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ORIGINAL ARTICLE

Characterization of stress degradation products of curcumin and its two derivatives by UPLC–DAD–MS/MS

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Abstract Curcumin and its two derivatives of PB-3 and DY-1 were subjected to the forced degradation studies under the conditions of hydrolysis (acidic and alkaline), oxidation, photolysis, and thermal stress recommended by ICH Q1A (R2) by means of UPLC–DAD–MS/MS. Three analytes and their degradation products were separated on a column of ACQUITY UPLC® BEH C_{18} (100 mm × 2.1 mm, 1.7 μm) with an in-line filter prior to the column using acetonitrile and 10 mM ammonium acetate buffer (pH adjusted to 3.5) as a mobile phase. Both curcumin and DY-1 showed extensive degradation under alkaline condition and gave rise to two degradation products for curcumin and three for DY-1, respectively, while no degradation product was observed under other tested conditions. PB-3 was found to be unstable in acid and alkaline conditions, two degradation products in acidic hydrolytic condition and one in alkaline condition were obtained, while it was stable in photolytic, oxidative and thermal stress conditions. The degradation products of three analytes were characterized as follows by analyzing the mass fragmentation patterns of curcumin and mass analysis of the degradation products: ferulic acid and vanillin for curcumin, 3-methoxyl-4-[3-(1-tetrahydropyrrolyl)propoxyl] benzoic aldehyde, 2,2-di-(1-phenylmethyl)-3-oxo-5-[3-methoxyl-4-(3-tetrahydropyrolylpropoxyl)] pent-4-enoic acid, and tetrapyrolyl propoxyl methoxyl phenyl prop-2-enoic acid for PB-3, 3-oxo-5-[4-hydroxyl-5-methoxyl-3-(1-morpholinylmethyl)] pent-4-enoic aldehyde, 3-oxo-5-(4-hydroxyl-5-methoxyl-3-(1-morpholinylmethyl)) pent-4-enoic acid and

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2-hydroxyl-3-methoxyl-5-(7-(3-methoxyl-4-hydroxylphenyl)-3,5-dioxo-4,4-dimethyl hept-1,6-dienyl) benzoic acid for DY-1, respectively. The degradation pathways of curcumin and its derivatives were presented in addition.

1. Introduction

Curcumin is a bright yellow-colored phenolic compound which was discovered in *Curcuma longa* L. (Zingiberaceae) rhizomes in 1815 (Gupta et al., 2013). Cultivated in India, Southeast Asia, and other tropical areas, *C. longa* L. is a major food ingredient and herbal medicine, also known as tumeric (Martin et al., 2012). Curcumin is known to exhibit antioxidant, anti-inflammatory, anti-microbial and anti-carcinogenic activities (Anand et al., 2007). The anticancer effect of curcumin is mainly mediated through induction of apoptosis. The anti-inflammatory, anticancer and antioxidant roles may be clinically exploited to control rheumatism, carcinogenesis and oxidative stress-related pathogenesis (Ishita et al., 2004). The therapeutic effect on the cardiovascular system and digestive system has also been reported (Azuine and Bhide, 1994).

Curcumin has many clinical activities; however, application of curcumin is limited by its poor oral absorption and rapid elimination (Zhao et al., 2011). It is insoluble in water at low pH and physiological pH and hydrolyzed at high pH. It is also very susceptible to photochemical degradation (Tønnesen and Karlsen, 1985) though it exhibits good optical and electrical properties owing to a highly p-electron delocalized system and symmetric structure (Liu et al., 2012; Sagnou et al., 2011). In our work, two derivatives PB-3 and DY-1 were synthesized, and their structures are shown in Fig. 1. Compared with curcumin, the ring of phenol hydroxyl groups was transformed into ether to retard its metabolism in the body, and 1-propyl-piperidine was introduced into the other phenolic hydroxyl group to increase water solubility. Finally, the groups added between the two keto groups replaced both enolizable hydrogens, and it would be impossible to get tautomerism. Curcumin is believed to play an important role in mediating the antitumor activity. The antiproliferative activity of curcumin analogues was evaluated with five human tumor cell lines by MTT assay, including human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HepG2), human colorectal carcinoma (HCT116), human lung carcinoma (A549), and human fibrosarcoma (HT-1080). They showed certain antiproliferative effects in all five cell lines (Liu et al., 2013).

Several HPLC methods were published for determination of curcumin including in different formulations (Ma et al., 2007). LC–MS/MS method was usually applied to determine the trace amount of curcumin in biological samples (Wang et al., 2012). Wang et al. (1997) investigated the degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices, pointed out its degradation followed first-order kinetics, also identified the degradation products (DPs) in 0.1 M phosphate buffer, pH 7.2 and serum free medium. However, in all the above cases, much toward deducing the degradation pathways of curcumin was not reported. The International Conference on Harmonization (ICH) guidelines suggests stress studies on a compound to establish its inherent stability characteristics not only for identification of degradation products but also for understanding the stability of drug molecule. So, it is of great importance to know the complete degradation profile of curcumin and its derivatives PB-3 and DY-1.

