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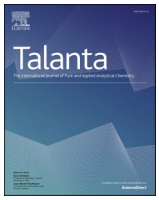

Simultaneous enantiomeric separation of linagliptin and clopidogrel by capillary electrophoresis for the individual, combined, and enantiomeric ecotoxicity evaluation on *Pseudokirchneriella subcapitata*

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ABSTRACT

An analytical methodology was developed in this work enabling the simultaneous enantioseparation of linagliptin and clopidogrel by Capillary Electrophoresis (CE). The use of 10.4 mg/mL carboxymethyl-β-CD (CM-β-CD) as chiral selector in 25 mM phosphate buffer (pH 7.0), at 25 °C and a separation voltage of 30 kV, made possible the chiral separation in 7.4 min, with resolution values of 3.9 and 2.7 for linagliptin and clopidogrel enantiomers, respectively. The analytical characteristics of the developed methodology were evaluated in terms of linearity, precision, trueness, LODs and LOQs, and found adequate for the enantiomeric determination of both drugs. The method was applied to study the stability under abiotic and biotic conditions and the ecotoxicity of both drugs on *Pseudokirchneriella subcapitata* at individual, combined, and enantiomeric level. Both studies were carried out for each enantiomer of linagliptin, racemic linagliptin, S-clopidogrel, racemic clopidogrel, a mixture of the active enantiomers (R-linagliptin and S-clopidogrel), and a mixture of both racemates. Results obtained showed that individual solutions of S-linagliptin, R-linagliptin, S-clopidogrel, RS-linagliptin, and a mixture of the active enantiomers of both compounds, were stable under abiotic and biotic conditions, regardless of the incubation time; however, the concentration of racemic clopidogrel after 96 h of incubation showed a decay which ranged from 18 to 24 % under abiotic and biotic conditions, respectively. Experimental determination of ecotoxicological parameters demonstrated that all compounds studied (individual enantiomers, racemates, the combination of active enantiomers, and racemic mixtures) are highly toxic to algae. In mixtures, different degrees of antagonisms and synergism were observed.

1. Introduction

In the last decades, contamination caused by pharmaceutical formulations has had a great impact on the environment due to their high use and even abuse in many cases. Drugs were detected at concentration levels within the nano or micro scale [1] and may have negative effects on non-target species, such as aquatic microorganisms. For this reason, drugs were considered emerging pollutants in the environmental field. It is known that more than half of the drugs commercialized are chiral [2]. This implies that although their enantiomers have the same

Abbreviations: 1-Ethyl-3-methylimidazolium L-lactate ([EMIm][L-Lact]), background electrolyte (BGE); Capillary Electrophoresis (CE), carboxymethyl-β-cyclodextrin (CM-β-CD); carboxymethyl-γ-cyclodextrin (CM-γ-CD), chiral ionic liquid (CIL); corrected peak areas (Ac), cyclodextrins (CDs); degree of substitution (DS), diode array detector (DAD); EC_{50} (effective concentration originating a 50 % inhibition), Electrokinetic Chromatography (EKC); Gas Chromatography (GC), hydrochloric acid (HCl); L-carnitine methyl ester bis(trifluoromethane)sulfonimide ([LCarn][L-Nft_2]), limits of detection (LODs); limits of quantification (LOQs), Liquid Chromatography (LC); methanol (MeOH), relative standard deviation (RSD); resolution values (Rs), sodium hydroxide (NaOH); sulphated-β-cyclodextrin (S-β-CD), tetrabutylammonium-L-aspartic acid ([TBA][L-Asp]); tetrabutylammonium-L-arginine ([TBA][L-Arg]), tetramethylammonium-L-glutamic acid ([TMA][L-Glu]).

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physicochemical properties, they may present different toxicity, distribution, and even bioaccumulation in the environment [1].

Organisms are exposed to different types of toxic pollutants (drugs, among many others) that can interact with each other. Therefore, the growing concern about the presence of pharmaceuticals in the environment is being included in the regulations. For example, the European Union (EU) is in the process of updating the Urban Wastewater Treatment Directive (Directive 91/271) [3] to enhance protection for both Europeans' health and the environment by addressing issues like pollution in smaller cities, stormwater overflows, microcontaminants, climate change, energy efficiency, and resource management. The new directive is expected to introduce stricter standards for nutrients and microcontaminants like pharmaceuticals, forcing us to carry out a more exhaustive surveillance and control of these pollutants in the natural environment. Despite this clear tightening of regulations, there is still a lack of awareness and knowledge about the risks of the presence of chiral drugs and the combined action of toxics in general [4]. In fact, there are various regulations to control the levels of these emerging pollutants present in wastewater [5]. Nevertheless, they do not distinguish between enantiomers, which reduces the validity of the results obtained. In fact, given the different characteristics that the stereoisomers of chiral pollutants may have, it is of great interest to study the effect of each enantiomer separately, or even to investigate the effect of mixtures of chiral contaminants because they are not isolated in the environment [6]. The use of racemic drugs, for which only one of the enantiomers is active, increases the environmental contamination. In this regard, the World Health Organization (WHO) recommends using pure active enantiomers and tolerate racemates only if they do not cause more side effects than the pure enantiomer. In addition, toxicological studies should be performed with individual enantiomers.

For all these reasons, it is necessary to develop chiral analytical methodologies allowing the individual enantiomeric determination of chiral drugs. Among the most used analytical techniques for chiral analysis, such as CE, Liquid Chromatography (LC) or Gas Chromatography (GC), CE has shown to have certain advantages over LC or GC for chiral analysis such the minimal volumes of reagents and samples necessary or for avoiding the use of expensive chiral chromatographic columns [7–12]. One of the most used strategies to achieve chiral separations in CE consists of the addition of a chiral selector to the separation medium, being cyclodextrins (CDs) the most employed due to their high availability at low cost compared to the chiral columns, as well as their high solubility in aqueous media and low UV absorbance [13–18]. The development of enantioselective methodologies by CE and their application to the evaluation of the stability and ecotoxicity of emerging chiral contaminants at enantiomeric level was reported for drugs (duloxetine, econazole, ivabradine, and ibrutinib), pesticides (sulfoxaflor and tetramethrin) and a cosmetic (panthenol) on different aquatic microorganisms, the microcrustacean *Daphnia magna* [19–21], the plant *Spirodela polyrhiza* [22–24], or the marine bacterium *Vibrio fischeri* [23,25]. In two of the works mentioned above, stability and ecotoxicity studies were carried out for two chiral drugs (duloxetine and econazole), individually and in their mixtures, one in the presence of *Daphnia magna*, and the other in the presence of *Spirodela polyrhiza*. The results showed that, in both cases, the EC_{50} values found (effective concentration originating a 50 % inhibition) were higher for the mixtures of the contaminants due to a synergistic effect between both chiral drugs, this demonstrating the importance of evaluating the combined ecotoxicity of mixtures of emerging chiral contaminants as they are simultaneously present in the environment as above mentioned.

Pseudokirchneriella subcapitata is widely used in ecotoxicological studies due to its high sensitivity to emerging contaminants, including pharmaceuticals. Its rapid growth cycle and essential role at the base of the aquatic food chain make it a reliable bioindicator for assessing the toxic effects of pollutants in aquatic ecosystems. Furthermore, its use is recommended by the Organisation for Economic Co-operation and Development (OECD) in Test Guideline 201 (OECD, 2006) [26], which establishes standardized protocols for toxicity evaluation in freshwater algae and cyanobacteria. Observing the literature, there are very few published studies in which *P. subcapitata* has been used as a model organism to evaluate the negative effect of pure enantiomers and racemic mixtures on aquatic organisms [27–30]. Only in one of these four articles [30] the stability of enantiomers was studied. However, the combined toxicological effect of the enantiomers on green alga has not been systematically investigated in any of the above mentioned previous works.

Linagliptin (8-[(3)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl) methyl]-3,7-dihydro-1H-purine-2,6-dione), a highly specific dipeptidyl peptidase-4 inhibitor, which is administered orally for the treatment of type II diabetes [31], and clopidogrel (methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno [3,2-c] pyridin-5(4H)-yl) acetate), a thienopyridine-type antiplatelet and antithrombotic drug that acts by inhibiting clot formation in coronary artery disease, peripheral vascular disease, and cerebrovascular disease [32], are chiral compounds with an asymmetric carbon, and therefore two enantiomers (Fig. 1). Both chiral drugs are marketed as pure enantiomer, R-linagliptin, and S-clopidogrel [33], respectively, which are the pharmacologically active enantiomers while S-linagliptin and R-clopidogrel are inactive and are considered enantiomeric impurities [34,35]. The separation of the enantiomers of linagliptin by CE was reported by May et al. using carboxymethyl-β-cyclodextrin (CM-β-CD) at a concentration of 4.7 mM in 70 mM sodium acetate buffer at pH 6.1 in an analysis time of 10 min and a resolution value of 1.5 [31]. The method was applied to the determination of the enantiomeric impurity in a bulk drug sample (R-linagliptin). In the case of clopidogrel, two studies reported its chiral separation. Fayed et al. described the separation of clopidogrel enantiomers by CE in an analysis time of 17 min using triethylamino-phosphoric acid at pH 2.3 as separation buffer in the presence of 5 % m/v sulphated-β-cyclodextrin (S-β-CD) as the chiral selector and applied the method to the analysis of S-clopidogrel and its impurities including R-clopidogrel in commercial bulk samples [36]. In the second study, our research group investigated the effect of ionic liquids (ILs) on the chiral separation of clopidogrel, demonstrating that, in general, the addition of ILs enabled to increase the enantiomeric resolution. The best chiral separation was obtained in less than 17.5 min with a resolution value of 3.4 with a dual system formed by 12.5 mM carboxymethyl-γ-cyclodextrin (CM-γ-CD) and 10 mM tetrabutylammonium-L-aspartic acid ([TBA][L-Asp]) in a 100 mM formate buffer at pH 3.0, a temperature of 25 °C and an applied voltage of 30 kV. The developed methodology was applied to the analysis of pharmaceutical formulations marketed as enantiomerically pure in S-clopidogrel [32]. However, to our knowledge, there is no chiral analytical methodology by CE enabling the simultaneous separation of the enantiomers of linagliptin and clopidogrel. Developing such enantiomeric simultaneous methodology would provide a powerful and rapid tool to assess their behavior and impact in combined systems of both toxics.

