*Journal of Chromatography*, 272 (1983) 333—340
*Biomedical Applications*
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1500

# QUANTITATIVE DETERMINATION OF O-(β-HYDROXYETHYL)-RUTOSIDES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

WILHELM KUHNZ, KARL ZECH, RUDOLF LUPP, GÜNTHER JUNG* and WOLFGANG VOELTER

*Institut für organische Chemie der Universität Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen (G.F.R.)*

and

F. MATZKIES

*Kurparkklinik, D-8740 Bad Neustadt/Saale (G.F.R.)*

(First received July 24th, 1980; revised manuscript received September 22nd, 1982)

## SUMMARY

A procedure for the quantitative determination of O-hydroxyethylated rutosides by high-performance liquid chromatography is described, which can be used for the detection of these modified flavonoids in human serum. Serum samples are processed by the addition of acetone, which removes most of the proteins. After passing the supernatant through a microcolumn of Amberlite XAD-2 and washing with water, the hydroxyethylated rutosides are eluted with methanol. The eluate is concentrated in vacuo. The methanolic solution of the residue is chromatographed on RP-8 columns using UV and fluorescence detectors. The mono- to tetrahydroxyethylated constituents and their corresponding aglycones could be separated with a step gradient, starting with a solvent system of water—methanol—acetic acid (70:30:6) followed by a mixture of water—ethanol—acetic acid (70:30:6). Alternatively, the rutosides can be separated by a linear gradient of water—acetonitrile. An almost linear calibration curve and about 80% recovery are obtained. A detection limit of 1 mg/l is achieved. Pharmacokinetic studies in human volunteers are described.

## INTRODUCTION

O-(β-Hydroxyethyl)-rutoside (HR) has been used successfully in the therapy of various diseases like venous insufficiency and radiation damage [1, 2]. In order to obtain reliable information about the pharmacokinetic characteristics of HR, a method had to be developed which allowed quantification of

0378-4347/83/0000—0000/$03.00 

the drug in human serum. The development of a suitable assay for HR in blood is a particularly difficult problem because HR is a mixture of rutosides, which are hydroxyethylated to a different degree (mono- to tetrahydroxyethyl) and at different positions [1]. High-performance liquid chromatography (HPLC) in a reversed-phase system proved to be an efficient and sensitive method to solve the problem. Processing of serum samples and chromatographic separation is described including a successful application to a pharmacokinetic study after intravenous administration of HR to human volunteers.

## MATERIALS

### *Chemicals*

The commercially available mixture of the drug O-(β-hydroxyethyl)-rutoside (HR) was provided by Zyma (Munich, G.F.R.). The individual components of this flavonoid mixture were obtained by purification on Sephadex LH-20, obtained from Pharmacia (Uppsala, Sweden). Subsequent acid hydrolysis of the separated compounds yielded their aglycones [3, 4]. The individual components were identified by gas chromatography and mass spectrometry [5].

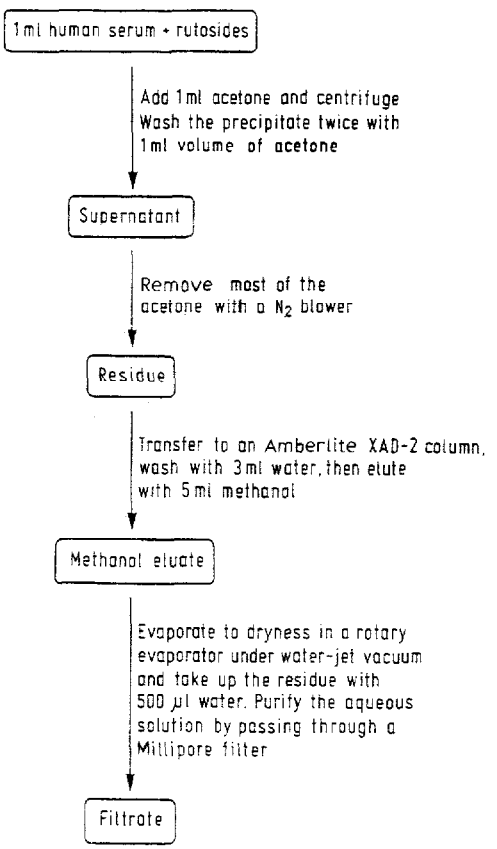


Fig. 1. Processing scheme for O-(β-hydroxyethyl)-rutosides in human serum.

