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An integrated platform for directly widely-targeted quantitative analysis of feces part I: Platform configuration and method validation

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ABSTRACT

Direct analysis is of great importance to understand the real chemical profile of a given sample, notably biological materials, because either chemical degradation or diverse errors and uncertainties might be resulted from sophisticated protocols. In comparison with biofluids, it is still challenging for direct analysis of solid biological samples using high performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Herein, a new analytical platform was configured by online hyphenating pressurized liquid extraction (PLE), turbulent flow chromatography (TFC), and LC–MS/MS. A facile, but robust PLE module was constructed based on the phenomenon that noticeable back-pressure can be generated during rapid fluid passing through a narrow tube. TFC column that is advantageous at extracting low molecular analytes from rushing fluid was employed to link at the outlet of the PLE module to capture constituents-of-interest. An electronic 6-port/2-position valve was introduced between TFC column and LC–MS/MS to fragment each measurement into extraction and elution phases, whereas LC–MS/MS took the charge of analyte separation and monitoring. As a proof of concept, simultaneous determination of 24 endogenous substances including eighteen steroids, five eicosanoids, and one porphyrin in feces was carried out in this paper. Method validation assays demonstrated the analytical platform to be qualified for directly simultaneous measurement of diverse endogenous analytes in fecal matrices. Application of this integrated platform on homolog-focused profiling of feces is discussed in a companion paper.

1. Introduction

Sustained efforts have been made by the analysts from all over the world to minimize sample preparation procedures which account for most of the errors and uncertainties during quantitative measurements [1]. Direct analytical methods exactly fulfill this requirement because they preclude all sample preparation procedures [2,3], and they could also prevent against the potential chemical degradation in biological matrices. Therefore, direct analysis is qualified for understanding the real chemical profile of a given biological sample. Direct analysis of biofluids, such as plasma, bile along with urine, has been extensively achieved on high performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) [4–7]; however, direct analysis is still challenging and annoying for solid biological samples, in particular fecal matrices. Feces are widely favored biological sources for metabolomics investigations because they possess non-invasive and more accessible properties [8] and contain a vast number of endogenous substances that could reflect the physiological status as well as the gut functional ecology [9]. Traditionally, progressive procedures such as extraction with certain organic solvents, centrifugation, and filtration, are usually involved for the sample preparation of the feces prior to being subjected onto LC-derived analytical platforms [8], indicating a time-consuming and laborious workload. Even worse, conventional extraction approaches, such as reflux and sonication, required a long time exposure of the endogenous substances to organic solvents (*e.g.*, methanol) and lights, resulting in significant risks of structural transformation and degradation for those instable metabolites, for instance methylation of bile acids (BAs) in methanol [10] and generation of vitamin D_3 because of sunlight exposure [11]. Moreover, the placement of the fecal extract either on the bench or in the auto-sampler might further advance the transformation of the targeted compounds.

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Therefore, it is urgent to propose a direct analysis strategy for feces to achieve high-throughput assay, and more importantly, to understand the inherent form of analytes in the solid biological samples.

Pressurized liquid extraction (PLE) has been proposed as an efficient and greener approach for solid samples due to the rapidity and outstanding extraction efficiency under elevated pressure and temperature. However, this technique cannot prevent against the potential degradation of analytes because the extraction was performed at high temperature and the analytes should undergo a long-time exposure to organic solvents either on the bench or in the auto-sampler prior to injection. Although some state-of-the-art apparatuses have been developed, the specialized PLE instrumentation still indicates an obvious dilemma for the online hyphenation between PLE module and LC–MS/MS that has been heavily utilized for separation and detection of those endogenous substances in biological matrices within the past decades [12–14]. Because noteworthy back-pressure can be generated during high flow rate fluid passing through a narrow tube, it is thereby feasible to construct a facile PLE module by employing a long PEEK tube to generate desired pressure for solid materials when extraction solvent is delivered at a high flow rate. We envision that this module can not only show comparable extraction efficiency with routine approaches, but also offer satisfactory compatibility with LC-derived platforms. Afterwards, attentions should be put onto finding a robust interface to transmit components from PLE into LC. Fortunately, the task suits well with the intrinsic principle of turbulent flow chromatography (TFC), which is a special solid phase extraction (SPE) technique and skillful at extracting low molecular constituents from torrential fluid according to turbulence inside the column packed with large particles (approximately 50–100 μm) [15]. It is worthwhile to mention that extensive peak broadening and resolution decreasing could be resulted from the introduction of TFC column; hence, multiple reaction monitoring (MRM) mode which is a superior function of triple quadrupole mass spectrometer and exhibits a great potential for simultaneous monitoring of those overlapped signals, was employed to resist the decrement of resolution induced by back flushing TFC column.

