



Identification and quantification of (5'R)- and (5'S)-8,5'-cyclo-2'-deoxyadenosines in human urine as putative biomarkers of oxidatively induced damage to DNA

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ABSTRACT

Biomarkers of oxidatively induced DNA damage are of great interest and can potentially be used for the early detection of disease, monitoring the progression of disease and determining the efficacy of therapy. The present work deals with the measurement in human urine of (5'R)-8,5'-cyclo-2'-deoxyadenosine (R-cdA) and (5'S)-8,5'-cyclo-2'-deoxyadenosine (S-cdA). These modified nucleosides had hitherto not been considered or investigated to be present in urine as possible biomarkers of oxidatively induced DNA damage. Urine samples were collected from volunteers, purified and analyzed by LC-MS/MS with isotope-dilution. R-cdA and S-cdA were detected in urine and quantified. Creatinine levels were also measured. In addition, we measured 8-hydroxy-2'-deoxyguanosine that is commonly used as a biomarker. This study shows, for the first time, that R-cdA and S-cdA exist in human urine and can be identified and quantified by LC-MS/MS. We propose that R-cdA and S-cdA may be well-suited biomarkers for disease processes such as carcinogenesis.

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1. Introduction

Oxidatively induced DNA damage comprising numerous products occurs in living organisms by endogenous and exogenous sources, and is implicated in a variety of disease processes including carcinogenesis and aging [1]. Among DNA lesions, 8,5'-cyclo-urine-2'-deoxynucleosides are unique in that they represent concomitant damage to both the sugar and base moieties of a nucleoside [2]. Most of the oxidatively induced DNA base lesions are repaired by base excision repair (BER). However, 8,5'-cyclo-urine-2'-deoxynucleosides cannot be removed from DNA by DNA glycosylases and are subject to nucleotide excision repair (NER) [3–5]. These compounds exist in living tissues and accumulate due to lack of DNA repair and various pathological conditions [2].

Biomarkers of oxidatively induced DNA damage are of great interest because of the involvement of this type of damage in disease processes, including carcinogenesis. Products of DNA damage can potentially be used as biomarkers for the early detection of disease,

Abbreviations: R-cdA, (5'R)-8,5'-cyclo-2'-deoxyadenosine; S-cdA, (5'S)-8,5'-cyclo-2'-deoxyadenosine; BER, base excision repair; NER, nucleotide excision repair; LC-MS/MS, liquid chromatography/tandem mass spectrometry; 8-OH-Gua, 8-hydroxyguanine; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; 8-OH-dA, 8-hydroxy-2'-deoxyadenosine; HPLC, high-performance liquid chromatography; SPE, solid phase extraction.

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monitoring the progression of disease and determining the efficacy of therapy. In addition, such biomarkers may be useful in establishing the effect of antioxidants and other drugs to reduce the risk of disease development. DNA damage can be measured in living tissues, mostly requiring invasive procedures. The use of urine instead as the measurement matrix offers an alternative non-invasive route that can readily be employed to assess DNA products as potential disease biomarkers. Among these products, 8-hydroxyguanine (8-OH-Gua) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) have received the most attention for measurement in DNA and especially in urine [1,6,7]. Techniques such as high-performance liquid chromatography (HPLC)/electrochemical detection and enzyme-linked immunosorbent assay (ELISA) were used for their measurement in urine. Mass spectrometric techniques with isotope-dilution have also been applied for the measurement of these and some other lesions [8–12]. In most studies, there has been a significant discrepancy between the results obtained with these techniques in different laboratories [7]. It is beyond the scope of the present work to review the vast literature on this subject. For this purpose, the reader is referred to some recent review articles [6,7,13].

In the present study, we hypothesized that there must be other modified nucleosides in human urine, resulting from oxidatively induced damage to DNA. To test this hypothesis, we decided to search for (5'R)-8,5'-cyclo-2'-deoxyadenosine (R-cdA) and (5'S)-8,5'-cyclo-2'-deoxyadenosine (S-cdA) that had hitherto not been considered or investigated to be present in human urine as possible biomarkers of oxidatively induced DNA damage.

