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Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry

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Vitamins B_2 and B_6 serve as cofactors in enzymatic reactions involved in tryptophan and homocysteine metabolism. Plasma concentrations of these vitamins and amino acids are related to smoking and inflammation, and correlate with other markers of immune activation. Large-scale studies of these relations have been hampered by lack of suitable analytical methods. The assay described includes riboflavin, five vitamin B_6 forms (pyridoxal 5′-phosphate, pyridoxal, 4-pyridoxic acid, pyridoxine and pyridoxamine), tryptophan and six tryptophan metabolites (kynurenine, kynurenic acid, anthranilic acid, 3-hydroxykynurenine, xanthurenic acid and 3-hydroxyanthranilic acid), cystathionine, neopterin and cotinine. Trichloroacetic acid containing 13 isotope-labelled internal standards was added to 60 μL of plasma, the mixture was centrifuged, and the resulting supernatant used for analysis. The analytes were separated within 5 min on a stable-bond C8 column by a gradient-type mobile phase containing acetonitrile, heptafluorobutyric acid and high concentration (650 mmol/L) of acetic acid, and detected using electrospray ionization tandem mass spectrometry (ESI-MS/MS). The mobile phase ensured sufficient separation and high ionization efficiency of all analytes. Recoveries were 75–123% and within-day and between-day coefficients of variance (CVs) were 2.5–9.5% and 5.4–16.9%, respectively. Limits of detection ranged from 0.05 to 7 nmol/L. The method enables quantification of endogenous plasma concentrations of 16 analytes related to B-vitamin status and inflammation, and may prove useful in large-scale epidemiological studies.

Vitamins B_2 and B_6 serve as cofactors in enzymatic reactions involved in tryptophan and homocysteine metabolism. Tryptophan is degraded primarily through the kynurenine pathway,[1] which is a cascade of reactions initiated by tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) followed by kynurenine formamidase.[2] Kynurenine thus formed may be further metabolized by vitamin B_2- or B_6-dependent enzymes to kynurenic acid (KA), anthranilic acid (AA), 3-hydroxykynurenine (HK), xanthurenic acid (XA) or 3-hydroxyanthranilic acid (HAA).[3–5] Several reactions in the homocysteine metabolism require vitamins B_2 or B_6 as cofactors. These include the vitamin B_2-dependent reaction that provides 5-methyltetrahydrofolate for homocysteine remethylation,[6] and the B_6-dependent transsulfuration pathway, where homocysteine is converted into cystathionine and further into cysteine.[6]

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In addition to sharing vitamins B_2 and B_6 as cofactors, the metabolism of both tryptophan and homocysteine is related to inflammation and immune activation.[7] IDO is induced by Th1-type cytokine interferon-γ, leading to increased degradation of tryptophan to kynurenine.[2] Recent studies have demonstrated that plasma levels of total homocysteine (tHcy) and the kynurenine/tryptophan ratio show a strong positive correlation with neopterin,[7,8] a sensitive marker of Th1-type immune activation.[9] Furthermore, low plasma[10,11] and serum[12] concentrations of the active form of vitamin B_6, pyridoxal 5′-phosphate (PLP), have been found in inflammation.

Smoking increases the risk of cardiovascular disease and other chronic diseases,[13] and has adverse effects on micronutrients status, including riboflavin[14,15] and vitamin B_6.[16] Accordingly, plasma concentrations of the nicotine metabolite cotinine, a measure of tobacco smoke exposure,[17] shows a strong, positive relation to plasma tHcy.[18] Serum cotinine is associated with reduced serum kynurenine/tryptophan ratio,[19] while data on the effect of smoking on serum neopterin concentrations are inconsistent,[20,21] possibly reflecting complex immunomodulatory effects of smoking.

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Methods have been developed for the separate determination of riboflavin,[22] vitamin B_6 species,[23] tryptophan and kynurenines (metabolites of the kynurenine pathway),[24–31] neopterin,[32–35] cystathionine[36–38] and cotinine.[39–41] However, no method has been developed that simultaneously determines these vitamins and biomarkers that converge on B-vitamin status and inflammation. Such developmental work would be a demanding undertaking because of the chemical diversity and wide plasma concentration range (~1 nmol/L to 100 μmol/L) of these compounds. However, a multiplexing method of these vitamins and metabolites would be useful in large epidemiological studies on B-vitamins, inflammation and chronic diseases, in particular if only a small sample volume is available.

We have previously described a liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay for the determination of vitamin B_2 and B_6 species in human plasma.[42] We here present a further development of this method to simultaneously determine riboflavin, five vitamin B_6 species, tryptophan and six kynurenines, cystathionine, neopterin and cotinine in 60 μL of plasma. Simple sample preparation and chromatographic run time of 5 min ensure high sample throughput.

