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Chiral ligand-exchange chromatography as the screening method for proposed modifications in exametazime synthesis to enhance diastereoselectivity

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Abstract

^{99m}Tc(V)-*d*,*l*-HM-PAO complex is well-known radiopharmaceutical for regional cerebral blood flow imaging. The proposed modifications in exametazime, hexamethylpropyleneamine oxime (HM-PAO) (4,8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bisoxime) synthesis, for reduction of intermediary reactant diiminebisoxime (DI) (4,8-diaza-3,6,6,9-tetramethylundecane-3,8-diene-2,10-dione bisoxime) concerned two reductants ($NaBH_4$ and KBH_4), two solvents (ethanol and 2-propanol), and three mole ratios of reactant/reductants (1:1, 1:1.5, and 1:2). The simultaneous analysis of diastereo–enantiomeric HM-PAO content, as well as the content of starting DI, in different reduction mixtures were performed using chiral ligand-exchange chromatography (CLEC). The separation of the samples of investigated reduction mixtures, obtained in the second step of HM-PAO synthesis, has been accomplished by using an achiral sorbent (RP-18) and a chiral mobile phase (CMP) containing copper(II) complex with *N*,*N*-dimethyl-L-phenylalanine (L-DM-PhA) as initial complex for CLEC. With 12 different reduction conditions, the obtained ratios of diastereoisomers *d*,*l*-HM-PAO*: meso*-HM-PAO varied from 69.2:30.8 to 15.9:84.1, in comparison to the reduction in routine synthesis of HM-PAO which gives an equal mixture of diastereoisomers. The ternary mixed complexes formation recorded spectrophotometrically on addition of HM-PAO or DI to the mobile phase with binary complex Cu(L-DM-PhA)$_2$, due to the evidence of bathochromic shift of 46 nm for λ_{max} with significant difference in absorptivity contributes to separation mechanism.

Keywords: Exametazime (HM-PAO) synthesis; Diastereoselective reduction; Chiral ligand-exchange chromatography (CLEC); Diastereo–enantio separation

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1. Introduction

This work concerns clinically important compound hexamethylpropyleneamine oxime (HM-PAO) (4, 8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bis-oxime). The complex ^{99m}Tc(V)-*d*,*l*-HM-PAO has been developed as brain-perfusion imaging agent, which is used on single-photon emission computerized tomography (SPECT) as Ceretec^{TM} [1,2]. Also, the investigation of brain tumors with ^{99m}Tc(V)-*d*,*l*-HM-PAO SPECT has been reported [3]. This complex has also been used extensively for in vitro ^{99m}Tc labeling of leukocytes and platelets [4,5] on re-injection and these labeled cells are used for imaging sites of infection and thrombi, respectively. Rocco et al. [6] used *d*,*l*-HM-PAO in cerebral extraction studies.

Up to date in HM-PAO routine synthesis [7–9] an equal mixture of the *meso*- and *d*,*l*-diastereoisomers is obtained and the yield of *d*,*l*-HM-PAO after repeated recrystallization is significantly low.

In this work, 12 reduction conditions are proposed, concerning different reductants ($NaBH_4$ and KBH_4), two solvents (ethanol and 2-propanol), and three mole ratios of reactant/reductants (1:1, 1:1.5, and 1:2) for the reduction of diiminebisoxime (DI) (4,8-diaza-3,6,6,9-tetramethylundecane-3,8-diene-2,10-dione bisoxime) which is also synthesized in our laboratory according to the first part of routine HM-PAO synthesis [8]. The goal of this work was to change reduction conditions in HM-PAO synthesis to enhance diastereoselectivity.

The analysis of HM-PAO diastereo–enantiomeric content in different reduction mixtures was carried out according to our previously published results [10], using a chiral eluent, containing the initial complex of Cu(II) with chiral selector *N*,*N*-dimethyl-L-phenylalanine (L-DM-PhA), and RP-18 column with the immobilized binary complex Cu(L-DM-PhA)$_2$ for ligand-exchange chromatography. Chiral ligand-exchange chromatography (CLEC) has been invented by Davankov et al. [11], and recently the review on CLEC has been published by Kurganov [12]. 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. The objective of this work is to establish the possibility of simultaneous monitoring the ratio of HM-PAO distereoisomers, obtained under different proposed reduction conditions, in the presence of starting reactant diiminebisoxime, by means of the recently published CLEC method which has been developed for HM-PAO isomeric purity determination [10].

