Published in final edited form as:
J AOAC Int. 2009 ; 92(2): 680–687.

Single-Laboratory Validation of a High-Performance Liquid Chromatographic-Diode Array Detector-Fluorescence Detector/ Mass Spectrometric Method for Simultaneous Determination of Water-Soluble Vitamins in Multivitamin Dietary Tablets

Pei Chen, **Renata Atkinson**, and **Wayne R. Wolf**
U.S. Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Food Composition and Methods Development Laboratory, Beltsville, MD 20705

Abstract

The purpose of this study was to develop a single-laboratory validated (SLV) method using high-performance liquid chromatography with different detectors [diode array detector (DAD); fluorescence detector (FLD); and mass spectrometry (MS)] for determination of 7 B-complex vitamins (B_1-thiamin, B_2-riboflavin, B_3-nicotinamide, B_6-pyridoxine, B_9-folic acid, pantothenic acid, and biotin) and vitamin C in multivitamin/multimineral dietary supplements. The method involves the use of a reversed-phase octadecylsilyl column (4 μm, 250 × 2.0 mm id) and a gradient mobile phase profile. Gradient elution was performed at a flow rate of 0.25 mL/min. After a 5 min isocratic elution at 100% A (0.1% formic acid in water), a linear gradient to 50% A and 50% B (0.1% formic acid in acetonitrile) at 15 min was employed. Detection was performed with a DAD as well as either an FLD or a triple-quadrupole MS detector in the multiple reaction monitoring mode. SLV was performed using Standard Reference Material (SRM) 3280 Multivitamin/ Multimineral Tablets, being developed by the National Institute of Standards and Technology, with support by the Office of Dietary Supplements of the National Institutes of Health. Phosphate buffer (10 mM, pH 2.0) extracts of the NIST SRM 3280 were analyzed by the liquid chromatographic (LC)-DAD-FLD/MS method. Following extraction, the method does not require any sample cleanup/preconcentration steps except centrifugation and filtration.

Vitamins are minor constituents of food that are essential for normal growth, self-maintenance, and functioning of human and animals. These compounds can be classified into 2 main groups: fat- and water-soluble vitamins. Seven members of the water-soluble vitamin B complex were investigated in the present assay: thiamin (vitamin B_1); riboflavin (vitamin B_2); niacin (vitamin B_3); pyridoxine (vitamin B_6); folic acid (vitamin B_9); pantothenic acid; and biotin. Ascorbic acid (vitamin C) was also included in the assay.

Today there is increased interest in accurately assessing the total dietary intake of vitamins from all sources, including foods and dietary supplements, and the demand for rapid, specific, and updated methodology for determination of vitamins is growing. A dietary supplement ingredient database (DSID) comprising analytical values is under joint

Corresponding author's pei.chen@ars.usda.gov.

development by the Agricultural Research Service (ARS) and the Office of Dietary Supplements (ODS)/National Institutes of Health (NIH; 1). Analytical values for the DSID will be generated by contract with commercial analytical laboratories.

Historically and still often currently, the B-complex vitamins are determined (particularly in conjunction with food labeling regulatory purposes) by microbiological assay methods available from the *Official Methods of Analysis* of AOAC INTERNATIONAL (2). While these microbiological methods are highly sensitive, they are also imprecise due to very detailed laborious procedures that are time-consuming and require multiple determinations to achieve the required precision for estimation of mean values. These microbiological procedures were developed more than 50 years ago and are generally outdated in light of current technology.

Various detection and preparation methods to determine water soluble vitamins have been reported in the literature to provide separation and quantitation of B-complex vitamins in several matrixes. The choice of method depends largely on the accuracy and sensitivity required, as well as interferences encountered in the sample matrix. While there are reports in the literature of high-performance liquid chromatographic (HPLC) methods for the determination of B-complex vitamins, these methods have not been rigorously validated and often target only one vitamin at a time (3–7). A limited number of methods for the simultaneous determination of more than one member of the B-complex have been reported (8–11), but none of them is specifically designed for multivitamin supplements in which the amount of a B vitamin typically ranges widely from 30 (biotin) to 20 000 μg/tablet (nicotinamide). Many of the reported methods require complex sample preparation steps, such as solid-phase extraction (SPE). Therefore, in support of the DSID project and a broader interest in updating methods for vitamins, this study reports a single-laboratory validation (SLV; 12) of a method for determining B-complex vitamins in multivitamin supplements using a single chromatographic run.

We have previously reported a method for simultaneous determination of 7 B-complex vitamins (thiamin, riboflavin, nicotinamide, pyridoxine, folic acid, pantothenic acid, and biotin) in multivitamin supplements using HPLC/tandem mass spectrometry (MS/MS; 13). Five of the vitamins (thiamin, riboflavin, nicotinamide, pyridoxine, and folic acid) were also determined using diode array detection (DAD); pantothenic acid and biotin do not have adequate sensitivity for detection by DAD. Information including linear ranges, linearity, limits of quantitation (LOQ), extraction efficiency, and recovery studies were included in that report (13).