EXPERIMENTAL

Materials

Riboflavin (purity >98%), PLP (98%), pyridoxal hydrochloride (99%), 4-pyridoxic acid (PA) (>99%), pyridoxine hydrochloride (>99%), pyridoxamine dihydrochloride (98%), tryptophan (98%), kynurenine (99%), KA (>98%), AA (>98%), HK (99%), HAA (>99%), neopterin (>98.5%) and cotinine (>98%) were purchased from Sigma-Aldrich. XA (>95%), cystathionine (>95%) and heptafluorobutyric acid (HFBA, 99.5%) were purchased from Fluka. Acetic acid (99.8%) and trichloroacetic acid (TCA) (99.5%) were from Merck, and acetonitrile (99.8%) from SDS. $^{15}N_1$-Neopterin (>98.8%) (Schircks Laboratories, Jona, Switzerland) and 12 deuterated species were used as internal standards. $^2H_8$-Riboflavin (98.3%), $^2H_3$-pyridoxal (99%) and $^2H_4$-pyridoxine (99%) were synthesized by Beta Chem (Leawood, KS, USA). $^2H_2$-PLP (95%) was kindly supplied as a gift by S. P. Coburn (Department of Chemistry, Indiana University – Purdue University, USA). $^2H_2$-PA (>98%), $^2H_4$-kynurenine (>98%), $^2H_2$-HAA (>98%) and $^2H_4$-AA (>98%) were from Buchem (Apeldoorn, The Netherlands). $^2H_5$-Tryptophan (>98%), $^2H_5$-KA (>99%) and $^2H_4$-cystathionine (>95%) were from C/D/N Isotopes (Pointe-Claire, Canada), while $^2H_3$-cotinine (99%) was obtained from Sigma-Aldrich. Bovine serum albumin (99%) was from Sigma-Aldrich. Doubly deionized water was used.

Individual stock solutions of analytes and internal standards were prepared in water, and stored at −80°C. The concentrations of the stock solutions were 100 μM for neopterin, riboflavin, KA, AA, HK, XA, HAA, $^{15}N_1$-neopterin, $^2H_5$-KA, $^2H_4$-kynurenine and $^2H_3$-cotinine, 150 μM for $^2H_8$-riboflavin, 200 μM for PLP, pyridoxal, PA, PM, PN and cystathionine, 400 μM for $^2H_2$-HAA, 500 μM for $^2H_2$-PLP, $^2H_3$-pyridoxal, and $^2H_4$-PN, 1 mM for cotinine, kynurenine, $^2H_2$-PA and $^2H_4$-AA and $^2H_4$-cystathionine, 5 mM for tryptophan and 20 mM for $^2H_5$-tryptophan.

With the exception of $^2H_2$-PA, which contained ~2% PA and $^{15}N_1$-neopterin, which contained ~0.5% neopterin, unlabelled compounds were not detected in the isotope-labelled internal standards. The amount of PA added to the sample matrix with $^2H_2$-PA was subtracted when calculating PA concentrations.

A fraction of a plasma pooled from four persons, three non-smokers and one smoker, was spiked with 20 nmol/L of riboflavin, pyridoxal, pyridoxine and pyridoxamine, 50 nmol/L of PA, 100 nmol/L of PLP, neopterin, cotinine, KA, AA, XA, HK, HAA, 200 nmol/L cystathionine, 1 μmol/L kynurenine and 50 μmol/L tryptophan. Analyte concentrations of the unspiked plasma, calculated using the standard addition method by using the difference in response between the spiked and unspiked plasma and averaging 32 measurements obtained on 32 different days, were 8.2 nmol/L for riboflavin, 40.2 nmol/L for PLP, 8.0 nmol/L for pyridoxal, 24.0 nmol/L for PA, <0.05 nmol/L for pyridoxine, <0.1 nmol/L for pyridoxamine, 66.9 μmol/L for tryptophan, 1.53 μmol/L for kynurenine, 44.7 nmol/L for KA, 12.5 nmol/L for AA, 36.2 nmol/L for HK, 15.8 nmol/L for XA, 37.6 nmol/L for HAA, 12.4 nmol/L for neopterin, 0.326 μmol/L for cystathionine and 359 nmol/L for cotinine. The concentration of the spiked plasma was calculated as unspiked plus spiking level. The unspiked and spiked plasma were aliquoted and stored at −80°C and were used as calibrators in routine assay calibration. The calibrators with added analytes were freshly prepared at regular intervals of 14 days over more than 12 months. A constant ratio between endogenous and added vitamers indicates stability of the endogenous vitamins during storage. A plasma pooled from six healthy individuals are used for quality control in routine analysis.

For the matrix effect experiments we used spiked plasma from eight healthy subjects, and for the recovery and imprecision experiments, we used unspiked and spiked plasma from a healthy blood donor.

The study has been examined by the Institutional Review Board (REK Vest) and found to be of the Quality Control Category, which under the Norwegian regulations in force is exempt from full review by the Board. The Board thus has no objection to the use of plasma samples in this study.

Sample collection and processing

EDTA plasma was obtained by collecting blood into Vacutainer tubes (Becton Dickinson), giving a final EDTA concentration of 4 mmol/L. The samples were immediately put on ice, and centrifuged (2000 *g*, 4°C for 10 min) within 60 min. The plasma was then processed further or stored at −80°C until use.

