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Note

Anion-exchange high-performance liquid chromatography of technetium-labeled phosphonoacetic acid skeletal imaging agent preparations

MOHAMED A. ABDELNASSER, EDWARD DEUTSCH and WILLIAM R. HEINEMAN*

Biomedical Chemistry Research Center, Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221-0172 (U.S.A.)

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Technetium (Tc) radiopharmaceuticals are widely used for skeletal imaging in the field of nuclear medicine [1,2]. The most efficacious technetium bone imaging agents in current use are made with diphosphonate ligands such as hydroxyethylidine diphosphonate (HEDP), methylene diphosphonate (MDP), dimethylamino disphosphonate (DMAD), hydroxymethyl diphosphonate (HMDP), or dicarboxypropane diphosphonate (DPD). High-performance liquid chromatographic (HPLC) studies of different Tc–diphosphonate radiopharmaceuticals formed with HEDP [3,4] MDP [5,6], and DMAD [7] have shown that these preparations are mixtures containing a number of Tc–diphosphonate complexes in varying amounts. These complexes are oligomeric or polymeric in nature [8,9]. Moreover, while not yet conclusively proven, it appears that the smaller oligomers or polymers are superior skeletal imaging agents as compared to the larger ones [10,11]. One way to maximize the formation of smaller species is to modify the diphosphonate ligand to disfavor the formation of polymers. This can be achieved by replacing one $-PO_3H_2$ group of the diphosphonate ligand by a –COOH group since the carboxylate moiety is less able to bridge two metal centers than is the phosphonate moiety. This replacement converts the prototypical diphosphonate ligand MDP ($[O_3P-CH_2-PO_3]^{4-}$) into phosphonoacetic acid, PAA ($[O_3P-CH_2-COO]^{3-}$). A brief report has appeared indicating that ^{99m}Tc-labeled PAA mixtures can provide skeletal images in animals [12].

In this work ^{99m}Tc–PAA complexes were prepared by reduction of pertechnetate with sodium borohydride in the presence of PAA. An anion-exchange HPLC separation of these ^{99m}Tc–PAA complex mixtures has been developed as the first

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step in a systematic study of the ^{99m}Tc–PAA system. This separation will enable in future studies of (a) the effect of reaction conditions such as pH, time, and ligand/metal ratio on the relative concentrations of individual Tc–PAA complexes to be evaluated and (b) the individual Tc–PAA complexes to be isolated for biological distribution studies in test animals.

EXPERIMENTAL

Reagents

Water was distilled, deionized, and photolyzed with a Nanopure–Organicpure system (Sybron Barnstead, Boston, MA, U.S.A.). Unless otherwise noted, all chemicals were reagent grade. PAA, $(HO_2CCH_2)P(O)(OH)_2$, was 98% pure (Alfa Products, Danver, MA, U.S.A.). $K^{99}TcO_4$ was prepared from $NH_4{}^{99}TcO_4$ (Oak Ridge Labs., Oak Ridge, TN, U.S.A.) by metathesis with KOH and was purified by several recrystallizations from warm water [13]. $^{99m}TcO_4^-$ was obtained by elution of commercially available $^{99}Mo/^{99m}Tc$ generators (Cintichem/Union Carbide, Tuxedo, NY, U.S.A.) with 0.9% aqueous NaCl solution provided by the generator manufacturer. Sodium acetate and sodium chloride were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

Preparation of $NaBH_4$-reduced ^{99m}Tc–PAA reaction mixtures

^{99m}Tc–PAA complexes were prepared as follows: 0.2 ml of a 50% PAA solution, 1 mCi of $^{99m}TcO_4^-$, and 0.1 ml of 40 m*M* $K^{99}TcO_4$ were placed in a small vial. Purified water was then added as necessary to bring the total volume to 1.2 ml. An 0.1-ml aliquot of freshly prepared 12% $NaBH_4$ in 0.1 *M* KOH was added dropwise over 5 min. The reaction mixture was allowed to stir for an additional 30 min prior to injection in the HPLC system. Final concentrations of reagents in the mixture are 40 m*M* for Tc and 0.6 *M* for PAA.

