1 LC-MS/MS determination of tralopyril in water samples

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Abstract

A targeted analytical method was established to determine tralopyril (4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1*H*-pyrrole-3-carbonitrile) in water. This compound has been recently introduced as a biocide in ship antifouling paints, becoming a potential new environmental contaminant. The method presented here allows for the first time the direct determination of tralopyril in environmental samples without the need of a pre-concentration step. The injected sample is separated by a 30 min HPLC-gradient on a reversed phase column and the compound identified and quantified by negative ion LC-MS/MS. Tralopyril solutions in DMSO, seawater, river Glatt water and E3 medium (used for zebrafish experiments) were analysed to demonstrate the applicability of the method. The method provides good retention time reproducibility and a quantitation limit (LOQ) of 0.025 µg L⁻¹ for DMSO, seawater and E3 exposure medium and 0.05 µg L⁻¹ for river Glatt water. Calculated tralopyril half-lives were 6.1 h for seawater, 8.1 h for river Glatt water and 7.4 h for E3 medium.

1. Introduction

Biofouling is a natural occurring process that typically results in the undesirable attachment and growth of living organisms (biofouling) on immersed surfaces. This may lead to i) the deterioration of diverse aquatic equipment and structures, ii) the increase of frictional drag between a ship hull and the water leading to increased fuel consumption and iii) the dissemination of invasive species through ship traffic. Antifouling systems (AFS) have been developed to prevent this phenomenon but the leaching of biocides from AFS into water has been responsible for deleterious impacts in the environment. Currently, it is required that

emerging biocides maintain a good antifouling (AF) performance towards target organisms and, on the other hand, have a low environmental impact. Rapid transformation to less toxic products, a low tendency to bioaccumulate and a low toxicity to non-target organisms are desirable characteristics of environmentally friendly AF-biocides (IMO 1999).

Tralopyril (4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1*H*-pyrrole-3-carbonitrile) is an emerging AF-biocide marketed as a non-persistent and biodegradable substance. It is considered an ecologically safer alternative to former hazardous biocides used in AFS, such as tributyltin (Thomas and Brooks 2010; Mert et al. 2014). It is used to control biofouling by barnacles, hydroids, mussels, oysters and polychaetes and it is the main active substance of the AFS ECONEA[®] (Janssen PMP, Belgium). Furthermore, this compound was recently accepted (April of 2014) for inclusion under the Biocide Products Regulation (BPR, Regulation EU No. 528/2012) as antifoulant, meaning that its use will most likely become widespread in Europe. Information available in the literature includes chemical properties, degradation half-lives (3 and 15 hours in seawater at 25 and 10 °C, respectively) and efficacy of AFS containing this active ingredient (Kempen 2011). To our best knowledge, the only reports available on the toxicity of tralopyril regard effects on the larvae of the acorn barnacle *Balanus amphitrite* (Kempen 2011), the marine bacteria *Shewanella algae* (Martín-Rodríguez et al. 2014) and few other marine invertebrate taxa (Oliveira et al. 2014).

The work presented aimed to develop a robust method for the quantification of tralopyril in aqueous samples not only to be used in the context of environmental monitoring (sea and riverine waters) but also in toxicity bioassays (in this case the E3 medium for zebrafish embryo toxicity test, addressed elsewhere). Moreover, the Biocidal Products Committee requested further validation data regarding the levels of tralopyril in several ecological compartments

namely, seawater, sediments, fish and shellfish (BPC, ECHA/BCP/002/2014). Based on the ISO norm 15181-6 (ISO 2012), we established, in a first trial, an HPLC-fluorescence method that allowed us to perform water-based tralopyril analyses with a limit of quantitation (LOQ) of 2 $\mu\text{g L}^{-1}$. However, a previous study estimated that the average environmental tralopyril concentration expected for a seawater marina could be 0.4 $\mu\text{g L}^{-1}$ (Oliveira et al. 2014), requiring a lower LOQ and thus a more sensitive analytical method. Other techniques, i.e. GC-MS and HPLC-UV, were unsuccessfully tried for the quantification of this biocide (data not shown). The method described here uses, for the first time, negative ion LC-MS/MS to robustly determine tralopyril in water-based samples.

2. Material and Methods

2.1. Chemicals and solutions

Tralopyril (PESTANAL®, analytical standard) and dimethyl sulfoxide (DMSO, >99.5% purity) were purchased from Sigma-Aldrich (Buchs, Switzerland). Acetonitrile and methanol HPLC gradient-grade purity was obtained from Acros or Fisher Scientific, nanopure water from Barnstead NANOpure (Skan, Allschwil, Switzerland) and the 25% aqueous ammonia solution from Merck (Darmstadt, Germany). The salts for the E3 medium (NaCl, KCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaHCO_3) were ordered from Sigma-Aldrich.

