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Stability studies of tetracycline in methanol solution

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Abstract

The stability of tetracycline in methanol solution was investigated by UV–visible spectroscopy, HPLC and TLC methods. After dissolution in methanol, tetracycline decomposed rapidly under the influence of light and atmospheric oxygen, forming more than fourteen different degradation products. None of the previously reported degradation products, such as the epi- and anhydro-compounds, were detected as the final degradation products. The molecular structures for eight of the compounds were suggested by their product-ion mass spectra. A degradation sequence was proposed for the reactions of tetracycline with methanol. A new HPLC–MS mobile phase was developed, which solved the clogging problem at the interface between the HPLC and MS chamber and enabled a high separation efficiency. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Mass spectrometry; Stability studies; Tetracyclines; Antibiotics; Methanol

1. Introduction

Tetracycline antibiotics are known to be unstable, often changing from yellow to brown over time [1–4]. The stability of tetracyclines has been studied by several different methods [5–10]. Although more complex degradation schemes have been suggested and some unknown degradation products have been observed [5,11–16], the only degradations that have been thoroughly studied are epimerization, which is a steric rearrangement in the configuration of dimethylamine group at C-4, and oxidation of the phenolic group present on the tetracycline nucleus [16]. No studies on the stability of tetracycline have been reported since the late 1980s.

Since methanol is used in most tetracycline analytical work as a dissolution, extraction and elution solvent, and is also used as an ingredient in several topical medicines containing tetracyclines, stability studies of tetracyclines in methanol solution are important and require investigation.

In this paper, the stability of tetracycline in methanol solution was investigated by UV–vis spectrometry, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) combined with UV–vis and mass spectrometry.

2. Materials and methods

2.1. Standards, solvents and reagents

Tetracycline free base was obtained from Sigma; the remaining tetracyclines were obtained from

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Table 1
Chemical information on tetracyclines

Name	Lot no.	Purity (%)
Tetracycline free base	115H0560	99.0
Anhydrotetracycline·HCl	A0097378	99.1
4-Epi-anhydrotetracycline·HCl	A0095119	99.5
4-Epi-tetracycline·HCl	A0078456	99.9
Chlorotetracycline·HCl	A0082854	99.20

Acros Organic; all standards were in powder or crystalline solid form. Chemical information from the manufacturers is provided in Table 1.

The stock solutions were prepared with HPLC-grade methanol (J.T. Baker, Philipsburg, NJ, USA): 300 ppm each of tetracycline free base (TC), anhydrotetracycline·HCl (ATC), 4-epi-tetracycline·HCl (ETC), and 4-epi-anhydrotetracycline·HCl (EATC), and a mixture containing 100 ppm each of ATC, ETC, and EATC. All stock solutions were stored at −11°C in plastic bottles wrapped in aluminum foil. Fresh stock solutions were prepared each month. Working standards were prepared daily by diluting the stock solutions with methanol to the desired concentration. The solutions were protected from light during use.

Other solvents and chemicals employed for this study were either HPLC-grade or analytical reagent grade. Organic solvents and ammonium hydroxide were obtained from Merck. Solutions of saturated disodium EDTA (Na_2EDTA) (Mallinckrodt, Paris, KT, USA) and oxalic acid (J.T. Baker) were prepared with distilled water. The pH of the Na_2EDTA solution was adjusted to 9.0 with 42% sodium hydroxide. The pH of oxalic acid and trifluoracetic acid were adjusted with ammonium hydroxide to the desired values. All solutions for HPLC analysis were filtered through a 0.45-μm FP Vericel membrane (Gelman Sciences, Ann Arbor, MI, USA) before use.

2.2. Degraded sample

Tetracycline free base was dissolved in methanol (300 ppm). The solution was kept at room temperature and exposed to room light. Analyses were performed after the solution had been stored for 6 months.

2.3. UV–vis spectrometry

A Hitachi U-2000 UV–vis spectrophotometer with a scan rate of 200 nm/min and spectral resolution of 1 nm from 200 to 1000 nm was employed for this study. The UV–vis spectra of 10 ppm tetracycline methanol solutions were measured.

