Spectrophotofluorometric Analysis of 3′,4′,7-Tris[*O*-(β-hydroxyethyl)]rutoside in Urine

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Received April 21, 1977, from the *College of Pharmacy, University of Cincinnati Medical Center, Cincinnati, OH 45267.* Accepted for publication December 2, 1977.

Abstract □ A sensitive procedure was developed for 3′,4′,7-tris[*O*-(β-hydroxyethyl)]rutoside (I) in urine. The method is based on the fluorescence behavior of the I–aluminum complex in absolute methanol. This complex has activation and emission wavelengths of 420 and 480 nm, respectively. Optimum conditions for the reaction were investigated. The fluorescence was linear (r = 0.998) in the range of 0.1–4.0 μg of I/ml. At concentrations below 0.1 μg/ml, a shift in the emission wavelength was observed. Replicate studies (n = 9) of spiked urine samples, each containing 0.4 μg of I/ml, showed good precision with a relative standard deviation of 0.009. Overall percent recovery (± *SEM*) from five urine samples was 99.5 ± 1.34%. Following a single 500-mg po dose of I to individuals, only traces of I were found in the urine. However, β-glucuronidase treatment of urine resulted in a total cumulative urine excretion of 26.53 mg of I after 78.6 hr.

Keyphrases □ 3′,4′,7-Tris[*O*-(β-hydroxyethyl)]rutoside—spectrophotofluorometric analysis in urine □ Spectrophotofluorometry—analysis, 3′,4′,7-tris[*O*-(β-hydroxyethyl)]rutoside in urine

3′,4′,7-Tris[*O*-(β-hydroxyethyl)]rutoside[1] (I), a bioflavonoid, is widely used in Europe for the treatment of some pathological conditions associated with the increased permeability or fragility of blood capillaries such as varicosis and chronic erythema. For pharmacokinetic studies on I and its dosage forms, a simple, yet sensitive, analytical procedure for I in urine is needed.

Flavonoids form complexes in the presence of aluminum chloride (1). 3′,4′,5,7-Tetrahydroxyflavonol-3-rutinoside, for example, was determined by precipitating the drug–aluminum complex with ammonia, followed by redissolving the complex in acetic acid and measuring absorbance after addition of potassium acetate solution (2).

Previous determinations of I in blood or urine were based on quantitative TLC. Wienert and Gahlen (3) irradiated the chromatogram by UV light and quantitated the spots by visual comparison of the fluorescence produced. Foerster and Ziege (4) sprayed the chromatograms with methanolic aluminum chloride and measured the resulting fluorescence densitometrically. The time required for the elution was 1–6 hr. The UV absorption spectra of the hydroxyethylrutosides were determined in the presence of aluminum chloride (5). Recently, liquid scintillation counting was used to study ^{14}C-I metabolism (6).

This paper reports the spectrophotofluorometric analysis of I in urine based on its complex formation with aluminum chloride in absolute methanol.

EXPERIMENTAL

Apparatus—The following instruments were used: a ratio spectrophotofluorometer[2] with 1-cm cells and excitation exit, emission entrance, and rotary exit slit widths of 2 mm; and an analytical balance[3]. The spectrophotofluorometer was calibrated daily using a solid fluorescence standard[4].

Materials and Reagents—The following were used: I[5] (mp 175°), 0.1 *M* aluminum chloride[6] (anhydrous, reagent grade) in absolute methanol[6] (water content 0.05%), and β-glucuronidase solution, prepared by dissolving 0.139 g of β-glucuronidase[7] (bovine liver, 15–50 U/g) in 10 ml of distilled water.

Preparation of Standard Curve—Weigh accurately ~25 mg of I and dissolve in 100.0 ml of absolute methanol. Prepare standard solutions containing 1–40 μg of I/ml. Pipet 1.0 ml of the standard solution into a 10-ml glass-stoppered volumetric flask, add 6.0 ml of aluminum chloride reagent, and dilute with absolute methanol to the mark. Warm the flask in a constant-temperature water bath at 50° for 15 min, cool to room temperature, and measure the fluorescence at λ_{act} 420 nm and λ_{emis} 480 nm against a blank.

Assay of I in Urine—Pipet 1.0 ml of urine into a 50-ml volumetric flask and dilute to volume with absolute methanol. Pipet 1.0 ml of this solution into a 10-ml glass-stoppered volumetric flask and proceed as described under *Preparation of Standard Curve*, beginning with "... add 6.0 ml of aluminum chloride reagent...."

