

A quantitative HPLC–MS/MS method for studying internal concentrations and toxicokinetics of 34 polar analytes in zebrafish (*Danio rerio*) embryos

Stephan Brox · Axel P. Ritter · Eberhard Küster ·
Thorsten Reemtsma

Received: 2 April 2014 / Revised: 16 May 2014 / Accepted: 26 May 2014 / Published online: 20 June 2014
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Abstract An analytical method using high-performance liquid chromatography–tandem mass spectrometry was developed to determine internal concentrations of 34 test compounds such as pharmaceuticals and pesticides in zebrafish embryos (ZFE), among them, cimetidine, 2,4-dichlorophenoxyacetic acid, metoprolol, atropine and phenytoin. For qualification and quantification, multiple reaction monitoring mode was used. The linear range extends from 0.075 ng/mL for thiachlorid and metazachlor and 7.5 ng/mL for coniine and clofibrate to 250 ng/mL for many of the test compounds. Matrix effects were strongest for nicotine, but never exceeded $\pm 20\%$ for any of the developmental stages of the ZFE. Method recoveries ranged from 90 to 110 % from an analysis of nine pooled ZFE. These findings together with the simple sample preparation mean this approach is suitable for the determination of internal concentrations from only nine individual ZFE in all life stages up to 96 h post-fertilization. Exemplarily, the time course of the internal concentrations of clofibric acid, metribuzin and benzocaine in ZFE was studied over 96 h, and three different patterns were distinguished, on the basis of the speed and extent of uptake and whether or not a steady state was reached. Decreasing internal concentrations may be due to metabolism in the ZFE.

Keywords Bioconcentration · Toxicodynamic · Absorption, distribution, metabolism and excretion · Larvae · Electrospray · Toxicity

Introduction

The zebrafish (*Danio rerio*, Hamilton–Buchanan, 1822) embryo (ZFE) is a well-established experimental model in different fields, such as embryology, pharmacology, toxicology and ecotoxicology, to study development and gene function [1–5]. In the above-mentioned areas of research, ZFE are also used to determine effect concentrations of chemicals in the contexts of chemical safety, drug discovery and environmental risk assessment [6–8]. Furthermore, the use of the ZFE is an alternative to acute toxicity tests with adult fish [9, 10]. In routine toxicity tests, the concentration of the test compound in the exposure solution resulting in lethal or other effects for 50 % of the ZFE (LC₅₀ or EC₅₀ values) is usually determined [6, 8, 11–13]. Differences in the LC₅₀ or EC₅₀ values of the test compounds may be partly due to different uptake, metabolism or elimination rates i.e. the toxicokinetics [14]. To study this, it is indispensable to determine the internal effect concentration [15].

Different chromatographic techniques have been used to determine internal concentrations in ZFE, such as gas chromatography for more hydrophobic compounds [16–18] and liquid chromatography for more polar compounds, e.g. for ibuprofen [19], an isoflavone [20], drugs [5] or other compounds such as chlorpromazine and testosterone and their metabolites [21]. Toxicokinetic studies using high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS) have also been performed with other test organisms, such as *Daphnia magna* [22]. However, most of the HPLC–mass spectrometry (MS) methods used in this context focus on single compounds.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-014-7929-y) contains supplementary material, which is available to authorized users.

S. Brox · A. P. Ritter · T. Reemtsma (✉)
Department of Analytical Chemistry,
Helmholtz Centre for Environmental Research—UFZ,
Permoserstr. 15, 04318 Leipzig, Germany
e-mail: thorsten.reemtsma@ufz.de

E. Küster
Department Bioanalytical Ecotoxicology,
Helmholtz Centre for Environmental Research—UFZ,
Permoserstr. 15, 04318 Leipzig, Germany

Toxicokinetic studies with ZFE would require repeated samplings of test organisms within the first 5 days of development, including very early life stages (e.g. 24 h post-fertilization (hpf)) to be able to thoroughly analyse the uptake kinetics [18, 23]. To minimize the analytical efforts (a) only a low number of individual ZFE should be required for one analysis and (b) the analytical procedure should ideally require minimal effort and time. Both the number of organisms used and the analytical effort could be significantly reduced if toxicokinetic tests were performed with mixtures of test compounds, assuming that their concentrations are low enough to avoid mutual interferences. Under these circumstances, the analytical procedure should allow analysis of a large number of analytes simultaneously.

Most research to date has been done with lipophilic substances. These substances include many anthropogenic environmental contaminants, such as polycyclic aromatic hydrocarbons [23, 24], pesticides [16] and polybrominated diphenyl ether [25]. Such compounds are thought to be taken up passively and quite efficiently, so comparatively high internal concentrations are reached and the analysis can be done with a very low number of individuals [23]. The uptake of less lipophilic and more polar substances such as many pharmaceuticals has been studied less. Their uptake might be lower, but their effects can nonetheless be specific and multiple and could affect fish in the environment [26]. Therefore, a method has been developed and validated which allows the simultaneous analysis of up to 34 test compounds of high to moderate polarity. Method development included the conditions of exposure and washing of the ZFE, extraction of the test compounds and their quantification by HPLC–MS/MS. The simple sample preparation and extraction procedure presented reduces the analytical effort. Furthermore, the method developed is suitable to determine the concentrations of the test compounds in the exposure solution. The feasibility of the final method is illustrated by applying it to study the uptake of three test compounds in ZFE. The method developed can support toxicology studies and can be used for toxicokinetic studies in ZFE and, likely, also in other test organisms.

