

**A NEW VALIDATED SIMULTANEOUS RP- HPLC ASSAY METHOD FOR
ESTIMATION OF TWO FLAVONES (APIGENIN AND GENKWANIN) IN API DRUGS**

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ABSTRACT

A reversed-phase high-performance liquid-chromatography method has been successfully developed for the simultaneous determination of Apigenin and Genkwanin in API drugs. The RP-HPLC method employed a Symmetry C18 reversed phase column (100 × 4.6 mm×3.5µm) with an isocratic mixture of Acetonitrile and 0.1% glacial acetic acid buffer in the ratio of (45:55; v/v) as the mobile phase. The column temperature was kept at 30°C. The flow rate was 1.0 mL/min and detection was by means of a PDA detector at wavelength of 207 nm. All the active components were successfully eluted with mean retention times of 2.02 and 5.12 min for Apigenin and Genkwanin respectively. The method was found to be linear ($R^2 > 0.99$), precise (RSD NMT 2.0%), accurate (recoveries 98.0–102.0%), specific, rugged and robust. The validated method can be used in routine quality control analysis of bulk drugs without any interference by excipients.

KEYWORDS: Apigenin, Genkwanin, RP-HPLC, Method development, Validation.

INTRODUCTION

Flavonoids are a family of polyphenolic compounds synthesized by plants with a similar structure, are divided into subclasses, including anthocyanidins, flavanols, flavanones, flavonols, flavones and isoflavones. Several beneficial properties have been attributed to these dietary compounds, including antioxidant, anti-inflammatory, and anti-carcinogenic effects. One of the molecule that has gained considerable interest as beneficial agent for human health with cancer preventive and therapeutic properties is plant flavone, apigenin.^[1]

Apigenin chemically known as 4', 5, 7, -trihydroxyflavone, with molecular formula $C_{15}H_{10}O_5$ whose molecular weight is 270.24g/mol. IUPAC name is 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one. In nature apigenin also exists as a dimer, biapigenin, mainly isolated from the buds and flowers of *Hypericum perforatum* (Fig.1), which has neuroprotective effects.^[2] Apigenin is abundantly present in common fruits such as grapefruit, plant-derived beverages and vegetables such as parsley, onions, oranges, tea, chamomile, wheat sprouts and in some seasonings. One of the most common sources of apigenin consumed as single ingredient herbal tea is chamomile, prepared from the dried flowers from *Matricaria chamomilla*.^[3] Apigenin is a naturally occurring plant

flavone, abundantly present in common fruits and vegetables is recognized as a bioactive flavonoid shown to possess anti-inflammatory, antioxidant and anticancer properties. Epidemiologic studies suggest that a diet rich in flavones is related to a decreased risk of certain cancers, particularly cancers of the breast, digestive tract, skin, prostate and certain hematological malignancies. It has been suggested that apigenin may be protective in other diseases that are affected by oxidative process such as cardiovascular and neurological disorders, although more research needs to be conducted in this regard. Other sources for apigenin include beverages such as wine and beer brewed from natural ingredients. Apigenin is commonly present as a constituent in red wine. Like red wine, beer also provides a good source of apigenin.^[4-5] In natural sources, apigenin is present as apigenin-7-O-glucoside and various acylated derivatives.^[6]

Genkwanin is an O-methylated flavone, a type of flavonoid. It can be found in the seeds of *Alnus glutinosa* and the leaves of the ferns *Notholaena bryopoda* and *Asplenium normale*. Genkwanin chemically known as 4',5-Dihydroxy-7-methoxyflavone, with molecular formula $C_{16}H_{12}O_5$ whose molecular weight is 284.27g/mol. IUPAC name is 5-hydroxy-2-(4-hydroxyphenyl)-7-methoxychromen-4-one (Fig. 2).

