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A SIMPLE METHOD FOR THE CORRECTION OF BILIARY EXCRETION CURVES DISTORTED BY THE BILIARY DEAD SPACE*

MARCELO G. ROMA, ERNESTO A. LUQUE, RAÚL A. MARINELLI and EMILIO A. RODRÍGUEZ GARAY†

Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Rosario, Rosario, Argentina

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Abstract—Biliary solute concentrations measured at the tip of the cannula suffer a delay with respect to bile flow due to the transit time through the biliary tree volume. This study proposes a simple method, which is valid under variable bile flow conditions, to correct the distortion introduced by the biliary tree volume on the kinetic curves of the biliary excretion rate. The biliary transit time (t_t) was calculated as the time needed to excrete a bile volume equal to the biliary tree volume by means of the interpolation of biliary cumulative volume versus time curves. Such t_t permits one to estimate the canalicular concentration at time t, interpolating the biliary concentration curves at time t-t_t. The product between the estimated canalicular concentration and the bile flow allows the calculation of the corrected biliary excretion rate. This method was evaluated by a comparison between biliary excretion rate curves of [^{14}C]taurocholate ([^{14}C]TC) injected as a bolus under basal and sodium dehydrocholate (DHC)-induced choleresis conditions. Since the canalicular excretion rate of [^{14}C]TC is considered independent of bile flow, the significant differences observed in its excretion kinetics under both conditions were attributed to distortion due to the biliary tree volume. After the correction, both curves showed a significant overlapping. This result indicates that the method improves the time–course representation of canalicular events in biliary excretion kinetic studies.

Biliary kinetic studies are usually required to characterize the mechanisms involved in the bile formation and the role of the liver as eliminating organ for endogenous and exogenous compounds [1–4]. For this purpose, the bile is collected through a bile duct cannula in consecutive time periods. The excretory rates of the compounds under study are commonly calculated from bile flow and solute concentration values measured at the tip of the cannula. However, solute concentration suffers a delay with respect to bile flow on account of transit time through the collecting bile ductules and ducts and also the cannula employed. In this way, the excretion rates thus calculated do not reflect the changes suffered by the biliary output at the canalicular level.

Several methods have been reported to determine the capacity of the biliary tree under various experimental conditions [5–9]. However, no reliable methods have been proposed to correct the distortion introduced by the biliary tree space on the excretory curves including cases in which the bile flow changes during the study. Lack of a dependable method to make that correction has forced investigators to choose no correction at all rather than an arbitrary and possibly incorrect procedure [10].

Therefore, in this paper we describe a simple method for correction of the excretory curves distorted by transit through the dead space of the biliary tree. In addition, an experimental procedure to ascertain the validity of the correction method is also presented.

THEORY

The biliary excretion rate of a compound usually is estimated from bile solute concentration $C_b(t)$ and bile flow $F(t)$ values measured in bile collected through a bile duct cannula at time t. Thus, the biliary excretion rate uncorrecting for the dead space of the biliary tree (ER_{unc}) is calculated as:

$$ER_{unc} = C_b(t)F(t) \quad (1)$$

Since the transit time through the biliary tree (t_t) is not negligible, solute concentration in the canaliculus at time t is detected with some delay in the bile collected from a bile duct catheter. Assuming that this delay is uniform, average canalicular solute concentration (C_c) may be related to biliary concentration in the following way:

$$C_c(t) = C_b(t + t_t) \quad (2)$$

Transit time may be easily calculated when bile flow is constant as:

$$t_t = V_{BT}/F \quad (3)$$

where V_{BT} is the sum of the volume present in the biliary tree volume and the polyethylene cannula.

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† Correspondence: Dr Emilio A. Rodríguez Garay, Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas - U.N.R., Suipacha 570, 2000 Rosario, Argentina.

