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Alkaline degradation of lyophilized DMSA prior to labeling with ^{99m}Tc: Identification and development of the degradation pathway by HPLC and MS

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ABSTRACT

Introduction: Complexes of technetium-99m (99mTc) with meso-dimercaptosuccinic acid (DMSA) have been widely used as diagnostic agents in nuclear medicine. The degradation products (DP) of DMSA formed under different forced conditions have been identified through HPLC-DAD and LC-MS^n studies. In this study, the DMSA kit was subjected to forced degradation under hydrolysis conditions as prescribed by the International Conference on Harmonization (ICH) guideline Q1A.
Methods: Chromatographic separation was accomplished on a reverse phase Shim-Pack VP-ODS (150 mm × 4.6 mm; 5 μm) analytical column using the gradient elution method. LC-MS^n analysis was performed using an Esquire 3000 Plus ion trap mass spectrometer, operating under electrospray ionization (ESI).
Results: No products were found under acidic or neutral stress conditions. All the products found were identified through LC-MS^n analyses and their fragmentation pathways were proposed. The DMSA standard degraded into an adduct DMSA dimer (2DMSA[-2H + Na]$^+$) and adduct DMSA bound to fumaric acid and dithioglucolic acid (DTGA). In the DMSA kit, the degradation products were dimers and trimers of DMSA with tin. A possible degradation pathway is presented.
Conclusions: This method proved to be convenient and effective since it provided fast and efficient separation of DMSA from its degradation products. The degradation studies carried out were able to delineate the stability of the DMSA standard and the DMSA kit.

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1. Introduction

Complexes of 2,3-dimercaptosuccinic acid (DMSA) with technetium-99m (^{99m}Tc) have been very widely used for many years in nuclear medical diagnosis [1]. The application of ^{99m}Tc-DMSA as a diagnostic agent in nuclear medicine is dependent on the chemical conditions of its preparation. DMSA labelled with ^{99m}Tc under acidic conditions can be used for renal scintigraphy or renal function diagnosis [2]. On the other hand, it has been reported that ^{99m}Tc-DMSA complexes prepared under alkali reaction conditions might be useful for tumor imaging and have been applied for lung cancer, breast cancer and thyroid carcinoma [3,4].

Like other ^{99m}Tc-labeled radiopharmaceuticals, ^{99m}Tc-DMSA is prepared just before injection from ^{99m}Tc-pertechnetate and a lyophilized reagent containing the ligand of DMSA (the DMSA kit). It is common knowledge that impurities of DMSA are important with respect to the quality of a kit like ^{99m}Tc-DMSA [2].

Spies et al. and Staník et al. studied the importance of DMSA stability and the consequences on complex formation in aqueous solution, characterizing the structure of impurities with nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectroscopy [5,6]. Spies et al. concluded that, in potassium hydroxide solution, complexes of the meso ligand racemize to give complex compounds of racemic 2,3-dimercaptosuccinic acid dimethylester [5]. Staník et al. concluded that the degradation of DMSA is most significant at alkali pH. They observed oxidized and dimerized forms of DMSA and fumaric acid. The progression of instability was less substantial and slower under neutral reaction conditions. At acidic pH, the concentration of soluble DMSA decreased with time. The ligand forms a complex with $SnCl_2$ at alkali pH (pH 8.8); its chemical formula was determined to be $Sn(DMSA)_2$ [6].

High performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS) has been used to delineate the structure of ^{99g}Tc(V)DMSA and ^{99g}Tc(III)DMSA [4,7]. The structure of the ^{99m}Tc(III)DMSA complex was suggested by Moretti et al. [8] after a study on the concentration ratio between Tc-DMSA. When this ratio is

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1:1, complexes III, IV, V are obtained using 2 carboxyls, 1 SH and 1 carboxyl, or 2 SH; the other ligands are four hydroxyls. In each case, the technetium is hexacoordinated. When DMSA is in excess. the $Tc(DMSA)_2$ complex is obtained. Thus, they were able to conclude that only one hexacoordinated complex has reproducibly high renal cortical affinity (^{99m}Tc(III)DMSA) and that the quality of this renal agent depends on the preparation conditions.

The identity of ^{99m}Tc(V)DMSA complexes was extensively studied by Blower et al. through various analytical techniques: thin-layer chromatography (TLC) under various conditions, clearly distinguishing ^{99m}Tc(III)DMSA and ^{99m}Tc(V)DMSA but failing to distinguish ^{99m}Tc(V)DMSA and $^{99}TcO(DMSA)_2]^-$. These observations were substantiated by more discriminating HPLC methods over a wide pH range. Similarly, electrophoresis over a wide pH range failed to distinguish the two complexes. The electrophoresis results imply that they share the same ionizable groups with the same pKa values and the same charge even when the carboxylate groups are fully associated and fully dissociated [9]. The presence of three isomers was confirmed by HPLC using the aqueous trifluoroacetic acid/acetonitrile gradient system. Thus, ^{99m}Tc(V)DMSA has the formula $[TcO(DMSA)_2]^-$ [9].

Forced degradation studies (stress studies) are usually part of a drug development strategy, undertaken to elucidate the intrinsic stability of the drug. Such studies are conducted under more severe and exaggerated conditions than those usually used for long-term stability tests [10,11].

