RAPID RESOLUTION LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF CHLOROGENIC ACID IN ECHINACEA EXTRACTS

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Abstract

This study presents a development and validation of a new, fast, efficient and cost effective reversed-phase rapid resolution liquid chromatography (RP-RRLC) method for determination of chlorogenic acid in echinacea extracts. The optimum separation with symmetrical peak shape and good index purity of the analyte was achieved on a Poroshell 120 EC-18 (50 mm x 3 mm; 2.7 µm) analytical column, mobile phase consisted of acetonitrile/(water with 1 % phosphoric acid), (10/90, *V*/*V*)in isocratic elution with flow rate of 1 mL/min and UV diode-array detection (UV-DAD) at 325 nm. The developed method was validated by testing specificity, selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The calibration curve of chlorogenic acid followed Beer's law within the range 28.97 ng - 362.19 ng (R^2 = 0.9994). The LOD was 0.29 pg, while LOQ was 0.96pg. The intra-day precisions was evaluated for the retention time, peak area and peak height and the calculated values for relative standard deviations (RSD) were 0.21 %, 0.11 % and 0.22 %, respectively. The mean recoveries ranged from 98.75 to 104.63 % and RSD was less than 0.23 %. The developed method was successfully applied for identification and quantification of chlorogenic acid in three samples of echinacea extracts, taken from local pharmacies.

Keywords: RP-RRLC, UV-DAD, method development, validation.

Introduction

Echinacea species are perennial plants which originate from North America. There are nine *Echinacea* species, three of which are used for medical purposes: *Echinacea angustifolia* DC, *Echinacea purpurea* (L.) *Moench* and *Echinacea pallida* (Nutt.). About 80 % of commercial products such as tinctures, alcoholic and non-alcoholic extracts, capsules, tablets, syrups, teas, beverages and the like are produced by *Echinacea purpurea*. The latest research shows that *Echinacea* species have anti-inflammatory, wound-healing and immune system stimulating effects against bacterial and viral infections (Ma et al., 2011). *Echinacea* speciescontain polysaccharides, flavonoids, caffeic acid derivatives,essential oils, polyacetylenes, alkylamides and miscellaneous chemicals (Cozzolinoet al., 2006). According to the United States Pharmacopoeial Convention,caffeic acid, cichoric acid, chlorogenic acid, dicaffeoylquinic acids, echinacoside and dodecatetraenoic acid isobutylamides are found in *Echinacea* species (Members of the USP, 2009).

Chlorogenic acid is an ester of caffeic acid and quinic acid (Fig. 1) (dos Santos et al., 2006).

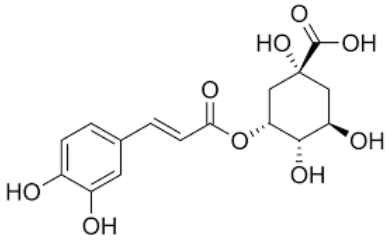

Figure 1. Chemical structure of chlorogenic acid

Although in the name it has the word "chloro", the chlorogenic acid does not contain chlorine. Its name comes from the Greek word which means light green, because of the green colour it receives in oxidation (Kremret al., 2016). Chlorogenic acid has many beneficial properties such as antioxidant activity, anticarcinogenic potential and may also slow the release of glucose into the bloodstream after a meal (Bakuradzeet al., 2011; Tunnicliffeet al., 2011). Additionally, chlorogenic acid has anti-inflammatory, antibacterial and anti-obesity properties (Chagas-Paula et al., 2011). Standardized methods with more analytical techniques were developed for the quantification of chlorogenic acid, but high-performance liquid chromatography (HPLC) remains one of the techniques that have the widest use and give accurate results (Craig, et al., 2016; Naegele, 2016; Brown et al., 2011;Pellati et al., 2004). The aim of this paper was to develop a fast, simple and accurate analytical method, for qualitative and quantitative determination of chlorogenic acid as one of the most active components in echinaceaextracts using Rapid Resolution Liquid Chromatography (RRLC) and ultraviolet diode array detection (UV-DAD).

