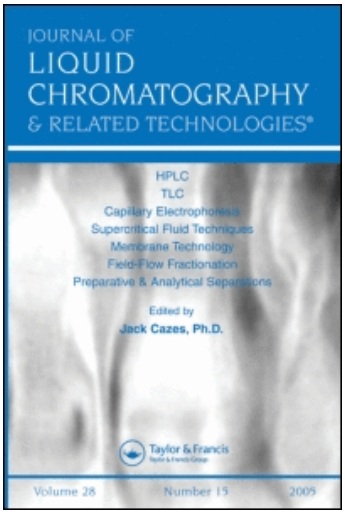

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SEPARATION OF THE STEREOISOMERS OF HEXAMETHYL-PROPYLENEAMINE OXIME (HM-PAO) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The Resolvosil BSA-7, Chiracel OD, and Chiralpak AD columns were examined to determine whether one might be suitable for the separation of enatiomers of the ligand hexamethyl-propyleneamine oxime (HM-PAO) and/or its technetium-99m complex. Using hexane/IPA as eluent, partial separation (R_S = 0.96) of the d- and l-ligand enantiomers was achieved on the OD column, but as the meso-form of the ligand was eluted midway between the d- and l-enantiomers. There was excellent separation of the technetium-99m complexes of d- and l-HM-PAO on the OD column, and partial separation (R_S = 0.90) of the meso- and d-complexes. Only partial resolution of the Tc-99m complexes of d,l- and meso-diastereoisomers was achieved on the AD column, but this column provided baseline resolution of all three stereoisomeric forms of the ligand. Thus, the OD column could be used for HPLC analysis of Tc-99m complexes of HM-PAO stereoisomers, while the AD column is suitable for analysis of the ligand stereoisomers.

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INTRODUCTION

While the influence of stereochemistry on the pharmacological action of drugs is well known [1,2], there have been relatively few examples of stereochemical effects of biodistribution properties of the technetium-99m complexes employed in diagnostic nuclear medicine [3,4]. One notable example in recent years is the technetium complex of hexamethyl-propyleneamine oxime (HM-PAO). This ligand, shown in figure 1, has two chiral centers, giving rise meso-, d-, and l-isomers. Synthesis of the ligand in a 2-step process gives an equal mixture of the meso- and d,l-diastereoisomer [5]. This mixture was labeled with technetium-99m to give, in high yield, a lipophilic Tc(V) complex with zero net charge [6], which is, presumably, a mixture of the three stereoisomers shown in figure 1. When administered intravenously to laboratory animals [7] and man [7,8], this

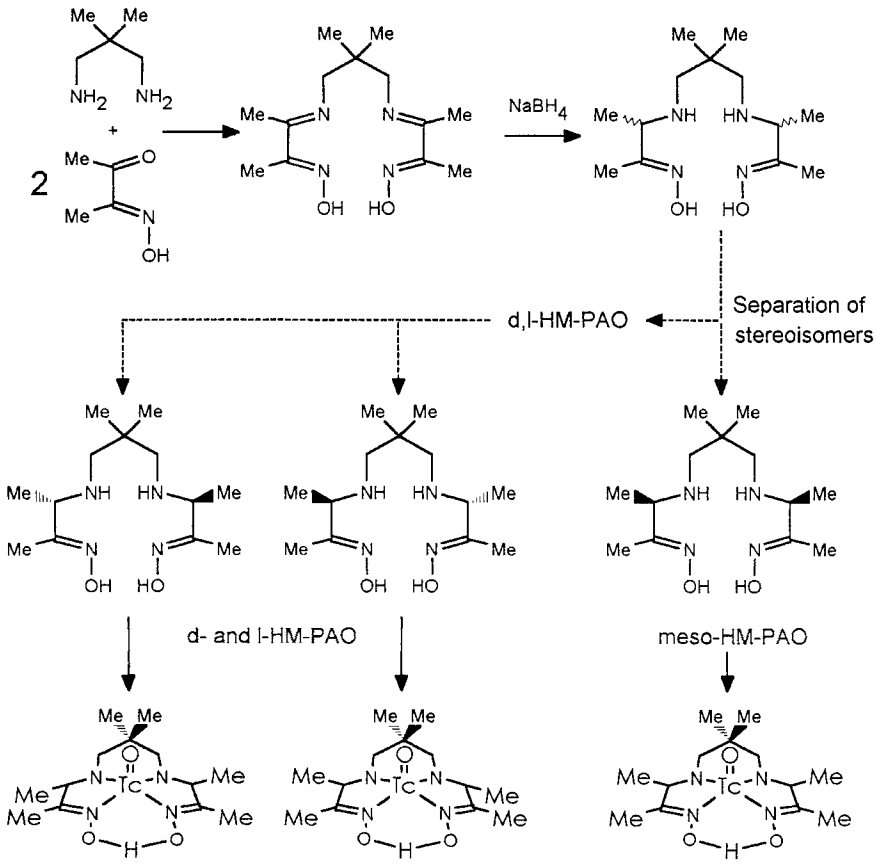

Figure 1. The synthesis of HM-PAO, and the structures of the stereoisomers of the ligand and its technetium complex

