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Fate of haem after parenteral administration of haem arginate to rabbits

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Rabbits were injected either intravenously or intramuscularly with [^{14}C]haem arginate and [^{59}Fe]haem arginate (haem 5 mg kg^{-1}). The main part (80%) of AUC_{INF} of labelled haem was associated with the β-phase, $T_{\frac{1}{2}}$ being about 6 h. Only 1% of the haem dose had been taken up by the red blood cells. In contrast, the iron moiety from the haem molecule was effectively utilized. Thirty days post-injection of [^{59}Fe]haem arginate, 40% of the dose after intravenous injection and 60% after intramuscular injection was circulating with the red cells. Radioactivity was shown to concentrate in the liver, where haem is mainly metabolized and eliminated. An accumulation of haem in the adrenals was also evident. Haem itself did not concentrate in the bone marrow, and a negligible amount of radioactivity was recovered from brain, implying a poor penetration of the blood brain barrier.

Haem[1], a complex of iron and protoporphyrin, serves as the prosthetic moiety of a number of haemoproteins such as haemoglobin, myoglobin and cytochromes. Porphyrias and sideroblastic anaemias are clinical disorders which are characterized by defects in the enzymes involved in the biosynthesis of haem leading to haem deficiency (Heilmeyer 1966; Kappas et al 1983). A replacement treatment with haematin[2] has been suggested for acute hepatic porphyric attacks. This treatment has in practice been associated with some complications such as coagulopathy and thrombophlebitis (Dhar et al 1975; Morris et al 1981). In contrast, the recently introduced haem arginate[3] compound (Normosang) has proved safe and effective in that treatment (Tenhunen et al 1985; Mustajoki et al 1985). The compound is more stable than haematin and has, for example, been shown in-vitro to serve as an equally good substrate as physiological haem for the haem-degrading enzyme system, haem oxygenase and biliverdin reductase (Tenhunen et al 1985). Knowledge of the behaviour of haem after parenteral administration is limited. In the present study its distribution to various organs, metabolism and elimination have been studied after intravenous and intramuscular administration of haem arginate to rabbits.

[1] The term haem is used here to indicate an iron-protoporphyrin IX compound irrespective of the oxidation state of the iron.

[2] Haematin (haemin hydroxide) is a reaction product of haemin and sodium carbonate.

[3] The term haem arginate means here the reaction product of haemin and L-arginine in a mixture of propylene glycol, ethanol and water.

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MATERIALS AND METHODS

Labelled haem arginate (Normosang) solutions

[^{14}C]Haem was prepared in-vitro from reticulocyte-rich freeze-thawed rat erythrocytes by incubation with [4-^{14}C]δ-aminolevulinic acid (Goldstein et al 1968). Specific activities in separate batches ranged from 0·40 to 1·66 mCi mmol^{-1} (0·61–2·55 μCi mg^{-1}). [^{59}Fe]Haem was achieved by administering $^{59}FeSO_4$ to anaemic rats as described by Thomas et al (1971). ^{14}C-Labelled and ^{59}Fe-labelled haem arginates were prepared by using [^{14}C]haem and [^{59}Fe]haem, as haem sources. Haem [^{14}C]arginate was prepared by using L-[^{14}C]arginine purchased from New England Nuclear. The labelled haem arginate solutions were prepared freshly for each injection.

Administration to rabbits and sample collection

The labelled haem arginate solutions (containing haem 25 mg mL^{-1}), were diluted with physiological saline (1:5) and administered intravenously or intramuscularly at a dose of 5 mg haem kg^{-1} to Californian white rabbits, 2·5–4·2 kg. The amount of radioactivity administered per rabbit was 12–19 μCi of [^{14}C]haem and 4–10 μCi of [^{59}Fe]haem. The fate of arginine in the haem arginate solution was assessed by giving haem [^{14}C]arginate (7–19 μCi) intravenously to one group of rabbits and [^{14}C]arginine (11–22 μCi) to another group. Immediately

after administration of the compounds the rabbits were transferred to metabolic cages where they had free access to food and water. Blood samples were taken from the ear veins at various intervals and the heparin plasma and red blood cells were separated by centrifugation at 1500*g* for 10 min. The red blood cells were washed twice with physiological NaCl solution. Samples for determination of haematocrit values were taken from all rabbits. Urine and faeces were collected as long as measurable amounts of radioactivity were found. One group of animals was killed 24 h after treatment with either [^{14}C]haem arginate (n = 3) or [^{59}Fe]haem arginate (n = 3). Various organs were excised, weighed and stored at −20 °C until analysed. Another group of animals was kept in metabolic cages as long as radioactivity could be detected in their blood.

