IN VIVO CLEARANCE OF ETHOXYCOUMARIN AND ITS PREDICTION FROM IN VITRO SYSTEMS

Use of Drug Depletion and Metabolite Formation Methods in Hepatic Microsomes and Isolated Hepatocytes

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ABSTRACT:

The pharmacokinetics of ethoxycoumarin have been characterized using steady-state plasma concentrations achieved after administration of this compound, at a series of infusion rates, into the hepatic portal vein of rats. The clearance of ethoxycoumarin could be described by a one-site Michaelis-Menten kinetic model with V_{max} and unbound K_M values of 495 nmol/min/standard rat weight (SRW) and 3.6 μM, respectively, and an intrinsic clearance (CL_{int}, V_{max}/K_M ratio) of 137 ml/min/SRW (where SRW is 250 g). Urinary excretion experiments, using both ethoxycoumarin and hydroxycoumarin, demonstrated that 7-hydroxycoumarin, the metabolite frequently measured in *in vitro* studies, accounted for 26% of the metabolism of ethoxycoumarin. *In vitro* studies with hepatic microsomes and isolated hepatocytes were undertaken to characterize the kinetics of both hydroxycoumarin formation and ethoxycoumarin depletion and to compare the utility of these methods for predicting *in vivo* clearance. In both *in vitro* systems, hydroxycoumarin formation displayed biphasic kinetics, with a high-affinity/low-capacity component (with V_{max}, K_M, and CL_1 terms) and a low-affinity/high-capacity component (with a CL_2 term) that was not saturated over the substrate concentration range studied (0.5–100 μM). The use of scaling factors to relate *in vitro* and *in vivo* data showed that, although microsomal and hepatocyte V_{max} values were comparable (26 and 17 nmol/min/SRW, respectively), both were substantially lower than the *in vivo* value. However, scaling of the *in vitro* CL_{int} values, by taking into account the fraction of ethoxycoumarin metabolized to hydroxycoumarin, yielded *in vivo* predictions of 127 and 122 ml/min/SRW (representing 93 and 89% of the observed CL_{int} value) for microsomes and hepatocytes, respectively. The depletion of ethoxycoumarin (1–1.5 μM) with time in both microsomes and hepatocytes displayed a monoexponential decline and predicted *in vivo* CL_{int} values of 53 and 117 ml/min/SRW (representing 39 and 85% of the observed value), respectively. Therefore, both *in vitro* systems can accurately predict ethoxycoumarin CL_{int} values using hydroxycoumarin formation rates, providing the importance of this pathway in total clearance is taken into account. Moreover, these results demonstrate that, even when the complete metabolic fate of the compound under investigation is unknown, isolated hepatocytes can be successfully used to predict *in vivo* CL_{int} values by measurement of substrate depletion with time.

Ethoxycoumarin is a commonly used probe substrate for CYP[1] activity *in vitro,* and it has been recommended as a marker of metabolic competence for a number of *in vitro* systems, including microsomes (Skett *et al.*, 1995), isolated hepatocytes (Blaauboer *et al.*, 1994), precision-cut liver slices (Bach *et al.*, 1996), and various heterologous expression systems (Waxman *et al.*, 1991). Its initial popularity was based on the fluorescent properties of the metabolite 7-hydroxycoumarin (formed by an *O*-deethylation reaction), which allowed the development of a simple, sensitive, and accurate assay procedure (Greenlee and Poland, 1978). This reaction is catalyzed by

a variety of members of the CYP superfamily of enzymes present in both human (Waxman *et al.*, 1991) and rat (Ryan and Levin, 1990) liver. In human liver, CYP2E1 and CYP1A2 are believed to be the forms responsible for the high-affinity/low-capacity and low-affinity/high-capacity sites, respectively, for ethoxycoumarin *O*-deethylation (Yamazaki *et al.*, 1996); the range of activity between individual livers is relatively small, on the order of 3-fold (Beaune *et al.*, 1986; Stevens *et al.*, 1993). In untreated rats, the situation is less clear, because the two forms showing the highest activity, CYP1A1 and CYP2B1, represent minor amounts of total CYP (Ryan and Levin, 1990).

Although the kinetics of ethoxycoumarin have been well characterized in several *in vitro* systems (Boobis *et al.*, 1986; Rogiers *et al.*, 1986; Fry *et al.*, 1992; Bayliss *et al.*, 1994; Worboys *et al.*, 1995), there have been no investigations undertaken to describe the pharmacokinetics *in vivo*. Moreover, it is unclear how important the *O*-deethylation pathway is in the overall metabolic fate of this substrate. Recent studies with rat liver slices indicated that there are a number of other primary metabolites of ethoxycoumarin (Ball *et al.*, 1996).

Therefore, because the use of ethoxycoumarin *in vitro* is likely to continue, there is a need to establish the relationship between the *in vitro* data generated and the pharmacokinetics *in vivo*. This can be best achieved by assessing the ability of *in vitro* systems to quantitatively predict *in vivo* pharmacokinetic behavior.