In the previous report of this method (13), problems were encountered with determination of ascorbic acid, due to its reaction with minerals in the test material, which were mutually extracted into the analytical sample. Because multivitamin/multimineral supplement tablets are a high priority in development of the DSID, further research has resolved the problem, and ascorbic acid was successfully added to the suite of vitamins applicable to this method. Because the modifications consisted mainly of factors that would not affect the chromatographic/detection aspects of the previous methods, most of the information for the suite of vitamins previously reported is still valid to the expanded group. In addition, a

second HPLC system has been utilized that included a DAD and a fluorescence detector (FLD) in addition to the previous HPLC system with dual DAD and mass spectrometry (MS) detection. Each of these systems allows collection of multiple types of information in the same chromatographic run. Pyridoxine and riboflavin have much stronger fluorescence signals compared to DAD detection, and better analytical performance can be obtained with an FLD for these 2 vitamins.

In this report, additional validation information from both systems is compared to summary data from the first report with the HPLC-DAD/MS system (13) to constitute an SLV of this expanded method for determination of multiple water-soluble vitamins in dietary supplements. The method provides a high degree of chromatographic resolution and reproducibility, and provides quantitative determination.

Experimental

Reagents

a. *Water*.—Optima grade (Fisher Scientific, Pittsburgh, PA).

b. *Acetonitrile*.—Optima grade (Fisher Scientific).

c. *Formic acid*.—MS grade (Sigma/Aldrich, St. Louis, MO).

d. *Reference standards*.—Pure samples of B-complex and ascorbic acid vitamin standards were obtained from U.S. Pharmacopeia (USP; Rockville, MD).

e. *Test material*.—Samples of Standard Reference Materials (SRM) 3280 Multivitamin/Multimineral Tablets were obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD), while this SRM was under development. Final assigned values for the SRM were not yet available at time of this work, but use of this material for the SLV studies provided a stable, homogeneous test material whose general commercial availability will make it suitable for use by other laboratories to test proper implementation of this method.

f. *HPLC mobile phases*.—For the LC-DAD/MS system, mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. For the LC-DAD-FLD system, mobile phase A was 0.01% formic acid in water and mobile phase B was 0.01% formic acid in acetonitrile.

g. *Preparation of buffer solution*.—1.73 mL of 85% H_3PO_4 was added to 1 L water, then 7.5 M NaOH was added until pH 3 was reached. The buffer solution was adjusted to pH 2 by adding H_3PO_4 and was then degassed prior to extraction by sonication while under vacuum for 1 h followed by purging with N_2 gas for 30 min.

Apparatus

a. *HPLC system*.—Two Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA) were utilized, each consisting of a quaternary pump with a vacuum degasser, a thermostatted column compartment, an autosampler, and a DAD. One of the systems, used in the previous report (13), was equipped with the mass spectrometer described below The second system (System 2) was equipped with an

additional fluorescence detector (Model G-1321A, Agilent Technologies), and also with a thermostatted autosampler compartment to hold the analysis samples at a lower temperature (4°C) during an automated HPLC run. This lower temperature is a critical requirement for stability of the ascorbic acid extracts.

b. *Mass spectrometer*.—Micromass Quattro Micro triple-quad mass spectrometer (Waters Corp., Milford, MA).

c. *Analytical column*.—Hydro-RP C18 (4 μm particle size, 250 × 2.0 mm) reversed-phase octadecylsilyl column (Phenomenex, Torrance, CA) in combination with a Column-Saver™ precolumn filter (MAC-MOD Analytical, Inc., Chadds Ford, PA) was used.

d. *Mortar grinder*.—Retsch Rm-100 (Retsch GmbH & Co. KG, Haan, Germany).

LC Conditions

Chromatographic separation with the LC-DAD/MS system used in the previous report (13) was achieved using a programmed gradient mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, as follows: 0–5 min, isocratic at 100% A; 5–15 min, linear gradient from 100:0 A:B (v/v) to 50:50 A:B (v/v); 15–17 min, linear gradient from 50:50 A:B (v/v) to 5:95 A:B (v/v); 17.1 min, back to 100% A; 17.1–25 min, 100 % A (column equilibration). The flow rate used was 0.25 mL/min and the injection volume was 5 μL for all the analyses. The total run time was 25 min. For the later work done with the LC-DAD-FLD system, the higher level of mobile phase formic acid required for optimum MS conditions was lowered to (A) 0.01% formic acid in water and (B) 0.01% formic acid in acetonitrile. The same programmed gradient mobile phase conditions were used as for the previous LC-DAD/MS work. The main change in the LC-DAD-FLD system was the availability of the thermostatted autosampler, which allowed a temperature of 4°C to be maintained in the sample tray. The cooled autosampler condition was critical to avoid rapid oxidation of vitamin C in the presence of minerals from multimineral supplements. Vitamin C in the extraction solution was stable for 5–6 h under these conditions. Extraction with pH 2 buffer was also important; vitamin C disappeared quickly at pH 3.0.