Above all, a new analytical platform was constructed for directly quantitative analysis of various targets in fecal matrices by online hyphenating PLE, TFC, and LC–MS/MS (Fig. 1). Steroids, especially BAs, along with eicosanoids and porphyrins serve as primary chemical categories in feces, and they intervene in a wide range of metabolic pathways and various physiological processes, *e.g.*, lipid absorption and metabolism, and immunity [16], indicating pivotal roles for clinical diagnosis of various diseases. However, their chemical diversity raises a great challenge for comprehensive fingerprinting of biological samples, and the extremely broad concentration ranges along with the instability of those hydrophobic metabolites (*e.g.*, bilirubin- and vitamin D-derivatives) further strengthen the difficulty for accurate quantification. Therefore, these three chemical classes were selected as analytes-of-interest to validate the applicability of online PLE-TFC-LC–MS/MS platform. In this part, the configuration of the integrated platform, method validation, and simultaneous determination of 24 analytes are involved, whereas a companion paper presents the application for homolog-focused profiling of feces with various sources.

2. Experimental

2.1. Chemicals and materials

Prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were provided by Cayman Chemicals (Ann Arbor, MI, USA). Cholic acid (CA), hyodeoxycholic acid (HDCA), tauro-

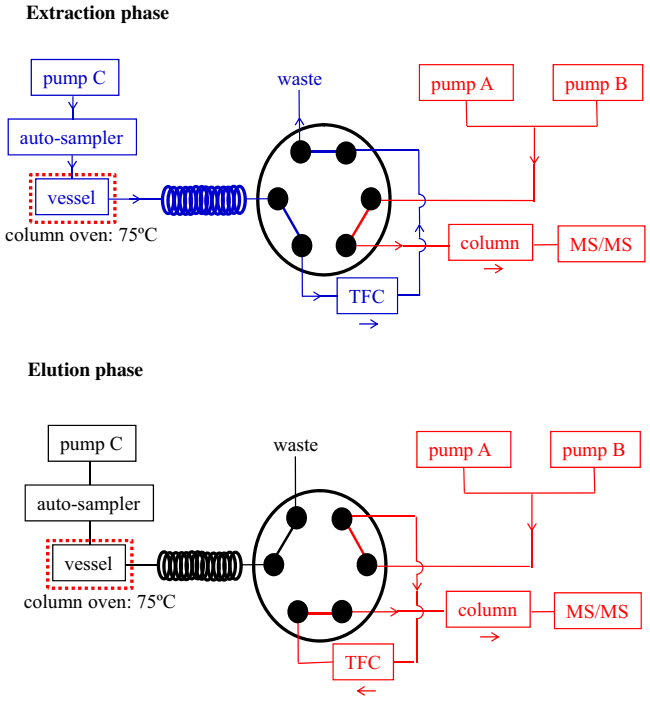

Fig 1. Schematic diagram of the home-made online PLE-TFC-LC–MS/MS analytical platform. An entire measurement was fragmented into extraction phase (0–2 min) and elution phase (2–40 min) by alternating the valve positions.

hydeoxycholic acid (THDCA), deoxycholic acid (DCA), cholesterol, estradiol, estriol, estrone, methyltestosterone, cortisol, cortisone, and mestanolone were obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), chenotaurocholic acid (CTCA), taurocynocholic acid (TCCA), 3α-hydroxy-oxo-5β-cholanoic acid-*N*-[2-sulfoethyl] amide (sulfoethyl amide-CA), and testosterone, as well as bilirubin were supplied by the State Key Laboratory of Natural and Biomimetic Drugs, Peking University (Beijing, China). Two natural products, including euscaphic acid (IS1) and oxypeucedanin (IS2), were purchased from NICPBP and severed as the internal standards for MRM-mediated quantification with negative and positive polarities, respectively. Purities of all authentic compounds, 24 ones in total, were determined to be greater than 98% by LC–MS/MS, whereas the structural confirmation was carried out *via* ^{1}H- and ^{13}C NMR spectroscopy.

LC–MS grade acetonitrile (ACN) and formic acid were obtained from Thermo-Fisher (Pittsburgh, PA, USA). Dimethylsulfoxide (DMSO) was supplied by Merck (Darmstadt, Germany). Ultra-pure deionized water was prepared using a Milli-Q Integral water purification device (Millipore, MA, USA).

2.2. Fecal sample collection

To ensure the applicability of the developed analytical platform, four sets of fecal samples were collected from six SD rats (G-a), eight ICR mice (G-b), eight ApoE-/-C57BL/6J mice (G-c), and eight ApoE-/-C57BL/6J mice (G-d), respectively. All animals were supplied by Vital River Laboratories (Beijing, China) and acclimated in laboratory at temperature of 23 ± 1 °C with 12 h-light/dark cycle and 50% relative humidity. Mice from G-c group received high fact diet (0.2% cholesterol and 15% fat added) for seven days after lab acclimation, whereas G-d mice were fed with standard chow [17]. Then, all animals were housed in separate metabolic cages and free access to