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On-line coupling of immobilized cytochrome P450 microreactor and capillary electrophoresis: A promising tool for drug development☆

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ABSTRACT

In this work, the combination of an immobilized enzyme microreactor (IMER) based on the clinically important isoform cytochrome P450 2C9 (CYP2C9) with capillary electrophoresis (CE) is presented. The CYP2C9 was attached to magnetic SiMAG-carboxyl microparticles using the carbodiimide method. The formation of an IMER in the inlet part of the separation capillary was ensured by two permanent magnets fixed in a cassette from the CE apparatus in the repulsive arrangement. The resulting on-line system provides an integration of enzyme reaction mixing and incubation, reaction products separation, detection and quantification into a single fully automated procedure with the possibility of repetitive use of the enzyme and minuscule amounts of reactant consumption. The on-line kinetic and inhibition studies of CYP2C9's reaction with diclofenac as a model substrate and sulfaphenazole as a model inhibitor were conducted in order to demonstrate its practical applicability. Values of the apparent Michalis–Menten constant, apparent maximum reaction velocity, Hill coefficient, apparent inhibition constant and half-maximal inhibition concentration were determined on the basis of the calculation of the effective substrate and inhibitor concentrations inside the capillary IMER using a model described by the Hagen–Poisseulle law and a novel enhanced model that reflects the influence of the reactants' diffusion during the injection process.

1. Introduction

New drug development is a dynamic process requiring constant innovation. Hundreds of thousands of compounds are tested every year, yet only tens of them become clinically used drugs. One of the main reasons for the high elimination of candidate compounds are safety issues, with approximately 75% of them being dose-related, which are considered to be predictable using appropriate pharmacological profiling during the preclinical phases of drug development [1,2]. Enormous effort is therefore being made to develop new *in vitro* and *in vivo* methods in order to reduce the tremendous costs of clinical trials [3,4].

Cytochrome P450 enzyme (CYP) assays represent an integral part of these tests, which can to a certain extent predict dosage and inappropriate drug–drug interactions. CYPs are mostly hepatic, membrane bound enzymes which play a key role in a drug's metabolism. Their main biological role is to functionalize xenobiotics in order to facilitate their excretion; as a result they modulate effective drug concentration. On the other hand many drugs modulate CYP activity, which can have lethal consequences [5]. Early determination of the precise relationship between the candidate drug and all significant CYP isoforms is thus of the utmost importance [6,7]. Today CYP assays are being used as early in the drug development process as possible, which results in an extensive number of compounds tested. This fact is driving the development of new rapid, cost-effective and high-throughput methods [8].

The immobilization of biomolecules is a well-established and still rapidly evolving bioanalytical field that has supported tremendous advances in life sciences [9]. In the fields employing enzyme assays, two main reasons have led to the wide usage of immobilization: the need for enhanced stability of the enzyme, and recyclability compared to the free enzyme [10]. In recent years immobilized enzyme reactors (IMERs) have attracted a great deal of attention, especially for their easy on-line coupling with analytical instruments such as HPLC and capillary electrophoresis (CE), benefiting both from the full automation of the measurements and significant savings in enzymes and reactants.

In this work, the main objective was to develop a magnetic particles-based IMER for kinetic and inhibition studies of the

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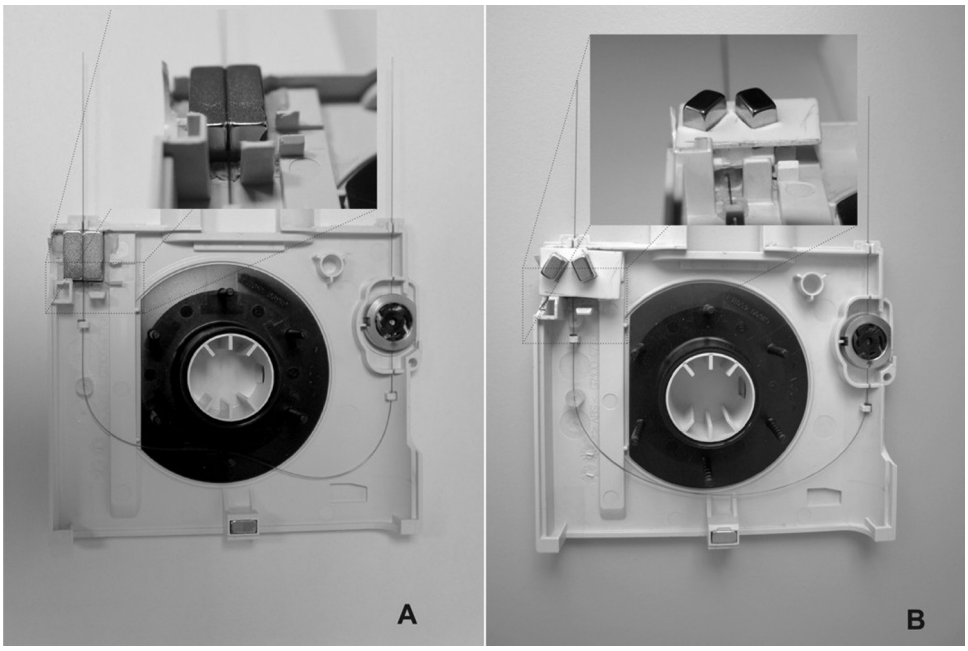

