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Allantoin in human plasma, serum, and nasal-lining fluids as a biomarker of oxidative stress: Avoiding artifacts and establishing real in vivo concentrations

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Abstract

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Keywords

concentrations, human, vivo, allantoin, real, establishing, artifacts, avoiding, stress, oxidative, biomarker, fluids, lining, nasal, serum, plasma

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Original Research Communication

Allantoin in Human Plasma, Serum, and Nasal-Lining Fluids as a Biomarker of Oxidative Stress: Avoiding Artifacts and Establishing Real *in vivo* Concentrations

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Christopher Chen,³ Frank J. Kelly,⁴ and Barry Halliwell¹

Abstract

Urate is the terminal product of purine metabolism in primates, including humans. Urate is also an efficient scavenger of oxidizing species and is thought to be an important antioxidant in human body fluids. Allantoin, the major oxidation product of urate, has been suggested as a candidate biomarker of oxidative stress because it is not produced metabolically. Although urate is converted to allantoin under strongly alkaline pH, such conditions have been used in the past to facilitate extraction of allantoin. We evolved a method for the determination of allantoin concentrations in human plasma and serum by gas chromatography–mass spectrometry without such artifact. With this method, we show that alkaline conditions do indeed cause breakdown of urate, leading to significant overestimation of allantoin concentration in human samples. By using our alternative method, serum samples from 98 volunteers were analyzed, and allantoin levels were found to be significantly lower than was previously reported. The *in vivo* utility and sensitivity of our method was further evaluated in human nasal-lining fluids. We were able to demonstrate an ozone-induced increase in allantoin, in the absence of increases in either ascorbate or glutathione oxidation products. *Antioxid. Redox Signal.* 11, 1767–1776.

Introduction

URATE is generated during purine metabolism *via* the oxidation of hypoxanthine and xanthine by xanthine oxidase (EC 1.14.21.1), but it cannot be further oxidized enzymatically in the human body and so accumulates to high levels in body fluids. Ames *et al.* (2) revealed that urate is a scavenger of hydroxyl radicals, hemoglobin-derived oxidizing species, and singlet oxygen *in vitro*. Urate has *in vitro* antioxidant activity against several reactive oxygen, nitrogen, and chlorine species (2, 3, 14, 30, 33, 34). As much as 60% of the total free radical scavenging capacity of human plasma has been attributed to urate, making urate potentially an impor-

tant antioxidant in human body fluids (32). Attack of a range of reactive species on urate produces allantoin, which has therefore been suggested as a biomarker for oxidative stress (13, 19).

Prior methods for the detection of allantoin in samples of human body fluids are based on alkaline hydrolysis of allantoin to allantoate, followed by acid hydrolysis of allantoate to glyoxylic acid and urea. Glyoxylic acid in turn forms the chromophore glyoxylate 2,4-dinitrophenylhydrazone when incubated with 2,4-dinitrophenylhydrazine (Rimini-Schryver reaction), which can be quantified spectrophotometrically or with high-performance liquid chromatography (HPLC) (13). The accuracy of methods based on this procedure is critically

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dependent on the removal of urate and glyoxylic acid during sample cleanup (18, 20, 21, 23). Furthermore, lack of internal standards can make these methods sensitive to volume changes potentially caused by evaporation during sample derivatization (20). More recently, a gas chromatography–mass spectrometry (GC/MS) method, originally developed for the quantification of allantoin in animal urine (8), has been modified for use with human samples (29). Several clinical investigations have used related GC/MS methods to quantify allantoin levels (10, 29, 35–37) but this method is not yet widely used because of the lack of analytic validation of some of the techniques. Direct chromatography as well as complex enzyme-cycling methods have also been proposed (5, 7).

Because of its polar character, allantoin is not retained well during reverse-phase chromatography or by using most solid-phase extraction (SPE) technologies. To overcome this problem, strongly alkaline conditions (either 50 mM NaOH or 6 M NH₄OH) are often used (8, 29). Although these alkaline conditions facilitate retention of allantoin on anion-exchange matrices, urate is unstable at high pH and can be oxidized to allantoin (11, 19). However, the extent to which this artifact might affect measurement of allantoin in body fluids is uncertain.

Here we reveal that the artifact is significant, describe a method to avoid it during isotope-dilution GC/MS determination of allantoin in plasma and serum samples, establish a true reference range for allantoin levels in human serum, compare the measurement of allantoin with that of F₂-isoprostanes as a biomarker of oxidative stress, and illustrate the use of allantoin measurements to measure oxidative stress in human nasal-lining fluids (NLFs) during a controlled exposure to ozone.

Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Allantoin was from Fluka. Sodium hydroxide and acetonitrile (HPLC grade) were from Merck AG (Darmstadt, Germany). [1,3-¹⁵N₂] urate was from Cambridge Isotope Laboratories (MA), and Allantoin-U-¹⁵N₄ 50% was from Icon Isotopes (NJ).

Preparation of ¹⁵N-labeled allantoin standard

Heavy isotope-labeled allantoin for use as internal standard was prepared by digesting [1,3-¹⁵N₂] urate with excess uricase from *Bacillus fastidiosus* (Fluka) by following the procedure reported by Chen *et al.* (8) with slight modifications. In brief, 50 ml of 1 mM [1,3-¹⁵N₂] urate stock solution was prepared in 0.25 mM sodium hydroxide (NaOH) and incubated at 37°C in the presence of 0.5 U of uricase. [¹⁵N]-labeled allantoin was purified by preparative HPLC on an Agilent 1100 Series prep HPLC system equipped with a 250-mm Agilent Zorbax SB-C18 Prep HT (7 μm) column. The purity of the [¹⁵N]-labeled allantoin standard was assessed with HPLC to be >95% (data not shown). The identity of the [¹⁵N]-labeled allantoin derivatives was confirmed with GC/MS.

Quantification of allantoin by isotope dilution

During the derivatization of allantoin with *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA),

three silicon-containing *tert*-butyldimethylsilyl (TBDMS) groups are added to each molecule of allantoin (8). Silicon has two stable and relatively abundant heavy isotopes [²⁹Si (+1) and ³⁰Si (+2); 4.67% and 3.1% abundance, respectively]. The addition of heavy isotopes of silicon during derivatization causes part of the allantoin signal (398 m/z) to be shifted to higher mass numbers. The resulting nonlinearity of the calibration curve does not affect the quantification of allantoin by stable isotope dilution (8, 29).

Allantoin sample extraction and derivatization

Our GC/MS method was modified from that of Chen *et al.* (8). Internal standard (25 μl of 13 μM [¹⁵N]-labeled allantoin) was added to 25 μl of either aqueous allantoin standards, human plasma, serum, or human nasal-lavage fluid (NLF) samples. Spiked samples were simultaneously deproteinized and extracted by addition of 100 μl of acetonitrile followed by thorough vortexing of samples. Samples were then centrifuged (20,000 g, 4°C, 5 min), and supernatants were dried under N₂. After drying, 50 μl of MTBSTFA in pyridine (1:1 vol/vol) was added, and the derivatization reaction was facilitated by incubation at 50°C for 2 h.

