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Chiral ligand-exchange chromatography for diastereo-enantio separation of exametazime

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Abstract

The diastereo-enantio separation of isomeric mixtures of exametazime (HM-PAO) by liquid chromatography is described using an achiral sorbent (RP-18). A chiral eluent with the initial complex of Cu(II) and the optically active selector N,N-dimethyl-*l*-phenylalanine (*l*-DM-PhA), based on the ligand-exchange principle, has been applied. The separation is based on the presence of the immobilized binary complex Cu(*l*-DM-PhA)$_2$ and formation of mixed ternary complex. The optimal mole ratio of Cu(II):*l*-DM-PhA is 1:4, the pH should be between 4.1 and 4.2 and up to 0.8 mM of triethylamine is added for column presaturation with the initial complex. The elution order has been defined using isolated *l*-HM-PAO via *l*-HM-PAO L(+)tartrate and *meso*-HM-PAO obtained by repeated recrystallization from the isomeric mixture of HM-PAO. Complete resolution between all isomers (R_S from 2.14 to 3.91) and partial resolution for *meso*$_{EE}$/*l*-HM-PAO ($R_S = 0.83$) has been obtained. This means that the proposed chiral ligand-exchange chromatography (CLEC) can be used for determination of the isomeric purity of HM-PAO. This as an alternative method for resolution measurements with chiral columns.

Keywords: Column liquid chromatography; Ligand-exchange chromatography; Chiral separation; Exametazime (HM-PAO)

1. Introduction

The increased demand for enantiopure drugs has led to the development of a variety of stereoselective separation technologies. The influence of stereochemistry on the pharmacological effect of drugs is well known. However, there have

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been relatively few data of stereochemical effects on biodistribution properties of the technetium-99m complexes employed in diagnostic nuclear medicine [1,2]. One notable example, in recent years, is the technetium-99m complex of (±)-(*RR,SS*)-4,8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bisoxime (Exametazime, HM-PAO). This ligand, shown in Fig. 1, has two chiral centers, giving the *meso-(R,S)*, *d-(R,R,)* and *l-(S,S)* isomers. Synthesis of the ligand gives an equal mixture of the *meso-* and *d,l-*diastereoisomers and their separation was achieved by repeated recrystallization [3].

The complex ^{99m}Tc-*d,l*-HM-PAO is widely used and well-established radiopharmaceutical in the evaluation of regional cerebral perfusion (rCBF) [4,5] for the diagnosis and management of various brain disorders, such as stroke, vascular disease, epilepsy, dementia and degenerative disease. It is also useful in the labelling of leucocytes for the localization of inflammations and infections [6]. The technetium complexes from the two diastereoisomeric forms of the ligand, displayed different in vivo properties. The complexes had similar brain uptake, but the *meso*-form exhibits far faster cerebral washout than the *d,l*-complex. Hence, it is important for data interpretation to know the proportion of the each of the stereoisomers. The determination of the stereoisomeric purity of the separated diastereoisomers has been described using the analytical chiral HPLC columns [7].

In the present paper the resolution of the *meso*-, *d*- and *l*-HM-PAO using RP-18 column and a chiral eluent, containing the initial complex of Cu(II) with chiral selector N,N-dimethyl-*l*-phenylalanine for ligand-exchange chromatography, has been studied. Chiral ligand-exchange chromatography (CLEC) has been invented by Davankov [8], and recently the review [9] on CLEC has been published by Kurganov. This method, due to the use of the variety of chiral selectors, which form initial complexes of different stability with metal ions, enables enantioresolution of numerous solutes of different chemical structures and the relationships derived for CLEC should be valid for any other chromatographic system involving solute-selector interactions.

2. Experimental

2.1. Instrumentation

All analyses were carried out on the Hewlett-Packard HPLC system consisted of a Model HP 1050 Series (including: quaternary pumping system, autosampler with automatic injector and variable sample loop and solvent cabinet with

