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A simple and easy method for determination of meloxicam in rat muscle and plasma

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Abstract

The aim of this paper is to provide a simple and easy method for determination of meloxicam in rat muscle and plasma, based on dissolution of tissue samples with Solvable™ and measurement with a column-switching high performance liquid chromatographic system. After tissue and plasma samples were extracted with Solvable™ and methanol, samples were injected into a precolumn (Hypersil ODS) in 0.05 M phosphate buffer (pH 3), and after clean-up and enrichment the analytes were transferred to an analytical column (YMC Pack Pro C18) by back-flushing with 0.05 M phosphate buffer (pH 6)-methanol (60:40, v/v). The eluate was monitored with a UV-detector set at 360 nm. Coefficients of determination (r^2) for muscle and plasma were both more than 0.999. The limits of quantification (LOQ) in muscle and plasma were 50 ng/g and 20 ng/mL, respectively. The accuracy and precision were achieved in the range of 50-2500 ng/g in muscle and 20-2500 ng/mL in plasma. Our method could be successfully applied to evaluate the pharmacokinetics of topically administrated meloxicam.

Keywords : Meloxicam, Column-switching, Solvable™, Muscle

Introduction

Meloxicam is a nonsteroidal anti-inflammatory drug (NSAID) which inhibits cyclooxygenase (COX), the enzyme responsible for the first step in synthesis of various prostaglandins (PGs) from arachidonic acid. COX exists as two isoforms, COX-1, which is constitutively present in almost all cells and produces PGs concerned with protection of the gastric mucosa and kidney functions, and COX-2, which is induced by cytokines or hormones in inflammatory processes [1]. Although meloxicam selectively inhibits COX-2, the COX-1 isoform is also affected to some extent [2-5]. To overcome associated side effects and local disorders, we have paid attention to transdermal administration and have focused on analytical methods capable of determining drug levels in target tissues such as muscle to evaluate the pharmacokinetics of topically administrated meloxicam. To evaluate large numbers of samples, a simple and easy method would have obvious advantages. A num-

ber of approaches have been published for the determination of meloxicam in muscle, making use of radiation measurement with carbon-14-labeled meloxicam [6,7] and liquid chromatography-tandem mass spectrometry [8-11]. However, these methods are unsuitable for use with large numbers of samples because they employ carbon-14-labeled meloxicam that is not commercially available. Furthermore, preparation of samples in these methods is technical and tedious because of requiring manual operations such as homogenization, solvent or solid phase extraction and evaporation and so on.

Therefore, we have developed a simple and easy method for measuring meloxicam in rat muscle and plasma using a tissue solubilizer and automated column-switching high performance liquid chromatography (HPLC). As a tissue solubilizer, Solvable™, the proprietary name for a mixture of dodecyltrimethylamine oxide, secondary alcohol ethoxylate and sodium hydroxide, is used. By

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using this reagent, the extraction of drugs from tissues is very easy without physical homogenization, because tissues can be dissolved. The approach has already been successfully applied for determination of meso-tetra(hydroxyphenyl) chlorine and porfimer sodium in tissues and simple methods for measuring drugs in tissues have been established [12,13]. Moreover, the column-switching HPLC technique has many advantages, including ease of sample preparation and automated analysis. The automated column-switching liquid chromatographic system allows direct injection of samples with clean-up and enrichment steps performed by on-line liquid-solid extraction in a short precolumn, featuring exclusion of macromolecules and simultaneous extraction of low-molecular-mass analytes. The present report describes a simple and easy method for measuring meloxicam using Solvable™ and automated column-switching HPLC. This method has been successfully applied to provide pharmacokinetics data for diverse pharmaceutical studies.

