Journal of Chromatography A, 1216 (2009) 3851–3856

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Two-dimensional liquid chromatography–ion trap mass spectrometry for the simultaneous determination of ketorolac enantiomers and paracetamol in human plasma
Application to a pharmacokinetic study

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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form 13 February 2009
Accepted 23 February 2009
Available online 3 March 2009

Keywords:
Chiral separation
Two-dimensional liquid chromatography
Pharmacokinetics
Paracetamol
Ketorolac

ABSTRACT

A bioanalytical method was developed for the simultaneous determination of paracetamol and ketorolac enantiomers in human plasma using two-dimensional liquid chromatography–mass spectrometry. Separation was first achieved in a reversed-phase C18 column by using a gradient solvent system consisting of 0.1% aqueous formic acid and acetonitrile (ACN). The effluent between 8.9 and 9.9 min, corresponding to phenacetin and racemic ketorolac peaks, was transferred to a polysaccharide-based chiral column (ChiralPak AD-RH) by using a six-port switching valve. Ketorolac enantiomers were subsequently separated on the chiral column using an isocratic mobile phase composed of ACN/0.1% formic acid 50:50 (v/v). The total run-time was less than 18 min. This innovative strategy prolongs the lifetime of chiral columns by avoiding damages due to the sample matrix. The detection was carried out with an ion trap mass spectrometer equipped with an electrospray ionisation source. The tested ranges were 0.05–20 μg/ml for paracetamol and 0.005–2 μg/ml for each ketorolac enantiomer. This method was fully validated and showed good performances in terms of trueness (80–110%) and precision (6.7–13.2%). The mean extraction recoveries were 60%, 72% and 76% for paracetamol, *R*-ketorolac and *S*-ketorolac, respectively. Finally, this procedure was successfully applied to a pharmacokinetic study.

1. Introduction

Ketorolac, [(±)-5-benzoyl-1,2-dihydro-3H-pyrrolo [1,2a] pyrrole-1-carboxylic acid], is a nonsteroidal antiinflammatory drug (NSAID) which has a strong analgesic activity [1]. The drug can be administered intravenously, intramuscularly or orally as the water-soluble tromethamine salt (Toradol, Hoffmann-La Roche, Reinach, Switzerland) to treat moderate pain. After absorption or injection, the salt dissociates into the anion form of ketorolac at physiological pH [2]. Ketorolac possesses a chiral center and is marketed as a racemic mixture of (+)*R* and (−)*S* enantiomers. The chiral carbon is within the pyrolle ring and thus differs from most other NSAIDs, in which the chiral carbon is within the 2-arylpropionic acid side-chain [3]. The pharmacological activity resides in the (−)*S* enantiomer, whereas the (+)*R* enantiomer has little or no activity. The kinetics of the two enantiomers of ketorolac is substantially different. Because the two enantiomers have different pharmacodynamic and pharmacokinetic profiles, pharmacokinetic data should be provided for the separate enantiomers.

Paracetamol has antipyretic and analgesic potential similar to NSAIDs; however, it is different from the NSAIDs because it lacks antiinflammatory, antiplatelet and gastrotoxic activities [4]. Paracetamol is often combined with NSAIDs in the management of acute [5,6] and chronic pain [7]. The rationale underlying the practice of combining drugs in pain management is based mainly on the consideration that combining drugs that act at different receptors and on different pain mechanisms may enhance pain relief. NSAIDs block peripheral biosynthesis of prostaglandins by inhibiting the cyclooxygenase (COX) enzyme, whereas paracetamol acts mainly on the brain and spinal cord; nevertheless, the exact mechanism of action of the latter is still unknown [8]. However, consensus on whether a combination of NSAIDs and paracetamol offers a clinical improvement in analgesia is still lacking [9] and more clinical studies are needed to confirm the utility of the combination.

