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High-performance liquid chromatographic determination of 9-(3-pyridylmethyl)-9-deazaguanine (BCX-34) in biological fluids

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Abstract

9-(3-Pyridylmethyl)-9-deazaguanine (BCX-34), a new purine nucleoside phosphorylase inhibitor, has selective immunosuppressive activity with potential therapeutic value in T-cell-mediated diseases. We now report a sensitive, specific and reproducible method for measurement of 9-(3-pyridylmethyl)-9-deazaguanine in biological fluids using high-performance liquid chromatography (HPLC). 9-(3-Pyridylmethyl)-9-deazaguanine was extracted from plasma using perchloric acid precipitation followed by passage through Sep-Pak C_{18} cartridges (average extraction efficiency, 64.6%). Standard curves were linear over the range of interest (28–1120 ng/ml in plasma and 200–4000 ng/ml in urine, $r^2>0.999$). Within-day and between-day coefficients of variation were less than 8%. The limit of quantitation was 28 ng/ml in plasma and 200 ng/ml in urine. This HPLC method should be useful in future clinical studies with this drug.

Keywords: 9-(-3-Pyridylmethyl)-9-deazaguanine

1. Introduction

9-(3-pyridylmethyl)-9-deazaguanine (BCX-34, Fig. 1) is a novel inhibitor of purine nucleoside phosphorylase (PNP, EC 2.4.2.1). The development of 9-(3-pyridylmethyl)-9-deazaguanine proceeded from the rational design of PNP inhibitors using computer-assisted molecular modeling, based on the three-dimensional structure of the enzyme determined by X-ray crystallography [1,2]. PNP catalyzes the reversible phosphorolytic cleavage of ribo- and 2′-deoxyribonucleosides of hypoxanthine and guanine [3,4]. Patients lacking this enzyme are profoundly deficient in T-cell function, while maintaining normal or exaggerated B-cell function [5–7]. PNP inhibitors may be useful in the treatment of T-cell-mediated diseases including cutaneous T-cell lymphoma (CTCL), autoimmune diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus,

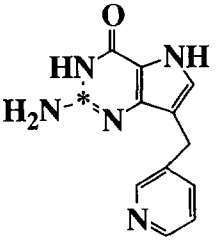

Fig. 1. Structure of 9-(3-pyridylmethyl)-9-deazaguanine (BCX-34; peldesine); the asterisk indicates ^{14}C.

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psoriasis, and type I diabetes), T-cell leukemia and the prevention of organ transplant rejection [8]. The PNP inhibitor, 8-aminoguanosine, has been shown to inhibit proliferation of MOLT-4 (a T-lymphoblastic cell line) in vitro in the presence of 2′-deoxyguanosine [9]. In vivo, 9-(3-pyridylmethyl)-9-deazaguanine can inhibit a T-cell-mediated inflammatory response in the dinitrofluorobenzene-induced contact-delayed hypersensitivity mouse ear edema model [2]. In clinical studies, 9-(3-pyridylmethyl)-9-deazaguanine dermal cream has been applied to affected areas of the skin in 8 CTCL patients and in 14 psoriasis patients as a new topical therapy [1,10]. These initial clinical studies provide the basis for undertaking further studies with systemic delivery of 9-(3-pyridylmethyl)-9-deazaguanine in the treatment of CTCL and other T-cell-mediated diseases.

The purpose of the present study was to develop a sensitive, specific, and reproducible HPLC method that would enable examination of plasma and urine 9-(3-pyridylmethyl)-9-deazaguanine concentrations in clinical pharmacokinetic studies. This method has been demonstrated to be useful in the measurement of plasma and urine concentrations of 9-(3-pyridylmethyl)-9-deazaguanine in an initial pre-phase I pharmacokinetic study in three patients with CTCL receiving intravenous infusions and oral administration of radiolabelled 9-(3-pyridylmethyl)-9-deazaguanine.

