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Enhanced Aqueous Stability of Hirsutenone with Antioxidant

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ABSTRACT − The instability of hirsutenone (HST), a potential therapeutic candidate for the treatment of atopic dermatitis (AD) and ovarian carcinoma, is one of the main concerns for the development of drug product. In the present study, aqueous stability of HST was investigated by kinetic analysis, and the effect of several factors covering temperature, nitrogen gas (N_2) flushing, and selection of proper antioxidant was compared. Cosolvent system composed of distilled water and methanol (9:1 v/v) was used as a vehicle to dissolve HST at the concentration of 200 μg/mL. Samples of aqueous solution were prepared under the absence or presence of antioxidants, such as ascorbic acid (AA), sodium edetate (EDTA), and ascorbyl palmitate (AP), and subjected for stability test. The degradation of HST in aqueous solution was followed by the first order kinetics with an extremely short half life of less than a week at room temperature, and was accelerated as the temperature increased. N_2 flushing brought a little enhancement in stability compared to control solution, but the effect was insufficient. The addition of AA and EDTA (0.1%) significantly enhanced the stability of HST at 40°C, but the addition of AP (0.01%) was limited due to its water insolubility and revealed no promising result. The stability of HST was increased proportionally by the amount of AA added, showing the difference in degree of stabilization as an order of magnitude. Finally, we conclude that HST was stabilized by the addition of a suitable antioxidant, suggesting AA as the most effective stabilizer.

Key words − Hirsutenone, Atopic dermatitis, Stability, Antioxidant, Ascorbic acid

Hirsutenone (HST), a diarylheptanoid isolated from the bark of *Alnus japonica* (Askawa et al., 1970), has been introduced as a potential therapeutic candidate for the treatment of atopic dermatitis (AD) by inhibiting T cell activation by blocking dephosphorylation of nuclear factor of activated T cells (Joo et al., 2009). This natural immunomodulator may also enhance the apoptotic effect of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) on ovarian carcinoma cell lines by increasing the activation of the caspase-8- and Bid-dependent pathways and the mitochondria-mediated apoptotic pathway, leading to caspase activation (Lee et al., 2010). Recently, several approaches for the development of HST-containing formulations have been studied in AD animal model induced by contacting allergens like house dust or diphenylcyclopropenone (DPCP). The topical application of conventional cream containing HST (1%) drastically reduced the clinical skin severity scores of AD-like skin lesions in NC/Nga mice, revealing decreased levels of IgE, eosinophil count, and other Th2-related cytokines and inflammatory factors (Jeong et al., 2010). In addition, elastic liposomal cream formulation of HST was developed for the purpose of AD treatment in NC/Nga mice. The addition of Tat peptide increased the skin permeation of HST and brought a significant improvement in both skin severity score and immune-related responses for the levels of nitric oxide synthase, cyclooxygenase-2, IL-4, IL-13, immunoglobulin E, and eosinophils (Kang et al., 2011).

However, the stability of HST is one of the main concerns for further development of drug product since the instability of diarylheptanoid compound is generally acknowledged. In the aspect of molecular structure, HST is very similar to curcumin that is well-known as an unstable diarylheptanoid. Earlier study on the stability of curcumin in various pH buffer solutions reported the mechanism of degradation due to the oxidation of double bond, resulting in several degradation products of vanillin, ferulic acid and feruloyl methane by the analysis of HPLC and GC/MS (Wang et al., 1997). In another research about the effect of curcumin on cytochrome P450 and glutathione S-Transferase activities in rat liver, it was observed that curcumin was stabilized by the addition of antioxidants such as ascorbic acid, glutathione, N-acetyl L-cysteine, etc. (Oetari et al., 1996).

