Moment Analysis of Metabolic Heterogeneity: Conjugation of Benzoate with Glycine in Rat Liver Studied by Multiple Indicator Dilution Technique

ANDREAS J. SCHWAB, LEI TAO, MANJINDER KANG, LINGJIE MENG, and K. SANDY PANG

McGill University Medical Clinic, McGill University Health Centre, Montreal, Canada (A.J.S.), and Departments of Pharmaceutical Sciences (L.T., M.K., L.M., and K.S.P.) and Pharmacology (K.S.P.), Faculties of Pharmacy and Medicine, University of Toronto, Toronto, Canada

ABSTRACT

Metabolic zonation was assessed with the multiple indicator dilution (MID) technique in the single-pass perfused rat liver with use of moment analysis of the formed metabolite (M) data. During single-pass, retrograde rat liver perfusion with 17 μM benzoate, a bolus containing tracer preformed metabolite (PM) (1H]hippurate was injected rapidly into the hepatic vein at 20 min postperfusion, followed by injection of a second bolus containing [14C]benzoate at 30 min. Both doses also contained noneliminated reference indicators (51Cr-labeled RBCs, 125I-labeled albumin, [14C]- or [3H]sucrose, and 2H2O). The steady-state extraction ratio of benzoate, the area under the curve (AUC) and its mean transit time (MTT) during retrograde flow were identical to those previously observed for prograde flow. Values of AUCPM and MTTPM and AUCM were also similar to previously published prograde data, but the MTTM with retrograde perfusion was smaller than that for prograde perfusion. This, according to theory based on the tubes-in-series model, was consistent with perivenous enrichment of glycination activity when transport of drug was even and when the ratio of drug influx/efflux coefficient exceeded that for metabolite. Similar benzoate transport in periportal, homogeneous and perivenous isolated rat hepatocytes existed, and the influx/efflux coefficients (partition ratio) of benzoate from MID indeed exceeded that of hippurate. However, metabolism by zonal hepatocytes failed to reveal the anticipated metabolic zonation, and this is likely due to the shallow gradient of metabolic activity. The study demonstrates that moment theory is useful in delineating the perivenous enrichment of glycine conjugation activity.

Drug removal by the liver involves the microcirculation, binding-debinding, transport, metabolism, and excretion, and is a distributed-in-space phenomenon that is modulated by zonation. A common method for probing enzyme zonation in the absence of a transport barrier is single-pass prograde/retrograde perfusion of the rat liver preparation (Pang and Terrell, 1981; St-Pierre et al., 1989). With prograde perfusion, perfusate enters the portal vein and exits at the hepatic vein, first recruiting upstream and then downstream enzymes. With retrograde perfusion, flow enters the hepatic vein, exits the portal vein, and exits the hepatic vein, first recruiting upstream and then downstream enzymes. With retrograde perfusion, flow enters the hepatic vein and exits the portal vein such that zonal distributions of enzymes appear reversed (Pang and Terrell, 1981; Pang et al., 1983). A more elaborate perfusion protocol is the dual perfusion of the hepatic artery (HA) and portal vein, or the hepatic vein (HV). If the substrate is delivered via HA single-pass to the liver with blank perfusate flowing retrogradely (HAHV), substrate will be confined to the periportal (PP) space. Conversely, with prograde flow of blank perfusate entering the portal vein (HAPV), substrate delivered from HA is swept across the entire liver (Pang et al., 1988). The removal rate constants for the respective regions may be obtained upon normalization of the metabolic activity to the cellular water spaces accessed (Chiba et al., 1994).

By contrast, assessment of metabolic heterogeneity behind a transport barrier in the intact liver is more complex. The metabolic rates will depend on the transport of the drug and metabolite and whether the enzyme distribution is periportal (PP) or perivenous (PV). It is known that if transport across the hepatocyte membrane is poor, the fate of formed and preformed metabolites may differ (Sato et al., 1986; deLannoy and Pang, 1987; Schwab and Pang, 1999). Although there exist a few reports on the use of metabolite data to assess enzyme zonation after pulse injection of drug to the liver.
perfused rat liver preparation (Mellick et al., 1994; Ballinger et al., 1995), the basis for estimation has not been rigorously developed theoretically. A recent theoretical treatise had examined the influence of enzyme zonation on the AUC and MTT of drugs and metabolites and the transmembrane transport when the liver is viewed as two half-parallel tubes arranged in series, or the tubes-in-series model (Schwab and Pang, 1999). Not unexpectedly, the mean residence time of the formed metabolite (MTT_M) depended on both transporter activities and localization of enzymes.

In this communication, we hypothesized that moment analysis of the metabolite is useful for examination of enzyme heterogeneity. Benzoate metabolism by glycine conjugation was chosen to illustrate the principle. A perivenous (zone 3) predominance of glycine conjugation has been inferred from HAPV and HAHV liver perfusion studies (Chiba et al., 1994). The metabolite, hippurate, undergoes saturable, barrier-limited transport into the rat liver, and is neither metabolized nor excreted into bile (Yoshimura et al., 1998). Benzoate also undergoes barrier-limited transport but is transported linearly and rapidly into hepatocytes at concentrations below 1 mM (Schwab et al., 2001). Transport is likely attributed to the monocarboxylic acid transporter 1 (Tamai et al., 1999) that transports l-lactate and is evenly distributed in the acinus (Staricoff et al., 1995). Although the mitochondrial space for metabolism constitutes an added, intracellular compartment (Chiba et al., 1994; Schwab et al., 2001), the attributes are ideal for the testing of the principles of moment analysis on enzyme zonation. In this study, a bolus containing tracer preformed [3H]hippurate (PM) was injected rapidly into the hepatic vein of the rat liver at 20 min post-perfusion to characterize the hippurate transfer coefficients. This was followed by a second injection containing [14C]benzoate at 30 min to characterize the transfer and metabolic coefficients of benzoate. Both injected doses also contained nonelminated reference indicators (51Cr-labeled RBCs, 125I-labeled albumin, [14C]- or [3H]sucrose, and 2H2O). The derived retrograde outflow data (17 ± 1 μM benzoate) were analyzed according to the barrier-limited, variable transit time model of Goresky (Goresky et al., 1973) and were compared with those observed previously for prograde flow under first-order conditions (<200 μM benzoate) (Schwab et al., 2001). However, due to our inability to encompass heterogeneity into the Goresky model, the previously developed tubes-in-series model (Schwab and Pang, 1999) was extended to encompass heterogeneity in mitochondrial metabolism behind transport barriers. The expectations were the unchanged area under the curve for preformed (PM) and formed (M) hippurate, an unchanged MTT_PM for PM, but a smaller MTT_M for formed hippurate for retrograde flow, since metabolic activity is distributed in the perivenous region. We further studied zonal transport and metabolism of benzoate with enriched, zonal rat hepatocytes.

