CrossMark

Anti-inflammatory choline based ionic liquids: Insights into their lipophilicity, solubility and toxicity parameters

Ana M.O. Azevedo [a], Susana P.F. Costa [a], Ana F.V. Dias [a,b], Alexandre H.O. Marques [a,b], Paula C.A.G. Pinto [a], Katharina Bica [c], Anna K. Ressmann [c], Marieta L.C. Passos [a], André R.T.S. Araújo [a,d], Salette Reis [e], M. Lúcia M.F.S. Saraiva [a,*]

[a] LAQV, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[b] Escola Superior de Saúde, Instituto Politécnico da Guarda, Avenida Rainha D. Amélia, S/N 6300-749 Guarda, Portugal
[c] Vienna University of Technology, Institute of Applied and Synthetic Chemistry, Getreidemarkt 9/163, A-1060 Vienna, Austria
[d] Unidade de Investigação para o Desenvolvimento do Interior, Instituto Politécnico da Guarda, Avenida Dr. Francisco de Sá Carneiro, 50, 6300-559 Guarda, Portugal
[e] UCIBIO, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

ARTICLE INFO

ABSTRACT

The impact on *in vivo* efficacy and safety of two novel ionic liquids based on the association of choline with non-steroidal anti-inflammatory drugs, ketoprofen and naproxen forming IL-APIs, was evaluated. Their lipophilicity, solubility and toxicity were assessed aiming the illustration of the pharmaceutical profile and potential toxic impact.
Partition coefficient was determined using micelles of hexadecylphosphocholine and UV–Vis derivative spectroscopy. Additionally, solubility in phosphate buffer pH 7.4 was measured using a modified shake flask method and UV–Vis spectroscopy as detection technique. Ultimately, toxicity was considered resorting to a fully automated cytochrome c oxidase assay based on microfluidics. The obtained results demonstrated that the IL-APIs' drug format has the ability to interact with biological membranes and also improves solubility up to 58 times. Moreover, it was evidenced that, although being a nutrient, choline influences the IL-APIs' toxicity. The studied anti-inflammatory IL-APIs exhibited promising properties regarding their incorporation in pharmaceutical formulations.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

The pharmaceutical industry faces nowadays unprecedented challenges and amendments as a consequence of strong demands related with economic viability and environmental sustainability. The investment on research and development is forecast to decline since the risks associated with drug discovery seem to not be in accordance with the current economic demands. It is known that 90% of the experimental drugs cannot reach the market mainly due to lack of efficacy, toxicity and inadequate pharmacokinetics [1,2]. Indeed, approximately 40% of the novel drug candidates fail to obtain approval because of poor pharmaceutical properties such as reduced solubility or decreased permeation across the blood-brain barrier [3]. Moreover, the demands regarding the environmental impact of drug discovery are on the frontline of the principles of Green Chemistry [4].

Typically, the pharmaceutical industry relies on crystalline forms of active pharmaceutical ingredients (APIs) with claimed advantages in terms of purity, thermal stability, manufacturing and handling. However, solid APIs often suffer from low solubility and polymorphism conversion, which can have a negative impact on drug bioavailability and ultimately on its efficacy [2,5]. The issue of efficacy is quite important considering that this is the most frequent reason for failure in phase II clinical trials [6]. Salt formation is the most common and cost-effective strategy to overcome the problems faced by solid forms of APIs, being estimated that around 50% of all drugs utilized in the pharmaceutical industry are salts with improved properties regarding the corresponding ionizable drugs [5,7].

Ionic liquids (ILs) emerged as a new class of compounds with peculiar properties that make them suitable for distinct pharmaceutical applications [8]. In the past few years, ILs have been explored as reaction media for the synthesis of APIs [9], as pharmaceutical solvents [10] and as part of drug delivery systems [11,12]. Additionally, several authors have reported ILs that are themselves the APIs (IL-APIs) [13–17]. From the pharmaceutical point of view, an IL approach in the design of novel APIs seems to be appropriate as it enables a large number of possible cation-anion combinations while providing singular properties unreachable in solid salts, namely improved solubility and absence of

Abbreviations: APIs, active pharmaceutical ingredients; chol [KTP], choline ketoprofenate; chol [NAP], choline naproxenate; CytCox, cytochrome c oxidase; FeC, ferrocytochrome c; HDPC, hexadecylphosphocholine; ILs, ionic liquids; IL-APIs, pharmaceutically active ionic liquids; KTP, ketoprofen; NAP, naproxen; SIA, sequential injection analysis.
  * Corresponding author.
    E-mail address: lsaraiva@ff.up.pt (M.L.M.F.S. Saraiva).

polymorphic forms [5,18,19]. Moreover, the appropriate combination of cations and anions empowers the chemical manipulation of compounds with specific purposes related with the manufacturing process, the stability of formulations and their bioavailability [18,20]. The research in this field has been focused mostly on the synthesis of new IL-APIs, their physico-chemical characterization and *in vitro* assessment of the expected pharmacological activity [14,17,21]. As far as we know there are relatively few studies exploring the pharmaceutical properties of IL-APIs for prediction of their *in vivo* behavior and there is still little information regarding the toxicity of these compounds [16,22–26].

Thus, in this work, we studied for the first time the lipophilicity, solubility and toxicity of two anti-inflammatory IL-APIs based on choline (Fig. 1). The selected IL-APIs include distinct anions with well-known analgesic, anti-inflammatory and anti-pyretic activity, namely ketoprofen (KTP) and naproxen (NAP) [27], combined with the choline cation, an essential nutrient that is necessary for many critical functions in the human body [28].

The lipophilicity of the anti-inflammatory IL-APIs was assessed through the calculation of partition coefficient (expressed as log *D*) by derivative spectroscopy. The determination was based on the study of the interaction of the selected IL-APIs with a membrane model aiming to evaluate the distribution of drugs between aqueous and lipid phases. It is nowadays accepted and demonstrated that membrane models are more adequate for this purpose than biphasic solvent systems like octanol-water since they mimic the lipid bilayer of biological membranes [29,30]. Indeed, biomimetic membrane models are able to consider the hydrophobic, hydrogen bond, dipole-dipole and electrostatic interactions between drug and membrane, whereas octanol-water systems can only model nonpolar interactions [31,32]. In the present work, micelles of hexadecylphosphocholine (HDPC) were used as membrane model since they are easy and fast to prepare, have high stability and few spectral interferences, and circumvent the use of toxic organic solvents [33,34].

