British Journal of Nutrition (2009), **101**, 794–797
doi:10.1017/S0007114508047739

Short Communication

A new approach to the assessment of marginal vitamin A deficiency in children in suburban Guwahati, India: hydrolysis of retinoyl glucuronide to retinoic acid

Pulin C. Sarma[1], Bhabesh C. Goswami[1], Krishna Gogoi[2], Harsha Bhattacharjee[2] and Arun B. Barua[3]*

[1]*Department of Chemistry, Gauhati University, Guwahati 781014, Assam, India*
[2]*Sri Sankaradeva Nethralaya, Beltola, Guwahati 781028, Assam, India*
[3]*Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA*

(Received 26 February 2008 – Revised 13 June 2008 – Accepted 16 June 2008 – First published online 9 September 2008)

The objective of the present study was to determine marginal vitamin A deficiency (VAD) by testing the hydrolysis of retinoyl glucuronide (RAG) to retinoic acid (RA) in children. Previous studies in rats showed that hydrolysis occurred when rats were vitamin A deficient. Children (*n* 61) aged 3–18 years, were divided into two groups, I and II. Blood was collected from the children in Group I (*n* 19) who were not dosed with RAG. Children in Group II (*n* 42) were administered all-*trans* retinoyl glucuronide (RAG) orally, and blood was collected 4 h after the dose. All serum samples were analysed for retinoids and carotenoids. RA was detected in serum only when serum retinol was $<0{\cdot}85$ μmol/l. Thus, hydrolysis of RAG to RA occurred in children with VAD or marginal VAD. Serum retinol was $<0{\cdot}35$ μmol/l in twenty-one children, 0·35–0·7 μmol/l in twenty-three children, 0·7–0·9 μmol/l in eleven children and >1 μmol/l in six children. Mean serum retinol in sixty-one children was 0·522 (SD 0·315) μmol/l. Mean β-carotene (0·016 (SD 0·015) μmol/l) was far below normal compared to the level of lutein (0·176 (SD 0·10) μmol/l) in sixty-one children. A low β-carotene level might be due to a low intake of carotene but high demand for vitamin A. The RAG hydrolysis test may prove to be a useful approach for the determination of marginal VAD with no clinical or subclinical signs of VAD. High prevalence of VAD amongst certain communities in Assam cannot be ruled out.

Vitamin A deficiency: Retinoyl glucuronide hydrolysis test: Serum retinol: Serum cartene:: Children

Vitamin A is an essential micronutrient for several life processes. Besides vision, it is required for growth, reproduction, cellular differentiation, development and immunity[1]. Man obtains vitamin A from foods such as milk, liver and eggs. Vegetarian people depend solely on provitamin A carotenoids, such as β-carotene, for their supply of vitamin A. Vitamin A deficiency (VAD) is a public health problem in many low-income countries, especially in Africa and South-East Asia, affecting young children and pregnant women the most. VAD is the leading cause of preventable blindness in children and increases the risk of disease and death from severe infections. In pregnant women,VAD causes night blindness and may increase the risk of maternal mortality[2].

The criteria for VAD are based on many factors such as population-based surveys, cross-sectional surveys, baseline values of intervention studies, and detection of signs of clinical xerophthalmia. Measurement of circulating retinol level represents the most common biochemical measure of vitamin A status. Due to homeostatic control, the level of vitamin A does not change when the liver store is adequate. Only during hepatic depletion of vitamin A stores does serum retinol concentration (serum retinol) fall[1,3]. However, the acute-phase response may occur during times of infections and trauma resulting in depressed serum retinol[4]. As subclinical infections are common in subjects with VAD, serum retinol may not be a good indicator for vitamin A status. Response to an oral dose of retinyl esters or 3,4-didehydroretinyl acetate known as relative dose response[5] and modified relative dose response[6], respectively, are accepted methods for vitamin A status determination. Both methods have advantages and disadvantages, and other methods using stable isotopes have been reported[7]. When the vitamin A status is adequate, retinoyl glucuronide (RAG), a water-soluble form of vitamin A, is neither absorbed nor hydrolysed to retinoic acid (RA) by man or rats[8,9]. However, when the vitamin A status is below normal, RAG is hydrolysed to RA in rats[8,9]. This test coined by the late James Allen Olson as the 'RAG hydrolysis test' has not been tested in human subjects with VAD.

