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QUANTIFICATION OF N-ACETYLNEURAMINIC ACID(NEU5AC) IN HUMAN MILK USING N-GLYCOLYLNEURAMINIC ACID AS INTERNAL STANDARD BY LC-MS/MS

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ABSTRACT

A rapid, reliable and sensitive LC-MS/MS method for the determination of N-acetylneuraminic acid (Neu5Ac) in human milk was developed and validated. Samples were hydrolysed with mild hydrochloric acid and analysed by LC-MS/MS on Thermo hypersilgold C-18 analytical column. The analytical procedure was found to be more selective, accurate, precise and linear. The method accuracy was 99.9 % and the mean precision (RSD) was 3.51 % and the calibration was linear from 0.1 to 10 µg mL^{-1} (R^2> 0.99), the lowest limit of quantification (LLOQ) was 0.1 µg mL^{-1} . N-glycolylneuraminicacid (Neu5Gc) was used as an internal standard. This method was proved as promising method for the determination of Neu5Ac in human milk samples.

KEYWORDS : N-acetylneuraminic acid (Neu5Ac). N-glycolylneuraminic acid (Neu5Gc). LC-MS/MS. Human milk

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INTRODUCTION

Feeding breast milk is the best source of nutrient for infants. It contains all the elements needed for a normal growth and development of infants and able to inhibit the growth of pathogens [1] by the effect of bacteiocin. Along with other nutrient, N-acetylneuraminic acid (Neu5Ac) is a promising nutrient during the period of rapid neural growth and brain development in the new born infants. New born babies may not be having required level of Neu5Ac to meet the high demand of brain sialylglycolyl conjugate synthesis [2,3]. Neu5Ac supplementation to the animal model experiments shows improvements in learning, brain development and memory power [3,4]. Human milk is one of the richest sources of Neu5Ac while compared to other sources like bovine or formulated milk products.

There are many methods have been reported for Neu5Ac in human milk and milk products such as colorimetric technique [2,5-7] , and HPLC with fluorometry [8-10]. Using high performance anion exchange chromatography coupled with pulsed amperometric detector [11], LC-MS/MS [12] method also reported for the determination of Neu5Ac in milk products. Many researchers were used Warren's TBA method to find total sialic acid in different matrix [13]. As per literature survey no LC-MS/MS method was reported for the quantification of Neu5Ac in human milk samples.

The main objective of this study is to develop a new analytical method to determine Neu5Ac in human milk using n-Glycolylneuramininc acid (Neu5Gc) as internal standard by LC-MS/MS technique. Neu5Gc chemical properties are very similar to Neu5Ac and absence of Neu5Gc in human milk allowed us to use Neu5Gc as internal standard.

EXPERIMENTAL

Chemicals and materials

Standard Neu5Ac and Neu5Gc (99.9%) procured from Sigma Aldrich (St Louis, Missouri USA) and methanol (LC-MS grade) procured from Lab scan. Ultrapure ammonium formate (LC-MS grade) procured from Biosolve chemicals Netherland. Formic acid was procured from Fluka Germany. Acetic acid, sulphuric and hydrochloric acid were procured from Merck chemicals and all analysis was carried out using ultra pure milli Q water.

Preparation of stock and standard solutions

Standard stock of Neu5Ac 100 µg mL^{-1} was prepared in milli Q water. From the standard stock, working standard was prepared by diluting to eight different concentrations ranging from 0.1 to 10 µg mL^{-1} for calibration purpose. Working internal standard (WIS), 35 µg mL^{-1} of Neu5Gc was prepared from neat material. While preparing calibration, standard 100 µl of WIS was added and each standard contain 3.5 µg mL^{-1} of Neu5Gc. All standard stock solutions were kept at -20˚C and working standards were prepared freshly before analysis.

Sample Collection

Human milk samples were obtained from 50 healthy women (Bangalore, India) aged between 20 to 35 years with different stages of lactation. The donors were primiparous or multiparous women with average socio-economic status consuming a common Indian diet, vegetarian or non vegetarian. Milk samples were collected manually in a sterilized plastic container from both breasts by the donors. The collected human milk samples were stored at -20˚C until analysis.

