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Quantification of Urinary Etheno-DNA Adducts by Column-Switching LC/APCI-MS/MS

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Lipid peroxidation induced etheno-DNA adducts are promutagenic and have been suggested to play a causal role in the development of human cancers. Therefore, human biomonitoring of etheno-DNA adducts in urine has been suggested as a potential marker for oxidative stress-related DNA damage. For quantitative determination, a column-switching LC/APCI-MS/MS method was developed for simultaneous analysis of εAde, εdC, and εdA in human urine. Quantitative validation parameters (precision, within-day repeatability, and between-day reproducibility) yielded satisfactory results below 10%. Limit of quantification for εAde, εdC, and εdA was 5.3 fmol, 7.5 fmol, and 1.3 fmol on column, respectively. Mean urinary excretion rates of a six healthy volunteers were 45.8 pmol εAde/24 h, 96.8 pmol εdC/24 h, and 18.1 pmol εdA/24 h. The demonstrated levels of performance suggest a future applicability of this method to studies of cancer and other diseases related to oxidative stress in humans. To our knowledge, this is the first method described that allows simultaneous determination of εAde, εdC, and εdA in human urine samples. (J Am Soc Mass Spectrom 2006, 17, 605–610)
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Etheno modified DNA bases, such as εdA, originating from known human carcinogens vinyl chloride [1] or ethyl carbamate [2], are also generated endogenously by reactions of DNA with products derived from lipid peroxidation and oxidative stress [3]. These DNA adducts have miscoding potential and specific repair pathways supporting the hypothesis that ε-DNA adducts play a causal relationship in carcinogenesis [4]. Furthermore, several studies have found elevated ε-DNA adduct levels in disorders known as risk factors for cancers [5, 6]. Therefore ε-DNA adducts have been proposed as biomarkers for human cancers associated with certain lifestyles or chronic inflammations [4]. Upon DNA repair, ε-DNA adducts are excreted in the urine and could, in that respect, be used to investigate the body burden of DNA damage.

The analytical procedures for urinary DNA adducts are challenging indeed. The presence of many interfering substances in concentrations far greater than those of the DNA adducts demands high selectivity and low limit of quantitation for unequivocal identification and quantification. Methods using high-performance liquid chromatography (HPLC) with fluorescence detection have been used for εdA determination [4, 7] after immunoaffinity clean-up and a very tedious sample preparation. Furthermore, the internal standard was measured separately. Several etheno-DNA adducts have been determined in urine from healthy humans using gas chromatography-electron capture negative chemical ionization mass spectrometry (GC/EC-NCI/MS) [8–11]. Gas chromatography offers the advantage of greater peak resolution compared to conventional LC. However, the requirement for derivatization before analysis is a major limitation. Recently, LC-MS have been employed in determination of etheno adducts from human urine using electrospray ionization (ESI) [12–14]. Atmospheric pressure chemical ionization (APCI) has been used for analysis of εdA and 1,N^2-εdG in vitro [15] and of εdA in human urine [16].

In this paper, we report that solid-phase extraction (SPE) for preconcentration combined with HPLC column switching tandem mass spectrometry can quantify urinary etheno-DNA modifications. This approach demonstrates progress in the simultaneous analysis of urinary εAde, εdC, and εdA in terms of specific identification and the stringent sensitivity requirements needed for low-level analysis.

Methods

Materials

Glacial acetic acid was from Merck KgaA (Darmstadt, Germany) while methanol and 25% aqueous ammonia were supplied from J. T. Baker (Deventer, The Nether-

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lands). Purified water was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA). All other solvents were of analytical grade. Lithium acetate dihydrate was from Sigma-Aldrich Co. Ltd. (Steinheim, Germany).

3,N^4-etheno-2′-deoxycytidine (εdC), 1,N^6-ethenoadenine (εAde), 1,N^6-etheno-2′-deoxyadenosine (εdA), and the stable isotope-labeled equivalents [$^{15}N_3$]-εdC, [$^{15}N_5$]-εAde, and [$^{15}N_5$]-εdA used as internal standards (ISTD), were all synthesized in our laboratory by the method of Green and Hathway [17] 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 the standard and the internal standard were also identical. Concentrations were determined by measuring the UV-absorbance in water (εdC; $\varepsilon_{272\ nm}$ = 12,000 $M^{-1}cm^{-1}$ [18], εAde and εdA; $\varepsilon_{260\ nm}$ = 10,300 $M^{-1}cm^{-1}$ [17]).

