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Short Communication

The production and evaluation of a radioligand and antiserum for the radioimmunoassay of sub-nanogram per millilitre concentrations of lamivudine

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Introduction

This paper describes the synthesis of a ^{3}H-lamivudine radioligand, and the production and evaluation of an avid antiserum suitable for the sensitive and specific determination of lamivudine by means of radioimmunoassay (RIA). The immunogens used to elicit antibody production in Soay ewes were characterized using matrix-assisted laser desorption time-of-flight mass spectrometry (MALD TOF MS) and/or UV spectroscopy.

Lamivudine [(2R-*cis*)-(−)-4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone, Fig. 1] is an important and novel dideoxynucleoside analogue that is currently being developed for the treatment of infections caused by the Hepatitis B and Human Immunodeficient Viruses (HIV-1 and HIV-2). Lamivudine has shown activity against Hepatitis B [1] and a range of HIV-1 and HIV-2 isolates *in vitro*, [2–4] and has shown promising anti-HIV activity *in vivo* during clinical trials [5]. Furthermore, the clinical trials have demonstrated that lamivudine has good bioavailability (82%) and is well tolerated by volunteers [6].

Within HIV infected peripheral blood monocytes (PBMC), lamivudine is phosphorylated to the active lamivudine triphosphate (Fig. 1) which acts as a specific chain terminator of reverse transcription. Consequently, the concentrations of the latter, rather than parent drug, may need to be determined to establish the relationship between dosing and efficacy.

Intracellular levels of lamivudine triphosphate can be quantified following its complete hydrolysis, by acid phosphatase, to parent lamivudine. An analytical method employing HPLC-UV (lower limit of quantification, 10 ng ml^{-1}) has been developed and applied successfully to the determination of lamivudine in serum taken from HIV infected patients [7]. However, this method is not sufficiently sensitive to detect the possible sub-nanogram levels of lamivudine derived from the lamivudine phosphate present in purified extracts of PBMC. Since immunoassay offers potentially better sensitivity a competitive RIA has

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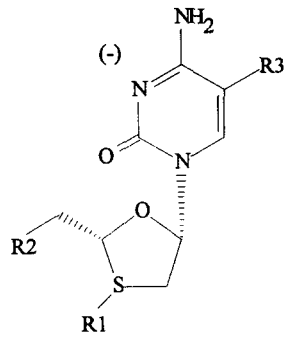

Compound	R1	R2	R3
Lamivudine	-	OH	H
(+/–) Lamivudine hapten (GR130001)	-	OH	COOH
^{3}H-lamivudine radioligand	-	OH	^{3}H
Trans-sulphoxide metabolite		OH	H
Cis-sulphoxide metabolite		OH	H
Monophosphate metabolite	-	$PO_4^{2-}Na^{2+}$	H

Figure 1
Chemical structures for lamivudine [(2R-*cis*)-(−)-4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone], the carboxy-lamivudine hapten, the radioligand and the lamivudine metabolites.

been developed to enable the determination of these low concentrations. The indirect analytical approach was employed because it was not possible to use lamivudine triphosphate for the synthesis of a suitable hapten.

The final validation of the RIA for the determination of lamivudine triphosphate in chromatographically purified extracts of PBMC, its application, and the clinical significance of the results, will be described elsewhere. The antisera, radioligand and methods described in the current paper may also be applicable to the determination of lamivudine in plasma, urine and other biological fluids.

Experimental

Chemicals

1,1′-Carbonyldiimidazole (CDI), triethylamine, N-bromosuccinimide and dimethylformamide (DMF) were purchased from Aldrich. Methanol, dichloromethane, acetic acid and ethyl acetate were from Fisons Analytical. Acetonitrile and 20% (m/m) palladium/charcoal were obtained from Rathburn and Engelhard, respectively. Tritium labelling service was performed by Amersham International. Bovine serum albumin (BSA), bovine thyroglobulin, HCl-washed charcoal, dialysis tubing, cytosine, uracil, cytidine (and its mono-, di- and triphosphorylated derivatives) were obtained from The Sigma Chemical Co. Freund's incomplete non-ulcerative adjuvant was from Guildhay Antisera (Evans BCG intradermal vaccine was added, according to Guildhay instructions, to prepare complete adjuvant). Gelatine was provided by Croda Colloids.

Lamivudine and the lamivudine monophosphate were provided by the Chemical Development Division, Glaxo Research and Development, UK. The hapten (GR130001X, Fig. 1) used to synthesize the immunogens was provided by BioChem Therapeutic Inc. (Quebec, Canada). All buffer salts were obtained from BDH (analytical reagent grade).

Deionized water, used for the preparation of the RIA working reagents, was obtained from an Elgastat purification system (>14 MΩ cm).