Sample preparation and extraction

One hour before extraction, milk samples were taken from -20˚C and it brought down to room temperature. Milk sample were diluted 100 times with milli Q water. In 400 µL of diluted milk sample, 100 µL of 35 µg mL^{-1} Neu5Gc

(Internal standard) was added and it was mixed with 500 µL of 60 mM of hydrochloric acid, hydrolysed at 80°C for 30 min.
After hydrolysis the solution was immediately cooled in an ice bath, centrifuged at 10,000 rpm for 5 minutes. The solution was filtered through 0.22 µm PVDF filter and used for LC-MS/MS analysis.

LC-ESI-MS/MS analysis

Agilent 6460 ESI Jet stream LC-MS/MS was employed for all experiments. A Thermo hypersilgold C-18 column (150mm × 4.6mm ID × 3µ) was used. The mobile phase A was constituted using 0.3% of formic acid in water and mobile phase B was constituted using 5 mM ammonium formate in 90% methanol and 10% water. A gradient mode (time 0 to 3min: 80/20(A/B); 3.1 min: 00/100; 4min: 00/100; 4.1 to 7 min: 80/20) with 0.5 mL/min flow , the column temperature maintained at 30°C and an injection volume of 2 µl were used as analytical condition.

The MS/MS system consisted of a triple quadrupole mass spectrometer equipped with electro spray ionisation (ESI) source with jet stream technology, operated in negative ion mode. Mass Hunter workstation software version B.02.01 was used for instrument control and data acquisition.

ESI with jet stream technology showed good sensitivity for milk matrix compared to normal ESI – ve. Jet stream technology with high temperatures and high gas flow rates allow faster evaporation of mobile phase leading to quick formation of aerosol. The evaporation of aerosol droplets are faster and it allowed maximum number of ions to enter into the MS. The optimum MS conditions are summarised in Table 1 and 2. The selected reaction monitoring (SRM) mode was used for quantification of Neu5Ac and the detector response delta EMV were set at 300 V.

Table 1
Optimized source parameter

Source parameter	Optimized Value
Gas Temperature(˚C)	350
Gas Flow (L/min)	5
Nebulizer gas (psi)	45
Sheath gas Temperature (˚C)	400
Sheath gas Flow (L/min)	11
Capillary voltage (V)	4000
Nozzle voltage (V)	0

Table 2
Optimized transitions for Neu5Ac and Neu5Gc

Compound	SRM Transitions (m/z)	Fragmentor(V)	Collision Energy (V)	Dwell time (µs)
Neu5Ac	308.1 – 87.0	62	8	300
	308.1 – 170.1	62	8	300
Neu5Gc	324.0 – 116.0	74	12	300
	324.0 – 87.1	74	20	300

Selectivity
The selectivity was evaluated by analysing 20 blank soya milk samples. Soya milk sample is free of Neu5Ac and Neu5Gc and it was hydrolysed for selectivity analysis.

Linearity and Lower Limit of Quantification (LLOQ)
The eight point calibration curve was constructed by plotting peak area ratio (y) of Neu5Ac to Neu5Gc versus Neu5Ac nominal concentration (x) . Linearity was evaluated through Neu5Ac concentration range 0.1 µg mL^{-1} to 10 µg mL^{-1} (0.1, 0.2, 0.3, 0.5, 1.0, 2.5, 5.0 and 10 µg mL^{-1}). Weighted least-square linear regression analysis was used to determine the slope, intercept and correlation coefficient.
The lower limit of quantification (LLOQ) was defined as the lowest milk Neu5Ac concentration that yield a signal-to-noise (S/N) ratio >10, with acceptable precision and accuracy (<20%)