Apparatus and conditions

The HPLC system (Bioanalytical Systems, West Lafayette, IN, U.S.A.) consisted of a Waters M-45 pump, 0.1-ml Rheodyne Model 7125 injection valve, CS-3 instrument rack, PG-5 pressure gauge, and PD-1 pulse damper. The analytical column (250 mm×4.0 mm) contained Aminex A-27 anion-exchange resin consisting of styrene-divinylbenzene copolymer with quaternary ammonium functional groups (Bio-Rad Labs., Rockville Center, NY, U.S.A.). The unreacted pertechnetate was removed by an AE-Pellionex anion-exchange resin (Cat. No. 9262, Whatman Chemical Separation, Clifton, NJ, U.S.A.) in a guard column (7.0 mm×2.1 mm).

The γ-ray activity of eluting ^{99m}Tc–PAA complexes was measured with a scintillation detection system (Harshaw Chemical, Solon, OH, U.S.A.).

The mobile phase was an aqueous mixture of 0.5 *M* sodium acetate and various amounts of sodium chloride. The pH was adjusted by adding the required amount of acetic acid. The solution was filtered through a 0.2-μm GA-8 filter (Gelman Sciences, Ann Arbor, MI, U.S.A.) and deaerated prior to use. The flow-rate was maintained at 0.2 ml/min.

Because the volume of the quaternary ammonium anion-exchange resin (Aminex A-27) varies with changes in ionic strength of the mobile phase, the analytical column was repacked for each variation in mobile phase ionic strength. Since changes in the pH of the mobile phase within about two units had no visible effect on the resin volume, the column was repacked only when the mobile phase pH was varied beyond this range.

Gravity flow columns were packed with AG MP-1 macroporous anion-exchange resin (Bio-Rad Labs.).

RESULTS AND DISCUSSION

Development of mobile phase

^{99m}Tc–diphosphonate complexes with the diphosphonate ligands HEDP [3], MDP [5], and DMAD [7] have been separated in this laboratory on columns of Aminex A-27 anion-exchange resin with 0.85 *M* sodium acetate solution (pH 8.4) as the mobile phase. However, no separation of the ^{99m}Tc–PAA complex mixtures of Aminex A-27 could be achieved on this resin with mobile phases consisting of sodium acetate in concentrations between 0.5 and 2.0 *M* at pH values between 4.5 and 9.0 (using acetic acid for pH adjustment). Experiments on AG MP-1 macroporous anion-exchange resin (which has properties similar to those of Aminex A-27 resin) under gravity flow conditions showed that addition of sodium chloride to 0.5 *M* sodium acetate mobile phase increased the anion-exchanging power of the mobile phase sufficiently to elute and separate the Tc–PAA complexes in the mixture. This effect was also observed for the Aminex A-27 resin. Hence, further experiments on Aminex A-27 were carried out using sodium chloride in the mobile phase.

To investigate the possibility of ligand exchange between chloride and PAA anions, visible absorption spectra were taken of two diluted samples of a typical ^{99m}Tc($NaBH_4$)–PAA preparation (pH 1.35); the first sample was diluted in purified water and the second in the mobile phase solution. The two spectra are almost identical implying that no significant amount of chloride exchange occurs.

Addition of a ^{99m}Tc–diphosphonate complex mixture to a solution of a pH value significantly different from the pH of formulation of the mixture could cause the components of the mixture to convert to other compounds that are stable in the new pH environment. Therefore, it is important that the mobile phase has sufficient buffering capacity. The pH values of some HPLC-isolated components of different pH reaction mixtures were found to have the same pH (5.5) as the mobile phase, indicating that the mobile phase effectively buffers the injected aliquots of reaction mixture during the separation procedure.

Effect of mobile phase ionic strength, pH, and temperature

HPLC with sodium chloride added to the mobile phase gives chromatograms in which six ^{99m}Tc-containing components can be identified. The retention times of these components decrease with increasing ionic strength, as shown by the series of chromatograms in Fig. 1.

