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A New Method for D-Glucaric Acid Excretion Measurement That Is Suitable for Automated Instruments

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Urinary excretion of D-glucaric acid (uGA) is an index of type II hepatic microsomal enzyme induction, indirectly revealing possible organic effects of some drugs and environmental pollutants. However, its determination is often cumbersome. We suggest a new, fast microanalytical method for uGA determination in which β-glucuronidase (BG; EC 3.2.1.31) activity inhibition produced by uGA-derived 1,4-D-glucarolactone is measured. With use of purified BG, the method is suitable for centrifugal analyzers, allowing assay of >100 samples per day. Moreover, the method measures uGA more accurately than other enzymatic methods based on BG inhibition. The within-day CV ranges from 7.9% to 4.6% (uGA 31.55–121.31 μmol/L); the between-day CV ranges from 11.5% to 5.0% (uGA 26.09–124.10 μmol/L). The detection limit is 6.0 μmol/L. The standard curve is linear from 10 to 200 μmol/L. Mean analytical recovery is 100%. Comparison with the method of Simmons et al. (Clin Chim Acta 1974;51:47–51) gave a correlation of $r = 0.978$, $y = 1.40x - 2.81$. Reference intervals were established in a healthy population sample of 369 people (165 under 14 y), and uGA, expressed in micromoles per gram of creatinine, was higher in women than in girls or in males.

Additional Keyphrases: *effects of drugs, environmental pollutants · hepatic microsomal enzyme induction · centrifugal analyzer · reference interval · sex- and age-related effects · monitoring therapy · enzyme purification*

Different xenobiotic substances, including environmental pollutants and drugs, can activate the drug-metabolizing system in liver microsomes and are metabolized mainly by the cytochrome P-450 system of mono-oxygenases and other enzymes of the smooth endoplasmic reticulum (*1*).

After exposure to an exogenous lipophilic substrate, the activities of substrate-specific mono-oxygenases increase, generating oxygen radicals that hydroxylate (so-called phase-I reaction) the exogenous substrate to metabolites, which undergo further enzymatic conjugation (e.g., to glucuronide) in the phase-II reaction and are then excreted.

Assessment of functional induction of the hepatic microsomal enzyme system is therefore valuable to predict the individual response to therapy, reveal individual or group exposure to chemicals, and correlate it with changes in metabolic activation and deactivation of hepatotoxic agents and carcinogens (*2, 3*).

The methods used to assess induction of the hepatic microsomal enzyme system are:

1) in vitro determination of specific enzyme content and activity in liver biopsy specimens (*4–6*);

2) in vivo antipyrine or theophylline clearance, indicators of phase-I reactions (*7, 8*);

3) serum γ-glutamyltransferase (EC 2.3.2.2) determination (*9–11*);

4) urinary excretion of 6β-hydroxycortisol, indicator of phase-I reactions (*8, 12, 13*); and

5) urinary excretion of D-glucaric (or D-saccharic) acid (uGA), indicator of phase-II reactions (*14–18*).[3]

Demonstrably (*2, 3, 14–18*), uGA is a suitable indicator of liver microsomal enzyme induction, thus giving indirect information on the presence of foreign toxic substances (*12, 19, 20*), although a recent paper (*21*) questions this assumption and suggests that uGA is a less-sensitive index than antipyrine clearance, at least for low doses of phenobarbital.

uGA can be quantified either by chromatography (*22–24*), which is complex and not particularly favored in a clinical laboratory, or by enzymatic methods (*25–31*).

These rely on two different principles:

1) uGA in urine is in equilibrium with 1,4- and 1,6-glucarolactone (GL). Boiling urine at an acidic pH transforms uGA into 1,4-GL. uGA can be indirectly measured by means of the competitive inhibition that 1,4-GL exerts on the enzymatic activity of β-glucuronidases (EC 3.2.1.31) (BGs) on adequate substrates, such as phenolphthalein-β-D-glucuronide and 4-nitrophenyl-β-D-glucuronide (NP-G) (*25–30*).

2) Treatment of uGA with a bacterial extract containing glucarate dehydratase (EC 4.2.1.40) and ketodeoxyglucarate aldolase (EC 4.1.2.20) leads to quantitative formation of pyruvate, which is then assayed by use of lactate dehydrogenase (EC 1.1.1.27) (*31*).

Table 1 lists advantages and disadvantages of both groups of methods.

For sample throughput to be high, tests based on enzymatic inhibition (*26–30*) are usually preferred. Here we report a new method for uGA assay, which is faster, yielding about 100 uGA determinations per day, but is as precise and accurate as previous procedures.

