*Section A-Research paper*

# SIMULTANEOUS ESTIMATION OF INULIN AND ESCULETIN IN AN AYURVEDIC FORMULATION USING RP-HPLC AFTER DERIVATIZING THE INULIN

**Gouri.V.Kanabaragi, Alisha Syeda, Muddukrishna B S, Gundawar Ravi, Niharika Gaddam, S. G. Vasantharaju***

Department of Pharmaceutical Quality Assurance, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal-576104, Udupi, Karnataka

**Corresponding Author*:**
Dr. SG Vasantharaju
Department of Pharmaceutical Quality Assurance,
Manipal College of Pharmaceutical Sciences,
Manipal Academy of Higher Education,
Manipal-576104, Udupi, Karnataka.
E-mail: sgvasanth65@gmail.com
Mobile No.: +919880106983

## ABSTRACT

A simple, sensitive, precise, and accurate RP-HPLC assay method was developed for simultaneous estimation of Inulin and Esculetin in a formulation containing Chicory. Inulin is a polysaccharide and due to a lack of chromophore, it is to be derivatized before analyzing. Hence was opted to derivatized Inulin using Seliwinoff's reagent. The chromatographic separation was achieved using $C_{18}$ Phenomenex Hyperclone BDS column (250×4.6mm, 5µ). The mobile phase containing a mixture of 10 mM of phosphate buffer (pH 3.0) and acetonitrile (35: 65 v/v) at a flow rate of 1ml/min was used and the response was measured at 364nm (isosbestic point) using a PDA detector. The retention time of Esculetin and Inulin is found to be 3.7 and 7.1 minutes respectively with a resolution of 8.5. The method was linear over the concentration range of 0.05-0.8 µg/ml for Esculetin and 0.4-3 µg/ml for derivatized Inulin with a correlation constant of $\geq 0.999$. The method was found to be precise, and robust within the acceptable limits. The LOD and LOQ were to be 0.006308 µg/ml and 0.01912 µg/ml for Esculetin and 0.06131 µg/ml and 0.1857 µg/ml for derivatized Inulin which indicates the sensitivity of the method. Accuracy was determined at 80%, 100%, and 120% and the results obtained were within limits. The relative standard deviation was not more than 2.0%. The % recovery of Esculetin and Inulin in herbal preparation were found to be 0.2% and 58% respectively.

**Keywords:** Inulin, Esculetin, Derivatization, HPLC, Validation.

## INTRODUCTION

Polysaccharides, coumarins, flavonoids, sesquiterpene lactones (lactucin and lactucopicrin), tannins, and other phytochemicals are found in chicory root. Inulin is a fructose polymer with a -(2-1)-glycosidic bond that makes up to 68 percent of the total components in fresh chicory roots.

Esculetin belongs to the class of coumarins, featuring two hydroxyl groups at carbons 6 and 7 that serve as candidates for O-methylation or O-glycosylation. Esculetin has powerful antioxidant and photoprotective properties.

Inulin is a kind of dietary fiber that has a strong prebiotic impact and immunostimulant effects. Their application in the production of functional food and low caloric nutrition formulas constantly increases nowadays, hence Inulin content in foods and adulteration needs to be regulated. Hence Reverse – Phase High Performance Liquid Chromatography (HPLC) is the most preferred method.

Esculetin has a variety of biological properties, including the suppression of xanthine oxidase activity, anti-oxidant activity, anticancer action, and antioxidant activity. Inhibiting the development of human breast cancer cells. As a result, it is critical to build a basic and efficient approach for quantitative analysis of these components.

## EXPERIMENTAL

### Material and Methods

Instrument

Shimadzu HPLC controlled by LC solution software, Shimadzu LC 20 AD equipped with, Pump LC-20 AD, Autoinjector, SIL-20 AC HT, Column oven: CTO-10ASVP, Photodiode array detector: SPD M-10A VP, System controller: SCL-10A VP were used for the study.

Materials

Inulin from Chicory and Esculetin were purchased from Sigma Aldrich, Bangalore. All chemicals and reagents used were of analytical grade. Ultra-purified water was prepared the in lab using Merck Water Purification system throughout the work. Chicory capsules were purchased from XOVAK Pharma, India.

