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Abstract: Human pharmaceuticals and their residues are constantly detected in our waterbodies, due to poor elimination rates, even in the most advanced waste water treatment plants. Their impact on the environment and human health still remains unclear. When phytoremediation is applied to aid water treatment, plants may transform and degrade xenobiotic contaminants through phase I and phase II metabolism to more water soluble and less toxic intermediates. In this context, peroxidases play a major role in activating compounds during phase I via oxidation. In the present work, the ability of a plant peroxidase to oxidize the human painkiller diclofenac was confirmed using stopped flow spectroscopy in combination with LC-MS analysis. Analysis of an orange colored product revealed the structure of the highly reactive Diclofenac-2,5-Iminoquinone, which may be the precursor of several biological conjugates and breakdown products in planta.

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12/10/15

Dear Prof. Aga,

please find attached our manuscript “Emerging pollutants and plants - metabolic activation of Diclofenac by peroxidases” for publication in your journal.

It is a cooperative work between three institutes with different areas of expertise and tries to unravel the initial activation step of diclofenac in plants. This is important, since phytoremediation of diclofenac and other pharmaceuticals becomes more and more relevant for small waste water treatment facilities. It is an original and novel finding, since we are able to demonstrate that, different from the well studied mammalian system, plant peroxidases perform this important step. Peroxidases are of similar abundance in plants as P450 monooxygenases. We are able to demonstrate that a special metabolite rises from the activation of Diclo by POX, that might initiate the further metabolism of the compound in plants.

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We hope that the manuscript is appropriate for publication by the JHM and are looking forward to favorable reviews.

For the authors

Sincerely

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Highlights:

- First report of a plant peroxidase oxidizing the human painkiller diclofenac
- Enzymatic reaction followed by stopped flow spectroscopy
- Identification of reaction product Diclofenac-2,5-Iminoquinone by LS-MS/MS and TOF high resolution mass spectrometry with accurate mass determination

***Novelty Statement**

Novelty statement:

This is the first report of a plant peroxidase oxidizing the human painkiller Diclofenac and its identification as Diclofenac-2,5-Iminoquinone by LS-MS/MS and TOF high resolution mass spectrometry with accurate mass determination. The paper utilizes an enzymatic reaction followed by stopped flow spectroscopy with a horseradish hairy root culture.

Emerging pollutants and plants - metabolic activation of Diclofenac by peroxidases

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Abstract

Human pharmaceuticals and their residues are constantly detected in our waterbodies, due to poor elimination rates, even in the most advanced waste water treatment plants. Their impact on the environment and human health still remains unclear. When phytoremediation is applied to aid water treatment, plants may transform and degrade xenobiotic contaminants through phase I and phase II metabolism to more water soluble and less toxic intermediates. In this context, peroxidases play a major role in activating compounds during phase I via oxidation. In the present work, the ability of a plant peroxidase to oxidize the human painkiller diclofenac was confirmed using stopped flow spectroscopy in combination with LC-MS analysis. Analysis of an orange colored product revealed the structure of the highly reactive Diclofenac-2,5-

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Iminoquinone, which may be the precursor of several biological conjugates and breakdown products in planta.

Keywords: Diclofenac, iminoquinone, peroxidase, stopped flow spectroscopy, LC-MS

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Introduction

The occurrence, fate and possible effects of pharmaceuticals in the environment have been topic of interest for more than a decade. It has become obvious that the production and use of some of human pharmaceuticals may equal or even exceed the quantities of e.g. agrochemicals and hence, their occurrence in an increasing number in surface waters and ground water reserves cannot longer be overlooked [1].

Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) (DCF) is one of the most prominent compounds in the class of micropollutants or microcontaminants found ubiquitously. It is a widely used non-steroidal anti-inflammatory drug (NSAID) frequently used as prescription free pain killer for many purposes. For medication it is either administered in form of tablets or as topical gels. Once ingested or dermally available after application on the skin, DCF undergoes rapid metabolisation. In the human body, 65-70 % of the orally ingested dose is excreted via urine and 20-30 % in feces as the unaltered drug or as major metabolites [2]. Metabolism proceeds via oxidation reactions delivering a number of hydroxylated metabolites: 4′-hydroxy-DCF and 5´-hydroxy-DCF, 3′-hydroxy-DCF and 4′,5-dihydroxy-DCF [3,4]. The majority of these activated compounds is further bound to glucuronic acid and excreted via urine as conjugates.

Like other pharmaceuticals and personal care products (PCPs), DCF enters the environment mainly through our wastewater treatment systems, which hold only limited capacities for degradation and removal of a broad spectrum of pharmaceutical compounds. And like many other PPCPs, DCF leaves the WWTPs unaltered or as metabolites that can be even more toxic in the environment than the parent compound itself. E.g. for DCF, Diniz and coworkers (2015) reported a higher toxicity for some of its by-products occurring during photolysis towards zebrafish (*Danio rerio*) [5].

