SELECTED RETINOIDS: DETERMINATION BY ISOCRATIC NORMAL-PHASE HPLC

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Retinol (ROL), retinal (RAL) and retinoic acid (RA) are physiologically active forms of vitamin A. All-trans retinoic acid (ATRA) can be formed by oxidation from all-trans retinal (ATRAL). Isomerization of RA is considered to be an important metabolic pathway of retinoids. RA isomers transactivate various response pathways via their cognate nuclear receptors that act as ligand inducible transcription factors. The aim of this study was to establish a rapid and simple method for determination of ATRA, 13-cis retinoic acid (13CRA) and ATRAL by HPLC. In our laboratory, we slightly modified the method of MIYAGI et al. (2001) and separated ATRA, 13CRA and ATRAL by simple isocratic normal phase HPLC. Both retinoic acid isomers and ATRAL were eluated within 13 min and all components were well resolved. The coefficients of variation (C.V.) for RAs and RAL were from 3.0 to 5.4 %.

Biochemical role of retinoids

Class of retinoids includes naturally occurring derivates of vitamin A as well as synthetic analogues with or without vitamin A activity (SUN and LOTAN 1999). Retinol (ROL), retinal (RAL) and retinoic acid (RA) are physiologically active forms of vitamin A (REIFEN and WASANTWISUT 1998). All-trans retinoic acid (ATRA) can be formed by oxidation from all-trans retinal (ATRAL, Fig. 1). In human body, ATRAL can either be synthetised by oxidation of dietary ROL or by metabolic cleavage of β-carotene (PALACE et al. 1998). The natural retinoids are known for their susceptibility to isomerization (cis- or trans-isomeric forms). ATRA isomerizes to 13-cis-retinoic acid (13CRA), 9-cis-retinoic acid (9CRA), and 9,13-di-cis-retinoic acid. In vivo, the same four isomers were presented in human plasma after applications of 9CRA. Isomerization is considered to be an important metabolic pathway of RA because it results in metabolites with different mechanisms of action (LANVERS et al. 1998). RA isomers transactivate various response pathways via their cognate nuclear receptors. Another step in RA metabolism is oxidation to 4-oxo-metabolites by cytochrome P450, the main metabolic pathway after aplication of pharmacological doses of RA (LANVERS et al. 1996).

ROL is transported in human plasma bound to retinol-binding proteins (RBP), which further interacts with transthyretin, the protein that transports the thyroid hormones. Transport of RA occurs almost entirely through nonspecific binding to plasma albumin. Once in cell, retinoic acid is bound to cellular retinoic acid binding protein (CRABP-I and CRABP-II) and retinol to cellular retinol binding proteins (CRBP-I and CRBP-II) (REIFEN and WASANTWISUT 1998, PALACE et al. 1999). Retinoids exert most of their effects by binding to two subtypes of nuclear retinoid receptors, the all-trans retinoic acid receptors (RAR α, RAR β, RAR γ) and the 9-cis retinoic acid receptors (RXR α, RXR β, RXR γ). The RARs are activated with both ATRA and 9CRA, whereas RXRs are capable to bind specifically 9CRA. These retinoid receptors are known to belong to steroid/thyroid/retinoid hormone superfamily of nuclear receptors that act in nucleus as ligand inducible tran-

scription factors (Sun and Lotan 1999, Evans and Kaye 1999).

Retinoids are involved in both growth and differentiation of cells (normal and also transformed), they play an important role in vision (in the form of RAL), embryogenesis and reproduction (Clagett-Dame and DeLuca 2002), apoptosis (Xu et al. 2002) and various aspects of the immune system (Brtko et al. 2000). Retinoids are considered to be a promising class of agents for the chemoprevention or treatment of skin disorders and malignant diseases. Among them, isotretinoin (13CRA) is the most effective retinoid for the prevention of non-melanoma skin cancers in high-risk patients in clinical trials (Niles 2002).

ATRA is also a potent inhibitor of cell proliferation or inducer of differentiation, but the use of it in the treatment of cancer is hampered by its toxicity and probably by its increased metabolism. The use of retinoids to suppress tumour development has been investigated in several animal models and clinical trials of carcinogenesis including skin, breast, oral cavity, lung, hepatic, gastrointestinal, prostatic and bladder cancers as well as thyroid cancer (Allen and Bloxham 1989, Reifen and Wasantwisut 1998, Sun and Lotan 1999). Treatment of acute promyelocytic leukemia (APL) with ATRA alone or in combination with chemotherapy yields in a complete remission as high as 85-95% (Wang and Chen 2000, Wang 2002, Chen et al. 2002).

Grunwald et al. (1998) and Schmutzler and Köhrle (2000) have found that redifferentiation therapy with 13CRA can induce radioiodine uptake in some patients with radioiodine negative thyroid carcinoma tumour sites. Several studies have shown that retinoids are very efficient agents against breast cancer. They can inhibit the growth of many human hormone-dependent breast cancer cells (Fontana 1987). Anzano et al. (1994) have found that 9CRA is much more potent than ATRA for suppression of carcinogenesis *in vivo*, both as a single agent or in combination with antiestrogen tamoxifen. Hou et al. (1998) have found the serum vitamin A levels significantly decreased in the metastatic breast cancer group, especially in liver metastatic women. That author has suggested a postoperative vitamin A suplementation that might have potential benefit to metastatic breast cancer patients.

