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Structural Identification of Nonvolatile Dimerization Products of Glucosamine by Gas Chromatography−Mass Spectrometry, Liquid Chromatography−Mass Spectrometry, and Nuclear Magnetic Resonance Analysis

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The degradation profile of glucosamine bulk form stressed at 100 °C for 2 h in an aqueous solution was studied. Column chromatography of acetylated product mixture led to isolation of two pure compounds (**1b** and **2b**) and a mixture of at least three isomers (**3b**). **1a** and **2a** were identified as 5-(hydroxymethyl)-2-furaldehyde (5-HMF) and 2-(tetrahydroxybutyl)-5-(3′,4′-dihydroxy-1′-*trans*-butenyl)-pyrazine, respectively, by utilizing a variety of analytical techniques, such as GC-MS, LC-MS, on-line UV spectrum, ^{1}H and ^{13}C NMR, and DEPT, as well as ^{1}H−^{1}H COSY. **3a** was identified as 2-(tetrahydroxybutyl)-5-(2′,3′,4′-trihydroxybutyl)pyrazine, commonly known as deoxyfructosazine. In addition, glucosamine solid dosage form was exposed to 40 °C/75% relative humility for 10 weeks. Methanol extract of glucosamine solid dosage form was analyzed after acetylation by LC-MS, resulting in degradants **3b** and **4b**. **3a** and **4a** were, therefore, determined as deoxyfructosazine and 2,5-bis-(tetrahydroxybutyl)pyrazine (fructosazine), respectively. Furthermore, the mechanisms of formation of identified degradation products are proposed and briefly discussed.

KEYWORDS: Glucosamine; 2-amino-2-deoxy-D-glucose; solid dosage form; degradation; GC-MS; LC-MS; NMR

INTRODUCTION

Glucosamine, 2-amino-2-deoxy-D-glucose, is an amino sugar found in various plant tissues and animal muscles (*1*). It is the most fundamental building block required for the biosynthesis of some classes of compounds including glycolipids, glycoproteins, glycosaminoglycans, and proteoglycans, building blocks of human cartilage (*2*, *3*). Glucosamine and chondroitin, a copolymer of *N*-acetylglucosamine and glucuronic acid, are found in and around the cells of the cartilage in human joints. Therefore, glucosamine is a basic subunit of cartilage and the other structures by serving as an essential constituent of these glycosaminoglycans (*3*). It is reported as a precursor for collagen production and has been known to aid in rebuilding damaged cartilage (*4*). It has chondroprotective and antiarthritic effects (*3*, *4*). Currently, glucosamine is marketed as a dietary supplement to enhance the repair and synthesis of cartilage and connective tissue. It is reported that the U.S. retail market for nutritional supplements containing glucosamine or chondroitins is more than $1 billion per year; the demand for bulk glucosamine has been growing in excess of 20% annually, and global consumption exceeds 5000 metric tons (*5*).

Studies on the stability of bulk drugs and/or drug products provided information about how their quality is influenced by various factors, for example, temperature, humidity, pH, and light (*6*). This information helps for determination of recommended storage conditions and for establishment of shelf lives and retest periods.

In the stability study of glucosamine, several works have been reported for the isolation and/or identification volatile compounds formed during glucosamine degradation (*1*, *8*, *9*). Separation and identification of the volatile compounds was done primarily by using GC-MS. Shu (*8*) has studied the degradation products in thermal degradation of glucosamine in aqueous solution (150 °C) with different pH conditions. In that study, it is reported that 3-hydroxypyridine, 1*H*-pyrrole-2-carboxaldehyde, furanones, and hydroxyketones were the major volatile degradants. Under dry conditions, pyrazines, pyridines, pyrroles, and furans were identified from glucosamine thermal degradation (*1*, *9*). The major compounds generated when glucosamine was pyrolyzed (200 °C for 30 min) were 2-(2-furyl)-6-methylpyrazine and 2-acetylfuran (*1*). It is reported that a longer heating time (200 °C, 4 h) generated different major degradation products, such as 2-methylpyrazine and 2,5-dimethylpyrazine (*9*).

However, compared with studies on volatile degradation products of glucosamine, only a few studies have been reported on nonvolatile degradation products. In addition, all of the

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previous studies were focused on the stability of glucosamine at different pH parameters by the addition of hydrochloric acid, sodium hydroxide, or ammonia (*7*, *10*, *11*). In strong acid condition (3 M hydrochloric acid), no identifiable decomposition products were identified (*10*). Amino group protonation provides an intermediate resistance to acid-catalyzed enolization, epimerization, and elimination reactions, which gives glucosamine stability in acidic condition. In contrast, it is reported that substantial decomposition occurred in less acidic conditions (<0.5 M hydrochloric acid, in the presence of glycine).

The degradation profiles of a drug substance are critical to its safety assessment and optimization of the formulation. Therefore, to support the studies on the stability of pharmaceutical formulations with the active ingredient glucosamine, understanding of glucosamine degradation is first required. The objective of this study was to identify nonvolatile compounds generated from the thermal degradation of glucosamine in aqueous solution by using GC-MS, LC-MS, on-line UV spectrum, and NMR techniques. In addition, the degradation profile of glucosamine solid dosage form was studied, and the mechanisms of the formation of the identified degradation products were proposed.

