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Determination of ketoprofen enantiomers in plasma and exudate by liquid chromatography-Mass spectrometry

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ABSTRACT

A liquid chromatographic-mass spectrometry method for the determination of ketoprofen enantiomers in swine/cat plasma and exudate is described. Plasma /exudate proteins were precipitated with acetone-trichloroacetic acid and centrifuged. The supernatant (~ 500uL) was filtered through a centrifuge filter tube before it was diluted and injected into the LC-MS/MS. The recovery for both R and S ketoprofen varied from 97 to 98% in plasma and exudate. The limits of quantification of R and S were 10 ng/mL and the limit of detection was 5 ng/mL for both R and S in plasma and exudate, based on 0.5 mL swine plasma or exudate.

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KEYWORDS

Ketoprofen;
Enantiomers;
LC-MS;
MS/MS;
HPLC;
Chiral separation.

INTRODUCTION

Ketoprofen (KTP) is a non-steroidal anti-inflammatory drug of the 2 aryl propionate family that has analgesic, anti-inflammatory and antipyretic properties[1]. KTP is a chiral compound that contains an asymmetrical carbon atom and exists in two enantiomeric forms[2]. Most of the clinical effect of KTP has been attributed to the S-enantiomers, but the R-form may have significant analgesic activity[3]. Enantiomers have very similar or identical physico-chemical properties and it is therefore difficult to distinguish between them in an achiral environment. On the other hand, the pharmacokinetic properties of the two enantiomers may differ profoundly. This arises because the body is a chiral environment. Studies of the pharmacokinetics of KTP should therefore always measure and quantify the separate enantiomers and not "total drug".

KTP is a well-described anti-inflammatory and analgesic agent in veterinary and human medicine. The published analytical methods are though rather complicated, and not suited for the small sample. For large sample series, a simple and rapid extraction and clean-up method is very beneficial, as this allows for a higher sample throughput and thereby comparative studies across species.

Several analytical methods based on high performance liquid chromatography (HPLC) with UV absorbance, capillary electrophoresis or enantiomers concentrations in plasma, including liquid-liquid extraction, dialysis[4-8] and solid-phase extraction[9]. Also the bioanalytical analyses of ketoprofen by mass-spectrometry (MS) based techniques have been performed using GC/MS[10]. To the GC/MS technique, derivatization is required. These methods are generally time-consuming.

Liquid chromatography mass-spectrometry (LC-MS/MS) is a more sophisticated technique allowing a very effective isolation of analyte ions from the noise-producing matrix. No derivatization is required. Only one publication utilizing LC-MS/MS for detection of KTP enantiomers[11]. In addition to the separation column, an automated solid-phase extraction system is inserted in the analytical line in this method. A minimum of 1 ml plasma is required for the analysis.

The aim of this work was to develop and validate a very simple, sensitive and reliable LC-MS/MS method for KTP enantiomers, based on 0.5 mL sample or less, and at the same time maintain the sensitivity necessary for pharmacokinetic studies in animals. The sample pretreatment described can also be used for analyzing the total concentration of KTP.

EXPERIMENTAL

Materials and reagents

Samples of drug free swine and cat plasma and exudate were used as control material and for spiking with KTP.

All chemicals and solvents were of analytical or HPLC grade. Racemic KTP (Sigma Co. St. Louis, MO, USA) and S (+)-KTP (Aldrich, Steinheim, Germany) stock standard solutions (l mg/mL) were prepared by dilution with methanol. The working standard solutions were made by aliquots from these stock solutions diluted with methanol. Stock and working standard solutions were stored at +4 °C.

Spin-X centrifuge tube filter units 0.22 μm, nylon type (Costar, NY, USA) were used for filtration.

Trichloroacetic acid (TCA) was supplied by Merck, (Darmstadt, Germany), and 0.15 % TCA in acetone was prepared by dissolving 85 g TCA in 15 g water (stock solution) and mixing 150 μL of this stock solution with 100 mL acetone.