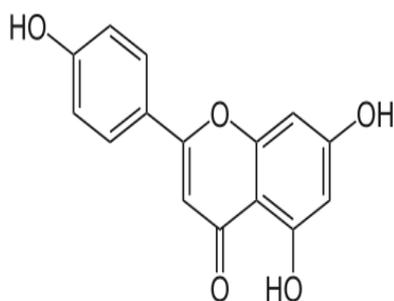


Fig.1: Apigenin

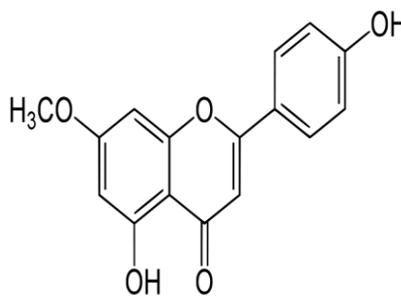


Fig.2: Genkwanin

In developing countries where the challenge of substandard drugs and drug counterfeiting is enormous, there is the need for methods which are accurate, cost effective, easy to use, rapid and require the use of non-sophisticated equipment in order to facilitate easy identification and quantitation of the active components in multi-component Drugs and formulations. The main objective of this work, therefore, is to develop and validate a new, simple, accurate, linear, precise, specific, robust, rugged and cost effective RP-HPLC assay method for simultaneous estimation of Apigenin and Genkwanin. Literature survey revealed that several methods were used to analysis of Apigenin and Genkwanin, in formulations, human urine and human blood serum. These methods include Simultaneous estimation of assay in single method. The aim of this study is performing very simple method in terms of mobile phase and program to analysis Apigenin and Genkwanin in API drugs.

MATERIALS AND METHODS

Instrument

Chromatographic separation was performed with High performance liquid chromatography having Waters, equipped with 2695 with PDA detector. Chromatograms and data were recorded by means of Empower 2 Software.

Chemicals and Reagents

Analytical grade glacial acetic acid, acetonitrile, methanol was procured from the Merck.

Stationary Phase

Analytical Column: Symmetry C18 column (100mm x 4.6mm x 3.5 μ m). was used.

Mobile phase

Mix the 45 volumes of Acetonitrile, 55 volumes of Buffer (0.1% glacial acetic acid).

Diluent

Mix the 30 volumes of Acetonitrile, 70 volumes of Methanol.

Preparation of standard solution

About 30 mg of each Apigenin and Genkwanin standards was accurately transferred in a 100mL volumetric flask and dissolved in 70 mL of diluent and made up with

diluent. Take 16.7 mL of this solution into 100mL of mobile phase was added to obtain 0.05mg/mL of Apigenin and Genkwanin standard solution.

Preparation of Sample solution

About 30 mg of each Apigenin and Genkwanin samples was accurately weighed in a 100mL volumetric flask and dissolved in 70 mL of diluent and made up with diluent. Take 16.7 ml of this solution into 100mL of mobile phase was added to obtain 0.05mg/mL of Apigenin and Genkwanin sample solution.

Chromatographic Condition

The established chromatographic conditions included a mobile phase of Acetonitrile: 0.1% glacial acetic acid in water(45:55 v/v), a Symmetry C18 (250 \times 4.6 mm \times 5 μ m,) stationary phase and a flow rate of 1 mL/min. Wavelength of detection was 207 nm and mode of elution was isocratic. Temperature was kept constant at 30°C. Injection Volume 20 μ l. These conditions gave the best resolution of peaks and separation of components.

Method Validation

A full method validation was performed according to guidelines set by the USFDA & ICH Guidelines.^[7-9] The validation of this procedure was performed in order to evaluate the method in terms of Specificity, System suitability, Method precision, Linearity, accuracy and Solution stability, Ruggedness and Robustness.

Specificity

Equal volume (20 μ l) of standard preparation and test preparation were separately injected into the chromatograph. Chromatograms were recorded and measured the responses for major peaks.

System suitability

Equal volume (20 μ l) of standard preparation was injected into the chromatograph. Chromatograms were recorded and measured the responses for major peaks.

