Electrophoretic Analysis of Different Technetium-99m (SnCl₂) Methylene Diphosphonate Complexes

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Technetium-99m MDP was prepared from MDP kits from several different sources. The resulting [⁹⁹ᵐTc]MDP preparations were analyzed by electrophoresis. The results demonstrated the presence of at least four different ⁹⁹ᵐTc complexes in these preparations. Modified kit preparations were analyzed to show the effects of concentration and pH on formation of the impurities. The electrophoresis results were correlated with scintillation camera imaging studies in rabbits and suggest that hydrolysis of MDP to phosphate and methylphosphate results in formation of ⁹⁹ᵐTc complexes with poor biological behavior as bone scanning agents.

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**MATERIALS AND METHODS**

Diphosphonate complexes of technetium-99m (⁹⁹ᵐTc) have been shown to be effective bone imaging agents and have been used in clinical nuclear medicine for more than a decade (1). Although the chemical structure of these complexes have been under extensive investigation by Van Den Brand et al. (2) and others (3,4), the structure of these complexes is still not clearly understood.

Our laboratory compunds an “in-house” methylene diphosphonate (MDP) kit for clinical studies which contains 10 mg medronic acid and 1.15 mg stannous chloride in 1.0 ml saline, at a pH of 6.0. Each lot of kits is tested for sterility and pyrogenicity and two paper chromatograms are run on each labeled product before use (saline and acetone) to assure the absence of subsequent free pertechnetate and reduced hydrated unbound technetium. Nevertheless, we have noticed occasional liver uptake in bone scintigraphy which did not correlate with paper chromatography. The same problem has also been found with commercial kits (5). As a result, we investigated our [⁹⁹ᵐTc]MDP preparations for different complexes using electrophoresis. Also each complex was evaluated as a skeletal imaging agent.

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**MATERIALS AND METHODS**

Methylene diphosphonate and stannous chloride were purchased commercially.* Sterile water in all our MDP products was obtained† and electrophoresis was carried out‡ using acetate buffer pH = 7.0 and Whatman paper No. 1. Each test was run at 5 mA per strip and for a sufficient time (~2 hr) to permit the [⁹⁹ᵐTc]pertechnetate (reference strip) to travel 15 cm towards the anode. After electrophoresis, each paper was cut into 2.5 mm-wide segments and each segment was counted in a sodium iodide well detector.

To study the electrophoretic patterns, a total of nine MDP kits (three from Mallinckrodt, three from Squibb, and three “in-house”) were labeled with ~150 mCi of ⁹⁹ᵐTc in 3 ml of saline and electrophoresed.

To study the effect of MDP and SnCl₂ concentration we labeled a number of MDP kits containing different amounts of medronic acid and stannous chloride in 1 ml saline, pH = 6.0. Table 1 shows the amount of each chemical used. All kits were labeled with ~150 mCi of ⁹⁹ᵐTc in 3 ml of saline and analyzed.

To study the effect of pH, different MDP kits were compounded so that each contained 10 mg of medronic acid and 1.15 mg of stannous chloride at the following pH values: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. These kits were all labeled with ~150 mCi of ⁹⁹ᵐTc in 3 ml of saline and analyzed.

To study the effect of phosphate and methylphos-
phate, two sets of MDP kits were compounded as follows: The first set contained 10 mg medronic acid, 1.15 mg stannous chloride, and 5.0 mg sodium phosphate at pH = 6.0. The second set contained 10 mg medronic acid, 1.15 mg stannous chloride, and 5.0 mg methylphosphate which was synthesized using the method reported by Ford-Moore and Williams (6). Both sets were labeled with ~150 mCi of $^{99m}$Tc in 3 ml of saline.

To study the effect of age, a set of MDP kits were prepared containing 10 mg of medronic acid and 1.15 mg stannous chloride, and 1 ml of saline at pH = 6.0. Electrophoresis study was performed on two of the kits immediately after preparation and labeling with 150 mCi of $^{99m}$Tc in 3 ml of saline. The labeling was repeated when the kits were 1 and 2 mo old.

To measure the $p_Ka$ value of medronic acid, 200 mg of MDP were dissolved in 50 ml of distilled water and titrated with a solution of 0.1 N sodium hydroxide which was added from a burette. The pH of the solution and the volume of sodium hydroxide solution were recorded after addition of each drop.

