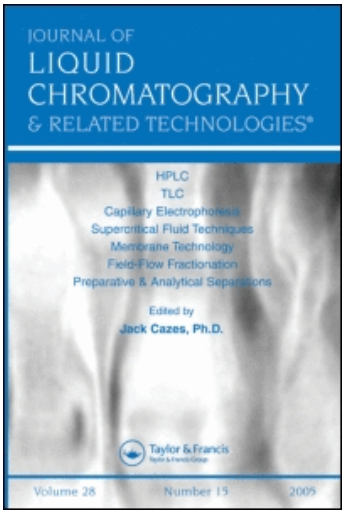

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DETERMINATION OF RETINOL AND RETINOIC ACID IN CAPILLARY BLOOD BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simplified method for quantitation of retinol in capillary blood by high-performance liquid chromatography (HPLC) is described. Serum (10-20 μl) obtained by centrifugation of capillary blood is extracted with a solvent mixture of isopropanol-dichloroethane, and the extract is directly injected into a HPLC system to quantitate retinol. The lower limit of quantitation is 0.25 ng retinol, thus permitting quantitation of serum or plasma level of 8 μg or more retinol/L. Although endogenous retinoic acid cannot be analyzed, retinoic acid, following an oral dose, can be extracted along with retinol in presence of acetic acid and quantitated. The lower limit of quantitation of retinoic acid is 0.5 ng, thus permitting quantitation of 50 ng or more of retinoic acid/ml serum or plasma. The small sample size required, their simple preparation, and rapid analysis make this method well suited for clinical studies. The method should be very useful for monitoring retinol level in blood of patients suspected of vitamin A deficiency or of retinol and retinoic acid levels in patients undergoing retinoic acid therapy for skin disorders or acute promyelocytic leukemia.

INTRODUCTION

Vitamin A is an essential micronutrient for several life processes. Vitamin A is required for growth, reproduction, vision, differentiation, hematopoiesis, bone development, and pattern formation [1]. Vitamin A deficiency is a major public health problem in many developing countries [2]. Deficiency of vitamin A results in defective vision, including blindness [2]. It is a major cause of morbidity and mortality among children in developing countries [2-4]. Retinol (ROL) and retinoic acid (RA) are naturally occurring forms of vitamin A that are continuosly circulating in human blood. Although blood ROL level does not reflect vitamin A status, blood levels of vitamin A continue to be the most commonly used biochemical measure of the vitamin A status of individuals and populations, even with all the limitations in their interpretation [2]. Measurement of plasma or serum retinol level is an essential part in the dose-response tests that have proved very useful for indirectly assessing inadequate liver stores of vitamin A [5,6]. All-trans-RA and 13-cis-RA are currently being prescribed for the treatment of various skin disorders [7] and acute promyelocytic leukemia [8]. Monitoring RA levels in the blood of such patients is often necessary and useful. There are a number of methods available for the analysis of ROL only or of ROL, carotenoids, and tocopherols [9]. Because the concentration of endogenous RA is negligible when compared with endogenous ROL in the blood, simultaneous analysis of RA and ROL in blood poses a problem. Using as much as 0.5 ml or more of serum, simultaneous quantitation of ROL with endogenous [10] or exogenous [11] RA has recently been reported. In 1993, we reported simplified procedures for the extraction and HPLC analysis of ROL, carotenoids, and tocopherols in human

serum [12]. Although endogenous RA could not be detected in the small volumes of sera used, we demonstrated the possibility of using RA as an internal standard in this assay [12]. In this paper, we demonstrate that modifications of the extraction and HPLC procedures have allowed analysis of ROL singly or simultaneously with RA in very small volumes of blood.

MATERIALS AND METHODS

All experiments were carried out under yellow light in laboratories illuminated with Gold fluorescent lights (F40GO made by Philips, USA). Retinoids and their solutions were kept under argon at -20°C.

