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Radioimmunoassay for the measurement of S9788 in serum and microdialysis samples

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Abstract

S9788, 6-[4-(2,2-di-(fluorophenyl)-ethylaminol)-1-piperidinyl]-*N,N'*-di-2-propenyl-1,3,5-triazine-2,4-diamine, is a novel compound designed to reverse tumour multidrug resistance associated with cancer chemotherapy. A specific and sensitive radioimmunoassay has been developed for the analysis of S9788 in serum samples and adapted for samples obtained by microdialysis. The limit of quantitation is 0.2 ng ml^{-1} in perfusion medium and there is no cross reactivity of the antibody with known metabolites of the parent compound or with certain cytotoxic compounds likely to be coadministered with S9788. Maximum probe recovery during microdialysis was 66% at a flow of 1 μl min^{-1}, using Ringer/BSA (70 mg ml^{-1}) as the perfusion medium. The assay has sufficient sensitivity, precision, accuracy and specificity for the analysis of rat and human serum and microdialysis perfusate samples. The assay has been successfully applied to the determination of S9788 in rat plasma (total concentration) and the microdialysate of the same samples. © 1997 Elsevier Science B.V.

Keywords: Radioimmunoassay; Microdialysis; S9788; Validation; Pharmacokinetics

1. Introduction

Multidrug resistance (MDR) is a phenomenon observed with certain types of tumours during treatment with chemotherapeutic agents. The MDR phenotype is associated with the over expression of a membrane bound glycoprotein, known as P-glycoprotein (Pgp). It is reported to function as an ATP driven intracellular efflux pump which causes a decrease in intracellular concentrations of cytotoxic drugs, rendering them ineffective [1]. Inhibition of the Pgp pump may thus enhance the therapeutic effect during treatment. S9788, 6-[4-(2,2-di-(fluorophenyl)-ethylaminol)-1-piperidinyl]-*N,N'*-di-2-propenyl-1,3,5-triazine-2,4-diamine, is a novel compound under development for its potential use in MDR (Fig. 1). It is a highly lipophilic compound (log *P* 4.2) and has an in vitro plasma protein binding of 98%. In order that S9788 can function as an

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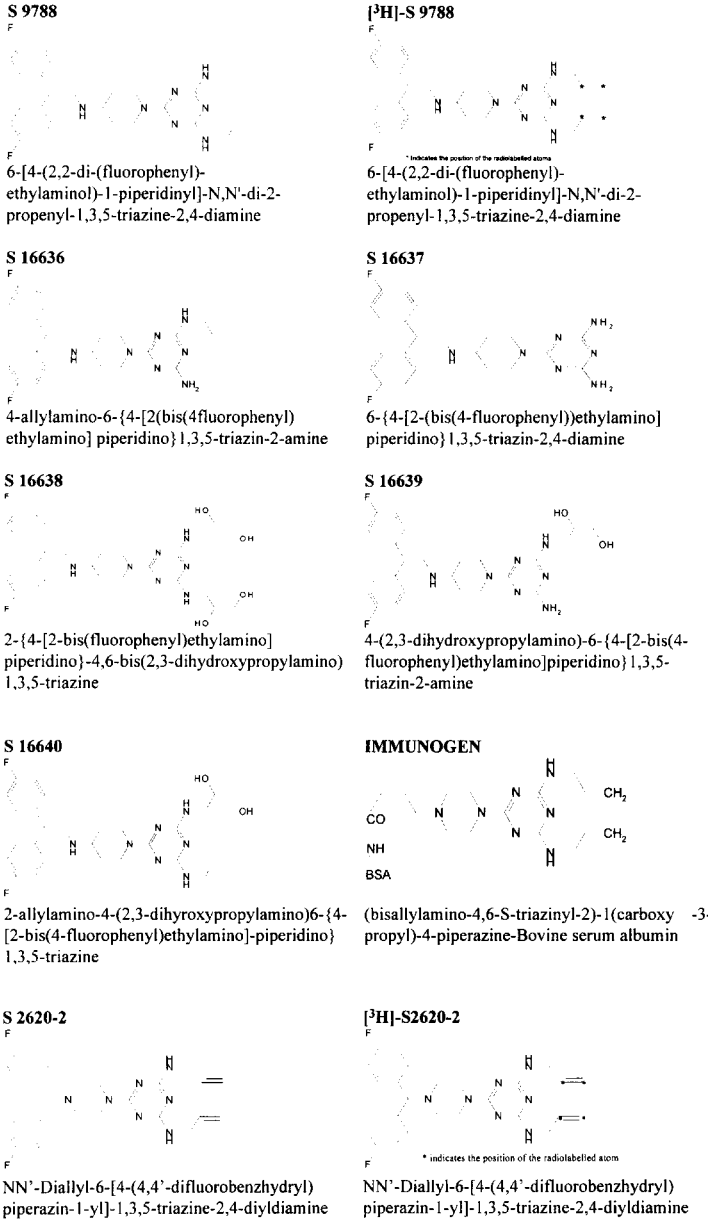

Fig. 1. Structures of S9788 and its metabolites, S2620-2, radioligands and immunogen used in the development of the immunoassay.

antagonist for this drug efflux, it should be present, certainly at the extracellular level in the interstitial spaces within solid type tumours, and preferably at the intercellular level. This would enable it to act on both sides of the cell membrane where the glycoprotein is located. One of the objectives of this study was therefore to develop a technique which would enable S9788 to be measured within the tumour.