Method Precision

Equal volume (20 μ l) of six different standard preparation solutions were injected into the chromatograph. Chromatograms were recorded and measured the responses for major peaks.

Linearity

Equal volume (20 μ l) of standard preparation was injected at different concentration 25% to 150% into the chromatograph. Chromatograms were recorded and measured the responses for major peaks.

Accuracy

It was obtained by Recovery studying using the standard addition method, Equal volume (20 μ l) of Accuracy at 50%, 100% and 150% solutions were injected into the chromatograph. Chromatograms were recorded and measured the responses for major peaks.

Solution stability

It was obtained by Solution stability study using the standard prepared solution. 20 μ l of initial, after 4 hours, 6 hours, 8 hours, 12 hours and 24 hours sample solutions were injected into the chromatograph. Chromatograms were recorded and measured the responses for major peaks.

Ruggedness

Equal volume of standard preparation was injected into the chromatograph by two different analysts in two different days. Chromatograms were recorded and measured the responses for major peaks.

Robustness

It is a Method parameter study. Equal volume of standard preparation was injected into the chromatograph by the change of two method parameters. Chromatograms were recorded and measured the responses for major peaks.

RESULTS AND DISCUSSION

Method development and optimization Mobile phase selection

Preliminary studies with several solvent systems were performed to select the most effective solvent system for the separation of the Flavones. The selection of these solvents as possible mobile phases depended on factors such as cost of solvents, polarities of solvents and that of the analytes of interest and the solubility of the analytes. Solvents such as methanol, Acetonitrile, and Glacial

acetic acid at various pH values, as well as combinations of these solvents were tried. The mobile phase of Acetonitrile and glacial acetic acid was tried in different proportions. However, an isocratic mixture of Acetonitrile and 0.1% glacial acetic acid in the ratio of (45:55; v/v) was chosen as the mobile phase because it produced the best resolution of peaks, peak symmetry and separation of all components within the least retention times. Mean retention times of 2.02 min and 5.12min.

Stationary phase selection

The polarities of the analytes of interest were taken into consideration when choosing the stationary phase. As the drug molecules are polar or moderately polar, reversed phase stationary phases were tried. A Symmetry C18 reversed phase column (100 \times 4.6 mm \times 3.5 μ m) was chosen in order to reduce the time of interaction between the stationary phase and the analytes. This helped to reduce analysis time as there is reduced affinity of the analytes for the stationary phase, and increased interaction of the analytes with the mobile phase.

Wavelength Selection

In simultaneous estimation of two Flavones isobestic wavelength is used. Standard solution of Apigenin and was prepared by weighing 30mg of Apigenin was weighed and transferred in to 100mL volumetric flask and dissolved in 70mL of diluent and then make up to the mark with the diluent and prepare 50 μ g /mL of solution by diluting 16.7mL to 100ml with diluent and standard solution of Genkwanin was prepared by weighing 30mg of Genkwanin was weighed in to 100mL volumetric flask and dissolved in 70mL of diluent and then dilute up to the mark with mobile phase and prepare 50 μ g /mL of solution by diluting 16.7mL to 100mL with diluent. The wavelength of maximum absorption (λ_{max}) of the drug of 50 μ g/mL solution in diluent were scanned using PDA within the wavelength region of 190–400 nm against diluent as blank. The λ_{max} was found to be 205 nm for Apigenin, 210 nm for Genkwanin and finally 207nm was selected for the combination and peak purity is also passed. Peak purity graph is as shown in fig 3.