Materials and Methods

Benzoic acid, hippoc acid, and bovine serum albumin (fraction V) were obtained from Sigma-Aldrich (St. Louis, MO). [14C]Benzoic acid (specific activity, 110 mCi/mmol) and the [2-3H]glicine (specific activity, 43.4 Ci/mmol) used for the synthesis of hippurate (Yoshimura et al., 1998) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and DuPont Canada, Inc. (Markham, ON, Canada), respectively. [54Cr]Sodium chromate (specific activity 5392 mCi/mg), [125I]-labeled serum albumin (specific activity 0.46 mCi/mg), and 2H2O (>99.98% pure) were procured from Merck Frosst (Montreal, QC, Canada). [14C]Sucrose (4.95 mCi/mmol) and [3H]sucrose (12.3 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Digitonin was obtained from Fluka (Buchs, Switzerland). Collagenase was purchased from Roche Diagnostics (Oakville, ON, Canada). All reagents were used from glass-distilled, high-performance liquid chromatographic grade, or the highest purity available (Fisher Scientific, Mississauga, ON, Canada).

Transport and Metabolic Studies in Isolated and Zonal Rat Hepatocytes

Isolation of Homogeneous, Periportal (PP), and Perivenous (PV) Rat Hepatocytes. Enriched PP and PV hepatocytes and homogeneous hepatocytes from male, Sprague-Dawley rats (304 ± 28 g; Charles River Canada, St. Constant, QC, Canada) were prepared according to the digitonin/collagenase perfusion method of Lindros and Penttila (1985), with modifications (Tan et al., 1999). The rats were housed in accordance with approved protocols of the University of Toronto Animal Committee, kept under artificial light on a 12:12-h light/dark cycle, and allowed free access to water and food ad libitum. Hepatocyte viability was assessed by Trypan blue exclusion, and averaged 93 ± 1.4% for the transport studies and 94 ± 2.8% for the metabolism studies. Zonal enrichment was defined with respect to the activities of alanine aminotransferase and glutamine synthetase, assayed by a commercially available kit (Sigma-Aldrich) and by a standard UV method (Tan et al., 1999; Tirona et al., 1999), respectively; the PP/PV ratio was 1.5 for alanine aminotransferase and 12 for glutamine synthetase. Protein was assayed by the method of Lowry et al. (1951).

Uptake of Benzoate by Isolated and Zonal Rat Hepatocytes. Uptake studies were conducted as outlined previously (Tirona et al., 1999). All buffers were pregressed with Carbogen (95% O2, 5% CO2; Canox Gas, Mississauga, ON, Canada). After preincubation in an atmosphere of Carbogen for 10 min at 37°C, uptake of [14C]benzoate (1 and 400 μM with 215,000 ± 102,000 dpm/ml) and [3H]sucrose (385,000 ± 301,000 dpm/ml), an interstitial space marker, by PP (n = 3) and PV hepatocytes (n = 3) and isolated (homogeneous) hepatocytes (n = 4) was examined over the course of 1 min in 1.67 × 106 cells. Samples were removed at 15, 30, 45, and 60 s for rapid centrifugation through a layer of silicon oil (100 μl, density 1.02 g/ml) into the lowest layer of 50 μl of 3 N NaOH. After removal of the tips into a 20-ml glass scintillation vial and left overnight, 50 μl of 3 N H2SO4 was added to neutralize the sample. After the addition of scintillation fluid (Ready Safe; Beckman Coulter, Canada, Mississauga, ON, Canada), the radioactivity of the cell and the supernatant (25 μl) were quantified in a liquid scintillation spectrometer (LS8001; Beckman Coulter Canada). The extracellular volume entrapped in the cellular compartment was found by the [3H]sucrose content, and a correction was made in the determination of cellular contents on [14C]benzoate uptake.

Metabolism of Benzoate in Zonal Rat Hepatocytes. Cell suspensions of PP or PV hepatocytes, preincubated at 37°C for 10 min, were added to mixtures of [14C]benzoate and unlabeled benzoate to result in concentrations of 480,000 ± 136,000 dpm/ml and 1 μM in 1.6 to 1.7 × 106 cells/ml. Duplicate samples (100 μl) were retrieved simultaneously at various times (0.5–7.5 min) directly into a 1.5-ml microcentrifuge tube containing 0.5 ml of acetonitrile (and internal standard, methoxybenzoic acid) for HPLC analysis using a reverse-phase Beckman Ultrasphere column as previously described (Chiba et al., 1994; Cong et al., 2001; Schwab et al., 2001). The samples were mixed well and stored at −20°C until analysis. After thawing and centrifugation, 200 μl of the sample was injected directly onto the HPLC column. Standards of known, different counts were processed in an identical fashion for construction of the calibration curve.
Rat Liver Perfusion

Male Sprague-Dawley rats (n = 8; 296 ± 9 g; livers were 11 ± 2.2 g) served as liver donors. In situ single-pass liver perfusion under retrograde flow was carried out as previously described (St-Pierre et al., 1989) with perfusate (12 ml/min) at 37°C entering via the hepatic vein and exiting via the portal vein; the hepatic artery was ligated. Perfusion consisted of bovine erythrocytes (20%), freshly obtained and washed (kind gift of Ryding-Regency Meat Packers, Toronto, ON, Canada), 5% bovine serum albumin, and 17 mM glucose (Baxter Travellon Laboratories, Deepport, IL) in Krebs-Henseleit bicarbonate solution (pH 7.4). Perfusion was gassed simultaneously with 95% oxygen/5% carbon dioxide (Matheson, Mississauga, ON) and oxygen (BOC Gases, Whitby, ON, Canada), and the pH, monitored by an "on-line" flow-through pH electrode (Thermo Orion, Boston, MA), was maintained at 7.4 by adjusting the proportion of the two gas supplies. Three to five inflow and outflow perfusate samples, collected during steady state, were used for determination of the average input (C_in) and output (C_out) perfusate concentrations of unlabelled benzoate and hippurate; both benzoate and hippurate do not distribute into red blood cells (Yoshimura et al., 1998; Schwab et al., 2001). Bile samples were collected for the first 15 min and at 5- to 10-min intervals thereafter up to 60 min to monitor the excreted radioactivity and the bile flow; the total recovery relative to the age input (C_in) and output (C_out) perfusate concentrations of unlabelled benzoate (17 ± 1.3 μM) in a composition otherwise identical to that of the perfusate and was introduced into the inflow system by an electronically controlled injection valve; subsequent outflow samples were continuously being collected for a total of 180 and 280 s, respectively, for the first and second injections, as described previously (Schwab et al., 2001). The hematocrits of the perfusate plasma was separated from red blood cells by centrifugation, and the contained [3H] and [14C] radioactivities in outflow perfusate and bile were assayed by high-performance liquid chromatography, as previously described (Chiba et al., 1994; Schwab et al., 2001). Perfusion plasma samples and bile were assayed by high-performance liquid chromatography, as previously described (Chiba et al., 1994; Schwab et al., 2001). The fit to the Goresky model will provide the transport parameters for hippurate (first dose) and benzoate (second dose) as well as the mitochondrial transfer and metabolic rate constants. However, we have not yet found a consistent way to model enzyme zonation in the Goresky-type model. Hence, we had an alternate approach for the detection of enzyme zonation from prograde and retrograde MID data by extending the existing theory on the tubes-in-series model (Schwab and Pang, 1999; Fig. 1A). However, with the present precursor-product pair, benzoate and hippurate, glycine conjugation occurs within the mitochondria (Gatley and Sherratt, 1977), evoking consideration of an extra pool (Fig. 1B). Results of MID studies by Schwab et al. (2001) indeed confirmed the involvement of a deep compartment for metabolism. Hence, the theory was extended to reflect this (see Appendix 1). Moments may then be calculated for this model, and the results are summarized in Table 1.