2. Materials and methods

2.1. Materials

Nuclease P1, snake venom phosphodiesterase and alkaline phosphatase were purchased from United States Biological (Swampscott, MA), Sigma Chemical Co. (St. Louis, MO) and Roche Applied Science (Indianapolis, IN), respectively. Water for LC-MS/MS and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ), and Burdick and Jackson (Muskegon, MI), respectively. S-cdA was from Berry & Associates (Ann Arbor, MI).

2.2. Stable isotope-labeled internal standards

R-cdA- $^{15}\text{N}_5$, S-cdA- $^{15}\text{N}_5$ and 8-OH-dA- $^{15}\text{N}_5$ were isolated and purified using dATP- $^{15}\text{N}_5$ (Medical Isotopes Inc., Pelham, NH, USA) as described [14]. 8-OH-dG- $^{15}\text{N}_5$ was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

2.3. Collection of urine samples and purification

Urine samples were collected from 18 female and male volunteers in the Biochemical Science Division at the National Institute of Standards and Technology (NIST) as the first sample in the morning before food intake. This study has been approved by the Office of the Chief Counsel for NIST. Aliquots of each urine sample (1 mL) were mixed with aliquots of internal standards, R-cdA- $^{15}\text{N}_5$ (0.13 pmol), S-cdA- $^{15}\text{N}_5$ (0.16 pmol), 8-OH-dA- $^{15}\text{N}_5$ (0.15 pmol) and 8-OH-dG- $^{15}\text{N}_5$ (22 pmol). The samples were centrifuged at 1000g for 10 min. The supernatant fractions were separated and filtered using nylon syringe filters (0.22 μm) (NALGENE Labware, Thermo Fisher Scientific, Rochester, NY). The solid phase extraction (SPE) procedure was performed on an extraction manifold (20 positions) (Waters Corp., Milford, MA) using Oasis[®] HLB 3cc Extraction Cartridges (Waters Corp., Milford, MA) that were activated with 1 mL of methanol, and then dried for 10 min under vacuum and washed with 2 mL of HPLC-grade water. The filtered supernatant fractions of urine samples were loaded onto extraction cartridges. Vacuum was applied to the manifold until all urine sample passed through the SPE cartridge. The cartridge was then washed with 2 mL of HPLC-grade water. Retained material was eluted with 1 mL of 30% methanol. Extracted samples were dried in a SpeedVac and then dissolved either in 100 μL of water or 10 mM Tris buffer (pH 7.5). Three aliquots of the samples in Tris buffer were treated with 22 U of alkaline phosphatase at 37 °C for 1 h. Another three aliquots were hydrolyzed with a mixture of nuclease P1, phosphodiesterase I and alkaline phosphatase according to a procedure published previously [15]. Prior to LC-MS/MS analysis, all samples were filtered using Millipore Microcon Ultracel YM-3 ultrafiltration membranes (Millipore, Bedford, MA) with molecular mass cutoff of 3 kDa.

2.4. Analysis by LC-MS/MS

LC-MS/MS analyses were performed on a Thermo-Scientific Accela High Speed LC system coupled to a Thermo-Scientific Finnigan TSQ Quantum Ultra AM triple quadrupole MS/MS system with a heated electrospray-ionization source. Samples (40 μL , no waste mode) were injected on a Zorbax SB-Aq rapid resolution narrow-bore LC column (2.1 \times 150 mm, 3.5 μm particle size) (Agilent Technologies, Wilmington, DE) with an attached Agilent Eclipse XDB-C8 guard column (2.1 \times 12.5 mm, 5 μm particle size). The autosampler and column temperatures were kept at 15 °C and 40 °C, respectively. Mobile phases A and B were water and acetonitrile, respectively. A gradient analysis of 3%

(vol/vol) of B/min starting from 98%A/2%B (vol/vol) was used. After 6 min, B was increased to 90% in 0.1 min and kept at this level for 30 min to secure removal of impurities from the column and then another 20 min at 2% to equilibrate the column. The flow rate was 500 $\mu\text{L}/\text{min}$ and the total analysis time was 56 min. Mass calibration of the MS/MS system and automated tuning for R-cdA and S-cdA analysis were performed as described previously [16]. Tuning for the analysis of 8-OH-dG and 8-OH-dA was achieved similarly by injection of aliquots of 0.05 mM solutions of these compounds and by selected-reaction monitoring (SRM) of the transitions m/z 284 \rightarrow m/z 168 and m/z 268 \rightarrow m/z 152, respectively.