Microdialysis [2] is a technique which allows continuous sampling of extracellular fluid in vivo. A probe incorporating a semi-permeable mem-

brane is positioned in a blood vessel or tissue and is continuously perfused with an isotonic solution. This permits the diffusion of substances between the area of interest and the perfusate along a concentration gradient. The diffusion is characterised by the molecular weight cut-off of the probe membrane, so that molecules such as plasma proteins cannot pass into the perfusate due to their large molecular weight. Microdialysis has been used for measuring free extracellular concentrations of various compounds. Most studies to date have involved hydrophilic compounds, but by altering the composition of the perfusion medium, lipophilic compounds can also be monitored [3].

With sampling volumes in the order of 10–20 μl and only 2% of the total S9788 free to cross the membrane, it is not feasible to use the established techniques of HPLC [4] and GC for measuring microdialysate levels of S9788. A radioimmunoassay method has been developed for the measurement of S9788 in human plasma and serum and this method has been adapted for microdialysis samples.

This paper describes the radioimmunoassay procedure based on rabbit polyclonal antibodies for the quantitation of S9788 in plasma and serum, and its adaptation and application to the measurement of S9788 microdialysate samples.

2. Materials and methods

2.1. Chemicals

S9788 was obtained from Technologie Servier (Orleans, France). Tritiated S9788-2 (bismesylate salt form, specific activity 110 Ci $mmol^{-1}$, purity 99%) was supplied by CEA (Paris, France). Disodium hydrogen orthophosphate, citric acid monohydrate, sodium chloride, potassium chloride, calcium chloride and sodium azide were purchased from BDH (Poole, UK) and were all of analytical grade. Bovine Serum Albumin (BSA) Fraction V powder and activated charcoal were purchased from Serva (Biowhittaker, Wokingham, UK), Dextan T70 from Pharmacia (Milton Keynes, UK) and tissue culture water from Sigma (Poole, UK). Freunds complete adjuvant was purchased from Difco/Osi (Elancourt, France) and Freunds incomplete adjuvant from Sigma Aldrich (St. Quentin-Fallavier, France). Ultima Gold™ scintillation cocktail was purchased from Packard (Pangbourne, UK). All other chemicals were of reagent grade or better and used as received.

Table 1
Cross reactivities of compounds obtained with S9788 antiserum

Compound	Cross index (I%)
S16636	0.80
S16637	0.30
S16638	0.10
S16639	0.08
S16640	0.37
Vincristine	No displacement[a]
Adriamycin	No displacement[a]

[a] No displacement of antiserum visible on the serum standard curve. ED_{50} value off-curve therefore it was not possible to calculate the exact value.

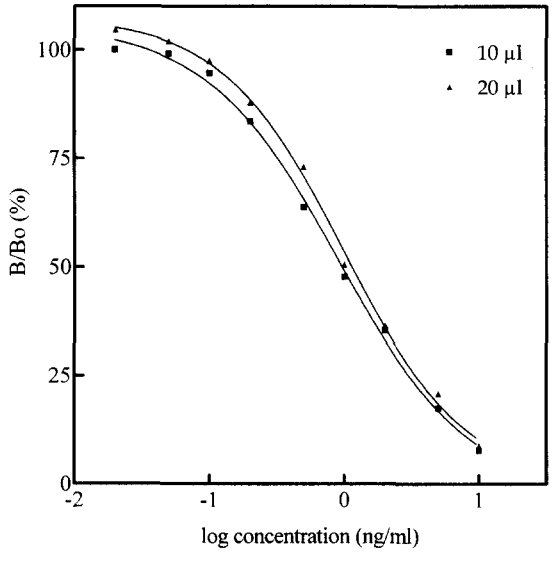

Fig. 2. Preparation of the standard curve in perfusion medium using differing assay volumes of 10 and 20 μl.

Table 2
Inter/intra-assay precision and accuracy for the determination of S9788 in perfusion medium

	Perfusion medium concentration (ng ml^{-1})	n^a	Determined concentration mean ± S.D. (ng ml^{-1})	CV (%)	Accuracy (%)
Intra-assay	0.2	6	0.19 ± 0.03	14.6	−5.6
	1	6	1.04 ± 0.10	10.1	3.9
	3	6	3.21 ± 0.37	11.6	7.1
Inter-assay	0.25	10	0.24 ± 0.03	13.3	−4.8
	0.5	10	0.49 ± 0.04	7.5	−1.2
	1	10	1.04 ± 0.06	5.7	4.4
	3	10	3.18 ± 0.26	8.2	6.1

[a] Each determination in triplicate.

