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Hepatocyte-based flow analytical bioreactor for *online* xenobiotics metabolism bioprediction

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Abstract

The research for new in vitro screening tools for predictive metabolic profiling of drug candidates is of major interest in the pharmaceutical field. The main motivation is to avoid late rejection in drug development and to deliver safer drugs to the market. Thanks to the superparamagnetic properties of iron oxide nanoparticles, a flow bioreactor has been developed which is able to perform xenobiotic metabolism studies. The selected cell line (HepaRG) maintained its metabolic competencies once iron oxide nanoparticles were internalized. Based on magnetically trapped cells in a homemade immobilization chamber, through which a flow of circulating phase was injected to transport nutrients and/or the studied xenobiotic, *off-line* and *online* (when coupled to a high-performance liquid chromatography chain) metabolic assays were developed using diclofenac as a reference compound. The diclofenac demonstrated a similar metabolization profile chromatogram, both with the newly developed setup and with the control situation. Highly versatile, this pioneering and innovative instrumental design paves the way for a new approach in predictive metabolism studies.

Keywords

Analytical bioreactor, HepaRG cells, magnetically labeled cells, metabolization test, hepatocytes

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Introduction

A thorough literature review reveals a large panel of biomedical applications involving iron oxide nanoparticles (IONPs).[1–4] Among them, the most usual application is their use as a contrast agent to generate high-quality data for magnetic resonance imaging (MRI).[5,6] Thanks to the possibility of anchoring specific entities (functionalized groups, peptides, aptamers, etc.) onto their surface, IONPs are able to target specific cells, allowing the development of powerful tools for medical and diagnostic imaging. The specific IONP labeling is widely detailed in the literature for MRI tracking,[7,8] but nowadays, research also exploits this ability for targeted drug delivery,[9–11] targeted hyperthermia,[12] and magnetic separation.[6]

Recently, new innovative and original IONP applications have also been reported for tissue engineering and tri-dimensional cell cultures.[13–16] Based on the magnetic properties of iron oxide, magnetically labeled cells of interest are controlled and driven by an external magnetic field.[17,18] Such research has been used for cardiovascular tissue construction,[19] tubular structures,[20] and 3D culture systems of the hepatocyte cell line.[21]

Researchers in the field of analytical chemistry have also introduced the use of magnetic nano (or micro) particles in their developments of new analytical tools in numerous applications. IONPs are now used in amperometric

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biosensors to allow working electrode surface modifications on which enzymes[22,23] or antibodies[24,25] are chemically linked to the IONPs' surfaces and retained on the electrode surface using magnets localized behind the electroactive surface. The advantage of this strategy is that the electrode surface is removable and easily regenerated. Electrochemical magnetic immunosensors have been used for the quantification of clinically significant biomarkers, such as ceruloplasmin.[26–28] Other analytical applications of IONPs are (i) in *online* enzymatic bioreactors in capillary electrophoresis,[29] (ii) in the development of magnetic nanoporous microparticles for analytical purposes,[30,31] and (iii) in magnetic molecular imprinted polymers.[32,33]

Currently, predictive xenobiotic metabolism studies represent a crucial key point in new drug candidate development or in assessing chemical compound toxicity present in the human environment (air, water, foodstuffs, etc.). Depending on the model, they can either detect/ identify the generated metabolites, evaluate the toxicity of the compound and/or the metabolites generated, demonstrate the inhibition or induction of enzymes, or attempt to predict in vivo pharmacokinetics.[34]

For the aforementioned scientific methodologies, miscellaneous in vitro models, or instrumental tools, are used, such as (i) microsomes,[35] (ii) genetically engineered eukaryote and prokaryote cells,[36,37] (iii) primary hepatocytes,[34,38] (iv) hepatocyte cell lines, such as HepG2 and HepaRG,[39,40] (v) liver slices, (vi) isolated and perfused liver,[41] and (vii) monitoring redox processes (such as metabolism mimicking) in electrochemical cells (EC) in cyclic voltamperometry or combination between an EC cell and mass spectrometry (MS).[42,43] Nowadays, research trends promote innovative bioreactor-based approaches for high-throughput drug screening and discovery in the pharmaceutical field. The main goal is to mimic, as closely as possible, the in vivo liver conditions.[44–47] These systems are principally focused on the culture and viability aspect of hepatocytes and tissues to create an artificial liver environment. Several of the aforementioned metabolism assessment tools analyze the generated metabolites via an *off-line* mode.

The *online* mode is widespread in analytical chemistry and covers a large panel of applications. This configuration offers several advantages: chromatographic separation coupled with selected detectors, unique run sample processing, automation, and so on.[48,49] Drug metabolization oxidative simulation through EC/MS perfectly exemplifies the trend of coupling *online* analytical characteristics and biological phenomena.

In this context, the present article introduces an innovative analytical flow bioreactor based on the magnetic immobilization of hepatocytes in a manifold, resulting in a hepatocyte "bed" able to generate metabolites. This created design is able to perform metabolism studies in *off-line* or *online* mode (when coupled with a chromatographic system).