MATERIALS AND METHODS

Materials and Reagents. D-Glucosamine hydrochloride, acetic anhydride, pyridine, acetic acid, and standard 5-(hydroxymethyl)-2-furaldehyde (5-HMF) were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent grade ammonium hydroxide and HPLC analytical grade solvents including hexane, ethyl acetate, methanol, and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Deuterated chloroform for NMR was purchased from Aldrich Chemical Co. (St. Louis, MO). Water was in-house deionized water. Silica gel (130−270 mesh, 60A, Aldrich Chemical) was used for column chromatography. Solid dosage form containing glucosamine (375 mg of glucosamine per tablet) was prepared by New Products Development of GlaxoSmithKline Consumer Healthcare.

Glucosamine Degradation Reaction. Glucosamine (100 g) was dissolved in water (150 mL). The mixture was placed in a 250 mL round-bottom flask and heated for 2 h in oil bath at 100 °C. The cooled mixture was filtered using filter paper (VWR Scientific, West Chester, PA) with the addition of methanol (150 mL × 2) to remove the remaining glucosamine. After filtration, the solution was concentrated to dryness by rotary evaporator (Büchi rotavapor R-205). The residue (6.4 g) was acetylated with acetic anhydride/pyridine (5:3 v/v) at room temperature overnight. The acetylated mixture was concentrated to dryness, dissolved with water, and then extracted with ethyl acetate (50 mL × 3).

Isolation of Degradation Products of Glucosamine. The ethyl acetate extract was subjected to column chromatography with silica gel eluted with hexane and ethyl acetate (2:1 → 1:1 → 1:2 → 1:5 → 1:10 → 100% ethyl acetate, v/v). A total of six fractions were collected. Fraction 1 was rechromatographed with silica gel eluted with hexane and ethyl acetate (5:1 → 4:1 → 3:1 → 2:1, v/v) to yield **1b** (24 mg).

After removal of solvent, fraction 2 yielded **2b** (237 mg). Fraction 3 was further purified with a silica gel column eluted with hexane and ethyl acetate (1:1 → 1:1.5 → 1:2, v/v) and provided **3b** (13 mg). The fractions were monitored by thin-layer chromatography (TLC) using a development solution [1:1 and 1:5 (v/v), hexane/ethyl acetate] and visualized by 10% sulfuric acid and Dragendorff reagent, which is a spray color reagent specifically for alkaloids (*12*).

Sample Preparation of Glucosamine Solid Dosage Form for LC-MS. Glucosamine tablets were exposed to 40 °C/75% relative humility for 10 weeks. The tablets were ground and extracted with methanol. The extract was concentrated, and the residue was acetylated with acetic anhydride/pyridine (5:3, v/v) at room temperature overnight.

High-Pressure Liquid Chromatography (HPLC) and LC—Mass Spectrometry (LC-MS) Analysis. The high-performance liquid chromatography was performed with an Agilent model 1100 series system.

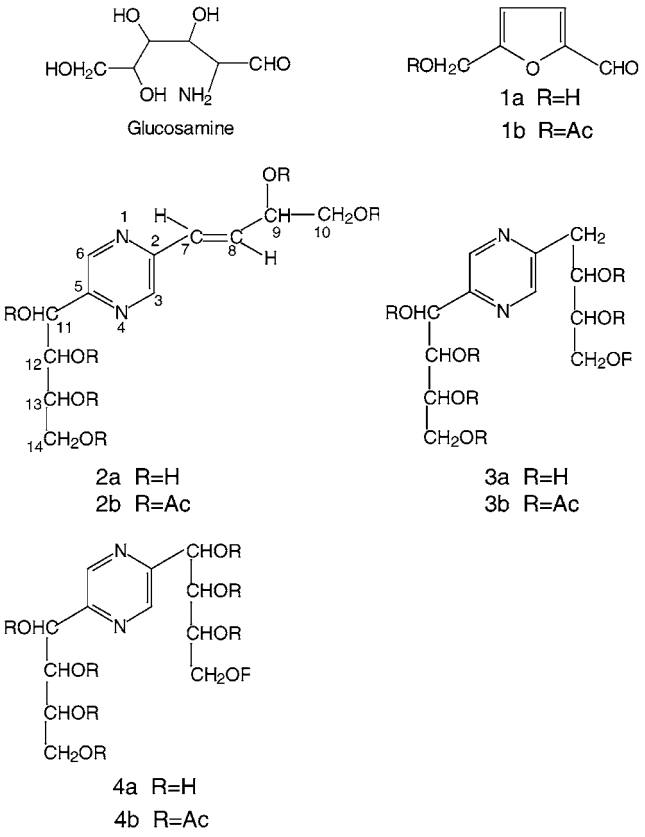

Figure 1. Structures of glucosamine and its degradation products.

An Xterra RP_{18} column (5 μm, 4.6 × 250 mm, Waters) was used. Solvent A was 10 mM ammonium hydroxide with 2% acetonitrile (pH 9.82 adjusted with acetic acid), and solvent B was acetonitrile. The gradient program was initiated with 65% solvent A and 35% solvent B, then linearly increased to 65% solvent B in 20 min, maintained for 5 min, and equilibrated for 10 min with 65% solvent A and 35% solvent B prior to the next injection. The flow rate was 1 mL/min, and the column temperature was kept at 30 °C. Injection volume was 20 μL. The wavelengths were set at 254 and 275 nm for UV detection with a bandwidth of 4 nm. LC-MS system consists of an Agilent model 1100 HPLC series with a degasser, binary pump, autosampler, column heater, diode array detector, and a Finnigan LCQ Deca ion-trap mass spectrometer (San Jose, CA) with APCI source operated in positive ion mode. The column, mobile phases, and temperature program were programmed to be the same as those of the HPLC analysis mentioned above. The operating parameters for the APCI source were as follows: sheath gas flow rate at 85 arbs; auxiliary gas flow rate at 14 arbs; vaporizer temperature at 505 °C; capillary temperature at 300 °C; discharge voltage at 4.25 kV; capillary voltage at 35 V; and discharge current at 5.96 μA.

