Extractive Biotransformation for Production of Metabolites of Poorly Soluble Compounds. Synthesis of 32-Hydroxy-Rifalazil

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Running title:
Rifalazil metabolite synthesis using mouse liver microsomes

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396 words in the Introduction
2319 words in the Results and Discussion

ABBREVIATIONS: MLM, mouse liver microsomes; EtOAc, ethyl acetate
ABSTRACT

A novel reaction system was developed for the production of metabolites of poorly water-soluble parent compounds using mammalian liver microsomes. The system includes the selection and use of an appropriate hydrophobic polymeric resin as a reservoir for the hydrophobic parent compounds and its metabolites. The utility of the extractive biotransformation approach was demonstrated for the production of a low yielding, synthetically-challenging 32-hydroxylated metabolite of the antibiotic rifalazil, using mouse liver microsomes. In order to address the low solubility and reactivity of rifalazil in the predominantly aqueous microsomal catalytic system, a variety of strategies were tested for the enhanced delivery of hydrophobic substrates, including the addition of mild detergents, polyvinylpyrrolidone, glycerol, bovine serum albumin, and hydrophobic polymeric resins. The latter strategy was identified as the most suitable for the production of 32-hydroxy-rifalazil, resulting in up to 13-fold enhancement of the volumetric productivity compared to the standard aqueous system operating at the solubility limit of rifalazil. The production process was optimized for a wide range of reaction parameters; the most important for improving volumetric productivity included the type and amount of the polymeric resin, cofactor recycling system, concentrations of the biocatalyst and rifalazil, reaction temperature, and agitation rate. The optimized extractive biotransformation system was used to synthesize 32-hydroxy-rifalazil on a multimilligram scale.
INTRODUCTION

Rifalazil (1) (Figure 1) is a new rifamycin derivative, exhibiting potent activity against a variety of mycobacteria and Gram-positive bacteria (Rothstein et al., 2003). The primary metabolites of rifalazil in humans and mice are 32-hydroxy-rifalazil (2) and 25-deacetyl rifalazil, while 30-hydroxy-rifalazil (3), 25-deacetyl rifalazil, and 30-hydroxy-25-deacetyl rifalazil are major products of metabolism in dog (Mae et al., 1999; Hosoe et al., 1996). 32-Hydroxy-rifalazil, being a major metabolite in humans, is an important synthetic target to provide material necessary for its toxicology evaluation and use as an analytical standard in preclinical and clinical studies. Previously, milligram quantities of 32-hydroxy-rifalazil were successfully isolated and purified from mouse urine after oral administration. However, to achieve this production, approximately 100 g of rifalazil was dosed to 25 mice over a period of several months, corresponding to an overall isolated yield of approximately 0.03% (Mae et al., 1999).

A possible alternative to this tedious and time-consuming procedure is in vitro preparative synthesis of the metabolite using liver microsomes, which have been shown to produce 32-hydroxy-rifalazil with up to 6% estimated yield (Mae et al., 1996 and 2000). The major obstacle in practical implementation of this synthetic strategy is the very low solubility (approximately 10 mg/l) of rifalazil in the predominantly aqueous reaction medium required for microsomal incubations. Simple linear scale-up of a low-yielding microsomal reaction at such a low concentration of the parent drug would result in unacceptably high costs of metabolite production even at milligram scale, due to the high cost of the catalyst and cofactor regeneration.

Published data describing the application of delivery vehicles for hydrophobic substrates in reactions catalyzed by liver microsomes or other liver fractions is quite limited. It has been shown that microsomes have very low tolerance to a variety of organic cosolvents such as methanol, dimethyl sulfoxide, and acetonitrile, which are typically used to deliver hydrophobic substrates.
Even after dilution of these cosolvents to approximately 1 % v/v or above the catalytic activity of microsomes is substantially reduced (Chauret et al., 1998). Moreover, such cosolvent concentrations are also not sufficient to appreciably increase the solubility of many hydrophobic drugs in the primarily aqueous reaction medium. Therefore, the main objective of this work was to develop a reaction system to improve compound delivery and increase productivity for microsomal transformations of poorly soluble substrates. Candidate methods were evaluated for preparative synthesis of 32-hydroxy-rifalazil on a multimilligram scale.
MATERIALS AND METHODS