Theory

The theory pertaining to fits to the hippurate and benzoate outflow data according to the Goresky model is based on homogeneous enzyme and transporter functions but heterogeneous flow behavior (Fig. 1). The equations that have been described in detail for prograde flow are presently utilized but will not be repeated here (Schwab et al., 2001). The fit to the Goresky model will provide the transport parameters for hippurate (first dose) and benzoate (second dose) as well as the mitochondrial transfer and metabolic rate constants. However, we have not yet found a consistent way to model enzyme zonation in the Goresky-type model. Hence, we had an alternate approach for the detection of enzyme zonation from prograde and retrograde MID data by extending the existing theory on the tubes-in-series model (Schwab and Pang, 1999; Fig. 1A). However, with the present precursor-product pair, benzoate and hippurate, glycine conjugation occurs within the mitochondria (Gatley and Sherratt, 1977), evoking consideration of an extra pool (Fig. 1B). Results of MID studies by Schwab et al. (2001) indeed confirmed the involvement of a deep compartment for metabolism. Hence, the theory was extended to reflect this (see Appendix 1). Moments may then be calculated for this model, and the results are summarized in Table 1.

Enzyme Zonation within the Tubes-in-Series Model. The rate constant for metabolic transformation is again treated as a function of the relative position within the acinus, denoted by τ (where τ = 0 represents a position adjacent to the portal venule, and τ = 1 represents one adjacent to the central venule) to represent enzyme zonation. Conversely, rate constants for transmembrane transport (k_{13}, k_{31}, k_{25}, k_{52}, k_{34}, and k_{43}) are treated as constants to represent homogeneously distributed transporter activity. This assumption is validated, at least for k_{13}, in the zonal-hepaticocyte uptake study of benzoate. For the formulation of Laplace transforms and moments of outflow profiles, the sinusoid was considered as two half-sinusoids arranged in series, each with a transit time of 0.5 MTT_{ref}. A stepwise change of k_{45} at τ = 0.5 was assumed such that

\[ k_{45}(τ) = (1 + r)k_{45}, \quad 0 < τ < 0.5 MTT_{ref} \]

(1)

\[ k_{45}(τ) = (1 - r)k_{45}, \quad 0.5 MTT_{ref} < τ < MTT_{ref} \]

(2)

where k_{45} is the length-averaged value of k_{45}(τ), the metabolic rate constant, and r is a heterogeneity parameter with values between −1 and 1. As defined previously, positive values of r denote a predominantly periporal enzymic distribution and negative values of r denote a predominantly perivenous enzymic distribution (Schwab and Pang, 1999). Special cases include exclusively periporal (PP) enzyme distribution (r = 1) and exclusively perivenous (PV) enzyme distribution (r = −1); for r = 0, the enzyme distribution is even or uniform. Various values of r ranging from −1 to +1 were used to
explore the impact of intermediate enzyme zonation on the moments of drugs and metabolites. As shown previously, the overall unit impulse response of subsystems connected in series is given as the convolution of the individual unit impulse responses (Lassen and Perl, 1979; Bronikowski et al., 1987; Schwab et al., 2001). Laplace transforms and AUCs are obtained as the products of those of the outflow profiles of the subsystems, whereas MTTs are their sums.

For the PP case \((r = 1)\), the overall outflow profile of the metabolite is the convolution of the outflow profile of the metabolite formed from the parent drug from an upstream partial sinusoid or first half-tube (where \(k_{43}\) is twice the length-averaged value) and the outflow profile of an existing (preformed) metabolite for a downstream partial sinusoid. For the PV case \((r = -1)\), the overall outflow profile of the metabolite is the convolution of the outflow profile of the parent drug from an upstream partial sinusoid where no conversion takes place \((k_{43}\) is set to zero), and the outflow profile of the metabolite formed from the parent drug from a downstream partial sinusoid (where \(k_{45}\) is set to twice the length-averaged value). With stepwise increasing or decreasing enzyme activity, the moments for the metabolite outflow curves are evaluated as follows,

\[
\text{AUC} = \text{AUC}_{PP} + \text{AUC}_{PM,PP}
\]

\[
\text{MTT} = \frac{(\text{MTT}_{PP} + \text{MTT}_{PM,PP}) \text{AUC}_{PP} \text{AUC}_{PM,PP} - (\text{MTT}_{PP} + \text{MTT}_{PM,PP}) \text{AUC}_{PP} \text{AUC}_{PM,PP}}{\text{MTT}_{PP} \text{AUC}_{PM,PP} + \text{MTT}_{PM,PP} \text{AUC}_{PP} \text{AUC}_{PM,PP} + (\text{MTT}_{PP} + \text{MTT}_{PM,PP}) \text{AUC}_{PP} \text{AUC}_{PM,PP} + (\text{MTT}_{PP} + \text{MTT}_{PM,PP}) \text{AUC}_{PP} \text{AUC}_{PM,PP}}
\]

where \(\text{AUC}_{PP}, \text{AUC}_{PM,PP}\) and \(\text{AUC}_{PM,PP}\) are expressions for the AUC of the parent drug, the formed metabolite (subscript M), and the preformed metabolite (subscript PM), respectively, for the portal part of the acinus, and \(\text{AUC}_{PP}, \text{AUC}_{PM,PP}\) and \(\text{AUC}_{PM,PP}\) are the corresponding values for the portal part. The mean transit times \(\text{MTT}_{PP}, \text{MTT}_{PM,PP}, \text{MTT}_{PP,PM}\) and \(\text{MTT}_{PM,PM}\) are defined equivalently. The algebraic expressions (see Appendix) were derived using MathView software (Waterloo Maple Inc., Waterloo, ON, Canada) on a Power Macintosh computer.

