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LC-APCI(-)-MS determination of 1-chloro-2,4-dinitrobenzene, a model substrate for glutathione S-transferases.

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ABSTRACT: 1-Chloro-2,4-dinitrobenzene (CDNB) is widely used as a model substrate for measuring enzyme activity of glutathione S-transferases in toxicity studies and in studies focusing on the metabolic capacity of different test systems. To allow the quantification of CDNB at low, non-toxic concentrations, we developed a sensitive liquid chromatography-mass spectrometry (LC-MS) technique, which is based on electron capture ionization using atmospheric pressure chemical ionization (APCI) in negative ion mode. Gas phase reactions occurring under atmospheric pressure produce specific ions that allow direct CDNB quantification down to 17 ng/ml in water. Using the new technique, we were able to verify CDNB exposure concentrations applied in two typical toxicity studies with early life stages of the common model organisms, zebrafish (*Danio rerio*) and a zebrafish embryonic cell line (Pac2).

Introduction

The electrophilic compound 1-chloro-2,4-dinitrobenzene (CDNB) is used among others as a chemical reagent during the manufacturing of azo dyes, and as an algacide in the coolant water of air-conditioning systems. In contact with skin, CDNB induces allergic contact dermatitis. Relatedly, it was shown to be effective as an immunostimulant and in treatment of viral warts¹. The compound is also widely used as a model substrate for activity studies of glutathione S-transferases (GSTs). The GSTs are a family of enzymes that plays a major role in detoxification of electrophilic compounds, such as drugs and environmental pollutants, by catalyzing the conjugation reaction of the electrophilic moiety with

glutathione (GSH). Connected to this major role in phase II metabolism, the level of GST activity is frequently used to define the biotransformation capacity of biological test systems^{2,3}.

However, *in vivo* studies that focus on biotransformation (i.e. phase II metabolism) require a substrate concentration that does not induce toxicity in the test organism - otherwise the biotransformation enzymes might be adversely affected. Therefore, when using CDNB as a substrate for GSTs, it is frequently required to measure CDNB at low, non-toxic concentrations in biological matrices or exposure solutions in order to verify and monitor the chemical load. Traditionally, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection has been used to measure CDNB concentrations³. This technique exploits the fact that biotransformation of CDNB by GSTs results in the formation of a GSH-conjugate (DNP-SG, $\lambda_{\text{max}} = 340 \text{ nm}$) which can be spectrophotometrically distinguished from the parent compound (CDNB, $\lambda_{\text{max}} = 280 \text{ nm}$)^{4, 5}. However, the lowest limit of quantification (LOQ) for CDNB with this technique was reported to be 200 ng/ml⁵.

Available toxicity data on aquatic organisms, such as guppy (*Poecilia reticulata*) and rotifers (*Brachionus calyciflorus*), report a half-maximal lethal (LC_{50}) CDNB concentration of 200-1'300 ng/ml¹. The HPLC-UV LOQ of 200 ng/ml⁵ is very close to the CDNB LC_{50} . As the non-toxic concentration is significantly lower than the LC_{50} , a more sensitive technique is needed to be able to quantify the frequently used CDNB at low, non-toxic concentrations. For this reason, we were interested in developing a more sensitive method for its quantification. While GC-MS has traditionally been used for halogenated aromatic compounds, the most powerful technique to access chemical compounds in biological matrices and environmental samples, without major sample preparation, is HPLC coupled to a high-resolution mass spectrometer (HR-MS). Currently, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources are the predominantly used interfaces. Both techniques enable the ionization and transfer of the analytes into the gas phase. Typically, ionization occurs based on acid/base reactions (protonation and deprotonation in the positive and negative ion mode, respectively), either in the liquid (ESI) or the gas phase (APCI). Thus, the ionization efficiencies for non-polar molecules that are not prone to protonation/deprotonation reaction, such as CDNB, are usually poor with both interfaces. However, nonpolar nitroaromatic compounds have been shown to undergo dissociative and

non-dissociative electron-capture (EC) ionization with APCI in the negative ion mode⁶. We demonstrate that this feature can be exploited for the quantification of CDNB down to the 17 ng/ml range.