In-situ bile collection

Rabbits were anaesthetized with pentobarbitone 35 mg kg^{-1} i.p. The bile duct was cannulated and the bile flow was allowed to stabilize for 15 min. [^{14}C]haem arginate and [^{59}Fe]haem arginate were administered intravenously and bile samples were collected at half-hour intervals for 7·5 h. The samples were kept in ice, protected from light, weighed and stored at −80 °C until analysed.

Determination of total radioactivity

The amount of total ^{14}C in the samples was measured in a 1215 Rack Beta liquid scintillation counter (LKB Wallac). Plasma and urine samples were counted in an organic scintillator solution (Lumagel). Blood, haem, bile and homogenized tissue and faeces samples were solubilized and bleached with H_2O_2 if necessary before scintillator solution was added. The amount of total ^{59}Fe was determined using an LKB-Wallac 80000 automatic gamma sample counter. Aliquots of solubilized plasma, blood, urine, bile and homogenized tissue and faeces were counted directly in suitable vials. In calculation of per cent of radioactive dose found in plasma and red blood cells, the blood volume was assumed to be 7·7% of the body weight, and by haematocrit determinations the red blood cell volume was averaged to 40% and the plasma volume to 60%.

Determination of haem

Haem not covalently bound to proteins is extractable from biological materials by using acidified organic solvents. [^{14}C]Haem and [^{59}Fe]haem were extracted from liver and bile samples according to Sedman & Tephly (1982). The recovery both of [^{14}C]haem and [^{59}Fe]haem was about 90%. The eluted sample containing haem was separated by TLC. Silica gel plates (Merck 60F254) of 0·2 mm thickness were used. Chloroform–methanol–formic acid (85 : 15 : 1·2) proved to be the most suitable solvent system. After development the plate was cut into 1 cm pieces and their activity was determined by liquid scintillation ([^{14}C]haem) or gamma counting ([^{59}Fe]haem). Unlabelled haem standard, which had recently been twice recrystallized, was applied concurrently with the sample and the haem spot was identified by visualization.

Pharmacokinetic calculations

Curve fitting and compartment modelling for each rabbit were performed using a least squares computer program (Brown & Manno 1978). The total plasma clearance (CL) was calculated by the equation CL = dose/AUC, where AUC is the area under the plasma concentration-time curve corrected for infinity. The distribution volumes were calculated using the conventional formulae (Ritschel 1976).

RESULTS

The radioactivity-time curves determined in plasma after [^{14}C]haem arginate and [^{59}Fe]haem arginate injections of 5 mg kg^{-1} were identical, implying the

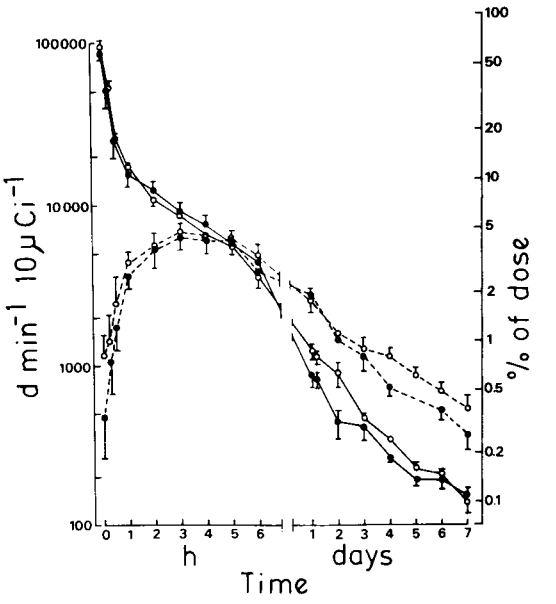

FIG. 1. Radioactivity in plasma at different times after intravenous and intramuscular administration of [^{14}C]haem arginate and [^{59}Fe]haem arginate to rabbits. Mean ± s.e.m. of 3 rabbits. Key: ○– – –○, ^{14}C i.m.; ○——○, ^{14}C i.v.; ●– – –●, ^{59}Fe i.m.; ●——●, ^{59}Fe i.v.

