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Development of three parallel Cytochrome P450 enzyme affinity detection systems coupled on-line to gradient HPLC

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Running Title Page:

Running Title: On-line detection of ligands in mixtures to three Cytochrome P450's

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List of non-standard abbreviations:

BSA: Bovine Serum Albumin; DEX: Dexamethasone; EAD: Enzyme Affinity Detection; FIA: Flow Injection Analysis; FLD: Fluorescence detector (for HPLC); HRS: High Resolution Screening; IPA: Isopropylalcohol; MeCN: Acetonitrile; MeOH: Methanol; NADPH: β-Nicotinamide adenine dinucleotide phosphate; βNF: β-Naphthoflavone; PB: Phenobarbital; PEG6000: Polyethylene glycol 6000.

Abstract:

A High Resolution Screening (HRS) technology is described, in which gradient HPLC is connected on-line to three parallel placed bioaffinity detection systems containing mammalian Cytochrome P450's (CYP's). 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 CYP EAD system was optimized for enzyme, substrate and organic modifier (isopropylalcohol, methanol and acetonitrile) in flow injection analysis (FIA) mode. Characteristic CYP ligands were used to validate the CYP EAD systems. IC_{50} values of the ligands were measured and found to be similar to those obtained with conventional microtiter-plate reader assays. Detection limits (n = 3; S/N = 3) of potent inhibitors ranged from 1 - 3 pmol for CYP1A activity, 4 - 17 pmol for CYP2B activity and 4 - 15 pmol for CYP3A activity. The three optimized CYP EAD systems were subsequently coupled to gradient HPLC and used to screen compound mixtures for individual ligands. In order to increase analysis efficiency, finally a HRS system was constructed in which all three CYP EAD systems were coupled on-line and in parallel to gradient HPLC. The triple parallelized CYP EAD system was shown to enable rapid profiling of individual components in complex mixtures for inhibitory activity to three different CYP's.

Introduction:

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

When many compounds are to be screened for their affinity to CYP's, often *in vitro* High Throughput Screening (HTS) methodologies are used (Masimirembwa et al., 2001). Usually, the inhibition of fluorescent metabolite formation from probe substrates is employed 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 for this type of mixtures (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 bio-affinity detection methods for ligands of e.g. the estrogen receptor, phosphodiesterases, acetylcholine esterases, angiotensin converting enzymes and antibodies have been described (Schenk et al., 2003, Van Elswijk et al., 2003). We recently developed a novel HRS bio-affinity detection system to screen on-line for compounds with affinity for rat liver microsomal CYP1A activity (Kool et al., 2005). In this HRS CYP-EAD system, CYP1A induced rat liver microsomes and the substrate ethoxyresorufine were added post-column to the HPLC eluate to allow a metabolic reaction for a period of time. The CYP1A mediated conversion of ethoxyresorufine to the highly fluorescent product resorufine was monitored continuously by fluorescence detection, thus allowing the on-line screening of individual inhibitors of CYP1A in mixtures. For CYP substrate and inhibitor binding and for drug-drug interaction screening, however, it is desirable to determine the inhibitory potential of compounds towards multiple relevant CYP's, ideally simultaneously.

In the present study, a HRS triple CYP Enzyme Affinity Detection (CYP EAD) system was developed for this purpose. The system consists of three parallel configured CYP EAD systems, with rat liver microsomes as principal source of CYP's. For proof of principle, CYP1A, 2B and 3A activities, respectively using β-naphthoflavone (βNF), phenobarbital (PB) and dexamethasone (DEX)-induced rat liver microsomes 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 CYP1A EAD system recently described (Kool et al., 2005). Finally, the three CYP EAD systems were incorporated on-

line and in parallel in a gradient HPLC apparatus. The entire HRS triple CYP EAD system was then used for the simultaneous screening of inhibitors for each of the three CYP's. The current HRS-methodology could be employed in the late drug discovery and early drug development stages for the screening of lead compounds or for related metabolic mixtures.

Methods:

Materials:

Ethoxyresorufin, Tween 20, polyethyleneglycol 6000 (PEG_{6000}), aminopyrine, α-naphthoflavone (α-NF), miconazole and nifedipine were purchased from Sigma (Zwijndrecht, The Netherlands). Caffeine and metyrapone were obtained from Aldrich (Zwijndrecht, The Netherlands). β-Naphthoflavone (β-NF) was supplied by Acros (Den Bosch, The Netherlands). β-Nicotinamide adenine dinucleotide phosphate (NADPH) tetra sodium salt was purchased from Applichem (Lokeren, Belgium). Trifluoroacetic acid (TFA) was bought from Merck (Schuchardt, Germany). Methanol (MeOH), acetonitrile (MeCN) and 2-propanol (iPA) came from Baker (Deventer, The Netherlands) and were of HPLC reagent grade. All other chemicals were of the highest purity grade commercially available.

β-Naphthoflavone (βNF) and phenobarbital (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 prior to homogenization. Protein concentrations in the β-NF and PB induced rat liver microsomes were 13.1 mg/ml and 9.9 mg/ml, respectively. Dexamethasone (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 the University were applied.

Apparatus:

The configuration of the CYP EAD systems in flow injection analysis (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 CYP EAD system coupled on-line to gradient reversed-phase HPLC is shown in Figure I. The CYP EAD systems are operated by mixing CYP enzymes (in rat liver microsomes), substrate and co-factor (NADPH) continuously with a carrier solution (FIA mode) or with an HPLC effluent (HPLC mode). Superloop A, C and E contain CYP enzymes (from DEX, PB and βNF induced microsomes, respectively) for the three EAD systems (i.e. with CYP3A, CYP2B and CYP1A). For mixing of the probe substrates and co-factor with the respective CYP in every reaction coil, the substrates (BTFC, pentoxyresorufin and ethoxyresorufin) were continuously pumped through superloop B, D and F for the respective CYP EAD system. The co-factor, needed for each EAD system, was present in the substrate containing superloops B, D and F. As enzyme, substrate and co-factor 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 CYP's 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 CYP EAD system at the time and operated in FIA mode was used. In this setup, compounds injected via an autosampler are directly introduced in the three EAD systems. When operated in HPLC mode (Figure I), the autosampler was replaced by a gradient

HPLC system with two additional post-gradient pumps in order to compensate for the increasing concentration of organic modifier during the HPLC gradients.

