Journal of Hazardous Materials 265 (2014) 133–141

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Automated evaluation of pharmaceutically active ionic liquids' (eco)toxicity through the inhibition of human carboxylesterase and *Vibrio fischeri*

Susana P.F. Costa [a], Vanessa D. Justina [a], Katharina Bica [b], Maria Vasiloiu [b], Paula C.A.G. Pinto [a,*], M. Lúcia M.F.S. Saraiva [a,*]

[a] *REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, n° 228, 4050-313 Porto, Portugal*

[b] *Vienna University of Technology, Institute of Applied and Synthetic Chemistry, A-1060 Vienna, Austria*

HIGHLIGHTS

- IL-APIs toxicity on humans and aquatic environment was evaluated by inhibition assays.
- The inhibition assays were implemented through automated screening bioassays.
- Automation of bioassays enabled a rigorous control of the reaction conditions.
- EC_{50} obtained provide vital information on IL-APIs safety and potential use as drugs.

ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 4 November 2013
Accepted 24 November 2013
Available online 1 December 2013

Keywords:
Pharmaceutically active ionic liquids
Human toxicity
Vibrio fischeri
SIA

ABSTRACT

The toxicity of 16 pharmaceutical active ionic liquids (IL-APIs) was evaluated by automated approaches based on sequential injection analysis (SIA). The implemented bioassays were centered on the inhibition of human carboxylesterase 2 and *Vibrio fischeri*, in the presence of the tested compounds. The inhibitory effects were quantified by calculating the inhibitor concentration required to cause 50% of inhibition (EC_{50}). The EC_{50} values demonstrated that the cetylpyridinium group was one of the most toxic cations and that the imidazolium group was the less toxic. The obtained results provide important information about the safety of the studied IL-APIs and their possible use as pharmaceutical drugs. The developed automated SIA methodologies are robust screening bioassays, and can be used as a generic tools to identify the (eco)toxicity of the structural elements of ILs, contributing to a sustainable development of drugs.

1. Introduction

Ionic liquids (ILs) are salts formed by ions [1] that only started to be noticed when their use as solvents revealed advantages [2], due to distinctive physical properties (1st generation) [3,4]. The increasing development in this area enabled the synthesis of ILs with specific physical and chemical properties, suitable for a variety of applications (2nd generation) such as organic and inorganic synthesis, biocatalysis [2], nanotechnology [1] and pharmaceutical industry.

In the pharmaceutical field, ILs were initially applied to improve the performance of drugs, in particular their solubility, bioavailability and stability [5]. Meanwhile, given the similarities between common ILs and active pharmaceutical ingredients (APIs) or API precursors, some researchers studied their biological properties [3]. It was found that different groups of ILs with well-established applications present pharmacological activity, namely

Abbreviations: ILs, ionic liquids; IL-APIs, pharmaceutical active ionic liquids; APIs, active pharmaceutical ingredients; SIA, sequential injection analysis; HCE, human carboxylesterase; MUA, 4-methylumbeliferyl acetate; BACl, benzalkonium chloride; BASal, benzalkonium salicylate; $BETf_2N$, benzethonium bistriflimide; BECl, benzethonium chloride; BED, benzethonium docusate; BESal, benzethonium salicylate; CetPyCl, cetylpyridinium chloride; CetPySal, cetylpyridinium salicylate; emimCl, 1-ethyl-3-methyl-imidazolium chloride; emimSal, 1-ethyl-3-methyl-imidazolium salicylate; NaSal, sodium salicylate; NaD, sodium docusate; $(Hex)_3(TDec)PTf_2N$, trihexyltetradecylphosphonium bistriflimide; $(Hex)_3(TDec)PCl$, trihexyltetradecylphosphonium chloride; $(Hex)_3(TDec)PD$, trihexyltetradecylphosphonium docusate; $(Hex)_3(TDec)PSal$, trihexyltetradecylphosphonium salicylate; DMSO, dimethylsulfoxide.

* Corresponding authors. Tel.: +351 220428670; fax: +351 226093483.
E-mail addresses: ppinto@ff.up.pt (P.C.A.G. Pinto), lsaraiva@ff.up.pt (M.L.M.F.S. Saraiva).

0304-3894/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jhazmat.2013.11.052

antimicrobial activity [6–8]. Recently, the synthesis of ILs with biological activity, resulting from the combination of APIs, was described (3rd generation). The ions can both present biological activity or the counterion can have the ability to reduce the adverse effects of the API or to change the solubility of the molecule, for example [9]. Several studies confirm that IL-APIs can result on increased bioactivity of the involved APIs [10–12].

Considering the future utilization of IL-APIs as pharmaceuticals, it is vital, not only to evaluate their therapeutic potential and action mechanism, but also their possible adverse effects on humans. Furthermore, the environmental risk of these compounds should also be assessed. IL-APIs, as well as conventional pharmaceuticals, have low volatility, which indicates that aquatic environment is probably the principal pathway for the release of these compounds in the environment and consequently one of the most affected by their toxicity [13,14]. Thus, the analysis of the impact of these possible new drugs should be performed at an early stage of their development and, as far as we know, this has not been performed yet. In this work, the toxicity of IL-APIs was studied through the adaptation of bioassays developed for conventional ILs.

Enzymatic inhibition assays have already proved to be a good choice for the prediction of ILs' impact on humans. Generally, enzymes like acetylcholinesterase and carboxylesterases showed to be good biomarkers of toxicity [15–17]. In particular, carboxylesterases play an important role on human metabolism, since they perform the detoxification of many agrochemicals and pharmaceuticals and the hydrolysis of several endogenous substrates. Two major human carboxylesterases have been identified, HCE1 and HCE2. These enzymes were applied to the evaluation of the toxicity of various compounds, namely pesticides and drugs [18–20] and human carboxylesterase 2 inhibition revealed good results in the prediction of ILs' toxicity [21].