### General Procedure

Derivatization of marker

Esculetin is a simple coumarin that can be analyzed using $C_{18}$ column. Hence derivatization is not required. Since Inulin is a high molecular weight polysaccharide special column are required for its analysis. Direct analysis of the molecule on $C_{18}$ is not possible. Trials were also carried out on $C_8$ column as well but it was not been detected.

Preparation of Seliwinoff's reagent

50mg of Resorcinol was weighed and diluted in 33 ml of 0.1N HCl and the volume was made up with water.

Derivatization Procedure

Pipetted out from the working stock and prepared various concentrations and made up the volume with Seliwinoff reagent. Heated these solutions in a boiling water bath for 30 secs. Reddish pink color will appear upon completion of the reaction.

Determination of $\lambda_{max}$

Standards of Esculetin and derivatized Inulin were analyzed under Ultraviolet (UV) spectrophotometry. Suitable wavelength was selected for analysis by determining the isosbestic point. Esculetin and derivatized Inulin of concentration 10µg/ml each were taken and UV spectrum was recorded from 200- 800nm. The spectrum was overlaid and the isosbestic point was found to be 369nm.

HPLC Method validation

Specificity

Blank i.e. mobile phase and a standard solution containing Esculetin and derivatized Inulin were injected twice into the HPLC system and the chromatograms were recorded.

System suitability

1ml was withdrawn from Esculetin, Inulin standard stock solution. Made up the volume with diluent and injected into HPLC.

Linearity

A series of standard solutions for the mixtures of Esculetin and derivatized Inulin was prepared

i.e., 0.05, 0.1, 0.2, 0.4, 0.8 µg/ml for Esculetin and 0.4, 0.8, 1, 2, 3 µg/ml for derivatized Inulin

Range

The range is the interval between the upper and lower levels of the analyte that have been determined with precision, accuracy, and linearity using the method as written. The range of an analytical procedure was found to be 0.05-0.8 µg/ml for Esculetin and 0.4-3 µg/ml for derivatized Inulin.

LOD & LOQ

The LOD and LOQ of the developed method were calculated based on the standard deviation of the response and the slope

LOD = 3.3 σ/S and LOQ = 10 σ/S

Accuracy

The accuracy of the method was performed based on the recovery of known amounts of analyte by standard spiking method. Accuracy was performed at 3 levels of 80%, 100% and 120% of standard concentration. 0.16µg/ml, 0.2 µg/ml, 0.24µg/ml for Esculetin and 0.8 µg/ml, 1 µg/ml, 1.2 µg/ml for derivatized Inulin.

Precision

Repeatability or intra- day precision, Intermediate or inter-day precision were performed. 6 injections of standard mixture solution were injected and the retention time and peak area of the drugs were analyzed. Mean, SD and RSD were calculated.

Robustness

Parameters are varied over a known range and their effect on the results are determined. These include: Flow rate, Column oven temperature and Mobile phase composition

## RESULTS AND DISCUSSION

Method validation was performed and validated as per ICH guidelines for the following parameters:

System suitability

1ml was withdrawn from Esculetin and derivatized Inulin standard stock solution. Make up the volume with diluent and inject them into HPLC. Results are displayed in Table 1.

Specificity

Blank i.e. mobile phase and a standard solution containing Esculetin and derivatized Inulin were injected twice into the HPLC system and the chromatograms were recorded. The peak purity of the standard solution of Esculetin and Inulin was generated with the help of Shimadzu system equipped with a PDA detector.

Linearity and Range

A series of standard solutions for the mixtures of Esculetin and derivatized Inulin were prepared as stated below.

0.05, 0.1, 0.2, 0.4, 0.8 µg/ml - Esculetin

0.4, 0.8, 1, 2, 3 µg/ml – derivatized Inulin

Each standard concentration was injected in triplicate and the chromatograms were recorded for all the linearity standards under optimized conditions. The calibration curve was prepared by plotting the mean peak area against the concentration standard. The slope, Y-intercept correlation coefficient for Esculetin, and derivatized Inulin were reported. Results are displayed in Table 2 and Table 3.