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doi:10.1016/j.chroma.2009.02.071

A randomised placebo-controlled crossover clinical study in healthy human volunteers was conducted to evaluate the effect of combining paracetamol and ketorolac on different experimental pain models. Therefore, an assay was needed for the determination of these drugs in the human plasma to characterise their pharmacokinetic profiles. Several direct and indirect stereoselective methods for the analysis of ketorolac enantiomers have been published [10–13]. Indirect methods that require derivatisation may contribute to the degradation of paracetamol, which is slowly hydrolysed under abnormal conditions (heat, pH, etc.) to a mixture of *p*-aminophenol and acetic acid [14]. Moreover, racemisation of ketorolac may occur during indirect racemic assays that require precolumn derivatisation under strong basic conditions [15]. A direct stereoselective method should then be preferred for the simultaneous analysis of paracetamol and enantiomers of ketorolac.

The chiral resolution of ketorolac in biological samples has been carried out using several chiral columns, particularly those based on proteins (α_1-acid glycoprotein, AGP) [10–12]. The major problem in using these columns when mass spectrometry (MS) detection is conducted is the incompatibility of the mobile phase with the most frequently used ion sources, electrospray and atmospheric pressure chemical ionisation, due to the use of nonvolatile buffers. Furthermore, protein-based stationary phases require highly aqueous mobile phases, which considerably reduces the sensitivity of the MS detection [16]. Alternatively, polysaccharide-based stationary phases, used for NSAID chiral separation in normal-phase conditions, are frequently used with mobile phases consisting of only organic solvents that require a postcolumn addition of additives to avoid the risk of explosion inside the ion source and to improve ionisation of drugs [17].

Thus, an amylose tris(3,5-dimethylphenylcarbamate)-derived stationary phase, that could be used under reversed-phase conditions using a mixture of aqueous and organic solvents, was selected. To the best of our knowledge, no study has been published characterising the separation of ketorolac enantiomers using this column.

The present study describes a two-dimensional liquid chromatography–mass spectrometry (LC–MS) method that allows the simultaneous analysis of paracetamol and the two ketorolac enantiomers. The method consists of a gradient reversed-phase LC method on a C18 column coupled with a stereoselective, isocratic, reversed-phase LC method on polysaccharide chiral column. The assay was fully validated and applied to an in vivo study.

2. Experimental

2.1. Chemicals

Racemic (*R*,*S*)-ketorolac tromethamine (purity >99%) and phenacetin (internal standard, I.S.) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Paracetamol was from Siegfried (Zofingen, Switzerland). Formic acid (98–100%) was obtained from Riedel-de Haën (Seelze, Germany) and HPLC-grade acetonitrile (ACN), ammonium hydroxide solution (32%), hexane and ethyl acetate were purchased from Merck (Darmstadt, Germany). Purified water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Two-dimensional LC–MS

The chromatographic equipment, an Agilent 1100 Series LC system (Agilent, Paolo Alto, USA), was composed of a quaternary pump, a vacuum degasser, an autosampler, a thermostated column compartment and a six-port switching valve. An additional Agilent Series 1100 LC isocratic pump was added to the system for the column-switching configuration. An Agilent ChemStation software package was used for instrument control, data acquisition and data handling. The first-dimension separation (nonstereoselective) was carried out on a Discovery C18 column (150 mm × 2.1 mm I.D., particle size 5 μm) from Supelco (Bellefonte, PA, USA), coupled with a guard column with the same stationary phase (20 mm × 2.1 mm I.D, particle size 5 μm). The second-dimension separation (enantioselective) was achieved on a ChiralPak AD-RH column (150 mm × 2.1 mm I.D., particle size 5 μm) from Chiral Technologies (Exton, PA, USA).

For both columns, the analysis was carried out at room temperature using a mobile phase composed of ACN/0.1 aqueous formic acid. In the C18 column, a gradient elution was used, in which the mobile phase composition was changed from 10–90% to 80–20% (ACN/0.1% formic acid) in 3 min, maintained at 80–20% between 3 and 6 min and was changed to the initial composition between 6 and 7 min. The flow rate was 0.2 ml/min.

