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# Development of a Stability Indicating Method for Simultaneous Analysis of Five Water-Soluble Vitamins by Liquid Chromatography

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**Abstract**

**Background:** Water-soluble vitamins are often included simultaneously in pharmaceutical formulations as food complements or in parenteral nutrition mixtures. Given their sensitivity to heat, light or pH variations, it is important to study their stability using validated stability indicating methods. We thus aimed to validate a liquid chromatography (LC) stability-indicating method for the simultaneous quantification of 5 water-soluble vitamins.

**Methods:** We analyzed four water-soluble B vitamins (nicotinamide, pyridoxine, folic acid, cyanocobalamin) and ascorbic acid using a LC method with diode array detector. They were separated on a C18 stationary phase under gradient elution of solvent A [0.2% of metaphosphoric acid in water and acetonitrile 98:2] and solvent B (100% acetonitrile). All vitamins were subjected to forced degradation conditions and we showed that the obtained degradation products didn't interfere with the vitamins.

**Results:** The method allows the separation of the 5 water-soluble vitamins in a 30 minute run without any interference from the breakdown products obtained with acid/alkaline solutions, hydrogen peroxide, temperature and light. It meets all the qualitative and quantitative criteria for validation with an acceptable accuracy and good linearity.

**Conclusion:** This stability-indicating method can be used for carrying out stability studies of water-soluble vitamins in pharmaceutical preparations.

**Keywords**: water-soluble vitamins, liquid chromatography, stability indicating method, analytical validation, diode array detector

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## Introduction

Vitamins are a broad group of organic compounds required to maintain normal eukaryotic cellular and metabolic functions. Vitamins are natural constituents of food and a well-balanced diet supplies all of the required water-soluble and fat-soluble vitamins. However, an appropriate vitamin supplementation is sometimes necessary in case of active disease processes, stress, increased physical activity, low food quality, inadequate nutritional intake, etc [1–3]. For outpatients, vitamins are supplied in pharmaceutical or dietary supplements (injections, tablets, capsules, syrups). In hospitals, vitamins can also be provided in the mixtures of total parenteral nutrition (TPN) prepared specifically for some patients, like neonates and children in intensive care units. It is thus of great importance to have information about the storage life of these formulations which may contain several vitamins, especially nicotinamide (B3), pyridoxine (B6), folic acid (B9), cyanocobalamin (B12) and ascorbic acid (vitamin C).

In order to determine the storage life conditions of such supplements or parenteral nutrition admixtures, it is necessary to have information about their stability under the influence of various environmental factors. Stability studies have to be performed in compliance with the International Conference Harmonization guidelines [4] and with the methodological guidelines for stability studies of hospital pharmaceutical preparations [5], which recommend the development of stability-indicating analysis methods. Such methods are useful to assess the behavior of active substances (i.e vitamins) and their breakdown products over time.

Many analytical methods used for the determination of vitamins and preservatives have already been described. Most of them use liquid chromatography (LC) with different detection methods like UV–visible with variable wavelength or diode array detection (DAD) [1, 6, 7], ultraviolet-mass spectrometry (UV–MS) [2] and fluorescence (FLD) [3, 8]. Other separative methods, like for example capillary electrophoresis (CE), can also be used [9].

However, most of these methods were developed in order to evaluate individually the stability of each vitamin present in pharmaceutical medications such as tablets, capsules and oral solutions. Several methods for the simultaneous determination of vitamins have been reported [6, 7, 10–23] but as reported by Abano et al [23], none of these methods were able to simultaneously quantify all the water-soluble vitamins and often many analyses have to be performed. Moreover, most of them preclude simultaneous determinations involving ascorbic acid, which is widely present in the multivitaminic complex products, because of coeluting issues [17, 24]. Lastly, very few are stability indicating [16, 19, 20]. In the work of Vidovic et al [16], the determination of folic acid and cyanocobalamin was missing and Jin et al [19] analyzed seven vitamins in two runs without cyanocobalamin. Mass spectrometry can also be an interesting alternative [20] for the dosage of vitamins, but is not as readily available in all laboratories as UV-visible detection.

The aim of our study was to develop and validate a high-pressure liquid chromatography (HPLC) stability-indicating method for the simultaneous quantification of vitamin C, vitamin B3, vitamin B6, vitamin B9 and vitamin B12 in an aqueous solution without interference from breakdown products in one run.

# Materials and methods

## Reagents and materials

Solvents used for chromatographic analysis were acetonitrile (ACN) (CAS n°: 75–05-8 purity: ≥ 99.9 %, Sigma-Aldrich- USA) and metaphosphoric acid (MPA) (CAS n°: 37,267–86-0 purity 36 % (m/m, Sigma- Aldrich- USA). Deionized water (Versylene®) was purchased from Fresenius, France.

Reagents used for forced degradations were sodium hydroxide concentrate (NaOH) (CAS number 1310–73-2, Fixanal Fluka- Germany) and Hydrogen chloride (HCl) (CAS number 7647–01-0, Fluka analytical- Germany), Hydrogen peroxide ($H_2O_2$ 110V) (CAS number 7722–84-1, Cooper-France).