Materials and methods

Chemicals, reagents and standards

All chemicals and solvents were purchased from Biosolve (Valkenswaard, The Netherlands), Sigma-Aldrich (Munich, Germany), ICN Biomedicals (Eschwege, Germany), USP (Basel, Switzerland) and Dr. Ehrenstorfer (Augsburg, Germany) at the highest available purity (more than 95 %). For further information, see the electronic supplementary material. Stock solutions of all 34 analytes were prepared in pure methanol for HPLC–MS/MS analysis and were stored at -20°C . The stock solutions were combined and diluted in

methanol to obtain a working solution of all analytes at a concentration of $1\ \mu\text{g}/\text{mL}$. Calibration standards were prepared from this working solution with methanol/water (50:50, v/v) in the range from 0.01 to 250 ng/mL.

The chemicals for ZFE exposure were dissolved in ISO standard dilution water [27] without the use of organic solvents. All substances were well below their maximum water solubility.

Instrumental conditions and data analysis

HPLC–MS/MS was performed using a 1260 Infinity HPLC system (Agilent Technologies, Böblingen, Germany) and a QTrap 5500 mass spectrometer (AB Sciex, Darmstadt, Germany) with a TurboIonSpray interface, all controlled by Analyst (version 1.5.2, AB Sciex). The HPLC system was equipped with a degasser, a pump with a binary high-pressure gradient mixer, a thermostated autosampler (temperature set to 4°C) and a column oven (set to 30°C). The injection volume was $5\ \mu\text{L}$. Separation was performed on a Atlantis T3 reversed-phase column ($2.1\ \text{mm}\times 50\ \text{mm}$, $3\ \mu\text{m}$; Waters, Eschborn, Germany) with a SecurityGuard C₁₈ guard column ($4\ \text{mm}\times 2.0\ \text{mm}$; Phenomenex, Aschaffenburg, Germany) using 5 mM ammonium acetate in water (solvent A) and pure methanol (solvent B) as eluents at a flow rate of $500\ \mu\text{L}/\text{min}$. The gradient was as follows: 0.0 min, 10 % solvent B; 0.5 min, 10 % solvent B; 3.5 min, 50 % solvent B; 5.0 min, 100 % solvent B; 10.0 min, 100 % solvent B; 10.5 min, 10 % solvent B; 14.0 min 10 % solvent B.

Multiple reaction monitoring was performed with dwell times of 10 ms for the mass transitions used for quantification and 2 ms for the mass transitions used for qualification. Three time periods were set (two using switching between positive mode and negative mode, and one using only positive mode) for the detection of 34 analytes.

The source parameters for both modes were as follows: gas 1 at 50 psi, gas 2 at 50 psi and curtain gas at 40 psi. The source temperature was 550°C , the ionization voltage was 5,500 V (positive mode) and $-4,500\ \text{V}$ (negative mode) and the entrance potential voltage was 10 and $-10\ \text{V}$, respectively. Compound-specific MS parameters (declustering potential, collision energy and collision cell exit potential) are given in Table S1. The electron multiplier voltage was set to 2,500 V for both polarities, and data analysis was performed using Analyst version 1.5.2. A $1/x$ weighted regression was used for determination of calibration curves. Before integration, the chromatograms were smoothed with a factor of 3 and the peak integration was checked visually.

ZFE exposure experiments

For ZFE exposure, ZFE were identified according to Kimmel et al. [28] using a light microscope. Only ZFE which reached

the four-cell stage were used. In this article, an organism up to 96 h post-fertilization is called a ZFE. All experiments were conducted according to EU and national animal welfare regulations but resembles standard conditions [29]. The maintenance of the adult zebrafish is described in the electronic supplementary material. ZFE were exposed in a 20-mL glass vial with a metal cover plate and a red rubber/TEF septum (Th. Geyer, Hamburg, Germany). The experiments were started approximately 4 h post-fertilization. Each replicate consisted of nine ZFE per vial and 18 mL exposure solution ("exposed ZFE"). The vials were placed in a climatic chamber (26 ± 1 °C) with horizontal agitation at 75 rpm (Edmund Buehler, Hechingen, Germany) and a light/dark cycle (14 h/10 h). Triplicate samples were analyzed after 24, 48, 72 and 96 h of exposure and processed separately.

For quality assurance, two different negative controls were run in parallel to the exposed ZFE: (a) ZFE were exposed to pure ISO water ("control ZFE") and (b) the stability of the test compounds was checked by parallel incubation without ZFE. Samples of the exposure medium were taken at the beginning (initial concentration) and at each sampling point during the experiment (stability control). All samples were stored at -18 °C until sample preparation.

ZFE used for the determination of matrix effects and method recovery were incubated in ISO water without the test compounds.

Determination of washing efficiency

For the investigation of the washing efficiency, ZFE were incubated as control ZFE (72 h in ISO water). The ZFE (72 h post-fertilization, hatched) were then exposed to a mixture of different chemicals for a maximum of 25–30 s. It was assumed that such a short exposure time would prevent significant uptake of the test compounds into the ZFE. The efficiency of washing was tested using five solutions with increasing methanol content ranging from 0 to 20 % (v/v). The efficiency was compared with that of non-washed samples.