Range

The range is the interval between the upper and lower levels of the analyte which have to be determined with precision, accuracy, and linearity using the method as written. The range of an analytical procedure was found to be 0.05-0.8 µg/ml for Esculetin and 0.4-3 µg/ml for derivatized Inulin.

LOD and LOQ

The LOD and LOQ were determined by the linearity studies. To compute the LOD and LOQ, the residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines were considered. The results of the same were represented in Table No 7.

Accuracy

Preparation of sample

Accuracy of method was performed based on the recovery of known amounts of analyte by standard spiking method. A known amount of standard drug was spiked to the pre-analysed standard samples and the % recovery of the drug was determined. Accuracy was performed at three levels of 80%, 100% and 120% of the standard concentration. The three concentration levels were 0.16 µg/ml, 0.2 µg/ml, 0.24µg/ml for Esculetin and 0.8 µg/ml, 1 µg/ml, 1.2 µg/ml for Inulin. The percentage recovery was calculated from the peak area of each drug. Results are displayed in Table 4 and Table 5.

Precision

Repeatability or intra-day precision

6 injections of a standard mixture of 1 µg/ml of derivatized Inulin and 0.2 µg/ml of Esculetin were injected into the system and the retention time and peak area of both the drugs were analyzed on the different days. Mean, SD, and RSD were calculated. Results are displayed in Table 6.

Limit of detection and limit of quantification:

The LOD and LOQ of the developed method were evaluated based on the standard deviation and the slope. The calculated LOD and LOQ values of the described method are tabulated as follows:

Robustness

Robustness was evaluated by changing the various HPLC parameters.

APPLICATION TO FORMULATION

Average weight of 10 capsules was taken and an equivalent amount of powder was weighed. Weighed 0.4g of encapsulated chicory powder in 100ml volumetric flask and make up the volume with methanol and water separately. Sonicate the solutions for 30 minutes with frequent shaking and keep for maceration for 24 hours. The extract was filtered and 0.5 ml of the filtrate of each was mixed with 50:50 methanol in water solution.0.1 ml of the above solution was pipetted out and made up the volume with Seliwinoff reagent and treated. The above solution was then injected into HPLC.

%Assay = (sample abs/std abs) X (std conc/ test concentration) x 100

**ACKNOWLEDGMENT**

The authors are thankful to the Manipal College of Pharmaceutical Sciences for providing technical support during the research.

## Conclusion

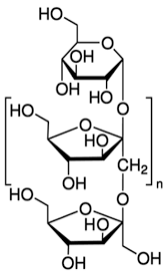
Inulin being a polysaccharide special column were required for its estimation. Hence, we opted to derivatize Inulin by simple way to estimated it in chicory. A simple, sensitive, precise, accurate assay method was developed using RP-HPLC for simultaneous estimation of Esculetin and Inulin. The method was validated and all it were found to be within the acceptance criteria. The above method was applied for the formulation assay was determined for Esculetin and Inulin and it was found to be 0.2% and 58% respectively.

## REFERENCES

1. Shoaib M, Shehzad A, Omar M, Rakha A, Raza H, Sharif HR, et al. Inulin: Properties, health benefits, and food applications, Carbohydrate Polymers 2016;147:444–54.
2. USP 29-NF 24 The United States Pharmacopoeia and The National Formulary: The Official Compendia of Standards 29/24. Rockville, MD: The United States Pharmacopeial Convention.
3. Barclay T, Ginic-Markovic M, Cooper P, Petrovsky N. Inulin-a versatile polysaccharide with multiple pharmaceutical and food chemical uses, Journal of Excipients and Food Chemicals. 2016;1(3):1132.
4. Abed SM, Ali AH, Noman A, Niazi S, Ammar AF, Bakry AM. Inulin as prebiotics and its applications in food industry and human health; a review, International Journal of Agriculture Innovations and Research, 2016;5(1):88-97.
5. Esculetin [Internet]. Pubchem.ncbi.nlm.nih.gov.
6. Mcrary, W. L., & Slattery, M. C. (n.d.). THE COLORIMETRIC DETERMINATION OF FRUCTOSAN IN PLANT MATERIAL
7. Sánchez-Viesca F, Gómez R. Reactivities involved in the Seliwanoff reaction. Modern Chemistry. 2018;6(1):1-5.
8. http://myweb.liu.edu/~nmatsuna/che122/exp20.pdf

9. ICH Harmonised Tripartite Guideline. Validation of analytical procedures: text and methodology Q2 (R1). InInternational conference on harmonization of technical requirements for registration of pharmaceuticals for human use 2005. Switzerland: Geneva.

