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Cytochrome P450 bio-affinity detection coupled to gradient HPLC: On-line screening of affinities to cytochrome P4501A2 and 2D6

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Abstract

Here we describe novel on-line human CYP1A2 and CYP2D6 Enzyme Affinity Detection (EAD) systems coupled to gradient HPLC. The use of the systems lies in the detection of individual inhibitory ligands in mixtures (e.g. metabolic mixtures or herbal extracts) towards two relevant drug metabolizing human CYPs. The systems can rapidly detect individual compounds in mixtures with affinities to CYP1A2 or 2D6. The HPLC–EAD systems were first evaluated and validated in flow injection analysis mode. IC50 values of known ligands for both CYPs, tested both in flow injection and in HPLC mode, were well comparable with those measured in microplate reader formats. Both EAD systems were also connected to gradient HPLC and used to screen known compound mixtures for the presence of CYP1A2 and 2D6 inhibitors. Finally, the on-line CYP2D6 EAD system was used to screen for the inhibitory activities of stereoisomers of a mixture of five methylenedioxy-alkylamphetamines (XTC analogs) on a chiral analytical column.

Keywords: Bio-affinity detection; On-line; HPLC; Cytochrome P450

1. Introduction

Cytochromes P450 (CYPs) constitute the most important enzymes involved in the biotransformation of drugs and other xenobiotics. Amongst these, is a vast array of clinically, toxicologically and physiologically important compounds [1–3]. In humans, hepatic CYP1A2, 2C9, 2C19, 2D6 and 3A4 are the most important isoforms in xenobiotic metabolism [4–6]. Of those isoforms, CYP2D6 is considered one of the most important enzymes, because of its polymorphic nature [7–9]. Genetic polymorphisms may result in differences in substrate specificity, loss of protein function, increased enzymatic activity or even different (adverse) effects of drugs [7,10]. Despite the fact that CYP2D6 represents only approximately 2–4% of total human hepatic CYPs, it plays an important role in the oxidation of drugs and xenobiotics, metabolizing about 30% of the drugs that are currently on the market [11,12]. Although not as pronounced as in the case of CYP2D6, interindividual differences in CYP1A2 may also result in different enzymatic activities towards drugs and xenobiotics [13]. These interindividual differences are extensively studied and associated with differences in cancer susceptibility towards environmental and food-derived carcinogenic compounds [14]. The enzyme is for example induced by cigarette smoking or by charcoal-broiled meat ingestion [15,16].

CYP inhibition studies are often performed on new chemical entities (NCEs) during the drug discovery and development process. Rapid high-throughput screening approaches are frequently utilized in order to keep pace with the increasing numbers of NCEs, generated by combinatorial chemistry and natural compound libraries [17,18]. The current CYP inhibition screens are usually based on a single enzyme paradigm for compound–compound interactions [19]. This actually means that NCEs inhibiting the metabolism of a probe substrate for a specific CYP also will inhibit other substrates for that enzyme. Thus, also potential drug–drug interactions can be evaluated

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for each enzyme of interest. One of the drawbacks in this strategy is that in case of mixtures of compounds (e.g. combinatorial libraries, natural compounds or metabolic mixtures), no conclusion can be drawn about the individual compound(s) in the mixtures that are responsible for the inhibitory potential towards the CYP screened for. To evaluate the active compounds in mixtures, usually HPLC separation followed by affinity screening of all separate fractions is performed [20]. One strategy to overcome this cumbersome process is the use of High-Resolution Screening (HRS), which stands for the on-line affinity detection after HPLC separation [21,22]. HRS systems in which compounds can be screened for affinity towards soluble receptors (e.g. the estrogen receptor) and enzymes (e.g. phosphodiesterases) in one bio-assay already exist [22,23]. Recently, new HRS methodologies for the screening of compounds with affinity for CYP1A, CYP2B and/or CYP3A enzymes in rat liver microsomes were developed [24,25]. Although being very sensitive and capable of rapidly identifying the CYP inhibitors in mixtures, these methodologies still lack the added specificity that could be obtained when using heterologously expressed human CYPs.

This article describes the development of two CYP Enzyme Affinity Detection (EAD) systems coupled on-line to gradient HPLC. One EAD system utilizes heterologously expressed human CYP2D6 as enzyme source and the second heterologously expressed human CYP1A2, thus giving rise to two truly specific human CYP EAD systems. Continuous mixing of enzyme, a non-fluorescent substrate and the eluent from a HPLC system in a reaction coil is the basis of this HRS methodology. In the reaction coil a continuous formation of a fluorescent product takes place, which is measured at the end of the reaction coil. Eluting CYP ligands temporarily inhibit the enzymatic formation of the fluorescent product, thereby allowing for detection of the inhibition potential of the ligands. Both the CYP1A2 and 2D6 EAD systems were firstly optimized and validated in flow injection analysis (FIA) mode and subsequently coupled to gradient HPLC for on-line screening of individual CYP inhibitors in mixtures. Finally, the CYP2D6 EAD system was coupled on-line to isocratic chiral HPLC for stereoselective screening of CYP2D6 inhibitors in mixtures.

2. Experimental

2.1. Chemicals

7-Methoxy-4-(aminomethyl)-coumarin (MAMC) was synthesized by Onderwater et al. [26]. Methoxyresorufin, Tween 20 and Tween 80, saponin (from Quillaja Bark), glycerol, polyethyleneglycol 6000 (PEG6000), polyethyleneglycol 3350 (PEG3350), quinidine, sparteine, tripolidine, dextromethorphan and α-naphthoflavone (αNF) were purchased from Sigma (Zwijndrecht, The Netherlands). Proadifen was purchased from SmithKline&French laboratories (Herts, United kingdom), quinine from Fluka (Zwijndrecht, The Netherlands) and caffeine and sodium cholate from Aldrich (Zwijndrecht, The Netherlands). Codeine and phenacetin were purchased from Brocades (Maarssen, The Netherlands). β-Naphthoflavone (βNF) was supplied by Acros (Den Bosch, The Netherlands). β-Nicotinamide adenine dinucleotide phosphate tetra sodium salt (NADPH) and TRIS (hydroxymethyl) aminomethane were obtained from Applichem (Lokeren, Belgium). Methanol (MeOH) and acetonitrile (ACN) were purchased from Baker (Deventer, The Netherlands) and were of HPLC reagent grade. All other chemicals were of the highest purity grade commercially available.

