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RESEARCH ARTICLE

Bioavailability of flumequine and diclofenac in mice exposed to a metal-drug chemical cocktail. Evaluation of the protective role of selenium.

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Abstract

Background and Purpose: Organisms, including humans, are subjected to the simultaneous action of a wide variety of pollutants, the effects of which should not be considered in isolation, as many synergies and antagonisms have been found between many of them. Therefore, this work proposes an in vivo study to evaluate the effect of certain metal contaminants on the bioavailability and metabolism of pharmacologically active compounds. Because the most frequent entry vector is through ingestion, the influence of the gut microbiota and the possible protective effects of selenium has been additionally evaluated.

Experimental Approach: A controlled exposure experiment in mammals (*Mus musculus*) to a "chemical cocktail" consisting of metals and pharmaceuticals (diclofenac and flumequine). The presence of selenium has also been evaluated as an antagonist. Mouse plasma samples were measured by UPLC-QTOF. A targeted search of 48 metabolites was also performed.

Key Results: Metals significantly affected the FMQ plasma levels when the gut microbiota was depleted. Hydroxy FMQ decreased if metals were present. Selenium minimized this decrease. The 3-hydroxy DCF metabolite was not found in any case. Changes in some metabolic pathways are discussed.

Conclusions and Implications: The presence of metals in the mouse diet as well as the prior treatment of mice with an antibiotic mixture (Abxs), which deplete the gut microbiota, has a decisive effect on the bioavailability and metabolism of the tested pharmaceuticals and dietary selenium minimize some of their effects.

KEYWORDS
diclofenac, drug metabolism, flumequine, metals, mice

Abbreviations: Abx, pretreatment with an antibiotics mixture; DCF, diclofenac; FMQ, flumequine; PACs, pharmacologically active compounds; QTOF, quadrupole time of flight.

1 | INTRODUCTION

Biological responses of organisms to environmental factors, such as chemical or biochemical pollutants, can be assessed using environmental bioindicators or laboratory models (Gago-Tinoco et al., 2014; García-Sevillano et al., 2014). Because humans and mice share a central route of entry, the digestive tract, from which ingested contaminants are distributed throughout the body via blood flow to different parts of the body (Gómez-Ariza et al., 2011), the use of mammals as laboratory models allows the results obtained to be transferred to humans, despite the fact that mice and humans have different gut microbiota communities.

The analysis and distribution of pollutants and their metabolites in living organisms is of great importance because of their different harmfulness, based not only on their concentration. Pollutants may interact through antagonistic or synergistic mechanisms that must be considered for proper evaluation of biological responses (García-Barrera et al., 2012). The use of controlled exposure experiments to environmental pollutants of different chemical groups (a "chemical cocktail") to assess the actual effects of pollutants in the environment (Van Den Brink et al., 2005) would be a good approximation for proper evaluation of the biological response of organisms.

In recent years, the traditional concern about "classical" pollutants, such as metals and pesticides, is shifting to a much more diverse and broad group of substances that constitute the so-called emerging pollutants. Their effect on biota, including humans, has been, and is being, extensively studied, not only as harmful but also as biologically active substances. In this context, pharmacologically active compounds (PACs) constitute a large group of emerging pollutants with potentially harmful effects on the environment and human health. It has been found that conventional wastewater treatment plants (WWTPs) do not adequately remove the residues of PACs in wastewater, resulting in their persistent incorporation to environmental waters (Deblonde et al., 2015) and thus are included in the existing biota. Many analytical methods have been developed to determine PACs and their main metabolites in all types of environmental samples, from water and sediments to animals and plants. However, studies on the effects of the presence of chemical cocktails of PACs and metals on biological responses are scarce (Cedergreen, 2014), despite the fact that studies in earthworms (*Eisenia fetida*) (Huang et al., 2009) have shown the effects of the presence of ciprofloxacin, an antibiotic widely used for human and veterinary therapeutic purposes, on copper distribution, accumulation and toxicity in this organism.

Although there are several papers on the metabolism of PACs (Anadón et al., 2008), serious and rigorous information on the synergistic or antagonistic effects of other chemicals on the metabolism of pharmaceutically active compounds (PACs) is very limited (Rodríguez-Moro et al., 2022).

On the other hand, the main way of entry of major pollutants into the animals is their ingestion. For this reason, the influence of gut absorption should also be considered. The human microbiome comprises a complex, dynamic and equilibrated bacterial community that plays a pivotal role in health, interfering with the host's vital physiological, metabolic and immunological functions (Kandel Gambarte & Wolansky, 2022; Martel et al., 2022). Alterations in microbial composition and diversity, known as "dysbiosis," are associated with an increased risk of certain diseases such as allergies, intestinal inflammatory problems, diabetes, obesity and also neurological and cognitive disorders via the gut–brain axis, (Borre et al., 2014; Collado et al., 2015; Hsiao et al., 2013; Stilling et al., 2014).

Some researchers have shown that gut microorganisms can directly or indirectly affect the bioavailability of oral drugs such as by changing intestinal properties. Gut microbial enzyme activity can directly influence the bioavailability of oral drugs by affecting their metabolism (Goldman et al., 1974), first-pass effect (McCabe et al., 2015) or enterohepatic recirculation (Liang et al., 2015). Thus, it is important to consider the influence of gut microbiota in the presence of other chemicals such as metals or metalloids (Zhang et al., 2021).

In addition, recent studies demonstrate the influence of gut microbiota on selenium status and expression of selenoproteins in mice (Hrdina et al., 2009) and that dietary selenium affects the expression of selenoproteins influenced by the gut microbiota in mouse (Kasaikina et al., 2011) and other mammals (Lv et al., 2015). However, these studies focused on the evaluation of enzymatic activity and the determination of low molecular weight selenium species such as inorganic selenium, selenomethionine, and selenocysteine and selenoproteins.

What is already known

- Pollutants may interact through antagonistic or synergistic mechanisms on biological responses of animals.
- Gut absorption could affect bioavailability and metabolism of PACs.

What does this study add

- Information on the synergistic or antagonistic effects of metals on the metabolism of PACs.
- Evaluation of the protective role of seluniumand gut microbiota on PACs metabolism changes.

What is the clinical significance

- Possible synergistic and antagonistic effects on mammalians PACs metabolism must be considered.
- Physiological undesirable effects of metals and gut microbiota could be minimized by dietary selenium.

The combined effects of exposure to the pesticide dichlorodiphenyldichloroethylene (DDE) and selenium on the metabolome of *Mus musculus* have been studied (Rodríguez-Moro et al., 2019). Likewise, some exposure experiments have been carried out for Se–Cd and Hg–Se mixtures (García-Sevillano et al., 2015). Moreover, there are studies that establish a relationship between oral exposure of metals and gut microbiota in mice (Zhai et al., 2017) and that the latter acts as a barrier against chronic exposure to heavy metals (Collado et al., 2015). Some metabolomics, metallomics and metataxonomics studies involving the influence of microbiota and the antagonistic effect of Se on metal toxicity have been previously published (Arias-Borrego et al., 2022; Callejón-Leblic, Selma-Royo, Collado, Abril, & García-Barrera, 2021; Callejón-Leblic, Selma-Royo, Collado, Gómez-Ariza, et al., 2021; Parra-Martínez et al., 2022). However, the consequences of pharmacological bioavailability and metabolism under these conditions have not been previously described.

We consider this study a good approach to addressing living beings' exposure to a wide variety of pollutants released into the environment and the multiple pathways through which they enter organisms in our soil, oceans, and rivers.

