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Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

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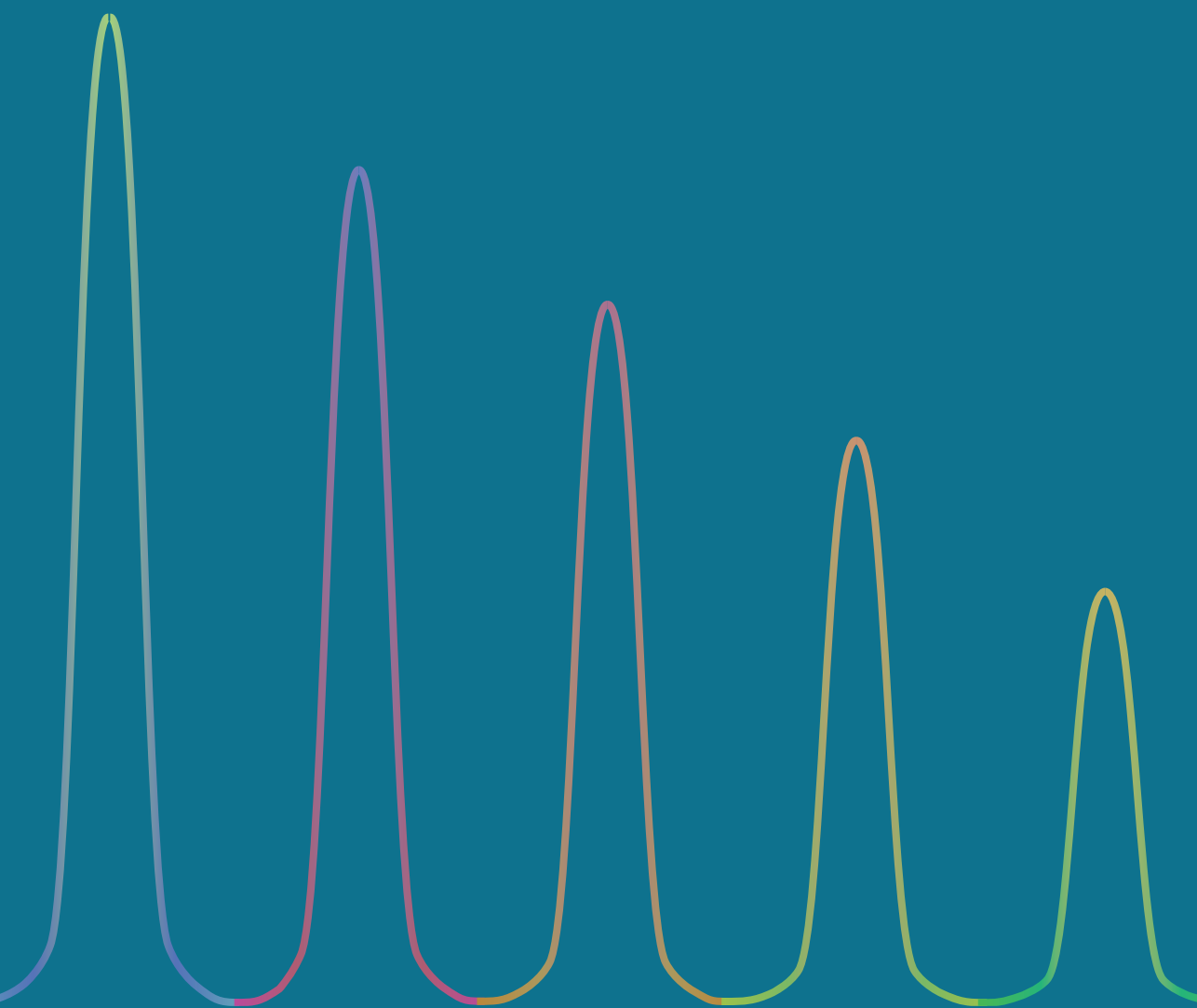

Chapter 2

Mass spectrometric quantification of glycosylated metabolites of cholesterol analogues using (isotope) standards

To be incorporated in revised form in an invited review

Chapter 2 - Mass spectrometric quantification of glycosylated metabolites of cholesterol analogues using (isotope) standards

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To be incorporated in revised form in review.

Contributions:
H.N.J.M. : author, performed described experimental biochemistry and analytical chemistry
P.W. : (BioSyn, LIC); synthesis of (isotope labeled) GlcChol, Glc7DHC and $GlcD_3$
K.K. : synthesis of (isotope labeled) GlcChol and GlcDesm
M.M. : advise with LC-MS/MS
M.J.F. : advise with sample preparation
D.E.C.B. : collaboration on extraction and measurements of metabolites in skin
C.M.B. : (LACDR); collection full skin samples
M.A. : co-supervision
J.M.A. : supervision

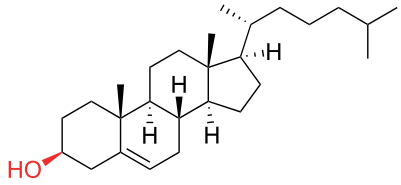
Abstract

Cholesterol (Chol) is an essential structural membrane lipid and an important precursor for metabolites such as oxysterols, bile acids and steroid hormones. The two analogues that resemble cholesterol very closely are its two precursors 7-dehydrocholesterol (7DHC) and desmosterol (Desm). The retaining β-glucosidase glucocerebrosidase (GBA) is able to transglucosylate the glucose moiety from glucosylceramide, the natural substrate of the enzyme, onto cholesterol, thus producing glucosylated cholesterol (GlcChol). A similar reaction is expected for its structural analogues, allowing the formation of glucosylated-7-dehydrocholesterol (Glc7DHC) and glucosylated desmosterol (GlcDesm). Expected is that Glc7DHC can be converted into glucosylated vitamin D (glucosylated cholecalciferol, $GlcD_3$) under influence of UVB irradiation (305 nm). To study these tentative glucosylated sterols, sensitive quantitative methods are required. We here report the development of a (sensitive) LC-MS/MS method to quantify Glc7DHC, $GlcD_3$ and GlcDesm in tissues and body fluids.

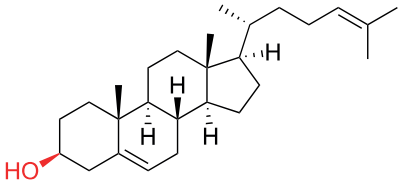
Introduction

The membrane-associated retaining β-glucosidase glucocerebrosidase (GBA, EC 3.2.1.45) is known for its deficiency in the lysosomal storage disorder Gaucher disease (GD) [1]. Defects in GBA impair its ability to degrade glucosylceramide (GlcCer) into glucose (Glc) and ceramide (Cer), resulting in lysosomal storage of GlcCer [2, 3]. Besides its function as a hydrolase, GBA has been shown to also catalyze transglycosylation [4-6]. This ability allows the formation of retinyl-β-D-glucoside (GlcRet) [4] and 1-*O*-cholesteryl-β-D-glucopyranoside (GlcChol) [5, 6]. Cholesterol (Chol) possess various structural analogues, with the sterols 7-dehydrocholesterol (7DHC) and desmosterol (Desm) showing the closest resemblance, both being the direct precursors of cholesterol in the cholesterol biosynthesis [7-9]. The existence of these closely related molecules, invites the investigation of the potential formation, and natural occurrence of glycosylated desmosterol (GlcDesm) and glucosylated 7DHC (Glc7DHC). As 7DHC is converted into vitamin D (cholecalciferol, D_3) under influence of thermal dependent rearrangement of the double bonds and UVB irradiation (305 nm) [10, 11], and as both 7DHC and D_3 have a secondary hydroxyl-group attached to their sterol scaffold, it makes D_3 an additional candidate for potential transglucosylation as well. Here we present LC-MS/MS methods for quantification of Glc7DHC, $GlcD_3$ and GlcDesm based on ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) using $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm as the internal standards, which were synthesized by Dr. Patrick Wisse and Ken Kok (from the Bio-organic Chemistry Department, LIC) for this particular purpose.