As representative model systems for toxicity studies, we focus on zebrafish early life stages and zebrafish cell lines (Pac2), due to their versatile application possibilities in life and environmental sciences. In both exposure systems, this technique allows measuring the whole range of exposure concentrations in a typical toxicity study, down to non-toxic concentrations.

Experimental

Chemicals and reagents: 1-Chloro-2,4-dinitrobenzene (CDNB) and ammonia were purchased from Sigma Aldrich (United States) with $\geq 99\%$ purity, and methanol and ethanol (for HPLC, gradient grade, $\geq 99.8\%$) from Fisher Scientific (United States). Nanopure water was obtained from Barnstead Nanopure, Fisher Scientific (United States). Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (United States). Reconstituted water (294.0 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 123.2 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 64.7 mg/l NaHCO_3 , and 5.7 mg/l KCl; ISO 15088:2007(E); 2007) and Leibovitz L-15 medium (Invitrogen, United States) supplemented with 5 % fetal bovine serum (FBS; PAA Laboratories, Switzerland) were prepared freshly.

Mass spectrometry: For method development, CDNB was directly infused into the mass spectrometer at a concentration of 1 $\mu\text{g/ml}$ and a flow rate of 15 $\mu\text{l/min}$. The measurements were performed on a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific Inc.) using an APCI source, operated in negative ion mode (APCI(-)). Full scan data were acquired over a mass range of 50-300 m/z with a resolving power of 30'000 at m/z 200. Capillary temperature was set to 250 °C, APCI vaporizer temperature to 250 °C, sheath gas flow to 30, aux gas flow to 10.

To evaluate the method, two 13 point calibration series were prepared over a concentration range of 0 (blank) to 2000 ng/ml (0, 1, 5, 10, 125, 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 ng/ml) in five replicates, which covered the fish LC_{50} from literature (200-1'300 ng/ml¹). The first calibration curve was prepared in reconstituted water with 0.01% ethanol to mimic the condition of a typical assay with zebrafish. For this, CDNB stock solutions were prepared in warm ethanol and then spiked into samples of reconstituted water. The second calibration curve was prepared in L15 medium supplemented with 5 % FBS and end concentration of 0.5 % DMSO to mimic the condition of a typical cell line assay. To

prepare the samples, CDNB stock solutions were prepared in DMSO and then spiked into samples of L15 medium with 5 % FBS. A slightly larger concentration range was chosen for the calibration curve in L15 medium with 5% FBS because we assumed a higher non-toxic concentration and LC_{50} in cell lines, as indicated by preliminary toxicity tests.

Chromatography: The samples were injected onto a Poroshell 120 EC - C18 column (2.7 μ m particle size, 2.1x100 mm – Agilent, Switzerland) and separated at a flow rate of 200 μ l/min using a 2 min linear gradient from 80 % solvent A (10 % methanol in water, 0.1 % ammonia) to 100 % solvent B (100 % methanol, 0.1 % ammonia) followed by a washing step (2 min, 100% solvent B) and a re-equilibration step (4 min, 80 % solvent A), giving a total runtime of 8 min.

Verification of exposure levels in CDNB toxicity studies:

To verify CDNB concentrations used in a typical CDNB toxicity study with zebrafish early life stages, working solutions with the concentrations of 3, 2, 1.2, 1, 0.7, 0.5 and 0.25 μ g/ml were prepared in ethanol. The working solutions were then diluted by a factor of 10^4 with reconstituted water to obtain the nominal exposure concentrations of 300, 200, 120, 100, 50 and 25 ng/ml and a final ethanol content of 0.01 %.

To verify CDNB concentrations used in a typical CDNB toxicity study with zebrafish cell lines (Pac2), working solutions with the concentrations of 320.0, 256.0, 204.8, 163.8, 131.1 and 83.9 μ g/ml were prepared in DMSO, and then diluted by a factor of 200 with L15 medium supplemented with 5 % FBS, to obtain the nominal exposure concentrations of 1600, 1280, 1024, 819.2, 655.36 and 419.43 ng/ml and a final DMSO content of 0.5 %.