Gas Chromatography—Mass Spectrometry (GC-MS) Analysis. The GC-MS system consists of an Agilent model 6890N GC system equipped with a capillary column (HP-5MS, 0.25 mm i.d. × 30 m length × 0.25 μm film thickness, Agilent) and coupled with an Agilent 5973N mass selective detector. The oven temperature was programmed from 200 °C (2 min) to 320 °C (8 min) at a rate of 8 °C/min. The injector temperature was maintained at 300 °C. The flow rate of carrier gas was 1 mL/min. The injection volume of sample was 1 μL without split. Mass spectra were obtained by total ion current (TIC) at 1011.8 EM voltage, with a 3 min solvent delay. The mass scan ranged from 50 to 650.

Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis. ^1H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT-135 (100 MHz), and $^1H-^1H$ COSY (400 MHz) were recorded on a JEOL NMR spectrometer in $CDCl_3$. Proton chemical shifts were referenced using tetramethylsilane as internal reference, and ^{13}C chemical shifts were referenced to the solvent.

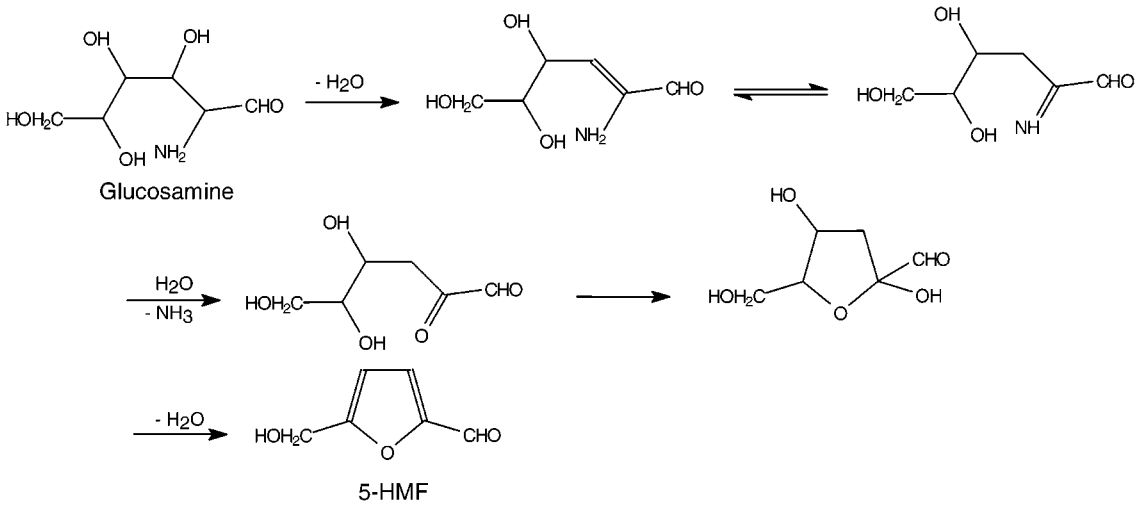

Figure 2. Proposed mechanism for the formation of **1a** from glucosamine degradation.