The majority of *in vitro* predictions of *in vivo* clearance have used metabolite appearance data; the kinetics of metabolite formation are characterized in hepatic microsomes and/or isolated hepatocytes, and CL_{int} is calculated from the V_{max}/K_M ratio and subsequently scaled to *in vivo* units (Houston, 1994; Iwatsubo *et al.*, 1996). This method has proven to be extremely useful for a wide range of compounds, from low-clearance compounds such as caffeine and tolbutamide (Hayes *et al.*, 1995; Ashforth *et al.*, 1995) to higher-clearance compounds such as diazepam and ondansetron (Zomorodi *et al.*, 1995; Worboys *et al.*, 1996). The main disadvantage of this approach is that it requires knowledge about the importance of the particular metabolic pathway under study in the overall metabolic clearance of the compound. For many drugs, particularly new candidate drugs under development within the pharmaceutical industry, such knowledge is not available and thus the use of this established *in vitro*/*in vivo* prediction method is limited. Therefore, we have examined an alternative prediction method that is based on monitoring the depletion of parent compound in the incubation medium with time and thus requires no *a priori* knowledge of the metabolic fate of the drug.

The aims of the present study were therefore twofold. The first goal was to establish the pharmacokinetics of ethoxycoumarin *in vivo* in rats and to define the importance of 7-hydroxycoumarin formation in the metabolic fate of this compound. The second aim was to compare the use of drug depletion and metabolite appearance methods and assess the accuracy of these *in vivo* predictions for ethoxycoumarin clearance in rats, using two commonly used *in vitro* systems, namely hepatic microsomes and freshly isolated hepatocytes.

Materials and Methods

Animals. Male Sprague-Dawley rats (230–260 g) were obtained from the Biological Sciences Unit, University of Manchester, and housed (two or three/cage) on a bedding of sawdust. They were fed with CRM rat diet (IMS, Congleton, UK), allowed water *ad libitum,* and housed at a room temperature of 20–21°C, in a humidity of 45–55%, and with a 12-hr dark/light lighting cycle.

Chemicals. All of the chemicals used in this study were purchased from either Sigma (Poole, UK) (including 7-ethoxycoumarin, 7-methoxycoumarin, and 7-hydroxycoumarin) or BDH (Lutterworth, UK).

***In Vivo* Pharmacokinetic Studies.** Rats ($N = 30$) were cannulated in the carotid artery (Harns and Ojeda, 1974) and hepatic portal vein (Akrawi and Wedlund, 1987) and allowed 24 hr to recover. Animals received a bolus dose of ethoxycoumarin (1–20 μmol/kg in polyethylene glycol 400/propylene glycol, 9:1) into the hepatic portal vein, followed immediately by an infusion of ethoxycoumarin *via* the same route, at a rate of either 8, 12, 24, 40, or 80 μmol/hr/kg (0.15 ml/hr, using the same vehicle, for 3 hr). This dosing regimen was well tolerated by the animals. The loading dose and infusion rates were calculated to yield a range of theoretical steady-state plasma concentrations, based on the volume of distribution (520 ml/kg) and the clearance (40 ml/min/kg) determined in preliminary iv bolus studies (Hayes *et al.*, 1995). Blood samples (200 μl) were taken from the carotid artery over the infusion period ($N = 6$, 30-min intervals), and plasma was obtained.

***In Vivo* Urinary Recovery Studies.** To determine the amount of ethoxycoumarin converted to 7-hydroxycoumarin, rats ($N = 3$) were treated with ethoxycoumarin (3, 30, or 30 μmol/kg, ip) and housed in metabolism cages for urine collection over 48 hr. To establish the importance of the urinary excretion route and the extent of further metabolism of this metabolite, additional rats ($N = 3$) were treated with 7-hydroxycoumarin (3, 30, or 130 μmol/SRW) and housed in metabolism cages for urine collection for 48 hr. A balanced cross-over design was used in both cases, allowing 48 hr between studies.

Hydroxycoumarin Formation in Microsomes and Hepatocytes. Hepatic

microsomes were prepared and hepatocytes were isolated as described previously (Hayes *et al.*, 1995). Hepatic microsomes (0.5 mg protein/ml, $N = 4$) were preincubated (2 min) with ethoxycoumarin (final concentration, 0.5–100 μM; added as 5 μl in DMF) at 37°C in a shaking water bath. The time and protein concentration used were both within the linear region for reaction conditions. The reaction was started by the addition of NADPH (10 μl of a 20 mM solution in water), and the final incubation volume was 1 ml (in 0.07 M phosphate buffer, pH 7.4). Incubations were terminated (4 min) by the addition of 10 μl of concentrated hydrochloric acid. All incubations were carried out in duplicate.

