

1,5-Anhydroglucitol - a marker for ante mortem hyperglycaemia?**Cornelius Hess, Frank Musshoff, Burkhard Madea**

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Key words: diabetes – hyperglycaemia – 1,5-anhydroglucitol – LC-MS/MS**Abstract**

Aim: 1,5-Anhydroglucitol (1,5-AG), the 1-deoxy form of glucose, competes with glucose for reabsorption in the kidneys. Our objective was to develop a liquid chromatography tandem mass spectrometric (LC-MS/MS)-method for the determination of 1,5-Anhydroglucitol, a possible marker for ante- and post mortem hyperglycemia, in serum.

Material and Methods: After a protein precipitation and a dilution step samples were separated isocratically over a polar NH₂-endcapped column. Mass spectrometric detection was made by atmospheric pressure chemical ionization with negative ionization, multiple reaction monitoring mode with 2 ion transitions was used. Serum of 166 volunteers was used to assess first data about reference concentrations in human serum.

Results and Discussion: Validation of the assay showed linearity from the limits of detection (0,34 µg/ml) up to 50 µg/ml. Precision data were in accordance with the guidelines. Diabetics with a constant high blood glucose concentration showed significantly lower serum concentrations of 1,5-AG than non-diabetics in the living and after death.

1. Introduction

For the post mortem determination of a hyperglycaemic coma, some parameters are either difficult to interpret or just unusable. Glucose might be an unreliable factor because it is rapidly metabolized into lactate by glycolysis. Therefore post mortem biochemical evaluation of glucose metabolism has to be based on combination of glucose and lactate levels in vitreous humour (VH) or cerebrospinal fluid. However, the thresholds for this so called “sum formula of Traub” are discussed controversially. Glycated hemoglobin (HbA1c) is not a good indicator of glycaemic control over shorter periods.

1,5-Anhydroglucitol (1,5-AG) (structure in figure 1) is a non-metabolizable glucose analogue (1-deoxy-form) and competes with glucose for reabsorption in the kidneys.

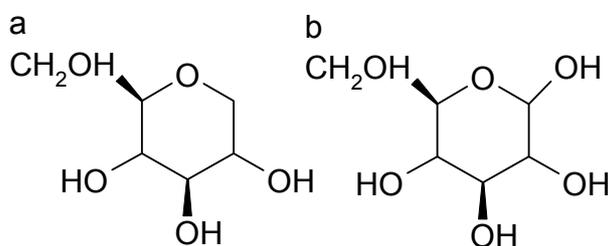


Fig. 1. Structures of 1,5-Anhydroglucitol (a) and Glucose (b).

The normal plasma concentration can be dramatically decreased by inhibition of tubular reabsorption during periods of hyperglycemia. Therefore, diabetics with a constant high blood glucose concentration show significantly lower serum concentrations of 1,5-AG than non-

diabetics, while urine concentrations of 1,5-AG of diabetics exceed those of non-diabetics. 1,5-AG is tightly associated with glucose fluctuations and postprandial glucose and predicts more accurately rapid changes in glycaemia than HbA1c or fructosamine [1].

The GlycoMark® enzymatic assay was developed for the determination of 1,5-AG in plasma [1]. Furthermore HPLC methods with pulsed amperometric detection [2] or labor intensive mass spectrometric methods by GC-MS [3] or LC-MS/MS with APC ionization after cation-exchange chromatography or with the use of HILIC (hydrophilic interaction liquid chromatography) [4] were described.

Our objective was to develop a fast and simple liquid chromatography tandem mass spectrometric (LC-MS/MS)-method for the determination of 1,5-AG in serum and urine and to compare our reference values with those measured with the enzymatic assays in literature. Furthermore 1,5-AG could be used as a marker to prove an ante-mortem hyperglycaemia.

2. Material and Methods

2.1. Sample preparation

50µl plasma/urine was treated as follows: after addition of 10µl of internal standard (200µg/ml 1,5-Anhydro-D-[¹³C₆] glucitol) the proteins were precipitated with 200µl methanol. The sample was then vortexed for 10s and centrifugated for 10min at 3000 rpm. The supernatant was diluted 1:5 with acetonitrile and 10µl of the diluted extract was injected into the chromatographic system.

2.2. LC-MS/MS-parameters

Studies were made on an Agilent series LC coupled with an Applied Biosystems API4000 QTrap Mass Spectrometer. Chromatographic separation were carried out with a Phenomenex Luna® NH₂ 100Å, 150*2mm, 3µm particle size and a Phenomenex NH₂, 4*2mm Guard Column. An isocratic flow (0.5 ml/min) with a mixture of acetonitrile and water (80:20, v/v) for 6 min was used. Molecules were ionized by Atmospheric Pressure Chemical Ionization (APCI) in negative mode and the following ion transitions in multiple reaction monitoring mode were used for qualifying and quantifying 1,5-AG and its internal standard: 162.9 - 112.7 (target) and 162.9 - 101.0 (qualifier) for 1,5-AG and 168.9 -105.0 for the internal standard.

2.3. Method validation

The method was validated in human plasma in accordance to the guideline of the Society of Toxicology and Forensic Chemistry (GTFCh). For stability studies, blood of 10 volunteers was centrifuged immediately, stored at -20°C and measured immediately, 24h, 48h, 72h, 7d and 14d after sampling.

2.4. Real samples

Blood (n=166, 21 known diabetics) and urine (n=14) of real traffic cases was centrifuged and freezed at -20°C until analysis for 1,5-AG which was conducted not later than 72h after arrival at our laboratory. Blood of 21 non-diabetic and 29 diabetic (3 in a status of hyperglycaemic coma with a sum formula of Traub > 450 mg/dl in VH) deaths from different sites of the bodies (femoral vein left or right, heart blood) was collected, freezed at -20°C until analysis for 1,5-AG.