Allantoin GC/MS instrumentation and analysis

We used a Hewlett–Packard 6890 gas chromatograph (Agilent Technologies, CA) connected to a HP5973 mass selective detector. The injection port was kept at 250°C and the GC/MS interface temperature was 290°C. Separations were performed on an Agilent Ultra 2 fused silica capillary column (12 m length, 0.2 mm internal diameter). Helium was used as the carrier gas at a flow rate of 1 ml/min. Derivatized product (1 μl) was injected in splitless mode. Initial column temperature was 100°C and was increased rapidly (40°C/min) to 150°C. Column temperature was then increased to 198°C at a rate of 4°C/min. Finally, the column temperature was increased to 300°C at a rate of 30°C/min, and this temperature was held for another 2 min. Ions were generated by using the electron-ionization mode at 70 eV, with the ion source maintained at 230°C.

Sample preparation using alkaline SPE for evaluation of extraction artifacts

The method of Pavitt *et al.* (29) was used with modifications. In brief, allantoin standards, urate standards, or plasma samples (100 μl) were spiked with 100 μl of 13 μM [¹⁵N]-labeled allantoin standard and diluted with 900 μl of H₂O. Samples were made alkaline by addition of 50 μl of 1 M aqueous NaOH and applied to preequilibrated Waters Oasis MAX-SPE cartridges (Oasis Water Corp., MA). Columns were washed 3 times with 1 ml of water followed by one wash with 1 ml of methanol, and allantoin was eluted by using 1 ml of 0.8 M acetic acid in pure methanol. Samples were dried under N₂, derivatized, and analyzed as described earlier.

NaOH incubation

Pure standards containing 400 μM urate were prepared in 10 mM Tris at pH 7.5. Urate standards or plasma samples (25 μl) were spiked with 25 μl of [¹⁵N]-labeled allantoin internal standard and diluted with 250 μl of water. Samples were incubated for 10 min at room temperature after addition

of 12.5 μl of 1 M aqueous NaOH. Incubations were stopped by addition of 1 ml of 0.8 M acetic acid in methanol.

Preparation of partially lysed plasma samples

For the *in vitro* comparison of sensitivity and correlation between allantoin and total F_2 -isoprostanes (free + esterified) in human plasma samples, we recruited eight volunteers (four male, four female subjects) younger than 40 years (mean age, 31.8 ± 4.7 years). Before enrollment, all subjects gave informed consent in accordance with the approval granted by the Institutional Review Board of the National University Hospital, Singapore. Subjects were required to fast for at least 8 h before blood collection. Blood samples (10 ml) were collected in Na-EDTA-coated vacutainer tubes. Plasma was prepared from one tube by centrifugation at 3,000 rpm for 10 min at 4°C, 15 ml of 5 mM indomethacin (in ethanol) per 1 ml plasma, and 20 ml per 1 ml plasma of 2 mM BHT (in ethanol) was added, and samples were stored at -80°C until used for analysis. Great care was taken to avoid any degree of lysis in these plasma samples. A second vacutainer was frozen immediately without first removing blood cells, leading to rupture of blood cells (fully lysed blood). For each volunteer, we then prepared plasma samples representing seven different degrees of lysis by mixing unlysed plasma with 0, 0.5, 1, 5, 10, 15, and 20% fully lysed blood from the same volunteer. Samples were mixed, incubated at room temperature for 20 min, and subsequently stored at -80°C until determination of allantoin and total F_2 -isoprostanes.

Urate determination

Urate concentrations were determined with HPLC with UV detection. Samples (30 μl) were diluted with water to a final volume of 150 μl . Diluted samples were filtered through a centrifugal molecular-weight cut-off spin-filter device (Nanosep; 10 kDa MWCO). The deproteinized filtrate was directly injected into an Agilent Series 1100 analytic HPLC system connected to a 250-mm Zorbax SB-C8 (5 μm) analytic column. The mobile phase was 2 mM $\text{NH}_4\text{H}_2\text{PO}_4$ at pH 2.95 (isocratic, 1 ml/min flow). Urate was monitored at 293 nm by using a diode array detector. Under these conditions, urate eluted at ~ 7 min and was not confounded by other serum constituents. Each sample was injected 3 times, and the peak area averaged. A zero to 400 μM standard curve, prepared from pure urate samples, was used for quantification. NLF urate and allantoin determinations were made in samples (400 μl) pretreated with 700 μl of 15 mM ethylenediaminetetraacetic acid.

Glutathione and glutathione disulfide determination

Glutathione and glutathione disulfide concentrations in recovered NLF were determined by using the glutathione disulfide reductase-dithiobisnitro-benzoic acid recycling assay, as previously described (27).

Ascorbate and dehydroascorbate determination

Ascorbate and total vitamin C determinations were made on nasal-lavage samples pretreated with the metal chelator desferrioxamine mesylate (DES) and the synthetic antioxidant butylated hydroxytoluene (BHT) on collection, as previously

described (24), before storage at -80°C . On the day of analysis, samples were thawed on wet ice and acidified with metaphosphoric acid (final concentration, 5%) and lipid extracted with heptane, before analysis with reverse-phase HPLC with electrochemical detection (400 mV, 100 nAmp) (17). Determination of the total vitamin C pool was performed after sample preincubation with the reductant dithiothreitol at a final concentration of 0.2% (1 h at room temperature), before sample acidification and lipid extraction as outlined earlier (12). The concentration of dehydroascorbate (DHA) in samples was calculated by subtracting the ascorbate concentration from that of the determined total vitamin C pool.

Determination of F_2 -isoprostanes levels

Samples were extracted and derivatized by using a previously described method (22). In brief, before analysis, the plasma samples were thawed at room temperature. Mixed heavy isotopes, 8-iso-PGF $_{2\alpha}$ -d $_4$ and IPF $_{2\alpha}$ -VI-d $_4$, all prepared in ethanol, were added to plasma and mixed. Plasma (1 ml) was hydrolyzed at 37°C for 30 min with 1 ml of 1 M potassium hydroxide prepared in methanol for the release of esterified lipids. Methanol, 5 M HCl, and 40 mM formic acid (pH 4.6) were added and mixed. The samples were extracted and derivatized as follows. MAX SPE (Oasis Water Corp.) cartridges were used to purify the prepared samples, by washing with 2% ammonium hydroxide and then by methanol: 20 mM formic acid (pH 4.6) mix (40:60 vol/vol), hexane, and hexane:ethyl acetate (70:30 vol/vol). Thereafter, hexane:ethanol:acetic acid (20:29.4:0.6 vol/vol) was loaded into the cartridge and collected for total F_2 -IsoPs analysis.

Samples were then dried under ultrahigh-purity nitrogen gas, derivatized with DIPEA and PFBBR at room temperature for 30 min and dried again under nitrogen gas. Afterward, acetonitrile and BSTFA with 1% TMCS were added and incubated at room temperature for 2 h. The derivatized samples were then dried and reconstituted in iso-octane and incubated at room temperature for 20 min for GC-MS analysis (Hewlett-Packard 5973N and 6890; Agilent Technologies).

