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Determination of peplomycin in mouse tissues and biofluids by radioimmunoassay

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Abstract: Peplomycin, an antitumour antibiotic analogue of bleomycin, was measured in mouse tissues using a rapid radioimmunoassay. Antiserum, obtained by immunizing rabbits with peplomycin–bovine serum albumin conjugate, showed no significant cross-reactivity with the closely related peplomycin analogues bleomycin and liblomycin, nor with a number of other structurally unrelated antitumour drugs. The assay is sensitive and can detect peplomycin levels as low as 2 ng ml^{-1}. The relative intra- and inter-assay standard deviation is ≤5%, indicating good assay reproducibility. Peplomycin levels in mouse tissues were easily determined without extraction. Fifteen minutes after administration of a single intraperitoneal dose of peplomycin at 8.5 mg kg^{-1} (1/10 of LD_{50}), high drug levels were found in plasma (46 μg ml^{-1}), kidneys (38 μg g^{-1}), urine and bladder (32 μg ml^{-1}), followed by gastrointestinal tract (13 μg g^{-1}), lung (8 μg g^{-1}), spleen (3.7 μg g^{-1}), heart (3.6 μg g^{-1}), gall bladder (2.7 μg g^{-1}), liver (2 μg g^{-1}), and brain (0.6 μg g^{-1}). The total amount of drug in all these organs accounted for more than 80% of the dose administered. We conclude that the radioimmunoassay is sensitive and reproducibile and is an ideal tool for measuring peplomycin in tissues and biofluids for pharmacological studies.

Keywords: *Peplomycin; radioimmunoassay; tissue drug level analysis; biofluid peplomycin level analysis.*

Introduction

Peplomycin (Fig. 1) is a biosynthetic analogue of bleomycin that substitutes a 3-[(*s*)]-1′-phenylethylamino]-propylamine group at the terminal amine site [1]. Peplomycin has more potent antineoplastic activity and lower pulmonary toxicity than do bleomycin and other structurally-related analogues produced to date [2, 3]. There is, therefore, a need for a more complete understanding of the pharmacokinetics and tissue distribution of this analogue. Analytical methods reported so far often lack the required selectivity and sensitivity or have not been shown to be applicable for routine determination of drug levels in biological fluids and tissues. For example, microbiologically based assays have been reported to be useful for determining peplomycin levels in mouse tissue following a single injection of a very high dose (100 mg kg^{-1}) of drug [1]; however, the relevance of this technique is questionable as the therapeutic dose is only 5 mg kg^{-1} [3]. Enzyme immunoassays and radioimmunoassays (RIAs) have been reported that are more sensitive than the microbiological assay, but their applicability for determining drug concentration in specific tissues is unknown [4–7]. We have therefore developed an RIA for determining peplomycin levels in biological fluids and tissues of mice administered therapeutic doses of drug (8.5 mg kg^{-1}) based on 1/10 LD_{50} [1].

Experimental

Chemicals and materials

Peplomycin sulphate was kindly provided by Bristol Laboratories of Bristol Myers Squibb (Syracuse, NY). Freund's adjuvant, bovine serum albumin (BSA) and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma (St Louis, MO). Norit A charcoal was obtained from Fisher Scientific (Pittsburgh, PA), gelatin from Eastman Kodak (Rochester, NY), dextran T-70 from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and CM-Sephadex C-25 from Pharmacia Laboratory Separation, Pharmacia (Piscataway, NJ). $Na^{125}I$ was obtained from Isotex (Houston, TX). BD2F1 female mice weighing 16–20 g were purchased from Charles Rivers Lab (Wilmington, MA). Male New Zealand rabbits weighing 2–2.5 kg

