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ORIGINAL ARTICLE

O-(β-hydroxyethyl)rutosides determination by micellar flow injection (FI)-spectrofluorimetry

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KEYWORDS

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Abstract A simple, eco-friendly, sensitive and economic flow injection spectrofluorimetric method was developed for the determination of O-(β-hydroxyethyl)rutosides. The procedure was based on the use of an anionic surfactant such as sodium dodecyl sulfate to provide an appreciable O-(β-hydroxyethyl) rutosides fluorescence enhancement, increasing considerably the sensitivity of detection. All the variables affecting the fluorescence intensity were studied and optimized. The flow rate was 5 mL/min with detection at 450 nm (after excitation at 346 nm). A linear correlation between drug amount and peak area was established for O-(β-hydroxyethyl)rutosides in the range of 0.01–200 μg/mL with a detection limit of 0.001 μg/mL ($s/n=3$). Validation processes were performed by recovering studies with satisfactory results. The new methodology can be employed for the routine analysis of O-(β-hydroxyethyl)rutosides in bulks as well as in commercial formulations.

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1. Introduction

Hydroxyethylrutosides (Fig. 1, oxerutins, O-β-hydroxyethyl-rutosides, HR) are hydroxyethyl acetylations of rutoside, semisynthetic derivatives of *Sophora japonica* L. (Leguminosae), a well-known traditional Chinese medicinal herb, officially listed in the Chinese Pharmacopoeia [1]. Its buds and fruits have been used as hemostatic agent in traditional Chinese medicine. Pharmacological studies and clinical practice have demonstrated that *S. japonica* L. has anti-tumour, anti-fertility and anti-cancer activities [2,3] and the active constituents of the herb are flavonoid components. The flavones from its buds and pericarps were discovered as hemostatic constituents [4,5].

HR has been used in the treatment of varicose veins and other disorders as hemorrhoids which cause blood to pool. It is in a class of medications known as flavonoids and works by repairing damage to capillaries and making them less susceptible

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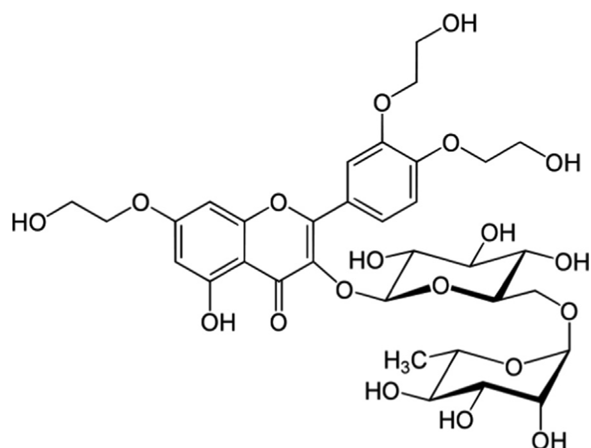

Fig. 1 Chemical structure of troxerutin.

to subsequent damage. HR helps to stabilize cell walls, making them less permeable, so that fluids are passed between cells and blood cells at normal levels. This prevents blood from pooling in the veins, which is the main cause and symptom of the varicose vein disorder.

A number of methodologies have been developed to determine unlabeled HR and their metabolites (Table 1) such as TLC, circular dichroism, and spectrofluorimetry [6–8]. More reliable HPLC with UV, diode array detection or fluorescence detection methods were developed to monitoring different flavonoids in various body fluids of humans and animals [9–11]. More recently, HPLC with electrochemical detection was used for the determination of 7-monohydroxyethylrutoside in plasma [12]. British, European and US Pharmacopoeias [13–15] do not have a method for determining HR in raw drug or pharmaceuticals.

The flow injection (FI) technique is very useful for the rapid determination of many samples because it can accomplish high sampling automatism with good precision. Moreover, an ideal analytical method for routine analysis and quality assurance should be simple, cost-effective, robust, precise and accurate, and have a high sample analysis frequency [16,17]. These automatic techniques offer significant advantages for the determination and the monitorship of one analyte (e.g. the active ingredient) and therefore can be applied to routine analysis.

