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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF THE ANTINEOPLASTIC AGENT TRICYCLIC NUCLEOSIDE 5'-PHOSPHATE AND ITS DISPOSITION IN RABBIT

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SUMMARY

Anion-exchange and reversed-phase high-performance liquid chromatographic procedures are described for the assay of the antineoplastic agent tricyclic nucleoside 5'-phosphate (TCNP) and its metabolite tricyclic nucleoside (TCN) in biological fluids. Disposition of TCNP has been studied in rabbit. TCNP is eliminated from blood and plasma with a biologic half-life of about 7.5 h. Apparent volume of distribution is 43.2 l/m² and total body plasma TCNP clearance is 67.8 ml/min/m². TCNP is hydrolyzed by plasma and probably other tissues to TCN which is present in blood and plasma at about one-tenth the concentration of TCNP. There is no accumulation of TCNP or TCN in blood or plasma over 2 days of administration. In 24 h 2.4% of a dose of TCNP is excreted in bile of a rabbit with a cannulated bile duct as unchanged TCNP and 30.7% as TCN. TCN is excreted in bile at an initial concentration half the maximum solubility of TCN in rabbit bile. Excretion of TCNP and TCN over 24 h in the urine of a rabbit with a cannulated bile duct is 1.5% and 5.2% of the dose, respectively.

INTRODUCTION

Tricyclic nucleoside 5'-phosphate [6-amino-4-methyl-8-(β-D-ribofuranosyl)-pyrrolo(4,3,2-*de*)pyrimido(4,5-*c*)pyridazine-5'-phosphate] (Fig. 1) is an antitumor agent with activity against several animal tumor model systems including L1210 and P-388 murine leukemias, CD8F1 mammary carcinoma and the human MX-1 mammary tumor xenograft [1, 2]. Tricyclic nucleoside 5'-phosphate is structurally related to certain naturally occurring 7-deazapurine nucleosides with antitumor activity. Tricyclic nucleoside 5'-phosphate is currently undergoing clinical evaluation as an antitumor agent in humans. As a preliminary step to studies in humans we developed sensitive high-performance liquid chromatographic (HPLC) assay procedures for tricyclic nucleoside

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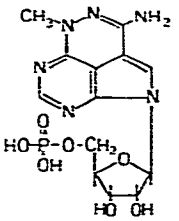


Fig. 1. Structure of tricyclic nucleoside 5′-phosphate.

5′-phosphate and its dephosphorylated metabolite tricyclic nucleoside in biological fluids. The disposition of tricyclic nucleoside 5′-phosphate in rabbit has been studied.

EXPERIMENTAL

Drugs

Tricyclic nucleoside 5′-phosphate (NSC 280594) and tricyclic nucleoside (NSC 154020) were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. Aminopyrine and 4-nitropyridine were obtained from Aldrich, Milwaukee, WI, U.S.A.

Animal and patient studies

Male New Zealand white rabbits weighing between 2 and 3 kg were injected intravenously with tricyclic nucleoside 5′-phosphate at a dose of 100 mg/m^2 (5.5 mg/kg) dissolved in 0.9% NaCl adjusted to pH 7.0 with 1 *N* NaOH. Injection was over a 1-min period into a peripheral ear vein using a vein infusion set with winged adapter (Miniset, Travenol, Deerfield, IL, U.S.A.). Tricyclic nucleoside 5′-phosphate was administered on two consecutive days in order to study possible accumulation of drug. Blood was collected at different times from a peripheral ear vein of the other ear into heparinized tubes. Plasma from a portion of the blood was collected immediately by centrifugation at 4°C. Plasma and blood were stored frozen at —70°C until assay. Rabbits used for biliary excretion studies were anesthetized with pentobarbital and a polyethylene cannula (PE 160, Intramedic, Clay Adams, Parsippany, NJ, U.S.A.) was inserted in the bile duct. Animals were allowed to recover for 3 h before giving tricyclic nucleoside 5′-phosphate and bile was collected over a 24 h period from the exteriorized bile cannula. Urine was collected using a pediatric Foley balloon catheter (French Size 10, Bard, Murray Hill, NJ, U.S.A.) inserted into the bladder through the urethra. Separate groups of rabbits were used for blood pharmacokinetic studies and biliary and urinary excretion studies.

