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Short communication

Analytical methods for assay of ellagic acid and its solubility studies

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Abstract

Ultra-violet (UV) spectrophotometric and high performance liquid chromatographic methods for quantitative determination of ellagic acid (EA), an antioxidant were developed. The analytical methods were validated for linearity, accuracy, intra- and inter-day variability, and precision. EA was eluted using a polyethylene glycol (PEG) column with mobile phase composed of acetonitrile and 5 mM potassium dihydrogen orthophosphate buffer pH 2.5 (80:20, v/v). The UV and high performance liquid chromatography (HPLC) methods have lower detection limits of 0.2 and 0.1 μg/ml, respectively. Because of the increasing pharmaceutical interest in phytochemicals and their solubility problems, solubility studies for EA were also carried out. Various organic solvents and surfactants were screened for assessing solubility of EA and the compound was found to be soluble in some pharmaceutically acceptable solvents like triethanolamine, polyethylene glycol 400 and *N*-methyl pyrrollidone (NMP). Aqueous and pH dependent solubility of EA were determined using UV and HPLC methods, respectively.

Keywords: Antioxidants; Ellagic acid; Analytical method; HPLC; Validation; Solubility

1. Introduction

A number of dietary factors are known to exhibit anticarcinogenic and antioxidant activities [1–7]. The established safety of these phytopharmaceuticals which are showing good clinical activity has infused a great interest in industry and academia alike. The challenge now is to incorporate these promising molecules into dosage forms that will standardize their usage for prevention and therapy of diseases. One such compound is ellagic acid (EA), dilactone of hexahydroxydiphenic acid, found in raspberries (1500 μg/g dry weight), strawberries (630 μg/g dry weight), cranberries (120 μg/g dry weight), walnuts (590 μg/g dry weight), pecans (330 μg/g dry weight), and other plant foods. EA has been investigated by researchers for a series of pharmacological properties ranging from antioxidant, antimutagenic, anticancer and apoptosis inducing activity. Aqueous solubility of a drug determines its concentration at the site of absorption and hence its systemic bioavailability in case of orally administered drugs. The estimation of solubility of a drug in water and various solvents is crucial in the process of development of a pharmaceutical formulation. Here, we report development of analytical methods for quantitative estimation of EA and their use in the drug's solubility estimation studies.

EA is a highly thermodynamically stable molecule (Fig. 1), with four rings representing the lipophillic domain and four phenolic and two lactone groups (which can act as hydrogen bond donor and acceptor, respectively) representing the hydrophilic part. Formulation and administration of EA into a pharmaceutical dosage form require its solubilization in safe and pharmaceutically acceptable solvents.

The ultra-violet (UV) absorption spectra of EA in methanol and at various pH values have been reported [8,9]. However, a validated UV spectrophotometric method for quantification of EA finds no mention in the literature. A UV spectrophotometric method was developed and validated, which is simple, rapid and economical. Reverse phase high performance liquid chromatography (HPLC) methods for identification and quantification of EA are available from published literature [10,11]. All the reported HPLC meth-

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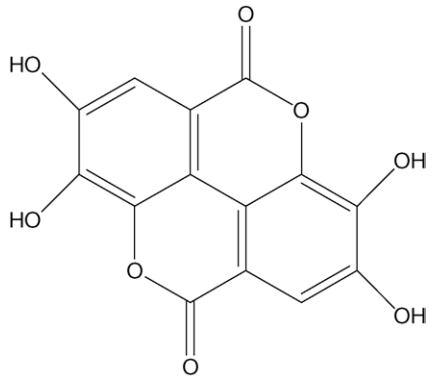

Fig. 1. Structure of EA (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione).

ods use a C18 column to elute EA. One of the problems that we encountered using a C18 column was peak tailing.

EA is reported to be insoluble in water and sparingly soluble in alcohol [12]. The solubility of EA was estimated in water and methanol using the validated UV analytical method. Solubility of EA at various physiologically relevant pH values was determined using HPLC method. Also, qualitative solubility of EA was determined in some solvents regularly used in pharmaceutical industry.

2. Experimental

2.1. Chemicals

EA (assay 95%) was purchased from Sigma, USA. Potassium dihydrogen orthophosphate and orthophosphoric acid were obtained from Loba Chemie, India and Merck, India, respectively. Solvents and mobile phases used for HPLC were of HPLC grade obtained from Rankem, India. Solvents used for solubility studies were of analytical grade. Ultra pure water (SG water purification system, Germany) was used to prepare all samples and buffers.

2.2. Instrumentation and analytical conditions

2.2.1. Ultra-violet spectrophotometric method

UV absorbance of EA was measured using a UV spectrophotometer (Beckman DU 640i, USA) at 255 nm using 1 cm quartz cuvette.