Freshly isolated hepatocytes ($N = 4$) were preincubated in Williams Medium E, in a shaking water bath, at 37°C. Ethoxycoumarin (0.5–100 μM, added as 5 μl in DMF) was incubated in conical flasks (25 ml) containing 2.5 ml of Williams medium E, and reactions were initiated by the addition of 0.5 ml of hepatocyte suspension, to yield a final cell density of 0.25×10^6 cells/ml. The time and cell density used were both within the linear region for reaction conditions. Reactions were terminated after 10 min by snap-freezing in liquid nitrogen. All incubations were carried out in duplicate.

Ethoxycoumarin Depletion in Microsomes and Hepatocytes. Microsomes ($N = 4$) were preincubated (2 min) with 1.5 μM ethoxycoumarin (added in 5 μl of DMF) in a shaking water bath at 37°C, and reactions were started by the addition of an NADPH-regenerating system (0.74 mg of NADP, 1.94 mg of isocitric acid, 0.5 units of isocitrate dehydrogenase, and 10 μmol of magnesium sulfate in 50 mM Trizma buffer). Incubations were terminated at 2-min time intervals over 20 min, by addition of chloroform. All incubations were carried out in duplicate.

Freshly isolated hepatocytes and ethoxycoumarin (1 μM) were preincubated separately as described above, and reactions were started by the addition of 0.5 ml of hepatocytes, to yield a cell density of 0.25×10^6 cells/ml. Reactions were terminated at 20-min time intervals over 100 min, by immersion in liquid nitrogen.

Assay for Hydroxycoumarin. Microsomal and hydrolyzed hepatocyte incubates (1000 units/ml glucuronidase, type H1 from *Helix pomatia,* containing sulfatase activity, in 60 mM sodium acetate, pH 4.5, for 2 hr at 37°C) were centrifuged (2500 rpm, for 10 min), and 1-ml aliquots were added to 2 ml of 0.2 M glycine buffer (pH 10). The fluorescence of the samples was then measured (Perkin-Elmer LS-5 fluorimeter) by using excitation and emission wavelengths of 375 and 452 nm, respectively. A standard was also assayed with blank microsomes or hepatocytes, and the samples were quantified as described by Lake (1987). It has been demonstrated that the 3-hydroxy metabolite of ethoxycoumarin has no fluorescent properties under alkaline conditions (Jung *et al.*, 1985). Aliquots of urine were hydrolyzed and hydroxycoumarin was quantified as described above, with an additional chloroform extraction step (5 ml, with mixing for 10 min) and back-extraction into the alkaline buffer (Greenlee and Poland, 1978). The limit of detection was 0.01 μM, with linearity extending to at least 100 μM; reproducibility was 0.5–2.3% in the range of 0.3–50 μM.

Assay for Ethoxycoumarin. Samples of plasma and incubates were extracted with chloroform (5 ml, with mixing for 10 min), with the addition of internal standard (7-methoxycoumarin, 10 μl of a 2.5 mM solution in DMF). The organic layer was evaporated to dryness at 40°C under oxygen-free nitrogen and reconstituted in mobile phase (50 μl), and 25 μl were injected into an HPLC system consisting of an SAS Hypersil column with a mobile phase of 0.2 M acetic acid/acetonitrile (55:45, v/v), at a flow rate of 1 ml/min. The eluent was monitored at a UV wavelength of 325 nm; the retention times of 7-ethoxycoumarin and 7-methoxycoumarin were 7.5 and 11 min, respectively. Standard curves for ethoxycoumarin in blank plasma/incubation medium were used to quantify the samples. The limit of detection was 0.3 μM, linearity in the assay extended to at least 200 μM, and reproducibility was 3–5.1% in the range of 1–100 μM.

Determinations of Plasma Protein Binding of Ethoxycoumarin and Blood/Plasma Concentration Ratios. Ethoxycoumarin (in DMF) was evaporated to dryness under nitrogen before the addition of either plasma or fresh blood. The samples were then treated as described in detail earlier (Zomorodi *et al.*, 1995). For both determinations, aliquots were extracted for HPLC analysis as described above. The concentration range of ethoxycoumarin studied was 1–400 μM, and each concentration was assessed in triplicate.

Data Analysis. Kinetic analysis of the metabolite appearance data and *in*

vivo data was undertaken using a least-squares nonlinear regression program (Siphar; Simed, Créteil, France). The *in vivo* data were best described by a one-site Michaelis-Menten model (eq. 1),

$$C_{SS} = \frac{K_M \cdot R}{V_{max} - R} \quad (1)$$

where C_{ss} is the ethoxycoumarin concentration at steady state and R is the infusion rate. The *in vivo* CL_{int} was calculated from the V_{max}/K_M ratio divided by the unbound fraction in blood.

Eq. 2 was used to estimate f_m,

$$f_m = \frac{f_{e, EC \to HC}}{f_{e, HC}} \quad (2)$$

where $f_{e, EC \to HC}$ is the fraction of hydroxycoumarin excreted in urine from rats dosed with ethoxycoumarin and $f_{e, HC}$ is the fraction of hydroxycoumarin excreted in urine from rats treated with hydroxycoumarin. This method accounts for any further metabolism and/or nonrenal excretion that may occur in addition to conjugation (Houston, 1986).

