A new validated HPTLC method for quantitative determination of 1, 5-dicaffeoylquinic acid in *Inula crithmoides* roots

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Abstract: 1, 5-Dicaffeoylquinic acid (1, 5-DCQA), a potent HIV-1 integrase inhibitor, is currently undergoing an evaluation as a promising novel HIV therapeutic agent. This work aims at developing an accurate, rapid, repeatable and robust HPTLC method for the determination of 1, 5-DCQA in its natural sources. 1, 5-DCQA is the major component of the *n*-butanol fraction, the most biologically active hepatoprotective fraction, of *Inula crithmoides* roots extract. Thus, it will be of interest to evaluate the plant roots as a potential source of 1, 5-DCQA using a fully validated HPTLC method. The percentage of 1, 5-DCQA in the studied plant (0.035% w/w) was found to be approximately similar to those previously determined in other antioxidant herbal drugs, in which 1, 5- DCQA is the main phenolic constituent. The results obtained showed that the described HPTLC method is suitable for routine use in quality control of herbal raw material, extracts and pharmaceutical preparations containing 1, 5-DCQA. No HPTLC method has been reported in literature for the determination of 1, 5-DCQA in medicinal plants.

Keywords: *Inula crithmoides* L. roots; high performance thin layer chromatography (HPTLC); 1, 5-Dicaffeoylquinic acid (1, 5-DCQA).

INTRODUCTION

Many reports have proven a wide spectrum of biological activities of DCQA including; antiviral, anti-HIV (Gu *et al.,* 2007) and hepatoprotective. DCQA derivatives showed marked antioxidant activity as they were found to be potent scavengers of the well known free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and are more potent than butylated hydroxyl toluene (BHT) which was used as a positive control (Hung *et al.,* 2006). Survey of literature revealed that some methods have been developed for the qualitative and quantitative determination of DCQA in different matrices including liquid chromatography coupled with mass spectroscopy (LC-MS). HPLC is also used for the determination of DCQA in herbal extracts, but after being separated by counter current chromatography (Shi *et al.,* 2007; Tong *et al.,* 2008). On the other hand, HPTLC method can be applied for the whole extract without any pre-treatment, thus reducing the experimental time and the possible loss of compounds during sample treatment. Moreover, we can analyze several samples on the same plate for rapid screening of different plants. As well as the simultaneous chromatography of samples and standards under identical conditions leads to excellent analytical accuracy and precision. Thus HPTLC is the method of choice for the analysis of plant extracts. It's worth mentioning that, no HPTLC method has been reported so far for the determination of 1, 5-DCQA in medicinal plants. Accordingly, the aim of this work is to develop an accurate, specific, repeatable and robust HPTLC method for the determination of 1, 5-DCQA in plant extracts.

MATERIALS AND METHODS

Plant material

Inula crithmoides L. was collected in April 2006 during the flowering stage, from Rosette, Alexandria, Egypt. The plant was identified by comparison with voucher sample verified by Dr. Nabil El-Hadidy, Professor of Plant Taxonomy, Faculty of Science, Cairo University. A Voucher specimen is deposited in the Pharmacognosy Department, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt.

Chromatographic conditions

Sample and standard solutions for the HPTLC analyses were applied in the form of bands on pre-coated HPTLC silica gel plates by means of Linomat IV automated spray-on band applicator. The mobile phase consisted of Ethyl acetate: Methanol : Water: acetic acid (30 : 5 : 4 : 4 drops) and 40 ml of mobile phase were used for development. Ascending development of the plates was carried out in (20cm X 20cm) Camag HPTLC twin trough development chamber saturated with the mobile phase. The optimized time for chamber saturation with the mobile phase was 30 min. at room temperature. The mobile phase was left to run to a distance of 8 cm beyond the origin for plate development. The development time was 25 min at room temperature. After development, the plates were dried in air for 5 min. Densitometric scanning was performed using Camag TLC scanner III in the

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reflectance-absorbance mode at λ 324 nm and was operated by WINCATS software (V. 3.1). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. The slit dimension was 6mm X 0.1mm. The monochromator bandwidth was 20nm and the scanning rate was 10mm s^{-1}. The peak areas of chromatograms were determined by WINCATS software (V. 3.1).

Chemicals

HPTLC analyses were performed on Merck (Darmstadt, Germany) 20x10 cm HPTLC silica gel 60F-254 (0.25 mm) plates. 1, 5-DCQA was supplied by Sigma, Aldrich, Germany.

Quantitative determination of 1, 5- DCQA in plant extract

Preparation of standard solution: 10 mg of standard 1, 5-DCQA were weighed accurately, quantitatively transferred into a 10 ml volumetric flask, dissolved in methanol and volume was adjusted to 10 ml mark.