2.2. Microsomal protein preparation

2.2.1. CYP1A2

The plasmid pCWori+ containing the modified CYP1A2 sequence fused to the rat cytochrome P450 NADPH-reductase [27], was transformed in *E. coli* strain DH5α by heat shock. Expression and membrane isolation was performed as follows: A single ampicillin-resistant colony of *E. coli* (DH5α) transformed with the fusion plasmid expression construct [27,28] was grown over night at 37 °C in 20 ml Luria–Bertani (LB) medium containing 100 μg/ml ampicillin. This pre-culture was used to inoculate 1 L of Terrific Broth (TB) medium, supplemented with 100 μg/ml ampicillin, 1.0 mM thiamine, 0.5 mM δ-ALA, 0.10 μg/ml riboflavin and 3 ml of a trace elements solution [10 mM $FeCl_3$, 1 mM $ZnCl_2$, 1.2 mM $CoCl_2$, 0.9 mM $NaMoO_4$, 0.7 mM $CaCl_2$, 0.6 mM $CuCl_2$, 0.8 mM H_3BO_3 in 10 M HCl]. Protein expression was induced directly and after 24 h with 1.0 mM IPTG. Cultures were grown at 28 °C with shaking at 125 rpm for 48 h. Cells were harvested by centrifugation at 4000 rpm for 30 min at 4 °C. All subsequent steps were carried out at 4 °C. The pelleted cells were resuspended in 50 ml PBS buffer (10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl) followed by centrifugation at 4000 rpm for 20 min. The supernatant was removed and the pelleted cells were resuspended in approximately 40 ml TSE buffer (75 mM Tris–HCl buffer, pH 7.5, containing 250 mM sucrose and 0.25 mM EDTA). Cells were subjected to three cycles of disruption with a cooled French pressure cell (1000 psi). The disrupted cells were centrifugated at 4000 rpm for 10 min to remove intact cells. The supernatant was further centrifugated at 100,000 × *g* for 1 h. The resulting pellet was resuspended in 2 ml PBS buffer and frozen at −80 °C.

2.2.2. CYP2D6

E. coli membranes containing CYP2D6 and human CYP NADPH-reductase were produced according to Keizers et al. [29].

2.3. Equipment

The configuration of the individual CYP EAD systems in flow injection analysis (FIA) and HPLC mode was similar to that described recently for the on-line CYP1A EAD system by Kool et al. [24]. A general scheme of a CYP EAD system coupled on-line to gradient reversed-phase HPLC is shown in Fig. 1. All experiments were performed with a Gilson 234 autosampler

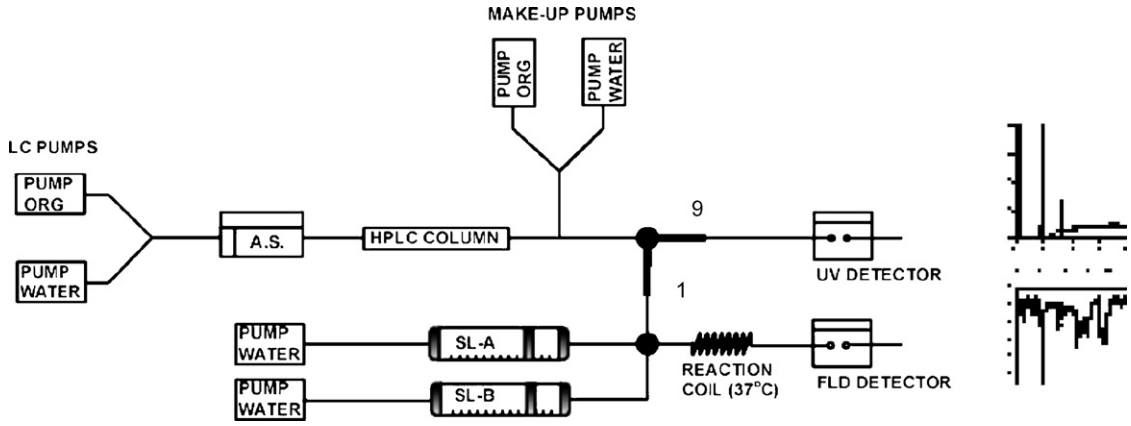

Fig. 1. Schematic view of a CYP EAD system in HPLC mode. Superloop-A (SL-A) and superloop-B (SL-B) are used to deliver enzyme and substrate to the reaction coil, respectively. Ligands are introduced into the system by a gradient reversed phase HPLC system. Ligands temporarily inhibit fluorescent product formation, which is monitored with a fluorescence (FLD) detector. After HPLC, the make-up pumps produce a counteracting gradient, resulting in a CYP EAD compatible constant organic modifier concentration. The eluent is then split 1:9 (90% to UV detection and 10% to CYP EAD). AS = autosampler.

(Villiers-le-Bel, France) equipped with a Rheodyne (Bensheim, Germany) six-port injection valve (injection loop, 40 μl). An Agilent 1100 (Waldbronn, Germany) series fluorescence detector (λ_{ex} 530 nm; λ_{em} 586 nm for P450 1A2 EAD and λ_{ex} 370 nm; λ_{em} 470 nm for P450 2D6 EAD) was used for detection, while Knauer K-500 HPLC pumps (Berlin, Germany) were used to deliver the carrier solution in flow injection analysis mode and to control the reagent (enzyme and substrate) containing Pharmacia 50 ml superloops (Uppsala, Sweden). A Shimadzu CTO-10AC column oven (Duisburg, Germany) controlled the temperature of the enzymatic reaction. Knitted PEEK reaction coils (0.5 mm i.d.; 1.59 mm o.d.; internal volumes of 25, 75, 150, 300, 500 and 800 μl) were tested for the on-line enzymatic reactions prior to detection. When the enzyme affinity detection assays were coupled to chromatographic systems, Knauer K-500 HPLC pumps, a 1/9 flow-splitter (5 cm length, 50 μm i.d., 375 μm o.d. and 5 cm length, 25 μm i.d., 375 μm o.d. fused silica connected to a T-piece) and an Agilent 1100 series UV detector (220 nm) were additionally used in the system. All hardware was integrated in one HRS-system by Kiadis B.V. (Groningen, NL) and was controlled by software developed by Kiadis B.V.