Both the hepatocyte and microsomal metabolite appearance data were best described by a two-site Michaelis-Menten model (eq. 3),

$$v = \frac{V_{max} \cdot S}{K_M + S} + CL_2 \cdot S \quad (3)$$

where v is the rate of metabolism, S is the substrate concentration, and CL_2 is the clearance for the second site, which was not saturated with the concentrations used. The clearance for the first site (CL_1) was calculated from V_{max}/K_M, and the formation clearance for ethoxycoumarin metabolism to hydroxycoumarin (CL_{HC}) was calculated from the sum of CL_1 and CL_2. To account for other routes of metabolism not assayed, the f_m must be taken into account to estimate total ethoxycoumarin *in vitro* CL_{int} (Houston, 1986), as in eq. 4,

$$CL_{int} = \frac{CL_{HC}}{f_m} \quad (4)$$

The AUC for the drug depletion data *in vitro* was calculated using the trapezoidal rule to the last time point, with extrapolation to infinity using the terminal rate constant and the concentration at the last time point. In these studies, *in vitro* CL_{int} was calculated by dividing the amount of substrate in the incubation medium at time 0 by the AUC value. For both *in vitro* methods, an initial ethoxycoumarin concentration that ensured nonsaturating conditions was used (see *Discussion*).

The *in vitro* data (V_{max} and clearance terms) were scaled to *in vivo* units using either the microsomal scaling factor (660 mg microsomal protein/SRW) or hepatocellularity (1.2×10^9 cells/SRW), as appropriate (Carlile *et al.*, 1997). *In vivo* values for K_M and hence CL_{int} were obtained from hepatic portal infusion studies to simulate oral administration (thus eliminating hepatic blood flow as a rate-limiting step in hepatic clearance), without concern for incomplete absorption from the intestinal tract. Implicit in this approach is the assumption that the venous equilibration model adequately describes hepatic events (Wilkinson, 1987). The *in vivo* and scaled *in vitro* data are expressed per SRW (250 g).

Results

***In Vivo* Pharmacokinetic Studies.** A constant-rate infusion of ethoxycoumarin into the hepatic portal vein, together with a bolus dose, produced steady-state plasma concentrations within 30 min, which were maintained for the 3-hr experiment duration. Maximal fluctuations in plasma concentrations ($N = 6$/animal) were <11% for individual animals. The average steady-state concentrations for each group of rats, receiving one of the five different infusion rates, are shown in fig. 1. The data for these 30 animals show a curvilinear relationship between the steady-state concentration achieved and the infusion rate used, which can be best described by simple one-site Michaelis-Menten kinetic model. The V_{max}, K_M, and CL_{int} values
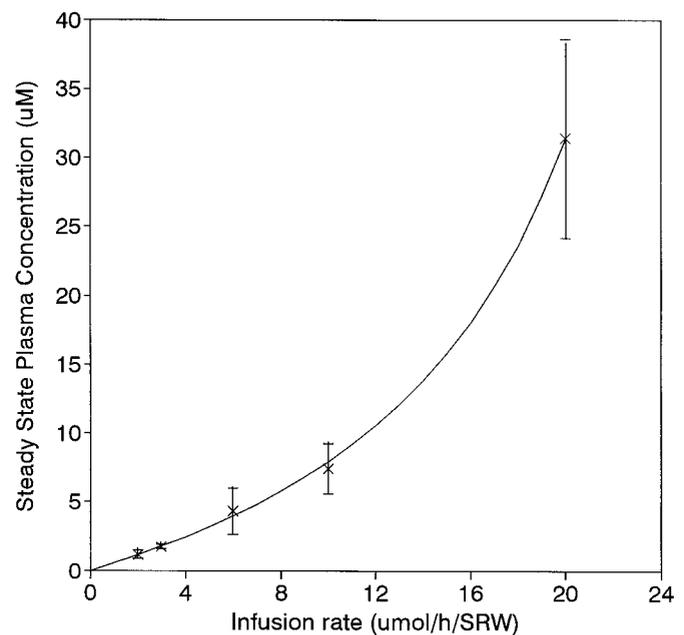

FIG. 1. *Relationship between ethoxycoumarin steady-state plasma concentrations and the infusion rate.*

Data on total drug concentrations (mean ± SD) for five groups of rats (N = 30) are shown. *Solid line*, nonlinear regression line, with V_{max} of 495 nmol/min/SRW and K_M of 16.5 μM.

obtained for these data by nonlinear regression analysis are shown in table 1.

Ethoxycoumarin is bound to plasma proteins, with an unbound fraction of 0.22 ± 0.1, and has a blood/plasma concentration ratio of 0.98 ± 0.12. Both of these values are concentration independent over a concentration range of 1–400 μM. Therefore, the CL_{int} and K_M values for unbound blood concentrations of ethoxycoumarin are 137 ml/min/SRW and 3.6 μM, respectively. Using the venous equilibration model (Pang and Rowland, 1977), the hepatic clearance and the extraction ratio can be calculated (using 20 ml/min/SRW as a measure of hepatic blood flow) as 12 ml/min/SRW and 0.6, respectively. Thus, ethoxycoumarin can be classified as an intermediate-extraction drug, and its hepatic availability, defined as (1 − extraction ratio), is 0.4. As a consequence of the appreciable first-pass effect for ethoxycoumarin, the use of systemic iv administration would significantly underestimate clearance.

