High-performance Liquid Chromatography of Water-soluble Vitamins

Part 3.* Simultaneous Determination of Vitamins B_1, B_2, B_6, B_{12} and C, Nicotinamide and Folic Acid in Capsule Preparations by Ion-pair Reversed-phase High-performance Liquid Chromatography

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An ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method for the separation and simultaneous determination of thiamine HCl (vitamin B_1), riboflavin (vitamin B_2), nicotinamide, pyridoxine HCl (vitamin B_6), cyanocobalamin (vitamin B_{12}), ascorbic acid (vitamin C) and folic acid in capsule preparations is described. The above seven active substances were extracted from the preparations within 5 min using an electronically controlled extraction apparatus. Ion-pair reversed-phase HPLC was carried out on LiChrosorb RP-18 using methanol - water - concentrated phosphoric acid - octanesulphonic acid as the eluent. All seven active ingredients were separated in less than 4 min. The detection limits were 1–2 ng for all vitamins; however, amounts of 5–10 ng were required for the quantitative HPLC determination of the vitamins. The proposed HPLC method is suitable for the determination of these active ingredients in pharmaceutical preparations such as multivitamin capsules and has a coefficient of variation between 1.1 and 3.2%. The ease of extraction, accuracy and sensitivity of the method and the time required for analysis are emphasised.

Keywords: *High-performance liquid chromatography; ion-pair reversed-phase high-performance liquid chromatography; water-soluble vitamins; multivitamin capsules*

The determination of vitamin C and the B-group of vitamins, including B_1, B_2, B_6 and B_{12}, has been reported in Parts 1 and 2 of this series.[1,2] In this paper the development of an ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method for the separation and determination of the above five vitamins plus nicotinamide and folic acid is described. Many methods for the determination of water-soluble vitamins using different physical, chemical and biological methods have been published,[3–5] and the advantages and disadvantages of these methods have been discussed.[1,2] The HPLC separation and determination of water-soluble vitamins in vitamin and multivitamin preparations and in biological fluids is now well established.[6–16] However, the HPLC determination of the vitamins in pharmaceutical preparations and in biological materials is often complicated by the large excess of inactive ingredients, and by the low concentrations of the vitamins in biological fluids (nanogram range). This paper describes an ion-pair reversed-phase HPLC method for the separation and simultaneous determination of vitamins, B_1, B_2, B_6, B_{12} and C, nicotanamide and folic acid in the nanogram range.

Experimental

Apparatus and Conditions

Electronically controlled extraction apparatus

This apparatus (W. Krannich, Göttingen, FRG) is composed of the glass set for the simultaneous extraction of three samples described previously.[1] The good reproducibility of the extraction of many active substances from preparations using this apparatus has been demonstrated.[1,2,17,18]

High-performance liquid chromatograph

A Knauer compact liquid chromatograph with a spectrophotometric detector and a syringe-loaded loop injection valve (internal volume 50 µl) were used. A 250 × 4 mm i.d. column of Vertex LiChrosorb RP-18 (10 µm) was used for the reversed-phase chromatography. The chromatograms were recorded on a Knauer recorder with a 100 mV span set at a chart speed of 10 mm min^{-1}. The following conditions were maintained: detection wavelength = 254 nm; pressure = 140 bar; flow-rate = 1.5 ml min^{-1}; room temperature = 22–25 °C; injection volume = 5–25 µl; and detector sensitivity = 1.28 a.u.f.s. The eluent consisted of methanol - water - 85% phosphoric acid (55 + 45 + 1) plus 65 mg of octanesulphonic acid in 101 ml.

Materials and Reagents

The six vitamin and folic acid standards were obtained from Pfizer Pharmaceutical (Cairo, Egypt). The following capsules were specially prepared for this study to contain exactly the labelled amounts.

Preparation A. Multi-vitamin capsules containing 3 mg of vitamin B_1, 2 mg of vitamin B_2, 20 mg of nicotinamide, 10 mg of vitamin B_6, 2 µg of vitamin B_{12} and 50 mg of vitamin C per capsule.

Preparation B. Multi-vitamin capsules containing 4 mg of vitamin B_1, 4 mg of vitamin B_2, 25 mg of nicotinamide, 0.5 mg of vitamin B_6, 1.5 µg of vitamin B_{12}, 60 mg of vitamin C and 1 mg of folic acid.

