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Bile acid kinetics in man studied by radio thin-layer chromatography and densitometry coupling

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Abstract

A method based on coupling of the techniques of radioscanning a TLC plate and densitometry has been developed for the determination of pool sizes and fractional turnover rate of bile acids in man after intraduodenal administration of ^{14}C-labelled acid. The validity of the method has been checked by comparison of the results obtained with those of an enzymatic spectrophotometric analysis, and a measurement of the radioactivity by liquid scintillation counting, after elution of the separated bile acid from a TLC plate. Advantages of the proposed method over the previous one include a reduced number of manipulations, the possibility of automation, a better reproducibility, and the possibility of elaborating the radiometric data obtained for the primary bile acid for better characterising its metabolism inside the enterohepatic circulation. © 1997 Elsevier Science B.V.

Keywords: Bile acids; Cholic acid; Deoxycholic acid; Radio thin-layer chromatography; Kinetics

1. Introduction

In the last few years considerable attention has been devoted to the biodynamics of bile acids (BA) in patients with hepatobiliary diseases. The primary BA, cholic and chenodeoxycholic acids, are synthesised from cholesterol and conjugated with glycine or taurine in the liver cells. The secondary BA, deoxycholic and lithocholic acids are formed by intestinal bacterial enzymatic 7-dehydroxylation of primary acids. All BA are actively and almost completely absorbed in the intestine and return to liver via the portal blood. Since hepatocytes are provided with an efficient uptake system, BA are found only at a trace level in the systemic circulation (μmol/l). Therefore, BA continuously circulate from the liver to the intestine playing their physiological functions (enterohepatic circulation). Hepatobiliary and intestinal diseases alter these processes: the former diseases induce BA accumulation in the systemic circulation, the latter ones cause abnormal losses of BA with the stools. The intensive study of BA and of their enterohepatic circulation has provided valuable information in the fields of intestinal digestion and absorption, of diseases of the liver and biliary tract, and of the metabolism and excretion of lipids such as cholesterol, from the body.

Measurements of the size and kinetics of the bile

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acid pool in man, especially for the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), have generally been based on the isotope dilution technique, introduced by Lindstedt in 1957 [1]. To this purpose a tracer dose of ^{14}C- or ^{3}H-labelled bile acid is administered intravenously or orally and the exponential decay of the specific activity is measured in duodenal samples collected at regular intervals, for at least 4 days. The assumptions are that a simple compartment is present and that this is in a steady-state condition [2]. In addition to the isotope-dilution method, other well-established methods for measuring BA synthesis are those based on the fecal acidic sterol balance [3] and the conversion of 26-[^{14}C]cholesterol to $^{14}CO_2$. Recently, the results obtained by the three methods in hypertriglyceridemic and control subjects have been compared by Duane [4].

The administration of radioactive tracers cannot be largely employed, particularly in certain groups of patients such as pregnant women or children, due to a possible radioactivity hazard. Therefore, other procedures have been introduced, using stable isotope-labelled [^{13}C]- and [^{2}H]-BA and measuring the relative abundances in biological fluids by gas chromatography–mass spectrometry-isotope ratio [5–7]. The main drawback of this measurement lies in the use of expensive instrumentation, not commonly available in most hospital laboratories.

The aim of this paper is to describe and validate a method based on the coupling of radiochromatography and densitometry for the determination of pool sizes (p) and fractional turnover rates (k) of bile acids in man, after intraduodenal administration of ^{14}C-labelled acid. The validity of the method has been checked by comparison of the results obtained with those of another independent method, previously used in our laboratory. This method is based on TLC separation of the unconjugated acids, determination of the radioactivity by liquid scintillation counting, and of the mass by an enzymatic spectrophotometric analysis, after scraping the gel and eluting the BA under examination. The proposed method is much simpler than the previous one, allowing large-scale studies on BA pools in man under different pathological and pharmacological conditions.

