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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRICYCLIC NUCLEOSIDE AND TRICYCLIC NUCLEOSIDE 5'-PHOSPHATE IN BIOLOGICAL SPECIMENS

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SUMMARY

An isocratic, sensitive, high-performance liquid chromatographic assay was developed for the determination of the tricyclic nucleoside 1,4,5,6,8-pentaazaacenaphthylene-3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl 5'-monophosphate (TCN-P; NSC 280594) and its dephosphorylated metabolite TCN (NSC 154020). Separation was obtained using a C_{18} Sep-Pak precolumn, a reversed-phase column, and a mobile phase of phosphate buffer—methanol (87.5:12.5, v/v) containing 0.0025 *M* tetrabutylammonium hydroxide. The absorbance of both TCN and TCN-P was monitored at 280 nm with a sensitivity limit of 10 ng/ml. The recovery was 54 ± 6% and 51 ± 8% (mean ± S.D.) from plasma for TCN and TCN-P, respectively. Rapid enzymatic dephosphorylation of TCN-P in plasma and tissue samples was prevented by adding a large excess of adenosine 5'-monophosphate. The assay was used to determine plasma and tissue concentrations of TCN-P and TCN after administration of TCN-P to cancer patients in a Phase I clinical study.

INTRODUCTION

The tricyclic nucleoside, 1,4,5,6,8-pentaazaacenaphthylene-3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl (TCN; NSC 154020) is a new anticancer agent in the class of nucleosides active against implanted murine tumors and L1210 leukemia [1]. Tricyclic nucleoside 5'-phosphate (TCN-P; NSC 280594) is the more water-soluble phosphate ester of TCN (Fig. 1). Both agents inhibit DNA synthesis and are toxic to cells in the S phase of the growth cycle. TCN-P is enzymatically dephosphorylated by ecto-5-nucleotidase, actively transported into cells, and then rephosphorylated by adenosine kinase to the cytotoxic form [2—4]. TCN-P is currently being investigated in five major cancer centers

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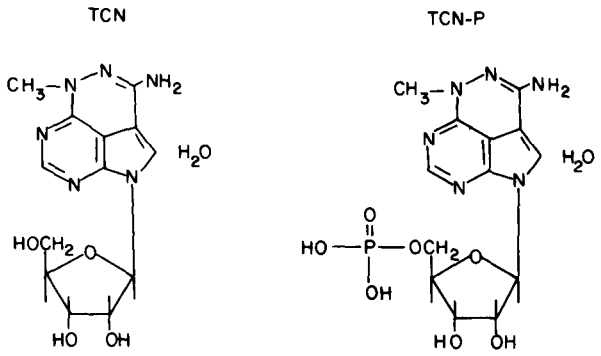

Fig. 1. Clinical names and structures of 1,4,5,6,8-pentaazaacenaphthylene-3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl (TCN, left) and its 5'-monophosphate (TCN-P, right).

in the U.S.A. as part of Phase I clinical studies. A method was needed to determine both TCN-P and its metabolite TCN in biological fluids and tissues at concentrations below 100 ng/ml.

The method described herein uses a concentration step to attain the required sensitivity, a reagent to retard dephosphorylation of TCN-P, and an ion-pair reagent to prolong retention times and allow the use of an isocratic system. Previously published methods did not address the problem of dephosphorylation in a biological matrix, and were not sufficiently sensitive for a pharmacokinetic study [5, 6].

MATERIALS AND METHODS

Reagents

TCN and TCN-P were obtained from Investigational Drug Branch Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.; Lot No. 3, Lot No. MS-01-188). Adenosine 5'-monophosphate (AMP) was supplied by Sigma (St. Louis, MO, U.S.A.), HPLC-grade methanol, acetonitrile and water by J.T. Baker (Phillipsburg, NJ, U.S.A.), reagents for sodium phosphate buffer by Fisher Scientific (Fairlawn, NJ, U.S.A.) and tetrabutylammonium hydroxide (TBA) (1 *M* in methanol) by Eastman-Kodak (Rochester, NY, U.S.A.).

