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Liquid Chromatography-Mass Spectrometry to Monitor the Labeling Process Stability and Identify Derivatives during the Preparation of ^{188}Re-MN-16ET: A Radiopharmaceutical for Hepatoma

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Abstract

The labeling process stability of a radiopharmaceutical, radio isotope rhenium-labeled *N*-[2-mercaptoethyl]-3-aza-19-ethyloxycarbonyl-3-[2-mercaptoethyl] octadecanoate (^{188}Re-MN-16ET) for treatment of hepatocellular carcinoma (HCC) from its precursor, triphenylmethyl (Ph_3C-) group protected-MN-16ET with $^{188}ReO_4^-$ was surveyed and identified the derivatives from both reactant and product. After incubation for various specified periods of up to 6 h under various labeling reaction conditions, including acetic acid or mixed with normal saline, room temperature or a reaction temperature of 100°C, and the radiolytic effect of Re-188, the levels of protected-MN-16ET and Re-MN-16ET in solutions were analyzed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to study the impacts of the labeling process on their structural stabilities and the derivatives that arise from decomposition when preparing ^{188}Re-MN-16ET from stock solution. This information is the key to determine whether ^{188}Re-MN-16ET is structurally accurate and suitable for its intended use. The results showed that the major factor that impacts the structural stability of the product, Re-MN-16ET, is $^{188}ReO_4^-$; however, a thermal (100°C) effect on the precursor, protected-MN-16ET, was also observed. After 1 h incubation with $^{188}ReO_4^-$ (100°C), the abundances of Re-MN-16ET and protected-MN-16ET were reduced by approximately 15% and 25%, respectively. The major decomposition products were Re-MN-16COOH and intra-molecular disulfide derivative. Moreover, the auto-radiolysis effect of Re-188 (60 mCi) on ^{188}Re-MN-16ET was not clearly observable until 140 h. On the other hand, a stability study of Re-MN-16ET in plasma showed that 8% remained after incubation for 1 h; the metabolite was Re-MN-16COOH, which is identical to the hepatic bio-transformed product.

Keywords: Labeling process stability; Re-MN-16ET; Radiolysis; Decomposed derivative; Radiopharmaceutical

Introduction

Hepatocellular carcinoma (HCC) is a tumor disease with a high fatality rate for humans worldwide; it is mainly found in Asian and Africa. In Taiwan, especially, HCC is the second leading cause of cancer death (2011), with around 7,000 deaths reported and 8,000 new cases yearly [1]. Although surgery is the major approach to cure of HCC completely, fewer than 10% of HCC patients in Taiwan can undergo direct surgery [1]. In general, it is necessary to integrate two or more methods to treat hepatoma in addition to surgery, depending on the stage and class of the tumor. Feasible treatment methods of HCC include transcatheter arterial embolization (TAE), radiotherapy, chemotherapy, and ablation to reduce the size of tumor and the side effects of medicines [1,2].

A ligand containing dithio-amido-amine tetradentate (N_2S_2) and long alkyl carboxylate ethyl ester functional groups, namely H3MN-16ET (*N*-[2-mercaptoethyl]-3-aza-19-ethyloxycarbonyl-3-[2-mercaptoethyl] octadecanoate), is an excellent chelator of transition metal ions (such as Re); it also dissolved readily in lipiodol (an iodinated ethyl ester of fatty acids derived from poppy seed oil that is applied by injection as a radio-opaque contrast agent [3]) to form Re-MN-16ET/lipiodol. If the radionuclide ^{188}Re is involved in the oily agent, ^{188}Re-MN-16ET/lipiodol can serve as a radiopharmaceutical with both β^- radiotherapy and TAE efficacies to treat of liver tumors [4-6]. Preclinical studies have demonstrated the feasibility of this treatment method, and Phase I clinical trials are currently being conducted.

Reversed phase high performance liquid chromatography-tandem mass spectrometry (RP-HPLC-MS/MS) analytical methods for Re-MN-16ET (the non-radioactive active pharmaceutical ingredient, or API) and its precursor, triphenylmethyl (Ph_3C-) group protected-H_3MN-16ET (protected-H_3MN-16ET), have been established; these methods have been applied to investigate the metabolism pathways of Re-MN-16ET by hepatic enzyme system and the forced degradation stability of protected-H_3MN-16ET respectively [7,8]. The results showed that protected-H_3MN-16ET is unstable under acidic/alkaline (0.05 M HCl or NaOH aqueous), thermal (80°C), and UV irradiation (254 nm) conditions for up to 24 h [8].