Quantitation

Quantitation was done by external standard calibration and by standard addition.

Fluorescence Detection Conditions

Pyridoxin was detected by the FLD at 310 nm excitation (EX) and 390 nm emission (EM), and riboflavin at 450 nm EX and 530 nm EM.

MS Conditions

MS was performed with electrospray ionization in the multiple reaction monitoring (MRM) positive ion mode. Quadrupole 1 was fixed at a set parent ion, quadrupole 2 was used as a collision chamber to induce fragmentation, and quadrupole 3 was fixed at a set daughter ion. MRM is the preferred mode for quantitation since it usually achieves the best possible specificity and signal-to-noise (S/N) ratio for a given analyte.

The desolvation gas (N_2) flow rate was 650 L/h, the cone gas (N_2) flow rate was 50 L/h, and the collision gas (Ar) was tuned to a pressure of 2.3×10^{-3} torr. Source temperature was set at 100°C and desolvation temperature 280°C. For the electrospray source, the capillary voltage was set at 3.5 kV, the extractor voltage at 2 V, and the RF lens voltage at 0.2 V. For the analyzer, the low mass Resolution 1 and high mass Resolution 1 were 15 V and ion energy (IE) 1 was 0.5 V; the entrance was 0 V and the exit was 5 V; the LM Resolution 2 and HM Resolution 2 were 15 V, and IE 2 was 5 V. The multiplier voltage was 700 V. The cone voltages and collision energies, respectively, for the vitamins were as follow: thiamin-20 and 12 V; riboflavin-25 and 23 V; nicotinamide-10 and 12 V; pyridoxine-20 and 14 V; folic acid-20 and 22 V; pantothenic acid-10 and 12 V; and biotin-17 and 16 V. Vitamin C was not measured by this system.

DAD Conditions

The DAD wavelengths (nm) used for vitamin determinations were as follow: thiamin 260; riboflavin 270; nicotinamide 260; pyridoxine 292; folic acid 280; and ascorbic acid 241.

Sample Preparation

For LC-DAD/MS, a composite of 20 tablets was accurately weighed and ground to a uniform powder with the mortar grinder for 15 min. A portion slightly over 1/10 tablet (0.1527 g) was weighed, transferred into a red-colored 50 mL volumetric flask (Pyrex® 55640, Corning Inc., Lowell, MA) and brought to volume with 10 mM phosphate buffer (pH 2.0). The final volume was then adjusted according to the actual sample weight to make the extraction level equal to 500 mL for 1 tablet. The flask was sonicated in the dark for 20 min. Then a portion of the supernatant was decanted into 15 mL centrifuge tubes and centrifuged at 5000 *g* for 10 min. The supernatants were then filtered through 0.2 μm polytetrafluoroethylene (PTFE) syringe filters and labeled as A500×. The extracted samples were stored at −80°C until tested. Most B-complex vitamins are more stable in acidic compared to basic conditions, thus extraction of these vitamins in acidic conditions is recommended. Details of sample preparation for the LC-DAD/MS studies are discussed in the previous report (13).

For LC-DAD-FLD, 50 mL phosphate buffer (pH 2) was added to 75 mg of the 20 tablet composite finely ground for LC-DAD/MS and shaken vigorously. The mixture was sonicated at room temperature for 10 min. A small aliquot (1–1.5 mL) of diluted extract was filtered through a 0.2 μm PTFE syringe filter. Compared to the previous study (13), the extraction buffer was lowered to pH 2.0 to improve the stability of extracted ascorbic acid.

Recovery Studies

Two experiments described in the previous report (13) were used to determine the extraction efficiency of the sample preparation. In the first experiment, a second extraction was performed on the residues of the first extraction, and the water-soluble vitamin contents of both extractions were determined. In the second experiment, known amounts of each B-complex vitamin under investigation (50, 100, and 200% of the estimated value) were added to the respective aliquots of the tablet powder. The extraction procedure was then performed on the spiked samples (3 sets). The extractions were diluted 2× and analyzed immediately.

Extraction efficiency and recovery results shown in Table 1 are from the previous report (13).

Standard Solutions

Approximately 10.0 mg each of the USP standards for ascorbic acid, thiamin, riboflavin, nicotinamide, pyridoxine, folic acid, pantothenic acid, and biotin were accurately weighed. Thiamin, nicotinamide, pyridoxine, and pantothenic acid were brought to a concentration of 1.0 mg/mL and riboflavin to 20 μg/mL with 10 mM phosphate buffer (pH 2.5). Folic acid and biotin were brought to 1.0 mg/mL with 10 mM phosphate buffer (pH 9.0). These stock vitamin solutions were stored at −20°C.

Calibration curves were generated by serial dilution of the standard stock solutions. A rough estimate of the vitamin content of the proposed SRM 3280 was used to determine the concentration ranges of standards to be investigated for the different vitamins.

