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A new HPLC method for the direct analysis of triglycerides of dicarboxylic acids in biological samples

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Abstract

Dicarboxylic acids (DA) are alternate lipid substrates recently proposed in parenteral nutrition. Two new derivatives of DA, a triglyceride of sebacic (TGC10) and one of dodecanedioic (TGC12) acid have been synthesised in order to reduce the amount of sodium given with the unesterified forms. The present paper describes a rapid and direct high-performance liquid chromatographic method (HPLC) for the analysis of these substances in both plasma and urine. Thirty-six male Wistar rats were rapidly injected with 64 mg of TGC10 or 53 mg of TGC12. The triglycerides and their products of hydrolysis were measured in plasma samples taken at different times. For the dose of 500 ng the intra-assay variations ranged from 6.80±0.35% for TGC10 to 18.6±3.20% for TGC12 and the inter-assay variations were from 4.44±2.21% for TGC10 to 15.0±6.72% for TGC12. The detection limit for both triglycerides was 5 ng. This rapid and direct HPLC method could have practical implications in monitoring the concentration of both triglycerides and free forms of DA in biological samples of patients who might benefit from the administration of these substances during parenteral nutrition regimens.

Keywords: Dicarboxylic acids; Dodecanedioic acid; High-performance liquid chromatography; Sebacic acid; Triglycerides

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1. Introduction

An increasing interest has been recently focused by physicians on the use of alternate lipid substrates in parenteral nutrition in order to both minimize side-effects and optimize the nutritional benefits of this therapeutic approach. In particular, an alternate fuel substrate could be useful in clinical conditions in which plasma triglyceride lipolysis is impaired and/or long chain fatty acid oxidation is altered; as happens in sepsis, acute pancreatitis and decompensated diabetes mellitus with acidosis. Among the substrates investigated, great attention has been devoted to the use of inorganic salts of dicarboxylic acids (DA) such as sebacic (C10) and dodecanedioic (C12) acids [1–5] because of their favourable characteristics in parenteral nutrition. These include a rapid plasma clearance from tissues with respect to traditionally used lipid substrates, such as long chain triglycerides; a low urinary excretion; a good caloric equivalent (i.e. 7.18 kcal/g of C12 oxidized) and a high oxidation rate with prevention of fat accumulation in the liver [6–8]. In particular, even-numbered DA might be useful in those clinical conditions in which alternative gluconeogenic substrates, such as amino acids, are preferentially used with a consequent increase in protein catabolism, as in type 2 diabetes mellitus. Therefore, the use of DA in combination with other lipid substrates might improve lipid metabolism through the increased oxidation of acetyl-CoA in Krebs' cycle.

However, besides the advantageous aspects of DA, it must be borne in mind that their administration also provides a certain amount of sodium. In fact, if 79.2 g of C12 are administered over 24 h providing 568.74 kcal/24-h, 15.4 g of sodium and 2.15 l of water for solubilization are also given. It should be noted that this volume of solution can also contain 430 g of glucose (20% glucose solution), corresponding to 1612 kcal/24-h, and at least 107.5 g of amino acid equal to 430 kcal/24 h. Therefore, the caloric intake with 2.15 l of such solution should totally correspond to 2610 kcal/24-h. Other options could include long- or medium-chain triglycerides as partial substitution of glucose.

In order to reduce the amount of sodium administered with DA, two newly synthesized derivatives of DA, a triglyceride of sebacic (TGC10) and one of dodecanedioic acid (TGC12), which are still water soluble in sodium salt form, have been prepared. In those triglycerides the amount of sodium content is half of that given with C10 or C12 alone, since one -COOH group of each molecule of DA present in the triglyceride is esterified with each of the three alcoholic groups of the glycerol.

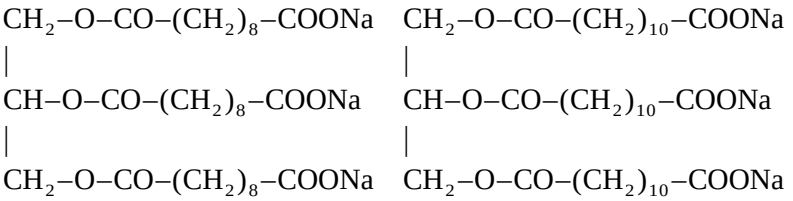
Our group previously described a HPLC method for measuring plasma DA concentration [7], and a rapid method for the determination of both medium chain fatty acids and triglycerides in plasma and urine [9]. However, no data are reported in the literature on the simultaneous determination of DA free forms and triglyceride concentration in blood and urine.