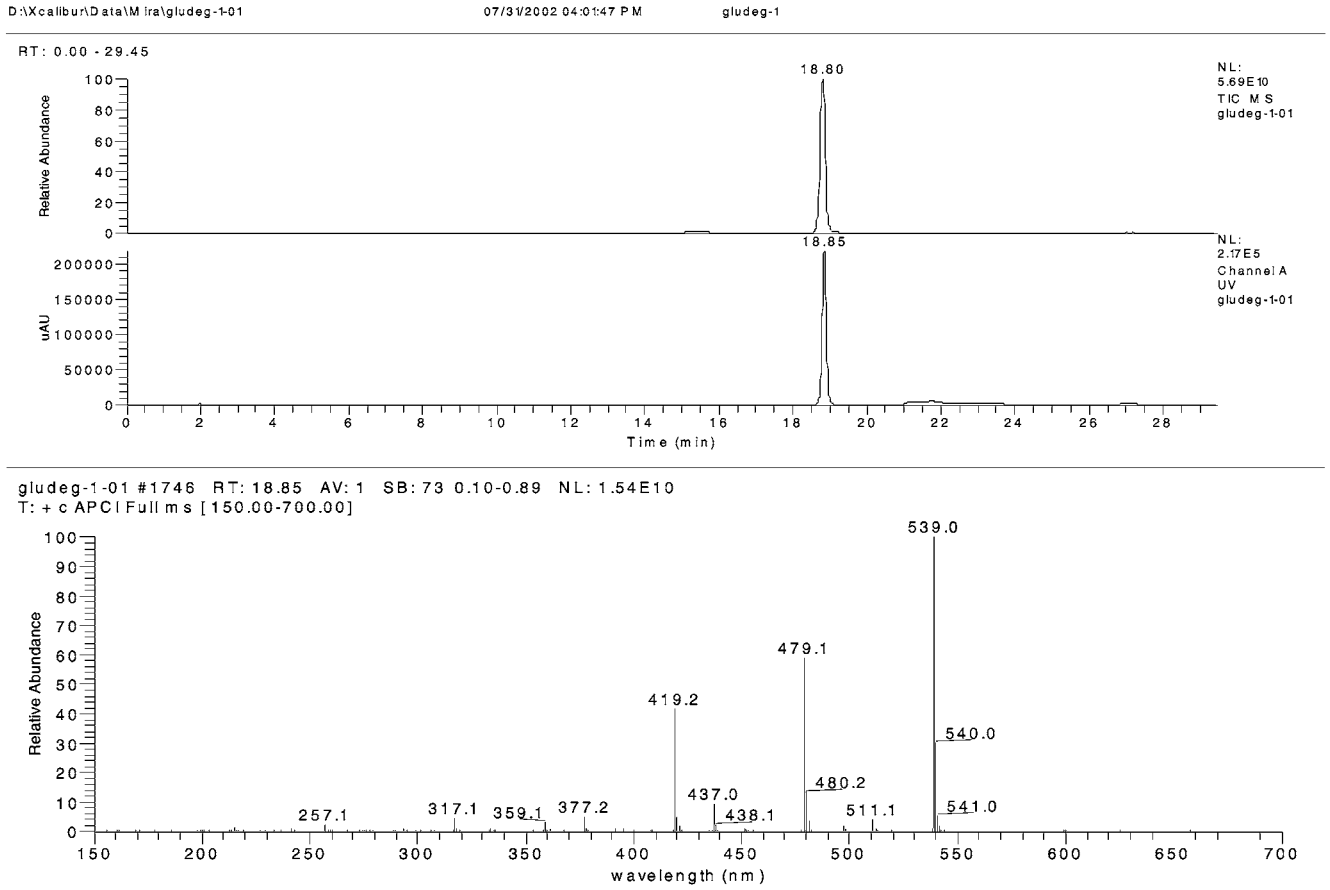

Figure 3. TIC trace (top) and APCI-(+)-MS spectrum (bottom) of **2b** from glucosamine degradation.

^{1}H and ^{13}C NMR data of **2b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (2H, s, H-3, H-6), 6.78 (2H, m, H-7, H-8), 6.13 (1H, d, H-11), 5.70 (2H, m, H-9, H-12), 5.30 (1H, m, H-13), 4.30 (4H, m, H-10, H-14), 2.20, 2.15, 2.10, 2.09, 2.04, and 1.94 (18H, s, 6 × CH_3 in Ac); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.59 (s), 170.57 (s), 169.95 (s), 169.80 (s), 169.75 (s), 169.00 (s) (6 × C═O in Ac), 150.12 (s, C-2), 149.22 (s, C-5), 142.59 (d, C-3), 142.22 (d, C-6), 131.09 (d, C-7), 128.91 (d, C-8), 72.01 (d, C-11), 71.23 (d, C-12), 70.50 (d, C-13), 68.38 (d, C-9), 64.54 (t, C-14), 61.83 (t, C-10), 21.00 (q, Me), 20.79 (q, Me), 20.76 (q, Me), 20.68 (q, 2 Me), 20.38 (q, Me).

RESULTS AND DISCUSSION

The structures of glucosamine and its degradation products are shown in **Figure 1**. Major degradation products were pyrazine derivatives containing four carbons with acetylated sugar side chains attached to C-2 and C-5 of the ring, respectively. **1b**, **2b**, and **3b** were identified from degraded glucosamine raw material. **3b** and **4b** were found as degradation products of glucosamine solid dosage form.

1a was identified as 5-HMF, by TLC comparison with standard 5-HMF, by retention time in HPLC spectrum, and by on-line UV spectrum with maximum absorption at 276 nm. In the previous studies, 5-HMF has been identified from various conditions of glucosamine such as basic (ammonia or sodium hydrochloride solution) and acidic (hydrochloric acid in the presence of glycine) (*7*, *10*, *13*). It is reported that the formation of 5-HMF was increased under acidic condition; however, the

