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Note

New ion-pair reagent for the high-performance liquid chromatographic separation of B-group vitamins in pharmaceuticals

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The advent of multi-vitamin analysis by high-performance liquid chromatography (HPLC) can possibly be attributed to the use of ion-exchange principles[1]. Although the future of these techniques can not be entirely disregarded, reversed-phase chromatography has largely superceded ion-exchange and many of the underlying principles and practicalities are well known[2]. The introduction of ion-pairing reagents has, perhaps more than anything else, allowed a greater versatility and scope in controlling vitamin assays[3]. Separations of B-group vitamins have often been used by manufacturers of stationary phases to illustrate the efficiency of their products.

The question may be asked why further studies are warranted in pharmaceutical analysis. However, this can be answered by the fact that no single chromatographic scheme to date has managed to allow a complete profile of all the vitamins. Each analytical application has different demands and existing procedures may not be sufficient to cope with the problems. A new procedure may therefore be of benefit to some analysts who have failed to achieve their own particular analysis through documented schemes. In addition the new ion-pair reagent, sodium dioctylsulphosuccinate, used in this work is considerably cheaper than traditional ion-pair reagents such as the alkylsulphonates. It also changes the elution order of the vitamins adding a new dimension to the "art" of vitamin separation. The reader is therefore introduced to an additional method to solve analytical problems should the need arise.

EXPERIMENTAL

Apparatus

The HPLC apparatus consisted of a WISP 410B autosampler, a M6000A reciprocating pump, a Model 440 dual wavelength detector (280 and 254 nm), a Model 430 data module (integrator) all supplied by Waters Assoc. (Milford, MA, U.S.A.). The column was a Waters Assoc. μBondapak C_{18} (10 μm) with a pellicular Corasil guard column placed between the injector and analytical column. For solvent programming studies a Waters Assoc. Model 680 programmer was used in which case a second M6000A pump was required. Fluorescence measurements were made using a Schoeffel FS970 detector (Westwood, NJ, U.S.A.) at 436 nm (excitation) and 550 nm (emission, cut-off filter).

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Reagents

Solvent A of the HPLC mobile phase was prepared by dissolving 1 g of sodium dioctylsulphosuccinate (Sigma, St. Louis, MO, U.S.A.) in 170 ml of methanol. A volume of 10 ml concentrated formic acid was added and diluted with distilled water to 800 ml. The pH was checked and adjusted if necessary to 2.5 by dropwise addition of 1 *M* potassium hydroxide. The solution was then taken to 1 l with water and filtered through a Whatman 0.5-μm (47 mm diameter) PTFE filter (Maidstone, U.K.) and degassed by vacuum. The solution foamed extensively.

For solvent programming studies solvent B consisted of 45% methanol with pH adjusted to 4.6. To minimise re-equilibration times, solvent B also contained the same amount (1 g) of ion-pair reagent. In certain instances where vitamin C was present in the sample together with riboflavin-5'-phosphate, solvent A was changed to contain 5% methanol to achieve greater resolution at the front of the chromatogram.

Sample preparation and standardisation

Solutions of intravenous injections were prepared for analysis by simple dilution with water to yield concentrations of 50–500 mg/l for each vitamin of interest. At these concentrations the chromatograph could be operated at the useful gain setting of 0.2 a.u.f.s. Unfortunately in some preparations the various components existed in widely differing concentration and a single dilution was inexpedient. The availability of two detectors, each at different attenuations, say 0.2 and 0.02 a.u.f.s. was often helpful in overcoming this problem.

Multi-vitamin capsules were emptied into a volumetric vessel and diluted to a similar concentration. Warming of the solution (to dissolve the riboflavin in particular) was generally warranted followed by filtration through 0.5-μm (13 mm diameter) PTFE filters (Micro Filtration Systems, Dublin, CA, U.S.A.). Vitamin tablets were first crushed and then treated in a similar manner to the capsules. Tablets or preparations containing proteinaceous material were dissolved in hot (60°C) water then precipitated using 5% trichloroacetic acid (TCA) (to pH 4.4) prior to filtration and analysis.

