Journal of Chromatography, 382 (1986) 284—289
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3260

Note

Microdetermination of vitamin A in human plasma using high-performance liquid chromatography with fluorescence detection

A.J. SPEEK*

TNO-CIVO Toxicology and Nutrition Institute, Department of Clinical Biochemistry, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

C. WONGKHAM, N. LIMRATANA and S. SAOWAKONTHA

Khon Kaen University, Faculty of Medicine, Khon Kaen 40002 (Thailand)

and

W.H.P. SCHREURS

TNO-CIVO Toxicology and Nutrition Institute, Department of Clinical Biochemistry, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

(First received February 19th, 1986; revised manuscript received May 20th, 1986)

In many studies, vitamin A (retinol) has received considerable attention as it is known to have an influence on metabolic processes and to play a specific part in rod vision [1]. Also, a possible role in the prevention and therapy of cancer is acknowledged [2, 3]. Furthermore, in many developing countries vitamin A deficiencies occur among children during the weaning period, which constitutes a public health problem owing to the possibly resulting xerophthalmia, which may cause blindness [1, 4].

Currently, vitamin A levels in blood plasma are measured using either direct spectrophotometric, colorimetric and fluorometric methods [5], or high-performance liquid chromatographic (HPLC) methods with UV [6—10] or fluorometric detection [11, 12]. However, these methods have several drawbacks. Direct optical methods may be subject to interference from endogenous compounds and are time-consuming. Generally, the HPLC methods require at least 100 μl of plasma. In addition, many methods require evaporation of the extracting solvent and redissolving of the residue since the extraction and HPLC solvents are not compatible [7—11]. This step, even if performed under

0378-4347/86/$03.50

nitrogen, may cause loss of vitamin A and, moreover, it makes large-scale routine analysis more time-consuming.

As part of a nutritional intervention study [13], plasma vitamin A levels had to be determined in a large group of Thai children of which only a few capillaries filled with whole blood were available. In this connection, we developed an HPLC method for the determination of vitamin A in only 5 μl of plasma. After dilution of plasma with physiological saline followed by deproteinization with ethanol, vitamin A was extracted with *n*-hexane. This extract was directly injected onto a normal-phase HPLC column. The fluorometric detection permitted vitamin A determination down to concentrations below those normally occurring in human plasma.

EXPERIMENTAL

Apparatus

HPLC was performed using a Waters Model 6000 A constant-flow pump, a Waters Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a Shimadzu Type RF-530 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan). A Knauer stainless-steel column (4.6 mm I.D.) was modified to a length of 8 cm and laboratory-packed with Polygosil 60-5, 5 μm (No. 8283, Macherey-Nagel, Düren, F.R.G.) by the balanced-density slurry technique on a column-packing apparatus designed at our institute, using a Haskel pump Type DSTV 150 (Ammann Technik, Stuttgart, F.R.G.). The slurry and packing solvents were methylene chloride and *n*-hexane, respectively (Nos. 6050 and 4367, Merck, Darmstadt, F.R.G.). Elution profiles were displayed on a Kipp BD-8 recorder (Kipp & Zonen, Delft, The Netherlands).

Sample collection

Capillary blood (finger-prick) was obtained from Thai children in the age range of 3—6 years (living in six villages in the Sakhon Nakhon province, Northeastern Thailand) in heparinized 75-μl capillaries (No. HI 9100275, Hirschmann Glasgerätebau, Eberstadt, F.R.G.) and stored in the dark at 6°C for not more than 3 h. Plasma and cells were then separated using a micro-haematocrit centrifuge. Plasma was collected in a 0.4-ml sample vial (No. 0010 036.004, Merck) and stored in the dark at —20°C for analysis within one month.

Plasma extraction

The extraction of plasma was carried out in a room protected from direct sunlight. Using a micropipette, 5 μl of plasma were brought in a 50 × 6 mm glass tube containing 95 μl of physiological saline (9 g/l sodium chloride). The contents were mixed with 100 μl of ethanol containing 1.5 g/l butylated hydroxytoluene (BHT) and then thoroughly vortex-mixed for 1 min with 200 μl of *n*-hexane containing 0.25 g/l BHT. The tube was centrifuged at 1000 *g* for 5 min and stored in the dark at —20°C for HPLC analysis within 24 h.

Standardization

A stock standard solution of vitamin A (all-*trans*-retinol, No. 95144, Fluka, Buchs, Switzerland) was prepared by dissolving ca. 50 mg in 100 ml absolute ethanol. The vitamin A concentration of this solution was determined by measuring the absorbance at 325 nm of a 100-fold dilution in a suitable spectrophotometer using $A^{325}_{1\,cm} = 1832$ for a 1% (w/v) solution of all-*trans*-retinol in absolute ethanol [14].

A working standard solution was obtained by a dilution of the stock standard solution with *n*-hexane to a concentration of ca. 50 nmol/l. This solution and the stock standard solution were stored in the dark at −20°C and were stable for at least one month.