Material and methods

Reagents and Chemicals

HPLC-grade methanol, acetonitrile and water, as well as pure analytical standards of chlorogenic acid (95 %) and phosphoric acid (85.5 %) were purchased by Sigma-Aldrich (Germany). Three different preparations obtained by extraction of echinacea were used as test material: non-alcoholic extract, herbal drops of echinacea (aqueous-alcoholic extract) and capsules produced in Italy and Germany that were procured from local pharmacies.

Equipment

The chromatographic analysis was performed on an Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with: vacuum degasser (G1322A), binary pump (G1312B), autosampler (G1329B), a column compartment (G1316A), UV-VIS diode array detector (G1316B) and ChemStation software. For the better dissolving of the stock solutions an ultrasonic bath “Elma” was used. The investigations were carried out using Poroshell EC 120-C18 (50 mm x 3 mm; 2.7 µm) analytical column produced by Agilent Technologies (USA).

Preparation of Standard Solutions

The stock solution was prepared by dissolving 0.0061 g of the pure analytical standard of chlorogenic acid with a mixture of methanol/water (80/20, *V/V*) in a 10 mL volumetric flask. The prepared standard solution was ultrasonized in an ultrasonic bath of type "Elma" for 15 minutes, in order to better dissolve of the analytical standard.

For determination of the linearity a series of working standard solutions was prepared by taking 100, 250, 500, 750, 1000, and 1250 µL of the standard solution in the measured flasks of 10 mL. The flasks were filled-up with a mixture of methanol/water in a volume ratio of 80/20. Each working standard solution was injected three times with a volume of 5 µL.

Preparation of Sample Solutions

Sample solution 1 was prepared by measuring 5.0393 g of the non-alcoholic extract of echinacea in a 10 mL volumetric flask, filled-up to the mark with the mixture of methanol/water (80/20, *V/V*). The sample was degassed for 15 min in an ultrasonic bath, and after cooling, 2 mL of this solution,was transferred in a 10 mL volumetric flask and filled-up to the mark with the mixture of methanol/water (80/20, *V/V*). Sample solution 2 was a pure aqueous-alcoholic extract (plant drops of echinacea). Sample 3 was prepared by measuring 1.14 g of echinacea capsules in a 10 mL volumetric flask, filled-up to the mark with the mixture of methanol/water (80/20, *V/V*). The prepared sample was dissolved by ultrasonification for 15 min. Before HPLC analysis, all samples were filtered through 0.45 µm Iso-Disc PTFE syringe filters (Supelco, Sigma-Aldrich, Germany).From all sample solutions, three injections were performed with 5 µL each.

Sample preparation for recovery determination

The recovery of the method was determinated by dissolving 5.0393 g of sample 1 in 10 mL volumetric flask and filled-up to the mark with methanol/water (80/20, *V/V*) and ultrasonificated for 15 min. Into three flasks (10 mL), 2 mL from this solution were transferred, and known concentration (7.24, 14.49 and 28.97 µg/mL) of the analytical standard was added into each flask, than filled-up to the mark with methanol and water in the volume ratio 80/20. All the sample solutions were filtered through 0.45 µm syringe filters and the three injections were performed with 5 µL each for all cases.

