

Study on ^{99m}Tc-Labeling Mechanism of Bz-MAG3

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Bz-MAG3의 테크네슘표지 기전에 관한 연구

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^{99m}Tc-MAG3는 세뇨관 배설을 하는 신장 기능 영상용 방사성의약품으로서, 가운데 ^{99m}Tc이 있고 1개의 유황과 3개의 질소가 아래쪽 면에 정사각형으로 배치되고 정점에 산소가 위치한 4각 피라미드형을 하고 있다. 그러나 시판되는 신장 영상용 MAG3는 반응성이 강한 -SH기에 의한 부산물 생성을 방지하기 위해 benzoyl기로 보호되어 있으므로 보통 조건으로는 잘 표지되지 않고 10분 정도 끓여 주어야 한다. 본 실험에서는 실온에서도 benzoyl기를 잘 끊어지게 하는 이미다졸, 아그마틴, 피리딘 등과 같은 염기성 촉매제를 가하고 여러가지 pH에서 실온에서 반응시켜 본 결과, benzoyl기가 끊어지면서 ^{99m}Tc과 -SH기가 반응하는 첫번째 반응이 일어난 후에도 산성에서 약알칼리성 범위에서는 중간체가 상당량 생성되고 알칼리성(pH 10.5)이 되어야 최종 산물로 재빨리 넘어가는 것을 발견하였다. 또한 glucarate, medronate, phthalate, acetate 등 여러가지 다른 transchelating agent를 사용하여 같은 반응을 시킨 후에도 생성된 중간체의 HPLC 피크가 같은 retention time (Rt)을 보이는 것으로 보아 이 중간체의 화학구조내에는 transchelating agent를 포함하지 않는 것으로 보인다. 최종 생성물과 중간체의 1mM 시트테인 용액 중에서의 안정도 시험을 하여 본 결과 최종 생성물은 매우 안정하나 중간체는 불안정한 것을 발견하였다.

주요 단어 : MAG3, ^{99m}Tc-labeling, General base catalysis

INTRODUCTION

^{99m}Tc(V)-MAG3 is an excellent renal imaging agent replacing for ¹³¹I or ¹²³I labeled o-iodohippuric acid¹⁻⁴). Its chemical structure has been reported as square pyramidal oxotecnium (V) chelated with one sulfur and three nitrogen groups of mercaptoacetyltiglycine resulting in net -2 charge⁵). To protect highly reactive sulfhydryl group of MAG3, benzoylthioester of MAG3 (Bz-MAG3) is used as ligand. To label Bz-MAG3 with ^{99m}Tc, the benzoyl

group should be removed by harsh condition-boiling for 10 min. In spite of its excellent stability of ^{99m}Tc complex, this boiling procedure restrained using Bz-MAG3 as a bichelating agent for labeling antibody or other heat-sensitive proteins.

A kit was made using benzoyl group removed MAG3 and showed good labeling yield and purity⁶). According to the results obtained by benzoyl group removed MAG3, high pH(>10) and enough incubation time (15 min) is necessary to complete the reaction. And excess benzoyl group removed MAG3 produced side product⁷.

According to the chemical structure of ^{99m}Tc -MAG3, we can predict that benzoyl group of Bz-MAG3 should be removed first, then sulfhydryl group of MAG3 will bind to ^{99m}Tc quickly because of its high nucleophilicity, finally nitrogen atoms of three amide groups will bind successively. Although the nitrogens of amide groups are very poor nucleophiles, the electrophilicity of reduced technetium and chelate effect will drive the reaction to finish⁸). And the final product might be very stable.

At this point we can think about certain base catalyzer that can accelerate the labeling speed of ^{99m}Tc to Bz-MAG3 by increasing the rate of hydrolysis of thiobenzoyl ester and the basicity of amide nitrogen at room temperature. If the catalyzer can improve the labeling speed enough, then possibly we can use the Bz-MAG3 as bichelating agent for antibody or heat-sensitive proteins. We labeled Bz-MAG3 at various pH, using different kind of base catalyzers and transchelating agents at room temperature, and found that some base catalyzers can accelerate the formation of intermediate and the intermediate can be converted to final product quickly at high pH (10.5).

