CLIN. CHEM. 27/3, 422-426 (1981)

# Improved Determination of D-Glucaric Acid in Urine

**Klaus Jung,[1] Dietmar Scholz,[1] and Gottfried Schreiber[2]**

Using a simplex optimization procedure, we have elaborated a sensitive and reliable micromethod for determining D-glucaric acid, based on inhibition of glucuronidase activity by 1,4-D-glucarolactone, produced from D-glucaric acid by heating. The maximum attainable difference between glucuronidase activity in the reaction mixture with and without D-glucaric acid was used as an optimization criterion. From the optimization scheme, 1 mmol/L 4-nitrophenyl-β-D-glucuronide and pH 5.0 were found to be optimal conditions for determination of D-glucaric acid with this test. In contrast to other similar methods, in which a logarithmic calibration curve is used, we calculate D-glucaric acid with a linear calibration curve by using the Dixon (*Biochem. J.* **55:** 170, 1953) plot. The coefficient of variation for the method (within-run and between-day precision) was from 3.1 to 5.8%. Men excrete more D-glucaric acid than do women per 24 hours, but we saw no sex-related differences in excretion of D-glucaric acid when it was expressed in terms of urinary creatinine.

**Additional Keyphrases:** *hepatic microsomal enzyme activity · enzyme induction · spectrophotometry · inhibition of β-glucuronidase · sex-related effect · reference interval*

The microsomal enzyme system of the liver can be activated by various drugs (*1–6*). Thus the biotransformation of endogenous and exogenous substances in the human organism and the biological availability of drugs are decisively influenced. Determination of GA[3] excretion in urine has proved to be a suitable index to microsomal enzyme activity of the liver (*7*). Marsh (*8*) described a method for GA based on the inhibition of GLUC activity by GA after this had been transformed into the GLUC inhibitor GL by boiling.

A disadvantage of this method is the logarithmic calibration curve given by Marsh (*8*) and used also by all subsequent authors (*1–6, 9, 10*), which is linear only in a relatively narrow range and may lead to low precision (*4*). Therefore, we elaborated a sensitive method by simplex optimization which, by using the Dixon plot, permits determination of GA with a linear calibration curve over a wide range.

## Materials and Methods

### Samples

Urine samples obtained from healthy adults were stored at −20 °C, if the assay was not done on the day of collection.

### Apparatus

Eppendorf photometer 1101 M, Eppendorf pipettes, thermoblock 2764 (all from Eppendorf Gerätebau Netheler & Hinz, Hamburg, F.R.G.)

pH-meter, Model MV 87 (VEB Präcitronic, Dresden, G.D.R.)

### Reagents

Sodium acetate, acetic acid, NaCl, and KOH were supplied by VEB Laborchemie, Apolda, G.D.R., and 4-nitrophenyl-β-D-glucuronide by Serva Feinbiochemica, Heidelberg, F.R.G. Potassium hydrogen D-glucarate was prepared according to Bose et al. (*11*). The melting point of doubly recrystallized potassium hydrogen D-glucarate was 188–191 °C and corresponded to the reported value (*12*). GLUC used was prepared from rat liver according to Lin et al. (*13*) and stored in portions at −20 °C. The solutions mentioned below were made up in triply distilled water. We adjusted the pH of solutions at 37 °C, using calibration buffers produced according to the U.S. National Bureau of Standards.

*Acetate buffer,* 250 mmol/L, pH 5.0. Dissolve 3.4 g of sodium acetate in about 80 mL of water, adjust to pH 5.0 with acetic acid, dilute with water to 100 mL. Stable for 14 days at 4 °C.

*Substrate solution* (250 mmol/L acetate buffer, 2.5 mmol/L 4-nitrophenyl-β-D-glucuronide). Dissolve 19.7 mg of 4-nitrophenyl-β-D-glucuronide in 25 mL of acetate buffer. Stable for two days at 4 °C.

