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Application of Differential Pulse Polarography to the Assay of Vitamins

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The determination of low concentrations of the vitamins B_2, nicotinic acid, nicotinamide, ascorbic acid, K_1 and K_3 in pure solutions by use of differential pulse polarography has been studied. The approximate limits of detection achieved for the different vitamins varied from 0·01 to 1 p.p.m., which is 10 to 100 times better than for d.c. polarography. A comparative determination of vitamin B_2 and nicotinamide in three complex multivitamin preparations showed that differential pulse polarography is more selective than d.c. polarography. With the former method the nicotinamide can be determined directly, *i.e.*, without prior separation of interfering substances.

Most of the vitamins can be determined polarographically.[1] Even the vitamins of the A and D groups give polarographic waves in solvents such as benzene - acetonitrile,[2] dioxan - water[3] or dimethylformamide.[4]

In textbooks that deal with methods of vitamin assay, several polarographic procedures are given, especially for determining water-soluble vitamins.[5,6] However, most of the applications are for pharmaceutical preparations in which the vitamins are present in relatively high concentrations. Only for ascorbic acid are polarographic procedures given even for determinations in biological materials such as fruits and vegetables. In those instances when the method is applicable it is often claimed to be simple, specific and accurate. One limitation of polarography has been the relatively low sensitivity attainable. The method is best suited to determinations at concentrations between about 1×10^{-5} and 2×10^{-3} M.[7] Analysis of 10^{-6} M solutions can be performed but only under favourable conditions and in the absence of interfering substances. In order to obtain accurate results it is advantageous if the waves are in the form of easily measurable steps, which condition occurs in the concentration range between 10^{-4} and 10^{-3} M. The lower limit can, however, be considerably extended by the use of other voltammetric methods. The application of a pulse technique to polarography results, for instance, in the possibility of detecting reversibly reduced species at concentrations as low as 10^{-8} M, and irreversibly reduced species at 5×10^{-8} M.[8]

The polarographic method is rather selective, as waves are observed in finite ranges of potentials. A 100-mV interval between two half-wave potentials can be sufficient to permit measurement of the corresponding wave heights if the concentration ratio is not less than 10:1. The differential pulse technique is even more efficient because it yields peak shapes that approximate closely to the theoretically predicted derivative of an ordinary d.c. polarogram. It thus enables one to obtain the maximum possible resolution between closely spaced waves. A 40-mV interval between two peak potentials can be enough for the corresponding peak heights to be measured, even if the concentration ratio is as high as 10^4:1.[9]

The applications of modern polarographic methods to vitamin assay have hitherto been very few; they include the determination of riboflavine by a.c. polarography,[10] and riboflavine, thiamine and nicotinamide in pharmaceutical preparations by cathode-ray polarography.[11] The reason for this deficiency is probably the complex instrumentation that is required for use with modern techniques. Recent advances in commercial instrumentation have, however, provided equipment at a price equivalent to that of the older d.c. polarographs, with which both modern pulse polarography and classical d.c. polarography can be carried out.

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Experimental

Instrumentation

D.c. polarograms were recorded with a Radiometer Polariter PO4 instrument and differential pulse polarograms with a PAR, Model 174, polarographic analyser. A saturated silver - silver chloride electrode (d.c. polarography) and a saturated calomel electrode served as reference electrodes, a platinum wire being employed as auxiliary electrode. All potentials given are referred to the saturated calomel electrode. The solutions were de-aerated with oxygen-free nitrogen and controlled thermostatically at 25·0 ± 0·1 °C.

A controlled drop time of 2 s was used for the differential pulse polarography. Although longer drop times increase the ratio of faradaic to capacitative current to a maximum, the drop time of 2 s was chosen because it gave better reproducibility (greater drop stability).

Reagents

The vitamins menadione (K_3), nicotinic acid, nicotinamide and riboflavine prepared for biochemical use were obtained from E. Merck AG, Darmstadt. Phytomenadione (vitamin K_1) was obtained from Sigma Chemical Company.

Lithium hydroxide. Pure.

All other chemicals used were of analytical-reagent grade.

