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Short communication

Validation of an isocratic LC method for determination of quercetin and methylquercetin in topical nanoemulsions

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Abstract

The aim of this study was to validate an isocratic LC method for the quantification of either quercetin (Q) or methylquercetin (MQ) incorporated in topical nanoemulsions. The analyses were performed at room temperature on a reversed-phase C_{18} column using a mobile phase composed of methanol/water (70:30, v/v) and trifluoracetic acid 0.1% at 0.8 mL min^{-1}. The detection was carried out on a UV detector at 368 or 354 nm for Q and MQ, respectively. The linearity, in the range of 0.15–1.5 μg/mL, presented a determination coefficient (r^2) higher than 0.99, calculated by the least square method for both flavonoids. No interferences from the excipients (egg-lecithin or octyldodecanol) were detected. The R.S.D. values for intra- and inter-day precision experiments were lower than 2% for both flavonoids. The recovery ranged from 98.9% to 103.46% for Q and from 98.9% to 102.92% for MQ.

Keywords: Quercetin; Methylquercetin; Nanoemulsions; Liquid chromatography; Validation

1. Introduction

In recent years, the antiviral activity of polyphenolic compounds has been extensively investigated. The effect of polyphenols, especially flavonoid compounds, against different virus strands has been well documented [1–4]. Several studies have been focused on the effect of these compounds over Herpes Simplex Virus (HSV) since they are important human pathogens [5]. For instance, it has been shown that quercetin causes a concentration-dependent reduction in the infectivity of HSV-1 and reduces the intracellular virus replication [2]. The ability of flavonoids to link the viral protein and interfere in the synthesis of viral nucleic acid seems to be the main mechanism involved in the antiviral activity [5].

However, the use of flavonoids (as aglycones) in pharmaceutical products is compromised by their low water solubility. The incorporation of poorly soluble drugs in nanoemulsions has received increasing attention as colloidal carriers for topical delivery [6–8]. Nanoemulsions are fine dispersions of oil-in-water (o/w) in which the poorly soluble drugs could be dissolved in the oil core and/or adsorbed on the o/w interface [9,10]. In fact, the incorporation of drugs in such systems could increase the skin permeation rate and enhance the topical effect due to prolonged residence time in the uppermost skin layers due to the large surface area and low surface tension of the oil droplets [11,12].

In this context, the development of nanoemulsions for flavonoid topical administration intended for antiviral activity is currently under study by our research group. Several reports have described the validation of LC methods for quantifying flavonoids; however, most of them have been focused on their separation in food, medicinal plants or biological fluids (for a recent review, see de Rijke et al. [13]). To our knowledge, no studies of the quantification of flavonoids in lipid nanoemulsions have been reported to date. Thus, the aim of the present study was to validate an isocratic LC method, in accordance with ICH [14], for the determination of quercetin (Q) or methylquercetin (MQ) in nanoemulsions for topical use.

2. Experimental

2.1. Chemical and reagents

Egg-lecithin (Lipoid E-80®) was kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Octyldodecanol was

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Table 1
Final composition of nanoemulsions (%, w/w)

	Q	MQ
Q	0.1	–
MQ	–	0.1
ODD	8.0	8.0
Egg-lecithin	2.0	2.0
Purified water to	100	100

obtained from Delaware (Porto Alegre, Brazil). Q raw material was purchased from Galena (Campinas, Brazil). Q reference standard (>98%) was purchased from Sigma–Aldrich (São Paulo, Brazil). MQ was isolated from *Achyrocline satureioides* Lam D.C. (Asteraceae) and identified by different methods including UV, IR, and NMR as previously reported [15]. The purity of MQ was higher than 99%. Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). Methanol LC grade was obtained from Merck (Darmstadt, Germany). Trifluoracetic acid was obtained from Nuclear (Diadema, Brazil).