### Data Treatment

For the multiple indicator dilution data, the concentrations of radiolabels in the outflow perfusate were normalized to the respective doses, yielding fractional recoveries (or concentration/dose). The fractional recovery-integral (or area under the curve, AUC) and area under the moment curve (AUMC, or the integral of the product of fractional recovery and time at mid-intervals) of the outflow data of benzoate and hippurate were estimated by the spline function of IMSL (Visual Numerics, Houston, TX). The ratio of AUMC/AUC furnished the mean transit time, MTT. Tracer recoveries were obtained by multiplying AUCs with perfusate flow and were virtually complete for the noneliminated reference indicators and the \([^{3}H]\)hippurate and \([^{14}C]s\)benzoate doses; the latter recovery was assessed as the summed recoveries for\([^{14}C]s\)benzoate and \([^{14}C]s\)hippurate.

### Modeling of Hepatic Hippurate and Benzoate Disposition

The outflow profiles of the nonmetabolized indicators, \(^{35}Cl\)-labeled erythrocytes, \(^{125}I\)-labeled albumin, \(^{[3}H]\)- or \([^{14}C]s\)sucrose, and \(^{3}H_2O\), were evaluated by linear superposition as described previously (St-Pierre et al., 1989; Xu et al., 1990; Schwab et al., 2001). The kinetic model for the transport and metabolism of benzoate in the perfused rat liver has previously been validated (Schwab et al., 2001). In the absence of an established whole-organ model that includes enzyme zonation, the data were fitted to the homogeneous-enzyme model of Goresky, as done previously for the prograde experiments (Schwab et al., 2001). The outflow profiles (impulse responses) were calculated using an algorithm based on an approximation of the reference indicator curves by exponential sums as previously described (Schwab, 1984; Schwab et al., 2001).

The fit of outflow data from the first MID injection of tracer hippurate according to the Goresky model yielded the transfer coefficients for hippurate transport, \(k_{25}\) and \(k_{52}\), and the parameter \(\gamma_{rel,HH}\), derived from the ratio of interstitial to vascular distribution spaces of hippurate, \(\gamma_H\). These values \((k_{25}\) and \(k_{52}\) and \(\gamma_H))\) were assigned as the parameters for \([^{14}C]s\)benzoate transport and for the interstitial-vascular distribution space ratio of hippurate for the same liver. Fitting of the \([^{14}C]s\)benzoate and \([^{14}C]s\)hippurate profiles from the second MID injection furnished the transfer coefficients for benzoate transport, \(k_{13}\) and \(k_{31}\), the exchange parameters between intracellular pools, \(k_{24}\) and \(k_{42}\), the coefficient for metabolism, \(k_{45}\), and the parameter \(\gamma_{rel,B}\), derived from the ratio of the interstitial to the vascular distribution space of benzoate \(\gamma_B\).

For analysis of outflow profiles, transport functions (impulse responses) of the liver were obtained by numerical deconvolution of experimental outflow profiles with the transport function of the combined injection and collection devices (the catheter transport function), using an algorithm obtained from the National Simulation Resource in Mass Transport and Exchange, University of Washington (Seattle, WA). The theoretical outflow dilution profiles of noneliminated reference indicators were obtained by convolution of calculated transport functions with the catheter transport function.
TABLE 1

Expected behavior of moments (zeroth moment or AUCM and first moment or MTTM) of the generated metabolite with prograde/retrograde perfusion (k_mM > k_pM = 0; Fig. 2) for which the mitochondrial compartment is present for metabolite formation (see Fig. 1B).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition</th>
<th>Enzyme Distribution</th>
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<tbody>
<tr>
<td>AUCM</td>
<td>(k_1/k_31)(1 + k_3/k_42) &lt; k_2/k_32</td>
<td>Prograde = Retrograde</td>
</tr>
<tr>
<td></td>
<td>(k_1/k_31)(1 + k_3/k_43) &gt; k_2/k_32</td>
<td>Prograde = Prograde</td>
</tr>
<tr>
<td>MTTM</td>
<td>(k_1/k_31)(1 + k_3/k_42) &lt; k_2/k_32</td>
<td>Prograde = Retrograde</td>
</tr>
<tr>
<td></td>
<td>(k_1/k_31)(1 + k_3/k_43) &gt; k_2/k_32</td>
<td>Prograde &gt; Retrograde</td>
</tr>
</tbody>
</table>

(Yoshimura et al., 1998), using an algorithm for numerical integration (QDAG from IMSL; Visual Numerics). Convolution with the catheter transport function was incorporated into the algorithm for calculating the outflow profiles of benzoate and hippurate (Schwab et al., 2001).

Statistics
All data were presented as mean ± S.D. Analysis of variance was used to compare differences in the mean values. A P value of < 0.05 was viewed as significant.

Results
Uptake and Metabolism of Benzoate by Zonal Hepatocytes. The amounts of benzoate accumulated within hepatocytes, estimated as the radioactivity in cell pellets, were linear with time within 1 min of sampling. The regression slopes yielded similar uptake rates for the PP, PV, and homogeneous isolated rat hepatocytes at the low BA (1 μM) and high (400 μM) concentrations tested (Fig. 2). The results paralleled those for the uptake of t-lactate, the model substrate of monocarboxylic acid transporter 2, in zonal rat hepatocytes (Staricoff et al., 1995). The disappearance of benzoate (1 μM) in the incubation mixture upon incubation was similar for both PP and PV hepatocytes (n = 4 preparations each). The rate of formation of hippurate was linear up to 5 min of incubation, and interpretation was based on data gathered up to 3 or 5 min (Fig. 3). No difference was found for hippurate formation from PP (5.86 ± 1.88% initial concentration/min) and PV (5.25 ± 1.46% initial concentration/min, P > 0.05) hepatocytes, nor for the decay rate constants of BA (0.0676 ± 0.0220 min⁻¹ for PP cells and 0.0563 ± 0.0162 min⁻¹ for PV cells) in the incubation system (P > 0.05).

