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A validation study comparing accelerator MS and liquid scintillation counting for analysis of ^{14}C-labelled drugs in plasma, urine and faecal extracts

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Abstract

A comparison has been made between accelerator mass spectrometry (AMS) analysis and liquid scintillation counting (LSC) of plasma, urine and faecal samples containing ^{14}C-labelled drugs. In an in vitro study in which human plasma was spiked (the term spiked is used in Section 2.6) with ^{14}C-Fluconazole (^{14}C-FL) over a concentration range of 0.1–2.5 dpm/ml, a correlation coefficient of 0.999 was determined for AMS analysis versus extrapolated LSC data. No significant day to day (or inter-day)variation was seen ($P < 0.05$ by ANOVA). Coefficients of variation for these analyses ranged from 2.68 to 6.50%. In vivo studies in which rats were given a high (11.5 μCi/kg) or low (18.1 nCi/kg) radioactive dose (to model an exposure of 0.9 μSievert to man) of ^{14}C-Fluticasone propionate(^{14}C-FP) showed that there was also a good correspondence between AMS and LSC data. A mass balance study in a single rat given the 0.9 μSievert human modelling dose of ^{14}C-FP demonstrated that over 80% of the dose was excreted in the faeces by 96 h; less than 1% of the administered dose was excreted in the urine. The limit of reliable measurement of drug related material, above background concentrations, by AMS analysis in this study was approximately 0.1 dpm/ml for plasma, 0.01 dpm/ml for urine without any sample extraction or concentration and 0.01 dpm/ml for faecal extracts. The data reported here demonstrate that AMS is an ultrasensitive and reliable method for analysing ^{14}C-labelled drugs in human and animal body fluids.

Keywords: Accelerator mass spectrometry; Low dose radioactive studies; Drug metabolism; Mass balance

1. Introduction

^{14}C-Labelled drugs are widely used in the pharmaceutical industry to study their absorption, distribution, metabolism and excretion (ADME) in

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both animals and humans to establish pharmacokinetic and metabolism parameters. This information is of use in determining the metabolic fate of the drug and in confirming the validity of the animal species used in toxicology studies.. ^{14}C is the radiolabel of choice because of its stability once chemically incorporated into a drug molecule. ^{14}C has been measured in biomedical research for nearly 50 years by decay counting, primarily liquid scintillation counting (LSC) by virtue of the fact that this isotope is a low energy β-emitter [1]. The use of ^{14}C however has a number of practical limitations due to the long half-life of this isotope (5740 years). In order to have relatively short sample counting times very many ^{14}C atoms must be present in the sample, typically of the order of 5–10 picomoles ^{14}C/ml of plasma or urine [2]. For drugs that are administered to humans at microgram doses or where only low specific activity drugs can be prepared such as biologics, analysts often struggle to develop sensitive detection methods to measure for example plasma concentrations of radioactivity.

In the mid-1970s, two groups published independently of each other, a technique called accelerator mass spectrometry (AMS), to measure radiocarbon (^{14}C) in order to date archaeological samples [3,4]. AMS uses a tandem Van de Graaff accelerator to provide the potential energy to strip off the outer valency electrons of negatively ionised atoms in order to generate positively ionised atoms which can be separated post-acceleration using conventional mass spectrometric techniques. For AMS measurement of carbon, the three isotopic species viz ^{14}C, ^{13}C and ^{12}C are separated from each other by virtue of their differing energies and mass/charge ratio for each isotopic species. AMS counts individual atoms in a sample rather than radioactive decay events and is therefore the most sensitive analytical technique yet devised (AMS is comprehensively reviewed in [5]).

In 1990 the first paper in which AMS was used to measure ^{14}C in biological samples was published [6]. Since that time a number of biological AMS papers have been published focussing primarily on the use of AMS in toxicology or cancer research [7–13]. The use of AMS in pharmaceutical research has been discussed extensively but little original data has been published, except for a short communication demonstrating the feasibility of using AMS for drug measurement [14].

We report here an extensive validation study on a laboratory comparison of AMS versus liquid scintillation counting (LSC) which provides assurance that the much enhanced sensitivity of AMS can be used to detect and quantitate much lower levels of ^{14}C compared with LSC methods. The AMS and LSC results were obtained from the following types of sample (a) human plasma fortified with varying amounts of ^{14}C-Fluconazole (^{14}C-FL), an anti-fungal agent in general medical use and (b) rat plasma, urine and faecal extracts obtained after dosing animals with high and low radioactive doses of the drug ^{14}C-Fluticasone propionate in general medical use for the treatment of asthma and rhinitis (^{14}C-FP-target dose 100 μg/kg for both groups). In addition to studying the precision of analysing replicate samples, day to day variability was also examined using randomly mixed samples processed as batches on separate days.