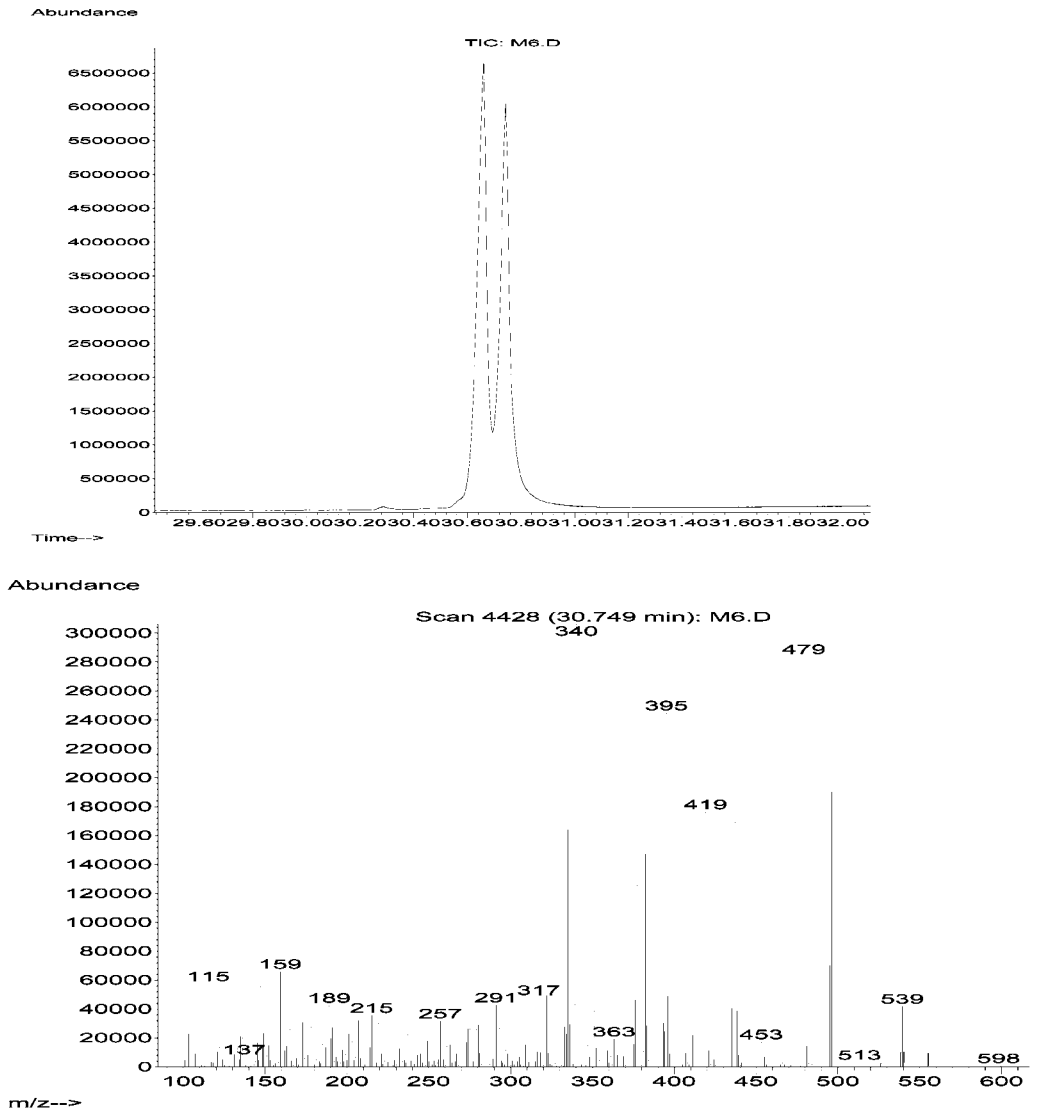

Figure 4. TIC trace (top) and GC-MS spectrum (bottom) of **3b** from glucosamine degradation.

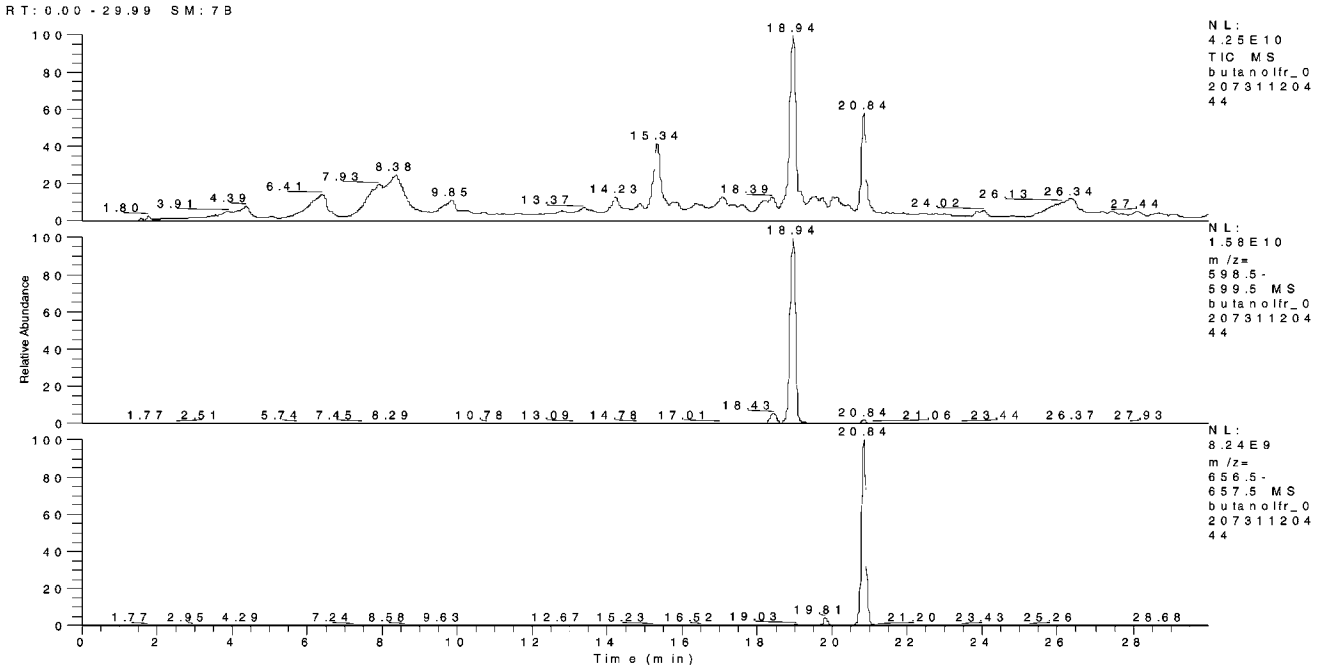

Figure 5. TIC trace (top) and extracted ion traces (bottom) of degradation products (**3b** and **4b**) from glucosamine solid dosage form.