Maximum DCF concentrations in municipal wastewaters range around 7 µg/l but vary greatly between and also within countries, with significantly higher concentrations in hospital or manufacturers wastewaters [6]. These concentrations depend mainly on the consumption of DCF which is between 195 and 940 mg per inhabitant and year in different countries [7]. WWTPs efflux concentrations for DCF range between 0.12 and 4.7 µg/l. For 5-OH-DCF, one of the most abundant DCF metabolites, Langford and Thomas (2011) reported a concentration of 3.7 µg/l in Norway [8]. DCF

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concentrations found in surface water hardly ever exceed 100 ng/l. In ground and drinking water the amount of DCF to date is very low or below detection limits (1–7 ng/l) [9].

With concentrations of these compounds ranging from traces to ppb levels the ecological impact of pharmaceuticals in the environment is considered to be rather low, although its risks might be underestimated due to the lack of information on fate and effects of these molecules. Traditional ecotoxicological testing mostly focuses on single compounds, rather than a mixture of compounds with unknown properties, although that is much more likely to appear in the field than a single compound scenario. DCFs increasing relevance in ecotoxicology recently was recognized by the European Union that has classified it, together with two estrogenic hormones as novel priority substances and added it to a watchlist [10]. Compounds on the watchlist have to be monitored by the EU member states for at least four years in surface waters.

Data on the ecotoxicological effects of DCF exist mainly from animal and aquatic invertebrate studies but are scarce for plants and algae. Different biomarkers and endpoints have been used to elucidate and monitor the ecotoxicological effects of DCF. Observed effect concentrations for different biomarkers vary greatly between species and exposure time [11,12].

For few PPCPs uptake and metabolism into plant cells has been suggested eg. for Triclosan [13]. Huber and coworkers (2012) reported the detoxification of DCF in plant tissues, where hydroxylation is followed by conjugation with glucose [14]. The glucosylation of the OH-DCF was confirmed by Bartha et al (2013) who extended the spectrum of analytes towards the formation of 4-OH-glutathionyl-diclofenac in *Typha latifolia* [15], a species that is frequently used in the remediation of heavy metal contaminated wastewaters [16,17]

All these reports indicate that DCF metabolism follows general principles of plant xenobiotic metabolism. An oxidation reaction which is referred to as a Phase I reaction activates the molecule for further metabolism (conjugation, transport) in Phase 2. This activating oxidation is thought to be catalyzed by either P450 monooxygenases or peroxidases [18].

Plant peroxidases (POX, EC 2.5.1.18) represent a class of enzymes with multiple functions in plant metabolism. The super protein family of Heme Peroxidases can be divided into three large classes: POX catalyze the polymeric oxidation of phenolic units during lignin synthesis and cell wall formation. Furthermore POX are crucial

during growth processes, fruit development, ethylene formation as well as detoxification and general stress response [19,20,21]. In many cases POX are involved whenever reactive oxygen (ROS) species occur within plant tissues. There is strong evidence that POX contribute in both processes – the elimination of ROS on one hand, and its production on the other hand [22].

The involvement of plant peroxidases in the detoxification of xenobiotics has been demonstrated in previous studies. Agostini and coworkers (2003) could show the capacity of peroxidases to degrade the toxic pesticide 2,4-dichlorophenol (2,4-DCP) in a cell culture of turnip (*Brassica napus)* [23]. Stiborová and coworkers (2000) studied the catalytic properties of microsomal peroxidases for oxidizing the model compounds N-nitrosodimethylamine (NDMA), N-nitrosomethylaniline (NMA), aminopyrine and 1-phenylazo 2-hydroxynaphthalene (Sudan I) [24]. Samokyszyn and coworkers (1995) showed a peroxidase-catalyzed oxidation of PCP to the electrophilic tetrachloro-1,4-benzo-quinone [25].

The present study derived from the observation of the formation of an orange colored product *in vitro,* when diclofenac was incubated with a crude protein extract from *Armoracia rusticana* in the presence of hydrogen peroxide. Using different approaches, we could clearly demonstrate the oxidation of diclofenac by plant peroxidases and elucidate the nature of the reaction product.

2. Material and Methods

2. 1. Horseradish root cell culture

A hairy root culture of horseradish (*Armoracia rusticana* L.) that had been transformed by *Agrobacterium rhizogenes* strain A4 was grown in Erlenmeyer flasks in 100-ml full-strength Murashige and Skoog medium containing thiamine and inositol for 10 days as previously described [26]. Plant material was used for the extraction of a POX containing enzyme fraction.

2. 2. Stopped flow spectrometry

Concentrations of reagents were determined by preparing solutions from a known mass of substance, apart from the following reactants whose concentrations were determined spectrophotometrically based on their extinction coefficient: HRP (ε=102

