THE USE OF THE DR CALUX® ASSAY FOR IDENTIFICATION OF NOVEL RISKS

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Introduction

History has shown that novel risks are always detected by their effects in animals and humans. Incidents subsequently resulted in the identification of a novel compound or the presence of a known compound at an unexpected place. Dead and diseased chickens actually led to the discovery of dioxins in fat drippings of cow hides treated with chlorophenols[1]. Later on, in 1999, effects in chickens resulted in the discovery of the mixing of over 200 kg PCB-oil in fat used for animal feed[2,3]. But even very recently, the presence of melamine in dog feed was only detected after several animals had died. It is clear that this is not a desirable situation and that screening of samples should ideally prevent the death or illness of animals and humans. In this regard, chemical analytical methods are suitable for the detection of known toxic compounds. However, the identification of novel emerging risks can only be guaranteed by the use of bioassays. At the same time there is a serious fear that the application of bioassays will result in many false-positive results, since not every compound showing a positive response, is necessarily a risk for the consumer. This requires the development of proper strategies in order to discriminate between potential risks and real risks, including the rapid identification of the bioactive compounds.

The DR CALUX® has shown its value as a screening tool for dioxins and dl-PCBs in the food chain and the environment. This is primarily based on its high-throughput properties and relatively simple sample clean-up, making it a cheap alternative for the GC-HRMS standard method. In 2007 its application resulted in the discovery of dioxins in fat due to the use of contaminated hydrochloric acid for the production of gelatin[4]. However, this assay, although designed for dioxins and dl-PCBs, can detect also other compounds that bind to the Ah-receptor and subsequently activate the Ah-receptor pathway. This may include other more persistent compounds like brominated dioxins and PBBs but also less persistent pollutants like polyaromatic hydrocarbons, and even various natural compounds present in food. The latter includes e.g. various metabolites of indole-3-carbinol formed in the stomach and the group of furocoumarins present in various vegetables and citrus fruits. In practice, the clean-up procedure based on an acid silica clean-up, sometimes followed by an activated carbon column, determines the selectivity of the assay for some of the more persistent chemicals.

The application of this bioassay for the potential detection of novel emerging risks, threatening our food chain and potentially our health, offers another valuable feature of the application of bioassays in combination with sophisticated confirmation techniques. Recently we used the bioassay to identify the furocoumarin bergapten as the compound responsible for the positive response of marmalade extracts[5]. The present paper presents two cases where the screening of samples resulted in a positive screening result that was not supported by the GC/HRMS confirmation method. An approach is suggested for the elucidation of the bioactive compounds in these samples, which may potentially present a risk for the consumer.

Materials and Methods

DR CALUX® assay

Grass samples were mixed with methanol/water (85/15) and extracted with hexane/diethyl ether (97/3). The extract was reduced to a small volume and eluted on an acid silica column using hexane/diethyl ether. The eluate was dried in the presence of 40 µl DMSO, mixed with culture medium and applied to the cells. After 24 h the luciferase was released from the cells and the concentration determined. Each test series contained a number of reference samples with a known amount of dioxins.

Purification of extracts

Extracts eluting from the acid silica column were transferred to a Florisil column, that was eluted with hexane (mo-PCBs), hexane/dichloromethane (10/90 v/v) (no-PCBs) and dichloromethane (dioxins). Eluates were mixed with 40 µl DMSO, evaporated under nitrogen and than mixed with culture medium. In a second approach, acid silica eluates were evaporated until approximately 50 µL, redissolved into 1 mL of pure hexane and purified via an aluminium oxide column. Columns were washed and the extract quantitatively transferred to the columns and

then eluted with hexane, dichloromethane/hexane 10/90 (v/v) and dichloromethane. The 3 fractions were solvent exchanged into 40 µL of DMSO under nitrogen and mixed with 2mL culture medium.

HRGC/HRMS analysis of the standards

Samples were analysed for dioxins and dl-PCBs as described previously. PAHs were also analysed by GC/HRMS. Data were transferred to BaP-equivalents using relative potency factors based on the carcinogenic potency of the different congeners[6].

Results

RIKILT applied the DR CALUX® assay for about ten years. Responses obtained with the assay are compared to a set of reference samples and declared negative or suspected, the latter requiring confirmation by GC/HRMS. During this period several cases were obtained with false-positive results that indicate the presence of other compounds able to bind to the Ah-receptor. It should be stressed that contrary to e.g. the furocoumarins present in citrus fruits[5], the compounds responsible for the false-positive response survive the acid silica clean-up step.