The HepaRG cell line was selected as the hepatocyte source for this work. Since 2002, the number of publications on this cell line has been increasing constantly.[50,51] Briefly, the HepaRG cells have the particularity of expressing and maintaining a large panel of phase I and phase II enzymes[52,53] and, therefore, are considered to be a valuable alternative to the primary hepatocytes and a promising in vitro model for performing xenobiotic metabolization studies.[40,54–57]

Materials and methods

Chemicals and reagents

William's E medium (WE) without phenol red, trypsin, Dulbecco's phosphate buffered saline (D-PBS), phosphoric acid, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, ammonium formate, and diclofenac (DCF) sodium salt were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Cell culture flasks were obtained from Greiner Bio One (Wemmel, Belgium). Acetonitrile and formic acid were purchased from Biosolve BV (Valkenswaard, the Netherlands). Sunitinib was supplied from LC Laboratories (Woburn, Massachusetts, USA) and *N*-desethyl sunitinib from Biozol (Eching, Germany). All chemicals were analytical grade or higher. Ultra-pure water was obtained using a Milli-Q purification system from Merck Millipore (Overijse, Belgium).

Stock and standards solutions of reference compounds

Diclofenac salt was solubilized in purified water at 5 mM (stock solution) and filtered on a 0.22-µm polyvinylidene difluoride (PVDF) membrane (Merck Milllipore). A working solution at a final concentration of 250 µM was obtained by dilution with modified media.

Sunitinib free base was solubilized in methanol (stock solution at 2 mM). After filtration on a 0.22-µM PVDF membrane, a working solution (final concentration 20 µM) was obtained by dilution in a modified medium (methanol residual concentration 1%).

Diclofenac is the main reference substance for the experiments. Indeed, this molecule is described in the literature as a reference for targeting CYP2C9 and gives 4′-hydroxydiclofenac as a main metabolite. Moreover, its metabolism in HepaRG cells has already been reported in the literature[34,58–65]

Sunitinib was retained as a second reference substance because it is metabolized into a single primary metabolite (*N*-desethyl sunitinib).

Magnetic nanoparticles

Superparamagnetic anionic IONPs (8.4 $\pm$ 2.2 nm particle diameter) were kindly provided by the NMR and Molecular Imaging Laboratory of the General, Organic and Biomedical Chemistry Unit (Professor L Vander Elst) of the

University of Mons (Belgium). Synthesis details and characterization techniques are listed in the references.[66,67] Resulting IONPs show carboxylated functions anchored onto the surface of an iron oxide core (magnetite) leading to a global negative charge. The final concentration of iron used to perform the HepaRG cell labeling step in the experiments was 2 mM in WE without phenol red.

Cell culture

HepaRG cells were obtained from Biopredic International (Saint-Grégoire, France) and were seeded in 25 cm^2 flasks at 2.4×10^4 cells/cm^2. Cells were cultured in growth medium provided from Biopredic International corresponding to WE supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, and 5×10^{-5} M hydrocortisone hemisuccinate. After 2 weeks, the medium was changed to a differentiation medium provided by the manufacturer, corresponding to an enriched WE medium with 2% dimethylsulfoxide, for 2 more weeks. This second medium allows cell differentiation into two different cell types: biliary-like and hepatocyte-like cells.[50] The medium was renewed every 2 or 3 days. Cell culture was carried out under +37°C and 5% CO_2 atmosphere conditions.

Magnetic labeling and immobilization of HepaRG cells

After a 17-h incubation time with the selected anionic IONPs ([Fe] = 2 mM), fully differentiated HepaRG cells (between passage numbers 17 and 19) were washed twice with D-PBS and harvested. Once in suspension, trypsin was neutralized with growth medium. The cell suspension was centrifuged at 500*g* for 3 min at 4°C (with a Hettich refrigerated centrifuge (Analis, Suarlee, Belgium). The supernatant was discarded, and the cells were resuspended with WE medium without phenol red. The final cell suspension was manually injected by a syringe surrounded by an Ibidi Luer Lock Connector Female (Proxylab sprl, Beloeil, Belgium) into the silicone tubing (BioPharm Plus Masterflex® (platinum-cured), Fisher, Aalst, Belgium). A homemade compact (8 × 5 × 3 cm (length × width × height)) wooden holder was designed (see Figure 1). Its role was to gather (with ease and reproducibility) the three essential elements of the bioreactor in the same location: the labeled cells, the tubing, and the magnets. The wooden holder encompasses two movable iron bars where neodymium magnets (adhesive force 1.1 kg/magnet; from Supermagnete Webcraft GmbH, Gottmadingen, Germany) are located which allow the positioning of the magnets as close as possible to the silicone tubing. The magnetic field generated around the tubing is able to retain the magnetically labeled cells and consequently give rise to the expected hepatocyte "bed." The length of the immobilization chamber depends on the number of magnets used. The tubing has an internal diameter of 1.6 mm and a total length of 41 cm. This tubing was selected due to its gas permeability (O_2 and CO_2) and its biocompatibility.