*KOH solution,* 2 mol/L. Dissolve 11.2 g of KOH in water and dilute to 100 mL. Unlimited stability.

*Working enzyme solution.* Dissolve a portion of GLUC stored at −20 °C in sufficient water that an absorbance of about 1.000 is attained after 15 min activity determination.

*D-Glucaric acid,* 10 μmol/L. Dissolve 15.51 mg of potassium hydrogen D-glucarate in 250 mL of 10 mmol/L HCl. Dilute 4 mL of this solution with 46 mL of 10 mmol/L HCl, boil for 45 min, and use this solution for calibration (see Table 1). The solution must be freshly prepared daily.

### Procedures

The determination of GA is based on the principle that, by boiling the urine at pH 2.0, the GA is transformed into the GLUC inhibitor GL. From its inhibition of GLUC activity, the GA concentration is determined with a calibration curve. Because other substances in urine may inhibit GLUC as GL does, the concentration of GA, determined thus, consists of the actual GA concentration and an apparent GA concentration that is ascribable to the inhibitors of other nature in urine (*8*). Therefore, GLUC activity is in addition determined with urine boiled at pH 8.0, a pH at which GA is not transformed into GL. From this sample an apparent GA concentration is calculated from the calibration curve. The difference in the concentrations determined under these two conditions is the true concentration of GA.

We performed the determination of GA as detailed in Table 1. To determine optimal conditions for the assay, we varied the substrate concentrations and pH values and used a simplex optimization procedure. Details are given in *Results* and in the legends of the figures.

We adjusted the pH of diluted urines by adding 4 mmol/L KOH and 4 mmol/L HCl, respectively. Volume changes were not allowed for, because they were less than 0.5%.

[1] Department of Experimental Organ Transplantation, University Hospital Charité, Humboldt University Berlin, Leninallee 49, DDR-1017 Berlin, G.D.R.

[2] Central Laboratory, Friedrichshain City Hospital, Leninallee 49, DDR-1017 Berlin, G.D.R.

[3] Nonstandard abbrevations used: GA, D-glucaric acid; GL, 1,4-D-glucarolactone; GLUC, β-glucuronidase (EC 3.2.1.31).

Received July 23, 1980; accepted Dec. 8, 1980.

## Table 1. Protocol for Determining the Concentration of GA in Urine

*I. Preparation of urine*

Centrifuge the urine (500 × *g*, 10 min). Dilute 1.0-mL portions of urine with 9.0 mL of 20 mmol/L HCl and 9.0 mL of water, respectively, adjust to pH 2.0 and pH 8.0, respectively. Boil for 45 min. Then cool, readjust to pH 4.0 and to pH 6.0, respectively.

*II. Assay*

| | Sample | Sample blank | Reagent blank | Calibration standards | |
|---|---|---|---|---|---|
| | | | | Without GA | With GA |
| | | mL | | | |
| Substrate | 0.2 | 0 | 0.2 | 0.2 | 0.2 |
| Urine alkaline- or acid-treated) | 0.2 | 0.2 | 0 | 0 | 0 |
| Water | 0 | 0.3 | 0.2 | 0.2 | 0 |
| Standard | 0 | 0 | 0 | 0 | 0.2 |

Mix and incubate for 10 min at 37 °C. Start the reaction in samples and calibration standards by adding 100 μL of working enzyme solution. Stop the reaction with 50 μL of KOH solution after 15 min, add 100 μL of enzyme solution to reagent blank, mix well, and measure *A* at 405 nm in a cuvette of 1-cm lightpath between 5 and 15 min.

