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Kinetic study of derivatives of phenylcarbamic acid enantiomers in rabbit blood serum using an on-line coupled column liquid chromatographic system

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An on-line coupled HPLC system is described for the kinetic study of the enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-alkoxyphenylcarbamic acid in rabbit blood serum. The method involves three steps: (i) off-line pre-concentration and cleanup step, (ii) analytical separation of the racemate on a reversed-phase stationary phase, and (iii) separation of the enantiomers on a teicoplanin aglycone chiral stationary phase (Chirobiotic Tag). The limit of the determination with standard solutions was approximately 5.0 µg/ml. *In vitro* degradation studies of enantiomers have demonstrated differences in the concentration of the enantiomers after the treatment. The rate constants of *R*(–)- and *S*(+)-forms of enantiomers (the same as the position of alkoxy chain) were, not significantly different. The number of carbon atoms had an influence on the degradation kinetics.

1. Introduction

Alkoxy-substituted esters of phenylcarbamic acid form a group of potential drugs employed in the local anaesthesia [1–3]. In this paper we describe an on-line coupled column liquid chromatographic system (achiral–chiral) to determine the enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-alkoxyphenylcarbamic acid in rabbit blood serum. The method involves three steps: (i) an off-line pre-concentration and clean-up step, (ii) analytical separation of the racemate on a reversed-phase stationary phase, and (iii) the separation of the enantiomers on a teicoplanin aglycone chiral stationary phase (Chirobiotic Tag). The developed method was used for the study of *in vitro* degradation of alkoxy substituted esters of phenylcarbamic acid in rabbit blood serum. The influence of the number of carbon atoms in the alkoxy chain and position on the degradation of selected enantiomers in rabbit blood serum was studied.

2. Investigations, results and discussion

The enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-alkoxyphenylcarbamic acid have a different local anaesthetic activity. The methoxy- and ethoxy-derivatives are slightly less almost equally active in the surface anaesthesia when they are compared with cocaine as a standard. The activity of all three position isomers begins to increase since the propoxy substitution (C₃) and decrease since the octyloxy substitution (C₈) (mean cut off effect). The maximum activity is reached at the 2-hexoxy, which is 166.6-times more active than cocaine. All the 4-substituted derivatives are appreciably less active than the corresponding 2- and 3-isomers [4].

From a previous work [5, 6], which concerns the pharmacological appreciation of derivatives of phenylcarbamic acid with alkoxy substitution in 2-, 3- and 4-position followed, that enantiomers with a number of carbon atoms between 4 and 7 in the alkoxy substituent are most active. Therefore, enantiomers with low activity (C₁, C₁₀) and the compound with the highest activity (C₆) were used for the kinetic study.

The retention factors and the resolution of the enantiomers on a teicoplanin aglycone chiral stationary phase (Chirobiotic Tag) in polar-organic mode is well documented [3]. A good resolution ($R_{ij} > 1.3$) was obtained when methanol (100 ml (v)) containing 17.5 mmol/l acetic acid and 4.8 mmol/l diethylamine was used as the mobile phase. The structure of teicoplanin aglycone indicates that the mechanism of separation would include π – π complexation, hydrogen bonding, dipole stacking, steric interaction and inclusion complexation. The different interaction of two enantiomeric forms with the CSP leading to chiral discrimination can be expressed as the difference in the free energy $-\Delta_{1,2}\Delta G^\circ$ calculated from the separation factor α according to eqs. (1) and (2)

$$-\Delta G_{1,2}\Delta G^\circ = \Delta_2 G^\circ - \Delta_1 G^\circ \quad (1)$$

$$-\Delta_{1,2}\Delta G^\circ = RT \ln k_2/k_1 = RT \ln \alpha \quad (2)$$

The results given in Table 1 show the very small energy differences, which are needed for the chromatographic resolution of selected enantiomers. Obviously the binding of two enantiomers to a given chiral site may involve different amounts of energy simply because one of the enantiomers, for steric reasons, might be forced to adopt an energetically less favourable conformation.

