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Evaluation of an enzyme-immunometric assay for serum α-glutathione S-transferase

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SUMMARY. A commercially available enzyme-immunometric assay for serum α-glutathione S-transferase (GST) was evaluated. Endogenous serum α-GST diluted linearly within the calibration range. However, we recommend that the sample and second antibody reagent are always added sequentially in the assay to avoid hook effect. Between-assay variability was below 7% across the calibration range and the upper limit of the reference range in adults ($n = 219$) was 11·4 μg/L. Within-individual variability in serum α-GST concentrations measured over a 4–6 week period in 4 healthy adults was small. Serum α-GST concentrations did not change significantly 6 h after a therapeutic dose of paracetamol.

Studies in two patients after liver transplantation showed that serum α-GST is a better discriminant of acute changes in liver function than conventional tests. Serum α-GST concentrations were unaffected by gross muscle damage, extra-hepatic inflammation, or haemolysis and thus appear to be more liver specific than transaminase activities. The effect of renal impairment on serum α-GST concentrations requires further investigation.

Additional key phrases: serum α-GST; enzyme-immunometric assay; method validation; liver function; tissue specificity; ALT; AST

The glutathione S-transferases (GSTs) (E.C. 2.5.1.18) form a complex family of enzymes, primarily involved in the modification of xenobiotics and some endogenous substrates by catalysing their conjugation to reduced glutathione. Some may also exhibit selenium-independent glutathione peroxidase activity.[1,2] In man, α-GST is found primarily within hepatocytes, comprising approximately 5% of the total soluble cytoplasmic protein mass, although it is also present in the kidney, adrenal gland and testis.[3] It is a dimeric protein made up of two subunits (B_1 and B_2) and may exist as a homodimer (B_1B_1 and B_2B_2) or heterodimer (B_1B_2).[2] A genetically based nomenclature for GST has now been proposed[4] distinguishing both the subunit composition of individual isoenzyme as well as allelic variants encoded at the same gene locus (denoted by capital letters). In this new classification, the α-class dimers described above are referred to as A1-1, A2-2 and A1-2, respectively. However, since most published research on GST has referred to B_1 and B_2 subunits, the latter designation is followed in this report.

The relatively small size of α-GST (mol. wt ≈ 50 000) results in its rapid release following hepatocellular damage. This property, together with the restricted distribution of α-class GST in tissues other than liver, the high concentration of the enzyme within hepatocytes, its short plasma half-life (approximately 1 h) and wide distribution across the liver lobule should make α-GST an ideal marker for monitoring acute changes in hepatocellular integrity.[2] Monitoring α-GST has been found to offer diagnostic and/or prognostic advantages over conventional liver function tests (LFTs) in a variety of acute clinical circumstances.[5–13] Serum α-GST concentrations also appear to correlate better with the histopathological

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features of chronic conditions such as chronic active hepatitis, even when conventional transaminase activities are normal.[14–16]

Published methods for the measurement of α-GST have usually been either non-specific and susceptible to interference from endogenous substances (enzymatic assays) or too time-consuming.[2] However, a time-resolved immunofluorometric assay has recently been described.[17] We have evaluated a commercial enzyme-immunometric assay for the quantitation of total serum α-GST.

MATERIALS AND METHODS

α-GST enzyme-immunometric assay

We used a commercially available enzyme-immunometric assay for the quantitation of serum α-GST (Biotrin International, Stillorgan, Dublin, Ireland). Two procedures are described by the manufacturers and these are outlined below.

