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Analysis of Vitamins A and E in the Plasma of Animals by High-Performance Liquid Chromatography

Poziom witamin A i E w plazmie zwierząt określony metodą HPLC

INTRODUCTION

An often disregarded part of modern chemistry is the separation of compound mixtures. For the analysis of biologically significant materials, the use of high-performance liquid chromatography (HPLC) has proved successful. HPLC methods continue to find higher success rates for measurement of metabolites as regards the nutrition of farm animals. In the analysis of lipophylic vitamins, colorimetric methods have until recently been employed, and later also column liquid chromatography. The HPLC method, however, has several undeniable advantages, such as: speed and repeatability of distribution, low consumption of samples, elimination of interfering influences, and the possibliity of separating even thermolabile liquid and lipid materials. The literature describes a number of procedures for independent measurement of vitamins A and E by the HPLC method.

Biery et al. (1) and De Leenheer et al. (4) describe procedures of separation which allow simultaneous assay of vitamins A and E with the use of the synthetic analog for vitamin E, tocol, as an internal standard. This work compares results of analyses of vitamin A and E in animals blood plasma, obtained by the HPLC method and by standard spectrophotometric methods (3).

MATERIALS AND METHODS

Chemicals. Vitamin DL-retinol and alpha-tocopherol, as well as internal standard retinyl acetate and tocopheryl acetate, came from the firm BASF (Ludwigshafen, Germany), methanol p.a. from Lachema (Brno, CSFR) and n-hexane p.a. from international Enzymes Ltd. (Great Britain). Blood plasma obtained from clinically healthy cows.

Sample preparation. 100/µl of plasma was piped into centrifugal test tubes 75 × 10 mm. 10/µl of ethanol, containing a known quantity of retinyl acetate (1 µg/ml) and tocopheryl acetate (50 µg/ml) as internal standards was added to the plasma. After agitation, 500/µl of n-hexane was added and mixed by hand mixer. After centrifuging for 5 min at 3,000 r/min 400 ml of supernatant was transferred to a 10 ml test tube. The supernatant was evaporated by nitrogen at

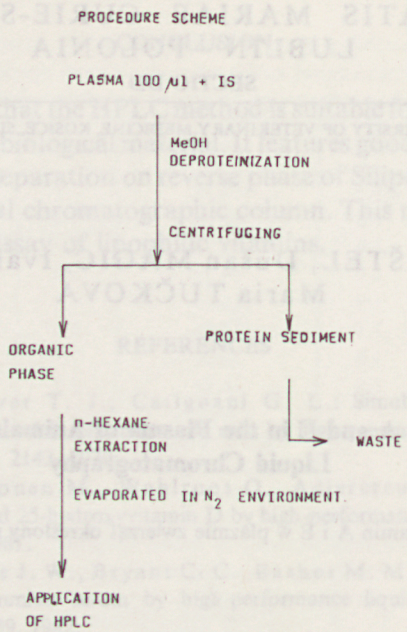

Fig. 1. The scheme of isocratic method procedure
Schemat przygotowania próbek plazmy do oznaczeń chromatograficznych

a 50°C. The remainder was dissolved in 100/µl of methanol and injected into the chromatographic column.

High-performance liquid chromatography. The liquid chromatograph system SP 8,700, with UV-VIS detector with variable wavelength, model 770 from SPECTRA PHYSICS (Santa Clara, CA, USA) was used for separation. At the intake valve we connected a 50 µl loop. Vitamins were analysed by isocratic reverse-phase chromatography on CGC glass column (150 mm × 3.2 mm I.D.) packed with Separen Six, C 18.5 mm from Laboratorni pristroje (Prague, Czech Republic). The mobile phase for vitamins determination was methanol/water (94.3:5.7) with flow rate of 1.5 ml/min and column pressure of 16 MPa. Monitoring was at an optimum wavelength of 294 nm. Fig. 1 represents a summary of the working procedure.

RESULTS AND DISCUSSION

On the basis of preliminary measurement with equal stationary phases, a number of procedures for the separation of vitamins was tested. Conditions for separation of standard mixtures, by changing the composition of linear gradient and appropriate wavelength of the UV-VIS detector, were optimized. A typical chromatogram of standard solution containing retinol, alfa-tocopherol and IS retinyl acetate and tocopheryl acetate is in Fig. 2. Finally, a chromatogram of calves blood plasma extract was obtained, when at the flow rate of 1.5 ml/min a complete analysis was accomplished in 10 min. (Fig. 3). In the run of one internal standard – retinyl acetate – the analysis took just 6 min. The separation