Experimental

1. Materials and reagents

Meloxicam [4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide] was generously provided by Nippon Boehringer Ingelheim Co., Ltd. (Hyogo, Japan). Piroxicam [4-hydroxy-2-methyl-N-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], as an internal standard,

was purchased from SIGMA-ALDRICH Japan Co., Ltd. (Tokyo, Japan). Meloxicam patches were prepared by NIPRO PATCH Co., Ltd. (Saitama, Japan). Solvable™, a mixture of dodecyldimethylamine oxide, secondary alcohol ethoxylate and sodium hydroxide in water, was purchased from Perkin-Elmer (Groningen, Netherlands). HPLC reagent grade methanol and water were purchased from Kokusan Kagaku Co., Ltd. (Tokyo, Japan). Other reagents used were of analytical grade.

2. Equipment

The chromatographic system with column-switching consisted of a type 1100 series quaternary pump, a 1100 series thermostatic compartment set at 50°C, a 1100 series on-line degasser, a 1100 series variable-wavelength UV-vis absorbance detector operated at 360 nm, and a 1100 series automatic sampler equipped with two electrically actuated six-port Rheodyne valves, one for sample injection and the other for column-switching. The apparatus was all obtained from Hewlett-Packard (California, USA). The clean-up and enrichment process took place on a Hypersil ODS (2.1×20 mm, I.D., Agilent Technol., California, USA) and separation on a YMC-Pack Pro C18 (4.6×75 mm, I.D., YMC, Kyoto, Japan). Both columns incorporated porous silica gel bonded covalently with octadecyl groups. Data acquisition and integration of the chromatographic peaks were carried out using HPLC system control soft-