Volunteers and study design

For serum baseline determination, 98 volunteers (49 male, 49 female subjects) were included in the serum baseline study. Volunteers were aged between 40 and 96 years, with a median age of 65.5 (mean, 64.4 ± 12.4) years. Demographics such as age, gender, ethnicity, and education were collected, as well as health indicators such as weight, height, and physical activity. Before enrollment, all subjects gave informed consent in accordance with the approval granted by the local Ethics Board (Domain Specific Review Board, Singapore). Subjects were required to fast for at least 8 h before serum collection.

Human nasal-lavage samples for the determination of allantoin were obtained from a double-blinded, crossover control ozone challenge study performed at the University Hospital of Northern Sweden, Umeå (24). In this study 15 healthy subjects (seven male, eight female subjects; mean age, 24 ± 2.6 years), all nonsmokers with no preexisting allergic or respiratory disease, were exposed on three separate occasions, once to filtered air, and twice to 0.2 ppm of ozone for 2 h, with separate exposures at least 3 weeks apart. Ozone exposures were performed after either a 7-day treatment with a vitamin

C and E cosupplement [500 mg vitamin C, 100 mg vitamin E, (RRR)- α -tocopherol acetate] or a placebo compound, with the final tablet taken with the subject's breakfast 1 h before entering the exposure chamber. Full details of the exposure protocol and exposure setup were published previously (24). Nasal-lavage baseline samples were obtained from subjects immediately before entering the chamber, 1 h into the actual exposure (1h-E), immediately after the 2-h exposure (0 h after exposure, 0h-PE), and 6 h after the end of the challenge (6 h after exposure, 6h-PE). Informed consent was obtained from each volunteer, and the Ethics Committee of Umeå University approved the study in accordance with the Declaration of Helsinki.

Samples for allantoin determination in biologic fluids

The method was initially validated for both plasma and serum samples by using pooled reference samples. Initial method development and *in vitro* evaluation of extraction artifacts used pooled samples kept in the laboratory for the purpose of quality control and method development. Evaluations of clinical baseline levels, detection limits, spike recovery, and precision were carried out by using serum samples collected from healthy volunteers (see earlier). Serum samples were collected into 10-ml serum-separator tubes. Separation of serum from blood cells was achieved by centrifugation at 2,000 *g* at 4°C for 10 min. Samples were stored at -80°C before analysis.

Nasal-lavage samples were collected by using the metered nasal-spray method (25). In brief, five 10×0.1-ml volumes of 0.9% saline were sprayed into each nostril. The recovered material was pooled and transferred into a sterile plastic receptacle maintained on wet ice. The lavage was self-administered by the subject after demonstration of the procedure, with the aspirates collected after each 1-ml instillation to limit the instilled volume and dwell time of the saline in the nose. In total, the protocol took ~2 min per nostril, with a total instilled volume of 10 ml. The recovered aspirate was filtered through a sterile 100- μ m pore nylon filter to remove mucus aggregates before centrifugation at 400 *g* at 4°C for 15 min to isolate the cell-free fraction. Nasal-lavage samples for the determination of urate and allantoin were treated the metal (Chelator EDTA): 0.4 ml of lavage, plus 0.7 ml 1.5 mM EDTA, before storage at -80°C.

Statistical analysis

Data were analyzed by using SPSS for Windows (SPSS Inc., Chicago, IL) and the R software environment for statistical computing and graphics (R Foundation for Statistical Computing, Wien, Austria). Serum allantoin values were not normally distributed (Shapiro-Wilk normality test); therefore, data are presented as median (5–95% percentiles), unless otherwise specified. Serum urate data were normally distributed and are therefore summarized as means with standard deviations. All antioxidant and oxidation marker concentrations in NLF were nonparametric and are hence expressed throughout as median concentrations with the interquartile range. Significant-difference testing was performed by using the Mann-Whitney *U* test at $p < 0.05$ or Student's *t* test for all unpaired data. For the NLF in which data were paired, comparisons between the groups were performed by using the Wilcoxon signed-rank test.

Results

Assay development

Despite extensive testing of reverse-phase and ion-exchange materials, allantoin was not retained satisfactorily on any of the SPE matrices tested unless alkaline conditions were used (data not shown). We therefore developed a modified GC/MS procedure that allowed us to avoid SPE entirely. Samples were spiked with [¹⁵N]-labelled allantoin as internal standard, extracted into acetonitrile, and dried under N₂ for analysis with GC/MS. The detection limit (the concentration at which the peak area exceeded the baseline noise by at least a factor of 10) was determined in human serum by using allantoin-U-¹⁵N₄ (yielding a triply labeled ion with an *m/z* of 401) to be <2 pmol (data not shown). When applied to

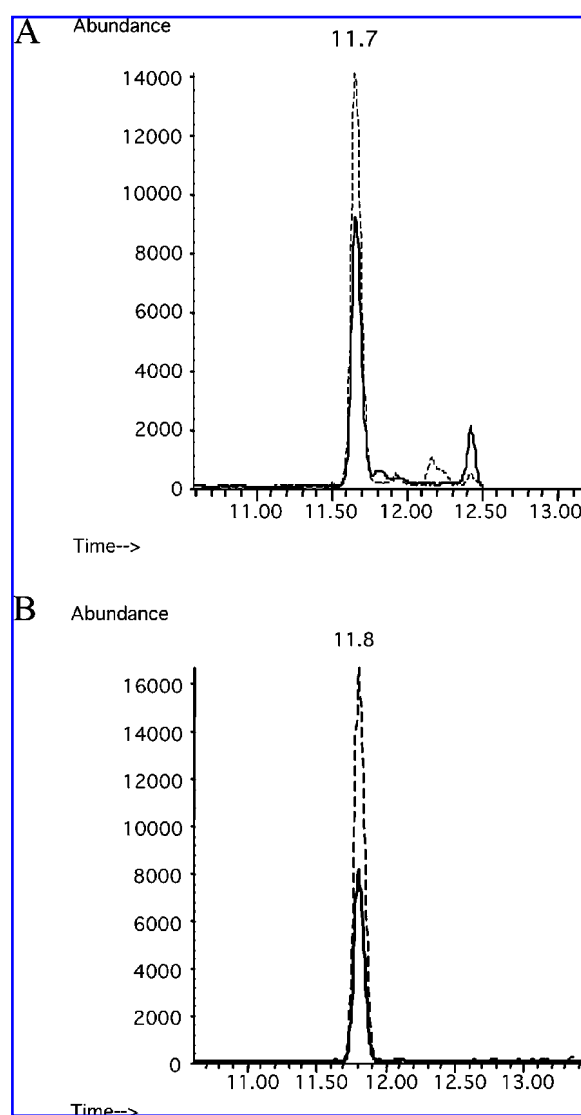


FIG. 1. Quantification of allantoin by isotope dilution. Peaks of endogenous allantoin from extraction of 25 μ l of human serum (A) and nasal lavage fluids (B) without alkaline solid phase extraction. Quantifier ion at 398 *m/z* (solid line) and internal standard ion at 400 *m/z* (dotted line) are shown.