*III. Calculation*

$$\mu\text{mol/L} = \frac{\left(\frac{1}{A_1} - \frac{1}{A_2}\right)}{\left(\frac{1}{A_3} - \frac{1}{A_4}\right) \times \frac{1}{C}} \times F, \text{ where}$$

- $A_1$ = absorbance of the sample of acid-treated urine minus absorbance of the respective sample blank and minus reagent blank
- $A_2$ = absorbance of the sample of alkaline-treated urine minus absorbance of the respective sample blank and minus reagent blank
- $A_3$ = absorbance of calibration standard with GA minus reagent blank
- $A_4$ = absorbance of calibration standard without GA minus reagent blank
- C = concentration of standard, μmol/L
- F = dilution factor of urine

If C and F are 10, the following formula will result:

$$\mu\text{mol/L} = \frac{\left(\frac{1}{A_1} - \frac{1}{A_2}\right)}{\left(\frac{1}{A_3} - \frac{1}{A_4}\right)} \times 100$$

*Calculations.* For statistical analysis of the results we used Student's *t*-test.

## Results

### Analytical Variables

According to the described principle of GA determination, we studied factors influencing the analytical reliability of the method, both the conditions to be chosen for transforming GA into its lactone and for determination of GLUC activity in the presence of GA.

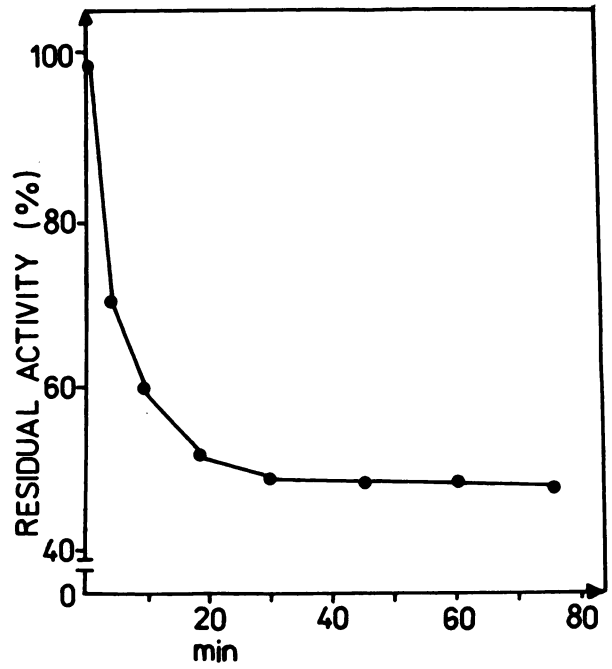


Fig. 1. GL production from GA as a function of duration of boiling

13.3 μmol of GA per liter was dissolved in 10 mmol/L HCl, boiled for different intervals, and the inhibition of GLUC activity by these fractions was determined

*Influence of pH and boiling time on GL production.* In model studies with GA we studied the transformation of GA into its lactone with respect to pH and boiling time, because data on optimal conditions are contradictory (*8, 9*). After boiling GA for 30 min the extent of GLUC inhibition increased only little (Figure 1). Thus 45 min suffices to ensure maximal transformation of GA into GL. pH 2.0 can be considered to be the optimal pH (Figure 2).

*Influence of pH and substrate concentration on GLUC activity.* In the presence of GL the pH optimum for GLUC activity is shifted (Figure 3). The difference in reaction rates with and without GA was greatest in the pH range between