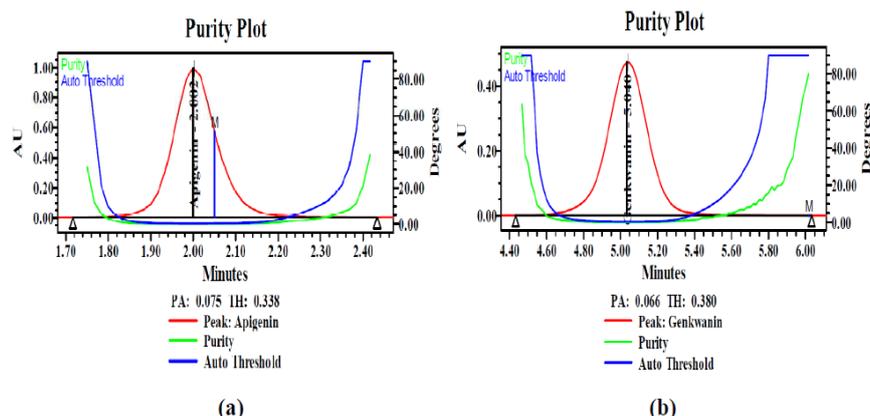


Fig .3: Peak purity Chromatograms of (a) Apigenin and (b) Genkwanin

Mass Spectral Analysis

As per the analysis conducted by MASS analysis. The m/z value of Apigenin was 271.08 in +ve mode, 269.16 in -ve mode, the m/z value of Genkwanin was 285.12 in

+ve mode, 283.11 in -ve mode respectively. The Mass spectrums of Apigenin and Genkwanin is as shown in fig 4.



Fig. 4: Mass spectrums of (a &b) Apigenin and (c&d)Genkwanin

Method Validation

Specificity

Peak purities higher than 99% were obtained for all two Flavones in the chromatograms of sample solutions depicting that the method was very specific to the two

Flavones under consideration. There were no interfering peaks on the retention times of the Flavones in the presence of excipients. This was very evident in the chromatograms of the sample (**Fig. 5**).

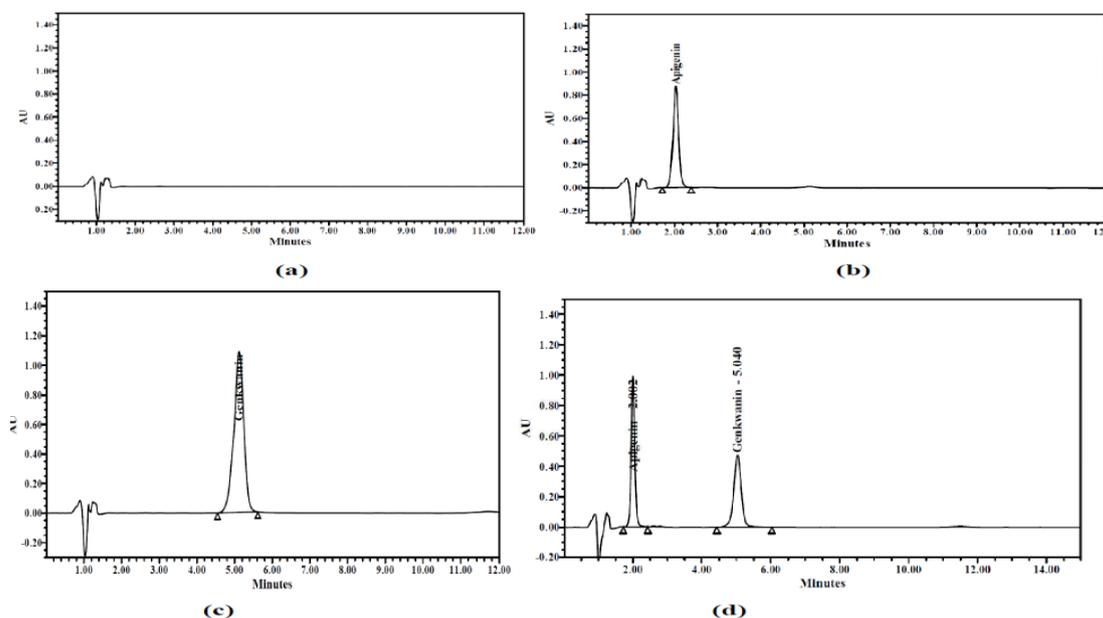


Fig. 5: Specificity Chromatograms of (a)Blank (b) Apigenin (c)Genkwanin(d) Spiked.