The methanol used for the chromatographic separation was purchased from E. Merck (Darmstadt, FRG). The water used for the chromatography was prepared by glass distillation and filtration with a membrane filter. All other chemicals were of the best grade commercially available.

Procedure

Preparation of standard solutions (pure substances)

A 10 mg mass of each standard substance was weighed to 0.01 mg and dissolved in 100 ml of methanol - water (80 + 20). A 1 ml aliquot of each solution was diluted such that 1 ml contained 3–4 µg of vitamin B_1, 2–4 µg of vitamin B_2, 20–25 µg

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of nicotinamide, 5–10 μg of vitamin B_6, 1.5–2 μg of vitamin B_{12}, 50–60 μg of vitamin C and 1–2 μg of folic acid.

Preparation of standard mixture solution

A solution of the seven active ingredients was such that 1 ml of the solution contained 5 μg each of vitamin B_1, B_2 and C, 10 μg each of nicotinamide, vitamin B_6 and folic acid and 20 μg of vitamin B_{12}.

Preparation of samples (pharmaceutical preparations)

The contents of one capsule of each preparation were pulverised and transferred accurately into the fully automated extraction apparatus and extracted three times with a total volume of 20 ml of methanol - water (8 + 2). A 10 ml aliquot of this solution was concentrated such that 1 ml of the solution contained 0.75–1 μg of vitamin B_{12}. The other 10 ml of the extracted solution was diluted such that 1 ml of the solution contained 3–4 of μg vitamin B_1, 2–4 μg of vitamin B_2, 20–25 μg of nicotinamide, 0.5–10 μg of vitamin B_6, 50–60 μg of vitamin C and 1 μg of folic acid, depending on the vitamin content of the investigated preparation.

Calculation

The percentage recovery of the vitamins in the investigated preparations can be calculated from calibration graphs determined under the same conditions or by the external standard method according to the following equation:

$$\% \text{ of nominal content} = \frac{CR\ Ps}{Cs\ PR} \times 100$$

where CR is the mass of reference compound used (μg or ng), Ps is the peak area of the active substance (cm^2), Cs is the mass of the active substance present (μg or ng, calculated from the stated amount in the preparation), and PR is the peak area for the reference compound (cm^2).

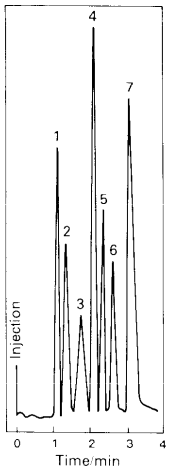

Fig. 1. Typical high-performance liquid chromatogram of the seven vitamins separated from the standard mixture solution. 1, Ascorbic acid (vitamin C), 0.125 μg; 2, cyanocobalamin (vitamin B_{12}), 0.5 μg; 3, riboflavin (vitamin B_2), 0.125 μg; 4, nicotinamide, 0.25 μg; 5, folic acid, 0.25 μg; 6, pyridoxine HCl (vitamin B_6), 0.25 μg; and 7, thiamine HCl (vitamin B_1), 0.125 μg. Conditions: column, Knauer LiChrosorb RP-18, 10 μm, 250 mm × 4 mm; pressure, 140 bar; flow-rate, 1.5 ml min^{-1}; detector, UV, 254 nm; sensitivity, 1.28 a.u.f.s; eluent, methanol - water - 85% phosphoric acid (55 + 45 + 1) plus 65 mg of octanesulphonic acid in 101 ml; volume injected, 25 μl

Results

Fig. 1 shows the HPLC separation pattern of the seven vitamins obtained using the standard mixture solution. Fig. 2 shows the separation of the active substances from Preparation B. Table 1 gives the retention times of the investigated substances and Table 2 illustrates the reproducibility of the HPLC determination of the seven compounds. The coefficients of variation based on six injections of standard and sample solutions are summarised in this table. Table 3 presents the results of the HPLC determination of the drugs in the two investigated capsule preparations.