Sequential assay procedure

Stock calibration material was diluted in phosphate buffered saline with 0·25% v/v Tween 20 (assay buffer) to yield concentrations in the range 1–50 μg/L. Serum samples and quality control material were both diluted 1 in 5 in assay buffer. One hundred microlitres of appropriately diluted calibrators, quality controls and serum samples were added in duplicate to microtitre plates pre-coated with affinity purified polyclonal IgG antibody raised against purified total hepatic α-GST (probably cross-reacting with all α-GST dimeric forms). The assay plates were incubated at 25°C for 1 h with shaking (DSG Titretek plate shaker, speed 6). Then, the plate was washed four times with 200 μL assay buffer and 100 μL of the second antibody (polyclonal anti-α-GST IgG conjugated to horseradish peroxidase) resuspended in assay buffer was added to each well. The plate was then incubated for a further hour at 25°C with shaking. After four further washes in assay buffer, 200 μL of liquid tetramethylbenzidine substrate was added to each well and incubated for 10 min at 25°C with shaking. The reaction was then stopped with 50 μL 1 M sulphuric acid and the optical density read at 450 nm (650 nm reference) on a Vmax Kinetic Microplate Reader (NovoLabs). This sequential assay procedure was adhered to for all the studies described below unless stated otherwise.

Simultaneous assay procedure

Conditions were identical to the sequential assay protocol except that the addition of second antibody-HRP conjugate (100 μL) followed the addition of 100 μL of appropriately diluted serum samples, calibrators and quality control material and the incubation time was 1 h. The simultaneous assay procedure was only followed in a comparison of linearity and precision with the sequential procedure.

Assay performance

Linearity

A serum sample from a liver transplant recipient with an α-GST concentration of about 3000 μg/L was serially diluted and analysed by both sequential and simultaneous assay procedures in order to compare linearity.

Precision profile

Duplicate α-GST measurements on clinical samples from 38 consecutive assays run on different days over a 2 month period were used to determine a precision profile across the calibration range. Duplicate values were classified into 12 concentration ranges (bins) and the bin mean, standard deviation and coefficient of variation calculated according to the method of Raggatt.[18]

Intra- and inter-assay variability

Alpha-GST was purified to homogeneity from human liver as verified by SDS-PAGE and isoelectric focusing. The absence of both μ- and π-GST was confirmed by Western blot analysis of the purified protein. This purified hepatic α-GST was added to a pool of normal human serum to yield final concentrations of about 7·5, 25, 50 and 125 μg/L α-GST and assayed 28 times in a single batch to assess intra-assay variability. Inter-assay variability was determined by analysing these samples in 24 consecutive assays (single kit batch), run on different days over a period of one month.

Reference range and biological variability of α-GST

Blood samples were collected from 219 adult blood donors (aged 18 to 65 years) in order to determine the adult reference range. At least 15 sequential samples were collected at 09·00 am in the morning from each of four healthy adult volunteers over a period of between 34 and 42 days in order to provide a preliminary indication of the biological variability.

Blood samples were also collected from eight healthy volunteers before and 6 h after the ingestion of 1 g paracetamol in order to assess the influence of a therapeutic dose on serum α-GST concentrations.

Tissue specificity studies

Serum samples were collected from patients with a variety of clinical conditions in order to assess the tissue specificity of changes in serum α-GST.

Liver transplant recipients

Sequential serum samples were prospectively collected from two liver allograft recipients to compare the characteristic pattern of changes in α-GST and conventional LFTs seen during hepatocellular damage associated with acute allograft rejection following orthotopic liver transplantation.

Patients with renal dysfunction

Serum samples from 33 patients with normal liver function but elevated serum creatinine concentrations (median 383 μmol/L; range 130 to 1364 μmol/L) were collected following routine urea and electrolyte analysis.

Patient with rhabdomyolysis

Serial samples were collected from a patient with clinically documented rhabdomyolysis and a peak serum total creatinine kinase (CK) activity greater than 400 times the upper limit of the reference range.

Patients with rheumatoid arthritis

Serum samples were collected from 40 patients with rheumatoid arthritis: 20 with raised serum C-reactive protein (CRP) (median 31 mg/L; range 8–104 mg/L) and 20 with normal serum CRP concentration (<6 mg/L). These were included primarily to assess the influence of extra-hepatic inflammatory disease on α-GST measurements. Many of these samples were also known to contain high levels of IgM rheumatoid factor (up to 1250 IU/mL) and, therefore, also served to assess any influence of this potential source of interference in the α-GST immunoassay (see below).