Water-soluble vitamins were pharmaceutical raw materials provided as powders: B3 (CAS number 98–92-0), B6 (CAS number 58–56-0), B9 (CAS number 59–30-3) by DSM (Heerlen, the Netherlands), C (CAS number 50–81-7) by Pfannenschmidt (Hamburg, Germany) and B12 (CAS number 68–19-9) by Sigma-Aldrich (Darmstadt, Germany).

## Preparation of stock and sample solutions

Each powder vitamin was weighed on a precision scale (Secura 224–15, Sartorius, France) and dissolved in appropriate solvent to get stock solutions: Vitamin C in aqueous solution with 0.2 % MPA, vitamin B9 in alkaline solution (0.01N NaOH), vitamins B3 and B6 in water.

The stability of each stock solution was checked and a shelf life of 15 hours at 4 °C was validated.

## Preparation of calibration and validation standards

Five calibration standards (CS) (0.5, 1, 5, 25 and 50 mg/L) and four validation standards (VS) (0.5, 2.5, 15 and 30 mg/L) were prepared each day during the three days of the validation process by dilution of each stock solution in deionized water.

## Chromatographic equipment

The analyses were performed using a Jasco 2000 liquid chromatographic system composed of a PU-2080 Plus pump, and an AS-2055Plus auto-sampler coupled with a diode array detector (MD –2018 Plus Jasco France, Bouguenais, France). The separation was achieved using a 5 µm Nucleodur HTEC C18 column (250/4.6 mm ID) (Macherey-Nagel, Germany).

## Chromatographic parameters

The mobile phase was a mixture of two phases A and B used in a gradient mode.

- Phase A was composed of a mixture of water acidified with 0.2 % metaphosphoric acid (m/v) and ACN (98/2 v/v), adjusted at pH = 3.5 with NaOH 10N solution.
- Phase B consisted of 100 % ACN.

The solutions were filtered and degassed under vacuum in an ultrasonic bath before use.

The vitamins were analyzed with a gradient elution method at a flow rate of 0.75 mL/min as follows: 0–7 min: 100 % A; 7-20 min: 100 % A to 87 %; 20-23 min: 87 % A to 100 %; 23 min to end of run: 100 % A.

The injection volume was of 20 µl.

Each vitamin was quantified at a specific wavelength: 200 nm (B3 and B6), 245 nm (C), 280 nm (B9) and 360 nm (B12).

## Method validation

The analytical validation was conducted according to the recommendations of the ICH Q2(R1) (International Conference on Harmonization) [4] and the guidelines proposed by the French Society of Pharmaceutical Sciences and Techniques according to Hubert et al [25, 26].

This methodology is based on the study of accuracy called “total error”, which corresponds to the addition of systematic error (trueness) and random error (precision) obtained with validation standards. “Total error” signifies to the difference between the true value and the current value back-calculated from a validation standard solution analyzed with the analytical method. Precision (repeatability, intermediate precision) expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Trueness expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted either as a conventional true value or an accepted reference value.

Validation was performed during three consecutive days. Each day, blank samples, five CS and four VS prepared in triplicate were analyzed. All validation criteria are accepted within a range of ± 10 % (15 % error is allowed for the limit of quantification) [26].

Both limits of quantification (LOQ) and detection (LOD) were calculated according to the ICH method Q2 (R1) [4] and Hubert et al [25] after computation of the standard deviation on blank samples (water alone), and the obtained LOQ and LOD were checked experimentally.

The homogeneity of variance of the results of calibration lines is verified using the Cochran test. All statistics and computations were performed using Excel software (Microsoft® Office).

To demonstrate selectivity, the UV spectrum of the individual vitamins obtained by chromatography was compared to its reference spectrum in the ChromNav® software data basis. Also, a high peak purity and adequate resolution from the adjacent peaks were verified by the software.

The specificity was verified: it is defined as the ability of this method to discriminate between compounds of closely related structures which are likely to be detected, e. g. breakdown products [5]. Hence, starting point of our experiment was the degradation of each individual vitamin working standard substance under different stress conditions: alkaline and acid under heating degradations, oxidative degradation and photodegradation [5, 27].

We adapted the temperature and the heating time in order to obtain a moderate degradation of the vitamins making it possible to visualize the molecule and its primary breakdown products.

For each degradation assay, a blank (solvent of dilution without vitamins) was prepared. Each degradated vitamin solution was compared to the standard solution at the same initial concentration.

## Alkaline/acid and temperature degradation

Vitamin solutions at 40 mg/L were subjected to changes in pH by separately adding chlorhydric acid and sodium hydroxide at different concentrations (0.1N, 0.5N, 1N), and by heating them to 80 °C during 60 minutes except for vitamin C which was exposed to 40 °C during 40 minutes to avoid a fast and total degradation of the molecule.

After pH neutralization, the solutions were directly analyzed without dilution at a concentration 10 mg/L for each vitamin.

