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Assessment of ionic liquids' toxicity through the inhibition of acylase I activity on a microflow system

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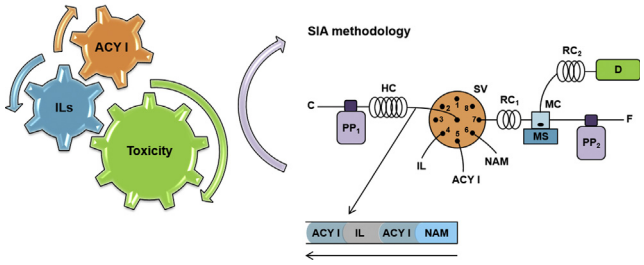
HIGHLIGHTS

- An automated ACY I assay based on SIA was developed for the first time.
- The inhibition assay was applied to the toxicity assessment of eight ILs.
- The results evidenced that ILs' structural elements can influence its toxicity.
- The ACY I assay proved to be an interesting toxicity screening tool.

GRAPHICAL ABSTRACT



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ABSTRACT

Acylase I (ACY I) plays a role in the detoxication and bioactivation of xenobiotics as well in other physiological functions. In this context, an automated ACY I assay for the evaluation of ionic liquids' (ILs) toxicity was developed. The assay was implemented in a sequential injection analysis (SIA) system and was applied to eight commercially available ILs. The SIA methodology was based on the deacetylation of *N*-acetyl-L-methionine with production of L-methionine, which was determined using fluorescamine. ACY I inhibition in the presence of ILs was monitored by the decrease of fluorescence intensity. The obtained results confirmed the influence of ILs' structural elements on its toxicity and revealed that pyridinium and phosphonium cations, longer alkyl side chains and tetrafluoroborate anion displayed higher toxic effect on enzyme activity.

The developed methodology proved to be robust and exhibited good repeatability (RSD < 1.3%, $n = 10$), leading also to a reduction of reagents consumption and effluents production. Thus, it is expected that the proposed assay can be used as a novel tool for ILs' toxicity screening.

1. Introduction

In the past fifteen years, ionic liquids (ILs) have been used in different fields (Moniruzzaman et al., 2010; Ho et al., 2014;

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Marrucho et al., 2014) as a greener alternative to conventional organic solvents, because of their ability to interchange cation-anion combinations. Accordingly, these chemical compounds can exhibit peculiar properties, such as negligible vapor pressure, non-flammability, high chemical and thermal stability (Wasserscheid and Welton, 2008; Heckenbach et al., 2016). The growing interest in ILs envisions an increase of their manufacture and use at industrial scale, which may result in the release of these compounds into the environment. Although ILs can reduce air pollution risk, even the hydrophobic ones present some water solubility (Freire et al., 2007) and can cause aquatic and soil contamination through wastewater discharges or leaching of landfills (Bubalo et al., 2014). It is therefore essential the assessment of these compounds toxicity and biodegradability prior to their indiscriminate use. A few steps have been done in this direction with some information already available in the literature concerning ILs' environmental impact, particularly their toxic effect on several biological elements (Heckenbach et al., 2016). Green algae (Deng et al., 2015), bacteria (Costa et al., 2015a), fish (Pretti et al., 2006), crustacean (Costa et al., 2015b) and duckweed (Santos et al., 2015) are some of the living organisms most used to evaluate the aquatic toxicity. On the other hand, terrestrial toxicity studies have been focused on the assessment of ILs' ability to sorption onto soils (Matzke et al., 2009) as well as their effect on plants (Pawlowska and Biczak, 2016) and earthworms (Luo et al., 2010).

Although most of the (eco)toxicological assays have been performed on biological elements of higher levels of complexity, there is still a limited knowledge about ILs' action on the cellular and subcellular levels. *In vitro* experiments with cell lines (e.g., HT-29 and CaCo-2 human cell lines) (Frade et al., 2007) and enzymatic inhibition assays (Costa et al., 2016; Dong et al., 2016) proved to be useful for this purpose. These tests enabled the identification of potential toxicophore structures, providing additional information for the design of safer compounds (Arning et al., 2008). Enzymatic inhibition assays are generally simple and result in reduction of both analysis time and costs. The enzymatic inhibition assays already proved to be an adequate alternative for the evaluation of ILs toxicity (Pinto et al., 2011; Cunha et al., 2015). Moreover, the substitution of animals when feasible enables to overcome some disadvantages of animal experimentation like requirement of skilled operator, time consuming protocols and high cost. Furthermore, the animals are sometimes submitted to pain, distress and death during the experiences (Doke and Dhawale, 2015). The development of these alternative methodologies has contributed to a considerably reduction on the animals use and promotion of the three Rs (reduction, refinement and replacement) implementation in the laboratory experimentation (Blaauboer et al., 1998).

