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Sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of meloxicam in human plasma

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Abstract

Meloxicam was quantified in human plasma after a single 15 mg oral dose of the drug was given to 26 healthy volunteers. An Applied Biosystems Sciex API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using TurboIonSpray (TIS) in the positive ion mode, was used. Protein precipitation with acetonitrile was followed by C_{18} reverse phase liquid chromatography and tandem mass spectrometry. The mean recovery for meloxicam was 92% with a lower limit of quantification of 8.96 ng/ml. Piroxicam was used as the internal standard. This assay method makes use of the increased sensitivity and selectivity of tandem mass spectrometry (MS–MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of meloxicam in human plasma than has previously been described.

Keywords: Meloxicam

1. Introduction

Meloxicam, 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl) - 2H - 1,2 - benzothiazine - 3 - carboxamide-1,1-dioxide, is a potent non-steroidal anti-inflammatory drug of the enolic acid class of oxicam derivatives. It is indicated for the treatment of rheumatoid arthritis, osteoarthritis, and other joint diseases. Its therapeutic benefits combined with a good gastrointestinal tolerability are well-documented [1–4].

A number of analytical methods were published for the determination of meloxicam in biological fluids, making use of reverse phase high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection (346–364 nm) [5–9]. Various sample preparation techniques were employed, including protein precipitation [6], protein precipitation combined with liquid–liquid extraction [9] resulting in limits of quantification ranging from 121 to 40 ng/ml.

Velpandian et al. developed a HPLC method for the determination of meloxicam in biological samples. They used piroxicam as the internal standard (I.S.), reverse phase chromatography and UV de-

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tection at 364 nm and were able to detect a minimum concentration of 29 ng/ml in plasma [5].

These methods were not sensitive enough for pharmacokinetic studies where plasma concentrations have to be assayed for a period of about five half-lives. It was therefore decided to develop a new method involving the use of a mass-selective detector with mass spectrometry–mass spectrometry (MS–MS) capabilities in tandem with liquid chromatography (LC) to increase the sensitivity and selectivity which would allow for more rapid chromatography and sample clean-up. This is the first description of an LC–MS–MS assay method for the quantitation of meloxicam in human plasma. The calibration range was between 8.96 and 2059 ng/ml. The lower limit of quantification (LLOQ) of this method was 8.96 ng/ml for meloxicam, which enables the determination of the analyte for the full indicated pharmacokinetic profile of the drug (120 h) allowing the determination of more than five half lives after a single oral dose of 15 mg meloxicam. The assay method is well suited for the analysis of samples in studies generating large numbers of samples.

2. Experimental

2.1. Materials and chemicals

A Phenomenex® Luna C_{18} (2) 5 μm, 150×2 mm analytical column (Torrance, CA, USA) was used for compound retention. The mobile phase consisted of acetonitrile–aqueous formic acid (0.2%) (65:35, v/v, pH 3.1), delivered at 0.3 ml/min by an Agilent Series 1100 quaternary pump at ambient temperature. Automatic injection (10 μl) was done by a Perkin-Elmer Series 200 autosampler. Detection of meloxicam and piroxicam (I.S.) was achieved using an Applied Biosystems API 2000 LC–MS–MS apparatus (Applied Biosystems, Ontario, Canada) fitted with a TurboIonSpray source.

HPLC-grade acetonitrile and methanol (B&J Brand™) were purchased from Baxter (Muskegon, MI, USA). Formic acid (BDH, UK) was used without further purification. A Millipore Elix 5 reverse osmosis and Milli-Q® (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA) were used to purify water.

Meloxicam was obtained from Cipla (Mumbai). Piroxicam was obtained from the FARMOVS-PAREXEL pure substance reference library.

2.2. Preparation of standards and quality control samples

A meloxicam stock solution was prepared by dissolving meloxicam in methanol to obtain a desired concentration. This stock solution was used to spike a pool of blank human plasma which was serially diluted with blank human plasma (1:1, v/v) eight times to obtain calibration standards (STDs) spanning a range between 2059 and 8.96 ng/ml ($2\times C_{max}$ and LLOQ). Similarly, quality control standards (QCs) were prepared (using the same methodology) spanning a range between 1719 and 11.7 ng/ml. Sufficient calibration standards and quality control standards were prepared to validate the method and to serve as standards and controls during the assay of all study sample batches. Aliquots of the standards and quality controls were stored together with the study samples at −20 °C until used for sample processing.