Table 3
Inter/intra-assay precision and accuracy for the determination of S9788 in rat plasma

	Plasma concentration (ng ml^{-1})	n^a	Determined concentration mean ± S.D. (ng ml^{-1})	CV (%)	Accuracy (%)
Intra-assay	0.25	6	0.20 ± 0.04	18.6	−19.6
	1	6	0.86 ± 0.06	6.8	−14.3
	3	6	3.26 ± 0.06	1.6	8.7
Inter-assay	0.25	10	0.24 ± 0.05	19.8	−4.0
	0.5	10	0.48 ± 0.06	12.2	−5.0
	1	10	1.06 ± 0.07	6.8	5.7
	3	10	3.12 ± 0.20	6.7	5.9

[a] Each determination in triplicate.

2.2. Preparation of the immunogen

The immunogen was prepared from a structurally similar drug S10328-1, (bisallylamino-4,6-S-triazinyl-2)-1(carboxy-3-propyl)-4 piperazine), as S9788 does not possess a carboxyl group which is necessary for coupling to the carrier protein. The carboxyl group of S10328-1 was activated by a carbodiimide to couple to the amine groups of the tyrosyl residues of bovine serum albumin (BSA). The condensation reaction was carried out in an aqueous medium because the dialkylcarbodiimide used forms a soluble urea salt in water. S10328 (2.98×10^{-4} mol) dissolved in 3.5 ml of propyleneglycol, water, dimethyl sulphoxide (10:10:15 v/v/v) was added to *N*-(dimethylamino 3-propyl)-*N'*-ethylcarbodiimide chlorohydrate (2.98×10^{-4} mol) in water (1 ml). BSA (2.98×10^{-4} mol) in water (3 ml) was added dropwise to this mixture.

The resulting mixture was stirred for 24 h at ambient temperature, kept at 4°C for 10 h then dialysed against water at ambient temperature for 48 h. The final product was freeze dried. The purity of the conjugate was assessed after dialysis, by silica gel chromatography using a system of dioxan, water, acetic acid (90:10:1 v/v/v). The conjugate obtained was characterised by UV spectrometry at 252 nm corresponding to an absorption maximum of S10328 in methane sulphonic acid (15.4 M).

2.3. Immunisation

Immunisation was performed in six male New Zealand white rabbits. The immunogen (0.5–1.0 mg) was suspended in physiological saline (1 ml) emulsified with Freund's complete adjuvant (1 ml). This suspension was injected intradermally in multiple ante- and retroscapular sites. Booster

injections of 0.1–0.2 mg of immunogen were given every 4–8 weeks as an emulsion in Freund's incomplete adjuvant.

Blood samples were collected from the marginal ear vein 7–10 days after each booster injection, and the titre of the antiserum determined. After the final injection, the rabbits were bled; the serum samples were separated by centrifugation at $3000 \times g$ for 10 min and aliquots were stored at -70°C prior to use.

2.4. Antiserum titre determination

Assay buffer: phosphate buffer (0.16 M, pH 6.4) containing citric acid (0.034 M), sodium azide (0.01 M), EDTA (0.003 M), Tween 80 (0.001%) and BSA (1 g l^{-1}). Tracer: S2620-2, a structurally similar compound to S9788 was used as the tracer. [^{3}H]S2620-2 (specific activity: 80–100 Ci $mmol^{-1}$) diluted in assay buffer, (5000–10 000 dpm 100 μl^{-1}). Separation suspension: charcoal (2.4 g l^{-1}) and dextran T70 (0.2 g l^{-1}) in assay buffer.

To determine the titre of the antiserum, 100 μl each of assay buffer, tracer and various antiserum dilutions (1:50–1:5000) were mixed and incubated at ambient temperature for 3 h. The separation of free and bound ligand was achieved by the addition of a dextran coated charcoal suspension whilst mixing in an ice water bath. After incubation for 15 min and centrifugation at 4000 rpm for 15 min at 4°C, 100 μl of the supernatant was removed, mixed with scintillation cocktail (6 ml) and the bound radioactivity (B) was counted. The total activity (T) was determined by radioactivity measurement of 100 μl of [^{3}H]S2620-2 in buffer. The mean response was measured and the percentage of bound radioactivity to the antiserum (% B/T) determined using the following equation.

$$\%B/T = \frac{B - \mathrm{NSB}}{T}$$

where B is the maximum binding and NSB the non specific binding of the tracer. The titre is defined as the final dilution of the antiserum in the assay at which 50% of the added tracer is bound.

2.5. Determination of the antibody apparent affinity constant

Increasing quantities of [^{3}H]S2620-2 (2800 to 200 000 dpm) were incubated with 100 μl of assay buffer and 100 μl of antiserum at the working dilution of 1:500. The same procedure described above was used for separation and counting. The apparent affinity constant for the binding of [^{3}H]S2620 to the antibodies was determined from a Scatchard plot.

2.6. Antiserum cross reactivity determinations

The cross reactivity of the antiserum in the complete assay was determined in the presence of the known metabolites of S9788, and with vincristine and adriamycin, two anti-cancer compounds likely to be used with S9788. The concentration of chemotherapeutic agent (0.1–1000 ng ml^{-1}) and S9788 metabolites (0.1–500 ng ml^{-1}) was determined by reference to a standard curve for S9788 prepared over the range 0.02–10 ng ml^{-1}. The cross reactivity of the metabolite/cytotoxic compound, and thus the specificity of the antiserum was calculated using Abraham's Cross Index [5].

$$I\% = \frac{\mathrm{ED}_{50}\ \mathrm{S9788} \times \text{molecular weight (P)}}{\mathrm{ED}_{50}(\mathrm{P}) \times \text{molecular weight S9788}} \times 100$$

where (P) represents the metabolite or cytotoxic agent and ED_{50} the estimated concentration of compound which produces 50% binding of the labelled ligand to the antiserum.