***In Vivo* Urinary Recovery Studies.** Administration of ethoxycoumarin resulted in the renal excretion of <1% of the dose in the unchanged form. Table 2 presents the recovery of hydroxycoumarin, after deconjugation, in urine after administration of either ethoxycoumarin or hydroxycoumarin. After administration of ethoxycoumarin, only relatively minor amounts (approximately 17%) were present at each dose level. Studies after administration of hydroxycoumarin itself demonstrated that 65% of the initial dose could be recovered in the urine as hydroxycoumarin after hydrolysis, indicating the importance of further metabolism and/or nonrenal excretion of this metabolite. Therefore, the true f_m for ethoxycoumarin metabolism to hydroxycoumarin (using eq. 4) is 0.26 and the formation clearance for this route is 36 ml/min/SRW.

Hydroxycoumarin Formation in Microsomes and Hepatocytes. Hydroxycoumarin formation in both microsomes and hepatocytes under initial rate conditions demonstrated biphasic kinetics, characterized by a high-affinity/low-capacity component (described by V_{max}, K_M, and hence CL_1) and a low-affinity/high-capacity component that

TABLE 1

In vivo disposition parameters for ethoxycoumarin

	Based on Total Drug Concentration[a]	Based on Unbound Drug Concentration[b]
V_{max} (nmol/min/SRW)	495 ± 15	495
K_M (μM)	16.5 ± 1.1	3.6
CL_{int} (ml/min/SRW)	30	137
CL_H (ml/min/SRW)	12	

[a] Steady-state blood concentration data from 30 rats (blood/plasma concentration, 0.98); parameter values (mean ± SE) from nonlinear regression analysis are shown.
[b] Steady-state concentration data divided by the plasma unbound fraction of 0.22.

TABLE 2

Urinary recovery of hydroxycoumarin (after 48 hr) from rats administered either ethoxycoumarin or hydroxycoumarin ip

Dose	Hydroxycoumarin Excreted in Urine	
	Ethoxycoumarin Administration	Hydroxycoumarin Administration
μmol/kg	*%*	
3	19 ± 5	61 ± 3
30	14 ± 2	64 ± 9
130	17 ± 1	69 ± 6
Mean	17	65

Data are presented as mean ± SD (N = 3).

was not saturated within the concentration range used (described by CL_2). Fig. 2 presents kinetic profiles for hydroxycoumarin formation from typical microsomal and hepatocyte preparations, and the kinetic parameters obtained from these studies are presented in table 3. The K_M values were virtually identical in the two *in vitro* systems; moreover, because of this low value the corresponding CL_1 parameter accounted for approximately 96% of the microsomal clearance and 92% of the hepatocyte clearance. Thus, the importance of the high-affinity component in ethoxycoumarin *O*-dealkylation is comparable in these systems.

Ethoxycoumarin Depletion in Microsomes and Hepatocytes. In both microsomes and hepatocytes, the decline in ethoxycoumarin concentrations over the time course of incubation was monoexponential, as shown in fig. 3 for typical microsomal and hepatocyte preparations. In both cases, incubations were carried out over a time period greater than one half-life. The half-lives differed substantially between the systems (table 4), mainly as a result of the different incubation volumes used (1 ml and 3 ml for microsomes and hepatocytes, respectively). When CL_{int} was estimated from the AUC values, similar values were obtained for these unscaled parameters (table 4).

The first time point in these studies corresponds to the time used to calculate the initial rates of hydroxycoumarin formation in the studies presented above. In the former samples, hydroxycoumarin formation was determined in addition to ethoxycoumarin depletion. There was no significant difference ($p < 0.01$ by *t* test) between these formation rates and those determined in the initial rate study at the same substrate concentration.

