

Characterization of the Rat α -Glutathione S-Transferase (GST) ELISA™ (Biotrin) Using Rat Hepatoma (H4IIE) Cells

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ABSTRACT

The release of intracellular proteins, such as GST, into the culture medium can be used as an indicator of cell death. The GST assay has been used *in vitro*, however some important aspects of the assay have not been evaluated. The present study characterizes the GST ELISA for use as an assay to effectively measure cell death in H4IIE cells. The primary objectives of the present study were to determine if the assay could accurately measure GST in cell culture medium containing 20% serum, assess metabolic stability of GST in media at 37°C, investigate freezer stability of GST at -80°C, and evaluate the importance of plate mixing. To determine if the assay could accurately measure GST in cells that had become detached from plates, but remained intact after compound exposure, H4IIE cells were seeded into 96-well plates (10,000 cells/200 μ L/well). Cells were lysed immediately with digitonin (DIG, 1 mM) or frozen at -80°C. Total GST was determined in both DIG-treated and frozen suspensions of cells. No significant difference was found between the two treatments. GST stability in a cell matrix was evaluated at 37°C by treating cells with 1 mM DIG to obtain maximum GST release. Aliquots were collected at 0, 24, 48, and 72 hr for GST determination. GST recovered from media relative to GST at time 0 was 79% at 24 hr, 74% at 48 hr, and 63% at 72 hr. Stability of GST at -80°C was determined by adding known amounts of purified GST standard (0, 2, 10, 20, and 40 μ g/L) into media. GST was determined on day 0 and used as a reference. After 2 weeks GST was 95% of day 0, followed by 81% after one month, 76% after two months, and 65% after 6 months. To evaluate the importance of plate mixing during the ELISA assay, GST standards were assayed with and without mixing. Absorbance values were increased 15% as a result of plate mixing. In conclusion, the GST ELISA is a reliable assay for assessing cell death *in vitro*. It is reproducible, highly specific, stable, and because of its 96-well format, lends itself to high throughput analyses.

INTRODUCTION

Release of intracellular proteins such as α -glutathione S-transferase (GST), into the outer milieu through disruption of cell membranes, can provide information on cell death. Because α -GST is found primarily in liver parenchymal and renal proximal tubule cells, it provides a highly specific serum marker for hepatotoxicity and a highly specific urine marker for renal toxicity. Standard markers for liver toxicity such as LDH, ALT, and AST are based on enzyme activity. If a test compound directly interferes with the enzyme through protein binding, sensitivity would be significantly reduced. In contrast, the GST ELISA is based on the actual amount of GST protein present, not its activity. This combined with the mechanics of the assay, which includes multiple wash steps, significantly reduces compound-related interference while maintaining sensitivity.

The GST ELISA kit is commercially available from Biotrin International and is used for quantitating rat GST in various biological fluids such as blood, urine, and tissue culture medium. The ELISA is based on addition of sample, antibody-enzyme conjugate, and substrate to the assay plate coated with anti-rat GST. The resultant color intensity is proportional to the amount of GST in the sample. The kit offers a number of advantages over other membrane leakage markers including its high specificity and 96-well format, which is important for high throughput analyses.

Although the GST assay has been used successfully with some *in vitro* systems, its primary use has been with *in vivo* blood and urine samples. In order for the GST assay to be used routinely in *in vitro* applications, several experimental parameters must be examined and characterized. In the present study, a number of important parameters were investigated in order to validate the GST ELISA for detecting cell death in the rat hepatoma (H4IIE) cell line. The endpoints characterized included assay linearity and sensitivity, stability of GST in the culture medium at 37°C in the presence of living cells, stability of GST in -80°C freezer storage, effective cell lysis for obtaining total GST values, the effect of centrifugation on cell supernatant analysis, and the need for plate mixing during the GST assay.

METHODS

Cell Culture Conditions

Flat bottom 96-well plates were seeded with 10,000 - 20,000 cells (H4IIE) in 200 μ L of medium and allowed to equilibrate for 24 hr at 37°C. The culture medium consisted of Modified Eagle Media supplemented with bicarbonate, pyruvate, and 20% serum (i.e., 10% calf and 10% fetal bovine). After the 48 hr equilibration period the cells were treated with test compounds in 200 μ L of medium for 24, 48, or 72 hr. Following each exposure period, cell supernatants were collected and frozen for later analysis.

GST ELISA Procedure

The rat α -GST ELISA kits were purchased from Biotrin International. The procedure was performed according to the manufacturer's suggestions. Cell culture supernatants were thawed and centrifuged at 3000 RPM using a Beckman Allegra™ 6R centrifuge to pellet residual cellular debris. Sample medium was diluted using sample dilution buffer as provided by Biotrin. GST standards were prepared to concentrations of 2, 10, 20, 40, 50, and 100 μ g/L. Medium and GST standards were added to the ELISA plate to initiate the assay. Plates were incubated at room temperature for 1 hr with gentle, uniform mixing using a Thermolyne Model 50800 Roto-Mix™.

