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Coupled column chromatography for separation and determination of enantiomers of phenylcarbamic acid derivatives in serum

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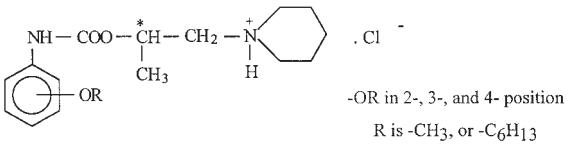
Columns packed with vancomycin coupled to an achiral C18 column and β-cyclodextrin were used for the separation and the determination of enantiomers of alkoxysubstituted esters of phenylcarbamic acid in blood serum. The method involves off-line SPE, the separation of the racemate on a reversed-phase stationary phase, and the separation of the enantiomers on a chiral stationary phase. The limit of detection was 1.0 μg/ml for the vancomycin column and 10.0 μg/ml for the β-cyclodextrin column in standard solution. *In vitro* degradation studies of enantiomers demonstrated a difference in the concentation of the enantiomers after the treatment. It was found that the rate constants of R(−)- and S(+)-forms of enantiomers are not significantly different.

1. Introduction

Alkoxysubstituted esters of phenylcarbamic acid are potential local anaesthetic drugs [1, 2]. The enantiomeric separation of phenylcarbamic acid derivatives can be performed by means of different chromatographic techniques, including GC, TLC, and LC. In LC numerous chiral stationary phases can be used for the separation of the enantiomers. Some chiral stationary phases consist of cellulose derivatives especially cellulose tris-3,5-triphenylcarbamate [3]. Others are based on the use of cyclodextrin [4] and α_1-acid glycoprotein [5]. Some π-complex type columns were also reported to be suitable for achieving the chiral separation of derivatives of phenylcarbamic acid [6].

In this work, a coupled achiral-chiral two column system was developed for the separation and the determination of the enantiomers of alkoxysubstituted esters of phenylcarbamic acid in serum. To optimise the on-line two column system the effect of the mobile phase composition for the achiral column, the dimension of the achiral column, direction of the flow in the achiral column, on the chromatographic resolution and the efficiency of the separation have been investigated.



2. Investigations, results and discussion

2.1. Chiral stationary phase

The selectivity of the separation and retention behaviour of the enantiomers on the chiral stationary phase as a function of the composition of the mobile phase (the organic modifier concentration, the amount of acid and base in the mobile phase) in polar-organic mode is well documented in the literature [7]. As it is evident from a previous work [8], the polar-organic mode is suitable for the separation of the enantiomers of 3- and 4-alkoxysubstituted esters of phenylcarbamic acid using a vancomycin column. A resolution ($R_{ij} > 1.2$) for enantiomers of 3- and 4-alkoxysubstituted esters of phenylcarbamic acid was obtained when a mixture of methanol/acetonitrile 80/20 (v/v) containing 17.5 mmol/l acetic acid and 7.94 mmol/l diethylamine was used as the mobile phase. The selectivity factor was about 1.25 with a resolution of 1.5–1.6. The elution order of enantiomers was S(+)-enantiomer before R(−)-enantiomer. The limit of determination was approximately 1.0 μg/ml.

Enantiomers of derivatives with alkoxysubstitution in 2-position on the aromatic ring were not separated on macrocyclic (vancomycin and teicoplanin) chiral stationary phases in the polar-organic mode [8, 9]. The enantioseparations of these analytes were achieved by β-cyclodextrin chiral stationary phases in the reversed-phase mode [10]. Good separation conditions were obtained using the mobile phase acetonitrile/0.1% triethylamine acetate pH = 5.5 (acetic acid) 10/90 (v/v). The selectivity factor for studied analyte (2-hexoxyphenylcarbamic acid derivate) was about 1.21 with a resolution of 1.1. The elution order was S(+)-enantiomer before the R(−)-enantiomer. The limit of determination was approximately 10.0 μg/ml.

2.2. Achiral column

The achiral column has the function to separate the racemate of analyte from the matrix compounds. The racemate was removed in an on-line system. To optimise the achiral system, the effects of the mobile phase composition, the

Table 1: Effect of the length of the achiral column and the direction of the flow of the mobile phase for desorption of analyte from the analytical reversed-phase column on the total time analysis, values of the resolution of enantiomers (R_S), and number of theoretical plates (N) on the vancomycin chiral stationary phase

	Separon SGX C18 (3,0 mm I.D., 5 μm)	
	L = 150 mm	L = 30 mm
Straight-flush		
Total time of analysis	28–30 min	–
R_{ij}*	0.6–0.7	–
N*	$(4.4–4.6) \times 10^3$	–
Back-flush		
Total time of analysis	28–30 min	20–23 min
R_{ij}*	0.95–1.0	1.1–1.2
N*	$(8.5–8.8) \times 10^3$	$(9.2–9.4) \times 10^3$

* The values of R_{ij} and N were calculated for the separation of the enantiomers using chiral stationary phase

dimension of the achiral column and the direction of the flow of mobile phase in achiral column were studied.

