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TISSUE SLICES REVISITED: EVALUATION AND DEVELOPMENT OF A SHORT-TERM INCUBATION FOR INTEGRATED DRUG METABOLISM

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ABSTRACT:

This work details the development of a model for the rapid evaluation of drug metabolism in an integrated fashion using in situ architecture of the liver. A Krumdieck tissue slicer was used to generate slices from 10-mm cores of rat liver (approximately 250-μm thick). Initial unsuccessful efforts with 6-well plate-based incubation were overcome with the use of a dynamic (rotating) incubation in 23-ml liquid scintillation vials containing titanium mesh supports for the slice. Incubation of 1 slice/5 ml of a Krebs-Henseleit solution buffered with HEPES showed a <2% increase over the initial 25% release of lactate dehydrogenase over 2 h of incubation at 37°C under ambient oxygen conditions. Coupled *O*-dealkylase and conjugative metabolism of alkoxycoumarin derivatives was shown to be linear for both 7-methoxy- and 7-ethoxycoumarin (100 μM) with a low amount of nonconjugated 7-hydroxycoumarin (7-HC) at all time points. Metabolic profiles for 7-methoxy- and 7-ethoxycoumarin were compared between slice and microsomal incubations generated from the same tissue. The use of 7-HC as a primary substrate not only provided an assessment of the capacity-based differences in oxidative versus conjugative metabolism but also capacity-based differences in glucuronidation and sulfation. These studies underscore the physiological fact that phase I metabolism has a lower capacity for substrate metabolism than phase II metabolism. Additionally, this technique provides a model for examination of pharmacodynamic and pharmacokinetic influences in the context of maintenance of the in situ architecture of the liver.

The mammalian liver has the capacity to metabolize a variety of endogenous and xenobiotic compounds. It is important to appreciate the roles of phase I and phase II biotransformations in the integrated metabolic profile of a compound. This sequence of events, operating in isolation or in concert, can dramatically alter the disposition of a compound within an organ system or the body as a whole. Given the major role that the liver plays in the metabolism of foreign compounds, it is prudent to consider development of in vitro methods using this tissue. Commonly used in vitro preparations for animal studies include perfused liver, hepatocytes, subcellular fractions, reconstituted purified enzyme fractions, and liver slices. Any of these preparations may be useful for the study of hepatic biotransformation; however, like all in vitro models, there are limits to each method (reviewed in Fisher et al., 1995; Kane and Thohan, 1996; Worboys et al., 1996; Toutain et al., 1998; Hashemi et al., 1999). A chronology of liver slice development and the various systems that have been adapted from the inception of tissue slices as a paradigm to study metabolism has been reviewed (Ekins, 1996).

The use of liver slices is a relatively new addition to the battery of in vitro models to evaluate the metabolism of xenobiotics. As with any developing or new technology, there are improvements and alterations effected to optimize this methodology. Liver slices have been used as an alternative in vitro method for the assessment of hepatic drug metabolism (Barr et al., 1991a,b; Kane and Thohan, 1996). Use of liver slices provides decided advantages over previous in vitro techniques because this preparation allows for maintenance of the functional acinar architecture of the liver and has displayed drug metabolism over a span of hours to days. Maintenance of the functional architecture, simultaneous analysis of human and test animal tissue, and a direct comparison of metabolites using subcellular fractions from the same tissue sample have made this a very versatile method for the study of drug disposition in vitro.

Improvements in the methodology of tissue slice generation and incubation have been reviewed in attempts to harmonize research efforts; however, there are still only a limited number of studies that use this paradigm in drug safety and toxicity assessment (Fisher et al., 1995; Ekins, 1996). An overview of compounds that have been recently studied in liver slices include: testosterone (Ekins et al., 1996), tacrolimus (Ueda et al., 1996), caffeine, tolbutamide, phenytoin, ondansetron, diazepam (Worboys et al., 1997), olanzapine (Murphy et al., 1998), clenbuterol (Zalko et al., 1998), and thio-tepa (Dale et al., 1999). Alkoxycoumarins as model substrates have been extensively used to characterize drug-metabolizing activities in various species (Walsh et al., 1995; Ball et al., 1996; Ekins, 1996; Kane and Thohan, 1996; Carlile et al., 1999; Hashemi et al., 1999; Thohan et al., 1999).

