Determination of flavonoids in pharmaceutical preparations using Terbium sensitized fluorescence method

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Received 12 May 2009; Revised 13 Sept 2009; Accepted 20 Sept 2009

ABSTRACT

Background and the Purpose of the Study: The aim of this study was development and validation of a simple, rapid and sensitive spectrofluorimetric method for determination of total flavonoids in two topical formulations of Calendula officinalis, Ziziphus Spina-christi and an oral drop of Hypiran perforatum L. The proposed method is based on the formation of terbium (Tb^{3+})-flavonoids (quercetin as a reference standard) complex at pH 7.0, which has fluorescence intensely with maximum emission at 545 nm when excited at 310 nm.

Method: For ointments masses of topical formulations were weighed and added to ethanol-aqueous buffer (pH 10.0) and the resulting mixtures were shaken and then two phases were separated by centrifugation. Aqueous phases were filtered and then diluted with water. For Hypiran drops an appropriate portion was diluted with ethanol and then aliquots of sample or standard solutions were determined according to the experimental procedure.

Results: Under the optimum conditions, total concentrations of flavonoids (as quercetin equivalent) in three tested formulations were found to be 0.204 mg/g (for Dermatin cream), 0.476 mg/g (for Calendula ointment) and 13.50 µg/ml (for Hypiran drops). Analytical recoveries from samples spiked with different amounts of quercetin were 96.1-104.0 % with RSD % of less than 3.5.

Conclusion: The proposed method which requires a simple dissolution step without any matrix interferences provided high sensitivity and selectivity and was easily applied to determine total flavonoids in real samples of three investigated formulations with excellent reproducibility.

Keywords: Sensitized fluorescence, Flavonols, Total flavonoids content, Terbium

INTRODUCTION

Calendula officinalis, Ziziphus Spina-christi and Hypiran perforatum L. (known as St. John's wort) have become a source of medicine for the treatment of many diseases including dermatitis, exema and mild to moderate depression. Published data strongly indicate that beside of sterols, carotenoids and saponins, the pharmacological effects of these plants are related to active important constituents including some flavonoids, particularly those with the quercetin structure (1-3).

Flavonoids are a group of natural polyphenolic compounds widely distributed in the medicinal plants, vegetables, fruit juices and beverages. Flavonoids, and particularly quercetin derivatives, have received more attention as dietary constituents during the last few years (5, 6). Considering the importance of biological activity of flavonoids, their identification and determination in plant tissues, will play an important role to control their quality and safety for clinical applications. Therefore, there is always a need for simple, sensitive, accurate, rapid and reproducible analytical method for the determination of flavonoids in the formulations and in biological fluids. Methods for the determination of flavonoids in medicinal plants are reversed-phase high-performance liquid chromatography (7, 8) and capillary electrophoresis (9) and spectrophotometry (10). These techniques mostly require expensive and complicated instrumentation, lengthy and complicated preparation procedures (extraction or derivatization), and as a result are not widely used in routine laboratories and the development of more simple and effective methods are necessary.

Luminescence spectrometry offers possibilities of sensitive and selective detection, by the use of lanthanide sensitized luminescence. The specific strong fluorescence of lanthanide ions, which usually occurs as a result of intramolecular energy transfer through the excited triplet state of the

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ligand to the emitting level of the lanthanide ion, is characterized by large Stokes shifts, narrow emission bands and long fluorescence lifetimes (11). As the result of spectral and temporal discrimination of the analytical signal, background fluorescent emission interferences from the complex matrices are eliminated, selectivity and detection limits are improved. Therefore, lanthanide ions have been used as a fluorescent probes to determine the biomolecules (12, 13).
In a previous work, it was reported that quercetin can be complexated with Terbium cations (at pH 7.0) to form a stable product and consequently emission at 545 nm could be enhanced (14). In the present work, the Tb-quercetin chelate was used as the luminescent probe for the determination of total flavonoids in these pharmaceutical preparations, based on Terbium Sensitized Fluorescence (TSF) and the total flavonoids content was expressed in terms of quercetin equivalent (QE) which could be used in the quality control of the pharmaceutical formulations. The investigated formulations are:
Calendula ointment containing 1.5% of total extract of Calendula officinalis flowers including carotenoids, flavonoids and saponosids. Dermatin cream prepared from the extract of Lotus leave, Ziziphus Spina-christi, containing plant sterols, flavonoids and steroid saponins. Hypiran drops prepared from the extract of Hypericum perforatu ml. plant, known as St. John׳s wort (SJW).

