Simultaneous determination of vitamins A and D3 in dairy products by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Abstract. A potential method for simultaneous determination of vitamin A and vitamin D3 (25-hydroxyvitamin D3) in fresh milk samples is addressed. The method is based on combination of high performance liquid chromatography and mass spectrometry during the course of analysis. The method applied for determination of vitamins A and D3 on eighteen (18) different fresh milk samples using liquid chromatography along with tandem mass spectrometry. The work describes the suitability of the proposed method for the simultaneous determination of both vitamins using LC-MS/MS as a specific and quantitative technique. The vitamins of milk were separated by C18 Thermo gold column (100mm x 4.6mm x 5μm) with a flow rate of 1ml/min (using an isocratic mobile phase). The method was validated using duplicate analyses, relative recovery experiment, and comparative analysis with control samples. Liquid- liquid extraction was employed as a pre-concentration step with n-hexane - dichloromethane mixture (90%:10%) as an extraction solvent. The molecular ions (m/z) appeared near 286 and 385nm and for the base peaks were appeared near 255 and 355nm for vitamins A and D3. Good correlation coefficients were obtained, 0.9999 for vitamin D3 and 0.9994 for vitamin A. The limit of detection and the limit of quantification were found to be 0.09ng/ml and 0.54ng/ml for vitamin D3 and 0.32ng/ml and 1.8ng/ml and for vitamin A. The proposed method showed excellent recoveries, about 98% for both vitamins A and D3.

1. Introduction

A vitamin is an organic compound required by an organism as a vital nutrient in limited amounts [1]. Vitamins generally act as bio-catalysts, reacting with proteins to create metabolic active enzymes that in turn produce thousands of significant chemical reactions throughout the body [2]. However, 13 identified vitamins have been identified as essential to humans. They are classified as fat soluble vitamins such as A, D, E and K and water soluble vitamins such as C and B-group. Vitamin A is a group of nutritionally unsaturated hydrocarbons, which include retinol, retinal, retinoic acid, and several pro-vitamin A carotenoids, among which β-carotene is the most important [3]. Vitamin D helps the body in the absorption of calcium, needed for the normal development and maintenance of healthy teeth and bones. It also helps also maintain proper blood levels of calcium and phosphorus in the blood[4].

To date, high performance liquid chromatographic ultraviolet/visible (HPLC–UV/Vis) detection for vitamins A, D and carotenes, and fluorescence (HPLC–Fl) detection for vitamins A, E and K have been the methods of choice for the analysis of fat soluble vitamins in milk [3] [5]. Both vitamins were usually assayed individually due to their chemical diversity and varying levels within samples. However, recently an UPLC–UV/Vis method was reported for the simultaneous determination of all trans-retinol, α-tocopherol and β-carotene in milk [6]. High performance liquid chromatography–
tandem [7] mass spectrometry (HPLC–MS/MS) is widely accepted as the benchmark for quantification of a wide range of non-volatile compounds at the parts-per-million (mg/kg) and parts-per-billion (μg/kg) levels [8], this is due to its superior sensitivity and selectivity compared to single quadruple (MS), 3D (Paul) ion trap (MS), and time of flight (ToF) instruments. HPLC–MS/MS has been used for the analyses of vitamins A, D, E, and K in breast milk [10], and other food stuffs, [11][12], infant formula [13][14] and human blood serum [15][16].

2. Experimental

A chromatographic system of model API 300 Applied Bio-system consisting of an Agilent stable-bond C18 Thermo gold column with in-built detector was used. The guard and analytical columns were mounted in a thermo stated column compartment set at 30 °C. The peak areas were integrated automatically by computer using Analyst version 1.4 (AB Sciex) software program. Other apparatus used included a centrifuge, normal evaporator and vortex.

2.1 Apparatus

A chromatographic system of model 3200 ABSciex supplied with an Agilent stable-bond C18 Thermo gold column with in-built MS detector was used. The guard and analytical columns were mounted in a thermo stated column compartment set at 30 °C. The peak areas were integrated automatically by computer using Analyst version 1.4 (AB Sciex) software program. Other apparatus used included a centrifuge, normal evaporator and vortex.

2.2. Materials

Pure vitamins A, D3 and A- acetate (Sigma- Aldrich Chemicals) with analytical grade methanol, ethanol, hexane, dichloromethane, formic acid and acetonitrile were used.

2.3. Procedure for high-performance liquid chromatography

2.3.1. Chromatographic conditions

Solutions and mobile phases were prepared for the time of use. The mobile phase used was methanol:acetonitrile: de-ionized water: formic acid (68:30:2:0.1 v/v/v/v) with a pH= 3.5. The analytical column used was C18 Thermo gold (100mm x 4.5mm x 5μm). All analysis was performed under isocratic conditions at a flow-rate of 1.0 ml/min and at room temperature.

