Simultaneous determination of retinol and α-tocopherol by high pressure liquid chromatography in micro-volumes of serum

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ABSTRACT

The lack of biochemical data to indicate the prevalence of marginal vitamin A deficiency (VAD) in the country is largely because of the difficulty of obtaining enough serum for analysis, especially from malnourished children. Efforts were therefore made to establish a high-pressure liquid chromatography system (HPLC) for the determination of retinol in micro-volumes of serum. Since our previous studies showed that tocopherol could be simultaneously determined in the same system, studies have been carried out for the determination of both vitamins using retinol acetate and -tocopherol acetate as internal standards. Trials were carried out to determine the most suitable sample treatment procedures and chromatographic system including composition of the mobile phase for handling 20 μl of serum. The HPLC system proposed enables successful separation and quantitation of retinol and α-tocopherol and their respective internal standards, retinol acetate and -tocopherol in less than 14 minutes. Reproducibility studies carried out with pooled sera showed a within day and between day variation of less than 8% and 13% respectively for retinol, whilst variations for α-tocopherol were higher, ranging from 8-16%. The proposed method is currently being applied to the determination of retinol and α-tocopherol in a group of malnourished children under six years of age.

INTRODUCTION

Vitamin A is not only essential for vision and eye health but is also critical for child health and survival. Recent studies suggest that ill health and risk of death from some infections are increased in children who are not clinically deficient but whose vitamin A body stores are depleted. There is therefore a continuing global emphasis on the need to assess the vitamin A status of population groups. It has been reported that vitamin A deficiency (VAD) is not a major problem in Malaysia, but this has been based on the lack of eye signs and no large scale biochemical data are available to indicate marginal deficiencies (Tee,

1993). The lack of biochemical data is largely because of the difficulty of obtaining enough biological material (eg serum) for analysis, especially from malnourished children.

This laboratory has been carrying out a series of studies into the development of improved methods for the determination of vitamins in food and serum using HPLC. A method previously developed for the estimation of retinol, tocopherol and carotenoids by HPLC (Tee & Khor, 1995) required an amount of 200 µl or more of serum to obtain reliable results. Trials carried out showed great variability in results when using lesser amounts of serum. It would obviously be advantageous if the volume of serum used can be reduced as biological samples, especially from children, are difficult to obtain and many biochemical tests cannot be done because of shortage of sample. This paper reports the establishment of a HPLC method that enables the simultaneous determination of retinol and -tocopherol in micro-volumes of serum. Experiments carried out on various aspects of the method are described, including sample extraction procedure, mobile phase composition, the use of internal standards (retinol acetate and α-tocopherol acetate) and reproducibility studies.

MATERIALS AND METHOD

HPLC conditions

A Gilson liquid chromatography system was used, comprising a 305 piston pump connected to a 805S manometric module and a 234 autoinjector with a 500 µl interchangeble sample loop. The detector used was a 119 UV detector with dual wavelength detection at 325 nm at 0.002 absorbance unit full scale and 295 nm at 0.002 absorbance unit full scale. The pump and detector were controlled by the 715 system controller software which saved, viewed and enabled re-integration of all stored chromatographic data. All chromatograms and results analysed were then recorded on a Hewlett Packard 5L laser printer. A stainless steel 30 cm by 3.9 mm I.D. 10 µm µBondapak C_{18} column was used for the chromatographic separation. This was preceded by a Sentry guard column holder housing a disposable guard column insert which was packed with the same material as that in the analytical column.

Chemicals and reagents

Solvents used for preparation of mobile phase for liquid chromatography were of HPLC grade and ultra pure water of resistivity of around 18 megohms centimetres (MΩ - cm) was used. Ethanol used for standard preparation and sample extraction was HPLC grade. Retinol and -tocopherol used as standards and retinol acetate and -tocopherol acetate as internal standards were from Sigma Chemicals (USA). Ascorbic acid used was analar grade. Petroleum ether (b.p. 40 - 60°C) used for sample preparation was analytical grade as HPLC grade was not available in the market. The solvent was redistilled to prevent interference with retinol peak during the chromatographic run.

Preparation of mobile phase

Two mobile phase systems were used in the study. The first was a mixture of acetonitrile, methanol and ethyl acetate (88:10:2, v/v) developed by Tee, Lim & Chong (1994) and Tee & Khor (1995). The second mobile phase comprises a mixture of methanol and water (95:5, v/v). All mobile phases solutions were filtered through a 47 mm, 0.45 µm nylon membrane filter and subsequently degassed for 15 minutes with an ultra-sonic bath. The analytical column was washed with at least 100 to 150 ml of mobile phase and then equilibrated at a flow rate of 2 ml per minute before injecting 100 µl of the standard mixture.