Furthermore, the radiometric data obtained for the study of p and k of cholic acid, have been used also for validating a model which could be very useful for predicting alterations in the enterohepatic circulation associated with gastrointestinal diseases.

2. Experimental

2.1. Materials

[Carboxyl-^{14}C]cholic acid, in ethanol solution (specific activity, 51.35 mCi/mmol) was purchased from NEN (New England Nuclear Research Products, Boston, MA, USA). [Carboxyl-^{14}C]deoxycholic acid, sodium salt, in aqueous solution containing 2% ethanol (specific activity, 55 mCi/mmol) was obtained from Amersham (Little Chalfont, UK). Cholic acid, deoxycholic acid, and cholylglycine hydrolase (EC 3.5.1.24), from *Clostridium perfringens*, (100 U/mg of protein) were from Sigma (St. Louis, MO, USA).

Bond Elut reversed-phase octadecylsilane-bonded silica cartridges (100 mg) and a Vac-Elut vacuum system were obtained from Analytichem International (Harbor City, CA, USA). All other solvents and reagents were of analytical grade. Chromatography was carried out on 20×20 cm plates of silica gel 60 without fluorescent indicator, 0.25 mm thick (Merck, Darmstadt, Germany). The kit Sterognost-3 Pho was purchased by Nycomed (Oslo, Norway).

2.2. Bile samples

An exact dose of [carboxyl-^{14}C]cholic or [carboxyl-^{14}C]deoxycholic acid (160–185 kBq), in 5 ml of ethanol–saline solution (5:95, v/v), was administered intraduodenally to the patients, through a duodenal tube. All subjects were studied as inpatients at the Department of Internal Medicine and Gastroenterology of the University of Bologna. Five biliary samples were obtained by duodenal drainage 8, 24, 48, 72, and 96 h after administration. Gallbladder emptying was induced by intravenous cerulein infusion. Bile samples were stored at −20°C until analysis.

2.3. Bile acid hydrolysis

Since the labelled tracer was administered in the free form, bile samples were hydrolysed according to

the following procedure: 1-ml aliquots of bile were treated with 4 ml of 0.3 *M* acetate buffer, pH 5.6, 0.4 ml of 0.2 *M* sodium EDTA, 0.4 ml of 0.2 *M* mercaptoethanol, and 0.6 ml of aqueous cholylglycine hydrolase suspension (1000 U/ml). The mixture was incubated for 1 h at 37°C under constant stirring, and the hydrolysis was stopped by adding 0.4 ml of 0.5 *M* sodium hydroxide. The mixture was adsorbed on a Bond Elut cartridge, which was previously conditioned with 3 ml of methanol and 5 ml of distilled water. After washing the cartridge with 5 ml of water, the bile acids were eluted with 4 ml of methanol. The eluate was taken to dryness at 50°C under nitrogen and the residue redissolved in an appropriate volume of methanol.

2.4. Measurement of specific radioactivity: previous method

The assay proposed by Iwata and Yamasaki [8] was routinely used in our laboratory. Known volumes of bile methanol extracts, together with methanol standard solutions of bile acid, were applied as streaks to the TLC plates, which were developed by ascending chromatography at room temperature, into saturated tanks containing the solvent mixture proposed by Hofmann for free acids: acetic acid, *n*-propanol, benzene, carbon tetrachloride, diisopropyl ether, isoamyl acetate (5:10:10:20:30:40, v/v). The migration distance was 15 cm. After development, the plates were dried in hot air and sprayed with a 1:1 mixture of concentrated sulphuric acid and acetic anhydride, only in the zone where a standard sample had been applied. Colour development was achieved by oven heating the plates at 120°C for 10 min. The areas of silica gel contiguous to the coloured bile acid spot (including samples, standards and a silica blank) were marked out in the undeveloped lanes, scraped off, and the bile acid eluted from the silica by shaking with methanol. The eluate was then evaporated to dryness and the residue reconstituted with 0.5 ml of methanol.