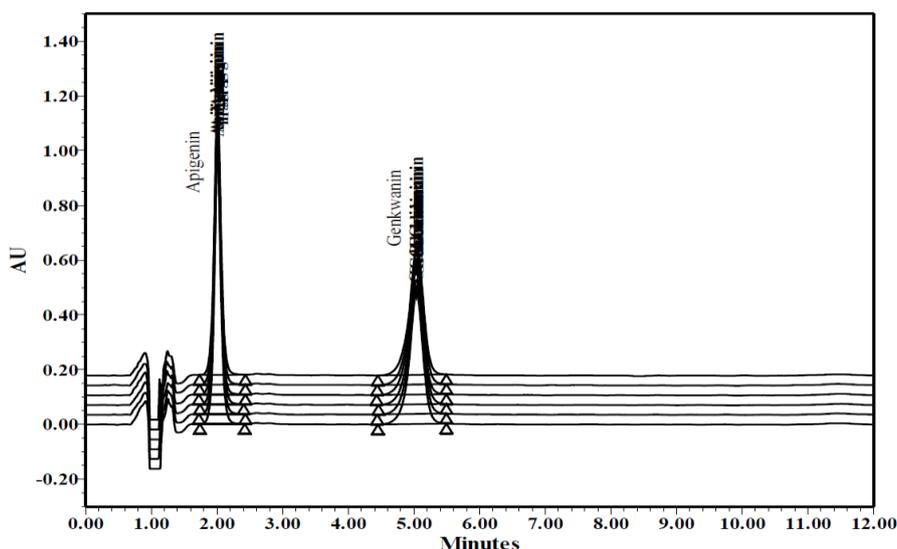
System precision

0.05mg/ml standard solution was prepared to calculate the precision for the developed method. The prepared solution was injected into injector (n=6) at same concentrations and same chromatographic conditions.

The chromatograms were recorded. The values are given in Table-1. %RSD for the values calculated is 0.10% for apigenin and 0.15% for Genkwanin. So, the developed method shows precision. The chromatograms are following fig 6.

Table 1: System precision data for Apigenin and Genkwanin

S.No.	Apigenin	Genkwanin
1	7294567	7203170
2	7283328	7176619
3	7275497	7200559
4	7287282	7191623
5	284725	7180677
6	7275660	7186862
Average area	7283510	7189918
Standard Deviation	7264	10615
% of RSD	0.10	0.15

**Fig .6: System precision %RSD Chromatogram of Apigenin and Genkwanin****Method Precision**

0.05mg/mL of six different standard solution were prepared to calculate the precision for the developed method. The six prepared solutions were injected into injector (n=1) at same concentrations and same chromatographic conditions. The chromatograms were

recorded. The values are given in Table-2. %RSD for the values calculated is 0.14% for apigenin and 0.19% for Genkwanin. So, the developed method shows precision. The chromatograms are following fig 7.

Table 2: Method precision data for Apigenin and Genkwanin

Preparation S.No.	Apigenin	Genkwanin
1	7257284	7172744
2	7238736	7155720
3	7247393	7163584
4	7267145	7161840
5	7259039	7149251
6	7251038	7132617
Average area	7253439	7155959
Standard Deviation	9925	13888
% of RSD	0.14	0.19

