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Novel use of accelerator mass spectrometry for the quantification of low levels of systemic therapeutic recombinant protein

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Abstract

Although ^{14}C-labelling has been routinely used for small molecules, this technique is not routinely applied to therapeutic proteins due to difficulties of incorporating the label into the protein to a sufficiently high specific activity. An analytical method known as accelerator mass spectrometry (AMS) offers an extremely sensitive method of ^{14}C quantification, thereby enabling ^{14}C-labeling methods to be applied to therapeutic protein detection. The therapeutic protein CAT-192 (metelimumab), a human anti-TGFß1 monocloncal antibody was manufactured in the presence of ^{14}C-precursors resulting in a low specific activity product (1.4% ^{14}C incorporation). [^{14}C]-CAT-192 was administered to rats (1 mg/kg and 222, 22 and 2.2 dpm/kg) and serum samples were collected. ^{14}C in serum samples from the 2.2 dpm dosing was not detectable but samples from the 22 and 2220 dpm doses were measured by AMS and by ELISA for comparison. By both ELISA and AMS bioassay, the half-lives approximated 140 h (S.E.M. 15 h). The estimates of clearance were also comparable, 7.3 and 4.6×10^{-4} ml/h/g (S.E.M. 6.6 and 5.1×10^{-5}) for ELISA and AMS, respectively. The estimated limit of quantification (LOQ) was approximately 1 ng/ml, about 15 times lower than the ELISA LOQ of 15.6 ng/ml.

Keywords: Accelerator mass spectrometry; Therapeutic protein; ELISA; Anti-TGFß1 monocloncal antibody; Radiolabelled protein

1. Introduction

In the United States, 70% of new biopharmaceuticals approved in 2000–2002 were recombinant proteins [1]. The majority of these biopharmaceuticals were recombinant versions of well-understood proteins. More recent developments, however, are of less well-known proteins and consequently clinical development times are extending [1]. An increasing number of new developments are being designed with altered pharmacokinetics [2] and so a good understanding of the metabolic fate of biopharmaceutical proteins in humans is becoming increasingly important.

Recombinant proteins are sometimes identical or so closely related to naturally-occurring proteins that it is very difficult to distinguish them analytically. Even if the protein is sufficiently different from those occurring naturally, developing suitable assay techniques can be challenging. LC–MS methods have been used [3] but other techniques such as immunoassays [4] are still required in many cases. Immunoassays however, suffer from a lack of specificity and sensitivity. Iodination of proteins with ^{125}I alongside standard radiotracer methodologies have been used with some success, particularly with recombinant versions of naturally-occurring proteins [5]. ^{125}I however, is a γ-emitter and so administration to humans is problematic. When developing a small molecule (New Chemical Entity-NCE) metabolism and pharmacokinetic studies are routinely performed using a drug synthesised with an enriched level of ^{14}C. The radioactivity emitted from the ^{14}C label is then used to trace the fate of the compound in laboratory animals or in humans. Such an approach with therapeutic proteins however, is more difficult than with NCEs. Whilst it is possible to chemically synthesise a radiolabelled NCE to high specific activity, this is not so easy with recombinant proteins, which are produced from living systems such as fermentation [6] or possibly whole plants [7]. Feeding a living system with ^{14}C-precursor rarely results in a product with a specific activity comparable with that made by a synthetic chemist. The difficulty with using relatively low levels of radioactivity in recombinant protein radiotracer studies is one

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of detection. The routine method of detecting ^{14}C is liquid scintillation counting. Energy released during radioactive decay is transferred to a fluor, which then emits photons of light. Since ^{14}C has a half-life of 5730 years, the rate of radioactive decay is such that approximately 4.3×10^9 atoms of ^{14}C are required to generate one disintegration per minute [8].

