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The protagonism of bioanalytical methods in high-throughput drug discovery

Edited by
Marcela Cristina De Moraes, Fernando Goncalves De Almeida and Luzineide Wanderley Tinoco

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The protagonism of bioanalytical methods in high-throughput drug discovery

Topic editors

Marcela Cristina De Moraes — Fluminense Federal University, Brazil

Fernando Goncalves De Almeida — Omass therapeutics, United Kingdom

Luzineide Wanderley Tinoco — Federal University of Rio de Janeiro, Brazil

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Caterina Temporini,
University of Pavia, Italy

*CORRESPONDENCE
Marcela Cristina de Moraes,
mcmoraes@id.uff.br

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Editorial: The protagonism of bioanalytical methods in high-throughput drug discovery

Marcela Cristina de Moraes[1]*, Fernando Gonçalves de Almeida[2] and Luzineide Wanderley Tinoco[3]

[1]BioCrom, Departamento de Química Orgânica, Instituto de Química, Universidade Federal Fluminense, Niterói, Brazil, [2]OMASS Therapeutics, Oxford, United Kingdom, [3]Instituto de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro, Brazil

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Editorial on the Research Topic
The protagonism of bioanalytical methods in high-throughput drug discovery

Protein-ligand interactions are essential for the regulation of biological processes, such as signal transduction, gene regulation, cellular metabolism, and immunoreaction. The term ligand encompasses nucleic acids, cofactors, metals, other proteins, peptides, amino acids, lipids, and drugs. Protein function can be regulated by its interaction with specific ligands through different mechanisms. Protein-ligand interaction studies are crucial for understanding the regulation of the biological function of proteins, elucidating potential biological targets, as well as discovering bioactive compounds in the drug development process. Within this context, this Research Topic aims to highlight different aspects of analytical techniques as a useful tool to develop new, rapid, and reliable ligand screening assays.

Two original research manuscripts, one review, and one mini-review on analytical assays for ligand screening are collected in this Research Topic, covering assays for ornithine decarboxylase inhibitor screening, on-flow enzymatic inhibitor screening through liquid chromatography methods, liquid chromatography coupled to mass spectrometry (LC-MS) method to screen human kallikrein (KLKs) inhibitors, and a study on salt concentration to improve the separation performance of biomarkers for transporter protein inhibition. In the following paragraphs, each published manuscript is presented and briefly described.

The ornithine decarboxylase (ODC) enzyme belongs to the polyamine biosynthetic pathway, catalyzing the decarboxylation of ornithine to putrescine. Polyamines (putrescine, spermidine, and spermine) are essential growth factors in eukaryotic cells, but their high levels are associated with carcinogenesis (Gerner and Meyskens, 2004) and Alzheimer's disease (Mäkitie et al., 2010). Consequently, ODC is considered a biological target for developing new drugs for the treatment of several diseases. Tinoco et al. (2022) summarized the methods based on radiolabeling, colorimetric assays using auxiliary enzymes to detect CO_2 or H_2O_2 release, chromatographic-based methods with putrescine derivation, mass spectrometry, circular dichroism, and fluorescence techniques. The authors highlight the demand for the development of high-throughput assays for the screening of ODC inhibitors. Since ornithine and putrescine (substrate and product) cannot be directly monitored by spectroscopic techniques, derivation or conversion procedures into spectrophotometrically detectable species are mandatory, resulting in low throughput assays.

Kallikreins (KLKs) are a subgroup of the serine protease enzyme family that play a crucial role in biological fluids (plasma kallikrein, KLK 1B) and tissues (tissue KLK, KLK-15). Unregulated levels of KLK expression may be associated with various diseases. For instance, KLK 1B plasmatic high concentrations or hyperactivity perpetuates cardiovascular disease (Kolte and Shariat-Madar, 2016). Other examples of KLK-related diseases include Alzheimer's and Parkinson's disease (Diamandis et al., 2000), inflammatory skin disorders (Di Paolo et al., 2021), and cancer (Kryza et al., 2016). The manuscript published by De Carvalho et al. in the present Research Topic describes the KLK immobilization on Sepharose-NHS as a micro-column for the development of an offline screening assay. KLK activity was monitored by quantifying the formed product (7-amino-4-methyl coumarin, AMC). After conducting kinetic assays, the method was validated for screening purposes using leupeptin as a reference inhibitor. Immobilized KLK exhibited stability through several cycles and it represents a promising alternative to the traditional fluorescence microplate assays.

Enzyme inhibition is known as an approach to the development of new drugs for the treatment of several pathological conditions, such as inflammation, diabetes, microbial infections, HIV, neglected diseases, and others (Geronikaki, 2020). Therefore, the development of analytical assays for high-throughput screening assays that enable the evaluation of large libraries as potential enzyme inhibitors can accelerate the drug development process (de Moraes et al.). In this realm, on-flow assays based on liquid chromatography (LC) can be highlighted. De Oliveira et al. summarized the applications of on-flow LC-based assays for monitoring the enzyme catalytic activity and the affinity/retention ligands. Since most of the applications use the immobilized biological target, immobilization methods and solid supports for enzyme immobilization are discussed. Activity- and affinity-based assays, including frontal affinity chromatography, zonal affinity chromatography, and ligand fishing applications are presented. The versatility of on-flow set-ups, the possibility of automation, and enzyme reuse are highlighted as the main factors associated with the emerging success of LC-based on-flow screening assays.

Taurine and glycochenodeoxycholate sulfate (GCDCA-S) can be used as probes for evaluating pharmacokinetic drug-drug interactions (DDI) involving renal organic anion transporters OAT1 and OAT3 inhibition in humans (Tsuruya et al., 2016). Both molecules are highly polar, hydrophilic compounds, and incompatible with conventional reverse phase LC mode. Wouters et al. reported the use of high salt concentrations to improve separation performance with suppressor technology in LC-MS using hydrophilic interaction liquid chromatography (HILIC) separation. The use of high salt concentration solvent modifiers to tune resolution and subsequently mitigate the MS incompatibility of high molar ammonium acetate was achieved by a post-column mobile phase modulation approach. The proposed method resulted in an up to a 10-fold increase in detection sensitivity.

The published manuscripts on this Research Topic nicely highlight some of the state-of-the-art achievements and challenges concerning the contribution of bioanalytical methods in high-throughput drug discovery. It is our hope that this Research Topic will encourage readers to apply different bioanalytical technologies to advanced applications in medicinal chemistry.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Anderson R. Moraes De Oliveira,
University of São Paulo, Brazil

REVIEWED BY
Gabriella Massolini,
University of Pavia, Italy
Feng-Qing Yang,
Chongqing University, China

*CORRESPONDENCE
Marcela Cristina De Moraes,
mcmoraes@id.uff.br

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On-flow enzymatic inhibitor screening: The emerging success of liquid chromatography-based assays

Pamella Christina Ortega De Oliveira, Renato Côrrea Lessa, Millena Santana Ceroullo, Camila Anchau Wegermann and Marcela Cristina De Moraes*

BioCrom, Laboratório de Cromatografia de Bioafinidade e Química Ambiental, Departamento de Química Orgânica, Instituto de Química, Universidade Federal Fluminense, Niterói, Brazil

Enzymes are targets commonly explored in screening assays aiming to discover new leads in the drug development process. Among the diverse assay models to identify new enzymatic inhibitors, on-flow assays based on liquid chromatography (LC) can be highlighted. In these approaches, the ligand-enzyme interaction can be examined by monitoring the catalytic activity or the affinity/retention. Most applications use the biological target immobilized in solid supports resulting in the acquisition of an immobilized enzymatic reactor (IMER). Coupling IMERs to LC or mass spectrometry (MS) systems allows monitoring enzyme activity online and studying binding events between target and ligands. On-flow screening assays present many advantages for the hit-to-lead process, such as the possibility of system automation, reusability, and high stability. This review covers articles from the last decade that combine the use of varied immobilization methods on different solid supports and several equipment setups in on-flow systems, emphasizing the performance and capacity of recognizing and identifying biologically active compounds in various matrices.

KEYWORDS

screening, activity-based assay, affinity-based assay, on-flow, enzyme reactors

1 Introduction

Several analytical approaches have been applied for the screening of potential enzyme inhibitors, such as colorimetric (Rennó et al., 2012) and fluorescent (Liu et al., 2018a) assays. However, these methods present some advantages. Colorimetric and fluorescent assays are only suitable for monitoring reactions in which the substrate and products have significant difference in their spectrophotometric properties. Additionally, fluorescence signal could be also interfered by background absorption and auto-fluorescence in biological samples (Cheng and Chen, 2018).

More recently, new reliable methodologies have been developed to improve the understanding of enzyme-inhibitor interaction and allow the high-throughput screening

of large libraries. Within this context, methodologies that involve solid-supported enzymes have emerged as promising due to cost-saving, reusability, increased stability, and operational optimization.

Immobilized Enzyme Reactors (IMERs, occasionally named immobilized capillary enzyme reactors, ICERs) can be defined as devices that contain the target enzyme, physically confined or localized, with retention of its catalytic activity. At this condition, the immobilized enzyme can be used repeatedly and continuously, and the catalytic reaction can occur under on-flow or static conditions (with the paused flow) (Wouters et al., 2021). Different solid support can be used to create IMERs, such as silica particles (Yuan et al., 2020), magnetic particles (Ximenes et al., 2022), monolithic supports (De Moraes et al., 2014; Wang et al., 2018), and silica fused capillaries (Vanzolini et al., 2013; Seidl et al., 2019).

IMERs can be essential parts of the analytical workflow for screening assays, and can be employed online or offline to an analytical technique, such as liquid chromatography (LC). However, offline approaches generally are time-consuming and involve more sample manipulation in multi-step workflows. On the other hand, IMERs can be prepared by immobilizing the target enzyme in solid supports suitable to LC systems, exhibiting rapid mass transfer capacity, low backpressure, and adequate efficiency, besides being mechanically and chemically stable (Temporini et al., 2016). Furthermore, the solid support should be able to retain the target enzyme even in the on-flow conditions. In this case, on-flow approaches, in which the binding event occurs within the liquid chromatographic system for instance, can be developed. Examples of solid supports suitable for LC systems include open tubular (de Castro et al., 2022) and packed fused silica capillaries (De Moraes et al., 2014), monolithic supports (Kubota et al., 2017), and immobilized artificial membranes (IAM) (Russo et al., 2018). On-flow screening assays based on liquid chromatographic methods furnish high-throughput, automation, and mimic the biochemical system more accurately, given that *in vivo* ligand-enzyme interactions occur in an on-flow system.

Concerning the on-flow liquid chromatography-based screening assays, the terms online and inline are used for methods that do not require manual transference of the analytes (Minnich et al., 2016; Calleri et al., 2021). Online analytical setup refers to a platform configuration where the aliquots periodically collected in a first dimension are automatically analyzed in a second dimension (de Moraes et al., 2013), whereas inline set-up involves the in-series connection of the IMER and the analytical instrument (Ximenes et al., 2022), with the continuous and total transfer of the analytes.

Herein, we presented a review focused on on-flow enzymatic inhibitor screening assays based on liquid chromatographic methods from 2012 to 2022. The aim of this contribution is to help the pharmaceutical research community to select the most promising approach for the preparation of the IMER with the biomolecule of interest, and to evaluate the ideal instrumental setup for the assay to be developed, considering the objective of the screening assays and the available equipment. Initially, the importance of enzymes as biological targets in the drug development and discovery process is highlighted, followed by a summary of the most common immobilization methods to produce IMERs compatible with LC systems. Finally, an overview of the affinity- and activity-based on-flow screening assays will be covered. Outside the scope of this review are capillary-electrophoresis methods and screening assays involving biomolecules other than enzymes.

2 Molecular basis of enzyme inhibition

Enzymes are involved in several biochemical processes and therefore are essential for living beings. These classes of proteins are extraordinary biocatalysts and can bind to specific substrates and promote several reactions with a high rate of conversion.

Efficient therapies can be achieved by using enzymes as drugs or in diagnostics (Farhadi et al., 2018; Rufer, 2021). On the other hand, the lack, overexpression, or malfunction of a particular type of enzyme can be associated with many diseases making these macromolecules a hallmark group for drug development and target (Robertson, 2007).

Several diseases require the use of enzyme inhibitors, indeed, a substantial part of the drugs applied for clinical purposes are enzyme inhibitors (Drews, 2000; Hopkins and Groom, 2002; Robertson, 2007). The clinical treatment of many diseases such as cancer (Dhokne et al., 2021), neurological disorders (Saxena and Dubey, 2019), infections (Zhang et al., 2021), viruses (Hľasová et al., 2021; Kharkwal et al., 2021), and others, usually involves enzymes as the drug target. In fact, in the last few years (2015–2020), FDA has approved several drugs that act as enzyme inhibitors, those classes include kinase inhibitors for cancer therapy, CY3PA4 inhibitors for neurological, metabolic disorders, and infections, and others (Bhutani et al., 2021a).

Enzymatic inhibition aims to cause any change in the protein conformation, usually by blocking its active site and limiting enzymatic activity. The main challenges in this process are designing molecules with high specificity and being able to act under physiologic conditions (Bhutani et al., 2021b). In this case, a large group of compounds is routinely submitted to screening assays as potential enzymatic inhibitors (Davies et al., 2021).

Understanding the mechanisms involved in enzyme-inhibitor interaction is central to the design of new compounds as potent enzymatic inhibitors. In a biochemical reaction, when the concentration of the enzyme is kept constant and the substrate concentration is increased, the Michaelis-Menten equation (Eq. 1) provides parameters that can be

expressed as a function of the substrate concentration and offer information about the kinetics of enzyme-substrate interaction at specific experimental conditions. The parameters V, V_{max}, S, and K_m are respectively defined as the reaction rate, maximum reaction rate, substrate concentration, and Michaelis-Menten constant. The last one, K_m, is also defined as the substrate concentration at half of the maximum velocity and is an intrinsic parameter for each enzyme-substrate system. In general, the enzymatic reaction follows the same behavior: the first-order reaction when the reaction rate is proportional to substrate concentration, and it is a zero-order reaction when the enzyme is saturated by substrate (Sirajuddin and Saqib Ali, 2013; Cornish-bowden, 2015).

$$V = \frac{V_{max}S}{K_m + S} \quad (1)$$

Michaelis-Menten equation.

When an inhibitor is added to an enzymatic reaction, new parameters should be considered, such as inhibitor concentration and inhibition constant (K_i). The value of half maximal inhibitory concentration (IC_{50}) provides the inhibitor concentration required to decrease at half the rate of the enzyme activity, while K_i is the dissociation constant describing the binding affinity between the enzyme and the inhibitor. The deductions of IC_{50} and K_i and their thermodynamic considerations are already properly described in the literature and are out of the scope of this review (Copeland, 2000; Burlingham and Widlanski, 2003; Buker et al., 2019; Georgakis et al., 2020).

The process of inhibition depends on the molecular dynamics between the inhibitor (I), the enzyme (E), and the substrate (S), and the types of inhibition can be fundamentally classified into reversible and irreversible.

Reversible inhibition should lead to the regeneration of the enzyme activity in the media and can be classified into four categories: competitive when the I binds directly to the E; non-competitive, when I binds equally to the free enzyme E and the ES complex; uncompetitive which means that the inhibitor binds exclusively to the ES complex; and mixed inhibition when I binds to both E and ES, but not equivalently as in non-competitive type (Buker et al., 2019).

An example of a competitive inhibitor is Statin which, according to the FDA, is classified as a group of marketed drugs used for cholesterol being effective to reduce the rates of myocardial infarction and mortality (Dimmitt et al., 2018). Among Statin group, the active compounds include Simvastatin (IC_{50} 11 nmol/L), Fluvastatin (IC_{50} 28 nmol/L), Atorvastatin (IC_{50} 8 nmol/L), and Rosuvastatin (IC_{50} 5 nmol/L). Those compounds are responsible for inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) and consequently reducing the levels of mevalonate, a metabolite of cholesterol synthesis (Corsini et al., 1999; Istvan, 2002; Rosendo et al., 2007; Dimmitt et al., 2018).

Donepezil is a drug clinically used for Alzheimer's disease that acts as an acetylcholinesterase (AChE) non-competitive and mixed competitive inhibitor. The multistable modes of interaction with the enzyme allow that donepezil blocks the active site of the enzyme as well binds to the enzyme-substrate complex with similar stability (Silva et al., 2020).

Unlike reversible inhibitors, irreversible inhibitors covalently bind to the enzyme and obstruct its regeneration into free form. This phenomenon can be seen through the low values of the dissociation constant (in the range of nmol/L) (Palmer and Bonner, 2007). There are several examples of irreversible covalent inhibitors in clinical use today, such as omeprazole (Johnson et al., 2010; De Cesco et al., 2017). Omeprazole inhibits the activity of the H^+/K^+ ATPase through the disulfide bonds formed with cysteine residues, as a consequence of the linkage between the omeprazole and the enzyme, the levels of gastric acid are reduced in the stomach (Cartee and Wang, 2020; Gehringer, 2020).

3 Immobilization methods

On one hand, enzymes combine some ideal features, like selectivity and high activity combined with mild condition processes, such as aqueous medium and atmospheric pressure. On the other hand, these biomolecules are susceptible to denaturation due to temperature and pH value variations, the presence of organic solvents, etc. The enzyme in its free form is highly soluble in the reaction medium, which is excellent for the catalytic activity, however, it results in considerable instability and prevents its reuse. Enzyme immobilization on solid supports promotes stabilization and easy recovery of these biomolecules, converting the initial homogenous catalytic system into a heterogeneous one, facilitating the isolation step and obtaining considerably high catalytic activity.

Some studies have shown that enzyme immobilization could provide an extension of the optimal pH and temperature ranges of the enzyme activity compared to the free enzyme (Liu et al., 2018b, 2018a). Also, the wide range of materials used as solid support for enzyme immobilization provides cost reduction and better operational stability, besides avoiding time-laborious steps (Hanefeld et al., 2009; Franssen et al., 2013; Liu et al., 2017). For instance, screening assays of lipase inhibitors demonstrated that the immobilized enzyme on Fe_3O_4@TiO_2/CRL magnetic nanoparticles presented better activity in all evaluated conditions such as temperature, time, stability, and reusability (Liu et al., 2020).

An enzyme can be attached to a solid support through different methodologies, including physical and chemical approaches, as illustrated in Figure 1.

