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Detection of DNA-bound advanced glycation end-products by immunoaffinity chromatography coupled to HPLC-diode array detection

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Sugars and sugar degradation products are formed during food processing, but also endogenously *in vivo*. *In vitro*, nucleosides and DNA react readily with these carbonyl compounds during the formation of the two diastereomers of N^2-carboxyethyl-2′-deoxyguanosine ($CEdG_{A,B}$), leading to a loss of DNA integrity. Only little is known about DNA glycation *in vivo* and about the influence of nutrition on CEdG formation. In this study, we developed a sensitive method to analyze DNA glycation by HPLC. For this purpose, immunoaffinity chromatography (IAC) using a polyclonal antibody against N^2-carboxyethylguanine (CEguanine) was coupled to HPLC-DAD. In some samples, peak identity was confirmed by LC-MS/MS. The recovery of CEguanine from the IAC columns was 52.5% ± 3.6 ($n = 4$). Thus, it was possible for the first time to detect $CEdG_{A,B}$, N^2-carboxyethylguanosine ($CEG_{A,B}$), and CEguanine in 11 human urine samples. However, due to imprecision of IAC, valid quantification of the adducts could not be achieved. Furthermore, CEdG was also detected in the DNA of cultured human smooth muscle cells (SMCs) and bovine aorta endothelium cells (BAECs). In BAECs, $CEdG_{A,B}$ were found by HPLC-DAD and LC-MS/MS after immunoaffinity purification, whereas in SMCs DNA-advanced glycation end-products were only detected with the more sensitive LC-MS/MS method.

Keywords: Advanced glycation end-products / DNA / Immunoaffinity chromatography / Maillard reaction

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1 Introduction

In heated food stuffs, sugars and other reactive carbonyl compounds react readily with proteins and amino acids, leading to the formation of Maillard products [1]. *In vivo*, similar reactions take place resulting in advanced glycation end-products (AGEs). AGEs accumulate *in vivo* during aging and particularly in patients with diabetes or renal failure where they play a major role in the development of complications [2–4], such as atherosclerosis [5], nephropathy, or retinopathy [6].

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Abbreviations: AGEs, advanced glycation end-products; **BAEC,** bovine aorta endothelium cell; **CEdG,** N^2-carboxyethyl-2′-deoxyguanosine; **CEguanine,** N^2-carboxyethylguanine; **CEG,** N^2-carboxyethylguanosine; **IAC,** immunoaffinity chromatography; **MRM,** multiple reaction monitoring; **SMC,** smooth muscle cell

Maillard products taken up from nutrition may increase the burden of systemic AGEs and thus contribute to their proinflammatory activity [7, 8]. Furthermore, reactive carbonyl compounds are formed in heat-treated food and can promote systemic AGE formation after intake [9, 10].

More recently, it was shown that DNA reacts with sugars and sugar degradation products *in vitro* in a similar way as proteins [11–13]. The reaction products, which have been isolated so far, are almost exclusively adducts of 2′-desoxyguanosine, which are alkylated at the exocyclic amino group, such as the two diastereomers of N^2-carboxyethyl-2′-deoxyguanosine ($CEdG_{A,B}$, Scheme 1) or N^2-carboxymethyl-2′-deoxyguanosine [11, 14]. There is strong evidence that DNA-AGEs cause loss of the genomic integrity and are therefore potentially genotoxic: consequences of DNA glycation are, for example, depurination [15], single-strand breaks, and an increase in mutation frequencies [16]. Mutations which are caused by DNA glycation are insertions, deletions [17], and transposition involving mammalian chromosomal elements [18]. However, little is known

about the occurrence of DNA-AGEs in cellular systems and *in vivo*. $CEdG_{A,B}$ proved to be good markers for DNA-bound AGEs, because they are DNA-glycation products which are formed *in vitro* under physiological conditions and can derive from many different carbonyl precursors [19–21]. A polyclonal antibody was raised against N^2-carboxyethylguanine (CEguanine) which was used in an ELISA to monitor DNA glycation *in vitro* [19], but the assay was not sensitive enough for the analysis of cellular DNA or other biological samples. Using a mAb against the same target, it was possible to measure carboxyethylated nucleobases in 121 human urine samples [22], thus providing the first evidence for the presence of DNA-AGEs *in vivo*.

