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A radioimmunoassay procedure for tallysomycin S_{10b} in human plasma and urine

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Summary. A simple and sensitive radioimmunoassay (RIA) procedure was developed and validated for the analysis of tallysomycin S_{10b} in human plasma and urine. The assay utilized antisera developed in rabbits, ^{125}I-tallysomycin as the radioligand, and dextran-coated charcoal to separate free and bound antigen. The antibody was specific in that it did not cross-react with either tallysomycin A or bleomycin. The lower limit of quantification was 5 ng per ml plasma or urine, and the linear range of the assay was 5–320 ng tallysomycin S_{10b} base per ml plasma or urine. The within-day assay variability (%RSD) for plasma and urine was 11% at a concentration of 50 ng per ml, and 3% and 7% for plasma and urine, respectively at 200 ng per ml. Within-day accuracy ranged from 100% to 108% of the theoretical value. Tallysomycin S_{10b} was stable in human plasma and urine at concentrations of 20 and 160 ng per ml for at least 7 months when stored at −20° C. The method was applied to the analysis of plasma and urine samples from a patient given tallysomycin S_{10b} as part of a phase I study.

Introduction

Tallysomycin S_{10b} (Fig. 1) is a new, water-soluble, basic glycopeptide with antibiotic and antitumor activities [6, 7]. It is a semibiosynthetic tallysomycin compound formed by the addition of 1,4 diaminobutane to cultures of *Streptoalloteichus hindustanus*. The naturally produced tallysomycins A and B differ from tallysomycin S_{10b} in the nature of the amine side-chain attached to the 2,4′ bithiozolyl-2,4 carboxylic acid portion of the molecule. The sidechain in tallysomycin S_{10b} is 1,4-diaminobutane. Both tallysomycins A and B contain spermidine in the terminal portion of the side-chain, while tallysomycin A has an additional L beta lysine in the subterminal portion of the chain. Tallysomycin S_{10b} has the same chromophore as bleomycin, but differs from the latter by the absence of a methyl group in one of the amino acids, the presence of two additional hydroxyl groups, a unique sugar, 4-amino-4,6 dideoxy-L-talose, and a 1,4-diaminobutane side-chain.

Tallysomycin S_{10b} had comparable or greater activity than bleomycin in a variety of murine tumor systems, and was about twice as potent as bleomycin [8]. In single- and multiple-dose toxicological studies in mice and dogs, nephrotoxicity was the most consistent and prominent drug-related alteration and was the dose-limiting toxicity [3]. The compound appeared to produce less pulmonary toxicity than bleomycin in mice and rats at doses approaching the maximum tolerated levels [8]. In dogs, the pulmonary toxicity of the two drugs was comparable [3].

Because of the low initial dose (1.25 mg/m^2) projected for phase I trials in cancer patients, a sensitive, rapid and specific radioimmunoassay (RIA) procedure was required for definition of the pharmacokinetics of tallysomycin S_{10b} in humans. This report describes an RIA procedure for tallysomycin S_{10b} that is suitable for measuring concentrations of the drug in human serum and urine.

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Materials and methods

Chemicals. The following chemicals were obtained from the sources indicated: sodium-^{125}I, Amersham Searle, Arlington Heights, Ill; sodium azide and chloramine T, Eastman Kodak Co., Rochester, NY; activated charcoal, RIA grade, Becton Dickinson Co., Orangeburg, NJ; sodium metabisulfite and KI, Matheson Coleman and Bell, Norwood, Ohio; gelatin, Difco Laboratories, Detroit, Mich; Sephadex C-25, Sephadex G-25, and dextran T-70, Pharmacia, Piscataway, NJ; Freund's adjuvant and bovine serum albumin, Calbiochem-Behring Corp., LaJolla, Calif; l-ethyl-3-(dimethylaminopropyl) carbodiimide, Aldrich, Milwaukee, Wis; human serum, Interstate Blood Bank, Philadelphia, Pa; tallysomycin S_{10b} (lots 124F10, 80F751), tallysomycin A (lot 87F11), and bleomycin (lot 82L104), Bristol Laboratories, Syracuse, NY. Other chemicals were reagent grade.

All reagents were prepared in water generated by a Milli-Q^{TM} water system, Millipore Corp., Bedford, Mass.

