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Simultaneous quantification of 21 water soluble vitamin circulating forms in human plasma by liquid chromatography-mass spectrometry

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ABSTRACT

This manuscript reports a validated analytical approach for the quantification of 21 water soluble vitamins and their main circulating forms in human plasma. Isotope dilution-based sample preparation consisted of protein precipitation using acidic methanol enriched with stable isotope labelled internal standards. Separation was achieved by reversed-phase liquid chromatography and detection performed by tandem mass spectrometry in positive electrospray ionization mode. Instrumental lower limits of detection and quantification reached <0.1–10 nM and 0.2–25 nM, respectively. Commercially available pooled human plasma was used to build matrix-matched calibration curves ranging 2–500, 5–1250, 20–5000 or 150–37500 nM depending on the analyte. The overall performance of the method was considered adequate, with 2.8–20.9% and 5.2–20.0% intra and inter-day precision, respectively and averaged accuracy reaching 91–108%. Recovery experiments were also performed and reached in average 82%. This analytical approach was then applied for the quantification of circulating water soluble vitamins in human plasma single donor samples.

The present report provides a sensitive and reliable approach for the quantification of water soluble vitamins and main circulating forms in human plasma. In the future, the application of this analytical approach will give more confidence to provide a comprehensive assessment of water soluble vitamins nutritional status and bioavailability studies in humans.

1. Introduction

Several water soluble vitamin metabolites are involved in cellular metabolism, mainly as coenzymes, to prevent metabolic disorders. Most of them are essential for energy metabolism. The phosphorylated forms of thiamine (vitamin B_1) play a key role in the Krebs cycle [1,2]. Riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxal 5′-phosphate (circulating form of vitamin B_6), 5-methyl tetrahydrofolate (circulating form of vitamin B_9) and biotin (vitamin B_8) are involved in oxidation/reduction reactions, fatty acid and neurotransmitters synthesis or one carbon metabolism [3–6]. Flavin mononucleotide (FMN), flavin adenosine dinucleotide (FAD) and NAD, NADP carry electron/proton for chain reactions whereas pantothenic acid, precursor of coenzyme A [7] indirectly acts as acyl group donor to form holo-acyl carrier protein [8]. At last, 5-methyl tetrahydrofolate methylates homocysteine to form methionine [9].

Because all these vitamers are directly involved in various body functions, their deficiency will impact human health. Supplementation is therefore highly recommended for targeted populations such as pregnant women, lactating women, infants, elderly and athletes to either prevent diseases and/or risks such as cardiovascular risk [10], anemia, cognitive impairment [11], neural tube defect [12,13] or improve physical performance [14].

Depending on the compound of interest, some analytical standards have been well established years ago and are widely applied to quantify them in food. Microbiological methods have been traditionally used and, although this is currently changing, they are still part of reference standards used for food control in different parts of the world [15,16]. Although these type of assays have been applied to the analysis of human serum or plasma, they present the disadvantage of providing a global result, without the possibility of differentiate between native vitamin used in food supplementation and its metabolites, which reduces the field of application in clinical research. Other techniques are also applied for the analysis of

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food, such as ELISA [17], radio immunoassay [18], protein-binding or biosensor based [19–21] with the same disadvantage on the differentiation of metabolites. Fluorescence detection combined with liquid chromatography has been extensively used to quantify B_1 [22], B_2 [23], B_6 [24,25] and biotin [26] in foods and biological fluids but also used to validate multi-analyte methods in food matrices. For example, Heudi et al. reported a single chromatography for the quantification of 8 water soluble vitamins used for fortification in premixes samples [27]. In the past ten years, liquid chromatography hyphenated with tandem mass spectrometry (LC-MS/MS) demonstrated to be a more and more attractive technique with various advantages such as improved sensitivity and higher resolution for the analysis of water soluble vitamins. The vitamins used for fortification and/or their naturally present vitamers are then quantified individually [28,29] or in multi-analyte methods [30–35].

Only few methods were established for the analysis of water soluble vitamins in biological fluids such as human milk [24,36], human plasma [23] or urine [37], where single vitamin was quantified. More recent studies demonstrated the trend to go further into multi-analyte methodologies [38]. For instance, in 2012, Hampel et al. quantified four different water soluble vitamins represented by five analytes in human milk by LC-MS/MS [39]. Multi-analyte methodologies in human serum or plasma are less common. Only B_2 and B_6 are currently quantified together in human plasma [30]. It was never reported that a unique analytical approach could help performing the simultaneous quantification of B_1, B_2, B_3, B_5, B_6, B_8 and B_9 and their main circulating forms in human plasma. This can be of a great advantage when vitamer profiling is required in large longitudinal studies.

Expanding our knowledge towards the metabolic fate of vitamins is the key to understanding their actual contribution in biological activities. In the present paper, we report for the first time a novel chromatography method for the separation of 21 water soluble vitamins and main circulating forms in human plasma. For this purpose, a methodology based on LC-MS/MS is proposed to detect, identify and quantify these compounds.