Statistics

The LC-DAD/MS data were collected and processed by Waters MassLynx 4.0 software, which also served as the controlling software for the LC-DAD/MS system. The data were further processed using Microsoft Excel. The LC-DAD-FLD data were collected with Agilent 1100 ChemStation software and further processed using Microsoft Excel.

Results and Discussion

Chromatography

The HPLC parameters were selected after screening a number of columns and solvent systems with different gradient profiles. The conditions selected in the developed method gave excellent separation within 20 min. As reported previously (13), typical LC/MS-MRM and LC-DAD chromatograms for the SRM 3280 are shown in Figures 1 and 2. Retention time (t_R) values (min) of the examined vitamins were: (*1*) thiamin, 2.14 (DAD) and 2.26 (MRM); (*2*) ascorbic acid, 3.38 (DAD); (*3*) nicotinamide, 4.76 (DAD) and 4.80 (MRM); (*4*) pyridoxine, 5.43 (DAD) and 5.56 (MRM); (*5*) pantothenic acid, 14.61 (MRM); (*6*) folic acid, 15.16 (DAD) and 15.28 (MRM); (*7*) riboflavin, 16.07 (DAD) and 16.19 (MRM); and (*8*) biotin, 16.65 (MRM).

Method Validation

The method was validated with respect to repeatability (n = 6) between-day precision (n = 7), long-term precision (n = 3), LOQ, and linearity. An external standard calibration method was used. Details on linearity, calibration, and LOQs were reported previously (13) are shown in Table 2. The LOQs were estimated at S/N = 10 (13). It is worth noting that the LOQs obtained may not represent the best LOQ for each individual vitamin investigated. A multicomponent analytical method is not optimized for each individual component. This is especially true for MRM detection because the MS conditions for our instrument were not optimized for each individual vitamin. Some compromises in conditions were made to accommodate measurement of all vitamins in a single chromatographic run. The MS

detector was deliberately detuned for nicotinamide and pantothenic acid (lowered cone voltage) to accommodate the much higher concentration of these 2 vitamins.

Reproducibility

Reproducibility of both DAD and MRM results of the LC-DAD/MS analyses of the NIST SRM 3280 over a longer period of time are shown in Table 3. Each result represents the mean and standard deviation of 21 individual instrumental measurements (injections) of the same sample preparation over a 7-day period. Samples were run on 3 different dates over a 7-month period, each date representing a completely different sample set and sample preparation. For determination of B vitamins in multivitamin supplements, DAD detection should be used when applicable since we did not find significant advantages for using MRM detection. In the case of thiamin and folic acid, MRM detection was not appropriate under our conditions. MS detectors, although more expensive and difficult to maintain and operate compared to DAD, can measure vitamins without UV-sensitive chromophores, such as pantothenic acid and biotin, that are impossible to measure by DAD in a complex matrix. Another reason for using MS is to transfer the method to stable isotope-dilution measurement in the future to accurately determine the true content of each vitamin. Stable isotope-dilution MS is considered to be potentially the most accurate method available. Parallel work on this approach has been reported elsewhere (14).

Repeatability

Repeatability of the method was evaluated by independently analyzing 6 different bottles of the SRM 3280 test material (Table 4). Whole bottles, approximately 30 tablets, were ground and analytical aliquots analyzed separately. The excellent agreement of values across the bottles, shown in Table 4, represents good repeatability and confirms the homogeneity of the test material from bottle to bottle.

Development of a Modified Method to Include Ascorbic Acid

Modifications to the method to stabilize ascorbic acid in the extracts included extraction at a slightly lower pH (2.0) and, most importantly, adding a thermostatted sample chamber held at 4°C to the autosampler for the second HPLC system, allowing ascorbic acid to be determined within 5–6 h after the extraction. Along with the new data for ascorbic acid, the vitamins pyridoxine and riboflavin were determined by fluorescence detection with greater sensitivity than when using a DAD. Thiamin and nicotinamide were also determined by a DAD with this system. Data for these 5 vitamins with the LC-DAD-FLD System 2 are given in Table 5. These results were obtained by a second analyst in the laboratory, thereby testing the portability of the method.

Accuracy

Since the assigned values for SRM 3280 were not available at the time of this study, accuracy was assessed by the method of standard additions (Table 5). Accuracy tests were performed with the addition of USP vitamin standards at approximately 50, 100, 150, and 200% of the expected vitamin concentration of the analyzed samples. A total of 5 separate samples from the same bottle of SRM 3280 were analyzed on separate days. The 0 addition

samples were also used to determine vitamin content for each separate sample based on a composite calibration curve. Individual calibration curves representing 4 points on each curve were re-run on each of the 5 analysis days (resulting in 20 points for the composite calibration curve). Values for each point of the 5 separate daily standard addition curves (0 addition plus 4 levels of additions) resulted in 25 points in a 5-day composite standard addition curve for each vitamin. A ratio of the slopes of these 2 composite curves (addition and calibration) was then used to obtain a value for recovery of the added vitamin over the range of standard additions. If the recovery of an added vitamin is complete, these 2 slopes should be identical. This recovery data is also given in Table 5, and shows that within experimental error the calibration and standard additions slopes were identical.