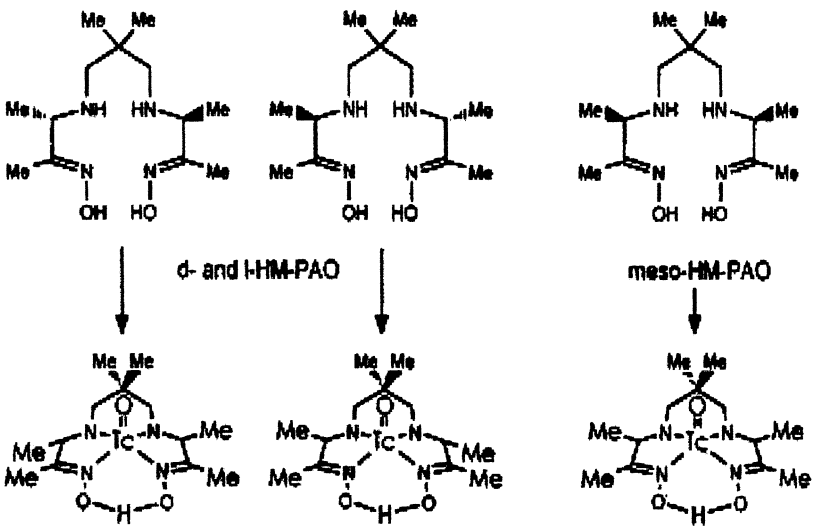

Fig. 1. The structures of the stereoisomers of HM-PAO and its technetium complexes.

degaser and heater). UV-Visible variable wavelength detector is HP 1100 Series. Separation is performed on analytical column LiChroCart Superspher 100-RP-18 (125 mm × 4 mm ID), particle size 5 μm (Merck, Darmstadt, Germany).

2.2. Reagents and solutions

N,N-dimethyl-*l*-phenylalanine (*l*-DM-PhA) 99%, copper(II) sulphate pentahydrate 99%, triethylamine (TEA) 99.5% were from Aldrich (Milwaukee, WI). Acetonitrile and methanol HPLC-grade (Merck, Darmstadt, Germany) were used. Acetic acid, sodium carbonate, L(+)-tartaric acid and diethyl ether were all analytical-reagent grade from Merck. Water was purified to HPLC quality with a Millipore-Q RG Ultra Pure Water System (Millipore, Milford, USA).

HM-PAO was synthesized in our Laboratory: ^{1}H NMR (d_6-DMSO) δ 10.3 (2H. s. OH), 3.3 (2H. s. NH), 3.12 (2H. q. CH), 2.12 (4H. q. CH_2), 1.64 (6H. s. CH_3), 1.07 (6H. d. CH_3), 0.78 (6H. s. CH_3). *Meso*-HM-PAO was separated by repeated recrystallization from hot acetonitrile, while *l*-HM-PAO was obtained via precipitation of *l*-HM-PAO L(+)-tartrate by treatment with an aqueous solution of the salt with an excess of solid sodium carbonate, followed by extraction of free base into diethyl ether. For *l*-HM-PAO specific rotation was $[\alpha]_D^{25} = -2.49^\circ$, for concentration 4 g (100 ml)$^{-1}$ in methanol. After filtration of *l*-HM-PAO L(+)-tartrate, *d*-HM-PAO and *meso*-HM-PAO remain in the supernatant.

The mobile phase was prepared by dissolving copper(II) sulphate pentahydrate (0.0867 g) and *l*-DM-PhA (0.2705 g) in 10 and 20 ml of HPLC-grade water, respectively. The solutions were mixed and allow to stand 30 min, then diluted with water up to 500 ml, giving the concentrations of Cu(II) and *l*-DM-PhA of 0.7 and 2.8 mM, respectively. Next the pH was adjusted to 4.1–4.2 with concentrated acetic acid and triethylamine (maximum 0.8 mM). This pH adjustment has been carried out by adding triethylamine and concentrated acetic acid, one after another, in small aliquots (5 or 10 μl) to obtain final triethylamine concentration to prevent precipitation of Cu(II). The pH value of the mobile phase should not exceed 4.3, since Cu(II) is precipitated. The eluent was allowed to equilibrate for 24 h before use.

Standards (*meso*- and *l*-HM-PAO) as well as the samples of isomeric mixtures, were dissolved in the eluent to provide a concentrations of about 1 μmol ml^{-1} (0.28 mg ml^{-1}), and filtered through a 0.45 μm Millipore filter membrane.

2.3. Chromatographic conditions

The analytical column was presaturated with the initial complex [Cu(*l*-DM-PhA)$_2$] in the eluent for at least 4 h (flow rate 0.6 ml min^{-1}) and stored during 24 h before use. Thereafter, the column was conditioned by flushing it with the eluent for ca. 60 min. The injection volume was 5 μl. The UV detector was set at 225 nm, the flow rate of the eluent was 0.6 ml min^{-1} and the column temperature was 30 °C.

