Kinetic Analysis of Technetium-99m d,1-HM-PAO Decomposition in Aqueous Media

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Technetium-99m-d,1-hexamethylpropyleneamine oxime [^{99m}Tc]d,1-HM-PAO) is a neutral-lipophilic chelate formed from a kit in high yield by stannous reduction of Na$^{99m}TcO_4^-$. Of three methods used to analyze the purity of the ^{99m}Tc complexes, a single strip method using ether as the developing solvent was the most rapid and simple to perform. The lipophilic chelate converts to $^{99m}TcO_4^-$ and other hydrophilic products with time and this limits the useful lifetime of the preparations. The rate of decomposition of [^{99m}Tc]d,1-HM-PAO increased in the presence of excess stannous ion and at pH >9.

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Technetium-99m-d,1-hexamethylpropyleneamine oxime ([^{99m}Tc]d,1-HM-PAO) is a neutral-lipophilic chelate that is under investigation to assess regional cerebral blood flow in patients with neurological disorders (*1–6*). This agent can be used with conventional single photon emission computed tomography brain imaging because it does not redistribute significantly in the brain for several hours after its initial localization (*3,4,6*). This is in contrast to the iodine-123-labeled amphetamines which demonstrate major redistribution within the first 2 hr after injection (*7–9*).

Technetium-99m-d,1-HM-PAO rapidly diffuses across the blood brain barrier (~80% first-pass extraction efficiency) at normal flow rates (*10*). Once deposited the major fraction of the chelate is retained at the original site. Neirinckx et al., proposed that the mechanism of retention involves slow conversion of the lipophilic chelate to hydrophilic products in the presence of glutathione (*11*). Although the aqueous in vitro degradation rate is slow, the instability of [^{99m}Tc]d,1-HM-PAO in the vial requires that it be administered within 30 min of its formulation. To better understand the conversion process and those factors that influence chelate stability we undertook this study to measure, at the tracer level, the effects of various chemical conditions (e.g., pH, buffer, ligand concentration, etc.) on [^{99m}Tc]d,1-HM-PAO stability in an aqueous medium. The majority of the studies used chelates that were isolated by ether extraction to eliminate the influence of any residual compounds required for chelate formation.

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MATERIALS AND METHODS

Formulation of [^{99m}Tc]d,1-HM-PAO

Technetium-99m-d,1-HM-PAO was prepared from a kit (Ceretec, Amersham International plc, Amersham, Buckinghamshire, England) containing 0.5 mg of d,1-4, 8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bisoxime (d,1-HM-PAO), 7.6 μg stannous chloride dihydrate, and 4.5 mg sodium chloride, freeze-dried and stoppered under nitrogen. $^{99m}TcO_4^-$ (pertechnetate) was obtained from a commercial ^{99m}Mo/^{99m}Tc generator (Mallinckrodt, Inc., St. Louis, MO) that had been eluted in the past 24 hr (*10*). To ensure the proper formulation of the [^{99m}Tc]d,1-HM-PAO, the manufacturer's package insert suggests that 5 ml of sodium [^{99m}Tc]pertechnetate in 0.9% NaCl containing 25–35 mCi (925–1,295 MBq) should be injected into the vial and eluate more than 2 hr old should not be used. The final concentration of d,1-HM-PAO was 0.1 mg/ml in the pH range 9-10.

Extraction of [^{99m}Tc]d,1-HM-PAO with Ether

The lipophilic [^{99m}Tc]d,1-HM-PAO chelate was isolated from the other kit formulated compounds by extracting 1 ml of the kit solution with 1 ml of diethyl ether vortexed for 1 min (ether/buffer partition coefficient of HM-PAO is 0.25 ± 0.02: Communication from Brian Higley, Amersham International plc, Amersham, Buckinghamshire, England). Fifty microliter of the ether layer (top) containing only the lipophilic

[^{99m}Tc]d,l-HM-PAO chelate were pipetted into a fresh test tube. The ether was evaporated to dryness in an ambient atmosphere using a stream of N_2. One-half milliliter of aqueous media was added to dissolve the chelate residue.

Analysis of ^{99m}Tc Complexes

The percents of the primary lipophilic complex, the hydrophilic complex species, free pertechnetate, and hydrolyzed-reduced (Hyd-Red) ^{99m}Tc were measured using three analytical methods.

1. Three-strip method. Previously described by Neirinckx et al. (*12*) this method determines the quantities of $^{99m}TcO_4^-$, the hydrophilic ^{99m}Tc species, Hyd-Red ^{99m}Tc, and lipophilic [^{99m}Tc]d,l-HM-PAO in physiological saline solution. It involves a combination of three chromatographic systems: (a) a silica gel instant thin-layer chromatography strip (ITLC-SG, Gelman Sciences, Inc., Ann Arbor, MI) developed with methyl ethyl ketone (MEK); (*6*) ITLC-SG developed with 0.9% W/V aqueous sodium chloride, and (c) Whatman No. 1 paper or Solvent Saturation Pads (Gelman Sciences, Inc., Ann Arbor, MI) developed with 50% V/V aqueous acetonitrile.

2. Ether paper chromatography. The samples were applied to either Whatman No. 1 paper or Solvent Saturation Pads and developed with diethyl ether (*13*). Only the lipophilic complex migrates with ether to the solvent front ($R_f = 1.0$) while $^{99m}TcO_4^-$, Hyd-Red ^{99m}Tc, and the hydrophilic species remain at the origin ($R_f = 0$).