$mM^{-1}cm^{-1}$ at 402 nm; [27], H_2O_2 (ε=39.4 $mM^{-1}cm^{-1}$ at 240 nm; [28]).The purity of peroxidase was determined by the RZ-value ($RZ=A_{\lambda=403nm} / A_{\lambda=280nm}$) and yielded 2.8. Pre-steady-state kinetic data, *i.e.*, a single-turnover of the peroxidase enzymatic cycle, were monitored on a stopped-flow spectrometer [29] model SX.18MV-R (Applied Photophysics, Leatherhead, U.K). Experiments were carried out at 23.5 °C in pH 5.5 McIlvaine-buffer, *i.e.*, 0.1 M citrate/0.2 M phosphate buffer [30, 31], which is commonly used to assess peroxidase activity (32,33,34] Isolation of enzyme intermediates was optimised in the conventional dual-syringe system by mixing equal volumes of reactants. Peroxidase HRP-Compound I was prepared using a 1:1 molar ratio of HRP-E and H_2O_2 [27]. HRP-CI was formed after 0.7 s and stable for approximately 3 s. Reactions of HRP-CI with diclofenac (dissolved in McIlvaine-buffer containing 1 % DMSO) were performed by sequential (four-syringe) stopped-flow spectrometry. Reactants were mixed by driving the syringes by compressed air (8 bar) until they were mechanically stopped and the solution was forwarded to a 20 µL optical cell (10 mm pathlength) resulting in a dead time of 0.00128 s. Spectral changes were monitored on a diode array as 3D data arrays (wavelength/absorbance/time) and analysed by Acorn Pro K Global Analysis Software (Applied Photophysics Ltd., Leatherhead, UK) and Specfit/32 Global Analysis System (Spectrum Software Associates, Marlborough, MA, USA). The number and identity of peroxidase-intermediates in the dataset was identified by singular value decomposition (SVD) and evolving factor analysis (EFA). A kinetic model was fitted accordingly and solved by non-linear least-square fitting procedures in combination with numerical integration methods (Levenberg-Marquardt Algorithm) by the least squares criterion (i.e., to find a set of parameters that result in a minimal Square Sum of all residuals). Pseudo first order rate constants (k_{obs}) were obtained from the fitting procedure plotted against diclofenac concentration to obtain the second order rate constants (k) from the slope of the linear regression of the data (mean of 5 measurements).

2.3. Analysis

LC–MS/MS analysis was carried out on an HPLC system (Varian ProStar 210, Varian, Walnut Creek, CA, USA) coupled to an ion trap mass spectrometer (Varian 500-MS, Walnut Creek, CA, USA), operated in the positive ion mode as described previously

[14]. The HPLC eluent was introduced to the mass spectrometer using a pneumatically assisted electrospray source. The interface was adjusted to the following conditions: capillary voltage, 40 V; needle voltage, 4,000 V; spray shield voltage, 600 V; nebulizer gas pressure, 50 psi; drying gas pressure, 30 psi; drying gas temperature, 300 °C. A 20-µl aliquot of each sample was directly injected into the HPLC system. MS/ MS spectra were obtained by collision-induced dissociation using helium as the collision gas. The mass transitions for identification of analytes were 296→250 for DCF and 310→292 for the unknown metabolite. HPLC conditions were as follows: the column was a Phenomenex (Darmstadt, Germany) HYDRO-RP column (C18, polar endcapped; particle size 5 µm; 4.6×200 mm ID). Diclofenac and its metabolites were separated using a linear gradient of eluents: buffer A (H_2O, 0.1% formic acid) and buffer B (acetonitrile, 0.1% formic acid). Elution gradient: 0–2 min 97% buffer A (isocratic); 2–10 min 95% buffer B (linearly increasing); 10–12.5 min 95% buffer B (isocratic); 12–12.5 min 97% buffer A (linear decreasing); 12.5–17 min 97% A (isocratic). The flow rate was 0.3 ml/min. All solvents used for LC–MS were of the highest grade available, filtered and degassed before use.

For accurate mass determination of metabolites a second LC-MS setup was used consisting of an Agilent HPLC systems series 1260 Infinity (Waldbronn, Germany) The system was coupled to an Agilent TOF-MS system series 6230 equipped with a Jet Stream ESI interface (Agilent Technologies, Santa Clara, CA, USA). For separation the same system of solvents and the same column was used as described above. The Jet Stream ESI source was used in negative mode with the following conditions: gas temperature 325 °C, drying gas flow 7.1 L/min, nebulizer gas pressure 45 psi, sheath gas temperature 250 °C, sheath gas flow 5.5 L/min, capillary voltage 2 kV, fragmentor 175 V. Nitrogen was used as both drying and sheath gas. For accurate mass measurements an online reference mass correction was done with Agilent´s API-TOF Reference Mass Solution Kit, including the compounds purine and HP-0921. Thus, a mass tolerance <5 ppm was achieved. Samples were analyzed in extended resolution mode with a low-mass range (100-1700 m/z) in full mode.

2.4. Extraction of POX containing enzyme fraction

A cytotosolic enzyme extraction was prepared according to the method described by Bartha and coworkers [15]. Shortly, 3 g of frozen plant material was ground to a fine powder which was transferred to a precooled beaker and extracted with buffer (0.1 M

Tris/HCl pH 7.8, 5 mM EDTA, 5 mM dithioerythritol DTE, 1 % NonidetP40, 1 % insoluble polyvinylpyrrilidone PVP K90) for 30 minutes on ice prior to centrifugation at 20,000 rpm. Proteins in the resulting crude extract were precipitated by addition of ammonium sulphate in two steps of 40 and 80 percent of saturation.