Accelerator mass spectroscopy (AMS), developed in the 1970's as a carbon dating technique for use in archaeology separates and counts atoms of ^{12}C, ^{13}C and ^{14}C. Detection therefore does not rely on relatively infrequent decay events. AMS can therefore measure a fraction of one dpm and is at least a thousand times more sensitive that liquid scintillation counting [9]. The use of AMS therefore opens up the possibility of administering very low levels of ^{14}C-labelled protein to humans, thus minimising radioactive exposure, whilst having sufficient analytical sensitivity to overcome the use of low specific activities.

AMS has been previously used to detect very low levels of ^{14}C-compound in humans [8] but there are no reports of it being used with respect to recombinant proteins. The present study constitutes an investigation into the use of AMS for the detection and quantification of therapeutic protein (CAT-192) in serum. Since this is the first time such an approach has been attempted, the experiments were conducted in the laboratory rat, rather than in humans.

CAT-192 (metelimumab) is a human anti-TGFß1 monocloncal antibody. Transforming Growth Factor beta-1 protein (TGFß1) plays an important role in biological processes such as immune surveillance and wound healing. ^{14}C-CAT-192 was administered to three groups of four rats. The dose level was kept constant at 1 mg/kg and the radioactive dose was altered to achieve doses of 3.7, 0.37 and 0.037 Bq/kg (222, 22 and 2.2 dpm, respectively). It should be noted for comparison, that a typical radioactive dose in a rat study with a NCE is around 1–2 MBq/kg. Blood serum samples were taken periodically after dosing and analysed by AMS and ELISA.

2. Methods

2.1. Preparation of radiolabelled CAT-192

A 10% volume spike (370 kBq) of ^{14}C labelled amino acids (Amersham-Pharmacia, UK, at 1.85 MBq/ml) was prepared in cell culture media and 0.2 μm filtered. The CAT-192 producing cell line was diluted 1:2 into media containing the ^{14}C amino acids at approximately 2×10^5 cells/ml, into two, T-175 flasks. Five percent carbon dioxide in air was bubbled into each flask, which were sealed and left static in a 36.5 °C room for 2 weeks, at which point the cell viability was approximately 70%.

Supernatant (160 ml) was clarified using 0.45 μm syringe filters and loaded onto five, 1 ml HiTrap protein A columns (Amersham-Pharmacia, UK) pre-equilibrated with 10 ml of 50 mM glycine, 250 Mm NaCl pH8.0 buffer. The HiTraps were washed with 10 ml of the glycine buffer that had been used for pre-equilibration followed by 1 ml of 0.1 M citrate pH 3.75. CAT-192 was eluted from each column with 3 ml of the citrate buffer, and was collected into tubes containing 300 μL of 1 M Tris–HCl, 1.5 M NaCl, 1 Mm EDTA, pH 8.0 to neutralise the citrate buffer.

The product was dialysed against 1 l of PBS for 60 h using a 3500 MWCO slide-a-lyzer cassette (Pierce, UK) at 4 °C. The sample was recovered and the concentration determined as 0.669 mg/ml on an HP8453 UV/vis spectrophotometer (Agilent Technologies, UK) at 280 nm and 340 nm subtraction. The ^{14}C-CAT-192 was characterised using SEC HPLC, and shown to be mainly intact 150 kDa immunoglobulin (there was a little breakdown present but no aggregation), and by reduced SDS-PAGE which confirmed the results obtained by UV/vis.

Forty microliters of the ^{14}C-CAT-192 was added to 160 μl of microscint, in duplicate in a 96 well microtiter plate, and counted on a Packard TopCount liquid scintillation counter with 92% efficiency, for 5 min. The average counts per minute were 561.75 dpm, which equated to 251.6 Bq/ml. Based on a spike of 370 KBq of ^{14}C amino acids, there was a 1.4% incorporation of the ^{14}C into the CAT-192, at a specific activity of 370 Bq/mg, equal to 55.5 MBq/mmole of CAT-192.