In this paper, the bioavailability and metabolism in mice of two commonly used pharmaceuticals (diclofenac and flumequine) with a high environmental relevance are studied. Mice were exposed to a cocktail of metal drugs, including cadmium, arsenic and mercury, and the potential antagonist effect of selenium as well as the influence of the gut microbiota was evaluated.

The purpose of this study is to initiate a line of research that will analyse synergism and antagonism between different types of substances and examine their effects on mammalian physiology and metabolism.

2 | METHODS

2.1 | Chemicals and reagents

Diclofenac, 3-hydroxydiclofenac, 4-hydroxydiclofenac, 5-hydroxydiclofenac, flumequine, $^{13}C_3$-flumequine, d_4-diclofenac, $CdCl_2$ and As_2O_3 were purchased from Sigma-Aldrich (Madrid, Spain); $HgCl_2$ was supplied by Supelco-Merck (Madrid, Spain) and Flumesyva powder (10% [w/w] flumequine) was acquired from Laboratorios SYVA (León, Spain).

Acetonitrile and methanol (LC-MS grade) and formic acid (analytical grade) were purchased from VWR (Barcelona, Spain). For all preparations and dilutions, ultrapure deionized water (Milli-Q Plus water system, Millipore, Billerica, MA, USA) was used.

Stock solutions of diclofenac, 3-hydroxydiclofenac, 5-hydroxydiclofenac and d_4-diclofenac (1000 μg·mL^{-1}), flumequine (400 μg·mL^{-1}) and $^{13}C_3$-flumequine (500 μg·mL^{-1}) were prepared by dissolving the substances in methanol; 4-hydroxydiclofenac (100 μg·mL^{-1}) was supplied as a solution in acetonitrile. All the stock solutions were stable for at least 2 months at 4°C. Working solutions were prepared daily by adequate dilutions using ultrapure water.

2.2 | Chromatographic separation and mass spectrometry determination

Chromatographic analysis was conducted in an Acquity® UPLC (Waters, Milford, MA, USA) coupled to a Xevo® G2S QTOF mass spectrometer (Waters, Micromass, Manchester, UK). An Acquity® BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) at 25°C using water (A) and methanol (with 0.1% [v/v] formic acid) (B) as mobile phase at a flow rate of 0.4 ml·min^{-1} for 17 min was used. The following gradient elution programme was applied to achieve the separation: (1) $t = 0$ min 70% A; (2) $t = 3.0$ min 45% A; (3) $t = 10$ min 45% A; (4) $t = 13$ min 10% A; (5) $t = 15$ min 70% A and (6) $t = 17$ min 70% A; 10 μl of the extracted sample were injected into the column, and detection was carried out in positive/negative ionization, with an electrospray source at 2.0/−2.0 kV capillary voltage, 120°C source temperature and 400°C desolvation temperature.

Data were acquired using a reference spray (lockSpray®) to certify accuracy and reproducibility. Leucine enkephalin m/z $[M + H]^+$ 556.2771 and m/z $[M - H]^-$ 554.2615 were used as references. The lockSpray® frequency was set at 30 s for mass correction of the analyte. The lockSpray® capillary was set at 1.6 kV.

Under these conditions, the target analytes of three diclofenac hydroxylated metabolites with similar structures and comparable m/z values were clearly distinguished. Additionally, target analytes were isolated with low limits of detection and quantification, unambiguous determination and/or quantitative analysis of selected compounds. Table 1 shows the corresponding retention times for the target analytes along with their theoretical and experimental m/z.

The software MassLynx® (version 4.1) was used to process the data, whereas target metabolites were identified using Chromalynx® XS (Waters, Micromass, Manchester, UK), which identifies previously defined metabolites. In this case, a targeted search of 48 candidates was performed based on previous metabolites reported in the bibliography (Fu et al., 2017; Parshikov & Sutherland, 2012; Sarda et al., 2014). This identification was based on exact mass (error <5 ppm), chlorine isotopic patterns for diclofenac (DCF), metabolites and the MS/MS fragments obtained in the second function by comparing them to those previously reported in literature. The targeted search was conducted in both positive and negative ionization.

TABLE 1 Retention times and theoretical and experimental *m/z* for the target analytes (ESI^+ ionization).

	t_R (min)	$m/z_{theor.}$	$m/z_{exp.}$
$^{13}C_3$-flumequine	4.09	265.0979	265.0974
Flumequine	4.09	262.0879	262.0879
d_4-diclofenac	12.32	300.0558	300.0492
Diclofenac	12.41	296.0245	296.0241
3OH-diclofenac	6.35	312.0194	312.0186
4OH-diclofenac	6.62	312.0194	312.0179
5OH-diclofenac	7.21	312.0194	312.0179

2.3 | Data and statistical analysis

This work complies with *BJP*'s recommendations and requirements (Curtis et al., 2022) on experimental design and analysis. Statistical analysis (one-way ANOVA and nonparametric analysis) of data from plasma of the studied compounds in different groups of mice submitted to the exposure test was carried out using Minitab® 16.1.0 software (Minitab Inc., State College, PA, USA). The group size represents the number of independent values, and statistical analysis was performed based on these independent values. All obtained data were used in the statistical analysis. To test whether there were significant differences in FMQ and DCF levels found in different exposure groups, statistical data were analysed using Minitab® Statistical Software. First, to compare two sets of variables, a normality test was used to determine whether each dataset was well modelled by a normal distribution. If the normality test had a P value >0.05, a one-way ANOVA statistical test with Tukey comparison was performed using mean values of plasma concentration levels. When $P < 0.05$, a less accurate nonparametric test (Mood's median test) was applied using the median of the concentration values in the dataset. Twelve groups consisting of $n = 5$ animals were considered for statistical analysis.

2.4 | Exposure experiences

This investigation was performed with the consent of the Ethical Committee of the University of Córdoba (Spain) and the regional government (Code Num. 02-01-2019-001), in accordance with current European Union regulations. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Male *M. musculus* mice 8 weeks old of the consanguineous strain BALB/c (Charles River, Barcelona, Spain) were used as model organism in mammalian exposure experiments. Studies were designed to generate groups of equal size, using randomisation and blinded analysis. Mice were divided into six test groups: (1) control group with a regular diet, (2) control group with diet supplemented with selenium, (3) diclofenac and flumequine supplemented regular diet, (4) diclofenac, flumequine and selenium supplemented diet, (5) arsenic, cadmium, mercury, diclofenac and flumequine supplemented diet and (6) arsenic, cadmium, mercury, diclofenac, flumequine and selenium supplemented diet. In addition, the influence of gut microbiota dysbiosis on the bioavailability and/or metabolism of the pharmaceuticals has been assessed in different groups (group B) within each of the aforementioned groups. Thus, a total of 12 groups consisting of five animals were considered (see Table 2 for details).

Mice were housed in individual cages under a controlled environment (photoperiod 12-h light/dark cycle, temperature 25 ± 2°C), with ad libitum access to drinking water and food, at the Experimental Animal Services (SAEx) from the University of Córdoba (Spain), under its qualified staff control. Individual caging allowed determination of actual water and food consumed and to infer the exact dose of compounds. After the acclimatisation period (3 days), the mice were submitted to a pre-exposure period for 7 days, during which some of the animals (B groups) received a combination of antibiotics (Abx) in the drinking water inducing gut microbiota depletion prior to the exposure assay. The antibiotic cocktail consisted of ampicillin 1%, metronidazole 1%, neomycin 1%, vancomycin 0.5% and an antifungal (amphotericin B, 10 $mg{\cdot}L^{-1}$) (Callejón-Leblic, Selma-Royo, Collado, Abril, & García-Barrera, 2021; Zarrinpar et al., 2018).

