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Application of the restricted-access precolumn packing material alkyl-diol silica in a column-switching system for the determination of ketoprofen enantiomers in horse plasma

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Abstract

The group of LiChrospher ADS (alkyl-diol silica) sorbents that make part of a unique family of restricted-access materials, have been developed as special packings for precolumns used in the LC-integrated sample processing of biofluids. The advantage of these sorbents lies in the direct injection of untreated biological fluids, that is without sample clean-up, the elimination of the protein matrix with a quantitative recovery together with an on-column enrichment. The present method is based on previous work applying UV detection at 260 nm for ketoprofen determinations. Plasma samples introduced to the ADS precolumn using a 0.1 *M* phosphate buffer, pH 7.0. After washing with the buffer the ADS column was backflushed with the mobile phase 0.01 *M* phosphate buffer–6% (v/v) 2-propanol–5 m*M* octanoic acid at a pH of 5.5, thus transporting the analytes to the chiral-HSA (human serum albumin) (100×4.0 mm) column where the separation of the ketoprofen enantiomers was achieved with a resolution factor of 1.4. The developed column-switching method was fully applicable to plasma injections.

Keywords: Column switching; Alkyl-diol silica columns; Enantiomer separation; Ketoprofen; Nonsteroidal anti-inflammatory drugs

1. Introduction

Ketoprofen [(±)-2-benzoylphenylpropionic acid] is a non-steroidal anti-inflammatory drug of the propionic acid group which also includes ibuprofen, naproxen and flurbiprofen. In human therapy ketoprofen has been used in rheumatology because of its analgesic and anti-inflammatory properties, and 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 [1–3]. Initial work from our groups involved the determination of ketoprofen in plasma using narrow-bore reversed-phase HPLC. For this purpose the residue of a diethyl ether extract of

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the acidified plasma samples was solubilized in the mobile phase and subsequently injected into the chromatographic system [4,5].

Conventional methods for analysis of the target molecule in plasma samples require extensive sample preparation and often introduce substantial experimental errors. In order to avoid time-consuming manipulations, a rapid, robust and reproducible method using an automated column-switching liquid chromatographic system for the determination of ketoprofen applying a reversed-phase column and UV detection was recently reported by our group [6,7].

A column-switching configuration employing specially developed precolumn phases enables the direct injection of untreated biological fluids into an HPLC set-up, followed by subsequent analysis of the ketoprofen analyte. The use of restricted-access material (RAM) phases is based on the complete non-adsorptive size-exclusion of macromolecules and on the simultaneous extraction of low-molecular-mass analytes. The plasma matrix compounds are quantitatively eluted in the void volume of the precolumn due to the restricted access given by the pore size of the packing.

The LiChrospher alkyl-diol silica (ADS) belonging to the RAM family [8–11] was developed as a special packing material for precolumns used in LC-integrated sample processing systems and applied to the determination of different drugs in biological matrices [12–15].

As is known, the measurement of drug levels in body fluids is carried out for a variety of reasons; e.g., quantitative bioanalysis is required at many steps of drug development. When the drug to be analysed is chiral the analysis becomes more complicated: enantiomers exhibit identical physicochemical characteristics but may behave entirely different in biological systems.

Ketoprofen possesses an asymmetric carbon atom and is therefore a chiral molecule existing as two enantiomers. The determination of the enantiomeric purity of drug substances has increased in importance during the last decade, along with the manufacture of chiral drugs. The enantiomeric composition of new molecules must be determined in view of the study on drug efficiency and safety. Reliable quantitative methods for the separation of drug enantiomers for process and purity control studies are thus a necessity. As enantiomeric differences in drug distribution, metabolism and excretion are common in view of pharmakinetic studies, it is highly desirable that bioanalysis of a chiral substance distinguishes between the stereochemical forms. Since the early 1980s, a number of approaches for the analytical separation of enantiomers have been developed mainly in the area of high-performance liquid chromatography and capillary electrophoresis.

In previous work from this group, precolumn derivatization was applied for the separation of ketoprofen enantiomers [16–18]. However, nowadays direct injection of the initial solution is often preferred [19–21], employing a variety of stationary phases [22–26].

