Monitoring urinary excretion of 5-hydroxymethyluracil for assessment of oxidative DNA damage and repair

Franca Bianchini, Janet Hall, Francesco Donato and Jean Cadet

Urinary excretion of oxidized nucleobases and nucleosides has been used as a biomarker of oxidative DNA damage and repair. Most studies have focused on the measurements of 8-oxo-7,8-dihydro-2′-deoxyguanosine; however, the urinary levels of other DNA modifications may represent useful indicators of oxidative stress. We developed a method for the determination of 5-hydroxymethyluracil (5-HMUra), consisting of the separation of the modified base in urine by HPLC and quantification by GC/MS in the selective ion monitoring mode. This experimental approach was subsequently validated in human samples, with the effect of storage and the inter- and intra-individual variations in 5-HMUra excretion being evaluated. Results showed that 5-HMUra is stable in samples frozen at –80 °C for at least 4 months. Inter-individual variations in 5-HMUra excretion were observed when the results were expressed either as nmoles excreted per kg per day (1.2–2.4) or corrected by creatinine values (7.2–12.2 nmoles 5-HMUra per mmoles creatinine). Intra-individual variability was low, varying slightly at different time collections for several individuals. Differences in the excretion of 5-HMUra in urine collected at three different 8-h intervals during the day were not significant and, in particular, the levels of 5-HMUra calculated from the overnight or the 24-h samples were highly correlated. These results indicate that monitoring urinary levels of 5-HMUra could be a suitable indicator of oxidative damage in human studies.

Keywords: *5-hydroxymethyluracil, urinary excretion, oxidative DNA damage, biomarkers.*

Abbreviations: *CV, Coefficient of variation; 8-oxoGua, 8-Oxo-7,8-dihydroguanine; 8-oxodGuo, 8-Oxo-7,8-dihydro-2′-deoxyguanosine; 5-HMUra, 5-Hydroxymethyluracil.*

Introduction

Oxidative damage to DNA is believed to be one of the major causes of ageing and age-related diseases, including cancer (reviewed in Ames *et al.* 1993). A variety of reactive oxygen forms can modify DNA bases, giving rise to derivatives some of which have been shown to have mutagenic properties (Marnett and Burcham 1993). Specific DNA repair enzymes ensure the removal of these lesions, thus reducing possible deleterious biological effects (Boiteux *et al.* 1992, Demple and Harrison 1994); the oxidized nucleobases or nucleosides released from DNA may then be easily excreted in the urine. Measurement of urinary excretion of oxidized DNA derivatives has therefore been suggested as a non-invasive biomarker for assessment of oxidative damage and repair (Bergtold and Simic 1988, Loft *et al.* 1993). Several human studies have been reported, in which the correlation between the urinary levels of oxidative DNA lesions and possible environmental modulators of oxidative stress, including smoking or dietary factors have been examined (Cathcart *et al.* 1984, Simic and Bergtold 1991, Loft *et al.* 1992, 1994, van Poppel *et al.* 1995, Verhagen *et al.* 1995). These studies have focused on the determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and thymine and thymidine glycols. However other DNA modifications, which may be of biological relevance, are formed in comparable amounts as a result of oxidative stress (Cadet 1994). Measurement of DNA adducts other than 8-oxodGuo could therefore allow a more complete assessment of oxidative damage.

