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A Radioimmunoassay for VP16-213 in Plasma

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Summary. *A radioimmunoassay for the semi-synthetic podophyllotoxin VP16-213 has been developed which is suitable for pharmacokinetic studies of the drug. A high titre antiserum was produced in a sheep in response to a VP16-213-BSA conjugate prepared using sodium periodate. Podophyllotoxin does not cross-react with the antiserum and VM26 cross-reacts to only a small extent (< 0.6%). In the absence of a high specific activity tritium label, a radioiodinated histamine ligand was produced which was only partially displaced from antibody by native drug. VP16-213 can be measured in plasma without prior drug extraction with a theoretical limit of detection of 5–10 μg/l. VP16-213 cis (picro) hydroxy acid is recognised by the antiserum to a greater extent than the drug itself. Thus, in order to eliminate any interference from the trans hydroxy acid metabolite chloroform extraction of plasma samples was carried out.*

Introduction

Pharmacokinetic studies on cytotoxic drugs have recently become a important part of the clinical use of these drugs [1]. In the past such studies have been limited by the lack of sensitivity and nonspecificity of the available methodology. However, since the advent of such techniques as radioimmunoassay (RIA) and high performance liquid chromatography (HPLC) comprehensive pharmacokinetic studies are now possible for a number of antineoplastic drugs. HPLC techniques, using UV detectors, for determining the semisynthetic podophyllotoxins VP16-213 and VM26 which are suitable for clinical pharmacokinetic studies have been described [5, 10]. The limit of detection of these methods is approximately 500 μg/l. The paper describes the production of reagents suitable for the development of a RIA for VP16-213 which is capable of measuring the drug in plasma at concentrations below 100 μg/l.

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Materials and Methods

Preparation of Immunising Conjugate. A VP16-213 bovine serum albumin (BSA) conjugate was prepared using the sodium periodate reaction [3]. 2.5 ml of the pharmaceutical preparation of VP16-213, equivalent to 50 mg of drug, and 50 μl ^{3}H-VP16-213 (specific activity 44 μCi/mg, 5.3 μCi/ml – kindly supplied by Sandoz Products Ltd) were mixed with 2 ml 0.1 M sodium periodate for 30 min. 0.2 ml ethylene glycol was added and after 5 min the solution was added to a solution of 200 mg BSA (Sigma, Fraction V) in 5 ml distilled water, adjusted to pH 9 with 5% (w/v) K_2CO_3 and the solution mixed for 45 min. Sodium borohydride (75 mg in 2.5 ml water) was added and left overnight when 2 ml formic acid (1 M) was added. After 1 h the pH was adjusted to 8.5 with 10% (w/v) amonium hydroxide. The solution was dialysed against distilled water (3 × 1 l changes) and the conjugate lyophylised. The molar incorporation of drug into protein was determined from the radioactivity detected in the dialysates and was 11.5 moles VP16-213 per mole protein.

Production of Antisera. Three sheep were immunised with the VP16-213-BSA conjugate. The priming injection consisted of 5 mg conjugate and 3 mg BCG vaccine (Glaxo) in 1 ml saline emulsified with 2 ml of non-ulcerative Freunds adjuvant [8] and was injected into six intramuscular sites (0.5 ml per site). The animals were injected at 4–6 monthly intervals following the prime with 2.5 mg conjugate without BCG and emulsified and injected in the same way. The animals were bled from the jugular vein at intervals following each injection, serum collected and stored at 4° C with 0.1% sodium azide added.

Preparation of VP16-213-histamine. Histamine (5 mg) was conjugated to VP16-213 (20 mg containing 2 μg ^{3}H-VP16-213) using the sodium periodate reaction already described for preparation of the immunising conjugate. At the end of the reaction, the mixture was purified on a Sephadex LH-20 column (1 × 20 cm) and 2.0 ml fractions of the aqueous eluate collected. Two UV absorbing peaks corresponding to the elution of radioactivity were eluted, and it was assumed that the second peak eluting at a volume of 16–22 ml

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Table 1. Procedure for RIA of VP16-213

	Volume of reagent added (µl)					
	Total counts tube	Non-specific binding tube	Maximum binding tube	Zero or antiserum dilution	Standard	Sample dilution
Reagent	–	500	–	400	300	300
Standard	–	–	–	–	100	–
Sample	–	–	–	–	–	100
Antiserum dilution	–	–	500	100	100	100
VP16-213-histamine ^{125}I	100	100	100	100	100	100
	Mix and incubate at 4° C for 2 h					
Dextran coated charcoal	–	200	200	200	200	200
	Mix, incubate at 4° C for 10 min, spin, aspirate supernatant and count charcoal pellet					

was the desired VP16-213-Hist. The concentration of tagged drug in the peak fraction was estimated from the amount of radioactivity to be approximately 300 µg/ml assuming a drug histamine ratio of 1:1.