The long-term goal of these studies was to refine liver slice use for

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a rapid assessment of bioactivation of model and investigational compounds that would serve as a more relevant predictor of in vivo metabolism. To this end, alkoxycoumarins were selected as model compounds for characterization of phase I, phase II, and integrated drug metabolism. These substrates provide a broad spectral analysis of cytochrome P450 (CYP[1])-related activity, glucuronidation, and sulfation. Remnant tissue from the preparation of liver slice cores was used to compare liver slice metabolism with microsomal CYP-mediated metabolism and glucuronosyl transferase activities.

Materials and Methods

Chemicals. 7-Ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), β-glucuronidase (glucarase), saccharic acid 1,4-lactone, phenylmethylsulfonyl fluoride, butylated hydroxy toluene, lactate dehydrogenase (LDH) determination (kit no. 228-UV), Waymouth's MB medium, Krebs-Henseleit buffer, and HEPES were purchased from Sigma (St. Louis, MO). 7-Methoxycoumarin (7-MC) was obtained from Aldrich (Milwaukee, WI). Coomassie blue reagent was purchased from Bio-Rad (Richmond, CA). All other chemicals were of the highest chemical purity commercially available.

Animals. Male Sprague-Dawley rats (180–220 g; Charles River Laboratories, Inc., Wilmington, MA) were used in these studies. Animals were housed in the Walter Reed Army Institute of Research (WRAIR) animal facility under standard operating conditions. Food and water were allowed ad libitum. Animals were restricted from food for 16 h before euthanasia. Animals were prepared for terminal surgical anesthesia with pentobarbital (60 mg/kg) by intraperitoneal injection. Livers were perfused with ice-cold 0.9% saline before excision.

Slice Generation. *Core preparation.* Livers were dissected into lobes, and cores were produced using a cylindrical stainless steel corer with an 11-mm internal diameter. Care was taken during the entire procedure to observe aseptic procedures where possible. Cores of tissue were transferred to ice-cold 0.9% saline and sliced.

Slice preparation and incubation. The slicing mechanism was placed into ice-cold saline, and the tissue core loaded into the holder mechanism of the Krumdieck tissue slicer (Alabama R&D, Munford, AL). Slice thickness (approximately 0.25 mm) was controlled via an adjustable base plate and variation in the weight on the tissue core. Slice thickness was periodically monitored using a microscope and a micrometer guide for 0.25 mm. Liver slices were generated by movement of the weighted tissue core sample over an oscillating razor blade. These slices were swept away to a collection chamber by a stream of saline. Using the tissue slicer in the above fashion, a slice was generated every 3 to 4 s. Slices were transferred to a shallow tray containing ice-cold 0.9% normal saline for selection and loading onto the incubation supports. Selection criteria were based upon the physical appearance of the tissue slice. Slices that had portions of the hepatic capsule associated with them were not used. These slices were recognizable from a folded or "curled-up" appearance. Slices that did not appear to be uniform in thickness or cellular integrity were also not used for experimental incubations.

Dynamic incubation. Titanium mesh supports (20 × 55 mm) were fitted to 23-ml liquid scintillation vials used for incubation. These mesh supports were stored in ethanol until use, at which time they were rinsed with distilled water, followed by incubation medium to ensure no alcohol was transferred with the mesh support. Slices were "loaded" onto supports by swirling the medium with a cotton-tipped applicator. Supports were blotted to ensure adherence of the tissue slice to the support and placed into 23-ml glass incubation vials containing 5 ml of oxygenated medium, pH 7.4. Vials were incubated horizontally in an enclosed incubator maintained at 37°C and rotated at 6 rpm using a modified hot dog cooker (AP Wyott, Baltimore, MD). After a 60-min preincubation, tissue supports were blotted and transferred to new vials containing fresh medium and substrate. Incubations were terminated by removal of the slice from the medium. Tissue slices were transferred to polypropylene microcentrifuge tubes that contained 1 ml of distilled water. Slices were homogenized using sonic disruption, and protein content was estimated by the method of Bradford (1976).