The duration of the assay was 2 weeks, and all mice (except control groups 1 and 2) received up to five pollutants: PACs in the food and/or metals in the drinking water. Animals from exposure groups 4 and 6 also received selenium (0.65 $mg{\cdot}kg^{-1}$) via a supplemental feed (Altromin Natrium-Selenit, Altromin Spezialfutter GmbH & Co., Lage, Germany). Estimated daily intakes ($mg{\cdot}kg^{-1}$) for PACs and metals were as follows: flumequine (625), diclofenac (20), arsenic (3), cadmium (0.1) and mercury (1). The dose was chosen after an exhaustive review of the literature (Callejón-Leblic, Selma-Royo, Collado, Gómez-Ariza, et al., 2021; García-Sevillano et al., 2013; Kashida et al., 2006;

TABLE 2 Mice exposure test groups (for details, see text).

GROUP		Abx	Se supplemented	Pharmaceuticals	Metals cocktail
1A					
1B		+			
2A	CONTROLS		+		
2B		+	+		
3A				+	
3B		+		+	
4A			+	+	
4B		+	+	+	
5A				+	+
5B		+		+	+
6A			+	+	+
6B		+	+	+	+

López-Pacheco et al., 2019; Rodríguez-Moro et al., 2020; Trombini et al., 2021) and based on our own expertise. The doses used are consistent with environmentally relevant concentrations and are a compromise between semirealistic conditions and the concentrations expected to have pharmacological effects. For the selection of doses, we considered the literature, our previous works and the possibility of bioaccumulation along the food chain (Chormare & Kumar, 2022; Nagpal et al., 2018; Nilsen et al., 2019; Zenker et al., 2014) and used doses below their LD_{50} to minimize possible synergistic effects that could enhance their toxicity.

After exposure, mice were individually anaesthetised by isoflurane (1.5%) inhalation and exsanguinated by cardiac puncture into a heparinized tube before being killed by cervical dislocation. Plasma samples were collected by centrifugation (725 × *g*, 30 min, 4°C) and stored at −80°C until their analysis.

2.5 | Vortex assisted sample plasma extraction procedure

To 50 µl of plasma sample in an Eppendorf tube, 5 µl of 1000 µg·L^{-1} aqueous solution of $^{13}C_3$-flumequine and d_4-diclofenac (internal standards, IS) and 500 µl of acetonitrile were added. The tube was subjected to vortex agitation (VORX-005-001, Labbox Labware; Premia de Dalt, Barcelona, Spain) for 3 min, followed by centrifugation (4°C) at 10,000 × g (Biocen 22R, Orto alresa; Daganzo, Madrid, Spain) for 15 min. The supernatant was decanted, 500 µl of acetonitrile were added, and the agitation/centrifugation steps were repeated. Combined supernatants were evaporated to dryness at room temperature using a speed vacuum system (Genevac™ MiVac Duo, Fisher Scientific, Madrid, Spain) and stored at −80°C until analysis. The extracts were reconstituted by the addition of 50 µl of a 1:1 methanol:water mixture and vortex agitation for 2 min. Reconstituted extracts were centrifuged (10,000 × *g*, 4°C) for 15 min, and 10 µl of the supernatant were injected into the chromatographic system for the analysis.

3 | RESULTS

3.1 | Optimization of the chromatographic-mass spectrometry (UPLC-ESI-QTOF-MS) procedure

For the quantitation of aqueous standards of flumequine (FMQ), diclofenac and 3-hydroxy, 4-hydroxy and 5-hydroxydiclofenac, internal standard calibration using $^{13}C_3$-flumequine and d_4-diclofenac were used. As the mobile phase, mixtures of water:methanol and water:acetonitrile were tested. Formic acid, acetic acid and ammonium formate were checked as additives in the mobile phase. Two chromatographic columns were tested: Acquity® UPLC BEH C18 (1.7-µm particle size, 500 × 2.1 mm) and Cortecs® UPLC C18 (1.6-µm particle size, 500 × 2.1 mm). Additionally, ESI ionization mode was evaluated in both positive and negative modes. Optimization of these experimental parameters led to the conditions shown earlier in the experimental section. Figure 1 shows a representative ion chromatogram for a 10 ng·ml^{-1} aqueous standard solution containing all selected compounds and internal standards.

3.2 | Optimization of plasma samples vortex assisted extraction method

Blank mouse plasma samples were spiked (100 ng·ml^{-1}) with selected compounds and the internal standards to optimize their extraction procedure. A preliminary study tested acetonitrile, methanol and their mixtures with 1:1(v/v) and 1:3 (v/v) water as extraction solvents. The mixtures provided lower recovery than the pure solvent, and the highest recovery was obtained using acetonitrile. Then, this solvent was selected for subsequent optimization procedures.

Different recoveries were obtained using variable volumes of acetonitrile: 30%–70% for 500 µl, 35%–85% for 1000 µl and 45–99% using 2× 500 µl, so this last condition was selected as optimal. No significant differences were observed in recoveries using extraction (vortex agitation) times between 3 and 5 min. Thus, an extraction time of 3 min was selected as optimal.

On the other hand, three different volumes (50, 100 and 150 µl) were evaluated to reconstitute the extracts after drying of the combined supernatants using a speed vacuum system. No significant difference was observed in recoveries in the results obtained, and 50 µl was selected as the optimal reconstitution volume in order to achieve better sensitivity for the analytical procedure. Finally, methanol, acetonitrile and 1:1 mixtures with water as reconstituting solvent was tested providing a 1:1 mixture methanol:water with better sensitivity and peak shape.

Table S1 shows all data from the optimization procedure.

3.3 | Validation of the proposed analytical procedure

The method was validated based on EURACHEM (European Analytical Chemistry) guide (Magnusson & Örnemark, 2014) considering the following quality parameters: linearity, sensitivity (instrumental and method limits of detection and quantitation), matrix effect (suppression or enhancement of ionization and overall matrix effect), precision and recovery (trueness). Blank plasma samples from laboratory mice were used as a matrix for method validation purposes.

A comparison of external calibration and matrix-matched calibration curves was conducted by spiking blank plasma extracts at 10 concentration levels (ranging from 0.5 to 500 ng·ml^{-1}) of selected analytes. In both cases, using $^{13}C_3$-flumequine and d4-diclofenac as internal standards, no matrix effect was detected.

Thus, external calibrations using internal standards were used to validate the proposed procedure. The corresponding linearity percentage and linear range values obtained for the chromatographic procedure are shown in Table 3.