2. Experimental

2.1. Instrumentation

All chromatographic analysis were carried out on HPLC system and with the same analytical column as used previously [10].

UV spectra were recorded using Uvicon 810/820 (Kontron, Switzerland) UV-Vis spectrophotometer, with 10 mm quartz cells. NMR Spectra were obtained using Varian Gemini 200 (^{1}H at 200 MHz; DMSO-d$_6$) with $SiMe_4$ as internal standard; δ in parts per million.

A standard pH-meter (Beckman) with a combined pH electrode pHC3001 (Radiometer) was calibrated with standard buffer solutions: hydrogen phthalate, pH 4.01 ± 0.02 (Radiometer S 1316), hydrogen phosphate, pH 7.00 ± 0.02 (Radiometer S 1326), and glycine/hydrochloric acid, pH 1.00 ± 0.02 (Merck).

2.2. Reagents and solutions

The reagents for CLEC method were quoted previously [10]. Water was purified to HPLC quality with a Millipore-Q RG Ultra Pure Water System (Millipore, Milford, USA).

In HM-PAO synthesis 2,3-butanedione monoxime, 2,2-dimethyl-1,3-propanediamine, sodium carbonate, acetonitrile (99%), ethanol (95%), benzene (98%), KBH_4, and $NaBH_4$ all p.a. (Merck) were used. The anhydrous solvents benzene, ethanol, and 2-propanol were prepared.

4,8-Diaza-3,6,6,9-tetramethylundecane-3,8-diene-2, 10-dione bisoxime was synthesized according to the first part of the routine synthesis [8], and starting ketooxime removed completely by a single recrystallization from benzene, giving a product for analysis (m.p., 134 °C; ^{1}H NMR (DMSO-d$_6$) δ 3.2 (4H s CH_2N), 1.9 (12H s CMe), and 1.0 (6H s CMe_2)).

Hexamethylpropyleneamine oxime synthesized in the Laboratory of Radioisotopes, Institute of Nuclear Sciences, Vinca, according to the Canning et al. [8]

method. The chemical characterization, as well as purification of *meso*-HM-PAO and isolation and purification of *l*-HM-PAO were reported previously [10].

The procedure for mobile phase preparation has been described previously [10], consisting of Cu(II) and L-DM-PhA concentrations 0.7 and 2.8 mM, respectively, with pH adjusted to 4.1–4.2 (triethylamine maximum 0.8 mM).

The purified DI, obtained from the first part of HM-PAO synthesis, as well as the standards (*meso*- and *l*-HM-PAO), were dissolved in the eluent, to provide the concentrations of 1.12 μmol ml^{-1} (0.3 mg ml^{-1}) and 1 μmol ml^{-1} (0.28 mg ml^{-1}), respectively and filtered through a 0.45 μm Millipore filter membrane for HPLC analysis.

2.3. Chromatographic conditions

The procedure for an analytical column (RP-18) presaturation with the initial complex [Cu(L-DM-PhA)$_2$] was reported previously [10]. 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. Aliquots (50 μl) of the reaction mixtures from the 12 reductions, were diluted with 5 ml of the mobile phase and analyzed.

2.4. UV spectrophotometry

For UV spectrophotometry analysis, the samples of pure DI and HM-PAO were dissolved in ethanol to provide final concentrations of 1.49×10^{-5} and 5×10^{-5} M, respectively. The samples of reduction mixtures, with estimated HM-PAO concentration of about 5×10^{-5} M, were diluted with ethanol.

The absorption spectra of DI and HM-PAO solutions in mobile phase, at the concentrations 1.9×10^{-4} and 2.0×10^{-4} M, respectively, were also recorded.

