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Uptake of dodecanedioic acid by isolated rat liver

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Abstract

The uptake of dodecanedioic acid (C12), a dicarboxylic acid with 12 carbon atoms, was studied in the isolated perfused rat liver. Fifty μmol of C12 were injected as a bolus into the perfusing liver solution. The concentration of C12 in perfusate samples taken over 2 h from the beginning of the experiments were analyzed by high performance liquid chromatography. An in vitro experimental session was performed to determine the binding curve of C12 to defatted bovine serum albumin. These data were then used to compute the perfusate C12 free fraction. The number of binding sites on the albumin molecule was equal to 4.29 ± 0.21 (S.E.), while the affinity constant was $6.33 \pm 0.87 \times 10^3$ M^{-1}. Experimental values of perfusate C12 concentration versus time were individually plotted and fitted to a monoexponential decay for each liver perfused. The predicted C12 concentration at time zero averaged 0.354 ± 0.0375 μmol/ml. Prom this value the apparent volume of distribution of C12 was obtained and corresponded to 153.02 ± 14.56 ml. The disappearance rate constant from the perfusate was 0.0278 ± 0.0030 min^{-1}. The C12 half life was 26.6 ± 2.3 min. The mean hepatic clearance from the perfusate was 4.08 ± 0.38 ml/min. In conclusion, C12 is quickly taken up by the liver so that in about 100 min it was completely cleared from the perfusate.

Keywords: Dodecanedioic acid; Dicarboxylic acid; HPLC; Isolated liver perfusion; Mathematical models

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1. Introduction

Mitochondrial β-oxidation is commonly considered to be the primary pathway of fatty acid (FA) oxidation; however, the contribution of alternate pathways to FA catabolism might be underestimated. Ω-oxidation of FA was first described by Verkade and Van der Lee [1]. It requires a microsomal ω-hydroxylation reaction followed by oxidation of ω-OH-fatty acid to dicarboxylic acid (DA).

In spite of the many investigations performed in this field [2–8] the quantitative contribution of peroxisomal β- and ω-oxidation to the oxidation of long chain fatty acids remains still unclear and controversial. Two recent studies [8,9], have shown that the ω-oxidation of palmitate in rat liver peroxisomes accounts for about 30% of the total palmitate oxidation. Therefore, dicarboxylic acids represent an important intermediate in one of the two pathways of monocarboxylic fatty acid oxidation.

It has been shown that dicarboxylic acids can be β-oxidized at the level of both liver and muscle [10–13]. However, the relative contributions of these organs and tissues remain to be clarified.

The aim of the present investigation was to study the uptake of dodecanedioic acid by isolated perfused rat liver. Among DA, dodecanedioic acid (C12) appears to be the most promising substance as alternate energy substrate for parenteral nutrition. In fact, compared with other DA with shorter chain length, such as azelaic (C9) and sebacic (C10) acids [14–24], C12 is excreted in the urine to a lower extent in both rats (3.9% of the administered dose) [25] and humans (7% of the administered dose) [26,27]. In addition, it is rapidly cleared from plasma by tissues and oxidized: C12 oxidation, expressed as percent oxidation, is around 35% in healthy control subjects [27].

2. Materials and methods

2.1. Materials

(1,12)^{14}Dodecanedioic acid (C12) (specific activity 117 mCi/mmol) was purchased from Amersham (Buckinghamshire, UK). Dodecanedioic acid and azelaic acid were from Sigma (St. Louis, MO, USA). Dodecanedioic acid was then purified by Real S.R.L., Como, Italy and was free from pyrogens and contaminants with a degree of purification, ascertained by gas-liquid chromatography and mass spectrometry, of 99.8%.

All other chemicals were of the highest purity available. A 0.4 mol/l solution of dodecanedioic acid, neutralised with NaOH, was used to prepare

the bolus, which was sterilized by ultrafiltration through 0.25 mμ Millipore filters (Molsheim, France) before administration.