Materials. Frozen mouse livers, pooled from a combination of both male and female mice, were obtained from Pel-Freez Biologicals (Rogers, AR). Mice were grown on a diet of dog food and sacrificed via carbon dioxide asphyxiation at about ten to twelve weeks of age. Upon harvesting, livers were immediately frozen on dry ice. Commercial liver microsomes from human, mouse and dog were obtained from Gentest (Woburn, MA). Glucose dehydrogenase and NADP were purchased from Biocatalytics (Pasadena, CA). Polymeric resins used in this work included Amberlite (Supelco, Bellefonte, PA), Sepabeads and Diaion resins (Mitsubishi Chemical Corp., Chesapeake, VA), and Bio-Beads (Bio-Rad Laboratories, Hercules, CA). Alkamuls EL-620 was obtained from Rhodia (Cranbury, NJ). Rifalazil was supplied by ActivBiotics, Inc. (Lexington, MA). All other reagents and chemicals were from Sigma (St. Louis, MO).

Preparation of Mouse Liver Microsomes (MLM). The preparation of MLM was done following a published protocol (Schenkman and Jansson, 1998). Each batch of prepared MLM was submitted to the carbon monoxide binding assay (Omura and Sato, 1964) and Bradford protein assay (Bradford M, 1976) to determine the overall CYP and protein content, respectively. The protein content and total CYP concentration in MLM suspensions were typically 70-150 mg/ml and 30-70 nmoles/ml, respectively, depending on the specific batch of mouse livers.

Production of 32-Hydroxy-Rifalazil. In a typical reaction, 10 g of resin (e.g., Amberlite XAD-16) was placed in a 250 ml reaction flask containing 75.6 ml of deionized water, 2 g of glucose, and 10 ml of 1 M K phosphate buffer, pH 7.4. To this mixture were added 8.4 ml of 3 mM NADP and 2.5 ml of 200 U/ml glucose dehydrogenase, prepared in 100 mM K phosphate buffer, pH 7.4. Freshly prepared MLM suspension was then added to achieve CYP concentration of 1600 pmoles/ml (typically 2.5 to 4.5 ml, depending on the activity of microsomes). The reaction was initiated by adding 1 ml of rifalazil solution in ethanol (10 mg/ml). The reaction mixture was
incubated for 20 h at 37 °C in a rotary shaker operating at 150 rpm. Daily reaction batches were typically run in multiple reaction flasks prepared as described above. After completion of the reaction the daily batches were combined and stored at -25 °C until a sufficient amount was collected for a large-scale isolation step. We verified that the storage of the reaction mixture under freezing for several days did not result in product losses.

**Solid Phase Extraction of 32-Hydroxy-Rifalazil.** In a typical isolation batch, a set of 57 reaction flasks, prepared as described in the preceding section, was removed from -25 °C freezer storage and melted in a 60 °C water bath. The contents of each flask were transferred into a 10 l polypropylene carboy. A total of 600 ml methanol was used to rinse the flasks, and the rinses were added to the carboy.

A depth filtration bed was prepared by mixing 107 g Celite 512 with 750 ml deionized water and quickly poured into a 2 l Gooch funnel over a 4 l vacuum flask. The suspension was vacuum filtered to remove the visible liquid, creating a homogeneous bed approximately 1.5 cm in thickness. The bed was covered with a polypropylene mesh.

To the 10 l carboy was added 110 g Celite 512 and swirled until homogeneous. This mixture was poured onto the filter bed in the Gooch funnel, while vacuum was applied and filtrate was collected into the vacuum flask. The resin/Celite cake obtained after filtration (approx. volume 1.5 l) was rinsed in the funnel with two volumes of 10% methanol/water and two volumes of 25% methanol/water, with vacuum applied during both rinses. HPLC analysis showed no traces of rifalazil or 32-hydroxy-rifalazil in the initial filtrate and subsequent rinses, which were therefore discarded.