5-Hydroxymethyluracil (5-HMUra) is one of the major oxidation products of thymine (Téoule and Cadet 1978) and has been demonstrated to be formed *in vivo* in animal and human DNA in comparable amounts to 8-oxodGuo (Djuric *et al.* 1991, 1992, Olinski *et al.* 1992, Malins 1993). This modified base is mutagenic in *Salmonella typhimurium* (Shirnamé-Moré *et al.* 1987), induces sister chromatid exchanges in mammalian cells (Kaufman 1989) and has cytotoxic properties (Waschke *et al.* 1975). A specific 5-HMUra-DNA glycosylase, distinct from the uracil-DNA glycosylase (Cannon-Carlson *et al.* 1989), has been found in eukaryotic cells (Hollstein *et al.* 1984, Boorstein *et al.* 1987) but is apparently absent in bacteria and yeast (Friedberg *et al.* 1978, Boorstein *et al.* 1987). However, in *Escherichia coli* the inducible AlkA enzyme, which repairs several modified DNA bases including 3-methyladenine, has recently been shown to excise both 5-HMUra and 5-formyluracil from DNA (Bjelland *et al.* 1994). The presence of such DNA repair pathways suggests that 5-HMUra is of biological relevance and its urinary excretion after removal from DNA could therefore represent a reliable biomarker for assessment of oxidative damage to biological cellular targets.

In the present study, a method developed for the determination of 5-HMUra in urine was applied to human samples. The effect of sample storage and inter- and intra-individual variations in 5-HMUra excretion over the course of a 24-h period and in repeat 24-h collections were investigated, in order to validate the analytical procedure and to select the best conditions for large-scale sampling in molecular epidemiological studies.

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MATERIALS AND METHODS

Chemicals

5-HMUra was purchased from Aldrich (St Louis, MO, USA) and [1,3-^{15}N, 5-d_2] 5-HMUra, the (M + 4) isotopically enriched internal standard was synthesized

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using the Cline method (Cline *et al.* 1959, Incardona *et al.* 1995). *N*-(*tert*-butyldimethilsilyl)-*N*-(methyl-trifluoroacetamide) was from Fluka (Fluka Chemie AG, Buchs, Switzerland) and all the other reagents were of analytical grade.

Sample collection

Three different studies carried out on 12 healthy volunteers (see Table 1) were designed:

(i) To evaluate the stability of 5-HMUra in stored samples, in which 24-h urine samples were collected from seven individuals, aliquoted and stored at –80 °C until the time of analysis, which was performed at different times after freezing (0, 2, 4, 8, 16 weeks).

(ii) To investigate the variation of urinary excretion of 5-HMUra over the course of 24 h, in which 10 subjects gave three urinary samples, collected at the following intervals: 8.00–16.00; 16.00–24.00; 24.00–8.00. The three collections were also mixed to obtain a sample corresponding to the 24-h collection. All but one of the subjects collected urine twice.

(iii) To assess the intra-individual variability in 5-HMUra excretion in which seven subjects collected a 24-h urine sample three times, approximately 1 month apart.

In all studies the total urine volume was noted prior to freezing and no additives were added. In studies (ii) and (iii) all samples were stored frozen for a maximum of 2 weeks before analysis. Creatinine analysis was performed on a Hitachi 717 autoanalyser (Boehringer-Mannheim France S.A., Meylan, France).

5-HMUra determination

5-HMUra determination was performed by GC/MS analysis, following an HPLC prepurification (Incardona *et al.* 1995). Briefly, 1 ml of urine, to which 100 pmoles of the (M + 4) isotopically enriched internal standard was added, was centrifuged, filtered and purified by HPLC. A semi-preparative octadecylsilyl silica gel column (250 × 10 mm, 5 µm particle size) (Interchim, Montluçon, France), equipped with an RP-18 guard column was used, eluted with 50 mM ammonium formate at a flow rate of 2 ml min^{-1}. Fractions containing 5-HMUra were collected and the dried samples derivatized with 100 ml of a mixture of acetonitrile / *N*-(*tert*-butyldimethilsilyl)-*N*-(methyl-trifluroacetamide) (1: 1, v/v), by incubating at 110 °C for 20 min. Silylated samples were then directly injected into GC/MS (injection volume 1 µl) in the splitless mode. Separations were achieved on a 5890 II gas-chromatograph (Hewlett-Packard, Les Ulis, France) equipped with a capillary column (30 m × 0.25 mm) coated with a film (0.25 µm thick) of methylsiloxane substituted by 5% phenylsiloxane (HP5-MS, Hewlett-Packard, Les Ulis, France), using helium as the gas carrier (constant flow = 1.6 ml min^{-1}). The injector and detector temperatures were 250 °C and 280 °C respectively; the oven temperature, after 5 min at 70 °C, was increased up to 275 °C, at a rate of 15 °C min^{-1}. Detection was obtained by MS analysis performed on a Hewlett Packard, Model 5972A detector using electron impact mass ionization (Hewlett-Packard, Les Ulis, France) in the selective ion monitoring mode. The ions recorded were 427.4 and 431.4 for 5-HMUra and the (M + 4) 5-HMUra internal standard, respectively. The detection limit of the procedure was 5 fmoles 5-HMUra injected on the GC/MS.

