



MEASUREMENT OF 8-OXO-2'-DEOXYGUANOSINE AND 8-OXO-2'-DEOXYADENOSINE IN DNA AND HUMAN URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROSPRAY TANDEM MASS SPECTROMETRY

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(Received 28 August 2000; Revised 19 December 2000; Accepted 19 December 2000)

Abstract—A method for the determination of 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine in DNA and urine by High Performance Liquid Chromatography (HPLC)-Tandem Mass Spectrometry is described. For the urine samples there is no sample preparation except for addition of buffer and internal standards followed by redissolution of precipitate containing 8-oxo-2'-deoxyguanosine and a centrifugation step before the samples are injected onto the HPLC column. The detection limit for 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine is approximately 0.3 nM corresponding to 7.5 fmol injected. Long runs, that is, > 50 samples, can be analyzed with only minimal loss of sensitivity. The concentrations excreted into urine samples from humans are between 1 and 100 nM for 8-oxo-2'-deoxyguanosine and below 0.3 nM for 8-oxo-2'-deoxyadenosine. In calf thymus DNA levels down to about 1 oxidized guanosine and adenosine per 10⁶ unmodified bases can be detected. High levels of 8-oxo-2'-deoxyguanosine were found, 30 per 10⁶ 2'-deoxyguanosine, levels of 8-oxo-2'-deoxyadenosine are at or below the detection limit. These findings indicate that High Performance Liquid Chromatography-Tandem Mass Spectrometry is a highly sensitive and specific method for analysis of oxidative DNA modifications in tissue as well as for analysis of excretion of oxidized nucleotides into urine that ensures a minimum artifact formation. © 2001 Elsevier Science Inc.

Keywords—8-oxo-2'-deoxyguanosine, 8-oxo-2'-deoxyadenosine, Oxidative stress, Nucleosides, Electrospray, Isotope dilution, Free radicals

INTRODUCTION

Oxidative modification of DNA has attracted considerable interest because such modifications are abundant, mutagenic, and because there are specific repair enzymes for some of the modifications [1]. The lesions, particularly the 8-hydroxylation of the guanine moiety of DNA, have attracted much interest and have been advocated as important pathophysiological mechanisms for ageing and cancer, two important health issues. Presently more than 100 different oxidative modifications in DNA have been described, mainly by gas chromatography-mass spectrometry (GC-MS). The electrochemical properties of 8-oxo-2'-deoxyguanosine (8-oxodG) are widely utilized to measure the lesions in nuclear and mitochondrial DNA and the excretion

of the modified nucleoside into urine. GC-MS has also been used for measurement of 8-hydroxy-guanine (8-oxoGua), however, artificial oxidation during sample preparation, workup, and analysis has been reported [2–7]. From a theoretical point of view the optimum method of analysis should include a minimum of sample preparation, fast analysis time, and limited exposure to oxygen combined with specific and sensitive detection. While HPLC-EC and GC-MS are used to analyze DNA for oxidative products, urinary analysis presents a more demanding task. HPLC-EC analysis of urine demands triple-column switching and separation before EC detection is feasible [8,9], or an immunochemical clean-up procedure, and still the analysis is limited to measurement of 8-oxodG, the only electrochemically detectable DNA lesion in urine.

To achieve these requirements we have developed a LC-MS/MS method that fulfills these demands and also shows potential for expansion with analysis of other oxidative modifications.

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In DNA the interesting point is to measure the fraction of 2'-deoxyguanosine (dG) and 2'-deoxyadenosine (dA) which has been modified, and thus it is necessary to measure both the unmodified and the oxidized deoxynucleosides. The concentration measured results from the balance between the rate of oxidation and the rate of repair. In urine the amount of excreted oxidation products per unit time is measured, representing an average measure of the oxidative stress to the total body DNA. This is independent of DNA repair [1] if steady state can be assured.