Viability assessment. Slice viability was assessed by the release of LDH from the tissue slice into the medium. LDH activity was determined in the medium and the slice homogenate according to the Sigma kit instructions (kit 228-UV). A kinetic change in absorbance at 340 nm was quantified using a Beckman DU 640 (Beckman, CA) according to the methods of Amador et al. (1963). Values were normalized as a percentage of total content of the marker enzyme.

Microsomal preparation. Tissue remnants (8–12 g) from the slice preparation procedure were used for subcellular fractionation and generation of microsomal fractions by the methods of Van der Hoeven and Coon (1974) and Halpert et al. (1983). Minced livers were weighed and placed in four volumes of a buffer consisting of 50 mM Tris, 0.1 M KCl, 1.0 mM EDTA, and 20 mM butylated hydroxy toluene, pH 7.4. Each liver sample was subjected to three 10-s bursts from a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) equipped with an ST-10 probe. Microsomal fractions were isolated and were then subdivided for single use and stored at −80°C.

Alkoxycoumarin Assay. *Microsomal assay.* Alkoxycoumarin *O*-dealkylase was measured using a 1-ml reaction mixture containing 0.5 mg of microsomal protein, 0.05 M Tris buffer, pH 7.4, with 1 mM EDTA, and 100 μM substrate. After a 5-min preincubation at 37°C, the reaction was initiated by the addition of an NADPH-generating system (5 mM G6P, 1 unit/ml G6PDH, 1 mM NADP, and 3 mM $MgCl_2$). Reactions were terminated by the addition of 5 ml of an ether/isoamyl alcohol mixture (ether/IAA, 1:0.014) to selectively remove 7-HC from the aqueous medium. This ether/IAA extraction mixture (5 ml) was added to 1 ml of incubation medium and shaken horizontally for 15 min on a reciprocating shaker. A 1-ml aliquot of the ether extraction mixture was then evaporated over 3 ml of glycine/NaOH (0.2 M; pH, >10.4) buffer to ensure the complete "back extraction" of the free metabolite. Authentic standards of 7-HC were used to generate a standard curve (2.5–1000 nM) used for quantification (excitation, 370 nm; emission, 450 nm), as described by Greenlee and Poland (1978).

Phase I metabolite determination from rat liver slices. A 1-ml sample aliquot was extracted in a similar fashion to microsomal incubations to quantify and selectively remove nonconjugated 7-HC (free-metabolite) from the aqueous medium. The remaining aqueous portion of sample extracts that contain conjugated 7-HC was analyzed as detailed below.

Phase II metabolite determination from rat liver slices. Glucuronide and sulfate conjugates in the aqueous phase of the initial extracts were subjected to specific hydrolytic enzymes to liberate free 7-hydroxycoumarin. Individual aliquot samples were hydrolyzed with 500 units of glucarase or 10 units of a crude sulfatase preparation containing 20 mM *d*-saccharic acid 1,4-lactone in a 0.2 M sodium acetate buffer (adjusted to pH 5.1). The liberation of 7-hydroxycoumarin after a 16-h incubation at 37°C was quantified by the extraction procedure described above for free 7-hydroxycoumarin quantitation. Using 7-HC as the starting metabolite allowed assessment of phase II activity in the absence of phase I metabolism. The use of 7-HC as a primary substrate required an additional cleanup extraction with ether/IAA extraction mixture before the addition of hydrolytic enzymes to minimize background interference from residual parent compound. The entire procedure for the quantitation of free 7-HC and conjugates could be accomplished within 48 h.

Protein Determination. Protein contents for tissue slices and microsomes were determined using the method of Bradford (1976). Concentrated reagent stock was diluted with double distilled water to generate a working solution according to the manufacturer's instructions. Standard curves were prepared from fresh stock solutions of bovine serum albumin. Standard curves were linear between 0.05 and 1.0 mg/ml.

Statistics. Statistical evaluations were carried out using the Student's *t* test; $p \leq 0.1$ was considered significant.