For reasons of economy we also propose a method to produce, through simple chromatographic purification of BG, an enzyme suitable for uGA determination that will be sufficiently sensitive to inhibition by 1,4-GL—although the method also works with a commercially available BG.

Materials and Methods

Specimen Collection and Preparation

Specimens of first morning urine from apparently healthy people were stored at room temperature if analyzed the

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[3] Nonstandard abbreviations: uGA, urinary D-glucaric acid; GA, D-glucaric acid; BG, β-glucuronidase; GL, D-glucarolactone; NP-G, 4-nitrophenyl-β-D-glucuronide; PBS, phosphate-buffered isotonic saline; CRE, creatinine; Con-A, concanavalin A.

Table 1. Outlines of Analytical Methodologies for Urinary D-Glucaric Acid Determination

Ref. no.	Method	Specimen preparation	BG[a] purification	Analytical steps	Calibration curve	Interference
25	Enzymatic inhibition	1)Acid-boiling 2)Alkali-boiling (blank)	Complete BG purification	Multistep manual	Exponential	Subtraction of blank value
22	Liquid chromatography	Not required	Not required	Multistep manual	Linear	By ascorbic acid
23	Gas chromatography	1) Acid-boiling 2) Silanization	Not required	One-step manual	Linear	Not described
26	Enzymatic inhibition	1) Acid-boiling 2) Alkali-boiling (blank)	Not required	Multistep manual	Exponential	Subtraction of blank value
27	Enzymatic inhibition	1) Acid-boiling 2) Alkali-boiling (blank)	Complete BG purification	Multistep manual	Linear	Subtraction of blank value
24	HPLC	Not required	Not required	One-step manual	Linear	By biol. matrices[b]
28	Enzymatic inhibition	Acid-boiling	Not required	Multistep manual	Linear	Not taken into account
31	Enzymatic detn. by conversion to pyruvate	Ion-exchange chromatography	Bacterial BG preparation	Multistep manual	Linear	Subtraction of blank value
30	Enzymatic inhibition	Acid-boiling	Not required	Multistep semiautomated	Linear	Not reported in abstract

[a] BG, β-glucuronidase. [b] Determination possible only for pure solutions.

same day or at −20 °C if analysis was delayed. Samples were centrifuged (10 min, 2000 × *g*) immediately before analysis.

Reagents

The following reagents were used: BG/arylsulfatase (EC 3.2.1.31/EC 3.1.6.1) from *Helix pomatia*, stabilized solution (code 127 698, lot 10003320; Boehringer, Mannheim, F.R.G.); BG from *Haliotis midae* (code 36–203, lot 32; Miles Italiana, Cavenago Brianza, Milano, Italy); BG from bovine liver (code G-0501); BG from *Patella vulgata* (code G-8132, lot 92F-3913); BG from *Helix pomatia*, partially purified by gel permeation chromatography (code G-1512, lot 73F-71851; Sigma Chemical Co., St. Louis, MO); NP-G (Boehringer); Sepharose CL-6B, Con-A Sepharose 4B (Pharmacia, Uppsala, Sweden); methyl-4-D-mannoside (grade III, Sigma); "Total Protein, Lowry Micro Method" kit (Sigma); "Creatinine Merckotest A" (Merck, Darmstadt, F.R.G.); and distilled water with a resistance of more than 10 MΩ, prepared with a "MilliQ" unit (Millipore, Bedford, MA).

Standards

The D-saccharic acid (glucaric acid; GA), monopotassium salt, used as a standard was supplied by Sigma and was prepared daily from a stock 200 μmol/L solution in distilled water (stable for 12 months at −80 °C).

Apparatus

The diluter used was a Model 2075 (LKB, Bromma, Sweden) and the pH-meter was a Model PW 9408 (Philips, Cambridge, U.K.). Throughout the study we used either a Cobas Bio or a Cobas Fara (Roche, Basel, Switzerland) centrifugal analyzer.

BG Purification

Dilute 1 mL of the BG original solution with 4 mL of phosphate-buffered saline (PBS), load on a Con-A Sepharose-CL-4B column (10 mL, bed height 5 cm), wash with four bed volumes of PBS, elute with one bed volume of methyl-4-D-mannoside, 20 mmol/L in PBS, collect fractions of the eluate, and test them for BG activity.

Analytical recovery of the enzyme activity is usually 80% (stable for at least six months if stored at 4 °C).

Alternatively, to save on the cost of Con-A-coupled resin, chromatograph at room temperature (25 °C), 5 mL of the BG original solution on a CL-6B-Sepharose column (72 mL, bed height 36 cm), with PBS (pH 7.4, phosphate 10 mmol/L) containing NaCl 130 mmol/L, KCl 2.7 mmol/L, $CaCl_2$ 0.9 mmol/L, and $MgCl_2$ 0.5 mmol/L as eluent. Collect and test fractions for BG activity in acetate buffer (pH 5.0, 0.8 mol/L) with NP-G, 1 mmol/L (final concentration), at 37 °C and read the absorbance at 401 nm.