The purpose of this work is to develop an alternative FI spectrofluorimetric methodology for the determination of total oxerutins in pharmaceuticals. The loss of sensitivity produced in flow-injection systems by dispersing the analyte into the carrier can be saved using a molecular fluorescence as a detector; additionally, a beneficial enhancement of natural analyte emission can be produced by micellar media.

Table 1 Methods for HR determination.

Instrumental methodology	Comments	LOD	LOL	RSD	Samples	Ref.
TLC	The inclusion capacity of cyclodextrins follows the order HP-β-CD > M-β-CD > β-CD > γ-CD. Rutin is more easily included by the studied cyclodextrins than venoruton. Other experimental conditions: pH = 9.7, 20 °C.	WD	WD	WD	Pure drug	[6]
Circular dichroism	After oral administration of 4 g HR the blood concentration of Venoruton has a maximum between 6 and 10 h and the drug can be detected by circular dichroism up to 25 h in blood. However, evaluation of the data for pharmacokinetics is not possible. $\lambda = 345$ nm.	WD	WD	WD	Serum and urine	[7]
Spectrofluorimetry	The method is based on the fluorescence behavior of the HR–aluminum complex in absolute methanol; $\lambda_{ex/em} = 420/480$ nm.	WD	0.1–4.0 µg/mL	1.34	Urine	[8]
HPLC-UV and fluorescence detection	A microcolumn of Amberlite XAD-2 is used to preconcentrating HR before the injection in LiChrosorb RP-8 column using solvent gradient ($\lambda_{ex/em} = 355/460$ nm).	1 mg/L	WD	8	Serum	[9]
HPLC-diode array detection	Reversed-phase HPLC is use for the determination of the biologically active plant phenolic compounds mangiferin, likviritin and dihydroquercetin. Other experimental conditions: C18 column; 290 nm.	0.2/0.5 µg/mL	0.5–20 µg/mL	3.8	Rat plasma and urine	[10]
HPLC-UV detection	Quercetin was evaluated by HPLC using kaempferol as internal standard; Other experimental conditions: C18 column; pH 3.5; flow-rate: 1.0 mL/min; 375 nm.	0.1 µg/mL	300 nM–3 mM	9.9	Human plasma	[11]
HPLC-electrochemical detection	MonoHER was analyzed by HPLC, using a reversed-phase ODS column, with a mobile phase consisting of 49% methanol and 51% of an aqueous solution containing 10 mM sodium dihydrogen phosphate (pH 3.4), 10 mM acetic acid and 36 mM EDTA.	WD	0.3–75 mM	19.5	Plasma	[12]
This work	Flow injection spectrofluorimetric method is used for HR determinations. Experimental conditions: $\lambda_{ex/em} = 346/450$ nm; pH 9.0; SDS: 20 mM; flow rate: 5 mL/min; sampling rate: 30 samples/h.	0.001 µg/mL	0.01–200 µg/mL	4.86	Pharmaceuticals	

LOD: Limit of detection, LOL: Limit of lineality, RSD: Relative standard deviation, TLC: Thin layer chromatography, CD: cyclodextrin, HP-β-CD: hydroxypropyl-β-cyclodextrin, M-β-CD: methyl-β-cyclodextrin, HPLC: High-performance liquid chromatography, UV: ultraviolet, MonoHER: 7-monohydroxyethyl rutoside, WD: without datum.

2. Experimental

2.1. Apparatus

All fluorescent measurements were carried out on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu, Analytical Instrument Division, Kyoto, Japan), equipped with an Xenon discharge lamp and 1 cm quartz cells. For flow measurements, an LC flow cell unit (12 μL cell) was used. The fluorescent emissions were measured at λ_{em} 450 nm, using a λ_{exc} 346 nm. An injection valve (Rheodyne Model 5041) was used for FIA configuration. Solutions were propelled by a peristaltic pump (Gilson Minipuls 3) with pumping tubes. All tubes connecting the different components of the flow system were PVC, 0.8 mm i.d. All pH measurements were made with a pH meter (Model EA 940, Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) with combined glass electrode.