Physical methods, considered one of the easiest and cheapest methodologies, encompass weak interactions such as electrostatic, hydrophobic, entrapment, and biospecific

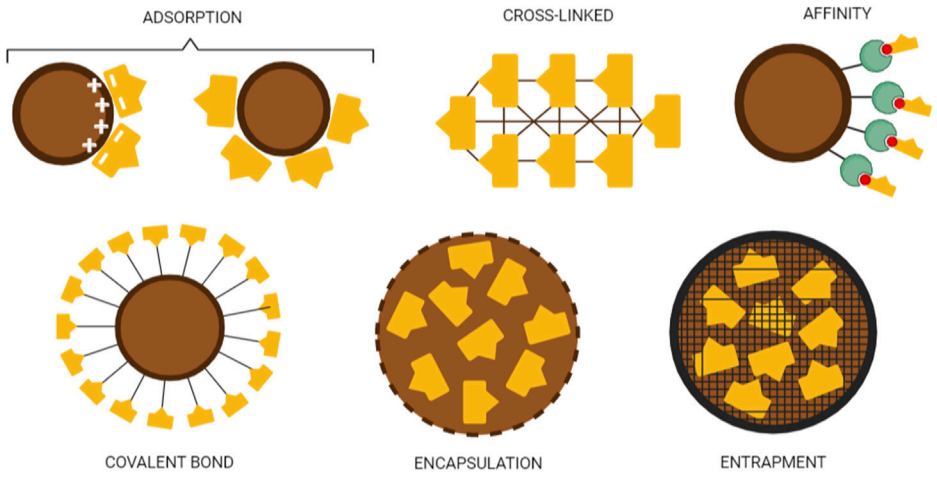

FIGURE 1
A schematic representation of different types of immobilizations.

adsorption. The main advantages of the physical methods arise from the simplicity of the immobilization procedure, demanding few equipment and reagents, and resulting in few changes in the enzyme conformation (Rodriguez et al., 2020). Physical immobilization is a reversible method, i.e., the enzyme is attached by weak interaction forces that can be ruptured releasing the biomolecule in solution. Depending on the purpose of immobilization this can be helpful because it is possible to retrieve the support. The reversible condition of this attachment represents a major drawback in on-flow systems, and it narrows the applicability of physical immobilization.

Hou et al (Hou et al., 2020) created a new stationary phase containing α-glucosidase immobilized in porous silica gel with liposome vesicles to screen bioactive compounds from herbal extracts. Based on the hydrophilic behavior of the biological target, it was encapsulated in the aqueous phase of the lipid bilayer. A control stationary phase was prepared in the same conditions but without the α-glucosidase enzyme. Differences in retention times of known α-glucosidase inhibitors in both stationary phases were used to validate the methodology before performing screening assays on Schisandra chinensis. In this case, this methodology explores the interactions between ligand and biological target and the ligand capacity to cross the double-layer membrane of liposomes, proving insight into the performance in studies involving cells and animals.

The biospecific adsorption immobilization procedure involves affinity binding. Usually considered as a subclass of physical immobilization, affinity immobilization is ruled by the bio-specific interactions between a tag (or entity) and a target (like an enzyme). The biomolecule attachment to the support surface is performed by using a second biological structure: at one end this structure attaches to the solid support surface, and at the other one, it binds to the target. In this methodology, the selective interactions between the tag and the target are explored, and the immobilization occurs in a nearly ordered orientation on the support surface. Therefore, that immobilization protocol can avoid random attachment and furnish minimal conformational changes. The main goal of affinity binding immobilization is to maximize the availability of the biological target active site (Mohamad et al., 2015; Reis et al., 2019; Zhang et al., 2019a; Rodriguez et al., 2020).

A classic example of this guided immobilization is the use of biotin, which can strongly bind to streptavidin/neutravidin/avidin, so the biotin pair intermediates the link between the solid support surface and target. In the proposal of a new miniaturized system, André et al. (André and Guillaume, 2021) performed the immobilization of arginase using this approach. Separately, neutravidin was covalently bonded on the support surface and the target was biotinylated. When in contact the pair biotin/neutravidin promoted a fast and strong binding between the solid support and the enzyme. The arginase capillary column produced showed to be stable, it retained 97% of activity after 3 months.

The second type of immobilization procedure is the chemical method, in which the enzyme is attached to the support surface by strong chemical bonds, more specifically, covalent bonds. Chemical immobilization methods avoid enzyme desorption, frequently observed in physical methods, mainly in on-flow assays. The main advantage of chemical immobilization lies in the fact that the target will be anchored in the solid support surface through specific functional groups (such as aldehyde, carboxylic acid, thiol, amine, epoxy, and maleimide) by the formation of a covalent bond (Mohamad et al., 2015; Bilal and Iqbal, 2019; Bilal et al., 2019), enhancing its stability.

Usually, in this configuration, the biomolecule is less susceptible to temperature and pH changes, and more stable in the presence of organic solvents. The chemical immobilization methods involve cross-linking and covalent bonds.

Due to their advantages, chemical methods represent the main procedure for enzyme immobilization in the development of on-flow inhibitor screening assays. Frequently, chemical immobilization methods require the use of a bifunctional reagent, namely a linker or cross-linker agent, which should react with the support surface by one end, leaving the other one free to react with functional groups present in the enzyme surface. Consequently, the immobilized enzyme exhibits more mobility favoring its conformational structure. The robustness brought by chemical immobilization comes with a price, to provide stability, the target must be irreversibly attached to the support, which means that there is no recovery of the enzyme (Meryam Sardar, 2015; Wahab et al., 2020).

One of the most used protocols for chemical immobilization involves the formation of Schiff bases using glutaraldehyde as a linking agent. This reaction can be performed under aqueous media and mild conditions which is very attractive for biochemical purposes (Jia and Li, 2015). Evaluating a new selective substrate for Beta-secretase1 (BACE1), De Simone et al (De Simone et al., 2014) used an amino monolithic disk to immobilize the biological target. The support surface was activated with glutaraldehyde, a molecule with an aldehyde in both ends, to then immobilize the BACE1. The BACE1 disk was coupled to the LC system. A known and a new inhibitor of this enzyme were used to validate the selective substrate. The on-flow assay presented a good response for screening potential inhibitors without having long incubation ties and aggregation. The application of this selective substrate avoided the interference of inhibitors with a strong fluorescent response. Not only was developed a fast, high throughput screening (HTS) assay but also a more selective one.

In an innovative strategy, Ximenes et al. (Ximenes et al., 2022) assemble an inline system using magnetic particles as support to immobilize human purine nucleoside phosphorylase (HsPNP). Glutaraldehyde was used as a spacer and cross-linking agent for the covalent immobilization of the target enzyme onto the magnetic particles (MPs) surface. The HsPNP-coated MPs were trapped inside a PEEK tube using a series of magnets and inserted into the on-flow system. The assay was successfully applied in the study of the fourth-generation Immucillin derivative (DI4G), a known inhibitor, determining IC_{50} and K_i values. The enzyme immobilization on magnetic particles proved to be a versatile approach in which offline and inline modes could be applied using the same target-support system. Other biomolecules were immobilized by covalent bond through the formation of Schiff bases, like as trypsin (Liu et al., 2019), human Thymidylate Kinase (hTMPK) and the human Nucleoside Diphosphate Kinase (hNDPK) (Ferey et al., 2019), cathepsin D (Cornelio et al., 2018), acetylcholinesterase (AChE) (Vandeput et al., 2015; Seidl et al., 2019), butyrylcholinesterase (BChE) (Seidl et al., 2019), Phospholipase A2 (PLA2) (Wei et al., 2021), and others.

In the realm of chemical immobilization techniques, Qiu and collaborators (Qiu et al., 2020) immobilized α-glucosidase using a Metal-Organic Framework (MOF), ZIF-90. This porous material can involve the enzyme in its structure by forming bonds between aldehyde groups from the organic part of ZIF-90 and amino groups from the enzyme, forming a "cluster" around the target. The microreactor containing the ZIF-90@α-glucosidase was coupled to an LC system, resulting in an automated screening system that was applied to screen selective inhibitors from *Dioscorea opposite* Thunb peel and other Chinese herbs extract (Qiu et al., 2020).

As illustrated in Table 1, the use of chemical immobilization through the formation of covalent bonds represents the main choice to attach the biological target to the solid support surface in the development of on-flow screening assays. The main reason lies in the stability furnished by this type of immobilization procedure, which is highly recommended for on-flow systems.

4 Solid supports for enzyme immobilization in the development of on-flow assays

Solid supports employed for enzyme immobilization aiming for the development of on-flow assays should attend the maximum as possible of the following desired attributes: 1) susceptible to specific chemical reactions for surface functionalization to achieve the desired enzyme immobilization; 2) appropriate superficial area dimension, enabling the enzyme immobilization avoiding overloading that could affect enzyme-ligand interactions; 3) compatible with high-pressure conditions in HPLC systems; 4) support green chemistry purposes to achieve environment-friendly experimental conditions; 6) good to the excellent cost-benefit relationship (Liu and Dong, 2020; Rodriguez et al., 2020; Wahab et al., 2020).

Different solid supports can be employed for enzyme immobilization in the development of LC-based on-flow screening assays. Silica-based supports (de Moraes et al., 2012, 2013; Vanzolini et al., 2013; da Silva et al., 2013; Hu et al., 2015; Silva et al., 2015; Vilela et al., 2015, 2018; Calil et al., 2016; Magalhães et al., 2016; Sarria et al., 2016; Ferreira Lopes Vilela and Cardoso, 2017; Cornelio et al., 2018; Lima et al., 2019; Medina et al., 2019; Qian et al., 2019; Seidl et al., 2019, 2022; Ferey et al., 2019; Chapla et al., 2020; Hou et al., 2020; de Castro et al., 2022) present a versatile and largely explored motif due presence of many hydroxyl groups on its surface. For example, those groups are readily reactive with 3-aminopropyltriethoxysilane (APTES), furnishing an amine-

TABLE 1 Different system setups, solid support, immobilization methods, and target analytes used in the development of LC-based on-flow screening assays.

Target	Experimental approach	Support	Immobilization	Target analytes	References
AChE	2D HPLC with dual IMER	poly (GMA-co-EDMA)	Covalent bond	TCM (*Corydalis yanhusuo*)	(Wang et al., 2018)
	2D HPLC-IMER-MS	Amino silica	Covalent bond	Known inhibitors (galanthamine, tacrine, and huperizine A) and NP (*Lycoris radiata*)	(Yuan et al., 2020)
	1D HPLC-ICER-DAD	Fused silica capillary	Covalent bond	Known inhibitors (galanthamine, tacrine, and propidium iodide) and Coumarin derivatives	(da Silva et al., 2013)
	1D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Galanthamine and NP (cyclohexanoids from *Saccharicola sp.)*	(Chapla et al., 2020)
	2D HPLC-ICER-MS	Fused silica capillary	Covalent bond	Known inhibitors (galanthamine and tacrine) and Coumarin derivatives	(Vanzolini et al., 2013)
	2D-LC-MS	Fused silica capillary	Covalent bond	Known inhibitors (galanthamine, tacrine, and donepezil) and NP (*Hippeastrum calyptratum*)	(Seidl et al., 2022)
	1D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Tacrine and 3-*O*-acetyl-*N*-benzylpiperidine derivatives	(Silva et al., 2015)
AChE and BACE	LC-MS	Fused silica capillary	Covalent bond	Galanthamine and BACE inhibitor	(Ferreira et al., 2021)
AChE and BChE	1D LC with parallel dual ICER	Fused silica capillary	Covalent bond	Galanthamine	(Seidl et al., 2019)
	1D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Known inhibitors (galanthamine and eserine) and Coumarin derivatives	(Vilela et al., 2014)
	1D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Metallic complexes of Cu (II) and Zn (II) with flavanones	(Sarria et al., 2016)
	1D HPLC-ICER-MS	Fused silica capillary	Covalent bond	Galanthamine and NP (botryane terpenoids from *Nemania bipapillata)*	(Medina et al., 2019)
	1D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Galanthamine, Aminonaphthoquinone Mannich Bases and their Cu (II) complexes	(Vilela et al., 2015)
α-glucosidase	2D HPLC-IMER-MS/DAD	Fe_3O_4@ZIF-67	Covalent bond	TCM (Xinyang Maojian tea)	(Wu et al., 2020)
	1D HPLC-IMER-UV	Silica with liposomes	Encapsulation	Known inhibitors (miglitol and acarbose) and TCM (*Schisandra chinensis*, Rhizoma Anemarrhenae, *Fructus Corni*, Rhubarb, Mulberry, *Ginkgo* Leaf, *Radix Paeoniae Rubra*)	(Hou et al., 2020)
	1D HPLC-UV	ZIF-90	Covalent bond	TCM (*Dioscorea opposita* Thunb., honeysuckle, Xinyang Maojian tea, and Radix Rehmanniae Praeparata)	(Qiu et al., 2020)
Arginase	1D Nano LC-DAD	Poly (GMA-co-EDMA)	Affinity bonding	Known inhibitors (caffeic acid phenylamide, chlorogenic acid, piceatannol, and nor-NOHA acetate) and NP (*Caesalpinia pulcherrima* (L.), *Sterculia macrophylla*, and *Spirotropis Longifolia*)	(André and Guillaume, 2021)
BACE1	1D LC-IMER	EDA-CIM disk	Covalent bond	Known inhibitors (inhibitor IV and uleine)	(De Simone et al., 2014)
	1D HPLC-ICER-MS	Fused silica capillary	Covalent bond	BACE inhibitor	(Ferreira Lopes Vilela and Cardoso, 2017)
BChE	1D HPLC-ICER-MS	Fused silica capillary	Covalent bond	Galanthamine, tacrine and uleine	(Vilela et al., 2018)
Cathepsin D	2D HPLC-IMER-FS	Fused silica capillary	Covalent bond	Pepstatin A, NP (*Almeidea* sp., *Hortia longifolia*, *Metrodorea nigra*, *Pilocarpus riedelianus*, *Neoraputia magnifica*, and *Lithraea molleoides*), and isolated NP compounds collection	(Cornelio et al., 2018)
Kringle-5	FAC	Amino silica gel	Covalent bond	trans-4-(aminomethyl) cyclohexane carboxylic acid, epsilon- aminocaproic acid, benzylamine, 7-aminoheptanoic acid, and L-lysine	(Bian et al., 2015)
Maltase, Invertase and Lipase	In-series reactors coupled with MS	Commercial magnetic beads	Covalent bond	Known inhibitors (caffeic acid, ferulic acid, and hesperidin) and TCM ("Tang-Zhi-Qing")	(Tao et al., 2013)
NME2	2D-HPLC-UV	Fused silica capillary	Covalent bond and Affinity attachment	Synthetic collection	(Lima et al., 2016)
	2D HPLC-ICER-DAD	Fused silica capillary	Covalent bond	(−)-epicatechin gallate	(Lima et al., 2019)

(Continued on following page)

TABLE 1 (*Continued*) Different system setups, solid support, immobilization methods, and target analytes used in the development of LC-based on-flow screening assays.

Target	Experimental approach	Support	Immobilization	Target analytes	References
NTPDase	2D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Suramin	(Magalhães et al., 2016)
	2D HPLC-ICER-UV/Vis	Fused silica capillary	Covalent bond	Suramin and gadolinium chloride	(Calil et al., 2016)
PNP	2D HPLC-IMER-UV/Vis	Fused silica capillary	Covalent bond	9-deazaguanine derivatives	(de Moraes et al., 2013)
	2D-HPLC-IMER-DAD	Fused silica capillary	Covalent bond	DI4G and 1*H*-1,2,3-triazole quinacrine derivatives	(de Castro et al., 2022)
	2D HPLC-IMER-UV/Vis	Fused silica capillary	Covalent bond	DI4G	(de Moraes et al., 2012)
	FAC	Fused silica capillary and epoxy-silica	Covalent bond	DI4G and synthetic compounds	(De Moraes et al., 2014)
	2D LC-DAD	Commercial magnetic beads	Covalent bond	DI4G and 1*H*-1,2,3-triazole quinacrine derivatives	(Ximenes et al., 2022)
TEM-1 beta-lactamase	FAC	Amino silica gel	Covalent bond	Penicillin G, cefalexin, and cefoxitin	(Chen et al., 2017)
Thrombin	ZAC	Amino silica gel	Covalent bond	Argatroban and TCM (*Radix Salviae Miltiorrhiae*)	(Shi et al., 2020)
TMPK and NDPK	2D HPLC with in-series dual IMER-MS	Silica beads	Covalent bond	—	(Ferey et al., 2019)
Trypsin	FAC	Fused silica capillary	Covalent bond	Matrine, oxymatrine, daidzin, and genistin	(Hu et al., 2015)
	FAC	Fused silica capillary	Covalent bond	Oxymatrine and matrine	(Qian et al., 2019)
XO	2D LC-ICER-UV/Vis—ZAC	Fused silica capillary	Covalent bond	Allopurinol and Ru (II) complexes	(Rodrigues et al., 2015)
	Ligand fishing	Free target		TCM (*Radix Salviae Miltiorrhiae*)	(Fu et al., 2014)
	1D HPLC–DAD–MS/MS.	Amino silica gel	Covalent bond	Allopurinol and TCM (*Lonicera macranthoides*)	(Peng et al., 2016)
	2D LC-ICER-UV/Vis	Fused silica capillary	Covalent bond	9-benzoyl 9-deazaguanine derivatives	(Rodrigues et al., 2016)
	1D UHPLC-ICER-MS	Fused silica capillary	Covalent bond	Allopurinol and NP compounds collection	(Rodrigues et al., 2020)

TCM, traditional Chinese medicine; NME2, Nucleoside diphosphate kinase b; XO, Xanthine oxidase; Poly (GMA-co-EDMA), Poly (glycidyl methacrylate-co-ethylene dimethacrylate); FAC, Frontal Affinity Chromatography; ZAC, Zonal Affinity Chromatography.

rich surface that can further react with glutaraldehyde (GA), yielding a terminal reactive aldehyde surface susceptible reactions with nucleophilic groups present on the enzyme surface (Figure 2B). Seidl et al. (2022) successfully explored this approach by immobilizing AChE in a fused silica capillary for inhibitors screening in bulbs of *Hippeastrum calyptratum*. Moreover, the readily available fused silica capillary might justify the majority of the works (>80%) employing silica-based supports for on-flow assays (Table 1).

Monolith-based supports (GMA-co-EDMA, GMA-co-GlyMA, and Acrylamine) (De Moraes et al., 2014; Wang et al., 2018; Zhang et al., 2019b) are described as a synthetically versatile motif (especially when comprising epoxy groups), compatible with a wide flow rate range and detaining a highly porous structure that leads to better interactions between the stationary phase and mobile phase (Figure 2A) (Faria et al., 2006; Groarke and Brabazon, 2016). The use of this material for enzyme immobilization in the development of on-flow assays was found in 10% of the works considered in this review. Based on the aforementioned, pepsin was covalently immobilized on a polymer monolith using glutaraldehyde as a linker by Zhang et al. (Zhang et al., 2019b) to conduct screening assays in nine different natural products.