Chromatographic equipment

The chromatographic system consisted of a Waters Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) at a flow-rate of 1 ml/min, a Rheodyne injector (Rheodyne, Berkeley, CA, U.S.A.) or a Waters Model U6K injector with a 100-μl loop, a precolumn, a Waters μBondapak C_{18} column (300 × 4.6 mm I.D.), a Waters Model 440 detector set at 280 nm, and a Linear strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.). Precolumns were either silica or C_{18} Sep-Pak cartridges obtained from Waters Assoc. The evaporator was an N-EVAP Model 112 obtained from Organization Assoc. (South Berlin, MA, U.S.A.).

Solutions

TCN and TCN-P stock solutions containing 50,000 ng/ml were prepared

every second day in deionized water and stored at 2°C. Stock solutions were serially diluted into the working range of 10—10,000 ng/ml. These solutions were injected directly onto the column. AMP was dissolved in water to a concentration of 10 mg/ml and stored at 2°C; its retention time of 6.3 min did not interfere with drug detection.

Plasma standards contained 2 ml blank plasma (Harper Hospital Blood Bank, Detroit, MI, U.S.A.), 0.2 ml TCN or TCN-P aqueous standards over a concentration range of 10—1000 ng/ml, and 0.2 ml AMP solution. This mixture was vortexed for 30 sec. Standards of each drug were used to establish a calibration curve for quantification of concentrations from patient specimens.

Mobile phase

To 1 l of 0.01 *M* phosphate buffer, pH 7.0, were added 2.5 ml of 1.0 *M* TBA. The pH of the solution was adjusted to 7.0 with phosphoric acid and poured through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). To 875 ml of this solution were added 125 ml methanol for the final mobile phase. Prior to use, the mobile phase was degassed by stirring under vacuum for 15 sec.

Extraction procedure

Because TCN-P was unstable in blood plasma, specimens were chilled in ice immediately after collection, centrifuged at 2800 rpm for 5 min (Beckman Instruments, Palo Alto, CA, U.S.A.; Model TJ5 centrifuge), AMP was added, and samples were stored at −18°C until analysis. Plasma (2 ml) containing 0.2 ml AMP solution was applied to a conditioned C_{18} Sep-Pak column, washed with 5 ml of 0.0025 *M* TBA—0.005 *M* phosphate buffer adjusted to pH 7.0 with phosphoric acid and eluted with 3 ml methanol. The methanol was evaporated in a water bath at 40°C under a stream of nitrogen, and the residue was redissolved in 100 μl water.

A 50-μl aliquot was injected onto the column.

Patients received 70 or 96 mg TCN-P per m^2 as a 5-min intravenous infusion. Blood samples were collected via an intravenous catheter and transferred to heparinized test tubes before therapy and at selected times thereafter.

From one patient who died 61 days after a single dose of 70 mg TCN-P per m^2, pleural fluid was obtained by pleuracentesis twenty days after dosage, and tissue specimens were obtained at autopsy.

Three male Sprague—Dawley rats were obtained from Charles River (Wilmington, MA, U.S.A.). All animals were anesthetized with 50 mg pentobarbital per kg body weight, given intraperitoneally. Tissue and plasma samples from one animal served as blanks. Two rats were administered 50 mg TCN-P per kg by rapid injection into a femoral vein and the animals were killed 15 min later by cervical dislocation. Selected tissue specimens were removed, mechanically disintegrated (Polytron, Brinkman Instruments, Westbury, NY, U.S.A.), an aliquot was taken, AMP added, centrifuged, and immediately extracted and analyzed such as plasma samples.

Calculations

The concentration of TCN-P or its metabolite TCN in plasma or tissue was calculated by dividing the peak height by the slope of the appropriate plasma

standard curve. The slope of each standard curve was determined as the mean ± S.D. of response factors (peak height divided by concentration) calculated for each standard concentration. Extraction efficiency (recovery) was determined by comparing peak heights of extracted plasma standards to peak heights of directly injected aqueous standards of corresponding concentrations.