2.3. Extraction procedure

The plasma samples (100 μl) were transferred to 1.5-ml Eppendorf® microfuge tubes (Eppendorf, Hamburg, Germany) and acetonitrile (200 μl) containing the internal standard (300 ng piroxicam/ml acetonitrile) added. The samples were immediately vortexed for 20 s, and centrifuged at 8000×*g* for 10 min using an Eppendorf 5416 high-speed centrifuge. The supernatant layer (100 μl) was transferred to an autosampler vial containing a glass insert and 10 μl injected onto the HPLC column.

2.4. Mass spectrometry

Electrospray ionisation (ESI) was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 70 and 50 (respective arbitrary values). The heated nebulizer temperature was set at 400 °C. The pause time was set at 5 ms and the dwell

time at 150 ms. The collision gas (N_2) was set at 3 (arbitrary value). The Applied Biosystems API 2000 LC–MS–MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 352 to the product ion m/z 115 for meloxicam and the transition of the protonated molecular ion m/z 332 to the product ion m/z 95 for the I.S. The instrument response was optimised for meloxicam by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. The same methodology was used to optimise the response of the instrument for the I.S.

Fig. 1 shows the product ion mass spectra obtained from collision-induced dissociation of the protonated molecular ions of meloxicam and piroxicam. Plausible fragmentation patterns presented in Fig. 1 are suggested but not proven.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.0 software.

2.5. Validation

The method was validated by analysing plasma quality control samples five times at eight different meloxicam concentrations, i.e. 1719, 860, 430, 215, 107, 53.7, 25.1, and 11.7 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing nine different concentrations spanning the concentration range of 2059 to 8.96 ng/ml. Calibration graphs were constructed using a quadratic regression (weighted with 1/concentration^2) of the drug peak-area ratios of the product ions of the analyte to the internal standard versus nominal drug concentrations. Several regression types were tested and the quadratic regression (weighted with 1/concentration^2) was found to be the simplest regression, giving the best results ($r^2 =$ 0.9951, for intra-batch validation).

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated by extracting ''blank'' normal human plasma from 10 different sources, reconstituting the final extract in injecting solvent containing a known amount of the analyte and I.S., analysing the reconstituted extracts and then comparing the peak areas of the analyte and I.S. [10].

Absolute recoveries of the analyte were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with meloxicam. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted system performance verification standard mixtures (prepared in the injection vehicle) representing 100% recovery.

Fifteen stability samples of the same concentration were injected at intervals during the first two validation batches to simulate the time of a batch run. By regression analysis of the peak-area ratios the cumulative time can be established.

3. Results and discussion

The mean absolute recoveries of meloxicam determined in triplicate at 1719, 215 and 53.7 ng/ml were 88.2%, 94.8% and 92.8%, respectively. The mean absolute recovery of piroxicam was 96%.

No matrix effect for meloxicam was observed for 10 different plasma pools tested. The peak areas in the chromatograms of the 10 reconstituted samples had a coefficient of variation of 4.0% for meloxicam and 3.59% for the I.S. indicating that the extracts were ''clean'' with no co-eluting compounds influencing the ionisation of the analyte and I.S.

The much higher selectivity of MS–MS detection allowed the development of a very specific and rapid method for the determination of meloxicam in plasma.

The LLOQ, defined as that concentration of meloxicam which can still be determined with acceptable precision (C.V.% <20) and accuracy (bias $<20\%$) was found to be 8.96 ng/ml with a signal-to-noise ratio of 15. Results of the intra-batch and inter-batch validation assays presented in Tables 1 and 2 indicate a valid calibration range of 8.96–2059 ng/ml for meloxicam. The back-calculated quality control standard concentrations of meloxicam (eight batches) showing the repeatability of the method (inter-day variation) is summarised in Table 3.

