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Short communication

Isocratic RP-HPLC method for rutin determination in solid oral dosage forms

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Abstract

A rapid and sensitive assay for quantitative determination of rutin in oral dosage forms based on isocratic reversed phase high performance liquid chromatography (RP-HPLC) was developed and validated. Using a C_{18} reverse-phase analytical column, the following conditions were chosen as optimal: mobile phase methanol–water 1:1 (v/v), pH 2.8 (adjusted with phosphoric acid), flow rate = 1 mL min^{-1} and temperature T = 40.0 °C. Linearity was observed in the concentration range 8–120 μg mL^{-1} with a correlation coefficient of 0.99982 and the limit of detection (LOD) = 2.6 μg mL^{-1}, and limit of quantification (LOQ) = 8.0 μg mL^{-1}. Intra- and inter-day precision were within acceptable limits. Robustness test indicated that the mobile phase composition and pH influence mainly the separation. The proposed method allowed direct determination of rutin in pharmaceutical dosage forms in the presence of excipients, but is not suitable for preparations where compounds structurally/chemically related to rutin may be present.
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1. Introduction

Rutin (3,3′,4′,5,7-pentahydrohyflavone-3-rhamnoglucoside) is a flavonoid of the flavonol type, consisting of the flavonol quercetin and disaccharide rutinose (rhamnose and glucose) [1]. It is found in many typical nutrimental plants (especially in buckwheat, apple and black tea) and is an important dietary constituent of food and plant-based beverages [2–3]. Like other flavonoid derivatives, which all display a remarkable array of biological and pharmacological activities, rutin exhibits antioxidant, antiinflammatory, anticarcinogenic, antithrombic, cytoprotective and vasoprotective activities [4–7]. By increasing the strength of the capillaries and reducing their permeability, rutin helps preventing hemorrhages and ruptures in the capillaries and connective tissues, and is therefore often used to treat chronic venous insufficiency, hemorrhages and epitaxis [8].

So far, a number of analytical techniques have been described for rutin determination [9–25]. For example, Pharmacopoeia Helvetica recommends direct spectrophotometric detection based on the strong absorption of ultra-violet (UV) light by conjugated double bounds and hydroxyl groups [9] and indirect spectrophotometric methods based on the formation of colored chelating complexes with metal ions were proposed [10–13]. However, liquid chromatography is currently preferred method of choice for determination of rutin and flavonoids in general. Established in the late 1980s [14], reversed phase RP-HPLC approaches aim at separating, identifying and quantifying rutin in crude plant material/extracts and in plant-based beverages [15–24], whereas relatively low number of assays is directed towards the detection of rutin in oral dosage forms [25]. Recently, Dubber and Kanfer described a HPLC based approach for rutin determination in a mixture of flavonoids in *Ginkgo biloba* solid oral dosage forms [26]. In their approach,

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separation was achieved on a minibore C_{18} column involving complex gradient profiles and with a relatively long retention time (about 10 min).

This study describes a rapid, accurate and precise method for determination of rutin in tablets using isocratic RP-HPLC. The method has been validated with respect to linearity range, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness. The proposed method has been applied to the analysis of rutin in commercially available pharmaceutical preparations.

2. Experimental

2.1. Solvents and chemicals

Methanol (HPLC grade), ethanol and phosphoric acid (analytical reagent grade) were from Merck (Darmstadt, Germany). Solvent mixtures were filtered and degassed before use. Rutin as a standard compound was acquired from Fluka (Buchs, Switzerland), Rutinion® forte tablets (containing 100 mg rutin) from Biomo Natur-Medizin, GmbH, and Veneton® capsules (containing 10 mg rutin and 130 mg dry extract Hippocastani semen adjusted to a content of 16% escin) from Diethparm, Kirchberg.

2.2. Equipment

Isocratic RP-HPLC was performed using a Hewlett-Packard (Beaverton, OR) chromatograph equipped with high-pressure binary pumps HP 1100, a Rheodyne model injector (sample loop 20 μL) USA, Hewlett-Packard (HP) 1100A UV detector, controlled by IBM PC Pentium Vectra XA computer. A Zorbax Extend-C_{18} reverse-phase analytical column (150 mm × 46 mm, i.d., 5-μm particle size), Agilent, USA was used. The temperature was maintained at 40.0 °C throughout the study. The data were analyzed using the Chem Station software package.

2.3. Chromatographic conditions

The mobile phase was a binary mixture of methanol–water, 1:1 (v/v), adjusted to pH 2.8 with phosphoric acid. The flow rate was 1 mL min^{-1}. Absorbance was monitored at $\lambda = 360$ nm.

2.4. Standard solutions

Standard stock solution of rutin was prepared by dissolving 10.2 mg of rutin in ethanol, yielding 25 mL of a concentration $c_{stock} = 0.41$ mg mL^{-1}. Series of dilutions were prepared by aliquoting 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 mL of the standard stock solution and diluted with the mobile phase to yield 10 mL of standard solutions containing 2, 4, 8, 20, 40, 80, 120 and 200 μg mL^{-1} of rutin, respectively.