Results and discussion

For the preparation of the standard solution, working solutions and test samples, a mixture of organic solvent methanol and water in a volume ratio of 80/20 was used, taking into account the research of Pellati et al. (2004) in which, among other things, they made selection of the best solvent for the extraction of phenolic compounds from the *Echinacea* root. In doing so, they examined the various concentrations of aqueous solutions of methanol and ethanol (50%, 60%, 70%, 80%, 90% and 100%), so that they found that higher water concentrations lead to hydrolysis of phenolic compounds and the best results were obtained by using 80% methanol in water. Preliminary chromatographic studies made on the Agilent Poroshell 120 EC-C18 column (50 mm x 3 mm; 2.7 µm) showed that optimal conditions for the separation of chlorogenic acid using reversed-phase rapid resolution liquid chromatography (RP-RRLC) were achieved with an isotactic elution with a mobile phase consisted of a mixture of acetonitrile and 1 % phosphoric acid in water with a volume ratio of 10/90, a flow rate of 1 mL/min and column temperature at 25 °C (Jakimoski, 2015). Therefore, the same chromatographic conditions were applied in the research. The chromatographic process was followed at a wavelength of 325 nm. In Fig. 2 is presented a chromatogram obtained from the analytical standard of chlorogenic acid under the defined experimental conditions. As can be seen from this figure, a narrow, high and symmetrical chromatographic peak was achieved.

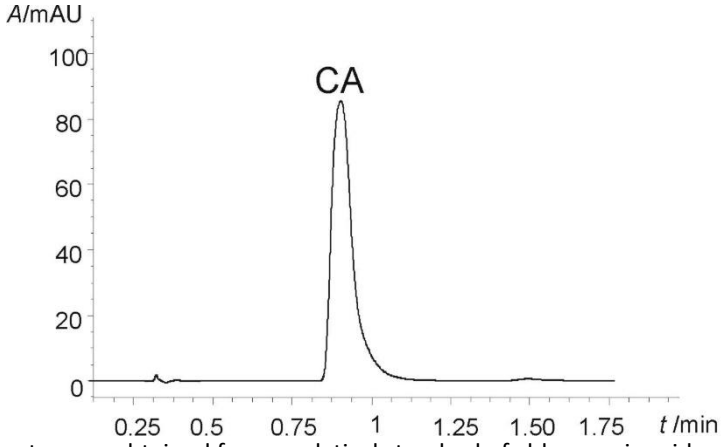

Figure 2. Chromatogram obtained from analytical standard of chlorogenic acid on the Agilent Poroshell 120 EC-C18 (50 mm x 3mm; 2.7 µm) column with acetonitrile/1 % phosphoric acid (10/90, *V/V*) as mobile phase, flow rate of 1 mL/min, column temperature at 25 ºC, and UV-detection at 325 nm

The proposed reversed-phase rapid resolution liquid chromatography method was successfully applied for the determination of the chlorogenic acid in echinacea extract samples. In Fig. 3 are shown the chromatograms obtained from the analyzed samples using the proposed method. The figure shows that the chromatographic peak of chlorogenic acid (CA) has symmetrical peaks shape and it is well separated from other components in the samples. The choice of the wavelength to monitor the chromatographic process was made based on the UV spectrum of chlorogenic acid recorded in a solution of acetonitrile and 1% phosphoric acid dissolved in water, with a volume ratio of 10/90 (Fig. 4). Thus, from the recorded UV spectra it can be noticed that the chlorogenic acid has two maxima, one at a wavelength of about 325 nm and the other smaller at a wavelength of 220 nm.

Because chlorogenic acid exhibits maximum absorption at 325 nm, the chromatographic process was followed at a wavelength of 325 nm.

