Urinary Lipoperoxides Quantified by Liquid Chromatography, and Determination of Reference Values for Adults

Joseph A. Knight,1,2 Stanley E. Smith,2 Vaughn E. Kinder,1 and Robert K. Pieper1

Urinary lipoperoxides, measured as the malondialdehyde–thiobarbituric acid adduct, were quantified by adapting to urine the liquid-chromatographic method of Wong et al. (Clin Chem 1987;33:214-20) to plasma. Reference intervals for untimed urine specimens from 121 men, ages 16 to 67 years, and 107 women, ages 15 to 55, were determined. Their concentrations differed significantly (P = 0.015), males having a mean (and SD) of 0.89 (0.35) nmol of malondialdehyde per milligram of creatinine, females 0.78 (0.30). In both groups, the values were slightly skewed to the higher values. Our early studies suggest that measuring urinary lipoperoxide may have advantages over plasma in studying certain disorders. The presence of other urinary chromophores or TBA-reactive substances stresses the need for chromatographic techniques when lipoperoxides are measured in biological samples.

Additional Keyphrases: malondialdehyde  •  lipid peroxidation  

sex- and age-related differences  •  urine

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Received December 28, 1987; accepted March 3, 1988.

Oxidative damage to unsaturated lipids (lipid peroxidation) is a well-established general mechanism for cellular injury (1-3). In addition to extensive experimental studies, increased lipid peroxidation has been reported in a wide variety of clinical and toxicological conditions, including acute myocardial infarction, stroke, diabetes mellitus, rheumatic disorders, hepatic diseases, burns, and toxicity by certain drugs, pesticides, and metals (4-12).

Lipoperoxides in serum or plasma are most frequently measured as the malondialdehyde–thiobarbituric acid adduct (MDA-TBA), a red complex with maximum absorption at 532 nm (13). It has been quantified by spectrophotometry (5, 14), fluorometry (15), and liquid chromatography (16). However, measurement by either spectrophotometry or fluorometry includes various interfering compounds, thereby decreasing the specificity of the method (17, 18). Wong et al. (19) recently reported an improved liquid-chromatographic procedure for measuring MDA in plasma, and reliable reference values for this method have now been published (20). MDA has also been measured in urine of rats by liquid chromatography (21, 22). In these studies, the

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1 Nonstandard abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid; LC, liquid chromatographic; DMSO, dimethyl sulfoxide; TEP, tetraethoxy propane; Cr, creatinine.
Table 1. Reference Values for Urine MDA (nmol/mg Cr)

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>Age range, y</th>
<th>Mean (and SD)</th>
<th>Range (±2 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>121</td>
<td>18–57</td>
<td>0.89 (0.35)*</td>
<td>0.19–1.59</td>
</tr>
<tr>
<td>F</td>
<td>107</td>
<td>15–55</td>
<td>0.78 (0.30)</td>
<td>0.16–1.38</td>
</tr>
</tbody>
</table>

*Significantly greater than females (P = 0.015).

Table 2. MDA Concentrations in Plasma and Urine of Five Renal-Transplant Patients

<table>
<thead>
<tr>
<th>Plasma MDA, μmol/L</th>
<th>Urine MDA, nmol/mg Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.44</td>
<td>1.48</td>
</tr>
<tr>
<td>0.52</td>
<td>1.91</td>
</tr>
<tr>
<td>0.72</td>
<td>1.58</td>
</tr>
<tr>
<td>0.72</td>
<td>1.10</td>
</tr>
<tr>
<td>0.49</td>
<td>2.32</td>
</tr>
<tr>
<td>Mean</td>
<td>0.58</td>
</tr>
<tr>
<td>Reference values</td>
<td>0.60 (0.21)*</td>
</tr>
</tbody>
</table>

* From ref. 20. *From Table 1.

The urinary excretion of MDA was noted to be increased by dietary fatty acids, and by the administration of corticoter-
in, epinephrine, adriamycin, and carbon tetrachloride.

In the current study, we describe an adaptation to urine of the improved liquid-chromatographic method for plasma (19). In addition, we determined reliable reference values on untimed urine specimens from men and women. Our early observations suggest that under some conditions, assay of MDA in urine may be preferred to assays in plasma.