Pool the fractions containing more than 5% of the total activity applied to the column. Chromatograph the pool at room temperature on a Con-A Sepharose-CL-4B column (10 mL, bed height 5 cm), wash with two bed volumes of PBS, then elute with one bed volume of methyl-4-D-mannoside, 20 mmol/L in PBS. Analytical recovery and stability of the purified enzyme are as above.

Study of the Specific Activity of BG

Add one volume of the diluted BG solution to one volume of NP-G, 5 mmol/L, and measure the activity as the absorbance increase per minute at 401 nm in the linear range. Measure the total protein content of the eluate by the "Total Protein, Lowry Micro Method" (see *Reagents*). Divide the absorbance increase per minute by the protein concentration to find the specific activity of BG per gram of protein.

K_m Determination for NP-G

For each BG preparation, dilute the enzyme solution in a tube with acetate buffer (1 mol/L, pH 5.0) so as to obtain a 0.2 absorbance unit increase in 10 min when mixed with an equal volume of NP-G, 2 mmol/L, in the same acetate buffer.

Prepare five tubes, each containing four volumes of acetate buffer (1 mol/L, pH 5.0) and NP-G concentrations of 6.0, 1.5, 0.85, 0.60, and 0.45 mmol/L, respectively. Add to each tube one volume of distilled water and one volume of diluted BG, to obtain final NP-G concentrations of 4.00, 1.00, 0.57, 0.40, and 0.30 mmol/L, respectively. Measure the absorbance increase per minute at 401 nm during the first 10 min.

Plot the results according to the Michaelis–Menten equation and calculate K_m values for each enzyme.

K_i Determination for GL When the Substrate Is NP-G

Prepare five tubes, each containing four volumes of acetate buffer (1 mmol/L, pH 5.0) and respective NP-G concentrations of 6.0, 1.5, 0.85, 0.60, and 0.45 mmol/L. Add to each tube one volume of 1,4-GL, obtained by boiling a 100 μmol/L GA solution with one volume of diluted BG. The final NP-G concentrations should be 4.00, 1.00, 0.57, 0.40, and 0.30 mmol/L, respectively. Measure the absorbance increase per minute at 401 nm during the first 10 min. Substitute results in the Michaelis equation modified for competitive inhibition and calculate K_i.

Preparation of Reagents for uGA Assay

Prepare sodium acetate buffers (1 mol/L and 2 mol/L, pH 5.0) according to standard procedures.

BG working solution: Dilute BG with acetate buffer (1 mol/L, pH 5.0) to have an absorbance of 0.2 *A* in 10 min when incubated with NP-G (1 mmol/L, final concentration).

Substrate (NP-G) working solution (1.72 mmol/L): Prepare substrate freshly each day. Dissolve 16.2 mg of NP-G in 30 mL of acetate buffer (1 mol/L, pH 5.0). This quantity suffices for 50 specimens.

GA standard stock solution (200 μmol/L): Dissolve 49.64 mg of D-glucaric acid, monopotassium salt, in 1000 mL of distilled water (stable for one year if stored at −80 °C).

Treatment of Standards and Urine Samples for uGA Determination

Adjust the pH to between 4.0 and 6.0 by adding glacial acetic acid to those centrifuged urine specimens whose pH exceeds 6.0. Aliquot 1 mL of each urine specimen and of standard solution into two sample-tubes, A and B; add to A 0.2 mL of 1.5 mol/L HCl and to B 0.2 mL of 1.5 mol/L KOH. Boil both aliquots for 45 min and cool them to room temperature. The resulting pH (*32*) will always be <2.0 for sample A and >8.0 for sample B. After boiling, the samples are stable for 4–5 h. Add 1 mL of acetate buffer (2 mol/L, pH 5.0), to both A and B, then add 0.2 mL of 1.5 mol/L KOH to A and 0.2 mL of 1.5 mol/L HCl to B. The resulting pH of the samples will be 5.0 ± 0.1.

Assay of uGA

Program the Cobas Bio or Fara at 37 °C: (*a*) to transfer 50 μL of each aliquot and 200 μL of NP-G substrate working solution in the reaction cell and to mix them for 5 min; (*b*) to add, as a starter reagent, 50 μL of BG working solution; and (*c*) to monitor the reaction at 401 nm for 10 min.

Programming for the Cobas Bio and Fara is shown in Table 2.

