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Note

High-performance liquid chromatography of water-soluble vitamins

II. Simultaneous determinations of vitamins B_1, B_2, B_6 and B_{12} in pharmaceutical preparations

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Vitamins of the B group including B_1, B_2, B_6 and B_{12} are classified as water-soluble vitamins. They are a complex group of substances whose chemical structures are not related. Numerous publications have appeared on the quantitation of single vitamins of this group using widely differing physical, chemical and biological methods. These methods can be used for production control both of the pure vitamins and of the vitamins in pharmaceutical preparations, when analysed individually. However, they are unsuitable for stability studies of these vitamins in drug formulations and for the determinations of quantities in the nanogram range, as in pharmacokinetic and bioavailability studies of these vitamins.

High-performance liquid chromatography (HPLC) provides a rapid, sensitive and accurate method for vitamin determination[1]. The HPLC separation and determination of vitamins of the B group is well documented[2–11]. Other methods are microbiological assay[12] and a chemical method[13]. None of the described HPLC or non-chromatographic methods satisfies the requirements for quality control and stability studies, of multivitamin preparations. We, therefore, wanted to develop a simple, quick, specific and sensitive HPLC method for the separation and determination of all four B vitamins in the presence of each other, enabling vitamins to be investigated in the nanogram range as is required for pharmacokinetic and bioavailability studies.

EXPERIMENTAL

Electronically controlled extraction apparatus

This apparatus (W. Krannich K. G., Göttingen, F.R.G.) consists of glassware for the simultaneous extraction of three samples, a thermostat, a cryostat, a computer and a cabinet which houses the valves, a nitrogen pump and a vacuum pump (Fig. 1). All sequences of operation, such as addition of the extracting agent, stirring, warming, cooling and filtering, are fully automated by means of an electronic control

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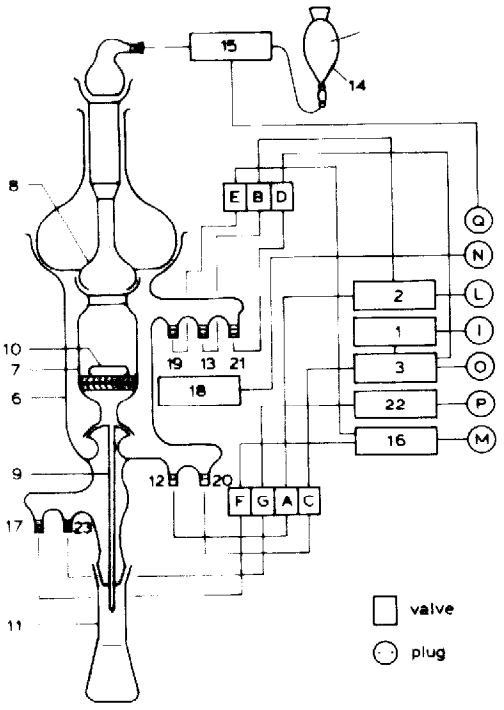

Fig. 1. Schematic diagram of the extraction apparatus. A–F = valves; I–Q = sockets (remote controlled); 1 = cryostat; 2 = thermostat; 3 = cooling vessel; 6 = main body; 7 = exchangeable filter; 8 = spherical ground joint; 9 = exit pipe; 10 = stirring device; 11 = measuring cylinder; 12 = juncture (thermost. fluid); 13 = juncture (air); 14 = solvent vessel; 15 = proportioning pump; 16 = air pump; 17 = juncture (air); 18 = stirrer mechanism; 19 = juncture (air); 20 = juncture (thermostat. fluid); 21 = juncture (thermost. fluid); 22 = vacuum pump; 23 = juncture (air/vacuum).

system. The good reproducibility of the extraction of many active substances from preparations has been demonstrated[14,15].

HPLC

A Knauer compact liquid chromatograph with a spectrophotometric detector, a stainless-steel column of Vertex LiChrosorb RP-18, 5 μm, 25 cm × 4 mm I.D., and a Knauer recorder were used. The following conditions were maintained: detection wavelength, 254 nm; pressure, 180 bar; flow-rate, 2 ml/min; room temperature, 22–25°C; injection volume, 5–25 μl; chart speed = 1 cm/min and detection sensitivity, 1.28 a.u.f.s. The eluents were methanol–water (80:20) and (50:50). These mobile phases were optimized in our laboratory.