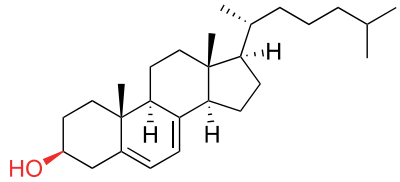
Chol



Desm



7DHC



D_3

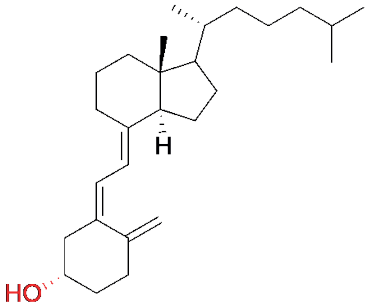


Figure 1. Chemical structures of cholesterol (Chol), desmosterol (Desm), 7-dehydrocholesterol (7DHC) and vitamin D (cholecalciferol, D_3).

Results

Quantification of Glc7DHC, $GlcD_3$ and GlcDesm by LC-MS/MS

Based on our previously developed LC-MS/MS procedure for quantitative detection of GlcChol [6], we developed a LC-MS/MS procedure for quantitative detection of Glc7DHC, $GlcD_3$ and GlcDesm. For this purpose, internal standards, $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm, were synthesized. This avoids the need for corrections for extraction efficiency, chromatographic behaviour and ionization efficiency during quantification. Lipids were extracted by Bligh and Dyer followed by butanol/water extraction.

For the quantification of Glc7DHC, $GlcD_3$ and GlcDesm in plasma, a calibration curve was performed in plasma by spiking the standard solution of Glc7DHC, $GlcD_3$ and GlcDesm. The calibration curve is: 0 – 0.1 – 0.5 – 1 – 2 – 4 – 10 – 20 – 50 – 100 – 200 nM. As internal standards $^{13}C_6$-Glc7DHC was used for quantification of Glc7DHC and $GlcD_3$ and $^{13}C_6$-GlcDesm for quantification of GlcDesm. A fixed amount of internal standard was added to the sample before extraction and the samples were extracted and measured on the same day. The ratio, area of analyte/area of internal standard, was plotted against the concentration of analyte in spiked plasma (Figure 2A and 2B). For GlcDesm a similar plot was made using the area from the transition of $^{13}C_6$-GlcDesm (Figure 2C).

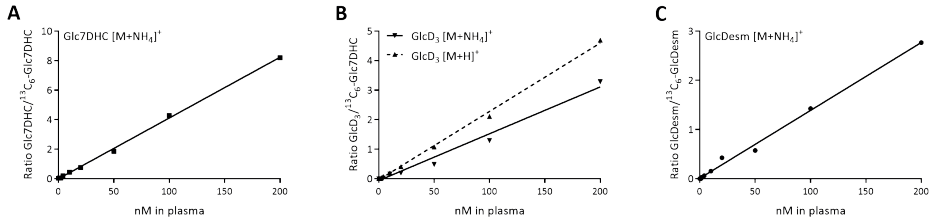


Figure 2. Linearity of Glc7DHC, $GlcD_3$ and GlcDesm in plasma. *A) Glc7DHC absolutely dominant transition $[M+NH4]^+$. B) $GlcD_3$ major transition $[M+H]^+$ and minor transition $[M+NH4]^+$. C) GlcDesm absolutely dominant transition $[M+NH4]^+$. X-axes represents the spiked concentration in plasma (nM), Y-axes represents ratio between analyte and the corresponds $^{13}C_6$-standard.*

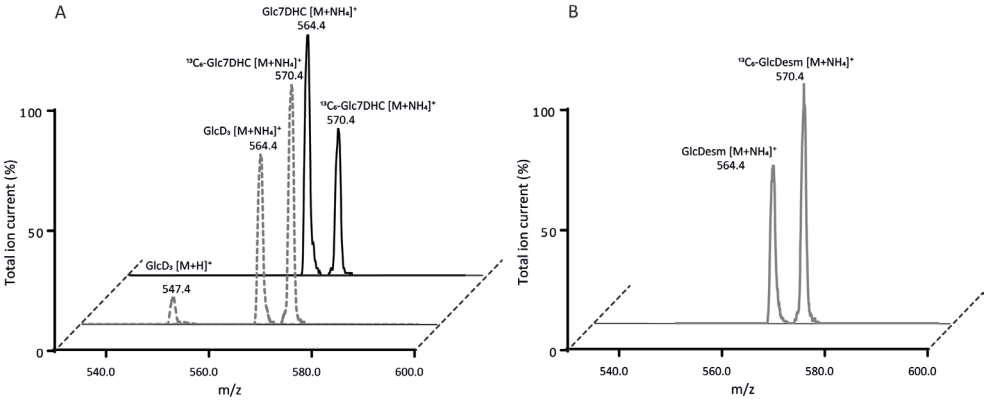
As for GlcChol [6], for all three compounds a linear response was obtained over the entire concentration range (Figure 2 and Table 1). The corresponding limit of detection (LOD) and limit of quantification (LOQ) for the compounds in plasma are given in Table 1. Calculation of the signal-to-noise (SN) was done utilizing the peak-to-peak method.

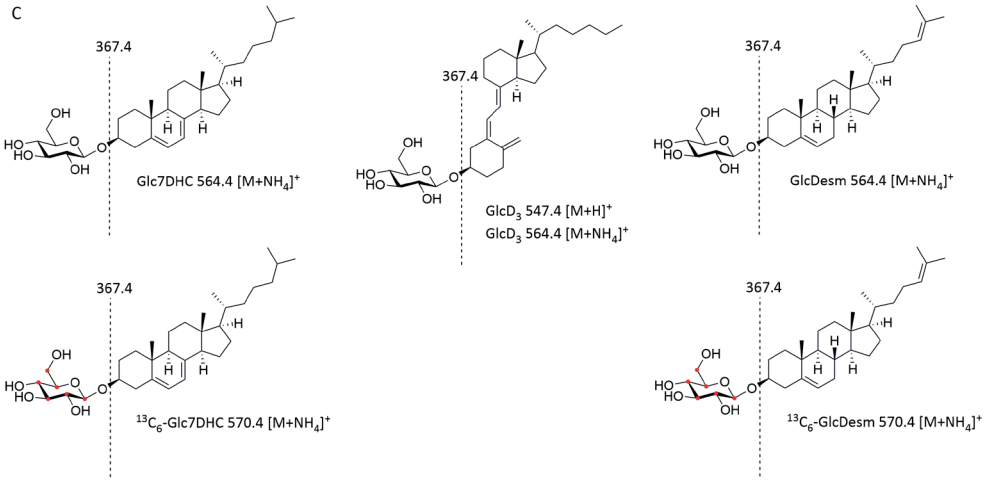
Table 1. Limit of Detection (LOD), Limit of Quantification (LOQ) and corresponding signal-to-noise ratios (S/N).

Compound	R^2	LOD (nM)	S/N	LOQ (nM)	S/N
Glc7DHC	0.999	0.1	3	0.3	10
$GlcD_3$ $[M+H]^+$ $GlcD_3$ $[M+NH_4]^+$	0.998 0.983	0.1	3	0.3	10
GlcDesm	0.995	0.1	5	0.5	10

Mass spectrometric analysis, fragmentation and elution spectrum

Mass spectra were recorded from the newly synthesized unlabelled Glc7DHC, $GlcD_3$ and GlcDesm and $^{13}C_6$- labelled Glc7DHC and GlcDesm, injected as a mixture of 10 µM each (Figure 3A and 3B). Under influence of collision-induced dissociation the compounds lose the glucose moiety, resulting in a product ion of *m/z* 367.4 (Figure 3C). The most abundant species for GlcChol, GlcDesm and Glc7DHC were ammonium adducts, $[M+NH_4]^+$. For D_3 no clear abundant species could be detected. So, both hydrogen adducts, $[M+H]^+$ and ammonium adducts, $[M+NH_4]^+$ are reported. The ^{13}C-isotopes are on the glucose molecule, therefore the daughter fragment of the compounds have the same *m/z* ratio as the not isotope compound. The expected *m/z* values for each compound are presented in Figure 3D. Even though the compounds have the same mass, each compound elutes at a different retention time (Figure 3E and 3F).