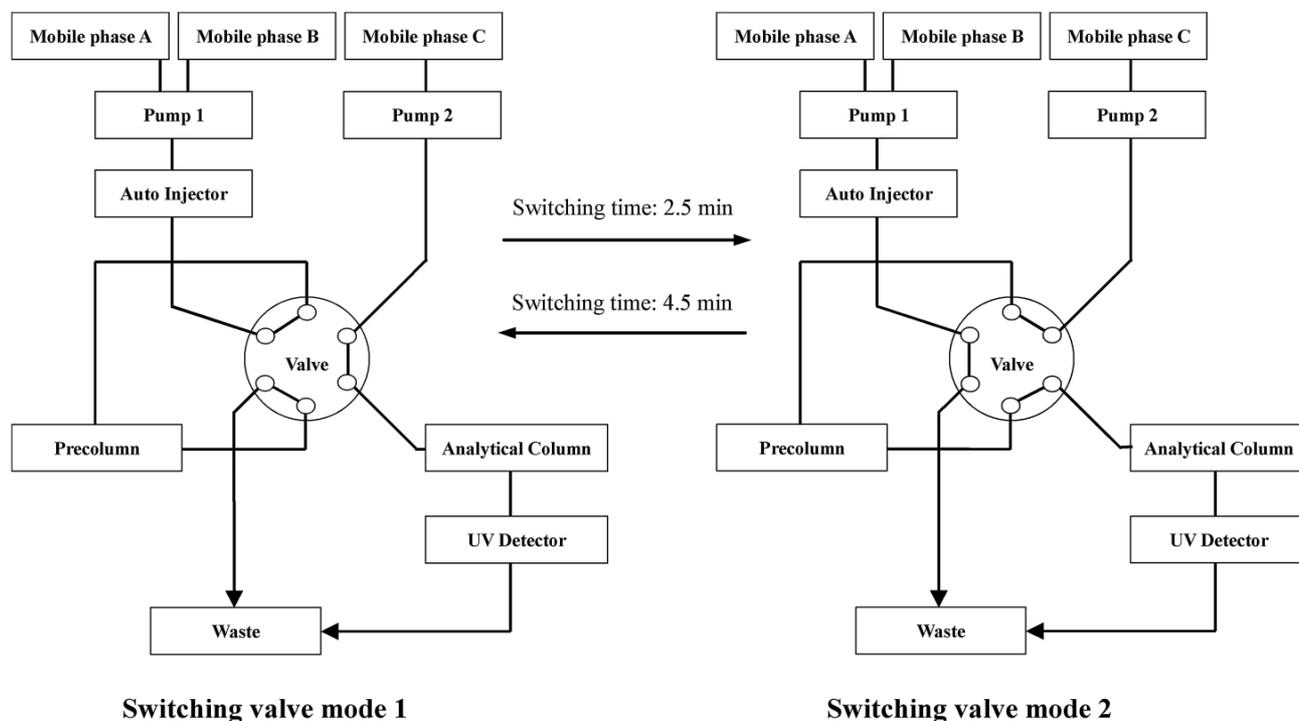


Figure 1. Schematic illustration of the column-switching HPLC system for on-line separation of meloxicam following direct injection of rat muscle and plasma samples. Mobile phases A, B and C are 0.05 M phosphate buffer (pH 3), methanol and 0.05 M phosphate buffer (pH 6)-methanol (60:40, v/v), respectively.

ware running on a compatible computer (Hewlett-Packard CHEM-STATION chromatographic management system).

3. Chromatographic system

The column-switching system is fully automated, with the autosampler performing all conditioning, washing, loading operations and data collection. The dual-column system is coupled via an electrically driven six-port switching column valve from the programmable autosampler to a reversed-phase analytical column, allowing clean-up and enrichment of the sample following direct injection. A schematic representation of the apparatus is shown in Figure 1.

In mode 1 of the switching valve, the sample was directly injected and eluted onto the precolumn, with a washing mobile phase using 0.05 M phosphate buffer (pH 3) at a flow-rate of 1 mL/min to remove the proteins and enrich the meloxicam from the sample. The analytical column was not connected to the autosampler and precolumn, and eluted with a separation mobile phase of 0.05 M phosphate buffer (pH 6)-methanol (60:40, v/v) at a flow-rate of 1 mL/min. At 2.5 min after injection the switching valve was turned to mode 2 (backflush mode), coupling the precolumn in line with the analytical column and allowing transfer of the enriched analytes from the precolumn to the analytical column with a separation mobile phase at a flow-rate of 1 mL/min. After backflush for 2 min the switching valve was returned to its original position so that separation was performed with a separation mobile phase at a flow-rate of 1 mL/min. The precolumn was rinsed with methanol for 9 min and further reequilibrated with a 0.05 M phosphate buffer (pH 3) eluent prior to injection of the next sample. Column temperature was kept at 50°C. The total analytical time, including data acquisition for a single analytical run, was approximately 18 min.

4. Sample preparation

Each muscle sample (0.2 g wet weight) was chopped into small pieces using a scissors, immersed in 1 mL of Solvable™ in a glass vial and sonicated in 42 kHz for 2 hr at about 40°C until the solution was clear without any visible muscle residue. 200 µL of 2 M phosphoric acid solution were added to glass vials. After mixing well, 100 µL aliquots were introduced into sample tubes, and 50

µL each of water and internal standard (300 ng/mL in methanol) were added, with 10 µL of 2 M phosphoric acid solution. After mixing well, the sample was centrifuged (5°C, 11000 g, 5 min), and the supernatant was filtered through a 0.2 µm microporous filter. The filtrate was transferred to injection vials, and 50 µL aliquots were injected into the HPLC system.

100 µL of plasma samples were introduced into sample tubes, and 50 µL each of water and internal standard were added, with 10 µL of 2 M phosphoric acid solution. After mixing well, the same operations as for muscle samples were carried out.

5. Preparation of standard samples

A stock solution of meloxicam prepared by dissolving meloxicam in methanol to obtain the desired concentration. Preparation of the standard sample followed the same procedure as for muscle and plasma samples using drug free muscle and plasma.

6. Calibration curves

Standard samples were prepared by transferring known amounts of the meloxicam in the range of 50-2500 ng/g and 20-2500 ng/mL. The peak area ratios of meloxicam and the internal standard were measured and plotted against the known concentration of meloxicam.