Table 1: Retention factors (k_1 , k_2), free energy differences ($-\Delta_{1,2}\Delta G^\circ$) of the selected enantiomers of alkoxy-substituted esters of phenylcarbamic acid obtained on the teicoplanin aglycone chiral stationary phase

	Chirobiotic Tag		$-\Delta_{1,2}\Delta G$ (J/mol)
	k_1	k_2	
2-Methoxy	13.38	16.24	475.11
2-Hexoxy	8.48	9.33	234.29
2-Decyloxy	6.62	7.24	219.57
3-Methoxy	14.19	16.10	309.72
3-Hexoxy	12.10	13.81	324.21
3-Decyloxy	8.90	10.33	365.44
4-Methoxy	15.29	17.24	294.40
4-Hexoxy	13.29	14.95	288.67
4-Decyloxy	10.52	12.00	322.84

k_1 , $k_2 \pm 0.15$ ($n = 3$)

$-\Delta_{1,2}\Delta G^\circ \pm 0.8$ J/mol ($n = 3$)

The achiral column served two functions. It focused the enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-alkoxyphenylcarbamic acid at the top of the column and separated the racemate from the other matrix compounds. Subsequently, the concentrated enantiomers were eluted into the teicoplanin aglycone chiral column (Chirobiotic Tag). In the next experiments, methanol was used as the mobile phase for the achiral column in which the analytes were sorbed (at the top of the achiral column) and the matrix components were eluted quickly with methanol (elution time less than 5 min).

In our experiments, a C18 achiral column of 30 mm length was used. The direction of the flow of the mobile phase for the desorption of analyte from the analytical reversed-phase column into the chiral column significantly influenced the resolution of enantiomers and efficiency of enantioseparation. A C18 column and backflush mode for the elution of analytes from the achiral into the chiral column were used for the on-line coupled system [7].

The performance of the on-line coupled column system (with teicoplanin aglycone chiral stationary phase – Chirobiotic Tag) was investigated with standard solutions of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-methoxy-, -hexoxy- and -decyloxy-substituted esters of phenylcarbamic acid racemate and spiked rabbit blood serum samples. The limit of determination with standard solutions was approximately 5.0 µg/ml and for serum 10.0 µg/ml. The recovery of off-line clean-up of analytes from spiked rabbit blood serum at a concentration level of 10.0 µg/ml was found to be 86–91% with a RSD of $\pm 8\%$ (3 measurements; chromatographic conditions: see Experimental).

Various esterase enzymes cause the degradation of the enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-alkoxyphenylcarbamic acid [7]. The developed HPLC method was applied to investigate the *in vitro* kinetics of enzymatic hydrolysis of the selected enantiomers. A standard solution of analyte racemate was added to the rabbit blood serum ($t = 0$ h) and the biological sample was incubated at 37 °C (time interval from 0 to 24 h).

First, an one year old rabbit blood serum was estimated. It is assumed that rabbit blood serum has another property (the activity of esterase decreased), when it was in a freezer longer than 1 year. In this case the selected enantiomers were not degraded. The time curves of *in vitro* degradation of 1-methyl-2-piperidinoethylesters of 2-hexoxy-substituted esters of phenyl carbamic acid are given in Fig. 1. The dependencies (A) have similar tendency for all selected enantiomers:

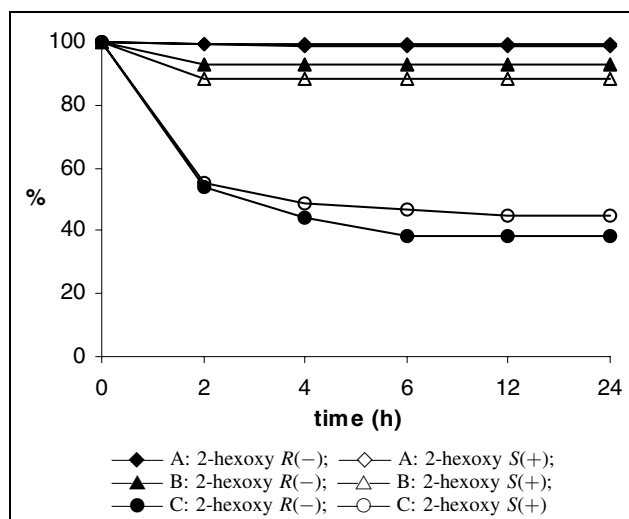


Fig. 1: Time course of the enantiomer of 1-methyl-2-piperidinoethylesters of 2-hexoxy-substituted esters of phenylcarbamic acid. (A) – the rabbit blood serum was one year old, (B) – after injecting of atropine into rabbit; (C) – the rabbit blood serum was fresh

In the second part of the study we used the rabbit blood serum, which was taken after addition of atropine into rabbits. In this serum the selected enantiomers with alkoxy-substitution in 2-position degraded within 2 h. The enantiomers with alkoxy-substitution in 3- and 4-position degraded within 4 h (Fig. 2). The experimental rate constants (k) are summarised in Table 2. The rate constants were determined using the linear dependence: $\ln(c/c_0) = f(t)$ (first order) and $1/c = f(t)$ (second order). According to the low value of r , it can be assumed that atropine injected into rabbits before sampling of serum has an influence on the rate of degradation of alkoxy-substituted esters of phenylcarbamic acid.

Finally, new fresh rabbit blood serum (without injection of atropine into rabbits) was used. The time curves of the *in*

Table 2: Rate constants of decomposition of R(–)- and S(+)-forms of the selected enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-substituted esters of phenylcarbamic acid in rabbit blood serum (after injecting of atropine into rabbits)

	First order section		Second order section	
	$k \times 10^6$ (s ⁻¹)	r	$k \times 10^2$ (l · mol ⁻¹ · s ⁻¹)	r
2-Methoxy R(–)	1.92	0.58	1.33	0.58
2-Methoxy S(+)	1.83	0.58	1.28	0.58
2-Hexoxy R(–)	1.14	0.58	0.94	0.58
2-Hexoxy S(+)	2.00	0.58	1.71	0.58
2-Decyloxy R(–)	1.67	0.58	1.59	0.58
2-Decyloxy S(+)	1.92	0.58	1.86	0.58
3-Methoxy R(–)	0	0	0	0
3-Methoxy S(+)	2.89	0.75	1.86	0.75
3-Hexoxy R(–)	0	0	0	0
3-Hexoxy S(+)	3.19	0.75	2.71	0.75
3-Decyloxy R(–)	0	0	0	0
3-Decyloxy S(+)	3.03	0.75	2.95	0.75
4-Methoxy R(–)	0.97	0.58	0.66	0.58
4-Methoxy S(+)	1.03	0.58	0.71	0.58
4-Hexoxy R(–)	1.81	0.75	1.49	0.75
4-Hexoxy S(+)	3.09	0.75	2.71	0.75
4-Decyloxy R(–)	1.72	0.75	1.63	0.75
4-Decyloxy S(+)	2.61	0.75	2.52	0.75

first order section: $k \pm 0.04 \times 10^{-6}$ s⁻¹ ($n = 3$)

second order section: $k \pm 0.03 \times 10^{-2}$ l · mol⁻¹ · s⁻¹ ($n = 3$)