Plasma (60 μL) was deproteinized by mixing with an equal volume of TCA in water (60 g/L) containing the isotope-labelled internal standards (100 nmol/L of $^2H_2$-PLP, $^2H_3$-pyridoxal, $^2H_2$-PA, $^2H_4$-pyridoxine, $^2H_5$-KA, $^2H_4$-AA and $^2H_2$-HAA, 200 nmol/L of $^2H_8$-riboflavin, $^2H_4$-kynurenine, $^2H_4$-cystathionine, $^{15}N_1$-neopterin and $^2H_3$-cotinine and 2 μmol/L of $^2H_5$-tryptophan). The precipitation step was carried out by a robotic workstation (MicrolabAT Plus, Reno,

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NV, USA). The solution was mixed by the robot, left for 60 min on ice, and finally centrifuged at 5796 g at 4°C for 15 min. The supernatant (60 μL) was transferred to a new vial which was placed in a cooled (8°C) autosampler. The samples were protected from light.

Instrumentation

We used an Agilent series 1100 HPLC system equipped with a thermostatted autosampler and degasser, and an API 4000 triple-quadrupole tandem mass spectrometer from Applied Biosystems/MSD SCIEX with electrospray ionization (ESI) source. A column switcher from Valco (type EMHA) was used to divert the flow to waste during the first 1.8 min of the run and between each injection. Analyst v.1.4.1 from Applied Biosystems/MDS SCIEX was used for data acquisition and analysis.

LC/MS/MS

Deproteinized plasma (50 μL) was injected into a Zorbax stable-bond C8 reversed-phase column (150 × 4.6 mm, particle size 3.5 μ) equipped with a similar guard column (12.5 × 4.6 mm, particle size 5 μ). The guard and analytical columns were mounted in a thermostatted column compartment set at 40°C.

The mobile phase consisted of three components, solution A (650 mmol/L acetic acid), solution B (100 mmol/L HFBA in A) and solution C (90% acetonitrile in water).

Samples were injected every 6.4 min, and analytes eluted at a flow rate of 1.3 mL/min by the following gradient: 0–0.14 min (98% A and 2% B), 2.2 min (78% A, 2% B and 20% C), 2.3 min (60% A, 2% B and 38% C), 3.3 min (40% A, 2% B and 58% C), 3.4–4.1 min (2% B and 98% C) and 4.2–5.0 min (98% A and 2% B). All gradient steps were linear. The column effluent was delivered to the mass spectrometer with no split.

Acquisition parameters were optimized by infusion of a 10 μmol/L standard solution of each analyte at a rate of 1 μL/min. Using a T-junction in front of the mass spectrometer, this solution was mixed with mobile phase delivered at a rate of 1 mL/min and with a composition corresponding to the retention time of the actual analyte. The parameters (Table 1) were optimized within four scan segments. Ion-spray (5500 V), curtain gas (10 psig), collision gas (4 psig), ion source temperature (650°C), ion source gas 1 and 2 (75 psig) and activated interface heater were identical for all segments.

Table 1. Retention times and instrument settings[a]

		Transitions, *m/z*					
Analyte	t_r, min	Precursor ion	Product ion	DT, ms	DP, V	CE, V	CXP, V
Scan segment 1, 0–2.70 min							
$^2H_2$-PLP	2.07	250.3	152.1	30	76	24	12
PLP	2.09	248.1	149.9	30	90	24	12
$^{15}N_1$-Neopterin	2.09	255.1	207.3	100	30	29	18
Neopterin	2.10	254.1	206.3	100	30	29	18
$^2H_4$-Cystathionine	2.40	226.9	137.9	30	20	20	12
Cystathionine	2.40	222.9	133.9	30	20	20	12
Scan segment 2, 2.70–4.25 min							
$^2H_2$-PA	3.38	186.2	150.0	30	61	31	12
PA	3.39	184.1	148.0	30	61	31	12
$^2H_3$-Pyridoxal	3.53	171.1	153.1	30	51	15	16
Pyridoxal	3.54	168.1	150.1	30	51	17	14
Pyridoxamine	3.70	169.3	134.1	30	46	29	14
$^2H_4$-Pyridoxine	4.01	174.1	138.1	30	66	29	10
Pyridoxine	4.06	170.1	134.1	30	66	29	10
$^2H_3$-Cotinine	4.11	180.2	100.9	30	50	35	10
Cotinine	4.12	177.2	80.2	30	50	34	10
HK	4.15	225.2	208.3	30	33	14	10
Scan segment 3, 4.25–4.75 min							
$^2H_2$-HAA	4.35	156.2	82.2	30	35	35	10
HAA	4.36	154.1	80.0	30	35	39	10
$^2H_8$-Riboflavin	4.47	385.4	251.1	30	90	30	6
Riboflavin	4.47	377.4	243.3	30	90	30	6
XA	4.47	206.0	160.0	30	45	25	12
$^2H_5$-KA	4.54	195.2	149.0	30	50	30	12
KA	4.54	190.3	143.9	30	45	27	12
$^2H_4$-Kynurenine	4.61	213.2	178.3	30	50	23	12
Kynurenine	4.62	209.1	174.3	30	40	21	12
$^2H_5$-Tryptophan	4.65	210.3	193.1	30	40	30	15
Tryptophan	4.65	206.3	189.1	30	40	30	15
Scan segment 4, 4.75–5.0 min							
$^2H_4$-AA	4.83	142.1	124.1	30	35	15	10
AA	4.86	138.1	120.1	30	35	15	10

[a] t_r, retention time; DT, dwell time; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.
Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; PA, 4-pyridoxic acid; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

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The analytes were detected in positive-ion multiple-reaction monitoring (MRM) mode with unit resolution at Q1 and Q3. The ion transitions for all analytes are shown in Table 1, which lists the protonated molecular ion $[M+H]^+$ and the product ion used for detection. Table 1 also shows the division of the chromatographic cycle into four scan segments, which each encompassed 2–11 ion pairs.

