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Application of an alkyl-diol silica precolumn in a column-switching system for the determination of meloxicam in plasma

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Abstract

The group of LiChrospher alkyl-diol silica (ADS) phases that make part of the unique family of restricted-access materials, have been developed as special packings used in the liquid chromatographic integrated sample processing of biofluids. The advantage of these phases lies in the possibility of direct injection of untreated plasma. An on-line elimination of the protein matrix is achieved with a quantitative recovery together with an on-column enrichment. The present method describes a hand-operated on-line switching high-performance liquid chromatographic system for the determination of meloxicam. Spiked plasma samples were introduced on the ADS precolumn using a 0.05 M phosphate buffer, pH 6.0. After washing with the buffer the ADS column was backflushed with the mobile phase 0.05 M phosphate buffer–30% (v/v) acetonitrile (ACN)–25 mM *t*-butylamine (TBA) at a pH of 7.0, thus transferring the analyte to the analytical column LiChrocart 125-4 LiChrospher RP-8. The eluent was monitored by a UV-detector set at 364 nm. The developed column-switching method is fully applicable to plasma injections.

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1. Introduction

Isoxicam, meloxicam (Fig. 1), piroxicam and tenoxicam, belonging to the group of non-steroidal anti-inflammatory agents (NSAIs) have analgesic, anti-inflammatory, and antipyretic properties. The most important representative, piroxicam, is used in musculoskeletal and joint disorders, whereas tenoxicam is used in the symptomatic management of osteoarthritis and rheumatoid arthritis. Published methods for the determination of their plasma concentrations involve complex procedures such as liquid–liquid extraction [1–7].

Conventional methods for the analysis of these analytes in plasma samples require extensive

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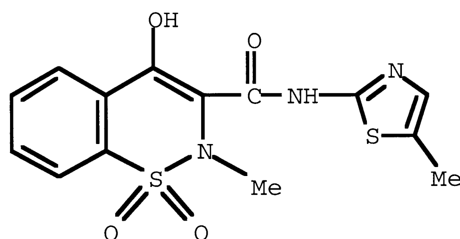

Fig. 1. Structure of meloxicam.

sample preparation and often introduce substantial experimental errors. In order to avoid time-consuming manipulations of extraction, a rapid, robust and reproducible method using a column-switching liquid chromatographic system was developed based on the application of an alkyl-diol silica (ADS) precolumn technique [8–14]. In previous work from this group, methods for the determination of ketoprofen and ketoprofen enantiomers in horse plasma were reported applying ADS precolumn material in a column-switching system [15–17].

The present method describes a hand-operated on-line switching high-performance liquid chromatographic system for the determination of meloxicam, a recently introduced NSAI drug for the relief of the pain and stiffness associated with osteoarthritis. Meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] exhibits anti-inflammatory, analgesic and antipyretic activities. The mechanism of action may be related to prostaglandin synthetase (cyclooxygenase) inhibition with a higher COX-2 selectivity than many other NSAIs. Meloxicam has been used frequently as an internal standard for the determination of other enolic acids but very few determinations of meloxicam as such are reported in literature [5,18].

2. Experimental

2.1. Reagents and chemicals

HPLC grade acetonitrile (ACN) was obtained from Panreac-Química (Spain). The amines used in the mobile phase were *N*,*N*-dimethyloctylamine (DMOA) and *t*-butylamine (TBA) from Acros Organics (Belgium) and Sigma-Aldrich (Belgium), respectively. Sodium dihydrogen phosphate, ACS grade from Merck Eurolab (Germany) was used for the preparation of the buffer. Tenoxicam and isoxicam were purchased from Sigma-Aldrich (Belgium), piroxicam was a gift from Pfizer (Belgium) and meloxicam was obtained from ID INDIS (Aartselaar, Belgium). All other chemicals used were of analytical grade and deionised water was used throughout.