One of the advantages of protein-based chiral sorbents is that separation is performed in the reversed-phase mode. Hence aqueous mobile phase systems compatible which those used with the ADS precolumns can be applied. A wide variety of chiral organic compounds of different structural types [27–31] have been resolved into their enantiomers on albumin-based columns. A method applying an avidin protein-conjugated column for direct injection analysis of ketoprofen enantiomers in plasma (run time 30 min) was described by Oda et al. [32].

2. Experimental

2.1. Reagents and standards

All solvents and chemicals used were of HPLC or analytical reagent grade and no further purification was carried out. Ketoprofen was purchased from Sigma (St. Louis, MO, USA); monobasic potassium phosphate, octanoic acid and 2-propanol were purchased from Merck (Darmstadt, Germany). The phosphate buffers were prepared by dissolving the appropriate amount in deionised water, brought to pH with a sodium hydroxide solution (20%, v/v).

2.2. Sample collection and preparation

A standard solution of ketoprofen at 420 μg/ml

(high range) in 0.1 *M* phosphate buffer, pH 7.0, was prepared, dilutions were made to provide two working standard solutions of 42 μg/ml (medium range) and of 4.2 μg/ml (low range). All solutions were stored in dark glassware at about 8°C, they were stable for at least 3 months.

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, as well as at 1, 2, 3, 4, 6, 8, 10, 12 and 24 h after intravenous administration of 1.0 g ketoprofen. Blood samples were collected in vacuum tubes (Venoject, Kimble-Terumo, Elkton, USA) containing lithium heparin as an anticoagulant. Plasma was separated by centrifugation at 2000 *g* for 2 min and stored at −20°C until analysed.

The calibration standard solutions were prepared by spiking 1000 μl drug-free horse plasma with varying volumes of 10, 30, 50, 70 and 90 μl, respectively, of the standard working solutions in 0.1 *M* phosphate buffer, pH 7.0, and made up to 100 μl with the latter buffer solution. A 60-μl volume of 1.8 *M* phosphate buffer, pH 7.0, was added to buffer the solution in order to eliminate small pH variations of the plasma samples. The samples to be analysed were prepared by adding 100 μl 0.1 *M* phosphate buffer, pH 7.0, and 60 μl 1.8 *M* phosphate buffer, pH 7.0, to 1000 μl of horse plasma. The prepared plasma solutions were filtered through a regenerated cellulose (grey, 0.2 μm, 13 mm, hold-up volume 28 ml) syringe filter (Chromacol, Herts, UK) and placed in 1.5-ml threaded vials (Merck) provided with screw caps with hole and slotted silicon/PTFE septa (Merck). Although not necessary a filtration step was performed to eliminate particles and small flocks, especially with older plasma samples, so as to eliminate blocking of tubing, injection valve and column filter.

2.3. Apparatus

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

2.4. Chromatographic conditions

After conditioning the precolumn LiChrocart 25-4 LiChrospher RP-18 ADS (Merck) with 9 ml 0.1 *M* phosphate buffer, pH 7.0, a volume of 80 or 500 μl, depending on the ketoprofen concentration in the horse plasma, was injected. The plasma matrix was then removed from the precolumn with 9 ml of the same buffer solution.

After 11 min the valve was switched and the analyte was transferred to the analytical column chiral-HSA, 100×4.0 mm, provided with a guard column, 10×3.0 mm (Advanced Separation Technologies, NJ, USA) applying a mobile phase consisting of 94% (v/v) 0.01 *M* phosphate buffer, 6% (v/v) 2-propanol and 5 m*M* octanoic acid at pH 5.5, at a flow-rate of 0.6 ml/min, and kept at 30°C. The UV-detector was set at 260 nm. Separation of the enantiomers was achieved with a resolution factor (R_s) of 1.4.

3. Results and discussion

3.1. Alkyl-diol silica precolumn

Before HPLC analysis macromolecular compounds have to be removed from the sample because of their precipitation by the high amounts of organic solvents and because of their binding to the surface of the packing material, leading to an irreversible increase in pressure and to a loss of the column capacity and selectivity. In an LC-integrated sample preparation set-up the sample first is fractionated into sample matrix and analytes by the use of the LiChrospher ADS precolumn. The latter possesses two chemically different surfaces. At the outer surface of the spherical particles hydrophilic, electroneutral diolgroups are bound preventing any interaction with proteins. This means that the protein matrix of a biological sample can be directly flushed into the waste as the precolumn applied excludes the macromolecules in the void volume. Meanwhile the analyte fraction having free access to the binding centers at the inner surface of the porous particles (pore size 6 nm) is selectively extracted and enriched at the stationary phase of the precolumn. In this way, the packing material provides a direct extraction

base, fully automated on-column enrichment and subsequent analytical separation of low-molecular target compounds from untreated plasma samples.