3. Results and discussion

The analyzed diaminedioxime HM-PAO has two chiral centers C-3 and C-9, and exists in two diastereoisomeric forms: a *d,l*-racemate mixture and *meso*-HM-PAO. Separation of HM-PAO isomers, by reversed-phase liquid chromatography has been accomplished by using achiral stationary phase and chiral mobile phase (CMP) containing copper(II) complex of optically active *l*-DM-PhA as initial complex for CLEC. During the column preparation, by passing the mobile phase, presaturation of the achiral stationary phase with initial complex Cu(*l*-DM-PhA)$_2$,was achieved. It is reasonable to assume that the diastereo-enantio separation of HM-PAO occurs on the column due to presence of immobilized binary complex Cu(*l*-DM-PhA)$_2$ and formation of ternary mixed complex, since diaminodioximes [10] similar to HM-PAO form complexes with copper(II).

The preliminary investigations were carried out using RP-18 column (250 mm × 4 mm ID, particle size 7 μm). The chromatogram for HM-PAO isomeric mixture, presented in Fig. 2, obtained with mobile phase which is prepared with mole ratio Cu(II): *l*-DM-PhA = 1:2 and without TEA, shows insufficient resolution ($R_s = 0.38$). Only two

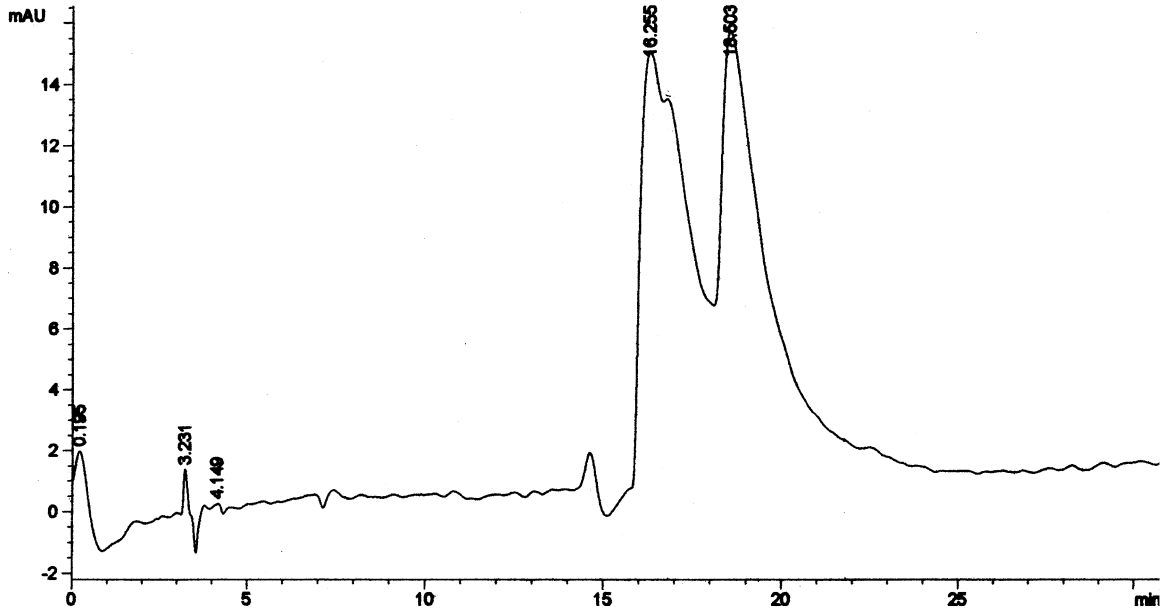

Fig. 2. The chromatogram of *meso-*/*d,l*-mixture of HM-PAO. Mobile phase with mole ratio Cu(II):*l*-DM-PhA = 1:2. Column RP-18 (250 mm × 4 mm ID).

broad peaks (later defined as *l*- and *d*-HM-PAO) were obtained (Table 1). There was only a slight indication of a third peak (a shoulder on descending part of the first peak, which was later defined as *meso*-HM-PAO peak). For all further experiments analytical column LiChroCart Superspher 100-RP-18 (125 mm × 4 mm, ID, particle size 5 μm) was used.

