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Note on the enzyme assay for urinary D-glucaric acid and correlation with rifampicin-induced mixed function oxidase activity

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Abstract. The enzyme assay for urinary D-glucaric acid is the simplest specific procedure for measuring drug-mediated hepatic enzyme induction in an ordinary laboratory without complex equipment. The problem of lactone conversion and interfering substances in the urine is examined. The normal range showed no significant difference between males (mean ± SD = 42.60 ± 19.80 μmol/day) and females (mean ± SD = 40.40 ± 31.50 μmol/day). Storage of urine at −20 °C longer than 6 months caused a decline in recovery probably due to further breakdown of D-glucarate. During rifampicin/streptomycin treatment a negative correlation was found between decline in antipyrine half-life (t½), a measure of mixed function oxidase activity, and rise in urinary D-glucaric acid ($r = -0.7614$, $p < 0.05$). However, in 63 patients receiving rifampicin/isoniazid therapy no rise in D-glucaric acid was detected. Isoniazid appears to be an inhibitor of the glucuronic acid pathway in man at the level of uronlactonase or glucuronolactone dehydrogenase.

Key words: D-glucaric acid (sugar acid) – enzyme induction – rifampicin – antipyrine half-life – isoniazid

Introduction

There are considerable differences in reported values of daily urinary D-glucaric acid excretion using the enzymatic procedure first introduced by Marsh [1963]. This largely stems from the complexity of the assay which although simplified [Simmons et al. 1974] continues to present several methodological problems. Its main clinical value is in the detection of drug-mediated hepatic microsomal enzyme induction [Aarts 1965, Hunter et al. 1973, Perry and Stamp 1984] which can be accomplished by simple laboratory equipment compared to the indices of antipyrine t½ and urinary 6B-hydroxycortisol now requiring gas liquid chromatography and radioimmunoassay. Plasma gamma glutamyltranspeptidase is in widespread use but is not specific for enzyme induction [Perry and Stamp 1984]. This study highlights practical problems of the enzyme method found over 5 years experience. In view of the discrepancies reported for normal excretion we record our findings and the effect of prolonged storage on recovery of D-glucaric acid which may have affected previous results [Fiedler et al. 1980].

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During anticonvulsant therapy a correlation was found between induction of the mixed function oxidase system and excretion of D-glucaric acid [Hunter et al. 1973, Lecamwasam 1975]. If this could be determined with rifampicin using antipyrine t½ as the test of oxidase activity it would increase confidence in the value of D-glucaric acid as an indicator of hepatic microsomal function even though part of the glucuronic pathway lies outside the microsome (Figure 1). The effect of isoniazid on D-glucaric acid excretion is observed during long term therapy with rifampicin.

Patients and methods

The principle of the enzyme method consists of the conversion of urinary D-glucaric acid, by boiling at acid pH, to glucaro-1,4-lactone a specific inhibitor of the enzyme β-glucuronidase. The lactone is incubated at 37 °C with β-glucuronidase and sodium phenolphthalein glucuronide, a substrate of the enzyme, at a specific pH. The reaction is stopped by adding an alkaline buffer and the free phenolphthalein measured spectrophotometrically. Absorption values are plotted against D-glucarate standards producing a sigmoid curve relationship (Figure 2). Urine test samples are additionally boiled at alkali pH producing minimal lactonization but conserving the presence of non-specific inhibitors of β-glucuronidase. The concentration of D-glucarate is obtained by