A schematic diagram of the developed two-dimensional LC system is presented in Fig. 1. In the initial configuration (Fig. 1A), the two columns were not connected. The sample was injected in the C18 column and the separation was performed under gradient conditions, while the chiral column was under isocratic conditions. The switch from the C18 column to the chiral column was achieved using the two-position, automatic switch valve from 8.9 to 9.9 min, corresponding to the elution of phenacetin and ketorolac peaks in the C18 column (Fig. 1B). The system then returned to its initial configuration (Fig. 1C). The switch had to be accomplished before elution of phenacetin in the C18 column because its retention time was very close to that of ketorolac. In the chiral column, phenacetin and the two ketorolac enantiomers were eluted isocratically with a mobile phase composed of ACN and 0.1% formic acid (50:50, v/v) at a flow rate of 0.15 ml/min. The effluents from both columns were directed into the mass detector by using a tee.

The chromatographic system was coupled to an ion trap mass spectrometer (Esquire 3000+, Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray ionisation source (ESI) working in positive ion mode. The ion count cumulative target for the ion trap mass analyser was 10,000, with a maximum accumulation time of 200 ms. Optimised ESI source voltages were as follows: spray needle at +4.2 kV, end-plate offset at −500 V, capillary exit offset at −200 V, skimmer 1 at −107.4 V. Further ion source parameters were 70 psi for the nebuliser gas and 11 l/min for the drying gas with a temperature of 350 °C. The following ions were retained for quantification: paracetamol *m*/*z* 152, phenacetin (I.S.) *m*/*z* 180 and ketorolac *m*/*z* 256.

2.3. Preparation of standard solutions, calibration curves and quality control samples

Ketorolac tromethamine (14.7 mg) was dissolved in 10 ml of methanol to provide a stock standard solution of 1.0 mg/ml of free acid racemic ketorolac. Paracetamol (10 mg) was dissolved in 10 ml of methanol to make a 1.0 mg/ml stock solution. These solutions were stored at −20 °C before use. Appropriate dilutions of ketorolac/paracetamol mixed in 1:5 ratio were daily prepared in methanol/water 50:50 to produce concentrations of 0.1/0.5, 0.5/2.5, 2/10, 5/25, 10/50, 20/100 and 40/200 μg/ml (racemic ketorolac/paracetamol) in stock solutions. Twenty-five microliters of each concentration were added to 225 μl of human plasma to give calibration standards in plasma at concentrations of 0.01/0.05, 0.05/0.25, 0.2/1, 0.5/2.5, 1/5, 2/10 and 4/20 μg/ml, respectively. A solution of phenacetin at 1.0 mg/ml in methanol was also prepared and further dilution at 2.5 μg/ml in methanol was achieved to prepare the working I.S. solution.

Quality control (QC) samples were prepared independently using the same procedure to yield final concentrations of 0.02/0.1, 0.1/0.5, 0.5/2.5 and 2/10 μg/ml (racemic ketorolac/paracetamol).

