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# Bioavailability and Biotransformation of Paralytic Shellfish Toxins Assessed by Permeability Assays Using Caco-2 Monolayers †

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**Abstract:** Caco-2 cells are well established models of intestinal epithelium, being routinely used to evaluate toxicity and bioavailability. Although usually overlooked, Caco-2 monolayers may also be used to assess biotransformation by epithelial cells, which may lead to significant changes in the composition and properties of the ingested matrix. In this work we characterize paralytic shellfish toxins (PSTs) extracted from the dinoflagellate *Gymnodinium catenatum* strain regarding their permeability through and biotransformation by polarized Caco-2 monolayers. The results show that biotransformation influences the apparent permeability measured for the different PSTs in the extract, and alters the extract's effective toxicity.

**Keywords:** *Gymnodinium catenatum*; paralytic shellfish toxins; Caco-2 monolayers; intestinal absorption; permeability assays; HPLC analysis



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## 1. Introduction

Paralytic shellfish toxins (PSTs) are responsible for acute and fatal human poisoning events worldwide [1,2]. Exposure to the PSTs occurs mainly through the ingestion of contaminated bivalve mollusks, which acquire high concentrations of toxins by feeding from toxigenic planktonic species [3]. The dinoflagellate *Gymnodinium catenatum* species has been associated with PSTs intoxication, which in the Portuguese coast is mainly due to decarbamoyl(dc) toxins (dcSTX, dc-gonyautoxin (GTX)-2&3 and dcNeoSTX), sulfocarbamoyl (C-1&2 and GTX-5) and hydroxybenzoate (GC-1 to -3) analogues [4].

To minimize the risks of exposure for humans, a regulatory PSTs limit of 800 µg of STX equivalents per kg of shellfish consumed has been used in many countries [5]. However, this current limit does not account for the distinct bioavailability of the different PSTs and biotransformation among them, which may significantly alter their in vivo cytotoxicity through oral ingestion. Only a few studies have so far addressed the prediction of the PSTs intestinal absorption in humans with epithelial cell models [6,7]. Using enterocyte-like Caco-2 monolayers, we have recently reported very low apparent permeability coefficient ($P_{app}$) values for the five PSTs analogues present in a crude extract prepared from a *G. catenatum* strain [7]. Although the PSTs were poorly absorbed, their proportions were

altered by the 90 min period of incubation with the cells, suggesting metabolic alterations of the PSTs by the Caco-2 cells [7].

The aim of this report is to highlight the importance of using Caco-2 monolayers differentiated on permeable filters to evaluate biotransformation by the intestinal epithelium. Metabolization by the epithelial cells has implications on the permeability observed for each PST, and on the in vivo human health risks.

## 2. Materials and Methods

The *G. catenatum* strain GCAT1_L2_16 isolated in the Portuguese northwestern coast (Aguda beach) in 2016 was obtained from the culture collection of the phytoplankton laboratory in the Institute for the Sea and Atmosphere (Portugal), following the conditions and procedures previously reported [7]. Caco-2 cells (ECACC 09042001, Salisbury, UK) at passage 93 were used on day 23 post-seeding (on 12-well Transwell inserts) for the transport experiments, following the protocols previously described [7,8]. Crude PSTs extract at 15% (*v*/*v*) in Hank's balanced salt solution (HBSS) was applied at the apical side to mimic the in vivo conditions of intestinal absorption, and incubated with the cell monolayer for 90 min at 37 °C. The PSTs profile of the crude extract, and samples collected from the Caco-2 permeability assay (apical and basolateral) were analyzed using an optimized high-performance liquid chromatograph (HPLC) method for the detection of the fluorescent derivatives of the toxins produced by pre-chromatographic oxidation with $H_2O_2$ (for details see Rodrigues et al. [7]).

## 3. Results and Discussion

### 3.1. Biotransformation of PSTs in Human Caco-2 Monolayers

The HPLC elution profile of the PSTs extract obtained from *G. catenatum* is shown in Figure 1A. Peak V was identified as the GTX-5 toxin by comparison with the elution of the certificate reference material [7]. Peaks I and IV were tentatively identified as dcGTX-2&3 and dcSTX, respectively, based on the retention times and relative abundance reported in the literature [9]. To study the biotransformation of the PSTs by human's intestinal epithelial cells, the elution profile of the PSTs from the apical and basolateral compartments of Caco-2 monolayers after incubation for 90 min was also analyzed (Figure 1B,C). Small variations were observed in the PSTs elution profile, maintaining the same components, but with changes in their relative abundance. The same trend was observed in both compartments, although the variations were more accentuated in the basolateral compartment.