High performance liquid chromatography with diode array detection (HPLC-DAD) and liquid chromatography coupled to multistage mass spectrometry (LC-MS^n) are becoming the most versatile technique for the identification of pharmaceutical degradation products and impurity profiling [12,13]. The complete degradation profile of the DMSA in the kit and the diagram of its degradation products have not been reported in the literature.

The present work describes the degradation behavior of DMSA and the DMSA kit under hydrolysis conditions (acid, alkaline and neutral), and the identification of degradation products and the fragmentation pathways of degradants using HPLC-DAD and LC-MS^n.

2. Methods

The compound DMSA and the DMSA kit were purchased from the Sigma Chemical Company (Canada) and the Nuclear Energy and Research Institute (Brazil), respectively. The composition of the DMSA kit is shown in Table 1.

All mobile phases were prepared from HPLC-grade chemicals and purified water delivered by an Elix 10 Millipore system (France). Acetonitrile, formic acid, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Merck (Germany). The mobile phases were filtered through a 0.22 μm PVDF membrane filter from Sartorius (Germany).

2.1. High performance liquid chromatography-diode array detector (HPLC-DAD)

An LC-20AT Prominence HPLC system equipped with a diode array detector and LC Solution data handling system (Shimadzu, Japan) was used for the analysis. The analytical column was a Shimadzu Shim-Pack VP-ODS (150 mm × 4.6 mm; 5 μm) column. Mobile phase A was 0.1% formic acid. Mobile phase B was acetonitrile. The flow rate was kept at 1.0 mL min^{-1} and the column was maintained at room temperature. The injection volume was 20 μL. Related substances were detected at 245 nm using a gradient HPLC method. The A:B (v/v) linear gradient elution program was 5%–60% at 15 min.

Table 1
Composition of the DMSA kit from IPEN.

DMSA kit	mg/kit
DMSA	1.00
$SnCl_2.2H_2O$[a]	0.44
Inositol	50.0
Ascorbic acid	0.70

[a] $SnCl_2.2H_2O$: Tin(II) chloride dihydrate.

2.2. Liquid chromatography-mass spectrometry (LC-MS^n)

LC-MS^n analysis was performed using an Esquire 3000 Plus ion trap mass spectrometer (Bruker Daltonics, USA), operating in positive electrospray ionization (ESI) mode. Data Analysis V4.0 was used for data acquisition and data processing. The HPLC conditions were the same as those described in the section "HPLC-DAD". The typical source conditions were: capillary voltage, 3.0 kV; cone voltage, 30 V; source temperature, 150 °C; desolvation temperature, 365 °C; desolvation gas flow, 540 L h^{-1}; cone gas flow, L h^{-1}. Highly pure nitrogen was used as the nebulizer gas. The collision energy was 15 eV.

2.3. Stress study of the DMSA standard and DMSA kit

Stress studies were carried out under hydrolysis conditions as mentioned in ICH Q1A guideline and suggested by Bakshi and Singh [10,11]. A stock solution (1 mg mL^{-1}) containing 10 mg DMSA was prepared in 10 mL of pure water.

2.4. Induced degradation by acid, alkaline and neutral hydrolysis

Acid and alkaline decomposition studies were performed by mix of the solution of DMSA standard or DMSA kit with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH, respectively. Neutral hydrolysis was performed with the DMSA standard and DMSA kit prepared in pure water. The stability chamber was maintained at 25 °C and a relative humidity of 75%.

2.5. Identification of degradation products by HPLC-DAD and LC-MS^n

The molecules of the degradation products were identified by HPLC-DAD and LC-MS^n. The molecular weights (MW) and the collisionally activated dissociation (CAD) were used for suggest the fragmentation pathways.

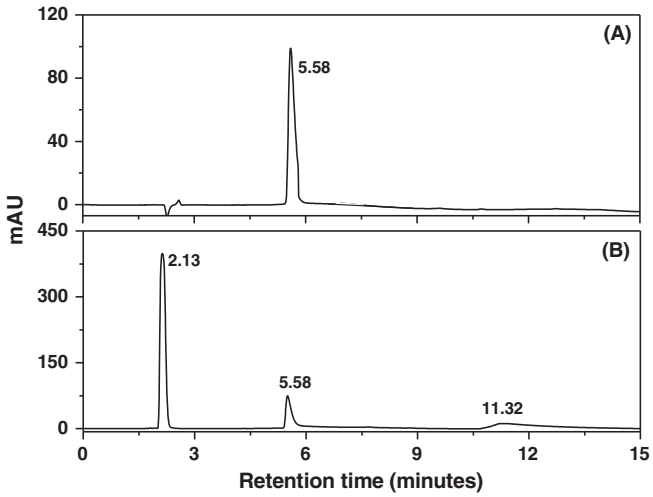

Fig. 1. Chromatograms. Concentration: 1 mg mL^{-1}. (a) DMSA standard and (b) DMSA kit. Shimadzu Shim-Pack VP-ODS column; 0.1% formic acid: acetonitrile, 1.0 mL min^{-1}, 20 μL injection volume and 245 nm detection wavelength.