RESULTS

Stability of aqueous standards

At 2°C TCN-P standards in aqueous solutions were stable for up to three days, but long-term studies revealed a complete decomposition after six months; TCN aqueous standards were stable over a four-week period at 2°C, and afterwards additional peaks were detected at 4 min retention time. TCN-P aqueous standards (10 μg/ml) were unaltered by heat (60°C) over 4 h. Long-term (three months) ultracold storage (—60°C) led to degradation. Light-protected samples were stable for one week at room temperature and for two months when stored at 2°C. We routinely prepared fresh aqueous standards every two days, and these were stored at 2°C.

Stability of plasma standards

In heparinized blood bank plasma TCN-P (1.0 μg/ml) was rapidly dephosphorylated with a half-life of 40 min (Table I). The disappearance of TCN-P was correlated in time to the appearance of TCN, and by 3 h the dephosphorylation reaction was nearly complete. When a 1000-fold excess of AMP was added to the plasma (Table I), the TCN-P concentration did not decrease during the 3-h study, and no TCN could be detected. When this experiment was repeated using fresh plasma from a volunteer, essentially the same results were obtained.

Chromatograms

Sample chromatograms of aqueous standards (A), control blood bank plasma

TABLE I

PERCENTAGE OF ADDED 1.0 μg TCN-P PER ml PLASMA AND APPEARANCE OF TCN AFTER INCUBATION AT ROOM TEMPERATURE

Time (min)	Without added AMP		With added AMP	
	TCN-P	TCN	TCN-P	TCN
0	100*	0	100	0
15	67.0**	14.9***	100.0	0
75	17.5	63.2	105.5	0
135	5.6	95.4	107.1	0
185	2.0	82.8	86.6	0

*Theoretical.