2.2. Animals, dosing and sample collection

All experiments were conducted in the UK in accordance with the Animals (Scientific Procedures) Act 1986. ^{14}C labelled CAT-192 was mixed with non-radiolabelled CAT-192 so that the rats could be dosed with 1 mg/kg CAT-192, at decreasing exposure to ^{14}C: group A (animals 1–4), 1.98 mg of CAT-192 was mixed with 20 μg of ^{14}C-CAT-192 and diluted with PBS to give 2 ml of material at 1 mg/ml CAT-192 at 3.7 Bq ^{14}C/mg. Group B (animals 5–8), 200 μg of the first formulation was added to 1.8 mg of CAT-192 and mixed with PBS to give 2 ml at 1 mg/ml CAT-192 and 0.37 Bq ^{14}C/mg. This second formulation was diluted in the same way as the first to give 1 mg/ml CAT-192 at 0.037 Bq ^{14}C/mg (group C, animals 9–12). All three stocks were 0.2 μm filtered (it has previously been determined that 0.2 μm filtration did not cause a loss of material) into autoclaved glass vials and sealed, in a class II containment cabinet. The material was frozen at −80 °C prior to shipping to the testing laboratory.

Twelve male Sprague–Dawley CD strain rats (purchased from Charles River UK Ltd.) were used for the study. The rats were housed in controlled environment facilities during the study, were of bodyweight range 324–363 g and aged about 8–11 weeks at the time of dose administration.

Single intravenous doses of ^{14}C-CAT-192 were administered (four rats per dose formulation) at a nominal dose level of 1 mg/kg. All doses were administered by bolus injection into the left caudal vein at a dose volume of 1 ml/kg. The radioactive dose administered to each rat was quantified from the volume of formulation given and its quoted radiochemical concentration. Blood samples (500 μl) were collected from the right caudal vein of each rat into plain microcentrifuge tubes at pre-dose and at each of the following times post dose: 0.5, 24, 48, 96 and 192 h. Samples were placed in a refrigerator until clotted (ca. 10 min) and centrifuged for 15 min at a speed of 3000 rpm to separate the serum. The serum was subsequently divided into two fresh microcentrifuge tubes and the clot discarded. All samples were stored at −20 °C prior to shipment and analysis.

2.3. ELISA assay

Samples from animals 1–3 (group A) 5–7 (group B) were analysed by a conventional sandwich ELISA. Briefly, Goat anti-human Fc (Jackson Immunoresearch, PA, USA) was immobilised onto 96 well Maxisorp microtiter plates (Nunc, NY, USA). After blocking with 2% Marvel-PBS, standards, sample dilution series and controls were incubated in duplicate and bound CAT-192 was detected with Sheep anti human κ-HRP (The Binding Site, UK). Bound antibody was visualised with TMB substrate (Sigma–Aldrich, UK). After stopping the reaction with 2 M H_2SO_4 the substrate was measured by UV absorption at 450, with 540 nm background correction on an MRX plate reader (Dynex Technologies, UK). Sample concentrations were determined by back calculation off the standard curve using proprietary software. The CAT-192 standard curve (Lin, Log) is prepared from a doubling dilution series from 2000 to 2 ng/ml, (linear from 500 to 15.6 ng/ml). The assay LOQ was 15.6 ng/ml.

2.4. Carbon content

The carbon content of each serum sample was measured on a C,H,N NA2100 Brewanalyser using 15 μl aliquots adsorbed onto Chromosorb W in a tin capsules. A calibration curve was obtained using urea standards (100 mg/ml).

2.5. Sample preparation for AMS analysis

Serum (40 μl) was placed into sample tubes together with 50 mg CuO. Australian National University (ANU) sugar (5–7 mg) with a precisely known ^{12}C:^{13}C:^{14}C ratio and synthetic graphite (2–3 mg) as a negative control were placed in separate sample tubes with CuO. All samples were dried and heat-sealed under vacuum and graphitised for AMS analysis as described in [10]. AMS analysis was as described by Sarapa et al. [11].