2. Experimental

2.1. Chemicals and reagents

9-(3-pyridylmethyl)-9-deazaguanine and ^{14}C-labeled 9-(3-pyridylmethyl)-9-deazaguanine [11] were obtained from BioCryst Pharmaceuticals (Birmingham, AL, USA). HPLC grade acetonitrile, methanol, ammonium sulfate, potassium hydroxide and potassium phosphate were obtained from Fisher Scientific (Fair Lawn NJ, USA). Perchloric acid was obtained from Aldrich (Milwaukee, WI, USA). Dimethyl sulfoxide was obtained from Sigma (St. Louis, MO, USA). Scintillation cocktail was obtained from Research Products International (Mt. Prospect, IL, USA).

2.2. Equipment and chromatographic conditions

The high-performance liquid chromatographic (HPLC) analysis was conducted using a Hewlett-Packard 1050 liquid chromatographic system. The eluent from the HPLC was monitored for ultraviolet absorbance at 233 nm at 0.001 AUFS with a Hewlett-Packard 1050 variable-wavelength detector. The detector output was recorded on a Hewlett-Packard Model 3396 integrator. A Pinkerton internal surface reversed-phase HPLC column, GFF-II-S5-80 (25 cm×4.6 mm I.D.), together with a 1 cm GFF guard column from Regis (Morton Grove, IL, USA), were used at ambient temperature. The mobile phase was prepared by mixing 100 m*M* ammonium sulfate (pH 6.0) with acetonitrile (85:15, v/v), then filtering through a 0.2-μm Super-200 membrane from Gelman Sciences (Ann Arbor, MI, USA), and finally degassing with high purity helium for 20 min. The flow-rate used in this study was 1 ml/min.

2.3. Sample pretreatment and recovery

2.3.1. Sample pretreatment

In order to quantitate the efficiency of extraction and obtain a standard curve, radiolabelled 9-(3-pyridylmethyl)-9-deazaguanine (20 ng, 10 000 DPM, based measured values) was added to 1-ml samples of drug-free plasma. Each plasma sample (1 ml) was mixed with 100 μl of 4 *M* cold (4°C) perchloric acid and placed on ice for 10 min, vortexed and then kept on ice for another 5 min. The mixture was subsequently centrifuged at 16 000 *g* for 10 min. The supernatant was removed, adjusted to pH 6.0–6.5 with a mixture of 4 *M* potassium hydroxide and 1 *M* dipotassium hydrogenphosphate, placed on ice for 10 min and then centrifuged at 16 000 *g* for 10 min. The supernatant was then removed for solid-phase extraction using a Sep-Pak C_{18} cartridge from Millipore (Milford, MA, USA). The Sep-Pak cartridge was conditioned with 10 ml of methanol followed by 10 ml of distilled and deionized water. The resultant supernatant was loaded onto a cartridge and washed with 5 ml of 5 m*M* ammonium sulfate (pH 9.0), followed by 10 ml of distilled and deionized water. After the cartridge was suctioned to near dryness under vacuum, 9-(3-pyridylmethyl)-9-deazaguanine was eluted into a 15-ml polypropylene tube with 5

ml of 10% methanol (pH 2–3). The eluates were adjusted to pH 6.0–7.0 with potassium hydroxide, dried under nitrogen and the residue then redissolved in 200 μl of mobile phase. The entire sample was passed through a 0.2-μm Acrodisc LC13 PVDF filter from Gelman Sciences (Ann Arbor, MI, USA). Finally, 100 μl of the filtrate was injected onto the HPLC column.

Urine samples were not extracted, but prepared as follows. Samples were initially centrifuged at 2800 *g* at 4°C for 15 min, diluted 1:1–1:20 with mobile phase, then filtered though 0.2-μm acrodisc filter prior to HPLC analysis.