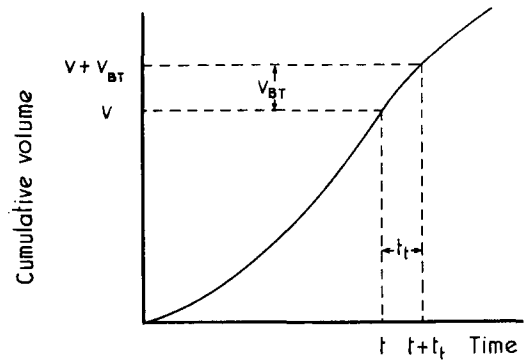

Fig. 1. Cumulative volume of collected bile plotted against time. As indicated in this graph, the biliary t_t is calculated as the time necessary to collect a bile volume equal to V_{BT}.

When the bile flow is not constant, assuming that choleresis does not modify V_{BT}, the delay t_t can be calculated from the time–course of the cumulative volume of bile collected, as illustrated in Fig. 1. When cumulative volume V is measured at time t, delay t_t is the time necessary for cumulative volume to reach the value $V + V_{BT}$. If $T(V)$ is the inverse function of $V(t)$ (it yields the time T necessary to reach a cumulative volume V), the delay t_t may be calculated as:

$$t_t = T(V + V_{BT}) - T(V) \quad (4)$$

Delay t_t is an implicit function of time because, when bile flow changes with time, t_t also changes. Incidentally, when bile flow remains constant, Eqn. 4 is reduced to Eqn. 3.

On the basis of the considerations described, the biliary excretion rate of a solute at the canalicular level (corrected biliary excretion rate ER_c) can be estimated as follows:

$$ER_c = C_c(t)F(t) = C_b(t + t_t)F(t) \quad (5)$$

MATERIALS AND METHODS

Animal preparation. Adult male Wistar rats weighing 250–350 g were used. The animals were anesthetized with sodium pentobarbital (50 mg/kg body wt, i.p.) and thus maintained throughout the experiments. A polyethylene catheter (PE-10, Intramedic, U.S.A.) was inserted into the bile duct. The femoral vein was also catheterized (PC-40, Rivero y Cía, Argentina). A tracheal cannula was systematically employed, and the rectal temperature was maintained at 37.5 to 38.0° with a heating lamp to prevent hypothermal alterations of the bile flow [11].

Experimental procedure. The biliary excretion rate of [^{14}C]taurocholate ([^{14}C]TC) (46.7 mCi/mmol, New England Nuclear, U.S.A.) was measured on each animal under basal conditions and following the injection of the choleretic agent sodium dehydrocholate (DHC) (Sigma Chemical Co., U.S.A.).

A bolus i.v. injection of [^{14}C]TC (0.5 μCi dissolved in 0.3 ml of saline and flushed with 0.2 ml of this isotonic solution) was administered. Bile was collected in 20-, 30- or 60-sec periods up to 13 min (basal choleresis). Then, the remaining radioactivity was allowed to be eliminated for about 60 min. A Ringer–Krebs–bicarbonate buffer enriched with bovine albumin (Sigma Chemical Co., U.S.A.) (3 mg/ml) and glucose (4 mg/ml) was infused during this period. At the end of this time interval, the bile flow did not differ from baseline values. Then, a bolus injection of a second similar dose of [^{14}C]TC was administered i.v. with the simultaneous incorporation of DHC (10 μmol/100 g body wt). Bile collection (DHC-induced choleresis) was performed in the same fashion as described above. At the end of the experiment the animal was killed by exsanguination, and the liver was removed, gently blotted on filter paper, and weighed.

Analytical procedures. The volume of the bile was measured by weight assuming a density of 10 g/ml. Bile flow was expressed as $\mu l \cdot min^{-1} \cdot (g\ liver)^{-1}$. Radioactivity in the bile was measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, U.S.A.). Bile aliquots were mixed in 1 M methylbenzethonium chloride (hyamine) in methanol and 10 ml of 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) toluene solution. An external standard method was used for quenching correction. Concentrations of ^{14}C in bile samples (basal and DHC-induced choleresis periods) (dpm/μl) were normalized to the total dpm excreted during each 13-min period of bile collection, giving units of μl^{-1}. Thus, the respective excretion rates were units of $min^{-1} \cdot (g\ liver\ wt)^{-1}$.