Standardisation was performed externally by preparing solutions of the vitamins (Sigma) in the correct concentrations. Solutions of samples and standards were not exposed to excessive light and were stored under refrigeration (4°C). They were discarded after one to two days. However, acidification to pH 5 improved the stability characteristics of the solutions if longer storage periods were desirable. In cases where the sample contained only a few vitamins, say thiamine, riboflavin and niacinamide, internal standardisation was used by adding determined quantities of one of the missing vitamins, in this instance niacin.

Chromatography

The mobile phase A was reticulated through the column for 1 h prior to chromatography to achieve ion-pair equilibration. Volumes of 50 μl of the samples and standard were the injected into the chromatograph at a flow-rate of 1.5 ml/min. The external standard solution was generally re-analysed following each set of five samples to guard against sensitivity or chromatographic drifts. Manual switching to solvent B was performed following the pyridoxine elution. However, when using au-

tomated solvent programming, curve 9 over 25 min was selected for optimum separation. Curve 9 achieved an initial slow rate of change in mobile phase composition (simulating the isocratic mode) followed by a rapid change to solvent B to elute vitamin B_1.

RESULTS

Fig. 1 illustrates a chromatogram at 280 nm of a series of B-group vitamin standards using manual switching of the solvent to ensure elution of the strongly adsorbed thiamine peak. The solvent changeover is indicated as B. Fig. 2 repeats the analysis of Fig. 1 but uses 254 nm to illustrate the different responses of the vitamins.

Fig. 3 shows an intravenous veterinary product chromatographed under the same conditions. The time of the solvent changeover and the elution position of vitamin B_{12} (cyanocobalamin) is indicated in the chromatogram by an asterisk. Vitamin B_{12}, which exists in small concentrations compared to the other vitamins and absorbs poorly above 230 mm is not visualised. A preservative, methyl paraben, is however observable in the chromatogram. Riboflavin observed in the sample is an impurity in the riboflavin-5'-phosphate, the major vitamin B_2 component.

