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Chiral capillary liquid chromatography based on penicillin G acylase immobilized on monolithic epoxy silica column

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ABSTRACT

An epoxy derivatized monolithic silica capillary column (100 μm i.d.) was used as a support for immobilization of penicillin G acylase (PGA), an enzyme used in the production of semisynthetic antibiotics. In order to allow for sensitive UV detection, the PGA-based monolithic capillary column was coupled to an open fused-silica capillary via a TFE (Teflon®) shrink tube sleeve (1 cm long, 300 μm i.d.), which proved to be a robust, dead-volume free and easily replaceable connector. This configuration resulted in a duplex fritless column for capillary liquid chromatography (CLC) and electrically assisted CLC (eCLC). In particular, using the driving pressure (2–12 bar) supplied by the commercial CE instruments, CLC separations could be obtained in short time due to the low column backpressure of the monolith. In particular, the developed stationary phase characterized by the chiral recognition ability of PGA, was successfully applied in enantioseparation of arylpropionic acids of pharmaceutical interest (*i.e.*, profens). As an example, by using a 7 cm long monolith capillary column, the enantioresolution (Rs > 3.0) of *rac*-ketoprofen was achieved in less than 2 min (pressure 12 bar) with a minimum plate height in the order of 20 μm and using as a mobile phase a 50 mM phosphate buffer pH 7.0. Validation data such as repeatability of retention time (intraday < 0.62, $n = 6$; interday < 1.62, $n = 9$; and column-to-column < 10.5, $n = 2$), linearity ($r^2 = 0.999$), and sensitivity (LOQ 0.25% (w/w) of (*R*)-ketoprofen with respect to (*S*)-ketoprofen) showed good method performance. The method was successfully applied to the determination of (*S*)-ketoprofen in pharmaceutical samples (tablets).

1. Introduction

There is an increasing interest towards microseparations using capillary electrochromatography (CEC) and capillary liquid chromatography (CLC). These methods combine the typical advantages of capillary electrophoresis (CE), such as low consumption of sample and solvents, with the opportunity of using stationary phases with various chemistry for improving selectivity as well as feasibility of hyphenation with mass spectrometry. In particular the separation mechanism involved in CEC merges that of zone electrophoresis of the analytes with their partitioning towards the stationary phase. Under these hybrid conditions the separation is the results of combination of high efficiency achieved by CZE with the selectivity of HPLC [1–3]. The stability of solvent flow in CEC is however a critical issue because it depends on the electroosmotic flow (EOF). Thus, in order to achieve the method reliability required in analytical separations, the charge of the CEC stationary phase and the mobile phase composition have to be carefully controlled. Differently from CEC, in CLC and/or micro-LC the mobile phase is only pushed by the pressure thus, under these conditions, all the weakness related to CEC flow stability *i.e.*, the demand for the maintenance of electrical conductivity of the mobile phase and long equilibration time of stationary phase, can be bypassed. A number of studies have shown that using commercial CE instruments equipped with an external pressure supply (<15 bar), CLC or micro-LC separation can be performed [4–6]. In particular, these applications can be advantageously carried out by using monolithic columns that fulfill the necessity to achieve acceptable linear velocity of mobile phase at relatively low pressure. Furthermore, the use of CE instrumentation to perform CLC experiments, offers the opportunity for the application of an electrical field to the pressure driven separation, thus providing improved control and fine tuning in analysis of charged solutes [7,8].

Very recently, the state-of-the-art in microseparation techniques has been reviewed and it has been concluded that monolithic silica capillaries are the preferred column types for CEC and CLC, in particular when chiral selectors are used for functionalization oriented to analytical enantioseparations. Besides the above

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mentioned advantages, the use of monolithic columns eliminates the need for frits retaining the stationary phase; in addition, monoliths are easy to be prepared, their surface can be modified using a variety of different approaches and they possess good permeability and good peak capacity [9–11].

