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The development and performance of a radioimmunoassay for the analysis of ZM 213,689, the major metabolite of meropenem—a carbapenem antibiotic—in plasma and urine

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Abstract

The development of a radioimmunoassay for the analysis of ZM 213, 689, the major metabolite of meropenem found in the plasma and urine of rat, dog and humans, is described. The assay is rapid in order to minimise the effect of degradation of meropenem to ZM 213,689 in biological samples and has a working range of 0.08–3.5 mg l^{-1} (RSD $\leqslant$ 15%). The antibody was specific for ZM 213,689 with cross-reactivity to meropenem of only 0.4%. The synthesis of the immunogen and radiotracer involved a novel approach due to the multifunctional nature of ZM 213,689.

Keywords: Meropenem; Radioimmunoassay; Metabolite; Pharmacokinetics

1. Introduction

Meropenem (Fig. 1) is a novel carbapenem antibiotic with a wide spectrum of in-vitro activity against Gram-positive and Gram-negative pathogens including anaerobes [1,2]. Early in-vitro studies [3] predicted that meropenem would be more stable to ring opening by human renal dehydropepsidase (DHP-I) than imipenem (an alternative carbapenem antibiotic) and consequently would not require the co-administration of a DHP-I enzyme inhibitor such as cilastatin. Later studies demonstrated that meropenem is partially metabolised in animals and humans to the ring opened β-lactam form, ZM 213,689 [4] (Fig. 1), which is excreted in the urine. Meropenem also undergoes chemical hydrolysis to give the same product.

As part of the clinical and pre-clinical safety evaluation program for meropenem, an assay was required to quantify ZM 213,689 in plasma and urine. A combination of very poor UV chromophoric characteristics (maximum absorption at less than 200 nm), zwitterionic structure and high polarity made analysis using standard extraction procedures and chromatography difficult and in-

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sensitive. Consequently an alternative analytical procedure, radioimmunoassay (RIA), was developed with the emphasis on speed and specificity.

2. Materials and methods

2.1. Reagents

All compounds were either of analytical reagent grade or used as received unless other wise stated. ZM 213,689, meropenem (purity > 97%, ZM 213,689 content < 0.2%), and ^{14}C-meropenem were obtanied from Zeneca Pharmaceuticals. Bolton and Hunter reagent was obtained from Amersham International (Amersham, Buckinghamshire, UK). *m*-Maleimidobenzoyl-*N*-Hydroxysulfosuccinimide ester (Sulfo-MBS) was obtained from Life Science Laboratories (Luton, UK). Freund's complete and incomplete adjuvants were supplied by Difco Laboratories (Detroit, MI). The assay buffer was prepared by dissolving sodium azide (0.1% w/v) and bovine gamma globulin (BGG, 0.2% w/v) in phosphate buffer (0.1 M, pH 7.4). Blood was taken from volunteers or animals into oxalated tubes and centrifuged to obtain plasma. Control urine was also collected. Both biomatrices were stored at −20°C.

2.2. High performance liquid chromatography conditions

For the monitoring of immunogen synthesis, the following high performance liquid chromatography (HPLC) conditions were used: Hypersil 3 μm MOS column (150 mm × 4.6 mm i.d. Hichrom Ltd., Reading, Berkshire, UK) and a mobile phase consisting of phosphate buffer (0.1 M, pH 7.4):acetonitrile (10:1 v/v) monitored at 280 nm, the λ_{max} value of meropenem. Meropenem eluted under these conditions at a capacity factor (k′) = 1.0. The gel permeation chromatography (GPC) conditions used to separate the meropenem conjugates of either bovine serum albumin (BSA) or keyhole lympet haemocyanin (KLH) from their reaction mixtures were: a Sephadex G25 column (20 cm × 2 cm i.d., Pharmacia Limited, Milton Keynes, UK) and phosphate buffer (0.1 M, pH 7.4) as the mobile phase, monitoring at 280 nm. The HPLC conditions for purifying the radiotracer were a Hypersil 5 μm MOS column (150 mm × 4.6 mm i.d.) and a mobile phase of phosphate buffer (0.013 M, pH 7.4):acetonitrile (80:20 v/v) at a flow rate of 1.0 ml min^{-1}. The radioactive products were detected using a Beckman 170 radioactivity monitor.