Results

Linear Incubation: Method Evaluation. Studies were conducted to optimize conditions for the development of an in vitro model that would provide rapid and reproducible data that may be compared with in vivo findings. This multistep process involving assessment of reproducibility, viability, and metabolic capacity must be performed

[1] Abbreviations used are: CYP, cytochrome P450; 7-EC, 7-ethoxycoumarin; 7-HC, 7-hydroxycoumarin; LDH; lactate dehydrogenase; 7-MC, 7-methoxycoumarin; IAA, isoamyl alcohol mixture.

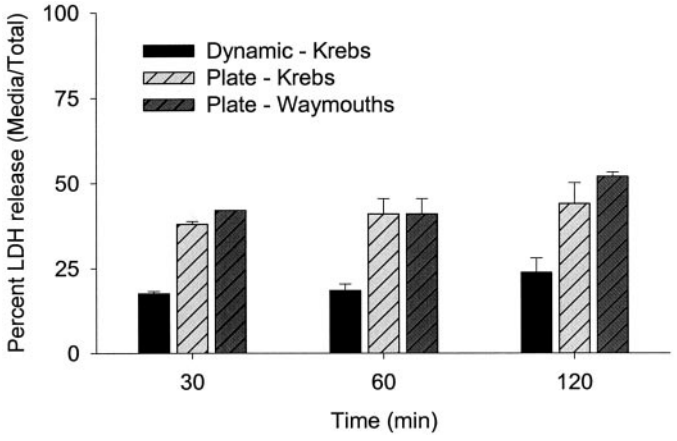

FIG. 1. *LDH release from liver slices using dynamic incubation.*

Figure 1 shows LDH release from control rat liver slices (n = 6 slices per determination).Values presented are mean ± S.D. Percentage of release shown corresponds to percentage of the total cellular content. This graph illustrates the high release of intracellular enzymes over the course of incubation of liver slices in the linear incubation (see *Materials and Methods*). These studies provided sufficient evidence regarding viability of the preparation to select the dynamic incubation as a method of study up to 120 min of incubation.

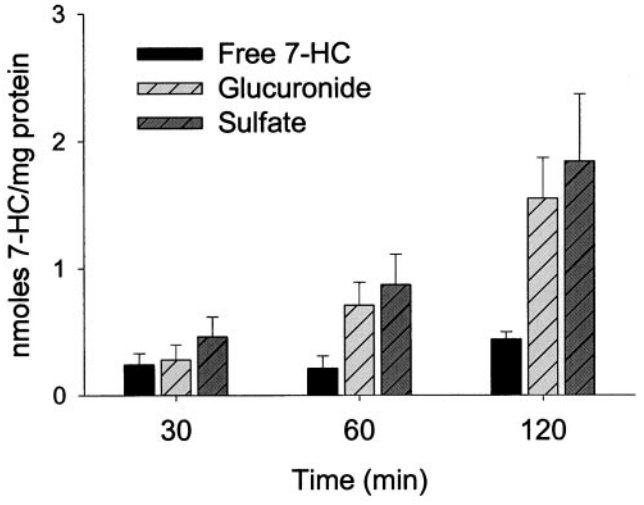

FIG. 2. *Integrated metabolism of 7-methoxycoumarin.*

Figure 2 depicts the rat liver slice metabolic profile for 7-methoxycoumarin (n = 7 rats). Values presented are mean ± S.D. Substrate concentrations of 100 μM were added after a 60-min preincubation period. Metabolites were assayed as detailed under *Materials and Methods*. Tight integration of metabolism was demonstrated by the low level of free 7-HC.