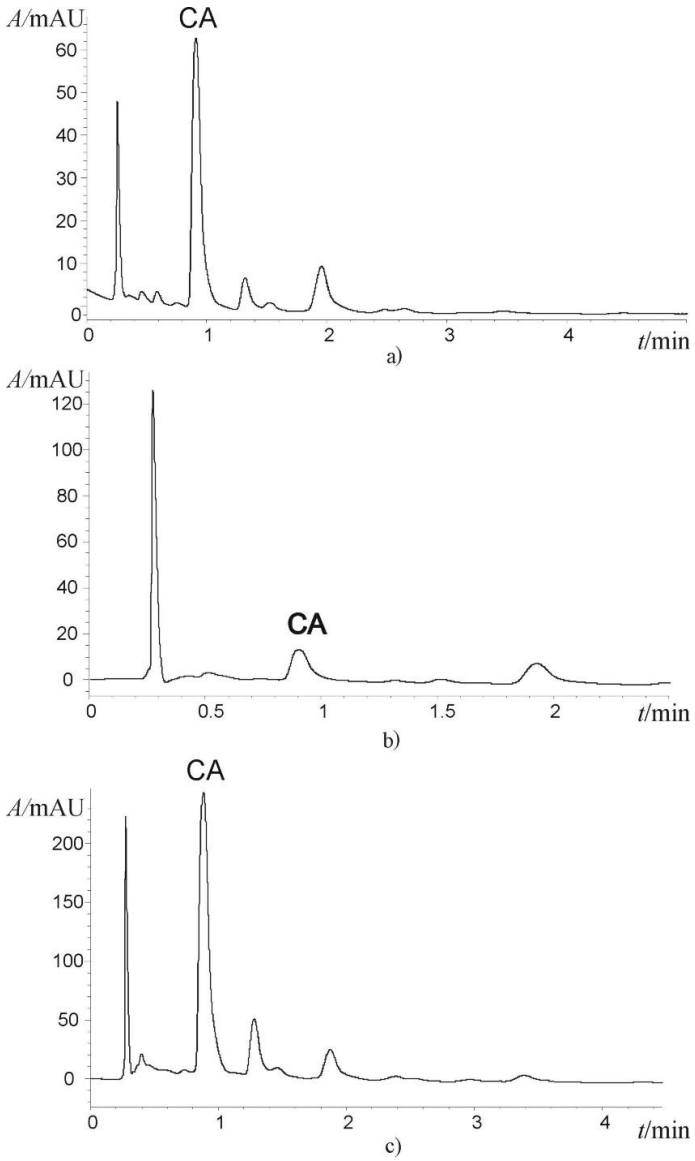

Figure 3. Chromatograms obtained from analyzed samples 1 (a), 2 (b) and 3 (c) using the developed method

For the method validation were determined: specificity, selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The specificity and selectivity of the method were estimated by identifying the peak of interest and the value obtained for the purity index. The identification of the analyte was performed by comparing the retention time of the analytical standard of chlorogenic acid with the retention time of the same component in the analyzedechinacea samples. The identification of the analyte was also confirmed by overlapping the absorption spectra of the pure analytical standard of the chlorogenic acid and the absorption spectrum of the same substance in the samples. The high value of the match factors (> 999) were evidence that the chromatographic peak was of the same substance. Under the stipulated chromatographic conditions the obtained value for column dead time was 0.26 min, and the mean value for the retention time of the analyte was 0.96 min. Hence, the calculated value for retention factor (*k'*) was 2.69 which is the optimum value for this parameter (Dong, 2006). For the estimation of the linearity of the method, calibration curves in a concentration range of 28.97 ng to 362.19 ng were constructed. The obtained results show that the curves followed Beer's law in the investigated range. The obtained values for multiple correlation coefficients (R^2 = 0.9994) revealed good linearity

of the developed method (Table 1). The limits of detection (LOD) was defined as the amount of analyte for which the signal-to-noise ratio (*S*/*N*) was 3, whereas the limits of quantification (LOQ) was defined as the amount of analyte for which *S*/*N* =10. The LOD and LOQ are listed in Table 1.