3. High pressure liquid chromatography (HPLC). Complex analysis used a Hamilton PRP-1 reversed phase column with Waters Associates dual pump gradient chromatographic system. Eluent radioactivity was detected with a NaI(Tl) scintillation detector connected to a ratemeter. Signals from the ratemeter were directed to a Hewlett-Packard Model 3390A integrator for peak integration. Only HPLC columns that had never been exposed to Sn^{2+} in the injectate were used to analyze ether-extracted material (i.e., Sn^{2+} is not soluble in ether).

(a) Gradient Elution System A. A gradient elution system using 0.01*M* KH_2PO_4 containing 1% CH_3OH at pH 7 (solvent A) and pure acetonitrile (solvent B) was used for the HPLC analysis. One and one-half minutes after the injection of a 10-μl sample using 100% solvent A, solvent B was increased until it reached a final concentration of 50% (V/V) at 5 min. The flow rate was maintained at 2 ml/min for all phases of the separation. In a typical run, $^{99m}TcO_4^-$ had a retention time of ~0.5 min, the hydrophilic ^{99m}Tc-species ~3.5–4.5 min, and the lipophilic complex ~7.5 min. Paper chromatography using 50% acetonitrile was used in these studies to determine the % of Hyd-Red ^{99m}Tc (which did not come through the column) and the correct % yield of each species (*12*).

(b) Gradient Elution System B. The same gradient elution method was used except deionized H_2O was used as solvent A instead of the 0.01*M* phosphate buffer.

Effects of 0.01*M* Phosphate or Bicarbonate Buffer

The lipophilic [^{99m}Tc]d,l-HM-PAO chelate isolated from the other kit compounds by ether extraction was mixed with 0.5 ml of aqueous media containing either 0.01*M* $NaHCO_3$, 0.9% NaCl, or 0.01*M* KH_2PO_4. The solutions were adjusted to pH 6 except the 0.9% NaCl solution. The rate of decomposition of the lipophilic ^{99m}Tc-chelate was measured at ambient temperature (24.7 ± 0.4°C, n = 95) under these conditions for 3 hr. The least square method was applied to obtain the line of best fit for % complex yield on a logarithmic scale versus time plot. The k_d represents the first-order rate constant for chelate decomposition and the $t_{½}$ expresses the half-life of this first-order rate reaction.

Effect of pH

The addition of 5 ml deionized H_2O or 0.9% NaCl to dissolve 0.5 mg d,l-HM-PAO produced a pH of ~9.0. To determine the effect of pH on the stability of ^{99m}Tc-d,l-HM-PAO in aqueous medium, the ether-extracted ^{99m}Tc complex was dissolved in 0.5 ml of 0.01*M* bicarbonate buffer at the desired pH and allowed to incubate at ambient temperature for 3 hr.

HM-PAO exists in two diastereisomeric forms, d,l- and meso-. Since [^{99m}Tc]meso-HM-PAO has much poorer cerebral retention than the d,l-isomer in both rats (*14*) and humans (*15*) (apparently due to a much slower in vivo trapping rate), some in vitro stability studies with the meso-isomer were also performed. The ^{99m}Tc complex of meso-HM-PAO was formed using 0.1 mg of meso-HM-PAO dissolved in 0.5 ml deionized H_2O, 100 μl of a saturated solution of stannous tartrate ($SnC_4H_4O_6$; ~10^{-4}*M*) (*16*), and 0.5 ml of sodium [^{99m}Tc] pertechnetate (10-15 mCi, 370-550 MBq). The lipophilic [^{99m}Tc]meso-HM-PAO complex was extracted by ether and then incubated with 0.5 ml of 0.01*M* $NaHCO_3$ buffer at pH 6. The mixture was allowed to stand at ambient temperature during the 24-hr study.

Effect of Excess Ligand on Stability

Ether-extracted [^{99m}Tc]d,l-HM-PAO was incubated for 3 hr with 0.01*M* $NaHCO_3$ buffer at pH 6 and containing 0 mg/ml, 0.1 mg/ml or 1 mg/ml of added free ligand at ambient temperature. The volume of the mixture was 0.5 ml. The % of the remaining lipophilic product was measured at different times by the three chromatography analytical systems.

Effects of EDTA or Tetraamine on Stability

The effects of two strong ^{99m}Tc chelating agents on the stability of the lipophilic chelate and their possible ligand exchange were determined by incubating ether-extracted [^{99m}Tc]d,l-HM-PAO in 0.01*M* $NaHCO_3$ and buffering at pH 6 with either 1 m*M* of ethylenediaminetetraacetic acid (EDTA) or, 1,5,8,12-tetraazadodecane (ta), a linear tetraamine. The percent of the ^{99m}Tc lipophilic chelate and other lipophilic products was measured by the three methods during the 3-hr study. The formation of ^{99m}Tc-ta by ligand exchange was determined by electrophoresis. Electrophoresis was performed with a Gelman Deluxe regulated power supply and chamber containing 0.05*M* $NaHCO_3$ buffer at pH 6.0 and Beckman paper electrophoresis strips (Type: S&S 2043A mgl) at 300V for 1 hr. Technetium-99m-ta is easily identified since it has a +1 charge (*17*) and migrates toward the cathode. This was unlikely to occur with [^{99m}Tc]d,l-HM-PAO or its decomposition products. Technetium-99m-ta could also be detected by HPLC since its retention time differed from the [^{99m}Tc] d,l-HM-PAO, ^{99m}Tc-hydrophilic species, and $^{99m}TcO_4^-$.

Effect of Sn^{2+}

Saturated solutions of stannous tartrate were prepared by adding the stannous compound to deionized H_2O which had been purged with N_2 for 30 min. N_2 was bubbled through the solution for an additional 15 min. Ether-extracted [^{99m}Tc]d,l-

HM-PAO or [^{99m}Tc]meso-HM-PAO was first dissolved in 0.01*M* bicarbonate buffer (pH 6) at ambient temperature, and different volumes of saturated $SnC_4H_4O_6$ (10 μl, 50 μl or 100 μl) were added. The final pH of the sample solution was 6.3 ± 0.1. The amount of lipophilic ^{99m}Tc complex and other radiochemical impurities were determined by the three-strip method and ether paper chromatography.