Materials and reagents

The four vitamin standards were obtained from Pfizer Pharmaceutical Co. (Cairo, Egypt). The following pharmaceutical preparations were obtained locally: (1) tablets with 10 mg vitamin B_1 and 10 mg vitamin B_2 per tablet; (2) dragées with 15 mg vitamin B_1, 15 mg vitamin B_2, 5 mg vitamin B_6 and 5 μg vitamin B_{12} per dragee;

(3) capsules with 4 mg vitamin B_1, 4 mg vitamin B_2, 0.5 mg vitamin B_6 and 1.5 μg vitamin B_{12} per capsule.

Methanol used for the chromatography was obtained from E. Merck (Darmstadt, F.R.G.). Water used for the chromatography was glass-distilled and filtered with a membrane filter. All other chemicals were of the best grade commercially available.

Procedure

Preparation of standard solutions (pure substances). The vitamins were dissolved in such a way that 1 ml of the methanol–water solution contained 50 μg vitamin B_1, 50 μg B_2, 15 μg B_6 and 5 μg B_{12}.

Preparation of samples (pharmaceutical preparations). Either one tablet, drageé or the content of one capsule was pulverized, transferred accurately to the fully automated extraction apparatus and extracted three times with a total volume of 30 ml methanol water (1:1). The extracts were concentrated in such a way that 1 ml of solution contained 1.5–5 μg vitamin B_{12} depending on the B_{12} content in the preparations investigated. These stock solutions were diluted for determination of vitamins B_1, B_2 and B_6 in such a way that 10 ml of solution contained 0.5 mg B_1, 0.5 mg B_2 and 62.5–167 μg B_6 depending on the B_6 content in the preparation investigated.

Calculation

The percentage recovery of vitamins can be calculated by the calibration line method or by the external standard method according to

$$\text{Recovery (\%)} = \frac{C_R \cdot P_s}{C_s \cdot P_R} \cdot 100$$

where C_R is the amount (μg or ng) of reference compound applied, P_s is the peak area (cm^2) of the active substance, C_s is the amount (μg or ng) of the active substance applied (calculated from the amount stated in the preparation) and P_R is the peak area (cm^2) of the reference.

Fig. 2. Typical chromatogram of the separation of the four vitamins from capsules. Conditions: column, Vertex LiChrosorb RP-18, 5 μm, 25 cm × 4 mm I.D.; mobile phase, methanol–water (50:50); flow-rate, 2 ml/min; pressure, 180 bar; detector, UV 254 nm; sensitivity, 1.28 a.u.f.s. Peaks 1–4 are vitamins B_1, B_6, B_{12} and B_2, respectively.

TABLE I

REPRODUCIBILITY OF HPLC DETERMINATION OF VITAMINS B_1, B_2, B_6 AND B_{12} CARRIED OUT WITH PURE SUBSTANCES

Detector sensitivity 1.28 a.u.f.s.; the results given are the means from six injections.

	Vitamin			
	B_1	B_2	B_6	B_{12}
Amount injected (ng)	50	250	250	250
Arithmetic mean, $\bar{x}$ (peak area in cm^2)	0.28	0.65	0.15	0.12
Standard deviation of a single value, S.D. (cm^2)	0.006	0.015	0.004	0.005
Coefficient of variation, C.V. (%)	2.1	2.3	2.7	4.2

RESULTS

Fig. 2 shows the separation pattern of the four vitamins from capsules, *e.g.*, preparation 3. Table I illustrates the reproducibility of the HPLC determination of the standard preparation of the vitamins. Table II gives the retention times of the four vitamins with the two used eluents, methanol–water (80:20 and 50:50). Table III presents the results of HPLC determination of the vitamins in the three pharmaceutical preparations.

Linear relationships between the peak areas and the concentrations for the four vitamins were found in the following ranges: 10–50 and 50–250 ng for vitamin B_1 and 20–100 and 100–500 ng for B_2, B_6 and B_{12}.

DISCUSSION

Table I shows that the HPLC method described is reproducible and can be used for the determination of the four B vitamins with a maximum coefficient of

TABLE II

RETENTION TIMES OF VITAMINS B_1, B_2, B_6 AND B_{12} ON LICHROSORB RP-18, 5 μm, 25 cm × 4 mm I.D.