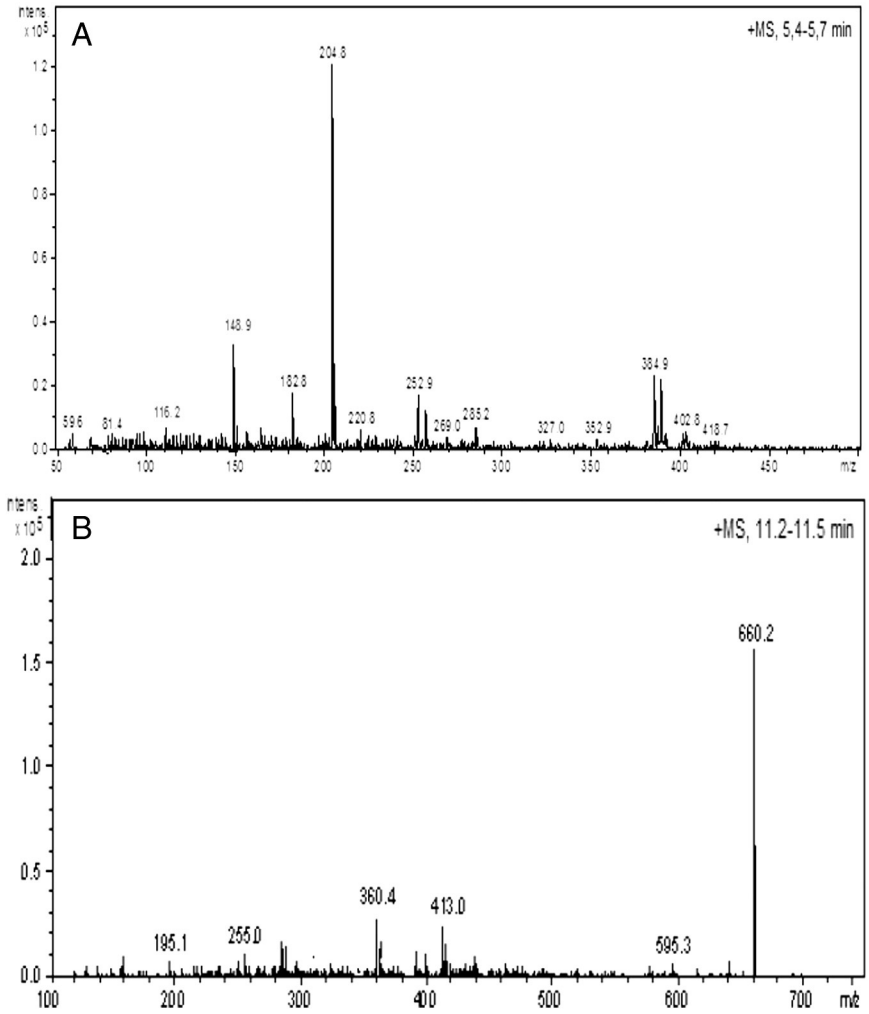

Fig. 2. (a) Mass spectrum of the peak with the retention time of 5.58 min in Fig. 1a. The ion m/z 204.8 corresponds to a DMSA adduct with a sodium ion $[M + Na]^+$. (b) Mass spectrum of the peak with the retention time of 11.32 min in Fig. 1b. The ion of m/z 660.2 is a trimer of DMSA attached to a tin atom $[3M + Sn]^+$.

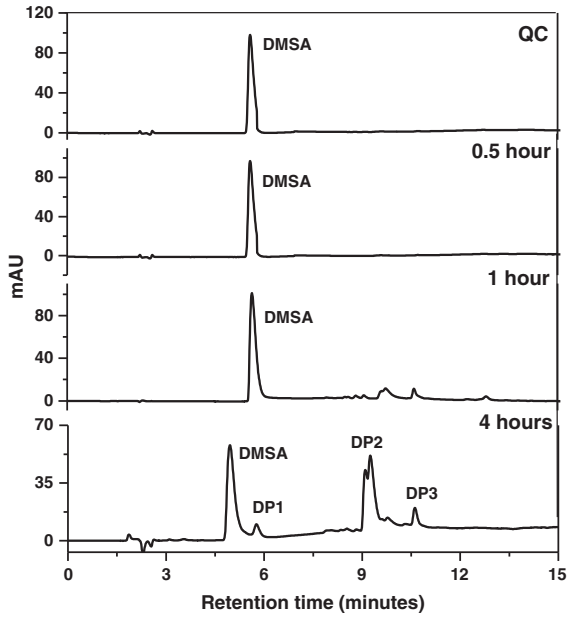

Fig. 3. Chromatograms of the DMSA standard and its degradation products obtained by HPLC-DAD after alkaline hydrolysis with 0.1 mol L^{-1} NaOH after 0.5; 1 and 4 h of treatment. Retention times: DMSA: 4.98 min; DP1: 5.59 min; DP2: 9.73 min; DP3: 10.61 min. DMSA standard concentration: 1 mg mL^{-1}. Quality control without the addition of NaOH (QC). Shimadzu Shim-Pack VP-ODS column; 0.1% formic acid: acetonitrile, 1.0 mL min^{-1}, 20 μL injection volume and 245 nm detection wavelength.