Assay of Creatinine (CRE)

CRE was determined with the "Creatinine Merckotest A" (see *Reagents*). Specimens from children younger than 6 y were first boiled for 4 h at pH 2.0, in capped tubes, to convert creatine to creatinine. The initial volume was restored by adding distilled water.

Calculation to Obtain the Concentration of uGA

For each standard and urine specimen compute $y = (1/A_A - 1/A_B)$, where A_A and A_B are respectively the absorbance after 10 min minus the absorbance after 30 s of reaction for the acid-treated (A) and the alkaline-treated (B) aliquot. Compute the regression curve and the coefficient of correlation, *r*, between concentration and (*y*) the values for the

Table 2. Programs Used in the Cobas Bio and Cobas Fara Centrifugal Analyzers for Urinary D-Glucaric Acid Determination

Cobas Bio		Cobas Fara	
1. Units	*A*	Measurement mode	Absorb
2. Calculation factor	1.000	Reaction mode	P-I1-SR1-AO
3. Standard 1 concn.	0	Calibration mode	Factor
4. Standard 2 concn.	0	Reagent blank	No blank
5. Standard 3 concn.	0	Wavelength, nm	401
6. Limit	0	Temperature, °C	37.0
7. Temperature, °C	37.0	Decimal position	4
8. Type of analysis	7	Unit	Δ*A*
9. Wavelength, nm	401	Sample vol., μL	50
10. Sample vol., μL	50	Diluent vol., μL	40
11. Diluent vol., μL	45	Reagent[a] vol., μL	200
12. Reagent vol.,[a] μL	200	Incubation time, s	300
13. Incubation time, s	300	M1, s	300
14. Start reagent[b] vol., μL	50	Start R1[b] vol., μL	50
15. Time of first reading, s	30.0	Diluent vol., μL	25
16. Time interval, s	200	First reading, s	30.0
17. No. readings	04	Readings number	4
18. Blanking mode	1	Interval, s	200
19. Printout mode	3	Conversion factor	1.00000
		Offs	0.00000
		Reaction direction	Increase
		Check	On
		Sample limit	No
		Test range: low, high	No
		Calcn. steps	1
		Calcn. step A	Endpoint
		Reading, first	1
		Reading, last	4
		Calibn. factor	1.00000
		Control, CS1, CS2, CS3	

[a] 4-Nitrophenyl-β-D-glucuronide working solution.
[b] β-Glucuronidase working solution.

standards. Calibrations are to be rejected as faulty if r is <0.998. Divide the uGA concentration by CRE concentration and express results as micromoles of uGA per gram of CRE.

Evaluation of the Method

Within-day precision: Three specimens (whose average uGA concentrations were measured to be 31.55, 69.56, 121.31 μmol/L) were analyzed 20 times in the same day.

Between-day precision: Three pools with measured uGA concentrations of 26.09, 76.57, 124.10 μmol/L, respectively, were prepared from urine samples from 200 randomly selected people (including both healthy and sick subjects), aliquoted, and stored at −15 °C. Aliquots were analyzed (in triplicate) on each of 20 days.

Detection limit: The detection limit of the method was calculated as 2.6 × the SD of 20 blank determinations, because the true blank is set to zero in each analytical batch.

Analytical recovery: Known amounts of GA (50 or 98 μmol/L final increase in concentration) were added to 20 different urine specimens and to a pool of 200 urine samples, which were then assayed.

Interference: An aliquot of the interferent substance to be tested was added (1/10 by vol) to a urine pool to obtain the following final concentrations: hemoglobin 5 g/L, bilirubin 80 mg/L, porphyrins 2 mg/L, ascorbic acid 2 g/L, glucose 100 g/L, and albumin up to 18 g/L. Each of these samples and a control aliquot, to which distilled water (1/10 by vol) was added, were assayed in triplicate.

Method comparison: uGA concentration was measured by both the present method and that of Simmons et al. (*26*) in urine samples from 152 people (including 21 apparently healthy people, 27 pregnant women, six drug addicts, 27 patients who were undergoing benzodiazepine treatment, and 71 patients on anti-epileptic medication). All samples were assayed in duplicate by each method, and data were subjected to regression analysis.

Reference Interval

We selected 183 males and 186 females (one to 65 y old) and categorized them by age, according to IFCC recommendations (*33*). All were apparently healthy people who were undergoing routine analyses at the Hospital of Desio, Milano, Italy. They had no smoking and (or) alcohol abuse habits and did not present any history of medication or drug consumption in the 30 days before specimen collection. The results of their uGA assay were evaluated according to IFCC recommendations (*34*).