2.2. Reagents

O-β-hydroxyethylrutosides was purchased from Lab. Novartis (Buenos Aires, Argentina). Sodium dodecyl sulphate (SDS) and hexadecyltrimethylammonium bromide (HTAB) were obtained from Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan). Triton X-100 (TX-100) was purchased from Merck (Darmstadt, Germany).

2.3. Preparation of standard solutions and samples

Standard solutions: HR standard solution containing 0.1 mg/mL was prepared by dissolving the reagent in water. Under these conditions, HR solution was found to be stable for several weeks when kept in dark. A standard working solution of 10 μg/mL was prepared daily by dilution of stock standard solution with dually distilled water and stored in a dark bottle. SDS solution (20 mM) and basic solution (20 mM borax) were prepared in dually distilled water.

Sample: Five capsules of Venorutón® (Lab. Novartis, Bs. As., Argentina) or five tablets of Veraldid® (Soubeiran Chobet, Bs. As., Argentina) were weighed (to obtain the average mass of tablets) and finely powdered. A portion of the powder, equivalent to 300 mg of HR (one capsule) or 250 mg (one tablet), was accurately weighed and dissolved in 80 mL of bidistilled water and filtered to remove insoluble material. The filtered solution was transferred to a 100 mL volumetric flask and completed to the volume with dually distilled water. The final concentration was about 3 or 2.5 mg/mL of HR. A solution of 0.1 mg/mL was prepared by dilution of the sample solution with bidistilled water. Further dilutions were done daily with bidistilled water.

A portion of Venorutón® gel, equivalent to 50 mg of HR, was accurately weighed and dissolved in 40 mL of bidistilled water and filtered to remove insoluble material. The filtered solution was transferred to a 50 mL volumetric flask and completed to the volume with bidistilled water. The final concentration was about 1 mg/mL of HR. A solution of 0.1 mg/mL was prepared by dilution of the sample solution with bidistilled water. Further dilutions were done daily with bidistilled water.

2.4. General procedure

A stream of sample or standard solutions containing HR was combined with the carrier stream prepared with SDS (20 mM) and buffer sodium tetraborate (pH 9) to obtain the optimal conditions for analyte fluorescent emission (Fig. 2). The drug contained in the sample or standards and the carrier stream interacted in the mixed coil (MC) and flowed to the fluorescence detector. The manual valve was switched in such a manner that allowed, in one position, to pass the carrier stream to the detector to generate the blank signal, and in the second position allowed to pass the sample/standards and carrier solution.

2.5. Validation procedure

In order to demonstrate the validity of this methodology, an accurate weight of commercial tablets (Veraldid®) powder containing 250 mg of HR was dissolved and transferred to a 100 mL volumetric flask and completed to the volume with water. A portion of 4 μL of this sample solution was transferred to 10 volumetric flasks of 10 mL. The general procedure was applied to six portions and the average quantity of HR determined was taken as a base value. Then, increasing quantities of HR were added to other four aliquots of sample and HR concentrations were determined by applying the addition standard method.

The same validation procedure was applied for Venorutón® capsules and gels.

3. Results and discussion

3.1. Fluorescence characteristics of HR in aqueous and micellar media

In order to study the spectral behavior of HR, systems were prepared containing aqueous solutions and micellar solutions of drug: anionic (SDS 0–20 mM), cationic (HTAB 0.9–9 mM) and non-ionic (TX-100 0.05–0.1 M). In aqueous solution, HR showed a fluorescent emission at 468 nm when excited at 346 nm (Fig. 3). In HTAB and TX-100 solutions, HR did not present beneficial