After each step the homogenate was centrifuged and the pellet was finally resuspended in 2.5 ml of 25 mm Tris/HCl buffer pH 7.8. In a last step the extracts were desalted on Sephadex PD-10 columns (Pharmacia, Germany).

2.5. Incubation of peroxidases with Diclofenac

Diclofenac was incubated with purified horseradish peroxidase or peroxidase containing enzyme preparations in the presence of hydrogen peroxide. Incubations were performed at 30 °C in sodium phosphate buffer at different pH values ranging from 2-8, containing 200 µM diclofenac, 18 µM hydrogen peroxide and 20 U of horseradish peroxidase or 50 µl of crude enzyme extract, respectively.

For the determination of the optimal pH of the reaction, the formation of an orange colored product was followed at 450 nm in a spectral photometer for 10 minutes.

3. Results

When incubated with HRP in the presence of hydrogen peroxide *in vitro*, DCF was rapidly converted into a product of dark orange color. The same reaction was observed when a crude enzyme extract of *A. rusticana* was used instead of pure HRP under the same experimental conditions.

Spectrophotometric analysis of the reaction revealed a maximum of absorption at 450 nm for the orange colored product. The rate of formation of this product was proportional to either enzyme or substrate concentration. Kinetic analysis revealed a pH-optimum of 5.5 in different buffer systems (data not shown). Based on these observations the enzymatic assay was transferred to stopped flow conditions.

Active intermediates of horseradish peroxidase (HRP) oxidized diclofenac. At pH 5.5, the reaction of pre-formed CI with diclofenac showed the formation of E via CII (Figure 1A, B and C). This was confirmed by the shift of the isosbestic point from 397 nm (CI/CII) to 411 nm (CII/E). Additionally, SVD (Figure 2 A,B) and EFA (Figure 2C) analysis identified three colored components, corresponding to CI, CII and E. Three V- (Figure 2A) and U-vectors (Figure 2B) varied consistently over all

wavelengths or time, respectively, and represented intermediates. The remaining vectors showed random fluctuations which indicated noise. The identification of three colored components justified the use of a model with two kinetic processes in the data set and therefore the kinetics were modelled by a two-step reaction: CI→CII→E (Figure 2D). This was supported by the quality of the kinetic fit to the kinetic data derived from the changes in the absorbances at 403 nm and 420 nm to the estimated model (Figure 2E) indicating the decay of CI (403 nm), formation of CII (420 nm), decay of CII (420 nm) and formation of E (403 nm). Additionally, the fitting residuals (Figure 2F) varied randomly and showed no systematic deviations over all wavelengths and therefore retained no significant colorimetric or kinetic information. Hence, the kinetic model CI→CII→E showed a good correlation with the dataset.

The pseudo-first-order rate constants (k_{obs}) for the reduction of CI by diclofenac were plotted against the concentration of diclofenac (Figure 3) and the second-order rate constants (k) were calculated from the slope of the data. The value of the correlation coefficient (R^2: 0.9982 for k_{CI} and 0.9976 for k_{CII}) indicated a good fit and therefore a high accuracy of the second-order rate constant. The low value of the y-intercept (0.5132 for k_{CI} and 0.0945 for k_{CII}) indicated pure preparations without contaminant electron donors present [27]. Second order rate constants (k) of the reduction of HRP-CI by diclofenac in comparison with other substrates are given in table 1.

For identification of the product formed during the enzymatic reaction the product was analyzed using LC-MS/MS and LC-TOF-MS detection. Mass spectrometric analysis of the incubation assay after protein precipitation revealed a dominant signal of m/z 310 that was only detected in the presence of diclofenac, hydrogen peroxide and the peroxidase (Figure 4). Fragmentation of m/z 310 during MS/MS experiments resulted in a mayor fragment of m/z 292.1 (insert in Figure 4) corresponding to the loss of water [35,36]. Based on this information obtained with an ion trap (ESI) the sample was further analyzed using an LC-system coupled to a TOF mass spectrometer. Under the same chromatographic conditions the signal of m/z 310 could be confirmed with an exact m/z of 310,0030.

4. Discussion

Stopped flow spectrometry was the method of choice to prove the capacity of HRP to oxidize DCF. Second order rate constants are in line with previous studies [37, 38].

Oxidation of DCF by peroxidases, namely myeloperoxidases, in mammalian activated neutrophils is described with different interpretation on the nature of the product [35,36]. Zuurbier and coworkers reported the formation of an intensively orange colored product when DCF had been incubated with myeloperoxidase in the presence of hydrogen peroxide. The reaction product shows a maximum of absorption at 452 nm and was identified as dihydroxyazobenzene via FAB-MS [36]. Even though an orange compound was formed by HRP in the presence of DCF and hydrogen peroxide in our study, it could not be identified as dihydroxyazobenzene, which was available as an analytical standard and characterized under identical analytic conditions standard. This goes, again in line, with the findings of Miyamoto et al [35] who were unable to confirm the structure of dihydroxyazobenzene in their study either.