2.3.2. Recovery

Radioactivity measurements were utilized to assess recovery, with ^{14}C-labelled 9-(3-pyridylmethyl)-9-deazaguanine being added as an internal standard to the plasma samples used for the standard curve and quality control. Fractions (1 ml) of eluent were collected following HPLC, into 7-ml scintillation vials in a RadiFrac fraction collector from Pharmacia Biotech (Piscataway, NJ, USA), and then mixed with 5 ml scintillation cocktail. The radioactivities of both pre-extracted and post-extracted samples were quantitated using a Beckman (Fullerton, CA, USA) LS 6000 liquid scintillation counter. The percentage of recovery of 9-(3-pyridylmethyl)-9-deazaguanine was calculated as: recovery (%)=[(DPM of post-HPLC fractions/injected volume)/(DPM of pre-extracted sample/volume)]×100.

2.4. Preparation of standard curve

9-(3-Pyridylmethyl)-9-deazaguanine was weighed accurately and then dissolved with dimethyl sulfoxide to 1 mg/ml. The stock solution was stored at −70°C and used within two months. This stock solution was further diluted 1:100 with distilled and deionized water to obtain solution I (10 μg/ml), which was used for the urine study, and 1:500 to obtain solution II (2 μg/ml), which was used for the plasma study. Working solutions I and II were prepared daily. Human plasma and urine were obtained from healthy volunteers.

For the plasma standard curve, solutions were prepared containing varying concentrations of 9-(3-pyridylmethyl)-9-deazaguanine in volumes between 14 to 560 μl. These were evaporated to dryness under a stream of nitrogen. The remaining residues were then dissolved in 1 ml of human plasma to obtain standard samples with concentrations ranging between 28 and 1120 ng/ml of 9-(3-pyridylmethyl)-9-deazaguanine.

For the urine standard curve, solutions were prepared containing concentrations of 9-(3-pyridylmethyl)-9-deazaguanine between 20 to 400 μl. These were evaporated to dryness under nitrogen. The residues were then dissolved in 1 ml of human urine to obtain standard samples, with concentrations ranging between 200 and 4000 ng/ml of 9-(3-pyridylmethyl)-9-deazaguanine.

2.5. Quantitation

9-(3-Pyridylmethyl)-9-deazaguanine was quantitated by measuring HPLC peak height produced by change in UV absorbance at the retention time where 9-(3-pyridylmethyl)-9-deazaguanine eluted. Prior to examination of an unknown, a standard curve was obtained by plotting the peak heights of extracted standard samples of 9-(3-pyridylmethyl)-9-deazaguanine against their known concentrations. The concentration of 9-(3-pyridylmethyl)-9-deazaguanine in each unknown sample was then determined using the standard curve.

2.6. Accuracy and precision

The within- and between-day precision as well as the accuracy, were determined using standard methods [12]. The within-day precision and accuracy were determined from five quality control samples at four different concentrations on the same day. The between-day precision and accuracy were determined from the quality control samples at the same concentrations, on different days.

2.7. Application

This method was applied to the determination of 9-(3-pyridylmethyl)-9-deazaguanine in plasma and urine from the pre-phase I pharmacokinetic study of three patients with CTCL. Patient plasma and urine samples were obtained from a clinical pharmacokinetic study of CTCL patients being treated

with radiolabelled 9-(3-pyridylmethyl)-9-deazaguanine. A more complete description of the patients and the pharmacokinetic study using radiolabelled 9-(3-pyridylmethyl)-9-deazaguanine is being reported separately.

Patients in this study initially received an intravenous (IV) infusion (over 30 min) of 9-(3-pyridylmethyl)-9-deazaguanine at a dose of 18 mg/m^2 mixed with ^{14}C-labelled 9-(3-pyridylmethyl)-9-deazaguanine (61.4 mCi/mmol, 99.9% radiochemical purity) and 14 days later received oral administration of the same dose and formulation of radiolabelled 9-(3-pyridylmethyl)-9-deazaguanine. For IV infusion, heparinized blood samples were drawn from each patient at pretherapy, and then at −0.25, 0, 0.17, 0.33, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 h after drug administration. The −0.25 and 0 h times represent samples collected at 15 min after the start of the intravenous infusion and the end of the infusion, respectively. For oral administration, the −0.25 and 0 h samples were omitted, and all other time points were the same as intravenous infusion. Each collected blood sample was immediately centrifuged. Plasma was aliquoted into 1-ml samples and stored at −70°C until analysis. Total voided urine was collected from each patient pretherapy, and at 0–2, 2–4, 4–8, 8–12, 12–24, 24–36, and 36–48 h the volume of urine for each collection was recorded. A 50-ml aliquot was retained and frozen at −70°C until analysis.