3. Results and discussion

3.1. HPLC-DAD and LC-MS^n analyses

A typical HPLC profile of the DMSA standard and DMSA kit aqueous solution is shown in Fig. 1. The DMSA standard contained no impurities (Fig. 1A), but the DMSA kit contained two impurities (Fig. 1B). The retention time of 2.13 min (Fig. 1B) can be attributed to ascorbic acid, a component of the DMSA kit as described in Table 1. The mass spectra relating to the chromatographic peak with a retention time at 5.58 and 11.32 minutes (Fig. 1B) are shown in Fig. 2.

Positive ionization mode by electrospray (ESI^+) and negative ionization mode by electrospray (ESI^-) were used to analyze DMSA and the DMSA kit. The results obtained for ionization in negative mode showed a significant increase in baseline noise compared to positive ionization mode (approximately three times higher). The deprotonated DMSA

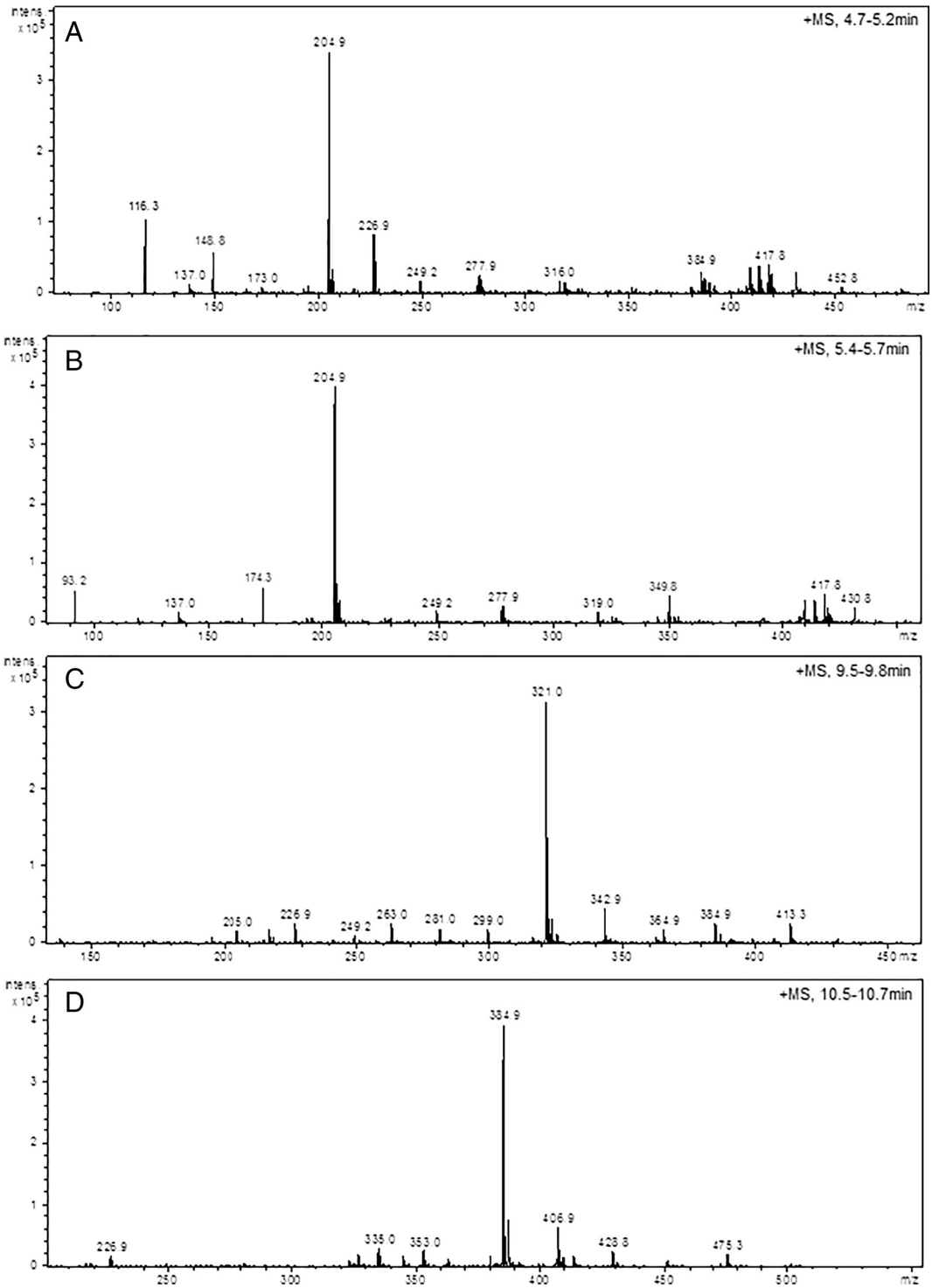

Fig. 4. Mass spectra obtained from the degradation products of DMSA after alkaline hydrolysis with NaOH shown in Fig. 3. (a) Sodium DMSA adduct. Degradation products: (b) dithioglycolic acid; (c) adduct of DMSA and fumaric acid and (d) adduct of DMSA dimer.

ion $[M + H]^-$ with m/z 181.1 showed low intensity compared with the intensity of other interfering ions. Thus, we opted for positive ionization mode for all analyses discussed here.

The mass spectra in Fig. 2A show that the ion m/z 204.8 corresponds to a DMSA adduct with a sodium ion $[M + Na]^+$, whereas the ion of m/z 660.2 (Fig. 2B) is a trimer of DMSA attached to a tin atom $[3M + Sn]^+$. The tin ion is from stannous chloride, a reducing agent, a component of the DMSA kit as described in Table 1.