Results and Discussion

Riboflavine (Vitamin B_2)

The electrochemical behaviour of riboflavine has been extensively studied. This vitamin forms a reversible redox system the half-wave potential of which is identical with the actual redox potential.[5] As hydrogen ions are consumed in the reduction, the potential is shifted to more negative values with increasing pH[12]

$$E_{\frac{1}{2}} = E^\circ + \frac{RT}{nF} \ln \frac{[H^+]^3 + K_1[H^+]^2}{[H^+] + K_2}$$

where $E^\circ = -0{\cdot}056$ V, $K_1 = 5 \times 10^{-7}$ and $K_2 = 0{\cdot}6 \times 10^{-10}$.

In acidic solution, a characteristic pre-wave appears on the polarogram, which is formed by the adsorption of the reduced form of riboflavine.[5] The polarogram is well developed over a wide pH range but a pH of about 7 is most often recommended for analytical purposes.

Calibration graph by d.c. polarography

The linear concentration range was measured with pure riboflavine solutions in 0·1 M phosphate buffer of pH 7·2. A 100 p.p.m. stock solution was prepared by dissolving 50 mg of riboflavine (previously dried in a vacuum desiccator over concentrated sulphuric acid) in 500 ml of water in a calibrated flask. Before making the volume up to the mark, 5 g of sodium salicylate were added in order to facilitate dissolution. From this solution a series of solutions was prepared by serial dilution, in the concentration range of riboflavine from 1 to 50 p.p.m. Each solution in turn was transferred into the polarographic cell, de-aerated and polarographed and two or three polarograms were recorded for each solution. The diffusion current (i_d) was linearly dependent on the concentration down to 1 p.p.m., which is very near the limit of detection (Fig. 1). The relative standard deviation of i_d/C was ±2·3 per cent. for the range 10–50 p.p.m., ±0·6 per cent. for 4–12 p.p.m. and ±3·3 per cent. for 1·0–2·0 p.p.m.

Calibration graph by differential pulse polarography

Fig. 1 shows a d.c. polarogram and a differential pulse polarogram of a 1 p.p.m. solution of riboflavine in 0·1 M phosphate buffer of pH 7·2. At this low concentration the d.c. polarogram is too poorly defined to be useful for the determination of the vitamin. The peak obtained by the pulse technique, on the other hand, is excellent for that purpose. However, when the concentration of the vitamin was diminished, the slope of the base-line changed, which made the evaluation of the peak height difficult even at a concentration of 0·2 p.p.m. (Fig. 2); this difficulty was not expected because the peak was well developed at the 1 p.p.m. level, but is probably caused by the adsorptive properties of riboflavine.

Although the double-layer charging current associated with the potential step will decay to zero, there is another effect that limits the sensitivity of differential pulse polarography.

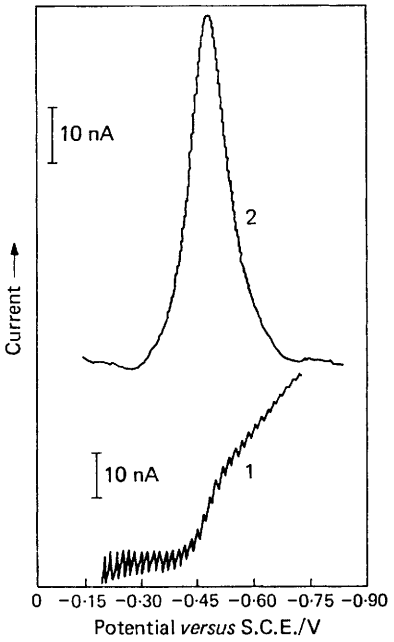

Fig. 1. A d.c. polarogram (1) and a differential pulse polarogram (2) of 1 p.p.m. of riboflavine in phosphate buffer of pH 7·2. Pulse amplitude 50 mV.

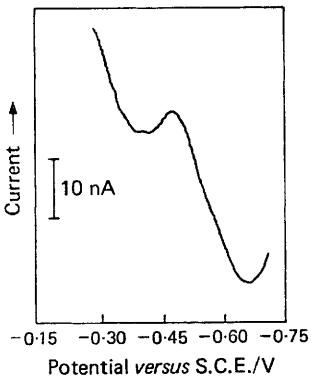

Fig. 2. A differential pulse polarogram of 0·2 p.p.m. of riboflavine in phosphate buffer of pH 7·2. Pulse amplitude 25 mV.

