Development of Three Parallel Cytochrome P450 Enzyme Affinity Detection Systems Coupled On-line to Gradient High-Performance Liquid Chromatography

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ABSTRACT:
A high resolution screening (HRS) technology is described, in which gradient high-performance liquid chromatography (HPLC) is connected on-line to three parallel placed bioaffinity detection systems containing mammalian cytochromes P450 (P450s). The three so-called enzyme affinity detection (EAD) systems contained, respectively, liver microsomes from rats induced by β-naphthoflavone (CYP1A activity), phenobarbital (CYP2B activity), and dexamethasone (CYP3A activity). Each P450-EAD system was optimized for enzyme, substrate, and organic modifier (isopropyl alcohol, methanol, and acetonitrile) in flow injection analysis mode. Characteristic P450 ligands were used to validate the P450-EAD systems. IC50 values of the ligands were measured and found to be similar to those obtained with conventional microtiter plate reader assays. Detection limits ($n = 3$; signal-to-noise ratio $= 3$) of potent inhibitors ranged from 1 to 3 pmol for CYP1A activity, 4 to 17 pmol for CYP2B activity, and 4 to 15 pmol for CYP3A activity. The three optimized P450-EAD systems were subsequently coupled to gradient HPLC and used to screen compound mixtures for individual ligands. Finally, to increase analysis efficiency, a HRS system was constructed in which all three P450-EAD systems were coupled on-line and in parallel to gradient HPLC. The triple parallelized P450-EAD system was shown to enable rapid profiling of individual components in complex mixtures for inhibitory activity to three different P450s.

Cytochromes P450 (P450s) are of major importance for the disposition and metabolism of drugs and other xenobiotics (Guenigerich, 2001). Therefore, it is of profound importance to determine the affinity of P450s for drugs as well as for their metabolites. Especially in drug discovery and development, new chemical entities and lead compounds, as well as their metabolites, must be evaluated for their affinities toward relevant P450 isoenzymes (Vermeulen, 2003).

When many compounds are to be screened for their affinity to P450s, in vitro high throughput screening (HTS) methodologies are often used (Masimirembwa et al., 2001). Usually, the inhibition of fluorescent metabolite formation from probe substrates is used for this purpose (Crespi and Stresser, 2000). These HTS-focused methodologies, however, cannot be used for the identification of individual ligands in compound mixtures. In these cases, mixtures have to be separated chromatographically before affinity screening of individual compounds can occur (Fura et al., 2004). Natural compound extracts, combinatorial chemistry libraries, and drug metabolite mixtures are representative of this type of mixture (Shin and van Breemen, 2001). Other methodologies, in which ligands are first captured by (immobilized) target biomolecules followed by their release and subsequent separation and detection (Hsieh et al., 1997), lack the possibility of identification of low-affinity compounds in the presence of high-affinity compounds.

About a decade ago, an on-line high resolution screening (HRS) concept based on a continuous-flow biochemical assay coupled on-line to HPLC and circumventing several of these disadvantages was described (Oosterkamp et al., 1994). Subsequently, HRS-based bioaffinity detection methods for ligands of, for example, the estrogen receptor, phosphodiesterases, acetylcholinesterases, angiotensin-converting enzymes, and antibodies have been described (Schenk et al., 2003; Van Elswijk et al., 2003). We recently developed a novel HRS bioaffinity detection system to screen on-line for compounds with affinity for rat liver microsomal CYP1A activity (Kool et al., 2005). In this HRS triple P450 enzyme affinity detection (P450-EAD) system, CYP1A-induced rat liver microsomes and the substrate ethoxyresorufin were added postcolumn to the HPLC eluate to allow a metabolic reaction for a period of time. The CYP1A-mediated
Materials. Ethoxyresorufin, Tween 20, polyethylene glycol 6000 (PEG6000), aminopyrine, \( \alpha \)-naphthoflavone (\( \alpha \)-NF), miconazole, and nifedipine were purchased from Sigma (Zwijndrecht, The Netherlands). Caffeine and metyrapone were obtained from Aldrich (Zwijndrecht, The Netherlands). \( \beta \)-NF was supplied by Acros (Den Bosch, The Netherlands). NADPH tetrasodium salt was purchased from Sigma (Zwijndrecht, The Netherlands). Aminopyrine, \( \alpha \)-naphthoflavone, miconazole, and dexamethasone (DEX) were used. First, the CYP2B and CYP3A EAD systems were developed and optimized in flow injection analysis (FIA) mode and thereafter, they were coupled individually on-line to HPLC. The CYP1A EAD system used was similar to the EAD system described recently for CYP1A activity (Kool et al., 2005). Finally, the three CYP450-EAD systems were incorporated on-line and in parallel in a gradient HPLC apparatus. The entire HRS triple CYP450-EAD system was then used for the simultaneous screening of inhibitors for each of the three CYPs. The current HRS methodology could be used in the late drug discovery and early drug development stages for the screening of lead compounds for related metabolic mixtures.

