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New ultrasensitive ^{32}P-postlabelling method for the analysis of 3,N^4-etheno-2′-deoxycytidine in human urine

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Abstract

Etheno–DNA adducts are generated from exogenous carcinogens such as vinyl chloride and urethane and also from endogenous lipid peroxidation products such as *trans*-4-hydroxy-2-nonenal (HNE). The present authors and others have established that 1,N^6-ethenodeoxyadenosine (εdA) and 3,N^4-ethenodeoxycytidine (εdC) are present in human urine and could be explored as biomarkers for monitoring whole-body oxidative stress. The present study reports on a new ultrasensitive ^{32}P-postlabelling/thin-layer chromatography (TLC) method for the analysis of εdC as deoxynucleoside in human urine. The urine samples were purified and enriched on a solid-phase silica C-18 column followed by a semi-preparative reverse-phase high-performance liquid chromatography. The purified sample was labelled with a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* (Dm-dNK) in the presence of 5′-bromo-2′-deoxyuridine (BrdU) as internal standard. The absolute sensitivity of the method was 0.1 fmol εdC detectable in 500 μl of human urine. The analysis of human urine samples from 15 healthy volunteers revealed a mean εdC level of 2.49 ± 1.76 (SD) fmol μmol^{-1} creatinine (range 0.66–6.42). By this non-invasive method, εdC in human urine could be explored as a biomarker for oxidative stress-related human diseases.

Keywords: *Urinalysis, 3,N^4-ethenodeoxycytidine, lipid peroxidation, human biomonitoring*

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Introduction

Etheno–DNA adducts such as 3,N^4-ethenodeoxycytidine (εdC) and 1,N^6-ethenodeoxyadenosine (εdA) are formed not only from the carcinogens vinyl chloride and urethane (Leithauser et al. 1990, Guengerich 1992, Swenberg et al. 1992, Fernando et al. 1996), but also from reactive alkenals generated via lipid peroxidation (LPO) (Chung et al. 1996, Nair et al. 1999). Etheno–DNA adducts were formed after peroxidation of arachidonic acid and liver microsomal membranes in the presence of LPO-inducing compounds (El Ghissassi et al. 1995). These miscoding lesions are elevated in various disease conditions where increased oxidative stress is induced by excess metal storage or chronic inflammation and infections (reviewed in Bartsch &

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Nair 2005). Elevated etheno–DNA adducts were found in hepatic DNA from patients suffering from primary haemochromatosis and Wilson's disease, resulting in metal-induced oxidative stress and a high risk of liver cancer in the former (Nair et al. 1998a). A very high ω-6 polyunsaturated fatty acid diet strongly increased etheno–DNA adduct levels in white blood cells, particularly in female subjects (Nair et al. 1997). Mice injected with RcsX cells, which cause overexpression of inducible nitric oxide synthase (iNOS) exhibited a sixfold higher etheno adduct levels in the splenic DNA compared with the controls (Nair et al. 1998b). Etheno–DNA adducts were also found to be increased in the affected tissue of chronic pancreatitis patients (Nair et al. 2006). These observations suggested that the etheno–DNA adducts accumulate in cancer-prone tissues as a result of chronic inflammatory processes causing oxidative stress and LPO.

Non-invasive detection methods such as urinalysis will expedite studies in humans aimed to elucidate aetiopathological factors that cause oxidative DNA damage. For these reasons we and others have established methods to study the formation and excretion of etheno–DNA base adducts. εdA and εdC were found to be present in human urine, and these could then be explored as biomarkers for assessing whole-body oxidative stress (Nair 1999, Chen et al. 2004, Hillestrom et al. 2004). The present method, involving immunoprecipitation, high-performance liquid chromatography and fluorescence detection (IP-HPLC-FD), was applied to evaluate the effects of some dietary factors on the formation of urinary εdA levels in non-smoking post-menopausal women (Hanaoka et al. 2002). Chen et al. have developed a gas chromatography (GC)/negetive ion chemical ionization (NICI)/mass spectrometry (MS) method for the detection of 3,N^4-ethenocytosine (εC) and 1,N^6-ethenoadenine (εA) in human urine (Chen et al. 2001, 2004). Also, a liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) method was set up for the urinalysis of εA and compared with a GC/NICI/MS method (Chen et al. 2004).

The present paper now reports on a new ultrasensitive ^{32}P-postlabelling/thin-layer chromatography (TLC) method for the analysis of εdC using a new approach of labelling deoxynucleosides by a multisubstrate deoxyribonucleoside kinase enzyme (dNK) in small amounts of human urine. dNK was cloned from *Drosophila melanogaster* (hence it is called Dm-dNK) (Johansson et al. 1999) and was shown to be capable of labelling εdC and 5′-bromo-2′-deoxyuridine (BrdU), the latter being used as an internal standard (IS). The present paper presents the details of the method protocol and its application to quantify εdC in human urine samples.

