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Note

High-performance liquid chromatographic determination of 2-β-D-ribofuranosylthiazole-4-carboxamide in urine and plasma

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2-β-D-Ribofuranosylthiazole-4-carboxamide (thiazole nucleoside) was synthesized [1] and evaluated as a potential compound against type I herpes virus, type 3 parainfluenza virus, type 13 rhinovirus and parainfluenza virus and is also reported as an active inhibitor of guanine nucleotide biosynthesis [2]. It was found that the compound exhibited antitumor activity against L1210 and P388 murine leukemias. Lewis lung carcinoma, a neoplasm refractory to many chemotherapeutic agents, was cured by treatment with the thiazole nucleoside over a broad range of doses (25—800 mg/kg) [3]. The drug was shown to arrest cells in the "S Phase" of the cell cycle and also inhibited the synthesis of RNA and DNA in P388 murine leukemia cells growing in culture [4]. The present study is aimed to measure the levels of the thiazole nucleoside in biological samples to be able to understand the fate of the drug in the biological system to permit pharmacological studies.

EXPERIMENTAL

Apparatus

Separation was achieved on a Model 6000A solvent delivery system, Model U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and a Model SF-720 Spectroflow monitor (Schoeffel Instruments, Westwood, NJ, U.S.A.). Peak areas, retention time, and concentrations based on standards were calculated with a Model 720 system controller and a Model 730 data module (Waters Assoc.). Absorption spectra were obtained using a Gilford Spectrophotometer Model 250 (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.).

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Column

The column used for reversed-phase high-performance liquid chromatography (HPLC) was 300 × 4 mm μBondapak C_{18} (Waters Assoc.). The column was prepacked with 10 μm (average diameter) porous silica particles to which octadecyl groups were covalently bonded through a Si—O—Si bond.

Reagents

The following reagents were used: ammonium formate (Sigma, St. Louis, MO, U.S.A.); trichloroacetic acid (Baker Chemical, Phillipsburg, NJ, U.S.A.); methanol (HPLC grade, Fisher Scientific, Fairlawn, NJ, U.S.A.); glass double-distilled water was used in preparing the buffers and all the other aqueous solutions. All solutions used in the HPLC system were filtered through a membrane filter (average pore size 0.22 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum immediately before use.

Drug

2-β-D-Ribofuranosylthiazole-4-carboxamide was obtained from Mrs. Ruth Davis, National Cancer Institute (Bethesda, MD, U.S.A.).

Buffer preparation

A 5 m*M* ammonium formate solution was freshly prepared in glass double-distilled water, filtered through a membrane filter and degassed under vacuum immediately before use in the HPLC system.

Sample collection and storage

Female B6DZF_1 mice (25—30 g) were used in all the experiments. The animals were housed under natural lighting and fed a standard laboratory chow (Wayne Lab. Animal Diets, Chicago, IL, U.S.A.) ad libitum. Each mouse was administered intraperitoneally (i.p.) with 0.25 ml of thiazole nucleoside in normal saline (20 mg/ml). Urine specimens (24-h) were collected in metabolic cages before and after administration of the drug without preservative and kept frozen. About 0.5 ml of blood was collected at different time intervals (0, 1, 2, 3, 4, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480, 600, 720, 1440 min) from the nasal sinus of the mice using heparinized micro-hematocrit capillary tubes, and immediately centrifuged for 2 min. Plasma was separated and stored in ice until the collection was completed and then kept frozen at −20°C.

Reversed-phase HPLC determination of thiazole nucleoside

The first 24-h urine specimen was diluted 1:100 with glass-distilled water whereas subsequent 24-h specimens were undiluted. All urine specimens were filtered through Millipore membrane filters. Plasma (5 μl) was placed in an Eppendorff microcentrifuge tube and 45 μl of 6% trichloroacetic acid were added to deproteinize the plasma, vortexed with 50 μl of water and centrifuged for 5 min. An aliquot of 20 μl was injected into the chromatograph. The drug was quantitated by its absorbance at 235 nm. The areas under the peaks were integrated with a Data Module, Model 730 and a system controller, Model 720 and the amount of the drug in the biological sample was calculated as follows.

$$\text{amount of drug per ml of sample} = \left[\frac{\text{Area test}}{\text{Area standard}}\right]_{\text{sample}} \times \text{amount of standard} \times \frac{\text{ml sample}}{\text{volume of standard}}$$

The peak was identified on the basis of retention time and co-chromatography of the authentic compound with test materials.