Knauer K-500 HPLC pumps (Berlin, Germany) were used to control the Pharmacia 150 ml superloops (Uppsala, Sweden) and to conduct the HPLC gradients. The superloops were kept on ice during operation. Agilent 1100 (Waldbronn, Germany) series fluorescence detectors were used for monitoring the fluorescent CYP EAD signals. An excitation wavelength (λ_{ex}) of 530 nm and an emission wavelength (λ_{em}) of 586 nm were used for measuring the fluorescence of the enzymatic product resorufin in case of the CYP1A EAD and the CYP2B EAD. For the CYP3A EAD, an excitation wavelength (λ_{ex}) of 409 nm and an emission wavelength (λ_{em}) of 530 nm were used for measuring the enzymatic product 7-HFC. Knitted PEEK reaction coils (to obtain optimal mixing) were used during the optimization process of the CYP EAD systems (0.50 mm i.d., 1.59 mm o.d.; internal volumes of 75, 100, 150, 200, 400 and 500 µl; representing different reaction times) prior to fluorescence detection. The temperature of the reaction coils (37°C) was controlled by a Shimadzu CTO-10AC column oven (Duisburg, Germany).

When the three CYP EAD systems were coupled in parallel to a single gradient HPLC, the effluent from the HPLC was directed through a 5-piece and splitted 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, while 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, NL) and was controlled by software developed by Kiadis B.V.

CYP Enzyme Affinity Detection in Flow-Injection Analysis mode:

The three CYP EAD systems are operated by continuous mixing of rat liver microsomes, substrate and co-factor (NADPH) with a carrier solution when operated in FIA mode and the effluent from an HPLC separation when operated in HPLC mode (Figure I). After mixing of the substrate with CYP's 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 CYP's, will cause a (temporary) 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, 7-benzyloxy-4-trifluoromethylcoumarin (BTFC) (Stresser et al., 2002) is used, yielding highly fluorescent 7-hydroxy-4-trifluoromethylcoumarine (7-HFC) 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 case of CYP2B and DEX-induced rat liver microsomes in case of CYP3A) and substrate solutions (pentoxyresorufin for CYP2B and BTFC for CYP3A, with NADPH as co-factor 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 CYP 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 prior to mixing of the carrier solution with the enzyme and co-factor/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 co-factor (NADPH; 40 μM). The inhibitor proadifen (injected in different amounts: 0, 6.8, 13.6, 27.3, 54.5, 109, 218, 436, 872 pmol) was used during the optimization process. The buffer used in the superloops consisted of 50 mM potassium phosphate buffer (pH 7.4 containing 2.5 mM $MgCl_2$). 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 in this case place in a 200 μl knitted PEEK tubing. The inhibitor ketoconazole (injected in different amounts: 0, 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200) was used as a reference compound during the optimization. For optimization, the substrate pentoxyresorufin for CYP 2B EAD was tested at concentrations ranging from 100 nM to 1800 nM (Figure IIa), while for CYP3A, substrate (BTFC) concentrations from 1 to 30 μM were tested (Figure IIb). The microsomal concentrations for the CYP 2B 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 CYP EAD systems from 100 to 500 μl, with steps of 100 μl. PEG_{6000} was tested for both systems in the range from 0.5 mg/ml up to 5mg/ml in steps of 0.5 mg/ml, while 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%.

CYP Enzyme Affinity Detection coupled to HPLC:

After optimization and validation of the CYP EAD systems in FIA mode, the CYP 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 CYP EAD system was done in a similar way as described previously for the CYP1A system (Kool et al., 2005). Finally, all three CYP EAD systems were connected and integrated into the triple CYP EAD system in HPLC mode (Figure 1).

HPLC separations were performed using a Phenomenex (Torrance, USA) stainless-steel column, 30 mm × 2 mm i.d., Luna 3µm particles, C18(2). An injection volume of 40 µl was used. The starting flow rate over the analytical column was 700 µl/min, H_2O: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 – 99 % (v/v) IPA gradient was applied in 15 min, followed by a post-gradient 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 equaled 400 µl/min, H_2O: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 make-up HPLC system was set at 1030 µl/min. H_2O:IPA (99:1) and was maintained during the 22 min post gradient. Finally, re-equilibration to starting conditions was performed in 0.5 min. The aqueous and organic modifier solution of the make-up HPLC system contained 100 mg/L Tween 20. The total flow rate of the entire HPLC system was kept constant at 1100 µl/min and contained a

constant IPA concentration of 7 % (v/v). During the evaluation and validation process of the individual CYP EAD systems in HPLC mode, the HPLC effluent was splitted in a 1:9 ratio. The 10 % (v/v) fraction of the flow being directed to the CYP EAD system and the 90 % (v/v) fraction to the UV detector. A mixture of different CYP2B ligands, i.e. 70 μM metyrapon, 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 CYP EAD systems in HPLC mode, the final triple HPLC CYP EAD system was constructed (Figure 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 make-up solutions, the HPLC effluent was split into the three CYP biochemical assays and a UV detector. Prior to 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 CYP's in every system, inhibitors of other CYP's present in the microsomal preparations were included in every CYP EAD system. In this way activities of interfering CYP's could be inhibited. (Stresser et al., 2002). To achieve this goal, the following inhibitors were added to superloop 1: ketoconazole (10 μM) and metyrapon (1.5 μM) for CYP1A EAD, ketoconazole (10 μM) and α-NF (400 nM) for CYP2B EAD and metyrapon (1.5 μM) and α-NF (400 nM) for CYP3A EAD. First, the triple HPLC CYP EAD system was tested for robustness and stability of the enzymatic

assays over time. It was found that the triple HPLC CYP 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 eight hours before they had to be refreshed. After eight hours, the enzymatic activity of the CYP2B and 3A assays declined approximately 20 %, while for CYP1A this decline was only 10 %. A mixture of different CYP ligands was used for validation of the triple parallelized CYP 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 caffein (25 mM). For CYP2B, the inhibitors metyrapon (300 μM) and proadifen (500 μM) and the substrate chloramphenicol (8 mM) were used. For CYP3A, the inhibitors ketoconazole (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 CYP Inhibition:

Microplate reader assay for CYP2B: The microplate reader assay for CYP2B activity was done in a similar way as done for CYP1A activity (Kool et al., 2005). In short, a mixture of 150 μl PB-induced rat liver microsomes (40 μg protein/ml) and pentoxyresorufin (300 nM) in potassium phosphate buffer (50 mM; pH 7.4) containing $MgCl_2$ (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 20 µl solution of test compound in H_2O and 80 µl of a solution containing NADPH (50 µM) in 3 % (v/v) IPA or (setup 2) a freshly prepared mixture of 20 µl solution of test compound in H_2O and 80 µl of a solution containing NADPH (50 µM), PEG_{6000} (0.5 mg/ml) and Tween 20 (100 mg/L) in 3 % (v/v) IPA. The fluorescence of resorufin was measured at λ_{ex} 530 nm (bandwidth 8 nm) and λ_{em} 580 nm (bandwidth 30 nm) on a Victor^2 1420 multilabel counter (Wallac, Turku, Finland).

Microplate reader assay for CYP3A: The microplate reader assays for CYP3A activity were performed in a similar way as for CYP1A and CYP2B activity, with minor modifications. A mixture of 150 µl DEX-induced rat liver microsomes (40 µg protein/ml) and BTFC (4.5 µM) in potassium phosphate buffer (50 mM; pH 7.4) containing $MgCl_2$ (2.5 mM) was used as enzyme and substrate mix, respectively. The fluorescence was measured at λ_{ex} 405 nm (bandwidth 8 nm) and λ_{em} 530 nm (bandwidth 30 nm).

Results:

In order to optimize and validate the CYP EAD systems, a FIA mode was used as 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 CYP inhibition.

Optimization of the CYP 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 CYP EAD's 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 CYP EAD systems in FIA mode.

Substrate optimization: Substrate optimization was conducted by injecting an appropriate test compound in different concentrations (depicted in Figure II) 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 (Figure IIa) 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 upto 9 μM (Figure IIb). Higher concentrations of BTFC only gave minor improvements in S/N-ratios, while the resolution decreased significantly. This decreased resolution was observed as band broadening of signals from injected inhibitors. A concentration of 9 μM is lower then 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, while the S/N-ratios did not increase. Therefore, a microsomal protein concentration of 70 μg/ml was used in both CYP 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: PEG_{6000} and Tween 20 were found to prevent peak broadening in both the CYP2B and 3A EAD system: 0.5 mg/ml PEG_{6000} (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 CYP EAD systems was also investigated in FIA mode. For the CYP2B and 3A EAD systems, similar effects were seen as described previously for CYP1A activity (Kool et al., 2005): 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 system.

Variability:

Inter-day and intra-day variability for the CYP EAD systems were determined in FIA mode under optimized conditions. Intra-day variability, determined by injecting 300 pmol metyrapone (causing 67 % inhibition of the enzyme activity in the CYP2B EAD system) in triplicate, at 2.5 hour time intervals was found to be 6.4 %. For inter-day variability, metyrapone (300 pmol) was injected in triplicate for three sequential days and found to be 7.3 %. The inter- and intra-day measurements for the CYP3A EAD system were conducted in a similar way, with 150 pmol ketoconazole (causing 67 % inhibition of the enzyme activity in the CYP3A EAD system) In this case, intra-day variability was 6.9 % while inter-day variability 8.2 %. With both CYP EAD systems these variabilities were within the normal range of bio-analytical screening methods (Oosterkamp et al., 1994; Schobel et al., 2001). For CYP1A, the inter- and intra-day variabilities have been previously described and were less than 3 % (Kool et al., 2005).

Validation of the CYP 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 bio-affinity assays. In order 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 literature are based on (single) human CYP systems, we used well accepted microplate reader formats in order to obtain reliable IC_{50} values of the test compounds used.

For the determination of IC_{50} values obtained with the on-line CYP EAD systems, the dilution factors of reference inhibitors were first determined. This was done in a similar

way as described recently for the CYP1A EAD system (Kool et al., 2005). Briefly, resorufin concentrations of 10 µM and 100 µM were injected in the CYP 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 CYP 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 Figure IIIa for the CYP2B system and in Figure IIIb for the CYP3A system in. The IC_{50} values obtained from the respective curves are shown in Table I. 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 I. Moreover, Table I 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 CYP 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.

On-line coupling of the CYP Enzyme Affinity Detection systems to HPLC:

Figure I shows the final configuration of the triple CYP 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 CYP EAD systems were coupled in parallel to gradient HPLC.

Typical bio-affinity 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 Figure IV and Vb, 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 were shown, because the injected mixture of seemingly three inhibitors showed actually four UV and four EAD peaks. LC-MS/MS confirmed that nifedipine ($[M + H^+] = 347$) was the third peak in the chromatogram, while 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 CYP EAD systems in HPLC mode. The corresponding IC_{50} values (constructed from 5 different concentrations), calculated in the same way as described above, are presented in Table I.

Finally, the CYP1A, 2B and 3A EAD systems were simultaneously coupled to a single gradient HPLC (Figure I) in order to examine a triple CYP EAD system for on-line

screening of individual compounds in mixtures in a panel of three CYP's. For this purpose, a mixture of 12 different CYP ligands was prepared. The ligands used are all known ligands for at least one of the CYP's. 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 VI shows an overlay of the bio-affinity signals for the CYP1A (Figure VIa), CYP2B (Figure VIb) and CYP3A EAD systems (Figure VIc) and the corresponding UV trace (Figure VId). The lowest EAD trace in every 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 CYP-containing Enzyme Affinity Detection (EAD) systems configured in parallel and on-line with gradient HPLC was the primary goal of this study. For this purpose, β-naphthoflavone (β-NF), phenobarbital (PB) and dexamethasone (DEX) induced rat liver microsomes were used as source of CYP1A, 2B and 3A, respectively. 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 of CYP EAD's specifically (Kool et al., 2005). Hence similar strategies were used to optimize the present EAD systems.

