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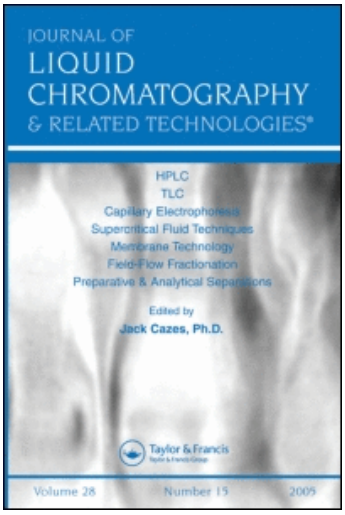

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Determination of Vitamin A in Infant/Adult Formula by HPLC-Isotope Dilution Mass Spectrometry

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Abstract: Vitamin A is an essential nutrient. Accurate determination of vitamin A in formulae or serum is a challenging problem, due to the labile properties of vitamin A and complexity of the matrices. For accurate and precise determination of vitamin A in formulae, a method of high performance liquid chromatography-isotope dilution mass spectrometry (HPLC-IDMS) was established with D_4-retinyl palmitate as an internal standard. This method was validated by a standard reference material. Coefficient of variation of this method is 2.85%, which is less than that of other literatures for determination of vitamin A in complex matrices (5–10%). This method was applied to participate in an international intercomparison. The result indicated that this method is an accurate and precise method for determination of vitamin A in complex matrices especially in formulae.

Keywords: High performance liquid chromatography, Infant/adult formula, Isotope dilution mass spectrometry, Retinyl palmitate, Vitamin A

INTRODUCTION

Vitamin A is an essential nutrient of all animal species for normal vision, growth, and cellular differentiation. These roles are critical during periods of proliferative growth and tissue development, such as infancy.

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Foods such as milk or infant formula are most commonly fortified with vitamin A in the form of retinyl palmitate, which is more stable and less susceptible to oxidation. The concentration of these vitamins after storage and manufacture needs to be checked in order to ensure correct intake and the accuracy of the label statements.[1]

Accurate determination of vitamin A in formulae or serum is a challenging problem, due to labile properties of vitamin A and complexity of matrices. The coefficient of variation (CV) in reported literatures often ranges from 5% to 10%. With liquid chromatography coupled with ultraviolet detector, determination of vitamin A in infant formulae,[1] human serum,[2] and breast milk[3] gave CVs of 5.7%, 7.1%, and 5%, respectively. With liquid chromatography coupled with mass spectrometry, determination of vitamin A in infant formulae[4] and human serum[5] gave CVs of 9% and 10%, respectively.

High performance liquid chromatography-isotope dilution mass spectrometry is a new technique, which was regarded as a candidate reference method,[6] because it applied the isotope labeled compound of analytes as the internal standard to circumvent variation caused by matrix effects and recovery rate. Determination of vitamin A usually involves a saponification reaction for transferring retinyl palmitate to retinol.[2–4] Due to recovery of retinol in this reaction and other steps in sample preparation was not negligible, an internal standard was often used. Since isotope labeled vitamin A has the identical chemical property of vitamin A, its recovery rate during preparation is equal to that of vitamin A, which makes it an ideal internal standard for determination. Due to the identical property of labeled and unlabeled analytes, they have identical retention time in liquid chromatography. Their determination was often carried out by mass spectrometry.

In-line calibration of mass spectrometry is essential to achieve more precise results. In bracketing calibration,[7] determination of a sample solution was bracketed by determination of a low concentration standard solution and a high concentration standard solution, which have a close signal to the sample solution. This method can circumvent the variation of conditions in mass spectrometry.

In this study, isotope labeled retinyl palmitate (the fortified form of vitamin A in formulae) was added before sample preparation. Bracketing calibration was applied for precise calibration. With this isotope labeled internal standard, a small CV of 2.85% was obtained. The result of determination was validated by determination of a standard reference material. This method was applied to participate in an international intercomparison with other national metrological institutes, in which various sample preparation methods and instruments were applied.

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EXPERIMENTAL

Reagents

All chemicals were of analytical reagent grade. Retinyl palmitate was purchased from Dr. Ehrenstorfer (Augsburg, Germany). D_4-Retinyl palmitate was brought from BUCHEM BV (Apeldoorn, Netherlands). Several packets of infant/adult formula were given by NIST (National Institute of Standards and Technology, US), which were Standard Reference Materials (SRM 1849). The formulae were stored at $-20^{\circ}C$ until required. Deionized water was used throughout.