On-instrument stability was inferred from stability samples that were prepared and included in the first two validation batches. No significant degradation

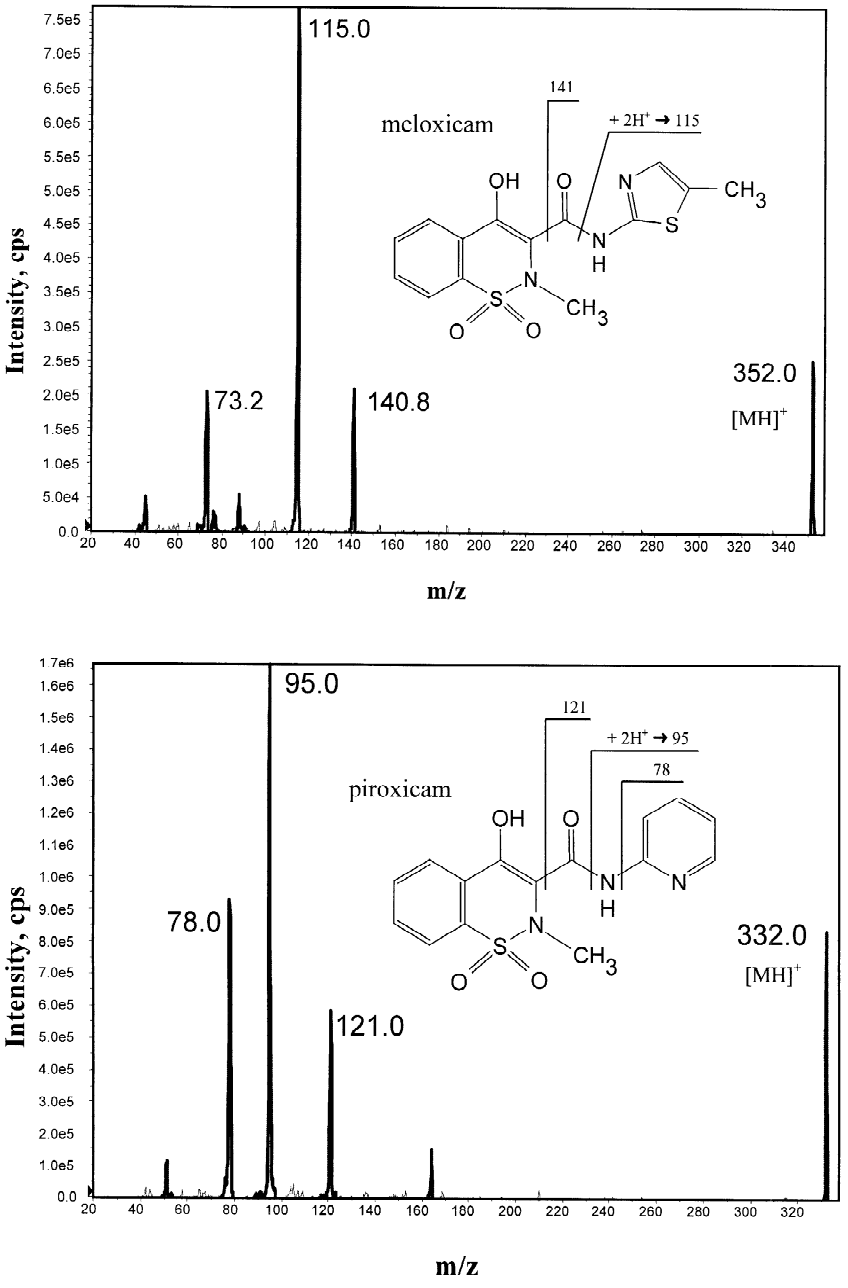

Fig. 1. Product ion mass spectra obtained from collision-induced dissociation of the protonated molecular ions of meloxicam and piroxicam.

could be detected in the samples (ambient temperature) left on the autosampler for at least 21 h.