Various approaches to stabilize a labile drug from oxidative degradation have been explored. Since the oxidation is catalyzed by the several factors involving the presence of heavy

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metals or dissolved oxygen and the exposure to high temperature, relevant methods such as the addition of chelating agent, the flushing with nitrogen gas and the lowering of storage temperature could be applied (Lee et al., 1994; Teeranachaideekul et al., 2007). Among these methods, the use of antioxidants is the most selective way for protecting the product from oxidative degradation in practical fields of pharmaceutical and food industries. Antioxidants are the most effective in stabilizing oxygen-sensitive drug formulation because they are oxidized themselves in place of the unchanged drug (Akers et al., 1985). Antioxidants work by one of three different mechanisms: First, preferentially undergoing oxidative degradation in place of the drug because of the higher standard oxidation potential (E^o) of the antioxidant; Second, serving as an acceptor of the free radical and inhibiting the free radical chain reaction process; Last, retarding the formation of free radicals. Water soluble antioxidants such as ascorbic acid and sodium bisulfite follow the first mechanism, whereas lipophilic antioxidants such as butylated hydroxyl toluene (BHT) and propyl gallate obey the second mechanism. And metal-sequestering agent like EDTA is an example of retardants of free radicals. For example, the instability problem of quinapril HCl in tablet and capsule was overcome by the incorporation of ascorbic acid, showing the result of enhanced stability at 45°C over a month (Murthy et al., 1988). It was also described that ascorbic acid and acyl ascorbate stabilized the aqueous degradation of catechin molecules by suppressing the oxidative degradation in the emulsion (Watanabe et al., 2011).

Therefore, in the present study, aqueous stability of HST was investigated by kinetic analysis. And the effect of several factors covering temperature, N_2 displacement, and selection of proper antioxidant was compared.

Materials and Methods

Materials

HST (purity >95% by HPLC) was supplied by Pharmacognosy Lab. at College of Pharmacy in Chung-Ang University. Ascorbic acid (AA), EDTA-Na, and ascorbyl palmitate (AP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals and solvents purchased from commercial sources were of analytical reagent grade.

Preparation of aqueous solutions of HST

Cosolvent system composed of distilled water and methanol (9:1 v/v) was used as a vehicle to dissolve HST at the concentration of 200 μg/mL. The solubility of HST in water is very limited, but increased greatly by the addition of cosolvent, e.g., 1215 μg/mL in 20% MeOH. However, since increasing the content of cosolvent generally decreases the degradation rate of drug (Suo et al., 2007), 10% MeOH solution was finally selected to minimize the influence of the organic solvent. Samples of aqueous solution were prepared under the absence or presence of antioxidants, designated as antioxidant-free (control) solution, ascorbic acid (AA)-added solution, sodium edetate (EDTA)-added solution, and ascorbyl palmitate (AP)-added solution, respectively. The water-soluble antioxidants such as AA and EDTA were added at the level of 0.01 to 1%. However, for the lipophilic antioxidant, AP was added in the range of 0.001 to 0.01% because of its limited solubility in the vehicle selected.

Stability study

Two milliliters of aqueous solution samples was transferred to 5mL teflon-capped vials and sealed with parafilm to shut off the evaporation of solvent, then stored at the constant temperature cabinet (Shaking Incubator, SI-900R, Jeio-Tech, Korea). To observe the temperature dependence of HST degradation, control solution was stored at different temperature of 25, 40, and 50°C. Meanwhile, all antioxidant-added samples were kept at the same temperature of 40°C to compare the effect of stabilizers. At appropriate time points, 200 μL of sample solution was removed from the vial and mixed with 200 μL of methanol. Fifty microliters of the mixture was injected for the HPLC analysis. On the other hand, nitrogen flushing study was conducted separately for control solution. The procedure was same as the above description except for the pretreatment that nitrogen gas (N_2) was flushed to the vehicle for 10 min at the flow rate of 20 mL/s to get rid of dissolved oxygen as possible.

HPLC assay of HST

The amount of HST in aqueous samples was determined by chromatographic analysis at room temperature. A solvent gradient elution was performed with solvent A of water and solvent B of acetonitrile. The starting mobile phase was 90% A with 10% B, and the linear gradient was run over 24 min to proportion of 60% A with 40% B, then returned to a final proportion of 90% A with 10% B at 30 min. The flow rate was 1mL/min. The HPLC system consisted of a pump (L-2130), UV detector (L-2400), a data station (LaChrom Elite, Hitachi, Japan), and a 15 cm C18 column (Shiseido, Tokyo, Japan). The column eluent was monitored at 280 nm, and HST peak was separated with a retention time of 21.0 min. The intra- and inter-day precision and accuracy were estimated by analyzing

three replicates containing HST at four different concentrations, i.e. 10, 20, 100 and 200 μg/mL.