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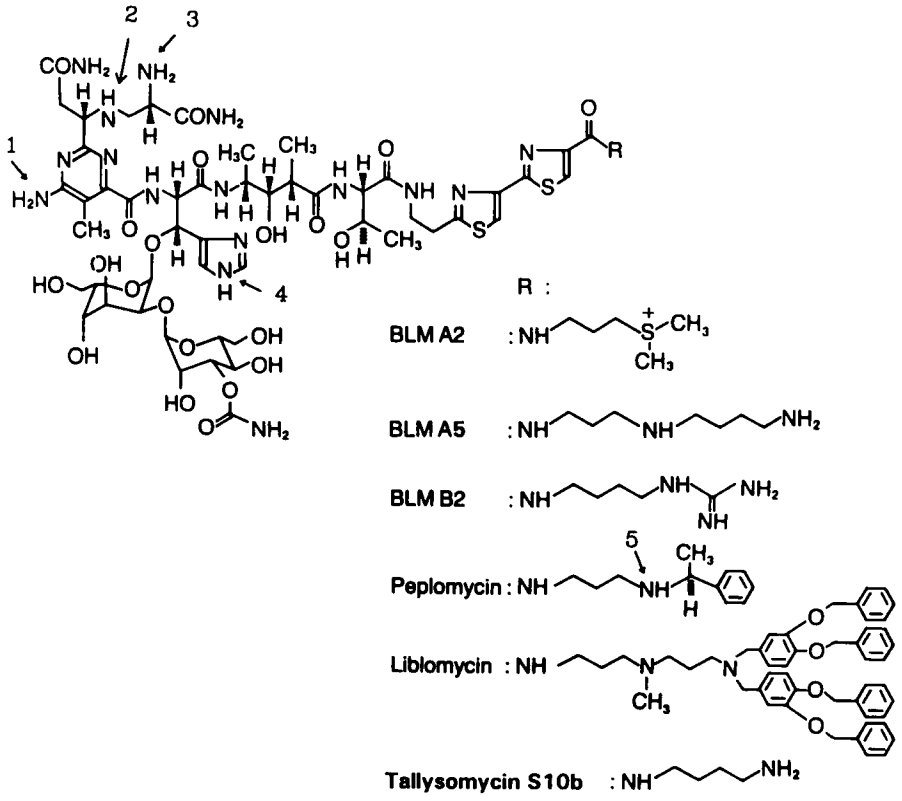

Figure 1
Structures of bleomycins, peplomycin, liblomycin, and tallysomycin S10b. The proposed possible sites for conjugation of peplomycin to BSA are indicated with arrows.

were purchased from Ray Nichols Rabbitry (Lumberton, TX). Millipore water (18 megohm-cm) was used throughout the entire study.

Preparation of peplomycin–bovine serum albumin conjugates

Bovine serum albumin (20 mg) and peplomycin (50 mg) were dissolved in 2.2 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.5, 0.01 M NaH_2PO_4–0.15 M NaCl). A 1 ml volume of the cross-linking agent, EDC (800 mg ml^{-1}), was then added dropwise with constant stirring. Stirring was continued for 1 h at room temperature and then at 4°C for 3 days. Cold (4°C) acetone (8 ml) was then slowly added, and the resulting solution centrifuged at 900*g* for 10 min at 4°C. The recovered precipitate was washed with 10 ml of 80% cold acetone, centrifuged, and lyophilized. The purity of the conjugated peplomycin was confirmed by thin-layer chromatography (TLC) utilizing silica gel F254 plates (Whatman, Clifton, NJ) and a solvent system of 1-butanol–acetic acid–water (2:1:1, v/v/v). A single spot at the origin was detected under ultraviolet light, indicating that no free form of peplomycin existed in the peplomycin–BSA conjugate preparation. The trinitrobenzenesulphonic acid method was used to quantify the primary amine content in the antigen and showed the conjugate contained 13 moles peplomycin per mole BSA [8].

