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HPLC method for enantioselective analysis of cloprostenol

Květa Kalíková [a], Eva Tesařová [a,*], Zuzana Bosáková [b]

[a] *Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, 128 43 Prague 2, Albertov 2030, Czech Republic*
[b] *Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, 128 43 Prague 2, Albertov 2030, Czech Republic*

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Abstract

A new HPLC method for the separation and quantification of cloprostenol enantiomers was developed. The optimized separation system consisted of Chiralcel OD-RH column and acetonitrile–sodium dihydrogenphosphate (pH 3.0; 20 mM) (33:67, v/v) as the mobile phase. Baseline resolution of (±)-cloprostenol ($R = 2.16$) was achieved and the analysis time did not exceed 10 min. Limits of detection and quantification were units of μmol/l at 274 nm. The respective values decreased an order of magnitude at 210 nm. The R.S.D. values obtained for the retention factor, peak area and peak height of each enantiomer were less than 2%. Conditions for semipreparative separation of the enantiomers can be achieved easily just by a small adaptation of the mobile phase composition.
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Keywords: Reversed phase HPLC; Enantioselective separation; Chiralcel OD-RH column; Validation; Prostaglandin analogue; Cloprostenol

1. Introduction

Prostaglandins are successfully employed in veterinary medicine. Reproductive management programs based on strategic use of prostaglandin F2α to induce and synchronize oestrus in post-partum dairy cows are widespread [1]. Repeated shortening of the oestrous cycle during early lactation in high-yielding dairy cows, however, could impair corpus luteum function and thus decrease fertility.

Cloprostenol is a synthetically prepared analogue of prostaglandin F2α. The preparation has ability to elicit luteolysis and uterine contraction; it is also used for synchronizations of rates and some other treatments. Chemical synthesis produces a racemic mixture of (±)-cloprostenol. However, (+)-enantiomer (see Fig. 1) was shown to have higher biological activity and only this enantiomer seems to exhibit luteolytic activity [2–4]. A chemoenzymatic synthesis of (+)-cloprostenol based on enantiospecific process catalyzed by the yeast *Kluyveromyces marxianus* has also been developed [5].

Repeated application of (+)-cloprostenol (sodium salt) affects progesterone concentrations that indicate function of corpus luteum in cows and influences milk production, parity and endometritis [1]. The positive impacts of cloprostenol on the induction of luteolysis but also its clinical side effects were observed in mares [6]. Cows with subclinical endometritis treated with cloprostenol (or cephapirin) had significantly increased relative pregnancy rate. The reproductive performance was improved just after a single treatment with these preparations [7]. The positive effect of cloprostenol on reproduction of cows diagnosed with endometritis seems to be the main reason for further investigations in this field nowadays [8].

Different effects of the racemic cloprostenol and its single enantiomer, (+)-cloprostenol, have been described by several authors [9–11] and are still a subject of extensive studies. In vitro investigations confirmed stereoselective binding of (+)-cloprostenol (and also of natural prostaglandin F2α) to be 150 times more potent than (±)-cloprostenol on corpus luteum cells and 10 times more potent on myometrium cells [12]. The significantly lower affinity of the racemic cloprostenol (as compared to (+)-enantiomer) to the tissue receptors was explained by a possible interference of the (−)-enantiomer from the racemate for the (+)-cloprostenol receptors [2].

In spite of the great interest in cloprostenol application and investigation of racemate versus pure enantiomer effects only few methods (HPLC) for its analysis can be found in the lit-

* Corresponding author. Tel.: +420 2 21951296; fax: +420 2 24919752.
E-mail address: tesarove@natur.cuni.cz (E. Tesařová).

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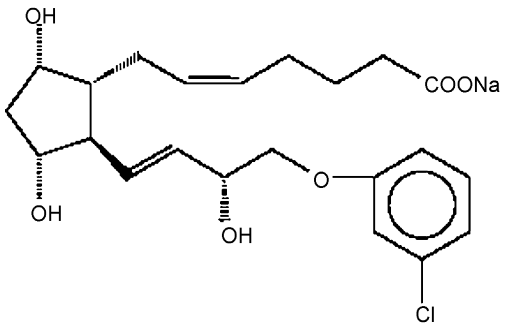

Fig. 1. Chemical structure of (+)-cloprostenol (sodium salt of [9α,11α,15*R*-trihydroxy-16-(3-chlorophenoxy)-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-oic acid]).