Table 1
Retention (k), separation (α) and resolution (R_s) for HM-PAO isomers with different mobile phase composition

Mobile phase composition	0.7 mM $CuSO_4 \cdot 5H_2O$ 2.8 mM *l*-DM-PhA			
Isomer	k	Isomers	α	R_s
l	5.41	*d*/*l*	1.89	3.91
$meso_{EE}$	6.19	*d*/$meso_{EE}$	1.65	3.13
$meso_{EZ}$	8.39	*d*/$meso_{EZ}$	1.22	2.14
d	10.24	$meso_{EZ}$/*l*	1.55	3.68
		$meso_{EZ}$/$meso_{EE}$	1.36	2.53
		$meso_{EE}$/*l*	1.14	0.83
Mobile phase composition	1.0 mM $CuSO_4 \cdot 5H_2O$ 2.0 mM *l*-DM-PhA without TEA			
Isomer	k	Isomers	α	R_s
l	4.03	*d*/*l*	1.17	0.38
$meso_{EE}$	4.21	*d*/$meso_{EE}$	1.12	–
$meso_{EZ}$	–	*d*/$meso_{EZ}$	–	–
d	4.73	$meso_{EZ}$/*l*	–	–
		$meso_{EZ}$/$meso_{EE}$	–	–
		$meso_{EE}$/*l*	1.05	–

Increasing the *l*-DM-PhA concentration in the eluent at the constant Cu(II) concentration, will shift the equilibrium of initial complex formation resulting in a higher concentration of Cu(*l*-DM-PhA)$_2$ complex. This implies that more interaction sites become available for the HM-PAO isomers to form ternary mixed-ligand complexes. The achieved separation for the same isomeric mixture, with mobile phase containing the initial complex in the mole ratio Cu(II):*l*-DM-PhA = 1:4 with the addition of TEA, is reported at Fig. 3, and gave three well defined peaks. The peak symmetry is improved by the addition of TEA [11] to the eluent, and consequently the enantiomeric resolution increases. Some of the experiments were performed with the addition of ACN or methanol to mobile phase containing initial complex Cu(*l*-DM-PhA)$_2$. The results showed that the addition of organic modifier ACN or methanol, even at low content (2.5% *v*/*v*), partly changed the interaction mechanism since the immobilized initial complex is removed from the surface, resolution decreased and lower reproducibility of the retention times was observed. In all other separations mobile phase without organic modifier was used.

To establish the order of elution the chromatogram of *l*-HM-PAO, prepared via *l*-HM-PAO L(+)-tartrate, is shown in Fig. 4a, while the chromatogram with two main peaks at Fig. 4b is obtained for supernatant in which *d*-HM-PAO and *meso*-HM-PAO remain. The first small peak (Fig. 4b, peak 1) corresponds to the residue of *l*-HM-PAO in supernatant. To define the peak that corresponds to *meso*-HM-PAO, a chromatogram was recorded of *meso*-HM-PAO, obtained by repeated recrystallization, for two samples with melting points 142 (Fig. 5a) and 145°C (Fig. 5b). For *meso*-HM-PAO two peaks are obtained, due to the isomerism of *cis–trans* type at oxime groups, defined as *meso*$_{EE}$ and *meso*$_{EZ}$ isomers, and corresponding peaks are the peak 2 and peak 3, respectively, as shown in Figs. 3 and 4b. The first peak (peak 2) corresponds to *meso*$_{EE}$ isomer since this isomer is thermodynamically preferred. Two *meso* species with determined melting points of 140 and 145°C, using differential scanning calorimetry, have been previously reported [12], as well as the characterization of crystalline and amorphous HM-PAO fractions. In Fig. 5a, two small peaks (peak 1 and peak 4) correspond to