2.2. Liver perfusion

Eleven male Wistar rats weighing in the range 80–120 g were used. The animals were fasting for 18 h before the experiment. On the day of the experiments the rats were anesthetized with an intraperitoneal injection of Nembutal (4.5 mg/100 g body weight) at 09:00 h. The bile duct and portal vein were cannulated with PE 10 and PE indwelling Braunula, respectively. The inferior vena cava was then ligated after sectioning the superior vena cava in the thoracic cavity. During surgery and transfer to the perfusion apparatus, the liver was continuously perfused with Krebs-Henseleit buffer [28] containing 0.1% (w/v) glucose and 2% (w/v) fatty acid-free bovine albumin, and maintained at 37°C while oxygenated with 95% O_2 and 5% CO_2. The liver was then perfused with 70–80 ml of recirculating Krebs-Henseleit medium, at a rate of 20 ml/min at 37°C. Dodecanedioic acid was injected into the perfusate as a 50 μmol bolus, dissolved in 1 ml of buffer solution containing 6% defatted serum albumin. The perfusate was oxygenated with 95% O_2 and 5% CO_2 using the apparatus described by Hamilton et al. [29]. Liver perfusion was maintained for 120 min. Perfusate samples (0.5 ml) were collected at 0.5, 5, 10, 15, 25, 40, 60, 80, 100, 120 min, immediately frozen, and kept at −20°C until analysis.

Liver viability was assessed by bile flow, bile salt secretion, oxygen extraction and gross appearance.

2.3. Dicarboxylic acid analysis

One-hundred μg of azelaic acid was added to 1 ml of each perfusate sample as an internal standard. Proteins were precipitated with 5 mg of trichloroacetic acid and dicarboxylic acids extracted twice with 8 volumes of ethylacetate, maintaining the solutions at 60°C for 15 min. The combined extracts were dried in a GyroVap apparatus (Howe, mod GV1, Gio. DeVita, Rome, Italy), operating at 60°C, coupled with a vacuum pump and a gas trap FTS System (Stone Ridge, New York, USA).

2.4. HPLC analysis

The extracted solutes were dissolved in 0.5 ml acetonitrile/methanol (1:1, v/v) and added to 20 mg of *p*-bromophenacylbromide and 70 μl of *N,N*-diisopropylethylamine as catalyst. The mixture was heated to 60°C for 15 min. The derivatives were dissolved in a final volume of 1 ml of

acetonitrile/methanol (1:1, v/v) and an aliquot of 10 μl was automatically injected into a liquid chromatograph (Hewlett-Packard 1050) with an HP 3396A integrator and a scanning spectrophotometer operating in the 190–600 nm wavelength range (light source: deuterium lamp, noise $< 2.5 \times 10^{-5}$ AU peak-to-peak at 254 nm with 4 nm bandwidth, flowing water at 1 ml/min).

Dicarboxylic acid derivatives were separated on an LC-18, 4.6 mm i.d., 25 cm length, 5 μm particle size, reversed phase column (Supelco Inc., Bellefonte, PA, USA). The HPLC conditions were as follows: solvent A, bidistilled water/methanol (1:1, v/v); solvent B, acetonitrile; after 15 min isocratic elution with 15% acetonitrile, a gradient elution was performed from 15% to 100% of B in 80 min. The flow rate was 1 ml/min, UV detector operating at 255 nm, chart speed 0.2 cm/min, range of absorbance from −0.300 to 1.000 absorbance units (AU).

2.5. Equilibrium dialysis

Bovine serum albumin (BSA) essentially free from fatty acids was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Binding of dodecanedioic acid to defatted BSA was determined by equilibrium dialysis according to Ashbrook et al. [30] as previously described [23]. Briefly, we used a 5-cell Spectrum Equilibrium Dialyzer (Spectrum Medical Industries Inc., Los Angeles, CA, USA). The teflon cells contained 1 ml compartments separated by a dialysis membrane (Spectra/Por, 47 mm diameter, molecular weight cut-off 12 000–14 000, Spectrum Medical Industries Inc., Los Angeles, CA, USA). Albumin does not cross the dialysis membrane. C12 reached equilibrium within 2 h at 37°C in the absence of albumin. We chose a 3 h incubation period.