**Recovery of 32-Hydroxy-Rifalazil from the Resin.** The resin/Celite cake and Celite bed were transferred to a 4 l glass bottle and mixed with 1.5 l methanol for extraction. The extraction was performed by placing the bottle in a water bath at 50 °C for one hour, with vigorous agitation.
every 10-15 minutes during this time. The mixture was filtered through a 2 l Gooch funnel under vacuum, and the extract (filtrate) was collected. The solids were slurried in ethyl acetate (EtOAc) and returned to the 4 l bottle. Extraction was repeated 4 x 1.5 l of EtOAc, using the same procedure as for the methanol extraction. The methanol extract was evaporated to remove methanol, leaving about 700 ml of aqueous suspension that was extracted with 500 ml EtOAc. All EtOAc extracts were pooled, giving a final volume of about 6.5 l. The EtOAc extract pool was evaporated under vacuum to solids. External temperature during evaporation did not exceed 50 °C. The solids were dissolved into 25 ml methanol and filtered through a 25 mm, 0.45 µm PTFE syringe filter. The filter was washed twice with 3-5 ml methanol, and the washings were pooled with the filtrate to give approximately 36 ml of a dark blue solution, which was submitted to preparative HPLC purification.

**Preparative HPLC.** The chromatographic purification was done in two separate runs, each using 18 ml of the methanol concentrate. The chromatographic system consisted of two Shimadzu LC-8A liquid chromatograph pumps, a Shimadzu SPD-10AV UV/VIS detector, a Shimadzu SCL-10A System Controller, a Waters PrepLC Universal Base and cartridge hardware, and a Waters Symmetry C18 radial compression cartridge column assembly (two 40 x 100 mm cartridge columns with a guard column, all 7 µm average particle size). An initial radial compression of 500 psi was obtained using deionized water as the hydraulic medium. Preparative HPLC runs were done using a gradient elution with water/acetonitrile containing 0.1% trifluoroacetic acid. The gradient was from 25% to 40% acetonitrile (0–3 min), constant 40% acetonitrile (3–5 min), from 40% to 46% acetonitrile (5–35 min), from 46% to 50% acetonitrile (35–45 min), from 50% to 100% acetonitrile (45–46 min), and then constant 100% acetonitrile for 10 min. The flow rate was 50 ml/min with detection at 620 nm. Fractions were collected into 50 ml polypropylene tubes, each containing 5 ml of 500 mM phosphate buffer, pH 7.8, to
neutralize the acid in the mobile phase, and analyzed by analytical HPLC. Collected fractions containing 32-hydroxy-rifalazil (approximately 200 ml) were evaporated under reduced pressure at 50 °C to remove acetonitrile. The remaining liquid (approximately 100 ml), was extracted twice with 100 ml portions of EtOAc and the extracts were pooled. The pooled extract was back-extracted three times with 100 ml portions of deionized water to remove inorganic salts. The desalted extract was evaporated under reduced pressure at 50 °C to solids, and quantitatively transferred to a tared 2 ml polypropylene vial using 200-300 µl washings of methanol. Finally, the methanolic product concentrate was evaporated in vacuum to solids, the vial was weighed, and net weight was calculated.

**Analytical HPLC.** HPLC analysis was performed on a Shimadzu 10AVP system with a PDA UV detector. Chromatography was accomplished at 40 °C using a Phenomenex Columbus C18 column (250 × 4.6 mm, 5 µm) with isocratic elution using the mobile phase composed of 1 : 1 v/v acetonitrile and citrate perchlorate buffer pH 4.3 (0.1 M sodium perchlorate monohydrate, 0.01 M anhydrous citric acid, and 0.01 M trisodium citrate dihydrate).

