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Successive determination of thiamine and ascorbic acid in pharmaceuticals by flow injection analysis

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Abstract

A simple and rapid fluorimetric method for the determination of mixtures of thiamine and ascorbic acid is proposed. The procedure is based on the oxidation with mercury(II) of the B_1 and C vitamins to form thiochrome (TC) and quinoxaline derivate, respectively. Both reaction products exhibit fluorescence at the same wavelengths ($\lambda_{ex} = 356$ and $\lambda_{em} = 440$ nm). The procedure is optimised in a flow injection (FI) system and applied with excellent results in the determination of B_1 and C vitamins in commercial pharmaceutical preparations. The calibration graphs were linear over the range 2–100 $\mu g\,ml^{-1}$ for thiamine and 5–100 $\mu g\,ml^{-1}$ for ascorbic acid. The throughput was 25 samples per hour.

Keywords: Ascorbic acid; Thiamine; Pharmaceuticals; Successive determination; Flow injection

1. Introduction

Thiamine (vitamin B_1), a water-soluble vitamin, is a natural nutrient present in many foods and is also added as an essential nutrient. It has been used for the prevention and treatment of beriberi, neuralgia, etc. in medical doses or vitamin B_1-enriched food or drinks. It is necessary for carbohydrate metabolism and for the maintenance of neural activity.

The chemical method most widely used involves the reaction between vitamin B_1 and potassium hexacyanoferrate(III) in alkaline solution, followed by extraction of the thiochrome (TC) formed in aqueous phase into an organic phase, which is then measured fluorimetrically [1]. This procedure is the official U.S.P. method and has been automated by flow injection (FI) with fluorimetric and chemiluminescence detection [2,3]. Other FI methods accomplish the oxidation of thiamine to fluorescent TC using Hg(II) [4], Cu(II) [5], strong anionic resins loaded with hexacyanoferrate(III) [6] and electrochemical oxidation [7]. The on-line UV-irradiation of thiamine with photometric [8] and fluorimetric detection [9] and the derivatization reaction of the primary amine group with *o*-phthalaldehyde in the presence of 2-mercaptoethanol using fluorimetric detection [10] have also been proposed.

Ascorbic acid (vitamin C), a water-soluble vitamin, is an important micronutrient and plays many physiological roles [11]. Fruit and vegetables constitute the

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principal source of vitamin C in most human diets, where it occurs as L-ascorbic acid (AA) and its oxidised form, dehydro-L-ascorbic acid (DHAA), both of which are biologically active.

Many FI methods have been reported for the determination of ascorbic acid in pharmaceutical preparations, food products, and biological samples using different detection systems [12]. The FI spectrophotometric methods involve redox reactions with AA, in which a coloured compound is formed or decomposed in a redox reaction [13,14]. The FI methods with electroanalytical detectors are mainly based on the inherent redox chemistry of AA and reagents [12]. Chemiluminescent methods in flow systems have involved the oxidation of AA by iron(III), hexacyanoferrate(III) or permanganate, or photochemical oxidation, followed by the interaction with another chemiluminescent reagent such as luminol [15,16] or lucigenin [17,18]. Methods based on derivatization reactions have been developed to improve the selectivity for total ascorbic acid (AA and DHAA). Usually, AA is first converted to DHAA by means of a suitable oxidising agent and then condensed with a selective reagent, such as *o*-phenylendiamine (OPDA), to form a fluorescent quinoxaline derivate. The OPDA method was originally developed by Deutsch and Weeks [18], but has been modified by many investigators and used widely [19–21]. Chung and Ingle [20] have proposed a kinetic procedure based on the rapid oxidation of AA by mercury(II) chloride, measuring the condensation rate between DHAA and OPDA.

The determination of several analytes in the same sample using flow injection analysis has a number of potential advantages because of the flexibility of FI assemblies and their typically high throughput, modest reagent consumption and adaptable sensitivity (dependent on the particular detector employed).

The high flexibility of FI has fostered the development of a wide variety of multi-determinations based on a number of chemical systems [22,23]. However, the assemblies involved are occasionally highly complex and use several detectors. The ideal assembly for multi-determinations should be fairly simple and use reliable, straightforward chemical methods and, whenever possible, a single injector and detector. FI manifolds using only one detector usually make use of splitting, merging points, internally coupled valves or open–closed configurations [24].

In this work, a FI system for the successive fluorimetric determination of thiamine and ascorbic acid is proposed. The method is based on the oxidation of both vitamins with mercury(II). Thiamine forms the fluorescent product (thiochrome) directly, while AA is oxidised to DHAA, which then reacts with OPDA to yield a fluorescent quinoxaline derivative. Both products were successively directed to the fluorimeter and detected at the same excitation and emission wavelengths.