AUC and MTT For the Tubes-in-Series Model. The solutions for AUC and MTT are shown in eqs. 3 and 4. For a drug such as benzoate, which entails metabolism within a sequestered pool (see Fig. 1B), the apparent tissue to plasma partitioning of drug was given by (k_1/k_31)(1 + k_3/k_42) due to the additional mitochondrial pool, whereas that for the preformed metabolite was given by k_2/k_32, and the ratio of these values yields the relative permeability characteristics of the drug and metabolite (Table 1). The area under the curve of the formed metabolite, AUCM, was found to be constant for prograde and retrograde flow directions regardless of whether (k_1/k_31)(1 + k_3/k_42) > k_2/k_32 or (k_1/k_31)(1 + k_3/k_42) < k_2/k_32. By contrast, the MTTM would change according to the values of (k_1/k_31)(1 + k_3/k_42) versus k_2/k_32 and the flow direction (Table 1). These relationships were similar to those found in an earlier treatise when the tubes-in-series model was first developed in the absence of the mitochondrial compartment (Schwab and Pang, 1999).

Moment Analysis of Hippurate and Benzoate in MID Studies. The steady-state extraction ratio of benzoate (0.6) for retrograde flow (Table 2) was similar to that observed previously for prograde flow (data of Schwab et al., 2001). Representative outflow profiles of tracer [3H]hippurate (first injection), of tracer [14C]benzoate and its metabolite, [14C]hippurate (second injection), and of the noneliminated indicators are shown in Figs. 4 and 5, respectively, for 17 μM benzoate in the inflow perfusate. The outflow profiles of the noneliminated indicators (labeled erythrocytes, albumin, sucrose, and 3H2O) for the first and the second injections in outflow (portal venous) perfusate were increasingly dispersed. The total recoveries of the 51Cr-, 125I-, 14C-, and 3H-radiolabels and of 3H2O in the venous outflow samples were virtually complete (Table 3). All of the injected [3H]hippurate returned to the vasculature as [3H]hippurate, and the [14C]benzoate returned as unchanged [14C]benzoate or [14C]hippurate, suggesting that benzoate was metabolized.

Fig. 2. Uptake of benzoate by periportal (n = 3), homogeneous (n = 4), and perivenous (n = 3) isolated rat hepatocytes for 1 and 400 μM benzoate. Data are mean ± S.D.

Fig. 3. Lack of zonal difference in metabolism of benzoate (1 μM) by PP and PV hepatocytes (1.6–17 × 10⁶ cells/ml) in formation of hippurate. The disappearance of benzoate from PP (▲) and PV (○) cells was accounted for by formation of hippurate in PP (●) and PV (▼) cells. Data are mean ± S.D. of four preparations each.
exclusively to hippurate and that both benzoate and hippurate were not significantly excreted.

The mean transit times of the vascular noneliminated indicators differed slightly between the two injections made at 20 and 30 min for the present studies (Table 4). The data, when compared with previous prograde data of Schwab et al. (2001), revealed that the values for total water volumes (sum of both cellular and extracellular water spaces) were greater than unity (Table 4). The apparently high value is due to the distention of the vasculature during retrograde flow, as described by St-Pierre et al. (1989) and Xu et al. (1990). The MTTs for labeled red blood cells, albumin, and sucrose were all increased with retrograde flow, especially for data of the first injection, although changes in the second injection were attenuated. The reason for the difference between the first and second injections was unknown, but may be due to a greater compliance with the slightly longer perfusion time. Higher sinusoidal volumes and sucrose (for the first injection) and albumin (for the second injection) Disse spaces were observed for retrograde flow (Fig. 6).

There was no change in the mean transit times for benzoate (MTT) and the preformed hippurate (MTT PM) (Table 4). Although values for MTT PM were similar to that of labeled sucrose, the shapes of the curves differed due to partial entry of hippurate into the rat liver (Fig. 4). The \(^{3}H\)hippurate profile crossed over and then peaked lower and earlier than the labeled sucrose curve and exhibited a more delayed downslope. The mean transit time of formed hippurate (MTTM) in the present study was greater than MTT PM but was much lower than that of Schwab et al. (2001) for prograde flow (\(P \leq 0.05\)). The PP/PV ratio of MTT M exceeded unity. Moreover, values of MTT M (67 \(\pm\) 7 s) significantly exceeded \(P \leq 0.05\) the summed MTTs for preformed HA and BA (52 \(\pm\) 6 s). The same trend was also observed upon reexamination of the prograde data of Schwab et al. (2001); MTT M (114 \(\pm\) 32 s) was significantly greater \(P \leq 0.05\) than the sum of the MTTs for preformed HA and BA (56 \(\pm\) 13 s).

**Model Fits to Outflow Profiles of \(^{3}H\)Hippurate According to Goresky’s Model.** The outflow profile of hippurate obtained after injection of \(^{3}H\)hippurate during retro-
TABLE 3
Recoveries of noneliminated reference indicators and labeled hippurate and benzoate after injection of MID doses of \[^{3}H\]hippurate and \[^{14}C\]benzoate during retrograde perfusion were compared with those published by Schwab et al. (2001) for prograde perfusion.

<table>
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<tr>
<th>Tracer</th>
<th>Catheter-corrected MTT (s)</th>
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<tr>
<td>Hippurate</td>
<td>[^{3}H]Cr-labeled RBCs</td>
</tr>
<tr>
<td>First Injection: Hippurate</td>
<td></td>
</tr>
<tr>
<td>Retrograde (n = 8)</td>
<td>18.0 ± 2.4</td>
</tr>
<tr>
<td>Prograde (n = 7)</td>
<td>11.0 ± 2.5*</td>
</tr>
<tr>
<td>Second Injection: Benzoate</td>
<td></td>
</tr>
<tr>
<td>Retrograde (n = 8)</td>
<td>14.5 ± 2.7†</td>
</tr>
<tr>
<td>Prograde (n = 7)</td>
<td>10.8 ± 2.9*</td>
</tr>
</tbody>
</table>

*, †, ‡ P < 0.05, vs. retrograde data for first or second injection.
‡ P < 0.05, vs. first injection retrograde data.
Data were mean ± S.D.

Fig. 6. Distention of the vascular spaces during retrograde perfusion (light bar) over prograde perfusion (dark bar). * significantly different, with P < 0.05.

TABLE 4
Transit times of outflow profiles for the noneliminated reference indicators and preformed hippurate and benzoate during retrograde perfusion were compared with those of Schwab et al. (2001) for prograde perfusion of benzoate.