Results and Discussion

Assay validation

Typical HPLC chromatograms for HST assay are shown in Figure 1. Even though peak resolution was acceptable, there was a little interference with adjacent minor peaks which could not be separated by changing either mobile phase ratio or flow rate during gradient method. So calculation of peak height was employed to avoid the influence of interference peak. A representative calibration curve of peak-height ratio of HST versus HST concentration resulted in the regression equation as y=184.06x-12.32 (r^2 = 0.9994) in the range of 10-200 μg/mL. The limit of quantitation was approximately 10 μg/mL at the signal to noise ratio of 10.8 : 1.

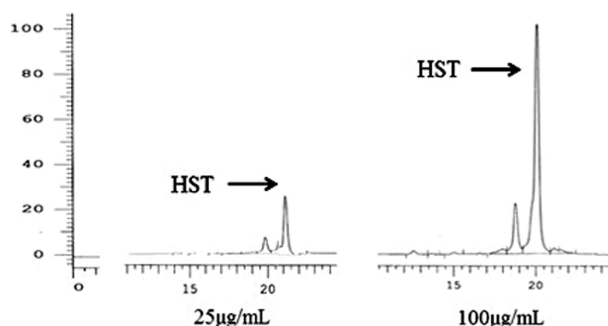

Figure 1. Typical HPLC chromatograms of HST.

Table I. *Intra- and inter-day accuracy and precision for HST assay*

Spiked concentration (μg/mL)	Measured concentration (Mean ± SD, μg/mL)	Precision (R.S.D)[a]	Accuracy (% DEV)[b]
Intra-day variation			
10	9.76 ± 0.35	3.59	2.42
20	19.72 ± 0.91	4.60	1.4
100	103.2 ± 0.67	0.65	-3.20
200	219.2 ± 4.49	2.05	-9.61
Inter-day variation			
10	10.01 ± 0.27	2.73	-0.13
20	20.65 ± 1.28	6.17	-3.26
100	102.98 ± 0.94	0.92	-2.98
200	213.31 ± 8.76	4.11	-6.66

[a]R.S.D, relative standard deviation: (SD × 100/mean).
[b]% DEV, percentage deviation calculated by (spiked concentration-measured concentration) × 100/spiked concentration.

Accuracy and precision data for intra- and inter-day variation are listed in Table I. For accuracy, the range of intra- or inter-day variation (% DEV) was calculated as maximum as -9.61 and -6.66, respectively, at 200 ug/mL. The other variations including precision were within 5% deviation, except for inter-day precision (% RSD) as 6.17. All validation data were acceptable, based on the criteria for both accuracy as within 15% deviation (DEV) and precision as within 15% relative standard deviation (RSD) from the nominal values (United States Pharmacopeia, 2008; Shah et al., 1992).

Thermal stability of HST

Samples of control solution were stored at three different temperatures to observe the thermal degradation kinetics and the temperature dependence. As shown in Figure 2, the degradation of HST was very fast and followed by the first order kinetics as Eq. 1:

$$C/C_0 = \exp(-kt) \quad (1)$$

where C/C_0 represents the fraction of remaining HST in the aqueous solution at any time t, and k is the rate constant. The rate constant of degradation was calculated from the slope to best-fit the experimental results by the regression function of Microsoft Excel 2007 (Table II). The half lives were extremely short, revealing less than a week at room temperature. As expected, the degradation was accelerated as the temperature increased. This phenomenon is consistent with a rule of thumb, stating that reaction is accelerated two-fold by 10ºC increment in temperature as previously reported elsewhere (El-Badry et al., 2009; Martin et al., 1993; Suo et al., 2007).

The temperature dependence of HST degradation was analyzed by the Arrhenius equation as follows:

$$k = A \cdot \exp(-E_a/RT) \quad (2)$$

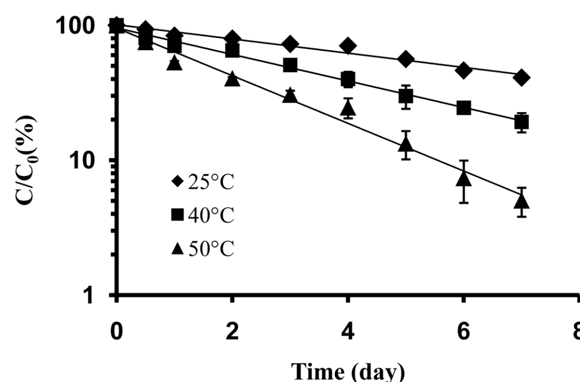

Figure 2. First-order plots for HST degradation in aqueous solution at different temperatures. Value represents for mean ± SD (n=3).