2. Experimental

2.1. Reagents

All chemicals were of analytical reagent grade and demineralized water from a Milli-Q system was used in all experiments.

Aqueous standard solutions (1×10^{-3} M) of vitamin B_1 were prepared by dissolving thiamine hydrochloride, previously dried, in ultrapure water and adjusted to pH 4 with hydrochloric acid; this solution was stable for 3 months if kept refrigerated. Working standard solutions were prepared daily from the stock solution by appropriate dilution with ultrapure water.

Ascorbic acid standard solutions (1×10^{-3} M) were prepared daily no more than 3 h prior to use by dissolving AA (Sigma) in 0.01 M perchloric acid. Working solutions of lower concentrations were prepared by appropriate dilution with ultrapure water. All solutions were kept in amber-coloured bottles in the dark.

Mercury(II) chloride solutions were prepared from $HgCl_2$ (Merck) and adjusted to pH 4 with HCl.

OPDA stock solution (0.1 M) was prepared in water daily.

2.2. Apparatus

A Hitachi F-3010 spectrofluorimeter was used to record spectra and carry out fluorescence measurements. A Gilson Miniplus-3 peristaltic pump was used to introduce the reagents into the system. An Omnifit rotary valve and a Hellma (176.052 QS, inner volume 25 μl) flow cell were also used.

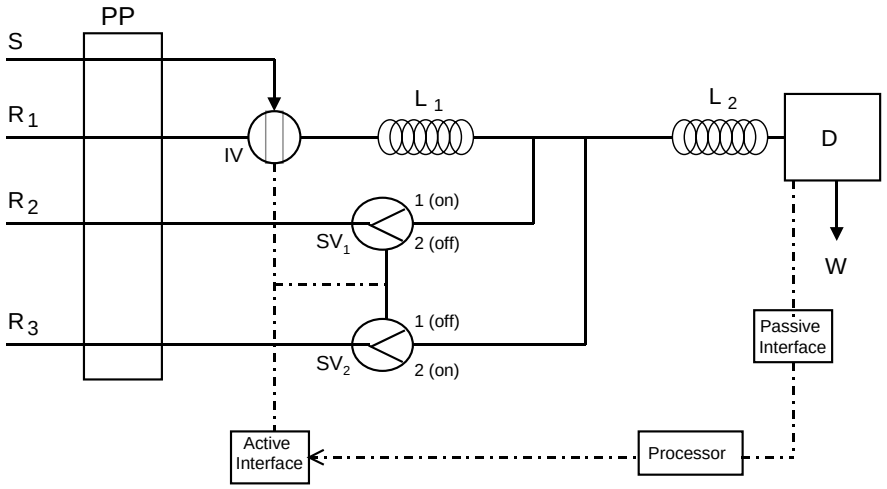

Fig. 1. Flow injection manifold for the determination of the vitamin B_1 and C. PP, peristaltic pump; IV, injection valve; SV_1 and SV_2, selection valves; S, sample; R_1, 1.5×10^{-3} M mercury(II) solution in 1% (v/v) Triton X-100; R_2, 0.2 M phosphate buffer of pH 12; R_3, 5×10^{-2} M OPDA solution and 0.2 M acetate buffer of pH 4.

2.3. Manifold and procedure

The schematic diagram of the instrumental set-up is shown in Fig. 1.

The sample is injected into the carrier (1.5×10^{-3} M Hg^{2+} and 1% (m/v) Triton X-100) with the aid of a rotary valve with a loop of 185 μl. Two selection valves (SV_1 and SV_2) synchronised with the injection valve (IV) permit either the phosphate buffer (pH 12.0) stream or the OPDA (buffered at pH 4.0) stream to be alternatively joined to the sample plug. The fluorescent products are formed in the reaction coil L_2 and then directed to the detector. The signal corresponding to thiamine and AA are alternatively obtained measuring the fluorescence at 440 nm with excitation at 356 nm.

2.4. Preparation of assay solutions

2.4.1. Tablets

Ten tablets are powdered and an amount equivalent to 20 mg of the vitamins was weighed accurately and dissolved in ca. 100 ml of water. The solution was filtered through a Whatman no. 1 filter paper and the filtrate and two washings, each of 20 ml, were collected in a 1000 ml calibrated flask and diluted to volume with water.

