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Simultaneous quantification of α-lactalbumin and β-casein in human milk using ultra-performance liquid chromatography with tandem mass spectrometry based on their signature peptides and winged isotope internal standards☆,☆☆

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ABSTRACT

In recent years, there is an increasing need to measure the concentration of individual proteins in human milk, instead of total human milk proteins. Due to lack of human milk protein standards, there are only few quantification methods established. The objective of the present work was to develop a simple and rapid quantification method for simultaneous determination of α-lactalbumin and β-casein in human milk using signature peptides according to a modified quantitative proteomics strategy. The internal standards containing the signature peptide sequences were synthesized with isotope-labeled amino acids. The purity of synthesized peptides as standards was determined by amino acid analysis method and area normalization method. The contents of α-lactalbumin and β-casein in human milk were measured according to the equimolar relationship between the two proteins and their corresponding signature peptides. The method validation results showed a satisfied linearity ($R^2 > 0.99$) and recoveries (97.2–102.5% for α-lactalbumin and 99.5–100.3% for β-casein). The limit of quantification for α-lactalbumin and β-casein was 8.0 mg/100 g and 1.2 mg/100 g, respectively. CVs for α-lactalbumin and β-casein in human milk were 5.2% and 3.0%. The contents of α-lactalbumin and β-casein in 147 human milk samples were successfully determined by the established method and their contents were 205.5–578.2 mg/100 g and 116.4–467.4 mg/100 g at different lactation stages. The developed method allows simultaneously determination of α-lactalbumin and β-casein in human milk. The quantitative strategy based on signature peptide should be applicable to other endogenous proteins in breast milk and other body fluids.

1. Introduction

Human breast milk contains 1–2% protein mainly constituted of whey protein and casein [1–4]. The α-lactalbumin and β-casein are the major whey protein and casein. Their contents are *circa* 0.45% and 0.25%, respectively [5–7]. β-Casein is rich in phosphorylated serine [8], which can improve the intestinal absorption of calcium [9,10]. α-Lactalbumin is the carrier of calcium, magnesium, manganese, sodium, potassium and zinc [11–13]. The peptides derived from α-lactalbumin are reported to have anti-bacterial effects [14]. Human milk is a gold standard for infant formula. Instead of total protein and amino acids, the infant formula manufacturers tend to adjust the single protein content to achieve a similar level in human milk. Therefore, it is necessary to investigate the major human protein content.

Most of the quantification methods of protein in human milk are based on the total protein determination using Kjeldahl method. The protein content can be quantified by separation of protein nitrogen from non-protein nitrogen after precipitation with trichloroacetic acid [15,16]. The content of whey protein can be quantified after precipitation of casein at isoelectric point [17].

Some studies have tried to quantify the individual proteins in human milk using ion-exchange chromatography [5], gel electrophoresis [5,18], immunological methods and reversed-phase chromatography [7]. However, the target proteins weren't baseline separated due to the complex matrix in human milk [7]. The poor limit of detection with UV detector and the lack of available standards for human milk proteins limited the quantification of the individual target protein in human milk.

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The aim of this study was 1) to search and select the signature tryptic peptides for human milk α-lactalbumin and β-casein using ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-TOF); 2) to design the stable isotope-labeled internal standards based on the sequences of the selected signature peptides; and 3) to establish and validate a quantification method for human milk α-lactalbumin and β-casein using ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-TQ-MS) with multiple reaction monitoring (MRM).

2. Materials and methods

2.1. Reagents

Ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide (IAA), hydrogen chloride (HCl) and human α-lactalbumin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and formic acid were HPLC grade and purchased from Merck (Darmstadt, Germany). Recombinant porcine trypsin (EC 3.4.21.4) without protease inhibitors was obtained from Yaxin Biotechnology Co., Ltd. (Shanghai, China). Stable isotope-labeled [$^{13}C_6$, ^{15}N]-Leucine (L*) and [$^{13}C_5$, ^{15}N]-Valine (V*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was generated from a Milli-Q Gradient A 10 system (Millipore, Bedford, MA, USA).

2.2. Peptides and their purity

The peptides CELSQLLK (signature peptide for human milk α-lactalbumin, SPA, UniProt no.: P00709), AKQFTKCELSQL*L*KDIDGYGGIA (stable isotope-labeled internal standard for human milk α-lactalbumin, ISA), VMPVLK (signature peptide for human milk β-casein, SPB, UniProt no.: P05814) and YTKGRVMPV*L*KSPTIPFFDPQIPKL (stable isotope-labeled internal standard for human milk β-casein, ISB) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China).

