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Development and validation of a microsomal online cytochrome P450 bioreactor coupled to solid-phase extraction and reversed-phase liquid chromatography

Sebastiaan M. van Liempd[a], Jeroen Kool[a], Jelle Reinen[a], Tim Schenk[b], John H.N. Meerman[a], Hubertus Irth[b], Nico P.E. Vermeulen[a,*]

[a] *LACDR-Division of Molecular Toxicology, Department of Pharmacochemistry, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*
[b] *Kiadis B.V., Groningen, The Netherlands*

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Abstract

The development and validation of an online cytochrome P450 (CYP)-based bioreactor coupled to automated solid-phase extraction (SPE) and gradient HPLC separation is described. The analytical method was checked on intra- and inter-day repeatability of the ethoxyresorufin-*O*-demethylation (EROD) reaction with CYP 1A1/1A2 containing β-NF induced rat liver microsomes as an enzyme source. These experiments showed that CYP activity was linearly decreased with 16% over an 11 h period. Inter-day measurements had a CV of 9.1%. Furthermore, K_m and V_{max} values of the EROD reaction, measured with the bioreactor, were 2.72 ± 0.46 μM and 7.9 ± 0.5 nmol/min/mg protein, respectively. These were in good correspondence with K_m and V_{max} values, measured with standard batch assay, which amounted 0.66 ± 0.08 μM and 6.4 ± 0.2 nmol/min/mg protein respectively. In conclusion the newly developed analytical method can be used effectively and at a microliter scale for online generation, extraction and separation of metabolites.

Keywords: Cytochrome P450; Online bioreactor; Biocatalysis; Drug metabolism; Metabolic profiling

1. Introduction

Membrane-bound cytochromes P450 (CYPs) are the most important enzymes involved in metabolism of drugs and other xenobiotics [1]. In mammalians, including humans, the majority of CYPs can be found in the endoplasmatic reticulum of hepatic tissue. However, CYPs are also expressed in other organs and tissues, like kidneys, lungs and brain. CYPs may be involved in three types of metabolic reactions, namely monooxygenase, oxygen reductase or substrate reductase reactions [2]. Of these, monooxygenase reactions are the most common. Examples are aromatic and aliphatic hydroxylation, heteroatom oxygenation and dealkylation, epoxidation of double bonds and cleavage of esters [3].

Studying CYP metabolism is important for pharmacological, toxicological or environmental reasons. Environmental factors and polymorphisms of CYPs may cause variability in the pharmaco-/toxicokinetics of drugs or nutrients [4–6]. CYPs can also be involved in prodrug activation, e.g. in the case of codeine. Toxic effects induced by drugs or environmental chemicals can also result from CYP metabolism. In the latter case, metabolites formed, often interact with macromolecular biomolecules. These interactions may include covalent binding to proteins or DNA which can lead to severe pathologies. Furthermore, metabolites formed by CYPs can have pharmacological or toxicological effects at sites different from the target site of the parent compound. In the case of two or more drugs, inhibition of CYPs can lead to hazardous drug–drug interactions. Clearly, it is not only important to identify CYP-related biological properties of the parent compounds, but also of the metabolites formed.

* Corresponding author. Tel.: +31 20 4447590; fax: +31 20 4447610.
E-mail address: npe.vermeule@few.vu.nl (N.P.E. Vermeulen).

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Several types of assays have been developed to investigate CYP activity including CYP inhibition assays [7–9]. Alternatively, identification of metabolites formed by CYPs as well as metabolic profiling is often performed. For this purpose, metabolite incubation mixtures are usually extracted and subsequently introduced in HPLC, coupled to various detection techniques [10,11]. This often impels automatic procedures for incubation of compounds and subsequent extraction and separation of metabolites. An online bioreactor, coupled to solid-phase extraction (SPE) and HPLC might therefore, be very useful to fulfill this demand.

Until now, some bioreactors have been developed which use membrane bound CYPs as catalyst in order to carry out stereo- or regioselective metabolic reactions for biosynthetic purposes [10,11]. However, these bioreactors usually have large volumes (≥100 ml), which makes them less suitable for the biotransformation of small quantities of compounds. Some bioreactors make use of hepatocyte cultures on various supports [12–15]. A disadvantage of these systems is that the production of metabolites is low and the metabolic reactions of single CYPs cannot be investigated. A rather elegant system, reported recently, makes use of chip technology to metabolize substrates and to trap CYP metabolites on a microliter scale [16]. Finally, a stirred cell bioreactor has been described which makes use of ultra-filtration for sample clean up [17,18]. As yet, all mentioned bioreactors lack online coupling between analyte trapping and HPLC separation, which excludes online metabolite generation and identification. Recently, however, Friedrich et al. described a method using online sample preparation coupled to HPLC separation for analysis of offline CYP derived metabolites [19,20].