Thus, the aim of the present paper is to describe a simple and rapid HPLC method for the analysis of both TG and free forms of DA, which can be easily applied to biological samples.

2. Materials and methods

2.1. Reagents and chemicals

Suberic (C8), azelaic (C9), sebacic (C10) and dodecanedioic (C12) acids were purchased from Sigma (St. Louis, MO, USA). DA triglycerides as sodium salts were purchased from Real S.r.l., Como, Italy. Molecular weight of trisebacoylglycerol was 644 in the acidic form and 710 as sodium salt, while that of tridodecanedioylglycerol was 728 as acid and 794 as sodium salt. Their formulas are indicated below:



All other chemicals used were purity available quality or of the highest purity available.

A 90 mmol/l solution of TGC10 and a 66.7 mmol/l solution of TGC12 sodium salts were used for the intravenous bolus injection. The solutions were sterilized by ultrafiltration through 0.25 μm Ø Millipore filters (Molsheim, France) before administration.

2.2. Experimental protocol

Thirty-six male Wistar rats (bred at the Catholic University animal facilities in Rome) weighing between 160 and 190 g were used in all the experiments. At least 1 week before the experiment, the animals were housed in pairs in a light-controlled room, at an ambient temperature of 22°C to allow monitoring of body weight gain and to ensure normal growth characteristics.

During this period, the animals consumed standard laboratory food and tap-water ad libitum. Twenty-four hours before the study the animals were fasted. The rats were rapidly injected intravenously, through the vein of the tail, with either 64 mg of TGC10 or 53 mg of TGC12 diluted in 1 ml of bidistilled water. The rats were anesthetized with ethyl ether and blood samples were drawn in duplicate, using two different animals, by cardiac puncture at 5, 10, 20,

30, 40, 50, 60, 80 or 120 min after the bolus injection; the animals were then killed by cervical dislocation.

Heparinized blood samples were immediately centrifuged at $4000 \times g$ and plasma was frozen at -20°C until analysis. Eight rats, four injected with TGC10 and four injected with TGC12 as described above, were allocated to individual metabolic cages for the collection of 24-h urine.

The study followed the guidelines set forth by the Catholic University in-house animal experimentation ethical committee.

2.3. Dicarboxylic acid analysis

2.3.1. Plasma samples

One hundred μg of azelaic acid were added to 1 ml of each plasma sample as an internal standard. Proteins were precipitated with 5 mg of trichloracetic acid and dicarboxylic acids extracted twice with eight 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).

2.3.2. Urine samples

Samples (0.5 ml) from 24-h urine were added to 50 μg azelaic acid as internal standard and then treated with cation-exchange resin (Dowex 50 W-X4, 100–200 μm mesh, H^+) to remove salts, concentrated under reduced pressure and filtered through a Millipore HV (0.45 μm) filter. The samples were acidified to pH 1–2 with 4 mol/l HCl, extracted twice with ethylacetate and evaporated in the GyroVap, as previously described.

2.3.3. 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; Avondale, PA) with a HP 3396A integrator and a scanning spectrophotometer operating in the 190 to 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). 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).

The peak purities were confirmed by both retention time and UV-spectra analysis.

2.4. Recoveries of standards

Aliquots from 25 to 200 μg in 100 μl acetonitrile–methanol (1:1, v/v) of TGC10 and TGC12 standards were added to 1 ml of plasma from five untreated rats to measure the recovery of the individual standards. Both the triglycerides were extracted and analyzed as described above.

2.5. Calibration curve

Amounts from 30 to 1000 ng in acetonitrile/methanol (1:1, v/v) of each triglyceride standard derivatives were directly injected into the HPLC to perform a calibration curve. Each concentration was injected in triplicate, and the mean value was used as final.

2.6. Statistics

Data are presented as mean±standard deviation (S.D.), unless otherwise specified.

Regressions of peak area against injected amount ($Y = a + bX$) were linearly fitted to the experimental points. The F ratio (the ratio of explained to unexplained variability adjusted for the degrees of freedom) and the P value indicate the reliability of the data. The detection limit of TGC10 and TGC12 standards was taken at least as twice the background noise level at the highest sensitivity of the instrument (1250 pA/cm). The value of the the plasma curves of TGC12 was obtained using a biexponential decay equation: $y0 + A1e\hat{}(-x/t1) + A2e\hat{}(-x/t2)$ based on the model shown in Fig. 1.

