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Determination of ghrelin and desacyl ghrelin in human plasma and urine by means of LC-MS/MS for doping controls

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Abstract

The hunger hormone ghrelin (G) is classified as prohibited substance in professional sport by the World Anti-Doping Agency (WADA), due to its known growth hormone releasing properties. The endogenous bioactive peptide consists of 28 amino acids with a caprylic acid attached to serine at position 3. Within this study it was aimed to develop methods to determine G and desacyl ghrelin (DAG) in plasma and urine by means of LC-MS/MS. Two strategies were applied with a bottom-up approach for plasma and top-down analyses for urine. Both sample preparation procedures were based on solid-phase extraction for enrichment and sample clean-up. Method validation showed good results for plasma and urine with limits of detection (LODs) for G and DAG between 30 and 50 pg/mL, recoveries between 45-50 %, and imprecisions (intra- and inter-day) between 3 – 24 %. Plasma analysis was also valid for quantification with accuracies determined with ~100 % for G and ~106 % for DAG. The minimum required performance level for doping control laboratories is set to 2 ng/mL in urine, and the herein established method yielded acceptable results even at 5 % of this level. As proof-of-concept, plasma levels (G and DAG) of healthy volunteers were determined and ranged between 30 and 100 pg/mL for G and 100 – 1200 pg/mL for DAG. In contrast to earlier reported studies using ligand binding assays for urinary G and DAG, in this mass spectrometry-based study no endogenous urinary G and DAG were found, although the LODs should enable this.

1. Introduction

The use of growth-promoting substances such as somatotropin in sport is prohibited by the regulations of the World Anti-Doping Agency (WADA) [1].

Since 2015, WADA's list of banned substances has also explicitly mentioned the so-called "growth hormone secretagogues" such as ghrelin (G) and its mimetics, which were banned due to their growth hormone-releasing effect [2].

G is an endogenous peptide hormone that is secreted in the human body, especially in the stomach, and plays an important role in appetite regulation ("hunger hormone"), growth hormone secretion, insulin secretion, and cardiovascular processes [3].

The substance was first discovered in 1999 by Kojima et al., who isolated and identified the hormone as an endogenous ligand of the growth hormone secretagogue receptor (GHSR) [3]. Through its potent action at the GHSR, it causes a release of somatotropin from the anterior pituitary gland. Somatotropin has a direct influence on lipolysis and glucose release from the liver and, via the release of insulin like-growth factor 1 (IGF-1), it mediates multiple anabolic effects such as skeletal growth and protein synthesis. [3]

Physiologically, however, the main function of G seems to be to stimulate appetite by acting as the most important orexigenic hormone, transmitting hunger signals to the brain and stimulating food intake [2, 3].

In structural terms, G is a peptide hormone consisting of 28 amino acids, whose active form carries a covalently bound octanolic (caprylic) acid on $serine_3$ (Tab. 1) [3]. It is formed from the precursor protein proghrelin by acylation and subsequent cleavage into G and obestatin, which owns opposed properties. Acylation of G is essential for binding to the GHS receptor, thus, the desoctanoyl or deacyl ghrelin (DAG), which is also circulating in the blood, does not bind to the receptor and does not mediate a growth hormone secreting effect, accordingly [3].

In the circulation, the deacylated form is the predominant one, while acylated G is present only at about 10-20% [2, 4, 5]. Due to the hydrolytic and enzymatic cleavage of the octanoyl group, an increase in the total concentration of G and DAG can be observed when the acylated G is supplied exogenously [6]. Both forms not only circulate freely in the plasma, but are also bound to plasma proteins to a considerable extent [7]. Thus, an interaction of octanoylated G with albumin has already been described, which also influences its

hydrolytic properties and biological activity, but both acylated and deacylated G seem to bind primarily to lipoproteins [7].

The release of somatotropin and the associated lipolytic and anabolic effects suggest a potential misuse of G and its mimetics in sports. A possible complication in the detection and evaluation of G as an endogenously administered substance could offer another potential aspect of its use for doping purposes (in contrast to other clearly xenobiotic growth hormone releasing factors). Several methods are available to determine G and DAG in biological fluids, which already use LC-MS/MS after efficient sample preparation. [8-11] In terms of sensitivity, all of these assays show comparable results, strongly related to the expected endogenous levels in pg/mL-concentrations. Noteworthy, to the best of our knowledge, no doping control assay for G and DAG and no MS-based assay for urinary G and DAG exists so far. Urinary concentrations are reported only for ligand binding assays. [5, 12]

Materials

The commercially available plasma Octaplas (200 mL for intravenous infusion) was obtained from Octapharma GmbH (Langenfeld, Germany) and served as the biological matrix. Additionally, pooled human plasma from Innovative Research (Novi, MI, USA) was used. As reference substances G (human), (desoctanoyl-)G (human) and ($^{13}C_6$-Leu^5)-G (human) were purchased from Bachem (Bubendorf, Switzerland). For solid-phase extraction Oasis HLB (1 ccm, 30 mg) and MAX (1 ccm, 30 mg) from Waters (Milford, CT, USA) were used. A modified sequencing-grade trypsin from Promega (Walldorf, Germany) was used for the enzymatic digestion of the peptides. Ammonia, ammonium bicarbonate, formic acid, dimethyl sulfoxide (DMSO), bovine plasma / serum and sulfosalicylic acid were obtained from Sigma Aldrich (Steinheim, Germany), and the acetonitrile was from VWR International (Darmstadt, Germany). For the production of the reagents and eluents, high purity water was used, produced by a Barnstead™ GenPure™- plant of the company Thermo Scientific™ (Waltham, MA, USA).