Immunization procedure

Two rabbits were each immunized at several subcutaneous sites with 1.3 mg peplomycin–BSA conjugate emulsified in 1.3 ml of 50% complete Freund's adjuvant in 0.9% NaCl. An additional two rabbits were each immunized with 1.0 mg unconjugated peplomycin. Booster injections made in incomplete adjuvant, using half the initial immunization dose were given on a bimonthly schedule. After 3 months of immunization, peplomycin antibody was determined by its binding ability to ^{125}I-labelled peplomycin. Under the assay conditions described in 'Radioimmunoassay procedure', titre is the highest dilution of the peplomycin antiserum (1:12,000) that binds approximately 50% of the ligand. Blood was collected from rabbits' ear veins into tubes

containing heparin 1 week after each booster injection. Plasma was separated after centrifugation and stored in small portions at −70°C for testing the presence and immunoreactivity of peplomycin antibody.

Iodination procedure

Peplomycin was labelled with ^{125}I using a modified chloramine-T method described by Broughton and Strong [6]. Briefly, to 10 μl of 1 mCi $Na^{125}I$, 10 μl of chloramine-T (5 mg ml^{-1}) and 10 μl peplomycin (1 mg ml^{-1}) were added, gently mixed, and incubated for 2 min at room temperature. The reaction was stopped by adding 10 μl of sodium metabisulphite (12 mg ml^{-1}) and 10 μl of potassium iodide (20 mg ml^{-1}). All the solutions were made in 0.1 M borate buffer, pH 9.0. The mixture was then applied onto a cation exchange column containing CM-Sephadex C-25 (0.9 × 20 cm). Unreacted $Na^{125}I$ was eluted with 0.1 M ammonium formate (pH 6.4). ^{125}I-labelled peplomycin and unreacted peplomycin were then eluted with 1 M ammonium formate (pH 6.4). The specific activity of the product was estimated to be 14.9 mCi μmol^{-1}.

Drug administration

Six mice were intraperitoneally administered peplomycin (8.5 mg kg^{-1}), a dose 1/10th the LD_{50} [1]. With the exception of urine and bladder, which had to be pooled, all blood and tissues were collected, stored, and processed individually. Urine from all the mice was collected onto filter paper during the 15 min after injection. The filter paper was also used to absorb any urine excreted when the mice were killed. The filter paper was then soaked in 5 ml water for 30 min. Fifteen minutes after peplomycin injection, blood was drawn from the inferior vena cava into tubes containing heparin. The blood was immediately centrifuged (900*g*, 15 min), and the resulting plasma was frozen at −20°C. Brain, heart, lung, kidneys, liver, spleen, gall bladder, stomach, small intestine, and bladder were removed and, with the exception of bladder, homogenized in four volumes (1:5, wt/vol) of ice-cold water. The homogenate was further diluted (gastrointestinal tissue, 1:500; kidney, 1:200; lung, 1:100; liver, 1:20; and spleen, heart, brain, and gall bladder, 1:10) with water just before being assayed. All of the urinary bladders were placed into 2 ml ice-cold water and homogenized. The 5 ml of urinary product from the filter paper and the 2 ml bladder homogenate were combined and further diluted (1:1000) for assay.

Tissues, plasma, and urine pooled from six mice injected with 0.9% NaCl as the control group were similarly processed.

Radioimmunoassay procedure

Both peplomycin antiserum and ^{125}I-peplomycin were diluted immediately before use with 0.1% gelatin dissolved in 0.01 M PBS, pH 7.0. To 4-ml polypropylene tubes (Sarstedt, Princeton, NJ), was added 0.1 ml each of the following reagents: 0.01 M PBS (pH 7.0), ^{125}I-peplomycin (0.45 pmol/15,000 cpm), antiserum (1:12,000 dilution, v/v), and diluted tissue homogenate or peplomycin solution containing 1, 2.5, 5 and 10 ng in a total assay volume of 0.5 ml. A similar amount of appropriately diluted tissue homogenate from the control mice was added to the assay tubes containing these standard amounts of peplomycin. All samples were assayed in triplicate.