Interference studies

The influence of potential endogenous interferents on serum α-GST measurements was further investigated in a series of recovery experiments.

Lipaemia

Intra-lipid was added to normal human serum to give a triglyceride concentration of 63 mmol/L.

Icterus

Five hundred microlitres, 100 mM NaOH containing 30 g/L bovine serum albumin was saturated with unconjugated bilirubin (Sigma Chemical Company). This was diluted 1/100 in an aliquot of the serum pool to give a pool with a bilirubin concentration of 591 μmol/L.

Haemolysis

Freshly donated whole blood was lysed by repeated freeze-thawing giving a homogenous lysate with a haemoglobin concentration of 15·2 g/dL.

The normal serum pool, icteric and lipaemic serum pools and the whole blood haemosylate was spiked to a target concentration of 100 μg/L purified human hepatic α-GST. The three spiked pools containing the potential interferents were then mixed with the spiked normal serum pool to yield the following proportions of interferent—100%, 80%, 60%, 40%, 20%, 10%, 5%, 0%. The recovery of spiked α-GST in these serum mixtures was then measured.

Paraproteins

In our earlier study of α-GST in liver allograft recipients,[5] samples from one patient with a low concentration of serum IgM paraprotein appeared to be a source of assay interference. This interference was suspected from highly erratic changes in daily α-GST measurements. This was investigated by assessing the recovery of purified α-GST added to the patient's serum and to sera from non-transplant patients with known serum paraproteins of immunoglobulin classes IgG, IgA or IgM. The latter were selected to provide a broad range of paraprotein concentrations.

RESULTS

Assay pereformance

Figure 1 illustrates a marked 'hook effect' associated with high α-GST concentrations in the simultaneous assay procedure—the maximum absorbance occurring at a concentration of approximately 60 μg/L. By contrast, in the sequential assay the optical density reached a plateau at α-GST concentrations above 100 μg/L. In view of this finding, only the sequential procedure was used in all other evaluations.

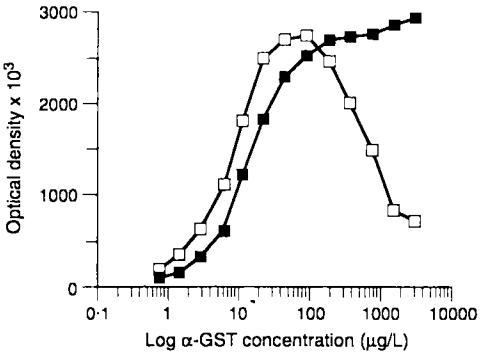

FIGURE 1. *Comparison of assay linearity for simultaneous (□) and sequential (■) assay protocols. GST = glutathione S-transferase.*

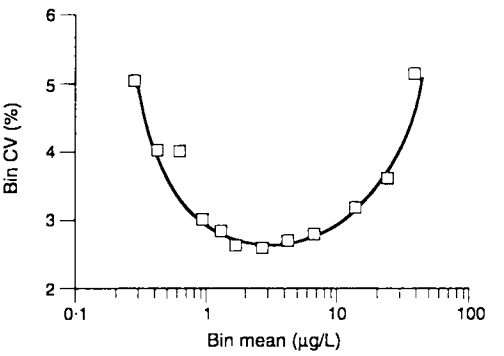

FIGURE 2. *Precision profile for the sequential assay determined by the analysis of variability between sample duplicates. Bin = concentration range; CV = coefficient of variation.*

The detection limit assessed as the mean plus 2·5 times the standard deviation of eight α-GST measurements of assay bufer alone was 0·5 μg/L.

The precision profile is shown in Fig. 2. For all α-GST concentrations within the range 0·2 to 50 μg/L (equating with serum GST concentrations of 1–250 μg/L), the mean coefficient of variation was less than 6%.

Table 1 shows the results of the precision study. The inter-assay coefficient of variation (CV) only exceeded 10% at a concentration of 7·5 μg/L (Table 1).