2.2. Instrumentation

The liquid chromatographic system consisted of two pumps (Shimadzu LC-9A, Germany) and two Valco injectors (C6W, Valco Europe, Switzerland), one equipped with a home-made 100 μl sample loop and the other provided with the ADS pre-column instead of a sample loop. The analytical column was kept at 35 °C in a column oven (Shimadzu CTO-6A). All switching handling was done by hand and the obtained signal was monitored by a UV-detector (LaChrom L-7420, Merck Eurolab). The Shimadzu C-R6A Chromatopac integrator was used for data handling. In the peek tubing between the two valve injectors a low-dead volume filter (A315, Upchurch Scientific, USA) was placed with a 2 μm frit 0.062 (A-101X, Upchurch Scientific), so as to protect the filters of the ADS precolumn and tubing from blocking. This on-line filter was replaced after about 4 ml of plasma injection.

2.3. Alkyl-diol silica precolumn

Before HPLC analysis, macromolecular compounds have to be removed from the sample because of their precipitation by higher amounts of organic solvents and their binding on the surface of the packing material. LiChrospher ADS materials consist of a family of special reversed phase sorbents [8,9,19] for LC-integrated sample preparation of biological fluids, these precolumn packing materials having been developed in a cooperation between Merck Eurolab and K.-S. Boos [8–13]. At the outer surface of the spherical particles, hydrophylic, electroneutral diol

groups are bound, preventing interactions with the protein matrix. The latter can be directly flushed into the waste. The inner surface, with the hydrophobic C-18 alkyl chains, is freely accessible for low molecular analytes. The analytes are thus selectively extracted and enriched on the inner surface. Thus the packing material provides a direct extraction-base on-line, an on-column enrichment and subsequent analytical separation of low-molecular compounds from untreated plasma samples.

2.4. Mobile phase

The pH of the mobile phases (pH Meter 691, Metrohm, Herisau, Switzerland) was adjusted utilising a solution of sodium hydroxide 20% (g/v) or phosphoric acid 20% (v/v), next brought to volume.

2.5. Chromatographic conditions

After conditioning the precolumn LiChrocart 25-4 LiChrospher RP-18 ADS (Merck Eurolab) with 3.2 ml 0.05 M phosphate buffer, pH 7.0 (MF I, pump 1), a volume of 100 μl spiked horse plasma was injected. The plasma matrix was then removed from the precolumn with 6.4 ml of the same buffer solution.

After 8 min the valve was switched and the analyte was transferred to the analytical column LiChrocart 125-4 LiChrospher RP-8 (Merck Eurolab), provided with a guard column 4-4 applying a mobile phase (MF) consisting of 0.05 M phosphate buffer, ACN (70–30 v/v) and 25 mM TBA brought at pH 7.0, at a flow-rate of 0.8 ml/min (MF II, pump 2). The stronger elution power of this eluent causes the analytes to be desorbed from the precolumn and to be transferred on top of the analytical column. Column temperature was kept at 35 °C. The UV-detector was set at the wavelength obtained from on-line spectra of each compound (about 360 nm):

Isoxicam	352 nm
Meloxicam	364 nm
Piroxicam	358 nm
Tenoxicam	372 nm

2.6. Standard solution preparation

A standard solution of meloxicam at 0.7 mg/ml in methanol was prepared. A 100 μl volume of sodium hydroxide (20% g/v) was added to 50 ml of the previous solution to facilitate dissolution. Stock solutions of the other oxicams were prepared identically. Dilutions in 0.5 M phosphate buffer pH 7.0 were made to provide working standard solutions (range 1: 35 μg/ml, range 2: 7 μg/ml, range 3: 1.4 μg/ml and range 4: 0.28 μg/ml). All solutions were stored in dark glassware at room temperature.

The calibration standard solution was prepared by spiking 1000 μl drug-free horse plasma with varying volumes of respectively, 20, 40, 60, 80 and 100 μl of the standard working solutions in 0.5 M phosphate buffer, pH 7.0, then made up to 100 μl with the latter buffer solution. Another volume of 100 μl 0.5 M phosphate buffer pH 7.0 was added to buffer the solution in order to eliminate small pH variations of the plasma samples; moreover, this also opens the possibility to include an internal standard, if necessary.