2.2.2. High performance liquid chromatography method

HPLC analysis was done using Shimadzu Class LC-VP HPLC system with Class VP software, coupled to a UV detector (SPD-10Avp) (Shimadzu, Japan). All separations were performed using Supelco Discovery® HS polyethylene glycol (PEG) column, 25 cm × 4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) maintained at 40 °C. EA was eluted isocratically at a flow rate of 1 ml/min using mobile phase consisting of 5 mM potassium dihydrogen orthophosphate pH 2.5 and acetonitrile (20:80, v/v). The mobile phase was prepared fresh on the day of use, filtered through 0.45 μm nylon filter and deaerated by sonication for 30 min. Absorbance of eluent was measured at 254 nm.

2.3. Preparation of standard solutions

For UV method, a standard solution of 100 μg/ml was prepared by dissolving accurately weighed quantity of EA in methanol and working standards were prepared by dilution of this standard solution with pH 7.4 phosphate buffer. For HPLC, standard solution and working standards were prepared in methanol.

2.4. Method validation

The analytical methods were validated according to the guidelines of the international conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH).

2.5. Linearity

Calibration curve was generated using six concentrations (in triplicates) of EA in the range of 0.5–8 μg/ml for both the methods. Linearity was evaluated by linear regression analysis.

2.6. Precision

Precision of the assay method was determined by repeatability (intra-day) and intermediate precision (inter-day). Intermediate precision was estimated by comparing the assays on three different days. Repeatability was assessed by analyzing six sample solutions of 8 μg/ml under the same operating conditions over a short interval of time.

2.7. Accuracy

Accuracy was determined by recovery of known amounts of EA standards in triplicate.

2.8. Limit of detection (LOD)

LOD was found by successive dilution of a standard solution in pH 7.4 phosphate buffer and methanol for UV and HPLC methods, respectively.

2.9. Equilibrium solubility determination

The equilibrium solubility of EA in water, phosphate buffer pH 7.4, methanol, and phosphate buffer pH 7.4 with methanol (80:20, v/v) was determined by shake flask method. An excess amount of EA was added to 5 ml of solvent, sonicated for 10 min and maintained at 37 °C in shaking water

bath (Julabo SW 21, Germany) at 100 rpm. Aliquots were withdrawn every 24 h for five days and centrifuged (Sigma 3 K30, Germany) at 28,500 × *g* for 10 min. After an appropriate dilution in phosphate buffer pH 7.4, the concentration of EA in the supernatant was determined by UV absorbance at 255 nm. Assays were performed in triplicate.

2.10. pH dependent solubility determination

Solubility of EA in buffers of different pH (pH 1.0, 4.5, 5.5, 6.5 and 7.5) was investigated by adding an excess amount of EA in screw capped vials at an experimental temperature of 37 °C and 100 rpm in shaking water bath for 48 h. The samples were centrifuged at 28,500 × *g* for 10 min. After an appropriate dilution in methanol, the concentration of EA in supernatant was determined by HPLC at 254 nm. Assays were performed in triplicate.

2.11. Qualitative estimation of EA solubility in organic solvents

A number of organic solvents were screened to assess the solubility of EA by adding successive amounts of EA to a known volume of solvent with stirring and/or bath sonication.

3. Results and discussion

3.1. UV method

The proposed UV method allows rapid and economical quantification of EA. In methanol, EA showed absorbance maximas at wavelength of 255 and 360 nm while it exhibits additional maxima at 276 nm in phosphate buffer pH 7.4 due to the ionization of phenolic hydroxyl group. The LOD was found to be 0.2 μg/ml. The correlation coefficient of 0.9998 indicates compliance with the Beer–Lamberts law. Percentage relative standard deviation (% R.S.D.) shows the validity for repeatability (Table 1). Both the intra- and inter-day relative standard deviations of QC standards were less than 3% over the selected range (Table 2). A good accuracy of the method was verified with recovery values of 98–101%.

Table 1
UV and HPLC method validation parameters

Parameters	UV ($n = 3$)	HPLC ($n = 5$)
Linearity range (μg/ml)[a]	0.5–8.0	0.5–8.0
Linearity	0.9998 ± 0.0001(0.0058)[b]	0.9982 ± 0.0022(0.22)[b]
Slope	0.1531 ± 0.0051(3.3)	61247.4 ± 4186.8(6.8)
Intercept	0.003 ± 0.0023	6599.1 ± 7293.9
Repeatability	99.95 ± 1.90	100.16 ± 1.24

[a] Linearity established using six concentrations ($n = 3$).
[b] Values are mean ± S.D. (R.S.D.) of *n* number of calibration curves.

