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Original Article

PREPARATION, DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF APIGENIN IN APIGENIN–HYDROGENATED SOY PHOSPHATIDYLCHOLINE (HSPC) COMPLEX

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ABSTRACT

Objective: To Develop simple, sensitive UV – visible spectrophotometric method for determination of Apigenin (APG) in Apigenin – Hydrogenated Soy Phosphatidylcholine (HSPC) Complex.

Methods: The APG –HSPC Complex (phytosomes) were prepared by dissolving both APG and HSPC in 20 ml mixture of 1, 4 – dioxane: methanol at a ratio of (14:6) by refluxing and complex produced by solvent evaporation method. The spectrophotometric detection of APG was done at the absorption maximum (λ max) of 335 nm and 268 nm using methanol as solvent. The developed method was validated as per International Conference on Harmonization (ICH) guidelines.

Results: APG content in APG – HSPC Complex was found to be 82.86±0.90% and 76.89±0.84% at 335 nm and 268 nm. APG exhibited good linearity in concentration range 2 – 12 µg/ml (r^2>0.99) at 335 nm and 2 – 14 µg/ml (r^2>0.99) at 268 nm. Precision and mean recoveries were found to be in the range of (% RSD 0.0981 & 0.0989) and (% RSD 0.0829 & 0.1116) and 94.67±2.52 % & 86.56±1.90 % at 335 nm and at 268 nm. The limit of detection (LOD) and limit of quantification (LOQ) was found to be (0.0106µg/ml & 0.0322µg/ml) and (0.0259µg/ml & 0.0757µg/ml) respectively.

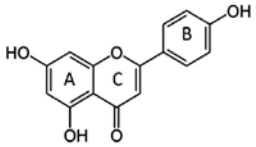
Conclusion: The developed method was found to be minimal, specific, economic, reliable, accurate, precise, and reproducible that used as a quality control tool for analysis of APG.

Keywords: UV–Visible spectrophotometer, Apigenin, Solvent evaporation, Apigenin-Hydrogenated Soy Phosphatidylcholine, Method validation.

INTRODUCTION

Apigenin (APG), chemically known as (5, 7 – dihydroxy – 2 - (4 - hydroxyphenyl) – 4H – 1 – benzopy – ran – 4 – one) a yellow compound with molecular formula ($C_{15}H_{10}O_5$) whose molecular weight is (MW: 270.24 g/Mol) as shown in [fig. 1]. Naturally, it exists as dimer biapigenin as well as apigenin – 7- 0 – glucoside and various acylated derivatives that recognize it to the subcategory of flavone. This flavonoid is abundant in common fruits such as grapefruits, plant derived beverages, and vegetables such as parsley, oranges, onions, tea, chamomile and wheat sprouts etc [1]. Although APG is common one in variety of plants, some that are widely marketed as dietary and herbal supplements, in addition APG to some extent is a potent inhibitor of the cytochrome P450 (CYP) enzyme system that is responsible for metabolism of considerable pharmaceutical drugs [2]. APG exhibits wide range importance, it has been isolated from different plants as Salvia officinalis L. [3], Chamomila recutita L. [4], Elsholtzia regulosa [5], Perilla frutescens L. [6], Coriander sativum L. [7], Stachys tibetica [8]and also reported its many biological and pharmacological effects include antimicrobial [9], antioxidant [10-12], anti-tumor [13-14], anti-inflammatory[15-16] antiproliferative [17], antiviral[18], and antidiabetic [19].

However, APG solubility in water is up to 2.16µg/ml and 0.001 – 1.63 mg/ml in hydrophilic and non – polar solvents leading to poor absorption in the gastrointestinal tract. It is hydrophobic in nature and freely soluble in methanol, ethanol, dimethylsulfoxide and dimethylformamide[20]. In a wide range of HPLC and HPTLC techniques were reported for estimation of apigenin with other flavonoid in human urine[21], dog and rat plasma[22-24], tablets[25],Turnera aphordisiaca W.[26], Glycyrrhiza glabra L.[27], Euphorbia prosptrata[28] at an around 350 nm. The literature survey revealed that no simple UV – visible method has been reported for individual quantitative estimation of APG in prepared APG – HSPC complex. Hence we have developed novel sensitive, specific, economic, reliable, accurate, precise, and reproducible UV method for quantitative estimation of APG in prepared complex at a wavelength of 335 nm and 268 nm and validate according to ICH guidelines.