Predictions of *In Vivo* Kinetics from *In Vitro* Data. The V_{max} and CL_{int} values from the *in vitro* studies were scaled to *in vivo* units using either the microsomal protein recovery index or hepatocellularity (table 5). For the hydroxycoumarin formation data, a correction for the f_m for this pathway was made. Comparison with the corresponding *in vivo* data indicates different levels of success for both the incubation method and the *in vitro* system used. The predicted CL_{int} values from both hepatocyte studies were excellent, *i.e.* 85 and 89% of the actual CL_{int} value for ethoxycoumarin depletion data and hydroxycoumarin formation data, respectively. The metabolite appearance
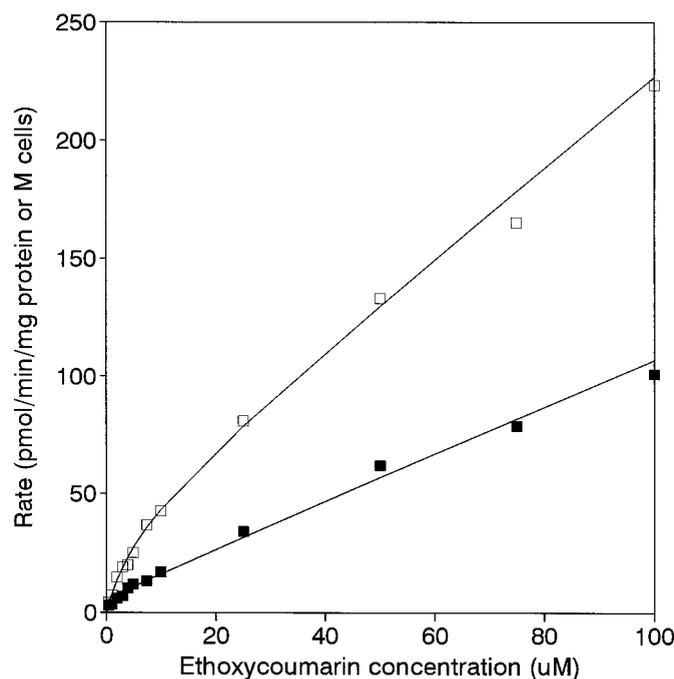

FIG. 2. *Relationships between the rate of* O-*deethylation of ethoxycoumarin and substrate concentrations in typical microsomal (□) and hepatocyte (■) preparations.*

For this particular microsomal preparation, V_{max} was 39 pmol/min/mg protein, K_M was 1 μM, and CL_2 was 1.9 μl/min/mg protein; for this particular hepatocyte preparation, V_{max} was 7 pmol/min/10^6 cells, K_M was 1.5 μM, and CL_2 was 1 μl/min/10^6 cells.

TABLE 3

Michaelis-Menten parameters obtained for hydroxycoumarin formation in hepatic microsomes and isolated hepatocytes

	V_{max}	K_M	CL_1	CL_2	CL_{HC}
	pmol/min	*μM*	*μl/min*	*μl/min*	*μl/min*
Microsomes[a]	39.8 ± 9.8	0.9 ± 0.1	47.7 ± 17.4	2.1 ± 1.0	49.8 ± 18.6
Hepatocytes[b]	14.1 ± 6.1	0.8 ± 0.5	24.3 ± 2.1	2.1 ± 0.7	26.4 ± 16.8

Data are presented as mean ± SD (N = 4).
[a] V_{max} and clearance terms are expressed per milligram of microsomal protein.
[b] V_{max} and clearance terms are expressed per 10^6 cells.

data measured in microsomes also accurately predicted the *in vivo* value (93%), whereas the ethoxycoumarin depletion data substantially underpredicted the CL_{int} value (39%).

Discussion

The pharmacokinetics of ethoxycoumarin have been defined *in vivo* in rats, with particular emphasis placed on characterizing the importance of the *O*-deethylation pathway. 7-Hydroxycoumarin, which is formed *via* this metabolic route, is the species monitored by most workers using this substrate as an *in vitro* probe substrate (Yamazaki *et al.*, 1996; Beaune *et al.*, 1986; Stevens *et al.*, 1993; Boobis *et al.*, 1986; Rogiers *et al.*, 1986; Fry *et al.*, 1992; Bayliss *et al.*, 1994; Worboys *et al.*, 1995). The urinary excretion data demonstrate that this pathway is of relatively minor importance *in vivo*, with only 26% of an administered dose of ethoxycoumarin being converted to this metabolite. Recent work by Ball *et al.* (1996) with rat liver slices indicated that there are several metabolites of ethoxycoumarin in addition to hydroxycoumarin. Indeed, Jung *et al.* (1985) identified 3-hydroxy,7-ethoxycoumarin as a primary metabolite of ethoxycoumarin *in vitro,* and previous work (Cohen, 1979) with coumarin in rats
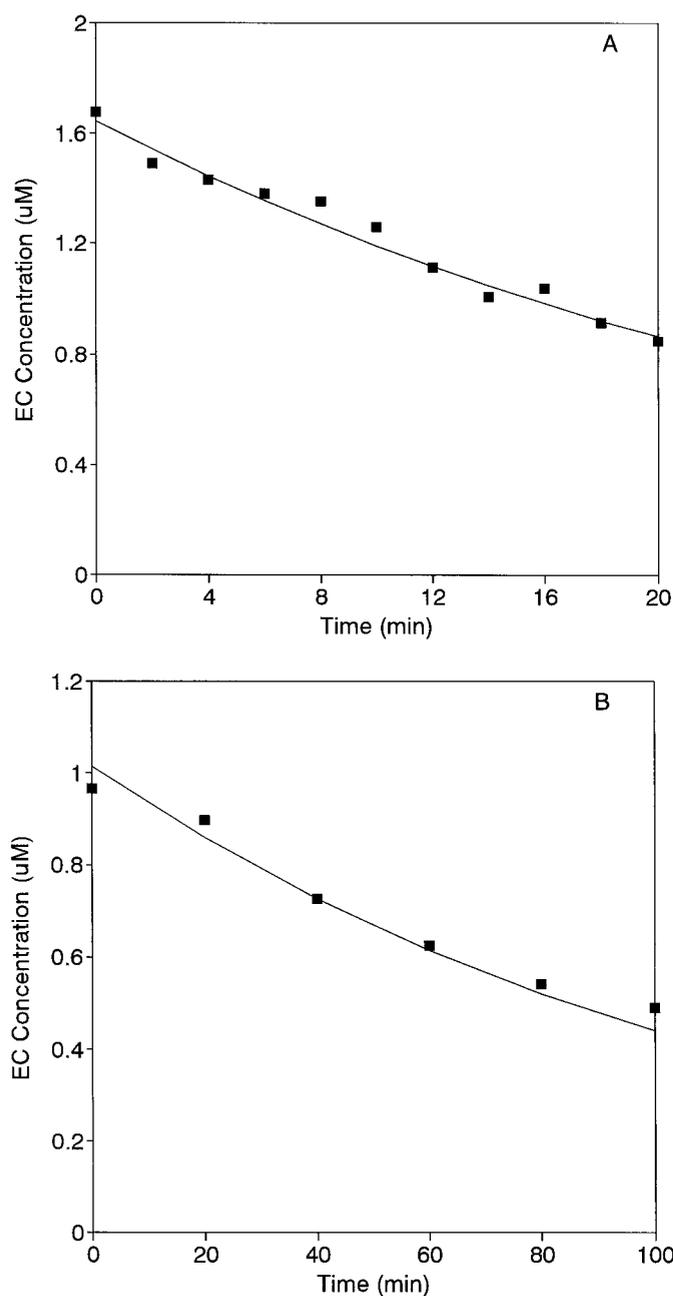
Fɪɢ. 3. *Time courses for the depletion of ethoxycoumarin (EC) in typical microsomal (A) and hepatocyte (B) preparations.*