The aims of this work were: i) to develop an analytical methodology by CE enabling, for the first time, the simultaneous enantiomeric separation of the two chiral drugs, linagliptin and clopidogrel, in order to provide a rapid tool for determining the real concentrations of the individual enantiomers in culture media necessary for stability and ecotoxicity studies at enantiomeric level, and ii) to apply the developed methodology to evaluate the stability of each enantiomer of the compounds studied under abiotic and biotic conditions, and conducting the first comprehensive ecotoxicity assessment of them, at enantiomeric and racemic level, as well as in mixtures of both racemic compounds and of both pharmaceutical active enantiomers, on the freshwater alga *Pseudokirchneriella subcapitata*. This organism was particularly suitable in this work for several reasons: sensitivity to emerging contaminants allowing the detection of toxic effects even at low concentrations; environmental relevance as a representative species of aquatic ecosystems, because its response to the compounds studied helps to evaluate potential environmental impacts; and the scarce data previously

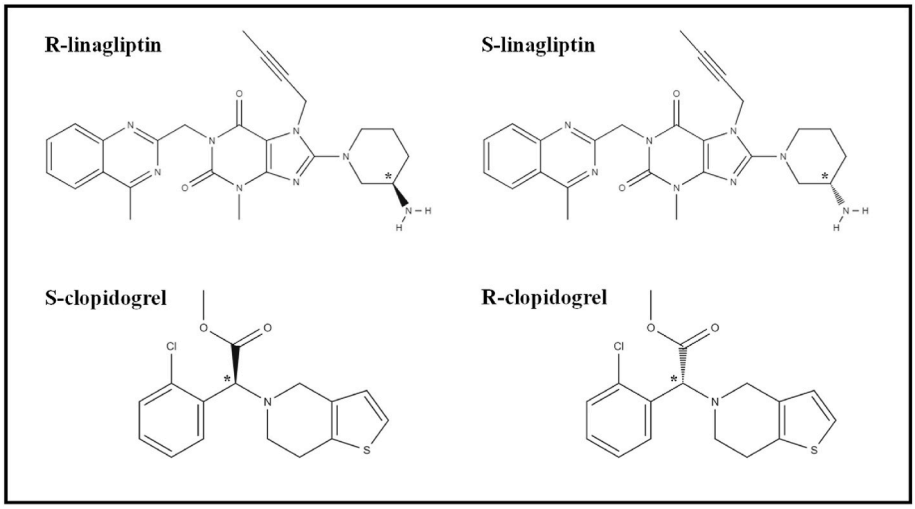

Fig. 1. Chemical structures of the enantiomers of linagliptin and clopidogrel.

reported on the study of ecotoxicity of chiral toxics at enantiomeric level on this non-target organism.

2. Materials and methods

2.1. Chemicals

Boric acid, glacial acetic acid, orthophosphoric acid 85 %, sodium hydroxide (NaOH), 1-ethyl-3-methylimidazolium L-lactate ([EMIm][L-Lact]), and RS-clopidogrel were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-cyclodextrin (β-CD) was from Fluka (Buchs, Switzerland), and CM-β-CD (degree of substitution (DS) 3.0) and CM-γ-CD (DS 3.5) from Cyclolab (Budapest, Hungary). Methanol (MeOH) and hydrochloric acid (HCl) 37 % were from Scharlab S.L. (Barcelona, Spain). The ultrapure water (Millipore Milli-Q system, Bedford, MA, USA) was employed. Tetrabutylammonium-L-aspartic acid ([TBA][L-Asp]), tetrabutylammonium-L-arginine ([TBA][L-Arg]), tetramethylammonium-L-glutamic acid ([TMA][L-Glu]), and L-carnitine methyl ester bis(trifluoromethane)sulfonimide ([LCarn][L-Nft_2]), were synthesized at the Center for Applied Chemistry and Biotechnology (CQAB) from the University of Alcalá, Spain, (their synthesis was described in two previous works [37,38]). R-linagliptin was purchased from MedChem Express (Monmouth Junction NJ, USA), S-linagliptin from Toronto Research Chemicals Canada (North York, ON, Canada), and S-clopidogrel from TargetMol (Wellesley Hills, MA, USA).

2.2. Pre-culture conditions for Pseudokirchneriella subcapitata

P. subcapitata immobilized in alginate pearls and the concentrated nutrient solution medium were purchased from MicroBioTests (Gent, Belgium). Demobilizations of cells, culture medium preparation and inoculum growth conditions were carried out following the manufacturer's guidelines and the OECD Test Guide 201 open system [26].

Preculture of algae cells were done according to the procedure published in our previous work [39]. Briefly, demobilized algal cells were cultured in Erlenmeyer flask using 25 mL of culture medium, continuous light, and agitation. Biomass in exponential growing phase was used as inoculum for exposure test.

2.3. Analytical method

Buffer solutions were prepared by dissolving the appropriate volume in the case of phosphate and acetate buffer or the appropriate amount of boric acid and adjusting to the respective pH values with 1 M NaOH. Milli-Q water was used to complete the volume necessary to reach the desired buffer concentration. The appropriate amount of each CD was dissolved in the respective buffer solution to obtain the desired background electrolytes (BGE). The dual systems were prepared weighting the respective amounts of CDs and Chiral Ionic Liquids (CILs) to achieve the desired concentrations dissolving them in the buffer separation medium.

Stock solutions of R-linagliptin and S-linagliptin at 1000 mg L^{-1} in a 30 % aqueous solution of MeOH, and of RS-clopidogrel and S-clopidogrel at 2000 mg L^{-1} in MeOH were prepared. Working solutions were obtained by diluting the appropriate volume of the corresponding stock solutions in water. All standard solutions as well as working solutions were stored at 3–5 °C. Prior to injection into the CE equipment, all standard solutions and samples were degassed in an ultrasonic bath.

Electrophoretic experiments were performed on an Agilent 7100 CE system from Agilent Technologies (Waldbronn, Germany) controlled with the HP ^{3D}CE ChemStation software that included data collection and analysis. A diode array detector (DAD) was employed as detection system. Separations were achieved in uncoated fused-silica capillaries (58.5 cm total length (50 cm effective length) x 50 μm internal diameter) from Polymicro Technologies (Phoenix, AZ, USA), in positive polarity.

Each new capillary was rinsed with 1 M NaOH for 30 min, Milli-Q water for 15 min followed by 60 min with the respective background electrolyte (BGE) (at 1 bar pressure). At the beginning of each working day, the capillary was conditioned with 0.1 M NaOH for 5 min, Milli-Q water for 5 min, buffer solution for 5 min, and finally, with the BGE for 10 min. At the end of the day, the capillary was flushed with NaOH 0.1 M and Milli-Q water, for 5 min and 10 min, respectively. To ensure the repeatability between injections, the capillary was flushed with Milli-Q water for 2 min, 0.1 M of NaOH for 2 min, 2 min with Milli-Q water, and with BGE for 5 min.

2.4. Stability assessment

Stability was evaluated by triplicate for the enantiomers (except for R-clopidogrel as it is not commercially available) and racemates of each compound individually. In addition, it was also evaluated for the mixture of the active enantiomers and the mixture of the racemates of both compounds. The concentrations studied were 2, 5, and 50 mg L^{-1} per enantiomer of linagliptin, and 7, 10, and 30 mg L^{-1} per enantiomer of clopidogrel.

All assays described were performed both in the absence of microorganism *P. subcapitata* (abiotic assays) and in the presence of the algae cells (biotic assays). All samples were incubated in culture medium for

96 h both in absence of light and under controlled irradiation.

The concentrations of enantiomers were measured at the initial time (0 min) and at the end of the exposure time (96 h).