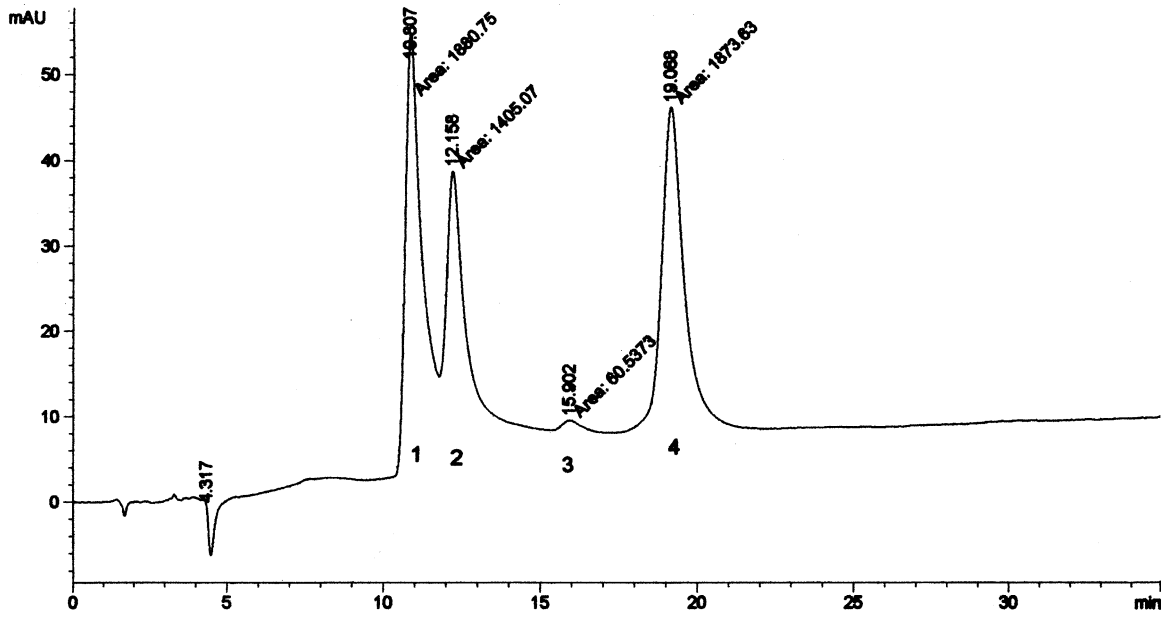

Fig. 3. The chromatogram of *meso*-/*d*,*l*-mixture of HM-PAO. Mobile phase with mole ratio Cu(II):*l*-DM-PhA = 1:4. Column LiChroCart Superspher 100-RP-18 (125 mm × 4 mm ID).