Picafluor 30 liquid scintillation cocktail was obtained from Packard Inc.

Preparation of radioimmunoassay working solutions and calibration standards

Radioimmunoassay (RIA) diluent consisted of 0.1% (m/v) gelatine and 0.05% (m/v) sodium azide in 0.1 M phosphate buffer (pH 7.4) solution.

A solution containing 5% (m/v) BSA was prepared by mixing equal volumes of the assay diluent and a stock solution containing 10% (m/v) BSA and 0.05% (m/v) sodium azide in deionized water. Each solution was stored at approximately 4°C. The 10% (m/v) BSA solution was replaced monthly; whilst the assay diluent and 5% (m/v) BSA solutions were replaced weekly.

Working solutions of the ^{3}H-lamivudine radioligand and anti-lamivudine serum (described below) were prepared on each day of analysis. The working radioligand solution was prepared by diluting an aliquot of the stock reagent with assay diluent to give a radioactive concentration of approximately 333 Bq (20,000 dpm) 50 μl^{-1}. The working solutions of anti-lamivudine sera were prepared by diluting each antiserum to its working titre (corresponding to approximately 50% of maximum radioligand binding) in assay diluent.

Primary stock standard solutions containing 1 mg lamivudine ml^{-1} were prepared monthly in deionized water. Working calibration standards containing 0.1–100.0 ng lamivudine ml^{-1} of assay diluent (n = 11) were prepared from this stock on each day of analysis. A zero standard was also included in each standard series. All solutions containing lamivudine were kept at 4°C and protected from light.

Phase-separation of antibody-bound and free (unbound) radioligand was performed using a 0.1% (m/v) dextran T70 coated 1.0% (m/v) HCl-washed charcoal suspension in assay diluent. Freshly prepared suspension was stirred for approximately 15 min and then stored at 4°C for at least 2 h before resuspension and use in the RIA. This reagent was used within 24 h of preparation.

Apparatus

Purification procedures performed during the preparation of the ^{3}H-lamivudine radioligand used preparative silica TLC plates (Whatman, 20 × 20 cm, 60A PK6F); silica gel (Merck 9385); a Gilson 305 HPLC pump in conjunction with an Applied Biosystems 757 UV detector and a Radiomatic Flo-one Beta CR radiodetector; Anachem analytical and preparative S5 ODS2 columns, and a Technicol Cyclobond 1 2000 Ac chiral column. The chromatographic conditions are described below.

Nuclear magnetic resonance spectroscopy was performed using either Bruker AC 300 MHz or Varian XL 200 MHz spectrometers. Chemical ionization (CI) and MALDTOF MS were performed in Finnigan 4600 single quadrupole and VG Analytical TofSpec spectrometers, respectively. Ultra violet (UV) absorption spectroscopy was carried out in a Varian Cary 1E spectrophotometer.

Liquid scintillation counting was accomplished in a Denley Delta counter that transmitted cpm data to a Viglen 386SX personal computer running the MULTICALC data-acquisition and reduction program (Wallac). The weighted four parameter logistic (4PL) algorithm was employed to convert radioactivity measurements into concentration values (ng ml^{-1}).

Methods

Antiserum production

Preparation of the immunogens. The immunogens were prepared by conjugating the hapten GR130001X, the 5-carboxy derivative of (±)-lamivudine (Fig. 1), to bovine serum albumin (BSA) or bovine thyroglobulin using 1,1′-carbonyldiimidazole (CDI) in a two stage reaction scheme (Fig. 2). The CDI: hapten:protein molar ratios in the reaction mixtures were 100:50:1 and 1000:500:1 for BSA and thyroglobulin, respectively.

Hapten and CDI reagent were dissolved in separate 1.5-ml aliquots of anhydrous DMF. The CDI solution was added, dropwise, to the hapten solution under continuous stirring and the reaction allowed to proceed for approximately 4 h at ambient temperature. The reaction mixture, containing excess unreacted CDI reagent and the activated hapten, was then transferred, dropwise, to a continuously stirred solution containing the required protein in 3 ml of 0.1 M phosphate buffer (pH 8.0). The conjugation reaction between the activated carboxylic acid group on the hapten and primary amino groups in the protein was allowed to proceed for approximately 15 h (overnight) at ambient temperature.