Blood and urine samples

For validation and method development, plasma and urine samples were used from healthy volunteers without any known diseases or medication. Written consent and

approval of the local ethical committee was obtained (No.: 047/2021). Ten plasma and ten urine samples were collected in sum. Four of these urine and plasma samples were collected nearly simultaneously (within one hour time period) in order to monitor plasma levels and the corresponding urine concentrations.

Methods

Sample preparation plasma: To each 250 µl of plasma, 10 µl of a 0.1 ppm solution of the internal standard ([$^{13}C_6$]Leu^5)-G) were added and mixed. To separate the non-specific binding of G to albumin and other plasma proteins, 5 µl of a 5 % ammonium hydroxide solution were added. Precipitation of larger proteins was then performed by adding 75 µl of sulfosalicylic acid solution (100 mg/ml). The precipitate was separated by centrifugation at 17 000 x *g* for 10 min.

For solid-phase extraction, the Oasis HLB cartridge was first washed with 1 ml of acetonitrile, conditioned with 1 ml of water, then the samples were added and the cartridge was washed with 1 ml of water. Elution was performed with 600 µl of acetonitrile/water (40/60; v:v) in Eppendorf Protein Lo-Bind Tubes. Afterwards, the eluate was evaporated to dryness in a vacuum centrifuge.

The dried eluate was reconstituted in 50 µl of ammonium bicarbonate buffer (50 mM) each, and 10 µl of a trypsin solution (100 ppm) and 10 µl of ACN were added. Trypsin digestion was performed overnight in a heating block at 37 °C with continuous shaking at a speed of 500 rpm. To stop the digestion, 5 µl of 2 % acetic acid was added and, after centrifugation (9000 x *g*, 2 min), the sample was ready for injection.

Sample preparation urine:

To 1 mL of urine, 10 µl of a 0.1 ppm solution of the internal standard ([$^{13}C_6$]Leu^5)-G) and 20 µl of an ammonium hydroxide solution (25 %) were added and mixed. The sample was transferred to a mixed-mode SPE cartridge (Waters, MAX, 30 mg, 3 ccm), which was conditioned with 1 ml of methanol and 1 ml of water. The cartridge was washed with 1 ml of methanol/water (70/30) and finally eluted with 0.6 ml of methanol acidified with 5 % of formic acid. The eluate was evaporated to dryness in a vacuum centrifuge and the dry residue

was reconstituted with 100 µl of water with 1 % of acetic acid. After centrifugation (9000 x *g*, 2 min) the sample was ready for LC-MS.

Liquid chromatography - tandem mass spectrometry

LC-MS/MS analysis was performed on a Thermo Scientific Vanquish™ UHPLC system coupled to a Thermo Scientific Orbitrap Exploris™ 480 mass spectrometer.

Injected samples were first trapped for 2 min at 400 µl/min on an Accucore Phenyl/Hexyl trapping column (3x10 mm, 2,6 µm, Thermo Bremen, Germany) using an isocratic flow with formic acid (0.1 %). After 2 min the flow was directed to the analytical column (Poroshell 120 EC-C18, 3x50 mm, 2.7 µm, Agilent Technologies, Waldbronn, Germany) with a flow rate of 400 µl/min. Eluent A consisted of 0.1% formic acid and DMSO 1% in water, Eluent B consisted of 0.1% formic acid in ACN, with an addition of 1% DMSO. The separation followed a gradient from 0 % of B for 2 min (trapping), increased to 80 % B in 8 minutes afterwards, then increased to 90 % B within 2 min and finally equilibrated at the initial concentration of 0 % B for 2 min. The overall runtime was 14 min.

The voltage of the needle during positive electrospray ionization was set to 3 kV, the temperature of transfer capillary and gas was 300 °C. A full-MS scan in a range from *m/z* 400 to 1700 as well as a targeted MS2 (PRM) experiment at a resolution of 60,000 FWHM (full width at half maximum) was performed. For PRM experiments the isolation window of the quadrupole was set to 2 *m/z* and the resolution was 30,000 FWHM. The optimized collision energies for G, DAG and the internal standard (ISTD) are shown in Tab. 1. The instrument was calibrated according to the manufacturer`s instructions.