Apart from mammals, fungi are known to possess peroxidases able to utilize DCF as a substrate. Zhang and Geissen (2010) reported the in vitro degradation of DCF by a crude lignin peroxidase, produced from the white rot fungus *Phanerochaete chrysosporium,* although no information on the nature of the product is given [39].

Gröning and coworkers could observe the formation of Diclofenac-2,5-Iminoquinone under aerobic conditions in bioreactors in the presence of DCF and an active microbial community isolated from river sediments [40]. They report a fast and effective degradation of DCF up to a concentration of 70 µM. Higher doses caused a decline in degradation rates, probably due to an increased toxicity of DCF towards the bacteria. Forrez and coworkers (2010) achieved the formation of Diclofenac-2,5-Iminoquinone via oxidation of DCF in aqueous solution within a matrix of biological produced manganese dioxide (MnO_2). Under these conditions 5-10 percent of degraded DCF was present as the iminoquinone metabolite [41]. Additionally its formation was observed in the presence of ozone in aqueous solution of DCF [42].

It is postulated that Diclofenac-2,5-Iminoquinone is formed via the reactive precursor 5`OH-Diclofenac which might be converted spontaneously in aqueous solution. Due to its high reactivity and its ability to bind proteins covalently the iminoquinone itself is hepatotoxic in mammals [35].

In the present study, LC-MS analysis of the orange compound in fact points towards the formation of the Diclofenac-2,5-iminoquinone. Its fragmentation pattern during MS/MS experiments revealed the loss of an H_2O molecule resulting in a very dominant fragment of m/z 292 as described elsewhere in different systems [35,40,41]. Accurate mass determination by a TOF mass-spectrometer further confirms the

formation of Diclofenac-2,5-iminoquinone since the m/z of 310.0030 corresponds to the postulated molecular formula of $C_{14}H_{10}O_3NCl_2$ based on the ChemCalc algorithm [43] with minimal deviation. Based on these data we suggest the formation of this metabolite catalyzed by plant peroxidases for the very first time. Whether this involves the previous formation of 5´OH-Diclofenac remains unclear as this metabolite has not been observed in horseradish hairy roots yet.

A majority of the DCF-Iminochinon will undergo rapid further metabolism in phase II and yield the observed glycosyl- and glutathionyl-conjugates. Hence, although working with a model system, it may be concluded that plant aided remediation of waste waters might lead to higher removal rates of DCF, and to a lower burden to the aquatic environment. The presence of the respective POX, its inducibility to enhance metabolism, and the practical implementation of capable plant species require further studies.

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Captions and legends

Table 1: Second-order rate constants (k) of the reduction of HRP-CI by diclofenac in comparison with other substrates.

Figure 1: Reduction of 1µM HRP-CI by 50 molar equivalents of diclofenac in McIlvaine-buffer at pH 5.5. Spectrokinetic data depicting the transition from CI (blue spectrum; 0.00128 s; λmax(↑): 403 nm) to CII (red spectrum, 2.359 s; λmax(↑): 418.1 nm, 527 nm and 555 nm) to E (green spectrum, 43.8 s; λmax(↑): 403 nm, 498 nm); Isosbestic points (↑): 397 nm (CI/CII), 411 nm (CII/E), 425 nm (CI/E). (A) Raw data (B) Soret band (C) Q-band.

---colored print---

Figure 2: Reduction of 1 µM HRP-CI by 50 molar equivalents of diclofenac in McIlvaine-buffer at pH 5.5. Three (A) V- and (B) U-vectors (i.e., intermediates) were identified by SVD analysis. (C) EFA-calculated absorbance profiles of intermediates (i.e., CI, CII and E). (D) Concentration profiles of the kinetic evolution of intermediates. (E) Kinetic trace of absorbance at 403 nm (i.e., decay of CI and formation of E) and 420 nm (i.e., formation and decay of CII) accompanied by predicted data from the CI→CII→E model. (F) Fitting residuals from the fit of the data to the model CI→CII→E.

---colored print----

Figure 3: Kinetics of the reduction of pre-formed HRP-CI by diclofenac in pH 5.5 McIlvaine Buffer.

Figure 4: LC-MS/MS (+) analysis of an enzymatic assay containing Diclofenac, H_2O_2 and HRP (A) and without HRP (B) in SIM for m/z 310. Insert in A shows the fragmentation pattern of m/z 310 at 7,6 minutes.

Table

substrate	k_{CI} ($M^{-1}s^{-1}$)	k_{CII} ($M^{-1}s^{-1}$)	reference
diclofenac	2.35×10^4	8.85×10^2	
diclofenac	6.05×10^5	9.75×10^1	Dunford, 1999; oxidation by MPO
ferulic acid	4.8×10^7	1.3×10^7	Henriksen et al, 1999
indole-3-propionic acid	6.6×10^3	4.0×10^2	Henriksen et al, 1999
p-aminobenzoic acid	6.5×10^3	4.9×10^2	Henriksen et al, 1999
ferrocyanide	1.4×10^7	2.1×10^5	Henriksen et al, 1999