Apparatus

A Finnigan LTQ LC-MS (linear ion trap) equipped with ion source of APCI (atmosphere pressure chemical ionization) was used for LC-MS analysis. A balance from Sartorius (CP324S, Max = 320 g, d = 0.1 mg, Germany) was used for samples greater than 1 g. A balance from Mettler Toledo (UMX2, Max = 2.1 g, d = 0.1 μg, Switzerland) was used for samples between 3 mg to 1 g. Black curtains were equipped in laboratories to avoid exposure of samples to bright light throughout the entire process.

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Stock Solutions

The amount of the added solution was determined by weight rather than by volume.

The stock solution of reference material containing 30 μg/mL of retinyl palmitate was prepared. Retinyl palmitate of 3.00 mg was accurately weighed, and dissolved in 100 mL of ethanol containing 3.0 mg of BHT (butylated hydroxytoluene) as a protection reagent.

The stock solution of isotope labeled material containing 30 μg/mL of D_4-retinyl palmitate was prepared. D_4-retinyl palmitate (3.00 mg) was accurately weighed, and dissolved in 100 mL of ethanol containing 3.0 mg of BHT as a protection reagent.

Sample Preparation Protocol

Weighing

A test portion (2.000 g) from a packet of infant/adult formula was weighed into a flask, spiked with D_4-retinyl palmitate stock solution and dissolved

by 6 mL of 60 ~ 70°C water. The amount of D_4-spiked retinyl palmitate approached the amount of retinyl palmitate within a test sample (W), which was determined by preliminary experiments. For example, 60 μg of retinyl palmitate in 2 g of test sample was determined by preliminary experiments, which meant $W = 60\,\mu g$. Therefore, the volume of spiked D_4-retinyl palmitate stock solution (30 μg/mL) was 2 mL [60 μg/(30 μg/mL) = 2 mL].

Saponification

Pyrogallol of 20 mL (15 g/L, dissolved in ethanol) and 10 mL potassium hydroxide (0.5 g/mL) was added. With filled sufficient nitrogen, the solution was capped and placed under nitrogen at 28°C for 16 h.

Extraction

The sample was extracted by petroleum ether (30 ~ 60°C) in separator funnels for 3 times. The organic phase was filtered through anhydrous sodium sulphate (~3 g). The filtrate was evaporated to ~10 mL. It was dried by nitrogen and was dissolved into methanol of 5 mL.

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Standard Solutions

Preparation of High Concentration Standard Solution

Retinyl palmitate of which the amount approached $1.1 \times W$, and D_4-retinyl palmitate of which the amount approached W were added into a flask. After being treated by the preparation protocol described above (dissolved in water, saponification and extraction), this solution was a high concentration standard solution containing retinol and D_4-retinol with ratio of 1.1:1.

Preparation of Low Concentration Standard Solution

Retinyl palmitate of which amount approached $0.9 \times W$, and D_4-retinyl palmitate whose amount approached W were added into a flask. After treated by the preparation protocol described above, this solution was a low concentration standard solution containing retinol and D_4-retinol with ratio of 0.9:1.

Determination

Each sample solution of a test portion was determined 6 times by LC-MS, and each determination was embraced by a low concentration

standard solution and a high concentration standard solution for calibration. Typically, the determination order for one sample solution was "*L*, *S*, *H*, *S*, *L*, *S*, *H*, *S*, *L*, *S*, *H*, *S* and *L*", in which *L* meant the low concentration standard solution, *S* meant the sample solution, and *H* meant the high concentration standard solution.

Chromatographic separations were performed at 25°C on a ZORBAX SB-C_{18} column (50 mm × 2.1 mm i.d.), 1.8 μm particle size (supplied by Agilent Technologies). The mobile phase was composed of 75% phase A (methanol:acetonitrile 1:1 containing 0.1% acetic acid) and 25% phase B (0.1% acetic acid) and was delivered at a flow rate of 0.22 mL/min. Mass spectral identification was carried out with an atmosphere pressure chemical ionization interface and an ion-trap mass analyzer. The mass spectrometer was operated in the positive ion mode. $(M+H)^+$ ions at m/z 269 and 273 were monitored for retinol and D_4-retinol, respectively. The nitrogen drying gas temperature was set at 350°C. The sheath gas flow rate was 25 arb (a unit of Finnigan's instrument), and auxiliary gas flow rate was 10 arb. Spray voltage was 3.5 kV.