After 1 hr incubation, plates were washed with Biotrin wash buffer and a Dynatech Ultra-Wash automatic plate washer. Two series of 6 washes were performed per wash step. Solution was removed from each well by blotting plates on absorbent paper toweling after each wash. Conjugating reagent was added and plates were incubated at room temperature for 1 hr with mixing. After another wash step, substrate was added and plates were incubated at room temperature for 15 minutes. The reaction was stopped with the addition of 1N sulfuric acid. Plates were read at 450 nm/650 nm reference using a Packard 96-well plate reader (SpectraCount™).

GST Stability at -80°C

GST standards were prepared in complete H4IIE medium at concentrations of 0, 2, 10, 20, and 40 μ g/L. Standards were aliquoted into microcentrifuge tubes and stored frozen at -80°C. Standards were kept in the freezer and analyzed at various time points (0, 0.5, 1, 2, and 6 months) for GST using the method outlined above.

METHODS

GST Stability at 37°C

Cells were seeded into 96-well plates at a density of 10,000 cells/200 μ L/well and allowed to establish growth for 24 hr at 37°C, 5% CO₂. After 24 hr, medium containing 1 mM digitonin was added to wells (200 μ L/well) in order to lyse cells and release total GST into the medium. Cells were observed under the microscope to ensure complete lysis of cellular membranes.

Plates were kept in the incubator for 3 days at 37°C, 5% CO₂. Medium was removed from each plate (30 μ L/well) at various time points (0, 1, 2, and 3 days) and stored frozen at -80°C. GST in medium was determined with the ELISA kit.

Efficiency of Cell Lysis: Comparison of Cells Frozen in Suspension Compared to Cells Treated with Digitonin

H4IIE cells were seeded into 96-well plates at a density of 14,000 cells/well. While cells were in suspension, plates received 1 mM digitonin treatment to lyse cells. Additionally, plates containing cell suspensions without digitonin were frozen at -80°C to lyse cells. Total GST amount in both plates was determined.

Medium from digitonin-treated and frozen plates of cell suspensions was also used to investigate the importance of centrifugation on cell supernatant analysis and total GST determination. Medium from both digitonin-treated cells and frozen cell suspensions was aliquoted into separate 96-well plates.

One set of plates was centrifuged for 5 min at 3000 RPM using a Beckman Allegra™ 6R centrifuge to pellet residual cellular debris, which could potentially interfere with the ELISA. Medium from the other set of plates was not centrifuged. Both centrifuged and non-centrifuged medium was analyzed for total GST.

Effect of Plate Mixing

GST standards were prepared in complete H4IIE medium at concentrations of 0, 2, 10, 20, 40, and 50 μ g/L. GST ELISA plates were analyzed for GST with and without plate mixing during incubation steps. Medium was analyzed for GST with the ELISA kit.

Rat Urine Stability With and Without Stabilizing Buffer

GST standards were prepared in control rat urine at concentrations of 2, 10, 20, 40, 100, 200, and 400 μ g/L with and without Biotrin urine stabilizing buffer. Standards were aliquoted into microcentrifuge tubes and stored frozen at -80°C. Standards were kept in the freezer and analyzed for GST at various time points (0, 5, and 32 days) using a 5-fold dilution with sample diluent. Final standard values after dilution were 2, 20, 40, and 80 μ g/L.

