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Short communication

Quantification of steroid glycosides from *Hoodia gordonii* in porcine plasma using high performance liquid chromatography–mass spectrometry

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ABSTRACT

An HPLC–ESI–MS/MS method using collision induced dissociation – multiple reaction monitoring was developed for the quantification of eight *Hoodia gordonii* steroid glycosides and their metabolites in porcine plasma samples. The method was validated for the three most important glycosides and was successfully applied also for the related glycosides and metabolites. The limits of quantification were 0.04 ng ml^{-1} for the two main steroid glycosides and 0.1 ng ml^{-1} for the detiglated metabolites. These limits are sufficiently low to allow monitoring the concentration–time profiles in plasma after feeding *H. gordonii*. The standard deviations of the intra-day measurements were better than 20% for concentrations below 5 ng ml^{-1} and better than 10% for concentrations above 5 ng ml^{-1}. The method was successfully applied to plasma samples collected from a porcine pharmacokinetics study.

1. Introduction

Obesity is a risk factor for a number of diseases including high blood pressure, high cholesterol and asthma [1,2]. Recent studies suggest that approximately 300,000 deaths per year in the USA can be attributed to obesity [3]. A healthy diet associated with exercise is clearly beneficial for reducing bodyweight and products that safely help consumers to control their calorie intake could significantly contribute to this [4].

Hoodia gordonii is a perennial, succulent plant from the Apocycnacaea family indigenous to the arid regions of Southern Africa. In the past, several species of *Hoodia* were included in a research program undertaken by the Council of Scientific and Industrial Research (South Africa). Their research showed that *H. gordonii* contained a range of steroid glycosides that decreased food intake and body weight in animals [5]. A number of these steroid glycosides have been identified by different authors [6]. Some of the more common *H. gordonii* steroid glycosides are shown in Fig. 1.

In recent literature several methods for identifying and quantifying *H. gordonii* (*H.g.*) steroid glycosides in raw plant material have been described [6–11]. Also, a rapid method for quantifying *H.g.*-12 (also referred to as P57AS3 or P57) and *H.g.*-22 in plasma has recently been published [12]. However, *Hoodia* extracts contain a number of other steroid glycosides that are structurally very similar to *H.g.*-12 and *H.g.*-22 [7–9]. Moreover, recent *in vitro* studies suggest that *H.g.*-12 and its homologues rapidly metabolise *in vivo* [11,13]. To get insight into the physiological behaviour and the mechanism of action of the steroidal glycosides it is important to be able to determine the concentration of all these compounds in blood samples collected after intake of *H. gordonii*.

The subject of this paper was to develop and validate an analytical method for the quantification of steroidal glycosides originating from *H. gordonii* and their metabolites in porcine plasma at ng g^{-1} concentration levels. Such a method will enable studies aimed at understanding the metabolic pathway of *Hoodia* steroid glycosides in the body. The series of compound studied were the aglycone, seven steroidal glycosides having various (de-oxy) sugar chains and six metabolites obtained from *H. gordonii*.

2. Experimental

2.1. Materials and methods

2.1.1. Standards and chemicals

The *H. gordonii extract* was obtained from Cognis Iberia S.A.U. (Barcelona, Spain). Using the previously developed LC–UV method