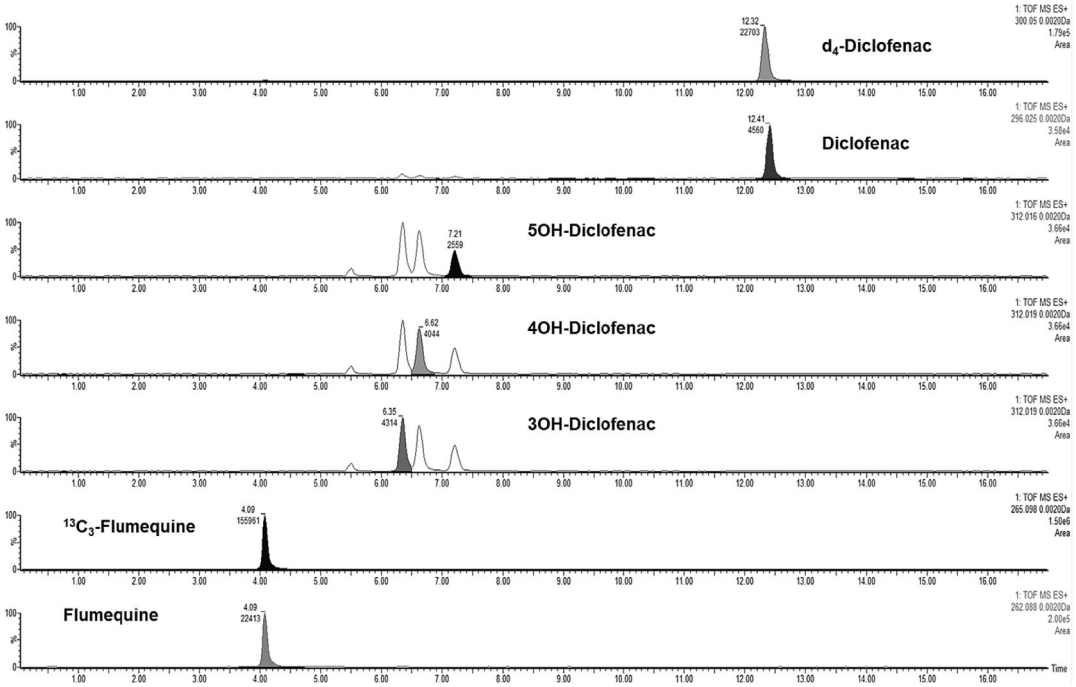

FIGURE 1 Extracted ion chromatograms (XIC) for a 10 ng·ml^{-1} aqueous standard solution containing the selected compounds and the internal standards.

TABLE 3 Linear ranges and instrumental and method limits of detection and quantitation for the selected analytes.

Compound	Linearity[a] (%)	Linear Range[a] (ng·ml^{-1})	ILOD[b] (ng·ml^{-1})	ILOQ[b] (ng·ml^{-1})	MLOD[b] (ng·ml^{-1})	MLOQ[b] (ng·ml^{-1})
FMQ	99	0.76 (0.9)-500 (2.3)	0.24	0.80	0.23	0.76
DCF	99	3.0 (3.2)-500 (6.1)	1.1	3.7	0.9	3.0
3OH-DCF	98	3.7(7.3)-500 (4.5)	1.2	4.0	1.1	3.7
4OH-DCF	98	3.4 (6.0)-500 (6.8)	1.2	4.1	1.1	3.4
5OH-DCF	97	4.1(8.2)-500 (5.3)	1.3	5.2	1.2	4.1

[a]Concentration (%RSD). Ten concentration levels; n = 3.
[b]n = 6.

TABLE 4 Evaluation of the precision (% RSD) for the proposed procedure at two concentration levels. (internal standard concentrations 100 ng·ml^{-1}, n = 6).

	Repeatability		Intermediate precision	
	50 ng·ml^{-1}	500 ng·ml^{-1}	50 ng·ml^{-1}	500 ng·ml^{-1}
Flumequine	1.21	3.22	2.53	4.62
Diclofenac	4.53	8.51	5.23	6.38
3OH-diclofenac	7.25	4.65	14.3	17.1
4OH-diclofenac	11.3	6.58	7.25	10.2
5OH-diclofenac	5.26	2.63	16.3	6.50

Instrumental detection and quantitation limits were calculated as the minimum concentrations of analyte peaks with signal-to-noise ratios of 3 and 10, respectively. Limits of detection and quantitation of the methods were carried out on blank plasma samples (n = 6).

To evaluate the precision of the method, repeatability (within a single day) and intermediate precision (within three days) were studied at two concentration levels (50 and 500 ng·ml^{-1}) and n = 6. A precision study was performed using actual concentration predictions from the validation standards selected for the analytical assay and calculating the relative standard deviation (RSD) (Table 4). In all cases, the values obtained in all cases were less than 15% RSD, which is usually considered an acceptance criterion for bioanalytical methods (Naidis & Turpeinen, 2009).

Accuracy was evaluated by recovery assays on spiked samples submitted to the extraction procedure and measured using external calibration using internal standards. Recoveries were calculated at three concentration levels for the analysed compounds. Spiked blank plasma samples at 10, 100 and 500 ng·ml^{-1} final concentrations (in triplicate) were submitted to the extraction procedure, and the results obtained were evaluated (Table 5).

4 | DISCUSSION

4.1 | Bioavailability of flumequine

It has been reported (Plakas et al., 2000) that after oral dosing, peak plasma concentrations of flumequine are reached within14 h. Absorption and elimination half-lives are 4.9 and 22 h, respectively. After ingestion, more than 80% of FMQ remains unmetabolized, with the major metabolite being 7-hydroxyflumequine. However, in several studies, the parent drug was usually the only residue detected (Haagsma et al., 1993; Samuelsen & Ervik, 1997). FMQ is excreted in urine and faeces as glucuronide conjugates of the parent drug and 7-hydroxyflumequine. In our study, FMQ plasma concentrations were very high, probably due to the high dose administered during the experiment.

Table 6 shows the FMQ concentrations found in the plasma samples of the different groups corresponding to the exposure tests. It is remarkable that exposure to selected metals resulted in a higher dispersion in flumequine plasma levels for exposed animals (Groups 5 and 6). From a qualitative point of view, group means for individuals with Abxs pretreatment (B groups) appear slightly higher except for the 3B group (without metals) that showed the lowest FMQ levels for all tests. Furthermore, selenium generally does not have a major effect on global plasma levels. Figure S1 shows the scatter plot of the data for individuals in each exposure group.

Statistical significance was analysed based on the normal distribution of datasets using either one-way ANOVA or nonparametric analysis. Table 7 shows the corresponding results obtained. As can be seen, significant differences in FQM plasma levels were found only in two experiments (shaded cells) involving mice with prior Abxs pretreatment (groups 3B, 4B and 5B). The presence of metals significantly affected FQM plasma levels when the gut microbiota was depleted with a marked decrease when metals were not present in the diet. The absence of dietary supplemental selenium affects animals submitted to Abx pretreatment and fed diets lacking the metal. This suggests that the ingestion of selenium supplemented feed favours FMQ absorption for animals submitted to Abxs pretreatment, but an antagonistic effect was observed in the presence of the metal cocktail.

4.2 | Bioavailability of diclofenac and their hydroxy-metabolites

Diclofenac is eliminated primarily by metabolism (Davies & Anderson, 1997; Todd & Sorkin, 1988). The biotransformation of diclofenac takes place partly through glucuronidation of the intact molecule but mainly through single and multiple hydroxylation and/or methoxylation, giving rise to several phenolic metabolites (3-hydroxy-, 4-hydroxy-, 5-hydroxy-, 4,5-dihydroxy- and 3-hydroxy-4-methoxydiclofenac), which are largely converted to glucuronic conjugates. Two of these metabolites (3-hydroxy and 4-hydroxy) are biologically active, but to a much lesser degree than the parent drug (Wiesenberg-Boettcher et al., 1991). The two main DCF metabolites are 4-hydroxydiclofenac (more than 50%), which is produced by the CYP2C9 enzyme, and 5-hydroxydiclofenac, which seems to be the product of some enzymes: CYP2C8, CYP2C18, CYP2C19, CYP2B6, among others (Dorado et al., 2008); 60%–70% of administered diclofenac is eliminated in the urine, and 30% is excreted in the faeces. The terminal half-life of diclofenac is approximately 2 h, although the apparent half-life, including all metabolites, is 25.8–33 h (Todd & Sorkin, 1988).

TABLE 5 Recovery percentages obtained at three concentration levels for the selected analytes from spiked blank plasma samples (n = 3).

	10 ng·ml^{-1}	100 ng·ml^{-1}	500 ng·ml^{-1}
FMQ	98 ± 1	97 ± 3	95 ± 4
DCF	97 ± 3	99 ± 4	96 ± 2
3OH-DCF	99 ± 2	98 ± 1	97 ± 3
4OH-DCF	99 ± 3	98 ± 2	98 ± 4
5OH-DCF	88 ± 1	97 ± 3	97 ± 5

TABLE 6 Bioavailability of flumequine in the exposed mice: average plasma concentrations from five individuals and standard deviations (data from measurements in triplicate).

