A SIMPLE, SPECIFIC, AND PRECISE HPLC METHOD FOR THE MEASUREMENT OF MELOXICAM IN BIOLOGICAL FLUIDS

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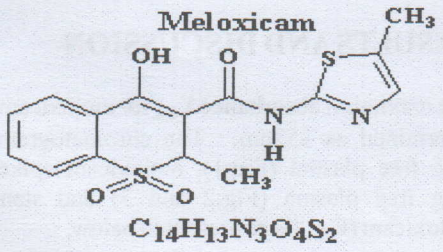
ABSTRACT: An HPLC assay method for the measurement of meloxicam in biological fluids was developed and validated at UVAS, Lahore and Quality Assurance Laboratory, Lahore College for Women University, Lahore. Meloxicam (Sigma) was used as external standard. The mobile phase comprising of phosphate buffer and acetonitrile (42:62, v/v) was pumped into Water 1525 Binary HPLC Pump at the rate 0,5ml/min. Separation was achieved by using a reversed phase C18 column (Phenomenex, particle size 5 µm; 4.6 mm × 150 mm).The meloxicam spiked in plasma was separated by a simple solvent extraction process.. The samples were introduced through an injector valve with a 10 µl sample loop. Oven temperature was set at 25 °C. The meloxicam was detected at 352 nm by using a Water 2487 dual absorbance detectors. The distinct similar peaks were visible in chromatograms of meloxicam external standard and meloxicam separated from plasma at retention time of around 7.4 minutes. Similarity between peaks indicated specificity. Eighty eight---ninety percent recovery of meloxicam from the plasma indicated accuracy. The intra- and inter-day assay had indicated precision. The method was linear and reproducible in the range of 0.08 - 6 µg/mL (r^2 = 0.999). This HPLC method was successfully applied for measurement of meloxicam in different biological samples.

Keywords: *Meloxicam; Simple Sample preparation, HPLC; C.18 and UV-detection*

INTRODUCTION

Non-steroidal Anti-Inflammatory Drugs (NSAIDs) are the agents having analgesic, antipyretic, anti-inflammatory and antiplatlets effects. These reduce pain, fever and inflammation in humans and animals. NSAIDs have now become among the most frequently used drugs (Wynne 1993). These drugs have analgesic, antipyretic action at lower doses and anti-inflammatory actions at higher doses (Neal *et al.*, 2005). NSAIDs are usually used as an adjunct to antimicrobial therapy in veterinary practice (Shpigel, 1994 and Deleforge *et al.*, 1994.)

Meloxicam [4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine -3-carboxamide-1,1-dioxide, UH-AC 62 XX; is a NSAID of the acidic enolcarboxamide class.



$C_{14}H_{13}N_3O_4S_2$

It has been reported as a safe substitute of diclofenac sodium which has been banned for veterinary use in Pakistan and India due to its relay toxicity associated with the catastrophic decline in vulture population of the subcontinent. (Oaks, *et al.*, 2004, Oaks 2006, Swan *et al.*, 2006).

Meloxicam preferentially inhibits cyclooxygenase-2, which is induced by inflammatory stimuli in pathophysiological conditions, rather than cyclooxygenase-1, which is responsible for physiological processes, (Churchill *et al.*, 1996).Despite the structural relationship to other NSAIDs the introduction of the methyl group in the thiazolyl moiety of meloxicam has facilitated the formation of metabolites that undergo fast elimination, leading to a shorter $t_{1/2}$ (half life), in comparison with piroxicam and tenoxicam. The metabolites do not change renal blood flow and therefore have no capability for nephrotoxicity (Schmid *et al.*,1995a). These findings are in accordance with the observations in the rat kidney during sub acute and chronic toxicity studies, where no nephrotoxic effects could be detected after therapeutic doses (Woolf, 1989; Schmid *et al.*, 1995). Meloxicam is registered for use in small animals in the United

Kingdom and USA. The government of Pakistan has recently registered meloxicam injections for veterinary use.