3. Results

The HPLC separation of a synthetic mixture of dicarboxylic acids (suberic acid, C8; azelaic acid, C9; sebacic acid, C10 and dodecanedioic acid, C12) and of the triglycerides of both sebacic and dodecanedioic acids is reported in Fig. 2. A HPLC chromatogram of DA and TGC10 or TGC12 extracted from the plasma of two rats are shown in Fig. 3A and B.

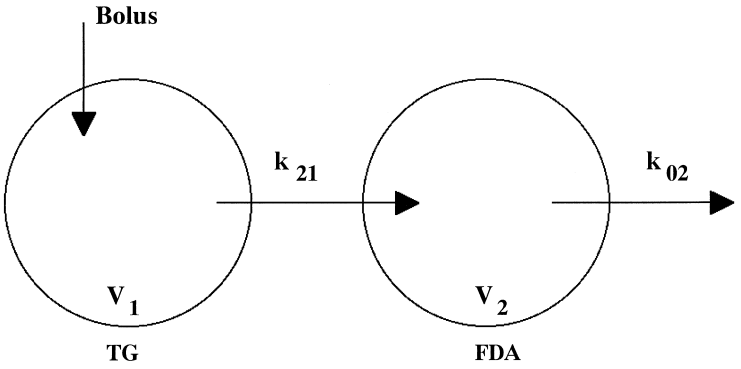


Fig. 1. A diagram of the final model chosen to represent the kinetics of sebacic and dodecanedioic acids: transfer (hydrolysis) of TGDA to DA is assumed to be produced by the action of an active carrier (enzyme) linearly dependent on the concentration of the precursor. Elimination (tissue uptake) of DA is linearly dependent on the amount of available free DA and a proportionality constant k_{02}. The volumes of distribution of TG and free DA are, respectively, V_1 and V_2.

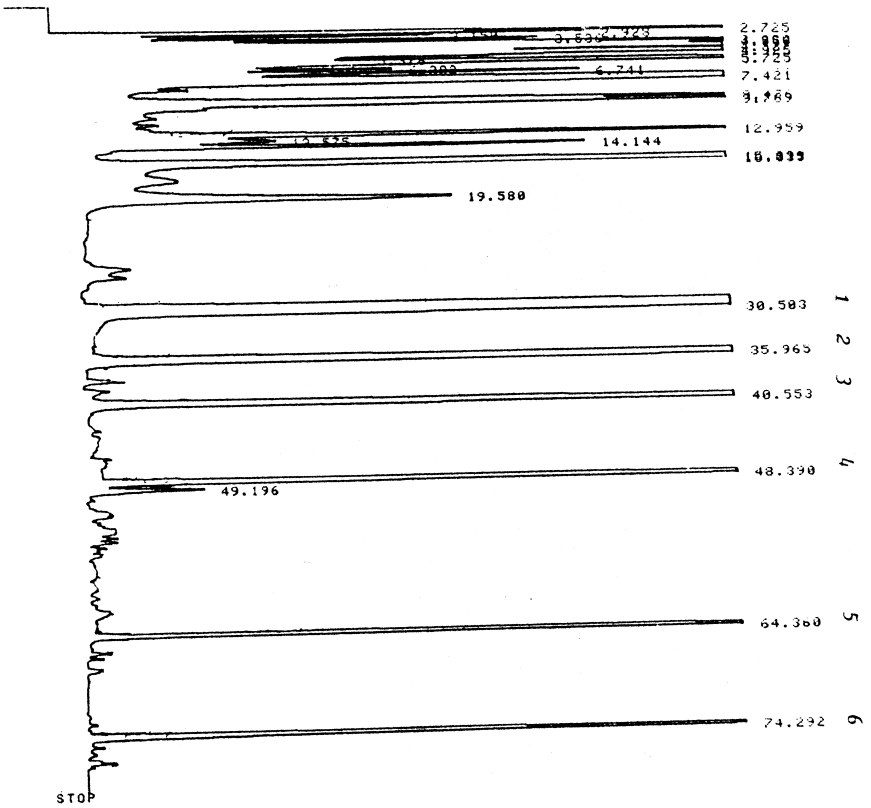


Fig. 2. HPLC separation of a mixture of standard dicarboxylic acids and their triglycerides. 1, Suberic acid (C8); 2, azelaic acid (C9); 3, sebacic acid (C10); 4, dodecanedioic acid (C12); 5, 1,2,3-tridecanedioylglycerol (TGC10); 6, 1,2,3-tridodecanedioylglycerol (TGC12).