2.5. Ecotoxicology test

The toxic effect of the enantiomers, racemates, and mixtures, was determined based on the algal population growth in pure cultures of *P. subcapitata* for 3 days, by monitoring the natural fluorescent emission of the culture in microplates, according to the procedure explained in our previous work [39]. Algal cells were cultured in microplates within a growth chamber maintained at 22 ± 2 °C under constant light exposure, with the light intensity set at 6000 lux. The growth of *P. subcapitata* was tracked over a 72-h period by measuring the *in vivo* chlorophyll fluorescence in the microplates. This was done in the presence of various concentrations of enantiomers, racemates, and mixtures, alongside a control group (without toxicants). Each experimental condition was achieved in triplicate across three independent sets of experiments.

The growth rate of the algae in each of the tested conditions together with the real concentrations of the enantiomers of chiral drugs, which were determined according to the procedure described in section 2.3, were used for the calculation of the EC_{50}, EC_{20} and EC_{10} parameters.

2.5.1. Equations for the calculation of toxicity parameters of individual toxicants and mixtures

To determine the parameters of acute toxicity, the median-effect-isobologram method was employed, utilizing the concentration-response inhibition curve [19,22]:

$$\frac{f_a}{1-f_a} = \left(\frac{D}{D_m}\right)^m$$

Here D refers to the concentration of the toxicant affecting a fraction f_a; Dm stands for the median effective concentration (EC_{50}), and m is the coefficient that characterizes the shape of the dose-effect curve. Fitting experimental data to this equation also provides EC_{20} and EC_{10} values.

The study of combination effects involved mixtures at various concentration levels. To assess these effects, the combination index (CI) values were computed using the following formula, which applies to chemical combinations at a given inhibition percentage [19,22]:

$$(CI)_x^n = \sum_j^n \frac{(D)_j}{(D_x)_j} = \sum_j^n \frac{(D_x)_{1-n} - \left\{[D]_j \middle| \sum_j^n [D]\right\}}{(D_m)_j \left\{(f_{ax})_j \middle| \left[1-(f_{ax})_j\right]\right\}^{1/mj}}$$

$(CI)_x^n$ is the combination index for n chemicals at a certain x inhibition; $\left\{[D]_j \middle| \sum_j^n [D]\right\}$ is the ratio of a given (j) chemical inducing a x inhibition in combination and; $(D_m)_j \left\{(f_{ax})_j \middle| \left[1-(f_{ax})_j\right]\right\}^{1/mj}$ is the dose of each compound alone producing the same effect. The value of CI parameter indicates synergism (CI < 1), additivity (CI = 1), or antagonism (CI > 1).

These calculations of toxicity parameters were made for the individual enantiomers of linagliptin, racemic linagliptin, S-clopidogrel, racemic clopidogrel, a mixture of the active enantiomers of both compounds (R-linagliptin and S-clopidogrel), and a mixture of both compounds in their racemic forms.

2.6. Data treatment

Migration times, peak areas, and resolution values (Rs) were obtained by Chemstation software from Agilent Technologies. To improve data reproducibility, corrected peak areas (Ac) were used (peak area divided by the corresponding retention time of each enantiomer). Excel Microsoft and Statgraphics Centurion XVII software were used for the experimental data analysis, calculate all required parameters and statistical tests, and Origin Pro8 for the composition of graphs. CompuSyn software [40] was used for the calculation of dose-effect parameters.

3. Results and discussion

3.1. Development of an EKC methodology for the simultaneous separation of linagliptin and clopidogrel enantiomers

In order to achieve the simultaneous separation of the enantiomers of linagliptin and clopidogrel in the shortest analysis time and with the highest enantioresolution values, the conditions previously optimized by our research group for the enantiomeric separation of clopidogrel [32] were tested. In this case, a dual system consisting of an anionic cyclodextrin, and a CIL was employed: 18.4 mg/mL CM-γ-CD + 10 mM [TBA][L-Asp] in 100 mM formate buffer at pH 3.0, a temperature of 25 °C, a separation voltage of 30 kV and an injection of 50 mbar × 10 s. Although under these conditions, clopidogrel enantiomers were separated with an enantiomeric resolution of 3.4 in 17.5 min, an enantioresolution of 0.7 was obtained for linagliptin, results that did not improve in absence of the CIL. For this reason, other dual systems which showed good results for the separation of clopidogrel enantiomers [32] were assayed under the same conditions (CM-γ-CD and different CILs ([TBA][L-Asp], [TMA][L-Glu], [TBA][L-Arg], [LCarMe][L-Ntf_2], [EMIm][L-Lact]). As the results obtained were not satisfactory, the separation conditions reported for the chiral separation of linagliptin were tested [31] (7 mg/mL CM-β-CD in 70 mM acetate buffer at pH 6.1, 25 °C, 28 kV, a hydrodynamic injection of 50 mbar × 5 s, and a capillary of 50 μm × 56 cm effective length). However, enantiomers of clopidogrel were not separated and linagliptin eluted with the electroosmotic flow (EOF), i.e., the results described in the literature could not be reproduced. Different buffers were then tested at different pH values: phosphate buffer at pH 5.0 and 7.0, acetate buffer at pH 6.1, and borate buffer at pH 9.0, at different concentrations, all of them under the same conditions (14.9 mg/mL CM-β-CD, a temperature of 30 °C, a separation voltage of 30 kV, a hydrodynamic injection 50 mbar × 10 s, a capillary of 50 μm × 50 cm effective length and a wavelength of 205 ± 30 nm (corresponding to the absorption maximum for clopidogrel), without reference). Among all these conditions, only the use of a 40 mM phosphate buffer (pH 7.0) enabled the separation of the enantiomers of clopidogrel and linagliptin. However, the peaks of linagliptin still eluted very close to the EOF. Then, aimed to eliminate the solvent peak, the effect of the detection wavelength was studied. Thus, under the previous experimental conditions, the wavelength corresponding to the absorption maximum of linagliptin ($\lambda_{linagliptin}$: 228 nm) was employed with a reference wavelength of 350 ± 100 nm in all cases and different bandwidths. Since significant differences were not observed for clopidogrel enantiomers at different bandwidths (Table S1), and the highest values for both peak area (10.7 and 23.0 for R- and S-linagliptin, respectively) and height (7.0 and 11.3 for R- and S-linagliptin, respectively) were obtained using a bandwidth of 4 nm for linagliptin enantiomers, this value was chosen as the optimum (see Table S1).

Once the most appropriate detection wavelength was chosen, the effect of different variables such as the injection volume, buffer and CD concentration, temperature, and separation voltage, was investigated. First, the effect of the injection volume was studied. Fig. 2 shows the results obtained when the pressure in the hydrodynamic injection was kept constant at 50 mbar and the injection time was modified (5, 7, and 10 s). It can be observed that, for both compounds, and as expected, increasing the injection time increased both the area (12 units for S-linagliptin and 4.8 units for S-clopidogrel, being these the enantiomers present at the highest concentrations) and the height (6.3 units for S-linagliptin and 1.2 units for S-clopidogrel, enantiomers present at the highest concentrations) of the peaks while maintaining the enantioresolution values (see Table S2). As a result, an injection of 50 mbar × 10 s was chosen as the optimum. The phosphate buffer concentration was next optimized by varying this parameter between 25 and 50 mM (25, 30, 40, 50 mM). As shown in Tables 1 and 2, and Fig. 3A, the buffer

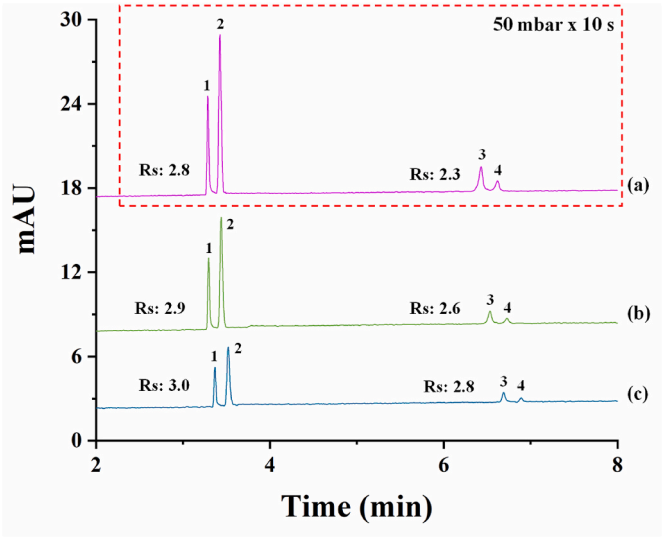

Fig. 2. Electropherograms corresponding to the simultaneous separation of the enantiomers of linagliptin and clopidogrel, using 14.9 mg/mL CM-β-CD in 40 mM phosphate buffer at pH 7.0 with hydrodynamic injection: **(a)** 50 mbar × 10 s; **(b)** 50 mbar × 7 s; **(c)** 50 mbar × 5 s. Other experimental conditions: temperature 30 °C; voltage: 30 kV; uncoated fused-silica capillary, 58.5 cm (50 cm effective length) x 50 μm i.d.; λ: 228 ± 4 nm. 1: 10 mg L^{-1} of S-linagliptin, 2: 20 mg L^{-1} of R-linagliptin; 3: 20 mg L^{-1} of S-clopidogrel; 4: 10 mg L^{-1} of R-clopidogrel.