Chromatographic conditions

The LC-MS-MS instrumentation used for the method development consisted of a Series 200 quaternary pump and autosampler (Perkin Elmer, Norwalk, USA) and an API 2000 LC-MS/MS system (Applied Biosystems, Ontario, Canada) equipped with a Turbo-Ion Spray ion source. The turbo probe vaporizer temperature of the interface was fixed at 425 °C. The MS was set to collect ion data in positive mode. Data were acquired in the multiple reaction monitoring (MRM) mode. The protonated molecular ion was *m/z* 255. The product ion *m/z* 209.1 was used for screening and quantification, while the ratios with the product ion *m/z* 105.2 were used for confirmation of the identity.

A precolumn filter A-318 with an A-102X frits (Upchurch Scientific,USA) was connected to the guard column. The column Chirobiotic R (Ristocetin A), 100 x 4.6 mm (Astec, NJ, USA. cat. N°. 13022) and guard column (20 x 4.0 mm) containing the identical sorbent, were operated at a constant temperature of 18 °C. The mobile phase consisted of a mixture of two solutions. Solution A consisted of 10 mM ammonium acetate added 0.3 mL/L formic acid and B was methanol. The mobile phase operating conditions are shown in TABLE 1.

TABLE 1 : Mobile phase operating conditions

Step	Total time (min)	Flow (uL/min)	Solution A (%)	Solution B (%)	TE#1
0	0.0	600	46	54	Open
1	4.0	600	46	54	Close
2	7.8	600	46	54	Close
3	10.0	600	46	54	Open

TE#1= time events.

After separation, the LC fluent was connected to a two-position micro electric valve actuator (Vici, Valco Instruments Co. Inc. Texas, USA) programmed in mode two by our provider. Thereafter, the LC fluent was split approximately 1:4 before entering the MS interface.

Sample pretreatment

A volume of 0.2 mL methanol or standard dissolved in methanol (to standards curves the corresponding volume of standard solution were diluted to 0.2 mL with methanol) and 0.7 mL 0.15 % TCA in acetone was added to 0.5 mL plasma or exudate. The mixture was mixed with a wirlimixer for 10 sec. After centrifugation for 4 min at 3600 rpm, approximately 0.5 mL of the supernatant was filtered through a Spin-X centrifuge filter with centrifugation for 1 min at 10000 rpm (5600 x g) with a Costar (USA) mini centrifuge. To 0.1 mL filtrated supernatant, 0.2 mL 1M ammonium acetate was added. After whirl - mixing for 2 sec, 50 μL was injected into the LC-MS-MS system at intervals of 10

min for the determination of KTP enantiomers.

Calibration curves and recovery studies

The precision, recovery and linearity for KTP enantiomers were determined by spiking cat and swine plasma and cat exudate samples with standard solutions to yield 0, 10, 15, 20, 25, 50, 100, 200, 400, 600, 1000, 4000 and 8000 ng/mL. The recovery was determined by comparing the appropriate peak areas from the spiked samples with those of standard solutions of KTP. The standards were prepared by diluting the stock standard with methanol to 0.2 mL, adding 0.5 mL water and then 0.7 mL 0.15 % TCA in acetone. After mixing, 0.1 mL of this solution was mixed with 0.2 mL 1 M ammonium acetate.

RESULTS AND DISCUSSION

The standard curves were linear in the investigated areas from 10 to 8000 ng/mL for R (-) and S (+) KTP. The linear correlation coefficients were r = 0.9999 for R (-) and S (+) KTP. The recovery and repeatability values for R (-) and S (+) KTP from spiked swine plasma, cat plasma and exudates are shown in TABLE 2.

Chromatograms obtained from drug-free cat plasma and from the corresponding samples spiked with KTP are shown in Figure 1.

Chromatograms of drug-free cat exudates and swine plasma spiked with KTP and also with the S- enantiomer only, in addition are shown in Figure 2. The S-KTP eluted first and R-KTP eluted secondly, using the described chromatographic conditions.