2.2. Preparation and characterization of nanoemulsions

Nanoemulsions were prepared using the spontaneous emulsification procedure [10]. This method consists of injecting an organic phase (ethanol) containing components of the oil core (ODD, egg-lecithin and Q or MQ) into the water phase under magnetic stirring (15 min). Subsequently, the organic solvent was removed by evaporation under reduced pressure at 40–45 °C. Table 1 shows the final composition of nanoemulsions containing Q or MQ at 1 mg/mL. Blank nanoemulsions were obtained, under the same conditions, in the absence of flavonoids.

The mean droplet size of the emulsions was determined by quasi-elastic light scattering after dilution in water and the electrophoretic mobility was measured with a zetasizer (HAS 3000, Malvern, England). The pH values of nanoemulsions were directly determined in samples (Micronal B374 potenciometer). The viscosity of the nanoemulsions was measured using a capillary viscosimeter. The measurements were performed at room temperature.

2.3. Chromatographic conditions and apparatus

The LC apparatus consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with a model LC-10AT pump, an SPD-10AV UV–vis variable-wavelength detector (set at 354 nm for MQ and 368 nm for Q), a SCL-10Avp system controller, Rheodyne 7725 injection valve with a 50 μL loop. Flavonoids were analyzed using a Shim-pack column CLC-ODS (M) RP-18, 5 μm, 250 mm × 4 mm i.d., connected with a precolumn Waters RP-18, 10 μm. The mobile phase consisted of a mixture methanol–water (70:30, v/v) acidified with 0.1% of trifluoracetic acid (TFA), filtered and degassed by suction-filtration through a nylon membrane, in isocratic flow. The LC system was operated at flow-rate of 0.8 mL min^{-1} and the sensitivity was 0.5 AUFS, at room temperature. All calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

2.4. Method validation

The validation was performed based on the ICH [14], taking into account the characteristics required for assaying dosage forms.

The specificity of the method was evaluated by analyzing solutions of the blank nanoemulsions obtained in the absence of Q or MQ. The system response was examined through the presence of interference or overlaps with the Q or MQ responses.

For linearity experiments, solutions of Q or MQ were prepared at five concentrations within the range of 0.15–1.50 μg/mL on 3 different days. The results were represented graphically, which allowed for the evaluation of the calibration curve and coefficient of determination. Limits of detection (LOD) and quantification (LOQ) were determined based on the standard deviation of the response and the slope, using the calibration curve data. Analysis was performed in triplicate.

The intra-day precision (repeatability) of the method was determined by analysis of three samples of Q or MQ for each point of the calibration curve (three replicates each), during the same day, under the same experimental conditions. Inter-day precision values were obtained by assaying freshly prepared solutions of Q or MQ on 3 different days.

Accuracy was evaluated by determining the method recovery. The accuracy experiments were performed applying the method to quantify Q or MQ in the presence of excipients of formulations. Next, blank nanoemulsions were spiked with known amounts of Q or MQ at different levels: low, medium and high, corresponding, respectively, to 0.81, 1.08, and 1.35 μg/mL. Samples were appropriately diluted and analyzed. The results represent the mean of recovery for three independent samples.

2.5. Q and MQ content

The determination of the content of Q and MQ in the nanoemulsions was carried out by means of the conditions described below. Nanoemulsions aliquots of 0.5 mL, containing Q or MQ, were appropriately diluted in methanol (final concentration of 0.5 μg/mL), filtered, and analyzed. Free Q or MQ was determined in a clear ultrafiltrate obtained through separation of the water phase using an ultrafiltration/centrifugation procedure (Ultrafree-MC 10,000 MW, Millipore) [16]. Samples of nanoemulsions were added to ultrafiltration membranes and centrifugated at 5000 rpm. The concentration of Q or MQ was determined in the ultrafiltrate. The association efficiency (%) was estimated by the difference between the total (flavonoids content) and free drug concentrations.

