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Letter

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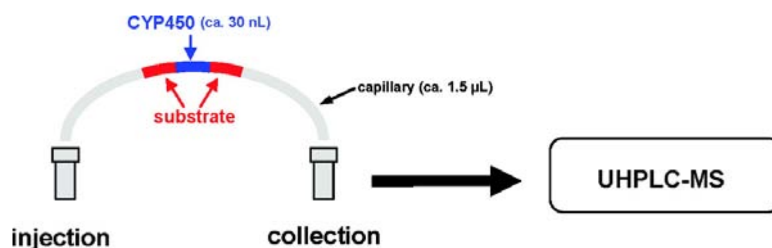

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Development of an In-Capillary Approach to Nanoscale Automated in Vitro Cytochromes P450 Assays

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Abstract: A method to perform nanoscale automated CYP450-based drug metabolism studies using a capillary as a reaction vessel is described. In-capillary assays consumed only ~30 nL of recombinant human CYP450 solution. Ultrafast analysis of substrates and metabolites was achieved off-line by ultrahigh-pressure liquid chromatography coupled to mass spectrometry. This approach was successfully applied to qualitative metabolism studies of six major CYP450 isozymes and CYP2D6 inhibition experiments.

Studying the oxidative metabolism of new chemical entities (NCEs[a]) mediated by cytochrome P450 (CYP450) isozymes is an important process of the drug discovery phase.[1,2] In this stage, in vitro CYP450 assays represent a common approach to such investigations. To determine its metabolic stability in presence of these enzymes and identify the formed metabolites, the compound of interest is generally incubated with human liver microsomes (HLM) or recombinantly expressed human CYP450 isozymes (rCYP450). Although higher in cost than HLM, using rCYP450 is advantageous because the role of individual isozymes in the oxidative conversion of a given compound can be determined separately. For CYP450-based drug−drug interactions (DDIs) studies, enzyme inhibition experiments are performed evaluating the effect of the compound of interest on CYP450(s) activity. To monitor this enzymatic activity, fluorogenic or more isozyzme-specific and clinically relevant CYP450 probe substrates are employed.[3,4] For all these in vitro CYP450 assays, detection of substrates (e.g., a NCE) and/or metabolites is generally achieved by the use of liquid chromatography (LC) coupled to various detectors (e.g., UV/vis, fluorescence, and mass spectrometry (MS))[5,6] or without separation by fluorescence spectrophotometry.[7,8] The CYP450 assays can be automated using 96 well-plates and robotic systems.[9,10] However, they are generally performed in an incubation volume of 100−500 μL, with a high CYP450 consumption (several microliters per incubation). Since in drug discovery the number NCEs is high, the classical in vitro approaches to evaluate their metabolic properties are therefore not cost-effective, especially if rCYP450 isozymes are employed.

Two strategies to reduce CYP450 enzyme consumption and automate the assays have been recently developed in our group. The first has already been published[11] and consists of immobilizing rCYP450 isozymes on a monolithic support. Automation of the drug metabolism studies is achieved by inserting the CYP450-based immobilized enzyme reactor (IMER) into a LC−MS system. To date, such an IMER can be reused for about 15 experiments, reducing CYP450 enzyme consumption.

In parallel, a second strategy for cost-effective and automated CYP450-based drug metabolism studies has been developed and is presented in this Letter. It uses a fresh CYP450 aliquot for each incubation but drastically reduces enzyme consumption compared to standard in vitro experiments. This is achieved by carrying out the CYP450 assays in a fused-silica capillary of a few microliters volume. Bao and Regnier proposed a similar approach using the capillary electrophoresis (CE) technique to automate the enzymatic assays.[12] In that strategy, known as electrophoretically mediated microanalysis (EMMA), the enzymatic assays are coupled with the subsequent electrophoretic separation and detection of analytes within the same capillary.[13] EMMA has been recently employed by Zhang et al. to perform CYP3A4-based kinetic studies detecting NADP formation by UV as a measure of enzymatic activity.[14] Their in-capillary approach necessitates preliminary CE experiments to determine suitable electrophoretic conditions for the mixing of the reactants and analytes separation. Furthermore, the online UV detection does not allow any identification (e.g., chemical structure) of the formed metabolite(s) and lacks sensitivity. All these limitations render the use of EMMA complex for CYP450-based drug metabolism studies particularly when dealing with numerous compounds.