erature. A laborious semipreparative method was designed that consisted of preparation of a diastereomeric mixture of methyl ester trismandelates of (±)-cloprostenol, their separation on a silica-column with trichloromethane–ethylacetate (2:1, v/v) and subsequent alkaline hydrolysis of the esters to obtain the enantiomers [13]. An achiral method was used to control cloprostenol content in pharmaceutical preparation (solution) using an Ultrasphere ODS column and methanol–phosphate buffer (pH 6.75; 0.02 M) (50:50, v/v) as mobile phase [14]. Separation of cloprostenol epimers (stereoisomers the configuration of which differs just in the 15 position) was described on silica gel (Zorbax SIL) stationary phase. Addition of small amount of water (less than 1%) to hexane-propan-2-ol mobile phase was shown to dramatically improve separation efficiency and in this way also resolution [15].

However, to the best of our knowledge a method for direct chiral separation of cloprostenol enantiomers (stereoisomers, which differ in configuration on all stereogenic centers) has not been described in the literature, yet. In view of the above-mentioned facts and extensive concern of pharmaceutical companies over chiral separation of the racemic mixture of cloprostenol, the aim of this work is to develop a simple analytical method for chromatographic separation and determination of cloprostenol enantiomers. The method is aimed for enantiomeric purity control but it should be easy switched to a semipreparative mode.

2. Experimental

2.1. Equipment

Measurements were performed with a liquid chromatograph (Pye Unicam, Cambridge, UK) consisting of a PU 4015 pump, a PU 4020 UV–Vis detector and a Rheodyne 7725 injector with a 20 μl loop (Cotati, CA, USA). CSW32 software provided by DataApex (Prague, Czech Republic) was used for the process control and data handling. Another liquid chromatograph (Waters, Milford, MA, USA) composed of a Waters 1525 binary HPLC pump, Waters 717 plus autosampler and Waters 2487 dual λ absorbance detector, with Breeze software, was used for study of reproducibility of the method. NCSS software (NCSS, Kaysville, UT, USA) was applied to perform one-way analysis of variance (ANOVA). Temperature was controlled *via* Mistral column thermostat (Spark, Emmen, The Netherlands).

The chiral stationary phases (CSPs) tested during the method development were Chirobiotic T (teicoplanin-bonded CSP), Chirobiotic TAG (teicoplanin aglycon-bonded CSP), Chirobiotic V (vancomycin-bonded CSP) and Chirobiotic R (ristocetin A-bonded CSP), all 250 mm × 4.6 mm i.d., particle size 5 μm. These macrocyclic antibiotic-based columns were manufactured by ASTEC (Whippany, NJ, USA). The flow rate used was 0.6 ml/min in reversed phase and polar-organic modes and 1 ml/min in normal-phase mode. Analyses were performed at laboratory temperature. Another type of chiral stationary phases used in our experiments was Chiralcel OD-RH column (column size 150 mm × 4.6 mm) with guard cartridge (cartridge size 10 mm × 4 mm) (Chiral Technologies Europe, France). The chiral stationary phase of both the column and the cartridge consisted of cellulose tris(3,5-dimethylphenylcarbamate) (see Fig. 2) coated on 5 μm silica gel. This column was thermostated at 20 °C. The flow rate was set to 0.7 ml/min. Detection was carried on at wavelength of the absorption maximum 274 nm. At this wavelength mobile phase constituents and potential impurities do not interfere. Nevertheless, detection sensitivity is usually higher at wavelengths' range of 190–215 nm where the interference takes place. Therefore, linearity, limit of detection (LOD) and limit of quantification (LOQ) were also determined at 210 nm.

2.2. Chemicals

Methanol (MeOH), *n*-hexane (hex), propan-2-ol (IPA) and acetonitrile (ACN) for HPLC were obtained from Sigma–Aldrich (Prague, Czech Republic). Triethylamine (TEA, purity > 99.5%) and glacial acetic acid (HAc, purity > 99%) were from Fluka (Prague, Czech Republic). Sodium dihydrogenphosphate dihydrate was purchased from Lachema (Brno, Czech Republic) and *ortho*-phosphoric acid (85%) from Fluka (Buchs, Switzerland). Water was prepared with a Milli-Q water purification system (Millipore, Milford, MA, USA).