Group	Exposure conditions	Flumequine (mg L^{-1})	SD (mg L^{-1})
3A	Pharmaceuticals	30	0.5
3B	Abx + pharmaceuticals	25	0.6
4A	Se + pharmaceuticals	37	0.5
4B	Abx + Se + pharmaceuticals	47	0.2
5A	Pharmaceuticals + metals	32	13
5B	Abx + pharmaceuticals + metals	42	11
6A	Se + pharmaceuticals + metals	40	16
6B	Abx + Se + pharmaceuticals + metals	48	8.5

TABLE 7 Influence of the different exposure conditions on the flumequine plasma contents (in parentheses experimental conditions for the analysed groups). Statistical analysis of significance accordingly to normality distribution of sets of data: one-way ANOVA or nonparametric analysis (shaded cells indicate groups with statistically significant differences) (median and mean values in mg L^{-1}) (for details, see text).

Metals effects												
Metals effect			**Metals effect (selenium)**			**Metals effect (depleted microbiota)**			**Metals effect (selenium & depleted microbiota)**			
	Median	***P* value**		**Median**	***P* value**		**Mean**	***P* value**		**Median**	***P* value**	
5A	37	1.000	6A	39	1.000	5B	42	0.024	6B	52	0.280	
3A	36		4A	42		3B	25		4B	44		
Effects when metals were not present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Mean	***P* value**		**Median**	***P* value**		**Median**	***P* value**		**Median**	***P* value**	
3A	30	0.419	3A	37	0.302	4A	42	1.000	3B	20	0.004	
4A	37		3B	20		4B	44		4B	44		
Effects when metals were present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Median	***P* value**		**Mean**	***P* value**		**Median**	***P* value**		**Mean**	***P* value**	
5A	37	1.000	5A	33	0.230	6A	39	0.248	5B	42	0.377	
6A	40		5B	42		6B	52		6B	48		

TABLE 8 Bioavailability of diclofenac, 4-hydroxdiclofenac and 5-hydroxydiclofenac for the exposed mice: average plasma concentrations from five individuals and standard deviations (data from measurements in triplicate) (all data in $\mu g \cdot L^{-1}$).

		DCF		4-OH DCF		5-OH DCF	
Group	**Exposure conditions**	**Conc.**	**SD**	**Conc.**	**SD**	**Conc.**	**SD**
3A	Pharmaceuticals	322	253	22	19	85	61
3B	Abx + pharmaceuticals	257	273	12	7.3	55	30
4A	Se + pharmaceuticals	646	429	42	38	154	88
4B	Abx + Se + pharmaceuticals	302	109	19	9.3	9.1	2.6
5A	Pharmaceuticals + metals	229	158	6.3	1.8	16	9.0
5B	Abx + pharmaceuticals + metals	343	191	14	3.5	31	6.8
6A	Se + pharmaceuticals + metals	836	937	27	6.1	57	28
6B	Abx + Se + pharmaceuticals + metals	647	314	38	21	72	36

It is remarkable that no 3-hydroxydiclofenac metabolite was detected in any of the plasma samples in our study, which is in accordance with previously reported studies in mice, including an extensive metabolite screening using ^{14}C-diclofenac (Sarda et al., 2012). Table 8 shows the average concentrations of diclofenac and OH- metabolites in plasma samples corresponding to the exposure test of the different groups. In general, a high intersubject variability in compound levels was obtained for all exposure conditions, so that only general trends could be extracted. Figures S2–S4 show the scatter plots of the data for individuals in each exposure group.

Trends showed that selenium supplemented mice had the highest plasma DCF concentrations regardless of dietary metal presence (groups 4A, 6A and 6B), but if the gut microbiota was depleted (4B), the plasma levels were similar to those that were not supplemented with selenium. Animals in these exposure groups also showed the highest plasma levels of 4-hydroxydiclofenac, indicating that the presence of dietary selenium increases the levels of this metabolite. Moreover, when the gut microbiota was depleted (4B), 4-hydroxydiclofenac levels were in the same order as in the animals exposed only to the PACs cocktail (3A). This is in accordance with the early report by Saitta et al. (2014), who demonstrated that the gut microbiota increases the metabolism (deglucuronation) and delays the excretion of diclofenac, and can lead to an increase in its toxicity (enterohepatic circulation).

According to the literature, the major metabolite in humans is 4-OH DCF (Dorado et al., 2003), but in our experience, the highest concentrations was found for 5-OH diclofenac.

Therefore, its plasma levels significantly decrease in mice exposed to metals (groups 5A and 5B) but dietary selenium minimizes these decreases (groups 6A and 6B). Abxs pretreatment (B groups) does not affect this metabolic pathway (B groups) except for the case of dietary selenium (group 4B).

TABLE 9 Influence of the different exposure conditions on the diclofenac plasma contents (in parentheses experimental conditions for the analysed groups). Statistical analysis of significance accordingly to normality distribution of sets of data: one factor ANOVA or nonparametric analysis (shaded cells indicate groups with statistically significant differences) (median and mean values in $\mu g \cdot L^{-1}$) (for details, see text).

Metals effects												
Metals effect			**Metals effect (selenium)**			**Metals effect (depleted microbiota)**			**Metals effect (selenium & depleted microbiota)**			
	Median	***P* value**		**Median**	***P* value**		**Mean**	***P* value**		**Median**	***P* value**	
5A	342	0.248	6A	239	0.158	5B	363	0.398	6B	747	0.280	
3A	370		4A	646		3B	257		4B	296		
Effects when metals were not present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Mean	***P* value**		**Median**	***P* value**		**Median**	***P* value**		**Median**	***P* value**	
3A	322	0.101	3A	369	0.302	4A	646	0.004	3B	91	0.343	
4A	646		3B	91		4B	296		4B	296		
Effects when metals were present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Median	***P* value**		**Mean**	***P* value**		**Median**	***P* value**		**Mean**	***P* value**	
5A	342	0.248	5A	278	0.230	6A	239	0.248	5B	363	0.158	
6A	239		5B	363		6B	747		6B	647		

TABLE 10 Influence of the different exposure conditions on the 4-hydroxydiclofenac plasma contents (in parentheses experimental conditions for the analysed groups). Statistical analysis of significance accordingly to normality distribution of sets of data: one-way ANOVA or nonparametric analysis (shaded cells indicate groups with statistically significant differences) (median and mean values in $\mu g \cdot L^{-1}$) (for details, see text).

Metals effects												
Metals effect			**Metals effect (selenium)**			**Metals effect (depleted microbiota)**			**Metals effect (selenium & depleted microbiota)**			
	Median	***P* value**		**Median**	***P* value**		**Mean**	***P* value**		**Median**	***P* value**	
5A	5.3	0.248	6A	32	1.000	5B	14	0.210	6B	32	0.031	
3A	15		4A	32		3B	10		4B	16		
Effects when metals were not present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Mean	***P* value**		**Median**	***P* value**		**Median**	***P* value**		**Median**	***P* value**	
3A	22	0.349	3A	15	0.302	4A	32	0.058	3B	11	0.058	
4A	33		3B	11		4B	16		4B	16		
Effects when metals were present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Median	***P* value**		**Mean**	***P* value**		**Median**	***P* value**		**Mean**	***P* value**	
5A	5.3	0.248	5A	6.5	0.005	6A	32	1.000	5B	14	0.039	
6A	32		5B	14		6B	32		6B	34		

Statistical analysis of DCF plasma levels (Table 9) reveals that the metal cocktail does not significantly affect its bioavailability. However, significant differences are obtained in groups with depleted microbiota in the Se-supplemented diet group compared with the groups with depleted microbiota. It seems that microbiota depletion significantly affects DCF levels.