Determination of retinoids by various normal phase HPLC methods

Currently, high–performance liquid chromathography has become the method of choise for the determination of retinoids. Quite a large number of reversed-phase HPLC (RP-HPLC) have been reported for analysis of polar and nonpolar retinoids, but there are only few normal-phase HPLC (NP-HPLC) procedures that allow simultaneous analysis of a mixture of RA and ROL geometrical isomers. Meyer et al. (1994) first described a simple isocratic NP-HPLC for the simultaneous analysis of endogenous 13CRA, ATRA, and ROL in human plasma. Separation was performed on a silica gel column (150 x 4.6 mm I. D., 5 μm particle size), the solvent system consisted of a mixture of n-hexane:2-propanol:acetic acid (1000 : 3.5 : 0.675, v/v) at a flow-rate of 0.9 ml/min. Meyer and co-workers found that 2–propanol content ranging from 2.5 to 6 ml/l is required for ROL symmetry and sufficient RA isomer differentiation (Meyer et al. 1994). In Meyer´s method, 500 μl of human plasma was used for quantitation of physiological concentration of RA isomers. Each retinoid was determined by UV detection at a wavelength of 350 nm, near an absorption maximum of all trans-retinol (ATROL) (325 nm). The mean physiological concentrations of ATRA, 13CRA, ROL, and 4-oxo-13CRA in human plasma are 1.35 μg/l, 1.79 μg/l, 533 μg/l (Meyer et al. 1994), and 3.68 μg/l, respectively (Lanvers et al. 1996). Because the concentration of retinol is ~ 300-fold higher than those of RA, ROL still absorbs sufficiently at 350 nm. The limits of detection were 0.5 μg/l in human plasma for RA isomers and 10 μg/l for ROL. Meyer et al. (1994) recommended less acidic extraction conditions (solution of water, n-hexane, acetic acid) in order to avoid hydrolysis of endogenous compounds of human blood, such as retinoyl-β-glucuronides. Lanvers et al. (1996) extracted retinoids at a pH values of 5. Barua (2001) found that only trace amount of RA was detected and very poor recovery of internal standard in the absence of acetic acid, was found. The arotinoid ethylsulfonic acid and acitretin have been used as the internal standards (Meyer et al. 1994, Lanvers et al. 1996, Barua 2001, Miyagi et al. 2001). Dzerk et al. (1998) investigated a light sensitivity of methanolic solution containing 9CRA and 4-oxo-

9CRA. They found that those standard solutions are very sensitive to white light, however, only approximately 10 % degradation of the compounds was observed after 4 h yellow light exposure.

Lanvers et al. (1996) improved the sample preparation and the HPLC method published by Meyer et al. (1994), and developed a method for determination of three physiologically important RA isomers (ATRA, 13CRA and 9CRA), their 4-oxo metabolites, and ATROL in human plasma. In comparison with previous method, Lanvers and co-worker have used binary multistep gradient composed of n-hexan : 2-propanol : glacial acetic acid (solvent A – 1000 : 2.5 : 0.675, solvent B – 1000 : 15 : 0.675) at a flow-rate 1ml/min. One of disadvantages using a solvent gradient is longer time for analysis and additional time required for equilibration of the column between runs. Run time each analysis was 45 min and time required for equilibration of the column between runs was 10 min. The experimental data of the recent studies (Lanvers et. al. 1996, Miyagi et. al. 2001) have shown that 9CRA in plasma from healthy human subjects is probably below limit of detection (0.5 μg/l).

Barua (2001) also modificated the method published by Meyer et al. [1994] and described a rapid (12 min) isocratic NP-HPLC analysis for the separation and quantitation of ROL and ATRA in human serum obtained from human subjects 1 h after an oral dose of ATRA (50 mg/person). Lipids from human serum (100 μl) were extracted with the mixture of hexane, ethyl acetate and 2-propanol in presence of acetic acid, followed by separation on short (100 x 3.6 mm I. D.) 3 μm silica column. Used mobile phase consisted of the same solvents (n-hexane : 2-propanol : acetic acid), but the proportions of solvents were different (1000 : 5 : 1, v/v) from the methods mentioned previously. By the method described by Barua (2001), it was able to separate a standard mixture of cis/trans isomers (13 cis-; all trans-) of ROL and RA. However, suitability of the procedure for the separation of cis/trans isomers in human serum has not been tested. Barua (2001) found that sample aliquot used during the study was not adequate for analysis of RA under normal physiological condition. Because of the short analysis time, the method should be useful to assess ROL in epidemiological studies.

The aim of this study was to establish a rapid and simple method for determination of ATRA, 13CRA and ATRAL by HPLC. A slightly modified method described recently by Miyagi et al. [2001] was used in our laboratory.