Binding was assessed at varying concentrations of dodecanedioic acid (from 0.5×10^{-5} mol/l to 1.0×10^{-2} mol/l) in phosphate buffer, at pH 7.4. Experimental evidence of reduced water solubility of dodecanedioic acid at concentrations higher than 3.0×10^{-2} mol/l led us to perform experiments with C12 levels not exceeding this value. C12 was added to one side of the chamber, and defatted BSA in the same salt solution at 0.36 mmol/l concentration was added to the other side. The rotation speed of the dialysis cells was 30 rev./min and temperature of the solution was kept at 37°C by a thermostatic bath (Haakes, Karlsruhe, Germany). At the end of the incubation period, aliquots of 500 μl were removed from each side of the equilibration chamber, and the radioactivity was measured by a Beta scintillation counter (Canberra-Packard, Model 1600TR; Canberra, CT, USA). The recovery of radioactivity ranged from 95–100%.

2.6. Statistical methods

Model fitting (both for albumin binding and for kinetics) was performed by unweighed least squares with a standard Levenberg-Marquardt algorithm.

Albumin binding was modelled by the one site (equivalently, one step) model according to Scatchard [31]. The model equation is reported in Table 1.

After correcting observed total concentrations for albumin binding, in order to obtain free C12 levels, dodecanedioic uptake by liver from the perfusate was modelled with a mono-exponential decay equation (Table 1). A model fit, with the relative parameter estimates, was obtained on each individual liver perfused. Sample mean and sample standard error have been computed on the model parameter estimates thus obtained.

Asymptotic parameter standard errors were obtained by inversion of the Hessian matrix at the optimum. All results are expressed as mean ± standard error, unless otherwise specified.

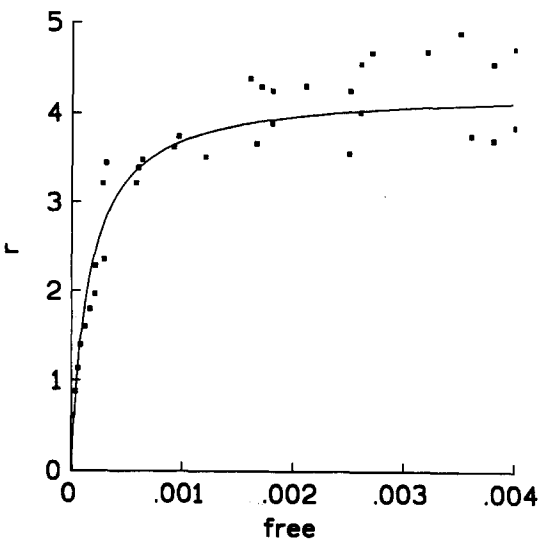

Fig. 1. Binding of dodecanedioic acid to bovine serum albumin (BSA). r is the molar ratio (mmol/mmol) between bound C12 and BSA. The points ($n = 37$) represent the experimental data and the line is the best theoretical model fitting the data.

Table 1
Mathematical models used

Albumin binding: the one site model used is

$$r = \frac{nKf}{1+Kf}$$

,

where
r [#], is the number of binding sites per albumin molecule;
K [M^{-1}], is the affinity of the single class of binding sites;
f [M], is the molar concentration of free dodecanedioic acid.

Kinetics: the uptake of dodecanedioic acid from the perfusate is described by the equation

$$\frac{dC}{dt} = -kC \quad C(0) = C_0$$

, solved as

$$C(t) = C_0 e^{-kt}$$

where
C [M], is the concentration of dodecanedioic in the perfusate;
t [min], is time;
C_0 [M] is the concentration at time zero;
k [min^{-1}] is the first order apparent plasma disappearance rate constant.