Reference range and biological variability of α-GST

Serum α-GST concentrations were log-normally distributed and the upper limit of the reference range (logarithmic mean + 2 SD) was 11·4 μg/L.

In all four healthy individuals monitored sequentially, the median morning serum α-GST concentration was 2 μg/L (range 1–4 μg/L). In the longest series of 18 sequential samples, collected from one individual over a period of 34 days, the mean (SD) serum α-GST concentration was 2·2(0·4) μg/L. There was no detectable pattern within individuals.

Serum α-GST concentrations did not change significantly 6 h after a therapeutic dose of paracetamol (3·5 ± 3·5 μg/L pre-dose and 3·2 ± 2·5 μg/L post-dose).

Tissue specificity and interference studies

The results of conventional LFTs and α-GST measurements following liver transplantation in two patients are shown in Fig. 3 (*a*,*b*). Both patients experienced episodes of acute allograft rejection within the first post-operative month.

TABLE 1. *Inter- (n = 24) and intra-assay (n = 28) variability for the sequential assay protocol*

Concentration of α-GST added (μg/L)	Purified α-GST added to serum pool			
	7·5	25*	50	125
Measured α-GST concentration				
Inter-assay				
Mean	7·9	25·1	58·2	135·0
SD	1·1	1·4	3·5	6·4
CV (%)	14·2	5·5	6·1	4·8
Intra-assay				
Mean	6·3	30·9	54·6	124·0
SD	0·2	1·0	1·2	5·8
CV (%)	2·9	3·1	2·2	4·7

*Two separate human serum pools were used to assess inter- and intra-assay variability.

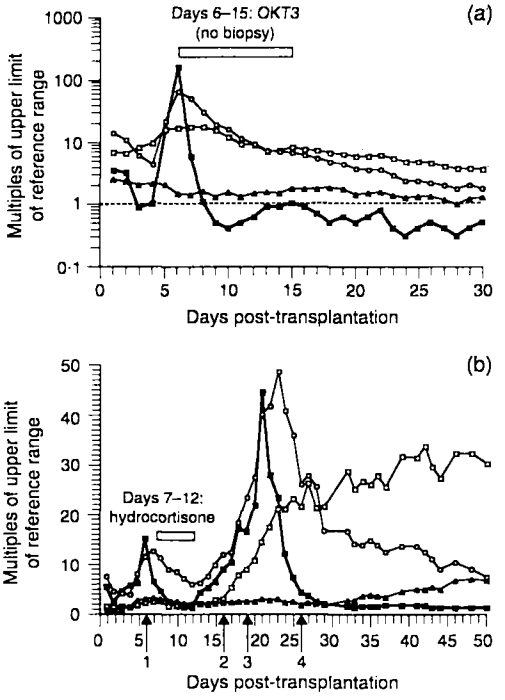

FIGURE 3. *Longitudinal changes in serum α-glutathione S-transferase (GST, ■), bilirubin (BILI, □), alkaline phosphatase (ALP, ▲) and alanine transaminase (ALT, ○) measurements in two patients (a and b) following orthotopic liver transplantation. All results are expressed as multiples of the upper limit of the respective reference range (broken line). (b) Biopsy findings (arrowed): (1) moderate rejection (treated); (2) mild rejection (no treatment); (3) mild/moderate rejection plus necrosis; (4) resolved acute rejection.*

In the first patient (*a*), all LFTs exceeded their respective reference ranges in the immediate post-operative period but only α-GST fell to within the reference range by day 3. Subsequently, there was a marked rise in α-GST as well as alanine transaminase (ALT) and bilirubin. This was associated with an episode of acute allograft rejection diagnosed clinically on day 6 which was successfully treated with the monoclonal anti-T cell antibody OKT3. Consistent with the resolution of rejection, α-GST concentrations fell rapidly to within the reference range by the third day of therapy and remained there throughout the first post-operative month. Conventional LFTs, however, failed to return to the reference range during this time.