2.4. On-line CYP1A2 and 2D6 EAD systems in FIA mode

A schematic representation of an on-line CYP enzyme affinity detection (EAD) system in FIA mode is shown and described by Kool et al. [24]. During the optimization process in FIA mode, the carrier solution consisted of water and was pumped at a flow rate of 100 μl/min. Flow injections were made on the carrier solution prior to mixing with enzyme (100 μl/min) and substrate (100 μl/min). Enzyme (CYP1A2 or CYP2D6) and substrate (methoxyresorufin) for CYP1A2 EAD or MAMC for CYP2D6 EAD) were housed at 0 °C in superloops. The CYP1A2 concentration was 0.35 nM, whereas the CYP2D6 concentration was 60 nM. The buffer that was used for the enzyme and substrate solutions consisted of potassium phosphate (50 mM, pH 7.4 for CYP1A2 and pH 7.8 for CYP2D6), containing 2.5 mM $MgCl_2$.

2.5. On-line CYP1A2 and 2D6 EAD systems in HPLC mode

Gradient HPLC separations were performed using a 30 mm length × 2 mm i.d. stainless-steel column (Phenomenex Luna 3 μ C18(2)). The chromatographic separations were performed using a decreasing flow rate gradient. The initial flow rate was set at 700 μl/min. A pre-gradient of 2 min was applied using water and acetonitril in a 95:5 ratio. Subsequently the percentage ACN was increased to 95% within 12 min. During this time period the flow rate dropped gradually to 100 μl/min. A post-column gradient of 10 min, H_2O:ACN (5:95), with a flow rate of 100 μl/min was applied. After chromatographic separation the column was equilibrated to the starting conditions in 0.5 min. In order to obtain a constant and bioassay compatible concentration of organic modifier in the P450 EAD system, an additional makeup gradient with an initial flow rate of 300 μl/min was added to the HPLC effluent. Throughout the chromatographic separation the total flow rate after dilution of the HPLC effluent was 1 ml/min. By using this approach the concentration acetonitrile introduced in the biochemical assay was 10%. During the chromatographic separation the flow rate of the makeup gradient was increased to 900 μl/min in 12 min and was maintained at this level during the post-gradient. During a 0.5 min equilibration period the flow rate was decreased to starting conditions. The aqueous and organic modifier solvents of the makeup gradient both contained 4 g/l Tween 20 in the case of CYP1A2 and 100 mg/l Tween 20 in the case of CYP2D6. The advantage of a decreasing flow rate gradient compared to a normal gradient when coupled on-line to a biochemical assay is that the dilution factor of the HPLC effluent increases with increasing organic modifier percentage. When applying a standard gradient, however, the dilution factor is constant. During a decreasing flow rate gradient polar compounds are diluted less then more apolar compounds. Consequently the

sensivity of the method is enhanced for these types of compounds.

2.6. Chiral chromatography coupled to CYP2D6 EAD

For the stereoisomer separation of a series of XTC-analogs, isocratic separations were performed on a 250 mm length × 4.6 mm chiral Cyclobond I 2000 RSP column (Astec). The separated compounds were simultaneously detected with UV detection and the CYP2D6 EAD. The chiral column was eluted with a 100 mM potassium phosphate buffer containing 0.1% triethylamine and 5% MeCN (pH adjusted to 4.0 with acetic acid) at a flow rate of 500 μl/min. To obtain a CYP2D6 EAD compatible pH, a make-up flow consisting of 300 mM TRIS (pH 10.5; 500 μl/min.), which was mixed with the HPLC effluent, ensured a final pH of 7.4 after equilibration in a 50 μl reaction coil. The diluted effluent was directed through to a T-piece and splitted in a 1:9 ratio using a flowsplitter. Ten percent of the flow was directed to the CYP2D6 EAD system and 90% was splitted to the UV detector.

2.7. Microplate reader assays for CYP inhibition

For CYP1A2, the fluorescence of the metabolic product resorufin was measured at excitation and emission wavelengths of 530 nm (bandwidth 8 nm) and 580 nm (bandwidth 30 nm), respectively, on a Victor^2 1420 multi-label counter (Wallac, Turku, Finland). Tests were performed under: (1) normal conditions and (2) conditions used in the P450 1A2 EAD system. In this way possible differences between the obtained IC50's were determined. Concentration ranges of test compounds were prepared by serial dilution of compounds dissolved in 50 μl DMSO with 150 μl DMSO. A mixture (150 μl) of CYP1A2 (0.2 nM) and methoxyresorufin (1.2 μM) in 50 mM potassium phosphate buffer (pH 7.4), containing 2.5 mM $MgCl_2$, was incubated for 15 min at 37 °C. Then, 75 μl of one of the following solutions was added to start the reaction: (1) (normal conditions): a freshly prepared mixture of 20 μl test compound in DMSO and 80 μl of a solution containing 50 μM NADPH in 10% ACN or (2) (CYP1A2 EAD conditions): a freshly prepared mixture of 20 μl test compound in DMSO and 80 μl of a solution containing 50 μM NADPH, 1 g/l PEG6000 and 4 g/l Tween 20 in 10% ACN. The initial linear increase in resorufin fluorescence was a marker of the enzymatic activity. Inhibition curves based on 11 compound concentrations and a blank were measured in quadruplicate for each test compound. IC50 values for each compound were determined with Prism3 software for both assay formats.

3. Results and discussion

This study describes the development of two on-line EAD systems for the screening of affinities of individual compounds in mixtures to human CYP1A2 and 2D6 isoenzymes. Both on-line EAD systems were first optimized and validated in FIA mode. Subsequent on-line coupling of the EAD systems to gradient HPLC allowed isoenzyme selective screening of affinities of individual compounds in mixtures as they eluted from the HPLC column. Both EAD systems presented in this study relied on *E. coli* expression systems for the supply of the CYP isoenzymes. Combination with relatively selective probe substrates, ensured sensitivity and total selectivity for both CYPs, this in contrast to the previously used CYP EAD systems containing microsomal CYPs [24]. The relatively specific substrates methoxyresorufin and MAMC were used as probe for monitoring the formation of fluorescent product (respectively resorufin by CYP1A2 and 7-hydroxy-4-(aminomethyl)-coumarin (HAMC) by CYP2D6). The post-column EAD systems are operated by simultaneous mixing of CYP, cofactor and substrate with the mobile phase in a reaction coil. The substrate reacts with the enzyme to form a fluorescent product. Inhibitors of this enzymatic reaction will temporarily inhibit the formation of fluorescent product, which is detected as a negative peak in the fluorescence readout. A scheme of a CYP EAD system in HPLC mode is shown in Fig. 1. For CYP2D6 EAD, quinidine was used as model inhibitor whereas α-naphthoflavone (αNF) served as model inhibitor for CYP1A2 EAD. The on-line EAD systems were first developed and optimized in Flow Injection Analysis (FIA) mode, before coupling to gradient HPLC.