This effect is due to the capacitative current that flows at a growing drop and is dependent on the different times, and potentials, at which measurements are made.[13] Hence, the changes in the double-layer capacity caused, for instance, by adsorption will be of decisive importance for the shape of the base-line. Strong adsorption not only of leuco-riboflavine but also of riboflavine itself has been demonstrated by using the a.c. polarographic method[14] (an a.c. wave of riboflavine at concentrations as low as 2×10^{-7} M can, as a consequence of this fact, be obtained[10]). For the best conditions for differential pulse polarography of riboflavine at low concentrations, a modulation amplitude of 25 mV and a scan rate of 2 mV s^{-1} were required. A calibration graph from 1 to 0·1 p.p.m. of riboflavine is shown in Fig. 3.

Nicotinamide

The reduction of nicotinamide corresponds to a 2-electron change and the wave is well defined in buffers of pH 8 or above. The half-wave potential is −1·75 V in 0·1 N sodium hydroxide solution.[15] For the quantitative determination, sodium hydroxide solution is usually recommended for use as supporting electrolyte.[15,16] The wave lies, however, near the discharge of the sodium ion, which makes the evaluation rather difficult. As the lithium ion has a lower reducing potential, lithium hydroxide was chosen instead of sodium hydroxide in this work, and Fig. 4 shows that 0·1 M lithium hydroxide solution is a much more satisfactory supporting electrolyte.

Calibration graph by d.c. polarography

A 1–100 p.p.m. concentration range of pure nicotinamide solutions was measured in 0·1 M lithium hydroxide solution. Two stock solutions of 100 and 1000 p.p.m. were prepared by dissolving 50 mg of nicotinamide in 500 and 50 ml of water, respectively. These solutions were diluted with lithium hydroxide solution to give appropriate concentrations of the vitamin. The wave heights (i_d) determined for the various calibration solutions were found to be linearly dependent on the concentration and the relative standard deviation of i_d/C was ±1·5 per cent. for the whole of the measured range. The limit of detection is well below

1 p.p.m., probably 0·2–0·3 p.p.m., *i.e.*, polarograms were similar to that for 1 p.p.m. of riboflavine (Fig. 1).

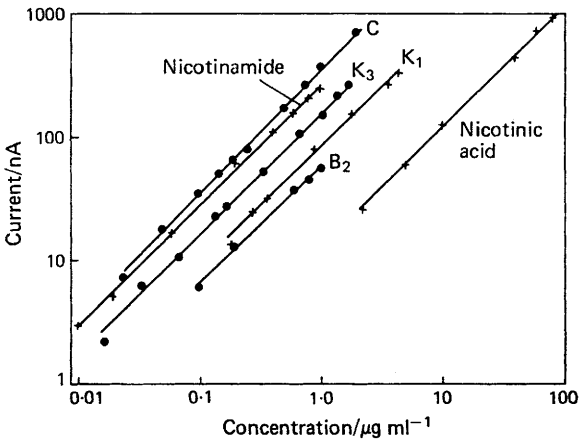

Fig. 3. Differential pulse-polarographic calibration graphs of the 1 to 2 lowest concentration decades for nicotinamide, nicotinic acid and vitamins B_2, C, K_1 and K_3. Each graph is normalised to constant pulse amplitude.

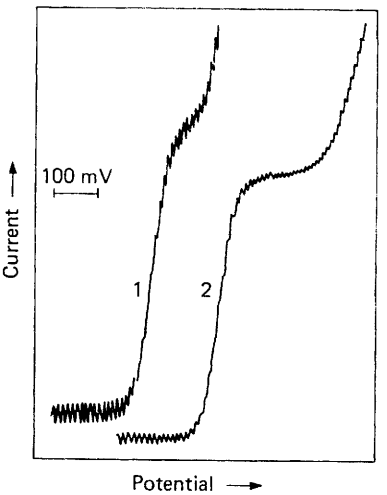

Fig. 4. D.c. polarograms of nicotinamide (8 p.p.m.): 1, in 0·1 M NaOH; and 2, in 0·1 M LiOH solution.

Calibration graph by differential pulse polarography

Concentrations up to 1 p.p.m. were studied with the pulse technique. The peaks were well developed in the range 0·1–1 p.p.m. and the detection limit was as low as about 0·01 p.p.m. At concentrations below 0·1 p.p.m. problems in the evaluation of the peak heights arose because of the changing background. Fig. 5 shows some peaks of the interval 0·1–0·01 p.p.m., which are highly reproducible, but the peak heights are not a linear function of the concentration if they are measured from the peak to the lowest part of the curve or to the mean of the lowest two parts of the curve.

