When we focused on the evolution of the patients of Mx group, we found that the efficiency of the tests in this group was slightly lower for GHYL (0.83), and lower for ALP (0.64) and that the interaction between GHYL and ALP did not increase the area under the ROC curve (Figure 3). Moreover, the multivariate logistic regression approach showed that the only predictive variable for metastases was GHYL ($P = 0.03$). We believe this apparent discrepancy reflects the different populations of patients that form the Mo and the Mx group. The Mx group is not as homogeneous as the Mo group, because some patients in the former group may indeed have metastases, even if at the moment these could be detected only by scintigraphy. Because scintigraphy is much more sensitive than the biochemical markers, the rate of false positives (true metastases despite the biochemical markers being in the normal range) will affect the efficiency of the tests when compared with their performance for the Mo and M+ groups.

In conclusion, the ROC curve and the multivariate logistic regression analysis show that also in the Mx group, some of whom converted to the M+ group, the only predictive marker for metastases remains GHYL.

We thank C. Modricky and K. Noris Suasres for calculating the areas under the ROC curves. Our research was supported by MURST (Italian Ministry of University and of Scientific and Technological Research).

References

PLASMA AND URINARY OXALATE AND GLYCOLATE IN HEALTHY SUBJECTS

L. Hagen, V. R. Walker, and R. A. L. Sutton

High-performance ion chromatography (HPIC) has been widely used for oxalate analysis and, more recently, for glycolate analysis. We describe a procedure for sample preparation in which the plasma ultrafiltrate is acidified during harvesting with a cation-exchange resin, and the chloride is removed before the ion chromatography, which is performed with a newly developed AS10 column. The same ultrafiltrate sample is analyzed for glycolate. For plasma oxalate, the mean recovery of sample in eluted fractions was 95-96%, and intra-assay CV was 6.2-8.1%. The reference interval (mean ±2 SD) for men was 0.8-3.2 μmol/L and for women, 1.0-2.6 μmol/L. For urinary oxalate, the reference interval for men was 175-560 μmol/day and for women, 107-432 μmol/day. For plasma glycolate, the mean analytical recovery was 96-98%, and the intra-assay CV was 2.4-6.2%. The reference interval for men was 1.9-7.5 μmol/L and for women, 1.4-7.4 μmol/L. For urinary glycolate, the reference interval for men was 0-1400 μmol/day and for women, 91-1001 μmol/day.

Additional Keywords: high-performance ion chromatography reference interval

The measurement of oxalate and, more recently, glycolate in plasma has been associated with several difficulties, resulting in variable and inaccurate determinations, often with an overestimation of plasma concentrations ($I-4$). A high-performance ion chromatography (HPIC) system has been used to quantify plasma oxalate ($I, 2, 5$). Basically, this system involves production of an ultrafiltrate of plasma (or serum), which may or may not be cooled and acidified; dilution (usually) with boric acid; and injection onto an HPIC AS4A ion-exchange column for elution with a carbonate/bicarbonate buffer. However, there have been problems with the recovery of oxalate from the sample and with the prevention of oxalate generation before the assay. Indirect isotopic studies indicate that oxalate concentrations in plasma are very low ($6, 7$) and, thus, great sensitivity is required in the assay. For glycolate, overestimation of plasma concentrations is the major problem ($4, 8, 9$).

Here we describe an improved sample-processing technique for determining plasma oxalate. For glycolate, we used a simple chromatographic method adapted from Wandzilak et al. ($10$).
Materials and Methods

Chemicals

All standard chemicals were Analar or finer quality. [14C] Oxalate and [14C] Glycolate were from Amersham (Oakville, ON), oxalic acid dihydrate was from BDH Chemicals Canada Ltd. (Vancouver, BC), glycolic acid was from Sigma Chemical Co. (St. Louis, MO), and cation-exchange resin (AG 50W-X8) was from Bio-Rad Laboratories (Mississauga, ON). Water was purified by a Milli-RO and a Milli-Q system from Millipore (Mississauga, ON).

HPIC Equipment

HPIC equipment was obtained from Dionex Corporation (Sunnyvale, CA) and included two anion-exchange columns (an AS10 analytical and guard column for plasma oxalate and an AS6A column (Carbo Pak PA1) and guard column for plasma glycolate), a micromembrane cation exchanger, and a Model 2000i conductivity detector. The Model 510 pump was from Millipore-Waters (Mississauga, ON), the SP4270 integrator was from Technical Marketing (Vancouver, BC), and the Shimadzu SIL-B automated injector (with temperature controller and automated switching valve) was from Fisher Scientific (Vancouver, BC). The Model Minipuls 2 peristaltic pump was from Terochem Labs. (Edmonton, AB).