7. Animal experimentation

Male hairless rats weighing about 200 g were purchased from Nihon SLC (Tokyo, Japan). Meloxicam patches (0.86 mg/body) of 4 cm diameter were applied to the abdomen, and muscle samples were collected from the site of application at 2, 4, 8, 12, 16 and 24 hr after their removal. Blood samples were collected from the abdominal vein and centrifuged to obtain plasma. All samples were immediately frozen and stored at -40°C prior to assay.

Results and Discussion

1. Preliminary experiments

1.1. Stability of sample solution in Solvable™

Extraction of meloxicam from muscle was carried out using Solvable™, a strong base reagent which can chemically dissolve tissues by hydrolysis. Therefore, use of this reagent is limited to

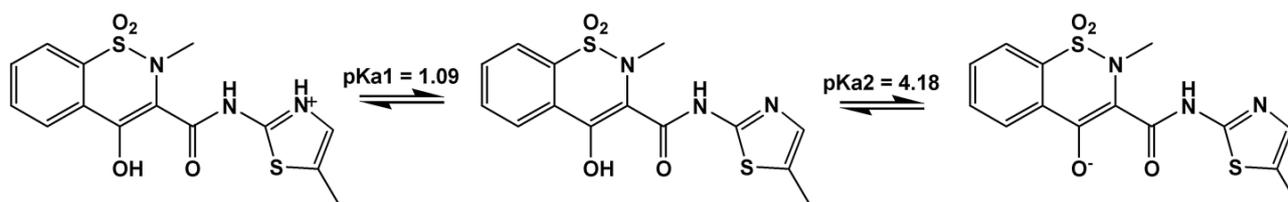


Figure 2. Chemical structure of meloxicam in acid-base equilibrium.

compounds that are stable in strong base solutions. The stability of meloxicam in Solvable™ for 4 hr at about 40°C was evaluated by comparing with samples in methanol. The ratio of peak area of meloxicam in Solvable™ to that in methanol was found to be 99.7%, confirming stability in Solvable™ during ultrasonic treatment and applicability for extraction from muscle. The operations do not require particularly skillful technique, in contrast to other established methods.

1.2. Determination of mobile phase and switching time

When developing a column-switching method, endogenous components that cause detectable interfering signals need to be removed and the target, in this case meloxicam, should be extracted from muscle and plasma by an adequate precolumn. The packing material used in our precolumn was ODS silica gel, so that hydrophobic molecules can be held well. As shown in Figure 2, the pKa1 and pKa2 of meloxicam are 1.09 and 4.18, respectively, and this molecule clearly exists in a fully un-ionized form between pKa1 and pKa2 that is most hydrophobic. Furthermore, no organic modifier was needed in order to prevent decrease of retention in this precolumn. Therefore, an acidic mobile phase (pH 3) was chosen with no organic solvent as a washing eluent for the precolumn.

To determine an adequate switching time and optimize chromatographic conditions, drug free muscle and plasma samples were injected onto the precolumn, and the elution profiles were assessed by direct connection of the precolumn with a UV detector at 360 nm indicating high absorbance. The endogenous components from muscle and plasma were completely removed from the precolumn within 2.5 min according to a visible analysis of the elution profile when the detector signal reached the baseline without any detectable interfering signals. We therefore chose the first valve switching time after 2.5 min. Since the total analytical process, including on-line extraction, transfer of analyte, and separation and monitoring, was carried out within 18 min for each sample.

2. Method validation

Specificity against interference from endogenous compounds found in muscle and plasma is generally established by analyzing independent sources, in this case drug free muscle and plasma. As shown in Figure 3, meloxicam and the internal standard could be clearly separated from endogenous compounds extracted with muscle and plasma. The retention times for meloxicam and the internal standard were 10.2 and 6.7 min, respectively.

