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Workshop 3.4

Identifying the causative agents: The use of combined chemical and biological strategies in monitoring programs*

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Abstract: The toxicity identification and evaluation (TIE) approach that identified unconjugated natural and synthetic steroid estrogens to be the major estrogenic component in domestic sewage treatment work effluent is described. Given the increasing popularity of TIE-based approaches in ecotoxicology research, a number of important considerations, limitations, and possible future directions of TIE studies are also discussed.

BACKGROUND

In 1994, Purdom and colleagues reported that all UK effluents were estrogenic to fish [1]. These effluents were mainly domestic (rather than industrial) in source, indicating that the estrogenic component(s) were likely to be domestic in origin, and were probably common to most of them. At the time that this research was initiated, there were no specific examples where an estrogenic effect on wild fish had been conclusively linked to a particular chemical emanating from sewage-treatment works (STW) effluents, although many chemicals (including ethynylestradiol and alkylphenolic chemicals) had been implicated. This project was therefore commissioned by the UK Environment Agency in order to identify and quantify the estrogenic substances present in domestic sewage effluent and, once identified, to assess their effects on fish at environmentally relevant concentrations [2].

GENERAL APPROACH

STW effluent composed of domestic and industrial waste release a complex and ill-defined mixture of chemicals, their metabolites, and biotransformation products into the aquatic environment. Although effluents have been tested for their toxicity to aquatic organisms, few chemicals within an effluent have been tested, on an individual basis, for their toxicity or endocrine-disrupting activity either in vivo or in vitro. As it was clearly not practical to identify, quantify, and test all the individual substances present in effluent, a toxicity identification and evaluation (TIE) approach was employed [3]. Using this bioassay-directed fractionation procedure, STW effluent was chemically separated into fractions of decreasing complexity, which were then assessed for estrogenic activity in vitro. Fractions identified as active in the bioassay were separated further until the causal compounds could be identified by GC/MS. This procedure, through a series of steps involving separation and resolution, simultaneously eliminates inactive compounds in the mixture, and isolates chemicals that are biologically active, without any preconceived ideas about their identity.

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SELECTION OF A SUITABLE BIOASSAY

Selection of a suitable in vitro bioassay for detecting estrogenic chemicals was important if the effluent fractionation scheme was to succeed. It was recognized from an early stage that the bioassay employed would have to be robust, reproducible, sensitive, and specific, with a clear endpoint. Moreover, given the possibility that a large number of fractions would need to be tested (we screened 500 fractions per effluent in triplicate), the bioassay would also need to be rapid, inexpensive to run, and high throughput. This meant that many of the established tests for estrogenicity were unsuitable, particularly those using extended in vivo techniques. At the commencement of the fractionation project we acquired the recombinant yeast estrogen screen [4]. Following validation, it became apparent that the yeast-based system was rapid (four days), sensitive (could consistently detect 2 ng 17β-estradiol/l in the assay medium), specific, reproducible, robust, and high-throughput (32 fractions could be tested in triplicate on a single 96-well microtitre plate), making it an ideal test system to screen large numbers of effluent fractions throughout the project. Moreover, the bioassay detects natural estrogens as well as known xenoestrogens; thus, we expected the bioassay to produce a quantitative measurement of estrogenic compounds regardless of the identity of the chemical (or chemicals) responsible for the estrogenic activity of STW effluent. An important consideration when selecting a bioassay is the amount of sample required to perform the assay, as the more sample that is used for biological testing, the less sample that is subsequently available for further analytical chemistry.

CRITERIA FOR SITE SELECTION AND SAMPLING

Seven domestic STW effluents were selected based on convenience of sample collection, continuity with previous sampling (caged fish) studies and to provide a range of treatment processes. Southend-on-Sea STW was selected for method development as it received only primary treatment, and therefore was expected to contain high concentrations of the estrogenic chemicals under investigation, and would provide a readily measurable response to enable isolation of the estrogenic fraction(s). Discrete samples were taken from each of the STWs, in preference to a 24-h bulked composite sample, as the stabilities of the estrogenic compounds at this stage were unknown. However, the consistency of the estrogenic activity in each effluent sample was determined on three separate occasions.

COARSE FRACTIONATION OF SEWAGE EFFLUENT

Neat ("whole" sample) effluent was collected and tested for estrogenicity, after which it was crudely fractionated to determine the contribution of the particulate ("filtered" sample) and volatile phase ("purged" sample) to the overall estrogenicity of the effluent. The effluent was also tested after C18 extraction in order to evaluate the contribution of organic compounds in the sample to the estrogenic activity. For the assay controls, the same procedure was carried out using distilled, deionized water to provide a "procedural blank", which was analyzed alongside the effluent fraction at each stage. At the five-fold dilution, the "whole" effluent sample from Southend-on-Sea contained estrogenic activity equivalent to approximately 10 ng 17β-estradiol/l. Our results also indicated that the estrogenic activity was mainly present in the dissolved phase, as extraction of the effluent liquid phase using a C18 SPE cartridge caused a >80 % reduction in estrogenic activity compared with the "whole" effluent.