Fig. 1. Modified CE cassette housing two NdFeB magnets, which determine the position of the IMER inside the capillary; insert – details of magnet setup. (A) attractive arrangement, (B) repulsive arrangement.

cytochrome P450 2C9 isoform (CYP2C9) and its hyphenation with CE. This clinically and pharmacologically important isoform represents approximately 20% of all CYP in human liver, and is responsible for metabolizing more than 10% of commonly used drugs [6]. Furthermore, this isoform was extensively studied in the previous research of our group by off- and on-capillary CE methodologies [11–13]. The method was optimized using diclofenac as a model substrate and sulfaphenazole as a model inhibitor, which are preferred substances for *in vitro* studies of CYP2C9 by the U.S. Food and Drug Administration [14]. Besides lowering reactant and enzyme consumption per analysis, the key pursued factors were set to achieve a rapid analysis time and full automation with sufficient sensitivity and accuracy to obtain valid kinetic and inhibition data comparable to those determined using traditional techniques. To the best of the authors' knowledge this is the first application of the CYP IMER-CE methodology, with a high relevance for high-throughput screening in the early stages of new drug development, especially after potential transfer to multi-capillary CE systems.

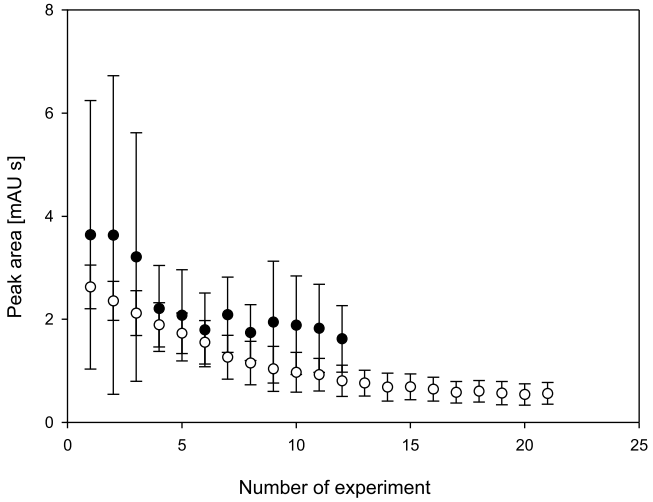

Fig. 2. Comparison of dependencies of relative CYP2C9 activity on number of subsequent measurements using IMER with attractive (●) and repulsive (○) arrangements. Each symbol is an average value of three independent analyses each performed on the individual IMER.

2. Materials and methods

2.1. Chemicals and reagents

Recombinant human CYP2C9 BACULOSOMES® Plus Reagent was obtained from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Diclofenac; 4′-hydroxydiclofenac; reduced nicotinamide adenine dinucleotide phosphate (NADPH); sulfaphenazole; solvents and all components of rinsing and immobilization solutions, incubation and storage buffers and background electrolyte (BGE) were supplied by Sigma (St. Louis, MO, USA). 1-μm-diameter SiMAG-carboxyl magnetic microparticles were obtained from Chemicell (Berlin, Germany). NdFeB magnets ($15 \times 7.5 \times 5$ mm) with polarization along the shortest dimension were obtained from MAGSY s.r.o. (Zlín, Czech Republic).

All solutions were prepared using deionized water from a Millipore Direct Q 5 UV system (Milford, MA, USA). Fresh incubation buffer and BGE (see below) were prepared each week and stored at 4 °C. 10 mM stock solutions of diclofenac, 4′-hydroxydiclofenac and sulfaphenazole were prepared in methanol and stored at −20 °C. Working solutions at a concentration of 200 μM were prepared from stock solutions every day by diluting with incubation buffer and kept at 4 °C. The mixture of reactants used in experiments were prepared at the specified concentrations, while the final methanol content was lower than 1% v/v, so as to have no effect on CYP2C9 activity.

2.2. Capillary electrophoresis

An Agilent 7100CE System (Waldbronn, Germany) equipped with a diode-array UV–vis detector was used for all analyses. Data was collected using the Agilent software ChemStation 1.8.1. The analyses were carried out in a 75 μm I.D., 375 μm O.D. fused silica capillary (33.5 cm total length, 25 cm effective length) from Polymicro Technologies (Phoenix, AZ, USA) thermostated at 30 °C. A