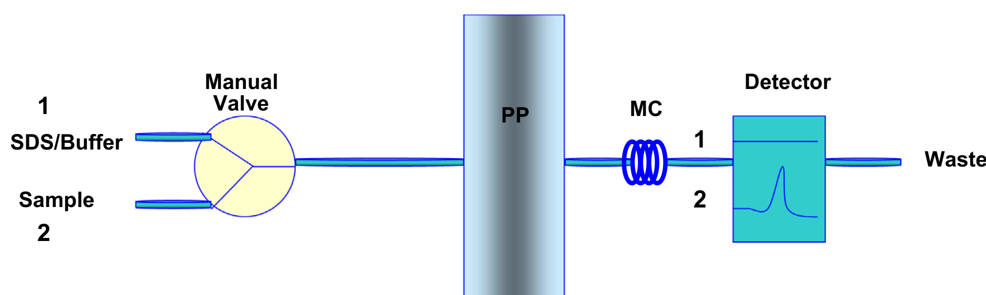

Fig. 2 FIA configuration. PP: Peristaltic pump. MC: Mixed coil.

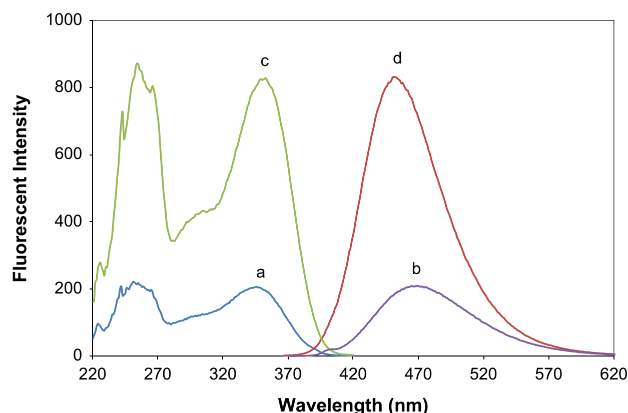

Fig. 3 Excitation and emission fluorescence spectra for HR. (a, b) HR in aqueous solutions; (c, d) in SDS micellar media. $C_{HR}=5$ μg/mL; $C_{SDS}=20$ mM. $\lambda_{exc}=346$ nm; $\lambda_{em}=450$ nm.

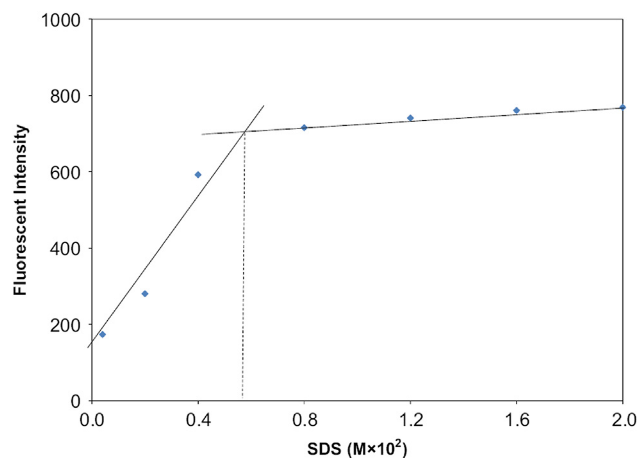

Fig. 4 Determination of experimental CMC value for HR–SDS system. $C_{HR}=5$ μg/mL. $C_{SDS}=0$–20 mM. $\lambda_{exc}=346$ nm; $\lambda_{em}=450$ nm. CMC value obtained: 0.57 mM.

changes in emission signals. When SDS micellar medium was used, a hypsochromic shift and a beneficial intensity increase were observed with a maximum of emission at 450 nm when excited at 346 nm. Experimental data showed an enhancement factor for the HR–SDS system of 4-fold with respect to HR fluorescence in water medium; thus, the anionic surfactant SDS was chosen for further analysis, at a concentration of 20 mM. Likewise, these wavelengths for HR–SDS system ($\lambda_{em}=450$ nm; $\lambda_{exc}=346$ nm) were selected to measure the fluorescence intensity for the subsequent assays.