In this study, we developed a sensitive method for the analysis of CEdG in genomic DNA and in human urine using immunoaffinity chromatography (IAC) coupled with HPLC-DAD. Thus, it was for the first time possible to show directly that the DNA-AGEs $CEdG_{A,B}$ are present in human urine samples as well as in cultured bovine aortic endothelial cells (BAECs) and human aortic smooth muscle cells (SMCs).

2 Materials and methods

2.1 Cell culture

Cell culture experiments were performed on two vascular cell lines: BAECs and human aortic SMCs. Cells were grown in DMEM with 10% fetal calf serum and 5 mM glucose for 1 wk. Then, half of the dishes were transferred in the same medium supplemented with 30 mM glucose, while the other half remained further in 5 mM glucose. After attaining confluency in about 1 wk, culture medium was removed and saved at −20°C. The cell layers were washed twice in PBS and used for DNA isolation.

Glycated nucleobases were analyzed three times in SMCs and four times in BAECs by HPLC/DAD and in each cell type twice by LC-MS/MS.

2.2 DNA isolation and hydrolysis

Immediately after harvesting, DNA was extracted quickly from the cell layers using the Wizard Genomic DNA purification kit (Promega). The amount of DNA was measured spectrophotometrically at 260 nm and its purity was confirmed by a ratio $OD_{260}/OD_{280} > 1.68$. The absence of DNA fragments was confirmed by obtaining a single high molecular band by agarose gel electrophoresis [23].

For hydrolysis [24], the DNA samples were dissolved in 100 µL of water, heated for 3 min at 100°C, and immediately cooled for 5 min on ice. The solutions were subsequently digested with 2 µL of nuclease P1 (Sigma, 2.5 U/µL) in 10 µL of ammonium acetate buffer (0.1 M, pH 5.3) for 2 h at 45°C; 4 µL of phosphodiesterase Type IV (Sigma, 0.1–0.2 U/mL) in 10 µL of 1 M ammonium bicarbonate (pH 8.0) for 2 h at 37°C; and finally with 8 µL of alkaline phosphatase (Boehringer, Mannheim, from calf intestine, grade II) for 1 h at 37°C. Finally, the samples were stored overnight at 4°C and applied the next day to affinity chromatography.

2.3 IAC

The immunoaffinity matrix was prepared according to the instructions of the manufacturer by covalently linking 5 mg protein A purified IgG (IgG) fraction from a polyclonal CEguanine antiserum [11] to 1.0 g CNBr-activated sepharose 4B (Pharmacia). Briefly, the IgG fraction was dialyzed against coupling buffer (16.8 g $NaHCO_3$ and 29.22 g NaCl/L H_2O, adjusted to pH 8.8). In the meantime, 1.1 g CNBr-sepharose 4B was washed with 200 mL of 1 mM HCl, centrifuged to remove the HCl and resuspended in 25 mL of coupling buffer. The IgG solution was added to 12 mL of the sepharose suspension and shaken for 2 h. Successful coupling was monitored by measuring the OD at 280 nm. The gel was washed with coupling buffer, covered with the uncoupled sepharose, and incubated with Tris buffer (15.56 g Tris hydrochloride and 29.22 g NaCl/L H_2O, adjusted to pH 8.0) for 2 h at room temperature or for 16 h at 4°C. The sepharose was then centrifuged and washed three times subsequently with washing buffer (8.20 g sodium acetate and 29.22 g NaCl/L H_2O, pH adjusted to 4.0) and Tris buffer. Immunoaffinity columns were prepared by packing 0.8 cm × 4 cm Poly-Prep® Chromatography Columns (BioRad) with the antibody-coupled sepharose.