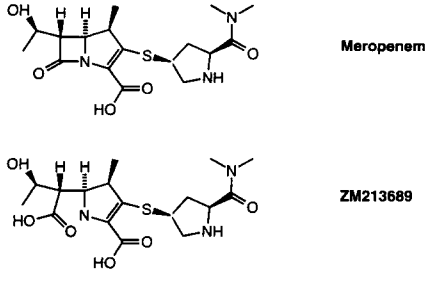

Fig. 1. Structures of meropenem and ZM 213,689

2.3. Synthesis of immunogen

Meropenem was linked through the proline nitrogen to either BSA or KLH using the reagent sulfo-MBS as the bridging group. The resulting conjugates were then converted into their respective ZM 213,689 immunogens by base hydrolysis. The details of the procedure used are outlined below.

Meropenem (12 mg, 31 μmol, containing approximately 7×10^5 dpm of ^{14}C-meropenem) was reacted with Sulfo-MBS (16 mg, 38 μmol) in borate buffer (0.1 M, pH 8.6). The pH was maintained at around 8.5 for 2.5 h at room temperature by the addition of sodium hydroxide solution (1.0 M). The disappearance of meropenem during the conjugation process was monitored by HPLC analysis of the reaction mixture.

The solution containing the meropenem–Sulfo-MBS adduct was split into two equal aliquots which were added to solutions of BSA (50 mg, 780 nmol) in phosphate buffer (0.1 M, pH 6.0, 2 ml) and KLH (25 mg, 12 nmol) in borate buffer (0.1 M, pH 8.6, 8.5 ml) respectively. These solutions were maintained at 4°C overnight and then purified by GPC.

The purified meropenem conjugates were hydrolysed to their respective ZM 213,689 conjugates by addition of an equal volume of borate buffer (0.1 M, pH 9.4). The degradation of meropenem to ZM 213,689 in the conjugate was followed by monitoring the decrease in absorbance of the solutions at 300 nm. This wavelength was selected due to the high ultraviolet absorbance of BSA and KLH at 280 nm (the λ_{max} value of meropenem) which effectively precluded detection of meropenem at this wavelength. The solutions were then concentrated by pressure dialysis using a membrane with a molecular weight cut-off of 10 000 Da (Amicon, Stonehouse, Gloucestershire, UK). The conjugation ratios of meropenem to KLH and BSA were determined by measuring the specific activity of the conjugates after the final purification, assuming complete recovery of protein throughout the synthesis.

2.4. Immunisation procedure

Eight sheep (four per immunogen) were immunised with subcutaneous injections of the immunogen mixed with Freund's complete adjuvant (1:3 v/v) at five sites on the back and hind limbs. Approximately 125 μg of immunogen (in 1.0 ml of emulsion) was used per immunisation. Subsequent booster immunisations employed Freund's incomplete adjuvant and were carried out at 6, 12 and 17 weeks after the primary immunisation. From week 12 onwards the mass of conjugate injected was reduced from 125 μg to 110 μg (BSA) and 60 μg (KLH) although the volume of emulsion was kept constant. Blood samples were typically taken between 10 and 21 days following an immunisation. However, a more rigorous sampling schedule was adopted after the third boost when blood samples were taken at 0, 4, 6, 10, 12 and 14 days. The maximum specific antibody levels were determined for all the antisera (diluted 1:10 v/v in assay buffer) using a ZM 213,689 radiotracer. Antisera showing high binding were further evaluated to determine the antibody titre (defined as the working dilution of antiserum which bound 50% of added radiotacer) and the cross-reactivity of meropenem.