\( \beta \)-NF- and PB-induced rat liver microsomes were prepared as described previously (Rooseboom et al., 2001) from five male Wistar rats (200–250 g) obtained from Hope Farms (Woerden, The Netherlands). The isolated livers were pooled before homogenization. Protein concentrations in the \( \beta \)-NF- and PB-induced rat liver microsomes were 13.1 mg/ml and 9.9 mg/ml, respectively. DEX-induced rat liver microsomes were also prepared as described previously (Rooseboom et al., 2001) except that 60 mg DEX/kg rat was used for induction. Protein concentration in the DEX microsomes was 12.9 mg/ml. For all animal experiments, standard ethical guidelines for animal care of Vrije University were applied.

**Materials and Methods**

**Apparatus.** The configuration of the CYP450-EAD systems in FIA and HPLC mode was similar to the EAD system described recently for CYP1A activity (Kool et al., 2005). A general scheme of the current triple parallelized P450-EAD system coupled on-line to gradient reversed-phase HPLC is shown in Fig. 1. The P450-EAD systems are operated by mixing P450 enzymes (in rat liver microsomes), substrate, and cofactor (NADPH) continuously with a carrier solution (FIA mode) or with an HPLC effluent (HPLC mode). Superloops A, C, and E contain P450 enzymes (from DEX-, PB-, and \( \beta \)-NF-induced microsomes, respectively) for the three EAD systems (i.e., with CYP3A, 2B, and 1A). For mixing of the probe substrates and cofactor with the respective P450s in every reaction coil, the substrates \[7\text{-benzyloxy-4 trifluoromethylcoumarin (BTFC)}, \text{pentoxyresorufin, and ethoxyresorufin}\] were continuously pumped through superloops B, D, and F for the respective P450-EAD system. The cofactor, needed for each EAD system, was present in the substrate containing superloops B, D, and F. As enzyme, substrate and cofactor are continuously added to the reaction coils; the substrates are converted into fluorescent products. Eluting ligands (i.e., both inhibitors and substrates) competing with the probe substrates for the active sites of the P450s concerned cause a (temporarily) decreased production of fluorescent product that is monitored by fluorescence detection.

For optimization processes and for the screening of pure compounds, only one P450-EAD system at a time and operated in FIA mode was used. In this setup, compounds injected via an autosampler are directly introduced in the P450-EAD system. The cofactor, needed for each EAD system, was present in the substrate containing superloops B, D, and F. As enzyme, substrate and cofactor are continuously added to the reaction coils; the substrates are converted into fluorescent products. Eluting ligands (i.e., both inhibitors and substrates) competing with the probe substrates for the active sites of the P450s concerned cause a (temporarily) decreased production of fluorescent product that is monitored by fluorescence detection.
The CYP2B EAD system is based on the ability of CYP2B to metabolize pentoxyresorufin into highly fluorescent resorufin. Similarly, for the CYP3A system used was a reference compound during the optimization. For optimization, the substrate pentoxyresorufin for CYP2B EAD was tested at concentrations ranging from 50 to 500 μM (in steps of 50 μM). The enzymatic product was monitored using a 1:1:2:6 by means of a flowsplitter. The two 10% (v/v) fractions were directed to the CYP2B EAD and the CYP3A EAD systems, whereas the 20% (v/v) fraction was introduced into the CYP1A EAD system. The remaining 60% (v/v) fraction was directed to the UV detector. All hardware was integrated in one system by Kiadis B.V. (Groningen, The Netherlands) and was controlled by software developed by Kiadis B.V.

P450 Enzyme Affinity Detection in Flow-Injection Analysis Mode. The three P450-EAD systems are operated by continuous mixing of rat liver microsomes, substrate, and cofactor (NADPH) with a carrier solution when operated in FIA mode and the effluent from an HPLC separation when operated in HPLC mode (Fig. 1). After mixing of the substrate with P450s in a knitted reaction coil, it is enzymatically converted into a highly fluorescent product. Eluting ligands (i.e., both inhibitors and substrates), which compete with the substrate for the active site of the respective P450s, will cause a decrease in formation of fluorescent product. The CYP1A EAD system used was similar to a previously described system (Kool et al., 2005). The CYP2B EAD system is based on the ability of CYP2B to metabolize pentoxyresorufin into highly fluorescent resorufin. Similarly, for the CYP3A EAD system, BTFC (Stresser et al., 2002) is used, yielding highly fluorescent 7-hydroxy-4-trifluoromethylcoumarin after debenzylation by CYP3A. The carrier solution initially used in the optimization process for the CYP2B and 3A EAD systems was water. The enzyme solutions (PB-induced rat liver microsomes in the case of CYP2B, and DEX-induced rat liver microsomes in the case of CYP3A) and substrate solutions (pentoxyresorufin for CYP2B and BTFC for CYP3A, with NADPH as cofactor in both solutions) were kept on ice in superloops and were added to the carrier flow at a flow rate of 100 μl/min each.

