HPLC Analysis of Tc-99m Iminodiacetate Hepatobiliary Agents and a Question of Multiple Peaks: Concise Communication

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The application of high-performance liquid chromatography (HPLC) to Tc-99m-labeled iminodiacetate hepatobiliary agents is described. The method is sensitive to variations of phenyl ring structure and gives different retention times for the different agents. Technetium-99m-labeled N-(2,6-diethylacetanilide)iminodiacetate from different sources had two components in differing amounts. It was determined that the second component, which is minor at pH above 6, is major when prepared at pH 4.5 or less. Raising the pH results in rapid conversion of the second component into the first. This observation was demonstrated also in plasma and would be expected to occur when the radiopharmaceutical is injected intravenously. The kinetics of appearance of the preparations in the bile when prepared at pH 4 and pH 6 were essentially the same.

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In contrast to the report of a single peak on high-performance liquid chromatography (HPLC) for Tc-99m-N-(2,6-dimethylacetanilide)iminodiacetate (Tc-dimethyl-IDA) (*1*), we observed multiple peak patterns for several other Tc-99m iminodiacetates in the evaluation of chromatography systems for routine quality control of these hepatobiliary agents (*2*). Since it was found that dilution of the kits before application to the chromatography strip resulted in coalescence of these peak patterns, we felt that the multiplicity was an artifact and not relevant to biological behavior. It was of interest, therefore, to evaluate the behavior of these radiopharmaceuticals on HPLC.

The comparison of the HPLC behavior of Tc-99m-N-(2,6-diethylacetanilide)iminodiacetate (Tc-diethyl-IDA) from two commercial and one in-house sources indicated two major components that varied significantly with supplier. Experiments were also carried out to determine the basis of the differences between the preparations.

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METHODS

Radiopharmaceuticals. Kits containing N-(2,6-diisopropylacetanilide)iminodiacetic acid* and N-(*p*-butylacetanilide)iminodiacetic acid,* and N-(*p*-isopropylacetanilide)iminodiacetic acid† were obtained commercially. N-(2,6-diethylacetanilide)iminodiacetic acid (A and S) was obtained from two commercial sources‡ and also prepared in house as previously described (*3*).

For experiments to study the effect of changing the stannous-to-stannic ion ratio, 35 mg of N-(2,6-diethylacetanilide)iminodiacetic acid was dissolved and pH adjusted to the desired value in 0.7 ml. Then 0.1 ml of a dilute acid solution of 0.35 mg of the reducing agent prepared from the appropriate combination of stannous and stannic chlorides was added and the pH readjusted if necessary. The kits were then frozen under vacuum until used.

The effects obtained with a further reducing agent were determined by adding 0.2 mg (0.1 ml of a 2 mg/ml solution in isopropyl alcohol) of $NaBH_4$ to 0.5 ml of a kit prepared at 3 ml total volume. The effect of chromate as an oxidizing agent was determined by the addition of 0.03 mg of potassium chromate (0.1 ml of a 0.3 mg/ml

aqueous solution) to a similar fraction of a kit.

Analytical methods. Reversed-phase HPLC was carried out on a 0.46 by 15 cm ultrasphere octadecylsilyl (ODS) column‖ using gradients consisting usually of 0.01 *M* sodium phosphate, pH 5.8, and acetonitrile (see figures for specific pH solvent composition and flow rate). The effluent passed through a 254 nm detector and then passed a crystal scintillation detector for continuous determination of radioactivity in the effluent. For the evaluation of plasma effects on radiochemical forms the radiopharmaceutical was diluted 1 to 10 in plasma. After the appropriate time, an equal volume of acetonitrile was added. The precipitated proteins were immediately separated using a desk-top centrifuge, and the supernatant was then injected.

Electrophoresis was carried out on paper strips at either pH 4.5 in 0.1 *M* sodium phosphate or pH 6.6 in 0.05 *M* sodium phosphate. Strips were run for 15, 30, 60, and 105 min at a total continuous voltage of 250.

Animal experiments. Sprague-Dawley rats were prepared for bile collection as described (*4*). After injecting 2 mCi of Tc-diethyl-IDA in 0.25 ml, bile was collected for 10-min periods from 0 to 50 min. Bile samples of 0.16 to 0.18 ml were diluted to 0.4 to 0.6 ml before HPLC injection. To determine the effects of pH on bile radioactivity appearance rates, bile samples were also collected from 0–2 min and then for 4-min periods for a total of 90 min. Preparations of Tc-diethyl-IDA at pH 4 and pH 6 were injected and the percent injected dose determined in each sample.