D

Compound	Mass	$[M+NH_4]^+$ (Parent)	$[M+H]^+$ (Parent)	Product ion (Daughter)
$^{13}C_6$-Glc7DHC	552.4	570.4	NA	367.4
Glc7DHC	546.4	564.4	NA	367.4
$GlcD_3$	546.4	564.4	547.4	367.4
$^{13}C_6$-GlcDesm	552.4	570.4	NA	367.4
GlcDesm	546.4	564.4	NA	367.4

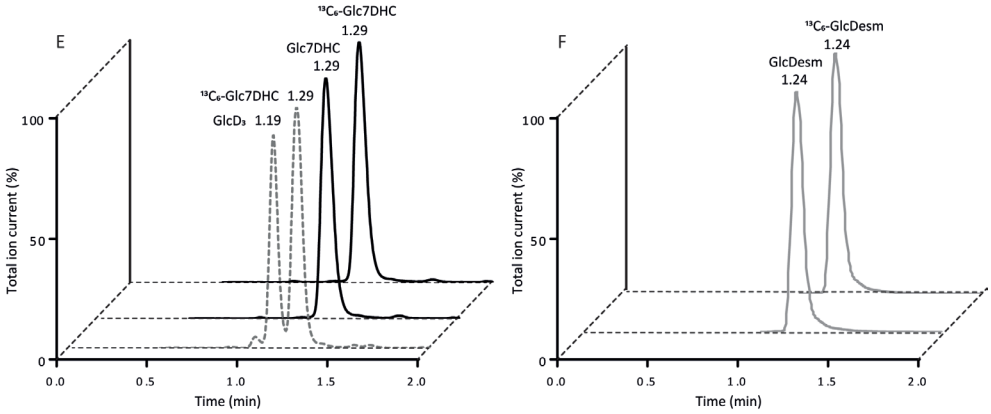


Figure 3. Mass spectrometric analysis. *A) MS-scan of Glc7DHC, GlcD$_3$ and $^{13}C_6$-Glc7DHC. B) MS-scan of GlcDesm and $^{13}C_6$-GlcDesm. C) Fragmentation pattern, the common product ion is 367.4 for all compounds loss of glucose moiety. D) Compounds mass,* m/z *values of parents and daughters. E) Elution pattern of Glc7DHC, GlcD$_3$ and $^{13}C_6$-β-Glc7DHC. F) Elution pattern of GlcDesm and $^{13}C_6$-β-GlcDesm.*

Intra/Inter assay variation

The precision of the quantification was determined by intra-assay and inter-assay variations. Figure 4 and Table 2 show the intra and inter-assay variation. The intra- and inter-assay variation was over all compounds on average 15.6% and 20%, respectively (ranges 5.9 – 32.8% for the intra-assay variation and 5.8 – 37.1% for the inter-assay variation. The ranges for Glc7DHC and GlcDesm are acceptable, as Glc7DHC ranges 5.9 – 10.6% and GlcDesm 14.7 – 23.7% for intra-assay variation and 6.2 – 14.1% (Glc7DHC) and 5.8 – 11.4% (GlcDesm) for inter-assay variation. Due to lack of a $^{13}C_6$-GlcD$_3$ internal standards the variations for GlcD$_3$ $[M+H]^+$ and GlcD$_3$ $[M+NH_4]^+$ ranges 8.2 – 32.8% for intra-assay variation and ranges 25.4 -37.1% for inter-assay variation.

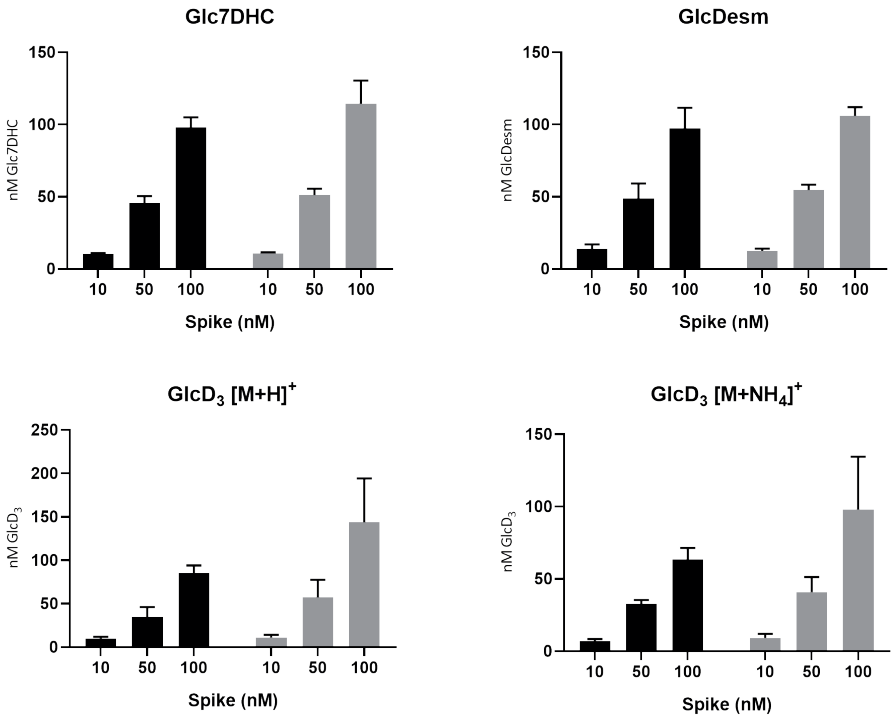


Figure 4. Intra-assay and inter-assay validation. *A) Left bars (black) represent intra-assay and right bars (grey) represent inter-assay validation. All samples were corrected for endogenous present analytes. X-axes represents the spiked concentration (nM), Y-axes represents the found concentration of the analyte in plasma (nM).*

Table 2. Intra-assay and inter-assay variation. In nM (n=5).

		10 nM			50 nM			100 nM		
Compound	**Assay**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**
Glc7DHC	Intra	10.4	0.6	5.9	45.6	4.8	10.6	98.1	6.9	7.0
	Inter	10.8	0.7	6.2	51.2	4.3	8.3	114.4	16.1	14.1
$GlcD_3$ $[M+H]^+$	Intra	10.0	1.9	19.2	34.7	11.4	32.8	85.2	8.9	10.4
	Inter	11.2	3.0	26.3	57.3	20.2	35.3	143.9	50.4	35.0
$GlcD_3$ $[M+NH_4]^+$	Intra	7.0	1.4	20.3	32.7	2.7	8.2	63.4	8.0	12.6
	Inter	9.2	2.8	30.6	40.9	10.4	25.4	98.1	36.4	37.1
GlcDesm	Intra	13.8	3.3	23.7	48.8	10.4	21.3	97.4	14.3	14.7
	Inter	12.6	1.4	11.4	54.7	3.6	6.5	106.0	6.1	5.8

Carryover

The carryover was determined by injecting twice methanol following the injection of pure standards (0.05 µM and 1 µM) or injection of standards spiked in biological samples. All 0.5 µM injections of pure standards and the standards spiked in biological samples revealed a carryover of zero. The injection of 1 µM pure $^{13}C_6$-Glc7DHC and pure Glc7DHC showed a carryover of ≤0.03% after the first methanol injection, and zero after the second methanol injection. Injection of 1 µM pure $^{13}C_6$-GlcDesm and pure GlcDesm showed a carry-over of ≤0.02% after the first and second methanol injection, and zero after the third methanol injection. Based on these findings injecting methanol twice between each set of GlcDesm samples is advised.

Storage of samples

Storage of skin. Several skin samples of the same healthy person were measured fresh (non-stored, extraction and measurement on same day) and stored at different temperatures: fridge (4°C), freezer (-20°C), freezer (-80°C) for 1.5 month. Lipids were extracted and measured on the same day as samples were taken out of storage. As shown by Figure 5 GlcChol is stable for all conditions, Glc7DHC on the other hand is not. Samples stored at -20°C and -80°C show higher level of Glc7DHC (pmol/g of tissue), indicating that due to storage the structure of other lipids rearranged into Glc7DHC. Detection of Glc7DHC was 60% less for -80°C stored skin compared to non-stored skin (data not shown). We propose to measure the skin samples fresh for the most reliable Glc7DHC measurement.