In the present study, penicillin G acylase (PGA) of *Escherichia coli* ATCC 11105 (EC 3.5.1.11), an N-terminal nucleophile hydrolase, was used as chiral selector immobilized on an epoxy derivatized monolithic capillary support to be used in CLC enantioseparations carried out by commercial CE instrument. PGA catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid and it is well known for its industrial application in the production of β-lactamic nucleus which is a building block in the synthesis of semi-synthetic penicillins [12]. In the last years the chiral selector properties of PGA have been fully investigated by immobilizing this enzyme on different HPLC silica supports [13–17]. The prepared analytical columns have been used for the resolution of several acidic racemic mixtures and the chromatographic data have been used to extend the understanding of chiral recognition mechanism governing the enantioselective binding of PGA-CSPs towards acidic aromatic compounds [18,19]. More recently the use of PGA as chiral selector in partial filling CE has been also investigated suggesting that the immobilization procedure used in the development of PGA-CSPs did not alter the enantioselective properties of the enzyme [20].

2. Materials and methods

2.1. Chemicals and reagents

Rac-ketoprofen, (*S*)-(+)-ketoprofen, *rac*-fenoprofen calcium salt hydrate, *rac*-flurbiprofen, *rac*-suprofen and thiourea were from Sigma–Aldrich (Milan, Italy); glycine was from Fluka (Buchs, Switzerland). Phosphoric acid, sodium hydroxide, ammonium sulfate, methanol, and all the other chemicals were from Carlo Erba Reagenti (Milan, Italy). Penicillin G acylase (PGA) crude extract from *E. coli* ATCC 11105 (EC 3.5.1.11) (2287 U/mL) was kindly provided by Recordati (Milan, Italy).

Water used for the preparation of sample solutions and buffers, was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

2.2. Immobilization of penicillin G acylase on monolithic epoxy silica capillary column

The immobilization procedure was carried out by following a previously described method [15]. Briefly, the monolithic epoxy silica capillary column Chromolith SpeedRod (60 cm × 100 μm i.d.) was provided as a research sample. The material comprises a silica gel skeleton containing mesopores of 13 nm and macropores of 2 μm diameter and column porosity higher than 80% [21]. The specific surface area was 323 m^2/g and as previously reported, the modification of the Silica-Rods was carried out with 3-glycidoxypropyltrimethoxysilane; the surface coverage (α_{epoxy}) was found to be 2.9 μmol epoxy groups per m^2 unmodified silica [17]. A 15 cm long piece of the capillary column was connected to an HPLC pump (Jasco PU-1585, Jasco Corporation, Tokyo, Japan) and was flushed at the flow rate of 1 μL/min for 2 h with a coupling solution composed of 50 mM phosphate buffer (pH 7.5) containing 1.875 M ammonium sulfate. PGA was dissolved (3 mg/mL) in the coupling solution and after filtration through 0.20 μm Minisart RC 25 membrane filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) it was applied to the column at 1 μL/min for 24 h. The column was back flushed every hour and it was finally washed for 2 h with a 50 mM phosphate solution (pH 7.0) and with a 1 M solution of glycine to block the remaining epoxide groups. The capillary column was stored in 50 mM phosphate pH 7.0 at 4 °C.

2.3. Solutions

Sample solutions of the analytes (arylpropionic acids such as ketoprofen, fenoprofen, flurbiprofen, and suprofen) were prepared at the concentration of 0.50 mg/mL in a mixture water:methanol 90:10 (v/v). Calibration graphs for the enantiomers of ketoprofen were obtained by linear regression analysis within a concentration range 0.0125–2.0 mg/mL (as *rac*-ketoprofen); triplicate injections were made for each calibration point and the peak area of the single enantiomers was plotted against the concentration. Furthermore, the linearity of the distomer (*R*)-ketoprofen was evaluated at impurity level in the presence of a constant amount of (*S*)-ketoprofen, by spiking the latter (at a final concentration of 1.0 mg/mL) with *rac*-ketoprofen in order to obtain concentration of (*R*)-ketoprofen in the range 0.0025–0.020 mg/mL (corresponding to 0.25–2.0%, w/w).