Collection and washing procedure for the ZFE

At the end of the exposure experiments, only alive ZFE were used for further analysis. They were transferred onto a metal mesh (size 2 cm \times 2.8 cm, pore size 0.25 mm, stainless steel, manually folded) with a plastic Pasteur pipette (Th. Geyer, Hamburg, Germany). ZFE which had not hatched at the time of sampling were manually dechorionated in their exposure medium using forceps before they were transferred onto the metal mesh. To reduce the number of samples that have to be analyzed, ZFE exposed to different chemicals were combined in a single mesh.

The ZFE on the mesh were immersed for 15 s in 50 mL stirred bidistilled water in a beaker. Afterwards, the mesh with the ZFE was dried using tissue paper, transferred into an Eppendorf tube (1.5 mL) and shock-frozen with liquid nitrogen. Unexposed ZFE which were used for determination of recovery or matrix effects were not washed.

Extraction of the analytes

Frozen ZFE were thawed at room temperature. Then, 450 μ L methanol and 50 μ L methanolic internal standard solution (ifosfamide) were added. The concentration of the internal standard solution varied depending on the dilution factor of the final methanolic extract; its target concentration in the prepared extract was 20–50 ng/mL. The tube containing the mesh and the ZFE was vortexed, and the analytes were extracted under sonication for 15 min. Afterwards, the mesh was removed and the precipitate was spun down by centrifugation at 14,000g (room temperature) for 10 min.

For control ZFE, 100 μ L of the supernatant was directly transferred into a 2-mL HPLC vial (Th. Geyer, Hamburg, Germany) and diluted with 100 μ L pure water. Extracts of exposed ZFE were diluted to the calibration range of the analyte. The final solution for HPLC analysis should contain water and methanol in a ratio of 50:50 (v/v).

Sample preparation for evaluation of matrix effects

For the study of matrix effects, 108 ZFE (equivalent to three replicates consisting of nine ZFE for four standard solutions in different concentrations) per life stage were collected in a metal mesh.

Overall, five different life stages between 4 and 96 h post-fertilization were investigated. Stages at 24 and 48 h post-fertilization were analysed with chorion and after manual dechorionation using forceps. The evaluation with an increased number of ZFE was performed using 216 and 432 ZFE at 48 and 96 h post-fertilization per replicate ($n=3$), respectively. Samples were extracted as already described using 6 mL pure methanol. For every sample, 500 μ L of the supernatant was used. Therefore, the aliquot was diluted with 500 μ L of an aqueous solution containing all analytes at specified concentrations. The resulting concentrations for the evaluation of matrix effects were 0, 5, 25 and 50 ng/mL (0.55–5.55 ng per organism) to cover the calibration range. Data for the 5 ng/mL level for coniine and clofibrate and for 50 ng/mL for metazachlor and diazepam were excluded because they did not fall into the respective linear range.

Sample preparation for determination of method recovery

For the recovery experiment, nine ZFEs per replicate ($n=3$) and life stage were collected in a metal mesh. The same life stages

were used as for the evaluation of matrix effects. Additional experiments were performed using 18 and 36 ZFEs at 48 and 96 h post-fertilization per replicate ($n=3$), respectively. For sample extraction, 500 μL of a methanolic solution containing all analytes (performed at three different concentrations) was used. These samples were prepared and extracted as described earlier. A 100- μL aliquot of the final extract was diluted with 100 μL water and used for quantitative analysis. The resulting concentrations for the method recovery were 5, 25 and 50 ng/mL (0.55–5.55 ng per organism). Again, data for the 5 ng/mL level for coniine and clofibrate and for 50 ng/mL for metazachlor and diazepam were excluded.

Preparation of exposure medium samples

Samples containing different analytes were combined in one sample for HPLC analysis to reduce the analytical effort. All aliquots taken from the exposure medium (initial concentration and stability control) were diluted to the calibration range of every analyte with a mixture of water and methanol (50:50, v/v). No internal standard was added.

Results and discussion

Development of the HPLC–MS/MS method

The final method allowed the simultaneous determination of 34 compounds (33 analytes and one internal standard) by HPLC–MS/MS (Table 1). The selection of the test compounds was based on polarity, environmental relevance and previously reported effects on the ZFE [6, 8] and involves mainly pharmaceuticals and pesticides.

Owing to the high selectivity of the multiple reaction monitoring detection, no full chromatographic separation of the analytes was required. Analyte detection was completed within 6.5 min (Fig. 1), and reinjection could be performed after 14 min. The test substances exhibited very different physicochemical properties, and therefore, electrospray ionization MS detection in positive mode and negative mode was required in different periods of elution for the highest sensitivity. The resulting limits of detection for pure standard solutions were determined as a signal-to-noise ratio of 3:1 and ranged from 0.025 to 5 ng/mL. The lower limit of quantification was defined as a signal-to-noise ratio of 5:1 and did not deviate by more than $\pm 20\%$ from the calculated value. The mean values of every other calibration points were within $\pm 15\%$ of the expected value. All calibration curves show high linearity ($r^2 > 0.99$). The linear ranges often exceeded two orders of magnitude (Table 1). The sensitivity of this method is appropriate for the determination of exposure and internal concentrations in the ZFE. Toxic effects on ZFE are observed

at external concentrations in the microgram per millilitre range [6, 8].