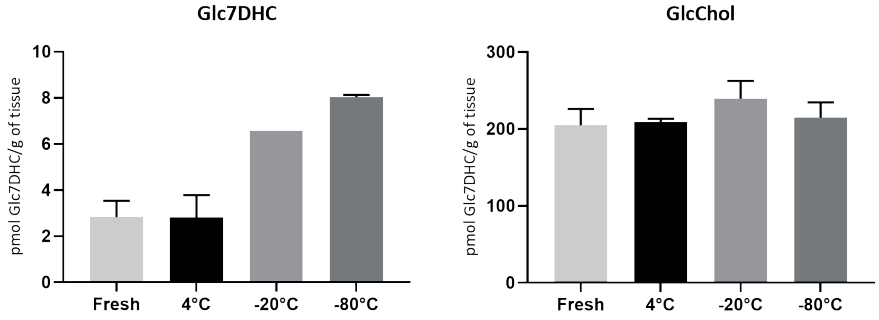


Figure 5. Glycosylated sterols in skin stored for 1.5 month at different conditions. *From left to right bars samples were fresh (non-stored), kept in fridge (4°C), freezer (-20°C) or freezer (-80°C). Measurement of Glc7DHC at -20°C shows no error bar due to sample loss.*

Storage of plasma. Plasma samples were stored for either 10 days at RT, fridge (4°C) and freezer (-20°C) or 14 days at freezer (-20°C) and freezer (-80°C). As shown by Figure 6 for Glc7DHC, GlcDesm, and GlcChol no significant difference in levels were noted, when stored at different conditions. As standard deviations are smallest for storage at -20°C and -80°C, storage at these temperatures is advised. $GlcD_3$ on the other hand seems less stable, and shows a variability between the $[M+H]^+$ and the $[M+NH_4]^+$ transition, especially for storage at -80°C. The spiked concentration of 10 nM was not detectable anymore for the $[M+NH_4]^+$ transition, indicating that the $[M+H]^+$ is the more suitable transition for measuring low concentrations.

Storage of breast milk. Breast milk samples, were stored for 14 days at freezer -20°C and -80°C. As shown by Figure 7 for Glc7DHC, GlcDesm, and GlcChol no difference in levels of the various lipids, when stored at different conditions were noted. $GlcD_3$ shows again less stability and shows, just as in the plasma, variability between the $[M+H]^+$ and the $[M+NH_4]^+$ transition. Again the $[M+H]^+$ is the more suitable transition for measuring low concentrations.

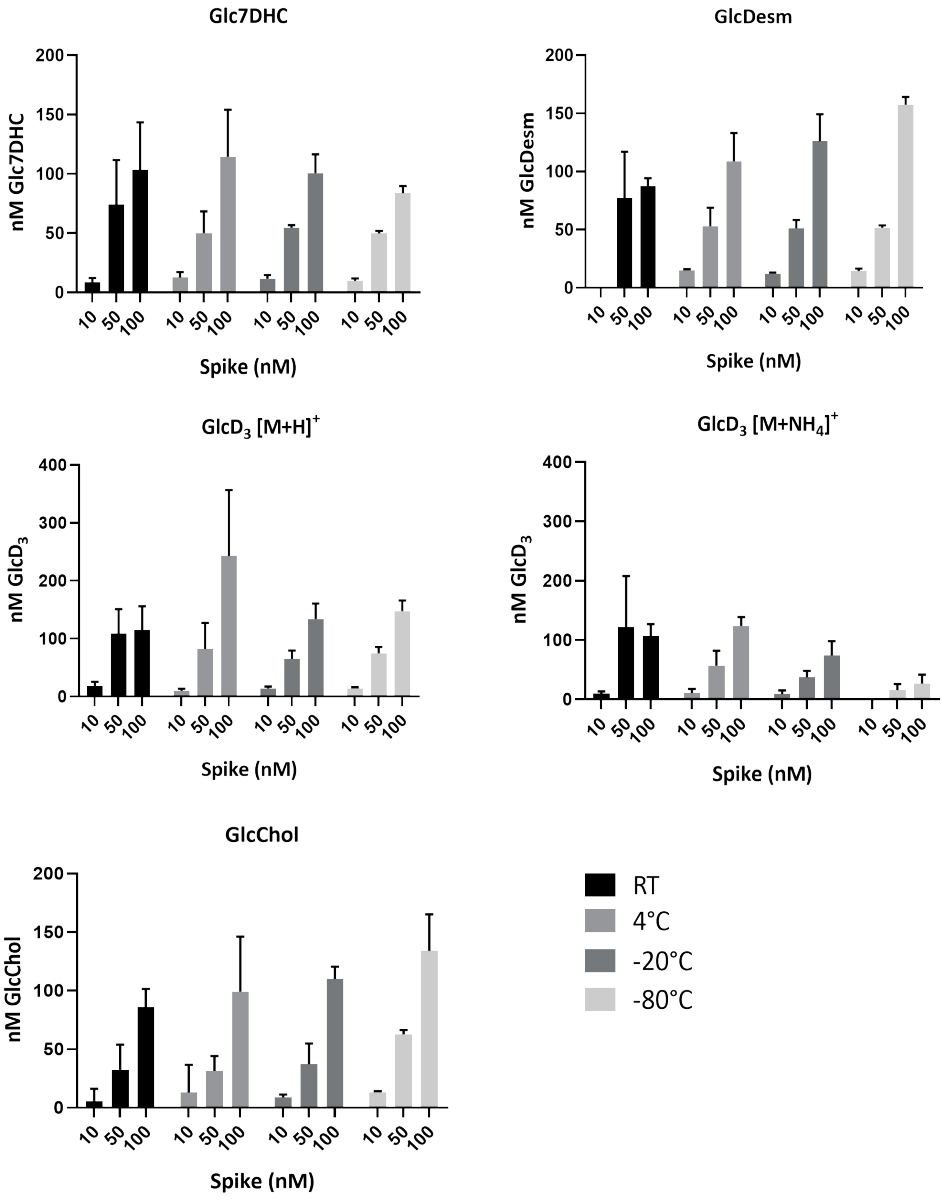


Figure 6. Glycosylated sterols in plasma stored at different conditions. *From left to right bars samples were kept at room temperature (RT), in fridge (4°C), freezer (-20°C) or freezer (-80°C). Presented data were calculated with correction for the appropriate blanks.*

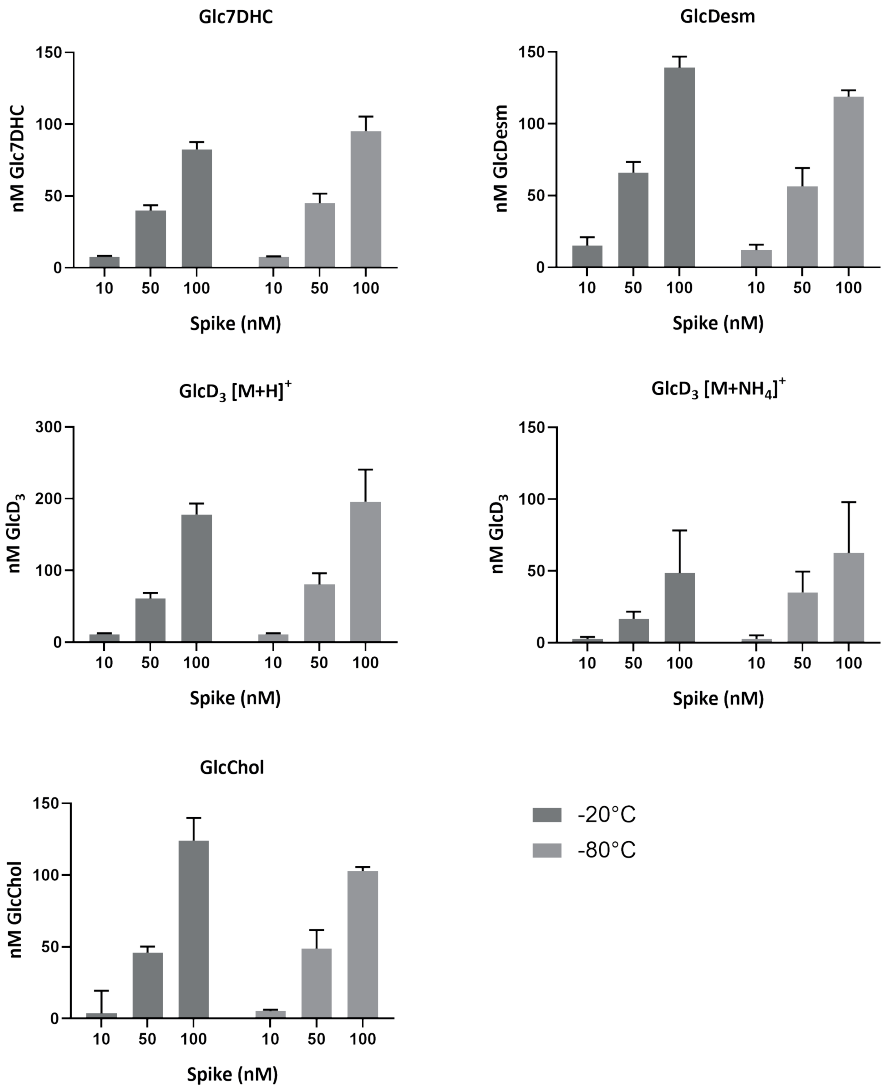


Figure 7. Glycosylated sterols in breast milk stored for 14 days at different conditions. *From left to right bars samples were kept at freezer -20°C or -80°C. Presented data were calculated with correction for the appropriate blanks.*

Impurities

The specific lipid standards were synthesized at the department of Bio-organic Synthesis. Minor impurities, as shown in Table 3, were observed when injecting 3 µL of 0.1 µM of standard on the LC-MS/MS. For all standards, the impurities are within acceptable percentage, with exception of an average 33% (27-39% respectively) impurity of GlcChol in the Glc7DHC, which might have to be purified or resynthesized in the future.