During the optimization processes of the P450 EAD systems, the carrier solution was pumped at a flow rate of 100 μl/min. Flow injections (40 μl) were made into the carrier solution before mixing of the carrier solution with the enzyme and cofactor/substrate solution. Substrate and enzyme concentrations, detergents, blocking agents, and concentrations of organic modifier (IPA, MeCN, and MeOH) were optimized in FIA mode as well.

As starting conditions for the CYP2B EAD system, superloop 1 contained PB-induced rat liver microsomes (20 μg protein/ml), and superloop 2 substrate pentoxyresorufin (0.70 μM) and cofactor (NADPH; 40 μM). The inhibitor proprafen (injected in different amounts: 0, 6.8, 13.6, 27.3, 54.5, 109, 218, 436, or 872 pmol) was used during the optimization process. The buffer used in the superloops consisted of 30 mM potassium phosphate buffer (pH 7.4, containing 2.5 mM MgCl2). The volume of the knitted reaction coil was 75 μl. For the CYP3A EAD system, superloop 1 contained DEX-induced rat liver microsomes (20 μg protein/ml) and superloop 2, substrate BTFC (4.5 μM) and NADPH (40 μM). The enzymatic reaction took place, in this case, in a 200-μl knitted poly(etheretherketone) tubing. The inhibitor ketoconazole (injected in different amounts: 0, 12.5, 25, 50, 100, 200, 400, 800, 1600, or 3200 μM) was used as a reference compound during the optimization. For optimization, the substrate pentoxyresorufin for CYP2B EAD was tested at concentrations ranging from 100 nM to 1800 nM (Fig. 2A), whereas for CYP3A, substrate (BTFC) concentrations from 1 to 30 μM were tested (Fig. 2B). The microsomal concentrations for the CYP2B and 3A EAD systems were tested in the range of 10 to 100 μg protein/ml, in steps of 10 μg protein/ml. Coil volumes were tested for both P450-EAD systems from 100 to 500 μl, with steps of 100 μl. PEG6000 was tested for both systems in the range from 0.5 mg/ml up to 5 mg/ml in steps of 0.5 mg/ml, whereas Tween 20 was tested in the concentration range of 50 to 500 mg/l (in steps of 50 mg/l). Three organic modifiers (MeOH, MeCN, and ACN) were tested in the range of 1 to 10%, in steps of 1%.

P450 Enzyme Affinity Detection Coupled to HPLC. After optimization and validation of the P450-EAD systems in FIA mode, the P450-EAD systems were individually evaluated and validated in HPLC mode before integrating them into a triple parallelized HPLC format. This optimization and validation of each individual P450-EAD system was done in a manner similar to that described previously for the CYP1A system (Kool et al., 2005). Finally, all three P450-EAD systems were connected and integrated into the triple P450-EAD system in HPLC mode (Fig. 1).

HPLC separations were performed using a Phenomenex (Torrance, CA) stainless steel column, 30 mm × 2 mm i.d., Luna 3-μm particles, C18(2). An injection volume of 40 μl was used. The starting flow rate over the analytical column was 700 μl/min, H2O/IPA (95:5), and was maintained for 4 min. The water and organic phase both contained 0.1% (v/v) acetic acid. Next a 5 to 99% (v/v) IPA gradient was applied in 15 min, followed by a postgradient time of 22 min. During the gradient, the flow rate gradually decreased to 70 μl/min. Subsequently, re-equilibration of the column to the starting conditions was performed in 5 min. To maintain a constant concentration of IPA after the HPLC column, a second gradient with an increasing flow rate was configured after HPLC separation. The initial flow rate equalled 400 μl/min, H2O/IPA (9:1), and was kept constant for 4.7 min. Next, a gradient with increasing flow rate profile was applied for 15 min. The final flow rate of the second makeup HPLC system was set at 1030 μl/min, H2O/IPA (99:1), and was maintained during the 22-min postgradient period. Finally, re-equilibration to starting conditions was performed in 0.5 min. The aqueous and organic modifier solution of the makeup HPLC system contained 100 mg/l Tween 20. The total