Taking into account the low solubility of the anions incorporated in the selected compounds it was evaluated if this property was improved in the IL format [35,36]. This was performed through the determination of thermodynamic solubility using the shake flask method. The solubility issue is of great importance since it is known that solubility affects both *in vitro* assay results and *in vivo* oral bioavailability. Indeed, poor aqueous solubility is one of the main causes for low systemic exposure and, consequently, lack of *in vivo* efficacy [3].

Considering the pharmaceutical potential of IL-APIs, their toxicity was evaluated resorting to a fully automated cytochrome *c* oxidase (CytCox) assay aiming to predict IL-APIs' safety. Enzymatic inhibition assays have already proved to be useful in clarifying the impact of ILs' structural elements on toxicity and the way they should be changed to decrease hazardous potential. The methodology presented the additional advantage of automation of the assay based on sequential injection analysis (SIA) as it guarantees a precise control of the reaction conditions and reduces the consumption of reagents as well as the production of hazardous effluents, being in good agreement with the present concerns of Green Chemistry [37,38].

With this work, it is then expected to contribute to the understanding of the potentialities of chol [KTP] and chol [NAP] to replace their APIs counterparts. For that, these compounds were submitted to new assays to evaluate more pharmaceutical properties and toxicity in order to complement the information gathered during their synthesis and physico-chemical characterization as well as their binding affinity data [26]. The comparison of the IL-APIs' profile with the respective starting materials aims to highlight some of the features of APIs in the IL format. Moreover, by contributing to the revealing of the drug-like nature of these compounds it is also expected to give early warning of potential difficulties in formulation, process development and safety that would otherwise increase development time/cost and delay their possible clinical introduction.

2. Experimental

2.1. Chemicals

All solutions were prepared using chemicals of analytical reagent grade and high purity water (Milli-Q water) with a specific conductance $< 0.1\ \mu S\ cm^{-1}$.

KTP (2-(3-Benzoylphenyl)propionic acid), Na [NAP] ((*S*)-6-Methoxy-α-methyl-2-naphthaleneacetic acid sodium salt), Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol), CytCox from bovine heart (EC 1.9.3.1), cytochrome *c* and DL-dithiothreitol (*threo*-1,4-Dimercapto-2,3-butanediol) were all purchased from Sigma-Aldrich and used as supplied. HDPC was obtained from Cayman Chemical and used without further purification. chol [KTP] and chol [NAP] (99%) were synthesized and characterized as reported in our previous work [26]. The tested IL-APIs were stored at room temperature in a carefully controlled anhydrous environment.

The partition coefficient and solubility assays were performed in phosphate buffer $0.1\ mol\ L^{-1}$, pH 7.4 ($I = 0.15\ mol\ L^{-1}$). A stock suspension of HDPC $600\ \mu mol\ L^{-1}$ was prepared daily in the described phosphate buffer solution and a suspension of HDPC $150\ \mu mol\ L^{-1}$ was prepared by suitable dilution of the stock suspension in the same buffer. In the toxicological assays, a Tris-HCl buffer $0.01\ mol\ L^{-1}$ (pH 7.0), containing KCl $0.12\ mol\ L^{-1}$ was used as carrier in the flow system. CytCox $0.2\ U\ mL^{-1}$ was reconstituted in Tris-HCl enzyme dilution buffer $0.01\ mol\ L^{-1}$ (pH 7.0), with sucrose $0.25\ mol\ L^{-1}$, and divided in 12 working solutions, which were stored at $-20\ °C$. Each aliquot of working solution was reconstituted with enzyme dilution buffer at a final concentration of $0.01\ U\ mL^{-1}$. Ferrocytochrome *c* (FeC) solution was prepared daily by the combination of 25 μL of DL-dithiothreitol $0.1\ mol\ L^{-1}$ solution, previously prepared, 13.5 mg of cytochrome *c* and water to a final volume of 5 mL.

In the partition coefficient assays solutions of chol [KTP] and KTP $250\ \mu mol\ L^{-1}$, chol [NAP] and Na [NAP] $800\ \mu mol\ L^{-1}$ were prepared
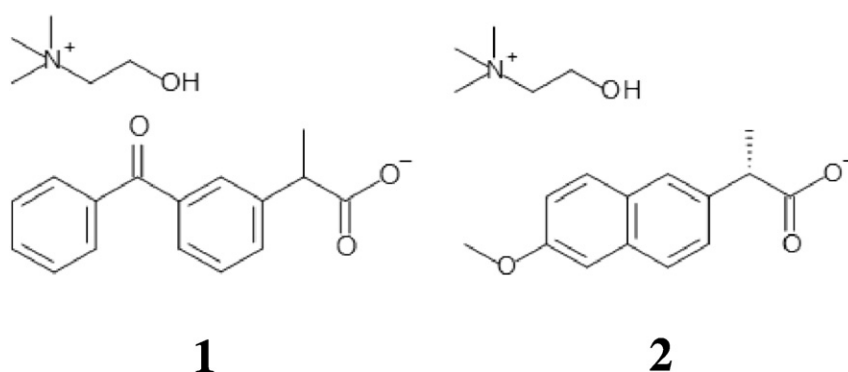

Fig. 1. Chemical structures of the studied IL-APIs: (1) choline ketoprofenate (chol [KTP]) and (2) choline naproxenate (chol [NAP]).

in phosphate buffer. For the toxicological assays solutions of the selected IL-APIs and respective starting materials were prepared in the same buffer, in increasing concentrations.

2.2. Instrumentation

In the batch assays, absorbance measurements were performed on a Synergy HT Multi-Mode (BioTek Instruments) microplate reader.

In the solubility studies a C-MAG HS 7 magnetic stirrer (IKA) was used to mix the aqueous and solid phases. The samples were centrifuged using an Allegra X-15R centrifuge (Beckman Coulter).