Effect of Oxygen

To study the effect of O_2 in the sample solution, the ether-extracted ^{99m}Tc-chelate was incubated with 0.5 ml of N_2-bubbled saline at ambient temperature. The mixture was purged with N_2 throughout the 3-hr study. Air-saturated physiological saline mixed with ether-extracted complex was used as the control group. The pH of all solutions were maintained at 6.

RESULTS

Comparison of Analytical Methods

The results using three-strip and the ether paper chromatography analytical systems were in good agreement except the % ^{99m}Tc measured as the lipophilic component was slightly lower in the system using ether as the mobile phase (Fig. 1). In contrast, HPLC analysis of the Ceretec vial contents using PRP-1 columns previously used to analyze solutions containing Sn^{2+} overestimated the % of ^{99m}Tc lipophilic chelate and underestimated the amount of pertechnetate (Fig. 1, Table 1). When PRP-1 columns that were used to analyze solutions of [^{99m}Tc]d,1-HM-PAO which had first been ether extracted from the vial components (i.e., the column remained Sn^{2+} free), the HPLC analysis were the same with both systems (Fig. 2).

TABLE 1
Differences Between HPLC and ITLC with Sn^{2+} Contaminated Column*

Time (hr)	n	% Free $^{99m}TcO_4^-$ HPLC	ITLC
1 h	4	10.6 ± 2.4	26.5 ± 7.3
2 h	4	18.4 ± 3.4	34.3 ± 2.5
3 h	2	24.4 ± 9.3	41.7 ± 0.1

* All values are mean ± s.d.

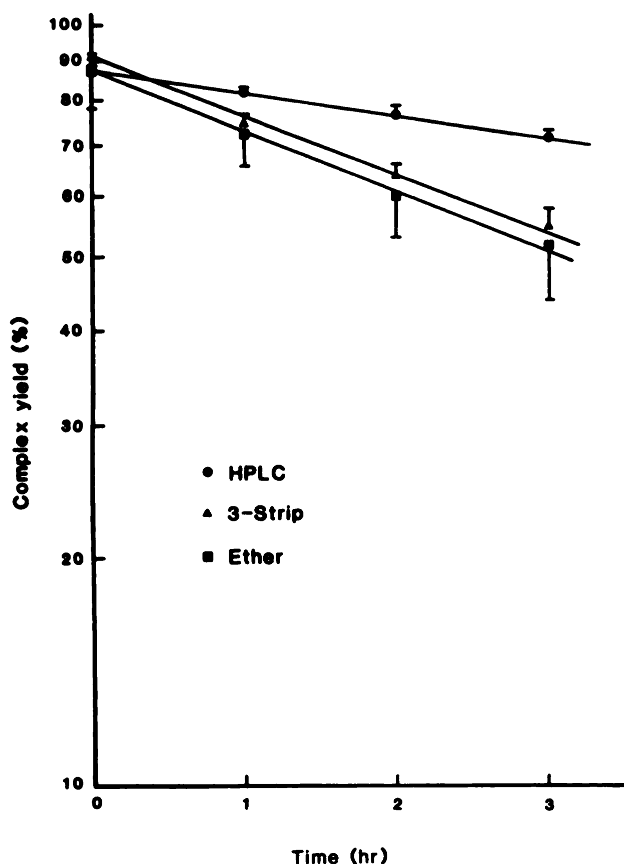

FIGURE 1
Rate of decomposition of kit-formulated [^{99m}Tc]d,1-HM-PAO measured by three analytical methods (mean ± s.d., n = 3). HPLC PRP-1 column was contaminated with stannous ion.

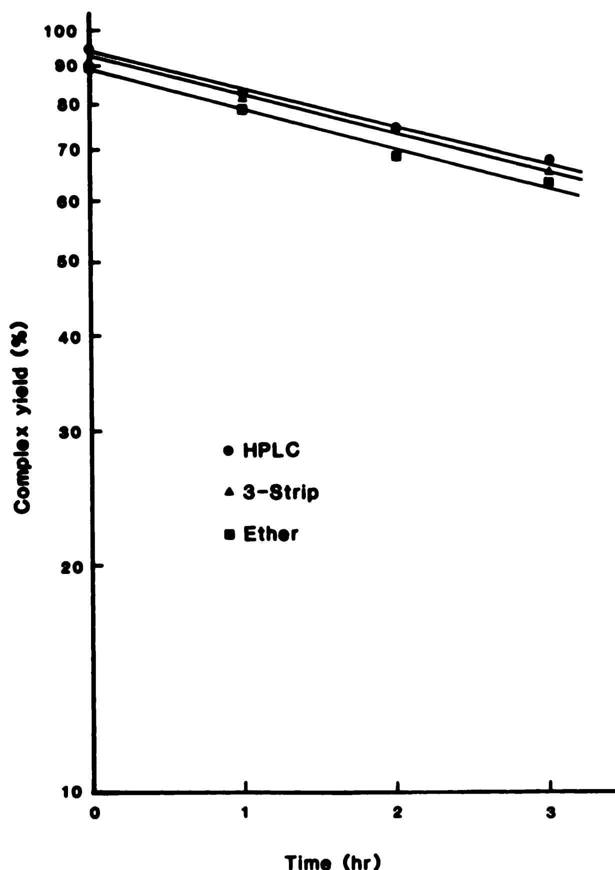

FIGURE 2
Rate of decomposition of ether-extracted [^{99m}Tc]d,1-HM-PAO measured by three analytical methods (mean ± s.d., n = 3). The k_d for HPLC, three-strip method, and ethyl ether method were 0.11 ± 0.03 hr^{-1}, 0.10 ± 0.03 hr^{-1}, and 0.11 ± 0.04 hr^{-1}, respectively.

