Low-pressure Liquid Chromatography With Electrochemical Detection for the Determination of Vitamin A in a Multi-vitamin Preparation

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Low-pressure liquid chromatography with electrochemical detection has been applied successfully to the determination of vitamin A in a liquid multi-vitamin preparation. Details of the stationary phase, column and other components are given. A well-jet cell containing a glassy carbon working electrode, operated in the oxidative mode, was used as the detection system. Optimisation of the chromatographic separation was achieved by varying the percentage of methanol in the mobile phase. For one of the columns investigated a mobile phase containing 65% methanol - 0.075 M acetate buffer (pH 5.0) gave a retention time of 2.8 min which was suitable for the determination of the vitamin in multi-vitamin samples; the coefficient of variation was found to be 3.8% (n = 10). The recovery of the vitamin was found to be within the manufacturer's specifications. The limit of detection was 300 pg injected (based on a signal to noise ratio of 3 : 1) which is much lower than that expected for vitamin A in pharmaceutical products.

Keywords: *Low-pressure liquid chromatography; electrochemical detection; vitamin A; retinol*

High-pressure (performance) liquid chromatography (HPLC) with electrochemical detection (LCEC) is now recognised as an extremely powerful analytical technique.[1–5] However, there appear to have been no reports on the application of low-pressure chromatographic systems with electrochemical detection to analytical determinations. Low-pressure liquid chromatography is commonly used as a preparative technique.[6–8] Low-pressure LCEC (subsequently referred to as LP-LCEC) should offer similar advantages to high-pressure LCEC, *i.e.*, high sensitivity and selectivity, but could also offer a fairly inexpensive alternative technique, particularly in areas where high resolution may not be required.

The purpose of this study was to investigate the possibility of using equipment normally applied to flow injection (FI), together with a suitable reversed-phase column for LP-LCEC, and to attempt to apply this to the determination of vitamin A in a pharmaceutical product; the electrochemical characteristics of this vitamin have been reported recently by Wring *et al.*[9] This paper describes the results of our investigations.

Experimental

Chemicals and Reagents

All chemicals were of analytical-reagent grade and were obtained from BDH unless stated otherwise. Vitamin A, (all-*trans*-retinol) was obtained from Sigma and used without further purification. This substance may degrade on storage (it must always be protected from light); hence the purity was calculated from the molar absorptivity and the absorbance measured at 325 nm.[10]

The Abidec multi-vitamin drops were a gift from Parke Davis (Pontypool, Gwent, UK); the stated concentration of vitamin A (retinol palmitate) was 4000 U per 0.6 cm^3; the other constituents of this product per 0.6 cm^3 were: vitamin D (calciferol), 400 U; vitamin C (ascorbic acid), 50 mg; vitamin B_2 (riboflavine), 400 μg; vitamin B_1 (thiamine hydrochloride), 1 mg; vitamin B_6 (pyridoxine hydrochloride), 500 μg; and nicotinamide, 5 mg.

The supporting electrolyte used in the mobile phase was prepared by mixing 1.5 M solutions of sodium acetate and acetic acid to give a pH of 5.0 (a pH meter was used). The resulting buffer was diluted with methanol and water to give the desired concentrations; the final electrolyte concentration was 0.075 M.

Instrumentation and Equipment

Electrochemical detection was performed with a Metrohm 641 VA potentiostat in conjunction with a Metrohm 656 electrochemical detector; this contained a wall-jet cell consisting of a glassy carbon working electrode, an Ag - AgCl reference electrode and a gold counter electrode.

The mobile phase was pumped through the system with a Gilson Minipuls 2 peristaltic pump at a flow-rate of 1.0 ml min^{-1}. A T-piece was placed between the pump and injector and a 15 cm length of PTFE tubing was attached to the side-arm; a small amount of air was trapped at the end of the tube by using a clamp and this served as a pulse damper. One of the columns was a 5 cm × 4 mm i.d. stainless-steel column obtained from Upchurch and was dry-packed with part of the contents of a C_{18} Sep-Pak cartridge (40-μm particle size, Waters Chromatography). The other column was a 5 cm × 3 mm i.d. glass column obtained from Anachem and was dry-packed with the same material as the stainless-steel column. The samples were injected through a Rheodyne Model 5020 low-pressure valve containing a 100-μl loop; the connections between the injector and detector were made with 0.5-mm PTFE tubing. The chromatograms were recorded with a Gould BS-271 potentiometric recorder.