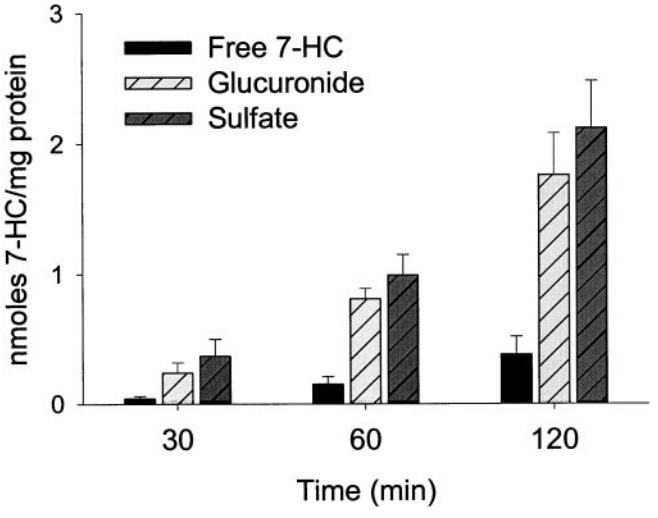

FIG. 3. *Integrated metabolism of 7-ethoxycoumarin.*

Figure 3 depicts the rat liver slice metabolic profile for 7-ethoxycoumarin (n = 7 rats). Values presented are mean ± S.D. Substrate concentrations of 100 μM were added after a 60-min preincubation period. Metabolites were assayed as detailed under *Materials and Methods*. Tight integration of metabolism was demonstrated by the low level of free 7-HC.

before being used with test compounds. Initially, the linear incubation method was evaluated for preparation viability using 6-well tissue culture plates, as adapted from Dogetrom (1993). Preincubation of liver slices, coupled with a short-term exposure to test compounds or marker substrates, was defined as an acute exposure and obviated the addition of bacteriostatic agents, such as gentamicin and/or penicillin-streptomycin in the incubation medium. Waymouth's MB medium was chosen initially; however, LDH release of greater than 40% after 30 min, which remained at this level over the 120-min incubation period, resulted in the search for alternatives (Fig. 1). As a secondary preference, Krebs-Henseleit buffer with HEPES was used. As in the case of Waymouth's MB medium, an LDH release of approximately 40% occurred after 30 min, staying at this level over the 120-min course of incubation (Fig. 1). Various permutations and combinations of experimental conditions were examined to provide a low background of LDH release. Typical patterns of LDH release showed an early release of LDH (~40%) followed by a dramatic increase over 120 min of incubation. These studies were suspended in lieu of an alternative incubation system.

Dynamic Incubation: Method Development. The dynamic incubation type described by Smith et al. (1985, 1987) and Barr et al. (1991a,b) was investigated as an alternative to the linear method. Krebs-Henseleit buffered with HEPES was used as the incubation medium. A 60-min preincubation period was used to acclimate the slices to the buffer medium and reduce the effects of hydrolytic enzymes in the slice incubation. Less than a 25% LDH release was routinely maintained throughout the course of studies conducted using Krebs-Henseleit incubation medium (Fig. 1). There was no significant increase in the release of LDH over the course of the incubation, whether or not the alkoxycoumarins were present (data not shown). This absence of significant LDH release was in direct contrast to results found using the linear incubation method.

Alkoxycoumarin Integrated Metabolism. Initial studies using time-dependent *O*-demethylation and *O*-deethylation of alkoxycoumarins were investigated to provide specific and sensitive measures of cytochrome P450 activity. An integration of phase I and phase II metabolism was supported by the low amount of unconjugated 7-HC (free 7-HC) relative to the total metabolite disposition (Figs. 2 and 3). Table 1 is a summary of the differential metabolism of 7-MC and 7-EC. A significant disparity in metabolite formation at the 30-min time point showed that *O*-demethylase activity was proceeding at a faster rate than *O*-deethylase activity. This faster rate of 7-HC production highlighted the time lag between phase I production of metabolites and phase II conjugation. Microsomal fractions isolated from these tissues were used to compare metabolic rates. Rat liver slice metabolism profiles for *O*-dealkylase did not qualitatively reflect the initial metabolic rates of 247 ± 81 and 268 ± 50 pmol/min/mg of protein for 7-MC and 7-EC, respectively, found in microsomal preparations. Microsomal metabolic rates showed no difference between *O*-demethylation and *O*-deethylation activities. After the initial 30-min time period, no difference was noted in microsomal metabolic rates upon comparison with those obtained in tissue slices. In liver slice incubations, the difference in initial rates of *O*-dealkylation

TABLE 1

7-HC generation from 7-MC and 7-EC O-dealkylase activity in liver slices

Table 1 represents an $n = 7$ rats. Values presented are mean nanomoles of 7-HC/milligrams of slice protein ± S.D. Total metabolism is a summation of free, glucuronide, and sulfate conjugates arising from alkoxycoumarin metabolism in rat liver slices. Substrate concentrations of 100 μM were added after a 60-min preincubation period. Metabolites were assayed as detailed under *Materials and Methods*.