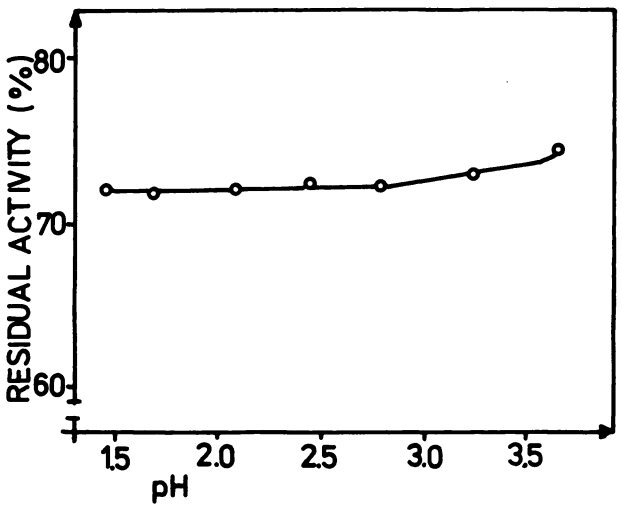


Fig. 2. Dependence of GL production on pH

2.5 μmol of GA per liter in 100 mmol/L glycine-HCl buffer of different pHs, boiled for 45 min, the pH readjusted to pH 5.0, and the GL production determined by measuring the inhibition of GLUC activity. Inhibitory influence of glycine on GLUC activity was corrected by blanks

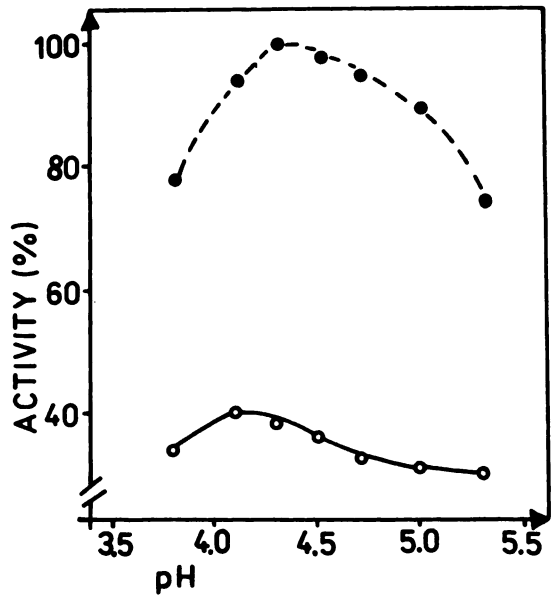


Figure 3. Influence of pH on GLUC activity in the presence of GL

Conditions are given in Table 1, but 13.3 μmol of GA per liter was used in this experiment, after boiling. Each point is the mean value of triplicates

pH 4.3 and 5.0. Inhibition of GLUC activity by GL depends on the concentration of 4-nitrophenyl-β-D-glucuronide (Figure 4).

*Simplex optimization.* The experiments mentioned above indicate that, as decisive variables, pH and substrate concentration influence the sensitivity of the test. To cover the dependence on both variables in common, we applied the simplex optimization procedure according to Nelder and Mead (*14*). As optimization value, the maximum attainable difference between GLUC activity in the presence and absence of GA in reaction mixture was used. From this optimization scheme 1 mmol/L 4-nitrophenyl-β-D-glucuronide and pH 5.0 were determined to be optimal conditions for the determination of GA (Table 2).

*Calibration.* Urine boiled at pH 2.0 and pH 8.0, respectively, competitively inhibits GLUC (Figure 5). So, from an enzyme-kinetics viewpoint, it is not understandable why logarithmic calibration curves were used for calculating GA concentration in the past.

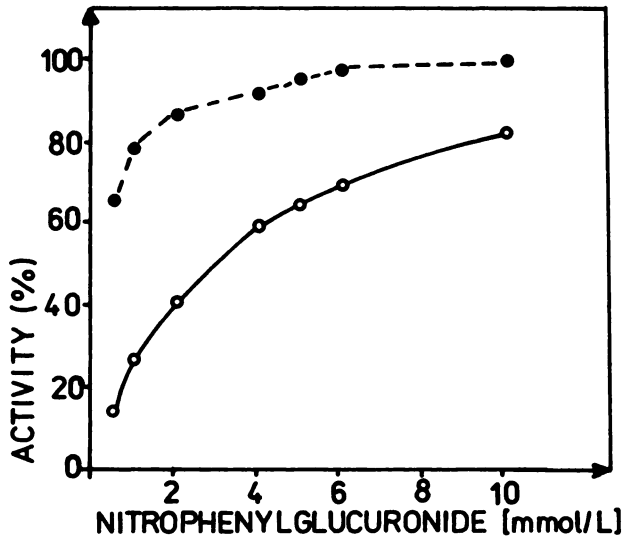


Fig. 4. Influence of substrate concentration on the GLUC activity in the presence of GL