Grass samples

RIKILT routinely uses the DR CALUX® assay to screen a number of dried grass and lucerne samples used as animal feed. This frequently results in samples that show an elevated response but rarely these samples contain elevated levels of dioxins or dioxin-like PCBs. Figure 1 shows a comparison of levels of PAHs and the response obtained in the DR CALUX® assay in samples tested in 2001. The response in the bioassay is expressed as the ratio of the response obtained with the sample and that of a citrus pulp sample containing 0.5 ng TEQ/kg. Since the lighter PAHs show only a poor response in the bioassay, we applied a kind of TEQ-principle on the levels of PAHs, based on their relative carcinogenic potency. When comparing the absolute levels of PAHs with the DR CALUX® response, a similar correlation is obtaind. It is evident that the correlation is rather poor and it seems unlikely that PAHs are responsible for the positive response. Furthermore, spiking of grass samples with high levels of PAHs like benzo(a)pyrene, benzo(k)fluoranthene, dibenzo(a,h)anthracene and chrysene showed that these PAHs do not pass the acid silica columns routinely used for the clean-up of samples.

For identification of the bioactive components, acid silica extracts were further purified on a Florisil column, using a procedure that separates dioxins from dioxin-like non-otho and mono-ortho PCBs. Only the extract that normally contains the dioxins showed a positive response. However, when the acid silica eluates were purified on an aluminium oxide column, part of the bioactive compounds eluted in the hexane extract, similar to dioxins and dl-PCBs. A similar amount ended up in the hexane/dichloromethane extract. Further studies are required to identify the responsible components.

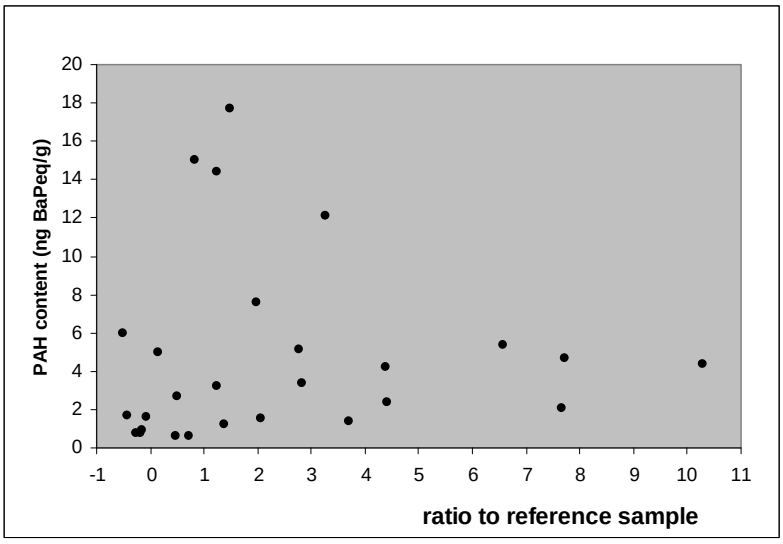

Figure 1. Comparison of the response in the DR CALUX® assay, in relation to a citrus pulp sample with 0.5 ng TEQ/kg, and the level of PAHs expressed in BaP-equivalents.

Table 1. Levels of PAHs, dioxins and dl-PCBs in 33 grass samples in comparison to the response in the DR CALUX® assay. The DR CALUX® response was transferred to bioassay equivalents (BEq) based on their relative response to a set of feed samples. GC/HRMS results represent lower bound results. PAH results were transferred to BaP equivalents.

Sample	PAHs µg BaPEQ/kg	PCDD/Fs ng TEQ/kg	dl-PCBs ng TEQ/kg	Total TEQ ng TEQ/kg	CALUX ng Beq/kg
1	1.1	0.0	0.1	0.1	0.0
2	3.0	0.0	0.1	0.2	0.0
3	41.2	0.0	0.1	0.1	2.4
4	0.0	0.0	0.0	0.0	0.0
5	1.9	0.0	0.1	0.1	0.0
6	0.0	0.0	0.1	0.1	0.0
7	0.2	0.0	0.1	0.1	0.4
8	11.6	0.1	0.1	0.2	0.8
9	0.1	0.0	0.1	0.1	0.0
10	1.0	0.0	0.0	0.0	0.0
11	4.3	0.0	0.1	0.1	0.2
12	2.5	0.0	0.1	0.1	1.9
13	2.1	0.0	0.0	0.0	0.0
14	1.7	0.0	0.1	0.1	0.0
15	1.8	0.0	0.1	0.1	2.9
16	2.4	0.0	0.1	0.1	0.0
17	0.1	0.0	0.0	0.0	0.0
18	0.0	0.0	0.0	0.0	0.0
19	2.9	0.0	0.2	0.2	0.0
20	12.6	0.2	0.3	0.6	2.3
21	0.0	0.0	0.1	0.1	0.3
22	1.6	0.1	0.1	0.2	0.7
23	0.8	0.0	0.1	0.1	0.2
24	0.1	0.0	0.1	0.1	0.1
25	2.7	0.0	0.1	0.1	0.4
26	2.7	0.0	0.1	0.1	0.2
27	12.5	0.1	0.1	0.2	4.0
28	48.8	0.2	0.3	0.5	3.3
29	40.8	0.3	0.3	0.6	3.5
30	20.3	0.4	0.3	0.6	1.9
31	19.8	0.2	0.3	0.5	1.9
32	10.2	0.4	0.3	0.6	2.7
33	2.3	0.0	0.1	0.1	0.1