Table 3. Impurities in synthesized standards.

Standard	Compound causing impurity:	Amount of impurity (%)
$^{13}C_6$-GlcChol	GlcChol $^{13}C_6$-GlcDesm	0.2 0.3
GlcChol	GlcDesm	1.1
$^{13}C_6$-GlcDesm	GlcDesm GlcChol	0.5 0.03
GlcDesm	GlcChol	0.1
$^{13}C_6$-Glc7DHC	Glc7DHC $^{13}C_6$-GlcChol	1.6 1.2
Glc7DHC	GlcChol	27-39
$GlcD_3$	GlcChol	0.3

Detection of glycosylated sterols in biological samples

The use of the described LC-MS/MS method allows detection and quantification of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm in several fluids and tissues.

Plasma. Within plasma of ten healthy individuals, five females, five males, ranging in age from 30 of 82 the glycosylated sterols of GlcChol (average 170 nM in plasma), Glc7DHC (average 0.54 nM in plasma) and GlcDesm (average 2.84 nM in plasma) could be detected (Figure 8A). $GlcD_3$ could not be detected. No correlation in relation to age was observed.

Breast milk. Two healthy lactating females donated breast milk for measurement. Female one has been lactating for 2.5 months, female two has been lactating for 21 months. As shown by Figure 8B both GlcDesm and GlcChol could be detected, Glc7DHC and $GlcD_3$ were not detected. The data shows the longer the female lactates, the more the levels of both GlcChol and GlcDesm decrease. For female 2 only traces of GlcDesm were detectable. The decrease is expected as levels of Chol and Desm in breast milk are known to decrease after lactating 30 days postpartum [12]. To confirm the data of Figure 8B a larger cohort is needed. We propose to measure breast milk samples of at least five different females at various times

postpartum, e.g., 1, 2, 3, 6, 12 and 24 months of lactation. This will allow the monitoring of the levels of GlcChol and GlcDesm within the breast milk over time.

Skin. As shown by Figure 8C in skin of three healthy female individuals GlcChol, Glc7DHC and GlcDesm are present. $GlcD_3$ could not be detected. The age of the examined individuals ranges from 21 – to 50 year, and skin colour ranges from white to dark (Table 4). The data implies age dependence for GlcChol and GlcDesm, as levels of patient number 1 and 3 with comparable age are similar. Glc7DHC seems to increase with increase skin pigmentation. In order to confirm this observation a larger cohort of samples is required. We propose at least three different people per skin colour, per age class.

Spleen. The presence of GlcChol and Glc7DHC were detected within both human spleen from healthy control as in tissue from Gaucher disease patients. Levels of both glycosylated sterols are affected by GD, as the levels differ significantly from the control spleens ($P<0.05$).

Table 4. Age and skin colour per patient (female).

Patient nr	Age	Skin colour
1	21	White
2	50	Tanned
3	23	Dark

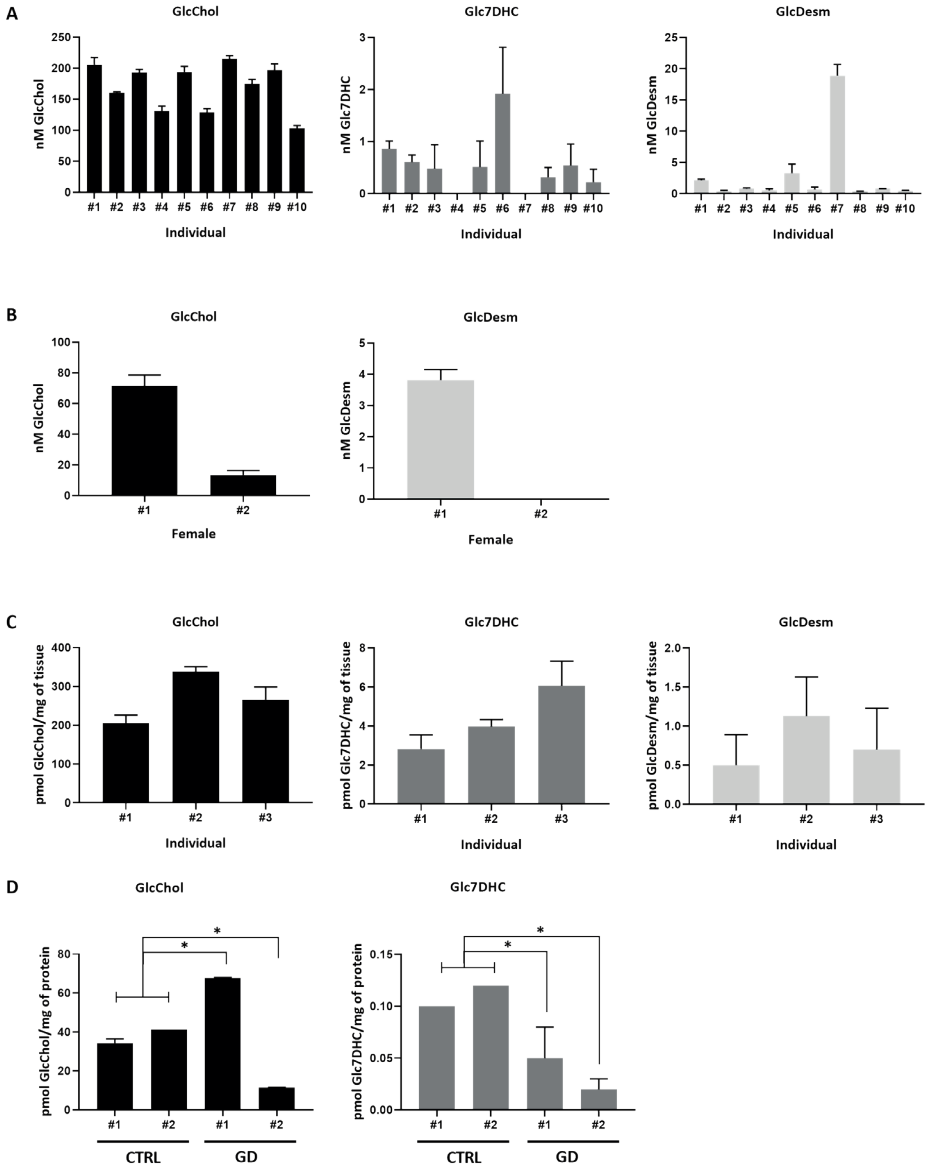


Figure 8. Glycosylated sterols in biological sample.

*A) GlcChol, Glc7DHC and GlcDesm in plasma of 10 healthy individuals. B) GlcChol and GlcDesm in breast milk of two lactating females. For female 2 only traces of GlcDesm could be detected. C) GlcChol, Glc7DHC and GlcDesm in skin of three healthy female individuals. D) GlcChol and Glc7DHC in control (CTRL) and Gaucher Disease (GD) spleen. *P<0.05.*

Discussion

In order to identify and quantify Glc7DHC, $GlcD_3$ and GlcDesm in biological materials we developed LC-MS/MS methods. The methods exploit isotope-labelled internal standards which are chemically identical to the analytes. This allows correction for variations due to extraction, minimizes matrix effects and LC-MS/MS performance. Resulting in an accurate, reproducible and sensitive assay. As no $^{13}C_6$-$GlcD_3$ is available, the precision for determining $GlcD_3$ is less accurate than the determination of Glc7DHC and GlcDesm. The synthesis of $^{13}C_6$-$GlcD_3$ would improve the described method for detection of $GlcD_3$. Furthermore, a $^{13}C_6$-$GlcD_3$ standard could help in tuning the method for a clear dominant transition for $GlcD_3$, either $[M+H]^+$ or $[M+NH_4]^+$, in order to improve the current LC-MS/MS method for measurement of $GlcD_3$. As $GlcD_3$ $[M+H]^+$ has shown to be more accurate, especially for low concentrations, in this thesis measurements for $GlcD_3$ will be performed and reported with the $[M+H]^+$ transition.