The objective of the present study was the development and validation of a novel, automated online, microliter scale liver microsomal CYP-based bioreactor, coupled to SPE and HPLC. Development of this system included the design of a CYP bioreactor unit and online SPE trapping for substrate and products of the ethoxyresorufin-*O*-demethylation (EROD) reaction. Furthermore, repeatability of the product signal with SPE–HPLC coupled online to the CYP bioreactor was assessed by means of inter- and intra-day measurements. Further validation of the system was performed by comparing enzyme kinetic parameters obtained by a standard batch assay with those obtained with the newly developed hyphenated system.

2. Experimental

2.1. Materials

Triethylamine (TEA), resorufin (RES) and ethoxyresorufin (EthRES) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Riedel de Häen (Seelze, Germany) supplied natrium hydroxide (NaOH), magnesium chloride ($MgCl_2$), potassium dihydrogenphosphate (KH_2PO_4) and dipotassium hydrogenphosphate (K_2HPO_4). β-Nicotinamide adenine dinucleotide phosphate (NADPH) tetra sodium salt was purchased from Applichem (Lokeren, Belgium). HPLC grade methanol (MeOH), Bakerbond C_8, C_{18} PolarPlus and C_{18} Standard SPE material were acquired from J.T. Baker (Deventer, The Netherlands). Luna $C_{18}(2)$ material was purchased from Phenomenex (Amstelveen, The Netherlands) and Polyethersulphone (PES) 0.22 μm membrane filters from Sterlitech (Kent, WA, USA). Knitted 1/16 in. × 0.75 mm PTFE reaction coils were obtained from VICI Jour (Amstelveen, The Netherlands). β-NF induced rat liver microsomes (further referred to as liver microsomes) were prepared as described by Rooseboom et al. [21]. Protein concentration of the microsomes was determined with the BioRad Protein Assay and amounted to 35 mg/ml.

2.2. Cytochrome P450 reactor unit

The online CYP bioreactor coupled to SPE–HPLC can be divided in two distinct parts namely a CYP reactor unit and a chromatographic unit (Fig. 1A). The final version of the CYP reactor unit consisted of a Knauer K-500 HPLC pump, a VICI Jour six-way dead-end switch valve and a VICI Jour 2-position 6-port switch valve. Both valves were made of N60 metal alloy and were electronically actuated. Microsomes, NADPH and wash solutions were kept on ice and delivered by hydraulic driven syringes called superloops with volumes of 50 ml (Amersham Biosciences). Compounds were injected with a Gilson 234 autoinjector equipped with a Rheodyne six-port injection valve (injection loop, 50 μl). In reaction mode NADPH, microsomes, and substrate were mixed in a volume ratio of 1:8:1 with a total flow rate of 400 μl/min. A knitted 1/16 in. × 0.75 mm PTFE reaction coil with a volume of approximately 880 μl was used for optimal mixing of the reaction mixture. Temperature of the reaction coil was set at 37 °C with a Shimadzu CTO-10AC column oven. Furthermore, the PEEK filter unit having a filtration area of 0.5 cm^2 (Fig. 2), was manufactured in-house. In order to filter out obstructing components in liver microsomes a 0.22 μm PES membrane filter which was embedded between PEEK support material with 150 μm pores, was used. After reaction, substrate and metabolites were trapped on a SPE column in potassium phosphate buffer (*K*Pi buffer) (pH 7.4) with a flow rate of 400 μl/min.

A two-position six-port switch valve (SV2) adjusted the change between the forward flow and the back flush flow (Fig. 1B). The washing of the reaction coil and filter unit was performed by subsequent flows of Milli-Q water and 0.1 M NaOH/1% (w/v) SDS and Milli-Q water on both sides of the filter (back flush flow). Each of these flows amounted 400 μl/min. NaOH/SDS was applied by a 50 ml superloop. A six-way dead-end switch valve (SV1) adjusted changes between reaction flow, *K*Pi-buffer flow and wash flows. Table 1 summarizes the events in time during a single bioreaction run.