Analytical recovery of standard thiazole nucleoside

An equal volume of thiazole nucleoside (2—20 m*M*) was added to urine or plasma and processed as described earlier and an aliquot of 20 μl of solution (10—100 μM) was analyzed by reversed-phase HPLC for quantitation.

Animal experiment

Thiazole nucleoside was injected into three mice in each group. Urine specimens (24-h) of the same day from all three mice were pooled and analyzed for drug content. Plasma levels of drug were analyzed in a total number of 21 mice, which were divided into 7 groups with 3 mice in each group. Blood was drawn 3 times in each mouse, i.e., a total number of 63 times were drawn from 21 mice at various time intervals as described earlier.

RESULTS AND DISCUSSION

UV absorption spectra of standard thiazole nucleoside (200 μM) were obtained at wavelengths ranging from 220—280 nm and the drug had an absorption maximum at 235 nm (Fig. 1).

Quantitation of thiazole nucleoside at different concentrations ranging from 20 pmol to 20 nmol (1 μM to 1 m*M*) showed that linearity was observed

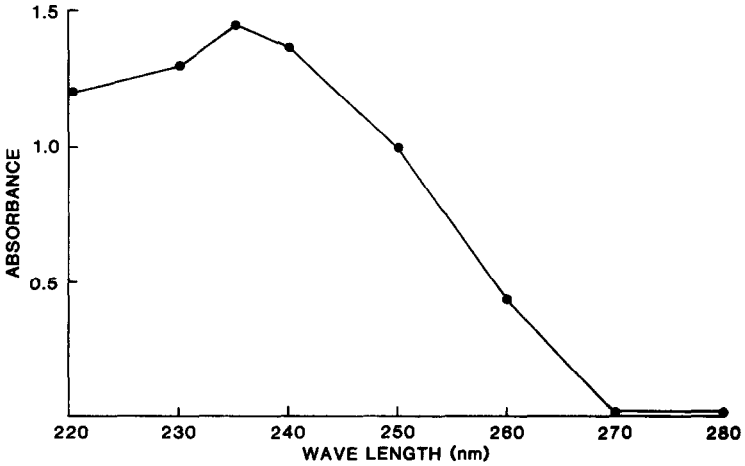

Fig. 1. UV absorption spectra of standard thiazole nucleoside (200 μM) measured in a Gilford Spectrophotometer Model 250.

TABLE I

PRECISION OF HPLC ANALYSIS FOR THIAZOLE NUCLEOSIDE USING AUTHENTIC COMPOUND

Thiazole nucleoside standards (nmol/ml)		
Injected	Recovered*	Average correction value** (%)
100	96.71	3.29
50	49.47	1.06
20	19.77	1.15
10	9.82	1.8
5	4.95	1

*Each value is the mean of three or more determinations.
**Average of the difference between injected and recovered thiazole nucleoside standard of three or more determinations.

in the concentration range from 0.2 to 2 nmol (10—100 μM). The peak areas corresponding to the concentrations of 0.2, 0.4, 1.0, and 2.0 nmol of the authentic drug were $0.8 \cdot 10^6$, $1.6 \cdot 10^6$, $4 \cdot 10^6$ and $8 \cdot 10^6$, respectively, and the high efficiency of the HPLC separation allows an extremely low detection limit of 20 pmol.

The reversed-phase HPLC analytical recovery of standard drug from biological samples gave excellent precision at concentrations easily obtained from small samples of biological fluids (Table I). Repeated injection of drug (0.1—2 nmol) at each of five concentrations gave average correction values (1—3%).