3.2. Forced degradation studies

3.2.1. Degradation products formed from DMSA standard hydrolysis

Degradation was not observed in the DMSA standard sample when it was subjected to acidic and neutral hydrolysis even after exposure to higher temperatures, for a longer period of testing (up to 24 h) and with increased strength of the acid solution (up to 2 mol L^{-1} HCl).

The chromatograms obtained for the DMSA standard by HPLC-DAD under stressed conditions after alkaline hydrolysis are shown in Fig. 3. The degradation products were well-resolved from DMSA and did not interfere with its determination. The degradation profile of DMSA after alkaline hydrolysis for 4 hours with 0.1 mol L^{-1} NaOH presented three degradation products with peaks at 5.59 min (DP1), 9.73 min (DP2) and 10.61 min (DP3). A shift in the DMSA retention time was observed from 5.8 min to 4.9 min after 4 h of alkaline hydrolysis with 0.1 mol L^{-1} NaOH, which may be related to the predominance of one of the DMSA stereoisomers with more hydrophilic characteristics [5].

The degradation products of DMSA obtained in the analysis by HPLC-DAD following alkaline hydrolysis (Fig. 3) were identified by LC-MS^n and the spectra obtained are shown in Fig. 4.

The peaks eluted at 4.98 and 5.59 min (Fig. 3), corresponding to the mass spectra of Fig. 4A and B, respectively. They are isomers with m/z 204.9. DP2 showed m/z 321.0 (Fig. 4C), while the DP3 showed m/z 384.9 (Fig. 4D). The use of multistage mass spectrometry (MS, MS^2, MS^3 and MS^4) allowed us to propose a diagram of the formation of the main degradation products of the DMSA standard after alkaline hydrolysis with 0.1 mol L^{-1} NaOH (Fig. 5).

LC-MS^n experiments enabled us to identify the molecular ion in the form of an adduct ion at m/z 204.9 ($[DMSA + Na]^+$). In the spectrum of the fragmentation ion m/z 204.9 (MS^2), we observed the presence of the fragment m/z 172.9, with loss of the SH group and the corresponding sodium adduct of mercaptosuccinic acid (MSA); the m/z 139.0 (MS^3) fragment with the loss of H_2S and group corresponded to an adduct ion of fumaric acid (AF) ($AF + Na]^+$).

The adduct ion of fumaric acid fragmented (MS^4) and resulted in fumaric acid m/z 116.9 (Fig. 5). The coordination of MSA with molybdenum (Mo) and tungsten (W) in aqueous solutions is described in the literature [14].

Fumaric acid is a major precursor used in the synthesis of DMSA and may be the result of incomplete synthesis present as impurities in the kit, or it may be a degradation product. Some researchers have studied the effect of the presence of fumaric acid in the kit and the resulting radiopharmaceutical [15–20]. Stanik et al. evaluated DMSA degradation under alkaline conditions to monitor the production of fumaric acid [3,4]. They observed that, after 21 days of storing DMSA in alkaline solution, the proportion of DMSA to fumaric acid was 1:1. The adduct DMSA dimer ($[2DMSA\text{-}2H + Na]^+$) was observed at m/z 384.9 (Fig. 5). The fragmentation of the ion of m/z 384.9 through a desulfurization process generated an ion with m/z 321.0 (MS^2), corresponding to an ion adduct of DMSA and fumaric acid. Experiments revealed an ion of m/z 139.0 (MS^3) and m/z 116.9 (MS^4), may also be generated by the diagram of degradation (Fig. 5). However, the actual structure is still an open question that can be answered by previous studies using such techniques as nuclear magnetic resonance (NMR) and high-resolution mass spectrometry capable of characterizing chemical species.

The ion m/z 115.0 was also observed following the fragmentation of fumaric acid (MS^3) (Fig. 5) and refers to acetylene dicarboxylic acid. Some manufacturers use acetylene dicarboxylic acid as a precursor for the synthesis of DMSA. The preparation of DMSA involves adding 2 mol of thiolacetic acid through the triple bond of acetylene dicarboxylic acid followed by alkaline hydrolysis [21]. Fig. 6 shows a proposed diagram of the formation of the main degradation products of the DMSA isomer of m/z 204.9 (DP1 in Figs. 1 and 4B) after alkaline hydrolysis with 0.1 mol L^{-1} NaOH. These isomers are dithioglycolic acid (DTGA).

