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## Note

# Determination of riboflavine in human urine by the use of a hydrophilic gel column

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The determination of the concentration of riboflavine in urine appears to be an ideal means for monitoring the dietary intake of this vitamin. Therefore, a simple and rapid method for the determination of riboflavine is needed. Smith[1,7] and Gatautis and Naito[2] introduced an high-performance-liquid chromatography method that allows the determination of riboflavine in urine with an analysis time of about 10 min per sample. We have reported that purine and pyrimidine bases, ascorbic acid and its oxidation products can be separated by isocratic elution from a column packed with a hydrophilic gel (Asahipak GS-320) using an aqueous buffer with pH 3.0.[3,4] Our results suggested that compounds which have conjugated double bonds or an aromatic nucleus will be reversibly adsorbed on the gel from the aqueous solution. Therefore, we have tried to determine riboflavine in human urine by elution with aqueous buffer from Asahipak GS-320H column. Good separation of riboflavine from fluorescent impurities could be obtained at pH 4.4 using propionate buffer as the mobile phase at 55°C.

## EXPERIENTAL

### *Materials and solutions*

Riboflavine, flavine mononucleotide sodium salt (FMN), and flavine adenine dinucleotide disodium salt (FAD) were purchased from Yashima Pharmaceutical (Osaka, Japan). Lumiflavine was prepared by photolysis of riboflavine in alkaline solution (0.05 m*M* in 0.2 *M* sodium hydroxide) by irradiation with a fluorescent light source for 30 min. The photolysate was mixed with an equal volume of a (0.15 *M* solution of propionic acid. 7α-Hydroxyriboflavine was a gift from Dr. K. Matsui. The mobile phase was prepared by dissolving 148 g of propionic acid and 32 g of sodium hydroxide in 2 kg of ultrapure water. The pH of the solution was 4.4.

### *Equipment*

The chromatographic system consisted of a constant delivery pump (TRI ROTAR-III; Jasco, Tokyo, Japan), an automatic sampler (Model KSST-60J; Kyowa

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Seimitsu, Tokyo, Japan) and a column packed with hydrophilic gel (Asahipak GS-320H, 25 × 0.76 cm I.D.; Asahi Chemical Industry, Japan). A Model FP-550F spectrofluorometer (Jasco) equipped with a flow cell (7 μl) was used to monitor fluorescence.

*Chromatographic separation and fluorometric determination*

A 50–250-μl volume of standard or sample solution was injected into the column, which was kept at 55°C. The mobile phase was pumped at a flow-rate of 1.5 ml/min and the fluorescence of the effluent was monitored with excitation at 450 nm and emission at 525 nm. Sample solutions of human urine were prepared by mixing equal volumes of fresh urine and a 5% metaphosphoric acid solution containing 0.5% (w/v) β-thiodiglycol. The mixture was centrifuged at 1500 *g* for 15 min. The supernatant was filtered through a disposable filter unit (ED-03, pore size 0.45 μm; Gelman Sciences Japan, Tokyo, Japan) and 50- to 250-μl of the filtrate was used for analysis.

## RESULTS AND DISCUSSION

Riboflavine was adsorbed rather strongly to the gel from phosphate buffer (pH 6–7), and the shape of the peak was unsymmetric. We tried to use the propionate

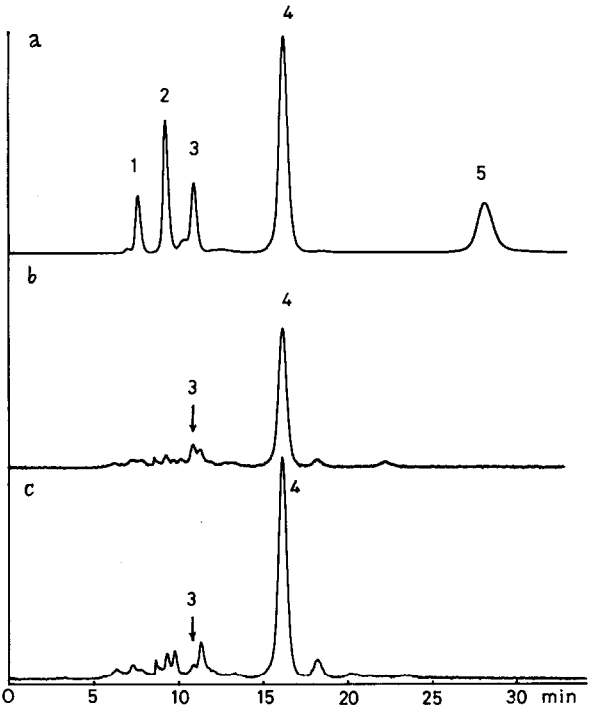


Fig. 1. Elution of a standard solution of riboflavine, riboflavine derivatives and urinary riboflavine from an Asahipak GS-320H column. Peaks: 1 = flavine adenine dinucleotide; 2 = flavine mononucleotide; 3 = 7α-hydroxyriboflavine; 4 = riboflavine; 5 = lumiflavine. Elution patterns of human urine samples are shown in b and c. for chromatographic conditions, see text.

buffer as the eluent, because the presence of propionate in the mobile phase will reduce non-ionic interaction and hydrogen bond formation of the solutes with the gel matrix. As shown in Fig. 1a, riboflavine was separated from its derivatives by isocratic elution from a column packed with a hydrophilic gel, using a propionate buffer of pH 4.4. The solutes were eluted in the order of decreasing polarity. Elution at higher temperature (55°C or 60°C) led to sharp peaks for riboflavine. The retention times were quite reproducible and the recovery of riboflavine added to a urine sample at a concentration of 1 μmol/l was 98.7±2.5% (S.D.; seven determinations). An amount of 0.1 μmol/l of riboflavine could be determined. Ohkawa *et al.*[5] found 7α-hydroxyriboflavine and 8α-hydroxyriboflavine in human urine and noticed that hydroxyethylflavine eluted just after riboflavine. Several fluorescent peaks could be observed in our chromatogram of human urine samples as shown in Fig. 1b and c, and the retention time of peak 3 coincided with that of 7α-hydroxyriboflavin. The comparison of the retention times of other peaks with those of standard samples will be reported later.

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