Discussion

Table 1 shows that the HPLC technique used permits the simultaneous separation of vitamins B_1, B_2, B_6, B_{12} and C, nicotinamide and folic acid in less than 4 min. Tables 2 and 3 show all that the described extraction and HPLC methods are

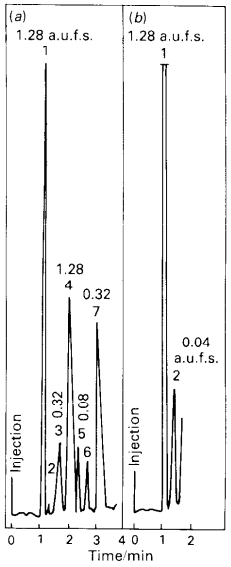

Fig. 2 (*a*) Typical high-performance liquid chromatogram of the separation pattern of the seven drugs from capsules (Preparation B). Conditions: as in Fig. 1, except sensitivity of detector (1.28 a.u.f.s. for 1 and 4; 0.32 a.u.f.s. for 2, 3 and 7; 0.08 a.u.f.s. for 5 and 6). Injected volume, 10 μl. 1, Vitamin C, 600 ng; 2, vitamin B_{12}, <1 ng; 3, vitamin B_2, 40 ng; 4, nicotinamide, 250 ng; 5, folic acid, 10 ng; 6, vitamin B_6, 5 ng; and 7, vitamin B_1, 40 ng. (*b*) Separation of vitamin B_{12} from the concentrated solution of preparation B. Volume injected = 100 μl (100 ng). Conditions: as in Fig. 2(*a*) except sensitivity of the detector = 0.04 a.u.f.s. 1, Vitamin C; and 2, vitamin B_{12}, 100 ng

Table 1. Retention times of the seven investigated vitamins separated by HPLC

Vitamin	Retention time/min	Capacity factor (k')
Vitamin C	1–1.1	6.0
Vitamin B_{12}	1.2–1.4	7.7
Vitamin B_2	1.5–1.8	10.0
Nicotinamide	2–2.1	12.7
Folic acid	2.2–2.4	14.3
Vitamin B_6	2.5–2.7	16.3
Vitamin B_1	3–3.2	19.7

Table 2. Reproducibility of HPLC determination of the seven vitamins in standard preparations. Detector sensitivity, 1.28 a.u.f.s. The results given are the means of six injections

Parameter	Vitamin						
	B_1	B_2	Nicotinamide	B_6	B_{12}	C	Folic acid
Amount injected/ng	125	125	250	250	500	125	250
Arithmetic mean ($\bar{X}$) of peak area/cm²	1.65	0.51	1.30	0.52	0.60	0.71	0.53
Standard deviation (s.d.) of single values/cm²	0.018	0.012	0.015	0.015	0.12	0.01	0.014
Coefficient of variation, %	1.1	2.4	1.2	2.9	2	1.4	2.65

Table 3. Results obtained for the HPLC determination of the vitamins in the capsule preparations A and B. The results are the means of six determinations (six extracts)

Preparation		Mass of vitamins present in each capsule/mg	Amount of vitamins found		
			$\bar{X}$	S.d./mg	C.v., %
A	B_1	3	3.15	0.07	2.2
	B_2	2	2.06	0.05	2.4
	Nicotinamide	20	20.4	0.45	2.2
	B_6	10	9.75	0.25	2.6
	B_{12}*	2 μg	1.95 μg	0.04 μg	2.1
	C	50	50.6	0.82	1.6
B	B_1	4	4.2	0.085	2.0
	B_2	4	4.2	0.092	2.2
	Nicotinamide	25	25.7	0.60	2.3
	B_6	0.5	0.55	0.007	1.3
	B_{12}*	1.5 μg	1.54 μg	0.03 μg	1.9
	C	60	61.5	1.15	1.9
	Folic acid	1	1.1	0.035	3.2