Sample preparation

The washing procedure at the beginning of the sample preparation is an important step to avoid carryover of the compound from the exposure medium into the sample extract. If washing is incomplete, the uptake of test substances may be overestimated, whereas a too extensive washing would lead to loss of substances by diffusion from the interior of the ZFE and, thus, to an underestimation of the internal concentration.

The efficiency of washing with aqueous solutions of increasing methanol content (0–20 %) was first tested for 26 compounds of different polarity using a short-term exposure (less than 30 s) to an external concentration of 2 $\mu\text{g}/\text{mL}$ (Fig. 2). It was assumed that such a short exposure time would prevent significant uptake of the test compounds into the ZFE. However, literature proving this assumption is not available. For 11 compounds, the peak area in the chromatogram was below the limit of quantification or the limit of detection, respectively. Concentrations above 0.25 ng per organism were recorded for only three analytes: benzocaine, verapamil and kresoxim-methyl. The remaining concentrations were about an order of magnitude lower than those found for non-washed ZFE (data not shown). Figure 2 also suggests a slight trend of increasing concentrations remaining after washing for later-eluted, less polar compounds. Stronger washing solutions with higher methanol contents (up to 20 %), however, did not lead to reduced concentrations in the extracts (Fig. 2). Methanol contents above 20 % are not recommended as they lead to coagulation of the ZFE on washing.

The remaining concentrations of less polar compounds may not necessarily indicate incomplete washing, but may also be due to rapid adsorption of the compounds to the outer surface of the ZFE, a process that is inevitable. Therefore, on the basis of these data, pure water was selected for washing.

The washing efficiency of pure water was further tested after short-term exposure to three compounds—clofibric acid, benzocaine and metribuzin—at elevated concentrations that correspond to the level used for uptake studies (10 and 50 $\mu\text{g}/\text{mL}$ external concentration). The remaining concentrations found after washing with pure water were compared with those found in extracts after 72 h of exposure (Fig. 3). For two of the three compounds, the concentrations remaining after washing were 10 % or less of the concentration detected after 72 h of exposure. This is a satisfactory result. Only for benzocaine was a mean concentration corresponding to 28 % found. Again, this may be due to rapid adsorption to the surface of the ZFE. Inversely, at least 70 % (benzocaine) and more than 90 % (other compounds) of the analytically determined concentrations in ZFE do clearly represent the

Table 1 Validation data for the 34 study compounds (further details on the compounds are provided in Table S1)

Number	Analyte	LOD (ng/mL)	Linear range (ng/mL)	Linear range (ng/organism) ^a	R ²
1	Imazapyr	0.10	0.50–250	0.055–27.77	0.9980
2	Paracetamol	0.25	0.50–150	0.055–16.66	0.9977
3	Theobromine	1.00	1.50–250	0.166–27.77	0.9991
4	Sulfamethoxazole	0.075	0.25–100	0.027–11.11	0.9974
5	Atenolol	0.10	0.25–150	0.027–16.66	0.9981
6	Theophylline	0.50	1.00–250	0.111–27.77	0.9984
7	Coniine	5.00	7.50–250	0.833–27.77	0.9983
8	Nicotine	2.50	5.00–250	0.556–27.77	0.9980
9	Sulfamethazine	0.025	0.10–66.66	0.011–7.41	0.9965
10	Caffeine	0.50	1.00–200	0.111–22.22	0.9980
11	Cimetidine	0.05	0.50–80	0.055–8.88	0.9959
12	Atropine	0.075	0.25–125	0.027–13.88	0.9983
13	Chloramphenicol	0.25	0.75–250	0.083–27.77	0.9978
14	Metoprolol	0.075	0.25–80	0.027–8.88	0.9971
15	Thiacloprid	0.025	0.075–50	0.008–5.55	0.9962
16	Ifosfamide	0.05	0.10–80	0.011–8.88	0.9966
17	Phenacetin	0.05	0.25–80	0.027–8.88	0.9961
18	2,4-Dichlorophenoxyacetic acid	0.10	0.25–250	0.027–27.77	0.9989
19	Cyclophosphamide	0.025	0.10–100	0.011–11.11	0.9976
20	Benzocaine	0.25	0.50–250	0.055–27.77	0.9977
21	Clofibric acid	0.25	0.50–250	0.055–27.77	0.9992
22	Colchicine	0.25	0.50–250	0.055–27.77	0.9976
23	Metribuzin	0.50	0.75–100	0.083–11.11	0.9978
24	Phenytoin	0.25	0.75–250	0.083–27.77	0.9992
25	Hydrocortisone	0.50	1.00–150	0.111–16.66	0.9967
26	Metazachlor	0.025	0.075–40	0.008–4.44	0.9959
27	Diazepam	0.05	0.10–40	0.011–4.44	0.9960
28	Verapamil	0.05	0.25–50	0.027–5.55	0.9962
29	Malathion	0.10	0.50–66.66	0.055–7.41	0.9964
30	Alachlor	0.25	0.75–175	0.083–19.44	0.9973
31	Kresoxim-methyl	0.10	0.50–125	0.055–13.88	0.9971
32	Clofibrate	5.00	7.50–250	0.833–27.77	0.9991
33	Triclosan	0.25	0.75–66.66	0.083–7.41	0.9958
34	Triclocarban	0.075	0.50–66.66	0.055–7.41	0.9959

LOD limit of detection

^a Using nine zebrafish embryos and an extract volume of 0.5 mL that is diluted by a factor of 2

respective internal concentrations after long-term exposure. On the basis of these data, pure water was used for washing as reported in previous studies [16, 18–20]. However, the washing conditions (stirred solution, large wash volume) are harsher than in most previous studies.