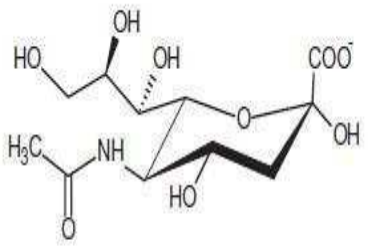
Precision, accuracy and recovery
The Precision and accuracy were determined by replicate analysis (n=6) of milk samples at four concentration levels of Neu5Ac (0.1, 0.7, 1.5 and 2.0 µg mL^{-1}).To evaluate intra and inter-assay precision and accuracy, three consecutive batches were analysed. Each batch contained a freshly prepared calibration curve and six replicates of four different concentrations of milk samples. Intra-day accuracy and precision were evaluated by analysing the samples at different time interval during the same day. Inter-day accuracy and precision were determined by repeated analysis of sample over 3 consecutive days. The accuracy was expressed as bias through calculating the percentages of difference between measured and nominal value (RME %), whereas the precision was expressed using relative standard deviation (RSD %). Precision and accuracy establish to the acceptable range $\leq$15 % [14].

Confirmation
According to the European commission decision 2002/65/EC [15] for the confirmation of analyte by LC-MS methods the following three criteria had to be met. (1) the retention time should be 2.5 % of the external standard solution ; (2) the signal-to- noise ratio (S/N) for each diagnostic ion should be $\geq$ 3:1 ; (3) the relative abundance of two reaction product ions of the sample was within acceptable range relative to the average external standard.

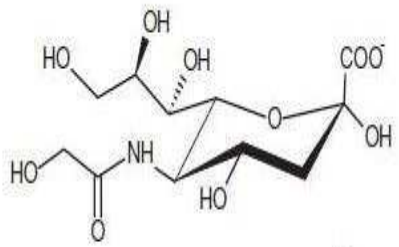
Stability
Stability was determined in two ways: (1) in solvent (stock solution) and (2) in matrix (spiked at milk at 2000 µg mL^{-1})

Fig. 1

Chemical Structure deprotonated A) Neu5Ac B) Neu5Gc



A



B

RESULTS AND DISCUSSIONS

Chromatography

The deprotonated chemical structure of Neu5Ac and Neu5Gc (IS) are given in Fig. 1. Deprotonated molecular ions of Neu5Ac and IS showed *m/z* 308.1 and 324 ([M - H]$^-$), respectively. The product ion scan spectra showed high abundance fragment ion at *m/z* 87.0 and 116 for Neu5Ac and IS, respectively. Therefore, multiple reaction monitoring (MRM) using transition at *m/z* 308.1 to 87.0 and 324.0 to 116.0 was used for quantification of Neu5Ac and IS, respectively. The typical LC-MS-MS MRM chromatograms of blank soya milk and sample spiked with Neu5Ac at lower limit of quantification (LLOQ) (0.1 µg mL^{-1}) are shown in Fig. 4. There was no interference peak at the retention time of Neu5Ac and IS, confirming the selectivity of the present method.

Selection of mobile phase and column

Prior to optimization of the mobile phase and column, many mobile phase combinations for the analysis of Neu5Ac were carried out. Among tested mobile phase combination 0.3% formic acid and 5mM of ammonium formate mobile phase gave excellent sensitivity and the gradient pump program also set within 7 minutes. The sensitivity of the method was high when gradient method with hypersil gold C-18 150mm × 4.6 mm ID × 3.0 µ column compared to other C-18 column.

Sample hydrolysis

Neu5Ac is majorly bound to oligosaccharides and glyco proteins in human milk sample. To quantify Neu5Ac in human milk by LC-MS/MS method, requires release the Neu5Ac before analysis from the oligosaccharides and glycoprotein's. Few methods were reported to release the bounded Neu5Ac which includes acid hydrolysis and enzymatic hydrolysis.