concentration did not exert generally a significant effect on the chiral resolution, although it influenced the values of peak area and peak height, which increased as the buffer concentration decreased. A slight increase in analysis time for each analyte was also observed when decreasing this concentration (0.3 min for linagliptin and 0.6 min for clopidogrel). In addition, the electric current values increased considerably with the phosphate buffer concentration (Tables 1 and 2), which can in turn unfavorably affect the repeatability and reproducibility of the method due to system heating by the Joule effect. For all these reasons, a 25 mM concentration of phosphate buffer at pH 7.0 was chosen as the optimum. To evaluate the effect of the CD concentration, values from 7.4 to 22.3 mg/mL of CM-β-CD were tested (7.4, 10.4, 14.9, 17.8 and 22.3 mg/mL). As can be seen in Tables 1 and 2 and Fig. 3B, the analysis time was longer when increasing the CM-β-CD concentration for both compounds (although this effect was greater for clopidogrel), and the same occurred for the electrical current observed. Regarding the effect of the CD concentration on the enantioresolution, it slightly increased with the concentration of CM-β-CD for clopidogrel. In the case of linagliptin, it remained practically constant up to a concentration of 14.9 mg/mL decreasing for higher concentrations and observing a single peak at 22.3 mg/mL. It was observed that the highest values for peak areas and heights for the enantiomers were obtained at concentrations of 10.4 and 14.9 mg/mL for linagliptin and 10.4 mg/mL for clopidogrel. For all these reasons, a CM-β-CD concentration of 10.4 mg/mL was chosen as the most appropriate.

The effect of the temperature was studied between 20 and 30 °C observing (Tables 1 and 2, Fig. 3C) that the enantioresolution values and the analysis times increased when decreasing the temperature. A value of 25 °C was chosen as the optimum as a compromise between the resolution values observed, the peak areas and heights obtained, the shape of the electrophoretic peaks, and the analysis time.

Finally, the effect of the separation voltage was studied between 20 and 30 kV (Tables 1 and 2, Fig. 4). The results showed that the analysis times decreased (from 6.4 min to 3.9 min for linagliptin and from 12.4 min to 7.4 min for clopidogrel) when increasing the voltage, although a slight decrease in the chiral resolution was also observed, especially for linagliptin (from 4.9 to 3.9). Since the decrease in analysis time was more important than the loss in the chiral resolution, 30 kV was chosen as the optimum separation voltage.

Under the final optimized conditions, the method developed in this work enabled the simultaneous enantiomeric separation of linagliptin and clopidogrel in about 7 min with resolutions of 3.9 and 2.7, respectively, being the analysis time corresponding to linagliptin of 4 min. These results significantly improve those obtained in the only previous work describing the chiral separation of linagliptin by CE in which an analysis time of 10 min and an enantiomeric resolution of 1.5 were reported (see Table S3 in supplementary material). In the case of clopidogrel, two previous works reported its enantiomeric separation in about 17 min with resolutions of 3.4 or higher (the exact value of resolution is not given). Since the method developed in the present work gave rise to an enantiomeric resolution of 2.7 in 7 min, it can be stated that it is advantageous compared with these previous works (see Table S3 in supplementary material). An additional advantage of the present work is that it is possible to simultaneously separate the enantiomers of both compounds for the first time. This point makes possible to study the stability and ecotoxicity of both compounds at enantiomeric level and under different conditions (biotic and abiotic) significantly simplifying the determination of real enantiomeric concentrations which can be achieved in a considerable shorter time than using a different methodology for each compound.

Table 1
Analysis time, resolution values, peak areas and heights for enantiomers, and currents corresponding to the separation of linagliptin enantiomers under different experimental conditions (1: S-linagliptin, at 5 mg L^{-1}; 2: R-linagliptin, at 10 mg L^{-1}).

Variable		Analysis time (min)	Rs	A_1[a]	A_2[a]	H_1[b]	H_2[b]	Current (μA)
Phosphate buffer concentration (mM)	**25**	**3.8**	**2.8**	**7.6**	**13.9**	**4.6**	**10.5**	**60**
*14.*9 mg/mL CM-*β-CD / 30 °C* / 30 kV	30	3.6	3.4	6.0	12.0	3.8	9.2	71
	40	3.6	3.0	5.5	11.1	3.7	5.1	80
	50	3.5	2.9	5.6	11.0	3.8	5.2	100
CD concentration (mg/mL)	57.4	3.3	2.4	4.0	9.6	2.5	3.4	54
25 mM *phosphate buffer* pH *7.0 / 30 °C* / 30 kV	**10.4**	**3.4**	**2.8**	**6.4**	**11.7**	**4.3**	**4.6**	**58**
	14.9	3.8	2.8	7.6	13.9	4.6	10.5	60
	17.8	3.7	1.1	5.7	12.8	3.8	9.1	67
	22.3	3.7	–	17.4		11.1		75
Temperature (°C)	20	4.3	3.4	7.8	14.0	3.3	8.6	47
25 mM *phosphate buffer* pH *7.0 / 10.*4 mg/mL CM-*β-CD* / 30 kV	**25**	**3.9**	**3.9**	**6.6**	**12.8**	**3.7**	**6.2**	**52**
	30	3.4	2.8	6.4	11.7	4.3	4.6	58
Voltage (kV)	20	6.4	4.9	11.1	20.9	4.0	12.1	30
25 mM *phosphate buffer* pH *7.0/ 10.*4 mg/mL CM-*β-CD / 25 °C*	25	5.0	4.8	8.2	15.9	3.7	9.7	41
	30	**3.9**	**3.9**	**6.6**	**12.8**	**3.7**	**6.2**	**52**

[a] A_1 and A_2: Peak areas corresponding to the S- and R-enantiomers, respectively.
[b] H_1 and H_2: Peak heights corresponding to the S- and R-enantiomers, respectively.

Table 2
Analysis time, resolution values, peak areas and heights for enantiomers, and currents corresponding to the separation of clopidogrel enantiomers under different experimental conditions (1: S-clopidogrel, at 20 mg L^{-1}; 2: R-clopidogrel, at 10 mg L^{-1}).

Variable		Analysis time (min)	Rs	A_1[a]	A_2[a]	H_1[b]	H_2[b]	Current (μA)
Phosphate buffer concentration (mM) *14.*9 mg/mL CM-*β-CD* / *30* °*C* / 30 kV	**25**	**7.9**	**2.7**	**6.0**	**2.5**	**1.5**	**0.7**	**60**
	30	7.4	2.7	6.1	2.2	1.6	0.6	71
	40	7.3	2.2	4.3	1.9	1.2	0.5	80
	50	7.4	2.7	4.2	1.8	1.1	0.5	100
CD concentration (mg/mL) 25 mM *phosphate buffer* pH *7.0* / *30* °*C* / 30 kV	57.4	5.8	2.2	7.0	3.3	1.9	0.8	54
	710.4	**6.2**	**2.5**	**7.4**	**3.7**	**2.3**	**1.0**	**58**
	1014.9	7.9	2.7	6.0	2.5	1.5	0.7	60
	1217.8	8.0	3.0	6.3	2.9	1.9	0.8	67
	1522.3	8.4	3.1	6.4	2.9	2.0	0.9	75
Temperature (°C) 25 mM *phosphate buffer* pH *7.0* / *10.*4 mg/mL CM-*β-CD* / 30 kV	20	8.2	3.2	9.7	4.9	2.5	1.1	47
	25	**7.4**	**2.7**	**12.8**	**5.6**	**3.2**	**1.3**	**52**
	30	6.2	2.5	7.4	3.7	2.3	1.0	58
Voltage (kV) 25 mM *phosphate buffer* pH *7.0* / *10.*4 mg/mL CM-*β-CD* / *25* °*C*	20	12.4	3.0	16.2	6.5	2.7	1.0	30
	25	9.5	2.9	14.2	5.9	3.0	1.2	41
	30	**7.4**	**2.7**	**12.8**	**5.6**	**3.2**	**1.3**	**52**

[a] A_1 and A_2: Peak areas corresponding to the S- and R-enantiomers, respectively.
[b] H_1 and H_2: Peak heights corresponding to the S- and R-enantiomers, respectively.

3.2. Analytical characteristics of the developed methodology

Once the simultaneous separation of linagliptin and clopidogrel enantiomers was optimized, the analytical characteristics of this methodology were evaluated in terms of linearity, precision, trueness, matrix interferences, and limits of detection and quantification (LODs and LOQs, respectively), being the results obtained grouped in Tables 3 and 4.

The linearity was studied by plotting the corrected peak area versus the concentration of each enantiomer in mg L^{-1}. As shown in these tables, a good linearity was obtained in all cases (R^2 values greater than 99.3 %) including the intercept the zero value (for a 95 % confidence interval in all cases). However, the linear range was wider for linagliptin (from 0.4 to 70 mg L^{-1} for each enantiomer) than for clopidogrel (from 5 to 40 mg L^{-1} for each enantiomer). Moreover, the experimental data fitted correctly to a linear model since the p-values were greater than 0.05 in all cases (ANOVA test). The Response Relative Factor (RRF, calculated by dividing the slope value of the majority enantiomer by the slope value of the minority enantiomer) that should be comprised between 0.8 and 1.2, was 1.00 for linagliptin enantiomers and 1.03 for clopidogrel enantiomers. These results suggested that the responses for both enantiomers were equivalent according to the European Pharmacopeia [41]. Therefore, their concentration ratio can be expressed as the ratio between their corrected peak areas.