Capacity factors (k') of the six major components are plotted versus the ionic

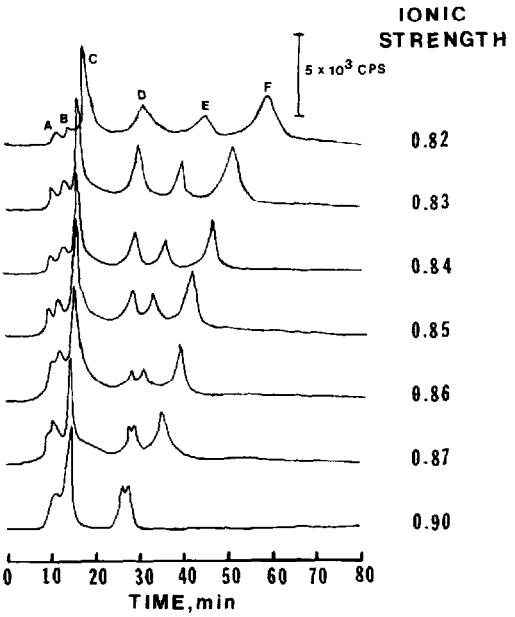

Fig. 1. Chromatograms of a ^{99m}Tc-PAA preparation obtained at different ionic strengths of the mobile phase. Mobile phase conditions: 0.5 *M* sodium acetate-acetic acid; ionic strength is adjusted by addition of sodium chloride; pH = 5.50; ambient temperature. Preparation conditions: pH = 1.35; 40 m*M* ^{99}Tc; 0.6 *M* PAA.

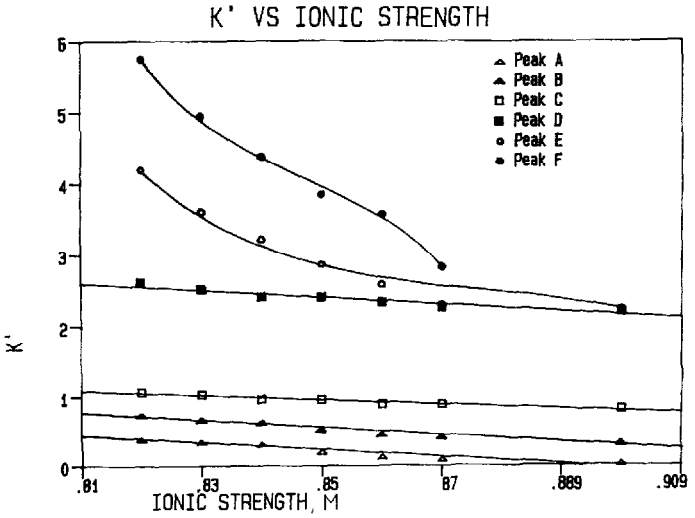

Fig. 2. Plots of k' values of chromatographic peaks in Fig. 1 versus ionic strength of the mobile phase. Each data point is an average of three trials.

strength of the mobile phase in Fig. 2. The order of elution of the sample components in anion-exchange HPLC is determined by the charge density of the molecules with the more weakly charged components eluting first. The effect of changes in the ionic strength of the mobile phase on the retention times of the

last two components (F and E) is significantly different from the effect on the retention times of the first four components (A, B, C, and D), as shown in Fig. 2. Further experiments were carried out at 0.83 *M* ionic strength.

The effect of pH of the mobile phase on the retention times was determined on the same ^{99m}Tc($NaBH_4$)–PAA complex mixture prepared at pH 1.35. The pH of the mobile phase was varied between 4.75 and 5.85 at 0.83 *M* ionic strength. Acetic acid was used to adjust the pH of the mobile phase without causing any considerable change in the ionic strength because of its minimal dissociation. Capacity factors for the six major components are plotted versus the pH of the mobile phase in Fig. 3. No significant change is observed for components A, B, C, and D, while the last two components are significantly affected by changes in the pH of the mobile phase.