Freshly distilled solvents were used, together with the resin Amberlite XAD-2, supplied by Serva (Heidelberg, G.F.R.).

*Blood samples*

The serum samples were obtained from hospitalized volunteers, whose identity was unknown to us. Serum samples were kept deep frozen and thawed at room temperature before use.

## METHODS

*Processing of serum samples*

In each case 1 ml of serum was processed (Fig. 1). After acetone precipitation of most of the serum proteins, chromatography of the residue was carried out on an Amberlite XAD-2 column (50 × 6 mm). The salts, free sugars and other interfering components were first removed with water; finally the rutoside mixture was eluted with methanol and concentrated in vacuo.

*High-performance liquid chromatography*

HPLC measurements were made with equipment from Waters Assoc., consisting of two pumps (type M 6000A), a solvent programmer (Model 660), an integrator and a UV detector (Model 440) with a fixed wavelength of

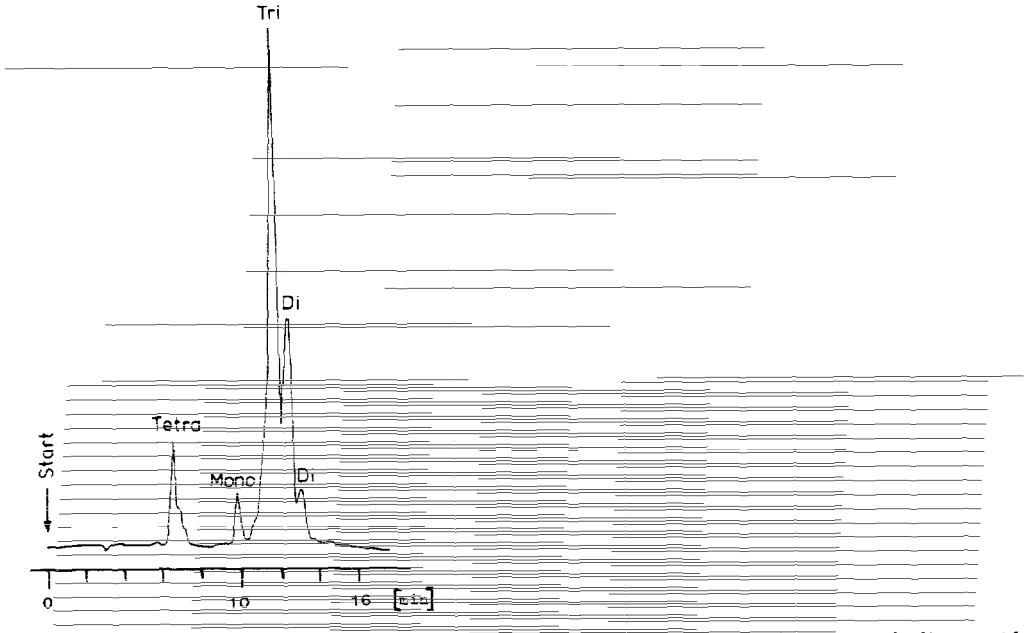


Fig. 2. HPLC chromatogram of the standard mixture of the O-(β-hydroxyethyl)-rutosides (HR). The assignment of the individual components is based on chromatographic comparison with the pure compounds of confirmed structure. Conditions: LiChrosorb RP-8 (10 μm, 250 × 4 mm, Merck); mobile phase, water–methanol–acetic acid (70:30:6); flow-rate, 0.9 ml/min; ambient temperature.

254 nm. Fluorescence detection was performed with an instrument from Schoeffel (Model FS 970) with an excitation wavelength of 355 nm, and emission at 460 nm and above. The fluorescence detector was connected to a Hewlett-Packard liquid chromatograph (type 1084B) equipped with an automatic sample applicator and integrator.