Data for the B-complex vitamins obtained using this modification of the method to include ascorbic acid (Table 5) were in agreement with the data from conditions used for previous report (Tables 1–3; 13), confirming expanded applicability of the method to include ascorbic acid in the suite of vitamins for this method.

Conclusions

This method was developed to be as simple and as versatile as possible, requiring no expensive SPE and no ion-pairing reagent in the HPLC procedure. The simplicity and versatility of the procedure is particularly suitable for applications in the pharmaceutical industry (e.g., multivitamin commercial quality control or for labeling). The method has potential to be easily adapted to determinations of these vitamins in fortified foods, which may contain added vitamins at levels similar to dietary supplements. This method will also be the basis for further studies and validation of methods for determining natural levels of vitamins in other food materials.

Acknowledgments

This research was supported by United States Department of Agriculture/ARS and the ODS/NIH under an Interagency Agreement.

References

1. Dwyer J, Picciano MF, Betz J, Fisher K, Saldanha L, Yetley EA, Coates PM, Radimer K, Bindewald B, Sharpless K, Holden JM, Andrews K, Zhao C, Harnly JM, Wolf WR, Perry C. J. Food Comp. Anal. 2006; 19:S108–S114.
2. Official Methods of Analysis. 18th Ed.. Gaithersburg, MD: AOAC INTERNATIONAL; 2006.
3. Albala-Hurtado S, Veciana-Nogues MT, Izquierdo-Puliddo M, Marine-Font A. J. Chromatogr. A. 1997; 778:247–253. [PubMed: 9299739]
4. Esteve MJ, Farre R, Frigola A, Garcia-Cantabella JM. J. Chromatogr. A. 1998; 795:383–387. [PubMed: 9528106]
5. Gregory J III, Sartain D. J. Agric. Food Chem. 1991; 39:899–905.
6. Yamanaka K, Matsuoka M, Banno K. J. Chromatogr. A. 1996; 726:237–240.
7. Wongyai S. J. Chromatogr. A. 2000; 870:217–220. [PubMed: 10722079]
8. Moreno P, Salvado V. J. Chromatogr. A. 2000; 870:207–215. [PubMed: 10722078]
9. Papadoyannis IN, Tsioni JK, Samanidou VF. J. Liq. Chromatogr. Relat. Technol. 1997; 20:3203–3231.

10. Chatzimichalakis PF, Samanidou VF, Verpoorte R, Papadoyannis IN. J. Sep. Sci. 2004; 27:1181–1188. [PubMed: 15537074]
11. Li HB, Chen F, Jiang Y. J. Chromatogr. A. 2000; 891:243–247. [PubMed: 11043784]
12. AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. Gaithersburg, MD: AOAC INTERNATIONAL; 2002.
13. Chen P, Wolf WR. Anal. Bioanal. Chem. 2006; 387:2441–2448. [PubMed: 17091235]
14. Chen P, Ozcan M, Wolf WR. Anal. Bioanal. Chem. 2007; 389:343–347. [PubMed: 17646973]