Out of the three types of LiChrospher RP-ADS phases, available as LiChroCART 25-4 cartridges, covering the whole range of hydrophobic capacity factors, the most suitable precolumn for a given analyte has to be determined in each specific case. Preliminary work [4], coupling the precolumn to a reversed-phase Ecocart column, and eluting the RP-4 ADS precolumn with 50 m*M* phosphate buffer, pH 7.0, yielded a very small capacity factor for ketoprofen. The system with the LiChrospher RP-18 ADS precolumn provided less disturbed chromatograms and more stable baselines than when applying RP-8 ADS and was therefore chosen for further work. The ADS column lifespan mounted up to 80 ml when processing horse plasma. Depending on the injection volume, ranging from 80 μl in the high range to 500 μl in the low range, some 300 injections can be made. The price of sample pretreatment is lower than or at least comparable to that of solid-phase extraction.

During the method development and the actual determination of ketoprofen about 300 ml horse plasma was injected and no changes of the chiral column performance were observed.

3.2. Human serum albumin (HSA) column

In recent years a large number of resolutions of racemic pharmaceuticals have been described by means of liquid chromatography employing protein phases. However, when the target molecule is present in a biological matrix various problems have to be overcome and often special techniques are required, especially in order to process the samples on-line.

Moreover, the efficiency and peak capacity of the HSA column, taking into account the interferences by endogenous plasma matrix components together with the low levels of analyte to be measured make sample pretreatment and fractionation of utmost importance.

The retention of ketoprofen enantiomers on an HSA-based HPLC chiral stationary phase coupled to an ADS packing was investigated so as to assess the utility of direct injection of plasma samples.

Before application onto the HSA column, ketoprofen samples were introduced to an ADS column for preconcentration purposes in a precolumn switching mode, the main aim being to perform direct analysis of plasma samples. Different parameters (temperature, organic modifier, phosphate buffer molarity and pH) influencing the separation of the ketoprofen enantiomers in spiked buffer solutions and in plasma were investigated in the LC-integrated sample preparation set-up with the RP-18 ADS precolumn and the HSA analytical column, connected with a six-port valve. The molarity (range 0.01– 0.05 *M* with 0.01 *M* intervals) was the most important parameter for the separation of the ketoprofen enantiomers. The pH (5.5–7.0), the organic modifier concentration (4–10% (v/v) 2-propanol), and the octanoic acid concentration (4–8 m*M*) had only a slight influence on the resolution. The values chosen were those offering the lowest retention for the ketoprofen enantiomers. Excellent recoveries were obtained when applying the volumes of washing fluid obtained from previous work for the determination of ketoprofen.

A mobile phase consisting of 2-propanol (6% v/v)–5 m*M* octanoic acid–0.01 *M* phosphate buffer, pH 5.5, at a flow-rate of 0.6 ml/min on the chiral-HSA column, protected with a guard column, in combination with an 0.1 *M* phosphate buffer, pH 7.0, as preconcentrating mobile phase on the ADS precolumn provided satisfactory retention and separation (resolution factor $R_s = 1.4$) for both ketoprofen enantiomers within 20 min.

The developed column-switching method was fully applicable to plasma injections and for the subsequent determination of the ketoprofen enantiomers.

3.3. Switching times

When developing a column-switching method, the switching times must be determined initially [33,34]: 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, second the switching time for the transfer step. The elution profile of the sample matrix on the precolumn applied was determined by direct connection to the

UV detector set at 260 nm — although other wavelength settings are to be considered as well — applying a given flow-rate. A 500-μl blank horse plasma volume was injected and the detector signal monitored. The fractionation step was considered complete when the detector signal reached the baseline. After injection of the buffered and filtered sample onto the precolumn, the unwanted matrix components are flushed directly into the waste. Depending on the injection volume, the time required for the sample washing step may be adapted. The complete elimination of matrix components must be achieved in order to prevent interferences with subsequent separation of the analyte, and to protect the HSA column. A guard column for the latter is therefore strongly recommended. However, high organic modifier solvent contents may cause buffer precipitation which can be the cause of clogging precolumns and tubing. 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-denaturing.