technetium complex displayed rapid uptake into the brain, with slow washout from this organ.

Separation of the ligand into the meso- and d,l-diastereoisomers was achieved by repeated recrystallization [9]. Analysis of the stereoisomeric purity of the separated diastereoisomers has been described using normal phase HPLC [5], and quantitation of the signals associated with minor differences in the ^1H nmr [10] and IR [11] spectra of meso- and d,l-HM-PAO. The technetium complexes from the two diastereoisomeric forms of the ligand displayed quite different in vivo properties in rats; the complexes had similar brain uptake, but the meso-form displayed far faster cerebral washout than the d,l-complex [12]. The Tc-99m complex of d,l-HM-PAO was also shown to possess these desirable properties in man [13], and has since become a the basis of a commercially-available pharmaceutical product; Ceretec™. The d,l-diastereoisomer complex has also been used extensively for *in vitro* Tc-99m labeling of leukocytes and platelets [14]; on re-injection, these labeled cells are used for imaging of sites of infection and thrombi, respectively. Recently, evidence has been presented which indicates that the cell labeling properties of the d- and l-enantiomer complexes are not identical [15]. Similarly, the brain uptake/retention properties of these two complexes may also be different [16,17]. Hence, it is important to the interpretation of data in studies involving Tc-99m HM-PAO to know the proportion of each of the stereoisomers.

As we needed to prepare d,l-HM-PAO for use by our colleagues in cerebral extraction studies [18], we found it necessary to develop reliable methods for the determination of stereochemcial purity. We now describe some new HPLC methods for the analysis of HM-PAO.

MATERIALS AND METHODS

HPLC-grade solvents were filtered and degassed prior to use. The HM-PAO (meso- and d,l-diastereoisomer mixture) was synthesized by the method described previously [5], with minor modifications. Meso-and d,l-HM-PAO were separated by repeated crystallization from acetonitrile as described previously [5,19]. The individual d- and l-enantiomers were separated by crystallization as their (-)-tartrate and (+)-tartrate salts (respectively) directly from the meso/d,l-mixture (eliminating the need for the initial isolation of the d,l-diastereoisomer, described previously [17]) as follows:

d-HM-PAO D-(-)-tartrate: D-(-)-tartaric acid (3.3 g, 22 mmol) was dissolved in hot ethanol (40 mL) and was added to a suspension of HM-PAO (meso/d,l-mixture, 6 g, 22 mmol) in ethanol (50 mL). The resulting mixture was heated to brief boiling, insoluble material was removed by filtration and washed with ethanol (10 mL). The yellowish solution was allowed to stand at room temperature for one week. The white crystalline solid which formed was isolated by filtration and washed with ethanol (3 x 25 mL) to give 6.23 g of a mixed isomer HM-PAO tartrate salt. The melting point of this product was 167-169 °C. The product was recrystallized a total of three times from ethanol, and the purity of product was monitored by melting point and ^1H NMR (D_2O) after each recrystallization. The purified product (0.41g) was obtained as short needles, mp 168-169.5 °C (lit. 17 167.5-168 °C). ^1H NMR (D_2O) δ 1.108 [s, 6H, $C(C\underline{H}_3)_2$], 1.432 and

1.458 (s, 12H, $CHC\underline{H}_3$), 1.858 [s, 6H, $C=N(OH)C\underline{H}_3$], 3.000 (q, 4H, $C\underline{H}_2NH$), 3.945 (q, 2H, $C\underline{H}CH_3$), 4.297 (s,2H, tartaric $C\underline{H}OH$). ^{13}C NMR (D_2O) 12.02 [$C(\underline{C}H_3)_2$], 16.28 [$\underline{C}(CH_3)_2$], 22.56 ($CH\underline{C}H_3$), 33.79 [$C=N(OH)\underline{C}H_3$], 55.25 ($\underline{C}H_2NH$), 59.05 ($\underline{C}HCH_3$), 74.31 and 74.48 (tartaric $\underline{C}HOH$), 155.34 [$\underline{C}=N(OH)CH_3$], 178.78 (tartaric $\underline{C}OOH$). d-HM-PAO D-(-)-tartrate was dissolved in water to a concentration of 2.5 g/100 mL to measure its specific rotation. $[\alpha]_D^{25}$ = -26.42° (lit. 17 -27.67°).