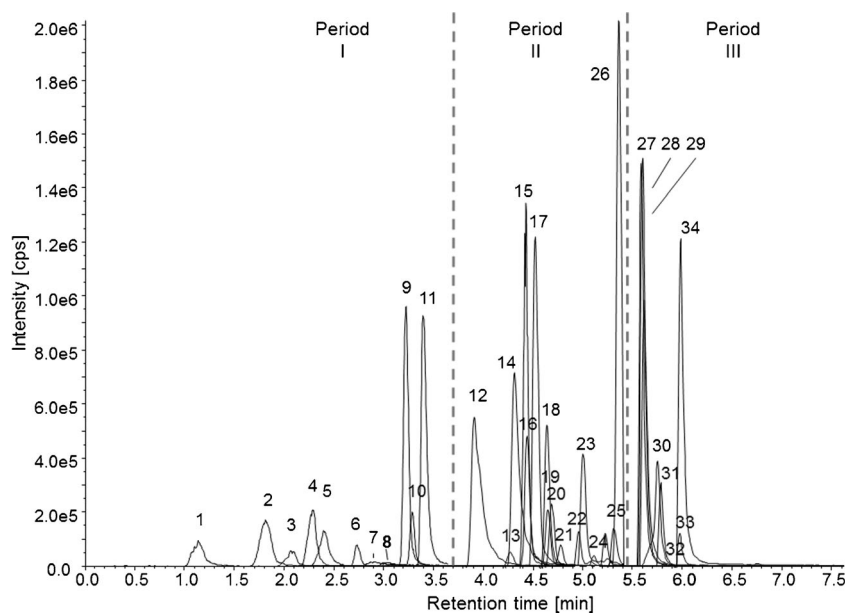
Matrix effects and method recovery

A critical aspect of quantitative analyses by HPLC–electrospray ionization MS/MS is possible matrix effects, i.e. the enhancement or suppression of analyte signals due to coeluted matrix components. Since the ZFE represents a developing organism, i.e. a dynamic system, this could result in

differential impact on matrix effects, especially in time-resolved analysis (Fig. 4). To check for matrix effects, ZFE extracts of nine individuals were used at five life stages (4–96 h post-fertilization) and were spiked with all 34 analytes at four concentrations (0, 5, 25 and 50 ng/mL). Furthermore, intact and dechorionated ZFE were compared at early life stages, and another set of experiments was performed with higher numbers of individuals (18 and 36 ZFE) at 48 and 96 h post-fertilization in the same final volume, leading to an increased matrix strength.

The mean signal intensity for all 34 test substances in all extracts of nine individuals relative to the signal in aqueous solution ranged from 90 to 110 % (Fig. 5a). No significant

Fig. 1 Multiple reaction monitoring chromatogram for the 34 compounds (standard, 10 ng/mL). For compound names, see Table 1. *Period I* in positive mode, *period II* and *period III* in positive mode and negative mode



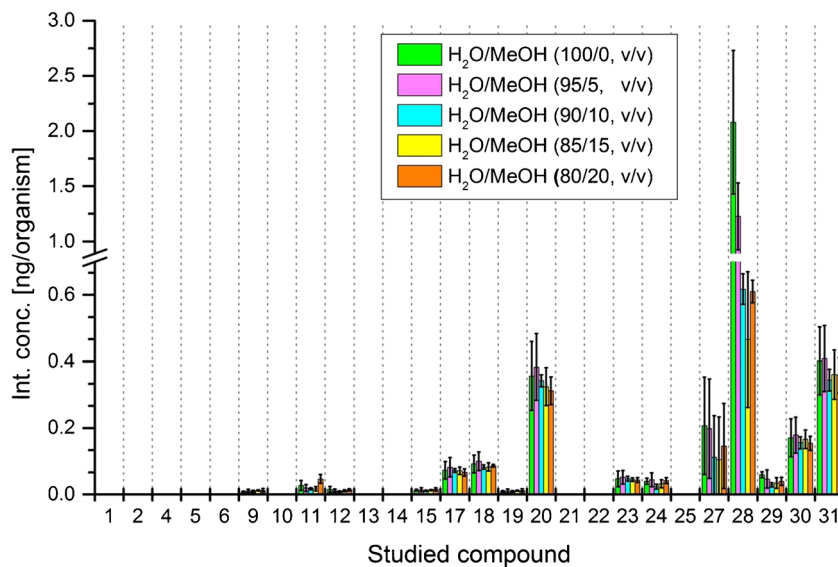
differences were found between ZFE in early life stages with and without chorion (Fig. S1). Slightly stronger matrix effects and higher variabilities were recorded for some of the 34 test substances when 36 individuals were extracted (Fig. 5c), i.e. in a four times stronger matrix. Here the signal of nicotine (compound 8) was increased by 11 %. These data are still acceptable and indicate that more than nine individuals could be extracted if the method sensitivity should require this. The results obtained with 18 ZFE are shown in Fig. S2.