EXPERIMENTAL PROCEDURES

Chemicals

dG, dA, and 8-oxodG were purchased from Sigma (St. Louis, MO, USA); 8-oxo-2'-deoxyadenosine (8-oxodA) was purchased from Berry & associates, Inc. (Dexter, MI, USA); [$^{15}\text{N}_5$] 8-oxodG and [$^{15}\text{N}_5$] 8-oxodA were synthesized by Dr. Ravanat (CEA Grenoble); [$^{15}\text{N}_5$] dG and [$^{15}\text{N}_5$] dA were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Alkaline phosphatase was purchased from Boehringer Mannheim (GmbH—Mannheim, Germany) (Cat. No. 713 023). Nuclease P₁ and desferrioxamin were obtained from Sigma.

Apparatus

The HPLC used was a Perkin Elmer Series 200 HPLC equipped with two pumps, autosampler, solvent cabinet, and vacuum degasser (Perkin Elmer, Norwalk, CT, USA). The HPLC was fully controlled by the mass spectrometer: a Sciex API 3000 triple quadrupole mass spectrometer equipped with a turboionspray source (Sciex, Thornhill, Canada). Between the outlet of the HPLC column and the electrospray inlet a Valco Nitronic-60 6-port diverting valve was placed and driven by a Valco standard electric actuator (Valco Instruments, Houston, TX, USA). Only the eluent fractions that contained the nucleosides were transferred into the mass spectrometer. The early eluting components were diverted to waste.

The HPLC separation was performed on an Interchrom Hypersil C18 HPLC column (150 × 2 mm, 5 μ) (H5C18#15M), and the column was protected using a Hypersil C18 guard column (10 × 2 mm, 5 μ) (CHO99961), both obtained from Interchim (Montlucon, France). For urine samples the mobile phase was 10 mM ammonium formate, adjusted to pH 3.75 with formic acid, and 2% acetonitrile. For hydrolyzed calf thymus DNA the mobile phase was 10 mM ammonium formate, adjusted to pH 3.75 with formic acid, and 1.5% aceto-

nitrile. The flow rate was 200 μl/min for both sample types and the injection volume was typically 25 μl.

Electrospray ionization was performed in the positive ion mode. For all nucleosides the $[\text{M}+\text{H}]^+$ was selected by the first mass filter. After collision activation the $[\text{M}+\text{H}-116]^+$ ions corresponding to BH_2^+ were selected by the last mass filter. Nitrogen was used as nebulizing, curtain, heater (7.5 l/min) and collision gas. The electrospray probe temperature was 450°C. Individual tuning files were used for the two nucleosides and their oxidation products to achieve maximum sensitivity. According to this the potentials, collision gas pressure, and the flow of the gasses to the ion source were changed during each run. In order to remove contributions from other compounds, the resolution was increased slightly in both the first quadrupole Q1 and the third quadrupole Q3.

Sample preparation

Urine. A solution containing each of the stable isotopes labeled dA, dG, 8-oxodA, and 8-oxodG was added to a 100 mM lithiumacetate buffer pH 6.4.

The frozen urine was thawed and diluted 1:1 with the buffer containing the internal standards. A possible precipitate containing 8-oxodG was redissolved by heating to 37°C for 10 min and Whirly mixing before the samples were centrifuged at 1600 × g for 10 min. Addition of the buffer to the samples resulted in a final concentration of 50 nM of each of the labeled standards in the sample.

Hydrolyzed calf thymus DNA. Ten U Nuclease P1 was added to 100 μl 300 μg/ml calf thymus DNA solution in 0.1 mM desferrioxamin. The mixture was heated at 37°C for 30 min. One U alkaline phosphatase was added followed by heating at 37°C for 60 min. Chloroform (50 μl) was added before the sample was centrifuged at 5000 × g for 15 min. Internal standards were added to the water phase. A run without addition of labeled 8-oxodA was performed to eliminate possible contribution from approximately 2% contamination of unlabeled 8-oxodA in the standard, because this was added in a concentration 20 times higher than the detection limit.

RESULTS

Daughter ion spectra of the four analytes are shown in Fig. 1A–D.

A HPLC-MS/MS chromatogram of the two nucleosides and their oxidation product is shown in Fig. 2 (panel A) together with chromatograms of a urine sample (panel B) and hydrolyzed calf thymus DNA (panel C). Each chromatogram is divided into four time windows.