TABLE 2

Number of Samples	Amount of drug (ng/mL)	R (-) KTP (%) SD	R (-) KTP (%) RC	S(+) KTP (%) SD	S (+) KTP (%) RC
Swine plasma					
6	25	1.3	98	1.3	98
6	100	1.1	97	0.8	97
6	400	0.9	98	0.7	98
Cat plasma					
6	400	0.7	97	0.5	97
6	1000	0.8	97	0.4	97
Cat exudate					
6	400	0.4	98	0.4	98
6	1000	0.3	98	0.4	98

SD = Standard deviation; RC = Recovery

The limit of detection for KTP enantiomers were calculated as three times the peak-to peak baseline noise (S/N = 3) from drug-free swine plasma. It was 3.5 ng/mL. The limit of quantification for either enantiomer was 10 ng/mL based on 0.5 mL plasma.

If the amount of plasma/exudate available was less than 0.5 mL, purified water was added to a total volume of 0.5 mL and the results were corrected for the

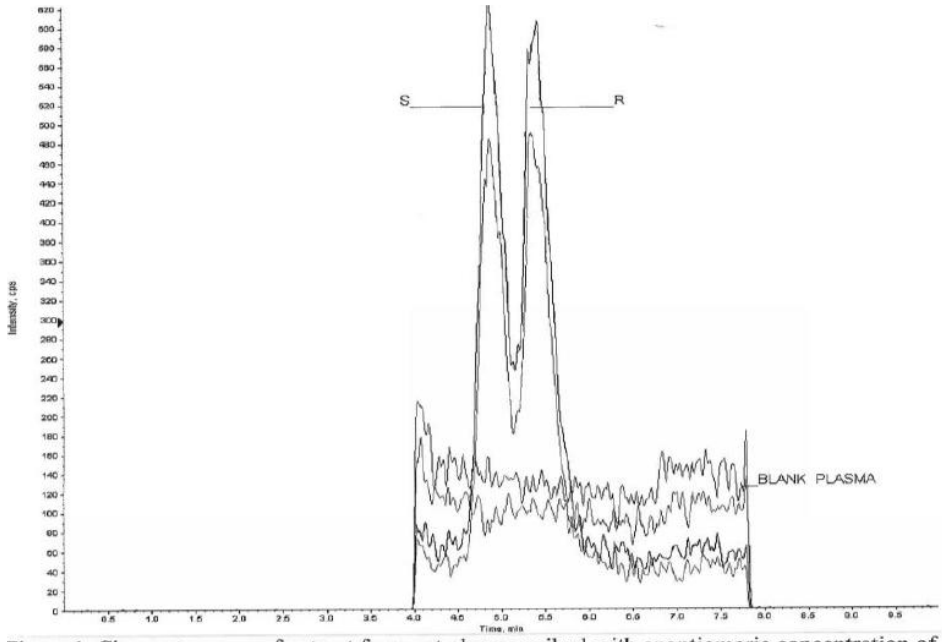

Figure1. Chromatograms of extract from cat plasma spiked with enantiomeric concentration of 25 ng/mL racemic ketoprofen and blank cat plasma. Chromatograms showed product ions *m/z* 105