	Free-7-HC	Total Metabolism	Free/Total Ratio
7-MC			
30 min	0.24 ± 0.09[a]	0.97 ± 0.22	0.24
60 min	0.21 ± 0.10	1.78 ± 0.31	0.11
120 min	0.44 ± 0.06	3.83 ± 0.62	0.11
7-EC			
30 min	0.04 ± 0.02	0.64 ± 0.15	0.06
60 min	0.15 ± 0.06	1.95 ± 0.19	0.08
120 min	0.38 ± 0.14	4.26 ± 0.50	0.09

[a] Denotes statistical significance, $p \leq 0.1$, between *O*-demethylase and *O*-deethylase activities.

resulting in the generation of free 7-HC was not measurable after 30 min.

Once a pharmacodynamic equilibrium had been reached, *O*-demethylase activity was 1.88 nmol/mg of slice protein/h, whereas the *O*-deethylase activity was 1.78 nmol/mg of slice protein/h. No statistical difference in the total rate of *O*-demethylase and *O*-deethylase activities in liver slices (sum total of free and conjugates) was observed, in contrast to levels of free 7-HC summarized in Table 1.

Metabolic rates for 7-HC were approximately 7-fold greater for phase II metabolism than for phase I metabolism (Fig. 4). Using 7-HC as the primary substrate, the average metabolic rate for phase II conjugation in rat liver slices was 12.5 nmol/h/mg of slice protein. Total phase I activity over a similar incubation period, using identical substrate concentrations, was 1.88 and 1.78 nmol/h/mg of slice protein for *O*-demethylase and *O*-deethylase activities, respectively.

Glucuronidation rates were linear over the 120-min incubation, whereas sulfation rates stabilized after the first hour. These results illustrated preferential glucuronidation in phase II conjugation reactions in liver slices. This study, in conjunction with the presence of only low levels of free 7-HC from *O*-demethylase and *O*-deethylase activities, confirms the observation that phase I is the determining factor in the integrated metabolism profile. Phase II conjugative metabolism has a far greater capacity than phase I metabolism.

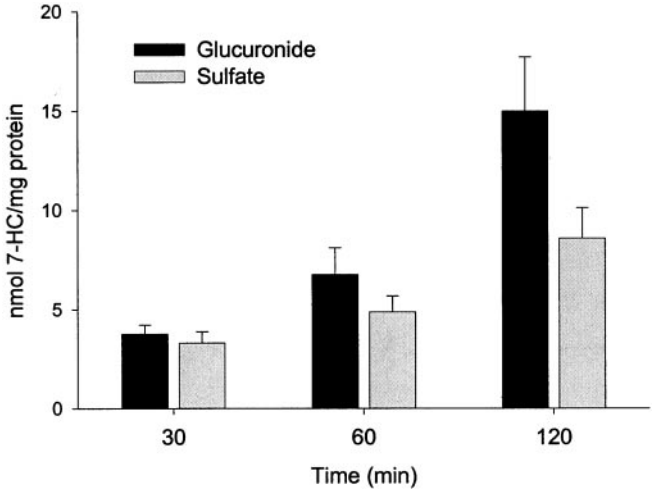

FIG. 4. *Phase II metabolism of 7-hydroxycoumarin.*

Figure 4 depicts the rat liver slice metabolic profile for phase II metabolism ($n = 7$ rats). Values presented are mean ± S.D. Substrate concentrations of 100 μM were added after a 60-min preincubation period. Metabolites were assayed as detailed under *Materials and Methods*. *, denotes statistical significance between glucuronide and sulfate conjugates, $p \leq 0.1$.