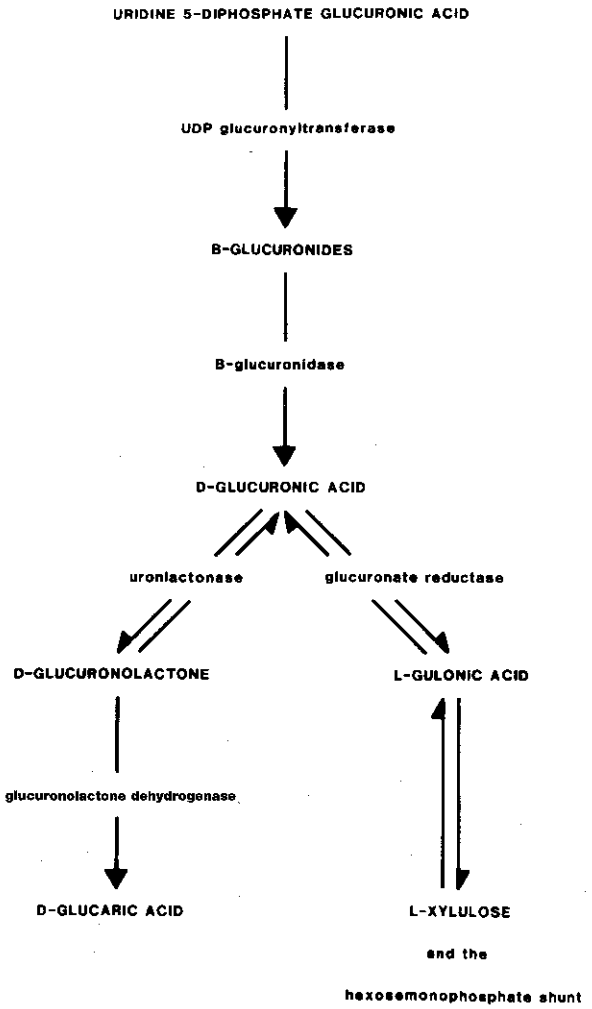

Fig. 1 Metabolism of free glucuronic acid in man (adapted from Miettinen and Leskinen [1970]).

subtracting the alkali boiled sample from the acid boiled sample both of which are read from the standard curve.

In our procedure a standard solution of calcium hydrogen glucarate 1000 µmol/l (Koch Light Laboratories Ltd.) was prepared. Sodium phenolphthalein glucuronide (Sigma Chemical Co.) was made up in a stock solution containing 55 mg in 10 ml of 0.5 M acetate buffer, pH 4.8. A twenty-fold dilution in 0.5 M acetate was made immediately before use. The working enzyme, buffers and procedure were followed as previously reported [Simmons et al. 1974]. Absorption was measured on a Gilford spectrometer at 555 nm. Samples were stored at −20 °C. Two internal urine standards were estimated in each assay after being stored as 5 ml aliquots. The first standard was obtained from a patient with a relatively low excretion of D-glucaric acid and the second standard contained 100 µmol/1 of glucarate added to the first. For construction of the standard curve six water standards containing 1000, 500, 100, 50, 10, 5 µmol/l of D-glucarate and a water blank gave seven points on the curve and this was repeated in each assay (Figure 2).

Table 1 Recovery of D-glucarate using 2M formate pH 3.3.

Incubation time in min at 37 °C	Enzyme conc. Fishman units per tube	Water blank absorbance	Urine standard µmol/l of D-glucarate	Urine standard + 100 µmol/l of D-glucarate
60	250	654	12.0	84.0
60	250	1292	14.0	89.0
40	125	913	21.0	84.0
40	125	821	11.0	74.5
40	125	846	15.0	82.0
60	250	1460	11.0	91.0
40	125	522	14.0	75.0
60	250	1158	21.0	76.0
60	125	639	20.0	98.0
60	125	639	20.0	86.0
40	250	591	15.0	77.0
40	250	781	5.0	57.0
40	250	942	8.0	55.0
40	250	563	5.0	37.0

Urine standards were measured during 10 months storage at −20 °C and the bottom three values were estimated after 6 months.

Table 2 Recovery of D-glucarate using 2M formate pH 2.

Incubation time in min at 37 °C	Enzyme conc. Fishman units per tube	Water blank absorbance	Urine standard µmol/l of D-glucarate	Urine standard + 100 µmol/l of D-glucarate
60	250	904	13.5	50
90	125	606	21.0	94
40	125	522	12.0	70
40	125	539	20.0	30
90	125	1043	28.0	47

Note the low recoveries in the second urine standard.

