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Short communication

Determination of serum retinol by reversed-phase high-performance liquid chromatography

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Abstract

A rapid, sensitive and specific high-performance liquid chromatographic method was developed for the determination of serum levels of retinol in humans. A direct serum injection technique after deproteinisation was used to avoid lengthy pretreatment steps which can result in degradation of retinol during analysis. The column used was CLC-ODS, the mobile phase was acetonitrile–water and detection wavelength was 328 nm. Deterioration in column performance was not observed even after injection of 300 samples. The lower detection limit was 10 μg/l. On analyzing a serum pool six times, a C.V of 0.7% was obtained. The method is quantitative, reproducible, rapid and highly accurate for routine analysis.

1. Introduction

Vitamin A (retinol) is essential for normal growth, vision, reproduction and epithelial differentiation in cases of malnutrition or in post-disease states especially in children [1,2]. The deficiency of vitamin A occurs and results in pathological complications such as chronic diarrhoea, low immune response, xerophthalmia and acute respiratory infections. To prevent such complications, determination of the retinol level in serum is essential so that, in cases of deficiency, proper medical treatment can be given. The acceptable serum vitamin A levels range from 200 to 700 μg/l [2,3].

The traditional methods for the determination of retinol in serum or plasma [4–6] are time-consuming, and non-specific with poor reproducibility because of oxidation, photoisomerisation, and loss of retinol due to imperfect extraction. Chromatographic methods, especially HPLC, have made the determination of vitamin A somewhat simpler. However, most of the published methods still require multiple steps, and facilities and precautions to avoid the loss of vitamin A during analysis [3,7–11]. Such facilities are generally not available in many developing countries. This is perhaps one of the reasons why very few studies have been conducted to determine vitamin A deficiency in Pakistan [12–16].

Sample preparation of biological fluids is time-consuming and a great source of error in an analytical method especially for the analytes which are light and/or air sensitive like retinol. The monograph [3] on vitamin A clinical studies includes methods which involve liquid–liquid extraction for sample preparation. These meth-

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ods, in spite of the precautions taken, are still not reliable. To overcome the problems of liquid–liquid extraction, Roth et al. [17], Wyss and Bucheli [18,19] and Rissler and Friedrich [20] have used injections of biological samples onto the chromatographic system using a number of precolumns and column-switching devices. These techniques, although useful for their particular purposes, require accessory attachments like sophisticated autosamplers with change-over valves, time relays, number of precolumns, etc. which increase the complexity of the method.

To monitor retinol deficiency, a simple and reproducible method for the determination of retinol levels in serum was required which could be performed in an ordinary laboratory equipped with an HPLC with improved recovery and without lengthy pretreatment steps.

2. Experimental

2.1. Materials and reagents

Centrifugation was performed on a centrifuge Machine 10500 × g from Abbott Diagnostics Division (Texas, USA). Injections were made with a 250-μl syringe from Scientific Glass Engineering (Vic., Australia). Acetonitrile and methanol were HPLC grade from BDH (Poole, UK) and Merck (Darmstadt, Germany). The above solvents were used without further purification. Water was double distilled using an all-glass still. The mobile phase was degassed by filtration and sonication. Retinol R-7632 standard was obtained from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Venous blood was collected (in a syringe) without anticoagulant and carefully wrapped in aluminum foil. Aliquots of blood were then transferred to a polystyrene capped centrifuge tube (1.5 ml) and centrifuged at 10 500 *g* to obtain a clear serum. All steps involved in sample preparation were carried out in subdued light thus avoiding light-induced degradation of retinol [1,7]. With the help of a micropipette (40–200 μl), 100 μl of the serum was transferred to a capped centrifuge tube (1.5 ml). It was then mixed and vortexed with 300 μl of acetonitrile for 90 s. The mixture was centrifuged again at 10 500 *g* for 60 s and the liquid phases were transferred to another capped centrifuge tube. The supernatant was injected onto the chromatographic system by puncturing the cap of the tube to avoid any loss of solvent through evaporation.