(±)-Cloprostenol and (+)-cloprostenol, purity 99.8%, were obtained from Nerapharm (Neratovice, Czech Republic). Stock

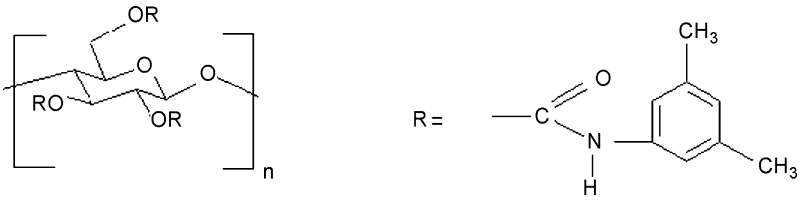

Fig. 2. Chemical structure of chiral stationary phase of Chiralcel OD-RH column.

solutions of (±)-cloprostenol and (+)-cloprostenol were prepared in concentration of 1 mg/ml using mobile phase as a solvent.

Various mobile phase compositions (with a variety of solvents and buffer components) were used in different separation modes tested with macrocyclic antibiotics-based CSPs (see Section 3). As the results obtained in these separation systems were less satisfactory all the tested mobile phase compositions are not listed here.

If the measurements were performed with Chiralcel OD-RH column acetonitrile–phosphate buffer mobile phases were used. The phosphate buffer was prepared by titration of 20 mM aqueous solution of sodium dihydrogenphosphate dihydrate with *ortho*-phosphoric acid to pH 3.0. Then the buffer was mixed with acetonitrile to reach the appropriate ratio (v/v) of ACN–phosphate buffer (pH 3.0; 20 mM). Certain variations of the optimized mobile phase composition, as well as of the separation conditions were part of the validation procedure.

2.3. Procedures

The hold-up volumes were measured by injection of MeOH or ACN to individual mobile phases used according to [16]. The retention factors (k) were calculated from the peak maxima.

One-way analysis of variance (ANOVA) statistical method was used for robustness testing. The method parameters, which could become significant sources of errors in practice, were chosen as variables. The significance level (α) was set to 0.05. The one-way analysis of variance compares the medians of two or more groups in order to determine if at least a median value of one group is different from the others. The tests are non-directional as the null hypothesis specifies that all medians are equal and the alternative hypothesis simply states that at least one median value is different [17]. If the statistical p-value is higher than the chosen significance level, the null hypothesis of equal medians is accepted.

3. Results and discussion

3.1. Optimization of the separation conditions

3.1.1. Selection of chiral stationary phase

In our preliminary experiments we examined the possibility to utilize chiral stationary phases (CSPs) based on macrocyclic antibiotics (MAs)—vancomycin, ristocetin A, teicoplanin and teicoplanin aglycon (TAG), for the separation of (±)-cloprostenol enantiomers. Quite promising results were obtained with the teicoplanin aglycon CSP. Satisfactory resolution ($R = 1.22$) was achieved in mobile phase composed of ACN–MeOH–HAc–TEA (90:10:0.1:0.1, v/v/v/v). From the above-mentioned separation systems, the best resolution of (±)-cloprostenol was obtained in normal-phase separation mode, in which the mobile phase was composed of hex–IPA–MeOH–TEA (60:15:25:0.5, v/v/v/v) ($R = 2.05$, $k_1 = 12.71$, $\alpha = 1.39$). Although baseline resolution of the enantiomers was achieved the run time was too long for practical use in these separation systems.

Table 1
The effect of ACN/buffer ratio on separation parameters of cloprostenol using Chiralcel OD-RH column; resolution, R, retention factor of (+)-enantiomer, k_1, selectivity, α

ACN–sodium dihydrogenphosphate (pH 3.0; 20 mM) (v/v)	R	k_1	α
40:60	1.10	0.58	1.17
33:67	2.16	1.63	1.21
30:70	2.26	3.03	1.20