The method recovery was determined by spiking ZFE at the same life stages before homogenization with methanol at the concentrations. The mean recovery from nine individuals, covering extraction and HPLC–MS/MS analysis, was in the range from 90 and 110 % for 33 of the compounds, and was

only slightly higher (112 %) for nicotine (Fig. 5b). Again, the stronger matrix (36 individuals per extract) led to a higher variability in the recovery (Fig. 5d). The mean recovery ranged from 80 to 110 % for 32 of the compounds, but was 128 % for nicotine and 73 % for triclocarban. Triclocarban was the least polar analyte in the test mix, with $\log K_{OW}$ of 4.71, suggesting that sorption to insoluble ZFE constituents, such as its membrane, may be responsible for the lower recovery of this compound. The stronger matrix also led to a lower but still acceptable reproducibility of the analyses (relative standard deviation from 2.1 to 9.4 %; $n=6$).

These data clearly indicate that the method developed is well suited to determine internal concentrations of each of the 33 study compounds at all developmental stages of the ZFE in

Fig. 2 Remaining concentration of the compounds investigated after short-term exposure (external concentration 2 $\mu\text{g/mL}$, 30 s) and washing with solutions of increasing methanol content. The concentrations of 11 compounds were below the limit of quantification. For compound names, see Table 1



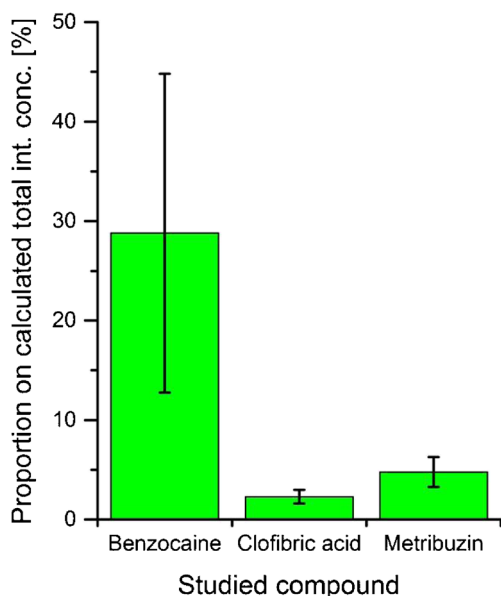
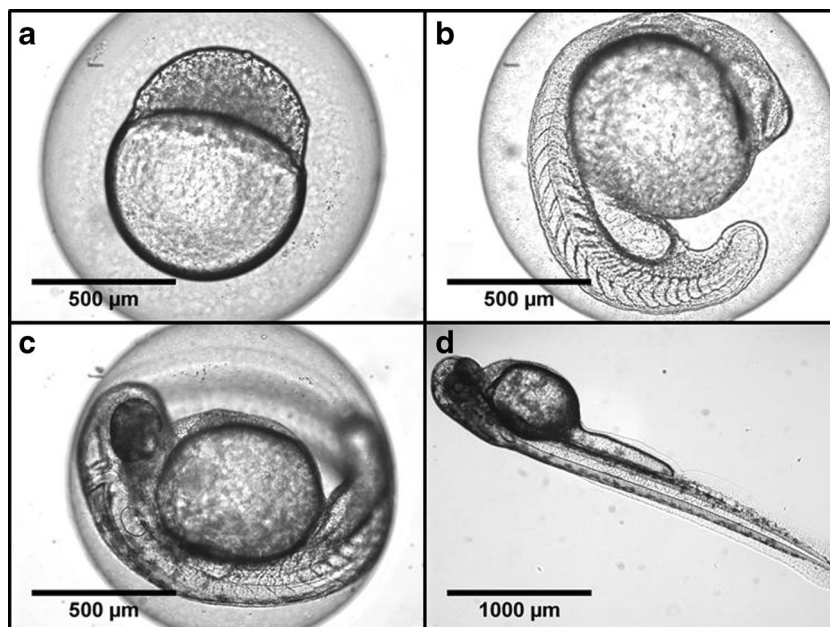


Fig. 3 Remaining analyte concentration of the study compounds after short-term exposure of 25–30 s (external concentration 10 and 50 $\mu\text{g}/\text{mL}$) and washing with pure water relative to the concentration found after 72 h of exposure to the same concentration (see Fig. 6)

the first 4 days of development. If higher sensitivity is required, the number of individuals used for the extraction could be increased by a factor of 4. Rather, in many of the experiments, the extracts of nine ZFE had to be diluted to approach the linear range of detection of the respective compound. With the need for nine ZFE to determine 34 compounds, in this method the number of ZFE needed is at the lower end, with other HPLC–MS methods using ten to 40 individuals [5, 20, 21, 30].