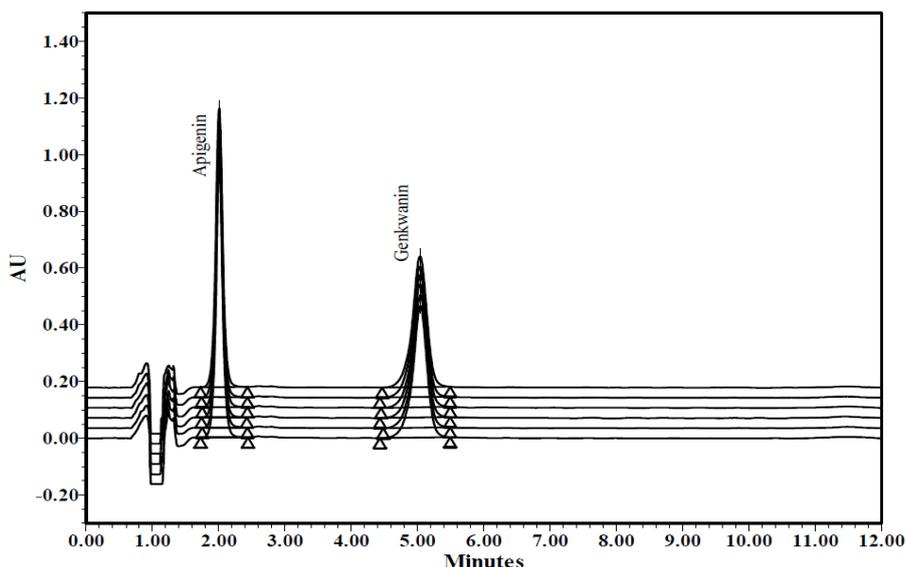


Fig. 7: Method precision% RSD Chromatogram of Apigenin and Genkwainin

Linearity

Regression analysis of the standard calibration graphs was used to determine the linearity of the developed method. The results obtained showed that the method is linear for the two flavones in the range of 12.25µg/mL to

75µg/mL for Apigenin and Genkwainin respectively with their coefficient of correlation (R^2) all approximately equal to 1 ($R^2 = 1$). The linearity graphs and results are presented in Fig.8 and Table 3.

Table 3: Linearity data for Apigenin and Genkwainin

Conc.(µg/ml)	Area of Apigenin	Area of Genkwainin
25	1850828	1828793
50	3442972	3368355
75	5458646	5378602
100	7259307	7168128
125	8987490	8874656
150	10703788	10581191
Cor. Coefficient(r^2)	0.999	0.999

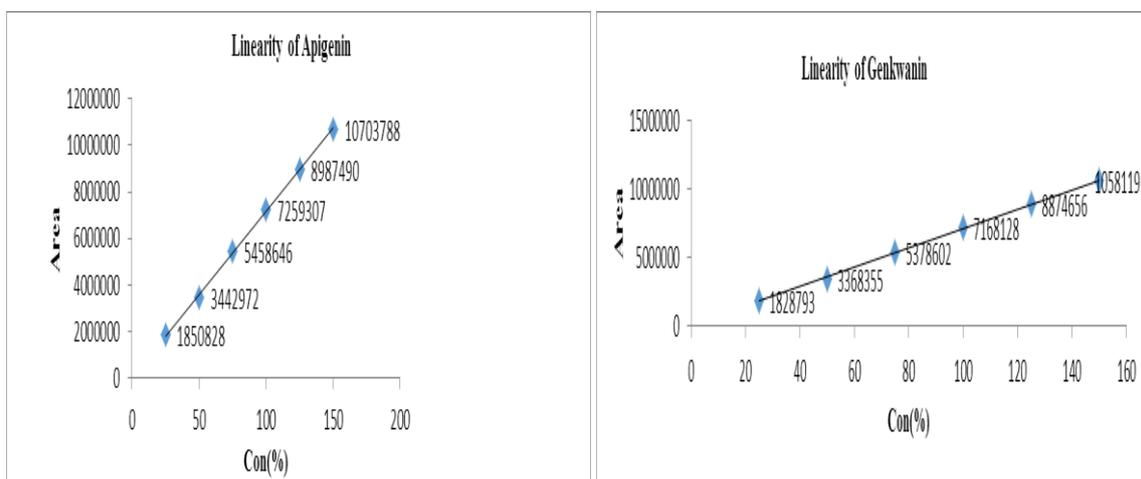


Fig.8: Linearity Graph for Apigenin and Genkwainin

Accuracy

The accuracy of method was determined by studying recovery at three different concentrations for two flavones, by replicate analysis (n = 3). Samples of known concentration (reference standard solutions) were analyzed and the measured values, from the respective

peak areas, were compared with the true values. The recovery was found to be within acceptable range ($100 \pm 2\%$) and the %RSD was also passed for all 9 determinations (NMT 2.0%). The results are summarized in Table 4.