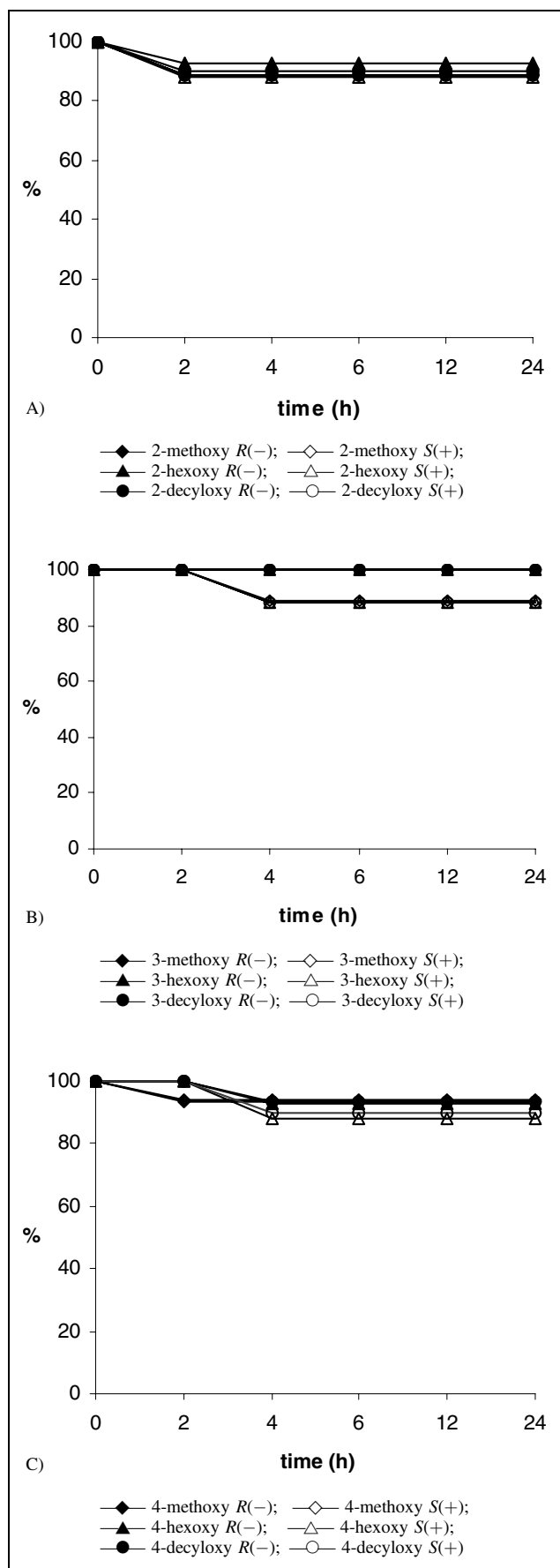


Fig. 2: Time course of the selected enantiomers of 1-methyl-2-piperidinoethylesters of 2-methoxy-, -hexoxy- and -decyloxy- (A); 3-methoxy-, -hexoxy- and -decyloxy- (B); 4-methoxy-, -hexoxy- and -decyloxy- (C) substituted esters of phenylcarbamic acid (after injecting of atropine into rabbits)

