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Urinary intermediates of tryptophan as indicators of the gut microbial metabolism

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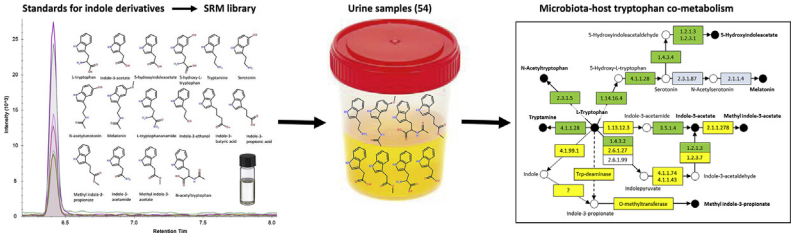
HIGHLIGHTS

- Generated SRM library for simultaneous quantification of 16 indole-derived metabolites.
- Application of UHPLC-SRM assays to explore an uncharted part of microbiota-modulated tryptophan metabolism.
- The first report and quantification of methyl indole-3-acetate (MIA) and methyl indol-3-propionate (MIP) in urine.
- Simultaneous urinary metabolic profiling of tryptophan and 7 intermediates of the tryptophan metabolism.

GRAPHICAL ABSTRACT



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ABSTRACT

While over 10% of the human metabolome is directly associated with the gut microbial metabolism, specific metabolites are largely uncharacterized. Therefore, methods for the identification and quantification of microbiota-associated metabolites in biological fluids such as urine or plasma are necessary in order to elucidate the molecular basis of host–microbiota interaction. In this study, we focused on the tryptophan metabolism, employing quantitative assays by ultra-high performance liquid chromatography (UHPLC) and tandem mass spectrometry, specifically selected reaction monitoring (SRM). Metabolite standards were utilized to generate SRM library for 16 intermediates of the tryptophan metabolism which were human endogenous as well as microbiota-associated based on the HMDB classification. Next, the SRM assays were utilized for screening in maternal urine samples and in dried urine specimens from neonates. The approach resulted in the discovery of microbiota-associated metabolites (methyl indole-3-acetate and methyl indol-3-propionate) previously unreported in urine samples and additionally in quantification of 8 intermediates of the tryptophan metabolism. To the best of our knowledge, this study represents the first attempt to explore previously unreported microbial

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metabolites in urine by UHPLC-SRM and novel methodology for simultaneous determination of microbiota-modulated component of Trp metabolism.

1. Introduction

The well documented evidence on important roles of microbial metabolic pathways in a mammalian host physiology includes the production of vitamin K and a group of B vitamins as well as the modification of bile salts [1,2]. However, currently available high-throughput sequencing methods have revisited the phenomenon of gut microbiota–host interaction, linking it to additional important physiological processes such as neurological behavior [3], immune homeostasis [4] and the energy metabolism [5]. On the other hand, molecular mechanisms behind these functions recently attributed to human microbiota are yet to be explored.

A widely applicable methodology for consistent identification and quantification of metabolites modulated by microbiota in biological fluids (e.g. urine, plasma) is a mandatory first step required to improve the understanding of host–microbiome interaction on the molecular basis. Microbiota-associated metabolites are either exclusively produced by gut microbiota, derived from microbial transformation of dietary components or jointly contributed by both mammalian cells and bacteria [6]. It is estimated that over 10% of the mammalian plasma metabolome is directly dependent on the microbiome [7], but specific metabolites as well as their physiological levels remains unknown.

The microbial influence on tryptophan metabolism in human has been studied intensively, as it presumably affects several key physiological processes and metabolic pathways, thus playing a pivotal role in human health [3,8,9]. Tryptophan (Trp), overall the least abundant amino acid in proteins [10], is necessary to maintain protein biosynthesis in humans and to ensure the synthesis of neurotransmitters (serotonin and melatonin) [11]. Since the metabolic activity of microbiota residing in the human intestine influences the availability of Trp and consequently also serotonergic signaling in the central nervous system, Trp presumably mediates communication between the gut and the brain and its involvement in the modulation of human behavior seems plausible [3]. A vigorous metabolism of Trp by microbial enzymes results in a range of indol-derived intermediates of which a handful have been characterized in biofluids i.e. 3-indoxyl sulfate and more importantly indole-3-propionate (IPA) or indole-3-acetate (IAA) [6,12,13].