An aliquot (30–50 μl) was used in duplicate to measure the mass of the bile acid by the 3-hydroxysteroid dehydrogenase assay, which is based on the measurement of the absorbance at 340 nm of the NADH produced as a result of oxidation of the 3-hydroxyl group on the bile acid nucleus. The concentration (m*M*) of the bile acid in each sample was estimated from a standard curve prepared for each plate. Another aliquot (50–100 μl) was taken in duplicate and added with 15 ml of Ready Solv NA (Beckman, Geneva, Switzerland) for the measurement of radioactivity by liquid scintillation counting.

2.4.1. Calculations

A plot of the natural logarithm of specific activity against time exhibits a $-k$ slope and a $\ln A_s(t_0)$ intercept. From $A_s(t_0)$ and the value of the injected dose (D), pool size can be calculated on the basis of $p=D/A_s(t_0)$ and, since the enterohepatic circulation is in a steady state, daily synthesis rate is given by $S=p\cdot k$; k has units of day^{-1} and is usually measured over a period of 5 days. The pool sizes have been expressed as mmol, and the synthesis rates as mmol day^{-1}. Results have been expressed as mean±standard deviation (S.D.).

2.5. Measurement of specific radioactivity: proposed method

Known volumes of bile methanol extracts together with one methanol standard solution of labelled bile acid, were applied as streaks 15–20 mm long to the TLC plates which were then developed by ascending chromatography at room temperature, into saturated tanks containing the solvent mixture: *n*-hexane, isobutylmethyl ketone, ethyl acetate, acetic acid (10:5:5:1, v/v). The migration distance was 15 cm. After development, the plates were dried in hot air and scanned for radioactivity (counts per minute, cpm) by means of a linear analyser (Berthold LB 282), which makes use of a position sensitive proportional counter to count the particles emitted from the chromatographic plate. The instrument is provided with a data acquisition system for processing the data of the measurement. Since the width of the entrance window is 15 mm, the bile extracts were applied to the plate as streaks at least 15 mm long, in order to perform the measurement with the maximum sensitivity and therefore to shorten the analysis time.

After the radiometric measurements, the layers were dipped into a solution of 5% phosphomolybdic acid in ethanol. Colour development was achieved by oven heating the plates at 120°C for 10 min. Bile acids appeared as blue zones on a pale yellow background. The readings were carried out within 1

h since colour development by direct densitometry (Camag, TLC-HPTLC 76500 Scanner) using a filter wavelength of 620 nm and a 0.3×5-mm slit. The peak areas, eventually corrected by the dilution factors, were used for determining the specific counting rates (R_S=cpm/peak area).

2.5.1. Calculations

The natural logarithm of R_S was plotted vs. time, and the slope (k) and intercept (b) of the regression line were calculated. The pool size was determined using the equation: $p=\text{mmol}_{std}\cdot(A-1)$ where mmol_{std} are the mmoles of the standard labelled bile acid introduced at the administration time and A represents the ratio $(\ln R_S)_{std}/b$. The standard radiotracer was considered highly reliable and its specific radioactivity, provided by the manufacturer, was taken as a 'true value'.

3. Results and discussion

3.1. Radio-TLC and densitometry

Fig. 1 shows the radiometric and densitometric profiles for a bile sample, collected 48 h after the administration of labelled CA, obtained under the conditions used in this study. The solvent proposed for the development of the TLC plate allowed a good resolution of CDCA and DCA without using high-performance TLC plates [9]. The radioactivity scan clearly shows the extent of transformation of CA to DCA. The procedure of dipping the layers into the detection mixture instead of spraying, has the advantage of achieving a more homogeneous distribution of the detection reagent, which results in a better reproducibility of the densitometric measurement.