The CYP EAD systems were first evaluated and optimized in FIA mode as 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 CYP inhibition. The present on-line FIA methodology is merely suitable to screen for the inhibitory potential of individual compounds in mixtures towards a panel of CYP's. Current 384-well and higher plate assay based HTS methodologies have uncontested throughput rates compared to the present CYP EAD systems in FIA mode, in which 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 CYP EAD systems. This was done, since rat liver microsomal systems were used for the present proof of principle study, while most inhibition parameters described in literature for CYP inhibition are based on (single) human CYP systems. Because induced rat liver microsomal preparations are not a validated matrix and because they are subject

to variations in CYP contents and activity, in CYP reductase activity and in many other factors, the microplate reader IC50 assays had to be conducted for comparison reasons.

In the optimization process, the additives PEG_{6000} and Tween 20 were found to prevent peak broadening in both the CYP2B and 3A EAD system and were thus used in these systems. Compared to CYP1A (Kool et al., 2005), CYP2B and 3A were much less tolerant towards 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 resolutions for CYP 2B and 3A EAD when compared to 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 (Figure VI). 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 CYP's 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 non-specific drug binding to microsomes was described recently (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 in 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 CYP 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 ensure therefore higher selectivities and sensitivities. Much higher protein concentrations (70 μg/ml) were found to be necessary in order to obtain sufficient S/N ratios for the EAD systems. Additionally, longer reaction coils were needed in order 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). For this CYP EAD system, also smaller reaction coil volumes were sufficient to obtaine good S/N ratios. These differences in optimal protein concentration and reaction coil volumes can be explained by the high turnover rate of ethoxyresorufine by CYP1A and the high concentrations of CYP1A isoenzymes in α-NF-induced rat liver microsomes, when compared to 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 the resolution significantly. This phenomenon is likely caused by increased dispersion of the injected sample when employing larger reaction coils. With CYP1A, a smaller reaction coil volume of 25 μl

was used because ethoxyresorufin O-dealkylation, the biochemical basis of the CYP1A EAD system, involves a high affinity and high turnover substrate (van Liempd 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 CYP 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 towards the organic modifiers tested then 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 CYP (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 Figure III and the resulting IC_{50} values in Table I. The differences between IC_{50} values obtained with the microplate reader assays in setup 1 and setup 2 were only significant in a few cases. Such differences are not un-common for microsomal CYP inhibition assays when measured with different methodologies (Crespi and Stresser, 2000). Taking this into consideration, the IC_{50} values obtained with both CYP EAD systems in FIA mode were well comparable with those of the microplate reader assays (Table I) and can thus even be used as initial IC_{50} value estimates.

When placing the optimized and validated CYP2B and 3A EAD systems on-line after HPLC, typical EAD bio-affinity chromatograms resulting from injections of

mixtures of inhibitory ligands of CYP2B and of 3A (Figure IV and Vb, respectively) show the applicability of theses EAD systems in HPLC mode to separate and subsequently identify the individual inhibitors. For CYP 2B, 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. As the UV chromatogram obtained for this mixture (Figure Va), actually showed four peaks. With LC-MS/MS, it was found that the unknown product was a breakdown product of nifedipine, being probably a nitroso-dehydronifedipine known to result from photo degradation of nifedipine due to sunlight (Grundy et al., 1994). The corresponding CYP3A EAD trace (Figure Vb) accurately identified all three reference ligands as well as the nifedipine breakdown product as inhibitors of CYP3A. These results clearly show the potential of the CYP EAD systems in HPLC mode to measure individual inhibitors in mixtures, which is not possible using other rapid CYP based bio-affinity screening methodologies (van Breemen et al., 1998; Ansede and Thakker, 2004). When reference compounds were injected in a concentration range, IC_{50} values could be constructed for individual inhibitors for both the 2B as the 3A EAD system (see Figure III and Table I). The IC_{50} values obtained with the EAD systems in HPLC mode were well comparable to those obtained with the microplate reader assays and the EAD systems in FIA mode. The CYP EAD systems could thus not only be used to screen individual compounds in mixtures for their inhibitory potencies, but also for the determination of IC_{50} values if the concentrations of the screened compounds are known. When using unknown compound mixtures, an evaporative light scattering detector might be used for estimating compound concentrations for the determination of the IC50 values.

A triple parallelized CYP1A, 2B and 3A EAD system in gradient HPLC mode was also constructed. It proved to be very robust as it could run continuously without failure. The long operating time with one batch of enzymes and substrates in the superloops (eight hours) afforded an efficient system for continuous measurements. It was demonstrated that even in a mixture of 12 CYP inhibitors, the triple parallelized CYP1A, 2B and 3A EAD system in gradient HPLC mode was able to individually identify the ligands and their relative inhibitory potency towards each of the three CYP systems (Figure VI). In contrast to HTS strategies (Kariv et al., 2002) or MS-based screening methodologies (Bu et al., 2001a; Bu et al., 2001b), our methodology does not only allow the simultaneous screening of the inhibition potential of compound mixtures towards three CYP's, but also the identification of the individual compounds in the mixtures by splitting the HPLC effluent to a mass spectrometer (MS). The HRS-technology developed combines the resolving power of HPLC with the sensitivity and selectivity of a bio-affinity assay. The ability to detect and identify CYP ligands on-line after HPLC separation eliminates much of the time and labour required for a fraction collection strategy. Instead of the rat liver microsomes combined with selective substrates as used in this study, human CYP based EAD systems could be used as well. The present triple configured HRS-platform may rapidly provide relevant data during the drug discovery and development processby profiling lead and drug candidate molecules as well as CYP based drug-drug interactions of metabolic mixtures.

