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Short communication

A simple rapid method of sample preparation for LC analysis of retinoyl β-glucuronide and retinoic acid in water-based creams

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Abstract

A simple method of sample preparation for LC analysis of retinoic acid (RA) and retinoyl β-glucuronide (RAG) in creams has been developed. Water-based cream of all *trans*-RAG, devoid of side effects but efficacious in the treatment of acne vulgaris, was found to be hydrolyzed to RA in a temperature dependant manner. The potential benefits of water-based RAG cream stored at room temperature for the treatment of acne and wrinkle is discussed.

Keywords: Retinoyl β-glucuronide; Retinoic acid; Hydrolysis; Cream

1. Introduction

All *trans*-retinoic acid (RA) (tretinoin; RA, Fig. 1) is an effective topical drug for the treatment of acne vulgaris and facial wrinkles. A disadvantage of RA cream or gel (RetinA® and Renova®, Johnson and Johnson) therapy is the side effects associated with it. Erythema (redness), peeling, burning/stinging and itching of skin occur in more than 90% of the patients [1]. Therefore, it is customary to begin treatment with very low concentration (0.025%) of RA in gels and creams, and increase the concentration gradually to 0.05–0.1%. Usually the side effects do not last for a long time, and disappear with prolonged use. A newer less irritating form, Retin-A Micro® where RA is entraped in microsphere systems has been introduced. All *trans*-retinoyl β-glucuronide (RAG; Fig. 1), an endogenous water-soluble metabolite of RA, is biologically active, but unlike RA, is much less toxic [2–8]. In clinical trials carried out on acne patients in USA [9] and India [10], RAG was found efficacious, but devoid of side effects. In this paper, a simple rapid method of sample preparation for LC analysis of retinoids in water-based creams is presented. The temperature dependent hydrolysis of RAG to RA in water-based creams and the potential benefit of this hydrolysis in creams for the treatment of acne and facial wrinkles is discussed.

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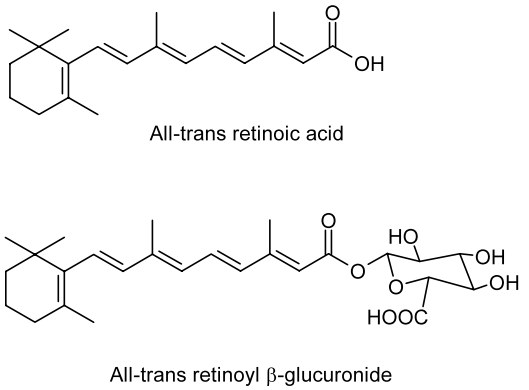

Fig. 1. Chemical structures of all *trans*-RAG and all *trans*-RA.

2. Experimental

2.1. Materials

Methanol, methylene chloride and butylated hydroxytoluene were supplied by Fisher Scientific Co., Fair Lawn, NJ. RAG was synthesized from RA according to the procedure published from this laboratory [11]. RA was obtained from BASF Corporation (Parispanny, NJ).

2.2. Preparation of standards

Standard solution of RAG was prepared by dissolving RAG (2–3 mg) in a few drops of methylene chloride and then diluting with methanol. Standard solution of RA was prepared in methanol. The concentrations of RAG and RA in solutions were determined from their absorption at 360 and 350 nm, using E 1%, 1 cm values of 1065 and 1510, respectively [12]. A Shimadzu model 2010 spectrophotometer was used. Standard curves were prepared for both RA and RAG by analyzing solutions of different concentrations by reversed phase linear gradient HPLC as described below.

2.3. LC analysis

Waters Associates (Milford, MA) LC equipment consisting of two pumps (Model 510), a photo-diode array detector (Model 996), a gradient control module, a Millennium 2010 chromatography manager, and an autosampler (WISP Model 717 plus) was used [12]. The computer was connected to a Hewlett-Packard Laser Jet printer.

For reversed-phase gradient LC analysis, the following system was used: a Microsorb-MV 3μ C_{18} column (4.6 mm × 10 cm) (Varian Analytical Instruments, Walnut Creek, CA) preceded by a guard column packed with C_{18} material, and a solvent mixture of methanol:water containing 10 mM ammonium acetate (7:3, v/v) (Solvent A) and methanol:methylene chloride (4:1, v/v)(Solvent B) [12]. A linear gradient of Solvent A (100%) to Solvent B (100%) was applied for 15 min, followed by isocratic elution with Solvent B for another 15 min. The solvent system was then reversed to initial conditions. The flow rate was 0.6 ml/min.