2.4. High-performance thin-layer chromatography

HPTLC analysis was performed using a scientifically operated charge couple device (CCD) detector [17,18]. Fluorescence detection mode was used with a 360 nm excitation source and a 550 nm band-pass filter to remove any undesired radiation below 500 nm and beyond 600 nm. A HPTLC Si-gel plate (Merck) was used as the stationary phase. Proper separation of the TC compounds could not be achieved with an untreated silica-gel plate due to severe tailing problems [19–22]. To increase the separation efficiency and decrease the tailing problem, all the plates were coated with saturated Na_2EDTA (pH 9.0) solution. The plates were reactivated by heating at 105°C for 2 h and were then stored in a desiccator before use. Methanol–dichloromethane–water (30:64:6, v/v/v) was used as the mobile phase. The TLC plate was developed in a saturated developing chamber until the solvent front travelled 7.5 cm. After separation, the plates were dried in air for 5 min, then submerged in a 30% solution of paraffin in *n*-hexane for 1 s. The plates were then dried in air for another 10 min to allow the *n*-hexane to evaporate completely, as any remaining hexane would increase the fluorescence background level.

2.5. HPLC

A TSP P4000 LC-Spectra system combined with a UV 3000 diode array multichannel detector (Thermo Separation) was used for HPLC–UV–vis analysis. Dual channel absorption detection mode was used at 360 and 425 nm in order to detect tetracycline and its degradation products, ETC, EATC and ATC, simultaneously with maximum sensitivity. UV–vis wavelength scan detection mode was also used to obtain the UV–vis spectra of each separated component.

For HPLC–MS analysis, an HP 1050 automated HPLC (Palo Alto, CA, USA) system with a variable

wavelength detector was used to perform the separation. A Finnigan TSQ 7000 triple quadrupole mass spectrometer (San Jose, CA, USA), equipped with an atmospheric pressure chemical ionization (APCI) interface, was used for mass spectrometric analysis. Both electrospray ionization (ESI) and APCI methods were employed in the positive ion mode for sample ionization. The original degraded sample was used for APCI analysis and the same sample was condensed for ESI analysis. Molecular structures of the degradation compounds were derived from their daughter ion mass spectra, which were obtained in tandem MS–MS detection mode. Since ESI is a much softer ionization method than APCI, the ESI method was used to generate protonated molecules or adducts before the selected m/z components were fragmented by collision with argon gas (CID of 25 eV).

An Apex I 10 cm×4.6 mm ODS 5 μm column, (Johns Chromatography, Littletown, CO, USA), was used for the HPLC separation. Due to the chelating effects of TC with metallic cations, the existence of even trace amount of metals on the packing material will cause peak tailing and/or irreversible absorption effects. Therefore, the column was treated with Na_2EDTA to remove all trace metals before use. Na_2EDTA aqueous solution (0.1 M) was flushed through the column at a flow-rate of 2 ml/min for 1 h before the first sample run. The column was then rinsed with water for 30 min to remove all Na_2EDTA residue and was re-conditioned by flushing with methanol for another 30 min. No further treatment was necessary during the experiments.

Two eluents were used for HPLC analysis: (A) methanol–acetonitrile–0.1 M oxalic acid (pH 2.0) with a gradient from 10:5:85, (v/v/v) to 22.5:30:47.5 (v/v/v) in 25 min. The solvent flow-rate was 2 ml/min; (B) methanol–acetonitrile–0.2 M CF_3COONH_4 (pH 2.50) with a gradient from 10:5:85 (v/v/v) to 30:45:25 (v/v/v) in 40 min. The solvent flow-rate was 1 ml/min.

3. Results and discussion

3.1. UV–visible

All previous studies concluded that color change in tetracycline is due to the formation of ATC and EATC [3]. The UV–vis spectra of tetracycline and its known degradation products, ATC, EATC and ETC, have been measured [23,24], and were confirmed by this work. The UV–vis spectrum of the TC standards mixture should display the simple addition of each component without extra features in the visible range. Fig. 1a and b show that all of our results agreed with the above conclusion. However, comparison of the UV–vis spectra of the degraded sample with the TC standards mixture (Fig. 1b and c) demonstrated that ATC, ETC, and EATC were not the only degradation products of tetracycline. The degraded sample showed strong absorption around 303 and 338 nm and a broad absorption band between 400 and 650 nm, whereas the TCs mixture had relatively low absorption at those wavelengths and did not show any absorption beyond 500 nm. Furthermore, the TCs mixture remained yellow rather that turning brown like the degraded sample. Although the same behavior has been observed previously [9]. No explanation as to the nature of the degradation products was offered. The UV–vis experimental results indicated that neither EATC nor ATC, but some unknown compounds with an absorption in the visible range, were turning the tetracycline solution brown.