2.4. Apparatus

The CLC and electrically assisted CLC (eCLC) separations were carried out using an Agilent ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany), equipped with a pressure supply (provided by nitrogen) at the inlet and outlet vials (max pressure 12 bar). The data were collected on a personal computer (integration software Agilent Rev. A. 09. 01).

The PGA-based monolithic capillary column was coupled to an open fused-silica capillary (50 μm, i.d., Composite Metal Service, Ilkley, UK) via a TFE (Teflon®) shrink tube sleeve 1 cm long (0.3 mm i.d. × 1.58 mm o.d., Supelco, Milan, Italy) as previously described *e.g.*, by Liu et al. [7] and by Zhang et al. [8]. In the present study, two different configurations were used: (i) a 14 cm long monolithic capillary was coupled to a 19 cm long open fused-silica capillary (the detection point is set at 10.5 cm from the end of the separation column); (ii) a 7 cm long monolithic capillary was coupled to a 26 cm open fused-silica capillary (the detection point is set at 1.5 cm from the end of the separation column).

2.5. CLC and eCLC conditions

In optimized CLC separations, the mobile phase was pushed at the pressure of 12 bar applied at the inlet vial (when the 14 cm long monolithic capillary was installed) or at the outlet vial (when the 7 cm long monolithic capillary was installed). Hydrodynamic injections were performed at 5 bar × 0.03 min (1.8 s). A 50 mM phosphate buffer (pH 7.0) solution was used as the mobile phase; the cartridge temperature was 25 °C and the detection wavelength was 200 nm. The eCLC experiments were performed by applying, during the run, voltages within the range 5–10 kV (depending on the specific application).

2.6. Analysis of pharmaceutical samples

Analysis of real samples, namely tablets containing (*S*)-ketoprofen trometamol (excipients: cellulose microcrystalline, corn starch, sodium starch glycolate, glyceryl palmitostearate, hypromellose, titanium dioxide, propylene glycol, macrogol 6000) was carried out by grinding 5 tablets with a pestle in a mortar. An amount equivalent to about 25 mg of the active pharmaceutical principle was treated with 25 mL of water:methanol 90:10 (v/v) by ultrasonication for 5 min at room temperature. Finally the sample was filtered through 0.20 μm RC filter to obtain a clarified solution. The samples were analyzed by chiral CLC and the quantitation was

carried out by comparison of the response of a reference standard solution of (*S*)-ketoprofen.

3. Results and discussion

The most recent developments in the field of nano-HPLC and CEC using chiral-modified silica-based monoliths have been recently summarized by Wistuba [22] and proteins are considered to be among the most attractive chiral selectors due to their selectivity in recognizing specific molecules. Despite this important feature, only a few CEC and CLC applications have been reported so far. Avidin was physically adsorbed on a silica-based monolithic capillary for CEC and CLC chiral separation of a number of compounds including arylpropionic acids [7]. Toyo'oka developed a protein-encapsulation technique in preparation of silica monolithic columns using bovine serum albumin and ovomucoid from chicken egg white, as chiral selectors [23–25]. In the present study, penicillin G acylase (PGA) was covalently immobilized on an epoxy silica capillary monolithic support, following previous studies performed on HPLC columns [17]. Those studies showed the good enantiorecognition properties of PGA immobilized on different supports towards the racemic mixtures of acidic compounds such as 2-aryloxyalkanoic and 2-arylpropionic acids [17–19]. The covalent immobilization on monolithic silica columns with epoxy functionalities resulted in the highest stability of the chiral selector and in improved chromatographic performances *i.e.*, enantioselectivity and efficiency.