## Oxidative degradation

Vitamin solutions at 100 mg/L were exposed to hydrogen peroxide solutions (3 %) and then heated to 25 and 80 °C during different times.

To avoid deterioration of the column by the hydrogen peroxide, vitamin solutions were diluted in water to a concentration of 10 mg/L just before analysis.

## Photodegradation

The photodegradation of the active ingredients was performed by exposure of the 100 mg/L vitamin samples to light in an climate chamber (ICH 240 Binder QCA) using a cool white light (400 to 800 nm wavelength, color 640) and UVA radiations (320 to 400 nm wavelength, color 09), at a distance of 15 cm from the radiation source. The samples were analyzed after dilution in water to 10 mg/L and the progress of degradation is followed every 30 minutes for up to 6 hours.

This intentional degradation was carried out on the experimental standards of vitamins in order to generate likely breakdown products and to demonstrate specificity and stability-indicating nature of the proposed analytical method.

This enable us to determine relative retention of breakdown products of each individual vitamin and confirm that these peaks do not interfere with any of the vitamins. All vitamins peaks in solution were also checked for peak purity with DAD detector.

We aimed to transpose our method to an external laboratory with another HPLC system (Flexar system with PDA Plus, Perkin Elmer). The transposition was performed during three consecutive days. Each day, blank samples, four CS (1, 5, 25 and 50 mg/L) and three VS (2.5, 15 and 30 mg/L) prepared in triplicate were analyzed.

## Quantification of vitamins in commercial products

In order to assess the workability of our method we performed preliminary analyses of water-soluble vitamins in 3 marketed products:

- Laroscorbine® 1 g/5mL intravenous injectable solution, containing vitamin C (batch F0600;; expiry date: 11/2020)
- Chlorhydrate de Pyridoxine Renaudin® 250 mg/5mL injectable solution containing vitamin B6 ((batch 205,548; expiry date: 03/2021)
- Soluvit® lyopilisat, which is a mixture of vitamin B1, B2, B3, B5, B6, B9, B12, C, H. (batch 10MG6324;; expiry date: 08/2018)

The three tested drugs were diluted in deionized water to obtain theoretical vitamins concentrations in the validated range of linearity.

# Results

## Selectivity

The analytical method allows the identification of each vitamin in a specific manner from the various sources of active molecule studied (raw material). The vitamins C, B6, B3, B9 and B12 had a retention time of 5.0–7.5–11.5–25.5 and 26.5 min respectively (Figure 1(a) and the resolution was always greater than 1.5 at the different wavelengths chosen.

## Specificity

In addition to the selectivity, the specificity was verified during the intentional degradation process to confirm the stability indicating nature of the developed method.

### Alkaline and acidic degradation

As shown in Figures 1(b)–1(c)–1(e), no degradation peak from the different vitamins interfered with the detection of their own parent molecule or with the other vitamins.

For vitamin C: 2 breakdown products were detected with retention times (RT) of 3.1 and 3.5 min respectively (Figure 1(b).

For vitamin B3: 1 degradation product was detected with a RT of 6 min just before the main peak of vitamin B6 (Figure 2).

For vitamin B12: several breakdown products that appear towards the end of the run were detected (Figure 1(e).

In Table 1 the percentages of vitamin degradation under acid/alkaline solutions and temperature are presented.

As shown in Table 1, vitamin C is completely degraded in the alkaline solution and the more acid the solution is, the more stable the vitamin is. Vitamin B3 is the most stable vitamin in acidic conditions and its degradation increases as NaOH concentration increases. Vitamin B12 is the most unstable vitamin in either conditions (acidic or alkaline solutions) unlike vitamin B6 which is the least degraded one. Vitamin B9 is more stable in basic than acid solutions.

### Oxidative degradation

The vitamins were degraded rapidly to more than 20 % of initial concentration, when they were subjected to 3 % of