Statistical analysis of the influence of different exposure conditions on 4-hydroxydiclofenac plasma content (Table 10) revealed a metal effect in depleted microbiota and Se-supplemented groups. Greater differences were observed in the data for groups submitted to the metal-drug chemical cocktail, with significant differences in plasma levels produced by Abx pretreatment and selenium

TABLE 11 Influence of the different exposure conditions on the 5-hydroxydiclofenac plasma contents (in parentheses experimental conditions for the analysed groups). Statistical analysis of significance accordingly to normality distribution of sets of data: one-way ANOVA or nonparametric analysis (shaded cells indicate groups with statistically significant differences) (median and mean values in $\mu g \cdot L^{-1}$) (for details, see text).

Metals effects												
Metals effect			**Metals effect (selenium)**			**Metals effect (depleted microbiota)**			**Metals effect (selenium & depleted microbiota)**			
	Median	***P* value**		**Median**	***P* value**		**Mean**	***P* value**		**Median**	***P* value**	
5A	10	0.248	6A	28	0.002	5B	31	0.374	6B	85	0.001	
3A	107		4A	152		3B	44		4B	10		
Effects when metals were not present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Mean	***P* value**		**Median**	***P* value**		**Median**	***P* value**		**Median**	***P* value**	
3A	85	0.136	3A	107	0.302	4A	152	0.001	3B	42	0.004	
4A	148		3B	42		4B	10		4B	10		
Effects when metals were present												
Selenium effect			**Microbiota effect**			**Microbiota effect (selenium)**			**Selenium effect (depleted microbiota)**			
	Median	***P* value**		**Mean**	***P* value**		**Median**	***P* value**		**Mean**	***P* value**	
5A	10	0.248	5A	15	0.023	6A	28	0.248	5B	31	0.015	
6A	28		5B	31		6B	85		6B	82		

supplementation (6B-4B). Furthermore, an effect of dietary selenium on the metabolism of DCF was observed in the groups with depleted microbiota (5B-6B). The presence of a quite functional gut seems to be decisive on DCF metabolism to 4-OH DCF when animals were exposed to the metal cocktail (5A-5B).

Statistically, 5-OH-DCF showed highly significant differences between groups (Table 11). Metals had significant effects when mouse diet was supplemented with selenium (gut microbiota depleted or not) (6A-4A and 6B-4B). When the metal cocktails were not present in the exposure groups, the microbiota affected the exposure group supplemented with selenium (4A-4B), and selenium had an effect if mice were submitted to Abxs pretreatment (3B-4B). When groups exposed to metals were statistically analysed, the significance of the microbiota effect (5A-5B) and the selenium effect on depleted animals (5B-6B) was confirmed.

4.3 | Targeted identification of diclofenac and flumequine metabolites

The use of high-resolution mass spectrometry allows targeted detection of substances that may be present when analytical standards are not available. Using Chromalynx® software, the possible presence of metabolites described in the literature for the analysed drugs has been studied. Table 12 shows potential DCF and FMQ metabolites that could be found in plasma samples according to the reviewed literature (Fu et al., 2017; Parshikov & Sutherland, 2012; Sarda et al., 2014).

Computational identification was based on exact mass (error <5 ppm), isotopic pattern of chlorine (Cl, for DCF metabolites) and MS/MS fragments obtained in a second function comparable to those previously reported in the literature. Tables 13 and 14 show the metabolites identified using Chromalynx®. Logically, 4-hydroxydiclofenac and 5-hydroxydiclofenac were also identified but were quantified using analytical standards, and their corresponding plasma levels are discussed above.

With respect to the metabolism of flumequine, its main metabolites (7-hydroxyflumequine and 7-oxoflumequine) appeared in all groups of exposed mice. These metabolites were not detected in the plasma extracts of only one animal from group 6B, suggesting that flumequine metabolism remains unchanged under different exposure conditions. However, Chromalynx® allows semiquantitative assessment of metabolites based on peak area ratio, which, although it does not allow real quantification, allows comparison between metabolites found in different groups. Inherent variation in the organism's metabolism must be taken into account, so only the most evident trends will be highlighted. The peak areas of the detected flumequine metabolites were corrected and normalized to the $^{13}C_3$-flumequine internal standard. Similar considerations were applied to the discussion of diclofenac metabolites using d_4-diclofenac as an internal standard.

Relative concentrations of 7-oxoflumequine (M48) were of the same order in all test animals, regardless of exposure conditions. In contrast, relative concentrations of hydroxyflumequine (M47) decreased appreciably when metals were added to the diet, a reduction being somewhat less pronounced if the animals' microbiota were depleted (5B and 6B). This clearly highlights the influence of metals on flumequine metabolism and the synergism of gut microbiota. The presence of selenium in the diet somewhat minimizes the plasma

TABLE 12 Targeted metabolites for diclofenac and flumequine.

	Compound	Elemental composition	m/z $[M + H]^+$	m/z $[M - H]^-$
M1	3-OH-DCF	C14H11Cl2N1O3	312.0194	310.0038
M2	4-OH-DCF	C14H11Cl2N1O3	312.0194	310.0038
M3	5-OH-DCF	C14H11Cl2N1O3	312.0194	310.0038
M4	(5,4)-OH ether glucuronide	C20H19O9N1Cl2	488.0515	486.0359
M5	(5,4)-OH glucuronide taurine	C22H24O11N2Cl2S	595.0556	593.0400
M6	4-OH glucose	C20H21O8N1Cl2	474.0722	472.0566
M7	(5,4)-OH-glucose acyl glucuronide	C26H29O14N1Cl2	650.1043	648.0887
M8	5-OH glucose taurine	C22H26O10N2Cl2S	581.0763	579.0607
M9	Indolinone 5-hydroxyglucuronide	C20H17O8N1Cl2	470.0409	468.0253
M10	(5,4)-OH acyl glucuronide	C20H19O9N1Cl2	488.0515	486.0359
M11	(5,4)-OH taurine	C16H16O5N2Cl2S	419.0235	417.0079
M12	Benzyl acyl glucuronide	C19H17O2N1Cl2	362.0715	360.0558
M13	1-beta-O-acyl glucuronide	C20H19O2N1Cl2	376.0871	374.0715
M14	2,3,4-Alpha/beta-O-acyl glucuronide	(cluster) C20H19O2N1Cl2	376.0871	374.0715
M15	Taurine-DCF	C16H16O4N2Cl2S	403.0286	401.0130
M16	Dihydroxy-DCF	C14H11O4N1Cl2	328.0143	325.9987
M17	DCF-hydroxy glucuronide	C20H19Cl2N1O9	488.0515	486.0359
M18	Hydroxy-methoxy-DCF	C15H13Cl2N1O4	342.0294	340.0143
M19	Hydroxy mercapturate	C19H18Cl2N2O6S	473.0335	471.0184
M20	Hydroxyribose	C19H19Cl2N1O7	444.0617	442.046
M21	Hydroxy deschloro cysteine	C17H17Cl1N2O5S	397.0625	395.0468
M22	Deschloro cysteine	C17H17Cl1N2O4S	381.0676	379.0519
M23	Hydroxyglucuronide indoline (Na^+)	C20H16Cl2N1O8Na	492.0229	490.0072
M24	Hydroxycysteine	C17H16Cl2N2O5S	431.0235	429.0079
M25	Hydroxyglucuronide indoline (Na^+)	C20H16Cl2N1O8Na	492.0229	490.0072
M26	Hydroxycysteine	C17H16Cl2N2O5S	431.0235	429.0079
M27	Hydroxyglucuronide (Na^+)	C20H18Cl2N1O9Na	510.0335	508.0178
M28	Acyl glucuronide	C20H19Cl2N1O8	472.0566	470.0409
M29	Transacylated hydroxyglucuronide (cluster)	C20H19Cl2NO9	488.0515	486.0359
M30	Acyl glucuronide (Na^+)	C20H18Cl2N1O8Na	494.0385	492.0229
M31	Transacylated glucuronide (cluster)	C20H19Cl2N1O8	472.0566	470.0409
M32	DCF-leucine	C20H22N2O3Cl2	409.1086	407.0929
M33	OH-DCF lactam dehydrated	C14H9NO2Cl2	294.0089	291.9932
M34	DCF-lactam sulfate conjugation	C14H9NO5Cl2S	373.9350	371.9194
M35	DCF-glycine	C16H14N2O3Cl2	353.0460	351.0303
M36	DCF-gycerol	C17H17NO4Cl2	370.0613	368.0456
M37	DCF-glutamic acid	C19H18N2O5Cl2	425.0671	423.0515
M38	DCF-ethanolamine	C16H16N2O2Cl2	339.0667	337.0511
M39	Unknown	C19H21NO6Cl2	430.0824	428.0668
M40	OH-DCF-ethanolamine	C16H16N2O3Cl2	355.0616	353.046
M41	OH-DCF-dehydrogenated	C14H9Cl2NO3	310.0032	307.9881
M42	DiOH-DCF-methyl-lactam	C15H11Cl2NO	324.0192	322.0038
M43	Methyl ester	C15H13Cl2NO2	310.0396	308.0245
M44	Hydroxy-methyl-DCF	C15H13Cl2NO3	326.0348	324.0194
M45	Sulfate conjugation-DCF	C14H11Cl2NO6S	391.9762	389.9611
M46	Sulfate conjugation, methylation-DCF	C15H13Cl2NOS	326.0173	403.9768