The different parts of the system were connected with 1/16 in. × 0.75 mm I.D. PEEK tubing. Exceptions were the

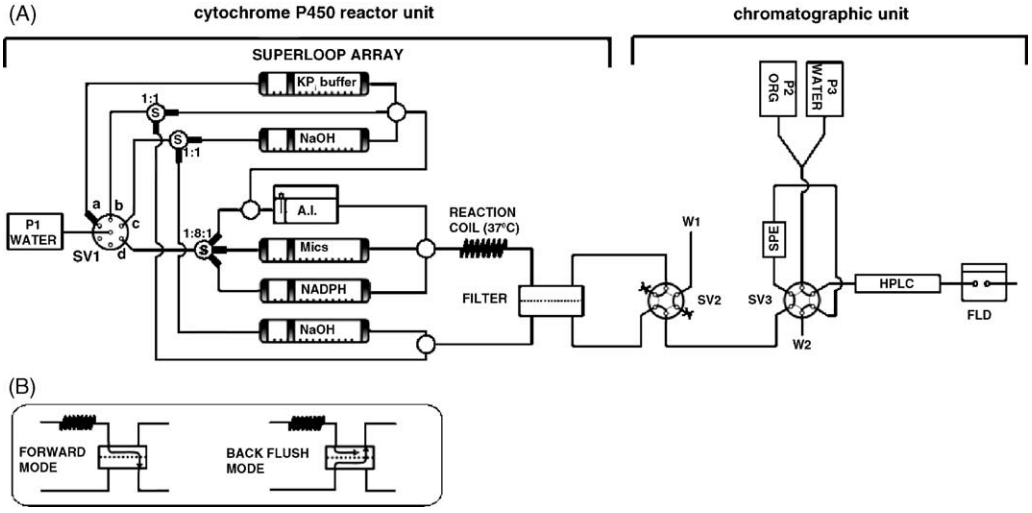


Fig. 1. (A) Schematic set up of the bioreactor. P1 and P3: water pumps, P2: organic solvent pump, SV1 to SV3: switch valves, S: flow splitter with indicated split ratios, Mics: microsomes, A.I.: auto-injector. W1 and W2: waste outlets, SPE: solid-phase extraction column, FLD: fluorescence detector. For SV2 and SV3: closed lines indicate position (**a**) and dotted lines indicate position (**b**). (B) Flow through the filter during SPE conditioning until reaction coil emptying (forward flow) and during washing of the filter (back flush flow).

connections between the SPE column, HPLC column and detector. Here 1/16 in. × 0.12 mm PEEK tubing was used. Flows were adjusted with the aid of flow restrictors as shown in Fig. 1A. Flow restrictors were made of 1/16 in. × 0.5 mm PEEK tubing with 50 μm I.D. × 375 μm O.D. fused silica inserts. These had an approximate backpressure of 60 bar at a flow rate of 400 μl/min. Flow rates, switch valves and injection procedures were regulated by Screencontrol software (Kiadis, Groningen).

2.3. Reaction conditions in the CYP reactor unit

All bioreactions were carried out at 37 °C in 50 mM *K*Pi buffer (pH = 7.4) containing 2.5 mM $MgCl_2$. NADPH concentrations in the superloop were always 400 μM, which resulted in coil concentrations of 40 μM. The ethoxyresorufin-*O*-dealkylation assay (EROD assay), which is based on the conversion of EthRES in fluorescent RES, was used. This reaction is specifically performed by CYP1A1/1A2 which are abundantly present in β-NF induced rat liver microsomes [22]. All RES and EthRES solutions were dissolved in 50% EthOH which lead to a final coil concentration of 5% EthOH. Blank injections contained 50% EthOH. Injection volume of all samples was 50 μl.

Enzyme kinetic measurements were carried out under the following conditions. First, the concentration of microsomes was determined which gave a linear production of RES over a 10 min period at the lowest EthRES concentration used in kinetic experiments. Hence, 50 μl 1.0 μM EthRES was incubated with 1.0, 0.5, 0.1 or 0.05% (v/v) microsomes and 400 μM NADPH in a ratio of 1:8:1. Subsequent measurements were performed with 0.05% microsomes. Substrate-dependent product formation was assessed in a total reaction volume of 500 μl with 40 μM NADPH, 0.04% microsomes and coil concentrations of EthRES varying in concentration from 100 nM to 10 μM. The incubations with different con-

Table 1
Table of events in the online bioreactor

Time (min)	Switch valve positions[a]			Flow rate P1(μl/min)	Filter flow	Events
	SV 1	SV 2	SV 3			
0	b	a	a	400	Forward	SPE conditioning
2	d	a	a	400	Forward	Injection of compounds
4	d	a	a	400	Forward	Emptying of injection coil in reaction coil
6	d	a	a	0	Forward	Wait time (e.g. 5 min)
11	a	a	a	400	Forward	Reaction coil emptying with *K*Pi buffer
15	c	b	b	800	Back flush	Start SPE/HPLC run Filter wash with NaOH
20	b	b	b	800	Back flush	Filter wash with water
40						End of run

[a] As indicated in Fig. 1A.