l-HM-PAO was obtained in a similar manner from HM-PAO (meso/d,l-mixture, 3.0 g, 11 mmol) and L-(+)-tartaric acid (1.7 g, 11 mmol). After a total of four crystallizations from ethanol l-HM-PAO L-(+)-tartarate (0.36 g) was obtained, mp 170-172 °C (lit. 17173-175 °C). ^1H NMR (D_2O) δ 1.095 [s, 6H, $C(C\underline{H}_3)_2$], 1.419 and 1.445 (s, 12H, $CHC\underline{H}_3$), 1.850 [s, 6H, $C=N(OH)C\underline{H}_3$], 2.986 (q, 4H, $C\underline{H}_2NH$), 3.927 (q, 2H, $C\underline{H}CH_3$), 4.278 (s,2H, tartaric $C\underline{H}OH$). ^{13}C NMR (D_2O) 12.88 [$C(\underline{C}H_3)_2$], 17.21 [$\underline{C}(CH_3)_2$], 23.85 ($CH\underline{C}H_3$), 34.66 [$C=N(OH)\underline{C}H_3$], 56.18 ($\underline{C}H_2NH$), 59.93 ($\underline{C}HCH_3$), 75.47 (tartaric $\underline{C}HOH$), 156.38 [$\underline{C}=N(OH)CH_3$], 179.80 (tartaric $\underline{C}OOH$). l-HM-PAO D-(+)-tartrate was dissolved in water in a concentration of 2.5 g/100 mL to measure its specific rotation. $[\alpha]_D^{25}$ = 27.06° (lit. 17 28.08°).

HPLC analyses of the ligand, and the preparation of Tc-99m complexes, were conducted using the free base form of the ligand. Tartrate salts were converted to the free base form by treatment of aqueous solutions of the ligand with excess solid sodium carbonate, followed by extraction of the free base into diethyl ether and evaporation of the organic solvent.

The Tc-99m complexes of HM-PAO stereoisomers were prepared by methods similar to those described previously [5,12,17,18]. The radiochemical purities (RCPs) of the complexes were determined by HPLC analysis using a reversed-phase system [18]. For certain studies, the complexes were purified by a solid-phase extraction procedure reported previously [18,20]. By this procedure, Tc-99m HM-PAO complexes were obtained in ethanolic solution; suitable for use in the normal phase HPLC studies.

Two HPLC systems were used:

System 1. Two Rainin Rabbit HPX pumps, controlled by a personal computer operating Gilson 712 software, fitted with a Kratos UV detector set at 230 nm.

System 2. A Spectra-Physics Model SP8700 HPLC system equipped with an ISCO V^4 UV/visible detector and a radiometric detector connected to a Spectra-Physics Model SP4270 integrator/recorder.

Three analytical chiral HPLC columns, Chiracel OD and Chiralpak AD (5 μm, 150 x 4 mm, Chiral Technologies, Inc.) and Resolvosil BSA-7 (7 μm, 150 x 4 mm, Alltech Associates, Inc.) were examined for their potential to separate all three stereoisomers of the ligand and/or their complexes. In all cases, column integrity was checked regularly using the standard compounds and chromatography conditions recommended by the suppliers.

RESULTS and DISCUSSION

There are to date very few reported examples of the resolution of Tc-99m enantiomeric complexes. Verbruggen et al resolved the two enatiomeric technetium complexes of the achiral ligand termed MAG_3 by formation of a pair of diastereoisomers by esterification with a chiral alcohol [21]. Recently, the rhenium analog of the brain perfusion radiopharmaceutical Tc-99m L,L-ECD [22] was resolved from the Re complexes of other ECD stereoisomers by HPLC using a cyclodextrin-based column [23]. We have previously reported the resolution of the enantiomeric complexes of a Tc-99m PnAO-nitroimidazole complex using the Chiracel OD column [24]. This column was selected for evaluation with HM-PAO as it has proved successful in the resolution of a wide variety of drug enantiomers [25]. Tc-99m HM-PAO stereoisomers were also studied on the Chiralpak AD and Resolvosil BSA-7 columns.