The study of matrix interferences was achieved by comparing the slopes corresponding to the external standard and the standard additions calibration methods (the same concentrations were prepared in this case as for the external calibration but in the culture medium). No matrix interferences were observed for linagliptin since the slopes obtained for both calibration methods did not show significant differences (p-values >0.05 for a confidence interval of 95 %). However, in the case of clopidogrel the contrary was observed (p-values <0.05 for a confidence interval of 95 %). Thus, the standard additions calibration method was employed for the quantitation of clopidogrel on *P. subcapitata* culture medium, whereas the external standard calibration method was used for linagliptin.

To assess the trueness of the method, the recovery values were determined for each enantiomer at two concentration levels, 5 and 50 mg L^{-1} for linagliptin enantiomers, and 10 and 30 mg L^{-1} for clopidogrel enantiomers. In all cases, recoveries obtained included the 100 % value (see Tables 3 and 4). Precision was also evaluated at the above-mentioned concentration levels and considering the instrumental repeatability, method repeatability, and intermediate precision. The relative standard deviation (RSD) values (%) obtained were lower than 4.0 and 5.8 % for analysis times and corrected peak areas, respectively (see Tables 3 and 4).

LOD and LOQ values were calculated using the signal-to-noise ratio (signal to noise ratio values of 3 and 10 were employed for LOD and LOQ, respectively) and experimentally determined. The calculated LOD values were 0.4 mg L^{-1} for S-linagliptin, 0.3 mg L^{-1} for R-linagliptin, 2.6 mg L^{-1} for S-clopidogrel, and 2.6 mg L^{-1} for R-clopidogrel while calculated LOQ values were 1.2 mg L^{-1}, 1.1 mg L^{-1}, 8.7 mg L^{-1}, and 8.8 mg L^{-1}, respectively. Experimental LOD and LOQ values were 0.3 mg L^{-1} and 0.4 mg L^{-1} for S- and R-linagliptin enantiomers, and 2.5 mg L^{-1} and 5 mg L^{-1} for S- and R-clopidogrel enantiomers.

Once the analytical characteristics of the method were evaluated and found adequate for the quantification of the enantiomers of linagliptin and clopidogrel, the method was applied to the evaluation of the stability under abiotic and biotic conditions and to predict the ecotoxicity of these drugs at an enantiomeric level on the green algae *P. subcapitata*.

3.3. Stability evaluation for linagliptin and clopidogrel

The stability of linagliptin and clopidogrel was evaluated in presence and absence of the microorganism *P. subcapitata*. These studies were carried out for solutions containing S-linagliptin; R-linagliptin (active); S-clopidogrel (active enantiomer); RS-linagliptin; RS-clopidogrel; a mixture of S-clopidogrel and R-linagliptin (active enantiomers); and a mixture of RS-clopidogrel and RS-linagliptin.

Stability was studied at different incubation times, from 0 to 96 h, under abiotic (only culture medium) and biotic (in presence of growing cells in the culture medium) conditions, i.e., in the absence and presence of the algae, respectively (see Fig. 5). First, S-clopidogrel showed be stable both in absence and presence of the *P. subcapitata* after 96 h (Fig. 5A (a,b,c)). However, for racemic clopidogrel a decrease in concentration by up to 20 % under abiotic conditions and 24 % under biotic conditions was obtained after 96 h of incubation (Fig. 5A (d,e,f)). For linagliptin, the same behavior was observed for both the pure enantiomer (R-linagliptin) and the racemate, no change over time under either abiotic or biotic conditions, i.e., this analyte remained stable in this aquatic medium and in presence of the microorganism along the time of experiment (Fig. 5B).

Finally, the same stability results were observed for linagliptin and clopidogrel enantiomers in the following two cases (Fig. 5C): i) for a mixture of the active enantiomers of both compounds (S-clopidogrel and R-linagliptin); and ii) for a mixture of both racemates. R-linagliptin was shown to be stable in presence of S-clopidogrel, and vice versa (Fig. 5C (a,b,c)), and also RS-linagliptin in presence of RS-clopidogrel (Fig. 5C (d, e,f)). However, racemic clopidogrel showed similar behavior to that observed in the absence of racemic linagliptin, i.e. RS-clopidogrel

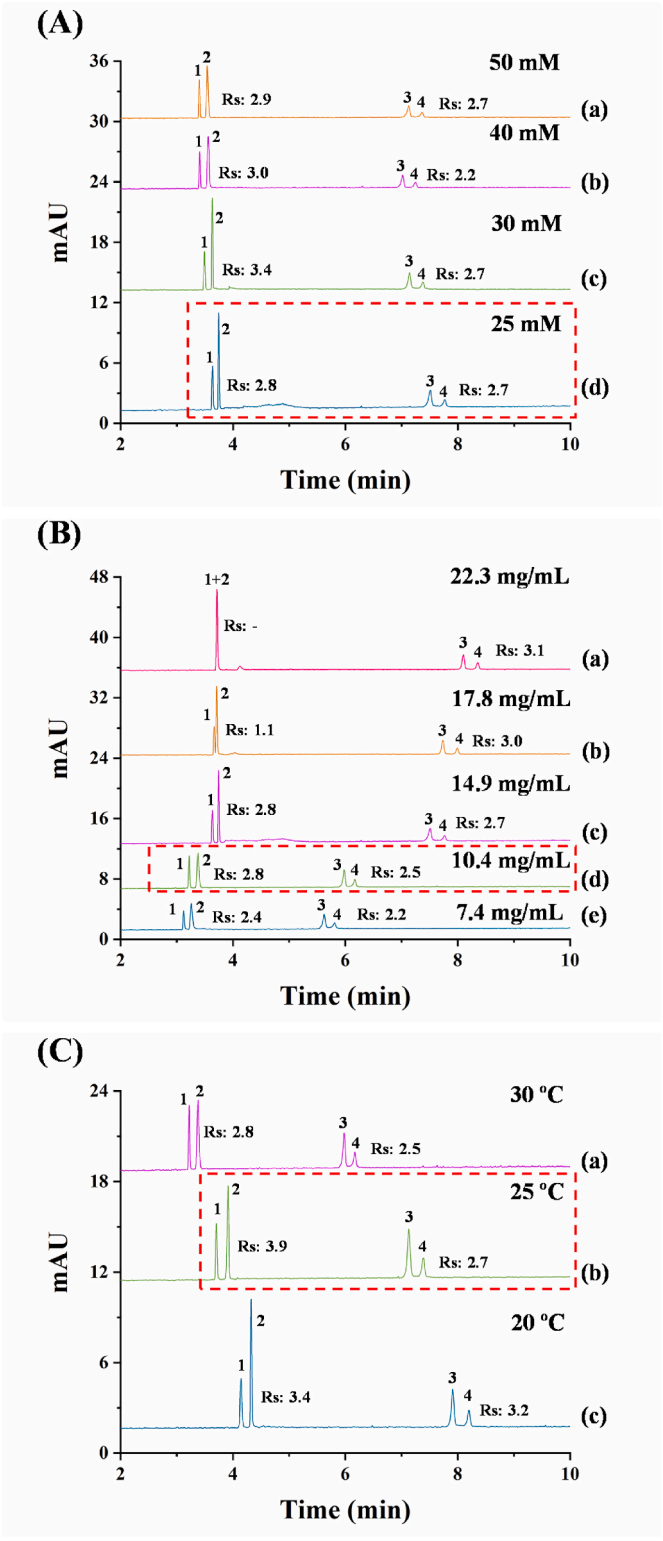

Fig. 3. Electropherograms corresponding to the simultaneous separation of the enantiomers of linagliptin and clopidogrel, using CM-β-CD as chiral selector in phosphate buffer at pH 7.0 for different: **(A)** buffer concentrations; **(B)** cyclodextrin concentrations, and **(C)** temperatures. Other experimental conditions as in Fig. 2a. 1: 5 mg L^{-1} of S-linagliptin, 2: 10 mg L^{-1} of R-linagliptin; 3: 20 mg L^{-1} of S-clopidogrel; 4: 10 mg L^{-1} of R-clopidogrel.