The reaction conditions used to prepare ^{188}Re-MN-16ET by labeling protected- H_3MN-16ET with Re-188 include a solution of acetic acid (70%), tartaric acid, normal saline, $SnCl_2$ aqueous buffer; $^{188}ReO_4^-$ (radioactivity 60 mCi) as the source of ^{188}Re; a temperature of 100°C; and a reaction time of 1 h (Figure 1) [4]. The issues that must be clarified for successful production of ^{188}Re-MN-16ET are whether the stock and product both resist transformation into derivative compounds; the identity of the derivatives; the cause of

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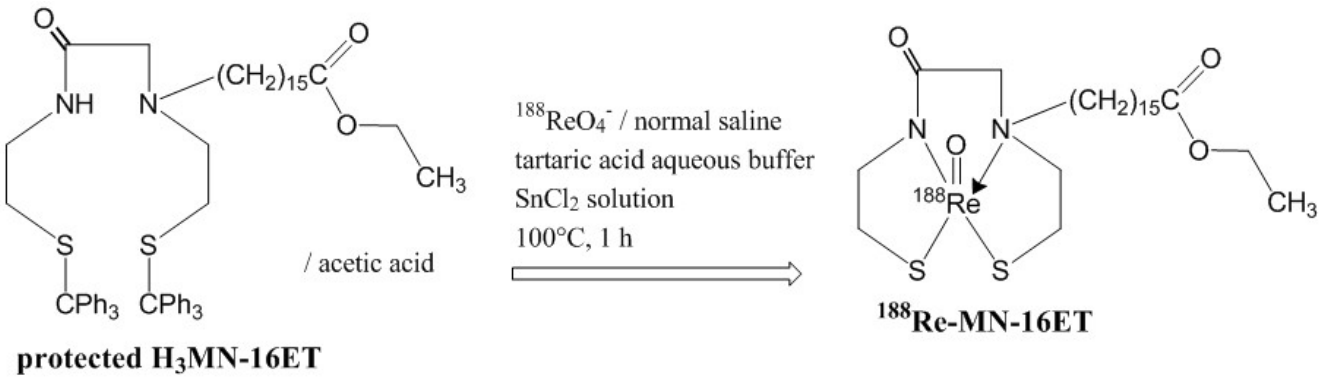

Figure 1: Scheme of the labeling process to prepare the API, ^{188}Re-MN-16ET, from the precursor, protected-H_3MN-16ET.

the transformations; and the exact total of ^{188}Re-MN-16ET produced. Furthermore, the radiolytic effect of Re-188 on ^{188}Re-MN-16ET itself to induce decomposition of the structure (auto-radiolysis) is another issue when considering the fate of ^{188}Re-MN-16ET in the liver, in addition to hepatic metabolization. These results are key to determine whether ^{188}Re-MN-16ET can safely and accurately execute its original intended mission and meet the requirements of 'Chemistry, Manufacturing, and Controls (CMC): "What you get is what you want" [9].

It is important to monitor the stability of a new chemical entity (NCE) in plasma. Information regarding the stability of an NCE in plasma enables the identification of clinical candidates with pharmacokinetic properties. An NCE that is rapidly degraded in plasma generally show poor *in vivo* efficacy [10,11].

In this study, the levels of protected-H_3MN-16ET and Re-MN-16ET (non-radioactive) were monitored, and their respective derivatives were identified using established RP- HPLC-MS/MS methods after incubation in acetic acid and normal saline media (at both room temperature and 100°C) respectively for up to 6 h to study the stabilities of the reactant and product of the ^{188}Re-MN-16ET labeling process. Furthermore, we sought to answer the questions of whether and how radioactive labeling agent $^{188}ReO_4^-$ affects the stabilities of both protected-H_3MN-16ET and Re-MN-16ET. After the Re-188 labeling process and lipiodol extraction to separate the aqueous and oil phases and obtain the product, ^{188}Re-MN-16ET / lipiodol, the auto-radiolysis effects of ^{188}Re (β^- particles [2.12 and 1.96 MeV] and a γ ray [155 keV] emitter with a half-life of 17.0 h) on ^{188}Re-MN-16ET were studied. There are few studies in the literature regarding the auto-radiolysis effects of labeling nuclides on radiopharmaceuticals (and their precursors), with the exception of F-18 (a positron-emitter) radio-medicines as positron emission tomography imaging agents [12,13]. Moreover, the stability of Re-MN-16ET in rat plasma was also surveyed.

Materials and Methods

Materials and reagents

Analytical-grade chemicals for LC-MS were used as received without further purification. Acetonitrile (HPLC and MS grade), acetic acid, and ammonium acetate were all purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Smart DQ3 reverse osmosis reagent water system (Merck Millipore, Billerica, MA, USA). *N*-[2-(triphenylmethyl) thioethyl]-3-aza-19-ethyloxycarbonyl-3-[2-(triphenylmethyl) thioethyl] octadecenoate (protected-H_3MN-16ET, the precursor) and non-radioactive Re-labeled ligand ($^{185/187}$Re-MN-16ET) were prepared as a yellowish, amorphous and red-brownish materials, respectively, by the Chemistry Division of the Institute of Nuclear Energy Research (INER, Taiwan). Both compounds were identified by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy using a 300 MHz Gemini 2000 instrument (Varian Inc.) and by infrared spectroscopy (FT-IR, Bio-Rad FTS-40). The purity criteria of the compounds were above 95% for quality control purposes using HPLC (Hitachi, L-7000). Radioactive $^{188}ReO_4^-$ dissolved in normal saline was obtained from an ^{188}W/^{188}Re generator. ^{188}Re-MN-16ET was obtained from an $^{188}ReO_4^-$ labeling process with protected-H_3MN-16ET and was extracted with lipiodol [5]. The radiochemical purity (RCP) of the ^{188}Re-MN-16ET/lipiodol product was analyzed to be >95%. Fresh rat plasma was acquired from healthy male Sprague-Dawley (SD) rats at 7 to 10 weeks of age (Bio LASCO Co, Taipei, Taiwan).