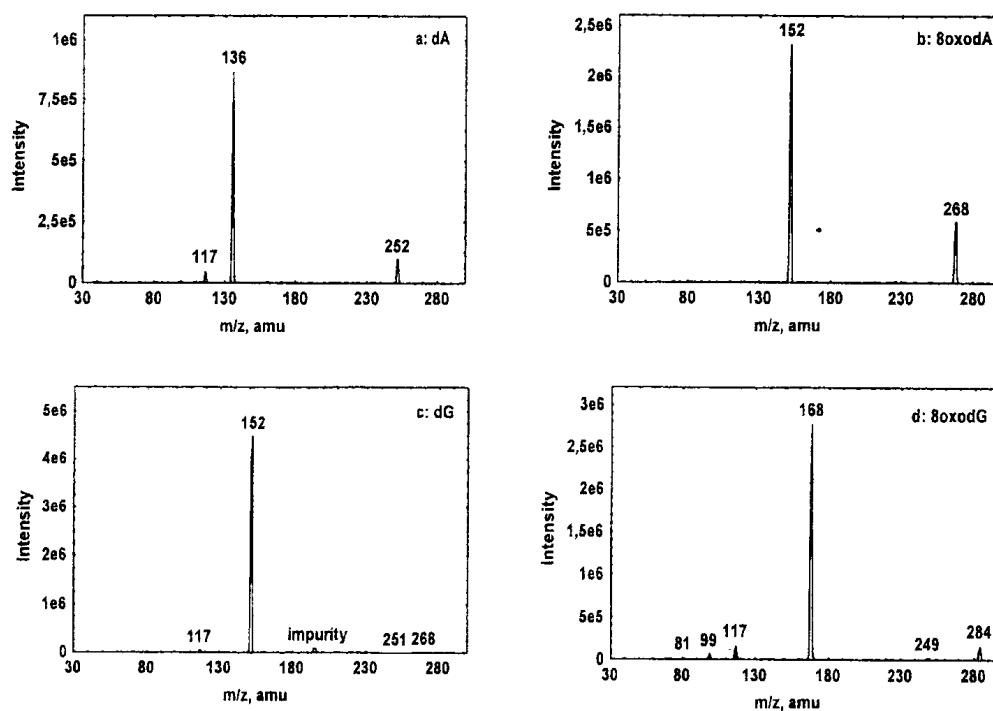


Fig. 1. Daughter ion spectra of $[M+H]^+$ of (A) 2'-deoxyadenosine, (B) 8-oxo-2'-deoxyadenosine, (C) 2'-deoxyguanosine, and (D) 8-oxo-2'-deoxyguanosine. The spectra are recorded by selecting the pseudo molecular ion ($[M+H]^+$) in the first quadrupole (Q1). After collision activation of the selected ions in the collision cell, the daughter ion spectra are recorded by scanning the last quadrupole (Q3). (Spectra recorded on a Sciex API 365 triple quadrupole mass spectrometer).

Time window 1 shows the 268/152 transition corresponding to dG, time window 2 the 284/168 transition corresponding to 8-oxodG, time window 3 the 252/136 transition corresponding to dA, and time window 4 the 268/152 transition corresponding to 8-oxodA.

The detection limits ($S/N = 3$) for 8-oxodG and 8-oxodA are approximately 0.3 nM corresponding to 7.5 fmol injected. The calibration curves for 8-oxodG and 8-oxodA are shown in Fig. 3A and 3B, respectively. The ruggedness of the method is shown in Fig. 4. In this experiment 50 samples were individually prepared from the same urine, that is, individually spiked with internal standards, diluted with buffer, precipitate dissolved, and centrifuged. After the runs the ratio between the peak area of the analyte and the internal standard was calculated. From a calibration curve the level of the analyte in the sample could be found. The coefficient of variation (CV) during injection of the 50 samples was 2.9%, and there was no change in the measured level during the 50 sample run for 37 h.