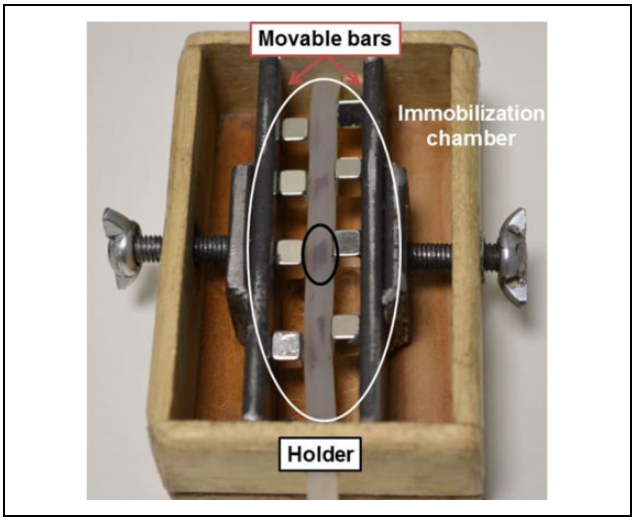

Figure 1. Picture of the immobilization chamber (8 × 5 × 3 cm). The two iron bars can be moved by screws in order to modify the distance between the magnets and the silicone tubing. Labeled HepaRG cells are magnetically stopped in front of the magnets (dark circle).

Once the cell immobilization was achieved in the immobilization chamber, a syringe pump (Harvard Apparatus, Les Ulis Cedex, France) was used to inject the working solutions through the flow bioreactor. Flow rate influence was assessed in order to determine a compromise value for the circulating phase flow which would be compatible with the metabolization test.

The approximate percentage of cell immobilization was evaluated with a Scepter 2.0 handheld automated cell counter (Merck Millipore). Cells were counted before injection and after their release from the holder. The comparison between labeled and unlabeled cells, concerning the impact following exposure to selected IONP, has been studied previously.[68]

Chromatographic methods

Samples were analyzed either with a Waters Acquity H-Class UPLC® System or with a Waters high-performance liquid chromatography (HPLC) chain (515 HPLC pump, 2996 photodiode array detector and a Rheodyne valve (Milford, Massachusetts, USA)). Both systems were controlled by the Empower® 3.0 software (Waters).

UPLC was used for different development steps as it allows faster analysis and lower solvent consumption than HPLC. However, HPLC was needed as well for the *online* setup tuning (see sections Online metabolism study and Metabolization test online) since the coupling was only achievable with the HPLC chain, because the back pressure generated with UPLC technology was too high.

Among numerous substances listed and considered by the scientific community as reference drugs for performing metabolization studies, diclofenac was selected as a reference compound. Indeed, diclofenac's metabolic pathway is widely described in literature.[55,60,61] Another pharmaceutical molecule (sunitinib) was included in this work; it belongs to the pharmacological class of tyrosine kinase inhibitors and also undergoes a cytochrome P450 metabolization.[69,70]

Diclofenac and metabolites. The chromatographic analysis was performed on an Acquity UPLC CSH Phenyl-Hexyl column (1.7 µm; 2.1 × 100 mm) at 40°C. The mobile phase was constituted of acetonitrile and water acidified with 0.1% formic acid (v/v). The percentage of acetonitrile was initially set at 43%, constantly increased up to 50% until 4.99 min and quickly reduced back to 43% at 5 min. The system was re-equilibrated for 1 min before the next injection. The total runtime was 6 min with a flow rate set at 0.5 mL/min. A diode array detector (DAD) was used and the wavelength was fixed at 275 nm.

The HPLC column used was an Atlantis® dC18 (5 µm; 4.6 × 250 mm). The mobile phase was made of 30% acetonitrile and 70% phosphate buffer 20 mM (v/v). The pH was adjusted to 7.4 with phosphoric acid with a Metrohm 827 pH meter (Herisau, Switzerland). Elution was performed following an isocratic mode set at 1.2 mL/min for 32 min. The DAD wavelength was also fixed at 275 nm.

Sunitinib. Sunitinib and *N*-desethyl sunitinib (metabolite) were separated on an Acquity UPLC BEH C18 (1.7 µm; 2.1 × 50 mm) column at 40°C. Ammonium formate 4 mM (pH adjusted to 3.2 with formic acid) and a combination of 90% acetonitrile/10% 4 mM ammonium formate (pH 3.2) were the mobile phase components. The percentage of this combination was initially set at 5% and immediately increased to 35% at 0.15 min. Thereafter, it was constantly increased to 37% until 0.8 min, linearly increased to 90% until 2.9 min, and quickly reduced to 5% between 3.02 min and 4 min. The flow rate was set at 0.4 mL/min. The total run time was 5 min (4 min for analysis and 1 min for re-equilibration). The wavelength of the DAD was fixed at 430 nm.[71]

Metabolism assays

Cells in suspension versus adherent cells in culture flasks: Metabolic activity assessment. Three different experimental conditions were tested in order to evaluate the HepaRG cell metabolization activity in suspension: the control situation (corresponding to adherent unlabeled cells), the unlabeled cells in suspension, and the labeled cells in suspension (see section Cell culture). These three conditions correspond to one independent culture, and a total of four independent cultures were used to perform this assay.