We included $^2H_8$-riboflavin, $^2H_2$-PLP, $^2H_3$-pyridoxal, $^2H_2$-PA, $^2H_4$-pyridoxine, $^2H_5$-tryptophan, $^2H_4$-kynurenine, $^2H_5$-KA, $^2H_4$-AA, $^2H_2$-HAA, $^2H_4$-cystathionine, $^{15}N_1$-neopterin and $^2H_3$-cotinine as internal standards. The response of each analyte for which a corresponding isotopologue was available as internal standard was calculated as the ratio between the signal intensity area for the analyte divided by the area for the isotopologue. Isotopologues were unavailable for the pyridoxamine, HK and XA, thus $^2H_4$-pyridoxine was used to calculate the area ratio for pyridoxamine, $^2H_2$-HAA for HK, and $^2H_5$-tryptophan for XA.

Matrix effects

We investigated matrix effects[43] in plasma by measuring peak areas in eight different plasma samples spiked after the protein precipitation step with 10 nmol/L of pyridoxal, pyridoxine, pyridoxamine, XA, HAA, riboflavin and neopterin, 50 nmol/L of PLP, PA, KA, AA and HK, 200 nmol/L of cystathionine and cotinine, 500 nmol/L of kynurenine and 10 μmol/L of tryptophan. The increases in peak areas of the analytes were compared with the respective areas measured in an aqueous standard solution spiked with the same concentrations. The matrix effects were calculated from the analyte peak areas as:

$$\text{Matrix effect} = \frac{[\text{Analyte peak area}]_{\text{spiked}} - [\text{Analyte peak area}]_{\text{endogenous}}}{[\text{Analyte peak area}]_{\text{standard solution}}} * 100\%$$

and from peak area ratios as:

$$\text{Matrix effect} = \frac{[\text{Analyte peak area/ISTD peak area}]_{\text{spiked}} - [\text{Analyte peak area/ISTD peak area}]_{\text{endogenous}}}{[\text{Analyte peak area/ISTD peak area}]_{\text{standard solution}}} * 100\%$$

Values lower or higher than 100 indicate ion suppression or enhancement, respectively.

Performance of the method

To assess linearity and limit of detection (LOD), we prepared solutions of 4% albumin in phosphate-buffered saline (PBS), to which all analytes were added to obtain 24 different solutions with final concentrations in the range 0.05–400 nmol/L for PLP, pyridoxal, PA, pyridoxine, pyridoxamine, riboflavin, KA, AA, HK, XA, HAA and neopterin, 0.5–4000 nmol/L for kynurenine, cystathionine and cotinine, and 0.05–400 μmol/L for tryptophan. The albumin solution was treated with active charcoal (16 mg/mL) in order to remove endogenous AA.

Signal-to-noise (S/N) ratios for each ion pair were calculated as S/N = (peak height − baseline)/SD(baseline) using the script supplied by Applied Biosystems (Analyst v.1.4.1), and LODs were determined from the linearity data as the lowest concentrations that gave peaks with S/N values higher than five.

To determine recovery and imprecision, we used plasma divided into three portions. One of these portions was spiked to medium concentrations (by adding 10 nmol/L of pyridoxal, pyridoxine, pyridoxamine, XA, HAA, riboflavin and neopterin, 50 nmol/L of PLP, PA, KA, AA and HK, 200 nmol/L of cystathionine and cotinine, 1 μmol/L of kynurenine and 16.7 μmol/L tryptophan) and one spiked to high concentrations (by adding 30 nmol/L of pyridoxal, pyridoxine, pyridoxamine, XA, HAA, riboflavin and neopterin, 150 nmol/L of PLP, PA, KA, AA and HK, 600 nmol/L of cystathionine and cotinine, 3 μmol/L of kynurenine, and 50 μmol/L of tryptophan). We analyzed 24 replicates at each analyte level (low, medium and high) in one analytical run, and recovery was calculated as:

$$\text{Recovery}(\%) = \frac{\text{Measured concentration} - \text{Endogenous concentration}}{\text{Added concentration}} \times 100$$

Within-day imprecision of the assay was calculated from the data of the recovery experiments. Between-day imprecision was determined by assaying the same samples on 19 different days over a period of 41 days.

Plasma concentrations of the analytes

EDTA-plasma samples from 94 anonymous, non-fasting, presumed healthy individuals (64% female), with a median (range) age of 56 (11–93) years, was donated by the blood bank at Haukeland University Hospital. These plasma samples were processed and stored as described to minimize preanalytical errors, and used for method validation only.