Following a 1-h incubation at room temperature, tubes were placed in an ice bath, and 0.5 ml of a 0.1% dextran–2% charcoal suspension in 0.01 M PBS (pH 7.0) was added. After a 15 min incubation at 4°C, the reaction mixture was centrifuged at 900*g* for 15 min, and the radioactivity of the supernatant was determined in a Packard PRIAS gamma counter. Two additional sets of assay tubes (to which no anti-peplomycin serum was added but instead was added a similar amount of diluted normal rabbit serum) were included in each assay to determine control binding and non-specific binding (A_0). The A_0 set additionally had the 500 μl charcoal suspension substituted with 0.01 M PBS, pH 7.0. After correction for nonspecific binding (212 ± 31 cpm $tube^{-1}$), the control binding was 15,182 ± 1177 cpm $tube^{-1}$.

Results and Discussion

Antibody immunoreactivity

Antibodies against peplomycin were detected in two rabbits immunized for 3 months with the peplomycin–BSA conjugate. After 6 months' immunization, the binding of the ^{125}I-peplomycin to peplomycin antisera from one rabbit approached a maximum; a dilution of the antiserum at 1:12,000 (v/v) binds 50% of ^{125}I-peplomycin (0.45 pmol/15,000 cpm). Under the assay conditions described above,

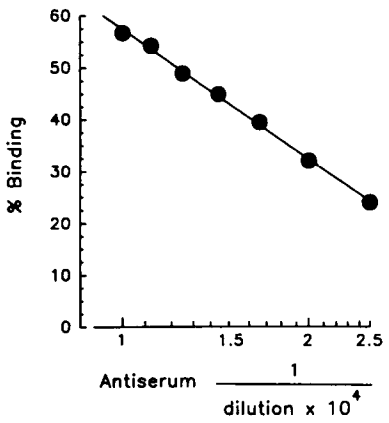

Figure 2
Immunoreactivity of peplomycin antisera. Under the assay conditions described in the Experimental section, the percentage binding of ^{125}I-peplomycin is proportional to various amounts of peplomycin anti-serum.

the percentage binding of ^{125}I-peplomycin was proportional to various amounts of anti-peplomycin serum present, as shown in Fig. 2. Chemically, the possible reactive sites of peplomycin conjugated to BSA are indicated on Fig. 1. Among these proposed five sites, the most reactive groups are probably the amine group attached to the pyrimidine ring (site 1) and the amine group of the β-aminoalanine moiety (site 3), and reactivity is dependent upon steric accessibility. The amine group of the pyrimidine ring and of the β-amino alanine moiety were also the sites proposed for bleomycin conjugation to BSA [9]. It is not likely that the conjugation would occur at the terminal group and/or the mannose moiety because the immunoreactive sites of a hapten are usually those distal to the site of conjugation with the carrier protein BSA [4]. Under the same assay conditions, the second rabbit achieved an antibody titre of 1:6200 dilution of the antiserum after 6 months' immunization. Further immunizations did not increase the titre, and the serum was then collected separately from these two rabbits, lyophilized, and stored at −70°C. Antibody without purification from the first rabbit was subsequently used to develop, characterize, and validate the RIA procedure and to determine the drug levels in mouse tissues. The two other rabbits immunized with unconjugated peplomycin over the same period of time as the above rabbits achieved a titre of only 1:12.

Effect of temperature

The effect of temperature on the drug–antibody binding reaction was studied at 4, 22 and 37°C. Under the assay conditions described above, the binding capacity was greatest at 22°C (82 ± 0.6%, mean ±SD, six replicates) and was less at other temperatures (77 ± 2.4% at 4°C, 62 ± 0.3% at 37°C). All subsequent assays were therefore performed at room temperature (22°C).