2.3.2 Standard Solutions

Three volumetric flasks (100mL each) of individual vitamins A, D3 and A- acetate stock solutions were prepared by dissolving 10mg of each in ethanol. The concentration of each was 1x 105 ng/mL. Serial solutions of vitamin A (50, 100, 200, 300, 400, 500, 750 and 1000 ng/mL) and vitamin D3 (5, 10, 20, 25, 30, 50 and 100 ng/mL) were prepared. A solution of 100ng/mL of vitamin A- acetate as an internal standard was also prepared.

2.3.3 Samples Preparation

1 g of each fresh milk sample was accurately weighed and transferred to a 10-mL plastic tube containing 1mL of internal standard (vitamin A- acetate) and 1 mL of 39% ethanolic sodium hydroxide solution. The mixture was then heated in a water bath at 60°C for 40 minutes. Extraction was performed by adding 4 mL n-hexane-dichloromethane mixture (90%:10%) and manually shook vigorously for 1 minute to yield two different layers, one organic and one aqueous layer. The hexane-dichloromethane extract was removed, washed twice with 2 mL water. The hexane-dichloromethane extracts was then taken and evaporated until dryness and filtered before acquisition with LC-MS/MS.
2.3.4. Calibration and linearity
Calibration curves were constructed in the range 5–100 ng/mL for vitamin D3 and 5–950.0 ng/mL for vitamin A to encompass the expected concentrations of measured samples. Curves were obtained by plotting the peak area of the corresponding pure vitamins against their concentrations. Linear calibration curves were generated by linear regression analysis and over the respective standard concentration ranges. The calibration curves equations for the two vitamins are:

\[ y = 0.00185x \ (R = 0.9999) \] for vitamin A

\[ y = 0.0028x \ (R = 0.9994) \] for vitamin D3.

2.3.5. Analytical recovery
Absolute recoveries of 5 different concentrations of both vitamin D3 (5–100 ng/ml) and vitamin A (5–950.0 ng/mL) in dairy products were determined by assaying the samples as described above. The peak areas of both vitamins were compared with those obtained from direct injection of the compounds dissolved in the processed blank sample.

2.3.6. Precision and accuracy
The precision and accuracy of the assay was ascertained based on analysis of quality control samples. Dairy product quality control sample concentrations for vitamin D3 and vitamin A were 5–100 ng/mL and 5–950 ng/mL, respectively. Five replicate quality control samples at each concentration were analyzed and the mean, standard deviations (SD) and coefficients of variation (C.V.) were calculated by standard statistical methods.

3. Results and discussion
Entire vitamin molecules were ionized by positive ionization mode. Fragmentation of the molecular ions produced fragment ions which were suitable for quantification. A full mass spectra scan protocol was studied using the following: Vitamin D3 parent mass 384.64 m/z, scan range of 0-377.2 m/z, quantification ions m/z 357.2 and m/z 355.2 and vitamin A, parent mass 286.45 m/z, scan range of 0-340 m/z, quantification ions 268 m/z and 255 m/z. Various extraction solvents were used such as hexane and dichloromethane, but hexane-dichloromethane mixture emerged as the best extraction solvent. Formic acid was also used in the mobile phase to enhance resolutions. During reconstitution of the evaporated prepared samples, the residue reconstitution was far better with mobile phase than with methanol due to the lower polarity of the vitamins (A and D3) in the sample. (see figure 1,2 and 3).
Fig 1. A typical Chromatograms for the simultaneous determination of vitamins A, D3 and internal standard. Where retention times of: internal standard = 1.9 min, vitamin A= 2.2min and vitamin D3 1.8min.
Fig 2. Mass spectra of vitamin A

Fig 3. Mass spectra of vitamin D3
Table 1. The average concentrations of Vitamin A (ng/mL) and Vitamin D3 (ng/mL) of each local milk and milk product (18 samples):

| S/NO | Type                  | Code     | Vitamin A (ng/ml) | Vitamin D3 (ng/ml) |
|------|-----------------------|----------|-------------------|-------------------|
| 1    | LOCAL                 | SPL001   | 46.35             | 14.60             |
| 2    | LOCAL                 | SPL002   | 6.47              | 4.91              |
| 3    | LOCAL                 | SPL003   | 39.80             | 8.88              |
| 4    | LOCAL                 | SPL004   | 12.55             | 13.65             |
| 5    | LOCAL                 | SPL005   | 30.00             | 10.00             |
| 6    | LOCAL                 | SPL006   | 7.83              | 10.36             |
| 7    | LOCAL                 | SPL007   | 7.01              | 6.76              |
| 8    | TEEBA FRESH LABANEH   | SPL009   | 9.67              | 2.69              |
| 9    | YOGHURT HAMMOUDEH     | SPL010   | 10.00             | 7.51              |
| 10   | TEEBA NATURAL YOGHURT | SPL011   | 16.20             | 6.78              |
| 11   | FRESH YOGHURT BALADNA | SPL0112  | 56.75             | 5.35              |
| 12   | JUICE MILK            | SPL013   | 8.87              | 33.9              |
| 13   | BANANA MILK HAMMOUDEH | SPL014   | 1.87              | 1.76              |
| 14   | SHANINA BALADNA       | SPL015   | 16.15             | 8.55              |
| 15   | TEEBA SHANEENA        | SPL016   | 10.30             | 9.01              |
| 16   | ACTIVIA               |          | 17.60             | 1.71              |
| 17   | ALMARAI MILK          |          | 10.78             | 8.29              |
| 18   | FRESH MILK HAMMOUDEH  |          | 8.89              | 1.88              |