3. Results and discussion

In a first step, the physico-chemical properties of nanoemulsions were evaluated. As can be seen in Table 2, the emulsification conditions yielded monodisperse emulsions

Table 2
Physico-chemical properties of nanoemulsions containing either Q or MQ

	Q	MQ
Droplet size (nm)	305 ± 13	307 ± 19
ζ-potential (mV)	−27 ± 6	−29 ± 5
Viscosity (cP)	1.96 ± 0.05	1.99 ± 0.005
pH	7.10 ± 0.04	7.30 ± 0.02

Table 3
Linearity, LOD, and LOQ of LC assay for Q and MQ

	Q	MQ
Concentration range (μg/mL)	0.15–1.5	0.15–1.5
Equation	$y = 163179x - 10769$	$y = 86975x - 2949.8$
r^2	0.9989	0.9992
LOD	0.0363	0.0354
LOQ	0.1209	0.1179

(IP < 0.2) with a typical droplet size of approximately 300 nm and a viscosity of approximately 2 cP. These data are in agreement with physico-chemical properties of nanoemulsions obtained through spontaneous emulsification procedure [10,16]. Concerning ζ-potential experiments, nanoemulsions presented negative values due to the presence of negatively charged phospholipids in egg-lecithin compositions, such as phosphatidylserine and phosphatidic acid at neutral pH of formulations (Table 2), as reported in previous literature [9,17].

The first analytical experiments were performed in order to evaluate whether nanoemulsion excipients could interfere over Q or MQ quantification since they are stabilized by egg-lecithin, which is a complex mixture of phospholipids (mainly phosphatidylcholine) combined with other substances such as carbohydrates, fatty acids, and triglycerides [18]. The mobile phase composition, as well as the other chromatographic conditions, was chosen in order to obtain efficient routine analysis. The specificity was carried out through the comparison of the peak retention time of the Q or MQ and a placebo (blank nanoemulsions). No interference of the emulsion excipients was noticed since no peak was detected after injection of blank nanoemulsion at set wavelengths (Fig. 1). In the employed chromatographic conditions, Q and MQ presented a retention time of approximately 6.4 and 7 min, respectively.

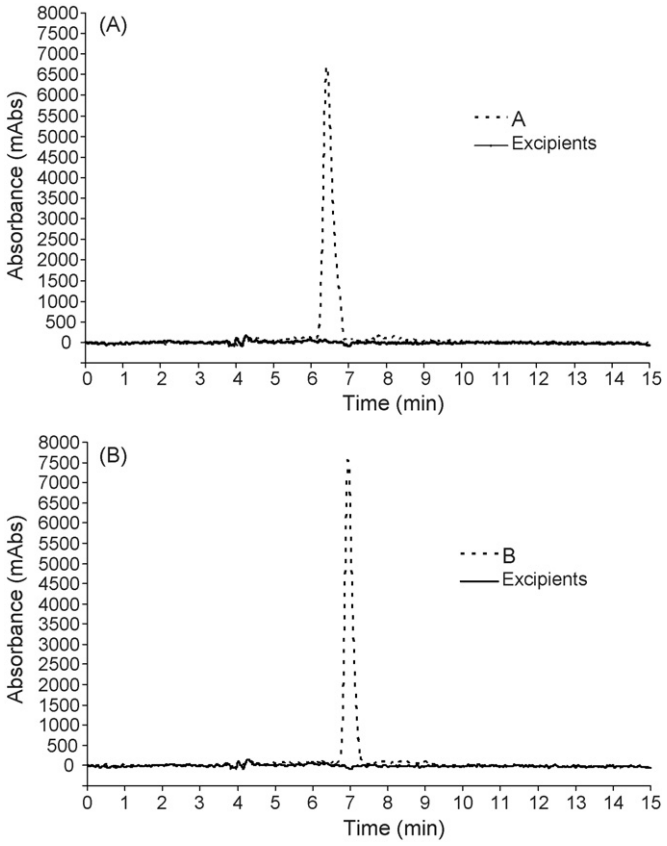

Fig. 1. Typical chromatograms of Q (A) and MQ (B) and mixture of formulation excipients.

