ELIMINATION OF PRAZIQUANTEL FROM WASTE WATERS BOTH IN LABORATORY AND REAL SCALE

MARSIK P., PODLIPNA R. and VANEK T.

Laboratory of Plant Biotechnologies, Institute of Experimental Botany AS CR, Rozvojova 313, Prague 6 – Lysolaje, 165 02, Czech Republic
E-mail : vanek@ueb.cas.cz

ABSTRACT

Accumulation and/or degradation of Praziquantel (PZQ) in plant organs was determined using *Phragmites australis in vitro* cultivated plants. The changes of PZQ content in cultivation media was monitored by HPLC analysis with PDA detection. In case of starting PZQ concentration 20 mg/l, 90 % was removed from liquid media within 21 days. Control blank samples (cultivation media without plants) showed no decrease of PZQ concentration caused by biotic degradation during the experimental period.

For the proving of PZQ uptake and metabolism in plant tissues, the qualitative LC/MS/MS analysis followed with LC/MS/MS HRES of leaf extract of in vitro growing *P. australis* was carried out. The analysis showed, that the PZQ can be transported to the aerial organs in plants.

Moreover, the results proved that the accumulated PZQ was partly metabolized in tissues. Twenty one potential metabolites were identified by LC/MS/MS analysis of the leaf extract. There were found compounds derived from both phase I and II of detoxification metabolism which corresponded to products of metabolic steps, which were previously described for other eukaryotic organisms. They include dehydrogenated, mono- and di-hydroxylated derivatives and their glycosylated (hexose) conjugates. The majority of the identified metabolites was belonged to monohydroxylated derivatives with prevalence of them with hydroxyl group on cyclohexyl moiety of the molecules. The compounds with hydroxyl group bounded to izoquinoline part were rarely occurred in the extract. Ions with mass corresponding to the PZQ hydroxylated products with loss of two hydrogens was also identified. The majority of sugar conjugates (II step metabolites) belong to mono-hexosylated metabolites; there was found only one assumed di-hexosylated compound in the trace concentration.

Laboratory results were confirmed in real conditions in the constructed wetland (CW), where PZQ content was determined in the waste water applied to the system. The system offers a promising possibility to use CW for PZQ removal from agricultural as well as domestic waste-waters. The amount of PZQ in CW decreased in dependence on duration of PZQ treatment and in the direction from inflow to outflow (10 sampling points). The PZQ concentration in water reached the detection limit after sampling point 7, but the majority of the PZQ was removed after the first three sampling points.

Keywords: praziquantel, waste water, constructed wetland, plants, *Phragmites*, metabolism, LC/MS/MS, *in vitro*

1. Introduction

At present, the global production of the human and veterinary pharmaceuticals strongly increases, together with their accessibility not only in developed countries, but due their availability and decreasing prices also in the Third World countries. Pharmaceutical pollution, however, is increasing especially in developed and industrial countries with high living standard and population

density. One of the most significant problems is persistence of the pharmaceutical residues and their metabolites in the environment. Many of them do not exhibit acute toxicity for water ecosystems, but have a cumulative effect on non-target organisms.

There are only a few reports concerning degradation of veterinary drugs in plants. Carvalho *et al*. (2014) give in their review an in-depth overview of the phytotoxicity of pharmaceuticals, their uptake and their removal by plants. Plant in vitro cultures can serve as suitable models to elucidate the mechanisms of uptake or/and transformation of different xenobiotics. In our previously study we described uptake and biotransformation of benzimidazole anthelmintics Albendazole and Flubendazole by reed cells (Podlipna *et al*., 2013). Praziquantel (PZQ) is a widely used anthelmitic drug against trematodes and Schistosomes both for human and veterinary purposes. It is included in the WHO Model List of Essential Drugs and global need is estimated to 424 million of tablets per year (http://apps.who.int/medicinedocs/en/d/Jwhozip48e/10.1.html), so environmental consequences can be serious. Fortunately, according to the recent literature (Mister I. *et al* 2014), these metabolites are significantly less active that starting PZQ, so they exhibit much lower effect on the environment and food chain than parent compound.