3.1. CYP1A2 and 2D6 EAD systems in FIA mode

3.1.1. Optimization of the CYP EAD systems

The optimization of the CYP EAD systems was done in a similar way as recently described for the optimization of the on-line rat liver microsomal CYP1A system [24]. For optimization, different parameters were looked at. These parameters were divided into three different categories, namely: (1) parameters that increase the sensitivity of on-line EAD systems; (2): additives that increase the resolution of the on-line systems by decreasing band broadening of injected ligands (i.e. test compounds) due to a-specific binding of these ligands or enzymes to reaction coils; (3) parameters that are automatically introduced in on-line post-column EAD systems (organic modifiers).

3.1.1.1. Parameters influencing the assay sensitivity. Assay sensitivity is influenced by temperature, enzyme concentration, substrate concentration (around the K_m value, a commonly used and accepted substrate concentration for biochemical assay formats) and reaction time. As expected for the CYP1A2 and 2D6 EAD systems, the maximum formation of fluorescent product was observed at 37 °C. K_m concentrations of the substrates methoxyresorufin and MAMC, determined in a microplate reader assay, were used as substrate concentrations in the respective reaction coils. The K_m value of methoxyresorufin for CYP1A2 was 0.40 μM ± 0.06 μM, and corresponded well with literature [30]. The presence of PEG 6000 (1 g/l) and Tween 20 (4 g/l) did not change the K_m value significantly (PEG and Tween are discussed in Section 3.1.1.2. For CYP2D6, the K_m value was also determined with and without using PEG 6000 and Tween 20, and was found to be 50 μM ± 6 μM which corresponded well with literature [29]. The effect of reaction time on the on-line enzymatic reactions was investigated by using reaction coils of different volumes fluvoxamine as probe inhibitor for CYP1A2 and quinidine for CYP2D6. For CYP1A2, S/N ratios

leveled off at reaction coil volumes higher than 150 μl. In the case of CYP2D6, similar effects were seen, but the S/N ratios leveled off at a reaction coil volume of 400 μl.

3.1.1.2. Parameters influencing the assay resolution. Resolution (peak width or band broadening of injected test compounds) is influenced by reaction coil length (reaction time) and additives that reduce a-specific binding of enzymes and ligands (e.g. Tween or PEG [24]). First, several additives were tested for their ability to increase resolution in both CYP EAD systems. It was found in both the on-line EAD systems that the polymers PEG 3325 and 6000 as well as the detergents Tween 20 and 80 and saponin increased the resolution of the on-line systems at increasing concentrations. Concentrations up to 1 g/l in case of PEG 3325 and 6000 increased the resolution of test compounds injected in both EAD systems. Higher concentrations did not alter the resolution anymore. Increasing concentrations of Tween 20 and 80 and saponin increased the resolution of test compounds injected in both EAD systems. In this case no optima were found since increasing concentrations of detergents also decreased the enzymatic activities up to complete enzyme inactivation. To determine the maximally allowable concentrations of these additives that can be used in the on-line assay formats, these additives were examined for their ability to reduce enzymatic activity using a microplate reader format. Up to 3 g/l PEG 3325 and 6000 did not alter enzymatic activity of both CYPs. In the case of CYP1A2, Tween 20 and 80, could be used for up to 6 g/l with retention of 50% of the original enzymatic activity. For CYP2D6, however, only amounts up to 150 mg/l Tween 20 or 80 could be used (Fig. 2a). A combination of Tween and PEG proved optimal for improving resolution in both CYP EAD systems, a result consistent with literature [24]. The optimal conditions were 4 g/l Tween 20 with 1 g/l PEG 6000 in the carrier solution for CYP1A2 EAD and 100 mg/l Tween 20 with 1 g/l PEG 6000 for CYP2D6. With these concentrations a maximum decrease in band broadening of injected test compounds in the on-line EAD systems was accomplished with a minimum destruction of enzymatic activity. The reaction time (coil volume; already discussed in Section 3.1.1.1) was also investigated to determine the effect on the resolution of the on-line EAD systems by using reaction coils of different volumes with fluvoxamine as probe inhibitor for CYP1A2 and quinidine for CYP2D6. For CYP1A2, the resolution decreased with increasing volumes of the reaction coil due to band broadening. In the case of CYP2D6, the same effect was seen. An optimum reaction coil volume combines two factors: (a) increased formation of fluorescent product from the respective substrates due to longer reaction times and (b) a larger band broadening of injected ligands due to larger reaction coils. These optima were also described previously in similar HRS systems [24,31].

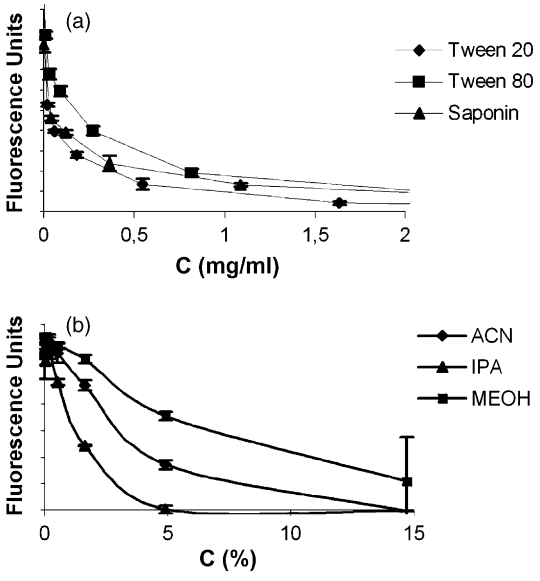

Fig. 2. Microplate reader assay based optimization results of (a) detergents for CYP2D6 and (b) organic modifiers for CYP1A2. Initial increases in fluorescence in the presence of the tested additives were measured.