3.2. HPTLC

Fig. 2 illustrates the HPTLC chromatograms for

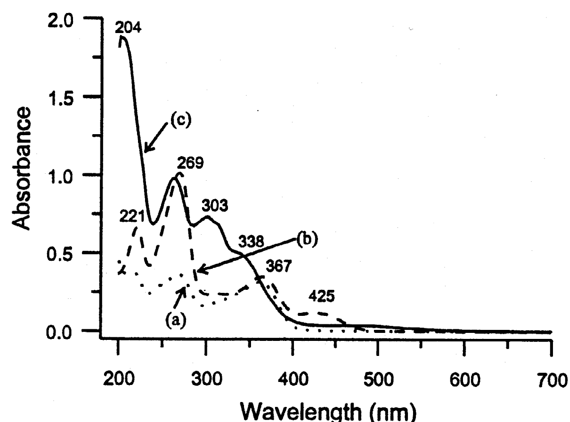

Fig. 1. UV–vis spectra of (a) TC (· · ·), (b) TC standards mixture (- - -) and (c) the degraded sample (———).

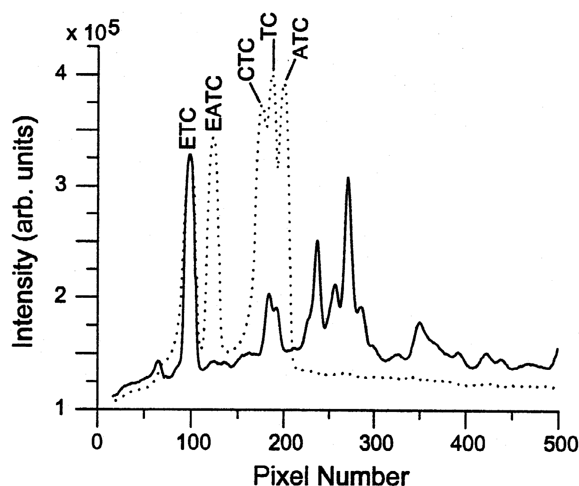

Fig. 2. HPTLC chromatograms of the degraded sample (———) and TC standards mixture (- - -).

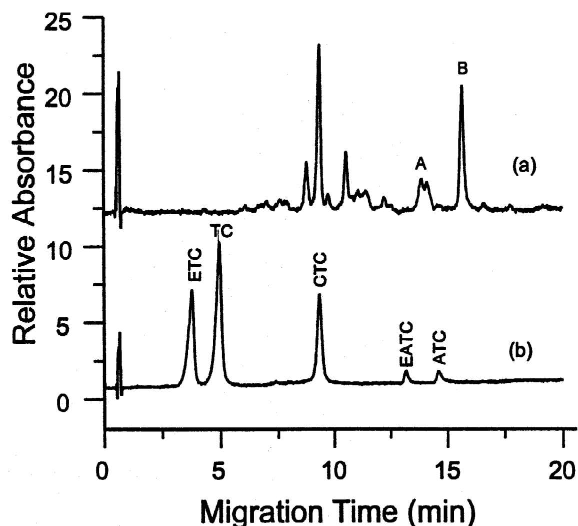

Fig. 3. HPLC chromatogram of (a) the degraded sample and (b) tetracycline standards mixture. Solvent A was used as mobile phase with UV–vis absorption detection mode at 350 nm.

the degraded sample and TC standards mixture. Compared with the TC standards mixture, more than ten fluorescent compounds were detected from the degraded sample, in which a very small amount of tetracycline remained. An intense chromatographic peak was detected in the area of the epi-tetracycline. However, the identities of the degradation products could not be determined using TLC.

3.3. HPLC

Two systems were used for the HPLC analysis. The first was an HPLC–UV–vis system with solvent A as the mobile phase. Fig. 3 shows the chromatograms of the tetracyclines and the degraded sample detected at 360 nm. The degradation of tetracycline in methanol solution was monitored over a 6-month period. After 10 days of storing in methanol, a significant amount of ETC, with tiny amounts of ATC and EATC, had formed. One month later, a significant amount of unknown components had formed as the concentration of ETC and TC decreased. No ATC or EATC was detectable at 425 nm. Meanwhile the relative peak area ratio of ETC to TC decreased from 33 to 27%. After storage in methanol for 6 months, no TC, ATC, EATC or ETC could be detected.

HPLC–UV–vis wavelength scan has been used to study the UV absorption properties of the degradation products. However, due to the interference of the mobile phase, UV absorbance features below 300 nm could not be distinguished from the background. All of the degradation products have absorption between 300 and 400 nm with several of them shifting toward shorter wavelengths. The components eluted with the solvent front and in peaks A and B (refer to Fig. 3a) had broad absorption bands extending into the visible range, indicating changes in the conjugation structure of the TC molecule.