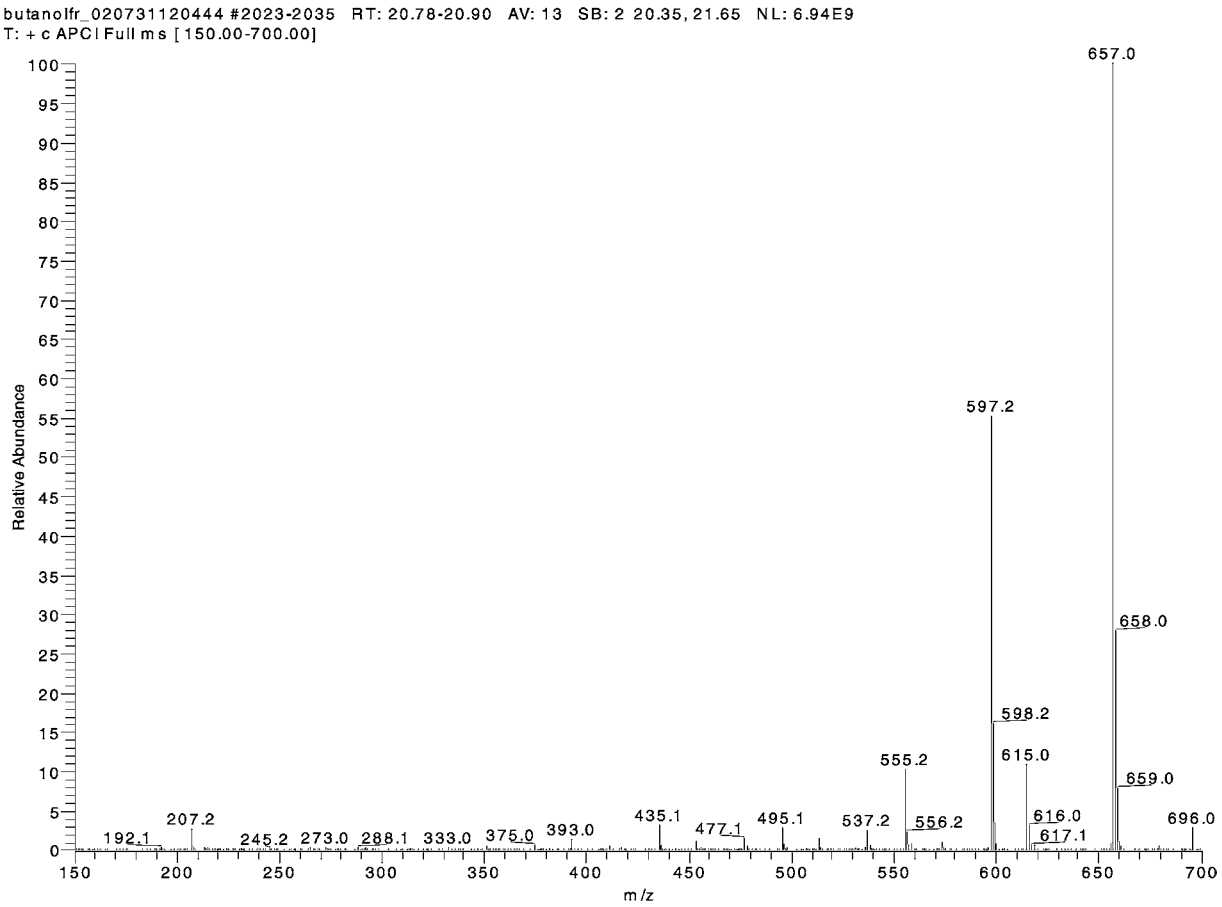

Figure 6. APCI-(+)-MS spectrum of **4b** identified from degraded glucosamine solid dosage form.

formation of pyrazine derivatives were greatly decreased (*13*). The formation mechanism of **1a** is proposed in **Figure 2**. From glucosamine, dehydration and deamination led to 3-deoxyhexosone, followed by cyclization. In its cyclic form, by loss of water it will give hydroxymethylfurfural.

2b was the major compound generated during the glucosamine degradation reaction. With the recent development of the atmospheric pressure ionization) (API)−mass spectrometry technique, on-line HPLC−photodiode array)-MS has become a powerful technique for identifying low-level degradation products in the stability study of drug substances. This technique gives an on-line mass spectrum and UV spectrum that can elucidate the chemical structures. Degradant **2b** was analyzed by LC-MS. The on-line UV spectrum of **2b** displayed maximum absorption at 300 nm, which is reported as the characteristic of disubstituted pyrazine conjugated with a vinyl group (*14*, *15*). The TIC trace and APCI-(+)-MS spectrum of **2** are shown in **Figure 3**. It gave a protonated molecular ion $[M + H]^+$ at *m*/*z* 539.0. The fragmental ions at *m*/*z* 479, 419, and 359 were due to the consequential loss of three molecules of acetic acid (HOAc, 60 amu), respectively. The fragmental ion at *m*/*z* 377 corresponds to the loss of an acetyl group ($CH_3C{=}O$) from the fragment ion at *m*/*z* 419 (419 − 377 = 42). From the fragment ion at *m*/*z* 377, other losses of two molecules of acetic acids (HOAc, 60 amu) are indicated by fragmental ions at *m*/*z* 317 and 257.

The chemical structure of **2b** was confirmed by NMR analysis. The interpretation of the ^1H and ^{13}C NMR data was based on DEPT and $^1H-^1H$ COSY spectra. ^1H NMR of **2** demonstrated signals at δ 2.20, 2.15, 2.10, 2.09, 2.04, and 1.94 (18H, 6s), which were correlated to six acetyl methyl groups. From the signals at δ 8.49 (2H, s), it was confirmed that **2b** has a 2,5-disubstituted pyrazine moiety. Signals at δ 6.78 (2H, *m*) indicated two alkenic protons in a double bond between C-7 and C-8 in the molecule (**Figure 1**). Six signals at δ 170.59 (s), 170.58 (s), 169.95 (s), 169.80 (s), 169.75 (s), and 169.00 (s) from ^{13}C NMR of **2b** suggested six carbonyl groups (C═O) in the molecules. Six signals at 21.00 (q, Me), 20.79 (q, Me), 20.76 (q, Me), 20.68 (q, 2 Me), and 20.38 (q, Me) indicated six acetyl methyl carbons in the moiety.