Materials and methods

5′-Bromo-2′-deoxyuridine (BrdU) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Superclean LC-18 solid-phase silica columns (500 mg, 3 ml) were obtained from Supelco (Supelco Park, Bellefonte, CA, USA). [γ-^{32}P]-adenosine triphosphate (ATP) with a specific activity of approximately 220 TBq mmol^{-1} (approximately 600 Ci mmol^{-1}) was purchased from Amersham Biosciences Europe GmbH (Freiburg, Germany). (Caution: [γ-^{32}P]-ATP is a hazardous radioactive compound and should be handled with sufficient protection and shielding.) 3,N^4-ethenodeoxycytidine (εdC) was synthesized from deoxycytidine by reaction with bromoacetaldehyde and purification by semi-preparative HPLC

(Guichard et al. 1993). The structure, ultraviolet light and mass spectra of the product are given in Figure 1.

The multisubstrate deoxyribonucleoside kinase was originally cloned from *Drosophila melanogaster* (Dm-dNK). The recombinant Dm-dNK was expressed in *Escherichia coli* as a fusion protein to glutathione *S*-transferase, purified as described, and kept at a stock protein concentration of 1 mg ml^{-1}. The recombinant enzyme preferentially phosphorylated the pyrimidine nucleosides thymidine, deoxycytidine and deoxyuridine, but phosphorylation of the purine nucleosides deoxyadenosine and deoxyguanosine was also efficiently catalysed (Johansson et al. 1999).

Preparation of urine samples

Briefly, urine samples were filtered through a 0.22-μm filter, and a 0.5-ml sample was spiked with the IS [2,8-^{3}H]-1,N^6-ethenoadenosine ([^{3}H]-εA). It was synthesized by reacting [2,8-^{3}H]-adenosine with bromoacetaldehyde and purified by semi-preparative HPLC (Nair 1999). The urinary protein was precipitated by adding cold ethanol and centrifuged at 4000 rpm for 10 min after standing at −20°C for 30 min, and

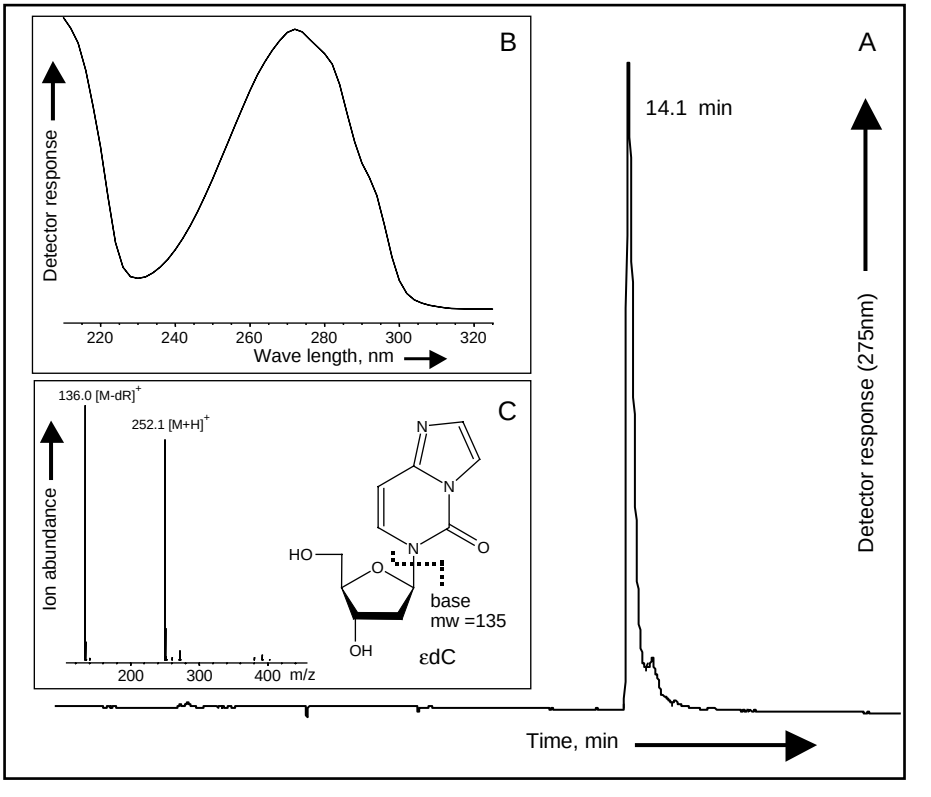

Figure 1. Structure of εdC, ultraviolet light and mass spectra of the synthesized standard. Purified εdC was injected into a liquid chromatography-mass spectrometer (Agilent, 1100). High-performance liquid chromatography was performed on a 30-cm C18 column (5 μ) using a linear gradient of water:acetonitrile 0–30% in 30 min at a flow rate of 0.8 ml min^{-1}: (A) chromatogram of εdC monitored at 275 nm; (B) online ultraviolet light spectrum of εdC; and (C) online mass spectrum of εdC (fragmentor at 40, positive electrospray).

