.0, adjusted with phosphoric acid), methanol, and acetonitrile (73:5:22, v:v:v) and was delivered at a flow rate of 1.5 ml/min. Eluents were measured using photodiode array detector set at 355 nm. Under these conditions, no interference in blank plasma or of commonly used drugs was observed. The relationship between the concentration of meloxicam in plasma and peak height ratio of meloxicam to the IS was linear over the range of 0.05-2.0 µg/ml. Intra-day and inter-day coefficient of variations (CV) and biases were ≤ 6.0% and ≤ 8.6%, and ±5.9% and ±5.3%, respectively. Extraction recovery of meloxicam and the IS from plasma was ≥80% and 98%, respectively. The method was applied to assess the stability of meloxicam under various clinical laboratory conditions. In processed samples, meloxicam was stable for at least 24 hours at room temperature (≥ 82%) and 48 hours at -20C (≥ 95%). In unprocessed sample it was stable for at least 24 hours at RT (≥ 82%), 16 weeks at -20ºC (≥ 87%), and after three freeze-thaw cycles (≥ 90%). The method is suitable for clinical and bioavailability investigation involving meloxicam concentration in the therapeutic range.

Keywords: Meloxicam, Piroxicam, Human plasma, HPLC

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INTRODUCTION

Meloxicam (CAS:71125-38-7, 4-hydroxy-2-methyl-N-(5-methyl-2-thiazoly)-2H-1,2-benzo thiazine 3-carboxamide-1,1-dioxide) is an oxicam derivative that belongs to nonsteroidal anti-inflammatory drugs and is widely used as pain reducing agent in various medical conditions[1,2]. Its oral bioavailability is around 90%, with a peak plasma concentration of 1.1±0.4 µg/ml at about 3-4 hours after the ingestion of 15 mg single therapeutic oral dosage[3,4].

Several analytical methods have been reported for the determination of meloxicam in pharmaceutical formulations, individually[5,6] or in combination with others agents, such as paracetamol[7], pridinol mesylate[8], and piroxicam[9]. Meloxicam level in human plasma or serum has been mainly determined by high performance liquid chromatography (HPLC) with ultraviolet detection (UV)[10-14], or liquid chromatography mass spectrometry (LCMS)[15,16]. Sample preparation included pre-column enrichment[13], solid phase extraction techniques[14] and protein precipitation[11,12]. Although LCMS assays have several advantages over HPLC assays, many laboratories prefer HPLC- UV assays because of low cost and better availability.

The present paper describes a simple and reliable HPLC method for the quantitative determination of meloxicam levels in human plasma. The method is based on protein precipitation using trifluoroacetic acid and acetonitrile and requires 0.5 ml human plasma. The method was applied to determine the stability of meloxicam under various conditions encountered in the clinical laboratory.

MATERIALS AND METHOD

Apparatus

Chromatography was performed on HPLC system (Waters Alliance 2695 Separation Module) consisting of quaternary pump, auto-sampler, column thermostat and a photodiode array detector. A reversed phase Symmetry shield RP18 (4.6 x 150 mm 5-µm) steel column with a guard pre-column Nova-pak C18 (4-µm) insert were used for the separation. The data were collected with a Pentium IV computer using Millennium Chromatography Manager Software.

Chemicals and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Meloxicam and piroxicam (USP reference standard) were purchased from Sigma-Aldrich Co, Steinheim, Germany. Acetonitrile, trifluoroacetic acid, methanol, and 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 Milli-Q System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase was composed of 0.025 M dibasic potassium phosphate (pH 6.0, adjusted with phosphoric acid), methanol, and acetonitrile (73:5:22, v:v:v). Before delivering into the system, the mobile phase 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 of 1.5 ml/min at room temperature and a run time of 10 minutes. Chromatograms were recorded at 355 nm using a photodiode array detector.