Liquid Chromatography

The set-up is shown in Figure 1. The liquid handling system consisted of two pumps. Pump 1: an Agilent 1100 binary HPLC pump (Agilent, Palo Alto, CA) connected to a PE autosampler and Pump 2: a single PE 200micro pump (Perkin Elmer, Norwalk, CT).

The components for the two-dimensional separation consisted of column 1 (C1), a Luna HPLC column C_{18}(2) (150 × 4.6 mm, 5 μm) protected with a C18 (ODS) guard column (4.0 × 3.0 mm); column 2 (C2) was a Synergi Polar-RP column (75 × 4.6 mm, 4 μm); column 3 (C3) was a Synergi Polar-RP column (150 × 4.6 mm, 4 μm). All columns were from Phenomenex (Torrance, CA). Constant column temperatures were achieved using an Agilent 1100 Column Oven (Agilent, Palo Alto, CA) and a Comfort Heto Chill Master (Holm and Halby, Brøndby, Denmark), maintaining C1 at 40 °C and C2 and C3 at 1 °C. Pump 1 was connected to eluent A:5 mM ammonium acetate (pH 5), and eluent B:100% methanol; Pump 2 was connected to eluent C:92.5% 5 mM ammonium acetate in methanol (vol/vol). Column switching was achieved using three Cheminert valves (Valco International, Schenkon, Switzerland): Valve 1 (8-port 4-position two-stream selection C5F), Valve 2 (8-port 4-position C5F), and Valve 3 (6-port 2-position C2), all fitted with micro-electric valve actuators.

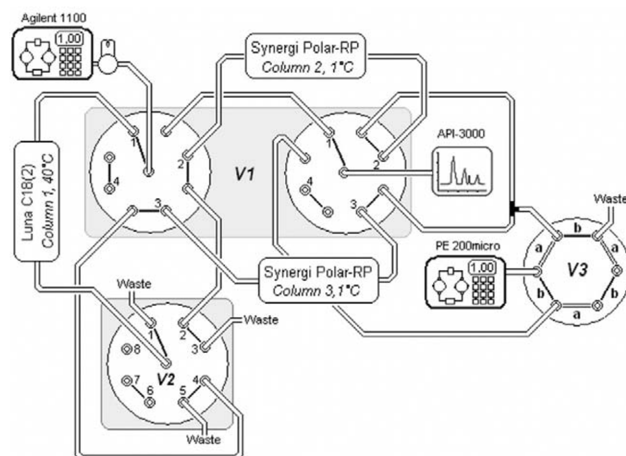

Figure 1. Schematic view of the analytical set-up.

Figure 1 (and Table in Supplementary Material section which can be found in the electronic version of this article.) show in detail the chromatographic conditions for the developed method. The experiment was conducted according to the following scheme: first, initial separation on the primary column (C1) by Pump 1 and re-equilibration of C2 + C3 by Pump 2; second, loading a heart-cut fraction containing εAde from C1 onto C2; third, gradient elution of C2 into the MS by Pump 1 and continued separation on C1 by Pump 2; fourth, sustained gradient elution of C2 into the MS and at the same time loading a heart-cut fraction containing εdC + εdA from C1 onto C3; fifth, gradient elution of C3 into the MS by Pump 1 and re-equilibration of C2 by Pump 2; and sixth, before injecting the next sample, washing + re-equilibration of C1 by Pump 1. Three valves accomplished the switching operations. Valve 1 was directing the gradient flow from Pump 1 through the columns. Valve 2 was programmed to multiple switches between waste and loading of the "heart-cut" fractions. Valve 3 was directing the isocratic flow from Pump 2 through the columns.

Mass Spectrometry

The mass spectrometer used was an API 3000 triple quadrupole (Sciex, Toronto, Canada) equipped with an APCI ion source (Heated Nebulizer). The vaporizer temperature was 500 °C. Nitrogen was used as nebulizer-, auxiliary-, and collision gas. Interface settings and gas pressures were manually optimized at the LC conditions prevailing when the analyte in question elutes into the MS. Detections were performed in MRM mode. "High-resolution mode" corresponding to a peak width of 0.5 u at half the maximum peak height (0.5 FWHM) was used in both the first quadrupole (Q1) and the third quadrupole (Q3).

Sample Handling

Aliquots of 24-h urine samples were obtained from 6 nonsmoking healthy men (age 21–48 y) consuming unrestricted diets, and stored at −20°C before analysis. The urine was collected as part of another study estimating oxidative stress from dietary intervention and was approved by the local ethics committee of Copenhagen (no. 01-099102).