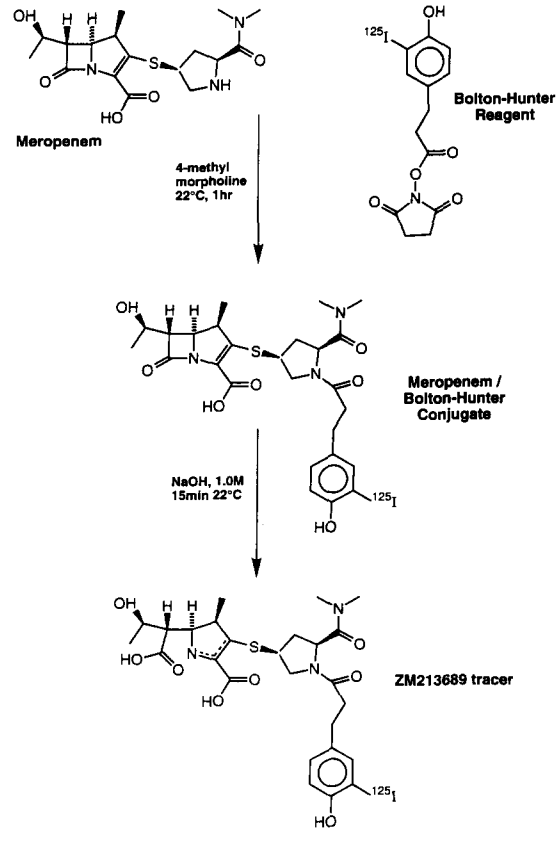

Fig. 2. Reaction scheme for the preparation of the ZM 213,689 radiotracer.

2.5. Synthesis of radiotracer

An iodinated ZM 213,689 tracer was prepared by linking meropenem through the proline nitrogen directly to Bolton and Hunter reagent (Fig. 2). The meropenem moiety was subsequently hydrolysed to ZM 213,689 prior to use in the immunoassay procedure. The following method was employed.

The solvent was removed from the Bolton and Hunter reagent (1 mCi) at room temperature using a stream of dry nitrogen (anhydrous calcium chloride) vented through a charcoal trap (approximately 7 min). Meropenem (1 mg, 2.6 μmol) was dissolved in 4-methyl morpholine (0.092 M, 1 ml) and an aliquot (40 μl) added to the vial containing dried Bolton and Hunter reagent. To minimise the hydrolysis of meropenem to ZM 213,689, the

meropenem/4-methyl morpholine solution was prepared just prior to use. The vial was vortex mixed for a few seconds and the reaction allowed to proceed for 1 h at room temperature. The reaction mixture was purified by HPLC with the meropenem/Bolton and Hunter conjugate (Fig. 2) eluting in a 1 ml fraction ($k' = 3$). The material, which had a specific activity of 2200 Ci $mmol^{-1}$, was hydrolysed to the corresponding ZM 213,689 radiotracer immediately prior to setting up the RIA. Hydrolysis was carried out by addition of half an equivalent volume of sodium hydroxide solution (1.0 M) to purified conjugate solution. The optimum hydrolysis time was found by sampling the reaction over 19 h at room temperature and determining the level of binding to antibody using the RIA procedure.

To verify that the correct reagent was being synthesised, the radiotracer perparation was repeated using the uniodinated form of Bolton and Hunter reagent, *N*-succinimidyl 3-(4-hydroxyphenyl)propionate. The products were extracted from the reaction mixture with ethyl acetate and the organic solvent evaporated with nitrogen at room temperature. The residue was freeze dried and the products analysed by positive fast atom bombardment mass spectrometry.

2.6. Radiotracer stability

The effects of temperature, concentration, solvent and container material on radiotracer stability were investigated. The unhydrolysed radio tracer (meropenem/Bolton and Hunter conjugate) was stored at temperatures from −70 to 4°C in methanol, water, acetonitrile, HPLC eluent (phosphate buffer (0.013 M, pH 7.4):acetonitrile (80:20 v/v)) or acetonitrile/water (50/50 v/v) at two radiochemical concentrations (10 and 300 mCi l^{-1}) in both glass and polyethylene containers. At periodic intervals, aliquots of the stored samples were removed, hydrolysed, diluted to a consistent radiochemical concentration and the level of specific binding remaining in each solution determined using the RIA procedure.