While compared to enzyme method, acid hydrolysis method is less expensive, effective but care to be taken with respect to analyte degradation. Four different acids were used to get optimum value of Neu5Ac in human milk. Formic, acetic, hydrochloric and sulphuric acid with different concentration from 10 to 100 mM were used for hydrolysis. Known amount of internal standard was added to human milk sample and it was hydrolysed with 10, 25, 50, 75 & 100 mM of sulphuric, hydrochloric, Acetic and formic acid at 80°C for 30 minutes. The degradation of internal standard in human millk for sulphuric acid was 16.5 % for 10 mM and it was gradually increased to 83.3 % for 100 mM. Whereas formic and acetic acid shows the degradation of 7 to 8 % with different acid concentration ranges. When hydrolysis was carried out with hydrochloric acid the degradation was within 2 % upto 50 mM concentration and it increased to 13.5 % of degradation when hydrochloric acid concentration increased to 100 mM. From this study hydrochloric acid was found to be best hydrolysing agent for the hydrolysis.

Hydrolysis with formic acid showed good recovery for Neu5Ac, but the time required for hydrolysis was more than hydrochloric acid. The recovery of Neu5Ac was higher in hydrochloric acid compared to other acids. To get the optimum temperature for hydrolysis, the sample was hydrolysed at different temperatures starting from 40 to 90 °C in the increment of 10°C and the optimum temperature was 80° C. Hydrolysis time was optimized with increment of 15 minutes starting initial zero to 120 minutes and the results showed 30 minutes was found to be optimum . Effect of acids and hydrolysis conditions were shown in Fig. 2 and 3.

Fig 2

Effect different acids on the hydrolysis of Neu5Ac

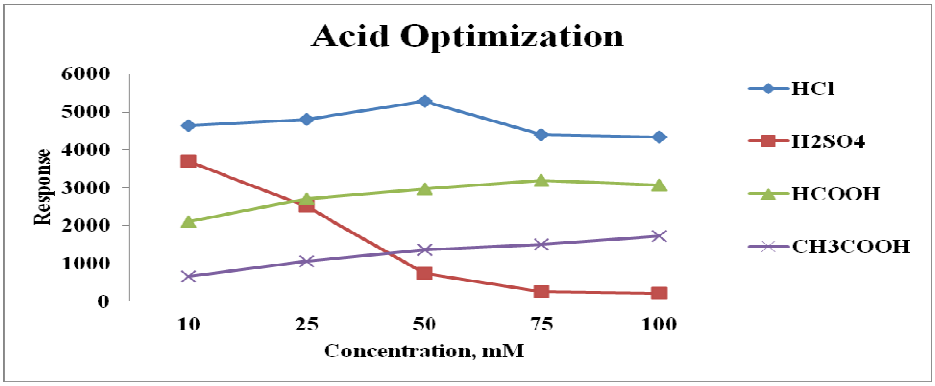
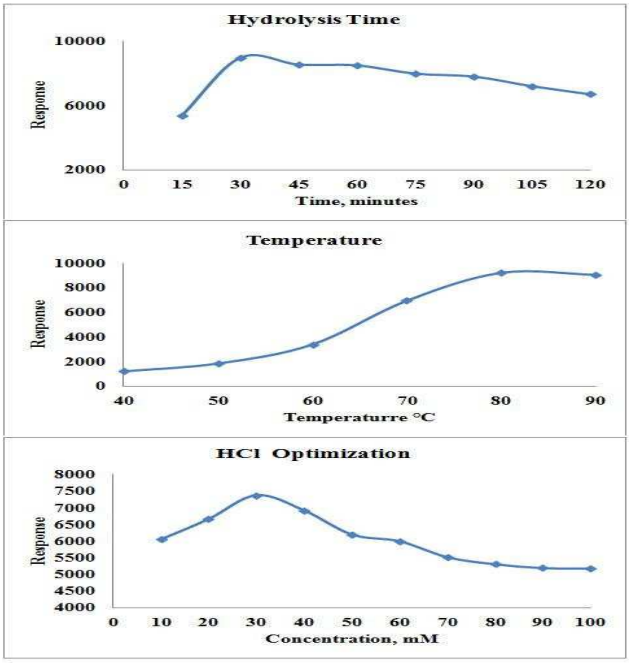


Fig 3

Effect of Time, Temperature and HCl concentration on the hydrolysis of Neu5Ac



Performance of the method

A method validation procedure was conducted to determine the response linearity, extraction efficiency, lower limit of quantification (LLOQ), precision and accuracy. The developed method shown to be no co eluting endogeneous interferences at the retention time of Neu5Ac and Neu5Gc, indicating selectivity of the method (Fig 4).

