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Clinical and forensic examinations of glycemic marker 1,5-anhydroglucitol by means of high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Postmortem diagnosis of diabetes and a diabetic coma can be difficult because of the lack of characteristic morphological findings. 1,5-Anhydroglucitol (1,5-AG), the 1-deoxy form of glucose, competes with glucose for reabsorption in the kidneys. Therefore, diabetics with a permanent hyperglycemia show significantly lower serum concentrations of 1,5-AG than non-diabetics. A liquid chromatography–mass spectrometric method for the determination of 1,5-AG in serum and postmortem blood was developed and validated according to international guidelines. Linearity was given between 1 μg/ml and 50 μg/ml. Recovery rates ranged between 70.8% and 89.8%, the limit of quantification of the procedure was 0.20 μg/ml, limit of quantification was 0.55 μg/ml. Serum of 199 diabetics and 116 non-diabetics and femoral blood of 31 diabetic and 27 non-diabetic deceased was measured. Average concentrations were significantly ($p < 0.001$) higher in non-diabetics compared to diabetics ante and postmortem. Seven of the diabetics may have died because of a hyperglycemic coma indicated by a sum formula of Traub > 450 mg/dl. 1,5-AG average concentrations in these deceased were not significantly different to diabetics which did not die because of a diabetic coma. Concentrations of 1,5-AG give a hint for not well controlled diabetes antemortem and postmortem and can be assumed as an additional and alternative information postmortem to the measurement of HbA1c or fructosamine.

1. Introduction

1,5-Anhydroglucitol (1,5-AG) (Fig. 1), the 1-deoxy-form of glucose, is ingested by nutrition and competes with glucose for reabsorption by the specific transporter SGLT4 in the kidneys [1]. The normal plasma 1,5-AG concentration can be decreased by inhibition of tubular reabsorption during periods of hyperglycemia. At normoglycemia 1,5-AG is maintained at constant steady state levels due to a large body reserve compared to the amount of intake by nutrition and due to lack of metabolism [1]. If blood glucose concentration exceed 10 mmol/l, which is the renal threshold for glucose, serum 1,5-AG concentration fall in close relation to the severity of glucosuria [2]. Therefore patients with diabetes show significantly lower serum concentrations of 1,5-AG than non-diabetics [2–5]. Measurement of 1,5-AG has been shown to be an alternative for the determination of glycated hemoglobin as it predicts more accurately rapid changes in glycemia (postprandial hyperglycemia) than HbA1c or fructosamine [4].

The postmortem determination of a hyperglycemic coma is quite difficult because of the lack of morphological findings and the fact that biochemical parameters are either difficult to interpret or just unusable [6,7]. Glucose might be an unreliable factor because it is rapidly metabolized into lactate by glycolysis after death. Therefore postmortem biochemical evaluation of glucose metabolism has to be based on the combination of glucose and lactate levels in vitreous humor (VH) or cerebrospinal fluid (CF) [8,9]. However, the thresholds for this so called "sum formula of Traub" are discussed controversially. Glycated hemoglobin (HbA1c) – although very stable after death [10] – is not a good indicator of glycemic control over shorter periods. For the determination of 1,5-AG in clinical serum samples, two commercially available enzymatic assays exist, the GlykoMark® assay [4] and the Determiner-L-1,5-AG® (Kyowa Medex, Japan) [11] which were compared recently, both showing comparable results [12]. In these assays, a hydrogen peroxide by-product is detected colorimetrically using a standard peroxidase assay after the oxidation of 1,5-AG to 1,5-anhydrofuctose by pyranose oxidase. Because it is also oxidized by pyranose oxidase, glucose could interfere with this reaction. The sample is first exposed to glucokinase to convert glucose to glucose-6-phosphate. Although these tests are sensitive and precise, disturbances by glucose, bilirubin, hemoglobin [11],