2. Materials and methods

2.1. Chemicals

Two batches of ^{14}C-FP with specific activities of 119 μCi / mg and 0.169 μCi / mg were prepared by Glaxo Wellcome Isotope Chemistry Department. The radiochemical and chemical purity of both batches of ^{14}C-FP was $>99\%$. Polyethylene glycol (PEG) 400 was supplied by ICN Biomedicals Ltd, Thame, Oxfordshire. Dimethyl sulfoxide (DMSO), was supplied by Sigma-Aldrich Company Ltd, Poole, Dorset. 0.9% (w/v). Saline for injection was obtained from Fresenius Ltd, Basingstoke, Hants. Heparinised blood collection tubes (10 ml) were supplied by LIP Ltd, Shipley, Yorkshire. STARSCINT™ liquid scintillation cocktail was purchased from Packard Bioscience, Pangbourne, Berkshire. Copper oxide wire (ACS) and cobalt powder (100 Mesh, 99.9%) from Aldrich Chemical Co were pre-baked at 500°C for 2–4 h before use. Zinc powder (100 Mesh 98.98%)

and titanium (II) hydride (325 Mesh, 98%) were also obtained from Aldrich Chemical Co. Graphitisation tubes, sample tubes, combustion tubes and culture tubes were all obtained from York Glassware Services, York and were all pre-baked at 500°C for 2–4 h before use. Aluminium cathodes were obtained from National Electrostatics Corp, Middleton, WI.

All solvents used were of either Analytical Reagent or HPLC grade.

2.2. AMS standards, controls and blanks

ANU sucrose was kindly provided by The Quaternary Dating Research Centre, Australian National University, Canberra, Australia and stored at room temperature prior to use. The certificated $^{14}C/^{12}C$ ratio for ANU sucrose provided was 1.5061 Times Modern where 1.0 Times Modern = 97.7 attomoles ^{14}C/mg C.

For each AMS run, in addition to cathodes containing graphitised samples, cathodes containing standards or blanks as shown in Table 1 were included.

Synthetic graphite (200 Mesh, 99.9999%, Alfa Aestar) was obtained from Johnson Matthey PLC, Royston, Hertfordshire and stored at room temperature prior to use. Poco graphite rod (1 mm diam) was obtained from National Electrostatics Corp.

Table 1
Standards, controls and blanks used for each AMS run

Cathode type	Purpose	Number used per AMS sample wheel
Cobalt	Process blank	1
POCO graphite[a]	^{14}C-depleted for instrument tuning	1
Pooled ANU sugar[b]	Standard normalisation	3
ANU sugar	Process control	3
Synthetic graphite	Machine blank	2
Synthetic graphite	Process control	2

[a] POCO, graphite rod.
[b] ANU, Australian National University.

2.3. Sample preparation for AMS analysis

In order to measure the ^{14}C content of samples by AMS, the carbon in the sample must first be converted to graphite. This was done according to the procedure of Vogel [15] and is summarised here for information.

2.3.1. Preparation of samples and standards

Samples, typically 60 μl for plasma and 100 μl for urine were placed in clean, baked quartz glass sample tubes together with pre-baked copper oxide wire (50 ± 10 mg) and the whole dried under vacuum using a Savant AES2010 Speed Vac for 30 min. The final carbon amount used for graphitisation was approximately 2 mg. For samples that had minimal carbon content eg methanolic faecal extracts, 100 μl of tributyrin (20 mg/ml in methanol) was added as a carrier. In addition, ANU sucrose (5–7 mg) or synthetic graphite (2–3 mg) were weighed out in separate sample tubes and copper oxide added.

2.3.2. Combustion (sample oxidation)

The quartz glass sample tube containing the dried sample and copper oxide was placed into a larger quartz glass combustion tube, which was heat-sealed under vacuum and heated at 900°C for 2 h in a Carbolite furnace. After combustion, the tubes were allowed to cool slowly to ambient temperature.

2.3.3. Graphitisation (sample reduction)

The pointed end of the larger combustion tube was placed in a plastic Y-manifold to the other arm of which was attached a silica glass graphitisation tube. The latter tube contained titanium hydride (15 ± 5 mg) and zinc powder (125 ± 25 mg). Inside the graphitisation tube was a culture tube containing cobalt powder (6.5 ± 0.5 mg). The combustion tube was dipped into an isopropanol/dry ice bath and the graphitisation tube into a bath of liquid nitrogen. The whole system was placed under vacuum and the carbon dioxide formed from the oxidised sample cryogenically transferred to the graphitisation tube by breaking the combustion tube tip. Once transferred, the graphitisation tube was heat-sealed under vacuum

and placed in a furnace and heated at 500°C for 4 h, followed by a further 6 h heating at 550°C before slow cooling to ambient temperature.

2.3.4. Packing of aluminium cathodes with graphite

Once the graphitisation process had been completed, the graphite was left in the sealed graphitisation tube until ready to be packed into a cathode. To pack the cathode, the graphitisation tube was opened and the culture tube containing the graphite adsorbed onto the cobalt catalyst removed. The cobalt/graphite was carefully tipped out into an aluminium cathode and compressed into place at 170–200 psi in a Parr Pellet Press to form a tablet of graphite within the cathode. After every pressing, the press plunger was cleaned by wiping it with tissue moistened with acetone. The pressed cathodes were then individually placed into labelled plastic capped tubes for storage at room temperature. When required for analysis, the samples were placed into a 134-position AMS sample wheel and the sample wheel stored under vacuum.