concentrated by vacuum centrifugation. The dried sample was redissolved in water and loaded onto an LC-18 solid-phase silica column (500 mg, 3 ml; Supelco). The columns were washed with 10 ml of 20 mM NaH_2PO_4 (pH 6.8) followed by 10 ml of water and 10 ml of 10% methanol (v/v) at 4°C in order to remove the bulk of normal nucleosides. The columns were brought to room temperature (20°C) and εdC was eluted twice with 2.5 ml of methanol:water (1:1, v/v) and concentrated by vacuum centrifugation. Before sample loading, the columns were prewashed with 10 ml of methanol followed by 10 ml of water. The dried samples were resolved on a reverse-phase HPLC with a Hypersil ODS column (250 × 8.0 mm, 5 μ) connected with an automated fraction collector (Agilent 1100 series, Waldbronn, Germany) using a linear water (A)/acetonitrile (B) gradient as follows: 0–8 min, 100% A; 8–20 min, 0–20% B in A; 20–30 min, 20% B isocratically; 30–40 min, 20–50% B in A; 40–45 min, 50% B isocratically; 45–50 min, 100% B. The flow rate was 2 ml min^{-1}. Fractions containing the IS (20.6 min) and εdC (21.9 min) were collected separately. The earlier eluting IS fraction was counted for the correction of recovery. The fraction containing εdC was concentrated by vacuum centrifugation and subjected to ^{32}P-postlabelling/TLC analysis. The HPLC system was cleaned after each sample analysis, and blank samples were randomly run to ensure that no residual contamination was present.

^{32}P-postlabelling and TLC analysis of εdC

Optimal pH of the kinase buffer, Dm-dNK protein concentration and the incubation time for the maximal labelling efficiency of εdC and BrdU were established (see the Results). For the final analysis, 1 fmol of BrdU as IS was added to each dried sample, obtained from semi-preparative HPLC. After addition of 5 μl kinase buffer (125 mM Tris-HCl, 25 mM $MgCl_2$, and 25 mM dithiolthreitol (DTT), pH 6.8), 2 μl of [γ-^{32}P]-ATP (10 μCi) and 0.5 μg of Dm-dNK protein, the mixture was incubated at 37°C for 2 h, centrifuged and spotted onto the left side of pre-washed polyethyleneimine (PEI)-cellulose plates at 1.5 cm from the margins. The PEI cellulose plates were developed by two-dimensional TLC (D1: 1 M acetic acid, pH 3.5; D2: saturated ammonium sulphate, pH 3.5). The plates were exposed to X-ray films (Fuji medical X-ray film, 100 NIF, 18 × 20 cm, Düsseldorf, Germany) in cassettes supported with intensifying screens at −80°C for 2 h. Sections of the TLC plates corresponding to the spots of the [^{32}P]-εdC-5′-monophosphate and [^{32}P]-BrdU-5′-monophosphate were cut out and radioactivity was determined by liquid scintillation counting. Background counts were determined from appropriate blank areas on the TLC plates and subtracted. A total of 1 fmol of εdC standard was also labelled in parallel and separated in a similar way. The fmol amount of εdC in the spots was calculated by the term:

$$(F_2/F_1) \times 100,$$

where

F_1 = [counts per minute (cpm) of εdC]/[cpm of BrdU (IS)] in standard
F_2 = [cpm of εdC]/[cpm of BrdU (IS)] in samples.

The urinary creatinine used for normalizing the εdC concentration was measured by a picric acid-based method using a kit (Sigma-Aldrich, Schnelldorf,

Germany) according to the supplier's protocol. The method is depicted schematically in Figure 2.

Results

εdC adduct analysis

Purification and enrichment of εdC in urine samples was achieved using a solid-phase silica column followed a semi-preparative reverse phase (RP)-HPLC. After samples were loaded, the columns were washed with 20 mM NaH_2PO_4 (pH 6.8) followed by water and 10% methanol to remove the bulk of the normal nucleosides by which εdC was not eluted. The fraction containing εdC and [^{3}H]-εA (IS) was eluted with methanol:water (1:1, v/v) and then was separated by a semi-preparative RP-HPLC. The retention times of [^{3}H]-εA (IS) and εdC were 20.6 and 21.9 min, respectively. The addition of [^{3}H]-εA as the IS correcting for recovery was found to be reproducible with a recovery rate of [^{3}H]-εA of 80% ± 10%.