The columns used were either LiChrosorb RP-8 (10 $\mu$m, 250 × 4 mm) or LiChrosorb RP-8 (5 $\mu$m, 100 × 4 mm), both from Merck (Darmstadt, G.F.R.). The step gradient was performed with two solvent systems: water—methanol—acetic acid (70:30:6, v/v, system A) and water—ethanol—acetic acid (70:30:6, v/v, system B). For separation of rutosides and aglycones in a single run, system A was applied for 6 min, then system B for 14 min, both at a flow-rate of 0.9 ml/min. Alternatively, for the separation of the rutosides a linear gradient of water (pH 3 adjusted with sulphuric acid)—acetonitrile was used. The samples were introduced in volumes of between 10 and 100 $\mu$l via a sample loop.

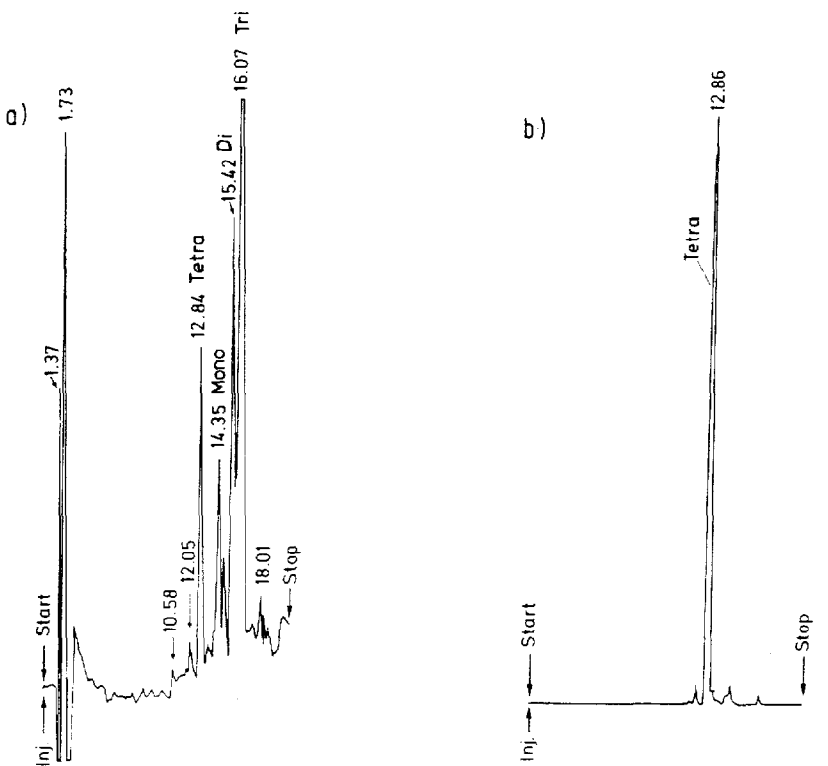


Fig. 3. (a) HPLC separation of HR. Conditions: LiChrosorb RP-8 (250 × 4 mm, 10 $\mu$m, Merck); mobile phase, gradient acetonitrile—water (pH 3); UV detection at 256 nm. (b) Conditions as in Fig. 3a but with fluorescence detection (excitation, 355 nm; emission, 460 nm).

## RESULTS

*Separation of aglycones and glycosides*

Preliminary experiments were carried out in order to optimize the separation of HR in mixtures with their aglycones. Excellent separation of the glycosides was achieved using solvent system A (Fig. 2). The glycosides could be separated as well with the solvent mixture acetonitrile—water (pH 3) under gradient elution (Fig. 3a and b). The rutosides and their corresponding aglycones can be readily separated in a single run when elution is performed with a step gradient (Fig. 4) with solvent system A followed by solvent system B.