The entire reaction mixture was subsequently dialysed against several changes of 0.1% (m/v) sodium carbonate for 36 h and

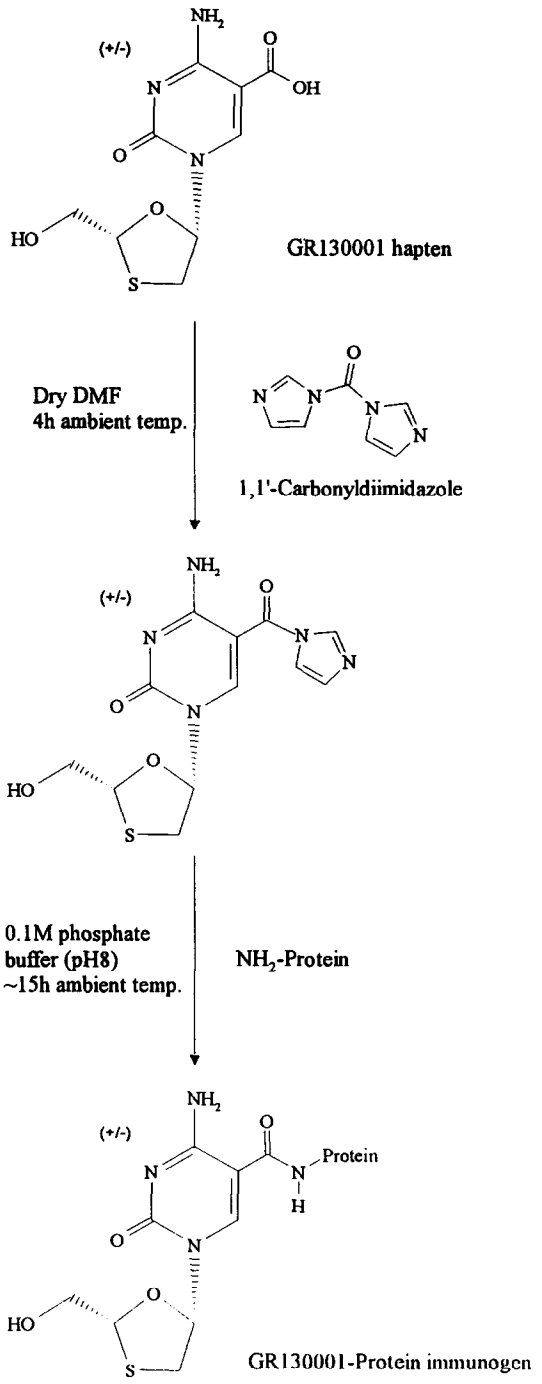

Figure 2
Synthetic route for the synthesis of the lamivudine immunogens.

then against deionized water for a further 24 h. Finally, the purified product was freeze-dried to constant mass and stored at 4°C.

Characterization of the immunogens. The extent of hapten conjugation to the BSA carrier protein was determined by means of UV spectroscopy (at 280 nm) and MALDTOF MS. The molecular mass of thyroglobulin (~660 kDa) was too great for it to be suitable for analysis by the latter technique; consequently, the GR130001-thyroglobulin immunogen was characterized by UV spectroscopy only.

For MADTOF MS, the GR130001-BSA immunogen and BSA were dissolved in separate solutions of 0.05 M sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) to give a final concentration of approximately 0.1 mg of protein ml^{-1}. For analysis, approximately 0.5–1.5 μl of protein containing solution was loaded onto the spectrometer's sample stage and allowed to evaporate to dryness before introduction into the instrument. Typically, spectra from 10–100 laser shots were averaged to produce a final mean spectrum.

Antiserum generation in Soay sheep. The GR130001-BSA and GR130001-thyroglobulin immunogens (5 mg dissolved in 1.5 ml sterile water per animal) were each emulsified with Freund's complete, non-ulcerative, adjuvant (3 ml per animal) and used to immunize, by the intramuscular and subcutaneous routes, four young Soay ewes (two per immunogen). The animals immunized with the GR130001-thyroglobulin conjugate were boosted 5, 8 and 13 months later with the same immunogen (2.5 mg per animal) emulsified in Freund's incomplete non-ulcerative adjuvant. The sheep immunized with the GR130001-BSA immunogen were boosted, in a similar manner, 9 and 12 months after the primary immunization.

Small (~10 ml) samples of blood were obtained from each sheep at regular intervals for monitoring lamivudine antibody development. Approximately 150 ml of blood was withdrawn 10 days after each booster immunization and the serum stored at −70°C.