Preparation of sample solution: Dried roots (250 g) of *I. crithmoides* were ground and exhaustively extracted using ethyl alcohol 95% (2Lx 3) at room temperature. The solvent was evaporated under reduced pressure giving the crude extract (7g). 2 g of the crude extract were weighed accurately, quantitatively transferred into a 10 ml volumetric flask, dissolved in methanol, the volume was adjusted with the same solvent and mixed thoroughly.

Preparation of the Calibration curve: According to the recommendations of the International Committee on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (Validation of Analytical Procedures 2005), a calibration curve was constructed using six analyte concentrations (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 µl $zone^{-1}$) of the TLC standard applied in duplicate, representing 3–8 µg of 1,5- DCQA. For routine analytical procedures, a three-point calibration curve within this range was used, produced by applying duplicate 3.0, 5.0 and 8.0 µl (3.0, 5.0, 8.0 µg) of the standard on each plate. The volumes applied for routine analysis were duplicates 3.0 µl, 5.0 µl and 8.0 µl of 1, 5-DCQA standard and duplicate 2 µl or 3 µl aliquots of sample solution.

Sample assay: Aliquots of 2 µl of the prepared sample solution were analyzed by the HPTLC method as described before under preparation of the calibration curve.

Method validation

Selectivity is the ability of an analytical method to assess unequivocally the analyte in the presence of other components in the sample. This will be accomplished through the separation between the peak of the analyte and other sample components. This definition should ensure identity, purity and exact potency (concentration) of the analyte.

Linearity is the relationship between the peak areas and the amount of substance applied. This was determined by constructing a calibration graph using increasing amounts of standard solution (3, 4, 5, 6, 7 and 8 µg/spot) where each concentration was plotted against the respective peak area (fig. 2). This was repeated six times as recommended by the ICH. The method was evaluated by its regression equation data (intercept and slope), correlation coefficient (r-value) and other statistical parameters shown in table 1.

In order to determine the limit of detection (LOD) and limit of quantitation (LOQ) the proposed method was applied for the assay of 1, 5-DCQA in concentration ranging from 80% to 120% of the working concentrations.

According to the ICH guidelines, the detection limit (DL) may be expressed as:

$DL = 3.3\ \sigma/S$

While, the quantitation limit (QL) may be expressed as:

$QL = 10\ \sigma/S$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S is estimated from the calibration curve. The estimate of σ was carried out based on the calibration curves, where the standard deviations of y-intercepts of regression lines were used as the standard deviation.

LOD and LOQ were proven experimentally by diluting known concentrations of standard 1, 5-dicaffeoylquinic acid solutions and the results were recorded in table 1.

Table 1: Linear regression data for the calibration curve of 1, 5-DCQA (n=6)

Linearity range (µg/spot)	2.4 - 9.6
Intercept (a)	16040
Slope (b)	5583.1
Correlation coefficient (r)	0.9975
S_a standard deviation of X	197.7634
S_b standard deviation of Y	1138.93
LOD (µg/spot)	0.6732
LOQ (µg/spot)	2.040

According to the ICH guidelines, precision must be considered through both, repeatability and intermediate precision. Repeatability was determined by independent repeated analysis of three different 1, 5-DCQA standard solutions in triplicates each and calculating the percent relative standard deviation (RSD%). The experiment was

repeated on the same day by the use of the same analytical procedure, the same equipment, the same type of plate and in the same laboratory. The results are shown in table 2. On the other hand, intermediate precision was performed by the analysis of five different concentrations of the standard solution, each repeated three times on different days. The results are shown in table 3. The relative standard deviation (RSD%) was calculated in order to assess the precision of the assay, which is specified by the ICH guidelines to be less than 2%.

Table 2: Precision (repeatability) of the HPTLC method for the determination of 1, 5-DCQA (Intra-day precision)

Experiment number	Concentration (µg/spot)	Mean ± SD	RSD %
1	5.255 5.428 5.242	5.3038 ± 0.10333	1.947
2	7.130 7.246 7.302	7.2260± 0.08773	1.214
3	7.747 7.839 8.011	7.8657 ± 0.13400	1.704

Table 3: Intermediate precision of the HPTLC method for the determination of 1, 5-DCQA (Inter-day precision)

Experiment number	Concentration (µg/spot)	Mean ± SD	RSD %
1	2.967 2.920 2.979	2.95533 ± 0.0312	1.056
2	3.998 3.992 3.952	3.98067 ± 0.0250	0.628
3	5.197 5.190 5.242	5.20967 ± 0.0282	0.541
4	6.253 6.214 6.168	6.21167 ± 0.0425	0.684
5	7.297 7.130 7.144	7.19033 ± 0.0926	1.288