Statistical analysis

Data in studies (i) and (iii) were first analysed by determining the coefficient of variation (CV). This was calculated for each subject as the square root of variance between different measurements divided by the mean of determinations; the mean CV for all individuals was then reported. The variation of measurements of 5-HMUra after different storage times [study (i)] and at different collections during the course of a 24-h period [study (ii)] was determined by an analysis of variance (ANOVA) for repeated measures (Neter *et al.* 1990). A test for linear trend using orthogonal polynomials (Snedecor and Cochran 1987) by the BMDP computer program (Dixon *et al.* 1990) was performed to evaluate whether there was a systematic increase or decrease of measurements over time [study (i)]. Spearman's rank correlation coefficients (r) (Snedecor and Cochran 1987) were estimated to assess the presence of correlations between different measurements for the same individual.

Subject	Sex	Age	Smoking status
LY1	F	59	NS
LY2	F	52	NS
LY3	F	38	NS
LY4	F	38	NS
LY5	F	49	S
LY6	F	39	S
LY7	M	36	S
GR5	F	37	NS
GR6	F	26	S
GR7	F	26	NS
GR11	F	26	S
GR12	F	24	S

Table 1. Characteristics of individuals.

Results

Table 2 reports the concentration of 5-HMUra, expressed as pmoles per ml urine, in samples analysed after different storage times [study (i)]. Surprisingly, the results show a slight decrease in 5-HMUra levels from the 0- to the 4-week time and subsequently an increase up to the 16-week time. These differences were significant by the analysis of variance for repeated measures, due to the low values found in the intermediate times with respect to the first and last storage times ($p < 0.001$); however, there was no systemic increase or decrease of successive measurements with increasing time of storage ($p > 0.1$). In addition, average CV was low (4.7% ± 0.8), indicating that differences between repeated measures could be attributable to variability between experiments. Repeated measurements ($n = 5$) of the same sample (number of samples = 7) were highly correlated ($r = 0.9$).

Figures 1 and 2 report the variations in the excretion of 5-HMUra in the three 8-h fractionated collections, compared with the 24-h / three urine samples [study (ii)]. Results are expressed as total nmoles 5-HMUra excreted per 8 h (Figure 1) or as nmoles 5-HMUra per mmole creatinine (Figure 2); panels a and b refer to the first or the second urine collection, respectively. A significant variation between individuals was found ($p = 0.02$ for total nmoles 5-HMUra excreted and

	pmoles 5-HMUra per ml urine					
Subject	0	2 weeks	4 weeks	8 weeks	16 weeks	Mean
LY1	80	76	70	75	75	75.2
LY2	38	35	33	34	34	34.8
LY3	44	40	38	40	41	40.6
LY4	73	72	70	76	80	74.2
GR5	74	69	66	68	75	70.4
GR6	58	56	53	57	57	56.2
GR7	108	107	106	102	112	107

Table 2. Urinary excretion of 5-HMUra measured at different times of storage.