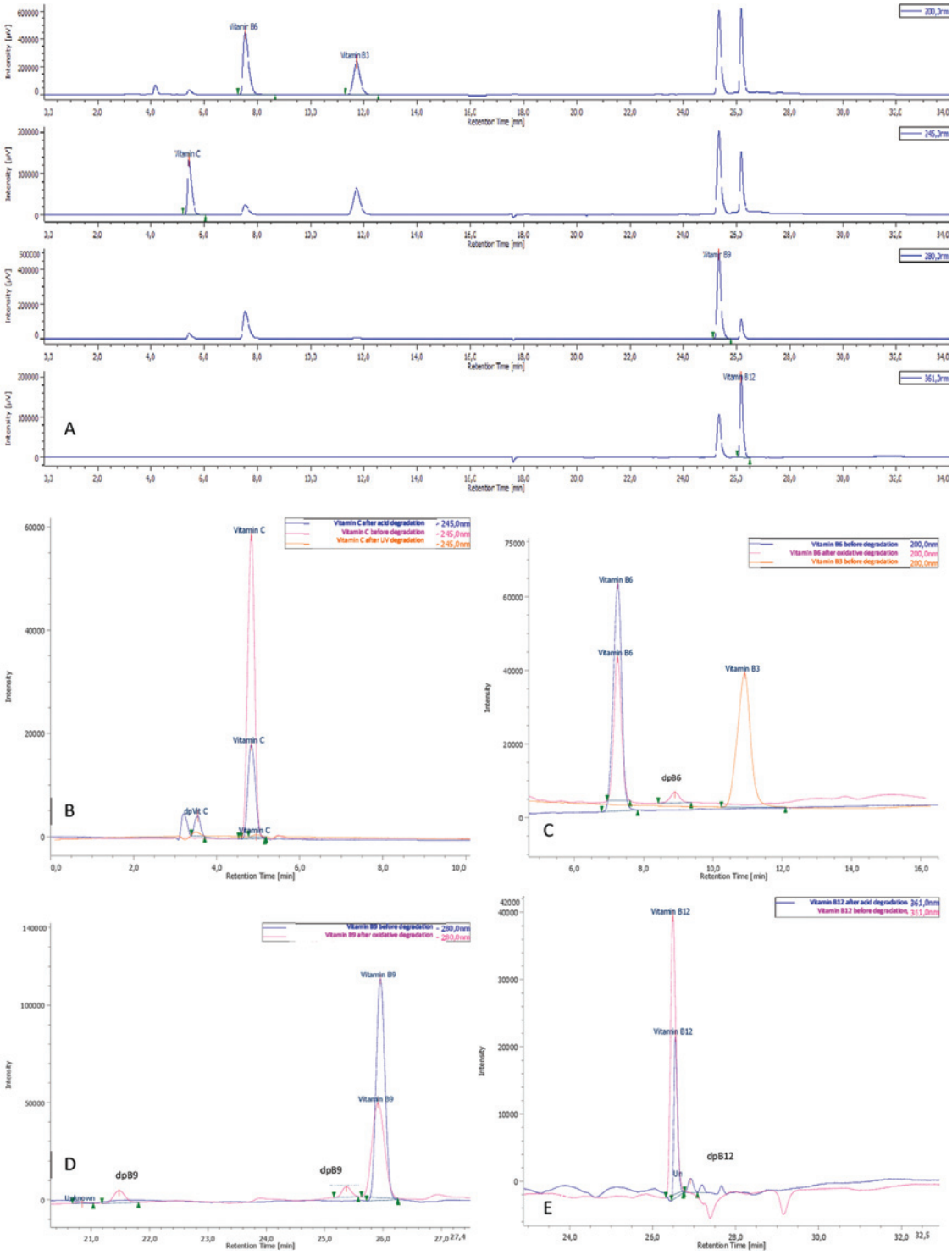


**Figure 1:** Chromatograms results of detection of vitamins. (A) Chromatograms of vitamins before degradation (vitamin B6 and vitamin B3 at 200 nm, vitamin C at 245 nm, vitamin B9 at 280 nm, vitamin B12 at 361 nm). (B) Chromatogram of vitamin C at 245 nm after acid and UVA degradation (vitamin C before degradation (purple line), after acid degradation with 0.5N HCl (blue line), after 1hour of exposition to UVA (orange line), dpVitC: degradation product of vitamin C). (C) Chromatogram of vitamin B6 at 200 nm degraded with 3 % solution of H2O2 (dpB6: degradation product of vitamin B6, vitamin B6 before degradation (blue line), vitamin B3 before degradation (orange line), vitamin B6 after oxidative degradation (red line)) at 200 nm. (D) Chromatogram of vitamin B9 after oxidative degradation (dpB9: breakdown products of vitamin B9, vitamin B9 before degradation (blue line) and vitamin B9 after degradation (red line)). (E) Chromatogram of vitamin B12 after acid degradation at 360 nm (vitamin B12 before degradation (purple line), vitamin B12 after degradation (blue line), dpB12: breakdown products of vitamin B12).