Preparation of tallysomycin S_{10b}-BSA conjugate. The bovine serum albumin (BSA) conjugate was prepared by procedures based on methods described by Broughton and Strong [1] and Broughton et al. [2] for the preparation of BSA conjugates of bleomycin and tallysomycin, respectively. BSA (20 mg/ml) was dissolved in 0.50 *M* (pH 7.5) phosphate-buffered saline (PBS) containing 45 mg of tallysomycin S_{10b} plus a trace amount (2×10^6 cpm) of ^{125}I-tallysomycin S_{10b}. A 1.0-ml solution of l-ethyl-3(dimethylaminopropyl) carbodiimide (800 mg/ml) was slowly added with constant stirring. After 1 h of mixing at room temperature the mixture was left at 4 °C for 3 days. A sample of

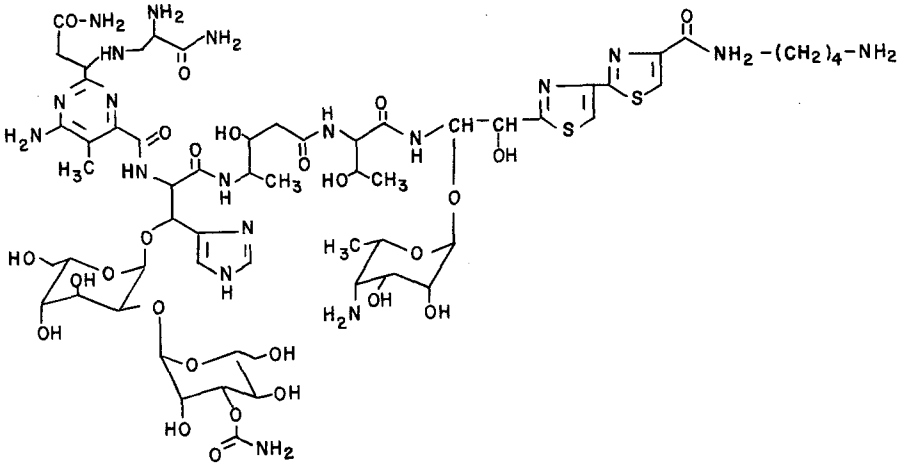

Fig. 1. Structure of tallysomycin S_{10b}

the mixture was removed for determination of radioactivity, and the remainder was dialyzed for 18 h at 4 °C against PBS. Additional purfication was achieved by chromatography on a Sephadex G-25 column (30 × 1 cm). The column was eluted with 0.01 *M* PBS (pH 7.5) at a flow rate of 1 ml/min, and thirty 1-ml fractions were collected. The UV absorbance (280 nm) and the amount of radioactivity in each fraction were determined. The tallysomycin S_{10b}-BSA conjugate eluted in fractions 17–19 and accounted for about 60% of the radioactivity applied to the column.

Immunization. The purified conjugate was emulsified in complete Freund's adjuvant and was injected into three New Zealand white rabbits by the procedure of Hurn and Landon [5]. Each rabbit received 0.5 mg in 2 ml by IM injection in each limb and by SC injections at several sites along the back. Four booster injections were given over 5 months. Antisera with a titer of 1 : 500 was obtained from one rabbit and was stored frozen at −20 °C.

Iodination of tallysomycin S_{10b}. Tallysomycin was iodinated with iodine-125, using a modification of the Hunter and Greenwood chloramine-T reaction [4] as described by Broughton and Strong [1]. To a 1-dram WISP vial containing a magnetic stirrer, the following ingredients were added with microliter syringes in the order indicated: 10 µl (1 mCi) sodium-^{125}I, 60 µl 0.1 *M* sodium borate buffer (pH 9.0), 10 µl tallysomycin S_{10b} base (1 mg/ml), and 10 µl chloramine T (5 mg/ml). The mixture was incubated for 3 min at room temperature. The reaction was terminated by adding 10 µl sodium metabisulfite (12 mg/ml) and 10 µl potassium iodide (20 mg/ml). The contents of the vial were applied to a Sephadex C-25 column (30 × 0.9 cm) covered with a lead sheet, prepared and equilibrated in 0.1 *M* ammonium formate buffer (pH 6.4). The column was eluted with 0.1 *M* buffer at a flow rate of 5 ml/min, and 3 ml fractions were collected. The column was then eluted (5 ml/min) with 1.0 *M* ammonium formate buffer (pH 6.4), and 30 additional 3-ml fractions were collected. A 20-µl sample of each fraction was taken for a determination of radioactivity. The fractions eluting with the 1.0-*M* formate buffer and containing radioactivity were combined, and 1.0-ml samples were stored at −20 °C.

