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Direct chromatographic study of the enantioselective biodegradation of ibuprofen and ketoprofen by an activated sludge☆

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ABSTRACT

The quantification of the enantiomeric fraction (*EF*) during the biodegradation process is essential for environmental risk assessment. In this paper the enantioselective biodegradation of ibuprofen, IBU, and ketoprofen, KET, two of the drugs most consumed, was evaluated. Biodegradation experiments were performed in batch mode using a minimal salts medium inoculated with an activated sludge (collected from a Valencian Waste Water Treatment Plant) and supplemented with the racemate of each compound. The inoculum activity was verified using fluoxetine as reference compound. The experimental conditions used (analyte concentration and volume of inoculum) were chosen according to OECD guidelines. In parallel, the optical density at 600 nm was measured to control the biomass growth and to connect it with enantioselectivity. Two RPLC methods for chiral separations of IBU and KET using polysaccharides-based stationary phases were developed. Novel calculations and adapted models, using directly the chromatographic peak areas as dependent variable, were proposed to estimate significant parameters related to the biodegradation process: biodegradation (*BD*) and *EF* values at given time, half-life times of (R)- and (S)-enantiomers, number of days to reach a complete *BD* and the minimum *EF* expected. The modelled *BD* and *EF* curves fitted adequately the data ($R^2 > 0.94$). The use of these new equations provided similar results to those obtained using concentration data. However, the use of chromatographic peak areas data, eliminates the uncertainty associated to the use of the calibration curves. The results obtained in this paper indicate that an enantiorecognition towards IBU enantiomers by the microorganisms present in the activated sludge used in this study occurred, being the biodegradation of (R)-IBU higher than that of (S)-IBU. For KET, non-enantioselective biodegradation was observed.

1. Introduction

Public environmental awareness has increased notably in recent years, due to the adverse effects that pollutants can produce not only on the environment but also on the human health. Among the emerging pollutants, drugs are probably the main concern of regulatory authorities because of their huge consume. Drugs, their metabolites and their degradation products could reach the environment mainly due to poor removal rates in Waste Water Treatment Plants (WWTP), and by improper disposal of unused medicines [1]. The presence of these compounds in the environment has been extensively reported [2–5]. However, although the great efforts made by regulatory authorities and scientific community, not much is known about the impact of this kind of compounds on the environment and the human health [6–10].

The majority of currently available drugs are chiral compounds and, even though the use of single enantiomers has increased during the last decades, many of these are marketed as racemates [11]. As a result of the enantioselective human metabolism, and also of the microbial biodegradation during the wastewater treatment process, these compounds are not present in the environment in their original racemic or enantiomeric form. For this reason, and due to the fact that enantiomers may have different toxicological and ecotoxicological properties compared to each other, the quantification of the enantiomeric fractions (*EF*) during the

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biodegradation process is essential for environmental risk assessment. However, there is not much research done on this subject.

Some studies have been focused on the direct *EF* quantification in WWTP influents and effluents [12–15]. Other approaches have been based on *in vitro* biodegradability assays. The OECD tests for ready biodegradability have been devised as screening methods to determine whether or not a chemical is potentially easily biodegradable. They simulate the degradation processes of chemicals in soils, surface waters or in activated sludge, generally in the presence of oxygen. A high concentration of the test substance (2–100 mg L^{-1}) is used in these tests [16]. The subsequent separation and determination of the individual enantiomers of the original compound (or its metabolites) in the test solution by analytical techniques, mainly liquid chromatography, allow the estimation of *EF*. Using this methodology, the enantioselective evaluation of biodegradation (*BD*) for amphetamines [14,17,18], β-blockers [17,19,20] and antidepressants [15,17,19,21,22] have been reported.

Ibuprofen (IBU) and ketoprofen (KET) are non-steroidal anti-inflammatory (NSAIDs) chiral drugs with anti-inflammatory and analgesic activities, being IBU one of the most popular clinically used drugs in the world [23]. Their therapeutic effects have been observed to reside almost exclusively in the (S)-enantiomers while the (R)-enantiomers are weakly active or inactive (e.g, (S)-IBU has been reported to be 160 times more active than (R)-IBU) [24,25]. In spite of the differences in their enantiomers activity, IBU and KET are commonly manufactured, dispensed and consumed as racemic mixtures [25].

The occurrence of the NSAIDs IBU and KET in WWTP influents and effluents from WWTPs of Spain [26,27], Australia [25,28] and Switzerland [29] has been reported. In these studies, the enantioselective biodegradation was evaluated by comparison of the *EF* values obtained in influents and effluents. (S)-IBU was found to be predominant in wastewater influents due to chiral inversion during human metabolism [25–28] and preferentially degraded during wastewater treatment [27–29]. Matomoros et al. [26], in the evaluation of the biodegradation of IBU in different WWTPs, found high variability on the *EF* values for constructed wetlands, being in the final step of the process (R)-IBU preferentially degraded. The authors attributed this behaviour to the presence of microorganisms and changes in the aerobic and anaerobic conditions in the WWTP could induce the chiral inversion of (R)-IBU to its optical antipode. Moreover, the chiral inversion degree seems to depend on the WWTP considered [28]. For KET, no significant changes in *EF* were reported [25,27].