METHODS

FIGURE 1
Diagram of Cell Culture & GST ELISA Procedures

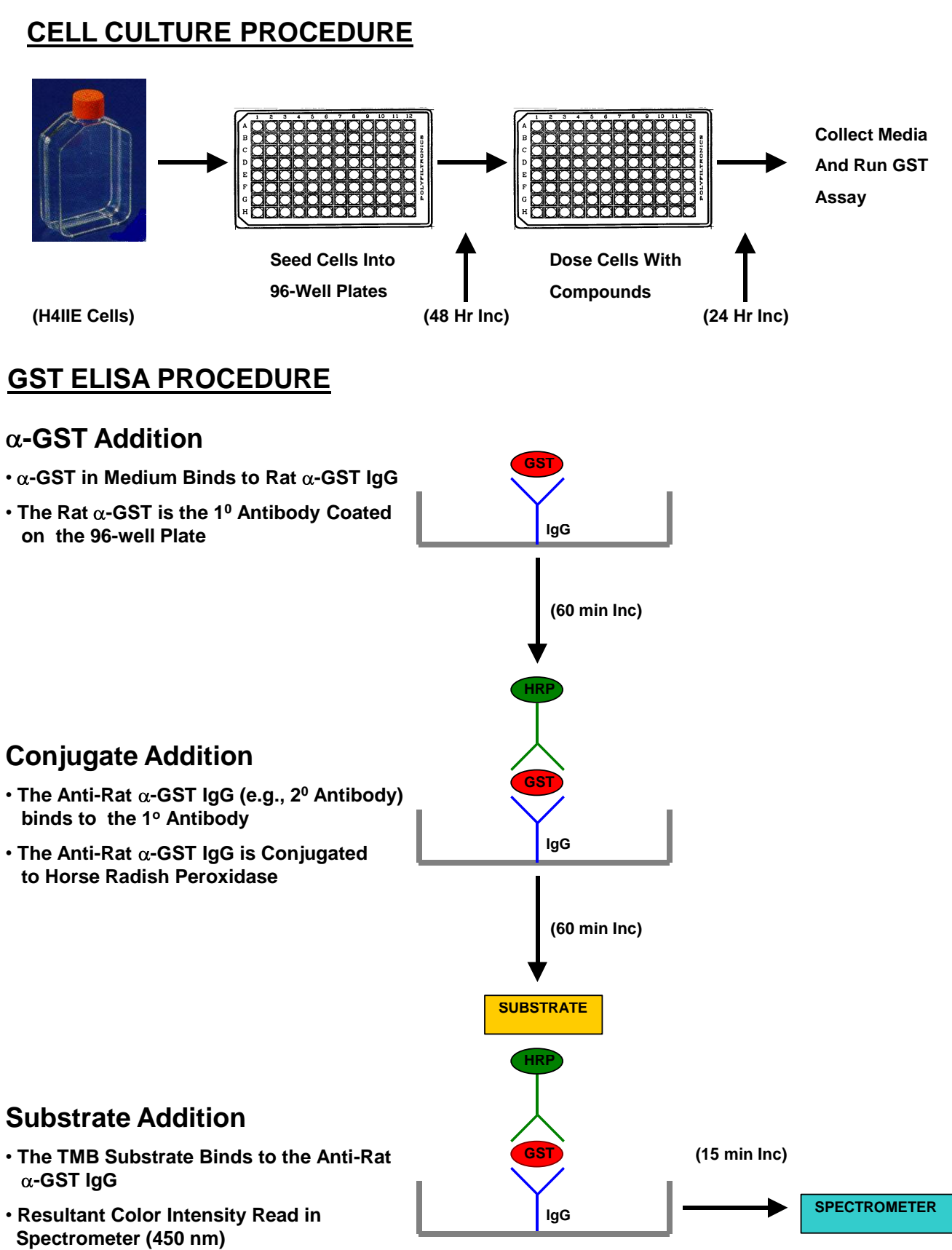


FIG. 1: The ELISA procedure is based on binding of α -GST to a 96-well plate coated with α -GST IgG. Sequential addition of antibody-enzyme conjugate and substrate results in color intensity which is proportional to the amount of α -GST in the sample.

FIGURE 2
Comparison of Exponential and Linear Regression Curves for GST Standards in Control Media