Table 1. Pharmacokinetic coefficients of the bi-exponential equations, fractions of total area under the plasma concentration time curve (AUC) associated with each phase, and derived pharmacokinetic parameters for disposition of haem after intravenous administration of [^{14}C] and [^{59}Fe]haem arginate (dose: 5 mg haem kg^{-1}, 10 μCi). Mean ± s.e.m. of 3 experiments.

Parameter		[^{14}C]Haem	[^{59}Fe]Haem
Intercept with ordinate (d min^{-1} mL^{-1})	A	111070 ± 7040	120710 ± 18590
	B	10950 ± 330	15140 ± 2900
Exponential coefficient (h^{-1})	α	4·29 ± 0·86	4·86 ± 0·41
	β	0·103 ± 0·010	0·132 ± 0·004
Fraction of AUC (%) associated with each phase	α	20 ± 1·6	18 ± 0·5
	β	80 ± 1·6	82 ± 0·4
k_{12} (h^{-1})		2·99 ± 0·66	3·36 ± 0·34
k_{21} (h^{-1})		0·48 ± 0·08	0·65 ± 0·02
k_{el} (h^{-1})		0·92 ± 0·13	0·98 ± 0·06
V_1 (mL)		183 ± 10	172 ± 27
V_{ss} (mL)		1300 ± 17	1060 ± 136

* k_{12} = distribution rate constant for transfer of drug from central to peripheral compartment, k_{21} = distribution rate constant for transfer of drug from peripheral to central compartment, k_{el} = elimination rate constant of central compartment, V_1 = volume of central compartment, V_{ss} = volume of distribution at steady state.

Table 2. Some model-independent pharmacokinetic parameters of haem following intravenous administration of [^{14}C] and [^{59}Fe]haem arginate. Mean ± s.e.m.

Parameter	[^{14}C]Haem	[^{59}Fe]Haem
$T_{\frac{1}{2}}$[a] (h)	6·9 ± 0·8	5·3 ± 0·2
Cl[b] (mL min^{-1})	2·77 ± 0·27	2·81 ± 0·43
AUC_{INF}[c]	122600 ± 16400	131800 ± 19200
Varea[d] (mL)	1620 ± 17	1290 ± 228

[a] Elimination half-life, plasma data up to 24 h.
[b] Total plasma clearance.
[c] Total area under the plasma concentration-time curve.
[d] Distribution volume by area (β) method.

occurrence of only haem and not liberated iron molecules in plasma (Fig. 1). Plasma concentrations could be measured for up to 7 days after intravenous administration of the labelled compounds. Since the main part of the activity was eliminated during 0–24 h and a good fit (r^2: 0·987 ± 0·002 after [^{14}C]haem and 0·998 after [^{59}Fe]haem) was obtained with a two-compartment open model, the results were used for calculation of pharmacokinetic parameters (Tables 1, 2). The decline of radioactivity in plasma follows the equation

$$Cp = Ae^{-\alpha \cdot t} + Be^{-\beta \cdot t}$$

where Cp is the concentration (activity) in plasma at time t, α and β are the exponential coefficients during the two phases, and A and B are the contributions of the respective exponential terms at t = 0 (intercepts with ordinate). These pharmacokinetic coefficients with derived constants and volumes of distribution are listed in Table 1. The main part (80–82%) of AUC_{INF} (total area under the plasma concentration-time curve) was associated with β-phase (Table 1) and the half-life of this phase was 6·9 h and 5·3 h following intravenous administration of [^{14}C]haem and [^{59}Fe]haem arginate, respectively (Table 2). Haem was distributed into a small central compartment of the same order as the plasma volume but distribution volumes during the steady states and elimination phase were 6 to 7-fold and 8 to 9-fold higher, respectively (Tables 1, 2).

