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The comparative disposition of [^{14}C]-fotemustine in non-tumourous and tumourous mice

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Summary. The distribution and excretion of radioactivity from [^{14}C]-fotemustine was examined in mice with melanomas at different stages of development to determine whether the disease state substantially alters the disposition of the drug and its metabolites. Normal BDF$_1$ mice and mice that had been subcutaneously grafted with B16 melanoma either 1, 3, 7, 14 or 21 days previously were used. The animals were killed at either 5 min, or at 3, 24 or 96 h after receiving an intravenous dose of [^{14}C]-fotemustine (20 mg/kg) and were examined either by whole-body autoradiography or by liquid scintillation counting of excreta and tissues of interest. The majority of the [^{14}C]-fotemustine dose was excreted in the urine, with similar amounts being measured in both non-tumourous animals (61.6% ± 13.1%) and tumourous mice grafted 14 days previously (67.2% ± 5.7%). Small amounts of radioactivity, again similar in both non-tumourous and tumourous mice, were recovered in the faeces (5.4% ± 5.6% and 3.6% ± 1.8%, respectively) and as carbon dioxide (7% ± 3.5% and 6.4% ± 1%, respectively), with minimal amounts being expired as chloroethanol (<1%). When mice were examined 5 min after dosing, there was extensive tissue distribution accounting for 75% ± 10% of the dose. The highest concentrations determined by both whole-body autoradiography and liquid scintillation counting were measured in the excretory organs, with 33 and 28 μgEq/g being found in the liver and kidney, respectively. High levels were also seen in the lung and plasma (19.8 and 19.5 μgEq/g, respectively). Analysis of variance indicated that groups of tissues, such as the excretory organs, blood and plasma or the pigmented tissues, showed distinct but inconsistent patterns. Only tumours at 14 and 21 days of development were suitable for examination, and these showed levels of 12.1 μgEq/g; however, the tumour-to-plasma ratio increased from between approx. 0.5 and 0.6 at 5 min to approx. 2 at 96 h after dosing, suggesting retention within the melanoma, whereas the ratio for the femur remained at approx. 1. Whole-body autoradiography showed that the distribution in the tumour was not uniform, but rather was concentrated in the peripheral area (presumably viable cells) as opposed to the central necrotic region. Thus, the high and sustained concentration of radioactivity found in the active cells of the melanoma may provide an explanation for the high efficacy of the drug.

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Introduction

Fotemustine, diethyl-1-[-3-(2-chloroethyl)-3-nitrosoureido]ethyl phosphonate, is a novel 2-chloroethyl nitrosourea derivative that has been found to have significant advantages over other compounds for the treatment of cancers such as malignant melanoma. In BDF mice with either L1210 leukaemia or subcutaneous B16 melanoma, the optimal dose was 20 mg/kg given intravenously. Subsequent clinical studies have also demonstrated that a 1-h intravenous infusion of 100 mg/m^2 for 3 consecutive weeks, followed by a 5-week rest period and, subsequently, a monthly maintenance dose for responders was the most effective schedule [7].

Many physiological changes can take place in diseases such as cancer, and some can have marked effects on the disposition of drugs [3]. This can result from impaired hepatic or renal function, changes in the plasma albumin/globulin ratio, anaemia, leukocytosis and adipose tissue deficiency. We therefore considered it worthwhile to compare the distribution and elimination of fotemustine and its metabolites in non-tumourous and tumourous animals using the radiolabelled compound (Fig. 1) by determining the distribution of radioactivity using both whole-body autoradiography and liquid scintillation counting. The elimination of radioactivity from the animals' excreta was also examined and compared between non-tumourous and tumourous mice. As the drug is indicated for the treatment of malignant melanoma, mice (BDF) bearing B16

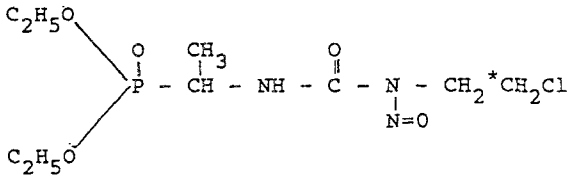

Fig. 1. Chemical structure of [^{14}C]-fotemustine. *, Position of the [^{14}C]-label

melanoma were considered to represent the most appropriate animal model for these studies.