(Continues)

TABLE 12 (Continued)

	Compound	Elemental composition	m/z $[M + H]^+$	m/z $[M - H]^-$
M47	7-Hydroxyflumequine	C14H12FNO4	278.0829	--
M48	7-Oxoflumequine	C14H10F1N1O4	276.0672	--

TABLE 13 Targeted metabolites for diclofenac and flumequine found in the mice individuals from the exposure tests without metals cocktail (positive ratio: number of individuals in which it has been detected).

Exposure group		Found metabolite	Retention time	Experimental *m/z* $[M + H]^+$	Theoretical *m/z* $[M + H]^+$	Positive ratio
3A	M2	4-OH-DCF	6.62	312.0178	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0178	312.0194	5/5
	M15	Taurine-DCF	6.33	403.0300	403.0286	3/5
	M28	Acyl glucuronide	6.60	472.0560	472.0569	3/5
	M33	OH-DCF lactam dehydrated	7.23	294.0103	294.0089	1/5
	M47	7-Hydroxyflumequine	3.55	278.0825	278.0829	5/5
	M48	7-Oxoflumequine	3.93	276.0668	276.0672	5/5
4A	M2	4-OH-DCF	6.62	312.0179	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0181	312.0194	5/5
	M11	(5,4)-OH taurine	4.25	419.0238	419.0235	4/5
	M15	Taurine-DCF	6.35	403.0290	403.0286	4/5
	M28	Acyl glucuronide	6.60	472.0564	472.0569	4/5
	M47	7-Hydroxyflumequine	3.68	278.0823	278.0829	5/5
	M48	7-Oxoflumequine	4.05	276.0670	276.0672	5/5
3B	M2	4-OH-DCF	6.62	312.0178	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0177	312.0194	5/5
	M8	5-OH glucose taurine	4.06	581.0890	581.0763	2/5
	M11	(5,4)-OH taurine	4.22	419.0238	419.0235	2/5
	M15	Taurine-DCF	6.26	403.0298	403.0286	2/5
	M28	Acyl glucuronide	6.60	472.0576	472.0569	2/5
	M36	DCF-Gycerol	13.73	370.0618	370.0613	1/5
	M40	OH-DCF-ethanolamine	12.60	355.0610	355.0616	3/5
	M47	7-Hydroxyflumequine	3.62	278.0821	278.0829	5/5
	M48	7-Oxoflumequine	3.98	276.0680	276.0672	5/5
4B	M2	4-OH-DCF	6.62	312.0177	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0180	312.0194	5/5
	M7	(5,4)-OH-glucose acyl glucuronide	14.34	650.1065	650.1047	4/5
	M8	5-OH glucose taurine	4.10	581.0790	581.0763	3/5
	M11	(5,4)-OH taurine	4.24	419.0220	419.0235	2/5
	M15	Taurine-DCF	6.30	403.0290	403.0286	1/5
	M28	Acyl glucuronide	6.58	472.0566	472.0569	3/5
	M47	7-Hydroxyflumequine	3.60	278.0822	278.0829	5/5
	M48	7-Oxoflumequine	3.90	276.0677	276.0672	5/5

concentrations of hydroxyflumequine in animals tested with the metal intake.

Qualitative changes in diclofenac metabolism were more evident between different exposure groups. Thus, the groups with the metals cocktail (groups 5 and 6) showed a lower number of diclofenac metabolites than the corresponding ones without metals, and in addition, distribution of metabolites was different if the gut microbiota was depleted or not. Indeed, for animals in groups 5A and 6A, one sample

TABLE 14 Targeted metabolites for diclofenac and flumequine found in the mice individuals from the exposure tests with metals cocktail (positive ratio: number of individuals in which it has been detected).

Exposure group		Found metabolite	Retention time	Experimental *m/z* $[M + H]^+$	Theoretical *m/z* $[M + H]^+$	Positive ratio
5A	M2	4-OH-DCF	6.62	312.0179	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0181	312.0194	5/5
	M27	Hydroxy glucuronide (Na^+)	13.65	510.0333	510.0335	1/5
	M47	7-Hydroxyflumequine	3.62	278.0821	278.0829	5/5
	M48	7-Oxoflumequine	3.93	276.0680	276.0672	5/5
6A	M2	4-OH-DCF	6.62	312.0178	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0178	312.0194	5/5
	M47	7-Hydroxyflumequine	3.58	278.0821	278.0829	5/5
	M48	7-Oxoflumequine	3.89	276.0680	276.0672	5/5
5B	M2	4-OH-DCF	6.62	312.0177	312.0194	5/5
	M3	5-OH-DCF	7.21	312.0180	312.0194	5/5
	M28	Acyl glucuronide	6.60	472.0566	472.0569	3/5
	M47	7-Hydroxyflumequine	3.65	278.0821	278.0829	5/5
	M48	7-Oxoflumequine	3.95	276.0680	276.0672	5/5
6B	M2	4-OH-DCF	6.62	312.0181	312.0194	4/5
	M3	5-OH-DCF	7.21	312.0178	312.0194	4/5
	M7	(5,4)-OH-glucose acyl glucuronide	14.5	650.1050	650.1047	5/5
	M15	Taurine-DCF	6.60	403.0296	403.0286	1/5
	M28	Acyl glucuronide	6.60	472.0566	472.0569	5/5
	M47	7-Hydroxyflumequine	3.67	278.0824	278.0829	5/5
	M48	7-Oxoflumequine	4.12	276.0675	276.0672	4/5

from group 5A showed only the DCF metabolite (besides the 4-OH and 5-OH ones) hydroxyl glucuronide (Na^+). A greater number of DCF metabolites were found in mice from 6B, for these exposure groups with metal cocktail (metal, selenium and microbiota depleted). As mentioned in Section 1, previous studies have already proven that DCF and certain metals have greater anti-inflammatory activity than DCF (Kovala-Demertzi, 2000), and a DCF-complex with mercury has also been described as an antibacterial agent (Refat et al., 2014). Therefore, the influence of the modification in its metabolic pathways due to the presence of metals should be considered. The presence of hydroxyl groups on the structure can enhance the interaction with metals through π-π interactions. Thus, hydroxyl DCF derivatives can easily form metal complexes (Yamada et al., 1990) that can lead to down-regulation of other metabolic pathways (Lonappan et al., 2016). Some researchers (González-Sarrías et al., 2013; Subramanian et al., 2018) have demonstrated that the gut microbiota influences drug transport by regulating substrate competition or the expression of transporters.