Subsequently our data shows stability of the analytes within biological samples such as plasma and breastmilk. The stability of the analytes within skin needs to be further studied. With the presented method, lipids could be extracted from the skin samples, while the skin is not homogenized. As the full skin is not homogenized a spiking experiment as executed for plasma and breastmilk is difficult to perform. So far, homogenization of skin has proven to be difficult. We tested cryogenic grinding with mortar and pestle within liquid nitrogen and mechanical lysis with glass beads in a FastPrep-24 (MP Biomedicals). The skin tissue is rigid and sticky, resulting in non-homogeneous material. A procedure to homogenize the full skin would allow the combination of the LC-MS/MS method with other assays like an ELISA measurement of Chol or 7DHC, or a fluorescence measurement for enzymatic activity.

A second aim of the investigation was to demonstrate the presence of Glc7DHC, $GlcD_3$ and GlcDesm in biological samples. The data shows that GlcChol can be detected in all samples, making it a good positive control. GlcDesm could be detected in breast milk and in low concentration in plasma and skin samples. Glc7DHC is present in plasma, skin and spleens of both healthy and GD affected samples. $GlcD_3$ could not be detected in any of the biological samples.

In conclusion, sensitive LC-MS/MS methods are established to identify and quantify Glc7DHC, $GlcD_3$ and GlcDesm in *in vitro* samples and biological materials. This allows further research of the biological presence and relevance of the glucosylated metabolites.

Experimental procedures

Materials

Plasma samples. AB-control plasma was obtained from EDTA-anticoagulated blood after centrifugation at 1750 g for 10 min and stored at -20°C until further use. Furthermore, ten EDTA plasma samples of random selected healthy individuals were collected by the Erasmus University Medical Centre. In agreement with the Erasmus MC Code of Conduct for responsible use, samples of patients who signed a noncooperation statement for the use of leftover patient material were excluded. The samples were aliquoted, coded and stored at 4°C. Thawed only once for analysis and measured within a week after collecting. All data were analyzed anonymously.

Breast milk. Two healthy lactating females donated their breast milk. One postpartum 2.5 months, one postpartum 21 months. Breast milk was directly extracted and measured for glucosylated metabolites of interest. The remaining breast milk was aliquoted and stored at -20°C until further use.

Skin. Breast skin from cosmetic surgery was obtained from a local hospital and used within 24 h after surgery. All human skin samples were obtained with consent. From the full thickness skin subcutaneous fat was removed with a surgical scalpel. Skin samples were directly extracted and measured for glucosylated metabolites of interest. The remaining skin was aliquoted and stored at -80°C until further use.

Spleen. Human spleens were obtained either as surgical specimens during therapeutic splenectomy or at autopsy. Clinical examination established the phenotype of the subjects. The spleens were stored at -80°C. Later, homogenates were prepared from the frozen material in water.

Pure grade solvents used for LC-MS/MS were ethanol purchased from Honeywell|Riedel-de Haën™ (Muskegon, USA), LC-MS-grade methanol, 2-propanol, water and HPLC-grade chloroform from Biosolve, LC-MS butanol from Merck KGaA (Darmstadt, Germany) and LC-MS quality ammonium formate from Sigma-Aldrich (St Louis, MO, USA).

Specific lipid standards. $^{13}C_6$-β-GlcChol [6], GlcChol [6], $^{13}C_6$-β-Glc7DHC, Glc7DHC, $GlcD_3$, $^{13}C_6$-β-GlcDesm and GlcDesm were synthesized at the Bio-organic Synthesis department (Leiden University, The Netherlands). The full description of the syntheses will be described separately.

Methods

Synthesis of glucosylated lipid standards. Synthesis of GlcChol and $^{13}C_6$-GlcChol were performed by Ken Kok following published procedures and their spectroscopic data are in agreement with those previously reported [6]. The synthesis of Glc7DHC, $^{13}C_6$-Glc7DHC and $GlcD_3$ was performed by Dr. Patrick Wisse, whereas the synthesis of $^{13}C_6$-β-GlcDesm and GlcDesm was performed by Ken Kok, both at Leiden Institute of Chemistry. The synthetic procedures and their spectroscopic data will be published somewhere else. All the lipid standards were kept frozen at -20°C in the dark.

Quantification of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm by LC-MS/MS. All experiments were performed on a Waters LC-MS instrument (ACQUITY UPLC H-Class coupled to Xevo-TQS-micro) runned by MassLynx 4.1 software, the data analyses was performed by TargetLynx software (Waters Corporation; Milford MA). Analytes were separated on a Acquity BEH C18 reversed-phase column (2.1 x 50 mm. particle size 1.7 µm) (Waters Corporation) by the following eluents: 2-propanol:water 90:10 (v/v) containing 10 mM ammonium formate (Eluent A); methanol containing 10 mM ammonium formate (Eluent B). The instrument settings used for GlcChol measurement are as described earlier [6]. A procedure for quantification of Glc7DHC, $GlcD_3$ and GlcDesm in plasma was developed using spiking of (isotope-encoded) standards, major adducted fragments were identified and the linearity, limit of detection (LOD) and limit of quantification (LOQ) of Glc7DHC, $GlcD_3$ and GlcDesm was established. For the measurement of Glc7DHC, $GlcD_3$ and GlcDesm the same settings were used, with a few adaptations (Table 5). Resulting in individual method for GlcChol, GlcDesm and a combined method for Glc7DHC and $GlcD_3$. These methods apply an isocratic elution of mobile phases, 10% eluent A and 90% eluent B at a flow rate of 0.250 mL/min for a duration of 5.5 min. To prevent system contamination the divert valve of the mass spectrometer was programmed to discard the UPLC effluent before 1.0 min and after 3.0 min. During the run the column temperature and the temperature of the auto sampler were kept at 23°C and 10°C, respectively. With exception for breast milk samples, as here the temperature of the auto sampler was kept at 20°C, to prevent lipid crystallization, which was observed when the auto sampler was at 10°C. For identification and quantification of the detected peaks, direct injection of 1 µM GlcChol, 1 µM GlcDesm, 1 µM Glc7DHC and 1 µM $GlcD_3$ in methanol and the internal standards of 1 µM $^{13}C_6$-β-GlcChol, 1 µM $^{13}C_6$-β-GlcDesm and 1 µM $^{13}C_6$-β-Glc7DHC in methanol were used.

As GlcDesm, Glc7DHC and $GlcD_3$ have the same mass (*m/z* 564.4>367.4 $[M+NH_4]^+$ and *m/z* 547.4>367.4 $[M+H]^+$), the compounds could only be separated based on Retention Time. LC-MS/MS settings were edited to a gradient method for better separation between the peaks of GlcDesm, Glc7DHC and $GlcD_3$ (Table 6). This method applies a mobile-phase gradient elution during 5.5 min run: 0.0 min 5% eluent A and 95% eluent B at a flowrate of 0.1 mL/min; 1.0 min 10% A, 90% B; 2.5 min 10% A, 90% B at a flowrate of 0.2 mL/min; 3.0 min 10% A, 90% B; 4.0 min 5% A, 95% B; 5.5 min 5% A, 95% B at a flowrate of 0.1 mL/min (Supplemental Figure 2). The eluent was diverted to waste between 0.0 - 1.0 min and 3.0 – 5.5 min to keep the source free of contamination.
Calibration curves. Calibration curves for Glc7DHC, $GlcD_3$ and GlcDesm were constructed by adding 0 – 0.1 – 0.5 – 1 – 2 – 4 – 10 – 20 – 50 – 100 – 200 nM of plasma to healthy control plasma. As internal standard 25 µL of 0.1 pmol/uL $^{13}C_6$-Glc7DHC or $^{13}C_6$-GlcDesm was added. Sample were extracted and measured on the same day.