In $^1H-^1H$ COSY spectra, correlations between H-11 at δ 6.13 and H-12 at δ 5.70, H-12 and H-13 at δ 5.30, and H-13 and H-14 at δ 4.30 were observed, indicating a moiety of $-CH-CH-CH-CH_2-$. Correlation between H-7 and H-8 at δ 6.78, between H-8 and H-9 at δ 5.70, and between H-9 and H-10 at δ 4.30 indicated a unit of $-CH{=}CH-CH-CH_2$ in degradant **2b**. Therefore, the chemical structure of **2b** can be concluded as shown in **Figure 1**.

The complexity of signals of H-7 and H-8 at δ 6.78 made it difficult to calculate their coupling constant. Thus, a decoupling experiment was performed. Decoupling the multiplet of signal at δ 5.70 (H-9) partially collapsed and simplified the multiplet of signal at δ 6.78 for H-7 and H-8 to a pair of doublets. The coupling constant between H-7 and H-8 was calculated as 15.6 Hz, indicating the double bond has the trans from. Therefore, **2a** was identified as 2-(tetrahydroxybutyl)-5-(3′,4′-dihydroxy-1′-*trans*-butenyl)pyrazine.

TLC of **3b** showed a single spot. However, GC-MS showed two separate peaks (**Figure 4**), and three separate peaks were shown in HPLC. It is assumed that three or more isomers seemed to exist in the two attached peaks in the GC-MS spectrum. They exhibited the same pattern of UV spectral maximum absorption at ∼275 nm, which implied that **3b** is a disubstituted pyrazine derivative (*14*−*16*). They had the same

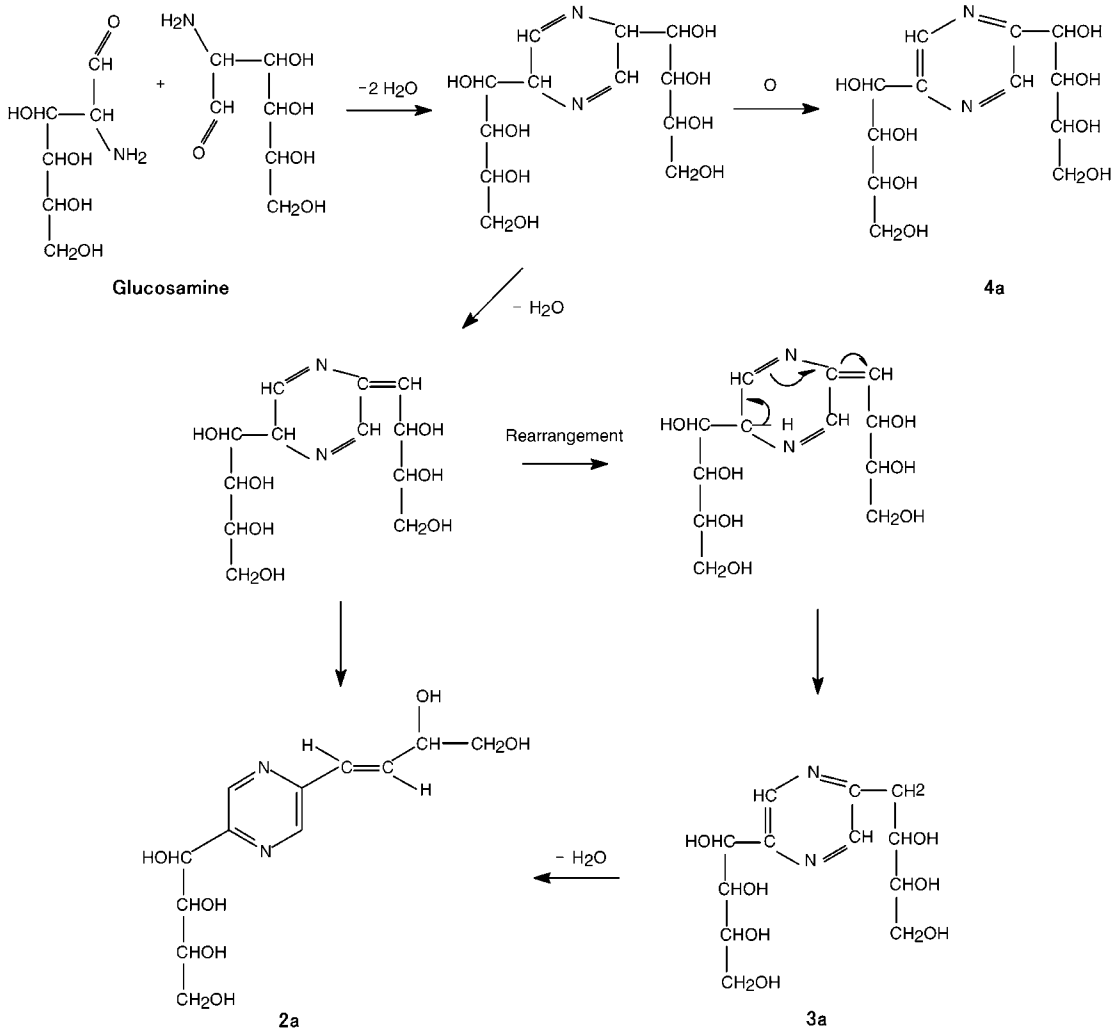

Figure 7. Proposed formation mechanism of **2a**, **3a**, and **4a** in glucosamine and glucosamine solid dosage form degradation.