Radioimmunoassay procedure. Unknown concentrations of tallysomycin S_{10b} in human plasma and urine were determined by a competitive protein-binding assay between ^{125}I-labeled drug and unlabeled drug for antibody binding sites. Free and bound drug were separated with dextran-coated charcoal. Appropriate volumes of tallysomycin S_{10b} standards in water (5–170 µl) were added in triplicate to 12 × 75 mm tubes to give 0.5, 1, 2, 4, 8, 16, 24, and 32 ng tallysomycin S_{10b} base per tube. Control human plasma or urine (0.1 ml) and appropriate volumes of PBS containing 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin (PBSA-Gel) were added to bring the total volume to 0.3 ml. Two additional sets of tubes without added unlabeled tallysomycin S_{10b} were prepared for determining nonspecific binding (NSB) and maximum binding (B_0). A 0.1-ml sample of ^{125}I-tallysomycin S_{10b} (approx. 30000 cpm) was added to all the tubes, including an extra set for determining the total counts per minute added (T_0). A 0.1-ml sample of the tallysomycin S_{10b} antiserum diluted 1 : 300 in PBSA-Gel was added to all tubes except the NSB and T_0 tubes. An additional 0.1 ml PBSA-Gel was added to the NSB tube in place of the antisera. The tubes were mixed on a vortex mixer, incubated for 15 min at 37 °C, and then placed in an ice-water bath for 15 min. With the tubes still in the ice bath, a 0.2-ml sample of dextran-coated charcoal (200 mg dextran T-70 and 2 g activated charcoal in 100 ml PBSA) was added, followed by 0.4 ml PBS. The tubes were mixed, incubated for an additional 5 min in the ice bath, and then centrifuged at 1800 *g* for 10 min at 5 °C. The supernatants were decanted into 12 × 75 mm glass tubes and were counted for 1 min in a gamma counter. Samples of plasma or urine were assayed in duplicate, following the same procedure as outlined for the standards, except that 0.1-ml samples were pipetted into the tubes in place of the 0.1 ml normal human plasma or urine.

Calculations. The raw counts (cpm) in the supernatant fractions were entered into a Hewlett Packard Model 3356 laboratory automation system where calculations were performed automatically. The bound radioactivity (B_1) for each sample or standard was calculated, and the ratio:

$$Y = \frac{B_1 - NSB}{B_0 - NSB}$$

was calculated and transformed to:

$$\text{logit } Y = \ln\left(\frac{Y}{1-Y}\right) = \ln\left(\frac{B_1 - NSB}{B_0 - B_1}\right)$$

The slope and intercept for the standard curve were obtained by linear regression of logit Y vs ln of the amount of standard in each tube. The amounts of tallysomycin S_{10b} in the samples were estimated by inverse prediction.

Validation. The specificity of the assay was estimated by preparing standard curves in human plasma and urine with the structurally related compounds bleomycin and tallysomycin A. The concentrations ranged from 6 to 160 ng/tube.

The lower limit of quantification of the assay was determined by obtaining samples of plasma and urine from ten donors. The samples were divided into duplicate samples of 1.0 ml each. To one tube of each pair was added 5.0 ng (5 µl) tallysomycin S_{10b} (test sample) in PBS. To the other tube was added 5 µl PBS (blank). All tubes were carried through the analytical procedure, and the measured responses (cpm) for each blank and test sample were recorded and subjected to a one-tailed paired-comparison *t*-test.

Within-day and between-day reproducibility and accuracy were determined by analysis of human plasma and urine samples prepared in bulk to contain 50 and 200 ng tallysomycin S_{10b} per ml. Samples (0.2 ml) of each matrix at each concentration were placed in individual tubes. One set of ten samples from each group was analyzed immediately. Additional samples were stored at −20 °C and were analyzed in triplicate on three separate occasions.

Storage stability. Quality control samples were prepared in bulk in human plasma and urine to contain 20 and 160 ng tallysomycin S_{10b} per ml. These samples were allocated to individual tubes, stored at −20 °C, and were analyzed in triplicate at various times over a 7-month period.

Application to patient samples. Blood samples were obtained before and at the following times after drug administration from a patient given the low starting dose of 1.25 mg tallysomycin S_{10b}/m^2 in a phase I study: 1, 5, 10, 20 and 30 min, and 1, 1.5, 2, 3, 4, 6, 8, and 24 h. The blood was placed in heparinized Vacutainer tubes, mixed, and centrifuged to separate plasma and cells. The plasma was removed immediately to a separate tube and stored at −20 °C until analyzed. A predose urine sample was obtained and total urine output was collected over the following intervals: 0–2, 2–4, 4–8, and 8–24 h. The urine was stored at 5 °C during the collection period, and samples were stored at −20 °C until analyzed. Written informed consent was obtained.

Results and discussion

The ^{125}I-tallysomycin S_{10b}, unreacted ^{125}I, and possible by-products were separated by the stepwise elution of a Sephadex C-25 column with 0.1 *M* and 1.0 *M* ammonium formate buffer. The ^{125}I-labeled tallysomycin S_{10b} was eluted with 1.0 *M* buffer and appeared to be stable for at least 5 weeks when stored at −20 °C, according to the reproducibility of standard curves over this time period.