The toxicity of IL-APIs and corresponding starting materials to CytCox was evaluated in a fully automated SIA system (Fig. 2) previously reported [39]. Spectophotometric measurements were performed resorting to a 6300 Jenway spectrophotometer model set at 550 nm and equipped with a 18 μL flow cell (Helma 178.711QS, Mülheim, Balden, Germany). A syringe module Bu1S from Crison Instruments S.A. (Allela, Barcelona, Spain) and a 10-port multiposition Cheminert™ selection valve were controlled by computer. A glass syringe of 5 mL total dispense volume (Hamilton Bonaduz AG, Switzerland) was coupled to the syringe equipment and driven by a stepper motor. Solenoid head-valves allowed the commutation of the syringe either to the manifold or to the carrier. All the components were connected by means of 0.8 mm i.d. PTFE tubing, which was also used for the holding and reaction coil (2 and 1 m, respectively). The data acquisition was recorded in a strip chart recorder (Kipp&Zonen BD 111) or via computer.

2.3. Assessment of partition coefficient by derivative spectroscopy

The study of the partitioning of the selected IL-APIs and corresponding starting materials between the aqueous and lipid (HDPC) phases was performed by derivative spectroscopy through a microplate assay described by Magalhaes and co-workers [34]. Buffered solution containing a fixed drug concentration (chol [KTP] and KTP 250 μmol L^{-1}, chol [NAP] and Na [NAP] 800 μmol L^{-1}) was added to increasing concentrations of HDPC ranging from 7.5 to 200 μmol L^{-1}. The corresponding reference suspensions were identically prepared in the absence of drug. The absorption spectra of samples and references were recorded at 37 °C in a microplate reader in accordance with the abovementioned protocol, in the range of 200–500 nm [34].

The mathematical treatment of the experimental data was performed using the Excel routine K_p calculator [34]. Briefly, this spreadsheet tool subtracts each reference spectrum from the respective sample spectrum to obtain corrected absorption spectra and subsequently determines the first, second and third derivative spectra. Derivatization allows the elimination of spectral interferences resulting from
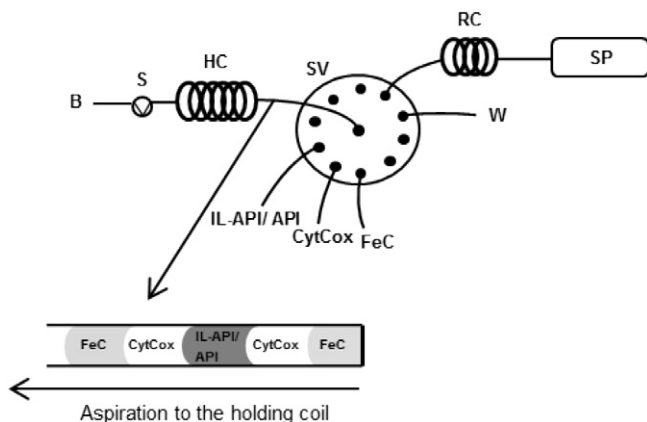

Fig. 2. Schematic representation of the SIA manifold. B: Tris-HCl buffer, 0.01 mol L^{-1} (pH 7.0), containing KCl 0.12 mol L^{-1}; S: syringe; HC: holding coil; SV: selection valve; RC: reaction coil; SP: spectrophotometer; W: waste; IL-API/API: chol [KTP] or KTP, chol [NAP] or Na [NAP]; CytCox: cytochrome c oxidase; FeC: ferrocytochrome c.

light scattered by the lipid micelles, enhance the ability to detect minor spectral features and improves the resolution of bands [33,40]. After this, the partition coefficients were calculated by fitting the following equation to the experimental data (D_t vs. [L]) using a non-linear least-squares regression method [34]:

$$D_t = D_w \frac{(D_l - D_w)K_p[L]}{1 + K_p[L]} \tag{1}$$

where D is the derivative intensity obtained from the absorbance values of: total amount of drug (D_t); drug distributed in the lipid (D_l) and in the aqueous (D_w) phase, respectively; [L] HDPC concentration (mol L^{-1}) and K_p is the partition coefficient expressed as mol L^{-1}. The dimensionless value was calculated from the division of the molar partition coefficient by the HDPC molar volume.

2.4. Determination of solubility by a shake flask method

The thermodynamic solubility of the synthesized IL-APIs and respective starting materials in phosphate buffer pH 7.4 was determined using a modified shake flask method. Samples were prepared by adding an excess amount of each drug to 1.5 mL of the buffer in screw cap vials. The vial contents were then mixed with a magnetic stirrer at 500 rpm for 8 h, allowing time for the system to reach equilibrium between the dissolved and solid compound. Afterwards, the saturated solution was separated from the precipitate by centrifugation at 10,000 rpm for 30 min. The resulting supernatant was appropriately diluted in phosphate buffer and the concentration of drug was measured spectrophotometrically in a microplate reader at the wavelength of maximum absorbance. For each drug, calibration curves were previously established using standard solutions of different concentrations of the drug in phosphate buffer. All the experiments were carried out in triplicate at 37 °C.

2.5. Evaluation of toxicity through automated CytCox assay

CytCox activity in the presence and in the absence of IL-APIs and corresponding starting materials was determined according to the analytical cycle optimized for that purpose [39]. The assays were performed at room temperature.

The real concentration of inhibitor in the flow system was calculated through the application of an experimentally determined dispersion factor correction. The results of the inhibition assays were presented as normalized CytCox activity, corresponding the maximum value 1 to CytCox activity in the absence of inhibitor. The effective concentration of the compound causing a decrease of 50% of enzyme activity was determined and represented as IC$_{50}$ value. The calculations were performed by means of linear or polynomial correlations established in the inhibition assays for each tested compound.

3. Results and discussion

3.1. Partition coefficient of anti-inflammatory IL-APIs

In this work, the lipophilicity of the synthesized IL-APIs was assessed through the determination of partition coefficients of drugs between aqueous and lipid phases. With this we intended to collect data that can be useful for the prediction and understanding of the passive diffusion processes of drugs across biomembranes. Considering the ionic nature of IL-APIs and the zwitterionic lipids as the major lipid components of eukaryotic membranes we resorted to neutral micelles of HDPC that enable the establishment of interactions with either lipophilic or hydrophilic drugs, contrary to other models like octanol-water [33].

The log D values of the tested IL-APIs and respective starting materials are listed in Table 1. As an example, the plot of the absorbance

Table 1
Partition coefficients (log D) (dimensionless) of IL-APIs and respective starting materials in micelles of HDPC at physiological conditions (pH 7.4 and 37 °C).