Standard curve

Figure 3 shows the ability of unlabelled peplomycin at concentrations in the range of 2–20 ng ml^{-1} in 0.01 M PBS to compete for the binding of ^{125}I-peplomycin to the peplomycin antisera. A linear curve is obtained by plotting the percentage binding inhibition versus the unlabelled peplomycin concentration on a logit–log graph. At peplomycin concentrations of 2, 5, 10 and 20 ng ml^{-1}, the percentage inhibition of antibody binding to ^{125}I-peplomycin under the standard assay conditions described were 37 ± 1.7, 67 ± 1.9, 81 ± 6.2 and 92 ± 0.5, respectively. Regression analysis was performed on a line that was calculated using the weighted regression of logit of percentage inhibition versus drug concentration. When 20 μl of 1:100 diluted drug-free mouse lung homogenate was added to the above various peplomycin concentrations, the standard curve was also linear and superimposed with that of the assays with 0.01 M PBS added (Fig. 3). Similarly, when assayed on six different days in eight separate assays, diluted gastrointestine, kidney, liver, spleen,

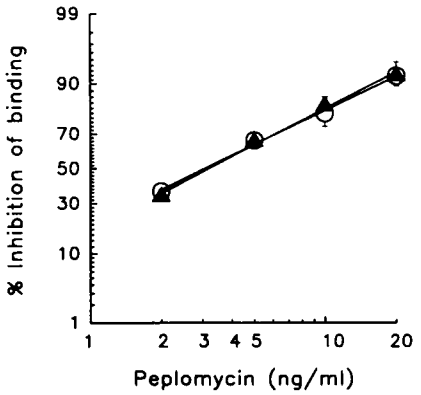

Figure 3
Standard curve of peplomycin by radioimmunoassay. Various amounts of peplomycin were prepared in 0.01 M PBS, pH 7.0 (○), r^2 = 0.9958 in diluted normal mouse lung homogenate (▲), r^2 = 0.9927. Data are presented as mean ±SD from assays performed on six separate days. The points without error bars have SD values <0.8. The assay is detailed in the Experimental section.

heart, gall bladder, brain, and bladder tissue (20 μl tube^{-1}) also had excellent linearity and reprodicibility (data not shown). No tissue-specific interference was observed in the assay of peplomycin.

Antibody specificity

Immunoreactivity of the peplomycin antibody to several structurally related as well as unrelated antitumour drugs was compared to immunoreactivity to peplomycin (Table 1). The test concentration chosen for each drug was based on clinically achievable plasma levels. Interestingly, the peplomycin antibody showed negligible affinity for the structurally related compounds bleomycin, liblomycin, or tallysomycin S10b. A previous report describing an RIA procedure for determining bleomycin A5 indicated that the antibody cross-reacted with several bleomycin-related compounds including bleomycin A2 and B2 and peplomycin [10]. The only structural difference between bleomycin and peplomycin is substitution of the terminal amine group of bleomycin for the 3-[(s)-1′-phenylethylamino]propyl-amino group in peplomycin (Fig. 1). Since our peplomycin antiserum did not cross-react with either bleomycin or bleomycin analogues, the immunoreactive group of peplomycin is specific and most likely located in the terminal group region. Because the metabolism of peplomycin has not been elucidated, the possible reactivity of its metabolites and the binding of the antibody cannot be ruled out. Therefore, the results of the RIA should be regarded as peplomycin equivalents but for ease will be referred to as peplomycin. However, the hydrolase activity should have a similar effect on peplomycin as on bleomycin. It is not likely that the small molecules generated from hydrolysis would react with peplomycin antibody.

Other commonly used anticancer drugs not structurally related to peplomycin, such as 5-fluorouracil (5-FU), cisplatin, methotrexate, etoposide (VP-16), doxorubicin, and vinblastine, showed no significant cross-reactivity, thus demonstrating excellent specificity of the peplomycin antibody. This assay will, therefore, be useful for peplomycin pharmacokinetic studies in cancer patients who receive a number of other coadministered chemotherapeutic agents.