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Mass fractions of retinyl palmitate in test samples (C in mg/kg) were calculated by the following formula:

$$C = \left[\frac{(RA_S - RA_L)(RW_H - RW_L)}{(RA_H - RA_L)} + RW_L\right] \times \frac{W_{\text{label}}}{W_{\text{sample}}} \times P$$

RA_S, RA_H, and RA_L are peak area ratios (relative areas) of retinol to D_4-retinol in sample solution, high concentration standard solution, and low concentration standard solution, respectively. RW_H and RW_L are mass ratios (relative weights) of retinyl palmitate to D_4-retinyl palmitate in high concentration standard solution and low concentration standard solution, respectively. W_{label} is mass (mg) of spiked D_4-retinyl palmitate in test sample. W_{sample} is mass (kg) of a test portion. P is the purity of retinyl palmitate as a reference material.

RESULTS AND DISCUSSION

Reaction and Calibration

The saponification reaction was shown as Figure 1. Retinyl palmitate in infant/adult formula and spiked D_4-retinyl palmitate were transferred to retinol and D_4-retinol, respectively, with identical recovery rate. Hence, peak ratios of retinol to D_4-retinol in MS analysis represent mass ratios of retinyl palmitate to D_4-retinyl palmitate in sample or standard solution before saponification.

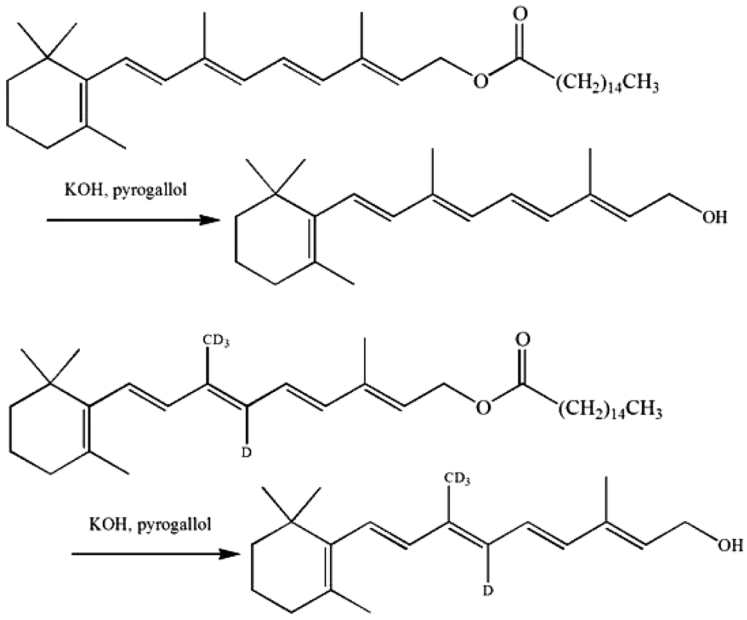

Figure 1. Saponification reaction. Retinyl palmitate and D_4-retinyl palmitate transferred to retinol and D_4-retinol, respectively.

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For accurate calibration, bracketing calibration was applied. Mass ratios of retinyl palmitate to D_4-retinyl palmitate in low concentration standard solution, sample solution, and high concentration standard solution were intentionally made to be 0.9, 1, 1.1, respectively, and a sample solution was detected just between a low concentration standard solution and a high concentration standard solution, which was called "bracketing". This calibration method can circumvent the variation of conditions in mass spectrometry, and minimize the uncertainty caused by the variation of mass spectrometry.

Optimization of Chromatography and Mass Spectrometry

The optimized condition of mass spectrometry was obtained by the optimization program in the Xcalibur data system of LC-MS, through flow injection of a standard solution of retinyl palmitate.

