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Short communication

Development and validation of a new high-performance liquid chromatographic estimation method of meloxicam in biological samples

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Abstract

A simple, HPLC method was developed to estimate meloxicam (COX-2 inhibitor) using piroxicam as the internal standard. The mobile phase containing methanol, acetonitrile and an aqueous solution of diammonium hydrogenorthophosphate (50 m*M*) in the ratio of 4:1:5 was pumped at the rate 1 ml/min. Lichrocart RP-18 (125×4 mm) was used as an analytical column and the analytes were detected at 364 nm using a UV detector. Acidified plasma samples were extracted with chloroform, evaporated to dryness, reconstituted in the mobile phase and then a volume of 10 μl of the prepared sample was injected in the column. The retention time of meloxicam and piroxicam was found to be 2.7 and 1.9, respectively. This method showed an accuracy of 102.3% at 0.52 μg/ml and was capable of detecting a minimum concentration of 0.029 μg/ml meloxicam from biological samples. The analytical method was successfully utilized for estimating meloxicam in biological samples.

Keywords: Meloxicam

1. Introduction

Meloxicam (Fig. 1), chemically: 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide is a newer NSAID in the group of enolic acids found to preferentially inhibit cycloxygenase-2 (COX-2). In vitro, meloxicam is found to be three times more effective in inhibiting the inducible COX-2 in cultured guinea pig peritoneal macrophages as compared to constitutive COX-1 of these cells [1]. Meloxicam was also reported to be a potent inhibitor of prostaglandin E_2 biosynthesis in vitro and in vivo. Engelhardt [2] extensively studied the pharmacodynamics of meloxicam on carrageenan induced edema, yeast induced pyrexia, visceral pain reflex and the uricosuric effect in rats. Its effects on bradykinin, PAF and acetylcholine induced bronchospasm in guinea pigs was also studied. Meloxicam was found to be effective against both osteoarthritis and rheumatoid arthritis in individual clinical trials [3]. In our laboratory, we were interested in evaluating the pharmacokinetic and pharmacodynamic profile of various formulations of meloxicam. The methods available in the literature for the estimation of meloxicam from biological fluids were complex

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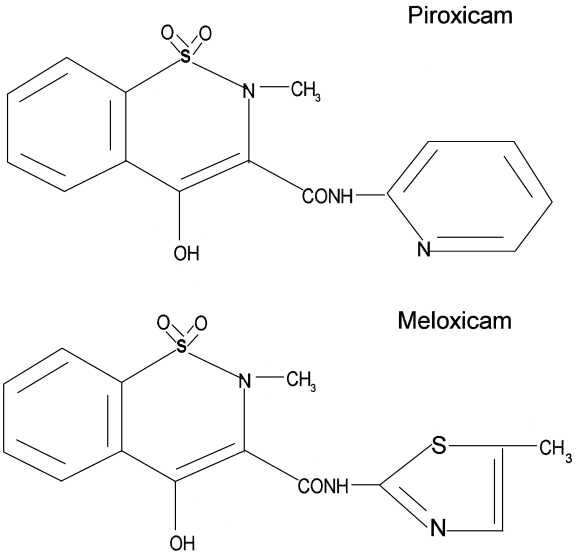

Fig. 1. Chemical structures of piroxicam (internal standard) and meloxicam.

and lacked an appropriate internal standard to control volume variability [4,5]. The objective of this study was to develop a simple, economic, rapid and sensitive estimation method for meloxicam from biological samples by high-performance liquid chromatography with UV detection.

2. Experimental

2.1. Chemicals

Meloxicam (standard) was given as a gift by M/s Unichem Laboratories Ltd. (Bombay, India) and piroxicam was obtained from Cipla Ltd. (Bombay, India). All solvents used were of HPLC-grade and were purchased from Spectrochem (Bombay, India) and E. Merck India Ltd. (Bombay, India). Diammonium hydrogenorthophosphate (anhydrous) was obtained from Loba Chemie (Bombay, India). Water (18.2 MΩ) used in the mobile phase was freshly prepared from Milli-Q (Millipore, Bedford, USA).