Compound		log $D \pm$ SD[a]
IL-APIs	chol [KTP]	5.44 ± 0.03
	chol [NAP]	5.44 ± 0.03
Starting materials	KTP	5.48 ± 0.02
	Na [NAP]	5.47 ± 0.02

[a] The values presented are the mean and the standard deviation of 3 replicates.

values and the 1st and 2nd derivative graphs for chol [KTP] at different micellar concentrations are presented in Fig. 3.

The similarity between the log D values of IL-APIs and corresponding starting materials indicated that the utilization of KTP and NAP in the IL format does not alter the ability to permeate biological membranes. The same trend was observed by Florindo and co-workers for a series of ILs based on ciprofloxacin and norfloxacin [24]. Moreover, Kawai et al. reported that choline based ILs can penetrate through cell membranes because their molecular structures resemble choline, which is transported across membranes for use as a precursor in the synthesis of acetylcholine and phospholipids [41].

KTP and NAP are weak acidic drugs that possess a carboxylic group that can undergo deprotonation (pK_a = 3.88 and 4.19, respectively). At the physiological pH (pH = 7.4) almost 100% of drug molecules are negatively charged, according to calculations made in Marvin Sketch

Calculator software from ChemAxon. The octanol-water distribution coefficients (log D) predicted by Marvin Sketch at pH 7.4 for KTP and NAP were 0.39 and −0.05, respectively. The discrepancy between experimental and calculated log D values can be explained by the fact of software does not take into account the ionic interactions established between the charged drugs and the polar head groups of the phospholipids of membranes. These observations highlight the advantage of using membrane models for the determination of partition coefficients since hydrophobic forces are not the only interactions driving the drugs partition, which also encodes electrostatic interactions.

3.2. Solubility of anti-inflammatory IL-APIs in phosphate buffer pH 7.4

The study of the solubility of the selected IL-APIs was conducted in phosphate buffer pH 7.4 with the objective of further elucidate the absorption of these compounds. This issue is of utmost importance since it is known that poorly water-soluble drugs tend to have incomplete absorption and subsequently low bioavailability. Thus, the thermodynamic solubility was measured by a modified shake flask method, ensuring that the equilibrium between the dissolved and solid drug was reached [42].

The comparison of the solubility at 37 °C of IL-APIs with the corresponding starting materials is depicted in Fig. 4.

As can be seen, both IL-APIs display greater solubility in phosphate buffer pH 7.4. chol [KTP] was 58 times more soluble than KTP and for chol [NAP], even when compared with Na [NAP], it was observed a 4-fold increase of its solubility. The observed differences can be related
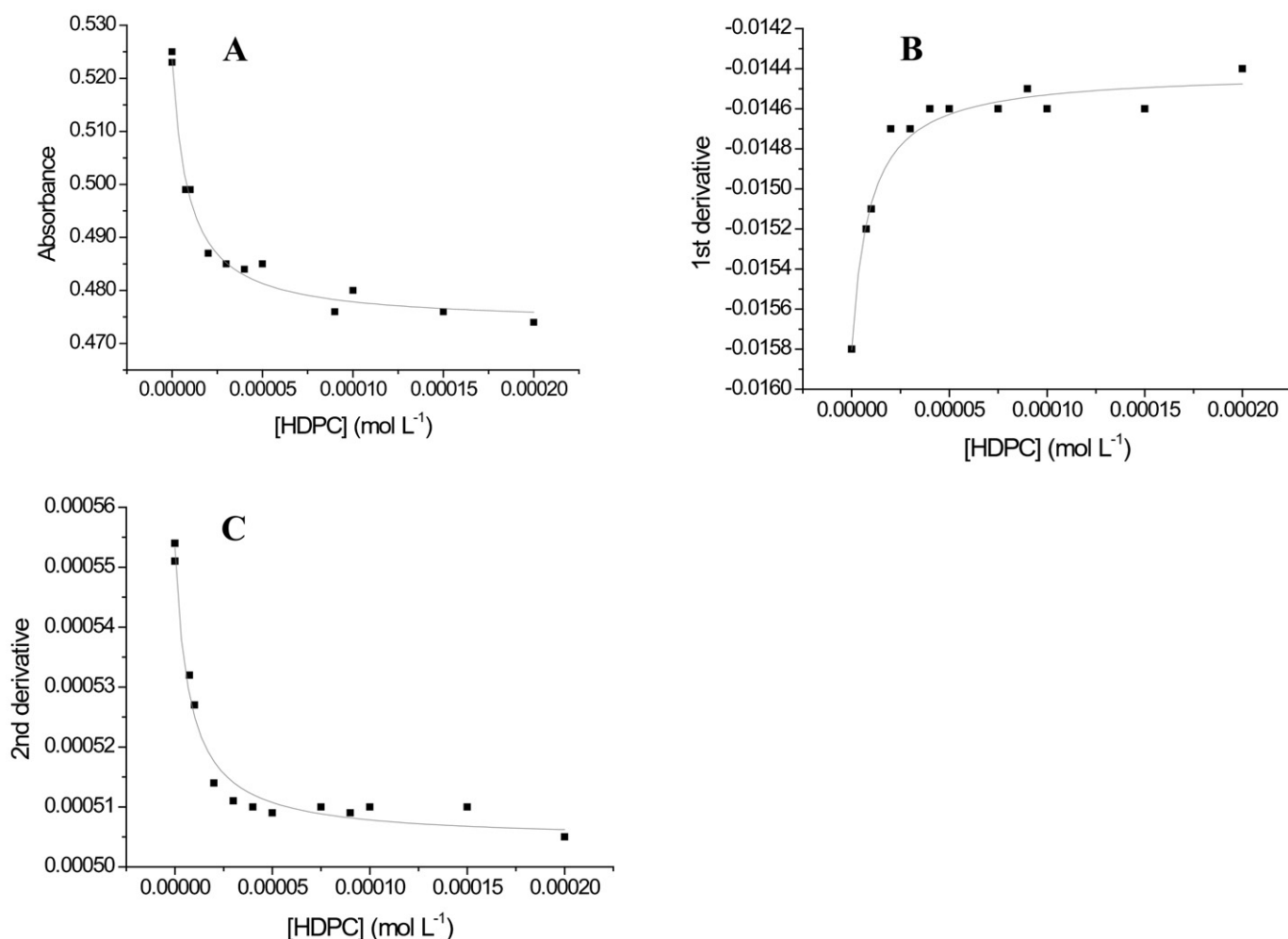