Taurine-DCF and DCF-acyl glucuronide were found in all groups 3 and 4 (without metals) samples. Among groups exposed to metal cocktails, taurine-DCF was detected in only one animal of group 6B and a sodium adduct of acyl glucuronide was detected in samples from animals of groups 4B and 6B, in mice with gut microbiota depletion. The taurine conjugation pathway, which together with hydroxylation is the most important metabolic pathway for diclofenac in mice (Sarda et al., 2012), disappears in the presence of dietary metals. As mentioned, the combination of depressed microbiota and a selenium-supplemented diet allowed this pathway in only one of five mice in group (6B).

The metabolic pathway of DCF-acyl glucuronide was observed to a greater or lesser extent in specimens from all test groups except for the presence of metals in the diet (groups 5A and 6A). This shows that, in this case, the presence of selenium in the diet seems to play a regulatory role in the presence of metals, because this pathway is practically intact in all specimens exposed to these conditions (group 5B and 6B).

The metabolite (5,4)-OH taurine was detected in all groups without metals except 3A. Thus, it appears that this metabolic pathway, which is not particularly favoured, becomes important after Abxs pretreatment or dietary selenium supplementation but results in all cases by the metal cocktail.

With respect to (5,4)-OH-glucose acyl glucuronide, it is an arene-oxide conjugate that undergoes fragmentation to hydroxyl-acyl glucuronides, hydroxyl-diclofenac and others (Sarda et al., 2012). This metabolite was detected only when the gut microbiota was depleted and the diet of mice was supplemented with selenium, independently

if metal cocktails were added to the diet or not (groups 4B and 6B). The presence of selenium appears to inhibit or minimize the metabolic fragmentation of this compound.

From a semiquantitative point of view, the corresponding corrected peak areas for DCF metabolites, in general, do not show any remarkable consequences due to more or less dispersed values and, moreover, at the same order of magnitude. It is only noteworthy that in the case where the mice were not subjected to the metal cocktail administration, the relative concentration of acyl glucuronide (M28) decreased about approximately 10-fold after Abx pretreatment (group 3B). The presence of selenium in the diet (group 4B) normalized the contents of this metabolite, despite Abx pretreatment, making them of the same order of magnitude as those found in animals with an intact gut microbiota.

It is clear that according to the data obtained, the ingestion of metal cocktails and/or the presence of selenium in the diet of mice alters the metabolism pathways qualitatively for diclofenac and only quantitatively for flumequine.

Throughout the paper, we ascribe the observed effects of Abx pretreatment of mice to dysbiosis of the gut microbiota. Nonetheless, the intestine is extensively linked to CYP metabolism and is accountable for extrinsic drug metabolism (Bezirtzoglou, 2012). However, because antibiotics can significantly alter the expression of CYPs within the intestinal epithelium, it cannot be ruled out that changes in epithelial metabolism, which may directly alter the first-pass metabolism of orally administered drugs, also contribute to the antibiotic effects of Abxs pretreatment.

When extrapolating these results obtained in mice to humans, it is important to consider the differences between the two species. One such difference is the composition of the intestinal microbiota, to which we attribute an important role in this work. Mice and humans share many anatomical, histological and physiological features of the gut but differ substantially in size, metabolism and diet. As a result, the composition of the gut microbiota differs significantly, both in the qualitative representation of taxa and the quantity of individual species (Hugenholtz & de Vos, 2018). Furthermore, only a fraction of the bacterial genes is common to both mice and humans. However, mice models are frequently utilized for translation to humans, and a straightforward explanation may be that there is no better option.

5 | CONCLUSIONS

Controlled experiments have been conducted on *M. musculus* with a "chemical cocktail" containing metals (arsenic, cadmium and mercury), pharmaceuticals (diclofenac and flumequine) as well as dietary selenium supplementation and/or the Abxs pretreatment of mammals. These experiments revealed the effect of these factors on the bioavailability and/or metabolism of the tested PACs.

The analytical method proposed and validated enabled the measurement of plasma levels of flumequine and diclofenac as well as 3-, 4- and 5-hydroxydiclofenac metabolites. Plasma levels and hence absorption of pharmaceuticals were clearly affected by the presence of metals. The significance of the obtained results was determined by one-way ANOVA or nonparametric statistical tests according to the data distribution (normal or not, respectively). Significant differences in FQM plasma levels were found only in two experiments involving previous Abx pretreatment of mice. The presence of metals significantly affected FQM plasma levels when the gut microbiota was depleted, with a marked reduction when metals were not present in the diet. The absence of dietary selenium supplementation affects the hydroxyflumequine levels in animals subjected to antibiotic (Abx) pretreatment. The addition of metals to the diet significantly reduces hydroxyflumequine levels, and selenium tends to minimize this reduction.

Regarding diclofenac and its main hydroxyl metabolites, a high variability was found between animals in the levels for all exposure conditions, so only general trends can be extracted. Mice fed with selenium supplementation presented the highest concentrations of DCF in plasma independent of the presence of metals in the diet, but if the gut microbiota was depleted, plasma levels were similar to those supplemented with selenium. Statistical analysis of plasma levels using ANOVA or nonparametric tests showed a statistically significant impact of Abx pretreatment on DCF levels only when participants were not exposed to the metal cocktail and lacked selenium supplementation.

The presence of selenium supplementation increased 4-hydroxydiclofenac plasma levels, but when the gut microbiota was depleted, the levels approached values found in animals exposed to PACs alone.

Statistically, 5-OH-DCF showed significant differences between groups. When the diet of mice was supplemented with selenium (independently from the gut microbiota), the metal had significant effects. When metal cocktails were not present, 5-OH diclofenac showed the highest plasma levels except in the gut microbiota depletion groups and selenium supplementation. When mice from the groups exposed to the metal cocktail were statistically analysed, the significance of the microbiota effect and the selenium effect on depleted animals were confirmed.

Finally, the computational identification of selected (48) target metabolites from the tested PACs showed that the presence of metal cocktails and/or selenium in the diet of mice as well as Abx pretreatment could be decisive on their metabolism. The number of metabolites identified was lower when animals were exposed to the metal-drug chemical cocktail and some metabolic pathways were markedly modified.

AUTHOR CONTRIBUTIONS

Noemí Aranda-Merino: Data curation (equal); formal analysis (equal); investigation (equal); validation (equal). **Antonio Marín-Garrido:** Data curation (equal); formal analysis (equal); software (equal); validation (equal). **Cristina Román-Hidalgo:** Formal analysis (equal); investigation (equal). **María Ramos-Payán:** Funding acquisition (equal); investigation (equal); project administration (equal); supervision (equal); writing—review and editing (equal). **Nieves Abril:** Funding acquisition (equal); methodology (equal); project administration (equal); writing—review

and editing (equal). **Rut Fernández-Torres:** Conceptualization (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Miguel Angel Bello-López:** Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

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. The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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