In this work, a novel method for performing in-capillary CYP450 assays is described. This approach allows automation of the assays and consumes few nanoliters of CYP450 solution (~30 nL), i.e., at least around 100−500 times less than classical in vitro assays. In contrast to the method proposed by Zhang et al., in this in-capillary approach no electric field is applied to mix the reactants or to separate substrate and/or metabolites within the capillary. Moreover, with the proposed method, analytes detection is not achieved online by UV, but the sample resulting from the in-capillary assay is collected for subsequent off-line analysis. The decoupling of the in-capillary enzymatic assay from the analysis is expected to offer various advantages. First, since the capillary is used as a reaction vessel only and mixing of the reactants is not achieved electrophoretically, direct transfer of classical in vitro incubation conditions (type, pH, and ionic strength of the incubation buffer, substrate and enzyme concentrations) to the in-capillary experiments should be possible for any compound and CYP450 isozyme. No preliminary experiments to the enzymatic assay are thus required. Second, off-line analysis of substrate and metabolite(s) should be achievable by LC−MS, the gold standard technique in drug metabolism studies,[15,16] allowing separation of analytes and direct metabolites identification, characterization, and quantification. To test these hypotheses, our approach was applied to qualitative CYP450-based drug metabolism studies with the most abundant CYP450 isozymes and to inhibition experiments with CYP2D6. Ultrahigh-pressure liquid chromatography (UHPLC) coupled to MS was employed to achieve ultrafast off-line analysis (i.e., less than 1 min).[17,18]

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[a] Abbreviations: CE, capillary electrophoresis; CYP450, cytochrome P450; DDIs, drug−drug interactions; EMMA, electrophoretically mediated microanalysis; ESI, electrospray ionization; HLM, human liver microsomes; IMER, immobilized enzyme reactor; IC_{50}, half-maximal inhibitory concentration; K_i, inhibition constant; K_m, Michaelis−Menten constant; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NCE, new chemical entity; rCYP450, recombinant human CYP450 isozymes; SRM, selected reaction monitoring; TOF-MS, time-of-flight mass spectrometry; UHPLC, ultrahigh-pressure liquid chromatography.

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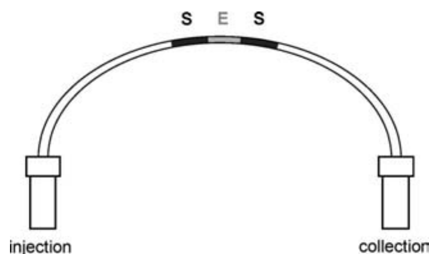

Figure 1. In-capillary CYP450 assays: injections of the reactants in the substrate−enzyme−substrate sequence (S−E−S).

All experiments presented below were performed in a fused-silica capillary of 1.4 μL (75 μm i.d., 32.5 cm length) coated with polyvinyl alcohol to reduce interactions between reactants and the capillary wall.[19] The capillary temperature was set at 37 °C. Incubation buffer was HEPES, 50 mM, set at pH 7.4. The cofactor NADPH was contained in the substrate(s) solution at 2.5 mM. Several strategies concerning the introduction of the reagents (substrate and CYP450 isozymes) into the capillary were investigated to optimize the enzymatic reaction and will be presented elsewhere.