Postlabelling of εdC and BrdU

A labelling assay using Dm-dNK in the presence of BrdU as IS was developed to convert εdC into its corresponding 5′-monophosphate. The addition of BrdU as IS provided a good control for labelling efficiency which was used for the correction in the routine analysis during the labelling step. Figure 3 shows the linearity of the ratio: [^{32}P]-εdC-5′-monophosphate:[^{32}P]-BrdU-5′-monophosphate when εdC ranging from 0.1–5.0 fmol was labelled in the presence of a fixed amount of

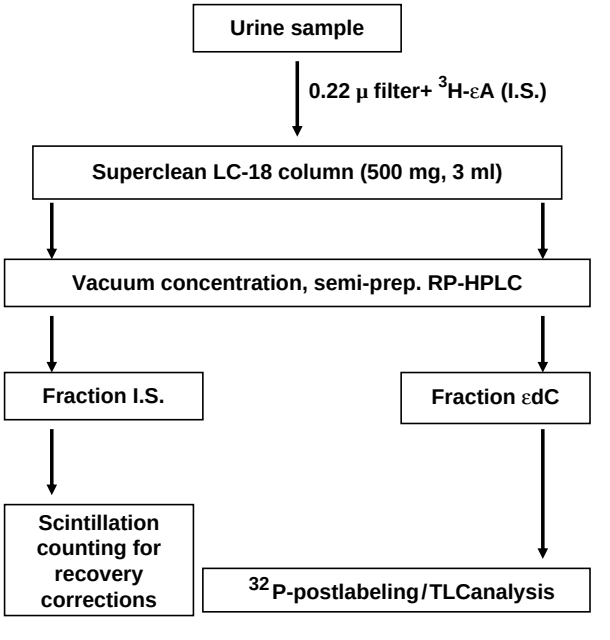

Figure 2. Scheme for the purification and enrichment of εdC from urine samples. [2,8-^{3}H]-1,N^6-ethenoadenosine (^{3}H-εA) is added as the internal standard.

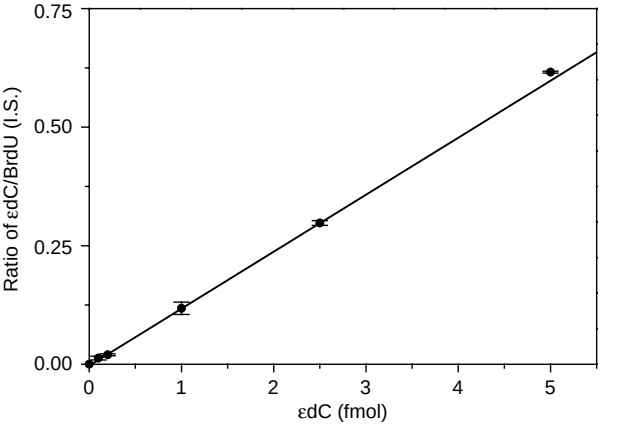

Figure 3. Linear relation of the εdC:BrdU ratio as a function of εdC concentration (a range of 0.1–5.0 fmol of εdC was labelled in the presence of a fixed amount of 1 fmol of BrdU in a 10-μl reaction mixture); assays were performed in triplicate.

BrdU (1 fmol). When calculated from the transfer of radioactivity on the basis of the specific activity of radiolabelled ATP, the labelling efficiency of BrdU was found to be >90%. Under these experimental conditions, the labelling efficiency of εdC was about approximately 40%. Initially, we have tried to use 2′-deoxyuridine (dU) as IS, however during the actual analysis of urine samples, we observed an interfering spot eluting close to that of dU (designated in Figure 7 as X). Hence, BrdU was introduced as IS that could be resolved on PEI cellulose plates (Figure 7).

For the optimization of the pH of the phosphorylation reaction, the pH of the kinase buffer was adjusted to 2.0, 5.0, 6.8 and 9.6 and the labelling efficiency for εdC and BrdU was tested. Maximal phosphorylation was observed at pH 6.8 (Figure 4), and this pH was further used in labelling experiments. The incubation time required for optimal labelling was investigated by incubating 1 fmol aliquots of εdC and BrdU

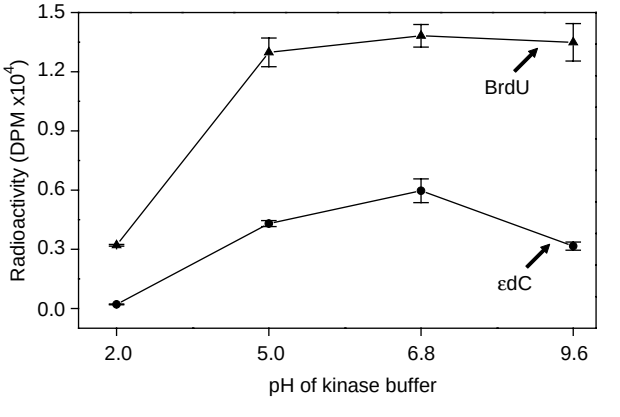

Figure 4. Determination of the optimal pH of kinase buffer for labelling εdC. A total of 1 fmol of εdC and BrdU (internal standard) were labelled by deoxyribonucleoside kinase from *Drosophila melanogaster* in a 10-μl reaction mixture with kinase buffer at pHs 2.0, 5.0, 6.8 and 9.6; assays were performed in triplicate.