Acylase I (ACY I), also known as *N*-acyl-L-amino acid amidohydrolase (EC 3.5.1.14), is a ubiquitous enzyme found in various mammalian tissues. It catalyzes the hydrolysis of *N*-acyl-L-amino acids into the corresponding free amino acid and fatty acid residue.

The deficiency of ACY I is characterized by accumulation of *N*-acetyl amino acids in the urine and seems to be associated to defects on brain metabolism and function. It is documented individuals with mutations on the ACY I gene with neurological impairment, including encephalopathy (Van Coster et al., 2005), psychomotor delay, among others neurological injuries (Sass et al., 2006). Moreover, the deregulation of ACY I expression seems to be present in some types of cancers, such as neuroblastoma (Long et al., 2011), renal cell carcinoma (Zhong et al., 2009), small cell lung cancer (Miller et al., 1989), hepatocellular carcinoma (Wei et al., 2014) and colorectal cancer (Shi et al., 2013). Therefore, ACY I appear to be a promising option to be used as prognostic biomarker and/or therapeutic target in cancer therapy. ACY I plays not only a main role in the regulation of cell viability and apoptosis, but also in the cytosolic breakdown of acetylated amino acids generated during intracellular protein degradation (Perrier et al., 2005). Furthermore, it has been suggested that ACY I may also participate in the detoxication or bioactivation of xenobiotics as well as in the interorgan processing of mercapturates (*N*-acetyl-L-cysteine *S*-conjugates) (Anders and Dekant, 1994; Stocker et al., 2012). The investigation of ACY I inhibitors might be an asset to improve the knowledge about the role of the enzyme on the metabolization processes, and potential consequences of the ACY I activity decrease. Cézanne was the only that evaluated the influence of some potential inhibitors on ACY I activity (Stocker et al., 2005). Therefore, additional studies need to be made to evaluate if it is in fact a practical biomarker to predict potential toxic mammals' xenobiotics.

Until now, the determination of ACY I activity has been performed in batch mode with drawbacks in terms of sample throughput, reagents consumption and operator intervention (Uttamsingh et al., 1998). In this context, it appears that flow techniques like sequential injection analysis (SIA) could be a good option for the implementation of the enzymatic assay and could further increase its potential for screening purposes. The advantages of the flow techniques bases on its operation mode. On SIA the analytical cycle consists on the sequential aspiration of sample and reagent solutions, reversion of the flow and propulsion of the reaction product to the detector where it is monitored. The selection valve and the propulsion device are controlled by computer. Therefore, it obtained an exquisite control of the reaction conditions, making SIA an interesting tool for the automation of procedures that demand exactly the same conditions in all the assays (Silvestre et al., 2011). Moreover, it is a robust and versatile technique which affords low reagents consumption and minimal effluents generation, being in agreement with the Green Chemistry principles (Lenehan et al., 2002). More recently, SIA emerged as a valuable alternative for the implementation of enzymatic procedures in ILs (Pinto et al., 2008), including for application in toxicity screening (Cunha et al., 2015; Costa et al., 2016).

Ultimately, in this work we intended to develop an automated SIA methodology for the evaluation of ILs' toxicity through the inhibition of ACY I. It is our purpose that the developed methodology can provide more information about the safety of the tested ILs and that can be used as an alternative approach to the batch procedures. Additionally, it is expected that the obtained results can help identify some toxicophore structures, contributing to the design of less toxic compounds.

2. Materials and methods

2.1. Reagents

All solutions were prepared using chemicals of analytical reagent grade and high purity water (Milli-Q water) with a specific conductance less than 0.1 μS cm^{-1}. All reagents were obtained from Sigma-Aldrich Co. and used without further purification. Fluorescamine (4-phenylspiro-[furan-2(3*H*),1-phthalan]-3,3′-dione) was kept under an inert atmosphere of nitrogen and protected from light. The tested ILs were stored in a carefully controlled anhydrous environment.

A phosphate buffer 100 mmol L^{-1} (pH 8.0) was used as carrier in the flow system.

A solution of ACY I from porcine kidney (grade II, 500–1500 U mg^{-1} protein), of approximately 54 U mL^{-1}, was prepared daily by dissolving the proper amount of the lyophilized enzyme in phosphate buffer 100 mmol L^{-1} (pH 7.0). Also daily, a stock solution of fluorescamine was prepared by dissolving 2.5 mg