![Fig. 2. Substrate optimization results of the P450-EAD systems in FIA mode. Optimization of the substrate pentoxyresorufin for the CYP2B EAD system (A) and for the CYP3A EAD system (B) with the substrate BTFC. For CYP2B, metyrapone was used as inhibitor; for CYP3A, ketoconazole was used.](image-url)
flow rate of the entire HPLC system was kept constant at 1100 µl/min and contained a constant IPA concentration of 3% (v/v). During the evaluation and validation process of the individual P450-EAD systems in HPLC mode, the HPLC effluent was split in a 1:9 ratio, the 10% (v/v) fraction of the flow being directed to the P450-EAD system and the 90% (v/v) fraction to the UV detector. A mixture of different CYP2B ligands, i.e., 70 µM metyrapone, 6000 µM aminopyrine, 7000 µM chloramphenicol, and 50 µM proadifen, was used for validation of the CYP2B EAD system in HPLC mode. For the CYP3A EAD system, a mixture of three CYP3A ligands was used, i.e., 28 µM ketoconazole, 52 µM miconazole, and 430 µM nifedipine. After evaluation and validation of the individual P450-EAD systems in HPLC mode, the final triple HPLC P450-EAD system was constructed (Fig. 1). For this system, a less steep gradient was chosen to obtain a higher separation efficiency. A gradient time of 30 instead of 15 min was used. After adding makeup solutions, the HPLC effluent was split into the three P450 biochemical assays and a UV detector. Before mixing with the biochemical reagents, a T-piece was inserted to add an aqueous Tween 20 (12 g/l) solution at 50 µl/min to the CYP1A EAD system. In this way peak broadening was substantially reduced in the CYP1A EAD system. To increase the specificities of the respective P450s in every system, inhibitors of other P450s present in the microsomal preparations were included in every P450-EAD system. In this way activities of interfering P450s could be inhibited (Stresser et al., 2002). To achieve this goal, the following inhibitors were added to superloop 1: ketoconazole (10 µM) and metyrapone (1.5 µM) for CYP1A EAD, ketocarzolone (10 µM) and a-NF (400 nM) for CYP2B EAD, and metyrapone (1.5 µM) and a-NF (400 nM) for CYP3A EAD. First, the triple HPLC P450-EAD system was tested for robustness and stability of the enzymatic assays over time. It was found that the triple HPLC P450-EAD system could be run in a stable and continuous fashion if the reaction coils and the detector cells were washed once a week with a 20% Tween 20 solution. The enzyme and substrate solutions in the superloops could be operated for at least 8 h before they had to be refreshed. After 8 h, the enzymatic activity of the CYP2B and 3A assays declined approximately 20%, whereas for CYP1A, this decline was only 10%. A mixture of different P450 ligands was used for validation of the triple parallelized P450-EAD system in HPLC mode. The mixture contained the CYP1A inhibitors ellipticine (200 µM), 9-hydroxyellipticine (200 µM), α-naphthoflavone (50 µM) and β-naphthoflavone (600 µM), and the substrates phenacetin (15 mM) and caffeine (25 mM). For CYP2B, the inhibitors metyrapone (300 µM) and proadifen (500 µM) and the substrate chloramphenicol (8 mM) were used. For CYP3A, the inhibitors ketocarzolone (250 µM) and miconazole (8 µM) with the substrate nifedipine (400 µM) were used. Of this mixture, a concentration range was prepared by serial dilution of the mixture dissolved in ethanol (50 µl) with 50% (v/v) aqueous ethanol (300 µl). The mixture and the serial dilutions were then injected in the triple parallelized CYP1A, 2B and 3A EAD system in gradient HPLC mode.

**Microplate Reader Assays for P450 Inhibition. Microplate reader assay for CYP2B.** The microplate reader assay for CYP2B activity was done in a manner similar to that for CYP1A activity (Kool et al., 2005). In short, a mixture of 150 µl of PB-induced rat liver microsomes (40 µg protein/ml) and pentoxyresorufin (300 nM) in potassium phosphate buffer (50 mM; pH 7.4) containing MgCl2 (2.5 mM) was incubated for 15 min at 37°C. Subsequently, 75 µl of one of the following solutions was added to start the reaction: setup 1, a freshly prepared mixture of a 20-µl solution of test compound in H2O and 80 µl of a solution containing NADPH (50 µM) in 3% (v/v) IPA; or setup 2, a freshly prepared mixture of a 20-µl solution of test compound in H2O and 80 µl of a solution containing NADPH (50 µM), PEG6000 (0.5 mg/ml), and Tween 20 (100 mg/ml) in 3% (v/v) IPA. The fluorescence of resorufin was measured at λ350 nm (bandwidth 8 nm) and λ580 nm (bandwidth 30 nm) on a Victor2 1420 multilabel counter (Wallac, Turku, Finland).