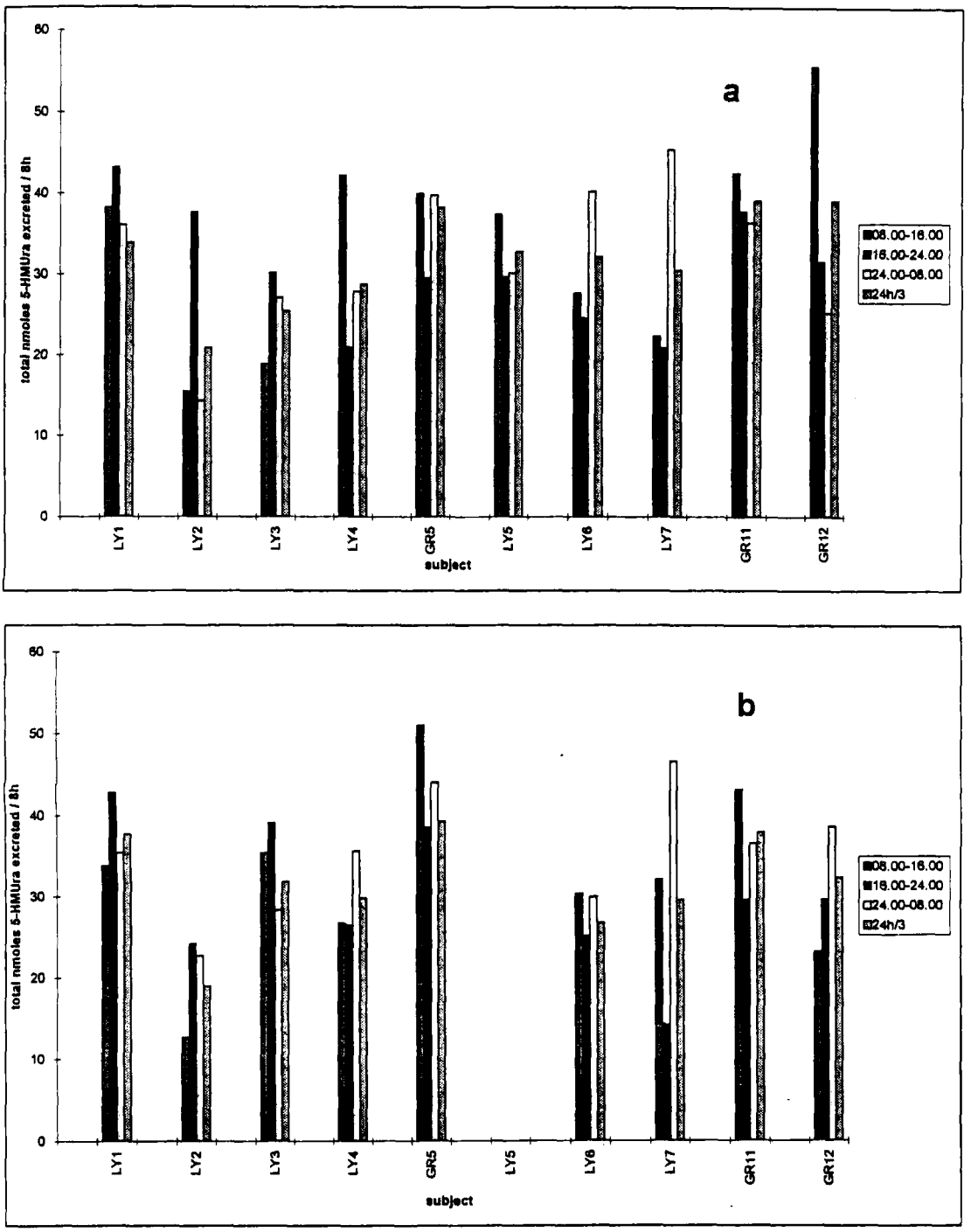

Figure 1. Urinary excretion of 5-HMUra in fractionated 8-h and 24-h collections, expressed as total nmoles 5-HMUra excreted per 8 h: (a) first collection, (b) second collection.

