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Profiling of urinary amino-carboxylic metabolites by in-situ heptafluorobutyl chloroformate mediated sample preparation and gas chromatography–mass spectrometry

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ABSTRACT

A novel 1,1,1,2,2,3,3-heptafluorobutyl chloroformate reagent (HFBCF) was examined for in-situ derivatization of amino-carboxylic metabolites in human urine. The arising reaction products exhibit greatly reduced polarity which facilitates combining the derivatization and liquid-liquid microextraction (LLME) from an aqueous urine into an isooctane phase and immediate gas chromatographic–mas spectrometric analysis (GC–MS). The sample preparation protocol is simple, proceeds without an alcohol excess and provides cleaner extracts than other urinary GC–MS based methods. Moreover, thiol metabolites bound in disulfide bonds can be released by reduction with tris(3-hydroxypropyl)phosphine (THP) prior to the developed derivatization and LLME step. In order to evaluate potential of the novel method for GC–MS metabolomics, reaction products of 153 urinary metabolites with HFBCF, particularly those possessing amino and carboxyl groups (56 amino acids and their conjugates, 84 organic acids, 9 biogenic amines, 4 other polar analytes) and two internal standards were investigated in detail by GC–MS and liquid chromatography-mass spectrometry (LC–MS). One hundred and twenty metabolites (78%) yielded a single product, 25 (16%) and 2 metabolites (2-methylcitrate, citrate) generated two and more derivatives. From the examined set, analytically applicable products of 5 metabolites were not detected; the derivatives of 3 metabolites were only suitable for LC–MS analysis. Electron ionization (EI) of the examined analytes contained characteristic, diagnostic ions enabling to distinguish related and isomeric structures. The new method was validated for 132 metabolites using two internal standards in artificial urine and with special attention to potential disease biomarker candidates. The developed sample preparation protocol was finally evaluated by means of a certified organic acid standard mixture in urine and by GC–MS analysis of 100 morning urines obtained from healthy patients (50 males and 50 females), where 112 physiological metabolites were quantified in a 25 μL sample aliquot. The quantification data for the set were satisfactory, most metabolites were found within the range reported in the reference human metabolome (HMDB) database and literature. The reported results suggest that the described method has been a novel promising tool for targeted GC–MS based metabolomic analysis in urine.

1. Introduction

Human urine is a complex biofluid of hundreds small polar metabolites that are excreted by kidney in a buffered aqueous medium containing high portions of inorganic salts, urea and other ionic organic species. The metabolite profile in urine virtually reflects metabolic processes in cells and, consequently, health and disease status of organism. As the urine collection is non-invasive, analysis of urine has been a widely used diagnostic tool and new strategies expanding the metabolite coverage by means of separation-based technologies coupled to mass spectrometry (MS) are of a great interest for targeted metabolite analysis, metabolite profiling, metabolic fingerprinting and metabolomics [1–3].

Gas chromatography coupled to mass spectrometry (GC–MS) has been an efficient tool for comprehensive urinary metabolite analysis due to its high separation efficiency, sensitivity and

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robustness [4,5]. In comparison to the concurrent liquid chromatography (LC) or capillary electrophoresis (CE) coupled to atmospheric pressure ionization (API) MS detection, GC–MS benefits from lower matrix effects of co-eluting components and library searchable electron ionization (EI) mass spectra. On the other hand, the application range of GC–MS is limited to sufficiently volatile and thermally stable analytes so that the small polar metabolic entities of primary concern in urine such as amino and carboxylic acids have to be derivatized prior to GC analysis. As the urinary analytes usually contain multiple active hydrogen containing functional groups, their effective comprehensive analysis requires use of general-purpose reagents, preferably those that allow direct conversion of analytes in urine, in situ.

Many derivatization methods were explored in the past for treating as much as possible urinary metabolites. Among them, two-step oximation-silylation approach was the most widely used, typically using methoxylamine (MO) and a subsequent silylation with a trimethylsilyl (TMS) or tert-butyldimethylsilyl (TBDMS) reagent [6–10]. The oximation step prior silylation converts aldehyde and keto groups into oximes and inhibits thus multiple peak formation of sugars and cyclization and decarboxylation of ketocarboxylic acids [4,5]. The silylation procedure requires strictly anhydrous conditions and was mainly efficient for GC–MS analysis of O-silylated compounds, typically sugars [11,12] and steroids [13]. However, silylated protic sulfur and nitrogen functional groups are much less stable and prone to hydrolysis in the presence of water traces. The metabolites having NH_2 group often provide mono and double-silylated forms that continue to react in the reagent excess. Moreover, they are themselves very reactive donors of the silylation group and often decompose in GC injector port or even on the large specific surface area of a GC capillary column [14]. These phenomena undoubtedly affect the yield of each metabolite derivative to a different extent and can be a source of biases that complicate correction and standardization of the obtained metabolomic data and may eventually lead to misinterpretations [15–17]. Despite the extensive use of the oximation-silylation procedures in GC–MS metabolite profiling for more than 4 decades, the described practical problems stimulate looking for alternative sample preparation strategies.