3.1. Preparation of the chiral stationary phase and coupling monolithic column-open capillary in CLC mode

The epoxy silica monolith in capillary format (60 cm × 100 μm i.d.) was here chosen as the support for immobilization of the PGA CSP as described in Section 2 on a 15 cm long piece of the column. The minimum column length to be set up in the cartridge of Agilent CE instrument is about 33 cm and although the silica monolithic support is characterized by a low backpressure, the linear velocity of mobile phase achieved by the pressure supplied by CE instrumentation (max 12 bar) was found to be low and not suitable for CLC applications. Only when monolithic capillaries shorter than 15 cm were used, the pressure supplied by commercially available CE instrument was able to provide a linear velocity of mobile phase within the range 0.5–1.5 mm/s (data not shown). In order to set up the shorter monolithic column in the CE cartridge, an open capillary section had to be connected in a duplex column modality. In a first investigation, a 14 cm long monolithic column (100 μm i.d.) was coupled to an open fused-silica capillary (19 cm long, 50 μm i.d.) via a TFE (Teflon®) shrink tube sleeve, which proved to be a robust, dead-volume free and easily replaceable connector. This configuration allowed a sensitive detection in the open capillary section instead of the in-column detection that, in this particular application, resulted to be problematic because of the high amount of the UV-absorbing PGA immobilized onto the monolithic support.

By applying the pressure of 12 bar the linear velocity of the mobile phase determined by the elution of thiourea (0.1 mg/mL) used as the flow marker, was 0.7 mm/s thus within the range of the values observed in packed CEC columns [26].

3.2. Chiral separation of arylpropionic acids in CLC and electrically assisted CLC (eCLC)

Arylpropionic acids (racemic mixtures) of pharmaceutical interest were selected as test compounds in evaluating monolithic silica PGA stationary phase under CLC conditions as convenient approach in the development of micro-enantioseparations. Our

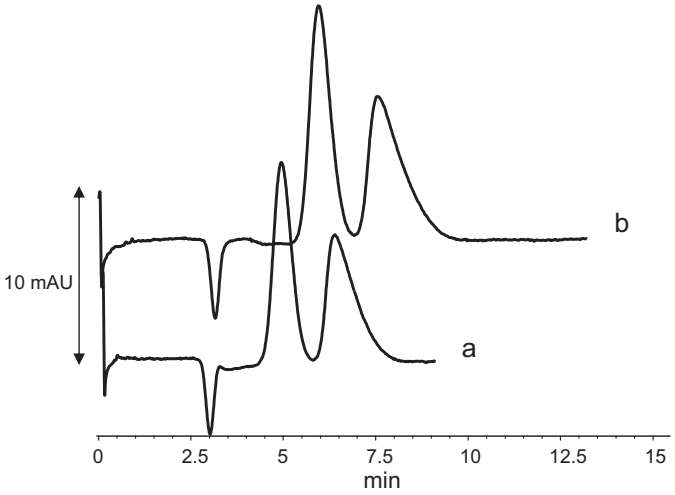

Fig. 1. (a) CLC separation of *rac*-ketoprofen using a duplex capillary constituted of a separation PGA-monolithic section (14 cm × 100 μm i.d.) and an open fused-silica capillary section (19 cm × 50 μm i.d.). The mobile phase was a 50 mM sodium phosphate buffer, pH 7.0. Pressure was 12 bar. Detection at 200 nm. The cartridge temperature was 25 °C. Hydrodynamic injection was performed at 5 bar × 0.03 min (1.8 s). (b) Electrically assisted CLC (eCLC) separation of *rac*-ketoprofen. Conditions as in (a); during the chromatographic run a voltage of 5 kV was applied.