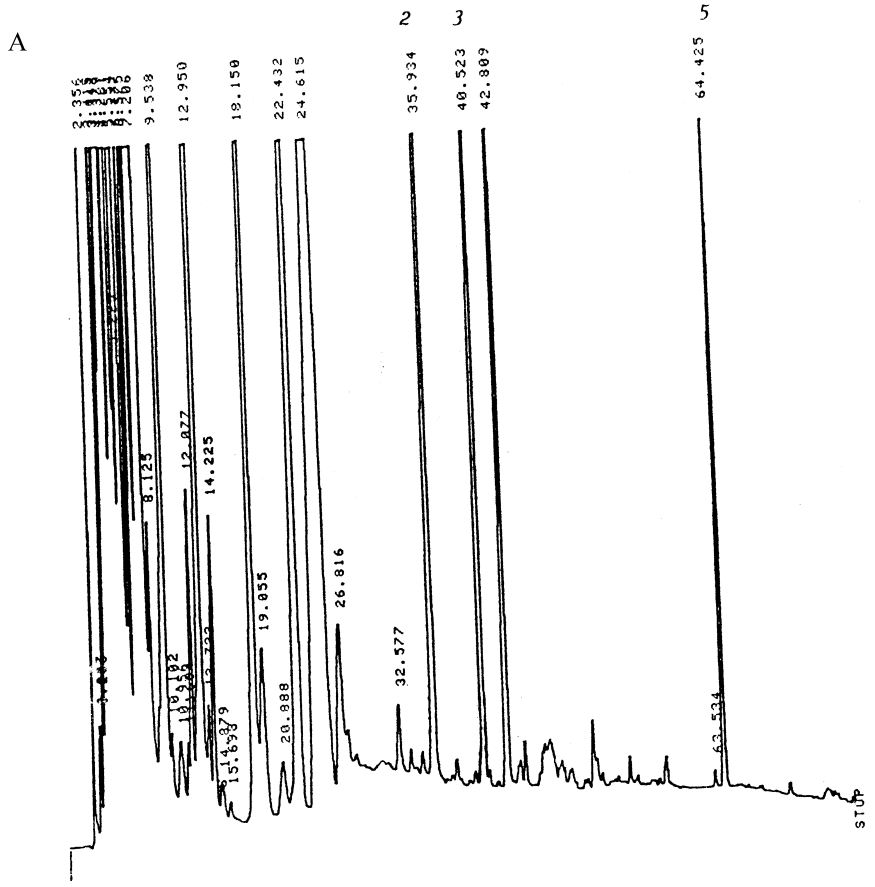


Fig. 3. HPLC separation of DA and TGDA extracted from a plasma of two rats i.v. injected with a TGC10 solution (A) or with a TGC12 solution (B). For the abbreviations used see Fig. 2.

The parameter estimates (intercept and slope) of the linear regression of peak area (pA* cm) on the injected amount (ng) and their standard errors were: for TGC10 -6.401 ± 1.022E5 and 3.387 ± 0.0115E4, with an R^2 of 95.34% ($P<0.001$); and for TGC12 -4.548 ± 8.622E4 and 3.427 ± 0.097E3, with an R^2 of 96.71% ($P<0.001$).

The intra-assay variations ranged from $6.80\pm0.35\%$ (mean$\pm$S.D.) for TGC10 and $18.6\pm3.21\%$ for TGC12 on 35 observations for the dose of 500 ng, while the inter-assay variations were from $4.44\pm2.20\%$ to $15.0\pm6.72\%$, for the same amount injected. The detection limit was 5 ng for both TGC10 and TGC12.

In Table 1 are shown the percentage recoveries of each standard (TGC10 and TGC12) added to plasma.