2.3. Standard preparation

A working standard was prepared by the following method: An equivalent of 25 mg of retinol was dissolved in 50 ml of methanol and 1.0 ml of this stock solution was transferred to a 10-ml volumetric flask and the volume was made up with acetonitrile. A 100-μl volume of the solution was transferred to another 10-ml volumetric flask and the volume was again made up with acetonitrile. The final solution thus contained 0.5 μg/ml of retinol. This was used as a standard injection. The standards were then treated like serum samples as described above in order to get similar conditions and concentrations for the external standard method. The retinol free sample was prepared by the method of De Ruyter and De Leenheer [7].

2.4. Chromatographic system

The analysis was carried out on LC-9A Liquid Chromatographic System (Shimadzu Corporation, Kyoto, Japan) equipped with double pumps, system controller, column oven, Rheodyne injector with 100-μl loop, variable-wavelength UV detector and CR4A Chromatopack integrator. An analytical column (5 μm, 250 × 4.6 mm I.D; CLC-ODS) and a guard column (5 μm, 10 × 4.5 mm I.D, CLC-G-ODS) packed with C_{18} sorbent (Shimadzu) were used for analysis. Acetonitrile–water (90:10, v/v), at a flow-rate of 2 ml/min was used as the mobile phase and the analytes were monitored at 328 nm. Other conditions were: column oven temperature 37°C, attenuation 4, chart speed 5 mm/min.

3. Results and discussion

Retinol is highly protein bound and is transported in the blood bound to retinol binding proteins (RPB) [2] resulting in low recoveries using on-line column techniques. In this method a simple pretreatment of 100 μl of serum sample by addition of 300 μl of acetonitrile gave excellent recoveries and clean chromatograms. Fig. 1a shows a chromatogram of retinol-free serum and Fig. 1b shows a typical chromatogram obtained from a serum sample following the conditions described in the Experimental section. The identification of the retinol peak was made on the basis of retention time. The selectivity of the method was checked by running samples on different wavelengths settings and plotting the peak area of the eluted peak vs. wavelength to assure the λ_{max} of retinol [11]. The retinol peak was eluted in less than 6 min, and as can be seen from the chromatogram, no other interferences were observed at the same retention time.

The calibration plot of retinol from serum using least square regression equation was linear within the range 150–750 μg/l; the equation of calibration being $y = 74.21x + 0.809524$, $r = 0.999919$. The lower limit of detection was 10 μg/l.

The analytical recovery of retinol from serum ranged between 97.4 and 100.9%. For this experiment, three known concentrations were added six times each in retinol free serum and results were analyzed. The results were then compared with the standards (Table 1). Six