MATERIALS AND METHODS

1. ^{99m}Tc -Labeling of MAG3

Bz-MAG3 is synthesized as reported⁹). Ten μl of 10 mg/ml Bz-MAG3 solution in dimethylsulfoxide (DMSO), 40 μl of 0.4 M glucarate (pH 5.6), 10 μl of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (1.5 mg/ml in 0.02 M HCl) and 200 μl of ^{99m}Tc -pertechnetate (1~2 mCi) were mixed in air-tight rubber-capped vial.

Forty μl of 1 M imidazole solutions pH adjusted to 10.5 (1 M sodium carbonate-bicarbonate), 8.5 (1 M sodium bicarbonate), 7.5 (1 M Sodium phosphate) or 6.5 (adequate amount of concentrated HCl) were added. After addition of all the reagents the vials were evacuated and incubated at room temperature. In stead of imidazole, same amount of other

catalyzers-1 M (4-aminobutyl) guanidine (Agmatine, Aldrich) pH adjusted to 12 by addition of 10 M NaOH or 1 M pyridine (pH 8.5 in 1 M sodium phosphate)-were used to test the catalysis mechanism. Glucarate was replaced by 0.4 M sodium phthalate (pH 5.6), 0.4 M sodium acetate (pH 5.6) or 0.4 M methylene diphosphonate (MDP, pH 5.6) to test the chemistry of transchelation and intermediate.

2. TLC of ^{99m}Tc -Labeled MAG3

To check the colloid formation ITLC (1 \times 10 cm, Gelman) was performed using 50% ethanol in 10% ammonium acetate as solvent. In this condition all the radioactivities except colloid were moved to solvent front. Reverse-phase TLC (RP-TLC, Analtech) was performed using 0.01 M sodium phosphate (pH 6.7) as solvent to check the formation of other components those are not eluted through HPLC column. All the ITLC and RP-TLC plates were scanned by TLC scanner (Imaging Scanner System 300, Bioscan).

3. HPLC of ^{99m}Tc Labeled MAG3

Five μl of ^{99m}Tc -labeled products were taken at each time point and frozen by dry ice immediately. Frozen samples were diluted with 200 μl of D.W. and 5~10 μl was injected to HPLC. Deltapak C18 reverse phase cartridge (10 mm \times 10 cm) in Waters RCM module was used. Gradient solvent system was used: 0% ethanol and 100% 0.005 M sodium phosphate (pH 6.7) for 4 min, ethanol was increased to 35% until 30 min and remained constant for five minutes, and decreased to 0 % for 1 minute. Flow rate of mixed solvent was 1 ml/min. Radioactivity was determined by NaI radioactivity detector. Each peak was cut by scissors and weighed to determine peak area.

4. Stability Test of Intermediate

Intermediate (peak 2) was made by same method described above by incubating at pH 8.5 with 0.1 M imidazole for 2 hr at room temperature. Five μl of each intermediate aliquot was added to 200 μl of 0.1

M sodium actate (pH 4.5), 0.1 M sodium phosphate (pH 7.0), 0.1 M sodium carbonate-bicarbonate buffer (pH 10.5) or 1 mM Cystein solution and incubated at room temperature.

Twenty μl of each incubation mixture was taken at each time and frozen by dry ice immediately. Each frozen sample was thawed and injected to HPLC immediately.