TABLE 1. ASSAY VARIABILITY AND RECOVERY

| Precision sample | Low | Medium | High |
|-------------------------|----------------------------|------------------|-------|
| N (repeated days) | 20 | 20 | 20 |
| Conc. (μM) | 1.19 | 2.43 | 14.60 |
| Total precision | | | |
| SD | 0.195 | 0.365 | 0.972 |
| CV | 16.4% | 15.0% | 6.7% |
| Within-run | | | |
| SD | 0.123 | 0.10 | 0.525 |
| CV | 10.3% | 4.1% | 3.6% |
| Spike recovery | | | |
| Spike (μM) | Observed (μM) | Recovery (%) | |
| 0 | 5.35 \pm 0.02 | | |
| 1 | 6.56 \pm 0.03 | 121.0 | |
| 2.5 | 7.70 \pm 0.02 | 93.9 | |
| 5 | 10.11 \pm 0.01 | 95.3 | |
| | Average recovery | 103.4 \pm 15.3 | |

Variability data were collected by using pooled serum samples at high, medium, and low concentrations. Two repeated runs of two aliquots each were analyzed daily for each of the three concentrations for determination of total and within-run variability in accordance with NCCLS guidelines. Spike recovery at each of three spike levels was determined for three independent samples.

pooled serum, plasma, or nasal-lavage samples from healthy subjects, our method yields clearly detectable concentrations of allantoin, and the selective ion chromatogram is free of confounding peaks (Fig. 1). Total variability and within-run variability were determined in pooled serum samples at low (1.2 μM), medium (2.4 μM), and high (14.6 μM) concentrations. This concentration range spans the majority of the clinical serum data observed (see later). Repeated data were collected in accordance with National Committee on Clinical Laboratory Standards (NCCLS) protocol EP5-A2 (28). The data showed acceptable inter- and intraday variabilities over the 20-day test period (Table 1).

It was previously suggested that salts, such as potassium phosphate and sodium acetate, can interfere with allantoin quantification (8). However, when serum samples were spiked with 1, 2.5, and 5 μM allantoin, we were able to quantitate accurately the allantoin spike levels in each case, even in the presence of relatively high endogenous allantoin levels, without the need for desalting. Recovery of spiked allantoin over this concentration range was \sim 95% (Table 1).

Assessment of artifactual conversion of urate to allantoin in previous alkali-based methods

First, we subjected analytic grade urate standards either to SPE in the presence of NaOH or to our modified GC/MS procedure, avoiding alkaline conditions. With our method, allantoin concentrations in pure urate standards (400 μM) are very low [0.27 \pm 0.03 μM ($n = 3$)]. However, after the alkaline SPE procedure for allantoin extraction, levels of 13.5 \pm 0.5 μM (or 3.38% urate) were detected in the urate standards. This increase in allantoin during SPE is associated with a decrease in urate from 399 \pm 2 μM before alkaline extraction to 381 \pm 2 μM after SPE. The increase in allantoin of 13.5 \pm 0.5 μM

therefore is associated with a comparable loss of urate of about 18 \pm 4 μM . This result is consistent with artifactual *in vitro* conversion of urate to allantoin. We also incubated human blood plasma with or without 50 mM NaOH, by using the same conditions used to facilitate binding of allantoin during SPE, and found that addition of NaOH to a final concentration of 50 mM was sufficient to increase allantoin concentrations from 2.4 to 14 μM . This suggests that the NaOH concentrations used during SPE of allantoin from plasma and serum in former methods can convert significant amounts of urate to allantoin. To confirm this, we used [1,3- $^{15}\text{N}_2$] urate spiking of plasma. When [1,3- $^{15}\text{N}_2$] urate is oxidized to allantoin, heavy [^{15}N]-labeled allantoin is produced, which is not normally present in the human body. Plasma samples spiked with [1,3- $^{15}\text{N}_2$] urate equivalent to 200, 300, or 400 μM were analyzed by using either our method or the reference GC/MS method, including alkaline SPE conditions. The SI-isotope-corrected 400–398 m/z ion ratio is calculated by subtracting 0.1523 from the experimentally observed ion ratio (8, 29). A corrected 400–398-m/z ion ratio larger than zero indicates conversion of the spiked [1,3- $^{15}\text{N}_2$] urate to [^{15}N]-labelled allantoin.

When [1,3- $^{15}\text{N}_2$] urate-spiked plasma samples were subjected to the alkaline SPE procedure, we found that the corrected ion ratio increased nonlinearly with the concentration of spiked [1,3- $^{15}\text{N}_2$] urate (Fig. 2). When plasma was spiked with high, but physiologically relevant levels of [1,3- $^{15}\text{N}_2$] urate (equivalent to 400 μM plasma urate), the corrected 400–398 m/z ion ratio was \sim 1.3. This indicates that under these conditions, the amount of ^{15}N -labelled allantoin produced by artifactual conversion of [1,3- $^{15}\text{N}_2$] urate exceeded the total amount of unlabelled endogenous allantoin in the sample. No [^{15}N]-labelled allantoin was detected when alkaline SPE conditions or spiking with [1,3- $^{15}\text{N}_2$] urate was omitted (Fig. 2).

Comparison of allantoin and F_2 -isoprostanes as marker of *in vitro* oxidative damage

To compare the utility of allantoin as a marker of oxidative damage with that of an established biomarker of oxidative damage, we used a simple *in vitro* paradigm of oxidative stress in human plasma. Hemoglobin in the presence of H_2O_2 has long been used to induce elevated levels of oxidative lipid peroxidation and is known to be capable of oxidizing urate with the formation of allantoin (2). However, even in the absence of added H_2O_2 , free hemoglobin favors peroxidation when added to lipid-containing systems (15).

To test the sensitivity of allantoin relative to a well-established and widely used marker of lipid oxidation, total F_2 -isoprostanes, we therefore chose to investigate the degree of correlation between total F_2 -isoprostanes and allantoin levels after incubation of plasma samples with increasing amounts of hemoglobin in the form of lysed whole blood. As expected, we found that with increasing amounts of hemoglobin, both total F_2 -isoprostanes and allantoin levels are robustly elevated [maximum \sim 300% and 700% of baseline, respectively (Fig. 3)]. We further found that, although a significant amount of scatter occurs, a moderately good correlation exists between total F_2 -isoprostanes and allantoin over the whole range of conditions tested (Pearson correlation coefficient, 0.69; $p < 0.0001$).