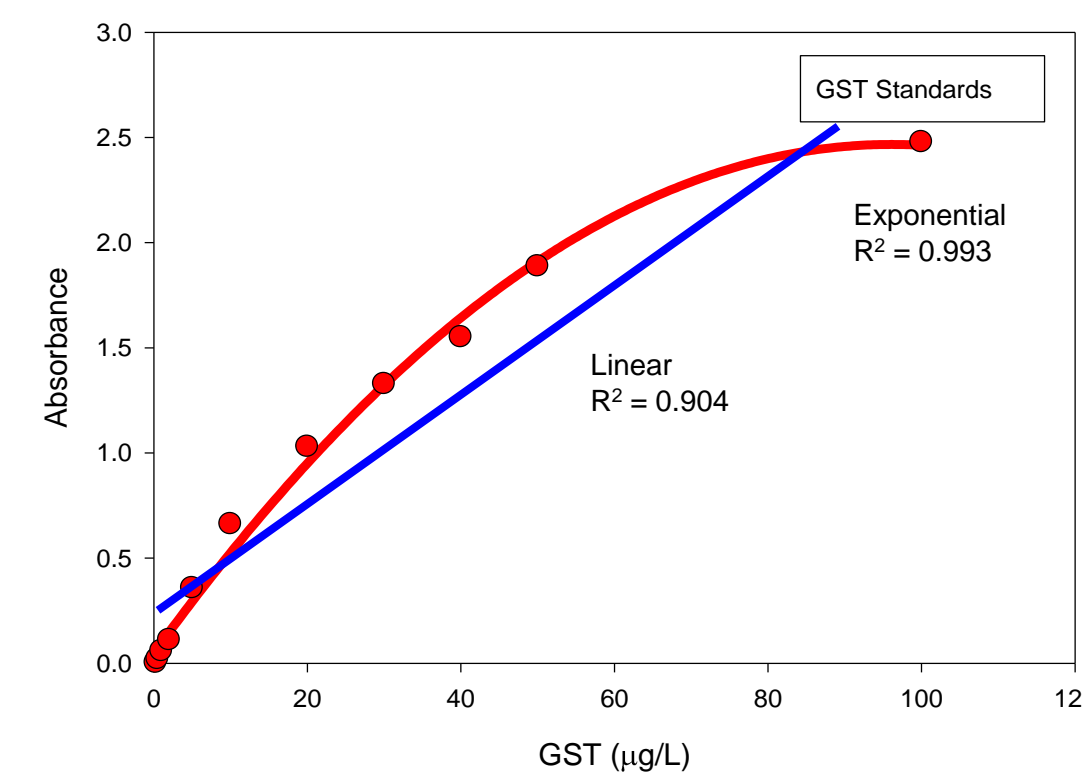


FIG. 2: GST Standards were prepared in control medium and plotted as GST (μ g/L) vs. absorbance. The red line represents the exponential regression curve and the blue line represents the linear regression curve. The exponential curve provides a much better fit of the data, especially at the higher GST concentrations. Values represent a mean of N=8. SEM not shown for clarity purposes. The %CV was \leq 8%.

FIGURE 4
GST Stability (Loss) Over Time at -80°C Relative to Day 0 Controls

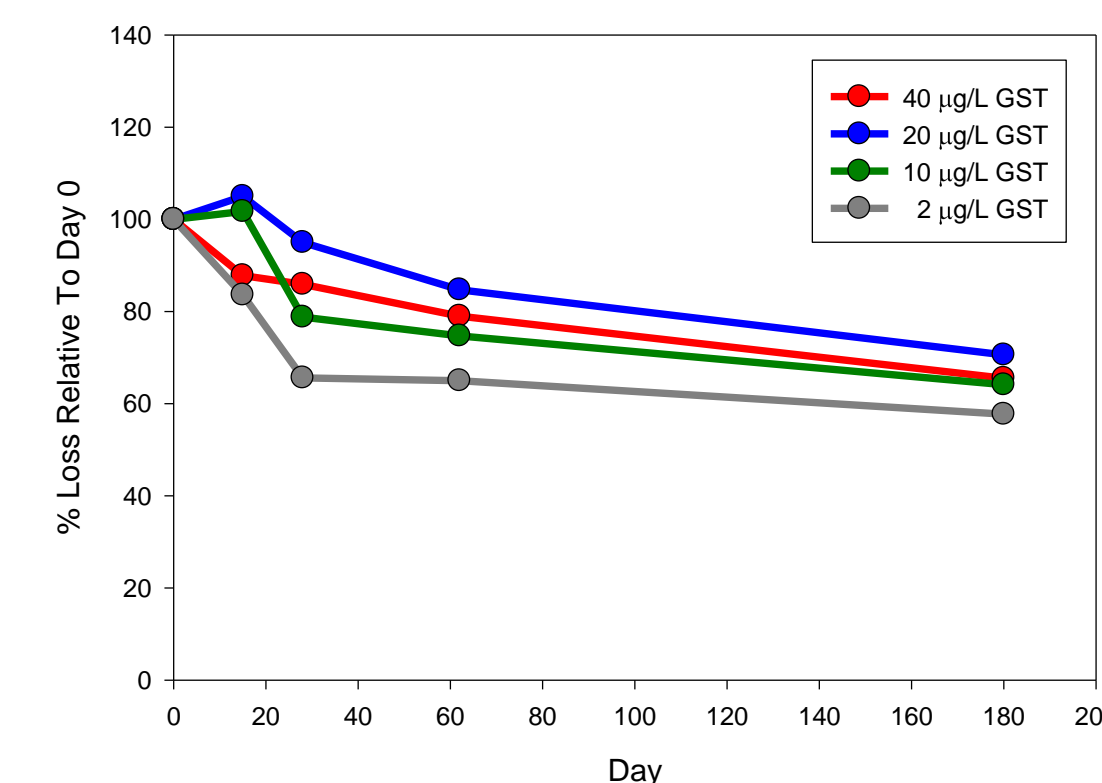


FIG. 4: GST standards were prepared in culture medium, stored frozen at -80°C, and analyzed at various time points to investigate GST loss over time. GST stability relative to controls was 95% at 2 weeks, 81% at 1 month, 76% at 2 months, and 65% at 6 months. Values represent the mean of N=3-4 wells. SEM not shown for clarity purposes (See FIG. 5). The %CV was \leq 11%.