The 24-h urinary excretion of TGC10 was 87.2 ± 19.8 μg and that of TGC12 was 355 ± 85.9 μg, respectively, corresponding to about 0.13% and 0.67% of the

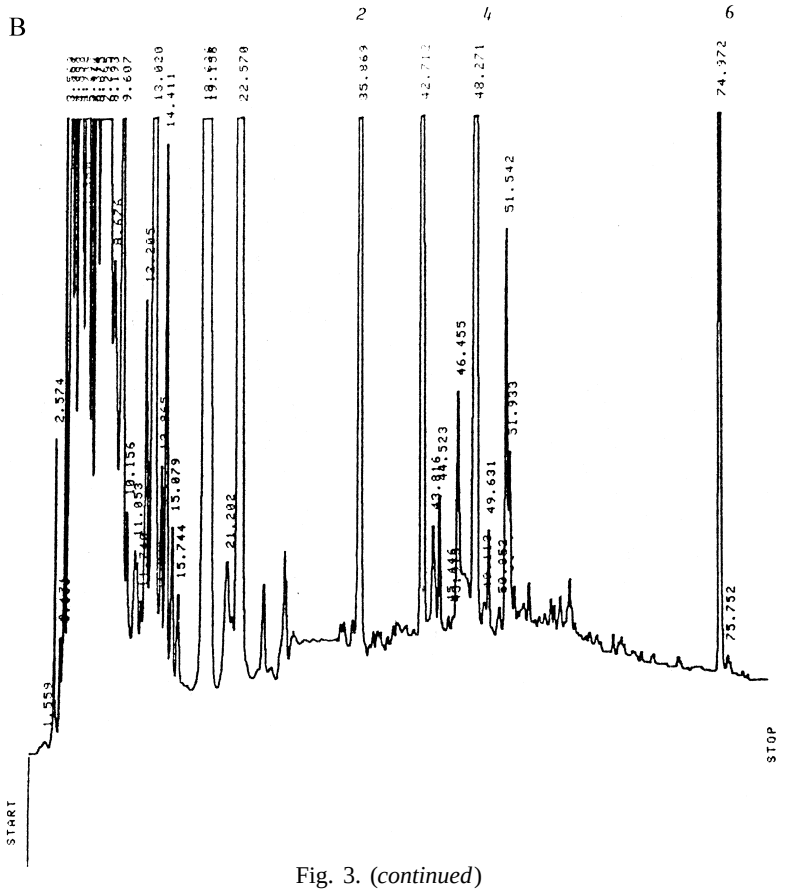


Fig. 3. (*continued*)

administered amount. In addition, small amounts of sebacic acid (37.8±10.5 μg) and dodecanedioic acid (502±64.9 μg) were excreted in the urine over 24 h.

Figs. 4 and 5 show the time course of TGC10 and TGC12 and their free form

Table 1
Recovery of standard TGC10 and TGC12 added to normal plasma samples (1 ml) expressed as percentage of the initial amount. The values represent the mean±S.E. of five determinations

μg added	TGC10	TGC12
200	97.46±0.88	86.76±0.65
100	96.25±0.73	85.09±0.71
50	94.51±0.61	83.84±0.46
25	93.40±0.92	82.55±0.77

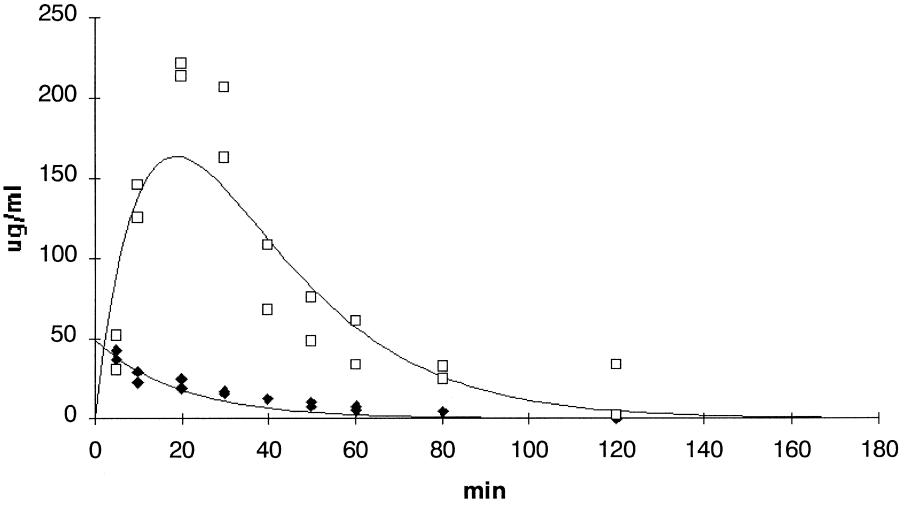


Fig. 4. Experimentally observed time–concentration points (solid diamonds: free DA; blank squares: TG) and model-predicted curves (lower: TG; upper: free DA) for C10.

plasma concentrations in the treated animals. Dodecanedioic acid peaked early, 10 min after TGC12 administration, and was rapidly cleared from plasma: at 30 min the dodecanedioic acid plasma level was very low. On the contrary, plasma concentration of sebacic acid reached the peak at 20 min following the i.v. bolus of TGC10 and it was eliminated from plasma more slowly.

