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Original Contribution

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QUANTIFICATION OF 1,*N*⁶-ETHENO-2'-DEOXYADENOSINE IN HUMAN URINE
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Abstract—1,*N*⁶-Etheno-2'-deoxyadenosine (ϵ dA) is one of several promutagenic DNA modifications arising from cellular oxidative metabolism. It is believed that these background DNA lesions may contribute to various diseases, such as cancer. Therefore, human biomonitoring of ϵ dA in urine could be a potential marker for oxidative stress-related DNA damage. Existing methods for quantifying urinary ϵ dA use ³²P postlabeling. We have developed a nonradioactive, fast, and easier method based on column-switching liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) in the positive mode. Differences in column temperatures were used to influence analyte retention and sample focusing. With multiple reaction monitoring (MRM) mode the afforded limit of detection was about 0.7 pM when starting with 3 ml of urine. The urinary excretion rates of ϵ dA from 28 nonsmoking and 5 smoking men were 10.0–99.6 pmol/24 h, and did not correlate with body weight, age, or plasma vitamin C concentration. The 5 smokers excreted 30.5 ± 8.5 and the 28 nonsmokers excreted 38.6 ± 2.4 pmol ϵ dA per 24 h, $p = .37$ (mean \pm SEM). The demonstrated level of performance suggests the future applicability of this method to studies of cancer and other diseases related to oxidative stress in humans. © 2004 Elsevier Inc. All rights reserved.

Keywords—Column switching, Liquid chromatography tandem mass spectrometry, Atmospheric pressure chemical ionization, DNA adducts, Human urine, 1,*N*⁶-Etheno-2'-deoxyadenosine

INTRODUCTION

Carcinogenesis includes changes in the genome caused by exogenous or endogenous factors. Increasingly, it has been understood that there is a long list of endogenous sources of mutagens among which are those formed during normal metabolic processes, in particular the utilization of oxygen by cells, and these have attracted much interest. The utilization of oxygen is often termed a double-edged sword, or the oxygen paradox. While oxygen is needed for sustaining life in aerobic organisms, reactive oxygen intermediates are

also generated in large amounts. If not contained or sufficiently controlled the resulting condition is referred to as oxidative stress [1]. Oxidative stress generates free radicals that modify macromolecules. DNA is considered the most important target, because it cannot be replaced and because it contains the “blueprint” of the cell. DNA adducts have been hypothesized as initial events in the multistep process of cancer [2]. Oxidative stress also leads to lipid peroxidation, the secondary products of which can generate exocyclic DNA adducts, e.g., 1,*N*⁶-etheno-2'-deoxyadenosine (ϵ dA). This product has been studied after exposure to vinyl chloride [3] and ethyl carbamate [4], and is known to have a high mutagenic potential [5].

The analytical procedures for DNA adducts are very challenging. For example, measurements of the most frequently studied modification, 8-oxodeoxyguanosine (8-oxodG) have been confounded by artifacts and other methodological problems. It has taken the joint efforts of

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about 25 laboratories 4 years to resolve this to an acceptable degree [6–11]. Furthermore, methodologies that can be applied to human samples are even more difficult, as target organ tissue is difficult or impossible to get. Finally, adducts are in such low concentrations that very high sensitivity is required, and the presence of many interfering substances demands high specificity of analysis. These factors contribute to the scarcity of available data on DNA adducts arising from lipid peroxidation [12,13]. For the measurement of ϵ dA in DNA digests, complicated cleanup procedures followed by very sensitive techniques have been established (see Ref. [14]), and also a simpler procedure for analysis of etheno adducts in DNA derived from experimental animal tissue has been published [15]. Analysis of ϵ dA excreted with urine is a particularly challenging task demanding high specificity and high sensitivity for unequivocal identification and quantification. Urine is a complex matrix containing a mixture of multiple compounds in concentrations far greater than those of the modified nucleoside. Therefore, considerable prepurification is required before analysis. Measurements of etheno-DNA adducts in human urine have been conducted using extensive immunoaffinity cleanup with HPLC–fluorescence detection (HPLC-FD) and the concurrent use of a radiolabeled standard to estimate analyte losses [12,13]. The combination of high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) provides the advantage of efficient separation and high selectivity. In this article, we report that solid-phase extraction (SPE) for preconcentration combined with column switching HPLC-MS/MS can quantify urinary ϵ dA. This approach demonstrates progress in the analysis of urinary ϵ dA in terms of specific identification and the stringent sensitivity requirements needed for low-level analysis. Atmospheric pressure chemical ionization (APCI) has also been demonstrated to be a highly sensitive alternative to the more frequently used electrospray (ESI) technique. To our knowledge, only a few applications have used APCI to monitor modified bases and nucleosides.

