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## Note

# Determination of water-soluble vitamins using high-performance liquid chromatography and electrochemical or absorbance detection

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Quantitative methods available for the determination of water-soluble vitamins involve enzymatic, microbiological and chemical procedures including electrochemical and absorbance techniques[1–3]. High-performance liquid chromatography (HPLC) has been shown to be a powerful approach for the trace analysis of vitamins[4,5]. As HPLC with electrochemical detection (ED) is especially suitable for the trace determination of electroactive compounds in complex matrices, it has been applied successfully to the trace analysis of vitamins[6–11]. The main advantage of ED systems is a higher sensitivity and better selectivity than those of absorbance methods. So far, no data have been published on the determination of folic acid and *p*-aminobenzoic acid by HPLC with ED. In this work, the separation and quantitation of these compounds and the simultaneous determination of four water-soluble vitamins were studied in order to extend the scope of analysis by HPLC with ED and to improve the sensitivity and selectivity of the method.

## EXPERIMENTAL

### *Apparatus*

An amperometric analyser (made in China) was used for cyclic voltammetry. A three-electrode cell system with a glassy carbon working electrode, a silver–silver chloride (saturated potassium chloride) reference electrode and a platinum auxiliary electrode was employed.

The HPLC system consisted of a Model 510 pump, a U6K injection valve and a Model 481 variable-wavelength detector (Waters Assoc.). The injection volume was 20 $\mu$l and the wavelength was 263 nm. A Zorbax ODS (5 $\mu$m) column (15 cm $\times$ 4.6 mm I.D.) (DuPont) was used, with a Model TL-5A thin-layer electrochemical cell (Bioanalytical Systems) and a laboratory-made bipotentiostat for amperometric detection. The bipotentiostat provided certain constant potentials for both working electrodes of the electrochemical cell and detected and amplified the currents of the working electrode. It was very stable, sensitive and convenient to operate. The chromatograms were obtained at ambient temperature with a mobile phase flow-rate of 1.0 ml/min.

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*Reagents*

All chemicals were of analytical-reagent grade, unless stated otherwise. All solutions were prepared with double distilled water. Nicotinamide was of biochemical-reagent grade and folic acid of chemical-reagent grade. The multivitamin tablets were commercially available products. A 9-vitamins-1 tablet contains vitamin A [2500 international units (i.u.)], $D_2$ (1000 i.u.), $B_1$ (2 mg), $B_2$ (1 mg), C (30 mg), $B_6$ (0.5 mg), E (1 mg), nicotinamide (10 mg) and dextro calcium pantothenate (1 mg). A 9-vitamins-2 tablet contains vitamin A (2500 i.u.), $B_1$ (2 mg), $B_2$ (1 mg), $B_6$ (1 mg), C (35 mg), $D_2$ (200 i.u.), E (1 mg), nicotinamide (10 mg) and dextro calcium pantothenate (2 mg). The vitamin contents of multivitamin–glucose power are not tabulated.

*Procedures*

All stock solutions were prepared at a concentration of 1 mg/ml in water and were stored at 277 K (ascorbic acid was dissolved in deoxygenated water). Just before the actual analysis, an aliquot was taken and diluted to the appropriate concentration.

The tablets were crushed to a fine powder and dissolved in water. The solution was filtered through a glass filter (porosity, 2–5 $\mu$m) and diluted to 25 ml with water. The glucose powder containing multivitamins was dissolved in water.

## RESULTS AND DISCUSSION

*Electrochemistry of water-soluble vitamins*

The amperometric behaviour of folic acid and *p*-aminobenzoic acid has been reported[12]. In this study, the electrochemical behaviour of folic acid and *p*-aminobenzoic acid was studied using a glassy carbon working electrode. Fig. 1 shows the cyclic voltammograms of ascorbic acid, folic acid and *p*-aminobenzoic acid in a conventional electrochemical cell. It can be seen that ascorbic acid yields an oxidation wave at about 0.25 V and both folic acid and *p*-aminobenzoic acid at about 0.97 V. Nicotinamide did not yield an oxidation wave in this potential range. With an increase in pH, the $E_p$ of ascorbic acid shifts to a more negative value but those of folic acid and *p*-aminobenzoic acid are hardly affected. The oxidation process of all three analytes is irreversible.

