Leptin identification and quantification in human and bovine milk and infant formulas

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OVERWIEW

• The LC-HR MS and HR MS/MS behavior of leptin was evaluated by the use of a Orbitrap Fusion tribrid analyzer. Bottom-up and top-down approaches were compared.

• We aimed to quantify leptin in human milk and infant formulas, so an immunoaffinity extraction was performed in comparison with direct detection.

• The protein analyte was detected in human milk at 6-7 µg L^{-1}.

INTRODUCTION

Leptin is a small protein (16 KDa) present in plasma and milk. It plays a key role in the regulation of body weight and its concentration is affected by many physio-pathological parameters especially body weight variation and lipodystrophic conditions[1]. Leptin is encoded by the obesity gene on human chromosome 7 and it is mainly secreted by adipocytes. It is possible that serum leptin concentration in breastfed infants is associated to early adipose tissue production and to the leptin levels in milk[2]. In order to understand the role of different newborn feeding (breastmilk, formulas, bovine milk) on infant leptin production and risk of obesity, we aimed to develop a selective nLC-HRMS method to evaluate leptin content in different milk matrices.

TOP-DOWN LC-MS

Top-down characterization and quantitation of intact leptin

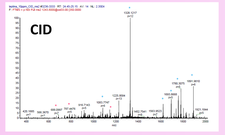


Figure 1: MS/MS: *m/z* 1244, z=13

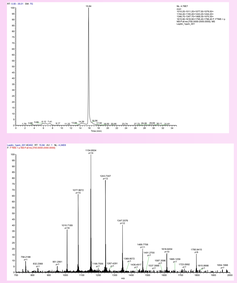


Figure 2: intact leptin chromatogram and full-Ms spectrum at 1000 ng mL^{-1}

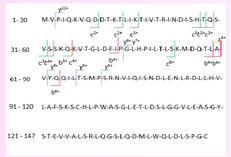
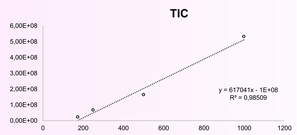


Figure 3: MS/MS fragmentation of standard recombinant methionyl human leptin,

MVPIQKVQDDTKTLIKTIVTRINDISHTQSV SSKQKVTGLDFIPGLHPILTLSKMDQTLAVY QQILTSMPSRNVIQISNDLENLRDLLHVLAF SKSCHLPWASGLETLDSLGGVLEASGYSTE VVALSRLQGSLQDMLWQLDLSPGC

METHOD 1



LLOQ 175 ng mL^{-1} (175 pg on-column); linearity 175-1000 ng mL^{-1}

Experimental

The human milk was prepared and purified following a immunoaffinity protocol in order to detect leptin as intact protein.

Sample preparation: purification of human milk with glycoLink micro immobilization kit and monoclonal antilep antibody (Sigma Aldrich). Make up 2,5 µL of antilep antibody in 97,5 µL of glikolink coupling buffer and add 11 µL of $NaIO_4$ 0.1 M for desalting step. Bind the antilep antibody to the column resin and equilibrate this latter with a pH 7-8 PBS buffer. Incubate overnight at 4°C 250 µL of human milk with binded antilep antibody. Elute analyte (leptin protein) with glicine HCl solution pH 2.5-3 0.2 M and neutralize the eluating with tris buffer 1M pH 8.5-9.

LC: Thermo Ultimate 3000 nano, column: Pepmap C18 2 µm, 100 Å, 75 µm x 25 cm column, HCOOH 0.1% in water - HCOOH 0.1% in ACN/H_2O 80/20 gradient 95:5-10:90 in 25', 0.3 µL min^{-1}, injection volume : 1 µL.

MS: Thermo Orbitrap Fusion with Easy-nanoLC source, FM and MS^2 acquisition.

DISCUSSION

At first human leptin as intact protein was extensively characterized by high resolution mass spectrometry in order to evaluate interferences. Full MS and CID/ETD MS/MS spectra (Figure 1) of multicharged positive ions of standard proteins were acquired. A top-down approach was used with the aim of reduce to a minimum sample manipulation. Accuracy, precision, LLOQ and linearity were then assessed. After immunoaffinity extraction in the samples analyzed (both human milk and infant formulas samples) an intense protein signal different from leptin was detected. After digestion of the immunoaffinity purified samples both qualifier (*m/z* 764) and quantifier (*m/z* 708) peptides from leptin were identified. These peptides were specifically calibrated using digested standard leptin (Figure 4).