SOLID-PHASE EXTRACTION USING C18 COLUMNS

Solid-phase extraction (SPE) columns containing C18 reversibly bind a broad range of organic compounds, which can be eluted with a range of different solvents according to their polarity, enabling the selective removal, and concentration of organic compounds from complex mixtures. By controlling the elution sequence of the compounds retained on the C18 matrix, the biologically active components can

be isolated within discrete fractions appropriate for higher-resolution procedures. Since most of the activity was retained on the C18 column, it appeared that the active compounds were not inorganic substances (which would not bind), but were organic substances. The organic compounds were eluted from the C18 column using a series of volumes of methanol/water mixtures (0, 25, 50, 75, 80, 85, 90, 95, and 100 % methanol), which were collected in separate vials. The estrogenic activity was found to elute within the 50 to 85 % methanol fractions. Elution of the SPE cartridges with solvents of low polarity to nonpolar did not yield any further activity, indicating that the compounds were mid-polar organics. However, as effluent contains numerous mid-polar organic constituents, which would elute in a similar manner on the C18 column, we could not ascertain whether the chemical(s) responsible for the estrogenic activity were similar at each site at this stage.

FINE FRACTIONATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Following their assessment for estrogenic activity, the active fractions were combined and reconcentrated prior to the next fractionation stage using high-performance liquid chromatography (HPLC). Gradient elution (40–100 % methanol) was employed to separate organic chemicals of medium polarity, so that the most polar (weakly retained) compounds were eluted before those of low polarity (more strongly retained). As the sample components were eluted from the separating column by the carrying solvent, they were monitored through an ultra-violet (UV) detector, and discrete fractions were collected at 1.5 or 1 min intervals over 45 min into separate vials which were then assessed for estrogenic activity. All the effluent samples analyzed produced a single highly estrogenic fraction, which eluted approximately 25 min after the start of the HPLC run, which suggested that the activity was due to a single compound, or a set of closely related compounds, that were eluted concurrently using the HPLC conditions employed. The UV profile of the chromatographic run indicated that the sample was still a highly complex mixture. Reversed-phase HPLC, at this stage, was unable to resolve the mixture into fractions composed of a few identifiable compounds. It was therefore impossible to determine whether the activity within the fraction was due to a highly abundant component, or whether it was due to minor components concealed within the UV profile. Attempts to identify the components present in the fraction by HPLC/MS were inconclusive, due to the complex nature of the mixture.

ANALYSIS OF ESTROGENIC FRACTION USING GC/MS

The active components within the effluent sample were transferable, by liquid/liquid extraction from the methanol/water HPLC fractions into DCM (nonpolar solvent), enabling their analysis by gas chromatography/mass spectrometry (GC/MS). The major components of the extracts were found to be a mixture of isomers of α-terpineol (a terpenoid alcohol widely used in detergents and cosmetics), which had been previously identified in sewage effluent. There were also around 20 unidentified minor components that occurred in all three extracts. Standards of the identified terpineol, of both pure and technical (an isomeric mixture) grade were purchased, and solutions spanning the concentration range present in the fraction were tested for estrogenicity in the yeast screen. None of the terpineols tested were found to be active in the yeast screen. Following the elimination of terpineols as the estrogenic components in the active fraction, it was considered necessary to focus on the large number of potentially active trace compounds. An additional fractionation step was developed using a "shallower" (55–60 % methanol) gradient in order to reduce the sample complexity further. Using the shallow HPLC elution gradient, the active fraction from the first HPLC run was resolved into two separate peaks of estrogenic activity, eluting between 29–31 and 31–33 min, respectively. The total estrogenic activity in the combined active samples was equivalent to around 100 ng 17β-E2/l in the neat effluent, indicating that the minor components in the fraction were potent estrogenic compounds. At this stage, it was also noted that the active fraction behaved in the yeast screen in a manner similar to steroid estrogens, in which

assay color development occurred rapidly relative to the xenoestrogens that we had tested. Based on this observation, it was postulated that the estrogenic component might be a steroidal compound.

BEHAVIOR OF STEROIDS IN THE FRACTIONATION SYSTEM

The behavior of steroids in the fractionation system was evaluated using the synthetic estrogen, ethynylestradiol (EE2). If steroids (or steroid-like compounds) were responsible for the activity in the effluent, we would expect their chromatographic retention time to coincide with that of the active fraction. Therefore, in separate chromatographic runs, EE2, procedural blanks and the active effluent fraction were injected onto the HPLC column, and fractions were collected at 1 min intervals using both gradients. The EE2 standard co-eluted with the estrogenic activity present in the effluent samples using the 40–100 % methanol gradient. In the 55–60 % gradient run, the EE2 standard coincided with the second peak of activity in the effluent sample. This result was confirmed using a standard solution containing estrone (E1), 17β-estradiol (E2) and EE2, which was analyzed under the same GC/MS conditions. The elution time of the standard compounds coincided with retention time of the peaks identified in the effluent extract. These results indicated that estrogenic steroids could be responsible for the estrogenic activity in domestic STW effluent. In both cases, the blanks were inactive, as expected.