The second method of analysis employed an HPLC–MS system using solvent B as the mobile phase. HPLC–MS is the best method to acquire information on molecular masses and chemical structures. However, the low volatility of oxalic acid has been shown to clog the interface between the HPLC column and the MS chamber [25] complicating HPLC–MS analysis. Various buffer solutions have been used for HPLC analysis of TCs, such as phosphorate [25–27] and acetate [28,29]. However, none of them are volatile enough to be injected directly into the MS chamber without clogging the interface. A new mobile phase was used in these studies: oxalic acid buffer solution was replaced with trifluoracetic acid (TFA) buffer solution. Testing

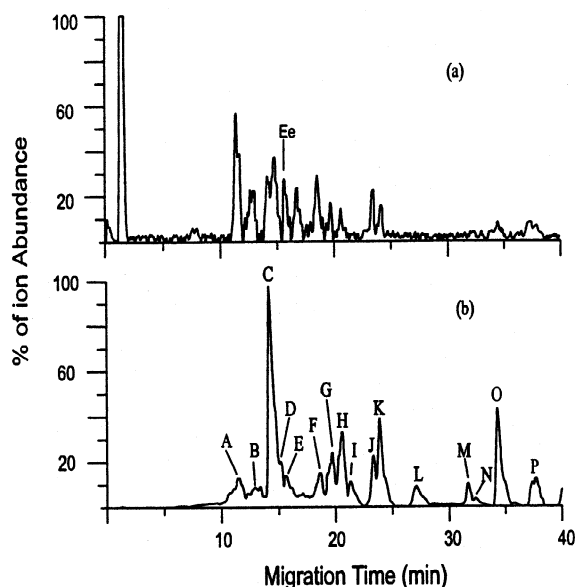

Fig. 4. Total ion chromatograms of the degraded sample ionized with (a) APCI and (b) ESI methods.

with the tetracycline standards demonstrated that the same separation efficiency could be achieved as with the previous mobile phase A. After the HPLC chromatographic separation, the samples were ionized by either ESI or APCI. Fig. 4 shows the chromatograms of the degraded sample measured with MS detector after (a) APCI and (b) ESI. Molecular mass information can be obtained from the MS spectrum corresponding to each chromatographic peak (Table 2).

None of the above molecular masses matched any of the previously suggested degradation products of tetracycline, such as ATC, EATC or ETC. Tetracycline itself was not detected. During the chromatographic and the ionization procedures, however, the possibility of reactions between the above tetracyclines and the mobile phase may exist. Therefore, the tetracycline standards mixture was analyzed with the same system to determine the existence of any possible reactions and products. Fig. 5 shows the chromatogram of the tetracycline standards measured

Table 2
HPLC–MS result of tetracycline degradation products

Peak no.	m/z=[Mass+H]$^+$ (% relative intensity)	
	ESI	APCI
Solvent front[a]	None	435 (100), 418[d] (98), 449 (96), 460[d] (89), 426 (78), 479 (64), 477 (50)
A	453[b] (100), 422 (60), 477 (23), 428 (20)	418[d] (100), 435 (56), 453 (18)
B[c]	477[b] (100), 410 (86), 426 (68)	450 (100), 426 (72), 477 (30)
C[c]	450[b] (100)	450 (100), 382 (40)
D[c]	450 (100), 478[b] (76), 426(72), 525 (34)	450 (100), 478 (95), 426 (69), 460[d] (45)
E	426[b] (100), 462 (62), 482 (44)	426 (100), 450 (68), 482 (52)
Ee	None	433 (100), 493 (96), 460 (83), 449 (72), 482 (68)
F[c]	493[b] (100), 424 (93)	493 (100), 431 (58), 460 (56)
G[c]	460[b] (100), 477 (58), 420 (34)	460 (100), 477 (30), 450 (22)
H[c]	508[b] (100), 491 (12)	491 (100), 508 (40), 447 (40), 460[d] (34)
I	552 (100), 464[d] (22)	none
J[c]	475[b] (100), 685 (42), 411 (36), 458 (17)	458 (100), 414 (88), 447 (84), 475 (54)
K[c]	464[b] (100), 447 (12)	447 (100), 464 (18)
L	306 (100)	None
M	332 (100)	None
N	354 (100), 306 (22)	None
0	378[b] (100)	378 (100), 361 (44)
P	360[b] (100), 378 (70)	343 (100), 360 (34)

[a] No m/z peaks were analyzed.
[b] m/z peaks were chosen for further HPLC–MS–MS analysis.
[c] Molecular structures have been derived from their daughter ion mass spectra.
[d] Possible intermediate compounds for the degradation sequence.

