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Quantitative separation of riboflavine from vitamin mixtures

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A method for separating riboflavine from vitamin mixtures is described. The acidified aqueous solution is passed through a small column of large particle size talc on which the riboflavine is selectively and quantitatively adsorbed. Successive washings with 0·01 N hydrochloric acid and 5–10% dioxan remove the other components almost completely. The vitamin, eluted with 20% dioxan, is measured spectrophotometrically.

VITAMIN formulations usually contain riboflavine in amounts large enough to permit determination using the distinguishing physical properties of fluorescence and light absorption at certain wavelengths. For simple preparations, where there is no interference from other components, direct measurement is possible; a typical example is the B.P.C. spectrophotometric assay of compound aneurine tablets.

With more complex formulations elaborate physical or chemical measures, or both, are necessary to eliminate the effects of other substances (see for example Koschara, 1935; Hodson & Norris, 1939; Brealey & Elvidge, 1956; Ferrebee, 1940; Conner & Straub, 1941; Klatzkin, Norris & Wokes, 1949).

We describe a successful separation of riboflavine from vitamin mixtures through its selective adsorption on a column of talc. The vitamin is then quantitatively eluted in a sufficiently pure state to permit viewing its full absorption spectrum.

The procedure was developed in the course of investigating the use of purified talc for the chromatographic separation of cyanocobalamin as a step in its determination. It is well known that talc has such a tenacious affinity for vitamin B_{12} as to preclude its use as a filter aid or lubricant for tablets (Merck Index, 1960). On the other hand talc is considered among the substances on which riboflavine is not adsorbed (Sebrell & Harris, 1954). Using talc, we found that riboflavine was adsorbed at the top of the column from aqueous solutions of vitamin B complex. Cyanocobalamin was then adsorbed as an immediately contiguous band and the remaining constituents passed through with the effluent. Cyanocobalamin was eluted with 10% dioxan while riboflavine required 20% dioxan, but the method was not generally applicable to the determination of vitamin B_{12}.

Experimental

Materials. Reagent grade dioxan checked to ensure that its absorbance at 267, 375 and 444 mμ does not exceed 0·03 (lower grades are sometimes effectively purified by charcoal treatment). Purified talc B.P. Hydrochloric acid 0·01 N.

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Effect of particle size. The degree of fineness of the talc was important; very fine talc, contrary to expectation, had little retentive power. This was observed initially; the yellow riboflavine band appearing a few mm below the top of the bed, leaving a white layer of the finest talc. In addition, the band of riboflavine adsorbed on ordinary purified talc was broad, easily washed down, and the eluate showed much extraneous spectrophotometric absorption. On coarse talc, left after elutriation, the riboflavine band was narrower, less easily eluted and the eluate gave curves more typical of the vitamin. For precision, reproducibility and to ensure a reasonable flow rate, all fine powder, therefore, had to be removed.

Preparation of talc bed. As much as 100–120 g of talc may be necessary to make a suitable bed because the coarse fraction forms only a small proportion of it. The mesh size is not critical.

After soaking the talc with water in a suitable Erlenmeyer flask, suspend it in a large volume of water and allow to settle. Decant the unsettled portion after 5 min. Repeat this operation several times until the supernatant no longer shows opalescence or suspended fine particles.* Pour a slurry of the talc sufficient to form a bed of about 80 mm into a column 12 × 200 mm with a stop cock connected to a suction pump and with a small cotton wool pledget at the bottom. Apply gentle suction to give a flow rate of about 80–100 drops (4–6 ml)/min and wash with water until the washings are clear.

The column may be used for several estimations provided each time it is adequately washed with water followed by about 30 ml each of 20% ethanol and water; this especially after passing coloured solutions such as B complex and liver extract, coloured tablets or aqueous preparations of fat and water soluble vitamins.

Effect of other substances. The adsorptive capacity of talc was reduced to a variable degree by the other materials present in solution with the riboflavine. This desorbing effect could be overcome by dilution, the extent of which was related to the nature and concentration of these materials. Thus an injection solution containing 10% of vitamin B_1, 20% of nicotinamide, 1% of vitamin B_6, 2% of procaine hydrochloride in addition to 0·2% of riboflavine, had to be diluted 100 times, while the same solution to which 10% of dry liver extract had been added required 400 times dilution to ensure that the riboflavine was held high enough on the column to permit adequate washing. A solution of lipotropic components containing acetylmethionine equivalent to 10% methionine, 10% inositol and 1% choline chloride with small amount of B complex factors including 0·03% riboflavine required only 4 times dilution. An aqueous mixture of fat and water soluble vitamins required 300 times dilution; it contained in 100 ml, 2 g of nicotinamide and vitamins A (as acetate or palmitate ester) 1 mega i.u.; D (calciferol) 0·2 mega i.u.; E

* The talc used in most of this work was British Drug House's "Talc, Purified by Acids"; measurement of the particle size by the Sedimentation Balance "Prolabo" revealed that 75% was larger than 4·8 μ, 50% than 8·1 μ and 10% than 15·8%. A sample of this talc, after processing as described, measured microscopically, 20–50μ.

((±)-α-tocopheryl acetate) 0·2 g; B_1 2·5 g; B_2 0·1 g; B_6 0·3 g; C 2·5 g together with polysorbate 80 5 g and propylene glycol 40 g. Solutions containing significantly higher concentrations of the surface-active agent could not be assayed by this method as the capacity of the talc to adsorb the riboflavine was then completely lost. Sugar and glycerol used to sweeten B complex syrups had a little desorbing effect and dilution was necessary mainly to reduce viscosity.

