Stress Degradation Studies on Embelin

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Ferreira and Laddha: Stress Degradation Studies on Embelin

Embelin, a naturally occurring benzoquinone, is obtained from fruits of *Embelia ribes,* which plays a vital role in the Ayurvedic system of medicine. The benzoquinone has diverse pharmacological and therapeutic properties and now constitutes a part of modern medicine. The aim of our study was to observe the effect of various stress conditions on this potential drug candidate. Embelin was subjected to stress conditions of acid and alkaline hydrolysis, oxidation, photolysis and thermal degradation. A significant degradation was found to occur by acid hydrolysis, oxidation and to a lesser extent under thermal stress and alkaline hydrolysis; the compound was found to be stable to photolytic stress. Further, the degradation product resulting from the acid hydrolysis was isolated and its structure was elucidated using spectroscopic techniques. Stress degradation studies on embelin provide an insight for its stability and storage considering the formulation aspects of modern medicine.

Key words: *Embelia ribes,* embelin, stress degradation

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) (fig. 1), is a naturally occurring benzoquinone is isolated from *Embelia ribes*. The fruits of this plant contain 2.5-3.1% of embelin on dry weight basis and finds its use in several Ayurvedic preparations like *Sanjivani Vati, Pippalyasavam, Dhanwantara ghritham*, *Vidangarishta, Kaisoraguggulu vatica*[1]. Embelin shows diverse pharmacological activities including chemo prevention in hepato-carcinogenesis observed in Wistar rats[2], antifertility effects[3], wound healing[4], antibacterial[5], free radical scavenging[6] and *in vitro* cytotoxic activity against B16 and XC cell lines[7]. It has been shown that embelin is a fairly potent, nonpeptidic, cell-permeable inhibitor of X-linked inhibitor of apoptosis protein[8,9] and hence can be considered as a potential drug candidate in modern system of medicine. Ammonium salt of embelin (ammonium embelate) is already marketed as an anthelmentic agent[10]. Considering
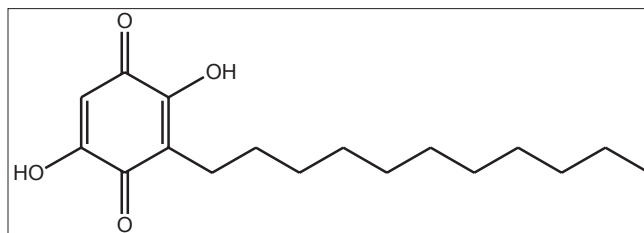

Fig. 1: Structure of embelin.

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its future applications in modern medicine, the objective of the current work was to carry out stress degradation studies to establish the inherent or intrinsic stability characteristics of the molecule and ascertain the degradation pathways. The parent drug stability guidelines by International Conference on Harmonisation (Q1AR) requires that the stress testing of drug substance should include the effect of elevated temperature, humidity, light, oxidizing agents, as well as the susceptibility across a range of pH values[11]. Stress degradation studies of various phytoconstituents such as forskolin and guggulosterone already been reported[12,13].

In this study, the effect of different stress conditions on the stability of the drug was observed using high-performance liquid chromatography (HPLC) analysis. The endeavor was to quantify degradation of embelin under various stress conditions. There is no report yet on these aspects for embelin.

The dried roots of *E. ribes* were obtained from local market of Mumbai, India and at Khalsa College, Mumbai; a voucher specimen was deposited in Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Mumbai. All the chemicals and solvents employed were of Analytical grade and procured from Merck Ltd., Mumbai or S. D. Fine Chemicals Ltd., Mumbai. Embelin was isolated from dried berries of *E. ribes* by an earlier reported method and its identity was confirmed with the aid of spectroscopic data[14]. The isolated compound was analyzed using a reported reverse phase high-performance liquid chromatography method comprising of a mobile phase of water containing 0.1% v/v ortho-phosphoric acid and acetonitrile (10:90) at a flow rate of 1.0 ml/min and employing ultraviolet (UV) detection at 286 nm[15]. Embelin shows a retention time of 7.3 min and the percent purity on the basis of area under curve was found to be 98.3% w/w. Embelin thus obtained was further used as working standard and subjected to stress degradation studies.