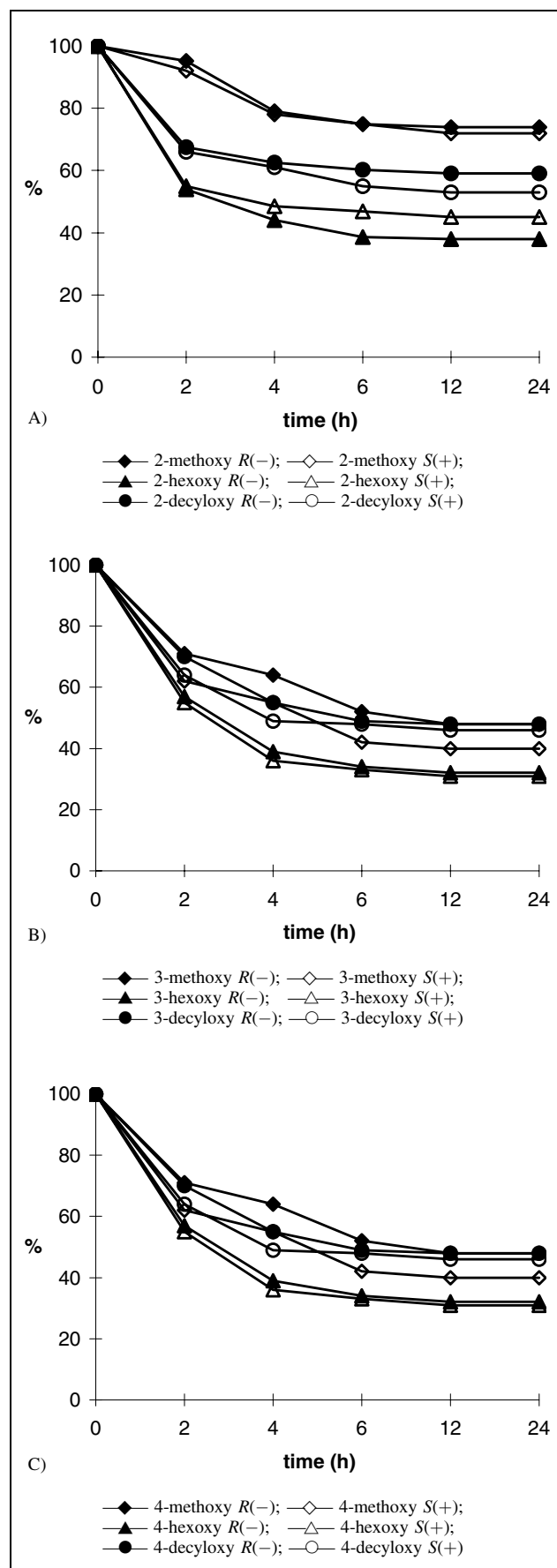


Fig. 3: Time course of the selected enantiomers of 1-methyl-2-piperidinoethylesters of 2-methoxy-, -hexoxy- and -decyloxy- (A); 3-methoxy-, -hexoxy- and -decyloxy- (B); 4-methoxy-, -hexoxy- and -decyloxy- (C) substituted esters of phenylcarbamic acid (the rabbit blood serum was fresh)

Table 3: Rate constants of decomposition of *R*(-)- and *S*(+)-forms of the selected enantiomers of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-substituted esters of phenylcarbamic acid in rabbit blood serum (fresh rabbit blood serum)

	First order section		Second order section	
	$k \times 10^5 \text{ s}^{-1}$	r	$k \times 10^2 \text{ l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$	r
2-Methoxy <i>R</i> (-)	1.89	0.88	0.22	0.91
2-Methoxy <i>S</i> (+)	0.74	0.91	0.26	0.91
2-Hexoxy <i>R</i> (-)	1.78	0.84	0.17	0.86
2-Hexoxy <i>S</i> (+)	0.96	0.85	0.15	0.87
2-Decyloxy <i>R</i> (-)	1.45	0.93	0.20	0.96
2-Decyloxy <i>S</i> (+)	0.95	0.93	0.28	0.96
3-Methoxy <i>R</i> (-)	1.27	0.88	0.22	0.91
3-Methoxy <i>S</i> (+)	0.93	0.90	0.25	0.93
3-Hexoxy <i>R</i> (-)	2.09	0.90	0.29	0.93
3-Hexoxy <i>S</i> (+)	1.61	0.87	0.21	0.90
3-Decyloxy <i>R</i> (-)	1.64	0.91	0.24	0.93
3-Decyloxy <i>S</i> (+)	1.03	0.90	0.22	0.96
4-Methoxy <i>R</i> (-)	1.73	0.90	0.19	0.93
4-Methoxy <i>S</i> (+)	1.11	0.92	0.20	0.95
4-Hexoxy <i>R</i> (-)	0.93	0.89	0.30	0.91
4-Hexoxy <i>S</i> (+)	1.57	0.85	0.21	0.88
4-Decyloxy <i>R</i> (-)	1.53	0.90	0.22	0.92
4-Decyloxy <i>S</i> (+)	0.85	0.90	0.29	0.96

first order section: $k \pm 0.06 \times 10^{-5} \text{ s}^{-1}$ ($n = 3$)

second order section: $k \pm 0.05 \text{ l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ($n = 3$)

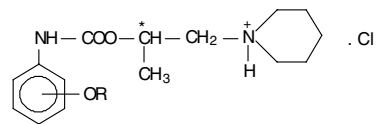
in vitro degradation of selected enantiomers are given in Fig. 3. During the first 2 h the decomposition of enantiomers was rather rapid, in the next 4 h it was decreased slower. After 6 h the concentration of selected enantiomers was constant. The experimental rate constants (k) on the assumption that the reaction of degradation is of the first or the second order are summarised in Table 3. It is evident that the course of the enzymatic degradation of studied enantiomers corresponds to the second order (higher value of r) with regard to the change of the concentration of the racemates during the study. The biological activity of both enantiomeric forms is connected with enzymatic degradation of selected enantiomers in biological material [7].