previous studies showed that the best conditions for chiral separation of arylpropionic acid on PGA-based HPLC columns, involved the use of 50 mM phosphate buffer (pH 7.0). Using this mobile phase, the chiral separation of the tested compounds was obtained both in CLC and eCLC mode. As an example in Fig. 1a is reported the enantioseparation of *rac*-ketoprofen using a duplex column (14 cm long monolithic section) and by applying a pressure of 12 bar. As expected, the efficiency of the system was found to be poor (number of theoretical plate < 1000, referred to the first eluting ketoprofen enantiomer, namely (*R*)-ketoprofen) because of the high extracolumn volume corresponding to the open capillary section (length 10.5 cm) used for connecting the separating capillary with the detection window. However a very good enantioselectivity of PGA towards arylpropionic acids was achieved resulting in typical enantioresolution values (Rs) for profens (*rac*-ketoprofen, *rac*-fenoprofen, *rac*-suprofen, etc.) higher than 1.5. In Fig. 1b is reported the enantioseparation of *rac*-ketoprofen under eCLC conditions (pressure 12 bar and voltage of 5 kV). It can be observed that the elution time of the neutral marker methanol does not significantly change after application of the electric field, suggesting that the contribution of EOF to the mobile phase velocity is negligible. Under these conditions, the elution time of anionic ketoprofen enantiomers (pK_a 4.6 [27]) increased compared to CLC conditions (Fig. 1a), because of their countercurrent electrophoretic migration. However the tested eCLC conditions did not provide any significant improvement of both separation efficiency and enantioresolution.

In order to improve the separation efficiency the length of the open capillary section of the duplex column was reduced using the short-end injection mode. In particular, in this configuration the separation column consisted of a 7 cm long PGA-monolith and it was set at the so-called short-end of the capillary cartridge. Using this design, the distance between the end of the monolithic separation column and the detection point, was only 1.5 cm long. Interestingly, although the length of the chiral stationary phase was reduced to 50% with respect to the previously considered design, significantly higher enantioresolution was obtained for each of tested compounds. The advantages achieved under these conditions were the lower backpressure and the reduction of the system-dead-volume, which corresponded to a gain in efficiency for the diminished longitudinal diffusion. The Van Deemter plot

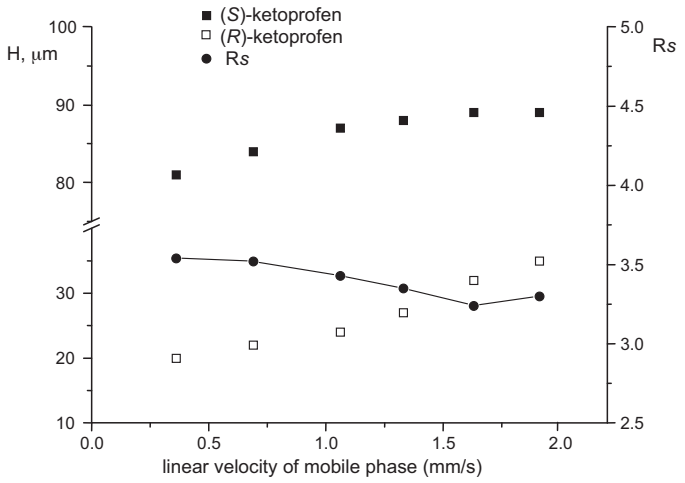

Fig. 2. Van Deemter plots of the enantiomers of ketoprofen and variation of the enantioresolution value (Rs) at different velocity of mobile phase. CLC was performed using a duplex capillary constituted of a separation PGA-monolithic section (7 cm × 100 μm i.d.) and an open fused-silica capillary section (26 cm × 50 μm i.d.). Other conditions as in Fig. 1a.