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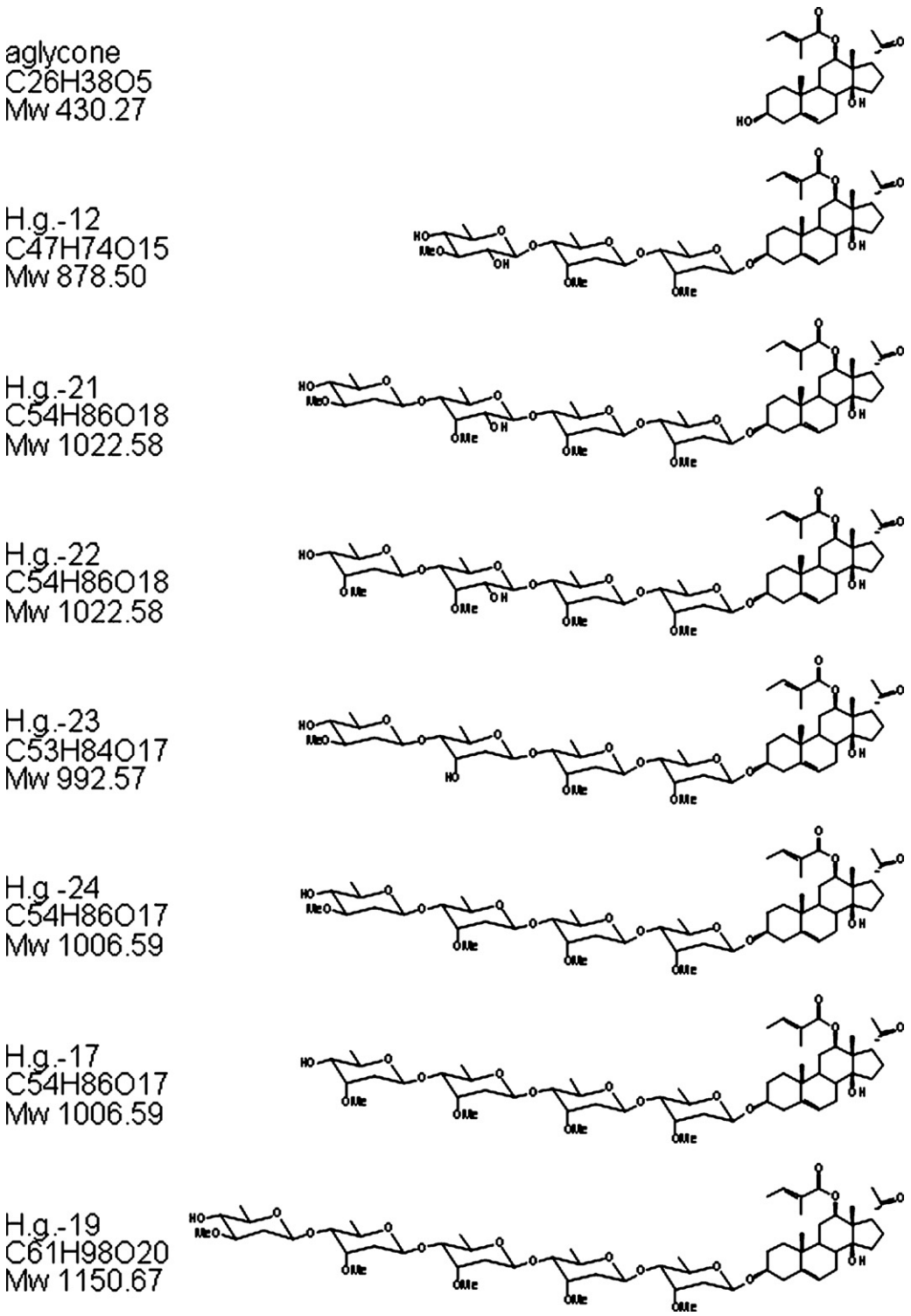

Fig. 1. Structures of the main *Hoodia gordonii* (*H.g.*) steroid glycosides. The *H.g.* molecules consist of a steroid core with a glycosidic chain esterified to tiglic acid (2-methylbut-2-enoic acid). Loss of the tiglate group is referred to as 'detiglation'.

[6], the total steroidal glycoside content of the extract was determined to be 87% divided over at least some 30 different compounds. *H.g.*-12, detiglated *H.g.*-12, *H.g.*-22 and the deuterium labeled *H.g.*-12 were purified or synthesized in-house. Porcine plasma was obtained from Innovative Research Inc. (Southfield, MI, USA). Methyl-tertiary butyl ether (MTBE), acetonitrile and methanol were of gradient grade (Merck, Amsterdam, the Netherlands). Formic acid was pro-analysis grade also from Merck. Demineralised water was prepared using a Milli-Q water purification device (Millipore, Bedford, NH, USA).