MATERIAL AND METHODS

Materials

Glacial acetic acid was from Merck KGaA (Darmstadt, Germany). Methanol and 25% ammonium hydroxide were supplied by J. T. Baker (Deventer, Holland). Purified water was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). All other solvents were of analytical grade. Lithium acetate dihydrate was from Sigma–Aldrich Company Ltd. (Steinheim, Germany).

1,*N*⁶-Etheno-2'-deoxyadenosine (ϵ dA) and the stable isotope-labeled equivalent [¹⁵N₅] ϵ dA used as internal standard (ISTD) were both synthesized in our laboratory by the method of Green and Hartway [16] with subsequent HPLC purification. The isotopic purity was determined by LC-MS/MS. No unlabeled adduct was present in the labeled analog (~0.01%). Absorption spectra of ϵ dA and the internal standard were also identical. Concentrations were determined by measuring the UV absorbance at 260 nm ($\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$) [16].

Liquid chromatography

The design of the setup is shown in Fig. 1. The liquid handling system consisted of a Perkin Elmer Series 200 Micro HPLC system equipped with an autosampler, two pumps, and vacuum degasser (Perkin Elmer, Norwalk, CT, USA) and a single Hitachi L-6000 pump (Merck KGaA, Darmstadt, Germany).

The components for the two-dimensional separation consisted of column 1, a Luna HPLC column C18(2) (75 × 4.6 mm, 5 μ m) protected with a C18 (ODS) guard column (4.0 × 3.0 mm), and column 2, a Synergi Polar-RP analytical column (150 × 4.6 mm, 4 μ m). All columns were from Phenomenex (Torrance, CA, USA). Constant column temperatures were achieved using a Universal-Thermostat (MicroLab, Aarhus, Denmark) and a Comfort Heto Chill Master (Holm & Halby, Brøndby, Denmark), maintaining column 1 at 30 °C and column 2 at 1 °C. A multistep gradient program of 5 mM ammonium acetate (pH 5) and 100% methanol was prepared for wash, transfer, and elution from the columns. Column switching was achieved using a six-port two-position switching valve and a four-position two-stream selection Cheminert C5F valve, both fitted with micro-electric valve actuators (Valco Instruments, Houston, TX, USA).

Figure 1 and Table 1 show in detail the chromatographic conditions for the developed column-switching HPLC method. Briefly, the sample was loaded onto column 1 that eluted to waste. The six-port valve was programmed to switch and elute a “heart-cut” fraction containing ϵ dA onto column 2. Immediately after the “heart-cut” of the ϵ dA fraction, column 1 was washed with a steep gradient to 100% methanol to elute the more hydrophobic impurities. Finally, after switching the flow back to column 2, a gradient of solvent A and B was applied, eluting ϵ dA to the detector. The pumps were programmed to return to the initial conditions after 49 min, thus preparing the system for the next injection. The Hitachi pump (pump 2) was connected to the two-stream valve, delivering a constant flow of 550 μ l/min of 95% 5 mM ammonium acetate in methanol (v/v) which was used for reequilibration of the columns.

The autosampler was programmed for 75 μ l injection volumes and dual postinjection flushes of the

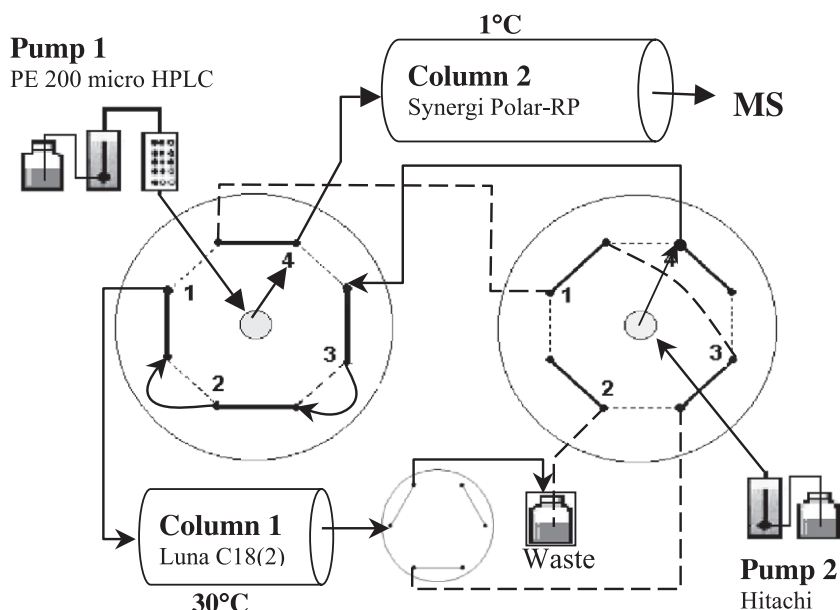


Fig. 1. Schematic view of the analytical setup. Valve position 4 is shown: separation of ϵ dA on column 2 (Synergi Polar-RP) and concurrent reequilibration of column 1 (Luna C18(2)). Solid lines represent active connections and the arrows show the direction of the flow while dotted lines are passive connections in the shown configuration.

syringe with solvent A to prevent carryover from the needle.