In order to study the enantioselectivity in the NSAIDs biodegradation, experiments under laboratory conditions have also been performed. In a laboratory-scale membrane bioreactor, the (S)-IBU was preferentially degraded, while for KET, a slightly higher degradation of (R)-KET was observed [30]. Buser et al. also found a faster degradation of (S)-IBU during the incubation in the laboratory of an influent from a WWTP containing IBU, with an activated sludge and under aerobic conditions [29]. Incubation of lake water fortified with racemic IBU also indicated a faster dissipation of the (S)-enantiomer [29].

Changes in the *EF* values during the biodegradation process have been mainly explained by the more rapid degradation of one enantiomer relative to the other (i.e. enantioselective degradation). However, it is well established that 2-arylpropionic acids undergo *in vivo* chiral inversion during mammalian metabolism, and also in several species of fungi and bacteria. Multiple enzymes are believed to play a role in the process, varying the degree and/or direction of chiral inversion in the different organisms [5,28]. Therefore, changes in the *EF* for ibuprofen and ketoprofen could be explained not only by enantioselective degradation, but also by chiral inversion or by the concurrence of both processes [5,28].

Chiral analysis of NSAIDs in the above-mentioned enantioselective studies was carried out mainly by gas chromatography-mass spectrometry after diastereomer formation with the chiral derivatising reagent (R)-1-phenylethylamine [25,28,30], or using a cyclodextrin-based chiral stationary phase [26,29]. Liquid chromatography-mass spectrometry has also been applied with a (R)-1-naphthylglycine based chiral stationary phase and a mixture of tetrahydrofuran- ammonium acetate in methanol as mobile phase [27]. In the literature, few references exist about chiral separations of IBU and KET in reversed phase conditions [31–36].

One of the main aims on this work is to evaluate the biodegradation of IBU and KET enantiomers to provide more information on this issue, due to the ambiguous results reported in the literature For this purpose, a ready biodegradability test (compatible with the OECD guidelines) is performed. Experiments are carried out in batch conditions using an activated sludge inoculum from a local WWTP which receives domestic, agricultural, livestock and industrial wastewater. The inoculum activity is checked using fluoxetine as reference substance. Abiotic assays and biomass growth control assays are also performed. For the separation of the IBU and KET enantiomers, several polysaccharide based chiral stationary phases in RPLC conditions were tested. Using the selected conditions, fit-for-purpose validation of the methods is performed.

Another aim of this work is to develop a new strategy based on the direct use of chromatographic peak areas of enantiomers (instead of their concentrations) to estimate significant parameters related to the biodegradation process such as: *BD* of enantiomers and *EF* values at a given time, half-life times of enantiomers, number of days to reach a complete biodegradation and the maximum enantioselectivity degree (e.g. |*EF*-0.5|) expected. The purposes of this strategy are: (i) to eliminate the uncertainty source associated to the calibration stage and (ii) to reduce the experimental effort and cost due to the elimination of the calibration step. For this purpose, novel equations to calculate and model *BD* and *EF* data based on peak areas as dependent variables are developed. The results obtained are compared with those obtained using conventional calculations/models and with previous reported results for biodegradation of IBU and KET enantiomers.

2. Material and methods

2.1. Chemicals and solutions

Racemic Ibuprofen (*rac*-IBU), (S)-(+)-ibuprofen ((S)-IBU), racemic ketoprofen (*rac*-KET) and (S)-(+)-ketoprofen ((S)-KET) were from Sigma-Aldrich (St. Louis, MO, USA). Fluoxetine hydrochloride (FLX) was kindly donated by Alter (Madrid, Spain).

All reagents were of analytical grade. Formic acid (98%), sodium dihydrogen phosphate monohydrate, sodium hydroxide, acetonitrile (ACN), isopropanol, ethanol and methanol (MeOH) (®Multisolvent, HPLC grade) were from Scharlau, S.L. (Barcelona, Spain).

Ultra Clear TWF UV deionized water (SG Water, Barsbüttel, Germany) was used to prepare solutions. Different solutions were tested for the preparation of the mobile phases. Aqueous solutions of formic acid (0.1%, v/v, pH 3.0) were prepared. Phosphate buffer (10 mM, pH 8.0) was prepared by dissolving the appropriate amount of sodium dihydrogen phosphate monohydrate in water and adjusting the pH with 2.5 M sodium hydroxide. The mobile phases were prepared by mixing 0.1% formic or 10 mM phosphate solutions with the tested organic modifier to obtain the working concentration.

The minimal salts medium (MSM) solution used in the biodegradation assays was prepared with the following composition per litre [19,20]: 2.1 g Na_2HPO_4 (Panreac Química, S.A., Barcelona,

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