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Flatworm models in pharmacological research: The importance of compound stability testing

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ABSTRACT

Flatworms possess adult pluripotent stem cells, which make them extraordinary experimental model organisms to assess *in vivo* the undesirable effects of substances on stem cells. Currently, quality practices, implying evaluation of the stability of the test compound under the proposed experimental conditions, are uncommon in this research field. Nevertheless, performing a stability study during the rational design of *in vivo* assay protocols will result in more reliable assay results. To illustrate the influence of the stability of the test substance on the final experimental outcome, we performed a short-term International Conference on Harmonization (ICH)-based stability study of cyclophosphamide in the culture medium, to which a marine flatworm model *Macrostomum lignano* is exposed. Using a validated U(H)PLC method, it was demonstrated that the cyclophosphamide concentration in the culture medium at 20 °C is lowered to 80% of the initial concentration after 21 days. The multiwell plates, flatworms and diatoms, as well as light exposure, did not influence significantly the cyclophosphamide concentration in the medium. The results of the stability study have practical implications on the experimental set-up of the carcinogenicity assay like the frequency of medium renewal. This case study demonstrates the benefits of applying appropriate quality guidelines already during fundamental research increasing the credibility of the results.

1. Introduction

Flatworms are known for their amazing regenerative capacity. Even a fragment as small as 1/279th of the size of the original individual has the capacity to regenerate into an entire animal (Newmark and Sánchez Alvarado, 2002). The key to the regenerative prowess of these creatures are totipotent stem cells, called neoblasts, distributed throughout the organism. Thus, neoblasts are the flatworm equivalent of somatic stem cells, making these remarkable creatures an excellent new model system for studying stem cell biology. *In vivo* studies of somatic stem cells are not easy in vertebrate models due to the fact that their experimental accessibility is relatively more challenging. Alternatively, non-mammalian model systems, such as flatworms, can be used to assess *in vivo* the effect of substances on stem cells. This has been done extensively in the past to test diverse pharmacological and carcinogenic compounds and recently their use has regained much interest (Alonso and Camargo, 2006, 2011; Best and Morita, 1982; Chan and Marchant, 2006; Demircan and Berezikov, 2013; Foster, 1963, 1969; González-Estévez et al., 2012a; Hall et al., 1986a,b; Isolani et al., 2012; Oviedo et al., 2008; Oviedo and Beane, 2009; Pagan et al., 2006, 2012, 2013; Plusquin et al., 2012a,b; Schaeffer et al., 1991; Schaeffer, 1993; Sánchez Alvarado, 2006; Simanov et al., 2012; Wu et al., 2012; Zhang et al., 2013). The power of these alternative animal models lies in their ease of culture and experimental accessibility. However, it is important to be aware that the specific test conditions, *i.a.* culture media, day/night regimes,

Abbreviations: CI, confidence interval; CP, cyclophosphamide; GLP, Good Laboratory Practices; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; ICH, International Conference on Harmonization; l.c., label claim; LoD, limit of detection; LoQ, limit of quantification; PDA, photodiode array; Ph. Eur., European Pharmacopoeia; r.h., relative humidity; S/N, signal-to-noise ratio; U(H)PLC, ultra (high) performance liquid chromatography; WHO, World Health Organization.

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type of animal containers, feeding scheme and exposure time, can affect the stability of the substance under investigation. To our knowledge, this aspect has never been mentioned in any past or recent reported studies. We use the flatworm model organism *Macrostomum lignano* (Ladurner et al., 2005, 2008; Mouton et al., 2009; Willems et al., 2014) for our toxicological studies. Being a small (about 1.5 mm) marine species, flatworms are cultured in plastic or glass (multiwell) dishes with artificial seawater media under a specific day/night light regime and temperature-controlled conditions. The effect of all aforementioned factors are usually not mentioned in the stability data sheet of toxic substances but, in a pharmaceutical context where regulatory GLP (Good Laboratory Practices) are requested, it is essential to rationally design high quality experiments to maximize the reliability of the outcomes (Verbeken et al., 2012; Vergote et al., 2008). Therefore, we initiated an analytical U(H)PLC stability study of cyclophosphamide (CP) under experimental conditions, which is used as a standard test compound for screening of genotoxicity and carcinogenicity using a flatworm model (Willems et al., 2014). The study design was based on the International Conference on Harmonization (ICH) guidelines (D'Hondt et al., 2012; European Medicines Agency, 1998, 2003). Moreover, to our knowledge, it is the first time an U(H)PLC method is described for evaluation of the stability of CP. In literature, both HPLC and HPTLC methods are available for testing the CP stability in different formulation types (Bouligand et al., 2005; Kennedy et al., 2010; Menard et al., 2003; Mittner et al., 1999).

The aim of this study was to quantitatively evaluate the influence of experimental conditions on the CP concentration in the used medium, hence on the final conclusions of the biological experiment. Using this study, we want to demonstrate the benefits of including appropriate quality practices already during research phases of fundamental science and encourage researchers to apply appropriate quality assurance practices to ensure the credibility of their results.