In order to permit comparison between quantitation of 9-(3-pyridylmethyl)-9-deazaguanine by the method described in this manuscript and the radioassay, 9-(3-pyridylmethyl)-9-deazaguanine was determined by both methods in plasma and urine.

For the radioassay used in the initial study, 20–100 μl of plasma or urine was mixed with 5 ml scintillation cocktail. The radioactivity in plasma or urine was determined by a Beckman LS 6000 liquid scintillation counter. The concentration of 9-(3-pyridylmethyl)-9-deazaguanine was then calculated based on specific activity of ^{14}C-labelled 9-(3-pyridylmethyl)-9-deazaguanine. In the initial study with administration of ^{14}C-labelled 9-(3-pyridylmethyl)-9-deazaguanine, plasma and urine samples were also evaluated by HPLC with examination of each eluted fraction to determine the presence of drug and metabolites.

3. Results

3.1. Chromatograms

Representative chromatograms from human plasma and urine are shown in Figs. 2 and 3. Examination of drug-free plasma and urine standard samples demonstrated no interfering endogenous peaks. The retention time of 9-(3-pyridylmethyl)-9-deaza-

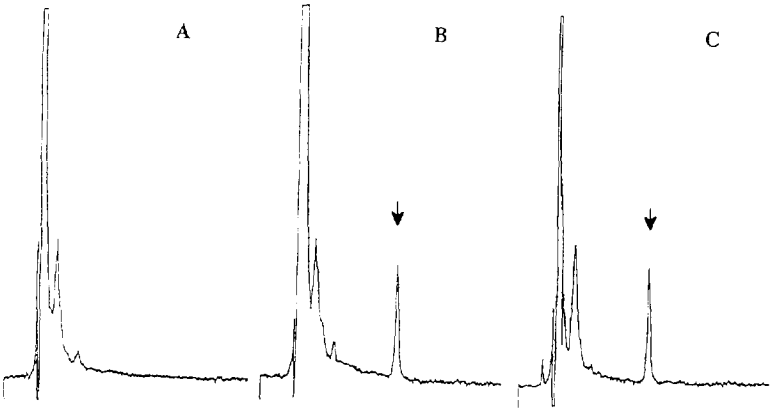

Fig. 2. Representative HPLC chromatograms of plasma (UV absorbance at 233 nm at 0.001 AUFS). (A) Blank plasma, (B) plasma spiked with 9-(3-pyridylmethyl)-9-deazaguanine, (C) plasma of patient who received 18 mg/m^2 9-(3-pyridylmethyl)-9-deazaguanine by oral administration, and ↓ indicates the peak of 9-(3-pyridylmethyl)-9-deazaguanine.

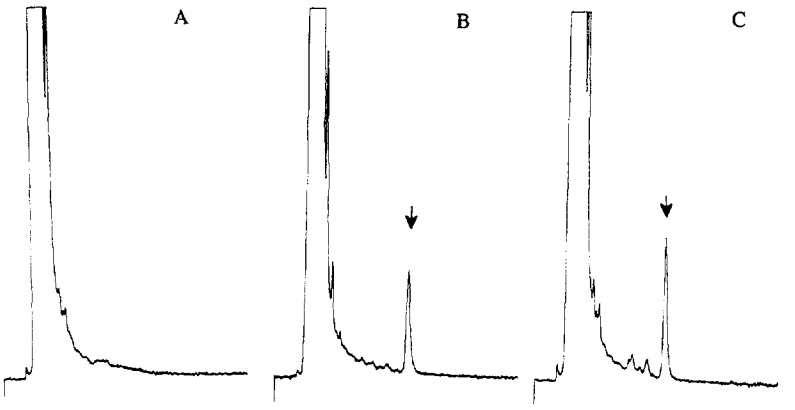

Fig. 3. Representative HPLC chromatograms of urine (UV absorbance at 233 nm at 0.001 AUFS). (A) Blank urine, (B) urine spiked with 9-(3-pyridylmethyl)-9-deazaguanine, (C) urine of patient who received 18 mg/m^2 9-(3-pyridylmethyl)-9-deazaguanine by oral administration, and ↓ indicates the peak of 9-(3-pyridylmethyl)-9-deazaguanine.