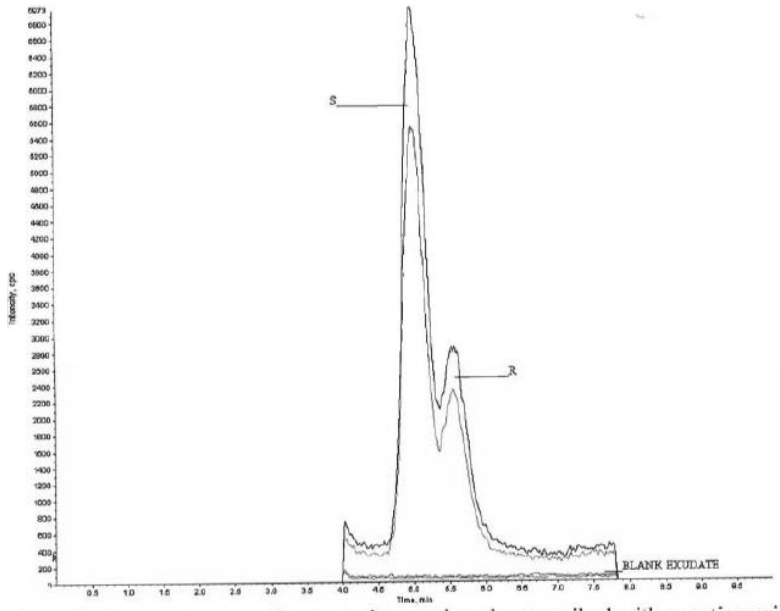

Figure 2. Chromatograms of extracts from swine plasma spiked with enantiomeric concentration of 200 ng/mL racemic ketoprofen and spiked additional with 250 ng/mL of S enantiomers. Also chromatograms of blank exudate. Chromatograms showed product ions *m/z* 105 and 209.

dilution factor.

Enantiomers have very similar or identical physicochemical properties and it is therefore difficult to achieve separation between R (-) and S (+) KTP. Improved separation was achieved with an increased the ammonium acetate concentration in the sample vial.

Also the addition of 0.3 mL/L formic acid to the 10 mM ammonium acetate in the mobile phase and a low column temperature (18 °C) improved separation.

The efficacy and selectivity of the Chirobiotic R column in the reverse phase mode was stable across the various sample series analyzed. The pH of the buffer is most important parameter in chiral selectivity in the reverse phase mode. Chirobiotic phases have been used in the reverse phase mode with buffers in a pH range of 3.5 to 7.0. The stability of the chiral stationary phase/analyte complex is dependent on the charge of the analyte. Because of the complexities of these interactions, it is necessary to observe the retention and resolution as a function of pH. It has been determined that the safest and most stable pH-ranges for the chirobiotic column phase R were 3.5 to 6.8.

Lower temperature results in higher resolution. Higher temperatures reduce tailing, decrease retention and can reverse elution order[12].

The use of a two position micro electric valve actuator prevented unnecessary mobile phase and the solvent front containing water - soluble co - extracts to reach the MS. Thereby, unnecessary contamination of the instrument could be avoided. It is essential that the micro electric valve actuator is programmed in mode two. The micro electric valve actuator appears favorable in all MS analyzes. The actuator was guided from data software under LC pumps (events). When the event is open, the mobile phase flows to waste. When the event is closed, the mobile phase flows to the MS until a new close event is given; hereafter, the mobile phase flows to the waste. However, the use of a micro electric valve actuator is not an absolute requirement to carry out the described method for KTP enantiomers.

We used acetone, methanol and TCA in the sample pretreatment in order to break protein binding of the ketoprofen enantiomers[5]. In this way the method analyzes the total KTP amount, and not only the free fraction of the enantiomers in plasma.

It is possible to use the sample pretreatment to analyze the total concentration (no enantiomers) of ketoprofen in plasma and exudate. But, the chromatographic conditions must in such cases be adjusted as described earlier[13] or equal methods for analyzing total KTP.

The LC-MS/MS method presented here is selective and robust, and the sensitivity satisfies the requirements for pharmacokinetic studies. No interfering peaks were found. The time needed for sample preparation

was short. We have analyzed over 1000 plasma/exudates samples using the method without changing the analytical columns resolution.

CONCLUSIONS

The applications presented here are a good example that LC-MS/MS can offer a number of significant advantages for the detection and quantification of KTP enantiomers in plasma and exudates samples compared to previously published methods. The advantage of the LC-MS technique lies in the combination of the separation capabilities of HPLC and the power of MS as an identification and confirmation tool with good sensitivity, selectivity, and quantitative capability. The LC-MS/MS methods generally require only a simple clean-up or only a dilution procedure and no derivatization. The validation data show that the methods performance is good and can be used for routine analysis.

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