Apigenin

Fig. 1: Structure of Apigenin

MATERIALS AND METHODS

Materials

Phospholipion 90H (Hydrogenated Soy Phosphatidylcholine) was received a gift sample from lipoid GmbH, Germany. Apigenin standard was purchased from Shandong Northwest Manufacturing Co., Ltd, China. All other chemicals used were of either pharmaceutical or analytical grade.

Methods

Preparation of Apigenin – HSPC complex

The complex was developed with APG and HSPC at a molar ratio of (1:2 w/w). Weighed amount of APG [Mol. Wt., 270.2] and HSPC [Mol. Wt., 790] were taken in 100 ml round bottom flask and 20 ml of 1, 4, - Dioxane: methanol mixture at a ratio of (14:6) was added. The mixture was refluxed at a temperature not exceeding 50^{0}C for 1 h to get a clear solution. The obtained solution was concentrated to dryness. The residue was dissolved in 50 ml of chloroform: methanol mixture at a ratio of (9:1). The chloroform solution was

evaporated to a small volume and the residue was poured into 100 ml of n-hexane. The resultant APG– HSPC complex (yield 79 % w/w) was kept in an amber colored glass bottle flushed with nitrogen and stored at room temperature till further analysis [29 - 32].

Method development

Instrument

Double beam UV – Visible spectrophotometer (JASCO V-630)

Preparation of standard stock solution

To find out the wavelength maximum absorption (λ max) of APG, the standard stock solution (1000 µg/ml) of APG was prepared by weighing accurately 5 mg of pure drug into a 5 ml volumetric flask and dissolved in a minimum quantity of methanol and the final volume was made up to mark with methanol.

Preparation of working stock solution

The working stock solution of APG (100µg/ml) was prepared and established by diluting 1 ml of standard stock solution to 10 ml with methanol.

Selection of (λ max)

The working stock solution was diluted with methanol to get (10 µg/ml) concentration (1 ml to 10 ml). This solution was scanned between the wavelength regions of 200 – 400 nm against methanol as blank. The UV spectra were shown in [fig. 2] and absorption curve showed two characteristic absorption maxima at 335 nm and 268 nm. Hence, both (λ max) were selected for analysis of APG. From working stock solution a series with a concentration range of 2 – 12 µg/ml at 335 nm and 2 – 14 µg/ml at 268 nm for preparation of calibration curves were obtained by dilution of stock solution with methanol (table 1, 2).

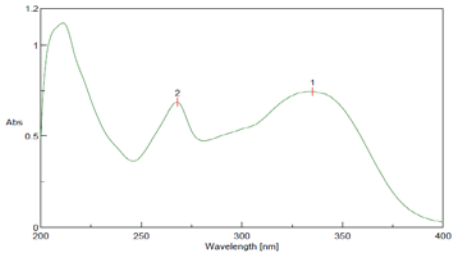


Fig. 2: UV spectrum of Standard APG in Methanol, UV spectrum represents standard solution of APG (10µg/ml) 1) λ max = 335 nm, 2) λ max = 268 nm

Table 1: Calibration data for APG (335 nm)

Concentration (µg/ml)	Absorbance at 335 nm
2	0.1395
4	0.2952
6	0.4458
8	0.6044
10	0.7969
12	0.9568
Regression equation	Y= 0.0821x – 0.0353
Correlation coefficient	0.9985

Table 2: Calibration data for APG (268 nm)

Concentration (µg/ml)	Absorbance at 268 nm
2	0.1683
4	0.3113
6	0.4673
8	0.6318
10	0.8190
12	0.9609
14	1.1210
Regression equation	Y= 0.0807x – 0.0053
Correlation coefficient	0.9990

Percent drug content

To determine the content of APG in APG – HSPC complex, the complex equivalent to 5mg of APG was weighed and transferred it to 5 ml volumetric flask; 2 ml of methanol was added, sonicated for 10 min and volume was adjusted to 5 ml using methanol. The solution was filtered through Whatman filter paper No.1; from this filtrate 1 ml was transferred to 10 ml volumetric flask and diluted with methanol to 10 ml to get the concentration of (100 µg/ml). The (100 µg/ml) solution was diluted with methanol to get a final concentration of (10 µg/ml). The absorbance was measured at 335 nm and 268 nm. The same procedure was followed for making the dilution of HSPC and it is used as blank. The analysis was repeated six times. The absorbance was mentioned in (table 3).