Prior to use, the column was washed with PBS containing 0.02% NaN_3. IAC was carried out at 4°C. Samples were buffered with 1 M phosphate buffer (pH 7.4) and applied to the IAC column. The column was washed with 10 mL of PBS/NaN_3 and 10 mL of water and glycated nucleobases were eluted with 8 mL of methanol. After use, the columns were washed with 10 mL of methanol and three times subsequently with 10 mL of washing buffer and Tris buffer. The columns were then stored in PBS/NaN_3 at 4°C.

The efficiency of the IAC step was checked regularly by analyzing a solution of CEguanine as positive control and PBS/NaN_3 as negative control.

2.4 HPLC-DAD and LC-MS/MS

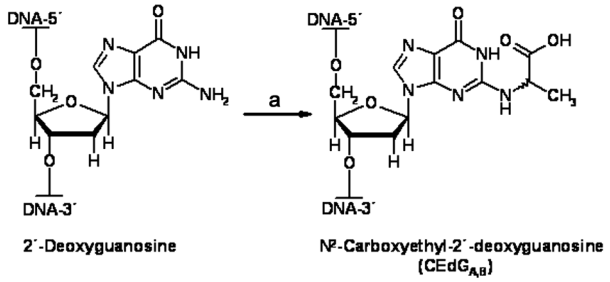
Methanol was evaporated from the eluate and the residue was resolved in 150 μL of water. An aliquot of 100 μL of the solution was injected into HPLC.

The HPLC-DAD system consisted of a Jasco PU-1580 HPLC Pump, a Jasco DG-980-50 3-Liner Degasser, a Jasco AS-1555 autosampler, and a Jasco MD-1510 Multiwavelength Detector. The compounds were separated on a CC 125/3 Nucleosil 100-5 C 18 column with an ammonium formate buffer (20 mmol, pH 4.0, solvent A), methanol (solvent B), and water (solvent C) gradient: 10% solvent A for the whole run time, 1.5–3.5% solvent B from 0 to 20 min, 3.5–30% solvent B from 20 to 25 min, 30–90% solvent B from 25 to 26 min, then continuing with 90% B from 26 to 31 min at a flow rate of 0.8 mL/min. The peaks were recorded at a wavelength range between 200 and 650 nm. The LC-MS/MS system consisted of a series 200 LC autosampler, an Agilent 1100 Quarternary pump, and an API 2000 MS/MS system with negative ESI (Applied Biosystems). The compounds were separated on a Purospher Star RP 18–e column (55 mm × 2 mm) with an ammonium acetate buffer (5 mM, solvent A), and ACN (solvent B) gradient: 10–90% solvent B from 0 to 7 min, 90% solvent B from 7 to 10 min, 10% solvent B from 10.1 to 15 min, at a flow rate of 0.3 mL/min.

Identification of the products was achieved in the multiple reaction monitoring (MRM) mode using the most intensive product ions *m/z* 149, 178, and 294 resulting from $[M-H]^-$ *m/z* 338 as qualifiers.

3 Results

In this study, we developed an analytical method to detect DNA-AGEs in genomic DNA as well as in human urine by HPLC. CEdG was used as a marker for DNA glycation because it is a reaction product that is formed from various carbohydrates and sugar degradation products [19–21] (Scheme 1). Using HPLC with DAD, it was not possible to detect the adducts in hydrolyzed cellular DNA or in human urine, but only the large excess of unmodified nucleosides was visible. Therefore, we added an IAC step prior to HPLC analyses. A polyclonal antibody had been raised against CEguanine, which shows high affinity to $CEG_{A,B}$, $CEdG_{A,B}$, CEguanine as well as *in vitro* glycated DNA [19]. The affinity of the antibody to different modified and unmodified nucleobases and nuclosides was determined by Seidel and Pischetsrieder [19]. For IAC, the IgG fraction, which was obtained by protein A purification of the polyclonal anti-CEguanine antiserum, was directly coupled to CNBr-activated sepharose. The samples were diluted in PBS before they were applied to the column. After extensive washing, carboxyethylated nucleobases and nucleosides were eluted with methanol. The eluted fraction was then applied to HPLC-DAD. Elution with methanol allows recovery of the IAC column which could be reutilized four to six times when urine was analyzed. When extracted and hydrolyzed cellular DNA was subjected to IAC, the stability of the column was better.



