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ORIGINAL RESEARCH

Direct HPLC analysis of ketoprofen in horse plasma applying an ADS-restricted access-phase

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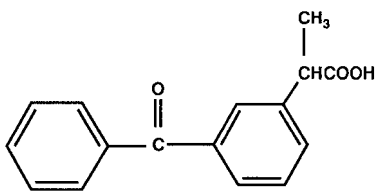
ABSTRACT: Making up part of the unique family of restricted access materials (RAM) the Lichrospher ADS (alkyl-diol silica) sorbents have been developed as special packing materials for precolumns used for LC-integrated sample processing of biofluids. The advantage of such phases consists of direct injection of untreated biological fluids without sample clean-up and elimination of the protein matrix together with an on-column enrichment. The plasma samples, with internal standard phenacetin added (not essential), were brought onto the precolumn (C-18 ADS, 25 μm, 25 × 4 mm i.d.) using a phosphate buffer, 0.1 M, pH 7.0. After washing with the buffer, the ADS column was backflushed with the mobile phase phosphate buffer 0.05 M pH 7.0: acetonitrile (80:20), thus transporting the analytes onto a reversed-phase column Ecocart 125-3 HPLC cartridge with a LiChrocart 4–4 guard column, both packed with LiChrospher 5 μm 100 RP-18; after separation detection was performed in UV at 260 nm. Essential features of the method include the novel precolumn packing, the absence of sample pretreatment, a quantitave recovery, good precision and accuracy, as well as a considerable reduction of analysis time compared to conventional manual methods applied in bioavailability studies.

INTRODUCTION

Ketoprofen [(+−)2-(3-benzoylphenyl)propionic acid] is a non-steroidal anti-inflammatory drug (NSAID) of the propionic acid class, which also includes pharmaceuticals such as ibuprofen, naproxen and fenoprofen. Ketoprofen is mainly used in human therapy in the treatment of arthritis because of its analgesic and anti-inflammatory properties. The FDA approved the use of ketoprofen in horses for the alleviation of inflammation and pain associated with musculoskeletal disorders.

Published methods for the determination of plasma ketoprofen concentrations involve complex procedures such as liquid–liquid extraction (Satterwhite and Boudinet, 1988) and SFE manipulations. Initial work from our group involved the determination of ketoprofen in plasma using reversed-phase HPLC, injecting the residue of a diethyl ether extract of the acidified plasma samples, solubilized in the mobile phase (Corveleyn *et al.*, 1996; Baeyens *et al.*, 1998a). In order to avoid time-consuming manipulations, a simple, rapid and reproducible method using an automated column-switching liquid chromatographic system for the determination of ketoprofen applying UV detection was reported (Baeyens *et al.*, 1998b).

The direct and repetitive injection of untreated biological fluids into an HPLC setup and the subsequent analysis of low-molecular weight analytes is rendered possible by a column-switching setup and special precolumn phases. The proposed method is based on the integrated sample clean-up configuration making use of the precolumn LiChrospher RP-18 ADS, 25 × 4 mm, connected via the electrically driven six-port valve from a



Ketoprofen

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Abbreviations used: ADS, alkyl-diol silica; NSAID, non-steroidal anti-inflammatory drug; RAM, restricted access materials.

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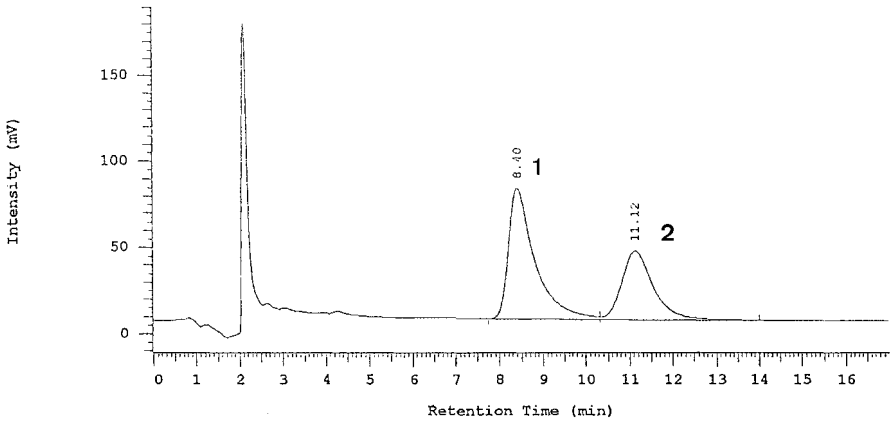

Figure 1. Typical chromatogram obtained after injection of 200 μL of spiked horse plasma with 3200 ng/mL ketoprofen (1) and the internal standard phenacetin (2).

programmable autosampler to the narrow-bore reversed-phase analytical column Ecocart LiChrospher 125-3 filled with LiChrospher 5 μm 100 RP-18, where an on-line determination of ketoprofen is performed.