If not stated otherwise, in-capillary experiments were performed as follows: after the capillary was conditioned with the incubation buffer, an hydrodynamic injection was carried out to introduce CYP450 isozyme and substrate in the substrate−enzyme−substrate sequence (50 mbar for 6 s for each reactant, corresponding to ~7% of the capillary volume). Reactants were flushed in the capillary with the incubation buffer and incubated for 2 min (Figure 1). Then the capillary content was flushed with water into a vial containing an acetonitrile/water mixture (1:1 v/v) to stop the enzymatic reaction, and the capillary was rinsed with the incubation buffer to restore initial conditions. The solution containing the incubation mixture was injected without pretreatment into a UHPLC system for rapid substrate and metabolites separation using a generic 5−95% acetonitrile gradient in an aqueous buffer solution set at pH 9.0. Depending on the purpose of the performed studies, UHPLC was coupled to electrospray ionization (ESI) tandem mass spectrometry (MS/MS) or to ESI-time-of-flight (TOF) MS.

The first goal of this work was to demonstrate that the proposed in-capillary method could be applied to any CYP450 isozyme to perform qualitative automated metabolic studies. For this purpose, six major recombinant human isozymes, involved in the oxidative conversion of ~90% of marketed drugs, were selected: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.[2] For each isozyme, the in-capillary assay was carried out using a probe substrate with a known metabolic pathway. Incubations were performed as described above, injecting substrates at 100 μM. Off-line metabolite analysis was achieved by UHPLC−MS/MS working in the selected reaction monitoring (SRM) mode to detect the expected major metabolites with high sensitivity and selectivity.

As shown in Figure 2, the in-capillary assays with the six CYP450 isozymes yielded the expected major metabolites of the selected substrates. An incubation time of 2 min and a substrate concentration of 100 μM resulted in satisfactory conditions for metabolite detection by MS/MS in the presence of unreacted substrate. To ensure that the observed probe substrate conversions were CYP450-catalyzed, control incubations were carried out in the absence of NADPH. In this case, no metabolites were detected (data not shown). The whole procedure (conditioning, injection, in-capillary enzymatic reaction, and off-line analysis) was completed in less than 10 min.