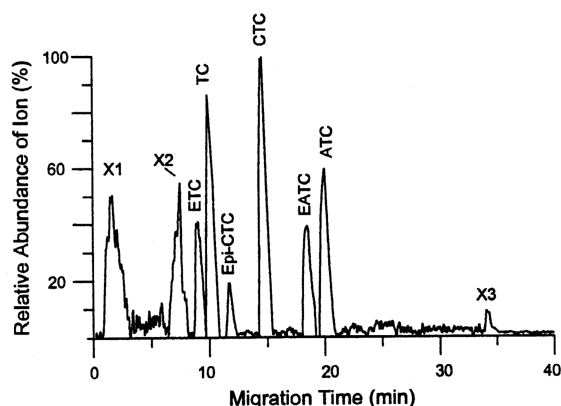

Fig. 5. HPLC chromatograms of TC standards mixture using solvent B as the mobile phase and ionized with ESI method.

with a mass detector after ESI. The corresponding *m*/*z* signals for each individual peak are illustrated in Table 3.

For all tetracycline standards, protonated molecule peaks dominated in the mass spectra. In addition, most had an extra peak for $[M+H]^+$ +41, most likely caused by the adsorption or addition of acetonitrile (M_r=41) onto the TC molecules. The spectra did not suggest the addition of methanol onto various tetracyclines during the chromatographic process. Three extra-unknown peaks were observed in the chromatograms of the tetracycline standards. Peak X1 was due to the solvent front. The mass spectrum of X1 was composed of small molecules with *m*/*z* less than 300. The mass spectrum of peak X2 had the same pattern as that of peak X1. The unknown compound in peak X3 had an *m*/*z* of 378 and the same migration time and mass spectra as peak O in the degraded sample. The product-ion mass spectrum of *m*/*z* 378 showed repeat units of *m*/*z* 72, possibly caused by some degree of polymerization of impurities in the solvent system. However, none of the three extra peaks were detected with UV–vis detection mode. The small molecules were most likely produced by impurities in the mobile phase rather than a reaction of TCs with the mobile phase. Except for peak O at *m*/*z* 378, no component observed in the degraded sample mass spectrum was detected in the TC standards mixture sample. The results indicated that all of the degradation components were formed prior to chromatographic separation, as opposed to the reaction of TC, ATC, EATC, or ETC with the mobile phase during the chromatographic or ionization processes.

Table 3
HPLC–MS result of tetracycline standards mixture with ESI method

Peak no.	M_r	*m*/*z* (% Relative intensity)
X1		Mixture
X2		Mixture
ETC	444	445 (100), 486 (18)
TC	444	445 (100), 486 (16)
Epi-CTC	478	479 (100), 445 (25)
CTC	478	479 (100)
EATC	426	427 (100), 468 (47)
ATC	426	427 (100), 468 (13)
X3		378 (100)

The above experimental results demonstrated that during long term storage in methanol solution, complex reactions occur which produce compounds not yet identified. To ascertain the nature of the degradation reactions, chemical structures of the products need to be determined. The major *m*/*z* components from each chromatographic peak were further analyzed with HPLC–MS–MS to elucidate their chemical structures. These *m*/*z* peaks (listed in Table 2b) were chosen because they were detected with both ESI and APCI methods and because they were the most intense peaks in the ESI mass spectra, except for the *m*/*z* peak from Peak D. Since Peaks C and D overlapped and the intensity of Peak C was much stronger, the *m*/*z* 450 on Peak D may come from Peak C. Meanwhile, the intensities of *m*/*z* 478 and 450 were similar; therefore, the *m*/*z* 478 peak was analyzed for Peak D.

Product-ion mass spectra were obtained for each selected *m*/*z* component. The MS–MS spectra are illustrated in Fig. 6. By analyzing the MS–MS spectra, eight molecular structures (Fig. 7) were postulated. For compounds H, J and K, the *m*/*z* were equal to $[M+NH_4]^+$ rather than $[M+H]^+$, evidenced by the easy loss of a group with *m*/*z*=17 from these components (Fig. 6). Previous MS work on tetracyclines has shown that the NH_2 group is not easily lost from $CONH_2$ [29]. Therefore, the most likely explanation for the NH_3 group was the addition of NH_4^+ from the mobile phase. Fig. 8 gives the detail procedures for elucidating these molecule structures. For each compound, stereoisomers are also possible.