2.5. Measurement of creatinine in urine

Creatinine concentration in urine was measured using Quanti-Chrom[™] Creatinine Assay Kit (DICT-500, BioAssay Systems, Hayward, CA) and SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA), utilizing the optimized Jaffe method, with colorimetric creatinine determination at 510 nm. Creatinine concentration was used for normalization of results expressed as μmol of modified nucleoside/mol of creatinine.

3. Results

The aim of the present work was to search for different modified DNA nucleosides that had hitherto not been considered or investigated to be present in urine as possible biomarkers of oxidatively induced DNA damage. We decided to look for R-cdA and S-cdA in human urine. These compounds are well known DNA lesions that are repaired by NER and cannot be subject to BER [2–5]. Previously, both R-cdA and S-cdA have been measured in human and animal tissues by LC-MS and LC-MS/MS with isotope-dilution [2,16]. Both techniques yielded similar levels of these compounds *in vivo*, which amounted to approximately 0.01–0.1 lesions/ 10^6 DNA nucleosides with the level of S-cdA being greater than that of R-cdA. We searched for R-cdA and S-cdA in urine of human subjects, applying a recently developed methodology using LC-MS/MS with isotope-dilution [16]. The levels of 8-OH-dG and 8-hydroxy-2'-deoxyadenosine (8-OH-dA) were also measured. Fig. 1 illustrates the structures of the investigated modified nucleosides.

Fig. 2 shows the full-scan product ion spectrum of commercially available S-cdA. This spectrum is characterized by a protonated molecular ion (MH^+) at m/z 250 and an intense fragment ion at m/z 164. The insert illustrates the fragmentation of the molecule. Two other minor ions at m/z 214 and m/z 136 are also present. Because of the most intense m/z 164 ion, the mass transition m/z 250 \rightarrow m/z 164 was used for selected-reaction monitoring of both R-cdA and S-cdA in urine samples. Accordingly, the m/z 255 \rightarrow m/z 169 transition was used for the internal standards R-cdA- $^{15}\text{N}_5$ and S-cdA- $^{15}\text{N}_5$. After purification of urine samples, the samples were lyophilized. Three samples were dissolved in an aliquot of water. Three others were treated either with alkaline phosphatase or with a combination of nuclease P1, phosphodiesterase I and alkaline phosphatase to check whether the modified nucleosides exist in urine as nucleotides or oligodeoxynucleotides. Each sample was analyzed by LC-MS/MS. The mass transitions m/z 284 \rightarrow m/z 168, m/z 289 \rightarrow m/z 173, m/z 268 \rightarrow m/z 152 and m/z 273 \rightarrow m/z 157 were used for selected-reaction monitoring of 8-OH-dG, 8-OH-dG- $^{15}\text{N}_5$, 8-OH-dA and 8-OH-dA- $^{15}\text{N}_5$, respectively. Fragment ions at m/z 168, 173, 152 and 157 represent protonated free base ions (BH_2^+) of 8-OH-dG, 8-OH-dA and their labeled internal standards, respectively [17,18]. The precursor ions are the protonated molecular ions (MH^+) of the compounds analyzed.