Eight-oxodA was not detected in any of the human urine samples that have been analyzed so far. In an attempt to see if 8-oxodA was present but below the normal detection limit, a human urine sample was concentrated $\times 25$ by freeze-drying. Even then no 8-oxodA was detected (data not shown) in spite of comparable

noise when running that sample and an unconcentrated sample.

In DNA samples the ratio between the oxidized and the nonoxidized forms of the deoxynucleosides can be expected to be in the range $1:10^5$ to $1:10^6$. With the current method the detection limits for the ratios 8-oxodG:dG and 8-oxodA:dA are about $1:10^6$.

It could be questioned whether the dynamic range of the mass spectrometer would be sufficiently large. If the dynamic range of dA and dG was sufficiently large, it was assumed that the range of 8-oxodA contra dA and 8-oxodG contra dG would also be sufficiently large, since the responses for all four deoxynucleosides were similar. Figure 5 shows the dynamic range for dA and dG. As can be seen, the dynamic range is about five to six orders of magnitude. This range is possible only by use of internal standards. Thus the dynamic range is sufficiently large to measure the ratio between oxidized and nonoxidized nucleosides in one run.

DISCUSSION

The purpose of the study was to develop a fast, sensitive, specific, and reliable method for the quantification of dG, dA, 8-oxodG, and 8-oxodA in urine, and to be able to measure the ratio between the oxidized and the

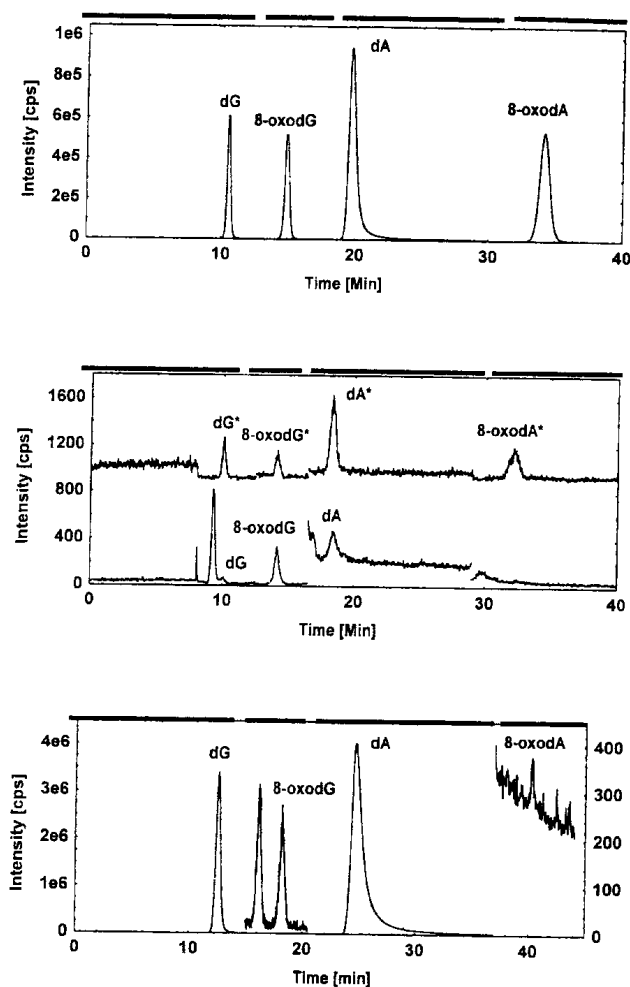


Fig. 2. HPLC-MS/MS chromatograms. Each chromatogram is divided into four time windows. Time window 1 shows the 268/152 transition corresponding to dG, time window 2 the 284/168 transition corresponding to 8-oxodG, time window 3 the 252/136 transition corresponding to dA, and time window 4 the 268/152 transition corresponding to 8-oxodA. (A) 10 μ M standard of dG, 8-oxodG, dA, and 8-oxodA. (B) Human urine sample. (C) Hydrolyzed calf thymus DNA. (The bars on top of each pane mark the length of each time window).

nonoxidized nucleosides in DNA. This type of direct method that measures more than one nucleoside has not been developed for urine before. Only a few papers have been published on measurement of modified nucleosides [10–14] or nucleobases [15,16] in DNA by HPLC or capillary electrophoresis [17] coupled with electrospray mass spectrometry. The measurement of modified nucleosides [10] or nucleobases [18] in urine by HPLC-MS, is even more scarce and requires cumbersome pre-treatment of the samples.