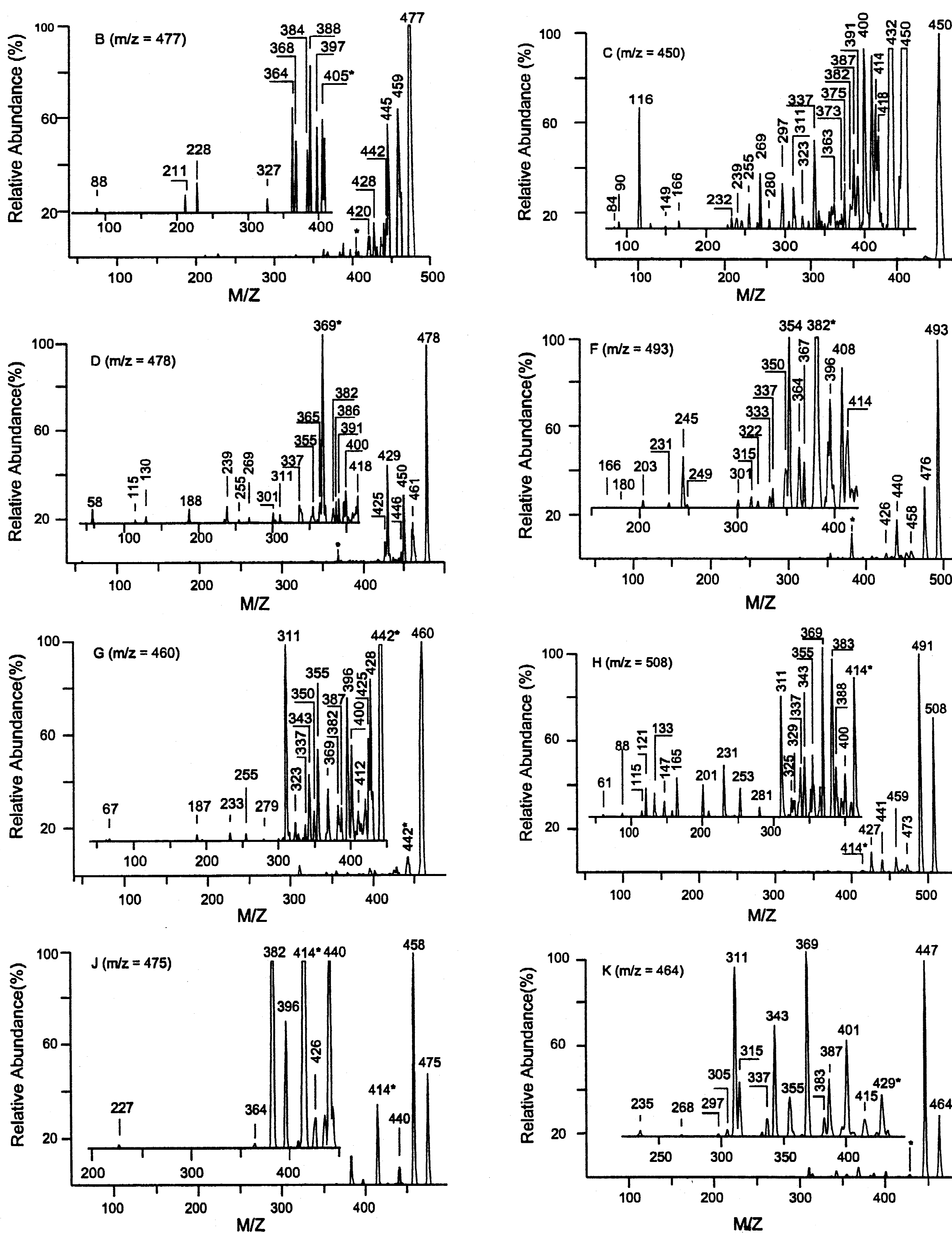

Fig. 6. MS–MS spectra of the selected degradation products.