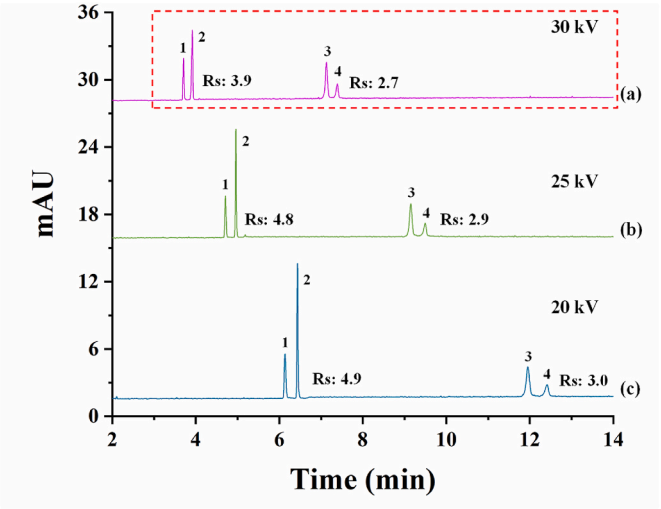

Fig. 4. Electropherograms corresponding to the simultaneous separation of the enantiomers of linagliptin and clopidogrel, using 10.4 mg/mL CM-β-CD in 25 mM phosphate buffer at pH 7.0 and 25 °C for different separation voltages: **(a)** 30 kV, **(b)** 25 kV, and **(c)** 20 kV. Other experimental conditions as in Fig. 2a. 1: 5 mg L^{-1} of S-linagliptin, 2: 10 mg L^{-1} of R-linagliptin; 3: 20 mg L^{-1} of S-clopidogrel; 4: 10 mg L^{-1} of R-clopidogrel.

decreased by about 16 % under abiotic conditions and by 18 % under biotic conditions after 96 h of incubation (Fig. 5C (d,e,f)).

3.4. Ecotoxicity evaluation

The experimental aquatic ecotoxicity parameters EC_{50}, EC_{20} and EC_{10} were obtained for the first time in this work for linagliptin and clopidogrel. Table 5 lists the ecotoxicological parameters obtained for the pure enantiomers, racemates and two specific combinations of active enantiomers and racemates tested. All the values were calculated using real concentrations of the enantiomers of both chiral drugs determined by CE.

It was noted that the EC_{50} values determined for the pure active enantiomers R-linagliptin (0.002 ± 0.001 mg L^{-1}) and S-clopidogrel (0.073 ± 0.001 mg L^{-1}) were below 1 mg L^{-1}, classifying them as highly toxic to aquatic organisms, in accordance with the European Regulation EC 1272/2008 [42]. In contrast, the EC_{50} value for S-linagliptin (1.355 ± 0.003 mg L^{-1}) falls in the range of toxic for aquatic life ($1 < EC_{50} < 10$ in mg L^{-1}).

The calculated toxicity of the racemic mixture RS-clopidogrel (EC_{50} = 0.059 ± 0.002 mg L^{-1}) indicates a very high negative effect of this compound on the growth of green algae population. Likewise, the racemic mixture RS-linagliptin gave an extremely high toxicity value (EC_{50} = 0.002 ± 0.001 mg L^{-1}), being equal to the result obtained for the pure active enantiomer R-linagliptin. Based on the EC_{50} values of the racemic mixtures obtained, RS-clopidogrel and RS-linagliptin can be considered very toxic compounds for aquatic life according to the European Regulation EC 1272/2008 [42].

Regarding the combined effect of these micropollutants, ecotoxicological parameters were also determined in this work for the first time, for the co-exposure of linagliptin and clopidogrel enantiomers. Ecotoxicity was determined for a specific combination of pollutants in a mixture of the pharmacologically active enantiomers of both drugs (S-clopidogrel + R-linagliptin) as well as for the more complex mixture prepared by combining both racemates (RS-clopidogrel + RS-linagliptin). Results grouped in Table 5 show that the mixture of S-clopidogrel and R-linagliptin, in EC_{50} ratio of the individual enantiomers, also exerts a very severe toxicological effect on the green algae population (EC_{50} = 0.063 ± 0.001 mg L^{-1}). At the ecotoxicological level, the combination formed by the mixture of the racemates RS-clopidogrel plus

Table 3
Analytical characteristics of the CE methodology developed for the determination of linagliptin enantiomers using 10.4 mg/mL CM-β-CD. Other experimental conditions as in Fig. 4a.

	S-Linagliptin		R-Linagliptin	
External standard calibration method[a]				
Range	0.4–70 mg L^{-1}	0.4–70 mg L^{-1}		
Slope ± t x S_{slope}	0.278 ± 0.004	0.278 ± 0.004		
Intercept ± t x $S_{intercept}$	0.024 ± 0.120	0.018 ± 0.134		
R^2	99.9 %	99.9 %		
Lack-of-Fit[b]	0.3292	0.2465		
Standard additions calibration method for toxicity studies[c]				
Range	0.4–70 mg L^{-1}	0.4–70 mg L^{-1}		
Slope ± t x S_{slope}	0.273 ± 0.004	0.273 ± 0.004		
R^2	99.9 %	99.9 %		
p-value of ANOVA[d]	0.2590	0.8394		
Trueness[e]	5 mg L^{-1}	50 mg L^{-1}	5 mg L^{-1}	50 mg L^{-1}
Recovery (%)	99 ± 5	98 ± 4	99 ± 2	97 ± 6
Precision	5 mg L^{-1}	50 mg L^{-1}	5 mg L^{-1}	50 mg L^{-1}
Instrumental repeatability				
Standard solution[f]				
t, RSD (%)	0.4	0.2	0.4	0.2
A_c, RSD (%)	3.9	3.8	3.4	3.6
Culture medium[g]				
t, RSD (%)	0.3	0.5	0.4	0.5
A_c, RSD (%)	1.5	4.4	1.4	3.6
Method repeatability				
Standard solution[h]				
t, RSD (%)	0.6	0.7	0.6	0.7
A_c, RSD (%)	3.9	4.0	3.6	3.5
Culture medium[i]				
t, RSD (%)	0.5	0.4	0.5	0.5
A_c, RSD (%)	2.0	3.9	2.3	3.1
Intermediate precision[h]				
Standard solution[j]				
t, RSD (%)	0.7	1.4	0.8	1.4
Ac, RSD (%)	5.1	4.2	5.1	3.0
Culture medium[k]				
t, RSD (%)	0.4	0.7	0.4	0.8
A_c, RSD (%)	3.1	5.2	3.4	5.7
LOD[l]	0.3 mg L^{-1}		0.3 mg L^{-1}	
LOQ[l]	0.4 mg L^{-1}		0.4 mg L^{-1}	

A_c: corrected peak area.
[a] Eleven standard solutions at different concentration levels injected in triplicate.
[b] p-value to confirm that experimental data fit properly to linear models.
[c] Addition of ten known amounts of RS-linagliptin standard solutions to the culture medium of *Pseudokirchneriella subcapitata*.
[d] p-values of ANOVA >0.05 at a confidence level of 95 % demonstrate the absence of matrix interferences.
[e] Evaluated as the mean recovery obtained from six standard solutions (n = 6) in the culture medium containing 10 mg L^{-1} and 100 mg L^{-1} of racemic linagliptin.
[f] Six repeated injections (n = 6) of standard solutions of racemic linagliptin at 10 mg L^{-1} and 100 mg L^{-1}.
[g] Six repeated injections (n = 6) of standard solutions of racemic linagliptin at 10 mg L^{-1} and 100 mg L^{-1} in the culture medium.
[h] Three replicates injected in triplicate (n = 9) on the same day of the standard solutions at 10 mg L^{-1} and 100 mg L^{-1} of racemic linagliptin.
[i] Three replicates injected in triplicate (n = 9) on the same day of the standard solutions at 10 mg L^{-1} and 100 mg L^{-1} of racemic linagliptin in the culture medium.
[j] Three replicates injected in triplicate (n = 9) during three consecutive days of the standard solutions at 10 mg L^{-1} and 100 mg L^{-1} for racemic linagliptin.
[k] Three replicates injected in triplicate (n = 9) during three consecutive days of the standard solutions at 10 mg L^{-1} and 100 mg L^{-1} for racemic linagliptin in the culture medium.
[l] Calculated experimentally.

RS-linagliptin constitutes a multicomponent mixture of 4 species (the 4 enantiomers of the two chiral drugs studied). This combination was found to be very toxic, with an EC_{50} value = 0.006 ± 0.001 mg L^{-1}, which also places it in the category of maximum toxicity to aquatic life.

Table 4
Analytical characteristics of the CE methodology developed for the determination of clopidogrel enantiomers using 10.4 mg/mL CM-β-CD. Other experimental conditions as in Fig. 4a.

