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Note

Liquid chromatographic determination and time—concentration studies of riboflavin in hemodialysate from uremic patients

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During recent years, the identification and assay of chemical compounds in physiological fluids has become an important diagnostic indicator for the characterization of metabolic disorders which are often associated with disease. High-performance liquid chromatography (HPLC) has been used extensively for this purpose. Recently, reversed-phase HPLC has been applied successfully to numerous biological and clinical assays, including the analysis of uremic hemodialysate fluid samples from the artificial kidney [1, 2], the determination of various classes of biochemically active compounds in urine [3—5] and blood plasma or serum (6—9], and the separation of derivatized amino acids [10].

Determinations of riboflavin in multivitamin preparations using ion-exchange [11], normal-phase [12] and reversed-phase columns [13, 14] have been described and reviewed [15]. Recently, an HPLC method for the determination of riboflavin in urine using fluorescence detection has been described [16, 17]. These methods are selective for riboflavin, but they have not been applied to uremic hemodialysate, nor have they involved the use of on-line pre-column enrichment. Short pre-columns have been employed in liquid chromatography as effective pre-column enrichment devices and to prevent strongly retained sample components from reaching the analytical column [18]. Cantwell [19] and Mohammed and Cantwell [20] have also used a

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pre-column containing Amberlite XAD-2 to facilitate on-line clean-up prior to the determination of preservatives and drugs in pharmaceutical syrups.

Vitamin supplements are routinely prescribed to kidney patients who undergo regular dialysis treatment. Loss of water-soluble vitamins via dialysis is well accepted, while the concentration of the excreted vitamins in dialysate is extremely small. This paper describes a rapid, simple and sensitive method for the determination of nanogram amounts of riboflavin in hemodialysate using a pre-column containing Corasil-C_{18}, an analytical reversed-phase column, and a solvent switching valve. Time—concentration profiles for riboflavin loss during hemodialysis for two patients are also included.

EXPERIMENTAL

Samples and sample preparation

Samples of uremic hemodialysate were collected from a male and a female patient suffering from chronic renal failure. These samples (approximately 500 ml each) were collected twice hourly throughout the 6-h dialysis treatment from the dialysate drain of a parallel-plate artificial kidney. Five hemodialysate samples were also obtained from five other patients. These samples were frozen and stored at −25°C in polyethylene containers until assayed.

Before chromatographic separation, the dialysate samples were thawed at room temperature and filtered through 0.2-μm Nalgene Filters (Sybron Corp., Rochester, NY, U.S.A.) to remove particulate matter.

Riboflavin was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. HPLC-grade methanol was obtained from Burdick and Jackson Labs., Muskegon, MI, U.S.A. HPLC-grade water was produced by a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA, U.S.A.). Standard solutions were prepared by dissolving weighed samples in deionized distilled water.

Apparatus

The schematic diagram of the liquid chromatograph used for the determination of riboflavin is illustrated in Fig. 1. Pump P_1 was a 5000 p.s.i. Mini-Pump supplied by Laboratory Data Control, Riviera Beach, FL, U.S.A. With the Teflon rotary valve V_1 (Type 50, Rheodyne, Cotati, CA, U.S.A.) in the position shown, pump P_1 pumped solvent 1 through valves V_1 and V_2 (Valco Instruments, Houston, TX, U.S.A.), as well as the pre-column C_1, and then to waste. Simultaneously, pump P_2, an SP8700 solvent delivery system (Spectra-Physics, Santa Clara, CA, U.S.A.) pumped solvent 2 through valve V_2, the analytical column C_2, and the fluorimetric detector (Fluoromonitor™ Filter Fluorometer, American Instrument Co., Silver Spring, MD, U.S.A.). A Corning 7-51 glass primary filter (370 nm) was used. The secondary filter was a Wratten No. 8 ($\geqslant$ 460 nm). The analytical column C_2 was constructed of 25 × 0.40 cm I.D. stainless steel, packed with 7-μm (average diameter) Zorbax BP-TMS spherical particles (DuPont, Wilmington, DE, U.S.A.). Pre-column C_1 consisted of 11.5 cm × 0.15 cm I.D. stainless-steel tubing dry-packed with 37—50 μm Corasil-C_{18} (Waters Assoc., Milford, MA, U.S.A.). An SP4100 (Spectra-Physics) computing integrator/printer-plotter was used to record all chromatograms and to calculate the analytical results.

