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Short communication

Kinetics and mechanism of 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside (THSG) degradation in aqueous solutions

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ABSTRACT

The hydrolytic kinetics and degradation mechanism of 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside (THSG) extracted from Radix *Polygoni Multiflori* (a commonly used officinal Chinese herbal *Heshouwu*), were investigated using reversed-phase high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS). The influences of pH (1.5–9.9), temperature (25–60 °C) and irradiation on the hydrolysis of THSG were studied in aqueous solutions. The results showed that the degradation of THSG was pH-, temperature- and irradiation-dependent and all followed first-order kinetics. The effect of temperature on the rate of THSG degradation was characterized using the Arrhenius equation. Maximum stability of THSG was found at pH 1.5 ($t_{0.5}$ = 47.57 d). THSG was unstable in alkaline and irradiation conditions. The active energy (E_a) of THSG degradation in aqueous solution at pH 6.8 (most frequently adopted extract solvent) under lucifugal and irradiation conditions was 47.7 kJ mol^{-1} and 25.3 kJ mol^{-1}, respectively. Three hydrolytic products of THSG were identified by LC–MS. *Cis–trans* isomerism took place under irradiation, and hydrolysis took place in acid–base conditions. Moreover, further oxidation on aglycon occurred after hydrolytic cleavage of phenolic glycoside in acidic conditions. The possible hydrolytic pathways of THSG are proposed.

1. Introduction

Radix *Polygoni Multiflori*, one of the authentic Chinese herbal drugs (HDs) in the Chinese pharmacopeia, has been recognized as an important health protection and nutritional supplement for thousands of years. Dozens of Chinese herbal drug preparations (HPDs), healthy tea and food containing Radix *Polygoni Multiflori* have been developed and sold in China and East Asia. As the bioactive and marker ingredient of Radix *Polygoni Multiflori*, 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside (THSG) has been proved to possess a variety of bioactivities. Recent pharmacological studies indicated that THSG has strong antioxidant and free radical scavenging activity [1–3]. In addition, THSG is indicated to reduce hyperlipidemia, prevent lipid peroxidation (LPO) and protect the cardiovascular system [4–6]. It is also effective in the prophylactic and therapeutic treatment of Alzheimer's disease [7,8].

Similar to other polyhydroxy stilbene compounds (such as resveratrol), the bioactivities of THSG have mainly been attributed to its antioxidant property, which is related to the polyphenolic unit (Fig. 1). This structure is also related to its instability [9–11]. THSG is thought to be oxidized and hydrolyzed, ultimately, resulting in degradative decomposition. Ban et al. discovered that THSG was unstable in extreme pH environments (4% sulfuric acid and 4% NaOH) [12]. However, to the best of our knowledge, there have been no reports on the chemical stability and degradation mechanism of THSG.

Since decoction is the most common dosage form of TCM, the stability of THSG in aqueous solution was investigated in this study for the first time. The study aimed to make available information on the inherent stability of THSG in aqueous solution under ICH guidelines and SFDA guidelines [13]. The stability research carried out under stress conditions (different temperatures, pH values, irradiation conditions) serves as facile models for understanding the degradation mechanisms of THSG in aqueous solution.

2. Materials and methods

2.1. Chemicals

THSG was isolated from Radix *Polygoni Multiflori* in the Research Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine. Silica gel column chromatography and recrystallization were adopted to separate and purify THSG. The TLC results showed a pure fleck in different solvent system. Following identification by LC–MS, ^{1}H NMR and ^{13}C NMR, the purity of THSG

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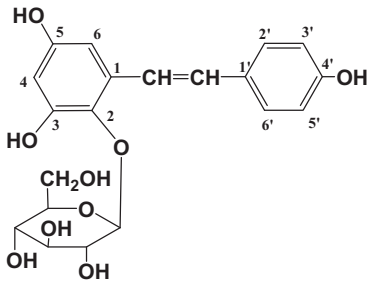


Fig. 1. The structure of THSG.

was found to be greater than 96.5% as determined by HPLC. The THSG reference was purchased from the National Institute for the Control of Biological and Pharmaceutical Drugs (Beijing, PR China, Batch No. 110844-200606). Acetonitrile was bought from Merck (Merck & Co., Inc., US). All other chemicals used in this experiment were analytical grade.