Some HPLC methods for determination of meloxicam in plasma have been reported. (Hormazabal *et al.*, 2006; Swan *et al.*, 2006; Velpandian *et al.*, 2000; Schmid *et al.*,1987). These methods required elaborate sample preparation, conventional extractions, dryings, reconstitutions, pre-column enrichment and radiant elusion processes. The method reported by Hormazabal *et al.*, (2006) required Liquid Chromatography-Mass Spectrometry.

The HPLC Method described below was developed and validated at Department of Pharmacology and Toxicology, UVAS Lahore/ Quality Assurance Laboratory, Lahore College for Women Universty, Lahore, was simple, specific, precise, accurate, reproducible and quicker.

MATERIALS AND METHODS

Experimental Drugs / Chemicals: Meloxicam standard was procured from the company Sigma which was employed as reference external standard in HPLC assay. Injectable formulation of Meloxicam manufactured by Intas Pharmaceutical Limited Matoda 382210 Dist. Ahmedabad, India was arranged. The acetonitrile, phosphoric acid (E. Merck Germany) and water used were of HPLC grade. All other chemicals of reagent grade were used.

Sample preparation: Stock solution of standard meloxicam was prepared in acetonitrile at concentration of 1mg/ml.

Sample-A: The solution at concentration of 5µg/ml was prepared with external standard meloxicam. It was subjected to same process up to filtration through 0.22 µm filter, as for samples described below.10µl of the aliquot was injected into HPLC system for the analysis.

Sample-B: Drug free human plasma was spiked with stock solution of standard meloxicam (1mg/ml) to prepare sample at concentration of 5µg/ml. HPLC grade Acetonitrile (1 ml) was added to 1ml plasma. The mixture was subjected to vortex mixing at high speed for 3 min, and then ultra centrifuged at 8000 xg for 15 min. The clear supernatant 1 mL mixed well with 1 mL of HPLC grade water and filtered through 0.22 µm filter. 10 µl of the aliquot was injected into HPLC system for the analysis.

Sample-C: Drug free human plasma was subjected to same process as for samples described above 10µl of the aliquot was injected into HPLC system for the analysis.

HPLC method: The maximum absorbance λ_{max} for meloxicam was determined by scanning in UV-Visible range of wave length. It was used as external standard. 10µl of sample (A) was injected in the HPLC system. The mobile phase comprising of phosphate buffer and acetonitrile (42:62, v/v) was pumped into Water 1525 Binary HPLC Pump 1525 at the rate 0,5ml/min Separation was achieved by using a reversed phase C18 column (Phenomenex, particle size 5 µm; 4.6 mm × 150 mm).The samples were introduced through an injector valve with a 10 µl sample loop Oven temperature was set at 25 °C. The meloxicam was detected at 352nm by using a Water 2487 dual absorbance detectors.. The distinct peak was visible in chromatograms of meloxicam external standard. The retention time observed for meloxicam was around 7.4 minutes. The sample B and C were treated in a similar fashion.

The method was validated to ascertain accuracy, specificity, precision and reproducibility. The samples at concentration of 6, 5,4,3,2.5,2,1,0.5,0.2,0.1,0.08,0.06 and 0.04 µg /ml were prepared by spiking meloxicam stock solution in the drug free human plasma. Similar samples were prepared for external standard by use of mobile phase. Ten µl of all these samples were subjected to proposed method. Standard curves were constructed by plotting concentration versus area in the chromatogram. Interday and intra-day assay(s) were also done.
The stability of stock solution of meloxicam at concentration of 1 mg/ml was carried out for six weeks at 4 °C and25°C. Freeze- Thraw Cycle Stability study was done by checking stability of meloxicam in frozen plasma spiked with drug. The assay of samples at0.8 and 3 µg were carried on days 1, 2, 4, 8, 16 and 24. All the determinations of concentration of meloxicam in plasma were performed by taking five reading.

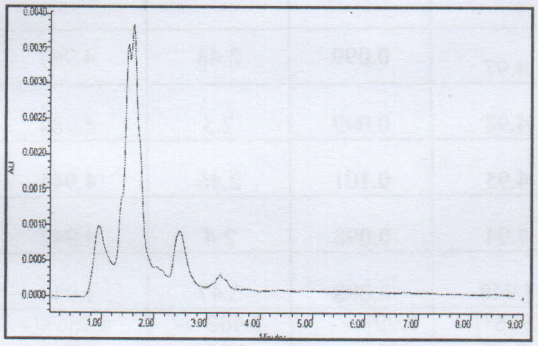
RESULTS AND DISCUSSION

The maximum absorbance λ_{max} for meloxicam was determined as 352nm. The chromatograms of drug free plasma (Fig;1), meloxicam spiked in drug free plasma (Fig;2 and 3) and standard meloxicam (Fig;4) are presented below.