On the basis of the ratio of AUCs after intramuscular and intravenous doses the mean bioavailability of intramuscular haem arginate was about 200% (Table 3). Administration of haem [^{14}C]arginate gave a quite different profile of the disappearance of labelled compound from plasma than administration of [^{14}C]haem or [^{59}]haem arginate (results not shown). The profile was identical with that following L-[^{14}C]arginine administration, indicating a splitting to haem and arginine as soon as the haem arginate compound reaches the blood.

Fig. 2 shows the amount of total radioactivity in red blood cells following [^{14}C]haem arginate and [^{59}Fe]haem arginate injections. During the first 6 h after administration of the compounds the time curves are identical, independent of label, indicating uptake by only haem in the blood cells. After this the profiles of the isotopes are different. At 48 h [^{14}C]haem total radioactivity reaches its maximum (about 1% of dose), which lasts for 10 days, whereafter the radioactivity declines to reach zero at an average of 70 days after administration. From the radioactivity of red blood cells 17·5 to 36·5% was in the haem of haemoglobin. In contrast, [^{59}Fe]haem total radioactivity reaches a maximum 30 days after administration (40% of dose for i.v., 60% of dose for i.m.). 240 days after [^{59}Fe]haem arginate intravenously or intramuscularly, 15% of the radioactivity was still circulating in the blood cells.

Table 3. Some pharmacokinetic parameters of haem derived from plasma concentrations following intramuscular administration of [^{14}C] and [^{59}Fe]haem arginate. Mean ± s.e.m.

Parameter	[^{14}C]Haem	[^{59}Fe]Haem
C max (d min^{-1} mL^{-1})	6204 ± 1103	6019 ± 1129
T max (h)	2·3 ± 0·3	3·2 ± 0·4
Ratio $AUC^{IM}_{INF}/AUC^{IV}_{INF}$	2·0	2·2

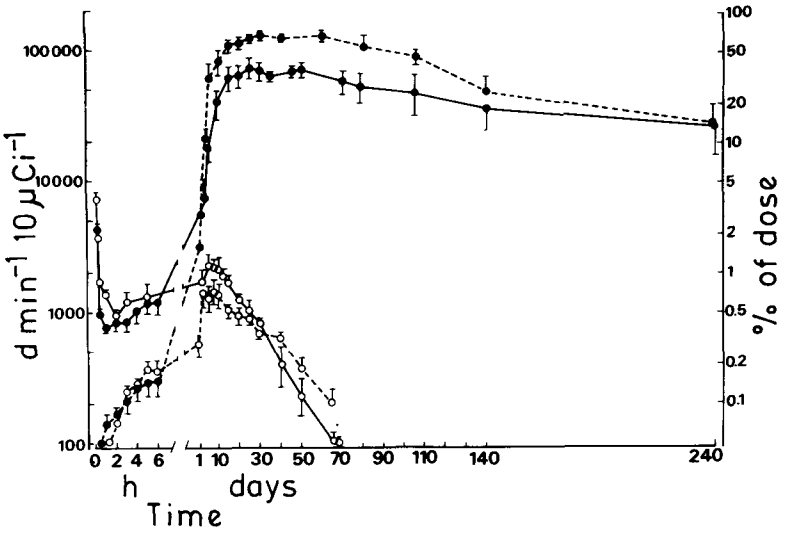

FIG. 2. Total radioactivity in red blood cells at different times after intravenous and intramuscular administration of [^{14}C]haem arginate and [^{59}Fe]haem arginate to rabbits. Mean ± s.e.m. of 3 rabbits. Key as Fig. 1.

Table 4. Distribution of radioactivity to various organs 24 h after administration of ^{14}C- and ^{59}Fe-labelled haem arginate to rabbits. The results are expressed as d min^{-1} g^{-1} 10 μCi^{-1} (mean ± s.e.m.). The number of rabbits = 3.