Lastly, magnetic particles (Figure 2C) have emerged as a promising alternative for enzyme immobilization in screening assays (Trindade Ximenes et al., 2021; Ximenes et al., 2022), representing 7% of the supports used for enzyme immobilization in the development of on-flow screening assays. Magnetic particles (MPs) coated with the target enzyme can be promptly recovered from the reaction medium, resulting in a simple process of enzyme reuse. Wu et al. (Wu et al., 2020) described the covalent immobilization of α-glucosidase onto the aminated MPs surface which was furtherly trapped inside a peek tube by an external magnetic field. Inhibitors from natural sources could be screened from complex natural matrices using the developed approach.

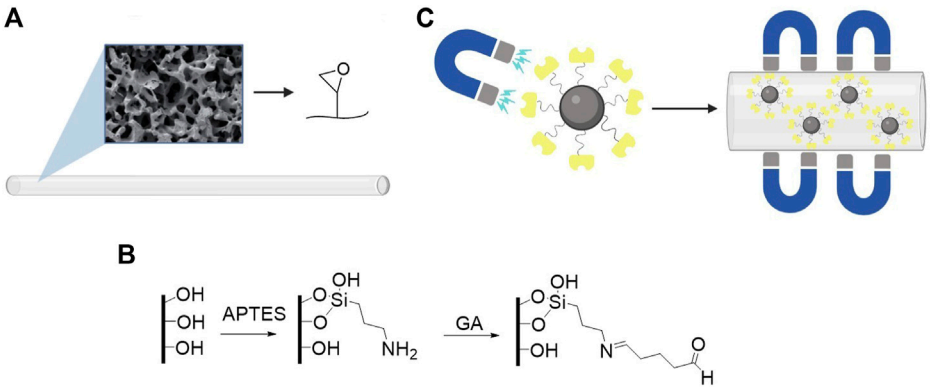

FIGURE 2
Examples of immobilization approach in the production of IMERs for on-flow screening assays: **(A)** Epoxy-based monolith; **(B)** Silica surface and an example of immobilization procedure using GA to form a Schiff base; and **(C)** Magnetic particles coated with the target enzyme are trapped inside a peek tube.

It should be mentioned that offline assays, which can employ different analytical techniques to monitor the protein-ligand interaction, are compatible with particularly interesting supports, such as nanotubes (Wang et al., 2015), zeolite (Tao et al., 2016), and hollow fibers (Zhao et al., 2021). In this review, supports compatible with LC on-flow assays are discussed.

5 On-flow screening methods

On-flow assays encompass analyses involving analyte separation, purification, identification, and quantification in a single, continuous, and automatized process (Calleri et al., 2021). Based on that, two possible on-flow approaches are applicable for screening assays and will be discussed in this review. The former employs activity-based methods, where the screening process occurs through enzyme activity monitoring. The second one employs affinity-based methods, where the screening process occurs through ligand-enzyme interactions.

The choice of approach to be explored in the development of an on-flow screening method essentially depends on the characteristics of the biological target and the assay purposes. When the biological target has a well-established catalytic function, activity-based assays provide a direct response to the interaction between the potential ligands and the catalytic site. In other cases, a broader approach, such as affinity-based assays, provides quick evidence of the interaction between the whole structure of the biological target and the potential ligands. Therefore, the desired modulating effect should be furtherly verified. Furthermore, when the interest of the screening assay is to isolate a ligand from a complex mixture, affinity-based approaches are essential.

The simplest analytical setup for on-flow screening assays employing HPLC encompasses the direct coupling of an IMER to a selective detector (Figure 3A). Mass spectrometers, for example,

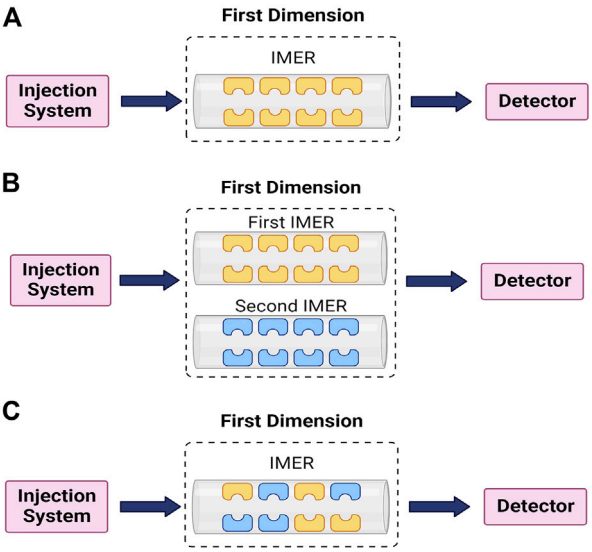

FIGURE 3
Simplified LC systems representations for on-flow screening assays with selective detector and absence of a separation step. **(A)** LC system with the IMER directly coupled to a selective detector. **(B)** LC system with two IMERs in parallel coupled to a selective detector. **(C)** LC system with the two different enzymes IMER directly coupled to a selective detector.

can distinguish enzymatic product, substrate, and inhibitor, even if they coelute, without further purification or separation steps, once the IMER does not possess any chromatographic resolution. The main advantage of this approach is fast analysis. However, in screening assays, the evaluated compound (potential inhibitor) coelutes to the quantified enzymatic product, which can result in signal suppression at the ionization source, making necessary to conduct routine control experiments.

Butyrylcholinesterase (BChE) was covalently immobilized into silica-fused capillaries by Vilela et al. (2018). The IMER obtained was coupled to a mass spectrometer and the enzyme activity was evaluated by monitoring the choline precursor ion in an analysis of only 3 min. IC_{50} and K_i of two known inhibitors (tacrine and galanthamine) were determined to validate the assay in ligands recognition.

Posteriorly, Seidl et al. (2019) described a simultaneous on-flow dual parallel enzyme assay based on two different IMERs containing AChE and BChE enzymes (Figure 3B). Both IMERs were inserted in parallel and placed between an LC system and a mass spectrometer. The resulting system allowed monitoring of the activity of both enzymes with only one single injection and in less than 6 min. Inhibition studies were conducted using galanthamine as a known inhibitor, through IC_{50} and K_i determination.

More recently, Vilela et al. (Ferreira et al., 2021) described the co-immobilization of beta-secretase1 (BACE1) and AChE in the same silica-fused capillary (Figure 3C), yielding a dual enzymatic assay by LC-MS. Galanthamine (AChE inhibitor) and β-secretase inhibitor (BACE1 inhibitor) were used to validated the analytical platform for screening of ligands. Each target enzyme exhibited selectivity and specificity for its substrate and inhibitor, and the co-immobilization did not affect the affinity of AChE and BACE1 for its ligands.

Other models of LC-based on-flow screening assays allow the use of more affordable detectors such as UV. This approach must include a second dimension in the equipment setup, which provides analytical separation of the residual substrate and the formed product. Despite requiring more sophisticated instruments, these assays can be considered more reliable for screening purposes because the detection of the product, and its subsequent quantification, is less susceptible to interferences than the previously mentioned methods.

Within this context, Magalhães et al. (2016) described the covalent immobilization of Nucleoside triphosphate diphosphohydrolase (NTPDase) into silica-fused capillaries. Activity assays were conducted using a multidimensional chromatographic method with a C8 column in the second dimension to provide the analytical separation of the substrates and products (Figure 4A). The described method was successfully employed in the evaluation and characterization of suramin as NTPDase inhibitor.

An online ligand fishing assay for screening AChE inhibitors was reported by Wang et al. (2018). AChE was immobilized on methacrylate-based monolithic capillaries and inserted in an LC-MS system for ligand fishing and identification from mixtures. To distinguish true ligands from false positives, a negative control-IMER was inserted in parallel to the IMER in the system (Figure 4B). Eight compounds were identified as AChE ligands in extracts of *Corydalis yanhusuo*, and their AChE inhibitory activities were further determined using an *in vitro* enzymatic assay.

More recently, an innovative platform for ligand screening in natural products that do not require pre-treatment steps of the sample was described by Seidl et al. (2022). An analytical column inserted in the first dimension (Figure 4C) provided the online microfractionation of the extract components, while the AChE-IMER in the second dimension yielded the inhibition profile of each chromatogram zone. As proof of concept, three known AChE inhibitors (tacrine, galanthamine, and donepezil) and an ethanolic extract obtained from the dry bulbs of *Hippeatrum calyptratum* were investigated. Specific regions in the chromatogram of the crude extract exhibited AChE inhibitory activity, demonstrating the method's capability to eliminate previous fractionation steps.

5.1 Activity-based assays

Activity-based on-flow assays are fundamental for the kinetic characterization and stability studies of the immobilized enzyme. When applied to ligand screening assays, they provide direct data regarding the modulation of the catalytic activity of the target enzyme by a ligand and, in a second moment, can furnish the inhibition mechanism and constants (K_i) involved in the enzymatic process. Moreover, the zonal elution mode allows the use of minimal amounts of samples per injection.

For screening purposes, after the initial characterization of the immobilized enzyme, known inhibitors are frequently evaluated as proof of concept. Rodrigues et al. (Rodrigues et al., 2020) developed a screening assay for the identification of xanthine oxidase (XO) inhibitors using an IMER-MS/MS platform. Kaempferol, a competitive XO inhibitor, was used as a known inhibitor as proof of concept and exhibited an IC_{50} of 4.50 μmol/L. After that, the new screening assay was applied in the evaluation of a library containing thirty natural compounds as potential inhibitors. Each chromatographic process took approximately 5 min. Dihydroartemisinin and artesunic acid were found to be strong XO inhibitors with IC_{50} of 1.90 μmol/L and 1.77 μmol/L, respectively. Pfaffic acid was identified as a weak inhibitor (IC_{50} = 57.90 μmol/L).

Recently, after gathering information about the *Mt*PNP-IMER stability, de Castro et al. (de Castro et al., 2022) conducted kinetic studies to access the K_M value and understand the binding parameters of the substrate regarding the immobilized enzyme. In brief, low K_M values stand for strong interactions between enzyme-substrate. The obtained values for free enzyme were 58.5 μmol/L and 40 μmol/L for inorganic phosphate and inosine substrates, respectively. While 748.6 μmol/L and 59.19 μmol/L were obtained for the IMER regarding the same substrates, respectively. That data pointed significant influence of the immobilization procedure and on-flow conditions over the enzymatic behavior against inorganic phosphate. Moreover, the authors also characterized the inhibition pattern of an Imucillin derivative (DI4G) as a

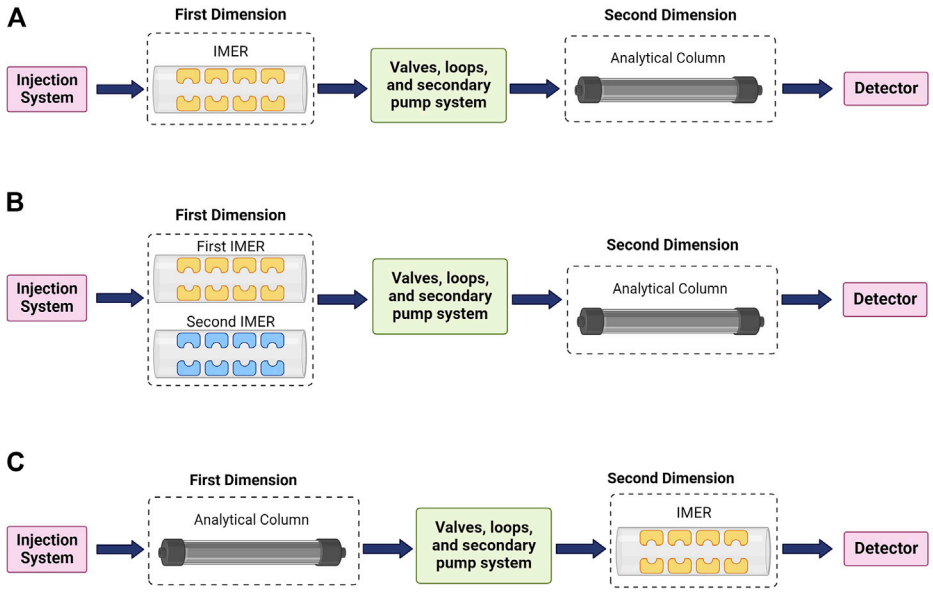

FIGURE 4
Simplified LC system representations for on-flow screening assays with emphasis on the dimension dispositions. **(A)** LC system containing an IMER in the first dimension coupled to an analytical column. **(B)** LC system with two parallel IMERs (a control and an AChE-containing IMER) in the first dimension and a separation column in the second one. **(C)** LC system setup to perform online microfractionation before inhibition assay.

competitive inhibitor. Lastly, the inhibition constant (K_i) of 34.8 nmol/L extracted from the plot, pointed out the selectivity of DI4G towards *Mt*PNP compared to *Hs*PNP, once K_i (*Hs*PNP) = 64.3 nmol/L.

5.2 Affinity-based assays

Affinity-based assays evaluate the binding events between the target biomolecule and a ligand. In a general form, the interactions can occur in any region of the enzyme, in an active site or not (not specific binding). Affinity-based assays can furnish the isolation of bioactive compounds from complex mixtures, the ligand ranking according to the strength of the interaction ligand-enzyme, and the determination of affinity constants. On-flow affinity-based assays encompass three different approaches: frontal affinity chromatography (FAC); zonal affinity chromatography (ZAC); and ligand fishing, as illustrated in Figure 5.

5.2.1 Frontal affinity chromatography

Frontal affinity chromatography (FAC) is an affinity chromatography technique capable to quantify the weak interactions between a ligand and a target through breakthrough curves, allowing determination of the dissociation constants (K_D), ranking ligands in a mixture, determination of the number of available binding sites, and investigation of binding sites.

This technique is based on the dynamic equilibrium state between the immobilized biological target and the ligand. In this assay, the mobile phase contains the ligand at a specific concentration, A_0. Since there is a continuous infusion of ligand into the affinity column, the chromatographic response is equivalent to a front followed by a plateau. In other words, at the beginning of the analysis, the eluting ligand interacts with the target promoting a delay. After that, a saturation of the active sites for binding is reached and from this point on there is no alteration in the ligand concentration eluting from the affinity column. A problematic point of this technique involves a large amount of sample required for the ligand's continuous infusion into the system (Kasai et al., 1986; Calleri et al., 2011; Kasai, 2021).

FAC assay provides valuable information regarding the bioaffinity column and the binding event, such as the dissociation constant (K_D), the number of active sites (n) present in the IMER, and the number of ligands interacting with the target (n_L). Exploring this approach, angiogenesis inhibitor Kringle 5 (Bian et al., 2015), TEM-1 beta-lactamase (Chen et al., 2017), trypsin (Qian et al., 2019), thrombin (Yang et al., 2017) and many other proteins were successfully studied. These parameters give information that allows ranking the ligands regarding their binding affinity to the biomolecule proving to be a useful tool to better understand binding events. Moraes et al. (De Moraes et al., 2014) proposed an assay using a mixture of inhibitors with different IC_{50} for

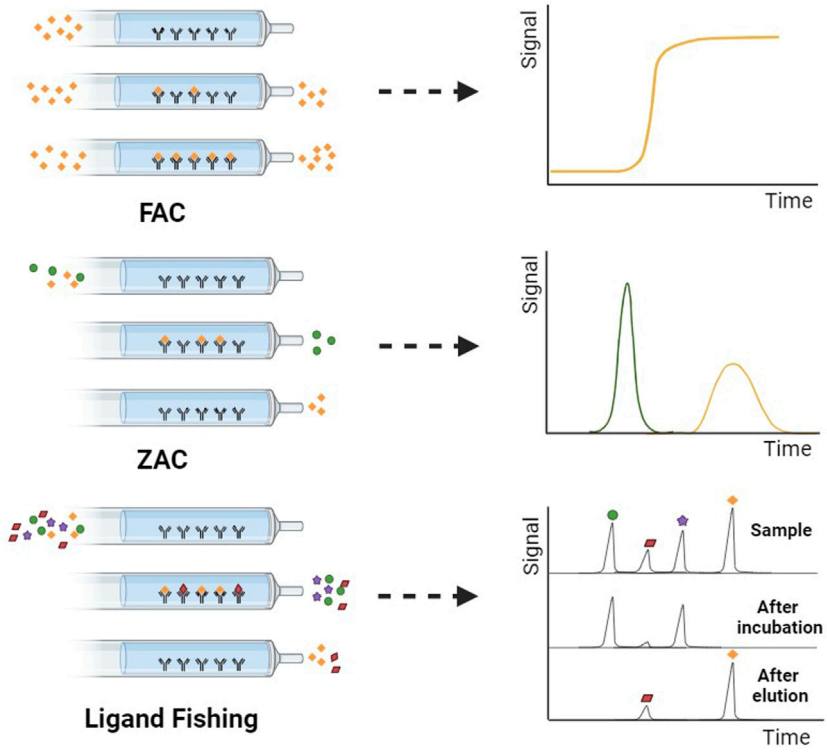

FIGURE 5
Representation of the main affinity-based chromatography.

human purine nucleoside phosphorylase inhibition to rank and point out ligands in a mixture. The developed system besides providing insight into interactions could also be used as a screening methodology for human purine nucleoside phosphorylase inhibitors.

5.2.2 Zonal affinity chromatography

Zonal affinity chromatography (ZAC) requires the injection of small quantities of the ligand. The introduction of potential ligands into the bioaffinity column allows binding events to take place, resulting in the increased retention time of substances that can interact and bind to the immobilized target. Likewise, the compounds that have no affinity for the target flow through the column and exhibited shortened retention times. In this assay, it is possible to obtain data regarding the affinity between ligand and biomolecule and the influence of external factors (such as temperature and mobile phase composition) in the ligand-target interaction (Chaiken, 1986, 1987). The extension and strength of interactions between the ligand and the biomolecule will affect the ligand's retention time. These alterations in experimental data can be translated to the retention factor (k), which can be described as a function of retention time and void time. Based on these relationships it is possible to obtain qualitative and quantitative information in a ZAC experiment. Different equations can be applied to determine binding extension, binding strength, characterization of binding sites, determination of the type of interaction, and other possibilities (Hage, 2017; Tao et al., 2018; Lecas et al., 2021).

Shi et al. (2020) optimize the thrombin immobilization into amino-functionalized silica gel to apply this affinity stationary phase in the screening of ligands by ZAC. After validating the methodology with a selective known inhibitor, the system was used to recognize active components present in the *Radix Salviae Miltiorrhiae* extract with potential anticoagulant activity. Three compounds (Cryptotanshinone, dihydrotanshinone I, and tanshinone IIA) were identified as thrombin inhibitors. In order to verify the biological activity, these three potential binders were isolated and tested by an anticoagulant *in vitro* experiment to confirm their response as thrombin inhibitors.