For quantification purpose, a stock solution of 1.0 mg/ml of embelin was prepared in methanol and stored in refrigerator. A set of standard solutions was prepared by diluting aliquots of the stock solution, with methanol to give concentrations ranging from 10.0 to 100.0 μg/ml. The calibration graphs were obtained in triplicate by plotting the peak areas against concentration of analyte and were found to be linear (equation of linearity: $y=82559x-86459$ with $r^2=0.994$).

To study the stability of embelin in solution, two different concentration solutions of 10 and 100 μg/ml were prepared from stock solution and stored at room temperature in tightly capped volumetric flasks protected from light for 6.0, 12.0, 24.0, 48.0 and 72.0 h, respectively. They were then analysed by HPLC and the chromatograms obtained were analysed for additional peaks, if any. The percentage relative standard deviation (%RSD) for the samples analysed at different elapsed assay times was found to be <2%, which proved the stability of the drug in solution state. In order to determine its susceptibility to different stress conditions embelin, was subjected to forced degradation studies under severe conditions of acid, base, oxidation, heat and light.

For acid and base degradation studies, accurately weighed 10 mg embelin was dissolved in 100 ml of methanol. 1 ml of this solution was incubated in dark (in order to exclude the possible effects of light on degradation) with 1 ml of methanol solution of 0.01, 0.1 and 1 N HCl for acid induced degradation and with 1 ml of methanol solution of 0.01, 0.1 and 1 N NaOH for base induced degradation studies. After 2 h the solutions were neutralized and volume made upto 10 ml. The resultant solutions were analysed by HPLC in triplicate.

For oxidative degradation studies accurately weighed 10 mg of embelin was dissolved in 100 ml of methanol. Subsequently, to each ml of the resultant solution 1 ml of hydrogen peroxide of concentrations 0.3, 3.0 and 30.0% v/v were added to three different tubes containing 1 ml of the stock solution and incubated in dark for 2 h and then the solution was heated in boiling water bath for 1 h to remove the excess hydrogen peroxide. The volume of the resultant solution was made up to 10 ml with methanol and analysed by HPLC in triplicate for measuring oxidative degradation.

The photochemical stability of the drug was studied by exposing the drug solution (1 mg/ml) to direct sunlight and UV radiation at 254 nm for 24 h. The resultant solutions were diluted to obtain a concentration of 10 μg/ml and analysed by HPLC in triplicate for degradation studies. Embelin (10 mg)

was stored at 80°, 90° and 100° for 1 h under dry heat conditions in a hot air oven for analysis of thermal degradation when subjected to dry heat. It was then dissolved in methanol to obtain a concentration of 10 µg/ml and analysed by HPLC in triplicate to study the degradation products. In all degradation studies, the average peak area of embelin, after application of three replicates was obtained and the content of residual embelin was calculated.

On treating the solution with 1, 0.1 and 0.01 N HCl for 2 h, the area of the peak corresponding to embelin decreased and an additional peak was observed at Rt 8.9 min (fig. 2a), indicating that embelin undergoes degradation under acidic conditions. However, the drug was found to be less susceptible to alkaline medium. On treating the solution with 1, 0.1 and 0.01 N NaOH for 2 h, the area of the drug peaks shows decrease in concentration when treated with 1N NaOH, however, no significant decrease was observed when treated with lower concentrations of alkali (fig. 2b). Embelin also undergoes oxidative degradation in presence of 0.3, 3.0 and 30.0% w/v of hydrogen peroxide when exposed for 2 h and the HPLC chromatogram shows an additional peak at Rt 2.5 min (fig. 2c). Exposure to dry heat indicated that the sample degrades completely when heated to a temperature of 90° for 15 min, degrades slowly when kept at 80° for 1 h and remains stable when kept at 60° for 24 h. Embelin was stable when exposed to sunlight and UV-254 nm for 24 h (fig. 2d and e). Based on the area under curve the concentration of embelin unaffected was calculated under various stress conditions (Table 1).