3. Sample analysis

3.1. Serum and plasma samples

A phosphate incubation buffer (0.016 M, pH 6.4) containing citric acid (0.034 M), sodium azide (0.003 M) and BSA (2 g l^{-1}) was used. A stock solution of S9788 bismesylate (1 mg ml^{-1}) was prepared in water and from this a set of calibration standards was constructed in control serum, by serial dilution, over the range 0.02–10.0 ng ml^{-1}. Quality control standards were similarly

Table 4
Inter/intra-assay precision and accuracy for the determination of S9788 in human serum

	Serum concentration (ng ml^{-1})	n[a]	Determined concentration mean ± S.D. (ng ml^{-1})	CV (%)	Accuracy (%)
Intra-assay	0.25	6	0.27 ± 0.04	16.0	8.4
	1	6	0.98 ± 0.07	6.9	−2.4
	3	6	2.80 ± 0.34	12.0	−5.9
Inter-assay	0.25	5	0.21 ± 0.03	14.5	−16.0
	1	5	1.02 ± 0.13	12.4	2.4
	3	5	3.14 ± 0.29	9.4	4.5

[a] Each determination in triplicate.

prepared in serum at concentrations of 0.2, 1 and 3 ng ml^{-1}. The antiserum dilution and [^{3}H]S9788 tracer solution were prepared in incubation buffer. The reagents were added to 5 ml polypropylene tubes (Sarstedt, Leicester, UK) as follows: 100 μl calibration standard, quality control or sample, 100 μl incubation buffer, 100μl [^{3}H]S9788 (15–20 000 dpm tube^{-1}) and 100 μl of antiserum (1:15 000 dilution). Non-specific binding (NSB) was determined by substituting the antiserum with an equal amount of incubation buffer. Control binding (Bo) was determined using control serum.

The samples were agitated using a vortex mixer and incubated overnight at 4°C. Free and bound ligand were separated by the addition of 1 ml of a dextran coated charcoal suspension (dextran T70 0.5 g l^{-1}, charcoal 6 g l^{-1} in incubation buffer). The suspension was incubated at 4°C for 15 min and centrifuged at 4000 × g for 15 min. An aliquot (1 ml) of the supernatant was removed, mixed with 4.5 ml of scintillation cocktail and the radioactivity measured for 2 min on a liquid scintillation counter (Packard Tri-Carb 22000 Beta Spectrometer). All samples were analysed in triplicate.

3.2. Microdialysate samples

The perfusion medium used for microdialysis was a Ringer solution consisting of 155 mM NaCl, 5.5 mM KCl, 2.3 mM $CaCl_2$ and BSA 70 mg ml^{-1} pH 7.4. The standards and samples were analysed as described above, except that the calibration and quality control standards were prepared using Ringer/BSA solution. Non-specific and control binding were determined with perfusion medium.

Microdialysis samples can vary in size depending on the length of the collection period, so it was necessary to check that this variation did not affect the assay. The standard sample volume used in analysis was 100 μl, with only the sample size altering. Standard curves using a volume size of 10 and 20 μl diluted up to 100 μl were used to assess the stability of the standard curve.

3.3. Data processing

A calibration curve of [(B−NSB)/(Bo−NSB)] versus logarithmic concentration of S9788 (ng ml^{-1}) was constructed for both assays using the RIASMART software package (Packard, Pangbourne, Berks). The concentration of S9788 in samples was then calculated from the calibration curves.

3.4. Assay validation

The limit of quantification (LOQ) was determined for both assay procedures by four standards of S9788 (0.05, 0.1, 0.2 and 0.3 ng ml^{-1}) prepared in control serum and in perfusion medium. Each solution was assessed with six replicates (each replicate measured three times). The concentration of S9788 was calculated from a calibration curve (0.02–10 ng ml^{-1}) prepared at the same time in control serum and perfusion

medium. The LOQ was established as the lowest concentration which could be detected with precision and accuracy levels of less than 20%.

Intra-assay variability was assessed, once the LOQ had been determined, from standards at concentrations of 0.25, 1 and 3 ng ml^{-1} (linear range of the calibration curve). Each solution was assayed with six replicates (each replicate analysed in triplicate), against a standard curve (0.02–10 ng ml^{-1}) prepared at the same time. Precision and accuracy values were again calculated.

Inter-assay accuracy and precision were calculated from quality control data obtained from separate assays.

4. Microdialysis

4.1. Apparatus

Flexible microdialysis probes (10 mm), made from regenerated cellulose dialysis fibres, with an internal diameter of 215 μm and a molecular weight cut-off of 6000 Da (Harvard Apparatus) were used. The perfusion medium was delivered by a variable flow syringe pump (Model 44, Harvard Apparatus, South Natick, MA). The probes were linked to the pump and collection vessel using fused silica tubing (75 μm i.d., 150 μm OD, Harvard Apparatus). All samples were collected in 1.5 ml Eppendorfs for appropriate dilution prior to analysis.