Preparation of standard and quality control samples

Stock solution of meloxicam (1mg/ml) and piroxicam (100 µg/ml) were prepared in methanol. They were then diluted with blank human plasma or mobile phase respectively, to produce working solutions of 10 µg/ml and 5 µg/ml, respectively. Nine calibration standards in the range of 0.05-2.0 µg/ml were prepared in human plasma. Four quality control (QC) samples (0.05, 0.15 1, 1.8 µg/ml) were prepared in human plasma. The solutions were vortexed for one minute, then 0.5 ml aliquots were transferred into Teflon-lined, screw-capped, borosilicate, 13 x 100 mm glass culture tubes (Fisher Scientific Co., Fairlawn, NJ, USA), and stored at –20ºC until used.

Sample preparation

Aliquots of 0.5 ml of calibration curve samples or QC samples were allowed to equilibrate to room temperature. To each tube, 50 µl of the IS working solution (5 µg/ml in mobile phase) were added and the mixture was vortexed for 10 seconds. Then, 100 µl of trifluoroacetic acid was added and vortexed for 10 seconds. After the addition of 5 ml of acetonitrile, the mixture was vortexed again for 5 min and then centrifuged for 15 min at 4200 rpm at room temperature. The organic layer was carefully collected into a clean tube and dried under a gentle stream of nitrogen (36ºC), and the residue was reconstituted in 150 µl mobile phase and centrifuged at 10000 rpm for 10 min at room temperature. The supernatant was transferred into an auto-sampler vial, and 100 µl were injected into the HPLC system with a run time of 10 min.

Stability studies

Forty aliquots of three QC concentrations (0.05, 0.15, and 1.8 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 (counter stability, 24 hours at room temperature), five aliquots were stored

at –20°C for sixteen weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed, reconstituted, and stored at room temperature for 24 hours or 48 hours at –20°C before analysis (auto-sampler 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 were 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[17]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Under the optimal experimental conditions, consisting of a mobile phase of dibasic potassium phosphate 0.025M (pH=6 adjusted with phosphoric acid), methanol, and acetonitrile (73:5:22, v:v:v) delivered at flow rate 1.5 ml/min, meloxicam, piroxicam and plasma components exhibited a well-defined chromatographic separation within 10 minutes run. The retention times of piroxicam (IS) and meloxicam and were around 4.8 and 7.6, respectively.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. In order to confirm method specificity, we screened six batches of blank plasma and eight frequently used medications (acetaminophen, omeprazole, ranitidine, nicotinic acid, ascorbic acid, caffeine, ibuprofen, and diclofenac) for potential interference. No endogenous component co-eluted with meloxicam and IS. Figure 1 depicts a representative chromatogram of drug free human plasma used in preparation of standard and QC samples.

Linearity, Accuracy, and Precision

Linearity of meloxicam was evaluated by analyzing ten curves of nine standard concentrations over the range of 0.05-2.0 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 meloxicam. Peak height ratios were subjected to regression analysis. Mean (SD) of co-efficient of correlation (r^2) was 0.9989 (0.0009). The accuracy of the calibration curves was confirmed by back-calculating

meloxicam concentration from the calibration curves (Table **1)**. All calculated concentrations were well within the acceptable limits (≤ 15%, except LLOQ ≤ 20%). Accuracy and precision were determined using four QCs (0.05, 0.15, 1.0, and 1.8 µg/ml). Inter-day precision and accuracy of the assay were determined over three different days. Intra-day (n=10) and inter-day (n=20) precision was ≤ 6.0% and ≤ 8.6%, respectively. The intra-day and inter-day accuracy (as bias) was ±5.9% and ±5.3%, respectively. Figure 2 represents an overlay chromatogram of four quality control samples and the results are summarized in Table 2.