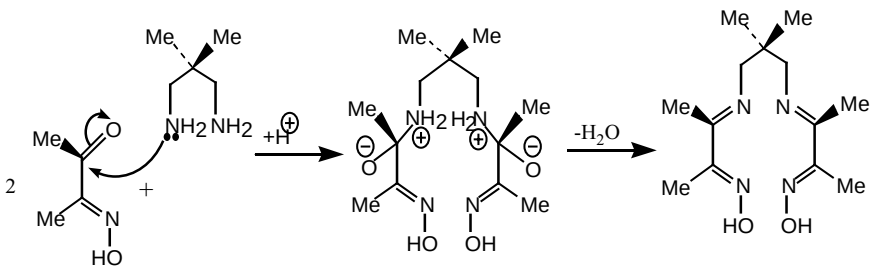
2.5. Proposed modifications for the reduction procedure in HM-PAO synthesis: reductions of 4,8-diaza-3,6,6,9-tetramethylundecane-3,8-diene-2,10-dione bisoxime with sodium boron hydride and potassium boron hydride in anhydrous ethanol and 2-propanol

1. *Reduction no.*1 (mole ratio; reactant/reductant, 1:1)
   - *Conditions a*. Anhydrous ethanol, $NaBH_4$.

     General procedure: The diiminebisoxime (5 mmol) was slurred in anhydrous ethanol (24.6 ml) at 0 °C. Sodium boron hydride (5 mmol) was added in portions over 30 min, and the mixture stirred at 0 °C for further 4 h. Water (4.8 ml) was added and the mixture stirred at 0 °C for 2 h. Then 50 μl aliquots for HPLC analysis were taken from reaction mixture. The further steps of synthesis were the same as proposed by Canning et al. [8]. Double recrystallization of crude HM-PAO product from hot acetonitrile provided the mixture of HM-PAO diastereoisomers free from major impurities.
   - *Conditions b.* Anhydrous ethanol, KBH_4, all other conditions are same as in general procedure.
   - *Conditions c.* Anhydrous 2-propanol, $NaBH_4$, all other conditions are same as in general procedure.
   - *Conditions d.* Anhydrous 2-propanol, KBH_4; after addition of potassium boron hydride at 0 °C, mixture was stirred at 20 °C for 4 h, and after addition of first portion of water mixture was stirred also at 20 °C for further 2 h.
2. *Reduction no.* 2 (mole ratio; reactant/reductant, 1:1.5)
   - *Conditions a*, *b*, *c*, and *d* are the same as the conditions in the procedures for reduction no. 1, respectively.
3. *Reduction no.* 3 (mole ratio; reactant/reductant, 1:2)
   - *Conditions a*, *b*, *c*, and *d* are the same as the conditions in the procedures for reduction no. 1, respectively.

3. Results and discussion

Routine synthesis [7,8] of the ligand HM-PAO is a two-step process, which first step is shown in Scheme 1. Condensation of 2,2-dimethyl-1,3-propanediamine with 2 eq. of 2,3-butanedione monoxime in anhydrous benzene, with catalytic amount of acetic acid, provided 4,8-diaza-3,6,6,9-tetramethylundecane-3,8-diene-2,10-dione bisoxime (yield ∼60%). In this addition–elimination reaction, fast nucleophilic attack to C=O double bond is followed by slow elimination of water.



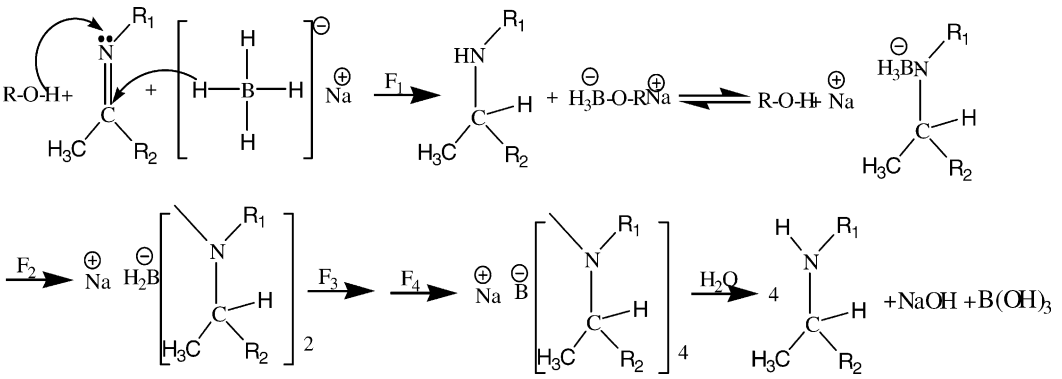
Scheme 1. Diiminebisoxime (DI) obtained from the first step in HM-PAO synthesis.