RESULTS

A comparison of HPLC patterns of Tc-99m-dimethyl-IDA, Tc-99m-diethyl-IDA, Tc-99m-diisopropyl-IDA, Tc-99m-*p*-isopropyl-IDA, and Tc-99m-*p*-butyl-IDA indicates that differences are fairly large between the 2,6-disubstituted complexes as a group and the two para substituted complexes as a pair (Fig. 1). Increasing retention times indicate greater lipophilicity and are consistent with increasing size of alkyl substituents. These results demonstrate the sensitivity of HPLC to structural changes.

The effect of changing gradients and flow rates is shown in Table 1. Separation of the main peaks of Tc-diethyl-IDA was maintained or increased by reducing the gradient from 10 to 50% acetonitrile to 20 to 40% while dropping the flow rate from 3.0 ml/min to 2.35 ml/min. The sharpness of the peaks was also maintained.

Other parameters were also studied. No changes were noted as the pH of the phosphate buffer component was varied from 5.5 to 6.8. Methanol or ethanol instead of phosphate buffer reduced retention times to less than 2 min and resulted in loss of peak resolution. Water as the primary solvent and methanol or ethanol as the secondary resulted in comparable retention times to phosphate buffer and acetonitrile, but with broadened peaks.

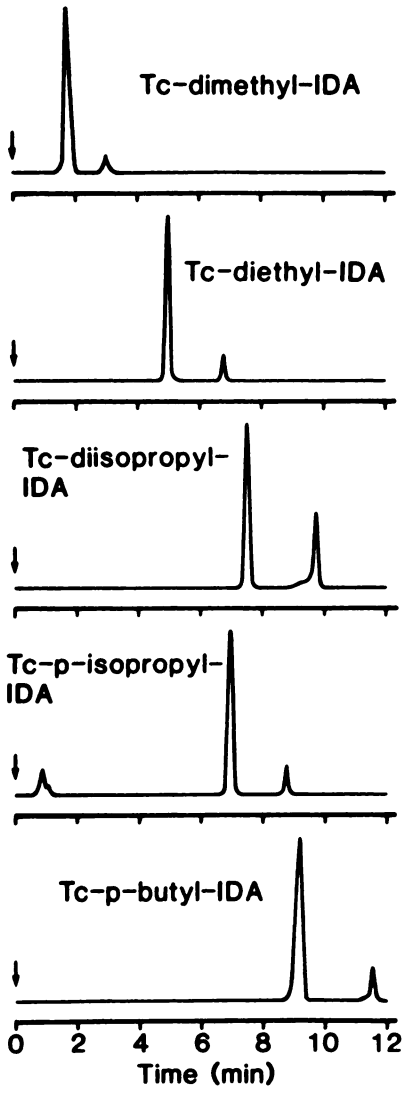

Fig. 1. HPCL retention times and typical peak patterns of Tc-99m iminodiacetate hepatobiliary agents. Conditions were 20 to 40% gradient in 10 min of 0.01 *M* phosphate, pH 5.8, and acetonitrile on a 0.46 × 15 cm ODS column. Mass detection at 254 nm indicated carrier iminodiacetate material at shorter retention times. Diethyl-IDA had retention time of 1.4 min when major peak of Tc-diethyl-IDA appeared at 5.0 min.

In general the pattern for the different agents was similar, with a major peak of 80 to 90% and a minor peak of 10 to 20%. However, evaluation of Tc-diethyl-IDA prepared from kits of manufacturer A‡ resulted in the same peaks, but with intensities of 45 and 55% in reversed order (Fig. 2, injectate). A review of differences that could account for the result indicated a kit pH of 4.6 for manufacturer A in contrast to 5.9 to 6.0 for in-house preparations, and 5.6 for manufacturer S.‡ In addition, kits of manufacturer A were sterilized by radiation. These differences suggested that responsible factors could be simply (a) pH, or perhaps (b) oxidation level

TABLE 1. RETENTION TIMES OF Tc-DIETHYL-IDA UNDER VARYING GRADIENT AND FLOW RATE CONDITIONS*

Condition				
Gradient	10–50%	15–45% B	18–42% B	20–40% B
Flow rate	3.0 ml/min	2.75 ml/min	2.5 ml/min	2.35 ml/min
Rentention time				
Peak 1	5.9 min	5.5 min	5.7 min	5.0 min
Peak 2	6.8 min	6.4 min	6.8 min	6.8 min

* Solvent A was 0.01 *M* phosphate, pH 5.8, solvent B acetonitrile. Gradient was over 10-min period. Column was 0.46 × 15 cm octadecylsilyl C-18 ultrasphere.

resulting from possible oxidative effect of the irradiation, or (c) differences in stability of oxidation level at different pH values.