Determination of Vitamin A in Abidec Drops by Low-pressure Liquid Chromatography With Electrochemical Detection

Aliquots of the Abidec drops (0.1 cm^3) were treated with potassium hydroxide solution (5% *m/V* in ethanol) (1.9 cm^3) for 20 min at 80 °C in sealed containers which were protected from light; this hydrolysed the retinol palmitate to retinol. Following hydrolysis, the alkaline solution was diluted 1 + 249 with the mobile phase. An aliquot (100 μl) of the latter dilute reaction mixture, containing retinol, was injected on to the small stainless-steel column containing the reversed-phase stationary phase mentioned above. The mobile phase used for the chromatographic separation consisted of 65% methanol - 0.075 M acetate buffer (pH 5.0) (original buffer pH); the electrochemical detector was set at a potential of +0.95 V

versus Ag - AgCl. The concentration of retinol in the final solution was calculated by reference to a calibration graph of peak current *versus* concentration of retinol (μg cm^{-3}); this was constructed from a set of external standards prepared from a stock solution of retinol dissolved in the mobile phase. The concentration of vitamin A in U cm^{-3} was calculated on the basis that 1 U cm^{-3} of vitamin A was equivalent to 0.3 μg cm^{-3} of retinol.[11]

Results and Discussion

Optimisation of Mobile Phase Composition and Applied Potential

In order to determine the optimum applied potential for electrochemical detection, following LP-LCEC, a hydrodynamic voltammogram was constructed for retinol with a mobile phase containing 65% methanol - 0.075 M acetate buffer (pH 5.0). For this study the column was removed from the system and the potential of the working electrode was increased in 50-mV steps between 0.7 and 1.3 V *versus* Ag - AgCl (Fig. 1). Retinol showed two separate waves, which is consistent with our earlier observations.[9] The highest currents were achieved at potentials greater than 1.1 V, but the level of noise was also highest at these potentials. Therefore, we chose a potential on the plateau of the first wave; a suitable value was found to be +0.95 V. It should be added that the position of the plateau on the two hydrodynamic waves was the same whether the eluent contained 65 or 95% methanol; however, the limiting currents were greater for the latter (results not shown).

Initial liquid chromatographic studies were performed with the stainless-steel column that had been dry-packed with the contents from a C_{18} Sep-Pak cartridge. Fig. 2 shows that retinol was suitably retained when the mobile phase contained 65% methanol - 0.075 M acetate buffer (pH 5.0); therefore, this was used in all subsequent studies. It should be added that similar investigations were performed with the small glass column containing the same stationary phase. However, the chromatographic peaks obtained were much broader than those obtained with the stainless-steel column, even with methanol concentrations higher than 65%. This may be due to larger dead-volumes at the end-fittings of this column and/or to differences in the types of frit employed. As the metal column was superior it was used in the remainder of this work.

Calibration, Linear Range and Limit of Detection for Standard Retinol Solutions

The calibration graph for retinol was found to be rectilinear over the range 6–180 ng injected. Although we were not attempting to carry out low-level detection of retinol, it was still of interest to ascertain the limit of detection of the method. Fig. 3 shows a chromatogram obtained for only 0.6 ng of retinol; this was recorded with a full-scale deflection of 5 nA. Based on a signal to noise ratio of 3 : 1 the detection limit was calculated to be 300 pg. Clearly, the technique has potential in applications where the determination of trace amounts of retinol is required.

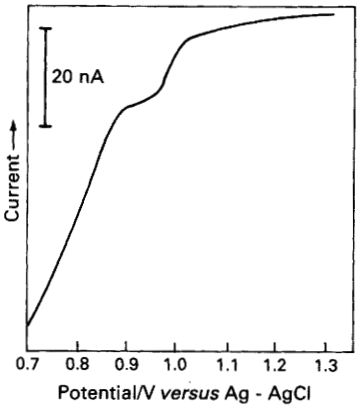

Fig. 1. Hydrodynamic voltammogram for retinol in 65% methanol - 0.075 M acetate buffer (pH 5.0); 60 ng of the vitamin were injected and the column has been removed from the system