Materials and methods

The [^{14}C]-fotemustine was synthesised at the Commissariat à l'Energie Atomique, France, to a specific activity of 53 mCi/mmol (Fig. 1). The purity of the radiolabelled material, checked prior to its use by high-performance liquid chromatography, was >92%. Non-radiolabelled fotemustine was supplied by Les Laboratoires Servier, France.

The BDF mice (Charles River, U. K.) were divided into different treatment groups, either non-tumourous animals or those that had been subcutaneously grafted with B16 melanoma according to the procedure of Geran et al. at [4] 1, 3, 7, 14 or 21 days prior to the dose of [^{14}C]-fotemustine.

For the examination of the elimination of radioactivity from [^{14}C]-fotemustine, four mice without tumours and four animals that had been implanted with B16 melanoma 14 days previously were used. The animals were housed individually in glass metabolism cages (Jencons Scientific, U. K.) designed for the separate collection of urine, faeces and expired carbon dioxide and chloroethanol. After a 24-h acclimatization period, each animal received an intravenous bolus injection of [^{14}C]-fotemustine, dissolved in a mixture of ethanol (10%) and 5% aqueous glucose solution in the lateral tail vein at a dose of approx. 20 mg/kg. Urine was collected at 3, 6 and 24 h and then daily, with separate daily collections of faeces, for up to 4 days after dosing. Chloroethanol was trapped in 2-ethoxyethanol (200 ml) contained in the first of two Nilox columns connected in series; the second Nilox column contained a 2-ethoxy-ethanol:ethanolamine (3 : 1, v/v) mixture (200 ml) for collection of all expired carbon dioxide. The respective trapping agents were changed daily for up to 96 h after dosing.

For determination of the tissue distribution of radioactivity from [^{14}C]-fotemustine, we used groups containing 12 mice each. The groups comprised non-tumourous animals and those grafted with tumours at 1, 3, 7, 14 and 21 days previously. From each group, three mice each were killed at 5 min and at 3, 24 and 96 h after dosing, and two of the mice from each of these groups were exsanguinated; subsequently, tissues of interest (tumour, thymus, liver, kidney, eye, skin, brain, lung and femur) were dissected, placed into preweighed containers and immediately reweighed.

The remaining animal from each of these groups was killed by ether anaesthesia, immediately mounted onto cork boards and frozen by immersion in a mixture of hexane: solid carbon dioxide. The boards were then stored at –70° C until sectioning, which was performed using a Cambridge Electrolinear sledge microtome (Cambridge Instrument Co. Ltd., U. K.) maintained at –20° C in a Bright cryostat (Bright Instrument Co. Ltd., U. K.). The sections (20 µm) were cut using a method based on that described by Ullberg [10], then freeze-dried and exposed to Agfa Osray M3 X-ray film at –20° C in a dark environment, for up to 74 days.

Quantitation of radioactivity from [^{14}C]-fotemustine was achieved by liquid scintillation counting using a Packard 300, 1500 or 2000 CA liquid scintillation counter with an automatic external standard for quench correction and conversion to disintegrations per minute (dpm). Urine (50 µl), chloroethanol-trapping fluid (1 ml) and carbon dioxide-trapping fluid (1 ml) were added directly to Optiphase "safe" (10 ml) liquid scintillation fluid (LKB Instruments Ltd., U. K.) for counting. Faecal samples were homogenised with a known amount of water and an aliquot (50 mg) was combusted using a Packard Tricarb Sample Oxidiser Model 306 prior to counting. Tissue samples were solubilised in a mixture of sodium hydroxide (80 g): Triton X-100 (100 ml): water (600 ml): methanol (300 ml) by incubation at approx. 50° C for up to 48 h. Accurately known weights of aliquots from the solubilised tissues (approx. 200 mg) were bleached with hydrogen peroxide overnight, if coloured, and was neutralized by the addition of 70% perchloric acid (200 µl) before the addition of Optiphase "safe" liquid scintillation fluid for counting.