**LC/MS Analysis.** LC/MS analysis was performed on a PE SCIEX API 2000 system with a PDA UV detector. The mass spectrometer was operated in positive ion mode with spray voltage set at 4500 V. The ion source temperature was set at 400 °C. Chromatography was accomplished at room temperature using a Waters Symmetry Shield RP18 column (150 × 4.6 mm, 3.5 µm) with the mobile phase initially composed of 60% of solvent A (0.1% trifluoroacetic acid in water) and 40% of solvent B (0.1% trifluoroacetic acid in acetonitrile). Elution was performed with a linear gradient from 40 to 70% B in 12 minutes, increased to 95%B in 0.5 min and hold for 1 min at a flow rate of 1 ml/min. The column was re-equilibrated for 2 min after programming back to the starting solvent mixture over 0.5 minute.
RESULTS

Selection of the Biosynthetic System

According to literature reports, rifalazil is converted to the 32-hydroxy metabolite in humans and mice (Mae et al., 1996, 1999, 2000). In initial experiments, we confirmed the ability of commercially available human and mouse liver microsomes to catalyze the conversion of rifalazil to 32-hydroxy-rifalazil on an analytical (microgram) scale (Figure 2, traces 2 and 3). The corresponding mass spectrum of the metabolite peak eluting at 24.2 min is given in Figure 3A, showing the molecular ion corresponding to hydroxylated rifalazil ($m/z$ 958) and fragments at $m/z$ 925 and $m/z$ 517 characteristic for 32-hydroxy-rifalazil. For comparison, HPLC analysis of rifalazil incubation with dog liver microsomes is shown in Figure 2 (trace 4), with the predominant metabolite peak eluting at 26.4 min. The mass spectrum of this metabolite is shown in Figure 3B. In addition to ions detected in 32-hydroxy-rifalazil (Figure 3A), this spectrum also has a signal at $m/z$ 907, which is unique for 30-hydroxy-rifalazil (Mae et al., 1999). This result is in agreement with previous reports that dog liver microsomes predominantly generate 30-hydroxy-rifalazil (Mae et al., 1999; Hosoe et al., 1996). Thus, both mass spectral data and HPLC retention times of rifalazil metabolites produced by human, mouse and dog microsomes are in good agreement with those reported previously, thus confirming the identity of predominant metabolites as 32-hydroxy-rifalazil (human and mouse microsomes) and 30-hydroxy-rifalazil (dog microsomes).

The biotransformation using human microsomes resulted in approximately 5% yield of 32-hydroxy-rifalazil on the microgram scale (estimated from the corresponding HPLC peak area), in good comparison with previously reported results (Mae et al., 2000). The reaction catalyzed by commercial mouse liver microsomes (MLM) gave approximately 2.5 to 3-fold lower estimated yield under similar conditions. Nevertheless, MLM can be readily prepared economically, and in
significant quantities from commercially available mouse livers (Schenkman and Jansson, 1998). It was confirmed that MLM prepared on site from frozen mouse livers in bulk possessed activity very similar to that of the commercial MLM, based on assay with both a known hydrophobic substrate, testosterone (conversion to 6β-hydroxytestosterone) and rifalazil (conversion to 32-hydroxy-rifalazil). Therefore, this catalyst was chosen for the preparative synthesis of 32-hydroxy-rifalazil as a more economical alternative to human liver microsomes.

**Process Engineering and Optimization for the Production of 32-Hydroxy-Rifalazil**

**Selection of Rifalazil Delivery System.** A wide range of different delivery systems were tested to introduce poorly soluble in water rifalazil in the microsomal reaction system. Two main types of delivery vehicles were evaluated: solubilizing agents and solid polymeric sorbents serving as reservoirs for the hydrophobic substrate. Results of testing on a variety of solubilizing agents and polymeric supports, including their effect on solubility and/or reaction loading of rifalazil and production of 32-hydroxy-rifalazil, are shown in Tables 1 and 2, respectively. Based on reaction models, the volumetric productivity of the reaction (amount of product generated in the unit reaction volume over a specified period of time) was selected as the critical parameter to evaluate the suitability of different delivery vehicles for the production of 32-hydroxy-rifalazil. Minimizing the reaction volume helps to significantly reduce the consumption of the expensive biocatalyst and components of the cofactor regeneration system, resulting in a substantial reduction of overall production costs, and improved scalability.