Chemicals and Reagents

All-trans RA, all-trans ROL, and all-trans retinyl acetate (RAC) were purchased from Sigma Chemical Co., St. Louis, MO. The purity of retinoids was checked by high performance liquid chromatography (HPLC). Whenever necessary, retinoids were purified by HPLC according to the procedure described [13]. HPLC grade methanol and acetonitrile and reagent grade dichloroethane and glacial acetic acid were purchased from Fisher Scientific Co., Fair Lawn, NJ.

Preparation of Standards

Concentrated solutions of retinoids were first prepared by dissolving about 1-2 mg of each pure retinoid in methanol. The solutions were diluted until an absorbace reading of 0.3-0.5 was reached at the wave length of maximum absorption. These solutions served as stock solutions. At the time of extraction of retinoids

in serum, the stock solutions were diluted 10-fold with a mixture of isopropanol/dichloroethane (2:1, v/v). These solutions, therefore, contained 0.2-0.5 ng retinoid/μl of the solution. The following E (1%, 1 cm) values were used: 1850 at 325 nm for ROL, 1500 at 350 nm for RA, and 1500 at 325 nm for RAC [9].

HPLC System

The HPLC system consisted of: a model 510 pump (Waters Associates, Milford, MA), a model V^4 absorbance detector (ISCO, Lincoln, NE), a dual-channel CR-4A integrator (Shimadju, Columbia, MD), and a Rheodyne injector (Cotati, CA).

For analysis of ROL only, a Microsorb-MV C_{18} 3-μm column (100 x 4.6 mm; Rainin, Woburn, MA) was used. For simultaneous analysis of ROL and RA, a Ultracarb 5 ODS30 (150 x 4.6 mm) column (Phenomenex, Rancho Palos Verdes, CA). A guard column of C_{18} material (Upchurch Scientific, Omaha, NE) preceded the main column. The mobile phase consisted of acetonitrile-dichloromethane-methanol (85:12:3) containing 0.1% ammonium acetate. Ammonium acetate must first be dissolved in methanol; then acetonitrile and dichloromethane were added. Dichloromethane can be substituted by the less volatile dichloroethane without affecting the analysis. The flow rate was 0.5 ml/min, and the detection wavelength was 325 nm when the Rainin column was used; the flow rate was 1 ml/min, and the detection wavelength was 335 nm when the Phenomenex column was used for simultaneous analysis of ROL and RA.

Collection of Blood and Preparation of Serum

Capillary blood was drawn from fingers by means of an Autolet II (Fisher Scientific, Fair Lawn, NJ) and 100-

μl micro pipets (DADE, American Hospital Supply Corp., Miami, FL). The tubes were sealed at the bottom, and centrifuged for 5 min at 2000 rpm at 4°C. The serum was pipetted out by means of a 50 μl Hamilton syringe (Cobert Associates, St. Louis, MO), and 10-20 μl of portions of serum were transferred into clear glass autosampler inserts (100 μl; Kimble/Kontes, Skokie, IL). The serum was used immediately, or stored at -20°C.

Venous blood was drawn by medical staff at the University Health Center.

Extraction of Retinol:

A solution containing retinyl acetate in isopropanol/dichloroethane (0.2-0.5 ng RAC/μl; 30 μl) was added to each 10 μl of serum in the insert. The insert was closed by means of a Parafilm (American National Can, Greenwich, CT). The mixture was vortexed for 30 sec, then centrifuged for 1 min, and 10-20 μl of the supernatant solution was injected into the HPLC system (Rainin column).

Extraction of Retinol and Retinoic Acid:

Retinoic acid was not extracted efficiently by the above procedure [12]. Hence, 5 μl of glacial acetic acid was added per 30 μl of isopropanol/dichloroethane in the above extraction procedure. After vortexing and centrifugation, 10-20 μl of the supernatant was injected into the HPLC system (Phenomenex column).