Chiracel OD column

An initial study involving d,l-HM-PAO and the Chiracel OD column was conducted by Daicel Chemical Industries Ltd at our request; it was reported that the d- and l-enantiomers could be resolved using this system [26]. Our initial studies with this column were conducted at ambient temperature using hexane/IPA (97:3) as eluent. Typical chromatograms are shown in figure 2a. The d,l-mixture is resolved into two peaks, with the l-enatiomer eluting first. The meso-isomer has a retention time midway between the d- and l-isomers. The separation of d- and l-isomers deteriorated on raising the proporation of IPA in the eluent. However, separation was improved by raising the column temperature to 40 ^{o}C and by the addition of 0.1% diethylamine to IPA; as shown in figure 2b [26]. While this HPLC system does not provide baseline separation (R_S for d,l-HM-PAO = 0.96), it might be suitable for a quantitative assay of isomer purity of the d- and l-enantiomers. Following personal communication of our results to colleagues, this HPLC system has been employed for the preparative separation of d- and l-enatiomers from the d,l-mixture, resulting in the comparison of the Tc-99m enantiomer complexes in an animal model [16]. It should be noted that we found there was some variation in the separation of d,l-HM-PAO with the batch number of the column obtained from the supplier[#] .The Chiralcel OD column was also examined with respect to the separation of the Tc-99m complexes of HM-PAO. As shown in figure 3, this system provided excellent separation of the d,l-diastereoisomer complexes. However, baseline separation of Tc-99m meso-HM-PAO from Tc-99m l-HM-PAO was not achieved. These data indicate that the Chiracel OD system can be used to separate d- and l-HM-PAO (ligand and complex) from a d,l-mixture, but will not fully resolve HM-PAO (ligand and complex) as a mixture of the diastereoisomers.

We have recently shown that certain Tc-99m propyleneamine oxime (PnAO) complexes can undergo inversion of the TcO core. Thus, if the ligand is unsymmetical about the central carbon atom, Tc-complexation results in the formation of an enantiomeric pair of complexes, which can interconvert rapidly in the presense of water

[#] These studies were conducted over a five year period. It was found that the separation of the ligand enantiomers deteriorated with columns purchased after 1991. After sharing our findings with Chiral Technologies Inc, it appears that differences may well be related to changes in column manufacture.

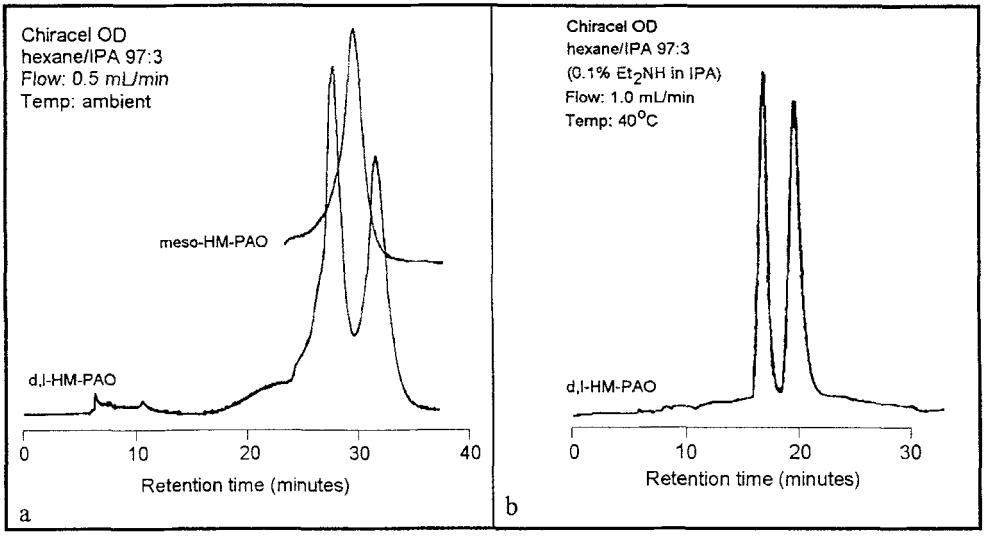

Figure 2. HPLC Chromatograms of meso- and d,l-HM-PAO (ligand) on the Chiracel OD column.