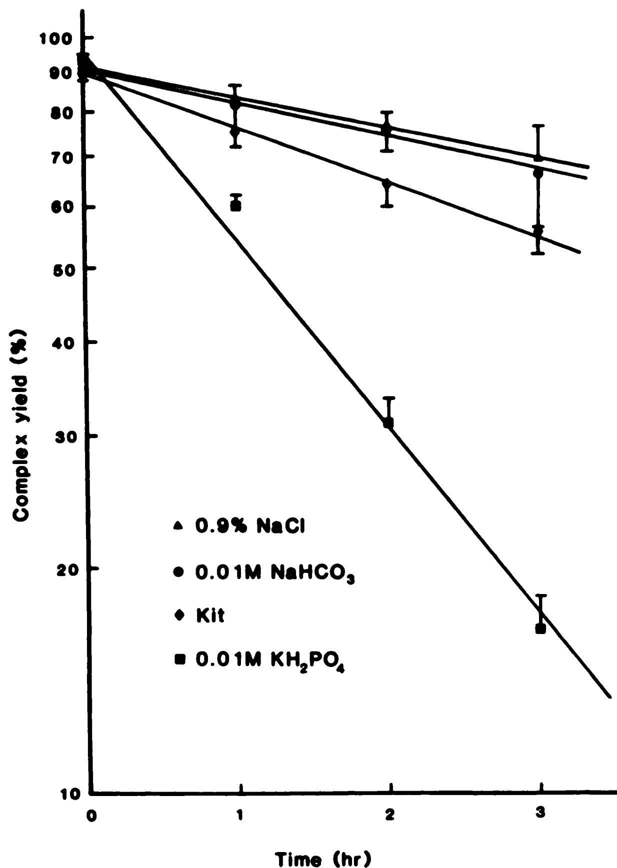

FIGURE 3
Stability of ether-extracted [^{99m}Tc]d,1-HM-PAO in different aqueous media and in the [^{99m}Tc]d,1-HM-PAO kit formulation. All solutions of ether-extracted ^{99m}Tc complexes were adjusted to pH 6 except 0.9% NaCl (pH-5.9 ± 0.4, n = 3). Measurement of yield was accomplished by the three-strip method. Each data point is the mean ± s.d. of three studies.

Effects of Phosphate Buffer on Stability

Figure 3 shows that the exclusive presence of 0.01*M* $NaHCO_3$ in the aqueous medium of ether-extracted complex had no effect on the rate of decomposition when compared to 0.9% NaCl. A significant acceleration of chelate dissociation was noted when phosphate buffer was used instead of bicarbonate buffer ($p < 0.01$) (Fig. 3). The principal decomposition product in phosphate buffer was pertechnetate (53.3 ± 9.3% at 1 hr and 79.2 ± 9.5% at 2 hr) with no evidence of increasing the formation of Hyd-Red ^{99m}Tc. By adding stannous tartrate (50 μl of saturated stannous tartrate) to the phosphate-buffered solutions the % pertechnetate was reduced to 1.4 ± 0.6% at 1 hr and 2.7 ± 0.7% at 2 hr. A higher % of the [^{99m}Tc]d,1-HM-PAO remained at 1 hr after ether extraction compared to the control studies (i.e., 89.4% versus 81.4%). A similar observation was not observed after addition of phosphate and excess Sn^{2+} directly to kit after its formulation.

Effects of Phosphate on HPLC Analysis

Analysis of the samples using the Gradient Elution System A (i.e., the aqueous solvent contained 0.01*M* KH_2PO_4) produced the chromatographic pattern shown in Figure 4A. Both [^{99m}Tc]pertechnetate and the lipophilic ^{99m}Tc-chelates were easily identified (i.e., peaks 1 and 3, respectively) while the hydrophilic ^{99m}Tc species were represented by multiple peaks and ~3.5–4.5 min retention times (Region 2, Fig. 4A). When Gradient Elution System B (containing no phosphate) was used, the hydrophilic ^{99m}Tc species showed a single peak with a 4-min retention time (Fig. 4B). These HPLC analyses (as well as the three-strip method) show that a minimal amount of hydrophilic species was present 3 min postformulation but by 30 min, substantially more of the hydrophilic species was present (Figs. 4 and 5). More of the hydrophilic component(s) were formed at 1 hr without significant change thereafter. Both $^{99m}TcO_4^-$ and secondary hydrophilic species were the principal impurities (<4% of Hyd-Red ^{99m}Tc was formed) during the first 60 min, however, after 1 hr, $^{99m}TcO_4^-$ became the major hydrophilic component (Fig. 5).