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Table 1
Summary of the quality control standard results for meloxicam as obtained during the intra-batch validation

Meloxicam concentration added (ng/ml)	Meloxicam ($n=5$) mean concentration found (ng/ml)	Precision (RSD, %)	Recovery (%)
11.7	12.7	3.0	108.6
25.1	28.3	3.5	113.0
53.7	55.7	3.8	103.8
107	112	1.8	104.0
215	232	2.2	108.0
430	456	1.3	106.0
860	908	1.9	105.6
1719	1791	2.0	104.2

When testing extraction procedures it is important to look at the cost effect of the method to be developed. Protein precipitation is the most cost-effective method if compared with solid-phase and liquid–liquid extraction methods. Protein precipitation with acetonitrile was tested and average recoveries above 90% were found for both analytes.

Different solutions were tested for optimum ionisation of the analytes and it was found that 0.2% formic acid gave the best result. The best resolution and peak shape were obtained with a mobile phase consisting of acetonitrile and 0.2% formic acid (65:35, v/v).

Piroxicam is structurally related to meloxicam and was tested as a possible internal standard. Ionisation, retention and extraction characteristics were found to be similar to that of meloxicam.

The retention times for meloxicam and piroxicam were ~2.5 min and ~2 min, respectively (Fig. 2). A total chromatography run time of 3 min made it possible to analyse a large number of samples in a batch. Fig. 3 shows comparative chromatograms at a concentration of 8.96 ng/ml (the LLOQ) and of a study sample at the late elimination phase (120 h after dose) of the pharmacokinetic profile for the analyte (a blank sample is also shown).

The method was employed to analyse plasma samples containing meloxicam obtained after a single oral dose of 15 mg meloxicam per treatment in 26 healthy volunteers. Concentration vs. time profiles were constructed for up to 120 h (Fig. 4). The

Table 2
Summary of the quality control standard results for meloxicam as obtained during the two inter-batch validations

Meloxicam concentration added (ng/ml)	Meloxicam ($n=10$) mean concentration found (ng/ml)	Precision (RSD, %)	Recovery (%)
11.7	12.8	4.5	109.4
53.7	58.2	4.0	108.4
430	468	5.5	108.8
860	892	3.3	103.8

Table 3
Summary of the back-calculated quality control standard concentrations of meloxicam (eight batches) showing the repeatability of the method (inter-batch variation)

Meloxicam concentration added (ng/ml)	Meloxicam ($n=16$) mean concentration found (ng/ml)	Precision (RSD, %)	Recovery (%)
11.7	12.1	8.9	103.6
53.7	56.7	7.4	105.7
430	472	4.7	109.7
860	892	4.6	103.7

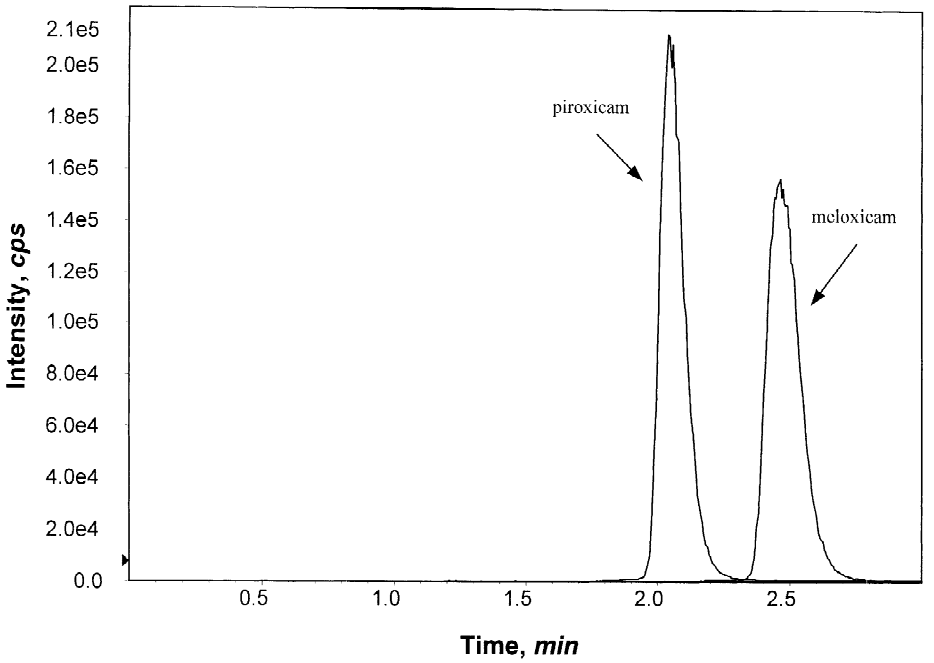

Fig. 2. A representative chromatogram of an extracted calibration standard to illustrate the retention times of the meloxicam (2059 ng/ml) and of the internal standard (piroxicam).