Scheme 1. Formation of $CEdG_{A,B}$ by the reaction of DNA with sugars and sugar degradation products; (a) nonenzymatic reaction with sugars or sugar degradation products.

The recovery of four different IAC columns was tested, each in duplicate, and was found to be 52.5% ± 3.6 SD, when about 1 μg of CEguanine was applied to the column. This number is in good accordance with results of similar methods reported in literature [25, 26].

The eluted fraction was then applied to HPLC-DAD. Under the HPLC conditions applied, CEguanine, CEG_A and CEG_B, and $CEdG_A$ and $CEdG_B$ can be detected. $CEdG_B$, however, could not fully be separated from interferents. The adducts were identified by comparison of retention time with those of the synthesized reference substances. Furthermore, carboxyethylated guanine derivatives show characteristic UV spectra which are different from the unmodified bases. These UV spectra were also used for peak identification. In some samples, peak assignment was further verified by LC-MS/MS. The method was then used in a pilot study to analyze glycated nucleobases in the urine samples of eight diabetic patients and three healthy controls. An HPLC-chromatogram of a urine sample from a diabetic patient is shown in Fig. 1. However, characteristic differences in the distribution pattern of the glycated nucleobases in diabetic *versus* healthy controls were not found by this method.

Furthermore, DNA glycation in cultured human SMCs and in BAECs was investigated. The analysis of genomic DNA requires enzymatic hydrolysis prior to HPLC analysis. Sample work-up may lead to degradation or *de novo* formation of CEdG. Thus, DNA denaturation, for which the DNA is heated for 3 min at 100°C, was identified as a critical step during work-up. Therefore, we measured the glycation rate of minimally glycated DNA by LC-MS/MS with and without including a DNA denaturation step. The glycation rate expressed by the ratio of CEdG (ng/mL) to 2′-deoxyguanosine (ng/mL) with denaturation was $38 \times 10^{-6} \pm 13$ ($n = 5$)