$p = 0.01$ for nmoles 5-HMUra per mmole creatinine), with no major differences between the two collections from the same individual. The variation between the three fractionated urine samples, taken at different times, was found to be non-significant by the analysis of variance for repeated measures ($p > 0.1$ in every case). The correlations between the 5-HMUra measurements in the overnight (24.00–8.00) compared with the 24-h collections are shown in Figure 3. A moderate ($r = 0.4$) or a high ($r = 0.9$) correlation was obtained when results were expressed as total nmoles 5-HMUra excreted per 8 h (panel a) or corrected by creatinine values (panel b), respectively.

Excretion of 5-HMUra for seven individuals on three separate days is reported in Table 3 [study (iii)]. Values range from 1.2 to 2.4 nmoles 5-HMUra per kg per day or from 7.2 to

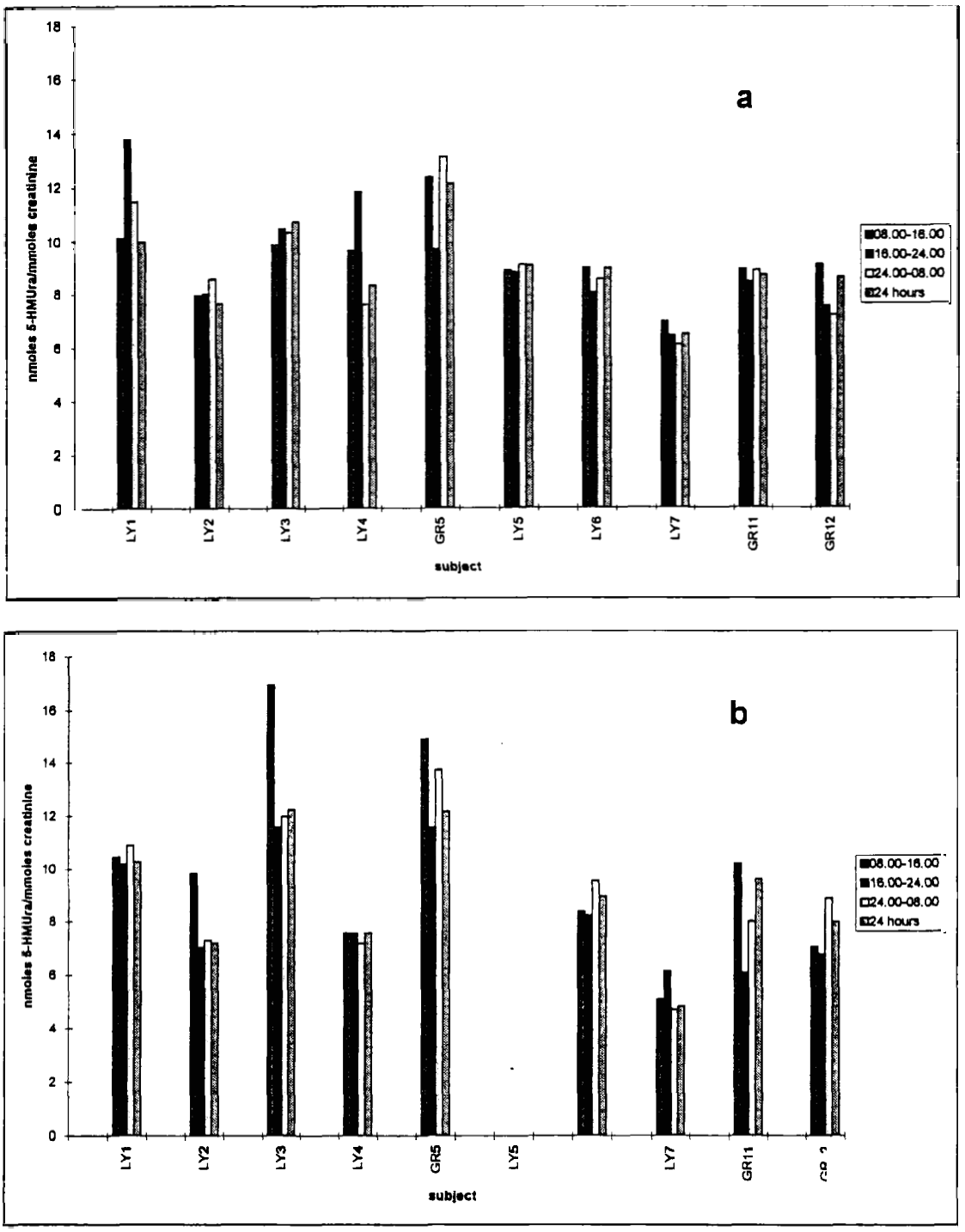

Figure 2. Urinary excretion of 5-HMUra in fractionated 8-h and 24-h collections, expressed as nmoles mmoles^{-1} creatinine: (a) first collection, (b) second collection.

12.2 nmoles 5-HMUra per mmoles creatinine. Measurements were highly correlated in any one subject, the Spearman's correlation coefficients being 0.8–0.9 or 0.7–0.9 when results were reported in kg day^{-1} or divided by mmoles creatinine, respectively. The means of CV were 9.0% ± 3.9 (nmoles kg^{-1} day^{-1}) or 7.7% ± 3.1 (nmoles mmoles^{-1} creatinine), indicating a low intra-individual variability in 5-HMUra excretion.

Discussion

In the present paper, 5-HMUra was analysed in human urine samples using a GC/MS quantification following a prepurification by HPLC. Such a technique provides a sensitive analytical procedure, allowing the detection of the adduct in only 1 ml of urine. Therefore, large volumes of urine do not need to be stocked. Analysis of samples stored at –80 °C