3.1.1.3. Parameters that are automatically introduced in on-line post-column EAD systems:. When coupling the CYP EAD systems to gradient HPLC, organic modifiers necessary for the separation are also introduced into the system. The influence of organic modifiers was therefore first evaluated in a microplate reader format. MeOH was found to decrease the enzymatic reaction of CYP1A2 by 50% at a concentration of 7% of MeOH, while in the case of IPA and MeCN these percentages were 3 and 6%, respectively (Fig. 2b). For CYP2D6, concentrations of 2, 3 and 3.5% for MeOH, IPA and MeCN were found, respectively. The enzymatic activity of CYP1A2 was markedly more stable compared to CYP2D6. The fact that the CYP1A2 used is a fusion protein with NADPH CYP reductase means that no separation from the CYP and the reductase after solubilisation [22] can occur [32]. This could attribute to the inherent stability of the present CYP1A2 fusion protein to detergents and organic modifiers. The present microplate reader assays were performed over a 6 min time period, while the biochemical reactions in the optimized on-line CYP EAD systems last for time periods of only 20 s for CYP1A2 and 80 s for CYP2D6. It was therefore expected that higher organic modifier concentrations could be used in the on-line biochemical assay formats. This was indeed observed in both the CYP EAD systems, where organic modifier concentrations twice as high as those in the microplate reader assay were found before a 50% decrease in enzymatic activity was found.

The final optimized conditions of the CYP1A2 EAD were as follows: The first superloop (SL-1) contained CYP1A2 (0.35 nM) in potassium phosphate buffer (50 mM, pH 7.4, 2.5 mM $MgCl_2$). The second superloop (SL-2) contained a solution of 40 μM NADPH, 1.2 μM methoxyresorufin and 1 g/l PEG 6000 in potassium phosphate buffer (50 mM, pH 7.4, 2.5 mM $MgCl_2$). The carrier solution or the decreasing flow rate gradient contained 4 g/l Tween 20, while a 100 μl reaction coil was used.

For CYP2D6 EAD, the optimized conditions were as follows: The first superloop (SL-1) contained CYP2D6 (60 nM) in potassium phosphate buffer (50 mM, pH 7.8, 2.5 mM $MgCl_2$). The second superloop (SL-2) contained a solution of 40 μM

Table 1
IC50 values of six CYP1A2 inhibitors measured with the CYP1A2 EAD system in FIA mode, four CYP1A2 inhibitors measured with the CYP1A2 EAD system in HPLC mode and six CYP1A2 inhibitors measured in two different batch assay formats

Inhibitor	CYP1A2 in FIA mode		CYP1A2 in HPLC mode		Microplate reader	
	IC50's (μM ± SEM; $n = 3$)	Det. Lim. (pmol; S/N = 3)	IC50's (μM; $n = 4$)	Det. Lim. (pmol; S/N = 3)	Setup 1 (μM ± SEM; $n = 4$)	Setup 2 (μM ± SEM; $n = 4$)
Ellipticine	0.007 ± 0.005	0.4	0.011 ± 0.005	32	0.026 ± 0.004	0.008 ± 0.001
9OH-Ellipticine	0.011 ± 0.006	0.4	0.004 ± 0.002	30	0.033 ± 0.004	0.010 ± 0.006
α-Naphtoflavone	0.06 ± 0.02	1.0	0.031 ± 0.020	50	0.06 ± 0.01	0.08 ± 0.02
Fluvoxamine	0.63 ± 0.10	18	0.15 ± 0.01	350	0.28 ± 0.03	0.46 ± 0.03
Phenacetin	87 ± 6	82	N.D.	N.D.	123 ± 15	154 ± 4
Caffein	5338 ± 78	8025	N.D.	N.D.	5262 ± 595	2619 ± 554

Detection limits for both on-line modes are also given. N.D., not determined. Setup 1: IPA, PEG and Tween 20. Setup 2: IPA. Det. Lim., detection limit.

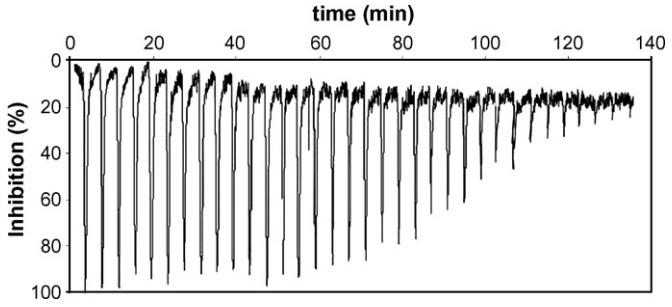

Fig. 3. Injections (triplicates) of fluvoxamine in the CYP1A2 EAD system in FIA mode. Total amounts of 18.4, 9.2, 4.6, 2.3, 1.2, 576, 288, 144, 72, 36 and 18 pmol were injected.

NADPH, 150 μM MAMC and 1 g/l PEG 6000 in potassium phosphate buffer (50 mM, pH 7.8, 2.5 mM $MgCl_2$). The carrier solution or the decreasing flow rate gradient contained 100 mg/l Tween 20, while a 400 μl reaction coil was used.

3.1.2. Validation of the CYP EAD systems

Several inhibitory ligands of both CYP EAD systems were injected (in triplicate) in concentrations yielding 0–100% enzyme inhibition. For this purpose, well known and often used inhibitory test compounds for both CYPs encompassing a wide range of inhibitory activities were selected (see Tables 1 and 2). A typical result obtained with fluvoxamine in the CYP1A2 EAD system is shown in Fig. 3. The following compounds, displaying a wide range of inhibitory potencies, were chosen for validation of the CYP1A2 EAD system: αNF, ellipticine, 9-hydroxy-ellipticine (9OH-ellipticine), fluvoxamine, phenacetin and caffeine. Concentration–response curves were obtained by plotting the concentration of the inhibitor in the reaction coil against the S/N ratio of the resulting response according to Kool et al. [24]. Fig. 4a shows a cumulative plot presenting the concentration–response curves of all inhibitors tested. The respective IC50 values and the detection limits are presented in Table 1. For caffein and phenacetin, quenching of fluorescence at high concentrations prevented the measurement of complete IC50 curves, but estimates of the affinities could still be made [24]. For potent inhibitors, such as αNF, ellipticine and 9OH-ellipticine, IC50 values obtained with the CYP1A2 EAD system were in accordance with those from the microplate assays (Table 1). When compared to the rat liver CYP1A EAD system, previously described [24], similar IC50's were found for the test compounds. Since rat liver CYP1A enzymes resembles human CYP1A2 [33,34], similar IC50's may be expected and therefore our experiments demonstrate the applicability of the present CYP1A2 EAD system in measuring IC50 values for human CYP1A2.