Assay of I Glucuronide in Urine—Pipet 3.0 ml of urine into a 15-ml glass-stoppered centrifuge tube, add 1.0 ml of β-glucuronidase solution, and incubate in a constant-temperature water bath at 37° for 24 hr. Centrifuge the mixture for 10 min at 2000 rpm, pipet 1.0 ml of the supernate into a 50-ml volumetric flask, and dilute to volume with absolute methanol. Pipet 1.0 ml of the resulting solution into a 10-ml glass-stoppered volumetric flask and proceed as described under *Preparation of Standard Curve*, beginning with "... add 6.0 ml of aluminum chloride reagent...."

RESULTS AND DISCUSSION

In the presence of aluminum chloride, I showed a yellow fluorescence in methanol. The fluorophore had activation and emission maxima of 420 and 480 nm, respectively (Fig. 1). These wavelengths remained

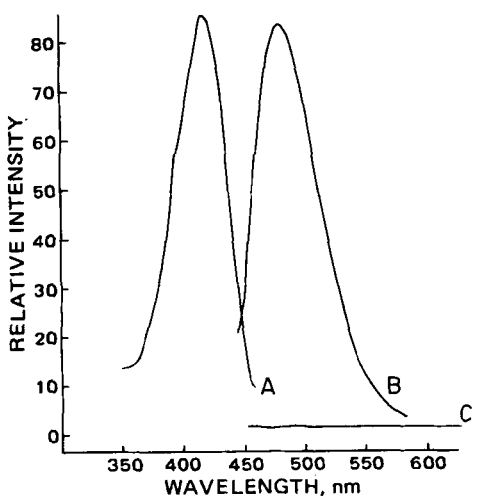

Figure 1—*Activation (curve A) and emission (curve B) spectra of I–aluminum chloride complex. Curve C is emission spectrum of I alone.*

[1] The International Nonproprietary Name is troxerutin.
[2] Aminco-Bowman, Silver Spring, Md.
[3] Model H-18, Mettler Instrument Corp., Princeton, N.J.
[4] Type F53, Carl Zeiss, Inc., New York, N.Y.
[5] Schaper and Brümmer, Salzgitter-Ringelheim, West Germany.
[6] Matheson, Coleman & Bell, Norwood, Ohio.
[7] Worthington Biochemical Corp., Freehold, N.J.

0022-3549/78/0800-1142$01.00/0

Table I—Reproducibility of Fluorophore Development of I in Replicate Spiked Urine Samples at 0.4 μg/ml

Sample	Relative Fluorescence[a]
1	43.5
2	43.5
3	43.2
4	44.4
5	43.8
6	43.8
7	44.1
8	43.2
9	43.8
Average	43.7
RSD	0.009

[a] With λ_{act} 420 nm and λ_{emis} 480 nm.

constant as long as the water content in the final test sample was less than 0.2% (v/v). Relatively large percentages of water, however, caused both wavelengths to shift toward shorter wavelengths. For example, at a water concentration of 10%, the activation and emission wavelengths shifted to 355 and 425 nm, respectively.

The concentration optimum of the aluminum chloride reagent was determined by adding varying volumes of 0.1 *M* methanolic aluminum chloride to a series of 10-ml volumetric flasks, each containing 1.0 ml of 0.5 μg of I/ml, and measuring the fluorescence against a blank after diluting each solution to volume with methanol and heating at 50° for 15 min. Each blank contained the same amount of reagent as in the solution studied. The maximum fluorescence was obtained when the concentration of the reagent was 0.06 *M* in the final test solution.

The reaction time and temperature were determined by following the fluorescence development at room temperature, 37°, and 50°. At room temperature, the maximum fluorescence was not obtained even after 100 min (Fig. 2). Heating accelerated the reaction; a heating time of 15 min at 50° was adequate to attain the maximum fluorescence, which remained stable for at least 105 min.

Under the proposed experimental conditions, a linear fluorescence response was obtained from 0.1 to 4.0 μg of I/ml (r = 0.998). At concentrations below 0.1 μg/ml, a shift in the emission wavelength to 465 nm