Derivatization with alkyl chloroformates (RCFs) has been increasingly popular approach. Unlike silylation, the RCFs smoothly convert highly polar functional groups into the corresponding *N*-carbamate and (*S*, *O*)-carbonate carboxylic esters in the presence of the corresponding alcohol and pyridine catalyst. The reaction is quickly stopped by depletion of the reagent and the arising products are simultaneously extracted into an immiscible organic solvent layer which is directly amenable to GC–MS analysis [18,19].

In urinary analysis, the simple and cost-effective RCF mediated derivatization and concurrent liquid-liquid microextraction procedure was first applied to GC profiling of 44 organic acids [20] and branched chain carboxylic, keto, hydroxy and amino acids useful in diagnosis of maple syrup urine disease [21]. However, succinic and glutaric dicarboxylic acid and their analogues being important markers of impaired enzyme functions underwent undesired internal cyclizations to unstable anhydrides in the first studies. The facile esterification of a wide range of di- and tri-carboxylic acids with methyl and ethyl chloroformates (MCF, ECF), but not with less reactive RCFs of higher alkyls, was resolved by repeated addition of the reagent in presence of sodium hydroxide [22]. The improved sample preparation protocol enabled to increase metabolite coverage for simultaneous profiling of amino carboxylic species and has been successfully applied for GC–MS metabolomic analysis in various biological matrices [23–32].

The promising analytical features of the RCF reagents encouraged, by analogy with the historical development of perfluoroacyl reagents (anhydrides or imidazoles), research of chloroformates with fluorinated alkyls of a various chain length. Vincenti et al. [33–36] synthesized and employed long-chain fluoroalkyl chloroformates (FCFs) in GC–MS analysis of small very polar water disinfection by-products. A short chain trifluoroethyl chloroformate (TFECF) was prepared and examined to chiral separation of a set of amino acids by Abe et al. [37,38]. Analytical properties of RCFs with a pentafluoropropyl (PFPCF) or heptafluorobutyl (HFBCF) moiety were extensively studied by Hušek and co-workers [39–41]. Unlike the common RCFs, the FCF reagents are more reactive and capable to transform the target protic functional groups under pyridine catalysis without a presence of analogous alcohol. FCFs with five or seven fluorine atoms are stable and exhibit excellent volatility enabling enantiomeric separations of amino acids [40–42].

The HFBCF reagent was found highly efficient in nonchiral & chiral profiling of amino acids in human serum [43,44]. The sample preparation protocol comprised three simple steps; (i) release of bound thiols by a novel reducing agent 2,3-dimercaptopropane sulfonate (DMPS), (ii) protein precipitation by perchloric acid and (iii) direct treatment of the supernatant with HFBCF in isooctane that simultaneously allows transfer of the nonpolar derivatization products into an immiscible organic phase. In this way, extraordinary clean extracts were obtained enabling plasma/serum GC–MS metabolite analysis in the full scan MS regime [43]. Moreover, amino acid derivatives with HFBCF exhibit excellent chiral separation properties on Chirasil-Val phases that outperform other RCF amino acid derivatives hitherto examined; more than 35 amino acid enantiomeric pairs were separated and analyzed by GC–MS in human serum, except the D,L enantiomers of arginine and cystine (not eluted) and proline (not separated), [44]. Furthermore, FCFs have been shown highly reactive towards alicyclic hydroxyl in steroids and tocopherols under anhydrous conditions [45].

The HFBCF-mediated sample preparation strategy undoubtedly indicates very good perspectives in GC–MS profiling of other acidic and multifunctional metabolites occurring in urine and which, to our knowledge, has not yet been investigated. However, for standardization of a novel, effective GC–MS metabolomics method, qualified experimental data sets must be acquired in order to guarantee comparability among the particular samples. This requires thorough knowledge of the properties not only of the obtained metabolite derivatives but also abundance of possible side products, their stability, possible metabolite inter-conversions and cross-reactions [15].

This study expands the current knowledge by development a new GC–MS method for urinary metabolomic analysis. To evaluate the new method, reaction products and analytical properties of more than 150 known urinary metabolites, particularly those possessing amino and carboxyl groups, were investigated. The developed sample preparation protocol involves an immediate conversion of disulfides into thiols by the tris(3-hydroxypropyl) phosphine (THP) reducing agent [46] followed by in situ treatment of urine with HFBCF under pyridine catalysis. The derivatives possessing highly non-polar HFB moieties are concurrently transferred from urine into an isooctane immiscible layer by the employed liquid-liquid microextraction principle (LLME). The developed sample preparation protocol was evaluated by means of the analyte calibration in artificial urine, by determination of 19 diagnostic metabolites in a certified urine standard, and finally, by GC–MS analysis of 100 morning urines obtained from control adult subjects of both genders.

2. Experimental

2.1. Urine samples

Aliquots of the morning, second-void urine samples were gathered from adult clinical staff subjects in the Faculty Hospital Ostrava