Preparation of standard solutions

Retinol, retinol acetate, -tocopherol and -tocopherol acetate of 100 µg per ml were prepared in HPLC grade ethanol. These standard solutions were kept refrigerated in amber containers and were found to be stable for 3-4 months.

Absorbance readings of each standard was also taken before each assay and the appropriate absorptivities (extinction coefficients) of De Ritter & Purcell (1981), Schudel, Mayer & Isler (1972) and Sigma Chemicals were used to calculate the exact concentrations of each compounds as given in Table 1. The preparation of all standard solution were carried out rapidly in a room with subdued light and with all windows tinted with a light-protective film. All sample extraction and analytical procedures were also carried out in this room.

Quantitation using internal standards

Internal standards were added to the sample at the beginning of the extraction procedure to compensate for losses at each step of the sample preparation. After processing as described in the extraction procedure, the final extract was filtered with a 4 mm, 0.45µm disposable membrane filter and then chromatographed for the simultaneous determination of the above vitamins.

Calibration curves for the two standards were prepared weekly and unknown samples quantitated by the software using the standard curves obtained. Quantitation was accomplished by comparing the peak

Table 1. Extinction coefficient of vitamin standards

Standards	Solvent (wavelength maxima, nm)	Extinction Coefficient	Reference
Retinol	Ethanol (325)	1570	Sigma Chemical Co.
Retinol acetate	Ethanol (325)	1550	Sober HA (1970)
α-tocopherol	Ethanol (292)	75.8	Schudel, Mayer & Isler (1972)
α-tocopherol acetate	Ethanol (298)	43.6	Sober HA (1970)

area of the analyte in the unknown to the peak area of the internal standard in the standard solution i.e. retinol to retinol acetate and α-tocopherol to α-tocopherol acetate. With internal standard data analysis, the system controller software automatically adjusts the amount of the analyte in unknown samples, in relation to the amount of internal standard added at the beginning of the whole procedure.

Extraction procedure

A 10% solution of vitamin C in ultra-pure water was prepared and 200 µl added to a 10 ml flask containing 0.065 µg/ml of retinol acetate and 23.03 µg/ml of -tocopherol acetate and made up to 10 ml with ethanol. The addition of ascorbic acid to ethanol has been reported to prevent deterioration of retinol and tocopherol during the extraction step (Driskell, Bashor & Neese, 1985; Tee & Khor, 1995). A volume of 20 µl of this solution was added to 20 µl of serum in a 8 mm x 75 mm extraction tube and mixed for 30 seconds to precipitate the protein. 200 µl of redistilled petroleum ether was then added to extract the retinol and -tocopherol from the sample using an electronic shaker for at least 2 minutes. The extract was then centrifuged at 1300 rpm for 5 minutes to separate the petroleum ether layer which was then removed with either a pasteur pipette or micropipette into a 1.5 ml, eppendorf tube. The extract was completely dried using a vacuum concentrator (Hetovac HS-1-110) for 10 minutes after which 200 µl of ethanol was added and then mixed with a vortex mixer. After a period of 5 to 10 minutes, the extract was filtered with a 0.45 µm disposable membrane filter into a disposable vial for chromatography using the Gilson's 234 autoinjector. Not more than 100 µl of the extract was injected as excess solvent would result in ethanol interfering in the HPLC chromatogram.

RESULTS AND DISCUSSIONS

Development of an internal standard method for the quantitation of retinol and α-tocopherol

Experiments were initially carried out using the procedure previously developed by this laboratory (Tee, Lim & Chong, 1994; Tee & Khor, 1995) for the simultaneous detection and quantitation of retinol, tocopherols and carotenoids, employing a mobile phase comprising a mixture of acetonitrile, methanol and ethyl acetate (88:10:2, v/v/v) to determine if the method could still be used if the volume of blood used is reduced to one-tenth. Aliquots of 20 µl of pooled serum were used and extracted with an equal volume of ethanol containing vitamin C. Extraction and evaporation of solvent was carried out as previously reported but the volume of petroleum ether was similarly scaled down. Results obtained showed poor reproducibility.