Materials and Methods

Subjects: Untimed specimens of urine were obtained from men and women laboratory employees, other hospital em-
ployees who were undergoing annual physical examinations, and from other individuals undergoing pre-employ-
ment evaluations. The urine specimens from this latter group were all negative when screened for various drugs. All individuals were working and considered to be in good health (Table 1). In addition, plasma from EDTA-anticoag-
ulated venous blood and untimed urine specimens were obtained from five renal-transplant patients, all of whom were on cyclosporine (Table 2). All specimens were stored at 4 °C until analyzed, usually within 24 h.

Apparatus: The liquid-chromatographic (LC) equipment has been recently described (19, 20). However, we used larger urine volumes, so the recorder sensitivity was decreased to 0.05 A full scale. In the current study, the line of the C18 column was prolonged and the quality of the chromatograms was improved by the following changes in technique:

(a) Each day before the initial sample injection, 100 μL of undiluted 99.9% dimethyl sulfoxide (DMSO) was injected into the LC apparatus. The system was then purged with mobile phase to cleanse it of any residual DMSO.

(b) Column life was further prolonged by decreasing the methanol concentration of the mobile phase from 400 mL/L to 350 mL/L. Although this prolonged the retention time of the MDA-TBA peak and resulted in slightly smaller and broader peaks, we saw no measurable differences in accuracy or precision. We used this concentration change only when the column had been used for some time and the MDA-TBA peak began to merge with the large earlier solvent/reagent front.

These two procedures prolonged the column life by approximately 25% over our previous experience (19, 20).

(c) We initially used polypropylene tubes for the liperox-
ide hydrolysis step, resulting in a small contamination peak readily seen in the blank sample (19). Efforts to remove this contamination were not completely successful. When glass tubes were substituted, there was no contamination (Figure 1A).

Reagents: All reagents, chemicals, and solvents were exactly as previously described (19). However, the tetrachlo-
ropropane (TEP) standard solutions were prepared as follows. The stock TEP solution (2.02 nmol/L) was prepared by diluting 25 μL of 1,1,3,3-tetraethoxypropene (Sigma Chemical Co., St. Louis, MO; cat no. T-9889, 97–98% anhydrous, relative density 0.91, relative molecular mass 220.3) to the mark in a 50-mL volumetric flask with ethanol/water solution (40/60 by vol) and stored at 4 °C. It was prepared bi-weekly.

TEP working standard solutions (0.60, 2.40, and 4.00 μmol/L) were prepared by placing 15, 60, and 100 μL of the TEP stock standard in three 50-mL volumetric flasks and diluting to the mark with the ethanol/water solution. These working standards, prepared bi-weekly, contained 0.36, 1.45, and 2.42 nmol/0.6 mL, and were stored at 4 °C.

Liperoxide hydrolysis and TBA reaction. In each analytical run, reagent blank, quality-control specimens, TEP working standard solutions, and urine samples are assayed. Pipet 3.0 mL of phosphoric acid solution into the required number of 13-mL screw-capped glass test tubes. Using a negative volume displacement pipetor, pipet 600 μL of distilled water (blank), TEP standards, and urine into the respective tubes and vortex-mix. Add 1.0 mL of TBA solution to all tubes. Pipet 400 μL of distilled water into the blank, standard, and urine tubes; the final volume is 5.0 mL. Cap all tubes tightly and vortex-mix for 20 s before placing them in a boiling water bath for 60 min. They are then cooled in an ice-water bath until the LC analyses are performed.

Because the MDA-TBA adduct is unstable at neutral or alkaline pH, the boiled samples are neutralized individually within 10 min of injection onto the LC column. Pipet 0.5 mL of each boiled sample into a polypropylene microtube that contains 0.5 mL of methanol–NaOH solution. The tubes are then capped, vortex-mixed, and centrifuged at 9500 × g for 90 s to sediment the precipitated proteins.