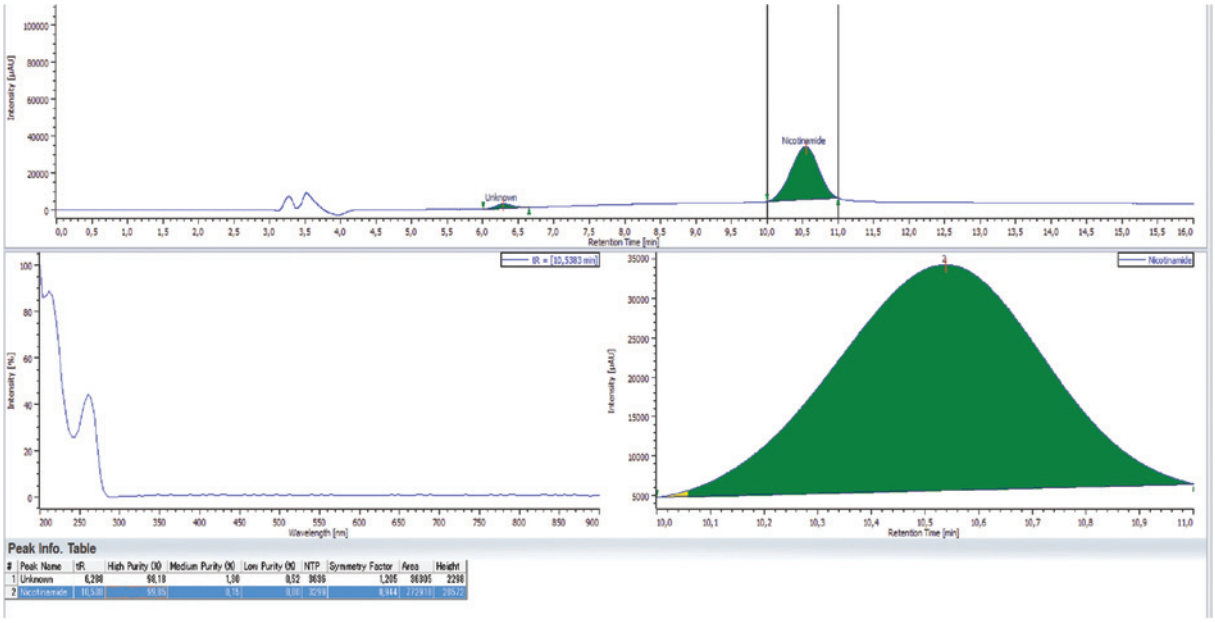


**Figure 2:** Chromatogram of vitamin B3 (nicotinamide) after acid degradation with peak purity information.

**Table 1:** Percentages of vitamins degradation in acid and basic solutions.

| Vitamin | Conditions | | % of degradation |
|---|---|---|---|
| Vitamin C | 40 °C/40 min | 0.1N HCl | 61 |
| | | 0.5N HCl | 44 |
| | | 1 N HCl | 35 |
| | | NaOH solutions | 100 |
| Vitamin B3 | 80 °C/60 min | 0.1N HCl | 11 |
| | | 0.5N HCl | 14 |
| | | 1 N HCl | 16 |
| | | 0.1 N NaOH | 39 |
| | | 0.5 N NaOH | 93 |
| | | 1N NaOH | 99 |
| Vitamin B6 | 80 °C/60 min | 0.1N HCl | 17 |
| | | 0.5N HCl | 14 |
| | | 1 N HCl | 18 |
| | | 0.1 N NaOH | 30 |
| | | 0.5 N NaOH | 20 |
| | | 1N NaOH | 10 |
| Vitamin B9 | 80 °C/60 min | 0.1N HCl | 34 |
| | | 0.5N HCl | 49 |
| | | 1 N HCl | 39 |
| | | 0.1 N NaOH | 33 |
| | | 0.5 N NaOH | 20 |
| | | 1N NaOH | 23 |
| Vitamin B12 | 80 °C/60 min | 0.1N HCl | 61 |
| | | 0.5N HCl | 87 |
| | | 1 N HCl | 87 |
| | | 0.1 N NaOH | 85 |
| | | 0.5 N NaOH | 100 |
| | | 1N NaOH | 100 |

$H_2O_2$ without heating for 60 minutes (or even less for vitamin B6) except for vitamin B3 which seemed more stable than the others.

The breakdown products of vitamin B9 and B6 are shown in Figures 1(c) and 1(d).

The Table 2 shows the different percentages of vitamins degradation with 3 % of $H_2O_2$.

**Table 2:** Vitamins degradation with 3 %H2O2.

| Vitamin | Conditions | Degradation (%) |
|---|---|---|
| Vitamin C | 25 °C/60 min | 50 |
| | 80 °C/60 min | 100 |
| Vitamin B12 | 25 °C/60 min | 55 |
| | 80 °C/60 min | 64 |
| Vitamin B6 | 25 °C/30 min | 20 |
| | 25 °C/150 min | 28 |
| | 80 °C/60 min | 62 |
| Vitamin B9 | 25 °C/60 min | 62 |
| | 80 °C/60 min | 100 |
| Vitamin B3 | 25 °C/60 min | 14 |
| | 80 °C/60 min | 23 |

### Photodegradation

After one hour of exposure to UVA and visible light, vitamin C was completely degraded (Figure 1(b) and vitamins B3, B6, B9 and B12 were partially degraded (over 50 %), however no breakdown products were detected at any wavelength for any of the vitamins.

Retention times of all vitamins and their breakdown products were summarized in Figure 3.

## Response function

Weighted linear regression with the inverse of value ($1/x$) is well suited to giving a precise estimation corresponding to already established data. Regression parameters are summarized in Table 3.

### Linearity

The measured concentrations were back calculated using the selected calibration model. For all vitamins, the relationship was linear as $r^2$ was always > 0.999. Thus, good linearity was shown over the whole concentration range.

### Limits of detection (LOD) and of quantification (LOQ)

The LOQ and LOD are shown in Table 3 for each vitamin.

### Precision, accuracy and trueness

Within-day precision or repeatability and between-day precision or intermediate precision are included in the pre-defined validation ranges ± 10 % and ± 15 % for the LOQ (Table 4). Due to the wide range of concentrations, weighting the signal with the inverse of concentration increased precision. The value of 100 % recovery was included in the 95 % confidence interval, and response variances at each concentration levels were homogeneous (Cochran test).