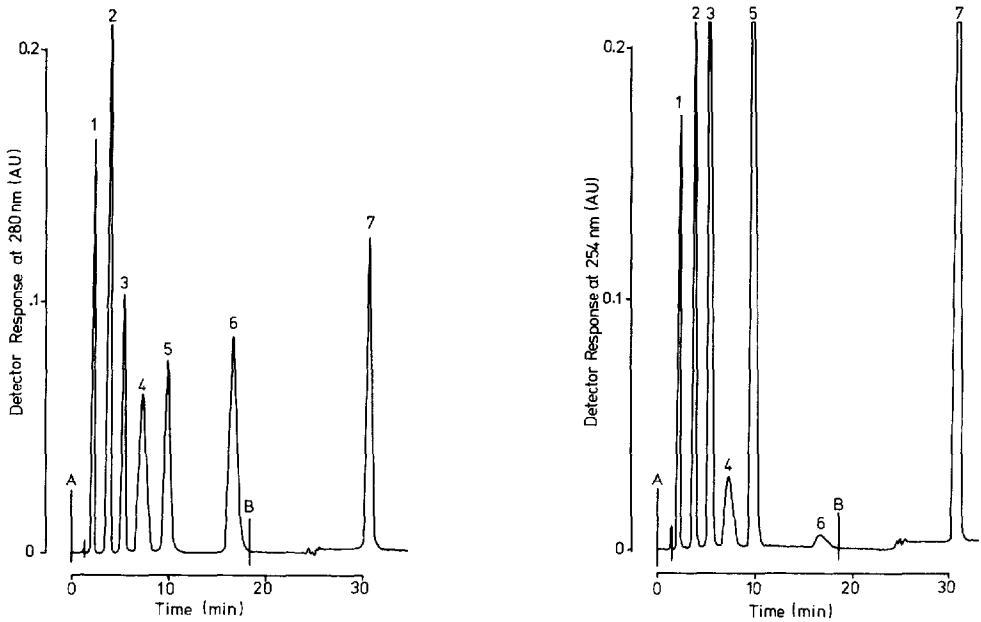

Fig. 1. Two step gradient chromatogram of B-group vitamin standards at 280 nm. Solvent A: methanol (17%), water (82%), formic acid (1%), sodium dioctylsulphosuccinate (1 g/l), pH adjusted to 2.5. Solvent B: methanol (45%), water (54%), formic acid (1%), sodium dioctylsulphosuccinate (1 g/l), pH adjusted to 4.6. Peak identities: 1 = riboflavin-5'-phosphate (0.025 mg/ml); 2 = riboflavin (0.05 mg/ml); 3 = niacin (0.4 mg/ml), 4 = folic acid (0.05 mg/ml); 5 = niacinamide (0.4 mg/ml); 6 = pyridoxine (0.1 mg/ml); 7 = thiamine (0.2 mg/ml). Other conditions: Wavelength = 280 nm (0.2 a.u.f.s.), flow-rate = 1.5 ml/min Manual changeover to solvent B indicated at approximately 19 min.

Fig. 2. Two-step gradient chromatogram of vitamin standards at 254 mm. Chromatographic conditions and peak assignments as described in Fig. 1 except wavelength changed to 254 nm (0.2 a.u.f.s.) to illustrate sensitivity changes and advantages of dual wavelength detection.

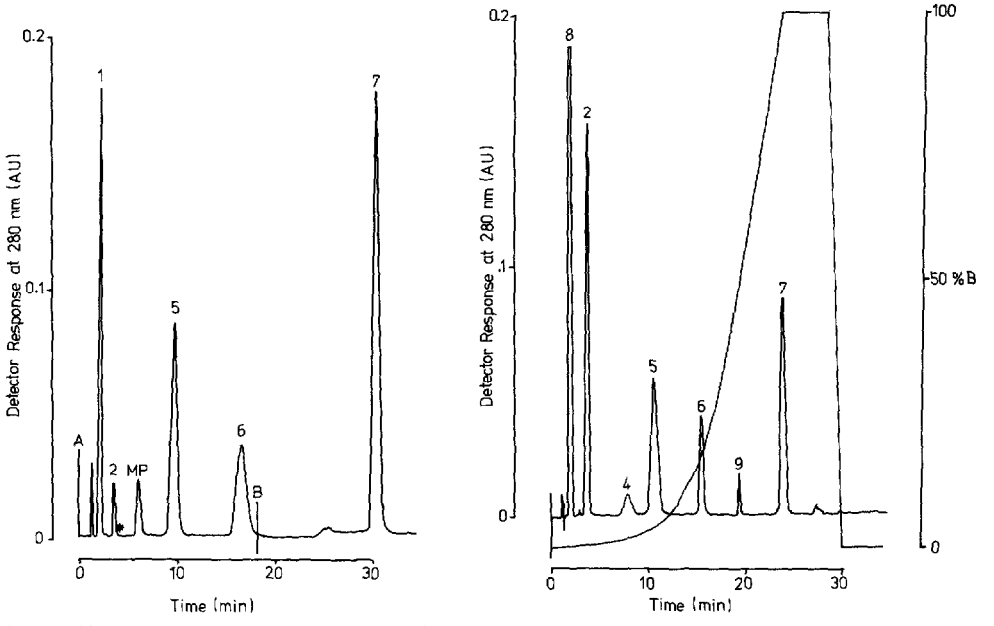

Fig. 3. Chromatograph at 280 nm of equine intravenous injection solution. Chromatographic conditions and peak identities as described in Fig. 1. MP = methyl paraben (preservative used in the formulation). The location of cyanocobalamin elution is indicated by an asterisk although the vitamin is not visible at 280 nm.

Fig. 4. Chromatogram at 280 nm of vitamin capsule using automatic solvent programming. Chromatographic conditions: Solvent A and B as described in Fig. 1. Solvent B programmed from 0 to 100% over 25 min using curve 9 as shown. 100% B maintained for 3 min then returned to 100% A over a 2 min period using linear gradient (curve 6). Peak identities: as described in Fig. 1 with the following additional assignments: 8 = ascorbic acid; 9 = pyridoxamine.

Fig. 4 demonstrates the use of automatic solvent programming. The programmer was taken through curve 9 over a 25-min interval as depicted in the figure. The sample was a multi-vitamin capsule which contained a portion of pyridoxamine as shown, and also ascorbic acid.