2.2. Analytical method

2.2.1. HPLC method

The analysis of THSG was performed using a Waters 600E HPLC system equipped with a 2487 UV-Detector (Waters Corporation, US) on a Waters SymmetryShield™ RP-C_{18} column (150 mm × 3.9 mm, 5 μm). The column was maintained at 25 °C, the detection wavelength was 320 nm, the flow rate was 1.0 mL min^{-1}, and injection volume was 10 μL. An isocratic elution was used with acetonitrile–water (24:76, v/v) for the stability studies. These methods allowed satisfactory chromatographic separation. No interference with THSG and/or its degradants was exhibited by blank injections.

2.2.2. HPLC–ESI/MS method

The degradants were identified by HPLC–MS (Agilent 1100 series HPLC, Agilent Technology, US; LCQ Iontrap MS, Finnigan Corporation, US). MS detection was performed using an electrospray ionization (ESI) source, both in positive and negative modes. The HPLC conditions for LC–MS analysis were achieved on a linear gradient of acetonitrile–water from 25% to 50% acetonitrile for 40 min. The column was kept at 25 °C, the flow rate was 1.0 mL min^{-1}, post-column split. The MS parameters were set as follows: auxiliary gas was 5 arbitrary units; sheath gas was 20 arbitrary units; spray voltage was 4.5 kV; capillary temperature was 300 °C. The mass spectrometer was programmed to perform full scans from 100 to 1000 m/z in order to obtain molecular ion signals as well as fragments or adducts of the possible degradation products.

2.3. Sample solution

To avoid contamination by microorganisms, all glassware was sterilized by autoclaving for at least 20 min at 120 °C prior to use. The THSG reference was dissolved in methanol to obtain a stock solution with a concentration of 500 μg mL^{-1} (stored at 4 °C in the dark). The final concentration of all stability samples was 10 μg mL^{-1}, which was obtained by mixing a 2:98 ratio of stock solution with the appropriate experimental solution. Seven solutions of different pH value (pH 1.5–9.9) were prepared using sodium hydroxide and hydrochloric acid prior to use, the pH values of all solutions were determined by a pH meter (DELTA 320, METTLER TOLEDO Corporation, Switzerland) equipped with a combination electrode, which was calibrated with primary buffer solution of pH 4.01, 6.86 and 9.18.

2.4. THSG stability studies

2.4.1. Effect of pH

To evaluate the effect of pH on hydrolysis of THSG, all stability samples at different pH values were sealed in screw-topped, light-proof test tubes and then kept at 25 °C. The samples were subjected to HPLC analysis to determine the content of THSG periodically. Observed rate constants (k_{obs}) at different pH values were obtained by a chemical kinetic equation to evaluate the influence of pH (1.5–9.9) on hydrolysis. Linear regression analysis was performed on the data using the computer software program Microsoft Excel 2007 (Microsoft Corp., US), with the given k_{obs} and half-life of THSG.

2.4.2. Effect of temperature

The influence of temperature on degradation was investigated in aqueous solution at pH 6.8 (most commonly used form of TCM herbal drugs). All samples were sealed in screw-topped, light-proof test tubes and placed in a high precision water bath capable of controlling the temperature within ±0.5 °C. Samples were periodically withdrawn and cooled to ambient temperature before injection. The kinetics of hydrolysis were investigated at 25 °C, 34 °C, 44 °C, 50 °C and 60 °C. An Arrhenius plot was adopted to analyze the influence of temperature on the rate of degradation.

2.4.3. Effect of irradiation

Samples were prepared in aqueous solution at pH 6.8 and kept in a stability chamber (KBF, WTB Binder, Germany) equipped with incandescent light (GLS-20W-C Philips, Netherland). Illuminance (E_n) was controlled at 660 ± 50 l× and real time monitored by a luminometer (TES-1332A digital-lux meter, TES, Taipei, China). The cumulative illuminance (E_{cum}) was calculated using the equation: $E_{cum} = \Sigma E_n \times \Delta t$. The samples were moved from the chamber and cooled before detection to quench the reaction. The content of THSG was determined by HPLC. To evaluate the influence of irradiation, k_{obs} and active energy (E_a) were also obtained at 25 °C, 34 °C, 44 °C, 50 °C and 60 °C similar to Section 2.4.2 by comparing with samples in dark conditions.