Analysis of variance was used to examine specimens for overall differences between non-tumourous and tumourous animals and to determine the effect of sacrifice time and age of tumour using the respective interactions. These analyses were performed using the GENSTAT 5 program [9].

Results

Excretion of radioactivity

The majority of the radioactivity from [^{14}C]-fotemustine was excreted in the urine of both non-tumourous (61.6% ± 13.1%) and tumourous mice (67.2% ± 5.7%) over a 4-day period after dosing. Moreover, the majority of the renal excretion of radioactivity occurred within the first 24 h after dosing (51.7% ± 19.1% and 59.6% ± 4.5% for non-tumourous and tumourous mice, respectively). Total faecal elimination accounted for only 5.4% ± 5.6% and 3.6% ± 1.8% of the radioactive dose in non-tumourous and tumourous mice, respectively, with slightly more being recovered as expired carbon dioxide (6.4% ± 1% and 7% ± 3.5%, respectively) and only minimal quantities (0.9% ± 0.7% and 0.7% ± 0.3%, respectively) being found in the chloroethanol traps (Fig. 2A, B). A further 3.8% ± 0.9% of the dose was recovered in the carcasses of non-tumourous mice vs 2.9% ± 0.2% in tumourous ani-

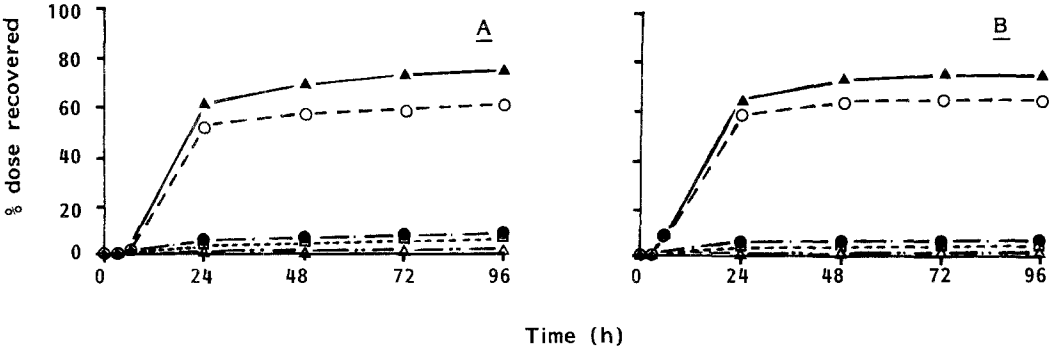

Fig. 2. A, B Excretion of radioactivity from **A** non-tumourous and **B** tumourous mice dosed intravenously with [^{14}C]-fotemustine: total, ▲; urine, ○; carbon dioxide, ●; faeces, □; chloroethanol, △