5.2.3 Ligand fishing

Ligand fishing assay is based on the affinity selection of a ligand from a complex sample by an immobilized biological target. This screening assay comprises 3 different steps: 1) Incubation—a bioactive separation, in which the biological target is incubated with the library containing the potential ligands, allowing the selective formation of ligand-target complexes; 2) Wash—once "fished out" the ligands, the

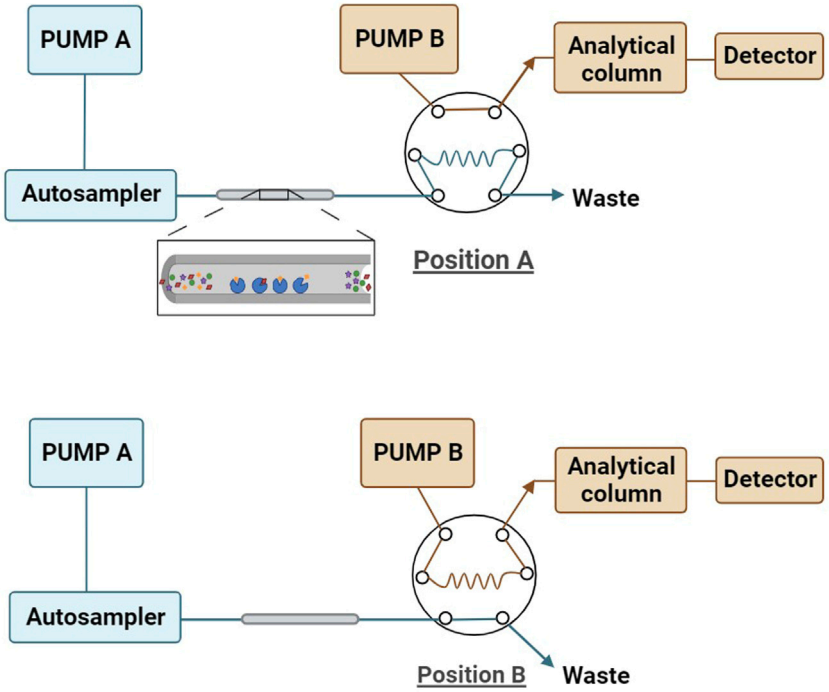

FIGURE 6
Representation of valves for an online ligand fishing assay.

ligand-target complex is extensively washed, so it possible to elute any ligand that weakly interacts with the biomolecule; 3) Elution—after the separation of the ligand-target complex from the unbound compounds via dialysis, magnetic extraction, ultrafiltration, etc.; the ligands are eluted and characterized by different analytical techniques.

Ligand fishing assays can be performed in offline and online mode when coupled to liquid chromatographic systems. It is possible to find studies that perform the ligand fishing assay but do not use this term to define the methodology. Sometimes, terminologies such as magnetic/affinity solid phase extraction and magnetic microbead affinity selection screening (MagMASS) are used to describe screening experiments that are based on the same principle of ligand fishing.

In 2007, Moaddel and collaborators (Moaddel et al., 2007) intended to use a new support—magnetic beads—for the immobilization of a model target, Human Serum Albumin (HSA) to perform an offline ligand fishing assay. Briefly, the immobilized target was incubated with a solution containing ligands and non-ligands. After magnetic separation, the supernatant was collected, and the HSA-coated magnetic beads were washed twice with a buffer solution. Then the supernatant was removed, and a mixture of buffer and the organic solvent was used to elute the retained compounds (with affinity for the HSA). The article describes a simple and effective assay to assess the capacity of HSA to differentiate known ligands from non-ligands in a mixture, and, specially, to demonstrate the potential of immobilizing the target onto the magnetic support. The application of magnetic beads as support for ligand fishing assay became an eminent choice of support (Trindade Ximenes et al., 2021). One reason for that relies on its execution, the target can be easily recovered by an external magnetic field.

However, the transposition of these principles to an on-flow system requires a more complex apparatus, which unfortunately represents more costs. The use of switch valves and loops allows the implementation of affinity assays in one dimension and chromatographic separation in another. Since the incubation occurs in an independent dimension, the unbound components present in the sample can be directed to waste, as illustrated in Figure 6. In Position A (Figure 6), the sample is inserted into the affinity column to allow the interaction between ligands and target, while in the second dimension the analytical column is being conditioned using pump B. Switching valves to Position B (Figure 6), the second configuration, the retained ligands are eluted and transferred to the analytical column for separation and identification. The change in valve position allows that first and second dimensions operate at the same time on different mobile phase compositions and flow rates. In a general mode, these are the steps of this affinity-based assay, however, valves and loops can differ from each experiment. Therefore, exists a diverse number of system setups to perform this assay.

The on-flow method proposed by Peng et al. (2016) employed four-port and six-port valves to screen and identify xanthine oxidase inhibitors from *L. macranthoides* extract. In the optimization process of the ligand fishing assay, it was established that methanol was the adequate solvent to elute the retained compounds. Even though it was used as an organic solvent for the elution of the retained ligands, the xanthine oxidase enzyme remained with high activity (96%) after ten consecutive cycles. The immobilization promoted better chemical stability to the target allowing its reuse.

Fu et al. (2014) developed an online ligand fishing assay using the free target, i.e., xanthine oxidase was not immobilized on a solid support. This approach was made possible by the ability of turbulent flow chromatography to retain small molecules and allow the easy elution of big structures, such as biomolecules. In this multidimensional setup was necessary the usage of three columns: in the first dimension, the first turbulent flow column separated the unbounded components of the *S. miltiorrhiza* from the ligand-target complex. The complex flowed to a loop to elute the binders and the eluent was inserted into the second turbulent flow column, where the small molecules (ligands) were retained, and the target easily eluted the column. After separation, the retained ligands were introduced in the third column to be chromatographically separated and identified. This 2D-affinity chromatography was able to recognize three potential inhibitors for the xanthine oxidase, in which two of which confirmed their inhibitory activity.

A different strategy for the ligand fishing assay was proposed by Tao et al. (2013). Three different targets (maltase, invertase, and lipase) were immobilized onto different magnetic particles. After that, permanent magnets were used in chambers containing the MPs coated with each target and connected in series. The developed multi-target affinity selection assay did not use valves; the samples were inserted by a peristaltic pump in a continuous flow. This simpler approach was able to identify seven ligands from a Traditional Chinese Medicine (TCM) plant extract for those three targets. Inhibition studies demonstrated that five out of seven ligands act as enzyme inhibitors.

The improvement of screening assays not only aims to provide reliable data but also to effectively contribute to shortening the time spent in the discovery of new bioactive compounds. The advances in technology demand investments and these online methodologies are no different, usually, they involve relatively expensive accessories and instruments which can be a major drawback to the usage of these techniques.

6 Conclusion

Regardless of the sample matrices, on-flow systems have been applied to screen potential inhibitors from a variety of targets. Most of the assays used solid-supported enzymes, and, therefore, this review presented the most relevant characteristics of the immobilization process, mentioning the advantages and drawbacks of each methodology always highlighting the most recurrent solid supports and immobilization types.

The versatility of on-flow setups was continually discussed through the many examples presented. Some recent studies enable to conduct automated selectivity studies concurrently with the inhibition assays, or even use two enzymes as biological targets concomitantly. Other setups encompass online microfractionation of complex matrices with subsequent evaluation of the inhibitory profile of each microfraction. These advances, as well as other examples discussed in this review, are examples of the emerging success of liquid-chromatography on-flow assays to screen and identify new inhibitors. The coupling of the separation capacity of the liquid chromatography technique with the high selectivity of ligand-target interaction furnishes a high throughput and reliable screening assay.

All bioassays described have proven to be powerful tools in the discovery of new inhibitors from natural or synthetic sources. The on-flow approach still has a lot to explore, new targets, and configurations. This shows that apart from the benefits there is still a lot of potential in studying this field.

Author contributions

PD designed the review, wrote the section about affinity-based assays and revised the whole manuscript. RL wrote the section about solid supports, on-flow screening assays, and activity-based assays. MC wrote about the methods for enzyme immobilization. CW wrote the introduction and the enzymes as special targets section. MD discussed the structure of the manuscript and contributed to writing the whole manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Marianne Fillet,
University of Liège, Belgium

REVIEWED BY
Roman Reminek,
Institute of Analytical Chemistry (ASCR), Czechia
Leandro Augusto Calixto,
Federal University of São Paulo, Brazil

*CORRESPONDENCE
Sebastiaan Eeltink,
sebastiaan.eeltink@vub.be

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Making high salt concentrations for optimal chromatography compatible with electrospray ionization mass spectrometry using an ion exchange membrane suppressor: Analysis of biomarkers for transporter protein inhibition as a case study

Sam Wouters[1,2], Ils Pijpers[3], Ninon Vanden Haute[1], Daniel Meston[1], Lieve Dillen[3], Filip Cuyckens[2] and Sebastiaan Eeltink[1]*

[1]Vrije Universiteit Brussel (VUB), Department of Chemical Engineering, Brussels, Belgium, [2]Janssen R&D, Drug Metabolism and Pharmacokinetics, Beerse, Belgium, [3]Janssen R&D, Bioanalysis Discovery and Development Sciences, Beerse, Belgium

This study reports on the potential of using ion-exchange suppressor technology in liquid chromatography—electrospray ionization mass spectrometry workflows. The aim was to use high salt concentrations to improve separation performance, while overcoming the resulting significant ion suppression during electrospray ionization. As a case study, we apply suppressor technology to the hydrophilic interaction liquid chromatography separation and detection of taurine and glycochenodeoxycholate sulfate, endogenous biomarkers for organic anion transporter protein inhibition. The desired chromatographic selectivity was achieved applying 100 mM ion-pairing agent, while competing ions negatively affecting MS sensitivity were actively removed post-column from the solvent *via* a charged partially permeable membrane and replaced with protons, resulting in an up to 10-fold increase in detection sensitivity.

KEYWORDS

ion suppression, biomarker analysis, organic anion transporter proteins, MS sensitivity, ion exchange suppressor

Introduction

Ion suppression in electrospray ionization is a major limitation in the techniques applicability, which is also very relevant for hydrophilic interaction liquid chromatography (HILIC) (Mallet et al., 2004; Volmer and Jessome, 2006). HILIC is based on partitioning of analytes between a water-enriched layer of the mobile phase that is immobilized on to a polar stationary phase and the low aqueous/highly polar organic mobile phase (Dejaegher et al., 2008; McCalley, 2017). The presence of buffer salts or ion-pairing agents in the mobile phase are routinely used to tune the retention behavior of charged analytes allowing to improve chromatographic resolution. While HILIC usually enjoys high detector response due to the volatility of the effluent, the increased ionic eluent strength tends to limit MS sensitivity due to charge competition during electrospray ionization (ESI). To address this long-standing limitation, our group recently explored the use of a membrane-based microfluidic chip for selective exchange of trifluoroacetate in HILIC and reversed-phase liquid chromatography (RPLC) mobile phases with formate and propionate prior to MS detection, aiming at greatly reducing adduct formation and significantly improving MS sensitivity in protein and peptide analysis (Wouters et al., 2021). The concept is based on suppressed conductivity detection, commonly used in ion chromatography, where selective post-column exchange of ions from mobile phases is achieved using so-called suppressor or stripper devices, prior to conductivity detection (Wouters et al., 2017; Wouters et al., 2019). In this work, we explore the use of high salt concentration solvent modifiers to tune resolution and subsequently mitigate the MS incompatibility of high molar ammonium acetate applying a post-column mobile-phase modulation approach.

We exemplify from a case study where the concept is applied to the separation of taurine and glycochenodeoxycholate sulfate (GCDCA-S), which are both highly polar, hydrophilic compounds and are therefore incompatible with conventional RPLC. As such, HILIC is better suited to separate these compounds, which are under investigation in the context of drug-drug interactions (DDI). Drug transporter proteins in the liver and kidney play an important role in the uptake and elimination of a wide range of chemicals. The inhibition of these transporters may cause significant DDI, which may increase the risk of adverse events through altered exposure of the combination of drugs (Palleria et al., 2013). Given the growing relevance of polypharmacy, and the increased attention for DDI from the regulatory agencies, the potential for DDI needs to be assessed in drug development as part of the safety program. Traditionally, *in vitro* DDI evaluations first identify any potential risk followed by clinical DDI studies with probe substrates for the transporter flagged *in vitro* (Chu et al., 2018). However, an emerging approach has been presented making use of endogenous biomarkers as read-out for *in vivo* DDI. Several endogenous compounds that can be measured in urine and plasma, have been evaluated as substrates for drug transporters in the liver and kidney (Tsuruya et al., 2016). Previous observation demonstrates that inhibition of two major renal organic anion polypeptide transporters (OAT1 and OAT3) decreases the renal clearance of taurine and GCDCA-S significantly, suggesting these compounds may serve as diagnostic biomarkers of OAT inhibition. While taurine and GCDCA-S are opportune analytes for monitoring OAT activity, they are not without analytical challenges.

Materials and methods

Chemicals and materials

Taurine, acetonitrile (ACN, LC-MS grade), ammonium acetate, ammonium hydroxide, and 10% sulfuric acid were purchased from Sigma-Aldrich (Bornem, Belgium). GCDCA-S, taurine-D4 and GCDC-D4 were obtained at Toronto Research Chemicals (Toronto, Canada). An Acquity UPLC BEH Amide 50 × 2.1 mm i. d. column packed with 1.7 µm particles was purchased from Waters (Milford, MA, United States). A planar dual-membrane chemically-regenerated anion suppressor fitted with sulfonated cation-exchange membranes (ACRS500) was purchased at Thermo Fisher Scientific (Sunnyvale United States).

QC sample preparation and processing

Since taurine and GCDCA-S are endogenously present in plasma (target matrix), Milli-Q water was used as a surrogate matrix for the calibration curve. Combined calibration samples for both analytes were prepared to analyze a range from 200 to 50,000 ng/ml for taurine and 20.0–5,000 ng/ml for GCDCA-S. Combined QC samples were prepared by spiking native pooled K_2EDTA human plasma, at a concentration of 20,000 ng/ml for taurine and 2,000 ng/ml for GCDCA-S. These QC samples were serially diluted in pooled K_2EDTA plasma. Together with the spiked QC samples, the pooled human plasma used for the preparation of the QC samples, was analyzed for the presence of endogenous concentrations. The nominal values of the QCs were calculated as the sum of the endogenous level plus the spiked amount. To minimize the matrix effect and avoid saturation of the detector of the mass spectrometer, all samples were 10-fold diluted with Milli-Q water before protein precipitation with 10-fold volume of acetonitrile. For taurine, a stable isotope labeled internal standard was available. For GCDCA-S the deuterated analogue of GCDC was used as structural analogue internal standard.

For parallelism evaluation, the endogenous concentration of the blank plasma used for the preparation of the QC samples was determined by interpolation of the response of the QC blank to

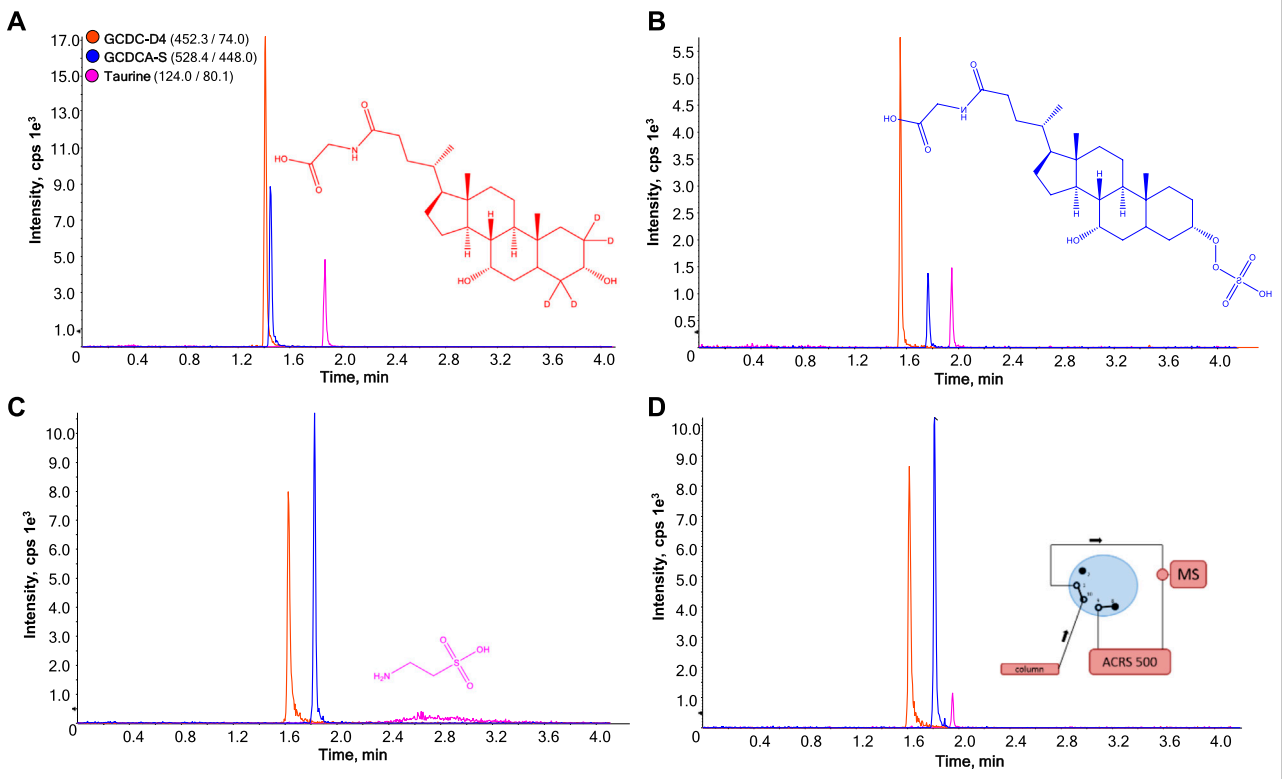

FIGURE 1
Effect of amount of ion pairing agent on HILIC-ESI-MS analysis of GCDC-D4 (1), GCDCA-S (2), and taurine (3) without **(A,B)** and with **(C,D)** on-line membrane suppression of ammonium acetate. The ammonium acetate concentrations are 10 mM **(A)** or 100 mM **(B, C,** and **(D)**. In **(D)** GCDC-D4 and GCDCA-S pass though the membrane suppressor prior to MS detection, whereas taurine is directed to the MS bypassing the suppressor by employing a heart-cutting approach programmed at 1.9 min.

the calibration curve in surrogate matrix. Secondly, a linear regression with a 1/x2 weighing was performed using the spiked concentration of analyte of the QC sample and the peak area ratios obtained. The endogenous level of the QC was determined by dividing the intercept with the slope of the curve. Parallelism is considered proven if the difference between both values is less than 20.0% and −20%.