The achieved limits of detection (LODs, based on SD of 5 measurements of replicate samples x 3) and the limits of quantitation (LOQs, based on SD of 5 measurements of replicate samples x 10) for both vitamins A and D3 are shown in table 2.

Table 2. Limits of detection and quantitation

| Replicate Samples | Conc. Of vitamin D3 (ng/ml) | Conc. Of vitamin A (ng/ml) |
|-------------------|------------------------------|----------------------------|
| 1                 | 1.710                        | 17.600                     |
| 2                 | 1.730                        | 17.700                     |
| 3                 | 1.770                        | 17.850                     |
| 4                 | 1.750                        | 17.900                     |
| 5                 | 1.790                        | 17.460                     |
| Average           | 1.750                        | 17.700                     |
| SD                | 0.032                        | 0.180                      |
| CV (%)            | 1.83                         | 1.02                       |
| LOD (3x SD)       | 0.096                        | 0.54                       |
| LOQ               | 0.32                         | 1.80                       |
| Relative error %  | 1.32                         | 0.70                       |
The absolute recovery was calculated by comparing the areas under the peaks obtained from standard working solutions with the peak-areas from standard samples. The recoveries of vitamins A and D3 were 98.08 %±1.01 and 98.00 %±1.26 in fresh milk samples, respectively.

Table 3. Recovery of vitamins A and D3 added to fresh milk samples

| Amount added (ng/ml) | Amount found (ng/ml) | Recovery % | Amount added (ng/ml) | Amount found (ng/ml) | Recovery % |
|---------------------|---------------------|------------|---------------------|---------------------|------------|
| Vit A               | Vit D3              | Vit A      | Vit D3              | Vit A               | Vit D3     |
| 50                  | 14.60               | 94.90      | 25                  | 12.55               | 24.80      | 99.20      |
| 50                  | 10.00               | 98.00      | 25                  | 9.67                | 24.80      | 99.20      |
| 50                  | 10.00               | 96.80      | 25                  | 12.55               | 24.10      | 96.40      |
| 50                  | 13.65               | 97.60      | 25                  | 7.83                | 24.30      | 97.20      |
| 50                  | 14.60               | 98.60      | 25                  | 9.67                | 24.50      | 98.00      |
| Mean %              |                     | 98.08      | Mean %              |                     | 98.00      |
| SD                  |                     | 0.99       | SD                  |                     | 1.23       |
| CV%                 |                     | 1.01       | CV%                 |                     | 1.26       |

The concentration of each sample was measured in duplicate. The range of average calculated concentrations were 1.76 ng/ml – 14.60 ng/ml and 1.88 ng/ml – 56.75 ng/ml for vitamins D3 and A respectively as listed in Table 1. Also the range for the standard concentrations were 5.22 ng/ml – 52.50 ng/ml and 44.00 ng/ml – 922.00 ng/ml for vitamins D3 and A respectively as listed in table 1. This shows that some of the samples have an appreciable amount of vitamins D3 and A, while some have not. This can either be as a result of pasteurization, nature of storage of the milk samples, addition of preservatives, elapsing of expiry date or possibly the exact amount of vitamins A and/or D3 was not added as claimed by the milk producers or that the amount added was not up to the minimum required as recommended by the international standard. This method showed satisfactory recovery of 98.08% for vitamin A and 98% for vitamin D3.

The present study revealed that there is little or no interference, hence no endogenous or exogenous substances were observed during the analysis. The retention times for vitamin D3 was in the range (1.66 - 1.76) min while elution range for vitamin A was (1.88 - 2.46) min.

Conclusion

The research work has potentially provided an accurate, precise, cheap, simple, reproducible and sensitive LC-MS/MS method for the simultaneous determination of vitamins A and D3 in dairy products. Vitamin A- acetate was used as internal standard for better resolution.

The internal standard used was found to be helpful in ensuring better resolution during the analysis. The method has been successfully applied for the simultaneous determination of Vitamins A & D3 in a set of Jordanian Diary products. From the results obtained we can deduce that the fresh milk samples contain an appreciable amount of vitamins A and D3 compared to long life products.

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