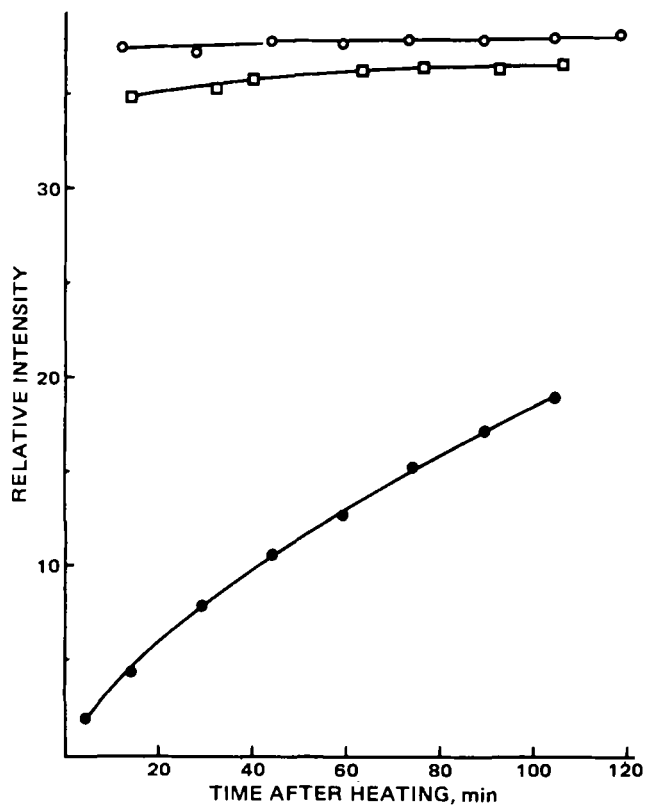

Figure 2—*Effect of heating on the I–aluminum chloride fluorophore. Key: ○, 15 and 30 min at 50°; □, 30 min at 37°; and ●, room temperature with time in hours after mixing.*

Table II—Recovery of I from Spiked Urine Samples

Amount Weighed, mg	Analyzed at Concentration Level, μg/ml	Amount Found, mg	Recovery, %
24.8	0.248	23.5	94.8
24.8	0.496	24.6	99.2
41.6	0.832	41.7	100.2
41.9	0.419	41.9	100.0
51.3	1.026	52.9	103.1
		Average	99.5
		SEM	1.34

was observed. A typical regression equation[8] was $F = 87.13C + 1.51$, where F is the relative fluorescence at λ_{act} 420 and λ_{emis} 480 nm and C is the concentration in micrograms per milliliter. The standard error (n = 5) of the estimates of the slope and the y-intercept were 4.05 and 0.70, respectively.

The reproducibility of the analysis of I in urine was determined by analyzing nine replicate spiked urine samples. At a concentration level of 0.4 μg of I/ml, the coefficient of variation was 0.009 (Table I).

Recovery studies of I from urine samples also were performed. The overall recovery of five spiked urine samples was 99.5% with a standard error of the mean of 1.34% (Table II).

The proposed method was applied to the analysis of I in urine of a human subject following oral I administration. This subject kept the same diet before and during the experiment and took no drugs or vitamins. An aliquot of urine collected before the administration of I (pretreatment urine) was assayed by the proposed method. Negligible fluorescence was observed before and after β-glucuronidase treatment. Therefore, this pretreatment urine served as the blank in subsequent measurements.

After oral administration of a single dose of 500 mg of I to the human subject, only traces of unchanged I were found in the urine. This result is in contrast to the findings of Wienert and Gahlen (3) who, by TLC, did not find traces of I in human urine following a 3600-mg oral dose of I.

When urine of the subject was treated with β-glucuronidase for 24 hr at 37°, following a modified procedure of Griffiths and Smith (7), and then analyzed by the proposed method, a cumulative urinary excretion of 26.53 mg (corresponding to 5.30% of the oral dose) was found in 78.6 hr (Fig. 3). Hackett *et al.* (6) reported that excretion *via* the renal pathway was virtually complete after 48 hr. These workers found, by liquid scintillation analysis, that excretion in human urine accounted for 3.05–5.97% of the administered oral dose of 300 mg of a mixture of *O*-(β-hydroxy-^{14}C-ethyl)rutosides[9].

Studies with ^{14}C-I in rats (5) showed that, following oral administration, two metabolites of I were found in rat urine: the 5′-*O*-glucuronide and a trace of an unidentified 5′-*O*-conjugate. These metabolites do not form fluorescent derivatives with aluminum chloride. No other metabolites of I were reported in rat urine. Although these data are not extrapolatable to humans, the fact that untreated human urine following oral administration of I gave extremely low fluorescence readings whereas the same volume of the same urine after β-glucuronidase treatment showed a tremendous increase in fluorescence at the activation and emission wavelengths of I suggests that the 5′-*O*-glucuronide of I is

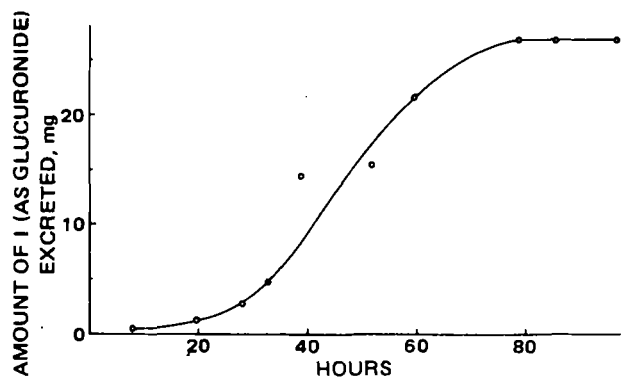

Figure 3—*Cumulative urinary excretion following a single oral dose of 500 mg of I in solution as measured after β-glucuronidase treatment of urine.*

[8] Wang Computer 700C and plotting output writer.
[9] Venuruton, Paroven preparations.

present in human urine. Further evidence was not obtained for the presence of this conjugate in human urine. However, Wienert and Gahlen (3) reported the presence of only one drug-related spot on the thin-layer chromatogram when they analyzed human urine following an intravenous administration of I.