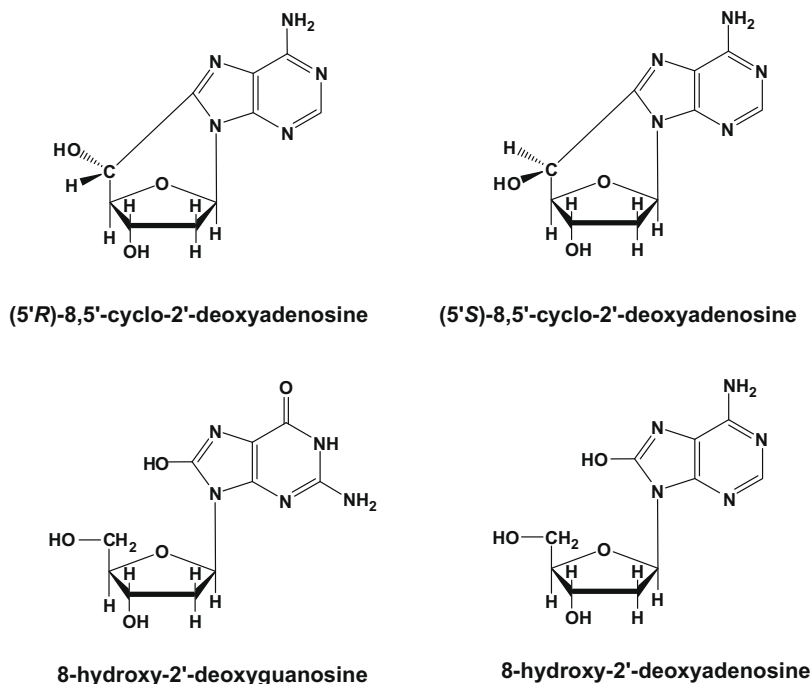


Fig. 1. Structures of modified nucleosides measured in this work.

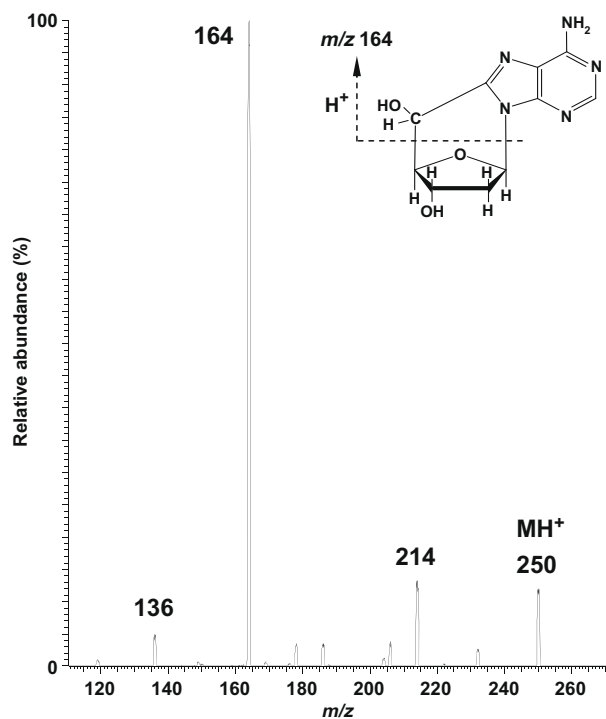


Fig. 2. Full-scan product ion spectrum of S-cdA.

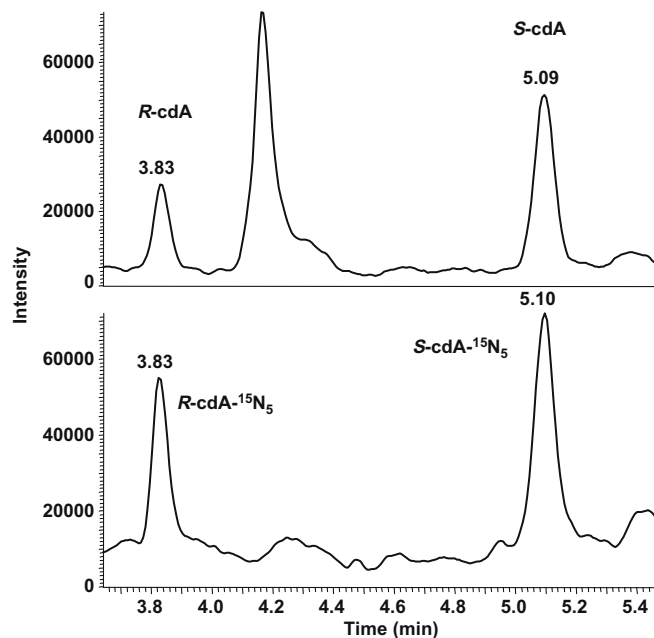


Fig. 3. Ion-current profiles of the transitions m/z 250 \rightarrow m/z 164 (R-cdA and S-cdA) and m/z 255 \rightarrow m/z 169 (R-cdA- $^{15}\text{N}_5$ and S-cdA- $^{15}\text{N}_5$). The origin of the peak at 4.2 min is unknown.