4.2. In vitro probe relative recovery

The probe was perfused with Ringer solution containing BSA (70 mg ml^{-1}) at flow rates of 0.5, 0.75, 1.0, 2.0 and 3.0 μl min^{-1}. The sample size was 20 μl for 0.5 and 0.75 μl min^{-1}, and 10 μl for the remaining flow rates. When changing the perfusion rate, the probe was perfused for 30 min, prior to determination, to allow the membrane to equilibrate at the new flow rate. Triplicate samples of 20 min duration were collected at 0.5 and 0.75 μl min^{-1}, and six samples of 10 min at 1.0, 2.0 and 3.0 μl, from a Ringer/BSA 2 μg ml^{-1} S9788 reservoir standard, maintained at 37°C in a water bath under constant stirring. The probe was then introduced into solutions of 2, 10 and 50 μg ml^{-1} S9788 and perfused at 1.0 μl min^{-1}. Six consecutive samples of 10 μl were collected from each concentration. The ratio of the concentration determined in the dialysate against the concentration of the surrounding solution was used as a measure of the recovery.

4.3. In vivo experimental plan

A group of 6 female Wistar rats were cannulated via the right carotid artery under general anaesthesia and allowed 24 h to recover before dosing. Under isoflurane anaesthesia they were then given a single infusion of S9788 (15 mg kg^{-1}) over 30 min directly into the jugular vein. Blood samples (0.125 ml) were taken at pre-dose, 0.5, 0.75, 1, 2, 4 and 8 h after which the volume of sample was increased to 0.25 ml at 24 and 32 h and 2, 3, 4, 5, 6 and 7 days after administration from the cannula inserted into the carotid artery. Samples were collected in heparinised tubes, plasma was obtained by centrifugation and stored at −20°C until analysis by radioimmunoassay. Samples were diluted with control plasma where necessary, to bring the plasma concentration of S9788 within the range of the calibration curve.

4.4. In vitro experimental plan

To measure the free fraction of S9788, aliquots of the plasma samples (obtained at 0.5, 0.75, 1, 4 and 8 h) were placed in Eppendorfs and maintained at 37°C in a water bath. The microdialysis probe was placed into each sample in turn and perfused at 1 μl min^{-1} with Ringer/BSA (70 mg ml^{-1}) for a period of approximately 30 min. Triplicate samples of 10 μl were collected from each sample. Ringer/BSA (90 μl) was added to each sample to achieve the volume necessary for radioimmunoassay analysis.

5. Results

5.1. Antiserum

The immunogen was characterised by UV spectrometry. The molar ratio of hapten molecules to protein was approximately 13 mol of S10328-1

per mol of BSA. The antiserum issued from the screening demonstrated a high affinity for [^{3}H]S9788 with an apparent affinity constant Ka equal to 8.4×10^{10} l mol^{-1} calculated from the Scatchard plot. Titration of the antiserum showed 50% binding (Bo/total counts) at a dilution of 1:15 000 for the assay in serum, but this was altered to 1:20 000 for the assay in the dialysate medium to obtain the same sensitivity. Aliquots were stored at −70°C prior to use.

5.2. Assessment of assay specificity

The cross reactivities, calculated using Abraham's Cross Index [5], were found to be < 1% for the metabolites of S9788 and for the two anticancer drugs likely to be co-administered with the compound. The results are shown in Table 1.

5.3. Effect of sample volume

Fig. 2 represents standard calibration curves in perfusion medium, prepared using a 10 and 20 μl sample size. The assay volume was maintained at 100 μl by the addition of Ringer/BSA (70 mg ml^{-1}). The shape of the curve and similar ED_{50} values indicate the assay is not affected by volume.

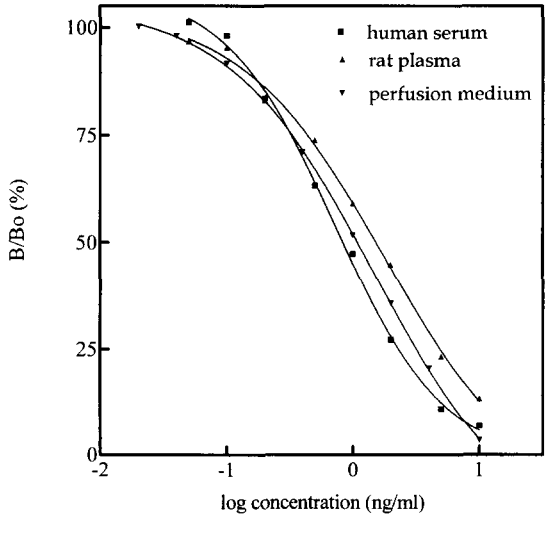

Fig. 3. Comparison of typical standard curves for S9788 prepared in perfusion medium, human serum and rat plasma.