We have previously argued that the urinary excretion of 8-oxodG, and possibly other DNA oxidation products, can be used as biomarkers of oxidative stress. The previous method for analyzing 8-oxodG in urine by HPLC-EC [8,19] requires a complicated set-up and mul-

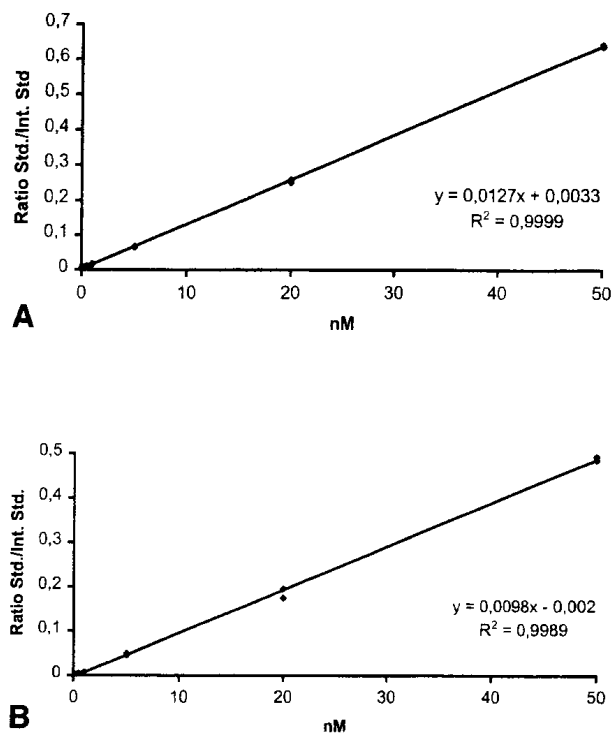


Fig. 3. Calibration curves obtained by plotting the ratio between the area of the analyte peak divided by the area of the internal standard peak as a function of the analyte concentration. (A) Calibration curve for 8-oxodA. (B) Calibration curve for 8-oxodG.

iple runs per sample and is limited to 8-oxodG analysis. Alternative methods require extensive cleanup of urine or elaborate procedures before GC-MS. The method developed in the present paper is fast, easily automated, allows use of internal standardization, measures two and potentially more oxidative DNA products, and is thus applicable to large scale epidemiological studies. A drawback of the method is that the equipment is expensive.

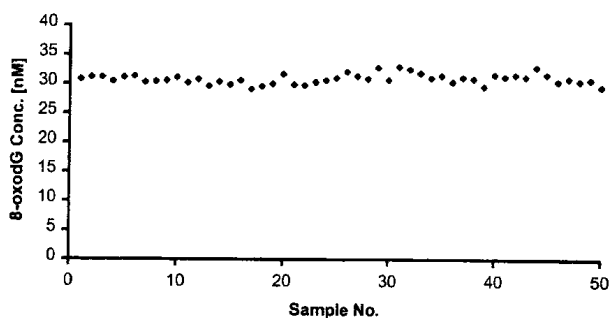


Fig. 4. Robustness of the method. The figure shows the variation on 50 injections of the same urine sample (Individual sample preparations). From the figure it can be seen that during overnight run of 50 samples that should give identical results, the intra-sample coefficient of variation (CV) is 2.9%.