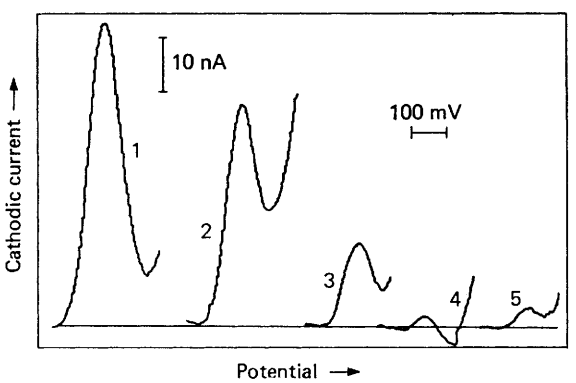

Fig. 5. Differential pulse polarograms of 1, 0·1; 2, 0·08; 3, 0·04; 4, 0·02; and 5, 0·01 p.p.m. of nicotinamide in 0·1 M LiOH solution.

The linearity was, however, improved by changing the pulse amplitude from 100 to 50 mV but the limit of detection was thereby impaired. Fig. 3 shows a calibration graph for the concentration interval 1–0·02 p.p.m. The scan rate was 2 mV s^{-1}, with pulse amplitude 25 mV in the range 1–0·1 p.p.m. and 50 mV in the range 0·1–0·02 p.p.m. The whole of the curve has been normalised to a 25-mV amplitude.

Nicotinic acid

Nicotinic acid gives a catalytic hydrogen wave in the pH range from 1 to 10. Buffers between pH 8 and 9 are normally used for the determination of nicotinic acid because the waves are relatively well developed in that range.[5] Both nicotinamide and nicotinic acid can be determined in the same solution; their sum is determined at pH 8·0 and nicotinamide at pH 13, at which pH the acid does not give a wave.[5] The linear concentration range for nicotinic acid (by d.c. polarography) is from about 40 to 250 p.p.m.[5]

Calibration graph by differential pulse polarography

A 0·1 M borate buffer of pH 8·7 was used as supporting electrolyte and concentrations up to 100 p.p.m. were studied. The sensitivity was not as good as that for nicotinamide and the limit of detection was about 1 p.p.m. when a pulse amplitude of 10 mV was used. The best linearity was obtained with an amplitude of 5 mV and a scan rate of 1 mV s^{-1}. Fig. 6 shows some peaks in the range 1–20 p.p.m. and Fig. 3 the entire calibration graph.

Ascorbic acid

The polarographic assay of vitamin C is stated to be more specific and to require fewer steps in sample preparation than colorimetric and titration methods.[1] The relatively low sensitivity of the method and the limited anodic voltage range of the mercury electrode have been the main limitations. The normal concentration range is between 25 and 250 p.p.m. of ascorbic acid.[6] Buffers with pH between 3·4 and 6 are normally used as supporting electrolytes. In more strongly acidic solutions the anodic wave has a very flat slope and lies too near the background current, and at higher pH values ascorbic acid is too readily oxidised.[6]

Calibration graph by differential pulse polarography

The supporting electrolyte was an acetate buffer saturated with sodium oxalate and its pH was 5·5.[6] Concentrations up to 25 p.p.m. were studied. Excellent peaks were obtained with concentrations down to 0·2 p.p.m. The peak half-width was 46 mV at 25-mV pulse amplitude, which is close to the theoretical value for a reversible 2-electron process.[17] A changing base-line made the evaluation troublesome at concentrations below 0·2 p.p.m., as shown in Fig. 7. If the peak heights are measured from the highest to the lowest parts of the curves, the value at 0·1 p.p.m. is half that at 0·2 p.p.m., but in order to ensure proportionality in the 0·1–0·025 p.p.m. range, the peak heights must be measured from the break points indicated by the arrows. The best modulation amplitude was 5 mV with respect to both the detection limit and the linearity. Fig. 3 shows a calibration graph for the range 0·025–2 p.p.m.