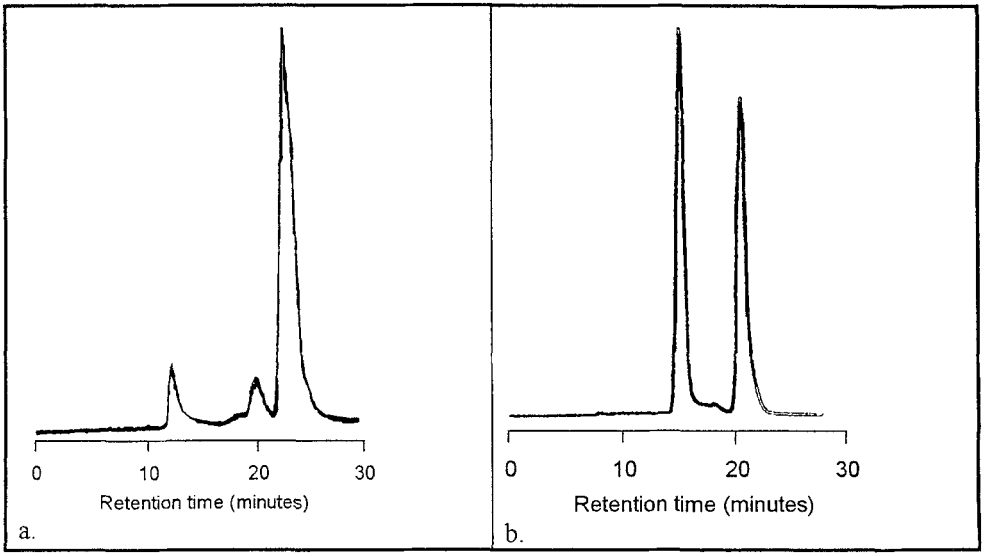

Figure 3. Separation of the Tc-99m complexes of HM-PAO on the Chiracel OD column

a. Chromatogram of the Tc-99m complexes from a meso-/d,l-mixture of HM-PAO (meso-form > 50%)

b. Chromatogram of Tc-99m d,l-HM-PAO

[Chiracel OD column hexane/IPA 85/15. Flow 1mL/min, temperature ambient]

[27]. In the case of the Tc-99m complex of d- or l-HM-PAO, a process in which the net result is inversion of the TcO core will give a product identical to the starting material. Thus, isolated fractions thought to be the complexes of either d- or l-HM-PAO should not convert to another HM-PAO complex over time unless racemization of the chiral center is involved. Water (to ~15%) was added to isolated HPLC fractions of Tc-99m d- and l-HM-PAO (from Chiracel OD HPLC (hexane/IPA 75:25) of Tc-99m d,l-HM-PAO). The solutions were allowed to stand at ambient temperature, and samples were reanalyzed over a period over several hours. While some peak broadening was observed (presumably due to the presense of water in the injectate), no additional peaks were formed.

Meso-HM-PAO can, in theory, form two technetium complexes, in which the two methyl substituents (adjacent to the nitrogen atoms) are both either syn- or anti- with respect to the Tc-oxygen atom (figure 4). The X-ray crystal structure of Tc-99 meso-HM-PAO [6] suggests that only one complex is formed (the syn-isomer), and conventional HPLC analyses of the Tc-99m complex have also indicated that only one complex is formed [28]. At all hexane/IPA solvent ratios employed in this study, Tc-99m meso-HM-PAO was observed as a single peak. Water (to ~15%) was added to an isolated HPLC fraction of Tc-99m meso-HM-PAO (from Chiracel OD HPLC (hexane/IPA 75:25)). As was the case with isolated d- and l-complexes, re-analysis showed that some peak broadening occured, but no additional peaks were formed. These data provide further evidence that only one of the two possible geometrical isomers of meso-HM-PAO Tc-complexes is formed. By comparison, another achiral linear tetradentate ligand, meso-ECD, gave two rhenium(V)O complexes (resolved on a cyclodextrin chiral column) [23]; which are, presumably, the syn- and anti-forms of the complex (figure 4).

In order to assign individual peaks as d- and l- , samples of these enantiomers were obtained by crystallization of the tartrate salts of the crude meso/d,l-stereoisomer mixture obtained on ligand synthesis. HPLC chromatograms of the isolated d- and l-enantiomers of HM-PAO (free base form) are shown in figure 5, and the chromatograms of the complexes obtained from these ligands are shown in figure 6. The elution order of the ligand enantiomers on the OD column was l- then d-. The chromatogram of the isolated d-HM-PAO ligand enantiomer shows a single peak, while that for the l-HM-PAO ligand enantiomer indicates that there is either meso- or d-isomer present as an impurity. For the complexes, the l-enantiomer again has a shorter elution time than the d-enantiomer. However, because the complexes have better separation than the ligands on this HPLC system, the isomer impurity obtained on recrystallization of HM-PAO L-(+)-tartrate salt can be seen to be the meso-form in the analysis of Tc-99m l-HM-PAO. In addition, the d-complex also displays some meso-complex as an impurity. From the chromatograms of the complexes, it was determined that the % meso-ligand remaining in the d- and l-samples are 17.8 and 12.5%, respectively.