In general, the adsorbant resins showed high biocompatibility for the MLM-catalyzed reactions, and significant increases in reaction productivity (Table 2). The best result was obtained with XAD-16, which gave 13-fold increase in volumetric productivity. Pretreatment of the resin by water/methanol wash, recommended by the manufacturer, did not affect the volumetric
productivity of the reaction. Based on these findings, untreated XAD-16 was chosen for all remaining production work.

Optimization of Reaction Parameters. In order to maximize the efficiency of the metabolite synthesis, we carried out a limited optimization of the most important reaction parameters that could affect the formation of 32-hydroxy-rifalazil. Improvements to the volumetric productivity of the reaction (and minimization of catalyst cost) was again used to guide the optimization study.

**Catalyst Concentration.** As expected, the amount of 32-hydroxy-rifalazil formed in the reaction increased with increasing concentration of added MLM. However, at high concentrations of MLM the reaction productivity started to decline. The optimal concentration of MLM was found to be approximately 1600 pmoles/ml of overall microsomal cytochrome P450 (CYP), which corresponds to 25-50 µl of MLM solution per ml of the reaction volume, depending on the CYP content in MLM.

**Reaction Time.** Liver microsomes are fairly unstable and irreversibly lose their catalytic activity in the course of the reaction (Chauret et al., 1998; Pearce et al., 1996). Stability experiments using rifalazil and testosterone as test substrates for measuring the catalytic activity of MLM showed that microsomes completely lost activity after 18-20 h incubation under reaction conditions. The addition of fresh MLM to the reaction after 20 h did not significantly increase product formation, possibly because of the adverse effect of high concentrations of MLM on the synthetic reaction, as discussed above. Therefore, all production scale reactions using MLM were run for 20 h before work-up and product isolation.

**Cofactor Recycling.** The enzymatic cofactor recycling system was another critically important component of the synthetic reaction catalyzed by the microsomal CYP. This NADPH cofactor is expensive and must be recycled *in situ* to reduce production costs. The glucose-6-phosphate dehydrogenase catalyzed oxidation of glucose-6-phosphate successfully regenerated
NADPH in the presence of polymeric resins. However, this system was not suitable for larger scale production because of the high cost of glucose-6-phosphate. An alternative cofactor recycling system based on glucose dehydrogenase and glucose also afforded acceptable levels of volumetric productivity for the MLM-catalyzed formation of 32-hydroxy-rifalazil, and was significantly more scalable and cost effective due to the much lower cost of glucose compared to glucose-6-phosphate. Therefore, this system was subsequently employed in all production-scale bioconversions of rifalazil to 32-hydroxy-rifalazil.

**Concentration of Rifalazil and Resin.** The effect of rifalazil concentration and amount of added resin on the product formation was also examined. It was determined that the volumetric productivity first increased with increasing content of rifalazil in the reaction mixture, and then leveled off at higher rifalazil concentrations exceeding 100 mg/l. The dependence of the reaction productivity on the amount of added polymeric resin showed a broad maximum at approximately 100 g/l. Resin amounts exceeding this level resulted in a lower productivity and complicated subsequent product isolation, presumably due to increased retention of rifalazil and its derivatives on the additional resin. Therefore, rifalazil and resin concentrations of 100 mg/l and 100 g/l, respectively, were chosen for production-scale reactions.

**Reaction Temperature and Stirring Rate.** The volumetric productivity of the reaction was not significantly affected by changing the reaction temperature (28 °C or 37 °C) or stirring rate (120, 150 or 250 rpm). Therefore, reaction temperature of 37 °C, and stirring at 150 rpm were employed for preparative bioconversions.