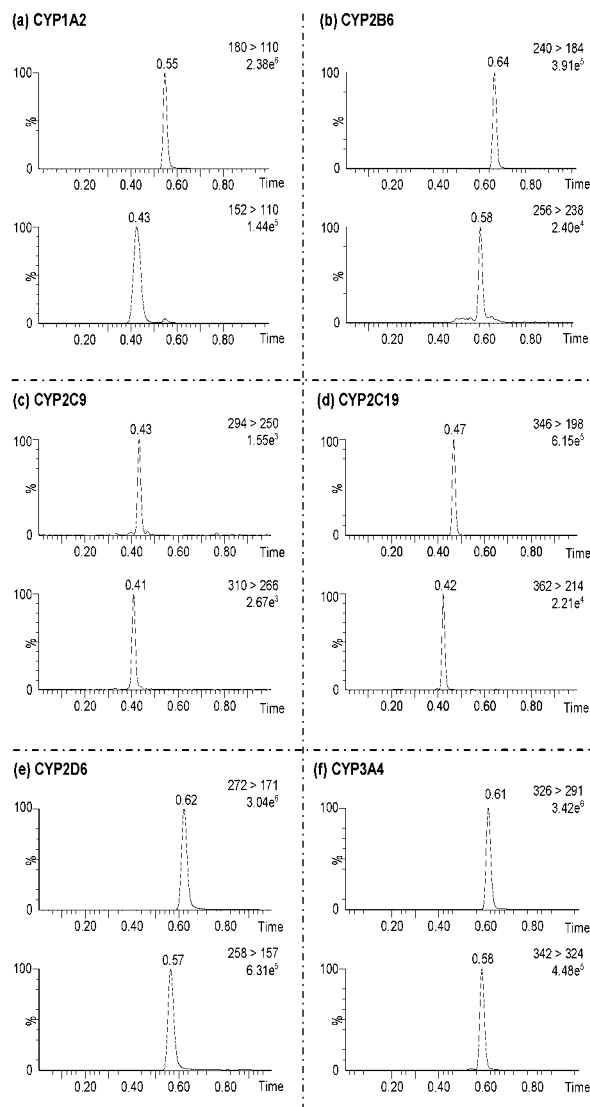

Figure 2. SRM chromatograms for the selected in-capillary CYP450 reactions: (a) phenacetin O-deethylation by CYP1A2; (b) bupropion hydroxylation by CYP2B6; (c) diclofenac 4′-hydroxylation by CYP2C9; (d) omeprazole 5′-hydroxylation by CYP2C19; (e) dextromethorphan O-demethylation by CYP2D6; (f) midazolam 1′-hydroxylation by CYP3A4. All analytes, except diclofenac and 4′-hydroxydiclofenac, were detected in the positive ESI mode.

The second goal of this study was to demonstrate that the proposed in-capillary method was compatible with an off-line analysis by UHPLC−TOF-MS. This compatibility is important because of the increasing use of TOF or hybrid quadrupole-TOF mass analyzers for nontargeted analysis of metabolites (e.g., metabolite identification studies).[20,21] For this purpose, further in-capillary experiments were performed with CYP2D6 and dextromethorphan. CYP2D6 was selected because of its involvement in the metabolism of clinically important drugs (e.g., opioids, β-blockers, and antidepressants) and its polymorphic activity, first described for the metabolism of debrisoquine.[22,23] Assays were carried out as described above using dextromethorphan at 100 μM. Control incubations were performed in the absence of the substrate and NADPH. After the assays, the content of the outlet vials was injected into an UHPLC−TOF-MS system for exact mass measurements of the formed metabolite(s).

The chromatogram obtained with the incubation was compared to the one of the control allowing identification of

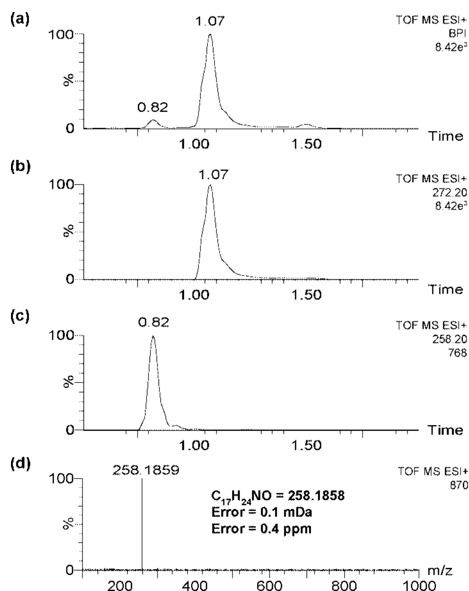

Figure 3. (a) Subtracted base peak chromatogram, extracted ion chromatograms for (b) dextromethorphan (t_R = 1.07 min) and (c) dextrorphan (t_R = 0.82 min) and (d) exact mass spectrum of dextrorphan. In comparison to UHPLC−MS/MS analysis, in this case the gradient time was extended to meet TOF-MS sampling rate criteria.

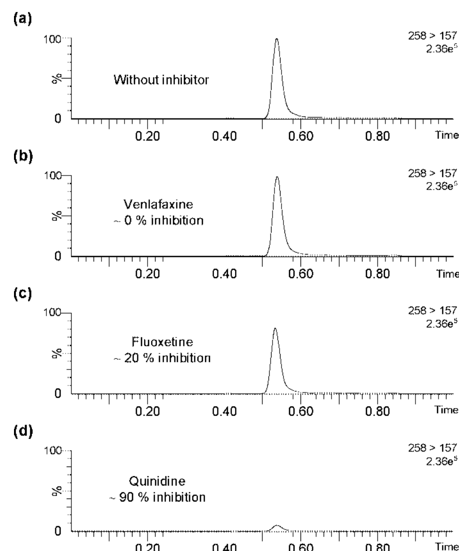

Figure 4. SRM chromatograms of dextrorphan for in-capillary CYP2D6 inhibition studies (a) in the absence of inhibitor and in the presence of 5 μM (b) venlafaxine, (c) fluoxetine, and (d) quinidine.