2.4. AMS analysis

The AMS sample wheel into which the graphite-containing cathodes were placed, was inserted into the ion source of the AMS instrument (5 MV 15SDH-2 Pelletron AMS system, National Electrostatics Corp.). The multi-cathode negative ion source (MC-SNICS) generated a caesium (Cs^+) ion beam that was accelerated onto each cathode's graphite surface in sequence. The resulting negative carbon ion beam contained $^{12}C^-$, $^{13}C^-$ and $^{14}C^-$ and other ions such as $^{16}O^-$. The isobar $^{14}N^-$ is unstable and therefore cannot interfere with the ^{14}C measurement.

The carbon ion beam was pre-accelerated, passed through a spherical electrostatic analyser and then progressed towards the injection magnet. Output of $^{12}C^-$ was typically 1–100 μA. The magnet was set to inject $^{12}C^-$(100 μs), $^{13}C^-$ (400 μs) and $^{14}C^-$ (0.1 s) ions sequentially at 68 keV; one combined measurement on each isotope in turn corresponded to one cycle. The carbon ion beam was accelerated towards the positive centre terminal of the tandem Pelletron accelerator through an Einzel lens. The terminal voltage used for this series of analyses was 4.5 MV with a particle energy of approximately 22.5 MeV. At the central terminal electrons were stripped from the carbon atom in an argon gas collision cell to yield positively charged carbon ions ($^{12,13,14}C^{+1-+6}$). C^{4+} ions were selected for measurement as these were the most abundant at this energy. These ions were accelerated away from the positive centre terminal and onwards towards the electrostatic quadrupole triplet and analysing magnet.

Immediately past the post-analysing magnet, $^{12}C^{4+}$ and $^{13}C^{4+}$ ions were measured as an ion current in offset Faraday cups. $^{14}C^{4+}$ Ions were passed down the high energy beam line, through an electrostatic quadrupole doublet and a cylindrical electrostatic analyser. From here, the positive ions entered a gas ionisation detector containing 10% v/v methane in argon where they were collected on anodes (four in total) which measured the energy loss and total energy of each ion. Other interfering non-$^{14}C^{4+}$ ions were generally prevented from entering the gas ionisation detector by the combinations of electrostatic analysers, magnets, slits and charge state separation. Vacuum pressures of approximately 10^{-9} Torr were maintained in the beam line and 10^{-6} Torr in the ion source. Ion transmission through the instrument was between 30–60%.

Overall time for analysis of each sample was between 5 and 12 min consisting of a burn-in time of 600 cycles (60.4 s) and sample analysis of either 1000 or 2500 cycles (100.7 or 252 s) conducted a minimum of three times and a maximum of five.

2.5. AMS acceptance criteria and calculations

For an AMS analysis result to be accepted the following criteria were applied;

1. the $[^{14}C]/[^{13}C]$ ratio for the international standard ANU sucrose which was run concurrently with the samples fell within $\pm 15\%$ of the accredited value which is 1.5061 Times Modern;
2. the pre-dose values lay within $\pm 20\%$ of Contemporary material, i.e. 1.16 Times Modern;

3. at least 1000 ^{14}C counts per run were collected in the ion detector; (samples with low (≤ 1 μA) or no beam current were rejected);
4. at least three measurements were made on each sample, where coefficients of variation for three concurrent $^{12}C/^{13}C$ ratios of less than 5% only were accepted.

In order to convert the AMS result to dpm / ml the following calculations were conducted. The AMS results were expressed as Fraction or Percentage Modern carbon (pMC), where Modern equals:

13.56 dpm/g C

or 0.01356 dpm/mg C

or 97.7 femtomole ^{14}C/g C (1 femtomole = 10^{-15} Mole)

or 97.7 attomole ^{14}C/mg C (1 attomole = 10^{-18} Mole)

Thus, pMC × 0.1356 = dpm ^{14}C/g C

and (dpm ^{14}C/g C) × (% w/v C in sample) = dpm ^{14}C/ml

assuming the density of the original biological sample to be 1 g/ml.

The $[^{14}C]/[^{13}C]$ ratio of a sample

$$= \frac{\text{Total } [^{14}C] \text{ (drug related material + biological sample)}}{\text{Total } [^{13}C] \text{ (drug related material + biological sample)}}$$

Thus, AMS measures the $^{14}C/^{13}C$ content of a sample derived from all sources of carbon in the sample. In order to determine actual ^{14}C content, the total carbon content in the sample needs to be known. Plasma was assumed to contain 3.5% w/v carbon and urine 1.0% w/v. This was confirmed using a C,H,N analyser.

2.6. In vitro spiking of ^{14}C-FL into human plasma

A stock solution of ^{14}C-FL containing 5000 dpm/ml (10 mg FL/ml) in 1:1 methanol/water was diluted into human plasma obtained from a volunteer to give a 5 dpm / ml solution (10 μg FL/ml). This stock solution was further diluted to give a ^{14}C-FL concentration range for AMS analysis of 0, 0.1, 0.25, 0.5, 1.0 and 2.5 dpm / ml above background 14°C present in control plasma. Eighteen replicates were prepared for each concentration; these were randomised for analysis and split into three batches. Each batch was taken through the graphitisation process on separate days and analysed on separate days to investigate if there was any day to day (or inter-day) variability associated with the sample processing or the analysis.