Figure 1

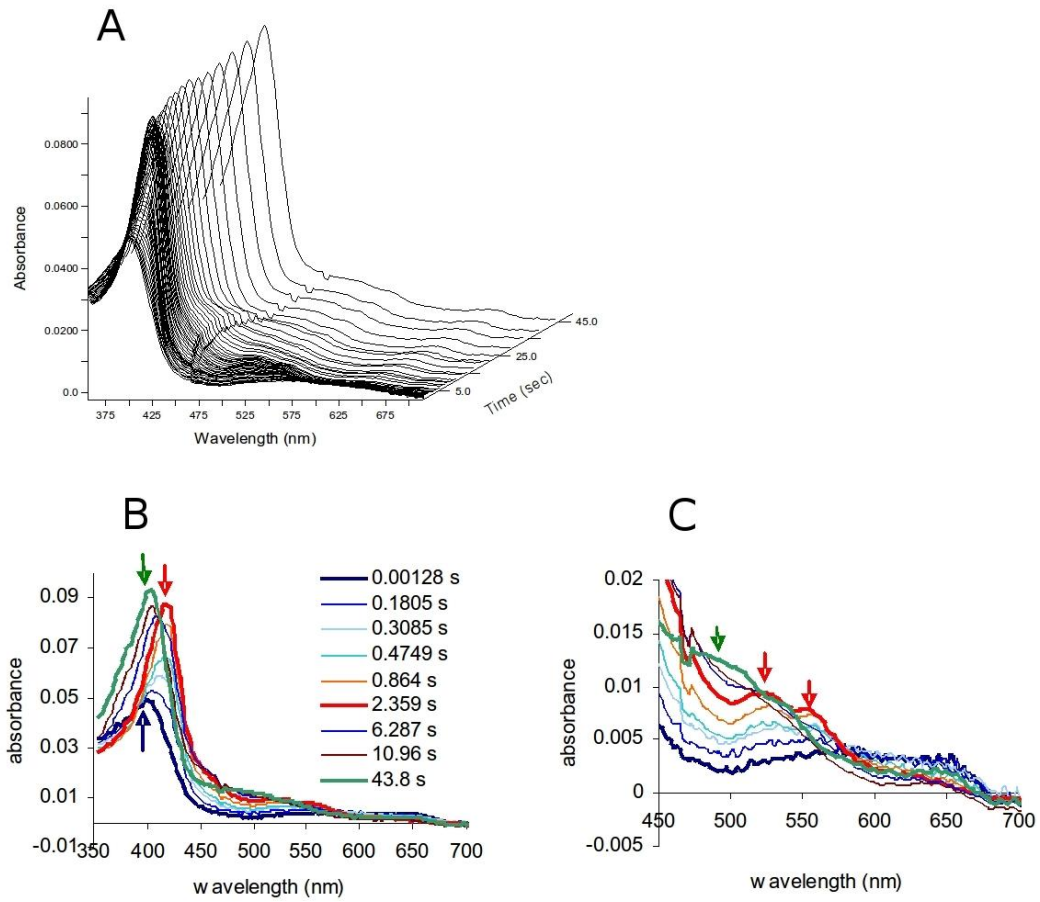

A
Absorbance
0.0800
0.0600
0.0400
0.0200
0.0
375
425
475
525
575
625
675
Wavelength (nm)
45.0
25.0
5.0
Time (sec)
B
absorbance
0.09
0.07
0.05
0.03
0.01
-0.01
350
400
450
500
550
600
650
700
w avelength (nm)
0.00128 s
0.1805 s
0.3085 s
0.4749 s
0.864 s
2.359 s
6.287 s
10.96 s
43.8 s
C
absorbance
0.02
0.015
0.01
0.005
0
-0.005
450
500
550
600
650
700
w avelength (nm)