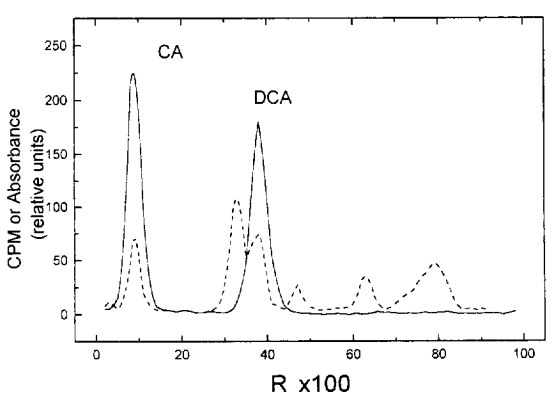


Fig. 1. Radiometric (continuous line) and densitometric (dashed line) profiles obtained for a bile sample collected 48 h after the intraduodenal administration of 173 kBq of [^{14}C]cholic acid.

The range of linearity was estimated by spotting equal volumes of solutions of CA at increasing concentrations and resulted in 0.1–5 nmol per spot. Within-day and between-day precision were better for the method based on TLC–densitometry than for TLC–spectrophotometry [8]. For example, the coefficient of variation (C.V.) of the within-day data ranged from 3 to 8% for the former method, and from 14 to 29% for the latter (n=20), over the analytical range considered.

As to the radiometric measurement, it must be pointed out that good precision was obtained only if the TLC layer was radioscanned before the colour development procedure. Actually, if the same volume of a solution of labelled CA (A_v=750 kBq/mmol) was spotted many times on different plates, which were submitted to ascending chromatography, air-dried and counted for radioactivity, the C.V. obtained for the measurements was lower than 3%. On the contrary, if the same set of observations was performed after the treatment with phosphomolybdic acid, the C.V. value increased up to 60%. This effect was probably due to a non-uniform distribution of the CA molecules inside the thickness of the layer, which resulted in a different self-absorption of the low-energy β particles emitted by ^{14}C.

3.2. Isotope-dilution study of CA kinetic parameters

Cholic acid decay curves measured in bile exhibited first-order kinetics. Linear regression correlation coefficients greater than 0.98 were obtained for all subjects. The sets of kinetic data obtained by the proposed method and the previous one performed on the same samples of bile, are reported in Table 1. The good agreement between the pool sizes and fractional turnover rates for cholic acid obtained by the two methods, confirms the validity of the proposed one. This latter, moreover, shows the following advantages: reduced number of manipulations

Table 1
Cholic acid turnover constants, pools and synthesis rate obtained from five patients by the proposed method (a) and the previous one (b)

k^a (day^{-1})		p^a (mmol)		S^a (mmol day^{-1})	
(a)	(b)	(a)	(b)	(a)	(b)
0.25±0.03	0.31±0.06	2.1±0.2	2.9±0.3	0.52±0.08	0.90±0.20
0.21±0.02	0.28±0.05	1.4±0.2	1.6±0.2	0.29±0.05	0.45±0.10
0.61±0.05	0.60±0.09	3.8±0.3	3.8±0.4	2.3±0.3	2.3±0.4
0.24±0.03	0.25±0.06	2.7±0.3	2.8±0.3	0.65±010	0.70±0.18
0.31±0.03	0.29±0.06	2.6±0.3	2.4±0.3	0.81±0.13	0.70±0.19

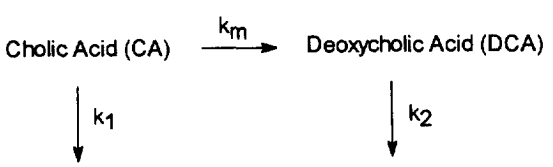
[a]Mean ($n=4$)±S.D.

(extractions, drawings, elutions, etc.); possibility of automation; better precision in the determination of bile acid mass and radioactivity.