When an aliquot of 20 μl of the filtrate of control urine or plasma was subjected to HPLC analysis, there was no non-specific peak corresponding to the thiazole nucleoside; a similar observation was also found in undiluted control urine. Retention times and peak areas of authentic drug and urine or plasma containing known amounts of drug were found to be the same at isocratic conditions (Fig. 2) and so no attempt was made to purify urine or plasma further for drug assay.

Analysis of the drug in 24-h urine specimens showed that more than 99% of the drug was excreted in the first 24 h and the remaining portion was completely excreted on the subsequent day (Table II). The drug could not be detected in the third 24-h urine specimen.

TABLE II

HPLC ANALYSIS FOR THIAZOLE NUCLEOSIDE IN URINE

Each value is the average of three independent runs. A total dose of 15 mg of drug is given to three mice and the 24-h urine collections of the same day were pooled.

Sample	Excretion of drug (mg)	
	Mean	S.D.
First 24-h urine specimen	14.97	± 0.29
Second 24-h urine specimen	0.023	± 0.004
Third 24-h urine specimen	0	0

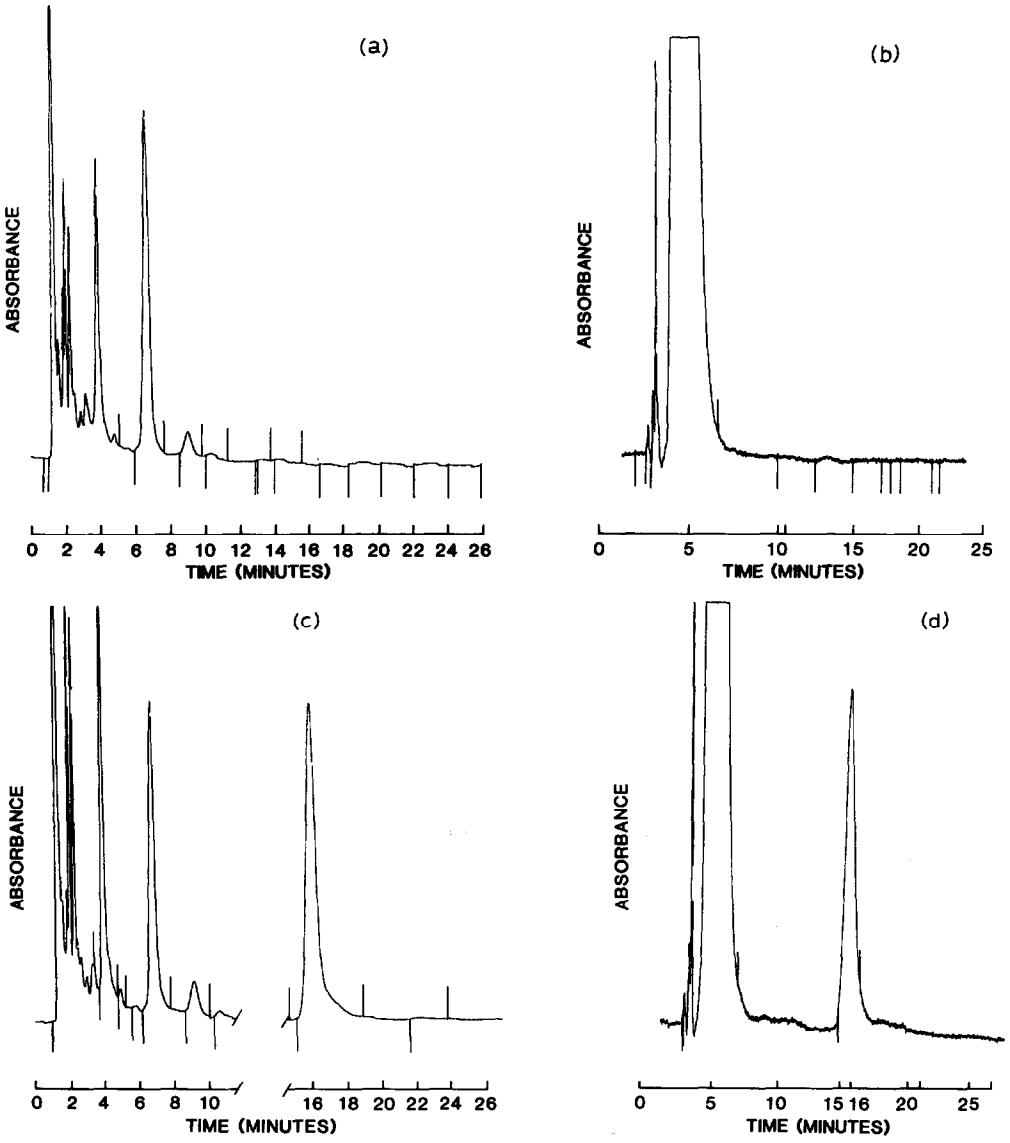

Fig. 2. High-performance liquid chromatograms obtained from mouse samples of (a) control urine; (b) control plasma; (c) urine containing drug; and (d) plasma containing drug. A total dose of 15 mg of drug was injected i.p. into 3 mice and 24-h urine collections of the same day were pooled. Sample, 20 μl equivalent to 0.2 μl or urine or plasma; column, μBondapak C_{18} (300 × 4 mm); buffer, 5 m*M* ammonium formate (native pH); flow-rate, 1 ml/min; detector, 235 nm; 0.01 absorbance; temperature, 24°C.