FIGURE 6
GST Stability (Loss) Over Time at 37°C Relative to Day 0 Controls Individual Plate Data

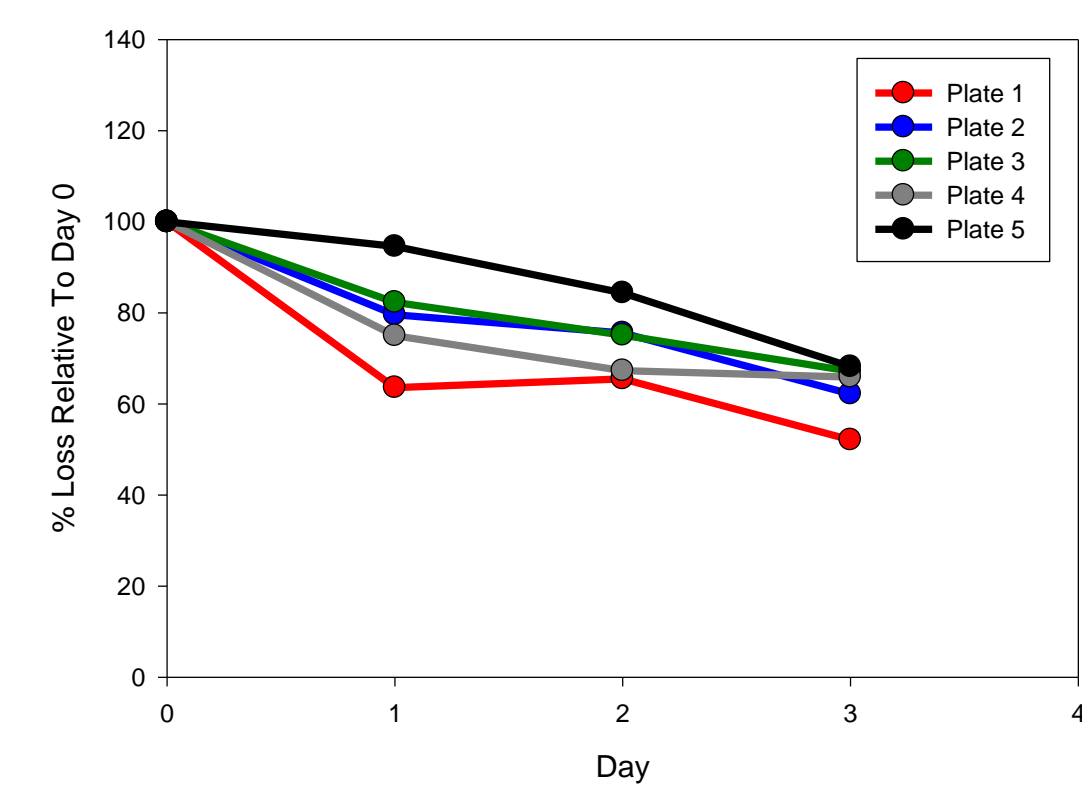


FIG. 6: H4IIE cells were treated with 1 mM digitonin to obtain total GST release. GST stability at 37°C was assessed by collecting media at 24, 48, and 72 hr after digitonin treatment (e.g., 0 hr). Values represent the mean of N=10-12 wells. SEM not shown for clarity purposes. The %CV was \leq 11%.