Instruments and methods

After incubation with acetic acid, normal saline and $^{188}ReO_4^-$ mixed solutions at both room temperature and 100°C for specified periods of time, respectively, the levels of $^{185/187}$Re-MN-16ET and protected-H_3MN-16ET were monitored by RP- HPLC (Chromolith® RP-18e end capped monolithic, Merck, Germany, 4.6 mm × 100 mm, and guard column) with diode array detection (DAD, wavelength at 250 nm) using Agilent ChemStation software (ed. 10.02); the identities of the derivatives were investigated using a tandem mass spectrometry system (4000 QTRAP®, AB Sciex, Concord, ON, Canada, with Analyst software 1.6.2). The HPLC-MS/MS analysis methods for protected-H_3MN-16ET and $^{185/187}$Re-MN-16ET were performed according to the established instruments parameters respectively [7,8]. The auto-radiolysis effect of Re-188 on ^{188}Re-MN-16ET was monitored over 140 h (6 days) by HPLC-tandem mass spectrometry of Re-MN-16ET. Moreover, the stability of non-radioactive Re-MN-16ET in SD rat plasma media and its metabolites were also investigated after incubation in rat plasma at 37°C for 4 h [10,11].

Results and Discussion

Labeling process stability of Re-MN-16ET

The retention time (t_R) of Re-MN-16ET on a C18 column was 23.1 ± 0.1 min with a theoretical plate number of 24,500 for HPLC analysis. The primary mass spectrum (MS^1) of non-radioactive $^{185/187}$Re-MN-16ET shows 675/677 with an intensity ratio of approximately 38: 62 (the isotopic abundance of Re); the product ion spectra of *m/z* 677 are 631, 613, 603, 395, etc. The structures of the fragment ions are shown in [7]. The HPLC-tandem MS analytical method for examination of the

metabolites of Re-MN-16ET was also applied to study of the stability of its labeling process with $^{188}ReO_4^-$.

Firstly, the chromatograms of Re-MN-16ET dissolved in acetonitrile and acetic acid for up to 6 h were compared to verify whether acetic acid affects the stability of Re-MN-16ET at room temperature (r.t). The peak area of Re-MN-16ET was consistent and did not decrease in either medium. Hence, Re-MN-16ET was stable in both acetic acid and acetonitrile. Secondly, we determined whether the labeling reaction temperature (100°C) would affect the structure of Re-MN-16ET. After Re-MN-16ET was incubated in acetic acid at 100°C for up to 6 h, the chromatographic peaks of Re-MN-16ET were repeatable, without any differences. The thermal effect of the temperature of 100°C on Re-MN-16ET was also excluded. In the next step, Re-MN-16ET was dissolved in acetic acid and mixed with normal saline solution so that the medium was similar to the labeling environment (acetic acid 70%). The levels of Re-MN-16ET did not decrease regardless of temperature.

Subsequently, the radiolytic and oxidative effects of $^{188}ReO_4^-$ on the structural stability of Re-MN-16ET were examined. Re-MN-16ET dissolved in acetic acid was mixed with $^{188}ReO_4^-$ in normal saline (60 mCi); the medium contained a total of 70% acetic acid at room temperature. The chromatographic peak areas of Re-MN-16ET solution incubated for various periods decreased gradually as the reaction proceeded. Furthermore, the tested solutions were also analyzed at 100°C. The slope of the decrease of the chromatographic peak areas was slightly sharper than at room temperature (Figure 2). Three decomposed products ($DP_{Re\text{-}L}$ 1 to 3) were detected in the Re-MN-16ET/$^{188}ReO_4^-$ tested solution by DAD at 250 nm (Figure 3). The t_R values of the three degraded derivatives were 16.0, 21.0, and 27.0 min, in sequentially, regardless of thermal effects, and the levels of the three derivatives increased as the reaction proceeded. $DP_{Re\text{-}L}$ 1 was the major product. For the first 1 h of the reaction Re-MN-16ET with $^{188}ReO_4^-$, the abundance of Re-MN-16ET was slightly reduced to 90%, and 85% at r.t. and 100°C, respectively. The thermal effect was not the major factor affecting the stability of Re-MN-16ET; however, it made a partial contribution, in tandem with the effects of $^{188}ReO_4^-$.

The identities of the three derivatives were determined using tandem mass spectra. For $DP_{Re\text{-}L}$ 1, the molecular ion $[M+H]^+$ m/z was 647/649 with the Re isotopic ratio pattern. The product ion spectra of 649 were 631, 613, 603, 395 and 367 etc. The major derivative of Re-MN-16ET was the product of the hydrolysis of the ethyl ester group to its carboxylic acid form. This result was same as that found for the metabolite M1 of Re-MN-16ET in a rat liver homogenate biotransformation study [7]. For $DP_{Re\text{-}L}$ 2, the molecular ion $[M+H]^+$ m/z was 691/693 with the Re isotopic mass ratio pattern. The product ions of 693 were 675, 629,