**Synthesis of 32-Hydroxy-Rifalazil**

The reaction conditions identified in the optimization study were applied for the production of 32-hydroxy-rifalazil on a preparative scale. Briefly, the reaction mixture was prepared by adding Amberlite XAD-16 resin to aqueous buffer, followed by components of cofactor regeneration.
system and freshly prepared MLM (see Materials and Methods for details). The reaction was initiated by the addition of rifalazil solution in ethanol at a final concentration 100 mg/l. The suspension was incubated for 20 h at 37 °C. After incubation the resin was separated by filtration, washed with aqueous methanol to remove impurities, and extracted with methanol and EtOAc. The extract was evaporated to dryness and purified by preparative HPLC.

HPLC analysis of the purified metabolite showed the presence of a single peak eluting at the retention time corresponding to 32-hydroxy-rifalazil, with the purity of at least 93% (Figure 2, trace 1). The mass spectrum and fragmentation pattern of the metabolite was identical to that determined for the prevalent hydroxylated metabolite formed in the presence of human liver microsomes (Figure 3A). These results confirm the identity of the product synthesized using MLM as 32-hydroxy-rifalazil.

The amount of material obtained as a result of purification was 11.4 mg, corresponding to approximately 1.4% isolated yield.
DISCUSSION

The use of mammalian liver fractions and metabolic enzymes is an increasingly useful strategy for the rapid synthesis of authentic drug metabolites early in the drug development process. However, the low aqueous solubility of many drug candidates, and poor compatibility of various organic cosolvents with metabolizing enzymes complicates many biocatalytic metabolite syntheses. The solubility of rifalazil in primarily aqueous media typically used for biotransformations catalyzed by MLM is only 10 mg/l, which corresponds to rifalazil concentration of 10 µM. In order to increase the reaction productivity to a synthetically useful level for preparative metabolite production, it is highly desirable to significantly increase the concentration of rifalazil available for the biotransformation. Since organic solvents cannot be used in concentrations sufficient to noticeably increase the concentration of rifalazil without destroying the catalytic activity of microsomes (Chauret et al., 1998), we surveyed and tested several alternative methods for improved delivery of hydrophobic compounds, which have been shown to be effective in similar oxidative biotransformations carried out by whole cell catalysts (Chien and Rosazza, 1980; Holst and Mattiasson, 1991; Vicenzi et al., 1997).

Several mild solubilizing agents can be used to increase the solubility of hydrophobic substrates in the reaction medium without destroying the activity of whole cell catalysts. The selection of solubilizing agents tested in this work included mild nonionic detergents Tween 80 and Alkamuls EL-620 (ethoxylated castor oil), polyvinylpyrrolidone of different molecular weights, and bovine serum albumin (Table 1). The selection also included glycerol, which is only a slightly better solvent than water but has been shown to provide a stabilizing effect on liver microsomes (Ichihara and Tanaka, 1989). All these agents were tested at several concentrations to cover a wide range of solubilization conditions and determine their effect on the catalytic activity of MLM. The data in Table 1 show that while the solubility of rifalazil was significantly
improved in the presence of these agents, the observed production of 32-hydroxy-rifalazil was either unchanged or considerably deteriorated. Thus, these solubilizing agents were ineffective in improving the biotransformation of rifalazil to the desired metabolite using MLM as a catalyst.

Solid sorbents have also been used as reservoirs for hydrophobic substrates, especially for delivery of inhibitory substrates and for biocatalysts with a low tolerance to standard solubilizing agents, such as organic cosolvents and detergents. The bioconversion is carried out in the presence of a hydrophobic solid support, which can adsorb the nonpolar substrate over a large interfacial area and deliver it to the biocatalyst, residing in the aqueous phase, at an equilibrium concentration. This technique is referred to as solid-phase extractive biotransformation (Holst and Mattiasson, 1991; Vicenzi et al., 1997). Frequently the product of the biotransformation can also be extracted back to the support, which may significantly facilitate subsequent isolation and purification steps.