*Liquid chromatographic separation and detection of water-soluble vitamins*

Fig. 2 shows the change in capacity ratios ($k'$) that occurred when the pH of the mobile phase was changed, and it can be seen that the $k'$ values of folic acid and *p*-aminobenzoic acid decreased with increasing pH owing to their enhanced polarity, whereas those of ascorbic acid and nicotinamide were hardly affected. As folic acid is insoluble in solutions with a pH below 5, the pH of the mobile phase was fixed at 6 in the subsequent experiments.

Fig. 2 also shows the influence of the methanol concentration in the mobile phase on the capacity ratios. With an increase in methanol concentration, the $k'$ values of folic acid and nicotinamide are reduced whereas those of ascorbic acid and *p*-aminobenzoic acid are influenced to minor extent.

A good separation of the four vitamins was achieved in with methanol–0.1 *M* phosphate buffer (pH 6.0) (1:4, v/v) at a flow-rate of 1 ml/min (Fig. 4A).

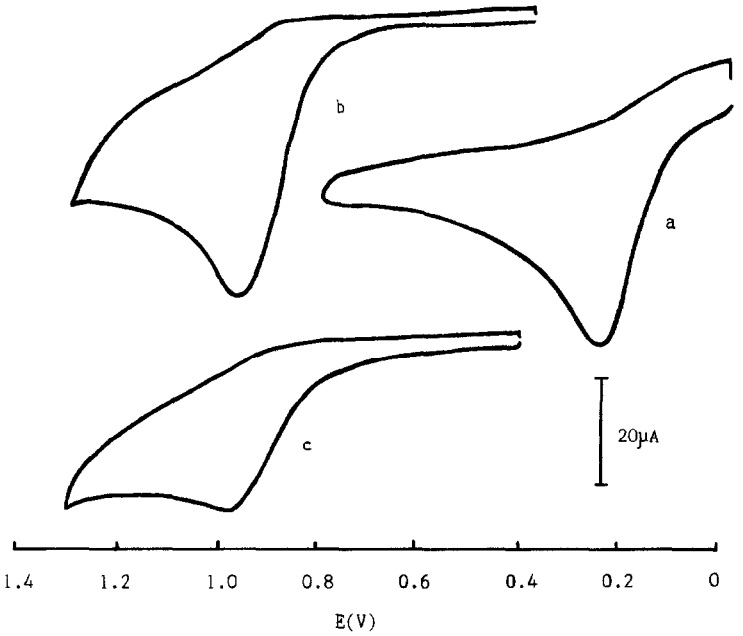


Fig. 1. Cyclic voltammograms obtained in 0.1 *M* phosphate buffer (pH 6.0). (a) 1 m*M* ascorbic acid; (b) 1 m*M* *p*-aminobenzoic acid; (c) 1 m*M* folic acid. Scan rate, 100 mV/s.

In order to determine the optimum potential for electrochemical detection, three hydrodynamic voltammograms were constructed (Fig. 3) by making injections of fixed volumes of the stock solutions and varying the potential between 0 and 1.3 V. It can be seen that ascorbic acid reaches its maximum current, whereas folic acid and *p*-aminobenzoic acid do not produce any current at +0.70 V but reach their

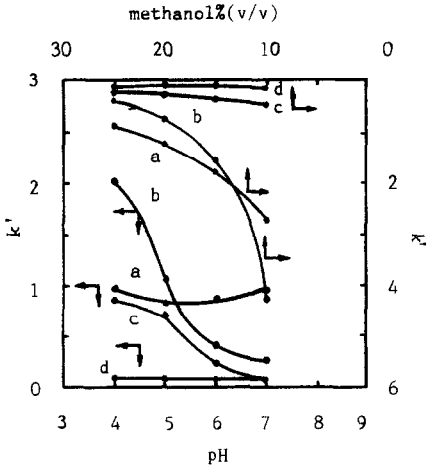


Fig. 2. Influence of mobile phase pH and methanol concentration in the mobile phase on capacity ratios. (a) Nicotinamide; (b) folic acid; (c) *p*-aminobenzoic acid; (d) ascorbic acid. Mobile phase: methanol–0.1 *M* phosphate buffer (1:3, v/v) for pH influence and methanol–0.1 *M* phosphate buffer (pH 6.0) for influence of methanol concentration.