Synthesis of the ^{3}H-lamivudine radioligand

The synthesis of the ^{3}H-lamivudine radioligand from lamivudine was performed in two stages (Fig. 3). In the first stage a 5-bromo derivative (Fig. 3, II) of lamivudine was synthesized as a reactive intermediate. This intermediate was subsequently specifically tritium-labelled by catalytic debromination under an atmosphere of tritium gas (Fig. 3, III). The two synthetic reactions, and the purification and characterization of the products are described below:

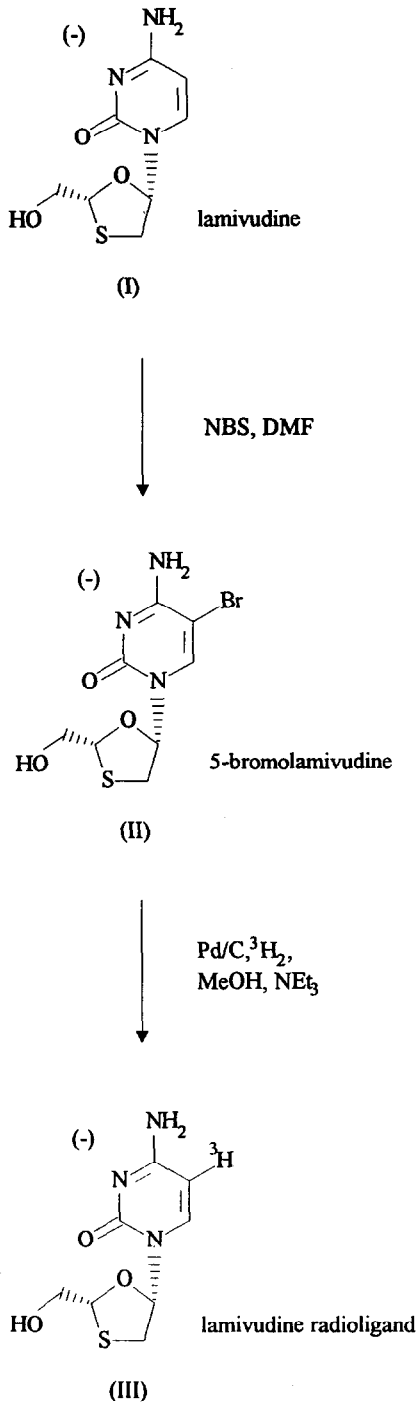

Figure 3
Synthetic route for the synthesis of the ^{3}H-lamivudine radioligand.

Stage 1: synthesis, purification and characterization of the 5-bromolamivudine intermediate. N-bromosuccinimide (275 mg, 1.55 mmol) was added, portion-wise over 5 min, to a cooled (0°C), continuously stirred solution of lamivudine (250 mg, 1.09 mmol) in anhydrous DMF (15 ml). The stirring was maintained for 30 min whilst the colourless solution rapidly developed a yellow colour; the reaction mixture was then left quiescent for a further 20 h at 0°C. The solvent was then removed *in vacuo* at <40°C, the residue purified by flash chromatography, using 20% (v/v) methanol in dichloromethane as the eluent. Further purification by preparative TLC on silica, with an ethyl acetate, methanol, acetic acid (8 + 1 + 1) eluent, yielded a cream solid (165 mg, 0.54 mmol), containing 5-bromolamivudine (Fig. 3, II).

Characterization. ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.38 (s, H, 6-H), 7.89 (s, 1H, NH), 7.02 (s, 1H, NH), 6.16 (m, 1H, 1-(5-H)), 5.21 (tr, 1H, 1-(2-H)), 3.9 to 3.7 (m, 2H, 1-(2-CH_2OH)), 3.48 and 3.18 (2m, 2H, 1-(4-H_2)). MS (CI NH_3 + ve) MH^+ 308/310.

Stage 2: synthesis, purification and characterization of the ^{3}H-lamivudine radioligand. Using previously optimized reaction conditions, 5-bromolamivudine (Fig. 3, II) (20 mg, 65 mmol) and triethylamine (20 ml) in methanol (4 ml) were stirred with 20% (m/m) palladium/charcoal (24 mg), under an atmosphere of tritium gas (185 GBq, 5 Ci), for 5 h. The mixture was filtered through a Supercel pad, which was subsequently washed with methanol. The crude product was purified by flash chromatography, using an ethyl acetate, methanol, acetic acid (8 + 1 + 1) eluent. This was followed by final purification by means of preparative reversed-phase HPLC on an S5-ODS2 (250 × 25 mm) column, with a mobile-phase gradient (over 45 min) consisting of 0–50% (v/v) acetonitrile in 0.2% (v/v) aqueous trifluoroacetic acid. The component eluting at the same retention time as lamivudine was collected as a series of fractions. The radiochemical purity of each fraction was determined by separate on-line analyses, and those with >99% radiochemical purity were combined, evaporated to dryness, and the residue dissolved in methanol (63 ml) to give a solution of [5-^{3}H]-lamivudine (2.34 GBq, 63.2 mCi) (Fig. 3, III).