To our knowledge to date, there have been no reported examples of using the extractive biotransformation for preparative-scale reactions using liver microsomes as a catalyst. Therefore, we performed a limited study to validate this approach for the hydroxylation of rifalazil using MLM and identify the most suitable hydrophobic support and reaction conditions. To this end, a wide range of different hydrophobic supports that are typically used for extractive biotransformations in whole-cell reaction systems were screened as substrate delivery vehicles for the hydroxylation of rifalazil catalyzed by MLM. Specifically, we examined various inert hydrophobic polystyrene-divinylbenzene supports, including Amberlite (Rohm & Haas), Sepabeads and Diaion resins (Mitsubishi), and Bio-Beads (BioRad). Different versions of these polymeric adsorbents differ in surface area, porosity, particle size, and hydrophobicity. All tested resins were compatible with good activity for both the microsomal catalysts and the cofactor regeneration systems, and afforded noticeable improvement in the production of 32-hydroxy-
rifalazil (Table 2). The best result for rifalazil hydroxylation was obtained with XAD-16, which
gave 13-fold increase in the reaction productivity and was used to scale-up the reaction to
produce 11.4 mg of 32-hydroxy-rifalazil.

In the absence of the resin, the conventional homogeneous microsomal reaction would
require running the synthesis reaction on a scale exceeding 100 l for production of a similar
amount of the metabolite. Due to the significantly increased volumetric productivity using the
extractive biotransformation strategy (Table 2), only approximately 8 l reaction was sufficient for
synthesis of suitable amounts of the low-yielding metabolite. Thus, the application of extractive
biotransformation for the production of metabolites from compounds with low aqueous solubility
offers substantial economy of the expensive microsomal catalyst. The strategy also simplifies the
reaction set-up, and significantly reduces the amount of effort involved in isolating the product.

Application of extractive biocatalysis has been well documented for reactions catalyzed by
microbial cells and isolated enzymes (for reviews, see Holst and Mattiasson, 1991; Kim et al.,
2007). In many cases this technique provided excellent results for biotransformation of
compounds sparingly soluble in water, such as steroids (Bhasin et al., 1976; Saunders et al., 1985)
or hydrophobic ketones and esters (Vicenzi et al., 1997; Wolberg et al., 2001). The presence of
solid resins can also be beneficial for controlling solvent concentration of reactants and products
that are toxic or inhibitory towards microbial cells or enzymes (Stark et al., 2003; Hua et al.,
2007). While the use of solid adsorbents for bioconversions catalyzed by liver cells or subcellular
fractions has not been previously reported, results of this study clearly demonstrate that this
strategy can be successfully applied for synthetic reactions catalyzed by liver systems.

In conclusion, we have developed and optimized a solid-phase extractive biotransformation
strategy applied to reactions catalyzed by liver microsomes. This strategy is compatible with
maintaining high catalytic activity for liver fractions, while substantially increasing the loading of
hydrophobic parent drugs. Thus, extractive biotransformation can substantially improve process productivity and serve as an efficient tool for preparative synthesis of low-yielding metabolites of compounds with very low water solubility. To demonstrate this, solid-phase extractive biotransformation was successfully applied for the production of 32-hydroxy-rifalazil on a multimilligram scale, resulting in 13-fold increase in volumetric productivity of the synthetic reaction.
Acknowledgement. Technical assistance of Lia Nilsen and Del Freeberg is highly appreciated.
REFERENCES


Footnotes.

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Legends for Figures

Figure 1. Structures of rifalazil and its hydroxylated metabolites, 32-hydroxy-rifalazil and 30-hydroxy-rifalazil.

Figure 2. HPLC traces of rifalazil and products of its incubations with mammalian liver microsomes. 1 – purified 32-hydroxy-rifalazil synthesized using extractive biotransformation with MLM; 2 – incubation with MLM; 3 – incubation with human liver microsomes; 4 – incubation with dog liver microsomes; 5 – rifalazil standard. Detection at 620 nm.