Furthermore, there is the possibility of elaborating the data obtained for the biodynamics of the bile acid under examination for getting further information on the kinetics of the metabolic pathway of the same bile acid and its secondary one. Performing all the measurements of a kinetic experiment on the same plate, makes the results obtained independent of the counting efficiency of the radiometric measurement and of the intensity of the colour developed by means of the chromogenic reagent. This is a great advantage in comparison to the previous method, because there is no need to determine the corresponding calibration curves.

The only disadvantage encountered using this method lies in the necessity of spotting, very often, the bile methanol extract at two different concentrations: the radioactivity measurement is performed on the more concentrated spot.

The minimum detectable radioactivity results about 0.7 Bq, for counting times of 60 min; since the administered dose of [^{14}C]cholic acid is normally 160–185 kBq, and the pools are in the range 1–3 mmol, $A_s(t_0)$, which obviously represents the maximum value of measurable radioactivity, ranges between 50 and 180 kBq/mmol. As a consequence, for



Scheme 1.

obtaining countings with a good statistic error (3%), it is necessary to spot a mass of CA of at least 10 nmol. This value lies out of the range of linearity for the densitometric quantitation and, therefore, an accurate measurement requires an opportune dilution of the extract.

3.3. Elaboration of the radiochromatographic data

Recent studies have revalued the role of deoxycholic acid, the dihydroxy bile acid produced from cholic acid through a reaction of 7α-dehydroxylation by intestinal bacteria, both in the pathogenesis of biliary cholesterol gallstones and, together with unknown factors, in the pathogenesis of some chronic cholestatic liver diseases [10]. DCA, highly hydrophobic and capable of solubilizing cellular membranes, seems to induce an increased secretion of cholesterol into bile [11] and, as a consequence, cholesterol supersaturation, nucleation and, finally, gallstone formation. In the last 20 years, the use of BA such as CDCA and ursodeoxycholic acid (UDCA) for the dissolution of cholesterol gallstones prompted many studies, but their mechanism is poorly understood [12]. For these reasons, the study of the intestinal BA metabolism is still attracting the interest of scientists for the need of fully understanding their role in the pathogenesis and the treatment of gastro-enteric affections.

We have tried to elaborate the radiochromatographic data obtained in the study of the pool and turnover constant of CA (k_{CA}) in order to better characterise the kinetics of CA and possibly to obtain information on the elimination constant of the secondary acid DCA. A simple monocompartmental model, reported in Scheme 1, was first proposed,

which is valid under the assumption that the transformation of CA into DCA is irreversible, as demonstrated by Lindstedt and Samuelsson [13] when studying the interconversion of CA and DCA in the rat. In the scheme k_1+k_m represents the turnover constant for cholic acid ($k_{CA}=k_1+k_m$) and k_2 is the turnover constant for deoxycholic acid (k_{DCA}). On the basis of the scheme the differential equations describing the kinetics are the following:

$$\frac{dA_{CA}}{dt} = -A_{CA}\cdot k_m - A_{CA}\cdot k_1 \quad (1)$$

$$\frac{dA_{DCA}}{dt} = A_{CA}\cdot k_m - A_{DCA}\cdot k_2 \quad (2)$$

where A_{CA} and A_{DCA} indicate the activity of cholic and deoxycholic acid, respectively. By solving Eq. (1) and Eq. (2), and introducing R, defined as the ratio of radioactivity of DCA to radioactivity of CA, we obtain:

$$R = \frac{k_m}{k_2 - k_m - k_1}\cdot[1 - \exp[-(k_2 - k_m - k_1)t]] \quad (3)$$

Once experimental R values at different times are available, which is possible from the radiochromatographic profiles obtained for the bile samples of the patients to whom [^{14}C]CA had been administered, k_m and the term $k_2-k_m-k_1$ can be evaluated. Since $k_{CA}=k_m+k_1$ has been computed from the measurement of R_s at different times, the values of k_2 and k_1 can be estimated.

It can be concluded that the elaboration of the radiometric data here described allows the estimation of all the kinetic constants relevant to the simple monocompartmental model.