Similar biochemical changes were seen in the second patient (Fig. 3*b*) in association with an early rejection episode on day 6. In this case, α-GST concentration increased two days earlier than conventional LFTs and 4 days prior to the histological diagnosis of rejection. Unlike ALT, serum α-GST fell rapidly to normal during augmented immunosuppression. Serum α-GST increased again on the thirteenth day, 1 day prior to a rise in ALT and 3 days prior to recurrent but apparently milder rejection. In the absence of treatment, rejection persisted and α-GST continued to increase in parallel with conventional LFTs. Eventually, the mild/moderate rejection appeared to resolve spontaneously without treatment and there was a corresponding rapid fall in α-GST and subsequent much slower fall in ALT. The persistent increase in bilirubin and ALP was compatible with the development of early chronic rejection in this patient.

In patients with renal dysfunction but normal conventional LFTs, no correlation was found between serum creatinine and α-GST concentrations. Serum α-GST concentrations were outside the reference range in two samples and serum creatinine concentrations in these cases were only marginally abnormal. The patient with the highest serum α-GST was receiving digoxin therapy but also had the highest serum ALT activity of 48 IU/L (upper limit of reference range 50 IU/L) in this group of patients. There was no clinical explanation for the raised α-GST concentration in the second patient.

Serial total CK and ALT activities as well as α-GST concentrations in a patient with rhabdomyolysis are shown in Fig. 4. Serum α-GST concentrations remained within the

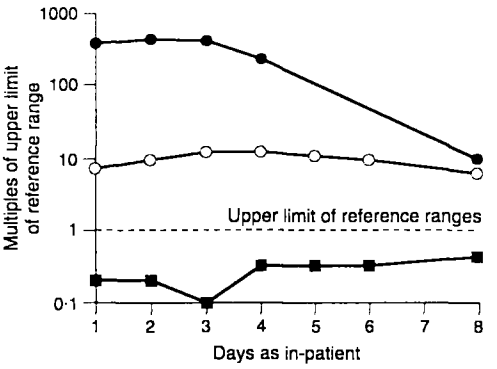

FIGURE 4. *Longitudinal changes in serum creatinine kinase (CK, ●), alanine transaminase (ALT, ○) and α-glutathione S-transferase (GST, ■) measurements in a patient with clinically diagnosed rhabdomyolysis.*

reference range, despite abnormal concentrations of ALT. Aspartate transaminase (AST) γ-glutamyltransferase (GGT) activities measured on the day of maximal CK activity (83 300 U/L: upper limit of reference range 195 U/L) were found to be 2121 U/L (upper limit of reference range 40 U/L) and 8 U/L (upper limit of reference range 38 U/L), respectively. Serum bilirubin and alkaline phosphatase measurements remained within their reference ranges throughout the patient's hospitalization.

Serum α-GST concentrations in rheumatoid arthritis patients with a raised serum CRP were not significantly different from either rheumatoid arthritis patients with a low CRP or healthy controls. However, four patients with a low CRP had serum α-GST concentrations greater than twice the upper limit of the reference range (27, 34, 42, and 83 μg/L) and all had particularly high IgM rheumatoid factor titres (214, 344, 1060, and 1250 IU/mL, respectively). Assay interference was confirmed in the first and last of these four samples, from the non-linear decrease in measured α-GST concentration upon serum dilution and from a 25 to 50% over-recovery of purified α-GST added to these samples. Serum α-GST concentrations were significantly higher in patients with rheumatoid factor titres greater than 70 IU/L (Fig. 5).

There appeared to be some under-recovery of α-GST in the presence of bilirubin (mean recovery 94%; 95% confidence interval (CI) 89–99%). However, there was no systematic increase in recovery as bilirubin was diluted out. There was no significant interference by either lipaemia (mean recovery 101%; 95% CI 98–104%) or haemolysis (mean recovery 102%; 95% CI 99–105%).