2.5. Characterization of degradation products

THSG and its degradation products formed under all the stress conditions were separated and identified by HPLC–ESI/MS. In addition, the spectrophotometric characteristics of THSG aqueous solution under each condition was studied by spectrophotometry (Cary50, Varian, US) from 200 nm to 600 nm to investigate UV behavior using appropriate blank solutions. The degradation products were deduced based on the combination of MS and spectrophotometric information. Furthermore, the degradation mechanism of THSG was proposed.

3. Results and discussion

3.1. Hydrolytic kinetics

For a given concentration of THSG under conditions of different temperature, pH value and irradiation, the linear relationship between the natural logarithmic remaining percentage of THSG ($\ln(C_t/C_0)$) and hydrolytic time were depicted, respectively. High correlation coefficients (r over 0.99) were observed from the kinetic plots under all experimental conditions, therefore, the hydrolysis of THSG could be modeled using first-order kinetics. The k_{obs} were obtained from the slope of the kinetic plots and active energy was calculated from the Arrhenius equation.

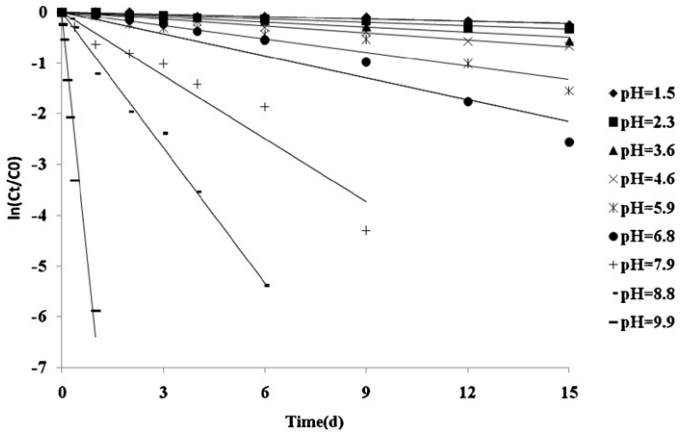


Fig. 2. Relationship of $\ln(C_t/C_0)$ and hydrolysis time for THSG at 25 °C in aqueous.

Table 1
Kinetic data of the hydrolysis of THSG at 25 °C in aqueous solutions.

pH	k (d^{-1})	r	$t_{1/2}$ (days)
1.5	0.0146	0.945	47.57
2.3	0.0224	0.986	30.94
3.6	0.0326	0.969	21.26
4.6	0.0457	0.997	15.17
5.9	0.0883	0.964	7.85
6.8	0.1440	0.962	4.81
7.9	0.4141	0.964	1.67
8.8	0.8898	0.995	0.78
9.9	6.3928	0.973	0.11

3.2. pH-rate profile of THSG

The change in THSG concentration *versus* time could be depicted as shown in Fig. 2. The k_{obs} and $t_{0.5}$ of THSG at different pH values are given in Table 1. It was shown that the maximum k_{obs} of degradation (pH 9.9) was 654 times higher than that of the minimum (pH 1.5). To better characterize the effects of pH (1.5–9.9) on the hydrolysis of THSG, the natural logarithm of k_{obs} was plotted in Fig. 3 against pH values. The k_{obs} were found to increase with pH. A linear regression between ln k and pH (r = 0.9562) was conducted to model the degradation characteristics of THSG in aqueous solutions.

As shown in Fig. 2, the most stable condition for THSG was found to be pH 1.5, while it was extremely unstable in alkaline conditions. It is thought that the hydrolysis was catalyzed both by hydrogen and hydroxyl ion. However, phenolic glycoside was more susceptible in alkaline conditions. Thus, the alkaline catalysis reaction was much steadier.