Table 1: Back-calculated meloxicam concentrations from ten calibration curves

Nominal level (µg/ml)	Calculated level (µg/ml) Mean (SD)	CV (%)	Bias (%)
0.05	0.0517 (0.0082)	15.8	3.4
0.1	0.1004 (0.0077)	7.7	0.4
0.2	0.2019 (0.0058)	2.9	0.9
0.4	0.4053 (0.0143)	3.5	1.3
0.8	0.7905 (0.0143)	1.8	-1.2
1.2	1.2116 (0.0346)	2.9	1.0
1.4	1.3900 (0.0324)	2.3	-0.7
1.6	1.5925 (0.0304)	1.9	-0.5
2.0	2.0104 (0.0285)	1.4	0.5

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

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

Nominal level(µg/ml)	Measured level (µg/ml) Mean (SD)	CV (%)	Bias (%)
Intra-day (n=10)			
0.05	0.0491 (0.0029)	6.0	-1.8
0.15	0.1588 (0.0037)	2.4	5.9
1.0	1.0475 (0.0472)	4.5	4.8
1.8	1.8639 (0.0593)	3.2	3.5
Inter-day (n=20)			
0.05	0.0475 (0.0041)	8.6	-5.3
0.15	0.1567 (0.0051)	3.2	4.3
1.0	1.0553 (0.0535)	5.1	5.2
1.8	1.8379 (0.0534)	2.9	2.1

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

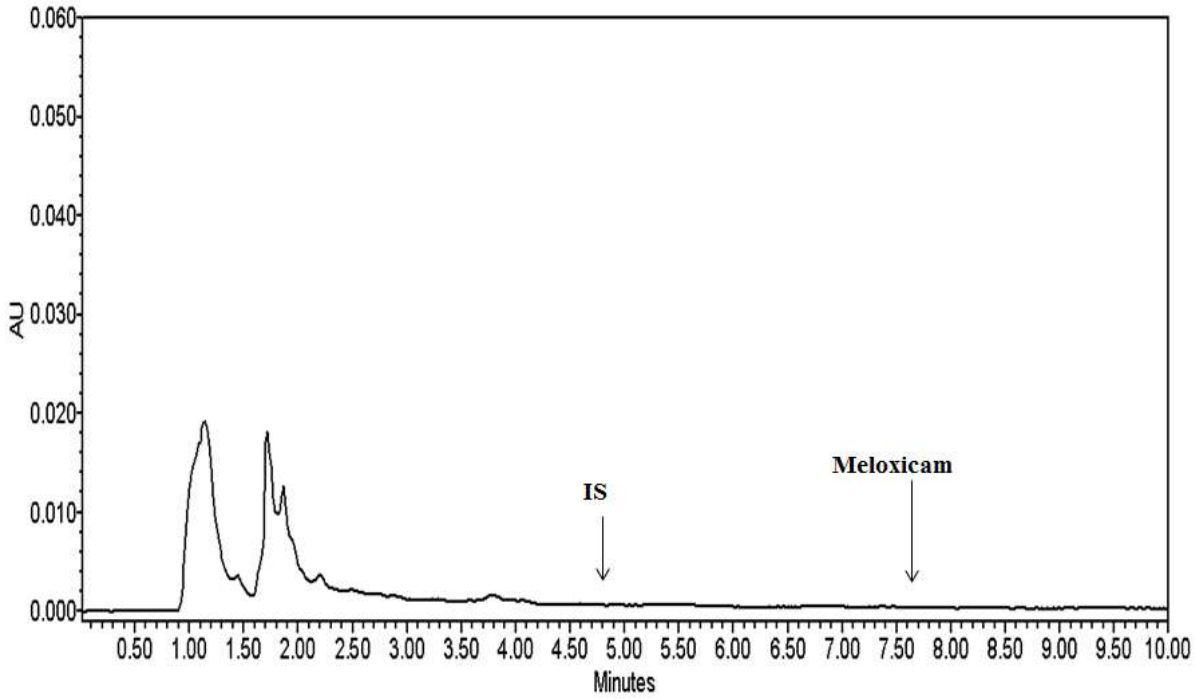

Figure 1: Representative chromatogram of a drug-free human plasma. The arrows indicate the retention times of meloxicam (7.6 min) and the internal standard (IS) (4.8 min).