Figures



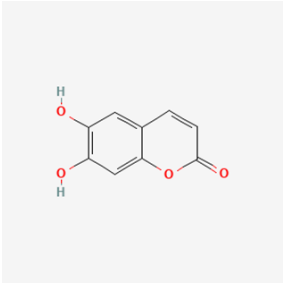




Fig 1 - Structure of Inulin

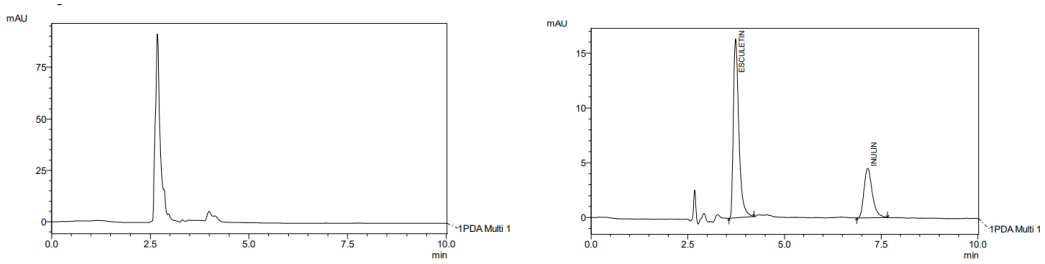



Fig 3 - Chromatogram of Blank

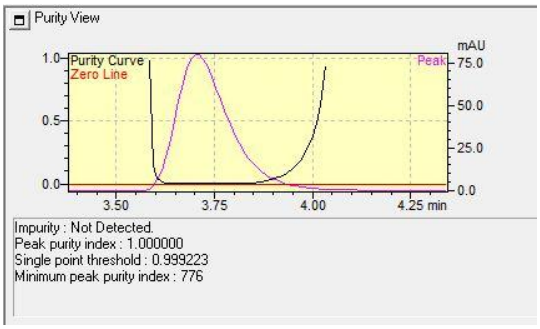



Fig 5 - Peak purity index of Esculetin

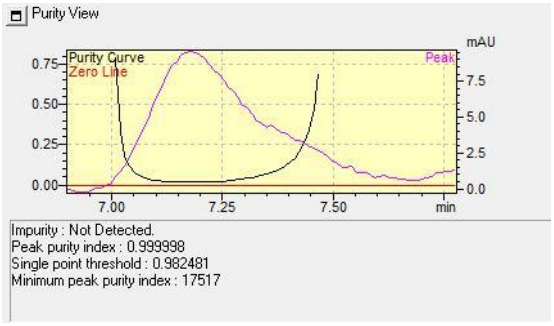



Fig 6 - Peak purity index of derivatized Inulin

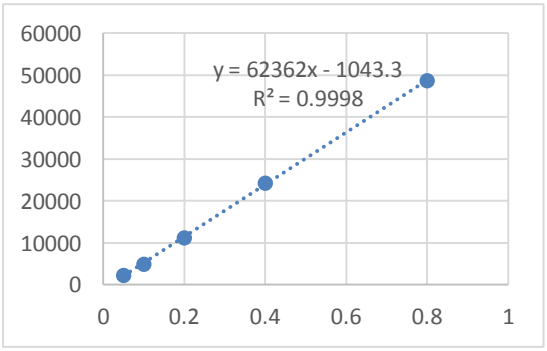



Fig 7- Linearity plot of Esculetin

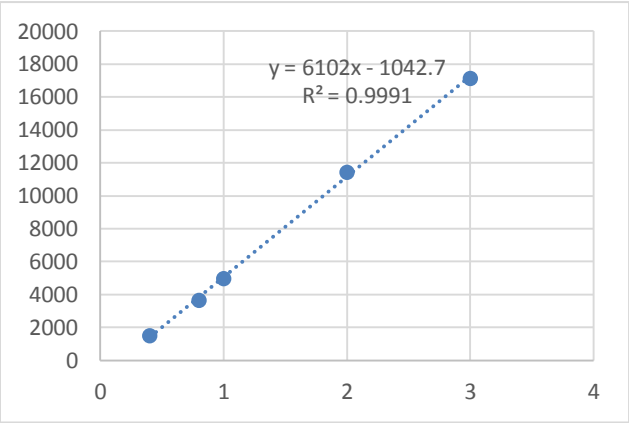



Fig 8 - Linearity plot of derivatized Inulin

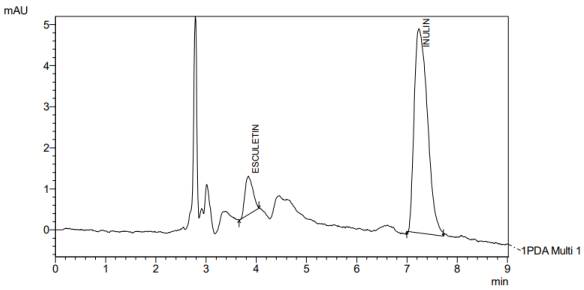



Fig 9-Chromatogram of formulation containing chicory powder

Tables

Table 1 – Results of System Suitability

| Parameter | Observation | | Acceptance criteria |
|---|---|---|---|
| | Esculetin | Derivatized Inulin | |
| %RSD of retention time of 6 injections | 0.14828 | 0.111841 | NMT 2% |
| %RSD of area of 6 injections | 0.29406 | 1.294399 | NMT 2% |
| Plate count | 3666.429 | 7340.465 | More than 2000 |
| Tailing factor | 1.795 | 1.606 | NMT 2.0 |
| Resolution | 8.5 | | NLT 2 |