Results

Choice of the Source of BG

As a first step to achieve good quality, we studied the K_m for NP-G and the K_i for GL of commercially available BGs (Table 3). BG from *Helix pomatia* (Boehringer) was used both as supplied and after our purification through affinity chromatography. The enzyme preparation obtained by affinity chromatography performed as well as the preparations (from the same source) that we purified by sequential purification through gel permeation chromatography, ion-exchange chromatography, *and* affinity chromatography on Con-A.

For good sensitivity to GL inhibitor, the K_i for 1,4-GL obtained by boiling GA solutions at pH 2.0, as well as the K_m for NP-G for a high catalytic velocity, should be as low as possible.

Table 3. Kinetic Characteristics of Tested β-Glucuronidases[a]

Source of enzyme	K_m μmol/L	K_i μmol/L	Specific activity, $A \times min^{-1} \times mg^{-1}$
Helix pomatia (Boehringer)	645	144	0.1998
Helix pomatia (Boehringer) after affinity chromatography[b]	712	69	0.3846
Bovine liver (Sigma)	3108	734	0.3978
Helix pomatia (Sigma) after exclusion chromatography	449	53	0.0133
Patella vulgata (Sigma)	407	92	0.0466
Haliotis midae (Miles)	525	332	0.0510

[a] See text for explanation. [b] Performed at the laboratory of Desio Hospital.

The K_m and K_i reported in Table 3 show that the best results are to be expected with BG from *Helix pomatia*, purified either by affinity chromatography in our laboratory or by gel permeation chromatography as is done by Sigma.

Use of Boehringer's BG purified by affinity chromatography offers advantages, both in terms of much higher specific activity and lower cost.

Bovine-liver BG performs much less satisfactorily with NP-G than with phenolphthalein-β-glucuronide as substrate.

Sample Preparation

Transformation of uGA into 1,4-GL is maximal when urine is boiled at a pH <2.5 (data not shown). The specimens must be diluted by 1.5 mol/L HCl or 1.5 mol/L KOH as little as possible, if one is to obtain good precision at the upper limit of the reference interval.

Reaction Conditions

It is important that the reaction conditions for uGA determination are set to have the greatest difference between the reaction rates of BG in acid-boiled or alkaline-boiled urines. This was the case under the following reaction conditions (data not shown): NP-G 1 mmol/L, acetate buffer 0.8 mol/L, pH 5.0—the same conditions used by Jung et al. (*27*).

Validation of the Method

Calibration curve: Plotting inhibition results according to Dixon (*35*) gives a linear calibration curve. Linear regression analysis of data from 30 sets of standards under different concentrations (Figure 1) confirms the linearity for uGA concentrations from 10 to 200 μmol/L. This is equivalent to the range obtained by the method of Jung et al. (*27*) and slightly lower than that of the enzymatic method of Marsh (*31*).

Detection limit: The detection limit for the method is 6.0 μmol/L, which is in agreement with that for most other methods (*26–28, 31*).

Within-day precision: Data from 20 replicates of uGA determinations at three different concentrations are reported in Table 4. The CV ranges from 4.6% to 7.9% at 121.31 and 31.55 μmol/L, respectively.

Between-day precision: Between-day precision at three different uGA concentrations was evaluated in triplicate. The data reported in Table 4 demonstrate that precision increases with increasing uGA concentrations.

Our CVs are higher than CVs reported by Jung et al. (*27*) but are similar to what was reported by Marsh (*31*), who

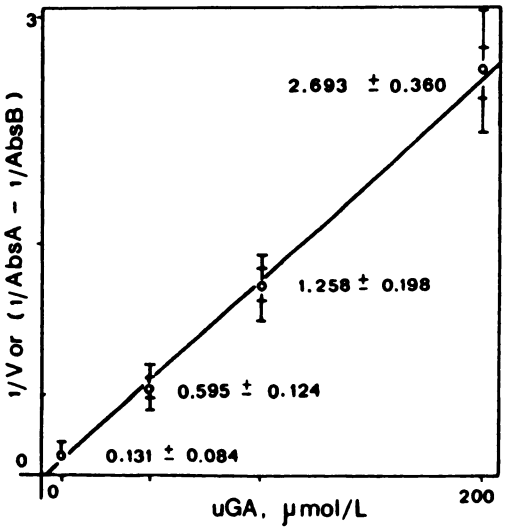

Fig. 1. Calibration curve for urinary D-glucaric acid (uGA) assay by the proposed method

Values determined during 30 days. *Points* (and *bars*) represent means ($1/A_a$[a] − $1/A_b$[b]) ± SD (both 1 and 2). Slope = 0.0134 (SD = 0.00013). Intercept = −0.0268 (SD = 0.0131). Standard error of estimate = 0.0570. [a]A, urine aliquot boiled at pH <2.0. [b]B, urine aliquot boiled at pH >8.0

analyzed quite a few replicates, and similar to the CVs reported by Colombi et al. (*28*) and by Sandle and Braganza (*36*), who used a low-pH enzymatic assay. Simmons et al. (*26*) do not report precision data for their method, although in our experience its precision is similar to that for the present method.