molecular weight and displayed almost the same fragmentation pattern in GC-MS spectra. In **Figure 4**, two fragmentation ions at *m*/*z* 555 and 539 were probably due to losses of acetyl ($CH_3C{=}O$) and $COOCH_3$ groups, respectively, from the molecular ion *m*/*z* 598. The fragmentation ion signals at *m*/*z* at 496, 479, 437, and 419 corresponded to the loss of [M − OAc − Ac], [M − HOAc − OAc], [M − OAc − OAc − Ac], and [M − HOAc − HOAc − OAc], respectively. It was not enough to prove the chemical structure of **3b** by only the information acquired from UV spectra and GC-MS spectra. However, compared with **2b**, degradant **3b** seemed to contain one more molecule of acetic acid than **2b**: 598 (MW of **3**) − 538 (MW of **2**) = 60 (HOAc, 60 amu), corresponding to the unit of acetic acid. This suggested that **3b** might be isomers having disubstituted pyrazine with different, acetylated sugar side chains, one of which contains a $-CH_2$ group. One of the deactylated isomers can be suggested as 2-(tetrahydroxybutyl)-5-(2′,3′,4′-trihydroxybutyl)pyrazine or deoxyfructosazine (**Figure 1**). The previous study regarding decomposition reactions of glucosamine (in acidified aqueous solution containing glycine) also mentioned these isomers but they were not positively determined (*10*). The number of isomers can be higher than assumed. Deoxyfructosazine has been identified from the reaction of glucose and ammonia as early as 1963 (*17*). Later, Tsuchida et al. identified deoxyfructosazine and its 6-isomer as browning reaction products between glucose and ammonia in a weakly acidic medium (*18*) and from nondialyzable melanoidin hydrolysate (*19*).

On the basis of the knowledge of glucosamine bulk drug degradation, the degradation profile of glucosamine solid dosage form, stored at 40 °C in 75% relative humidity for 10 weeks, was qualitatively analyzed by LC-MS. The TIC trace and extracted ion traces at *m*/*z* 598.5 and 656.5 are given in **Figure 5**, respectively. By UV spectra, the peak at RT 18.94 min was also identified to be a disubstituted pyrazine derivative from its maximum absorption at ∼275 nm (*14*−*16*). In addition, it demonstrated an equivalent fragmentation pattern in on-line MS spectra with that of **3b**, bearing four-carbon, acetylated acyclic sugar side chains with a methylene group on one of the side chains. Therefore, the peak at RT 18.94 (**3b**) was identified as peracetylated deoxyfructosazine.

As shown in **Figure 6**, on-line MS spectra of the peak at RT 20.84 gave a protonated molecular ion *m*/*z* at $[M + H]^+$ 657. The UV spectra maximum absorption at 275 nm suggested the molecule to be a disubstituted pyrazine. The fragmental ions at 597, 537, and 477 suggested the losses of [− HOAc], [−HOAc − HOAc], and [− HOAc − HOAc − HOAc], respectively. Also, several peaks showed the loss of [$CH_3C{=}O$], for example, from peaks at *m*/*z* between 597 and 555 and from peaks at *m*/*z* between 598 and 556. Compared with MS spectra of **2b**, 656 (MW of **4b**) − 538 (MW of **2b**) = 118 corresponded to the two units of CH_3COO- (59). Therefore, **4a** was identified as 2,5-bis(tetrahydroxybutyl)pyrazine or the so-called fructosazine. In previous studies, **4a** has been found in the various degradative conditions of glucosamine such as in the reaction of a glucosamine and lysine mixture and in glucosamine aqueous

ammonia (*20*, *21*). In addition, (polyhydroxyalkyl)pyrazine compounds such as 2,5-bis(tetrahydroxybutyl)pyrazine and 2-(tetrahydroxybutyl)-5 (or 6)-(2′,3′,4′-trihydroxybutyl)pyrazine have been reported to be formed in roasted peanuts and soy sauce (*16*, *22*).

In the present study, the structures of **2a**, **3a**, and **4a** were elucidated by analytical data obtained from NMR, MS, and UV. There is no attempt to synthesize these compounds for unequivocal structural elucidation; we will consider our identifications tentative.

The formation mechanism of pyrazine derivatives, major degradation products of glucosamine, is proposed in **Figure 7**. Pyrazine heterocycles are probably formed by self-condensation of glucosamines with two water molecules during thermal reaction, yielding polyhydroxydihydropyrazine. From an intermediate dihydropyrazine**,** 2,5-bis(tetrahydroxybutyl)pyrazine (**4a**) is generated by an oxidative process. Pyrazine, followed by dehydration and aromatization, gave **3a**. Alternatively, **3a** might also be formed from the dihydropyrazine intermediate by a double-bond shift initiated by deprotonation in the dehydropyrazine ring with aromatization and dehydration in the side chain as driving forces. **3a** was identified from both degradation of glucosamine raw material and glucosamine solid dosage form. On the other hand, **2a** was identified only in degraded glucosamine raw material (100 °C) but not in degraded glucosamine solid dosage form at a milder temperature (40 °C). As shown in **Figure 7**, **2a** has a chemical structure similar to that of **3a** but is further dehydrated. A stressed condition with higher temperature seemed to facilitate such a dehydration process and generated **2a** from **3a**.

From the preliminary data not presented in this study, many degradation products were observed in glucosamine and its solid dosage form with various factors such as pH, moisture, and temperature, but they are not identified so far. Further studies on glucosamine degradation might be needed for the better understanding of the glucosamine degradation profile.

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