MATERIALS AND METHODS

Reagents
A 10^{-2} M Terbium (Ш) solution was prepared by dissolving the appropriate amount of Terbium (Ш) Chloride hexahydrate ($TbCl_3$. 6 H_2O) (Acros Organics, USA) in double distilled water.
A stock solution (1000 µg/ml) of quercetin (for spectrophotometric study) (Sigma) was prepared in ethanol and a stock solution 100 µg/ml (for fluorimetric study), was prepared in 40 % v/v ethanol - water with the final pH of 10.0 for complete confidence of dissolution. For the experiments freshly diluted samples in water with less than 2 % of ethanol were used.
A 0.05 M tris-(hydroxymethyl) aminomethan-hydrochloric acid (Tris-HCl) buffer solution was prepared by dissolving a desired amount of Tris-base (Merck) in 90 ml of water, adjusting the pH to 7.0 with HCl and adjusting the volume to 100 ml with water.
Also 10 % v/v aluminum chloride hexahydrate ($AlCl_3$. 6 H_2O) (Merck) and 1.0 M of potassium acetate (CH_3COOK) (Merck) solutions were prepared in double distilled water.
Calendula ointment (Dineh Iran Company, Ghazvin, Iran), Dermatin cream (Barij Essence pharmaceutical company, Kashan, Iran) and Hypiran drops (Pursina Pharmaceutical company, Karaj, Iran) were obtained from a local pharmacy store.
All reagents and solvents were analytical grade and used without further purification. Double distilled water was used throughout this work.

Apparatus
Fluorescence spectra and intensity measurements were performed using a Shimadzu RF-540 spectrofluorimeter (Kyoto, Japan) equipped with a 150 W xenon lamp, using 1.0 cm quarts cell. The excitation and emission monochromator band width were 10 nm. The excitation wavelength was set at 310 nm and the fluorescence intensity was measured at 545 nm. All measurements were performed at 25 ± 0.1 °C using a thermostated cell holder and thermostatically controlled water bath (Rikakika, Japan). UV measurements (230-500 nm) were carried out at room temperature on a Shimadzu 1650 PC spectrophotometer. The pH of the solutions was measured with Metrohm model 654 pH meter (Herisau, Switzerland).

Methods
Sample preparation
Dermatin cream in the quantity of 0.2342 g and 0.1719 g of Calendula ointment were accurately weighed and dissolved in 40 % v/v ethanol-water (pH 10.0). Of different solvents which were investigated, it was found that 40 % v/v ethanol-water (pH 10.3) is the most efficient for dissolution of ointment and cream and this reagent is comparable with conditions of Terbium Sensitized Fluorescence. The mixtures were shaken for 15 min and then two phases (clear phase and lipid phase) were separated by centrifugation for 10 min at 3500 rpm. The aqueous phase was filtered using a 0.45 µm pore size membrane filter, transferred to a 100 ml flask and diluted with water. A 0.5 ml portion of Hypiran drops was transferred to a 100 ml flask and diluted with ethanol. Then, aliquots of sample; 0.1 ml for fluorescence measurements, 0.5 ml for spectrophotometry measurements, and standard solutions were determined according to the experimental procedure.

Experimental procedure
For Terbium Sensitized Fluorescence determination, the analytical procedure was as follows: to 5 ml volumetric flasks were added 2 ml of 1×10^{-2} M Tb^{3+} solution, 0.25 ml of 0.05 M Tris–HCl buffer (pH 7.0) solution and aliquots of sample or standard solutions where the final concentrations of quercetin were in the range of 0.01–2 µg ml^{-1}. The solutions were thermostated at 25 ± 0.1 °C and the fluorescence intensity was measured at 545 nm (after 5 min) using an excitation wavelength of 310 nm against a blank solution. Both emission and excitation slits were set at 10 nm.

Table 1. Determination of total flavonoids content (in quercetin equivalent) in pharmaceutical preparations obtained by Terbium Sensitized Fluorescence and spectrophotometry methods.

Sample type	Total flavnonoids content (in QE, mg/g)[a]	
	Terbium-sensitized fluorescence method	Spectrophotometric method (15)
Dermatin cream	0.204 ± 0.004 (1.96)[b]	0.222 ± 0.006 (2.70)
Calendula ointment	0.476 ± 0.007 (1.50)	0.499 ± 0.013 (2.60)
Hypiran drops	13.500 ± 0.460 (3.40)[c]	13.720 ± 0.196 (1.43)

[a] Average of three determinations ± SD.

[b] Numerical values in parentheses are the RSD (%) of three determinations.

[c] Total flavnonoids concentrations (as quercetin equivalent, µg/ml).

Determination of total flavonoids using specrto-photometric

Total flavonoids content of the samples were determined according to the reported colorimetric method (15) which is employed in the Iranian pharmaceutical companies. Briefly, aliquots of 0.5 ml of samples and standards were mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10% aluminum chloride hexahydrate, 0.1 ml of 1.0 M potassium acetate and 2.8 ml of double distilled water. After incubation at room temperature for 40 min, the absorbance of the reaction mixture was measured at 415 nm against a water blank on a spectrophotometer (Shimadzu, model 1650 PC) and quercetin as a standard. By using a seven point standard curve (0-40 µg/ml), the total flavonoids content of samples were determined in triplicates and the results were expressed as QE.