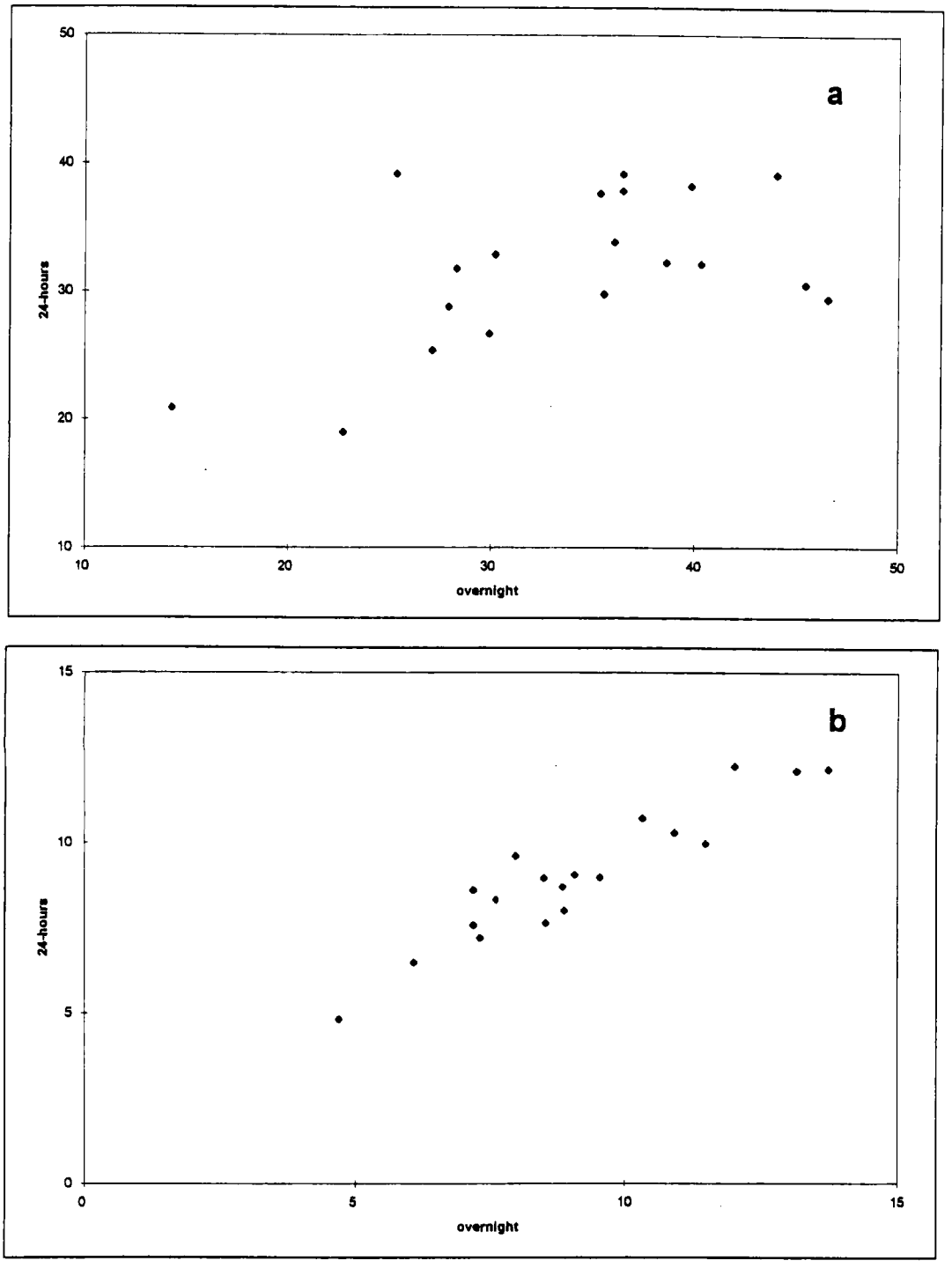

Figure 3. Correlations between 5-HMUra excretion in overnight (24.00–8.00) and 24-h urines, expressed as (a) total nmoles 5-HMUra excreted per 8 h or (b) nmoles 5-HMUra per mmoles creatinine.

for different times demonstrated no appreciable decrease in the amount of 5-HMUra, indicating that urine can be successfully frozen before analysis, which is a benefit if such measurements are to be incorporated into molecular epidemiological studies. Similar results have been reported for 8-oxodGuo, which was demonstrated to be stable for at least 2.5 years at –20 °C (Loft *et al.* 1994).

Urinary excretion of 5-HMUra over a 24-h period was significantly different between subjects, although the intra-individual variations in 5-HMUra levels observed during the three 8-h periods of collection over the course of a day were not significant, either when results were expressed as total nmoles excreted or corrected by creatinine. Comparison between the overnight and the 24-h samples indicates that

Subject	nmoles 5-HMUra per kg per day				Subject	nmoles 5-HMUra per mmole creatinine			
	1st	2nd	3rd	Mean		1st	2nd	3rd	Mean