Figure 2

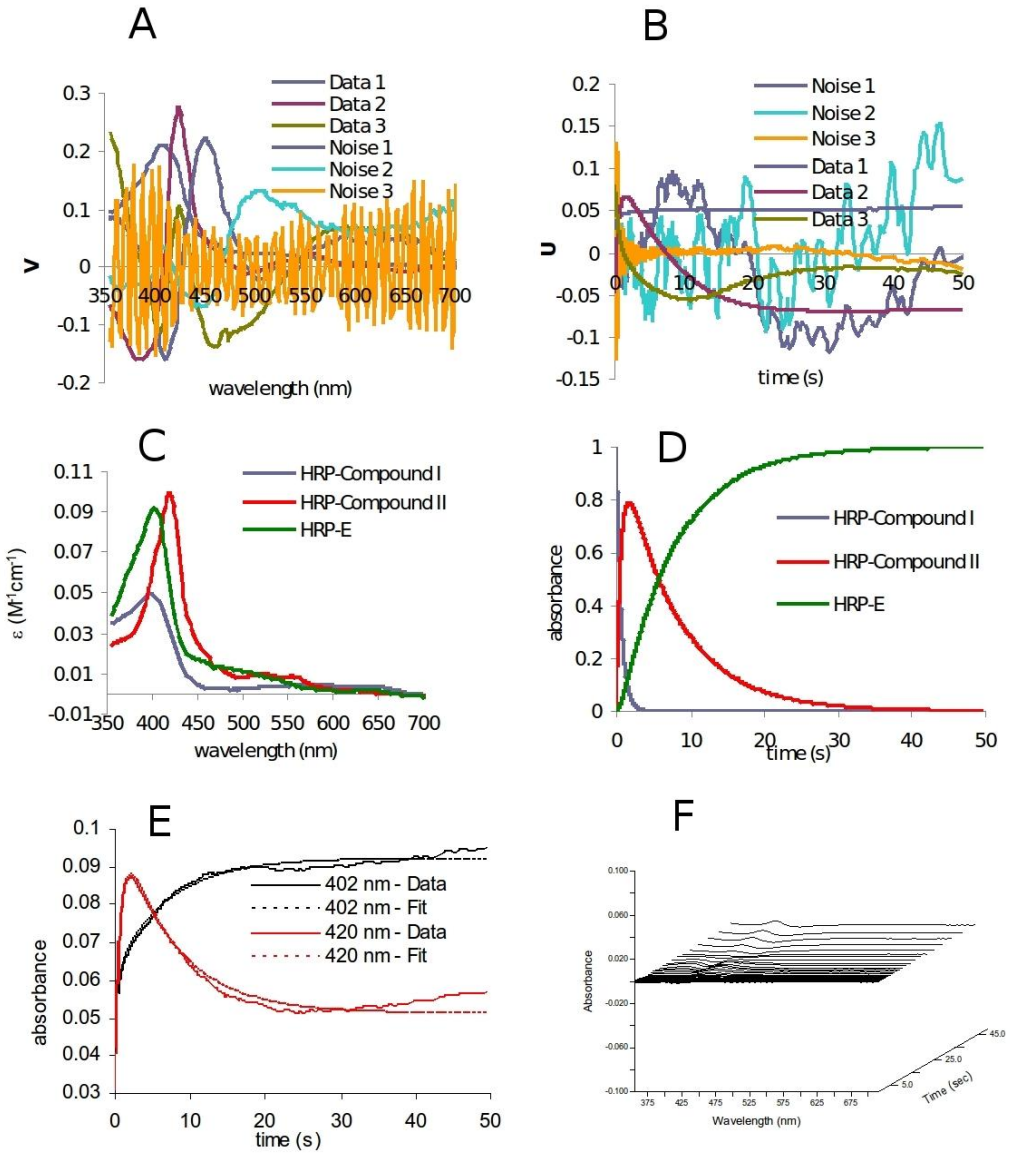

A
Data 1
Data 2
Data 3
Noise 1
Noise 2
Noise 3
v
wavelength (nm)
B
Noise 1
Noise 2
Noise 3
Data 1
Data 2
Data 3
u
time (s)
C
HRP-Compound I
HRP-Compound II
HRP-E
ε (M-1cm-1)
wavelength (nm)
D
HRP-Compound I
HRP-Compound II
HRP-E
absorbance
time (s)
E
402 nm - Data
402 nm - Fit
420 nm - Data
420 nm - Fit
absorbance
time (s)
F
Absorbance
Wavelength (nm)
Time (sec)