Suitable alternatives to MA CSPs are polysaccharide CSPs. The cellulose or amylose-based columns are frequently used in enantioselective separation nowadays [18–20]. Chiralcel OD-RH column (cellulose tris(3,5-dimethylphenylcarbamate) CSP) was proved to be more convenient for separation of cloprostenol enantiomers than the TAG CSP. Binary mobile phases composed of ACN and 20 mM phosphate buffer, pH 3.0 or 6.0, in various acetonitrile to the buffer ratios were tested. At high acetonitrile contents the analyte eluted almost with dead volume. Decreased organic modifier content resulted in increased retention, which was more pronounced at the buffer pH 3.0, and enantioresolution possibility. The final procedure of optimization of the mobile phase composition, ACN/buffer ratio, is shown in Table 1. The optimized separation conditions, as the compromise between resolution and analysis time, were: Chiralcel OD-RH column; mobile phase, ACN–sodium dihydrogenphosphate (pH 3.0; 20 mM) (33:67, v/v); flow rate 0.7 ml/min; column temperature 20 °C; detection wavelengths 274 nm and 210 nm. The chromatogram of the (±)-cloprostenol enantioseparation is shown in Fig. 3(A). Symmetrical peak shape, higher resolution values and short separation time were the most significant attributes of this reversed phase system. Chromatogram in Fig. 3(B) confirms that (+)-cloprostenol was eluted first. The elution order of the enantiomers was the same under any experimen-

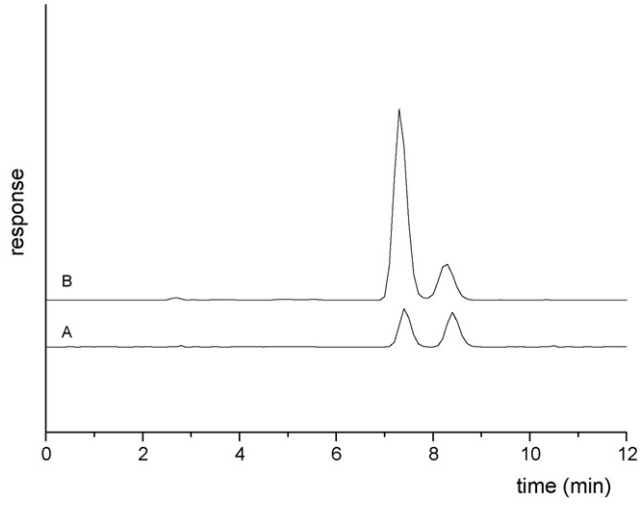

Fig. 3. Chromatograms of cloprostenol on the Chiralcel OD-RH column. Conditions: mobile phase acetonitrile–sodium dihydrogenphosphate (pH 3.0; 20 mM) (33:67, v/v); 0.7 ml/min; UV detection at 274 nm; column temperature 20 °C; samples: 0.1 mg/ml racemate of cloprostenol (A); racemate (0.1 mg/ml) enriched with (+)-cloprostenol (B).

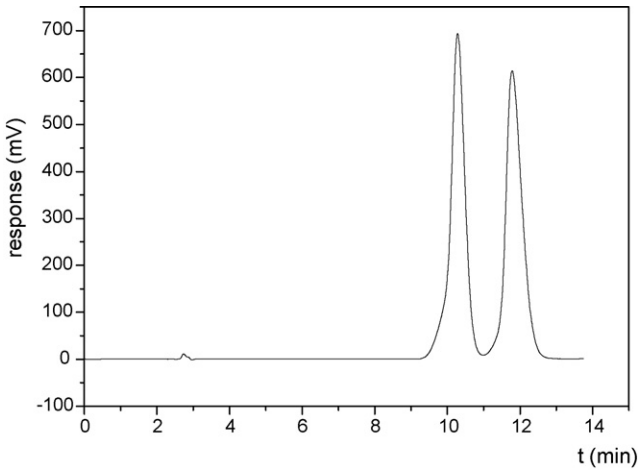

Fig. 4. Chromatogram of cloprostenol on the Chiralcel OD-RH column. Conditions: mobile phase acetonitrile–sodium dihydrogenphosphate (pH 3.0; 20 mM) (30:70, v/v); 0.7 ml/min; UV detection at 274 nm; column temperature 20 °C; injection volume 20 μl; sample: 5.5 mg/ml of cloprostenol racemate; enantioresolution 2.0.

tal conditions studied (also in separation system using the TAG CSP).