The optimization of the transfer step consists of peak compression of the analytes eluting in backflush mode from the precolumn. With columns applied in the reversed-phase mode, 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. The re-equilibration of the precolumn with the initial washing fluid and the injection followed by the fractionation of the next sample can be performed simultaneously to the separation step and detection of the preceeding sample. The overlap of sample preparation, analysis and reconditioning of the precolumn increases the overall sample throughput.

Nevertheless, ghost peaks or baseline abnormalities, originating from column-switching (eluent or pressure changes) have to be considered to eliminate interferences in the analytical separation. The HPLC system described was able to process about 30 horse plasma samples (typical chromatogram of spiked horse plasma in Fig. 1) per 24 h (cycle time 40 min). With precolumn equilibration and fractionation of the sample during the analysis step the sample throughput may even be doubled.

Due to more pronounced differences (pH, molarity, presence of octanoic acid) of the washing fluid for the sample preparation and the mobile phase

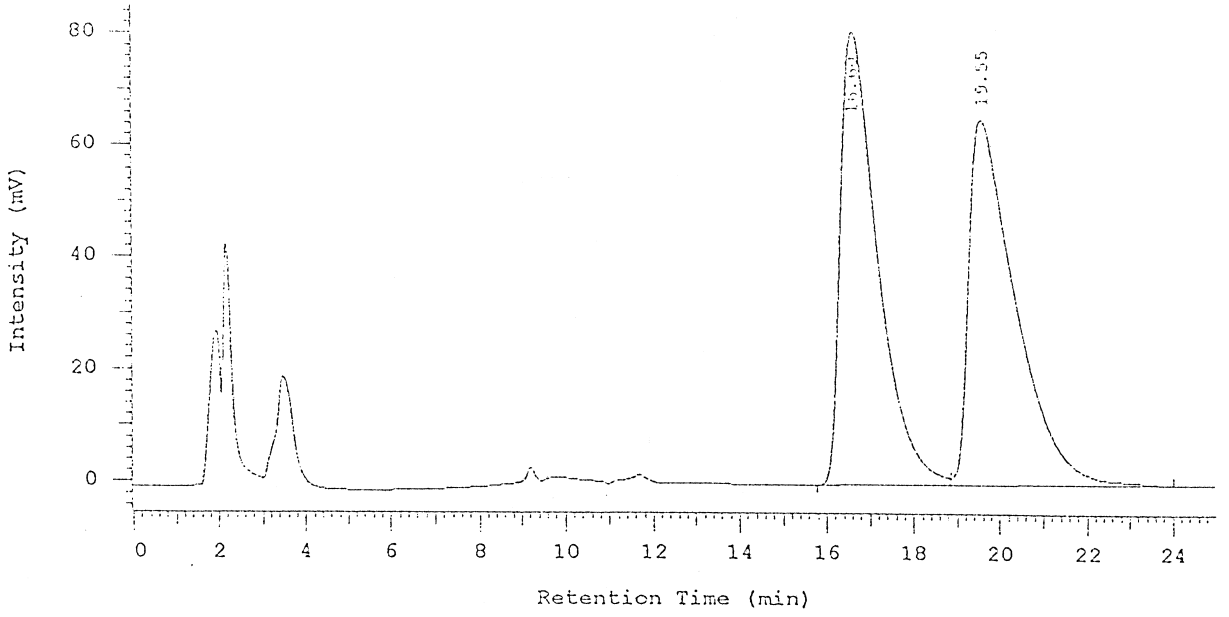

Fig. 1. Typical chromatogram obtained after injection of 500 μl of horse plasma spiked with 3600 ng/ml racemic ketoprofen. Retention times: *S*-ketoprofen 16.6 min, *R*-ketoprofen 19.6 min.