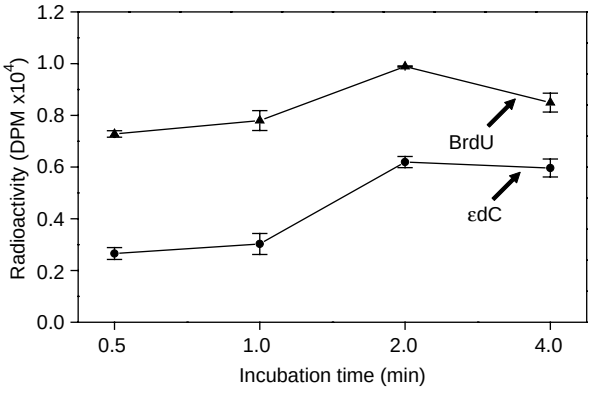

Figure 5. Determination of the optimal incubation time for the labelling of εdC. A total of 1 fmol of εdC and BrdU (internal standard) were labelled by deoxyribonucleoside kinase from *Drosophila melanogaster* in a 10-μl reaction mixture with incubation times of 0.5, 1, 2 and 4 h at 37°C; assays were performed in triplicate.

standards at 37°C for 0.5, 1, 2 and 4 h. The labelling efficiency reached its maximum at 2 h of incubation and decreased after 4 h (Figure 5); thus, a 2-h incubation time was used for further analysis.

The multisubstrate deoxyribonucleoside kinase (Dm-dNK) cloned from *D. melanogaster* was used for the first time for labelling of εdC and BrdU (IS). In order to optimize Dm-dNK protein concentration for labelling, protein concentrations ranging from 0.125 to 2.0 μg in a 10-μl reaction mixture were investigated. The highest labelling efficiency was observed in the presence of 0.5 μg of Dm-dNK protein in a 10-μl reaction mixture (Figure 6). Typical autoradiograms of εdC either as standard or found in human urine samples are shown in Figure 7. Co-migration of the corresponding spots isolated from PEI cellulose plates after TLC was further confirmed (Figure 8) by an established C-18 RP-HPLC system with modification (Sun et al. 2004).

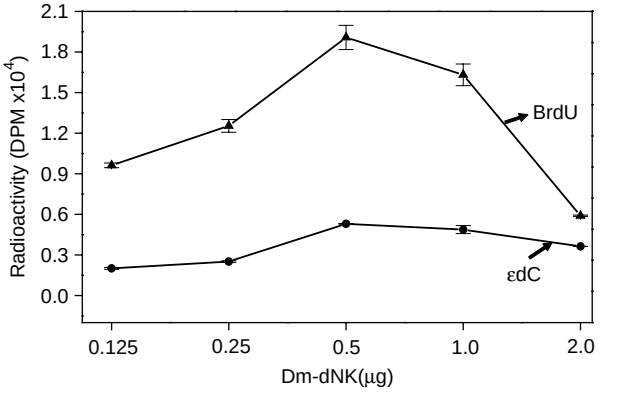

Figure 6. Determination of the optimal protein concentration of deoxyribonucleoside kinase from *Drosophila melanogaster* (Dm-dNK) for the labelling of εdC (1 fmol of εdC and BrdU (internal standard) were labelled by Dm-dNK ranging from 0.125 to 2.0 μg in a 10-μl reaction mixture); assays were performed in triplicate.

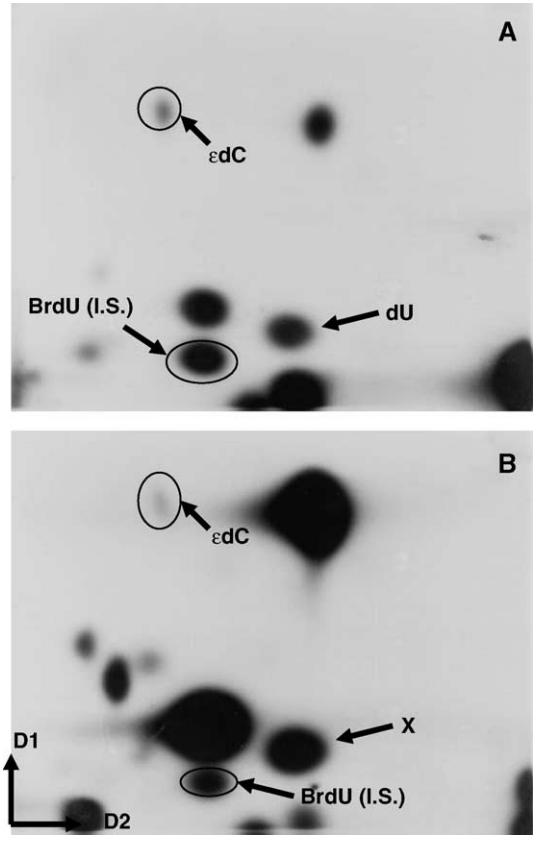

Figure 7. Typical autoradiograms of εdC on polyethyleneimine (PEI)-thin-layer chromatography (TLC) plates: (A) standards of εdC and BrdU (internal standard); and (B) a human urine sample. D1, 1 M acetic acid (pH 3.5); D2, saturated ammonium sulphate (pH 3.5); spot are designated as X elutes at the position of dU; undesignated spots are probably nucleosides present in the urine sample which did not interfere with εdC analysis.