Abbreviations: RA, retinoic acid; RAG, retinoyl glucuronide; serum retinol, serum retinol concentration (μmol/l); VAD, vitamin A deficiency.

* **Corresponding author:** Dr Arun B. Barua, 4737 S. Ellis Avenue, Chicago, IL 60615, USA, fax +1 515 294 0453, email abarua@iastate.edu

The aim of the present study was to determine whether RAG was hydrolysed to RA in children with low serum retinol. Appearance of RA following an oral dose of RAG might be a very useful indicator of vitamin A status in man. As reports on the concentrations of β-carotene and other carotenoids in subjects with VAD are scarce or not available, we also sought to determine the concentrations of carotenoids in these children.

Materials and methods

The study was conducted in the Chemistry Department, Gauhati University, and Sri Sankaradeva Nethralaya, an institute for the diagnosis and treatment of eye disorders, Guwahati, Assam, India.

Subjects

About 350 children were examined by physicians of Sankaradeva Nethralaya for their general health and eye condition in health camps set up in four different locations in the city of Greater Guwahati. One hundred children, selected at random, were further examined at Sankaradeva Nethralaya. Sixty-one children, forty-nine male and twelve female, aged 3–18 years, some with clinical signs of VAD, were selected for the study. Signed consent forms to agree to participate in the study and information about the food habit of the children were collected from parents. The study protocol was in accordance with ethical standards and was approved by the appropriate authorities at Gauhati University and Sankaradeva Nethralaya.

Experimental design

All chemical and analytical experiments were carried out under gold fluorescent light in the laboratories at the Chemistry Department, Gauhati University.

Chemicals and solvents

Anhydrous diethyl ether, acetonitrile, acetic acid, hexane, methanol, dichloromethane, ethyl acetate, 2-propanol, butylated hydroxytoluene and HPLC-grade solvents, methanol, dichloromethane and water were purchased from E. Merck India Ltd (Mumbai, India). The standard samples of retinol, RA, retinyl acetate, β-carotene and lutein were gifts from Hoffmann La Roche Co. (Basel, Switzerland), and were stored under nitrogen at −20°C. The purity of each standard was checked by HPLC before each analysis. All-*trans* RAG (95 % β, 5 % α anomer) was chemically synthesized and purified by the procedure described previously[10].

Spectrophotometry

UV–visible spectra were recorded on a Hitachi model U3210 spectrophotometer. The concentration of retinoids and carotenoids were determined using extinction coefficient values of 1845 at 325 nm for retinol, 1510 at 350 nm for RA, 1065 at 360 nm for RAG, 1560 at 325 nm for retinyl acetate (RAC), 2592 at 453 nm for β-carotene and 2550 at 445 nm for lutein[11].

Blood collection and oral dosing of retinoyl glucuronide

All the children (*n* 61) had a vegetarian breakfast 3 h before the start of the experiment. The children were divided into two groups. Venous blood (4–5 ml) was collected by certified nurses from the children in Group I (*n* 19) who were not dosed with RAG. Blood was protected from light and centrifuged immediately. The serum samples were kept frozen at −20°C until HPLC analysis. Children in Group II (*n* 42) were given an oral dose of RAG as described later. The children were not given any food until after blood draw.

The method of preparation and administration of RAG dose was the same as described previously[9]. The stock solution was appropriately diluted with maize oil to give a concentration of 50 mg (0·105 mmol)/ml oil. For oral dosing, 0·5 ml (25 mg RAG) or 1 ml (50 mg RAG) or 1·5 ml (75 mg RAG) of the oil was spread on a piece of bread and fed to each child.

For the RAG hydrolysis test, blood was collected as described earlier 4 h after the dose because it was found in rats that concentration of RA peaked at 4 h after the dose[8].

Extraction of carotenoids and retinoids

Carotenoids and retinoids were extracted by a previously published procedure[12]. The extraction efficiency was 95 ± 3 %. The extract was evaporated and the residue was dissolved in methanol–dichloromethane (3:2, v/v), and an aliquot was injected into the HPLC injector by means of a Hamilton syringe. The extraction and analysis of serum was carried out in duplicate, and if the two values were not close, it was repeated.