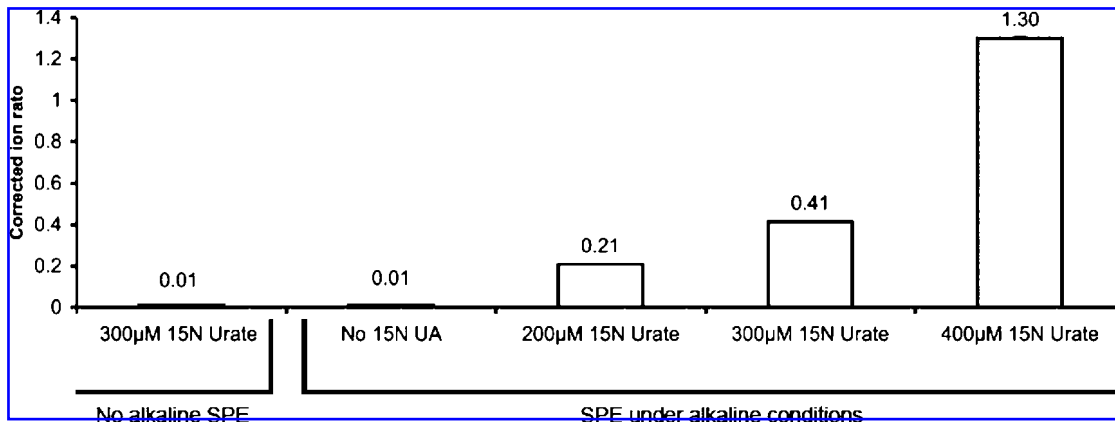


FIG. 2. Corrected ion ratio (400–398 m/z) as a function of $[1,3-^{15}\text{N}_2]$ urate concentration. Human plasma samples were spiked with increasing amounts of $[1,3-^{15}\text{N}_2]$ urate. No $[^{15}\text{N}]$ -labeled allantoin internal standard was added. Any ^{15}N -labeled allantoin detected, therefore, derives entirely from artifactual conversion for $[1,3-^{15}\text{N}_2]$ urate to allantoin. A corrected ion ratio of 1 indicates that at least as much ^{15}N -labeled heavy allantoin is present in the sample as is endogenous allantoin. When $[1,3-^{15}\text{N}_2]$ urate equivalent to $400\ \mu\text{M}$ is added, the ion ratio exceeds unity, suggesting that the extraction artifact in this case exceeds endogenous allantoin concentrations.

Baseline serum allantoin concentrations in human volunteers

We obtained fasting blood samples from 98 volunteers aged between 40 and 96 years. Baseline serum concentrations of allantoin in this cohort irrespective of gender were median $2.36\ \mu\text{M}$ with 5–95% percentiles (0.85 – 7.95) μM . A statistically significant trend to lower allantoin concentrations was found in women 2.18 (0.76 – 9.26) μM relative to men 2.77 (0.92 – 8.25) μM ($p = 0.032$, Mann–Whitney U test). Because urate is the precursor of allantoin, serum urate also was measured. A weak but statistically significant correlation was observed between serum urate and allantoin (Spearman's $\rho = 0.52$; $p < 0.001$).

Using allantoin formation as a biomarker

Ozone inhalation is well known to cause oxidative stress to the respiratory tract in humans. Ozone also has a high in-

trinsic reactivity to urate (26), and concentrations of this antioxidant have been shown to be decreased in human nasal lining fluids after controlled ozone exposures (25). In the latter study, although urate concentrations decreased by $\sim 30\%$, it was not possible to address whether this reflected the oxidation of urate *in vivo*, or its impaired transport onto the lung surface. In the current study, we observed a significant ozone-induced elevation of allantoin concentrations in human NLF samples after a high, but environmentally relevant dose (200 ppb for 2 h) ozone challenge after a period of vitamin C and E supplementation, or treatment with placebo (Fig. 4). The study was a crossover design, with volunteers being exposed in random order to both ozone and filtered air, with exposures separated by a period of at least 3 weeks. Baseline, preozone, and air concentrations did not differ significantly (Wilcoxon Signed Rank Test) for either urate or allantoin and were significantly correlated over the interval between exposures: Urate preair 36.5 (24.2 – 69.8) *versus* preozone 24.5 (14.0 – 62.7)

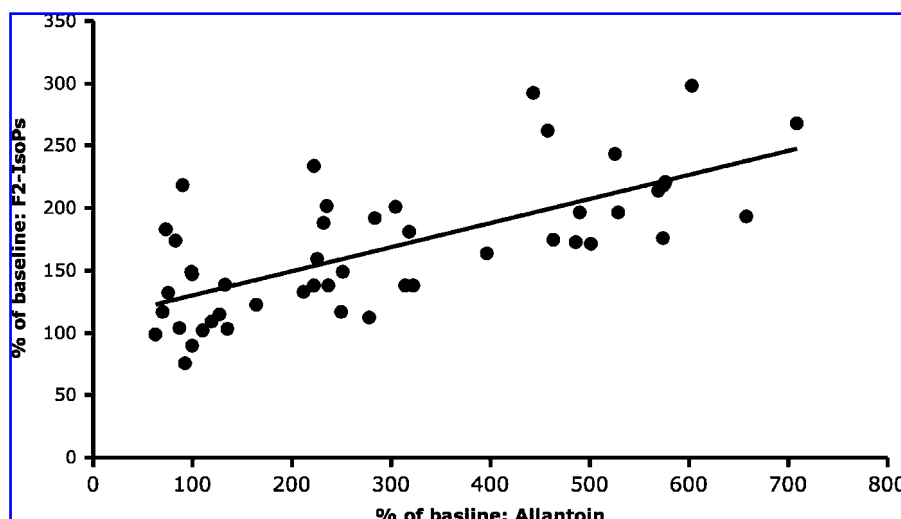


FIG. 3. Scatterplot comparing allantoin and F_2 -isoprostane levels in human plasma subjected to *in vitro* oxidative challenge. Scatterplot and best linear fit of F_2 -isoprostane and allantoin levels in plasma samples exposed to increasing amounts of hemoglobin. For each volunteer and condition, F_2 -isoprostane and allantoin levels were normalized to control sample from the same volunteer not exposed to any whole-blood lysate. Hemoglobin-induced increases in allantoin show moderately good correlation to those in total F_2 -isoprostanes tested (Pearson correlation coefficient, 0.69 ; $p < 0.0001$).