2.1.2. Standard preparation

Calibration standard solutions of the pure *H.g.*-12, detiglated *H.g.*-12 and *H.g.*-22 were prepared starting from a concentration of each steroid glycoside of 1 mg ml^{-1} in Milli-Q water/acetonitrile 50/50 (v/v). Subsequent dilutions were prepared

Table 1
Elution times, MRM conditions and product-ion ratios of *Hoodia gordonii* steroid glycosides and metabolites (cone voltage and collision energy optimized for each individual compound).

Steroid glycoside code	Elution time (min)	Precursor ion (*m*/*z*)	Cone voltage (V)	Product ion (*m*/*z*)	Collision energy (eV)	Product ion ratio (1/2)
Detiglated aglycone	25.89	396.3	30	1: 319.3 2: 337.3	1: 35 2: 25	2.0/1
Demethyl detiglated *H.g.*-12	28.72	789.5	80	1: 297.2	1: 50	n.a.
Detiglated *H.g.*-12 I	29.74	803.5	80	1: 311.2	1: 50	n.a.
Detiglated *H.g.*-12 II	30.46	803.5	80	1: 311.2	1: 50	n.a.
Demethyl diglated *H.g.*-22 I	31.04	933.5	80	1: 297.2	1: 50	n.a.
Demethyl diglated *H.g.*-22 II	31.30	933.5	80	1: 297.2	1: 50	n.a.
Demethyl diglated *H.g.*-22 III	31.73	933.5	80	1: 297.2	1: 50	n.a.
Detiglated *H.g.*-22 I	33.20	947.5	85	1: 455.2 2: 311.2	1: 50 2: 50	4.3/1
Detiglated *H.g.*-22 II	33.58	947.5	85	1: 455.2 2: 311.2	1: 50 2: 50	2.1/1
Detiglated *H.g.*-22 III	34.22	947.5	85	1: 455.2 2: 311.2	1: 50 2: 50	3.1/1
Aglycone I	33.99	478.4	30	1: 319.3 2: 337.3	1: 35 2: 25	1.4/1
Aglycone II	34.67	478.4	30	1: 319.3 2: 337.3	1: 35 2: 25	1.2/1
Demethyl *H.g.*-12 I	39.03	871.5	80	1: 297.2 2: 771.5	1: 50 2: 40	1.6/1
Demethyl *H.g.*-12 II	39.73	871.5	80	1: 297.2 2: 771.5	1: 50 2: 40	1.4/1
H.g.-12 I	41.08	885.5	80	1: 311.3 2: 785.5	1: 52 2: 40	1.4/1
H.g.-12 II	41.78	885.5	80	1: 311.3 2: 785.5	1: 52 2: 40	1.3/1
H.g.-21	42.97	1029.6	95	1: 455.3 2: 931.5	1: 55 2: 45	8.9/1
H.g.-22	43.92	1029.6	95	1: 455.3 2: 931.5	1: 55 2: 45	17.5/1
H.g.-23 I	45.27	999.5	90	1: 425.2 2: 899.5	1: 54 2: 45	1.7/1
H.g.-23 II	45.89	999.5	90	1: 425.2 2: 899.5	1: 54 2: 45	1.5/1
H.g.-23 III	46.31	999.5	90	1: 425.2 2: 900.5	1: 54 2: 45	1.8/1
H.g.-24 I	47.51	1013.6	95	1: 439.2 2: 914.5	1: 55 2: 45	1.1/1
H.g.-24 II	48.11	1013.6	95	1: 439.2 2: 914.5	1: 55 2: 45	1.0/1
H.g.-17	48.53	1013.6	95	1: 439.2 2: 914.5	1: 55 2: 45	1.2/1
H.g.-19	49.81	1157.5	110	1: 584.4 2: 1059.5	1: 55 2: 45	2.3/1
Deuterium labeled *H.g.*-12	40.72	895.5	80	1: 311.3 2: 785.5	1: 52 2: 40	1.5/1

Note: For several compounds more than one chromatographic peak representing different isomers could be observed. These peaks are identified by a I, II or III.

either in a solvent mixture consisting of aqueous 0.1% formic acid/acetonitrile/methanol 80/14/6 (v/v/v) or in porcine plasma to yield the following concentrations: 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng ml^{-1}. Each solution contained the internal standard, deuterium labeled *H.g.*-12, at a concentration of 50 ng ml^{-1}.