Comparison of neopterin assays

In order to compare neopterin concentrations obtained with this assay with results from enzyme-linked immunosorbent assay (ELISA) (IBL, Hamburg, Germany), we analyzed EDTA-plasma from 40 individuals (20% female, mean (SD) age of 57.8 (9.0) years). Plasma samples were processed and stored as described to minimize preanalytical errors, and used for method validation only.

RESULTS AND DISCUSSION

Metabolite profiling in large-scale epidemiological studies requires fast, multianalyte methods for measurement of biomarkers related to specific metabolic pathways or diseases. Methods optimized for such purposes may therefore include biomarkers that differ structurally and chemi-

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cally, while covering a wide concentration range. Multiplexing chemically different compounds places constraints on the procedures used for sample preparation to avoid degradation or loss of analytes.

In this article, we describe a method based on LC/MS/MS developed for fast analysis of a panel of analytes related to B-vitamin status and inflammation in 60 μL of plasma. The method exploits the efficient chromatography and strong ionization obtained with a mobile phase containing high concentration of acetic acid,[44] and includes riboflavin, five vitamin B_6 forms, tryptophan, six kynurenines, neopterin, cystathionine and cotinine.

Analyte stability, sample preparation and storage

We established procedures for sample treatment and storage, guided by published data on analyte stability. Most vitamin B_6[45–48] and B_2 forms,[49] tryptophan[50] and kynurenine[50] show some degree of instability in aqueous solutions when exposed to light. PLP is unstable in plasma at room temperature,[51] but is stable at −30°C.[52] In EDTA plasma, riboflavin is stable, and neither flavin adenine dinucleotide (FAD) nor flavin mononucleotide (FMN) is degraded to riboflavin.[49,53] Cotinine is considered to be stable.[17] Neopterin is stable in serum at −20°C for at least 6 months.[35] Analysis of 40 EDTA plasma samples with this assay and ELISA gave mean (SD) concentrations of 6.9 (4.8) nmol/L and 6.6 (3.4) nmol/L, respectively, with $p = 0.44$. This suggests that the present method (treatment of plasma with TCA at low temperature) did not oxidize 7,8-dihydroneopterin into neopterin.[35,54]

The calibrator plasma and aqueous stock solutions of analytes and internal standards were aliquoted and stored at −80°C, and no degradation was observed over a period of 1 year. Sample preparation included a simple protein precipitation step using TCA (giving a final concentration of 30 g/L) followed by an incubation time of 60 min at 0°C to optimize the yield of protein-bound PLP and pyridoxal.[42] All analytes and internal standards were stable for at least 48 h in the acidic supernatant when stored at 8°C in a dark vial in the autosampler.

Chromatography

The LC/MS/MS chromatogram of the spiked calibrator plasma is shown in Fig. 1 and the retention times are listed in Table 1. The mobile phase components and gradient were optimized in order to obtain short retention times and elution of 2–11 analytes within each of four separate scan segments. Short retention times are required for high sample throughput, but also have the advantage of minimizing the difference in retention time between isotopologues, which otherwise may become separated by liquid chromatography due to the isotope effect.[55–57] In the present assay, the retention times of isotopologues differed by 0.05 min or less. All analytes except pyridoxine, pyridoxamine and cotinine were detected in plasma from healthy subjects not exposed to tobacco smoke or taking supplements containing vitamin B_6.

Mass spectrometry and acquisition parameters

The mass spectrometer was used in the positive-ion MRM mode to optimize selectivity and sensitivity. All analytes gave a strong signal from the singly protonated molecule $[M + H]^+$, and we selected the most abundant product ion for all analytes except tryptophan, for which the second most abundant isotope was used in order to avoid saturation of the detector. The selected molecular transitions are listed in Table 1.

We optimized the acquisition parameters (Table 1) for each analyte, and divided the analysis into four scan segments in order to obtain optimal signal quality, which is related to the number of data points across each chromatographic peak.

There was carryover from pyridoxine into the pyridoxamine channel due to small mass difference combined with identical product ion, and from $^2H_2$-PA into the pyridoxal channel. The latter was probably caused by loss of water. This cross-talk caused no analytical interference because the actual peaks had different retention times (Fig. 1).

Matrix effects

The method performance, in terms of imprecision and accuracy, is influenced by matrix effects, which occur as ion suppression or ion enhancement.[43] Matrix effects may be reduced by sample clean-up or partly corrected for by the use of appropriate internal standards.[43,58] A more comprehensive sample clean-up was not feasible because of the chemical diversity of the analytes. We used isotope-labelled internal standards when obtainable.

The matrix effects in eight different plasma samples were found to vary in the range 14–123% (Table 2). The relative matrix effect (coefficient of variance (CV)),[43] which describes variation between samples, had an average value of 11%, range 6–22%.