Preparation of samples

A 1-ml volume of blood or plasma or 0.1 ml urine or bile was mixed with 4 ml ice-cold 0.5 *N* perchloric acid containing as internal standard 1 μg 4-nitropyridine for ion-exchange HPLC or 10 μg aminopyrine for reversed-phase HPLC. Protein was removed by centrifuging at 1000 *g* for 10 min at 4°C and

the supernatant applied to a 3-ml disposable octadecylsilane-bonded silica gel extraction column (J.T. Baker, Phillipsburg, NJ, U.S.A.). The column was washed with 4 × 2 ml water and adsorbed compounds eluted with 4 × 2 ml 10% water in methanol adjusted to pH 10.0 with ammonium hydroxide. Solvent was removed by evaporation under nitrogen at 30°C. The residue was dissolved in 200 μl water and 50 μl was taken for HPLC.

High-performance liquid chromatography

A Hewlett-Packard 1084B liquid chromatograph and variable-wavelength detector 798575A were used in the studies. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal and peak areas were integrated. Two HPLC procedures were developed. Ion-exchange HPLC employed a 25-cm Partisil-10 SAX anion-exchange column, particle size 10 μm (Whatman, Clifton, NJ, U.S.A.) and a 0—100% 15-min linear gradient of 0.25 *M* KH_2PO_4, 0.5 *M* KCl, pH 4.5, in 5 m*M* KH_2PO_4, pH 3.3, at a flow-rate of 1.5 ml/min. Reversed-phase HPLC employed a 25-cm LiChrosorb RP-18, 5-μm column (Merck, Darmstadt, G.F.R.) and a 5—100% 7.5-min linear gradient of methanol in 0.1 *M* sodium acetate pH 5.3, at a flow-rate of 1.5 ml/min. Eluting compounds were detected by their absorbance at 292 nm. Reversed-phase HPLC was used to measure tricyclic nucleoside 5′-phosphate and tricyclic nucleoside in blood, plasma and bile. Anion-exchange HPLC was used to measure tricyclic nucleoside 5′-phosphate in urine. Urine contained endogenous compounds which interfered with the detection of tricyclic nucleoside at 292 nm. Tricyclic nucleoside, although not tricyclic nucleoside 5′-phosphate, is fluorescent (excitation wavelength 370 nm, emission wavelength 453 nm) and tricyclic nucleoside in urine was detected by reversed-phase HPLC using a Varian Fluorichrom fluorescence detector (Varian, Walnut Creek, CA, U.S.A.).

Pharmacokinetic analysis

Non-linear least-squares regression analysis of the data to obtain pharmacokinetic parameters employed the NONLIN pharmacokinetic program [3].

RESULTS

Octadecylsilane bonded extraction columns provided a simple and rapid way of concentrating tricyclic nucleoside 5′-phosphate and tricyclic nucleoside from biological fluids. Efficiency of extraction of tricyclic nucleoside 5′-phosphate was 54% and of tricyclic nucleoside 100%. Columns could be used up to 3 times with no loss of efficiency. Two chromatographic procedures were developed for assaying tricyclic nucleoside 5′-phosphate. A method employing anion-exchange HPLC detected tricyclic nucleoside 5′-phosphate but tricyclic nucleoside was not retained by the column and eluted in the void volume with other endogenous compounds (Fig. 2). The limit of sensitivity for detection of tricyclic nucleoside 5′-phosphate by this method was 50 ng/ml. An alternative method employed reversed-phase HPLC on an octadecylsilane-bonded column and separated tricyclic nucleoside 5′-phosphate and tricyclic nucleoside (Fig. 3). The limit of sensitivity for detection of tricyclic nucleoside 5′-phosphate