2.1.3. Sample preparation

The target analytes are rather non-polar species with calculated Log Kow values ranging from 3.61 for *H.g.*-12 to 5.36 for *H.g.*-19. For such analytes liquid–liquid extraction using non-polar solvents is typically employed. Here we opted for the use of a two-

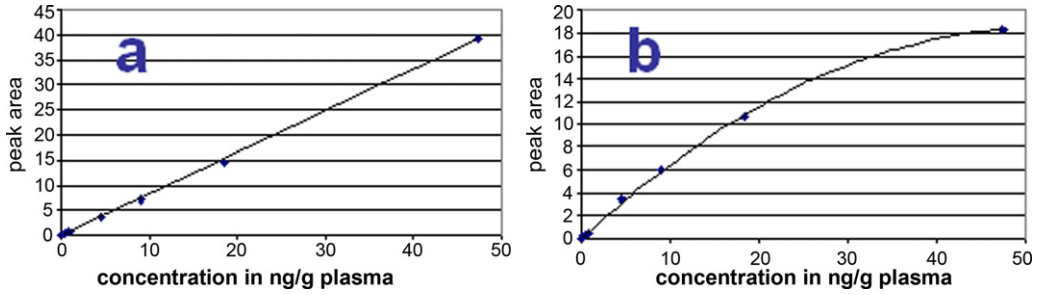

Fig. 2. Calibration curves for *H.g.*-12 analyzed with (a) and without (b) lithium chloride in the HPLC eluent. Details on the MS settings are given in Table 1.