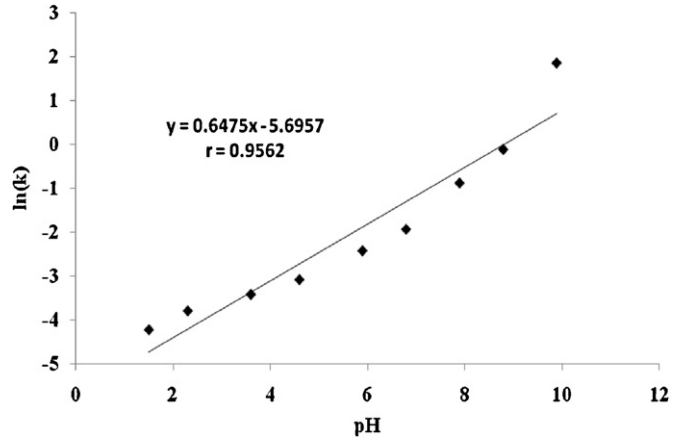


Fig. 3. Relationship between log (k) and pH for THSG at 25 °C in aqueous solutions.

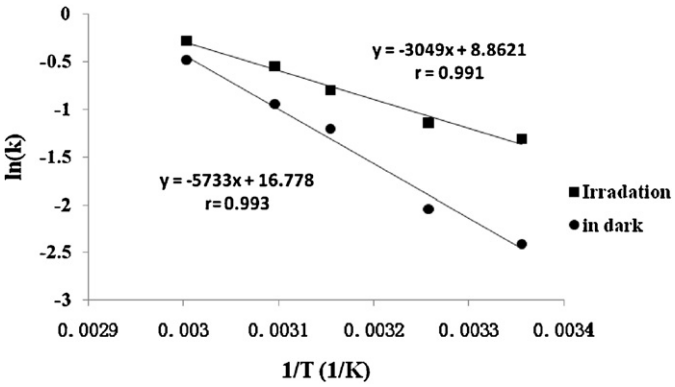


Fig. 4. Relationship between ln (k) and $1/T$ for THSG hydrolysis in aqueous solutions of pH 6.8.

3.3. Influence of temperature

The hydrolysis of THSG in neutral aqueous solution (pH 6.8) was monitored at a temperature range of 25–60 °C. The effects of temperature on the degradation rate of THSG were characterized by the Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{RT}$$

where A is the pre-exponential factor typical of THSG hydrolysis reaction, E_a is the activation energy (J mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), and T is the absolute temperature (K) [14,15].

According to the Arrhenius equation, the activation energy of THSG was calculated to be 47.7 kJ mol^{-1}, indicating that THSG in neutral aqueous solution (pH 6.8) was liable to degrade at ambient temperature.

As shown in Fig. 4, the degradation of THSG in aqueous solution (pH 6.8) at different temperatures followed first-order kinetics because a good linear relationship (r was above 0.99) was found between $\ln(C_t/C_0)$ and time. The k_{obs} obtained by kinetic equation increased as temperature increased.

3.4. Influence of irradiation

The degradation rate constant ($k_{obs(light)}$) of THSG at different temperatures under irradiation conditions was obtained from linear regression of $\ln(C_t/C_0)$ *versus* E_{cum}. The degradation reaction was observed to follow first order kinetics and was irradiation-dependent. Active energy ($E_{a\,light}$) was calculated by the Arrhenius equation to be 25.3 kJ mol^{-1}. This indicated that THSG was unstable under irradiation in aqueous solution of pH 6.8. The degradation reaction took place easily when THSG was exposed to light at ambient temperature.

3.5. Spectrometry analysis

3.5.1. In alkaline conditions (pH 9.9)

In alkaline conditions (pH 9.9), a red shift (from 320 nm to 349 nm) was observed initially. However, the spectra recorded at a later time were clearly blue shifted (from 349 nm to 280 nm) with respect to the initial wavelength (Fig. 5).

At first, rapid ionization of phenolic hydroxyls in alkaline solution led to an increase in electron cloud density of the benzene ring. The conjugation effect in the system was strengthened, leading to

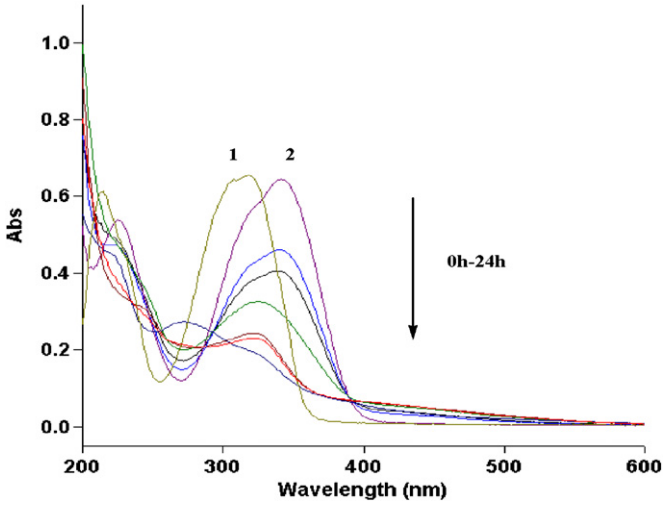


Fig. 5. Change of UV absorption with time of THSG hydrolysis in aqueous solutions (peak 1 in pH 6.8 and series peak 2 in pH 9.9).