Recovery experiments

A recovery solution containing 70 μmol/l retinol was prepared by dilution of the stock standard solution with dimethylacetamide (No. 803235, Merck). The recovery of vitamin A added to plasma was determined by analysing 5-μl aliquots of plasma to which 10 μl of the recovery solution per millilitre of plasma had been added. In this way the original vitamin A concentration was increased by 0.7 μmol/l of plasma.

High-performance liquid chromatography

HPLC analysis was carried out by injection of 100 μl of the *n*-hexane layer onto the Polygosil column using a calibrated syringe. The column was eluted isocratically with a mobile phase consisting of *n*-hexane—methylene chloride—2-propanol (90:10:1, v/v/v) (Nos. 4367, 6050 and 9634, respectively, Merck) at a flow-rate of 1.2 ml/min. The effluent was monitored using the fluorescence spectrophotometer set at the excitation/emission wavelength pair of 333/470 nm. The recorder was set at 10 mV full scale. The duration of the chromatographic run was ca. 5 min per sample.

The vitamin A concentration of the original plasma sample was calculated from peak heights with the working standard solution as reference. In routine analysis the working standard solution was run before each series of five samples.

RESULTS

Characteristics of the HPLC procedure

Typical HPLC elution profiles of the *n*-hexane extracts of plasma samples obtained from Thai children and of the working standard solution are shown in Fig. 1. The HPLC procedure described afforded an excellent separation of vitamin A. In blank experiments only a solvent peak was detected.

The fluorescence response of vitamin A was linear for concentrations of 0.06 up to 8 μmol/l plasma. At a signal-to-noise ratio of 3, the limit of detection of the vitamin A assay described in this paper is 50 nmol/l.

The frequency distribution of the vitamin A concentration in plasma of the children investigated is shown in Fig. 2. Extensive results of this study [13] will be published later.

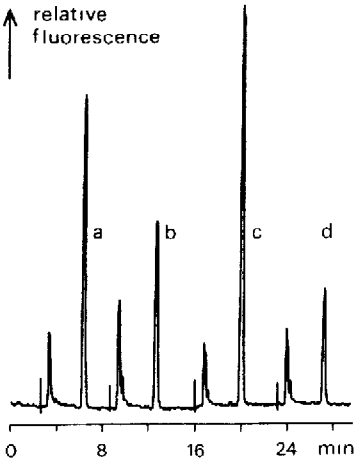

Fig. 1. Typical HPLC elution profiles of the working standard solution (a, 50 nmol/l) and of *n*-hexane extracts of plasma samples of Thai children (b, c and d: 1 19, 2.55 and 0.74 μmol/l vitamin A in plasma, respectively).

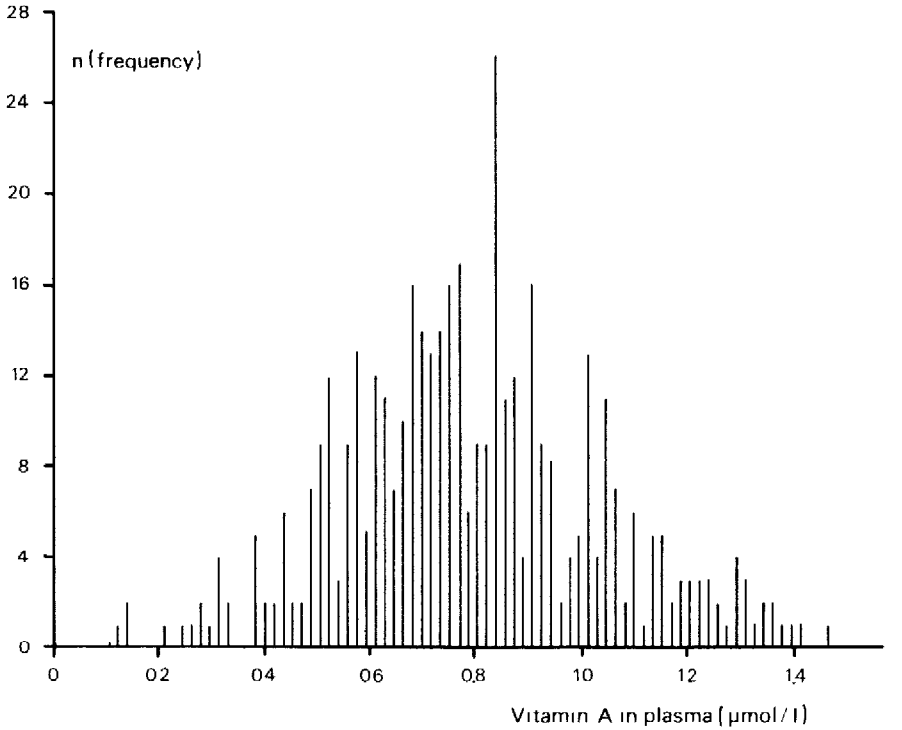

Fig. 2. Frequency distribution of the vitamin A concentration in plasma of the Thai children investigated. Total range, 0.12—1.47 μmol/l; average, 0.79 μmol/l; n = 429.