**Spiked plasma samples were prepared at time zero and extracted at designated times.

***TCN concentrations were converted to TCN-P equivalents which are shown here as a percentage of zero-time TCN-P.

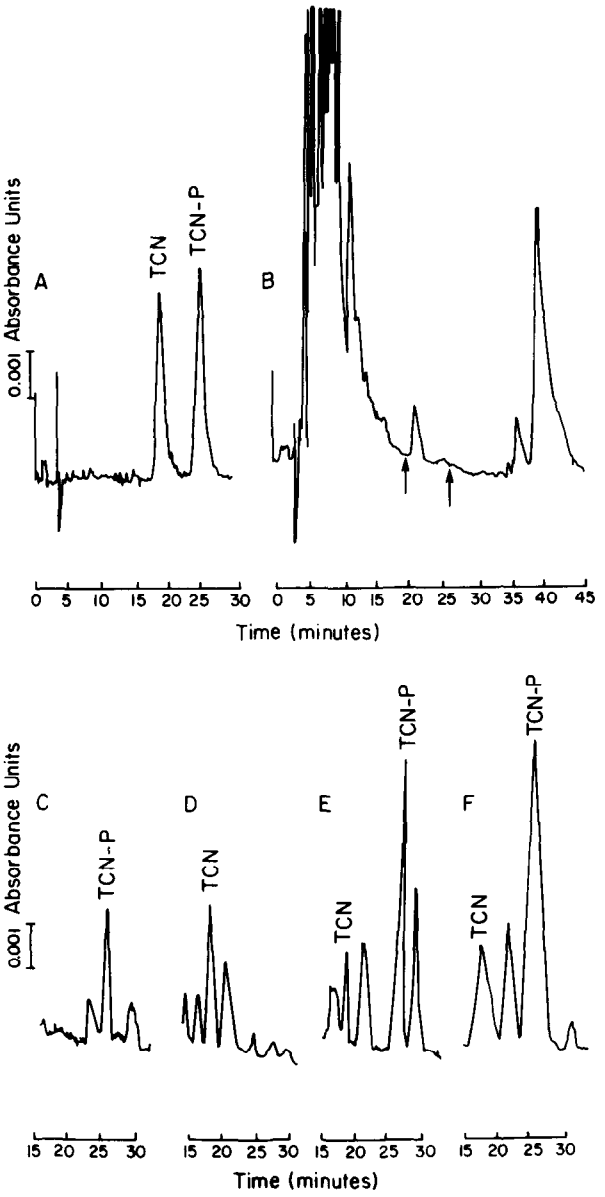

Fig. 2. Chromatograms obtained from (A) aqueous standards prepared with 10 μg/ml each; (B) control blank plasma; (C) control plasma containing 1.0 μg/ml TCN-P and 1.0 mg/ml AMP, processed 1 h after preparation; (D) patient's plasma 21 days, (E) pleural effusion fluid 20 days, and (F) liver metastases at autopsy 61 days after treatment with a single dose of 70 mg TCN-P per m^2 body surface area. The retention times for TCN and TCN-P were 18.6 and 25.5 min, respectively.

(B), spiked blood bank plasma (C) and patient plasma (D and E) are shown in Fig. 2. No interfering peaks were seen in control plasma, patient plasma obtained before administration of TCN-P, or in any of the blank tissue samples obtained from rats. The retention times for TCN-P and TCN were 25.5 and 18.6 min, respectively.

TABLE II

LINEARITY AND PRECISION OF THE HPLC ASSAY OF TCN-P ADDED TO WATER OR PLASMA

TCN-P aqueous standard (ng/ml)		Coefficient of variation (%)	TCN-P plasma standard (ng/ml)		Coefficient of variation (%)	Recovery[§] (%)
Theoretical	Found[*]		Theoretical[**]	Found[***]		
100	115.3	15.3	10	11.4	14.0	50
300	288.2	3.9	30	28.4	5.3	50
500	576.4	15.3	50	42.6	14.8	38
1000	1008.6	0.9	100	108.0	8.0	54
3000	2536.0	15.5	300	289.8	3.4	58
5000	4149.9	17.0	500	505.7	1.1	62
10,000	10,489.9	4.9	1000	1000.0	0.0	48
Mean ± S.D.		10.4 ± 6.84			6.7 ± 5.9	51 ± 8

[*]The slope calculated was 0.0347 ± 0.0046 mm/(ng/ml) (mean ± S.D.).
[**]Theoretical concentrations were based on the amount of TCN-P in water added to 2 ml plasma. AMP (1 mg/ml) was added to each milliliter of plasma to prevent dephosphorylation.
[***]The concentration found was calculated from the calibration curve for each of two plasma samples per concentration. The slope calculated was 0.352 ± 0.032 mm/(ng/ml) (mean ± S.D.).
[§]The percentage recovery was calculated from peak heights of plasma standards containing AMP divided by peak heights of standard solutions in water containing the corresponding concentrations.

TABLE III

LINEARITY AND PRECISION OF THE HPLC ASSAY OF TCN ADDED TO WATER OR PLASMA

TCN aqueous standard (ng/ml)		Coefficient of variation (%)	TCN plasma standard (ng/ml)		Coefficient of variation (%)	Recovery[§] (%)
Theoretical	Found[*]		Theoretical[**]	Found[***]		
300	321.3	7.1	50	50.0	0.0	45
1000	1164.7	16.5	100	123.1	23.1	55
5000	4819.3	3.6	500	446.2	10.8	58
10,000	8032.1	19.7	1000	876.9	12.3	57
Mean ± S.D.		11.7 ± 7.6			11.6 ± 8.2	54 ± 7

[*]The slope calculated was 0.0249 ± 0.0039 mm/(ng/ml) (mean ± S.D.).
[**]Theoretical concentrations were based on the amount of TCN in water added to plasma.
[***]The concentration found was calculated from the calibration curve for each of two plasma samples per concentration. The slope calculated was 0.260 ± 0.042 mm/(ng/ml) (mean ± S.D.).
[§]The percentage recovery was calculated from peak heights of plasma standards divided by peak heights of standard solutions in water containing the corresponding concentrations.