3.1.2. Semipreparative application of the separation system

The proposed enantioselective system could be also used for semipreparative separation of the cloprostenol enantiomers. Just a subtle change of mobile phase composition, i.e. decrease of acetonitrile to buffer ratio can substantially increase resolution. (This effect is, of course, accompanied by a certain extension of elution time.) Under these changed conditions the column can be overloaded while baseline enantioseparation is preserved. Fig. 4 shows the chromatogram obtained with higher sample injection using the mobile phase composed of ACN–sodium dihydrogenphosphate (pH 3.0; 20 mM) (30:70, v/v). The highest sample concentration, for which complete resolution could be achieved, was 5.5 mg/ml in this separation system. Further improvement in this way is possible. For example, another just slightly adopted mobile phase, in which the phosphate buffer was replaced by aqueous solution of acetic acid (all the other separation conditions were kept unchanged), was also tested. Enantioseparation with a resolution value of 2.14 was achieved in the mobile phase composed of ACN–0.1% acetic acid, pH 3.3 (30:70, v/v) and the analysis time did not exceed 12 min. Such mobile phase with volatile constituents should be better suited for (semi)preparative application.

3.2. Validation of the method

3.2.1. Stability of sample solutions

Stability of the sample solutions kept under various storage conditions was tested by the optimized HPLC method during the period of 3 weeks. Three equal solutions of (±)-cloprostenol were prepared by dilution of the stock solution with the mobile phase (ACN–sodium dihydrogenphosphate (pH 3.0; 20 mM) (33:67, v/v) to the final concentration 0.1 mg/ml. The first sample solution was stored at low temperature (in the refrigerator), the second one at room temperature and in darkness, and the last solution was held also at room temperature but on daily light. All the samples, no matter how they were stored, were proved to be stabile over the 3 weeks period.

3.2.2. Precision

In order to evaluate the precision of the HPLC method, repeatability and reproducibility of measurements were studied at the wavelength of 274 nm. The repeatability of the retention factors, peak areas and peak heights of the enantiomers were determined as relative standard deviations (R.S.D.) for 10 consecutive injections of the racemate with the concentration of 0.1 mg/ml. The following values were obtained: R.S.D. of the retention factors 0.11%, that of the areas 0.18% and R.S.D. of the peak heights 0.15% for the (+)-enantiomer. The respective values for the (−)-enantiomer were 0.13%, 0.22% and 0.17%. The reproducibility of the chromatographic data (retention factors, peak areas and peak heights) of the enantiomers was measured in 2 days, in two laboratories and on two different liquid chromatographs. The R.S.D. values obtained were 1.89% for the retention factor of (+)-enantiomer, 1.74% for the retention factor of (−)-enantiomer, 0.18% for the area of (+)-enantiomer, 0.49% for the area of (−)-enantiomer, and 1.16% and 1.59% for the heights of (+)-enantiomer and (−)-enantiomer, respectively. These results suggest that the method is suitable for both qualitative and quantitative analysis of the cloprostenol enantiomers.

3.2.3. Linearity

Linearity was studied at two wavelengths, 210 nm and 274 nm. At the wavelength of 274 nm the linearity was tested over the concentration range of 5.59×10^{-6} to 8.39×10^{-4} mol/l (2.50×10^{-3} to 3.75×10^{-1} g/l) for both enantiomers and at the wavelength of 210 nm over the range of 5.59×10^{-7} to 8.39×10^{-4} mol/l (2.50×10^{-4} to 3.75×10^{-1} g/l) for each cloprostenol enantiomer. Measurements at all concentration levels were carried out in triplicate and all values of peak areas were subjected to linear regression. Linear relationships between the peak areas and the concentrations of cloprostenol enantiomers were observed in the concentration range tested. The following linear regression equations were obtained:

- 274 nm

  (+)-cloprostenol :

  $$Y = 3.41 + 2.71 \times 10^{6}\ X \quad (r = 0.9999,\ \text{S.D.} = 7.76),$$

  (−)-cloprostenol :

  $$Y = 2.33 + 2.71 \times 10^{6}\ X \quad (r = 0.9999,\ \text{S.D.} = 8.82)$$

- 210 nm

  (+)-cloprostenol :

  $$Y = 133.37 + 2.21 \times 10^{7}\ X$$

  $$(r = 0.9997,\ \text{S.D.} = 151.07),$$

Table 2
Statistical *p*-values obtained from one-way ANOVA

Factor	*p*-value								
	(+)-Enantiomer				(−)-Enantiomer				Resolution
	Area	Height	Asymmetry	Efficiency	Area	Height	Asymmetry	Efficiency	
Temperature	0.11	0.06	0.10	0.42	0.73	0.05	0.18	0.15	0.18
Buffer pH	0.56	0.15	0.64	0.56	0.19	0.08	0.97	0.06	0.16
Buffer concentration	0.18	0.29	0.65	0.62	0.39	0.25	0.55	0.56	0.66
Acetonitrile content	0.73	0.03	0.18	0.08	0.59	0.03	0.73	0.43	0.03