**Optimization of the P450-EAD Systems in FIA Mode.** Additives, substrate and enzyme concentration and reaction time are important parameters influencing the robustness and sensitivity of on-line biochemical assays in general (Schenk et al., 2003) and also of P450 EADs specifically (Kool et al., 2005). Hence similar strategies were used to optimize the present CYP2B and 3A EAD systems. During every optimization step, the resolution and sensitivity obtained with known inhibitors (i.e., metyrapone for CYP2B and ketoconazole for CYP3A) were determined. The effects of IPA, MeCN and MeOH, later to be used in HPLC mode, were also tested on the performance of the P450 EAD systems in FIA mode.

**Substrate optimization.** Substrate optimization was conducted by injecting an appropriate test compound in different concentrations (depicted in Fig. 2) for every substrate concentration tested. For the CYP2B EAD system, a concentration of 600 nM the substrate pentoxyresorufin resulted in the highest S/N-ratios (Fig. 2A) and also in the highest resolution. In case of the CYP3A EAD system, the S/N-ratios increased significantly with concentrations of the substrate BTFC increasing from 1 up to 9 µM (Fig. 2B). Higher concentrations of BTFC only gave minor improvements in S/N-ratios, whereas the resolution decreased significantly. This decreased resolution was observed as band broadening of signals from injected inhibitors. A concentration of 9 µM is lower than the apparent Km of BTFC, which is above the limit of aqueous solubility as described for similar systems (Crespi and Stresser, 2000). For CYP2B, 600 nM pentoxyresorufin and for CYP3A, 9 µM BTFC were used further.

**Enzyme optimization.** When varying the microsomal protein concentrations in the CYP2B and 3A EAD systems, protein concentrations higher then 70 µg/ml were found to result in lower resolutions, whereas the S/N-ratios did not increase. Therefore, a microsomal protein concentration of 70 µg/ml was used in both P450 EAD systems.

**Optimization of reaction time.** For both the CYP2B and 3A EAD system, a reaction coil volume of 200 µl gave good S/N-ratios and was therefore used. Larger reaction coil volumes, i.e., up to 500 µl, increased the S/N-ratios slightly. This is probably due to the longer reaction times. The resolutions obtained, however, were reduced significantly.

**Additives.** PEG6000 and Tween 20 were found to prevent peak broadening in both the CYP2B and 3A EAD systems: 0.5 mg/ml PEG6000 (in superloop 2) and 100 mg/l Tween 20 (in the carrier solution) gave optimal resolution both for the CYP2B and the 3A EAD system while maintaining enzymatic activity.

**Optimization of organic modifier.** The effect of organic modifiers (IPA, MeCN, and MeOH) on the performance of the P450-EAD systems was also investigated in FIA mode. For the CYP2B and 3A EAD systems, effects similar to those described previously for CYP1A activity (Kool et al., 2005) were found: optimal concentrations of organic modifier were found to be 4.0% (v/v) IPA, 2.0% (v/v) MeCN, and 3.5% (v/v) MeOH for both the CYP2B and the 3A EAD systems.

**Variability.** Interday and intraday variability for the P450-EAD systems was determined in FIA mode under optimized conditions. Intraday variability, determined by injecting 300 pmol of metyrapone (causing 67% inhibition of the enzyme activity in the CYP2B EAD system) in triplicate, at 2.5-h time intervals, was found to be 6.4%. For

**Results**

To optimize and validate the P450-EAD systems, an FIA mode was used because this mode allowed a much more rapid optimization and validation process than the HPLC mode. The FIA mode might also be used to screen pure compounds for P450 inhibition.
obtained with the EAD systems in HPLC mode (described in the literature are based on (single) human P450 systems, we used well validated microplate reader setups and microplate reader assays were first performed with and without the addition of these reagents and compared. Since induced rat liver microsomal systems were used for this proof of principle study, and since most inhibition parameters described in the literature are based on (single) human P450 systems, we used well accepted microplate reader formats to obtain reliable IC_{50} values of the test compounds used.