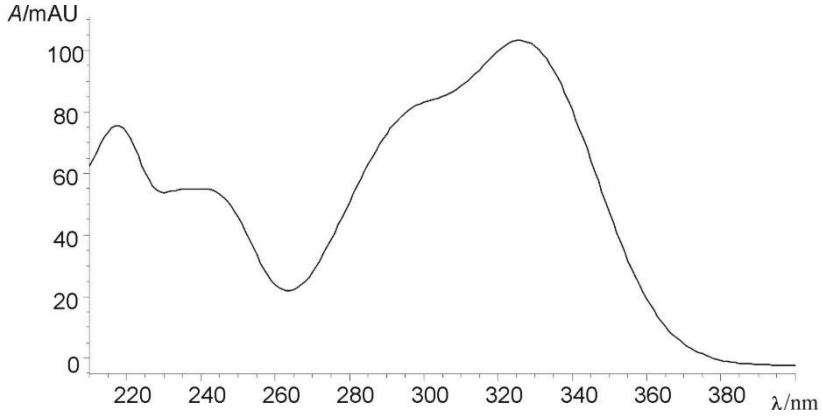

Figure 4. UV spectrum of chlorogenic acid in acetonitrile/1 % phosphoric acid (10/90, *V*/*V*) solution

Table 1. Linearity and sensitivity data for chlorogenic acid

	Linearity range (ng)	Regression equation	R^2	*LOD* (pg)	*LOQ* (pg)
Area	28.97 – 362.19	y = 3.8571x + 12.064	0.9994	0.29	0.96
Height		y = 0.6741x + 2.7508	0.9994		

In order to determine the precision of the elaborated method, expressed as intraday precision, 10 successive injections of the analytical standard with a mass concentration of 28.97 µg/mL were made. The calculated values for the relative standard deviation (RSD) for the peak area was 0.11 %, for the retention time 0.21 %, and for the peak height was 0.22 %. The obtained results show that the method has an excellent intraday precision. The accuracy of the method was confirmed by standard additions (Snyder, 1997). According to this method, a known amount of the analytical standard was added to sample 1, which already contains a certain amount of the analyte. Accuracy of the method was expressed as the deviation between the calculated mean value obtained by examination and the true value of the spiked amounts of the analyte into a sample 1 (Table 2). As it is shown in Table 2, the obtained values for recovery are within the following ranges 98.75 - 104.63 % (Table 2). Consequently, it can be concluded that the developed method is accurate enough and can be used for the quantitative determination of chlorogenic acid in echinacea extracts.

Table 2. Results from the recovery experiments (*n*=3)

m(analyte) before the ddition (ng)	*m*(analyte) added (ng)	*m*(analyte) after the ddition (ng)	***Recovery*** (%)	***RSD*** (%)
83.72	36.22	125.49 ± 0.31	104.63	0.23
	72.44	154.22 ± 0.21	98.75	0.08
	144.87	229.04 ± 0.21	100.22	0.06

Table 3. The content of chlorogenic acid in the analyzed samples

Sample	*m* (ng) ± SD (in V_{inj} = 5µL)	*RSD* (%)	Content of chlorogenic acid (ppm)
1	83.73 ± 0.23	0.27	166
2	19.50 ± 1.30	6.69	4.21
3	350.07 ± 34.38	9.82	638.44

The proposed method was successfully applied for the quantitative determination of chlorogenic acid in three commercial products designated as test 1, 2 and 3. The obtained mean values for the content of chlorogenic acid in the analyzed samples are shown in Table 3. As can be seen from the obtained results, the content of chlorogenic acid in the test samples, which are actually echinacea extracts, is very different and ranges from 4.21 to 638.44 ppm.

Conclusions

A new, fast, efficient and cost-effective reversed-phase RRLC method for the determination of chlorogenic acid in echinacea extracts has been developed. According to the experimentally obtained results, the optimal conditions for the qualitative and quantitative determination of the analyte in the analyzed samples were obtained using the Poroshell 120 EC-C18 (50 mm x 3 mm; 2.7 µm) analytical column, in isocratic elution mode with a mobile phase consisting of acetonitrile/1% phosphoric acid (10/90, *V/V*), 1 mL/min flow rate, column temperature at 25 ºC and UV detection at 325 nm. The obtained results revealed that the method is characterized by excellent linearity, precision and accuracy and it could be used for routine analysis of chlorogenic acid in echinacea extracts.

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