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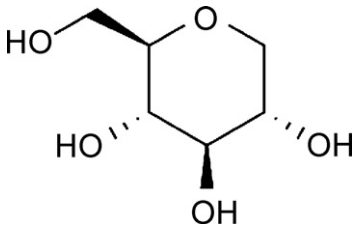

Fig. 1. Structure of 1,5-anhydroglucitol.

maltose [13] or myo-inositol [14] have been described. Tanabe et al. [15] used a flow-injection system, in which glucose and other interfering substances were adsorbed by an on-line anion exchange column prior to the enzyme reaction. Furthermore high performance liquid chromatography (HPLC) methods with pulsed amperometric detection after passing the sample through a 2- or 3-layer anion exchange column [5] or fluorometric detection after derivatization with benzoic acid [16] were described. Gas chromatographic mass spectrometric (GC–MS) [1,3] procedures for 1,5-AG were also described in the literature as well as LC–MS-methods monitoring 1,5-AG in negative ionization mode [17,18] with electrospray [17] or atmospheric pressure chemical ionization [14,18] after separation using a polar column (amide phase [17], hydrophilic interaction liquid chromatography with an amino-endcapped column [18], sulfonated styrene–divinylbenzene copolymer with zinc ions [14]) due to the polar character of 1,5-AG.

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 in order to investigate concentrations in living diabetics and non-diabetics and in the deceased. To our knowledge, the concentrations of 1,5-AG after death were not investigated yet and thus could give the possibility to prove hyperglycemia which have been occurred prior to death.

2. Experimental

2.1. Chemicals and reagents

1,5-Anhydro-D-glucitol was purchased from Sigma (Steinheim, Germany). The internal standard (IS) 1,5-anhydro-D-[$^{13}C_6$] glucitol (98 at% ^{13}C) was purchased from Omicron Chemicals (South Bend, USA). Water (from Riedel de Haen, Seelze, Germany), acetonitrile (from Merck, Darmstadt, Germany) and methanol (from Sigma, Steinheim, Germany) were HPLC grade.

2.2. Preparation of stock, standard and QC solutions

Stock solutions of 1,5-AG and the IS were prepared at 1 mg/ml in water HPLC grade. The IS working solution was prepared at 200 μg/ml water HPLC grade. 1,5-AG calibration standard working solutions were prepared to spike serum or postmortem femoral blood samples to reach the added concentrations of 1 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml and 50 μg/ml. Due to the endogenous presence of 1,5-AG in serum and postmortem samples it is not possible to acquire normal calibration curves. For the validation process, calibration curves were generated by the standard addition method. Quality control samples were prepared in water to spike serum samples to receive the added low (3 μg/ml), medium (15 μg/ml) and high (40 μg/ml) concentration.

2.3. Sample preparation

Sample preparation of Li et al. [17] was used: briefly, 50 μl of serum was spiked with 10 μl of internal standard (200 μg/ml 1,5-anhydro-D-[$^{13}C_6$] glucitol) and the proteins were precipitated with 200 μl of methanol. The sample was then vortexed for 10 s and centrifuged for 10 min at 10,000 × *g*. The supernatant was diluted 5-fold with acetonitrile and 10 μl of the diluted extract was injected into the chromatographic system.