guanine was approximately 10.2 min. The time for each chromatographic run was 20 min.

3.2. Extraction recovery

The overall recovery after extraction and HPLC was approximately 65% in plasma. Mean±S.D. of 9-(3-pyridylmethyl)-9-deazaguanine recoveries were 63.9±6.23% from 50 to 100 ng/ml and 64.6±5.65% from 200 to 500 ng/ml (n=8).

3.3. Assay validation

The standard curve for 9-(3-pyridylmethyl)-9-deazaguanine was linear over a concentration range from 28–1120 ng/ml in plasma and 200–4000 ng/ml in urine, with a correlation coefficient (r^2) of 0.999 for each. Coefficients of variation (C.V.) of slopes for standard curves in plasma and urine were 1.08 and 2.52% (n=6 for each), respectively.

For plasma, between-day variability, as expressed by between day C.V. for each quality control concentration, did not exceed 8% with 96.01–101.36% accuracy. Within-day variability, as demonstrated by within-day C.V. for each quality control concentration, did not exceed 7.5% with 96.24–106.94% accuracy (Table 1).

For urine, between-day C.V. for each quality control concentration did not exceed 6% with 99.93–106.17% accuracy. Within-day C.V. for each quality control concentration did not exceed 7% with 97.63–104.07% accuracy (Table 2).

There was no significant degradation of 9-(3-pyridylmethyl)-9-deazaguanine in plasma and urine observed in three months at −80°C. The extracts from plasma were stable for at least 12 h at 37°C and 48 h in the autosampler at room temperature.

The lower limit of quantitation (LOQ) was determined to be 28 ng/ml in plasma and 200 ng/ml in urine (C.V. <20%).

Table 1
Analytical reproduciblility for 9-(3-pyridylmethyl)-9-deazaguanine in plasma

Concentration (ng/ml)	Within-day (n=5) (mean±S.D.)	C.V.[a] (%)	Between-day (n=5) (mean±S.D.)	C.V. (%)
50.000	49.219±3.571	7.2	49.186±3.549	7.2
125.000	133.682±10.357	7.7	127.239±3.610	2.8
250.000	240.590±11.868	4.9	240.047±9.131	3.8
500.000	508.503±16.622	3.3	506.820±14.838	2.9
1000.000	1002.414±8.834	0.9	997.871±8.051	0.8

[a] Coefficient of variation.

Table 2
Analytical reproduciblility for 9-(3-pyridylmethyl)-9-deazaguanine in urine

Concentration (ng/ml)	Within-day ($n=5$) (mean±S.D.)	C.V.[a] (%)	Between-day ($n=5$) (mean±S.D.)	C.V. (%)
350.000	341.712±23.237	6.8	356.101±20.747	5.8
875.000	910.576±39.689	4.4	928.963±59.911	6.4
1750.000	1708.630±59.816	3.5	1748.780±60.061	3.4
3500.000	3568.366±80.570	2.3	3592.570±93.199	2.6

[a] Coefficient of variation.

3.4. Comparison of HPLC assay with the radioassay for quantitation of 9-(3-pyridylmethyl)-9-deazaguanine levels

As illustrated in Fig. 4A, there was excellent correlation between the HPLC assay and the radioassay for 9-(3-pyridylmethyl)-9-deazaguanine in plasma samples from the CTCL patients in the initial clinical pharmacologic study ($y=-2.6522+1.0710x$, $r^2=0.992$). Fig. 4B shows excellent correlation between both assays for urine samples from the same patients ($y=-1.2235+0.96691x$, $r^2=0.994$).