Capsule and sachet forms were similarly dissolved and appropriately diluted with ultrapure water prior to analysis.

3. Results and discussion

When thiamine is oxidised by Hg(II) in a FI system the largest yield of TC is obtained at a high Hg(II) concentration and in an alkaline medium. The time taken to reach equilibrium decreases with increasing Hg(II) and OH^- concentrations. The order of addition of the reagents is critical. If the thiamine is mixed with the base first, a low yield of TC is obtained upon addition of Hg(II). The addition of a large excess of Hg(II) to the thiamine solution apparently stabilises the latter by the formation of a complex which can then be oxidised to TC when the base is added.

The formation of a precipitate in a FI system with fluorimetric detection is unacceptable because of the lack of reproducibility of the measurements, Therefore, it was necessary to study the solubility of Hg(II) at alkaline pH. It was found that the $HgCl_2$ solutions are stable in the pH range 11–13 if 1% Triton X-100 is present.

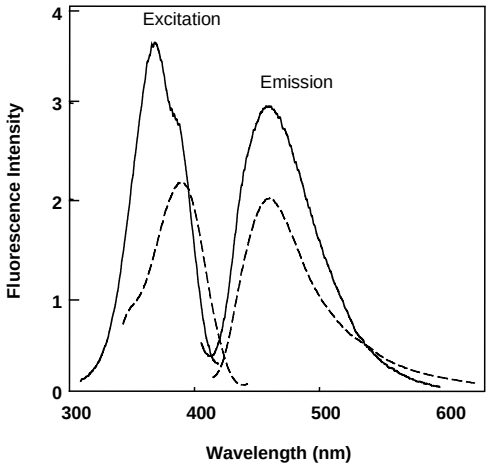

Fig. 2. Fluorescence spectra of the thiochrome (—) and quinoxaline (---). 3.9×10^{-5} M thiamine; 1.0×10^{-3} M Hg(II); phosphate buffer pH 12; 2.9×10^{-4} M ascorbic acid; 0.05 M OPDA; 1.0×10^{-3} M Hg(II); acetate buffer pH 4.

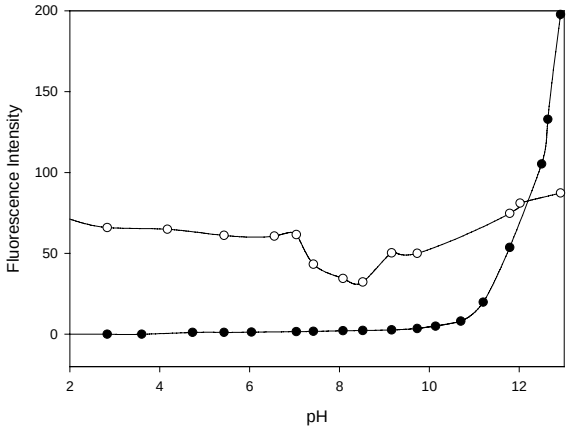

Fig. 3. Influence of pH on the conversion of thiamine to TC (●) and AA to the quinoxaline derivative (○).

The condensation between DHAA and OPDA yields a quinoxaline derivative which has a strong absorption band centred at about 366 nm whereas the reactants show no appreciable absorbance above 310 nm. The condensation product exhibits a high yellow fluorescence with the emission band centred at a wavelength of 435 nm.

Various oxidising agents have been use to converting AA to DHAA. Of these $HgCl_2$ was selected because it is inactive with OPDA and therefore, no reaction product is produced that absorbs in the wavelength region where the quinoxaline fluoresces.

Excitation and emission spectra of thiochrome and quinoxaline (Fig. 2) show that it is possible to carry out fluorescence measurement of both fluorophors at the same wavelength ($\lambda_{ex} = 356$, and $\lambda_{em} = 440$ nm).

3.1. Effect of chemical and FI variables

The effect of the chemical and FI variables on the fluorescence intensity obtained for each analyte was investigated by altering each variable in turn while keeping the others constant.

The efficiencies of the conversion of thiamine to thiochrome and AA to the quinoxaline derivative were measured at different pH values. The maximum fluorescence intensity was achieved in the pH range 12–13 for thiamine and AA (Fig. 3). However, the pH range 3.5–6.5 was selected for AA because in this pH range thiamine is not oxidised to thiochrome by Hg(II). Therefore, 0.2 M phosphate buffer (pH 12.0) and 0.2 M acetate buffer (pH 4.0) were selected because of their high buffering capacity at these pH.