Table 1 displays the dependence of peak separation of the technetium complexes on solvent composition (hexane/IPA ratio). Baseline separation of d- and l-complexes was achieved with all eluents with % hexane $\geq$ 25%. Good separation (R_S = 0.90) of meso- and d-complexes was obtained using 85% hexane.

meso-syn-complex meso-anti-complex

Figure 4. Schematic representation of the syn- and anti-forms of the Tc-complexes of meso-ligands, e.g. HM-PAO and ECD

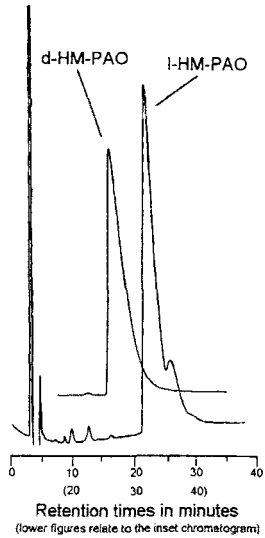

Figure 5. Analysis of isolated d- and l-enantiomers of HM-PAO (ligand) on the OD column.

Chiralpak AD column

Figure 7 displays a series of chromatograms obtained with samples of HM-PAO (ligand) on the Chiralpak AD column, eluted at room temperature with hexane/ethanol (containing 0.1% diethylamine). This system provides excellent separation of all three stereoisomers. In table 2 are listed the peak retention times and R_S values of d-, l-, and meso- peaks at several different hexane/ethanol ratios. By comparison, the Tc-99m complexes of HM-PAO were not resolved on this system. With isocratic elution and % EtOH >10%, the Tc-99m stereoisomer complexes co-eluted. At 10% EtOH, the meso-complex was observed as a shoulder to the d,l-peak. The complexes were not eluted using 5% EtOH, but using a shallow gradient elution profile (5 --> 10% of EtOH), the meso-peak could be separated ($R_S < 1$) from the d,l-peak.

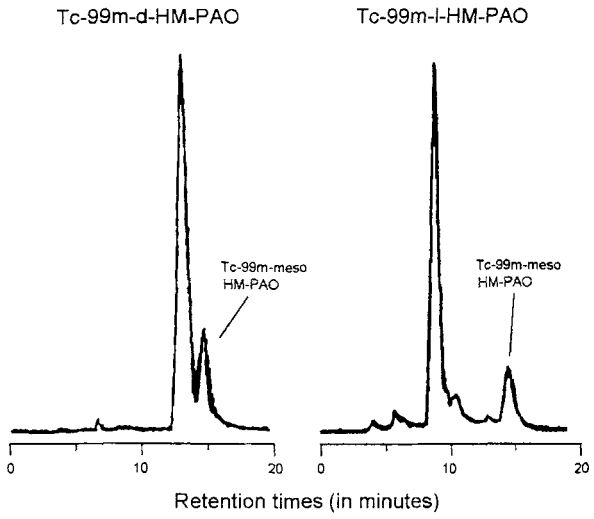

Figure 6. HPLC chromatograms of Tc-99m complexes from isolated d- and l-HM-PAO

Table 1. Separation of the Tc-99m complexes of meso-, d,- and l-HM-PAO on the Chiracel OD column at several solvent (hexane/IPA) ratios.

% hexane	R_S		% hexane	R_S	
	l and d	d and meso		l and d	d and meso
0	0.87	0.32	80	3.20	0.85
25	1.02	0.26	85	3.27	0.90
50	1.16	0.32	90	2.91	0.61
75	2.64	0.69			

Comparison of these results with those obtained on the OD column might appear to be somewhat surprising given the similarity between the OD and AD columns. Both columns have a silica gel base, to which is attached a polysaccharide based on a 1,4-glucose repeating unit derivatized (2-, 3- and 6-positions) with a 3,5-dimethylphenyl carbamate moiety. The fundamental difference is that the OD column is based on cellulose (β-1,4 linkage) and the AD system is based on amylose (α-1,4 linkage). This difference gives rise to very different three dimentional structures, and hence different resolving characteristics.