BOTTOM-UP LC-MS

Quantitation of leptin after hydrolysis

*MHWGTLCGFLWLWPYLFYVQA*VPIQK
VQDDTKTLIKTIVTR**INDISHTQSVSS**
KQKVTGLDFIPGLHPILTLSKMDQTLAV
YQQILTSMPSR**NVIQISNDLENLR**DL
LHVLAFSKSCHLPWASGLETLDSLGGVL
EASGYSTEVVALSRLQGSLQDMLWQLD
LSPGC

INDISHTQSVSS *m/z* 708, z = 2

NVIQISNDLENLR *m/z* 764, z = 2

Figure 4: natural human leptin primary sequence with digestion peptides used for quantification in evidence

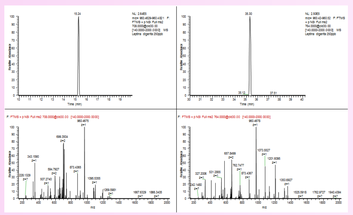
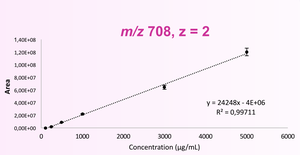


Figure 5: chromatograms and MS/MS spectra of *m/z* 708 and 764 ions

METHOD 2



LLOQ 100 ng mL^{-1} (100 pg on-column); linearity 175-5000 ng mL^{-1}

Experimental

The sample eluted from immunoaffinity columns was digested in order to detect marker peptides.

Sample preparation: digestion with Trypsin. Make up 200 mM dithiotreitol (DTT) in 50 mM ammonium bicarbonate for reduction step. Add 2.1 µL of DTT, heat solution at 60°C for 15 minutes. Remove from heat and cool for 5 minutes. Make up a 200 mM iodoacetamide (IAM) in 50mM ammonium bicarbonate for alkylation step. Add 4.4 µl of IAM and incubate the solution in the dark at room temperatures for 30 minutes. Add 2.5 µl of Trypsin solution (1 µg $µL^{-1}$). Digest overnight at 37°C.

LC: Thermo Ultimate 3000 nano, column: Accucore C4 2,6 µm, 150 Å, 75 µm x 15 cm column, HCOOH 0.1% in water - HCOOH 0.1% in ACN/H_2O gradient 95:5-1:99 in 98', 0.3 µL min^{-1}, injection volume : 1 µL

ESI MS: Thermo Orbitrap Fusion with Easy-nanoLC source, FM and data dependent analysis acquisition.

CONCLUSIONS

After digestion of purified samples leptin was quantified in samples (besides identification of interfering unknown proteins: lactoferrin and β-lactoglobulin). The method was applied to investigate about the presence of leptin in two different commercial infant formulas. In this kind of food supplements leptin resulted < LLOQ (1 µg/L).
Finally leptin concentration was quantified in human breast milk samples, obtaining values of 6-7 µg/L.
The developed method displays a better sensitivity compared to literature[3] and can be used for milk and plasma leptin dosage.
Data acquisition of new samples for statistical purposes is being implemented.

APPLICATION TO MILK SAMPLES

Evaluation of leptin in breast milk and commercial infant formulas

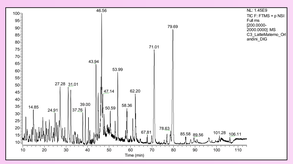


Figure 6: chromatogram of a digested breast milk sample

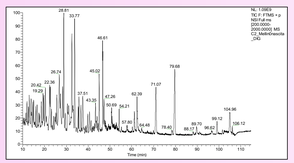


Figure 7: chromatogram of a digested infant formula sample

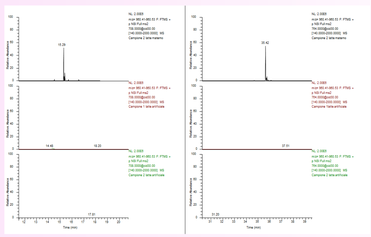


Figure 8: extracted chromatograms of *m/z* 708 and 764 ions: Breast milk vs. two different infant formula samples

RESULTS

Experimental: Leptin evaluation in real samples:

Human milk and infant formulas were provided from local pediatric hospital. In order to detect leptin concentrations < 0.1 fM, immunoaffinity extraction was performed (Experim., meth 1).
Intact leptin was not identified neither in breast milk nor in infant formulas, so the purified samples were subjected to trypsin digestion (Experim., meth 2).

Leptin quantitation in breast milk samples by MS/MS quantitation of *m/z* 708 ion: Sample A: 7.1 µg mL^{-1}; Sample B: 6.3 µg mL^{-1}

Leptin quantitation in infant formula samples by MS/MS quantitation of *m/z* 708 ion: Sample C < LLOQ; Sample D: < LLOQ.

Perspectives: to perform intact protein quantitation by enhancing method 1 sensitivity and by optimizing analyte protein immunoaffinity extraction.

References: 1) J.M. Friedman, J.L. Halaas. Leptin and the regulation of body weight in mammals. *Nature,* **395**, 763 (1998)
2) F. Savino, A. Sardo, L. Rossi, S. Benetti, A. Savino and L. Silvestro. Mother and Infant Body Mass Index, Breast Milk Leptin and Their Serum Leptin Values. *Nutrients,* **8**, 383 (2016)
3) Y. Wang, J.S. Heilig. Differentiation and quantification of endogenous and recombinant-methionyl human leptin in clinical plasma samples by immunocapture/mass spectrometry. *J. Pharm. Biomed. Anal.* **70**, 440 (2012)