Efficiency of the extraction

To investigate the efficiency of the extraction of vitamin A with *n*-hexane, 5-μl aliquots of three different plasma samples containing 0.3, 0.9 and 1.8

μmol/l, respectively, were mixed with saline and ethanol and thereafter extracted twice with *n*-hexane. Vitamin A appeared not to be detectable in the second *n*-hexane extract. This indicates that the first *n*-hexane treatment resulted in an optimal extraction of retinol from the ethanol extract of the plasma sample.

Stability of vitamin A after extraction

In order to investigate the stability of vitamin A in the *n*-hexane extract, several extracts from 5-μl aliquots of the same plasma were pooled and stored in the dark at −20°C. This pooled extract was analysed on four consecutive days. During storage no loss of vitamin A could be observed.

Precision and recovery

The recovery was determined in pooled plasma with and without addition of 0.7 μmol/l vitamin A twenty times on one day and as a single sample on ten consecutive days. The samples were stored in the dark at −20°C. The results are given in Table I. The coefficient of variation (C.V.) of the between-assay is somewhat higher than that of the within-assay, although both are sufficiently low. Table I also shows that the recovery of vitamin A added to plasma is acceptable.

TABLE I

PRECISION OF THE MICRODETERMINATION OF VITAMIN A IN HUMAN PLASMA

Precision type	Concentration (mean ± S.D.) (μmol/l)	Coefficient of variation (%)	*n*	Recovery* (mean ± S.D.) (%)
Within-assay	0.690 ± 0.027	3.9	20	96.4 ± 5.4
Between-assay	0.780 ± 0.035	5.0	10	97.0 ± 6.8

*Recovery experiments were performed as described in Experimental.

DISCUSSION

The method described in this paper enables a fast, sensitive and reliable determination of retinol in only 5-μl aliquots of human plasma. The method is suitable for large-scale routine analysis. Precision and recovery are good. Systematic errors due to oxidative degradation of retinol during sample handling are extracting solvents. Since extracting solvent and HPLC mobile phase are compatible, the extract can be directly injected onto the HPLC column.

As part of a nutrition intervention study [13] more than 2000 analyses were carried out in two months. The precision and recovery figures showed this micro-method to be reliable.

ACKNOWLEDGEMENTS

The current investigation was carried out within the scope of the bilateral Thailand—Netherlands Nutrition/Health Development Program in Northeastern

Thailand with financial support of the Netherlands Government and under the name of "Nutritional Supplementation Cooperation (NSC) Project". The authors are grateful to Dr. Nopadol Tongsopit, rector of the Khon Kaen University, Thailand, for providing the opportunity to carry out this work at the Khon Kaen University. The authors also thank Dr. J. Schrijver (TNO-CIVO) for critically reviewing the manuscript.

REFERENCES

1 J. Marks, A Guide to the Vitamins. Their Role in Health and Disease, MTP, Lancaster, 1975, p. 39.
2 R.W. Rawson, Nutr. Cancer, 2 (1980) 17.
3 C. Ip and M.M. Ip, Carcinogenesis, 2 (1981) 915.
4 F.H. Mrisho, in Practical Considerations for Childfeeding in East, Central and Southern African Countries, Workshop Arusha (Tanzania), 1981, p. 152.
5 International Vitamin A Consultative Group (IVACG), Biochemical Methodology for the Assessment of Vitamin A Status, The Nutrition Foundation, Washington, DC, 1982.
6 D.W. Nierenberg, J. Chromatogr., 311 (1984) 239.
7 G.L. Catignani and J.G. Bieri, Clin. Chem., 29 (1983) 708.
8 H.J.C.F. Nelis, J De Roose, H. Vandenbaviere and A.P. De Leenheer, Clin. Chem., 29 (1983) 1431.
9 D.W. Howells, I.R.F. Brown, O.G. Brooke and V. Newey, Ann. Clin. Biochem., 20 (1983) 308.
10 W.J. Driskell, J.W. Neese, C C. Bryant and M.M. Bashor, J. Chromatogr., 231 (1982) 439.
11 C.A. Collins and C.K. Chow, J. Chromatogr., 317 (1984) 349.
12 R. Mansourian, E. Shepperd and H. Dirren, Int. J. Vit. Nutr. Res., 52 (1982) 227.
13 R.J. Egger, M. Wedel and W.H.P. Schreurs, in F.P. Schelp (Editor), Health Problems in Asia and The Federal Republic of Germany. How to Solve Them?, Medizin in Entwicklungsländern, Vol. 21, Verlag Peter Lang, Frankfurt am Main, 1985, p. 307.
14 J. Boldingh, H.R. Cama, F.D. Collins, R.A. Morton, N.T. Gridgeman, O. Tisler, M. Kofler, R.J. Taylor, A.S. Wieland and T. Bradbury, Nature, 168 (1951) 598