Regarding environmental toxicity, *Vibrio fischeri* is one of the most applied aquatic organisms for the evaluation of toxicity of xenobiotics [22–24] being a standard (eco)toxicological bioassay in Europe (DIN EN ISO 11348) [25].

In this work, we decided for the automation of the assays through a flow technique, sequential injection analysis (SIA) [26], that originates versatile, low-cost and robust methodologies. SIA has been profitably applied in bioassays with ILs [29] and proved to be a suitable choice due to its ability to guarantee precisely the reaction conditions through computer control of analytical parameters [27–29].

Thus, automated methodologies based on SIA were applied to the assessment of toxicity of various IL-APIs on humans and in the aquatic environment. The selected IL-APIs include cations with antibacterial activity, namely cetylpyridinium, benzethonium, benzalkonium and trihexyltetradecylphosphonium (only 1-ethyl-3-methylimidazolium cation is neutral), combined with the salicylate anion, with well recognized activity (e.g. analgesic), or with very hydrophobic anions (e.g. bistriflimide or docusate).

The methodologies applied in this work were adapted from the assays developed before for carboxylesterase [21] and *V. fischeri.* [30]. The carboxylesterase assay was based on the hydrolysis of 4-methylumbeliferyl acetate (MUA) with formation of a fluorescent product [31]. In the *V. fischeri* assay, the influence of the compounds on the bioluminescence emitted by the marine gram negative bacterium was evaluated [25].

With this work, we intended to predict the (eco)toxicity of some IL-APIs and provide relevant data to identify ILs' structural elements, contributing to their safe utilization in the pharmaceutical field.

2. Materials and methods

2.1. Reagents

All solutions were prepared using chemicals of analytical reagent grade and high purity water (milli-Q) with a specific conductance <0.1 $\mu S\,cm^{-1}$ that was also used as carrier in the flow system in the carboxylesterase assay.

4-Methylumbeliferyl acetate (MUA), human carboxylesterase 2, dimethylsulfoxide (DMSO) and HEPES hemisodium salt were purchased from Sigma-Aldrich Cooperation.

Human carboxylesterase 2, 10 UG mL^{-1} was dissolved in Hepes buffer (10 mmol L^{-1}, pH 7.4) and divided in 10 working solutions which were stored at – 70 °C. Each aliquot of enzyme was dissolved in Hepes buffer and used in the inhibition assays, with a final concentration of 1 UG mL^{-1}. The stock solution of MUA (50 mmol L^{-1}) and the intermediate solution (10 mmol L^{-1}) were prepared in DMSO and the working standard (0.75 mmol L^{-1}) was diluted with mili-Q water to final concentration. The IL-APIs solutions were prepared by combining MUA (0.75 mmol L^{-1}) with increasing concentrations of IL-API, in water. The IL-APIs poorly soluble in water: $BETf_2N$, BED, $(Hex)_3(TDec)PTf_2N$, $(Hex)_3(TDec)PCl$, $(Hex)_3(TDec)PD$, $(Hex)_3(TDec)PSal$ and NaD were prepared similarly but using DMSO as solvent, at a final concentration of 35%.

In *V. fischeri* assay, a solution of NaCl 2%, pH 7 was used as carrier in the SIA system. Lyophilized *V. fischeri* and corresponding dilution solution were obtained from BioTox™ test kit. The IL-APIs tested were prepared in NaCl 2%, pH 7, in increasing concentrations. The poorly soluble IL-APIs were prepared in DMSO dissolved until 35% with NaCl 2%, pH 7. The positive control of the assays was performed with $CuSO_4$ (Fig. 1).

2.2. Synthesis and characterization

2.2.1. General remarks

All chemicals unless otherwise stated were purchased from commercial suppliers and used without further purification. Acetone and dichloromethane were distilled prior to use. Anhydrous solvents were obtained from a Puresolv-Anlage from It Innovative, Inc. BESal, BASal and CetPySal were synthesized as previously reported and analytical data were found to be in accordance with literature data [10].

^1H and ^{13}C NMR spectra were recorded on a Bruker AC 400 at 200 and 100 MHz, respectively, using the solvent peak as reference. *J* values are given in Hz. ^{13}C NMR spectra were run in proton-decoupled mode. IR spectra were recorded against an air background with 16 scans on a Biorad FTS 135 FT-IR spectrometer equipped with a specac ATR-unit. TLC-MS analyses were analyzed on a Bruker Esquire HTC ion trap mass spectrometer equipped with a camag TLC-MS interface. Thermogravimetric analysis was performed on a Netzsch TGA/DSC under helium. Samples between 5 and 10 mg were placed in open alumina pans and were heated from 25 °C to 500 °C with a heating rate of 10 °C/min. Decomposition temperatures ($T_{5\%dec}$) were reported from onset to 5 wt.% mass loss.

2.2.2. Synthesis

2.2.2.1. Benzethonium docusate. BECl (4.48 g, 10 mmol) and NaD (4.45 g, 10 mmol) were dissolved in 100 mL of acetone/water 1:1 and stirred over night at room temperature. The solution was concentrated to ~50 mL, diluted with water and repeatedly extracted with dichloromethane. The combined organic layers were washed with water until no more chloride ions could be detected (checked by addition of aqueous silver nitrate solution ($AgNO_3$ 1%), dried over sodium sulfate (Na_2SO_4) and concentrated under reduced pressure. Remaining solvent traces were removed under high