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Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 151–158
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Automated determination of selected water-soluble vitamins in tablets using a bench-top robotic system coupled to reversed-phase (RP-18) HPLC with UV detection

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Received 8 October 2002; accepted 21 October 2002

Abstract

The determination of folic acid, nicotinamide, nicotinic acid, riboflavin, riboflavin-5′-phosphate, pyridoxine and thiamine in tablets has been successfully automated using a bench-top robotic system (Zymark Tablet Processing Workstation II) coupled to reversed-phase (RP-18) HPLC with UV-detection. The new automated methods have been validated and were found to be applicable in routine analysis for all common types of multivitamin tablets. The precision is exemplified by relative standard deviations of 5.4% for folic acid, 2.1% for nicotinamide, 1.6% for pyridoxine, 0.7% for riboflavin and 1.1% for thiamine using film-coated tablets as sample. Mean recoveries obtained during spiking experiments were in the range of 95.2–103.9%.

Keywords: Water-soluble vitamins; Automation; Tablets; RP-18 HPLC

1. Introduction

Vitamins are an essential group of food ingredients which have to be supplied in sufficient amounts with diet. If this intake is insufficient or if special dietary requirements exist multivitamin preparations can be taken in order to prevent vitamin deficiency. Numerous such preparations, often formulated as effervescent or film-coated tablets, are available on the market. Correspondingly, manufacturers have to cope with a large number of analyses performed for quality control and product form development. These investigations typically require, among others, content uniformity determinations, and an investigation of stability of the tablet ingredients upon storage under different conditions. The large number of analyses required, and the need for an analytical method with a high reproducibility over a long period of time make the analysis of these tablet ingredients a promising target for automation. Automated analyses promise a major reduction in manual laboratory working time, and corre-

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00574-5

spondingly a significant cost reduction. Additionally, results of an automated method are less dependant on the experience of the analyst than those of a manual method. Thus, analytical methods are more easily transferred to subsidiary laboratories, and results tend to be more precise and reproducible. Examples for automation in vitamin analysis include the determination of vitamins A and E in milk-based products [1], and the determination of four water-soluble vitamins in tablets using continuous flow sample preparation [2].

The present work focused on the development of an automated method for the determination of folic acid, nicotinamide, nicotinic acid, riboflavin, riboflavin-5′-phosphate, pyridoxine and thiamine in tablets. Their contents in many tablets are adjusted at the Recommended Dietary Allowance, and typically range from 0.4 mg (folic acid) to 20 mg (nicotinamide). All seven compounds are soluble in aqueous solutions, and can easily be separated and determined by RP-HPLC with UV-detection.

An ideal automated method has to be very robust, reliable and applicable to all sample types without additional major manual sample preparations, posing high demands on robustness of instrumentation and working procedure. As a suitable instrument the bench-top robotic system Tablet Processing Workstation II (TPW II) has been chosen. Examples for the use of the TPW II in pharmaceutical analysis include the determination of a phenothiazine derivative, and roxifiban [3,4]. The TPW II is controlled via a personal computer, and offers mainly the following options for sample work up: Sample weighing, transfer of the sample from a test tube into the homogenisation vessel, addition of one to five different solvents, sample dispersion using a mechanical homogeniser with a 3 cm probe head, filtration of the resulting extract, dilution and injection of the extract onto a HPLC system. Using the software of the TPW II, these options can be combined forming a sample preparation method. This method can then be run automatically on up to 100 consecutive samples. All data generated, including sample weight, weights of the solvents added, homogeniser speeds and weight of the filtrate is documented by the software. This allows a control of the work up procedure, and the tracking of possible errors. The main difference to common laboratory practice is that solvent addition is controlled by weight and not by volume. Thus, densities of all solvents used must be known or have to be determined and entered into the software prior to analysis.

Equally important as a robust sample work up procedure is a reliable and stable chromatographic system for quantitation of the vitamins. Numerous reports about the simultaneous determination of water-soluble vitamins in various matrices using HPLC have been published, e.g. [5–7]. For the current work, a previously unpublished chromatographic system, using a reversed-phase (RP-18) column with a methanol–phosphate buffer as mobile phase, coupled to an UV-detector has been employed. The system has demonstrated the required robustness during routine use in our department over several years. Development and validation of the new methods are described below.