For assays with unlabeled or labeled cells in suspension, the cells were harvested and, once in suspension, trypsin was neutralized with growth medium. The cell suspensions were centrifuged at 500*g* and 4°C for 3 min. The supernatant was discarded and replaced by a 250 µM diclofenac solution (final concentration) in WE medium without phenol red for 5 h, and the cells were re-suspended in a culture flask.

For the control situation (adherent unlabeled cells), the cells were washed twice with D-PBS and incubated with 250 µM diclofenac for the same amount of time.

Each final metabolization medium was sampled and precipitated with 1/3 volume acetonitrile, centrifuged at 3300*g* for 15 min at 4°C and injected in UPLC (analysis in triplicate). Statistical analysis (Bonferroni's multiple comparison test) was carried out with GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA).

Modified culture media. Usually, complex media samples require acetonitrile precipitation before analysis in order to avoid any crystallization or column fouling of the UPLC and HPLC systems. As the aim here is to include the metabolic flow bioreactor in an *online* mode, the minimalist culture medium, able to still allow hepatocyte metabolization while being compatible with a chromatographic environment and without any sample preparation, was studied. In order to decrease the crystallization risk and to avoid the sample dilution, preliminary tests were done on two modified culture media in usual flasks: (i) WE medium without phenol red diluted with an equal volume of ultra-pure water or (ii) with D-PBS buffer.

Metabolization assays using 250 µM diclofenac as the final concentration in these two media were performed for 5 h. The resulting samples were directly analyzed by UPLC and compared to 100% WE medium (for pre-treatment method see section Diclofenac and metabolites).

Cell metabolic activity in the immobilization chamber. The assay consisted of studying the metabolization behavior of the selected compounds when they were injected into the flow injection system through the hepatocyte "bed," installed as depicted in section Cell culture. The immobilization chamber length depends on the number of neodymium magnets placed either side of the tubing. Eight cubic magnets (four each side) of 0.5 cm^3 were used for the present experiment. Metabolization assays were performed by filling the tubing with 250 µM diclofenac, or 20 µM sunitinib solution prepared with the selected modified medium (1/2 WE; 1/2 D-PBS), via the syringe pump at a flow rate of 0.4 mL/min. At this moment, the studied compounds were in contact with the HepaRG hepatocytes, and the flow was stopped in order to allow xenobiotic metabolization for 5 h for the diclofenac and 6 h for the sunitinib.

Online metabolism study. The ultimate goal was to develop an *online* metabolization tool. To reach this objective, the developed experimental protocols in sections Cell culture

and Chromatographic methods were adapted. This was done to drive generated products from the magnetically immobilized hepatocytes directly to a chromatographic system via a Rheodyne manual injection valve. Only diclofenac was tested as a reference substance and was injected into the tubing via the syringe pump at 0.4 mL/min flow rate. After 5 h of compound/hepatocyte exposure, the flow of the syringe pump was reactivated and set at 1 mL/min, the valve was switched, and the generated species continued their progress to the LC system.

Results and discussion

HepaRG cell immobilization

As shown in Figure 1, once inoculated in the silicone manifold, the magnetically labeled HepaRG cells reached the immobilization chamber in the wooden holder and were attracted, and retained, in the magnetic field created by the magnets. The two movable iron bars allowed the magnets to be as close as possible to the tubing and also allowed the cells to be released once the assay was finished.

The immobilization percentage of the magnetically labeled cells in the holder was assessed by cell counting and gave a high ratio of cell retention: $78\% \pm 9\%$ ($n = 5$).

Different mobile phase flow rates were tested with the syringe pump from 0.4 mL/min to 9.99 mL/min (maximum flow of the apparatus); none of them provoked a loss (visual aspect) of the labeled cell agglomerates into the holder (data not shown). The flow rate was fixed at 0.4 mL/min for further experiments since higher values did not bring real improvements.

The nonspecific labeling of anionic IONPs is described in the literature as a phenomenon applicable to all kinds of cells.[72] In this context, similar experiments were performed with other cell line types: epithelial intestinal cells (FHs74Int) and kidney proximal tubule cells (HK-2). They exhibited the same behavior when exposed to IONPs and comparable magnetic immobilizations in the wooden holder were observed (data not shown).