Radioiodination of VP16-213-Histamine. VP16-213-Histamine was radioiodinated using Iodogen [6]. 20 µg Iodogen (Pierce and Warriner, Chester, Cheshire, GB) were deposited on the bottom of a polypropylene tube (LP3P Luckham Ltd) from 20 µl of dichloromethane. 3 µg VP16-213-Histamine 10 µl 0.1 M borate buffer pH 8.2, and 1 MCi iodine^{125} (IMS 30, Amersham International Ltd Amersham, Buck, GB) were mixed in the tube for 3 min then transferred with 0.5 ml 0.05 M phosphate buffer pH 7.4 on to the top of a Sephadex G 10 column (1 × 10 cm) and the column eluted with assay buffer (see assay procedure). 0.5 ml fractions were collected into glass tubes and 10 µl aliquots of each fraction counted. Radioiodinated VP16-213-Histamine was eluted at a volume of 4–5 ml followed by free iodine at a volume of 8–10 ml.

Assay Procedure. The assay buffer used throughout the procedure was 0.05 M phosphate buffer pH 7.4 containing 6 g/l NaCl and 1 g/l gelatin (Sigma, St. Louis, USA). VP16-213-Hist I^{125} was diluted so that 10,000 cp 100 s were added to each assay tube. A dilution of antiserum was used so that 50% of immunoreactive label (maximum bound label) was bound to antibody, this dilution being derived from an antiserum dilution curve VP16-213 (supplied by Bristol Myers Co. Ltd, New York, USA, 98.2% purity) was dissolved in methanol at a concentration of 100 mg/l and stored at 4° C for use as the standard. This was diluted immediately before use with assay buffer to a concentration of 200 µg/l. Samples were assayed at three dilutions in duplicate. The reagents were added in the order indicated in Table 1 to LP3 tubes (Luckham Ltd, Burgess Hill, Sussex, GB) the contents mixed and incubated at 4° C for 2 h. Charcoal (Norit A) (25 g/l) coated with dextran T70 (Pharmacia) (2.5 g/l) [1] was used to separate the antibody bound fraction from the free fraction and following centrifugation, the supernatant was aspirated and the pellet counted (free fraction).

Results

Production of Antiserum

Antisera were produced in the three sheep immunised with the VP16-213-BSA conjugate, the presence of antibodies being detected with ^{3}H-VP16-213. Preimmunisation sera from the animals did not bind the label. One sheep G/S/810 produced antisera of higher titre than the other two and an antiserum obtained following the first booster injection (4 months after the prime) was used for developmental work. The titre used in the assay varied from 1 : 15,000 to 1 : 25,000 depending on different batches of iodinated label. The tritiated drug, although useful for screening purposes, was not of sufficiently high specific activity to use in the actual radioimmunoassay.

VP16-213-Histamine-^{125}I Label

Immunoreactive, high specific activity labels were produced by radioiodinating the drug-histamine conjugate. The binding of this label to VP16-213 antisera was only partially displaced by the drug itself although non-labelled VP16-213-Histamine caused complete displacement of antibody bound label (Fig. 1).

Radioimmunoassay

The standard curve obtained over a range of concentrations of 5 µg/l → 200 µg/l in the assay is shown in Fig. 2. Incorporating normal human serum into the assay tubes increased the binding of label significantly. The curves could be normalised by expressing each as a percentage of zero standard binding in the presence of 0, 10, and 50 µl normal human serum. Thus correction for protein effect could be achieved by including in the assay zero