3.5. Specificity of the analytical method

The peak at 10.2 min in HPLC chromatograms of plasma and urine from patients receiving 9-(3-pyridylmethyl)-9-deazaguanine was re-evaluated with liquid chromatography–mass spectrometry (LC–MS). The LC–MS study showed that this peak has the same molecular mass and fragmentation patterns as 9-(3-pyridylmethyl)-9-deazaguanine standard, suggesting that no metabolites co-elute with the intact drug form.

3.6. Application of method

The HPLC assay was applied to the measurement of 9-(3-pyridylmethyl)-9-deazaguanine following intravenous and oral administration of 18 mg/m^2. Fig. 5 shows the plasma concentration–time curves for 9-(3-pyridylmethyl)-9-deazaguanine after a 30-min continuous intravenous infusion and after a single oral administration of the same solution. Plasma concentrations of 9-(3-pyridylmethyl)-9-deazaguanine reached a peak at the end of intravenous

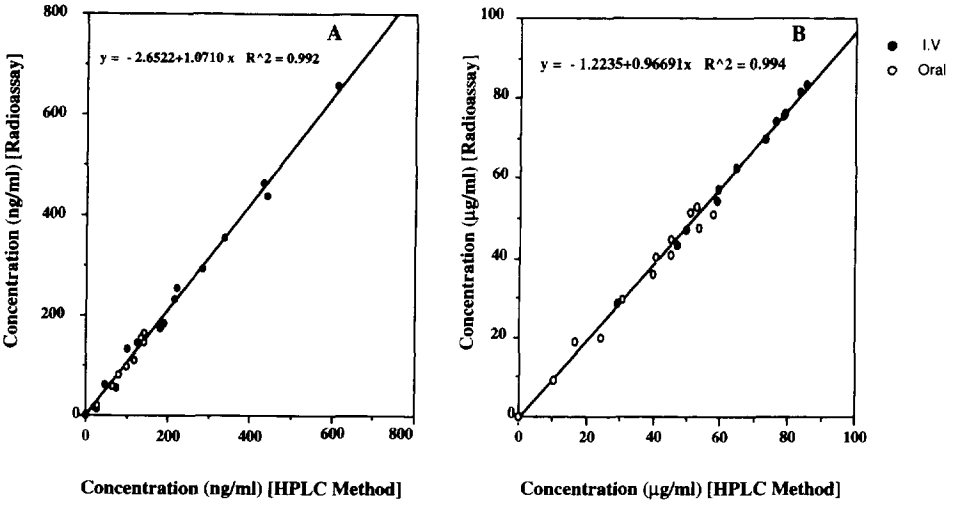

Fig. 4. Comparison of HPLC method and radioassay in quantitating 9-(3-pyridylmethyl)-9-deazaguanine in plasma (A) and urine (B) samples of patients who received 18 mg/m^2 9- (3-pyridylmethyl)-9-deazaguanine by oral and intra-venous administration.

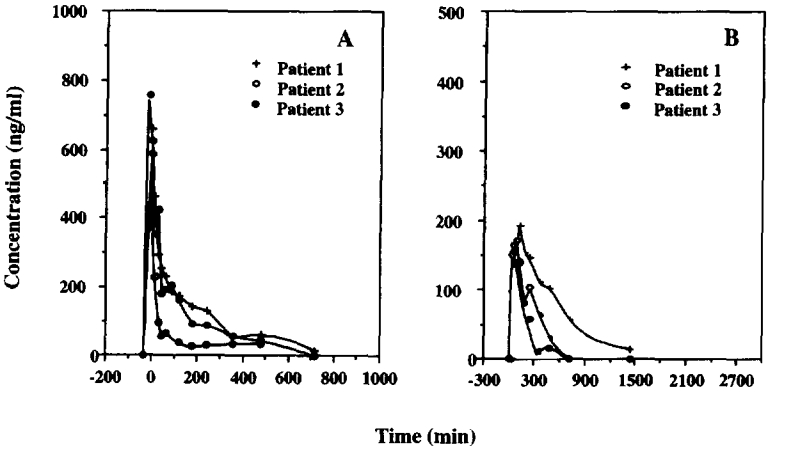

Fig. 5. Plasma concentration–time profiles in three patients following intravenous (A) and oral (B) administration of 18 mg/m^2 9-(3-pyridylmethyl)-9-deazaguanine. Plasma concentrations were measured by the HPLC method.