2.7. Excretion balance and pharmacokinetic studies in rats.

Male Han Wistar Rats, weights ranging from 250–300 g, were supplied by Charles River UK Ltd., Manston Road, Margate, Kent. The animals were divided into two groups; Group 1 rats were administered ^{14}C-FP at a dosage of 11.5 μCi/kg and Group 2 were administered ^{14}C-FP at a dosage of 18.1 nCi/kg.

2.7.1. Treatment of Group 1 Rats

^{14}C-FP was formulated in a solution of DMSO: PEG400: 0.9% (w/v) saline for injection in the proportions 40:40:20. The dose purity was checked by radio-HPLC and analysed against known standards of FP. The final concentration of FP was determined to be 37.4 μg/ml (5.2 μCi/ml).

Fifteen male rats were administered ^{14}C-FP by the intravenous route and the mean dosage to each rat was 83.6 μg/kg of FP (11.5 μCi/kg). The rats were given access to food and water ad libitum, before and after dosing. Blood samples were collected by terminal exsanguination via cardiac puncture, into heparinised collection tubes from 13 of the rats, pre-dose and at the following nominal timepoints after dosing: 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. The blood samples were centrifuged at 2000 × *g* for 5 min to separate the plasma.

A further two rats were placed into metabolism cages suitable for the collection of urine and faeces for 1 day prior to dosing and for 96 h after dosing to allow collection of control urine and faeces samples and post-dose samples at the following timepoints: 0–6, 6–24, 24–48, 48–72 and 72–96 h. After collection, all samples were stored frozen prior to analysis.

2.7.2. Treatment of Group 2 rats

^{14}C-FP was formulated in a solution of DMSO: PEG400: 0.9% (w/v) saline for injection in the proportions 40:40:20. The dose purity was checked by radio-HPLC and analysed against known standards of FP. The final concentration of FP was determined to be 65.1 μg/ml (8.8 nCi/ml).

Fifteen male rats were administered ^{14}C-FP by the intravenous route and the mean dosage to each rat was 134 μg/kg of FP (18.1 nCi/kg). The rats were then treated as for the Group 1 rats described above.

A further two rats were used for metabolism studies and were also given 134 μg/kg of FP (18.1 nCi/kg) and then treated as for the Group 1 rats.

2.8. Liquid scintillation counting of samples prepared for AMS analysis

2.8.1. In vitro prepared plasma samples fortified with ^{14}C-FL

One hundred microlitres of ^{14}C-FL (in duplicate) were added to 4 ml of STARSCINT™ liquid scintillation cocktail in scintillation vials and these were counted for ^{14}C-activity for 5 min on a Wallac 1409 scintillation counter. Apparent dpm values were determined by internal standardisation.

2.8.2. Analysis of Group 1 rat samples

One millilitre of each plasma and urine sample was aliquoted into scintillation vials along with 10 ml of STARSCINT™ liquid scintillation cocktail and these were counted for ^{14}C-activity for 30 min on a Packard Tri-Carb 2700TR counter (Packard Instruments, Brook House, 14 Station Road, Pangbourne, Berkshire). Absolute ^{14}C values were determined by external standardisation using a previously constructed quench curve.

Each faeces sample was homogenised with 20 ml of methanol (HPLC grade). The homogenates were then centrifuged and the methanolic supernatant removed. Aliquots of the supernatant (100:1) were added to 900:1 of distilled water and 9ml of STARSCINT™ liquid scintillation cocktail and this was analysed on the Packard Tri-Carb 2700TR liquid scintillation counter (LSC), for ^{14}C-activity by counting for 30 min.

Aliquots of all plasma and urine samples were then diluted 11 900-fold by two stage dilution (10:1 into a total of 1 ml followed by serial dilution of 42:1 to 5 ml) using human control plasma and urine, respectively. Aliquots of each methanolic extract of faeces were diluted 11 900-fold by two stage dilution using methanol. Following LSC analysis to ensure that the ^{14}C-activity of each diluted sample was < 20 dpm/ml, the samples were stored prior to analysis by AMS.

2.8.3. Analysis of Group 2 rat samples

One hundred microlitres of each plasma and urine sample was aliquoted into a scintillation vial along with 10 ml of STARSCINT™ scintillation cocktail and counted for radioactivity on a LSC.

Each faeces sample was homogenised with 20 ml of methanol. The homogenates were then centrifuged and the methanolic supernatant removed. Aliquots of the supernatant (1 ml) were added to 9 ml of STARSCINT™, scintillation cocktail and this was counted by LSC for ^{14}C-activity.

Aliquots of each methanolic extract of faeces were diluted using methanol (100-fold for 6–24 h collection, tenfold for 24–48 h collection, remainder undiluted). Following LSC analysis to ensure that the radioactive (^{14}C) activity of each diluted faeces extract or undiluted plasma and urine sample was < 20 dpm/ml, the samples were stored prior to analysis by AMS.