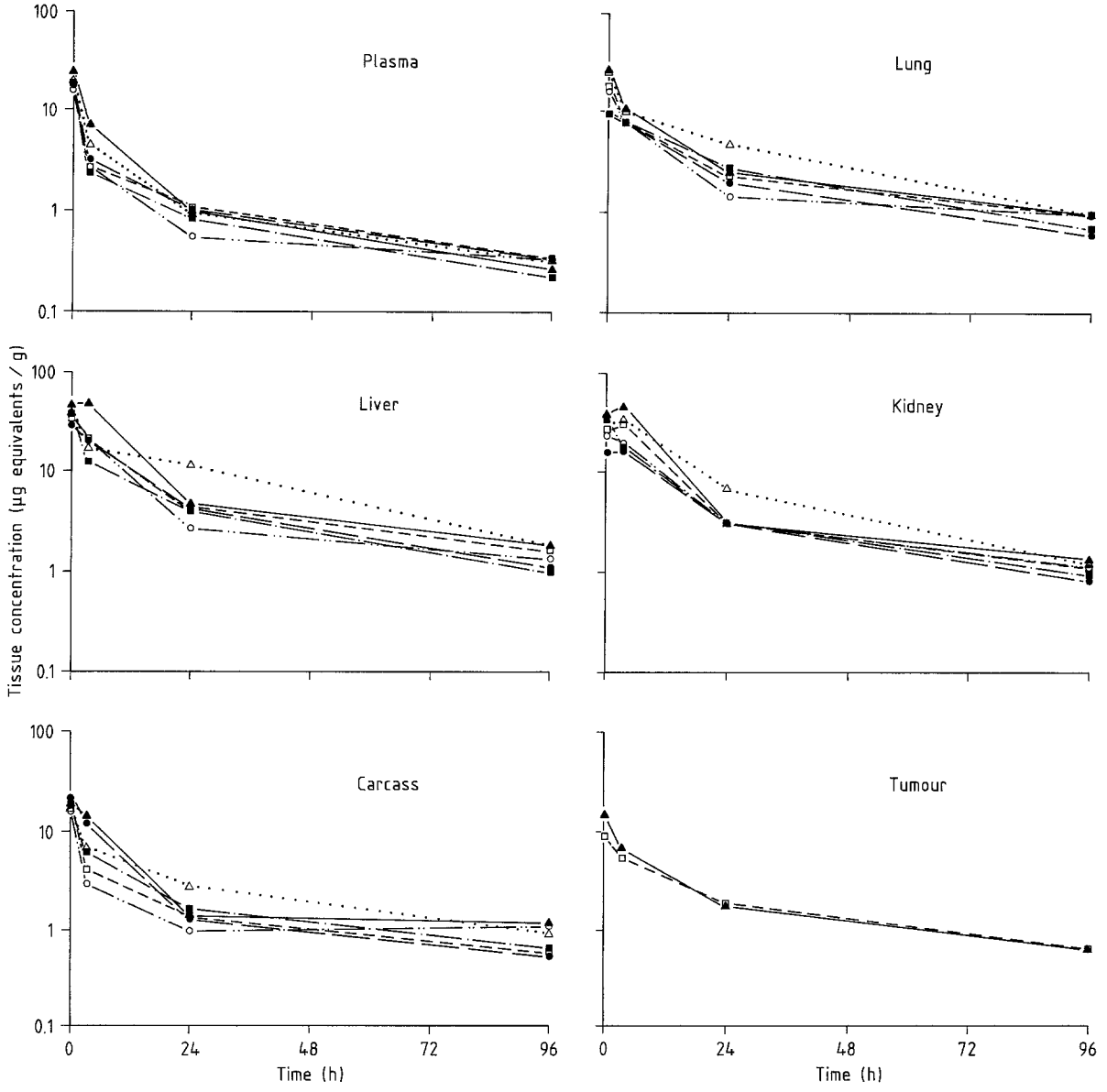

Fig. 3. Concentrations of radioactivity in different tissues and at different times after an intravenous dose (20 mg/kg) of [^{14}C]-fotemustine in mice with no melanoma (○) and in animals with melanomas at days 1 (△), 3 (●), 7 (■), 14 (□) and 21 (▲) after grafting

mals, giving an overall recovery of approx. 80% from both groups of mice. No statistical differences were seen by ANOVA between tumourous and non-tumourous animals for all parameters examined.

Quantitative tissue distribution

The tumour weights ranged from <1 mg at day 1 to approx. 3 g at day 21 after grafting. However, the average weight of the tumours at up to 7 days after dosing was only approx. 5 mg, making them difficult to dissect and analyse, mainly due to weight loss as a result of water evaporation during sample preparation. Thus, concentrations of radioactivity in these tumours must be viewed with this limitation in mind and, as a consequence, tumour concentrations are cited only for tumours at 14 and 21 days of development.