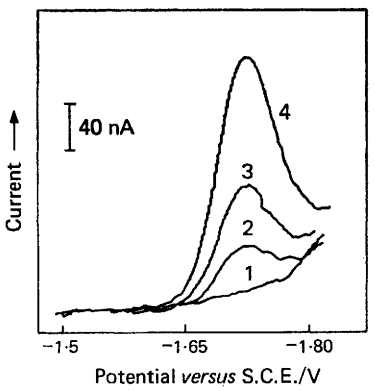

Fig. 6. Differential pulse polarograms of 1, 1; 2, 5; 3, 10; and 4, 20 p.p.m. of nicotinic acid in borate buffer of pH 8·7.

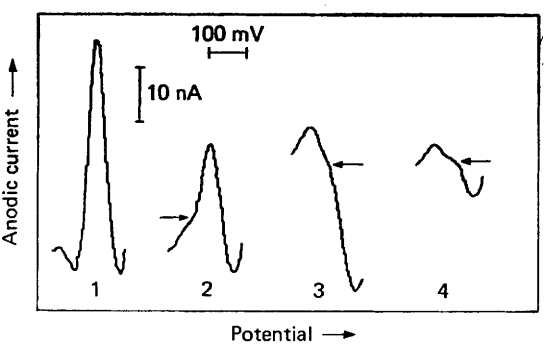

Fig. 7. Differential pulse polarograms of 1, 0·2; 2, 0·1; 3, 0·05; and 4, 0·025 p.p.m. of ascorbic acid at pH 5.5. Pulse amplitude 50 mV, peak potential +0·217 V.

Vitamin K_1 (Phytomenadione) and K_3 (Menadione)

Supporting electrolytes consisting of a 0·06 M solution of ammonium chloride in 75 per cent. propan-2-ol[5] or a 0·5 M solution of tetrabutylammonium iodide in acetonitrile[18] have been used for the polarography of vitamin K_1. Similar electrolytes have been used for vitamin K_3,[5] but, according to Patriarche and Lingane,[19] lower pH values are to be preferred because of the instability of vitamin K_3 in alkaline media.

Calibration graph by differential pulse polarography

Concentrations below 5 p.p.m. of vitamin K_1 in a 0·06 M solution of ammonium chloride in 75 per cent. propan-2-ol and concentrations below 2 p.p.m. of vitamin K_3 in 0·1 M acetate buffer of pH 5·0 containing 25 per cent. of methanol were studied. Very well developed peaks of vitamin K_1 were obtained in the concentration range 2–5 p.p.m. and Fig. 8 shows a peak at 2·7 p.p.m. Below about 0·5 p.p.m. (1 μM) the slope of the base-line makes the evaluation increasingly uncertain. This effect is also shown in Fig. 8, where some polarograms can be seen for concentrations near the limit of detection (about 0·1 p.p.m.). The pulse amplitude used was 50 mV.

The limit of detection for vitamin K_3 is as low as about 0·02 p.p.m. Fig. 9 shows two peaks at 0·069 and 0·17 p.p.m. (pulse amplitude 25 mV). Calibration graphs for both vitamins are shown in Fig. 3.

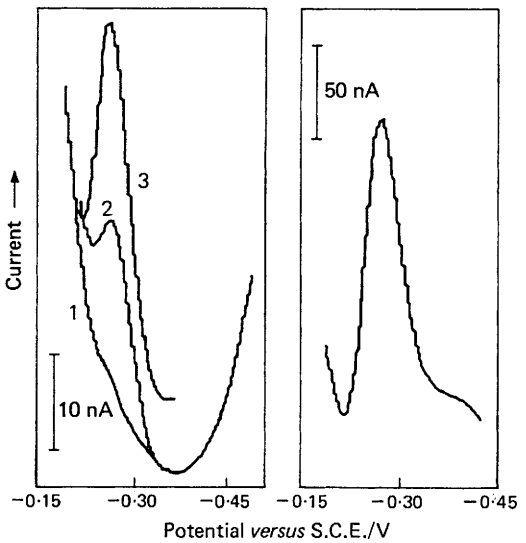

Fig. 8. Differential pulse polarograms of (left): 1, 0·09; 2, 0·18; and 3, 0·45 and (right): 2·7 p.p.m. of vitamin K_1 in 0·06 M solution of NH_4Cl in 75 per cent. propan-2-ol. Pulse amplitude 50 mV.