In 2006 RIKILT analyzed grass samples collected close to freeways for the presence of PAHs, dioxins and dl-PCBs. Some of the samples contained elevated levels of PAHs and in some cases slightly elevated levels of dioxins and dl-PCBs (Table 1). Testing of these samples with the DR CALUX® assay revealed relatively high responses in the DR CALUX® assay, which could not be accounted for by the levels of dioxins and dl-PCBs. In this case, the samples with the higher PAH levels show the higher CALUX responses, but the relationship is not conclusive (e.g. sample 8). These results indicate that in the case of dried samples, the drying process is not necessarily responsible for the result but that the samples were already contaminated before the drying.

Cholin chloride

In 2008 RIKILT tested two cholin chloride samples that showed an elevated response in the DR CALUX® assay. Cholin chloride is a widely used feed additive. Comparison of the response with that of a set of spiked feed samples indicated levels of 5-6 ng TEQ/kg (Figure 2). However, GC/HRMS analysis did not show the presence of dioxins or dl-PCBs. Further fractionation over aluminium oxide showed that the bioactive compounds eluted in the first hexane fraction. This is comparable to the behavior of chlorinated but also brominated dioxins. Initial data obtained with GCxGC/TOFMS indicate the presence of brominated compounds in these samples, including

bromophenols, known precursors of brominated dioxins. However, it remains to be elucidated whether these brominated compounds are also responsible for the positive response in the bioassay.

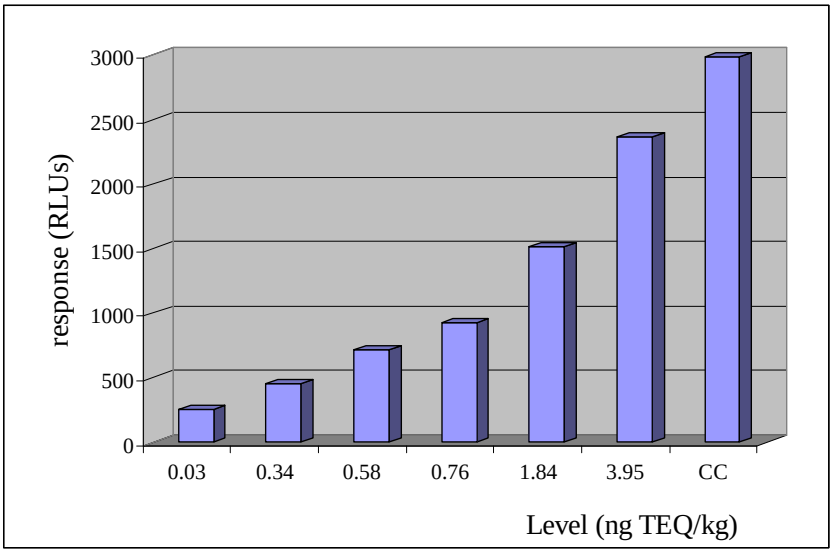

Figure 2. Response obtained in the DR CALUX® assay with a sample of cholin chloride in comparison to that of the reference feed samples, spiked with dioxins and dl-PCBs

Discussion

The use of bioassays, and more particular the DR CALUX® bioassay, for the identification of potential risks requires a sound strategy. The easiest approach is the identification in the final extracts of compounds that are already known to cause a positive response in the bioassay. This requires e.g. a library of compounds known to show a positive response in the test including information on their behavior during the clean-up of samples. Secondly, a strategy is required for the identification of previously unknown substances with dioxin-like properties.

Following identification of the bioactive compounds, further studies can be performed to evaluate the toxicity of the compounds and the potential significance for the consumer. Again, it should be stressed that not every compound causing a positive test result is necessarily a risk to the consumer. In addition to binding to the Ah-receptor, the resistance to degradation and accumulation of dioxins and dl-PCBs in the body is a very important factor in their toxicity. However, a positive response in the bioassay appears to fulfill at least one of the requirements for their adverse effects and should be an important trigger for follow-up studies.

Conclusion

The application of bioassays is not only a cheap and rapid alternative for sophisticated analytical methods, but also allows the elucidation of potential novel risks that may endanger the health of animals and people.

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