Table II. *Kinetic parameters of HST in aqueous solution at different temperatures*

Antioxidant-added	Temperature (°C)	Rate constant ($\times10^{-2}$, day^{-1})	Half life (day)
Antioxidant-free (Control)			
-	25	12.1	5.78
-	40	22.6	2.86
-	50	40.9	1.59
AA-added			
0.01%	40	22.3	2.89
0.1%	40	1.96	38.3
1%	40	0.28	251.4
EDTA-added			
0.1%	40	2.24	31.8
AP-added			
0.001%	40	23.7	2.62
0.005%	40	23.4	2.78
0.01%	40	22.6	2.94

*Initial HST concentrations were 200 µg/mL for all samples. Abbreviations are as follows: AA, ascorbic acid; EDTA, EDTA-Na; AP, ascorbyl palmitate.

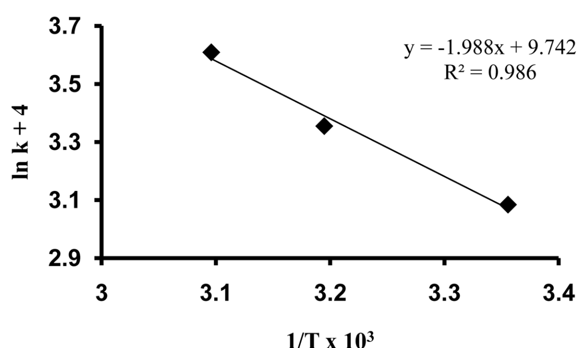

Figure 3. Arrhenius plot of degradation rate constant (k) versus reciprocal absolute temperature (1/T).

where A is the frequency factor, E_a is the activation energy, R is the gas constant, and T is the absolute temperature. In the semilogarithmic plot of rate constant (k) versus reciprocal absolute temperature (1/T) as shown in Figure 3, a linear relationship was confirmed with good correlation ($r^2 = 0.986$). Activation energies of the degradation can be calculated from the slopes of the regression line, resulting in the value of 16.53 kJ/mole. The higher the E_a is, the less the degradation reaction would be influenced by temperature. Curcumin has a similar structure of diarylheptanoid, as mentioned in earlier introduction part, in which E_a is 39.95kJ/mole for the reaction with m-chloroperoxybenzoic acid (m-CPBA) as an oxidative agent (Baek et al., 2011). The difference in E_a values suggests that HST is more potent antioxidative agent but less stable compared to curcumin. Curcumin prevents the peroxidation of linoleic acid that is easily degraded by oxidation, while HST carries potent activity on the scavenging superoxide free radical (Kuroyanagi et al., 2005; Jayaprakasha et al., 2006). Even though both HST and curcumin molecules are classified into a same diarylheptanoid, structural difference of curcumin having more methyl groups and double bonds than HST may generate this gap of thermodynamic stability.

Nitrogen gas flushing

Since diarylheptanoids including HST are susceptible to oxidation, nitrogen gas was flushed to the control solution to get rid of the dissolved oxygen (DO) in the vehicle. As depicted in Figure 4, N_2 flushing brought a little enhancement in stability compared to control solution, but the effect was insufficient. It might be due to the residual DO in the vehicle which catalyzed the oxidation reaction. From the literature (Butler et al., 1994), N_2 flushing is the most selective way to remove DO in comparison of four different methods: boiling at 1 atm, boiling under reduced pressure, purging with N_2, and sonication under reduced pressure. However, none of these physical techniques resulted in complete removal of DO, reporting that the DO was remained as 0.2~0.4 ppm after N_2 flushing for 20~40 min at the flow rate of 25 mL/s. Even though we tried with the addition of sodium sulfite as a chemical method to remove the DO, the stabilization was not accomplished (data not shown). Therefore, it was necessary to find alternative way for stabilization including the use of antioxidants.