IDENTIFICATION AND QUANTIFICATION OF ESTROGENIC COMPOUNDS BY GC/MS

Working on the premise that trace amounts of steroidal compounds were responsible for the estrogenicity observed in the effluent fraction, it was necessary to work with larger volumes of effluent (20 l) in order to concentrate the effluent samples further, and obtain sufficient quantities of substance to enable identification by GC/MS. The standard GC/MS library searching routine identified potent natural and synthetic steroidal estrogens in the extract, namely E1, E2, and EE2; all present in an unconjugated (free) biologically active form. Potential improvements to the GC/MS method, such as creating silyl derivatives of the sterols (less polar compounds) which were more amenable to gas chromatography, resulted in an improved peak shape for the steroids. However, this advantage was largely offset by a loss of sample during the additional manipulation. The results indicated that estrone and 17β-estradiol were present in the effluents at concentrations ranging from 1 ng/l up to 80 ng/l and 50 ng/l, respectively. Ethynylestradiol (up to 7 ng/l) was detected in one third of the samples collected, and was associated with effluents containing the highest steroid loads.

CONCLUSIONS

In this project, a TIE approach, modified for our specific needs, identified natural and synthetic steroidal estrogens as candidate compounds responsible for the estrogenic activity observed in domestic STW effluent [5]. Proficient chemical manipulation of the effluent sample, together with the use of a rapid, robust and high-throughput bioassay were key factors responsible for the success of the project. Despite the complexity of the composition of domestic effluents, only a small number of highly estrogenic compounds were identified. Three estrogenic steroids (E2, E1, and EE2) were positively identified in the active effluent fractions tested in the ng/l range. None of the activity identified was found to be associated with alkylphenolic chemicals. This finding was supported by direct measurements, indicating that alkylphenols, when present, were below the threshold for detection in the yeast screen.

The results suggested that the concentrations of EE2 detected in most of the samples were too low to account for the magnitude of the vitellogenic response observed in male fish exposed to domestic STW effluents. There was also little, if any, data to address the issue of sensitivity of fish exposed to natural estrogens via the water at the time. Hence, it was not possible to conclude whether the concentrations of E2 and E1, reported in this study, would, or would not, be estrogenic to fish. To address some of these issues, and thus put the results obtained from the fractionation studies into an environmental

context, in vivo laboratory tank trials were conducted in which rainbow trout and roach were exposed to low concentrations of steroid estrogens via the water. The results of these exposures demonstrated that the levels of steroidal estrogens present in STW effluent were estrogenic to fish, and that their effects were additive [6]. The previously reported observations of estrogenic activity in domestic effluents and some rivers could therefore be attributed to these hormones.

LIMITATIONS OF THE TIE APPROACH

The fractionation procedures described here are time-consuming and have a number of drawbacks. Toxicity was a problem associated with certain effluent fractions, which may have prevented the detection of estrogenic components within the samples. For example, a tentative identification of an estrogenic response at one concentration in the bioassay, may have been followed by a toxic effect at a higher concentration. Therefore, all fractions were tested using a range of volumes in an attempt to avoid complications associated with toxicity, and to observe dose-response effects. Changes in toxicity could result with each chemical manipulation of the sample, due to alterations in the bioavailability of toxins. Conversely, interactive effects may be reduced, as the mixtures were resolved into single components.

The results from the domestic effluent fractionation procedure illustrated that the majority of the activity in vitro occurred within a single fraction associated with natural steroidal estrogens. However, further increases in the concentration of the HPLC fractions tested in the yeast screen resulted in the appearance of other more weakly active fractions, which were also eluted in the mid-polar range. Most of the synthetic estrogens identified to date are reported to be lipophilic compounds which bioaccumulate in whole animals. Therefore, the potential contribution of these weakly active fractions to the estrogenic activity in vivo must not be underestimated, particularly in view of the fact that biological effects can be augmented by bioaccumulation, biomagnification, metabolism, and pharmacokinetics. Thus, the biological activity of a compound in vivo may be two or more orders or magnitude greater than they appear in vitro. It is therefore possible that in vitro assays may inherently underestimate the true environmental relevance of the "weaker" active components in the sample.

TIEs—THE WAY FORWARD

Given the power of TIE approaches to identify causal compound in complex mixtures, it is likely that future TIE studies will concentrate on samples with very different compositions (including landfill leachates, industrial effluents, agricultural runoffs, and pulp-mill effluents). New developments in bioassays will also broaden the range of measurable endpoints; many of which may be determined simultaneously using microarrays. In silico (QSAR) technology may assist in the identification of unknown metabolites and biotransformation products with inadvertent biological activity. Small-scale, short-term, in vivo assays (employing early life stage) may be developed for TIE studies using existing or novel model species, including invertebrates.

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