The volume of the biliary tree was calculated according to Barber-Riley [5], as modified by Häcki and Paumgartner [7], using [^{14}C]TC as marker. Volumes of the biliary tree were determined in each animal for both experimental conditions (basal and DHC-induced choleresis). In this way, individual measurements were used for correction of the corresponding excretory curves. In every case, the volume of bile excreted during the first 20 sec (lag of appearance of the injected marker in the canaliculus) was subtracted from the biliary tree capacity, as reported previously [7].

Processing of experimental data. The data employed to perform the calculation of the corrected biliary excretion rate were $V(t_i)$ and C_{bmi}. $V(t_i)$ represents the cumulative volume of bile collected throughout the experiment measured at the end of each period of bile collection (t_i). Bile collection periods were denoted as $i = 1, 2 \ldots n$, where $i = 1$ was the first sample collected after injection of marker. C_{bmi} was the mean marker concentration in bile sample i.

The time–course of the canalicular marker excretion rate may be calculated according to Eqn. 5. Thus, the mean value for each sample (ER_{cm}) was obtained by:

$$ER_{cmi} = \frac{1}{(t_i - t_{i-1})} \int_{t_{i-1}}^{t_i} C_b(t - t_t)F(t)\,dt \quad (6)$$

For this calculation, t_0 means the value of the lag of appearance of [^{14}C]TC in the canaliculus (transit time between injection and appearance of marker in bile). This lag includes the circulation delay and the transit time through the hepatocyte. A value of 20 sec

was used for t_0, as determined by Häcki and Paumgartner [7].

The integral in Eqn. 6 was numerically calculated by means of a gaussian quadrature [12]. The value of t_t for intermediate points within the integration intervals was determined using Eqn. 4. The values of $T(V)$ and $C_b(t)$ corresponding to intermediate points were obtained by interpolation of the experimental data using cubic splines [12]. Alternatively, two empirical functions fitted with a non-linear least squares regression program [13] were tried. Piecewise-linear functions [14] were used to fit cumulative volume data and a gamma-variate function [15] to fit biliary concentration data. The use of cubic splines was preferred because there were no detected differences in the final results using either method; the cubic splines were more direct and faster to calculate.

The bile flow $F(t)$ was determined as the time derivative of the interpolated $V(t)$ curve. This strategy was preferred to the direct interpolation of the mean bile flow data because cumulative volume measured at the end of each collection period may be considered exact. Instead, mean bile flow values are usually arbitrarily assigned to the midpoint of each interval without knowing the theoretical time dependence.

A BASIC program was written to implement all calculations. Its listing is available on request to the authors.

Statistical analysis. All data are presented as means ± SE. Paired *t*-tests were used to calculate the significance of differences between mean values, and $P < 0.05$ was considered significant. The superposition of excretory curves in both choleretic conditions (basal and DHC-induced choleresis) was estimated using a coefficient of variation (CV), defined as:

$$CV = \frac{[\Sigma\,(ER_{\text{basal i}} - ER_{\text{DHC i}})^2/(n-1)]^{1/2}}{\Sigma\, ER_{\text{DHC i}}/n}$$

RESULTS AND DISCUSSION

Experimental valuation of the correction method. In this study we described a procedure to correct the pattern of bile solute excretion rate distorted on account of transit along the biliary tree dead space. The validity of this procedure may be tested by study of the biliary excretion kinetics of a tracer solute whose canalicular excretion was independent of bile flow under both constant bile flow and induced choleresis conditions. In this way, all differences between both experimental curves should be attributed to the increased bile flow in the latter condition which promotes a decrease in transit time of the marker while it travels along the biliary tree.