Suspected assay interference by serum from a liver transplant recipient with an IgM paraprotein was confirmed by a 100 to 300% over-recovery of purified α-GST added to the patient's serum sample. However, eight further samples from non-transplant patients with IgG ($n=4$), IgA ($n=1$) and IgM ($n=3$) paraproteins at concentrations ranging from 10 to 64 g/L did not interfere in the measurement of α-GST.

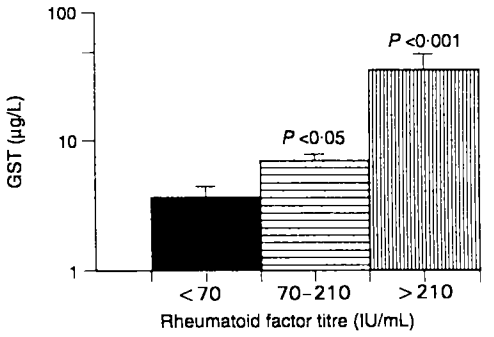

FIGURE 5. *Serum α-glutathione S-transferase (GST) concentrations in patients with rheumatoid arthritis according to the titres of serum rheumatoid factor (titres below 70 IU/L (n = 15), 70 to 210 IU/L (n = 6) and greater than 210 IU/L (n = 6)). Statistical differences between groups were determined by Student's t-test.*

DISCUSSION

We have evaluated a commercially available enzyme immunometric assay for the quantitation of total α-GST concentrations in human serum. Most published methods for measuring α-GST detect specific α-GST subunits whereas the antiserum used here was raised against total hepatic α-GST and, therefore, probably detects all α-GST dimeric forms. The relative clinical merits of measuring specific or total α-GST subunits has not been established although the use of antiserum that reacts with specific α-GST subunits has been advocated because the proportion of B_1B_2 heterodimer in liver can vary markedly.[2] In a previous study we have demonstrated the high sensitivity of the immunometric assay for monitoring hepatocellular integrity after human liver transplantation.[5] However, that study did not assess the organ specificity of serum total α-GST or involve any technical validation of the assay.

We recommend that the sequential assay protocol is followed since the simultaneous assay procedure results in a marked high dose 'hook effect' at serum α-GST concentrations greater than 60 μg/L (equivalent to 300 μg/L in serum).

The upper limit of the reference range for serum α-GST by this method was 11·4 μg/L; three to six times higher than that quoted for methods that detect specific homodimers of the enzyme (see Ref. 2). This probably reflects the fact that the antiserum used in the current method was raised against total hepatic α-GST. Serial morning α-GST concentrations measured over periods of 4–6 weeks in healthy adult volunteers showed very little variation. Further studies are warranted, however, to more fully assess the biological variability in serum α-GST. Such reproducibility is particularly important when monitoring daily changes in liver function.

Serum α-GST concentrations were unperturbed 6 h after a single therapeutic (1 g) dose of paracetamol. The measurement of serum α-GST at later time points after the dose will be necessary to confirm this result but previous studies have shown that patients who required treatment for paracetamol overdose consistently had raised serum α-GST as early as 4 h after the dose.[2] One of the potential applications of serum α-GST is likely to be in the pharmaceutical industry to monitor drug hepatotoxicity. However, it will be necessary to examine the effect of repeated therapeutic doses of paracetamol as well as other commonly used potentially hepatotoxic drugs on serum α-GST before this possibility can be realized.

We followed two adult liver transplant recipients longitudinally and a rise in α-GST was associated with episodes of acute liver allograft rejection confirming our previous observations.[5] In the second case, persistent mild rejection appeared to resolve spontaneously and α-GST concentrations fell accordingly to normal—remaining within the reference range despite the development of chronic allograft dysfunction. Compatible with the short *in vivo* half-life of α-GST (about 1 h), we found that concentrations of the enzyme fell more rapidly than conventional LFTs both immediately after successful transplantation and during resolution of rejection. This illustrates the potential value of this test.