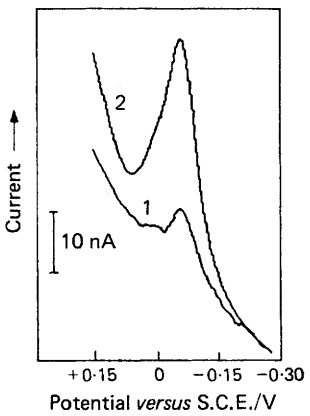

Fig. 9. Differential pulse polarograms of 1, 0·069; and 2, 0·17 p.p.m. of vitamin K_3 in 0·1 M acetate buffer of pH 5·0 containing 25 per cent. of methanol. Pulse amplitude 25 mV.

Comparative Determination of Vitamin B_2 and Nicotinamide in Three Complex Vitamin Preparations

In some instances, vitamins (especially vitamin B_2) can be determined directly, even in complex vitamin preparations, simply by adding supporting electrolyte to the sample, de-aerating the solution and recording a d.c. polarogram.[6] Because of the better selectivity of the differential pulse technique, it should be possible to increase the number of instances when such direct determinations can be made, and in order to verify this conclusion a comparison was made between the two techniques.

The declared contents of 100 ml of the vitamin mixtures analysed were as follows.

Vitalvin: thiamine hydrochloride, 11 mg; riboflavine, 7 mg; pyridoxine hydrochloride, 7 mg; nicotinamide, 0·11 g; ethanol, including the content of added wine, 10 g; sugar, 28 g; sodium phosphate; citric acid; ascorbic acid; apple syrup; blackcurrant syrup; raspberry syrup; and aromatic agents.

Roburan: caffeine, 0·27 g; vitamin A, 17 000 i.u.; vitamin D_3, 1700 i.u.; thiamine hydrochloride, 10 mg; sodium riboflavine phosphate, 15 mg; nicotinamide, 80 mg; pyridoxine hydrochloride, 7 mg; pantothenol, 27 mg; ascorbic acid, 0·5 g; sorbitol; and constituents.

Pharmaton: Thiamine hydrochloride, 11 mg; riboflavine, 7 mg; pyridoxine hydrochloride, 7 mg; nicotinamide, 0·11 g; disodium methylarsonate, 0·11 g; caffeine, 0·5 g; ethanol, 20 g; sugar, 33 g; sodium phosphate; citric acid; cherry syrup; aromatic agents; and colouring matter.

Determination of Vitamin B_2

Five 5·00-ml samples each of Vitalvin, Roburan and Pharmaton were pipetted into 50-ml calibrated flasks; 5 ml of phosphate buffer solution of pH 7·2 were added and to the last two flasks in each series of five samples, 1·50 and 2·00 ml of standard vitamin B_2 solution were added. The solutions were diluted to the marks with water and then about 15 ml were de-aerated in the polarographic cell and polarographed.

Vitalvin and Roburan gave well developed polarograms of vitamin B_2 and the concentrations of the vitamin were calculated (Table I). The polarograms obtained with the Pharmaton samples were, however, affected by the presence of an interfering substance, which made the evaluation inaccurate. Because of the high reducing potential of this substance it was assumed that it was a colour additive (possibly an azo compound) and as most edible colour additives are anions that it would be easy to remove by means of an anion exchanger. A chloride-saturated anion exchanger (Dowex 1 × 8, 50–100 mesh) was effective in removing it; 5·00-ml samples of Pharmaton were pipetted on to the column (1 × 4 cm) and the ion exchanger was washed five times with 5-ml portions of water. The eluates were collected in the calibrated flasks and polarographed as described above. The polarograms were then easily evaluated and the concentration of the vitamin was calculated (Table I).

TABLE I

RIBOFLAVINE CONTENT OF THE SAMPLES

Sample	Concentration of riboflavine, p.p.m.		
	Declared	Found by d.c. polarography	Found by differential pulse polarography
Vitalvin	7	6·61 ± 0·16	—
Roburan	15	14·52 ± 0·30	—
Pharmaton	7	6·90 ± 0·08*	(7·26)

* After separation of interfering substance.