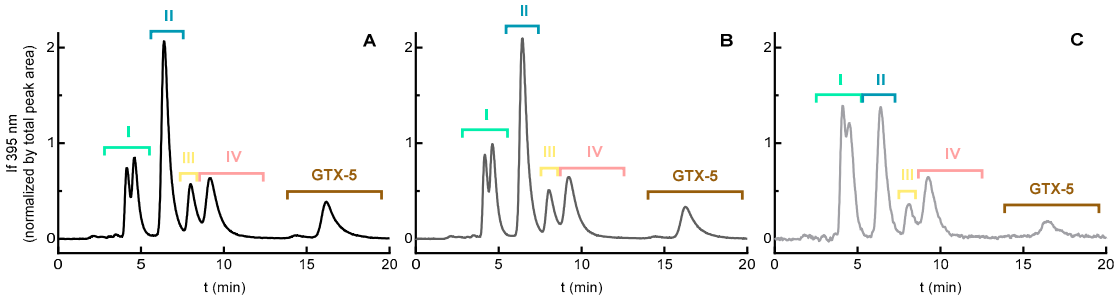


**Figure 1.** Representative elution profile ($\lambda_{ex}$ = 337 nm, $\lambda_{em}$ = 395 nm) for the *G. catenatum* crude extract (**A**), and after incubation with Caco-2 monolayers for 90 min in the apical (**B**) and in the basolateral (**C**) compartments. Data reproduced with permission from Rodrigues, E.T. et al., *Environ. Sci. Pollut. Res.*, published by Springer, 2021, [7].

The relative areas of all peaks are compared in Figure 2. In the crude extract, peak II corresponds to the most abundant toxin (35.4 ± 0.4%), peak I is the second most abundant (20.2 ± 0.5%), followed by peak IV (18.6 ± 0.2%), GTX-5 (15.6 ± 0.3%), and peak III (10.5 ± 0.2). After incubation for 90 min, the relative areas of the sample from the apical compartment show that although peak II remains the major component, the relative abun-

dance of peak I has increased to 24.5 ± 1.6% and that of peak III and GTX-5 has decreased to 9.2 ± 0.3% and 12.4 ± 0.3%, respectively. The variations observed upon incubation with the Caco-2 monolayer are more accentuated in the sample from the basolateral compartment, with peak I now becoming the most abundant PST 37.5 ± 1.1%, and peaks II, III and GTX-5 showing significant decreases in their relative abundance. The similarity in the trends observed for the variations in the relative amounts of PSTs in both the apical and basolateral compartments show that the variations are not due to a differential permeability, but rather, to biotransformation by the polarized Caco-2 cells. In fact, metabolization of those toxins in the liver, kidney and lung of a human was showed by Garcia et al. [10].

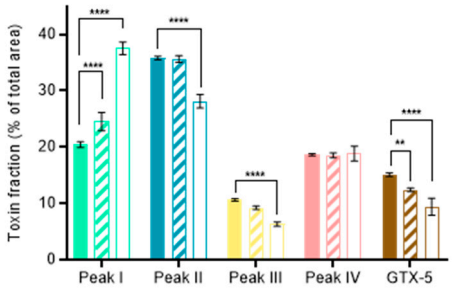


**Figure 2.** Relative area of the different peaks in the elution profile of the PSTs extract: crude extract (filled bars); and after 90 min addition of extract to the apical side of Caco-2 monolayer, sampled in the apical (pattern bars) and basolateral (open bars) compartment. Statistically significant differences from the crude extract are indicated by ** ($p \leq 0.01$) and **** ($p \leq 0.0001$) using two-way ANOVA.

Although not generally used, Transwell assays with Caco-2 monolayers on semi-permeable filters have been used by some authors to evaluate biotransformation and bioavailability of toxins [11,12]. These assays have several advantages when compared with cells on culture plates. On one side a monolayer of polarized and differentiated cells is obtained, better mimicking the intestinal epithelium [13]. In addition, as shown here, the direct access to the basolateral compartment significantly increases the sensitivity. This occurs as, in the apical compartment, the products of biotransformation are significantly diluted by sample components that have not interacted with the cells, the same being observed when using cells on culture plates. Finally, the use of Transwell assays allows evaluation of the apparent permeability of each sample component.

### *3.2. Implications of Biotransformation on Bioavailability and Toxicity*

The apparent permeability coefficient of each PST may be calculated from the relative amount that reaches the basolateral compartment. A low $P_{app}$ value was obtained for all PSTs from the *G. catenatum* extract studied in this work, ranging from $0.9 \times 10^{-7}$ cm/s for peak III and V (the later corresponding to GTX-5), to $2.7 \times 10^{-7}$ cm/s for peak I, with intermediate values obtained for peak II and IV. It should be noted that biotransformation leads to a higher apparent permeability of the toxins generated, while the reverse is observed for those that are metabolized. In the case of the PSTs from *G. catenatum* extract, the major biotransformation product (peak I attributed to dcGTX-2&3) has a relatively high toxicity [14], thus leading to an increase in the effective sample toxicity. The detailed prediction of toxin biotransformation and bioavailability was only possible as the toxin profile was obtained by HPLC analysis. If only the total concentration of toxins were analyzed, an intermediate bioavailability and toxicity would be obtained. This highlights the importance of characterizing the sample profile when studying natural extracts.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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