Alterations of pH alone appeared to account for the changing patterns observed. Chromatograms of Tc-diethyl-IDA prepared at pH 4, 5, and 6 indicate a graded conversion of the latter peak into the former as the pH increases (Fig. 3). Addition of 5-μl aliquots of 1 *N* NaOH also caused similar changes in the relative intensities of the peaks. These changes were rapid, since the altered pattern was observed as soon as 1 min after addition of the base. On the other hand, the change upon lowering of pH was slow. At 5 min after addition of hydrochloric acid, no change was observed, whereas after 90 min there was a partial return to the initial pattern.

Additions of sodium borohydride similarly converted the later peak into the earlier one. It was determined, however, that the addition of these amounts of sodium borohydride also caused similar pH changes and thus the reductive effect would have been masked. The effect of altered reduction potential was studied by preparation of kits with all stannous ion or stannous-to-stannic chloride ratios of 0.67 and 0.33. The HPLC patterns of tagged kits prepared at pH values of 4, 5, and 6 varied with pH as described, but were unchanged by the stannous-to-stannic ion ratios. The addition of small amounts of chromate as an oxidizing agent decreased the later peak slightly while producing some free [^{99m}Tc] pertechnetate, and further supports the suggestion that the later peak is not likely due to a higher oxidation level.

It was thought that the second peak resulting from lowered pH might have altered net charge. However, electrophoresis at pH 4.5 and 6.6 indicated only complexed technetium, with net anionic charge that migrated in both cases about 0.7 times that of bromcresol green.

Another question—the effect of time—resulted from the paper chromatography studies. Changes were observed over the first 30 min with paper chromatography and have been reported by others who used Sephadex G-25 methods (*5*). When Tc-diethyl-IDA prepared at pH 5.6 was subjected to HPLC chromatography, 5% or less of the early peak and 95% or more of the later peak were seen at 1.5 min after addition of the pertechnetate. After 15 min conversion to 60% early and 40% late peaks had occurred, and by 25 min 70% early and 30% late peaks were seen.

A question that is important to the clinical use of these agents is whether or not the additional variable peak alters the biodistribution. From the results described regarding the shift to the peak of shorter retention times

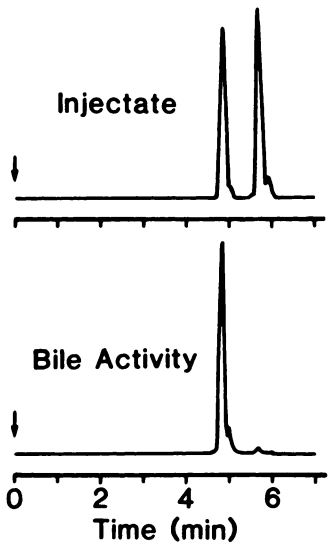

FIG. 2. Comparison of HPLC pattern of Tc-diethyl-IDA (manufacturer A)‡ as prepared, and radioactivity in rat bile from same preparation. HPLC conditions are as described in Fig. 1.

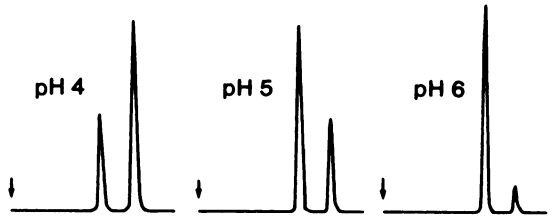

FIG. 3. HPLC patterns of Tc-diethyl-IDA prepared at pH values of 4, 5, and 6. HPLC conditions were as described in Fig. 1.