Table 3 shows the data from the calibration curves for Q or MQ fitted by plotting concentration versus the corresponding mean peak area. Satisfactory linearity was detected for both flavonoids in the 0.15–1.5 μg/mL range. The least square regression showed excellent correlation, higher than 0.998, which is considered highly significant for the method. The detection and quantification limits are also presented in Table 3. These parameters were calculated based on the standard deviation of the response and slopes of the calibration curves. The values remain quite similar for both Q and MQ. In the same manner, the precision of the method was assessed considering repeatability (intra-day analysis) and intermediate precision (inter-day analysis). The results were expressed as relative standard deviation

Table 4
Intra-day and inter-day precision of LC assay of Q and MQ

Concentration (μg/mL)	Q					MQ				
	Day 1 peak area (mV s)[a]	Day 2 peak area (mV s)[a]	Day 3 peak area (mV s)[a]	Intra-day R.S.D. (%)[b]	Inter-day R.S.D. (%)	Day 1 peak area (mV s)[a]	Day 2 peak area (mV s)[a]	Day 3 peak area (mV s)[a]	Intra-day R.S.D. (%)[b]	Inter-day R.S.D. (%)
0.15	17,428	17,144	17,053	0.74–1.76	1.14	11,794	11,668	11,873	0.96–1.71	0.88
0.30	36,973	35,973	35,809	0.52–1.83	1.74	22,724	23,031	22,664	0.77–1.42	0.86
0.60	87,421	84,941	87,907	0.85–1.51	1.83	48,715	47,843	47,060	1.03–1.88	1.73
0.90	135,288	131,213	131,458	0.31–0.88	1.72	74,869	74,760	73,153	1.05–1.75	1.29
1.50	238,620	233,201	236,933	0.57–1.36	1.17	12,8937	129,694	127,163	0.61–1.51	1.01

[a] The value represents the mean of three individual experiments.
[b] Range of R.S.D. considering 3 days of validation.

Table 5
Accuracy of LC assay for Q and MQ

Added (μg/mL)	Q		MQ	
	Found (μg/mL)	Recovered (%)	Found (μg/mL)	Recovered (%)
0.81	0.801	98.90	0.834	102.92
1.08	1.117	103.46	1.068	98.90
1.35	1.355	100.37	1.359	100.70

Table 6
Q and MQ content in nanoemulsions at 1 mg/mL

Nanoemulsion	Content (μg/mL)	R.S.D. (%)
Q	0.974 ± 0.003	0.308
MQ	0.984 ± 0.008	0.813

and are shown in Table 4. The intra- and inter-day precisions presented R.S.D. values lower than 2% for both Q and MQ, which were considered satisfactory for the purpose of the analysis [14].

The accuracy of the method was determined by the recovery test. As can be seen in Table 5, whatever the amount of flavonoids added in blank nanoemulsions, the recoveries ranged from 98.9% to 103.46% for Q and from 98.9% to 102.92% for MQ, indicating a good agreement between amounts added and found.

Finally, the method was used to evaluate the Q or MQ content in nanoemulsions obtained in this study (Table 6). The determination of both Q and MQ content demonstrated R.S.D. < 0.813% from triplicate analysis, indicating the precision of the validated method. In a last step, we investigated the location of flavonoids in nanoemulsions by estimating Q or MQ in the aqueous phase after an ultrafiltration/centrifugation procedure [16]. No Q or MQ were detected in the aqueous phase. Considering the LOQ described in Table 3, the association efficiency of Q or MQ in the oil core of nanoemulsions was nearly 100%. These findings could be related with the low water solubility of flavonoids [19] promoting the partitioning (encapsulation) of Q or MQ into oil core of nanoemulsions.

In conclusion, this paper shows a useful LC method to estimate, accurately and precisely, both Q and MQ incorporated in nanoemulsions. In the validated conditions, excipients of nanoemulsions did not interfere with the determination of flavonoids.

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