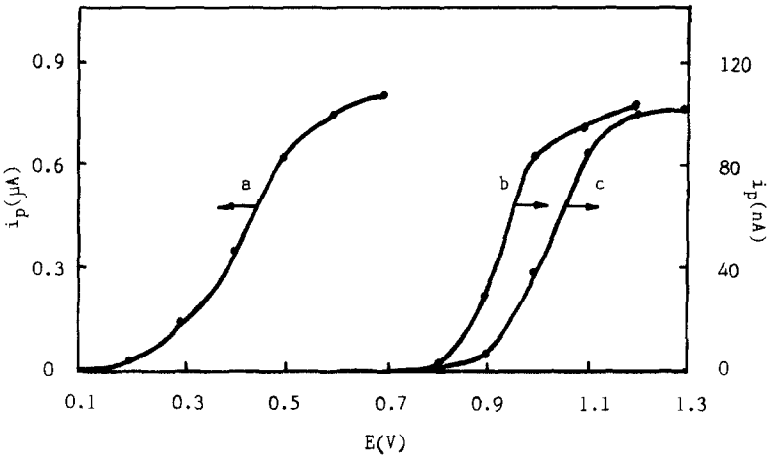


Fig. 3. Hydrodynamic voltammograms for vitamins (a) 20 ppm ascorbic acid; (b) 1 ppm *p*-aminobenzoic acid; (c) 15 ppm folic acid.

maximum current at +1.20 V. Higher potentials are disadvantageous in HPLC with ED owing to the higher background current, and therefore a potential of 0.60 V was selected for the determination of ascorbic acid and 1.10 V for folic acid and *p*-aminobenzoic acid. A parallel dual-electrode system was applied with two different oxidative potentials, allowing the simultaneous determination of three vitamins, one at 0.60 V and the other two at 1.10 V. Fig. 4B shows the chromatograms obtained.

Fig. 4 indicates that the HPLC–ED system offers superior selectivity over absorbance detection. It was possible to detect ascorbic acid directly in multivitamin samples and the chromatographic separation process can be avoided. Most absorbable and non-electrooxidized vitamins do not interfere in the HPLC–ED determination of the vitamins studied. As almost all vitamins absorb in a certain wavelength range, the analysis of multivitamin samples with absorbance detection can only be carried out following a good chromatographic separation. The results of this study demonstrate that the HPLC method with ED for determining vitamins is convenient, sensitive and selective.

Eight replicate injections of a stock solution containing 15 ppm of nicotinamide, 20 ppm of ascorbic acid, 5 ppm of *p*-aminobenzoic acid and 15 ppm of folic acid were carried out to determine the precision. Nicotinamide was detected with an absorbance detector and the others with the amperometric detector. The coefficient of variation of the peak height was 1.7% for nicotinamide, 4.7% for ascorbic acid, 3.0% for fotic acid and 2.2% for *p*-aminobenzoic acid.

The calibration graphs were linear over the ranges 20 pg–100 ng for ascorbic acid, 40 pg–200 ng for *p*-aminobenzoic acid and 200 pg–100 ng for folic acid with amperometric detection and 1–500 ng for nicotinamide with absorbance detection. The correlation coefficients were better than 99.90%. The detection limit is 20 pg for ascorbic acid, 40 pg for *p*-aminobenzoic acid and 0.2 ng for folic acid with amperometric detection and 0.1 ng for *p*-aminobenzoic acid, 0.2 ng for ascorbic acid, 0.6 ng for folic acid and 1 ng for nicotinamide with absorbance detection. This also reflects