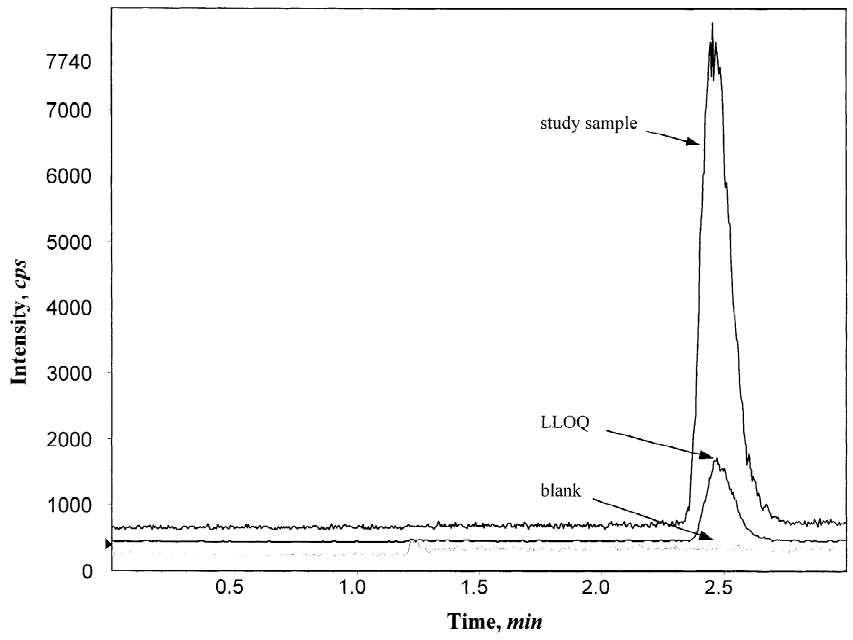

Fig. 3. High-performance liquid chromatograms of the calibration standard at the limit of quantification (LLOQ) containing 8.96 ng/ml meloxicam, of a blank sample and of a study sample at the late elimination phase (120 h after dose) of the pharmacokinetic profile for the analyte.

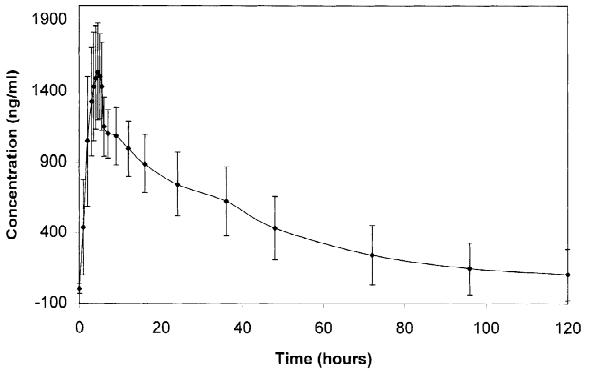

Fig. 4. A meloxicam plasma concentration vs. time profile as obtained after a single 15 mg oral dose of meloxicam (average of 26 subjects).

average maximum meloxicam plasma concentration was 1538 ng/ml. Meloxicam was rapidly absorbed leading to maximum plasma concentration at ~4.5 h. The elimination half-life of meloxicam was ~24 h.

4. Conclusion

A rapid, sensitive and highly selective method for the determination of meloxicam in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. This newly developed assay method was used in a clinical study in which 26 healthy volunteers were each given 15 mg meloxicam as a single oral dose. With an LLOQ of 8.96 ng/ml, pharmacokinetic profiles of the drug could be constructed for up to 120 h. The method is more selective than previously described methods and allows for a much higher sample throughput due to the short chromatography time (3 min) and simple sample preparation. Robust LC–MS–MS instrument performance was observed, with acceptable variation in instrument response within batches (no more than 5%). This method is an excellent analytical option for rapid quantification of meloxicam in human plasma.

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