Preparation of Resin

The cation-exchange resin (AG 50W-X8) contained traces of oxalate, which were removed with 100 mL/L HCl. The resin was washed with purified water, dried, and then stored in an airtight polystyrene container in the dark (stable for at least 4 months). For the silver cation-exchange resin, the AG 50W-X8 ion-exchange washed resin was mixed with aqueous AgNO3 (1 g of resin per 340 mg of AgNO3) and similarly washed, dried, and stored (stable for at least 1 month).

HPIC Conditions

Oxalate. The column was equilibrated with 40 mmol/L sodium tetraborate (pH ~9.4) at a constant flow rate of 1.0 mL/min. Background conductivity was suppressed by including 13.4 mmol/L H2SO4 delivered by a peristaltic pump set at a flow rate of 10 mL/min. A 150-μL sample was injected. The column eluate was monitored with the conductivity cell set at 3 μS full scale. The peak heights recorded were plotted against concentration. After 15 min, 50 mmol/L NaOH was pumped for 10 min to eliminate spurious peaks; then the original conditions were restored. Total run time was 35 min. [14C] Oxalate equilibrated with plasma was sampled at each phase of the procedure and percent recovery was calculated.

To determine the effect of ascorbic acid on plasma samples, we added ascorbate (100 and 500 μmol/L) to plasma and processed the samples by the above procedure. Ascorbic acid in concentrations of 500 μmol/L and 1 mmol/L was prepared in 100 mmol/L HCl to mimic plasma ultrafiltrate; these samples were processed similarly before injection onto the column to determine oxalate generation in the column itself.

Glycolate. Excess ultrafiltrate harvested for plasma oxalate determinations was analyzed by glycolate with an adaptation of the method described by Wanzilak et al. (10) for urine. The ultrafiltrate was diluted with an equal volume of purified water, and 150 μL was injected. HPIC conditions were identical to those for urine except that detector sensitivity was increased from 10 to 3 μS full scale. A standard curve was prepared from aqueous standards. [14C] Glycolate was also added to plasma and used to determine percent recovery.

Blood Sampling and Preparation

Thirty-nine apparently healthy subjects (14 men, 25 women) were maintained on their self-chosen diets before sample collection except that vitamin C supplements were withheld for at least 1 week. After a 14-h overnight fast, blood samples were obtained by venipuncture into heparinized tubes and were immediately centrifuged at 4 °C for 10 min (1800 × g). The cold plasma was then transferred to an Amicon membrane cone (molecular mass cutoff, 25 000 Da). The ultrafiltrate was then collected for 30 min at 4 °C (1800 × g) in 100 mg of washed cation-exchange resin. The resulting ultrafiltrate (pH ~1) was assayed or stored at −20 °C until assay. For the plasma oxalate assay only, 500 μL of ultrafiltrate was added to a tube containing 100 mg of silver cation-exchange resin and 0.5 mL of 0.5 mol/L boric acid and was vortex-mixed for 30 s. After centrifugation at 10 °C for 5 min (1800 × g), 150 μL of the supernate was injected onto the AS10 column. A standard curve was prepared by mixing aqueous oxalate with an equal volume of 0.5 mol/L boric acid to give oxalate concentrations ranging from 0.5 to 5 μmol/L.

Urine Sampling

Urine samples were collected from the fasting subjects at the same time as the blood samples. All subjects had also collected a 24-h urine sample, with HCl as preservative, on the previous day. The samples were stored in several portions at −20 °C until assay (<2 months). Samples were cleaned for chromatography with C18 Sep Pak cartridges (Millipore-Waters) as described elsewhere (10). For oxalate, urine samples (or standards) were diluted either 50- or 100-fold with 0.5 mol/L boric acid, and 150 μL was injected onto the AS10 column. The HPIC conditions for the urine assay were identical to those for plasma. For glycolate, samples were diluted with purified water (usually 20-fold dilution). Subsequent chromatography followed the procedure of Wanzilak et al. (10).

Results

Oxalate Assay

Figure 1 shows the chromatograms of plasma ultrafiltrate and urine oxalate as compared with those of aqueous standards. [14C] Oxalate was added to preparations to determine
the recovery of each assay. The overall median yield ± SD of recovered [14C]oxalate was 95% ± 2.1%.

Multiple assays (n = 9–16) of five 0.5-mL portions of prepared plasma ultrafiltrate yielded CVs of 6.2–8.1%. Repeat assays (after 6 months' storage) of 32 plasma ultrafiltrate samples with oxalate concentrations ranging from 1.2 to 9.1 μmol/L yielded a CV of 7.1%. The detection limit was 37.5 pmol injected (equivalent to 0.5 μmol/L in plasma).