The purity of peptide standards was measured by amino acid analyzer according to the method of Chinese national standard GB/T 5009.124-2003 with some modifications. The target peptide solution of 100 μL with theoretical concentration at 100 μmol/L was mixed with 400 μL water and 500 μL concentrated hydrochloric acid. The mixed solution was digested in oven at 110 °C for 22 h. After dried under nitrogen flow, the residues was dissolved in 0.1 mL hydrochloric acid (20 mmol/L) and analyzed by the amino acid analyzer (HITACHI, Japan). The ratio of the actual to theoretical concentration was considered as the purity of the target peptide standard.

2.3. Collections of human milk samples

The colostrum, transitional milk and mature milk were collected from 49 full-term women donors in Hangzhou, China during the first, second and sixth weeks after delivery. Each human milk sample was collected in a bottle with a breast pump. After homogenization by shaking, 10 mL milk samples were taken and stored individually at −20 °C until analysis. Surplus milk samples were pooled together and used for method development. All the milk samples were analyzed individually after the method had been established.

2.4. Ethics

The study protocol was approved by Ethics Committee of the College of Biosystem Engineering & Food Science, Zhejiang University. The approval no. was 2012006.

2.5. Sample preparation

The homogenized human milk sample was diluted with water at ratio of 1:100. 20 μL of the diluted human milk was mixed with 10 μL internal standard (1.5 μmol/L ISA and ISB in water), 10 μL DTT solution (50 mmol/L in water) and 835 μL water. The solution was incubated in water bath at 55 °C for 30 min. After the mixture was cooled to room temperature, 10 μL IAA solution (150 mM in water) was added to react for 30 min at room temperature in the dark. The solution was then added with 10 μL trypsin solution (200 μg/mL in 1 mmol/L HCl) and 100 μL NH_4HCO_3 solution (500 mmol/L) and digested in water bath at 37 °C for 2 h. The digestion was stopped by adding 5 μL formic acid. The solution was filtrated through 0.22 μm nylon filter before analysis.

2.6. Apparatus

The separation of tryptic peptides was carried out using the ACQUITY UPLC System equipped with ACQUITY UPLC binary solvent manager, ACQUITY UPLC sample manager, and ACQUITY UPLC column manager (Waters, Milford, MA, USA). The Column was ACQUITY UPLC BEH300 C18 column (1.7 μm particle size, 2.1 × 100 mm) (Waters, Milford, MA, USA), and equipped with a guard column of the same material. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The mobile phase flow rate was 0.3 mL/min. The injection volume was 5 μL. The column temperature was 40 °C. The LC elution program was linear gradient from 3% B to 40% B in 20 min, ramped up to 100% B in 0.1 min, then held at 100% B for 2 min, returned back to 3% B in 0.1 min and equilibrated at 3% B for 2.8 min. The total injection cycling time was 25 min.

TOF detection was performed on a Synapt G2 HDMS equipped with an electrospray ion (ESI) source (Waters, Milford, MA, USA). All data were acquired in MS^E mode under the electrospray positive ion (ESI^+) mode. Details of TOF conditions were as follows: capillary voltage, 3 kV; sampling cone voltage, 25 V; extraction cone voltage, 4 V; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas flow, 30 L/h; desolvation gas flow, 800 L/h; ramp trap collision energy, 15–35 V; lockspray reference compound, leucine-enkephalin.

The same UPLC system, column and solvents were used for the quantification with UPLC-TQ-MS. The LC elution program was linear gradient from 3% B to 32% B in 5 min, ramped up to 100% B in 0.1 min, then held at 100% B for 1 min, returned back to 3% B in 0.1 min and equilibrated at 3% B for 1.8 min. The total injection cycling time was 8 min.

Data acquisition were performed on a Xevo TQ MS equipped with an ESI source (Waters, Milford, MA, USA). The conditions of TQ MS were set as follows: capillary voltage, 3.0 kV; source temperature, 120 °C; desolvation temperature, 500 °C; cone gas flow, 50 L/h; desolvation gas flow, 900 L/h; argon collision gas pressure, 3×10^{-3} mbar. The precursor ions, product ions of signature peptides for human α-lactalbumin and β-casein and their other parameters were optimized (Table 1).

Table 1
Precursor/product ion and their corresponding fragment for target peptides of human milk α-lactalbumin and β-casein.

Protein	Sequence	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Fragment
β-Casein	VMPVLK	343.7	231.2	b2
			260.2	y2
			456.3	y4
	VMPV*L*K	350.2	231.2	b2
			267.2	y2
			469.3	y4
α-Lactalbumin	CELSQLLK	495.8	290.1	b2
			588.5	y5
			701.8	y6
	CELSQL*L*K	502.8	290.1	b2
			602.5	y5
			715.8	y6