A good linear relationship was found between the peak-area ratios of Neu5Ac to Neu5Gc versus the concentrations Neu5Ac ranging from 0.1 to 10 µg mL^{-1} with eight levels (Table 3). Linear regression analysis indicated that the coefficient of determination (r^2) was greater than 0.99. The precision of the method expressed as % RSD was below 7 percentages. The recovery of the method was found to be > 98 %.

Table 3
Linearity of Neu5Ac

Standard Concentration, µg mL^{-1}	Mean Ratio(n=6) (Standard area/Internal std area)
0.1	0.0475
0.2	0.0860
0.3	0.1188
0.5	0.1822
1	0.3929
2.5	0.9543
5	1.9227
10	3.8487

As human milk contains high amount of Neu5Ac, the recovery studies was carried out by using diluted human milk sample. Human milk sample was diluted to get 5 µg mL^{-1} level and four different concentration levels (0.1, 0.7, 1.5 and 2.0 µg mL^{-1}) of Neu5Ac were added and hydrolysis was carried out and analysed by LC-MS/MS. The recovery results were summarised in Table 4.

Table 4
Precision, accuracy and recovery of Neu5Ac at four different levels (n = 6)

Nominal concentration (µg mL^{-1})	Determined concentration (µg mL^{-1})	Accuracy (RME [a], %)	Intra-day Precision (RSD [b], %)	Inter-day Precision (RSD [b], %)	Recovery ,%
0.100	0.0984	-1.63	1.57	0.79	98.36
0.700	0.694	-8.32	1.57	2.79	99.16
1.500	1.555	3.64	6.09	4.47	103.6
2.000	1.970	-1.51	3.71	2.77	98.4

Relative Mean Error = (Determined concentration – nominal concentration)/nominal concentration x 100
Relative Standard Deviation = (standard deviation /mean) x 100

The precision and accuracy, % RSD and % RME, were < 9% (Table 4). According to Causon[14] , precision and accuracy are generally acceptable if RSD and RME are ≤ 15%, From the study (Table 4) shows the reproducibility of the method was good.

Confirmation

The retention time of Neu5Ac in fortified samples was within 2.5 % of the external standards. Ion ratios of the product ions quantification and confirmation were calculated and all of them were within the acceptance range. The lowest S/N for neu5Ac quantifying ion was 9.8 in fortified sample.