Inter-Intra assay variation. Different concentrations (respectively 10, 50 and 100 nM plasma) of non-labelled compound (Glc7DHC, $GlcD_3$, GlcDesm and GlcChol) were spiked into control plasma of a healthy subject. Samples were extracted in six-fold (five times with the $^{13}C_6$-labelled standards, $^{13}C_6$-GlcChol, $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm and one without $^{13}C_6$-labelled standards) and measured on the same day to determine the intra-assay variation, and extracted and measured on subsequent days for inter-assay variation.

Storage within plasma. Different concentrations (10, 50, 100 nM) of non-labelled compounds ($GlcD_3$, Glc7DHC, GlcDesm, GlcChol) were spiked into control plasma of a healthy subject. Control plasma of a healthy subject without any spike was also stored. Samples were stored for 10 days at three different conditions: freezer (-20°C), fridge (4°C), and room temperature. After 10 days of storage samples were extracted in four-fold (three times with the $^{13}C_6$-labelled standards, $^{13}C_6$-GlcChol, $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm and one without $^{13}C_6$-labelled standards) and measured on the same day. Samples were also stored for 14 days at two different conditions: freezer -20°C and freezer -80°C. After 14 days of storage samples were similarly extracted and measured as the samples which were stored for 10 days. Comparison showed for the samples stored at -20°C that storage of either 10 or 14 days revealed no difference (Supplemental Figure 1). Therefore, data for the -20°C was averaged and the -80°C data was included in Figure 6.

Storage within breast milk. Different concentrations (10, 50, 100 nM) of non-labelled compounds ($GlcD_3$, Glc7DHC, GlcDesm, GlcChol) were spiked into breast milk. Breast milk without any spike was also stored. Samples were stored for 14 days at two different conditions: freezer -20°C and freezer -80°C. After 14 days of storage samples were three times extracted in three-fold and measured on the same day.

Table 5. Instrument parameters for individual compound analysis. *Retention Time (RT)*

	GlcChol [6]	**GlcDesm**	**Glc7DHC/GlcD_3**
Capillary voltage	3.50 KV	3.50 KV	3.50 KV
Cone voltage	20 V	20 V	20 V
Source temperature	150°C	150°C	150°C
Desolvation temperature	450°C	450°C	450°C
Cone gas	50 L/h	50 L/h	50 L/h
Desolvation gas	950 L/h	950 L/h	950 L/h
Collision voltage	15 V	15 V	15 V
Type	MRM	MRM	MRM
Ion mode	ESI^+	ESI^+	ESI^+
Dwell time	0.100 s	0.165 s	0.040 s
Inter-channel delay	0.005 s	0.005 s	0.005 s
Inter-scan delay	0.005 s	0.005 s	0.005 s
Transitions:	**RT (min.):**	**RT (min.):**	**RT (min.):**
GlcChol	1.37		-
$^{13}C_6$-β-GlcChol	1.37		-
GlcDesm	-	1.24	-
$^{13}C_6$-β-GlcDesm	-	1.24	-
Glc7DHC	-		1.29
$^{13}C_6$-β-Glc7DHC	-		1.29
GlcD_3	-		1.19
Smooth method	Mean		
Smooth width	2		

Table 6. Instrument parameters gradient analysis. *Retention Time (RT)*

	GlcChol/GlcDesm/ Glc7DHC/$GlcD_3$
Capillary voltage	3.50 KV
Cone voltage	20 V
Source temperature	150°C
Desolvation temperature	450°C
Cone gas	50 L/h
Desolvation gas	950 L/h
Collision voltage	15 V
Type	MRM
Ion mode	ESI^+
Dwell time	0.054 s
Inter-channel delay	0.005 s
Inter-scan delay	0.005 s
Transitions:	**RT (min.):**
GlcChol	2.66
$^{13}C_6$-β-GlcChol	2.66
GlcDesm	2.40
$^{13}C_6$-β-GlcDesm	2.40
Glc7DHC	2.49
$^{13}C_6$-β-Glc7DHC	2.49
$GlcD_3$	2.31
Smooth method	Mean
Smooth width	2

Extraction method. Before extraction internal standards were added as either 25 or 50 µL of 0.1 µM $^{13}C_6$-β-GlcChol and/or $^{13}C_6$-β-GlcDesm and/or $^{13}C_6$-β-Glc7DHC each in methanol depending on *in vitro* or *in vivo* samples. The sample were subjected to protein precipitation by addition of methanol (MeOH) and chloroform ($CHCl_3$) (2:1. v/v) and shaken for 30 minutes at RT. Afterwards samples were spun down for 10 minutes at 13000 rpm and transferred to a new Eppendorf tube. The protein precipitation was followed by lipid extraction according to Bligh and Dyer [13] by addition of chloroform and water (final volumes: MeOH:$CHCl_3$:H_2O 1:1:0.9, v/v/v). The lower phase (chloroform) was taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. The isolated lipids were purified by butanol/water extraction (1:1, v/v) and the upper phase (butanol) was taken to dryness under vacuum at 45 °C in a *speed VAC concentrator plus*. The isolated lipids were resolved in either 75 µL or 100 µL methanol, stirred and sonicated for 30 seconds, stirred once more and centrifuged for 5 minutes at 13000 rpm. Samples are analysed by LC-MS as described above. Volume of injection for *in vitro* samples was 3 µL or 10 µL for *in vivo* samples.

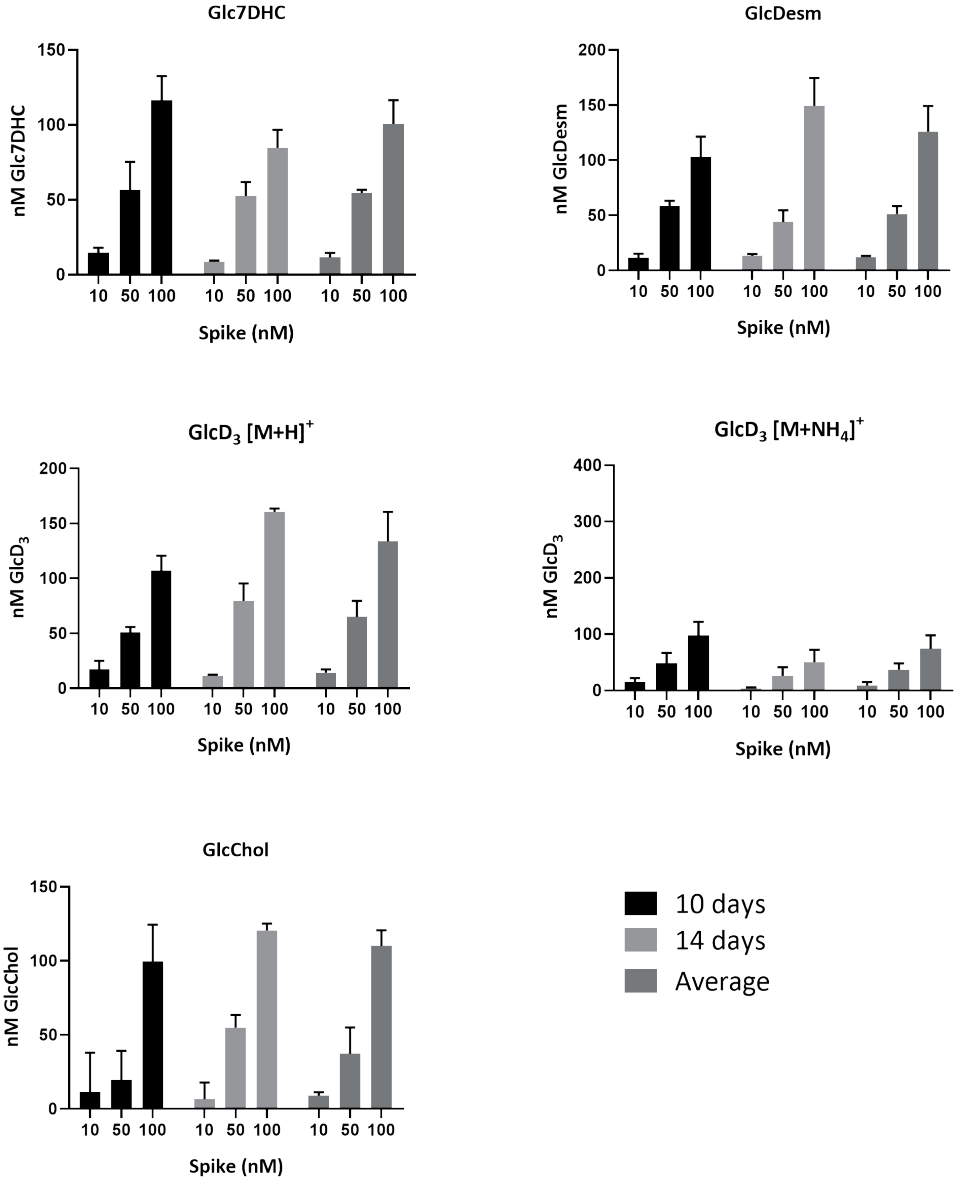
Measurement and quantification of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$ in human plasma, human spleen and human breast milk. The glucosylated sterols were extracted according to the above-described extraction method with a few modifications. Fifty µL of either plasma, homogenised spleen or breast milk was pipetted in an Eppendorf tube (2 mL) and internal standards were added as 25 µL of 0.1 µM $^{13}C_6$-β-GlcChol, 0.1 µM $^{13}C_6$-β-Glc7DHC and 0.1 µM $^{13}C_6$-β-GlcDesm each in methanol. An additional 425 µL methanol and 250 µL chloroform was added. Samples were stirred and left in the dark at room temperature for 30 min, mixed continuously and centrifuged for 10 min at 13000 rpm to spin down protein. The supernatant was transferred to a clean Eppendorf tube and 250 µL chloroform and 410 µL water was added. Samples were stirred shortly and centrifuged for 5 min at 13000 rpm. Lower phase (chloroform) was transferred to a clean Eppendorf tube and the upper phase was washed by addition of 500 µL chloroform. Subsequently, lower phases were pooled and taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. The residue was dissolved in 1.2 mL butanol/water (1:1, v/v) and mixed well. After centrifugation (10 min at 13000 rpm), the upper phase (butanol) was transferred to a clean Eppendorf tube and taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. Next the residue was dissolved in 75 µL of methanol by mixing and sonication. After centrifugation (5 min for human plasma samples and 10 minutes for human breast milk samples at 13000 rpm), samples were transferred into an MS vial and measured on the LC-MS/MS for determination of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$. As lipid crystallisation was observed in the breastmilk samples after LC-MS measurement, while breastmilk samples were kept at RT before measurement no crystallisation occurred. In order to prevent lipid crystallisation within the LC-MS/MS, breast milk samples were within the auto sampler kept at 20 °C instead of the usually 10 °C. Volume of injection was 10 µL.