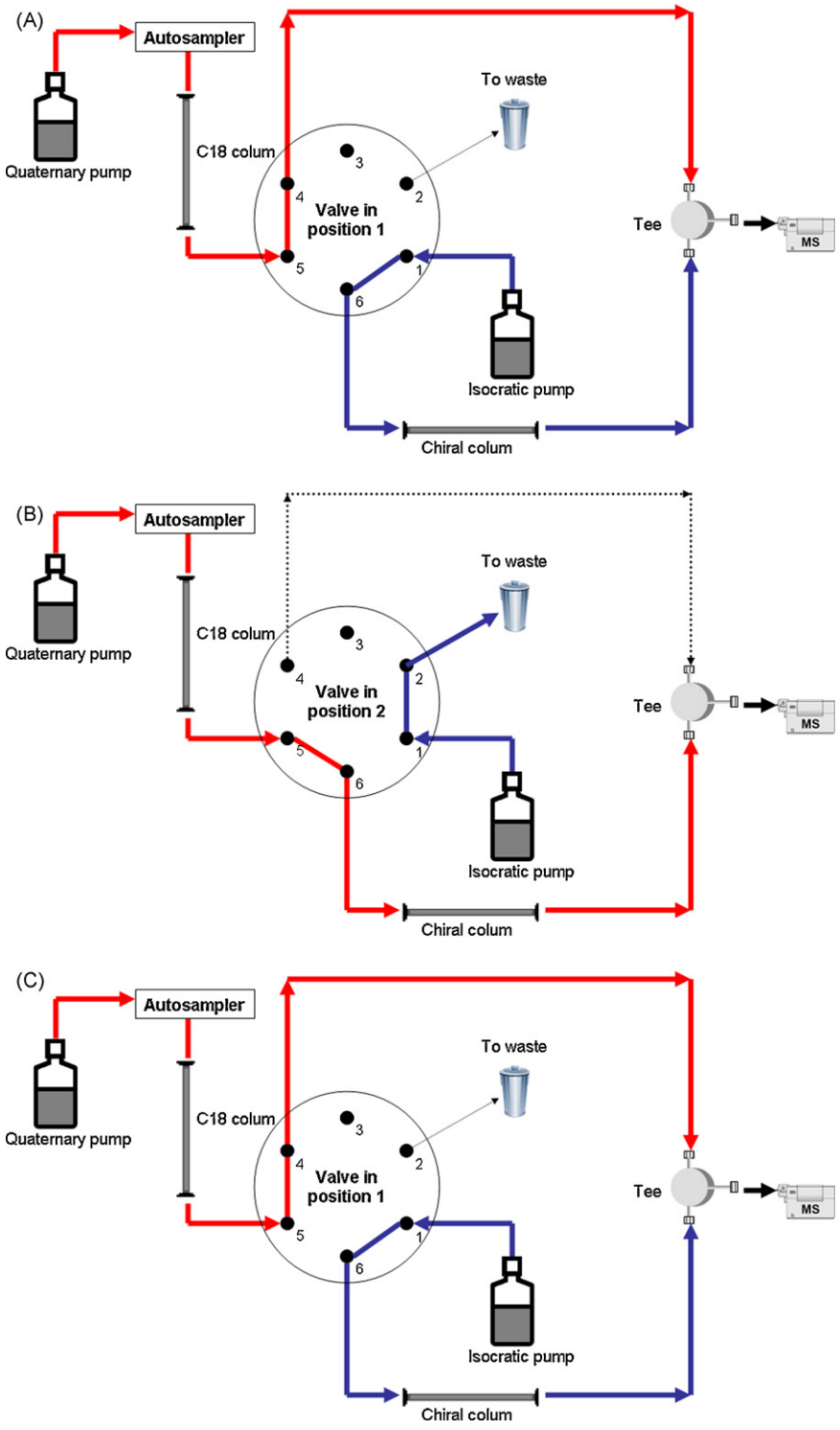

Fig. 1. Column switching configuration: (A) injection and separation on C18 column (from 0 to 8.9 min); (B) configuration of the system during the switch to the chiral column (8.9–9.9 min); (C) return to the initial configuration.

2.4. Sample preparation

2.4.1. Collection of plasma from human samples

Human blank plasma collected from whole blood, used for method development and validation, was obtained from the blood transfusion centre of Geneva University Hospitals (Geneva, Switzerland). Plasma samples were obtained from participants enrolled in a clinical study. Blood samples (12 ml) were drawn into tubes containing ethylenediaminetetraacetic acid (Becton–Dickinson, Franklin Lakes, NJ, USA) at 0.25, 0.5, 1, 2, 4, 6 and 24 h after drug administration. Plasma was obtained by centrifugation (900 × *g* for 15 min) and stored at −20 °C until analysis.

2.4.2. Extraction procedure

The plasma sample (250 μl) was spiked with 50 μl of I.S. (2.5 μg/ml of phenacetin) and acidified with 250 μl of ammonium

acetate/acetic acid 50 mM at pH 3.0. After the addition of 5 ml of hexane–ethyl acetate (50:50, v/v), the mixture was shaken for 15 min. After centrifugation (1300 × *g*, 5 min), the tubes were stored in a freezer (−80 °C) until the aqueous phase froze (30 min) and the upper organic layer was saved. This procedure was repeated twice and the organic layer was then added to the previous one and evaporated to dryness under a gentle stream of nitrogen. The dry residue was dissolved in 250 μl of mobile phase. The resulting solution was centrifuged (7000 × *g*, 5 min) and 20 μl was injected in the system.