Validation (plasma and urine)

The detection of G and DAG in urine and plasma in a concentration range of 0.05 – 4 ng/ml was validated. Since these are endogenous substances, the availability of analyte-free blank matrix is required and, here, the commercial plasma substitute Octaplas and blank urine from a healthy volunteer (tested as blank before) was used for certain validation parameters. In plasma, quantification was aimed while in urine, qualitative results were considered

sufficient. The quantification of G and DAG in plasma was performed by external calibration. The validation parameters for the urine assay follow the main criteria of the recent WADA documents. [13, 14] Qualitative result interpretation was applied here, because G is categorized in the prohibited as non-threshold substance with a MRPL of 2 ng/mL.

Specificity

Ten human plasma and urine samples from healthy volunteers were tested for the presence of G or DAG. The comparison of retention time and fragmentation pattern of certified reference substances served for a clear characterization.

Linearity

To investigate the functional relationship between measurand and concentration of the analyte, urine and plasma samples were fortified with increasing concentrations of the target analyte and measured (c=0, 0.2, 0.5, 1, 2, and 4 ng/ml). To check for linearity, the concentration was plotted against the peak area ratio (analyte/ISTD) and the regression coefficient determined.

Repeatability (intra-day imprecision/repeatability) in plasma

To determine the assay precision under repeatability conditions, three pooled human plasma samples were analyzed with exclusively endogenous, endogenous + 0.5 ng/mL and endogenous + 2 ng/mL G and DAG, respectively. Six aliquots each were processed, measured, and the relative standard deviation determined.

Laboratory precision (inter-day precision/intermediate precision)

Six aliquots from each pool (see repeatability) were measured on two different days and the relative standard deviation was determined.

Accuracy (plasma)

The systematic deviation of the detected concentration from the actual concentration was analyzed by adding the analytes to six blank plasma samples. The trueness was determined as the ratio of the measured concentration to the added concentration.

Limit of detection (LOD)

The detection limit for G was first estimated to range at about 30 pg/ml. For verification, the appropriate amount was added to six G-free human plasma samples (Octaplas) and urine, analyzed and confirmed to have a required signal-to-noise (S/N) ratio of at least 3.

For the determination of the detection limit of DAG, six analyte-free plasma (Octaplas) and urine were dosed with 50 pg/ml and measured, again with a required signal-to-noise ratio (S/N) of at least 3.

Limit of quantification (LOQ) for plasma

To establish the limit of quantitation of G, six human plasma samples (Octaplas), each containing 50 pg/ml, were spiked, analyzed and quantified. The S/N should be at least 10.

For DAG, six blank plasma samples (Octaplas) were dosed with 50 pg/mL and measured. The S/N was also required to be at least 10.

Limit of identification (LOI) for urine

Ten different urine samples were fortified at 2 ng/mL, corresponding to the minimum required performance limit (MRPL), and additionally at 1.0, 0.5, 0.25, 0.1 and 0.05 ng/mL and prepared as described above. [13] Identification performance was evaluated in all of these samples.

Recovery

To determine the recovery rate of the detection method, 4 ng/ml of G and DAG were added to a total of 6 human plasma and urine samples and processed according to the developed protocol. In another six samples, the analyte (resp. its hydrolysis product) was added immediately prior to analysis. The samples were analyzed and the recovery rate determined from the concentrations obtained. Since the corresponding hydrolysed peptide was not available for G, the parameter in plasma (bottom-up) was determined for the deacylated form only.

Robustness

The influence of different parameters on the final result of G detection was investigated by preparing and analyzing three samples each under the appropriate conditions. The

concentration was 4 ng/ml for all samples. The test parameters included for plasma a) the analysis according to protocol in bovine plasma, in human plasma, and in bovine serum; b) reduction of the trypsin solution concentration to 40 ppm (instead of 100 ppm); c) reduction of the trypsin digestion to 3 h (instead of overnight). For urine (top-down assay), 10 and 30 µL of ammonium hydroxide solution (instead of 20 µL) was added.

Carry-over

Immediately following a high concentration sample (8 ng/ml, 4 x MRPL), blank samples were analyzed and examined for the presence of the analytes due to possible carryover.

Results

As G is an endogenous peptide hormone, the simple qualitative detection in an athlete sample is not sufficient to uncover anti-doping rule violations. Thus, a robust and reliable quantification is required and subsequently, reference ranges in a representative population provide the basis to enable the establishment of a potential threshold. Above this threshold, the endogenous production of G and its metabolite is very unlikely and an exogenous administration is suggested. During method development it was obvious that the intact (non-digested) analytes own poor chromatographic properties due to the presence of 7 basic amino acids in the sequence. Therefore, the quantitative approach was designed under inclusion of an enzymatic hydrolysis step which yields the tryptic peptide $t_{1\text{-}11}$ with 11 amino acids and only one Arg residue at the C-terminus. This target peptide still includes the acyl-modification at position Ser_3 and the chromatographic properties of the hydrolysed peptides ($G_{1\text{-}11}$ and $DAG_{1\text{-}11}$) were enhanced considerably.