2. Materials and methods

2.1. Apparatus

A TPW II (Zymark, Hopkinton, USA), software version 2.0, equipped with custom-made amber reaction vessel and amber test tubes was used. Filters used were Zymark/Millipore Glassfiber APFB 1.0 μm, hydrophilic PTFE 0.45 μm two layer filters. The HPLC system consisted of a Jasco PU-1580 pump (Jasco, Tokyo, Japan), a Rheodyne sample injector with 20 μl loop integrated in the TPW II, a LiChrospher 60, RP-select B, 250×4.6 mm column (Grom, Herrenberg, Germany), and a Jasco UV-1575 detector. This set up used a single-wavelength UV-detector with the following timed wavelength sensitivity program in order to increase sensitivity, but a photodiode-array detector would be equally suitable: 0–6.5 min, 264 nm, range 0.16 (detection of nicotinic acid, nicotinamide and riboflavin-5′-phosphate); 6.5–10 min, 280 nm, range 0.08 (detection of pyridoxine); 10–17 min, 280 nm, range 0.04

(detection of riboflavin and folic acid); 17–25 min, 245 nm, range 0.04 (detection of thiamine). Data was processed using a VAX Multichrom V2.20h Chromatography Data System (LabSystems, Chesire, UK). Density of reagents has been determined using a DMA 38 densitometer (Anton Paar, Graz, Austria).

2.2. Chemicals and reagents

If not otherwise stated, all chemicals were of analytical grade quality and supplied by Fluka (Buchs, Switzerland). Millipore-Q water was used for all aqueous solutions.

Acidic extraction solution: water–acetonitrile–acetic acid (94:5:1, v/v/v), $\sigma_{23^\circ C} = 0.9934$ g cm^{-3}.

Alkaline extraction solution: ammonium hydroxide solution 0.3%, $\sigma_{23^\circ C} = 0.9960$ g cm^{-3}.

Mobile phase: phosphate buffer–methanol (4:1, v/v) (pH* 2.8).

Phosphate buffer: potassium dihydrogen phosphate (10.9 mM), 1-hexanesulfonic acid sodium salt monohydrate (5 mM), 1-octanesulfonic acid sodium salt monohydrate (4 mM) and triethyl amine (36 mM) in water, pH adjusted to 2.4 with ortho-phosphoric acid 85%.

Vitamin standard substances were certified and supplied by Roche Vitamins Ltd, (Basel, Switzerland) or Dr Ehrenstorfer GmbH (Augsburg, Germany). Standard solution was prepared with acidic extraction solution containing approximately the following vitamin concentrations: folic acid 5 μg ml^{-1}, nicotinamide 40 μg ml^{-1}, nicotinic acid 40 μg ml^{-1}, pyridoxine (as pyridoxine hydrochloride) 50 μg ml^{-1}, riboflavin 12 μg ml^{-1}, riboflavin-5′-phosphate (as riboflavin-5′-phosphate sodium) 40 μg ml^{-1}, and thiamine (as thiamine hydrochloride) 40 μg ml^{-1}. After addition of 5−10 mg ascorbic acid per 100 ml, the solution was stored protected from light and has been used for 48 h. Folic acid was first dissolved in ammonium hydroxide solution 2%, and was then diluted with the same volume acidic extraction solution.

2.3. Tablet samples

Three different types of multivitamin tablets (samples A–C), one film-coated and two effervescent tablet types, have been used for method development and validation. *Sample A:* film-coated tablets, selected ingredients per tablet five fat-soluble and nine water-soluble vitamins, eight minerals, 170 mg MgO and 550 mg $Ca_3(PO_4)_2$, total weight 1.6 g. *Samples B and C:* effervescent tablets, selected ingredients per tablet four fat-soluble and nine water-soluble vitamins, seven minerals, 1.5 g citric acid and 0.8 g Na_2CO_3, total weight 4.0 g. Sample B contained riboflavin, sample C riboflavin-5′-phosphate. Tablet samples were supplied by the formulation department of Roche Vitamins Ltd.

2.4. TPW II set up and sample processing

Tablets were put into sample tubes, larger tablets (samples B and C) were broken to fit. The following operations were carried out automatically by the TPW II. The first sample was transferred into the homogenisation vessel and homogenised after addition of acidic extraction solution (100 ml) or alkaline extraction solution (100 ml, determination of folic acid) using three 30 s pulses at 7000 rpm. During homogenisation, the homogeniser probe cycled several times from 1 cm above to the bottom of the vessel and back. After a soak time of 2 min, additional acidic extraction solution (50 ml for the determination of folic acid, 100 ml for the determination of all other vitamins) was added employing a rim wash of the vessel, and a second homogenisation by two 30 s pulses at 5000 rpm was carried out. After a settle time of 30 s, 5 ml of the resulting extract were filtered into an amber sample tube, and 20 μl of the filtrate were injected onto the HPLC system. Vessel and lines were flushed consecutively with water (300 ml) and acidic extraction solution (100 ml) prior to analysis of the next tablet. The whole process took about 20 min. Chromatographic separations were carried out at 30 °C using a mixture of phosphate buffer–methanol (4:1, v/v) (pH* 2.8) as mobile phase (2.2.) at a flow rate of

1 ml min^{-1}, resulting in a pressure of approximately 15 MPa.