2.6. Calculation of disintegrations per minute (dpm) from AMS data

AMS provides an isotope ratio (^{12}C:^{14}C) expressed as percent Modern Carbon, where 100 pMC equals 13.56 dpm/g C Thus, pMC × 0.1356 = dpm ^{14}C/g C and (dpm ^{14}C/g C) × (% w/v C in sample) = dpm ^{14}C/ml. The percentage carbon in the sample was measured by C,H,N analysis (see above).

2.7. Pharmacokinetic considerations

Serum concentrations were estimated from sampling at 0, 0.5, 24, 48, 96 and 192 h after dosing with CAT-192 using both ELISA and AMS methods. The values were entered into a Microsoft Excel worksheet and the following calculated for each animal for both assay methods: maximum observed serum concentration (C_{max}), half-life ($t_{1/2}$), clearance (Cl) and apparent volume of distribution (V). The concentration time profile was visually inspected to identify terminal elimination phases, to calculate half-life, clearance and volume of distribution. Areas under the curve (AUC—for clearance only) were calculated by the linear trapezoidal method and the area to infinity calculated using the point on the regression line at the time of the last quantifiable specimen for the estimate of the concentration (Cz). Geometric means and coefficients of variation were calculated for the maximum observed serum concentrations, means and standard errors of the mean for other measures.

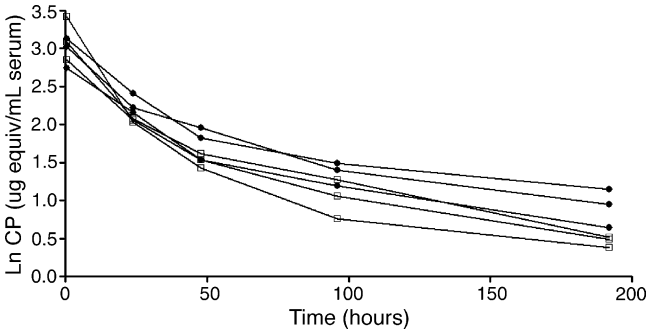

Fig. 1. Plot of Log CAT-192 concentration versus time as determined by ELISA. Group A (●) and group B (□) both dosed at 1 mg/kg but at different levels of radioactivity (see Fig. 2).

3. Results

All rats were administered 1 mg/kg CAT-192 but at 3 different levels of radioactivity; 3.7 Bq/kg (group A, animals 1–4), 0.37 Bq/kg (group B, animals 5–8) and 0.037 Bq/kg (group C, animals 9–12). Samples obtained from group C were below the limit of AMS detection and are therefore not considered further. For groups A and B the C_{max} was 21 μg/ml (CV 6%) and 25 μg/ml (CV 5%) for the ELISA and AMS methods, respectively, with the t_{max} always being at the first sampling time-point (30 min) following administration.

The profiles obtained with ELISA did not provide definitive terminal phases because the logarithm of concentration did not decline at a regular rate over the time of the last three samplings (Fig. 1). The gradient between the last two sampling times (96 and 192 h post-dose) were taken to represent the terminal elimination rate constant, but this procedure would be expected to overestimate the clearance and underestimate the half-life and apparent volume of distribution.

In contrast, the logarithmic concentration time profiles obtained from AMS analysis attained an identifiable terminal elimination phase. For every profile, three or more points appeared on the terminal phase, with a mean coefficient of determination of 0.84. For animal 8, there was rapid elimination of CAT-192 from the time of infusion, consistent with a different mechanism of clearance in this animal (Fig. 2). No samples from this animal were assayed by ELISA. By AMS analysis, the earlier attainment of the terminal phase, associated with a terminal elimination that is comparable to that obtained from ELISA results in a smaller apparent volume of distribution. Calculated from ELISA and AMS, respectively, the apparent volumes of distribution were 0.19 and 0.09 ml/g (S.E.M. 0.027 and 0.008) (Fig. 2).