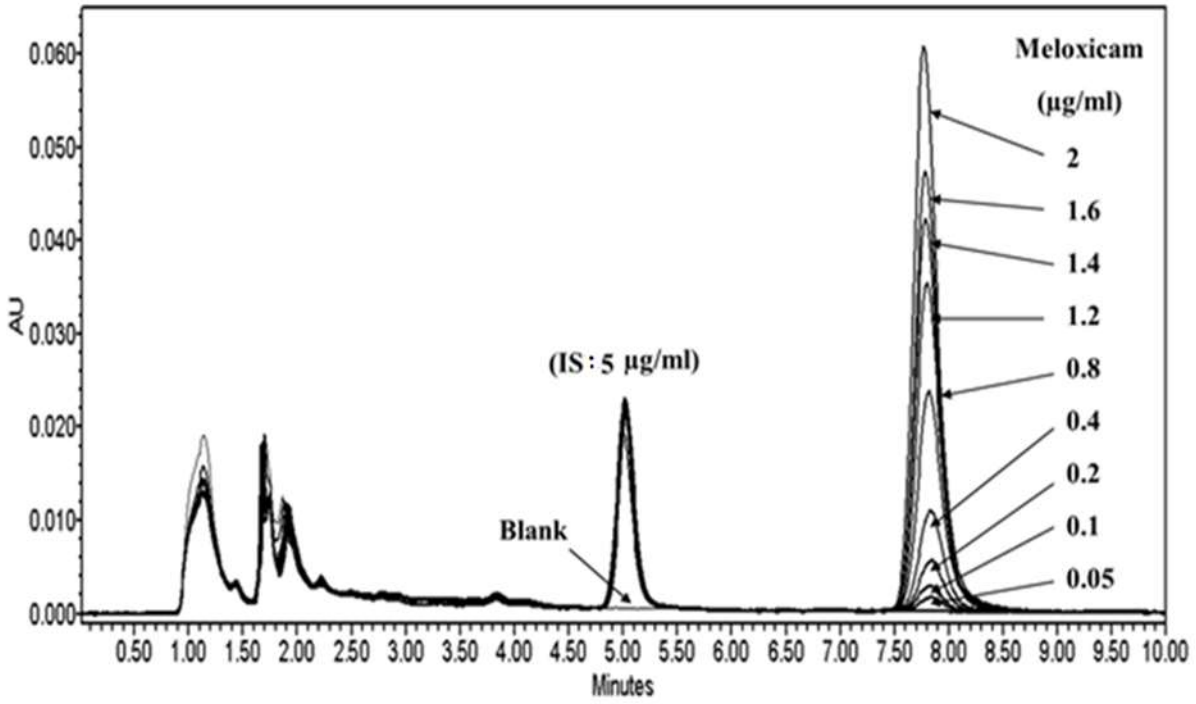

Figure 2: Overlay of chromatograms of extracts of 0.5 ml human plasma blank (B), spiked with one of nine concentrations of meloxicam (0.05, 0.1, 0.2, 0.4, 0.8, 1.2, 1.4, 1.6, and 2 µg/ml) and 5 µg/ml internal standard (IS).