Flow-rate: 2 ml/min. Pressure: 180 bar. $k' = (t_R - t_0)/t_0$, where t_R = retention time of the vitamin and t_0 = the retention time of the eluent (unretained peak). $\alpha = k'_2/k'_1$, where k'_2 is the capacity factor of vitamin B_2 and k'_1 is that of B_1, B_6 or B_{12}.

Vitamin	*Retention time (min)*		*Capacity factor, k'*	*Separation factor, α*
	Methanol–water (80:20)	*Methanol–water (50:50)*		
B_1	1.0–1.1	0.9–1.0	5.3	2.3
B_2	1.7–1.8	1.9–2.0	12.0	–
B_6	1.0–1.1	1.2–1.3	7.3	1.6
B_{12}	1.5–1.6	1.5–1.6	9.3	1.3

variation of 4.2%. The detector sensitivity of 1.28 a.u.f.s. indicates that at an 8 times higher sensitivity, *e.g.* at 0.08 a.u.f.s., amounts of ≈5–20 ng of the vitamins investigated could be determined. The determination of ≈5 ng vitamin B_1, of ≈10 ng B_2 and of 15–20 ng B_6 and B_{12} at this sensitivity is possible with an approximately 6–7 × noise amplitude at the peak maximum. However, a sensitivity not higher than 0.32 a.u.f.s. is recommended to achieve about 10 × noise amplitude at the peak maximum.

Table II indicates that, vitamins B_1, B_2 and B_{12} can be well separated with the eluent methanol–water (80:20, v/v). In this eluent a separation between vitamins B_6 B_1 is not possible because the two vitamines have the same retention time. On the other hand, the eluent methanol–water (50:50) permits the simultaneous separation of the four B vitamins. The four B vitamins can be separated only when the described conditions are strictly adhered to and the eluent is freshly prepared.

Table III shows the advantage of the combination of the extraction apparatus and the HPLC method in respect of the analysis of the four B vitamins. The four B vitamins can be determined in pharmaceutical preparations in about 15 min. The extraction apparatus allows the simultaneous extraction of the vitamins from three samples in about 5 min. The HPLC method described is rapid, accurate, sensitive and enables the determination of 5–20 ng of the vitamins with a coefficient of variation of $< 4\%$.

A great handicap is the simultaneous determination of 1–5 μg vitamin B_{12} in the presence of 4–15 mg B_1, B_2 and B_6. This problem can be avoided using one of the following possibilities: (1) by recording the peak of the three vitamins B_1, B_2 and B_6 at a low detector sensitivity and that of B_{12} at a higher one; (2) if the vitamin B_{12} concentration is not suitable for accurate analysis in samples with less than 20 ng per injection volume, the addition of 10–20 ng of B_{12} per injection volume is useful. In

TABLE III

QUANTITATIVE HPLC OF VITAMINS B_1, B_2, B_6 AND B_{12} IN PHARMACEUTICAL PREPARATIONS 1–3

The results given are the means from six determinations.

Preparation	*Vitamins present per unit*	*Amount of vitamin found*		
		$\bar{x}$ *(mg)*	*S.D. (mg)*	*C.V. (%)*
1	10 mg B_1	10.35	0.24	2.3
	10 mg B_2	10.42	0.19	1.8
2	15 mg B_1	15.60	0.28	1.8
	15 mg B_2	15.45	0.32	2.1
	5 mg B_6	5.30	0.11	2.1
	5 μg B_{12}	5.40*	0.14*	2.6
3	4 mg B_1	4.25	0.12	2.8
	4 mg B_2	4.30	0.10	2.3
	0.5 mg B_6	0.52	0.02	3.8
	1.5 μg B_{12}	1.6*	0.05*	3.1

* The amount of vitamin B_{12} is given in μg.

this case the evaluation took place on the basis of the resulting differential calibration line.

In comparison to the existing quantitative HPLC methods for the vitamin B group, the extraction and HPLC methods described have advantages with respect to the extraction time, chromatographic (separation) time and simplicity. Some of the previous methods require, for example, 10–30 min for the separation of vitamin B_6 and its derivatives[5,16] and about 15 min for the separation of B_1, B_2 and B_6. Our separations were made in less than 3 min.

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