2.5. Validation

The validation strategy was based on the guidelines of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [18]. The calibration ($k = 7$) and the QC ($k = 4$) samples were prepared on each validation day in triplicate ($n = 3$) and quadruplate ($n = 4$), respectively. The concentration range (0.01–4 μg/ml racemic ketorolac, 0.05–20 μg/ml paracetamol) was selected on the basis of preliminary results to cover the expected levels in clinical samples. Samples were prepared, extracted and analysed on 3 different validation days.

Method selectivity was assessed by analysing blank plasma from six different healthy volunteers. The lack of interfering peaks at the analyte retention time was considered as acceptable selectivity.

The trueness and precision of the method were determined with the QC samples, by recalculating the concentration with the daily response function established. Trueness was expressed as the ratio between the theoretical and the measured concentrations. Precision was expressed as the relative standard deviation (RSD) of the ratio of the intraday variance (repeatability) and interday variance (intermediate precision) on the theoretical value at each concentration level. The limit of quantification (LOQ) was estimated with a signal-to-noise (*S*/*N*) ratio of 10.

Overall recovery for ketorolac and paracetamol from human plasma was assessed at four concentrations (0.02/0.1, 0.1/0.5, 0.5/2.5 and 2/10 μg/ml) using the extraction procedure previously described and by comparing the analyte peak areas obtained from spiked plasma with those in mobile phase solutions.

The short-time stability of the analytes in reconstituted extracts during the run-time in the HPLC autoinjector was investigated by maintaining the samples in the sample rack of the autoinjector and injecting them into the HPLC system at 0 and 24 h at the temperature of the autoinjector (room temperature). No long-term stability studies were conducted here as previous works have shown that paracetamol and ketorolac have good stability in frozen plasma [12,19].

3. Results and discussion

Similar to many currently marketed drugs, ketorolac is available as a racemic mixture of stereoisomers. The chiral recognition of enantiomers by biological systems results in marked differences in biological activities, such as pharmacology, toxicology and pharmacokinetics. In the case of ketorolac, the (−)*S* enantiomer is responsible for the antiinflammatory and analgesic activities, whereas the (+)*R* enantiomer has little or no activity [3]. Moreover, the two enantiomers display different pharmacokinetics. For these reasons, it was desirable to develop a stereoselective assay for the study of their pharmacokinetic and pharmacodynamic properties.

In our initial investigations, a capillary electrophoresis (CE) method was developed for the simultaneous separation of paracetamol and ketorolac enantiomers using β-cyclodextrin as a chiral selector. Although CE has proven to be a successful technique for enantiomeric separation, it has relatively low sensitivity when it is used with simple UV detection [20,21]. Indeed, detection limits obtained using CE were not suitable for the in vivo kinetic determination of the studied drugs (data not shown).

For the direct stereoselective analysis of ketorolac by liquid chromatography, AGP has been the most frequently used column [10–12]. The latter is based on immobilised human plasma AGP on silica particles. In protein-based columns, the separation power may decrease with an increasing number of plasma samples and vary from one column to another [22], which was observed in our laboratory (data not shown). The other problem with the use of the AGP columns is their high cost and short lifetime. Typically, an AGP column shows acceptable performance for 600–700 injections of plasma extracts with routine use and proper maintenance [12]. Moreover, separation of ketorolac enantiomers using an AGP column requires high aqueous mobile phase percentage (>96%), which considerably reduces the sensitivity using MS. Considering these facts, a new amylose tris-(3,5-dimethylphenylcarbamate)-derived stationary phase (ChiralPak AD-RH) was selected. The RH series used in our study are for use in reversed-phase mode [23,24]. The enantiomeric separation is based on the formation of transient diastereomeric complexes between the functional groups of chiral analytes and the free amino and carbonyl groups of the polysaccharide [25]. The optimal mobile phase composition was ACN/0.1% formic acid 50:50 (v/v), allowing good enantiomeric resolution in a short time (less than 10 min). Preliminary experiments were conducted to evaluate the effect of the mobile phase pH and the organic solvent percentage on the chiral separation of ketorolac enantiomers (Fig. 2). The order of elution of ketorolac enantiomers was determined by separately analysing a solution of *R*-ketorolac (data not shown). As shown in Fig. 2, a dramatic decrease in the chiral

