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Identification of emerging pharmaceutical pollutants in a river impacted by an industrial effluent combining passive sampling and effect-directed analysis

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1. Introduction

In a French river impacted by urban and pharmaceutical wastewater treatment plants, authors reported the occurrence of strong reproductive alterations in fish showing the presence of endocrine disrupting compounds (EDCs) in water [1]. Passive sampling technique (Polar Organic Compound Integrative Sampler, POCIS) combined with *in vitro* mechanism-based bioassays were used to evaluate water contamination. Following the biological activity characterization of POCIS extract, target chemical analysis allowed the identification of several active compounds. Nevertheless, these molecules did not totally explain the biological activities [2]. To address this question, an Effect-Directed Analysis (EDA) method was performed in order to isolate and identify the active chemicals. Firstly, active fractions were analyzed by target chemical analysis allowing the detection of several steroids. However, for many highly active fractions, compounds responsible for these activities remained unknown. In this study, a LC-HRMS system (LC-QTOF) was used to identify these active compounds.

2. Materials and methods

Sample preparation and fractionation process

Investigations were conducted in surface water using polar organic compound integrative sampler (POCIS). POCIS sorbent was extracted using a sequential elution with 10 mL dichloromethane (Cl_2CH_2), 10 mL Cl_2CH_2/methanol mixture (50:50 v/v) and 10 mL methanol. POCIS extract was fractionated by Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) using C18 column. The gradient used was from 80/20 water/acetonitrile (v/v) to 100% acetonitrile at a flow rate of 1 mL/min. 40 fractions (F1 to F40) were collected (each 3 min) [3]. The fractions were evaporated to dryness and redissolved in acetonitrile for chemical analysis or in dimethylsulfoxid for biological tests.

Biological analysis

Table 1 presents the *in vitro* bioassays used to assess endocrine disrupting activities of each fraction.

Receptors	Cell lines	Principle	Reference ligands
Estrogen (**ER**)	MELN	MCF-7, ERE-LUC	17β-E2
Pregnane (**PXR**)	HG5LN-hPXR	GAL4RE-Luc/GAL4-hPXR	SR12813
Glucocorticoid (**GR**)	MDA-kb2)	MDA-MD-453,MMTV-Luc	Dexamethasone
Mineralocorticoid (**MR**)	HG5LN-hMR	GAL4RE-Luc/GAL4-hMR	Aldosterone
Progesterone (**PR**)	HG5LN-hPR	GAL4RE-Luc/GAL4-hPR	R5020

Table 1: **Reporter cell lines used for the detection of EDCs**

Non-target chemical analysis

- *Acquisition*

Non-target screening was performed by liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (Agilent 6540 LC-QTOF) using (data-dependent) auto-MS/MS acquisition mode. In the same run, the most abundant precursor ions are selected from a TOF mode scan and then fragmented in MS/MS mode (Q-TOF). Highly active fractions were analyzed using both positive and negative mode electrospray ionization (ESI).

- *Data analysis*

The data recorded was processed with MassHunter Qualitative Agilent software. Identification procedure starts by comparing compounds detected in the POCIS fraction with those of the corresponding blank fraction. The characterization of compounds was performed on peak only present in POCIS fractions. After

compounds selection, the elemental formula was generated for each candidate using Generate Formulas function of the software. Then, the MS/MS spectra of compound was compare to MS/MS spectra include in spectral libraries (e.g. Metlin, Forensics, MassBank). When no MS/MS spectra were reported, molecular formulas were searched in compound databases (e.g. Chemspider, SciFinder). In order to reduce the suspect list, only the more referenced molecule was selected as candidate. When available, the reference standard was purchased to confirm the identity of the compound.

3. Results and discussion

Sample fractionation allowed the isolation of strong GR and anti-MR activities in 14 fractions (fig 1). Firstly, target chemical analyses were performed on several fractions allowing the confirmation of the strong contribution of several steroids to some activities (e.g. for anti-MR activity: up to 100% for 6-methyl-prednisolone in F11, 65% for dexamethasone in F12, 30% for canrenone in F19 …) [2].

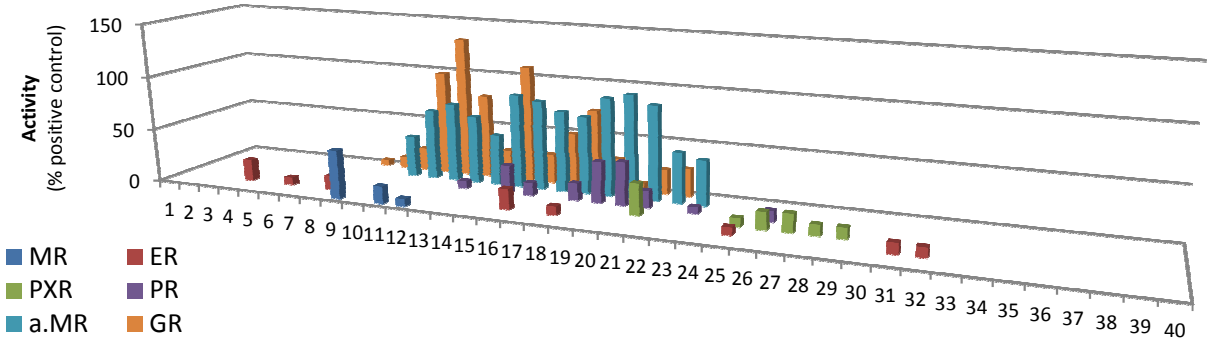

Fig 1: Toxicological profiling of RP-HPLC fractions of POCIS extracts

For many strong active fractions like F15, F20 and F21, compounds responsible for these activities remained unknown. To identify these active compounds a LC-HRMS (LC-QTOF) system was used. 128, 198 and 165 ions were detected in F15, F20 and F21, respectively. Our identification strategy allowed generating a suspect list including drugs, their metabolites and steroids. A selection of compounds was purchased and the confirmation of the identification is still under process. As an example, the presence of a drug used for the treatment of amyotrophic lateral sclerosis, riluzole, was already confirmed by these analyses in the most unexplained active fraction F15. In the same fraction, the presence of desoximetasone, a corticoid compound, was strongly assumed. Indeed, the fragmentation pattern of compound was correlated with MS/MS spectra of reference standard reported in Forensics library with a score higher than 97%. The endocrine disrupting activity of the identified chemicals is under investigations and results will be presented.

4. Conclusions

A strategy combining POCIS with EDA allowed the identification of several steroid target compounds as main contributors of GR and anti-MR activities. For unexplained active fractions, high resolution mass spectrometry was performed and allowed generating a list of candidate compounds. One of them is already structurally confirmed and the confirmation of endocrine disrupting activity is under investigation. For the other candidates structural identification and then the assessment of biological activity are ongoing. Nevertheless, the first investigation into the identification of non target compounds showed the presence of compounds that can have an effect on wildlife. The first results already demonstrated the usefulness of coupling EDA-based strategy with passive sampling technique to identify emerging pharmaceutical pollutants.

5. References

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