2. Materials and methods

2.1. Materials

Cyclophosphamide monohydrate (CAS No. 6055-19-2) was purchased from Sigma (Diegem, Belgium). Acetonitrile U(H)PLC grade (CAS No. 75-05-8) for the mobile phase was obtained at Fisher Chemical (Erembodegem, Belgium), while ultrapure water (18.2 MΩ × cm, CAS No. 7732-18-5) was produced using an Arium 611 VF water purification system (Sartorius, Vilvoorde, Belgium). HPLC glass vials with Teflon/polypropylene closures were purchased from Waters (Zellik, Belgium), polystyrene multiwell plates and covers from Novolab (Geraardsbergen, Belgium) and closable Quartz cuvets (10 mm) from Hellma GmbH & Co. (Müllheim, Germany).

2.2. Carcinogenicity assay

We developed an *in vivo* flatworm carcinogenicity bioassay, in which stem cell proliferation is used as an endpoint to assess the carcinogenic potential of compounds. A detailed description of this bioassay can be found in Willems et al. (2014) and is briefly summarized hereafter in order to understand which parameters were chosen for stability testing.

During the *in vivo* flatworm carcinogenicity assay, *M. lignano* cultures were grown in a medium consisting of artificial seawater enriched with a f/2 marine water solution (Guillard, 1975), under a 14 h day/10 h dark regime and fed *ad libitum* with the diatom *Nitzschia curvilineata* (PAE culture collection; UGent http://www.pae.ugent.be/collection.htm), as described by Ladurner et al. (2005, 2008), Mouton et al. (2009) and Rieger et al. (1988). The worms were exposed to a specific compound (CP in our case) dissolved in 4 ml of culture medium at 20 °C in polystyrene multiwell (typically six well) plates under the same day/night regime as described above. To achieve *ad libitum* feeding during the exposure time, diatoms were inoculated and allowed to grow on the multiwell plates two weeks prior to the start of the experiment. Exposure time can vary from two days to three months. Fig. 1 shows the model organism and the exposure condition in one well of a multiwell plate during the carcinogenicity assay.

In the considered *in vivo* carcinogenicity assay, the flatworms are exposed to a 28 μg/ml (100 μM) CP solution in culture medium during two weeks at 20 °C under a 14 h light/10 h dark cycle.

2.3. U(H)PLC-PDA method for determination of CP

The Acquity H-class U(H)PLC apparatus consisted of a quaternary solvent manager, an automatic sample injection system, combined with a flow through needle, a column heater and an ultra-performance LC photodiode array (PDA) detector, equipped with Empower 2 software for data acquisition (all from Waters, Zellik, Belgium). A BEH C18 column (Waters, 2.1 mm × 100 mm, 1.7 μm) was selected for isocratic separation using a mobile phase containing 75% ultrapure water and 25% acetonitrile. The flow rate was 0.3 ml/min and the total run time was 5 min. The injection volume was set at 2 μl. The BEH C18-column was thermostated at 40 °C (±3 °C) and the sample compartment at 25 °C (±5 °C). PDA-detection was performed from 190 to 400 nm, with quantification at 195 nm. Ultrapure water was used as needle rinsing liquid, as CP is water-soluble. All samples were prepared by dissolving an appropriate quantity of CP in ultrapure water. The CP reference sample (100% label claim (l.c.)) has a concentration of 28 μg CP/ml or 100 μM CP.

During method validation, the limit of quantification (LoQ, S/N = 10 according to the European Pharmacopoeia (Ph. Eur.)) was calculated to be 0.42 μg/ml (1.50% l.c.), while the limit of detection (LoD, S/N = 3 (Ph. Eur.)) was 0.13 μg/ml (0.45% l.c.). Injection of an over-concentrated CP sample (5 mg/ml) did not result in peaks in the subsequently injected blank solution (*i.e.*, <LoD), indicating there was no significant carry-over. The method was linear in the tested range from 3% l.c. to 200% l.c. (R^2 = 0.9998; ANOVA *F*-value = 23378). During the repeatability evaluation of the U(H)PLC-method, the relative standard deviation of six successive injections of a 100% l.c. reference sample was 1.44%. The performed stress stability study in acidic, basic, hydrogen peroxide and elevated temperature conditions, resulted in a similar impurity profile as previously described (Dhakane and Ubale, 2013). These results indicate the selectivity for CP of the new U(H)PLC-method, allowing the stability-indicating use of the method.

2.4. In use stability test of CP

In order to represent the production variability of the culture medium, three different batches of the medium, each containing 28 μg CP/ml or 100 μM CP, were used. For the *kinetic stability testing*, the three batches, as well as a placebo sample, *i.e.*, no CP in culture medium, were divided over different HPLC glass vials and stored in ICH conform storage rooms (Weiss Technik Belgium, Liedekerke, Belgium) at following conditions: 5 °C (50% relative humidity (r.h.)), 25 °C (60% r.h.) and 40 °C (75% r.h.), all protected from light. All the samples were analyzed using the previously described U(H)PLC-method at day T0, T1, T2, T5, T8, T10, T11 and T12. The freshly prepared reference sample consisted of a 28 μg/ml (100 μM) aqueous CP solution and was also used to test system suitability prior to analysis by evaluation of the plate number