2.7. Calculations

Concentrations of meloxicam were determined by using the linear regression equation from daily calibration curves, constructed by plotting peak areas versus concentration.

2.8. Determination of switching times

When developing a column-switching method, the switching times must be determined initially [20]: first, the switching time for the fractionating step, second the switching time for the transfer step and third the time needed for equilibration of the precolumn. The elution profile of the sample matrix on the precolumn applied was determined by direct connection of the precolumn to the UV detector set at 360 nm and a volume of 100 μl blank horse plasma was injected and transported by MF I; 0.05 M phosphate buffer–ACN (98–2% v/v) + 25 mM DMOA at pH 7.0, flow-rate 0.8 ml/

min. The fractionation step was considered complete when the detector signal reached the baseline (time $T_M = 3$ min), although it was taken into account that the signal of plasma was more pronounced at 260 nm (time $T_M = 3$ min) and that a complete elution of the plasma matrix is necessary before the transfer step can start. Thus for safety reasons a guard column 4-4 should be placed in front of the analytical column so as to prevent the analytical column from serious damage when contaminated with proteins, leading to a decrease of capacity and selectivity and to an irreversible increase of column pressure. The lifetime of the precolumn was equivalent to about 70 ml of horse plasma.

The elution profiles of the oxicams (1600 ng each) were controlled on the ADS RP-18 phase applying the above mentioned MF I. Obtained retention times were higher than 100 min even when employing 10% v/v ACN; the elution of the oxicam started at about 30 min (T_A: start of analyte elution). In this case, an ADS precolumn with smaller alkyl chains (RP-4 or -8) should be preferred, however, these were not available for the determinations. When the elution profile of the oxicams was investigated eluting with the MF: 0.05 M phosphate buffer–ACN (70–30 v/v)+25 mM DMOA brought at pH 7.0, retention times of 1.2–1.7 min were noted and the baseline was reached after about 4 min (T_T). T_T represents the earliest point at which the valve can be switched back to its original position so as to equilibrate the precolumn.

Calculation of switching times and abbreviations were used following the prescriptions of the LiChrospher ADS manual (Merck KGaA, Darmstadt, Germany). For the fractionation the calculation results in a minimum time of T_M+5 (= 8 min) applying a flow of 0.8 ml/min. For the transfer step a time of T_T+1 ($4+1=5$ min) was needed. As for the determination of meloxicam lower contents of ACN were applied and a time of 8 min was utilised practically. Calculation of equilibration time of the precolumn resulted—flow-rate at 0.8 ml/min—in a retention time of 2.3 min [21]. Taken into account the 1 pH-unit difference of the mobile phase applied, a time period of 4 min was applied in the determination. Run time of meloxicam analysis resulted in about 20 min (8+8+4); however, an optimum minimum time of 16 min could be used.

3. Results and discussion

3.1. Preliminary experiments

3.1.1. Back-flush versus forward-flush

The oxicams (1000 ng each) were injected on the precolumn and transported by MF I: 0.05 M phosphate buffer–ACN (98–2% v/v)+25 mM DMOA at pH 7.0, flow-rate 0.8 ml/min. After loading (8 min) the analytes are transported to the analytical column in forward-flush and in back-flush modes by turning the precolumn with the MF II: 0.05 M phosphate buffer–ACN (70–40 v/v)+25 mM DMOA brought at pH 7.0, flow-rate 0.8 ml/min. Tailing factor and peakwidth were calculated (Table 1). The obtained values and chromatograms demonstrated clearly the advantages of the back-flush mode. As the content of organic modifier in the mobile phase used for transfer and separation is higher than in the washing fluid, peak compression was achieved.