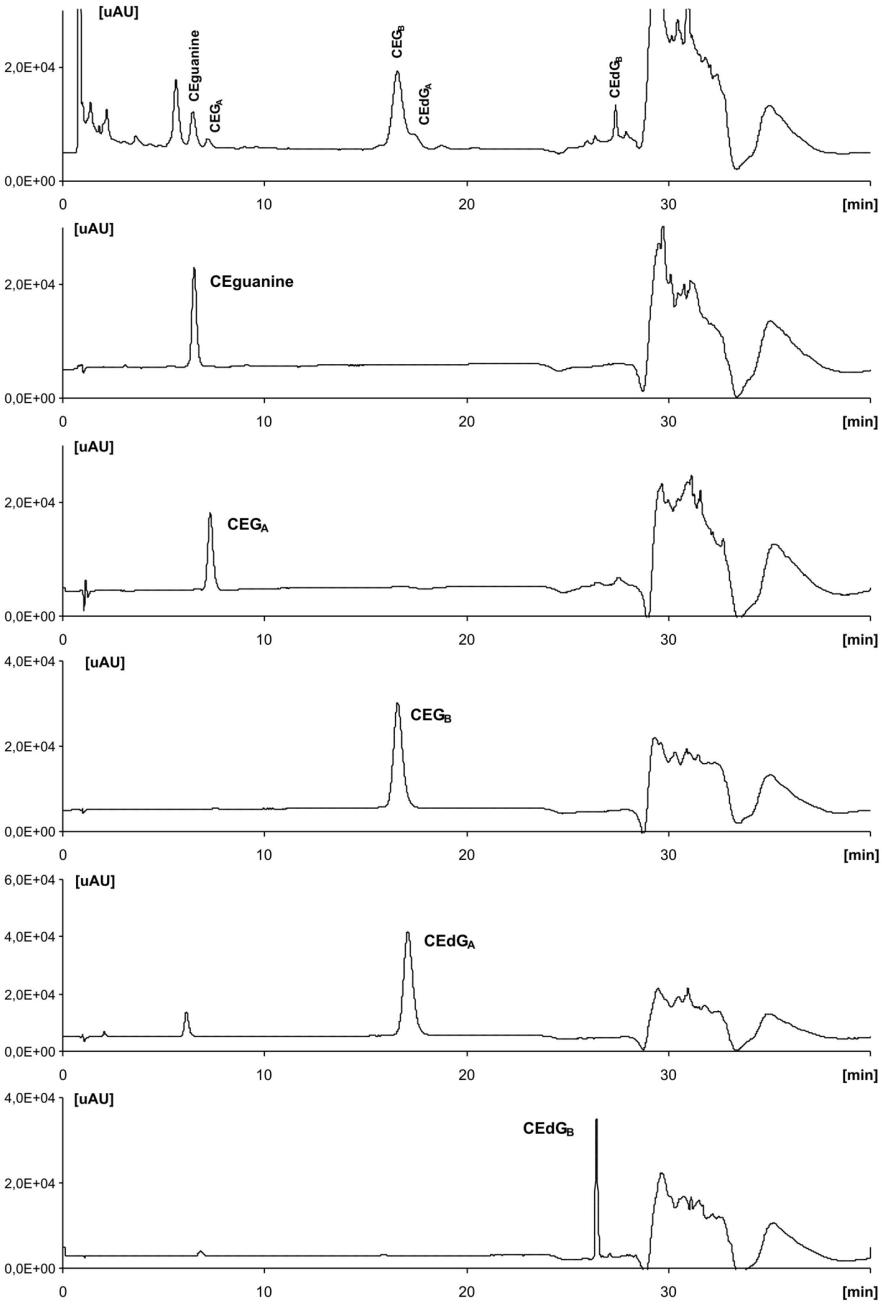


Figure 1. HPLC/DAD chromatograms of a human urine sample from a diabetic patient and of the synthesized reference compounds recorded at 254 nm.

and without denaturation $48 \times 10^{-6} \pm 19$ ($n = 6$). Thus, heating the isolated DNA for 3 min at 100°C did not lead to significant degradation or *de novo* formation of CEdG.

With this method, CEdG was detected in the DNA of BAECs, but not in SMCs. In some samples, the identity of CEdG was confirmed by LC-MS/MS. Using the more sensitive LC-MS/MS method, $CEdG_{A,B}$ were detected in SMCs as well as BAECs. Figure 2 shows a typical LC-MS/MS chromatogram of the two diastereomers of a synthesized $CEdG_{A,B}$ standard as well as of a sample of hydrolyzed DNA from human SMCs. The LC-MS/MS chromatograms were recorded in the MRM mode with *m/z* 338/178, *m/z* 338/294, and *m/z* 338/149 used as qualifiers. The full scan mass spectrum of CEdG and the product ion mass spectrum from *m/z* 338 are shown in Fig. 3.

4 Discussion

It is well known that sugars and sugar degradation products readily react with proteins to form protein glycation pro-