red shift of the maximum wavelength. However, the subsequent blue shift was thought to be induced by the formation of a new chromophore.

3.5.2. Under irradiation

In the irradiation test, a small blue-shift of the absorption maximum (from 320 to 290) was observed. This was thought to be attributed to a lower degree of symmetry and conjugation of the new product (Fig. 6).

3.6. Degradation products

There were three degradants formed in acidic, alkaline and irradiation conditions, respectively. As far as we known, no available information on the degradants of THSG have been reported in the literature. On the basis of the chemical structure of THSG and the analytical results (UV–vis spectrometry, HPLC–MS, HPLC–MS/MS), the degradation products of THSG in aqueous solution were deduced and are illustrated in Table 2.

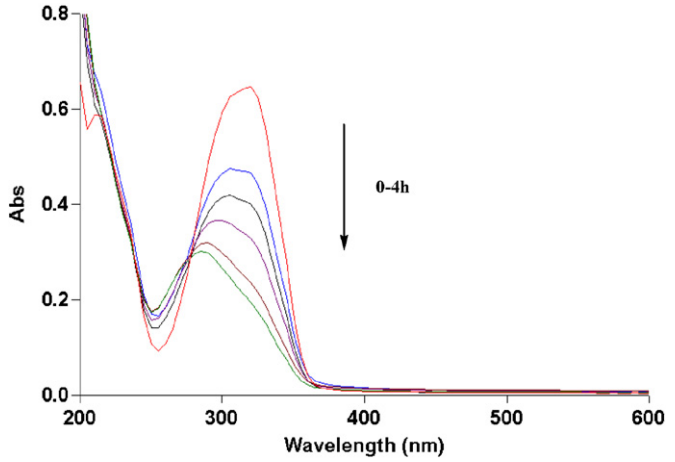
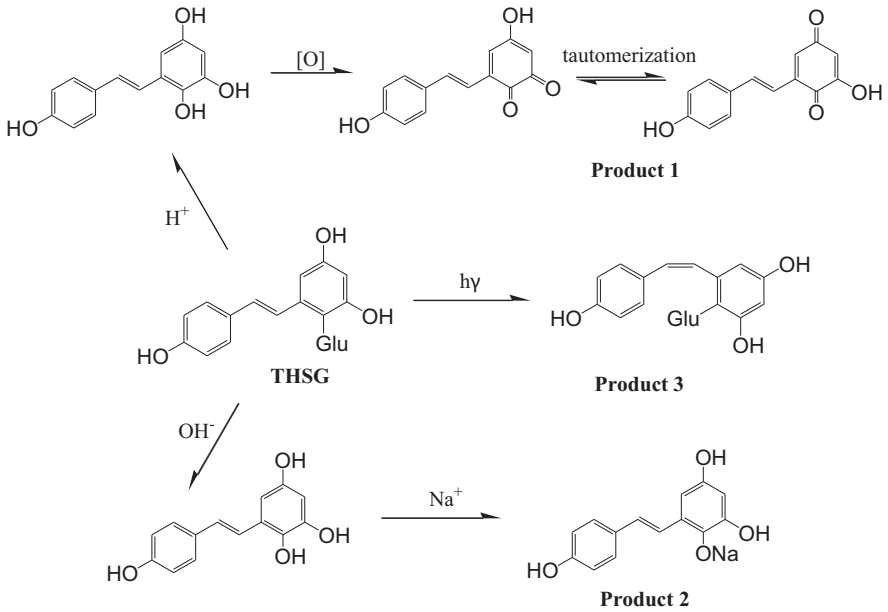


Fig. 6. Change of UV absorption with time of THSG hydrolysis in aqueous solutions in pH 6.8 under irradiation.