infusion with a mean peak level of 617.84 ng/ml. Plasma concentrations of 9-(3-pyridylmethyl)-9-deazaguanine reached a peak after oral administration at 60 min with a mean peak level of 154.90 ng/ml. Examination of the radioactivity following HPLC separation, demonstrated that more than 97% of the radioactivity in plasma was present as intact 9-(3-pyridylmethyl)-9-deazaguanine. A representative radiochromatogram of plasma is shown in Fig. 6A.

Fig. 7 shows the cumulative urinary excretion over 36 h after intravenous and oral administration of 9-(3-pyridylmethyl)-9-deazaguanine. 9-(3-Pyridylmethyl)-9-deazaguanine was rapidly excreted in the urine after intravenous administration, with 77.34±15.47% (mean±S.D.) of the administrated dose excreted within 12 h, 83.94±11.07% within 24 h and 85.67±11.13% within 36 h. With single oral administration of 9-(3-pyridylmethyl)-9-deazaguanine, 39.73±5.48% of the administered dose was excreted in urine within 12 h, 47.01±4.80% within 24 h and 49.72±4.00% within 36 h. Examination of the radioactivity following HPLC separation showed that more than 95% of the radioactivity in urine was

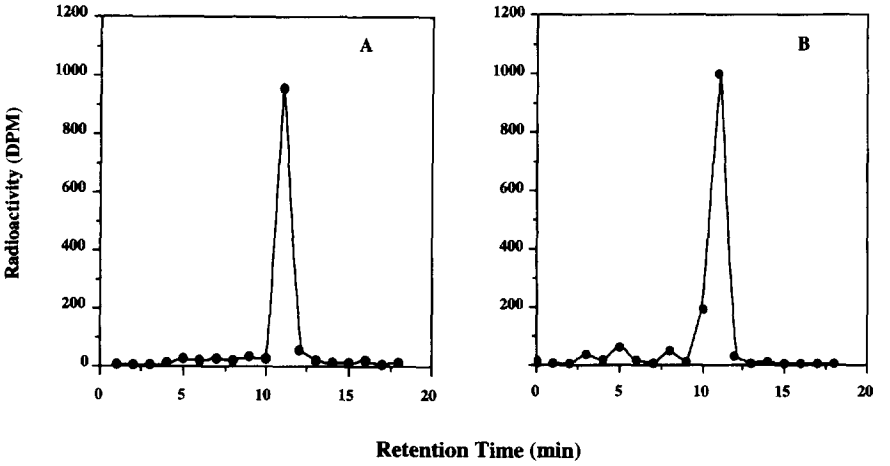

Fig. 6. Representative HPLC radiochromatograms of plasma (A) and urine (B) of patient who received 18 mg/m^2 9-(3-pyridylmethyl)-9-deazaguanine by oral administration.