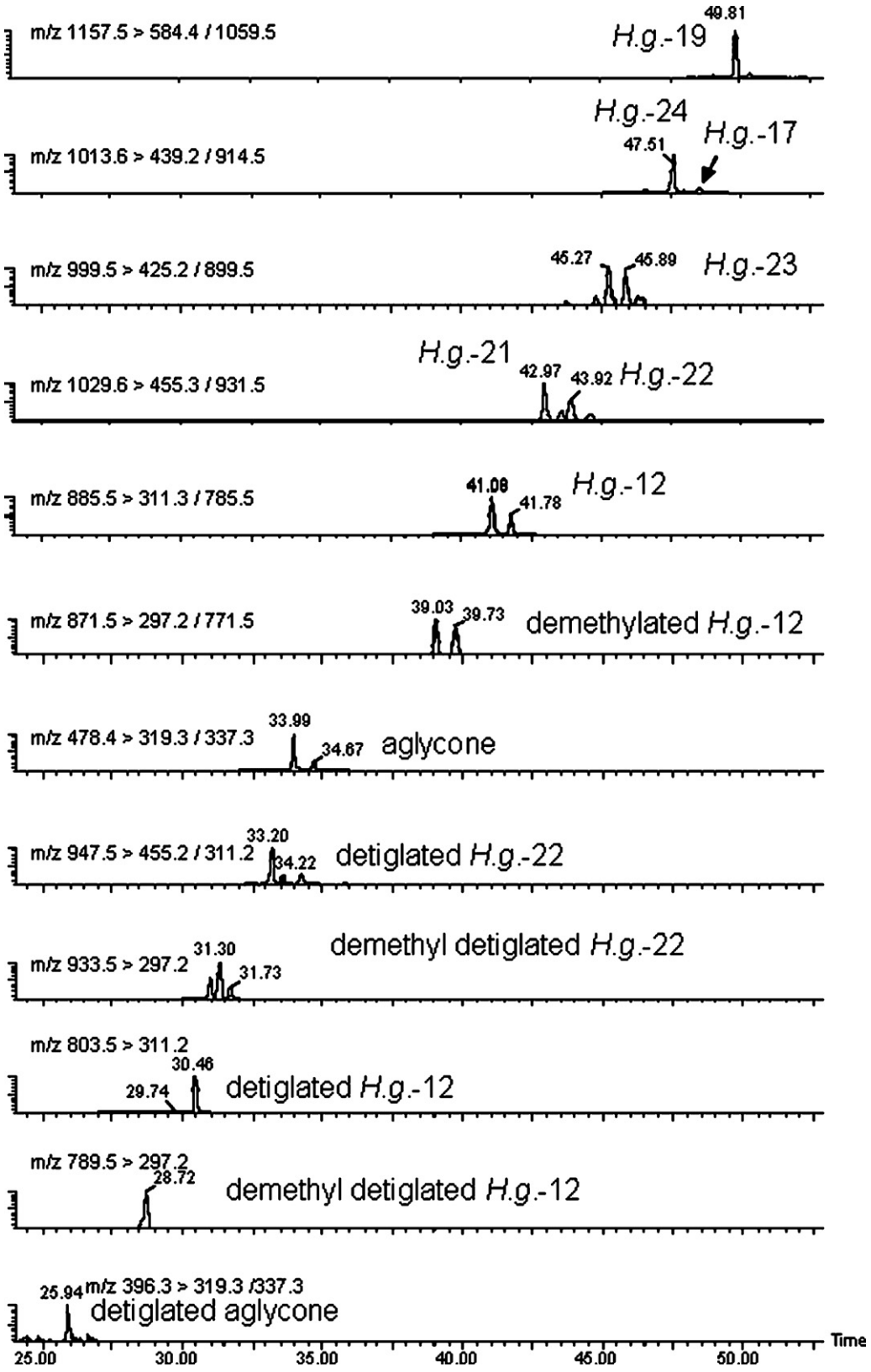

Fig. 3. Typical chromatogram of a plasma sample collected during a porcine intervention study. The traces represent the summarized MRM traces of two product ions monitored for each steroid glycoside or metabolite.

stage liquid/liquid extraction of plasma with MTBE. The best results were obtained when 500 μl of plasma was mixed with 50 μl of a 500 ng ml^{-1} internal standard solution consisting of the deuterium labeled *H.g.*-12. The sample and the internal standard stock solution were stored in melting ice. Next 5 ml of cold water was added and after vortexing for 10 s, 5 ml of cold MTBE was added. From this point on all further steps were carried out at room temperature. After vortexing for 5 s the mixture was shaken for 15 min at

Table 2
MS response factors for the *Hoodia gordonii* steroid glycosides relative to *H.g.*-12 (set to 100%).

Compound	Response factor	Relative standard deviation ($n=6$)
H.g.-12	1.00	1.3%
Aglycone	1.00	7.3%
H.g.-21	1.60	2.7%
H.g.-23	1.19	2.3%
H.g.-24	1.34	1.9%
H.g.-17	1.58	2.4%
H.g.-19	9.45	11.3%

Table 3
Linearity data of steroid glycosides in porcine plasma using LC–MRM–MS (concentration range 0.01–100 ng ml^{-1}).

Steroid glycoside	Limit of detection (LOD) (ng ml^{-1})	Limit of quantification (LOQ) (ng ml^{-1})	Linear regression coefficient (R^2)
Detiglated *H.g.*-12	0.05	0.1	0.9987
H.g.-12	0.01	0.04	0.9993
H.g.-22	0.01	0.04	0.9989

900 rpm using a mixing device. The layers were allowed to separate for 15 min and 4 ml of the upper MTBE layer was pipetted into a second test tube. Another portion of 5 ml MTBE was added to the first tube and after vortexing it was mixed again at 900 rpm for 15 min. After 15 min of stabilization 5 ml of the top MTBE layer was pipetted into the test tube containing the first extract. The 9 ml of MTBE was evaporated to dryness under a stream of nitrogen at 35 °C. After addition of 60 μl of methanol the tube was shaken at 400 rpm for 10 min. Then, 180 μl of 0.1% formic acid was added and the tube was shaken again at 400 rpm for another 10 min. Finally, the sample solution was pipetted into a 2 ml autosampler vial, treated ultrasonically for 5 min and stored at 15 °C until injection. Upon addition of the 0.1% formic acid solution a finely dispersed fat-in-water emulsion is formed in which the steroidal glycosides are homogeneously distributed. All samples were injected on the HPLC system within 24 h after preparation.