Mass spectrometry

The mass spectrometer used was an API 3000 triple quadrupole (Sciex, Toronto, Canada) equipped with an APCI source (Heated Nebulizer) in the positive mode. Nitrogen was used as nebulizer, auxiliary, and collision gas. The corona discharge current was set at 2 μ A and the vaporizer temperature was 410°C. Interface set-

tings and gas pressures were manually optimized. Detection was performed in MRM mode using the characteristic fragmentation from the protonated molecular ion to the protonated free base ion, $[M+H]^+ \rightarrow [BH_2]^+$, corresponding to the loss of deoxyribose. The transitions m/z 276 \rightarrow 160 and m/z 281 \rightarrow 165 were used for quantification of ϵ dA and the stable isotope-labeled equivalent ISTD, with dwell times set at 1100 and 500 ms, respectively. "High-resolution" modes corresponding to a peak width of 0.5 amu at half

Table 1. Gradient Elution Program and Schedule of Timed Events

Step	Time (min)	Flow (μ l/min)	Mobile phase ^a		Valve	Comments
			(% A)	(% B)		
0	0	800	98	2	Switch (position 1)	Sample load on column 1 and column 2 reequilibration
1	10	800	90	10		
2	13	600	89	11	Switch (position 2)	Heart-cut transfer at time 16.1–17.8 min
3	18	600	87.5	12.5	Switch (position 3)	Column 1 cleanup
4	20	1250	0	100		
5	21	700	92	8	Switch (position 4)	Separate/detect analyte of interest on column 2 and column 1 reequilibration
6	43	700	31	69		
7	45	700	95	5		
8	49	700	95	5	Switch (position 1)	System preparing for next injection
9	49	800	98	2		
10	54	800	98	2		

^a Mobile phase content. Solvent A: 5 mM ammonium acetate (pH 5), solvent B: 100% methanol.

the maximum peak height (0.5 FWHM) were used in both first (Q1) and third (Q3) quadrupole.

Sample handling

Aliquots of 24-h urine samples were obtained from 28 nonsmoking and 5 smoking healthy men (aged 21–56 years) consuming unrestricted diets and stored at -20°C prior to analysis. The urine was collected as part of another study estimating oxidative stress and plasma vitamin C approved by the local ethics committee of Copenhagen.

The frozen urine was thawed and 3 ml aliquots was diluted 1:1 in 5 mM ammonium acetate (pH 5). After being heated to 37°C for 5 min, the samples were centrifuged at 4000 rpm for 15 min. Internal standard (150 fmol) was added to all samples, which were loaded onto a Oasis HLB cartridge (Waters, Milford, MA, USA). The SPE column (6 ml, 200 mg) had been preconditioned with 3×1 ml of methanol and 3×1 ml of H_2O . Sample application was performed slowly under gravity flow. The column was washed with $2 \times$

1000 μl of water, followed by 2×750 μl of 10% methanol (aq) and subsequently dried under vacuum. The analyte was eluted with 2×800 μl of 60% methanol (aq), and the eluate was vacuum-centrifuged to dryness. The residue was redissolved in 250 μl of 0.5 M lithium acetate buffer (pH 6.4), and sonicated. An aliquot (75 μl) of the solution was injected into the HPLC system.

Method validation

The linearity and range of the calibration curve were established in a single series with nine levels of standards in duplicate. The calibration curve is expressed as the ratio of the area of the analyte peak to the area of the ISTD peak as a function of analyte concentration.

Reproducibility and recovery studies were performed to compile method performance. *Within-day* and *between-day* variances were estimated from three series of five human urine samples in duplicate. The urine samples were chosen to cover a broad diuresis range. Precision is expressed as the percentage of relative standard deviation (RSD, %).