(−)-cloprostenol :

$$Y = 125.40 + 2.22 \times 10^7\ X$$

$$(r = 0.9997,\ \ \text{S.D.} = 143.47)$$

where X is the concentration (mol/l) and Y is the peak area (mV s), r is correlation coefficient.

Somewhat worse linearity and higher standard deviation (S.D.) values at 210 nm are due to wider range of measurement and higher possibility of interference of impurities present in the separation system at this lower wavelength.

3.2.4. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were also calculated for two wavelengths, 210 nm and 274 nm. The limit of detection, expressed as a concentration at a signal-to-noise ratio 3:1, was calculated on the basis of the baseline noise, which was evaluated by recording the detector response over a period approximately ten times the widths of the peaks. The LOD was 1.15×10^{-6} mol/l (5.14×10^{-4} g/l) for (+)-enantiomer and 1.33×10^{-6} mol/l (5.94×10^{-4} g/l) for (−)-enantiomer at 274 nm. At the wavelength of 210 nm, the LOD was 1.33×10^{-7} mol/l (5.94×10^{-5} g/l) and 1.52×10^{-7} mol/l (6.79×10^{-5} g/l) for (+)-enantiomer and (−)-enantiomer, respectively. The signal-to-noise ratio of 10:1 was used to determine the limit of quantification. The obtained LOQ values were 3.85×10^{-6} mol/l (1.72×10^{-3} g/l) and 4.44×10^{-6} mol/l (1.98×10^{-3} g/l) for (+)- and (−)-cloprostenol, respectively, at 274 nm. At the wavelength of 210 nm, the LOQ was 4.44×10^{-7} mol/l (1.98×10^{-4} g/l) for (+)-enantiomer and 5.07×10^{-7} mol/l (2.27×10^{-4} g/l) for (−)-enantiomer. Both limits, LOD and LOQ, were approximately an order of magnitude lower if the analytes were detected at 210 nm than at 274 nm.

3.2.5. Robustness studies

One-way analysis of variance (ANOVA) statistical method was used for robustness testing. Our selected variable method parameters were: column temperature (19 °C, 20 °C and 21 °C), buffer pH (2.5, 3.0 and 3.5), acetonitrile content in mobile phase (± 2%) and concentration of the buffer (19.6 mM, 20.0 mM and 20.4 mM).

The robustness was determined from triplicate injections of 0.1 mg/ml cloprostenol racemic mixture. The effect of method parameters on resolution, peak areas, peak heights, efficiency and peak asymmetry were calculated. The hypothesis that errors resulted from a normal distribution was tested first. This hypothesis was accepted in all cases (at $\alpha = 0.05$). Consequently, the robustness of the method was examined using the one-way ANOVA. The calculated *p*-values are shown in Table 2.

This analytical method for measuring the cloprostenol enantiomers was proved to be robust to almost all the variations tested in this work, it means that *p*-values are higher than significance level ($\alpha = 0.05$). The factor of acetonitrile content in the mobile phase had effect on medians of resolution and peak heights (*p*-values are lower than 0.05). It reflected little change in retention of cloprostenol enantiomers by change of mobile phase composition. Accuracy and specifity were not determined as the aim of this work was a method for chiral separation of enantiomers of cloprostenol in pure racemic mixture.

4. Concluding remarks

Cellulose-based column Chiralcel OD-RH in combination with ACN–phosphate buffer mobile phase has been proved to be useful for fast and sensitive analysis of cloprostenol enantiomers. Basic validation parameters have been evaluated. The method can be applied in both the analytical and semipreparative separation modes. (+)-Enantiomer, which is therapeutically more active, is eluted first under the proposed conditions. This is advantageous especially for the semipreparative purposes. Chiral stationary phases based on macrocyclic antibiotics have been less suitable for enantioseparation of cloprostenol. Baseline separation on Chirobiotic TAG column can be achieved but the separation time is too long for practical application.

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