Reproducibility of ^{32}P-postlabelling/TLC

For the determination of intra- and inter-assay reproducibility, two human urine samples were randomly selected and analysed in triplicate by ^{32}P-postlabelling. For intra-assay, the resulting values for εdC (fmol μmol^{-1} creatinine) were 0.81, 0.66, 0.61 (mean ± SD; 0.69 ± 0.10) and 1.37, 1.24, 1.15 (mean ± SD; 1.25 ± 0.11). For the inter-assay, the values for εdC (fmol μmol^{-1} creatinine) were 0.68, 0.61, 0.58 (mean ± SD; 0.62 ± 0.05) and 1.21, 1.15, 1.01 (mean ± SD; 1.12 ± 0.10). The relative standard errors were not higher than 11% for intra- and inter-assay variations.

εdC levels detected in human urine

To validate this ^{32}P-postlabelling/TLC method, urine samples from 15 healthy volunteers were assayed. A typical autoradiogram of εdC detected in human urine is shown in Figure 7. The mean level of εdC (± SD) was 2.49 (± 1.76), ranging from 0.66 to 6.42 fmol μmol^{-1} creatinine (3.39–15.53 fmol ml^{-1} urine).

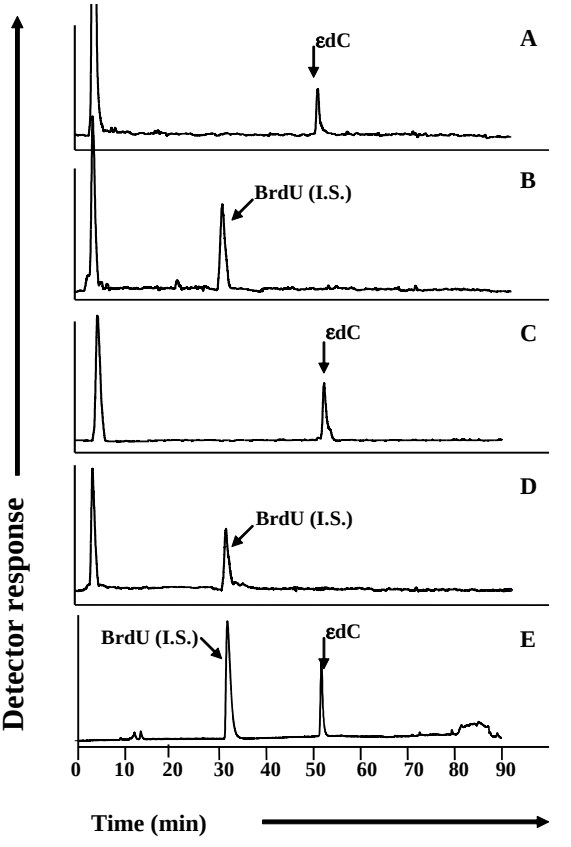

Figure 8. High-performance liquid chromatography profiles of [^{32}P]-BrdU-5′-monophosphate and [^{32}P]-εdC-5′-monophosphate isolated from the corresponding thin-layer chromatography spots: (A) standard of εdC; (B) standard of BrdU; (C) εdC from a human urine sample; (D) BrdU from the same urine sample; and (E) unlabelled BrdU-5′-monophosphate and εdC-5′-monophosphate standards as an ultraviolet light marker.

Discussion

Etheno adducts are formed in DNA bases after reaction with aldehydes such as HNE generated during the oxidative stress as LPO end-products. As discussed for other oxidized DNA bases, the presence of these etheno adducts in urine probably originates from the repair of damaged cellular DNA by nucleotide excision, from their formation in the deoxynucleoside pool or from DNA hydrolysis of dead cells with such adducts (Cooke et al. 2000). Therefore, the determination of etheno-bridged deoxynucleosides in urine can be explored as biomarkers for monitoring whole-body oxidative stress. For this purpose, sensitive and specific analytical methods are required for measuring precise levels of etheno adducts in small amounts of urine.