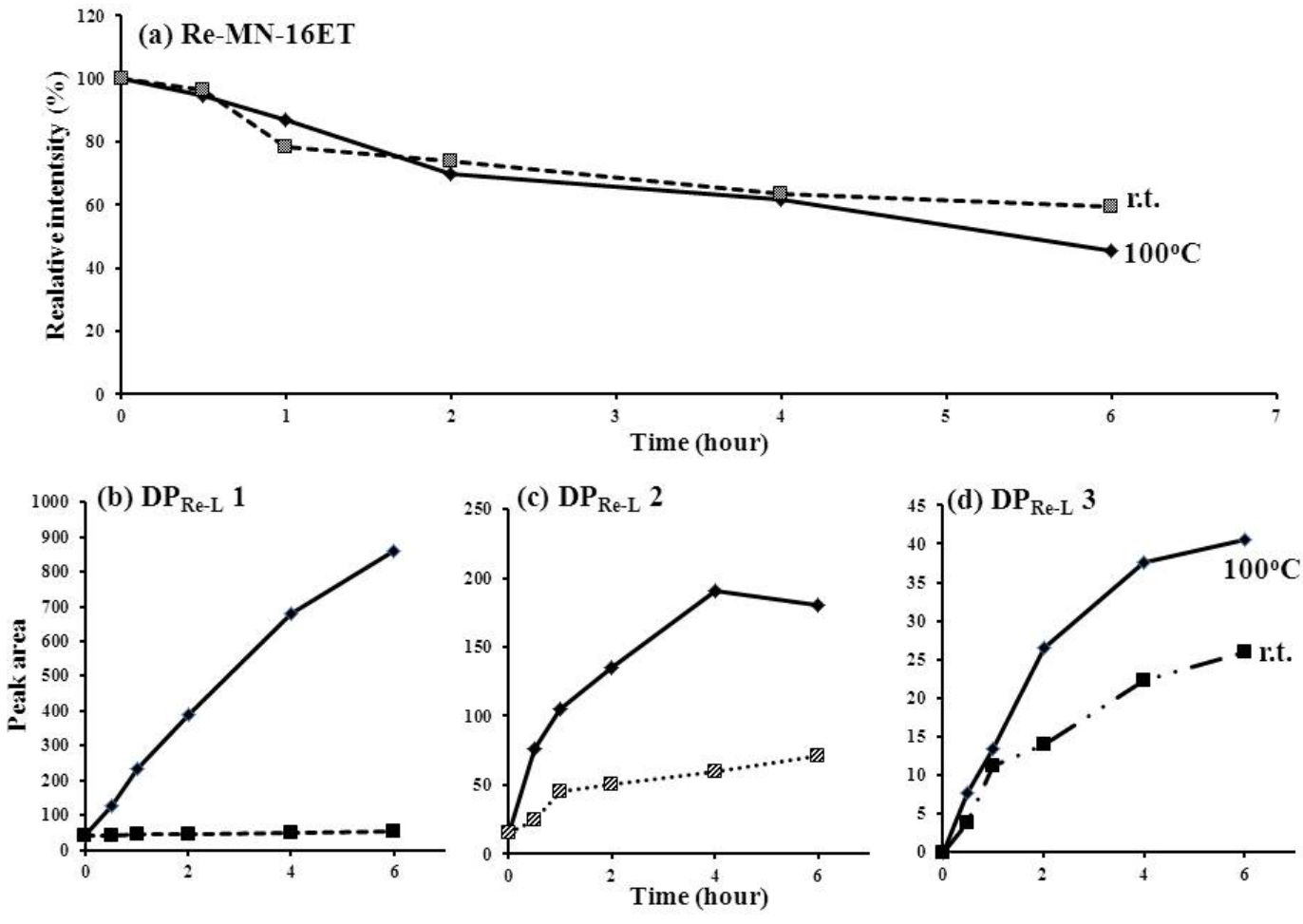

Figure 2: Level trends of the chromatographic peak areas for Re-MN-16ET and its derivatives incubated with $^{188}ReO_4^-$, 70% acetic acid, and normal saline medium. (The solid line represents100°C and the dotted line represents r.t.). (a) Re-MN-16ET; (b) to (d) $DP_{Re\text{-}L}$ 1 to 3, respectively.