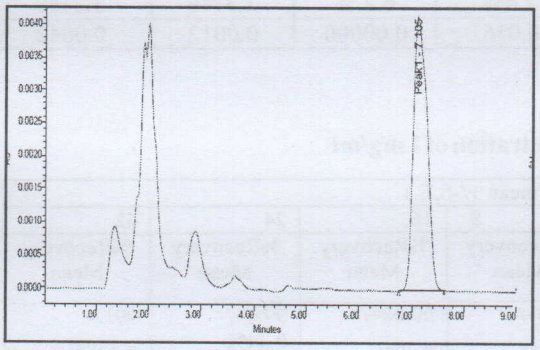
The distinct peaks were observed in the chromatograms of meloxicam separated from plasma at the retention time of around 7.4 minutes. The peak was similar to the peak observed with standard meloxicam. No interference peaks were observed with meloxicam spiked in plasma and meloxicam external standard at retention time of around 7.4 minutes, thus showing specificity.

The calibration curve was made and shown in Figure.5 .This curve showed linearity over a range of 0.08µg----6µg. with correlation coefficient > 0.99.

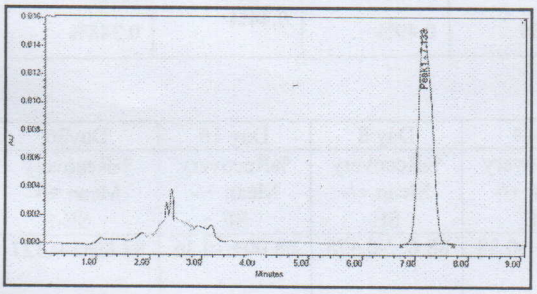
The limits of detection and quantification for meloxicam were 0.06 and 0.08µg/ml, respectively.



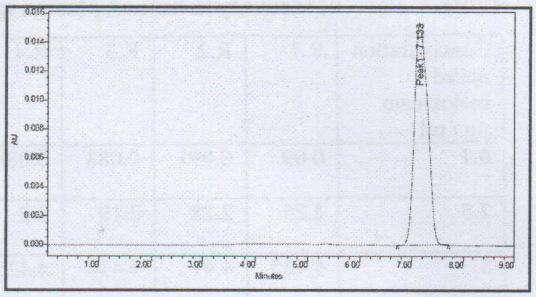
Fig; 1:Representative Chromatogram of blank plasma



Fig; 2:Representative Chromatogram of plasma spiked with meloxicam at 0.8 µg/ml

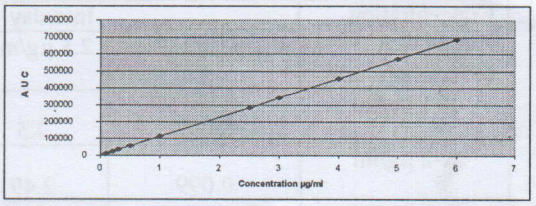


Fig; 3:Representative Chromatogram of plasma spiked with meloxicam at 3µg/ml



Fig; 4:Representative Chromatogram of standard meloxicam 3 µg/ml

Slope = 0.11467



Fig; 5 Standard curve for meloxicam spiked in plasma

The data regarding meloxicam recovered from plasma spiked with the drug is presented in the table 1. The Software SPS10 was used for statistical analysis. The recovery of 88.4% to 90.5% indicated that method was accurate. The pooled RSD of 7.93 indicated method was precise. The Intraday and intra day assay were carried out with pure meloxicam. The results presented in table2. These results had shown recovery in the range of 98.52-99.12 indicating that method was accurate, reproducible and precise for determination of pure meloxicam.

The results (table 3) of stability studies had indicated that stock solution of meloxicam @ concentration of 1 mg/ml was stable at least for six weeks at 4 °C and 25 °C. So there was no influence of storage on results.