3. Results and Discussion

3.1. LC-MS/MS experiments

In figure 2 chromatograms of serum of a non-diabetic (left) with 15.2 µg/ml 1,5-AG and a diabetic patient with 1.05 µg/ml 1,5-AG (right) are shown.

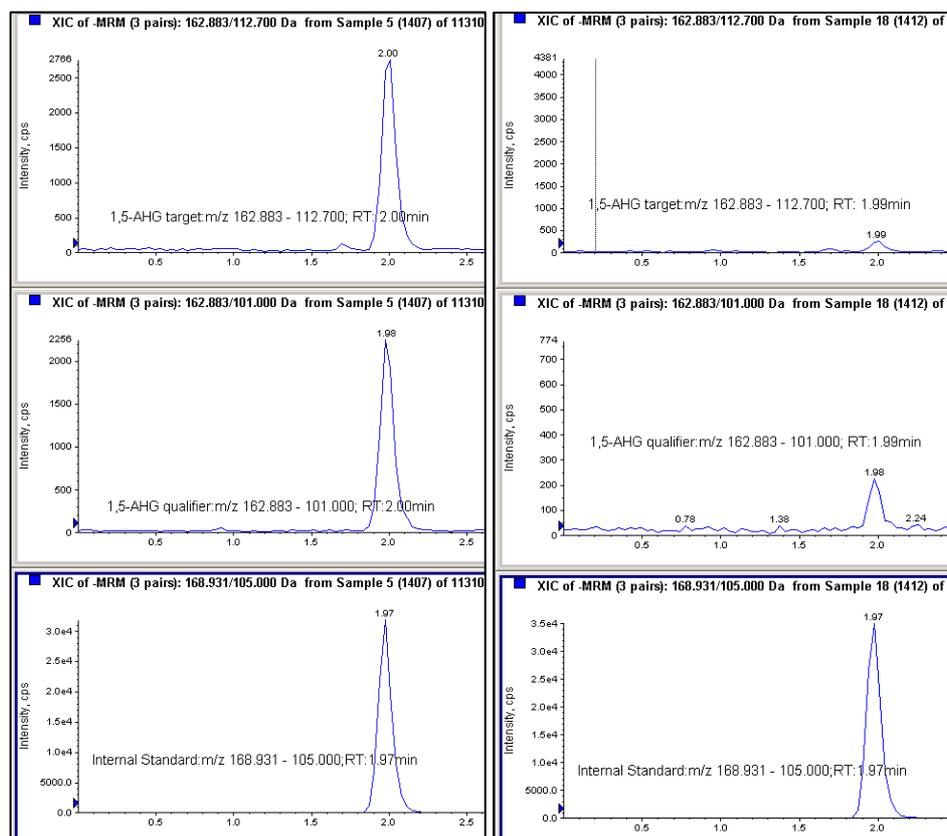


Fig. 2. Results of the LC-MS experiments.

3.2. Validation data

Validation of the assay showed linearity from the limit of detection (0,34 µg/ml) up to 50 µg/ml. Precision data at three concentrations (3 µg/ml, 15 µg/ml and 40µg/ml) were in accordance with the guidelines: intraday-precision 13,6%, 2,7% and 1,9%, inter-day precision 13,6%, 3,6% and 3,7%, accuracy bias 9,9%, 0,5% and -1,9%. Stability (decrease in concentration < 15%) of 1,5-AG is given until at least 2 weeks after sampling.

3.3. Reference concentrations in the living and in corpses

Reference values were in accordance with those measured with the enzymatic assays in literature. Diabetics with a constant high blood glucose concentration show significantly lower serum concentrations of 1,5-AG than non-diabetics in the living and after death.

Tab. 1. Reference concentrations in the living.

	Living, non diabetic. Blood [$\mu\text{g/ml}$]	Living, known diabetic, blood [$\mu\text{g/ml}$]	Living, non diabetic, urine [$\mu\text{g/ml}$]
mean	21.4	7.96	2.63
minimum	4.52	<0.34	<0.34
maximum	67.4	29.0	11.0
standard deviation	6.97	9.1	1.81
n	145	21	14

Tab. 2. Concentrations in corpses.

	Heart blood, non diabetic [$\mu\text{g/ml}$]	Heart blood, diabetic [$\mu\text{g/ml}$]	Heart blood, death due to hyperglycaemic coma (sum value > 450 mg/dl in VH) [$\mu\text{g/ml}$]	Femoral blood, non diabetic [$\mu\text{g/ml}$]	Femoral blood, diabetic [$\mu\text{g/ml}$]	Femoral blood, death due to hyperglycaemic coma (sum value > 450 mg/dl in VH) [$\mu\text{g/ml}$]
mean	15.2	5.49	5.76	17.8	3.84	6.86
minimum	5.3	0.06	0.94	4.1	0	0.63
maximum	40.0	25.2	8.46	38.4	12.1	10.9
n	21	29	3	18	27	3

4. Conclusion

Our LC-MS/MS procedure allows quantification of 1,5-AG within the physiological concentrations after a fast and simple protein precipitation step. The assay was validated in accordance to the GTFCh guidelines. Stability in plasma samples (decrease in concentration <15%) of 1,5-AG is given until at least 2 weeks after sampling. Reference values were in accordance with those measured with the enzymatic assays in literature. Concentrations in corpses were lower than in the living due to the time between death and sampling and were significantly higher in diabetics than in non-diabetics. So an antemortem unknown diabetes can be detected post mortem by an assay for 1,5-AG. Concentrations could not distinguish between deaths due to diabetic coma and other causes of death in diabetics. However, more data is needed to set reference ranges or thresholds in corpses.

5. References

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