# D-Glucaric acid as an index of hepatic enzyme induction by anticonvulsant drugs in man

Measurement of urinary D-glucaric acid (as inhibitor lactone), while not previously described in a pharmacological context, has been used as an index of hepatic microsomal enzyme induction (Aarts, 1965), particularly in epileptics receiving chronic anticonvulsant therapy (Okada, Matsui & others, 1969; Hunter, Maxwell & others, 1971; Latham & Richens, 1973; Latham, Millbank & others, 1973). However, Ishidate, Matsui & Okada (1965) suggested that the enzymatic estimation of urinary D-glucaric acid was unreliable because compounds other than the inhibitor D-glucaro-(1→4)-lactone (saccharolactone) interfered with the hydrolysis. Marsh (1963b) demonstrated the specificity of the inhibitory component, D-glucaro-(1→4)-lactone, to β-glucuronidase. D-Glucaric acid was thought to be the only natural precursor of a β-glucuronidase inhibitor formed by boiling urine in acid. Levvy (1952) demonstrated the potency of this inhibitor and although only one-third of the D-glucaric acid in the urine was converted to the inhibitor lactone *in vitro* the relatively slight inhibitory properties of some other naturally excreted metabolites were thought to be insignificant.

The validity of this technique for assessing the induction of hepatic enzymes by anticonvulsant drugs has now been investigated.

Standard saccharolactone inhibitor solutions (0·5–100 μM) were prepared from commercial saccharolactone. Duplicate 1 ml aliquots were incubated with 0·5 ml pH 5·2 acetic acid buffer, 0·25 ml 0·005M phenolphthalein β-D-glucuronide substrate and 100 μl β-glucuronidase for 1 h at 37°. [100 μl of a 0·54% (v/v) solution of β-glucuronidase extracted from *Helix pomatia* (Sigma Chemical Company, Type H-2, specific activity 89 000 Fishman units $ml^{-1}$) produces an uninhibited maximal absorbance of 1·00.] After incubation the reaction was stopped by the addition of 3·0 ml glycine buffer (pH 10·4) and the phenolphthalein liberated in the presence of varying concentrations of inhibitor was determined at 550 nm. The standard curve of % inhibition of β-glucuronidase activity against log concentration (μM) saccharolactone inhibitor was linear.

Standard D-glucaric acid solutions (10–1000 μM) were prepared from the monopotassium salt of D-saccharic acid. Before incubation, 5 ml aliquots of each standard solution were adjusted to pH 2·0 and boiled in a water bath for 40 min. After cooling, each standard was adjusted to pH 4·0 and the volume made up to 10 ml with distilled water. Aliquots (1 ml) were then incubated with β-glucuronidase as before. The standard curve of % inhibition of β-glucuronidase activity against log concentration (μM) D-glucaric acid was also linear. A parallel shift between this curve and that obtained with the commercial saccharolactone inhibitor showed that there was a 23% conversion of D-glucaric acid to the inhibitor D-glucaro-(1→4)-lactone on boiling.

Aliquots from 24 h urine collections were stored at −15° until required.

One portion (5 ml) of each urine specimen was adjusted to pH 2·0 and a second portion (5 ml) to pH 8·0 and both were placed in a boiling water bath. The alkaline sample was removed after 15 min and the acid sample after 40 min (Marsh, 1963a). After cooling, the acid sample was adjusted to pH 4·0 and the alkaline sample to pH 6·0 (Marsh, 1963a) before diluting to 10 ml with distilled water. Incubations of 1 ml aliquots of the specimens were performed as before in duplicate with a blank for both the acid and alkali samples. Hydrolysis of the substrate by β-glucuronidase was prevented in the blanks by the addition of 3·0 ml glycine buffer (pH 10·4) before incubation.

*Calculation.* The maximal attainable absorbance =1·00 or was adjusted by utilizing the absorbance of uninhibited standards incorporated with each batch of assays before calculating the % inhibition.

% inhibition = 1·00–(E–B) × 100 where E = mean absorbance B = blank.

For a given % inhibition of β-glucuronidase the concentration of inhibitor was found from the standard curve and the D-glucaric acid (as inhibitor lactone) calculated:

D-glucaric acid 24 $h^{-1}$ = (a × $d_2$) − A × $d_1$ × L

where a = concentration of acid potentiated inhibitor, A = concentration of alkali potentiated inhibitor, $d_1$ = initial dilution factor, $d_2$ = dilution factor of acid sample, L = volume of urine in litres.