Fig. 3. Absorbance (A), 1st (B) and 2nd (C) derivative data at 258, 274 and 288 nm, respectively, for chol [KTP] at different HDPC micellar concentrations.
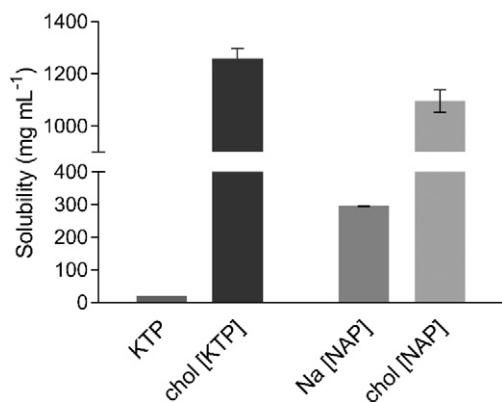
Fig. 4. Solubility of the synthesized anti-inflammatory IL-APIs and corresponding starting materials in phosphate buffer solution pH 7.4 at 37 °C.

to the fact that these compounds belong to the class of salts known as ILs and that the salt formation is one of the most frequently employed strategies to improve solubility and, ultimately, bioavailability of poorly water-soluble drugs [7,43]. Murti et al. demonstrated that chol [NAP] was 2 times more soluble than Na [NAP] [44]. However, the discrepancies between those and our results could be explained by the different temperature used (25 and 37 °C, respectively). Two other studies reported an increased solubility of IL-APIs containing the choline cation compared to the parent APIs [22,45]. Similar observations were made by Florindo and co-workers for IL-APIs based on fluoroquinolone drugs [24].

Moreover, from the analysis of the solubility values obtained for chol [NAP] and Na [NAP] it was possible to conclude that the selection of the most appropriate counter-cation enables the fine-tuning of the solubility of API. The higher solubility of chol [NAP] can be explained by the presence of the hydroxyl group in the alkyl chain of choline, which enhances the polarity and the hydrogen bonding capacity of the compound.

3.3. Toxicity of anti-inflammatory IL-APIs towards CytCox

Choline is a quaternary ammonium compound with vast industrial potential, considering the general idea of environmentally benign structure [46,47] and its role in the organism, from cell membranes to cholinergic neurotransmission [28]. Therefore, an increase number of new ILs incorporating the choline cation have been described [48–50]. Conversely, there is little information about the biodegradability and toxicity profile of these compounds.

The evaluation of chemicals' toxicity by means of enzyme inhibition presents advantages such as simplicity of laboratory implementation and data interpretation, reduction of costs and duration of the assays. In this section, we report the toxicity of new synthesized choline based IL-APIs through the inhibition of CytCox activity on a SIA system.

The inhibition profiles of the tested compounds are shown in Fig. 5 and the respective IC_{50} values are presented in Table 2. Due to solubility problems, it was not possible to determine the IC_{50} value of KTP.

Comparing the anions of chol [KTP] (IC_{50} = 21.32 mmol L^{-1}) and chol [NAP] (IC_{50} = 0.47 mmol L^{-1}), which confer the pharmacological activity to respective compounds, it was verified a higher toxic effect for the naproxen anion. The anions have a similar composition with slight differences in the arrangement of the two aromatic rings and the oxygen atom. It appears that naphthalene and terminal methoxy groups have a greater negative effect on enzyme activity than two phenyl groups linked by a ketone function. The higher IC_{50} value of chol [KTP] could be related to an easier degradation of the molecule at the ketone group. Additional studies are needed to confirm the hypothesis or identify other factors that might be involved in the toxicological mechanism. There is few information about NAP and KTP (eco)toxicity
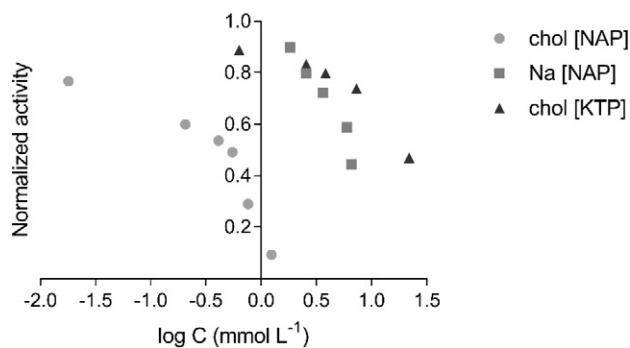

Fig. 5. Experimental toxicity data of the tested IL-APIs and respective starting materials in CytCox assay.

studies, especially with CytCox, to enable a reliable comparison with literature data. Farré et al. [51] studied the toxic effect of some drugs on the aquatic organism *Vibrio fischeri* and observed similar EC_{50} values for NAP and KTP, being the last one slightly more toxic. The results obtained for Na [NAP] (IC_{50} = 5.91 mmol L^{-1}) and chol [NAP] (IC_{50} = 0.47 mmol L^{-1}) demonstrated that the IL-API is more toxic than the corresponding starting material. The decrease of the IC_{50} value could also be related with solubility increase, which may contribute for its bioavailability enhancement. This in turn may explain the increase in CytCox activity inhibition. A similar inhibition trend was reported by our group for salicylate derived IL-APIs [23]. Despite the choline cation presented higher toxicity than sodium, the IC_{50} differences are small considering that sodium is a recognized inert ion, *i.e.*, it not contributes significantly to the molecule toxicity. Indeed, most of the works describe choline as a relatively safe compound due to its structure, *i.e.*, a quaternary ammonium cation incorporating a polar hydroxyl group in one of its alkyl side chains [48,52]. However, some studies have revealed that choline based ILs are not always harmless [49,53]. Santos et al., for example, observed that six of the ten tested ILs have hazardous potential [53]. The researchers also highlight the importance of the anion on ILs' toxicity, as demonstrated by our results for the tested IL-APIs. On the other hand, the higher IC_{50} values of the synthesized IL-APIs (up to 93 times) compared to zinc ion (IC_{50} = 230 μmol L^{-1}), a recognized CytCox inhibitor, suggest that these compounds are not strong inhibitors of enzyme activity [54]. Cooper and co-workers also reported lower IC_{50} values (in the nanomolar range) for nitric oxide than those obtained in the present work for chol [KTP] and chol [NAP] [55].

4. Conclusions

In this work, we studied the lipophilicity, solubility and toxicity of two anti-inflammatory choline based ILs with the aim of further elucidate their pharmaceutical profile and potential toxic effects.