Preliminary testing. Initially, sufficient sample is dissolved or diluted to contain 1 mg% of riboflavine. A preliminary test is then made to determine the dilution which ensures optimum adsorption while not rendering the final volume too large to take an unnecessarily long time to flow. A dilution higher than 1 mg% is made only if the adsorption area proves too broad or if partial elution does not permit sufficient washing for the complete removal of interfering substances.

The sample is passed through the talc and followed by sufficient 0·01 N hydrochloric acid and 10% dioxan to elute the other constituents. The amount of washing necessary must be determined for each type of product. The riboflavine is then eluted with 20% dioxan and the eluate, usually 10 ml or less, diluted to a concentration of 1 mg% before spectrophotometric examination at the three standard wavelengths of 267, 375 and 444 mμ against a blank solution prepared by appropriate dilution of the same eluant. The use of a special buffer for dilution is desirable but not essential since the pH of the eluates always lie between 2–8 within which range variation in spectral characteristics of riboflavine at these wavelengths is negligible (Brealey & Elvidge, 1956; Daglish, Baxter & Wokes, 1948).

The ratio of absorbance of the solution at 375 and 444 mμ to that at 267 mμ should lie within the limits 0·314 to 0·333 and 0·364 to 0·388 respectively as stated by the British Pharmacopoeia, otherwise the conditions should be readjusted.

General assay procedure. Carry out operations in a subdued light. Prepare a solution of the sample to contain about 1 mg% of riboflavine using 0·01 N hydrochloric acid. Filter through paper known not to adsorb riboflavine. If necessary dilute a sample size equivalent to 0·25 mg and allow to pass through the talc column at the rate of about 4–6 ml/min. Use a little water to wash the column walls free of sample into the bed then pass 30–60 ml of 0·01 N hydrochloric acid and 30–60 ml of 10% dioxan as determined by preliminary experiments. Add 20% dioxan until the yellow band nears the bottom of the column. When the coloured band reaches the stopcock, collect the effluent until all the yellow colour has flushed through the stem. Measure the eluate, add 2 ml of 0·01 N hydrochloric acid and dilute to 25 ml with water. Prepare a blank of the same volume of 20% dioxan diluted similarly to 25 ml.* Measure the absorbance at the maximum at 444 mμ and calculate the result using $E\,(1\%\ 1\ \mathrm{cm}) = 320$.

* If spectroscopically pure dioxan is available, omit measuring the eluate and directly dilute to 25 ml with 20% dioxan after adding 2 ml of 0·01 N hydrochloric acid; modify the blank accordingly.

Confirm the result and identity of riboflavine by measuring the absorbance at 375 and 267 mμ.

Results and discussion

The method was applicable to all the vitamin preparations examined; the results in Table 1 show that the recovery of riboflavine is 97–102%.

TABLE 1. ABSORPTION OF RIBOFLAVINE COMPARED WITH THAT OF THE VITAMIN MIXTURES FROM WHICH IT HAS BEEN SEPARATED

Product	Final concentration mg %	Absorption 267 mμ	Absorption 375 mμ	Absorption 444 mμ	Recovery %
Standard solution					
Direct	1	0·845	0·265	0·315	
Recovered	1	0·845	0·263	0·310	97
Parenteral solutions					
*B complex	1	over 2	0·360	0·322	
Riboflavine recovered	1	0·840	0·263	0·314	98·1
*B complex (same without procaine)	1	over 2	0·266	0·318	
Riboflavine recovered	1	0·830	0·264	0·308	96·3
*B complex with liver	1·1	over 2	0·655	0·475	
Riboflavine recovered	1·1	0·970	0·305	0·350	100
*B complex with lipotropic factors	1	over 2	0·325	0·350	
Riboflavine recovered	1	0·825	0·260	0·309	96·6
Oral liquid preparations					
B complex & vitamin C, Syrup	1	over 2	0·380	0·335	
Riboflavine recovered	1	0·840	0·260	0·310	97
*Aqueous mixture of fat & water soluble vitamins, Drops	1	over 2	1·200	0·395	
Riboflavine recovered	1	0·890	0·280	0·330	103·0
Tablets					
B complex (weak), uncoated	1·25	over 2	0·410	0·450	
Riboflavine recovered	1·25	1·10	0·350	0·410	102·5
B complex (weak), coated, coloured	1·25	over 2	0·376	0·440	
Riboflavine recovered	1·25	1·045	0·328	0·400	100
B complex (strong), coated, coloured	1·22	over 2	0·355	0·415	
Riboflavine recovered	1·22	1·05	0·330	0·390	99·9
Capsule					
B complex & lipotropic factors	1·25	over 2	0·456	0·440	
Riboflavine recovered	1·25	1·13	0·360	0·410	102·5

* Composition in the text.

In most of the methods cited for estimating riboflavine in complex vitamin mixtures fluorimetry is employed after either chemical treatment or a combination of chromatographic separation and chemical treatment. So far as could be traced no published procedure seems capable of giving a final solution sufficiently free from interfering substances to allow direct spectrophotometric measurement of riboflavine. Separation on talc as described by us makes this possible. This could be because talc, being one of the weakest adsorbents, allows the other constituents to pass freely and yet has sufficient affinity for riboflavine to provide a specific means for its separation. The method combines the advantages of a simple and rapid chromatographic separation with the speed and accuracy of spectrophotometry; it is also more specific and flexible than fluorimetric procedures.

The method gives a full spectral view of the riboflavine separated with its characteristic multiple peaks so that it is not necessary to check the results against a standard solution nor to compare them with those of

microbiological assays. Indeed, the method involves its own means of identifying the riboflavine and confirming its purity.

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