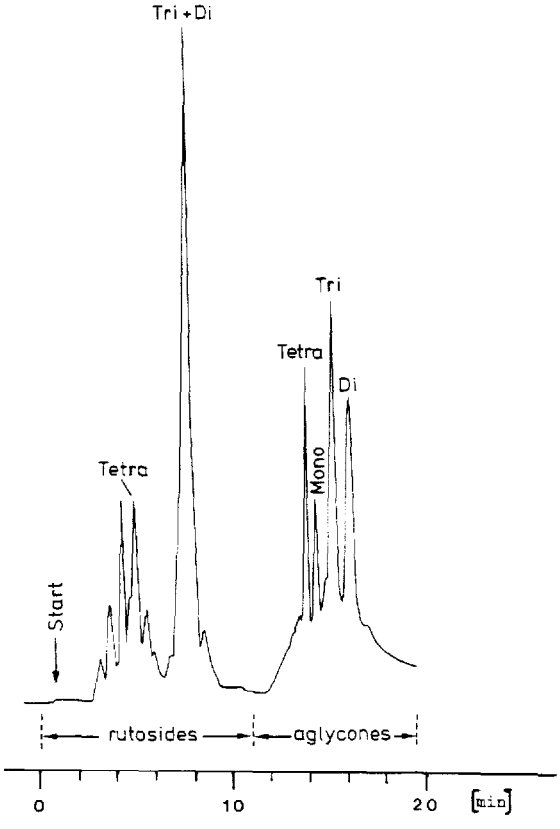


Fig. 4. HPLC separation of HR and HR aglycones in a single run. Conditions as in Fig. 2 but with a step gradient: system A (water—methanol—acetic acid, 70:30:6) for 6 min, then system B (water—ethanol—acetic acid, 70:30:6) for 14 min.

*Quantitative determination of HR in human serum*

In order to prepare a calibration curve quantities of 10, 20, 50, 100, 150, 200, 250 and 300 μg of HR were each added to 1 ml of human serum and the mixtures processed in accordance with the scheme shown in Fig. 1. The procedure was carried out twice for each quantity. A 10-μl volume of each filtrate was subjected to HPLC and an integrator used to measure the peak areas corresponding to the di- and tri-ethers on the one hand and the tetra-ether on the

other. Because only the tetra-ether displayed a reasonable fluorescence, the di- and tri-ethers were determined by UV absorption. In both cases we obtained calibration curves which displayed a linear correlation between the amount of HR added and the observed peak area. The recovery was determined by comparison of the areas given by pure HR and by HR determined in the serum; it was about 78% (±8). The limit of detection is at least 1 μg of HR per ml of serum, added before processing. Fluorescence detection of the tetra-ether was even more sensitive, with a detection limit of about 100 ng/ml serum.

*Detection of HR in human serum after intravenous administration*

In the first series of experiments we used the step gradient system and UV detection. Three male volunteers received a bolus injection of 1.5 g of HR at 8 a.m. Blood samples were then taken at 15, 30, 45, 60, 90 min and at 2, 3, 4, 6, 7, 12 and 24 hours. Each serum sample (1 ml) was processed as described in Fig. 1 and subjected to HPLC. The sums of the peak areas for the di- and tri-ethers were calculated on the basis of the calibration curve. The change in the blood HR level is shown in Fig. 5. The nature of the measured compounds was confirmed by collecting the di- and tri-ethers and identifying them by mass spectrometry [3—5]. In the second series the acetonitrile—water gradient system and fluorescence detection as well as UV absorption were used. Three different volunteers were treated as outlined above and serum concentrations of di- and tri-ether as well as those of the tetra-ether were determined. The results are outlined in Fig. 6.

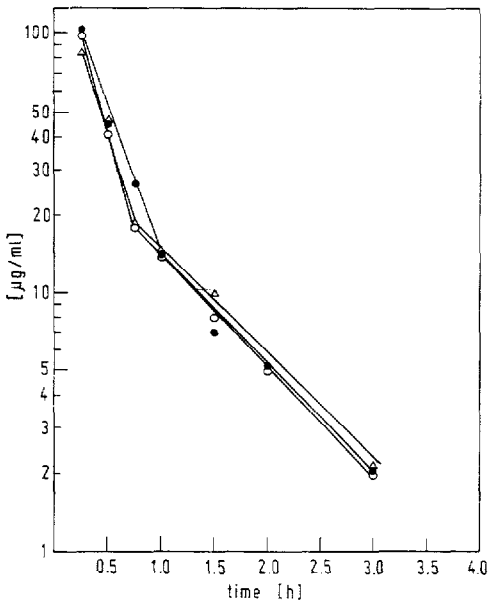


Fig. 5. Change in the blood HR level following intravenous injection of 1.5 g of HR for each of three male volunteers. The points are calculated from the peak areas of the di- and tri-ethers of HR.