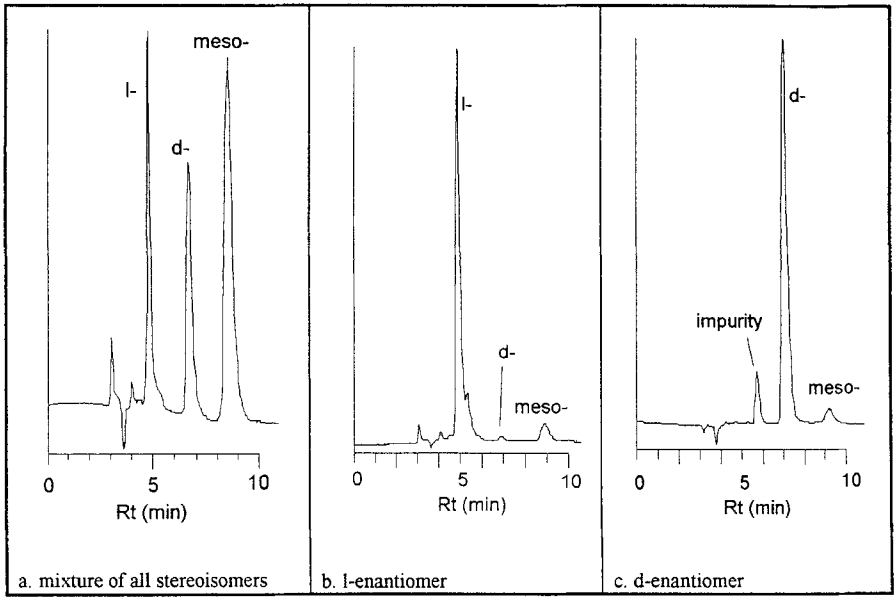

Figure 7. HPLC chromatograms of HM-PAO on the Chiralpak AD column.

Table 2. Separation of meso-, d,- and l-HM-PAO on the Chiralpak AD column at several solvent (hexane/EtOH) ratios.

% hexane	R_t (min)			R_s	
	l-	d-	meso-	l and d	d and meso
65	4.4	5.6	6.8	1.2	1.1
75	4.8	6.8	8.8	1.9	1.9
70	5.6	8.8	11.1	2.2	1.4
85	6.8	11.0	14.8	2.9	1.7

Chromatograms b and c in figure 7 show the results of ligand analyses on the AD column of two samples described in the previous section; l- and d-HM-PAO obtained by recystallization of the tartrate salts of the orginal meso/d,l-mixture. There is reasonably good agreement between the results of analyses of these samples as their Tc-99m complexes on the OD column, and as the free ligand on the AD column (these results are shown in table 3). For any given sample, the analytical results for the free ligand on the OD column and the complexes (formed from this ligand mixture) on AD column could only be similar provided that the avidity of the meso- and d,l-stereoisomers of HM-PAO for technetium are almost identical. The data given in table 3 would indicate that the HM-PAO stereoisomers have similar affinities for technetium.

Table 3. Comparison of the results of analyses of purified samples of d- and l-HM-PAO; analysis as free ligand on the Chiralpak AD column and as Tc-99m complexes on the Chiracel OD column.

sample/column	%		
	l-	d-	meso-
purified d- on OD	< 1	82.2	17.8
purified d- on AD[1]	6.1	74.3	19.6
purified d- on AD[2]	< 1	79.2	20.8
purified l- on OD	87.5	< 1	12.5
purified l- on AD[1]	87.3	1.8	10.9
purified l- on AD[2]	86.1	1.9	12.0

Key 1 - % l- includes the main peak and shoulder peak
2 - % l- includes the main peak but not the shoulder peak

In the chromatograms of HM-PAO ligand on the AD column, a shoulder to the main l-peak was observed (see figure 7). This peak is clearly seen in the samples of "purified" l-enantiomer, and, to a lesser extent, in samples which are mixtures of the stereoisomers. In the sample of "purified" d-enantiomer (figure 7c), the peak which appears to be l-HM-PAO has a R_t which corresponds to the shoulder peak rather than the main l-peak. impurity shown as l- in figure 7c . On labelling this sample with Tc-99m, it is clear that this impurity does not appear form Tc-99m l-HM-PAO complex (table 3). At present, the identity of this shoulder peak is not known. One possibility is that it is an oxime isomer. In HM-PAO, the oximes prefer to adopt the E-configuration, although E,Z- and Z,Z-isomers have been observed [29]. When isolated, Z-oxime HM-PAO isomers only give low yields of Tc-99m HM-PAO complexes [29].