Chromatography was optimized manually. Mobile phase was composed of phase A (methanol:acetonitrile 1:1 containing 0.1% acetic acid) and phase B (0.1% acetic acid). While phase B increased, the retention time of retinol was retarded. If phase B was less than 25%, a component within sample solutions was not separated from retinol. Therefore, 25%

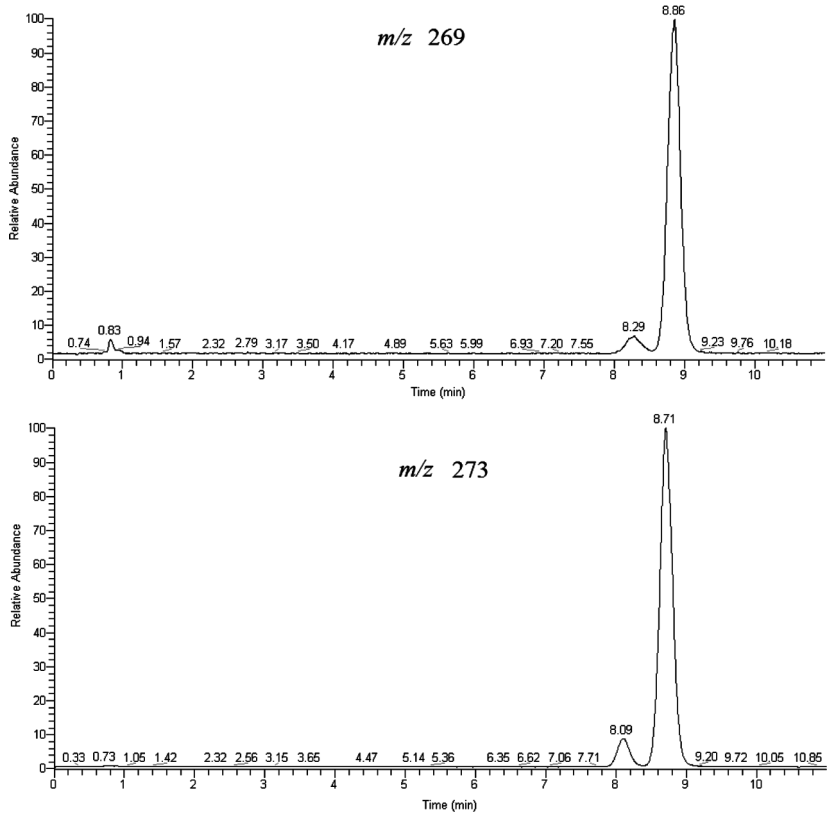

Figure 2. Liquid chromatography-mass spectrometry of sample solution from formula. Column: ZORBAX SB-C_{18} column (50 mm × 2.1 mm × 1.8 μm) mobile phase: 75% phase A (methanol:acetonitrile 1:1 containing 0.1% acetic acid) and 25% phase B (0.1% acetic acid). Flow rate: 0.22 mL/min. Ion source: atmosphere pressure chemical ionization (APCI). Mode: positive. $(M + H)^+$ ions at m/z 269 was for retinol. $(M + H)^+$ ions at m/z 273 was for D_4-retinol.

of phase B and 75% of phase A was chosen as the mobile phase. A chromatogram of the sample solution was shown in Figure 2.

In addition, determination time was greatly shortened by using Agilent's new column with particles in 1.8 μm diameter. Determination time with this new column was about 1/5 of a conventional HPLC column.

Determination of Standard Reference Material

SRM1849 is a standard reference material produced by NIST, and was applied as a test sample in an international intercomparison (CCQM-P78)

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organized by CCQM (Consultative Committee for Amount of Substance) in BIPM (International Bureau of Weights and Measures). National metrological institutes from USA, Korea, China, Thailand, and Mexico have participated in this intercomparison. By this intercomparison, the target value of vitamin A in SRM1849 was 16.6 ± 1.3 mg/kg (expressed in retinol).

The result determined by this LC-IDMS method was 16.432 mg/kg (expressed in retinol). Since deviation of this result from the target value is $\sim$1%, this method was validated for accurate determination.

Determination of Sample

Another international intercomparison (CCQM-K62/P78.1) was organized by CCQM. This method was applied to determine five samples from this key intercomparison. In this intercomparison, 20 packets of milk powder was given to each laboratory for determination of three kinds of vitamins. Only $6 \sim 7$ packets of samples was used for determination of vitamin A. This LC-IDMS method was applied, and its result is listed below.