Table 1
Cross-reactivity of peplomycin antisera with drugs structurally related and unrelated to peplomycin

Drugs	Conc. (ng assay^{-1})	Inhibition of binding* (%)
Peplomycin	1	56.3
Bleomycin	58	0.7
Liblomycin	4000	1.0
Tallysomycin S10b	10	0.2
Cisplatin	385	0
Doxorubicin	212	0
5-FU	3850	0.7
Methotrexate	5769	0
Vinblastine	27	0
VP-16	462	1.5

* Competitive inhibition of the 50% binding produced by ^{125}I-peplomycin (0.7 ng assay^{-1}) and peplomycin antiserum (1:12,000, v/v).

Accuracy and precision

On the same day, two peplomycin concentrations (2 and 20 ng ml^{-1}) were assayed in 0.01 M PBS, pH 7.0, or in control mouse homogenate of heart, lung, liver, or urine. Observed intra-assay concentrations showed excellent accuracy and good precision; they were all within 3% of nominal drug concentrations (Table 2). Similarly, these two drug concentrations in 0.01 M PBS were assayed on four different days within 2 weeks to define inter-assay accuracy and precision. The relative standard deviations of calculated values were 5% or less and were within 5% of actual

Table 2
Accuracy and precision of intra-assay and inter-assay of peplomycin

	Conc. (ng ml^{-1})		RSD* (%)	Deviation from nominal value (%)
	Nominal	Observed mean ± SD		
Intra-assay	2.0	1.96 ± 0.07	4	2
(n = 6)	20.0	20.50 ± 0.20	1	3
Inter-assay	2.0	2.10 ± 0.10	5	5
(n = 4)	20.0	19.90 ± 1.00	5	0.5

The assays were performed under the standard conditions described in the Experimental section.
* RSD = relative standard deviation.

Table 3
Peplomycin distribution in ɔuse tissues and biofluids

Organ	Peplomycin conc. ($\mu g\ g^{-1}$ tissue*)	($\mu g\ organ^{-1}$)	% of dose/organ
Plasma	46.1	69.1	37.0
Urine and bladder	31.9	26.6	20.0
Stomach and intestine	12.5	23.7	12.7
Kidneys	37.8	14.2	7.6
Liver	1.9	10.7	1.0
Lung	8.1	1.6	1.0
Spleen	3.7	0.7	0.4
Heart	3.6	0.7	0.6
Gall bladder	2.7	0.4	0.2
Brain	0.6	1.5	0.2
Total			80.7

*Tissues and biofluids were harvested from six mice 15 min after an intraperitoneal injection of peplomycin (8.5 mg kg^{-1}). Data are reported as mean μg peplomycin/g of tissue or μg peplomycin/ml for plasma and urine.

values at the two concentrations examined (Table 2).

Tissue distribution of peplomycin

Fifteen minutes after intraperitoneal injection of peplomycin (8.5 mg kg^{-1}), higher levels of that drug were found in plasma, urine, kidney, and gastrointestinal tract than were found in other organs (Table 3). Peplomycin concentration in lung tissue, a target of bleomycin-mediated toxicity, was relatively low compared with that found in gastrointestinal and kidney tissue. Expressed as a percentage of administered dose present in each organ, peplomycin distributed within 15 min as follows: gastrointestinal tract > kidneys ≫ lung > heart > spleen > gall bladder and brain. Interestingly, peplomycin, a water-soluble agent, was also found in brain tissue, although at an extremely low level.

Conclusion

Previous RIA procedures have been reported for the determination of peplomycin levels in plasma. The present report describes an RIA method suitable for determination of drug levels in not only plasma and urine but also a wide variety of tissues. The sensitivity of the assay is considered adequate for quantifying drug levels likely to be achieved after administration of therapeutic doses of peplomycin. In addition, the accuracy and reproducibility of the assay will enable us to use this procedure routinely for drug distribution and pharmacokinetic studies. Although human tissues and biological fluids such as plasma were not used in the present study, there is every reason to expect that the rabbit-derived antibody found to be useful for measuring drug levels in mouse tissues will also be useful for determining peplomycin levels in human tissues and fluids.

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