The relatively restricted distribution of the enzyme in human tissues[3] could theoretically offer advantages over conventional LFTs. Indeed, serial measurements of α-GST were found to remain persistently normal in a patient with clinical rhabdomyolysis and serum creatine kinase (CK) activities of up to 80 000 U/L, accompanied by markedly raised serum ALT and AST activities despite the absence of any other clinical or biochemical evidence of hepatocellular damage. Numerous studies in both animals[19,20] and humans[21,22] suggest that such elevated serum transaminase activities are due to release of muscle isoforms from damaged fibres. Transient hepatic dysfunction with elevated serum bilirubin concentration and prolonged prothrombin time has been reported in a series of patients with non-traumatic rhabdomyolysis.[23] Under such circumstances, where there is probably a true dysfunction—a transient rise in serum α-GST concentrations is likely.

Although immunohistochemical studies have shown that α-GST is detectable in the normal human kidney,[3] we found no relationship between serum α-GST and creatinine concentrations: α-GST concentrations were within the reference range in 31 of 33 samples from patients with renal dysfunction. There was no clinical explanation for the abnormal serum α-GST measurements in the two patients. The elevated enzyme levels could be due to active kidney tissue damage. The patient with the highest serum α-GST was receiving digoxin therapy. The serum concentration of digoxin, however, was not at concentrations likely to cause acute nephrotoxicity. The detection of increased α-GST concentrations in urine has been reported to provide a measure of proximal tubular damage in patients with raised serum creatinine levels attributed to cyclosporin-induced nephrotoxicity[24] but this is not reflected in increased serum concentrations. A more likely explanation for the raised serum α-GST in this case was perhaps mild occult liver disease as the patient also had the highest serum ALT activity of all 33 patients with renal failure.

The serum α-GST concentration in patients with rheumatoid arthritis and a raised serum CRP was not significantly different from that in patients with a normal CRP or healthy blood donors—confirming that α-GST is not a non-specific marker of inflammation. However, this study did alert us to a potentially important assay interference. Raised serum concentrations of α-GST were found in four patients and all four of these patients had normal CRP but raised IgM rheumatoid factor titres. In two cases, assay interference was confirmed by over recovery of added α-GST. It is well recognized that in immunoassays IgM rheumatoid factor can give rise to falsely elevated levels of analyte by binding to the Fc portions of the capture and/or detection antibodies.[25–27] Methods of sample pretreatment to overcome this analytical problem have been described,[28,29] but these are time-consuming. Reduction of the capture antibody to its Fab fragments[6] or use of avian capture/detection antibodies[26] may be better options.

In our previous study we observed highly erratic changes in daily α-GST measurements in a transplant recipient[5] and assay interference by serum was suspected. This interference was confirmed from the over-recovery (>100%) of added α-GST. Interference was probably attributable to the presence of low concentrations (<5 g/L) of an IgM paraprotein in the serum—a recognized potential source of interference in immunoassays.[30] In eight samples from non-transplant recipients with IgG, IgA and IgM paraproteins, at concentrations up to 64 g/L,

no interference in the α-GST immunoassay was observed suggesting that this interference is rare. Indeed, this was the only instance of interference out of more than 4000 samples tested.

It is known that GSTs have a bilirubin binding capacity which can interfere with activity measurements of the enzyme in serum.[2] It was, therefore, necessary to exclude the possibility that bilirubin may also affect the α-GST immunoassay. This would be particularly relevant in liver transplant recipients in whom serum bilirubin levels may exceed 500 μmol/L. Although there was a small (average 6%) under-recovery of α-GST spiked into icteric samples, there was no evidence of concentration-dependent interference by bilirubin at concentrations up to 591 μmol/L. It was also established that the assay was not susceptible to interference by either hyperlipidaemia or gross haemolysis.

In conclusion, monitoring serum α-GST offers several clinical advantages over conventional transaminase measurements as a marker of hepatocellular damage. In particular, the enzyme has a short *in vivo* half-life—allowing improved discrimination of acute changes in liver function over time. The test may also be more liver specific than the transaminases. Although this immunoassay is not as rapid as those used for conventional transaminase enzyme activity, it can be completed within 2·25 h and assay precision is satisfactory for routine daily monitoring.

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