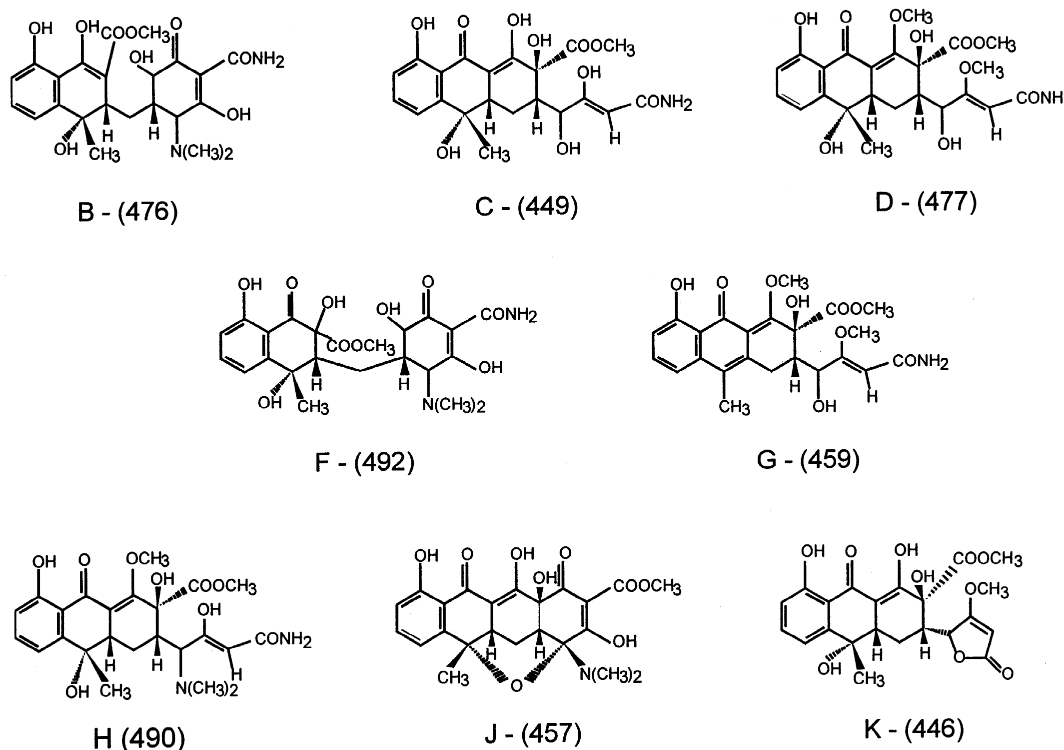

Fig. 7. Chemical structures of tetracycline degradation products.

Based on our measurements, however, it is impossible to determine with certainty which stereoisomers are present.

The chemical structures of degradation products with low masses were not elucidated due to the many possible interpretations of the molecular structures. UV–vis wavelength scan showed that the low *m/z* components had absorbance shifted toward shorter wavelengths, indicating a decrease in conjugation in the molecules.

The possible degradation sequence in Fig. 9 was derived from the proposed chemical structures of the degradation products,. The reactions start through the attack of methanol or oxygen on four reaction sites on TC, i.e. α, β, γ, or δ (see TC in Fig. 9). These initial attack sites have similar activities, resulting in a complex mixture of degradation products. Since the isomerization of TC to ETC occurs early in the degradation process, some products may come from the reactions of ETC with methanol. The dehydration of TC was hindered by the low pK_a of methanol. No MS–MS has been performed on the three intermediates depicted in Fig. 9. However, peaks at these *m/z* values were observed, perhaps due to these species (Table 2d).

The degradation of TC methanol solutions can be significantly decreased by storing them in a freezer (below −10°C) and preventing exposure to light. After 2 months in a freezer, only a slight amount of ETC had formed and there was no change in solution color.

4. Conclusions

The stability of tetracycline in methanol solution was studied using various techniques. More than fourteen new degradation products were observed to be formed after long-term storage of tetracycline methanol solution. Structures for eight of the degradation products were suggested from their MS–MS spectra. A chemical reaction sequence was proposed