The effect of Hg(II) concentration on the formation of both fluorophors was studied. The yields of thiochrome and quinoxaline increased with increasing Hg(II) concentration. A 1.5×10^{-3} M Hg(II) was selected because at this concentration level the solution is stable in alkaline medium in the presence of 1% Triton X-100 and the fluorescence peaks are high.

The influence of OPDA concentration was studied between 0.001 and 0.07 M. Fluorescence rapidly increased up to 0.05 M and then slightly increased (Fig. 4). An OPDA concentration of 5×10^{-2} M was selected.

The influence of FI variables was also studied. Increasing the residence time of the sample in the FI system led to an increase in fluorescence for thiamine and AA. Therefore, the flow-rates of the Hg(II) and buffer streams, and the length of L_1 and L_2 reactors were examined simultaneously. The best results were obtained when the Hg(II) solution and phosphate buffer or OPDA were pumped at 1.9 ml min^{-1} and the length of L_1 and L_2 were 150 and 200 cm, respectively,

The effect of the sample loop size was examined in the 35–250 μl range since fluorescence increased up to 185 μl and greater volumes produced a splitting in the thiamine peak. A 185 μl volume was selected.

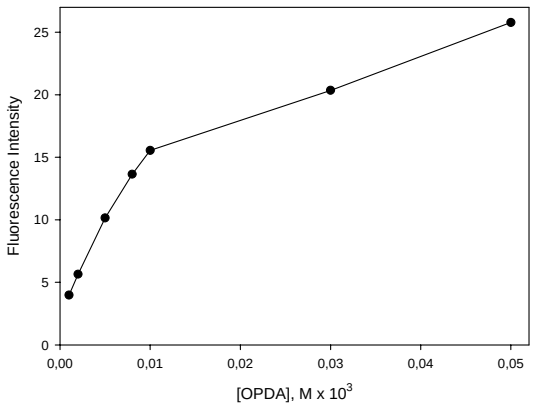

Fig. 4. Effect of OPDA concentration on the formation of the quinoxaline derivative.

3.2. Validation

The analytical method was validated in compliance with the guideline Q23 issued by the International Conference on Harmonization (ICH) [25]

3.2.1. Linearity

The calibration graphs were linear up to 100 μg ml^{-1} for both vitamins. Table 1 summarises the features of the calibration graphs.

3.2.2. Precision

The intra-day precision was tested with eleven repeated injections of two sample solutions containing the analytes at two concentration levels. The relative standard deviations (R.S.D.) were 0.47 and 0.51% at the 8 μg ml^{-1} level and 0.56 and 0.58% at the 20 μg ml^{-1} level for thiamine and AA, respectively.

The inter-day precision of the method was studied by analysing three identical samples (containing 8 μg ml^{-1} of each vitamin), injected six times every day, till 5 days, consecutively. The RSD were 1.8% for thiamine and 2.3% for AA.

Table 1
Analytical data for thiamine and ascorbic acid determinations

Analyte	Linear range (μg ml^{-1})	Slope ± s	Intercept ± s	r[a]
Thiamine	2–100	6.15 ± 0.05	4.92 ± 2.4	0.9991
Ascorbic acid	5–100	3.92 ± 0.06	5.70 ± 1.9	0.9989

[a] Correlation coefficient, $n = 15$.

3.2.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was studied from the equation LOD $= k\, s_B/m$, where the standard deviation (s_B) from 12 blank determinations, the slope of calibration curve (m) and $k = 3.3$ were used. The calculated LOD were 0.6 μg ml^{-1} for thiamine and 1.3 μg ml^{-1} for AA.

The LOQ was estimated using the above equation but taking now $k = 10$. The LOQ were 1.8 and 3.9 μg ml^{-1} for thiamine and AA, respectively.

Table 2
Analysis of synthetic mixture of the thiamine and ascorbic acid

Thiamine added (μg ml^{-1})	Ascorbic acid added (μg ml^{-1})	Thiamine found (μg ml^{-1})	Error (%)	Ascorbic acid found (μg ml^{-1})	Error (%)
5	10	4.91	1.8	9.84	1.6
5	50	4.92	1.5	49.40	1.2
5	100	5.05	1.0	102.0	2.0
10	5	9.84	1.6	4.90	1.9
10	50	9.86	1.4	48.95	2.1
10	100	9.09	1.0	98.10	1.0
50	10	49.0	2.0	10.13	1.3
50	50	50.85	1.7	49.10	1.8
50	100	49.55	0.9	101.5	1.5
50	5	50.55	1.1	5.08	1.6
100	5	98.4	1.6	4.93	1.3
100	10	98.0	2.0	9.90	1.0
100	50	101.8	1.8	50.8	1.6
100	100	99.1	0.9	98.5	1.5