It is supposed, that the position of the alkoxy chain (the same as the number of carbon atoms in alkoxy chain) has an influence on *in vitro* degradation of selected enantiomers in rabbit blood serum. From the statistical point of view (F -test was used, level of probability $\alpha = 0.05$) it was found that rate constants of *R*(-)- and *S*(+)-forms of enantiomers (Table 3) (as well as the position of the alkoxy chain) are not significantly different. On the other hand the number of carbon atoms has an influence on *in vitro* degradation. The 2-hexoxysubstituted ester of phenylcarbamic acid shows the slowest degradation (Table 3). It is assumed that the 2-hexoxysubstituted ester is the most active in rabbit blood serum [8–10].

3. Experimental

3.1. Materials

Racemic mixtures of 1-methyl-2-piperidinoethylesters of 2-, 3- and 4-alkoxyphenylcarbamic acid were prepared according to Pokorná et al. [2]. The organic solvents (methanol, acetonitrile and diethylamine) were of analytical grade (Merck, Germany), acetic acid was also of analytical quality (Lachema, Czech Republic).



Number of carbon atoms	2-Position		3-Position		4-Position	
	Analyte	R	Analyte	R	Analyte	R
C ₁	1v	—CH ₃	2v	—CH ₃	3v	—CH ₃
C ₆	4v	—C ₆ H ₁₃	5v	—C ₆ H ₁₃	6v	—C ₆ H ₁₃
C ₁₀	7v	—C ₁₀ H ₂₁	8v	—C ₁₀ H ₂₁	9v	—C ₁₀ H ₂₁

3.2. Sample preparation

The pre-column was dry packed and must be conditioned. The sorbent was conditioned with 2 ml of methanol, 1 ml of acetonitrile, and 10 ml of water. 0.5 ml of rabbit blood serum spiked with studied analyte (concentration 0.1 mg/ml) was injected into a C18 Sep-Pak cartridge. Then the sample was passed through the sorbent layer and washed with 1 ml of water and then with 1 ml of acetonitrile. Analyte retained by the sorbent was eluted with 1 ml of the mixture methanol (100 ml (v)) containing 17.5 mmol/l acetic acid and 4.8 mmol/l diethylamine.

3.3. Equipment

The measurements were carried out with Hewlett-Packard HPLC system (series 1100) consisting of a quaternary pump, an injection valve Rheodyne, switching valve Valco, and a photodiode array detector.

3.4. Chromatographic separation

The packing of the off-line cartridge was C18, Sep-Pak Vac 3cc (200 mg) (Waters, Germany). For the achiral column Separon SGX C18, 5 μm ($30 \times 3.2 \text{ mm I.D.}$) was used and the chiral column was a teicoplanin aglycone (Chirobiotic Tag, $250 \times 4.6 \text{ mm I.D.}$ 5 μm) (Astec, USA). With the off-line procedure, the enantiomers were extracted from rabbit blood serum and subsequently focused on the achiral C18 column. The achiral column was conditioned with mobile phase (methanol) before injection of the serum extract or standard solutions. After injection the achiral column was flushed with mobile phase during 5 min (flow rate 0.8 ml/min). Then the achiral column was connected via valve to the chiral column and analyte racemate was eluted with mobile phase for chiral column. For the teicoplanin aglycone chiral column (Chirobiotic Tag), the mobile phase methanol (100 ml (v)) containing 17.5 mmol/l acetic acid and 4.8 mmol/l diethylamine was used. The flow rate for achiral and chiral column was set at 0.8 ml/min. Injection volume was 20 μl and photodiode array detector at 240 nm was used.

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