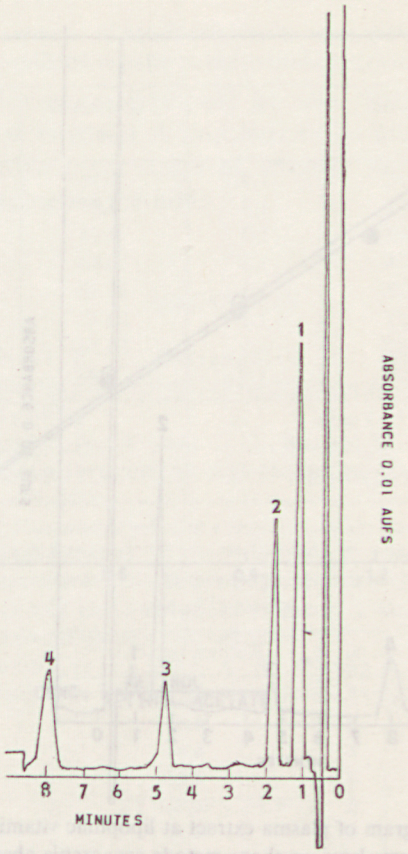

Fig. 2. Chromatogram of standard mixture of some vitamins and internal standards of isocratic elution. Respective peaks: 1 – retinol, 2 – retinyl acetate, 3 – alpha-tocopherol, 4 – tocopheryl acetate
Chromatogram standardowej mieszaniny witamin. Piki chromatograficzne oznaczają: 1 – retinol, 2 – octan retinolu, 3 – alfa-tokoferol, 4 – octan tokoferylu

capability of the employed column for the stated experiment is documented in Table 1 which presents relevant capacity factors of assayed vitamins. Assay values of recovery, as Table 2 documents, agree with the literature mentioned (2). Not of least significant is the repeatability of procedures used (Table 3) with regard to quantitative assay of tested vitamins in cow plasma. In the case of retinol, there was found a concentration in cow plasma of 3.6 μmol/l (coefficient of variation $v = 4.2\%$), while with alpha-tocopherol the value was higher than 10.0 μmol/l, though with higher coefficient of variation ($v = 5.5\%$). This fact is in some measure related to the detection limit at maximum sensitivity and at wavelength 294 nm, which is in agreement with the literature (4) at 0.349 μmol/l for vitamin A, and 1,84 μmol/l for vitamin E. In spite of the prolonged time of the analysis, which is to some extent related to a decrease in the flow rate of the linear

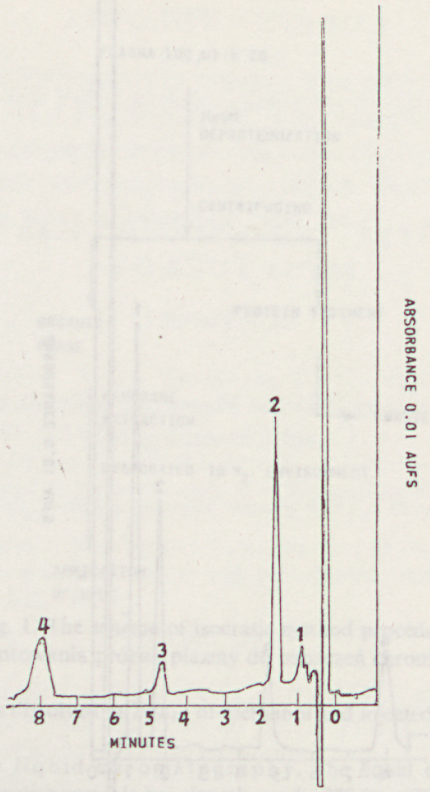

Fig. 3. Chromatogram of plasma extract at lipophilic vitamins isocratic mode
Chromatogram wyciągu plazmy krwi uzyskany metodę oznaczania obecności witamin rozpuszczalnych w tłuszczach

Tab. 1. Capacity rations (k') of chromatographic peaks
Współczynniki wydajności (k') pików chromatograficznych

Vitamins	(k')
Retinol	1.85
Retinyl acetate	3.54
Alpha-tocopherol	11.36
Tocopheryl acetate	19.51

Tab. 2. Analytical recovery of added lipophilic vitamins in plasma
Analityczny odzysk dodanych witamin lipofilnych w plazmie krów

Substances	$\bar{x}$ %	SD %	CV %	n	Limits (μmol/l)
Retinol	97.2	+2	2.1	5	1.047–5.235
Tocopherol	95.0	+1	1.0	5	2.3 –0.23