Fig. 3 illustrates the ion-current profiles of m/z 250 \rightarrow m/z 164 and m/z 255 \rightarrow m/z 169 transitions recorded during the analysis of a urine sample treated with alkaline phosphatase. Both R-cdA and S-cdA, and their internal standards were readily detected. The profiles of the transitions m/z 284 \rightarrow m/z 168 and m/z 289 \rightarrow m/z 173 are shown in Fig. 4, indicating the facile detection of 8-OH-dG and its internal standard. In general, the shapes of the signals of these transitions were excellent for quantification. In contrast, it was difficult to detect the 8-OH-dA signal in most

samples. Interfering peaks prevented an accurate quantification. Thus, this lesion was excluded from further consideration. There were no statistically significant differences between the levels of each of R-cdA, S-cdA and 8-OH-dG in all three sets of samples that were treated with either alkaline phosphatase or mixture of three enzymes or no enzymes. This indicates the absence of the nucleotide or oligodeoxynucleotide forms of these lesions in urine. Creatinine levels in urine samples were also measured using three independently prepared samples from each urine sample. Table 1

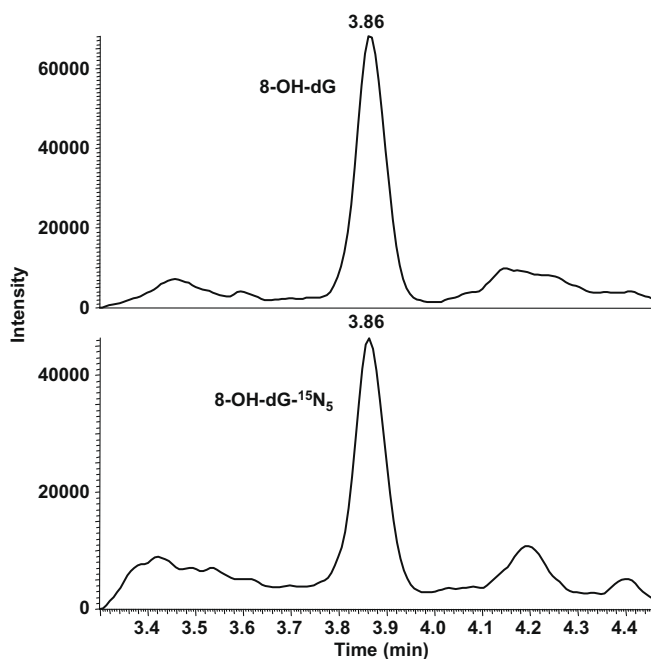


Fig. 4. Ion-current profiles of the transitions m/z 284 \rightarrow m/z 168 (8-OH-dG) and m/z 289 \rightarrow m/z 173 (8-OH-dG- $^{15}\text{N}_5$).

Table 1

Levels of R-cdA, S-cdA and 8-OH-dG in human urine ($\mu\text{mol/mol}$ creatinine). Uncertainties are standard deviations ($n = 3$).

	R-cdA	S-cdA	8-OH-dG
Females			
1	0.0284 ± 0.0013	0.0424 ± 0.0026	0.783 ± 0.113
2	0.0314 ± 0.0035	0.0399 ± 0.0015	1.073 ± 0.094
3	–	0.0311 ± 0.0083	3.364 ± 0.495
4	–	0.0111 ± 0.0008	–
5	0.0194 ± 0.0001	0.0441 ± 0.0032	1.777 ± 0.131
6	0.0221 ± 0.0047	0.0180 ± 0.0008	1.131 ± 0.067
Males			
1	–	0.0248 ± 0.0029	1.784 ± 0.126
2	–	0.0177 ± 0.0026	–
3	0.0275 ± 0.0022	0.0200 ± 0.0009	0.781 ± 0.015
4	–	0.0155 ± 0.0008	1.266 ± 0.136
5	–	0.0351 ± 0.0018	2.858 ± 0.169
6	0.0289 ± 0.0125	0.0091 ± 0.0003	–
7	–	–	4.517 ± 0.082
8	0.0694 ± 0.0057	0.0386 ± 0.0034	1.875 ± 0.074
9	0.0195 ± 0.0015	0.0157 ± 0.0008	1.429 ± 0.048
10	0.0276 ± 0.0012	0.0185 ± 0.0023	2.455 ± 0.128
11	0.0262 ± 0.0049	0.0387 ± 0.0027	1.150 ± 0.213
12	0.0268 ± 0.0013	–	1.429 ± 0.045

shows the levels of R-cdA, S-cdA and 8-OH-dG normalized to creatinine levels. Except for two samples, S-cdA was identified and quantified in all samples. Three samples did not yield satisfactory signals for the quantification of 8-OH-dG. In seven samples, R-cdA was not quantifiable. Some interfering signals prevented accurate quantification. Variations in the urine content between individual donors may be the cause of this phenomenon. The amounts of S-cdA or 8-OH-dG varied by about 4-fold between individuals, whereas the amount of R-cdA was similar except for one case. The difference between the amounts of cdAs and 8-OH-dG was up to two-orders of magnitude.