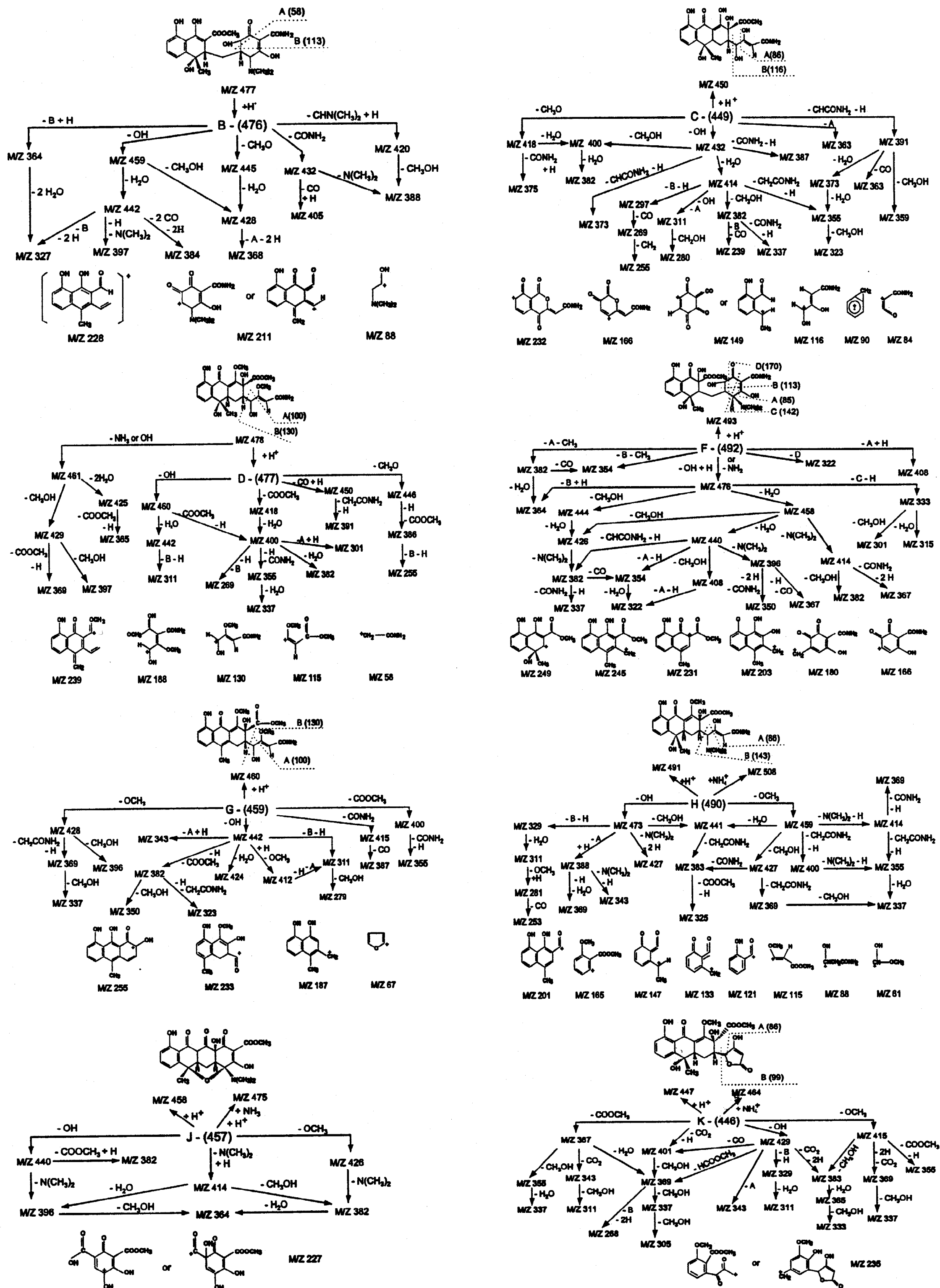

Fig. 8. Interpretation of the product-ion mass spectra.

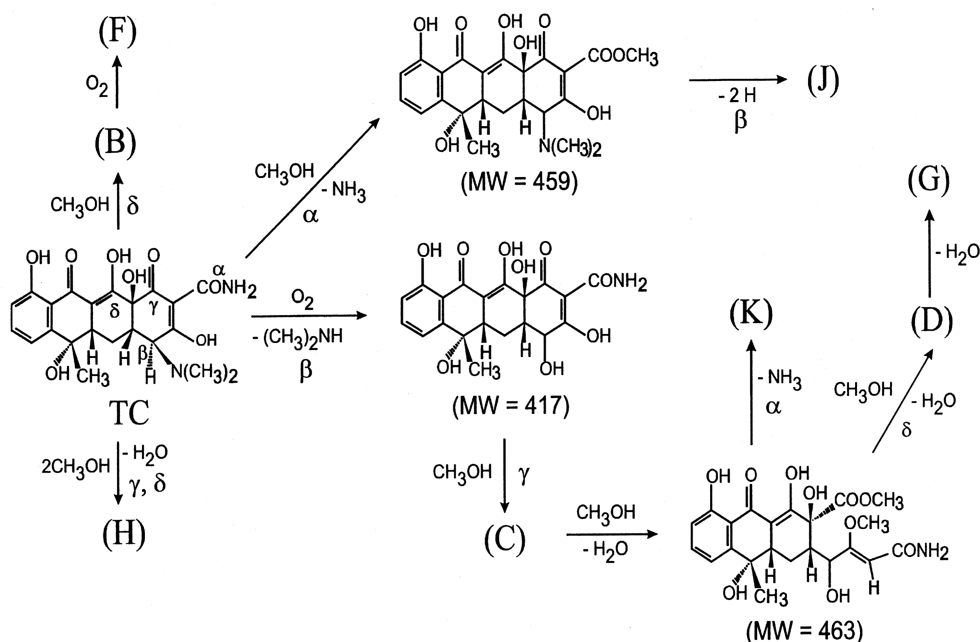

Fig. 9. Possible degradation sequence for tetracycline in methanol solution.

for the degradation of tetracycline in methanol solution. It was found that methanol plays a very important role in the degradation of tetracyclines through addition and substitution of the functional groups on TC.

The structures of these degradation compounds should be confirmed using other methods, such as NMR and IR. However, these techniques require significant amounts of pure compounds, and it is very difficult to separate enough samples for these analyses. Further studies are necessary to better understand tetracycline degradation processes as well as the biological effects and other properties of the degradation products.

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