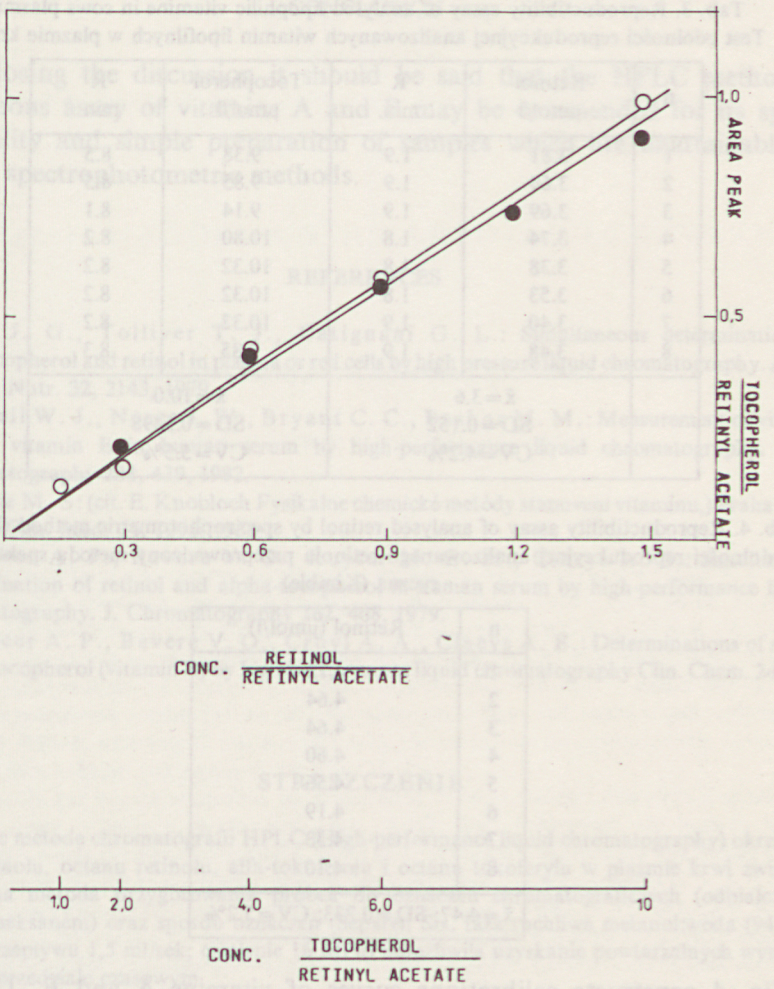

Fig. 4. Calibration curves for peak area ratio versus concentration ratio for retinol – retinyl acetate and alfa-tocopherol – tocopheryl acetate

Krzywe kalibracyjne dla stosunku: wielkość pola piku do stężenia dla retinolu – octanu retinolu oraz dla alfa-tokoferolu i octanu tokoferylu

gradient, when compared to the work of Driskell et al. (2) an improvement of separation was achieved. The advantage of this method is the use of less amount of physiological material as well as a longer lifetime of the column. Driskell et al. (2) ran up to 700 analyses in the same column. It is important to remember that it is possible to use a solution of internal standard in methanol for about 14 days, if preserved from light at 4°C.

Tab. 3. Reproductibility assay of analysed lipophilic vitamine in cows plasma
Test zdolności reprodukcyjnej analizowanych witamin lipofilnych w plazmie krów

n	Retinol µmol/l	R min.	Tocopherol µmol/l	R min.
1	3.81	1.9	9.38	8.5
2	3.69	1.9	9.85	8.5
3	3.69	1.9	9.14	8.1
4	3.74	1.8	10.80	8.2
5	3.38	1.8	10.32	8.2
6	3.53	1.8	10.32	8.2
7	3.40	1.9	10.32	8.2
8	3.48	1.9	9.85	8.3
	$\bar{x}=3.6$ SD = 0.152 CV = 4.2%		$\bar{x}=10.0$ SD = 0.5498 CV = 5.5%	

Tab. 4. Reproductibility assay of analysed retinol by spectrophotometric method (Kimble)
Test zdolności reprodukcyjnej analizowanego retinolu przeprowadzony metodą spektrofotometryczną (Kimble)

n	Retinol (µmol/l)
1	4.72
2	4.64
3	4.64
4	4.60
5	4.56
6	4.19
7	4.33
8	4.10
$\bar{x}=4.47$; SD = 0.233; CV = 5.2%	

Fig. 4 constructs calibrations curves of vitamins A and E. They were determined from surface proportions and concentration of relevant vitamins and internal standards. The equation of the calibration curve for vitamin A is $y=1.25x$, with correlation coefficient $r=0.9991$; for vitamin E, $y=0.098x$, with $r=0.9976$. The values of the correlation coefficients suggest a good linearity of calibration curves.

A comparison of the repeatability of the assay of vitamin A by HPLC with assay by spectrophotometry shows that the HPLC method features better repeatability (Tables 3 and 4). By the spectrophotometric method we got values represented in Table 4, for vitamin A of $\bar{x}=4.47$ µmol/l, with CV = 5.2%. Higher values of vitamin A in the spectrophotometric assay were probably caused by the interference of carotenoids. These effects are not present in the HPLC method because of the separation out of carotenoids in the column.

CONCLUSION

On closing the discussion it should be said that the HPLC method of simultaneous assay of vitamins A and E may be commended for its speed, repeatability and simple preparation of samples which are unattainable by common spectrophotometric methods.

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STRESZCZENIE

Stosując metodę chromatografii HPLC (High-performance liquid chromatography) określono poziom retinolu, octanu retinolu, alfa-tokoferolu i octanu tokoferylu w plazmie krwi zwierząt. Zastosowana metoda przygotowania próbek do oznaczeń chromatograficzych (odbiałczanie, ekstrakcja heksanem) oraz sposób oznaczeń (Separen Six, faza ruchliwa metanol:woda (94,3:5,7 szybkość przepływu 1,5 ml/sek; ciśnienie 16 MPa) umożliwiła uzyskanie powtarzalnych wyników w krótkim przedziale czasowym.