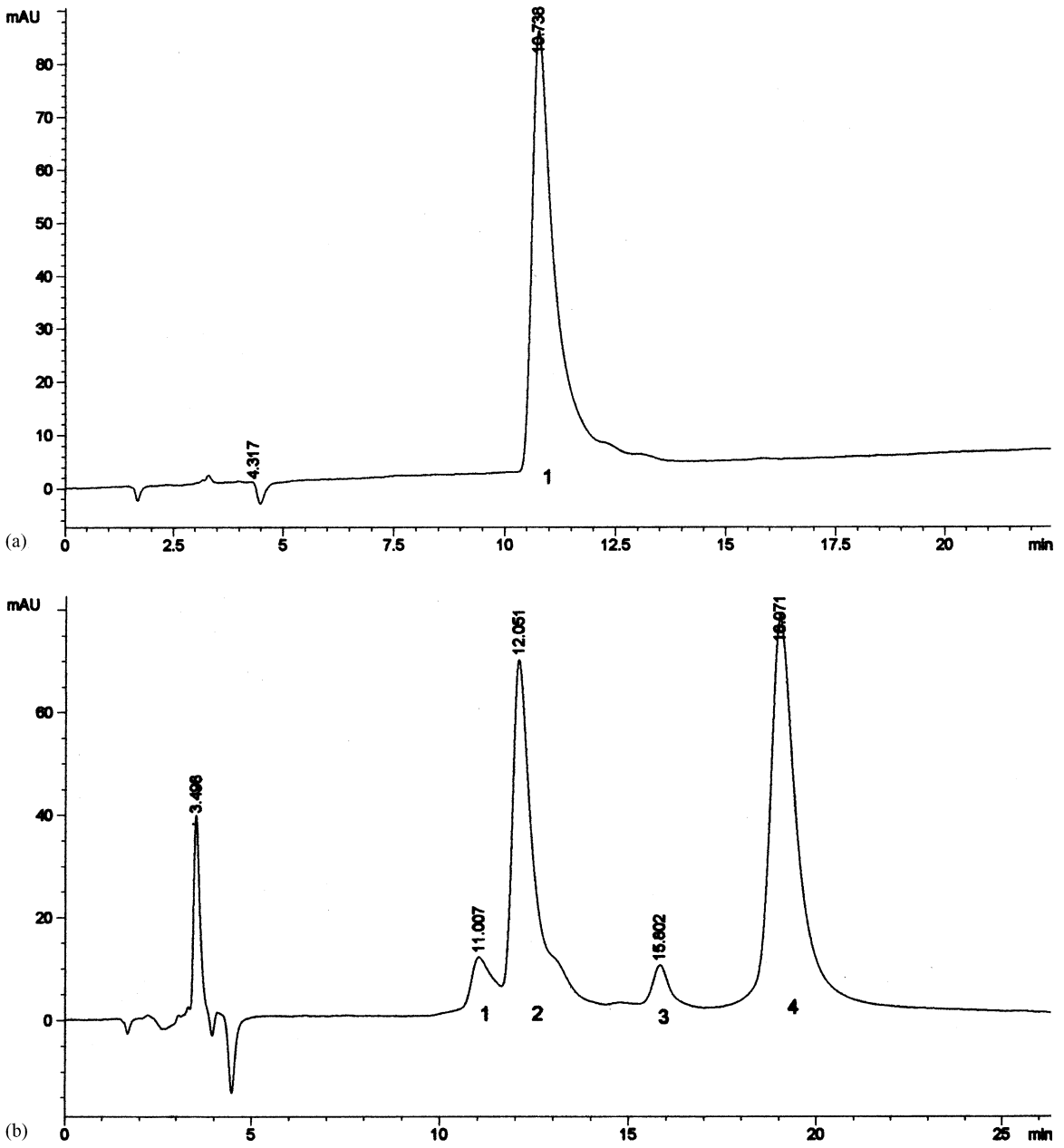

Fig. 4. (a) The chromatogram of *l*-HM-PAO. (b) The supernatant containing *meso*- and *d*-HM-PAO. Mobile phase and column are the same as for Fig. 3.

residue of *l*-HM-PAO and *d*-HM-PAO, respectively.

On the basis of peak's areas presented in Fig. 3, the percentage content of the isomeric mixture is: 35.6% *l*-, 26.9% $meso_{EE}$-, 1.6% $meso_{EZ}$-, and 35.7% *d*-HM-PAO. For the mixture of isomers, after repeated injections ($n = 6$), the values of RSD for peak's areas are: for *l*- (1.9%), for $meso_{EE}$-