	S-Clopidogrel		R-Clopidogrel	
External standard calibration method[a]				
Range	5–40 mg L^{-1}		5–40 mg L^{-1}	
Slope ± t x S_{slope}	0.061 ± 0.007		0.059 ± 0.006	
Intercept ± t x $S_{intercept}$	−0.17 ± 0.19		−0.15 ± 0.15	
R^2	99.3 %		99.5 %	
Lack-of-Fit[b]	0.0896		0.2023	
Standard additions calibration method for toxicity studies[c]				
Range	5–40 mg L^{-1}		5–40 mg L^{-1}	
Slope ± t x S_{slope}	0.043 ± 0.004		0.042 ± 0.006	
R^2	99.5 %		99.1 %	
p-value of ANOVA[d]	–		–	
Lack-of-Fit[b]	0.6193		0.2010	
Trueness[e]	10 mg L^{-1}	30 mg L^{-1}	10 mg L^{-1}	30 mg L^{-1}
Recovery (%)	102 ± 7	97 ± 7	101 ± 7	96 ± 6
Precision	10 mg L^{-1}	30 mg L^{-1}	10 mg L^{-1}	30 mg L^{-1}
Instrumental repeatability				
Standard solution[f]				
t, RSD (%)	0.2	0.3	0.2	0.3
A_c, RSD (%)	3.9	1.3	4.0	0.7
Culture medium[g]				
t, RSD (%)	1.0	0.7	1.0	0.7
A_c, RSD (%)	5.8	5.8	5.2	3.2
Method repeatability				
Standard solution[h]				
t, RSD (%)	1.2	1.3	1.2	1.4
A_c, RSD (%)	4.4	4.0	4.9	4.8
Culture medium[i]				
t, RSD (%)	1.0	0.4	1.0	0.4
A_c, RSD (%)	4.7	4.5	4.4	4.6
Intermediate precision				
Standard solution[j]				
t, RSD (%)	1.9	2.3	1.9	2.4
Ac, RSD (%)	4.3	5.8	5.8	4.8
Culture medium[k]				
t, RSD (%)	3.9	0.9	4.1	1.0
Ac, RSD (%)	4.6	4.8	5.0	4.8
LOD[l]	2.5 mg L^{-1}		2.5 mg L^{-1}	
LOQ[l]	5 mg L^{-1}		5 mg L^{-1}	

A_c: corrected peak area.
[a] Eight standard solutions at different concentration levels injected in triplicate.
[b] p-value to confirm that experimental data fit properly to linear models.
[c] Addition of seven known amounts of racemic clopidogrel standard solution to the culture medium of *Pseudokirchneriella subcapitata*.
[d] p-values of ANOVA >0.05 at a confidence level of 95 % demonstrate the absence of matrix interferences.
[e] Evaluated as the mean recovery obtained from six standard solutions (n = 6) in the culture medium containing 20 mg L^{-1} and 60 mg L^{-1} of racemic clopidogrel.
[f] Six repeated injections (n = 6) of standard solutions of racemic clopidogrel at 20 mg L^{-1} and 60 mg L^{-1}.
[g] Six repeated injections (n = 6) of standard solutions of racemic clopidogrel at 20 mg L^{-1} and 60 mg L^{-1} in the culture medium.
[h] Three replicates injected in triplicate (n = 9) on the same day of the standard solutions at 20 mg L^{-1} and 60 mg L^{-1} of racemic clopidogrel.
[i] Three replicates injected in triplicate (n = 9) on the same day of the standard solutions at 20 mg L^{-1} and 60 mg L^{-1} of racemic clopidogrel in the culture medium.
[j] Three replicates injected in triplicate (n = 9) during three consecutive days of the standard solutions at 20 mg L^{-1} and 60 mg L^{-1} for racemic clopidogrel.
[k] Three replicates injected in triplicate (n = 9) during three consecutive days of the standard solutions at 20 mg L^{-1} and 60 mg L^{-1} for racemic clopidogrel in the culture medium.
[l] Calculated experimentally.

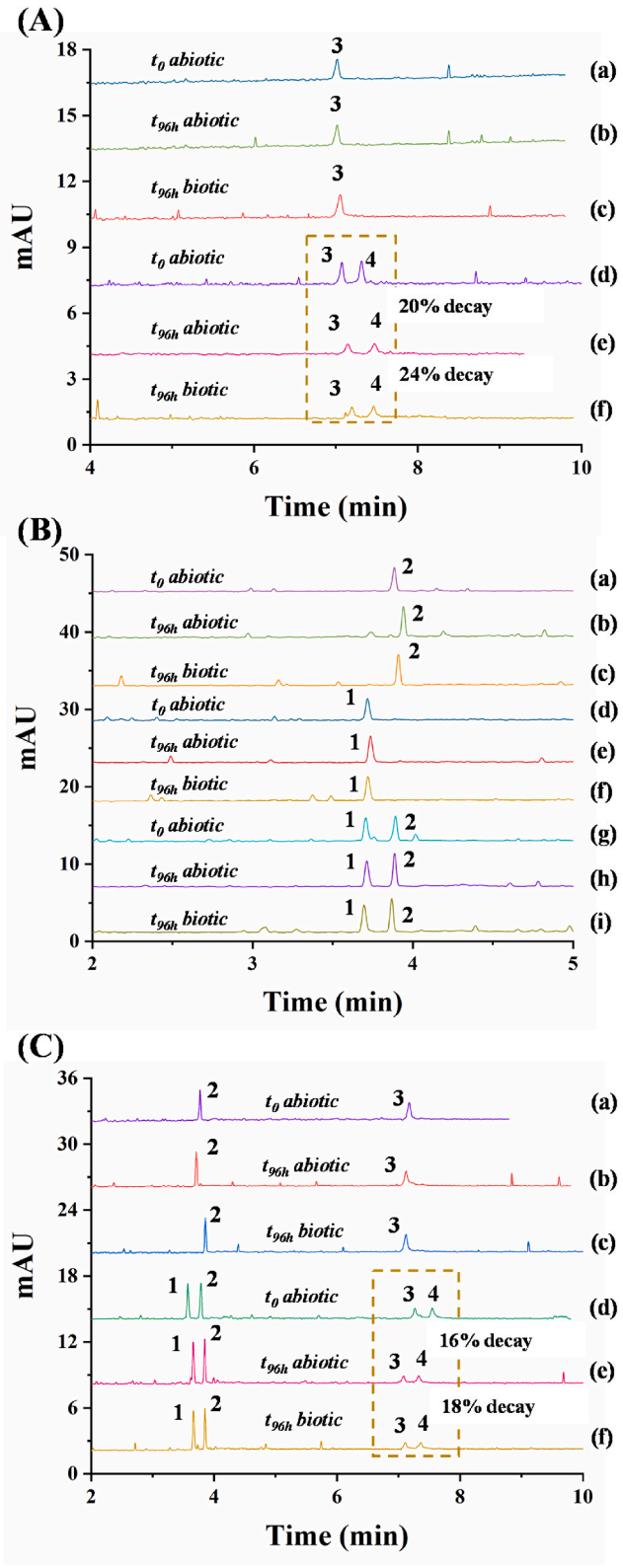

Fig. 5. Electropherograms corresponding to: **(A) (a,b,c)** 10 mg L^{-1} of S-clopidogrel, **(d,e,f)** 20 mg L^{-1} of RS-clopidogrel; **(B) (a,b,c)** 5 mg L^{-1} of R-linagliptin, **(d,e,f)** 5 mg L^{-1} of S-linagliptin, **(g,h,i)** 10 mg L^{-1} of RS-linagliptin; **(C) (a,b,c)** 10 mg L^{-1} of S-clopidogrel and 5 mg L^{-1} of R-linagliptin, **(d,e,f)** 20 mg L^{-1} of RS-clopidogrel and 10 mg L^{-1} of RS-linagliptin; under abiotic and biotic conditions on *Pseudokirchneriella subcapitata*. Experimental conditions as in Fig. 4a. 1: S-linagliptin, 2: R-linagliptin; 3: S-clopidogrel; 4: R-clopidogrel.

Table 5
Experimental ecotoxicity parameters on *Pseudokirchneriella subcapitata*. Values of EC_{50}, EC_{20} and EC_{10} (±SD) obtained for individual enantiomers, racemic mixture and their combination.

Enantiomers and mixtures	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{10} (mg L^{-1})	r^2
S-linagliptin	1.355 ± 0.003	0.134 ± 0.002	0.035 ± 0.001	0.97
R-linagliptin	0.002 ± 0.001	$6.44\ 10^{-4}$ ± $1\ 10^{-4}$	$3.79\ 10^{-4}$ ± $1\ 10^{-4}$	0.95
S-clopidogrel	0.073 ± 0.001	0.024 ± 0.002	0.012 ± 0.001	0.95
RS-clopidogrel	0.059 ± 0.002	0.025 ± 0.002	0.015 ± 0.001	0.96
RS-linagliptin	0.002 ± 0.001	$2.14\ 10^{-4}$ ± $1\ 10^{-4}$	$6.18\ 10^{-5}$ ± $1\ 10^{-5}$	0.95
S-clopidogrel + R-linagliptin[a]	0.063 ± 0.001	0.020 ± 0.002	0.010 ± 0.001	0.96
RS-clopidogrel + RS-linagliptin[b]	0.006 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.99

[a] Mix of pharmacologic active enantiomers.
[b] Combination of racemic mixtures.

Overall, our results on EC_{50} show that the pure enantiomers R-linagliptin and S-clopidogrel as well as all combinations studied have a very high toxicity to the alga *P. subcapitata*. Considering all the EC_{50} values obtained together with the calculated EC_{20} and EC_{10} values, it is possible to establish the following order of toxicity: RS- linagliptin > R-linagliptin > RS-clopidogrel + RS-linagliptin > RS-clopidogrel > S-clopidogrel + R-linagliptin > S-clopidogrel > S-linagliptin. The EC_{50} values for most of the compounds studied ranged between 0.002 ± 0.001 mg L^{-1} and 0.073 ± 0.001 mg L^{-1}, and only one compound (S-linagliptin) has an EC_{50} value = 1.355 ± 0.001 mg L^{-1} which is somewhat higher than the other compounds.