The present manuscript is aiming to describe the forced degradation of curcumin, PB-3 and DY-1 under hydrolysis (acid, alkaline), oxidation, photolysis and thermal stress conditions. A UPLC–DAD–MS/MS method will be developed to characterize the degradation products. The LC conditions will be optimized to separate the compound and its degradation products on a reversed-phase C18 column, characterization of degradation products and fragmentation pathways of degradants will also be described.

2. Materials and methods

2.1. Reagents and materials

Curcumin (purity 99.8%) was purchased from Sinopharm Co., Ltd. (Shanghai, China). PB-3 (purity 99.5%) and DY-1 (purity 99.8%) were synthesized and supported by Professor Jinhua Dong in the laboratory of medicinal chemistry of Shenyang Pharmaceutical University. Methanol, acetonitrile, and acetic acid were purchased from Fisher Co., Ltd. (Massachusetts, USA). Hydrochloric acid and sodium hydroxide were

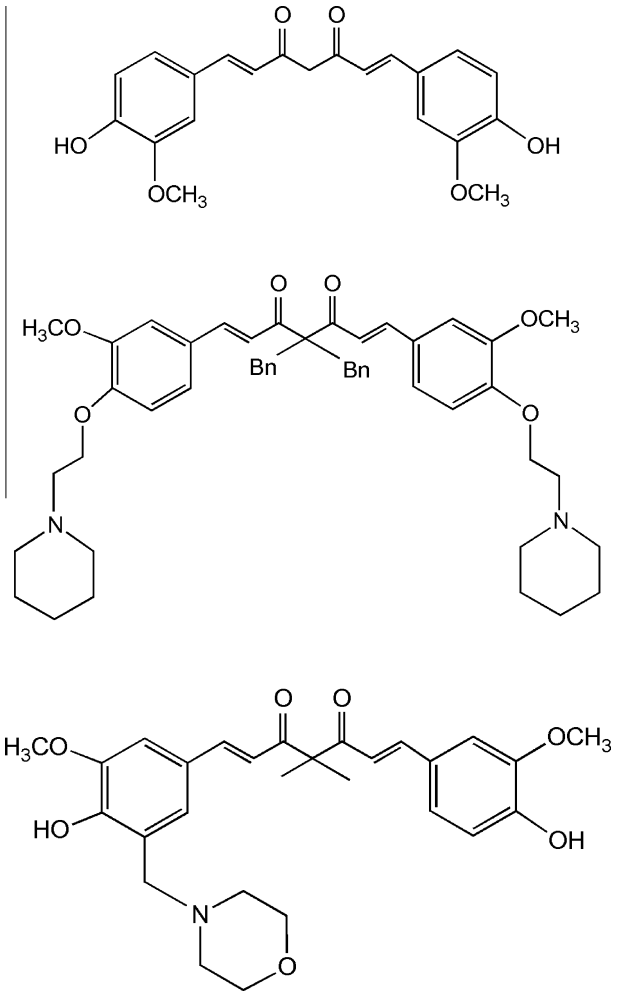

Figure 1 Chemical structures of curcumin, PB-3 and DY-1.

purchased from Damao Chemical Reagent Factory (Tianjin, China). Hydrogen peroxide (30%) was bought from Tianjin Fuyu Fine Chemistry Engineering Co., Ltd. (Tianjin, China). Methanol and acetonitrile were of HPLC grade and others were of analytical grade. HPLC-grade water was purified by a Milli-Q Reagent Water system (Millipore, Bedford, MA) used in preparing the aqueous solutions and the mobile phase throughout the experiments.

2.2. Instrumentation

Chromatographic analysis was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary pump solvent management system, micro degasser, an autoplate-sampler, and thermostatic column compartment. Chromatographic separation was carried out on a Waters ACQUITY UPLC® BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) with an in-line filter (0.22 μm) prior to the column. Triple-quadrupole tandem mass spectrometric detection was performed by Micromass Quattro Micro API mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. DK-S26 water baths were equipped with MV controller, Electro-thermostatic blast oven (DHG-9146A, Shanghai Jinghong Experimental Equipment Co., Ltd. Shanghai, China), and Hundred Thousandth Balance (AUW120D, SHIMADZU, Japan). A 50-W clear xenon lamp was employed as the light source for estimating the photolytic experiment (CEL-HXB F300, Beijing Zhongjiao Jinyuan technology co., Ltd. Beijing, China).