Recovery of urine standards stored at −20 °C was followed over 10 months (Table 1). Variations in enzyme concentration and incubation were performed to see if this would improve recovery. The effect of further lowering the acid boiled urine pH to less than 3.3 was also studied (Table 2). Twenty-four-hour urines were collected from normal subjects with an age range of 18–75 years who were not cigarette smokers and who were taking no drugs. Urine volumes were rejected if they were below 800 ml.

Eight Indian patients beginning rifampicin 450 mg and streptomycin 0.75 g i.m. daily for bone tuberculosis had antipyrine t½ measured one day before and at 10 days treatment. Twenty-four-hour urine D-glucaric acid was measured the day before each antipyrine t½. D-glucaric acid was also measured in 63 Indian and European outpatients receiving rifampicin 450–600 mg and

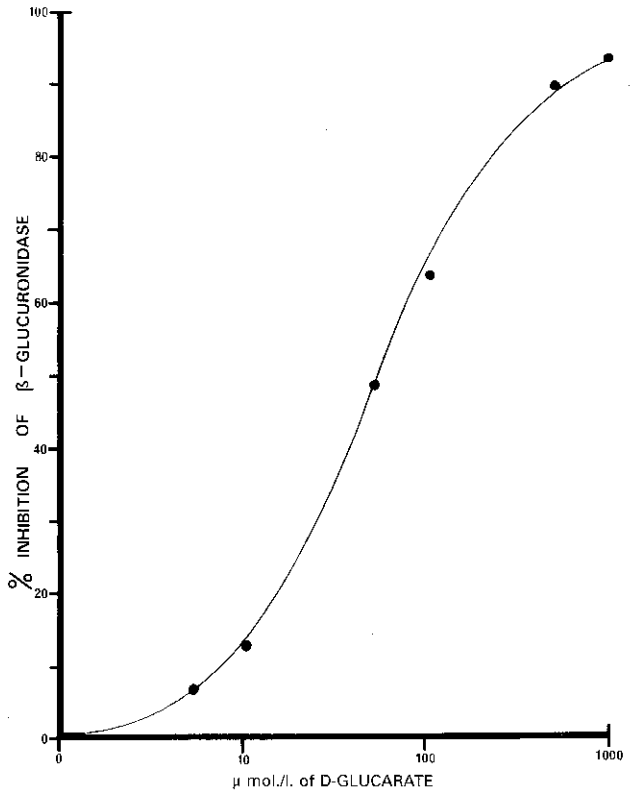

Fig. 2 Sigmoid curve relationship using water standards of D-glucarate for inhibition of β-glucuronidase.

isoniazid 300 mg daily after at least one month therapy. Antipyrine t½ was measured according to the method of Brodie et al. [1949], blood was drawn at intervals over 12 hours and t½ calculated by computer by the method of least squares. Informed consent was obtained for plasma t½ studies.

Results

Analysis of the assay

In order to measure 20 test samples and 2 internal urine standards a total of 197 tubes were required. Fourty-four for boiling urine at acid and alkali pH, 132 tubes for incubation with the enzyme (each sample requires duplicates and a blank to correct for endogenous urine color) and 21 tubes to carry the D-glucarate water standards and water blank through each assay. This required a full working day. A six-fold dilution of the test urines is necessary before final absorbance reading is made. Variations in enzyme activity with time seen in the absorbance values was the main reagent variability in the assay (Table 1). Non-specific inhibition of β-glucuronidase calculated from the alkali boiled tubes can be as high as 25 μmol/l.