Cells in suspension: Evaluation of metabolic activity

In a first step, the impact on HepaRG cells' xenobiotic metabolization was evaluated when they were in suspension in the culture flasks. In classical tests, HepaRG cells are used as adherent cells but the producer mentions the possibility of using them in suspension for metabolism studies. Peak areas of diclofenac metabolites were extracted from chromatograms (not shown at this stage to avoid redundancy with further results) and processed by statistical analysis. Data from adherent unlabeled cells served as a control situation and were set at 100%. Results obtained from Bonferroni's multiple comparison test (Figure 2) show significant differences between the control (unlabeled adherent cells) and labeled or unlabeled cells

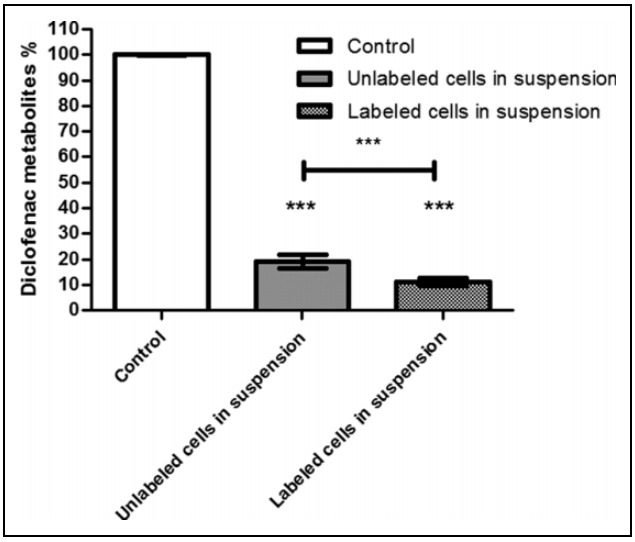

Figure 2. Histograms of diclofenac metabolites percentages for control (adherent unlabeled cells), unlabeled cells in suspension (no interaction with nanoparticles), and labeled cells in suspension (17 h incubation time with nanoparticles). Percentage of control situation was fixed at 100%. Significant differences (***, $p < 0.001$) between all tested conditions were observed using Bonferroni's multiple comparison test.

in suspension (***, $p < 0.001$). A significant difference was also observed between the unlabeled and labeled cells in suspension (***, $p < 0.001$). Compared to the 100% of the control situation, diclofenac metabolites peak areas exhibited a percentage of $19\% \pm 3\%$ and $11\% \pm 1\%$ for the unlabeled and labeled cells in suspension, respectively. The major activity decrease (nearly 80%) seems to result from the cell status itself (adherent or in suspension) and not from the incubation with nanoparticles.

As expected,[73] this outcome showed that unlabeled and labeled HepaRG cells preserve their metabolic activities in suspension. This observation is crucial, since magnetically immobilized cells within the flow bioreactor could be considered as being in a suspension status. Constant attention was paid to the major decrease in the xenobiotic metabolization activity, even though this was not considered a limiting factor for the flow bioreactor, since the trapped HepaRG cells in the flow bioreactor were still operating.

Development and evaluation of the modified medium culture

A metabolization test was carried out using a 250 µM diclofenac solution (final concentration) in each modified media. The obtained UPLC chromatograms (Figure 3) show that diclofenac and its metabolites appear in all tested media but differ in absorbance intensity. Furthermore, the peak shape was sharp and symmetrical.

Based on metabolite peak areas (Table 1), the D-PBS dilution gave higher peak areas than the ultra-pure water dilution and 100% WE medium. Interestingly, the 100%

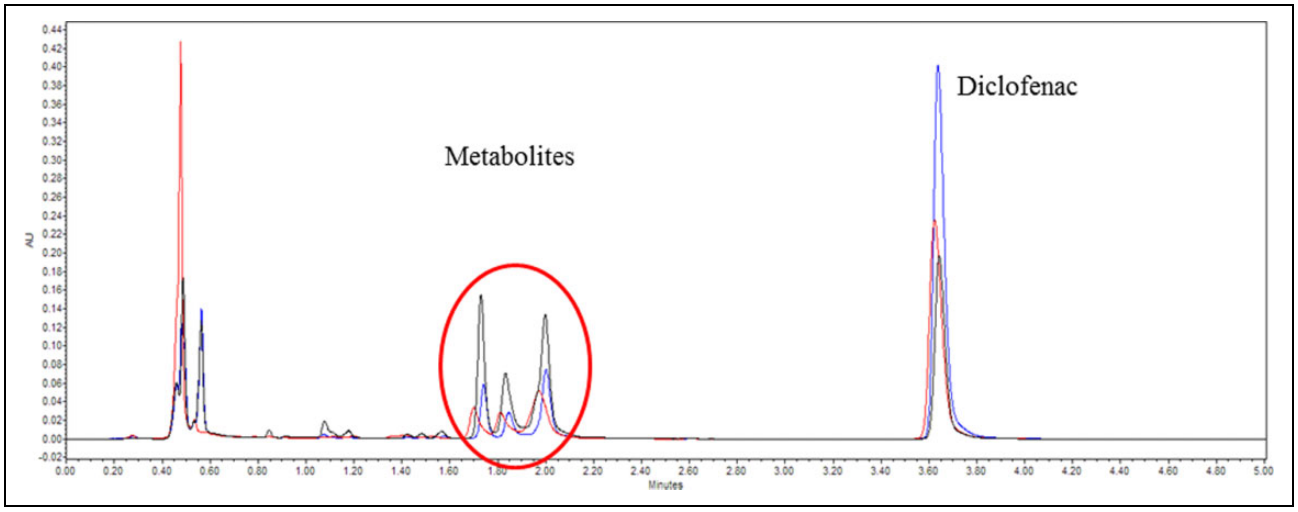

Figure 3. Obtained UPLC chromatograms after 5 h diclofenac metabolization in different modified media: WE medium without phenol red dilution (1:1) with D-PBS (in black) or water (in blue) and 100% WE medium without phenol red (in red). WE: William's E medium; D-PBS: Dulbecco's phosphate buffered saline.