The synthesis of IPA is probably preceded with the formation of indole from Try by tryptophanase of indole-producing microbiota [14]. Non-indole-producing species of microbes further modify indole using oxygenases to produce IPA [14,15]. A previous study demonstrated that the production of IPA in mammals depends entirely on commensal gut microbiota, specifically on the metabolic activity of *Clostridium Sporogenes* [15]. Since the discovery, IPA and several other known metabolites of microbial origin circulating in human blood [16] or present in the saliva metabolome [17] have been associated with a number of important physiological processes. Reportedly, IPA contributes to the maintenance of mucosal homeostasis and intestinal barrier function [18] while also functioning as a powerful antioxidant [19,20], neuroprotectant and a potential cure for Alzheimer's disease [21]. Clearly, these complex biological functions taking place in the human body are only marginally understood. To explain them, we need to obtain greater insight into the microbiota modulated metabolome by utilizing suitable methods.

IAA is endogenous metabolite in human, but its production in high amounts has been shown in diverse bacterial strains abundant in gut microbiota, such as *Bifidobacterium* and *Bacteroides* [22] and it has been identified as a marker of gut microbiota metabolic activity [23]. IAA and its derivative methyl indole-3-acetate (MIA) are also very well-known phytohormones [24] obtained in minute quantities (<50 pmol/g of FW) from a plant material [25–27]. Based on IAA and IPA examples we can speculate that the indole class of compounds may have important function as inter-kingdom signaling molecules regulating mammalian, bacterial, and plant signaling [13].

To date a large number of methods for the analysis of Trp and its metabolites have been developed. These formerly relied on liquid chromatography with UV absorbance [28] or fluorescent [29] detection. More recently, development in tandem mass spectrometry (MS/MS) technology, particularly selected reaction monitoring (SRM), delivered capability of multiplex, high sensitivity analysis in biological samples [25–27,30–34]. Similarly to this study SRM assays were developed to quantitatively profile 18 tryptophan metabolites in serum, urine, and cell culture supernatants [34]. However, to our knowledge this study is one of the first attempts using advantages of SRM technology to discover previously unreported microbial metabolites and it brings quantitative information on microbiota-modulated component of Trp metabolism in human urine.

In this study, an SRM library has been generated for a panel of indole derivatives listed in Table 1 and depicted in Fig. 1 and optimized UHPLC-SRM assays were applied to urine samples screening for Trp metabolism intermediates and novel microbial metabolites.

2. Material and methods

2.1. Chemicals and solvents

Chemical standards of L-tryptophan (Trp), 5-hydroxy-L-tryptophan (5HT), N-acetyltryptophan (NAT), tryptamine (TrpN), indole-3-acetate (IAA), methyl indole-3-acetate (MIA), indole-3-acetamide (IAM), melatonin (Mel), serotonin (Ser), 5-hydroxyindoleacetate (5HIAA), indole-3-ethanol (IEt), N-acetylserotonin (NASer), L-tryptophanamide (TrpAm), 3-methyl-2-oxindole (MOI), ethyl indole-3-acetate (EIA) and creatinine were purchased from IROA Technologies (cat. #MSMLS); methyl indole-3-propionate and indole-3-butyrate were purchased from Sigma Aldrich (cat. #S369764-10MG and 57310-5G-F, respectively) and indole-3-propionate was purchased from Alfa Aesar (cat. #L04877). Isotopically-labelled [^{2}H5]indole-3-acetic acid (^{2}H-IAA, cat. #031-1531) and [^{2}H5] indole-3-acetic acid methyl ester (^{2}H-IAAMe, cat. #031-1551) were purchased from OlChemIm Ltd. Czech Republic. All chemical s are listed in Table 1 and structures are shown in Fig. 1. Solvents for the preparation of the UHPLC mobile phase for the dilution of stock solutions and sample extraction, i.e. LC-MS grade methanol (cat. #9830-03), acetonitrile (cat. #9829-03) and 2-propanol (cat. #9827-03) were purchased from Avantor, LLC. Ultrapure water with a resistivity of >18 MΩ × cm was obtained using the Millipore purification system (Simplicity 185 system, mfr. #SIMS600CP, Millipore Corp.).