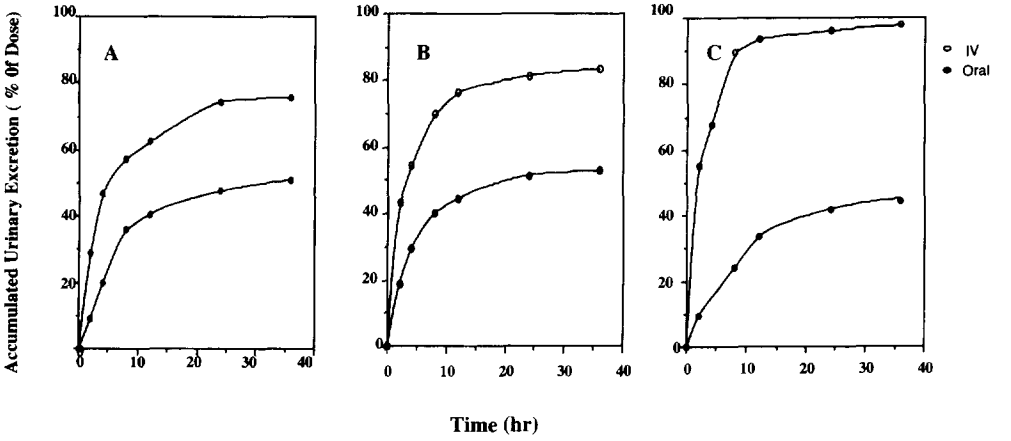

Fig. 7. Accumulated 9-(3-pyridylmethyl)-9-deazaguanine urinary excretion in patient 1 (A), 2 (B) and 3 (C) over 36 h following intravenous and oral administration of 18 mg/m^2 9-(3-pyridylmethyl)-9-deazaguanine. Urine concentrations were measured by the HPLC method.

intact 9-(3-pyridylmethyl)-9-deazaguanine. A representative radiochromatogram of urine is shown in Fig. 6B.

4. Discussion

With clinical studies currently underway to determine an effective and non-toxic schedule for administration of 9-(3-pyridylmethyl)-9-deazaguanine in CTCL patients, it is essential to have a sensitive, specific and reproducible method to measure 9-(3-pyridylmethyl)-9-deazaguanine in biological fluids. In the present study, we describe a method that should prove useful in further pharmacological studies with 9-(3-pyridylmethyl)-9-deazaguanine.

The HPLC method using an internal surface reverse phase column, was shown to provide satisfactory separation of 9-(3-pyridylmethyl)-9-deazaguanine from the endogenous plasma or urine components. Non-extracted plasma may be injected directly onto the HPLC column, but the sensitivity for determination of 9-(3-pyridylmethyl)-9-deazaguanine is relatively lower. The limit of quantitation was 200 ng/ml of 9-(3-pyridylmethyl)-9-deazaguanine, which was higher than most of the plasma concentrations of 9-(3-pyridylmethyl)-9-deazaguanine observed in this initial human pharmacokinetic study. We have found that a solid-phase extraction using Sep-Pak C_{18} cartridges resulted in increased sensitivity in measuring 9-(3-pyridylmethyl)-9-deazaguanine in plasma. The limit of quantitation was 28 ng/ml of 9-(3-pyridylmethyl)-9-deazaguanine. Washing the Sep-Pak with basic ammonium sulfate (pH 9.0) removed most endogenous substances that were not of interest. The specificity and reproducibility of the assay were also markedly improved. Accuracy and precision of the method were confirmed by determining known concentrations of 9-(3-pyridylmethyl)-9-deazaguanine in plasma and urine, both for within-day and between-day measurements. Accuracy was in an acceptable range (96.01–106.94%). The mean coefficients of variation were less than 8%. The assay showed good linearity (r^2>0.999) over the concentration range of interest (28–1120 ng/ml in plasma and 200–4000 ng/ml in urine). This method should be particularly useful for analyzing 9-(3-pyridylmethyl)-9-deazaguanine plasma and urine levels in the planned initial phase I oral dose-escalation study with this drug in CTCL patients.

Excellent correlation was found between the HPLC method and the radioassay. The HPLC assay of 9-(3-pyridylmethyl)-9-deazaguanine in biological fluids (based on UV absorbance) provides a reliable determination of intact 9-(3-pyridylmethyl)-9-deazaguanine in plasma and urine.

In conclusion, a sensitive, specific and reproducible method was developed for measurement of 9-(3-

pyridylmethyl)-9-deazaguanine in biological fluids. This method was successfully applied to the determination of 9-(3-pyridylmethyl)-9-deazaguanine in plasma and urine in pre-phase I pharmacokinetic studies and should prove useful in future pharmacological studies not utilizing radiolabelled drugs.

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