Recovery, Quantification and Calibration

ROL was first quantitated in five replicate serum samples. Next, measured quantities of ROL, RA, and RAC

were added to the serum sample and quantitated again to determine the percentage of recovery.

Standard curves of peak area ratios (compound of interest to internal standard RAC) versus concentration ratios were calculated for ROL and RA. Calibration mixtures contained differing amounts of ROL or RA with a constant amount of internal standard. Concentration of ROL and RA was selected to reflect expected concentrations in samples studied.

RESULTS

Chromatographic Separation

The separation of ROL present in 10 μl of extract prepared from 10 μl of serum with 30 μl of the solvent mixture of isopropanol and dichloroethane containing RAC as the internal standard is shown in Fig. 1. HPLC was performed on the Rainin 3μ column. There was baseline separation of ROL from RAC, and the analysis time was less than 5 min. Precipitation of proteins followed by vortexing and centrifugation resulted in a total volume of the final extract at 25-30 μl. Injection of 10 μl of this extract, therefore, was equivalent to about 3-4 μl of serum.

Unless extracts from large volumes of serum were injected, the endogenous level of RA present in 10-20 μl of serum extract could not be detected by our procedure (Fig. 2A). However, analysis of 10-20 μl of extract of serum obtained 0.5-3 h after an oral dose of 50-mg RA, could be detected and analyzed. The separation of RA from an unidentified compound present in serum was not satisfactory on the Rainin column. Therefore, for simultaneous analysis of ROL and RA in serum, the Phenomenex column was used. A chromatogram of 20 μl of

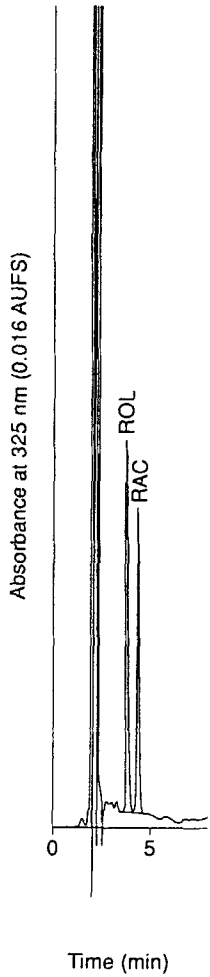

FIGURE 1. Chromatogram of HPLC analysis of retinol (ROL) in human serum: 10 μl of serum was extracted with 30 μl of the solvent mixture containing retinyl acetate (RAC) as internal standard, and 10 μl of the extract was injected directly.