2.5. Validation experiments

2.5.1. Linearity of the chromatographic system

Vitamin standard solutions of different concentrations were each analysed in duplicate. The values for the upper limits in Table 1 represent the highest concentrations tested. The detection limit was determined at a signal to noise ratio of approximately 3:1.

2.5.2. Linearity of the methods

Linearity of the entire determination was investigated by analysing increasing amounts of powdered film-coated tablets (sample A), and calculating the correlation between sample weight used for analysis and vitamin content found. For folic acid 7 different sample weights have been analysed ($n = 3$), for all other vitamins 11 different sample weights were analysed ($n = 3$). Tablets were milled prior to analysis in order to exclude varying homogeneity resulting from formulation. Except for the upper limits given for folic acid and nicotinamide, the values in Table 2 represent the ranges investigated.

2.5.3. Accuracy by recovery

Powder of film-coated tablets (sample A) and effervescent tablets (sample B) were spiked with vitamins at different levels (15–18 determinations over the range investigated). Tablets were milled prior to analysis in order to exclude varying homogeneity resulting from formulation. Vitamins were added to the tablet powder as solids, except for folic acid addition to sample B. In this case, the respective vitamin amount was dissolved in acidic extraction solution, and manually added to the TPW II homogenisation vessel directly after automatic sample transfer. *Sample A:* 0.750 g tablet powder (weight of one tablet 1.6 g) were used for each recovery experiment. Eighteen spiked samples (folic acid 15 samples) and 3 samples without vitamin addition were analysed. *Sample B:* 1.500 g tablet powder (weight of one tablet 4.0 g) were used for each spiking experiment. 0.94 mg citric acid and 0.5 g sodium carbonate were added to each sample in order to simulate the citric acid and sodium carbonate content of a complete tablet. Fifteen spiked samples and 3 samples without vitamin addition were analysed (Tables 3 and 4).

3. Results and discussion

3.1. Method development

During method development it became obvious that different work up procedures have to be used for determination of folic acid, and the other six compounds, respectively, due to differences in solubility and stability. While folic acid is best extracted at an alkaline pH, most of the remaining vitamins are more soluble under acidic conditions, and thiamine was even found to decompose in the alkaline extract. This resulted in the use of two different extraction solutions for the first homogenisation step, acidic extraction solution for nicotinamide, nicotinic acid, pyridoxine, riboflavin, riboflavin-5′-phosphate and thiamine, and alkaline extraction solution for folic acid. Some

Table 1
Linearity of the chromatographic system and detection limits

Vitamin	Linear range (μg ml^{-1})	R^2	Intercept	Slope	Detection limit (ng ml^{-1})
Folic acid	0.1–7.9	0.9993	−1251	17 035	25
Nicotinamide	0.1–128.7	1	−382	2794	50
Nicotinic acid	0.5–170.1	1	187	2827	50
Pyridoxine	0.6–60.7	1	33	4102	50
Riboflavin	0.03–42.5	1	−171	18 618	25
Riboflavin-5′-phosphate	0.5–41.8	1	−42	3731	50
Thiamine	0.5–42.6	0.9999	−3071	12 580	100

Table 2
Linearity of the methods for selected vitamins in film-coated tablets (sample A): correlation between vitamin content analysed and weight of tablet powder used for analysis

Vitamin	Linear range (mg vitamin per sample)[a]	Corresponding sample weight range (g)[a]	R^2	Intercept	Slope
Folic acid	0.1–0.8	0.5–3.0	0.9995	−0.003	0.26
Nicotinamide	1.2–54.7	0.1–4.5	0.9995	0.417	12.18
Pyridoxine	0.2–12.2	0.1–7.5	0.9999	0.014	1.62
Riboflavin	0.1–7.2	0.1–7.5	0.9999	0.015	0.97
Thiamine	0.1–7.4	0.1–7.5	0.9999	−0.034	0.99

[a] E.g., a sample content of 0.1 mg folic acid corresponds to 0.5 g tablet powder used for analysis; values rounded.