Fig. 5 indicates a chromatographic method to overcome the interference of riboflavin-5'-phosphate (the more soluble form of vitamin B_2) and ascorbic acid (vitamin C). The solvent A was more polar, containing only 5% methanol to slow down the early-eluting peaks and programming was achieved over 25 min using curve 9. The quantitation of riboflavin-5'-phosphate was best achieved using fluorescence detection to avoid solvent front disturbances. The preservative (benzyl alcohol) is not visible but its elution position is marked with an asterisk.

Fig. 6 illustrates a chromatogram of a vitaminised milk tablet containing proteinaceous and other milk solids material. The use of fluorescence for the riboflavin peak permits the full assay to continue despite the over-riding presence of other material in the sample.

As a test of accuracy, Table I compares the calculated and expected data for a multi-vitamin preparation. The calculated variant coefficients are also given, based upon five replicate analyses.

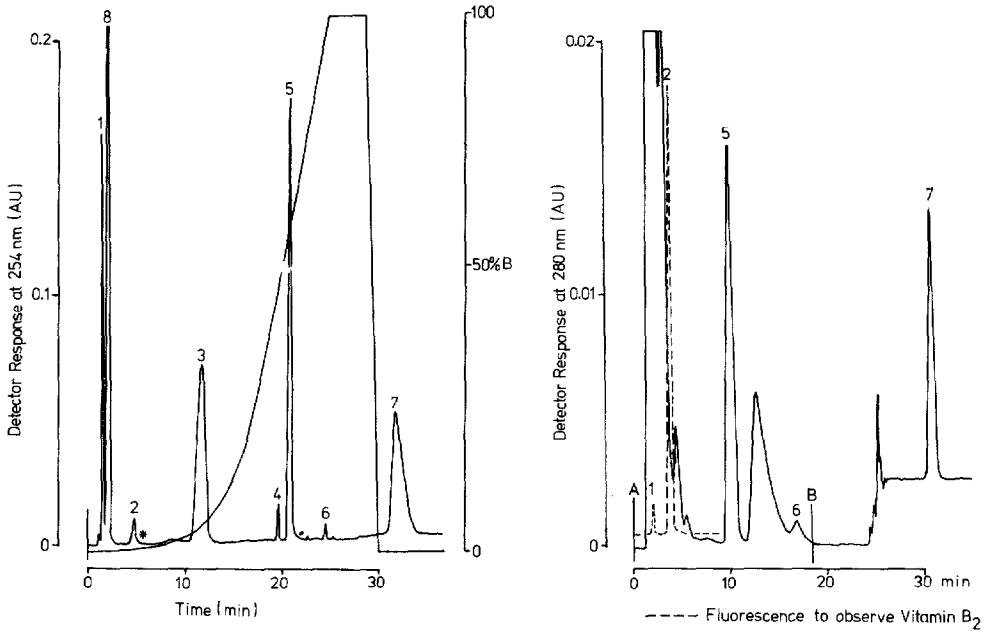

Fig. 5. Chromatogram at 254 nm of a vitamin injection solution using automatic solvent programming. Solvent A was modified to allow vitamin C resolution from riboflavin-5'-phosphate (flavin mononucleotide). Solvent A: methanol (5%), water (94%), formic acid (1%), sodium dioctylsulphosuccinate (1 g/l), pH adjusted to 2.5. Solvent B: unchanged. Chromatographic conditions and peak assignments as described in Fig. 4. Niacin (peak 3) being absent from the formulation was artificially added as an internal standard. Location of the benzyl alcohol (preservative) elution position is indicated by an asterisk.

Fig. 6. Vitaminised milk tablet formulation to illustrate the selective use of fluorometric detection for vitamin B_2. Chromatographic conditions: two-step gradient as described in Fig. 1. Detector sensitivity increased to 0.02 a.u.f.s. Peak identities as described in Fig. 1.