2.4. Preparation of water-based creams

The incorporation of RAG (0.16%) in water-based cream was done according to published procedure [9]. Freshly prepared creams were immediately transferred to 1/4 oz tubes and stored in four batches (three tubes/batch) in a refrigerator (4 °C), at room temperature (22 °C), and in two separate ovens at 37 and 50 °C.

2.5. Sample preparation and analysis of retinoids in RAG creams

A 100–120 mg portion of the cream was accurately weighed in a 13 cm long disposable culture tube. Methylene chloride (2 ml) and methanol (3 ml) were added. The tube was vortexed for 30 s and centrifuged for 1 min at $500 \times g$. The clear solution was kept at ice temperature To determine the initial concentration of RAG in freshly prepared creams (d 0), an aliquot of the extract was diluted with methanol, and the concentration of RAG was determined from its absorption at 360 nm (E 1%, 1 cm = 1065). Then, an aliquot of the dilute solution (o.d. 0.5–1 at 360 nm) was analyzed by LC to determine peak area and purity. Subsequent analysis of creams for RAG and any RA formed from hydrolysis of RAG stored at different

temperature was carried out in duplicates once a week for first 3 week, and then once a month.

3. Results

Analysis of RAG creams (160 mg/100 g cream) immediately after the preparation showed that the β-anomer (peak 1, Fig. 2A) predominated over the α-anomer (peak 2), and RA was initially absent. The reason for trailing of the α-anomer was not known. When the creams were stored in the refrigerator (4 °C), and analyzed, no hydrolysis of RAG to RA was observed up to 2 years. Analysis of RAG creams stored at room temperature (22 °C) showed the presence of trace of RA (2 mg/100 g cream) in the creams within a week. The concentration of RA increased slowly with time to 6 ± 1 (S.D.) mg/100 g cream in 3 weeks, and 28 ± 5 (S.D.) mg/100 g cream in 18 weeks (Fig. 3). The chromatograms of retinoids in creams on the day of preparation (Fig. 2A) and in creams stored at room temperature (22 °C) (Fig. 2B), 37 °C (Fig. 2C) and 50 °C (Fig. 2D) for 3 weeks, are shown in Fig. 2. The hydrolysis of RAG to RA was higher in creams stored at 37 °C, and highest in creams stored at 50 °C (Fig. 4) showing that the rate of hydrolysis of RAG to RA was temperature dependent. Both β- and α-anomers were found to be hydrolyzed at the same rate. The small peaks seen before the β-RAG peak were due to *cis* isomers that were also hydrolyzed to *cis*-RA that eluted just before *trans*-RA (Fig. 2D). The identity of the *cis* isomers was based on their absorption spectra obtained on the PDA detector, and conversion to *cis*-RA on treatment with β-glucuronidase.

3.1. Linearity, accuracy, precision and detection limits

Calibration curves were prepared by injecting samples containing 5 ng–1 μg of RAG and RA and plotting peak areas of each peak obtained at 350 nm. Linear correlation (0.99) between concentration and peak area ratio was obtained for both RAG and RA in the range stated. Precision of retention time and peak areas were measured

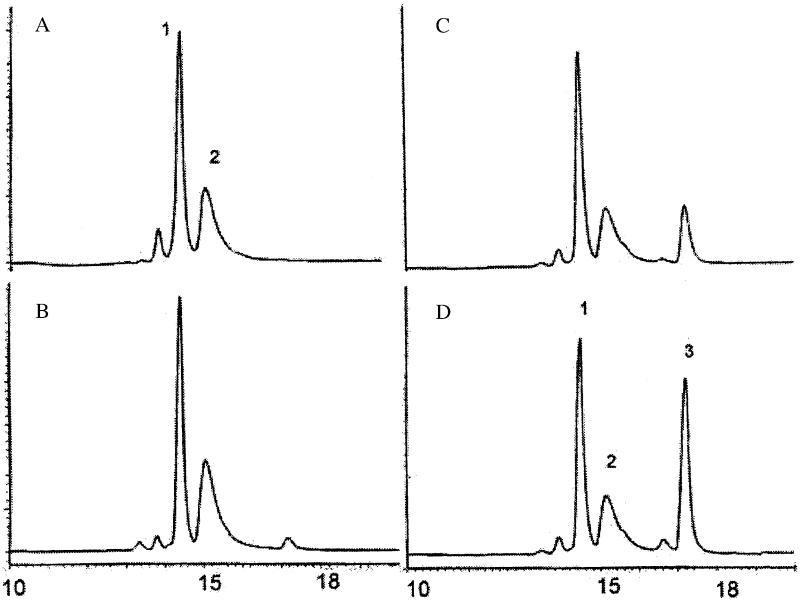

Fig. 2. LC Chromatograms of extracts of creams stored at different temperature for 3 weeks showing the temperature dependent hydrolysis of RAG to RA. (A) RAG cream analyzed on the day of preparation. Note the absence of RA; (B) cream stored at room temperature (22 °C); (C) cream stored at 37 °C; and (D) cream stored at 50 °C. Note increased formation of RA with temperature Peak identification: (1) RAG; (2) retinoyl α-glucuronide; (3) RA. The amounts of cream analyzed was not the same.

