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Determination of *in vitro* Produced Cichoriin from Callus Culture of *Cichorium intybus* L.

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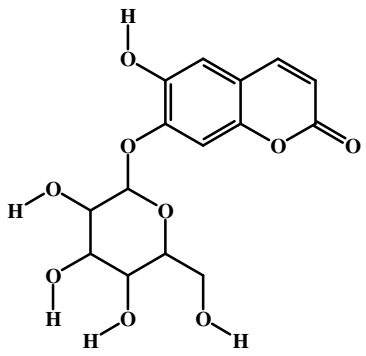
Reversed-phase high performance liquid chromatography (HPLC) has been developed for determination of *in vitro* produced Cichoriin from callus culture of *Cichorium intybus* L. Cichoriin was obtained from exhaustive methanolic extract of lyophillized callus tissue. Extract was chromatographed with ethyl acetate as mobile phase on a silica gel column. Ethyl acetate elution was followed by preparative thin layer chromatography. The extract was also processed by reversed-phase HPLC using C_{18} column. The proposed method was validated in accordance with the International Conference on Harmonization (ICH) Q2 (R1) validation guidelines by determining its selectivity, linearity, accuracy and precision. Based on the results, method is an effective choice to analyze this molecule.

Key Words: HPLC, ICH, Cichoriin, TLC.

INTRODUCTION

Plant derived natural products enjoys today a respectable position. Cichoriin ($C_{15}H_{16}O_9$) is a polyphenolic coumarin glycoside. The IUPAC name of cichoriin is 6-hydroxy-7-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-chromen-2-one. The chemical name of cichoriin is 7(β-D-glucopyranosyloxy)-6-hydroxy-2*H*-1-benzo-pyran-2-one (m.w. 340 g/mol). It is isomeric with esculin. It is crystalline in nature like needle with two molecules of water (m.p. 213-215 ºC).



Cichoriin is obtained as secondary metabolite from many genra and families of plants like asteraceae, compositae, solanaceae and oleacece. For example cichoriin have been found in extract of flowers of *Cichorium intybus* L. and leaves of *Nicotiana tabacum* L. Ito *et al.*[2] reported on component analysis of glycoside fraction of flue cured tobacco leaves. In this method, HPLC analysis of highly polar glycoside fraction yields cichoriin as polar glycoside. The proposed reversed HPLC method reports isocratic mode with mobile phase acetonitrile:water (50:50). Here cichoriin retent on 4.46 min with 20 min run time. This method solves real life problem with samples for example a fraction of a plant extract or the plant extract itself, because there will be many other compounds which may have comparable polarities, this will lead to peaks overlapping. In that case the intermediate isocratic method (20 min), described in this work, will be suitable for detection or comparison or separation of the cichoriin and its corresponding compounds. All parameters of method validation were assessed according to ICH Q2 (R1) guidelines[3].

EXPERIMENTAL

Procurement of plant material: The plants were obtained from field grown plants. Cotyledon, leaf, hypocotyl and root are used as explants for regeneration. The explants were washed in running tap water for 0.5 h. Then they were washed in an agitated solution of liquid detergent 2 % (v/v) for 2 min and rinsed with distilled water three times. Surface sterilization was also performed by immersion of the explants in 70 % (v/v) aqueous ethanol for 1 min followed by 0.1 % (w/v) mercuric chloride for five minutes.

Acetic acid (AR) was purchased from Qualligens chemicals Limited. Triethylamine (TEA) of analytical grade was purchased from Merck Limited. Potassium dihydrogen

phosphate (extra pure) was purchased from Fluka Limited. All solvents were HPLC grade and purchased from Qualligens chemicals Limited. HPLC Water of Fisher-scientific was used. 1.0 mL of TEA (peak modifier) was taken and prepared 0.1 % solution in water.

Extraction and isolation of cichoriin: Cichoriin was obtained from exhaustive methanolic extract of lyophillized callus tissue. Then extract was concentrated and aqueous residue was treated with chloroform[4]. Extract was chromatographed with ethyl acetate as mobile phase on a silica gel column. Ethyl acetate elution was followed by preparative thin layer chromatography (TLC). The extract was also processed by reversed-phase HPLC using C_{18} column.

Instrumentation and method: A CECIL high performance liquid chromatography (model 4200) was used with a UV-VIS detector (model 4201), a quaternary pump (model 4100), a manual, 20 mL syringe, degasser and column oven. Powerstream® software was used. An LUNA® C_{18} analytical column (25 cm × 4.6 mm, 5 µm) was used. All chromatographic runs were carried out in isocratic mode with a flow rate of 1.0 mL/min. The mobile phase consisted of eluent A (Fisher-scientific HPLC water) and eluent B (acetonitrile) in 50:50 ratios. The detector wavelength was set at 230 nm. The injection volume was 20 mL and the column and samples were kept at ambient temperature. Under these conditions, the retention time for cichoriin was found to be 4.46 min. The total run time for analysis is 20 min.