The accuracy of the method was validated using a standard addition analysis. This involves applying the analytical procedure to synthetic mixtures of the drug sample to which known quantities of the drug standard have been added. Thus, the sample solutions were spiked with two different, known, concentrations of 1, 5-DCQA standard. A 5 ml aliquot of sample solution of previously determined concentration (2.534 mg/ml) was added to a 5 ml of standard 1, 5-DCQA solution (2 mg/ ml) to give mix.1 and another 5 ml aliquot of the same sample solution was added to 5 ml of standard 1, 5-DCQA solution (4 mg/ml) to give mix. 2.

The original and the two mixed sample solutions (mix.1 and mix. 2) were analyzed on the same plate by application of triplicate 3.0µl and 2.0µl volumes, respectively, in addition to the three standards described earlier for routine analyses which were applied in duplicate. The difference between the expected concentrations and the found ones was calculated in order to judge the accuracy of the method. The results are presented in table 4.

Robustness tests examine the effect of the operational parameters on the test results. By introduction of small changes in the volume of the mobile phase, where the amount of mobile phase is changed in the range of ± 10%. Time from spotting till chromatography and from chromatography till scanning was varied from 0, 5, 10, to 15 min. The development distance was changed from 6.5 - 8.5 cm beyond the origin. Supplier (producer) of the TLC plate was changed; both brands Fluka and Merck were used. Robustness of the method was performed using three concentrations (3, 5 and 8 µg/spot) as shown in table 5.

RESULTS

Experimental conditions, such as scan mode, scan speed, mobile phase composition and wavelength of detection were optimized to provide accurate and precise results. Development using the mobile phase described above on the HPTLC silica gel plates produced compact, yellow bands of 1, 5-DCQA (R_F 0.55) viewed under a 324 nm UV light. The scan-densitogram obtained from the sample to be analysed showed a selective baseline separation between 1, 5-DCQA and other components in the sample.

Method validation

Selective baseline separation was found between 1, 5-dicaffeoylquinic acid and other matrix components as presented in the scan-densitogram obtained from a representative sample (fig. 1). The calibration curve (fig. 2) was constructed and the proposed method was evaluated using its correlation coefficient (0.9975) and other statistical parameters (table 1). The linear regression equation was found to be: Y = 16040 + 5583.1 X, where Y is the peak area and X is the amount of substance in µg/spot.

DISCUSSION

The results for limit of detection (LOD) and limit of quantitation (LOQ) are shown in table 1 and verified that the concentrations used for calibration curve construction lies above the minimum quantitation limit.

Table 4: Results of the standard addition experiments

Test solution	Concentration of 1, 5-DCQA in sample (µg/µl)	Concentration of standard 1, 5-DCQA added (µg/µl)	Concentration of 1, 5-DCQA expected (µg/µl)	Concentration of 1, 5-DCQA found* (µg/µl)	Recovery (%) ± SD	RSD%
Mix.1	2.534	2	2.267	2.11	93.0745 ± 0.239	3.64
Mix.2	2.534	4	3.267	3.14	96.1126± 0.128	2.00

*Each concentration is the average of three determinations.

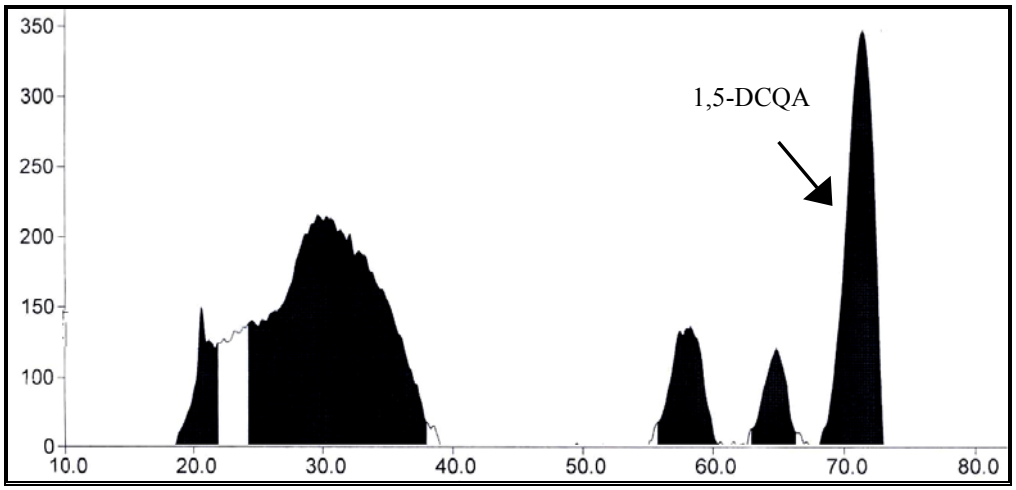

Fig. 1: HPTLC scan-densitogram showing the separation of 1, 5-dicaffeoylquinic acid (DCQA) from other matrix components in the sample at 324nm.