Analysis of the drug in plasma showed the presence of the drug within 1 min after administration, reaching a maximum by 10 min and steadily decreasing thereafter. The drug could be quantitated up to the 2-h specimen and it had disappeared completely from blood by 3 h (Fig. 3).

The reversed-phase HPLC method described for the separation and quantitation of thiazole nucleoside from biological samples with UV absorption detec-

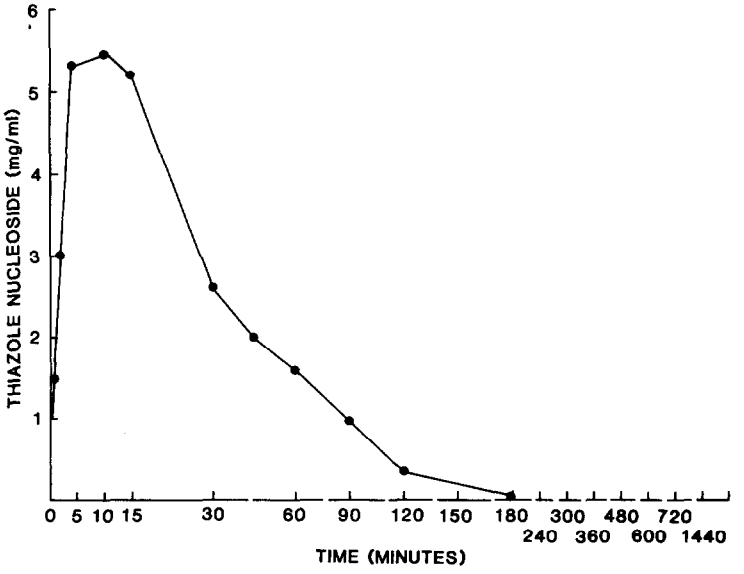

Fig. 3. Time course analysis of drug in plasma of mice using reversed-phase HPLC. A single dose of 0.25 ml of thiazole nucleoside (20 mg/ml) was injected i.p, into a total number of 21 mice, which were divided into 7 groups of 3 mice in each group. Blood was drawn 3 times in each mouse, i.e., a total number of 63 times were drawn from 21 mice at various time intervals as described in Experimental. All other conditions are the same as in Fig. 2.

tion is a rapid, efficient, selective, highly sensitive, non-destructive and quantitative method. There is no method available for the estimation of the drug in the literature. Thiazole is soon to enter Phase I study. The elucidation of this method will throw some light on pharmacology of the drug.

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