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Footnotes:

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Figure legends:

Figure I: *Schematic view of the triple and parallelized CYP1A , 2B and 3A EAD system in gradient HPLC mode. After HPLC, the make-up pumps produce a counteracting gradient, resulting in a constant CYP EAD compatible organic modifier concentration. The eluent is then split 1:1:2:6 to the CYP2B, 3A and 1A EAD systems and the UV detector, respectively. Eluting ligands cause a temporary inhibition of fluorescent product formation in the reaction coils, which is monitored with fluorescence (FLD) detectors. When operated in FIA mode (not shown), the HPLC system including the flow split is replaced by a carrier solution line. Ligands are then injected with an autosampler (A.S.) in the carrier solution and transported directly to a single P450 EAD system. SL = Superloop (all operated at a flow rate of 100 µl/min). Org = Organic modifier. SL A, C and E contain the enzymatic sources for CYP3A, 2B and 1A, respectively. SL B, D and F contain the substrates BTFC, pentoxyresorufin and ethoxyresorufin, respectively. SL B, D and F also contain the co-factor NADPH.*

Figure II: *Substrate optimization results of the CYP EAD systems in FIA mode. (A): Optimization of the substrate pentoxyresorufin for the CYP2B EAD system and (B) for the CYP3A EAD system with the substrate BTFC. For CYP2B, metyrapone was used as inhibitor; for CYP3A, ketoconazole was used.*

Figure III: *(A): IC50 curves (SD; n=3) for 5 different CYP2B ligands with the CYP2B EAD system in FIA mode. (B): IC50 curves (SD; n=3) for 3 different CYP3A ligands with the CYP3A EAD system in FIA mode.*

Figure IV: *CYP2B EAD trace of a mixture of 4 compounds injected in the CYP2B EAD system in HPLC mode (eluting compounds are: metyrapon (70 μM; 8.5 min), aminopyrine, (6000 μM; 9.5 min), chloramphenicol (7000 μM; 27.0 min) and proadifen (50 μM; 29.0 min)).*

Figure V: *(A) UV trace of a mixture of 3 compounds (and a breakdown product) injected in the CYP3A EAD system in HPLC mode (eluting compounds are: ketaconazole (28 μM; 14.5 min), breakdown product (19.0 min), nifidipine (430 μM; 21.5 min) and miconazole (52 μM; 25.0 min)). (B) Corresponding CYP3A EAD trace of the mixture depicted in B.*

Figure VI: *A mixture of 12 compounds at 3 different concentrations injected in the triple parallalyzed CYP1A, 2B and 3A EAD system in HPLC mode. The lowest EAD trace in each EAD chromatogram shows the highest injected mixture concentration. The other two EAD traces resulted from 16 and 256 times diluted mixtures, respectively. Injected compounds are: 1) methyrapon (300 μM; 7.5 min), 2) caffein (25 mM; 10.0 min), 3) 9-hydroxyellipticine (200 μM; 15.5 min), 4) ellipticine (200 μM; 19.5 min), 5) ketaconacole (250 μM; 20.0 min), 6) phenacetin (15 mM; 20.5 min), 7) chloramphenicol (8 mM; 23.0 min), 8) nifidipine (400 μM; 26.0 min), 9) proadifen (500 μM; 27.0 min), 10) miconazole (8 μM; 30.0 min), 11) α-napthoflavone(50 μM; 40.0 min) and 12) β-napthoflavone (600 μM; 41.0 min) . (A) P450 1A EAD trace (B) P450 2B EAD trace (C) P450 3A EAD trace (D) UV trace.*

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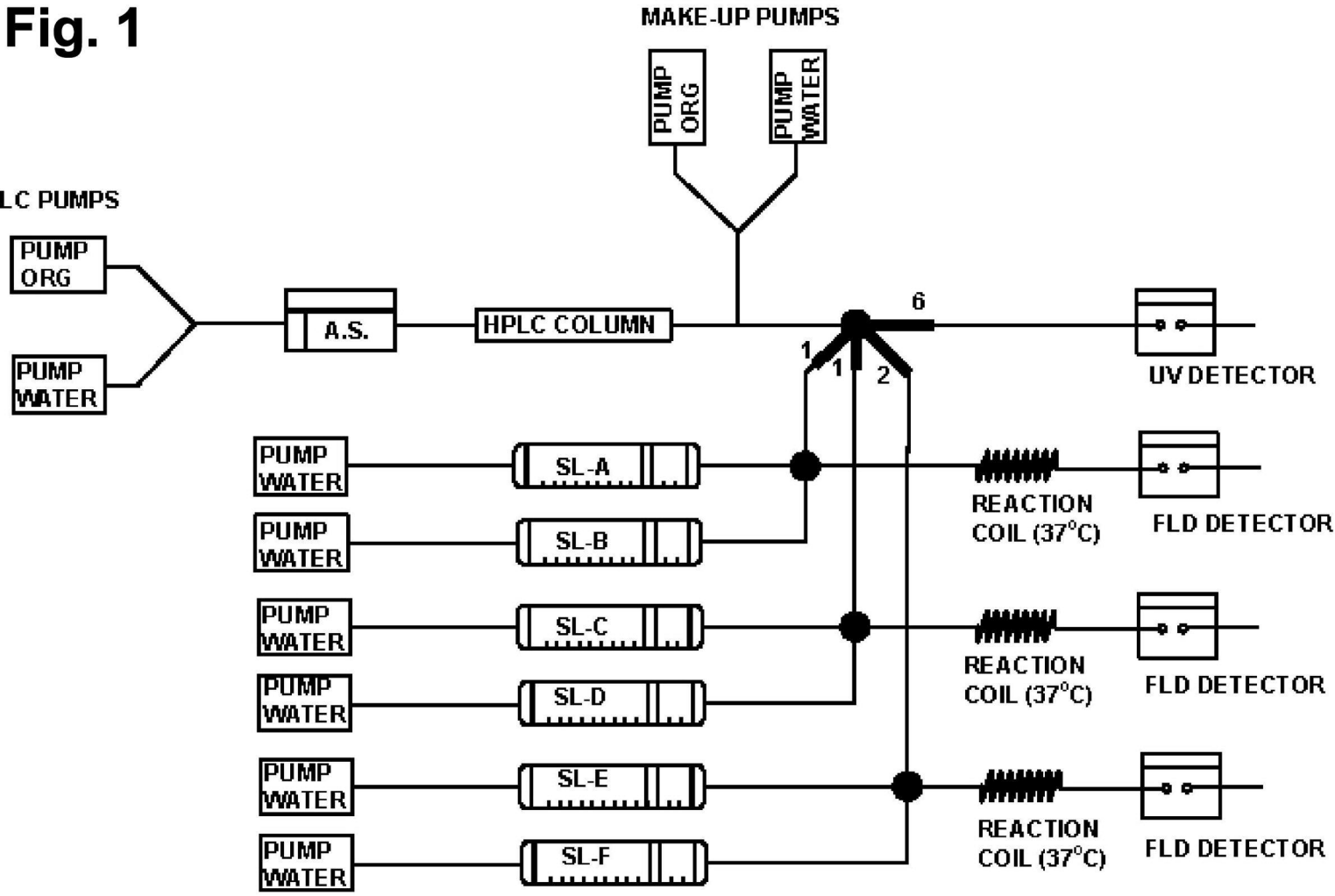
Table I: *CYP2B: IC50-values of 5 CYP2B inhibitors measured with the P450 2B EAD system in FIA mode, HPLC mode and in two different microplate reader formats. CYP3A: IC50-values of 3 CYP3A inhibitors measured with the P450 3A EAD system in FIA mode, HPLC mode and in two different microplate reader formats (Setup 1: ACN; Setup 2: ACN, PEG and T20).*