An HPLC system based upon the AD column provides a convenient method for the determination of stereoisomeric purity of samples of HM-PAO. This system was used to analyze the products from several tartrate salt recrystallizations of HM-PAO. Table 4 displays the results of one of these studies, showing that one stereoisomer can be isolated with high enantiomeric purity after just two recrystallizations starting from the reaction product.

Resolvosil BSA-7 column

Due to the sensitivity of this column to ethanol, the samples of Tc-99m d,l-HM-PAO used with this column were not subjected to the solid phase purification procedure, described above. While methods of HM-PAO complex stabilization are known [30], aqueous solutions of Tc-99m d,l-HM-PAO formed from the the commercially-available kit degrade rapidly to form one or more so-called secondary complexes [5,29,31-34]. A steady increase in the proportion of these secondary complexes was observed (as a broad

Table 4. Results of the analysis of one series of tartrate salt recrystallization samples on the Chiralpak AD column.

	% of each HM-PAO stereoisomer		
	d-	l-	meso-
1st crystallization	72.2	9.0	18.8
2nd crystallization	90.1	4.3	5.6
3rd crystallization	92.4	6.7	1.0

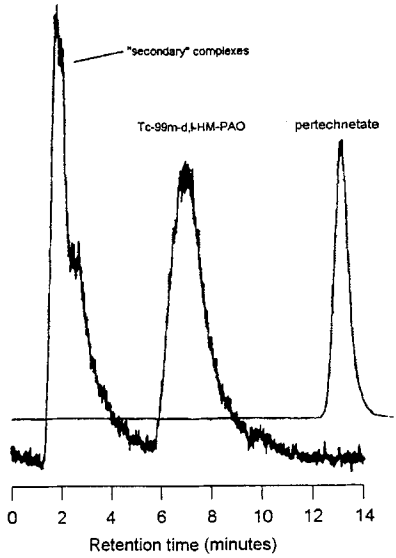

Figure 8. HPLC chromatogram of Tc-99m d,l-HM-PAO (4 hours post preparation) on the Resolvosil BSA system. A chromatogram of Tc-99m pertechnetate, obtained on the same system, is overlayed.

peak close to the void volume) on analysis of the aqueous solution of Tc-99m d,l-HM-PAO on the Resolvosil BSA-7 system over a period of several hours. However, this system fails to resolve the d- and l- enantiomer complexes, which appear as a broad peak at $R_t \sim 7.5$ minutes. Another major degradation product of Tc-99m d,l-HM-PAO prepared from the commercially-available kit is Tc-99m pertechnetate [31]. On the reversed-phase HPLC systems generally used for RCP determination of Tc-99m d,l-HM-PAO, pertechnetate appears in the void volume, and is indistinguishable from the secondary complexes. On the Resolvosil BSA-7 system, Tc-99m pertechnetate appears as

a sharp peak with greater retention than Tc-99m d,l-HM-PAO (figure 8). Therefore, while this HPLC system does not resolve the complexes of the d- and l-enantiomers, it has some potential for the rapid determination of radiochemical purity in those situations when it is desirable to distinguish between pertechnetate and the secondary complexes.

CONCLUSIONS

Three chiral HPLC columns were examined to determine their potential for the separation and analysis of HM-PAO stereoisomers. Tc-99m d,l-HM-PAO appeared as a single broad peak on the Resolvosil BSA-7 column. However, this peak was well separated from the radioactive impurities, Tc-99m pertechnetate and the so-called secondary complexes of Tc-99m d,l-HM-PAO. Therefore, while the Resolvosil BSA-7 column failed to separate the complex stereoisomers, it might be useful for routine analysis of the complex. The Chiracel OD column, using hexane/IPA as eluent, did separate the d- and l-HM-PAO ligand enantiomers. However, as separation was poor, and meso-HM-PAO was eluted midway between the enantiomers. The Tc-99m complexes of the d- and l-enantiomers are well separated on the Chiracel OD column, although there is only modest separation of the complexes from the meso- and d-ligands. Baseline separation of all three stereoisomers of the ligand was achieved on the Chiralpak AD column. This system does appear to be suitable for the routine analysis of the isomeric purity of HM-PAO samples. In addition, as semi-preparative versions of this column are commercially-available, it should be possible to isolate small quantities of HM-PAO enantiomers directly from the reaction mixture or from samples in which the proportion of one stereoisomer is increased through tartrate-salt recrystallization.

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