RP-TLC of ^{99m}Tc -MAG3 showed Rf 0.6 and ^{99m}Tc -glucurate showed Rf 1.0(Fig. 1). Other products

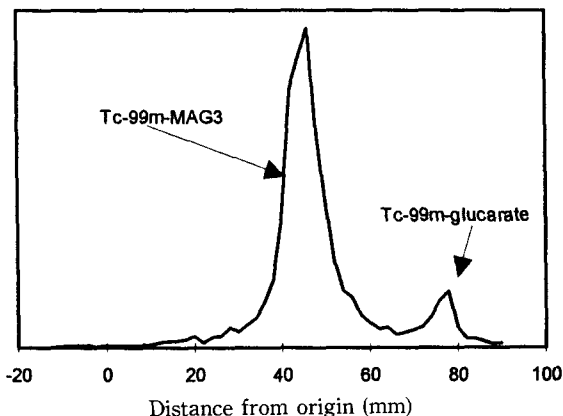


Fig. 1. Reverse phase TLC profile of ^{99m}Tc -MAG3 labeled at room temperature at pH 10.5 with 0.1 M imidazole for 1 hr.

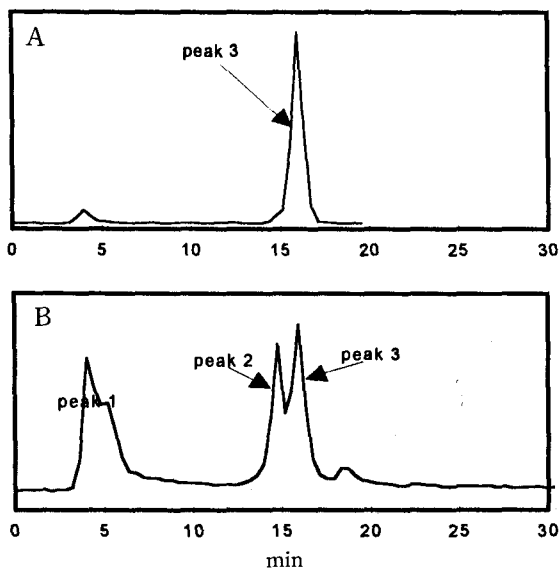


Fig. 2. HPLC profil of ^{99m}Tc -MAG3. X-axis shows time (min) and Y-axis shows radioactivity (arbitrary unit). A: Mallinkrodt Bz-MAG3 was labeled by boiling as company protocol, B: Bz-MAG3 was labeled in the presence of glucurate at room temperature at pH 11 for 1 hr.

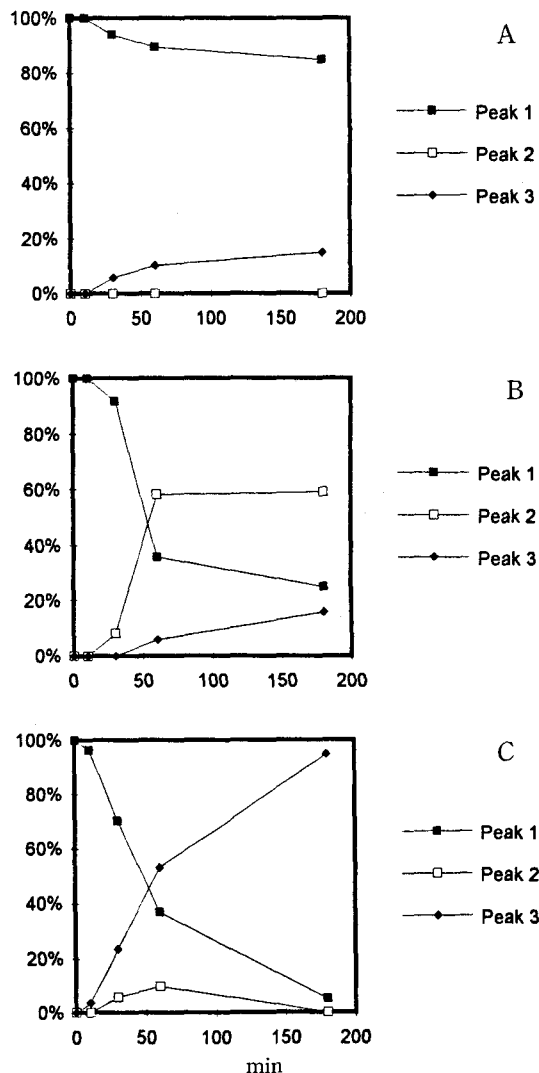


Fig. 3. Change of the peak ratio of each HPLC peak. X-axis shows time (min) and Y-axis shows the percentage of each peak. Bz-MAG3 was labeled at room temperature in the presence of glucurate at A: pH 6.5, B: pH 8.5, and C: pH 11.5.