As the Pharmaton sample could not be analysed by d.c. polarography without applying this separation procedure, it was subjected to differential pulse-polarographic analysis. Fig. 10 shows the polarograms before and after the addition of standard. The riboflavine peak is resolved but the background current changes after addition of the standard, which makes the evaluation very difficult. The peak heights measured to either of the broken lines in Fig. 10 gave results in accordance with those obtained by d.c. polarography after carrying out the separation procedure, but these lines are not satisfactory because similar lines cannot be drawn after the addition of standard. Hence, in this instance the method requires to be examined further.

Analysis of the Samples for Nicotinamide

Direct d.c. polarography of nicotinamide in multivitamin preparations is usually not possible because there is a greater risk of interference with the nicotinamide wave owing to the low reducing potential of the vitamin (−1·75 V); 0·50-ml samples were pipetted into 50-ml calibration flasks, 5 ml of supporting electrolyte (1 M lithium hydroxide solution) were added and the solutions were then diluted to the mark with water. After de-aeration the solutions were polarographed.

Nicotinamide could not be determined directly by d.c. polarography except in Roburan samples. A simple anion exchanger[6] (hydroxyl-saturated Dowex 1 × 8, 50–100 mesh) could, however, be used even in this instance to remove interfering substances. The results

TABLE II

NICOTINAMIDE CONTENT OF THE SAMPLES

Sample	Concentration of nicotinamide, p.p.m. Declared	Found by d.c. polarography	Found by differential pulse polarography
Roburan	80	83 ± 3	—
		88 ± 1*	—
Vitalvin	110	109 ± 3*	113
Pharmaton	110	113 ± 1*	111

* After separation on ion exchanger.

obtained are given in Table II. Fig. 11 shows differential pulse polarograms of the samples Vitalvin and Pharmaton that could not be analysed by d.c. polarography without previous separation of interfering substances. The base-lines (broken lines in Fig. 11) were drawn by using polarograms of pure supporting electrolyte. The measured results agreed well with the values obtained by d.c. polarography (Table II).

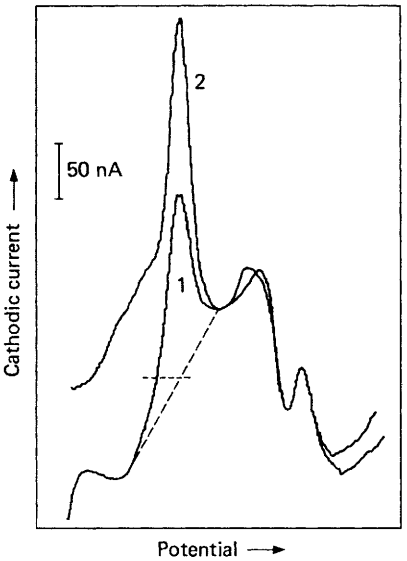

Fig. 10. Differential pulse polarograms of a complex vitamin preparation (Pharmaton) in phosphate buffer of pH 7·2: 1, before and 2, after addition of standard riboflavine solution. Broken lines denote arbitrarily drawn background currents.

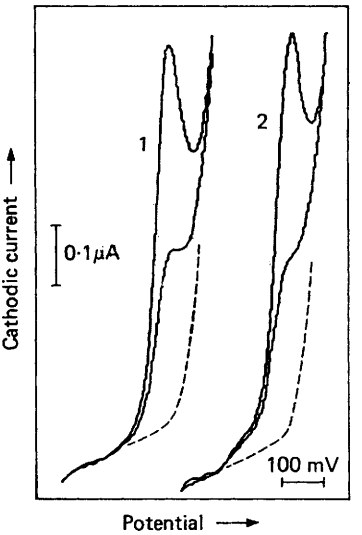

Fig. 11. Differential pulse polarograms of two complex vitamin preparations [Vitalvin (1) and Pharmaton (2)] in 0·1 M LiOH solution before and after addition of standard nicotinamide solution. Broken lines denote residual current.

Conclusion

The differential pulse-polarographic method shows promise. At the limit of detection for d.c. polarography the pulse technique gives excellent peaks and if the limit of determination is taken as about five times the limit of detection, it is possible to determine nicotinamide at 0·05, ascorbic acid and vitamin K_3 at 0·1, vitamins B_2 and K_1 at 0·5 and nicotinic acid at 5 p.p.m. The selectivity is also excellent as nicotinamide could be determined directly in the complex multivitamin preparations.

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