The mass spectrometric characterization was performed by means of product ion experiments of the dominant precursors using high resolution mass spectrometry. Fig. 1 shows the diagnostic product ions of the enzymatic hydrolysis products of $DAG_{(1\text{-}11)}$ acquired from the two-fold protonated precursor at *m/z* 622 resp. *m/z* 685 for $G_{(1\text{-}11)}$. In Fig. 2, the corresponding product ion mass spectra of the intact peptide chains are shown with

diagnostic product ions from the five-fold protonated precursors at *m/z* 649.6 for DAG and at *m/z* 674.8 for G, respectively.

Validation

The main results of the G and DAG validation are summarized in Table 2 (plasma) and 3 (urine).

Specificity

Endogenous G or DAG was clearly identified in all ten plasma samples from healthy volunteers. In accordance to the literature, DAG is found at much higher abundance than G. [3, 8, 10] Conversely, in none of the 10 urine samples, G or DAG was observed. The ISTD was detected in all samples.

Linearity

For both analytes and both matrices (plasma and urine), linear correlation between concentration and peak area ratio in the concentration range of 0-4 ng/ml could be shown. The coefficient of correlation R^2 obtained by linear regression was > 0.99 for G resp. DAG in both, plasma and urine.

Repeatability (intra-day imprecision/repeatability) in plasma

For the evaluation of the data, an outlier test according to Nalimov was carried out in order to remove possible systematic outliers from the data series. [15] For G, a good precision of the detection method could be shown for all concentrations. Endogenously, no G could be detected in Octaplas either; when 0.5 and 2 ng/ml were added, the relative standard deviation was about 3-5 % in each case. For the detection of DAG the method is less precise, here the relative standard deviation was between 8-24 % with repeated measurements. The lower precision for the detection of DAG is mainly due to the fact that only the octanoylated form of the ISTD was available as a certified reference and the reference of DAG to this standard is not entirely appropriate. Due to the missing octanoyl group, it behaves differently from the ISTD, resulting in variations in the results.

Laboratory precision (inter-day imprecision/intermediate precision) in plasma

To determine the precision under changing conditions, an initial outlier test according to Nalimov was also carried out for the data series of both days. Afterwards, the homogeneity of the variances was checked by means of the F-test and the differences in the mean values by means of the T-test, in order to determine an overall precision from the data of both days. For G, this was less than 10 % for all concentration ranges. For DAG, the mean standard deviation was between 6 and 21 %. Again, the precision of the detection of DAG in the low concentration range is limited.

Accuracy in plasma

The calculated accuracy was about 100 % for G and 106 % for DAG. Thus, systematic errors for the detection procedure of both analytes can be excluded.

Limit of detection (LOD)

Both analytes could be reliably detected at a concentration of 30 pg/ml with a S/N above the required value of 3 in plasma. In urine, approx. 50 pg/ml for G and DAG were reliably detected (see also LOI).

Limit of quantification (LOQ) in plasma

The limit of quantification could be set at a concentration of approx. 50 pg/ml for both analytes. In the corresponding concentration range, both could be quantified reliably with a S/N above 10.

Limit of identification (LOI) in urine

Table 2 summarizes the main results. At the MRPL, at 50 % MRPL, and at 25 % MRPL in all ten samples G and DAG were identified. For G, even at 100 pg/mL (5 % MRPL) 9 out of 10 samples showed clear signals. For DAG, also at 50 pg/mL (2.5% MRPL) 8 out of 10 samples were detected correctly. Fig. 3 shows the extracted product ion chromatograms of a blank urine and a blank urine fortified at 100 pg/mL of G and DAG. The signals at 3.7 min correspond to the diagnostic ion transition of *m/z* 649-717 for DAG resp. at 4.28 min of *m/z* 674-717 for G.

Recovery

The recovery rate in plasma is calculated with 46 % for the hydrolysis product of DAG. In urine, recoveries are determined to range at 45 % for G and 53 % for DAG.

Carry over

The carry over to the next injection was shown to range below 2 % of all target analytes.

Robustness

The examined parameters had different influences on the robustness. The matrix has a clear effect on the determination, e.g. the sensitivity in human plasma samples is better than in plasma samples from cattle. In bovine serum, the stability of the octanoyl group is visibly impaired, so that the proportion of DAG and desacyl-ISTD is correspondingly higher.

A reduction of the trypsin concentration has no influence on the overall result, but the sensitivity is significantly lower compared to the fixed concentration of 100 ppm. Regarding the time of digestion, it can be stated that approximately the same results are obtained with a digestion of 3 h and a digestion overnight. The detection method is therefore robust against these variations. In urine, the volume of the added ammonia between 10 and 30 µL shows no significant impact on the results.