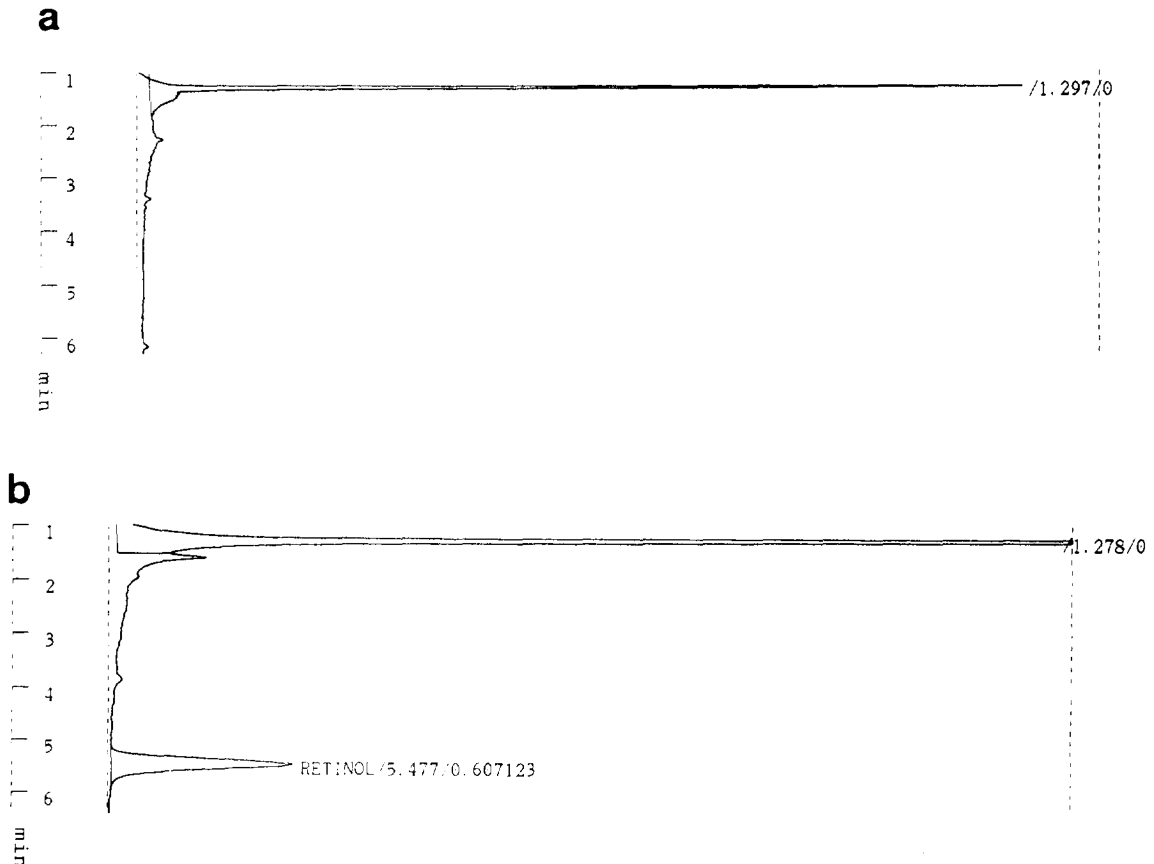

Fig. 1. (a) Chromatogram of a retinol-free serum. (b) A typical chromatogram of a serum sample.

Table 1
Analytical recovery of added retinol in retinol-free serum pool

Added (μg/l)	Recovered (mean) (μg/l)	Recovery (mean ± S.D., $n = 6$) (%)	C.V (%)
150	148.59	99.09 ± 1.21	1.22
300	295.48	98.54 ± 0.53	0.54
450	447.83	99.52 ± 0.60	0.60

replicate determinations of a serum pool resulted in a mean value of 659.61 μg/l, S.D. 5.20 and C.V. 0.7%.

The day-to-day reproducibility was determined following exactly the same injection procedure, measured over a period of 10 days for five different serum samples, stored at −18°C under nitrogen atmosphere. Results were (in μg/l ± S.D.) 634.80 ± 7.89, 606.47 ± 10.41, 563.34 ± 7.24, 650.00 ± 9.21 and 605.09 ± 11.97. The day-to-day precision (C.V.) being 1.24, 1.72, 1.28, 1.41 and 1.97, respectively.

The monograph published [3] by the International Vitamin A Consultative Group suggests that the method published by De Ruyter and De Leenheer be used for vitamin-A clinical studies. In comparison with the above method, our method is precise (0.7% against 2.5%) and more sensitive (10 μg/l against 50 μg/l) and involves only minimal sample preparation.

Table 2
Serum retinol concentration for some children

No	Age	Sex	Retinol (μg/l)
1	60 Months	Female	398.0
2	54 Months	Male	419.0
3	18 Months	Male	441.0
4	09 Months	Female	339.0
5	36 Months	Male	449.0
6	31 Months	Male	384.0
7	26 Months	Male	38.0[a]
8	42 Months	Female	105.0[a]
9	08 Months	Male	47.0[a]
10	18 Months	Male	123.0[a]
11	15 Months	Female	117.0[a]
12	36 Months	Female	134.0[a]

[a] Deficient.
These concentrations (< 150 μg/l) were calculated with a CR4A computing integrator (Shimadzu, Japan).