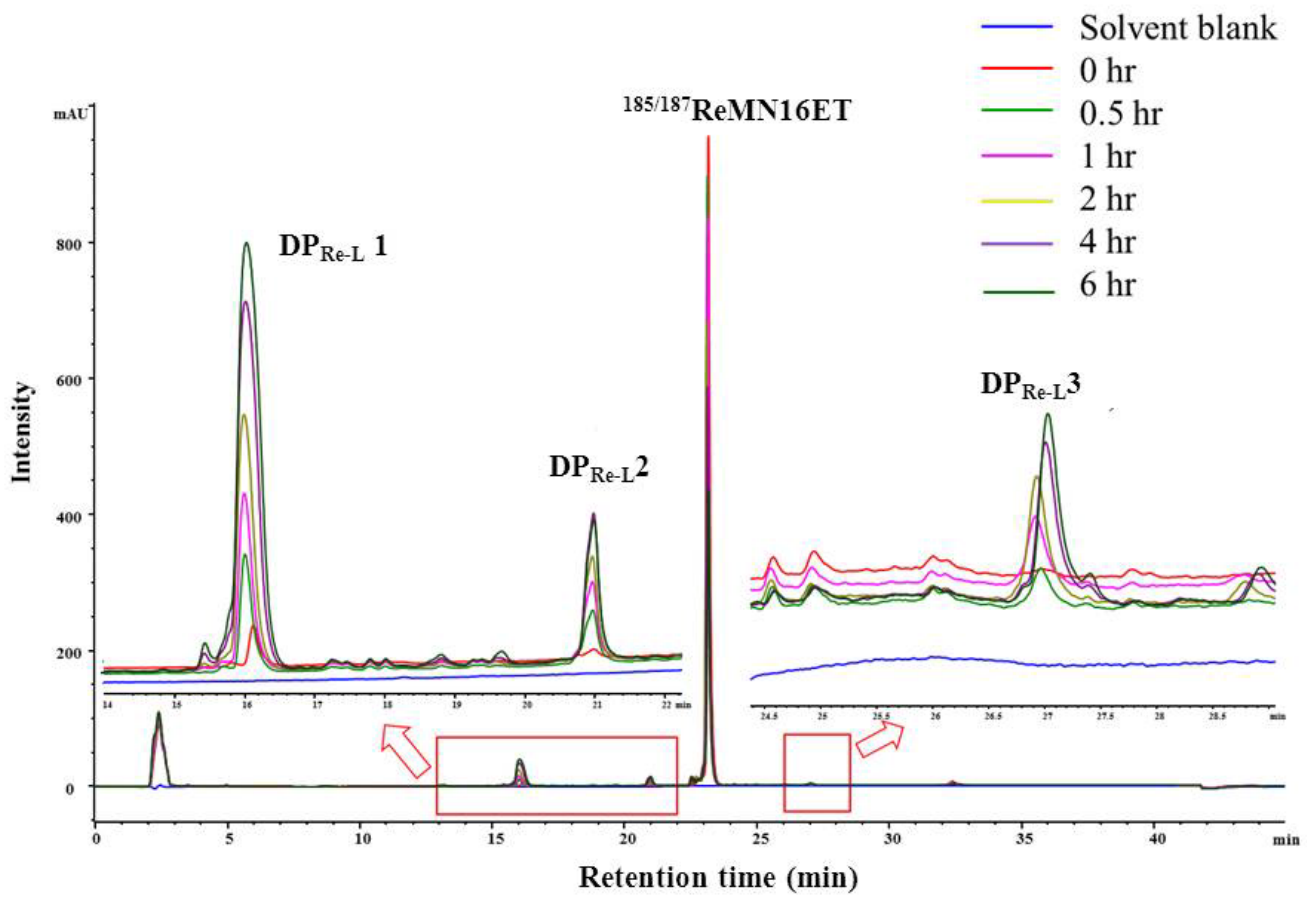

Figure 3: Chromatograms of the stability study ofRe-MN-16ET solution incubated for various durations with $^{188}ReO_4^-$ (r.t.). Three degraded product peaks were observed.

393, 365, etc. The structures of the fragments and the parent molecular ion were determined to account for the relationship between the mass spectra and the structures (Figure 4). Hence, DP_{Re-L} 2 was identified as an oxidized product bearing a hydroxyl group. The molecular ion m/z for DP_{Re-L} 3 was 475, without the characteristic mass pattern of Re; the fragment ions m/z were 447, 429, 415, 404, 401, 374, 358, 326, 312, etc. The fragment ions m/z and structural information respectively showed that the identity of DP_{Re-L} 3 was a de-ReO product of Re-MN-16ET. The third decomposed product also appeared during a photolysis study of protected-H_3MN-16ET (at λ=254 nm) with disulfide bonding [8]. DP_{Re-L} 1 and 2 were more hydrophilic than their parent API and impacted the solubility of Re-188 in lipiodol. DP_{Re-L} 3 could not chelate Re-188 efficiently; however, its abundance was insignificant.

Based on the results of the Re-MN-16ET stability tests under the Re-188 labeling process conditions, the primary factor affecting its structural integrity was the presence of $^{188}ReO_4^-$. This may be ascribed to oxidative and radiolysis effects of $^{188}ReO_4^-$. In the actual labeling process, $^{188}ReO_4^-$ is reduced by $SnCl_2$ gradually; therefore, its oxidative impact is less important. However, the radiolytic effect of ^{188}Re is continuous.

Labeling process stability of protected-H_3MN-16ET

The precursor of the radiopharmaceutical ^{188}Re-MN-16ET is H_3MN-16ET protected with Ph_3C- groups. The stability of this reactant during the Re-188 labeling process was also monitored by the same analysis method used to monitor the product to study the impurities and forced degradation [8].

The HPLC-tandem MS instrument parameters and the results for protected-H_3MN-16ET are reported in [8]. The t_R of protected-H_3MN-16ET is 30.6 ± 0.1 min, with a theoretical plate number of 8,500 for its HPLC analysis. The *m/z* of product ion for protected-H_3MN-16ET is 243; this shows that triphenylmethylium (CPh_3^+) is the most stable fragmentation ion from the parent molecule. First, the effects of acetic acid on protected-H3MN-16ET were studied. The compound was dissolved in acetic acid (100%) or acetonitrile and monitored for up to 6 h at r.t. The heights/areas of the chromatographic peaks of protected-H_3MN-16ET after incubation for various periods were repeatable and did not show a tendency to decline in either acetic acid or acetonitrile even after 6 h, on the basis on DAD detection at 250 nm. Hence, protected-H_3MN-16ET is stable in acetic acid medium.