Solid-phase extraction was performed as described previously with minor modifications [16]. The frozen urine was thawed and 2 mL aliquots were diluted 1:2 in 5 mM ammonium acetate (pH 5) and heated to 37 °C for 5 min. ISTD were added to all samples, which were loaded onto an Oasis HLB cartridge (Waters, Milford,

MA). 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 3.5 mL of water, followed by 2 × 750 μL of 10% methanol (aq) and subsequently dried under vacuum. The analytes were eluted with 2 × 950 μL of 75% methanol (aq), and the eluate was vacuum-centrifuged to dryness. The residue was redissolved in 160 μL of 0.5 M lithium acetate buffer (pH 6.4), and sonicated. This was done to redissolve precipitate-trapped adducts. An aliquot (75 μL) of the solution was injected into the HPLC system and dual post-injection flushes of the syringe with eluent A were performed to prevent carryover from the needle.

Validation Procedures

A validation program was executed according to the FDA guidelines [19]. The challenge in designing the validation protocol relates to the fact that no human urine can be found without detectable endogenous levels of the studied etheno adducts.

The linearity and range of the calibration curves were established in a single series over the working concentration range with nine levels of standards in duplicates. The calibration curves are expressed as the ratio between the areas of the analyte peak/the ISTD peak as a function of the analyte concentration. Two calibration curves that underwent the complete SPE procedure were simultaneously prepared in water and in urine. These were compared with a curve of directly analyzed standards.

Reproducibility and recovery studies were performed to compile method performance. The *within-day* and *between-day* variances were estimated from three series of six human urine samples in triplicate. The urine samples were chosen to cover a broad diuresis range (681–4500 mL/24 h). Precision is expressed as the percentage of relative standard deviation (RSD, %). To assess the effect of repeated freeze-thaw cycles on the analytes, injections were performed on fresh extracted aliquots. Then, the urine samples were refrozen and reanalyzed twice over the next three weeks as part of the reproducibility study. 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 levels (εAde: 100 and 400 fmol; εdC: 100 and 400 fmol, and εdA: 15 and 40 fmol) and extracted as a normal sample. However, in this particular experiment, the addition of internal standards to the spiked samples were first executed at the redissolvation step and will, in that respect, *not* compensate for any losses up till this point in the recovery study. The ISTD will, however, correct response variation originating from the ionization process. The unspiked urine samples were included in the set and their mean concentrations were used as blank estimations. Recovery is expressed as the percentage of recovery [({Mean observed concentration − blank concentration}/{spiked concentration}) × 100%].

Statistical Analysis

All results are reported as the means ± standard deviation (SD). STATISTICA version 6.0 (StatSoft, Inc., Tulsa, OK) was used for these analyses.

Results and Discussion

The evidence accumulated to date suggests that etheno adducts may be useful noninvasive biomarkers for lipid peroxidation related DNA damage. Urine is a complex matrix containing a mixture of multiple compounds in concentrations far greater than those of the modified adducts. As a consequence, interfering substances often hinder the direct determination of the analytes. The assays available, however, have required either extensive cleanup steps or very specific immunoaffinity columns. The methodology presented here consisted of a simple enrichment step with SPE followed by column switching HPLC and quantification by isotope dilution tandem mass spectrometry.

The strategy of column-switching is clearly attractive for the analysis of complex mixtures. It provides the optimum efficiency and selectivity for separation of compounds of interest, eliminating several sample preparation steps necessary for single column approach. A critical parameter is the selection and transference of the heart-cut fraction from the primary column to the secondary column. Selection is achieved by time-based valve switching assuming constant retention times. However, during a run the retention time can drift as a consequence of column degradation or pressure build-up. One way to overcome this problem is to use an isotope-labeled internal standard, since the analyte and the internal standard should behave in a chromatographically identical way, the internal standard can be used to deal with this "drifting peak" phenomenon because the calibration was 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. Because elution of the trapped compounds into the MS should be accomplished with the smallest possible solvent volume to maximize mass spectral response, the heart-cut fraction needs to be focused in a narrow band at the top of the secondary column. Therefore, cold temperature (1 °C) focusing on the two secondary columns (C2 and C3) was employed.