Doping controls

Although G represents a prohibited substance in professional sport, up to now no established doping control assay exists, which is approved to uncover the misuse of G in sports. As the endogenous peptide occurs in all plasma samples (as G and/or DAG) from athletes, obviously quantification is required in order to enable the interpretation of the origin of the peptide (administered or endogenously produced). In accordance to the existing literature, normal levels of G and DAG range from 0 to 0.2 ng/mL for G and from 0.1 to 1.5 ng/ml for DAG in plasma. [6, 8-12] Figure 4 shows the product ion chromatograms of a plasma sample from a healthy volunteer with endogenous G and DAG together with chromatograms from a fortified blank plasma (Octaplas at 200 pg/mL of G and DAG) for comparison. The corresponding chromatograms from the blank sample without fortification is shown in Fig. S1, indicating the absence of any interference in the used Octaplas matrix. With the small number of healthy volunteers in this study (n=10), these plasma levels were confirmed with the present method.

The measured concentrations in these samples range between 50-100 pg/mL for G and 100-1200 pg/mL for DAG. After administration of G, the levels of G and DAG increase up to 20 ng/mL, which was shown in earlier studies. [6] Noteworthy, for establishing valid thresholds from a reference population, much higher number of volunteers under defined conditions and controlled post administration studies are required.

In contrast, urine analysis shows a different outcome. Here, the described values from the literature (0-0.1 ng/ml for G and 0.05-1.0 ng/ml for DAG) were not confirmed and no endogenous G and DAG were detected. [5, 12] Also, the simultaneously collected plasma and urine samples, showed normal to high levels in plasma (80-100 pg/mL G, 170-700 pg/ml DAG), but nothing in corresponding urine samples. Noteworthy, this is the first mass spectrometric assay in urine and all former studies based on ligand binding assays (LBA). These LBAs may also detect other ghrelin-related degradation products (apart from DAG) and the sum of these metabolites are interpreted as urinary G and DAG in these assays. In the present study, endogenous urinary G and DAG were not detected in any sample with a LOD of approx. 50 pg/mL. Considering this fact, the minimum required performance level (MRPL) from the WADA at 2 ng/mL for urinary G might be a valid marker to uncover exogenous G administration, because these levels were never detected in urine samples from healthy volunteers. [13] But also here, a higher number of volunteers and controlled administration studies are required in order to get a valid threshold.

Conclusion

The administration of exogenous G will increase the secretion of growth hormone and is prohibited in professional sport accordingly. Although, G is not available as approved pharmaceutical product yet, the misuse is conceivable due to availability of G from manifold commercial sources. As G represents an endogenous hormone itself, simple qualitative detection in plasma or urine will not facilitate to uncover the surreptitious misuse in athletes. More intensive and complex quantification of G and its metabolite DAG under consideration of still nonexistent thresholds and reference levels are required to enable efficient doping controls. Within this study a potential analytical tools is provided, ensuring reliable measurement of G and DAG in plasma and urine. Noteworthy, this is the first, crucial step into the right direction only.

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Table 1: *Peptide sequences and basic mass spectrometric parameters*

Top-down	amino acid sequence	Precusror [*m/z*]	Charge state	Collision energy [%]	Product ions [*m/z*]	~Ret time [min]
G	GS**[oct]S**FLSPEHQRVQ QRKESKKPPAKLQPR	674.78	5+	25	717.2, 688.9	4.3
DAG	GSSFLSPEHQRVQQRKE SKKPPAKLQPR	649.56	5+	25	717.2, 688.9	3.7
ISTD	GS**[oct]S**F[$^{13}C_6$]LSPEH QRVQQRKESKKPPAKLQ PR	676.80	5+	25	718.9, 690.1	4.3
bottom-up						
$G_{(1-11)}$	GS**[oct]S**FLSPEHQR	685.86	2+	23	753.4, 666.3	7.6
$DAG_{(1-11)}$)	GSSFLSPEHQR	622.80	2+	21	753.4, 666.3	5.6
$ISTD_{(1-11)}$	GS**[oct]S**F[$^{13}C_6$]LSPEH QR	688.87	2+	23	753.4, 666.3	7.6

Table 2: Validation results for quantitative determination of G and DAG in plasma

	Ghrelin		Desacyl ghrelin	
specificity (n=10)	✓		✓	
linearity (0-4 ng/ml)	R^2= 0.9987		R^2= 0.9983	
precision/ repeatability (n=6)	endogenous endo. + 0.5 ng/ml endo. + 2 ng/ml	5 % 3 % 3 %	endogenous endo. + 0.5 ng/ml endo. + 2 ng/ml	8 % 24 % 15 %
Intermediate precision (n=6+6)	endogenous endo. + 0.5 ng/ml endo. + 2 ng/ml	3 % 7 % 3 %	endogenous endo. + 0.5 ng/ml endo. + 2 ng/ml	6 % 21 % 18 %
accuracy (n=6)	100 % (RSD=9 %)		106 % (RSD=8 %)	
LOD (n=6, S/N> 3)	30 pg/ml		30 pg/ml	
LOQ (n=6, S/N> 10)	50 pg/ml		50 pg/ml	
recovery (n=6+6)	-		46 %	

Table 3: Validation results for qualitative determination of G and DAG in urine [13, 14]