2.2. Equipment

A Waters (Model 510) solvent delivery pump (Waters, Milford, MA, USA) connected to a Rheodyne (No 77251) injector (California, USA) fitted with a 20-μl loop and a Waters 996 photodiode array detector (Waters) were used. For the data acquisition and integration, Waters Millennium-32 software operated by Pentium II (350 MHz) microprocessor (Intel Inc., USA) was used.

2.3. Chromatographic conditions

Lichrocart (125×4 mm; 5 μm particle size) RP-18 (Merck KGaA, Darmstadt, Germany) was used for analytical separation and Lichrocart (4×4 mm; 5 μm particle size) RP-18 (Merck KGaA, Darmstadt, Germany) used as a guard column. The mobile phase consisted of an aqueous solution of diammonium hydrogenorthophosphate (50 m*M*), methanol and acetonitrile in the ratio of (5:4:1, v/v). After mixing the solutions, the mobile phase was degassed under negative pressure through a 0.22-μm Millipore (Bedford, MA USA) filter. The flow-rate of the mobile phase was adjusted to 1 ml/min. The instrument was operated at an ambient temperature of 30.3 ±1.1°C. The UV detection was achieved at 364 nm and peak purity analysis was performed over a wavelength range of 200–400 nm.

2.4. Stock solutions and standards

Stock solutions were prepared by dissolving 5.2 mg of meloxicam in 100 ml of methanol. The internal standard was prepared by dissolving 9.19 mg of piroxicam in 100 ml of methanol. The stock solutions were stored in a freezer for a period of 3 weeks. On the assay day, the stock solution was brought to room temperature and diluted serially using the mobile phase. The meloxicam stock solution was diluted in the concentrations of 52, 5.2 and 0.52 μg/ml. Piroxicam was used in the concentrations of 9.1 and 0.91 μg/ml.

2.5. Extraction procedure

From the collected samples of heparinized blood, 100 μl of plasma was removed after centrifugation at 1300 *g* for 10 min, piroxicam (46ng) was added followed by 100 μl of 1 *M* HCl. The deproteinised plasma was extracted with 2 ml of chloroform. The

contents were vortexed for 3 min and 1 ml of the lower (organic) phase was removed and placed in a clean glass tube. The chloroform was evaporated at 60°C under a constant flow of nitrogen. The residue was reconstituted with 100 μl of the mobile phase and 10 μl of it was injected into the analytical column.

2.6. Validation parameters

2.6.1. Linearity

Volumes of 10 μl of each meloxicam standard of (52, 5.2, 0.52 and 0.052 μg/ml) were added into separate clean tubes using a Hamilton syringe (Reno, NV, USA). To each, 5 μl of internal standard solution (9.19 μg/ml) was added, and the solutions were dried in a stream of nitrogen. To this, drug free human plasma (100 μl) was added and vortexed. The calibration curves were plotted every day and subjected to analysis to determine the linearity.

2.6.2. Recovery

Meloxicam standards were added to the plasma in the final concentration of 5.2, 52 and 520 ng/0.1 ml level spiked with 46 ng of piroxicam. This was verified using water instead of plasma. The samples were subjected to the assay procedure described previously.

2.6.3. Determination of LOD and LOQ

The limit of detection (LOD) was set four times above the baseline noise (signal-to-noise>4). A reproducible lowest possible concentration, linear with the calibration curve having C.V.>20% was considered as the LOQ. The LOQ was repeated several times for confirmation.