2.7. RIA procedure

ZM 213,689 calibration standards were prepared in plasma, or urine diluted with phosphate buffer (0.1 M, pH 7.4) (1:50 v/v), at concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0313 mg l^{-1}, with blank matrix as the zero standard. Calibration standards were prepared fresh daily and maintained alongside samples at 4°C prior to analysis.

An aliquot of the meropenem/Bolton and Hunter conjugate (radioactive concentration approximately 10 mCi l^{-1}) was hydrolysed to the ZM 213,689 radiotracer with half an equivalent volume of sodium hydroxide (1.0 M) at room temperature for 10–15 min. The mixture was then diluted to approximately 4.2×10^8 dpm l^{-1} with assay buffer. The assay was carried out in duplicate in polystyrene tubes (55 mm × 12 mm i.d.). Standards or samples (10 μl), radiotracer (0.1 ml) and antiserum (0.1 ml, diluted with assay buffer 1:200 v/v)) were mixed and incubated at room temperature for 1 h. Bound radiotracer was precipitated by the addition of PEG (polyethylene glycol, molecular weitht 6000 Da) solution (0.5 ml, 27.5% w/v in deionised water). The tubes were vortex mixed until the incubate was homogeneous and immediately centrifuged (2800*g*, 15 min). After centrifugation the supernatant was aspirated to waste and the precipitate counted in a Nuclear Enterprises 1600 gamma counter (efficiency ≈ 70%) for 1 min.

The calibration data were fitted using a smoothed spline fitting routine using the percentage of radiotracer bound [$(B/T \times 100\%)$ against log concentration.

2.8. Matrix interference and limit of detection

Fifty control human plasma and urine samples from healthy volunteers and five plasma samples from patients with renal failure were analysed to determine any interference from the matrices. Urine samples were diluted with assay buffer (1:10 v/v) prior to analysis. Twenty control rat and 17 control dog plasma samples were also analysed for endogenous interference. The limit of detection for each type of matrix was set at the mean

response plus twice the standard deviation of the control samples.

2.9. Assay precision

Assay precision was determined using pooled human plasma. The intra-assay precision was determined by the analysis of ten separate calibration series within a single analytical run. The inter-assay precision was determined over a 28 day period by analysing a set of stored (− 70°C) calibration series.

2.10. Cross-reactivity

The cross-reactivity of meropenem in buffer as well as in rat, dog and human plasma was measured using calibration standards that had been prepared and maintained at 4°C and at room temperature for 3 h prior to analysis.

Human plasma was spiked with meropenem (0.25–150 mg l^{-1}) and the samples analysed against a ZM 213,689 calibration series using the RIA procedure. The concentrations of meropenem, and ZM 213,689 required to displace 50% of the bound counts (ED_{50}) were then determined. The cross-reactivity was calculated as the ratio of the ED_{50} concentrations of meropenem to ZM 213,689, expressed as a percentage.

3. Results

3.1. Synthesis of immunogen

ZM 213,689 could not be used directly for conjugation because of its multifunctional nature, namely two basic nitrogens of similar pK_a value and two carboxylic acid groups. Consequently, meropenem, with only a single basic nitrogen, was conjugated to the appropriate protein and subsequently ring opened to give the corresponding ZM 213,689 conjugate. The hydrolysis of meropenem conjugates to the corresponding ZM 213,689 conjugates was followed by measuring the change in UV absorption at 300 nm. From radioactivity measurements, the incorporation ratios for ZM 213,689 to BSA and KLH were 3:1 and 146:1 respectively.

3.2. Antibody production

The extent of radiotracer binding to antisera increased steadily throughout the immunisation program. However, the level of binding for a 1:10 dilution of antisera was poor, rising from approximately 10 to 35% using an impure radiotracer and non-optimised conditions. Consequently a more rigorous sampling regime was imposed following the third booster dose to determine the optimum time to harvest the antisera. This illustrated that the ZM 213,689 antibody levels increased rapidly following immunisation to a maximum at between 6 and 10 days (Fig. 3). The peak antibody levels were generally maintained for a maximum of only 3 days whereupon the levels decreased rapidly. Even with this level of sampling the highest antiserum titre was 1:200, serum S845-3.4, harvested from a sheep 12 days after the third immunisation with the ZM 231,689–KLH immunogen. However, this low titre was sufficient to allow 250 000 tests with this one non-terminal bleed and consequently all subsequent assay work was carried out using this antiserum.