2.1.4. Instrumentation

All analyses were performed on a Waters 2795 HPLC coupled to a Quattro-Premier triple quadrupole mass spectrometer (Waters, Etten-Leur, The Netherlands).

2.1.5. Chromatographic conditions

All analyses were performed on a 150 mm × 2.1 mm Zorbax Extend C18 column with a particle size of 5 μm (Agilent, Amstelveen, The Netherlands). Mobile phase A consisted of 0.1% formic acid in Milli-Q water, mobile phase B was acetonitrile and mobile phase C was methanol. The initial eluent composition was A/B/C 80/14/6 (v/v/v). All mobile phases contained 0.02 mM lithium chloride to improve the MS ionization properties (see below). After a 10 min hold a linear gradient was started to A/B/C 10/75/15 (v/v/v) in 35 min. The eluent was kept at this composition for 5 min and programmed back to the initial composition in 5 min. The system was allowed to re-equilibrate for 5 min. The total run time was 60 min. The eluent flow rate was 0.2 ml min^{-1} and the column temperature was set at 40 °C. The injection volume was 80 μl. The autosampler temperature was 15 °C. In order to overcome the possible negative effects of fatty material from the sample on the column performance, after each set of 25 samples a column clean-up was carried out by flushing the column with the solvents A/B/C in a ratio of 5/5/90 at an eluent flow of 0.2 ml min^{-1} for at least 4 h. With this method the column performance was stable for at least 1000 injections.

2.1.6. Mass spectrometric conditions

All measurements were carried out using high performance liquid chromatography coupled to quadrupole mass spectrometry by means of low-energy collision induced dissociation-MS/MS analysis (CID-MS/MS) using the multiple reaction monitoring (MRM) mode. The capillary voltage was set at 4 kV. The source temperature was kept at 100 °C and the nebulizer temperature at 250 °C. The desolvation and cone gas flows were 800 and 50 l h^{-1}, respectively. Argon was used as collision gas at a gas flow of 0.35 ml min^{-1}. The dwell time was set at 0.3 s for each steroidal glycoside, with an interscan time of 0.03 s. The optimum settings of the cone voltages and collision energies for the *H. gordonii* steroid glycosides were determined by repeated injections of the *Hoodia* extract in the initial eluent at a concentration of 1000 ng ml^{-1}. Successive analyses were performed in which the cone voltage was varied in the range of 10–120 V, with the collision energy being varied from 20 to 60 eV. Steroidal glycosides were identified based on their retention time, their molecular ion and the ratio of two different characteristic product ions for each steroidal glycoside, relative to those of the model compounds (see Table 1). A maximum variation of 0.1 min for the elution time and 10% for the product-ion ratio was accepted. Detailed information on the elution times, precursor ions, product-ions, cone voltages, collision energies and product-ion ratios is given in Table 1.

3. Results and discussion

3.1. Optimization of the extractions conditions and mass spectrometry settings

From the physico-chemical properties of the target analytes it is clear that simple protein-removal methods such as acetonitrile precipitation cannot be applied. The rather non-polar *Hoodia* steroidal glycosides will show a strong protein binding and are likely to be (partly) removed together with the proteins. Liquid/liquid extraction or SPE are probably more suited for the sample preparation.

Table 4
Within-day variation obtained in the analysis of a porcine plasma spiked with *Hoodia gordonii* extract analyzed on one day ($n=8$).

Meas. no	Concentration in ng g^{-1} plasma							
	H.g.-12	Aglycone	*H.g.*-21	*H.g.*-22	*H.g.*-23	*H.g.*-24	*H.g.*-17	*H.g.*-19
1	21.60	0.16	4.99	19.73	8.45	10.27	3.70	0.33
2	21.12	0.17	4.80	17.15	8.06	10.10	3.62	0.34
3	21.15	0.17	4.83	17.60	8.54	11.23	3.79	0.44
4	21.15	0.16	4.78	17.70	8.36	11.21	3.44	0.39
5	21.04	0.15	4.97	17.37	8.69	10.23	3.43	0.37
6	20.62	0.15	5.10	17.02	8.73	9.82	3.32	0.35
7	20.82	0.15	5.23	17.70	8.70	10.20	3.22	0.36
8	21.39	0.39	5.18	18.50	8.34	11.53	3.30	0.42
Average	21.11	0.19	4.98	17.84	8.48	10.57	3.48	0.37
Rel STD	1.46	44.40	3.52	4.95	2.71	6.07	5.93	10.74

Table 5
Precision and accuracy of the assay ($n = 5$).