Oxalate peaks were identified by comparing elution times with those of aqueous standards and collecting [14C]oxalate-supplemented plasma ultrafiltrates. In addition, an ultrafiltrate sample was incubated (pH 3, 37 °C for 30 min) with oxalate decarboxylase (11). This was then filtered (25-kDa cutoff filter) and injected onto the AS10 column after removal of chloride. No detectable peak was observed at the elution time for oxalate.

With the AS10 column, oxalate was eluted at ~15 min and with the AS4A column, at ~8–9 min. The longer elution time on the AS10 column allowed better separation than that found with the AS4A column. Although similar oxalate values were found with the AS4A column, the inadequate baseline separation meant that reproducibility and accuracy were not as good.

Ascorbic acid did not alter oxalate concentrations when added to plasma samples. Furthermore, there was no detectable oxalate peak when ascorbic acid was added to the column directly.

**Glycolate Assay**

Figure 2 shows the chromatogram of a plasma ultrafiltrate for glycolate compared with that for an aqueous standard.

CVs were 2.4% and 6.2% in samples measured 10 and 16 times, respectively. A repeat assay of 31 separate samples after 6 months' storage had a CV of 2.1%. The detection limit was 50 pmol injected (equivalent to 0.7 μmol/L in plasma).

Glycolate (2, 5, and 10 μmol/L) was added to plasma samples with initial glycolate concentrations of 7.5 and 17.1 μmol/L. The recovery ± SD of added glycolate in these six samples was 99.7% ± 11%. Recovery of the [14C]glycolate in plasma ultrafiltrate before and after elution was 98–103% and 96–98%, respectively (n = 11).

**Plasma and Urinary Oxalate and Glycolate Concentrations**

Fasting plasma oxalate and glycolate concentrations did not differ significantly between the sexes (Table 1). Fasting urinary oxalate/creatinine and glycolate/creatinine ratios and 24-h oxalate/creatinine ratios were also
similar in men and women. However, in absolute amounts, women excreted less oxalate than did men ($P < 0.01$). Absolute urinary glycolate excretion was not significantly different between sexes, but women had a greater glycolate/creatinine ratio than did men ($P < 0.01$). Table 2 shows urinary oxalate and glycolate concentrations in our subjects.

**Discussion**

We used an improved HPIC system to determine fasting plasma concentrations of oxalate and glycolate and fasting and nonfasting urinary concentrations of oxalate and glycolate in healthy normal subjects. Our plasma oxalate values were similar to those of Schwille et al. (5), who also used HPIC, and Kasidas and Rose (12) and Costello and Landwehr (13), who used enzymatic methods. These values, however, were lower than those of Petraru et al. (1), Politi et al. (2) (HPIC method), and Wilson and Liedtke (14) (enzymatic method). Our plasma glycolate values were considerably lower than those of others (3, 4, 9) and those reported by us previously (15). In 1989, we reported a plasma glycolate value (±SD) of 7.2 ± 0.95 μmol/L, which was similar to that reported by Marangella et al. (9) in 1991 (7.8 ± 1.7 μmol/L). Much higher values (150–190 μmol/L) were reported by Kasidas and Rose in 1979 (3) and Costello et al. in 1989 (4) (enzymatic method).

In the oxalate assay, several procedures improved the sensitivity and reproducibility of the assay. These included the removal of chloride as an initial step in separating oxalate from other anions in the blood and the use of the AS10 column, which gave better separation of the oxalate peak. We avoided the acidification of plasma before ultrafiltration (with HCl or cation-exchange resin) because this resulted in a poor recovery of oxalate (15).

We found that ascorbic acid did not generate additional oxalate when plasma samples were processed according to our protocol, which included attention to cooling, acidification of the ultrafiltrate, and addition of borate before injection and in the eluting buffer. Chalmers et al. (16) showed that in urine samples at room temperature and with pH $> 7$, oxalate is generated from ascorbate. Here, with borate buffer at pH $\sim 9.4$ as the eluting buffer, the resulting chromatograms showed a large peak in the first 4 min but no detectable peak at the elution time for oxalate. When NaOH was used instead of boric acid as the eluting buffer, oxalate was generated. This suggested that the borate in the buffer was acting as an inhibitor of oxalate generation from ascorbate for the brief time that ascorbate was in the column. Storage of plasma for long periods (≥ 6 months) did not alter oxalate concentrations.