2.3. Stress degradation studies

Stress degradation studies of curcumin, PB-3 and DY-1 were carried out under hydrolysis (acid and base), oxidation, photolytic and thermal forced conditions. The stock solutions of curcumin, PB-3, and DY-1 were prepared at the concentration of 0.2 mg mL^{-1} by dissolving accurately weighted 10 mg of three analytes in 50 mL of methanol. One milliliter of these three stock solutions was taken precisely out and put into a 10 mL volumetric flask, respectively. The tests of acidic and basic hydrolysis were carried out in 2 mL of hydrochloric acid solution (1 mol L^{-1}) and sodium hydroxide solution (1 mol L^{-1}), respectively. The hydrolysis processes were conducted at 60 °C for 5 h and controlled the concentration of each analyte at 20 μg mL^{-1} in a 10 mL volumetric flask. The oxidative degradation study was carried out in 2 mL of 30% of hydrogen peroxide at room temperature for 5 h and the concentration of each analyte was maintained at 20 μg mL^{-1} in a 10 mL volumetric flask. In the tests of photolytic and thermal studies, five milligrams of curcumin, PB-3, and DY-1 were put on the watch glasses and kept at 150 °C for the thermal experiment and exposed to the light of 4000 ± 500 lux for the photolytic experiment for 12 h, respectively. The blank samples were prepared without adding the analytes in each stress condition.

2.4. Preparation of sample solutions

The solutions obtained in the acidic and basic hydrolysis tests were cooled down to room temperature and neutralized with sodium hydroxide solution (1 mol L^{-1}) and hydrochloric acid solution (1 mol L^{-1}), respectively. Then, they were diluted to the mark with acetonitrile. The solutions obtained in the test of oxidative degradation were diluted to the volume of 10 mL round bottom flask with acetonitrile, and then diluted 20-fold with acetonitrile. The solutions obtained in the tests of photolytic and thermal degradation were diluted 200-fold with acetonitrile. Each sample was prepared in triplicate. All the solutions were filtered by 0.22 μm membrane filters and kept in refrigerator at 4 °C before UPLC–MS/MS analysis.

2.5. Chromatographic and mass spectrometric conditions

The HPLC method was developed on a ACQUITY UPLC® BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) with an in-line filter prior to the column by using acetonitrile (B) and 10 mM ammonium acetate buffer (pH adjusted to 3.5, B) as a mobile phase. The sample solution was scanned by photo diode array detector (DAD) in the mode of full length scan. The flow rate was set at 0.25 mL/min and the injection volume was 20 μL. The mobile phases for three analytes were varied in a gradient program and are presented in Table 1 in details. For curcumin acetonitrile (B) and buffer (A) were varied in a gradient program (T_{min}/A:B; $T_{0.01}$/85:15; $T_{8.0}$/5:95; $T_{10.0}$/5:95; $T_{10.5}$/85:15; $T_{13.0}$/85:15). For PB-3 the gradient program was (T_{min}/A:B; $T_{0.01}$/85:15; $T_{2.5}$/60:40; $T_{8.0}$/5:95; $T_{10.0}$/5:95; $T_{13.0}$/85:15). For DY-1 the gradient program was

Table 1 Mass spectrometric conditions for curcumin, PB-3 and DY-1.

Mass spectrometric conditions	Curcumin	PB-3	DY-1
Ionization source	ESI	ESI	ESI
Mode of detection	Negative	Negative	Negative
Capillary voltage	2.00 kV	1.00 kV	4.00 kV
Cone voltage	40 V	40 V	35 V
Temperature of capillary tube	110 °C	110 °C	110 °C
Nebulizer gas	Nitrogen	Nitrogen	Nitrogen
Auxiliary gas	Nitrogen	Nitrogen	Nitrogen
Flow rate of nebulizer gas	30 mL/min	30 mL/min	30 mL/min
Flow rate of auxiliary gas	450 mL/min	450 mL/min	450 mL/min
Collision gas	Argon	Argon	Argon
Collision energy	2 eV	2 eV	2 eV
Scan range (m/z)	100–2000	100–2000	100–2000

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(T_{min}/A:B; $T_{0.01}$/75:25; $T_{3.0}$/75:25; $T_{4.0}$/50:50; $T_{7.0}$/50:50; $T_{8.0}$/5:95; $T_{10.0}$/5:95; $T_{13.0}$/75:25). The mass spectrometric conditions are shown in Table 1.