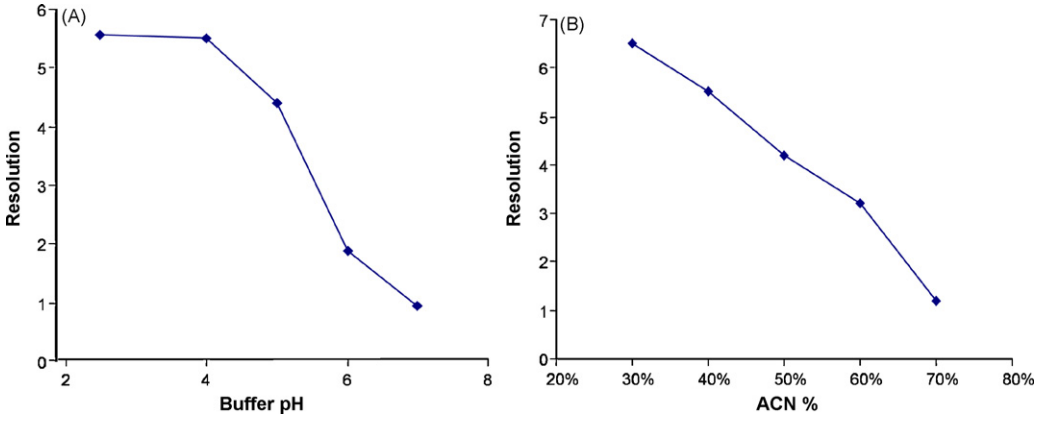

Fig. 2. Influence of (A) the buffer pH (B) ACN percentage, on ketorolac enantiomeric resolution. In (A), the optimisation of the buffer pH was achieved using 40% of ACN. In (B), the optimisation of the percentage of ACN was performed at pH 2.5.

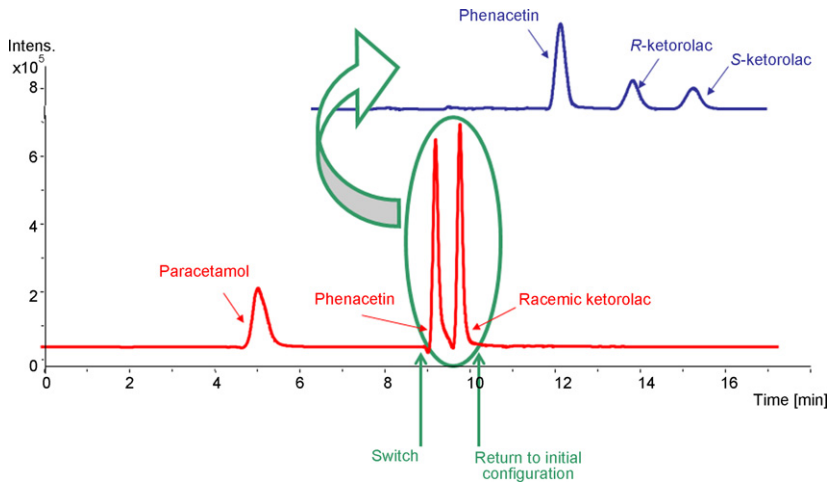

Fig. 3. Typical chromatograms of spiked plasma (0.5 μg/ml of racemic ketorolac + 2.5 μg/ml of paracetamol) without and with the switch.

resolution was observed when the mobile phase pH was increased from 2.5 to 7. In fact, it was shown that maintaining the chiral analytes neutral when working with polysaccharide stationary phases is fundamental for a good enantiomeric separation as these chiral stationary phases (CSPs) do not possess any ionic site susceptible to interaction with the charged compounds [24]. Because ketorolac is acidic ($pK_a = 3.54$), increasing the mobile phase pH will reduce the neutral form of the drug, which will weaken its interaction with the stationary phase. However, different results were obtained for ketoprofen using this column. In fact, enantioselective separation was only possible at pH 9, wherein the drug was in ionised form. On the other hand, enantioselectivity was observed at pH 2.0 and pH 9.0 for suprofen enantiomers but not at pH 5.5, which suggests that other factors such as modification in solvatation or changes in the structure of the CSPs could be important in the chiral recognition. Furthermore, ketorolac enantiomers resolution decreased as a function of the percentage of ACN while sensitivity increased. Thus, 50% of ACN was selected as a compromise between resolution and sensitivity.