2.7. Intravenous pharmacokinetic study

Male Wistar rats weighing 200 g were obtained from the Central Animal Facility of the All India Institute of Medical Sciences, New Delhi, India. They were anaesthetized with urethane at the dose of 1.2 ml/kg (25%, v/v). The jugular vein was cannulated and heparinized saline (0.5 ml) was injected. Initially, 0.4 ml of blood was collected and the plasma was separated by centrifugation. Meloxicam dissolved in 100 μl of chloroform (420 μg/ml) was placed in a tube and the chloroform removed by placing under a flow of nitrogen. The residue containing 42 μg of meloxicam was dissolved in 0.2 ml of plasma separated previously and injected through the jugular vein followed by 0.2 ml heparinized saline in the test animal. At intervals of 5, 15, 30, 60, 120, 240 and 360 min, 0.4 ml of blood was withdrawn and replaced by an equal volume of heparinized saline. The blood samples were centrifuged at 1300 *g* for 10 min. Plasma (100 μl) was subjected to HPLC as described previously.

2.8. Results

Under the given circumstances meloxicam and piroxicam showed retention times of 2.7 and 1.9 min, respectively (Fig. 2d). The capacity factors (k') of the meloxicam and piroxicam were 3.5 and 2.1, respectively; Using the 5σ method, the efficiency of the column (HETP) was calculated and the number of plates were found to be more than 1500 for meloxicam. The resolution of the meloxicam peak was calculated to be 2.83.

In the intravenous pharmacokinetic study, no interfering peaks were observed, at the retention times of meloxicam and its internal standard piroxicam even after 4 h in the detection wavelength used. The peak separation was found to be excellent in terms of peak sharpness, separation factor and plates (Fig. 2b and c).

The limit of detection (LOD) in this method was found to be 0.029 μg/ml and the limit of quantification (LOQ) was found to be 0.1 μg/ml. The inter-intra day variability (C.V.), was found to be 1.82 and 1.94%, respectively.

The concentration versus response (area) of the meloxicam in the standards was found to have linearity in the range of 0.52–52 μg/ml (r=0.9999). The response of the internal standard was linear in the range of 0.92–92 μg/ml (r=0.9982). The internal standard had a recovery of 94.9% at 92 μg/ml and 92.3% at 0.92 μg/ml.

The accuracy of the meloxicam estimation was found to be 102.3% at the level of 0.52 μg/ml, 104% at the level of 5.2 μg/ml and 97% at 52 μg/ml. The reproducibility of meloxicam standard at the concentration of 0.52 μg/ml was found to be