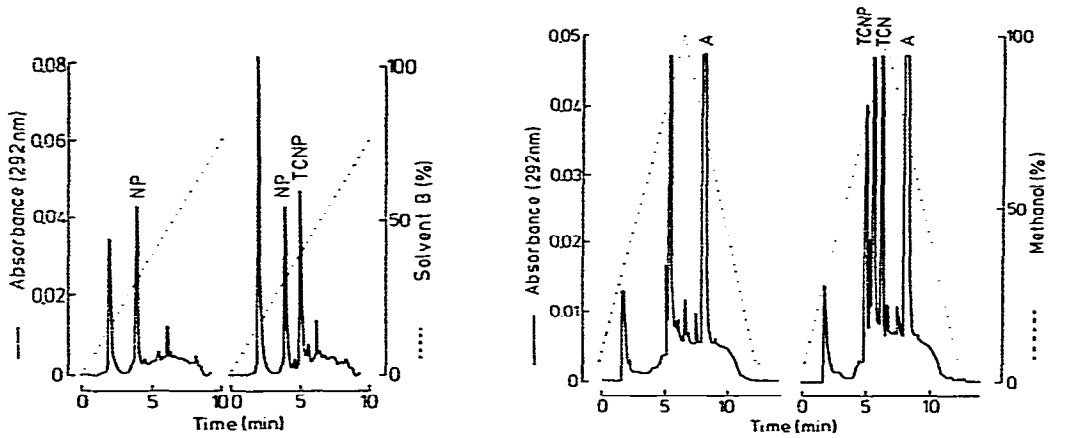
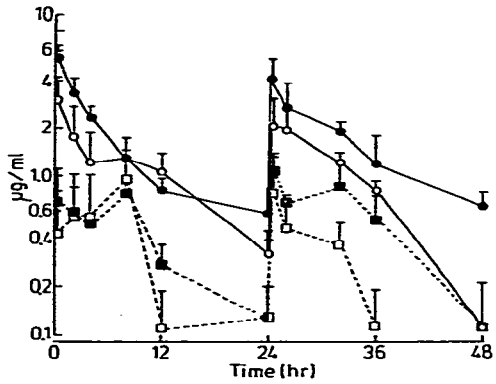


Fig. 2. Ion-exchange HPLC of human plasma to which had been added, left panel, 1 μg/ml 4-nitropyridine internal standard (NP); right panel, 1 μg/ml 4-nitropyridine internal standard and 1 μg/ml each of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside. Tricyclic nucleoside was not retained by the column and eluted in the void volume with other endogenous compounds. Chromatographic conditions: anion-exchange HPLC on a 25-cm Partisil-10 SAX column with a 0—100% linear gradient of 0.25 *M* KH_2PO_4, 0.5 *M* KCl, pH 4.5 (solvent B) in 5 m*M* KH_2PO_4, pH 3.3 (solvent A). Flow-rate, 1.5 ml/min. Detection by absorbance at 292 nm.

Fig. 3. Reversed-phase HPLC of human plasma to which had been added, left panel, 10 μg/ml aminopyrine internal standard (A); right panel, 10 μg/ml aminopyrine internal standard and 1 μg/ml each of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside (TCN). Chromatographic conditions: reversed-phase HPLC on a 25-cm C_{18} column with a 5—100% linear gradient of methanol in 0.1 *M* sodium acetate, pH 5.3. Flow-rate, 1.5 ml/min. Detection by absorbance at 292 nm.

in rabbit plasma was 50 ng/ml and for tricyclic nucleoside 25 ng/ml. The coefficient of variation of the method at 1 μg/ml in plasma was ± 4.3% for tricyclic nucleoside 5′-phosphate and ± 5.4% for tricyclic nucleoside. The assay was linear up to 100 μg/ml for both tricyclic nucleoside 5′-phosphate and tricyclic nucleoside.

Tricyclic nucleoside 5′-phosphate was administered to rabbits on two consecutive days at a daily dose of 100 mg/m². Blood and plasma concentrations of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside are shown in Fig. 4. Insufficient data points were obtained to accurately define an initial phase of tricyclic nucleoside 5′-phosphate distribution in either blood or plasma and the data were fitted to a one-compartment model. Tricyclic nucleoside 5′-phosphate concentrations in whole blood decreased with a half-life of 6.6 h on day 1 and 8.4 h on day 2. Tricyclic nucleoside 5′-phosphate concentrations in plasma were generally lower than in whole blood and fell with a half-life of 8.8 h on day 1 and 6.3 h on day 2. The apparent volume of distribution of tricyclic nucleoside 5′-phosphate (mean of day 1 and 2) calculated using plasma concentrations was 43.2 l/m² and plasma clearance 67.8 ml/min/m². Tricyclic nucleoside was found as soon as 5 min after administration of tricyclic nucleoside 5′-phosphate in both blood and plasma. Concentrations of tricyclic nucleoside were approximately one-tenth the



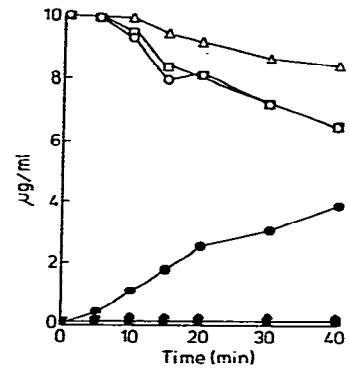


Fig. 4. Time course of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside (TCN) in rabbit blood and plasma. TCNP, 100 μg/m², was administered at 0 and 24 h by i.v. infusion over 1 min. (•) TCNP in blood, (▪) TCN in blood, (○) TCNP in plasma, (□) TCN in plasma. TCNP is shown by continuous lines, TCN by dotted lines. Each point is the mean of three rabbits. Bars are S.E. of mean.