Table 2
Estimated intra- and inter-day precision and accuracy of the analytical methods

Method	Concentration (μg/ml) ($n = 3$)	Intra-day		Inter-day[a]
		Accuracy (%)	Precision (%R.S.D.)	Precision (%R.S.D.)
UV	1	98.1	0.34	2.70
	3	100.7	0.87	1.79
	5	100.8	0.61	1.04
HPLC	1	101.7	6.87	3.50
	3	98.6	0.77	3.60
	5	103.0	1.09	2.33

[a] Three replicates each were analyzed on three consecutive days.

3.2. HPLC method

Many HPLC methods have been reported for identification and quantification of EA, all using reverse phase C18 column. Isocratic elution of EA using the reported methods resulted in tailing (asymmetry factor at 10% height of the peak being 1.7–1.8) and broadening of the peak, with an elution time of just 2 min, indicating poor retention. The recent use of new polar reverse phase PEG bonded column for phenolic drugs [13] that are poorly retained on the C18 column, promoted us to test this column for EA. The elution of EA on PEG bonded stationary phase using 5 mM potassium dihydrogen orthophosphate pH 2.5: acetonitrile (20:80, v/v) as mobile phase resulted in reduced tailing and a narrower peak, as shown in Fig. 2b. This improvement arises from a better retention of EA.

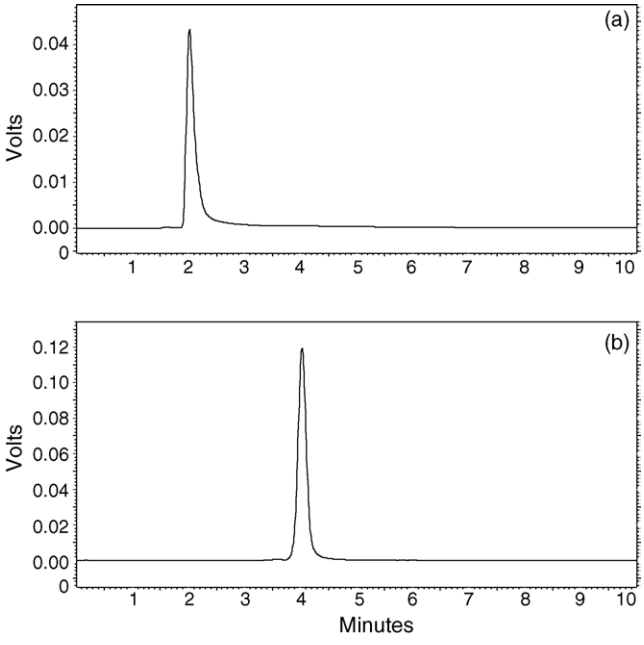

Fig. 2. Comparison of HPLC chromatogram (5 μg/ml, injected 5 μl) (a) C18 Merck (25 cm × 4.6 mm, 5 μm) and (b) supelco PEG column (25 cm × 4.6 mm, 5 μm) using 5 mM potassium dihydrogenphosphate solution (pH 2.5)–acetonitrile (20:80, v/v) at 1 ml/min and 40 °C.

The method has been optimized using different mobile phase compositions; methanol–0.2% phosphoric acid aqueous solution (35:65, v/v), 5 mM potassium dihydrogen orthophosphate (pH 2.5)–methanol (20:80, v/v), 5 mM potassium dihydrogen orthophosphate (pH 2.5)–methanol–acetonitrile (40:50:10, v/v), and 5 mM potassium dihydrogen orthophosphate (pH 2.5)–acetonitrile (20:80, v/v). The use of 5 mM potassium dihydrogen orthophosphate (pH 2.5)–acetonitrile (20:80, v/v) overcame the problem of peak tailing (asymmetry at 10% height of the peak reduced to 1.1) in PEG column with a retention time of 4 min. Improvement to this extent was not observed with C18 column (Fig. 2a).

The calibration curve for EA was constructed in concentration range of 0.5–8 μg/ml, showing good linearity with correlation coeffient of 0.9982. The limit of detection was found to be 0.1 μg/ml. Table 2 gives the results obtained for intra-day and inter-day precision. The percentage relative standard deviation of slopes for each standard curve obtained on three consecutive days was under limits, proving the intermediate precision of inter-day variation. The accuracy was found to be in the range of 98–103% indicating an agreement between the true and observed values.

3.3. EA solubility

Shake flask method was used to determine the equilibrium solubility of EA in water, phosphate buffer pH 7.4, methanol, and phosphate buffer with methanol (80:20, v/v). Samples were analyzed every 24 h by measuring UV absorbance at 255 nm. Equilibrium was reached within five days for all four solvents. EA showed poor aqueous solubility value of 9.7 μg/ml while it was higher in pH 7.4 phosphate buffer (33.1 μg/ml) because of the acidic nature of EA. Highest solubility (671 μg/ml) was found in methanol (Table 3). However, no significant increase in solubility of EA was seen on inclusion of methanol in phosphate buffer.