Dividing analyte area by internal standard area corrected for matrix effects for 10 of the 16 analytes (Table 2). The use of isotope-labelled internal standards reduced the relative matrix effect for some, but not all, analytes, and its average value was essentially unchanged (10%) (Table 2). Though the absolute matrix effect increased for some analytes (pyridoxamine, pyridoxine, riboflavin and KA) after dividing the analyte area by the area of internal standard, the relative matrix effects for these analytes were still low to moderate (7–13%), suggesting that quantification will still be adequate. It has been demonstrated that matrix effects are not always identical for different isotopologues,[59] which is consistent with our observations.

Assay validation

We assessed assay performance in terms of linearity, sensitivity, analytical recovery and imprecision.

The regression curves for area ratio vs. analyte concentration were linear for all analytes within the concentration ranges investigated, except for cystathionine and cotinine, which showed slightly nonlinear responses at concentrations above 1.33 and 1.0 μmol/L, respectively. Regression parameters for the linear ranges are shown in Table 3.

LODs (Table 3) were calculated from the linearity experiments. Eleven analytes had LODs in the picomolar range. The lowest sensitivities were obtained for tryptophan (400 nmol/L, 4 nmol/L when detecting the most abundant isotope) and kynurenine (7 nmol/L). The LOD values are comparable to or lower than those of published assays for riboflavin,[42,60] vitamin B_6 forms,[42,61–63] tryptophan and

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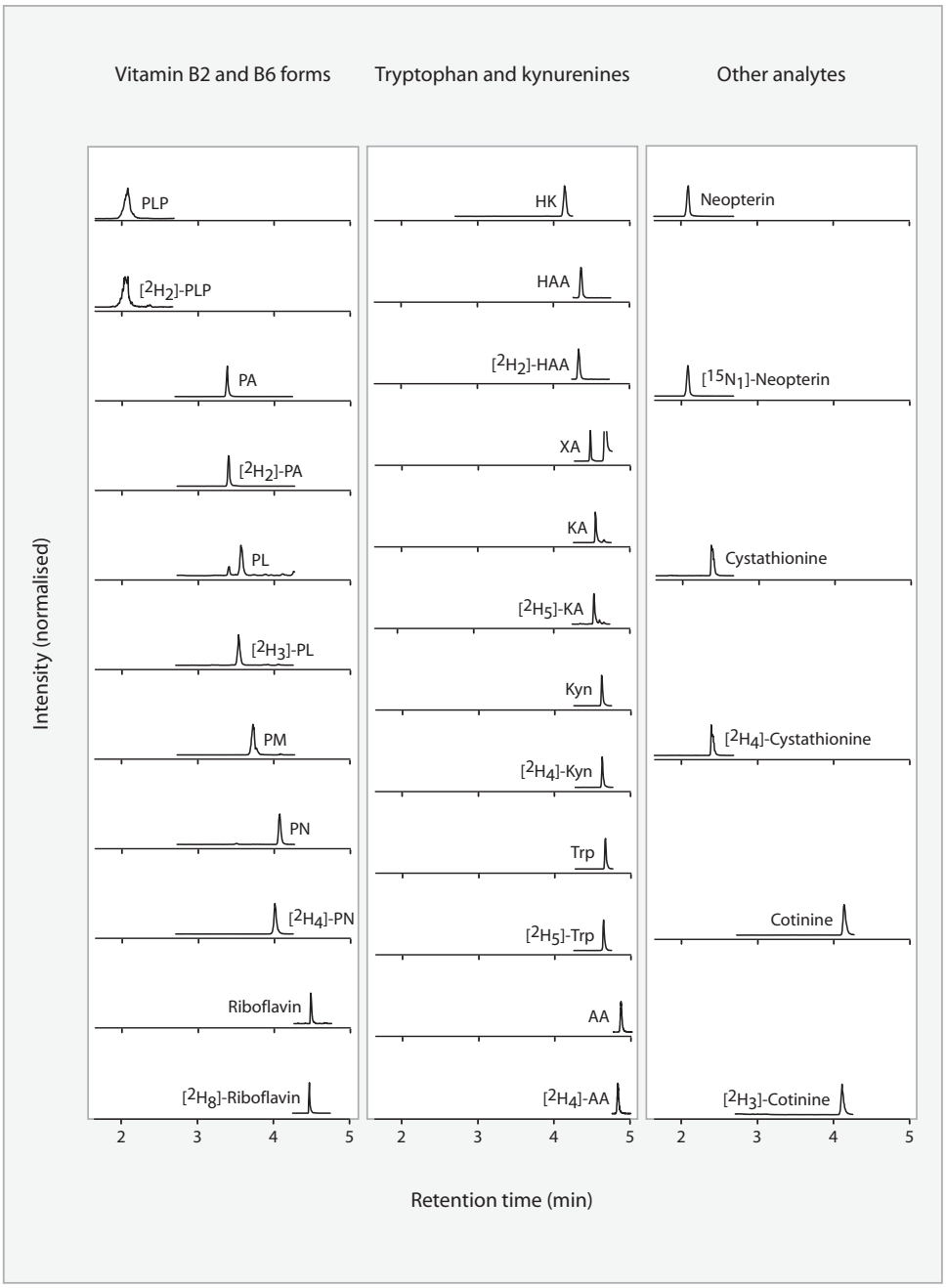