Moreover, the impact of the reaction temperature on protected-H_3MN-16ET was studied at 100°C in acetic acid solution for 6 h. The HPLC results showed that the heights/areas of the chromatographic peaks of protected-H_3MN-16ET decreased continuously (55% remained after 1 h of reaction); simultaneously, three decomposed products appeared with increasing levels. The t_R values of these products

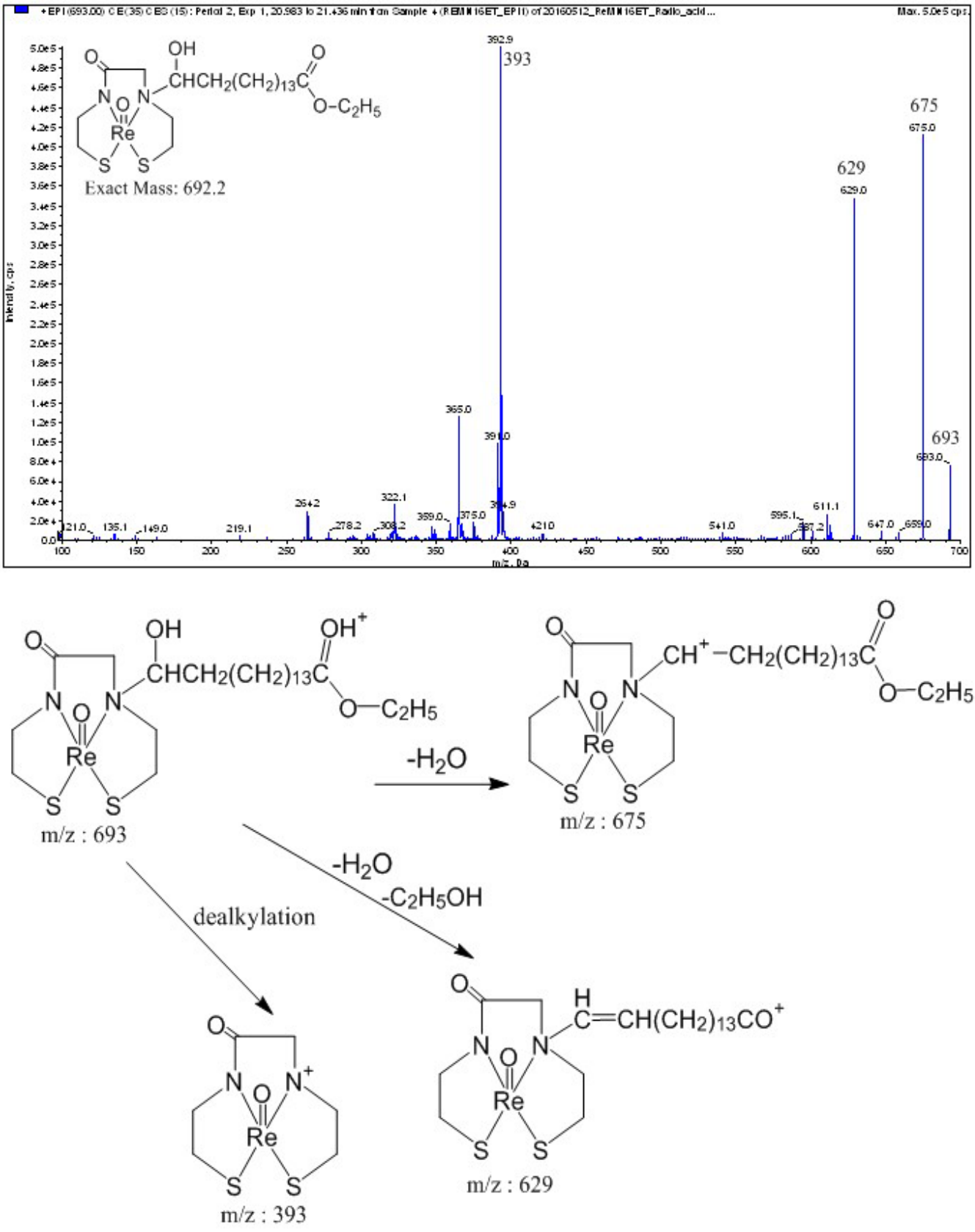

Figure 4: Fragmented mass spectra and proposed structures to illustrate the relationship between the spectra and the identity of DP_{Re-L} 2.

were 11.33, 12.71, and 19.45 min for DP_{p-L}1 to 3, in sequentially. The identities of DP_{p-L} 1 to 3 were determined based on their tandem mass spectra. The primary m/z (MS^1) for both DP_{p-L} 1 and 2 were 243; this indicated that both products contain the protecting group moiety, Ph_3C^+, because it is sufficiently stable to exist in the incubated solution of protected-H_3MN-16ET and be detected by mass spectrometry. The contaminated impurity in protected-H_3MN-16ET (Imp 1, 0.77%) with the same t_R and m/z as DP_{p-L} 1 was also determined to be X-CPh_3 (X: unknown) [8]; it might be Ph_3C^+ itself. Next, it was supposed that Ph_3COH might be the other of the decomposed products after it was protonized into $Ph_3COH_2^+$ by electrospray ionization (ESI); H_2O leaving from it instantly and m/z 243 was detected by the mass detector. The chromatogram of LC-MS for the triphenylmethanol reference standard material and mass spectra m/z=243 both confirmed that DP_{p-L} 2 was Ph_3COH rightly. The third decomposed product, DP_{p-L} 3, had the same molecular ion and fragmentation ions m/z as DP_{Re-L} 3. The hydrolyzed product of the ethyl ester into carboxylic acid was not found in the reaction system; therefore, acetic acid was not involved in the process, even at 100°C. It is believed that because acetic acid is a weak organic acid with low [H^+] activity, it cannot hydrolyze the ethyl ester group in the reaction solution; this result was different from acidic hydrolysis (0.05 M HCl) in forced degradation study of protected-H_3MN-16ET [8].