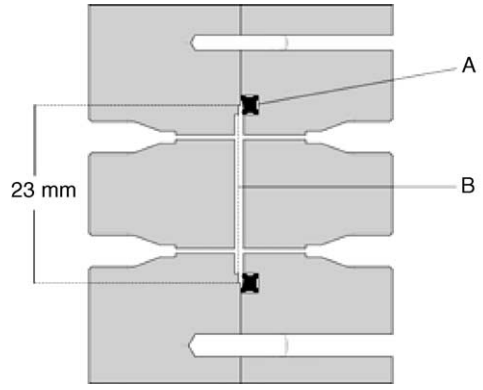


Fig. 2. Schematic representation of the filter unit. (A): Rubber seal to keep the filter and supports in place. (B): Filter and supports.

centrations of EthRES were allowed to react for 1.0, 3.0, 5.0 or 10.0 min. Different incubation times were achieved by stopping the flow through the reaction coil. Calibration of the amount of formed RES was established by injecting 50 μl of a series of RES solutions with the following concentrations: 0.1, 1.0, 3.0, 10 and 30 μM in duplo. One sequence consisted of incubation runs with one or two concentrations of EthRES each with four different incubation times and five concentration runs with RES in duplo. This sequence was repeated until each concentration of EthRES was evaluated. After each sequence, microsomes and NADPH were refreshed. For repeatability measurements, two succeeding reaction runs with 3 μM of EthRES or RES at a microsome concentration of 0.8% were carried out. In one reaction run EthRES was allowed to react for 5 min. Subsequently, the metabolites formed were trapped on the SPE unit and separated by HPLC. This was repeated four times with intervals of 3.5 h on four different days.

2.4. Enzymatic batch assays

A standard enzymatic batch assay was performed in order to compare the EthRES conversion in the CYP bioreactor [23]. The reaction conditions in the incubation mixture with respect to buffer, amounts of microsomes and NADPH were the same as coil concentrations in enzyme kinetic measurements in the reactor. The formation of RES was determined with a calibration curve of RES. The RES signal was monitored over a period of 10 min with a thermostated (37 °C) Shimadzu RF-5001PC spectrofluorophotometer ($\lambda_{excitation}$ = 530 nm; $\lambda_{emission}$ = 586 nm). Signals were recorded and slopes were calculated with accompanying RF-5001PC software.

2.5. SPE column packing

SPE columns were packed under the following conditions. A suspension of SPE material in acetone was applied under under-pressure in a stainless steel column (1.0 cm length × 3.0 mm I.D.) with one side closed by a 0.2 μm stainless steel screen. After filling, the column was sealed with a second, identical screen.

2.6. SPE evaluation

Breakthrough times and volumes were determined in order to validate the following SPE materials, i.e. 40 μm C_8, 40 μm C_{18} standard and 40 μm C_{18} PolarPlus Bakerbond and 10 μm Luna C_{18}(2). The substrate concentration was 10 μM whereas the microsome concentration was 0.8%. Breakthrough time (t_b) is defined as the difference in time between the start of the retained signal of the compounds (t_r), caused by the SPE material, and the start of the unretained signal (t_0). In order to determine t_0 and the time necessary for complete emptying of the reaction coil (t_e), the SPE column was taken out of the system and an Agilent 1100 series fluorescence detector ($\lambda_{excitation}$ = 530 nm; $\lambda_{emission}$ = 586 nm; further referred to as FLD) was connected to W2 (Fig. 1A). The extent of the unretained fluorescent signal of substrate and products was also verified in this way. The difference between t_e and t_0 is defined as net coil emptying time (t_{net}). Breakthrough times ($t_b = t_r - t_0$) of substrate and products of the EROD assay were assessed for different materials. By multiplying these times with the flow rate (400 μl/min), breakthrough volumes (V_b) could be calculated. Trapping efficiency (E%) of the SPE materials was determined by integration of area under the curves and calculated as follows:

$$E\% = \frac{\left(\int_{t_0}^{t_a} S_U \mathrm{d}t - \int_{t_a}^{t_e} S_R \mathrm{d}t\right)}{\left(\int_{t_0}^{t_e} S_U \mathrm{d}t\right)} 100\% \quad (1)$$

where S_R is the fluorescent signal of the retained compounds and S_U that of the unretained compounds, t_a is defined as the time where S_U equals S_R.