3. Results and discussion

3.1. Optimization of chromatographic conditions

During separation of the analytes by UPLC, adjustment of mobile phase pH is required so as to obtain an optimum resolution. Analytes can exhibit higher sensitivity in ionized form during MS analysis, but retained more strongly in unionized form in the case of LC. For instance, basic analytes such as amino compounds exist in protonated form at pH = $pK_a - 2$, whereas acidic analytes, such as carboxylic acids exist in deprotonated form at pH = $pK_a + 2$. Thus basic compounds undergo protonation at low pH, while acidic ones are deprotonated at high pH. In our work, the final pH was adjusted to 3.5 with acetic acid and resulted well in both UPLC and MS analysis.

Among the organic solvents, methanol and acetonitrile are the most widely used as mobile phase. Both produce enhanced response in mass studies (Jemal and Hawthorne, 1999). However, acetonitrile produced enhanced ionization and hence better sensitivity than methanol. Furthermore, it was also observed that all analytes with electron withdrawing groups and extended conjugation inherently were more ionized in the UPLC–MS/MS analysis. And it was also found existence of traces of ammonium acetic in the mobile phase can improve peak shapes. The LC–PDA studies were carried out to check the purity of prototype and each degradation product peak resolved in the UPLC–DAD chromatograms.

3.2. Degradation behavior, MS^n study and characterization of DPs of curcumin

Stress degradation studies of curcumin, PB-3 and DY-1 were performed under stressed conditions. As shown in Table 2, the degradation products of curcumin and its two derivatives exist in the acidic and basic hydrolysis, except those of DY-1 and curcumin in acid condition. However, there is nothing obviously changed under the oxidative, photolytic and thermal conditions.

3.2.1. Degradation behavior of curcumin

UPLC–DAD chromatograms of the curcumin standard solutions as well as of each degraded solution were compared with those of the corresponding and equivalently treated blank to identify peaks of degradation product, and nothing was found in the acid, photolytic, thermal and oxidative degradation samples. However, as shown in Fig. 2, unknown peak appeared in the retention time of 2.6 min in the alkaline degraded sample, and the MS full scan showed that there were two products whose m/z were 193(A1) and 151(A2) respectively. It can be inferred that the product m/z 193 is ferulic acid and m/z 151 is vanillin.

3.2.2. MS^n study and characterization of DPs of curcumin

3.2.2.1. A1. The observed m/z value of the protonated parent ion was 193. The MS–MS spectrum of the ion at m/z 193 displayed ions at 178, 149, 134 and 117. The ion at m/z 178 was formed by the loss of the methyl (−15 Da with a proton shift) from the parent ion 193; the ion at m/z 149 was formed by the loss of one carbon and two oxygens from the parent ion 193; further loss of one carbon and two oxygens from the ion at m/z 178 yields the ion at m/z 134; and further loss of hydroxy from the ion at m/z 134 yields the ion at m/z 117. The pathway was verified reasonably by those small degradation products observed in Fig. 3.

3.2.2.2. A2. The observed m/z value of the protonated parent ion was 151. The MS–MS spectrum of the ion at m/z 151 displayed ions at 136, 108 and 92. The ion at m/z 136 was formed by the loss of the methyl (−15 Da with a proton shift) from the parent ion 151; further loss of one carbon and one oxygens from the ion at m/z 136 yields the ion at m/z 108; and further loss of one oxygen from the ion at m/z 108 yields the ion at m/z 92. The pathway was verified reasonably by those small degradation products observed in Fig. 3.

3.2.3. Degradation pathway

The structural elucidation of degradation products revealed one susceptible site, carboxide. The carbonyl bond is susceptible to hydrolytic cleavage. During hydrolysis, the nucleophilic attack of water on the α-carbon of carboxide takes place, resulting in the cleavage of C—C bond to produce product A1. Further cleavage of C═C bond and oxidation from A1 formed product A2. The degradation products of curcumin in alkaline condition suggest hydrolysis is the main way, a proposed pathway described the degradation process in Fig. 4.

3.3. Degradation behavior, MS^n study and characterization of DPs of PB-3

3.3.1. Degradation behavior of PB-3

LC–UV chromatograms of the PB-3 standard solutions as well as of each degraded solution were compared with those of the corresponding and similarly treated blank to locate the peaks due to degradation products. Nothing was found in the photolytic, thermal and oxidative degradation samples. Some peaks were not detected by LC–MS, possibly due to poor ionizability. However, as shown in Fig. 2, unknown peaks appeared in the retention time of 1.25, 4.5 and 6.25 min in the acidic degraded sample, one big unknown peak appeared in the retention time of 2.2 min, and the MS full scan showed that there were two products whose mass weights were 527(B1) and 264(B2) respectively, only one product whose mass weight was 306(C1).