Table 1. Obtained values for peak areas of DCF metabolites with respect to tested media.

Tested media	DCF metabolite peak area units
Not modified (100% WE)	436,893
WE modified with D-PBS	976,665
WE modified with water	388,936

DCF: diclofenac; WE: William's E medium; D-PBS: Dulbecco's phosphate buffered saline.

WE medium did not give the highest peak areas, likely due to the effect of the 1/3 acetonitrile dilution.

Therefore, the D-PBS dilution was selected for the further experiments.

Metabolization tests of magnetically immobilized cells

Subsequent to the DCF or sunitinib exposure to the hepatocyte "bed," the tubing content was collected and injected into UPLC-DAD. As illustrated in Figure 4(a), DCF peak and DCF metabolite peaks were detected and denote the metabolic activity of the magnetically immobilized HepaRG cells.

In the case of sunitinib and its metabolite, an adsorption of the molecules onto the tubing walls was observed (data not shown). To desorb pharmaceutical compounds from the internal surface, the manifold was rinsed with methanol 100%. As shown in Figure 4(b), subsequent to this specific treatment, the chromatogram (blue line) highlights the presence of two peaks corresponding to sunitinib and its metabolite at their respective retention times. This result demonstrates, for the second time, the faculty of the immobilized hepatic cells to generate metabolites. In addition, it confirms that both were desorbed with methanol. A standard solution of 20 µM sunitinib and *N*-desethyl sunitinib (dark line) was injected without exposure to a metabolism test, was used as a second control for desorption, and permitted both peak identities to be proven.

Due to the aforementioned sunitinib adsorption phenomenon, only diclofenac was studied in subsequent experiments.

Metabolization test online

The final objective consisted of designing an *online* metabolization test by coupling different instrumental components to obtain the flow bioreactor setup illustrated in Figure 5: (1) syringe pump, (2) wooden magnet holder, (3) Rheodyne injection valve, and (4) HPLC system. The syringe pump delivered the selected modified medium (WE diluted in D-PBS) containing 250 µM DCF at a 0.4 mL/min flow rate throughout the immobilization chamber. This is where the HepaRG cells were previously magnetically trapped, giving rise to the previously described hepatocyte bioreactor. When the tested pharmaceutical compounds came into contact with the trapped heptocytes, the flow was stopped in order to allow the cells' metabolization process and the syringe content was replaced with D-PBS. After the 5-h incubation period, the syringe pump was reactivated at 1mL/min flow rate, and 50 s later, the Rheodyne valve was manually switched from the loading position to the inject position.

As illustrated in Figure 6(a), the proposed *online* setup was efficient and permitted the generation and detection of DCF metabolites in a single run. The DCF typical chromatogram indicated the presence of one major peak, corresponding to DCF, and a group of smaller peaks. As already noted in section Cells in suspension: Evaluation of metabolic activity, the area of the obtained metabolite peaks was smaller than in the control situation (see Figure