Substrates and products were washed on the SPE column with 40 mM *K*Pi application buffer (pH 7.4) during t_e. Trapping was also performed with Milli-Q water and buffers of pH 6.7 and 8.0. Trapped compounds were washed of the SPE column in a H_2O:MeOH gradient: in 5 min from 5% MeOH to 99% MeOH, 5 min constant at 99% and back to 5% in 0.5 min. Signals of compounds were detected with a second FLD. The SPE column was thermostated at 22 °C in a Shimadzu CTO-10AC column oven.

2.7. Chromatography

In application mode, compounds are brought from the reaction coil onto the SPE column, while in elution mode, compounds are eluted from the SPE and brought on the HPLC. A VICI Jour two-position six-port switch valve (SV3) was used to switch between these modes. Two Knauer K-500 HPLC pumps build the following H_2O:MeOH gradient: 6.0 min 5% MeOH, 12.0 min gradient to 99%, constant for

7.0 min at 99% and back to 5% in 0.5 min. An constant quantity of TEA (0.1%, v/v) was present during elution and the flow rate amounted 250 μl/min. HPLC separations were performed using a 150 mm length × 4.6 mm I.D. stainless-steel column (Intersil 5 μm ODS-2) with a 4 mm length × 4 mm I.D. guard column (Phenomenex C_{18} ODS). The columns were thermostated at 37 °C in a Shimadzu CTO-10AC column oven. Detection of fluorescent ligands was performed with the above-mentioned FLD.

2.8. Data processing and statistics

HPLC peaks were integrated with ACD/SpecManager 6.0 (Advanced Chemistry Development, Toronto, Canada). The drift in product formation and RES control signal in validation experiments was calculated by linear regression in GraphPath Prism 3.0 (GraphPad Software, San Diego, CA, USA). K_m and V_{max} values were derived from Hanes–Woolf plots which were obtained after transforming the Michaelis–Menten plot in GraphPath Prism 3.0.

3. Results and discussion

The aim of this study was the development and validation of a CYP-based bioreactor, coupled online to SPE and HPLC. The final construction of the newly designed analytical method is described in detail above and is shown in Fig. 1A. The CYP reactor unit consisted of superloops which supplied CYP-containing rat liver microsomes, NADPH and washing solutions. Furthermore, a filter unit was introduced to filter out substances which could obstruct the chromatographic unit. After SPE optimization the chromatographic unit was coupled to the CYP reactor unit by a switch valve system. The repeatability of the analytical method was subsequently determined by means of intra- and inter-day measurements. Finally, enzyme kinetic experiments of the EROD reaction were carried out in order to validate the method. With this setup for automated, online generation and separation of metabolites, a novel concept for metabolic profiling is introduced. Below, each of the validation steps is discussed briefly.

3.1. SPE selection

SPE was required in order to reconcentrate substrate and metabolites formed in the bioreactor unit. Therefore, various SPE supports were validated in reaction mode, which implies that breakthrough times of substrate and metabolic products were examined simultaneously. In this way, it was possible to examine influences on the SPE material of components in the microsomal fraction, which were not retained by the PES filter. Another advantage is that altering coil emptying times, mentioned above, could be taken in account as well. Retention properties of SPE material should preferably be such that t_b is larger than t_{net} [24]. In this way the complete coil contents will be trapped on the SPE column. Although all four tested SPE materials (Table 2) had lower a t_b than t_{net}, RES was trapped more than 99% on both 40 μm C_{18} PolarPlus, Bakerbond and 10 μm Luna C_{18}(2) materials. While Luna material showed good trapping capabilities it tended to clog under reaction conditions with 0.8% (v/v) microsomes. This effect is probably due to its small particle diameter (10 μm versus 40 μm of Bakerbond material), which causes proteins and other substances, that were not filtered out of the solutions, to clog the SPE column. With the other SPE materials, namely Bakerbond C_{18} PolarPlus, C_{18} Standard and C_8, the best trapping occurred with application buffers equal or higher then pH 7.4. Under these conditions, interactions between the product RES and hydrophilic, free OH-groups on the silica and hydrophobic interactions of the carbon moieties on the silica were optimal for adequate retention. Bakerbond C_8 and C_{18} Standard material caused insufficient retention of RES as shown in Table 2. The hydrophobic, non-ionized character of the substrate EthRES made that this compound was adequately trapped under every tested pH condition. C_{18} PolarPlus combined with a buffer of pH 7.4 was finally used for trapping of substrates and metabolites. When chromatograms, obtained with and without SPE, were compared, peak broadening was not observed with C_{18} PolarPlus SPE material.