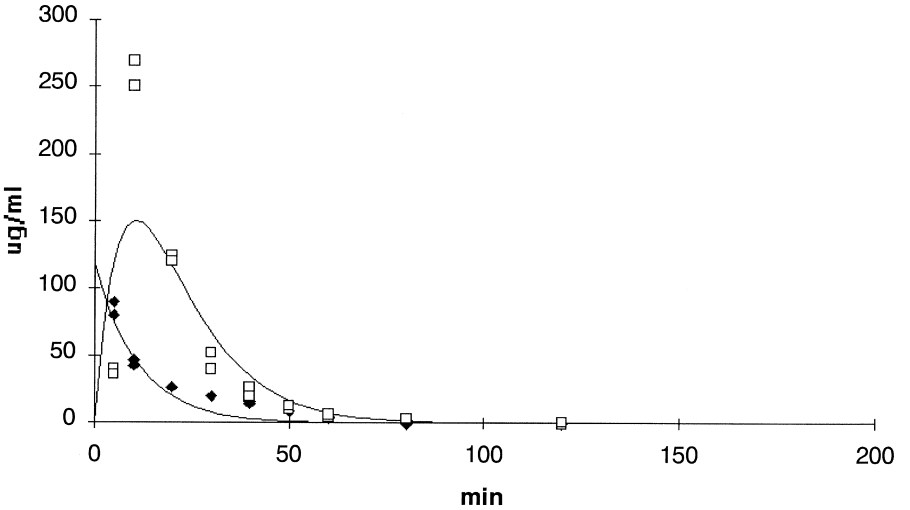


Fig. 5. Experimentally observed time–concentration points (solid diamonds: free DA; blank squares: TG) and model-predicted curves (lower: TG; upper: free DA) for C12.

4. Discussion

For the first time, in order to reduce the amount of sodium provided with DA administration, the triglycerides of DA, providing a twofold decreased amount of sodium, have been synthesised and administered in animals.

Sodium salts of triglycerides of sebacic and dodecanedioic acid are both soluble compounds, which however show different degree of hydrosolubility such as is observed for sebacic and dodecanedioic disodium salts. In fact, the water solubility of DA decreases with the elongation of the chain length.

The presence of three free carboxylic groups in the molecule of DA triglycerides allows to form derivatives which can be detected and well separated in HPLC. The range of linearity between peak area and nanograms injected of DA triglyceride standards is wide, giving from 10 to 2000 ng, and the detectability limit is low, for both TGC10 and TGC12.

Recovery of both TGC10 and TGC12 from plasma is good also when small amounts (in the order of 25 μg) of these triglycerides are added to 1 ml of plasma. Furthermore, the triglycerides of DA are rapidly hydrolyzed into their respective dicarboxylic acid free form and the urinary excretion of these DA triglycerides and of their products of hydrolysis is very small, being less than 1% of the administered dose.

The method described in this paper allows a direct HPLC analysis for the simultaneous measurement of the newly synthesised triglycerides of DA and free DA in plasma and urine without requiring any separation from other plasma lipids using thin-layer chromatography (TLC) or a chemical hydrolysis to liberate free DA. These latter, and in particular dodecanedioic acid, represent a promising lipid substrate in parenteral nutrition [1,7,8,10] especially in patients with sepsis and decompensated diabetes mellitus.

Even-numbered DA show similarity with carbohydrates due to the gluconeogenetic properties of succinic acid, which is formed during their β-oxidation [11]. In addition, succinic acid is a component of Krebs' cycle, which derives from the oxidation of certain amino acids (methionine, isoleucine, threonine and valine). Thus even-numbered DA can represent a way to activate the oxidation of other substrates, such as glucose and free fatty acids (FFA), which produce acetyl-CoA, the major oxidative substrate of Krebs' cycle.

In conclusion, the present paper showed a rapid and simple HPLC separation of triglycerides of dicarboxylic acids that will allow to ascertain the plasma clearance of these alternate lipid substrates during administration in patients requiring peculiar nutritional parenteral support. These substances represent an interesting form of energy delivery in human parenteral nutrition to be further investigated and the method described in this paper could be considered as a simple and important tool to monitor their concentration in plasma and urine.

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