The radiochemical purity, determined by on-line radio-detection, of the ^{3}H-lamivudine radioligand was >98%, and the specific activity determined by CI NH_3 positive ion mass spectroscopy, was 548 GBq $mmol^{-1}$ (14.8 Ci $mmol^{-1}$). The chemical purity was >99%,

determined by comparison to the area of a similar co-eluting amount of unlabelled lamivudine [chromatographic conditions: HPLC column, S5-ODS2 (250 × 4.6 mm dia.); mobile phase, 0.025 M disodium hydrogen phosphate containing 0.005 M triethylamine (solvent A) and 90% (v/v) acetonitrile in water (solvent B), at 2 ml min^{-1}, with gradient 0–30% (v/v) solvent B over 25 min; UV detection, 280 nm]. The optical purity (radiochemical and chemical) of the product was established by chiral HPLC [chromatographic conditions: HPLC column, Cyclobond 1 Acetyl (250 × 4.6 mm dia.); mobile phase, 0.2% (v/v) aqueous triethylamine, adjusted to pH 7.0 with glacial acetic acid]. The chromatographic results indicated that the radiolabelled compound was a single isomer that co-eluted with lamivudine ((−)-enantiomer); the (+)-enantiomer was not detected.

Characterization. ^3H NMR (EtOH and DMSO-d_6, 320 MHz, proton decoupled) δ 5.96 (s, 5-^3H).

The radiochemical purity of ^3H-lamivudine, measured by HPLC, decreased to approximately 94% after 6 months storage of a methanolic solution at −70°C. Repurification by preparative HPLC (described above) yielded a ^3H-lamivudine radioligand with >99% purity.

Radioimmunoassay of lamivudine

Duplicate assays were performed in 5-ml plastic tubes. In each instance the final volume was 0.5 ml that consisted of: 0.2 ml assay diluent, 0.1 ml 5% (m/v) BSA solution, 0.1 ml calibration standard, 0.05 ml working radioligand reagent and 0.05 ml working antiserum solution. Total radioactivity and non-specific binding (NSB) tubes were also set up with each assay batch. The total radioactivity tubes contained 0.45 ml of assay diluent and 0.05 ml working radioligand reagent, and the NSB tubes contained 0.35 ml of assay diluent, 0.1 ml 5% (m/v) BSA solution and 0.05 ml working radioligand reagent. All assay tubes were incubated overnight at 4°C; whereupon, phase-separation was performed by adding 0.5 ml of cold (4°C) dextran-coated charcoal suspension to each tube. After mixing, the tubes were centrifuged (20 min, 4°C) and 0.8 ml of supernatant decanted into scintillation vials containing 8 ml Picofluor 30 scintillation cocktail. The vials were then capped, shaken and individually counted for 5 min on the liquid scintillation counter.

Determination of antiserum titre and radioligand immunoreactivity

Antisera titres and radioligand immunoreactivity were determined using the standard RIA procedure described above, except that an additional volume of assay diluent was added instead of the calibration standard solution, and serial dilutions of antiserum replaced the working antiserum reagent.

Titres were determined for the anti-lamivudine sera taken before and after each immunization. The immunoreactivity of the ^3H-lamivudine radioligand was determined from the extent of binding of radioactivity to excess lamivudine antiserum.

Determination of potential sensitivity and calibration range

The potential sensitivity and calibration range of the lamivudine radioimmunoassay method was assessed from results obtained in a total of seven independent assays. These assays were run using the most promising antisera obtained from sheep 1 and 2 after the third booster immunization with the GR130001-thyroglobulin immunogen.

Cross-reactivity

The cross-reactivity of key metabolites and endogenous compounds with the most promising antiserum (obtained from sheep 2 after the third booster immunization) was assessed by determining the concentration of each compound that caused a 50% displacement (50% B/B_0) of the ^3H-lamivudine radioligand from the lamivudine antibodies relative to lamivudine itself.

Results and Discussion

Extent of hapten conjugation to the BSA and thyroglobulin carrier proteins

MALDTOF MS is a powerful technique for determining the molecular masses of proteins, and it has been successfully used by Wengatz *et al.* [8] and the present authors [9] for assessing the extent of hapten conjugation to carrier proteins during immunogen production. The MALDTOF mass spectra acquired in the current study for BSA and freeze-dried GR130001-BSA immunogen are presented in Fig. 4. These spectra each show several peaks