After these studies it was found out that embelin was more susceptible to oxidative and acid

degradation. Since oxidative degradation using peroxide follows a free radical mechanism, the degradation products so formed, can be nonspecific and hence emphasis was given on separation and identification of the acid degradation product. In order to isolate the acid degradation product, about 1 g of embelin was dissolved in 200 ml of methanol and 50 ml of hydrochloric acid was
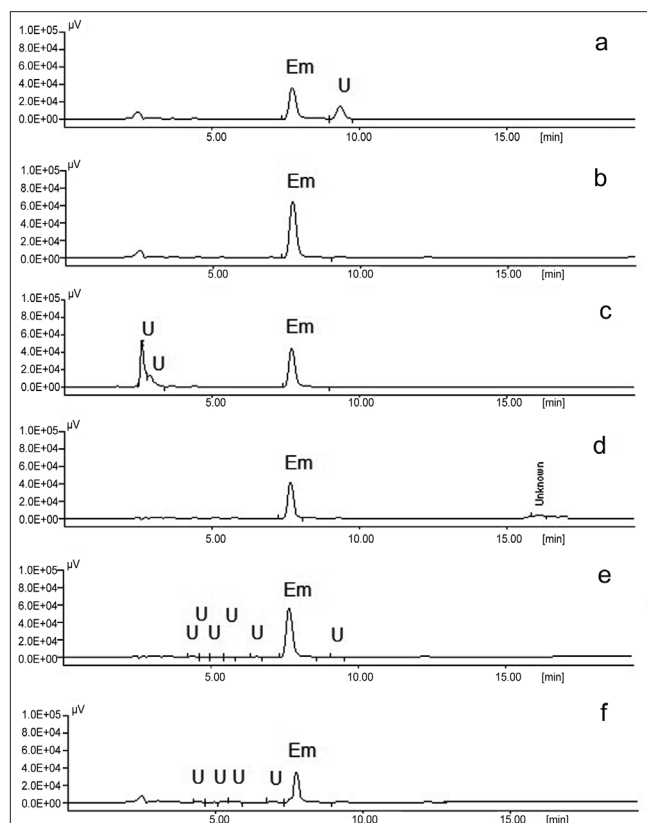

Fig. 2: HPLC chromatograms of embelin and its degradation products. HPLC chromatograms of degradation products of embelin on exposure to different stress conditions: (a)-acid (1N), (b)-base (1N), (c)-oxidation (3% H$_2$O$_2$), (d)-day light, (e)-ultraviolet, (f)-dry heat (80°).

TABLE 1: FORCED DEGRADATION OF EMBELIN

Condition	Time	% recovery	RT of degradation products
Acid (1 N)	2 h	70.07±0.21	8.9
Acid (0.1 N)	2 h	83.47±0.39	8.9
Acid (0.01 N)	2 h	99.41±0.19	8.9
Base (1 N)	2 h	94.09±0.27	-
Base (0.1 N)	2 h	99.09±0.43	-
Base (0.01 N)	2 h	99.30±0.32	-
Hydrogen peroxide (30% v/v)	2 h	37.00±0.25	2.5
Hydrogen peroxide (3% v/v)	2 h	73.17±0.21	2.5
Hydrogen peroxide (0.3% v/v)	2 h	90.63±0.19	2.5
Day light	3 weeks	85.76±0.16	4.4, 5.9, 6.5, 16.4
UV light (254 nm)	24 h	96.23±0.29	4.4, 5.2, 5.9, 6.5, 9.1
Heat (80˚)	1 h	40.65±0.34	4.4, 5.9, 6.5

RT=Retention time

added to the above solution, this mixture was stored in dark for 24 h. The mixture was neutralized by alkali, filtered and concentrated in vacuum. The residue thus obtained was loaded on silica gel (60#) and subjected to column chromatography using light petroleum ether and ethyl acetate as eluent. A 5% fraction of ethyl acetate in petroleum ether yielded orange coloured needles of the acid degradation product (235 mg) which was subjected to spectroscopic studies.

The spectroscopic data of the acid degradation product (Table 2) shows base peak of acid degradation product to be M+15 of the parent compound indicating addition of a methyl group to the parent structure which remains unaltered as observed from mass spectrometry fragmentation peaks at 169.1 and 141.1 (fig. 3). The proton nuclear magnetic resonance spectroscopy and carbon nuclear magnetic resonance spectra shows a peak at δ 3.86 and δ 56.5 respectively, corresponding to formation of a methoxy group, indicating that one of the two hydroxyl groups of embelin has undergone etherification. Since the hydroxyl group present at C-2 of embelin is sterically hindered by the undecyl chain present on adjacent C-3 carbon, it was concluded that the 5-hydroxyl group of embelin must have undergone etherification to form 2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone (methyl ether of embelin).