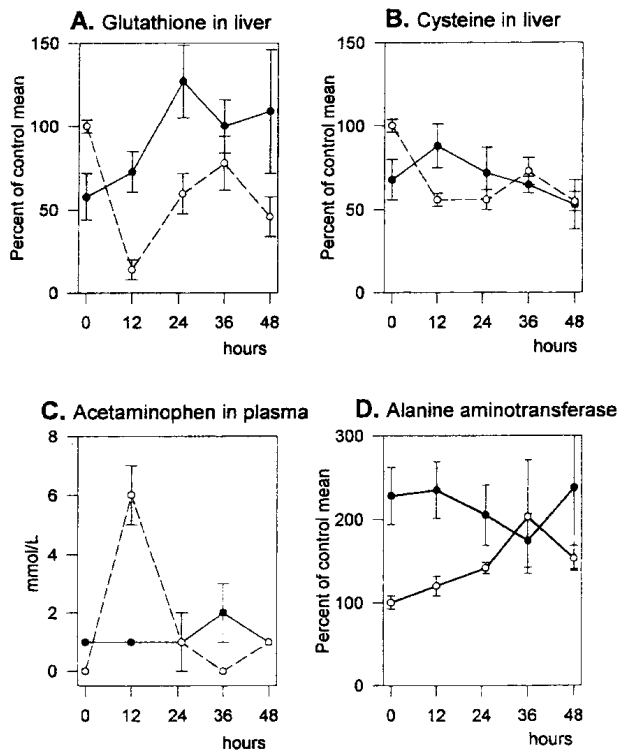


Fig. 3. The concentration of glutathione (panel A) and cysteine (panel B) in the liver, the concentration of acetaminophen (panel C) and alanine aminotransferase activity (panel D) in plasma before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pretreated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naive rats (○). Dots show means, error bars indicate standard error of the mean. Statistics: see table 1 and 2.

decreased from elevated levels of pretreated rats (fig. 4). In naive rats the mRNA level of γ -glutamylcysteine synthetase heavy chain subunit increased seven times above the control after 12 hr. The mRNA level of CYP1A2 (fig. 5A) fell to low values at 24 hr in both groups and was partially restored at 48 hr. Changes were much smaller for CYP2E1 mRNA (fig. 5B).

The mRNA levels of the cell-division related proteins Fos and Histone 3 were sharply increased at 12 hr in naive rats, both with highly significant interaction (fig. 5C and D). The mean proliferating cell nuclear antigen score (fig. 2A) was high before administration of the toxic dose in pretreated rats and increased further to a maximum after 24 hr and then declined, in naive rats, proliferating cell nuclear antigen positive cells were only found after 24 hr, and none after 48 hr. ^3H -Thymidine incorporation in DNA was significantly increased in only two naive rats at 24 hr (data not shown).

Discussion

This study compared hepatic responses to acetaminophen in naive and pretreated rats suffering a similar degree of acetaminophen toxicity. Using mortality as a measure of

toxicity, similar survival (fig. 1) of naive rats receiving 4.3 g/kg of acetaminophen and of pretreated rats receiving twice that dose indicates a redoubled increase in tolerance from pretreatment.

Liver damage is the most conspicuous but not the only toxic effect of an acetaminophen overdose. Although it is not known whether liver damage was the immediate cause of mortality, the increased tolerance resulting from pretreatment is likely to be related to changes in the response of the liver to the toxin.

Acetaminophen toxicity is mostly attributed to the reactive metabolite of acetaminophen NAPQI, which forms adducts with and inactivates proteins necessary for the function and survival of liver cells (Nelson 1990). Thus likely