The overall procedure is simple and the total analysis time is relatively short (less than 10 min) including sample preparation and chromatography. More than 300 human serum samples were quantified with this method and no deterioration in column condition, shift in retention time or increase in back-pressure was observed. Routine maintenance, i.e. cleaning of upper filter of guard column, may some times be necessary when direct injection of serum samples is applied.

The method proposed requires only small amounts of serum (100 μl) and is sensitive enough to detect retinol concentrations as low as 10 μg/l. The over all procedure is simple and the total analysis time is less than 10 min. This method also does not require an additional compound as internal standard because extraction procedures are not applied and recovery problems are not encountered. The advantages of our method are high speed, high precision, selectivity, lower detection limit and direct injection of serum with minimum sample pretreatment. It is thus believed to be superior to analytical procedures previously proposed for such application. Some serum retinol levels of children from different age groups are given in (Table 2).

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References

[1] R. Wyss, *J. Chromatogr.*, 531 (1990) 481.
[2] D.S. Goodman, M.B. Sporn, A.B. Roberts and D.S. Goodman (Editors), *The Retinoids*, Vol.2, Academic Press, New York, NY, 1984, p. 41.
[3] A monograph of the International Vitamin-A Consultative Group IVACG, The Nutrition Foundation, 888 Seventeenth Street, N.W. Washington, D.C 20006, USA.
[4] P.J. Garry, J.D. Pollack and G.M. Owen, *Clin. Chem.*, 16 (1970) 766.
[5] J.D. Pollack, G.M. Owen, P.J. Garry and D. Clark, *Clin. Chem.*, 19 (1973) 977.
[6] S. Futterman, D. Swanson and R.E. Kalina, *Invest. Opthalmol.*, 14 (1975) 125.
[7] M.G.M. De Ruyter and A.P. De Leenheer, *Clin. Chem.*, 22 (1976) 1593.
[8] A.P. De Leenheer, H.J. Nelis, W.E. Lambert and R.M. Bauwens, *J. Chromatogr.*, 429 (1988) 3.
[9] M. Vecchi, J. Vesely and G. Oesterhelt, *J. Chromatogr.*, 83 (1973) 447.
[10] C.E. Parkinson and I. Gal, *Clin. Chem. Acta*, 40 (1972) 83.
[11] A.P. De Leenheer, V.O. De Bevere, G.M. Marcel, De Ruyter and A.E. Claeys, *J. Chromatogr.*, 162 (1979) 408.
[12] Nutrition survey of West Pakistan (1965–66), Government of Pakistan, Health Division, Islamabad, Pakistan.
[13] S.J. Zuberi and K. Ibrahim, *J. Pak. Med. Assoc.*, 13 (1974) 71.
[14] Micronutrient survey of Pakistan, Government of Pakistan, Nutrition and Planning Development Division, Islamabad, Pakistan, 1977–78.
[15] K. Ibrahim, M.A. Yousufi, S.N. Hasnain, S.J. Zuberi, *J. Pak. Med. Assoc.*, 37 (1987) 117.
[16] M.M. Khan, M.D Thesis, Children Hospital, Pakistan Institute of Medical Sciences, Islamabad, Pakistan, 1993.
[17] W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, *J. Chromatogr.*, 222 (1981) 13.
[18] R. Wyss and F. Bucheli, *J. Chromatogr.*, 424 (1988) 303.
[19] R. Wyss and F. Bucheli, *J. Chromatogr.*, 456 (1988) 33.
[20] K. Rissler and G. Friedrich, *J. Chromatogr.*, 569 (1991) 375.