could not be identified by our RP-TLC condition. Commercialized Technescan MAG3 from Mallin-

krodt labeled by the company protocol showed Rt 16 min on HPLC (Fig. 2A). But when Bz-MAG3 was labeled in the presence of glucarate at room temperature at pH 10.5 for 1 hr it showed three major peaks: peak 1, Rt 4.5 min; peak 2, Rt 14.5 min; peak 3, Rt 16 min (Fig. 2B). Peak 1 is believed to be mixture of ^{99m}Tc-glucarate and some kind of intermediate, peak 2 is intermediate, and peak 3 is the same chelate with the renal agent. When the Bz-MAG3 was labeled at room temperature at pH 6.5, peak 2 was not found and formation of peak 3 was very slow (Fig. 3A). When incubated at pH 8.5, formation rate of peak 2 was increased but formation rate of peak 3 was not increased (Fig. 3B). By raising incubation pH to 11.5 we could see much faster formation of peak 3 but

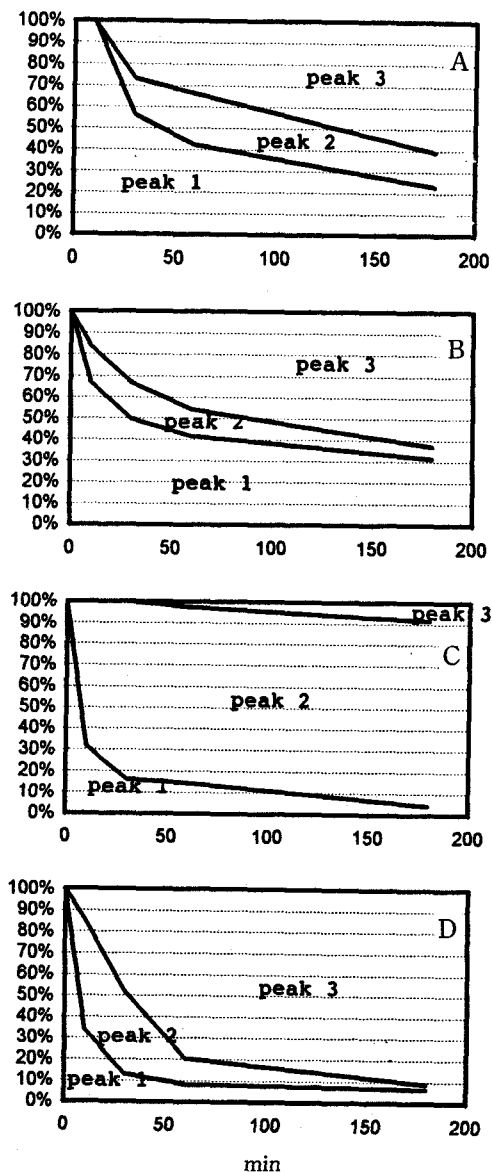


Fig. 4. Cumulative plotting of radioactivities of each peak by time. X-axis shows time (min), Y-axis shows percentage, and area between each curve shows the proportion of each peak. Bz-MAG3 was labeled at room temperature in the presence of 0.1 M imidazole and glucarate at A: pH 6.5, B: pH 7.5, C: pH 8.5 and D: pH 10.5.