Liquid-chromatographic analysis. The MDA concentra-
tion is determined by injecting 50 μL of each neutralized, centrifuged sample into the LC apparatus and measuring

![Fig. 1. Illustrative liquid chromatograms for urine liperoxides](image)

Fig. 1. Illustrative liquid chromatograms for urine liperoxides, obtained by spectrophotometric monitoring at 532 nm

Peak height (ordinate) = 0.05 A full scale, chart speed (abscissa) = 1 cm/min. (A) reagent blank; (B) TEP standard (reading, equivalent to MDA, 2.40 μmol/L or 1.45 nmol/0.6 mL); (C) most common urine pattern, but with variations in height of peak to the right of MDA; (D) one of several common variations for urine

* Recommendation from Dr. Steven H. Y. Wong, Farmington, CT.
the peak height at 532 nm as previously described (19). At a flow rate of 2.0 mL/min, the MDA-TBA adduct elutes at about 4.2 min (Figure 1).

**Urine creatinine (Cr).** Urine creatinine (Cr) concentrations (mg/dL) were determined in an Astra chemistry analyzer as described by the manufacturer (Beckman Instruments Inc., Fullerton, CA 92634).

**Urine MDA concentration.** The MDA concentration in urine is calculated by use of the following equation:

\[
\text{Urine MDA (nmol/mg Cr)} = \frac{\text{nmol} \times 1.67 \times 100}{\text{mg Cr}}
\]

where the value for nmol MDA/0.6 mL urine is taken directly from the standard curve and 1.67 is the conversion factor to correct the urine volume to 1.0 mL.

**Spectral analysis.** We performed a spectral study, using a urine specimen that showed multiple peaks (see Figure 1). We measured the peak heights at 512, 522, 532, 542, and 552 nm. The absorbance maximum, as expected, was at 532 nm; the absorbance at 522 nm was 95% of the 532 nm peak. The peak heights at both 512 and 542 nm were about 55% of the 532 nm peak. There was no absorbance at 552 nm.

**Sensitivity, precision, and recovery.** The detection limit of this method, determined by progressively diluting a pooled urine specimen with isotonic saline, is about 0.08 \(\mu\)mol/L. This concentration is well below that seen in all but the most dilute urine samples. We evaluated within-run and run-to-run precision by using two pooled urine specimens (Table 3). The pooled specimens for the run-to-run study were aliquoted and refrigerated at 4 °C. The MDA concentration in urine was stable for at least two weeks. Analytical recovery from urine averaged 96% (range 90–103%) when a TEP standard containing 1.66 \(\mu\)mol of MDA per liter was added to a urine specimen pool having a MDA concentration of 0.64 (0.04) \(\mu\)mol/L (n = 10).

**Tests for interference.** Addition of the following substances to normal urine, at the concentrations indicated, did not interfere in the assay: glucose (555 mmol/L), protein (2 g/L), acetoacetic acid (1.66 mmol/L), acetylsalicylic acid (1.66 mmol/L), acenomophen (2.0 mmol/L), phenobarbital (0.20 mmol/L), and amphetamine (6.0 mmol/L). Bilirubin has previously been shown not to interfere with the TBA reaction (19, 23), although biliverdin, present in icteric serum and bile, causes positive interference (23).

**Statistical analyses.** Data computations included the means, standard deviations, and Student's t-test. Outliers were removed according to Barnett (24); three outliers from both of the reference groups.

**Results and Discussion**

Increased lipid peroxidation, demonstrated most frequently in humans by increased MDA concentrations in serum or plasma, has recently been reported in a wide variety of clinical and toxicological conditions (4–12). Although urinary MDA concentrations have been measured in laboratory animals (21, 22), to our knowledge they have not been previously reported in humans.

In this paper we report our early studies on the measurement of MDA in human urine, including the establishment of reliable adult reference values, using a liquid-chromatographic method based on the procedure of Wong et al. (19). Slight modifications of this method have resulted in a significant simplification and improvement in urinary MDA measurement over the liquid-chromatographic techniques previously reported (21, 22). In this regard, Figure 1 demonstrates that, in contrast to plasma, multiple chromophores are present in urine. However, these are readily separated from each other, and the MDA-TBA peak is only rarely affected.