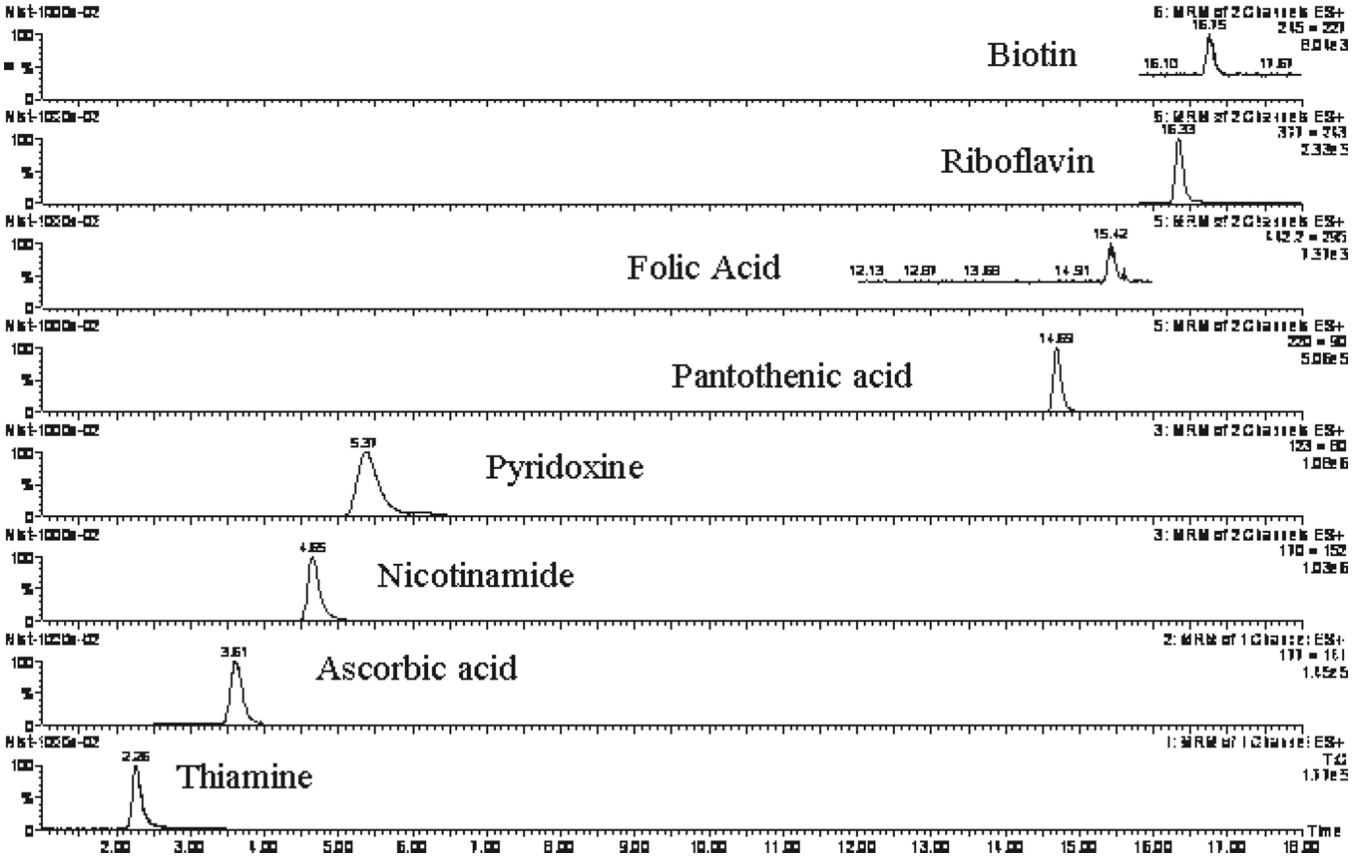

Figure 1.
LC/MS-MRM chromatogram of B vitamins and vitamin C of the SRM 3280.

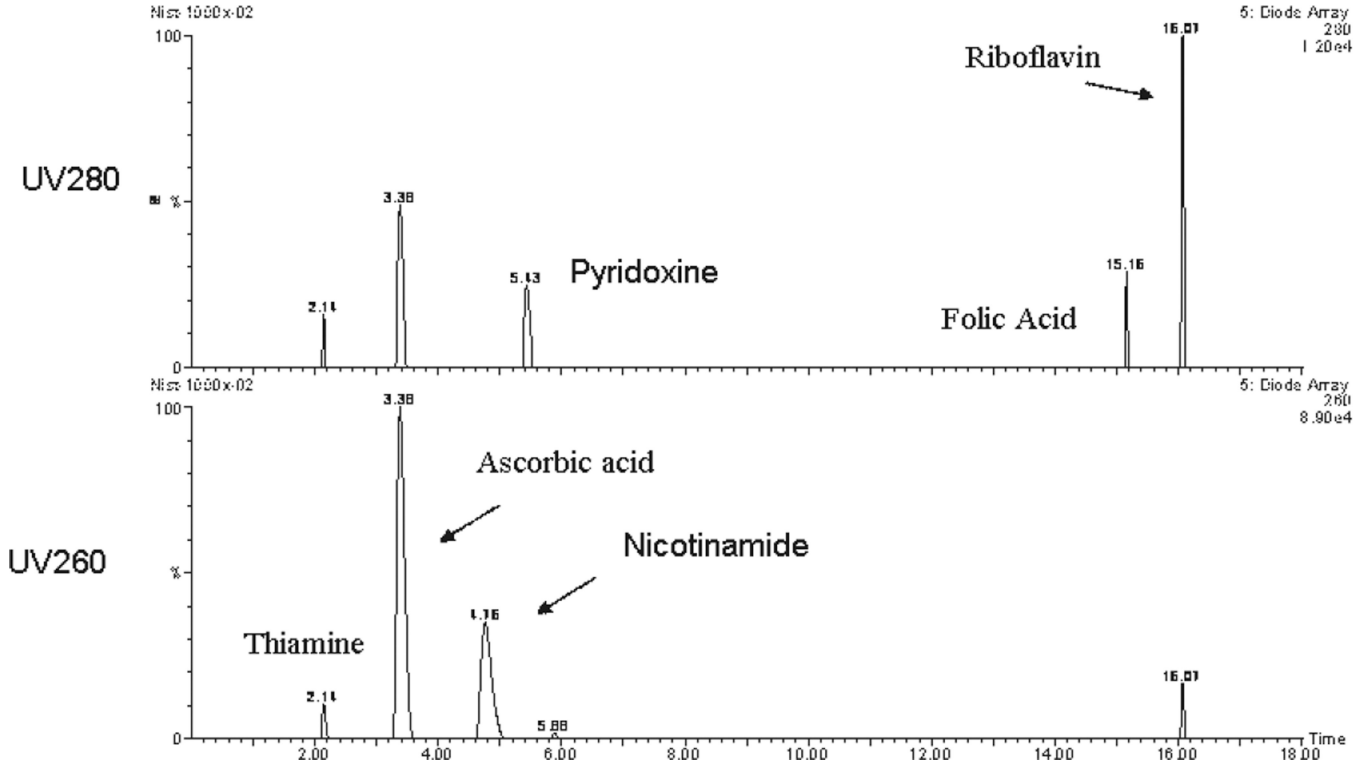

Figure 2.
LC-DAD chromatogram of B vitamins and vitamin C of the SRM 3280.