CL_{int} values in these particular preparations were 57 μl/min/mg protein and 99 μl/min/10[6] cells, respectively.

showed this closely related chemical to be hydroxylated in several positions but primarily at position 3 of the lactone ring. Therefore, there are several other metabolites likely to be quantitatively of equal, or more, importance, compared with 7-hydroxycoumarin.

By using different infusion rates with matching loading doses, a range of steady-state concentrations of ethoxycoumarin were obtained, which enabled the pharmacokinetics of this compound to be characterized. Ethoxycoumarin clearance *in vivo* could be adequately described by a classic Michaelis-Menten model with V_{max}, K_M, and CL_{int} values. The combination of a high unbound blood CL_{int} value of 137 ml/min/SRW and an unbound blood fraction of 0.22 results in hepatic extraction for ethoxycoumarin that is classified as intermediate (extraction ratio, 0.6). The K_M is quite low (3.6 μM, based on

TABLE 4
Kinetics of ethoxycoumarin depletion in microsomes and hepatocytes

	Initial EC Concentration	Half-life	AUC	CL_{int}
	μM	*min*	*nmol/ml/min*	*μl/min*
Microsomes[a]	1.54 ± 0.23	19 ± 8	42 ± 12	81 ± 30
Hepatocytes[b]	1.04 ± 0.17	88 ± 31	135 ± 31	98 ± 28

Data are presented as mean ± SD (*N* = 6).
[a] AUC and CL_{int} are expressed per milligram of microsomal protein (incubation concentration used, 0.5 mg protein/ml).
[b] AUC and CL_{int} are expressed per 10[6] cells (incubation concentration used, 0.25 × 10^{6}/ml.).

TABLE 5
CL_{int} and V_{max} values for ethoxycoumarin determined in vivo and from scaled microsomal and hepatocyte data

Study	V_{max}	CL_{int}	CL_{int} Prediction
	nmol/min/SRW	*ml/min/SRW*	*%*
In vivo	495	137	
Microsomal hydroxycoumarin formation	26	127[a]	93
Microsomal ethoxycoumarin depletion		53	39
Hepatocyte hydroxycoumarin formation	17	122[a]	89
Hepatocyte ethoxycoumarin depletion		117	85

[a] With f_m correction (see text).

unbound blood concentrations), and the enzymes involved in ethoxycoumarin metabolism have a relatively high capacity (V_{max}, 495 nmol/min/SRW). However, these are pooled parameters (Wagner, 1985) that describe the combined effects of at least two parallel pathways. The CL_{int} and V_{max} terms are the sums of the individual parallel pathways, and the K_M term is an average value for these pathways, weighted for their relative importance (Sedman and Wagner, 1974).