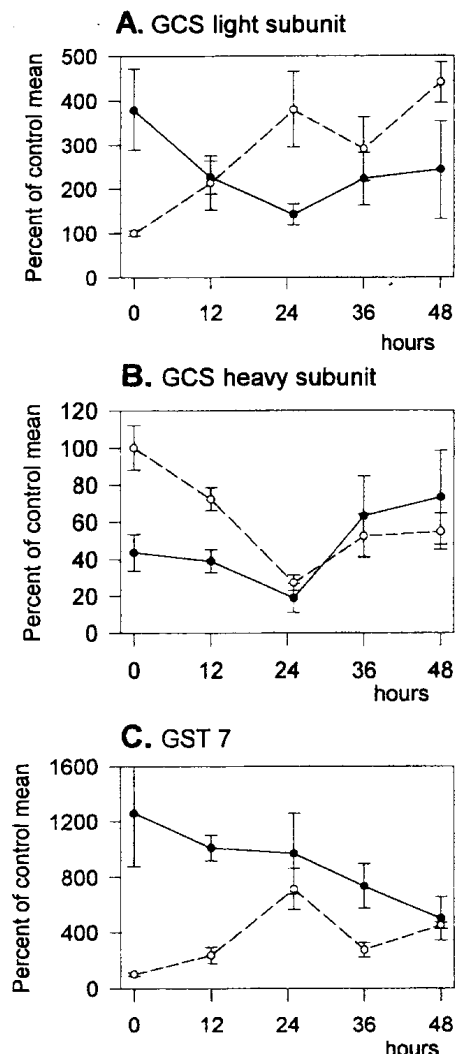


Fig. 4. Messenger RNA steady-state level in the liver of γ -glutamylcysteine synthetase light chain subunit (GCSlc) (panel A), heavy chain subunit (GCShc) (panel B), and glutathione-S-transferase (GST π subunit 7) (panel C) before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pre-treated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naive rats (○). Dots show means, error bars indicate standard error of the mean. Statistics: see table 1 and 2.

nately, when urine samples were run, dramatic ionization suppression was found in the early eluting peaks for some of the samples. The same phenomenon did not occur when the 10 mM buffer was used and thus, this eluent was selected. Post column addition of 2-propanol (40 μ l/min) could also increase the sensitivity by a factor of about two (data not given). However, this moderate increase in sensitivity, to our judgment, did not justify the more complicated setup.

To date, the majority of determinations of 8-oxoGua and 8-oxodG in tissue samples have been performed by GC-MS and HPLC-EC [9,19,21,25–38]. In urine the levels of 8-oxodG have mainly been measured by HPLC-EC [8,19,39–44].

The sensitivity obtained by using the current HPLC-MS/MS method is at the same level as the sensitivity for the best of the HPLC-EC methods often used for the detection of 8-oxodG. The HPLC-EC method is useful only for measurement of components that are electrochemically active. In contrast to this, a HPLC-MS/MS method is much more versatile. Another advantage of a HPLC-MS/MS method is that MS/MS detection is more specific than EC detection. Because of this the HPLC-EC method demands a comprehensive chromatographic separation. Furthermore, the possibility of using stable isotopically marked internal standards in mass spectrometry (isotope dilution) adds increased reliability to the method. In this method, $^{15}\text{N}_5$ -labeled internal standards are used. These are supposed to behave more similarly to the nonlabeled compounds than deuterated ones, providing an optimum internal standard. In addition, there is no risk of hydrogen exchange causing loss of deuterium. As the analyte and the internal standard should behave chemically identically, the internal standard can be used to compensate for losses during sample preparation and to compensate for signal suppression during ionization. The internal standard also has the same retention time as the analyte, and can thus be used as an extra control should the retention time of the analyte change during a run. The performance of the assay is satisfactory and long automated runs can be analyzed.

The performance of the described HPLC-MS/MS method (using an API 365 triple quadrupole mass spectrometer and 4% acetonitrile, 10 mM ammoniumacetate pH 4.8) has been compared to an already existing HPLC-EC method in our laboratory during an intercalibration exercise [45]. Oxidized and nonoxidized calf thymus DNA were hydrolyzed by the same method for both analyses. The results obtained for the two methods were similar (see Table 1).

A natural concern when injecting urine directly into a HPLC-MS system is how the chromatography is affected

Table 1. Comparison of LC-MS/MS and HPLC-EC

| Sample | Analysis method | Mean ($n = 3$) | s_{n-1} |
|----------|-----------------|------------------|-----------|
| Sample 1 | LC-MS/MS | 8.2 | 0.4 |
| | LC-EC | 7.0 | 0.5 |
| Sample 2 | LC-MS/MS | 6.0 | 1.0 |
| | LC-EC | 5.0 | 0.5 |
| Sample 3 | LC-MS/MS | 6.6 | 0.2 |
| | LC-EC | 7.2 | 3.3 |
| Sample 4 | LC-MS/MS | 5.4 | 0.5 |
| | LC-EC | 4.8 | 1.3 |