The second step in routine HM-PAO synthesis is the reduction of the two imine groups of DI with $NaBH_4$ in anhydrous ethanol (*T*: 0 °C and yield: 70–80%) provides HM-PAO as an equal mixture of the *meso*- and *d*,*l*-diastereoisomers. The reduction of DI is the step which determinate diastereisomeric ratio in HM-PAO synthesis. The mechanism of the reduction of imine group with $NaBH_4$ in protic solvent is shown in Scheme 2.

This work concerns the comparison between contents of HM-PAO diastereoisomers in the reaction mixtures obtained by 12 different reduction conditions. The reduction no. 1 is the routine synthesis [7,8], but with two differences proposed in this paper, instead of the 95% ethanol, anhydrous ethanol was used and also, the time of the reduction is prolonged from 2 to 4 h. All other reduction conditions are new proposed. With two reductants ($NaBH_4$ and KBH_4), two solvents (ethanol and 2-propanol), and three mole ratio of reactant/reductant (1:1, 1:1.5, and 1:2), the reduction of DI have been accomplished.

The chemical purity of samples, obtained from reaction mixtures, was checked by UV spectrophotometry in comparison to the spectra of pure HM-PAO (*d*,*l* or *meso*) and diiminebisoxime dissolved in ethanol to provide final concentrations of 5×10^{-5} and 1.49×10^{-5} M, respectively. The samples of reaction mixtures, with estimated HM-PAO concentration of about 5×10^{-5} M, were diluted with ethanol. The absorption spectra of pure HM-PAO with λ_{max} at 203 nm and DI with absorption maximum which is bathochromically shifted at 222 nm are presented in Fig. 1, using ethanol as a blank.

Absorption spectra of the samples of the reaction mixtures from reductions no. 1a and b are very similar (almost the same spectra of the samples from reductions no. 2a and b, as to those of reductions no. 1) indicating that these samples have almost none, or minimal content of starting reactant (DI). The replacement of ethanol with 2-propanol in reduction no. 1c, gave the considerably higher content of DI in comparison to reductions no. 1a and b, but significantly lower



Scheme 2. Reduction of imine group with $NaBH_4$.

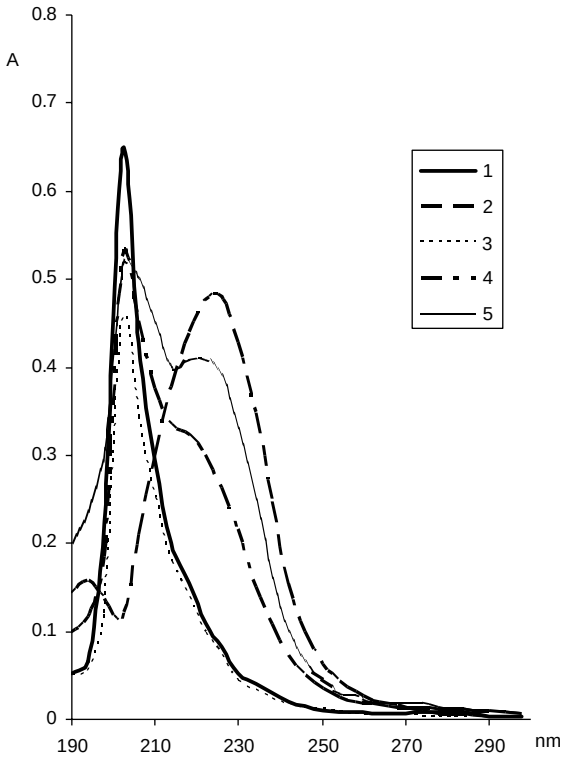


Fig. 1. Absorption spectra of HM-PAO (curve 1, 5×10^{-5} M), DI (curve 2, 1.49×10^{-5} M), and the samples of reaction mixtures from reductions 1a (curve 3), 1c (curve 4), and 1d (curve 5).

than in reduction no. 1d, since only the shoulder in spectrum could be observed. The absorption spectra of the reaction mixture from reduction no. 1d showed the highest content of starting DI (Fig. 1).