RESULTS

FIGURE 3
Effect of Plate Mixing on GST Absorbance Readings

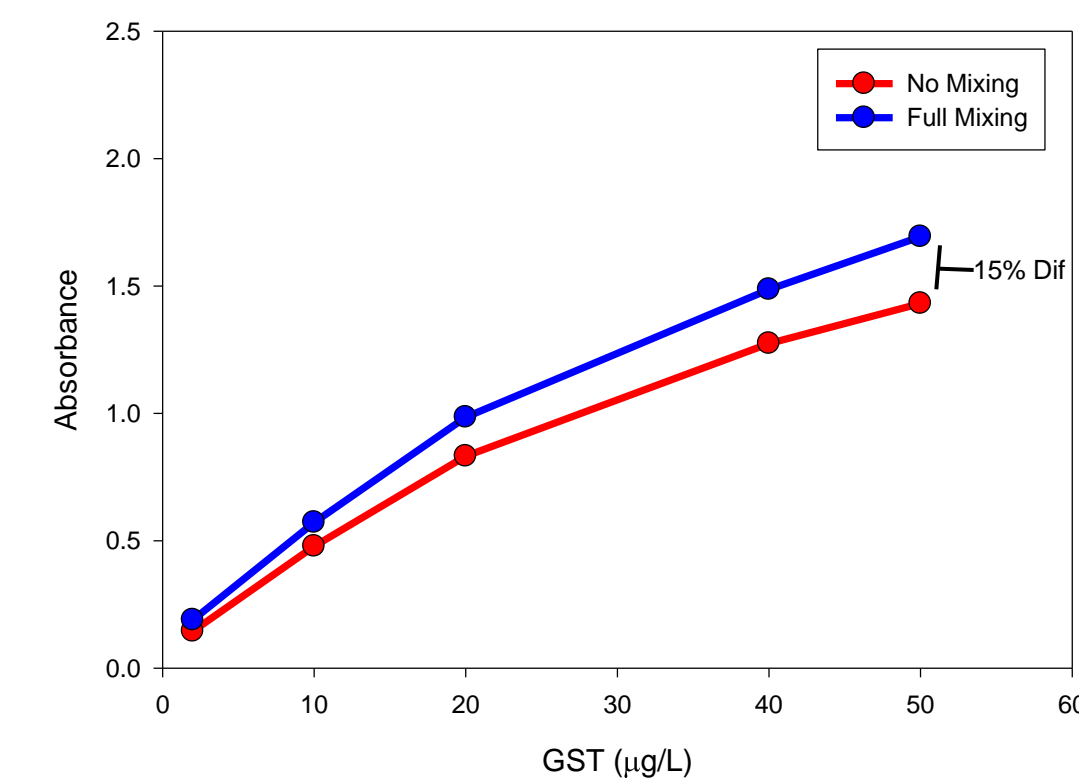


FIG. 3: To investigate the importance of plate mixing during incubation steps in the GST ELISA, GST standards were analyzed both with and without plate mixing. Mixing increased absorbance values 15% across all GST standard concentrations evaluated. Values represent a mean of N=3 experiments. SEM not shown for clarity purposes. The %CV was \leq 13%.

FIGURE 5
Relationship Between GST Concentration and GST Stability (Loss) at -80°C Relative to Day 0 Controls

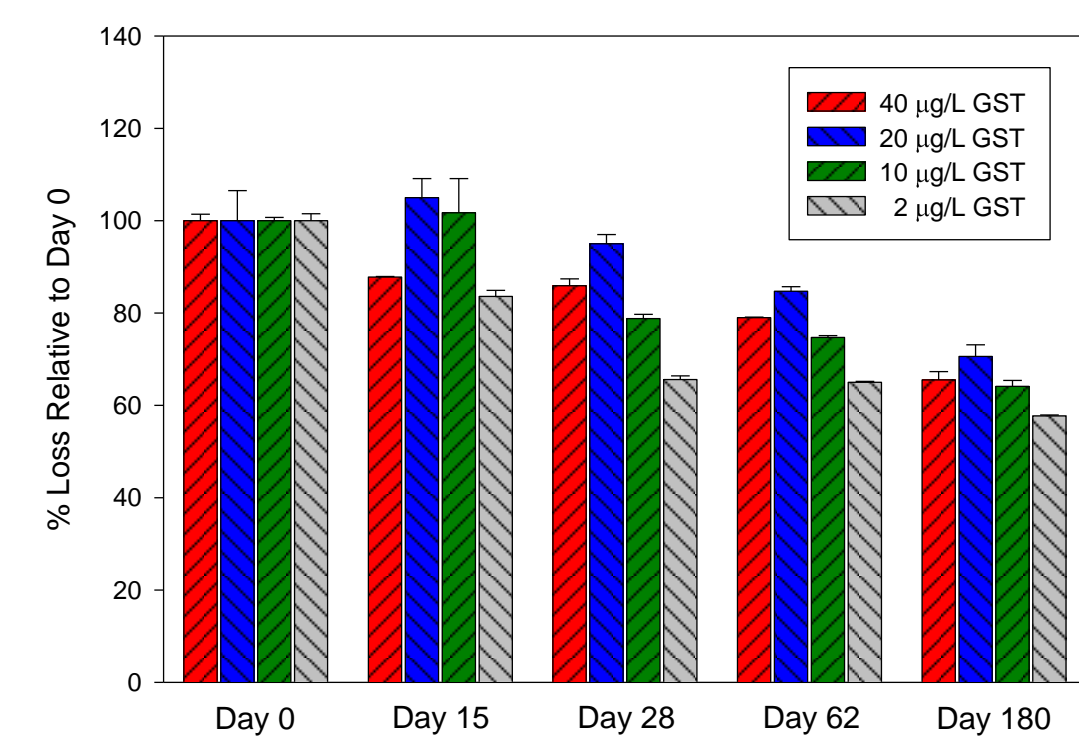


FIG. 5: GST standards prepared in culture medium and stored frozen were analyzed over time. The data in this graph represent GST loss relative to control for each individual GST concentration. These data demonstrate that higher GST concentrations in the medium (e.g., 40 μ g/L) are more stable compared to lower amounts (e.g., 2 μ g/L) at the same time point. Data points represent the mean of N=3-4 wells \pm SEM.