Table 1

Extraction efficiency and recovery of B vitamins at 50 mL/0.1 tablet level (13)

Parameter	Thiamin HCl	Nicotinamide	Pyridoxine HCl	Calcium pantothenate	Folic acid	Riboflavin	Biotin
E.E., %[a,b]	>99[c]	99.5 ± 2.3	99.5 ± 1.2	99.6 ± 0.9	>99[c]	96.7 ± 3.6	>99[c]
Recovery, %, w/ DAD	100.6 ± 1.2	99.7 ± 0.9	100.3 ± 2.7	NA[d]	96.2 ± 2.4	102.0 ± 2.4	NA
Recovery, %, w/ MRM	98.8 ± 1.6	99.7 ±1.9	95.8 ± 1.4	98.8 ± 1.3	100.9 ± 2.3	100.4 ± 0.5	96.0 ± 2.0
Extraction variability (RSD, %)[e] w/DAD	1.61	1.54	1.98	NA	5.48	2.01	NA
Extraction variability (RSD, %)[e] w/MRM	NA	2.04	1.44	1.67	6.54	1.82	2.48

[a] E.E. = Extraction efficiency; $n = 6$.

[b] The extraction efficiency was calculated with the following formula: E.E. % = 100*E1/(E1+ E2), where E1 is the amount of the respective vitamin measured in the first extraction, and E2 is the amount of the respective vitamin measured in the second extraction.

[c] The vitamin concentration in the second extraction is below its limit of quantitation at the 1× level.

[d] NA = Not applicable.

[e] RSD = Relative standard deviation; $n = 6$.

J AOAC Int. Author manuscript; available in PMC 2014 September 10.

Table 2

The investigated linear ranges[a], linearity[b], and limit of quantitation (LOQ)[b] values of the B vitamin standard curves[c,d]

Parameter	Thiamin HCl	Nicotinamide	Pyridoxine HCl	Calcium pantothenate	Folic acid	Riboflavin	Biotin
Investigated range, ng/μL	0.1–10.0	1.0–100.0	0.1–10.0	0.5–50	0.04–4.0	0.1–10.0	0.002–0.2
Linear range (DAD)	0.5–10.0	1.0–100.0	0.5–10.0	NA	0.2–1.0	0.1–10.0	NA
R^2 (DAD)	0.9991	0.9996	0.9995	NA	0.9995	0.9997	NA
Calibration curve (DAD)	$0.3308x - 43.63$	$0.3514x - 102.35$	$0.3790x - 11.00$		$0.4212x + 0.46$	$0.8234x - 2.51$	
LOQ (DAD), ng/μL	0.5	0.5	0.5	NA	0.2	0.1	NA
Linear range[c] (MRM)	0.1–2.0	1.0–100.0	0.1–10.0	0.5–50	0.2–1.0	0.1–10.0	0.02–0.2
R^2(MRM)	0.9969	0.9995	0.9990	0.9995	0.9997	0.9989	0.9995
Calibration curve (MRM)	$6.29e^{-3}x^2 + 133.53x + 12673.1$	$-1.90e^{-3}x^2 + 25.78x - 3266.22$	$-1.09e^{-3}x^2 + 209.80x + 6650.95$	$-9.56e^{-3}x^2 + 19.07x + 2391.62$	$2.08e^{-3}x^2 + 17.33x - 77.28$	$3.01e^{-4}x^2 + 29.87x - 1167.45$	$2.59e^{-2}x^2 + 36.08x + 1.16$
LOQ (MRM), ng/μL	0.02	0.1	0.02	0.02	0.05	0.02	0.02

[a] Expressed as R^2 – correlation coefficient and calibration curve equations. Linear equations for DAD and quadratic equations for MS.

[b] 5 μL injection volume.

[c] Quadratic fit extended the range to 10.0 for thiamin and 4.0 for folic acid.

[d] Ref. 13.

J AOAC Int. Author manuscript; available in PMC 2014 September 10.