Table 3: Estimation of APG in standard APG, APG – HSPC complex at 335 nm and 268 nm

λ max (nm)	Formulation	Concentration (µg/ml)	Purity ± SD*
335	Standard APG	10	101.36 ± 0.04
	APG – HSPC Complex	10	82.86 ± 0.90
268	Standard APG	10	102.25 ± 0.04
	APG – HSPC Complex	10	76.89 ± 0.84

SD* = Std. deviation

RESULT AND DISCUSSION

Method development

As APG is a flavonoid category of phytoconstituent that shows sparingly solubility in water and in other organic solvents too. For appreciable solubility of APG various solvents of (AR grade) in alone and combinations like hydro alcoholic solvent, ethanol, acetonitrile, 1, 4 – dioxane and tetrahydrofuran were tried for detection of wavelength, but after a long serious of experiments, it was found that, the methanol (AR grade) showed better solvent for APG solubility as well as stability point of view and method development purpose due to completely solubility of APG by sonication up to 1 to 2 min. Hence, methanol was used for detection of suitable wavelength and working concentration of APG. The absorption maxima (λ max) was found to be 335 nm and 268 nm in methanol as shown in [fig. 2]. In response to finding out the applicability of the developed method to an APG – HSPC Complex, a prepared APG – HSPC complex was studied at working concentration for quantification of percent drug content protocol of APG and it was quantified to be 82.86±0.90% and 76.89±0.84% at 335 nm and 268 nm at the standard working concentration level. Estimated content of APG in samples (complex) ranging 76 and 85% that is acceptable in any phytosomal formulations and may be also used as a quality control tool. Hence above developed method is optimized. This solvent was used for further method validation.

Method validation

The developed analytical method was validated for parameters as linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines.

Linearity

The working solution of APG from which different concentration range (2 – 12µg/ml) and (2 - 14µg/ml) for linearity was prepared in triplicates and analyzed at 335 nm and 268 nm. The calibration curve was created by plotting absorbance v/s concentration level for APG was found to be linear in at both wavelengths [fig. 3 and 4]. It shows an acceptable correlation between absorbance and concentration as per the above range of concentration with a correlation coefficient greater than >0.99 with a regression equation (y = 0.0821x – 0.0353) at 335 nm and (y = 0.0805x – 0.0042) at 268 nm in methanol said to be linear at both wavelengths [33].

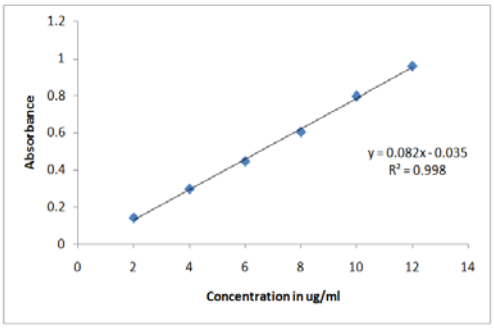


Fig. 3: Calibration Curve for APG at 335 nm

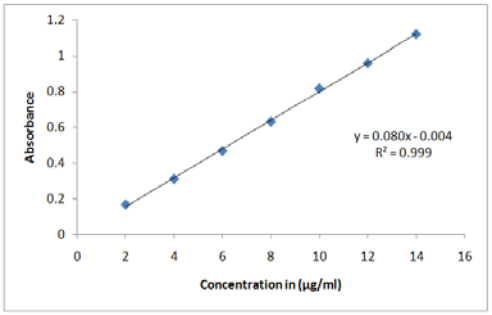


Fig. 4: Calibration Curve for APG at 268 nm

Precision

Method precision studies were done by recording absorbance of sample in two ways; (a) repeatability (intra-day) and (b) intermediate precision (inter-day).

Repeatability (Intra – day precision)

The intra-day precision was determined by recording absorbance of working concentration of (10µg/ml) APG at three different time points within day by six consecutive times at 335 nm and 268 nm reported % RSD (Relative Standard Deviation) less than 2 for APG within a day, which indicates that developed method is précised and thereby concerned with repeatability test that gives reproducible results as computed in (Table 4) [33].

Intermediate precision (Inter – day precision)

The inter-day precision was determined by recording absorbance of working concentration of (10µg/ml) APG at three different time points in three days by six consecutive times at 335 nm and 268 nm reported % RSD (Relative Standard Deviation) less than 2 for APG between days, which indicates that developed method is précised and results computed in (table 4) [33].