At 5 min after dosing, 75% ± 10.3% of the radioactive dose was recovered from tissues throughout the exsanguinated animals at all stages of tumour development, with the highest concentrations being found in the liver (33 ± 11.8 µgEq/g) and kidney (28 ± 12.6 µgEq/g). High concentrations were also found in the lung and plasma (19.8 ± 8.2 and 19.5 ± 5 µgEq/g). The lowest concentrations at 5 min after dosing were recovered in the eye (8.6 ± 4.9 µgEq/g), blood (9.21 µgEq/g) and femur (9.54 µgEq/g). The 14- and 21-day tumour concentrations at 5 min were also relatively high (12.1 ± 4.9 µgEq/g). There were no consistent overall differences in the concentrations of radioactivity in the respective tissues at different stages of tumour growth, and the radioactivity rapidly disappeared from these tissues such that only 3.6% ± 1.1% of the dose remained after 96 h. The decline of tissue levels of radioactivity essentially followed a biphasic decline, with an initial rapid phase of up to 24 h and a slower phase thereafter (Fig. 3).

However, analysis of variance revealed that certain tissues fell into distinct groups according to the concentra-

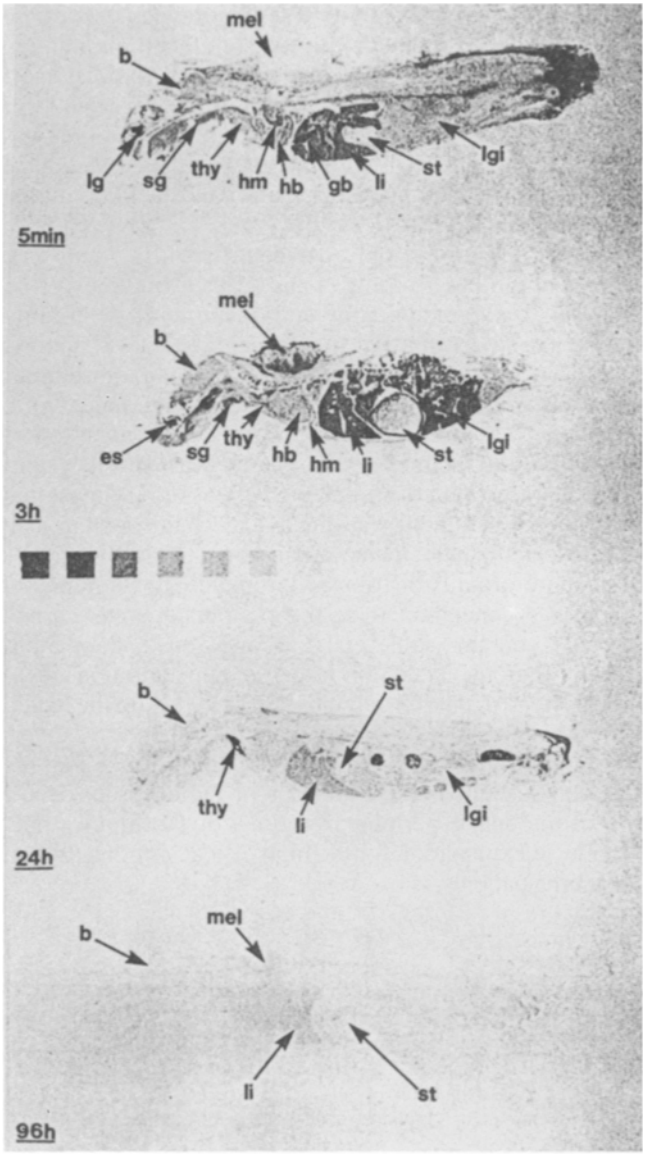

Fig. 4. Whole-body autoradiograph showing the tissue distribution of radioactivity from [^{14}C]-fotemustine at different times after an intravenous dose (20 mg/kg) in mice with B16 melanoma that was grafted 14 days previously: *b*, brain; *bl*, bladder; *bm*, bone marrow, *c*, caecum; *e*, eye; *es*, ethmoid sinuses; *fp*, faecal pellet; *gb*, gall bladder; *hb*, heart blood; *hm*, heart muscle; *k*, kidney; *lg*, lachrymal gland; *lgi*, large intestine; *li*, liver; *lu*, lung; *mel*, melanoma; *sc*, spinal cord; *sg*, salivary gland; *smi*, small intestine; *spl*, spleen; *st*, stomach; *te*, testis; *thy*, thymus

tions of radioactivity found at different times after dosing and at different stages of tumour development. Thus, the liver, kidney and lung all showed a similar pattern of distribution with time, but with a significantly higher mean concentration occurring in tumourous animals that was dependent on the age of the tumour. On the other hand, the blood and plasma showed an overall difference in distribution with time between tumourous and non-tumourous animals that appeared to be related to the age of the tumour, whereas the pigmented tissue (eyes and skin) showed no overall difference in the mean level between non-tumourous and tumourous animals, although there were tumour age differences that were related to the time after dosing. Interestingly, when the tumour-to-plasma ratio of the concentration of radioactivity for 14- and 21-day tumours was calculated, it increased from 0.54 and 0.61, respectively, at 5 min after dosing to 1.85 and 2.29, respectively, at 96 h after dosing, whereas that for femur remained at approx. 1.