(theoretical plate height, *H vs.* linear velocity of mobile phase, *u*) of ketoprofen enantiomers, is shown in Fig. 2. In the graph it is also depicted the variation of enantioresolution of *rac*-ketoprofen at different velocity of mobile phase. A minimum plate height value of 20 μm was observed at the flow rate of 0.35 mm/s, corresponding to the pressure of 2 bar. By increasing the applied pressure (within 2–12 bar), the linear velocity of the mobile phase was found to increase linearly. At the pressure of 12 bar the velocity of mobile phase resulted to be 1.9 mm/s and even if the plate height decreased to about 35 μm, the enantioresolution was still maintained well above the baseline. Under these conditions, the enantioseparation of *rac*-ketoprofen was accomplished in less than 2 min (2 min of equilibration time between consecutive injections). By assuming the column porosity as at least 0.80 [21], the volumetric flow rate *F* for the used 100 μm i.d. monolithic column, resulted to be 0.72 μL/min, as calculated by the equation given below:

$$F = \frac{u\pi dc^2\varepsilon}{4}$$

where *u* is the linear velocity of mobile phase, *dc* is the column diameter, and *ε* is the column porosity. The total peak volume of the first eluting peak (*R*-ketoprofen) was determined as about 0.3 μL. Since the extra-column volume introduced by the 1.5 cm × 50 μm i.d. capillary connection is 0.03 μL, the applied short-end design was found to fulfill the requirement that the cell volume should be comparable, or less, than one-tenth of the final volume of the peak in order to not to introduce peak dispersion [28]. In Fig. 3 is reported the simultaneous CLC separation of a mixture of racemic ketoprofen, suprofen and flurbiprofen at the pressure of 12 bar. Although the enantioresolution of each of the profens could be individually optimized by means of variation of the pH of the mobile phase, the achieved simultaneous baseline enantioseparation of the racemic drugs, proves the excellent chemoselectivity of the PGA-based monolithic CLC system.

3.3. Analytical performance and applications

In order to evaluate the opportunity to apply the CLC method in quantitative analysis, repeatability experiments were performed by determination of intraday (*n* runs = 6), interday (*n* = 9) and column-to-column (*n* columns = 2) RSD% of migration time of the first eluting enantiomer of three anti-inflammatory drugs, namely

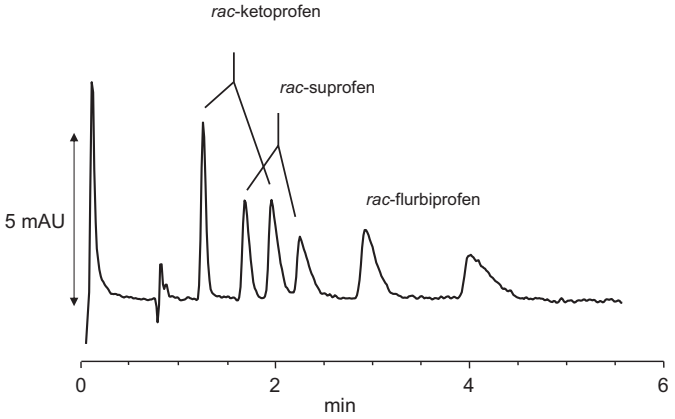

Fig. 3. Simultaneous enantioseparation of *rac*-ketoprofen, *rac*-suprofen and *rac*-flurbiprofen in CLC using a duplex column constituted of a separation PGA-monolithic section (7 cm × 100 μm i.d.) and an open fused-silica capillary section (26 cm × 50 μm i.d.). The applied pressure was 12 bar; other conditions as in Fig. 1a.

Table 1
Repeatability (RSD%) of retention time (t_r) of the first eluting enantiomer for three profens in CLC conditions.

	Intraday (*n* = 6)	Interday (*n* = 9)[a]	Column-to-column (*n* = 2)[b]
Ketoprofen	0.52	1.15	t_r 9.82 α 0.20[c]
Suprofen	0.58	1.15	t_r 10.5 α 0.22[c]
Flurbiprofen	0.62	1.62	t_r 10.4 α 0.20[c]

[a] For each day, three determinations were considered.
[b] RSD% was calculated for six replicated analysis on each of the two tested columns (*n* = 2); the total number of determinations was twelve.
[c] RSD% in column-to-column repeatability study, was given also for enantioselectivity factor (α).