Fig. 4 Life stages of the embryo
 a 5 h post-fertilization, b 24 h post-fertilization, c 48 h post-fertilization and d 72 h post-fertilization

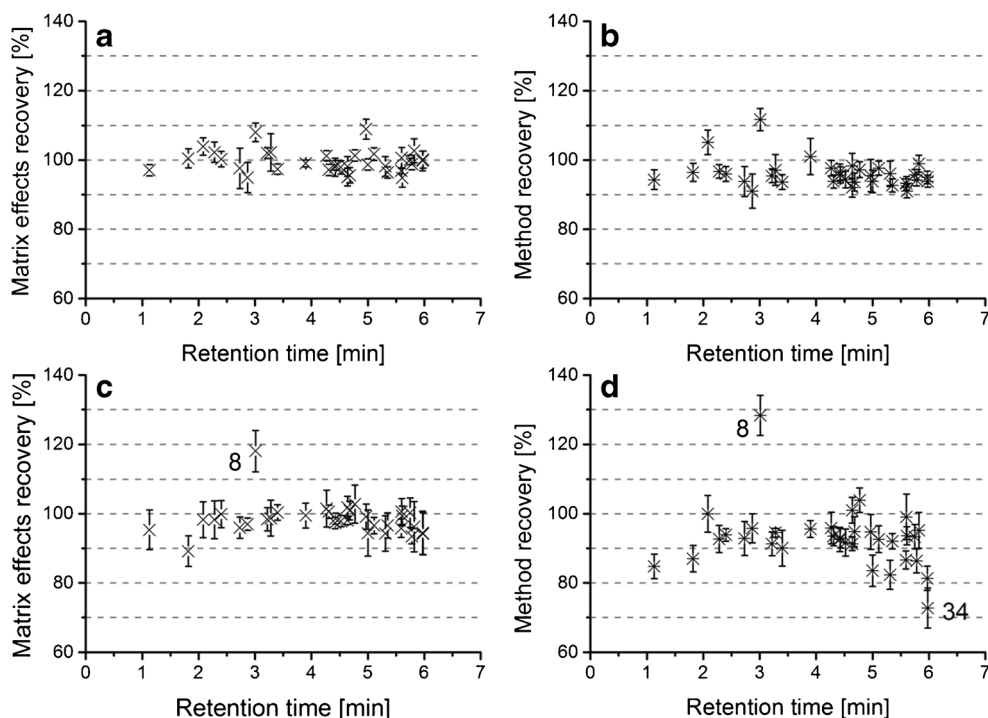


Quantification of internal concentrations in ZFE

The method developed was exemplarily applied to study the toxicokinetics of three test substances (clofibric acid and metribuzin at 50 $\mu\text{g}/\text{mL}$ and benzocaine at 10 $\mu\text{g}/\text{mL}$ external concentration) in ZFE. These concentrations were well below the respective LC_{50} values [6, 8], and screening experiments with one sampling point after 72 h of exposure showed that no effects on survival development, heartbeat frequency and movement were observed. Additionally, high internal concentrations were detected in the ZFE used for the screening experiments. Therefore, a time-resolved analysis is used to demonstrate potential applications of the method developed. Whereas the exposure experiments were performed separately for each compound owing to mixture toxicity, the ZFE from one sampling point were combined for workup and analysis, thus reducing the number of analyses. The exposure solutions were pooled as well. For the chromatograms of analyzed samples, see Fig. S3.

Figure 6 shows the time profiles of the internal concentrations (in nanograms per organism) gathered from two independent experiments with three parallel samplings for each of the test compounds. The same washing procedure was used for all life stages as described earlier. For all compounds, the time profiles show a high degree of similarity. In most cases (time/compound combinations), the concentration differences between the replicates do not exceed 25 %. In single cases, standard deviations higher than 25 % occurred. The analysis of variance between the two independent experiments for every time point shows good agreement except for clofibric acid after 48 h of exposure ($P < 0.05$). It has to be considered that the variability in the parallel samplings may reflect not

Fig. 5 **a** Matrix effects in extracts of nine embryos, **b** method recovery from nine embryos, **c** matrix effects in extracts of 36 embryos and **d** method recovery from 36 embryos. For compound names, see Table 1



only the analytical error but also differences in toxicokinetics. In all these cases, no analyte was detected in the control organisms and the external concentration in the exposure medium was constant over the exposure time (Fig. S4).

These data for the three test compounds generated from 72 single-exposure experiments, with nine ZFE each, demonstrate high precision of the whole approach, including exposure, the subsequent sample preparation and the final HPLC–MS/MS analysis. They also highlight that the use of nine individuals per experiment provides sufficient precision and that higher numbers of organisms are required only if higher analytical sensitivity is required.

The concentration–time profiles of the three test substances exhibit three different patterns: slowly increasing internal

concentration until the end of the test at 96 h (clofibric acid), rapid increase in internal concentration within 24 h with no significant change afterwards (metribuzin) and rapid increase in internal concentration within the first 24 h and decreasing concentration during longer exposure times (benzocaine).

A comparison of clofibric acid and metribuzin shows that the speed of uptake of the test compounds into the ZFE can differ widely. Whereas a steady concentration was reached for metribuzin in less than 24 h, more than 96 h was required for clofibric acid to reach its equilibrium concentration. The reason for these differences is unknown. In a study using three pesticides in older life stages of ZFE, it took between 20 h and more than 50 h to reach the steady-state concentration [16]. For clofibric acid, the slow uptake may be due to the dominance of its anionic form at neutral pH.