Stock solutions and DMSO standards were prepared by dissolving tralopyril in DMSO. The experimental solutions were obtained by diluting the stock solutions into the different media (seawater, river Glatt water and E3 medium). Both sea and river Glatt water were grab samples: the first was collected at Aveiro harbor, Portugal on the 14th of October, 2015 (salinity $\approx$ 35 psu

and pH = 7.8) and the river Glatt sample was collected in Dübendorf, Switzerland on the 2nd of November 2015 (pH = 8.1). The E3 medium (pH = 8.6) is used for zebrafish embryo exposures and was prepared as follows: a 60 times concentrated stock solution containing 17.2 g NaCl, 0.76 g KCl, 2.9 g CaCl₂·2H₂O plus 4.9 g MgCl₂·6H₂O was prepared in 1 L nanopure water. For the actual medium, 16.6 mL of this stock solution plus 9 mL of a 0.3 mM NaHCO₃ solution have to be added to 1 L of nanopure water (ISO 2007).

2.2. Sample Preparation

No sample preparation is needed since the method allows direct measurement of the target compound in the sample at environmentally relevant concentrations. Sample filtration is highly recommended in order to protect the HPLC column. Seawater and river Glatt water were filtered through a 0.45 µm disposable syringe filter (Macherey Nagel AG, Oensingen, Switzerland, Chromafil Xtra PTFE-45/25). There was no need for an internal standard, as tralopyril could be directly quantified using an external calibration, and because no ion suppression was observed in the water-based samples.

2.3. Chromatography

A sample volume of 40 µL was separated on a Waters XTerra reversed-phase column (2.1x100 mm, 3.5 µm particle size, Waters Schweiz, Baden-Dättwil, Switzerland) with the use of an Accela 600 HPLC pump fitted with an in-line degasser (Thermo Scientific, San Jose, USA), and a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). Sample temperature was kept at 18 °C.

Pre-mixed eluents were used for the gradient at a flow rate of 120 $\mu\text{L min}^{-1}$: A (99% nanopure water, 1% acetonitrile, total of 0.1% NH_3) and B (99% acetonitrile, 1% nanopure water, total of 0.1% NH_3). Starting conditions were 100% A during 5 min followed by a linear gradient to 100% B in 15 min which was kept for 3 min. Initial conditions were reached in 3 minutes and the column re-equilibrated for another 4 min, giving a total runtime of 30 min. The backpressure was < 150 bar at all times. Before and after each injection, the syringe and auto sampler valve were cleaned three times with nanopure water and methanol (1:1).

2.4. Mass spectrometry

Analytes eluting off the column were detected in negative ion mode using a TSQ Vantage triple quadrupole MS, fitted with a heated electrospray ion source (Thermo Scientific, San Jose, USA).

The collision energy (CE) for collisional activation was optimized to yield highest intensity fragments of the monoisotopic precursor ($m/z = 346.9$). Source settings were as follows: needle voltage -2500 V, interface and vaporizer temperatures were 150 and 180 $^{\circ}\text{C}$, respectively, sheath gas 40, ion sweep gas 2 and aux gas 5. The resolution of Q1 was set to 0.4 and 0.7 FWHM for Q3. The scan width was set to 0.5 m/z and the scan time to 0.05 sec.

Full scan acquisition gave the exact same isotopic distribution for the deprotonated molecular ion as the simulation (Figure 1). Three transitions were monitored in multiple reaction monitoring (MRM) mode during the whole acquisition. The transition m/z 346.9 $\rightarrow$ 78.9 was used for quantitation (quantifier), since it was the most intense, and the transitions m/z 348.9 $\rightarrow$ 131.0 and 348.9 $\rightarrow$ 268.0 as qualifier signals (see Figure 2). To prevent unnecessary MS

126 contamination, a divert valve switched from waste to MS after 2 min and back to the waste after
 127 26 min.

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 129 Figure 1. Comparison of measured (top graph) and simulated (bottom graph) tralopyril isotope distribution of the
 130 deprotonated molecular ion

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 133 Figure 2. Structure of the negatively charged tralopyril and most intense fragments generated, with the
 134 corresponding collision energies (CE) and the resulting sum formulas

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136 **2.5. Data processing and evaluation**

137 Peak areas were determined using XcaliburTM 2.2 software and manually adjusted before
138 transferring to Excel, together with the retention times, for further processing. Blank values were
139 obtained by measuring DMSO, sea and river water, and E3 medium. Blanks were subtracted
140 from samples before quantitation. Tralopyril half-lives in the different media were calculated
141 from fitted experimental data.

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143 **3. Results and Discussion**

144 **3.1. Mass spectrometry**

145 Tralopyril is a thermolabile compound, which deteriorates very rapidly. This is probably
146 the reason why to date no GC- or LC-MS methods have been reported. The key element of the
147 method presented here is that both the vaporizer and the capillary temperature were kept at
148 relatively low temperature (150 °C for the vaporizer and 180 °C for the capillary). The
149 chromatographic separation is reproducible with a retention time of 14.9 min and a
150 corresponding standard deviation of 0.12 (n = 128). To identify tralopyril, not only the retention
151 time was checked but also the ratios of the areas between the quantifier (m/z 346.9 → 78.9) and
152 the two qualifier signals. An average ratio (quantifier/qualifier) of 2.2 ± 0.2 for the fragment of
153 m/z 131.0 and 12.5 ± 1.6 for the fragment of m/z 268.0 were obtained (n = 87).