The use of RAM (restricted access material) phases is based on the complete non-adsorptive size-exclusion of macromolecules and on the simultaneous extraction of low-molecular weight analytes. The plasma matrix compounds are quantitatively eluted in the void volume of the precolumnn due to the restricted access given by the pore size of the packing. The LiChrospher ADS (alkyl-diol silica) belonging to the RAM family (Boos and Rudolphi, 1998a, b) was developed as a special packing material for precolumns used in an LC-integrated sample processing system (Boos *et al.*, 1995), and applied in the determination of different drugs in biological matrices (Vielhauer *et al.*, 1995; Yu and Westerlund, 1996).

EXPERIMENTAL

Chemicals. All solvents and chemicals used were of HPLC or analytical reagent grade and no further purification was carried out. Ketoprofen was purchased from Sigma Chemical Company (St. Louis, MO, USA), phenacetin from Rhône-Poulenc (Paris, France), monobasic potassium phosphate and acetonitrile (Lichrosolv) were purchased from Merck.

Equipment. The liquid chromatographic system used consisted of a pump (LaChrom® L-7100, Merck KGaA, Darmstadt, Germany), a programmable autosampler (LaChrom® L-7250, Merck KGaA) and a UV-vis detector (LaChrom® L-7420, Merck KGaA). The HPLC parts were connected through an interface (D-7000, Merck KGaA) with a Compaq Deskpro XL 5133 2 GB computer for data handling.

Standard and sample preparation. A standard solution of ketoprofen 400 μg/mL (high range) in phoshate buffer 0.1 M, pH 7.0, was prepared, and dilutions were made to provide two working standard solutions of 40 μg/mL (medium range) and 4 μg/mL (low range). The appropriate internal standard working solutions of phenacetin were prepared in phosphate buffer, 0.1 M, pH 7.0 with a concentration of 200 μg/mL (high range), 20 μg/mL (medium range) and 2 μg/mL (low range), respectively. All solutions were stored in dark glassware at about 8°C.

Blood samples (10.0 mL) were taken from the horse 5 min before drug administration and at 0, 2, 5, 10, 20 and 30 min, and at 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after i.v. administration of 1.0 g ketoprofen. Blood samples were collected in vacuum tubes (Venoject, Kimble-Terumo, Elkton, USA) containing lithium heparin as anticoagulant. Plasma was separated by centrifugation at 2000***g*** for 2 min and stored at −20°C until analysed.

Spiked plasma: the calibration standard solution was prepared by adding various volumes, respectively 10, 30, 50, 70 and 90 μL of the standard working solution in phosphate buffer, 0.1 M, pH 7.0, and made up to 100 μL with the latter, 100 μL appropriate working internal standard solution, 100 μL phosphate buffer, 0.6 M, pH 7.0, and 1000 μL drug-free plasma.

Unknown sample: 100 μL phosphate buffer, pH 7, 0.1 M, 100 μL appropriate working internal standard solution, 100 μL phosphate buffer, 0.6 M, pH 7.0, and 1000 μL plasma.

The prepared plasma solutions were filtered through a regenerated cellulose (gray, 0.2 mm 13 mm, hold-up volume 28 μL) syringe filter (Chromacol Ltd, Herts, UK) and placed in 1.5 mL threaded vials (Merck) provided with screw caps with a hole and slotted silicon/PTFE septa (Merck).

Chromatographic conditions. Due to the dosing system of the autosampler, different volumes (high range, 100 μL; medium range, 200 μL; and low range, 400 μL) can be injected in to the system with the standard syringe and brought onto the precolumn (C-18 ADS, 25 μm, 25 × 4 mm i.d.) using the phosphate buffer, 0.1 M, pH 7.0, as the transporting solvent. After washing the ADS column with about 6.0 mL phosphate buffer to eliminate the plasma matrix, the precolumn was put in backflush mode with a mixture of phosphate buffer 0.05 M, pH 7.0: acetonitrile (80:20), applying a flow rate of 0.8 mL/min thus transporting the analytes on the reversed-phase column Ecocart 125-3 HPLC (cartridge) with a LiChrocart 4-4 guard column, both packed with LiChrospher 5 μm 100 RP-18 (Merck) leading to separation. The analytical column was placed in a waterbath and kept at 35°C.