To study possible oxidation of different $^{99m}$Tc complexes, reduced hydrated unbound technetium was prepared as follows: 2.0 ml of deoxygenated water were added to a 0.1 ml solution of 0.1 N hydrochloric acid containing 1.0 mg of stannous chloride. The solution was neutralized to about pH = 7 by addition of 0.05 N solution of sodium hydroxide. To this cloudy solution was added ~150 mCi of $[^{99m}$Tc$]pertechnetate in 0.5 ml saline. This product was subjected to the same electrophoretic conditions as the above experiments. High pressure liquid chromatography (HPLC) was performed on a specially prepared 5-mo-old MDP kit which lacked stannous chloride but contained medronic acid 10 mg and saline 1 ml, pH = 7.0; HPLC was also performed on methylphosphate. This was done by using DEAE ion exchange column and a solution of 0.1 N phosphate 0.2 N sodium chloride pH = 7.0 as the mobile phase with flow rate of 0.5 ml/min and chart rate of 0.5 cm/min.

Normal rabbits of about 7.0 kg weight were injected i.v. 2–3 mCi of different MDP samples. Anterior and posterior views were recorded 2 hr postinjection by using a MEDX HS-37 gamma camera and collection of 600,000 counts for each image.

RESULTS

Electrophoresis results

Electrophoresis results on all three brands (Mallinckrodt, Squibb, and “in-house”) were consistent for each brand and a representative electrophoretogram is shown in Fig. 1. Mallinckrodt and in-house kits showed four major peaks (a), (b), (c), and (d) while Squibb kits showed at least one extra peak (e).

Effect of MDP and/or SnCl$_2$ concentration

The electrophoretic results using different amounts of medronic acid and stannous chloride shown in Table 1 were identical. A representative electrophoretogram is shown in Fig. 2B. These showed only two peaks (c) and (d). Therefore, the variation of concentration as described in Table 1 had no effect on the complex formation of MDP.
TABLE 1
Kit Formulation for Studying Effects of MDP and SnCl₂

<table>
<thead>
<tr>
<th>Concentration</th>
<th>MDP (mg)</th>
<th>SnCl₂ (mg)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.115</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.15</td>
<td>6.0</td>
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<td>5.75</td>
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<tr>
<td>20</td>
<td>0.115</td>
<td>6.0</td>
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</tbody>
</table>

Effect of pH

The electrophoresis results on the MDP kits at pH 5.5 or less were identical and showed only one peak which traveled the same as peak (c) (Fig. 2C). Similarly, the electrophoresis results on MDP kits with pH 6.5 or greater were identical and showed only one peak which is identical to peak (d) (Fig. 2A). The result of this experiment on MDP with pH = 6.0 (Fig. 2B) shows two peaks (c) and (d).

Effect of phosphate and methylphosphate

The electrophoresis results on MDP samples which had phosphate or methylphosphate as impurities showed multiple peaks (Fig. 3, A and B).

Effect of age

The electrophoresis results on freshly compounded MDP kits (Fig. 4A) shows only two peaks, (c) and (d). The results on 1- and 2-mo-old kits are shown (Fig. 4, B and C). These results show an increase in concentration.

FIGURE 2
Electrophoresis results on modified MDP kits at pH = 6.5 and more, pH = 6.0, and pH = 5.5 and less. All samples were produced by using freshly prepared MDP kits which were labeled with 150 mCi of 99mTc in 3 ml of saline.

FIGURE 3
Electrophoresis results on modified MDP kits with methylphosphate and phosphate as impurities.
of peak (a) and (b) as a function of the age of the kit.

Titration of MDP

The results of titration of medronic acid described in Methods are summarized showing pk$_3$ of about 7.0 (Fig. 5). This confirms the results reported previously (3).

Electrophoresis of unbound TcO$_2$

The reduced unbound technetium was subjected to the same electrophoretic conditions as the above experiments. The result of this experiment are shown, dem-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**FIGURE 4**
Electrophoresis results on different age MDP kits, which show increase in concentration of peak (a) and (b) as function of age of kit

![Graph D](image4)

**FIGURE 5**
Titration of medronic acid which shows pk$_3$ of ~7.0

...onstrating little or no electrolytic oxidation of technetium during this experimental condition (Fig. 6).

Imaging

The images obtained using samples whose electrophoresis results were presented in Figs. 2A, 2C, 3A, and 3B are shown in Figs. 7, 8, 9, and 10, respectively. Comparison of Figs. 7 and 8 suggests that both complexes (c) and (d) have similar skeletal imaging property. Likewise, Fig. 10 does not show anything significantly different from Figs. 7 and 8 which suggest peak (b) also has almost the same skeletal imaging property as peaks (c) and (d). The imaging studies performed on the commercial MDP samples revealed similar and normal skeletal images. HPLC results (Fig. 11) on aged MDP kits and methylphosphate using ion exchange column confirmed the existence of methylphosphate in aged kits. However, as illustrated in Fig. 9, excess of peak (a) in the sample causes accumulation of some activity in the liver.