To prolong the lifetime of our ChiralPak AD-RH column and because paracetamol showed no retention on this column, a C18 column (Supelco Discovery) coupled with a guard column was connected to the system. In the C18 column, the retention times were 5 min for paracetamol, 8.9 min for phenacetin (I.S.) and 9.9 min for racemic ketorolac. Only ketorolac and I.S. peaks were switched onto the chiral column. Using this procedure, the column remained clean and back-pressure stable during the entire process (more than 500 plasma extract injections). The total run-time from the injection to the data-acquisition stages, including the switch from the C18 to the chiral column, was less than 18 min (Fig. 3). The developed column-switching method can be applied to the direct enantioselective bioanalysis of chiral compounds as a strategy to prolong the lifetime of such expansive columns, as it avoids damages due to the sample matrix.

3.1. Validation results

Selectivity of the developed method was studied by analysis six different blank plasmas at the target m/z values. No interfering peaks were detected which indicate that to method is suitable for the simultaneous analysis of paracetamol and ketorolac in human plasma (Fig. 4).

To determine the best response function, different regression models (linear regression, linear regression on log-transformed data and weighted linear regression) were evaluated. The best calibration model was the log-transformation of the variables for paracetamol, and the $1/x^2$ weighted model for ketorolac. The tested ranges were 0.05–20 μg/ml for paracetamol and 0.005–2 μg/ml for each enantiomer of ketorolac.

The trueness and the intraday and interday precision data are presented in Table 1. All these parameters were found to be satisfactory. The LOQ, estimated according to the S/N ratio was 0.005 μg/ml for each ketorolac enantiomer and 0.001 μg/ml for paracetamol. The concentration of the lowest calibration sample for paracetamol was fixed at 0.05 μg/ml because the amounts in the clinical samples were very high.

The mean extraction recoveries (SD) were 60% (9.4), 72% (10.6) and 76% (14.1) for paracetamol, *R*-ketorolac and *S*-ketorolac, respectively. Because the recoveries were evaluated by comparing the absolute peak areas of the spiked plasma with those of the standards in reconstitution solvent, recoveries included matrix effects. Regarding the high percentage obtained for the extraction recoveries, it can be concluded that matrix effects are negligible. Finally, short-term stability was evaluated by maintaining the QC samples in the autoinjector at room temperature for 24 h and no degradation was observed.

3.2. Application of the LC–MS to clinical samples

The validated method was applied to the measurement of paracetamol and both enantiomers of ketorolac in healthy volunteers who had received an intravenous single dose of both drugs (paracetamol 1 g and racemic ketorolac 20 mg). This study was approved by the Ethics Committee of Geneva University Hospitals. Written informed consent was obtained from

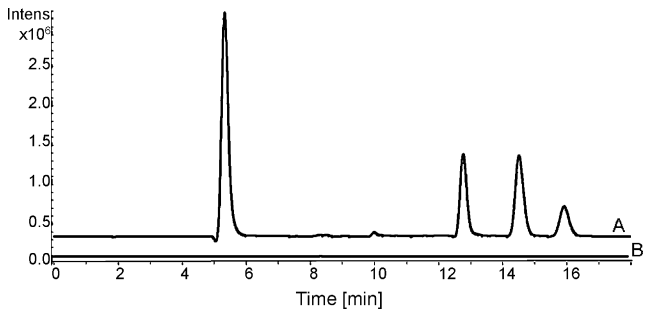

Fig. 4. (A) Chromatogram obtained in one volunteer 30 min after intravenous administration of paracetamol (1 g) and racemic ketorolac (20 mg) (B) blank plasma.

Table 1
Trueness, repeatability and intermediate precision of paracetamol and both ketorolac enantiomers.