From the previous work [9, 10] a good retention and symmetrical peaks were obtained using a mixture of methanol/water 90/10 (v/v) containing 6.8 g/l of sodium acetate as mobile phase. This mobile phase was not suitable for the separation of enantiomers on vancomycin and β-cyclodextrin columns. Therefore a mobile phase was used that was compatible with chiral stationary phases (methanol/acetonitrile 80/20 (v/v) containing 17.5 mmol/l acetic acid and 7.94 mmol/l diethylamine for the vancomycin column and acetonitrile/0.1% triethylamine acetate pH = 5.5 (acetic acid) 10/90 (v/v) for the β-cyclodextrin column) in which the separation of the analytes from matrix components was achieved. These mobile phases were suitable for achiral and chiral systems, but they were not suitable for an on-line coupled system because the values of resolutions of enantiomers were decreased ($R_S \sim 0.5–0.6$). In the next experiments methanol was used as the mobile phase for an achiral column in which the analytes were sorbed at the top of the column and the matrix components were eluted quickly (elution time less then 5 min).

The length of the achiral column influenced the total time of analysis. In our experiments, C18 columns of 150 mm or 30 mm length were used. The direction of the flow of the mobile phase for the desorption of the analyte from the analytical reversed-phase column into the chiral column influenced significantly the resolution of enantiomers and the efficiency of enantioseparation. The results obtained for long and short achiral columns with straight flush and back-flush direction of mobile phase are given in Table 1. From these results it can be seen that the short C18 column (L = 30 mm) and back-flush mode for the elution of analytes from the achiral into the chiral column were suitable conditions for an on-line coupled system.

2.3. On-line coupled column system

The performance the on-line coupled column system (with a vancomycin chiral stationary phase) was investigated with standard solutions of 3-; 4-hexoxy- and 3-; 4-methoxy-substituted esters of phenylcarbamic acid racemate and spiked rabbit blood serum samples (for 2-hexoxy-substituted esters of phenylcarbamic acid racemate a β-cyclodextrin chiral stationary phase without on-line coupled column system was used). The calibration with standard solutions in the range of 1.0–100 μg/ml (5 points) and in the range of 2.0–100 μg/ml (5 points) for serum was linear for standard solutions (r = 0.99) and for spiked serum (r = 0.98). The enantiomer ratio in the standard solutions was found to be 1.01 ± 0.05 (n = 15) and in serum 1.12 ± 0.06 (n = 10). The limit of determination with standard solutions was approximately 1.0 μg/ml and for serum 2.0 μg/ml. The recovery of off-line clean up of analytes from spiked serum at a concentration level 10 μg/ml was found to be 85–95% with RSD of 3–5%.

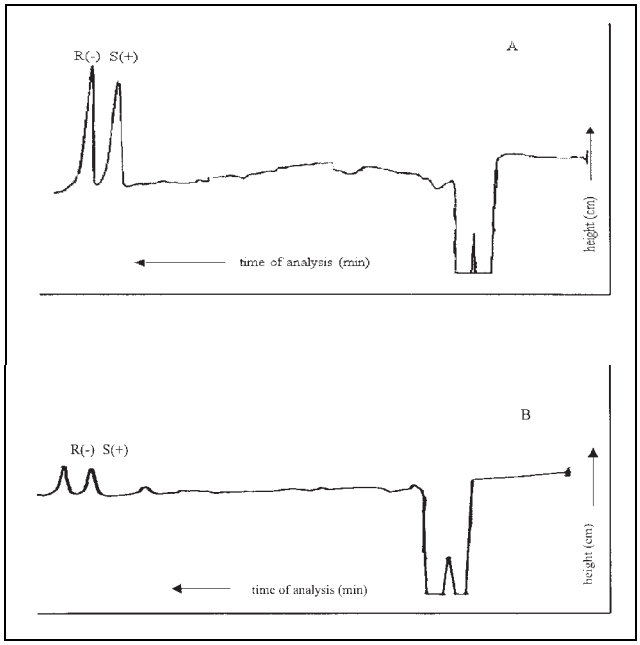


Fig. 1: Chromatograms of the standard solution of 1-methyl-2-piperidinoetylester-4-methoxyphenylcarbamic acid (A) and spiked blood serum (B) obtained on the coupled reversed-phase C18 and vancomycin chiral stationary phase

Fig. 1 shows the chromatograms of a standard solution of 1-methyl-2-piperidinoetylester-4-methoxyphenylcarbamic acid and spiked blood serum using the coupled C18 achiral and vancomycin chiral stationary phase.