The obtained results demonstrate the potential of the IL platform in the design of new APIs since the synthesized compounds not only show ability to interact with biological membranes but also present improved solubility. This can have a positive impact on drug bioavailability and ultimately on its efficacy. Moreover, the observed toxic alterations confirm the influence of IL-APIs' structural elements, namely the cation and anion, on their toxicity.

Still, further studies could be performed in order to confirm either the therapeutic effect or toxicity of the synthesized IL-APIs on humans.

Table 2
Results of the inhibition of cytochrome c oxidase by anti-inflammatory IL-APIs and corresponding starting materials expressed as IC_{50}.

Compound		Concentration range (mmol L^{-1})	IC_{50} (mmol L^{-1}) cytochrome c oxidase $\pm$ SD[a]
IL-APIs	chol [KTP]	0.64–21.9	21.32 $\pm$ 0.04
	chol [NAP]	0.02–1.24	0.47 $\pm$ 0.03
Starting materials	KTP	0.0004–0.006	>0.006
	Na [NAP]	1.82–6.55	5.91 $\pm$ 0.69

[a] The values presented are the mean and the standard deviation of 3 replicates.

References

[1] A. Moore, The big and small of drug discovery—biotech versus pharma: advantages and drawbacks in drug development, EMBO Rep. 4 (2003) 114–117, http://dx.doi.org/10.1038/embor748.

[2] N. Variankaval, A.S. Cote, M.F. Doherty, From form to function: crystallization of active pharmaceutical ingredients, AIChE J 54 (2008) 1682–1688, http://dx.doi.org/10.1002/aic.11555.

[3] L. Di, E.H. Kerns, Profiling drug-like properties in discovery research, Curr. Opin. Chem. Biol. 7 (2003) 402–408, http://dx.doi.org/10.1016/s1367-5931(03)00055-3.

[4] K. Alfonsi, J. Colberg, P.J. Dunn, T. Fevig, S. Jennings, T.A. Johnson, H.P. Kleine, C. Knight, M.A. Nagy, D.A. Perry, M. Stefaniak, Green chemistry tools to influence a medicinal chemistry and research chemistry based organisation, Green Chem. 10 (2008) 31–36, http://dx.doi.org/10.1039/b711717e.

[5] J. Stoimenovski, D.R. MacFarlane, K. Bica, R.D. Rogers, Crystalline vs. ionic liquid salt forms of active pharmaceutical ingredients: a position paper, Pharm. Res. 27 (2010) 521–526, http://dx.doi.org/10.1007/s11095-009-0030-0.

[6] J. Arrowsmith, Phase II failures: 2008–2010, Nat. Rev. Drug Discov. 10 (2011) 1, http://dx.doi.org/10.1038/nrd3439.

[7] D.P. Elder, R. Holm, H.L. Diego, Use of pharmaceutical salts and cocrystals to address the issue of poor solubility, Int. J. Pharm. 453 (2013) 88–100, http://dx.doi.org/10.1016/j.ijpharm.2012.11.028.

[8] I.M. Marrucho, L.P. Branco, L.P. Rebelo, Ionic liquids in pharmaceutical applications, Annu. Rev. Chem. Biomol. Eng. 5 (2014) 527–546, http://dx.doi.org/10.1146/annurev-chembioeng-060713-040024.

[9] T. Siodmiak, M.P. Marszall, A. Proszowska, Ionic liquids: a new strategy in pharmaceutical synthesis, Mini-Rev. Org. Chem. 9 (2012) 203–208.

[10] A.M.O. Azevedo, D.M.G. Ribeiro, P.C.A.G. Pinto, M. Lucio, S. Reis, M.L.M.F.S. Saraiva, Imidazolium ionic liquids as solvents of pharmaceuticals: influence on HSA binding and partition coefficient of nimesulide, Int. J. Pharm. 443 (2013) 273–278, http://dx.doi.org/10.1016/j.ijpharm.2012.12.030.

[11] D. Dobler, T. Schmidts, I. Klingenhofer, F. Runkel, Ionic liquids as ingredients in topical drug delivery systems, Int. J. Pharm. 441 (2013) 620–627, http://dx.doi.org/10.1016/j.ijpharm.2012.10.035.

[12] H.D. Williams, Y. Sahbaz, L. Ford, T.H. Nguyen, P.J. Scammells, C.J.H. Porter, Ionic liquids provide unique opportunities for oral drug delivery: structure optimization and in vivo evidence of utility, Chem. Commun. 50 (2014) 1688–1690, http://dx.doi.org/10.1039/c3cc48650h.

[13] R. Ferraz, L.C. Branco, C. Prudencio, J.P. Noronha, Z. Petrovski, Ionic liquids as active pharmaceutical ingredients, ChemMedChem 6 (2011) 975–985, http://dx.doi.org/10.1002/cmdc.201100082.

[14] R. Ferraz, L.C. Branco, I.M. Marrucho, J.M.M. Araujo, L.P.N. Rebelo, M.N. da Ponte, C. Prudencio, J.P. Noronha, Z. Petrovski, Development of novel ionic liquids based on ampicillin, Med. Chem. Commun. 3 (2012) 494–497, http://dx.doi.org/10.1039/c2md00269h.

[15] W.L. Hough-Troutman, M. Smiglak, S. Griffin, W. Matthew Reichert, I. Mirska, J. Jodynis-Liebert, T. Adamska, J. Nawrot, M. Stasiewicz, R.D. Rogers, J. Pernak, Ionic liquids with dual biological function: sweet and anti-microbial, hydrophobic quaternary ammonium-based salts, New J. Chem. 33 (2009) 26–33, http://dx.doi.org/10.1039/b813213p.

[16] P.C.A.G. Pinto, D.M.G.P. Ribeiro, A.M.O. Azevedo, V. Dela Justina, E. Cunha, K. Bica, M. Vasiloiu, S. Reis, M.L.M.F.S. Saraiva, Active pharmaceutical ingredients based on salicylate ionic liquids: insights into the evaluation of pharmaceutical profiles, New J. Chem. 37 (2013) 4095–4102, http://dx.doi.org/10.1039/c3nj00731f.

[17] K. Bica, C. Rijksen, M. Nieuwenhuyzen, R.D. Rogers, In search of pure liquid salt forms of aspirin: ionic liquid approaches with acetylsalicylic acid and salicylic acid, Phys. Chem. Chem. Phys. 12 (2010) 2011–2017, http://dx.doi.org/10.1039/b923855g.