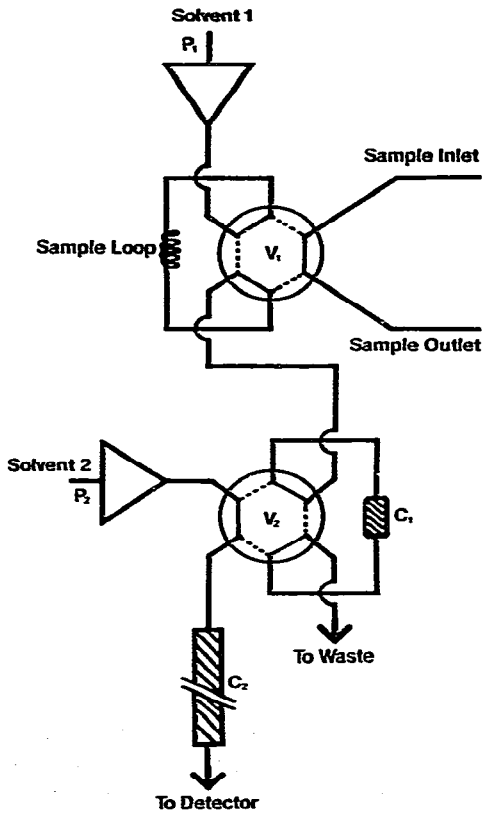

Fig. 1. Scheme of the liquid chromatograph.

Procedure

For the determination of riboflavin, solvent 1 consisted of an aqueous acetate buffer at pH 4.50 (10 mmol/l acetic acid taken to pH 4.50 with sodium hydroxide). Solvent 2 consisted of a mixture containing 65% solvent 1 and 35% methanol. With solvent 1 flowing at a rate of 2.0 ml/min, and solvent 2 passing through the analytical column at a flow-rate of 1.50 ml/min, a 1.0-ml sample of hemodialysate was injected. After 3.0 min, V_2 was switched to allow solvent 2 to pass through pre-column C_1, thus eluting riboflavin through both C_1 and C_2. Certain compounds which are strongly retained on C_1 and C_2 and which normally would elute well beyond riboflavin do not interfere with the analysis and are subsequently removed from both C_1 and C_2 by elution with 100% methanol. After 13 min, V_2 is switched back to its original position and the next injection can be made after a 5.0-min equilibration period.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram for riboflavin in uremic hemodialysate. Quantitation was based on a comparison with the standard curves obtained by injecting aqueous solutions of riboflavin. Both peak height and peak area measurements yielded linear calibration curves with correlation coefficients

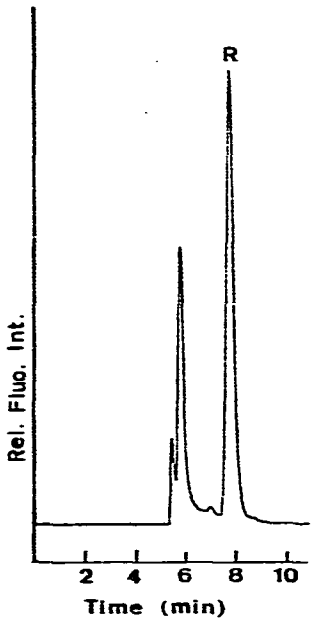

Fig. 2. Liquid chromatogram for 7.9 ng/ml riboflavin (R) in uremic hemodialysate.

of 0.999 for both peak height and peak area. Quantitative recovery was demonstrated with spiked hemodialysate, prepared by adding a known amount of riboflavin to a portion of blank hemodialysate. Recovery values reported in Table I are the averages of both height and area values.