Table 2
Properties of tested SPE materials

Material	Particle size (μm)[a]	Pore size (Å)[a]	Carbon load (%)[a]	End capping	t_b (min)[b]	V_b (μl)	E (%)
Luna C_{18}(2)	10	100	13	Y	5.2	2080[c]	99.6[c]
C_{18} PolarPlus	40	60	18	N	4.0	1600[d]	99.1[d]
C_{18} Standard	40	60	19	Y	2.8	1120[d]	74.6[d]
C_8	40	60	15	Y	1.9	760[d]	52.8[d]
Intersil ODS-2 (column)	5	150	19	Y			

[a] Material properties as indicated by manufacturer.
[b] t_{net} = 5.5 min (total emptying time of the reaction coil).
[c] Trapping was performed with Milli-Q water.
[d] Trapping was performed at pH 7.4.

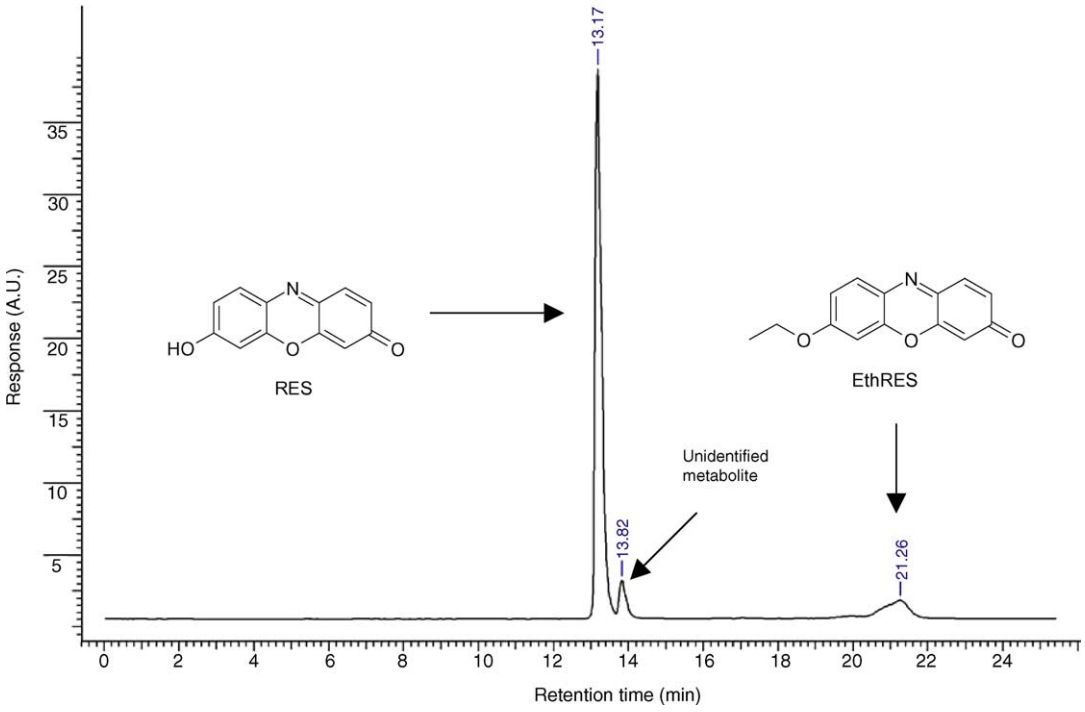


Fig. 3. Fluorescence detection chromatogram of EthRES (at 21.3 min), RES (at 13.17 min) and the unidentified metabolite (at13.82 min) after 3 min incubation. Coil concentrations were 3.0 μM EthRES, 40 μM NADPH and 0.04% rat liver microsomes.