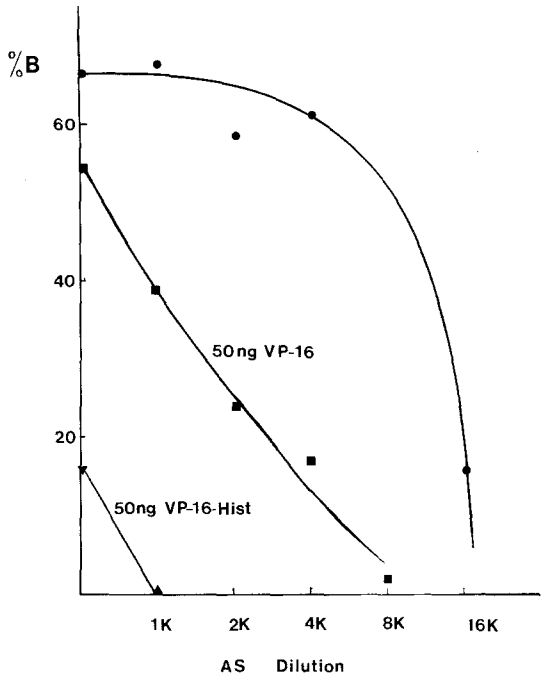

Fig. 1. The binding of VP16-213-Histamine-^{125}I to increasing dilutions of antiserum G/S/810 (●) and its displacement by 50 ng VP16-213 (■) and 50 ng unlabelled VP16-213-Histamine (▼)

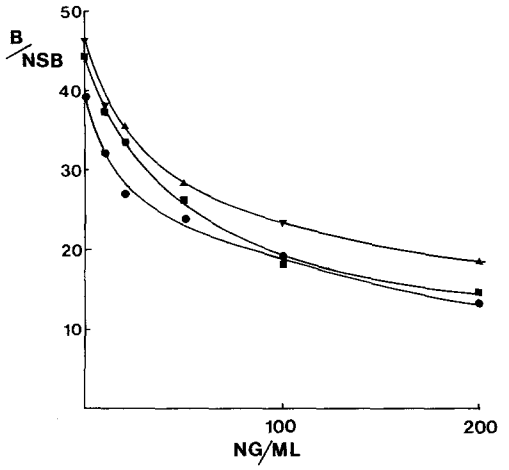

Fig. 2. VP16-213 standard curve in assay buffer (●) and with the addition of 50 µl (▼) and 10 µl (■) normal human serum

standard binding tubes containing the appropriate amount of normal human serum for each dilution of sample used.

Chloroform extraction of VP16-213 and subsequent radioimmunoassay was compared with the direct plasma assay of the drug. VP16-213 was extracted from 0.5 ml plasma or serum with 2 ml chloroform. Following mixing for 15 min, the upper layer was removed by aspiration and an aliquot of the chloroform layer (1 ml) was taken, dried down under nitrogen at 37° C and reconstituted in 0.5 ml assay buffer. A similar effect on the assay was seen with the addition of 100 µl of normal human serum extract as with normal human serum. This was corrected for by ensuring that all assay tubes contained the equivalent of 100 µl extracted serum or plasma. The calibration curve obtained ranged from 5–200 µg/l with a theoretical limit of detection of 5 µg/l.

The ability of structurally related compounds to displace the binding of label from antibody binding sites is shown in Table 2. Several structurally unrelated drugs which could be present in patients' samples did not displace the binding of label at concentrations of 100 mg/l Table 3.

A pool of samples from a patient who had received VP16-213 was assayed five times in five successive assays with and without chloroform extraction. The mean drug concentration on unextracted plasma was 4.63 ± 0.43 mg/l (CV = 9.3%)

Table 2. Cross-reactivity of structurally related substances with VP16-213 antiserum G/S/810

	Amount required for 50% inhibition	% cross reactivity
VP16-213	5.6 ng	100
VP16-213 cis (picro) hydroxyacid	1.5 ng	373
Podophyllotoxin	> 10 µg	< 0.056
VM26	> 1	< 0.56

Table 3. Unrelated compounds exhibiting no cross-reaction with VP16-213 antiserum G/S/810

Methotrexate	Salicylate
Cytosine arabinoside	Codeine
5-Fluorouracil	Morphine
Bleomycin	Diazepam
Adriamycin	Nitrazepam
Prednisolone	Tetracycline

Table 4. The recovery of VP16-213 from spiked normal human serum

	1,000 mg/l	100 µg/l
Unextracted	81.2% ± 16.9 n = 5, CV = 20.8%	81.8% ± 9.1 n = 4, CV = 11.1%
Extracted	96.4% ± 10.7 n = 5, CV = 11.0%	70.0 ± 12.7 n = 5, CV = 18.2%