In order to evaluate the loss of riboflavin during hemodialysis, samples of uremic hemodialysate fluid were collected at regular time intervals from two patients throughout the entire dialysis session. Concentrations of riboflavin in hemodialysate were determined by the HPLC method described in this paper and were plotted as a function of dialysis time as shown in Fig. 3. Our data for riboflavin correspond to our earlier time—concentration dialysis profiles [2], though our explanations now reflect dietary intake during the study, changes in transmembrane pressure and blood pressure during the study as well as our own level of understanding.

TABLE I

RECOVERY DATA FOR RIBOFLAVIN ADDED TO BLANK HEMODIALYSATE

Amount added (ng/ml)	Amount found (average) (ng/ml)	Recovery (%)
20.30	20.14 ± 0.01	99.2
16.20	16.12 ± 0.01	99.5
12.20	12.30 ± 0.02	100.8
8.10	8.02 ± 0.01	99.0
4.10	4.07 ± 0.02	99.3
2.00	2.03 ± 0.03	101.5

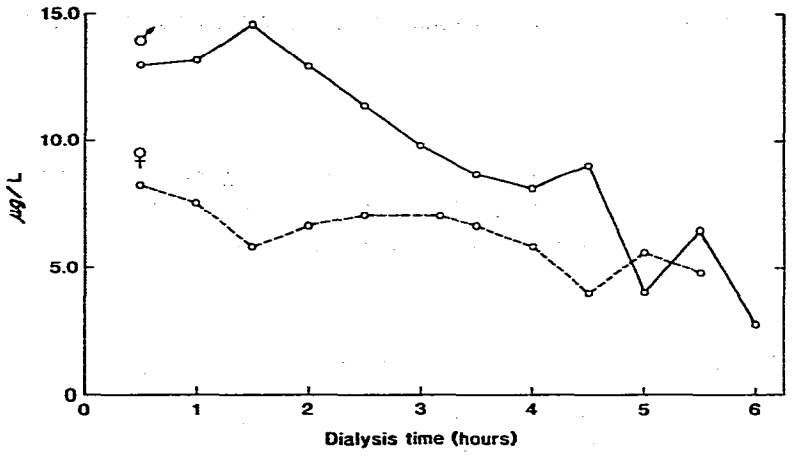

Fig. 3. Time—concentration dialysis profiles for riboflavin in hemodialysate of a male and a female patient.

With the ingestion of food (male: hours 1 and 4), the amount of riboflavin removed in the dialysate is increased and the time—concentration curve shows an upswing (hours 1.5 and 4.5) if blood pressure remains stable (Fig. 3, male), and a marked temporary downswing (hour 1.5) if blood pressure falls (Fig. 3, female). The oscillations at the end of the treatment and collection period are likely to reflect a combination of patient factors including the post-prandial state, the changing transmembrane pressure associated with membrane clotting, and the rate of change of body weight (due to the drop in total body water during treatment).

Riboflavin, the heat-resistant factor of vitamin B, is essential for normal growth and tissue maintenance, assisting in the metabolism of carbohydrates, fatty acids, and amino acids. With the recommended daily amount (RDA) for riboflavin being 0.8—2.6 mg for adults, and dialysis removing a large portion of this, vitamin supplements which include riboflavin would be justified. Riboflavin is primarily provided by dairy products and eggs, major sources of high biological proteins in the diets fed to the time—study subjects and patients with renal disease participating in nutritional therapy.

CONCLUSION

The method described in this paper permits nanogram quantities of riboflavin to be determined in uremic hemodialysate by direct injection using pre-column enrichment without sample pretreatment. Uremic hemodialysate was found to contain 2—20 ng/ml riboflavin. The use of a pre-column and a solvent switching valve eliminates the need for preliminary concentration and sample clean-up steps prior to injection. This method has been shown to be more sensitive by three orders of magnitude than the method previously applied to urine [16].

The time—concentration study presented as part of this investigation confirms our earlier data and emphasizes the need to pursue techniques for rapid determinations of nanogram quantities of vitamins in nutritional clinical

studies. Further delineation of the physiological role of vitamins in uremia is needed.

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