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

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KEYWORDS
O-(β-hydroxyethyl)rutosides; Micellar enhancement; Flow injection; Spectrofluorimetry; Pharmaceuticals

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 mg/mL with a detection limit of 0.001 mg/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 \textit{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 \textit{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...
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 monitoring 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.

<table>
<thead>
<tr>
<th>Instrumental methodology</th>
<th>Comments</th>
<th>LOD</th>
<th>LOL</th>
<th>RSD</th>
<th>Samples</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>The inclusion capacity of cyclodextrins follows the order HP-β-CD &gt; M-β-CD &gt; β-CD &gt; γ-CD. Rutin is more easily included by the studied cyclodextrins than venoruton. Other experimental conditions: pH=9.7, 20 °C.</td>
<td>WD</td>
<td>WD</td>
<td>WD</td>
<td>Pure drug</td>
<td>[6]</td>
</tr>
<tr>
<td>Circular dichroism</td>
<td>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.</td>
<td>WD</td>
<td>WD</td>
<td>WD</td>
<td>Serum and urine</td>
<td>[7]</td>
</tr>
<tr>
<td>Spectrofluorimetry</td>
<td>The method is based on the fluorescence behavior of the HR–aluminum complex in absolute methanol; $\lambda_{ex/em}=420/480$ nm.</td>
<td>WD</td>
<td>0.1– 4.0 μg/mL</td>
<td>1.34</td>
<td>Urine</td>
<td>[8]</td>
</tr>
<tr>
<td>HPLC-UV and fluorescence detection</td>
<td>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).</td>
<td>1 mg/L</td>
<td>0.2/ 0.5 μg/mL</td>
<td>0.5– 20 μg/mL</td>
<td>3.8</td>
<td>Rat plasma and urine</td>
</tr>
<tr>
<td>HPLC-diode array detection</td>
<td>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.</td>
<td>0.1 μg/mL</td>
<td>300 nM– 3 mM</td>
<td>9.9</td>
<td>Human plasma</td>
<td>[11]</td>
</tr>
<tr>
<td>HPLC-UV detection</td>
<td>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.</td>
<td>3.8</td>
<td>9.9</td>
<td>Human plasma</td>
<td>[11]</td>
<td></td>
</tr>
<tr>
<td>HPLC–electrochemical detection</td>
<td>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.</td>
<td>WD</td>
<td>0.3– 75 mM</td>
<td>19.5</td>
<td>Plasma</td>
<td>[12]</td>
</tr>
<tr>
<td>This work</td>
<td>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.</td>
<td>0.001 μg/ 0.01– 200 μg/mL</td>
<td>4.86</td>
<td>Pharmaceuticals</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 a 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 λem 450 nm, using a λexc 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 doubly distilled water and stored in a dark bottle. SDS solution (20 mM) and basic solution (20 mM borax) were prepared in doubly distilled 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.

Figure 2 (FIA configuration. PP: Peristaltic pump. MC: Mixed coil.)

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
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 fluorescence 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 FIA 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 \( \mu \)g/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-to-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 \( \mu \)g/mL, respectively.
3.4.3. Reproducibility
The diagram 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) ≤ 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 2 Validation of the proposed method for HR determination in commercial pharmaceutical formulae.

| Sample | Added μg/mL | Within-day |  | Between-day |  |
|--------|-------------|------------|----------------|------------------|
|        |             | Founda | Recoveryb (%) | RSD (%) | Founda | Recoveryb (%) | RSD (%) |
| Veraldid®, tablets | 0  | 0.13 | – | – | 0.14 | – | – |
|             | 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®, capsules | 1  | 2.07 | 107.99 | 3.21 | 2.10 | 110.00 | 3.87 |
|             | 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®, gel | 10 | 10.98 | – | – | 9.54 | – | – |
|             | 20 | 19.82 | 98.20 | 2.38 | 21.00 | 110.00 | 3.67 |
|             | 40 | 31.99 | 109.95 | 1.53 | 28.66 | 93.39 | 3.53 |
|             | 80 | 48.67 | 96.68 | 2.02 | 48.59 | 96.47 | 3.31 |
| aMean value, n = 6.  
| bRecovery = [(found−base)/added] ×100.

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

<table>
<thead>
<tr>
<th>Samplea</th>
<th>HR nominal quantity</th>
<th>HR found</th>
<th>E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsulesa</td>
<td>300 mg</td>
<td>309.85 mg</td>
<td>3.28</td>
</tr>
<tr>
<td>Gelb</td>
<td>2 g per 100 g</td>
<td>2.2 g</td>
<td>9.12</td>
</tr>
<tr>
<td>Tabletsb</td>
<td>250 mg</td>
<td>236.99 mg</td>
<td>5.20</td>
</tr>
</tbody>
</table>
| aVenorutón® (Novartis, Bs. As., Argentina).  
| bVeraldid® (Soubeiran Chobet, Bs. As., Argentina), HR content 250 mg, and 150 mg of magnesium ascorbate.
| cPercentual 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.
Acknowledgments

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