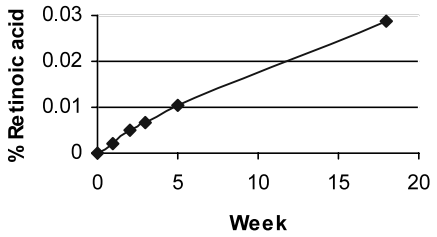

Fig. 3. Concentration of RA formed by hydrolysis of RAG (0.16%) in water-based creams stored at room temperature (22 °C) for 18 weeks.

from five replicate analysis of the same sample. The retention time and peak areas for RAG was 13.9 ± 0.02 (S.D.) min and $223\,054 \pm 7996$ (S.D.), respectively, and 17.1 ± 0.03 (S.D.) min and $15\,848 \pm 361$ (S.D.) for RA, respectively. The limit of detection was 1 ng (RAG) and 0.5 ng (RA).

4. Discussion

A simple method of sample preparation by dissolving water-based creams containing the retinoids, RAG and RA was developed. All the components in the creams are completely soluble in the solvent mixture of methylene chloride and methanol. Therefore, repeated extraction of the retinoids with organic solvents, necessary in the case of serum or tissue samples, was not necessary. Also, the chance of loss of retinoids during sample preparation was absent. In preliminary experiments, retinyl acetate ($t_R = 21$ min) that is routinely used as an internal standard in retinoid analysis [12], was used. The recovery of the internal standard from the solutions of creams was found to be $98 \pm 5\%$. However, because no extraction steps were involved, the internal standard was not used in routine cream analysis. Estimation of RAG and spiked RA in freshly prepared solutions of creams by spectrophotometry and by HPLC showed marginal errors of $\pm 4\%$ between the two procedures. The LC procedure described here is capable of simultaneously analyzing retinol, retinyl esters, RA and RAG [12]. In other experiments, the results of which are not presented here, it was also possible to simultaneously analyze spiked retinol, retinyl acetate and retinyl palmitate in creams. Therefore, the present procedure should be very suitable for analysis of creams containing, besides RAG and RA, retinol, retinyl palmitate or other retinyl esters. The present study shows that RAG in water-based creams is stable at refrigerator temperature (4 °C), but not at room or, higher temperature The hydrolysis of RAG to RA was temperature dependent.

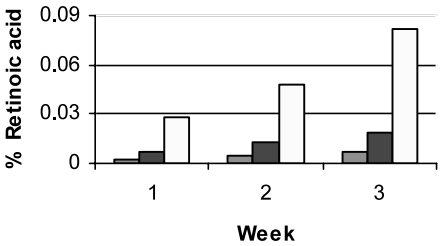

Fig. 4. Concentration of RA formed by hydrolysis of RAG (0.16%) in water-based creams after storage for 1, 2 and 3 weeks. Room temperature (22 °C): left column, medium shade; 37 °C: middle column, dark shade; and 50 °C: right column, light shade.

The present finding that RAG in water-based creams is slowly hydrolyzed to RA at room temperature reveals potential beneficial effects of water-based RAG cream for the treatment of acne or wrinkles. Even 2.4% RAG is devoid of side effects [9]. If RAG creams are stored in the refrigerator until the day of use by the patients, the creams will remain free from RA. If the patients store the creams at room temperature during use, RA will appear soon and its concentration will increase daily due to hydrolysis of RAG. In this way, the patient will be applying increasing concentrations of RA daily resulting in tolerance, and therefore free from any side effects. At the same time increasing concentration of RA in RAG creams should be more efficacious because RA therapy is dose dependent [13]. It has been reported that 0.025–0.05% creams might not be able to achieve the results as quickly or even the same degree of improvement as those who use 0.1% cream [13]. A few volunteers who have used RAG creams stored at room temperature have not experienced any side effects due to build up of RA in these creams. Further large scale study is warranted.

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