The ratio of the diastereoisomers was defined by CLEC method under the previously proposed conditions [10]. The chromatogram of DI (1.12 μmol ml^{-1}) is presented in Fig. 2. and confirmed that the same conditions for CLEC method proposed for diastereo–enantio separation of HM-PAO isomers could be used for screening starting reactant DI in HM-PAO synthesis. The retention time obtained for DI is between those of *meso*$_{EE}$- and *d*-HM-PAO but very close to that of *meso*$_{EZ}$-HM-PAO. As it has been reported previously [10], the content of *meso*$_{EZ}$-HM-PAO in isomeric mixture is very low (1.6%) and has no significant effect on ratio of percentage content of diastereoisomeres *meso*$_{EE}$-/*d*,*l*-HM-PAO.

The chromatogram of the sample from reaction mixture of reduction no. 2b, is shown in Fig. 3. In this chromatogram first peak corresponds to *l*-HM-PAO, second to *meso*-HM-PAO, third to DI, and fourth to *d*-HM-PAO with corresponding retention times of about 12.2, 13.5, 17.7, and 22.8 min, respectively. Diastereoisomeric percentage content was calculated according only to the sum of HM-PAO isomers peak's areas. This percentage content in reaction

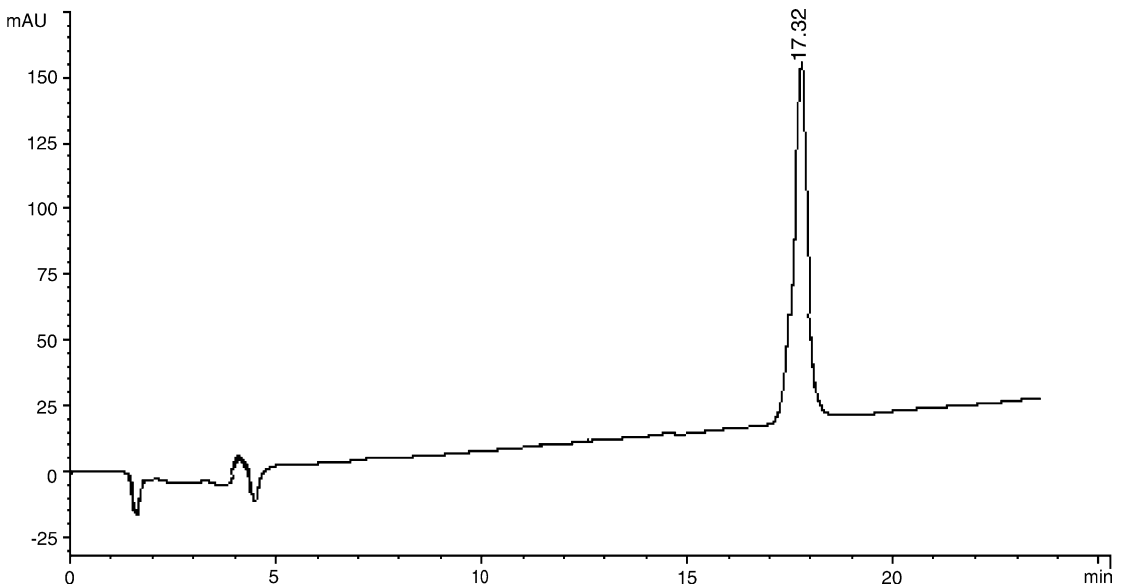


Fig. 2. The chromatogram of DI. Concentration, 1.12 μmol ml^{-1}.