In order to evaluate the effect of column and mobile phase temperature on the chromatographic separation of Tc–PAA complexes, the thermal stability of the complexes was first examined by heating a ^{99m}Tc–PAA preparation (prepared at room temperature, pH 1.45) to 60 °C for 1 h before being injected into the HPLC system which was maintained at room temperature. Comparison between the chromatogram obtained for this sample and that for an unheated sample of the same preparation (using both gamma and UV detection) showed no observable differences, indicating the stability of this preparation at temperatures up to 60 °C. Capacity factors are plotted versus the temperature of the HPLC system in Fig. 4. As a result of increasing the temperature from 25 to 55 °C, k' values of the first four components (A, B, C, and D) are slightly increased, while those of the last eluted components (E and F) are significantly increased. Although the amount of recovered activity decreases dramatically on going from 25 to 55 °C, the number of theoretical plates of the column, calculated using peak F, at 55 °C is three times as much as that calculated at 25 °C.

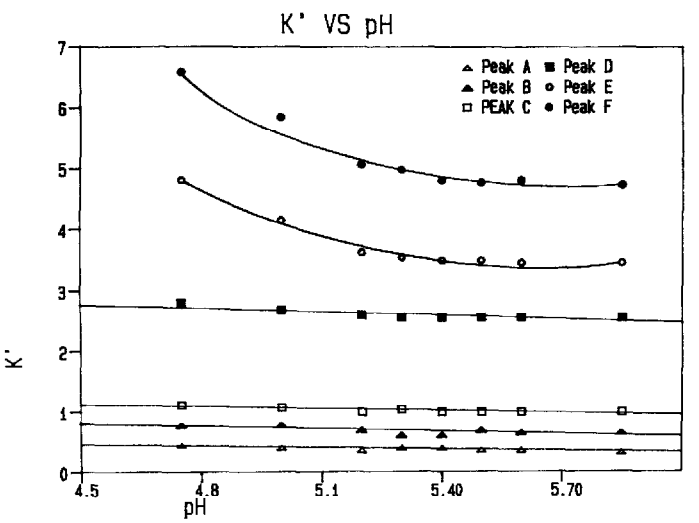

Fig. 3. Plots of k' values of chromatographic peaks in Fig. 1 versus mobile phase pH. Each data point is an average of three trials.

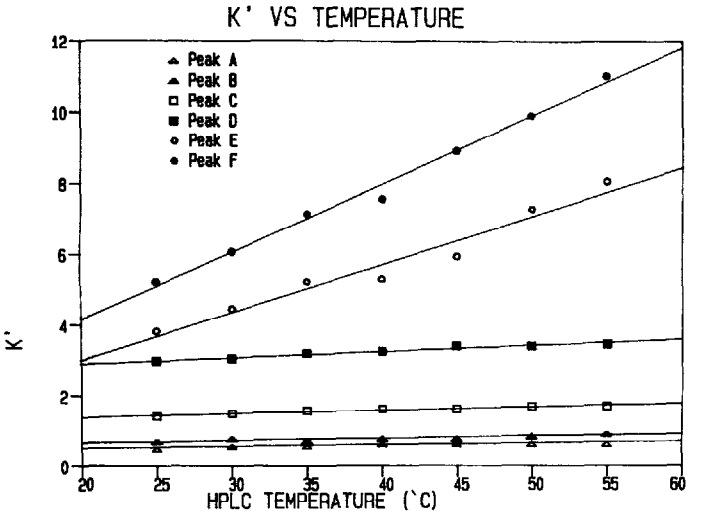

Fig. 4. Plots of k' values of chromatographic peaks in Fig. 1 versus temperature of the HPLC system. Each data point is an average of three trials.

The plots in Figs. 2, 3, and 4 show the effect of mobile phase ionic strength, pH, and temperature to be substantially greater on components E and F as compared to other components. The limited information available about the structures of these Tc–PAA complexes prevents a detailed explanation for the chromatographic behavior of these components. However, it is apparent that variations in both ionic strength and pH are changing the charge densities of the last two components in a similar manner.

CONCLUSIONS

As in the case of many diphosphonate ligands (e.g. MDP, HEDP, DMAD), $NaBH_4$ reduction of pertechnetate in the presence of the monophosphonate ligand, PAA, leads to the formation of complex mixtures with several ^{99m}Tc-containing components that can be separated by anion-exchange HPLC. The number of components separated, as well as the speed of separation, are affected by the pH, the ionic strength, and the temperature of the mobile phase.

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