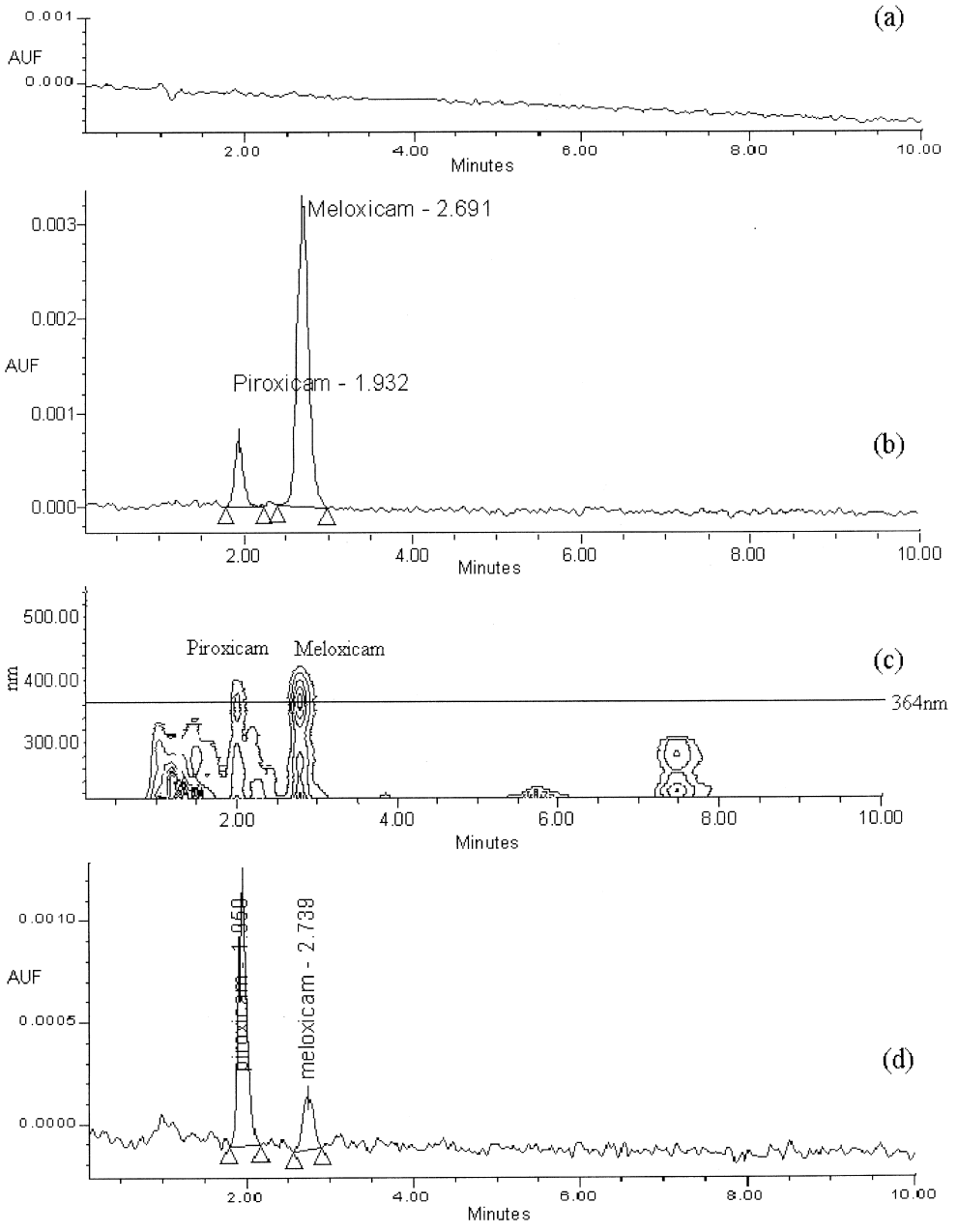

Fig. 2. Representative chromatograms of (a) Extracted blank rat's plasma, (b and c) plasma (meloxicam 1.78 μg ml^{-1}) obtained 15 min after a 0.21 mg kg^{-1} i.v. bolos dose and its contour plot showing well resolved peaks, (d) human plasma spiked with (5.2 ng/0.1 ml) of meloxicam and piroxicam (46 ng/0.1 ml).

0.52±0.01 μg/ml (C.V.<2%). The recovery of the internal standard from the extracted sample was found to be 92.7% (42.61±3.4ng) when 46 ng of the internal standard was added.

After the intravenous injection, meloxicam (0.21 mg/kg) showed two-compartment kinetics (Fig. 3). There was an initial rapid fall in the concentration followed by a long elimination profile. Using PCNONLIN version 4.0 (SCI Software, NC, USA) operated by a Pentium II (350 MHz) microprocessor (Intel