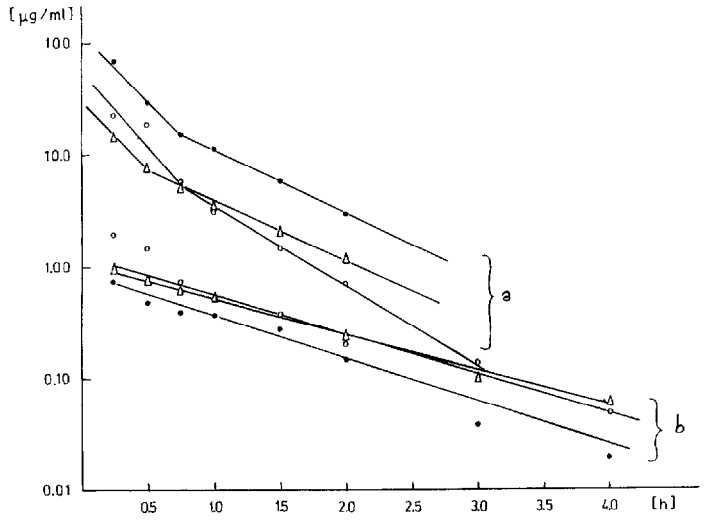


Fig. 6. Semilogarithmic concentration—time curves for each of three subjects following intravenous administration of 1.5 g of HR. (a) Di- and tri-ethers, (b) Tetra-ether.

## DISCUSSION

Two test series were carried out independently in each respect leading to identical results, thus confirming the reliability of the HPLC assay of O-(β-hydroxyethyl)-rutosides. Furthermore, the results of the pharmacokinetic studies after intravenous administration are in accordance with the results obtained by Förster [6] and Griffiths and Hackett [7], who used thin-layer chromatography and $^{14}$C-labeled HR. In addition, the HPLC results fully confirm the concentration—time curves of our group, obtained by circular dichroism measurements of serum samples after intravenous administration of HR [8].

The concentration—time curves of all six subjects are very similar in shape (Figs. 5 and 6). Measurable amounts of di- and tri-ethers are no longer present after 4 h. However, fluorescence detection clearly revealed traces of tetra-ether in all three subjects after 6—8 h. The biphasic elimination curve for the di- and tri-ethers could indicate a distribution of these compounds into deeper compartments. A closer examination of the serum chromatograms from the intravenous tests revealed the following differences in comparison with chromatograms of pure HR solutions in water and the serum HR standard solutions. Soon after intravenous administration of HR, a new UV-positive and fluorescent peak with a retention time of 12.1 min appears, which probably originates from a metabolite. This new peak decreases at the same rate as the HR components. Thus the possibility of accumulation of the metabolite can be excluded. The question of whether this metabolite is identical with those mentioned in the literature [7] to originate from biliary excretion will be the subject of further experiments.

REFERENCES

1 W. Voelter and G. Jung (Editors), O-(β-Hydroxyethyl)-rutoside, Experimentelle und klinische Ergebnisse, Springer, Berlin, 1978.
2 L. Farkas, M. Gabor and F. Kallay (Editors), Flavonoids and Bioflavonoids, Elsevier, Amsterdam, 1977.
3 W. Kuhnz and H. Rembold, in W. Voelter and G. Jung (Editors), O-(β-Hydroxyethyl)-rutoside, Experimentelle und klinische Ergebnisse, Springer, Berlin, 1978, p. 21.
4 W. Kuhnz, Ph.D. Thesis, University of Munich, 1979.
5 W. Kuhnz and H. Rembold, Biomed. Mass. Spectrom., 7 (1980) 269—272.
6 H. Förster, in W. Voelter and G. Jung (Editors), O-(β-Hydroxyethyl)-rutoside, Experimentelle und klinische Ergebnisse, Springer, Berlin, 1978, p. 43.
7 L.A. Griffiths and A.M. Hackett, in L. Farkas, M. Gábor and F. Kállay (Editors), Flavonoids and Bioflavonoids, Elsevier, Amsterdam, 1977, p. 325.
8 G. Jung, M. Ottnad and W. Voelter, Eur. J. Drug Metab. Pharmacokin., 3 (1977) 131.