LY1	1.78	1.69	1.98	1.82	LY1	9.96	10.95	10.28	10.4
LY2	1.49	1.15	1.36	1.33	LY2	7.62	7.96	7.19	7.59
LY3	1.58	1.99	2.04	1.87	LY3	10.7	12.24	9.4	10.78
LY4	1.29	1.35	1.33	1.32	LY4	8.31	9.28	7.56	8.28
GR5	2.22	2.26	2.11	2.2	GR5	12.1	12.15	10.98	11.74
GR6	2.1	2.43	2.11	2.21	GR6	10.15	11.3	9.18	10.21
GR7	1.77	1.55	2.06	1.79	GR7	8.33	7.66	8.04	8.01

Table 3. Urinary excretion of 5-HMUra in 24-h samples collected on three separate days.

overnight urines are representative of the 24-h collection, the measurements being highly correlated when results are corrected by creatinine, or only moderately correlated when absolute measurements are used. This observation can have important implications for human studies, as the overnight collection is easier to perform and avoids the problems of over- or under-collections likely in the 24-h samples. Our results are in agreement with those reported for urinary excretion of several catecholamines, where a correlation ranging from 0.54 to 0.81 for overnight and 24-h samples was observed (White *et al.* 1995). In addition, urine collection from 17.00 to 9.00 has previously been used for measurement of 8-oxodGuo in human samples (van Poppel *et al.* 1995).