The present paper now reports a new ultrasensitive ^{32}P-postlabelling method for the analysis of εdC as a deoxynucleoside in human urine. The analytical procedure appears to be superior to other methods reported, both in terms of overall specifity and sensitivity. The method is based on an effective purification and

enrichment of εdC in human urine samples; hereby, a solid-phase silica column chromatography followed by a semi-preparative RP-HPLC has been developed that allows the efficient separation of εdC and [^{3}H]-εA (IS) from the urinary matrix. The absolute sensitivity of the method was found to be 0.1 fmol of εdC detectable in 500 μl of human urine. Thus, it is two orders of magnitude higher than a currently reported GC/NICI/MS method with a sensitivity of 12 fmoles of εdC in 0.1 ml urine (Chen et al. 2004).

[^{3}H]-εA as an IS was added into each urine sample for correcting the absolute recovery of εdC after the purification steps. The final concentrations were calculated after correcting for the recovery. The reproducible recovery rate from 15 urine samples was found to be 80% ± 10% (mean ± SD). [^{3}H]-εA is a suitable IS because of its similar polarity and chromatographic properties as εdC. We also tried to use [^{3}H]-εdC to confirm the recovery of εdC, which was similar to that of [^{3}H]-εA. The use of [^{3}H]-εdC was not pursued further in order to avoid a possible contamination at the sensitive ^{32}P-postlabelling step.

Furthermore, in the labelling step, BrdU was used as an IS for correcting labelling efficiency so that εdC levels can be accurately quantified. BrdU was found to be best for IS in terms of both labelling efficiency and separability. Results on the intra- and inter-assay variabilities revealed a high reproducibility and accuracy. We still use a TLC method (Figure 2) as it is more sensitive than HPLC because TLC plates can be exposed for longer by autoradiography to detect the radiolabelled εdC.

The range of the εdC level detected per ml human urine was at least one order of magnitude lower than that reported by LC-MS/MS (Chen et al. 2004). These authors have also reported higher levels of another urinary ε-adduct, εdA, than reported by two different laboratories using either an immune-enriched HPLC-FLD method (Hanaoka et al. 2002) or an LC/APCI-MS/MS method (Hillestrom et al. 2004). At present it is not possible to evaluate the reasons for these discrepancies. εdC levels detected in the present study were one order of magnitude higher than the reported malondialdehyde-deoxyguanosine (M_1dG) level in human urine (Hoberg et al. 2004).

In our new ^{32}P-postlabelling/TLC assay for εdC as the deoxynucleoside, we used Dm-dNK enzyme for phosphorylation; Dm-dNK was reported to be a multisubstrate deoxynucleoside kinase, which in contrast to the other enzymes catalyses the phosphorylation of all the natural pyrimidine and purine deoxynucleosides with a preference for pyrimidine nucleosides. The catalytic rate of deoxyribonucleoside phosphorylation is, depending on the substrate, about ten to 100 times faster than what has been reported for other members of the deoxyribonucleoside kinase enzyme family (Johansson et al. 1999). The broad substrate specificity and high catalytic rate of Dm-dNK offers unique applications for ^{32}P-postlabelling assays.

There is increasing evidence for a role of reactive oxygen species and LPO in the aetiology of human cancers and the development of other chronic degenerative diseases (Hussain et al. 2003). A series of studies in animals and humans have demonstrated that etheno–DNA adducts are one of the lead markers for DNA damage produced endogenously as a result of persistent oxidative stress and LPO (Bartsch & Nair 2005). The development and application of sensitive and specific detection methods for this class of DNA adducts should provide valuable tools for investigating their role in human disease pathogenesis and its preventability.

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References

Bartsch H, Nair J. 2005. Accumulation of lipid peroxidation-derived DNA lesions: potential lead markers for chemoprevention of inflammation-derived malignancies. Mutation Research 591:34–44.

Chen HJ, Chang CM. 2004. Quantification of urinary excretion of 1,N^6-ethenoadenine, a potential biomarker of lipid peroxidation, in humans by stable isotope dilution, liquid chromatography-electrospray ionization-tandem mass spectrometry: comparison with gas chromatography-mass spectrometry. Chemical Research in Toxicology 17:963–971.

Chen HJ, Lin TC, Hong CL, Chiang LC. 2001. Analysis of 3,N^4-ethenocytosine in DNA and in human urine by isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry. Chemical Research in Toxicology 14:1612–1619.

Chen HJ, Wu CF, Hong CL, Chang CM. 2004. Urinary excretion of 3,N^4-etheno-2′-deoxycytidine in humans as a biomarker of oxidative stress: association with cigarette smoking. Chemical Research in Toxicology 17:896–903.

Chung F-L, Chen H-J, Nath RG. 1996. Lipid peroxidation as a potential endogenous source for the formation of exocyclic DNA adducts: a commentary. Carcinogenesis 17:2105–2111.

Cooke MS, Evans MD, Herbert LJ, Lunec J. 2000. Urinary 8-oxo-2′-deoxyguanosine-source, significance and supplements. Free Radical Research 32:381–397.

El Ghissassi F, Barbin A, Nair J, Bartsch H. 1995. Formation of 1,N^6-ethenoadenosine and 3,N^4-ethenocytosine by lipid peroxidation products and nucleic acid bases. Chemical Research in Toxicology 8:278–283.