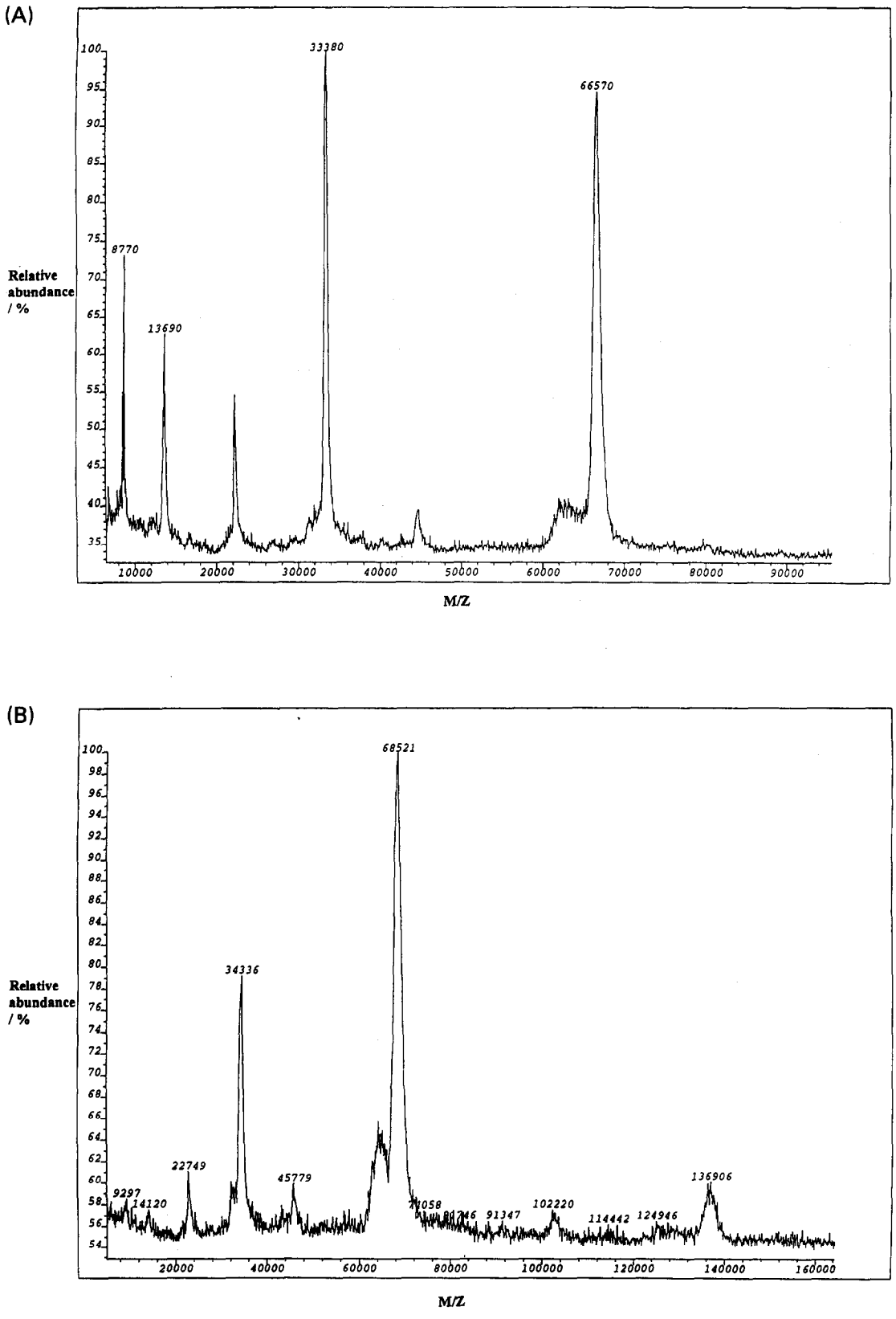

Figure 4
MALDTOF mass spectra for (A) BSA and (B) the GR130001-BSA immunogen.

that correspond generally to the $[M + 2H]^{2+}$ ($[M + nH]^{n+}$), MH^+ and $[2M + H]^+$ ($[nM + H]^+$) ions. The *m/z* data for the MH^+ ions from these spectra indicate that the mean relative molecular masses for BSA and the GR130001-BSA immunogen are approximately 66570 and 68512 Da, respectively. The difference between these figures is 1951 Da which corresponds to a mean incorporation of 7.6 moles of GR130001 per mole of BSA. The corresponding incorporation ratio determined by UV spectroscopy for this immunogen was approximately 6. The incorporation of hapten into the thyroglobulin immunogen, determined by UV spectroscopy only because the MALDTOF MS technique was not applicable, was approximately 67. These latter values obtained by UV spectroscopy are underestimates because the proteins have significant absorbance at 280 nm. In consequence the value determined by MALDTOF MS for the GR130001-BSA is likely to be more reliable.

Antisera titres and radioligand immunoreactivity

The sheep immunized with the GR130001-BSA and GR130001-thyroglobulin immunogens all gave measurable antibody responses, although the antisera obtained from the sheep receiving the GR130001-thyroglobulin immunogen yielded the highest titres. The most promising antiserum, obtained from sheep 2 after the third booster immunization with the GR130001-thyroglobulin immunogen, yielded a titre of 1/400 (in the working antiserum reagent). The corresponding titre for sheep 1 was 1/80.