Whole-body autoradiography

Whole-body autoradiography of non-tumourous and tumour-bearing mice confirmed the results of the quantitative studies by demonstrating rapid distribution of radioactivity from [^{14}C]-fotemustine to all tissues, even at early times after dosing, followed by a rapid elimination that left only trace levels at 96 h. As with the quantitative studies, it was not possible to identify tumours at 1, 3 and 7 days after grafting; however, by comparison with values for non-tumourous animals, we could show that the distribution of radioactivity from [^{14}C]-fotemustine was similar at all stages of tumour growth.

The distribution of radioactivity from the blood was rapid; at 5 min after dosing there was extensive distribution into all tissues, including the brain (Fig. 4). However, the radioactivity was not evenly distributed, with levels higher than those found in blood being measured in the liver, kidney and lachrymal gland. The concentration found in the muscle, lungs, salivary gland and thymus was similar to that measured in blood, whereas that determined in the brain, spinal cord and testes, although significant, was lower than that in blood. By 3 h after dosing, the distribution had markedly changed, with rapid elimination of radioactivity from the blood being reflected by a corresponding reduction in muscle, lung, brain, spinal cord and testes concentrations, but with retention occurring in the lachrymal gland, liver, kidney and salivary gland and apparent distribution of radioactivity into the thymus and intestinal contents being observed (Fig. 4). The radioactivity continued to decrease progressively from all tissues, although that in the bone marrow of the femur was still discernible at 24 h after dosing and traces could be detected in most tissues at 96 h after dosing.

At 14 and 21 days after grafting, the melanomas could be readily identified, and it was noteworthy that even at 5 min after dosing, radioactivity could be demonstrated in them (Fig. 4). However, the distribution was not uniform, with higher concentrations occurring around the periphery and relatively low levels, in the centre. The highest concentration of radioactivity within the tumour occurred at 3 h after dosing, again showing uneven distribution with high concentrations being detected around the edges, but at this point exhibiting evidence of ingress into the centre of the tumour (Fig. 4). However, areas remained into which relatively little radioactivity had distributed. At later times after dosing, the radioactivity level within the tumour had decreased, although the previously heavily labelled peripheral areas remained evident.