Figure 3. Mass spectra of rifalazil metabolites. A – peak eluting at 24.2 min in Figure 1, corresponding to 32-hydroxy-rifalazil (mouse and human liver microsomes); B – peak eluting at 26.4 min in Figure 1, corresponding to 30-hydroxy-rifalazil (dog liver microsomes).
Table 1. The Effect of Various Solubilizing Agents on the Solubility of Rifalazil and Production of 32-Hydroxy-Rifalazil catalyzed by MLM

<table>
<thead>
<tr>
<th>Solubilizing agent</th>
<th>Tested concentration range</th>
<th>Solubility of rifalazil, mg/l</th>
<th>Estimated relative volumetric productivity for 32-hydroxy-rifalazil</th>
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<tbody>
<tr>
<td>none</td>
<td>-</td>
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</tr>
<tr>
<td>Tween 80</td>
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</tr>
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<td>Alkamuls EL-620</td>
<td>0.01-1 % w/v</td>
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<td>0.6 d</td>
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<td>Polyvinylpyrrolidone, mw 10 KDa</td>
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<tr>
<td>Polyvinylpyrrolidone, mw 40 KDa</td>
<td>0.2-5 % w/v</td>
<td>&gt;100 c</td>
<td>1.1 d</td>
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<tr>
<td>Bovine serum albumin</td>
<td>1-20 mg/mL</td>
<td>&gt;100 c</td>
<td>0.6 d</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1-20 % v/v</td>
<td>10-50</td>
<td>0.3 e</td>
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\( ^a \) Reaction conditions: 100 mM potassium phosphate buffer, pH 7.4, containing 20 µl/ml MLM, 100 mg/l rifalazil, 0.4 U/ml glucose-6-phosphate dehydrogenase, 1.3 mM NADP, 3.3 mM glucose-6-phosphate and 1.3 mM MgCl\(_2\). Total reaction volume was 1 ml. Reaction temperature 37 °C, shaking rate 150 rpm, reaction time 16 h. Under these experimental conditions and using an MLM batch specific to this experiment, the volumetric productivity of the additive-free system was 0.6 mg product/L reaction volume. Volumetric productivity is determined as the amount of product generated in the unit reaction volume over a specified period of time.

\( ^b \) Estimated from the HPLC peak area.

\( ^c \) Estimated at the lowest tested concentration of the solubilizing agent.
d Estimated at the lowest tested concentration of the solubilizing agent; increasing concentrations of the agent resulted in a significant decrease of the estimated yield.

e At 20% glycerol.
Table 2. The Effect of Polymeric Support on the Production of 32-Hydroxy-Rifalazil

Catalyzed by MLM a

<table>
<thead>
<tr>
<th>Resin</th>
<th>Rifalazil concentration, mg/l</th>
<th>Estimated relative volumetric productivity for 32-hydroxy-rifalazil c</th>
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<tr>
<td>none</td>
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<tr>
<td>Sepabeads SP207</td>
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<td>6.5</td>
</tr>
<tr>
<td>Sepabeads SP825L</td>
<td>100</td>
<td>8.2</td>
</tr>
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</table>

a Reaction conditions: 100 mM potassium phosphate buffer, pH 7.4, containing 20 µl/ml MLM, 100 mg/l rifalazil, 5 U/ml glucose dehydrogenase, 0.25 mM NADP, 111 mM glucose. Total reaction volume was 1 ml. Reaction temperature 37 °C, shaking rate 150 rpm, reaction time 16 h. Polymeric resins were added to the reaction mixture separately from rifalazil. Under these experimental conditions and using an MLM batch specific to this experiment, the volumetric productivity of the resin-free system was 0.2 mg product/l reaction volume.

b Solubility limit.

c Estimated from the HPLC peak area.
Figure 1

Rifalazil (1)

32-Hydroxy-rifalazil (2)

30-Hydroxy-rifalazil (3)
Figure 2

The diagram shows a chromatogram with peaks indicating 32-hydroxy-rifalazil and 30-hydroxy-rifalazil. The chromatogram is labeled as follows:

1. Isolated product
2. Mouse microsomes
3. Human microsomes
4. Dog microsomes
5. Rifalazil standard

The x-axis represents time in minutes (10-80), and the y-axis represents mAU (0-9).
Figure 3