In complex samples, it is inevitable that some of the interfering compounds will be transferred together with the analytes of interest. By using two secondary columns, this problem was minimized, which allows final separation and subsequent MS detection of the εAde containing fraction alone, while initial separation of εdC and εdA from interfering peaks continues. This strategy has the advantage of optimizing valuable MS

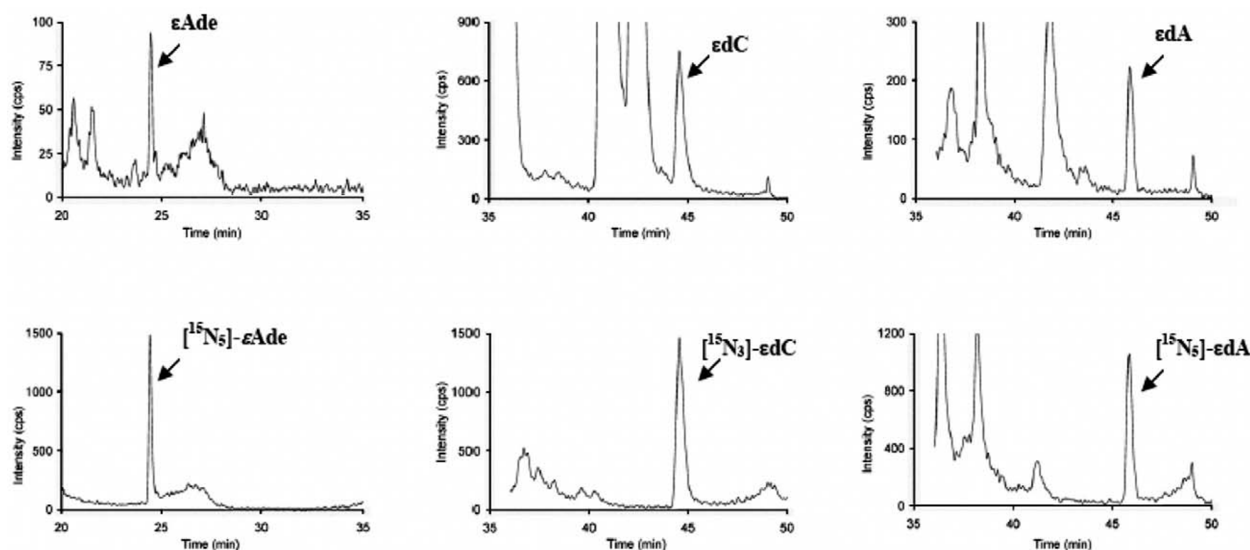

Figure 2. LC/APCI-MS/MS chromatograms of a real sample. Each chromatogram is divided into two time windows. Time window 1 shows the 158/117 (ISTD: 163/121) transitions corresponding to εAde. Time window 2 shows the 252/136 (ISTD: 255/139) and 276/160 (ISTD: 281/165) transitions corresponding to εdC and εdA, respectively. The urine sample contained 32.0 pM εAde, 79.0 pM εdC, and 8.9 pM εdA.

time compared to one secondary column approach. Furthermore, it allows a steeper gradient on each of the secondary columns and, consequently, sharper peaks and better ionization attributable to the higher content of organics in the final effluent composition.

To achieve high selectivity and S/N ratio, detection was performed in MRM mode. The overall signal sensitivity of εAde was highest in negative mode. Furthermore, when analyzing real samples, the background noise was also lower in negative mode, meaning that the sensitivity in terms of signal-to-noise was much higher in negative mode than in positive mode. For εdC and εdA, best sensitivity was obtained in positive mode. Due to the low intensity of other fragments from the collision-induced dissociation, and considering the low concentrations found in urine samples, no qualifier ions for analyte verification could be included in the final method.

During method development, two calibration curves were simultaneously prepared; one in water and one in urine to examine a possible influence of the urine matrix on slope and linearity. These were compared with a curve of directly analyzed standards. Urine matrix and SPE procedure did not influence slope and linearity of the calibration curve (data not shown; however, calculated accuracy of the concentrations were within ±5%). Therefore, calibration graphs were obtained directly by analyzing water standards without sample processing. Linearity was assessed using nine standard solutions in duplicates. Linear relationships were obtained for all three adducts in the following concentration range investigated: (LOQ-40.6 nM for εAde, LOQ-4.0 nM for εdC, and LOQ-4.0 nM for εdA) when a weighting factor of $1/x^2$ was used for all compounds. LOQ based on an S/N of 5:1 was about 70 pM, 100 pM, and 17 pM in the final sample concentrate for εAde, εdC, and εdA, respectively. This corresponded to urine concentrations of 5.6 pM, 8.0 pM, and 1.4 pM for εAde, εdC, and εdA, respectively. The LOQ for εAde was five times lower than reported by Chen and Chang [12], 10 times lower for εdC [11] and εdA [7], analyzing comparable urine volumes. Figure 2 presents representative chromatograms obtained for a urine sample.