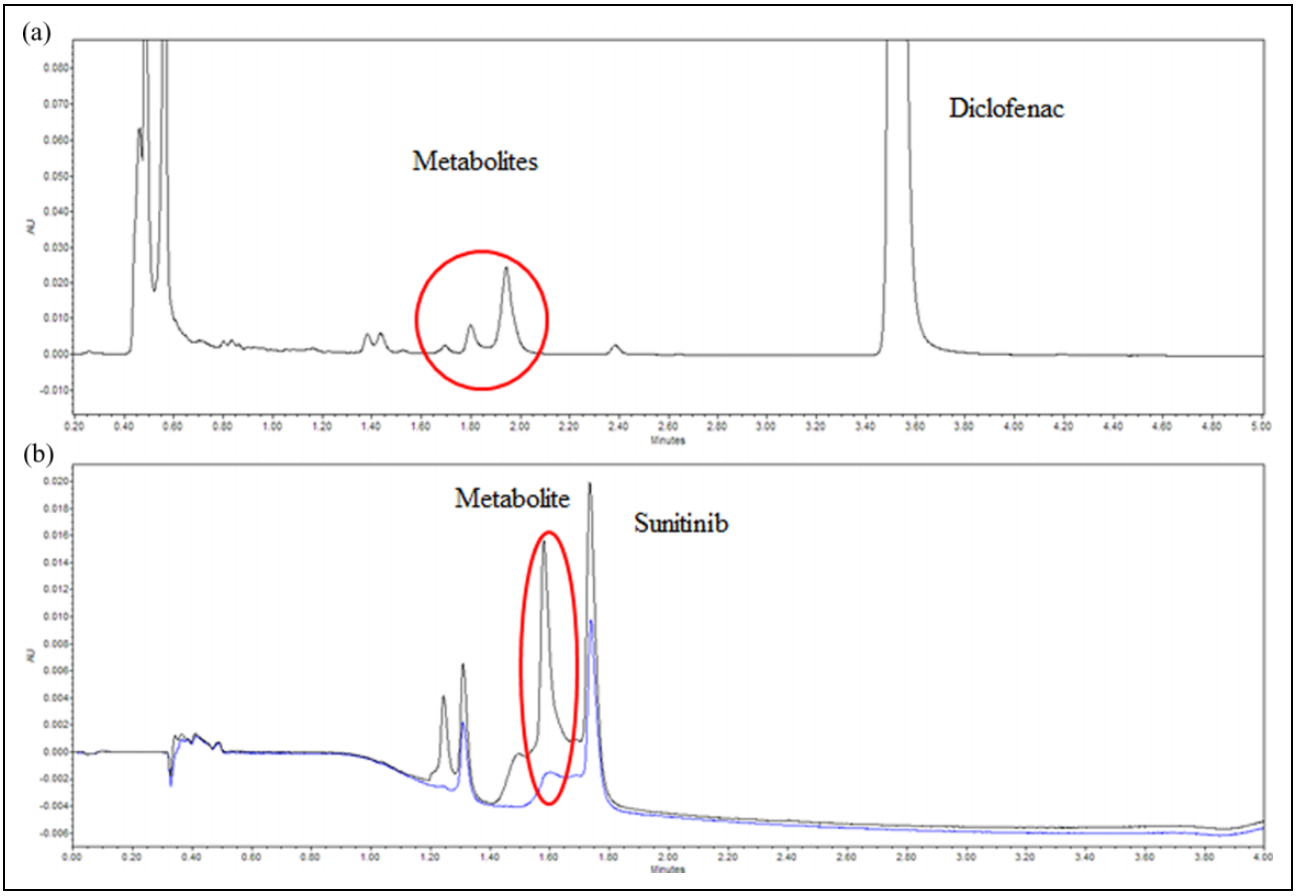

Figure 4. Generated UPLC chromatograms after drug metabolization of magnetically immobilized cells into our holder. (a) represents the 250 μM diclofenac metabolization sample. (b) shows the 20 μM sunitinib metabolization sample after tubing desorption by methanol (in blue) and the tubing control desorption sample of 20 μM sunitinib and *N*-desethyl sunitinib (in black).

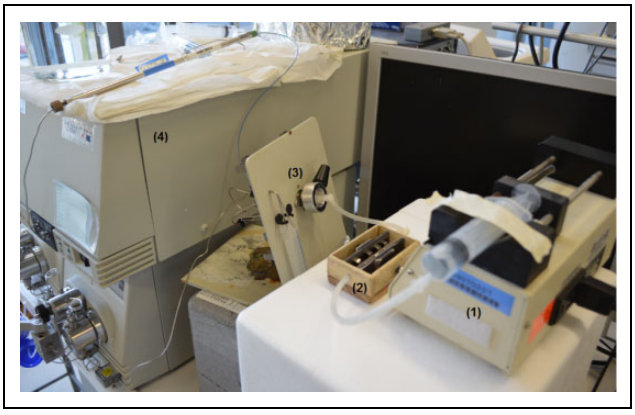

Figure 5. *Online* metabolization test design: (1) syringe pump, (2) wooden magnet holder, (3) Rheodyne injection valve, and (4) HPLC system. HPLC: high-performance liquid chromatography.

6(a)). The chromatogram shapes, however, were similar in both situations: all previously observed diclofenac metabolites were present.

In addition, the *online* mode offers the advantage of generating a larger amount of metabolites than the *off-line* injection mode and consequently facilitates compound detection. In *off-line* conditions, the total tubing content was emptied before analysis resulting in a final metabolite dilution. In *online* conditions, only the adequate volume corresponding to the immobilization chamber (metabolization space) was collected and injected leading to higher chromatographic peaks (approximately 60% more for the first metabolite group; the second was not detected in *off-line* conditions; see Figure 6(b)).

Conclusion

In the present work, an analytical flow bioreactor able to perform *online* xenobiotic metabolism studies has been successfully developed. The developed holder allows quick and appropriate magnet positioning close to the silicone manifold in order to create metabolically competent "bed cells" made of magnetically labeled HepaRG cells. In addition, the homemade wooden holder permits the fast and easy renewal of trapped cells from one test to another. This magnetic immobilization was also observed with other cell lines, namely FHs74Int and HK-2 cells, and therefore means that it can be inferred that such a setup could be transferred to all kinds of cells. The results showed that the profile shapes of the