3.1.2. Influence of amines

Due to the pyridinyl-structure of tenoxicam and piroxicam, amines are essential for good peak shapes in chromatography on the C8 reversed phase contrarily to meloxicam and isoxicam [22]. Inclusion of 25 mM DMOA to MF II (+40% v/v ACN), flow-rate 0.7 ml/min resulted not only in an improved symmetry of tenoxicam and piroxicam

Table 1
Back-flush and forward-flush obtained tailing peak factor (T) and width at half height ($W_{h/2}$)

Compound	Back-flush		Forward-flush	
	T	$W_{(h/2)}$ (s)	T	$W_{(h/2)}$ (s)
Isoxicam	1.7	15.9	2.5	28.4
Meloxicam	1.5	17.3	[a]	33.2
Piroxicam	1.6	14.7	2.4	26.8
Tenoxicam	1.8	12.9	2.5	26.8

[a] Peak doubling occurred.

peaks, tailing factors being, respectively, 1.2 and 1.4, but also in an improved resolution (1.7). Moreover, an increase in retention was noticed. When adding 25 mM DMOA to MF I and MF II, disturbing signals arose in the chromatogram [23,24] influencing the peaks of mainly piroxicam and tenoxicam when analysing low concentrations. The signals appeared to be due to the amine applied and by turning the valves. Replacement of 25 mM DMOA by 25 mM TBA in the mobile phases improved baseline shape. Further improvements were done by eliminating the amine MF I. Testing the Chromolith SpeedROD 18e 50-4.6 (Merck Eurolab) [25–27] with MF II (0.8 ml/min) without an amine demonstrated that tenoxicam and piroxicam eluted with an acceptable peak shape (tailing factor <1.4). To eliminate problems arising from adding amines to mobile phases, these columns should be recommended for analysing basic compounds.

3.1.3. Choice of pH

Initially the work started with a pH value of 7.0 for the MF I (0.7 ml/min). As a more stable baseline—in combination with the precolumn was obtained with a decrease of the pH, a pH value of 6.0 was applied to the determination of meloxicam.

Due to the acceptable peak symmetry of the oxicams at pH 7.0 of MF II, the pH was set at 7.0. When applying the MF II brought at pH 5.0 (0.7 ml/min) directly on the analytical column, a chromatogram was obtained with acceptable symmetry and a separation of all oxicams was obtained with excellent resolution (>2) within a chromatographic run time of about 16 min. This separation in combination with MFI and the precolumn could not be achieved., even by chenging switching times and adaptations of mobile phases.

3.1.4. Molarity

Different molarities (0.01–0.25 M) of the phosphate buffer were tested. Higher molarities slightly improved the peakwidth of the oxicams. The application of a phosphate buffer 0.25 M was omitted so as to avoid precipitation of the phosphate buffer constituents when in contact with ACN, to prevent the clogging of tubing and filters.

3.1.5. Acetonitrile content

An increase of ACN content in MF I resulted in a decrease of resolution between isoxicam and piroxicam. Other organic modifiers (methanol, isopropanol) were also tested and no improvement was obtained. An improvement of the baseline was observed when applying an MF I without ACN. For the determination of meloxicam no ACN was added to MF I. Moreover, no difference of recovery was observed.

3.1.6. Carryover

Except for peaks due to valve turning and solvent changing, sample peaks appeared in the chromatogram when only blanks were injected. Carryover peaks arose [28] comprising important phenomena when LC-methods are used for analysing a wide range of sample concentrations. Especially for piroxicam carryover showed up as mild to severe deviations from the standard curve. A standard curve of piroxicam (200–4000 ng/ml) was injected in a low-to-high sequence followed by a high-to-low sequence. When the injection was done from low to high a relative fair curve was obtained ($r = 0.96$). A reversed order resulted in an unacceptable linear regression ($r = 0.7$) together with errors $>30\%$ and in an unacceptable y-intercept. Due to the poor solubility of the oxicams, sample residues might remain in tubing, valves, injector needle etc. or even because of bleeding from the precolumn. All PEEK fittings were retightened and between injections the 0.5 M phosphate buffer pH 7.0 was added as washing fluid of the needle and sample loop; the injection system was regularly flushed with methanol. The washing of the precolumn with methanol also prolongs the column life-time.