Fernando RC, Nair J, Barbin A, Miller JA, Bartsch H. 1996. Detection of 1,N^6-ethenodeoxyadenosine and 3,N^4-ethenodeoxycytidine by immunoaffinity/^{32}P-postlabeling in liver and lung DNA of mice treated with ethyl carbamate (urethane) or its metabolites. Carcinogenesis 17:1711–1718.

Guengerich FP. 1992. Roles of the vinyl chloride oxidation products 1-chloro-oxirane and 2-chloroacetaldehyde in the in vitro formation of etheno adducts of nucleic acid bases. Chemical Research in Toxicology 5:2–5.

Guichard Y, Nair J, Barbin A, Bartsch H. 1993. Immunoaffinity clean-up combined with ^{32}P-postlabeling analysis of 1,N^6-ethenoadenosine and 3,N^4-ethenocytosine in DNA. In: Phillips DH, Castegnaro M, Bartsch H, editors. Postlabeling methods for the detection of DNA adducts. Science Publication Vol. 124. Lyon: International Agency for Research on Cancer. p. 263–269.

Hanaoka T, Nair J, Takahashi Y, Sasaki S, Bartsch H, Tsugane S. 2002. Urinary level of 1,N^6-ethenodeoxyadenosine, a marker of oxidative stress, is associated with salt excretion and ω-6-polyunsaturated fatty acid intake in postmenopausal Japanese women. International Journal of Cancer 100:71–75.

Hillestrom PR, Hoberg AM, Weimann A, Poulsen HE. 2004. Quantification of 1,N^6-ethenodeoxyadenosine in human urine by column-switching LC/APCI-MS/MS. Free Radicals in Biology and Medicine 36:1383–1392.

Hoberg AM, Otteneder M, Narnett LJ, Poulsen HE. 2004. Measurement of the malondialdehyde-2′-deoxyguanosine adduct in human urine by immuno-extraction and liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. Journal of Mass Spectrometry 39:38–42.

Hussain SP, Hofseth LJ, Harris CC. 2003. Radical causes of cancer. National Reviews in Cancer 3:276–285.

Johansson M, Van Rompay AR, Degreve B, Balzarin J, Karlsson A. 1999. Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of Drosophila melanogaster. Journal of Biology and Chemistry 274:23814–23819.

Leithauser MT, Liem A, Stewart BC, Miller EC, Miller JA. 1990. 1,N^6-ethenoadenosine formation, mutagenicity, and murine tumor induction as indicators of the generation of an electrophilic epoxide metabolite of the closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. Carcinogenesis 11:463–473.

Nair J. 1999. Lipid peroxidation-induced etheno–DNA adducts in humans. Exocylic DNA adducts in mutagenesis and Carcinogenesis. In: Singer B, Bartsch H, editors. IARC Scientific Publication Vol. 150. Lyon: International Agency for Research on Cancer. p. 55–61.

Nair J, Barbin A, Velic I, Bartsch H. 1999. Etheno–DNA-base adducts from endogenous reactive species. Mutation Research 424:59–69.

Nair J, Carmichael PL, Fernando RC, Phillips DH, Strain AJ, Bartsch H. 1998a. Lipid peroxidation-induced etheno–DNA adducts in the liver of patients with the genetic metal storage disorders Wilson's disease and primary hemochromatosis. Cancer Epidemiology Biomarkers Prevention 7:435–440.

Nair J, Gal A, Tamir S, Tannenbaum SR, Wogan GN, Bartsch H. 1998b. Etheno adducts in spleen DNA of SJL mice stimulated to overproduce nitric oxide. Carcinogenesis 19:2081–2084.

Nair J, Gansauge F, Beger H, Dolara P, Winde G, Bartsch H. 2006. Increased etheno–DNA adducts in affected tissues of patients suffering from Crohn's disease, Ulcerative colitis and Chronic Pancreatitis. Antioxidants and Redox Signaling 8:1003–10.

Nair J, Vaca CE, Velic I, Mutanen M, Valsta LM, Bartsch H. 1997. High dietary ω-6 polyunsaturated fatty acids drastically increase the formation of etheno–DNA base adducts in white blood cells of female subjects. Cancer Epidemiology Biomarkers Prevention 6:597–601.

Sun X, Nair J, Bartsch H. 2004. A modified immuno-enriched ^{32}P-postlabeling method for analyzing the malondialdehyde-deoxyguanosine adduct, 3-(2-deoxy-β-D-erythro-pentofuranosyl)-pyrimido [1,2-α]purin-10(3H)one in human tissue samples. Chemical Research in Toxicology 17:268–272.

Swenberg JA, Fedtke N, Ciroussel F, Barbin A, Bartsch H. 1992. Etheno adducts formed in DNA of vinyl chloride-exposed rats are highly persistent in liver. Carcinogenesis 13:727–729.