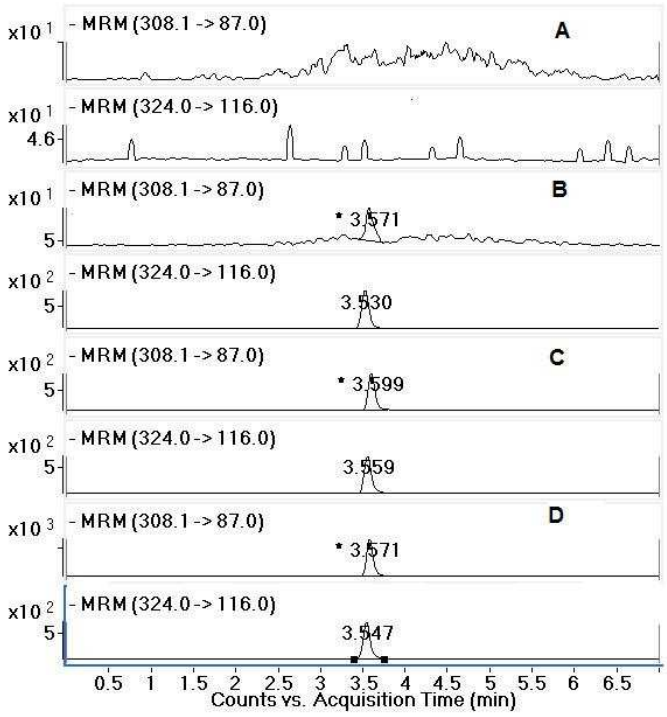
Internal standard

The extraction method involves acid hydrolysis step which is crucial with respect to acid concentration, hydrolysis temperature and time, small variation in these parameters may lead to wrong results. So to ensure the extraction procedure, known amount of internal standard should be added before starting the hydrolysis procedure.

One important requirement of internal standard is that the internal standard and the analyte should behave similarly both during the sample extraction and during the LC-MS/MS analysis. Available option for internal standard selection is (a) structurally related compound, (2) a structurally similar compound, (3) a stable isotope labelled compound. Since LC-MS/MS uses mass as a means of detection, the choice is of internal standard preferably isotopically labelled compound and since we were unable to get isotopically labelled Neu5Ac, we have chosen structurally related compound Neu5Gc as internal standard. Moreover the chemical properties of Neu5Gc were very similar to Neu5Ac and it was absent in human milk. Using Neu5Gc as internal standard the recovery was above 98 %.

Fig. 4

MRM of standard Neu5Ac (308.1 -> 87.0) and Neu5Gc IS (324.0 -> 116.0)
A) blank soya milk B)Extracted Neu5Ac in soya milk at LLOQ level C) Spiked Neu5Ac in human milk ,1 µg mL^{-1} D) Human milk sample



Stability

The stability of the stock solutions in the water was at least six months at - 20°C. The stock solution were analysed every month and the instrumental response were compared with the peak area obtained when the solution was freshly prepared. The acceptance criteria were comprised between 95 and 105% of the initial one. Standard solution of 5 µg mL^{-1} in water kept at room temperature and checked the response of the instrument, the response gradually decreased and on the fifth day analysis response dropped down to 54%. Spiked human milk samples of Neu5Ac at 2000 µg mL^{-1} stored at -20°C were analysed after 2, 4 &8 days. It was found that the recovery of the Neu5Ac had not obviously changed.

Application to sample

Human milk samples were analysed as following different groups colostrums (day2), transitional milk (day 15), mature milk (third month) and late lactation milk (10th month) samples were collected. The time of lactation was calculated as the days or months elapsed from delivery. Colostrums milk shows the highest level of Neu5Ac compared to any other milk. The level of Neu5Ac in milk is depends on the mother health, food habit etc. The results of few samples are summarized in Table 5.

Table 5

Results of Neu5Ac in human milk samples (n = 6)

Sample	Mean Content Mean ± SD [a] (µg mL^{-1})
Colostrums milk	1940 ± 120
Transition milk	1413 ± 100
Mature milk	910 ± 80
Late lactation milk	310 ± 55

a Standard Deviation

CONCLUSION

Earlier reported analytical methods for Neu5Ac was spectrophotometry technique, HPLC-Flourescent and GC-MS. Since spectrophotometer method widely used, it has certain limitation that many component may interfere with the assay while doing in complex milk matrix. HPLC with florescence gave good sensitivity for Neu5Ac but it require derivatization step which is more critical and time consuming. GC-MS method makes identity confirmation possible thus increasing the validity of the results but this method also requires derivatization to enhance the sensitivity. The developed LC-MS/MS method was more selective and sensitive since the detection is based on mass .It gives lower detection limit compare to other analytical techniques without any derivatization step which is more crucial with respect to time, temperature and moreover the derivatization reagents are costlier. This method can be applied to human milk, biological matrix and clinical trials to determine the Neu5Ac. The total run time was only 7 minutes and maximum samples can be analysed in a day. LC-MS/MS negative ESI mode was successfully applied for the quantification of Neu5Ac in human milk.

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