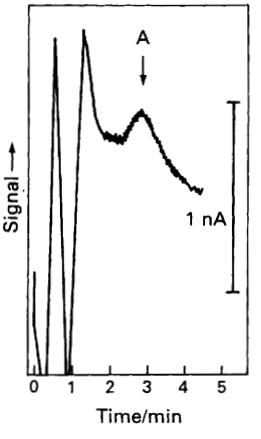

Fig. 3. Low-pressure LCEC chromatogram of 0.6 ng of retinol dissolved in the mobile phase. Current range, 5 nA. A, Vitamin A

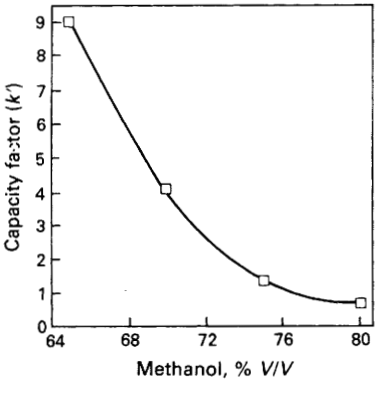

Fig. 2. Variation of capacity factors of retinol with the percentage of methanol in the mobile phase

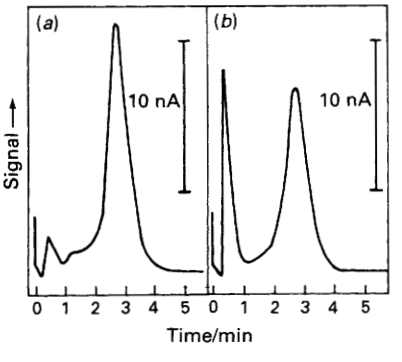

Fig. 4. Low-pressure LCEC chromatograms of (*a*) 60 ng of a retinol standard dissolved in the mobile phase and (*b*) Abidec drops following hydrolysis and dilution with the mobile phase. Current range, 50 nA

Table 1. Concentration of retinol in individual Abidec samples

Sample No.	Retinol found*/ U cm^{-3}
1	7237
2	6900
3	7320
4	7993
5	7320
6	7573
7	7490
8	7320
9	7227
10	7407
Mean:	7379
Standard deviation:	281
Coefficient of variation:	3.8%

* The retinol concentration in U cm^{-3} is equivalent to vitamin A as palmitate.[10]

Determination of Vitamin A in Abidec Drops by Low-pressure Liquid Chromatography With Electrochemical Detection

We were particularly interested in ascertaining whether the proposed LP-LCEC system could separate and detect vitamin A in a complex multi-vitamin preparation without a solvent extraction step. Solvent extraction and alkaline hydrolysis are both necessary stages in the spectroscopic method described in the British Pharmacopoeia;[12] these steps are time consuming.

Vitamin A is present in the Abidec drops as retinol palmitate and may contain some degradation products formed during storage; these forms of vitamin A required saponification with potassium hydroxide to produce retinol. Samples were treated as described earlier and injected on to the column. Fig. 4(*b*) shows that the retinol peak was well resolved from a large, early eluting peak; this was presumably due to the electro-oxidation of some of the other electroactive vitamins;[13,14] also shown for comparison is the peak obtained for standard retinol [Fig. 4(*a*)]. The method was evaluated by carrying out ten replicate determinations on individual aliquots of Abidec drops. Table 1 shows the results together with the calculated precision. The manufacturer's stated concentration of vitamin A is 4000 U per 0.6 cm^3, *i.e.*, 6667 U cm^{-3} and in this study a mean value of 7379 U cm^{-3} was found; therefore, the mean recovery was calculated to be 110%. This value is well within the expected range as the manufacturers actually add an excess over the stated value to allow for degradation of the vitamin during storage.[15] Therefore, the recovery and precision data (coefficient of variation = 3.8%) indicated that the proposed LP-LCEC method was reliable and may be suitable for the routine determination of vitamin A in Abidec drops.

Conclusions

The investigations described here indicate that LP-LCEC can be applied to the routine determination of vitamin A in a multi-vitamin preparation (Abidec multi-vitamin drops); solvent extraction is not necessary; hence the method is more rapid than that described in the British Pharmacopoeia.[12] This procedure may also be applicable to other multi-vitamin preparations containing vitamin A because these are likely to contain similar mixtures of the vitamins. The technique is fairly rapid, simple and reasonably inexpensive compared with the usual HPLC systems. We intend to evaluate this technique further for the determination of other fat-soluble and water-soluble vitamins present in pharmaceuticals. We also intend to investigate the possibility of automating the system described in this study.

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