With the aim of establishing the optimal SDS concentration allowing the best increase in HR fluorescent signal, experiments were performed by varying SDS concentration and maintaining constant other experimental conditions. The results obtained (Fig. 4) have highlighted that the CMC value of SDS has been diminished by the presence of HR, giving a value of 0.57 mM, being less than for the SDS solution [18].

3.2. *Influence of several experimental variables*

Fig. 5 depicts the effect of pH value on the fluorescence signal for HR. Analysis of spectrofluorimetric data for HR in SDS micellar

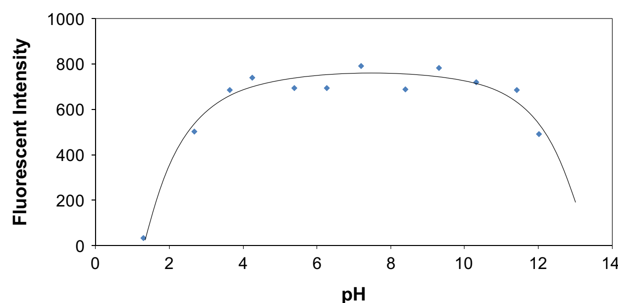

Fig. 5 Effect of pH on HR fluorescence intensity. $C_{HR}=5$ μg/mL; $C_{SDS}=20$ mM; $\lambda_{exc}=346$ nm; $\lambda_{em}=450$ nm.

solutions showed a maximal intensity in the pH range of 3–10 and a decrease to extremely acidic or alkaline pH. The working pH selected was 9, using sodium tetraborate as the buffer solution. The effect of the concentration of the buffer solution was studied. Fluorescence intensity remained constant in the maximum emission for buffer concentrations from 1 mM to 20 mM; higher concentrations led to a fluorescence decay. In order to maintain pH value in a constant optimal value, a concentration of 20 mM was selected and used in the following assays.

3.3. *FI system optimization*

Several experiments were done in order to study the effect of the length of the mixed coil and the flow rate of the FI system on the HR fluorescent signal. The presence of the mixed coil in the FIA manifold ensures adequate a mixture of SDS and the HR sample due to convective forces in play, guaranteeing the arrival to the detector of a homogeneous composition. The mixed coil length was evaluated in order to obtain the HR highest signal from 5 to 15 cm. The best results were obtained using an MC of 10 cm.

Then, experiments were realized by varying the flow rate. The results showed that the fluorescent signal increased with the increase of flow rate. For rates > 5 mL/min great turbulence was observed, due to the presence of SDS surfactant and the introduction of bubbles into the FI system, with consequent fluctuation in the fluorescent values. A flow rate of 5 mL/min was selected as optimal. Under these optimal conditions the sampling rate was 30 samples/h.

3.4. *Analytical performance and validation*

3.4.1. *Linearity*

The equation for the calibration graph was obtained by least-squares regression analysis, employing the areas of the analyte standard fluorescent signals: $F=0.6143C+6.68$, where F is the relative fluorescence intensity and C is the concentration of HR. The method was linear in the range of 0.01–200 μg/mL HR. The correlation coefficient (r^2) was 0.9959. The figures of merit obtained demonstrated the good performance of the calibration.

3.4.2. *Limit of detection and limit of quantification*

The limit of detection (LOD) was defined as the compound concentration that produced a signal:noise (*s/n*) ratio >3, while the limit of quantification (LOQ) of the assay was evaluated as the concentration equal to 10 times the value of the *s/n* ratio [19]. LOD and LOQ values for this method, based upon these criteria, were 0.001 and 0.01 μg/mL, respectively.

Table 2 Validation of the proposed method for HR determination in commercial pharmaceutical formulae.