Mono- and di-oxidized PZQ were identified as the main metabolites using liver microsomes (Huang *et al* 2010), isolated rat hepatocytes (Meier and Blaschke 2001) and in kingfish (Tubs *et al* 2008). However, the complete metabolite profile of PZQ remains unclear, including the identity of the phase II metabolites of PZQ (Wang *et al* 2014). The aim of this study has been evaluation of the plant potential to remove, accumulate and/or degrade PZQ, both in laboratory and real conditions and to prove the possibility of utilization of biological systems to clean both agricultural and domestic waste-waters.

2. Material and methods

Cultivation of *in vitro* plant culture of reed.

The seeds of reed (*Phragmites australis* (Cav.) Trin. ex Steud.) were obtained from wildly growing plants in the area of central Bohemia. The seeds were cleaned in 70 % ethanol for 1 min and sterilized by 1 % sodium hypochlorite supplemented by TWEEN 20 for 10 min. They were rinsed 3 times in sterile water and put on hormone-free solid Hoagland medium (Hoagland, 1920) with 40 g l^{-1} sucrose. Then they were cultivated aseptically in Magenta boxes under 16 hours light period at 24°C with 4-week subculture period. PZQ (in DMSO) was added in two concentrations (20 and 200 mg l^{-1}); the corresponding amount of DMSO was applied to the control plants. In blank samples, the medium containing the PZQ without plants was incubated.

Sample preparation and PZQ quantitation in laboratory experiments.

The explants were incubated with PZQ (20 and 200 mg l^{-1}) for 21 days. Three flasks for each time point and drug concentration were used. After the incubation, medium was taken up, placed into plastic tubes, frozen, and stored at -80°C. The washed plant organs were lyophilized, homogenized and extracted by orbital shaker in 10 ml methanol for 24 hours. The filtered extract was evaporated under vacuum and the solid residue was dissolved in 2 ml of methanol.

The medium was then applied to a SEP-PAK C_{18} cartridge (Waters, USA), eluted with 10 ml methanol and evaporated to dryness at 35°C under vacuum. The solid residue was dissolved in 1 mL methanol.

Content of PZQ was determined by HPLC on a reverse phase SiC18Biospher packed stainless steel column (250 × 4 mm). An isocratic gradient of acetonitrile (70 %) was applied for 10 min at a flow rate 1 ml min^{-1}. PZQ was determined by comparison of its spectra and retention time in extract samples with commercial standard using a PDA detector Jasco MD 1510. The quantitation was carried out at 217 nm.

Qualitative analysis of PZQ degradation products.

Main classes of degradation products of the first step metabolism of PZQ in plant tissues were analyzed using LC/MS/MS consisting of triple quadrupole mass spectrometer Q-Trap 4000 (AB Sciex, USA) and chromatographic unit Ultimate 3000 (Dionex, USA). Measurement was carried out on ESI source in positive mode, which gave more complex fragmentation and better sensitivity than negative mode. The metabolites were separated on Kinetex Phenyl-hexyl column (100 x 2.1 mm, 1.7µm, Phenomenex, USA) by using of mobile phase consisting of water with 5mM ammonium formate (A) and MeOH with formic acid (0.25% v/v) with flow rate 250 µl/min. Gradient elution was kept at 20% B, in 1 min, then it was increased to 100% B in 5 min and kept for 3 min. The system was equilibrated for 5 min. before the next analysis. Column temperature was set at 30°C.

MS conditions were optimized according to the modified method by Huang *et al*. (2010). The metabolite content in the extract was monitored using specific MRM transitions selected for the expected PZQ metabolites described before (Huang *et al*., 2010; Wang *et al*., 2014). At least three transitions were selected for each precursor ion. For the verification base on the MS/MS spectra interpretation, the IDA method (information dependent acquisition) was used. The exact mass of the detected ions was confirmed using the independent UPLC system working with identical chromatographic conditions coupled with HRES mass quadrupole Orbitrap spectrometer Q-Exactive (Thermo, USA).

Samples were prepared by the same way as those for HPLC/PDA quantitation. The samples were 10-time dissolved by mobile phase prior the analysis and 5 µl was injected into the system.