Concentration (ng ml^{-1})	Detiglated *H.g.*-12		*H.g.*-12		*H.g.*-22	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.1	16.2	126.8	10.7	112.6	12.1	89.4
0.5	11.6	106.3	8.3	97.3	9.2	92.5
1	9.3	94.6	6.0	91.7	6.4	88.5
10	7.2	98.2	4.3	101.5	5.2	91.6
100	4.1	97.3	1.7	98.4	2.5	89.1
500	2.5	99.1	1.3	95.6	1.6	84.5

Another problem with the relatively labile carbohydrate-like structures is the strong fragmentation that occurs in the MS source. The fragment intensity is concentration dependent which will result in highly non-linear calibration curves.

3.1.1. Addition of lithium chloride

In standard LC–ESI–CID–MS/MS experiments many product ions are generated, especially if sodium is added to the mobile phase [10,14]. Fragmentation might be helpful for identification of unknown steroidal glycosides, for quantification purposes however, the generation of multiple molecular ions and fragments in the MS source negatively influences the sensitivity and the linearity of the method. When lithium chloride was added to the eluent only one main molecular ion, the $[M+Li]^+$ ion, was generated in the MS source [11]. In the collision cell two fragment ions are generated, representing the loss of the tiglate group (if present) and the terminal disaccharide unit of the carbohydrate chain. In Fig. 2 the calibration curves of *H.g.*-12 are given generated with and without lithium chloride. The lithium adduct experiments showed good linearities in the relevant range.

3.1.2. Ionization efficiency

The accurate quantification of the steroidal glycosides requires the use of pure synthetic compounds and deuterated internal standards. However, the only purified standards available were the *H.g.*-12 and its detiglated form and the *H.g.*-22. Since no synthesized standards were available for the other steroidal glycosides the *Hoodia* extract was used instead. This extract was analyzed using both the HPLC–UV method as described by Janssen et al. [6] and by the HPLC–ESI–MS and CID-MS/MS (MRM) method we developed. In the LC–UV analyses it is reasonable to assume equal molar response factors for each of the analytes. Using one pure compound the levels of all steroidal glycosides can then be derived, and correction factors for the MS analyses can be calculated for each steroidal glycoside. The response factor of *H.g.*-12, which was available as the pure material, was arbitrarily set to 1.00. The response factors of other steroidal glycosides were calculated relative to *H.g.*-12. This approach failed for the metabolites as the levels of these species were too low for LC–UV analysis. Ionization-efficiency correction factors for the metabolites were set at 1.0 relative to *H.g.*-12. The results of these measurements are summarized in Table 2. The high correction factor of 9.45 for *H.g.*-19 negatively influences the sensitivity and accuracy of the method for this compound.

3.2. Evaluation of the method performance

The final method was validated according to the procedure described by Shah et al. [15].

3.2.1. Linearity and detection limits

The linearity was calculated by the analysis of a set of calibration standards of *H.g.*-12, detiglated *H.g.*-12 and *H.g.*-22 in porcine plasma. The concentration range studied ran from 0 to 1000 ng ml^{-1}. Deuterated *H.g.*-12 at a concentration level of 50 ng ml^{-1} was used as the internal standard. For the determination of the limit of detection samples spiked at 0.01 and 0.05 ng ml^{-1} were analyzed. One set of standards was measured in ascending concentration order followed by the other set in descending concentration order. The two sets were separated by a blank sample. The limit of detection (LOD) was calculated from the signal-to-noise ratio of the chromatographic peaks. The LOD was defined as the concentration that gave a chromatographic peak with a signal to noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration that could be measured with a maximum relative standard deviation for the precision of 20% and a maximum deviation from the nominal value in the accuracy/recovery measurements of 20%. The results of the measurements are summarized in Table 3. The calibration curves for *H.g.*-12, detiglated *H.g.*-12 and *H.g.*-22 showed a good linearity in the concentration range from 0.1 to 1000 ng ml^{-1}. The detection limits were approximately 0.01 ng ml^{-1} for *H.g.*-12 and *H.g.*-22 and 0.05 ng ml^{-1} for the detiglated *H.g.*-12. These values are low enough to measure physiologically relevant levels in complex sample matrices.