Fig. 2 shows the values of R as a function of time obtained for the patients to whom a tracer dose of [^{14}C]cholic acid has been administered. The values for the patients affected by a similar pathology have been indicated by the same symbol. In particular, the exponential curve shown in Fig. 2 well accounts for the trend of the values relative to the 12 patients with a nonfamilial hypercholesterolemia. The two patients, indicated by the full triangle symbol, who did not show any conversion of CA to DCA, were affected by primary biliary cirrhosis. These results are in satisfactory agreement with the findings by Williams et al. [14] who reported a remarkable

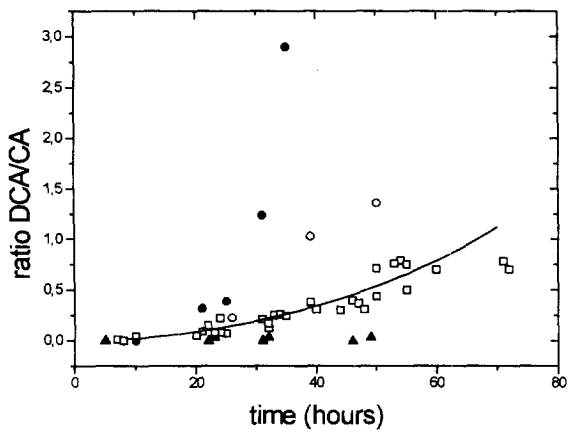
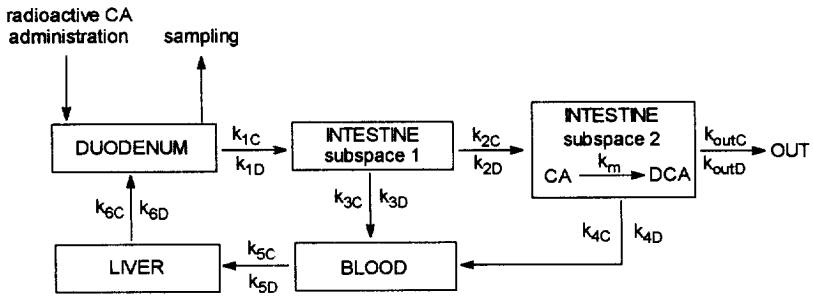


Fig. 2. Experimental values of the ratio between radioactivity of DCA and CA, respectively, as a function of time. (□) 12 patients with nonfamilial hypercholesterolemia; (▲) two patients affected by primary biliary cirrhosis; (○ and ●) a cholecystectomised patient before and after therapy with simvastatin, respectively.

increase of CA associated with a notable decrease of DCA in bile in this type of patient. If this trend of R could be confirmed for a statistically significant number of patients, the study of CA metabolism might be a valid tool for an early diagnosis of primary biliary cirrhosis.

At this point a question arises, i.e., if a single monocompartmental model can be considered a definite tool or only an acceptable approximation for studying the kinetics of the bile primary acids. A different model, based on a multicompartmental system much simpler than that proposed by Hofmann et al. [15] has been elaborated for describing the metabolism of cholic acid within the enterohepatic circulation. This model is shown in Scheme 2 where k_{iC} and k_{iD} represent the kinetic constants for CA and DCA, respectively, for the compartment i.

The system of differential equations derived from the model has been numerically resolved (Runge-Kutta, IV-order). The resulting trends of R and $\ln R_S$ vs. time are reported in Fig. 3. The analysis of these plots provides evidence of the good agreement of the calculated values with the experimental data relative to two of the 12 patients for which the exponential curve has been drawn in Fig. 2. These two series of values have been chosen on the basis of the best fit with the theoretical model. In particular, the trend of $\ln R_s$ vs. time, resulting from the proposed multicom-



Scheme 2.

partmental model, supports Lindsted's assumption, extended by other researchers to all BA [15], that the metabolism of CA follows a first-order kinetics under steady-state conditions. Actually, only from a certain time onwards the trend is linear and, consequently, the slope of the regression line representing the best fitting of the experimental data, gives a correct value of $k_{CA}=k_m+k_1$. However, the extrapolation of the linear portion of the curve down to $t=0$ leads to an underestimated value of $\ln R_S$ and therefore to an overestimation of the cholic acid pool (see Sections 2.4.1 and 2.5.1).