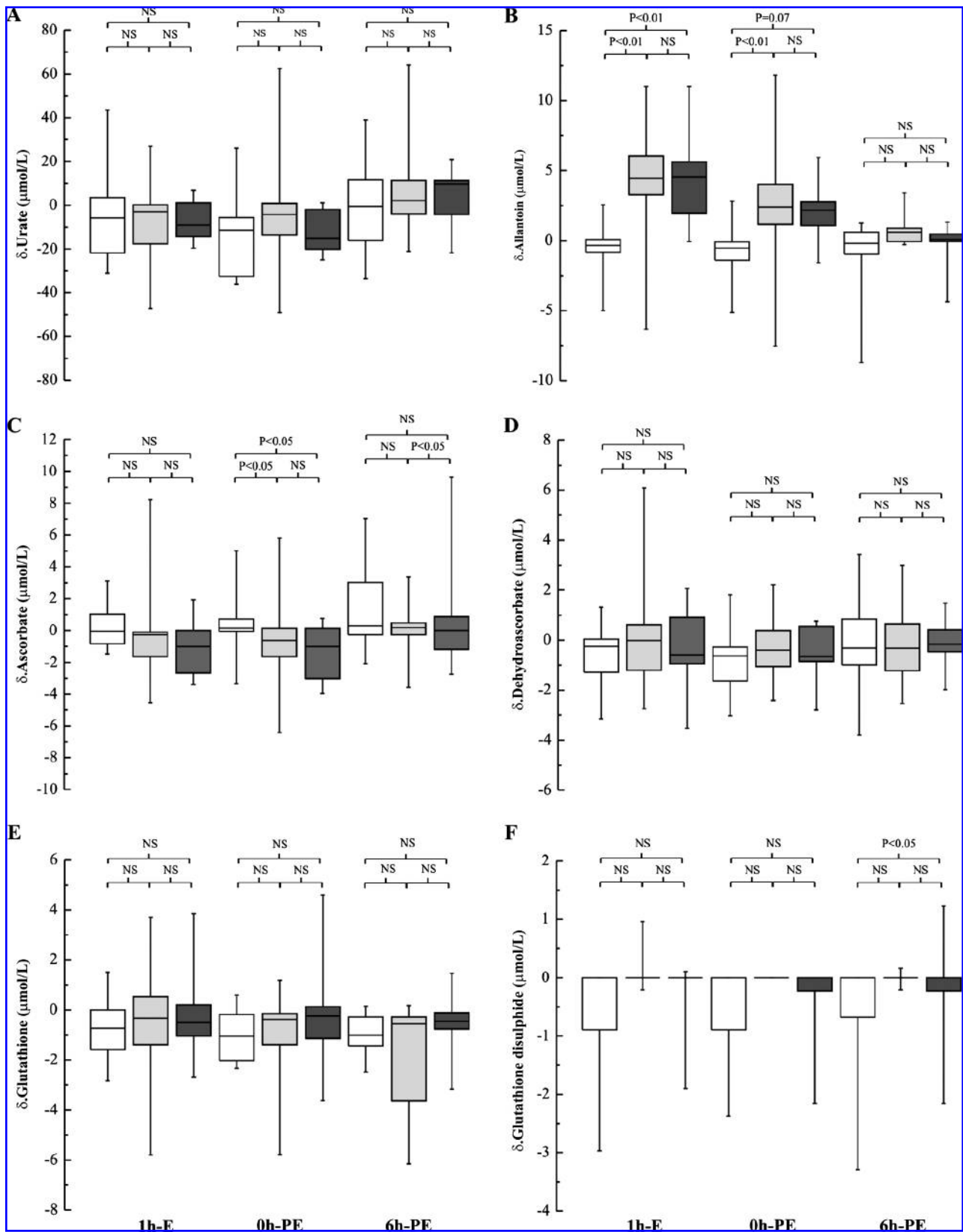


FIG. 4. Allantoin as a marker of ozone-induced oxidative stress. The molar change (δ) in nasal lavage fluid urate/allantoin (**A**, **B**), ascorbate/dehydroascorbate (**B**, **C**), and glutathione/glutathione disulphide concentrations (**E**, **F**) during and after ozone (*light grey boxes*, with placebo; *dark grey boxes*, after vitamin supplementation) and filtered air (*white boxes*) relative to preexposure concentrations. Data are presented as median values (*central line*) with the 25th and 75th percentiles (*lower and upper box boundaries*) and 95% confidence intervals (*whiskers*). Comparison of paired air and ozone concentrations was performed by using the Wilcoxon Signed Rank Test, with the *p* value indicated. NS, not significantly different from parallel air response; 1h-E, 1 h into the actual exposure; 0h-PE, immediately after exposure; and 6h-PE, 6 h after exposure.

μM ; $r = 0.61$; $p = 0.03$ (Spearman correlation), and allantoin: 1.4 (0.9–2.1) versus preozone 1.2 (0.9–1.9) μM ; $r = 0.60$; $p = 0.03$. The change in concentration of both urate and allantoin at the prescribed sampling points relative to the preexposure values are illustrated in Fig. 4A and B and demonstrate that although no significant loss of urate occurs during or after exposure, a significant increase in the concentration of allantoin 1 h into and immediately after exposure, returning to control concentrations 6 h after the challenge. Importantly, although the increase in allantoin immediately after exposure was diminished relative to the 1h-E time point, the individual magnitudes of change were highly correlated at these two sampling times: $r = 0.79$; $p = 0.01$. Supplementation for volunteers with oral antioxidants did not significantly reduce allantoin levels in NLF, indicating that antioxidant treatment was ineffective in preventing urate oxidation to allantoin (Fig. 4A and B). Although the evidence for an ozone-induced increase in allantoin was clear, increases in dehydroascorbate (Fig. 4D) and glutathione disulfide (Fig. 4F) was not observed during or immediately after exposure, with GSSG being largely below the detection limit in the NLF samples. Evidence was noted of a loss of NLF ascorbate 0h-PE, after both ozone challenges, suggesting that oxidation was occurring (Fig. 4C), but this was not matched by an increase in dehydroascorbate, probably reflecting the instability of this oxidation product at physiologic pH (6).

Discussion

Urate is present in blood and other body fluids at concentrations up to two orders of magnitude above those of allantoin and is easily oxidized to allantoin *in vitro* under alkaline conditions. Therefore, artifactual conversion of even a small percentage of urate to allantoin can lead to gross overestimation of allantoin concentrations. We have introduced a method to avoid this. The high sensitivity and specificity of GC/MS allow us to detect low amounts (pmol) of allantoin in very small samples (25 μl) without the need for solid-phase extraction. Our method is simple and rapid, with up to 60 samples being routinely processed in the laboratory in 1 day. We therefore believe that the determination of allantoin can now become more widely used as a routine method in clinical studies as a biomarker of oxidative stress.

When our method was applied to human serum samples, we detected allantoin at concentrations that were significantly lower than the values found by using earlier HPLC and GC/MS methods (Table 2). This is consistent with the assumption that at least some previous methods might be subject to artifacts. Our values are consistent with those detected by using the more recent HPLC methods by Lagendijk *et al.* (21) and Kandar *et al.* (18), both of which were designed to remove urate and other interfering substances before quantification with HPLC, a different approach from the one that we have taken here. By comparison with these methods, the method presented here has the advantage that it does not require any SPE step for the removal of urate before analysis, reducing time and resource requirement. Furthermore, by using our method, any loss or degradation during sample workup will affect the isotope-labeled internal standard in the same way as endogenous allantoin. The addition of heavy labeled internal standard before sample extraction therefore makes our assay highly robust against extraction- and

purification-related artifacts. We used a simple *in vitro* experiment to generate plasma samples exhibiting a relatively wide range of oxidative damage. With respect to the physiologic relevance of the degree of oxidation observed in this *in vitro* setting, it should be noted that it has previously been reported that F₂-isoprostane levels in certain pathologic situations can be more than twice as high as those of well-matched controls (*e.g.*, ref. 1). This indicates that the highest degree of *in vitro* oxidative damage imposed in our plasma samples (~300%) is comparable to levels that can indeed be observed *in vivo* under certain conditions, at least as far as F₂-isoprostanes are concerned. We acknowledge that this simple *in vitro* paradigm cannot predict the performance of allantoin relative to F₂-isoprostanes in the context of elevated *in vivo* oxidative stress. However, the relatively good correlation of allantoin with the more-established biomarker over the whole range of oxidative damage observed suggests that allantoin, at least in this *in vitro* setting, is a robust marker of oxidative damage, comparable to F₂-isoprostanes.