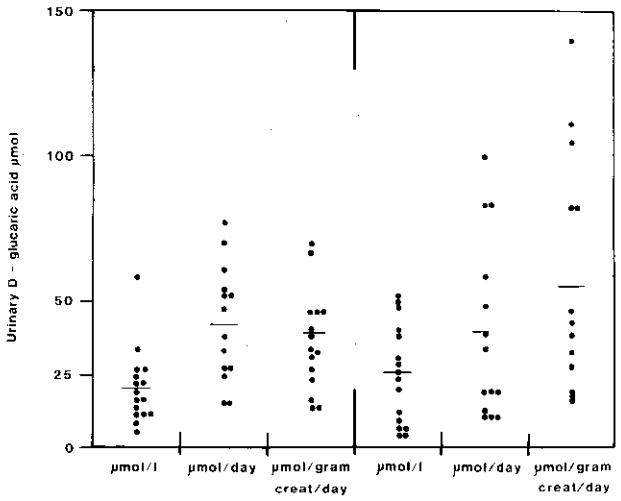

Fig. 3 Urinary D-glucaric acid excretion in males (left) and females. Horizontal line indicates the mean level.

The interassay reproducibility of urine standards is shown in Table 3. Two-fold variations were seen in the low value standard due to the effect of the alkali readings but the higher value second standard produced more consistent reproducibility and it appears that reading from the curve is most precise with values between 20 and 400 μmol/l, i.e., 20–80% inhibition of β-glucuronidase. Recoveries varied from 62–80% of added D-glucarate and alteration in incubation times and enzyme concentration did not improve this (Table 1). After 6 months storage at −20 °C there was a marked decline in recovery of both standards (Table 1). In Table 2 recovery using 2 M formate pH 2 was generally lower for the second urine standard. When the final pH after boiling was measured there were small differences between samples (unpublished data) but this could not acount for the large recovery differences.

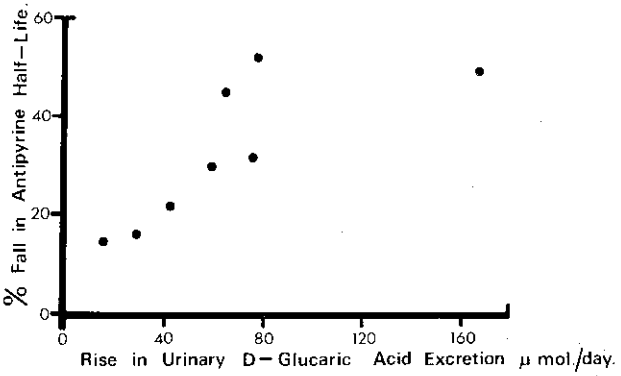

Fig. 4 Correlation of oxidase and glucuronic acid pathway induction by rifampicin ($r = -0.7614$, $p < 0.05$).

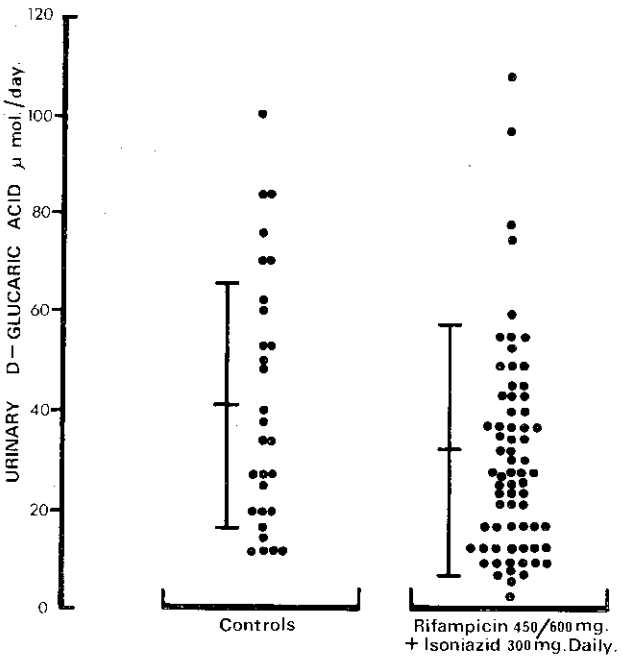

Fig. 5 Urinary D-glucaric acid excretion unchanged during rifampicin/isoniazid therapy. Vertical bars indicate mean ± SD.