The analytical intra-day and inter-day precisions are shown in Table 1. The coefficients of variation of the reproducibility study were generally below 10% RSD. These accuracy and precision determinations are comparable to those previously reported. Blank solvent samples analyzed within the batch verified no carryover from either urine samples or the highest standard sample. The recovery of the entire assay at both fortification levels for standards and human urine were in the range ±15% of nominal 100% recovery (86.7–97.3, 92.0–96.5, and 87.2–100.0% for εAde, εdC, and εdA, respectively) and standard derivations not exceeding 10%. The etheno adducts are stable in human urine throughout at least three freeze-thaw cycles. To assess the effect of long-term (>2 y at −20 °C) storage, one urine sample was analyzed and data compared to the εdA concentration measured previously. The εdA concentrations found were indistinguishable within method variation.

The main problem encountered by LC-MS/MS analysis relates to matrix effects, in particular ion suppression. Due to high variations in matrix effects from sample to sample and even analyte to analyte, one needs to address interindividual matrix effect. There-

Table 1. Validation results: Urine concentration (pM), within-day repeatability (RSD_W)[a] and between-day reproducibility (RSD_B) for each analyte

Sample	Diuresis (mL/24h)	εAde			εdC			εdA		
		Conc.	RSD_W	RSD_B	Conc.	RSD_W	RSD_B	Conc.	RSD_W	RSD_B
Urine A	1928	15.2	4.9	4.6	72.6	2.9	7.5	9.8	5.7	1.4
Urine B	2499	5.7	9.6	10.9	21.3	6.9	4.1	4.0	9.2	8.8
Urine C	4500	6.0	4.7	5.3	39.5	4.0	2.0	7.8	4.2	8.6
Urine D	1690	69.4	3.5	1.0	34.4	8.5	2.1	5.8	2.4	9.2
Urine E	859	55.0	2.0	2.0	97.4	5.0	4.3	27.2	4.0	8.8
Urine F	681	58.3	3.9	0.0	99.7	6.1	9.9	16.5	5.0	7.0

[a]RSD in %.

fore, it is important to evaluate matrix during method validation by using different batches of urine. The urine samples were chosen to cover a broad diuresis range. The use of stable isotope-labeled internal standards, which is expected to experience the same exact matrix effect as the analyte, will not eliminate the matrix effect but will ensure that the accuracy/precision of the method is unaffected. However, the matrix effect could significantly lower the response, affecting the achievable detection limit. Various degrees of ion suppression were observed by Chen and Chiu using ESI, the extent of decreased signals ranging from one-third to one-seventh [13]. APCI was chosen as the ionization mode for MS detection since APCI ion intensities are not as strongly affected by coeluting matrix constituents as in electrospray ionization [20, 21]. The signal intensity of the ISTD transitions was almost identical for a real sample compared to a standard, indicating negligible ion suppression, as identical amounts of ISTD has been added to both samples and standards.

The concentrations of the etheno adducts in human urine were: εAde, 34.9 ± 29.0; εdC, 60.8 ± 33.8; εdA, 11.9 ± 8.7 pM (n = 6). The corresponding levels of urinary excretion rates in the six subjects were in the ranges 14.2–117.3 pmol εAde/24 h, 53.2–117.8 pmol εdC/24 h, and 9.8–23.4 pmol εdA/24 h. The basal levels here are comparable with those of εdA [7, 16] and εdC [11] found previously. Slightly higher excretion levels of εAde than our finding have been reported by Chen and coworkers [9, 12]. The discrepancies in these findings are minor and may be attributable to experimental variation and differences in population and exposures. There was no relationship between excretion rate of εAde and either of the nucleoside adducts; however, although the samples numbers were limited, there seemed to be a correlation between εdC and εdA excretion.

Conclusions

In conclusion, this study is the first paper on simultaneous detection of the promutagenic etheno-adducts εAde, εdC, and εdA in human urine. The column-switching LC/APCI-MS/MS assay provides a useful tool for establishing determining factors and for investigations of the relationship to disease, in particular cancer development, and do not require radioactive standards or very specific antibodies. The method is a sensitive and reliable tool suitable for determination of the urinary excretion of εAde, εdC, and εdA, even at low levels, in humans. 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.

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