Table 3
Tolerance to different substances in the determination of vitamine B_1 and C[a]

Substance	Maximum tolerable [substance]/[AA][b]	Maximum tolerable [substance]/[thiamine][b]
Riboflavin	1	0.01
Biotin	10	0.01
Cyanocobalamin	10	10
Glucose	200	25
Pyridoxine	1	10
Ca^{2+}	50	100
Iodide	20	1
Saccharose	100	10
Fructose	500	100
Phenylalanine	500	50
Leucine	500	100
Histidine	200	10
Valine	500	50
Mg^{2+}	500	20
Citrate	50	0.1
Saccharine	50	20
Arginine	200	10
Calcium pantothenate	10	20
Nicotinamide	5	20

[a] Thiamine concentration, 10 μg ml^{-1}; ascorbic acid concentration, 10 μg ml^{-1}.
[b] Maximum ratio tested.

3.2.4. Accuracy

The accuracy of proposed method was tested with several synthetic mixtures containing both vitamins in different proportions. Thiamine/AA mixture in the ratios from 1:20 to 20:1 were analysed by the proposed FI procedure. The results obtained were excellent because the errors were always less than 3% (Table 2).

3.2.5. Specificity

As the procedure described is for application to pharmaceutical preparations, the response of thiamine and AA in the test mixtures containing a fixed concentrations of both analytes (10 μg ml^{-1}) and various concentrations of aminoacids, minerals, vitamins and excipients were studied. A substance was considered not to interfere if the variation in the peak height of thiamine or AA was less than 3%. Table 3 summarises the results obtained.

3.3. Applications

In order to study the validity of the method it was applied to the determination of thiamine and ascorbic acid in the commercially available pharmaceutical preparations listed in Table 4. The results obtained show that the thiamine and ascorbic acid contents, as measured by the proposed FI method were in excellent agreement with those obtained by the thiochrome fluorimetric method [2] for thiamine and the FI norit-OPDA method [26] for AA.

Table 4
Determination of thiamine and ascorbic acid in pharmaceutical preparations

Sample (supplier)	Thiamine		Ascorbic acid	
	Proposed method[a]	Reference method [6][b]	Proposed method[a]	Reference method [26][b]
Hidroxil (Almirall Prodesfarma)	242.1 ± 2.5 (mg/tablet)	245.2 (mg/tablet)	–	–
Prevalon (Abelló Farmacia)	49.5 ± 1.8 (mg/bag)	50.0 (mg/bag)	548.2 ± 2.6 (mg/bag)	545.0 (mg/bag)
Boi-K (Laboratorios Boi)	–	–	244.7 ± 2.8 (mg/tablet)	247.4 (mg/tablet)
Frenadol (Abelló Farmacia)	–	–	224.7 ± 1.6 (mg/bag)	228.5 (mg/bag)
Vitafardi (Fardi)	–	–	1031.7 ± 8.2 (mg/bag)	1015.6 (mg/bag)
Vicomin (Pfizer)	–	–	156.4 ± 1.2 (mg/bag)	153.1 (mg/bag)

Composition of samples: hidroxil: thiamine, 250 mg; piridoxine chlorhydrate, 250 g; hydroxicobalamin, 500 mcg. Prevalon: arginine aspartate, 1000 mg; glutamine, 150 mg; thiamine chlorhydrate, 50 mg; magnesium ascorbate, 50 mg; pyridoxine HCl, 50 mg; ascorbic acid, 500 mg; hydroxycobalamin, 50 mcg. Boi-K: ascorbic acid, 250 mg; K^+ ion 390 mg; saccharin, 2 mg; Sucrose, 530 mg. Frenadol: paracetamol, 650 mg; dextrometorfan hydrobromid, 20 mg; caffeine citrate, 30 mg; chlorfenamine maleate, 4 mg; ascorbic acid, 250 mg. Vitafardi: ascorbic acid, 1 g; hydroxycobalamin, 500 γ. Vicomin: retinol palmitate, 1250 IU; ascorbic acid, 150 mg; saccharin 11 mg.
[a] Mean of five determinations ± S.D.
[b] Mean of two determinations.

4. Conclusions

The results presented in this work demonstrate that the FI system proposed is very suitable approach for determining B_1 and C vitamins and binary mixtures thereof. The use of the FI technique allows automation and easy application of the method to the simple and rapid determination of thiamine and ascorbic acid contained in pharmaceutical preparation.

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