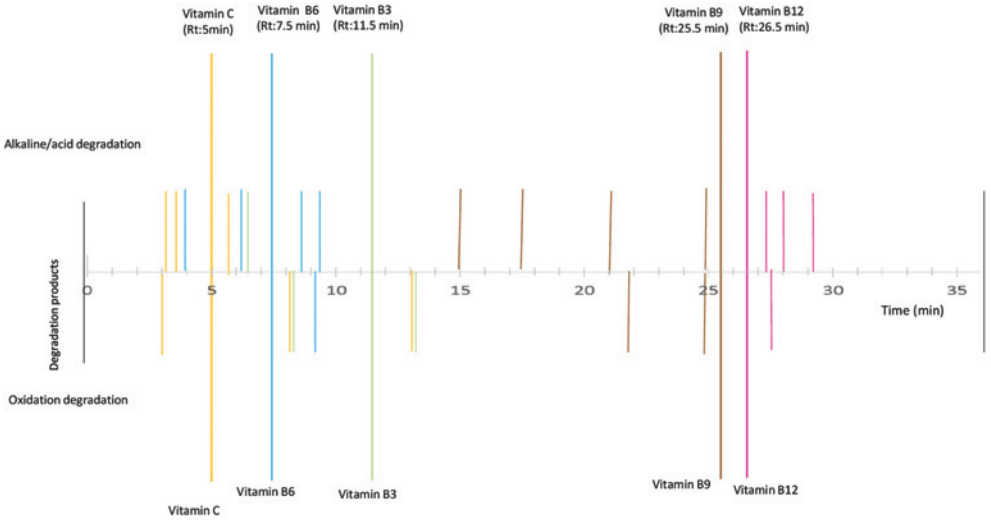


**Figure 3:** Linear representation of the retention times of all the vitamins studied and their breakdown products.

**Table 3:** Linearity validation of the method for each vitamin.

| | Average recovery interval (%) | Homogeneity of variances | Regression equation | Determination coefficient ($R^2$) | LOD (mg/L) | LOQ (mg/L) | Linear range (mg/L) |
|---|---|---|---|---|---|---|---|
| Vitamin C | [95.6; 100.2] | Accepted | *Y = 71370X−5484.85* | 0.9999 | 0.1 | 0.5 | 0.5–50 |
| Vitamin B6 | [99.0; 102.1] | Accepted | *Y = 127574X + 155.4* | 0.9999 | 0.1 | 0.5 | 0.5–50 |
| Vitamin B3 | [99.3; 104.2] | Accepted | *Y = 100117X + 425.89* | 0.9998 | 0.1 | 0.5 | 0.5–50 |
| Vitamin B9 | [97.6; 102.7] | Accepted | *Y = 89368X + 6376.33* | 0.9999 | 0.1 | 0.5 | 0.5–50 |
| Vitamin B12 | [97.1; 101.2] | Accepted | *Y = 30063X + 3246.15* | 0.9999 | 0.1 | 0.5 | 0.5–50 |

LOD: limit of detection
LOQ: limit of quantification

**Table 4:** Precision and accuracy validation of the method for each vitamin.

| [Vs] (mg $L^{-1}$) | | 0.5 | 2.5 | 15 | 30 |
|---|---|---|---|---|---|
| **Vitamin C** | mean intra-day precision (RSD, n = 3) % | 2.0 | 4.4 | 3.7 | 1.6 |
| | mean inter-day precision (RSD, n = 9) % | 2.5 | 5.7 | 4.4 | 4.1 |
| | Mean accuracy (relative bias) % | −6.3 | −5.1 | −1.8 | −1.8 |
| **Vitamin B6** | mean intra-day precision (RSD, n = 3) % | 5.0 | 2.0 | 4.0 | 2.1 |
| | mean inter-day precision (RSD, n = 9) % | 4.9 | 3.6 | 3.8 | 2.3 |
| | Mean accuracy (relative bias) % | 0.2 | −2.1 | −0.4 | 0.3 |
| **Vitamin B3** | mean intra-day precision (RSD, n = 3) % | 3.2 | 1.5 | 2.3 | 2.2 |
| | mean inter-day precision (RSD, n = 9) % | 7.8 | 4.4 | 6.5 | 5.0 |
| | Mean accuracy (relative bias) % | −4.6 | −3.6 | −4.3 | −5.2 |
| **Vitamin B9** | mean intra-day precision (RSD, n = 3) % | 6.4 | 1.7 | 3.5 | 2.3 |
| | mean inter-day precision (RSD, n = 9) % | 11.2 | 3.5 | 4.9 | 3.0 |
| | Mean accuracy (relative bias) % | −0.9 | 2.1 | −1.8 | −1.3 |
| **Vitamin B12** | mean intra-day precision (RSD, n = 3) % | 7.1 | 2.2 | 1.4 | 2.2 |
| | mean inter-day precision (RSD, n = 9) % | 11.2 | 7.8 | 2.4 | 2.5 |
| | Mean accuracy (relative bias) % | 4.6 | −1.1 | −3.7 | −0.2 |

The results of mean trueness are within the required validation limits of ± 10 % for all vitamins.