5.4. Assay sensitivity and precision

A maximum limit of 20% was used for the limit of quantification, intra and inter assay R.S.D., indicating that the lowest measurable concentration is 0.2 ng ml^{-1} in perfusion medium (Ringer/BSA 70 mg ml^{-1}). The upper limit was 10 ng ml^{-1}. The same criterion was applied to both serum and plasma curves yielding a limit of quantitation of 0.25 ng ml^{-1} and an upper limit of 10 ng ml^{-1}. The non-specific binding was calculated to be 2.0% of the total counts in the assay. The intra and inter assay precision and accuracy are shown in Tables 2–4.

5.5. Assay of S9788 in different media

Standard calibration curves for human serum, rat plasma, and perfusion medium are shown in Fig. 3. These curves were prepared using a 100 μl volume. The positions and shapes of the curves indicate that all three media are suitable for use in the radioimmunoassay.

Because rat plasma samples contained high concentration of drug which were outside the range of the standard curve they were diluted up to 1:500. This addition of matrix had no effect on the assay, and the absolute sensitivity was 20 pg per tube.

5.6. In vitro microdialysis

Fig. 4 illustrates the effect of increasing flow on analyte recovery. At 1 μl min^{-1} the relative recovery is 66% of the free concentration available for diffusion across the probe membrane (S9788 binds 92.5% in vitro to Ringer/BSA (70 mg ml^{-1})—(unreported data). The relative recovery is independent of concentration in the 2–50 μg ml^{-1} range, i.e. 0.15–3.5 μg ml^{-1} free concentration (Fig. 5).

Fig. 6 shows the plasma levels of S9788 for three rats over a 168 h period analysed by radioimmunoassay following intravenous infusion (30 min) of S9788 at 15 mg kg^{-1}. For one animal (rat 2), microdialysis levels of S9788 were determined from plasma samples over the first 8 h and

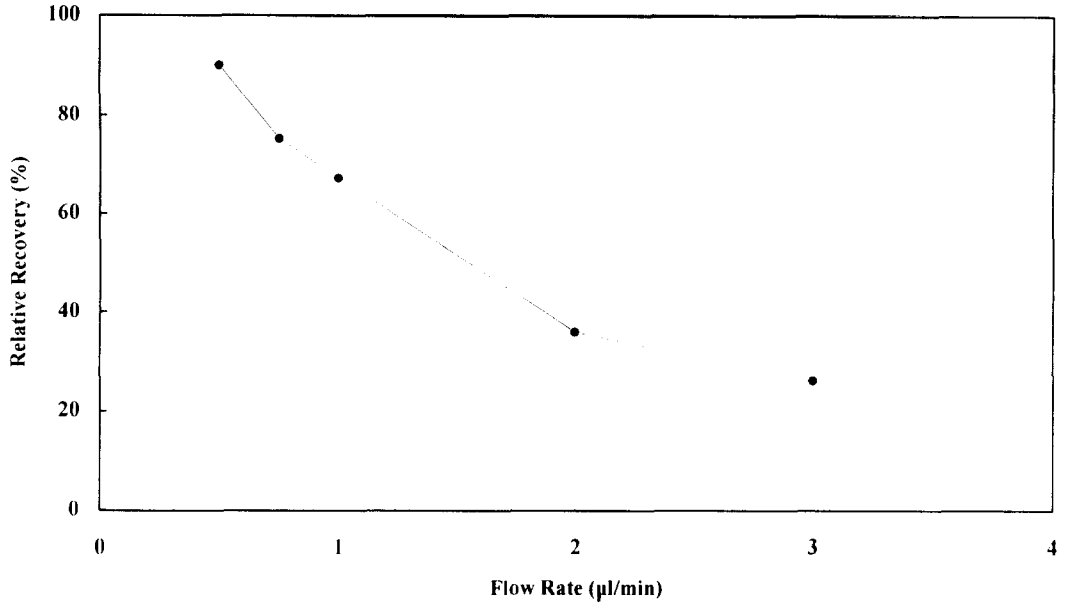

Fig. 4. Effect of flow rate on the relative recovery of S9788 for a 10 mm membrane length.