3. Results

For a new analytical method to be accepted for routine use a validation study should be conducted in which the new method is compared with an existing method. In the case of ^{14}C analysis, the current method, which has been used for approximately 50 years, is LSC which relies on radioactive decay. AMS also measures ^{14}C, but it does so on the basis of the mass/charge and energy difference of this isotope compared with ^{13}C and ^{12}C. As AMS is so much more sensitive than LSC, it is difficult to conduct analyses on samples having the same radioactive content. The relatively high levels of radioactivity detected by

Table 2
Comparison of liquid scintillation counting and AMS analysis for ^{14}C-FL spiked plasma samples and controls for samples split into three batches[a]

Target/nominal concentration (dpm/ml)	Batch	AMS result (dpm/ml)
0	1	0.5961
0	1	0.5937
0	1	0.5857
0	1	0.5666
0	1	0.5878
0	1	0.5662
0	2	0.5387
0	2	0.5717
0	2	0.5713
0	2	0.5724
0	2	0.5723
0	2	0.5551
0	3	0.5319
0	3	0.5822
0	3	0.5537
0	3	0.5630
0	3	0.5463
0	3	0.5593
0.1	1	0.6618
0.1	1	0.6372
0.1	1	0.6669
0.1	1	0.6686
0.1	1	0.6659
0.1	1	0.6114
0.1	2	0.6696
0.1	2	0.6390
0.1	2	0.6441
0.1	2	0.6582
0.1	2	0.6371
0.1	2	0.6334
0.1	3	0.6445
0.1	3	0.6515
0.1	3	0.6493
0.1	3	0.6440
0.1	3	0.7881
0.1	3	0.6445
0.25	1	0.8175
0.25	1	0.8056
0.25	1	0.8058
0.25	1	0.8028
0.25	1	0.8227
0.25	2	0.8364
0.25	2	0.8040
0.25	2	0.7780
0.25	2	0.7731
0.25	2	0.7874
0.25	2	0.7502
0.25	3	0.8029
0.25	3	0.7751
0.25	3	0.7820
0.25	3	0.7778
0.25	3	0.8001
0.25	3	0.8211
0.5	1	0.9878
0.5	1	1.0686
0.5	1	1.1786
0.5	1	SL[b]
0.5	1	1.0695
0.5	1	1.0848
0.5	2	1.0463
0.5	2	0.9931
0.5	2	1.0440
0.5	2	1.0367
0.5	2	1.0348
0.5	2	1.0112
0.5	3	1.0413
0.5	3	1.0468
0.5	3	0.9917
0.5	3	1.0176
0.5	3	1.0352
0.5	3	1.0003
1	1	1.6127
1	1	1.5475
1	1	1.4386
1	1	1.5671
1	1	1.5472
1	2	1.5763
1	2	1.5733
1	2	1.4903
1	2	1.5020
1	2	1.7898
1	2	1.4080
1	3	1.4737
1	3	1.4905
1	3	1.4231
1	3	1.4818
1	3	1.6254
1	3	1.5365
2.5	1	3.2967
2.5	1	SL
2.5	1	2.9289
2.5	1	2.7634
2.5	1	3.0575
2.5	1	2.9537
2.5	2	2.7711
2.5	2	2.8978
2.5	2	2.7372
2.5	2	2.9393
2.5	2	2.7689
2.5	2	2.8836
2.5	3	3.4297
2.5	3	2.8133
2.5	3	2.8449
2.5	3	2.8667
2.5	3	3.1200
2.5	3	2.8652

[a] Batches 1, 2 and 3 were processed and analysed by AMS on separate days. Six samples were analysed per dose level. Where less than six values appear these samples were lost during processing.
[b] SL means sample lost during processing.

LSC can easily saturate the gas ionisation detector of an AMS leading to memory effects.

For the in vitro spiked ^{14}C-FL plasma study, the LSC results have been obtained from samples that contain radioactivity at too high a level for AMS analysis. The LSC values are nominal values obtained by extrapolation from the more active samples counted by LSC. In addition, it should be noted that there is a background contribution of ^{14}C from the matrix being analysed. This can be seen in Table 2 where the blank plasma samples contain approximately 0.5 dpm/ml of radioactivity arising from ingestion of food containing ^{14}C.

To calculate the dpm/ml attributable to ^{14}C-FL, the radioactive value of the blank plasma as determined by AMS must first be subtracted. Analysis of variance to compare each of the three batches at each spiking level, processed and analysed on different days showed no statistical difference ($P > 0.05$). Combining replicates from each batch and plotting the LSC data against the AMS data, gives a linear relationship between the two methods ($r^2 = 0.999$) (Fig. 1). Table 3 demonstrates the mean, SDs and CVs for the sample values shown in Table 2.

Having established the correlation between LSC and AMS data using spiked plasma samples, an in vivo study in rats using intravenous administration of ^{14}C-FP at high and low doses of radioactivity was conducted. The compound selected, Fluticasone propionate, is administered at low therapeutics doses and is poorly absorbed from the gastrointestinal tract. Hence plasma levels can sometimes be difficult to determine.

Fig. 2a–c shows the rat plasma, urine and faecal extract radioactivity respectively after the intravenous administration of 83.6 μg ^{14}C-FP/kg (11.5 μCi/kg) (Group 1). For the AMS analysis, the collected samples were diluted 11 900-fold whilst for the LSC analysis samples were counted undiluted. A 11 900-fold dilution of the radioactive dose would be equivalent to 0.965 nCi/kg and this was a convenient sensitivity test of the technique. All AMS data points are the mean of quadruplicate aliquots of each sample.