<table>
<thead>
<tr>
<th>Data</th>
<th>[^{3}H]Cr-labeled RBCs</th>
<th>[^{125}I]labeled Albumin</th>
<th>Labeled Sucrose</th>
<th>[^{2}H]O</th>
<th>Hippurate (HA)</th>
<th>Benzoate (BA)</th>
<th>Hippurate + Benzoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Injection: Hippurate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrograde (n = 8)</td>
<td>0.16 0.21</td>
<td>0.21 0.45</td>
<td>0.08 0.36</td>
<td>0.16 0.46</td>
<td>0.11 0.36</td>
<td>0.08 0.36</td>
<td></td>
</tr>
<tr>
<td>Prograde (n = 7)</td>
<td>0.21 0.45</td>
<td>0.19 0.49</td>
<td>0.25 0.98</td>
<td>0.21 0.49</td>
<td>0.19 0.49</td>
<td>0.25 0.98</td>
<td></td>
</tr>
<tr>
<td>Second Injection: Benzoate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrograde (n = 8)</td>
<td>0.09 0.19</td>
<td>0.08 0.12</td>
<td>0.07 0.11</td>
<td>0.09 0.12</td>
<td>0.08 0.12</td>
<td>0.07 0.11</td>
<td></td>
</tr>
<tr>
<td>Prograde (n = 7)</td>
<td>0.10 0.13</td>
<td>0.11 0.13</td>
<td>0.10 0.10</td>
<td>0.05 0.10</td>
<td>0.10 0.10</td>
<td>0.10 0.10</td>
<td></td>
</tr>
</tbody>
</table>

*, † P < 0.05, prograde vs. retrograde.
‡ P < 0.05, prograde vs. retrograde.
Data were mean ± S.D. of fractions of complete recovery (AUC/perfusate rate).
Data were mean ± S.D.

Moment Analysis of Metabolic Heterogeneity 285

Simulations Based on Tubes-in-Series Theory. Simulations were made based on the analytical solutions for the MTTm (eq. 4) for prograde and retrograde flows and the values of \(k_{13}\), \(k_{31}\), \(k_{34}\), \(k_{43}\), and \(k_{45}\) for benzoate, and \(k_{25}\) and \(k_{22}\) in Table 5. The condition for simulation was identical to the experimentally obtained, fitted parameters that revealed that the partition ratio for benzoate (ratio of influx/efflux rate constants) \(k_{13}/k_{31}\) (1 + \(k_{34}/k_{43}\)) was greater than that for hippurate \((k_{25}/k_{52})\). The resulting PP/PV ratio of MTTM was plotted against \(r\), the heterogeneity parameter. The simulations (Fig. 7) predicted that the PP/PV ratio of MTTM exceeds unity for negative values of \(r\) and was less than unity for positive values of \(r\) for the tubes-in-series model. Both the simulation with the tubes-in-series model and the observed data suggest the pervenous abundance of benzoate glycine conjugation activity, as found by Chiba et al. (1994).
Metabolite behavior is highly complex and may differ from the kinetic behavior of a preformed metabolite entering an organ. Although the same transport function and enzyme(s) are involved in metabolite removal, perceptive differences exist due to the presence of a transport barrier for metabolite, barring the PM from entering and preventing M from leaving (deLannoy and Pang, 1987). The kinetic behavior of the formed metabolite (M) is highly dependent on the model of hepatic drug clearance: whether the well stirred model, the parallel tube model, or the dispersion model (St-Pierre et al., 1992; Pang, 1995) applies, and depending on whether the permeability of drugs and metabolite is high or poor.

According to the well stirred model, the kinetics of M is independent of drug behavior, and the MTTM is the sum of the MTTs for highly permeable drug and preformed metabolite (Chan et al., 1985; St-Pierre et al., 1992; Mellick et al., 1997). For both the parallel tube and dispersion models, however, the kinetic behavior of M is predicated on drug kinetic behavior (St-Pierre et al., 1992). The difference on metabolite formation between the dispersion model (dispersive flow and some mixing) and the parallel tube model (plug flow, nondispersive and no mixing) for flow-limited substrates in the absence of enzyme heterogeneity is, however, small, and a similar trend persists when enzyme heterogeneity is added (St-Pierre et al., 1992). For these reasons, lower values for MTTM are predicted for the parallel tube and dispersion models in comparison with the well stirred model for highly permeable drugs and metabolites (Roberts et al., 1988; St-Pierre et al., 1992; St-Pierre and Pang, 1993a,b; Mellick et al., 1997). When permeabilities of the drug and the metabolite are low, however, MTTM will exceed the sum of

---

**Table 5**

<table>
<thead>
<tr>
<th>Parameter for Benzoate</th>
<th>Parameters for Hippurate</th>
<th>Other Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{13}$, $k_{31}$</td>
<td>$k_{25}$, $k_{52}$</td>
<td>$k_{34}$, $k_{45}$</td>
</tr>
<tr>
<td>$P_{BA}$</td>
<td>$P_{HA}$</td>
<td>$t_0$ for First Injection</td>
</tr>
<tr>
<td>$m_{BA}/g$</td>
<td>$m_{HA}/g$</td>
<td>$t_0$ for Second Injection</td>
</tr>
<tr>
<td>1.58 ± 0.64</td>
<td>0.90 ± 0.67*</td>
<td>$0.24 ± 0.11$</td>
</tr>
<tr>
<td>0.30 ± 0.11</td>
<td>0.16 ± 0.05*</td>
<td>$0.04 ± 0.01$</td>
</tr>
<tr>
<td>0.12 ± 0.02</td>
<td>0.07 ± 0.02*</td>
<td>$0.02 ± 0.02$</td>
</tr>
<tr>
<td>0.0065 ± 0.0014</td>
<td>0.0002 ± 0.000002</td>
<td>$0.05 ± 0.05$</td>
</tr>
<tr>
<td>0.96 ± 0.39</td>
<td>0.082 ± 0.029</td>
<td>$0.09 ± 0.01$</td>
</tr>
<tr>
<td>0.033 ± 0.012</td>
<td>0.065 ± 0.029</td>
<td>$0.13 ± 0.07$</td>
</tr>
<tr>
<td>2.6 ± 1.2</td>
<td>4.3 ± 0.8</td>
<td>$S.D.$</td>
</tr>
<tr>
<td>2.6 ± 1.2</td>
<td>2.6 ± 1.2</td>
<td>3.5 ± 1.0</td>
</tr>
</tbody>
</table>

* Different from retrograde, $P < 0.05$.
the mean transit times of the drug and the preformed metabolite (Mellick et al., 1997), since poor permeability of the metabolite retards entry of preformed metabolite but extends the sojourn time of the metabolite formed in the organ. This is confirmed by calculations that show that barrier limitation of transport of the metabolite may increase MTTM beyond the sum of the mean transit times of the drug and the preformed metabolite, even when the transport of the precursor is not barrier-limited.