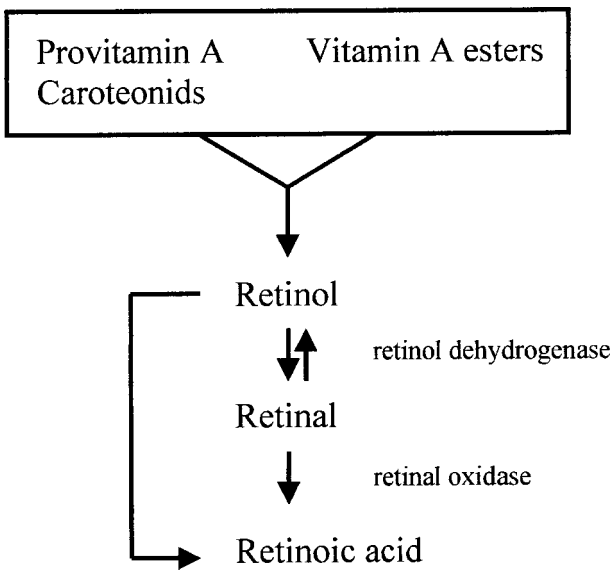

Fig. 1 Bioconversion of carotenoids and retinyl esters from dietary intake into diferent active forms of vitamin A (Palace et al. 1999).

Experimental and Discussion

Chemicals and solvents. All-trans-retinoic acid, 13-cis-retinoic acid, and all-trans-retinal were purchased from Sigma (St. Louis, MO, USA). Glacial acetic acid, n-hexan, 2-propanol and ethanol were purchased from Merck (Darmstadt, Germany). Glacial acetic acid, n-hexan and 2-propanol were of HPLC grade and ethanol was of analytical grade.

HPLC conditions. The Beckman HPLC system was equiped with a pump (Model 110), an ultra violet (UV) detector (Model 166), analog interface module 406, and System Gold software. Separation was performed on a silica gel column (Inertsil SILICA 100-5, 250 x 4.6 mm I. D., GL-Science Inc., Tokyo Japan) at a flow rate of 1 ml/min. The mobile phase consisted of hexane, 2-propanol, and glacial acetic acid at a ratio 1000 : 4.3 : 0.675. Standard solutions were separated in isocratic mode. Retinoids were determined by UV detection at a wavelength of 350 nm.

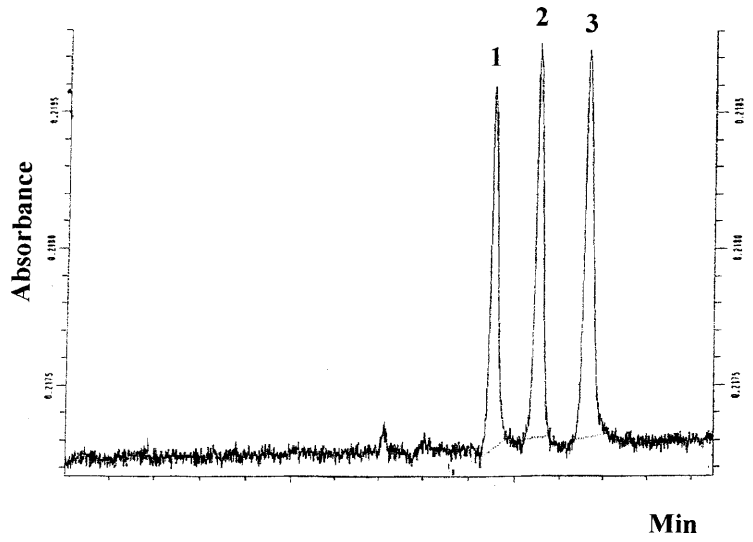

Fig. 2 Separation of selected retinoids by isocratic normal-phase HPLC (1 = all-trans retinal, retention time 9.54 min; 2 = 13-cis retinoic acid, retention time 10.55 min; 3 = all-trans retinoic acid, retention time 11.65 min).

Each retinoid was individually dissolved in ethanol (100 %) to produce a stock solution of 1 mg/ml. Working standards were prepared daily by serial dilutions of stock solutions with n-hexane to obtain concentration of 1000, 100 and 10 µg/l. They were kept in dark at 10 °C until analysis. The injection volume was 50 µl on each run. To avoid photoisomerization, all handling of retinoids was performed as quickly as possible under yellow light, and flasks containing retinoids were covered with aluminium foils.

Miyagi et al. (2001) established the method that enables full, the two-step oxidation process by which RA is synthetized from ROL. They used a linear gradient and reported suitable conditions for separate retinal isomers (13CRAL, 9CRAL, ATRAL), retinoic acid isomers (ATRA, 13CRA and 9CRA), their 4-oxo metabolites, ATROL, 13CROL. In our laboratory, we slightly modified that method reported by Miyagi et al. (2001) and separated ATRA, 13CRA and ATRAL by simple isocratic NP-HPLC. In our laboratory, both retinoic acid isomers and ATRAL were eluated within 13 min and all components were well resolved (Fig. 2). The coefficients of variation (C.V.) in five replicate analysis for RAs and RAL were from 3.0 to 5.4 %. Within-day the retention time of each retinoid was fully reproducible. Applicability of the method was repeatedly tested by using standard solutions as mentioned before.

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