For CYP2D6 EAD, the following inhibitors were used: quinidine, quinine, tripolidine, sparteine, dextromethorphan and codeine. The resulting IC50 curves are shown in Fig. 4c, and the calculated IC50 values together with literature IC50 values in Table 2. The IC50 values of the inhibitors quinidine and quinine obtained with the CYP2D6 EAD system were in accordance with IC50 values of quinidine (0.04 ± 0.01 μM) and quinine (0.76 ± 0.17 μM) determined previously by Keizers et al. [29]. No significant quenching was observed for the CYP2D6 inhibitors at the concentrations used for construction of the IC50 curves.

Table 2
IC50 values of six CYP2D6 inhibitors measured with the P450 2D6 EAD system in FIA mode, four CYP2D6 inhibitors measured with the P450 2D6 EAD system in HPLC mode

Inhibitor	CYP2D6 in FIA mode		CYP2D6 in HPLC mode	
	IC50's (μM ± SEM; $n = 3$)	Det. Lim. (pmol; S/N = 3)	IC50's (μM; $n = 4$)	Det. Lim. (pmol; S/N = 3)
Quinidine	0.028 ± 0.009	0.8	0.014	5
Quinine	0.57 ± 0.33	6	0.83	28
Triprolidine	2.3 ± 0.5	39	1.3	126
Sparteine	9.8 ± 2.8	93	N.D.	N.D.
Dextromethorphan	0.64 ± 0.18	19	2.5	80
Codeine	151 ± 29	3900	N.D.	N.D.

Detection limits for both modes are also given. N.D., not determined; Det. Lim., detection limit.

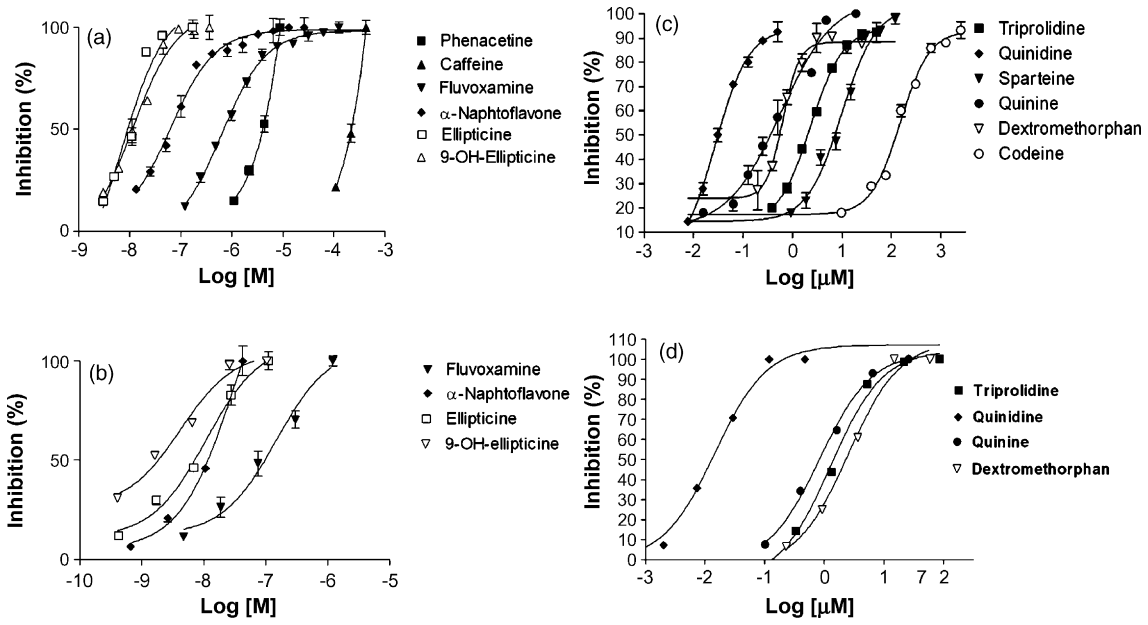

Fig. 4. (a) IC50 curves ($n = 3$) obtained for six different CYP1A2 ligands in the CYP1A2 EAD system in FIA mode. (b) IC50 curves ($n = 4$) obtained for four different CYP1A2 ligands in the CYP1A2 EAD system in HPLC mode. (c) IC50 curves ($n = 3$) obtained for six different CYP2D6 ligands in the CYP2D6 EAD system in FIA mode. (d) IC50 curves ($n = 1$) obtained for four different CYP2D6 ligands in the CYP2D6 EAD system in HPLC mode.

For both EAD systems, the concentrations below which the systems cannot detect inhibitors anymore are dependent upon the actual affinities of the inhibitors, their solubilities and their potential to show fluorescence quenching or enhancement at high concentrations. The detection limits (Tables 1 and 2) of the test compounds give indications regarding the applicability of the EAD systems and show that very low up to very high affinity inhibitors can be detected with both EAD systems. Both EAD systems in FIA mode were merely used for evaluation and validation purposes (before on-line coupling to HPLC). Nevertheless, it can concluded that both the CYP1A2 and the CYP2D6 EAD system in FIA mode can also be used to screen compounds for affinity and moreover relatively accurately measure the IC50 values of those compounds.

3.2. On-Line coupling of gradient HPLC to the CYP1A2 and 2D6 EAD systems

For on-line coupling of the CYP EAD systems after gradient HPLC (Fig. 1), two additional LC-pumps operating post-column with a counteracting gradient were employed, thus resulting in a constant concentration of organic modifier entering the biochemical assay. First, several inhibitors were individually injected in different concentrations in the CYP1A2 EAD system in HPLC mode. Representative superimposed chromatograms of different concentrations of αNF are shown in Fig. 5a. The CYP EAD responses were used for plotting IC50 curves (Fig. 4b) and calculation of the IC50 values (Table 1). In case of the potent CYP1A2 inhibitors αNF, ellipticine, 9OH-ellipticine and fluvoxamine, the IC50 values were in agreement with those obtained with the CYP1A2 EAD system in FIA mode as well as results from the microplate reader assay. For the low affinity inhibitors phenacetin and caffein, fluorescence quenching at high concentrations prevented accurate determination of complete IC50 curves. Moreover, very high concentrations of these compounds (overloading the analytical column) must be injected in order to construct the complete IC50 curves. Therefore, IC50 values of these compounds were not determined in HPLC mode. Similar effects were observed previously [24].