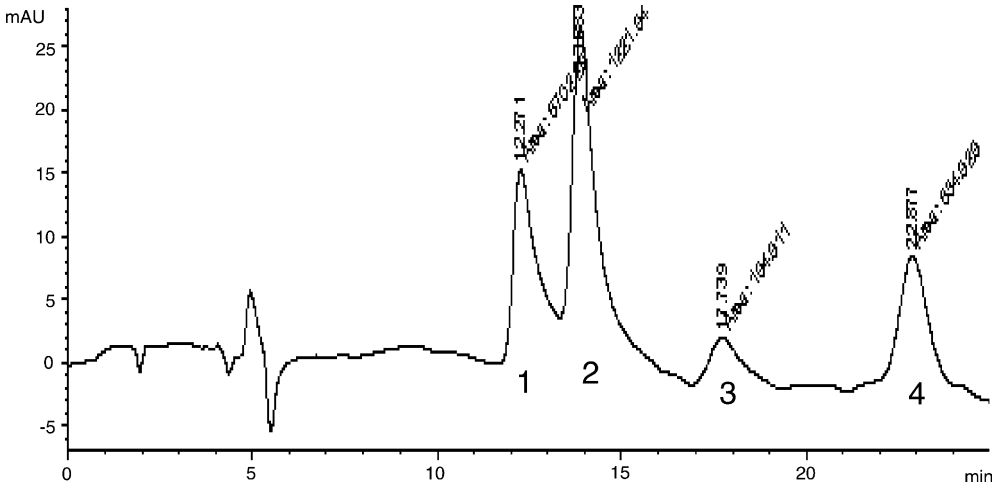


Fig. 3. The chromatogram of the sample of reaction mixture from reduction no. 2b.

mixture from reduction no. 2b for *meso*-:*d*,*l*-HM-PAO was 53.39:46.61. The chromatogram showed a little amount of the starting DI (Fig. 3, peak 3) which was in reference to the results of UV spectrophotometry. The results of CLEC method confirmed the significantly higher content of starting reactant DI (Fig. 4, peak 3) in the sample of reaction mixture of reduction no. 1d that was also in accordance with spectrophotometric analysis (Fig. 1, curve 5). Diastereoisomeric percentage content in the reaction mixture from reduction no. 1d for *meso*-:*d*,*l*-HM-PAO was 30.85:69.15.

The change of mole ratio reactant/reductant to 1:2 in reduction no. 3d resulted with the sample of reaction mixture with minimal content of starting DI (Fig. 5, peak 3). Diastereoisomeric percentage content in reaction mixture from reduction no. 3d for *meso*-:*d*,*l*-HM-PAO was 84.08:15.92.

For the chromatograms presented in Figs. 3–5, the values for separation factor for *d*-HM-PAO/DI and DI/*meso*$_{EE}$-HM-PAO varied from 1.18 to 1.33 and 1.28 to 1.32, respectively. The retention times of HM-PAO isomers, as well as that of DI were slightly different for the samples of dissimilar reaction mixtures

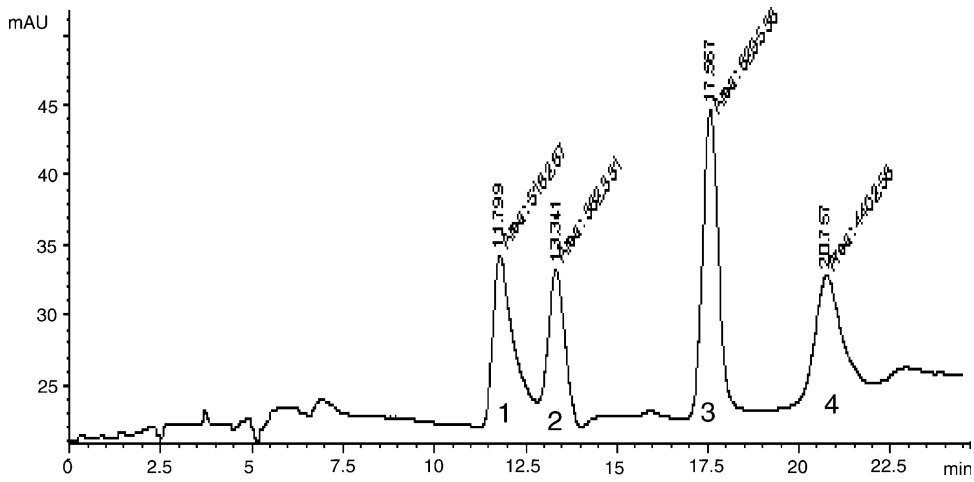


Fig. 4. The chromatogram of the sample of reaction mixture from reduction no. 1d.