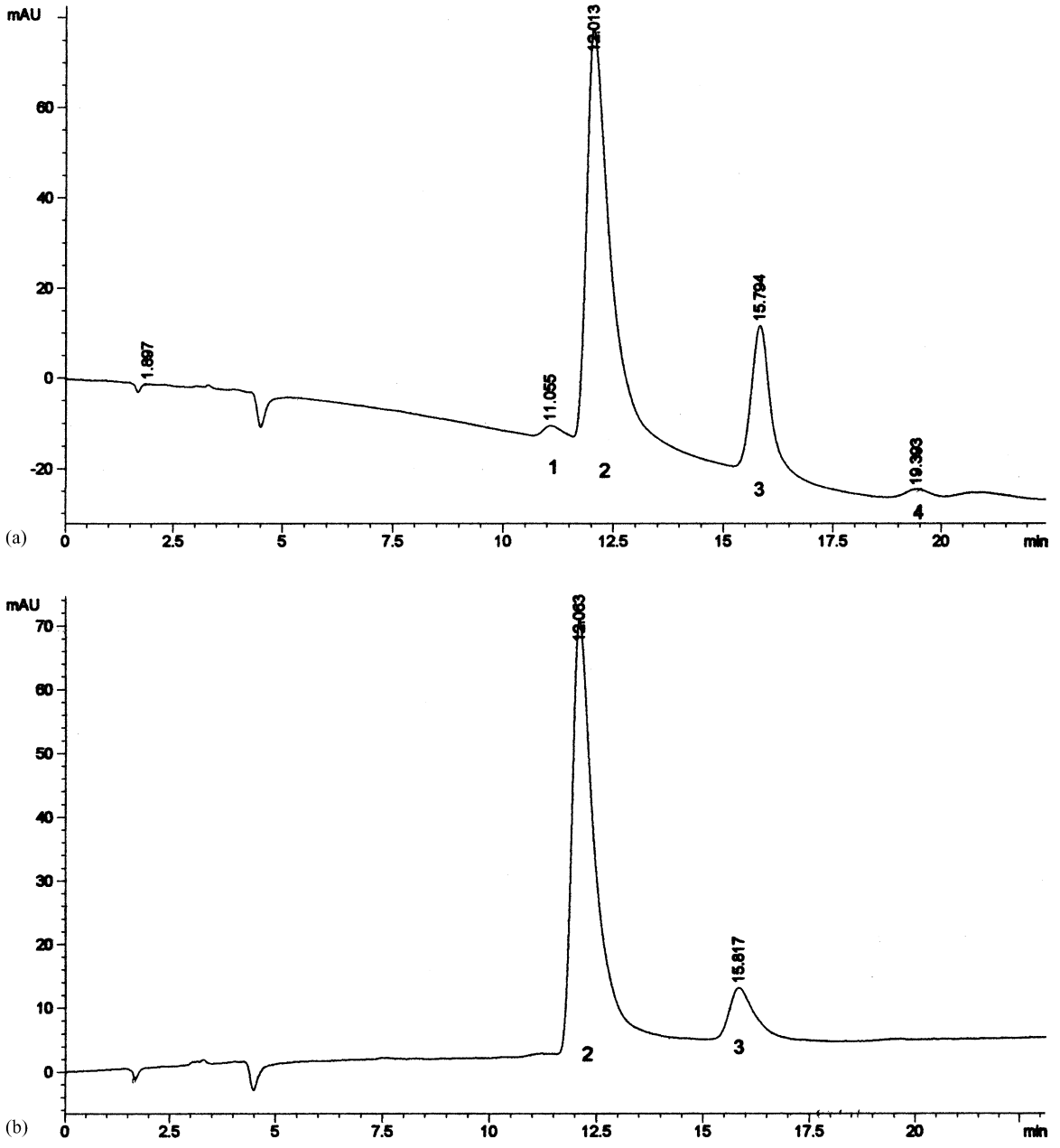

Fig. 5. (a) The chromatogram of *meso*-HM-PAO (m.p. 142 °C). (b) *meso*-HM-PAO (m.p. 145 °C). Mobile phase and column same as for Fig. 3.

(2.0%), for $meso_{EZ}$- (3.5%) and for *d*-HM-PAO (1.8%). The chromatogram shown in Fig. 4b, for supernatant gave the percentage content of: 4.1% residue of *l*-HM-PAO, 36.2% $meso_{EE}$-, 3.1% $meso_{EZ}$- and 56.6% *d*-HM-PAO. For the sample of *meso*-HM-PAO, with melting point 142°C, the residues of *l*- and *d*-HM-PAO are of 1.9 and 2%, respectively, while the contents of $meso_{EE}$- and

meso$_{EZ}$- HM-PAO are 74.6 and 21.5%, respectively. After the recrystallization of this sample, melting point is 145°C, and the content of *meso*$_{EE}$- is higher (87.5%) and *meso*$_{EZ}$-HM-PAO is lower (12.5%). For this sample, after repeated injections ($n = 6$) the RSD values were 0.67% for *meso*$_{EE}$- and 2.3% for *meso*$_{EZ}$-HM-PAO.

According to retentions times denoted in Fig. 3, the effects of mobile phase composition on k, α and R_S for isomers are summarized in Table 1. The mobile phase containing initial complex with the mole ratio Cu(II):*l*-DM-PhA = 1:4, provides the complete resolution between all isomers (R_S from 2.14 to 3.91) except for *meso*$_{EE}$- and *l*-HM-PAO ($R_S = 0.83$).

4. Conclusion

Reversed-phase chromatography, based on ligand exchange, with chiral mobile phase consisting of copper(II) and chiral amino acid *l*-DM-PhA is suitable for the separation and analysis of HM-PAO isomers. The proposed method could be successfully used for HM-PAO isomeric purity analysis, as the alternative to resolution with chiral columns. Nowotnik et al. [7] were examined the diastereo-enantio separation of HM-PAO and corresponding Tc-99m complexes, but using the three different chiral columns: Resolvosil BSA-7, Chiracel OD and Chiralpak AD. The best results for separation of *meso-, d-* and *l*-HM-PAO were obtained with Chiralpak AD, but the values of R_S (*d*/*l* 2.9 and *meso*/*d* 1.9) were significantly lower comparing to our results for corresponding values of R_S (*d*/*l* 3.91 and *d*/*meso* 3.13) with opposite elution order of *meso-* and *d*-HM-PAO. The CLEC method used in our experiments enable resolution even the geometric isomers *meso*$_{EE}$- and *meso*$_{EZ}$-HM-PAO. Also, this method can be suitable for enantioseparation of other aliphatic compounds with two function groups, for which the resolution via inclusion by β-cyclodextin is not efficient, since our investigations confirm no resolution for HM-PAO isomers.

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