TABLE 2. TIME COURSE OF CUMULATIVE PRECENT INJECTED DOSE IN RAT BILE OF Tc-DIETHYL-IDA PREPARED AT pH 4 and pH 6*

Time after injection (min)	pH 4	pH 6
2	0.03 (0–0.07)	0.1 ± 0.1
6	29.2 (27.0–31.0)	36.4 ± 8.2
10	62.5 (57.7–67.6)	64.6 ± 5.9
14	73.1 (68.2–77.6)	74.8 ± 5.1
18	79.2 (73.8–83.2)	79.5 ± 4.1
22	82.3 (76.8–88.0)	82.3 ± 3.1
26	84.3 (78.8–89.7)	84.1 ± 2.5
30	85.9 (80.3–89.7)	85.4 ± 2.1
62	91.0 (84.8–94.8)	90.6 ± 1.8

* Values at pH 4 are mean and range of cumulative percent injected dose for three rats. Values at pH 6 are mean and standard deviation for six rats.

as pH is raised, it would be expected that a similar change would take place when the injectate is equilibrated with plasma at pH 7.4. This possibility was studied by adding a pH 4 preparation of Tc-diethyl-IDA to plasma for short periods of time. The initial chromatogram showed 66% of the later peak and 34% of the earlier. After 1 min in plasma, 9% was found in the later peak and 91% in the earlier. After 2 min, the conversion was essentially complete.

These observations are also consistent with the chromatogram of a sample of rat bile radioactivity (Fig. 2). Although the injectate had the normal appearance of the low pH preparation, only the early peak is seen in the bile radioactivity chromatogram. Since normal recovery of the injected dose exceeds 90%, this finding also supports in vivo conversion of the later peak into the earlier.

Finally, the potential biological differences were studied by observing a time course of appearance of radioactivity in rat bile with Tc-diethyl-IDA preparations at pH 4 and pH 6. The results shown in Table 2 indicate quantitatively similar kinetics and are consistent with the rapid in vivo conversion of chemical forms.

DISCUSSION

The results of these studies indicate that HPLC is a sensitive method for the analysis of Tc-99m-iminodiacetate hepatibiliary agents. Those that differ in substitution on the phenyl ring can be separated from each other with no peak overlap. In addition, the appearance of pertechnetate at 1 min or less under conditions in which the agents appear at 2 to 10 min is large and unambiguous in contrast to some thin layer and paper chromatography methods.

In general the radiopharmaceuticals appeared largely as one radiochemical with usually a small second component in agreement with the Tc-dimethyl-IDA study (*1*). As a result of kits of Tc-diethyl-IDA that were prepared at different pH values, conditions in which a second peak is dominant were defined. This extra component does not appear to present a problem clinically, since conversion to a single radiochemical occurred rapidly upon raising the pH. This conversion occurs in plasma and was complete within 2 min. The overall fractions of the injected doses of pH 4 and pH 6 preparations appearing in the bile were unchanged, and the rate of appearance was not significantly altered. Only the early peak component of Tc-diethyl-IDA was found in the bile.

That the extra peak represents a different oxidation level was not supported by the alteration of the stannous ion reduction potential since no stannous-to-stannic ion ratio dependent changes were observed at any pH. Treatment of the low pH preparation with the oxidizing agent chromate did produce some free pertechnetate without increasing the later peak, further supporting that it is not caused by a higher oxidation level.

The time-course experiments after the addition of pertechnetate indicated that the later peak, which is dominant at low pH, is formed first and, thus, kinetically preferred. The rate of conversion appears to be pH-dependent, since formation of the early peak occurred over a 25-min period with the pH 5.5 preparation. Preparations at pH 4.5 or less, however, showed little change over periods of several hours after attaining a mixture of 60–70% later peak and 30–40% early peak.

The question of the identity of the extra component at lower pH remains. The results of electrophoresis indicate an unchanged net anionic charge. This appears to rule out protonation of a carboxyl group, which could give a neutral species. One possibility is protonation of both imino nitrogens of a structure involving a dihydroxy or technetium oxygen double bond with retention of the configuration of the carboxylate groups around the technetium. This would be consistent with expected protonation of the imino nitrogens at pH values below their pK_a of 6.1 (*7*), and also with the appearance of only a single additional component that can quickly return to the single structure of higher pH. Such a structure would also retain the net negative charge as observed. The structure, however, would involve eight-membered chelate rings, which are not favored, but are known (*8*). Another possibility would involve one carboxyl group and the imino nitrogen of each iminodiacetate moiety bound to the technetium with the other carboxylate group unbound. The pK of such carboxylic acid groups is less than 3, however, and no driving force for binding to the technetium would be expected over a pH 4 to pH 6 range. A final possibility would reflect the structure proposed by Loberg and Fields (*6*) of two iminodiacetate moieties and one technetium in each case but with