	[^{14}C]Haem i.v.	[^{14}C]Haem i.m.	[^{59}Fe]Haem i.v.	[^{59}Fe]Haem i.m.
Blood	1270 ± 67	2400 ± 982	3890 ± 1800	4500 ± 820
Kidney	3170 ± 23	3063 ± 143	11120 ± 1444	6343 ± 1118
Adrenals	5045 ± 15	7005 ± 1465	11553 ± 852	21653 ± 3738
Spleen	3173 ± 663	3827 ± 1314	11160 ± 1144	22953 ± 5257
Liver	17503 ± 848	32453 ± 1944	116553 ± 7257	58983 ± 8926
Heart	945 ± 185	1083 ± 242	2130 ± 306	3073 ± 584
Brain	87 ± 11	95 ± 8	100 ± 53	77 ± 38
Bone marrow (sternum)	2557 ± 299	4375 ± 2095	11223 ± 1693	11925 ± 860
Bone marrow (femurs)	1277 ± 118	1590 ± 520	6483 ± 1105	10853 ± 1638

Table 5. Total radioactivity and intact haem, expressed as percent of dose, in liver of rabbits 24 h after administration of 10 μCi of haem arginate.

Compound	Route	Hepatic total radio-activity (% of dose)	Hepatic haem (% of dose)
[^{14}C]Haem	i.v.	9·2 ± 0·6	5·6 ± 0·9
[^{14}C]Haem	i.m.	17·9 ± 1·4	14·8 ± 0·9
[^{59}Fe]Haem	i.v.	52·2 ± 4·2	5·5 ± 0·4
[^{59}Fe]Haem	i.m.	27·4 ± 2·7	10·5 ± 1·7

Number of experiments = 3.

Analysis of various organs of the rabbits killed 24 h after intravenous or intramuscular injection of [^{14}C]haem arginate revealed larger amounts of radioactivity after intramuscular than after intravenous injection (Table 4). The order of activity per g of tissues was liver ≫ adrenals > kidney = spleen = bone marrow (sternum) > blood = heart = bone marrow (femurs). A negligible amount of radioactivity was detected in the brain. When the rabbits had been treated with [^{59}Fe]haem arginate a different pattern of distribution of radioactivity was seen than after [^{14}C]haem arginate. The order of activity was liver ⋙ kidney = adrenals = spleen = bone marrow > blood = heart. The radioactivity found in brain was negligible. Two rabbits which had received [^{59}Fe]haem arginate were killed 106 days after its

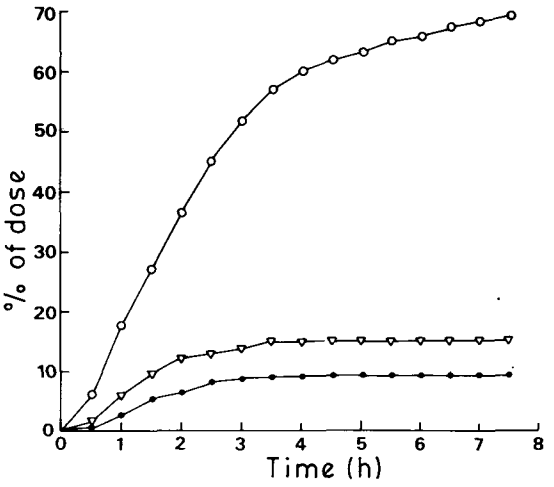

FIG. 3. Cumulative biliary excretion of total radioactivity and intact labelled haem after intravenous administration of [^{14}C]haem arginate and [^{59}]haem arginate to rabbits. Key: ○, [^{14}C]haem total activity; ▽, [^{14}C]haem intact; ●, [^{59}Fe]haem intact = total activity.

administration. The spleen showed a two-fold increase in activity compared with the 24 h value. Bone marrow and kidney had the same amount in the 24 h rabbits while the liver activity had decreased from 50% of the dose to 10%.