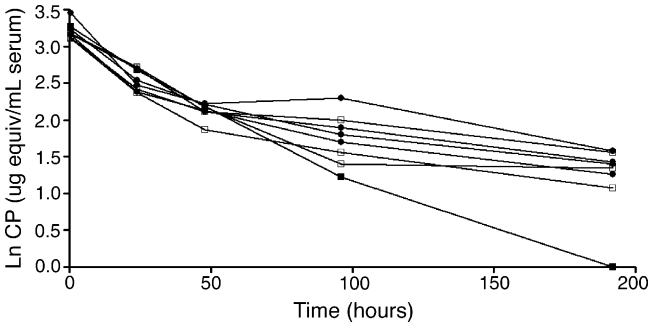

Fig. 2. Plot of Log CAT-192 concentration versus time as determined by AMS. Group A, 222 dpm/kg dosed (●) and group B (animals 5–7 (□) and animal 8 (■)) 22 dpm/kg dosed.

The LOQ for AMS is determined by the signal to noise, the noise being background ^{14}C. The natural abundance of ^{14}C is around 10^{-11}%, and AMS, used as described here, is capable of quantifying about 10% ^{14}C above this background [8]. For serum this equates to approximately 0.02 dpm/ml (not previously reported). In the current study therefore, for the highest radioactivity level dosed in group A (1 mg CAT-192 = 222 dpm) the LOQ was 100 ng/ml serum. The dose given to group A however, had been diluted 100 fold from the original [^{14}C]-CAT-192, manufactured from the culture. Thus if the undiluted [^{14}C]-CAT-192 had been administered, the LOQ would have been 1 ng/ml serum.

By both ELISA and AMS bioassay, the half-lives approximated 140 h (S.E.M. 15 h). The estimates of clearance were also comparable, 7.3 and 4.6×10^{-4} ml/h/g (S.E.M. 6.6 and 5.1×10^{-5}) for ELISA and AMS, respectively, although the mean amount of the AUC that was extrapolated for these estimates were 29 and 38%.

4. Discussion

The profiles provided by AMS attained a terminal elimination phase in every animal by 48 h, allowing full characterisation of pharmacokinetic values but these may not reflect the behaviour of the parent antibody. ELISA detects intact antibody (heavy and light chain present) but AMS detects denatured antibody, antibody fragments and metabolites. The difference in the concentration time profiles may reflect the differences in the chemical species detected by these two methods. In addition, the unusual profile for animal 8 (Fig. 2) as assessed by AMS may reflect some abnormality of the antibody injected since elimination is rapid and starts immediately. It should also be noted that the AMS measurements were made using whole serum samples. It is possible to separate parent protein using routine chromatographic methods such as HPLC or electrophoresis, and then determine the drug concentration by AMS. In so doing, background carbon is reduced and the LOQ is also reduced proportionally.

Further experiments are needed to validate the AMS method of in vivo bioassay for monoclonal antibody. These experiments would include comparison of AMS with evidence of biological activity of the parent, as well as dosing in sufficient amounts for detection by traditional assay methods and sampling for periods adequate for full characterisation of the profile.

An advantage of the current study design in particular and radiotracer studies in general, is that the radiotracer (^{14}C in this case) provides a means of universal detection and quantification. This is unlike, for example, LC–MS, where because of varying ionisation efficiencies, quantification is only possible by comparison with an authentic standard. Such an approach is routine with NCEs but for the reasons explained in the Introduction, such methodology is unusual with therapeutic proteins.

The results of the current study are preliminary and were designed to investigate the feasibility of the methodology. Nevertheless, although the incorporation rate of ^{14}C into the antibody was only 1.4%, it was possible to detect the ^{14}C tracer in rat serum after dosing only 0.37 Bq. Moreover, the theoretical LOQ was 1 ng/ml serum, which was approximately 15 times lower than the ELISA LOQ of 15.6 ng/ml.

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