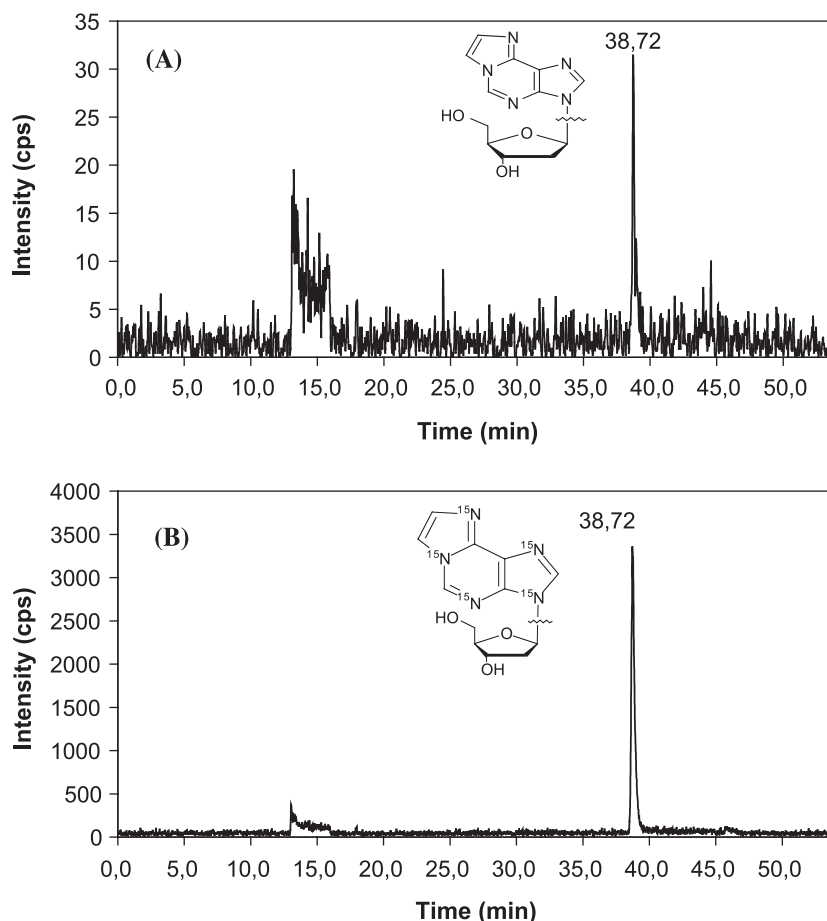


Fig. 2. LC/APCI-MS/MS chromatograms of a standard sample, containing 8.0 fmol/ml ϵdA and 0.6 nmol/ml $[^{15}\text{N}_5]\epsilon\text{dA}$ obtained by MRM at (A) m/z 276 \rightarrow 160 for ϵdA and (B) m/z 281 \rightarrow 165 for the internal standard $[^{15}\text{N}_5]\epsilon\text{dA}$. Retention time is 38.7 min. This figure illustrates a sensitivity of about 8 pmol/l when 75 μl is injected.

Recovery from SPE was determined by duplicate analysis of synthetic samples (water) and two urine samples from the reproducibility study. The samples were spiked at two concentrations (6.25 and 13.3 pM) of ϵ dA and extracted as a normal sample in parallel with the reproducibility study. ISTD was added to correct response variation originating from the ionization process. However, the addition of ISTD to the spiked samples was first executed at the redissolving step and will, in that respect, *not* compensate for any losses up to this point in the recovery study. The urine samples used were from the first validation set and their mean concentrations were used as blank estimations. Recovery is expressed as percentage recovery: $((\text{mean observed concentration} - \text{blank concentration}) / (\text{spiked concentration})) \times 100\%$.

RESULTS

Basal levels of ϵ dA in human urine were established by the method presented using a reasonably small urine sample volume of 3 ml. The experimental setup is depicted in Fig. 1, and the gradient elution in Table 1.

The detection limit based on a signal:noise ratio of 3:1 was about 8 pM in the final sample concentrate, corresponding to 600 amol injected. This afforded a concentration detection limit of 0.7 pmol/l urine. The

sensitivity of the assay in MRM mode is demonstrated in Fig. 2 (standard sample). The method was linear over the concentration range 8 pM to 0.3 μ M standard ϵ dA using 50 nM of ISTD. Figure 3 shows a working calibration curve. The overall recovery was between 86 and 104% and within the set requirements of $100 \pm 15\%$. The MRM profiles obtained from one of the purified urine samples are displayed in Fig. 4, which shows the ϵ dA trace (A) and the corresponding ISTD (B). As blank urines cannot be produced, suppression studies are not straightforward. The ISTD area of the standards and the urine samples were fairly constant, indicating minimal ion suppression from the sample matrix. Neither background noise nor basis level differed considerably between sample types, i.e., high urine volume to low urine volumes representing different levels of urine concentration. These facts combined with the high recovery cause us to anticipate the limit of detection for a real sample to be in the same range as the standards, which was also our experience from running urine samples.