Fourty eight hours solubility of EA at pH 1, 4.5, 5.5, 6.5 and 7.5 was determined by HPLC method (Fig. 3). Theoretical profile was generated using Henderson–Hasselbach equation for weak acids: $S/S_0 = 10^{(\mathrm{pH}-\mathrm{p}K_a)} + 1$ where S represents solubility of drug at a particular pH, pK_a is the dissociation constant and S_0 is solubility of unionized form of drug. EA, a weakly acidic drug shows an increase in solubility with pH. Experimentally generated profile was in close agreement with the theoretical profile based on the reported pK_a value of 5.6 [14]. EA is a weak acid with four phenolic groups and theoretically, should exhibit four acid dissociation constants [9]. Below pH 5.6, EA exists in a mono-deprotonated form [14] whereas, at pH higher than 5.6, deprotonation occurs at two hydroxyl group positions. It has been earlier reported that solubility of EA increases after it has lost two hydrogens per molecule [15]. Attempts to increase the solubility of EA by increasing the pH resulted in development of dark greenish brown color, which could be due to the hydrolysis of lactone moieties (pH 9.6, 10^{-5} M NaOH) [14].

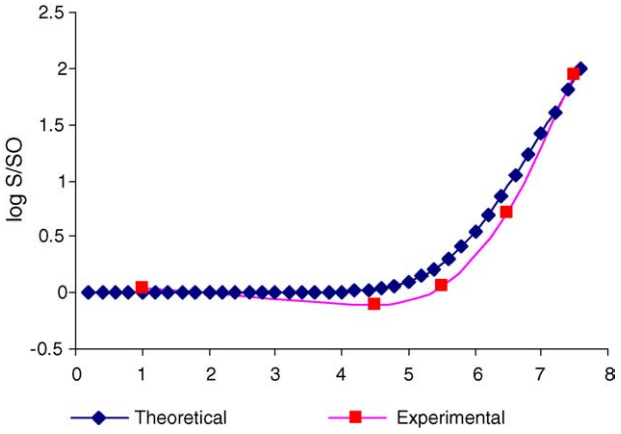

Fig. 3. Theoretical (based on a pK_a value of 5.6) and experimental pH dependent-solubility profiles for EA.

The solubility of EA in pyridine is well known by virtue of its ability of forming a charge transfer complex. Various organic solvents and surfactants were screened for solubilization of EA. The commonly used surfactants like sodium lauryl sulphate (1%, w/v), span20 (10%, w/v), tween 80, transcutol, cremophore EL (5%, w/v), pluronic PF68 (0.5%, w/v) did not solubilize EA. Table 4 enlists the approximate solubility of EA in various organic solvents screened. Acidic nature of EA suggests that it might be more soluble in basic solvents, which is confirmed by its solubilization in *N*-methyl pyrrollidone (NMP). NMP is recommended as a skin penetration enhancer for use in transdermal therapy in humans [16]. An interesting observation during this exercise was that EA was not soluble in triethanolamine (TEA) but rapidly solubilized on subsequent addition of a very small amount of water. This might be due to salt formation of EA with TEA in presence

Table 3
EA equilibrium solubility at 37 °C

Solvent	Equilibrium solubility (μg/ml)
Water	9.7 ± 3.2[a]
Phosphate buffer pH 7.4	33.1 ± 15.5
Phosphate buffer pH 7.4 with 20% methanol	41.8 ± 10.7
Methanol	671.7 ± 16.7

[a] Mean ± standard deviation (n = 3).

Table 4
Qualitative estimation of EA solubility in various organic solvent

Solvent	Millilitres of solvent required to dissolve 5 mg of EA
Ethanol	25
Pyridine	2.5
NMP	0.2
Triethanolamine + 50 μl water	0.15
DMSO	2.0
PEG 400	0.6

of water, since TEA is used for salt formation in injectable solutions and topical preparations [17]. The most encouraging result was obtained with polyethylene glycol (400) which is used as a vehicle in parenteral dosage forms [17]. PEGs are miscible with both aqueous and organic solvents. The presence of phenolic groups and lactone moiety in the structure of EA suggests higher chances of solubility in solvents with which it can form hydrogen bonds. Thus, hydrogen bonding might be playing a major role in solubilization of EA in PEG 400.

4. Conclusion

The developed UV and HPLC methods were found to be simple, accurate and precise for quantitative determination of EA. The elution of EA on PEG bonded stationary phase resulted in reduced tailing and narrower peak in comparison to commonly employed reverse phase C18 column. The equilibrium solubility of EA in water, pH 7.4 phosphate buffer and methanol was 9.7, 33.1 and 671 μg/ml, respectively. Being a weak acid, solubility of EA increased with a rise in pH beyond its reported pK_a value of 5.6. EA was found to have good solubility in NMP, TEA, and PEG 400. These solvents can thus be used for the formulation and administration of EA as an antioxidant.

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