Twenty-four hours after i.v. administration of [^{59}Fe]haem arginate, 52% of the radioactivity was recovered from the liver (Table 5). Haem accounted for only 5·5% of the dose, implying that the remaining activity found was stored labelled iron. After i.v. administration of [^{14}C]haem arginate only 9·2% of the dose remained in the liver. Extraction and determination of unchanged [^{14}C]haem showed that 5·6% of the dose was in the form of haem. The small amount of total activity found in the liver at 24 h after [^{14}C]haem arginate was due to the fact that 70% of the dose had been excreted into the bile 7½ h after i.v. administration (Fig. 3). About 10% of the dose was excreted in the form of haem. After i.v. and i.m. [^{14}C]haem arginate administration, 67 and 59%, respectively, was excreted in the faeces (Fig. 4). Of the [^{59}Fe]haem arginate 27% was excreted after i.v. and 10% after i.m. administration.

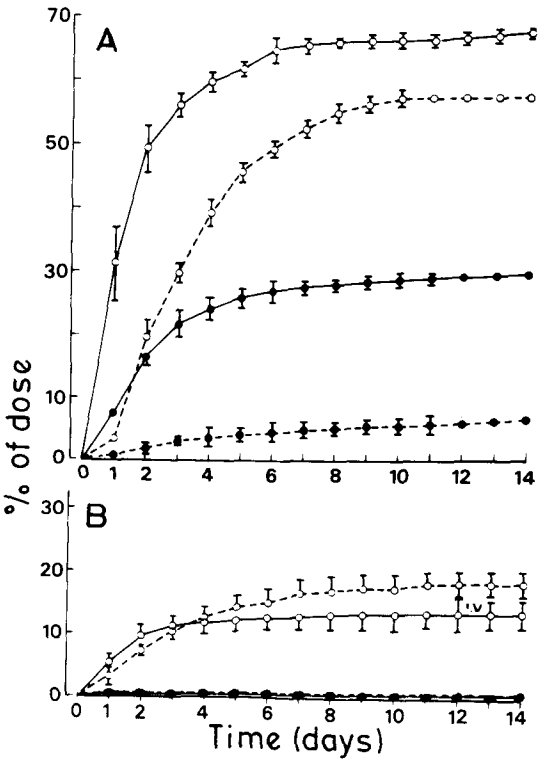

FIG. 4. Cumulative excretion of total radioactivity in (A) faeces and (B) urine after i.v. and i.m. injections of [^{14}C]haem arginate and [^{59}Fe]haem arginate. Key as Fig. 1.

Intact haem was not excreted into urine, since administration of [^{59}Fe]haem arginate failed to show detectable amounts of radioactivity in urine. After [^{14}C]haem arginate about 10% of the radioactivity was excreted in the urine, which means that haem in metabolized form is eliminated via the urine.

DISCUSSION

Circulating haem is taken up by two porphyrin binders, haemopexin and albumin. Haemopexin has a greater affinity for haem than albumin and binds at an equimolar ratio (Muller-Eberhard & Morgan 1975). Liem et al (1975) demonstrated in rabbits that an intravenous injection of haematin (12·5 mg kg^{-1}) strongly affected the plasma half-clearance time of haemopexin but not that of albumin. Of the plasma haemopexin 60–80% was removed, $T_{\frac{1}{2}}$ being 7·4 h and thereafter the remaining haemopexin was eliminated at a $T_{\frac{1}{2}}$ of 36 h. Interestingly, we found that the plasma haem elimination $T_{\frac{1}{2}}$ calculated after intravenous injection of 5 mg kg^{-1} of either [^{14}C]haem arginate or [^{59}Fe]haem arginate correlated completely with those described for haemopexin. Haemopexin has also been shown to transfer the haem from liver back to plasma (Schmid 1983) increasing the terminal half-life of haem. The main part (80%) of AUC_{INF} was associated with the β-phase. The elimination half-life of this phase was 6·9 h for [^{14}C]haem and 5·3 h for [^{59}Fe]haem. Calculation of $T_{\frac{1}{2}}$ during the terminal phase (up to 72 h) gave 33 h for [^{14}C]haem and 44 h for [^{59}Fe]haem. The two compartment open model used in the pharmacokinetic calculations of our study creates an approximation of a physiological system with numerous subsystems. Elimination from the two compartment system took place exclusively in the central compartment.