Table 2- Results of linearity studies of Esculetin

| Sr. No. | Concentration(µg/ml) | Area | | | Average |
|---|---|---|---|---|---|
| 1 | 0.05 | 2293 | 2281 | 2267 | 2280.333 |
| 2 | 0.1 | 4909 | 4937 | 4950 | 4932 |

| | | | | | |
|---|---|---|---|---|---|
| 3 | 0.2 | 11202 | 11239 | 11228 | 11223 |
| 4 | 0.4 | 24293 | 24271 | 24282 | 24282 |
| 5 | 0.8 | 48702 | 48749 | 48732 | 48727.67 |
| Regression Equation | | y = 62362x - 1043.3 | | | |
| Correlation Coefficient | | $R^2$ = 0.9998 | | | |
| Slope | | 62362 | | | |
| y-Intercept | | 1043.3 | | | |

Table 3- Results of linearity studies of derivatized Inulin

| Sr. No. | Concentration(µg/ml) | Area | | | Average |
|---|---|---|---|---|---|
| 1 | 0.4 | 1505 | 1520 | 1500 | 1508.333 |
| 2 | 0.8 | 3655 | 3698 | 3621 | 3658 |
| 3 | 1 | 4986 | 4990 | 4956 | 4977.333 |
| 4 | 2 | 11438 | 11442 | 11430 | 11436.67 |
| 5 | 3 | 17157 | 17138 | 17127 | 17140.67 |
| Regression Equation | | y = 6102x - 1042.7 | | | |
| Correlation Coefficient | | $R^2$ = 0.9991 | | | |
| Slope | | 6102 | | | |
| y-Intercept | | 1042.7 | | | |