Table 3
Accuracy by recovery investigated using powder of film-coated tablets (sample A)

Vitamin	Vitamin added (mg)[a]	Mean Recovery (%)	RSD (%)
Folic acid	0.3–1.0	97.6	10.5
Nicotinamide	9.5–39.0	96.3	1.5
Nicotinic acid	5.5–33.0	101.7	1.6
Pyridoxine	1.25–10.0	100.6	2.4
Riboflavin	0.7–5.5	96.2	2.7
Thiamine	0.7–6.0	99.8	3.7

[a] For each vitamin 18 determinations covering the range given were performed.

tablets, most notably effervescent tablets, can contain high amounts of organic acids, e.g. citric acid, which have a major influence on the pH during extraction of folic acid. Final extracts of film-coated tablets (sample A) for folic acid analysis usually had a pH of about 8.5, whereas the pH of extracts of effervescent tablets (samples B and C) for folic acid analysis was around 5.3. An influence of this acidic pH on extraction efficacy for folic acid has been investigated by control experiments in which the addition of acidic extraction solution after the first homogenisation step was replaced by addition of the same amount alkaline extraction solution, resulting in a final pH of 8.9. However, the folic acid amount found (0.44 ± 0.02 mg, $n = 6$) was not significantly different from the result of the original method. Thus, it was concluded that a variation of the pH of the final extract in the range of 5–9 has no relevant influence on the determination of folic acid.

Optimisation of sample homogenisation was adapted to the most important types of tablets, in our case film-coated, effervescent and chewable tablets. The latter two tablet types readily disintegrate and dissolve in an aqueous environment. Thus, optimisation was focused on film-coated tablets (sample A). In order to allow a fast analysis, the overall length of the sample preparation procedure, including the clean-up steps, should not exceed the duration of the chromatographic determination (20 min). This would allow the HPLC analysis of the sample

Table 4
Accuracy by recovery investigated using powder of effervescent tablets (sample B)

Vitamin	Vitamin added (mg)[a]	Mean recovery (%)	RSD (%)
Folic acid	0.15–0.75	96.9	2.7
Nicotinamide	7.0–40.0	95.5	1.9
Nicotinic acid	5.0–30.0	101.1	0.7
Pyridoxine	0.8–10.0	96.6	3.3
Riboflavin	0.75–5.0	95.2	2.1
Riboflavin-5′-phosphate	0.6–6.0	103.9	4.0
Thiamine	0.6–6.0	100.5	2.7

[a] For each vitamin 15–18 determinations covering the range given were performed.

extracts immediately after their filtration. Optimal results were obtained using two homogenisation steps with a soak time in between. The first step with cycling of the homogeniser probe cracked the tablet. During the following soak time eventually remaining pieces disintegrated, and the extraction process was completed during the second homogenisation step. In comparison with a manual sample work up, this automated procedure reduced laboratory-working time for the analyst by approximately 80%. A possible carry-over of vitamins has been investigated by analysing blank samples directly after tablet samples. It was estimated to be <0.03 mg for nicotinamide and <0.01 mg for all other vitamins (values below the linear range of the chromatographic system).

The chromatographic system had already been established for a manual method, and could be transferred without modifications. It had been applied in routine analysis in our department for several years, and was found to be very reliable and robust. A separation of all seven compounds investigated could be achieved in about 20 min (Fig. 1). No other ingredients of common multivitamin tablets interfered with the vitamin peaks.

3.2. Method validation

The two new automated methods have been validated with respect to linearity and limit of detection of the chromatographic system, linearity of the entire determination correlating sample weight and vitamin content, accuracy by recovery and precision.

3.2.1. Linearity of the methods

The linearity of the chromatographic system and the detection limits for the vitamins were determined by analysis of standard solutions (Table 1). For every vitamin, the linear range determined was found to cover the concentration range expected during routine analysis of multivitamin tablets. The linearity of the entire determination, correlating sample weight and vitamin