[18] W.L. Hough, M. Smiglak, H. Rodriguez, R.P. Swatloski, S.K. Spear, D.T. Daly, J. Pernak, J.E. Grisel, R.D. Carliss, M.D. Soutullo, J.J.H. Davis, R.D. Rogers, The third evolution of ionic liquids: active pharmaceutical ingredients, New J. Chem. 31 (2007) 1429–1436, http://dx.doi.org/10.1039/b706677p.

[19] K. Bica, R.D. Rogers, Confused ionic liquid ions-a "liquification" and dosage strategy for pharmaceutically active salts, Chem. Commun. 46 (2010) 1215–1217, http://dx.doi.org/10.1039/b925147b.

[20] A. Balk, U. Holzgrabe, L. Meinel, 'Pro et contra' ionic liquid drugs—challenges and opportunities for pharmaceutical translation, Eur. J. Pharm. Biopharm. 94 (2015) 291–304, http://dx.doi.org/10.1016/j.ejpb.2015.05.027.

[21] P.M. Dean, J. Turanjanin, M. Yoshizawa-Fujita, D.R. MacFarlane, J.L. Scott, Exploring an anti-crystal engineering approach to the preparation of pharmaceutically active ionic liquids, Cryst. Growth Des. 9 (2009) 1137–1145, http://dx.doi.org/10.1021/cg8009496.

[22] J.M.M. Araujo, C. Florindo, A.B. Pereiro, N.S.M. Vieira, A.A. Matias, C.M.M. Duarte, L.P.N. Rebelo, I.M. Marrucho, Cholinium-based ionic liquids with pharmaceutically active anions, RSC Adv. 4 (2014) 28126–28132, http://dx.doi.org/10.1039/c3ra47615d.

[23] S.P.F. Costa, V.D. Justina, K. Bica, M. Vasiloiu, P.C.A.G. Pinto, M.L.M.F.S. Saraiva, Automated evaluation of pharmaceutically active ionic liquids' (eco)toxicity through the inhibition of human carboxylesterase and Vibrio fischeri, J. Hazard. Mater. 265 (2014) 133–141, http://dx.doi.org/10.1016/j.jhazmat.2013.11.052.

[24] C. Florindo, A. Costa, C. Matos, S.L. Nunes, A.N. Matias, C.M. Duarte, L.P. Rebelo, L.C. Branco, I.M. Marrucho, Novel organic salts based on fluoroquinolone drugs: synthesis, bioavailability and toxicological profiles, Int. J. Pharm. 469 (2014) 179–189, http://dx.doi.org/10.1016/j.ijpharm.2014.04.034.

[25] Y. Miwa, H. Hamamoto, S. Hikake, Y. Kuwabara, A phase I, randomized, open-label, cross-over study of the pharmacokinetics, dermal tolerability, and safety of MRX-7EAT Etodolac-Lidocaine Topical Patch in healthy volunteers, J. Pain 14 (2013) S72.

[26] R. Ribeiro, P.C.A.G. Pinto, A.M.O. Azevedo, K. Bica, A.K. Ressmann, S. Reis, M.L.M.F.S. Saraiva, Automated evaluation of protein binding affinity of anti-inflammatory choline based ionic liquids, Talanta 150 (2016) 20–26, http://dx.doi.org/10.1016/j.talanta.2015.12.009.

[27] P.G. Conaghan, A turbulent decade for NSAIDs: update on current concepts of classification, epidemiology, comparative efficacy, and toxicity, Rheumatol. Int. 32 (2012) 1491–1502, http://dx.doi.org/10.1007/s00296-011-2263-6.

[28] S.H. Zeisel, K.A. da Costa, Choline: an essential nutrient for public health, Nutr. Rev. 67 (2009) 615–623, http://dx.doi.org/10.1111/j.1753-4887.2009.00246.x.

[29] C. Giaginis, A. Tsantili-Kakoulidou, Alternative measures of lipophilicity: from octanol-water partitioning to IAM retention, J. Pharm. Sci. 97 (2008) 2984–3004, http://dx.doi.org/10.1002/jps.21244.

[30] C. Pereira-Leite, C. Nunes, S. Reis, Interaction of nonsteroidal anti-inflammatory drugs with membranes: in vitro assessment and relevance for their biological actions, Prog. Lipid Res. 52 (2013) 571–584, http://dx.doi.org/10.1016/j.plipres.2013.08.003.

[31] J. Brittes, M. Lucio, C. Nunes, J.L.F.C. Lima, S. Reis, Effects of resveratrol on membrane biophysical properties: relevance for its pharmacological effects, Chem. Phys. Lipids 163 (2010) 747–754, http://dx.doi.org/10.1016/j.chemphyslip.2010.07.004.

[32] H. Ferreira, M. Lucio, B. de Castro, P. Gameiro, J.L.F.C. Lima, S. Reis, Partition and location of nimesulide in EPC liposomes: a spectrophotometric and fluorescence study, Anal. Bioanal. Chem. 377 (2003) 293–298, http://dx.doi.org/10.1007/s00216-003-2089-5.

[33] B. de Castro, P. Gameiro, J.L.F.C. Lima, C. Matos, S. Reis, Interaction of drugs with hexadecylphosphocholine micelles. Derivative spectroscopy, acid-base and solubility studies, Mater. Sci. Eng. C Biomim. Supramol. Syst. 18 (2001) 71–78, http://dx.doi.org/10.1016/s0928-4931(01)00372-1.

[34] L.M. Magalhaes, C. Nunes, M. Lucio, M.A. Segundo, S. Reis, J.L.F.C. Lima, High-throughput microplate assay for the determination of drug partition coefficients, Nat. Protoc. 5 (2010) 1823–1830, http://dx.doi.org/10.1038/nprot.2010.137.

[35] I.E. Shohin, J.I. Kulinich, G.V. Ramenskaya, B. Abrahamsson, S. Kopp, P. Langguth, J.E. Polli, V.P. Shah, D.W. Groot, D.M. Barends, J.B. Dressman, Biowaiver monographs for immediate-release solid oral dosage forms: ketoprofen, J. Pharm. Sci. 101 (2012) 3593–3603, http://dx.doi.org/10.1002/jps.23233.