reproducible and can be used for the extraction and determination of the seven investigated vitamins in multi-vitamin preparations with a maximum coefficient of variation of 3.2%. The detector sensitivity of 1.28 a.u.f.s used in the determination of the vitamins in standard solutions (Table 2) indicates that by using a higher detector sensitivity, *e.g.*, 0.08 or 0.04 a.u.f.s., about 5–10 ng of the vitamins could be determined. The determination of 5 ng of vitamin B_6 and 10 ng of folic acid at a detector sensitivity of 0.08 a.u.f.s is possible as is seen in the analysis of Preparation B (Fig. 2). Only when the described conditions with respect to column, eluent, concentration of the solution and sensitivity of the detector are strictly adhered to and the eluent is freshly prepared can the seven vitamins be separated. A major problem is the separation and determination of 1.5–2 μg of vitamin B_{12} in the presence of 4 mg of vitamin B_1, 4 mg of B_2, 25 mg of B_3 and 10 mg of B_6. Because of the large difference in the concentration of the B_{12} compared with the other vitamins, especially C and B_3, the problem of the determination of vitamin B_{12} in the presence of the other vitamins cannot be resolved by recording the vitamin B_{12} peak at a higher detector sensitivity than the other vitamins. This particular problem was overcome in two ways. The first method involved the determination of vitamin B_{12} in a concentrated solution which contained *ca.* 1 μg ml^{-1} of vitamin B_{12} (*cf. Preparation of samples*). In this instance the peaks of the other vitamins, including vitamin C, were recorded at a lower detector sensitivity than that used for vitamin B_{12}. The instrument used allows for this rapid change of sensitivity. The second method of determining vitamin B_{12} in the presence of higher concentrations of other vitamins involves the addition of about 20 ng of vitamin B_{12} per injection volume and the evaluation on the vitamin B_{12} on the basis of the resulting differential calibration line.[19]

Conclusions

This paper has shown the advantages of the combination of the extraction apparatus and an HPLC method with respect to analysis time and sensitivity. The seven vitamins can be simultaneously determined in pharmaceutical preparations in about 10 min. The extraction apparatus allows the simultaneous extraction of the vitamins from three samples in about 5 min. The HPLC method is very sensitive and can be used for the determination of 5–10 ng of the vitamins with a maximum coefficient of variation of 3.2% in the injected amount of the vitamins present in the investigated preparations.

References

1. Amin, M., and Reusch, J., *Fresenius Z. Anal. Chem.*, in the press.
2. Amin, M., and Reusch, J., *J. Chromatogr.*, in the press.
3. Beltagy, Y. A., and Soliman, R., *Z. Pharm. Pharmakother. Lab. Diagn.*, 1979, **118**, 1285.
4. Elenaey, E., and Soliman, R., *Talanta*, 1979, **26**, 1164.
5. Vasileva-Aleksandrova, P., and Neicheva, A., *Microchim. Acta*, 1979, **II**, 337.
6. Williams, R. C., Baker, D. R., and Schidt, J. A., *J. Chromatogr. Sci.*, 1973, **11**, 618.
7. Wittmer, D., and Haney, W. G., *J. Pharm. Sci.*, 1974, **63**, 588.
8. Knox, J. H., and Pryde, A. J., *J. Chromatogr.*, 1975, **112**, 171.
9. Callmer, K., and Davies, L., *Chromatographia*, 1974, **7**, 644.
10. Unger, K., and Nyamah, D., *Chromatographia*, 1974, **7**, 63.
11. Sood, S. P., Sartori, L. E., Wittmer, D. P., and Haney, W. G., *Anal. Chem.*, 1976, **48**, 796.
12. Wagner, E. S., Lindley, B., and Coffin, R. D., *J. Chromatogr. Biomed. Appl.*, 1979, **5**, 225.
13. Iwata, T., Yamaguchi, M., Hara, S., Nakamura, M., and Ohkura, Y., *J. Chromatogr.*, 1985, **344**, 351.
14. Lequeu, B., Guilland, J. C., and Klepping, J., *J. Anal. Biochem.*, 1985, **149**, 296.
15. Lui, A., Lumeng, L., and Li, T. K., *Am. J. Clin. Nutr.*, 1985, **41**, 1236.
16. Watada, A. E., and Tran, T. T., *J. Liq. Chromatogr.*, 1985, **8**, 1615.
17. Amin, M., Korbakis, Z., and Petrick, D., *Fresenius Z. Anal. Chem.*, 1976, **279**, 283.
18. Amin, M., *in* Bertsch, W., Hara, S., Kaiser, R., and Zlatkis, A., *Editors*, "Instrumental HPTLC," Hüthig, Heidelberg, 1980, pp. 9–37.
19. Amin, M., Sepp, W., *J. Chromatogr.*, 1976, **118**, 225.

NOTE—References 1 and 2 are to Parts 1 and 2 of this series.

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