Figure 1. Normalized LC/MS/MS chromatograms for biomarkers of B-vitamin status and inflammation in human plasma. The spiked plasma contained riboflavin, 28.2 nmol/L; PLP, 140.2 nmol/L; pyridoxal, 28.0 nmol/L; PA, 44.0 nmol/L; pyridoxine, 20.0 nmol/L; pyridoxamine, 20.0 nmol/L; tryptophan, 116.9 μmol/L; kynurenine, 2.53 μmol/L; KA, 144.7 nmol/L; AA, 112.5 nmol/L; HK, 136.2 nmol/L; XA, 115.8 nmol/L; HAA, 137.6 nmol/L; neopterin, 112.4 nmol/L; cystathionine, 0.526 μmol/L; cotinine, 359 nmol/L. Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; Kyn, kyurenine; PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PN, pyridoxine; Trp, tryptophan; XA, xanthurenic acid.

kynurenines,[26–28,31] neopterin,[32,33,35] cystathionine[36,38] and cotinine.[39–41] Defining the limit of quantification (LOQ) as 2 × LOD implies that the sensitivity of the assay enables quantification of endogenous concentrations of all analytes, including cotinine levels caused by passive smoking.[64]

Analytical recoveries were in the range 75–123% (Table 4). Similar recoveries have been reported for other assays measuring riboflavin,[42,49,60] vitamin B_6 forms,[42,62,65–67] tryptophan and kynurenines,[25,26,28,30,31] neopterin,[32,33,35] cystathionine[36–38] or cotinine.[39–41]

All within-day CVs were below 10%, and below 5% for 12 of the analytes, while the between-day CVs were somewhat higher (Table 5). Similar imprecision has been obtained with published assays for riboflavin,[42,49,60,68] vitamin B_6 forms,[42,61–63,65,66] tryptophan and kynurenines,[25,26,30,31,69] neopterin,[32,33,35] cystathionine[36,38] and cotinine.[39–41]

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Table 2. Matrix effects[a]

Analyte	Based on analyte area	Based on analyte/ISTD area ratio
PLP	64 (6)	94 (10)
Neopterin	66 (7)	93 (8)
Cystathionine	14 (22)	94 (16)
PA	93 (6)	99 (9)
Pyridoxal	70 (9)	116 (11)
Pyridoxamine	119 (10)	216 (11)
Pyridoxine	87 (8)	157 (7)
Cotinine	98 (12)	96 (10)
HK	123 (6)	131 (11)
HAA	88 (12)	97 (11)
Riboflavin	96 (8)	131 (9)
XA	54 (14)	106 (7)
KA	69 (15)	135 (13)
Kynurenine	45 (12)	96 (17)
Tryptophan	33 (20)	105 (8)
AA	89 (7)	101 (5)

[a] All values (%) are given as mean (CV) for eight different plasmas. Values lower than 100 show ion suppression, while values greater than 100 show ion enhancement.
Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; ISTD, internal standard; KA, kynurenic acid; PA, 4-pyridoxic acid; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

Table 4. Analytical recovery of the assay[a]

	Concentration[b]			Recovery[c], %	
	Endogenous	Added		Added	
Analyte	Low	Medium	High	Medium	High
PLP	61.9	50	150	97	96
Neopterin	14.7	10	30	116	108
Cystathionine[b]	0.220	0.200	0.600	103	103
PA	13.4	50	150	108	103
Pyridoxal	14.1	10	30	96	101
Pyridoxamine	ND[d]	10	30	119	115
Pyridoxine	ND[d]	10	30	113	110
Cotinine	ND[d]	200	600	106	97
HK	43.3	50	150	96	101
HAA	62.4	10	30	104	95
Riboflavin	8.3	10	30	109	106
XA	19.1	10	30	112	123
KA	46.7	50	150	110	105
Kynurenine[b]	1.36	0.500	1.500	77	75
Tryptophan[b]	81.1	16.7	50	117	115
AA	21.2	50	150	107	100

[a] n = 24 for all concentrations.
[b] Units are nmol/L, except for cystathionine, kynurenine and tryptophan, which are given as μmol/L.
[c] Data are given as mean values for 24 observations.
[d] ND, not detectable.
Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; PA, 4-pyridoxic acid; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

For the analytes for which isotopologues were unavailable as internal standards (pyridoxamine, HK and XA) we calculated assay performance by using several internal standards eluting close to these analytes. The internal standards which provided the best assay performance were $^2H_4$-pyridoxine for pyridoxamine, $^2H_2$-HAA for HK and $^2H_5$-tryptophan for XA.

The results of the assay validation experiments show that the assay performance characteristics compare favorably with those of existing assays. The short retention times ensure fast analysis and high sample throughput of 172 samples per 24 h. Analysis of about 15000 samples per year has demonstrated ruggedness of the method, and about 2000 samples could be analyzed on each column before replacement was required.