3.3. Radiotracer synthesis

The radiotracer was purified by HPLC with typically three peaks being observed (Fig. 4). Iodinated meropenem, meropenem and ZM 213,689 eluted at k' values of 2.7, 1.0 and 0 respectively.

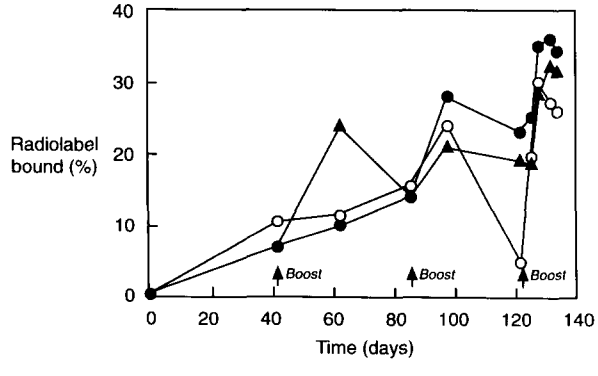

Fig. 3. The binding shown be three antisera obtained at various times throughout the immunisation schedule. Sheep were immunised with either BSA of KLH conjugates.

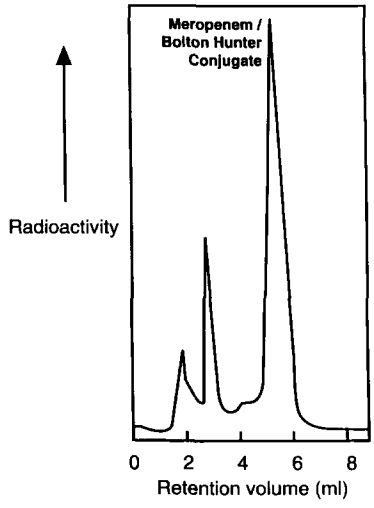

Fig. 4. Chromatogram illustrating the separation of radioactive products following the synthesis of the meropenem/Bolton and Hunter conjugate.

The radiochemical yield of iodinated meropenem was excellent, typically greater than 80%. The identity of the radiotracer was confirmed by reacting meropenem with *N*-succinimidyl 3-(4-hydroxyphenyl)propionate. The mass spectrum showed molecular ions for both the meropenem conjugate and meropenem. This was consistent with the major product of the reaction being meropenem linked to *N*-succinimidyl 3-(4-hydroxyphenyl)propionate through the proline nitrogen, as shown for the iodinated material (Fig. 2).

The hydrolysis of the meropenem conjugate to the ZM 213,689 radiotracer was monitored for 19 h following the addition of sodium hydroxide. The binding of radiotracer to antibody increased from 29% prior to hydrolysis to a maximal value of approximately 65% after 10 min. This level of binding was maintanied for at least 19 h. The relatively high initial level of binding is probably due to partial hydrolysis of meropenem to ZM 213,689 radiotracer on storage. A reaction time of 15 min was selected for routine hydrolysis.

3.4. Radiotracer stability

Extensive stability testing was undertaken using a wide range of storage conditions in both glass and polyethylene vials. The meropenem/Bolton and Hunter conjugate was least stable in methanol, acetonitrile and water when stored in glass vials at 4°C (Fig. 5). However, decomposition was less pronounced in HPLC eluent and acetonitrile:water (50:50 v/v), although this was still unsuitable for routine storage. Improved stability was observed for radiotracer stored in polyethylene vials compared to glass vials (data not shown) which may indicate some catalytic degradation on the glass surface. The effect of temperature and physical state of the stored radiotracer was pronounced and is clearly shown in Table 1. These data indicate that although temperature is an important factor in ensuring stability of the radiotracer, the physical form, or transition between forms, of the storage media can have an equal or greater effect. Thus the two samples stored at 4°C and −10°C which re-