Fig. 5. Stability of tricyclic nucleoside 5′-phosphate (TCNP) in rabbit blood and plasma at 37°C. Fresh heparinized plasma or blood was incubated with TCNP (10 μg/ml) with gentle shaking at 37°C. Open symbols are TCNP, closed symbols TCN. (•) Plasma alone, (▲) whole blood and (▪) plasma separated from blood after incubation with TCNP.

concentration of tricyclic nucleoside 5′-phosphate and appeared to fall at a rate similar to tricyclic nucleoside 5′-phosphate, although an estimate of half-life could not be obtained from the data. There was no accumulation of tricyclic nucleoside 5′-phosphate or tricyclic nucleoside in blood or plasma over 2 days of administration.

To determine the contribution of plasma and blood cells to the conversion of tricyclic nucleoside 5′-phosphate to tricyclic nucleoside fresh heparinized rabbit plasma or blood was incubated with tricyclic nucleoside 5′-phosphate, 10 μg/ml, at 37°C (Fig. 5). Tricyclic nucleoside 5′-phosphate was destroyed by plasma with the appearance of tricyclic nucleoside. Disappearance of tricyclic nucleoside 5′-phosphate from plasma was not affected by the presence of blood cells but tricyclic nucleoside was not found in plasma separated after incubation from whole blood. Disappearance of tricyclic nucleoside 5′-phosphate assayed in whole blood was slower than disappearance assayed in plasma and no tricyclic nucleoside was detected in whole blood. Similar findings were obtained with human blood and plasma (results not shown). Cooling blood and plasma to 4°C inhibited disappearance of tricyclic nucleoside 5′-phosphate. Tricyclic nucleoside 5′-phosphate was much more rapidly destroyed by hemolyzed blood than by plasma or non-hemolyzed blood (results not shown). Care should therefore be taken not to hemolyze blood during collection for pharmacokinetic studies of tricyclic nucleoside 5′-phosphate.

Biliary and urinary excretion of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside in rabbit with a cannulated bile duct is shown in Fig. 6. Small amounts of tricyclic nucleoside 5′-phosphate were found in bile during the first few hours after administration but in 24 h only 2.4% of the dose was excreted as unchanged tricyclic nucleoside 5′-phosphate. Much more tricyclic

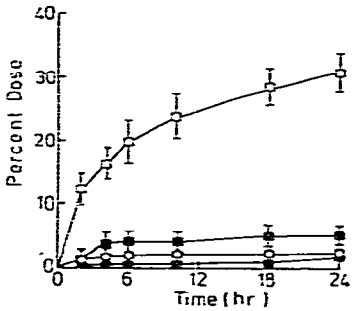


Fig. 6. Excretion of tricyclic nucleoside 5′-phosphate (TCNP) and tricyclic nucleoside (TCN) in rabbit bile and urine. TCNP (100 mg/m²) was administered to rabbit with a cannulated bile duct. Cumulative biliary excretion of (▪) TCNP and (▫) TCN. Cumulative urinary excretion of (•) TCNP and (○) TCN. Each point is the mean of three animals, bars are S.E. of mean.

nucleoside was excreted in bile, 30.7% of a dose of tricyclic nucleoside 5′-phosphate was excreted in bile in 24 h as tricyclic nucleoside. The concentration ratio of tricyclic nucleoside in bile to plasma over the first 2 h after administration of tricyclic nucleoside 5′-phosphate was approximately 230:1. The concentration of tricyclic nucleoside in bile collected over 2 h after administration of tricyclic nucleoside 5′-phosphate was (± S.E.M., n = 3) 115 ± 16.7 μg/ml. The maximum solubility of tricyclic nucleoside in rabbit bile at room temperature was determined by adding a concentrated solution of tricyclic nucleoside, 10 mg/ml, to rabbit bile to give a theoretical concentration in bile of 1 mg/ml, shaking the bile for 45 min at room temperature and removing undissolved drug by centrifugation. The maximum solubility of tricyclic nucleoside in rabbit bile was (± S.E.M., n = 3) 257 ± 35 μg/ml. Only small amounts of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside were excreted in urine in 24 h, 1.5% and 5.2% of the dose administered, respectively.