RESULTS AND DISCUSSION

Flourescence spectra

The UV-Vis absorbance and fluorescence spectra of the Tb-quercetin system have been previously described (14). The results indicated that when Tb^{3+} was added to the solution, the absorption spectrum of the complex is shifted to the long-wavelength region. Such bathochromic shift indicated that quercetin could form a stable complex with Tb^{3+} (14). Also, fluorescence spectra indicated that the addition of Terbium ions to the aqueous solutions of quercetin (pH 7.0) results in the appearance of the narrower and stronger new emission bands in the range of 450-600 nm (λ_{max} = 545 nm), characteristic of Terbium ion fluorescence due to the complex formation with quercetin, leading to energy transfer from quercetin to Tb^{3+}. These results showed that quercetin could be determined by the TSF method.

Optimization and analytical figures of merit

The experimental results indicated that Tris-HCl was the most suitable buffer and the maximum intensity of the fluorescence was obtained by 0.25 ml of 0.05 M of this buffer in a final 5 ml solution (pH 7.0).

The effect of the terbium concentration on the fluorescence intensity was also studied and fluorescence intensity maximum was obtained at a Tb^{3+} concentration of 4×10^{-3} M.

Under the above optimized conditions, a spectrofluorimetric method for determination of total flavonoids as QE was developed. The calibration graphs were found to be linear in the range of 0.01 and 2 µg/ml for quercetin and limit of detection calculated as $3S_b/m$ was found to be 0.002 µg/ml.

Determination of total flavonoids in pharmaceutical preparations

The TSF method successfully was applied for determination of the total flavonoid content, expressed in QE in the pharmaceutical preparations by using standard addition method. The spectral characteristics of the fluorescence spectra obtained for these preparations in comparison with the fluorescence emission spectrum of a quercetin standard solution indicated that there is no interference from the excipients and the emission peak of Tb^{3+}- quercetin complex at 545 nm can be quantified and expressed in terms of QE which can be used as a valuable index of the flavonoids content.

Table 1 shows the results for three replicated analyses. The total flavonoids content in Calendula ointment and Dermatin cream were 0.476 and 0.204 mg/g, respectively. The total flavonoids concentration in Hypiran drops was 13.50 µg/ml. As shown in table 1, the results obtained by the TSF and the spectrophotometry (commonly used as a reference method in the pharmaceutical companies for the assay of flavonoids) methods were in good agreement.

The accuracy of the method was determined by recovery studies. Recovery experiments on the clear solutions obtained by dissolution of the pharmaceutical preparations, spiked with different amounts of quercetin were also carried out (Table 2) and the obtained recoveries were between 96.1 % and 104.0 % with the RSD values less than 3.5%. It should be added that the recovery was calculated using.

Table 2. Recoveries of quercetin added to the pharmaceutical preparations.

Sample type	Amount added (mg/g)	Amount found (mg/g)[a]	Recovery (%)
Dermatin cream	0.857	1.030 ± 0.027 (2.60)[b]	96.8
	0.457	0.643 ± 0.008 (1.20)	96.1
	0.213	0.411 ± 0.006 (1.40)	97.2
Calendula ointment	0.151	0.624 ± 0.011 (1.76)	98.0
	0.076	0.551 ± 0.004 (0.72)	98.7
	0.038	0.513 ± 0.007 (1.40)	97.4
Hypiran drops[c]	1.000	14.52 ± 0.019 (0.13)	102.0
	0.750	14.23 ± 0.013 (0.09)	97.3
	0.250	13.76 ± 0.018 (0.13)	104.0

[a] Average of three determinations ± S.D.
[b] Numerical values in parentheses are the RSD (%) of three determinations and the amount found is sum of the added and found from standard addition method reported in Table 1.
[c] The given values µg/ml.

$$\left(\frac{\text{Total amount found – amount found from stadndard addition method}}{\text{Added amount}}\right)\times 100$$

A comparison of the length of the operations for the proposed fluorimetric method and a previous spectrophotometric method (15), and also other chromatographic methods (7-9), revealed that the time for each analysis is about 5 min which is very shorter than that of spectrophotometric (~ 40 min after separation of lipid phase of the pharmaceutical preparations which is required in both spectrofluorimetric and spectrophotometric methods) and also chromatographic methods (16, 17) and this is especially important when many samples should be analyzed daily. The procedure of sample preparation in comparison with other methods (11, 18) was very simple and only a simple pre-treatment step for separation of lipids before detection was required. It also gives good precision and accuracy and provides very low detection limit. Consequently, TSF may be the method of choice for determination of samples with very low flavonoid contents, where spectrophotometric method can not be used. The method was successfully applied to the determination of total flavonoid contents in the pharmaceutical preparations, with a simple dissolution, without interference from coexistent substances in the samples matrices.

ACKNOWLEDGMENT

The authors would like to thank the research affairs of the University of Tabriz for the financial support. We also thank Mrs Nazanin Gharib from Dineh Pharmaceutical Company for her technical assistance.

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