The coefficient of variation of determinations for 5 packets was 2.85%. Considering the uncertainty induced from purity (1%, RSD of determination of purity by spectrophotometry) and balance (0.002%, least weighing mass $m = 3$ mg, sensitive quantity of balance $\Delta m = 0.0001$ mg, uncertainty of balance $= \Delta m/m/\sqrt{3}$), the uncertainty of the result is 3.0% (the square root of sum of square of 2.9%, 1%, and 0.002%).

The result of international intercomparison (CCQM-K62/P78.1) for vitamin A is shown in Table 1 and the graph of value and uncertainty shown in Figure 3. Other national institutes had applied different sample preparation methods, such as extraction without saponification. They had also applied different determination methods, such as LC-MS and LC-UV. Although different methods were carried out in different countries, the results are consistent, which meant the HPLC-IDMS method for determination of vitamin A in this study is accurate and precise.

CONCLUSIONS

For accurate and precise determination of vitamin A in infant/adult formulae, high performance liquid chromatography-isotope dilution mass spectrometry was established. D_4-retinyl palmitate was used as an internal standard to circumvent consideration of the recovery rate. Bracketing calibration was applied to achieve precise determination. This method was validated by determination of a standard reference material. Coefficient of

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Table 1. Result of international intercomparison (CCQM-K62/P78.1) for vitamin A

Participant	Vitamin A (express as retinol, mg/kg)	u_c (combined uncertainty)	u_c (relative combined uncertainty)	U (expanded uncertainty, $k = 2$)	Sample reparation methods	Standard solution	Determination method
Lab 1	15.52	0.29	1.9%	0.57	Liquid-liquid extraction	IS	LC/MS
Lab 2	16.05	1.59	9.9%	3.18	Saponification	ES	LC/UV
Lab 3 (NIM)	16.66	0.5	3.0%	1.01	Saponification	IS	LC/MS
Lab 4	16.9	0.35	2.1%	0.7	Saponification	ES	LC/UV

Abbreviations are as follows: ES = external standard, IS = internal standards, LC = liquid chromatography, UV = ultraviolet absorbance detection, MS = mass spectrometric detection, NIM = National Institute of Metrology, China.

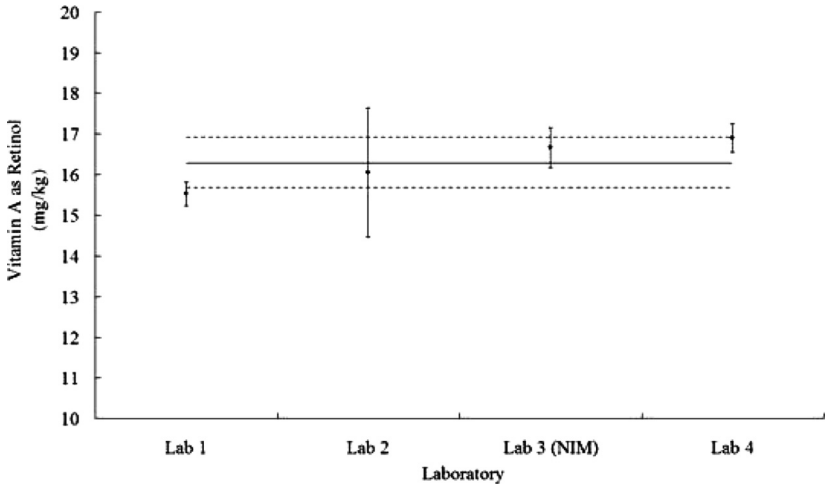

Figure 3. Result of international intercomparison (CCQM-K62/P78.1) for vitamin A. Values of vitamin A expressed as retinol were plotted (•). Error bars represent U (expanded uncertainties, cover factor $k = 2$) as reported by participants. Solid line was the average of values, and dotted lines represent the standard deviation of values.

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variation of this method is 2.85%, which is less than other literatures for determination of vitamin A in complex matrices. This method was applied to participate in an international intercomparison with other national metrological institutes, which uses various sample preparation methods and instruments. The consistent result of intercomparison also indicates that this method is an accurate and precise method for determination of vitamin A in complex matrices, especially in infant/adult formulae.

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