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3.2. Method performance

This method was initially developed for the measurement of tralopyril in DMSO. The LOQ with a signal-to-noise ratio of 10 was $0.025 \mu\text{g L}^{-1}$ (Figure 3). The calibration curve showed to be linear over 4 orders of magnitude from LOQ up to $5'000 \mu\text{g L}^{-1}$. Moreover, a $5 \mu\text{g L}^{-1}$ solution in DMSO was measured 5 times consecutively to assess the repeatability of the measurements. The results showed a good repeatability for retention time ($14.8 \pm 0.09 \text{ min}$) and for the quantitative determination ($4.8 \pm 0.59 \mu\text{g L}^{-1}$).

Tralopyril calibration curves in the three different aqueous media were subsequently acquired and LOQs determined: $0.025 \mu\text{g L}^{-1}$ for seawater and E3 medium and $0.05 \mu\text{g L}^{-1}$ for river Glatt water. A comparison between tralopyril standards in DMSO and in the aqueous media showed a significant difference in the slope. DMSO displayed lower sensitivity possibly due to its viscosity that might have led to the observed ion suppression whilst the aqueous media showed similar responses in between them (Figure 4).

Figure 3. Determination of the limit of quantitation in DMSO with a signal-to-noise ratio of 10 (quantifier, top trace)

Figure 4. Comparison of tralopyril calibration curves in the four different media tested.

3.3. Degradation of tralopyril in aqueous solution

Our data provides degradation rates for tralopyril in aqueous solutions, which is required to estimate the persistence of the compound in natural aquatic environments. In fact, persistence of tralopyril (together with bioaccumulation and toxicity) is an important criterion for identifying the hazard of this substance to human health and the environment. We found that tralopyril in DMSO was stable for at least seven days at room temperature, 8 weeks at 4 °C and one year at -20 °C. As the substance is rapidly hydrolysed, the samples in artificial seawater, natural river water or E3 medium had to be freshly prepared before injections. A 5 µg L⁻¹ standard in each of the three different media was used to determine the degradation of tralopyril over time at 18 °C (Figure 5). The sample was repeatedly injected over a time range of 13 to 16 hours. The measured tralopyril half-lives were 6.1 h for seawater, 8.1 h for river Glatt water and 7.4 h

for E3 medium. The value obtained for seawater is fairly in accordance with a previous study that reported a tralopyril half-life of 3 h at 25 °C and 15 h at 10 °C (Kempen 2011). This fact also confirms the thermolabile nature of tralopyril (already addressed in section 3.1.), by showing that tralopyril is degraded faster with increasing temperature. Due to its fast hydrolysis, tralopyril appears to be a promising AF-biocide with low persistence in the water. In comparison to other AF - biocides, tralopyril has a very short half-life in water. For example, the degradation half-lives of tributyltin (TBT) - one of the most used biocides in the history of AFS until its global ban in 2008 – were found to be in the range of 4 to 19 days in seawater and from a few weeks to several months in fresh water (Seligman et al. 1996). Likewise, Irgarol 1051, a well-known herbicidal additive used in copper-based AFS that is already restricted in some European countries (Thomas and Brooks 2010) is also known to be quite resistant to hydrolysis, as well as to heat and biodegradation (Okamura and Sugiyama 2004). Its reported half-life in seawater ranges from 100 to 350 days (Hall et al. 1999; Thomas and Brooks 2010; Thomas et al. 2002) although in the presence of humic substances it degrades much faster (Okamura and Sugiyama 2004). The present study reports the half-lives of tralopyril in three different aqueous media but further studies on tralopyril persistence should be performed in order to study the effect of light, pH, dissolved and suspended material, biodegradation, etc., and also to recognize possible persistence of toxic degradation products. For this reason and also because tralopyril was only included in the BPR since 2014 (BPC, 2014), it is still doubtful that environmental levels can already be measured. This is confirmed by the analysis of both sea and riverine water samples (Aveiro harbour, river Glatt) where no detectable tralopyril concentrations were found. Nevertheless, we plan to include tralopyril in further monitoring studies.

Figure 5. Degradation of tralopyril in seawater, river Glatt water and E3 medium

4. Conclusions

Our results show that the method described here is suitable to analyse tralopyril in DMSO, natural sea and riverine waters and E3 medium. Indeed, the major application of this method in the near future will be the analysis of environmental samples. Since tralopyril is already accepted under the Biocide Products Regulation it is expected that its use may rise in Europe as a substitute for other AF-biocides that have been discontinued or banned. Therefore, the method described herein might be used in monitoring programs dedicated to the assessment of water quality. Furthermore, it will be an appropriate and much needed method for obtaining real exposure concentrations in ecotoxicological studies.

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