The analytical intraday and interday precisions are listed in Table 2. The coefficients of variation of the reproducibility study were generally below 10% RSD, with the exception of urine B. However, this urine was analyzed in the first set and we believe that it can be attributed to some unidentified error. Because of the

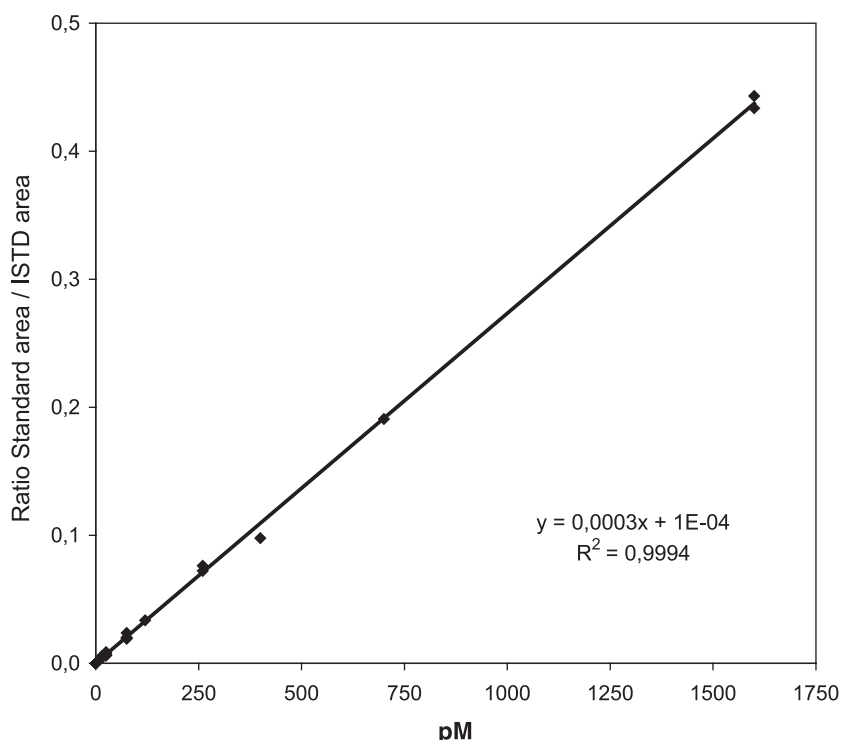


Fig. 3. Calibration curve obtained by plotting the ratio of area of the analyte peak to area of the internal standard peak as a function of analyte concentration.

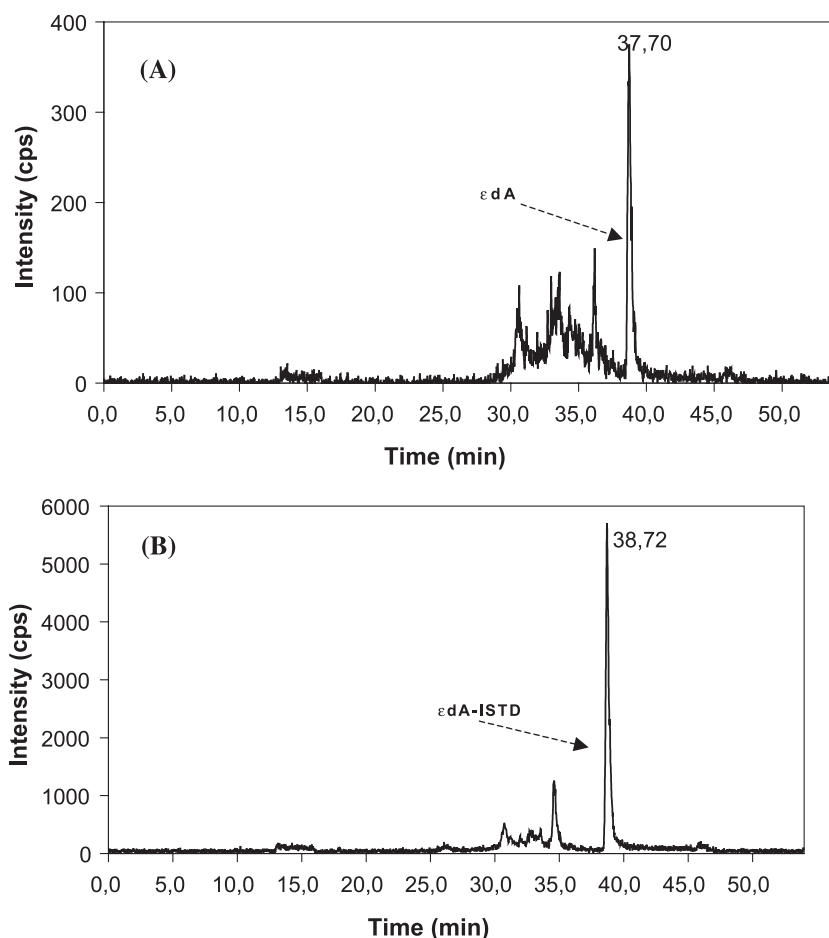


Fig. 4. LC/APCI-MS/MS analysis of ϵ dA in human urine eluting after 38.7 min. The traces are the MRM profiles of (A) ϵ dA and (B) ϵ dA-ISTD. To urine [$^{15}\text{N}_5$] ϵ dA was added, preconcentrated with an Oasis HLB SPE column, purified by a column-switching system, and analyzed under MRM mode as described under Material and Methods. The original volume of the sample was 3 ml, and the concentration factor was 12. The result corresponds to an ϵ dA concentration of 13.3 pM in the urine sample.

inexplicable result for urine B, it was decided to repeat the reproducibility study with a second validation set (urines F–J). Acceptable variances (RSD <10%) were found for all urines. Blank solvent samples analyzed within the batch verified no carryover from neither urine samples nor the highest standard sample.