3.3.2. MS^n study and characterization of DPs of PB-3

3.3.2.1. B2. The observed m/z value of the protonated parent ion was 264(benzoic aldehyde, 3-methoxyl-4-[3-(1-tetrahydro pyrrolyl)propoxyl]). The MS–MS spectrum of the ion at m/z 264 displayed ions at 112, 84 and 69. The ion at m/z 112 was formed by the loss of the 2-methoxyl-4-formylphenoxyl (−152 Da with a proton shift) from the parent ion 264; the ion at m/z 84 was formed by the loss of two methynes from the parent ion 112; further loss of one methyne from the ion at m/z 84 yields the ion at m/z 69.

3.3.2.2. B1. The observed m/z value of the protonated parent ion was 527(pent-4-enoic acid, 2,2-di-(1-phenylmethyl)-3-oxo-

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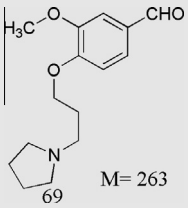
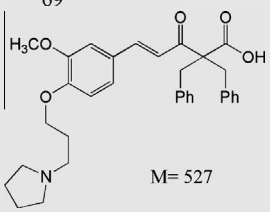
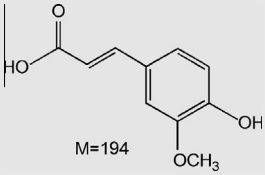
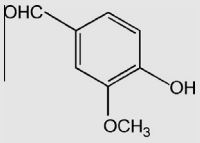
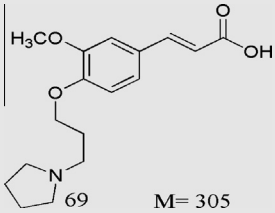
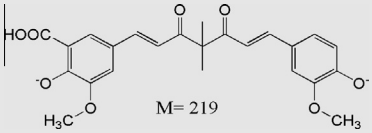
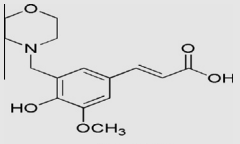
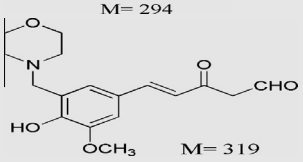
Table 2 Degradation products of curcumin, PB-3 and DY-1 under stressed conditions.

Stressed conditions	Compound	Structure of degradation production	Name of degradation production
Acidic hydrolysis	Curcumin	–	–
		 M= 263	Benzoic aldehyde, 3-methoxyl-4-[3-(1-tetrahydropyrrolyl)propoxyl]
	PB-3	 M= 527	Pent-4-enoic acid,2,2-di-(1-phenylmethyl)-3-oxo-5-[3-methoxyl-4-(3-tetrahydropyrolylpropoxyl)]
	DY-1	–	–
		 M=194	Ferulic acid
Basic hydrolysis	Curcumin	 M=152	Vanilin
	PB-3	 M= 305	Tetrapyrolyl propoxyl methoxyl phenyl prop-2-enoic acid
		 M= 219	2-hydroxyl-3-methoxyl-5-(7-(3-methoxyl-4-hydroxylphenyl)-3,5-dioxo-4,4-dimethyl hept-1,6-dienyl) benzoic acid
	DY-1	 M= 294	3-oxo-5-(4-hydroxyl-5-methoxyl-3-(1-morpholinylmethyl)) pent-4-enoic acid
		 M= 319	3-oxo-5-(4-hydroxyl-5-methoxyl-3-(1-morpholinylmethyl)) pent-4-enoic aldehyde
Oxidation	Curcumin	–	–
	PB-3	–	–
	DY-1	–	–
Photolysis	Curcumin	–	–
	PB-3	–	–
	DY-1	–	–

(*continued on next page*)

Table 2 (*continued*)

Stressed conditions	Compound	Structure of degradation production	Name of degradation production
Thermal	Curcumin	–	–
	PB-3	–	–
	DY-1	–	–

5-[3-methoxyl-4-(3-tetrahydropyrolylpropoxyl)]). The MS–MS spectrum of the ion at *m*/*z* 527 displayed ions at 435, 315, 112 and 84. The ion at *m*/*z* 435 was formed by the loss of the benzyl (−92 Da with a proton shift) from the parent ion 527; the ion at *m*/*z* 315 was formed by the loss of one benzyl and one carboxyl from the ion 435; further loss of 2-methoxyl-4-(3-oxopent-1,4-dienyl) phenoxyl free radical from the ion at *m*/*z* 315 yields the ion at *m*/*z* 112; and further loss of two methynes formed the ion at *m*/*z* 84.