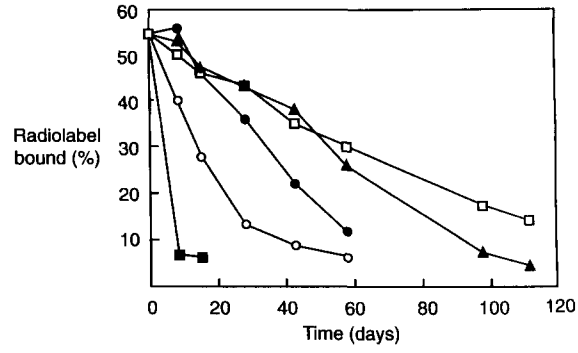

Fig. 5. The stability of radiotracer, stored in different solvents in glass vials at 4°C, measured by the binding of radiotracer to antisera: methanol (■); water (○);acetontrile (●); acetonitrile:water (50:50) (▲); HPLC eluent (□).

Table 1
Instability of ZM 213,689 stored in polyethylene vials as indicated by the decrease in binding to a fixed amount of antisera

Temperature (°C)	Physical form	Radioactive concentration (mCi l^{-1})	Decrease in binding (%)
−10	Liquid	10	3.9
4	Liquid	10	19.6
−70	Solid	10	23.5
−20	Solid	10	31.4
−10	Liquid	300	45.1

mained liquid showed greater stability than those which froze (Table 1).

The effect of temperature is clearly demonstrated by comparing the data for −10°C with those for 4°C (unfrozen) and the data for −70°C with those for −20°C (frozen). The concentration of the radioactive material also had a bearing on stability. The material stored at a concentration of 300 mCi l^{-1} was clearly less stable than the radiotracer stored at ⩽10 mCi l^{-1}.

For optimal stability of the radiotracer the following conditions were used: HPLC eluent, −10°C, polyethylene vials and a radiochemical concentration of 10 mCi l^{-1}.

3.5. Matrix interference and limit of detection

The response (mean ± standard deviation) from 50 control plasmas was equivalent to a ZM 213,689 concentration of 0.008 ± 0.002 mg l^{-1}. Plasma samples from patients with renal failure showed slightly greater interference (0.013 ± 0.005 mg l^{-1}). The interference from rat and dog plasma corresponded to 0.002 ± 0.002 and 0.003 ± 0.004 mg l^{-1} respectively. Control human urine showed a level of only 0.009 ± 0.004 mg l^{-1}. The limit of detection was based on the worst case (human plasma from renally impaired patients) and was set to 0.08 mg l^{-1} for all fluids/species.

3.6. Assay precision

The intra and inter-assay precision profiles for ZM 213,689 over the calibration range are shown in Fig. 6. The level of imprecision was slightly higher than usually observed with RIA methods. At best the assay gave an inter-assay RSD of 9% (0.25 mg l^{-1}) and, for practical purposes, the limits of quantification were set at an RSD of 15%, equivalent to a concentration range of 0.08–3.5 mg l^{-1}.

3.7. Cross-reactivity

The intrinsic specificity of the antiserum S845-3.4 was found to be very good with meropenem, showing only 0.4% cross-reactivity when measured using standards prepared in assay buffer, maintained at room temperature. However, when determined in human, rat and dog plasma, the apparent cross-reactivity for the same compound was found to have increased (Table 2). This was believed to be due to enzymatic activity, which is probably greater in the rat than in dog or human.

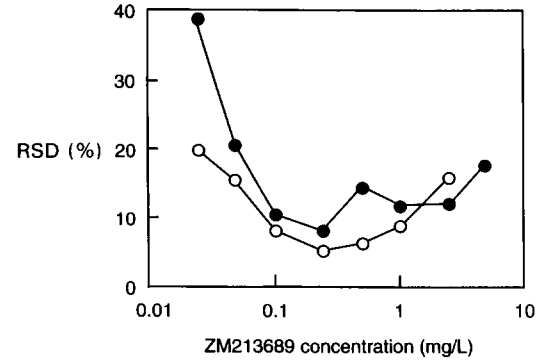

Fig. 6. Precision profile showing the inter-(●) and intra-assay (○) variations for the analysis of ZM 213,689 in human plasma.