The trends by $^{188}ReO_4^-$ (60 mCi) influenced the stability of protected-H_3MN-16ET at both r.t. and 100°C were shown in (Figure 5). The levels of protected-H_3MN-16ET decreased slowly at r.t. and quickly at 100°C to 90% and 75%, respectively, in 1 h. The three decomposed products were DP_{p-L} 1 to 3, as found in the thermal study described above.

It was believed that the protecting groups (CPh_3) on the sulfur ligands of protected-H_3MN-16ET leave easily at 100°C, becoming CPh_3^+ and Ph_3COH, regardless of the presence of $^{188}ReO_4^-$ in the labeling solution; however, $^{188}ReO_4^-$ promotes the reaction. The sulfur ligands on unprotected H_3MN-16ET were prone to couple quickly to form DP_{p-L}3, the disulfide derivative.

The actual Re-188 labeling reaction system contains $SnCl_2$ to reduce $^{188}ReO_4^-$ into reduced state of ^{188}Re. After the protecting groups (Ph_3C) leave the S-ligands, the freed sulfur atoms can chelate with reduced ^{188}Re to generate the desired product instead of coupling to form the disulfide derivative, DP_{p-L}3. Therefore, the decreasing tendency of the protected-H_3MN-16ET level does not indicate that it decomposed. The major driving force was the thermal effect; however, $^{188}ReO_4^-$ also had some effect.

Auto-radiolysis affects study of Re-188 on ^{188}Re-MN-16ET

The fate of ^{188}Re-MN-16ET in the liver depends on the auto-radiolysis effect of ^{188}Re in addition to biotransformation by enzymes in the liver. The auto-radiolysis effect may continuously impact the stability of the structure after $^{188}ReO_4^-$ is removed from the labeling reaction system, even until it is infused into the hepatic artery. The auto-radiolysis effect of ^{188}Re (60 mCi, about 59 ng mL^{-1}) on ^{188}Re-MN-16ET was monitored for up to 144 h (over 8 half-lives of ^{188}Re). The result showed that the levels of ^{188}Re-MN-16ET in the incubated solution were constant, and no derivatives were found in the chromatograms (DAD and MS detection). The radiation of Re-188 did not induce decomposition of ^{188}Re-MN-16ET.

Stability of Re-MN-16ET in plasma

Re-MN-16ET was metabolized in rat liver homogenate by thoroughly mixing it and the metabolites are carboxylic acid form (Re-MN-16COOH, M1 with m/z=649) and subsequently, the de-ReO-disulfide derivative (M2, m/z=447) [7]. In rat plasma, only one metabolite was found; 8% of the original Re-MN-16ET remained after incubation for 1 h. M1 was also found in rat plasma medium in the study, but not M2 involved (confirmed by the mass detector with extracted ion count (XIC)). It indicated that Re-MN-16ET could be bio-transformed into a more hydrophilic form in the circulation system of the body, but Re remained chelated by the ligand.

Conclusions

Figure 6 summarizes the identities of the derivatives found in the stability study of Re-MN-16ET and protected-H_3MN-16ET in various conditions. The primary impact on the structural integrity of the product, ^{188}Re-MN-16ET, during Re-188 labeling process arose from radiation of $^{188}ReO_4^-$ as well as from the temperature of 100°C