Our previous method (15) deproteinated the plasma by a variation of Hunter's method (17), which involves boiling plasma in dilute HCl for 5 min. This heating procedure may have been responsible for the higher

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**Table 1. Fasting Plasma Oxalate and Glycolate in Healthy Subjects**

<table>
<thead>
<tr>
<th></th>
<th><strong>Men (n = 14)</strong></th>
<th><strong>Women (n = 25)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>40 ± 10</td>
<td>39 ± 10</td>
</tr>
<tr>
<td><strong>Oxalate, μmol/L</strong></td>
<td>2.0 ± 0.2</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td><strong>Glycolate, μmol/L</strong></td>
<td>4.7 ± 0.4</td>
<td>4.4 ± 0.3</td>
</tr>
</tbody>
</table>

*Mean ± SD.*

**Table 2. Fasting and 24-h Nonfasting Urinary Oxalate and Glycolate in Healthy Subjects**

<table>
<thead>
<tr>
<th></th>
<th><strong>Men (n = 14)</strong></th>
<th><strong>Women (n = 25)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxalate/creatinine</td>
<td>0.017 ± 0.002 (0.004–0.030)</td>
<td>0.021 ± 0.002 (0.004–0.038)</td>
</tr>
<tr>
<td>Glycolate/creatinine</td>
<td>0.025 ± 0.003 (0.009–0.050)</td>
<td>0.030 ± 0.002 (0.009–0.051)</td>
</tr>
<tr>
<td><strong>24-h nonfasting</strong></td>
<td></td>
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<tr>
<td>Oxalate, mmol/day</td>
<td>0.368 ± 0.026 (0.010–0.040)</td>
<td>0.269 ± 0.017 (0.011–0.040)</td>
</tr>
<tr>
<td>Oxalate/creatinine</td>
<td>0.025 ± 0.002 (0.013–0.030)</td>
<td>0.025 ± 0.002 (0.013–0.030)</td>
</tr>
<tr>
<td>Glycolate, mmol/day</td>
<td>0.801 ± 0.111 (0.052 ± 0.004)</td>
<td>0.546 ± 0.047 (0.013–0.030)</td>
</tr>
<tr>
<td>Glycolate/creatinine</td>
<td>0.038 ± 0.004 (0.009)</td>
<td>0.052 ± 0.004 (0.009)</td>
</tr>
</tbody>
</table>

*Mean ± SE (and range, mean ± 2 SD).

*Significantly different from men, $P < 0.01$.

*Significantly different from men, $P < 0.05$. 

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**References:**

1. Petraru et al.
2. Politi et al.
5. Schwille et al.
7. Petraru et al.
9. Marangella et al.
10. Chalmers et al.
11. Petraru et al.
12. Politi et al.
15. Costello and Landwehr.
16. Chalmers et al.
17. Hunter's method.
plasma glycolate concentrations found previously, because heating may promote glycolate generation from precursors in plasma or contamination from the acid. Despite the differences between our earlier and current techniques, the recovery of [14C]glycolate was the same in each (~97%).

The technique described here for measuring plasma oxalate and glycolate is efficient, because a single sample ultrafiltrate can be used for both assays. Furthermore, the technique is sensitive, accurate, and reproducible.

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References

Interference of Carbamylated and Acetylated Hemoglobins in Assays of Glycohemoglobin by HPLC, Electrophoresis, Affinity Chromatography, and Enzyme Immunoassay

Cas W. Weykamp,1 Theo J. Penders,1 Carla W. M. Siebelder,1 Frits A. J. Muskiet,2 and Willem van der Silke2

In vitro-synthesized carbamylated and acetylated hemoglobins interfered in assays of glycohemoglobin by HPLC and electrophoresis but had no effects on results obtained by affinity chromatography and enzyme immunoassay. Correlations between long-term serum urea concentrations and glycohemoglobin percentages revealed that, in vivo, carbamylated hemoglobin equivalent to 0.063% of total hemoglobin is formed for every 1 mmol/L of serum urea. The use of acetylsalicylate, either chronically or in small doses (200-300 mg/day) or for 1 week at 2000 mg/day, did not cause significant interference from acetylated hemoglobin, formed in vivo. We conclude that interference from carbamylated hemoglobin explains only a small part of existing discrepancies between results of glycohemoglobin assays in current use. The interfering effect of acetylsalicylate in vivo with acetyl-CoA as substrate is as yet unknown.

Additional Keyphrases: variation, source of urea, acetylsalicylate

Glycohemoglobin is widely accepted as a valuable indicator for long-term diabetic control (1). However, the various methods currently used for its assay in clinical chemical laboratories show systematic differences between reported values (2). Solving this problem has become a major priority of the National Diabetes Group in the United States (3). In a European external