For comparison purposes, a further in vivo experiment was performed in rats in which the ^{14}C-FP administered dose was adjusted to give a radioactive dose equivalent to a human dose of 0.9 μSieverts adjusted for body weight. In Fig. 3 and Fig. 4 are shown the AMS analyses for rat plasma, urine and diluted faecal extracts from this dose level (equivalent to 18.1 nCi/kg, 134 μg ^{14}C-FP/kg). The data are expressed as either dpm/ml or ng ^{14}C-FP equivalents/ml. The majority of the administered dose for this drug is excreted in the faeces and very little in the urine. The peak urine radioactivity analysed here was no more than 1 dpm/ml. Comparing the results from this study with that shown in Fig. 2a shows that the plasma levels and excretion pattern are very similar even though the radioactive dosage administered in Group 2 was approximately 600-fold less than in Group 1.

4. Discussion

Radioisotope methods are used in mass balance studies, where it is desired to establish the rate and route of elimination of drugs from the body. For nearly 40 years, researchers have used LSC as the detection method of choice for these low energy β-emitters. ^{14}C is widely used for drug tracer studies because it can be stably incorporated into many organic drug molecules.

Accelerator mass spectrometry has been used for a wide range of scientific research [5]. Interestingly either because of cost or lack of knowledge of the methodology, AMS has not been widely used by the biomedical community. Pioneering work by a research group at the Lawrence Livermore National Laboratory, USA has demonstrated the usefulness of AMS particularly in toxicology studies and cancer research. Only a few papers have been published on the use of AMS in pharmaceutical research. AMS has considerable attractions over decay counting methods for the measurement of ^{14}C most notably the much-increased analytical sensitivity which can be up to 1 million-fold. AMS in contrast to LSC does not depend on radioactive decay of ^{14}C for a signal but on the mass difference between the three isotopes of carbon viz ^{14}C, ^{13}C and ^{12}C. Each of these is separated in the AMS by virtue of their

momentum, charge and energy. As a result isotope ratios can be measured down to 1 in 10^{16} making AMS the most sensitive analytical technique yet developed.

In order for AMS to be used in the drug development process it is necessary to validate the procedure against the currently used and accepted method, LSC. In this study we have examined a number of parameters.

4.1. AMS versus LSC for in vitro spiked samples

The sensitivity of AMS means that it is generally not possible to analyse the same sample by LSC and AMS. It is our experience that samples containing a maximum of 10–15 dpm/ml can be analysed by AMS whereas for LSC unless counting times are very long, this level of radioactivity cannot be accurately determined. For this reason it is necessary to take a stock solution containing relatively high levels of ^{14}C, measure the radioactive content by LSC and then dilute this stock solution down to levels which can be measured by AMS. The disadvantage of this approach is that pipetting inaccuracies could contribute to the overall imprecision of the analysis. Nevertheless as can be seen in Table 2 and Fig. 1, there is an excellent correlation between the two counting methods for the drug, ^{14}C-FL with a linear correlation coefficient of 0.999.

To examine possible day to day variation in processing or analysis, the in vitro ^{14}C-FL spiked plasma samples were randomly allocated to three separate batches and each batch was graphitised and analysed on different days. An analysis of variance was conducted for each concentration of drug processed in this way and no difference was found between any of the batch means ($P > 0.05$ for all batches and all concentrations) This provides re-assurance that sample batches can be split and processed separately. Statistical analysis of the in vitro spiked plasma shows small standard deviations and good coefficients of variation (Table 3). CV values of 15% or below are generally deemed satisfactory for an analytical method and AMS achieves this comfortably.

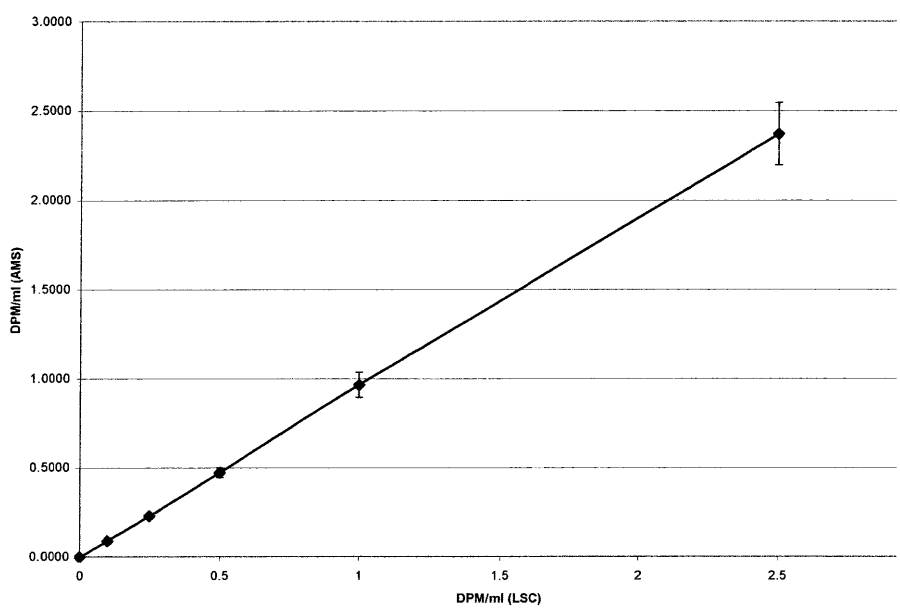

Fig. 1. Plot of radioactivity (Mean dpm/ml $\pm$ 1SD) determined by AMS against extrapolated LSC data from plasma samples fortified with ^{14}C-FL. Data plotted with plasma background substracted (data derived from Table 1).