Controls

Urinary excretion of D-glucarate was calculated in μmol/l, μmol/day and μmol/gram of creatinine day. The assay gives the initial result in μmol/l which is then corrected for recovery and either urine volume per twenty-four hours or creatinine excretion. Urinary creatinine excretion is lower in females than males [Vestergaard and Leverett 1958] thus when comparing these two groups this correction was not made. No significant difference was found between males (mean ± SD = 42.60 ± 19.80 μmol/day, n = 15) and females (mean ± SD = 40.40 ± 31.50 μmol/day, n = 14).

Table 3 Interassay reproducibility of urine standards using 2M formate pH 3.3.

Date	Urine standard μmol/l of D-glucarate	Urine standard + 100 μmol/l of added D-glucarate
11 June	12.24	84.15
18 June	14.03	89.47
25 June	21.30	84.28
9 July	10.90	74.70
23 July	15.00	82.10
30 July	11.12	91.30
Mean ± SD	14.10 ± 3.9	84.30 ± 5.9

Patients

When the rise in D-glucaric acid from pre-treatment levels was plotted against the % decline in antipyrine t½ over the first 10 day-period of treatment a significant negative correlation was found (Figure 4). However, the introduction of isoniazid during long term treatment in 63 patients produced no significant difference in D-glucaric acid excretion compared to normal controls (Figure 5).

Discussion

The modified urinary D-glucaric assay described is valid but remains cumbersome and time consuming. There is little possibility that it could be automated for large sample number in its present form. The first point of error is the six-fold dilution of test samples before measurement of lactone conversion. The problem of lactone conversion is itself a major potential source of error. Marsh [1963] boiled urine for 40 minutes at pH 2 and found a 30% conversion of D-glucarate to D-glucaro-1,4-lactone, the actual inhibitor of β-glucuronidase which is measured. Latham [1974] found 23% conversion at pH 2 and Simmons et al. [1974], from whom our method is derived, reported 18% conversion which increased to 29% at pH 3.6. However the latter do not indicate how they measured conversion to the lactone. We have confirmed that a final pH of 3.6 using formate buffer pH 3.3 gives satisfactory reproducibility of lactone inhibition (Table 3). Recovery was lower than reported by Simmons et al. [1974] and in a recent paper pH 3.8 held during lactone production and subsequent measurement with a Limpet β-glucuronidase has given the most satisfactory published results [Colombi et al. 1983]. These problems highlight the principal advantages of the anion exchange method in which there is no requirement for lactone conversion or sample dilution [Ishidate et al. 1965]. The GLC method still requires lactone conversion [Fiedler et al. 1980].

The use of standard D-glucarate solutions in each assay overcomes the problem of interassay differences in lactone conversion. However standards are run in water not urine and this may account for consistent under-recovery in the second internal urine standard. Ideally standards should be run in urine but in practice this will be difficult to achieve. A further assumption in this method was the requirement to run alkali urines in order to correct for compounds other than D-glucaric acid which might inhibit β-glucuronidase. This is supposed to take into account the effect of non-specific inhibition by other urinary metabolites including those of the drugs themselves. As the alkali-

boiled non-specific inhibitors are subtracted from the acid boiled samples, it assumes that these inhibitors are also acting at acid pH. This is not proven and as alkali inhibition can be as high as 25 μmol/l this accounts for the present under-recovery which would then correct to 90–95%.