3. Results

Fig. 1 shows the binding of dodecanedioic acid to defatted BSA at pH 7.4 and at 37°C. The experimental points ($n = 37$) were fitted with a one site/one step model, with affinity constant $K = 6.33 \pm 0.87 \times 10^3$ M^{-1} and with the number n of binding sites on the albumin molecule equal to 4.29 ± 0.21 (S.E.).

Experimental values of perfusate C12 concentration vs. time were individually plotted and fitted to the model given in Table 1 for each liver perfused. The fit of the model to the data was good in every case, the χ^2 ranging from 5.2 to 190.4.

Fig. 2 depicts the average concentrations of dodecanedioic acid in perfusion fluid vs. time. Fig. 3 shows the recorded perfusate concentration time-course together with the model-predicted time course, for a sample liver. The predicted C12 concentration at time zero averaged 0.354 ± 0.0375 μmol/ml. From this value the apparent volume of distribution of dodecanedioic acid was obtained and corresponded to 153.02 ± 14.56 ml.

The disappearance rate constant from the perfusate was 0.0278 ± 0.0030 min^{-1}. The dodecanedioic half life was 26.6 ± 2.3 min. The mean hepatic clearance from the perfusate was 4.08 ± 0.38 ml/min.

4. Discussion

The present work was designed to evaluate the degree to which the liver participates in the metabolism of dodecanedioic acid. As seen from the short half life of C12 in the perfusate, the liver's uptake of the diacid is brisk and essentially all the administered C12 is cleared from the medium within 1 and 1.5 h.

Preliminary to the kinetics experiment, the binding assay showed that an average of about 4.3 homogeneous sites for C12 are present on each defatted albumin molecule. This result, compared to the binding of up to 2.9 molecules of C12 per molecule of albumin, as seen in total plasma binding experiments [26], shows that some plasma constituents compete with C12 for albumin binding. This fact further supports the use of dodecanedioic acid in vivo, where its free fraction is higher due to competition, and its availability for tissue uptake is correspondingly increased.

Data reported in the literature about long chain free fatty acids (FFA), and in particular palmitate [32], using defatted bovine albumin in a system at 37°C and at pH 7.4, showed three classes of sites, two specific and one aspecific, with capacities and affinities respectively (3; 6.8×10^6 M^{-1}), (3;

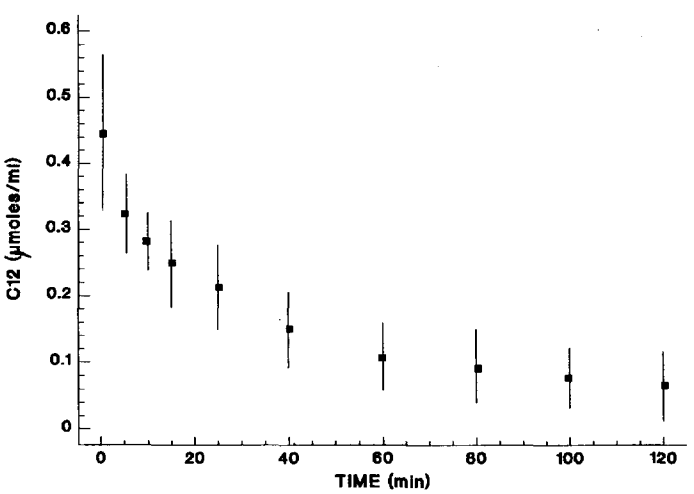

Fig. 2. Average perfusate concentration ($\pm$ S.D.) of free C12 (μmol/ml) vs. time (min) after bolus injection of 50 μmol of C12 into the perfusion solution.