The proposed multicompartmental model also allows determining the trends of R and $\ln R_S$ in blood which are shown in Fig. 4. As to the behaviour of $\ln R_S$ function, it can be noticed that the linear portion of the curve relative to blood is parallel to the curve relative to duodenum; the blood trend results only translated to lower values since the BA concentration in blood is much lower than that recovered in bile. Since we do not have any experimental values for the radioactivity of cholic and deoxycholic acids in samples of blood, the R values obtained for the bile samples of the two patients reported in Fig. 3 have been multiplied by a factor f that accounts for the different dilution ratios of CA and DCA between the blood and bile compartments, and plotted in Fig. 4. The resulting values well fit the theoretical trend forecast by the proposed model.

As a consequence, measurements carried out on blood samples should give the same information as those obtainable from bile samples, with the obvious advantage of using a less invasive technique, since few individuals tolerate repeated nasoduodenal intu-

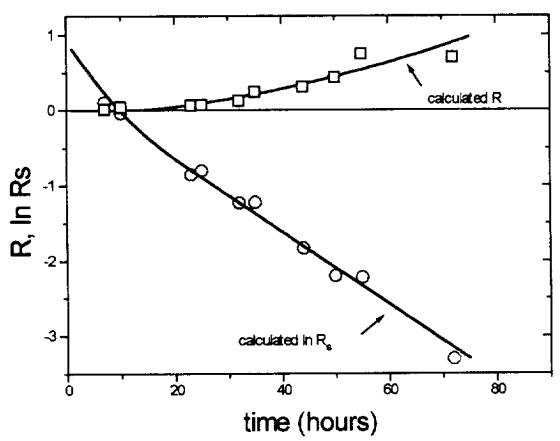


Fig. 3. Theoretical trend of R and $\ln R_S$ (bile) vs. time according to Scheme 2. (□ and ○) The experimental values obtained for two patients belonging to the group indicated by (□) in Fig. 2.

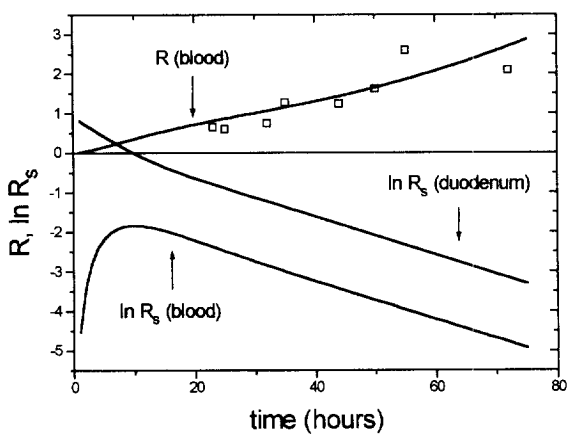


Fig. 4. Theoretical trend of $\ln R_S$ (blood) vs. time according to Scheme 2. For comparison, the theoretical trend of $\ln R_S$ relative to the duodenum and the values of R for blood (□), calculated by multiplying the experimental values relative to the same two patients of Fig. 3 by a factor f, are also reported.

bations. These findings are in agreement with those obtained by Stellaard et al. [5] and Everson [7], who demonstrated that kinetics from bile and serum for both CA and CDCA were very similar when administering BA labelled with stable isotopes to healthy subjects.

Despite the considerable limitations in available experimental data, we believe that the proposed model should be of notable utility. It could be used for predicting the behaviour of CA metabolism and for classifying alterations in the enterohepatic circulation in man in the presence of some pathological conditions.

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