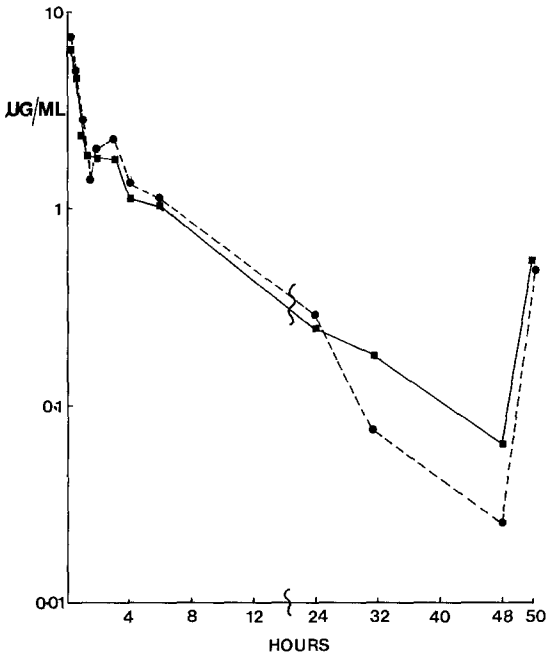

Fig. 3. Plasma VP16-213 levels in one patient given 100 mg IV infusion over 30 min, and 100 mg PO at 48 h measured by direct plasma RIA (■) and following chloroform extraction (●). No drug was detected in the pre-infusion sample

compared to 3.18 ; 0.35 mg/l (CV = 11.0%) for extracted plasma. The recovery of VP16-213 from normal human serum spiked with 1 mg/l and 100 µg/l of drug is shown in Table 4. The suitability of the RIA for pharmacokinetic studies is shown in Fig. 3. Blood samples were obtained at intervals from one patient given 100 mg VP16-213 as an iv infusion over 30 min and at 48 h, 100 mg of drug orally. VP16-213 plasma concentrations were measured directly and following chloroform extraction of the drug.

Discussion

The conjugate VP16-213-BSA was immunogenic in the three sheep immunised and an antibody response was observed following the priming injection which was considerably increased by subsequent booster immunisations.

The precise chemical nature of the conjugate is not known. The sodium periodate reaction has been widely used for producing conjugates where the hapten possessed adjacent (vicinal) hydroxyl groups [2, 3] which is the case for VP16-213. Antibodies to haptens are directed primarily to antigenic determinants on the molecule furthest form the site of conjugation to the carrier [7]. Since podophyllotoxin does not cross-react with the antiserum used in the assay, and VM26 cross-reacts only to a small extent, it can be assumed that the main antigenic determinant is the ethylidene glucosidic moiety of VP16-213. It can be speculated therefore that conjugation has proceeded at a point in the molecule other than the glucosidic residue.

The cis (picro) hydroxy acid derivative which was prepared by increasing the pH of a solution of VP16-213 [10] is recognised by the antiserum to a greater extent than the drug itself. A possible explanation for this, is that during conjugation the lactone ring of VP16-213 was converted to the hydroxy acid in the alkaline conditions used.

The disappointingly low detection limit for the assay can be explained by the partial displacement of iodinated VP16-213-Histamine by VP16-213 itself. This phenomenon has often been described [9] and is thought to occur because high avidity antibodies have been produced to the chemical bridge in the drug conjugate. These antibodies recognise the same chemical link in the iodinated label which cannot be displaced by drug alone. Work is in progress to produce a more suitable radioiodinated derivative of VP16-213 for use in the assay.

The reagents described in this paper are suitable for the radioimmunoassay of VP16-213 in plasma provided that appropriate procedures are adopted to overcome the non-specific protein effects observed on the addition of plasma or chloroform plasma extracts. The recovery of drug from spiked normal serum was over 80% except for the recovery of 100 µg/l following chloroform extraction. The inter-assay coefficient of variation was 10–20% and could reflect the difficulty of adequately correcting for the protein effect.

The difference in the results obtained for the sample pool assayed directly and following chloroform extraction suggest that an immunoreactive metabolite, e.g., cis/trans hydroxy acid is being measured in unextracted plasma. In the one patient studied very similar results were obtained for plasma assayed directly as for extracted plasma on samples taken up to 24 h following drug administration. At later time points when metabolites are likely to be present at higher concentrations direct assay results were higher than for the extracted assay.

The RIA of VP16-213 is potentially useful for long-term clinical pharmacokinetic studies specially where small sample volumes are available. As well as providing an alternative technique to HPLC the RIA may also prove useful as a sensitive detection system for HPLC.

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