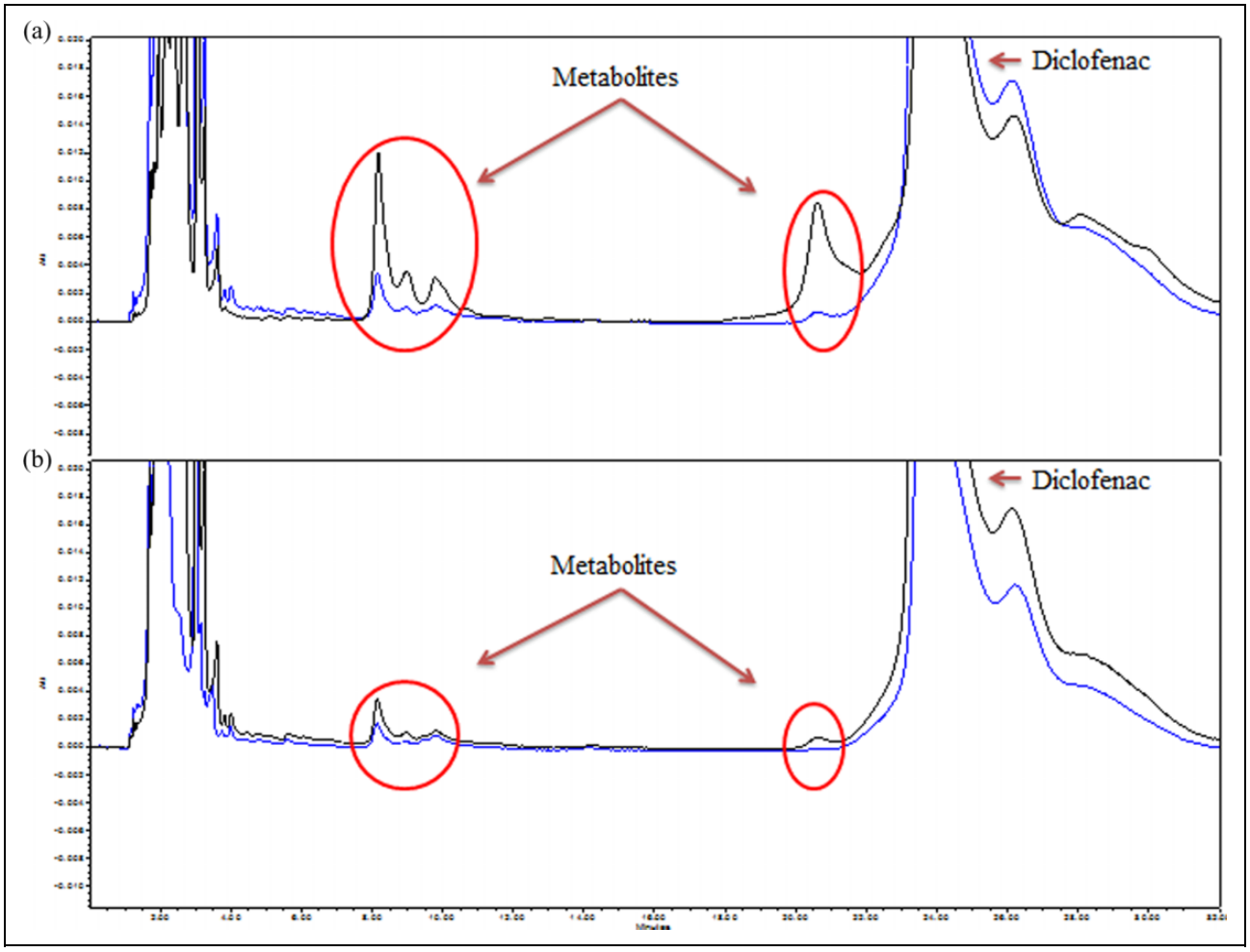

Figure 6. HPLC chromatograms of diclofenac metabolization with *online* injection. (a) shows the *online* result (in blue) compared to control situation in culture flask: adherent cells (in black). (b) compared the metabolization into the analytical bioreactor between *online* (in black) and *off-line* injections (in blue). HPLC: high-performance liquid chromatography.

obtained chromatograms were similar in the control (adherent unlabeled cells in a culture flask) and specific analytical bioreactor conditions, even though the detected diclofenac metabolite peak areas in the designed bioreactor were smaller than in the control situation. We speculate that this decrease (approximately 90% for labeled cells in suspension) is likely due to the change in the experimental conditions for HepaRG cells, from the adherent to suspension state.[73]

The described *online* setup combines a close bio-inspired approach and direct coupling with an LC without any sample pretreatment step. The concept is easy to use as it can be done in a short period of time, the blocked cells can be effortlessly removed, and a new batch of metabolically competent cells injected. Easily implemented, it could be used for predictive metabolism screening tests of drug candidates or drug-drug interactions. This is because it is able to provide reproducible results at an elevated throughput, thanks to there being no sampling collection step needed and the fact that the generated metabolites are immediately sent and processed in the LC-detector chain.

At this stage, additional assays have to be performed to consolidate the first semi-quantitative results and to improve the "metabolic-like" concept. More reference molecules or reference compound cocktails, also using other detection modes, have to be tested. Nevertheless, the initial objective, to build an *online* metabolically competent bioreactor, has been achieved. Pioneering and innovative, it paves the way for a new family of hepatic-like models.

Perspectives for further research works could include using several holders containing different types of magnetically immobilized cells. Such an association in series should firstly give way to metabolite generation with one kind of cell and subsequently be metabolized with another cell type, and so on, giving rise to a "multiple organ-like" system.[46]

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