3.2. Validation of the SPE–HPLC coupled CYP bioreactor

The analytical method was validated by measuring repeatability of product formation using the EROD assay which was determined with HPLC. The retention time of RES amounted 13.2 min and that of EthRES 21.3 min (Fig. 3). Linear regression analysis of these data showed a significant ($P < 0.01$) linear decrease in RES product formation of 16% over 11 h (Fig. 4). It is known that CYPs may loose 90% of their catalytic activity when stored at 25 °C [25], which is approximately 25 °C higher than storage temperatures used in these studies. Slopes and intercepts of linear regression curves of four intra-day measurements were overlapping within a 95% confidence interval. For inter-day EthRES product formation measurements a CV of 9.1% was calculated.

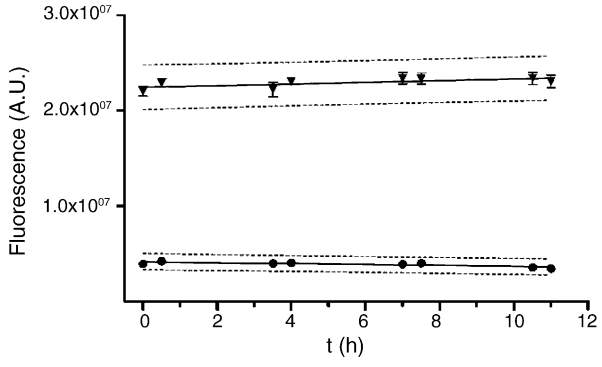
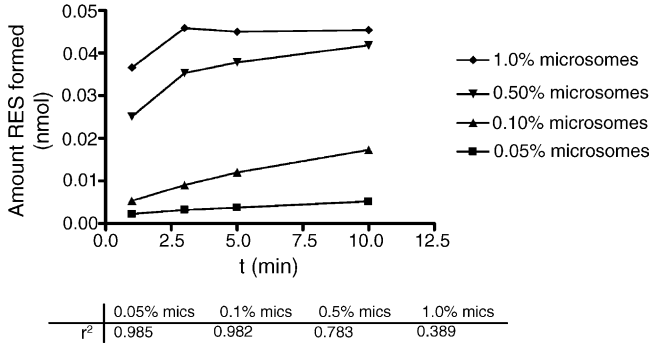


Fig. 4. Repeatability of the online bioreactor. Fluorescent RES signal due to product formation ((●), $n = 4$) and RES control signal ((▼), $n = 4$) were monitored over an 11 h period (intra-day measurements). Mean values of four intra-day measurements were used. Dotted lines indicate 95% confidence intervals.

To determine if the observed decrease (Fig. 4) in EthRES product signal was caused by obstruction of the PES filter, the product RES was injected ($n = 2$) after EthRES injections ($n = 2$). This injection sequence was repeated 4 times. According to linear regression analysis, the extent of this RES control signal did not vary significantly over this period ($P > 0.5$). Furthermore, slopes and intercepts of four intra-day sequences were again similar within a 95% confidence interval. Therefore, the small decrease in product signal is probably caused by slow degradation of microsomal CYP in the superloops. The CV of the intra-day RES control measurements amounted 3.4%, while the CV for the inter-day measurements was 4.9%. All the calculated CV values are well below the current criteria for biological method validation, i.e. ≤15% [26].

Interestingly, besides peaks of RES and EthRES, HPLC chromatograms showed a third peak, at 13.8 min (Fig. 3). Because blank injections gave no signal and because RES and EthRES gave single peaks at the respective retention times, it is assumed that this third peak is a second metabolite of EthRES. The nature and extent of formation of this metabolite needs to be determined.



	0.05% mics	0.1% mics	0.5% mics	1.0% mics
r^2	0.985	0.982	0.783	0.389

Fig. 5. Microsome concentration dependant linearity of RES production in time with a reaction coil concentration of 0.1 μM EthRES. The r^2 values of the curves are depicted in the table below the graph.

3.3. Optimization of conditions for enzyme kinetic measurements

In order to calculate reliable enzyme kinetics parameters it is necessary to have a linear relationship between substrate concentration and product formation in time. For this, microsome concentrations were varied from 0.05 to 1.0%. The substrate coil concentration was 0.1 μM EthRES, which was the lowest concentration used in enzyme kinetic measurements. A microsome concentration of 0.05% was sufficient to have a linear response (Fig. 5). With this microsome concentration all kinetic measurements were performed. For all EthRES concentrations used in enzyme kinetic experiments all slopes were linear with r^2 varying from 0.985 to 0.997. The non-linear response at higher microsome concentrations are caused by substrate depletion.