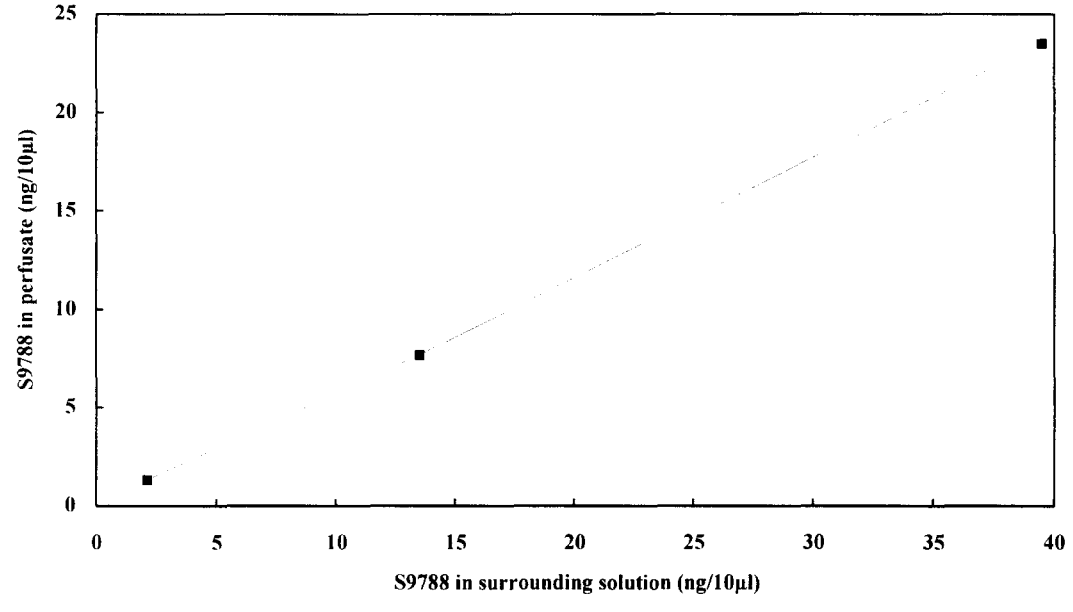

Fig. 5. S9788 perfusate concentration as a function of concentration in the surrounding solution for a 10 mm probe perfused at 1 µl min^{-1}.

these have been compared in Fig. 7 and Table 5. The results were corrected for probe recovery. The free concentration profile follows that of the total concentration profile. The AUC8 for total plasma S9788 and microdialysis are 5220.66 and 60.56 ng·h ml^{-1} respectively.

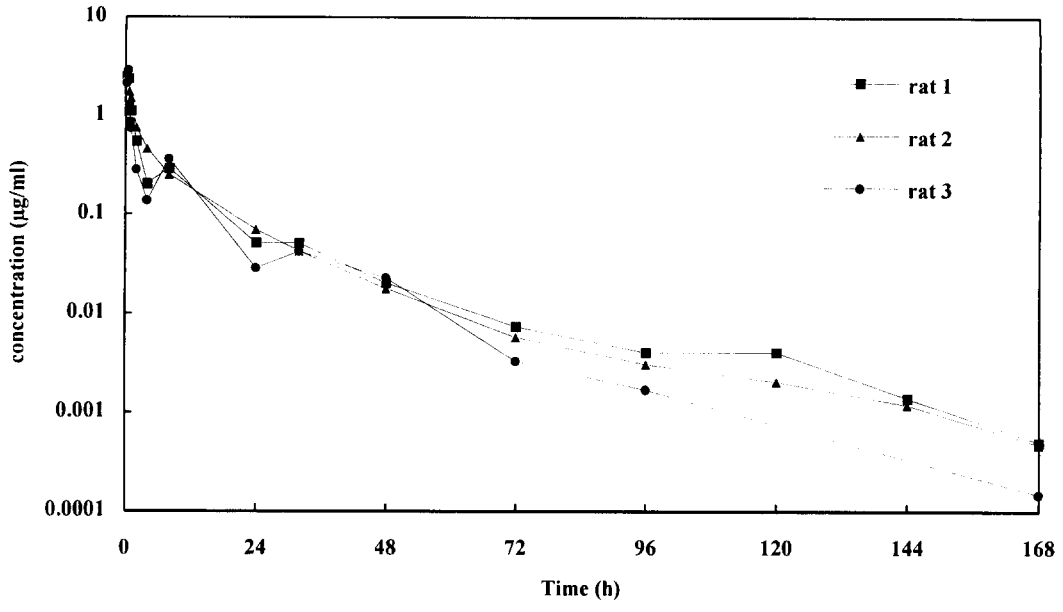

Fig. 6. Concentration–time profiles of S9788 in the plasma of three rats following an intravenous infusion of 15 mg kg^{-1} over 30 min.

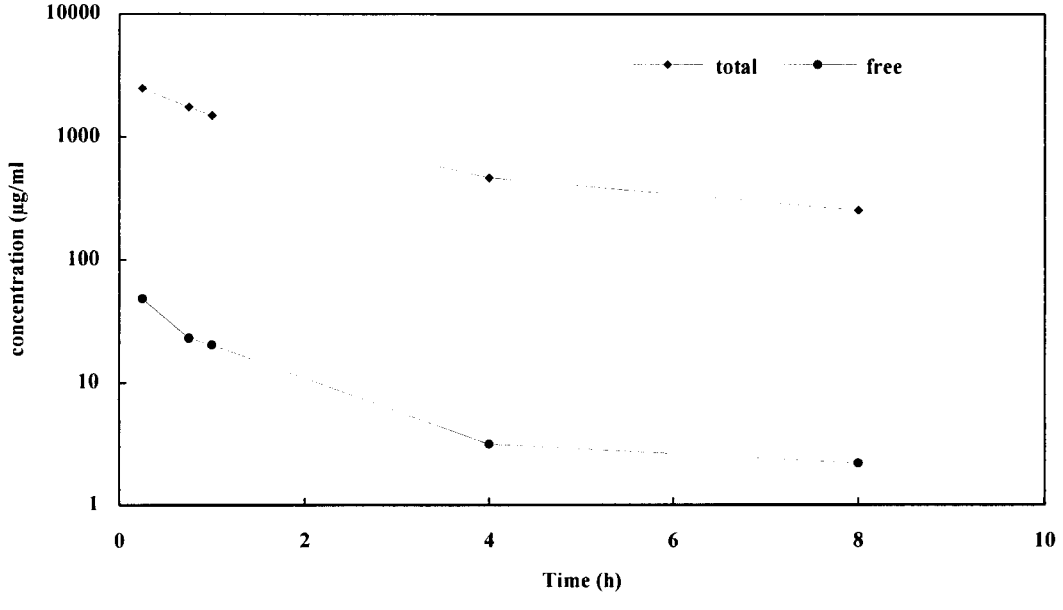

Fig. 7. Concentration (log)–time profile of S9788 in rat 2. Comparison of total plasma and corrected free dialysate levels following an intravenous infusion of 15 mg kg^{-1} over 30 min.