The mean ± SD immunoreactivity of the ^{3}H-lamivudine radioligand solution, measured in the presence of excess anti-lamivudine antibodies, was 83.4 ± 5.3% (n = 14). The corresponding mean ± SD non-specific radioligand binding in the same assays was 2.05 ± 1.13%.

Determination of potential sensitivity and calibration range

The antiserum obtained from sheep 2 after the third booster immunization with the GR130001-thyroglobulin immunogen afforded an RIA with a mean ± SD theoretical sensitivity (minimum detectable dose) in assay diluent of 0.22 ± 0.076 ng of lamivudine ml^{-1}. This value represents the imprecision (standard deviation) of the zero dose [10] measured in seven independent assay batches. The sensitivity was measured in assay diluent because this represents the intended assay matrix following chromatographic isolation of lamivudine triphosphate by HPLC. A mean ± SD calibration curve is presented in Fig. 5(A). The envisaged calibration range is approximately 0.4–100.0 ng lamivudine ml^{-1}. These limits correspond to the concentrations yielding acceptable reproducibility (generally <15% except for <20% of the lower limit of quantification) in a precision profile [11] (Fig. 5B) constructed from the mean intra-replicate precision of the analytical standards in seven independent assays. The potential sensitivity afforded by the antiserum obtained from sheep 1 after its third booster immunization was approximately 1.0 ng of lamivudine ml^{-1}. Consequently, the more sensitive antiserum obtained from sheep 2 was used for all further assays. The final validation of the complete HPLC-RIA method, its application, and the clinical significance of the results, will be reported elsewhere.

Cross-reactivity

Metabolism studies in the dog have shown that drug-related material is excreted, as unchanged drug and metabolites in approximately equal amounts, predominantly in the urine following oral and intravenous administration of lamivudine. The principle metabolite identified was lamivudine sulphoxide [12] (Fig. 1). Pharmacokinetic studies in patients with HIV infection have shown that following oral administration of lamivudine the majority of the drug (approximately 70%) is excreted unchanged in the urine with the sulphoxide being the most predominant metabolite produced (Glaxo Research and Development, unpublished results). In addition, lamivudine is phosphorylated intracellularly by kinases to produce the monophosphate (Fig. 1), diphosphate and the triphosphate metabolites.

The potential specificity of the RIA was assessed by determining the extent of cross-reactivity effected by key endogenous compounds that are either related to lamivudine or are likely to be present intracellularly, and the monophosphate and sulphoxide metabolites (Fig. 6 and Table 1).

The low values (range, 0.06–0.7%) for cross-reactivity obtained with the lamivudine metabolites clearly indicate that the antiserum is highly selective for parent lamivudine com-

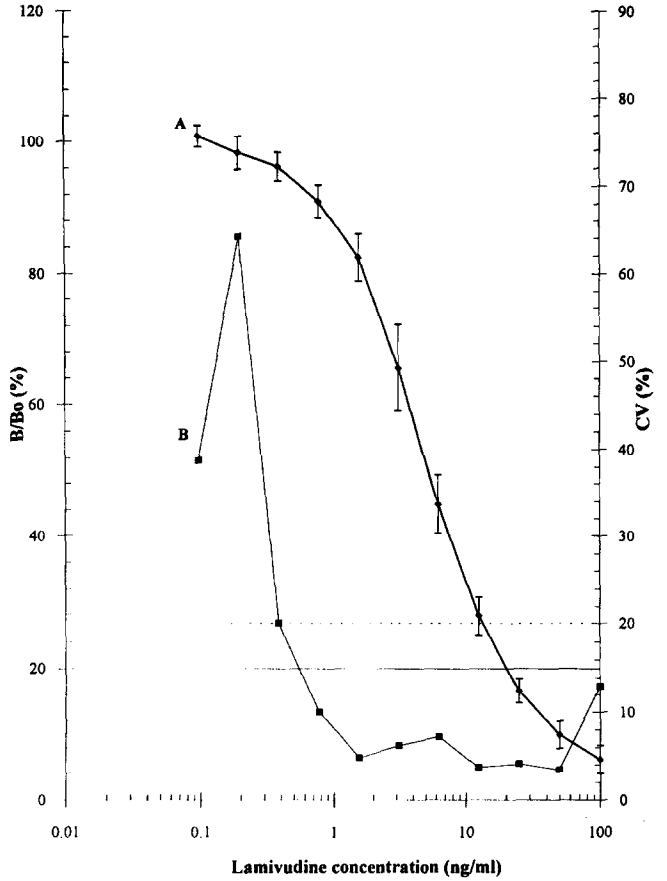

Figure 5
(A) Mean ± SD calibration curve constructed from the B/B_0 data obtained by the RIA of lamivudine, in the intended assay diluent matrix, in seven independent assay batches, and (B) the corresponding precision profile depicting the mean intra-replicate variation in back-calculated concentration values for each of the analytical standards.

pared to its metabolites. Indeed, low cross-reactivity was anticipated because each compound differs from the parent lamivudine structure at sites distant from the point of conjugation used during immunogen synthesis [13]. The lack of any detectable cross-reactivity (<0.01%) with the endogenous compounds confirms the functional specificity of the method. It is anticipated tht the cross-reactivity of the antiserum with the diphosphate and triphosphate metabolites would be even less than with the monophosphate.