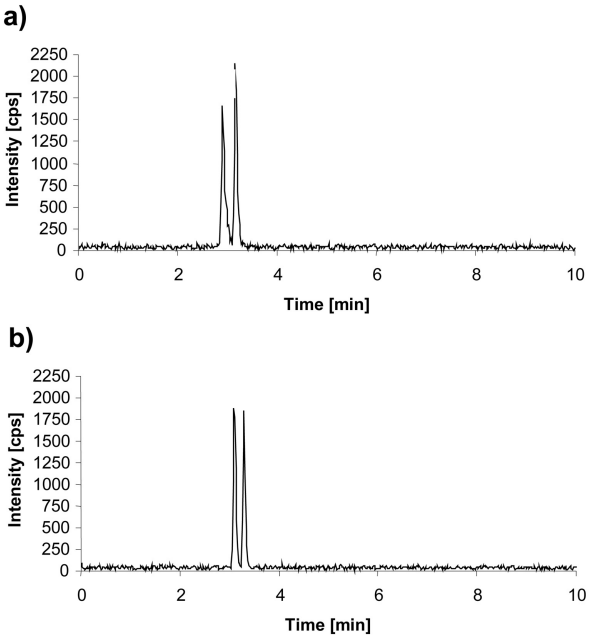


Figure 2. Detection of $CEdG_{A,B}$ in the DNA from cultured human aortic SMCs by LC-MS/MS. Identification was achieved in the MRM mode; *m/z* 338/178 (shown), *m/z* 338/294, and *m/z* 338/149 were used as qualifiers. (a) $CEdG_{A,B}$ standard solution, containing both synthesized diastereomers (10 ng/mL) (b) chromatogram of the DNA extract, isolated from human aortic SMCs.

ducts (Maillard products). Sugar degradation products comprise short-chain carbonyl compounds such as glyceraldehyde and/or substances with dicarbonyl structure, such as methylglyoxal or 3-deoxyglucosone. Sugar degradation products are formed during thermal treatment of sugar containing food, such as coffee roasting or bread toasting. The consumption of sugar degradation products through heated food can considerably contribute to the systemic pool of reactive carbonyl compounds and, as a result, may enhance the formation of AGEs *in vivo*.

Only little is known about similar glycation reactions involving sugars or sugar degradation products and DNA. *In vitro*, it was shown that the exocyclic amino group of DNA is readily glycated leading mainly to the formation of $CEdG_{A,B}$. *In vitro*, glycated DNA showed major structural and functional damage, for example depurination [15], single-strand breaks, and increased frequency of point mutations [16]. More detailed analysis revealed gross DNA alterations, such as insertions and deletions and the development of multiple species from a single cell as a result of the reaction of glucose-6-phosphate with plasmid DNA [17]. When glycated plasmid DNA was transfected to murine lymphoid cells, an increased frequency of INS-1 transposition [18] was detected. Thus, DNA glycation *in vivo* may lead to similar changes in the DNA structure and functionality as observed for *in vitro* glycated DNA.