For the determination of IC_{50} values obtained with the on-line P450-EAD systems, the dilution factors of reference inhibitors were first determined. This was done in a manner similar to that described recently for the CYP1A EAD system (Kool et al., 2005). In brief, resorufin concentrations of 10 μM and 100 μM were injected in the P450-EAD systems to measure peak heights of the resorufin standards. Next, the same resorufin concentrations were added to the carrier solution. The increase in fluorescence was subsequently compared with the peak heights of the injected resorufin samples. Peak heights thus obtained were 34 and 30% (n = 3) of the baselines of the resorufin concentrations injected with the CYP2B and 3A EAD systems, respectively. This implies resorufin concentrations of 11.3 and 10.0% of the injected concentrations in the reaction coil of the CYP2B and 3A EAD systems, respectively. Each inhibitory test compound was then injected in different concentrations (prepared by serial dilution of 400-μl solutions with 400-μl carrier solution), starting at a concentration causing 100% enzyme inhibition in the P450 EAD system and ending at a concentration giving S/N ratios of 3. The cumulative results for all inhibitory test compounds are shown as IC_{50} curves in Fig. 3A for the CYP2B system and in Fig. 3B for the CYP3A system. The IC_{50} values obtained from the respective curves are shown in Table 1. For comparative purposes, the IC_{50} values obtained with the EAD systems in HPLC mode (described in the following section) and in microplate reader setup are also shown in Table 1. Moreover, Table 1 also provides the lowest detectable inhibitor concentrations for all tested compounds (i.e., the detection limits at S/N = 3). Inhibitor concentrations that led to significant quenching of the fluorescent signal in the P450-EAD systems were not used for calculating the IC_{50} values (Kool et al., 2005). Aminopyrine and chloramphenicol showed quenching at concentrations higher than 15 and 10 mM, respectively.

Validation of the P450-EAD systems in FIA mode. For validation purposes, the CYP2B and 3A EAD systems in FIA mode were evaluated as to sensitivity and robustness of the respective bioaffinity assays. To investigate possible effects of reagents necessary to reduce peak broadening, microplate reader assays were first performed with and without the addition of these reagents and compared. Since induced rat liver microsomal systems were used for this proof of principle study, and since most inhibition parameters described in the literature are based on (single) human P450 systems, we used well accepted microplate reader formats to obtain reliable IC_{50} values of the test compounds used.

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On-line Coupling of the P450 Enzyme Affinity Detection Systems to HPLC. Figure 1 shows the final configuration of the triple P450-EAD system coupled on-line to gradient HPLC. The optimized conditions for the CYP1A EAD system, previously described (Kool et al., 2005), were also taken as optimized conditions in the present study. The CYP2B and 3A EAD systems were first optimized as described above and were then coupled individually to the gradient HPLC and validated by testing them with individual inhibitors injected in different concentrations and by injecting mixtures of inhibitors. Finally, all three P450-EAD systems were coupled in parallel to gradient HPLC.

Typical bioaffinity chromatograms resulting from injections of mixtures of inhibitory ligands of CYP2B and of 3A, in the respective EAD systems in HPLC mode, are shown in Figs. 4 and 5B, respectively. For the CYP3A EAD system in HPLC mode, a mixture of three CYP3A ligands was injected. In this case, both the EAD trace and the UV trace are shown, because the injected mixture of seemingly three inhibitors actually showed four UV and four EAD peaks. LC-MS/MS confirmed that nifedipine ([M + H^+] = 347) was the third peak in the chromatogram, whereas the second peak was an unknown product with [M + H^+] = 329.

All reference compounds used in the CYP2B and 3A EAD systems in FIA mode were also used to validate the P450-EAD systems in HPLC mode. The corresponding IC_{50} values (constructed from five different concentrations), calculated in the same way as described above, are presented in Table 1.

Finally, the CYP1A, 2B, and 3A EAD systems were simultaneously coupled to a single-gradient HPLC (Fig. 1) to examine a triple P450-EAD system for on-line screening of individual compounds in mixtures in a panel of three P450s. For this purpose, a mixture of 12 different P450 ligands was prepared. The ligands used are all known ligands for at least one of the P450s. A concentration range of the mixture was prepared and serial dilutions were subsequently injected in the triple parallelized CYP1A, 2B, and 3A EAD system in gradient HPLC mode. Figure 6 shows an overlay of the bioaffinity signals for the CYP1A (Fig. 6A), CYP2B (Fig. 6B), and CYP3A EAD systems (Fig. 6C) and the corresponding UV trace (Fig. 6D). The lowest EAD...
physiological concentrations. The other two EAD traces in each EAD chromatogram shows the highest injected concentrations. The other two EAD traces in each EAD chromatogram resulted from 16 and 256 times diluted mixtures, respectively.

Discussion

The development of a HRS platform with three P450-containing EAD systems configured in parallel and on-line with gradient HPLC was the primary goal of this study. For this purpose, EAD systems configured in parallel and on-line with gradient HPLC and DEX-induced rat liver microsomes were used as sources of CYP1A, 2B, and 3A, respectively. Additives, substrate and enzyme concentrations, and reaction time are important parameters influencing the robustness and sensitivity of on-line biochemical assays in general (Schenk et al., 2003) and of P450-EADs specifically (Kool et al., 2005). Hence similar strategies were used to optimize the present EAD systems.