The ϵ dA excretion rates ranged from 10 to 99.6 pmol per 24 h in normal subjects, with urine concentrations ranging from 2.3 to 105 pmol/l urine. Furthermore, in the sample population the excretion was close to normal distribution (Fig. 5), with no apparent polymorphisms. The method presented provides sensitivity that is main-

Table 2. Validation results

Sample	Diuresis (ml/24 h)	ϵ dA concentration (pmol/l)	N	Within-day precision		Between-day precision	
				SD _w (pmol/l)	RSD _w (%)	SD _b (pmol/l)	RSD _b (%)
Urine A	745	39.5	6	1.0	2.4	2.78	7.0
Urine B	2471	9.7	10	1.7	17.8	2.55	26.4
Urine C	4346	15.6	9	0.9	5.8	1.13	7.2
Urine D	1693	35.5	6	1.9	5.3	3.33	9.4
Urine E	990	12.1	6	0.6	4.7	1.24	10.2
Urine F	874	30.3	6	1.0	3.3	0.8	2.7
Urine G	2343	12.7	6	0.7	5.4	0.0	0.0
Urine H	1018	38.6	6	0.8	2.2	1.0	2.7
Urine I	5254	2.3	6	0.2	6.5	0.2	6.7
Urine J	3075	14.5	6	0.2	1.6	0.4	2.4

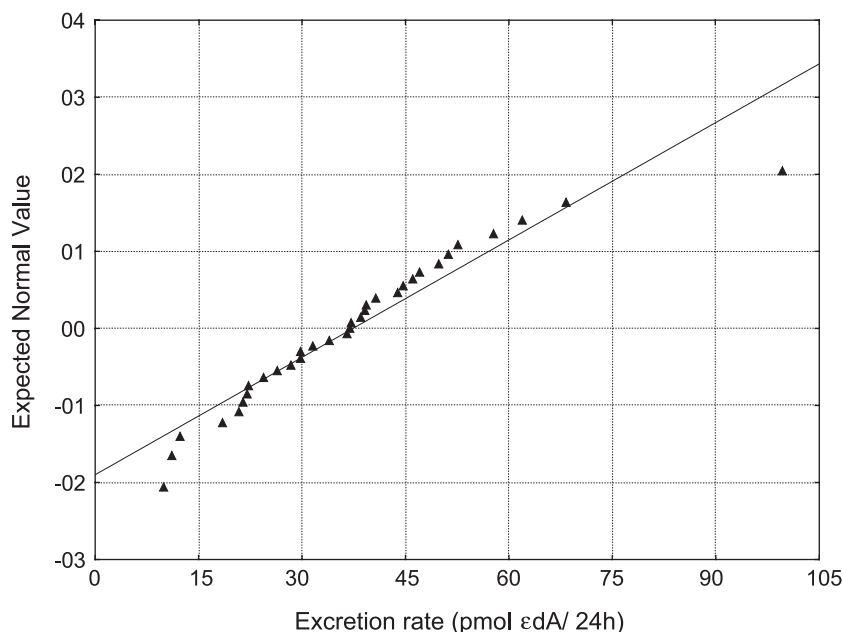


Fig. 5. Plot of excretion rates of ϵ dA in 24-h urines from 38 male volunteers. The straight line gives the expected normal distribution of the observed excretion values and the points give each of the observed values transformed to the normal distribution value (Y-axis). If the observed values followed the normal distribution all points would be on the straight line. The points do not deviate significantly from the expected values on the straight line, $p > .05$.

tained after a minimum of 80 injections. There were no significant differences between smokers and non-smokers. The excretion rate in smokers was nonsignificantly smaller than that in nonsmokers: smokers excreted 30.5 ± 8.5 pmol ϵ dA and the 28 nonsmokers excreted 38.6 ± 2.4 pmol ϵ dA per 24 h (means \pm SEM, $p = .37$).

There was no significant correlation of ϵ dA with age ($r = 0.02$, $p > .05$), body weight ($r = -0.01$, $p > .05$), or plasma vitamin C concentration ($r = 0.11$, $p > .05$).