rac-ketoprofen, *rac*-suprofen and *rac*-flurbiprofen; the results are summarized in Table 1. In order to prove the ability of the method in accurate quantitation of active enantiomers of pharmaceutical compounds, ketoprofen was chosen as the suitable analyte being a widespread drug also administered as a pure enantiomer being its activity resides almost exclusively in the (*S*)-(+)-isomer. Linearity of the response at the wavelength of 200 nm was evaluated using the chiral CLC method within the concentration range 0.0125–2.00 mg/mL. Triplicate injections were made for each calibration solution and the mean peak area values (*Y*) were plotted *vs.* the corresponding concentrations (*C*) to obtain the regression lines as follows:

(*R*)-ketoprofen $Y = 0.3564(\pm 0.0199) + 169.5539(\pm 0.15514)C \quad (r^2 = 0.999)$
(*S*)-ketoprofen $Y = 0.5851(\pm 0.02269) + 171.2879(\pm 1.7694)C \quad (r^2 = 0.999)$

Furthermore, *rac*-ketoprofen and (*S*)-ketoprofen solutions were mixed in order to obtain artificial solutions containing final (*S*)-ketoprofen concentration of 1.0 mg/mL in the presence of the distomer (*R*)-ketoprofen within the range 0.25–2.0% (w/w). The chiral CLC analysis showed a complete separation of the enantiomers at impurity levels (Fig. 4) thus confirming the ability of the method in quantitation of the distomer with a LOQ that can be reasonably assumed as 0.25% (w/w). The quantitation of (*S*)-ketoprofen was finally carried out in a pharmaceutical sample (tablets) by performing three analyses as described in Section 2 and by setting the purchased (*S*)-ketoprofen reference to 100%. The analyzed samples resulted to be enantiomerically pure and the obtained results were in agreement with the declared content: assay, 98.4 ± 1.8%. Accuracy was then assessed by recovery of (*S*)-ketoprofen spiked at 150%

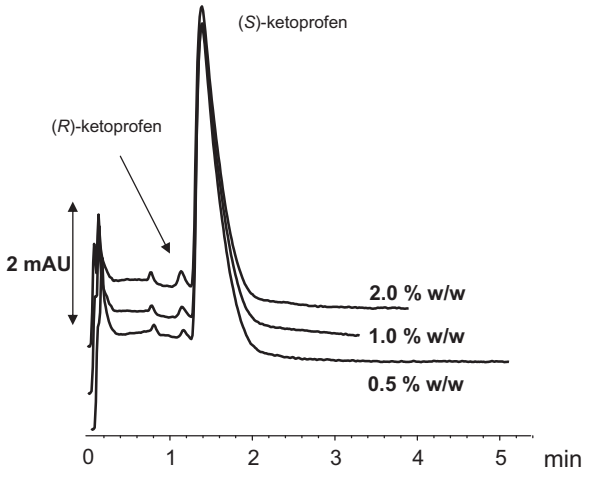

Fig. 4. CLC of (*S*)-ketoprofen (main drug) spiked with (*R*)-ketoprofen at impurity levels. Conditions as in Fig. 3.

level of the nominal concentration declared in the pharmaceutical formulation. The recovery value was found to be 101.4%, RSD 2.5%, $n = 3$.

4. Conclusions

The results of the present study confirmed the chiral discrimination ability of PGA after immobilization on monolithic support. Very fast enantioselective analysis of arylpropionic acids was achieved under CLC conditions using a short separation column connected to a transparent open capillary section allowing for high detection sensitivity at 200 nm. The proposed duplex column configuration showed to be a simple and robust technical approach for the application of CLC using commercial CE instrumentation. The potential of the proposed system was tested by performing a validation study, including quantitative applications to a real pharmaceutical sample and analysis at impurity level (on spiked samples); the achieved good results accounted for the reliability of CLC carried out using commercial CE instrumentation.

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