The P450-EAD systems were first evaluated and optimized in FIA mode because this mode allowed a much more rapid evaluation and optimization process than when HPLC mode is used. The FIA mode may in principle also be used to screen pure compounds for P450 inhibition. The present on-line FIA methodology is merely suitable to screen for the inhibitory potential of individual compounds in mixtures toward a panel of P450s. Current 384-well and higher plate assay-based HTS methodologies have uncontested throughput rates of 20 to 60 samples can be obtained at maximum. Microplate reader-based assay formats were used to compare and validate the results obtained for the on-line P450-EAD systems. This was done because rat liver microsomal systems were used for the present proof of principle study, whereas most inhibition parameters described in the literature for P450 inhibition are based on (single) human P450 systems. Because induced rat liver microsomal preparations are not a validated matrix and because they are subject to variations in P450 content and activity, P450 reductase activity, and many other factors, the microplate reader IC50 assays had to be conducted for comparison reasons.

In the optimization process, the additives PEG6000 and Tween 20 were found to prevent peak broadening in both the CYP2B and 3A EAD systems and were thus used in these systems. Compared with CYP1A (Kool et al., 2005), CYP2B and 3A were much less tolerant toward Tween 20. CYP1A still functioned properly at Tween 20 concentrations up to 670 mg/l in the carrier solution (Kool et al., 2005). The lower concentrations of Tween 20 did result in higher

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**TABLE 1**

IC50 values of five CYP2B inhibitors measured with the P450 2B EAD system and three CYP3A inhibitors measured with the P450 3A EAD system in FIA mode, HPLC mode, and two different microplate reader formats

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>FIA EAD</th>
<th>HPLC EAD</th>
<th>Microplate Reader</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (n = 3) Detection Limit (S/N = 3)</td>
<td>IC50 (n = 1) Detection Limit (S/N = 3)</td>
<td>Setup 1 IC50 (n = 4) Setup 2 IC50 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>μM ± S.E.M. pmol</td>
<td>μM pmol</td>
<td>μM ± S.E.M.</td>
</tr>
<tr>
<td>CYP2B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metyrapon</td>
<td>0.30 ± 0.02 4</td>
<td>0.60 ± 0.01 60</td>
<td>0.28 ± 0.09 0.22 ± 0.07</td>
</tr>
<tr>
<td>Prazidilen</td>
<td>0.24 ± 0.10 17</td>
<td>0.40 ± 0.21 240</td>
<td>0.41 ± 0.21 0.21 ± 0.05</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>75 ± 13 877</td>
<td>93 ± 7760 7760</td>
<td>80 ± 9 61 ± 4</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1062 ± 66 9933</td>
<td>N.D. N.D.</td>
<td>608 ± 43 402 ± 53</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>617 ± 390 4750</td>
<td>826 ± 7600 7600</td>
<td>455 ± 38 321 ± 71</td>
</tr>
<tr>
<td>CYP3A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketacozazole</td>
<td>0.33 ± 0.04 15</td>
<td>0.28 ± 0.17 120</td>
<td>0.28 ± 0.01 0.24 ± 0.01</td>
</tr>
<tr>
<td>Miconozol</td>
<td>0.11 ± 0.01 4</td>
<td>0.13 ± 0.19 32</td>
<td>0.10 ± 0.005 0.094 ± 0.004</td>
</tr>
<tr>
<td>Nifedipin</td>
<td>6.3 ± 4.5 200</td>
<td>3.89 ± 4.2 1600</td>
<td>9.0 ± 1.3 9.7 ± 2.2</td>
</tr>
</tbody>
</table>

N.D., not determined.
resolutions for CYP2B and 3A EAD compared with no detergent, but the highest resolution and, consequently, the least tailing was obtained in the CYP1A EAD system, in which the highest concentration of Tween 20 was used (Fig. 6). Previously reported on-line biochemical assays did not use detergents to prevent band broadening (Oosterkamp et al., 1994; van Elswijk et al., 2003), mainly because these assays are based on soluble protein targets (i.e., antibodies and the angiotensin-converting enzyme). Membrane-bound P450s apparently cause severe band broadening by sticking to walls of the reaction coils due to the lipophilic membranes (Kool et al., 2005). Eluting lipophilic compounds may in turn show additional retention by adsorbing to the microsomes and the walls of the reaction coils. Similar nonspecific drug binding to microsomes was described previously (McLure et al., 2000). Although the EAD systems use fairly low concentrations of microsomes, adhesion to reaction coils is still a major problem. When no measures are taken to prevent adhesion of microsomes, the amount of adhered microsomes builds up in time. This is contrary to off-line batch systems, in which microsomal adhesion only becomes a problem at much higher concentrations. PB- and DEX-induced rat liver microsomes were used in the present study for CYP2B and 3A affinity screening; however, it has to be noted that these enzyme sources are not fully selective for these two isoforms. Moreover, the selectivity of pentoxyresorufin (for CYP2B) and BTFC (for CYP3A) is not 100% (Burke et al., 1994; Kobayashi et al., 2002; Stresser et al., 2002). Nevertheless, these microsomes were chosen for a proof of principle demonstration of parallel P450 affinity screening, because we reasoned that absolute selectivities were not essential for this purpose. The increased CYP2B (Ryan and Levin, 1990) and 3A (Choudhuri et al., 1995) levels upon induction of rats with PB and DEX gave significantly higher turnover rates of the respective substrates pentoxyresorufin and BTFC and therefore ensure higher selectivities and sensitivities.