Table 1
Recovery of ketoprofen enantiomers from spiked horse plasma (values are the mean of three determinations performed at each concentration level)

Range (ng/ml)	Conc. enantiomer (ng/ml)	Recovery (%)	
		S-Ketoprofen	*R*-Ketoprofen
High	18 000	100.0	102.0
Medium	1800	100.2	100.2
Low	180	97.5	92.1

required for chromatography on the chiral column, the time periods were extended in comparison with the ketoprofen determination applying the reversed-phase EcoCart column.

3.4. Recovery

The recoveries of ketoprofen from spiked samples, determined at three different concentrations, were calculated by comparing the obtained peak areas with those from aqueous solutions (Table 1). Mean values of 99.2 and 98.1% for *S*-ketoprofen and *R*-ketoprofen, respectively, were obtained when the calculation was performed employing peak areas. These values demonstrate the excellent extraction efficiency of the ADS precolumns as the recovery of the drug from the biological matrix is quantitative, the addition of an internal standard not being necessary.

3.5. Linearity

The relationship between peak area and drug concentration in plasma samples spiked with known drug amounts covering the three concentration levels (high, medium and low range) was investigated. Due to the high recovery of ketoprofen, excellent linearity was obtained in the three working ranges indicating that no internal standard is required (mean values *S*-ketoprofen: $r = 0.9997$; *R*-ketoprofen: $r = 0.9993$).

3.6. Intra- and inter-day variations

Due to the automation and integration of the sample clean-up, the LC-integrated sample preparation system proved to be highly reproducible. The relative standard deviations for an intra-day ketoprofen enantiomer determination of three solutions (each spiked 10 times) were lower than 3%.

Inter-day relative standard deviations (from 0.3 to 6%) were measured at six different ketoprofen concentrations. The obtained values are shown in Table 2.

3.7. Limit of quantitation and limit of detection

The lowest concentration that can be quantified with acceptable accuracy (20% RSD) and precision was 16 ng/ml for *S*-ketoprofen. An *S*-ketoprofen concentration of 5 ng/ml plasma was considered the limit of detection (Table 3). As injection volumes and integration parameters were different in the three concentration ranges the obtained limit values are slightly different. The values for *R*-ketoprofen were of 18 ng/ml for the limit of quantitation and of 10 ng/ml for the limit of detection. The latter was calculated on a basis of three times the area of interfering signals arising in the chromatogram with a capacity factor close to the k' values of the

Table 2
Results of ketoprofen inter-day determinations ($n = 3$) in horse plasma samples ($n = 6$) after intravenous administration

Range	*S*-Ketoprofen		*R*-Ketoprofen	
	Concentration (ng/ml plasma)	RSD (%)	Concentration (ng/ml plasma)	RSD (%)
High	14 140	0.6	14 340	0.4
	10 240	0.4	10 510	0.3
Medium	2454	2.8	1063	6.0
	583	0.4	197	2.0
Low	121	3.7	121	2.1
	100	7.0	30	1.7

Table 3
Limit of quantitation (LOQ) and limit of detection (LOD) for ketoprofen enantiomers

Range	Concentration enantiomer (ng/ml)	*S*-/*R*-Ketoprofen	
		LOQ (ng/ml)	LOD (ng/ml)
High	2000–20 000	1600/1600	160/200
Medium	200–2000	63/65	5/10
Low	20–200	16/18	5/10

respective ketoprofen enantiomers. Calibration graphs were established with three replicates for each concentration value. The performance analytical characteristics (analytical sensitivity, limit of quantitation and limit of detection) were calculated from the calibration data sets using the ALAMIN program [35–37]. The latter has been developed for the calculation of performance characteristics of an analytical method from the standard calibration data set. From the calibration curve of a series of replicates of different standard solutions applying the linear regression analysis, linearity, sensitivity, detection limit, determination limit and precision can be directly calculated with a small number of experiments, only. The obtained values were checked practically and compared utilizing spiked plasma and a good agreement was found.