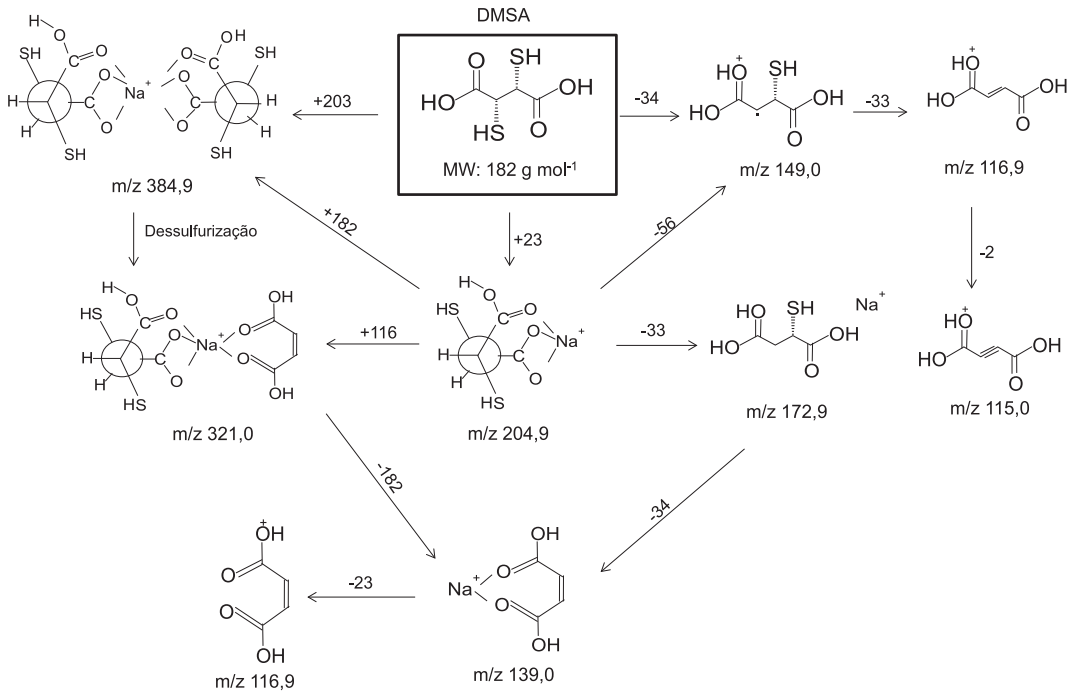

Fig. 5. Diagram for the formation of degradation products of DMSA after alkaline hydrolysis with 0.1 mol L^{-1} NaOH. The numbers indicated on the arrows refer to the gain (+) or loss (−) of ion mass.

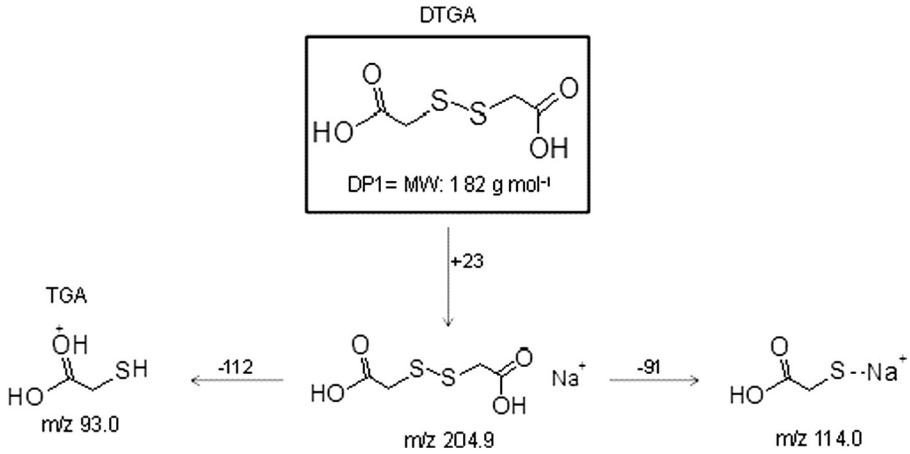

Fig. 6. Diagram for the formation of degradation products of DTGA after alkaline hydrolysis with 0.1 mol L^{-1} NaOH. The numbers indicated on the arrows refer to the gain (+) or loss (−) of ion mass.

The MS^2 spectrum of this ion produces a product ion m/z 93.0 (Fig. 6), characteristic of breaking the disulfide bridge, resulting in the thioglycolic acid (TGA).

The ion m/z 114.0 may be related to sodium thioglycolate. The presence of degradation products can be observed in DMSA in alkaline solutions that have a yellow color [22]. It should be noted that the degradation products and impurities detected can be considered as potential chelating agents. They can form complexes with ^{99m}Tc and subsequently increase the radiochemical impurity in radiopharmaceuticals [3,4].

3.2.2. Degradation products formed from DMSA kit hydrolysis

The results of the HPLC-DAD analysis of the DMSA kit after alkaline degradation with 0.1 mol L^{-1} NaOH are shown in Fig. 7.

The degradation times evaluated were 0.5, 1 and 4 h. The chromatogram showed DMSA at 5.62 min and degradation products at 8.21, 9.11 and 10.90 min. The identification of these degradation products was performed using LC-MS^n.

The degradation products DP1, DP2 and DP3 presented ion m/z 300.0, 480.1 and 661.0, respectively (Fig. 8). The results of MS^2, MS^3 and MS^4 performed for the ions m/z 300.0, 480.1 and 660.1 allowed us to propose a formation diagram for the main products of alkaline degradation of the DMSA kit, as shown in Fig. 9.

The coordination of the tin ion (Sn), from the reducing agent stannous chloride (compositions of DMSA kit -Table 1), can be observed with DMSA in all the degradation products after alkaline hydrolysis of the DMSA kit. The degradation diagram proposed in Fig. 9 shows some tin compounds. The precursor ion m/z 300.0 generated the ion m/z 255.2 resulting from the loss of the COOH group (45 Da). The ion m/z 480.1 (DMSA dimer with tin) generated the product ions m/z 434.1 (loss of COOH), m/z 388.2 (loss of two COOH groups), m/z 343.1 (loss of three COOH groups) and m/z 297.0 (loss of four COOH groups). The ion m/z 660.1 corresponds to a trimer of DMSA with tin.