Table 3. Limits of detection and linearity

			Regression parameters		
Analyte	Calibration range[a]	LOD[b], nmol/L	Slope	Intercept	r^2
PLP	0.05–400	0.2	0.0119	0.0247	0.9963
Neopterin	0.05–400	0.7	0.0044	0.0188	0.9950
Cystathionine	0.5–1333	0.5	0.0064	0.0799	0.9935
PA	0.05–400	0.5	0.0095	0.0423	0.9959
Pyrodixal	0.05–400	0.2	0.0126	0.0294	0.9962
Pyridoxamine	0.05–400	0.1	0.0109	0.0098	0.9979
Pyridoxine	0.05–400	0.05	0.0144	0.0401	0.9939
Cotinine[c]	0.5–1000	1	0.0125	0.0619	0.9978
HK	0.05–400	2	0.0016	-0.0106	0.9813
HAA	0.05–400	2	0.0053	-0.0085	0.9978
Riboflavin	0.05–400	0.2	0.0067	0.0063	0.9969
XA	0.05–400	0.5	0.0101	-0.0403	0.9894
KA	0.05–400	0.4	0.0055	0.0094	0.9954
Kynurenine	0.5–4000	7	0.0058	0.0727	0.9790
Tryptophan	0.5–400	400[d]	0.142	-0.0487	0.9986
AA	0.05–400	0.7	0.0108	0.0214	0.9975

[a] nmol/L for all analytes except tryptophan, where the concentrations are in μmol/L.
[b] LOD, limit of detection, defined as S/N ratio >5.
[c] For cotinine 1 nmol/L = 0.18 μg/L.
[d] This may be reduced to approximately 4 nmol/L by monitoring the most abundant isotope.
Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; PA, 4-pyridoxic acid; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

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Table 5. Imprecision of the assay

	Within-day CV (N = 24)[a], %			Between-day CV (N = 19), %		
Analyte	Low	Medium	High	Low	Medium	High
PLP	2.8	3.0	2.6	5.9	6.0	7.1
Neopterin	4.6	2.9	3.7	10.0	6.9	6.2
Cystathionine	2.5	2.3	2.9	5.4	6.0	6.4
PA	4.1	4.0	2.7	8.2	11.1	6.7
Pyridoxal	4.6	4.0	2.8	5.7	4.8	6.3
Pyridoxamine		6.9	8.6		15.2	12.7
Pyridoxine		3.3	4.9		7.2	7.3
Cotinine		2.3	2.9		5.5	6.2
HK	4.0	4.4	4.8	10.0	9.8	9.7
HAA	3.8	5.0	3.2	12.3	12.1	12.9
Riboflavin	6.2	6.0	5.5	13.2	12.6	11.3
XA	9.5	7.5	6.9	16.9	16.1	14.6
KA	4.5	2.9	3.0	10.5	9.5	8.5
Kynurenine	4.2	4.7	3.1	6.4	8.1	8.7
Tryptophan	3.8	3.5	2.5	5.7	6.0	5.7
AA	3.0	1.8	2.0	9.6	5.0	4.9

[a] Data from the recovery experiments.
Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; PA, 4-pyridoxic acid; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

Plasma concentrations of the analytes

The median analyte concentrations (5, 95 percentiles) in plasma samples from 94 non-fasting, presumed healthy individuals are summarised in Table 6. The median (5, 95 percentiles (nmol/L/μmol/L)) kynurenine/tryptophan ratio was 24.2 (12.9, 52.4). Similar analyte levels have been found by others for riboflavin,[42,49,70] vitamin B_6 forms[42,61,65,70,71] and cystathionine.[37,72–75] The obtained concentrations for neopterin are somewhat higher than published,[76] which might be due to preanalytical instability. The obtained plasma concentrations of tryptophan and kynurenine are similar to those reported by others, but for other kynurenines, published data are sparse and conflicting.[19,27,30,31,69,77–81]

Table 6. Analyte concentrations in nonfasting, presumed healthy persons[a]

Analyte	Median[b]	5, 95 percentile[b]
PLP	37.2	14.2–181.6
Neopterin	8.4	5.4–25.4
Cystathionine	0.238	0.103–0.976
PA	24.3	11.6–265.5
Pyridoxal	10.0	4.8–55.4
Pyridoxamine	<0.1	0.0–0.0
Pyridoxine	<0.05	0.0–0.0
Cotinine	2.1	0.0–1779
HK	24.8	12.5–65.8
HAA	23.3	9.4–55.4
Riboflavin	11.7	5.2–66.8
XA	11.4	4.1–32.8
KA	35.4	20.4–93.2
Kynurenine	1.62	0.97–2.86
Tryptophan	63.1	42.8–88.7
AA	12.7	6.7–33.4

[a] n = 94.
[b] Units are nmol/L for all analytes except tryptophan, kynurenine and cystathionine, which are given as μmol/L.
Abbreviations: AA, anthranilic acid; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; KA, kynurenic acid; PA, 4-pyridoxic acid; PLP, pyridoxal 5′-phosphate; XA, xanthurenic acid.

CONCLUSIONS

This high-throughput LC/MS/MS method combines a unique mobile phase, containing high concentration of acetic acid, with tandem mass spectrometry. This gives the separation and sensitivity needed to quantify endogenous plasma levels of biologically related, but chemically heterogenous, compounds spanning a wide concentration range. The method includes 16 analytes related to B-vitamins and inflammation, and may be useful for large-scale profiling in epidemiological studies.

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