TABLE I

DETERMINATION OF VITAMINS IN TABLETS

All parameters as in Fig. 4.

| *Sample* | *Ascorbic acid* | | | | *Nicotinamide* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Label claim (mg)* | *Found* (mg)* | *C.V.** (%)* | *Recovery (%)* | *Label claim (mg)* | *Found* (mg)* | *C.V.** (%)* | *Recovery (%)* |
| 9-Vitamins-1 | 30 | 29.88 ± 1.13 | 3.80 | 99.60 | 10 | 9.91 ± 0.02 | 2.10 | 99.10 |
| 9-Vitamins-2 | 35 | 34.75 ± 1.08 | 3.10 | 99.29 | 10 | 9.95 ± 0.18 | 1.80 | 99.50 |
| Multivitamin – glucose | – | 0.20 ± 0.005 mg/g | 2.50 | 99.40 | – | – | – | – |

* Mean ± standard deviation of six replicate sample treatments.

** C.V. = coefficient of variation.

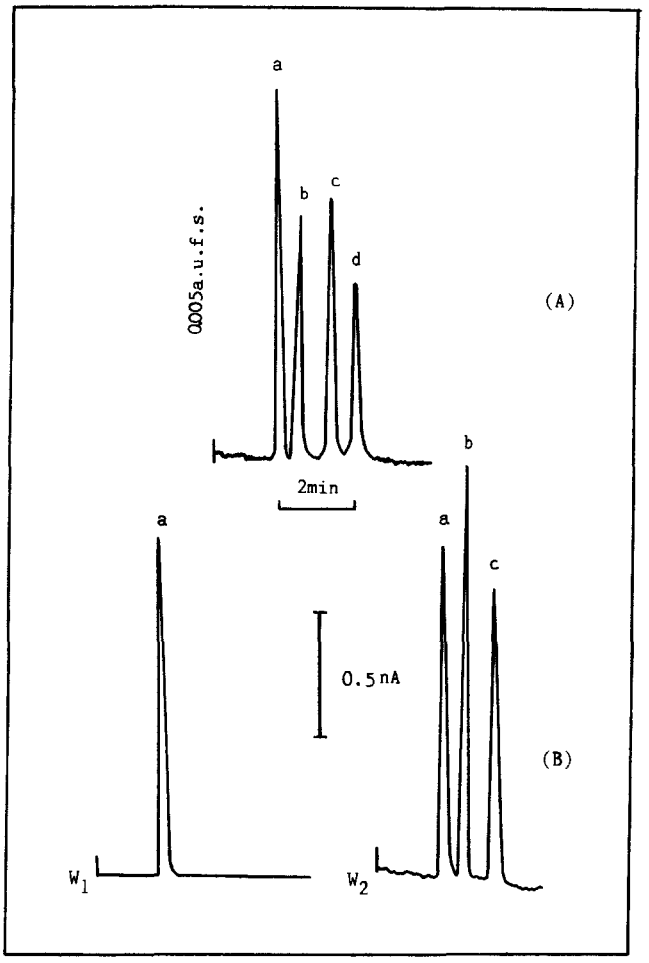


Fig. 4. Chromatograms of vitamins. (A) Absorbance detection. (a) 0.2 ppm ascorbic acid; (b) 0.1 ppm *p*-aminobenzoic acid; (c) 0.5 ppm folic acid; (d) 0.5 ppm nicotinamide. (B) Parallel dual-electrode detection. $E_1$ = 0.60 V ($W_1$); $E_2$ = 1.10 V ($W_2$). (a) 0.1 ppm ascorbic acid; (b) 0.05 ppm *p*-aminobenzoic acid; (c) 0.2 ppm folic acid; 0.5 ppm nicotinamide.

an improvement in sensitivity for determining vitamins by HPLC–ED compared with absorbance detection.

We determined ascorbic acid and nicotinamide in tablets and glucose powder containing multivitamins (Table I). Ascorbic acid was detected by amperometric detection and nicotinamide by absorbance detection. The contents found agreed well with the label claim and the reproducibility of this procedure was acceptable.

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