3.4. Enzyme kinetic measurements

Kinetic parameters of the EROD reaction, catalysed by the β-NF induced rat liver microsomes, were established with the CYP bioreactor and compared with values obtained by standard EROD batch assays. The product formation in the CYP bioreactor was measured by assessing RES production at four different time points (i.e. 1, 3, 5 and 10 min) with EthRES concentrations varying from 1 to 100 μM. With this data, the rate of product formation could be established. These rates were used for the online determination of K_m and V_{max} values. Typical Michaelis–Menten and Hanes–Woolf plots of both batch and online assays are shown in Fig. 6. Batch assays showed a K_m of 0.66 ± 0.08 μM and a V_{max} of 6.4 ± 0.2 nmol/min/mg protein ($n = 2$). K_m and V_{max} values obtained with the online CYP bioreactor amounted, respectively 2.72 ± 0.46 μM and 7.9 ± 0.5 nmol/min/mg protein ($n = 2$), which is in relatively good accordance with the corresponding parameters obtained with batch assays. The discrepancy between the K_m values obtained in batch and the bioreactor is probably caused by a wrong assumption of the reaction volume in the reaction coil. Because of diffusion, the reaction volume in the coil is probably higher

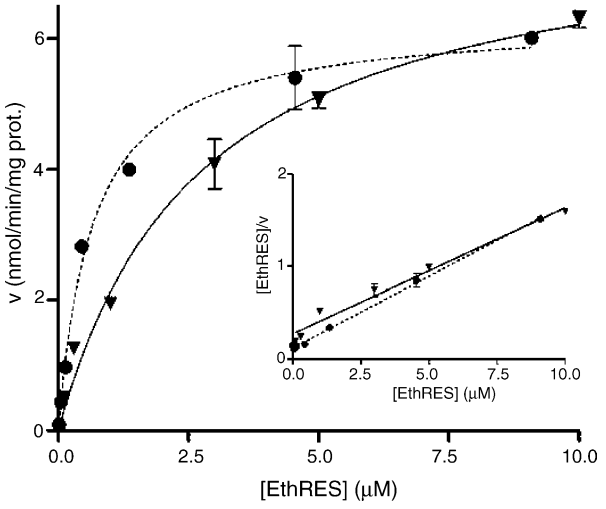


Fig. 6. Michaelis–Menten plots of the EROD assay reaction in batch (●) and online bioreactor (▼) setup. K_m and V_{max} values were derived from the Hanes–Woolf plots (inset).

than 0.5 ml. Literature values for β-NF induced rat liver microsomes are also in the same order of magnitude (e.g. $K_m = 0.99$ μM and $V_{max} = 33.96$ nmol/min/mg protein) [27]. The difference in V_{max} between bioreactor and literature can be explained by different ratios of CYPs per mg protein in the microsomes since V_{max} is calculated with the total amount of protein. The differences in kinetic parameters between bioreactor and batch or literature are acceptable for practical use. The unknown, second metabolite seen in Fig. 3 followed Michaelis–Menten kinetics as well. Furthermore, calibration curves of RES were linear in the concentration ranges used both for the CYP bioreactor ($R^2 = 0.989$) and batch assays ($R^2 = 0.992$). Based on comparison of enzyme kinetic parameters the system can be considered as an efficient CYP bioreactor.

4. Conclusions

A novel online CYP bioreactor coupled to SPE–HPLC was developed and validated. The designed analytical method provides a tool for efficient enzymatic conversion of substrates and automated, online trapping and separation of substrates and formed products. After each bioreaction-separation run, the system can be cleaned in order to remove remnants of the previous run. For validation, repeatability and enzyme kinetic experiments were carried out. Inter- and intra-day measurements showed that the present online CYP bioreactor can effectively perform enzymatic reactions, that the metabolites formed can be trapped efficiently and online separated with HPLC in a automated and repeatable way. In addition, enzyme kinetic parameters could be determined online and they were found to be comparable to those determined with a standard enzymatic batch assay. The metabolic activity of CYP was linearly decreased with 16% during 11 h

of operation. This setup makes it possible to circumvent laborious manual operations such as incubation, extraction and sample cleanup.

The present online CYP bioreactor, coupled to SPE and gradient HPLC is potentially applicable for bioaffinity screening with high resolution screening (HRS) techniques [28–30], for the assessment of metabolic profiles and metabolic stability of compounds as well as for prodrug activation, all being important issues in drug discovery and development. This CYP-based bioreactor may thus become a novel tool in drug discovery and development as well as in safety assessment research.

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