Sn-DMSA complexes have been reported in the literature by Moretti et al. [8]. They were concerned with the great variability in the biodistribution of ^{99m}Tc-DMSA in tissues and studied the nature

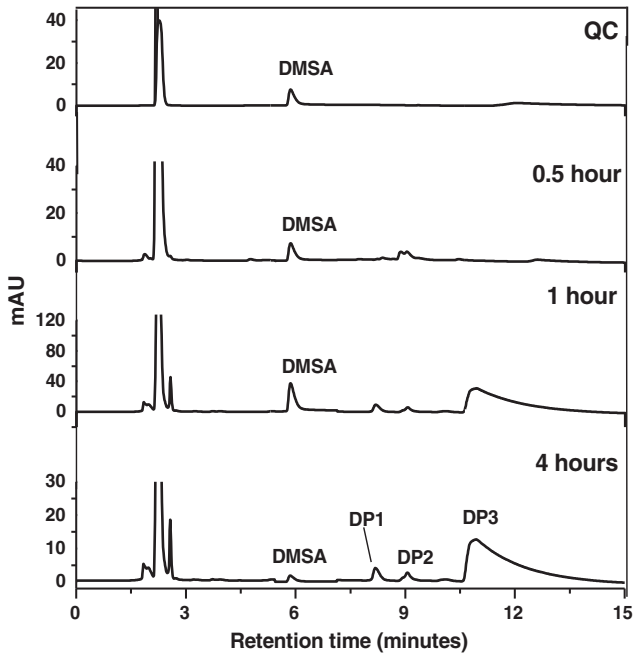

Fig. 7. Chromatograms of the DMSA kit and its degradation products (DP) obtained under alkaline conditions (0.1 mol L^{-1} NaOH). Retention times: DMSA: 5.62 min; DP1: 8.21 min; DP2: 9.11 min; DP3: 10.90 min. DMSA concentration: 1 mg mL^{-1}; after 0.5, 1 and 4 h of treatment. Quality control without the addition of NaOH (QC).