Parameter	Ghrelin	Desacylghrelin
Specificity	10	10
MRPL (2 ng/mL)	10/10	10/10
50% MRPL (1 ng/mL)	10/10	10/10
25% MRPL (500 pg/mL)	10/10	10/10
12.5 % MRPL (250 pg/ml)	9/10	10/10
5 %MRPL (100 pg/mL)	9/10	10/10
2.5% MRPL (50 pg/mL)	3/10	8/10
Carry over	< 1 %	< 1 %
Stability (24h)	ok	ok
Recovery	45 %	53 %
Robustness	ok	ok

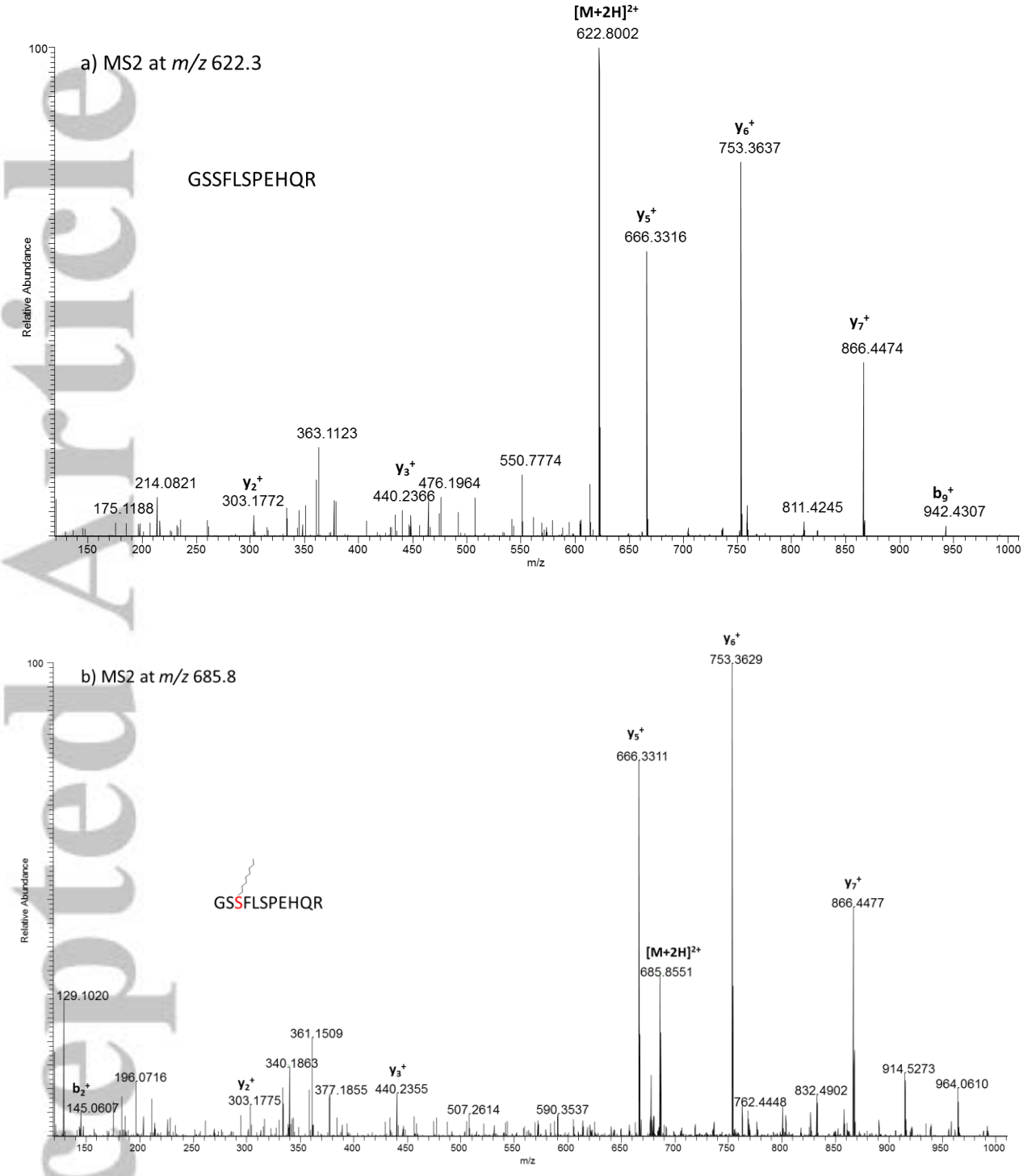

Fig. 1: Product ion spectra of the enzymatic hydrolysis product of a) $DAG_{(1\text{-}11)}$ at *m/z* 622.8 resp b) $G_{(1\text{-}11)}$ at *m/z* 685.8 with the abundant, diagnostic product ions y_5^+ and y_6^+.