DISCUSSION

Among the biochemicals used in the world today, perhaps none has received more analytical attention than the vitamins. The literature is full of information concerning the determination of the vitamins in pharmaceuticals, clinical samples and food products. Any summarisation of the current status of vitamin analysis must

TABLE I

ACTUAL AND MEASURED VITAMIN LEVELS FOR A MULTI-VITAMIN PREPARATION

Vitamin	*Expected (mg/ml)*	*Found (mg/ml)*	*C.V. (%)*
Thiamine	2.05	1.96	2.3
Riboflavin-5'-phosphate	0.75	0.73	2.9
Riboflavin	0.25	0.26	1.3
Niacinamide	5.00	5.04	1.3
Pyridoxine	0.55	0.56	1.2
Pyridoxamine	0.05	0.04	2.2
Folic acid	1.75	1.78	2.8

necessarily be left to multi-volume texts which are now approaching encyclopaedic scale[4,5].

HPLC has certainly revolutionised the analysis of both fat- and water-soluble vitamins and is now the method of choice in most analytical situations. However, unlike the amino acids and other biochemical classes the vitamins are not a structurally or functionally related series of compounds and can present some interesting problems when they are analysed concurrently, even for HPLC. For the B-group vitamins no single analytical protocol has managed to overcome the problems associated with total analysis of this class of compounds for several reasons:

(1) The vitamins do not respond to a single detection wavelength.

(2) There are invariably chromatographic overlaps of certain pairs of vitamins even under conditions of solvent programming. Solvent programming has been of immense help in overcoming this difficulty and also in adjusting the elution volumes (k') of early and late eluting peaks. However, it is generally observed that as two interfering vitamins are resolved by a set of analytical conditions it is often at the expense of decreased resolution in another section or the chromatogram.

(3) The vitamins are not found in samples within a uniform concentration range. Their biopotencies and subsequent "recommended daily allowances" are widely scattered. Some of the vitamins must therefore exist in the samples in small quantities while others are at macroscopic levels.

(4) Each B-group vitamin is not necessarily a single entity but may have many bioactive forms. For example vitamin B_6 activity is exhibited by pyridoxine, pyridoxal, pyridoxamine and a host of other species[6].

The work described here is aimed towards the analysis of pharmaceutical preparations which can be essentially regarded as pure solutions of the B-vitamins. This situation is the simplest incidence of this type of analysis because, other than the periodic incorporation of preservatives or excipients, there are few of the problems encountered with clinical and food samples. Sample clean-up to remove proteins, fats and other interferences is unnecessary and the vitamins are often in high concentration. The total effort can therefore be applied to ensure that interferences between the vitamins of interest are avoided during the chromatography.

Figs. 1–5 demonstrate the success of the described chromatographic system in achieving the analyses. However, the unwary analyst can encounter difficulties because many of the vitamin components are very sensitive to small changes in solvent or column characteristics. Care is required in each new application to ensure correct separations. This is best controlled by multi-wavelength detection or, in the future, perhaps using diode-array detector scanning. For many applications the vitamin range may be limited to fewer components in which case simplifications to the chromatographic operating system may be used to ensure a more rapid assay. This may even be achievable using purely isocratic conditions if thiamine is absent.

A major strength of the described method lies in the elution order of the vitamins. Riboflavin elutes early in the chromatogram rather than at its usual location towards the end. (Using other ion-pair reagents, riboflavin will only traverse the column at higher methanol concentrations[2].) The fact that riboflavin is among the few vitamins with a selective detection mode (fluorescence or colorimetric detection) allows the method to be used where early eluting excipients would otherwise interfere. This is well illustrated in Fig. 6. In many other methods[7] the early peaks are the B_6

group or niacin which do not possess selective detection wavelengths. However, these methods often have merits which may be desirable such as the use of low wavelengths (for pantothenate) which is not possible using dioctylsulphosuccinate. The work of Kothari and Taylor[8] is singled out as a useful alternative procedure where good resolution of vitamin B_{12} is possible, although at the expense of early elution of other vitamins and poor thiamine peak shape. The described separation also involves a simple two-step change of solvent.

One of the ultimate aims of this study is to extend the applicability of the method to other, more difficult, sample matrices such as vitamin fortified food products. This will require additional work to establish quantitative recoveries and also the integrity of the vitamin peaks in the presence of other interferences. It may also involve the use of post-column reactions to create selective detection and the use of higher resolution (5 μm) columns.

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