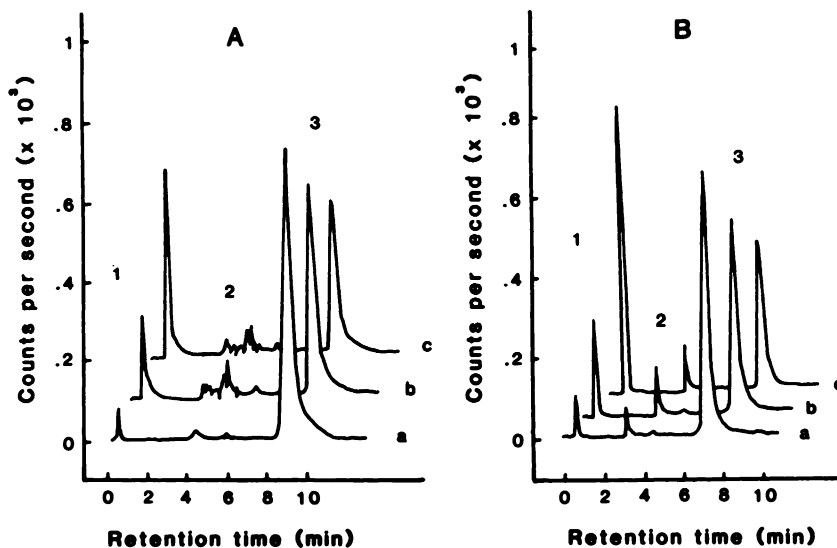

FIGURE 4
HPLC chromatographs of [^{99m}Tc]d,1-HM-PAO using 0.01*M* KH_2PO_4 buffer (A) and deionized H_2O (B) as initial eluents. 1: free pertechnetate, 2: [^{99m}Tc]d,1-HM-PAO hydrophilic complex, 3: [^{99m}Tc]d,1-HM-PAO lipophilic complex. a: 3 min postextraction, b: 1 hr postextraction, c: 3 hr postextraction (For HPLC conditions, see text).

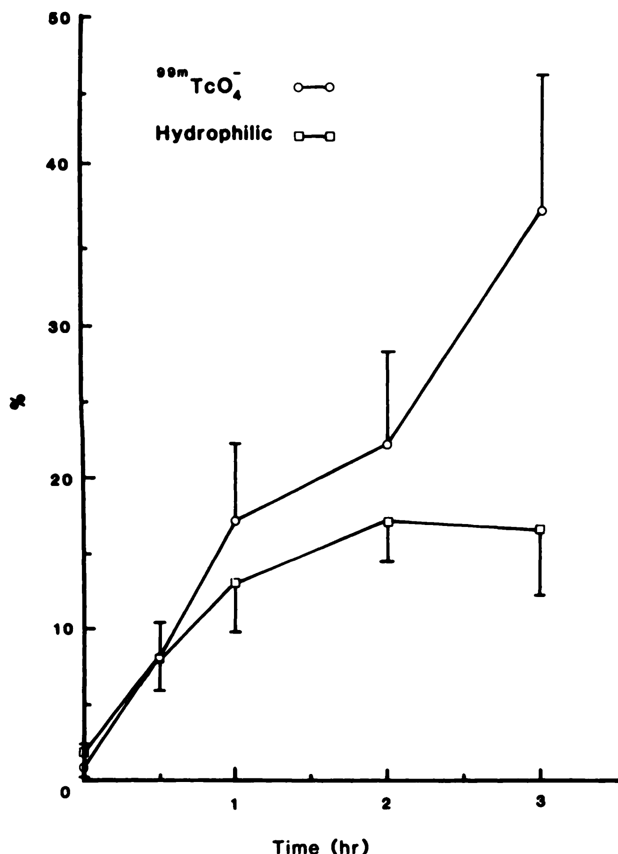

FIGURE 5
Percent of free pertechnate and hydrophilic components after adding $^{99m}TcO_4^-$ to d,1-HM-PAO kit. Analysis of hydrophilic species was measured by HPLC using phosphate buffer in the elution gradient system (mean ± s.d., n = 5).

Kinetics of [^{99m}Tc]d,1-HM-PAO Conversion

The conversion of the lipophilic component to $^{99m}TcO_4^-$ and the other hydrophilic species occurs by first-order kinetics for the first 3 hr in either the ether-extracted samples or the vial contents (Fig. 3). The slope of the complex yield in kit versus time plot was −0.074 ± 0.010 hr^{-1}. The k_d and $t_{1/2}$ for this first-order process were 0.17 ± 0.02 hr^{-1} and 4.1 ± 0.6 hr, respectively. The initial mean yield of [^{99m}Tc]d,1-HM-PAO at 2 min postformulation of 20 kits was 93.5 ± 1.5%.

pH Effects

The effect of pH on the stability of ether-extracted [^{99m}Tc]d,1-HM-PAO is shown in Table 2. The pH of the aqueous media buffered with 0.01*M* sodium bicarbonate had little effect on the stability of lipophilic chelate over the pH range of 4–8 with pH 6 producing the most consistent and lowest rate of decomposition. The chelates decomposition rate increased significantly as the pH increased above 9.

The ether-extracted [^{99m}Tc]meso-HM-PAO in 0.01*M* bicarbonate buffer (pH 6) had a much lower rate of decomposition compared to the d,1-isomer (k_d = 0.014 ± 0.004 hr^{-1} versus 0.11 ± 0.03 hr^{-1}).

Effects of Excess Uncomplexed Ligand

The presence of excess d,1-HM-PAO promoted slightly greater chelate stability. At a concentration of 1 mg/ml d,1-HM-PAO, k_d decreases to 0.050 ± 0.012 hr^{-1} from 0.11 ± 0.03 hr^{-1} without ligand excess at pH 6 in 0.01*M* $NaHCO_3$ ($p < 0.001$) (Table 3). In contrast by adding excess d,1-HM-PAO in unbuffered solutions decreases the chelate's stability (*18*). Unbuffered solutions of d,1-HM-PAO (i.e., ≥0.1 mg/ml) had a pH ≥9, and by increasing the concentrations of d,1-HM-PAO caused a corresponding increase in the solutions's pH.

Effects of ta or EDTA

The presence of either 1 m*M* ta or EDTA in solution at pH 6 had no significant effect on the stability of [^{99m}Tc]d,1-HM-PAO (Table 4). HPLC and the three-strip method of analysis demonstrates that the major decomposition products were the same, even when these strong chelating agents were present. There is no evidence of ^{99m}Tc-ta formation by either HPLC (Fig. 6) or electrophoresis (Fig. 7) as the lipophilic chelate converted to hydrophilic products.