2. Experimental

2.1. Chemicals

HPLC grade water, LC-MS grade acetonitrile (ACN), LC grade methanol (MeOH), LC grade ethanol (EtOH), LC grade isopropanol, trichloroacetic acid (TCA) and dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Acetic acid (Ac.Ac.) for analysis, ascorbic acid (Asc.Ac.) and LC grade heptafluorobutyric acid (HFBA) were from Fluka/Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

Standards and stable isotope labelled standards were purchased from various suppliers:

thiamine monophosphate (TMP) chloride dihydrate, thiamine pyrophosphate (TPP), FMN sodium salt hydrate, FAD disodium salt, nicotinamide (NM), nicotinic acid (NA), nicotinuric acid (NUA), pantothenate calcium, pyridoxal 5′-phosphate (PLP) hydrate, pyridoxal (PL) hydrochloride, pyridoxamine 5′-phosphate (PMP), pyridoxic 4-acid (Pyr) were purchased from Sigma-Aldrich Chemie GmbH. Pyridoxamine (PM) dihydrochloride, para-aminobenzoyl glutamic acid (*p*-ABGA) from Fluka/Sigma-Aldrich Chemie GmbH. Thiamine hydrochloride, riboflavin, pyridoxine (PN) hydrochloride, biotin were purchased from Supelco (Buchs, Switzerland), nudifloramide from Toronto Research Chemicals (Toronto, Canada), folic acid (FA) and 5-methyl tetrahydrofolic acid (5-Me THF) were purchased from Schircks Laboratories (Jona, Switzerland).

Nicotinamide-[d_4], nicotinic acid-[d_4], nicotinuric acid-[d_4], pyridoxine-[d_2] were purchased from CDN isotopes (Darmstadt, Germany). Pyridoxamine-[d_3], riboflavin-[$^{13}C_4$, $^{15}N_2$], thiamine-[$^{13}C_4$] hydrochloride, biotin-[d_2] were purchased from Isosciences (King of Prussia, USA). Nudifloramide-[d_3], para-aminobenzoyl glutamic acid-[d_4] (*p*-ABGA-[d_4]) were purchased from Toronto Research Chemicals. Pantothenic acid-[$^{13}C_6$, $^{15}N_2$] was purchased from Fluka/Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) and folic acid-[$^{13}C_5$] and 5-methyl tetrahydrofolic acid-[$^{13}C_5$] (5-Me THF-[$^{13}C_5$]) from Merck Eprova (Schaffhausen, Switzerland).

2.2. Standard solubility and stability in solution

Based on our previous experience with vitamins used for fortification, standards (STDs) were first weighted and dissolved individually in HCl, 0.1 M to study their solubility and stability. Due to partial solubility of some compounds in this solution, various solvents were explored to obtain the best solubility and stability conditions. Stock standard solutions were then aliquoted and kept at −20 °C. Each aliquot was used for standard stability studies that were performed at following time points: $t = 0$ (freshly prepared standards), $t = 2$ weeks, $t = 6$ weeks, $t = 10$ weeks, $t = 12$ weeks. For non-stable compounds at 2 weeks, an additional stability study was performed at $t = 1$ week.

At each time point, fresh stock solutions were prepared for each analyte to build a fresh calibration curve with 4 calibration points containing all analytes in solution. An aliquot of each analyte was thawed and used to prepare a standard mix solution at 2 concentration levels falling within the 4-point calibration curve. By comparison with the calculated and theoretical concentration, it was concluded if the stock solution was stable or not.

2.3. Standard solutions

Based on solubility/stability studies, individual standards and internal standard (ISTD) for method validation were weighed and dissolved in different solvents or solutions as given in Table 1. Individual standard stock solutions were then combined and diluted with H_2O to obtain the standard solution mixes to build up the calibration curve. Individual ISTD stock solutions were combined and diluted with H_2O to obtain an ISTD mix. ISTD mix was prepared in order to spike the matrix to obtain a final ISTD concentration corresponding to STD6, equivalent to 50 × STD1 analyte concentration.

2.4. Plasma samples

A pooled human plasma and 20 human plasma single donors samples were obtained from Innovative Research (King of Prussia, USA). Single donors were not restricted to any specific diet. Pooled human plasma was used for the development and validation of the analytical approach. Individual human plasma samples were only used to apply this methodology. All samples were stored at −80 °C previous to analysis.

2.5. Sample preparation

Plasma samples (pooled and unknown samples) were thawed and vortexed for homogenization. An aliquot of 200 μL was taken into an amber vial and spiked with 20 μL of ISTD mix (or 20 μL of dedicated STDs mix containing all ISTDs in order to build the calibration curve and QC samples in the matrix). Then, 800 μL of a mixture methanol containing 1% Ac.Ac.:H_2O (9:1, v/v) were added. The mixture was vortexed for 10 s, left stand for 5 min at room temperature to allow protein precipitation. The samples were centrifuged at 12,000 × *g* for 15 min at 4 °C in a Sigma 3-16K centrifuge. The supernatant was transferred into amber vials and evaporated

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