The presence of an additional precursor pool such as the mitochondrial space, where conjugation of benzoic acid takes place (Fig. 1B), will extend the mean transit times of the drug and the formed metabolite but will not affect that of the preformed metabolite. However, the presence of the additional mitochondrial pool would not affect the trends of the expected changes on the AUCM and MTTM with enzyme zonation (Table 1). Our observation that MTTM for formed hippurate greatly exceeded the sum of the MTTs of benzoate and preformed hippurate is only suggestive of the poor permeability of hippurate. This finding is in agreement with the present transfer constants for hippurate, as well as those found in previous MID experiments (Yoshimura et al., 1998; Schwab et al., 2001), showing that the influx clearance of hippurate (P25S) was less than the perfusate flow rate.

Interpretation of the AUC and MTTM data of the present study depends on verifying the viability of the preparation at the times of both injections during retrograde flow. In previous experiments with retrograde perfusion of rat livers under similar conditions, measured rates of bile flow or oxygen consumption were not different from values obtained with prograde perfusion (St-Pierre et al., 1989; Xu et al., 1990), and inflow venous pressures were unchanged (St-Pierre et al., 1989) or only increased minimally (Xu et al., 1990). The observed difference in the hematocrit (Table 2) should not impact on liver viability or processing of benzoate and hippurate since both are not bound to red blood cells. However, distention was a hallmark of retrograde perfusion (St-Pierre et al., 1989; Xu et al., 1990), and this was evident from the blobbing of the curve form (shifting of maximum concentration to a lower value and at a later time) with retrograde flow. Of note, the MTT and the volumes of the vascular indicators were enlarged, and cellular water space, estimated by difference, was reduced for retrograde flow when compared with prograde data (Fig. 5), as found previously (St-Pierre et al., 1989; Xu et al., 1990). However, vascular volume distention during the second injection was much less severe than that for the first injection, indicating an adaptation process. The AUC and MTT of preformed species (hippurate or “PM” and benzoate) remained unchanged with both perfusion directions (Tables 3 and 4), and this finding strongly suggests that parameters pertaining to the formed metabolite, AUCM and MTTM, are not influenced by the changes in the vasculature. The AUCM was unchanged, with reversal of flow direction (Table 3). The MTTM for retrograde flow was significantly shorter than that for prograde flow (Table 4; \( P < 0.05 \)), and the partition ratio (influx/efflux) of benzoate exceeded that for hippurate. The data are consistent with the theoretical prediction for a perivenous distribution of benzoate conjugation activity (Table 1). This metabolic heterogeneity suggested by moment analysis reflects that there is zonation of the enzyme, benzyoyl CoA ligase (Schwab et al., 2001). Moreover, due to the rapid transport of benzoate, transport would not affect the overall conjugation rate of benzoate since it is not the rate-determining step (Schwab et al., 2001).

The investigations on uptake corroborated with previous evidence on the lack of zonal transport of benzoate (Fig. 2), but the metabolic studies with zonal hepatocytes failed to provide the expected higher perivenous distribution of benzoate conjugation activity (Fig. 3). A lack of in vitro substantiation was also observed for the glutathione S-transferase (GST) activities toward 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrylic acid, both in incubation studies and Western blotting of immunoreactive Ya and Yb2 GSTs with zonal rat hepatocytes (Tirona et al., 1999). The observations were explained by the cross-contamination of the zonal hepatocytes by cells from other regions and the lack of a steep gradient for conjugation activity despite the fact that the activities are higher in the perivenous region. Indeed, the speculation was confirmed with use of PP and PV lysates for incubation studies and Western blotting when these provided a sharper enrichment and less cross-contamination between PP and PV cells; the higher GST activity in PV cells became apparent (Tirona et al., 1999). Hence, the lack of metabolic zonation for glycine conjugation within zonal hepatocytes in the present study is not definitive of a lack of zonation in the intact organ. This is primarily due to a limitation of the method for the preparation of enriched cells.

The tubes-in-series model, although less quantitative than the Goresky approach in incorporating flow heterogeneity, was nonetheless useful for the study of metabolic heterogeneity in the intact liver with the indicator dilution technique in progradely and retrogradely perfused livers. Although it is well known that the sinusoidal transit time is heterogeneous (Pang et al., 1994), the predictions afforded by the tubes-in-series model on the ranking of transit times should be preserved. In addition to the tubes-in-series model, another model, the tanks-in-series model (Gray and Tam, 1987; Saville et al., 1992) exists and may be another useful representation, since the impulse function resulting from an injection was similar to that for Goresky’s model. However, the tanks-in-series model is an empirical model, and the number of compartments (N) necessary to describe the data would vary according to the drug (Gray and Tam, 1987). It was found that N approached a value of 2 for red blood cells, albumin, and lidocaine (Gray and Tam, 1987; Saville et al., 1992), and a similar, tanks-in-series model with N = 2 was able to fit the data of estrone sulfate and estrone in the recirculating perfused rat liver preparation (Tan et al., 2001). In fact, our simulations based on the preliminary development of this model with two compartments (one for upstream and one for downstream) showed that characteristics exceeding similar to the tubes-in-series model were obtained (see Fig. 7). The predictions on the changes of the AUCM and MTTM for the tanks-in-series model with permeability and flow direction (data not shown) were similar to that of the tubes-in-series model (Table 1). These models may be useful approaches and further developed as a zonal model (Abu-Zahra and Pang, 2000) to encompass enzyme and transport heterogeneity for the description of zonal drug metabolism.