Having established and validated a method for the determination of allantoin in serum and plasma, free from artifactual sample oxidation, we next examined allantoin concentrations in NLF samples obtained from healthy subjects exposed to ozone, with and without supplementation with vitamin C and E. Previous work demonstrated ozone-dependent oxidation of plasma (9) and nasal-lining fluid urate *ex vivo* (16), as well as evidence of decreased urate concentrations *in vivo* (25). Whereas previous studies demonstrated a loss of urate during and immediately after ozone exposure, in

TABLE 2. COMPARISON OF THIS STUDY WITH PREVIOUS STUDIES MEASURING ALLANTOIN IN HUMAN SUBJECTS

| Method | Ref. number | No. of subjects | Allantoin (μM) |
|-----------|-------------|----------------------------|--|
| HPLC | 12 | 2 | 7.5 \pm 4.6 |
| HPLC | 10 | 7 | 18.6 \pm 3.8 |
| HPLC | 20 | 99 | 2.4 \pm 0.6 |
| HPLC | 21 | 18 | 12.4 \pm 20.6 |
| HPLC | 18 | 171 | 15.7 \pm 7.9 |
| HPLC | 19 | Male (180) Female (145) | 16.7 \pm 8.3 15.8 \pm 7.2 |
| HPLC | 3 | 40 | 20.9 \pm 3.1 |
| HPLC | 16 | 19 | 20.3 \pm 8.1 |
| HPLC | 15 | 24 | 22 \pm 12 |
| HPLC | 14 | 30 | 4.67 \pm 2.99 |
| HPLC | 4 | 6 | 3.5 \pm 1.0 |
| GC/MS | 27 | Male (56) Female (78) | 13.4 [7.4–46.8] ^a 10.8 [3.7–31.2] ^a |
| GC/MS | 34 | 15 | 13.6 \pm 6.3 |
| GC/MS | 32 | 35 | 12.6 \pm 6.3 |
| GC/MS | 33 | 21 | 15.9 \pm 6.9 |
| GC/MS | This study | Male (49) Female (49) | 2.77 (0.92–8.25) ^b 2.18 (0.76–9.26) ^b |
| Enzymatic | 26 | 30 | 8.2 \pm 3.1 |

Values listed for each study are baseline levels established for healthy controls by using various methods and published in the literature. Values are listed as mean \pm SD when such data were reported in the original publication.

^aValues in square brackets are 95% confidence intervals for mean baseline levels.

^bValues in brackets are 5th and 95th percentiles for median values.

the current study, no such loss was apparent. The evidence that allantoin concentrations increased at these times, however, illustrates that the loss of urate might be being masked by rapid-repletion kinetics into the nasal-lining fluids after the sequential lavages.

Interestingly, induction of *in vivo* oxidation of urate to allantoin in the presence of ozone was much more robust than that of either ascorbate to dehydroascorbate, or glutathione to glutathione disulfide in the same samples. This was the case for ascorbate, even after vitamin C supplementation, which has previously been shown to induce a transient three- to fourfold increase in nasal-lining fluid ascorbate concentrations (4). This suggests that urate may be a more relevant antioxidant in this particular setting, a result consistent with both its rapid reaction rate with ozone (26) and its relatively high abundance compared with other low-molecular-weight antioxidants in NLF as well as serum (31, 32), even after supplementation with ascorbate (26). Given this dominant role of urate relative to ascorbate and glutathione in the NLF, the observation that supplementation of volunteers with oral ascorbate before ozone exposure is ineffective in preventing urate oxidation to allantoin is perhaps not surprising. The direct evidence of urate oxidation by ozone in the nasal-lining fluids also raises the possibility that the upper airways may effectively scrub a significant proportion of inhaled ozone, limiting its interaction with more-sensitive distal lung cells (9). Together these data highlight the utility of allantoin as a biomarker of urate oxidation *in vivo*, providing a tool for estimating the ozone-scavenging capacity of the upper airway as well as a marker of oxidative damage in plasma and serum. The method described here should allow the more frequent use of allantoin measurements to assess oxidative damage accurately *in vivo*.

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Abbreviations

BSTFA, *N,O*-bis-(trimethylsilyl) trifluoroacetamide; DIPEA, *N,N*-diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide; NCCLS, National Committee on Clinical Laboratory Standards; NLF, nasal-lavage fluid; PFBBr, *penta*-fluorobenzylbromide; SPE, solid-phase extraction; TBDMS, *tert*-butyl-dimethylsilyl; TMCS, trimethylchlorosilane.

Author Disclosure Statement

No competing financial interests exist.