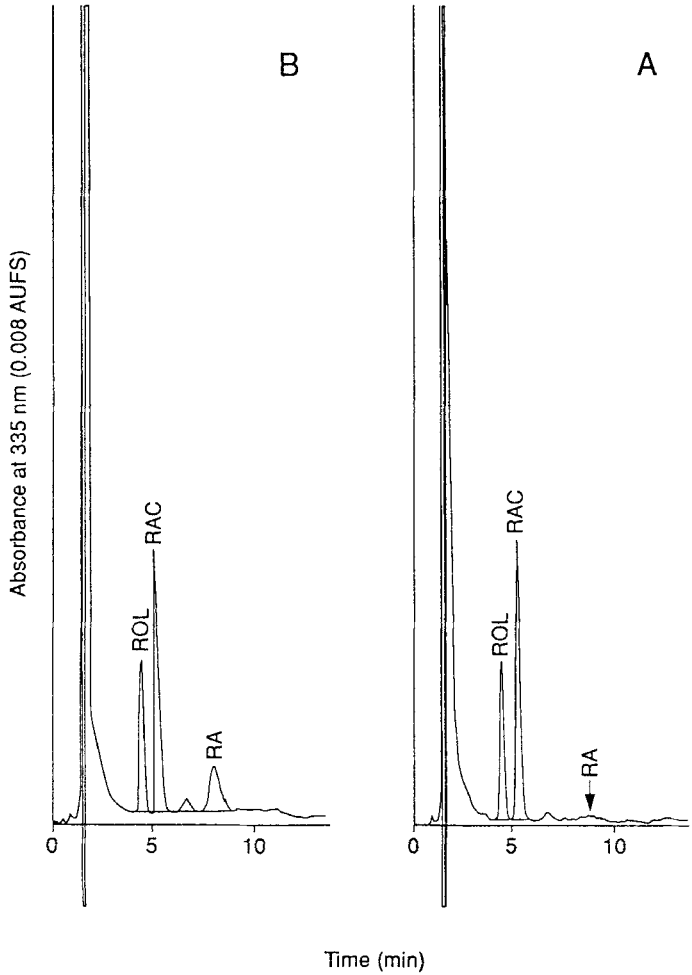

FIGURE 2. Chromatogram of HPLC analysis of retinol (ROL) and retinoic acid (RA) in human serum: 20 μl of serum was extracted with 60 μl of the solvent mixture containing acetic acid and RAC (internal standard), and 20 μl of the extract was injected directly. A, before; and B, 1 h after an oral dose of 50-mg RA.

the extract from 20 μl of serum (processed with 60 μl of the solvent mixture) obtained 1 h after a 50-mg dose of RA is shown in Fig. 2B.

Limit of Quantitation and Detection

Retinol: With both the Rainin and the Phenomenex column, there was a linear relationship between peak areas obtained and the concentration over the range of 0.25 ng to >10 ng of ROL. The satisfactory lower limit of quantitation on the Rainin column was 0.25 ng of ROL, although about 0.1 ng of ROL could be detected. The Phenomenex column was not as sensitive as the Rainin column, and the limit of quantitation on this column was 0.5 ng of ROL.

Retinoic acid: The lower limit of satisfactory quantitation of RA on the Phenomenex column was found to be 0.5 ng RA, equivalent to 50 ng/ml of serum. The lower limit of detection of RA, however, was found to be 0.1 ng of RA.

Recovery

The recovery of added ROL in five replicate serum samples was 101 ± 3%. The recovery of added RA in presence of acetic acid was similarly determined in five replicate analysis, and was found to be 98 ± 5%. The recovery of the internal standard RAC was found to be 99 ± 2%.

Precision of The Extraction Procedure

The within-run and between-run imprecision of the extraction procedure for ROL was estimated by repeated

TABLE I

Comparison of Retinol Level in Sera Obtained from Venous and Capillary Blood

Capillary blood	Venous blood
μg/dl Serum	
38.65 ± 3.8	38.46 ± 2.5

analysis of three serum samples over a period of 2 wk. The within-run and between-run results were found to be 31.4 ± 0.8 μg/dl and 34.2 ± 1 μg/dl, respectively.

Comparison of analysis of Venous and Capillary Blood

Sera (10 μl) each obtained from capillary blood and venous blood were extracted under identical conditions and analyzed on the Rainin column by HPLC. The results are shown in Table I. There was no significant difference in the values obtained from the two blood samples.

DISCUSSION

We reported recently a simplified extraction procedure for the simultaneous analysis of retinol, tocopherols, and carotenoids in serum by vortexing and centrifuging 50-100 μl of serum with a mixture of isopropanol/dichloromethane and injecting 25-100 μl aliquots of the extract [12]. The extraction procedure eliminated the need for repeated solvent extraction, evaporation of solvent, and reconstitution of the residue. This resulted, not only in reduction of analysis time, but also in chances of loss of analytes and

isomerization and decomposition of the otherwise labile retinoids. Although the procedure described in this research for extraction of ROL is similar to the published procedure, a modification was made for the simultaneous extraction of RA with ROL. We found that acetic acid needs to be added to the serum for efficient extraction and recovery of retinoic acid. In our previous method [12], we demonstrated that, whereas the recovery of ROL was 101 ± 4.7%, the recovery of RA was only 67.4 ± 1.5%. In this study, we found that, in the presence of acetic acid, the recovery of RA was 98 ± 5%. Besides the modification in the extraction procedure, we have made other modifications of the assay procedure. It is now possible to quantitate ROL in very small volumes of blood that can be obtained from capillaries. We found no difference in serum ROL content of capillary and venous blood (Table 1). Because larger volumes of blood are often difficult to obtain in large-scale population study, especially amongst infants, the present methodology should be very valuable in such studies. Moreover, retinyl acetate, which is readily available and which separates very well from retinol with the modified solvent composition, can be used as an internal standard.