The standard curves in human plasma and urine, plotted as ln concentration (ng/tube) vs logit, were linear over a concentration range of 0.5 to 32 ng tallysomycin S_{10b} per tube, or 5 to 320 ng/ml (Fig. 2). The lower limit of quantification, defined as that concentration having a response

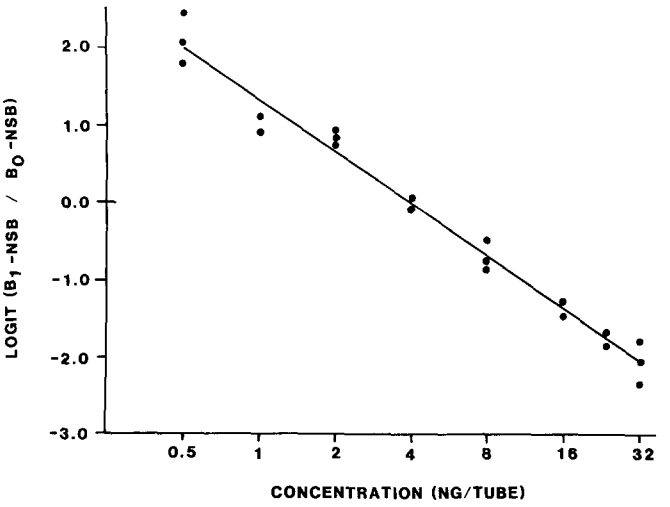

Fig. 2. Ln-logit plot of a typical standard curve for tallysomycin S_{10b} in human plasma

significantly different from that given by an identical sample containing no tallysomycin S_{10b}, was 5 ng/ml human plasma or urine. This is equivalent to 0.5 ng per assay tube, since 0.1-ml samples were analyzed.

The within-day assay variability (% RSD) for plasma and urine was 11% at a concentration of 50 ng per ml, and 3% and 7% for plasma and urine, respectively, at a concentration of 200 ng/ml. Accuracy ranged from 100% to 108% of theory. Between-day variability (% RSD) was 4% or less at concentrations of 50 and 200 ng/ml plasma. Between-day accuracy was 98%–102% for the high concentration and 119%–120% for the low concentration. Between-day variability for urine was 10%–12% at 50 ng per ml and 4%–7% at 200 ng per ml. Between-day accuracy was 102% and 105% and 102% and 107% for the low and high concentrations, respectively. Tallysomycin S_{10b} was stable in human plasma and urine at concentrations of 20 and 160 ng per ml for at least 7 months when stored at −20 °C. Therefore, samples can be stored for a considerable period of time prior to analysis.

The antibody to tallysomycin S_{10b} did not cross-react with the structurally-related compounds, tallysomycin A or bleomycin, at concentrations up to five times the upper linear limit of the assay (320 ng/ml). This assay was intended for samples from a phase I study in cancer patients, but since patients receiving concomitant drugs were excluded from this study, the cross-reactivity with other antitumor agents was not evaluated. Individual constituents or portions of the tallysomycin molecule were not available for defining specifically the cross-reactivity of the antibody and that portion of the molecule it reacted with. Additional studies will be required to establish this as well as the possible reactivity with as yet undefined metabolites.

The results of applying this RIA procedure to plasma samples from a cancer patient given 1.25 mg tallysomycin S_{10b}/m^2 by IV injection are shown in Fig. 3. Peak plasma concentrations were about 180 ng/ml and fell to 8 ng/ml by 8 h and to below the limit of quantification (5 ng/ml) by 24 h. Less than 10% of the administered dose was recovered in the urine in 24 h, most of the drug being excreted within the first 8–12 h. Serum creatinine and creatinine

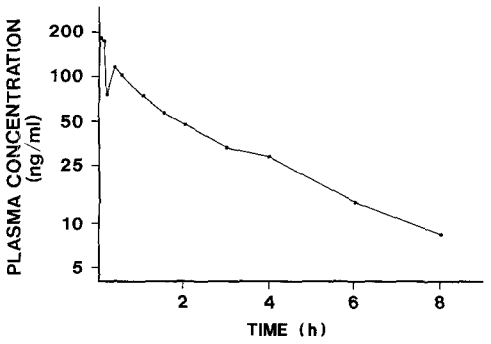

Fig. 3. Plasma concentrations of tallysomycin S_{10b} in a cancer patient as a function of time after IV administration of 1.25 mg tallysomycin S_{10b}/m^2

clearance were within normal ranges before and after drug administration. These results suggest either significant metabolism of tallysomycin S_{10b} or nonrenal elimination of a major portion of the administered drug.

The antibody prepared to tallysomycin S_{10b}, although of low titer, was sufficient to allow determination of the concentrations of tallysomycin S_{10b} in the plasma and urine of cancer patients receiving low doses of the drug in a phase I study.

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