Stress degradation studies on embelin were carried out and it was found that embelin was susceptible to oxidative, acid and to lesser extent thermal degradation. Embelin when treated with 1 N HCl for 2 h undergoes 30% degradation as compared to 1 N NaOH which shows only 6% degradation. It also undergoes 63% degradation when stored in presence of oxidizing agents such as 30% H_2O_2 indicating its liability to oxidizing agents. When stored in dry heat at a temperature of 80° for 1h the compound undergoes 60% degradation (fig. 2f). The HPLC technique employed for its estimation is a good measure for indicating stability of embelin, since it resolves the degradation products under all stress conditions. It was observed that embelin etherifies when stored in alcohol (methanol or ethanol) in presence of acid to form corresponding ethers of embelin. (spectroscopic data for the ethyl ether is not mentioned). These findings may help in formulation studies of the product considering

TABLE 2: SPECTROSCOPIC DATA OF ACID DEGRADATION PRODUCT OF EMBELIN

Characterization method	Values obtained
IR υ max (KBr) cm^{-1}	3353 (Ar-OH), 2920 (Ar-H), 2852 (C-H), 1638, 1599 (C=O), 1205 (−OCH$_3$)
Mass spectra	[M+1]$^+$309
MS-MS	[M+1]$^+$169.1, 141.1
^{1}H-NMR (CDCl$_3$, 400 MHz)	δ 0.5 (t, 3H, R-CH$_3$), 1.25 (s, 20H,-CH$_2$), 3.86 (s, 3H, O-CH$_3$), 7.23 (d, 1H, Ar-H)
^{13}C-NMR (CDCl$_3$, 125 MHz)	14 (C-17), 22 (C-16), 21 (C-7), 27 (C-8) 31 (C-15), 30 (C-14, C-13, C-12, C-11, C-10, C-9), 56.5 (C-18), 102 (C-6), 119 (C-3), 151 (C-2), 161 (C-5), 181 (C-4), 182 (C-1)
Melting point	118°

IR=Infra red, MS-MS=tandem mass spectrometry, H-NMR=proton nuclear magnetic resonance spectroscopy, C-NMR=carbon nuclear magnetic resonance
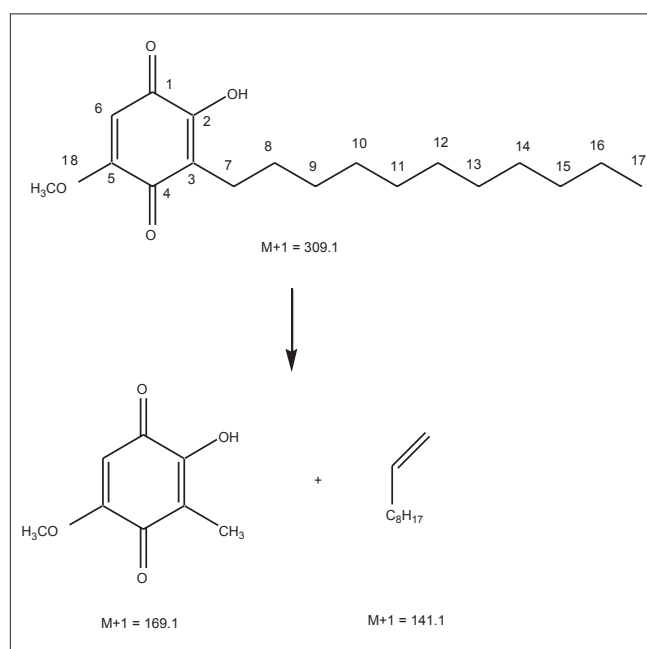

Fig. 3: Mass spectrometry fragmentation pattern ofacid degradation product of embelin.

its applications in modern as well as traditional medicine. When stored under acidic condition care should be taken to use a solvent other than alcohols to prevent etherification. The relevance of these studies in predicting the stability of embelin in Ayurvedic formulations is that they are known to contain self-generated alcohol which under acidic conditions may undergo etherification to form corresponding ether of embelin. In conclusion, the stress degradation studies on embelin have been carried out, which may provide an insight for its stability and storage considering the formulation aspects of modern medicine.

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