Table 4-Recovery analysis of Esculetin

| | | Sample | Amount added | | Total amount | Peak Area | Amount Found | Mean amount | Amount recovered | %Recovery |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Test | Standard | | | | | | |
| Esculetin | 80 | A | 0.2 | 0.16 | 0.36 | 21163 | 0.356 | | | |
| | | B | 0.2 | 0.16 | 0.36 | 21119 | 0.355 | 0.355 | 0.158 | 98.826 |
| | | C | 0.2 | 0.16 | 0.36 | 21149 | 0.355 | | | |
| | 100 | A | 0.2 | 0.2 | 0.4 | 23349 | 0.391 | | | |
| | | B | 0.2 | 0.2 | 0.4 | 23018 | 0.385 | 0.388 | 0.194 | 97.060 |
| | | C | 0.2 | 0.2 | 0.4 | 23138 | 0.387 | | | |
| | 120 | A | 0.2 | 0.24 | 0.44 | 26041 | 0.434 | | | |
| | | B | 0.2 | 0.24 | 0.44 | 26039 | 0.434 | 0.433 | 0.236 | 98.713 |
| | | C | 0.2 | 0.24 | 0.44 | 26049 | 0.434 | | | |

Table 5 -Recovery analysis of Derivatized Inulin

| | | Sample | Amount added | | Total amount | Peak Area | Amount Found | Mean amount | Amount recovered | %Recovery |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Test | Standard | | | | | | |
| Inulin | 80 | A | 1 | 0.8 | 1.8 | 9803 | 1.777 | | | |
| | | B | 1 | 0.8 | 1.8 | 9748 | 1.768 | 1.777 | 0.789 | 98.747 |
| | | C | 1 | 0.8 | 1.8 | 9859 | 1.786 | | | |
| | 100 | A | 1 | 1 | 2 | 11072 | 1.985 | | | |
| | | B | 1 | 1 | 2 | 11034 | 1.979 | 1.983 | 0.991 | 99.156 |
| | | C | 1 | 1 | 2 | 11069 | 1.984 | | | |
| | 120 | A | 1 | 1.2 | 2.2 | 15665 | 2.738 | | | |
| | | B | 1 | 1.2 | 2.2 | 15508 | 2.712 | 2.715 | 2.262 | 100.184 |
| | | C | 1 | 1.2 | 2.2 | 15403 | 2.695 | | | |

Table 6 -Precision results

| Precision | | Intra-day | | Inter-day | | | |
|---|---|---|---|---|---|---|---|
| | | RT | Area | RT | Area | RSD ≤ 2% | Pass |
| | Inulin | 0.111 | 1.29 | 0.18 | 1.32 | | |
| | Esculetin | 0.148 | 0.29 | 0.11 | 0.33 | | |

Table 7 -LOD & LOQ

| | Esculetin µg/ml | derivatized Inulin µg/ml |
|---|---|---|
| Limit of Detection | 0.006308 | 0.06131 |
| Limit of Quantification | 0.01912 | 0.1857 |

Table 8 -Robustness results

| | | | |
|---|---|---|---|
| Robustness | Change in flowrate | | |
| | Esculetin | | |
| | 0.9 ml/min | 1ml/min | 1.1 ml/min |
| | 0.39524 | 0.1668 | 0.35976 |
| | Inulin | | |
| | 0.9 ml/min | 1ml/min | 1.1 ml/min |
| | 1.443 | 0.54375 | 1.27529 |
| | Change in Column oven temperature | | |
| | Esculetin | | |
| | 15 $^0$C | 20 $^0$C | 25 $^0$C |
| | 0.42992 | 0.1668 | 0.32964 |
| | Inulin | | |
| | 15 $^0$C | 20 $^0$C | 25 $^0$C |
| | 1.20528 | 0.54375 | 0.2033 |
| | Change in mobile phase composition | | |
| | Esculetin | | |
| | 37:63 | 35:65 | 33:67 |
| | 0.429924 | 0.166797 | 0.329635 |
| | Inulin | | |
| | 37:63 | 35:65 | 33:67 |
| | 1.11467 | 0.54375 | 1.49781 |

Table No. 9- % Recovery of Inulin in the formulation

| Sr. No. | % Recovery | |
|---|---|---|
| | Esculetin | Inulin |
| 1 | 0.2 | 58.03 |
| 2 | 0.203 | 58.15 |
| 3 | 0.21 | 58.09 |
| Average | 0.204333 | 58.09 |
| SD± | 0.005132 | 0.06 |