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RAPID HPLC METHOD DEVOLOPMENT AND VALIDATION OF ESCULIN

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ABSTRACT

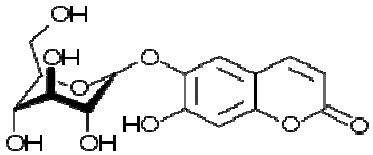
Esculin (6,7-dihydroxycoumarin-6-o-glucoside) is a coumarin derivative found in *Aesculus hippocastanum L* (Horse-chestnut). The present study was undertaken to develop and validate analytical method and to estimate the concentration of Esculin. Rutin was taken as internal standard. The Retention time of Esculin t_R = 5.58 min .The LLOQ of Esculin was found to be 0.025 µg/mL after injection of 20 µL of the calibration standard samples. The mean percent accuracy value for samples was 95.46 %. Correlation coefficient =0.99.

KEY WORDS: Esculin, HPLC, method development.

1.INTRODUCTION

Esculin (6,7-dihydroxycoumarin-6-o-glucoside) is a coumarin derivative found in *Aesculus hippocastanum* L. (Horse-chestnut). Their seeds have long been used to treat inflammatory and vascular problems. In Brazilian folk medicine, the tea prepared from the crushed seeds is used to protect against kidney stones and stomach pain. Esculin is known to be a 5- and 12-lipoxygenase inhibitor and to inhibit the production of leukotrienes and 5-hydroxyeicosatetraenoic acid through the lipoxygenase pathway(Kaneko, 2003). In 2007, Zhao used the dopamine-induced cytotoxicity model in human euroblastoma SH-SY5Y cells to demonstrate that esculin inhibited dopamine-induced caspase-3 cleavage and decreased cell death, overproduction of ROS, morphological changes of nuclei and damage to antioxidant enzymes (Zhao, 2007). Esculin scavenges hydroxyl radicals and inhibits lipid peroxidation in the rat liver (Kaneko, 2007) and displays anti-inflammatory activity in both zymosan and carrageenan induced paw edema in mice (Stefanova, 1995). The gastroprotective effect of esculin was also observed in rats by Martin (1991) in cold-restraint stress and pylorus ligation-induced ulcer models.

To our knowledge, no analytical method has been developed and validated to measure the concentration of esculin. We here in report the development and validation of a sensitive HPLC assay to quantify Esculin.



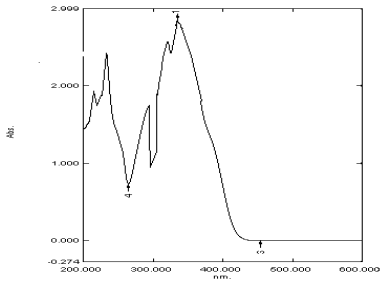


Fig-1. Absorption maximum of Esculin at concentration of 100 µg/ml.

2.MATERIALS AND METHODS

2.1. HPLC method development

2.1.1. Chemicals and reagents: Esculin was purchased from Yucca Enterprises (Mumbai). Methanol, Water and Glacial Acetic Acid were all of HPLC grade purchased from Finar chemicals (Ahmedabad).

2.1.2 HPLC instrument and chromatographic conditions: The Waters HPLC system consists of a UV-Vis detector 2487. The column and HPLC instrument was maintained at room temperature. The reverse phase chromatography was performed on analytical Hibar C18 column (150 mm x 2.0 mm, 5 µm) with an optimized mobile phase, Methanol:Water:Glacial Acetic Acid (30:70:0.1v/v). The flow rate was 1 ml/min and the injection volume was 20 µL. The UV detector was set at a single wavelength of 342 nm and AUFS at 1.000.

2.1.3. Stock solutions and standards: Primary stock solution of Esculin and Rutin (1 mg/ml) was prepared with mobile phase and it was diluted 100 times with mobile phase to obtain a working solution of 10µg/ml. This working solution of Esculin (10µg/ml) was diluted with mobile phase to give working solutions with concentrations of 0.01,

0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50µg/ml for the preparation of calibration and quality control (QC) samples. Esculin calibration standard samples were prepared with the concentration 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5µg/ml. For each sample of 500 µL, 100 µL of internal standard was added, and 20 µl of this solution was injected into HPLC for analysis. QC samples of Esculin at low (0.05 µg/ml), medium (0.5 µg/ml) and high (5 µg/ml) were freshly prepared to evaluate accuracy and precision of this HPLC method.

2.2 HPLC method validation

2.2.1. Specificity and selectivity: The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank mobile phase, sample with Esculin and internal standard Rutin.

2.2.2. Sensitivity and specificity: The lowest limit of quantification (LLOQ) was determined as the minimum concentration that could be accurately and precisely (± 5%) quantified. The lowest limit of detection (LLOD) was defined as the amount that could be detected with a signal-to-noise ratio of 4.

2.2.3 Linearity: Calibration curves of thirteen concentrations of Esculin ranging from 0.01 to 5 µg/ml were assayed. Blank mobile phase were analyzed to confirm the absence of interferences. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.99 or greater.