It was thus felt necessary to explore the use of internal standards which are often used to help correct for small preparation errors and for minor evaporation of sample injection solvent. It has been recognized, however, that gross errors in sample preparation will not be compensated.
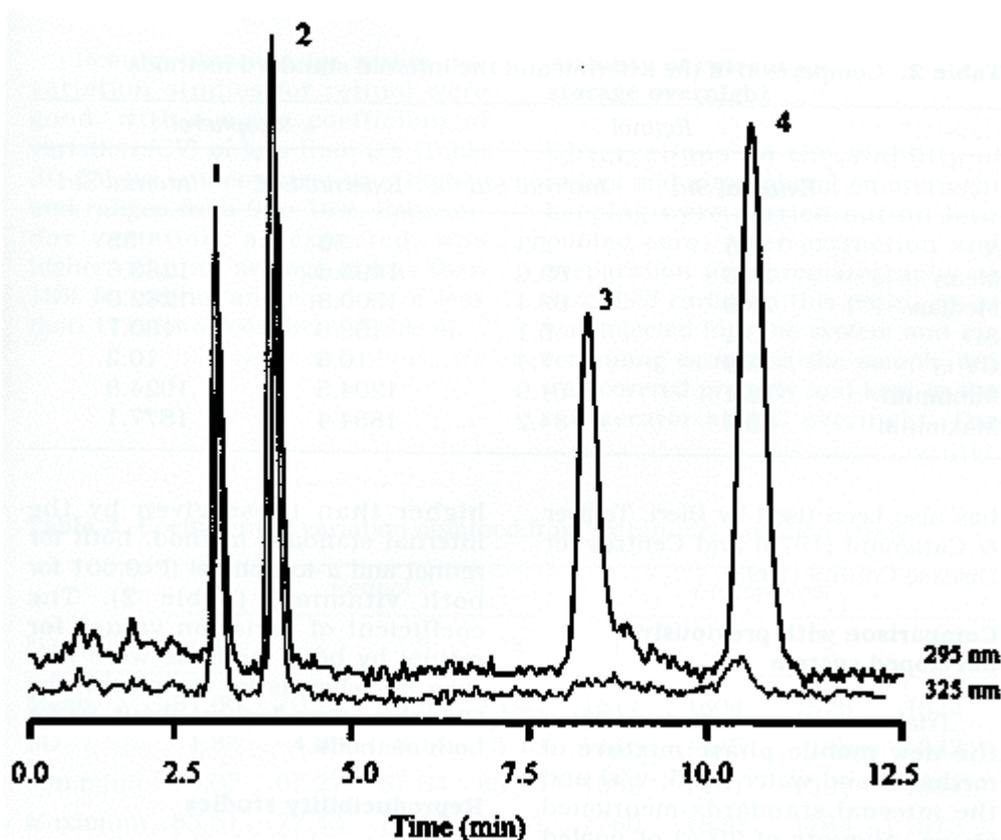
Figure 1. HPLC chromatogram of standard mixture. Retinol and a-tocopherol were detected at 325 nm and 295 nm respectively at 0.002 AUFS. Other chromatography conditions are as given in text.

1 = retinal; 2 = retinal acetate; 3= α-tocopherol; 4 = α-tocopherol acetate.

Bieri, Tolliver & Catignani (1979), Centres for Disease Control (1993) and Zahur, Fielden & Frost (1993) had reported the utilisation of retinol acetate as an internal standard for the detection of retinol and -tocopherol acetate for α-tocopherol. Trials carried out using the mobile phase previously used in this laboratory (Tee & Khor, 1995) showed that the system was not suitable for use as it could not separate retinol, α-tocopherol and the 2 internal standards.

Further trials were therefore carried out to establish a suitable mobile phase. A solution of 100% methanol was found to give poor separation for the above four vitamins but the introduction of water from 1% to 5% improved separation, the optimum mobile phase being 95% methanol: water, giving complete separation within 14 minutes. Retinol was eluted at 2.8 minutes, retinol acetate at 3.6 minutes, α-tocopherol at 8.1 minutes and -tocopherol acetate at 10.5 minutes (Figure 1). This mobile phase

Table 2.   Comparison of the external and the internal standard methods

	Retinol		α-tocopherol	
	External Std	Internal Std	External Std	Internal Std
N	55	53	50	53
Mean	78.6	69.6	1493.6	1280.6
Median	78.6	69.4	1500.3	1262.0
SD	3.1	5.1	158.8	130.7
CV	3.9	7.4	10.6	10.2
Minimum	72.1	61.9	1204.5	1024.8
Maximum	85.9	84.2	1854.4	1877.1

has also been used by Bieri, Tolliver & Catignani (1979) and Centres for Disease Control (1993).

Comparison with previously developed system

Trials were then carried out using the new mobile phase mixture of methanol and water (95:5, v/v) and the internal standards mentioned above. Aliquots of 20 μl of pooled sera were used in these experiments and were processed and chromatographed as described in the section on Materials and Method. With the chromatography conditions as described, the detection limits for the assay were 0.1457 ng for retinol and 0.5055 ng for -tocopherol. The same pooled sera (200 μl aliquots) were also analysed using the previously developed method of Tee & Khor (1995) where the vitamins were separated using a mixture of acetonitrile, methanol and ethyl acetate (88:10:2, v/v/v) and quantitated using external standards.