Analytical recovery: Fifty and 98 µmol of GA per liter was added to two sets of 20 different urine samples or to the same pool of 200 urines. uGA was determined in triplicate, and analytical recovery was ~100% (Table 5).

Interference: No interference was observed by added bilirubin up to 80 mg/L, porphyrins up to 2 mg/L, ascorbic acid up to 2 g/L, or glucose up to 100 g/L. Hemoglobin below 5 g/L and albumin below 15 g/L do not interfere if the specimens are centrifuged at 1000 × *g* for 5 min after acid-boiling to remove turbidity.

Comparison of methods: The overall correlation of results for 152 different samples between our method and that of Simmons et al. (*26*) is good ($r = 0.978$). As reported in Figure 2, the proposed method (y) shows concentrations higher than those obtained by Simmons et al. (x), the equation for the line being $y = 1.40x - 2.81$ ($S_{y \cdot x} = 42.83$, SD of slope = 0.02, and SD of intercept = 4.16).

Reference Interval

Results of uGA determinations on subjects of the population described above, submitted to the Kolmogorov–Smirnov test (*34*) for normality of the frequency distribution, did not show any deviation from normality for either sex. Data referring to age groups of apparently healthy people, categorized by sex, are reported in Figure 3.

In children younger than 14 y, uGA excretion (expressed in µmol per gram of CRE) does not differ in males (81 subjects, mean value 13.77) and females (84 subjects, mean value 14.59). Values for uGA excretion did not change (Table 6) in the group of 102 men older than 14 y (14.39), whereas higher ($P < 0.001$) values were observed for women older than 14 y (102 subjects, mean value 18.70). The latter also have higher interindividual variability than men, as also reported by March et al. (*37*).

The reference intervals, 2.5 and 97.5 fractiles, were calculated by parametric methodology (*34*) for females older than 14 y, females younger than 14 y, and for males. The fractiles and their 90% confidence intervals are reported in Table 6.

Discussion

This method for measurement of uGA excretion is quicker and much simpler than the procedures for uGA determination proposed so far (*22–28, 31*), while providing similar linearity, precision, accuracy, and analytical sensitivity (*25–31*). Our method allows many tests (80–100/day) to be performed routinely; hitherto, the maximum rate was 40–60 determinations per day (*26–27*).

To achieve these features we perform the enzymatic determination of uGA with use of a purified BG, whose reaction on the substrate NP-G can be easily monitored at pH 5.0 (the pH of the reaction solution), especially in a centrifugal analyzer.

This fact makes unnecessary the usual stopping of the NP-G enzymatic hydrolysis by adding NaOH (*26, 27*) to the reaction solution to increase its pH to alkaline values before reading. In fact, at pH 5.0 the NP-G hydrolysis by BG produces an absorbance increase linearly related to the concentration of the 4-nitrophenol released and measurable between 400 and 405 nm (the nonspecific absorbance of urine is too high below 400 nm). Under these conditions and in the absence of inhibitors, NP-G enzymatic hydrolysis produces a typical absorbance increase of ~0.20 *A* at 401 nm.

BGs from bovine liver and from *Helix pomatia* as crude extracts need to be purified, because they are not suitable to measure uGA under the conditions described. Purification of BG from *Helix pomatia* (Boehringer) is very simple in comparison with the purification specified in some methods,

Table 4. Precision of the Urinary D-Glucaric Acid (uGA) Assay

	uGA concentration, µmol/L		
n	Mean	SD	CV, %
Within-day			
20	31.55	2.50	7.9
20	69.56	4.70	6.8
20	121.31	5.58	4.6
Between-day			
20 (3)	26.09	3.01	11.5
20 (3)	76.57	6.72	8.8
20 (3)	124.10	6.20	5.0

In parentheses: number of replicates for each of n determination.

Table 5. Analytical Recovery of Urinary D-Glucaric Acid (uGA)

	uGA concn, µmol/L		
No. of samples	Added	Mean recovered (and SE, SD)	Mean uGA recovered, µmol/L (and SE, SD)
Recovery for different urine samples[a]			
20	50	49.81 (3.13, 5.42)	99.6 (6.27, 10.86)
20	98	102.19 (6.66, 11.53)	104.3 (6.80, 11.78)
Recovery for a pool of urine samples[b]			
20	50	50.48 (3.36, 5.82)	100.9 (6.72, 5.94)
20	98	98.01 (3.39, 5.88)	100.0 (6.78, 6.00)

[a] Each of the 20 urine samples was divided into three aliquots and assayed in triplicate.