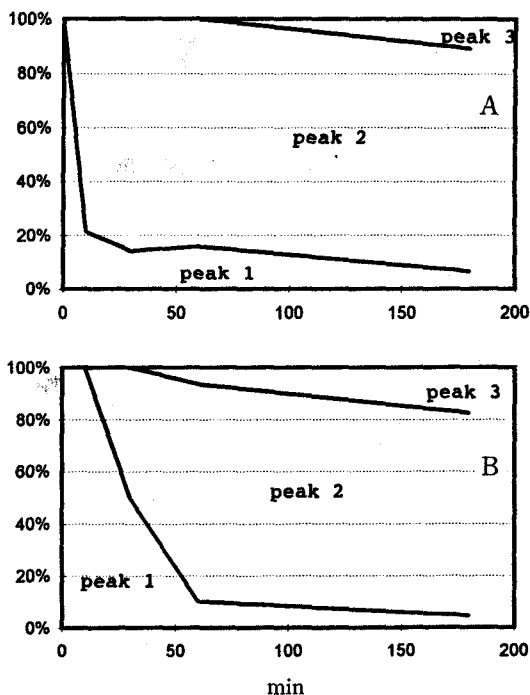


Fig. 5. Cumulative plotting of radioactivities of each peak by time. X-axis shows time (min), Y-axis shows percentage, and area between each curve shows the proportion of each peak. Bz-MAG3 was labeled at room temperature in the presence of glucarate and A: 0.1 M agmatine and B: 0.1 M pyridine. The final pH of each reactant was 9.0 and 8.5, respectively.

very little amount of peak 2 (Fig. 3C).

Imidazole is a well-known acid or base catalyzer at physiological condition because its pI is within physiological pH. To increase the reaction rate of the ^{99m}Tc labeling of Bz-MAG3, we added pH-adjusted imidazole solution to reacton mixture and checked the formation of each peak. Final concentration of imidazole was 100 mM and pH's were 6.5, 7.5, 8.5 and 10.5. Comparing with Fig. 3A when imidazole was added peak 1 disappeared much faster, peak 3 formed much faster, and we could see peak 2 at pH 6.5 (Fig. 4A). At pH 7.5 there was no prominent difference with pH 6.5 but formation of peak 3 from peak 2 slightly increased (Fig. 4B). We could see a very rapid disappearance of peak 1, extensive accumulation of peak 2, and a little forma-

tion of peak 3 at pH 8.5 (Fig. 4C). At pH 10.5 peak 1 disappeared similar speed with pH 8.5 but the main product was peak 3 (Fig. 4D).

In stead of imidazole we tested (4-aminobutyl) guanidine (agmatine, Aldrich) that contains quaternary and primary amine and pyridine that contains heterocyclic amine to catalyze the reaction. The result shows that both the agmatine and pyridine catalyze the formation of peak 2 but don't catalyze the formation of peak 3 (Fig. 5).

To see the pH dependence of the formation of peak 3 from peak 2, peak 2 was prepared by incubation of Bz-MAG3 with ^{99m}Tc -glucarate in the presence of 100 mM imidazole at pH 8.5 for 2 hrs. The resulting reaction mixture was added to 0.1 M carbonate-bicarbonate buffer (pH 10.5), 0.1 M