Excretion of 5-HMUra seems to be stable with time for all subjects. The analysis of the CV shows that the intra-individual variability of urinary 5-HMUra measurements on different days is not higher than that expected by chance, either when the results are expressed as nmoles kg^{-1} day^{-1} or when the 5-HMUra urinary level is related to creatinine concentration. Our results for 5-HMUra are in agreement with those reported for other oxidation compounds currently used as markers of oxidative damage. Excretion of 8-oxodGuo was in fact found to be similar between urine collected 4 months apart in 20 individuals (r = 0.53) (Park *et al.* 1992) and in one subject consuming a diet with similar caloric intake during months (Simic and Bergtold 1991). Accordingly, the urinary excretion of thymidine glycol, but not thymine glycol, did not vary considerably among different collections for the same individual (Cathcart *et al.* 1984).

Our results demonstrate that 5-HMUra is excreted in human urine at relatively high levels. These values (approximately 1.5 nmoles 5-HMUra per kg per day or 10 nmoles 5-HMUra per mmoles creatinine) are consistent with a previous report for the urinary detection of this adduct (Faure *et al.* 1993) and higher than those reported for other DNA modifications measured in human urine. Urinary excretion of 8-oxodGuo is approximately 200–300 pmoles kg^{-1} day^{-1} (Bergtold and Simic 1988, Shigenaga *et al.* 1989, Loft *et al.* 1992), while the excretion of the base 8-oxo-7,8-dihydroguanine (8-oxoGua) has been reported to be approximately 3 nmol kg^{-1} day^{-1} (Bergtold and Simic 1988) or 4 nmoles per mmole creatinine (Suzuki *et al.* 1995). Urinary levels of thymine and thymidine glycols are approximately 400 and 150 pmoles kg^{-1} day^{-1} or 2.4 and 0.6 nmoles per mmole creatinine, respectively (Cathcart *et al.* 1984, Cao and Wang 1993).

Our results suggest therefore that 5-HMUra may be formed in high amounts in human DNA and that an active repair process may be responsible for the elevated levels in urine. Other possible pathways for the presence of 5-HMUra in urine should, however, be considered. It has been suggested that urinary excretion of free bases can be greatly affected by diet as, once ingested, they can be absorbed from the intestine (Prevost *et al.* 1990, Park *et al.* 1992). In humans, however, 8-oxoGua excretion does not seem to vary substantially with diet (Suzuki *et al.* 1995) and thymine glycol, another modified DNA base, is unabsorbed from the diet in rats (Cathcart *et al.* 1984). The contribution of the diet to the urinary excretion of 5-HMUra remains to be fully assessed. The urinary excretion of modified nucleosides could reflect the degradation of oxidatively damaged DNA during cell turnover (Lindahl 1993). It has recently been shown that urinary 8-oxodGuo can also result from the dephosphorylation of 8-oxodGuo monophosphate, derived from the degradation of 8-oxodGuo triphosphate in the nucleotide pool of human cells, and which cannot be rephosphorylated (Hayakawa *et al.* 1995). Further work is therefore necessary to better understand the origin and the significance of oxidized DNA bases and nucleosides in urine.

In conclusion, our results indicate that 5-HMUra could represent, in addition to other DNA modifications, an appropriate biomarker for the assessment of oxidative DNA damage and repair, suitable for application in molecular epidemiological studies. This modified base is excreted in human urine at levels comparable or higher than those observed for other oxidative modifications, and is a relatively stable individual parameter. In addition, an overnight instead of a 24-h urine collection can be used, thus facilitating the sample collection and therefore the integration of this biomarker in human studies.

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