CYP2B						
	FIA EAD		**HPLC EAD**		**Microplate reader**	
Inhibitor	**IC50's (µM ± SEM; n=3)**	**Detection limit in pmol (S/N=3)**	**IC50's: (µM; n=1)**	**Detection limit in pmol (S/N=3)**	**Setup 1: IC50's (µM ± SEM; n=4)**	**Setup 2: IC50's (µM ± SEM; n=4)**
Metyrapon	0.30±0.02	4	0.60	60	0.28±0.09	0.22±0.07
Proadifen	0.24±0.10	17	0.40	240	0.41±0.21	0.21±0.05
Chloramphenicol	75±13	877	93	7760	80±9	61±4
Phenobarbital	1062±66	9933	N.D	N.D	608±43	402±53
Aminopyrine	617±390	4750	826	76000	455±38	321±71
CYP3A						
Ketaconazole	0.33±0.04	15	0.28±0.17	120	0.28±0.01	0.24±0.01
Miconazole	0.11±0.01	4	0.13±0.19	32	0.10±0,005	0.094±0,004
Nifedipine	6.3±4.5	200	3.89±4.2	1600	9.0±1.3	9.7±2.2

Fig. 1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PUMP WATER
SL-A
PUMP WATER
SL-B
REACTION COIL (37°C)
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SL-D
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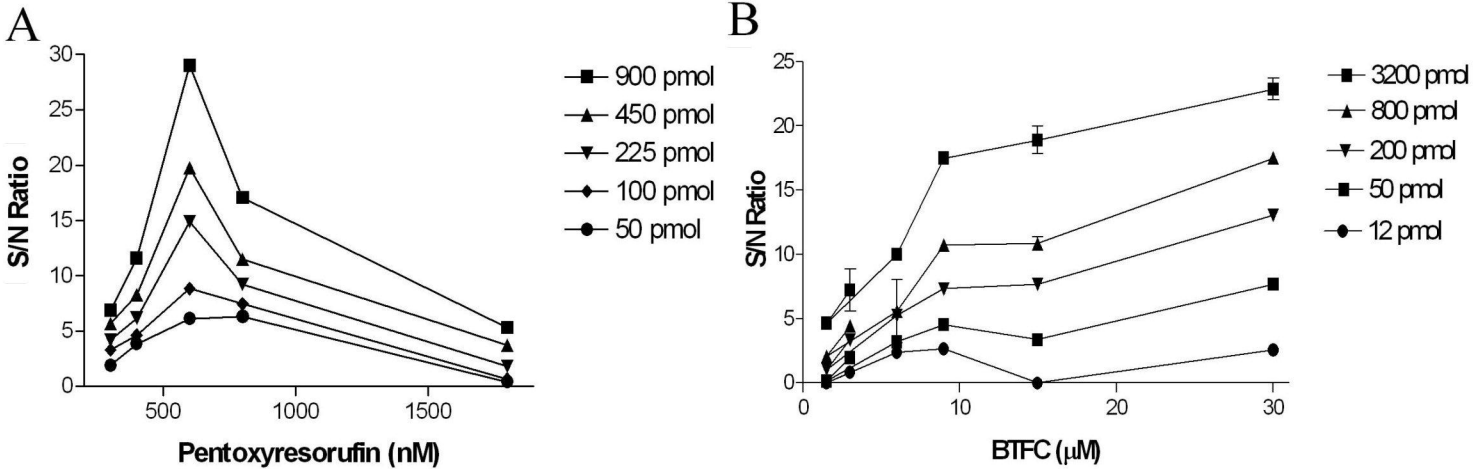
Fig. 2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100 pmol
50 pmol
B
S/N Ratio
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15
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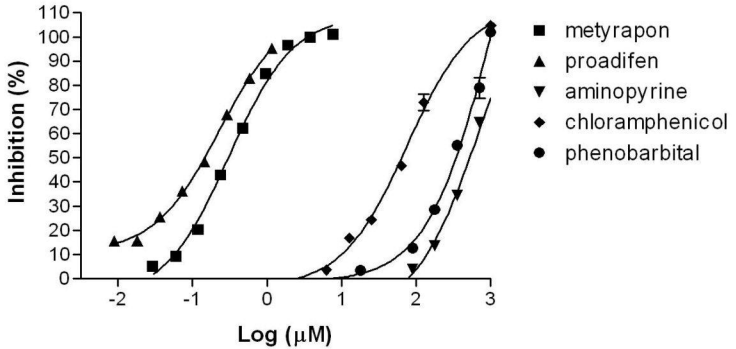
200 pmol
50 pmol
12 pmol