Reproducibility was tested by incorporating aliquots from one urine specimen as an internal standard with each batch of assays. This specimen produced a mean 83 ± s.d. 1·48(6)% inhibition of β-glucuronidase by the acid-treated sample (coefficient of variation = 1·78%). The mean D-glucaric acid concentration (as inhibitor lactone) of this specimen was 437 ± s.d. 45·8(11) μmol 24 $h^{-1}$ (coefficient of variation = 10·5%). Incorporation of the internal standard did not significantly reduce the variance ratio of two urine specimens assayed with it (F>0·2). However, although the variance ratio in terms of % inhibition of β-glucuronidase between the two test specimens was not significantly different ($P>0·05$), the results expressed as D-glucaric acid (acid potentiated inhibitor) showed a highly significant difference ($P<0·001$) between the variance ratios. In addition, the coefficient of variation ratios of D-glucaric acid/% inhibition of β-glucuronidase increased as the inhibition of β-glucuronidase by the specimens increased (3·71, 4·68 and 5·90 for the specimens producing 57, 76 and 83% inhibition of β-glucuronidase respectively). The implication is that lower D-glucaric acid concentrations may be calculated more accurately. One reason is that dilution of specimens to obtain satisfactory absorbance magnifies any error in the subsequent calculation. Furthermore, specimens producing the highest inhibition of β-glucuronidase are likely to incur most error from the semilogarithmic standard curve.

Two aliquots (5 and 10 ml) of a 24 h urine collection from a patient receiving mixed anticonvulsant therapy were passed through a Dowex 1X–8 anion exchange column after mixing with an equal volume of 0·1M sodium borate (Ishidate & others, 1965). The first fraction was collected by passing 150 ml 0·05M sodium borate-0·02M sodium sulphate buffer through the resin. D-glucaric acid was then eluted with 20 ml 0·05M sodium borate-0·1M sodium sulphate. A final wash was performed with the second buffer to ensure that all the D-glucaric acid was removed. The fractions collected were subsequently assayed for β-glucuronidase inhibition and D-glucaric acid (as acid potentiated lactone inhibitor). The D-glucaric acid fraction was much more inhibitory to β-glucuronidase than the pre- and post- rinse fractions (Table 1).

Table 1. *Inhibition of β-glucuronidase by 3 fractions collected from a Dowex 1X-8 ion exchange column after treatment with 5 ml and 10 ml of urine from a patient receiving anticonvulsant therapy.*

| Volume of urine | Pre-rinse fraction. 150 ml weak borate buffer | | D-Glucaric acid fraction. 20 ml strong borate buffer | | Post-rinse fraction. 20 ml strong borate buffer | |
|---|---|---|---|---|---|---|
| | Mean % inhibition | μmol inhibitor lactone | Mean % inhibition | μmol inhibitor lactone | Mean % inhibition | μmol inhibitor lactone |
| 5 ml | 11·0 | <1·0 | 59·5 | 17·4 | 9·0 | <1·0 |
| 10 ml | 17·5 | 1·0 | 70·5 | 36·3 | 22·5 | 1·4 |

Approximately twice the D-glucaric acid was removed when the volume of urine was doubled. These two findings confirm that the inhibitor of $\beta$-glucuronidase is D-glucaro-(1→4)-lactone.

Diazepam, chlordiazepoxide, carbamazepine, ethosuximide, methsuximide, phenytoin (and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, its main metabolite), phenobarbitone and primidone did not interfere with the assay when added to the urine. Similarly, excess glycine, (+)-tartaric acid, L-ascorbic acid, D-glucuronic acid and increasing the concentration of urea in the urine did not interfer with the assay.

The results presented support the use of the enzymatic assay of urinary D-glucaric acid as an index of hepatic enzyme induction in patients receiving antiepileptic drugs.

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*Department of Clinical Pharmacology,* A. N. LATHAM
*St. Bartholomew's Hospital,*
*London, EC1A 7BE, U.K.*

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# The micellar weights of the series dioctyl-to-didodecyl phosphatidylcholine

The effect of prolonged ultrasonication on aqueous sols of natural phosphatidylcholine (PC) has been reported independently by Attwood & Saunders (1965), Saunders (1966) and Huang (1969), and while there is agreement on a micellar weight of about $2{\cdot}0 \times 10^6$, differing opinions on the structure of the micelle are held. Huang argues for a bileaflet structure enclosing a core of solvent, whereas Saunders proposes that the particles are fragments of bilayers, the sheet of molecules being held firmly folded by head group interactions between the phosphate and choline ions.

The micellar weights of a series of synthetic dioctyl-to didodecyl-phosphatidylcholines ($C_8$ to $C_{12}$PCs) supplied by Dr. F. C. Reman of the Biochemistry Department University of Utrecht have been determined by light scattering. Reman, Demel & others (1969) have reported on the haemolytic activity of this series.

Two light scattering instruments were used. One used a low power He-Ne laser ($\lambda = 632{\cdot}8$ nm) as the light source (Pugh, 1970; Pugh & Saunders, 1971) and the other had a mercury lamp ($\lambda = 546{\cdot}1$ nm).

The samples were dissolved in a small amount of moist ether, shaken in water and the ether removed by treatment in a vacuum rotary evaporator at room temperature (20°), followed by bubbling with nitrogen. All samples were sonicated for 90 min at the maximum cavitation frequency (about 20 kHz) by a 60W Mullard generator with a titanium probe. The samples were immersed in an ice bath and nitrogen