DISCUSSION

The purpose of this study was to develop a specific and sensitive method to establish basal levels of the ϵ dA in human urine. The methodology consists of a simple prepurification step with SPE followed by column-switching HPLC and quantification by isotope dilution tandem mass spectrometry. In practical terms the method requires extractions of 3 ml of urine and a HPLC-MS/MS runtime of about 1 hour. This method provides sufficient specificity and sensitivity and is a clear improvement compared with the previous method based on immunoprecipitation with HPLC-FD detection [13], a method that requires extensive sample preparation. Also, the analysis presented is based on instrumentation and substances that are all commercially available. The ISTD is not commercially available; however, it can be synthesized from commercially available starting substances and established protocols.

The critical issue for the ϵ dA assay in urine is avoiding matrix effects arising from the required use of preconcentration. Enrichment of ϵ dA was performed on a polymeric Oasis HLB SPE column, which gave a cleaner sample than a normal C18 SPE column (data not shown). Varying the washing and elution solvents optimized the extraction procedure. Despite a substantial reduction in sample complexity, several interfering peaks could still be found in the urine preparations. This problem was further confounded by a high level of variability of potential interferences in different samples. Therefore, an additional cleanup was required before LC-MS/MS analysis.

A coupled HPLC column approach was clearly attractive for isolating a single analyte or small groups of components from a complex mixture like urine. Thus the first column functions to enrich analytes and eliminate interfering matrix compounds. A small fraction of the effluent from column 1 was selectively transferred in forward mode (heart-cutting) to column 2. The trapped fraction was then separated on the second column and eluted to the mass spectrometer for analysis. One additional advantage of this system was the utility of including a quick washing step (e.g., position 3 in Table 1) that minimizes carryover.

To maximize mass spectral response, elution of the trapped compound into the mass spectrometer should be accomplished with the smallest possible solvent volume. When the columns were operated at room temperature,

band broadening and tailing were observed, leading to insufficient retention on the second column. Several approaches were evaluated to refocus the transferred zone at the top of the second column. We tested remixing of the “heart-cut” fraction with eluant A to reduce solvent strength before entering the second column but this was not successful. We discovered that retention of nucleosides on reversed-phase columns dramatically increases at lower temperatures. Therefore, cold temperature (1°C) trapping was employed for efficient retention and maximum sensitivity. Further lowering of the temperature would, however, result in too high backpressure, as the aqueous mobile phase would freeze.

Matrix effects and ion suppression are always an issue of concern in quantitative methods based on HPLC-MS/MS. APCI was chosen as the ionization mode in MS detection as APCI ion intensities are not as strongly affected by co-eluting matrix constituents as in electrospray ionization [17,18]. Preliminary work revealed suppression of the MS signal for the very concentrated urines when using a Zorbax Eclipse XDB-C18 as the second column. The same phenomenon did not occur when a Synergi Polar-RP column (an ether-linked phenyl phase with polar endcapping) was used. One explanation could be an insufficient difference in retention and elution characteristics between the two reversed-phase columns, the Luna C18(2) and the Zorbax Eclipse XDB-C18. The “high-resolution mode” (0.5 FWHM) of the Sciex API 3000 was used. Although it lowered the signal intensity compared with “unit resolution mode” (0.75 FWHM), no decrease in S/N ratio was observed owing to the significant decrease in chemical noise.

Furthermore, the possibility of using stable isotope-labeled ISTD in mass spectrometry (isotope dilution) increases the reliability of the method. In the coupled-column configuration the “heart-cut” is achieved by time-based valve switching, assuming that the analyte retention time and peak width are constant. However, during a run the retention time can drift as a consequence of column degradation or pressure buildup. This means that the analyte can move either partially or totally out of the time window and hamper the quantification. Because the analyte and ISTD should behave chromatographically identical, the internal standard can be used to deal with this “drifting peak” phenomenon, as the calibration is based on area ratios. Furthermore, isotope-labeled ISTD can be used to compensate for losses during sample preparation and to compensate for variation and suppression of ionization. The use of [M+5] ϵ dA prevents any interfering contribution from the isotopic analog from the natural analyte.

Whereas our method measures the excretion of the lipid peroxidation modification of the nucleoside, it has recently been reported that urinary excretion of the

corresponding modified base (1,*N*⁶-ethenoadenine, ϵ A) can be quantified with stable isotope dilution capillary gas chromatography–negative ion chemical ionization mass spectrometry (GC/NICI-MS) [19,20]. With the GC-MS method the quantification limit is about 31 pM ϵ A compared with 0.7 pM ϵ dA with our HPLC-MS/MS method. Cheng *et al.* report urinary excretion rates of ϵ A of about 1 nmol/24 h (assuming 2 l urinary output per 24 h), whereas we report that ϵ dA is excreted in amounts of about 30 pmol/24 h, a ratio of modified nucleoside to modified base of 1:30. Evidently, the measurement of the modified nucleoside requires higher sensitivity; in our case this was done by using a larger urine volume for pre-concentration, 3 mL versus 0.1 mL in Cheng *et al.*'s method. Earlier we studied excretion of the 8-oxo modified base and nucleoside of guanine into urine and found that the ratio of modified nucleoside to modified base is about 1:10 [21]. The origin of the modified base/nucleoside is not fully established. We hypothesize that the ratio of the nucleoside/base modification represents the ratio between DNA repair by the glycosylase OGG1 and nucleotide excision repair.