3.3.2.3. C1. The observed *m*/*z* value of the protonated parent ion was 306(tetrapyrolyl propoxyl methoxyl phenyl prop-2-enoic acid). The MS–MS spectrum of the ion at *m*/*z* 306 displayed ions at 112, 84 and 69. The ion at *m*/*z* 112 was formed by the loss of the benzene ring part (−194 Da with a proton shift) from the parent ion 306; the ion at *m*/*z* 84 was formed by the loss of two methynes; further loss of one methyl yields the ion at *m*/*z* 69.

3.3.3. Degradation pathway and mechanism

Similar to curcumin, hydrolysis is also the main mechanism of the degradation of PB-3. The two benzyls strengthened between the two carboxide. So product B1 was formed by the cleavage of C═C bond and piperidine rearrangement after water attack. Further cleavage of C═C bond yields product B2, while C3 is a result of the loss of the two-benzyl part. A proposed pathway described the degradation process in Fig. 4.

3.4. Degradation behavior, MS^n study and characterization of DPs of DY-1

3.4.1. Degradation behavior of DY-1

LC–UV chromatograms of the DY-1 standard solutions as well as of each degraded solution were compared with those of the corresponding and similarly treated blank to locate the peaks due to degradation products. Nothing was found in the acidic, photolytic, thermal and oxidative degradation

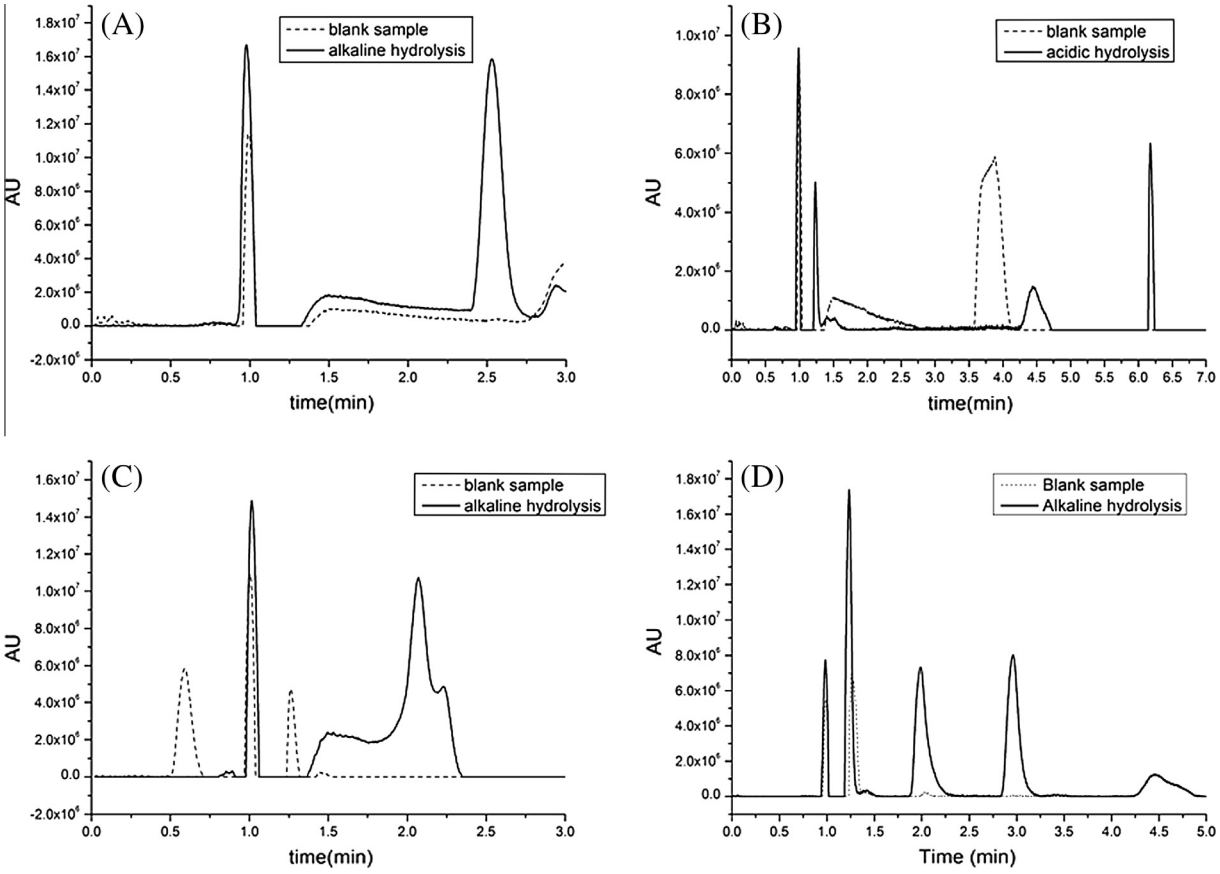


Figure 2 Chromatogram of the blank sample and alkaline hydrolysis sample of curcumin (A); blank sample and acidic hydrolysis sample of PB-3 (B); blank sample and alkaline hydrolysis sample of PB-3 (C); blank sample and alkaline hydrolysis sample of DY-1.