Relationships between detector response and drug concentration in muscle and plasma samples were also investigated. These samples are prepared by adding drug free muscle or plasma to known amounts of meloxicam. Coefficients of determination (r^2), obtained by applying a linear regression model, were 0.999 for

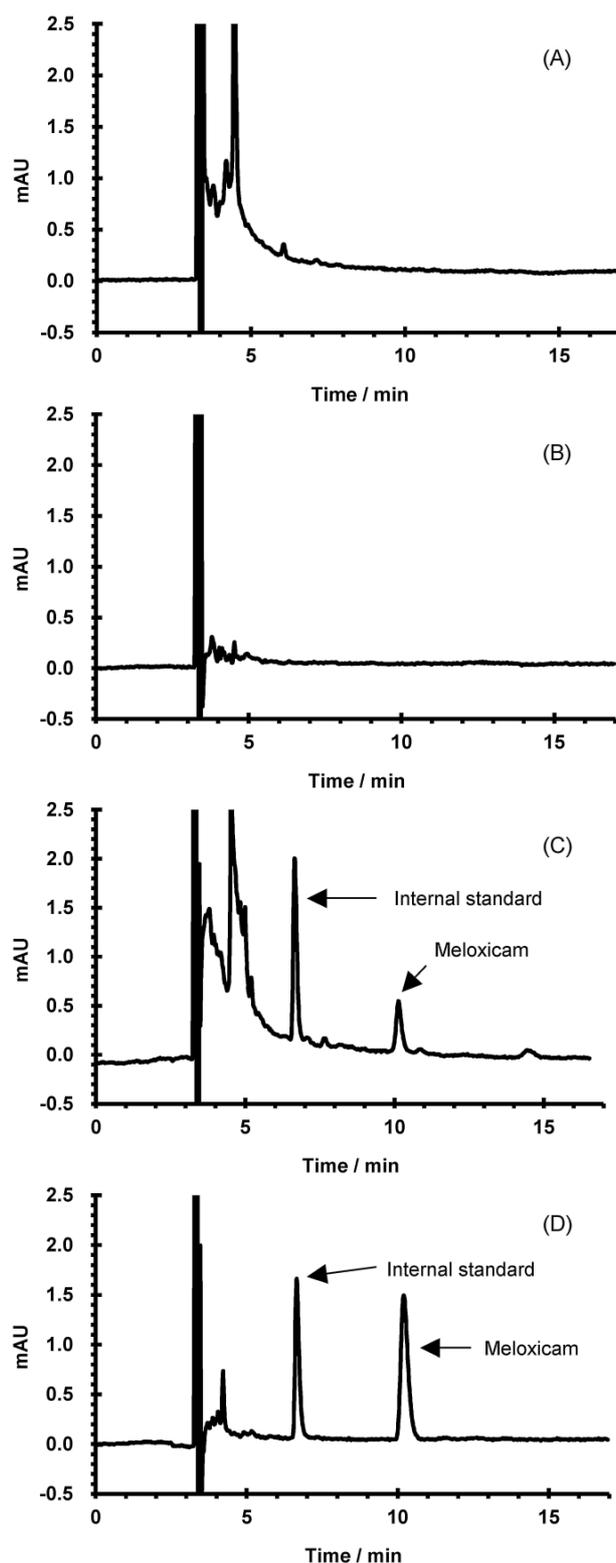


Figure 3. Representative chromatograms of (A) drug free muscle, (B) drug free plasma, (C) muscle sample (500 ng/g) and (D) plasma sample (500 ng/mL).

Table 1. Precision and accuracy for quantification of meloxicam in rat muscle and plasma.