The proximity to normal distribution for the excretion of ϵ dA and the excretion rate is in accordance with the pilot study done by Hanaoka *et al.* [13]. The excretion rates in smokers and nonsmokers have not previously been investigated. In malignant and nonmalignant lung tissue, DNA levels of ϵ dA were about 50% lower in smokers, not reaching statistical significance [22], and together these findings strongly point to no increase in this type of oxidative stress in smokers. This is at clear variance with the well-established increase in 8-oxodG in smokers [23]. The reason for this discrepancy is obscure; however, the present methodology will make it possible to gain further insight.

There was no significant correlation with age ($r = 0.02$, $p > .05$), body weight ($r = -0.01$, $p > .05$), or plasma vitamin C concentration ($r = 0.11$, $p > .05$). In their study, Chen and Chiu [19] found that the excretion of etheno modified base ϵ A was lower in smokers compared with nonsmokers, although not statistically significant. Interestingly they report levels in urine that are about 30 times higher than those we report on the corresponding modified nucleoside. This observation of a differential effect on the modified base versus the modified nucleoside, if it can be substantiated, could give rise to the hypothesis that smoking has a differential influence on different DNA repair pathways. The observation of such a differential effect of smoking on DNA damage is intriguing; however, it also speaks against an origin of the modified bases/nucleosides in dead cells or cell turnover. Also, it emphasizes the point of view put forward by Dizdaroglu *et al.* [24] that

measurement of DNA damage should not be limited to a single lesion.

The known factors that determine ϵ dA tissue levels and excretion are limited. A pilot study by Hanaoka et al. found an association between salt excretion, intake of ω 6-polyunsaturated fatty acids, and ϵ dA excretion in urine [13]. Very high levels of etheno DNA adducts were found in females after high dietary intake of ω 6-polyunsaturated fatty acids [25]. Also there are indications that ϵ dA is involved in human colon carcinogenesis [26].

The biological variations for ϵ dA urinary excretion range about 10-fold, indicating a large variation in oxidative stress from lipid peroxidation. In the limited population we examined, as part of the proficiency testing of this method, age and body weight were not determining factors for urinary ϵ dA excretion. The finding that smokers appeared to excrete lower amounts of ϵ dA needs to be confirmed by a larger study, as does the differential effect on different lesions. It could be interesting to address whether the gender-specific effect also applies to the excretion rates of ϵ dA in urine, and if urinary ϵ dA excretion rate correlates with other markers of lipid peroxidation. Various etiological factors that influence lipid peroxidation need to be evaluated with the monitoring of specific repair products such as ϵ dA.

In the cell, modified DNA is repaired by specific systems liberating bases and deoxynucleosides into the urine [27,28]. The finding of background levels of ϵ dA in tissues and body fluids from unexposed humans and animals [15,29] suggests an endogenous origin, which makes ϵ dA a promising tool as biomarker. Monitoring of etheno adducts, as excreted repair products in urine, could therefore be important in the noninvasive quantitative assessment of endogenous related DNA damage in clinical and public health studies. In urine the amount of excreted adduct per unit time represents an average measure of the whole-body burden of oxidative stress under steady-state conditions independent of DNA repair [30].

We conclude that ϵ dA urinary excretion varies about 10-fold in normal subjects; it is not increased in smokers and there is no correlation to anthropological measures. The LC/APCI-MS/MS method developed is useful for establishing determining factors and for investigations of the relationship to disease, in particular cancer development, and do not require radioactive standards. The method is a sensitive and reliable tool suitable for the determination of the urinary excretion of ϵ dA, even at the low levels, in humans. The beneficial aspects of the method include: (1) the use of SPE to enrich and clean up ϵ dA from extraneous compounds, (2) reduction of signal suppression effects from matrix

components by the use of column switching and APCI-MS, (3) peak focusing by low-temperature reversed-phase chromatography, (4) the high sensitivity of triple quadrupole tandem mass spectrometry in the MRM mode, and (5) an isotopically labeled ISTD for robust method performance.

The method is validated with respect to linearity, precision, and accuracy. The analysis of real samples was demonstrated and this assay may be useful for large-scale studies. This approach can easily be modified for tissue measurements and can be performed on urinary samples stored in biobanks.

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