The volume of central compartment (V_1) was identical with the volume of plasma which means that the timed plasma samples were taken at sufficiently short intervals to produce a reliable reference volume (V_1). Other distribution volumes, at steady state (V_{ss}) and by area (β) method (V_β) are more drug-specific values than V_1. V_{ss} is the sum of the volumes of all compartments in 'pseudo-distribution equilibrium' (see Rominger & Wolf 1982). The volumes of distribution are useful in estimating the plasma concentration when the amount of drug in the body is known or estimating the dose required to produce a wanted drug concentration in plasma.

The red blood cells, independent of label used, contained the same amount of radioactivity during the first 6 h after administration of haem arginate, suggesting binding or incorporation of haem to the blood cells. 48 h post-injection of intravenous or intramuscular [^{14}C]haem arginate the radioactivity peak of the cells was reached. At that time about 1% of the dose was recovered from the red blood cells. The profile of the time course curve of radioactivity implies that the labelled haem remained in the red blood cells for their whole life-time, which according to Platt et al (1979) is 69 days in rabbits, and was removed only with the removal of senescent red cells from the circulation. In contrast to [^{14}C]haem arginate, the administration of [^{59}Fe]haem arginate caused an increase of the radioactivity in the red cells up to 30 days post-injection. At that time, 40% of the dose after intravenous injection and 60% of the dose after intramuscular injection was circulating with the red cells. Evidently the iron from [^{59}Fe]haem was more effectively utilized after the intramuscular route of administration than after the rapid intravenous injection. Since the liver is the site where haem is mainly eliminated by its uptake from haemopexin (Smith & Morgan 1978), a process which is saturable-, time-, energy- and temperature-dependent (Smith & Morgan 1981), and since albumin is not taken up by the liver even when haem circulates in excess (Liem 1974), it is likely that the rapid intravenous injection of haem arginate caused a concentration of haem which exceeded the uptake and storage capacity of the liver. This is supported by the finding that 4 days post-injection, 20% of the intravenously but only 3% of the intramuscularly administered ^{59}Fe had been excreted into faeces, reflecting the iron-sparing capacity. Of the administered [^{14}C]haem arginate dose, about 60% was excreted in bile as bile pigments consisting of three different bilirubin conjugates and biliverdin (data not shown). In addition, intact haem, not normally found in rabbit bile, appeared at the amount of 7–15% of the dose in bile during the first hours post-injection. In agreement, Petryka et al (1977) found a dose-dependent excretion of haem in bile after intravenous administration of 5–40 mg kg^{-1} of haematin to rats.

The organ distribution of radioactivity was extensive and, even with some exceptions, independent of route of administration and of label used 24 h after administration of haem arginate. The accumulation of radioactivity in the adrenals was only second to the liver and separation of medulla and cortex revealed an even distribution of the radioactivity. A poor penetration of haem through the blood-brain barrier was evident, since a negligible amount of radioactivity was detected in the brain. This minimal activity might be due to contamination of blood. Haem itself was not shown to concentrate in the bone marrow. The radioactivity values were of the same order as those of blood. Iron however, was clearly accumulated in those organs which are important for erythropoiesis, destruction of senescent erythrocytes and for iron storage, i.e. bone marrow, liver and spleen. High amounts of ^{59}Fe radioactivity was also found in the kidney. None of the radioactivity, however, was excreted into urine in the form of intact haem or iron. This is in agreement with observations of Pimstone et al (1971) that the kidney of rabbits possesses an effective enzymatic mechanism for conversion of haem to bile pigments. The haem derivatives detected as ^{14}C-radioactivity in urine are therefore bile pigments formed in liver cells or in proximal tubular cells of the kidney as a function of haem oxygenase and biliverdin reductase activities (Tenhunen et al 1970).

The pharmacokinetic data with the microparameters do not give clear information as to which specific organs or tissues the haem is slowly distributed, but the distribution of radioactivity in various organs shows that major components of the peripheral compartment are the well perfused organs of which liver and spleen are important sites in the metabolism of haem. Haem may have a greater role in the functions of adrenals and kidneys than we presently know.

Haem arginate (5 mg kg^{-1}) was more effectively utilized by the organism after intramuscular injection than after a rapid intravenous injection. In the light of these findings the slow intravenous infusion route now in use in clinical treatment with haem arginate seems justified.

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