2.4. In-vitro kinetic study

The developed HPLC method was applied to investigate the *in vitro* kinetics of enzymatic hydrolysis of the enantiomers of 3-; 4-methoxy and 2-, 3-, 4-hexoxysubstituted esters of phenylcarbamic acid. The standard solution of analyte racemate was added to the blood serum (t = 0) and the biological sample was incubated at 37 °C (time interval from 0 to 24 h). The time curves of the *in vitro* degradation of enantiomers of analytes in blood serum are given in Fig. 2. These curves demonstrate the difference in the concentrations of the enantiomers after the treatment. The R(−)-forms were degraded slower than the S(+)-forms. The biological activity of both forms is connected with their degradation in biological material. The experimental rate constants (k) on the assumption that the reaction of degradation is of first or the second order are summarised in Table 2.

The rate constants were determined using the linear dependences: $\ln(c/c_0) = f(t)$ (first order) and $1/c = f(t)$ (second order). It is evident that the course of the enzymatic degradation of studied compounds corresponds to

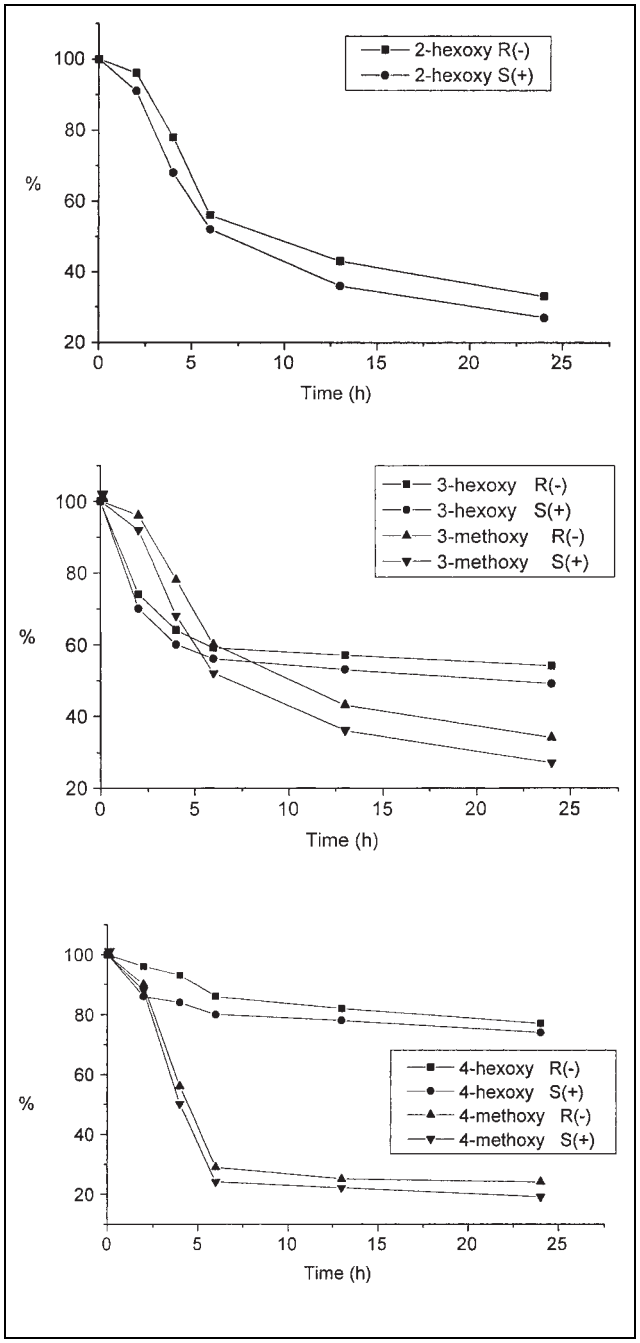


Fig. 2: Time course of the enantiomers of 1-methyl-2-piperidinoethylesters of 2 hexoxy (A); 3-hexoxy and 3-methoxy- (B); 4-hexoxy- and 4-methoxy- (C) substituted esters of phenylcarbamic acid

the second order with regard to the change of the concentration of the racemates during study (the correlation coefficients are close to 1). F-test was used for the evaluation of results (level of probability $\alpha = 0.05$). It was found that rate constants of R(−) and S(+) forms are not significantly different.