The developed analytical method can therefore be considered accurate and precise.

### Transposition

The transposition of this method was confirmed with good linearity ($R^2 > 0.999$) and an acceptable accuracy and trueness (error included in ± 10 % interval). The linear regression was obtained without weighting and results of linearity and accuracy are summered in Tables 5 and 6.

During the transposition, the regression equations possessed higher slope values than those obtained during the initial validation, which can be explained by the difference between the two chromatographic devices (detectors).

### Quantification of vitamins in commercial products

The results of vitamins' analyses in the three commercial products are shown in Table 7.

The measured concentrations varied from theoretical concentrations by 0.9 % to 18.8 %.

## Discussion

The developed LC-DAD method is a stability-indicating method that allows the simultaneous quantification of five water-soluble vitamins (ascorbic acid [vit C], nicotinamide [B3], pyridoxine [B6], folic acid [B9] and cyanocobalamin [B12]) in a single run of 30 minutes. Its development was challenging, especially because of the unstability of water-soluble vitamins stock solutions and

**Table 5:** Transposition: regression function.

| | Regression equation | $R^2$ | Linear range (mg/L) |
|---|---|---|---|
| Vitamin C | $Y = 107{,}706.7X - 3613.9$ | 0.9993 | 1–50 |
| Vitamin B6 | $Y = 280{,}375.5X + 100{,}763$ | 0.9994 | 1–50 |
| Vitamin B3 | $Y = 223642X + 67{,}428$ | 0.9997 | 1–50 |
| Vitamin B9 | $Y = 177{,}345.7X + 24{,}304$ | 0.9999 | 1–50 |
| Vitamin B12 | $Y = 58028X - 4132$ | 0.9998 | 1–50 |

**Table 6:** Transposition: accuracy results.

| [Vs] (mg $L^{-1}$) | | 2.5 | 15 | 30 |
|---|---|---|---|---|
| **Vitamin C** | mean intra-day precision (RSD, n = 3) % | 2.64 | 2.96 | 1.65 |
| | mean inter-day precision (RSD, n = 9) % | 4.81 | 7.24 | 4.23 |
| | Mean accuracy (relative bias) % | −3.66 | −4.63 | −7.14 |
| **Vitamin B6** | mean intra-day precision (RSD, n = 3) % | 0.59 | 1.55 | 0.31 |
| | mean inter-day precision (RSD, n = 9) % | 5.75 | 2.28 | 0.33 |
| | Mean accuracy (relative bias) % | 2.01 | 3.21 | 2.48 |
| **Vitamin B3** | mean intra-day precision (RSD, n = 3) % | 1.73 | 1.15 | 0.21 |
| | mean inter-day precision (RSD, n = 9) % | 2.41 | 1.24 | 0.63 |
| | Mean accuracy (relative bias) % | −3.54 | 3.15 | 2.63 |
| **Vitamin B9** | mean intra-day precision (RSD, n = 3) % | 0.82 | 0.77 | 1.71 |
| | mean inter-day precision (RSD, n = 9) % | 1.92 | 1.71 | 2.42 |
| | Mean accuracy (relative bias) % | 0.21 | 1.85 | 0.44 |
| **Vitamin B12** | mean intra-day precision (RSD, n = 3) % | 1.86 | 2.32 | 2.06 |
| | mean inter-day precision (RSD, n = 9) % | 4.46 | 1.89 | 2.13 |
| | Mean accuracy (relative bias) % | 3.89 | 2.62 | 2.52 |

**Table 7:** Results of vitamins analysis in three commercial products.

| Specialty | Form | Quantified vitamin(s) | Theoretical concentration (µg/mL) | Measured concentration (µg/mL) (mean ± sd) | IC95% | Accuracy (average, n = 3) % |
|---|---|---|---|---|---|---|
| Laroscorbine® 1 g/5 mL | Injectable solution | C | 20 | 18.1 ± 0.318 | 0.360 | −9.6 |
| Pyridoxine Renaudin® 250 mg/5 mL | Injectable solution | B6 | 50 | 41.9 ± 0.833 | 0.942 | −16.2 |
| Soluvit® | Lyophilized powder | C | 50 | 40.6 ± 0.324 | 0.366 | −18.8 |
| | | B6 | 2 | 2.02 ± 0.009 | 0.010 | 0.9 |
| | | B3 | 20 | 17.4 ± 0.063 | 0.070 | −12.9 |
| | | B9 | 40 | 38.3 ± 2.017 | 2.283 | −4.2 |
| | | B12 | 0.5 | < LOQ | - | - |

the large number of tested molecules. The chromatographic parameters were optimized step by step to separate vitamins from their breakdown products. Consequently, several assays with variation of flow rate and phase mobile composition were originally tested. Finally, the developed method is quite simple with a fixed flow rate, four gradient levels and mobile phase buffered at pH3.5 with metaphosphoric acid. Due to the instability of the vitamins, a particular attention was payed to the stock solution preparation. Vitamin C stock solution had to be stabilized with metaphosphoric acid (MPA), and vitamin B9 in an alkaline solution.