Table 3

LC-DAD and LC/MS-MRM results of B vitamin contents in the NIST SRM 3280[a,b,c] (mg/tablet) over a 7-month period

Date	Name	Thiamin HCl	Nicotinamide	Pyridoxine HCl	Calcium pantothenate	Folic acid	Riboflavin	Biotin[d]
10/2005[e]	DAD	1.89 ± 0.04	21.83 ± 0.36	2.53 ± 0.02	NA	NA	2.000 ± 0.004	NA
	RSD, %	2.01	1.63	0.83	NA	NA	0.20	
	MRM	1.93 ± 0.03	24.09 ± 0.86	2.70 ± 0.02	13.63 ± 0.10	NA	2.126 ± 0.024	NA
	RSD, %	1.70	3.57	0.63	0.73	NA	1.13	NA
04/2006[f]	DAD	1.78 ± 0.06	23.14 ± 0.83	2.63 ± 0.09	NA	0.703 ± 0.049	2.027 ± 0.047	NA
	RSD, %	3.25	3.59	3.49	NA	6.96	2.30	NA
	MRM	NA	22.98 ± 0.78	2.67 ± 0.12	13.38 ± 0.10	0.839 ± 0.074	2.099 ± 0.070	43.9 ± 7.3
	RSD, %	NA	3.40	4.67	7.41	8.75	3.35	16.73
06/2006[f]	DAD	1.74 ± 0.07	21.44 ± 0.40	2.72 ± 0.13	NA	0.794 ± 0.062	1.985 ± 0.035	NA
	RSD, %	4.18	4.89	4.87	NA	7.81	1.76	
	MRM	NA	22.80 ± 0.70	2.84 ± 0.13	13.48 ± 0.44	0.986 ± 0.151	2.262 ± 0.111	40.1 ± 4.04
	RSD, %	NA	3.08	4.60	4.89	15.33	4.89	10.06

[a] Quantitated at 1× level (equivalent to 1.0 L extraction solvent/tablet). Each date represents a different extraction of different batch of samples.

[b] Standardized for mg/tablet (average tablet weight = 1.527 g.)

[c] The values for DAD and MRM results are means ± standard deviation (SD).

[d] Biotin is expressed as μg/tablet.

[e] The means and SD are calculated based on 3 injections/sample over 7 days. Each sample was injected 21 times. The method was not ready for folic acid and biotin analysis at this time.

[f] The means and SD are calculated based on 3 injections/sample over 7 days. Each sample was injected 21 times.

Table 4

Repeatability of the LC-DAD and LC/MS-MRM results of B vitamin contents in the NIST SRM 3280[a,b,c,d] (mg/tablet) for 6 independent batches

Batch	Thiamin HCl	Nicotinamide	Pyridoxine HCl	Calcium pantothenate	Folic acid	Riboflavin	Biotin[e]
1	1.89 ± 0.032	21.8 ± 0.36	2.53 ± 0.02	NA	NA	2.00 ± 0.01	NA
2	1.93 ± 0.039	24.1 ± 0.86	2.71 ± 0.02	13.62 ± 0.099	NA	2.13 ± 0.02	NA
3	1.97 ± 0.029	23.1 ± 0.83	2.62 ± 0.09	NA	0.70 ± 0.05	2.03 ± 0.05	NA
4	1.99 ± 0.007	23.0 ± 0.78	2.67 ± 0.12	13.38 ± 0.99	0.84 ± 0.07	2.10 ± 0.07	43.9 ± 7.3
5	2.03 ± 0.038	21.4 ± 0.40	2.72 ± 0.13	NA	0.79 ± 0.06	1.99 ± 0.04	NA
6	1.98 ± 0.007	22.8 ± 0.70	2.84 ± 0.13	13.48 ± 0.44	0.99 ± 0.15	2.26 ± 0.11	40.1 ± 4.04

[a] Quantified at 1× level (equivalent to 1.0 L extraction solvent/tablet).

[b] Standardized for mg/tablet (average tablet weight = 1.527 g).

[c] Values are mean ± SD.

[d] DAD was used for thiamin HCl, nicotinamide, pyridoxine HCl, folic acid, riboflavin; LC/MS-MRM was used for calcium pantothenate and biotin (not UV chromophores).

[e] Biotin is expressed as μg/tablet.

Table 5

Water-soluble vitamin content of SRM 3280 multivitamin/multimineral tablets using LC-DAD-FLD System 2[a]

Parameter	Thiamin HCl	Nicotinamide	Pyridoxine HCl	Ascorbic acid	Riboflavin
Curve[b]	1.64 ± 0.07	21.36 ± 0.33	2.54 ± 0.10	63.03 ± 1.49	1.86 ± 0.09
Standard addition[c]	1.93 ± 0.039	21.41 ± 2.48	2.66 ± 0.11	61.98 ± 1.26	2.03 ± 0.19
Recovery, %	103.4	101.0	92.5	101.3	94.5

[a] Second analyst using HPLC System 2, pyridoxine and riboflavin determined by fluorescence detection, other vitamins by use of the DAD. Quantified at 1× level (equivalent to 1.0 L extraction solvent/tablet).

[b] Results calculated using standard calibration curve, mean ± SD.

[c] Results calculated using standard addition, mean ± SD.