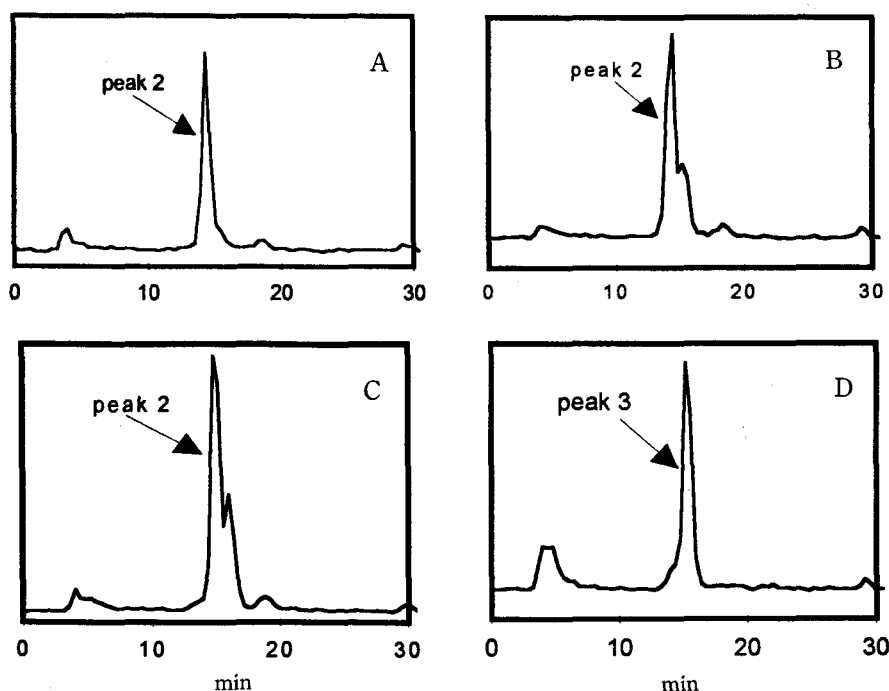


Fig. 6. HPLC profiles showing pH dependent change of intermediate peak. X-axis shows time (min) and Y-axis shows radioactivity (arbitrary unit). A: Intermediate peak was made by incubating Bz-MAG3 with ^{99m}Tc in the presence of glucarate and 0.1 M imidazole at pH 8.5 for 2 hrs. Intermediate peak was incubated at B: pH 4.5 for 30 min, C: pH 7.0 for 30 min, and D: pH 10.5 for 1 min.

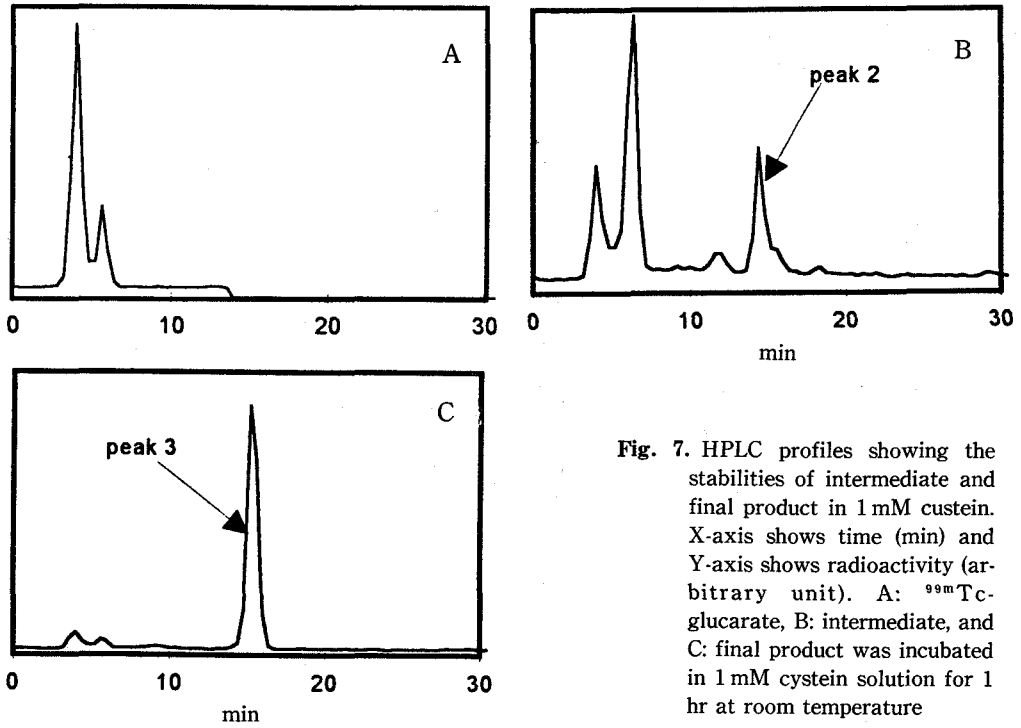


Fig. 7. HPLC profiles showing the stabilities of intermediate and final product in 1 mM cysteine. X-axis shows time (min) and Y-axis shows radioactivity (arbitrary unit). A: ^{99m}Tc -glucarate, B: intermediate, and C: final product was incubated in 1 mM cysteine solution for 1 hr at room temperature