Appendix: Tubes-in-Series Model

Moments for a Metabolite when Formation Occurs in the Mitochondria. The partial differential equations
describing the movement of tracers are as follows:

\[
\frac{\partial C_1}{\partial t} + \frac{\partial C_1}{\partial \tau} = k_{31}c C_3 - k_{13}C_1 + \frac{1}{Q} \delta(t) \delta(\tau) \quad \text{(A1)}
\]

\[
\frac{\partial C_2}{\partial t} + \frac{\partial C_2}{\partial \tau} = k_{23}c C_3 - k_{22}C_2 \quad \text{(A2)}
\]

\[
\frac{\partial C_3}{\partial t} - \frac{k_{43}C_1}{\theta'} + k_{43}C_4 - (k_{31} + k_{43})C_3 \quad \text{(A3)}
\]

\[
\frac{\partial C_4}{\partial t} = k_{34}C_3 - (k_{43} + k_{45})C_4 \quad \text{(A4)}
\]

\[
\frac{\partial C_5}{\partial t} = \frac{k_{25}C_2}{\theta'} + k_{45}C_4 - k_{55}C_5 \quad \text{(A5)}
\]

where \( C_1 \) and \( C_2 \) are dose-normalized tracer concentrations (fraction of dose per milliliter) of the parent drug and metabolite, respectively, in the expanded (sinusoidal + interstitial) plasma space; \( C_3 \) and \( C_4 \) are the corresponding dose-normalized concentrations of drug and metabolite in the cytosol of hepatocytes; \( C_5 \) is the dose-normalized concentration of drug in mitochondria; \( t \) is time; \( \tau \) is a space variable representing the cumulative transit time of a reference indicator from the entrance point of the sinusoid (equal to the ratio of the cumulative expanded plasma space to sinusoidal flow); \( Q \) is the flow of per fusate fluid through the liver; and \( \theta' = \theta(1 + \gamma_{ref}) \), where \( \theta \) is the ratio of the accessible cellular water space to the sinusoidal volume and \( \gamma_{ref} \) is the volume ratio of extracellular space of the reference indicator to the sinusoidal volume.

Laplace transformation and elimination of the Laplace transforms of \( C_3, C_4, \) and \( C_5 \) yields the following system of ordinary differential equations in \( \tau \):

\[
\frac{d\hat{C}_1}{d\tau} = \left[ s + \frac{k_{31}k_{34}k_{43}k_{52}}{(s + k_{31})(s + k_{34})(s + k_{43})(s + k_{45})} \right] \hat{C}_1
\]

\[
- \delta(\tau) \quad \text{(A6)}
\]

\[
\frac{d\hat{C}_2}{d\tau} = \left[ s + \frac{k_{13}k_{34}k_{45}k_{52}}{(s + k_{31})(s + k_{34})(s + k_{43})(s + k_{45})} \right] \hat{C}_1
\]

\[
- s \left( 1 + \frac{k_{25}}{s + k_{52}} \right) \hat{C}_2 \quad \text{(A7)}
\]

where \( \hat{C}_1(s) \) and \( \hat{C}_2(s) \) are the Laplace transforms of \( C_1 \) and \( C_2 \), respectively, and \( s \) is the Laplace variable. The solutions are

\[
\hat{C}_1(s) = \frac{1}{Q} \exp[-\lambda_1(s)\tau] \quad \text{(A8)}
\]

\[
\hat{C}_2(s) = \frac{k_{13}k_{34}k_{45}k_{52}[\exp(-\lambda_1(s)\tau) - \exp(-\lambda_2(s)\tau)]}{Q[k_{31}(s + k_{52})(s + k_{34}) - (s + k_{31})(s + k_{43})(s + k_{45})]}
\]

\[
- k_{25}[k_{34}k_{43} - (s + k_{31} + k_{34})(s + k_{43} + k_{45})] \quad \text{(A9)}
\]

where the exponential coefficients, \( \lambda_1 \) and \( \lambda_2 \), are

\[
\lambda_1(s) = s + \frac{k_{34}k_{43} - (s + k_{34})(s + k_{43} + k_{45})}{k_{34}k_{43} - (s + k_{31} + k_{34})(s + k_{43} + k_{45})}k_{13} \quad \text{(A10)}
\]

\[
\lambda_2(s) = s \left( 1 + \frac{k_{25}}{s + k_{52}} \right) \quad \text{(A11)}
\]

The area under the curve (AUC) or zeroth moment for drug is obtained from the following equation:

\[
\text{AUC} = \int_0^\infty C_1(t) dt
\]

\[
= \lim_{s \to 0} \hat{C}_1(s) \quad \text{(A12)}
\]

The recovery (survival fraction, or availability, \( F \)) is obtained as the product of AUC and the plasma flow rate, \( Q \).

\[
F = \frac{\text{AUC}}{\text{MTT}} \quad \text{(A13)}
\]

Substitution of eq. A8 into eqs. A12 and A14 yields the following analytical expressions for the zeroth (AUC) and first (MTT) moments of the parent drug:

\[
\text{AUC} = \frac{1}{Q} \exp\left( - \frac{k_{13}k_{34}k_{45}k_{52}}{k_{31}k_{34} + k_{31}k_{43} + k_{31}k_{45}} \tau \right) \quad \text{(A15)}
\]

\[
\text{MTT} = \left[ 1 + \frac{k_{13}k_{31}k_{34}k_{43} + (k_{43} + k_{45})^2}{(k_{31}k_{34} + k_{31}k_{43} + k_{31}k_{45})^2} \right] \tau \quad \text{(A16)}
\]

Equations A15 and A16 are equivalent to those presented previously (Rose et al., 1977).

The moments for the preformed metabolite are:

\[
\text{AUC}_{\text{PM}} = \frac{1}{Q} \quad \text{(A17)}
\]

\[
\text{MTT}_{\text{PM}} = \left( 1 + \frac{k_{25}}{k_{52}} \right) \tau \quad \text{(A18)}
\]

For the formed metabolite, the following analytical expressions are obtained similarly from eq. A9:

\[
\text{AUC}_M = \frac{1}{Q} \left[ 1 - \exp\left( - \frac{k_{13}k_{34}k_{45}k_{52}}{k_{31}k_{34} + k_{31}k_{43} + k_{31}k_{45}} \tau \right) \right] \quad \text{(A19)}
\]

\[
k_{31}k_{34}k_{45} + k_{31}k_{45} + k_{31}k_{45}
\]

\[
- k_{25}[k_{34}k_{43} + k_{34}k_{45} + k_{34}k_{45}]
\]

\[
\text{MTT}_M = \left[ 1 + \frac{k_{25}}{k_{52}} \right] \left( 1 - \exp\left( - \frac{k_{13}k_{34}k_{45}}{k_{31}k_{34} + k_{31}k_{45} + k_{31}k_{45}} \tau \right) \right) \quad \text{(A20)}
\]

\[
+ \left\{ \frac{1 + \frac{k_{25}}{k_{52}}}{1 - \exp\left( - \frac{k_{13}k_{34}k_{45}}{k_{31}k_{34} + k_{31}k_{45} + k_{31}k_{45}} \tau \right)} \right\} \quad \text{(A21)}
\]
Moment Analysis of Metabolic Heterogeneity

289

References

Address correspondence to: Dr. K. S. Pang, Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, Ontario, Canada M5S 2S2. E-mail: ks.pang@utoronto.ca