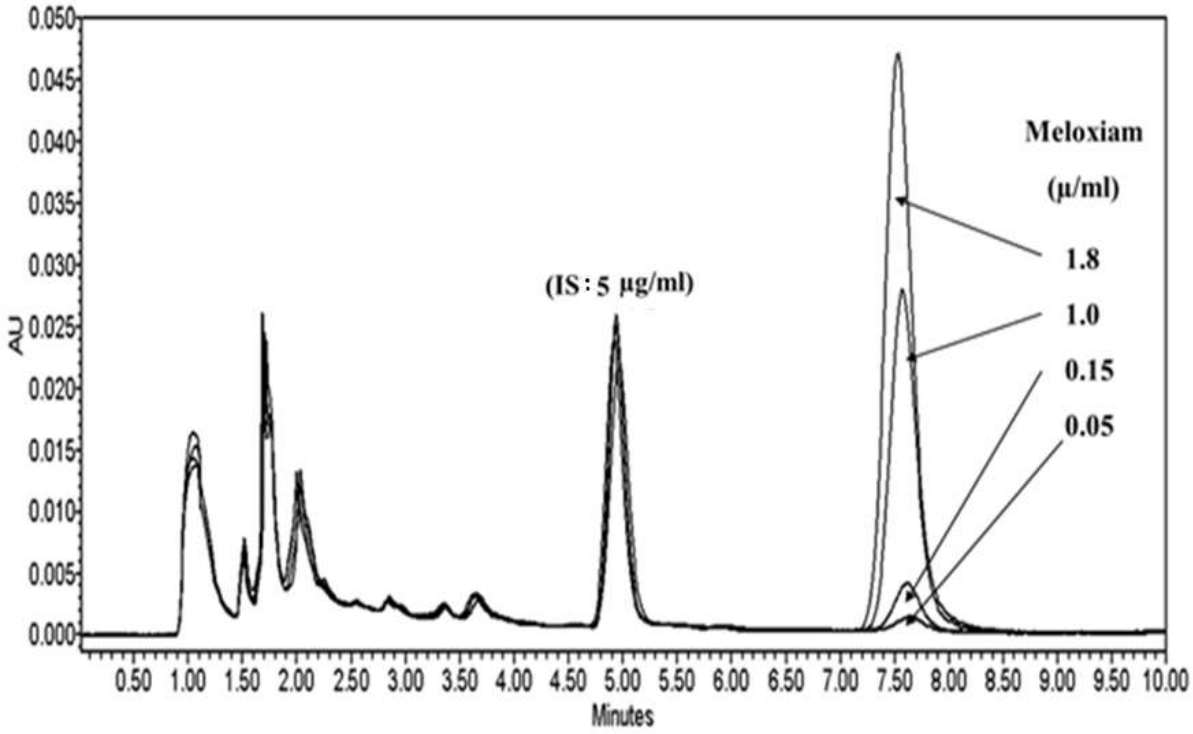

Figure 3: overlay of chromatograms of four human plasma quality control samples containing meloxicam (0.05, 0.15, 1.0, and 1.8 µg/ml) and 5 µg/ml internal standard (IS).

Recovery

Recovery of meloxicam was assessed by direct comparison of peak area of plasma and mobile phase samples, using five replicates of each of four QCs (0.05, 0.15, 1.0 and 1.8 µg/ml). Similarly, the recovery of the IS was determined by comparing the peak height of the IS in five aliquots of human plasma spiked with 50 µl of IS (5 µg/ml) with the peak areas of equivalent samples prepared in the mobile phase. The results are presented in **Table 3.**

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

Concentration (µg/ml)	*Human Plasma	*Mobile Phase	**Recovery (%)
Meloxicam 0.05	1368 (47)	1507 (50)	91
0.15	4061 (161)	5089 (61)	80
1.0	25245 (354)	31611 (531)	80
1.8	49894 (4243)	57337 (291)	87
Internal Standard 5.0	201182 (3278)	205368 (1347)	98

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

Stability

Meloxicam stabilities in processed and unprocessed plasma samples were investigated. No

significant change in chromatographic behavior of meloxicam or the IS were observed. Meloxicam in processed samples (0.05, 0.15, and 1.8 µg/ml) was found to be stable for 24 hours at room temperature (≥ 95%) and 48 hours at -20°C (≥86%). Meloxicam in unprocessed plasma samples was stable for at least 24 hours at room temperature (82-90%), for at least sixteen weeks at -20°C (≥ 87%), and after three freeze-and thaw cycles (≥ 90%). Table 4 summarizes the results of stability studies.

Table 4: Stability of meloxicam under various clinical laboratory conditions

Nominal level(µg/ml)	Unprocessed		Processed		Freeze-Thaw		
	24 hrs RT	16 wks 20 °C	24 hrs RT	48 hrs 20 °C			
0.05	82	112	95	93	90	94	90
0.15	90	92	102	92	102	99	96
1.80	86	87	101	86	104	96	97

Data represents 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 24 hours at room temperature (24 hrs RT), or after freezing at –20 ºC for 16 weeks (12 wks. –20 ºC), or 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).

CONCLUSION

In summary, the described HPLC method for determining therapeutic range meloxicam concentration in human plasma is simple, precise, and accurate. It has been applied for studying meloxicam stability under various clinical laboratory conditions. It is suitable for clinical and bioavailability investigations.

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