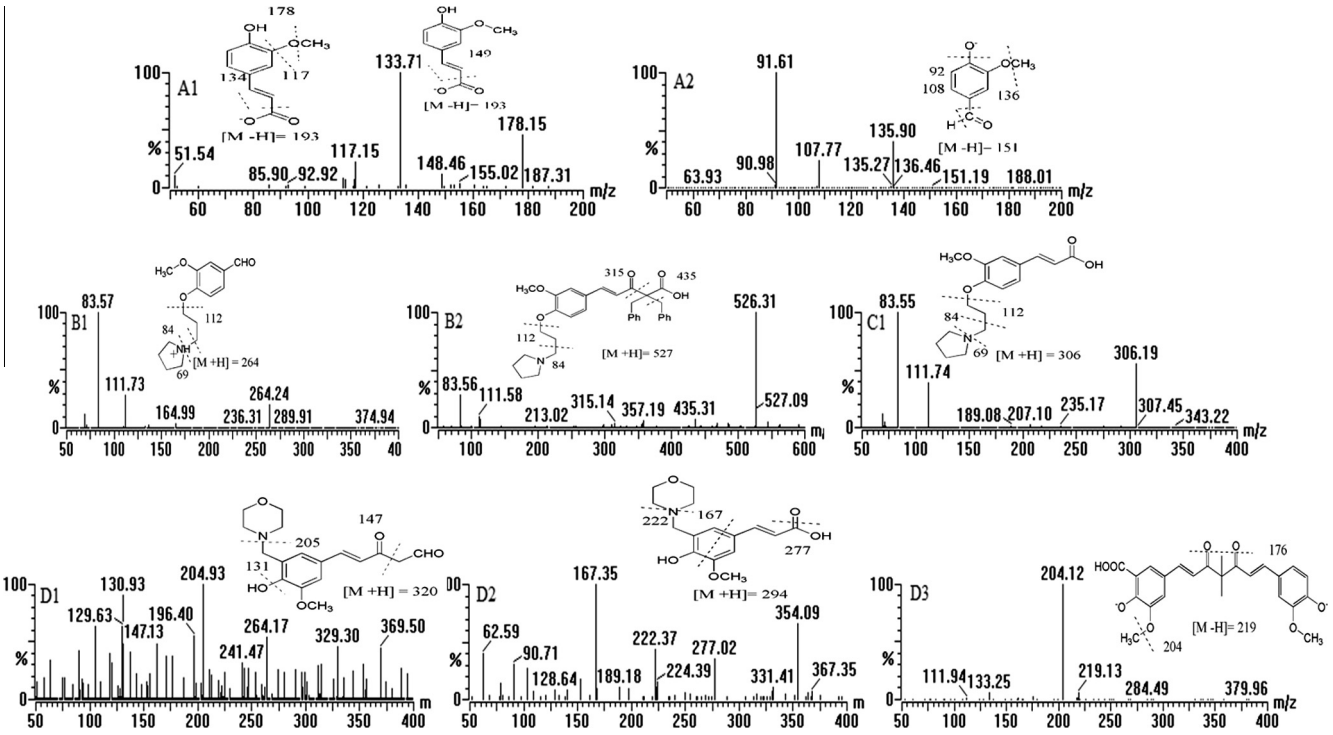


Figure 3 MS^2 spectrum of fragments of eight degradation products in degraded samples under stressed condition.