4. Discussion

In early studies, thymine glycol, 2'-deoxythymidine glycol and 8-OH-dG have been measured in human and rat urines as a possi-

ble assay for oxidatively induced DNA damage [19,20]. Subsequently, many laboratories reported the measurement of 8-OH-dG and 8-OH-Gua in urine. The origin of 8-OH-dG and 8-OH-Gua in urine has been a controversial issue [6,13]. It has been suggested that the excretion of these lesions and others into urine were a result of DNA repair and not of dietary origin [13,19,21,22]. Cell death/turnover, diet and repair in the nucleotide pool may also contribute to the presence of DNA lesions in urine [6,13]. 8-OH-Gua in urine may be due, at least in part, to BER, which releases free bases from DNA rather than nucleosides such as 8-OH-dG [23]. However, it is not clear as to how DNA repair would release 8-OH-dG. There is some *in vitro* evidence that 8-OH-dG and 2'-deoxythymidine glycol are subject to NER [24]. Subsequent studies reported no involvement of NER in the *in vitro* repair of 8-OH-dG, although this pathway has not been ruled out [25]. Oligodeoxynucleotides in human urine do not appear to contain 8-OH-dG [26]. This points to the absence of NER of 8-OH-dG or hydrolysis of 8-OH-dG-containing oligodeoxynucleotides prior to excretion into urine or within urine.

Our results unequivocally show that R-cdA and S-cdA are present in human urine and can be identified and quantified using LC-MS/MS with isotope-dilution. They exist as free nucleosides rather than as nucleotides or within an oligodeoxynucleotide. This finding is on a par with a previous report that showed the absence of 8-OH-dG-containing oligodeoxynucleotides in human urine [26]. Our methodology possesses the specificity and sensitivity to measure R-cdA and S-cdA in human urine and permits simultaneous measurement of 8-OH-dG as well. The purification of urine prior to LC-MS/MS is simple and uses commercially available cartridges. The availability of the stable isotope-labeled internal standards enables accurate measurements. The measured levels of R-cdA and S-cdA were lower than that of 8-OH-dG by approximately two-orders of magnitude. The *in vivo* levels of R-cdA and S-cdA in human and other mammalian tissues amount to approximately 0.01–0.1 lesions/ 10^6 DNA nucleosides [16]. Generally, the level of 8-OH-dG in DNA of mammalian cells vary from 1 to 10 lesions/ 10^6 DNA bases, depending on the methodology and laboratory [27]. The difference between the level of R-cdA or S-cdA and 8-OH-dG in mammalian cells is approximately two-orders of magnitude. This ratio is quite similar to that found in urine, indicating the excretion of these compounds into urine according to their levels in tissues. The range of the level of 8-OH-dG found in human urine in the present work is similar to those reported previously [6,28,29]. R-cdA and S-cdA may be removed by NER from DNA in oligodeoxynucleotides. The presence of these lesions in urine as free nucleosides points to hydrolysis of such oligodeoxynucleotides prior to excretion into urine, or, perhaps, within urine. In agreement with previous work on 8-OH-dG mentioned above, R-cdA and S-cdA in urine may not have a dietary origin. However, repair in the nucleotide pool cannot be excluded at present.

In conclusion, this study shows, for the first time, that R-cdA and S-cdA exist in urine as free nucleosides and can be positively identified and accurately quantified by LC-MS/MS with isotope-dilution. Unlike most of other oxidatively induced DNA lesions investigated thus far, R-cdA and S-cdA are repaired by NER. Thus, their presence in urine indicates the excretion of NER products into urine, and can be a measurable entity for the assessment of NER of oxidatively induced DNA damage. We propose that R-cdA and S-cdA may be alternative well-suited biomarkers for disease processes such as carcinogenesis.

Acknowledgments

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endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose. We thank the members of the Biochemical Science Division, NIST, who volunteered to donate urine samples.

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