Characterization of cichoriin: Characterization of cichoriin was carried out using various spectral studies. Spectral studies of isolated cichoriin and authentic cichoriin were compared and found to be identical. IR data of authentic and isolated cichoriin was compared and obtained λ_{max} in cm^{-1} 3295, 2934, 1595, 1452, 1125.

Preparation of standard solution: Stock solution of cichoriin was prepared in acetonitrile and Fisher-scientific HPLC water (50:50) at a concentration of 100 µg/mL. The working standard solution was prepared to adjust a final concentration of cichoriin by acetonitrile and HPLC water (50:50). The final solutions were filtered by nylon membrane filters 0.45 µm (Milipore®).

Preparation of sample solution: Isolated cichoriin was diluted to volume with acetonitrile and HPLC water (50:50). The final solutions were filtered by nylon membrane filters 0.45 µm (Milipore).

Calibration curve: Stock solutions were prepared in diluent (water 50 %-acetonitrile 50 %) at 100 µg/mL concentration. The calibration curves (n = 5) constructed, for cichoriin was linear over the concentration range of 100-20 µg/mL. Peak areas (y) of each compound were plotted *versus* each compound concentration (x) and linear regression analysis was performed on the resultant curve. The linearity for the relationship between peak area and concentration was demonstrated by a correlation coefficient (r^2) greater than 0.9999.

Recovery, precision and accuracy: Recovery is expressed as the amount of analyte found as a percentage to the theoretical amount thought to be present in the medium. The absolute recoveries of cichoriin from analytical solution were evaluated in three different concentrations with triplicate analysis. The spiked samples were prepared in triplicate at three levels over a range of 50-150 % of the target concentration. The mean recovery should be between 98-102 %. The RSD % should be less than 2 %. Precision and accuracy were evaluated by calculating the percentage of relative standard deviation (RSD %).

RESULTS AND DISCUSSION

HPLC assay development: Cichoriin was found to exhibit four UV absorption maxima (λ_{max}) at 215, 230, 290 and 350 nm. Cichoriin has higher intensity at 230 nm so this wavelength is selected as absorption maxima. HPLC columns are typically selected on the basis of hydrophilic and hydrophobic properties of the analyte of interest. Cichoriin is slightly hydrophilic compound. It is separated from reversed-phase columns with mobile phases containing higher organic content. Typical reversed-phase columns such as Kromacil® C_{18} and Luna® C_{18} are therefore used for these separations as a result from these non-polar columns. Cichoriin are eluted faster. As part of the assay development process, several columns were tested including typical reversed-phase columns, as well as different mobile phases.

The best result was obtained with the Luna® C_{18} analytical column (25 cm × 4.6 mm, 5 µm) water and acetonitrile in 50:50 ratios. Convenient retention time with excellent peak shape and good reproducibility was obtained when compared to the other analytical columns that were explored. The retention time for cichoriin in isocratic elution was 4.46 min. The parameters for the proposed HPLC method was given in Table-1.

TABLE-1
PARAMETERS OF THE PROPOSED HPLC METHOD

Stationary phase	LUNA® C_{18} (250 × 4.6) mm
Mobile phase A	Water (HPLC grade)
Mobile phase B	Acetonitrile (HPLC grade)
Elution	Isocratic run
Retention time	4.46 min
Run time	20 min
Detection	230 nm
Injection volume	20 µL
Column temperature	Ambient

Assay validation: Several criteria associated with assay validation were undertaken according to ICH guidelines[3] so as to define the method's system suitability testing, selectivity, linearity, precision, accuracy and recovery from analytical solutions.

System suitability testing: System suitability testing of the assay was investigated by injecting six replicates of the standard. The mean, standard deviation and RSD % for each set of data was calculated. The RSD % is less than 1 %.

Selectivity: A good analytical method should be able to measure the analyte accurately in the presence of suspected interferences such as blank, diluents and degradation products. Fig. 1 shows chromatographic base-line separation of cichoriin. Peak 5 in Fig. 2 demonstrates that no interferences were found at the retention time of pure cichoriin due to other compounds of comparable polarities.

Linearity: The correlation coefficient (r^2) for standard calibration curves was greater than 0.999 and the RSD % for each concentration studied was less than 2 %.