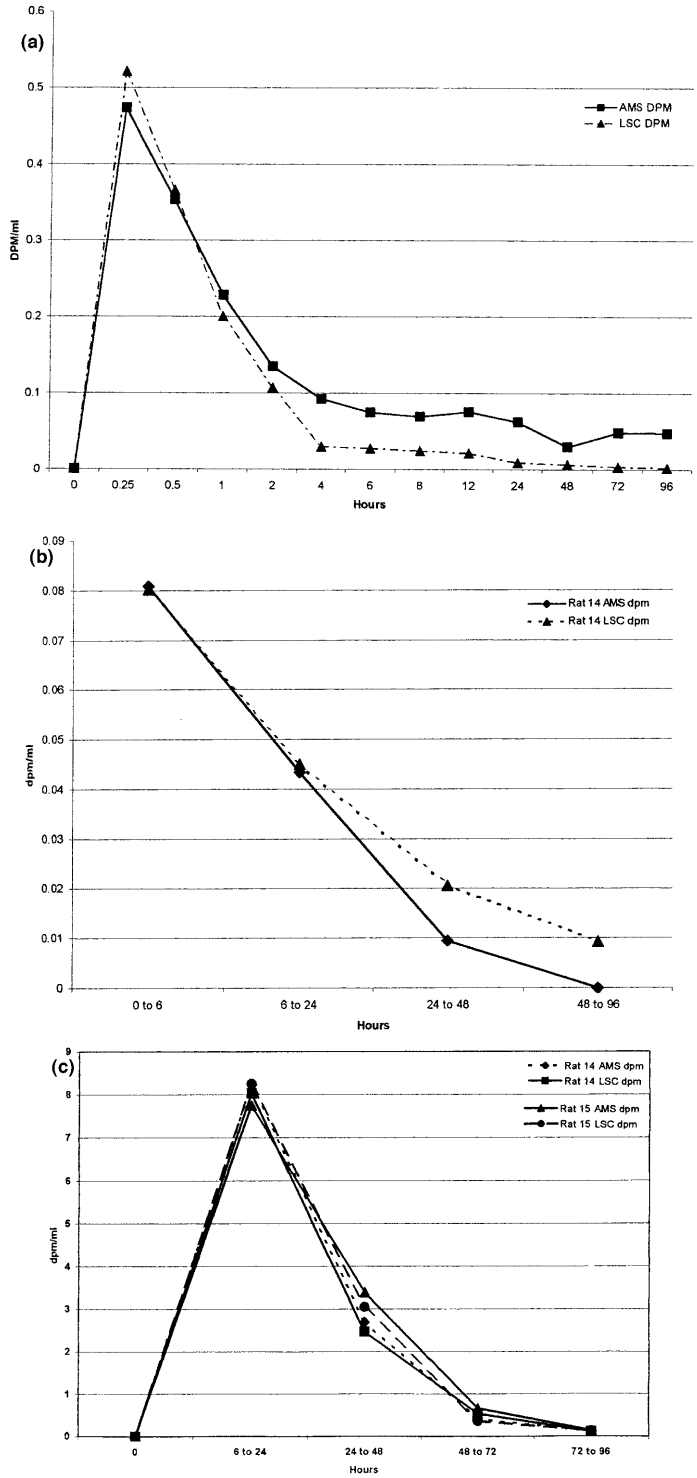

Fig. 2. Radioactivity comparison of AMS versus LSC for rat samples after dosing with 11.48 μCi/kg of ^{14}C-FP (83.6 μg/kg). AMS samples were diluted 11 900-fold for analysis whilst the LSC data is an extrapolation from the undiluted sample counts (a) plasma-composite profile from 13 rats; (b) urine, data from one rat only; (c) methanolic faecal extracts, data from two rats. AMS results are from quadruplicate analyses of the same sample.

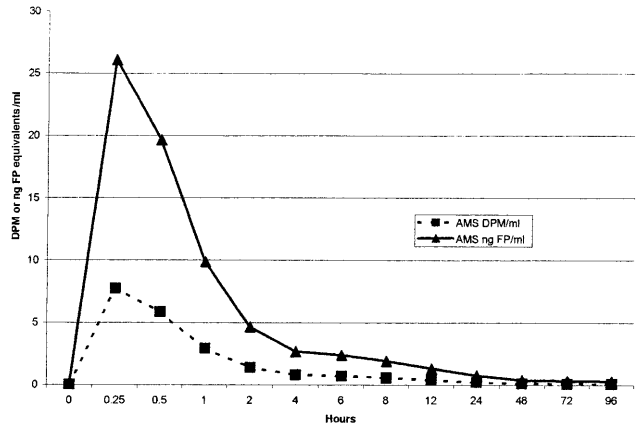

Fig. 3. Plasma time curve for rats dosed with 1 microSievert ^{14}C-FP (87.38 nanoCuries/kg, 134 μg/kg).