Nominal concentration (ng/g or ng/mL)	Muscle			Plasma		
	Concentration found (ng/g, mean \pm SD, n=5)	RSD ^a (%)	Accuracy ^b (%)	Concentration found (ng/mL, mean \pm SD, n=5)	RSD ^a (%)	Accuracy ^b (%)
20	-	-	-	22.6 \pm 3.2	14.0	112.8
50	51.0 \pm 6.6	12.9	102.0	47.3 \pm 6.1	12.8	94.7
100	100.8 \pm 3.8	3.7	100.8	97.1 \pm 2.2	2.3	97.1
250	253.0 \pm 15.1	6.0	101.2	-	-	-
500	484.2 \pm 13.1	2.7	96.8	506.1 \pm 18.8	3.7	101.2
2500	2492.4 \pm 19.5	0.8	99.7	2496.9 \pm 76.2	3.1	99.9

-: not assayed.

^aRSD (%) = 100 \times (SD / mean)

^bAccuracy (%) = mean concentration found / nominal concentration \times 100

muscle and 0.999 for plasma. Linearity for muscle was $y = 0.000828x + 0.0106$ and for plasma was $y = 0.00325x + 0.00496$, where y and x were the peak area ratio (area for meloxicam / area for the internal standard) and the meloxicam concentration (ng/g or ng/mL), respectively.

Accuracy of meloxicam, in the range of 50-2500 ng/g in muscle and 20-2500 ng/mL in plasma, were calculated by comparing the obtained concentration with the nominal concentration. The precision was estimated using a relative standard deviation (RSD) of quintuplicate results. As shown in Table 1, accuracy and RSD of all samples were within 100 \pm 15% of the expected values. Since accuracy and RSD ranged within 100 \pm 15% for all levels of muscle and plasma, adequate accuracy and precision were obtained in the wide ranges of 50-2500 ng/g for muscle and 20-2500 ng/mL for plasma.

The limits of quantification (LOQ) in muscle and plasma, defined as the lowest concentration which could be determined with accuracy and RSD within 100 \pm 20%, were found to be 50 ng/g and 20 ng/mL, respectively.

On-instrument stability was determined by comparing freshly prepared samples with prepared samples left on the autosampler of the automated HPLC system for 72 hr. No significant degradation could be detected in the muscle and plasma samples after this time period.

3. Application of the assay

This method could be successfully applied for the present pharmacokinetic study of meloxicam after transdermal application of patches in rats. Figure 4 shows the mean concentration profiles in muscle and plasma. C_{max} values were approximately 1900 ng/g and 1600 ng/mL, respectively. Meloxicam was absorbed leading to maximum muscle and plasma concentrations at 4 and 12 hr, respectively.

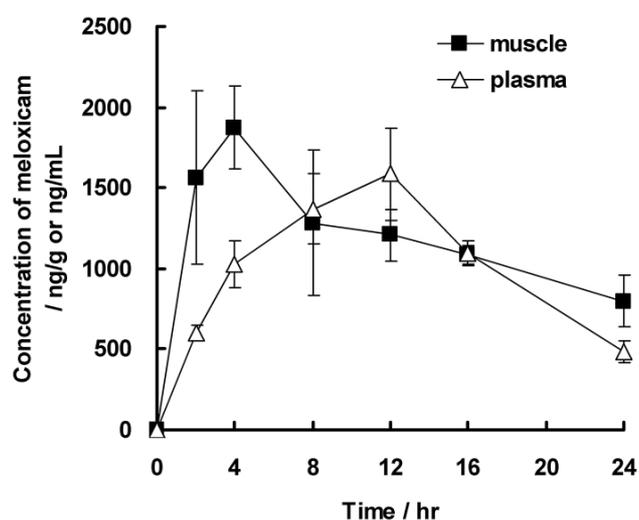


Figure 4. Concentration-time profile of meloxicam after application of transdermal patches in rats. Each value is a mean \pm SD (n=3).

Conclusion

We have established a simple and easy method for the determination of meloxicam in muscle and plasma using SolvableTM and a column-switching HPLC technique. This method has major advantages in eliminating tedious manual extraction and minimizing manipulation. Moreover, this has great potential to apply to the determination of meloxicam in other tissues, such as skin, stomach, liver and so on. The method could here be successfully applied to evaluate pharmacokinetics of topically administrated meloxicam.

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