HPLC system and operating condition

The HPLC system consisted of two pumps (Shimadzu, M-10 AVP), a photodiode array detector (model PDA 10 ATVP) and a personal computer connected to a Hewlett Packard Laser Jet 5 printer. The software used was class M10A.

Simultaneous analysis of retinoids and carotenoids was carried out with a reversed-phase gradient HPLC method on a Supelcosil LC-8 (5 μ) 25 cm × 4·6 mm column preceded by a guard column of C_{18} material.

The gradient programme was a slight modification of a published procedure[12]. A linear solvent gradient of methanol–water (85:15, v/v) containing 10 mM-ammonium acetate and acetic acid (0·01 %) to methanol–dichloromethane (4:1, v/v) was applied for 12 min. The initial flow rate of 0·6 ml/min was changed to 0·8 ml in 12 min, to 1 ml in 16 min, maintained at 1 ml until 23 min, and then reduced to 0·6 ml in the next 2 min. The gradient was reversed (25 min) to initial conditions in 10 min and the column was allowed to equilibrate for 10 min. Double channel chromatograms (550–190 nm) were monitored at 450 nm for carotenoids and at 330 nm for retinoids. The tray containing sample vials was maintained at 5°C.

Calculation

The vitamin A and carotenoid concentrations were determined from the standard curves generated for each analyte. A linear least square regression analysis was performed for each analyte and the standard curve was repeated if the correlation

coefficient was below 0·99. Statistical analysis was carried out by Student's *t* test[13]. The detection limit for RA, retinol, lutein and β-carotene was 0·1, 0·06, 0·02 and 0·18 ng, respectively.

Results

The majority of the children included in the study belonged to parents who had settled in suburban Guwahati, Assam, from neighbouring states. Many lived in slums around the city. The parents belonged to a low socio-economic group. The majority of them were vegetarians and their staple food consisted mainly of dal, roti and aloo (lentils, flat bread and potatoes) with occasional consumption of other vegetables and fruits. Carotenoids in the diet constituted the primary source of vitamin A.

Serum retinol and retinoyl glucuronide hydrolysis test

Serum retinol is expressed as μmol/l. The average serum retinol in all the children included in the present study (*n* 61) was 0·522 (SD 0·315). Serum retinol was $<0·35$ (*n* 21), 0·35–0·7 (*n* 23), 0·7–0·9 (*n* 11) and >1 (*n* 6). Twelve children with serum retinol 0·244 (SD 0·128) showed clinical signs of VAD, fifteen children with serum retinol 0·405 (SD 0·193) had watery eyes, eye aches and headaches, twenty-eight children with serum retinol 0·583 (SD 0·226) showed no clinical signs of VAD. Thus, the children, excluding only six children, in the study group were either deficient or marginally deficient as their serum retinol was $<0·7$.

Oral dosing of RAG to forty-two children with serum retinol of 0·21 (SD 0·088) (*n* 17), 0·454 (SD 0·089) (*n* 14) and 0·856 (SD 0·089) (*n* 3) resulted in 0·019 (SD 0·016), 0·018 (SD 0·017) and 0·006 (SD 0·012) μmol RA/l in serum, respectively. RA was not detected in the serum of eight children with serum retinol $>0·856$. No significant difference was observed in the concentration of RA in serum of children with serum retinol 0·21 and 0·45 ($P>0·96$), but the difference between these two groups of children and children with serum retinol 0·85 was significant ($P<0·01$). The data pointed towards serum retinol 0·85 as the approximate cut-off value for hydrolysis of RAG to RA. RA was characterized from retention time and nature of UV-spectrum that were identical with standard RA. Dose size of 0·052, 0·105 and 0·157 mmol RAG resulted in serum RA concentration of 0·004 (SD 0·002) (*n* 14), 0·052 (SD 0·04) (*n* 18) and 0·022 (SD 0·008) (*n* 4) μmol/l, respectively, mean serum retinol in these children being 0·375, 0·272 and 0·41, respectively. RA level was highest when RAG dose size was 0·1 mmol and mean serum retinol was lowest in the subjects.

HPLC profiles of serum carotenoids revealed that the carotenoid peaks were very weak indicating below normal levels of carotenoids. Attempts were made to identify only two carotenoids, lutein and β-carotene, by comparison of retention time and UV–visible spectrum with standards. The lutein level was low (0·176 (SD 0·10) μmol/l; range 0·005–0·475 μmol/l). The level of β-carotene was surprisingly very low (0·016 (SD 0·015) μmol/l; range 0–0·035 μmol/l) in the majority of the children.