3.2. Quantitative determination of meloxicam in spiked horse plasma

3.2.1. Recovery

The recoveries of meloxicam (and bias values) from spiked samples (Table 2), at four different concentrations, were calculated by comparing the

Table 2
Recovery of meloxicam

Concentration (ng/ml)	Recovery (%) ($n = 3$)	R.S.D. (%)
2064	99.2	1.72
1032	101.8	0.37
482	97.9	2.05
206	101.1	1.32

obtained peak areas from spiked horse plasma with those from aqueous solutions. Each solution was prepared in triplicate and one injection from each solution was performed. From the recovery covering four different concentrations, a mean value of 99.8% was obtained. These values demonstrate the excellent extraction efficiency for meloxicam from the ADS precolumn. Moreover, as the recovery is quantitative the addition of an internal standard is not necessary.

3.2.2. Linearity

The relationship was investigated between detector response (peak areas) and drug concentration in plasma spiked with known meloxicam amounts, ranging from 5 to 3500 ng/ml, in four different ranges, each employing five calibration points. Table 3 gives the coefficients of determination (r^2) obtained by applying a linear regression model. Linearity ($n = 1$) was also established from 50 to 3500 ng/ml with a seven point calibration set. A typical chromatogram of blank horse plasma and one spiked with 27.5 ng/ml meloxicam is shown in Fig. 2.

Table 3
Linearity of meloxicam signal

Range (ng/ml) plasma	Linear regression coefficient r^2 ($n = 3$)
700–3500	0.9996
140–700	0.9983
25–140	0.9937
5–25	0.9451[a]
50–3500	0.9997[a]

[a] $n = 1$.

3.2.3. Intra-day variations

Intra-day assays at five concentrations were performed on freshly prepared plasma samples (spiked six times). Calculation of the relative standard deviation (R.S.D.) was performed on peak areas of meloxicam, ranging from 0.5 to 3.0, utilising hand-operated injection and switching; however, the variations proved to be acceptable (Table 4).

3.2.4. Inter-day variations

Inter-day R.S.D. were measured from the analysis of pooled plasma spiked with meloxicam at five different concentrations. The spiked plasma solution for injection was prepared and injected only once, and was determined on 3 different days. The obtained values are shown in Table 4.

3.2.5. Limit of quantitation and limit of detection

A meloxicam concentration of 3 ng/ml plasma was considered as the limit of detection. The latter was calculated on the basis of three times the area of disturbing signals arising in the chromatogram with a capacity factor close to the k′-value of meloxicam. When decreasing the flow of MF II till 0.6 ml/min and increasing the injection volume up to 500 μl (washing step increased to 10 min) the limit of detection was decreased down to 1 ng/ml.

The limit of quantitation, being the lowest concentration that can be quantified with acceptable accuracy (R.S.D. 20%), was 20 ng/ml.

3.2.6. Internal standard

As the recovery of meloxicam is quantitative, the addition of an internal standard is considered not necessary. This was also demonstrated in previous work on the determination of ketoprofen [15]. In cases the application of an internal standard is preferred, the latter can be included in the 100 μl 0.5 M phosphate buffer pH 7.0, added in the sample preparation. The determination of accuracy was based upon the recovery of known amounts of analyte spiked into the sample matrix.

When lowering the ACN content to 20% v/v in the MF II, tenoxicam could be determined quantitatively as there are no interfering peaks. Piroxicam or isoxicam elutions were still slightly