[b] A pool of 200 urine samples was divided into three aliquots. Each aliquot was tested in triplicate for 20 days.

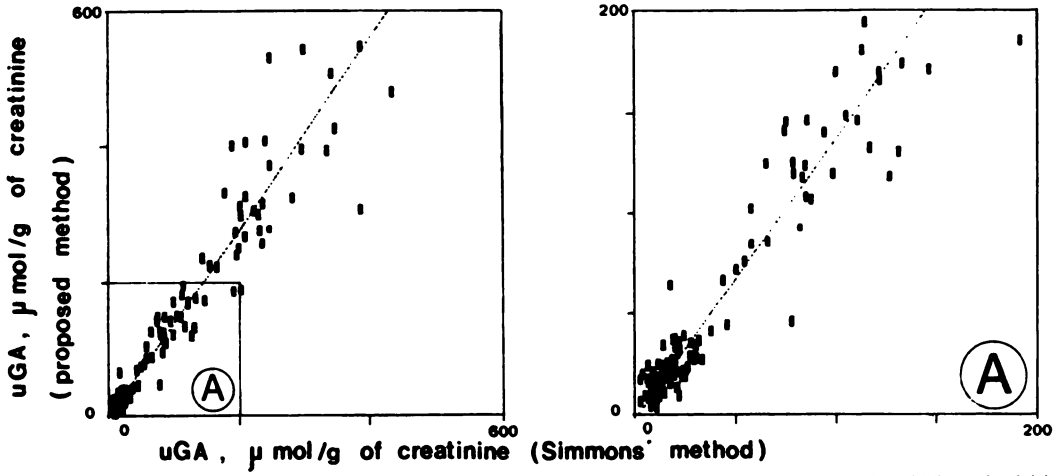

Fig. 2. Comparison of urinary D-glucaric acid (uGA) determination between the proposed method (*y*) and another (*26*) method (*x*)
Inset A shows the linear range of the proposed method. Slope = 1.40 (SD = 0.02); intercept = −2.81 (SD = 4.16); $S_{y.x}$ = 42.83; n = 152; *r* = 0.978

namely those for BG from rat liver (*25, 27*) and mouse kidney (*38*). In fact, it can be easily performed in any clinical chemistry laboratory with a single chromatographic step, passing BG through Con-A Sepharose (see *Methods*); alternatively, commercially available BG purified by gel permeation chromatography (Sigma) can be used.

With our method we have obtained uGA values ~40% higher than those obtained by the method of Simmons et al. (*26*) (see Figure 2). One reason could be that their method, like other methods based on BG inhibition, may be inaccurate because of the underestimate produced by nonspecific BG inhibition, as stated by Fiedler et al. (*39*). In fact, at the usual pH (5.0) of a BG assay, uGA (non-inhibitory) in the alkaline-treated aliquot is partly converted to GL (inhibitory) during the incubation and reaction intervals, which usually total 30–60 min (*31, 39*). Our method avoids such a nonspecific inhibition because the aliquots remain only briefly at pH 5.0 before reaction (about 15 min total for transfer operations and incubation) and because the use of an automated analyzer allows the reaction time to be shortened to 10 min. Up to a GA concentration of 200 μmol/L, GL production in the B aliquots is negligible, as proved by lack of BG activity inhibition in alkali-treated GA standards.

Moreover, Marsh (*31*) has reported that uGA conversion to pyruvate and subsequent enzymatic determination by means of lactate dehydrogenase gives higher values than those obtained by a modified method of Simmons et al. (26). Marsh (*31*) considered the former to be more reliable. These data support our results.

Reported normal reference values for uGA excretion differ greatly (*37, 39–41*), probably owing to the different methods used, to differences arising from standardization with GA or 1,4-GL, and to the small reference groups studied.

Because of its inherent specificity, the gas–liquid chromatographic method developed by Gangolli et al. (*23*) seems to provide the most reliable results for a comparison, even though they studied only six subjects. These authors reported a mean uGA excretion of 28.3 μmol/24 h (7.02 mg/24 h), equivalent to 18.9–21.8 μmol per gram of CRE (assuming the mean daily excretion of creatinine to be 1.5 g in the male and 1.3 g in the female). The mean uGA values obtained for a large sample population by our method (14.07 μmol/g CRE