Figure 1C shows the most common pattern noted, although there was considerable variation in the concentration of the chromophore immediately following the MDA-TBA peak. In addition, Figure 1D demonstrates the complexity of chromatograms often seen in some urines and points to the analytical problems of measuring MDA in biological specimens by spectrophotometry or fluorometry (17, 18). However, the addition to urine of glucose, protein, bilirubin, acetoacetic acid, phenobarbital, amphetamine, acetylsalicylic acid, and acetaminophen does not interfere with the assay.

Table 3 shows the within-run and run-to-run precision, which is similar to that reported for plasma (19, 20). The detection limit is about 0.08 \(\mu\)mol/L, a value considerably lower than seen for all but the most dilute urine specimens.

We measured MDA in untreated urine specimens from 107 women, ages 15 to 55 years, and 121 men, ages 16 to 67 years (Table 1). It is apparent that reference values for men significantly exceed those for women \((P = 0.015)\), a finding in agreement with recently reported values for plasma (20). We also studied five renal-transplant patients, all of whom were on cyclosporine medication and doing well clinically (Table 2). In all cases, the concentrations in plasma were within the reference interval (20). However, two urine samples showed moderate MDA elevations, two others were at the upper limit of the reference interval, and the fifth exceeded the reference mean. This mini-study suggests that urinary MDA measurements may be a more sensitive and specific indicator of lipid peroxidation than concentrations in plasma, at least in disorders of renal function. Whether these increased values for MDA represent mild cyclosporine toxicity, normal immunological effects, or other as-yet-undefined effects is not known. An extension of the studies reported here is needed to better understand these findings.

**References**

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Evaluation of a New Strategy for Detection of Thyroid Dysfunction in the Routine Laboratory

Rhys John, Robert Henley, Geoffrey Lloyd, and George H. Elder

We assessed the use of a new strategy for detecting thyroid disorders, utilizing a sensitive assay for concentrations of thyrotropin (TSH) and free thyroid hormone in serum as follow-up tests. Of 1279 patients who were not on thyroxin (T4) replacement treatment, 82% could be classified as euthyroid and would require no further tests. In patients who were on T4 replacement, 41% fell into the euthyroid category and would require no further tests. Using this strategy to replace our existing strategy of free thyroxin as a "first-line" test would reduce the proportion of patients who would require one or more follow-up tests from 49% to 24%.

Additional Keyphrases: thyrotropin • thyroxin • thyroid status • diagnostic efficiency

Recent developments in production of monoclonal antibodies have brought about a rapid change from competitive-binding assays for thyrotropin (TSH) to immunometric assays. This has produced many advantages, in that immunometric assays have short incubation times and are more robust. The labeling of antibodies with a nonradioactive substance such as europium (2), an enzyme (2), or an acridinium ester (3) has also made it possible for TSH assays to be performed in laboratories previously not licensed for work with radioactive materials. The increased sensitivity inherent in immunometric assays has allowed TSH concentrations in normal subjects to be distinguished from those in clinically thyrotoxic patients, so that interest has been aroused in the possible use of TSH as a "front-line" screen for thyroid disease, as proposed by Caldwell et al. (4). By this procedure, patients would be classified in one of five groups, depending on their basal concentrations of TSH and free thyroid hormones. If their TSH concentration in serum was within the reference interval, they would be classified as being euthyroid and have no further estimations performed. With an increased concentration of TSH, FT4 would be quantified to distinguish between overt and subclinical forms of hypothyroidism. With an undetectable concentration of TSH, either an FT4 test alone or an FT3 and an FT3 assay would be performed to distinguish hyperthyroidism from subclinical hyperthyroidism. Using this procedure, Caldwell et al. showed that they could reduce by about 50% the number of analyses needed to arrive at a diagnosis with no decrease in diagnostic accuracy and without additional delay.

In the present study we have evaluated this new strategy in patients' samples referred to a routine clinical chemistry department. We defined the biochemical thyroidal status of our patients on the basis of their TSH concentration, followed by determinations of FT4 and FT3 concentrations. The TSH assay we used is a new two-site immunoenzymometric assay in which enhanced chemiluminescence is the detection system used for quantification. We previously reported a clinical evaluation of this assay (5) and have now extended it to a much larger number of patients' samples referred for thyroid-function testing.