LC-MS instrumentation and settings

Separations were performed on a Shimadzu LC30 system (Shimadzu, Kyoto, Japan). Mobile phases consisted of 10 mM or 100 mM aqueous ammonium acetate (A), and ACN as the organic modifier (B). A binary pump was used to deliver a solvent gradient from 95% to 50% B from 0.3 to 3.5 min at a flow rate of 0.6 ml/min. The BEH amide column was thermostatted in a column compartment at 50°C. The suppressor was installed after the column prior to the MS detector and a 12.5 mM sulfuric acid in 10% ACN as regenerant flow was applied at 1.2 ml/min in counter flow using an isocratic pump (Shimadzu). A 10-port, 2-position switching valve (VICI, Schenkon, Switzerland) was used to bypass the suppressor. Detection was performed on an API 4000 triple quadruple MS (AB Sciex, Toronto, Canada) equipped with an electrospray ionization interface. LC-MS/MS was performed in the negative ionization mode at −4.5 keV with single reaction monitoring of the Q1/Q3 transitions at m/z: 452.3/74.0 for GCDC-D4, 528.3/448.3 for GCDCA-S and 124.0/80.0 for taurine and 128.0/80.0 for taurine-D4 while applying declustering potentials of −100/−100/−45 V, collision energies −42/−42/−28 and collision cell exit potentials −25/−25/−5 V, for GCDC-D4/GCDCA-S/taurine (−D4), respectively. Data acquisition and analysis were controlled using Analyst 1.7 software (AB Sciex, Toronto, Canada).

Results and discussion

HILIC-ESI-MS method development and use of post-column modulation

The incorporation of internal standards for quantitative analysis is recommended to adequately control experimental drift and correct for detector response variability. As such,

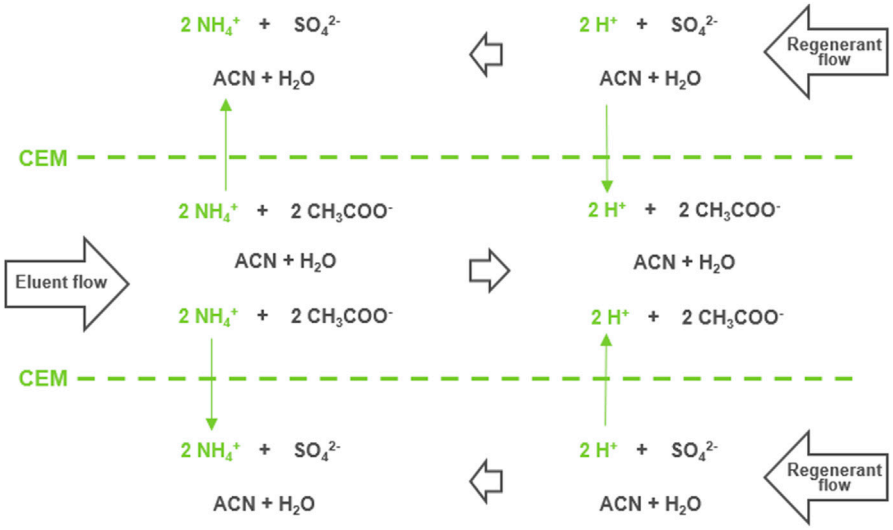

FIGURE 2
Schematic representation of the exchange process taking place in the planar double-sided flow-through membrane suppressor which features a central eluent channel, separated by cation-exchange membranes from a regenerant channel above and below.

isotopically labelled GCDC-D4 was included in the sample mixture as a structural analogue of GCDCA-S and taurine-D4 as stable isotope labeled internal standard for taurine. In the calibration samples prepared in MilliQ water, GCDC-D4 did not fully compensate for the matrix difference between the surrogate matrix and target matrix when applying 10 mM ammonium acetate. Altering the concentration from 10 mM to 100 mM resulted in an improved matrix effect compensation. Figures 1A,B show a representative chromatogram of the HILIC-ESI-MS analysis while applying 10 mM and 100 mM ammonium acetate to the mobile phase, respectively.

While improving resolution, increasing the ammonium acetate content led to a sharp 6.4-fold reduction in MS sensitivity due to ion suppression for GCDCA-S. Taurine was less sensitive to changes in retention but also undergoes substantial (3.2-fold) reduction in MS sensitivity. Figures 1C exemplifies the effect when integrating a cation-exchange membrane suppressor post-column prior to the ESI interface, targeting the depletion of ammonium ions from the HILIC mobile phase. The exchange in the membrane suppressor schematically shown in Figure 2. Positively charged ammonium ions from the effluent flow diffuse through the partially permeable negatively-charged membrane into the regenerant flow and protons from the regenerant flow diffuse into the effluent flow converting ammonium acetate to acetic acid. A dilute solution of a strong acid (sulfuric acid) was applied in the regenerant as a source of protons. The regenerant runs countercurrent with respect to the eluent flow at twice the flowrate to maximize the concentration gradient and thereby promote the complete conversion of ammonium acetate to acetic acid. Details on construction and functioning of this type of suppressor can be found in a recent review by Haddad *et al.* (Wouters et al., 2017). For details on how to further influence the exchange rate in such concentration-gradient-driven devices, we refer to earlier work (Wouters et al., 2016; Wouters et al., 2021). 10% acetonitrile was added to the regenerant to prevent loss of analytes due to interaction with the polymeric membrane.

Applying 100 mM ammonium acetate and integrating post-column modulation prior to MS led to a substantial increase in the detector response for GCDCA-S, presenting a 6.9-fold increase in peak height and 10.2-fold increase in peak area, despite an increase in peak width by a factor 1.4, which is to be expected with the added dead volume of the suppressor. However, the peak shape of taurine was strongly distorted, likely caused by the zwitterionic character of taurine and consequently, ionic interactions between the positively charged amine on taurine and the negatively-charged membrane. Post-column infusion of 10% v/v ammonium hydroxide between the column eluent and the ion-stripper lead to deprotonation of the carboxylate of taurine, inducing ionic repulsion of taurine from the negatively charged membrane. This led to a strong decrease in tailing factor but also a decrease in the intensity of taurine, again due to competitive ion-suppression in ESI from the additional ammonium ions (result not shown). To overcome the chemical incompatibility of taurine with the ion-stripper a flow diverter valve was integrated, allowing taurine to selectively bypass the ion-stripper, as demonstrated in Figures 1D. This led to a substantial decrease in tailing factor. Limitations would be that such approach is only possible when sufficient retention-time difference is established between both compounds, as is the

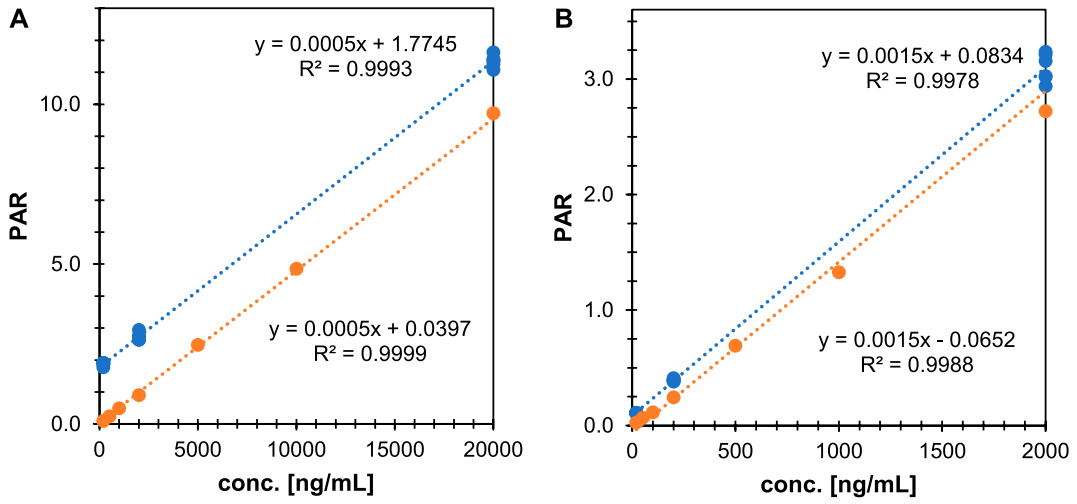

FIGURE 3
Parallelism evaluation of the HILIC-suppressor-ESI-MS with heart-cut of taurine for the evaluation of linearity, precision, and accuracy of **(A)** taurine and **(B)** GCDCA-S in QC samples in human plasma (blue circles) as well as surrogate matrix samples (orange circles).

case in our experiment, and taurine will still suffer to some extent from ion-suppression, as it is still eluting at higher concentrations of ammonium acetate.

Determination of the suitability of the methodologies for quantitative analysis

While taurine and GCDCA-S have been analyzed previously by LC-MS as potential endogenous biomarkers (Peng et al., 2014; Tsuruya et al., 2016; Shen et al., 2017; Horvath et al., 2020), they have not been validated for use in clinical studies. As GCDCA-S and taurine are endogenously present in plasma the calibration curve cannot be made in this matrix, instead water was selected as surrogate matrix. For the optimized method, we determined the robustness, linearity, and precision and confirmed the parallelism between the calibration samples and the authentic analyte in the biological matrix. The accuracy and precision were evaluated with QC samples in six-fold and the dilution integrity shows whether a dilution of the QC samples in water can be used. The criteria for a successful robustness run are: 80.0% ≤ accuracy ≤ 120.0%, 0% ≤ coefficient of variation (CV) ≤ 20.0%. Figure 3 shows the calibration lines for a standard curve in the surrogate matrix as well as for QCs spiked in plasma. Quantitative analysis of taurine and GCDCA-S with the proposed assay could fulfill regulatory acceptance criteria, with taurine determined between 200–50000 ng/ml and GCDCA-S between 20–5,000 ng/ml. For both compounds the accuracy was within criteria (taurine: 95.4%–100.0% and GCDCA-S:100.0%–115.8%) and the %CV was maximum 6.2% for taurine and 3.8% for GCDCA-S. The parallelism between the surrogate matrix and human plasma was -9.0% for taurine and −7.1% for GCDCA-S evaluated as the difference in the endogenous concentrations obtained with surrogate matrix and the target matrix. The method was qualified for the quantification of taurine and GCDCA-S concentrations in plasma originating from clinical studies.

Concluding remarks

Herein, we have provided a proof-of-concept showing the advantages one can expect when implementing suppressor technology for the analysis of taurine and GCDCA-S, diagnostic biomarkers for OAT inhibition. Membrane-suppressor technology can be easily integrated into existing LC-MS workflows and has the potential to provide dramatic increases in analyte detector response in the case MS detrimental mobile phase conditions are used. As such, suppressors may provide a facile methodology to implement in methods applying less “MS-friendly” buffers allowing one to tune selectivity using high concentration ion-pairing agents, without compromising MS detection sensitivity. A current limitation was observed when applying the concept to a zwitterionic compound, where unwanted interactions with the membrane introduced retention in the suppressor. We hypothesize that the alteration of membrane properties would allow suppressors to be tuned to give optimal signal amplification for specific analytes. Additionally, post-column buffering of the pH would provide flexibility when analyzing zwitterionic compounds, such as amino acids, opening several interesting opportunities in proteomic applications as well as for oligonucleotide analysis. The addition of a simple diverter valve allows to switch the application of the suppressor chip between well separated analytes within one chromatogram.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

SW, IP, LD, FC, and SE contributed to conception and design of the study. SW, IP, NV, and DM contributed to the methodology, analysis and validation. SW and IP wrote the first draft of the manuscript. SW, IP, LD, FC, and SE contributed to the writing of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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EDITED BY
Quezia B. Cass,
Federal University of São Carlos, Brazil

REVIEWED BY
Surabhi Chandra,
University of Nebraska at Kearney, United States
Robert Stephen Phillips,
University of Georgia, United States

*CORRESPONDENCE
Luzineide W. Tinoco,
lwtinoco@ippn.ufrj.br

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Analytical assays to evaluate enzymatic activity and screening of inhibitors for ornithine decarboxylase

Luzineide W. Tinoco*, Bruno Maia da Silva Santos, Jhones Matheus da Silva Soares and Fernanda Gadini Finelli

Instituto de Pesquisas de Produtos Naturais Walter Mors, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to produce putrescine, the first step in the metabolism of polyamines (putrescine, spermidine, and spermine), which are essential growth factors in eukaryotic cells. ODC is active as a homodimer and depends on pyridoxal 5′-phosphate (PLP) as a cofactor. An increase in the concentration of polyamines has been associated with carcinogenesis. Therefore, there is much interest in identifying inhibitors of this pathway as potential chemotherapeutic and chemopreventive agents. The best-known inhibitor of mammalian ODC is α-difluoromethylornithine (DFMO), a highly selective compound that alkylates Cys-360 (a residue of the ODC active site). Although DFMO was initially developed for the treatment of cancer, the World Health Organization recommends its use in combination with nifurtimox for the treatment of human African trypanosomiasis. Considering the importance of ODC as a promising target for the treatment of various types of cancer and other infectious diseases, choosing the right method for screening potential inhibitors can help to accelerate the discovery of new drugs. Several methods for the determination of ODC activity are found in the literature. Among these, we can mention analysis with radioactive markers, colorimetric assays using auxiliary enzymes to detect CO_2 or H_2O_2 release, chromatographic separations with putrescine derivatization, mass spectrometry, and spectroscopic techniques. In this review, the main analysis methods used will be described, highlighting their advantages and disadvantages, as well as identifying the most promising methods for high-throughput screening.

KEYWORDS

radiolabeling, CO_2 release, putrescine oxidation, TNBS colorimetric assay, HPLC, mass spectrometry, spectroscopic assays

1 Introduction

Ornithine decarboxylase (ODC, EC 4.1.1.17) is an enzyme that limits the rate of the polyamine biosynthetic pathway. Polyamines play an important role in the cellular homeostasis of most living organisms, and ODC has been reported in several species (Miller-Fleming et al., 2015; Bateman et al., 2021). ODC, a pyridoxal 5′-phosphate (PLP) dependent enzyme, catalyzes the decarboxylation of L-ornithine to putrescine *via* Schiff base formation (Figure 1). Putrescine is the precursor of spermidine and spermine in the first step of polyamine biosynthesis (Michael, 2016). Elevated expression of ODC, as well as abnormally high levels of polyamines, are correlated with the occurrence of many diseases, including cancer and Alzheimer's (Gerner and Meyskens, 2004; Mäkitie et al., 2010; Miller-Fleming et al., 2015; Somani et al., 2017; Igarashi and Kashiwagi, 2019; Holbert et al., 2022; Ivanov and Khomutov, 2022). Consequently, ODC is considered an oncogenic enzyme and a target for the treatment of several diseases (McCann and Pegg, 1992; Marton and Pegg, 1995; Mukhopadhyay and Madhubala, 1995; Casero and Marton, 2007; Smithson et al., 2010a; Colotti and Ilari, 2011; Chakraborty et al., 2013; Somani et al., 2017; Perdeh et al., 2020; Sweeney, 2020; Khan et al., 2021). ODC is a homodimer in a dynamic equilibrium between the active dimeric form and the inactive monomeric form (Solano et al., 1985; Mitchell et al., 1988; Mitchell and Chen, 1990; Coleman et al., 1994; Chai et al., 2020). The two identical active sites in the homodimer are formed at the dimer interface by residues from the N-terminal domain (Lys69) of one subunit and the C-terminal domain (Cys360) of the other (Tobias and Kahana, 1994; Chai et al., 2020). Lys69 was biochemically identified as the covalent binding site for PLP, while Cys360 is involved in covalent adduct formation with DFMO (α-difluoromethylomithine) (Poulin et al., 1992; Coleman et al., 1994; Grishin et al., 1999). In addition, other important interactions at the binding site have also been described, such as Glu274, which stabilizes the positive charge on the pyridine nitrogen of the PLP, increasing the ability to

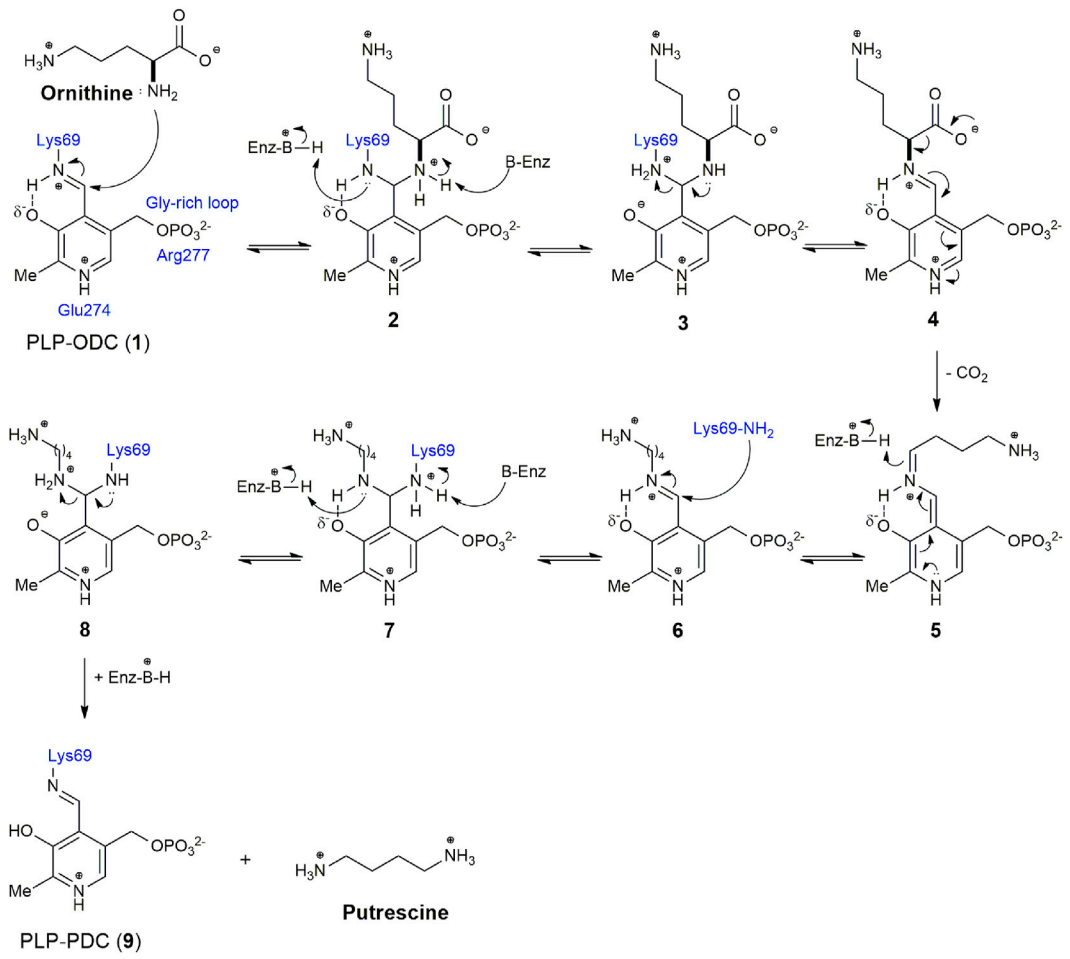

FIGURE 1
Reaction mechanism of Ornithine decarboxylase (ODC) with the pyridoxal 5′-phosphate (PLP) cofactor.