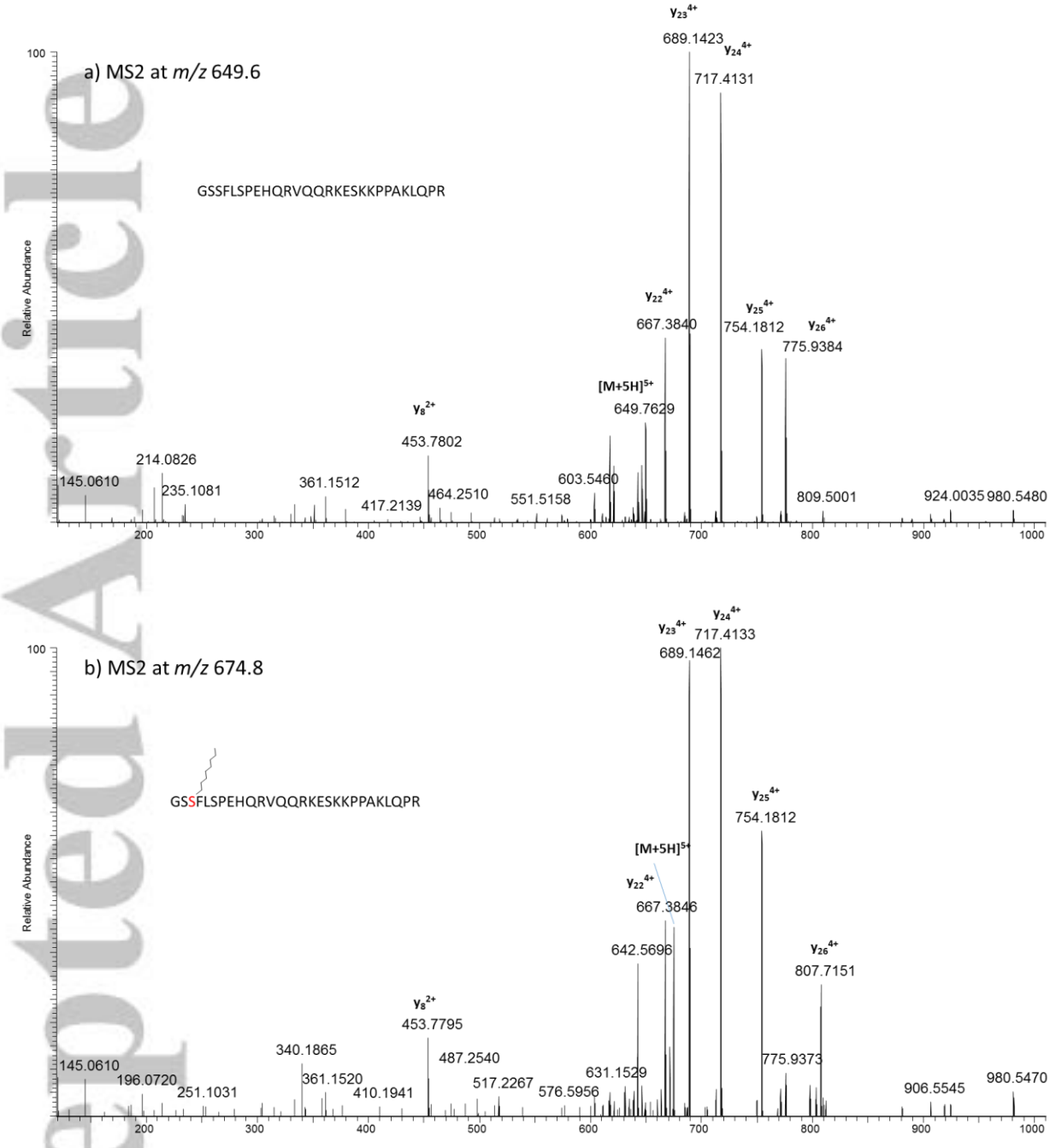

Fig. 2: Product ion spectra of intact a) DAG at *m/z* 649.6 resp b) G at *m/z* 674.8 including the abundant, diagnostic product ions y_{23}^{4+} and y_{24}^{4+}.