Figure 3

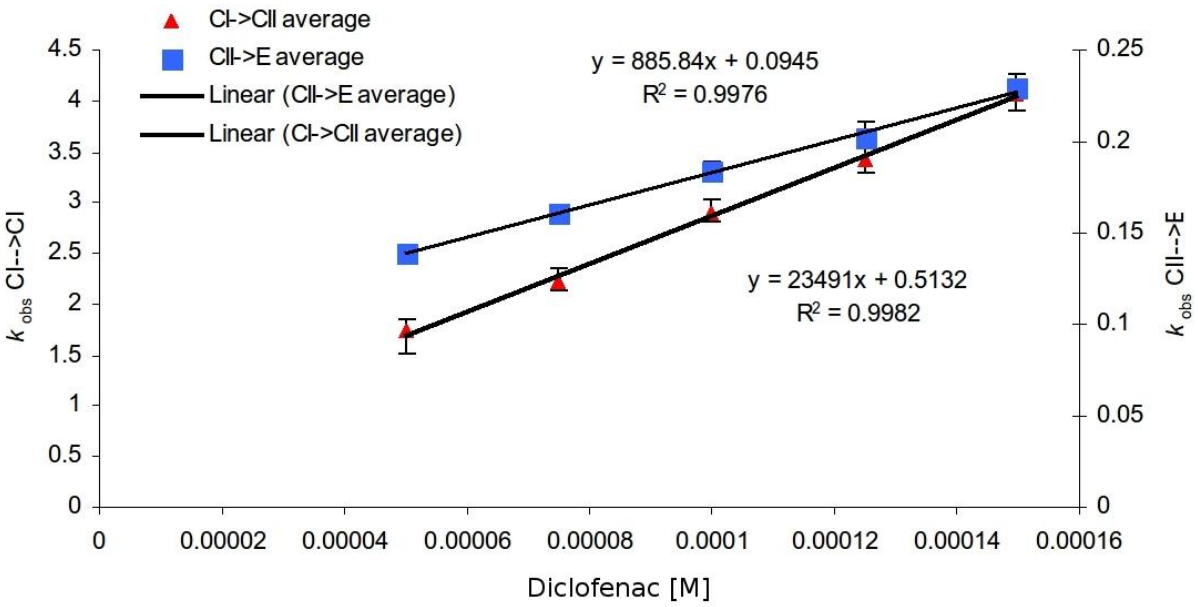
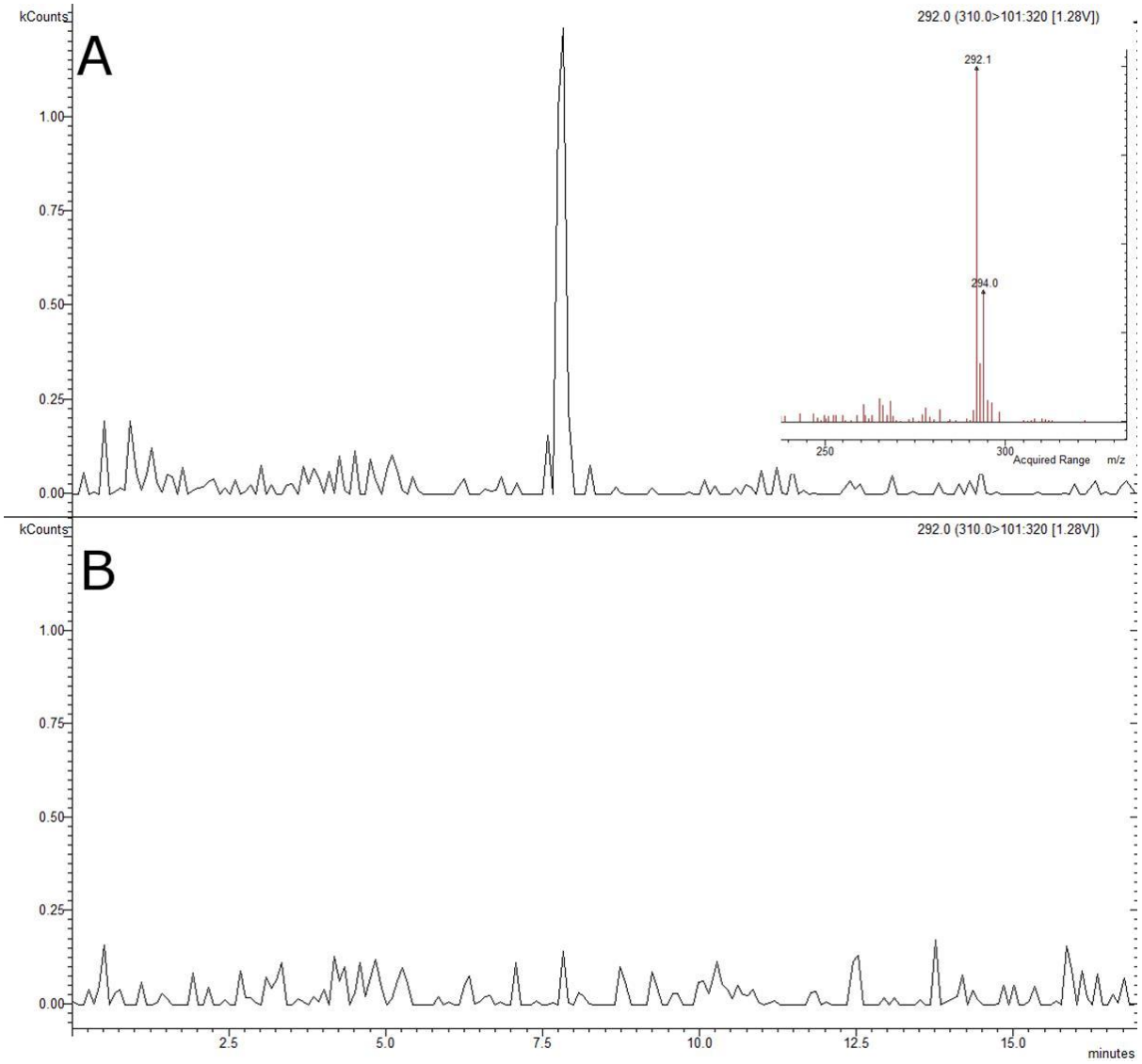

CI->CII average
CII->E average
Linear (CII->E average)
Linear (CI->CII average)
y = 885.84x + 0.0945
R2 = 0.9976
y = 23491x + 0.5132
R2 = 0.9982
kobs CI-->CI
kobs CII-->E
Diclofenac [M]
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0.05
0.1
0.15
0.2
0.25
0.00002
0.00004
0.00006
0.00008
0.0001
0.00012
0.00014
0.00016

Figure 4


A
kCounts
292.0 (310.0>101:320 [1.28V])
292.1
294.0
250
300
Acquired Range
m/z
1.00
0.75
0.50
0.25
0.00
B
kCounts
292.0 (310.0>101:320 [1.28V])
1.00
0.75
0.50
0.25
0.00
2.5
5.0
7.5
10.0
12.5
15.0
minutes