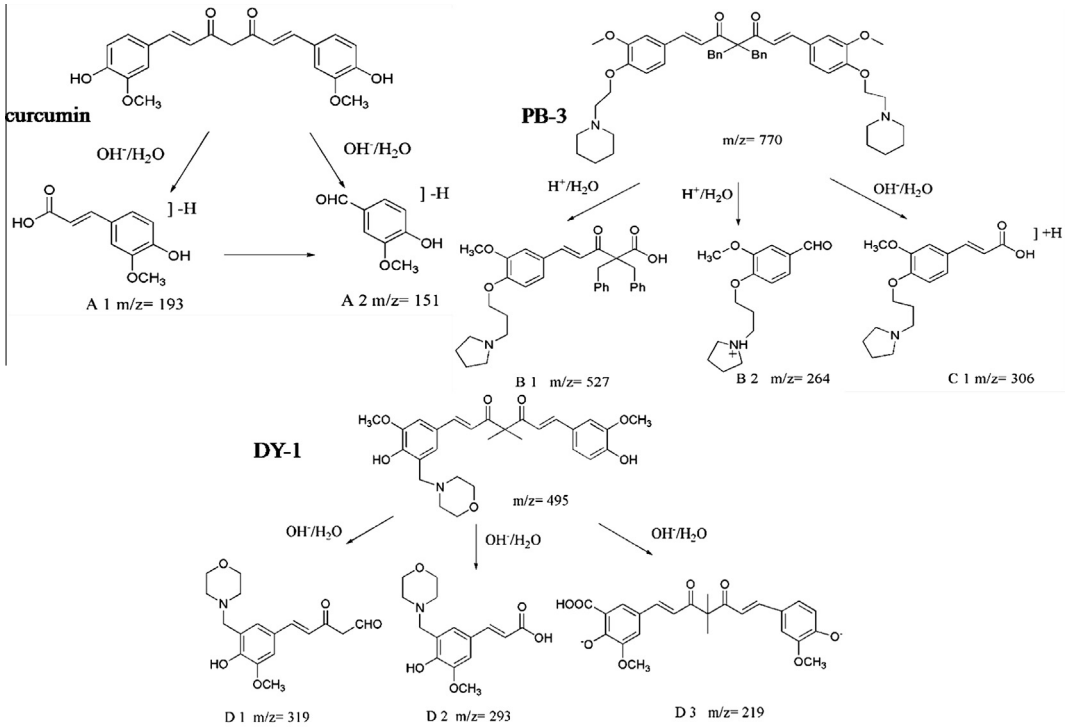


Figure 4 Degradation pathway of curcumin, PB-3 and DY-1 in stressed conditions.

samples. Some peaks were not detected by LC–MS, possibly due to poor ionizability. However, as shown in Fig. 2d, unknown peaks appeared in the retention time of 2.0 and 3.0 min in the alkaline degraded sample, and the MS full scan showed that there were three products whose mass weights were 319(D1), 294(D2) and 219 respectively.

3.4.2. MS^n study and characterization of DPs of DY-1

3.4.2.1. D1. The observed *m*/*z* value of the protonated parent ion was 319(3-oxo-5-(4-hydroxyl-5-methoxyl-3-(1-morpholinyl methyl)) pent-4-enoic aldehyde). The MS–MS spectrum of the ion at *m*/*z* 319 displayed ions at 204, 147 and 131. The ion at *m*/*z* 204 was formed by the rearrangement after splitting of α

ring (−115 Da with a proton shift) from the parent ion 320; the ion at m/z 147 was formed by the loss of one glyoxyl and one methyl; and further loss of one oxygen yields the ion at m/z 131.

3.4.2.2. D2. The observed m/z value of the protonated parent ion was 293(3-oxo-5-(4-hydroxyl-5-methoxyl-3-(1-morpholinyl methyl)) pent-4-enoic acid). The MS–MS spectrum of the ion at m/z 293 displayed ions at 277, 222 and 167. The ion at m/z 277 was formed by the loss of one hydroxy (−17 Da with a proton shift) from the parent ion 294; the ion at m/z 222 was formed by splitting of α ring from the ion 277; rearrangement of benzene ring from ion 277 formed the ion at m/z 167.

3.4.2.3. D3. The observed m/z value of the protonated parent ion was 219(2-hydroxyl-3-methoxyl-5-(7-(3-methoxyl-4-hydro xylphenyl)-3,5-dioxo-4,4-dimethyl hept-1,6-dienyl benzoic acid). The MS–MS spectrum of the ion at m/z 219 displayed ions at 204 and 176. The ion at m/z 204 was formed by the loss of one methyl (−15 Da with a proton shift) from the parent ion 219; the ion at m/z 176 was formed by the loss of one carbon and one oxygen.

3.4.3. Degradation path way and mechanism

The degradation mechanism of DY-1 is also similar to that of PB-3. Unlikely, the water attack didn't damage the morpholine but caused cleavage of the C═C bond to yield product D1. Further oxidation of the aldehyde group formed D2. D3 is a little complex because it is double charged, caused by the loss of morpholine. A proposed pathway described the degradation process in Fig. 4.

4. Conclusions

In this study, the degradation behavior of curcumin and its two derivative PB-3 and DY-1 were studied by subjecting the three compounds to various stress conditions recommended by ICH for the first time. The results showed that curcumin and DY-1 undergo an extensive degradation under alkaline condition and stable to acidic, photolytic, thermal and oxidative stress conditions; except from alkaline degradation, PB-3 also undergoes extensive degradation under acidic and alkaline conditions, but stable to photolytic, thermal and oxidative stress conditions. This study is a typical example of the development of a stability indicating assay, established following the recommendations of ICH guidelines. A total of 8 degradation products were characterized and a comprehensive mass fragmentation pathway of the drug was established based on UPLC–MS/MS data results.

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