Discussion

This work details the development of a model and its utility for assessment of integrated drug metabolism in liver slices. During development of a rugged research platform, one must optimize and assess reproducibility, viability, and metabolic capacity. These studies evaluated the applicability of the liver slice incubation to assess drug metabolism profiles using incubation profiles of less than 120 min.

Liver slice viability was assessed by LDH release quantified as a percentage of the total liver slice LDH content. The relationship between LDH release and viability has not been clearly established; however, the degree of leakage of this enzyme into the incubation medium has been a generally accepted marker of parenchymal cell membrane integrity (Barr et al., 1991a,b; Fisher et al., 1995; Ekins et al., 1996; Olinga et al., 1997). Since no threshold has been established for enzyme release and viability, background levels for intracellular enzyme release should be kept low so that increases in enzyme release may be reliably quantified. Histological evaluation by Smith et al. (1987), and later by Kane and Thohan (1996), confirmed observations of cellular damage at the cut faces of the liver slice; however, integrity of the deeper cellular architecture was maintained. LDH release can be enhanced by the physical trauma resulting of slice handling and type of incubation (Dogetrom, 1993; Fisher et al, 1995).

Composition of the incubation medium can also affect the outcome of metabolism-based studies. Waymouth's MB media and fetal calf serum are commonly used tissue culture components that contain a number of nucleophiles (sulfhydryl containing compounds and serum proteins) and antioxidants that stabilize the preparation and promote cellular growth; however, they can also result in the complication of metabolite identification or underestimation of the quantitation of toxicological events. A Krebs-Henseleit solution, supplemented with HEPES, has been shown to support drug metabolism in the absence of complex tissue culture media with supplemental components (Berthou et al., 1989; Dogetrom and Rothuzien, 1993; Steensma et al., 1994; Ekins et al., 1995; Worboys et al., 1995; Kane and Thohan, 1996). Using 6-well plates (linear method), Krebs-Henseleit buffer supplemented with HEPES did not provide any advantage in viability over the more complex Waymouth's MB medium. Due to potential future issues with metabolite identification and toxicological event quantitation, it was decided to proceed with Krebs-Henseleit buffer supplemented with HEPES as the incubation medium of choice. In contrast to the 50% release of LDH observed using the linear method, an LDH release of less than 25% was found using the dynamic incubation method. Augmenting that contrast was the fact that, over the course of the dynamic incubation, there was no significant increase in release of LDH, in agreement with Ekins et al. (1996).

Dynamic incubation of rat liver slices to quantify time-dependent *O*-demethylation and *O*-deethylation of alkoxycoumarins were investigated to provide specific and sensitive measures of CYP activity. The major metabolite of *O*-demethylation and *O*-deethylation activities of CYP is 7-HC, which may then be conjugated with glucuronic acid or sulfate moieties (Matsubara et al., 1983; Ball et al., 1996). These metabolites provide a sensitive and analytically stable assessment of integrated drug metabolism. Inclusion of 7-HC as a primary substrate allowed for quantification of phase II conjugation in the absence of phase I metabolism. These studies confirmed the integrated nature of drug metabolism in liver slices and that the rate of integrated metabolism was limited by the capacity of CYP and phase II enzymes in naive, untreated, rat liver slices. Integration of the drug metabolism process has been used previously as an alternative indicator of via-

bility (Barr et al., 1991a,b; Fisher et al., 1995; Ekins et al., 1996). Patterns for phase I metabolism were found to be linear and qualitatively similar between liver slice and microsomal preparations in that there were no significant differences in overall average rates of metabolism between *O*-demethylase and *O*-deethylase activities.

That an increased rate of *O*-demethylase activity versus *O*-deethylase activity was observed at the 30-min interval in liver slices but not in microsomal preparations illustrates the importance of physicochemical considerations and the utility of liver slices to investigate these issues. These findings highlight the importance of pharmacokinetic effects, such as substrate uptake, metabolism, and metabolite elimination in a more physiologically relevant system in vitro. Differences in *O*-demethylation and *O*-deethylation activity may have been previously overlooked since microsomal metabolism studies traditionally focus on K_m and V_{max} and, functionally, are not intended to differentiate metabolic rates based upon physicochemical and pharmacokinetic characteristics.