Fig. 3

A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proadifen
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B

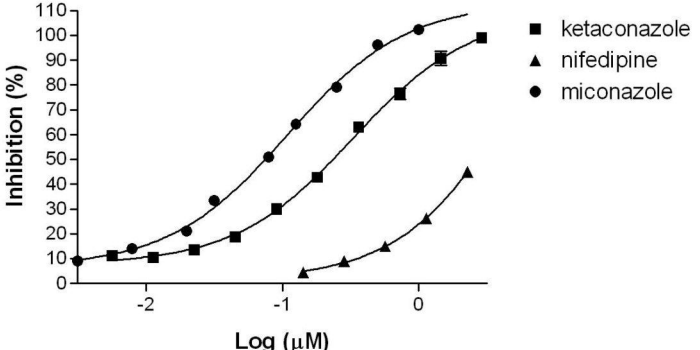
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Fig. 4

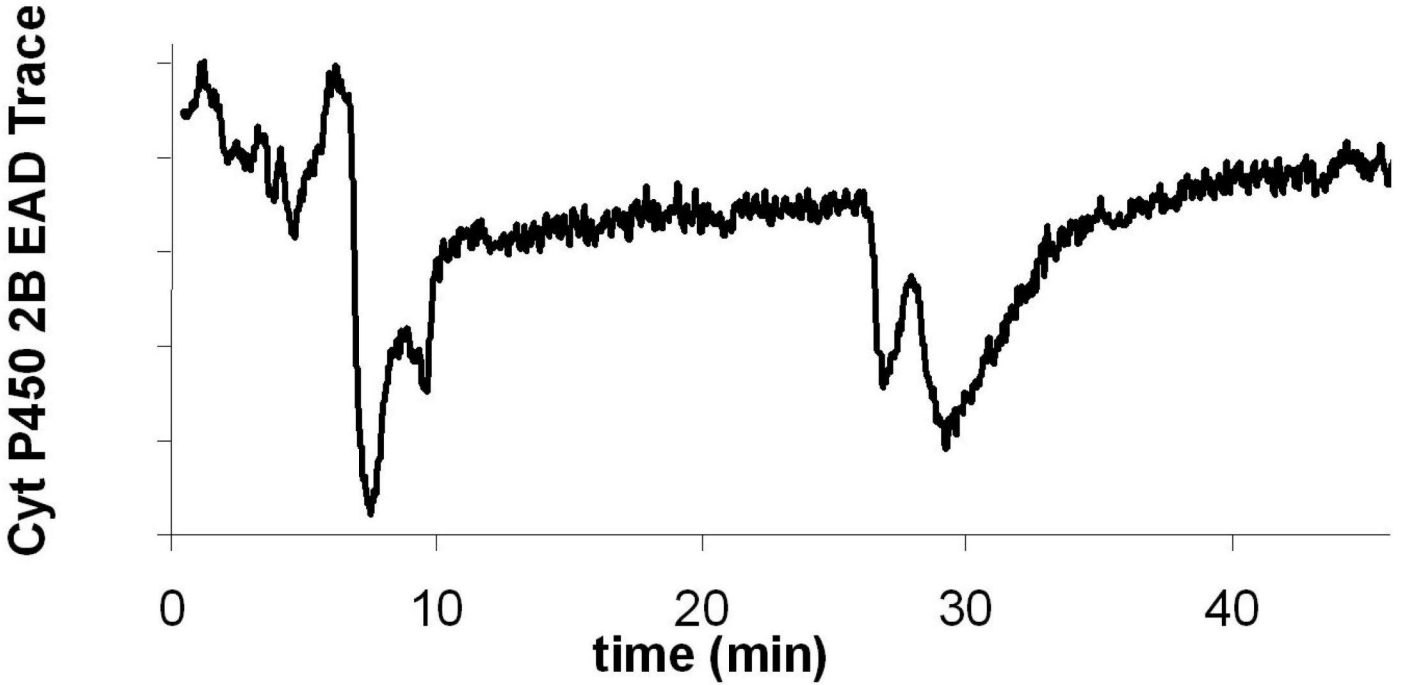

Fig. 5

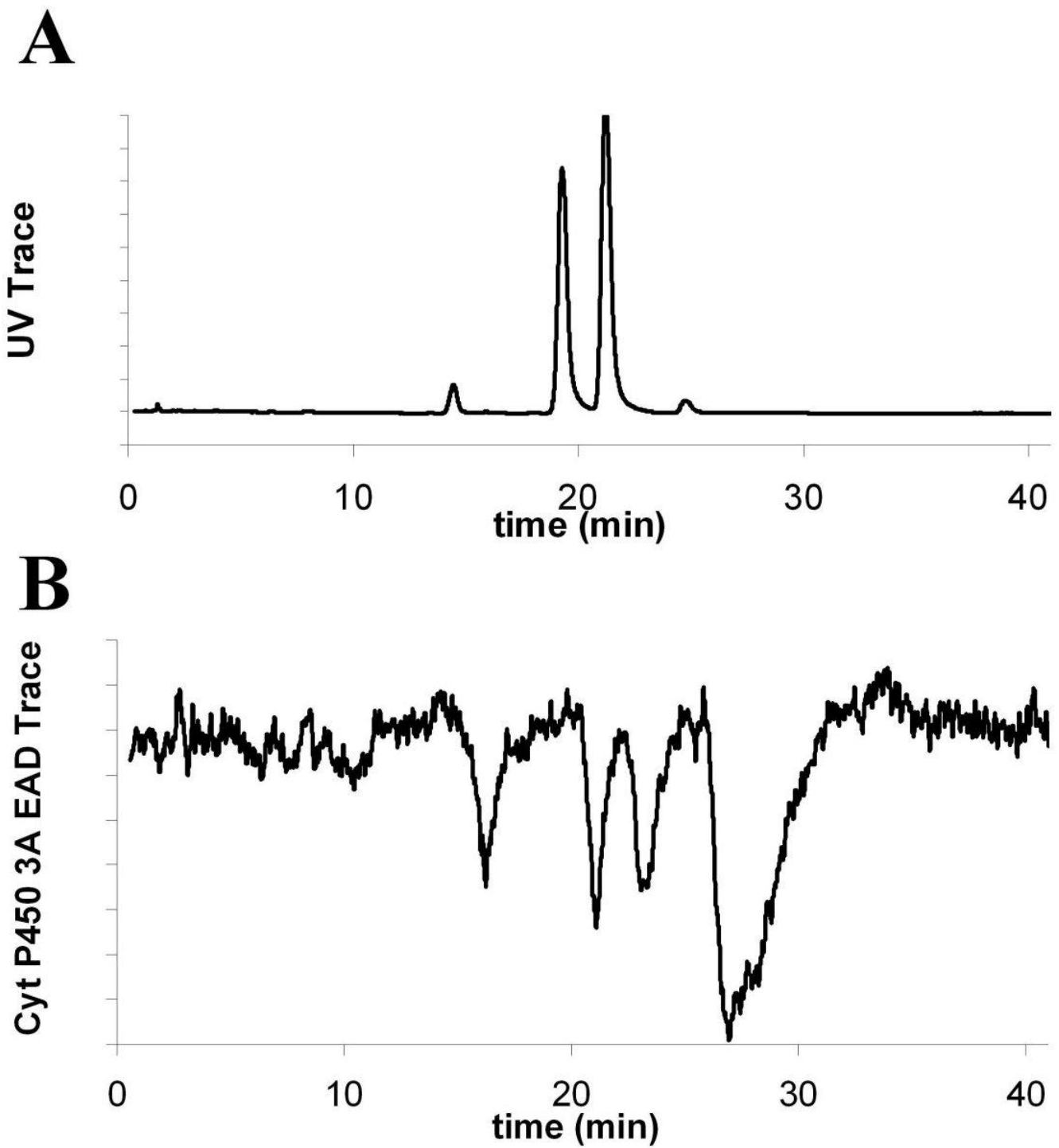
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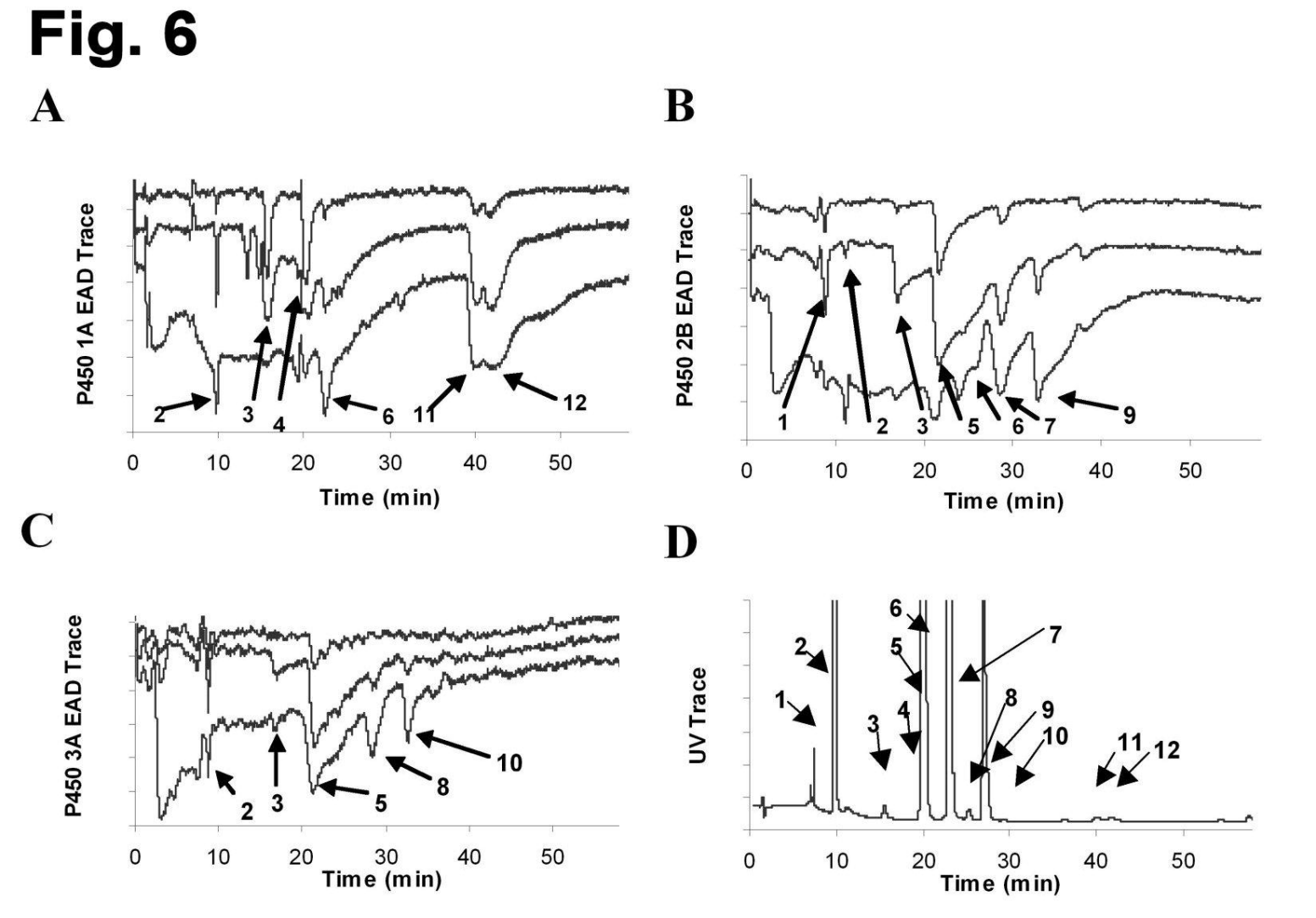
Fig. 6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