Effects of Excess Sn^{2+}

The addition of excess stannous ions to ether-extracted [^{99m}Tc]d,1-HM-PAO accelerated the disappearance rate of the lipophilic chelate (Table 5). Even the addition of 10 µl of a N_2-bubbled saturated stannous tartrate solution to 0.5 ml of the ligand solution significantly decreased the amount of lipophilic chelate at 1 hr. The addition of 50 µl caused most of the lipophilic chelate to disappear at 1 hr when ether was used as the mobile phase. Results of the three-strip method indicates that a significantly higher quantity of the lipophilic component remained at 1 hr (Table 5). Greater than 40% of the activity migrated with MEK and was not $^{99m}TcO_4^-$, suggesting that the large amount of secondary

TABLE 2
Effect of pH on Ether-Extracted [^{99m}Tc]d,1-HM-PAO Stability*

pH	4	5	6	7	8	9	10
Slope	−0.57 ± 0.011	−0.058 ± 0.019	−0.048 ± 0.013	−0.054 ± 0.009	−0.081 ± 0.018	−0.113 ± 0.021	−0.341 ± 0.047
k_d (hr^{-1})	0.13 ± 0.02	0.13 ± 0.04	0.11 ± 0.03	0.12 ± 0.02	0.18 ± 0.04	0.26 ± 0.05	0.78 ± 0.11
t ½ (hr)	5.4 ± 0.9	5.6 ± 1.7	6.6 ± 1.8	5.7 ± 1.0	3.9 ± 0.9	2.7 ± 0.5	0.9 ± 0.1

*Values are mean ± s.d. of five studies. Yields were measured by HPLC.

TABLE 3
Effects of Added Free Ligand Concentrations on Ether-Extracted [^{99m}Tc]d,1-HM-PAO Yield*

	Control†	0.1 mg/ml†	1 mg/ml†
Slope	−0.048 ± 0.013	−0.032 ± 0.008	−0.022 ± 0.005
k_d (hr^{-1})	0.11 ± 0.03	0.07 ± 0.02	0.05 ± 0.01
t ½ (hr)	6.6 ± 1.8	10.0 ± 2.5	14.4 ± 3.0

* Measurement of yield was accomplished by HPLC after adjusting to pH 6. Data are presented in mean ± s.d. of five studies.
† Each sample contained an additional 0.025 ± 0.02 mg/ml of free ligand since some HM-PAO was extracted in the ether (i.e., its ether/buffer partition coefficient = 0.25 ± 0.02, communication from Brian Higley, Amersham International plc, Amersham, Buckinghamshire, England).

^{99m}Tc-species formed were lipophilic enough to be soluble in MEK but not in ether. By adding excess Sn^{2+}, a large amount of [^{99m}Tc]d,1-HM-PAO was converted to Hyd-Red ^{99m}Tc (>45% when 100 μl of saturated stannous tartrate was added).

The introduction of excess Sn^{2+} to the ether-extracted ^{99m}Tc-meso-HM-PAO caused less decomposition than what was observed with the d,1-isomer, even when as much as 100 μl of $SnC_4H_4O_6$ were added (Table 6), using either the three-strip or ether paper chromatography methods.

Oxygen

The disappearance rate of [^{99m}Tc]d,1-HM-PAO in N_2-purged solutions (pH 6) had a $k_d = 0.105 \pm 0.004$ hr^{-1} that did not differ significantly from what was observed in air-saturated solutions ($k_d = 0.109 \pm 0.024$ hr^{-1}).

DISCUSSION

Data on the three analytic methods indicates that any one can be used to determine the % purity of the neutral-lipophilic ^{99m}Tc-chelate in aqueous solutions. The single strip method using ether as the eluting solvent and was the most rapid and simple to perform even though it gave lower than normal values than the three-strip method. HPLC analysis of [^{99m}Tc]d,1-HM-PAO was very sensitive but its application must be validated against other analytic methods. Contamination of the reversed-phase PRP-1 column with small amounts of stannous ion could result in significant errors (Table 1). To obtain accurate results it is imperative to eliminate any excess stannous ion during the HPLC analyses.

TABLE 4
Effects of Multidentate Chelating agents (conc: 1 m*M*) Ether-extracted [^{99m}Tc]d,1-HM-PAO Yields*

	EDTA	ta
Slope	−0.047 ± 0.011	−0.033 ± 0.010
k_d (hr^{-1})	0.107 ± 0.026	0.076 ± 0.024
t ½ (hr)	6.7 ± 1.7	9.7 ± 2.6

* Yields were measured by HPLC after adjusting to pH 6. Values are presented in mean ± s.d. (n = 3).

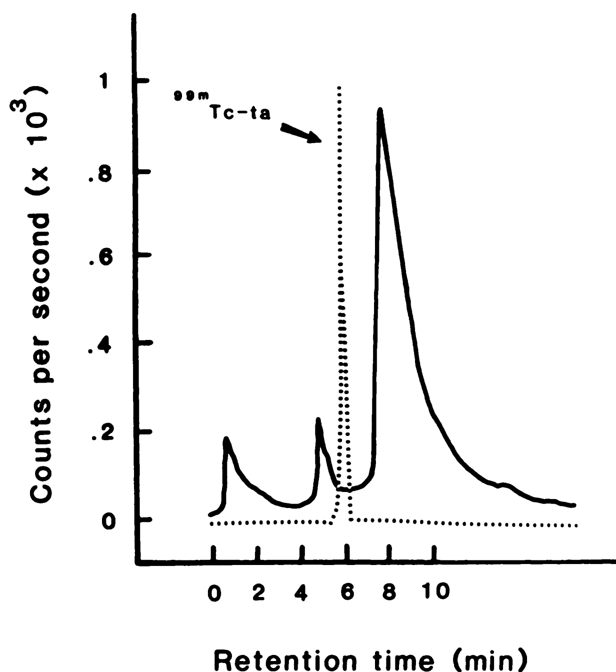

FIGURE 6
HPLC analysis of ether-extracted [^{99m}Tc]d,1-HM-PAO incubated with 1 m*M* linear tetraamine and HPLC chromatograph of ^{99m}Tc-ta (For HPLC conditions, see text).