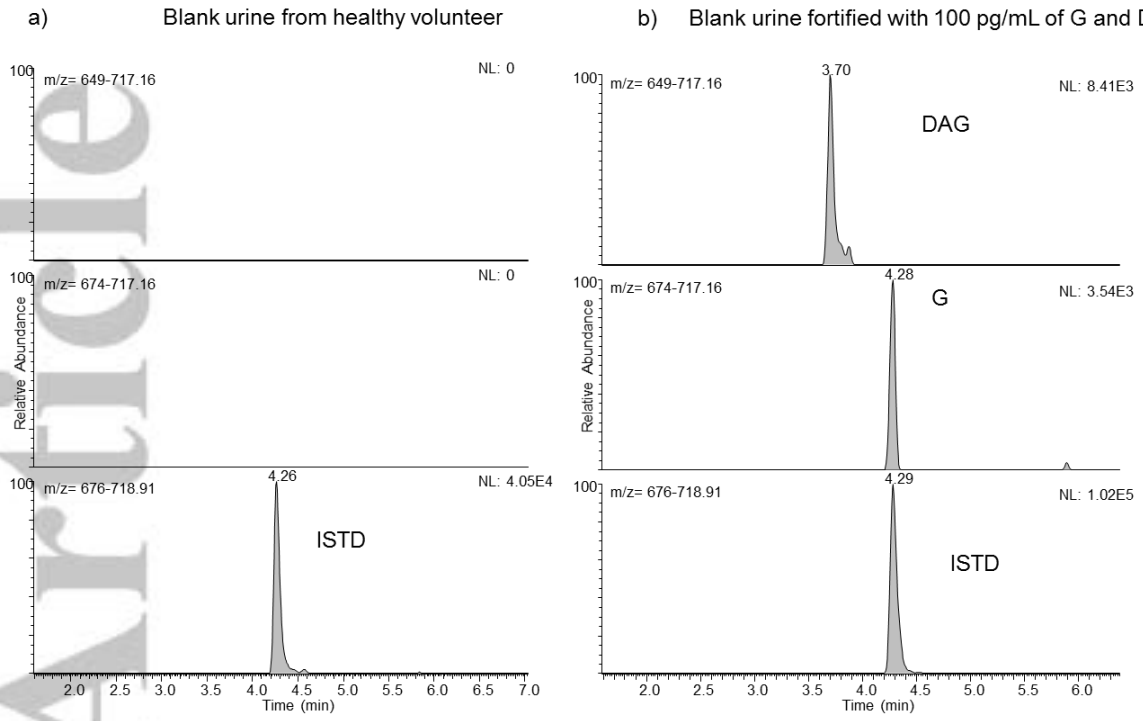

Fig 3: Product ion chromatograms of urine samples prepared according to the top-down approach with a) a blank urine (left) and b) a blank urine fortified with 100 pg/mL of DAG and G.

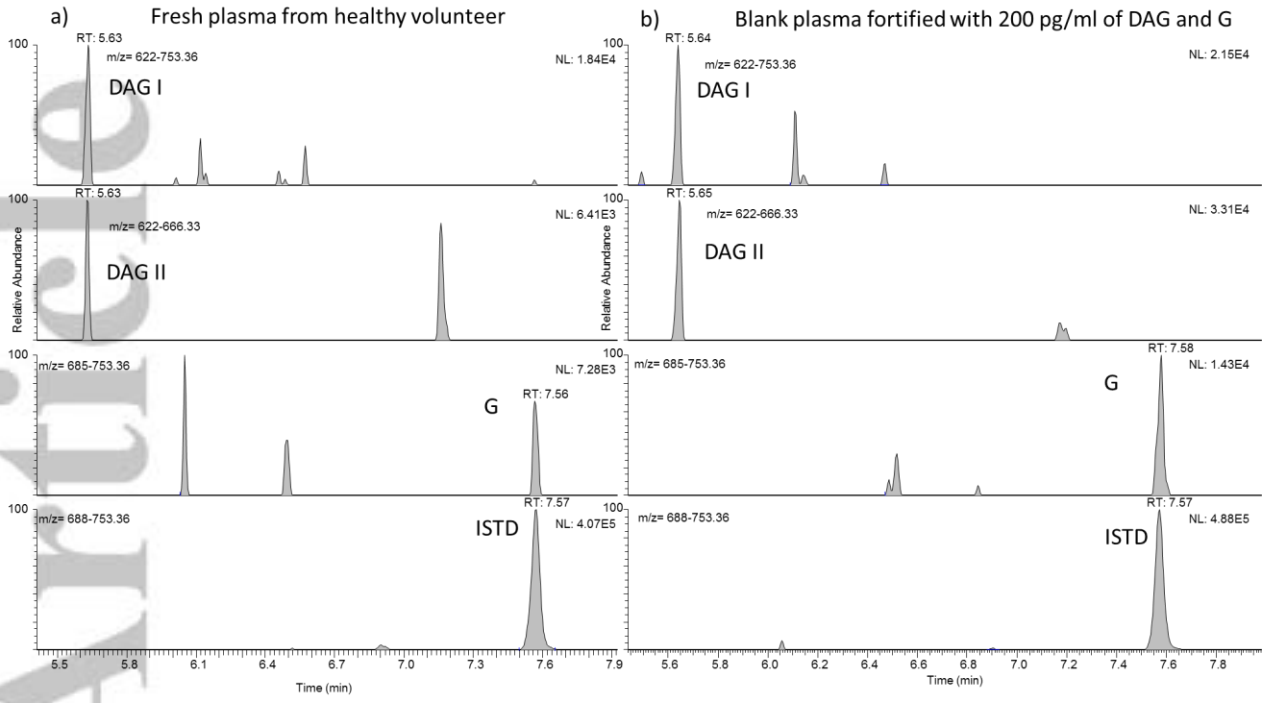

Fig 4: Product ion chromatograms of plasma samples prepared according to the bottom-up approach with a) a fresh plasma sample from a healthy volunteer with endogenous DAG and G (left) and b) a blank plasma (Octaplas) fortified with 200 pg/mL of DAG and G.