FIGURE 7
Mean GST Stability (Loss) Over Time at 37°C Relative to Day 0 Controls

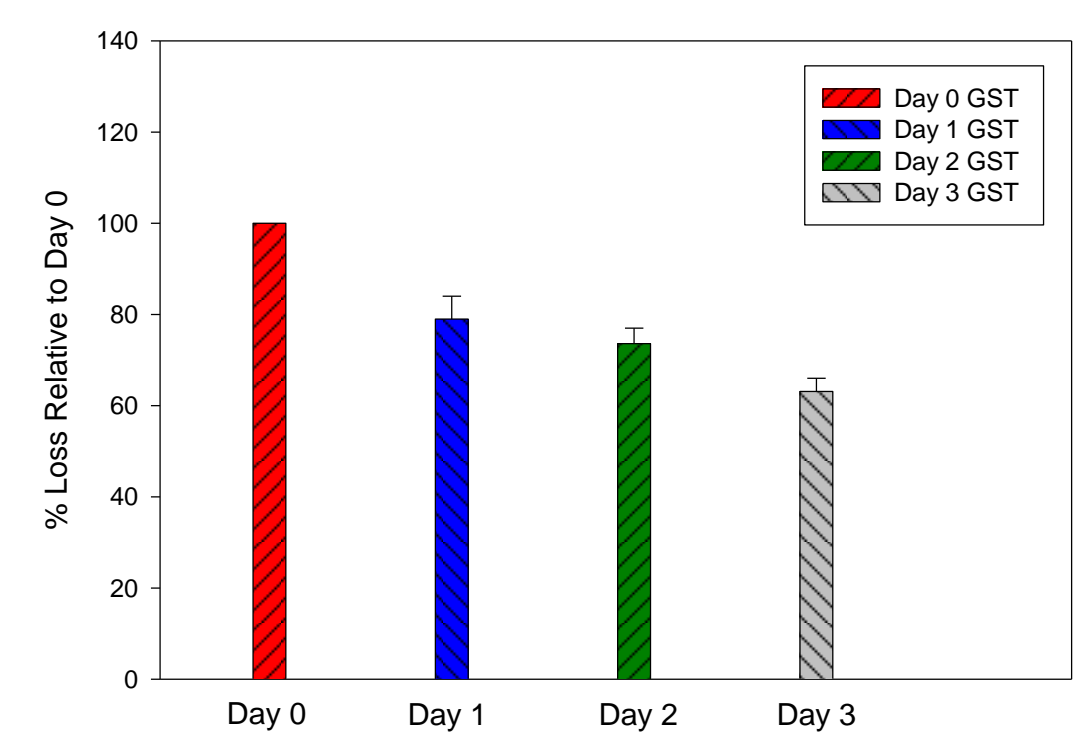


FIG. 7: GST release from H4IIE cells lysed with 1 mM digitonin was assessed at 24, 48, and 72 hr after digitonin treatment (e.g., 0 hr). GST stability relative to controls was 79% at 24 hr, 74% at 48 hr, and 63% at 72 hr. Values represent a mean of N=5 experiments \pm SEM (See FIG. 6).

FIGURE 8
Relationship Between GST Concentration and Rat Urine GST Stability at -80°C Relative to Day 0