Phosphate ion affected the stability of the lipophilic chelate as shown in Figure 3 and the HPLC analysis shown in Figure 4A demonstrates the presence of impurities. In the absence of excess stannous ions, 0.01*M* phosphate buffer increased the rate of dissociation, with $^{99m}TcO_4^-$ the primary product. The addition of excess stannous tartrate to solutions containing phosphate maintained a high % of the lipophilic chelate for ≥1 hr by reducing the $^{99m}TcO_4^-$ product to reform the chelate. The origin of the multiple hydrophilic peaks (Region 2–Fig. 4A) observed when 0.01*M* phosphate buffer was used as Solvent A in the HPLC gradient elution system is not yet understood. However, since only a single well-defined peak at 4.0 min was observed when phosphate was not in the Gradient Elution System B, this indicates that only one secondary ^{99m}Tc species was formed (aside from $^{99m}TcO_4^-$). This suggests that the low labeling efficiency of leukocytes using [^{99m}Tc]d,1-HM-PAO in phosphate-buffered saline might be improved if the incubating solution did not contain phosphate (*19,20*).

Although the pH of the aqueous medium in the range of 4–8 had no significant effects on the stability of the lipophilic complex, the rate of its decomposition increased when the pH exceeded 9 (Table 2). The addition of [^{99m}Tc]pertechnetate to freeze-dried Ceretec kits re-

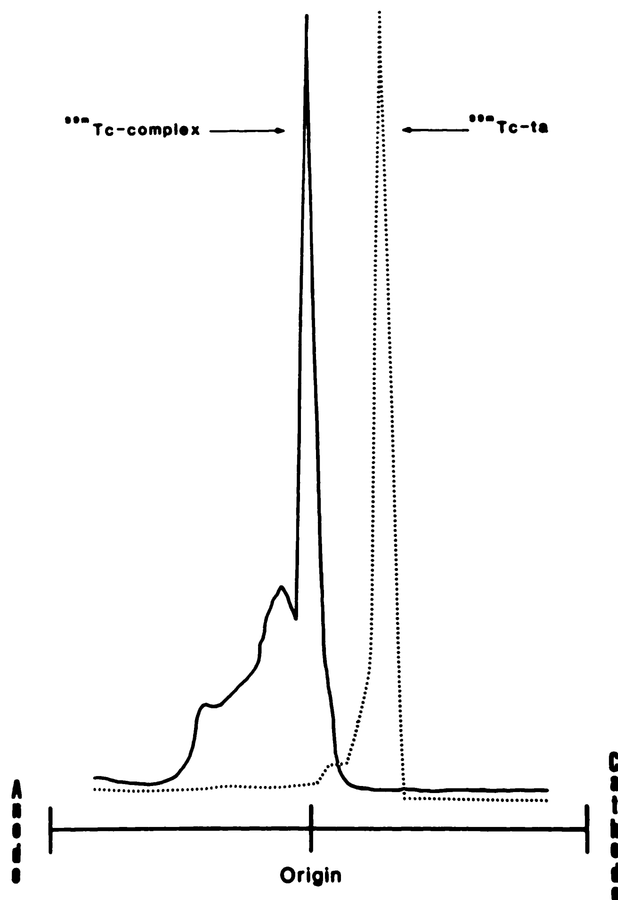

FIGURE 7
Electrophoresis of ether-extracted [^{99m}Tc]d,1-HM-PAO incubated with 1 mM linear tetraamine and electrophoresis of ^{99m}Tc-ta (For electrophoresis conditions, see text).

sults in pH of ≥9.0 suggesting that pH played a role in the dissociation kinetics of [^{99m}Tc]d,1-HM-PAO formulated from a kit.

Excess ligand in the aqueous solution exhibited a protective effect on chelate stability. At concentrations of 0.1 mg/ml and 1 mg/ml, the rate of [^{99m}Tc]d,1-HM-PAO decomposition decreased (Table 3). In the absence of buffer, however, pH became the dominant factor in stabilizing the complex.

The observation that neither 1 mM EDTA nor 1 mM ta accelerated the dissociation of the lipophilic chelate or its conversion to hydrophilic ^{99m}Tc-forms suggests that molecules containing carboxylic acid or amine groups exerted no effect on the [^{99m}Tc]d,1-HM-PAO stability. If decomposition of the chelate were to proceed by simply releasing the Tc (V) core into the aqueous media, it is reasonable to expect formulation of at least some [^{99m}Tc]EDTA or ^{99m}Tc-ta. This is particularly true for ^{99m}Tc-ta, since ta is known to rapidly form a very stable and easily identified chelate with Tc in the +5 oxidation state (i.e., TcO_2ta^{1+}) (*17*). Since transchelation to neither ta nor EDTA occurred, we can assume that [^{99m}Tc]d,1-HM-PAO did not undergo measurable dissociation with the release of Tc in the +5 oxidation state.