2.5. Sample solutions

Sample solutions were prepared by transferring a mass of powder equivalent to the average mass of one Rutinion forte tablet into a 250 mL volumetric flask. Two hundred milliliter of ethanol was added and the mixture was sonicated for 15 min at room temperature. The flask was then filled to volume with ethanol and the extract was filtered through a 0.2 μm Millipore membrane filter. One milliliter of this extract was transferred to a 10 mL volumetric flask and filled to volume with the mobile phase to give a final rutin concentration of 40 μg mL^{-1}. Sample solutions from Veneton capsules were prepared by transferring the content that is equivalent to the average mass of one capsule into a 50 mL volumetric flask. Twenty-five milliliter of warmed ethanol was added and the mixture was sonicated for 30 min. Thereafter, it was filled up to volume with warmed ethanol and cooled to room temperature. After cooling, the volumetric flask was refilled up to the mark with ethanol and the solution was filtered through Whattman No. 1 filter paper. 2.5 mL of this extract was transferred into a 10 mL volumetric flask, filled to volume with the mobile phase and filtered through a 0.2 μm Millipore membrane filter. The final concentration of rutin in the extract was 50 μg mL^{-1}.

Stability of the sample solutions was tested 24, 48 and 72 h after preparation and storage at 4.0 and 25.0 °C. Stability was assessed by comparing the chromatographic parameters of the solutions after storage with the same characteristics of freshly prepared solutions.

2.6. Assay validation

The proposed RP-HPLC method was validated according to the International Conference on Harmonization guidelines [27]. All measurements were performed in triplicates.

Linearity was evaluated in the range 2.0–200 μg mL^{-1}. Peak area *versus* rutin concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration were determined. Limits of detection and quantification were determined from the calibration curve using the following expressions: $3\sigma/S$ and $10\sigma/S$, where σ is the standard deviation and S is the slope of the calibration curve.

Method precision was evaluated by repeatability (intra-day) and intermediate precision (inter-day). Each level of precision was investigated by three sequential replicates of injections of rutin concentrations of 8, 40 and 120 μg mL^{-1}. Repeatability was evaluated on the same day, while intermediate precision was determined by comparing the assays for 5 days.

Accuracy of the proposed method was evaluated through the percentage of recovery of known amounts of rutin added to solutions of the commercial product (Rutinion). The analyzed samples were spiked with extra 80, 100 and 120 μg mL^{-1} of standard rutin solution. Accuracy was calculated from the following equation: [(spiked concentration − mean concentration)/spiked concentration] × 100.

For robustness test, the response surface method (RSM) was carried out to obtain comprehensive information and to investigate the response around the nominal values of the factors [28]. In particular, the impact of the following factors: the percentage of methanol in the mobile phase, pH of the mobile phase, the flow rate and the column temperature on the peak area of rutin were determined.

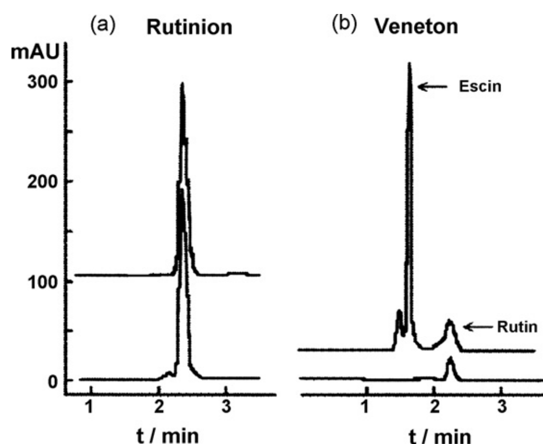

Fig. 1. Chromatograms recorded for rutin as a reference standard (lower curves): (a) Rutinion® forte tablets and (b) Veneton® capsule. Conditions: Zorbax C18 column, mobile phase methanol–water 1:1 (v/v), pH 2.8 (adjusted with phosphoric acid), flow rate = 1 mL min^{-1}, injector loop volume = 20 μL, UV detection at λ = 360 nm.

3. Result and discussion

3.1. Method optimization

To optimize the operating conditions for isocratic RP-HPLC detection of rutin a number of parameters such as the column type, mobile phase composition, pH, effectors (phosphoric or acetic acid) and the flow rate were varied. Conditions giving the shortest retention time (t_r = 2.3 min) and no apparent drug decomposition: C_{18} reverse-phase analytical column, mobile phase methanol–water 1:1 (v/v), pH 2.8 (adjusted with phosphoric acid), flow rate = 1 mL min^{-1} and temperature T = 40.0 °C, were chosen as optimal. A representative chromatogram is shown in Fig. 1 (note that different standard concentrations of rutin are presented because of the high concentration of escin in Veneton capsules).

3.2. Method validation

3.2.1. Linearity, limit of detection and quantification

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was found in the concentration range 8–200 μg mL^{-1}, as confirmed by the correlation coefficient of 0.99982. The peak area (y) is proportional to the concentration of rutin (x) following the regression equation $y = 23800x - 32$. The experimentally derived LOD and LOQ for rutin were determined to be 2.8 and 8.0 μg mL^{-1}, respectively.