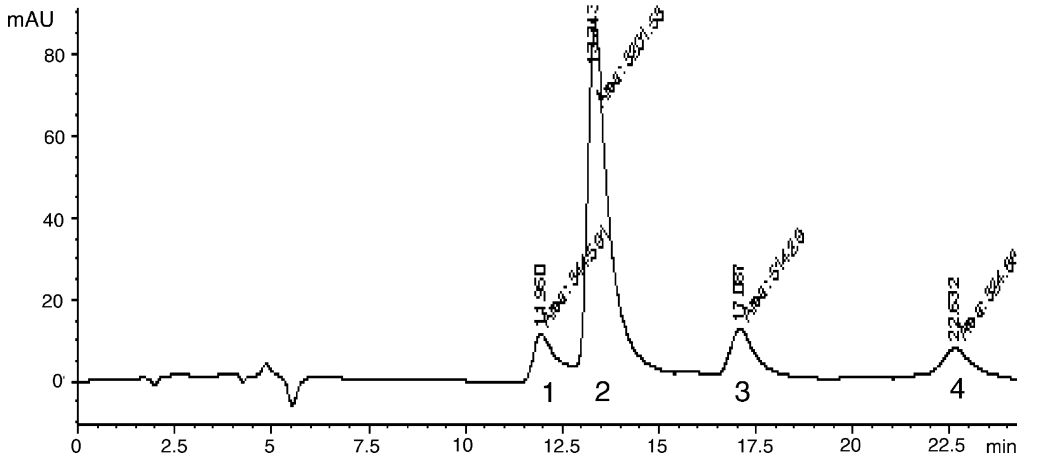


Fig. 5. The chromatogram of the sample of reaction mixture from reduction no. 3d.

since samples contained different residue of reductants and solvents and also due to presence of other impurities of the reduction process. These slightly higher values for the retention times for all HM-PAO isomers in comparison to those reported previously for purified HM-PAO isomeric mixtures [10] could be expected since crude samples from reaction mixtures, with present reactant DI, were analyzed. The most significant difference obtained for *d*-HM-PAO could be explained with elution order of DI, which was between *meso*- and *d*-HM-PAO, and since DI content in the reduction no. 1d was significantly higher than of reductions no. 2b and 3d, that could caused faster elution of *d*-HM-PAO.

The results obtained with 12 reactions conditions for reduction of 4,8-diaza-3,6,6,9-tetramethylundecane-3, 8-diene-2,10-dione bisoxime, are shown in Table 1. The attained diastereoisomeric ratio confirmed that the mild reductant KBH_4, in disparity to stronger reductant $NaBH_4$, in the same solvent (ethanol), is more favorable giving the lower percentage of *meso*-HM-PAO in comparison to that obtained with $NaBH_4$, and under such condition the slightly higher degree of diastereoselective reduction, favoring *d*,*l*-diastereoisomer was obtained but only for mole ratio 1:1. The utilization of the new solvent 2-propanol confirmed that the significantly higher degree of diastereoselective reduction was established for each reducing agent. This solvent decreases the reducing power of both reductants, as could be expected [13,14]. The stronger reducing agent ($NaBH_4$) in 2-propanol produced a higher content of *meso*-HM-PAO, while KBH_4 gave significant improvement in *d*,*l*-HMPAO yield but only for mole ratio 1:1.

Table 1
Percentage content of *meso*-HM-PAO in the reaction mixtures from reductions no. 1, 2, and 3 with the conditions a, b, c, and d

Reduction no. (mole ratio)	% *meso*-HM-PAO			
	Conditions			
	a	b	c	d
1 (1:1)	56.85	42.25	62.44	30.85
2 (1:1.5)	62.71	53.39	74.24	64.10
3 (1:2)	73.49	68.55	83.24	84.08