If [^{99m}Tc]d,1-HM-PAO did not dissociate by releasing Tc(V) into aqueous media, then converting this lipophilic chelate to hydrophilic products must involve more complex reactions than simple dissociation of the metal from the chelate. Neirinckx et al. (*11*) reported that glutathione, a reducing agent, rapidly accelerates the decomposition of [^{99m}Tc]d,1-HM-PAO in solutions. The presence of excess stannous ion (Table 5) also enhanced the rate of conversion of lipophilic [^{99m}Tc]d,1-HM-PAO to hydrophilic and Hyd-Red ^{99m}Tc-species (Table 5) suggesting that the rapid conversion of [^{99m}Tc]d,1-HM-PAO was accelerated by redox reactions.

The relatively slow decomposition ($k_d = 0.11 \pm 0.03$ hr^{-1}) of the ether-extracted [^{99m}Tc]d,1-HM-PAO in aqueous solutions (Table 3) was not regulated by reducing agents since none were present in the ether-extracted chelate studies. The rate of conversion of the lipophilic chelate to hydrophilic species was unaffected by the presence or absence of oxygen, eliminating it as a factor in the decomposition process.

In contrast, [^{99m}Tc]meso-HM-PAO, was ~10 times more stable in aqueous solutions than the d,1 isomeric

TABLE 5
Effect of Reducing Agent on Ether-Extracted [^{99m}Tc]d,1-HM-PAO Yields[†]

	% Lipophilic		
	3 min	1 hr	2 hr
Control[†]	88.9 ± 1.4/90.1 ± 0.5	79.0 ± 7.2/81.4 ± 10.2	68.6 ± 9.4/75.1 ± 4.5
Sn^{2+}[‡]	84.4 ± 8.7/89.8 ± 6.4	50.8 ± 6.9/64.7 ± 10.8	33.4 ± 5.4/45.4 ± 5.7
Sn^{2+}[§]	90.1 ± 4.2/92.6 ± 2.2	29.4 ± 9.9/43.6 ± 8.4	17.4 ± 6.3/42.1 ± 6.9
Sn^{2+}[¶]	87.8 ± 3.8/91.7 ± 0.6	8.3 ± 3.1/43.5 ± 5.1	6.0 ± 0.9/36.7 ± 7.6

* Yields were measured by paper chromatography (ethyl ether method/three-strip method). Values are mean ± s.d. of three studies.
† 0.01M $NaHCO_3$ buffer at pH 6.
‡ 10 μl Saturated $SnC_4H_4O_6$ in 0.5 ml 0.01M $NaHCO_3$ at pH 6.
§ 50 μl Saturated $SnC_4H_4O_6$ in 0.5 ml 0.01M $NaHCO_3$ at pH 6.
¶ 100 μl Saturated $SnC_4H_4O_6$ in 0.5 ml 0.01M $NaHCO_3$ buffer at pH 6.

TABLE 6
Effect of Reducing Agent on Ether-Extracted [^{99m}Tc]meso-HM-PAO Yields*

	% Lipophilic		
	3 min	1 hr	2 hr
Control†	97.8 ± 1.3/96.1 ± 0.4	94.1 ± 0.8/93.1 ± 0.4	88.7 ± 2.2/89.8 ± 1.2
Sn^{2+}‡	97.4 ± 0.1/95.8 ± 0.9	88.7 ± 5.8/90.2 ± 3.7	82.4 ± 5.7/84.8 ± 5.0
Sn^{2+}§	97.8 ± 0.2/95.1 ± 1.0	83.3 ± 2.8/89.2 ± 3.1	76.6 ± 2.9/81.1 ± 3.2
Sn^{2+}¶	98.1 ± 0.4/96.1 ± 0.5	75.6 ± 8.0/83.5 ± 6.3	63.3 ± 12.6/72.8 ± 11.4

* Yields were measured by paper chromatography (ethyl ether method/three-strip method). Values are mean ± s.d. of three studies.
† 0.01*M* $NaHCO_3$ buffer at pH 6.
‡ 10 μl Saturated $SnC_4H_4O_6$ in 0.5 ml 0.01*M* $NaHCO_3$ at pH 6.
§ 50 μl Saturated $SnC_4H_4O_6$ in 0.5 ml 0.01*M* $NaHCO_3$ at pH 6.
¶ 100 μl Saturated $SnC_4H_4O_6$ in 0.5 ml 0.01*M* $NaHCO_3$ buffer at pH 6.

form (i.e., $k_d = 0.014 \pm 0.004\ hr^{-1}$ versus $0.11 \pm 0.03\ hr^{-1}$ at pH 6, respectively). The rate of decomposition of [^{99m}Tc]meso-HM-PAO was increased by glutathione (*11*) and excess Sn^{2+} (Table 6) but to a lesser extent than the d,l-isomer. Jurison et al. (*13*) showed that both the meso- and d,l-HM-PAO ligands form chelates where Tc is present as the TcO^{3+} core. The basic ligand-metal complexation structures of the d,l- and meso-isomers are practically identical except for the orientation of the carbon-3 (C-3) and carbon-9 (C-9) methyl groups near the apical oxygen atom (*13*). The data suggests that the stereochemistry at the C-3 or C-9 sites on the ligand backbone was a principal factor in the reactions involved in converting the lipophilic d,l-chelate to hydrophilic products.

In summary, these experiments and results provide some insight about the chemical factors affecting the in vitro stability of the neutral-lipophilic [^{99m}Tc]d,l-HM-PAO chelate. Its kinetic stability against dissociation in aqueous media was high. When decomposition occurred, $^{99m}TcO_4^-$ and a secondary hydrophilic product were formed (up to 1 hr) after which pertechnetate became the major hydrophilic species. Instability of the complex could be promoted by excess stannous ion that catalyzes acceleration of the decomposition rate. The stability of the neutral-lipophilic chelate therefore, may be improved by buffering the storing solutions at pH <9 and eliminating or minimizing excess Sn^{2+}.

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