2.2.4 Precision and accuracy: In order to assess the intra- and inter-day precision and accuracy for the assay, Esculin QC samples at low, medium and high concentrations were prepared as described above. The intra-day precision of the assay was assessed by calculation the coefficient of variation (CV) for the analysis of QC samples in three replicates. And inter-day precision was determined by the analysis of QC samples on three consecutive days. Accuracy was calculated by comparing the averaged measurements and the nominal values and was expressed in percent. The criteria for acceptability of precision were that the relative standard deviation (RSD) for each concentration level should not exceed ± 15%. Similarly, for accuracy, the averaged value should be within ± 15% of nominal concentration except for the LLOQ, where the limit was ±20%.

3. RESULTS AND DISCUSSION

3.1 Method development: Esculin was scanned in UV-Vis range (200-600 nm) in Shimadzu UV-Visible spectrophotometer (UV-1800). The maximum absorbance was found to be at 342nm (Fig. 1) and hence this wavelength has been used for detection in HPLC. The mobile phase used for the assay achieved optimal separation of Esculin and I.S. Rutin without interference from the other components (Fig-3).

3.2. HPLC method validation

3.2.1. Specificity and selectivity: Fig.4 represents chromatograms of Esculin and I.S. Rutin and fig.3 blank mobile phase. No interference of endogenous peaks with Esculin or Rutin at their respective retention times (Esculin t_R = 5.58 min, Rutin t_R= 3.89 min) in samples was observed.

3.2.2. Sensitivity: The LLOQ of Esculin was found to be 0.025 µg/mL after injection of 20 µL of the calibration standard samples. The mean percent accuracy value for samples was found to be 93.073 and precision coefficient of variation was below 2 % at the LLOQ.

3.2.3 Linearity of calibration curve: The calibration curves for Esculin were linear over the concentration of 0.005-5 µg/mL. The mean (± SD) regression equation from three replicate calibration curves on different days was y = (0.1046 ± 0.002547)x + (-0.00004842 ± 0.02114) with correlation coefficient r^2= 0.994 ± 0.025.

3.2.4 Precision and accuracy: Table-1 shows the precision and accuracy in the range of 0.25-25 µg/ml. and the results indicate that the present assay has very good accuracy and precision.

4. CONCLUSION

A simple, sensitive, accurate and precise HPLC method was developed and validated for the first time to quantify Esculin. This method can be applied to quantify the esculin in various biological samples also.

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Table 1: Accuracy and precession during intraday, interday analysis

	Conc. (µg/ml)	Trail 1	Trail 2	Trail 3	Mean	SD	Accuracy %	cv%
Intraday	0.25	0.235	0.234	0.229	0.233	0.003	93.073	1.408
	2.5	2.422	2.408	2.357	2.396	0.034	95.835	1.417
	25	24.210	23.845	24.596	24.217	0.375	96.868	1.550
Interday	0.25	0.229	0.242	0.237	0.236	0.007	94.400	2.778
	2.5	2.390	2.417	2.374	2.394	0.022	95.747	0.907
	25	24.750	24.143	23.941	24.278	0.421	97.112	1.734

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Fig. 2: Standard graph of Esculin

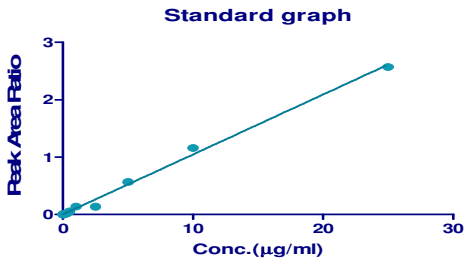


Fig. 3: Chromatogram of blank mobile phase

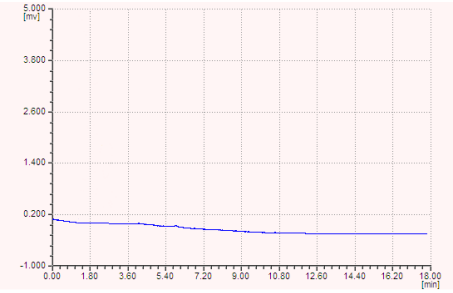


Fig. 4: Chromatogram of Esculin and Rutin in mobile phase

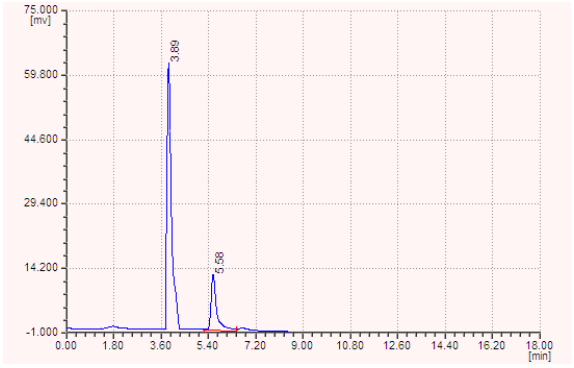
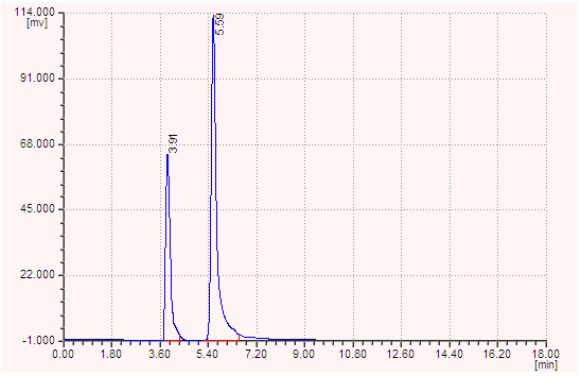


Fig. 5: Chromatogram of esculin and rutin



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