The cross-reactivity experiment using plasma was repeated with standards either freshly prepared or stored at 4°C prior to analysis. Under these conditions a substantial decrease in apparent cross-reactivity was observed in all three species. In human, the cross-reactivity was restored to the value found in the absence of plasma (0.4%). These results indicate that the higher cross-reactivity observed in plasma samples at room temperature (approximately 3–5%) is an artefact due to the decomposition of meropenem to ZM 213, 689. No enzymatic inhibitors were investigated because the level of cross-reactivity for samples maintained at 4°C was considered acceptable. Phosphate buffer (0.1 M) at pH 7.4 is

Table 2
Cross-reactivity (%) of meropenem in human, rat and dog plasma stored at different temperatures prior to analysis

Temperature (°C)	Species		
	Human	Rat	Dog
4	0.4	1.2	0.9
22	2.9	4.8	3.1

used in the assay because this is the buffer type and pH for which meropenem is most stable (degradation half life between pH 4 and 8 of around 24 h at 37°C).

4. Discussion

ZM 213,689 is distinguished from meropenem by having a cleaved β-lactam ring. However, ring cleavage also exposes a secondary amine in addition to the existing proline nitrogen. As a consequence, ZM 213,689 could not be used directly to produce a single selective tracer or immunogen using Bolton and Hunter reagent or Sulfo-MBS respectively. Therefore, meropenem was used to prepare a radiotracer and immunogens, which were subsequently hydrolysed to the appropriate ZM 213,689 analogue.

The bridging group MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide) has been used to prepare protein conjugates for various analytes [5,6]. However, MBS has a low aqueous solubility, requiring reactions to be carried out in organic solvent. A more recent derivative used here, Sulfo-MBS, facilitates the same reactions but in a totally aqueous environment.

The variation in antibody titre shown for serum harvested from sheep on sequential days after immunisation is not an observation that is generally found in the literature. It may be that workers carry out this procedure routinely and simply fail to report it. Most reports either indicate the time after immunisation that antisera were collected (e.g. Ref. [7]) or simply give no details at all (e.g. Refs. [8–10]). However, during the development of an antiserum against 9-desglycinamide-8-arginine vasopressin an increase in specific antibody levels was demonstrated for up to 107 days following immunisation in rabbits [11]. This prolonged increase in antibody levels is in marked contrast to the data reported here and it further demonstrates the need to systematically monitor antibody production in order to maximise the yield of antisera. Predicting the time course of antibody production however is likely to be compound-, immunogen- and species-dependent.

Conjugation reactions with Bolton and Hunter reagent are usually carried out in borate buffer (0.1 M, pH 8.6) [12]. However, meropenem is unstable in borate buffer, with a degradation half life of approximately 10 min at 37°C. Therefore, the reaction was carried out using 4-methyl morpholine, a non-nucleophilic base, which was sufficiently basic to catalyse the reaction without significantly degrading meropenem to ZM 213,689.

The instability of meropenem was minimised in the assay by use of a short incubation time (1 h) and by storing standards and samples at 4°C during sample preparation and prior to analysis.

The limit of detection was set at 0.08 ml l^{-1} which is high for an RIA employing a high specific activity ^{125}I-radiotracer (2200 Ci $mmol^{-1}$). This reflected the very high ZM 213,689 concentrations in plasma, which were higher than originally predicted, and the desire to avoid extensive sample dilutions.

The RIA procedure detailed here has been successfully used in several studies to determine the disposition of ZM 213,689 following intravenous infusion of meropenem in healthy volunteers [13] and renally impaired patients [14]. In healthy volunteers given an infusion of meropenem (0.5 g over 30 min [13]), the ZM 213,689 plasma concentrations were generally found to be ten times less than those of meropenem. Consequently, despite the low cross-reactivity (0.4%), which may in part be due to residual ZM 213,689 in the bulk meropenem, there will be a small overestimation of approximately 4% in the ZM 213,689 concentrations in this situation. However, with renally impaired patients, the ratio of ZM 213,689 to meropenem plasma concentrations increases, such that any meropenem has a negligible contribution to the measured concentration of ZM 213,689.

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