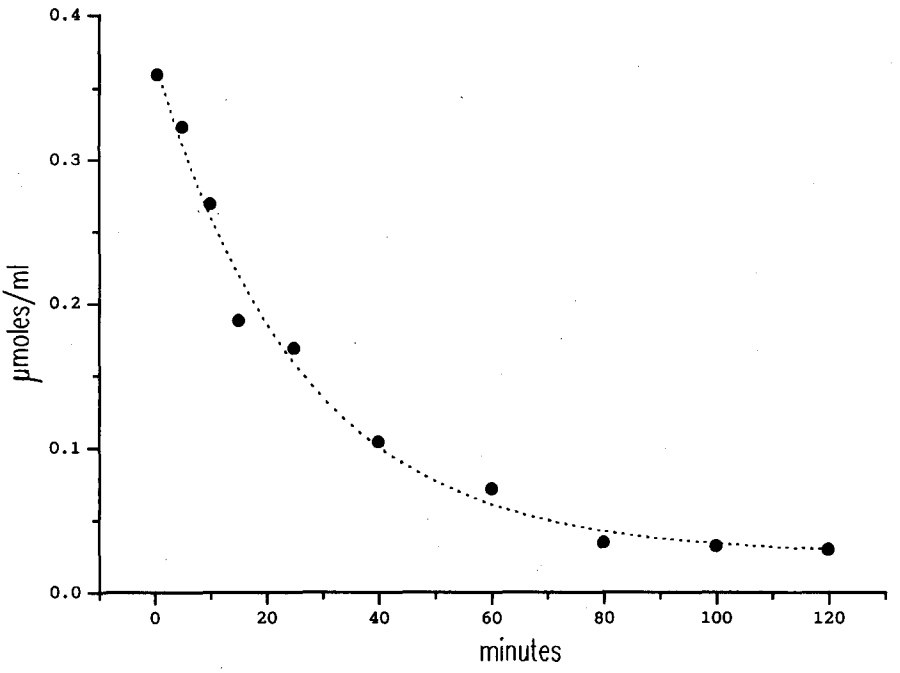

Fig. 3. Recorded perfusate concentration time-course (solid circles) and model-predicted time course (continuous line) for a sample perfused liver.

$5.0 \times 10^5\ M^{-1}$) and (aspecific, 63; $9.3 \times 10^2\ M^{-1}$). Lauric acid, a free fatty acid with 12 carbon atoms like C12, shows 5 different classes of sites [33] with affinity constants ranging from $2.4 \times 10^6\ M^{-1}$ to $1.9 \times 10^5\ M^{-1}$. These data indicate that dodecanedioic acid is much less albumin-bound, and therefore is available to a far greater amount for uptake as free acid, than long chain FFA or, even, than medium chain FFA with the same chain length as C12. Since it is known that it is the free form of a substrate which is taken up by the tissues, C12 should be easily cleared from plasma by the organs and tissues.

The experimental design used in the present investigation demonstrates a rapid C12 uptake by the liver. However, the relative amount of oxidized dodecanedioic acid compared to the amount stored in the liver remains to be quantified. However, it is helpful to note that during the late experimental period the levels of dodecanedioic acid in the perfusate are very low, which would indicate the absence of a major recirculation component. Another question concerns the form of C12 storage in the liver. Data from the literature [10] suggest that C12 can be accumulated for energy purposes as glycogen via succinyl-CoA formation.

In addition, studies performed in both animals [25] and humans [26,29] indicate that dodecanedioic acid is a promising alternate energy substrate for parenteral nutrition because, unlike other shorter chain dicarboxylic acids, it is excreted with urine to a small extent, and is quickly oxidized in man to a high degree [26]. The metabolic advantages that dodecanedioic acid shares with the other dicarboxylic acids, over the conventional monocarboxylic lipids, are its complete oxidation to acetyl-CoA, and the formation of succinyl-CoA [10], providing simultaneously both fuel and intermediate compounds for the Krebs cycle. As a gluconeogenic precursor, C12 seems to be a promising energy substrate, particularly in those cases in which there is a cellular depletion of glycogen, as in decompensated diabetes mellitus or sepsis. Further, it is easily soluble in water, making its administration and conservation easier with respect to lipid emulsions.

In conclusion, these data confirm that all of the administered dodecanedioic acid is taken up by the liver in this preparation. Further investigations will be needed to clarify the fate of this diacid once taken up by the liver.

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