3.2.2. Precision

Precision data on the intra- and inter-day variation for three different concentration levels are summarized in Table 1. Both inter- and intra-day R.S.D. were less than 2%, indicating a sufficient precision.

3.2.3. Accuracy

Rutin recovery from pharmaceutical dosage forms after spiking with 80, 100 and 120 μg mL^{-1} of additional standard was 101.1% with R.S.D. below 2% for all analyzed concentrations (Table 2), confirming the accuracy of the method.

3.2.4. Robustness

For the robustness test, three-dimensional graphs (3D) were constructed, as shown in Fig. 2. Three-dimensional graphs represent peak area dependence on: the percentage of methanol and pH of the mobile phase (Fig. 2a); percentage of methanol and the flow rate of the mobile phase (Fig. 2b); the flow rate of the mobile phase and the temperature of the column (Fig. 2c). Effects of the selected factors were evaluated over a range of conditions by determining the maximum rutin response (quantification). The 3D graphs indicate that chromatographic behavior of rutin was mostly influenced by the mobile phase composition and pH. Highest response was obtained for methanol concentrations within 45–65% and pH range 2.5–3.0. Among the studied factors, the flow rate had a minor influence on rutin peak area and was kept at the value of 1 mL min^{-1}. The column temperature influences the retention time but has no significant impact on the peak area. It was therefore maintained at 40.0 °C throughout the study.

3.2.5. Analysis of pharmaceutical formulation

Validity of the proposed method was tested for pharmaceutical preparations by assaying Rutinion forte tablets (labeled to contain 100 mg of rutin) and Veneton capsule (10 mg of rutin). The shape of the peaks was not altered by substances present in the matrix. For Veneton capsule, sufficient separation between the peaks of rutin and escin is achieved by the proposed method (R_s = 2.1). The content of rutin is found to be (99.5 ± 0.8) mg, and (10 ± 0.2) mg for Rutinion and Veneton, respectively, in accordance with suggested limits of USP 24 (Official Monographs, p. 297).

Stability of rutin in the sample solutions was evaluated to verify whether spontaneous degradation occurred within 3 days. The results were calculated as the percentage of non-degraded rutin at the specified time intervals. Rutin from Rutinion tablets showed less than 5% degradation at both investigated tem-

Table 1
Precision of the assay for three different concentrations of rutin: intra-day and inter-day variations

	Repeatability (intra-day variation)			Intermediate precision (inter-day variation)		
Rutin taken (μg mL^{-1})	8.0	40.0	120.0	8.0	40.0	120.0
Rutin found (μg mL^{-1})	8.2	39.5	122.0	7.8	41.0	117.5
S.D. (μg mL^{-1})	0.15	0.40	1.05	0.15	0.70	2.00
R.S.D. (%)	1.83	1.01	0.85	1.92	1.71	1.70

All values derived from n = 7 independent measurements.

Table 2
Accuracy of the developed method

Spiked concentration ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$) mean ± S.D.	Recovery (%)	Accuracy (%)	R.S.D. (%)
80	81 ± 1.26	101.2	1.25	1.55
100	98 ± 0.63	98.0	2.00	0.64
120	121 ± 1.34	100.8	1.83	1.11

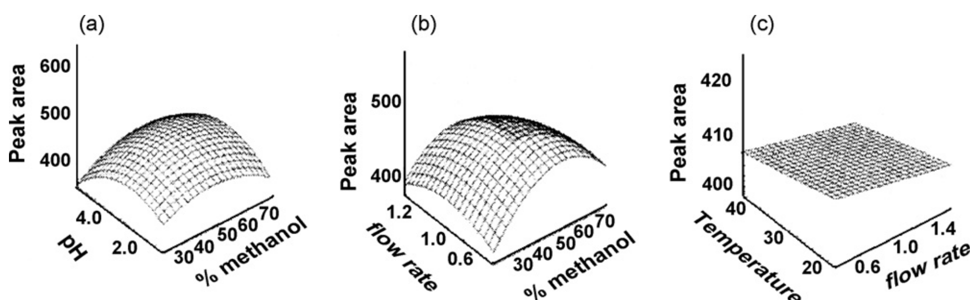

Fig. 2. Three-dimensional graphs. (a) Peak area = f (methanol percentage in the mobile phase, pH), (b) peak area = f (methanol percentage in the mobile phase, flow rate) and (c) peak area = f (flow rate, temperature).

peratures. In contrast, rutin from Veneton capsules showed a considerable degradation (10.5%) after 72 h at 25 °C.

4. Conclusion

Isocratic RP-HPLC provides a convenient and efficient method for determination of rutin in dosage forms. Sample preparation was simple, both samples were stable at 4.0 °C for 3 days and no tedious clean up was required. There was no interference of excipients and escin in the examined products, thus no additional extraction or separation procedures were required. The method shows the necessary speed (retention time lower than 5 min), accuracy and precision for rutin determination in dosage forms. This method is not suitable for preparations where other compounds structurally/chemically related to rutin may be present. In such cases, unequivocal peak identification is not possible by using UV absorption for detection. In such preparations, complete separation of flavonoids can be achieved only in complex gradient profiles.

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