Two types of *in vitro* studies have been undertaken with both hepatic microsomes and freshly isolated hepatocytes. In both systems, hydroxycoumarin formation rates demonstrated biphasic kinetics, which can be characterized by a high-affinity/low-capacity component (with V_{max}, K_M, and CL_1 terms) and a low-affinity/high-capacity component that was not saturated (with a CL_2 term), in agreement with several previous studies (Boobis *et al.*, 1986; Rogiers *et al.*, 1986; Fry *et al.*, 1992; Bayliss *et al.*, 1994). The microsomal and hepatocyte K_M values (0.9 and 0.8 μM, respectively) were comparable, as were the contributions of CL_1 to the overall clearance (96% and 92% in microsomes and hepatocytes, respectively). The observation that these K_M values are approximately 5-fold lower than the *in vivo* value based on the unbound blood concentration (3.6 μM) is not surprising, in view of the pooled nature of the *in vivo* parameter. It indicates that the K_M values for the other pathway(s) are higher than the K_M value for hydroxycoumarin formation.

Scaling factors for microsomal protein recovery (660 mg/SRW) and hepatocellularity (1.2 × 10^{9} cells/SRW), which relate the microsomal and hepatocyte data to the *in vivo* conditions (Houston, 1994), were applied to both V_{max} and CL_{int}. The scaled V_{max} values from microsomes and hepatocytes (table 5) are comparable to each other but substantially underpredict the *in vivo* value (by 20-fold). This result is probably a consequence of the f_m for the hydroxycoumarin pathway, which accounts for only approximately one fourth of the metabolism, and the lack of equivalence *in vivo* of the two-site model for hydroxycoumarin formation (the V_{max} term refers to the high-affinity/low-capacity site *in vitro*). Correction of the *in vitro* CL_{int}

values for the f_m for the hydroxycoumarin pathway yields values that are comparable to the *in vivo* CL_{int} data for both microsomes and hepatocytes (predictions of 93 and 89%, respectively, of the *in vivo* parameter). A similar approach was used previously for caffeine predictions (Hayes *et al.*, 1995), but in that case the f_m value was considerably higher.

The clearance values for ethoxycoumarin *in vivo* disposition (table 1) and hydroxycoumarin formation *in vitro* (table 3) formed part of a compilation used to assess the utility of *in vitro* systems for predicting *in vivo* metabolic clearances (Houston, 1994). Ethoxycoumarin was one of the few drugs whose clearance was not well predicted with either hepatocytes or microsomes. Now that the importance of the 7-hydroxylation pathway is better understood, the explanation for this previously reported underprediction is obvious. The case of ethoxycoumarin highlights the importance of establishing valid f_m values for incorporation into the scaling of *in vitro* data to obtain *in vivo* parameters. A number of other confounding issues were identified in previous studies, including product inhibition, futile (non-metabolism-related) binding, extrahepatic metabolism, and use of inappropriate scaling factors (Houston, 1994; Ashforth *et al.*, 1995; Carlile *et al.*, 1997).

The *in vitro* ethoxycoumarin depletion-time studies provide data that are amenable to scaling in the same fashion as the hydroxycoumarin formation data, allowing some interesting comparisons to be made. Although both the microsomal and hepatocyte CL_{int} values from the metabolite appearance studies accurately predict CL_{int} *in vivo*, this is not the case for the alternative approach. Hepatocyte depletion data also accurately predict the actual CL_{int} value, indicating that hepatocytes can be successfully used for prediction studies whether the metabolic fate of the compound of interest is known or not. In contrast, however, the corresponding microsomal data substantially underpredict the *in vivo* CL_{int}. The exact reasons for this are unknown, but some speculations can be made. In the microsomal study, although the initial EC concentration (1.5 μM) is slightly higher than the corresponding concentration in the hepatocyte study (1 μM) and the microsomal K_M for hydroxycoumarin formation, it is substantially lower than the unbound *in vivo* K_M for ethoxycoumarin (3.6 μM); therefore, this concentration difference is unlikely to play a major role. Previous comparisons between microsomes and hepatocytes have indicated that the former system is prone to product inhibition in some cases, namely with phenytoin (Ashforth *et al.*, 1995) and diazepam (Zomorodi *et al.*, 1995). The present studies indicate that such a phenomenon is of little importance when hydroxycoumarin formation is monitored, although it is possible that one of the quantitatively more important metabolites formed from ethoxycoumarin may cause some inhibition of its own metabolism in microsomes. However, because these other metabolites of ethoxycoumarin have not been identified and their relative importance is unknown, it is not possible to test this speculation further.

In conclusion, the present study characterizes for the first time the pharmacokinetics of ethoxycoumarin in rats. Hydroxycoumarin formation accounts for a relatively small fraction of ethoxycoumarin metabolism. Although this finding is of interest and is crucial for the extrapolation of *in vitro* kinetic data on hydroxycoumarin, it is of minor concern to investigators using ethoxycoumarin as a marker of the metabolic competence of their particular *in vitro* systems. The present studies also demonstrate the value of *in vitro* drug depletion data for *in vivo* prediction studies. The studies with hepatocytes provide unequivocal evidence that clearance can be predicted successfully from data describing either metabolite appearance or drug depletion. The latter approach has obvious advantages when the metabolic fate of the drug under investigation is not known.

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