Conclusions

A ^{3}H-lamivudine radioligand and several anti-lamivudine sera have been successfully produced and evaluated for the sensitive and selective radioimmunoassay (RIA) of lamivudine. The radioligand was immunoreactive (means ± SD = 83.4 ± 5.3%, n = 14) with all the antisera produced. The best antiserum (obtained from one Soay ewe after administration of the third booster immunization with a GR130001-thyroglobulin immunogen) yielded a titre (corresponding to 50% maximum radioligand binding) of 1/400 (in the

Table 1
Cross-reactivity values for the lamivudine metabolites and key endogenous compounds

Compound	Cross-reactivity (%)
Lamivudine	100.00
Monophosphate metabolite	0.69
Trans-sulphoxide metabolite	0.08
Cis-sulphoxide metabolite	0.06
Cytosine*	<0.01
Uracil*	<0.01
Cytidine*	<0.01
Cytidine-5′-monophosphate*	<0.01
Cytidine-5′-diphosphate*	<0.01
Cytidine-5′-triphosphate*	<0.01

* Estimated values because the highest concentration of test compound did not cause any detectable displacement of radioligand from the antibodies.

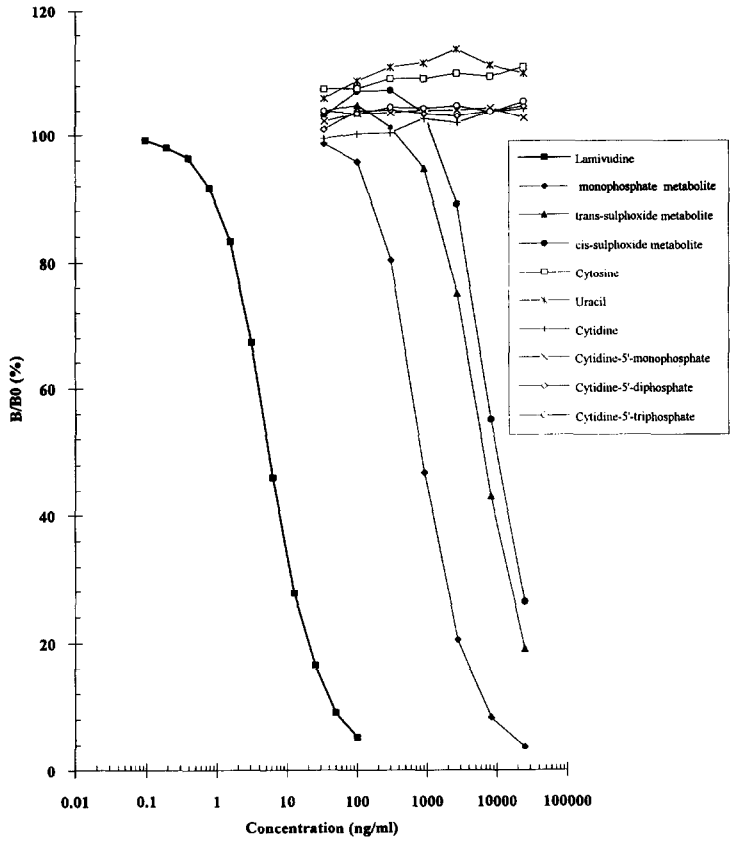

Figure 6
Cross-reactivity curves for lamivudine metabolites and key endogenous compounds. The lamivudine curve represents mean data from two independent assays used to obtain results for the test compounds. (The antiserum was obtained from sheep 2 after the third booster immunization with the GR130001-thyroglobulin immunogen.)

working antiserum reagent), and afforded a theoretical assay sensitivity of 0.22 ± 0.076 ng lamivudine ml^{-1} in assay diluent. The envisaged working calibration range is approximately 0.4–100.0 ng lamivudine ml^{-1}. The cross-reactivity of key endogenous compounds and metabolites of lamivudine with this antiserum was less than 0.7%. This low value indicates that the method should be suitably selective for the determination of lamivudine produced by enzymatic hydrolysis of lamivudine triphosphate in chromatographically purified preparations of PBMC.

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