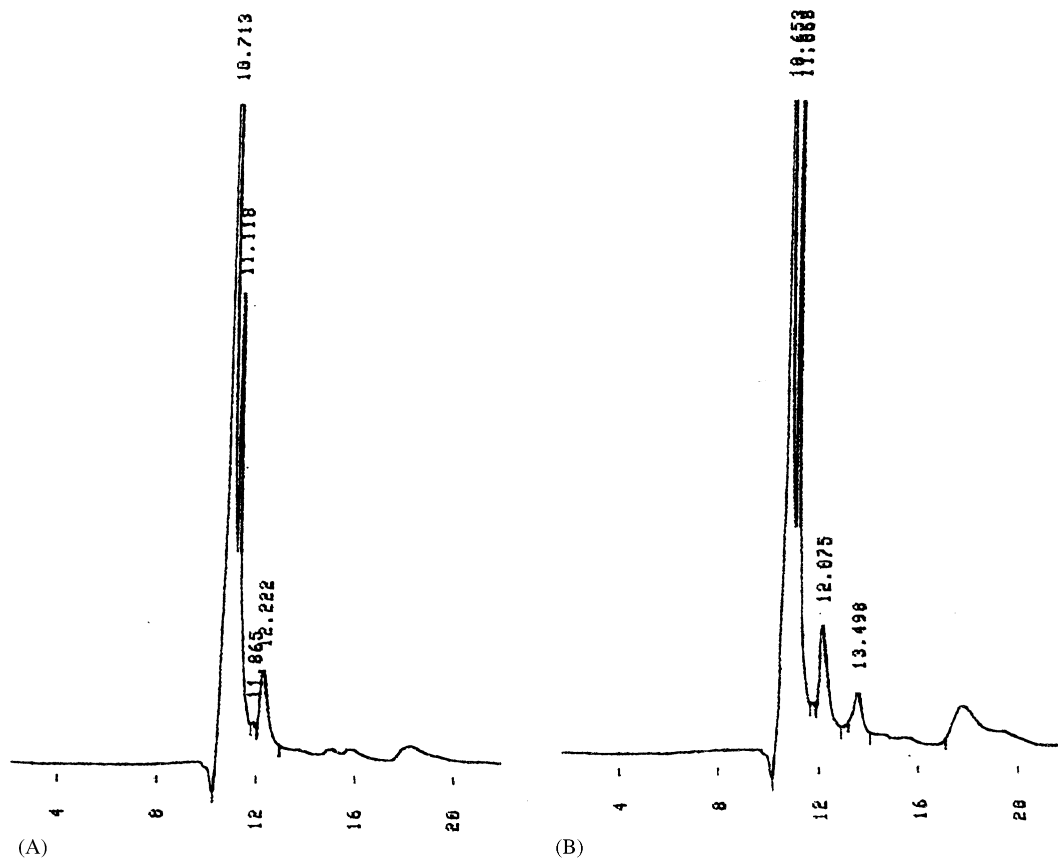

Fig. 2. (A) Typical chromatogram obtained after injection of 100 μl blank horse plasma. (B) Typical chromatogram obtained after injection of 100 μl horse plasma spiked with 27.5 ng/ml meloxicam ($t_r = 13.5$ min), (relative fluorescence emission signal vs. time (min.))

Table 4
Intra-day and inter-day variations

Intra-day		Inter-day	
Concentration (ng/ml)	R.S.D.	Concentration (ng/ml)	R.S.D.
2924	0.46	2177.8	1.52
1548	2.73	1151.4	3.61
861	3.02	513.2	1.42
399	2.18	294.5	8.29
96.6	1.42	71.3	3.53

disturbed by system peaks. The recovery from plasma was measured by spiking drug-free horse plasma with known amounts (2000, 1000 and 500 ng/ml) of tenoxicam. The mean values of recovery were, respectively, 97.7, 98.1 and 97.0%. Disadvantage of applying the internal standard tenoxicam in the above described method was an increase in run time of about 100%.

4. Conclusion

As conventional sample preparation is time-consuming and expensive, the use of the surface reversed-phase precolumn packing material ADS proved to enable the development of LC-integrated sample clean-up for drug determination in a biological matrix. Because of the total elimination

of the protein matrix, the analyte recovery is quantitative and no internal standard is needed.

Preliminary work was carried out in an on-line system, hand-operated, to investigate the possibilities of determining oxicams. The determination of meloxicam is satisfactory and proved that an investigation and application is possible with conventional chromatographic instruments thus eliminating the manual time-consuming extraction handling. Although the system is not continuos but well flexible, a most acceptable analysis time of about 20 min for the determination of meloxicam was achieved.

A coupled-column system, automatically or even hand-operated, using ADS precolumn packings should have a broad application in the analysis of biological or related fluids.

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