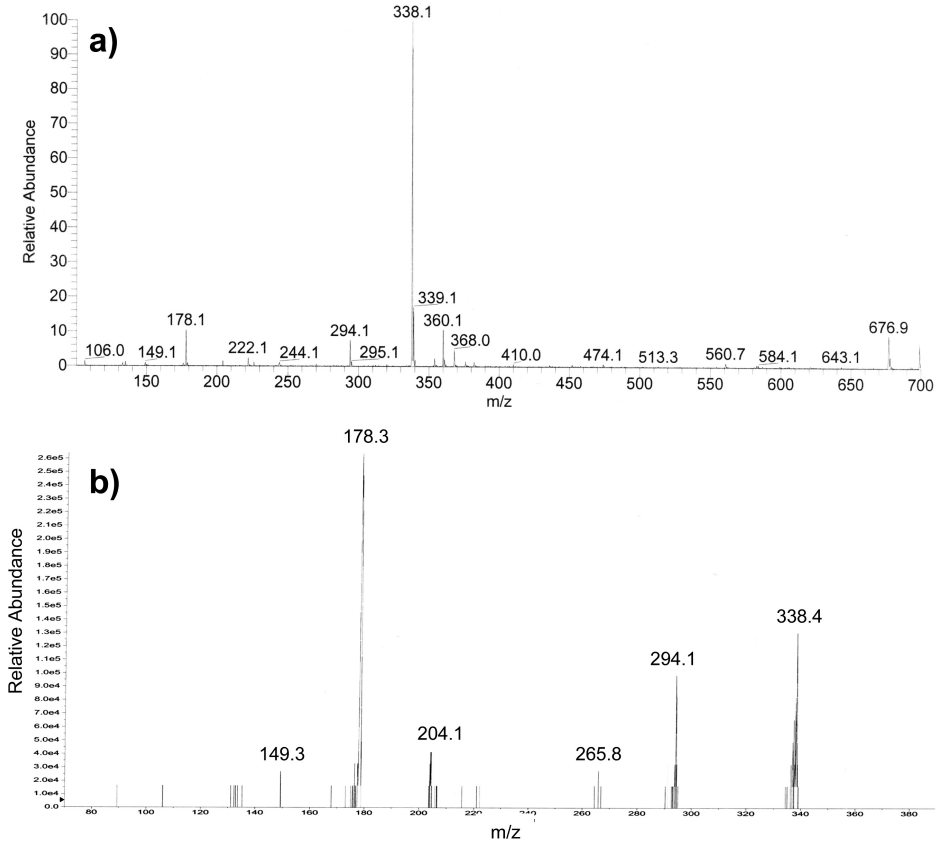


Figure 3. LC-MS/MS spectra of CEdG (a) full scan mass spectrum; (b) product ion mass spectrum from *m/z* 338.

However, sensitive analytical methods are sparse which allow the detection of DNA glycation in cellular DNA or in human urine so that it has not been clear so far if these *in vitro* studies are of relevance *in vivo*. Therefore, in this study, we developed a sensitive method to detect low levels of CEdG and other glycated nucleobases by coupling IAC to HPLC/DAD. The goal was to establish a method that can be used if LC-MS/MS is not available. IAC is commonly applied to purify or enrich DNA adducts of genomic DNA or biological samples prior to ELISA, HPLC, ^{32}P postlabeling or GC/MS [27, 28]. Particularly HPLC analysis of DNA adducts require an efficient purification step because the large excess of unmodified DNA bases would otherwise overlay the DNA adducts. IAC provided selective purification, since unmodified DNA bases and other main components of the biological samples could largely be removed in a single step so that HPLC analysis with DAD becomes possible. However, IAC also showed major disadvantages. First, a recovery of about 50% was found which impairs the sensitivity of the method. Second and more importantly, considerable inter and intra-batch variation was observed which prevented valid quantification. Particularly direct application of diluted urine led to a considerable decline in the column performance. Therefore, we do not recommend IAC-HPLC for quantification of glycated nucleobases in urine samples. For quantification, ELISA using the mAb against CEguanine could be applied or a LC-MS/MS method with a stable isotope-labeled standard should be established. On the other hand, IAC-HPLC allowed for the first time the direct proof of concept that DNA is glycated in living cells and *in vivo*. Furthermore, IAC-HPLC can be a complementary method to identify the nature of glycated nucleobases, which had been measured by ELISA, and differentiate between the different adducts: the antibodies, which were used for IAC purification, show affinity not only to $CEdG_{A,B}$, but also to CEguanine, which is the depurination product of CEdG, and to $CEG_{A,B}$, which are the analogous derivatives of guanosine. The method was then used to investigate if glycated nucleobases and nucleosides are present in urine samples of eight diabetic patients and three healthy controls. In all samples, carboxyethylated nucleobases were detected. Thus, previous results could be confirmed when carboxyethylated nucleobases were measured in 121 human urine samples by ELISA [22]. A great variation in the distribution pattern of the carboxyethylated nucleobases was observed, but characteristic differences between healthy controls and diabetic patients were not obvious.

In the second part of the study, DNA-glycation was analyzed in cultured human SMCs and in BAECs. In both cell types, CEdG was detected; in the SMCs, however, it was only by the more sensitive LC-MS/MS method. These results show that in an intact cellular system, sugars and sugar degradation products can indeed pass the nuclear membrane and react with the genomic DNA. The differences in the cell type indicate differences in the glycation rate or in the repair efficiency.

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5 References

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