3. Experimental

3.1. Chemicals

Racemic 1-methyl-2-piperidinoetylesters of 2-, 3-, and 4-alkoxyphenylcarbamic acid were prepared by standard procedures [1, 2]. Solvents (methanol and acetonitrile) of HPLC grade and other chemicals (acetic acid, diethylamine, triethylamine) of analytical grade were supplied by Merck (Germany).

Table 2: Rate constants of R(−)- and S(+)-forms of enantiomers of 1-methyl-2-piperidinoethylesters of 2-hexoxy-; 3-hexoxy- and 3-methoxy-; 4-hexoxy- and 4-methoxy-substituted esters of phenylcarbamic acid in blood serum

	First order section		Second order section	
	$k \cdot 10^5$ (s^{-1})	r^2	k ($l \cdot mol^{-1} \cdot s^{-1}$)	r^2
2-Hexoxy R(−)	1.29 ± 0.24	0.934	0.16 ± 0.02	0.969
2-Hexoxy S(+)	1.40 ± 0.27	0.934	0.19 ± 0.02	0.975
3-Hexoxy R(−)	0.51 ± 0.14	0.768	0.51 ± 0.21	0.761
3-Hexoxy S(+)	0.58 ± 0.23	0.766	0.65 ± 0.22	0.825
3-Mexoxy R(−)	1.27 ± 0.21	0.949	1.27 ± 0.14	0.978
3-Mexoxy S(+)	1.38 ± 0.22	0.962	1.48 ± 0.15	0.984
4-Hexoxy R(−)	0.28 ± 0.06	0.908	0.22 ± 0.05	0.919
4-Hexoxy S(+)	0.25 ± 0.09	0.897	0.21 ± 0.07	0.824
4-Mexoxy R(−)	1.58 ± 0.57	0.813	2.01 ± 0.63	0.849
4-Mexoxy S(+)	1.81 ± 0.65	0.814	2.36 ± 0.77	0.865

3.2. Equipment

The HPLC chromatographic system Hewlett Packard (series 1100) consisted of a quaternary pump, and injection valve Rheodyne, a switching valve Valco, and a photodiode array detector.

3.3. Chromatography

The chiral stationary phases were vancomycin (Chirobiotic V, 250 × 4.6 I.D., 10 μm) (Astec, USA) for separations of 3-, 4-alkoxysubstituted derivatives and β-cyclodextrin (LichroCart ChiraDex, 250 × 4 I.D., 5 μm) (Merck, Germany) for enantioseparations of 2-alkoxysubstituted derivatives. Further, a column (150 × 3.2 I.D., 5 μm) or (30 × 3.2 I.D., 5 μm) containing C18 (Separon SGX C18) (Tessek, Czech Republic). For the achiral column a mobile phase containing methanol was used. A mixture of methanol/acetonitrile 80/20 (v/v) containing 17.5 mmol/l acetic acid and 7.94 mmol/l diethylamine was used as the mobile phase for the vancomycin column. A mobile phase acetonitrile/0.1% triethylamine acetate pH = 5.5 (acetic acid) 10/90 (v/v) was used for the enantioseparation on the β-cyclodextrin column. The flow rate for the achiral and chiral columns was set at 0.5 ml/min. Injection volume was 20 μl. A photodiode array detector at 240 nm was used.

3.4. Sample preparation

The sorbent was conditioned with 2 ml of methanol, 1 ml of acetonitrile, and 10 ml of water. Blood serum (0.5 ml) spiked with studied analytes (concentration 10 μg/ml) was injected into a Sep-Pak (200 mg) (Waters, Germany) cartridge. Then the sample was passed through the sorbent layer and washed with 1 ml of water and then with 1 ml of acetonitrile. Analytes retained by the sorbent were eluted with 1 ml of the methanol mixture containing 17.5 mmol/l acetic acid and 7.94 mmol/l diethylamine. 20 μl of eluate were injected into the achiral column.
The achiral column was conditioned with the mobile phase (methanol) before injection of serum extract or standard solutions. After injection the achiral column was flushed with the mobile phase for 4 min. Then the achiral column was connected via valve to the chiral column and the analyte racemate was eluted with the mobile phase.

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