The recovery of meloxicam near to 90% during study of Freeze- Thaw Cycle indicated that sample of plasma collected after administration of drug to animals could easily be stored at -20 °C for a period of one month (table 4). It is better to perform analysis of meloxicam in plasma within four weeks of collection.

Table. 1: Percent recovery in human plasma spiked with meloxicam external standard

Concentration added meloxicam µg / ml	R.1	R.2	R.3	R.4	R.5	Mean R (+/-S.D.)	% R	C.V%(RSD)	Variance
0.1	0.09	0.091	0.088	0.088	0.091	0.089+/- 0.01	89%	11.24%	0.00011
2.5	2.25	2.28	2.19	2.2	2.27	2.23+/- 0.19	90.5%	8.5%	0.04
5	4.435	4.41	4.418	4.425	4.43	4.42+/- 0.18	88.4%	4.04%	0.03

R=Recovery of meloxicam in µg / ml C.V. coefficient of variation =RSD = Relative standard deviation

Table 2: Intraday and intra day variability of samples prepared with meloxicam external standard

Concentration Added meloxicam / ml	Interday			Intraday		
	0.1 µg/ml	2.5 µg/ml	5 µg/ml	0.1µg/ml	2.5µg/ml	5 µg/ml
R-1 µg/ml	0.097	2.5	4.97	0.099	2.48	4.964
R-2 µg/ml	0.099	2.49	4.98	0.099	2.5	4.984
R-3 µg/ml	0.101	2.46	4.95	0.101	2.46	4.944
R-4 µg/ml	0.098	2.47	4.94	0.098	2.4	4.942
R-5 µg/ml	0.098	2.47	4.948	0.098	2.47	4.948
Mean R (+/- S.D.)	0.0986+/- .0.0014	2.478+/- 0.015	4.956+/- .0.13	0.099+/- .0011	2.462+/- 0.034	4.956+/- 0.065
% R	98.6%	98.52%	99.12.%	99. %	98.52%	99.12.%
C.V%(RSD)	1.42.%	0.61%	2.6%	6.2.%	1.37%	1.31%
Variance	0.000002	0.00022	0.016	0.00000	0.0012	0.0042

R=Recovery of meloxicam in(µg) µg / ml

Table 3: Stability stock solution of meloxicam @ concentration of 1 mg/ml

	Mean (%)concentration of meloxicam recovered (mean +/-S.E.)						
Days	1	2	4	8	16	24	42
	%Recovery Mean	%Recovery Mean	%Recovery Mean	%Recovery Mean	%Recovery Mean	%Recovery Mean	%Recovery Mean
4 °C	100.16+/- 0.444%	99.48+/- 0.53%	99.08+/- 0.43	99.0+/- 0.53%	99.08+/- 0.49%	99.0+/- 0.44%	99+/- 0.348%
25 °C	100.16+/- 0.444	99.9+/- 0.53%	99.88+/- 0.43%	9.33+/- 0.53%	99.28+/- 0.49%	99.20+/- 0.44%	99.13+/- 0.348%

Table 4: Freeze- Thaw Cycle

Plasma defreeze.	Day 1	Day 2	Day4	Day 8	Day 16	Day30
Plasma spiked with meloxicam and stored @-20 °C	%Recovery Mean +/- SE	%Recovery Mean +/- SE	%Recovery Mean +/- SE	%Recovery Mean +/- SE	%Recovery Mean +/- SE	%Recovery Mean +/- SE
Freeze- Thaw Cycle@3 µg	90.6+/-0.56	90.05+/-0.43	89.44+/-0.38	88.6+/-0.378	88.00+/-0.36	87.89+/-0.327
Freeze- Thaw Cycle @0.8 µg	88.6+/-1.03	90.05+/-0.91	89.40+/-1.04	88.6 +/-0.51	90.05+/-0.61	89.44+/-1.04

CONCLUSION: The above validated HPLC method was simple, specific, precise accurate, reproducible and quicker. This method was successfully used for measurement of meloxicam in plasma of dog, horses, donkey, buffalo and goats during research work on pharmacokinetics of meloxicam in different species.

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