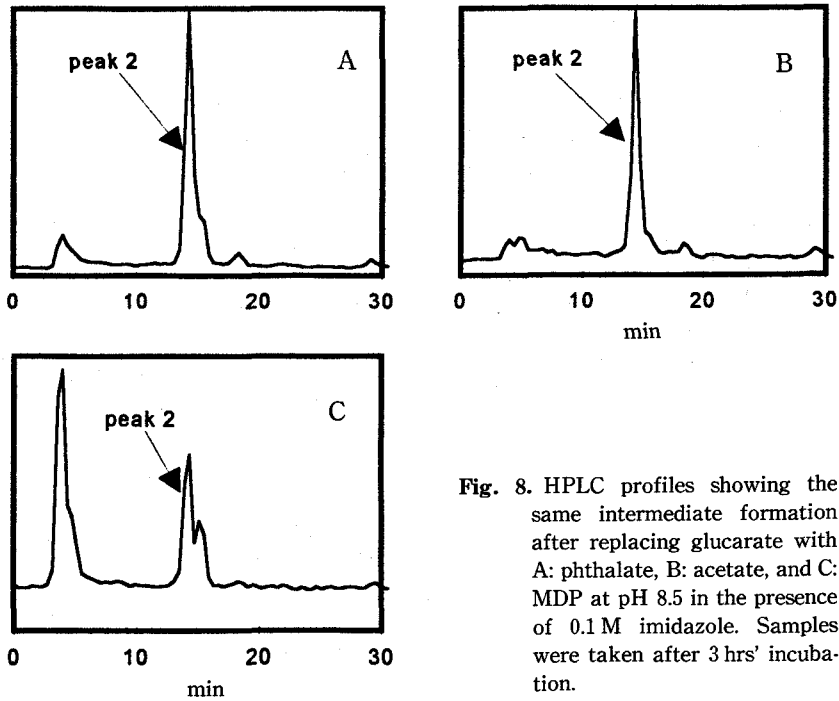


Fig. 8. HPLC profiles showing the same intermediate formation after replacing glucarate with A: phthalate, B: acetate, and C: MDP at pH 8.5 in the presence of 0.1 M imidazole. Samples were taken after 3 hrs' incubation.

sodium acetate buffer (pH 4.5) and 0.1 M sodium phosphate buffer (pH 7.0) and incubated 1 min or 30 min and tested by HPLC. At pH 10.5 the peak 2 converted to peak 3 completely in 1 minute (Fig. 6B), and 10% and 25% of peak 2 converted to peak 3 at pH 4.5 and pH 7.0, respectively, after 30 minutes (Fig. 6 CD). According to this result, peak 2 is most stable at pH 8.5, at lower pH it is converted to peak 3 slowly, at higher pH it is converted to peak 3 very quickly. This result is consistent with Fig. 3 that shows faster formation of peak 3 in spite of slower formation of peak 2 at pH 6.5 and 7.5 than at pH 8.5

Stabilities of peak 2 and peak 3 in 1 mM cysteine solution were tested after making them with above condition. Peak 3 was very stable (Fig. 7C) but peak 2 was not stable in 1 mM cysteine solution (Fig. 7B). Peak 2 was converted to 3 other peaks showing earlier retention time (4.1, 6.4 and 12 min, Fig. 7B).

We tested phthalic acid, acetic acid and methylene diphosphonate (MDP) in stead of glucarate as a transchelating agent to see whether they will make same intermediate and found same Rt intermediate with that of glucarate (Fig. 8). Phthalic acid and acetic acid showed somewhat similar product formation rate with glucarate, although acetic acid produced 73% colloid (Fig. 9AB).

However MDP showed much slower decrease of peak 1 and slower increase of peak 2 (Fig. 7C).