Table 4: Accuracy data for Apigenin and Genkwainin

Accuracy% Level	%Recovery of Apigenin	%Recovery of Genkwainin
Accuracy-50% Run-1	99.34	99.85
Run-2	99.07	98.87
Run-3	99.54	98.76
Accuracy -100% Run-1	99.54	99.56
Run-2	98.81	99.27
Run-3	98.41	98.51
Accuracy -100% Run-1	99.59	99.13
Run-2	99.33	99.33
Run-3	99.37	99.75
Average for 9 %Rec. determinations	99.19	99.23
STDV for 9 %Rec.determinations	0.375	0.455
%RSD for 9 %Rec.determinations	0.38	0.46

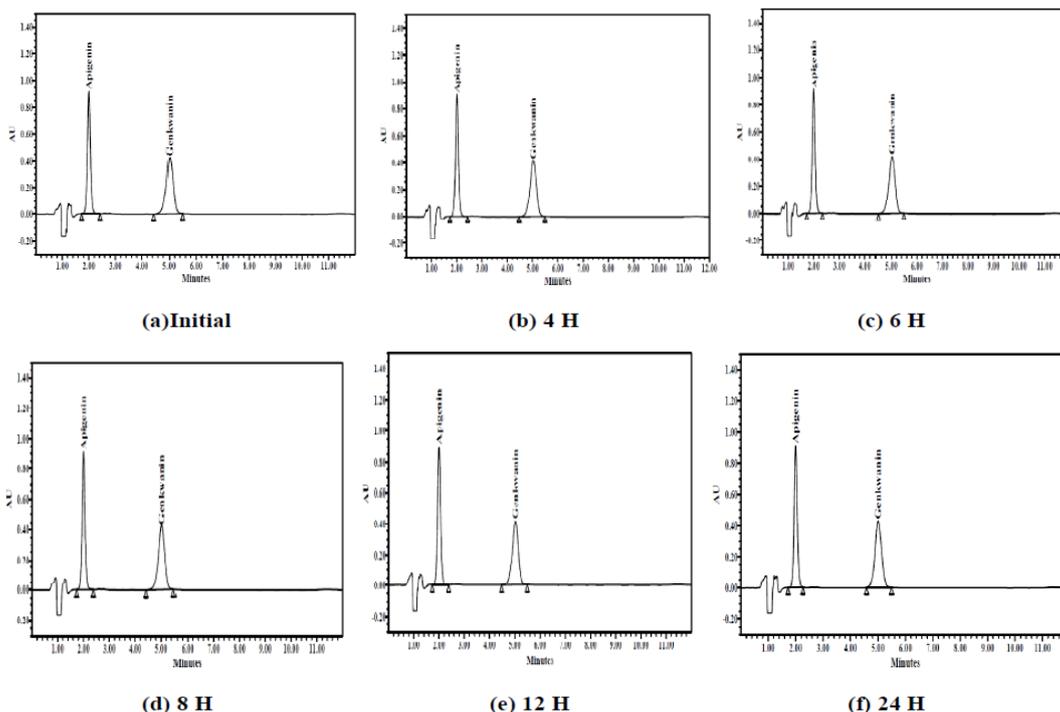
Solution stability

Standard and sample solutions stored in a capped volumetric flask on a lab bench under normal lighting conditions for Initial, 4 H,6 H,8 H,12 H and 24 H were injected(n=1) into the chromatograph. This results shown

to be stable with no significant change in progesterone concentration during this period. The % assay of each period should not differ by more than $\pm 0.5\%$ to the initial Assay value.The Chromatograms and data are smmarized in fig 9 and table 5.