Precision and accuracy (recovery): The precision and

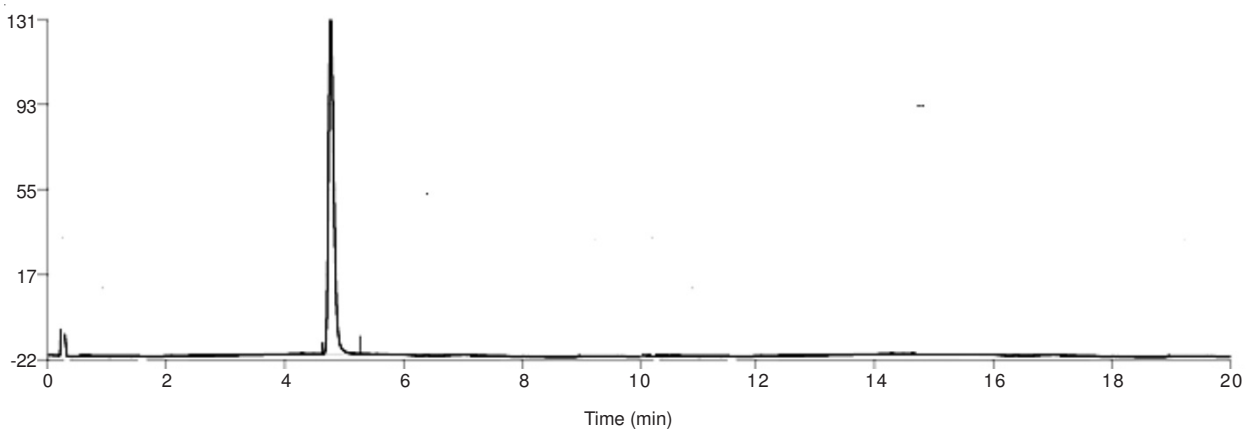


Fig. 3. Schematic chromatogram of pure cichoriin

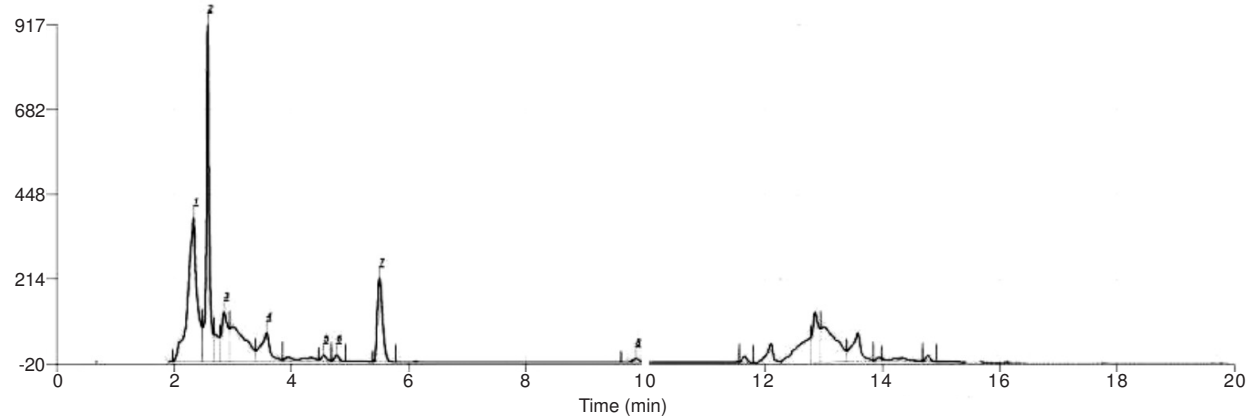


Fig. 4. Schematic chromatogram of cichoriin from callus extract of *Cichorium intybus*

accuracy parameters were determined from freshly made quality control standards in three different concentrations (50-150 %). Table-2 shows intra- and inter-day precision (RSD %) and accuracy of this assay method. Both intra- and inter-day precisions were less than 5.0, 3.0, 7.0 and 2.0 % for cichoriin. Both intra- and inter-day accuracies ranged from 99.7-106.8 % for cichoriin.

TABLE-2
INTRA-DAY AND INTER-DAY PRECISION (RSD %) AND ACCURACY

	Intra-day		Inter-day	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
50 µg/mL	1.3	99.7	0.8	101.8
80 µg/mL	0.9	100.3	1.1	99.9
100 µg/mL	1.4	99.81	1.3	101.25
120 µg/mL	1.7	101.51	0.6	101.3
150 µg/mL	0.8	99.3	1.6	100.8

Sample analysis: A convenient and rapid HPLC assay method has been developed for the determination of isolated cichoriin. The assay provides a linear response across a wide range of concentrations. Low intra-day and inter-day, RSD % coupled with excellent recovery was obtained.

The HPLC assay method proposed is accurate and precise for the determination of cichoriin. This proposed HPLC method is also helpful for quality control of cichoriin in natural products.

Conclusion

This method solves real life problem with samples for example a fraction of a plant extract or the plant extract itself, because there will be many other compounds which may have comparable polarities, this will lead to peaks overlapping. In this study the intermediate isocratic method (20 min), described which found to be suitable for detection or comparison or separation of the cichoriin and its corresponding compounds. All parameters of method validation were found to be according to ICH Q2 (R1) guidelines[3].

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