2.4. Chromatographic and mass spectrometric conditions

Studies were made on a Shimadzu (Duisburg, Germany) LC 20 series (binary pump, degasser, controller and autosampler) coupled with an Applied Biosystems API 4000 QTrap Mass Spectrometer (Applied Biosystems, Darmstadt, Germany). Chromatographic separation was carried out with a Phenomenex (Aschaffenburg, Germany) Luna® NH_2 analytical column (150 mm × 2 mm, 3 μm particle size) and a Phenomenex NH_2 (4 mm× 2 mm) 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. Mass spectrometry parameters were optimized by infusing a 10 μg/ml solution of 1,5-AG or the internal-standard directly into the ion source and automatically optimizing the following parameters with Analyst 1.5.1 software: optimized ion transitions in multiple reaction monitoring (MRM) mode were 162.8–112.7 (target) and 162.8–101.0 (qualifier) for 1,5-AG and 168.9–105.0 for the IS. Declustering potentials were −20 V for 1,5-AG and −25 V for the IS, collision energies were −10 V (target) and −7 V (qualifier) for 1,5-AG transitions and −12 V for the IS. Cell exit potentials were −7 V for 1,5-AG and −5 V for the IS. Data were acquired using Analyst Software 1.5.1 (Applied Biosystems, Darmstadt, Germany). Calibration curves were generated using the peak area ratio of 1,5-AG target peak to IS target peak and were fitted with a linear regression. Optimized voltages and gas settings were as follows: curtain gas 10 psi, ion spray voltage −4500 V, temperature 450 °C, ion source (gas 1) 50 psi, ion source (gas 2) 40 psi, entrance potential −10 V.

2.5. Method validation

The method was validated in human serum in accordance to the guideline of the Society of Toxicology and Forensic Chemistry (GTFCh) [19,20]. Parts of the procedure were also validated in postmortem whole blood.

2.5.1. Selectivity

Serum or postmortem femoral blood samples of six different donors/deceased were analyzed. Due to the endogenous presence of 1,5-AG ante and postmortem, blank samples for 1,5-AG are not available. Samples were investigated for peaks interfering with the detection of the IS by the procedure described above. In addition, water was spiked with the IS and was analyzed for peaks deriving from the IS and interfering with the detection of 1,5-AG.

2.5.2. Linearity

Calibration standards were assayed by spiking serum or postmortem femoral blood samples with aqueous standards to obtain the six added concentrations listed in Section 2.2. Because of the endogenous presence of 1,5-AG, calibration curves were generated by using the standard addition method both in serum and postmortem femoral blood. Matrix calibration curves were repeated 6 days in a row ($n = 6$). To compare aqueous and matrix calibration curves, aqueous calibration standards were prepared in the same concentrations as the added concentrations of the matrix calibration curves.

2.5.3. Accuracy and precision

Accuracy and precision data were only collected in serum as sample matrix. The QC concentrations (Section 2.2) of the analyte were assayed against a linear regression model by using the standard addition method. The weighted calibration curve was used for the estimation of accuracy and precision after subtraction of the initial concentration and extrapolation to the *y*-axis using the program Valistat®. The calculated values at each concentration were averaged and the percentage bias was calculated to estimate accuracy. The intraday and interday precision (relative standard deviation) was assessed from a comparison of the analysis of two control samples at each QC concentration on eight consecutive days. One-way analysis of variance was used for precision calculations.

2.5.4. Analytical limits

Analytical limits data were collected in water. Water was spiked to reach the same concentrations as the final extract of the seven calibration levels 0.1 μg/ml, 0.2 μg/ml, 0.25 μg/ml, 0.5 μg/ml, 0.7 μg/ml, 1.0 μg/ml, and 1.5 μg/ml in the range of the expected limit of detection. Calibration curves were generated using the peak area ratio of weaker 1,5-AG qualifier peak to IS target peak and were fitted with a linear regression.

2.5.5. Matrix effects and recovery

Matrix effects within an LC–MS/MS method are direct or indirect variations in the signal area caused by the presence of unintended analyte or other interfering substances in the matrix. Matrix effects and recovery rate were determined in serum and postmortem femoral blood. The method by Matuszewski et al. [21] was modified due to the endogenous presence of 1,5-AG as follows: five different matrices were divided into two parts. One part of the five matrices was spiked with standards to obtain added low – and high – concentration samples and extracted following the usual protocol. Additionally the five matrices were prepared five times without any addition of analyte. The other part was precipitated without addition of analyte, however, the extract was spiked with the two concentrations of the standard after the precipitation step. The low and high concentrations of the analyte were 2.5 μg/ml and 25 μg/ml. As a third part of the experiment, five standards of the analyte in water HPLC grade which had the same added concentrations as the spiked serum samples were prepared and measured.