DISCUSSION

Tricyclic nucleoside 5′-phosphate was synthetized as a water-soluble derivative of tricyclic nucleoside [4]. Tricyclic nucleoside 5′-phosphate and tricyclic nucleoside have similar antitumor activity [2]. The present study shows that tricyclic nucleoside 5′-phosphate is dephosphorylated in vivo by (an) enzyme(s) present in rabbit and human plasma. Cellular ecto-5′-nucleotidase may also contribute to the formation of tricyclic nucleoside from tricyclic nucleoside 5′-phosphate [5]. Human erythrocytes, which lack ecto-5′-nucleotidase [5], and rabbit erythrocytes do not contribute to the dephosphorylation of tricyclic nucleoside 5′-phosphate by whole blood. Erythrocytes take up tricyclic nucleoside almost as rapidly as it is formed from tricyclic nucleoside 5′-phosphate by plasma and in vitro no tricyclic nucleoside could be detected in plasma separated from whole blood after incubation with tricyclic nucleoside 5′-phosphate. In vivo tricyclic nucleoside is found in plasma at low concentrations suggesting that there could be saturation of the erythrocyte uptake process. This could be due to the rapid breakdown of tricyclic nucleoside

5′-phosphate to tricyclic nucleoside by tissues in addition to plasma. Some tricyclic nucleoside might be formed from tricyclic nucleoside 5′-phosphate during collection and separation of plasma. Tricyclic nucleoside is rephosphorylated by erythrocyte adenosine kinase to form tricyclic nucleoside 5′-phosphate [6]. This probably accounts for the apparently slower rate of tricyclic nucleoside 5′-phosphate disappearance from whole blood than from plasma. The fact that some destruction of tricyclic nucleoside 5′-phosphate was apparent in whole blood without appearance of tricyclic nucleoside suggests that other metabolites are formed which are not detected by the assay procedure. Schweinsberg et al. [7] have identified three oxidation products of tricyclic nucleoside in addition to tricyclic nucleoside 5′-phosphate, formed when tricyclic nucleoside is incubated with human erythrocytes. Metabolites, apart from tricyclic nucleoside, were not detected in vitro or in vivo by our assay procedures.

Tricyclic nucleoside 5′-phosphate administered to rabbit disappeared slowly from blood with a half-life of approximately 7.5 h. Tricyclic nucleoside 5′-phosphate concentrations in plasma are somewhat lower than in blood but fall at the same rate. Tricyclic nucleoside concentrations in blood and plasma are much lower than tricyclic nucleoside 5′-phosphate but fall at about the same rate. The results suggest that there might be an equilibrium between plasma and erythrocyte tricyclic nucleoside 5′-phosphate and tricyclic nucleoside. Only small amounts of tricyclic nucleoside 5′-phosphate and tricyclic nucleoside were excreted in rabbit urine, 1.5% and 5.2% of the dose in 24 h, respectively, in rabbits with a cannulated bile duct. Some tricyclic nucleoside 5′-phosphate was excreted in rabbit bile, 2.4% of the dose in 24 h, but relatively large amounts of tricyclic nucleoside, 30.7% of the dose in 24 h. Tricyclic nucleoside is relatively insoluble. The maximum solubility of tricyclic nucleoside in rabbit bile at room temperature is 257 μg/ml. This is about twice the concentration of tricyclic nucleoside seen in rabbit bile in the first 2 h after administration of tricyclic nucleoside 5′-phosphate. Although the rabbit has a gallbladder, bile did not appear to be concentrated but flowed continuously during the 24-h collection period. It is possible that under normal conditions bile is stored and concentrated in the gallbladder and if so the solubility of tricyclic nucleoside could be exceeded. Studies in dog have shown extensive excretion and crystallization of tricyclic nucleoside in the bile duct following administration of tricyclic nucleoside at doses of 5—25 mg/kg [8]. In the present study rabbits were given tricyclic nucleoside 5′-phosphate at 5.5 mg/kg. It is possible that crystallization of tricyclic nucleoside in rabbit bile might occur with higher doses of tricyclic nucleoside 5′-phosphate.

ACKNOWLEDGEMENT

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