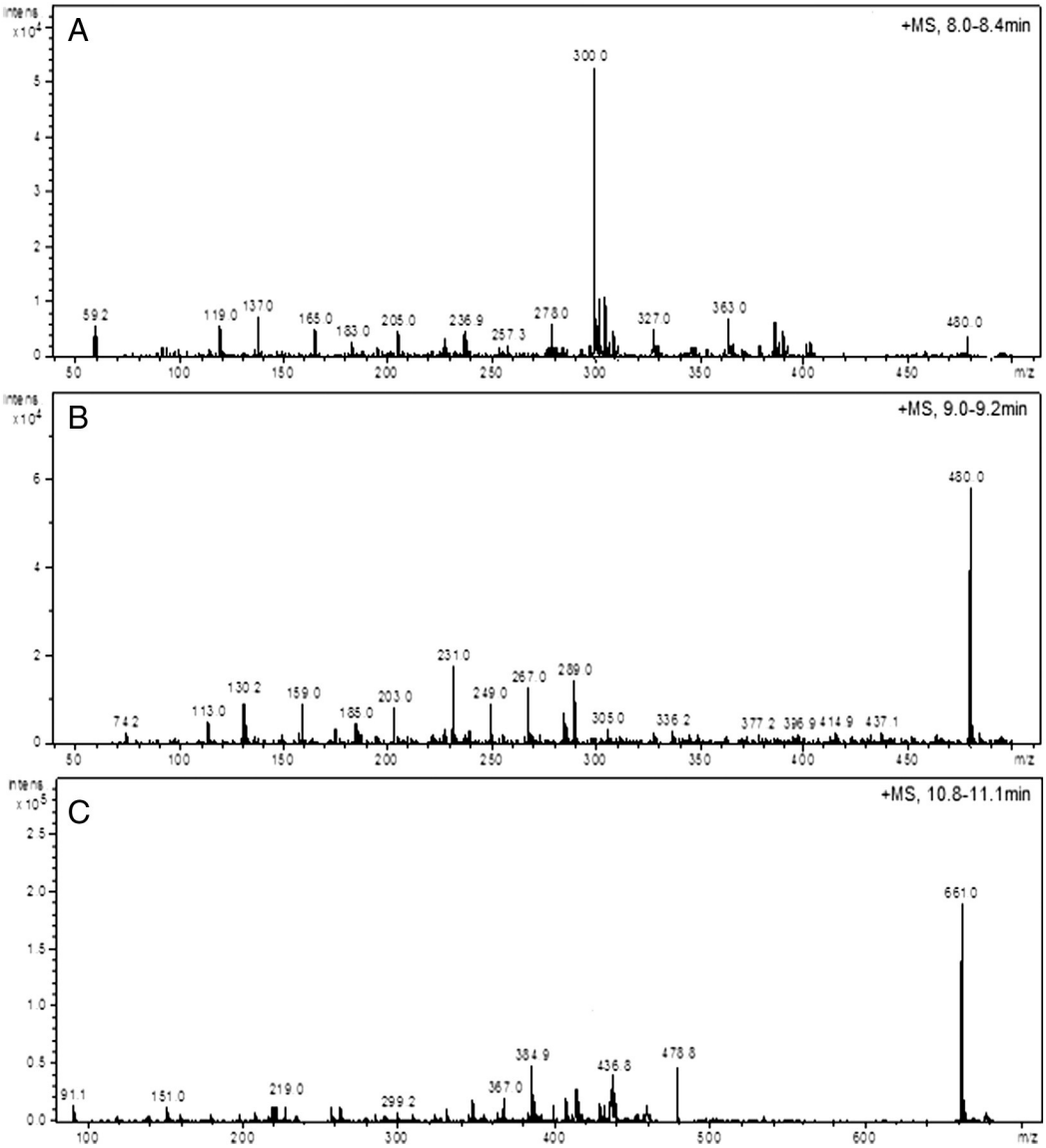

Fig. 8. Mass spectra obtained from the degradation products of DMSA RL after alkaline hydrolysis with NaOH. shown in Fig. 7. (A) DP1, (B) DP2 and (C) DP3. Analysis conditions: full MS, cone voltage: 30 V, capillary voltage: 3.0 kV and desolvation temperature: 365 °C.

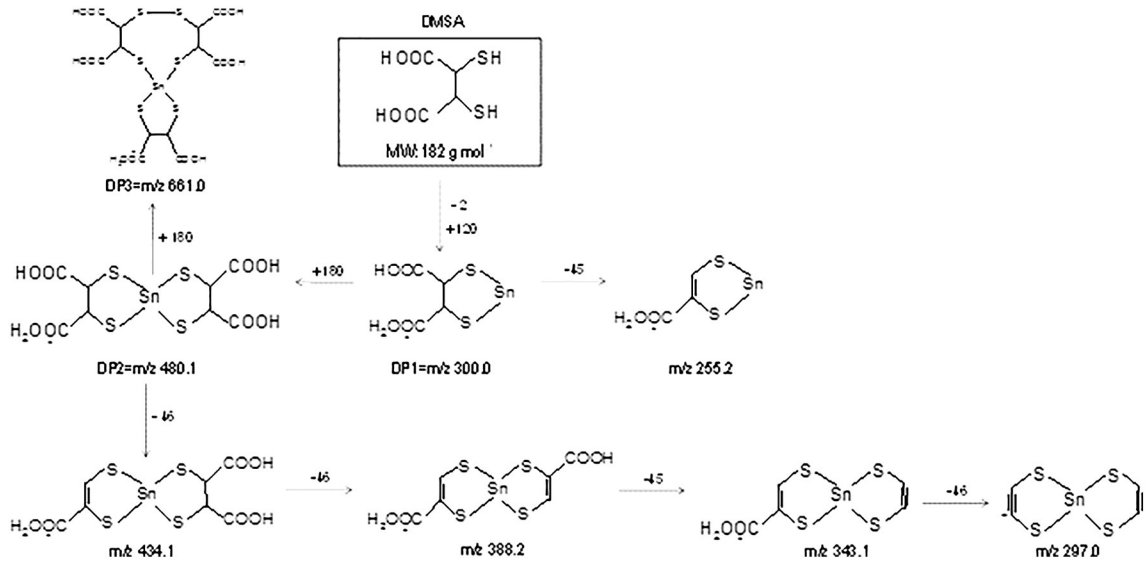

Fig. 9. Diagram of the formation of DP of DMSA kit after alkaline hydrolysis with 0.1 mol L^{-1} NaOH. The numbers indicated on the arrows refer to the gain (+) or loss (−) of ion mass.

of the complexes formed after labeling with ^{99m}Tc; they concluded that the amount of stannous chloride (reducing agent) should be optimized. The best choice is to keep the DMSA:Sn ratio at 3:1 and to use a stabilizer in the preparation of the kit in order to ensure the reduction of the technetium by tin. The presence of the Sn-DMSA complex promotes liver uptake on scintigraphic images and the background variation[8]. Additionally, Galvez et al. studied the labeling of DMSA with ^{99m}Tc without the use of an exogenous reducing agent. They observed that DMSA itself may reduce the pertechnetate to form ^{99m}Tc- DMSA very quickly [23].

4. Conclusion

In order to propose degradation pathways of the DMSA standard and DMSA kit for radiopharmaceuticals, a new analytical method for the rapid detection and identification of degradation products under stressed conditions (acidic, neutral and alkaline) using two chromatographic techniques (HPLC-DAD and LC-MS^n) was investigated. This method proved to be convenient and effective since it provided fast and efficient separation of DMSA from its degradation products.

The degradation studies were able to delineate the stability of the DMSA standard and the DMSA kit. They were stable with respect to hydrolytic acid and neutral stress; however, they were unstable in alkaline medium and some degradation products (DP) were found. In the DMSA standard, DP1, DP2 and DP3 were proposed be an adduct DMSA dimer, and adducts of DMSA with fumaric acid and dithioglycolic acid, respectively. In the DMSA kit, the mean degradation products were dimers and trimers of DMSA with tin.

We conclude that analysis by HPLC-DAD and LC-MS^n can be used to study the stability of the DMSA standard and the DMSA kit by identifying and quantifying impurities and degradation products.

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