TABLE 1 Methods to assess Ornithine Decarboxylase (ODC) activity and Inhibition.

Method	Advantages	Drawbacks	References
Radiolabeling	Allows monitoring of reaction course. No sample pre-treatment required	Requires labeled ^{14}C substrate or DFMO and a liquid scintillation spectrometer. Expensive for HTS.	Kobayashi (1963), Jones et al. (1972), Beaven et al. (1978), Smith and Marshall (1988), Henley et al. (1996), Vanisree and Shyamaladevi (2006), Schultz et al. (2020)
CO_2 consumption by coupled enzymes (PEPC-MDH)	Allows monitoring of reaction course. It can be performed in an automated system with 384-well microplates. It has already been used to analyze more than 3,000 compounds	Need a commercial system for bicarbonate detection and N_2 atmosphere for analysis The cost of the kit can make analysis by HTS unfeasible	Scriven et al. (1988), Smithson et al. (2010b), Liao et al. (2015), Chai et al. (2020), Smithson et al. (2010a)
Putrescine oxidation: H_2O_2 released - Colorimetric	Produces a colored complex that absorbs at 505 nm, which can be analyzed in a plate reader. Allows monitoring of reaction course	Use of the soy amine oxidase enzyme from the purified soy plant (purification step) or directly from the crude extract (possibility of interference by other enzymes)	Badolo et al. (1999), Das et al. (2015)
Putrescine oxidation: H_2O_2 released -Luminescence	Enables detection of putrescine on a picomolar scale. It can be adapted for luminescence detection in a microplate reader. Allows monitoring of reaction course	It requires an initial step of extracting the phosphocellulose paper. May be interfered with by other polyamines and other enzymes when used with cell extracts	Fagerström et al. (1984), Wang and Bachrach (2000)
Adducts of putrescine with trinitrophenyl groups	Use of cheap and affordable reagents	It takes several steps to prepare the sample, with many sources of errors that depend heavily on the analyst's skills. It only assesses the end point of the enzymatic reaction. The cost of 2,4,6-trinitrobenzenesulfonic acid (TNBS) is high for HTS and not widely available	Luqman et al. (2013), Nilam et al. (2017), El-Sayed et al. (2019), Villa-Ruano et al. (2019), Chai et al. (2020)
HPLC assays	HPLC resolution, specificity and sensitivity	Cost, long analysis time, and the need for laborious sample pre-treatment. It only assesses the end point of the enzymatic reaction	Kabra et al. (1986), Legaz et al. (2001), Kvannes and Flatmark (1987), Bito et al. (2019), Beeman and Rossomando (1989), del Rio et al. (2018), Xiao et al. (2009)
MS assays	MS analysis is fast and reproducible. Detection in the fmol range	Laborious sample pre-treatment. It only assesses the end point of the enzymatic reaction. Expensive equipment	Ducros et al. (2009), Liu et al. (2011), Liu et al. (2011), Gaboriau et al. (2003), Gaboriau et al. (2005)
Circular Dichroism	Allows monitoring of reaction course. No need for special reagents. No sample pre-treatment required	As many organic compounds absorb in the 201 nm region, it cannot be used with mixtures of compounds, nor for screening inhibitors. It cannot be performed on microplates. Expensive equipment and low sensitivity	Brooks and Phillips (1996)
Fluorescence Assays	Allows monitoring of reaction course. Can be analyzed on a microplate reader. No sample pre-treatment required	Low solubility of cucurbit [6]uril (CB6)	Nilam et al. (2017)

withdraw electrons from the ring, and Arg277, which is necessary for the binding of the high affinity of PLP through interaction with the cofactor 5′-phosphate (Figure 1) (Osterman et al., 1995, 1997; Brooks and Phillips, 1997).

Due to its importance in several cellular functions and wide distribution in several species, since the 1970s there has been a great therapeutic interest in the inhibition of ODC, with the consequent depletion of polyamine biosynthesis (Mamont et al., 1978; Metcalf et al., 1978). According to the mechanism of the ODC reaction shown in Figure 1, the enzyme and its reaction can be inhibited by cofactor (PLP), substrate (ornithine), or product (putrescine) analogues. α-difluoromethylornithine (DFMO), an ornithine analogue, is the most important and best-known inhibitor of ODC. DFMO, marketed as Eflornithine, belongs to the WHO Model List of Essential Medicines to treat sleeping sickness (WHO 2022). Although it is not effective for the treatment of cancer alone, it has been widely used in combination with other drugs. It is in several clinical trials as an adjuvant or chemopreventive agent and appears to be highly useful in the treatment of neuroblastoma (Kim et al., 2017; Alhosin et al., 2020; Schultz et al., 2021). In addition to the cofactor, substrate, and ODC reaction product analogues, other classes of inhibitors (benzothiazoles, indoles, bisbiguanide, and dithioamidine) with different binding modes were identified, indicating allosteric sites in the enzyme. The identification of these new classes of inhibitors was made from a high-throughput enzymatic screening with 316,114 different molecules, demonstrating the importance of this methodology for the identification of more selective and potent inhibitors for ODC, with improved pharmacokinetic properties (Smithson et al., 2010a). There are several methods in the literature to assess ODC activity. The most used methods are based on the detection of radiolabeled CO_2 or radiolabeled DMFO linked to ODC, on the capture of CO_2 for the conversion of NADH into NAD with the use of Phosphoenolpyruvate Carboxylase

(PEPC) and Malate Dehydrogenase (MDH) enzymes, on the chemiluminescence or colorimetric detection of H_2O_2 produced by putrescine oxidation by amine oxidases, and in derivatization for analysis by high-performance liquid chromatography (HPLC) or mass spectrometry (MS) and fluorescence. However, we did not find a compilation of existing methods in the literature, which makes it difficult to choose the most appropriate method to assess ODC activity and screening for inhibitors. Therefore, we will make a brief description of the main methods found (Table 1), seeking, whenever possible, to present their advantages and disadvantages and the possibility of application in high-throughput screening (HTS).

2 Methods to assess ODC activity and inhibition

2.1 Radiolabeling

The measurement of $^{14}CO_2$ release from [^{14}C]carboxyl-labeled amino acids is widely used as a method of assay for decarboxylase activity (Kobayashi, 1963). For *in vitro* ODC activity, the usual procedure is to incubate the enzyme sample in a medium with EDTA, sodium/potassium phosphate buffer, pyridoxal phosphate, and the [1–^{14}C]-L-ornithine in a vial sealed with the cap containing the paper discs impregnated with hyamine/sodium hydroxide to capture the release of radiolabeled carbon dioxide. The enzymatic reaction is stopped by the addition of sulfuric/citric acid. After an additional incubation period to complete $^{14}CO_2$ absorption, the caps are removed and transferred to a scintillation vial containing scintillation fluid. Measurements of disintegrations per minute are made in a liquid scintillation spectrometer. The specific ODC activity is expressed as nmol CO_2 released/min/mg protein (Jones et al., 1972; Beaven et al., 1978; Smith and Marshall, 1988; Henley et al., 1996; Vanisree and Shyamaladevi, 2006; Schultz et al., 2020).

Another method to monitor the ODC activity is by using α-[5–^{14}C]Difluoromethylornithine, an irreversible ODC inhibitor that forms a covalent bond with the enzyme. For *in vitro* ODC activity, mouse tissues are treated with α-[5–^{14}C]DFMO and incubated in the enzymatic reaction medium. After precipitation of protein by addition of acid and centrifugation, the pellet is dissolved in hydroxide sodium solution, mixed with scintillation fluid, and has the radioactivity counted in a liquid scintillation spectrometer (Seely et al., 1982). For *in vivo* controlling, the labeled ODC can be monitored by autoradiographic examination of mouse kidneys or livers after administration of α-[5–^{14}C]DFMO (Pegg et al., 1982; Pösö and Pegg, 1983; Zagon et al., 1984).

2.2 CO_2 consumption by coupled enzymes (PEPC-MDH)

The most used method for the detection of CO_2 produced by decarboxylases, such as ODC, makes use of CO_2 consumption through reactions with the enzymes Phosphoenolpyruvate Decarboxylase (PEPC) and Malate Dehydrogenase (MDH), known as PEPC-MDH (Figure 2). From the CO_2 produced by the decarboxylase reaction, PEPC catalyzes the condensation of bicarbonate with phosphoenol pyruvate to form oxaloacetate. In the next step, MDH (using NADH as a cofactor) reduces oxaloacetate to form malate and NAD^+. NADH absorbs light at 340 nm, but NAD^+ does not. The presence of CO_2 in the reaction system leads to a decrease in light absorbance, used to assess the production of CO_2 (Scriven et al., 1988). These reactions can be performed using commercial kits, standardized for CO_2 detection (Smithson et al., 2010b; Liao et al., 2015; Chai et al., 2020). This assay allows the monitoring of the reaction in real-time and is considered by some authors as a good candidate for adaptation to HTS (Smithson et al., 2010b; Chai et al., 2020). Smithson et al. (Smithson et al., 2010b), optimized this assay using a commercial system for the detection of bicarbonate, carried out the experiments in N_2 atmosphere, performed the analyzes in an automated system in 384-well microplates, and optimized all the conditions reactions to evaluate 3,600 compounds. This method was used to screen a library of 316,114 compounds as inhibitors of *T. brucei* ODC. New classes of ODC inhibitors were identified that probably bind to new non-active sites of the enzyme, demonstrating the importance of this type of screening for the identification and development of new inhibitors (Smithson et al., 2010a).

2.3 Putrescine oxidation: H_2O_2 released

2.3.1 Colorimetric

This method is based on the oxidation of putrescine by soyabean amine oxidase (SAO), does not require polyamine purification, and can be performed in a 96-well plate for analysis in a plate reader. In the ODC reaction, the concentration of putrescine formed is determined by the amount of H_2O_2 generated by the reaction with SAO. Oxidation of 1 mol of putrescine by SAO generates 1 mol of H_2O_2 which reacts with 4-aminoantipyrine and phenol catalyzed by horseradish peroxidase to produce a colored complex that absorbs at 505 nm (Figure 2). Negative control can be done by replacing protein or substrate with buffer in the ODC enzyme reaction mix (Badolo et al., 1999; Das et al., 2015). In these assays, the soy amine oxidase enzyme was isolated from a soy plant and used in crude (Das et al., 2015) or purified form (Badolo et al., 1999), which increases the test performance by one more step. Furthermore, the possibility of test compounds acting as inhibitors of one of the additional enzymes should always be considered in HTS assays, which could lead to false positives for ODC.

2.3.2 Luminescence

The luminescence-based method was developed for the determination of total polyamines in biological samples (Fagerström et al., 1984) and adapted to determine ODC activity in cell extracts (Wang and Bachrach, 2000). This method consists in

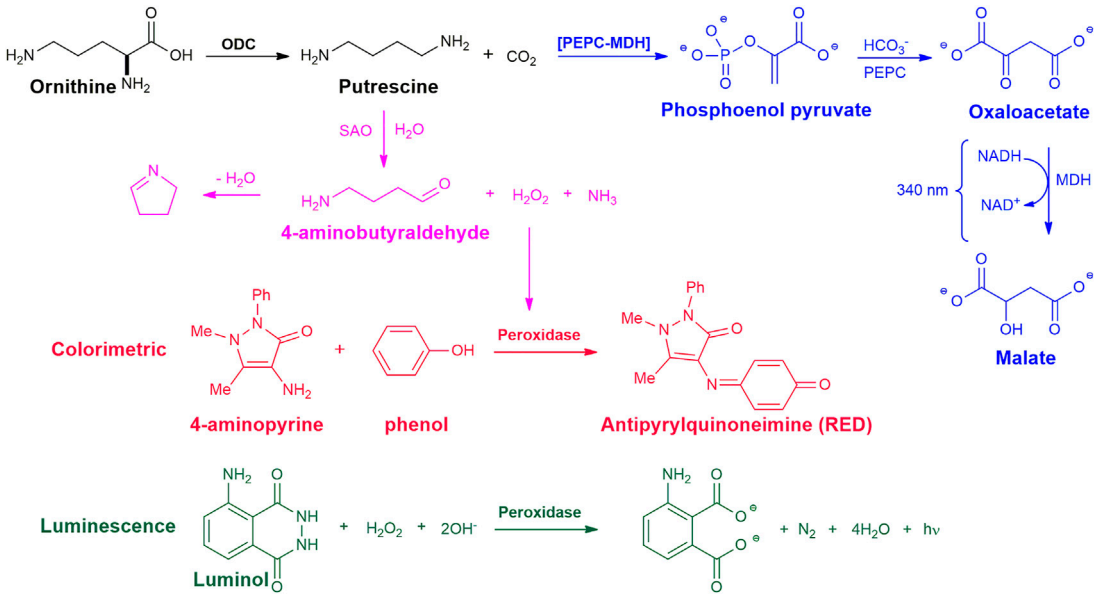

FIGURE 2
Chemical reactions for the enzymatic assay of ODC activity. *Blue* - detection of CO_2 formation, with Phosphoenolpyruvate Decarboxylase (PEPC) and Malate Dehydrogenase (MDH) enzymes. *Magenta* - detection of putrescine formation with soyabean amine oxidase (SAO) and horseradish peroxidase enzymes; *Red* - colorimetric analysis, *Green* - luminescence.

the oxidation of putrescine with a diamine oxidase, producing H_2O_2. The quantity of H_2O_2 formed is determined by chemiluminescence from the reaction with luminol and a peroxidase. Cell extracts are incubated with L-ornithine and placed on phosphocellulose paper strips. After drying, the contaminants are removed by washing the paper with ammonium hydroxide. To elute the putrescine, the paper is placed in a buffer solution containing magnesium sulfate and kept under agitation. After elution, the solution containing the putrescine is oxidized by a diamine oxidase generating H_2O_2. The hydrogen peroxide formed is subjected to a reaction with luminol and a peroxidase (Figure 2). This simple analytical method has the sensitivity of conventional assays based on the use of radioactive L-ornithine (Wang and Bachrach, 2000). Despite the relative simplicity and sensitivity of detecting putrescine on a picomolar scale, this method requires an initial step of extraction from paper, the value measured to produce putrescine may be interfered with by other polyamines and depends on other enzymes.

2.4 Adducts of putrescine with trinitrophenyl groups

Luqman and colleagues (Luqman et al., 2013) developed a colorimetric method to determine the formation of putrescine by ODC. This method is based on the solubility differences between substrates and product adducts and was developed aiming at the use of low-cost reagents and usual laboratory equipment. It is an endpoint method based on the reaction of picrylsulfonic acid (2,4,6 trinitrobenzenesulfonic acid, TNBS) with amino groups at alkaline pH forming colored trinitrophenyl (TNP) groups, resulting in water-soluble TNP-ornithine-TNP and adducts of TNP-putrescine-TNP soluble in 1-pentanol, which can be separated by phase extraction. The absorbance of the upper layer, containing TNP-putrescine-TNP, should be read at 426 nm. The difference in absorbance between the sample with and without enzyme will be related to the amount of putrescine formed, which must be compared with a standard putrescine plot for quantification. For the inhibition assay, a control set without the inhibitor needs to be compared to samples with the inhibitor. Despite being an inexpensive method and being applied by several authors (Nilam et al., 2017; El-Sayed et al., 2019; Villa-Ruano et al., 2019; Chai et al., 2020), it is not suitable for high-throughput screening with many samples. The method is time-consuming and laborious due to the need for several vortex mixing and centrifugation steps, in addition to the many transfers between new sets of tubes. To ensure the correct TNP-putrescine-TNP formation reaction and extractions, small aliquots must be pipetted many times correctly, giving many sources of errors that depend heavily on the analyst's skills.

2.5 HPLC assays

Due to its great resolution, specificity, and increased sensibility, HPLC assays are among the most reliable methods

for the detection of polyamines. The major drawbacks are the cost, long analysis time, and the need for a laborious pretreatment of the sample. Several methods describe the detection and analysis of polyamines through HPLC analysis, but only a few have been applied to monitor ODC activity. In general, the enzymatic reaction is terminated by the addition of $HClO_4$, before centrifugation for protein precipitation. The amine-containing supernatant is then used for derivatization and analysis.

This pretreatment and a classical derivatization procedure using NaOH and benzoyl chloride was recently performed by Liu and coworkers for the screening of ODC inhibitors (Chai et al., 2020). The dansylation of the diamines can also be performed using the amine-containing supernatant, saturated sodium carbonate solution, and dansyl chloride. After derivatization, a pre-purification of the mixture using a Bond-Elut C18 cartridge was performed before HPLC analysis (Kabra et al., 1986; Legaz et al., 2001). *o*-Phthalaldehyde (OPA) can also be used for amine derivatization from the mixture of the neutralized supernatant, OPA, a borate buffer, and mercaptoethanol for reagent stabilization. A fluorescence detector with standard filters for OPA was used for detection (Kvannes and Flatmark, 1987; Bito et al., 2019). Tritium-labeled ornithine was used for the detection of ODC activity, obtained directly from the murine embryonic submandibular gland. After complete disruption of the sample in phosphate buffer (pH 7.3), followed by centrifugation, analysis was performed by HPLC equipped with a scintillation counter (Beeman and Rossomando, 1989). Other recent derivatizations include carbamoylation with isobutyl chloroformate (Ah Byun et al., 2008), derivatization with diethyl ethoxymethylenemalonate (del Rio et al., 2018), and the use of commercially available derivatization kits (Xiao et al., 2009).