References

- Aleynik SI, Leo MA, Aleynik MK, and Lieber CS. Increased circulating products of lipid peroxidation in patients with alcoholic liver disease. *Alcohol Clin Exp Res* 22: 192–196, 1998.
- Ames BN, Cathcart R, Schwiers E, and Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci U S A* 78: 6858–6862, 1981.
- Aruoma OI and Halliwell B. Inactivation of alpha 1-antiproteinase by hydroxyl radicals: the effect of uric acid. *FEBS Lett* 244: 76–80, 1989.
- Behndig AF, Blomberg A, Helleday R, Kelly FJ, and Mudway IS. Augmentation of respiratory tract lining fluid ascorbate concentrations through supplementation with vitamin C. *Inhal Toxicol* 21: 250–258, 2009.
- Benzie IF, Chung W, and Tomlinson B. Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant:antioxidant balance studies. *Clin Chem* 45: 901–904, 1999.
- Buettner GR and Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* 145: 532–541, 1996.
- Causse E, Pradelles A, Dirat B, Negre-Salvayre A, Salvayre R, and Couderc F. Simultaneous determination of allantoin, hypoxanthine, xanthine, and uric acid in serum/plasma by CE. *Electrophoresis* 28: 381–387, 2007.
- Chen XB, Calder AG, Prasitkusol P, Kyle DJ, and Jayasuriya MC. Determination of ¹⁵N isotopic enrichment and concentrations of allantoin and uric acid in urine by gas chromatography/mass spectrometry. *J Mass Spectrom* 33: 130–137, 1998.
- Cross CE, Motchnik PA, Bruener BA, Jones DA, Kaur H, Ames BN, and Halliwell B. Oxidative damage to plasma constituents by ozone. *FEBS Lett* 298: 269–272, 1992.
- Doehner W, Schoene N, Rauchhaus M, Leyva-Leon F, Pavitt DV, Reaveley DA, Schuler G, Coats AJ, Anker SD, and Hambrecht R. Effects of xanthine oxidase inhibition with allopurinol on endothelial function and peripheral blood flow in hyperuricemic patients with chronic heart failure: results from two placebo-controlled studies. *Circulation* 105: 2619–2624, 2002.
- Ellerbe P, Cohen A, Welch MJ, and White E. The stability of uric acid in ammonium hydroxide. *Clin Chem* 34: 2280–2282, 1988.
- Esteve MJ, Farre R, Frigola A, and Garcia-Cantabella JM. Determination of ascorbic and dehydroascorbic acids in blood plasma and serum by liquid chromatography. *J Chromatogr B Biomed Sci Appl* 688: 345–349, 1997.
- Grootveld M and Halliwell B. Measurement of allantoin and uric acid in human body fluids: a potential index of free-radical reactions *in vivo*? *Biochem J* 243: 803–808, 1987.
- Grootveld M, Halliwell B, and Moorhouse CP. Action of uric acid, allopurinol and oxypurinol on the myeloperoxidase-derived oxidant hypochlorous acid. *Free Radic Res Commun* 4: 69–76, 1987.
- Halliwell B and Gutteridge JMC. *Free radicals in biology and medicine*, 4th ed. Oxford: Oxford University Press, 2007.
- Housley DG, Mudway I, Kelly FJ, Eccles R, and Richards RJ. Depletion of urate in human nasal lavage following *in vitro* ozone exposure. *Int J Biochem Cell Biol* 27: 1153–1159, 1995.
- Iriyama K, Yoshiura M, Iwamoto T, and Ozaki Y. Simultaneous determination of uric and ascorbic acids in human serum by reversed-phase high-performance liquid chromatography with electrochemical detection. *Anal Biochem* 141: 238–243, 1984.
- Kand'ar R, Zakova P, and Muzakova V. Monitoring of antioxidant properties of uric acid in humans for a consideration

- measuring of levels of allantoin in plasma by liquid chromatography. *Clin Chim Acta* 365: 249–256, 2006.
19. Kaur H and Halliwell B. Action of biologically relevant oxidizing species upon uric acid: identification of uric acid oxidation products. *Chem Biol Interact* 73: 235–247, 1990.
 20. Kock R, Delvoux B, and Greiling H. A high-performance liquid chromatographic method for the determination of hypoxanthine, xanthine, uric acid and allantoin in serum. *Eur J Clin Chem Clin Biochem* 31: 303–310, 1993.
 21. Legendijk J, Ubbink JB, and Vermaak WJ. The determination of allantoin, a possible indicator of oxidant status, in human plasma. *J Chromatogr Sci* 33: 186–193, 1995.
 22. Lee CY, Huang SH, Jenner AM, and Halliwell B. Measurement of F₂-isoprostanes, hydroxyeicosatetraenoic products, and oxysterols from a single plasma sample. *Free Radic Biol Med* 44: 1314–1322, 2008.
 23. Mikami T, Kita K, Tomita S, Qu GJ, Tasaki Y, and Ito A. Is allantoin in serum and urine a useful indicator of exercise-induced oxidative stress in humans? *Free Radic Res* 32: 235–244, 2000.
 24. Mudway IS, Behndig AF, Helleday R, Pourazar J, Frew AJ, Kelly FJ, and Blomberg A. Vitamin supplementation does not protect against symptoms in ozone-responsive subjects. *Free Radic Biol Med* 40: 1702–1712, 2006.
 25. Mudway IS, Blomberg A, Frew AJ, Holgate ST, Sandstrom T and Kelly FJ. Antioxidant consumption and repletion kinetics in nasal lavage fluid following exposure of healthy human volunteers to ozone. *Eur Respir J* 13: 1429–1438, 1999.
 26. Mudway IS and Kelly FJ. Modeling the interactions of ozone with pulmonary epithelial lining fluid antioxidants. *Toxicol Appl Pharmacol* 148: 91–100, 1998.
 27. Mudway IS, Stenfors N, Duggan ST, Roxborough H, Zielinski H, Marklund SL, Blomberg A, Frew AJ, Sandstrom T, and Kelly FJ. An in vitro and in vivo investigation of the effects of diesel exhaust on human airway lining fluid antioxidants. *Arch Biochem Biophys* 423: 200–212, 2004.
 28. NCCLS. *Clinical and laboratory standards I. Evaluation of precision performance of quantitative measurement methods; approved guideline-second edition*. NCCLS document EP5–A2. Clinical and Laboratory Standards Institute: PA, 2004.
 29. Pavitt DV, de Fonseca S, Al-Khalaf N, Cam JM, and Reaveley DA. Assay of serum allantoin in humans by gas chromatography-mass spectrometry. *Clin Chim Acta* 318: 63–70, 2002.
 30. Simic MG and Jovanovic SV. Antioxidation mechanisms of uric acid. *J Am Chem Soc* 111: 5778–5782, 1989.
 31. van der Vliet A, O'Neill CA, Cross CE, Koostra JM, Volz WG, Halliwell B, and Louie S. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 276: L289–L296, 1999.
 32. Wayner DD, Burton GW, Ingold KU, Barclay LR, and Locke SJ. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxy radical-trapping antioxidant activity of human blood plasma. *Biochim Biophys Acta* 924: 408–419, 1987.
 33. Whiteman M and Halliwell B. Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antitrypsinase inactivation by ascorbic acid: a comparison with other biological antioxidants. *Free Radic Res* 25: 275–283, 1996.
 34. Whiteman M, Ketsawatsakul U, and Halliwell B. A re-assessment of the peroxynitrite scavenging activity of uric acid. *Ann N Y Acad Sci* 962: 242–259, 2002.
 35. Yardim-Akaydin S, Kesimer M, Imren E, Sepici A, Simsek B, and Torun M. Urate oxidation during percutaneous transluminal coronary angioplasty and thrombolysis in patients with coronary artery disease. *Clin Chim Acta* 362: 131–137, 2005.
 36. Yardim-Akaydin S, Sepici A, Ozkan Y, Simsek B, and Sepici V. Evaluation of allantoin levels as a new marker of oxidative stress in Behcet's disease. *Scand J Rheumatol* 35: 61–64, 2006.
 37. Yardim-Akaydin S, Sepici A, Ozkan Y, Torun M, Simsek B, and Sepici V. Oxidation of uric acid in rheumatoid arthritis: is allantoin a marker of oxidative stress? *Free Radic Res* 38: 623–628, 2004.

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3. Raymond C.S. Seet, Chung-Yung J. Lee, Wai Mun Loke, Shan Hong Huang, Huiwen Huang, Woan Foon Looi, Eng Soh Chew, Amy M.L. Quek, Erle C.H. Lim, Barry Halliwell. 2011. Biomarkers of oxidative damage in cigarette smokers: Which biomarkers might reflect acute versus chronic oxidative stress?. *Free Radical Biology and Medicine* **50**:12, 1787-1793. [[CrossRef](#)]
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