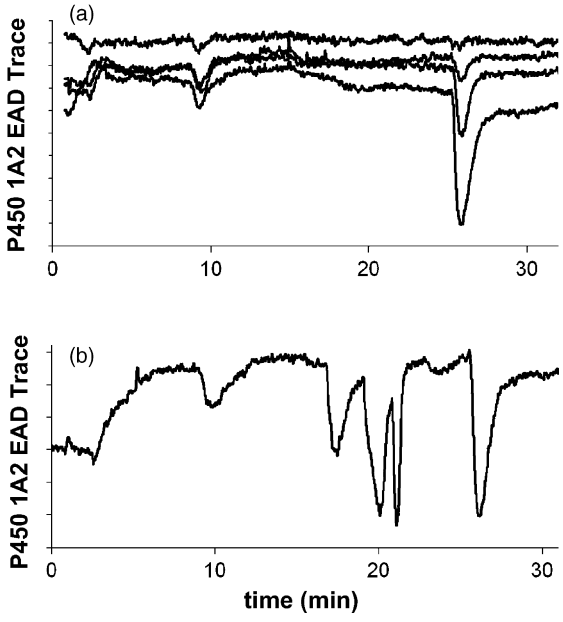

Fig. 5. (a) Superimposed CYP1A2 EAD traces of α-NF injected in different amounts (50, 150, 500 and 1500 pmol from top to bottom chromatogram, respectively) in the on-line CYP1A2 EAD HPLC system. (b) CYP1A2 EAD trace of a mixture of four compounds injected in the CYP1A2 EAD HPLC system (injected compounds are subsequently: (1) 9-OH-ellipticine (100 pmol; 17.0 min); (2) fluvoxamine, (10 nmol; 20.0 min); (3) phenacetin (2000 nmol; 21.0 min); and (4) α-NF (1000 pmol; 26.0 min)).

Mixtures of ligands for CYP1A2 were also injected into the CYP1A2 EAD system in HPLC mode. Fig. 5b shows a typical CYP1A2 EAD trace resulting from a mixture of αNF, fluvoxamine, 9OH-ellipticine and phenacetin. Due to measures to prevent band broadening (i.e. the use of organic modifiers, PEG and Tween), similar resolutions were obtained as in previous on-line biochemical assay formats that used soluble enzymes [23] or receptors [22] instead of the membrane bound CYP enzymes.

Similar experiments were performed for CYP2D6 EAD in HPLC mode. Representative superimposed chromatograms of different concentrations of the inhibitor tripolidine are shown in Fig. 6a. IC50 values of several CYP2D6 inhibitors measured with this system with corresponding IC50 curves are shown in Table 2 and Fig. 6d, respectively. The IC50 values were in agreement with those obtained with the CYP2D6 EAD system in FIA mode. The IC50 values obtained for quinidine and quinine were also compared with literature values and found to be in accordance as well [29,35]. The IC50 values of the low affinity compounds (sparteine and codeine) were not determined because of the same reasons as for the low affinity compounds in the case of the CYP1A2 EAD system in HPLC mode. Next, the CYP2D6 EAD in HPLC mode was used for screening mixtures. A typical result is shown in Fig. 6b with a mixture containing the inhibitors codeine, sparteine, quinidine and tripolidine. The resolution obtained was lower than that of the CYP1A2 EAD system. This was mainly due to the lower enzymatic conversion rate of the probe substrate MAMC, obligating the use of longer reaction coils thereby causing more band broadening.

The resolution obtained with the EAD systems in HPLC mode is both dependent on the EAD part and the chromatographic part of the complete systems. For example: Figs. 3 and 5 nicely show the resolutions obtained in case of CYP1A2, while

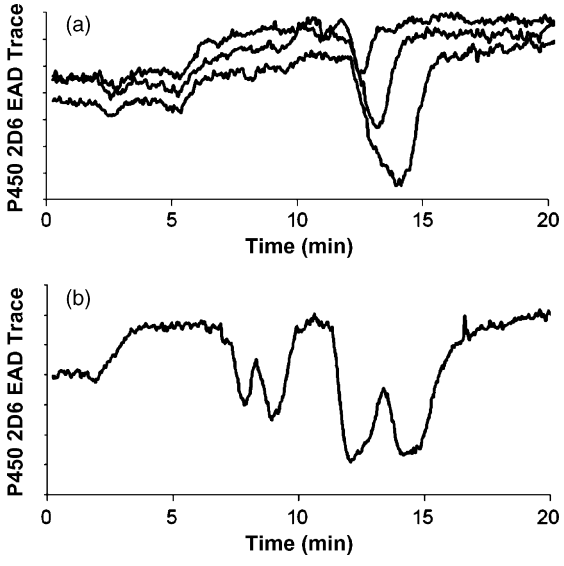

Fig. 6. (a) Superimposed CYP2D6 EAD traces of tripolidine injected in different amounts (5, 20 and 200 nmol) in the CYP2D6 EAD system in HPLC mode. (b) CYP2D6 EAD trace of a mixture of four compounds injected in the CYP2D6 EAD system in HPLC mode (injected compounds are: (1) codeine (2 μmol; 7.5 min); (2) sparteine, (250 nmol; 9.0 min); (3) quinidine (1 nmol; 12.0 min); and (4) tripolidine (50 nmol; 14.5 min)).