Accuracy

Accuracy was evaluated by performing a recovery study experiments, by estimations of % mean recovery of sample solutions of APG with three different levels (80%, 100%, 120%) were determined in a triplicate absorbance sand % recovery was computed in (Table 5). The (%) recovery values are 85% - 95% within the acceptable range that shows good recovery values at two wavelengths, hence exhibit accuracy of the developed method [33].

LOD & LOQ

LOD (limit of detection) is the lowest concentration of analyte in a sample that an analytical process can reliably differentiate from background levels and LOQ (limit of quantification) is the lowest concentration of calibration curve that can be measured with an acceptable accuracy and precision. In this method LOD and LOQ were based on the standard deviation of the response and the slope of the calibration curve using equations LOD = 3.3 S/M and LOQ = 10 S/M, where S is the standard deviation of the absorbance of the sample and M is the slope of the calibration curve.

Table 4: Intraday and interday precision results of APG at 335 nm and 268 nm

Intra/Inter – day Absorbance (335 nm) ± SD (10µg/ml)	% RSD (Mean)	Intra/Inter – day Absorbance (268 nm) ± SD (10µg/ml)	% RSD (Mean)
(Intra – day)		(Intra – day)	
0.7969 ±0.0003		0.8199 ±0.0003	
0.8001 ±0.0008	0.0981	0.8205 ±0.0008	0.0829
0.8147 ±0.0011		0.8399 ±0.0008	
(Inter – day)		(Inter – day)	
0.7969 ±0.0003		0.8199 ±0.0003	
0.8306 ±0.0011	0.0989	0.8576 ±0.0001	0.1116
0.8568 ±0.0009		0.8984 ±0.0024	

RSD* = Relative Std. deviation

Table 5: Results of Accuracy studies for APG at 335 nm and 268 nm

λ max (nm)	Level of recovery (%)	Recovery (%)	Mean ± SD*
	80	97.25	
335	100	92.20	94.67 ±2.52
	120	94.58	
	80	88.50	
268	100	84.70	86.56 ±1.90
	120	86.50	

SD* = Std. deviation

The sensitivity of measurement of APG by this developed method was determined in terms of the limit of detection (LOD) and limit of quantification (LOQ). The LOD for APG at two wavelengths were found to be 0.0106µg/ml and 0.0249µg/ml, respectively, and LOQ for APG at two wavelengths were found to be 0.0322µg/ml and 0.0757µg/ml respectively. Hence developed method was found to be sensitive. Optical characteristics and validation parameters are presented in (table 6).

Table 6: Optical characteristics and validation parameters

Parameters	Results	Results
λmax	335 nm	268 nm
Beer's law range (µg/ml)	2 - 12	2 - 14
Regression equation (y = mx + c)	y = 0.0821x – 0.0353	y = 0.0805x –0.0042
Correlation coeffient (r^2)	0.9985	0.9990
Slope (m)	0.0821	0.0805
Intercept (c)	0.0353	0.0042
Accuracy	94.67±2.52%	86.56±1.90%
Precision (%RSD)	0.0981	0.0829
Intra – day	0.0989	0.1116
Inter – day	0.0106	0.0249
LOD (µg/ml)	0.0322	0.0757
LOQ (µg/ml)		

CONCLUSION

A simple, specific, economic, reliable, UV spectroscopic method was developed and validated as per ICH guidelines. The APG was considered to be completely soluble in methanol. The absorption maximum (λ max) was found to be 335 nm and 268 nm. The good

linearity was found to be within concentration range of 2 – 12 µg/ml and 2 – 14 µg/ml with a correlation coefficient (r^2) greater than 0.99 and regression equation of the curve was found to be y = 0.0821x – 0.0353 at 335 nm and y = 0.0805x - 0.0042 at 268 nm. The precision (Intra – day and Inter – day) data represents good reproducibility with relative standard deviation (% RSD) lower than 2.0 % that ensure the method is précised. Mean recovery values at different concentrations were found to be in the range 85% - 95% indicates the accuracy of the method. LOD and LOQ for APG were found to be 0.0106 µg/ml & 0.0322µg/ml and 0.0259 µg/ml & 0.0757µg/ml at 335 nm and 268 nm respectively. Hence, the developed method is accurate, precise, reproducible and used as a quality control tool for analysis of APG in phytosomal formulations. And it gives less time consuming method alternative to HPLC.

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CONFLICT OF INTEREST

All authors have none to declare.

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