4.2. High radioactive dose in vivo rat studies with dilution of samples

In this series of experiments a high dose of ^{14}C-FP was given to rats and plasma, urine and faeces collected. Faecal pellets were extracted with methanol to recover the drug plus metabolites. Aliquots of all samples were analysed by LSC whereas a 11 900-fold dilution was made of each of the samples for the AMS analysis. This dilution is equivalent to dosing with 1 nCi/kg of labelled drug. A good correlation was again found between the LSC and the AMS results (see Fig. 2). In the case of the plasma analyses, the ^{14}C-FP drug concentration fell to a level that was lower than the ^{14}C-content of the plasma itself (0.28 dpm/ml).

4.3. Low radioactive dose in vivo rat studies

The UK Administration of Radioactive Substances Advisory Committee (ARSAC) require permission to be sought to administer radioactive drugs to humans. However there is an exemption

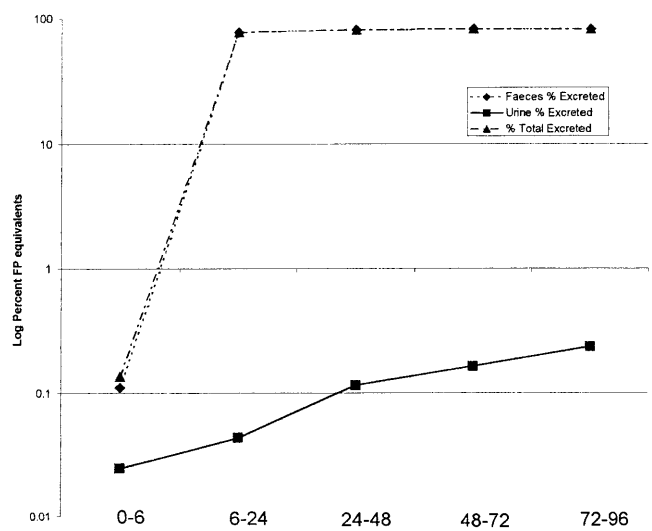

Fig. 4. Cumulative percentage of ^{14}C-FP equivalents excreted in urine and faeces from a single rat dosed with 1 μSievert.

Table 3
Mean, standard deviation and coefficient of variation for ^{14}C-FL spiked plasma samples and controls analysed by AMS[a]

Target or nominal concentration (dpm/ml)	Mean	SD	CV
0	0.567	0.018	3.177
0.1	0.656	0.036	5.501
0.25	0.797	0.021	2.686
0.5	1.041	0.045	4.380
1	1.533	0.089	5.791
2.5	2.938	0.191	6.507

[a] Note that the control plasma value (0 dpm) has not been subtracted.

criteria stating that for doses of 1.0 μSievert or below no application needs to be made. For this reason an in vivo rat study was conducted in which a dose equivalent to that which would produce a human exposure of 0.9 μSieverts ^{14}C-FP was administered, which for this drug was approximately 5.5 nCi/animal. The excretion pattern (Figs. 3 and 4) was similar to that seen in the high radioactive dose study (Fig. 2) as were the concentrations of drug in plasma. The levels of radioactivity in this experiment are too low to measure by liquid scintillation counting. The limit of detection of drug appears to be approximately 0.01 dpm/ml above background in urine without any concentration step.

This validation study represents the first comprehensive study in which AMS has been used for drug analysis and compared with LSC. The sensitivity difference between the two techniques makes it difficult to directly compare the two methods on the same samples. Nevertheless there is an excellent correspondence between diluted samples measured by AMS and more concentrated samples measured by LSC.

A number of previous publications have indicated the potential of AMS as an analytical technique in drug development. The work presented here shows that (a) it is possible to conduct low dose radioactive studies even in environments where high dose studies have been conducted previously (b) that levels of radioactivity as low as 0.1 dpm/ml for plasma and 0.01 dpm/ml for urine can be reliably measured, above background determinations, we have recently conducted studies in plasma and have detected 0.01 dpm/ml of drug in a plasma background of 0.47 dpm/ml (unpublished results) (c) that sample volumes of a few microlitres can be analysed (d) there are no inter-day variability effects in sample processing or analysis and (e) highly reproducible data can be obtained on replicates containing the same amounts of radioactivity.

In a recent paper comparing AMS versus LSC for quantitating the excretion of atrazine and its metabolites after dermal application to humans, the authors found AMS to give slightly lower values of radioactivity than LSC [12]. In our studies we found a correlation coefficient of 0.999 for our in vitro spiked plasma data set i.e. there was no difference between the two methods.

In conclusion we believe that AMS is a validated method that can be used for both pre-clinical and clinical studies to aid the drug development process. The ability to analyse radioactivity in small volumes means that serial bleeds could be taken from laboratory animals for ADME and PK studies rather than using several animals per time point. In addition using low radioactive doses in human Phase I or Phase II studies means that such studies can be made radiologically safer as well as reducing the amounts of radioactivity discharged into the environment.

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