Discussion

Lack of sensitivity of plasma retinol to early changes in body reserves has led to the quest for other indicators, clinical, biochemical, histological and dietary assessment, of vitamin A status[1,5,6]. Clinical signs of VAD are not seen when serum retinol is 0·7–3 μmol/l[1,3]. Clinical indicators of VAD appear when serum retinol falls below 0·35. During marginal vitamin A status when serum retinol is between 0·35 and 0·7, no clinical signs of VAD are observed, but the subjects are responsive to supply of vitamin A[3]. Therefore, information about serum retinol indicating marginal VAD would be very useful for the treatment and prevention of early stages of xerophthalmia[3].

We report here for the first time that detection of RA in the blood 4 h after an oral dose of RAG can be an indicator of marginal VAD. None of the forty-two children dosed with RAG showed any side-effects, indicating the safety of RAG for man. Orally administered RAG is not hydrolysed to RA by human subjects with normal vitamin A status[9], but is hydrolysed to RA only during VAD as was found in the present study in man[9]. Furthermore, RA is quickly metabolized. Therefore, the RAG hydrolysis test might be a safe useful new approach for assessing marginal vitamin A status in children. Further studies are needed to determine cut-off values for RA in relation to vitamin A status.

VAD increases the incidence of common childhood infections such as diarrhoeal disease and measles[4,14]. VAD is a major contributor to the mortality of vitamin A-deficient children under the age of 5. Improving vitamin A status by supplementation enhances the resistance to disease and can reduce mortality from all causes by 23 %[15]. In 2001 during supplementation of vitamin A in the state of Assam, under WHO and UNICEF's Vitamin A Global Initiative Program, paediatricians and nutritionists launched protests against the programme when thirty children died and many fell sick after ingestion of vitamin A[16]. The programme was stopped. To our knowledge, there are no available data on serum/plasma retinol concentration in children in Assam in WHO's global database[17] or elsewhere. The present study provides some data on serum retinol and carotenoid concentrations.

The present study was aimed at determining marginal VAD from hydrolysis of RAG to RA in a selected group of children belonging to an economically backward population that includes slum dwellers and tea garden labourers in Assam. As expected, children with clinical signs of VAD showed serum retinol $<0·35$, but children considered healthy with no obvious clinical signs of VAD showed marginal serum retinol $<0·7$. The present data showed that the children were in need of vitamin A and they were supplemented. Studies by WHO and UNICEF show that the benefit of supplementation with a large dose of vitamin A to children outweighs any adverse temporary side-effects such as nausea, vomiting and headache[2,15].

It should be emphasized that the present study was carried out in a small group of children in one city only and the study did not include any children below the age of 3 who were most vulnerable to VAD. It is warranted that vitamin A status is monitored as part of public health programmes in Assam and other states in India. High prevalence of VAD amongst certain communities in the state of Assam cannot be ruled out.

British Journal of Nutrition

It has not yet been established if plasma concentration of β-carotene can indicate vitamin A status in man. Liver vitamin A concentration is not proportional to liver carotenoid concentration[18]. Very few studies have been made to determine both plasma retinol and β-carotene levels in children. In the present study, we found that all the sixty-one children showed β-carotene level that was far below values reported by others[19]. The level of lutein was also low but somewhat satisfactory. There can be several reasons for the observed difference in lutein and β-carotene levels in the serum. An increased demand for vitamin A by the deficient children resulting in rapid conversion of any available β-carotene, but not of lutein, to vitamin A seemed to be a reasonable explanation for the observed difference.

Acknowledgements

This paper is dedicated to the memory of James Allen Olson (1924–2000). We gratefully acknowledge the parents and children who participated in the study. We thank the faculty and staff of the Chemistry Department, Gauhati University, and the physicians of Sri Sankaradeva Nethralaya, for valuable help during the study. P. C. S. carried out the chemical and analytical work. B. C. G. supervised and assisted P. C. S. in laboratory work and data analysis. K. G. and H. B. selected the subjects, carried out health examination including evaluation of clinical signs of xerophthalmia, and collected and processed blood from subjects. A. B. B. developed the protocol, guided the research, and made the final data analysis. All authors contributed to the final version of the manuscript. None of the authors have a conflict of interest. Supported in part by Nutritional Sciences Council, Iowa State University, from a Wilfred and Elizabeth Martin Life Sciences Fund (Iowa State University Foundation), Ames, USA.