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ACKNOWLEDGMENTS

Presented at the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, New York meeting, 1977.

Nature of Amorphous Aluminum Hydroxycarbonate

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Received November 7, 1977, from the *Department of Agronomy and the ‡Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907. Accepted for publication December 6, 1977.

Abstract □ The titration of sodium carbonate with aluminum nitrate is shown to produce amorphous aluminum hydroxycarbonate. This compound is not stoichiometric, although the maximum carbonate to aluminum ratio appears to be 0.5. The pH conditions for achieving the maximum carbonate content are concentration dependent. A model for the particle surface at the solution interface is proposed. This model accounts for the presence of carbonate directly coordinated to the aluminum and carbonate adsorbed by electrostatic forces. Sodium is present in the diffuse layer and is, therefore, not an integral part of the structure.

Keyphrases □ Aluminum hydroxycarbonate, amorphous—prepared by titration of sodium carbonate with aluminum nitrate, physicochemical nature studied, model for particle surface at solution interface proposed □ Antacids—amorphous aluminum hydroxycarbonate, prepared by titration of sodium carbonate with aluminum nitrate, physicochemical nature studied, model for particle surface at solution interface proposed

Aluminum hydroxide gel used as an antacid usually contains carbonate. Carbonate is part of the structure of aluminum hydroxide gel (1) and contributes significantly to the high rate of acid neutralization (2) as well as gel stability (3). Previously, the term carbonate-containing aluminum hydroxide gel was used to describe the highly reactive gels used as antacids (2, 4, 5). However, this gel is now well enough understood to justify its description as amorphous aluminum hydroxycarbonate.

The production of amorphous aluminum hydroxycarbonate is usually performed in basic media to achieve a high concentration of carbonate in the system during precipitation. However, most studies of the hydrolytic precipitation of aluminum hydroxide are performed in acidic media (6, 7). Therefore, a study of the precipitate produced by titrating sodium carbonate with aluminum nitrate was undertaken. Special emphasis was placed on the precipitation conditions favoring the incorporation of carbonate. Further points of interest were the adsorption of sodium cation by the amorphous aluminum hydroxycarbonate and the elution of anions and cations during washing.

Aluminum nitrate was selected as the aluminum salt for precipitation because nitrate does not coordinate with aluminum as does carbonate (5) and, therefore, does not compete with carbonate for adsorption by the gel.

EXPERIMENTAL

Potentiometric titrations were performed by placing 100 ml of either a sodium carbonate or a sodium hydroxide solution in a 400-ml jacketed beaker. The temperature was controlled to ±0.1°, and the solution was stirred[2] at 2000 rpm. Aluminum nitrate solution was added at a controlled rate[3], 2 ml/min. The solution pH was monitored and recorded continuously.

Preliminary experiments were performed to observe the effect of temperature, addition rate, and reactant concentration. Variation of these precipitation factors did not affect the general shape of the titration curves. However, the position of the inflection points was concentration dependent. Excellent reproducibility of titration curves was obtained.

The equivalent aluminum oxide content was determined by chelatometric titration (8). The carbonate content, determined by gasometric displacement using the Chittick apparatus[4] (9), was expressed as the molar ratio of carbonate to aluminum.

IR analysis[5] (10) was performed using potassium bromide pellets after the gel was dried under vacuum at room temperature. All samples were amorphous by X-ray diffraction.

Gels were washed with equal volumes of 0.1 *M* sodium sulfate to determine if the anions in the gel were exchangeable.

The rate of acid reactivity was determined by pH-stat titration at pH 3.0 (11).

The sodium content was determined by atomic absorption spectroscopy[6].

The effect of washing was determined by adding a volume of deionized water equal to the volume of the gel, mixing, and centrifuging. Successive washes were performed by reconstituting the cake with a volume of water equal to the original volume of the gel and repeating the process.

The charge on the gel particles was determined by observing the direction of motion of the particles during electrophoresis[7].

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[2] Stedi-Speed stirrer, Fisher Scientific, Pittsburgh, Pa.
[3] Solution metering pump, model 746, Beckman Instruments, Fullerton, Calif.
[4] Sargent-Welch Scientific Co., Skokie, Ill.
[5] Model 180, Perkin-Elmer Corp., Norwalk, Conn.
[6] Model 290B, Perkin-Elmer Corp., Norwalk, Conn.
[7] Zeta meter, Zeta Meter, Inc., New York, N.Y.

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