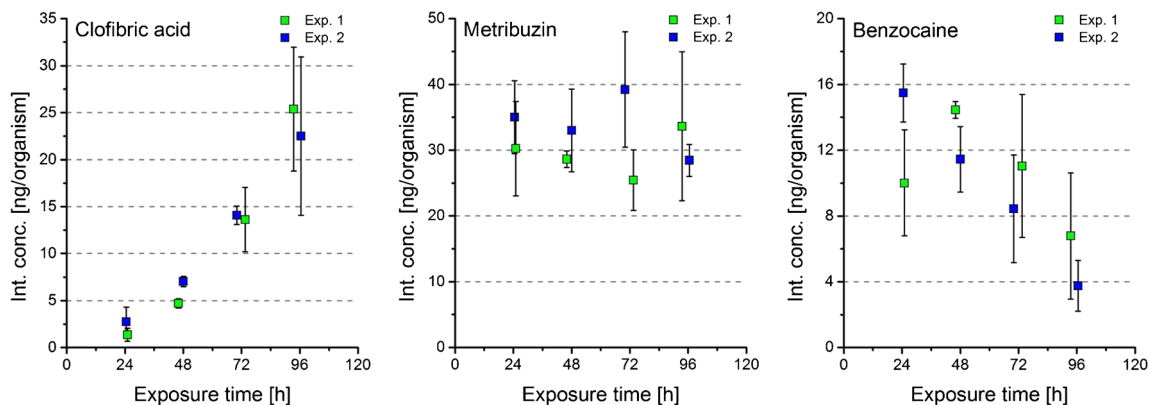


Fig. 6 Internal concentration–time profiles of embryos exposed to clofibric acid, metribuzin (external concentration 50 $\mu\text{g}/\text{mL}$) or benzocaine (external concentration 10 $\mu\text{g}/\text{mL}$) in two independent experimental series

Several reasons may lead to a decrease in the internal concentration of benzocaine in older life stages of the ZFE, such as enhanced excretion from or an active transport out of the organism [31] or metabolism in the organism. As the data in Fig. 6 are presented as mass per organism, a decreasing concentration cannot be due to the growth of the ZFE. The elimination of benzocaine has been reported in the literature for other fish species [32] as have decreasing internal concentrations in ZFE for other compounds [23]. Future toxicokinetic studies should include the analysis of metabolites to elucidate further the processes leading to decreasing internal concentrations. The formation of metabolites may then be linked to the evolution of certain metabolic enzymes in early life stages of the ZFE.

The three compounds investigated exhibit different patterns of the development of the internal concentration over time, illustrating the need for further extensive uptake studies to understand better biological effects in ZFE. A time-resolved determination of internal concentrations would help to understand, for example, the fate of chemicals in the ZFE. This would allow a thorough comparison of the uptake and the hazard evaluation of test compounds in adult zebrafish and fish embryos or adult fish of other species.

Conclusion

The experimental and analytical approach developed in this study allows an efficient analysis of internal concentrations of test compounds in ZFE using HPLC–MS/MS. No significant matrix effects were observed, and external calibration could be used for quantification of 34 analytes. In addition, the method recovery demonstrates that the workflow developed is suitable to determine internal concentrations in all life stages of ZFE. Washing of the ZFE after exposure is essential to avoid carry-over from the exposure medium. Water was shown to be sufficient for this process if polar compounds are studied. Owing to the large number of analytes for which the whole approach was validated, this method offers a broad range of applications.

The data for three of the 34 study compounds show a large diversity in the time courses of the internal concentrations over time. More extensive toxicokinetic studies comparing uptake of a larger number of structurally diverse compounds and involving different developmental stages of the ZFE will be needed to elucidate factors governing the temporal development of the internal concentrations of toxicants in ZFE. A method suitable for this purpose has been provided here.

Acknowledgments This work is part of the research topic “Chemicals in the Environment” (CITE) within the research programme of the Helmholtz Centre for Environmental Research–UFZ. We thank C. Petzold and J. Müller for their support in laboratory work and S. Scholz for helpful discussions on ZFE testing and toxicokinetics.

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Stephan Brox studied food chemistry in Braunschweig, Germany, and is currently a Ph.D. student in the Department of Analytical Chemistry at the Helmholtz Centre for Environmental Research—UFZ in Leipzig, Germany. His research focuses on the uptake of environmental contaminants in zebrafish embryos and combines analytical and ecotoxicological issues.



Axel P. Ritter studied chemistry in Leipzig, Germany, and received his Ph.D. degree in 2011 from the University of Regensburg, Germany. From 2011 to 2013, he was a postdoctoral researcher at the Helmholtz Centre for Environmental Research—UFZ in Leipzig, Germany. Currently, he works as a research scientist at MVZ Labordiagnostik Dessau in Dessau, Germany.



Eberhard Küster is a research scientist and workgroup leader in the Department Bioanalytical Ecotoxicology at the Helmholtz Centre for Environmental Research—UFZ in Leipzig, Germany. His main research interest is the evaluation of fish embryos as alternatives to fish tests. His recent work focuses on the toxicokinetics of pollutants in zebrafish embryos and reactions to toxins at the zebrafish embryo proteome level.

Thorsten Reemtsma is Head of the Department of Analytical Chemistry at the Helmholtz Centre of Environmental Research—UFZ in Leipzig, Germany, and Professor for Analytical Chemistry at the University of Leipzig. He has been active for several years in developing analytical methods for organic trace analytes from environmental matrices, and in applying these methods to study the occurrence and fate of contaminants, in cooperation with engineers, (micro)biologists and ecotoxicologists.