References

1. Ross AC (1999) Vitamin A and retinoids. In *Modern Nutrition in Health and Disease*, pp. 305–328 [ME Shils, JA Olson, M Shike and AC Ross, editors]. Baltimore, MD: Williams and Wilkins.
2. World Health Organization (2008) Micronutrient deficiencies. Vitamin A deficiency. http://www.who.int/nutrition/topics/vad/en/index.html (accessed February 2008).
3. West KP Jr (1994) Vitamin A deficiency. Its epidemiology and relation to child mortality and morbidity. In *Vitamin A in Health and Disease*, pp. 585–614 [R Blomhoff, editor]. New York: Marcel Dekker.
4. Thurnham DI, McCabe GP, Northrop-Clewes CA & Nestel P (2003) Effects of subclinical infection on plasma retinol concentrations and assessment of prevalence of vitamin A deficiency: meta-analysis. *Lancet* **362**, 2052–2058.
5. Underwood BA (1990) Methods for assessment of nutritional status. *J Nutr* **120**, 1459–1463.
6. Tanumihardjo SA (2004) Assessing vitamin A status: past, present and future. *J Nutr* **134**, 290S–293S.
7. Furr HC, Green MH, Haskell MJ, *et al.* (2005) Stable isotope dilution techniques for assessing vitamin A status and bioefficacy of provitamin A carotenoids in humans. *Public Health Nutr* **8**, 596–607.
8. Barua AB, Duitsman PK & Olson JA (1998) The role of vitamin A status in the conversion of all-trans retinoyl β-glucuronide to retinoic acid in male Sprague–Dawley rats. *Nutr Biochem* **9**, 676–681.
9. Goswami BC, Reida AK, Ivanoff KD, Barua AB & Olson JA (2003) Intestinal absorption and metabolism of retinoyl β-glucuronide in humans and of 15-[^{14}C]-retinoyl β-glucuronide in rats of different vitamin A status. *Nutr Biochem* **14**, 703–709.
10. Becker B, Barua AB & Olson JA (1996) All-trans retinoyl β-glucuronide: new procedure for chemical synthesis and its metabolism in vitamin A-deficient rats. *Biochem J* **314**, 249–252.
11. Barua AB, Furr HC, Olson JA & Bruman RB (2000) Vitamin A and carotenoids. In *Modern Chromatographic Analysis of Vitamins*, vol. 84, 3rd ed., pp. 1–74 [AP De Leenheer, WI De Lambert and F Van Bocxlacer, editors]. New York: Marcel Dekker.
12. Barua AB & Olson JA (2000) β-Carotene is primarily converted to retinoids in rats *in vivo*. *J Nutr* **130**, 1996–2001.
13. College of St Benedict & St John's University (2008) Student's *t*-tests. http://www.physics.csbsju.edu/stats/t-test.html (accessed May 2008).
14. Kongsback K, Wahed MA, Friis H & Thilsted SH (2006) Acute-phase protein levels, diarrhoea, *Trichuris trichura* and maternal education are predictors of serum retinol: a cross-sectional study of children in a Dhaka slum, Bangladesh. *Br J Nutr* **96**, 725–734.
15. UNICEF (2007) Vitamin A supplementation: a decade of progress. http://www.unicef.org (accessed February 2008).
16. Mudur G (2001) Deaths trigger fresh controversy over vitamin A programme in India. *Br Med J* **323**, 1206.
17. World Health Organization (2008) Micronutrient deficiencies. Vitamin A deficiency. www.who.int/vmnis/vitaminA/data/en/index.html (accessed February 2008).
18. Olson JA (1984) Serum levels of vitamin A and carotenoids as reflectors of nutritional status. *J Natl Cancer Inst* **73**, 1439–1444.
19. Furr HC & Clark RM (2004) Transport, uptake, and target tissue storage of carotenoids. In *Carotenoids in Health and Disease*, pp. 409–426 [NI Krinsky, ST Mayne and H Sies, editors]. New York: Marcel Dekker.