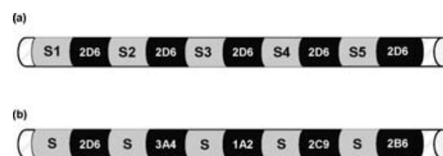

Figure 5. Proposed strategies for the multizones approach using a single capillary: (a) multisubstrate (S1−S5) approach with a single CYP450 isozyme (e.g., CYP2D6) and (b) multi-isozyme approach with a single putative substrate S.

dextrorphan and dextromethorphan peaks (Figure 3). The in-capillary formed metabolite dextrorphan was correctly identified by its elemental composition because of the exact mass measurement. This shows the potential of the presented in-capillary method combined with off-line UHPLC-TOF-MS analysis for confident metabolite identification of compounds with unknown metabolic fate.

Finally, the applicability of the in-capillary method to CYP450 inhibition studies was evaluated. To this end, the proposed approach was tested for its ability to correctly rank three known competitive CYP2D6 inhibitors (quinidine, fluoxetine, and venlafaxine) according to their inhibition potency. The O-demethylation of dextromethorphan into dextrorphan was employed as catalytic marker of CYP2D6 activity. Assays were performed as described above injecting a solution containing dextromethorphan and the selected inhibitor at 5 μM each. Inhibition was estimated by comparing the enzymatic activity in the presence and absence of the inhibitor (100% enzymatic activity). As shown in Figure 4, venlafaxine at 5 μM had no significant effect on CYP2D6 activity while fluoxetine weakly inhibited this isozyme (~20%). With quinidine, known to be a very potent CYP2D6 inhibitor, a strong reduction of CYP2D6 activity (~90%) was observed. The ranking of the three inhibitors obtained with the in-capillary method was in agreement with the literature.[24,25] Further assays were performed with quinidine at two additional concentrations, namely, 0.1 and 0.5 μM, to investigate the dependence of CYP2D6 activity on the inhibitor concentration. In agreement with classical in vitro inhibition experiments, a dose-dependent behavior was observed (data not shown).

In conclusion, the proposed in-capillary approach was found suitable for performing automated CYP450 assays for studying drug metabolism and DDIs. The results presented above are comparable, from a qualitative point of view, to those obtained with classical in vitro drug metabolism studies (e.g., production of expected metabolites, ranking of CYP2D6 inhibitors). Further investigations are in progress to evaluate the ability of the method to provide quantitative and reproducible results in rCYP450 kinetics and inhibition studies (e.g., determination of K_m, IC_{50}, and K_i values). A main advantage of the proposed in-capillary assays is the very low enzyme consumption with respect to classical in vitro approaches. In contrast to EMMA, with the proposed strategy the capillary is used as a reaction vessel only and the metabolite(s) analysis is performed off-line. As shown by the drug metabolism studies presented here, this concept allows the direct transfer of classical incubation conditions to the in-capillary assays and can be applied to any compound and CYP450 isozyme. Furthermore, ultrafast separation of analytes with a generic chromatographic method and metabolite(s) identification and characterization have been achieved by UHPLC coupled to appropriate MS systems.

In this work, all experiments were performed in a single capillary. To increase the productivity of this strategy, a zone of a given CYP450 isozyme followed by one of a putative substrate can be injected repeatedly into the capillary (Figure 5) allowing the testing of (i) multiple putative substrates with one specific isozyme (multisubstrate approach) and (ii) the metabolic fate of a given compound in the presence of multiple CYP450 isozymes (multi-isozyme approach). Finally, a very promising high-throughput strategy to study the CYP450-based metabolism of a large number of molecules in drug discovery is in development and consists of the simultaneous usage of multiple capillaries in parallel.

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