The metabolism of D-glucaric acid is shown in Figure 1. Variations in excretion could be due to a shunting effect either to or from L-xylulose caused by the drugs independent of enzyme induction. A more satisfactory approach may be the ratio of D-glucaric to L-xylulose in urine [Lake et al. 1982]. Glucuronolactone dehydrogenase is not a microsomal enzyme and so may not be susceptible to classic drug-mediated enzyme induction. Thus the rate limiting effect of this enzyme on conversion of D-glucuronolactone to D-glucaric acid may decrease the sensitivity of this pathway to induction. We have been unable to detect a difference in daily excretion between males and females in agreement with others [Fiedler et al. 1980, March et al. 1974] but Simmons et al. [1974] reported lower values in females.

Rifampicin alone or in combination with streptomycin induces D-glucaric acid excretion at the start of therapy [Perry and Stamp 1984] and the correlation with decline in antipyrine t½ shown here lends further support to its use as a simple urine indicator of hepatic mixed function oxidase induction similar to the anticonvulsant model [Hunter et al. 1973, Lecamwasam 1975]. However, the problem becomes more complicated when isoniazid is the combining drug. On introduction of isoniazid early in therapy D-glucaric acid excretion is reversed to pre-treatment levels [Perry and Stamp 1984] and here we show this reversal persisting in a large population of tuberculous treated patients throughout long term rifampicin/isoniazid therapy (Figure 5). Our observations in Indian patients show that antipyrine t½ on the other hand continues to be shortened [Perry and Jenkins 1986] although others using European subjects with more frequent slow acetylator status for isoniazid have shown reversal of this decline [Brodie et al. 1982].

It is not clear at what level of enzyme inhibition isoniazid is acting in the glucuronic acid pathway although a microsomal action is suggested, however since β-glucuronidase is raised in plasma during combined therapy [Perry et al. 1978] the enzymes uronlactonase or glucuronolactone dehydrogenase seem the likely level for the inhibition of rifampicin-mediated D-glucaric acid excretion.

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Pharmacokinetic study of ioxaglate, a low osmolality contrast medium, in patients with renal failure

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Abstract. Determination of changes in plasma concentration of ioxaglate in patients with renal failure made it possible to demonstrate that distribution and clearance of this contrast medium, as in the case of classic uroangiographic products, conform to an open two-compartment model with clearance from the central compartment. Various pharmacokinetic parameters were calculated. The metabolic clearance of ioxaglate was lower for all 6 patients studied, as compared with results for 3 normal subjects tested, but less marked than with iodamide, a contrast medium involving tubular secretion.

Key words: ioxaglate – pharmacokinetics – renal failure

Introduction

Various studies have been undertaken in an attempt to reduce the osmolality of contrast media [Allen 1951, Benness 1982]. One approach has led to the synthesis of monoacid hexaiodinated dimers represented by ioxaglic acid. Experimental and clinical studies have been performed in an effort to assess its tolerance and the quality of diagnostic data. However, the pharmacokinetics of this product has been studied only in the normal subject [Laporte, unpublished pharmacokinetic study of AG 62–27, Laboratoire Guerbet, 1980] [Laporte et al. 1980].

To our knowledge, the only uroangiographic contrast media previously subjected to pharmacokinetic study are iodamide in both normal subjects and those with renal failure and diatrizoate in normal subjects [Taenzer et al. 1973].

Reprint requests to Dr. M. Bourin.

The aim of the present work is to carry out pharmacokinetic study of ioxaglate in subjects with renal failure.

Material and methods

Subjects

The study involved 9 subjects from 41 to 85 years of age. Subjects 1, 2 and 3 presented no signs of renal failure, subject 9 was in chronic hemodialysis and the others were suffering from chronic renal failure.

Endogenous creatinine clearance, calculated according to the nomogram of Kampmann [Laporte et al. 1980], ranged between 0 and 70 ml/min for subjects with renal failure. The characteristics of the 9 subjects are indicated in Table 1.

Contrast medium

Hexabrix®, a meglumine and sodium ioxaglate solution containing 320 mg/ml of iodine, has an osmolality of only 600 mOsm/kg and a pH close to 7.30.

Ioxaglate is a monacid dimer with two aromatic nuclei, each containing three atoms of iodine.