2.6 MS assays

Given the importance of detecting very low concentrations (traces) of polyamines in biological fluids, many chromatographic methods have been used, mainly based on HPLC separation with fluorometric detection (Yoshida et al., 2004), GC separation (Li et al., 2008), and HPLC with MS detection (Ducros et al., 2009; Liu et al., 2011). The detection by HPLC/Q-TOF MS of benzoylated polyamines allowed the determination of very low concentrations of polyamines in urine (Liu et al., 2011). Mass spectrometry without chromatographic separation was used by Gaboriou and colleagues to detect and quantify biologically relevant polyamines after dansylation. In the Atmospheric Pressure Chemical Ionization (APCI) mass spectra, the positive ions for each dansylated polyamine were generated after optimization by flow injection analysis (FIA). FIA together with MS detection by monitoring selected ions has greatly increased the sensitivity of polyamine detection. The method proved to be linear over a wide range of polyamine concentrations allowing detection in the fmols range. The method was considered fast, efficient, economical, reproducible, and simple enough to allow its routine application (Gaboriau et al., 2003). Subsequently, this method was used to detect the putrescine formed *in vitro* during the decarboxylation of L-ornithine by ODC present in cell extracts of a mouse hepatoma cell line, allowing detection of ODC activity as low as 0.05 nmol/mg (Gaboriau et al., 2005).

2.7 Circular dichroism

L-ornithine is chiral and when decarboxylated by ODC it is converted to putrescine, achiral. This difference in chirality makes it possible to use circular dichroism to monitor ODC reactions. As an amino acid, L-ornithine generates a positive signal at 201 nm in the CD spectrum due to the interaction of the carboxylate group with chiral C_α. This signal is dependent on the concentration of L-ornithine, with a linear decay of its intensity along the ODC reaction. The monitoring of ODC reactions by CD is very simple, requiring only buffer (phosphate or tris–less interference in the spectrum), substrate, PLP, and enzyme. However, it has the limitation that several organic compounds absorb around 201 nm and cannot be used with cell extracts or mixtures of compounds (Brooks and Phillips, 1996). In addition, it cannot be analyzed on microplates, which makes it difficult to perform many analyses simultaneously.

2.8 Fluorescence assays

A fluorescence-based assay to continuously monitor ODC activity was developed based on the principle of the tandem supramolecular enzyme assay. Tandem assays rely on pairs of reporter compounds with different affinities for a macrocyclic receptor and a fluorescent dye. This difference in macrocycle affinity causes a change in the spectroscopic properties of the fluorescent dye. In tandem enzymatic assays, the macrocycle receptor interacts with the substrate or enzymatic reaction product, leading to a displacement of the fluorescent dye. The change in fluorescence to be detected will depend on the difference in affinity of the substrate or product for the macrocycle compared to the fluorescent dye (Ghale and Nau, 2014). In the assays carried out by Nilam et al. (Nilam et al., 2017) *trans*-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (DSMI) was used as a fluorescent dye and cucurbit [6]uril (CB6) as the macrocyclic receptor (Nau et al., 2009; Li et al., 2012). L-ornithine has little affinity for the macrocycle, so DSMI is preferentially encapsulated by the receptor (CB6) and is strongly fluorescent. With the conversion of L-ornithine into putrescine by the ODC, the putrescine, with greater affinity for the macrocycle, will be encapsulated, continuously releasing the DSMI, with a decrease in the fluorescence intensity with the

course of the reaction. This assay can be adapted for reading on microplates and allowed the determination of the enzymatic kinetic parameters of the ODC, using the known inhibitors DFMO and (−)-epigallocatechin (EGCG) as positive controls (Nilam et al., 2017).

3 Perspective for HTS

Despite the importance of ODC as a therapeutic target, the identification of new inhibitors from the screening of large libraries of compounds is very limited by the difficulty in assaying ODC activity in a high-throughput manner. This has been a major challenge for decarboxylases, as both the substrate and the product are not directly detected by spectroscopic techniques, needing derivatization or conversion processes into spectrophotometrically detectable complexes. Among the assays presented, only the PEPC-MDH and the fluorescence assay proved to be suitable for HTS due to their biological relevance, sensitivity, robustness, and economic viability (Blay et al., 2020). However, just in the PEPC-MDH the experimental and automation conditions were evaluated and optimized to meet the criteria for a high-quality HTS. Given the difficulties of HTS assays for ODC based on enzymatic reactions, a promising alternative would be to perform HTS assays based on ligand-enzyme processes (Bergsdorf and Ottl, 2010), employing libraries of compounds or molecular fragments (Erlanson, 2012) – which have shown to be very promising in the last few decades– developing several compounds that are in clinical phase trials and some drugs on the market (Erlanson et al., 2016).

Author contributions

LT Planned the organization of the manuscript, wrote and revised BS Wrote topic about HPLC JS Wrote topic about Adducts of putrescine with trinitrophenyl groups FF Wrote and revised the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Luzineide Wanderley Tinoco,
Federal University of Rio de Janeiro, Brazil

REVIEWED BY
Felipe Moura A. da Silva,
Federal University of Amazonas, Brazil
Angela De Simone,
University of Turin, Italy

*CORRESPONDENCE
Carmen Lúcia Cardoso,
cccardoso@ffclrp.usp.br

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Activity assay based on the immobilized enzyme kallikrein and mass spectrometry

Daniella Romano De Carvalho[1], Bruna Barbosa Laurentino[1], Camila Loreta Rocha[1], Jeroen Kool[2], Govert Somsen[2], Erika Amstalden van Hove [2†] and Carmen Lúcia Cardoso[1*]

[1]Departamento de Química, Grupo de Cromatografia de Bioafinidade e Produtos Naturais, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil, [2]Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam Institute of Molecules, Medicine, and Systems, Vrije Universiteit Amsterdam, Amsterdam, Netherlands

Deregulated activity and expression of human kallikreins (KLKs) may be involved in various pathologies, so these enzymes are an attractive biological target for identifying molecules that can modulate KLK activity. This identification involves applying fast and efficient screening methods. This work describes an off-line assay with mass spectrometry (MS) detection that uses KLK immobilized on Sepharose-NHS as a micro-column configuration (IMER-KLK-Sepharose-NHS). The mass spectrometry used has an ion trap analyzer and electrospray ionization (EIS). The HPLC-MS method for quantifying KLK activity was developed. The enzymatic assay conditions were optimized, and the IMER-KLK-Sepharose-NHS kinetic parameter (K_{Mapp} = 15.48 ± 3 µmol L^{-1}) was evaluated. Finally, the method was validated by using leupeptin as a reference inhibitor (IC_{50} = 0.85 ± 0.10 µmol L^{-1}). The developed method was able to identify the reference inhibitor and can be an alternative for screening KLK inhibitors.

KEYWORDS

immobilized enzyme, HPLC-MS, off-line assay, kallikerin enzyme, ligand screening assay

Introduction

Kallikreins (KLKs) are a group of serine protease enzymes that play an important role in biological fluids and tissues. KLKs can be divided into two groups: plasma kallikrein (KLK 1B) and tissue KLKs (KLK1–15) (Prassas et al., 2015). KLK 1B is exclusively expressed by hepatic cells and is part of the plasma kallikrein-kinin system, which, among other things, participates in the processes of fibrinolysis, blood coagulation, inflammation, cancer, and pathologies related to the cardiovascular system (Schmaier and Mccrae, 2007; Costa-Neto et al., 2008). Under physiological conditions, plasma kallikrein serves as a cardioprotective enzyme. However, its increased plasma concentration or hyperactivity perpetuates cardiovascular disease (CVD) (Kolte and Shariat-Madar, 2016). Tissue KLKs comprise human tissue kallikrein (KLK1) and 14 kallikrein-related peptidases (KLK2-15)

(Prassas et al., 2015) that share genetic and protein structural similarities. Amino acid identity among human tissue KLKs ranges from 40 to 80% (Yousef and Diamandis, 2001).

Tissue KLKs are present in several tissues of the human organism, and their unregulated expression may be associated with various diseases (Costa-Neto et al., 2008). For example, in patients with Alzheimer's and Parkinson's disease, KLK6 has decreased expression in brain tissue (Diamandis et al., 2000; Bayani and Diamandis, 2012). The human kallikrein-related peptidases 5 (hKLK5), 6 (hKLK), 7 (hKLK7), 8 (hKLK8), and 14 (hKLK14) are also involved in inflammatory skin disorders (Di Paolo, Diamandis and Prassas, 2020) with abnormal desquamation and inflammation, such as atopic dermatitis (AD), psoriasis, and the rare disease Netherton syndrome (NS) (Zani et al., 2022). In addition, KLKs are dysregulated in most solid tumors, especially in hormone-dependent tumors (Kryza et al., 2016). KLK3 also known as prostate specific antigen (PSA) is used as a biomarker for prostate cancer because this enzyme is overexpressed in these types of cancers and KLK3 presents dysregulated expression in breast tumors (Yu et al., 1995). KLK2 is overexpressed in prostate tumors (Darson et al., 1997). KLK7 overexpression in colon cancer tissues is involved in cancer cell proliferation (Walker et al., 2014).

Given that some enzymes underlie pathologies that affect humanity, searching for molecules that can inhibit them is a relevant topic when searching for a treatment or cure for these pathologies (Copeland, 2013). In this scenario, KLKs are important targets when it comes to developing drugs that can inhibit their activities or restore their expression (Kolte and Shariat-Madar, 2016; Xie et al., 2020). This is particularly relevant in the context of anticancer studies because KLKs have been shown to modulate cancer cell multiplication (Shang et al., 2014; Walker et al., 2014). The PROSTVAC vaccine, developed for prostate cancer treatment, is an example of a therapeutic resource that targets KLK3 and multiple T cell co-stimulatory molecules (TRICOM) (Madan et al., 2009).

Developing tools that allow enzyme inhibitors to be discovered and developed is therefore essential. In this sense, enzyme immobilization is an important tool for screening ligands that can potentially inhibit enzymes. Assays with immobilized enzymes offer advantages over the use of enzymes in solution—they allow enzymes to be better handled and reused, and they increase enzyme stability under varying conditions (Brena, González-Pombo and Batista-Viera, 2013). Additionally, enzyme immobilization allows enzymatic activity modulation to be monitored in the presence of libraries of molecules (synthetic or natural) and complex matrixes, like crude extracts (Singh et al., 2014). Moreover, enzyme immobilization enables high-throughput screening (HTS) of ligands from such matrixes (De Simone et al., 2019). Indeed, different enzyme immobilization protocols (Rodrigues et al., 2021) and ligand screening assays, ranging from online (flow) to offline assays, have been developed (Wubshet et al., 2019; Cieśla and Moaddel, 2016; Zhuo et al., 2016; Cheng and Chen, 2018; de Moraes, Cardoso and Cass, 2019; Q. Chen et al., 2021; Trindade Ximenes et al., 2021; de Oliveira et al., 2022).

In online assays, ligands are analyzed and screened simultaneously (Zhuo et al., 2016). These assays basically consist of an immobilized enzyme reactor (IMER) coupled to an automated system, such as high-performance liqbib_Chen_et_al_2021buid chromatography (HPLC) or capillary electrophoresis (Cheng and Chen, 2018; G. Y. Chen et al., 2021), which can be coupled with different detection techniques, including ultraviolet (UV) (da Silva et al., 2013), fluorescence (Hai et al., 2011), electrochemical detection (Sun, Gao and Jin, 2006), and mass spectrometry (MS) (Vilela and Cardoso, 2017).

HPLC-MS is considered the most powerful analytical tool for analyzing polar compounds, including peptides, phenols, polymers, and biomolecules (Loos, Van Schepdael and Cabooter, 2016). HPLC-MS combines separation by HPLC with the broad analysis capability of MS. The advantages of HPLC-MS include the need for a small amount of sample, high resolution power, high molecular specificity, and high detection sensitivity (Beccaria and Cabooter, 2020). The ionization mode is crucial for the performance of HPLC-MS method depending on the analytes targets, between others the electrospray ionization source (ESI) is the main ionization mode analysis for thermally labile, nonvolatile, and polar compounds. (Beccaria and Cabooter, 2020). Specifically, ESI ionization source and ion trap mass analyzer (IT) has been successfully used in IMER coupled with HPLC-MS. For example, Beta secretase1 and acetylcholinesterase (AChE) were co-immobilized and used to dual-ligand screening (Vilela, Narciso dos Reis, and Cardoso 2021) AChE-IMER (Vanzolini et al., 2013) used to screening coumarin derivatives and butyrylcholinesterase (BChE)-IMER and AChE-IMER used in on-flow mass spectrometry dual enzyme assay (Seidl et al., 2019).

On the other hand, off-line assays entail independent screening and analysis steps. However, the detection techniques mentioned above can be used in offline assays, as well (Zhuo et al., 2016; Zhang et al., 2018).

Numerous supports, such as magnetic particles (Wang et al., 2018; Q. Chen et al., 2021), silica gel microspheres (Shi et al., 2015), and Sepharose (Zofair et al., 2020), can be used to construct bioreactors that can be applied in off-line assays. Previously, we immobilized the KLK enzyme on Sepharose-NHS solid support and employed it in ligand screening assays during which we used a 96-well flat-bottom microplate to measure fluorescence (Carvalho et al., 2021). The immobilized KLK showed excellent operational and storage stability and proved an efficient tool for identifying KLK inhibitors (Carvalho et al., 2021).

Bearing the advantages of enzyme immobilization and the growing search for KLK inhibitors in mind, we present an off-line

assay based on KLK immobilized on Sepharose-NHS as a micro-column configuration and MS detection. We will show that this bioreactor, IMER-KLK-Sepharose-NHS, exhibits excellent stability, can be reused, is a useful tool in off-line ligand screening, and can be combined with an HPLC-MS method.

Materials and methods

Chemical and reagents

Porcine pancreas kallikrein (KLK, 250 units), *N*-hydroxysuccinimidyl-Sepharose® 4 Fast Flow, CBZ-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Phe-Arg-AMC), leupeptin hemisulfate salt (Acetyl-Leu-Leu-Arg-al), and 7-amino-4-methylcoumarin (AMC) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Buffer components and other chemicals were acquired from Sigma-Aldrich, Merck (Darmstadt, Germany), Synth (São Paulo, Brazil), or Acros (Geel, Belgium). The water that was used in all the preparations was obtained from a MILLI-Q® system (Millipore®, São Paulo, Brazil). All the chemicals and solvents used here were analytical or HPLC grade and were employed without any further purification.

Instrumentation and system configuration for analyses

HPLC-MS analyses were carried out on a NexeraXR high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with three LC 20ADXR pumps, an SIL-20A automatic injector, a DGU-20A degasser, a 10-port two-position high-pressure switching valve (Valco Instruments Co. Inc., Houston, United States), and a CBM-20A system controller. The HPLC system was coupled to ion trap mass spectrometer, model AmaZon speed dual ion funnel, QIT technology (IT-MS) (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source (ESI). The system was controlled by Bruker Compass Hystar software (version 4.5, Bruker Daltonics Inc., Billerica, United States). Data were acquired and analyzed by using the Compass DataAnalysis software (version 4.3, Bruker Daltonics Inc., Billerica, United States).

Preparation of IMER-KLK-sepharose-NHS micro-column

KLK from porcine pancreas (100 μg ml^{-1}) was covalently immobilized on *N*-hydroxysuccinimidyl-Sepharose® 4 Fast Flow (Sepharose-NHS, solid support) as previously described (Carvalho et al., 2021). After immobilization, 80 mg of the immobilized enzyme was placed in a micro-column device (Mobitec, Göttingen, Germany), to give IMER-KLK-Sepharose-NHS. The micro-column was washed with 50 mmol L^{-1} Tris-HCl buffer (pH 8.0), and stored in the same buffer solution at 4 C. Control columns containing 80 mg of Sepharose-NHS (without enzyme) were prepared as described above.

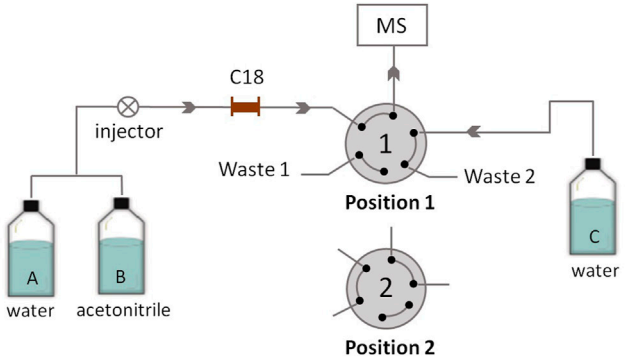
IMER-KLK-sepharose-NHS micro-column off-line assay

Aliquots of 120 μl of 10 mmol L^{-1} ammonium acetate solution (pH 8.0), and 50 μl of 200 μmol L^{-1} Z-Phe-Arg-AMC substrate were added to the micro-column and incubated under gentle agitation at room temperature for 5 min. After incubation, the reaction medium was filtered and collected. Then, 20-μL aliquots of the reaction mixture (filtered) were transferred to vials, diluted with 80 μl of 10 mmol L^{-1} ammonium acetate solution (pH 8.0), and analyzed by HPLC-MS. At the end of each reaction, the IMER-KLK-Sepharose-NHS micro-column was washed with 5 ml of Milli-Q water (three times), followed by 5 ml of 5 mmol L^{-1} ammonium acetate solution (pH 8.0) three times. At the end of each reaction, the immobilized KLK micro-column was washed with 5 ml of Milli-Q water (three times), followed by 5 ml of 5 mmol L^{-1} ammonium acetate solution pH 8.0 (for 3 times). Blank samples were prepared under the same reaction conditions by using control micro-columns (without enzyme). All the experiments were performed in triplicate.

The optimization of IMER-KLK-Sepharose-NHS off-line assay conditions included the effect of the reaction time and ammonium acetate solution concentration on the enzymatic activity. The effect of ammonium acetate solution concentration was evaluated at concentrations ranging from 2 to 15 mmol L^{-1} (pH 8.0). For this, the immobilized KLK micro-column was previously washed with 5 ml of each concentration of ammonium acetate pH 8.0 (three times) and left under conditioning for 10 min at each concentration. The reaction time was evaluated for incubation times ranging from 5 to 40 min. The enzymatic reactions were carried out under the same conditions described above, and Z-Phe-Arg-AMC was employed as substrate. The reproducibility of the IMER-KLK-Sepharose-NHS activity assay was verified by carrying out five consecutive reaction cycles.