When compared using t-test, results obtained by the external standard method were significantly higher than those given by the internal standard method, both for retinol and α-tocopherol ($P < 0.001$ for both vitamins) (Table 2). The coefficient of variation values for retinol by both methods were less than 8% whereas for tocopherol, the CV values were higher, about 10 for both methods.

Reproducibility studies

Blood samples that were sent to the laboratory for determination of serum lipid profile were pooled for these studies. Only samples which were clear and from apparently healthy subjects were pooled and fibrin clots removed by centrifugation. Aliquots of the pool sera were then kept in 0.5 ml eppendorf tube for storage at -20°C and each tube discarded once thawed.

Within-day and between-day variation studies were carried out using 0.02 ml aliquots of the pooled sera. The extraction procedure used is as described earlier in this report and chromatographed using mobile phase of 95% methanol:water.

Results obtained for within-day variation studies for retinol were good, with a mean coefficient of variation (CV) of less than 8% (Table 3). CV for -tocopherol was higher and ranged from 9 to 16%. Between-day variation, as expected, was higher; with an average of less than 13% for retinol and equal to or less than 11% for α-tocopherol (Table 4).

Stability of extract on storage overnight

Observations on the stability of retinol and α-tocopherol on overnight keeping were carried out on four pooled sera. After extraction and preparation for chromatography as described earlier in this paper, 80 μl was injected into the system and the remaining extract in the sample vial was covered properly and kept in the refrigerator at 4°C overnight. The

Table 3: Coefficient of variation obtained from within-day variation study

	Retinol				α-tocopherol			
Day	1	2	3	4	1	2	3	4
Mean	81.60	70.75	74.53	73.44	1511	1604	1575	1524
SD	1.85	4.09	5.14	5.18	146	145	195	242
Minimum	79.07	67.27	67.64	65.83	1383	1464	1307	1344
Maximum	83.51	77.43	78.95	78.61	1760	1830	1765	1913
CV	2.27	5.78	6.90	7.05	9.64	9.07	12.41	15.87

Values in table were obtained from 5 analyses per day

Table. 4: Coefficient of variation obtained from between-day variation study

	Retinol		α-tocopherol	
	Pool 1	Pool 2	Pool 1	Pool 2
Mean	71.67	60.03	1535	1678
SD	8.66	7.34	166	128
Minimum	54.38	46.73	1299	1450
Maximum	83.51	70.50	1913	1918
CV	12.08	12.23	10.79	7.65

Values in table were obtained from analysis of the 2 pools for 4-5 days (5 replicate analyses per pool)

Table 5: Retinol values on storage overnight

Pool	1		2		3		4	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Mean	81.60	83.05	70.75	73.34	75.65	82.12	58.60	59.47
SD	1.85	3.48	4.09	7.76	5.11	2.88	3.85	3.09
CV	2.27	4.19	5.78	10.58	6.75	3.51	6.57	5.20

A total of 5 replicate analyses were carried out for each pool serum

Table 6: α-Tocopherol values on storage overnight

Pool	1		2		3		4	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Mean	1511	1366	1604	1791	1718	1477	1468	1731
SD	146	68	145	604	360	126	119	359
CV	9.66	4.98	9.04	33.72	20.95	8.53	8.11	20.74

A total of 5 replicate analyses were carried out for each pool serum

next day, this stored extract was similarly chromatographed.

Results obtained showed that the extract on keeping overnight had a CV of less than 11% for retinol (Table 5). The average CV for the 4 days was 5.3% on day one as compared with a CV of 5.9% on day two showing that retinol was still stable on keeping overnight. Results obtained for -tocopherol (Table 6), on the other hand, were more variable, as can be seen from the CV values.

Conclusion

A reversed-phase HPLC method is proposed for the separation and quantitation of retinol using retinol acetate as the internal standard in micro-volumes of serum. A C_{18} column was used with a mobile phase of 95% methanol:water and the peaks detected at 325 nm. If it is desirable, tocopherol can be simultaneously determined by using a dual wavelength detector, setting the second wavelength at 295 nm. Tocopherol can be quantitated using α-tocopherol acetate as the internal standard. Both retinol and tocopherol and their internal standards can be separated using the proposed chromatographic system in 14 minutes. Greater caution and technical skill would be required when using the micro-volumes of serum. The use of a vacuum concentrator to evaporate solvents is very important to minimise loss of the vitamins analysed. As observed from the larger variations in values

obtained for tocopherol determination, there should be even greater caution when using this method for the determination of this vitamin.

When compared with the method previously developed by the authors (Tee & Khor, 1995) using 200 µl of serum and the external standard method, results obtained using the proposed method requiring only 20 µl of serum were significantly lower. This was observed for both retinol as well as tocopherol.

This established method is being used for the determination of level of retinol in a group of malnourished children under six years selected for the rehabilitation programme based on their poor growth rate.

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