Degradation product 1 was detected as +ESI $m/z = 243$ and −ESI $m/z = 241$. Neutral loss of 164 amu was found. The C2 glucosidic bond was cleaved due to acid catalysis hydrolysis. The C2–C3 o-di-hydroxybenzene of aglycon was oxidized steadily to C2–C3 o-dibenzoquinone (1-4′-hydroxy-phenylethyl-5-hydroxy-2,3-o-benzoquinone). C2–C5 p-dibenzoquinone (1-4′-hydroxy-phenylethyl-3-hydroxy-2,3-p-benzoquinone) was produced due to keto-enol tautomerism with C5 hydroxyl. Thus, degradation product 1 was deduced to be 1-4′-hydroxy-phenylethyl-5-hydroxy-2,3-o-benzoquinone and/or 1-4′-hydroxy-phenylethyl-3-hydroxy-2,3-p-benzoquinone.

Degradation product 2 was detected as +ESI $m/z = 268$ and −ESI $m/z = 243$. Neutral loss of 162 amu was thought to be one glucose molecule lost from THSG. The C2 glucoside was liable to be hydrolyzed in alkaline conditions. Influenced by the C3 and C5 hydroxyl, C2 hydroxyl showed enhanced acidity. It was salified steadily to form a sodium salt which hampered further oxidation. Degradation product 2 was deduced to be 2,3,5,4′-tetrahydroxystilbene sodium salt.

Degradation product 3 was detected as +ESI $m/z = 407$ and −ESI $m/z = 405$. Similar to THSG, the base peak $m/z = 245$ in the +ESI MS^2



Scheme 1. Proposed degradative pathway of THSG in aqueous solution.

Table 2
The MS analysis result of THSG degradation products.

Conditions	Name	Retention time (min)	Positive ion (*m*/*z*)	Negative ion (*m*/*z*)	Supposed structure
Acidic	Product 1	20.5	243	241	1-4′-Hydroxy-phenylethyl-5-hydroxy-2,3-o-benzoquinone or 1-4′-hydroxy-phenylethyl-3-hydroxy-2,5-p-benzoquinone
Alkaline	Product 2	8.3	267	243	2,3,5,4′-Tetrahydroxystilbene sodium salt
Irradiation	Product 3	11.7	405	407	*Cis*-2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside
–	THSG	14.2	405	407	*Trans*-2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside

spectrum was generated by loss of 162 amu from the precursor ion. However, degradation product 3 and THSG showed different retention times on single HPLC analysis. It is thought that degradation product 3 (*cis*-2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside) and THSG (*trans*-2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside) were *cis–trans* isomers. The stereospecific blockade of the *cis*-form of THSG hindered the hydrolysis of the glucosidic bond. *Cis*/*trans* isomerization on the C=C skeleton was also reported [16].

3.7. Degradation pathway

Following identification of all degradation products, the degradation pathway of THSG is depicted in Scheme 1. In acidic conditions, oxidation of the aglycone occurred after hydrolytic cleavage of the glucosidic bond. Salification of the aglycone of THSG occurred in sodium hydroxide solution. *Cis–trans* isomerization of THSG occurred under irradiation conditions. *Trans*-THSG exists in nature and is converted to *cis*-THSG.

4. Conclusion

The stability of THSG in aqueous solution was studied following the ICH and SFDA guidelines. The degradation of THSG was found to be pH-, temperature- and irradiation-dependent. Degradation of THSG followed first-order kinetics. The half-life at 25 °C was predicted to be 47.57 d at pH 1.5 and 0.11 d at pH 9.9. The activation energy of THSG in neutral aqueous solution (pH 6.8) was calculated to be 47.7 kJ mol^{-1} and 25.3 kJ mol^{-1} under lucifugal and irradiation conditions, respectively. These results indicated that THSG was unstable under alkaline or irradiation conditions. It is suggested that THSG should be extracted, isolated and kept in acidic conditions and protected from light.

The degradation mechanism was thought to be acid- and base-catalyzed hydrolysis and oxidation in THSG aqueous solution under lucifugal conditions. Isomerization took place under irradiation. Three degradation products were formed and were separated and identified successfully by HPLC, LC–MS, and UV spectrometry. The stability research on THSG in aqueous solution could provide reliable information on the preparation, application and storage of HDs and HPDs containing THSG.

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