changes in chloro, aquo, or hydroxo groups in a seven or more coordinate structure. Characterized complexes of technetium include seven and eight coordinate as well as the more common six (*9*). Increasing pH would then be expected to result in a displacement of chloride if chloride ions were part of the complex; or if an aquo group were part of the structure, conversion to a hydroxyl ligand, although the latter possibility would result in increased negative charge on the complex. Changes in simple groups would, however, be consistent with the small HPLC elution difference, the rapidity with which the conversion takes place upon raising the pH, and the decreased lipophilicity indicated by the direction of the HPLC retention time change.

The effect of pH on the radiochemical purity resulting from the chelation of technetium by phenethyliminodiacetic acid was studied by Fields, et al. (*7*). A shift to longer retention times at higher pH was observed when pH 4 and pH 8 preparations were compared. This finding is in the opposite direction to our observations. However, their elution time differences between major peaks were 7 min while ours differ by 1.5 min. Although direct comparisons are not possible, it seems that their change, which shows increased lipophilicity, may indicate components differing in technetium-to-ligand ratios, whereas our conversion indicates a smaller change in increased polarity, which may be more consistent with small group changes in a seventh coordination position.

FOOTNOTES

* New England Nuclear, North Billerica, MA.
† Diagnostic Isotopes.
‡ Amersham Corp (A) and Solco Nuclear (S).
‖ Altex Scientific.

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REFERENCES

1. FIELDS AT, PORTER DW, CALLERY PS, et al: Effect of ligand structure and radiolabeling methodology on radiochemical purity of Tc-iminodiacetates. *J Nucl Med* 19: 694, 1978 (abst)
2. FRITZBERG AR, HUCKABY D: Development and results of routine quality control procedures for Tc-99m iminodiacetate hepatobiliary agents. In *Radiopharmaceuticals II, Proceedings of 2nd International Symposium on Radiopharmaceuticals, Seattle, WA,* VJ Sodd, DR Allen, DR Hoogland, et al, Eds. 1979, pp 545-554
3. KLINGENSMITH WC, FRITZBERG AR, KOEP LJ: Comparison of Tc-99m-diethyl-iminodiacetic acid and I-131 rose bengal for hepatobiliary studies in liver-transplant patients: Concise communication. *J Nucl Med* 20: 314-318, 1979
4. FRITZBERG AR, WHITNEY WP, KLINGENSMITH WC: Hepatobiliary transport mechanism of Tc-99m-N, α-(2,6-diethylacetanilide)-iminodicarboxylic acid (Tc-99m-diethyl-IDA). In *Radiopharmaceuticals II, Proceedings of 2nd International Symposium on Radiopharmaceuticals, Seattle, WA,* VJ Sodd, DR Allen, DR Hoogland, et al, Eds. 1979, pp 557-586
5. FONDA U, PEDERSEN B: ^{99m}Tc-diethyl-HIDA. A contribution to the study of its structure. *Eur J Nucl Med* 3: 87-89, 1978
6. LOBERG MD, FIELDS AT: Chemical structure of technetium-99m-labeled N-(2,6-dimethylphenylcarbamoylmethyl)-iminodiacetic acid (Tc-HIDA). *Int J Appl Radiat Isot* 29: 167-173, 1978
7. FIELDS AT, PORTER DW, CALLERY PS, et al: Synthesis and radiolabeling of technetium radiopharmaceuticals based on N-substituted iminodiacetic acid: Effect of radiolabeling conditions on radiochemical purity. *J Label Comp Radiopharm* 15: 387-395, 1978
8. MARTELL AE, CALVIN M: *Chemistry of the Metal Chelate Compounds,* New York, Prentice-Hall, Inc, 1952, pp 138-139
9. DEUTSCH E: Inorganic Radiopharmaceuticals. In *Radiopharmaceuticals II, Proceedings of 2nd International Symposium on Radiopharmaceuticals, Seattle, WA.* VJ Sodd, DR Allen, DR Hoogland, et al. Eds. 1979, pp 129-146