For this purpose, [^{14}C]TC uncorrected excretory curves obtained under basal and DHC-induced choleresis were compared to those corrected, applying the method which was described. Figure 2A shows a representative experiment in which the non-corrected excretion rate of the marker under DHC-induced choleresis reached its maximum earlier and rose to a greater value than under basal conditions. When data were processed (Fig. 2B), both curves showed a significant overlapping. The values of the coefficient of variation, which affords a quantitative estimation of the differences between the biliary excretion curves previous to and after the correction, are presented in Table 1. The significant reduction obtained after the correction can be considered indicative of the appropriateness of the method proposed.

TC satisfies properly the conditions required for a marker in the validation procedure although its canalicular excretion mechanism is still a subject of discussion. This endogenous bile salt has been reported to be actively transferred to the canaliculus [16, 17]. In that case, its canalicular transport should not be affected by a possible lowering of the gradient across the hepatocyte canalicular membrane when an increased bile flow exists. Alternatively, in the case of passive translocation (facilitated diffusion) as was also suggested [18, 19], TC is a bile salt that promotes the formation of micelles [20] and, therefore, its free concentration gradient across the canalicular membrane would not be affected by our experimental conditions. In fact, we estimated that the TC biliary concentration under maximal induced choleresis was well above (> 10 mM) the critical micellar concentration (~ 2–5 mM) [20, 21], and its dilution by water excreted in excess may not be capable of affecting free (intermicellar) TC concentration in the canaliculus. In agreement with those considerations, no significant difference was observed between the total amounts of [^{14}C]TC recovered during the whole bile collecting period under basal and DHC-induced choleresis conditions.

On the other hand, DHC (a synthetic triketocholanic bile acid) was selected as a tool to induce an increase in bile flow due to its lack of competition with TC for the canalicular transport system [22]. In addition, DHC has a high choleretic efficiency and a very weak cytotoxic effect at the dose employed here [23].

Biliary tree volume. The biliary tree volume is an important parameter in the calculation algorithm proposed. As anticipated, there were no significant differences between the values obtained for this parameter during basal and DHC-induced choleresis conditions (1.45 ± 0.07 μl/g liver wt vs 1.32 ± 0.09 μl/g liver wt; N = 5). Indeed, the method used to measure the biliary tree volume determines the washout volume contained within the biliary tree prior to the secretion of the marker to the canaliculus [7]. As the time of arrival to the canaliculus for DHC is not expected to be shorter than that of TC used as marker, a distension of the biliary tree as a result of the choleresis induced by DHC should have no effect on the calculation of the biliary dead space.

The values of biliary tree volume obtained in this study were lower than those reported by Häcki and Paumgartner (2.30 ± 0.11 μl/g liver wt) who injected [^{14}C]TC simultaneously with a dose of unlabeled TC [7]. The transit time of the marker through the hepatocyte has been considered to be an important factor capable of explaining the different values reported when different markers are employed [5, 8]. Our finding of a lower value for the biliary tree volume when using DHC as a choleretic agent may be interpreted by a longer hepatocyte transit time of