Table 1. Recovery of ketoprofen from spiked horse plasma

Range	Concentration of ketoprofen (ng/mL)	Recovery of ketoprofen (%)	Recovery calculation, ratio of ketoprofen: phenacetin
High	38700	96.4	93.8
	10170	97.8	98.6
Medium	3870	95.2	92.8
	1017	97.5	97.2
Low	387	91.0	95.0
	101.7	101.6	103.5

The UV detector was set at 260 nm; the retention times were respectively about 8.5 min for ketoprofen and 11.0 min for phenacetin. The precolumn was reconditioned with about 6 mL phosphate buffer, 0.1 M, pH 7.0.

RESULTS

LiChrospher ADS

LiChrospher RP-18 ADS has a pore size of approximately 6 nm (physical diffusion barrier) and excludes macromolecules larger than 15 kDa in the void volume. 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. At the outer surface of the spherical particles are bound hydrophilic, electroneutral diol groups, preventing interactions with the protein matrix. The inner surface, covered by hydrophobic C-18 alkyl-chains, is freely accessible for low molecular weight analytes. Thus the packing material provides a direct extraction-base, fully automated, on-column enrichment and subsequent analytical separation of low-molecular compounds from untreated plasma samples.

In LC-integrated sample preparation the sample is first fractionated into sample matrix and analytes by the use of the precolumn. This means that the protein matrix of a biological sample can be directly flushed into the waste, the analyte fraction meanwhile being selectively extracted and enriched on the stationary phase of the precolumn.

Out of the three types of LiChrospher RP-ADS, covering the whole range of hydrophobic capacity factors, the most suitable precolumn for a given analyte has to be determined in each specific case. The ADS RP-4 was omitted because of the small capacity factor of ketoprofen when eluting with 50 mM phosphate buffer pH 7.0. The retention of ketoprofen was shorter and the peak-form significantly better on the ADS RP-18 precolumn compared to the RP-8 phase. The system with the LiChrospher ADS RP-18 precolumn provided less disturbed chromatograms and more stable baselines.

Switching times

When developing a column-switching method, initial switching times have to be determined (Majors *et al.*, 1996), first the switching time for the fractionating step expressed in minutes or as a volume of washing liquid completing the sample preparation and coupling the precolumn to the analytical column, and second the switching time for the transfer step. The elution profile of the sample matrix on the precolumn was determined by direct connection to the UV detector set at 260 nm, applying a given flow-rate. A 500 μL blank plasma volume was injected and the detector signal monitored. The fractionation step was considered complete when the detector signal reached the baseline. Depending on the injection volume, the time required for the sample preparation step may be adapted.

The complete elimination of matrix components has to be achieved in order to prevent interference with the subsequent separation of the analyte as well as to protect the analytical column. A guard-column for the latter is therefore strongly recommended. The precolumn lifetime amounts to about 80 mL of biological matrix when processing horse plasma.

The optimization of the transfer step consists of peak compression of the analytes eluting from the precolumn. With reversed-phase columns, peak compression can be achieved by ensuring that the content of organic modifier in the mobile phase used for transfer and separation is higher than in the washing fluid. However, high organic modifier solvent contents may cause buffer precipitation, which can be the cause of clogging precolumns and tubings. To avoid protein precipitation, the concentration of the organic modifier, the pH and the ionic strength of the washing fluid applied for the sample loading must be non-denaturating. As the run time of the analytical

Table 2. Linearity of ketoprofen extracted from spiked horse plasma (performed on different days; $n \geq 3$)

Range	Concentration of ketoprofen (ng/mL)	Injection volume (μL)	Linearity area of ketoprofen	Linearity ratio of area of ketoprofen:phenacetin
High	4000–40000	100	0.9994	0.9995
Medium	400–4000	200	0.9999	0.9991
Low	40–400	400	0.9990	0.9988

Table 3. Inter-day determination of ketoprofen in horse plasma samples

Calculation of area		Calculation of ratio/area	
Ketoprofen (ng/mL)	RSD (%)	Ketoprofen (ng/mL)	RSD (%)
32248	1.4	32521	0.9
11131	2.0	11190	2.5
3254	0.9	3227	0.8
1003	7.0	1009	3.6
299	0.4	299	1.3
100	6.8	107	10.4

separation is about 15 min (greater than the run time of the sample preparation) the fractionation of the next sample can be performed simultaneously with the analyzing step of the preceeding sample analysis (Fig. 1). The overlap of sample preparation, analysis and reconditioning of the precolumn increases the overall sample throughput. However, ghost peaks or base-line abnormalities, originating from column-switching (eluent or pressure change) have to be considered to eliminate interferences with the analytical separation. The HPLC system described was able to process about 40 horse plasma samples per 24 h. With precolumn equilibration during run analysis the sample throughput may even be increased up to 60 samples.