Discussion

The objective of this study was to establish whether the changes in the physiological parameters of animals that

result from implantation of B16 melanoma could be reflected by changes in the disposition of [^{14}C]-fotemustine. Therefore, the excretion of radioactivity from [^{14}C]-fotemustine was compared between non-tumourous and tumourous mice and the distribution of radioactivity was examined in the tissues of mice with B16 melanoma at different stages of development. Although the value obtained for total radioactivity can represent a mixture of the drug and its metabolites, sometimes making interpretation difficult, it can be advantageously used for compounds such as the nitrosoureas, which produce short-lived reactive intermediates, since it provides a means of detecting reactive products that bind covalently to endogenous macromolecules. [^{14}C]-Fotemustine was therefore radiolabelled in such a position (Fig. 1) that the reactive intermediates would retain the radiolabel.

The excretion of radioactivity from [^{14}C]-fotemustine was similar for both non-tumourous and tumourous animals, with the majority of the dose (61.6% and 67.2%) being recovered in the urine, most of which (>50%) was excreted within the first 24 h. The extensive renal excretion of radioactivity was typical of that of other radiolabelled chloroethyl nitrosoureas [2, 5, 8]. Furthermore, only minimal proportions of the dose (<1%) were recovered as chloroethanol, consistent with previous studies in which radiolabelled chloroethanol was given [6], but the recovery of significant proportions (>10%) as radiolabelled carbon dioxide would suggest removal of the chloroethyl moiety and subsequent degradation [2, 5].

Both whole-body autoradiography and quantitation of tissue distribution showed no consistent trend for changes in the disposition of radioactivity, although analysis of variance of the latter did indicate some patterns with changing age of tumour in some tissues. The interpretation of these findings is somewhat difficult, but it is noteworthy that the most apparent differences were observed in the excretory organs. The most common pattern of events was a parabolic effect of tumour age in animals with 1- and 21-day tumours, which showed higher levels whereas in mice with 3-day tumours, values tended to be similar to those measured in animals without a tumour. The levels measured on day 1 could be a reflection of trauma due to tumour implantation, whereas those found on day 21 were probably due to the fact that a significant proportion of the animal's body weight resulted from the tumours, considering that doses were given on a body-weight basis. These changes tended to be subtle, and it can thus be concluded from both the tissue distribution and the excretion of radioactivity from [^{14}C]-fotemustine that disposition of the drug is probably only marginally affected by the physiological changes brought about by the growth of the melanoma.

These studies did, however, show that radiolabelled products were rapidly and extensively distributed from the blood into the tissues of animals. The highest concentrations were found in organs associated with the elimination of drugs (liver and kidney); again, this finding is similar to that reported for other nitrosoureas [1]. The relatively high levels of radioactivity measured in the lachrymal and salivary gland would indicate avid uptake by the secretory organs, but it was noteworthy that there was no evidence of binding to melanin in the uveal tract of the eye. The high concentration of radioactivity found in the contents of the intestinal tract, combined with the significant elimination of radioactivity in the faeces (approx. 5%), could suggest some biliary excretion of radiolabelled products of fotemustine. However, the extent of biliary excretion was low, which is consistent with the low molecular weight of fotemustine (315.7 atomic mass unit) and its likely metabolic products.

Melanomas could only be identified in animals that underwent tumour grafting 14 and 21 days previously, but whole-body autoradiography and quantitative determinations of tissue distribution showed significant distribution into these tumours. Furthermore, the plasma-to-tumour ratio increased from between approx. 0.5 and 0.6 at 5 min to approx. 2 at 96 h after dosing, suggesting retention of radioactivity in the melanoma. The fact that similar retention was not seen in the pigmented eye would suggest that retention in the tumour was due to a mechanism other than normal electrostatic melanin binding. The radioactivity was not uniformally distributed throughout the tumour, but rather was concentrated in the peripheral areas, which probably contain viable cells, as opposed to the central necrotic regions. Thus, the effective concentration of radioactivity from [^{14}C]-fotemustine is likely to be much higher than would be indicated from the quantitative determinations of the whole tumour, and we postulate that the high and sustained concentrations of radioactivity occurring in the active peripheral regions of the tumour may provide an explanation for the high efficacy of this drug in melanoma patients.

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