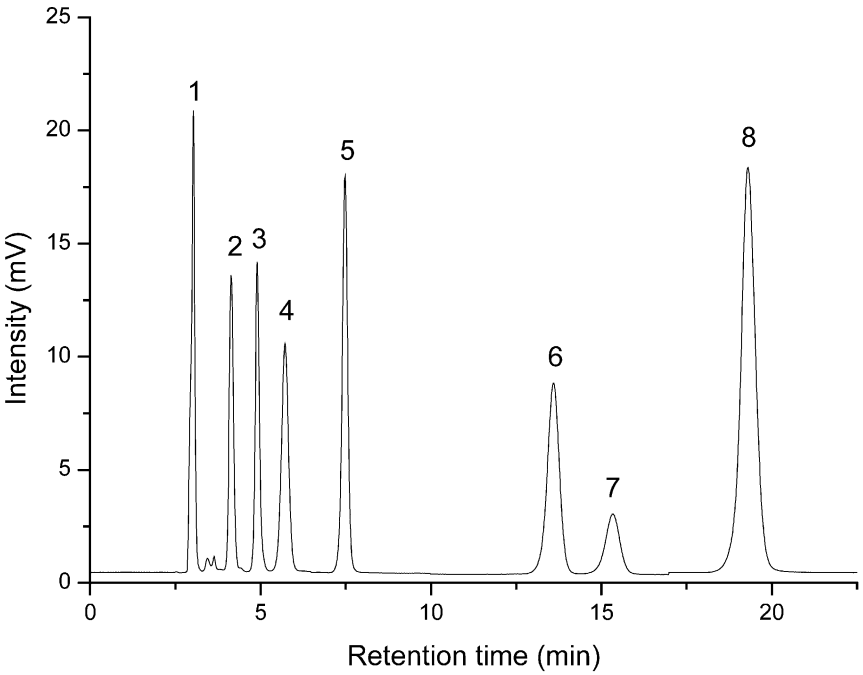

Fig. 1. Chromatogram of standard solution: 1: ascorbic acid (not quantified), 2: nicotinic acid (878 ng), 3: nicotinamide (828 ng), 4: riboflavin-5′-phosphate (693 ng), 5: pyridoxine (970 ng), 6: riboflavin (234 ng), 7: folic acid (94 ng), 8: thiamine (884 ng).

content analysed, has been investigated for selected compounds using powder of film-coated tablets (sample A, Table 2). It could be shown that the correlation is linear over a wide range covering all common tablet weights and vitamin contents. Thus, possible volume errors resulting from different amounts of tablet material added to the homogenisation vessel are negligible.

3.2.2. Accuracy of the methods

The accuracy of the methods has been investigated by spiking experiments using powder of film-coated tablets (sample A, Table 3) and effervescent tablets (sample B, Table 4). In order to enhance the precision with which the recovery of small vitamin additions could be determined, the tablet powder weight used for analysis equalled less than 50% of the weight of an entire tablet. Mean recoveries were in the range of 95.2–103.9% with corresponding relative standard deviations $\leqslant 4\%$. Only the recovery of folic acid from powder of film-coated tablets (sample A) varied by 10.5%. Obviously, this was due to the small amounts added which could not be weighed with sufficient precision. Recovery from powder of effervescent tablets (sample B), when folic acid was added as pipetted aliquots from a stock solution, varied only by 2.7%.

3.2.3. Precision of the methods

The precision of the new automated methods (Table 5) has been investigated using film-coated tablets (sample A) and effervescent tablets (sample C). No tablets containing nicotinic acid were available for analysis. This vitamin is only seldomly added to tablets in place of nicotinamide. With the exception of the results for vitamin B_2 (riboflavin and riboflavin-5′-phosphate), amounts analysed were higher than the label claim. This is in good agreement with the fact that vitamin amounts added by the manufactures are usually slightly higher than the label claim, e.g., accounting for a decomposition of vitamins during tablet storage. The precision of the methods is characterised by relative standard deviations ranging from 0.7% (riboflavin in film-coated tablets) to 5.4% (folic acid in film-coated tablets). Considering an inhomogeneity of the vitamin content of unknown magnitude due to tablet formulation, the precision of the methods is even greater.

3.3. Conclusion

The determination of selected water-soluble vitamins in tablets has been successfully automated. The new methods were shown to be suitable for application in routine analysis. Benefits of the automation include analysis of the sample extract immediately after extraction, and

Table 5
Quantitation of vitamins in multivitamin tablets ($n = 9$)

Vitamin	Label claim (mg)	Vitamin analysed (mg)	RSD (%)
Film-coated tablets (sample A)			
Folic acid	0.40	0.42	5.4
Nicotinamide	20.0	20.81	2.1
Pyridoxine	2.43	2.62	1.6
Riboflavin	1.70	1.62	0.7
Thiamine	1.55	1.58	1.1
Effervescent tablets (sample C)			
Folic acid	0.40	0.44	3.7
Nicotinamide	18.0	19.07	2.6
Pyridoxine	2.00	2.11	3.3
Riboflavin-5′-phosphate	2.00	1.94	4.2
Thiamine	1.44	1.62	0.9

a significant reduction of manual laboratory working time by approximately 80% with corresponding cost savings.

Acknowledgements

We wish to thank Dr Christian Fizet, Roche Vitamins Ltd, Basel, for continuous strong support of the project, and Dr C. Grammes for supplying sample tablets.

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