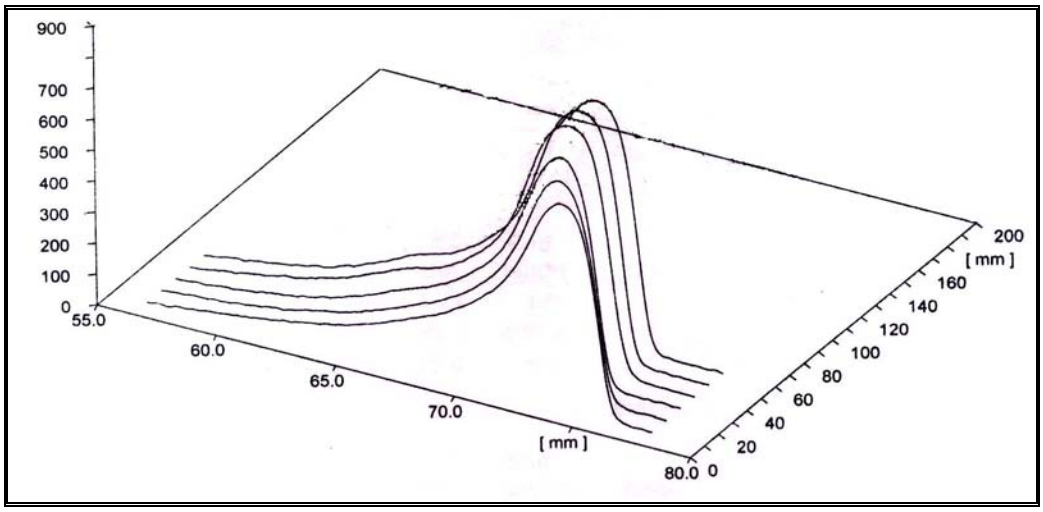

Fig. 2: Densitogram of pure single peak of standard 1, 5 DCQA

The data of repeatability (table 2) and intermediate precision (table 3) obtained RSD% values of less than 2% indicating a good precision. The accuracy of the method was assessed by means of % recovery and % RSD (table 4) which were found to be within accepted values. Also RSD% listed in table 5 is accepted and shows that the proposed method is robust.

CONCLUSION

It has been shown that, validation data for this new quantitative HPTLC method has been shown to achieve selectivity, linearity, precision (intra-day and inter-day) for replicate analyses, accuracy, robustness, detection and quantification limits as stated in the ICH guidelines.

Table 5: Robustness testing

Parameter	S.D.*	R.S.D. %*
Time from spotting to chromatography	0.099	1.53
Time from chromatography to scanning	0.043	0.74
The development distance	0.246	3.82
TLC plate supplier	0.118	3.64
Amount of mobile phase	0.129	3.49

*Average of S.D. of three concentrations.

The precision and accuracy values of the HPTLC method compare favorably with those obtained with HPLC (high performance liquid chromatography) (Krizman *et al.,* 2007; Pajero *et al.,* 2004) methods developed recently for assay of 1, 5-DCQA in different matrices.

The results meet the Guidelines of the International Conference of Harmonization (ICH) (Validation of Analytical Procedures-2005) for validation of analytical procedures used for drugs and drug products.

Application

The described HPTLC method is suitable for routine use in quality control of herbal raw material, extracts and pharmaceutical preparations containing 1, 5-DCQA. This method is sensitive, rapid and sample treatment is very simple. The time and cost for analysis per-sample basis is low, because up to six samples can be analyzed in duplicate with the three standards (also in duplicate) needed for preparation of the calibration curve in a single run on the same plate, rather than performing a sequence of injections of the samples and standards in HPLC. This simultaneous chromatography of samples and standards under identical conditions leads to excellent analytical accuracy, precision and reproducibility. Cost of solvent purchase and disposal is very low because no more than 30 ml of mobile phase is required for development of the plate in the development trough. Its worth to mention that, the percentage of 1, 5-DCQA in the studied plant (0.035% w/w) was found to be approximately similar to those previously determined in other antioxidant herbal drugs, in which 1, 5- DCQA is the main phenolic constituent (Krizman *et al.,* 2007; Pajero *et al.,* 2004).

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