Quantitative analysis of PZQ in field experiments

The water samples (1 l) were collected in the selected places of hybrid CW and stored at -18°C. The water was filtered using nylon membrane filter (0.45 µm, Cronus) under vacuum and aliquot of 250 ml was then acidified with acetic acid (pH 2.5) and applied to SPE Oasis HLB cartridges (200 mg, Waters, USA). The polar impurities were removed by 10 ml of acidified water and PZQ was eluted with 10 ml of MeOH. After evaporation by stream of N_2, the samples were dissolved with mobile phase before the analysis. The reed plant samples were collected at the end of both horizontal and vertical CW unit. Lyophilized tissues were ground to fine powder and about 0.2 g of the sample was 3 times extracted with 1.5 ml MeOH by sonication followed by shaking (1h). Extracts were centrifuged (10 min, 13000 rpm, centrifuge Hettich Universal 32 R, Germany) and the supernatant was evaporated under nitrogen. The dried crude extract was then dissolved in water, acidified to pH of 2.5 and purified by SPE by the same way as the water samples described above. For the quantitation, the commercial standard (purity of >98%, VETERANAL^{TM}, Sigma, Czech Republic) was used.

Quantitative analysis of water and plant samples collected in CW was carried out using the same LC/MS/MS instrumentation (Q-Trap 4000, AB Sciex, USA) as it was used for the qualitative analysis.

3. Results

The degradation of PZQ in in vitro system started 3 days after the application. The decrease of PZQ concentration was slower for the higher initial concentrations (200 $mg.l^{-1}$). The application of lower PZQ concentration (20 $mg.l^{-1}$ led to 90% decrease of PZQ in the medium (Fig. 1). After the experiments the plants exhibited no symptoms of stress.

In order to eliminate potential PZQ metabolites of non-plant origin (e.g. microbial), PZQ degradation products were isolated from aerial parts reed plants cultivated in vitro conditions. The potential metabolites were selected on the basis of molecular masses of predicted PZQ products involved in the phase I of xenobiotic detoxification. Only the compounds, which exact mass was in accordance with standard retention times in the independent UPLC conditions, were considered to be potential PZQ metabolites. This additional verification restricted the number of proposed metabolites to

several products of the preferred detoxification steps typical for plant organisms. Monohydroxylated PZQ derivatives were found as the most frequently occurred group. Among them, the oxidation products with the hydroxyl group situated on the cyclohexyl moiety with characteristic product ion 203 were the most abundant, while the oxidation on izoquinoline part (typical product ions 201 and 219) was less preferred (Wang *et al*., 2014). Three proposed di-oxidized PZQ metabolites with hydroxyl groups on both cyclohexyl ring as well as izoquinoline skeleton were also detected. Number of detected ions corresponding to the hydroxylated PZQ with loss of two hydrogens probably resulted from subsequent dehydrogenation described for I detoxification step of PZQ in mammals (Huang *et al*., 2010; Wang *et al*., 2014). The sugar conjugates of all proposed I step metabolites were detected previously as mono-hexosylated compounds; only one assumed di-hexosylated ion of dehydrogenated monohydroxyl PZQ was found with very low relative abundance.

The PZQ content in the water of the CW decreased in dependence on duration of PZQ treatment and in the direction from inflow (sampling point 1) to outflow (sampling point 10). It was below detection limit after sampling point 7.

The content of PZQ in tissues of the exposed plants growing in CW was measured in roots, stems and leaves of common reed (*P. australis*). The content of the PZQ in the roots varied from 17 to 1502 ng.mg^{-1} DW and in the leaves was almost comparable (10-1502 ng.mg^{-1} DW).