The presented data suggest that under the proposed conditions using KBH_4 in 2-propanol with mole ratio reactant/reductant = 1:1 (reduction no. 1d), higher yield (69.15%) of *d*,*l*-HM-PAO was obtained, thus expecting significantly higher yield of pure *d*,*l*-HM-PAO, after repeated recystallization. Reduction under the conditions, with same solvent and reductant, but with mole ratio of reactant/reductant = 1:2 (reduction no. 3d) provide higher yield (84.08%) of *meso*-HM-PAO. Almost the same percentage content of *meso*-HM-PAO (83.24%) was also obtained with stronger reductant in the same solvent and mole ratio. In the reductions, which have been carried out in the same solvent 2-propanol, with both reductants, the content of starting DI was essential, indicating slow reduction and due to that the selectivity of mild reductant was higher. According to achieved data, it could

be estimated that prolonged time for reduction no. 1d, would decrease DI content and with expected similar diastereoselectivity. In the reductions, which have been carried out in ethanol for almost all used mole ratios of reactant/reductant, as well as for both reductants, the content of *meso*-HM-PAO was always higher except for reduction no. 1b with KBH_4 for mole ratio 1:1.

Reversed-phase chromatography, based on ligand-exchange, with a chiral mobile phase (CMP), consisting of copper(II) and chiral amino acid L-DM-PhA, which was used previously for presaturation of achiral RP-18 column, is suitable as the screening method for the reduction step in HM-PAO synthesis. Separation of HM-PAO isomers and the detection of starting reactant diiminebisoxime occurs on the column due to the presence of immobilized binary complex Cu(L-DM-PhA)$_2$ and formation of ternary mixed complex of both HM-PAO and DI. The formation of these complexes was confirmed by recording the absorption spectra of HM-PAO and DI in mobile phase (Fig. 6, curves 2 and 3, respectively) in comparison to spectra of binary complex in mobile phase (Fig. 6, curve 1). The both ternary complexes have the same absorption maximum which is bathochromically shifted at 300 nm with significantly difference in absorptivity. The higher absorptivity of DI ternary complex is due to presence of two conjugated systems in diiminebisoxime.

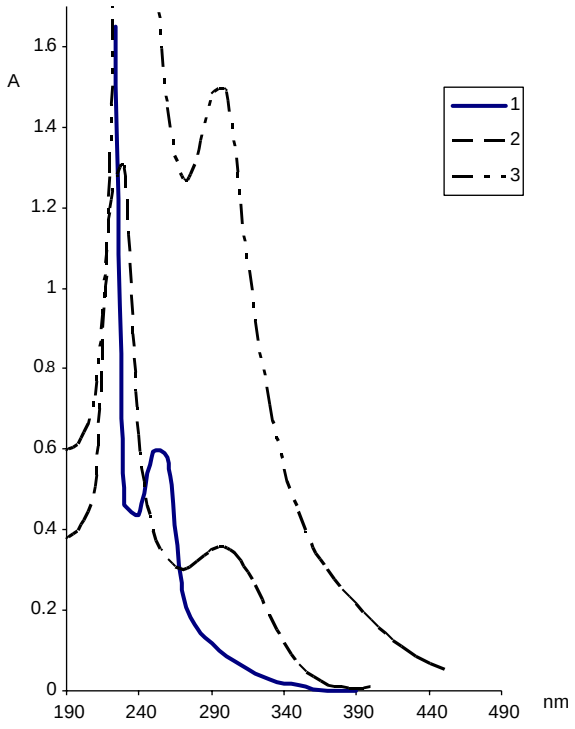


Fig. 6. Absorption spectra of: binary complex in mobile phase (curve 1), HM-PAO (2.0×10^{-4} M) in mobile phase (curve 2), and DI (1.9×10^{-4} M) in mobile phase (curve 3). Mobile phase with Cu(II) and L-DM-PhA of 0.7 and 2.8 mM, pH = 4.3, 0.8 mM TEA.

4. Conclusion

This work has demonstrated that CLEC is useful, simple, and rapid technique which can be used as the screening method for proposed modifications in HM-PAO synthesis, since the content of starting reactant diiminebisoxime as well as the ratio of HM-PAO diastereoisomers can be monitored simultaneously. The proposed conditions for reduction of diiminebisoxime, which include selection of reductant and solvent, as well as the mole ratio of reactant/reductant, could enhance diastereoselectivity for both HM-PAO diastereoisomers.

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