The method we developed is precise (repeatability, intermediate fidelity and reproducibility) and accurate for all the vitamins studied. It is also specific and selective with a capacity to identify and separate the five studied vitamins simultaneously and to separate them from their breakdown products. It has a good linearity on the concentration range

studied (0.5–50 µg/mL) with a coefficient of determination superior to 0.999. Our method allows the detection and the quantification of water-soluble vitamins at low levels (with LOD = 0.1 µg/mL and LOQ = 0.5 µg/mL).

There are many HPLC methods published concerning the simultaneous determination of vitamins; however, most of these methods are not stability indicating [8, 13–15, 21, 22] which renders them improper for use during stability studies, and some of them are unable to quantify all the water-soluble vitamins simultaneously. Indeed, Vidović et al [16] developed a stability indicating method for the simultaneous quantification of 7 water-soluble vitamins in multivitamin syrup preparations but it isn't able to analyze vitamins B9 and B12. Also, Jin et al [19] analyzed seven vitamins in two runs without cyanocobalamin whereas Maia et al [18] developed a stability indicating method only for vitamin C in semisolid pharmaceutical/cosmetic formulations. Some others publications mention using a MS-MS detector [14, 20] which involves a specific and expensive equipment not available in all laboratories for routine analyses.

Our method has a potential broad range of applications, ranging from the analysis of vitamins in dietary supplements and "nutraceuticals" to the quantification and stability study of vitamins in TPN preparations for hospitalized or ambulatory patients.

The human diet does not always contain the amount of vitamins needed for the normal development and maintenance of body functions. Therefore, fortification of certain food products, in particular those which are the sole source of nutrition (infants and clinical nutrition), is needed to ensure an adequate intake of vitamins. In this way, at hospital, parenteral nutrition (PN) is often prescribed for neonates, especially premature infants who cannot be fully fed orally or enterally [28]. It ensures the nutritional and quantitative needs of the child and it must contain the vitamins. The administration can be carried out with the help of vitamin "ration" with complete formulation as Cernévit® (water-soluble mixed micelles), or with non-complete formulations: as Soluvit® (water-soluble vitamin mixture), Polyvitamin® Hydrosol (vitamins A, D, E, five water-soluble B vitamins and vitamin C) [29].

This method is an essential first approach and was developed to allow stability studies of vitamins in nutritional mixtures either in hospital preparation (parenteral nutrition) and/or on industrial food supplements. However, it has to be completed by a study of the matrix effect, specific to each application. Indeed, this matrix effect may explain the accuracy biases observed on some marketed products we analyzed. Its simplicity of implementation and a short analysis time make it very "accessible" by many control laboratory to conduct, in good conditions, this sort of study [30].

Using a stability-indicating method to study the stability of vitamins mixed in parenteral nutrition bags will give information as to whether they can be prepared in advance and therefore help better manage the supply of care units. Uccelo-Barreta et al [31] reported that the vitamins (B1, B2, B3 and B6) were stable at 48 hours in presence of salt and trace elements. In the work of Bouchoud et al [32], the vitamins A, E and C remained stable for one week under refrigeration. However, other studies are mainly focused on ions and amino acids [30, 33–39] but not on all vitamins and their interactions.

Moreover, as suggested in the 2011 European legislation [40], dietary supplements may contain several vitamins, with amounts referring to the recommended nutrient intake (RNI). Nevertheless, the recommended amounts of vitamin B12 are very low, at 2 µg/L in drinks and 4 µg/g in other foodstuffs [40], and thus potentially undetectable with our method, unlike other vitamins. However, it seems to be an issue for any method developed to analyze water-soluble vitamins [20, 22] where authors decided to exclude this vitamin in their studies. The MS detection used in the work of Yong Hu et al [41] to develop a non stability-indicating method with a limit of quantification equal to 1.2 $10^{-2}$ µg/mL was interesting. Nevertheless, some products can contain vitamin B12 at higher levels and can thus be quantified with our method [29, 42].

## Conclusion

The method presented in this article can be useful for carrying out stability studies of water-soluble vitamins in solutions if, like for all methods, the absence of matrix effect is verified or taken into consideration. It may also be possible to use this method to quantify other vitamins such as thiamin in order to meet the hospital preparations needs, but further studies are needed to confirm this hypothesis.

This method using liquid chromatography with diode array detection is able to analyze simultaneously five water-soluble vitamins (C, B3, B6, B9 and B12) in aqueous solutions. In addition, this method is stability-indicating. The products resulting from the degradation of the five vitamins are separated from each original molecule,

which makes it possible to use this method to carry out stability studies in mixtures with several water-soluble vitamins. Despite the number of vitamins and breakdown products, the duration of the analysis (30 minutes) remains acceptable, allowing several samples to be tested per day.

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