The observation that there was no metabolic rate differential seen in microsomes is to be expected considering that the microsomal system is a "rate-optimized" situation in which there are no physiological barriers with saturating concentrations of both cofactor and substrate. These comments do not detract the validity of microsomal and subcellular fraction-based metabolism profiles. Quite the contrary is evidenced by the fact that many mechanistic studies performed using microsomal and other reconstituted systems could not have been performed any other way. The liver slice methodology, due to its design and similarity to the in situ condition, could yield rates that are different. Metabolic rate differentials between liver slices and microsomes may be calculated based upon microsomal yield and slice homogenate protein content; however, this comparison would only serve to reinforce the differences between these metabolic systems. It is important to note that metabolic rates should not be simplified into a function of the enzyme at saturating conditions, as with rate-optimized systems. It rather would be more appropriate to view these rates as a more dynamic process involving transport, uptake, affinity, and enzyme turnover. Using the method discussed in this work, early effects on these pharmacokinetic variables can be assessed.

The persistence of low levels of free 7-HC in liver slice incubations over 2 h demonstrated a tight integration of metabolism. Walsh et al. (1995) and Ball et al. (1996) detailed the presence of low levels of free 7-HC and time-dependent integrated metabolism up to 8 h. This early tight metabolic integration illustrates an important pharmacokinetic phenomenon of substrate accessibility to the enzyme site. The 7-MC is less polar and marginally more lipophilic than 7-EC and would, therefore, diffuse through lipid membranes and account for the increased initial rate of metabolism. Differential substrate access in liver slices was initially reported by Ekins et al. (1995). Their findings, using 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3*H*)-quinazolinone, supported the proposition of the existence of a diffusional barrier in liver slices that may hinder substrate access to the metabolizing enzymes. Worboys et al. (1997) examined the issue of diffusional barriers by quantifying the metabolism of a series of chemicals with distinct physicochemical characteristics. Slice content of drugs correlated positively with their lipophilicity. Microsomal fractions do not have diffusional barriers and thus represent a functional divergence from the physiological situation. It would, therefore, be reasonable to expect differences in metabolic rates between microsomes and liver slices, just as in vivo studies may not be matched in microsomal model systems. Chemical dissolution of the collagen support matrix of the liver during the preparation of hepatocytes can reduce physiological barriers and thus affect membrane permeability. Ekins et al. (1995) and Carlile et al. (1999) detailed the efficiency of phase I and II reactions in hepatocytes, microsomes, and liver slices. These investigators support the proposition that metabolic rates determined using liver slice preparations more accurately represent the pharmacokinetic/pharmacodynamic aspects of in vivo drug metabolism than is observed with other in vitro preparations. Furthermore, to accurately compare the metabolic rates between various in vitro preparations, one must be able to estimate the substrate concentration at/near the enzyme. This task has posed a challenge when comparing hepatocellularity with activity and normalized values between microsomal fractions, hepatocytes, and liver slices (Worboys et al., 1995, 1997; Carlile et al., 1999).

In summary, patterns for phase I metabolism were qualitatively similar between rat liver slice and microsomal preparations in that there were no significant differences in overall average rates of metabolism between *O*-demethylase and *O*-deethylase activities. Using 7-HC as a phase II probe substrate confirmed liver slice capacity-limited metabolism with the predominance of glucuronidation (100 μM 7-HC). Time-dependent linear patterns of metabolite formation were observed regardless of integrated *O*-dealkylase or conjugative metabolic activity. These studies underscore that phase I metabolism has a lower capacity for substrate metabolism than phase II metabolism. Liver slices are a more reflective in situ paradigm, with respect to pharmacokinetic/pharmacodynamic influences, than other in vitro techniques. This model would be readily applicable for the investigation of physiological and environmental factors that effect drug metabolism, such as age, gender, circadian rhythm, nutritional status, disease state, and enzyme induction or inhibition via endogenous or exogenous compounds.

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