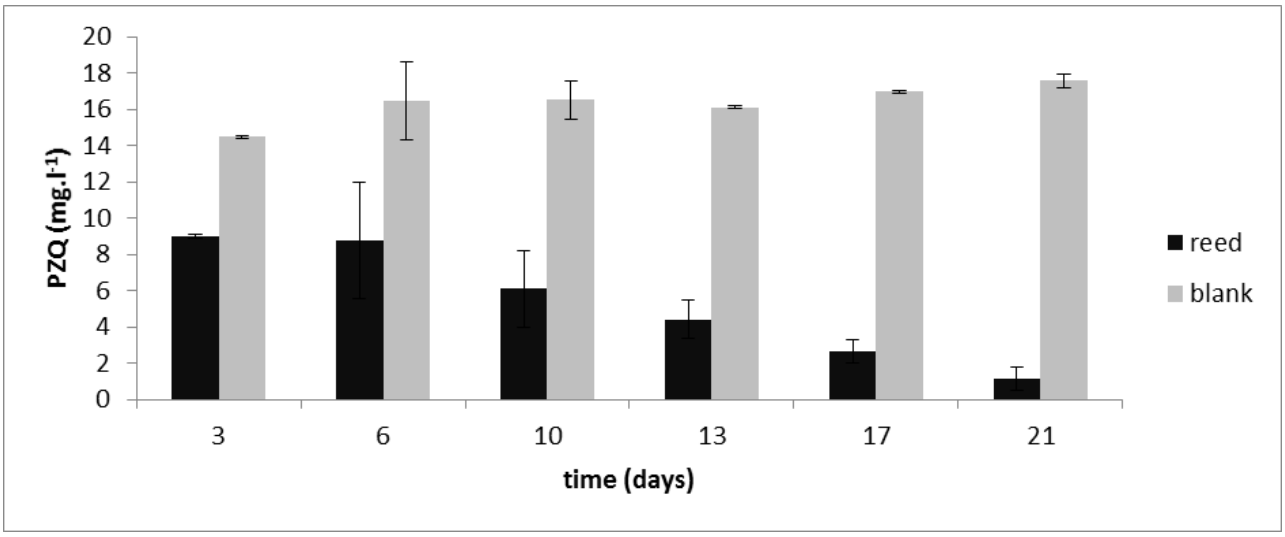

Figure 1: Uptake of PZQ (initial concentration 20 mg.l^{-1}) from liquid medium by reed (*Phragmites australis* (Cav.) Trin. ex Steud.) cultures in vitro. The blank represents the control boxes without plants.

4. Conclusions

In laboratory conditions, up to 90% of PZQ applied at concentration 20 mg/l, which is well above the real concentrations, was up-taken by the *Pragmites australis* plants, and thus removed from liquid media. Moreover, more than 30% of accumulated PZQ was further metabolised (21 identified products), both Phase I and II detoxification metabolisms. Taking into account that PZQ metabolites are generally less active than the starting compound (Mister *et al* 2014), much lower effects of these derivatives to the environment and food chain can be expected in comparison with the parent compound.

The achieved results thus prove the high potential of this phytoremediation system for the removal and detoxification of Praziquantel, both from agriculture and domestic waste waters.

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REFERENCES

1. Carvalho P. N., Basto M. C. P., Almeida C. M. R. and Brix H. (2014), A review of plant-pharmaceutical interactions: from uptake and effects in crop plants to phytoremediation in constructed wetlands: Environ. Sci. Pollut. R., **21**, 11729-11763.
2. Hoagland D. R. (1920), Special articles - Optimum nutrient solutions for plants. Science, **52**, 562-564.
3. Huang J., Bathena S.P.R. and Alnout Y. (2010), Metabolite profiling of praziquantel and its analogs during the analysis of in vitro metabolic stability using information-dependent acquisition on a hybrid triple quadrupole linear ion trap mass spectrometer. Drug Metab. Pharmacokinet., **25**, 487–499.
4. Meier H. and Blaschke G. (2001), Investigation of praziquantel metabolism in isolated rat hepatocytes. J. Pharm. Biomed. Anal., **26**, 409–15.
5. Meister I., Ingram-Sieber K., Cowan N., Todd M., Robertson M., Meli C., Patra M., Gasser G. and Keiser J. (2014), Activity of Praziquantel Enantiomers and Main Metabolites against Schistosoma mansoni. Antimicrob. Agents Ch., **58**, 5466–5472.
6. Podlipna R., Skalova L., Seidlova H., Szotakova B., Kubicek V., Stuchlikova L., Jirasko R., Vanek T. and Vokral I. (2013), Biotransformation of benzimidazole anthelmintics in reed (Phragmites australis) as a potential tool for their detoxification in environment. Bioresource Technol., **144**, 216-224.
7. Tubbs L., Mathieson T. and Tingle M. (2008), Metabolism of praziquantel in kingfish Seriola lalandi. Dis. Aquat. Org., **78**, 225-233.
8. Wang H., Fang Z.-Z. Zheng Y., Zhou, K., Hu Ch., Krausz K.W., Sun D., Idle J.R. and Gonzalez F.J. (2014), Metabolic profiling of praziquantel enantiomers. Biochem. Pharmacol., **90**, 166-178.