3.2.2. Within-day variation

The within-day variation was determined by analyzing eight samples of plasma spiked with the *Hoodia* extract at a concentration of 100 ng ml^{-1} within one day. The results of the analyses are summarized in Table 4. The standard deviations of all steroidal glycosides except the aglycone are within 20%. The poor result found for the aglycone is most likely caused by the low intensity of the peak of this compound which in turn is caused by both its low level and the poor ionization characteristics of this non-polar analyte.

3.2.3. Precision and accuracy

The precision was determined by analyzing plasma samples spiked with the *Hoodia* extract at a concentration of 100 ng ml^{-1}. Duplicate samples were prepared and analyzed on five days in a time period of five weeks. The accuracy was determined by analyzing five replicate preparations of porcine plasma spiked with the three reference standards at concentrations of 0.1, 0.5, 1, 10, 100 and 500 ng ml^{-1}. The deuterium labeled *H.g.*-12 internal standard concentration was 50 ng ml^{-1} in all samples. The results of the measurements are given in Table 5. Statistical evaluation of the results indicated an acceptable precision and accuracy for all steroidal aglycones, except for the detiglated *H.g.*-12. For this compound, at a concentration of 0.1 ng ml^{-1}, the accuracy was 126.8% and thus outside the acceptance limit of 120% [15].

3.2.4. Stability

No significant changes could be detected in the concentration of the steroid glycosides in liquid/liquid extracts of porcine plasma spiked at concentration levels of 0.5, 5 and 50 ng ml^{-1} stored for 24 or 72 h at 15 °C or −20 °C. The variations were within the reproducibility of the method. The stability of the glycosides in plasma stored at −80 °C was not investigated.

3.2.5. Carry-over

The percentage carry-over was measured in a blank sample injected directly after the 1000 ng ml^{-1} measurement. It was found to be below 0.01% for all analytes.

3.2.6. Robustness

The method was used to analyse over 2000 porcine plasma samples from an intervention trial [16]. The HPLC column was replaced only once. Elution times remained stable, even after a large number of injections. The cone of the MS source was cleaned once per week, i.e. typically after some 100 samples (including calibration line samples, quality control samples and blanks). Typical ion chromatograms obtained for a plasma sample collected during the trial are given in Fig. 3. Besides the named steroidal glycosides and metabolites, a number of isomers could be observed. This resulted in a total of 25 compounds to be quantified in one single analysis. The concentration levels found in the plasma samples varied from 0.14 ng ml^{-1} for the detiglated aglycone to almost 700 ng ml^{-1} for the detiglated species of *H.g.*-12. Good peak shapes are seen for all steroid glycosides, while most isomers were baseline separated.

4. Conclusions

An HPLC–ESI–MS/MS method was developed for the quantification of *H. gordonii* steroidal glycosides and their metabolites in porcine plasma. With the method a total of 25 compounds could be quantified in one run. Careful optimization of the extraction conditions was needed to prevent the removal of the fat-soluble hydrophobic glycosides during sample clean-up. The lower limits of detection and quantification varied between 0.01 and 0.1 ng ml^{-1} and are low enough to monitor relevant changes in plasma concentrations. The accuracy is better than 80% for *H.g.*-12 and *H.g.*-22 for concentrations between 0.1 and 500 ng ml^{-1}. A similar accuracy is also found for the detiglated *H.g.*-12. The precision was better than 20% enabling accurate monitoring of the time–concentration profiles of the steroid glycosides in the porcine plasma samples. The method is rugged, requires minimal maintenance or MS-source cleaning and was successfully applied to over 2000 samples collected from an intervention study in pigs.

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