Compound	Concentration (μg/ml)	Trueness (%)	Repeatability RSD (%)	Intermediate precision RSD (%)
Paracetamol	0.1	80.1	10.3	10.3
	0.5	106.0	9.4	10.7
	2.5	104.6	7.8	8.1
	10	105.1	9.5	9.5
R-Ketorolac	0.01	95.2	8.9	8.9
	0.05	87.8	11.2	11.2
	0.25	100.0	8.8	11.9
	1	110.4	11.5	12.2
S-Ketorolac	0.01	95.5	12.3	12.3
	0.05	94.3	10.7	10.7
	0.25	97.7	6.7	9.2
	1	108.8	11.6	13.2

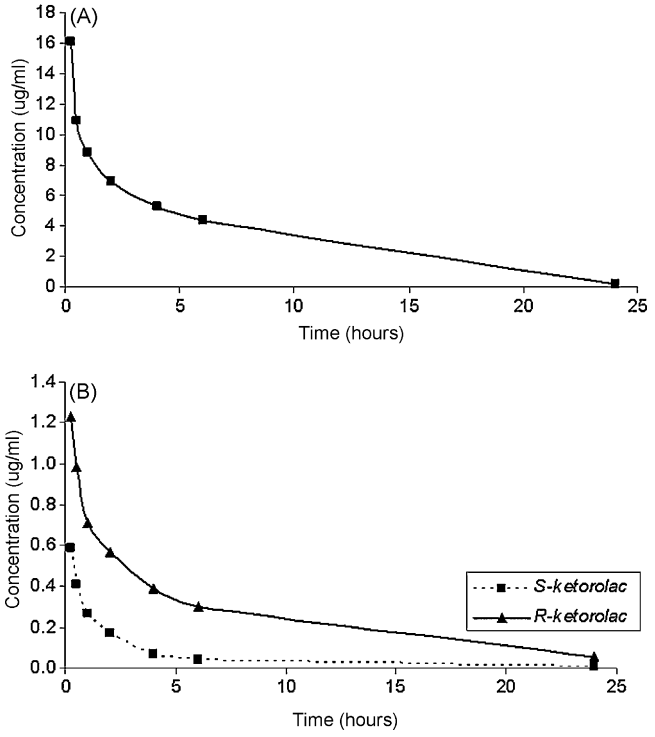

Fig. 5. Plasma concentration-time profile of: (A) paracetamol and (B) *S*-ketorolac and *R*-ketorolac in one volunteer after intravenous administration of paracetamol (1 g) and racemic ketorolac (20 mg).

all participants. Using this analytical method, the concentration of paracetamol and ketorolac were measured up to 24 h for all subjects after drug administration. A representative chromatogram from one volunteer 30 min after drug administration is presented in Fig. 4 and examples of the pharmacokinetic profiles for paracetamol and ketorolac enantiomers are shown in Fig. 5.

Pharmacokinetic data were obtained with WinNonlin 5.1 (Pharsight, Mountain View, CA, USA) using a noncompartmental model. The mean half-life ($t_{1/2}$) of *S*-ketorolac (active enantiomer) was 5.1 h and the mean area under the concentration–time curve (AUC) was 1.3 μg h ml^{-1}. Mean $t_{1/2}$ of *R*-ketorolac was 5.2 h and mean AUC was 4.7 μg h ml^{-1}. Mean $t_{1/2}$ for paracetamol was 3.8 h and mean AUC was 49.6 μg h ml^{-1}. These results are consistent with the previously published data [1,2,26].

4. Conclusion

A sensitive, accurate and precise bioanalytical method for the simultaneous determination of paracetamol and ketorolac enantiomers in human plasma was developed. The assay was based on two-dimensional LC–MS and involved two different columns (C18 and chiral stationary phase). The present approach allowed prolonging the chiral column lifetime by switching only the peaks of interest into the latter one. Overall assay, from the injection to data acquisition including the switch from the C18 to the chiral column, was performed in less than 20 min. The method was fully validated and applied to a pharmacokinetic study in healthy human volunteers. The expected differences in the pharmacokinetics of the ketorolac enantiomers were clearly observed.

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