Standard curves

Representative standard curves for TCN-P and TCN are shown in Tables II and III, respectively. For TCN-P the slopes of the aqueous (0.0347) and plasma (0.352) standard curves were essentially parallel, and the ten-fold difference in slope was due to the twenty-fold concentration step and a 51% recovery. The linearity of these curves was assessed by linear regression analysis of concentration versus peak height; for TCN-P the correlation coefficients were 0.9991 for aqueous standards and 0.9998 for plasma standards.

For TCN the slopes of aqueous and plasma standard curves were 0.0249 and 0.260, respectively, and the corresponding correlation coefficients were 0.9949 and 0.9974. The mean recovery from extraction was 54%.

Table IV describes the tissue distribution of both TCN and TCN-P studied in rats. The assay allowed detection of TCN in brain tissue and high concentrations of both drugs in the pancreatic gland.

TABLE IV

TCN AND TCN-P CONCENTRATIONS IN VARIOUS BODY FLUIDS AND TISSUES OF TWO RATS 15 min AFTER RECEIVING 50 mg TCN-P PER kg INTRAVENOUSLY AS A PUSH INJECTION

Material	Concentrations (μg/ml of μg/g)	
	TCN	TCN-P
Red blood cells	5.6	12.9
Blood plasma*	2.6, 6.1	6.6, 6.0
Brain	4.4	n.d.**
Pancreas	43.2	118.5
Spleen	9.6	n.d.
Kidneys	36.9	n.d.
Small intestine	8.7	31.7
Large intestine	17.3	36.0
Carcass	2.1	24.4

*Separate determinations for specimens from two animals.
**n.d. = concentrations not detected.

DISCUSSION

A variety of mobile phases revealed short retention times below 20 min and were insufficient to separate the drugs from interferences. However, when 0.0025 *M* tetrabutylammonium hydroxide was added to the aqueous buffer—methanol mobile phase, satisfactory sensitivity and resolution were achieved. A previously described extraction procedure employed 20% trichloroacetic acid (TCA) to precipitate proteins [5]. Repeated trials in this laboratory, however, revealed dephosphorylation and degradation of TCN-P immediately after TCA was added. Polar solvents, e.g. methanol, ethyl acetate or diethyl ether, allowed recovery of only 10% of added TCN-P from plasma. A silica Sep-Pak column conditioned with acetonitrile—water (90:10, v/v) and eluted with water revealed a drug recovery of only 18%. The most reproducible, sensitive results were obtained using an ion-pair reagent and C_{18} Sep-Pak precolumn.

The method described here was not subject to interferences from a number of concomitantly administered drugs such as digitalis or penicillin. In a further application of this assay, we have measured concentrations of TCN-P as small as 100 ng/ml incubated in human tumor stem cell assays [7, 8]. The method described herein is practical for use in any laboratory with HPLC capability for clinical plasma and tissue drug level measurement, as well as preclinical animal and in vitro studies.

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REFERENCES

1 W.R. Cobb, A.E. Bogden, S.D. Reich, T.W. Griffin, D.E. Hilton and D.J. LePage, Cancer Treat. Rep., 67 (1983) 173—178.
2 P.D. Schweinsberg, H.G. Taylor and T.L. Loo, Proc. Amer. Assoc. Cancer Res., 20 (1979) 678.
3 P.D. Schweinsberg and T.L. Loo, Proc. Amer. Assoc. Cancer Res., 21 (1980) 129.
4 P.D. Schweinsberg and T.L. Loo, J. Chromatogr., 181 (1980) 103—107.
5 Investigational Drug Branch, National Cancer Institute, Clinical brochure, Tricyclic nucleoside 5′-phosphate (NSC 280594), Bethesda, MD, 1983, pp. 1—18.
6 P.D. Schweinsberg, R.G. Smith and T.L. Loo, Biochem. Pharmacol., 20 (1981) 2521—2526.
7 R. Schilcher, C. Haas, J. Young and L.H. Baker, Proc. Amer. Soc. Clin. Oncol., 24 (1983) 1145.
8 A.W. Hamburger and S.E. Salmon, Science, 197 (1977) 461—463.