Antioxidant screening

The addition of antioxidants to minimize the oxidation of a labile active ingredient is well-known. Antioxidant prevents

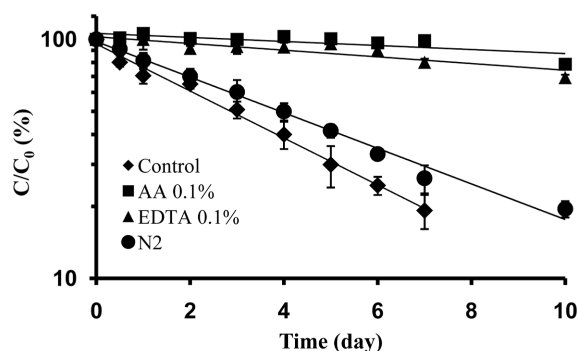

Figure 4. Effect of antioxidant and N_2 displacement on the chemical stability of HST in aqueous solution at 40°C. Value represents for mean ± SD (n=3).

the oxidative processes that occur on exposure to oxygen or in the presence of free radicals. Various kinds of antioxidants such as ascorbic acid (AA), alpha lipoic acid, EDTA, propyl gallate, ascorbyl palmitate (AP), carotene, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are available (Cort et al., 1974; Damian et al., 2010; Everett et al., 1996; Murthy et al., 1988; Packer et al., 1994). In this study, AA and EDTA were used as hydrophilic antioxidants, and AP as hydrophobic antioxidant. As shown in Figure 4, the addition of AA and EDTA at the level of 0.1% significantly enhanced the stability of HST at 40°C. Kinetic parameters like degradation rate constant and half life are listed in Table II, together with the results of control and AP-added solutions. Half life of HST was 10-fold extended by the addition of water-soluble antioxidants, manifesting the better result of AA than that of EDTA at the same level of 0.1% addition. Stabilization effect of AA was proven by Murthy et al., in which the chemical stability of quinapril HCl was improved by the incorporation of antioxidant during production process. However, in case of AP-added solution, no promising result was obtained due to the lower level of antioxidant addition. The highest level of AP addition was limited to 0.01% because of its water insolubility. At the same level of 0.01% addition, both AP-added and AA-added solutions brought a poor result in HST stabilization. It was considered that minimal effective concentration was around 0.1% level, regardless of the type of antioxidant.

AA concentration dependency

Figure 5 shows the concentration dependency of AA for stabilization of HST at 40°C. The lowest level of 0.01% did not influence on stabilization, giving the very similar pattern to that of control solution. However, at the highest level of 1% addition, the drug remained stable over three weeks at the same temperature. In comparison, at the medium level of 0.1% addition, HST was stable enough for a week, but started to decompose afterwards. This means that AA preferentially undergoes oxidative degradation in place of the active ingredient, HST. According to Akers et al., most of water-soluble antioxidants follow this pre-oxidative mechanism. It is reported that AA degrades in a first-order fashion and the rate constant is 0.348 per day at 40°C (Yang et al., 2006), which means the half-life of about 2 days. Therefore, the sudden decrease for HST content after 7 days in 0.1% AA addition was due to the reduced concentration of AA itself. Calculating the residual amount of AA after 7 days, the active level of AA is under 0.01%. On the other hand, the stability of HST was increased proportionally by the amount of AA added (Table II). The difference in degree of stabilization was an order of magnitude, e.g., 10-fold increase in AA addition decreased the rate constant about a tenth. Finally, it is suggested that HST could be stabilized by the addition of enough amount of antioxidant.

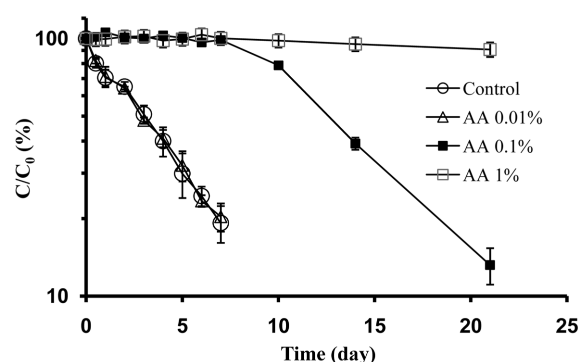

Figure 5. Dependence of HST stability on the concentration of ascorbic acid (AA). Value represents for mean ± SD (n=3).

Conclusion

The degradation of HST in aqueous solution was followed by the first order kinetics with extremely short half life of less than a week at room temperature, and was accelerated as the temperature increased. However, it was stabilized by the addition of a suitable antioxidant, suggesting that AA was the most effective stabilizer at sufficient level of the amount.

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