Table 5
S9788 total and free plasma concentrations in the plasma of 3 rats following i.v. infusion of 15 mg kg^{-1} over 30 min

Time (h)	Concentration (ng ml^{-1})			Concentration (ng ml^{-1})[a]
	Rat 1	Rat 2	Rat 3	Rat 2
0.25	2470	2490	2110	48.5
0.5	2340	2580	2850	N/A
0.75	837	1740	1260	22.9
1	1110	1490	741	20.3
2	550	754	282	N/A
4	202	461	140	3.14
8	290	250	361	2.18
AUC8 (ng·h ml^{-1})	4444.82	5220.66	5050.37	60.56

Microdialysis results are corrected for probe recovery.
[a] Values determined by microdialysis.
N/A sample size too small for analysis.

6. Discussion

Radioimmunoassay is a widely used technique in bio-analysis. It is advantageous because of the low sample volumes required [6] and high sample throughput, although it can suffer from a lack of selectivity due to cross reactivity with metabolites and endogenous chemicals. The assay described here has been successful for both small pharmacokinetic studies and microdialysis, because samples with volumes of 10 μl for microdialysis can be analysed with an absolute sensitivity of 0.2 ng ml^{-1} (20 pg per tube) and in the rat, a complete pharmacokinetic profile over 168 h (15 time points) can be established.

Preparation of the immunogen was carried out using a structurally similar compound, S10328, because S9788 does not possess the carboxyl function necessary to couple to the carrier protein (BSA). S10328 coupled to the BSA in a ratio of 13:1 providing an effective immunogen. Affinity constant values (K) for an antiserum normally range from 10^9 to 10^{11} l mol^{-1} [7]. This assay produced antiserum with a K value of 8.4×10^{10} l mol^{-1}, indicating sufficient binding to the radioligand. The resulting antibody dilution for the assay in human and rat serum was set at 1:15 000, but dilution had to be increased to 1:20 000 to maintain the same sensitivity in Ringer/BSA. Antibody specificity was determined using the known metabolites of S9788, and two anticancer drugs, vincristine and adriamycin. Cross reactivities of $< 1\%$ were obtained, demonstrating good selectivity and specificity of the antibody.

Previous microdialysis work (Ungerstedt et al., unpublished work), has shown that the sampling of lipophilic compounds is difficult due to poor recoveries and the problems of binding to the microdialysis device (probe membranes, tubing). Carnheim et al. [3] demonstrated that by altering the composition of the perfusion medium, the probe recoveries might increase. S9788 is lipophilic and poor recoveries ($< 15\%$) were experienced using Ringer perfusion medium due to the low solubility of the compound in aqueous solution. However, by adding BSA (70 mg ml^{-1}) to the Ringer solution perfusion medium, a suitable environment has been produced for the drug to bind to the BSA and remain in solution although the method of action is not clear [3]. The BSA used in the above experiments still contains the associated fatty acids and the levels of both could be sufficient to solubilise the drug in the fatty acid–albumin complex. This medium is suitable for direct analysis by radioimmunoassay, producing a similar curve in both shape and sensitivity, to that of human serum and rat plasma (Fig. 3).

There is a relationship between the flow rate of the perfusate and the relative recovery [8]. Flow rates of 0.5–3.0 μl min^{-1} show that the relative recovery of S9788 is inversely proportional to the flow rate, i.e. by increasing the flow, the concentration of the recovered substance decreases. At a flow of 1 μl min^{-1}, the recovery was 66% whilst at 3 μl min^{-1} recovery was reduced to 26%. The relative and absolute recovery was found to be independent of concentration in the range 2–50 μg ml^{-1}. There was a small probe to probe variation in recovery, therefore recovery in each probe should be determined separately prior to use in vivo, as the characteristics of each probe change. The sample volumes of 10 and 20 μl obtained at the various flow rates were of a suitable size for use in radioimmunoassay.

When free S9788 was obtained from rat samples by microdialysis, the profile of free concentration followed the total concentration over an 8 h period. S9788 is 98% plasma protein bound, and correcting for protein binding and probe recovery, the free concentration levels correspond closely to the theoretical free concentration calculated from the total plasma values.

In conclusion, a sensitive and selective radioimmunoassay technique has been developed for the analysis of S9788 which is ideal for microdialysis due to the low sample volumes obtained. Samples can be analysed directly as long as the overall volume is maintained (100 μl for this assay).

A combination of microdialysis with a modified perfusion medium, and a sensitive radioimmunoassay, enables the highly protein bound drug S9788 to be measured in vitro.

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