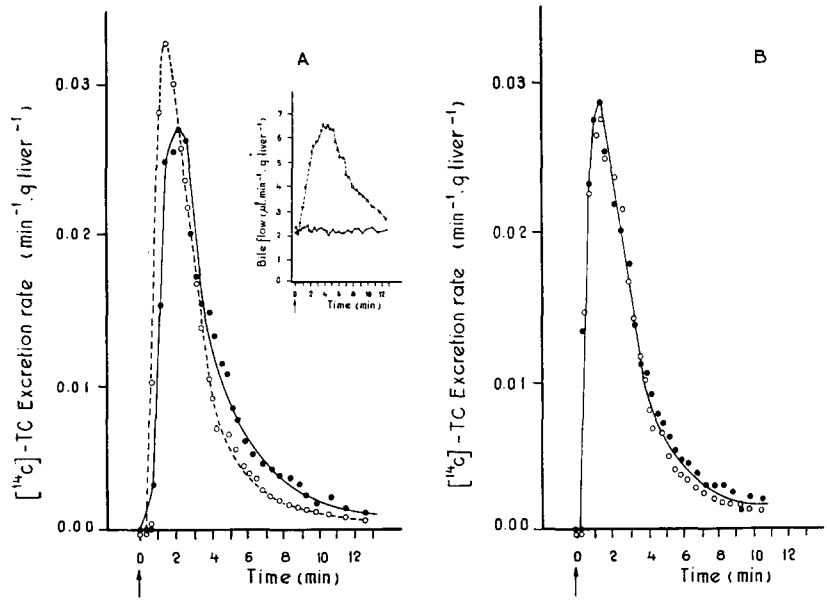

Fig. 2. Representative experiment of the biliary excretion rate of [^{14}C]TC under basal (●) and DHC-induced (○) choleresis conditions. (A) Prior to the correction (calculated by Eqn. 2). (B) After the correction (calculated by Eqn. 6). The inset shows the bile flow time–course in both choleresis conditions. All points were plotted at the midpoint of each collection period. Arrows indicate bolus administration of [^{14}C]TC.

Table 1. Coefficients of variation previous to and after the correction of biliary excretion rate curves of [^{14}C]TC

	Coefficient of variation (%)	
	Mean ± SE*	Range
Uncorrected curves	46.7 ± 3.5	36.1–54.0
Corrected curves	20.0 ± 2.4	13.2–27.4

* Values for five experiments. The differences were statistically significant ($P < 0.005$).

[^{4}C]TC when TC is employed as a choleretic agent due to competition between the marker and TC for the canalicular carrier. In agreement with this assumption, we found in preliminary experiments that simultaneous injection of [^{14}C]TC and unlabeled TC in a dose similar to that used by Häcki and Paumgartner diminished significantly (about 30%) the total tracer amount excreted in bile in comparison with the experiments where [^{14}C]TC was administered alone. In contrast, an equal dose of DHC did not affect the total amount of [^{14}C]TC excreted (data not shown). This result is in agreement with the study of Meijer *et al.* [22] who demonstrated that these two bile salts do not share a common biliary transport system. Furthermore, when in a previous report we used TC instead DHC as a choleretic [24], the biliary tree volume value was coincident with the value reported by Häcki and Paumgartner [7].

General conclusions. Biliary dead space correction is necessary in those kinetic studies that require bile sampling periods of the same order as the dead space transit time in order to describe properly the canalicular events. In our experiments, basal transit times ranged between 40 and 55 sec. This led to a correction when sampling periods were shorter than about 10 min, in which more than 9% of the volume collected corresponded to the biliary tree washout. The correction is particularly necessary when excretory patterns of xenobiotics or endogenous compounds capable of inducing osmotic choleresis are compared. An adequate correction would allow a comparison of the excretory patterns at the canalicular level, which may be quite different from that observed distally under those situations.

Even though the present results may improve kinetic analysis of liver excretory processes, more physiological models should be developed. The proposed method assumes no changes in the biliary tree volume due to distensibility in choleresis conditions. Besides, it was considered that no net reabsorption or secretion of fluid occurred within the bile ducts, capable of modifying the transit time through the biliary tree. Finally, it was assumed that there was no heterogeneity in the biliary transit times, i.e. a bolus at canalicular level would be excreted as a bolus, but delayed. This is a highly simplified picture of the biliary tree architecture, but the inclusion in the proposed method of such heterogeneity and the other factors discussed above is limited by poor knowledge of the biliary tree morphology and physiology. Further advances in these subjects may allow investigators to improve the proposed method. In spite of its simplicity, because of the satisfactory results obtained in the experimental valuation, this procedure may be a practical and suitable approach to performing corrections of biliary excretion kinetic curves, allowing us to improve the time–course representation of canalicular events in biliary secretion studies.

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