Table 5: Solution stability data for Apigenin and Genkwainin

Flavones	Initial	4 hours	6 hours	8 hors	12 hours	24 hours
Apigenin	7335327	7325138	7295716	7283513	7277624	7247511
%Assay	99.33	99.25	99.54	99.63	99.62	99.30
Genkwainin	7218466	7208853	7193296	7156844	7156765	7121468
%Assay	99.51	99.58	99.36	99.52	99.42	99.19

**Fig. 9: Solution stability Graphs for Apigenin and Genkwainin****Ruggedness**

Ruggedness of the method was evaluated by performing the sample analysis in six replicates using different analyst on different days. System suitability of the standard solution was checked at each day and each

analyst and data was found to be within the acceptable range. The %RSD values of less than 2.0%. This indicate that the method adopted is rugged. The data of Ruggedness was shown in table 6.

Table 6: Ruggedness data for Apigenin and Genkwanin

Name of Flavone	Day-1			Day-2			Ana-1	Ana-2
	%RSD	%RSD	%RSD	%RSD	%RSD	%RSD	%RSD	
	Ana-1	Ana-2	Ana-1&2	Ana-1	Ana-2	Ana-1&2	Day-1&2	Day-1&2
Apigenin	0.31	0.29	0.29	0.67	0.74	0.68	0.54	0.57
Genkwanin	0.34	0.52	0.44	0.52	1.23	0.99	1.50	1.47

Robustness

Robustness of the method were investigated by varying the instrumental conditions such as the Mobile phase change (± 2.0 volumes), Column flow change (+

0.1ml/min), System suitability of the standard solution was checked at each variable condition and data was found to be within the acceptable range. (% RSD NMT 2.0). The data of Ruggedness was shown in table 7.

Table 7: Robustness data for Apigenin and Genkwanin

Parameter	Apigenin		Genkwanin	
	Avg. area (n=6)	%RSD	Avg.area (n=6)	%RSD
Flow rate(mL/min)	0.9	8051288	0.25	7983364
	1.0	7651288	0.26	7416697
	1.1	6560868	0.17	6494259
Mobile Phase change	53:47	7442056	0.91	7826760
	55:45	7651288	0.26	7416697
	57:43	7344535	0.32	7222138

Batch Analysis

Prepared about 0.05 mg/mL of Apigenin and Genkwanin standard solution and 0.05mg/mL of three different Batches of Apigenin and Genkwanin API sample

solution. The % assay of each batch should be within the limit (98.0% to 102%). The data are summarized in table 8.

Table: 8 Batch analysis data for Apigenin and Genkwanin

Name of the Flavone	STD avg.area (n=6)	Batch-I avg.area (n=2)	Batch-II avg. area (n=2)	Batch-III avg.area (n=2)
Apigenin	7463419	7361681	7368061	7593654
%Assay	---	99.25%	99.34%	99.60%
Genkwanin	7147785	7019127	7047374	7283484
%Assay	---	99.49 %	99.76%	99.73%

CONCLUSION

An accurate, simple, linear, specific and precise reverse phase HPLC assay method with PDA detection for the simultaneous assay quantification of Apigenin and Genkwanin has been developed and validated. In this method solid buffers are not used. So the column goes for the longer periods. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC-MS and GC-MS. These methods are complicated, costly and rather time consuming than a simple HPLC-PDA method. The assay was linear from 12.25 μ g/mL to 12.25 μ g/mL. In the % recovery is 100 \pm 2 and % RSD is NMT 2.0% it meets criteria according to ICH Guideline. Thus, the proposed HPLC assay method can be successfully applied for the routine quality control analysis of Apigenin and Genkwanin. And also useful for formulation analysis of Apigenin and Genkwanin.

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