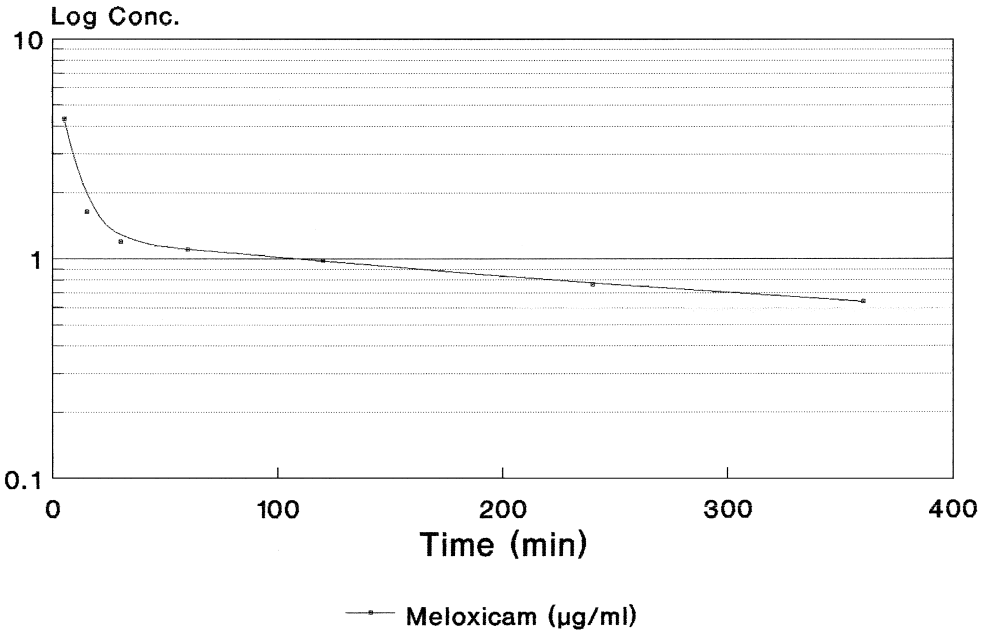

Fig. 3. Meloxicam concentration versus time in rat plasma sample after 0.21 mg/kg (in 200 µl plasma) intravenous bolus dosing.

Inc., USA) the pharmacokinetic parameters were calculated (Table 1) and the biexponential function best describing the plasma concentration–time data was

$$C_p = 8.3109e^{-11.8462t} + 1.233e^{-0.113525t}$$

3. Discussion

A pharmacokinetic study of intravenous meloxicam was conducted previously in horses by Lees et al. [4] using HPLC connected with spectroflurometric detection and a cocktail of solvents as the mobile phase. The column used was kept at 40°C and fitted with a precolumn switching module. Pharmacokinetic studies conducted by Busch et al. [5] also used a combination of methanol, acetonitrile, glacial acetic acid and heptanesulphonoic acid with UV detection at 355 nm using an external standard method. To the best of our knowledge, there is no simple method available for the estimation of meloxicam using an appropriate internal standard.

Table 1
Calculated i.v. pharmacokinetic parameters in rats ($N=4$)

Parameter	Estimate	Standard error
$AUC_{0-\infty}$ (µg ml^{-1} h)	11.56680	0.3227
Alpha (h^{-1})	11.8462	0.263563
Beta (h^{-1})	0.113525	0.004223
A (µg ml^{-1})	8.310921	0.171958
B (µg ml^{-1})	1.233479	0.013719
C_{max} (µg ml^{-1})	9.5444	0.178638
Cl	0.172909	0.004829
AUMC (µg h^2 ml^{-1})	95.766952	6.332697
V_{ss}	1.431591	0.018607
MRT (h)	8.279465	0.320080

This study was undertaken to simplify and to optimize a method for the rapid estimation of meloxicam in small volume biological fluids (<150 µl). In the alkaline mobile phase the separation of meloxicam was found to be excellent as compared to the reported acidic mobile phases in the C_{18} column. In the mobile phase used for the separation, meloxicam showed a maximum absorbance at a specific wavelength of 364 nm. Therefore, the same wavelength was used for analysis to achieve maximum sensitivity (Fig. 2c). Piroxicam, a close structural analog of meloxicam was found to be an ideal internal standard in terms of chromatography, extractability and detection. Moreover, it also showed good UV spectrum which can be detected at 364 nm

(Fig. 2). As far as extraction is concerned, a simple chloroform extraction produced a good recovery after denaturing the plasma proteins with 1 *M* HCl. The separated chloroform layer can be easily evaporated by blowing nitrogen at a slightly elevated temperature of 50–60°C. Injecting standards in pure methanol produced slight peak broadening where as it was not observed with the mobile phase.

In the pharmacokinetic studies we have adopted a modified approach by mixing the drug with the plasma of the test animal and re-injecting the same in the animal. This method can be utilized for other drugs which are not soluble in water and ethanol, thereby the interference of other solvents in the pharmacokinetic study can be avoided. To conclude, a simple, economic, modified procedure was developed to estimate meloxicam in biological samples by HPLC. This method would be useful for the biopharmaceutical studies on meloxicam.

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