3.8. Profile

The present study was designed to examine the stereoselective fate in vivo of racemic ketoprofen after rectal administration to healthy horses. A plasma concentration–time profile after intravenous administration of 1 g ketoprofen is shown in Fig. 2. After i.v. administration the *S*-(+)-enantiomer concentrations in plasma were higher than the *R*-(−)-enantiomer concentrations and the $AUC_{0-12\ h}$ (AUC = area under the curve) for the *S*-(+)-enantiomer was significantly higher than for the *R*-(−)-

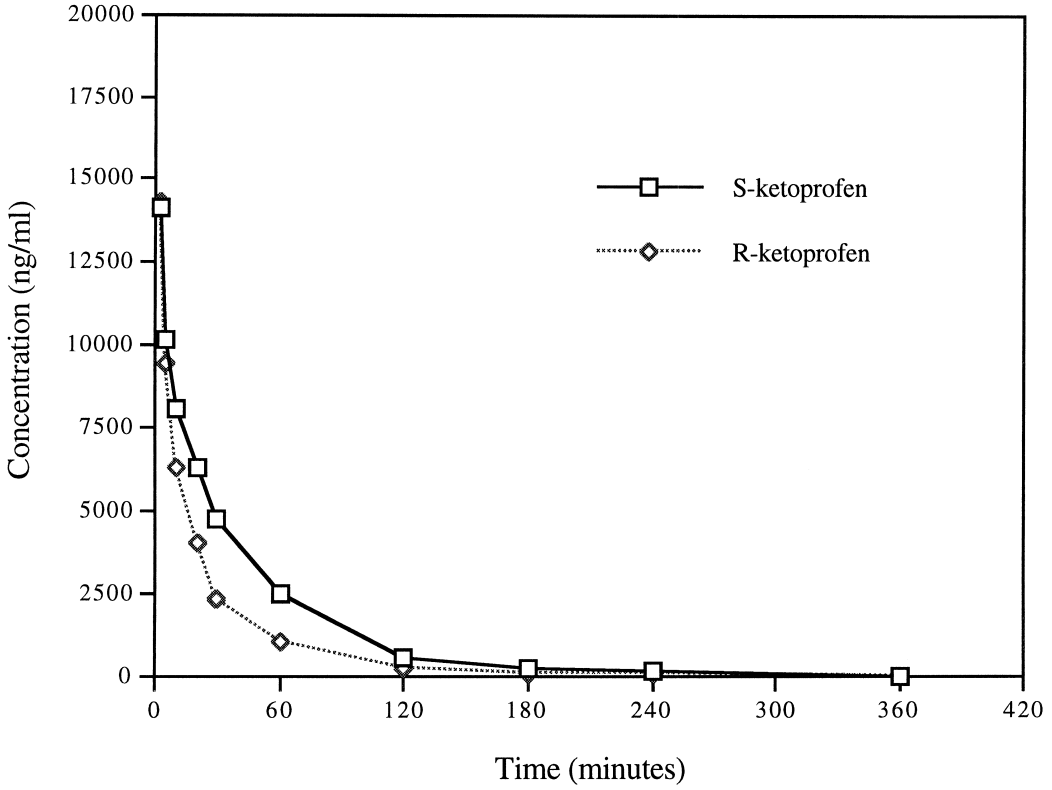

Fig. 2. Plasma–concentration time profile after intravenous administration of 1000 mg racemic ketoprofen to a healthy horse.

enantiomer. Following rectal administration C_{max} and $AUC_{0-12\ h}$ were significantly higher for the *S*-(+)- than for the *R*-(−)-enantiomer. Bioavailability after rectal administration was low. Since there was no significant difference in bioavailability between the two enantiomers, it is assumed that no presystemic inversion from *R*-(−) to *S*-(+) occurs after rectal administration of racemic ketoprofen to horses [38].

4. Conclusion

As conventional sample preparation is time-consuming and expensive, the use of the novel surface reversed-phase precolumn packing materials ADS proved to enable the development of LC-integrated sample clean-up for routine determinations of a variety of drugs belonging to different pharmaceutical groups and for drug enantiomers measurement in a biological matrix. No manual sample clean-up — except possibly a filtration step — is necessary allowing direct injection of untreated biological plasma samples. Because of the total automation and hence the safer handling of infectious biological fluids, there is the benefit of high sample throughput resulting in cost reduction.

Moreover precision, accuracy and sensitivity of the assay improve, due to the minimum manipulations of the biological sample. Because of the total elimination of the protein matrix, the recovery of the analyte is quantitative and no internal standard is required.

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