We also found that retinoic acid can be quantitated simultaneously in small volumes of serum obtained from volunteers taking a single 50-mg oral dose of RA. The method should find usefulness in analyzing ROL and RA in the blood of patients undergoing RA therapy. Such patients usually take daily 1 mg/kg body weight of RA for the treatment of acne [7] or 45 mg/m^2 body for the treatment of acute promyelocytic leukemia [8]. Because ROL and RA can be simultaneously quantitated, the method should be useful for monitoring serum ROL level of patients undergoing RA therapy when there is a danger of defective vision.

Analysis of ROL on the Rainin 3μ-column is very sensitive and rapid. Because the flow rate is only 0.5 ml/min and the analysis time is 5 min, less solvent is required. The column is also relatively inexpensive. The method should, therefore, be useful for the analysis of retinol in large-scale epidemiological studies in developing countries where cost often limits studies.

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REFERENCES

1. M. B. Sporn, A. B. Roberts, and D. S. Goodman, The Retinoids, Raven Press, New York, 1994

2. B. A. Underwood,"Vitamin A in Human Nutrition:Public Health Considerations" in The Retinoids, M. B. Sporn, A. B. Roberts, D. S. Goodman, eds., Raven Press, New York, 1994, pp. 211-227.

3. A. Sommer, I. Tarwotjo, E. Djunaedi, A. A. Loeden, K. P. West, R. Tilden, L. Mele, and the ACEH Study Group, Lancet, 1, 1169-1173 (1986)

4. G. H. Beaton, R. Martorell, R. L'Abbe, B. Edmonston, G. MeCabe, A. C. Ross, and B. Harvey, Final Report to CIDA, International Nutrition Program, University of Toronto, Toronto, 1992

5. H. Flores, F. Campos, C. R. C. Araujo, and B. A. Underwood, Am.J.Clin.Nutr., 40, 1281 (1984)

6. S. Tanumihardjo, Y. Muhilal, D. Permaeshi, Z. Sulaiman, D. Karyadi, and J. A. Olson, Am.J.Clin.Nutr., 52, 1068 (1990)

7. G. L. Peck and J. J. DiGiovanna, "Synthetic retinoids in Dermatology" in The Retinoids, M. B. Sporn, A. B. Roberts, D. S. Goodman, eds., Raven Press, New York, 1994, pp.631-658

8. W. K. Hong and L. M. Itri,"Retinoids and Human Cancer" in The Retinoids, M. B. Sporn, A. B. Roberts, D. S. Goodman, eds., Raven Press, New York, 1994, pp.597-630

9. H. C. Furr, A. B. Barua, and J. A. Olson, "Analytical Methods" in The Retinoids, M. B. Sporn, A. B. Roberts, D. S. Goodman, eds., Raven Press, New York, 1994, pp.179-209

10. E. Meyer, W. E. Lambert, and A. P. De Leenheer, Clin. Chem., 40, 48-57 (1994)

11. J. Creech Kraft, C. Eckhoff, W. Kuhnz, B. Lofberg, and H. Nau, J.Liq.Chromatogr., 11, 2051-2069 (1988)

12. A. B. Barua, D. Kostic, and J. A. Olson, J.Chromatogr., 617, 257-264 (1993)

13. A. B. Barua and H. C. Furr, Methods Enzymol., 213, 273-281 (1992)

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