Measurement and quantification of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$ in human skin. Full thickness skin was collected and stored over night at 4°C. In the morning of the subsequent day skin was cut in small pieces (100-150 mg) with a surgical scalpel. Internal standards were added as 50 µL aliquots of 0.1 µM $^{13}C_6$-β-GlcChol, 0.1 µM $^{13}C_6$-β-Glc7DHC and 0.1 µM $^{13}C_6$-β-GlcDesm each in methanol. An additional 450 µL methanol and 600 µL chloroform was added. Samples were stirred for 30 seconds alternating 1 min of sonication in a bath sonifier with melting ice, for four times. Subsequently, samples were left in the dark at room temperature for 1.5 h, mixed continuously. Samples were sonicated for 1 min in a bath sonifier with melting ice, twice, alternating 30 seconds of stirring and centrifuged for 10 min at 13000 rpm to precipitate protein. The supernatant was transferred to a clean Eppendorf tube and between 390 µL and 440 µL water, depending on the mg of tissue, was added for Bligh and Dyer extraction. Samples were stirred shortly and centrifuged for 5 min at 13000 rpm. Lower phase (chloroform) was transferred to a clean Eppendorf tube and the upper phase was washed by addition of 600 µL chloroform. Subsequently, lower phases were pooled and taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. Butanol/water extraction was performed as described for plasma samples. Next the residue was dissolved in 100 µL of methanol by mixing and sonication. After centrifugation (10 min at 13000 rpm), samples were transferred into an MS vial and measured on the LC-MS/MS for presence of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$. Volume of injection was 10 µL.

Statistical analysis. Values in figures are presented as a mean ± S.D. Data were analyzed by unpaired Student's t-test. P values of <0.05 were considered significant (*$P<0.05$).

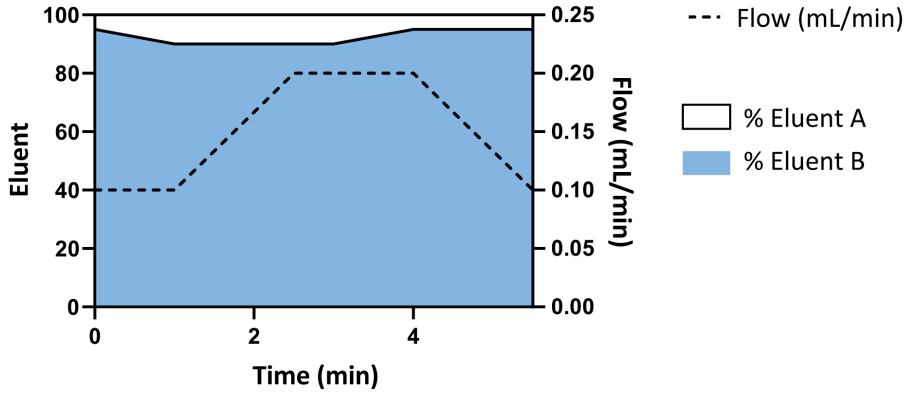
Supplementary information

Supplemental Figure 1. Glycosylated sterols in plasma stored for different days. *Samples were stored in freezer (-20°C). From left to right bars samples were kept in storage for 10 days, 14 days, average of both times of storage. Presented data were calculated with correction for the appropriate blanks.*

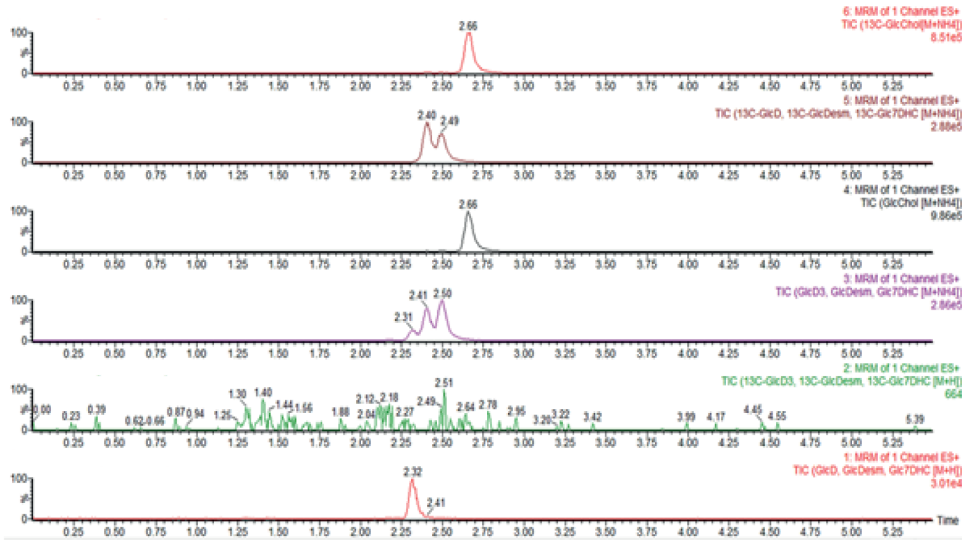


Supplemental Figure 2. Gradient elution and chromatogram. *A) Displays the gradient elution of the gradient analysis. B) Displays the chromatograms of the elution of $^{13}C_6$-GlcChol $[M+NH_4]^+$ (RT 2.66), $^{13}C_6$-GlcGlcDesm $[M+NH_4]^+$ (RT 2.40), $^{13}C_6$-Glc7DHC $[M+NH_4]^+$ (RT 2.49), GlcChol $[M+NH_4]^+$ (RT 2.66), $GlcD_3$ $[M+NH_4]^+$ (RT 2.31), GlcDesm $[M+NH_4]^+$ (RT 2.40) and Glc7DHC $[M+NH_4]^+$ (RT 2.49) and $GlcD_3$ $[M+H]^+$ (RT 2.31).*

A



B



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