For details see Fig. 3

**Table 2. Data for Simplex Optimization of GA Determination**

| Vertex no. | Variables: 4-Nitrophenyl-β-D-glucuronide, mmol/L | Variables: pH | Response, ΔA | Vertices retained from previous simplex |
|---|---|---|---|---|
| 1 | 5.00 | 4.30 | 0.184 | — |
| 2 | 8.00 | 4.30 | 0.151 | — |
| 3 | 6.50 | 4.99 | 0.248 | — |
| 4 | 3.50 | 4.99 | 0.341 | 1,3 |
| 5 | 1.25 | 5.34 | 0.346 | 1,3 |
| 6 | 2.75 | 6.03 | 0.194 | 3,5 |
| 7 | 3.31 | 5.60 | 0.178 | 3,5 |
| 8 | 3.13 | 4.82 | 0.260 | massive contraction |
| 9 | 3.88 | 5.17 | 0.253 | massive contraction |
| 10 | 1.25 | 5.34 | 0.342 | massive contraction |
| 11 | 0.50 | 4.99 | 0.429 | 8,10 |
| 12 | −1.19 | 4.91 | 0 | 8,10 |
| 13 | −1.38 | 5.52 | 0 | 10,11 |
| 14 | 2.00 | 4.99 | 0.265 | 10,11 |
| 15 | −0.25 | 5.16 | 0 | 10,11 |
| 16 | 1.44 | 5.08 | 0.360 | 10,11 |
| 17 | 0.69 | 4.74 | 0.384 | 11,16 |
| 18 | −0.25 | 4.65 | 0 | 11,17 |
| 19 | 1.02 | 4.97 | 0.464 | 11,17 |
| 20 | 0.83 | 5.23 | 0.410 | 11,19 |
| 21 | 0.79 | 5.10 | 0.443 | 11,19 |
| 22 | 1.30 | 5.10 | 0.435 | 19,21 |
| 23 | 1.10 | 5.07 | 0.453 | 19,21 |

The vertices are listed in the order in which experiments were performed. The concentrations are final concentrations in reaction mixture. The coordinates of vertices 1, 2, and 3 were calculated by the method according to Long (*20*) with the step sizes of 0.8 for pH and 3 for substrate. As optimization criterion we used the difference of reaction rates in reaction mixture with and without GA (4 μmol/L as final concentration). The optimization procedure of Nelder and Mead (*14*) was used. Values given are mean values of triplicates.

As Figure 6 shows, there is no linearity in logarithmic plotting. Besides, the inhibition curves of urine boiled at alkaline and acid pH are not parallel, although this is a necessary supposition for a correct calculation in this case. When the two inhibition curves are plotted according to Dixon (*15*), however, two straight lines with different slopes and the same intercept are obtained (Figure 6*B*). Thus GA concentration can be calculated by use of this plot. The calibration curve is linear up to an original GA concentration of 25 μmol/L, corresponding to a final concentration of 10 μmol/L in the reaction mixture. Because, as a rule, 10-fold diluted urine (with water or HCl solution) is used in the determination, concentrations up to 250 μmol/L can be correctly determined. Higher concentrations require further dilution of the sample.

*Analytical recovery.* We assessed recovery by adding 40 μmol of GA per liter to an urine with an initial GA concentration of 33.6 μmol/L. For 10 assays, the mean recovery was 98.3 (SD 3.78%).

*Precision.* Table 3 shows our precision data.