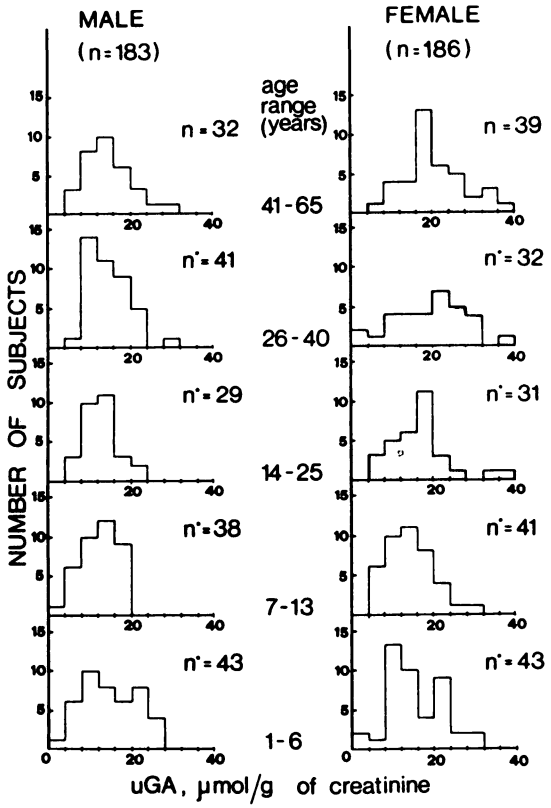

Fig. 3. Urinary D-glucaric acid (uGA) excretion by apparently healthy males and females according to age groups
x = uGA, μmol/g of creatinine. *y* = number of subjects. Data are for males, and for females <14 y, except in the three graphs top right, which are for females older than 14 y

Table 6. Reference Values for Urinary D-Glucaric Acid (uGA) Excretion in Morning Urines from Apparently Healthy People

Group (and age range, y)	No. of subjects	uGA concentration, μmol/g of creatinine: Mean	SD	0.95 reference limits (0.90 confidence interval for each limit)
Males (1–65)	183	14.07	5.34	3.6 to 24.5 (2.5–4.7) (23.4–25.6)
Females (1–13)	84	14.59	6.26	2.3 to 26.9 (0.4–4.2) (25.0–28.8)
Females (14–65)	102	18.70	7.91	3.2 to 34.2 (0.9–5.5) (31.9–36.5)

in 183 males and 18.70 μmol/g CRE in 102 females older than 14 y) agree reasonably well with their results.

Only Colombi et al. (*40*) report reference values derived from a large population of adults. They found a mean uGA excretion of 26.94 μmol per gram of CRE for 402 men, 29.67 μmol/g for 171 women. The higher values they obtained in comparison with our results can be expected, because their method (*28*) cannot measure the effects of nonspecific BG inhibitors in the matrix (*25, 31*).

Fiedler et al. (*39*) and Colombi et al. (*40*) also published values for uGA excretion per gram of CRE that were higher for women than for men, but no data from children were reported.

Steinberg et al. (*29*), who reported uGA excretion in mmol per mole of CRE, found a wider reference interval for children younger than 15 y than for adults. We did not find such a difference when we treated the children's urine samples to transform the excreted creatine into creatinine (see *Methods*); in fact, low values for creatinine in urine from children younger than 6 y, because of incomplete transformation of creatine to creatinine, give rise, by calculation, to apparently high results for uGA excretion.

In conclusion, the procedure described here for enzymatic measurement of uGA excretion is characterized by good sensitivity, precision comparable with that reported for other methods (*28, 31*), good specificity, accuracy excelling that for existing methods (*26, 28*), and faster analysis.

Finally we would like to remember that it is still an open question as to what is the best indicator of hepatic microsomal enzyme induction. Price et al. (*21*) support the view that, for low doses of phenobarbital, antipyrine clearance is more sensitive than 6β-hydroxycortisol or uGA. According to Sandle and Braganza (*36*), uGA would be a poor indicator of pancreatic monooxygenase induction. Recent reports (*8, 17, 18*) underline the usefulness of uGA determination as a marker of enzyme induction after administration of anticonvulsant drugs (*17*) and (or) any of a long list of other drugs (*18*). The correlation of uGA with 6β-hydroxycortisol varies in different reports, depending on the administered drugs (*13, 17, 18*). These differences can probably be explained with the hypothesis that "these tests probably reflect the activity of different but overlapping parts of the microsomal enzyme system" (*8*). This same author (*8*) stresses the potentially useful application of uGA determination for studies of microsomal enzyme function in occupational medicine. A more substantiated decision on the use of these tests can be made when a much larger number of people is studied as compared with the small groups investigated hitherto. Considering this important goal, the usefulness of our method is confirmed by the ease with which it permits, for health control or epidemiological studies, the follow-up of large populations of special subjects such as pregnant women, drug addicts, and people exposed to environmental toxic substances.

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