Chlorogenic Acid as a Model Compound for Optimization of an In Vitro Gut Microbiome-Metabolism Model †

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Abstract: It has been believed that the metabolism of xenobiotics occurred mainly by the cytochrome P450 enzyme system in the liver. However, recent data clearly suggest a significant role for the gut microbiota in the metabolism of xenobiotic compounds. This microbiotic biotransformation could lead to differences on activation, inactivation and possible toxicity of these compounds. In vitro models are generally used to study the colonic biotransformation as they allow easy dynamic and multiple sampling over time. However, to ensure this accurately mimics communities in vivo, the pre-analytical phase requires optimization. Chlorogenic acid, a polyphenolic compound abundantly present in the human diet, was used as a model compound to optimize a ready-to-use gut microbiome biotransformation platform. Samples of the in vitro gastrointestinal dialysis-model with colon stage were analyzed by liquid chromatography coupled to high resolution time-of-flight mass spectrometry. Complementary screening approaches were also employed to identify the biotransformation products.

Keywords: gut microbiome; in vitro gastrointestinal dialysis model; 16S rDNA sequencing; liquid chromatography-mass spectrometry

1. Introduction

The microorganisms that live inside and on the human body outnumber the human cells by a factor of ten [1–6]. The gastrointestinal tract (GIT) accommodates between 10^{12}–10^{13} microorganisms with the colon harboring the majority of bacteria of the GIT. Firmicutes and Bacteroidetes are the two dominant phyla present in the human GIT, accounting for 90% of the gut diversity [5,7–10]. The gut microbiota is responsible for various developmental, immunological and nutrition host functions [11,12].
The gut microbiome, the cumulative genome content of the gut microbiota, is estimated to contain 100 to 300-fold more genes than the human genome [1,3,5,13,14] and therefore is considered an additional metabolic organ [3,7,15,16]. Important differences between hepatic and bacterial metabolism have been described. Metabolic pathways by the liver cytochrome P450 enzymes (CYP450) lead to the conversion of lipophilic compounds into more hydrophilic compounds, while the majority of biotransformations encoded for by the gut microbiome are reductive and hydrolytic reactions. In addition, the gut flora is responsible for demethylation, deacetylation, decarboxylation, acetylation, dealkylation, dehalogenation, dehydroxylation, deamination, and oxidation/dehydrogenation [1,17–20]. Direct and indirect interactions can influence the activity and toxicity of xenobiotics [20]. Conversion of a prodrug to the active compound, inactivation, detoxification, change of efficacy and direct binding to xenobiotics or dietary molecules are examples of direct interferences [11,21]. Examples of indirect interferences are competition and/or inhibition of host enzymes by microbial metabolites, the alteration of host genes expression and reactivation of drugs by deconjugation of the phase II metabolite after enterohepatic circulation [11,21]. Varying health-promoting compounds present in the human diet such as polyphenols are characterized by a poor absorption in the small intestines, leading to extensive biotransformation of these xenobiotics by the gut microbiota [7,17].

In vitro GIT models offer an alternative approach to in vivo studies to investigate the colonic biotransformation of xenobiotics as they allow multiple sampling overtime. In vitro models of the GIT can simulate the different parts of the digestive system by adjusting pH, temperature, enzymes and peristalsis. The gut flora is mimicked using a fecal suspension [22–24]. However, in vitro models are not fully representative of in vivo conditions. Therefore, it is important to optimize the pre-analytical phase to mimic the in vivo situation in order to obtain a high level of physiological significance [18,22]. During this study, the gastrointestinal dialysis model with colon (GIDM-colon), developed and validated by Breynaert et al., was used to investigate the colonic biotransformation of the polyphenolic compound chlorogenic acid [25]. As chlorogenic acid is a polyphenol abundantly present in the human diet of which the gastrointestinal metabolism has already been extensively investigated by multiple studies, it was selected as model compound to optimize a ready-to-use setup to study the colonic metabolism of xenobiotics [25–33].

2. Materials and Methods

The colonic biotransformation of chlorogenic acid was investigated using the GIDM-colon [25]. A blank (no chlorogenic acid) and negative control (no fecal suspension) sample were included as controls in each experiment. The pH was set at 2, 7.5 and 5.8 for the gastric, small intestinal and colon-stage respectively, together with the appropriate enzymes. Temperature was kept at 37 °C and during the colon-stage, dialysis cells were placed in an anaerobic environment.

In order to further optimize the in vitro GIDM-colon system, a thorough investigation was conducted to determine effects on the bacterial composition of the fecal samples used to inoculate the colon system, aiming to minimize changes in bacterial composition between initial donation of fecal samples by individuals (meeting certain inclusion criteria) and final biotransformation experiments in the in vitro GIDM-colon system. Thus, the processing procedure of the fecal suspension, used during the colon-stage, was optimized. Two incubation media, Wilkins-Chalgren Anaerobe Broth (WCB) and a sterile phosphate buffer (0.1 M, pH 7) were investigated. The fecal slurry was incubated for 17 h in the media and samples were taken before and after incubation and analyzed for their bacterial composition by 16S rRNA gene sequencing. Furthermore, the effect of incubation time in the chosen medium on the bacterial concentration was also investigated. A pooled fecal slurry was incubated 24 h in the chosen medium and samples were taken every 2 h whereafter the anaerobic CFU/g was determined.

In view of optimal preparation of samples for accurate analysis of colon metabolites by liquid chromatography coupled to high-resolution mass spectrometry, the sample preparation procedure after GIDM-colon experiments was optimized. Four different sample preparation methods (SPP) on the samples received from the colon-stage were evaluated: all methods included the addition of methanol (MeOH) combined with centrifugation steps (14,000 rpm, 8 min), freeze-drying (reconstituted in MeOH, sonicated 45 min), sonication (60 min) or extraction by vortex mixing (60 s).
Sample preparation methods were evaluated based on the number of identified biotransformation products with MS/MS fragmentation, and the time and effort needed for sample preparation. Samples were analyzed by liquid chromatography coupled to high-resolution accurate-mass mass spectrometry (LC-QTOF-MS), including both complementary suspect and non-targeted screening workflows.

3. Results and Conclusions

Phosphate buffer was chosen as preferred incubation medium for this study. The medium demonstrated a closer resemblance to the initial in vivo bacterial composition, while WCB was found to introduce substantial changes. Furthermore, the anaerobic bacterial concentration remained stable during 24 h of incubation in phosphate buffer. In future experiments, no incubation is needed prior to the GIDM-colon which will lead to a reduction in total time of the experiment and reduced risk of introduction of changes to the initial bacterial composition.

Results of the investigation of different SPP prior to LC-QTOF-MS analysis, clearly showed freeze-drying to be the inferior, as lower relative peak areas of the identified biotransformation products were produced. Centrifugation, sonication and extraction SPPs showed similar relative peak areas. Furthermore, only 53% of the identified compounds showed MS/MS fragmentation by data dependent MS/MS using freeze-drying, while 63% was reached using the centrifugation or extraction method and 69% using the sonication method. Extraction was chosen as SPP for future experiments as a shorter time period is needed to obtain LC-suitable samples. In total, 23 colonic biotransformation products of chlorogenic acid were identified after digesting 78 mg chlorogenic acid in the optimized GIDM-colon system using liquid chromatography coupled to high resolution mass spectrometry.

This study contributed to the development of a ready-to-use platform that can be used to investigate the gastrointestinal fate of different xenobiotic compounds and/or to compare interindividual differences in colonic biotransformation between different populations (e.g., fecal samples of patient groups).

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References


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