## Excretion of GA by Healthy Persons

To determine reference intervals, GA was determined in

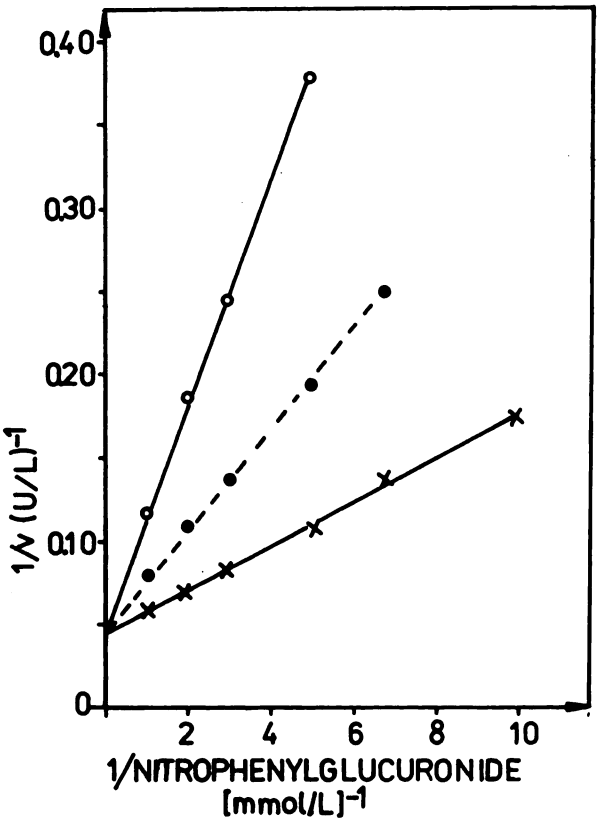


Fig. 5. Competitive inhibition of GLUC activity by boiled urine
Urine was boiled at pH 2.0 and pH 8.0, respectively, and then the influence was investigated on the GLUC activity for other conditions, see Table 1. X—X without addition of urine; ●—● with alkaline-treated urine; O—O with acid-treated urine

the 24-hour urines from 18 healthy women and 21 healthy men. Distributions of GA excretion, estimated by use of the Kolmogorov–Smirnov test (*16*), did not show any significant deviation from a gaussian distribution. Men excreted more GA ($p < 0.001$) than did women if the 24-h period was used as the basis of reference (Table 4).

## Discussion

Recently, workers in various fields of medicine have been increasingly concerned with problems of enzyme induction (*7*). Two relatively simple methods are proposed for revealing enzyme induction (*7, 17*): measurement of the urinary output

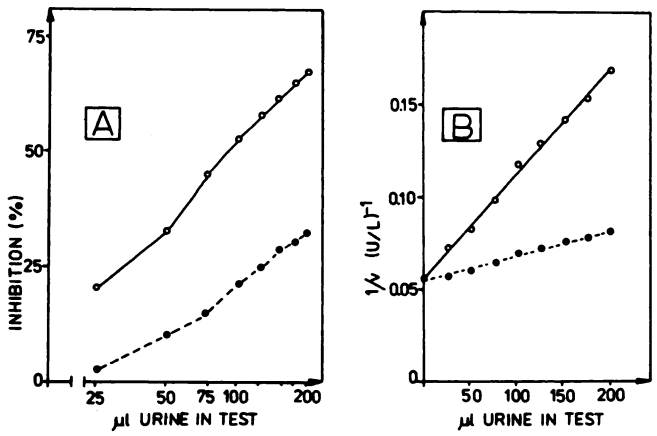


Fig. 6. Influence of amount of urine on GLUC activity
Alkaline-treated (●—●) and acid-treated (O—O) urine. Logarithmic plot (*A*); Dixon plot (*B*)

**Table 3. Precision Data**

| n | µmol/L $\bar{x}$ | SD | CV, % |
|---|---|---|---|
| | Within-run | | |
| 10 | 29.7 | 1.22 | 4.11 |
| 10 | 72.7 | 2.53 | 3.48 |
| 20 | 232.9 | 7.10 | 3.05 |
| | *Between-day* | | |
| 18 | 34.7 | 2.00 | 5.76 |