Comparison between the LC-MS/MS (results obtained on a Sciex API 365 triple quadrupole mass spectrometer) method and an LC-EC method for the determination of ratio between 8-oxodG and dG in calf thymus DNA. (Units: 8-oxodG/ 10^5 dG)

by buildup of fully retained compounds on the HPLC column, and how often the ion source needs to be cleaned. Our precolumns are usually changed for about every 200 injections, and the analytical column is back-flush eluted with 50% methanol for about every 100 urine samples. To reduce contamination of the ion source an automatic switching valve is placed between the column outlet and the ion source. The first eluting fraction of the urine is diverted to waste, and only the fraction containing the nucleosides is diverted to the mass spectrometer. Because there will still be some unwanted components left in the sample fraction, which is eluted into the mass spectrometer, the sensitivity will drop when the ion source becomes contaminated. By overnight run of urine sequences, the sensitivity of the mass spectrometer drops by around 5%. As the lowest levels of 8-oxodG found in urine are close to our limit of quantification (LOQ), it is necessary to clean the ion source frequently. As this is a fairly quick procedure, it is easily compensated for by the amount of time saved by having a short sample preparation and the amount of money saved by not using, for example, solid phase extraction columns.

The levels of 8-oxodG in the urine samples analyzed so far show a large variation from 1 nM to 100 nM, some of the variation being due to the variation in urinary output. It has not been possible to detect 8-oxodA in urine even after 25 \times concentration. A small signal, less than three times the noise level and with the same retention time as 8-oxodA, was seen in a few samples (Fig. 2C). This indicates that very small amounts of 8-oxodA could be present. This finding is in contrast to the findings by GC-MS, where high levels of both 8-oxodG and 8-oxodA have been reported in DNA [32,46]. The very low levels or absence of 8-oxodA in urine as well as calf thymus DNA and "normal" levels of 8-oxodG point to the possibility that the high levels found by GC-MS analysis are due to artifacts from oxidation during derivatization or the GC-run.

CONCLUSION

The presented HPLC-MS/MS method has shown sensitivity comparable to the best HPLC-EC methods, both concerning analysis of urine and DNA. In comparison to HPLC-EC methods, it is much more versatile as it can measure several different nucleosides in one run. The presented method requires no sample preparation for urine samples except for centrifugation, dilution, and dissolution of precipitate. Derivatization at elevated temperatures is not required. This decreases the risk of artifactual formation of oxidized bases during sample preparation and analysis. The possibility of using stable isotope labeled internal standards adds an increased reliability to the method.

The method is suitable for measurement of 8-oxodG as well as 8-oxodA in urine, but the latter was not detected in any of the human urine samples analyzed so far, not even if concentrated $\times 25$. This in combination with very low levels or absence of 8-oxodA in calf thymus DNA, point at potential problems with artifact formation from GC-MS analysis.

Acknowledgements — This work was supported by The Danish Medical Research Council, BAT, Roche Basel, Fine Vitamins and Chemicals Division, and European Framework V Project DNAge (Contract no. QLK6-CT-1999-02002).

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ABBREVIATIONS

- 8-oxodA—8-oxo-2'-deoxyadenosine
 8-oxodG—8-oxo-2'-deoxyguanosine
 8-oxoGua—8-oxo-Guanine
 BH₂⁺—The protonated nucleobase
 CE—Capillary Electrophoresis
 CID—Collision Induced Dissociation
 dA—2'-deoxyadenosine
 dG—2'-deoxyguanosine
 EC—Electrochemical Detection
 GC-MS—Gas Chromatography-Mass Spectrometry
 Gua—Guanine
 HPLC—High Performance Liquid Chromatography
 LOQ—Limit of Quantification
 MRM—Multiple Reaction Monitoring
 MS/MS—Tandem Mass Spectrometry
 SRM—Selected Reaction Monitoring
 Q1—The first quadrupole
 Q3—The third quadrupole