DISCUSSION

We can consider two possibilities about the ^{99m}Tc-labeling reaction of Bz-MAG3 at pH 6.5 at room temperature. One is that the formation of peak 2 is rate determining step to go final product (peak 3), and the other is that the final product is formed without making intermediate (peak 2). It was found that the first hypothesis is correct by successive experiments. The fact that formation of peak 2 was increased and peak 3 was not increased (Fig. 3B)

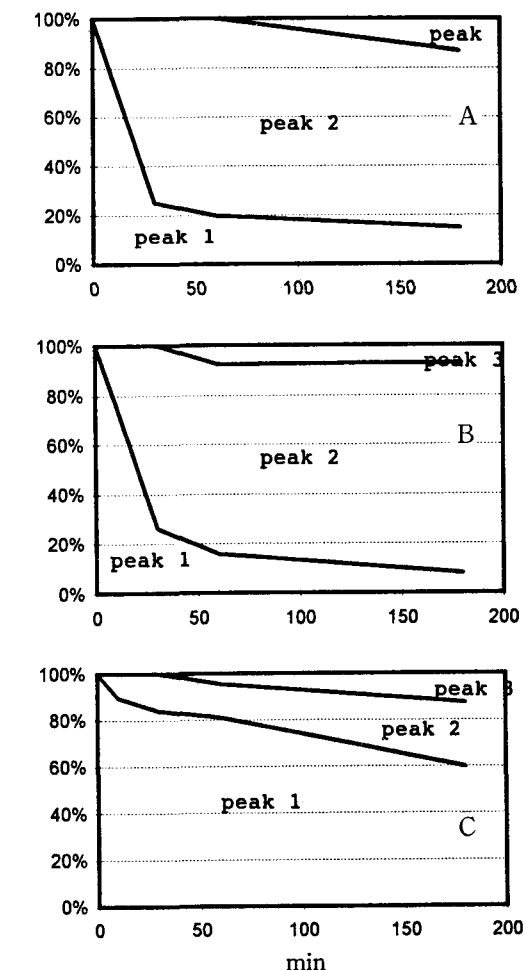


Fig. 9. Cumulative plotting of radioactivities of each peak by time. X-axis shows time (min), Y-axis shows percentage, and area between each curve shows the proportion of each peak. A: Phthalate, B: Acetate and C: MDP was used in stead of glucarate. Incubation was done at pH 8.5 in the presence of 0.1 M imidazole at room temperature.

means that the rate determining step is formation of peak 3 from peak 2 at pH 8.5. However, it was found that both the formation of intermediate and final product were increased but the intermediate was not accumulated because of its conversion to final product was fast at pH 11.5 (Fig. 3C)

It was found that imidazole catalyses the early

step of labeling so that intermediate formation and successive final product formation increase. Imidazole acts as strong catalyzer for formation of peak 2 however doesn't catalyze formation of peak 3 from peak 2. Formation of peak 3 was dependent to pH. Agmatine and pyridine catalyzed formation of peak 2. These results means that the formation of intermediate is general base catalyzed reaction, however the formation of final product is not general base catalyzed reaction but pH dependent reaction.

Peak 3 was stable but peak 2 was not stable in 1 mM cystein solution. This result can be related with direct ^{99m}Tc -labeled antibody. ^{99m}Tc is bound to sulfhydryl group of antibody and become more stable by making complex with other nearby functional groups in direct ^{99m}Tc -labeled antibody. If the functional groups are amide groups then the situation is very similar to MAG3. In that case the stability of direct ^{99m}Tc -labeled antibody will show variable stabilities in cystein solution by different incubation pH or time. Actually it has been reported that direct ^{99m}Tc -labeled antibody shows different stability in cystein solution by features of antibody or labeling method⁹⁾.

All the other transchelating agents, e.g. phthalic acid, acetic acid, and MDP produced same intermediate with that of glucarate. This means that the chemical structure of intermediate does not contain transchelating agent as ligand. So the speed of conversion from intermediate to final product may not be dependent to transchelating agent.

CONCLUSION

In this experiment, it was found that ^{99m}Tc -labeling Bz-MAG3 occurs in several steps at room temperature. The first step is general base catalysis reaction but the last step is dependent only to pH.

Addition of general base catalyzer showed ac-

cumulation of intermediate that is unstable in 1 mM cystein solution. Finally transchelating agent itself is not included in intermediate.

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