**Table 4. Urinary Excretion of GA by Healthy Persons**

| | n | GA excretion, mean (SD) µmol/24 h | mmol/mol creatinine |
|---|---|---|---|
| Men | 21 | 70.9 (21.0) | 3.96 (1.34) |
| Women | 18 | 48.5 (16.6) | 3.96 (1.21) |

of GA and determination of γ-glutamyltransferase (EC 2.3.2.2) activity in serum. But because liver affections may also cause increased γ-glutamyltransferase activity in serum (*18*), the diagnostic value of γ-glutamyltransferase for detecting enzyme induction is limited, and determination of GA excretion would appear to be preferable in studies of enzyme induction.

The method we proposed here represents an optimized GA determination by means of the GLUC inhibition test. All authors who used this principle of GA determination hitherto have not considered that not only GL, but also other inhibitors in urine, competitively inhibit GLUC activity. The determinations were made with tests which involve a high substrate concentration. Although a maximum GLUC activity is measured, because of the competitive inhibition mechanisms of GL the test becomes relatively insensitive for determination

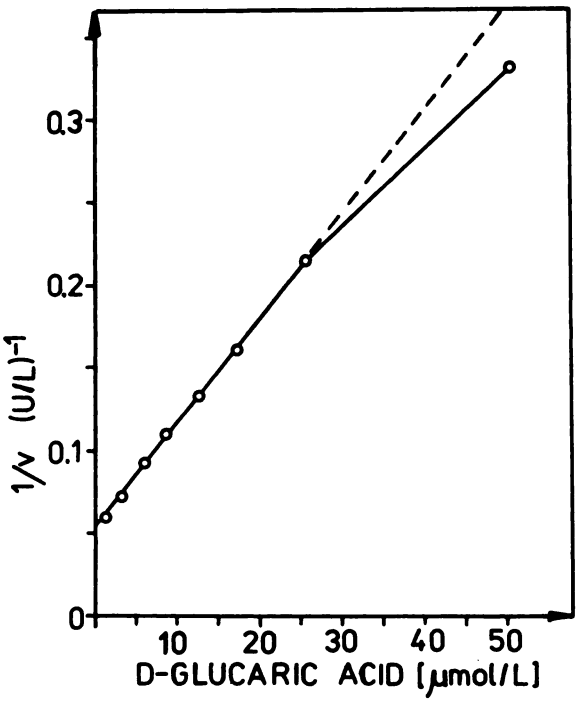


Fig. 7. Calibration curve for the determination of GA
Concentrations given refer to original concentrations. For other conditions see Table 1

of GA. For these reasons as well, other authors recommended a column-chromatographic method (*19*).

Compared with the traditional methods (*8–10*) of determining GA with the GLUC inhibition test, our modifications of the method have the following advantages:

1. The calibration curve is linearized by applying the Dixon plot. Our data prove that the method has high precision.

2. The method offers high analytical sensitivity. As a rule, the determination can be made with diluted urine. This makes possible such tests in small laboratory animals, to determine GA in urine in the framework of studies of enzyme induction.

3. The method is a micromethod and one person is able to make about 50 to 60 determinations a day.

Further to characterize the proposed method, we measured normal GA excretion. Published normal values, given in the literature as mean values, are clearly different (*7*): they lie between 13 and 98 μmol/24 h. We believe that this is mainly due to unsatisfactory methods. Our results show a sex-related difference in urinary output of GA if related to 24-hour excretion, as reported by other authors (*9, 10*), but in terms of creatinine (wt GA/wt creat.) this dependence is not observed, because men excrete more creatinine than do women.

## References


1. Aarts, E. M., Evidence for the function of D-glucaric acid as an indicator for drug induced enhanced metabolism through the glucuronic acid pathway in man. *Biochem. Pharmacol.* **14**, 359–363 (1965).