Much higher protein concentrations (70 μg/ml) were found to be necessary to obtain sufficient S/N ratios for the EAD systems. In addition, longer reaction coils were needed to generate enough fluorescent product in the on-line assays. For the CYP1A EAD system, the microsomal protein concentration was found to be optimal at a much lower concentration (20 μg/ml; Kool et al., 2005). Also, for this P450-EAD system, smaller reaction coil volumes were sufficient to obtain good S/N ratios. These differences in optimal protein concentration and reaction coil volumes can be explained by the high turnover rate of ethoxyresorufin by CYP1A and the high concentrations of CYP1A isoenzymes in α-NF-induced rat liver microsomes, compared with the CYP2B and 3A concentrations in PB- and DEX-induced rat liver microsomes (Guengerich et al., 1982). Large reaction coil volumes, i.e., up to 500 μl (resulting in longer reaction times), decreased the S/N ratios slightly but decreased the resolution significantly. This phenomenon is likely caused by increased dispersion of the injected sample when using larger reaction coils. With CYP1A, a smaller reaction coil volume of 25 μl was used because ethoxyresoru-
fin O-dealkylation, the biochemical basis of the CYP1A EAD system, involves a high-affinity and high-turnover substrate (van Liemden et al., 2005). With the CYP2B and 3A EAD systems, increasing IPA, MeCN, or MeOH concentrations led to decreasing S/N ratios with a concomitant increase in the resolution. Higher concentrations of organic modifier allow more effluent to be introduced into the P450-EAD systems when coupled on-line to HPLC. Therefore, compromises have to be made between resolution, S/N ratios, and amounts of HPLC effluent and, thus, introduced sample. As observed previously (Kool et al., 2005), CYP1A was more stable toward the organic modifiers tested than CYP2B and 3A. When MeOH is used as organic modifier, problems with affinity determinations of amine-containing ligands may arise due to possible condensation reactions with formaldehyde formed by P450 (Yin et al., 2001). This effect can be prevented by the use of reduced glutathione.

For validation purposes, first the CYP2B and 3A EAD systems in FIA mode were evaluated as to sensitivity and robustness. The cumulative results for all test compounds are shown in Fig. 3 and the resulting IC50 values in Table 1. The differences between IC50 values obtained with the microplate reader assays in setup 1 and setup 2 were only significant in a few cases. Such differences are not uncommon for microsomal P450 inhibition assays when measured with different methodologies (Crespi and Stresser, 2000). Taking this factor into consideration, the IC50 values obtained with both P450-EAD systems in FIA mode were well comparable with those of the microplate reader assay (Table 1) and can thus even be used as initial IC50 value estimates.

When placing the optimized and validated CYP2B and 3A EAD systems on-line after HPLC, typical EAD bioaffinity chromatograms resulting from injections of mixtures of inhibitory ligands of CYP2B and of 3A (Figs. 4 and 5B, respectively) show the applicability of theses EAD systems in HPLC mode to separate and subsequently identify the individual inhibitors. For CYP2B, all injected compounds were separated and could individually be judged for their inhibitory potential. For the CYP3A EAD system in HPLC mode, a mixture of three CYP3A ligands was injected. Because the UV chromatogram obtained for this mixture (Fig. 5A) actually showed four peaks, with LC-MS/MS it was found that the unknown product was a breakdown product of nifedipine, probably a nitroso-dehydronifedipine known to be formed by P450 (Yin et al., 2001). This effect can be prevented by the use of reduced glutathione. For validation purposes, first the CYP2B and 3A EAD systems in FIA mode were evaluated as to sensitivity and robustness. The cumulative results for all test compounds are shown in Fig. 3 and the resulting IC50 values in Table 1. The differences between IC50 values obtained with the microplate reader assays in setup 1 and setup 2 were only significant in a few cases. Such differences are not uncommon for microsomal P450 inhibition assays when measured with different methodologies (Crespi and Stresser, 2000). Taking this factor into consideration, the IC50 values obtained with both P450-EAD systems in FIA mode were well comparable with those of the microplate reader assays (Table 1) and can thus even be used as initial IC50 value estimates.

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