The exposure concentrations were chosen to cover the range between the LC_{50} and non-toxic concentrations, by considering data from literature¹ and running range finding experiments. For the quantification of CDNB in the respective exposure medium, 1 ml of medium was sampled at the beginning of each experiment and stored at 4 °C until further processing.

Data processing and evaluation: Method development, generation of theoretical isotope patterns and evaluation of the mass spectrometric data was done using Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific Inc.). Following the measurements, peak areas were extracted from the datasets, using a mass tolerance of 5 ppm. The integrated peaks (unsmoothed) were manually reviewed. The sum of all peak

areas corresponding to the dissociative and non-dissociative EC ions (Figure 1, A, B, C, D and E) were used for the linear regression. For this, the CDNB concentration (x) was fitted against the peak area (y) and a regression was performed without forcing the line to go through zero (SI Figure 1). The LOD and LOQ were calculated based on the linear calibration curve ($y=a+bx$) according to the following equations: $LOD=3S_a/b$ and $LOQ=10S_a/b$, where x is the concentration, y is the peak area, S_a is the standard deviation of the y -intercepts of the regression lines ($n=5$) and b is the slope of the calibration curve.

Results and Discussion

CDNB is a nonpolar compound that lacks proton-accepting and proton-donating functional groups. Thus, ionization of this molecule via acid/base reactions is not likely to occur. However, we were able to exploit the fact that CDNB undergoes electron capture processes in the APCI interface, thus allowing the determination of CDNB in liquid media. Electrons from the corona discharge in the APCI interface can interact with the nebulizer gas producing gas-phase low-energy electrons. Electron-affine molecules in the gas phase capture those low-energy electrons and form negative ions. This electron capture mechanism allows the measurement of electron-affine compounds in APCI(-) with high sensitivity^{7, 8}.

CDNB undergoes dissociative and non-dissociative EC in APCI(-)

It has been reported previously that aromatic compounds containing a halogen and/or a nitro-group can undergo dissociative electron capture in APCI(-)^{6, 9}. An interaction of the analyte ion or the neutral analyte with an oxygen or a superoxide ion can lead to a substitution of the halogen and/or the nitro-group by oxygen⁹. Indeed, we observed dissociative EC ions at $m/z = 183.00$ and 171.98 that showed a substitution of the chloride and nitro-group, respectively (Figure 1A, E). The good agreement between the measured and theoretically generated isotope profiles (Figure 2A, E) supports the proposition that CDNB undergoes dissociative electron capture in APCI(-), forming the predicted ions (Figure 1A, E).

Since the nitro aromatic system of CDNB has electron accepting capabilities, non-dissociative electron capture ionization should be possible⁶. Indeed, we observed a peak at $m/z = 201.98$, which corresponds to the negatively charged molecular radical ion $[M]^\bullet-$ (Figure 1D, Figure 2D). However, the isotopic distribution of the measured ion does not agree with the theoretically generated isotope profile. In addition, a further ion $[M-H]^-$ with the $m/z = 200.97$ is present in our measurements (Figure 1C, Figure

2C). Its isotopic distribution clearly overlaps with the isotopic pattern of the molecular radical ion. By subtracting the theoretical isotope intensities of the $[M-H]^-$ from the measured isotope pattern, a pattern emerges that agrees with the theoretical isotope distribution of the $[M]^-$ (SI Table 1). Additionally, we observed a solvent (methanol) cluster $[M+\text{solvent}-H]^-$ with an $m/z = 233.00$, whose identity was confirmed by the theoretically generated isotope profile (Figure 1B, Figure 2B).

The dissociative substitution of the chloride group by oxygen is the predominant reaction within the APCI source producing by far the strongest ion. The remaining ions contribute only little to the overall signal. Nonetheless, as ion formation in the APCI interface is a chemical gas phase reaction, the ratio of the ions produced might vary depending on the pressure gradient and source settings of the MS and the concentration used.