[36] M. Turk, D. Bolten, Formation of submicron poorly water-soluble drugs by rapid expansion of supercritical solution (RESS): results for naproxen, J. Supercrit. Fluids 55 (2010) 778–785, http://dx.doi.org/10.1016/j.supflu.2010.09.023.

[37] P.C.A.G. Pinto, A.D.F. Costa, J.L.F.C. Lima, M.L.M.F.S. Saraiva, Automated evaluation of the effect of ionic liquids on catalase activity, Chemosphere 82 (2011) 1620–1628, http://dx.doi.org/10.1016/j.chemosphere.2010.11.046.

[38] P.C.A.G. Pinto, S.P.F. Costa, J.L.F.C. Lima, M.L.M.F.S. Saraiva, Automated high-throughput Vibrio fischeri assay for (eco)toxicity screening: application to ionic liquids, Ecotoxicol. Environ. Saf. 80 (2012) 97–102, http://dx.doi.org/10.1016/j.ecoenv.2012.02.013.

[39] S.P.F. Costa, B.S.F. Martins, P.C.A.G. Pinto, M.L.M.F.S. Saraiva, Automated cytochrome c oxidase bioassay developed for ionic liquids' toxicity assessment, J. Hazard. Mater. 309 (2016) 165–172, http://dx.doi.org/10.1016/j.jhazmat.2016.02.005.

[40] C. Bosch Ojeda, F. Sanchez Rojas, Recent applications in derivative ultraviolet/visible absorption spectrophotometry: 2009–2011: a review, Microchem. J. 106 (2013) 1–16, http://dx.doi.org/10.1016/j.microc.2012.05.012.

[41] K. Kawai, K. Kaneko, H. Kawakami, T. Yonezawa, Bioinspired choline-like ionic liquids: their penetration ability through cell membranes and application to SEM visualization of hydrous samples, Langmuir 27 (2011) 9671–9675, http://dx.doi.org/10.1021/la201914h.

[42] E. Baka, J.E.A. Comer, K. Takacs-Novak, Study of equilibrium solubility measurement by saturation shake-flask method using hydrochlorothiazide as model compound, J. Pharm. Biomed. Anal. 46 (2008) 335–341, http://dx.doi.org/10.1016/j.jpba.2007.10.030.

[43] A.T. Serajuddin, Salt formation to improve drug solubility, Adv. Drug Deliv. Rev. 59 (2007) 603–616, http://dx.doi.org/10.1016/j.addr.2007.05.010.

[44] S.K. Murti, On the Preparation and Characterization of Water-soluble Choline Salts of Carboxylic Acid Drugs, University of Missouri-Kansas City, Kansas City, MO, 1993.

[45] C. Florindo, J.M. Araujo, F. Alves, C. Matos, R. Ferraz, C. Prudencio, J.P. Noronha, Z. Petrovski, L. Branco, L.P. Rebelo, I.M. Marrucho, Evaluation of solubility and partition properties of ampicillin-based ionic liquids, Int. J. Pharm. 456 (2013) 553–559, http://dx.doi.org/10.1016/j.ijpharm.2013.08.010.

[46] J. Gorke, F. Srienc, R. Kazlauskas, Toward advanced ionic liquids. Polar, enzyme-friendly solvents for biocatalysis, Biotechnol. Bioprocess Eng. 15 (2010) 40–53, http://dx.doi.org/10.1007/s12257-009-3079-z.

[47] Z.Y. Li, X.X. Liu, Y.C. Pei, J.J. Wang, M.Y. He, Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins, Green Chem. 14 (2012) 2941–2950, http://dx.doi.org/10.1039/c2gc35890e.

[48] X.D. Hou, Q.P. Liu, T.J. Smith, N. Li, M.H. Zong, Evaluation of toxicity and biodegradability of cholinium amino acids ionic liquids, PLoS One 8 (2013), e59145. http://dx.doi.org/10.1371/journal.pone.0059145.

[49] K.D. Weaver, H.J. Kim, J. Sun, D.R. MacFarlane, G.D. Elliott, Cyto-toxicity and biocompatibility of a family of choline phosphate ionic liquids designed for pharmaceutical applications, Green Chem. 12 (2010) 507–513, http://dx.doi.org/10.1039/b918726j.

[50] K.D. Weaver, M.P. Van Vorst, R. Vijayaraghavan, D.R. MacFarlane, G.D. Elliott, Interaction of choline salts with artificial biological membranes: DSC studies elucidating cellular interactions, Biochim. Biophys. Acta Biomembr. 1828 (2013) 1856–1862, http://dx.doi.org/10.1016/j.bbamem.2013.03.017.

[51] M.L. Farre, I. Ferrer, A. Ginebreda, M. Figueras, L. Olivella, L. Tirapu, M. Vilanova, D. Barcelo, Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: methods and preliminary results including toxicity studies with Vibrio fischeri, J. Chromatogr. A 938 (2001) 187–197, http://dx.doi.org/10.1016/s0021-9673(01)01154-2.

[52] S.P. Ventura, F.A. e Silva, A.M. Goncalves, J.L. Pereira, F. Goncalves, J.A. Coutinho, Ecotoxicity analysis of cholinium-based ionic liquids to Vibrio fischeri marine bacteria, Ecotoxicol. Environ. Saf. 102 (2014) 48–54, http://dx.doi.org/10.1016/j.ecoenv.2014.01.003.

[53] J.I. Santos, A.M.M. Goncalves, J.L. Pereira, B.F.H.T. Figueiredo, F.A.E. Silva, J.A.P. Coutinho, S.P.M. Ventura, F. Goncalves, Environmental safety of cholinium-based ionic liquids: assessing structure-ecotoxicity relationships, Green Chem. 17 (2015) 4657–4668, http://dx.doi.org/10.1039/c5gc01129a.

[54] S.S. Kuznetsova, N.V. Azarkina, T.V. Vygodina, S.A. Siletsky, A.A. Konstantinov, Zinc ions as cytochrome c oxidase inhibitors: two sites of action, Biochem. Mosc. 70 (2005) 128–136, http://dx.doi.org/10.1007/s10541-005-0091-6.

[55] C.E. Cooper, M.G. Mason, P. Nicholls, A dynamic model of nitric oxide inhibition of mitochondrial cytochrome c oxidase, Biochim. Biophys. Acta Bioenerg. 1777 (2008) 867–876, http://dx.doi.org/10.1016/j.bbabio.2008.03.021.