DISCUSSION

Electrophoresis shows that not only our nonlyophilized "in-house" MDP kits, but also the MDP kits from Mallinckrodt and Squibb consist of at least four different complexes when labeled with $^{99m}$Tc. This may not be
FIGURE 6
Electrophoresis result on "reduced unbound" technetium demonstrating little or no electrolytic oxidation of technetium "Reduced Technetium"

surprising since most of the weak chelating agents are likely to exhibit similar behavior. However, the question immediately arises as to which complex has the best skeletal imaging properties. Unfortunately, it is difficult to do preparative work with electrophoresis to study the biological behavior of each complex individually. In addition, the HPLC analysis on no-carrier-added $^{99m}$Tc-labeled MDP using ion exchange columns has been reported to exhibit only one peak (3). Therefore, we chose to use electrophoresis in our experiment and attempted to find the condition under which only one peak forms. Initially, the effect of concentration and/or molar ratio of MDP compared with stannous chloride was studied. Several MDP kits containing different amounts of MDP and stannous chloride at pH = 6.0 were prepared. Electrophoresis on labeled products show the presence of only two peaks, (c) and (d). Peaks (a) and (b) were not observed at all. This is interesting and surprising since the formulation of some of the kits employed in this experiment are identical to the formulation we use to compound our in-house kits, [already shown to consist of four peaks (Fig. 1C)]. Repetitive electrophoresis studies revealed (Fig. 4, A–C) that this difference was due to the age of the kit, i.e., in a freshly prepared kit the concentration of peaks (a) and (b) are very little; however, with age, the concentration of the peaks (a) and (b) increases. This strongly suggests that some chemical processes are taking place in the MDP kit even before addition of pertechnetate. Consequently, possible hydrolysis of methylene diphosphonate to phosphate and methylphosphate was investigated. It is known that neither phosphate nor methyl phosphate form soluble peaks with either Sn(II) or Tc(IV), although when a mixture of either phosphate or methylphosphate with MDP was labeled with $^{99m}$Tc an increase in quantity of peak (b) and peak (a) was observed, respectively. In both

FIGURE 7
Posterior view of rabbit imaged with Sample 2A

FIGURE 8
Posterior view of rabbit imaged with Sample 2C
cases very little of peak (c) and (d) was observed. This suggests that the presence of peak (a) and (b) is due to the presence of methylphosphate and phosphate respectively, both of which can be produced by hydrolysis of methylene diphosphonate.

\[
\text{HO—P—CH}_2—\text{P—OH} + \text{H}_2\text{O} \rightarrow \text{HO—P—OH} + \text{CH}_3—\text{P—OH}
\]

In another study we demonstrated that the pH can effect complex formation of MDP with $^{99m}$Tc. We labeled MDP using standard Sn(II) reduction at pH ranging from 4.5 to 7.5. Electrophoresis on these samples showed that at pH below 5.5 formation of peak (c) (Fig. 2C) and at pH above 6.5 formation of peak (d) (Fig. 2A) is favored, while both peaks are favored at pH = 6.0 (Fig. 2B). It is reported that at ionic strength of 0.1 and 0.25, $pK_3$ of MDP is 7.00, and 6.88, respectively (7). Physiological saline which is the solution of interest in our experiment has ionic strength of about 0.15. This means that $pK_3$ of MDP in our media is $\sim$ 6.96. The result of our titration of MDP (Fig. 5) is very similar with the result reported by Tanabe et al. (4) confirming the $pK_3$ of about 7 but also showing that MDP begins to lose its third acidic hydrogen at about pH 6. This is consistent with
our observation on the complex formation of MDP. This indicates that at pH below 6, \( \text{MDP}^{-2} \) is a responsible species for complex formation, however at pH 6 and above \( \text{MDP}^{-3} \) is available for complex formation and has a higher formation constant than \( \text{MDP}^{-2} \).

As far as skeletal imaging is concerned, peaks (b), (c), and (d) have similar and not significantly different properties (Figs. 7, 8, and 10). However, the presence of methylphosphate in MDP kits results in an increase of peak (a) which in turn results in accumulation of activity in the liver. We wish to emphasize that none of the samples, including Sample 3A, had reduced hydrated unbound activity using standard chromatography systems. Also, electrophoresis results on reduced hydrated unbound technetium (Fig. 6) eliminates the possibility of electrolytic oxidation of technetium under these experimental conditions. Therefore, observations of occasional liver uptake in bone scintigraphy may be due to presence of methylphosphate in MDP kits; however, further investigation in this area is needed.

**FOOTNOTES**

* Sigma Chemical Company, St. Louis, MO.
† Travenol Laboratories, Inc., Deerfield, IL.
‡ Gelman Deluxe Electrophoresis Chamber Model 51211 and Health kit regulated power supply Model PS-4. Gelman Sciences, Inc., Ann Arbor, MI.
§ Waters Associates, Milford, MA.

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**REFERENCES**