2. Hunter, J., Carella, M., Maxwell, J. D., et al., Urinary D-glucaric-acid excretion as a test for hepatic enzyme induction in man. *Lancet* **i**, 572–575 (1971).

3. Davidson, D. C., McIntosh, W. B., and Ford, J. A., Assessment of plasma glutamyltranspeptidase activity and urinary D-glucaric acid excretion as indices of enzyme induction. *Clin. Sci. Mol. Med.* **47**, 279–283 (1974).

4. Latham, A. N., D-Glucaric acid as an index of hepatic enzyme induction by anticonvulsant drugs in man. *J. Pharm. Pharmacol.* **26**, 284–286 (1974).

5. Smith, S. E., and Rawlins, M. D., Prediction of drug oxidation rates in man: Lack of correlation with serum gamma-glutamyl transpeptidase and urinary excretion of D-glucaric acid and 6-β-hydroxycortisol. *Eur. J. Clin. Pharmacol.* **7**, 71–75 (1974).

6. Herzberg, M., Fishel, B., and Wiener, M. H., Hepatic microsomal enzyme induction and its evaluation in a clinical laboratory. *Isr. J. Med. Sci.* **13**, 471–476 (1977).

7. Siest, G., Batt, A. M., and Galteau, M. M., Les examens biologiques témoins indirects de l'activité des enzymes du metabolisme des médicaments. Place de l'acide glucarique et de la gamma-glutamyltransférase. *Ann. Biol. Clin.* **35**, 425–432 (1977).

8. Marsh C, A., Metabolism of D-glucuronolactone in mammalian systems. Identification of D-glucaric acid as a normal constituent of urine. *Biochem. J.* **86**, 77–86 (1963).

9. Simmons, C. J., Davis, M., Dordoni, B., and Williams, R., Urinary D-glucaric acid assay by an improved enzymatic procedure. *Clin. Chim. Acta* **51**, 47–51 (1974).

10. March, J., Turner, W. J., Shanley, J., and Field, J., Values for urinary excretion of D-glucaric acid by normal individuals. *Clin. Chem.* **20**, 1155–1158 (1974).

11. Bose, R. J., Hullar, T. L., Lewis, B. A., and Smith, F., Isolation of the 1,4- and the 6,3-lactones of D-glucaric acid. *J. Org. Chem.* **26**, 1300–1301 (1961).

12. Kiliani, H., Neues aus der Zucker-Chemie (VI. Mitteilung). *Ber. Dtsch. Chem. Ges.* **58**, 2344–2362 (1925).

13. Lin, W. C., Orcutt, M. L., and Fishman, W. H., Purification and characterization of mouse kidney β-glucuronidase. *J. Biol. Chem.* **250**, 4737–4743 (1975).

14. Nelder, A. J., and Mead, R., A simplex method for function minimization. *Comput. J.* **7**, 308–313 (1965).

15. Dixon, M., The determination of enzyme inhibitor constants. *Biochem. J.* **55**, 170–171 (1953).

16. Storm, R., *Wahrscheinlichkeitsrechnung, mathematische Statistik und statistische Qualitätskontrolle.* Fachbuchverlag, Leipzig, 1969, pp 167–171.

17. Hildebrandt, A. G., Roots, I., Speck, M., et al., Evaluation of in vivo parameters of drug metabolizing enzyme activity in man after administration of clemastine, phenobarbital or placebo. *Eur. J. Clin. Pharmacol.* **8**, 327–336 (1975).

18. Rosalki, S. B., Gamma-glutamyl transpeptidase. *Adv. Clin. Chem.* **17**, 53–107 (1975).

19. Ishidate, M., Matsui, M., and Okada, M., Biochemical studies on glucuronic acid and glucaric acid. 1. Quantitative chemical determination of D-glucaric acid in urine. *Anal. Biochem.* **11**, 176–189 (1965).

20. Long, D. E., Simplex optimization of the response from chemical systems. *Anal. Chim. Acta* **46**, 193–206 (1969).