Dissociative EC in APCI(-) allows to measure CDNB load in common toxicity studies

Having successfully ionized CDNB by electron capture in APCI(-), we investigated whether this approach would be suitable and sensitive enough to identify and measure CDNB in two typical toxicity studies with different exposure media. CDNB is a lipophilic compound ($\log K_{ow} = 2.17$)¹⁰ which can be dissolved in organic solvents but is poorly soluble in water. However, in toxicity studies with aquatic organisms or cells, aqueous solutions are predominantly used as exposure medium.

First, to separate the analyte from other components present in the exposure medium, an 8 min HPLC method was established. In this gradient separation, CDNB elutes at 2 min. No carryover of the analyte was observed in blank samples. Second, to test if our method is suitable for toxicity studies, we measured CDNB dilution series in reconstituted water and L15 medium substituted with 5 % FBS.

In both media, peak areas and concentrations showed a linear relationship within the analyzed concentration range (1-2000 ng/ml) (SI Figure 1). In reconstituted water, we were able to detect CDNB down to the concentration of 5 ng/ml (LOD) and to quantify it down to the concentration of 17 ng/ml (LOQ). The LOQ of our method is 11 times lower than the previously reported LC_{50} for fish (200 ng/ml)⁵ which implies that we should be able to monitor the CDNB concentration in the exposure medium down to the non-toxic concentrations. Considering literature on CDNB toxicity in different aquatic organisms¹, we have chosen an exposure concentration range of 300 to 25 ng/ml for toxicity studies with zebrafish

early life stages. By using the developed method, we determined the actual CDNB concentrations prior to the exposure experiments (SI Table 2A), which allows a better interpretation of toxicity results.

In L15 medium substituted with 5 % FBS, we were able to detect CDNB down to the concentration of 17 ng/ml (LOD) and to quantify it down to the concentration of 57 ng/ml (LOQ). Preliminary range finding experiments indicated, that the CDNB toxic range for Pac2 cell lines is higher as compared to the fish embryos. For this reason we selected a higher exposure concentration range of 1600 to 420 ng/ml. The calibration curve was suitable to verify the concentrations in this typical cell toxicity assay (SI Table 2B).

During the toxicity study, the actual CDNB concentration deviated by up to 33 % in water and up to 27 % in L15 medium with 5 % FBS from the nominal concentration in both exposure media due to handling. Through the correction of the nominal concentration the toxic effects can be better correlated with the toxicants concentration and facilitate the design of further experiments.

Conclusion

We showed that APCI(-) is a powerful technique which can be applied to quantify nonpolar, nitroaromatic compounds, such as CDNB, through solvent adduct formation, dissociative and non-dissociative electron capture ionization. Considering that CDNB is widely used in toxicity research, especially as a model substrate for GST activity studies, a sensitive technique for compound quantification is of high importance to allow for a high degree of flexibility in experimental design.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Ethical approval

All applicable international, national, and/or institutional guidelines were followed.

171 **Figures**

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Figure 1: Structural formula of 1-chloro-2,4-dinitrobenzene (CDNB) and proposed chemical formula of ions produced with APCI(-)-MS electron capture (A, B, C, D and E). Below the relative abundance of the ions (A, B, C, D and E) is shown including an in-frame zoom on the low abundance ions.

Figure 2: Mass spectra of ions produced with APCI(-)-MS electron capture A, B, C, D and E (structural formula shown in figure 1), and a table with the parts per million (ppm) difference (Δ) between the measured and predicted mass to charge ratio (m/z). The measured isotopic distribution is shown on top of the predicted isotopic distribution in case of A, B and E. In case of C and D, the overlapping isotope pattern of the ions is shown at the top and the theoretical isotope distributions for ions C and D, respectively is shown at the bottom. An in-frame zoom on m/z of 202.95-203.00 is shown to emphasize the presence of both, C and D, isotope patterns within the measurements.

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