Analysis of the IMER-KLK-sepharose-NHS activity by HPLC-MS

HPLC analyses were carried out by using an Ascentis® Express C18 micron guard cartridge (5 mm × 2.1 mm x 2.7 μm, Supelco, Bellefonte, PA, United States) connected to a 10-port/two-position valve in the HPLC-MS system (Figure 1); the mobile phase consisted of water (solvent A) and acetonitrile (solvent B). Initially, with the valve in position 1, the enzymatic



Mobile phase (A:B, v/v)	Flow rate (mL min^{-1})	Time (min)	Event	Valve position
70:30	0.200	0.0 – 2.0	Enzymatic reaction analysis	1
10:90	0.500	2.1 – 5.0	System washing step	2
70:30	0.200	5.1 – 6.0	Return to initial condition	1

Obs: Pump C flow rate C = 0.200 mL min^{-1}

FIGURE 1
Schematic representation of the HPLC–MS system for monitoring the IMER-KLK-Sepharose-NHS activity. Source: Designed by the authors.

reaction products were eluted from the column with an initial mobile phase composed of 30% solvent B at a flow rate of 0.2 ml min^{-1} for analysis in the mass spectrometer. After 2.0 min, the valve was switched to position 2; solvent B was increased to 90% at a flow rate of 0.5 ml min^{-1}, which was maintained until 5 min, to wash the column and to remove excess substrate. At 5.1 min, the valve was switched to position 1 again, and the mobile phase was returned to the initial condition. The total run time was 6 min. Pump C (solvent water) at a flow rate of 0.2 ml min^{-1} was used to deliver the mobile phase to the MS during the column washing step. All the analyses were performed at 21 C (controlled room temperature), and the injection volume was 5 µl. The ESI ionization parameters were as follows: positive ionization mode, capillary voltage = 4500 V, end plate voltage = 550 V, drying gas flow = 7 L min^{-1}, drying temperature = 270 C, and nebulizer pressure = 27 psi. The product of the enzymatic reaction, AMC, was monitored at *m/z* 176 $[M + H]^+$.

Method qualification

Method qualification was performed on the basis of literature criteria (FDA 2011). Method linearity was evaluated by constructing a standard curve; AMC at the following concentrations was used: 50, 70, 100, 200, 300, 400, 500, 600, 800, and 1,000 nmol L^{-1}. For this purpose, from a standard 10 mmol L^{-1} AMC solution (in DMSO), the AMC solutions at concentrations ranging from 50 to 1,000 nmol L^{-1} were prepared in 10 mmol L^{-1} ammonium acetate solution. For the analytical curve, the solutions were prepared in triplicate from the concentrated stock solutions, and 5-µL aliquots were injected into the HPLC-MS system as described above. The analytical curve was constructed by linear regression of the plot of the chromatogram area of the extracted ions with *m/z* 176 $[M + H]^+$ as a function of the concentration. Method selectivity was evaluated by buffer analysis under the same conditions described above, but without adding AMC. The limit of quantification and the limit of detection were evaluated from the injection of AMC solutions at concentrations of 50, 30, 10, 5, and 2.5 nmol L^{-1}. The -limit of quantification was defined as the lowest concentration that produced a signal-to-noise value higher than ten times that response obtained by analyzing the blank (buffer without AMC), while the limit of detection was defined as the lowest concentration of an analyte in a sample that can be consistently detected with a stated probability and could be reliably differentiated from the baseline. When based on signal-to-noise establishing the minimum concentration at which the analyte can reliably be detected. Typically, acceptable signal-to-noise ratios are 2:1 or 3:1. (FDA 2011). The intra- and inter-day precision and accuracy were evaluated by analyzing samples at three different concentration levels (50, 500, and 1,000 nmol L^{-1}); each concentration level was prepared and analyzed in quintuplicate.

IMER-KLK-sepharose-NHS kinetic constant (K_{Mapp})

The IMER-KLK-Sepharose-NHS kinetic constant (K_{Mapp}) was determined by using different Z-Phe-Arg-AMC concentrations (4–1,200 µmol L^{-1}) in 10 mmol L^{-1} ammonium acetate solution, pH 8.0. After incubation for 5 min, the reaction medium was filtered and collected, and 20-µL aliquots of the

solution were transferred to vials, diluted with 80 µl of 10 mmol L^{-1} ammonium acetate solution (pH 8.0), and analyzed by HPLC-MS as describe above. The enzymatic activity was monitored by quantifying the AMC that was formed (*m/z* 176 $[M + H]^+$). Data were fitted by using nonlinear regression into a Michaelis-Menten plot, and K_{Mapp} values were obtained with the GraphPad Prism 8.0 software.

Inhibition studies

Inhibition studies were carried out by using leupeptin as standard inhibitor. To determine their half maximum inhibitory concentration (IC_{50}), 110-µL aliquots of 10 mmol L^{-1} ammonium acetate solution (pH 8.0), 10 µl of leupeptin (1–2,500 µmol L^{-1}), and 50 µl of the substrate Z-Phe-Arg-AMC at 200 µmol L^{-1} were added to the micro-column and incubated under gentle agitation at room temperature for 5 min. After incubation, the reaction medium was filtered and collected, and 20-µl aliquots of the solution were transferred to vials, diluted with 80 µl of 10 mmol L^{-1} ammonium acetate solution (pH 8.0), and analyzed by HPLC-MS as describe above. The enzymatic activity was monitored by quantifying the AMC that was formed (*m/z* 176 $[M + H]^+$). The activities in the presence (A_i) and absence (A_0) of the inhibitor were compared, and the percentage of inhibition was calculated by the expression: $\%I = 100—[(A_i/A_0) \times 100]$. The inhibition curve was constructed by plotting the percentage inhibition *versus* the corresponding leupeptin concentration, and the IC_{50} value was determined by plot sigmoidal curve build by log [leupeptin] *versus* % inhibition with the GraphPad Prism 5.0 software.

Results and discussion

Assays based on immobilized enzyme coupled to HPLC–MS systems have emerged as a promising alternative to colorimetric screening assays in microplates. Here, we developed an off-line assay with MS detection by using KLK immobilized on Sepharose-NHS as a micro-column configuration. We prepared the immobilized enzyme by a batch approach. After we covalently immobilized KLK onto Sepharose-NHS, we packed it into micro-column devices, to obtain IMER-KLK-Sepharose-NHS.

HPLC-MS analysis method development

We performed chromatographic analysis in the isocratic elution mode with a mobile phase composed of water/acetonitrile; a C18 micron guard cartridge (5 mm × 2.1 mm × 2.7 µm) was used. To eliminate the substrate excess and to avoid possible problems caused by ionization suppression and/or contamination in the MS ionization source, we added a clean-up step, as described in Figure 1.

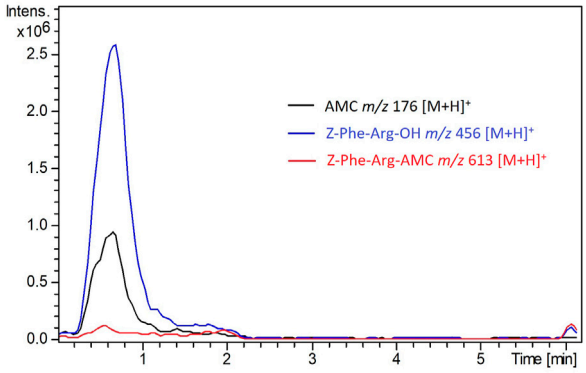


FIGURE 2
Overlap of peaks in the extracted ion chromatograms recorded for the IMER-KLK-Sepharose-NHS activity. See HPLC-MS conditions described in the experimental section Source: Designed by the authors.

Figure 2 shows the chromatogram corresponding to the analysis of the IMER-KLK-Sepharose-NHS enzymatic reaction by the proposed method. Under the employed conditions, we eliminated the interference caused by excess substrate, but the products AMC and Z-Phe-Arg-OH co-eluted. In this case, there was no need for high-resolution chromatographic separation even though the analytes co-eluted because we later identified them according to their respective *m/z* ratio. Therefore, this method allowed us to monitor the KLK activity by fast analysis, which lasted only 6 min. In routine analyses, we evaluated the IMER-Sepharose-NHS enzymatic activity by monitoring the ion with *m/z* 176 $[M + H]^+$, which refers to the AMC product.

Method qualification

We evaluated method linearity by constructing a calibration curve for the AMC product. The curve showed a linear response and $R^2 = 0.99$ ($n = 3$) for AMC concentrations ranging from 50 to 1,000 nmol L^{-1} ($y = 5.07 \times 10^7 x + 8.85 \times 10^5$); the relative standard deviation (RSD) values were below 15% for the triplicates. When we injected only the buffer under the same assay conditions, no signal due to AMC emerged, which demonstrated method selectivity. The limits of detection and quantification for AMC were 5 and 30 nmol L^{-1}, respectively. The intra- and inter-day precision values were expressed in RSD and ranged from 5 to 15%. The intra-day accuracy of the method ranged from 85 to 110%. The inter-day accuracy lay between 87 and 101%. Thus, the developed HPLC–MS method can be applied to monitor the immobilized KLK activity.

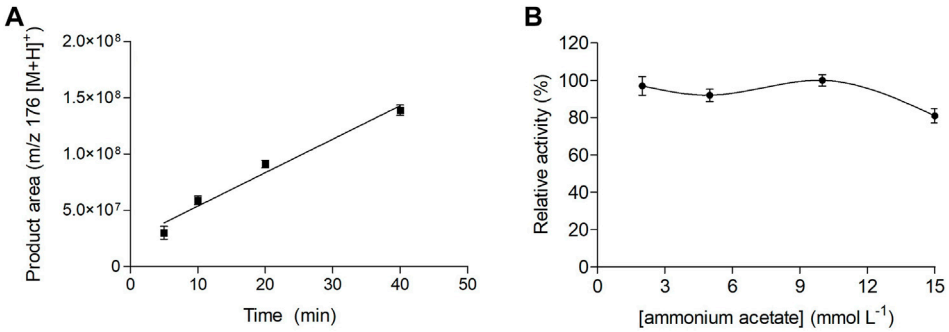


FIGURE 3
Effect of reaction time **(A)** and ammonium acetate solution concentration **(B)** on the IMER-KLK-Sepharose-NHS activity. Source: Designed by the authors.

IMER-KLK-sepharose-NHS off-line assay conditions

We evaluated the reaction conditions for the IMER-KLK-Sepharose-NHS activity assay, namely reaction time, ammonium acetate solution concentration, and immobilized KLK micro-column washing steps. We evaluated the IMER-KLK-Sepharose-NHS activity at different incubation times (Figure 3A). We obtained a linear response for AMC formation as a function of reaction time, which showed that the proposed assay model can be used to monitor the IMER-KLK-Sepharose-NHS activity. Among the analyzed conditions, incubation for 5 min was sufficient to measure the enzymatic activity, so we chose this time for further analyses.

Figure 3B shows how the ammonium acetate solution concentration, pH 8.0, affected the immobilized KLK activity. The immobilized enzyme remained active between 2 and 10 mmol L^{-1} ammonium acetate. In contrast, IMER-KLK-Sepharose-NHS showed lower activity at higher ammonium acetate concentration (15 mmol L^{-1}).

Hence, the optimized assay conditions for the IMER-KLK-Sepharose-NHS activity off-line assay were incubation time of 5 min and 10 mmol L^{-1} ammonium acetate solution (pH 8.0).

Another important parameter to evaluate is the clean-up step of the immobilized KLK micro-column, to ensure that it can be reused and that results are reproducible. KLK immobilized on Sepharose-NHS had previously been shown to have excellent reusability after simple washing steps with washing buffer (10 mmol L^{-1} Tris-HCl, pH 8.0) (Carvalho et al., 2021). Here, we adjusted the clean-up step to the conditions used during MS detection, and we replaced the washing buffer with 5 mmol L^{-1} ammonium acetate (pH 8.0) 0. The final clean-up conditions of the IMER-KLK-Sepharose-NHS were 5 ml of Milli-Q water followed by 5 ml of 5 mmol L^{-1} ammonium acetate solution (pH 8.0). The washing procedure proved adequate for cleaning the immobilized KLK micro-column. In routine analyses, we repeated this washing procedure three times.

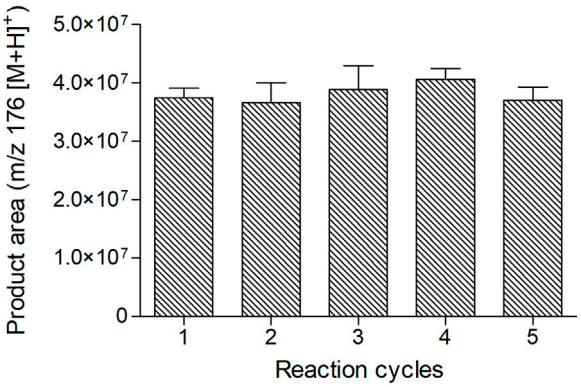


FIGURE 4
IMER-KLK-Sepharose-NHS reusability. Source: Elaborated by the authors.

In addition, we tested IMER-KLK-Sepharose-NHS in repeated reaction cycles (Figure 4), which showed excellent reproducibility in five consecutive reactions (CV = 4.3%, n = 5) and indicated that the proposed model assay was suitable for monitoring the IMER-KLK-Sepharose-NHS activity. The immobilized enzyme showed good stability and remained active throughout the assay.

IMER-KLK-sepharose-NHS kinetic constant (K_{Mapp})

We determined the kinetic parameter K_{Mapp} for Z-Phe-Arg-AMC hydrolysis by IMER-KLK-Sepharose-NHS under the conditions established for the enzymatic reaction and for the HPLC–MS analysis method. The K_{Mapp} value was 15.48 ± 3 μmol L^{-1} (Figure 5), corroborating the value previously obtained by the fluorescence assay on microplates of 10.3 ± 0.9 μmol L^{-1}

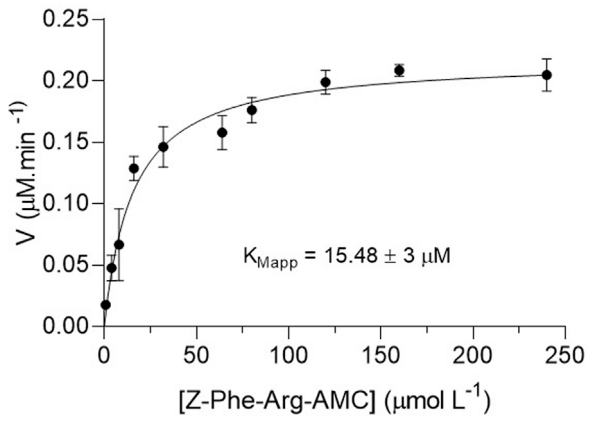


FIGURE 5
Michaelis-Menten plots of the product area obtained for IMER-KLK-Sepharose-NHS by using Z-Phe-Arg-AMC as substrate. Source: Elaborated by the authors.

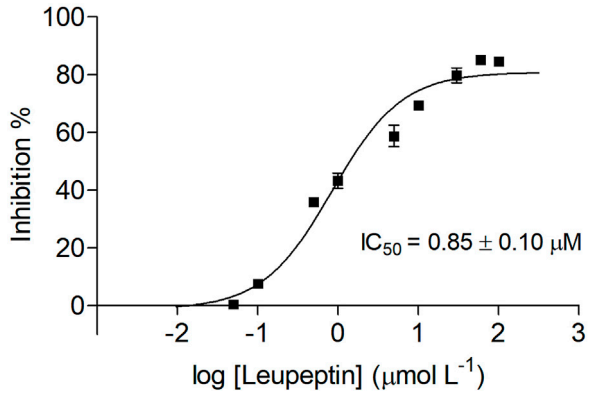


FIGURE 6
Dose-response curve plot of inhibition percentage for IMER-KLK-Sepharose-NHS in the presence of leupeptin. Source: Designed by the authors.

(Carvalho et al., 2021). Thus, we selected 40 µmol L^{-1} substrate (corresponding to more than twice the K_{Mapp}) for the inhibition studies.

Inhibition studies

As a proof of concept, we used the peptide leupeptin as a reference inhibitor to validate the off-line assay employing IMER-KLK-Sepharose-NHS as a tool for screening ligands. To determine the inhibitory potency, we evaluated the IMER-KLK-Sepharose-NHS activity in the presence of increasing leupeptin concentrations and obtained IC_{50} of 0.85 ± 0.10 µmol L^{-1} (Figure 6), which is in the same order of magnitude as the value obtained by the microplate assay and fluorescence detection IC_{50} of 0.13 ± 0.01 µmol L^{-1} and to IC_{50} obtained for the free enzyme in solution (IC_{50} = 1.62 ± 0.18 µmol L^{-1} (Carvalho et al., 2021). The IC_{50} values vary when the test conditions are modified, so it is a relative comparison parameter (Holdgate, Meek and Grimley, 2018). In general, IMER-KLK-Sepharose-NHS was able to identify the reference inhibitor and proved to be effective in determining the IC_{50} parameter.

Conclusion

KLK immobilized on Sepharose-NHS as a micro-column configuration is a useful approach for measuring KLK activity; exhibits satisfactory stability; allows the enzyme to be reused; and can be combined with an HPLC–MS method off-line. We determined the KLK activity by quantifying the AMC product through the proposed HPLC–MS method. The developed assay was also able to identify the known KLK inhibitor leupeptin, demonstrating that it can be used for screening inhibitors. Thus, the off-line assay with MS detection reported here represents a good alternative to the fluorescence microplate assays.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

DC: methodology, validation, formal analysis, investigation, writing—original draft, BL: formal analysis, validation. CR: writing—original draft formal analysis. EA: software, writing—review GS: writing—review. JK: writing—review. CC: conceptualization, funding acquisition, project administration, resources, visualization, supervision, writing—review and editing.

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responsibility of the author(s) and do not necessarily reflect the views of FAPESP.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frans.2022.1018115/full#supplementary-material

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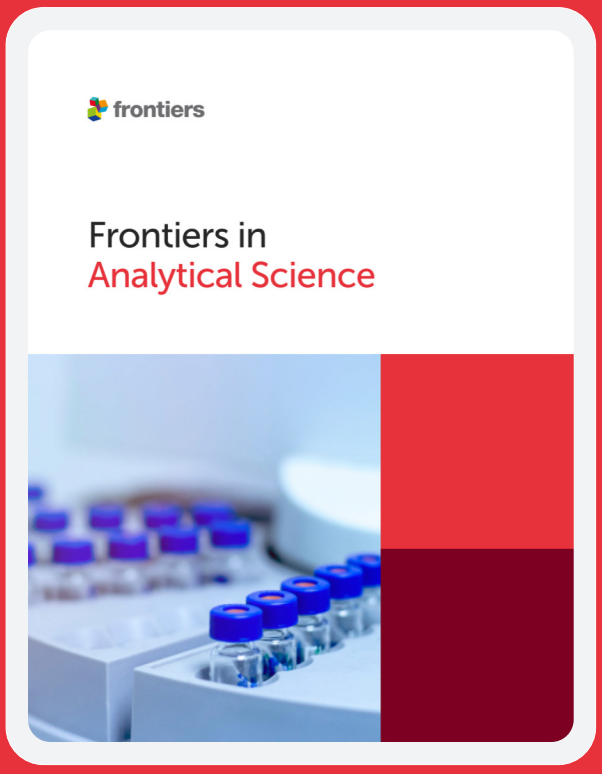

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