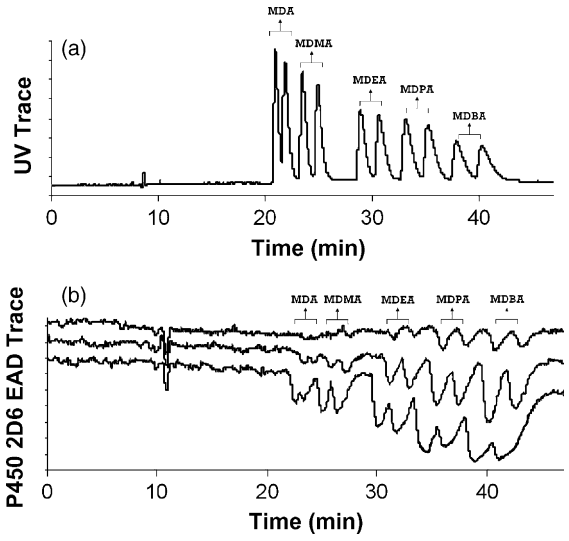

Fig. 7. (a) UV trace of a chiral HPLC separation of a mixture of 5 methylenedioxy-alkyl-amphetamines. (b) Corresponding superimposed CYP2D6 EAD traces of the same mixture injected at three different concentrations (10 mM; 2.5 mM and 0.5 mM).

Fig. 7 demonstrates the extra band broadening of the EAD system when comparing the peak widths in the EAD line and the chromatographic line (UV trace).

Connecting the EAD systems to chromatographic separations with very high resolutions obviously will result in higher resolutions in the EAD systems, which is desirable for screening of real life samples. For detection of individual inhibitors, however, baseline separations of EAD peaks are not needed, as superimposed peaks also allow identification of multiple inhibitory compounds in mixtures (see Fig. 5B and Figs. 5B and 7). For screening of mixtures with (potential) CYP1A2 or CYP2D6 inhibitors, however, it is not likely to find large numbers of inhibitors per mixture. It is concluded that the two on-line human CYP EAD systems in gradient HPLC mode constitute new valuable tools in the drug discovery and development area. The systems can efficiently be used for the sensitive and reproducible screening of inhibitory properties of individual compounds in mixtures (e.g. metabolic mixtures) towards human CYPs.

3.3. On-Line coupling of chiral HPLC to the CYP2D6 EAD system

The inhibitory properties of a set of CYP2D6 ligands with different alkyl substituents varies with the chain length of the alkyl group [36]. Moreover, different stereoisomers of a ligand may inhibit isoenzymes in different ways, which in turn may lead to varying pharmacokinetic and drug–drug interactions characteristics of the two stereoisomers [37]. Methylenedioxy-alkylamphetamine (MDMA, also known as XTC), for instance is a known chiral substrate for CYP2D6 and also are the chiral analogs methylenedioxy-ethylamphetamine (MDEA) and methylenedioxy-amphetamine (MDA), which are high affinity CYP2D6 substrates [38]. In the present study, chiral HPLC was used to separate the methylenedioxy-alkylamphetamine (MDAA) enantiomers, which were then

analyzed for CYP2D6 affinity using the post-column on-line CYP2D6 EAD. When injecting a mixture of MDA, MDMA, MDEA, methylenedioxy-propylamphetamine (MDPA) and methylenedioxy-butylamphetamine (MDBA), these analogs were baseline separated into 10 stereoisomers by chiral HPLC (Fig. 7a). Corresponding superimposed CYP2D6 EAD traces show the inhibitory properties of all individual stereoisomers injected at three different concentrations (Fig. 7b).

From the CYP2D6 EAD traces it can be concluded that the enantiomeric forms of each analog have more or less the same inhibitory potency for CYP2D6. The chainlength of the N-substituent, however, affects the affinity of the ligand giving higher inhibitory potencies with longer chain-length [36,38]. According to Sadeghipour and Veuthey [39], who used similar chromatographic separations of the different analogs together with polarimetric detection, the *R*-(−)-isomers eluted prior to the *S*-(+)-isomers. Polarimetric detection allows the relative affinity appointments of the enantiomers. It is demonstrated that the on-line coupling of chiral HPLC to CYP2D6 EAD results in a valuable system capable of separating mixtures of MDAA's into their respective enantiomers and screen each individual enantiomer for affinity towards CYP2D6.

4. Conclusions

This study shows the applicability of recombinant human CYPs for on-line Enzyme Affinity Detection (EAD) after gradient HPLC. It was demonstrated that recombinant human CYP1A2, fused to the NADPH CYP-reductase as a single membrane-bound protein, as well as recombinant human CYP2D6 co-expressed with NADPH CYP-reductase, could successfully be used on-line as EAD systems in Flow Injection Analysis (FIA) mode as well as in gradient HPLC mode. After optimization of both systems, they were validated with known ligands for both CYPs. The IC50 values of the ligands, determined with either EAD system in FIA mode, corresponded well with IC50 values measured with traditional microplate reader assays. For the CYP1A2 EAD system, detection limits of 0.4–1.0 pmol were found for potent CYP1A2 inhibitors (αNF, ellipticine and 9OH-ellipticine). For CYP2D6, the detection limit of the ligand with the highest affinity, i.e. quinidine, was 0.8 pmol. When the CYP EAD systems coupled on-line to gradient HPLC were used to screen mixtures of compounds, it was shown that both systems were able to separate the ligands prior to sensitive and reproducible determination of individual affinities for the CYP used. The results given clearly show that both EAD systems are capable of not only detecting inhibitors very sensitively, but also detecting very low to very high affinity inhibitors in a range that is compatible for pharmaceutical screening purposes. Moreover, with the CYP2D6 EAD system coupled on-line to chiral column chromatography, we were able to separate five methylenedioxy-alkylamphetamine analogs into their enantiomers and subsequently to screen their individual CYP2D6 affinity on-line. We conclude that the current CYP EAD systems in HPLC mode can be used for the sensitive and reproducible screening of individual substrates and inhibitors in mixtures of compounds (e.g. metabolic mixtures) to determine human CYP enzyme affinities and/or possible drug–drug interactions rapidly and efficiently. As the EAD systems in FIA mode can only handle a maximum of 40–60 samples per hour, the EAD systems are not ideal for High-throughput Screening (HTS) purposes. When compared to other CYP screening methodologies, the strength of the present methodology lies in the profiling of individual compounds in mixtures that gave a hit in HTS. Compared to the dereplication procedures nowadays used, our approach is not only less labor intensive and time costly, but also allows identification of co-eluting inhibitory compounds and allows the comparison of peak shapes of inhibitory compounds (EAD trace) with all compounds eluting from HPLC (UV or MS trace). Also, EAD systems are less prone to oxidation effects, which can occur during handling of collected fractions in dereplication processes. Therefore, the EAD systems are ideally suited in certain parts of the drug discovery and development process, i.e. by identifying undesired CYP-inhibitory metabolites in metabolic mixtures of lead compounds.

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