The methodology applied in the study of mixtures (widely contrasted in previous works) allows determining not only the combined toxicity (EC_{50}, EC_{20} and EC_{10} values), but also the type of interaction between the components of the toxic mixture, as well as the degree or level of interaction established between them. In this context, the combination index (CI) has been determined for the racemic mixture RS-linagliptin, the mixture of pharmacologically active enantiomers S-clopidogrel + R-linagliptin and the racemic mixture RS-clopidogrel + RS-linagliptin for the complete range of effect level. Results are shown in Fig. 6 A and **B**, where the discontinuous red line marks the additive effect, corresponding to CI = 1.

Fig. 6A allows to observe that the racemic mixture RS-linagliptin describes a complete interaction curve ranging from very strong synergism to very strong antagonism. At low effect levels (concentrations below EC_{10}) a very strong synergistic effect between the two enantiomers of linagliptin is observed. Synergism changes from very strong to moderate around EC_{50}, which rapidly changes to strong antagonism at high effect levels above EC_{90}.

Fig. 6B shows the interaction profile obtained for the mixture of pharmacologically active enantiomers (S-clopidogrel + R-linagliptin). In this case, only antagonism was obtained, and the degree of interaction increased with the negative effect observed until it doubles above EC_{90}. The interaction profile obtained for the mixture of the two racemates (RS-clopidogrel + RS-linagliptin) is also shown in Fig. 6B. A complete curve with antagonism at low doses and effect levels (up to EC_{10}) followed by synergism is also observed in this case. The synergistic effects change from moderate to strong values around the EC_{50}, with this effect being predominant for the racemate mixture.

Currently, there are no specific studies on the toxicological effects of linagliptin on aquatic organisms. However, linagliptin belongs to the class of imidazopyrimidines, heterocyclic compounds that include carbon and nitrogen. Its imidazopyrimidine structure is central to its function as an inhibitor of the DPP-4 enzyme, which prevents the

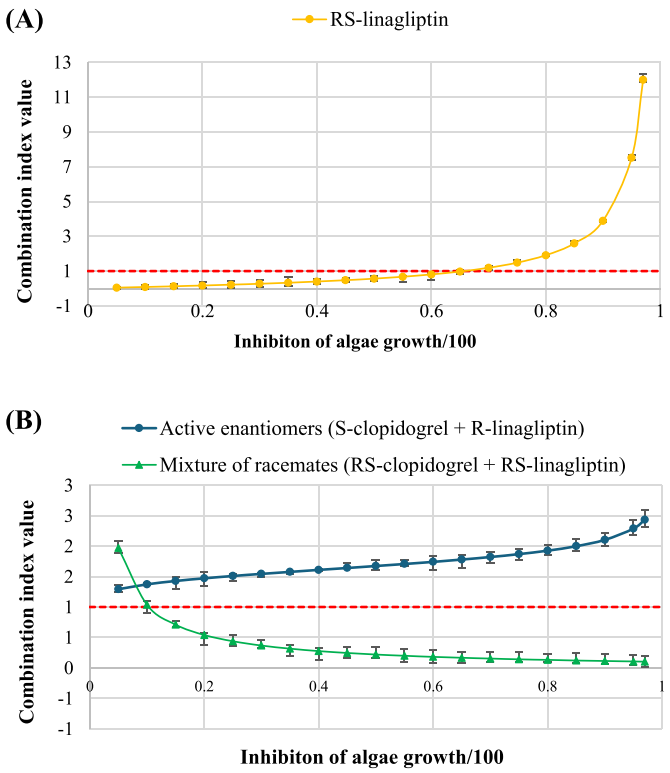

Fig. 6. Combination index obtained for different effect levels in mixtures of linagliptin and clopidogrel on *Pseudokirchneriella subcapitata*: **(A)** Racemic mixture (RS-linagliptin); **(B)** Mixture of pharmacologically active enantiomers (S-clopidogrel + R-linagliptin) and mixture of racemates (RS-clopidogrel + RS-linagliptin). Error bars correspond to 95 % confidence interval.

breakdown of incretin hormones and improves glucose regulation in people with type 2 diabetes. This makes imidazopyrimidine derivatives, such as linagliptin, valuable in pharmaceutical treatments to control blood sugar [43].

There are herbicides based on imidazopyrimidines, which act by inhibiting essential enzymes in amino acid synthesis, thus interfering with plant growth. An example is imazamox, an acetolactate synthase (ALS) inhibitor, used in crops such as soybeans and cereals to control weeds. These herbicides are effective at low doses, but raise environmental concerns, especially because of their impact on non-target organisms such as algae. Netherland et al. [44] concluded that imazamox showed no significant toxic effects on several algal species at concentrations up to 0.040 mg L^{-1}, although they recommend further studies with other species.

No experimental work has been reported on the toxicological effects of clopidogrel on aquatic organisms. Clopidogrel is a drug used as an antiplatelet agent, and its release into the environment may occur via wastewater effluents, raising concerns about its effects on aquatic ecosystems. Although not as common as other pollutants such as antibiotics or hormone products, clopidogrel and other cardiovascular drugs may have sublethal or chronic effects on aquatic ecosystems.

Some works were focused on the occurrence and impact of pharmaceuticals on different aquatic organisms, including clopidogrel, but toxicological parameters (EC_{50}) were estimated by toxicological modelling, not experimentally. These studies analyzed the effect of exposure to compounds such as clopidogrel, which can interfere with photosynthesis, growth and reproduction of algae. Escher et al. [45] estimated the value of EC_{50} = 1.608 mg L^{-1} for clopidogrel using the Quantitative Structure-Activity Relationship (QSAR model) [46] with the aim to assess the ecological risk of hospital effluents. The QSAR method is based on the premise that compounds that have similar chemical structures tend to have similar biological and toxicological effects. By using previous experimental data on the activity of known chemicals, QSAR models predict the toxicity of new compounds without experiments. In the same line, the work of Roveri et al. [47] determined the presence of pharmaceuticals and illicit drugs in a beach area of Brasil and the ecological risks of these compounds to the aquatic environment. Authors estimated EC_{50} of clopidogrel using the Ecological Structure Activity Relationships Program (ECOSAR, v 2.0) [48]. Here, the estimated EC_{50} value for clopidogrel was 3.15 10^5 mg L^{-1} for green algae. Based on the two previous studies together with the experimentally calculated EC_{50} values in the present work, the models used greatly underestimated the toxicity of RS-clopidogrel or S-clopidogrel but also it was not determined which chiral form of the compound occurs in real aquatic ecosystems.

Regarding to the type and degree of interaction of the mixtures studied in this work, no previous work on the combined ecotoxicity of the pharmaceuticals compounds studied has been found to contrast the results obtained.

Pharmaceutical compounds have been identified as harmful and toxic to algae [49]. Previous findings revealed that the toxicity of pharmaceuticals combined exposure varies according to the specific type of compound involved and produced varying effects across different organisms, even in the same algal species. In this line, the combined ecotoxicity on algae was most studied for antibiotics, resulting mostly synergistic effect both for binary mixtures [50,51] and multicomponent mixtures [52].

4. Conclusions

Stability and toxicity assessment was carried out for linagliptin and clopidogrel enantiomers. For this purpose, a chiral analytical methodology was developed allowing for the first time the simultaneous separation of the enantiomers of both compounds by CE in 7.4 min with enantioresolution values of 3.9 and 2.7 for linagliptin and clopidogrel, respectively. The methodology developed consisted of a 25 mM phosphate buffer at pH 7.0 with 10.4 mg/mL CM-β-CD.

Stability was evaluated over 96 h under abiotic and biotic conditions. R-linagliptin and linagliptin racemic, and S-clopidogrel, showed be stable, also in the case of the mixture of the pure enantiomers (R-linagliptin and S-clopidogrel), under both abiotic and biotic conditions while RS-clopidogrel showed decay rates from 18 to 24 % under all studied conditions.

The toxicity of pure and mixed enantiomers for both compounds has been determined for the first time in this work. All of them have shown toxicological activity on the growth of *P. subcapitata*, with RS-linagliptin being the most toxic followed by R-linagliptin with similar EC_{50} values of 0.002 ± 0.001 mg L^{-1}. In general, all the tested enantiomers and mixtures were highly toxic to the green microalgae, and only S-linagliptin showed a somewhat lower toxicity (EC_{50} = 1.355 ± 0.003 mg L^{-1}). RS-linagliptin was synergistic at low effect levels, tending to be antagonistic above EC_{50}. The mixture of S-clopidogrel + R-linagliptin only showed antagonistic effects with varying intensity, which gradually increased with the level of negative effect observed. The mixture RS-clopidogrel + RS-linagliptin showed antagonistic effects at low effect levels that rapidly changed to synergistic effects for increased concentrations.

CRediT authorship contribution statement

Laura García-Cansino: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Karina Boltes:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **María Luisa Marina:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **María Ángeles García:** Writing – review & editing, Writing – original draft, Validation,

Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.127992.

Data availability

No data was used for the research described in the article.

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