Recovery

When adapting the time-consuming diethyl ether extraction of acidified plasma to the LC-integrated column switching technique, the recovery of the applied internal standard naproxen reached only 20%, probably due to its high protein binding properties, the recovery of ketoprofen already being satisfactory.

Increasing the molarity of the washing fluid from 0.05 to 0.1 M improved the recovery of naproxen to about 40%; further molarity increase was omitted in order to avoid precipitation in the mobile phase. As the mean value for the recovery of phenacetin from plasma, performed at three concentration levels, was 99.8% and as an acceptable separation was obtained, the latter compound was used as the internal standard.

The recoveries of ketoprofen from spiked samples (Table 1), at six different concentrations, were calculated by comparing the obtained peak areas with those from aqueous solutions. A mean value of 96.7% was reached when peak areas of ketoprofen were used and of 96.8% when calculation was performed employing peak area ratios of ketoprofen/phenacetin.

Linearity

The relationship was investigated between detector response (peak areas) and drug concentration in plasma samples spiked with known ketoprofen amounts, ranging from 40 to 40.000 ng/mL, in three different ranges (five calibration points), each with the appropriate internal standard concentration. Evaluation of the linear regression coefficient for each range (Table 2) and calculations based on ketoprofen peak areas and on peak area ratios ketoprofen/internal standard proved that an internal standard is not necessary. Moreover, the relationship between injection volume and peak areas, as expected, proved to be reliable (ketoprofen 1 μg/mL, injection volume 10–100 μL, $r = 0.9996$).

Intra-day variations

Due to the automation and the integration of the sample clean-up, the LC-integrated sample preparation system turned out to be highly reproducible. Intra-day assay at three concentrations was performed on freshly prepared plasma samples (spiked 10 times). Calculation of the relative standard deviation performed on peak areas of ketoprofen, ranging from 0.3 to 0.8%, proved the variations to be acceptable. The values obtained with peak area ratios were slightly higher, from 0.4 to 1.2%.

Inter-day variations

Inter-day relative standard deviations were measured at six different ketoprofen-spiked plasma concentrations. The obtained values are shown in Table 3. As a conclusion it can be stated that the values obtained with ketoprofen peak areas are lower than those after calculations based on peak area ratios.

Limit of quantitation and limit of detection

The limit of quantitation, being the lowest concentration that can be quantified with acceptable accuracy, was 10 ng/mL. A ketoprofen concentration of 2 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 ketoprofen. These limits were established by a 400−μL injection. The cited limits may be lowered by injecting larger volumes.

Internal standard

Evaluation of the above-mentioned values was obtained after calculation performed on peak areas of ketoprofen and on peak area ratios of ketoprofen/phenacetin. These values demonstrate the excellent extraction efficiency of the ADS precolumns, as the recovery of the drug from the biological matrix is quantitave and the accuracy of the

injection volume satisfactory, hence the addition of an internal standard is considered not necessary.

DISCUSSION

It is expected that the developed system may not only be applied to the determination of ketoprofen, but also to the assay of other drugs from various pharmaceutical groups.

In initial experiments the recoveries from plasma of some representatives of the barbiturate group, analgesics, local anesthetics and xanthines were controlled and proved to be satisfactory. Only the detector wavelength and the concentration of the organic modifier needed adaptation.

The essential features of the method are the novel precolumn packing LiChrospher ADS, with the advantage of direct and repetitive injection of untreated plasma samples, except for a filtration step. Moreover there is a potential for safer handling of possibly infectious biological fluids. An on-column enrichment of analytes with elimination of the protein matrix and a quantitave recoverage of ketoprofen is achieved. Due to the quantitative elimination of the matrix, the application of an internal standard can be omitted. As the ADS column exhibits a long life-span there is a low cost per sample. A considerable reduction of the analysis times compared to manual methods for bioavailability studies is obtained together with an excellent linearity, good precision and accuracy.

A coupled-column system using ADS precolumn packings should have a broad application in pharmacokinetics, drug-monitoring and screening; further work in this area is in process.

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