Sample	Added μg/mL)	Within-day			Between-day		
		Found[a]	Recovery[b] (%)	RSD (%)	Found[a]	Recovery[b] (%)	RSD (%)
Veraldid®	–	0.13	–	–	0.14	–	–
tablets	0.1	0.21	110.00	4.75	0.19	92.10	4.86
	0.2	0.29	99.05	4.43	0.32	110.00	4.09
	0.4	0.52	105.08	3.90	0.53	109.96	3.89
	–	1.09	–	–	1.13	–	–
Venorutón®	1	2.07	107.99	3.21	2.10	110.00	3.87
capsules	2	2.91	95.51	3.98	3.02	101.15	3.63
	4	5.11	102.75	2.81	5.16	104.02	2.98
	8	8.88	98.52	2.42	8.76	97.89	3.08
Venorutón®	–	10.98	–	–	9.54	–	–
gel	10	19.82	98.20	2.38	21.00	110.00	3.67
	20	31.99	109.95	1.53	28.66	93.39	3.53
	40	48.67	96.68	2.02	48.59	96.47	3.31
	80	90.35	100.44	1.26	87.62	97.02	2.94

[a]Mean value, n = 6.
[b]Recovery = [(found−base)/added] ×100.

3.4.3. Reproducibility
The fiagram was registered for different standards of HR: (a) 2 μg/mL; (b) 3 μg/mL; (c) 4 μg/mL; (d) 6 μg/mL; and (e) 8 μg/mL. The triplicate signals demonstrated good reproducibility for the developed methodology.

3.4.4. Accuracy and precision
The within-day and between-day precisions of the method, based on repeatability, were determined by replicating injections ($n=6$) on five sample solutions of different commercial formulations prepared by the standard addition method at different concentration levels. A relative standard deviation (RSD) $\leq 4.86\%$ was obtained in all cases. The within-day and between-day recoveries were in the range of 92.10–110.00% (Table 2).

3.4.5. Specificity
The presence of other active drugs with which pharmaceuticals containing HR can be formulated (such as magnesium ascorbate), or of the common excipients of the analyzed formulations, was observed not to cause spectral interferences. This is an important advantage of the method, since it allows for the analysis of HR without a prior separation step.

3.4.6. Robustness
This was evaluated by challenging each operational parameter of the proposed method such as concentration of SDS, pH and flow rate. It was found that none of these variables significantly affected the analytical parameters of the method. This provides an indication of the reliability of the proposed method during normal usage, and so it can be considered robust.

3.5. Applications: determination of HR in pharmaceuticals

To assess the utility of the proposed method, several pharmaceutical preparations in different physical forms were analyzed by applying the general procedure. Results are included in Table 3. All values were in good agreement with the nominal values.

Table 3 Analysis of HR in pharmaceutical samples by the developed spectrofluorimetric method ($n=6$).

Sample[a]	HR nominal quantity	HR found	E^c (%)
Capsules[a]	300 mg	309.85 mg	3.28
Gel[a]	2 g per 100 g	2.2 g	9.12
Tablets[b]	250 mg	236.99 mg	5.20

[a]Venorutón® (Novartis, Bs. As., Argentina).
[b]Veraldid® (Soubeiran Chobet, Bs. As., Argentina), HR content 250 mg, and 150 mg of magnesium ascorbate.
[c]Percentual relative error (calculated considering that the preparations contain the amount reported by the manufacturing laboratories).

4. Conclusions

Taking into account the characteristics of the FI-spectrofluorimetry used in this work, the proposed methodology for the determination of HR is more simple, sensitive and rapid than the procedures previously reported. A sampling rate of 30 samples/h was attained, without including the pretreatment sample time. The use of SDS micellar system provides a simple means to enhance the fluorescence from HR giving about 4-fold increase in sensitivity and improves the limit of detection without further sample manipulation. The proposed method shows good accuracy and reproducibility, compared with the other methods described (see Table 1) and has been applied to the determination of HR in pharmaceuticals, since there is no interference from excipients or additives that might be found in different commercial formulations. Due to the small volumes of sample and reagents employed and their nontoxic characteristics, this method contributes to a clean or environmentally friendly analytical chemistry.

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