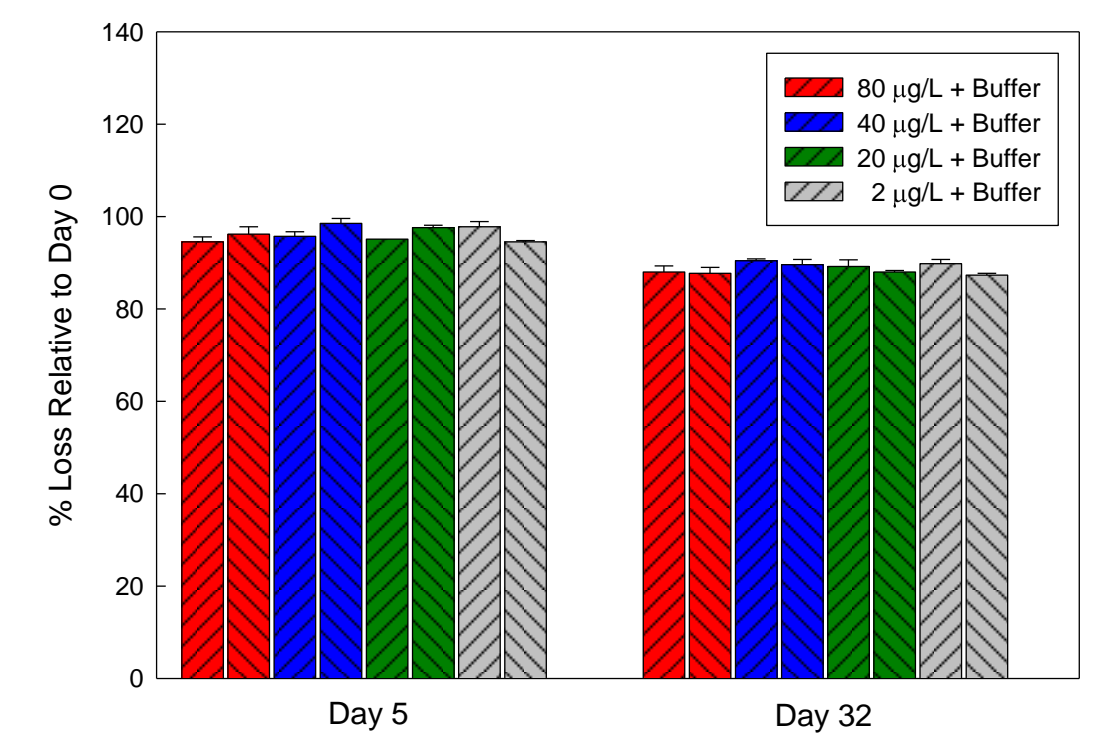


FIG. 8: GST standards were prepared in control rat urine with and without stabilizing buffer, stored frozen at -80°C, and analyzed over time to investigate GST loss. GST was stable in both urine preparations. GST relative to controls was 96% at day 5 and 89% at 1 month. Values represent a mean of 5-6 \pm SEM.

TABLE 1
Comparison of Cell Lysis Treatments and Centrifugation in GST ELISA

TREATMENT	GST ABSORBANCE VALUES		% DIFFERENCE
	Centrifuged	Not Centrifuged	
Freezing	0.636	0.632	→ 0.7%
Digitonin	0.619	0.639	→ 3.0%
% DIFFERENCE	2.7%	1.1%	

TABLE 1: Total GST released in cells by freezing or digitonin treatment was assessed. No significant difference was noted between either lysing procedure or centrifugation procedures. Values are mean of N=20. SEM not shown. CV% \leq 12%.

SUMMARY

The α -GST ELISA is a reliable assay for assessing cell death *in vitro*. It has been used as an effective measure of cell death in immortalized cell lines, rat primary hepatocytes, and rat urine samples. GST amounts (μ g/L) can be determined using exponential regression analysis, which provides a more accurate fit of data than linear regression.

The GST enzyme is quite stable in freezer storage at -80°C for the first two weeks, but declines steadily beyond one month. Higher GST concentrations in the medium appear to be more stable compared to lower GST amounts at -80°C storage. Cell lysis by freezing is equally effective as lysing cells with digitonin. Stability of GST in a cell matrix at 37°C decreases significantly over 3 days, with ~20% lost within the first 24 hours.

Plate mixing during GST incubation steps increases absorbance readings by 15%, but centrifugation of cell supernatants prior to sample analysis does not appear to be necessary. Lastly, freezer stability of GST in urine containing stabilizing buffer was the same as urine without stabilizing buffer, and GST loss was similar to that in medium.

CONCLUSIONS

Careful characterization of the α -GST ELISA for *in vitro* determination of cell death has led to its use as an integral part of screening efforts to detect hepatic toxicity early in the drug discovery process. The ELISA is a fitting assay for use in medium-to-high throughput screening, largely due to its high degree of reproducibility and convenient 96-well format. Interference from compounds or serum proteins in the medium has been minimal, likely due to the fact that the ELISA assay measures GST protein amounts and not enzyme activity. Traditional toxicity markers such as LDH, ALT, or AST can give erroneous results due to such interfering factors, as well as enzyme stability problems.

Since the α -GST is found primarily in liver parenchymal and renal proximal tubule cells, it also provides a highly specific serum marker for hepatotoxicity and a highly specific urine marker for renal toxicity *in vivo*. Used in conjunction with other markers such as μ -GST (Yb1), which is found primarily in the renal distal tubule cells, it is possible to accurately localize the site of injury. Thus, the GST kit can be a very powerful tool for assessing and localizing damage *in vivo*, as well as allowing simultaneous comparison of the *in vivo* condition to *in vitro* results.

REFERENCES

• Biotrin International Ltd. (10/99). Rat Alpha GST EIA, Enzyme Immunoassay (Kit Insert). Product Number BIO64RAT (96-well format), Document Code RAT-126-03.