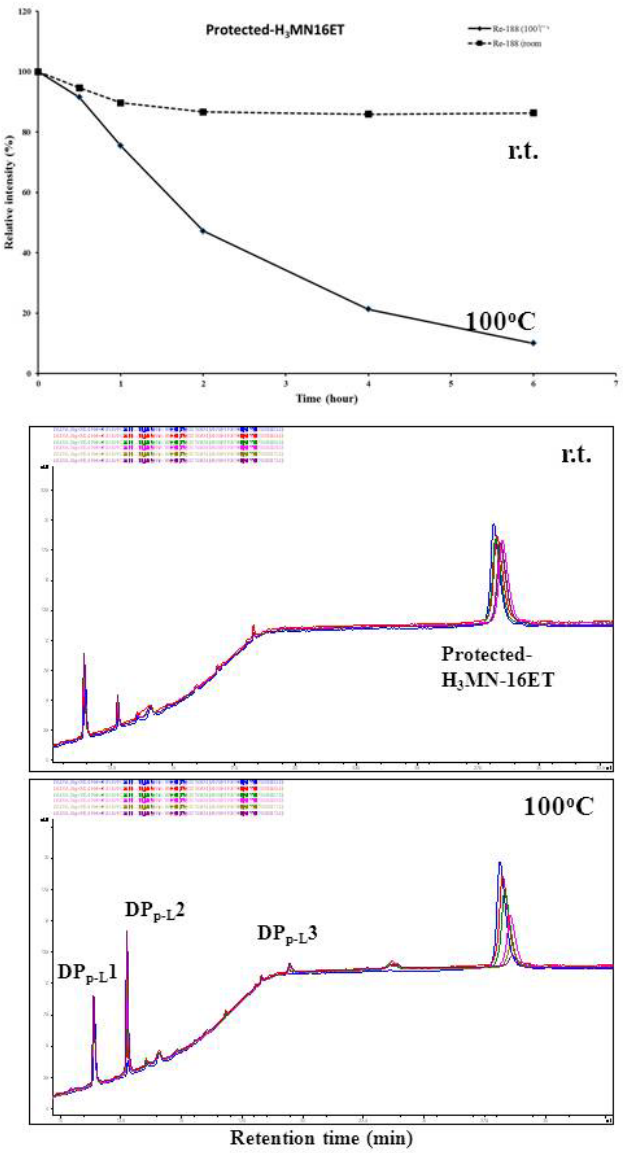

Figure 5: (a) The level trends of protected-MN-16ET incubated with $^{188}ReO_4^-$ (the solid line represents100°C and the dotted line representsr.t.); (b) and (c) chromatograms of protected-MN-16ET solution incubated for various durations.

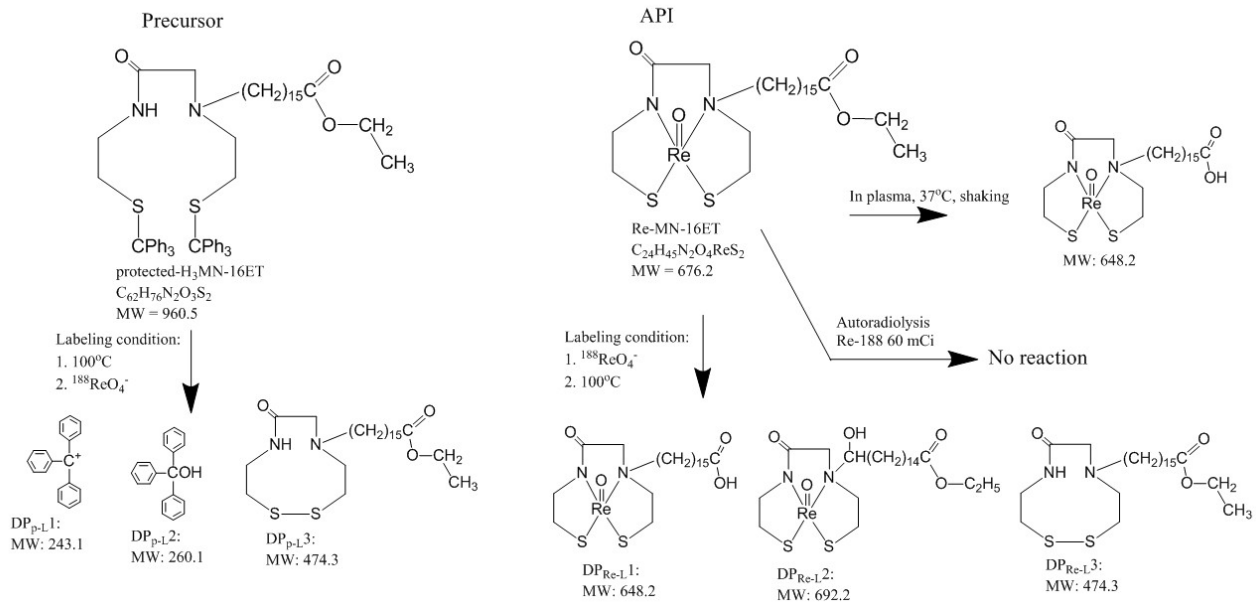

Figure 6: Summary of the identities of the derivatives found during the stability studies of Re-MN-16ET and protected-MN-16ET in various conditions.

to a minor degree; this induced hydrolysis of the ethyl ester to its carboxylic acid form. Up to 85% of the product remained after the labeling reaction was complete (1 h). However, the derivative ^{188}Re-MN-16COOH also dissolved in lipiodol and played the same role as ^{188}Re-MN-16ET, even though the solubility of ^{188}Re-MN-16COOH in lipiodol is less than its parent; however, it is present in minor amount. The protecting groups, Ph_3C-, detached from the sulfur ligands in the stock; this was mostly due to thermal effect of the temperature of 100°C, with a partial contribution from $^{188}ReO_4^-$. The freed S atoms then chelated with the reduced Re-188 ion, which ultimately produced the desired product during the labeling process. The radiation of Re-188 (60 mCi) didn't induce structural deterioration of ^{188}Re-MN-16ET, even when the radioactivity was <1/300 of the original value (over 8 half-lives). These results confirmed that the labeling process is suitable to prepare ^{188}Re-MN-16ET for application in the treatment of HCC. The studies of the stability and derivatives of Re-MN-16ET in plasma indicated that Re was firmly complexes with the ligand with a more hydrophilic metabolite when it was dissolved in plasma. This provides a pathway for clearance of the medicine via urine.

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