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<p>(21) International Application Number: PCT/IE94/00050</p> <p>(22) International Filing Date: 17 October 1994 (17.10.94)</p> <p>(71) Applicant (for all designated States except US): SYNCOR INTELLECTUAL PROPERTIES LIMITED [IE/IE]; 93 The Rise, Mount Merrion, County Dublin (IE).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): DOYLE, John, Martin [IE/IE]; 27 Kill Abbey, Deansgrange, County Dublin (IE). KILTY, Cormac, Gerard [IE/IE]; 34 Dundela Park, Sandycove, County Dublin (IE).</p> <p>(74) Agent: ANNE RYAN & CO.; 60 Northumberland Road, Ballsbridge, Dublin 4 (IE).</p>		<p>(81) Designated States: AU, BR, CA, CN, CZ, FI, HU, JP, KR, NO, NZ, PL, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report.</i></p>
<p>(54) Title: STABILISING MEDIUM FOR αGST IN URINE FOR USE IN AN ENZYME IMMUNOASSAY</p>		
<p>(57) Abstract</p> <p>Stabilising medium for urinary αGST contains a stabilising amount of a non-enzyme protein, such as a mixture of equal amounts (w/v) of bovine serum albumin and gelatin hydrolysate, chelating agent and a buffer, such that the medium has a pH in the range 7.0 - 7.5, the medium being effective to prevent loss of αGST immunological activity. The stabilising medium can be used to store urine samples at temperatures of the order of -20 °C without any loss of αGST immunoreactivity of the type observed in samples which are stored without such a stabilising medium. The stabilising medium also improves the immunoreactivity of αGST when added to fresh urine which is stored temporarily at 2-8 °C prior to assay for up to two hours.</p>		

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Description

Stabilising medium for α GST in urine for use in an enzyme immunoassay

Technical Field

- 5 This invention relates to a stabilising medium for alpha glutathione S transferase (α GST) in urine and the use thereof in an enzyme immunoassay for α GST.

Background Art

- 10 Glutathione transferases (GSTs) are enzymes which are found in highly varying amounts in human tissues. The enzymes form three major classes, designated α , π and μ . These three classes of enzyme are quite distinct in their properties.

- 15 α GST is found in the proximal tubule region of the kidney and is released into the urine in normal individuals, as confirmed by enzyme immunoassay and western blot analysis (Campbell, J.A.H. *et al* (1991) Cancer (Philadelphia), 67, 1608-1613). Any event which precipitates proximal tubule damage may cause the release of α GST into urine leading to an increase in normal urinary levels. Thus, an elevation of urinary α GST levels may be indicative of proximal tubule damage
- 20 (Sherman, R.A. *et al.* (1985) Uremia Investigation, 8, 111-115). Recent work has shown that cisplatin induced proximal tubule damage in Wistar rats is associated with elevated levels of urinary α GST activity and decreased serum creatinine clearance (Stojanov, M. *et al.* (1994) Clin. Chem., 14, 1125), and that acute tubular necrosis and
- 25 renal transplant infarction in humans result in a rapid increase of both α and π GST levels (Sundberg, A.G.M. *et al.* (1994) Nephron 67, 308-316).

The ability to use urine as a sample of a body fluid for the detection and determination of an enzyme indicative of kidney damage

and, in particular, a particular region of the kidney is an advantage, especially because no invasive collection of the body sample is required. In general, one wishes to estimate α GST in patients who are seriously ill and minimisation of any unnecessary trauma is very
5 desirable.

Traditionally, radioimmunoassay has been used for estimating α GST in urine with the attendant disadvantages of using a radio-labelled substance.

10 Frequently, it is not possible to carry out the necessary estimation of urinary α GST for some considerable time, such as days, after a sample has been collected. Accordingly, it is often necessary to store the urine sample at very low temperatures, typically of the order of -20°C . However, it is found that on storing urinary α GST at such
15 low temperatures leads to a loss of immunoreactivity and thus a poor sensitivity of any immunoassay. This loss of immunoreactivity is most likely due to freeze-thaw denaturation.

Accordingly, there is a need for a medium which enables one to store α GST in urine at temperatures of the order of -20°C without any substantial loss of immunoreactivity of α GST in an immunoassay used
20 to detect the α GST, when required.

Disclosure of Invention

Accordingly, the invention provides a stabilising medium for α GST in urine, which comprises a stabilising amount of a non-enzyme protein, a chelating agent and a buffer, such that the medium has a pH
25 in the range 7.0-7.5, and the medium being effective to prevent loss of α GST immunological activity.

The stabilising medium according to the invention can be used to store urine samples at temperatures of the order of -20°C without any loss of immunoreactivity. However, additionally, the stabilising
30 medium according to the invention is found to improve

immunoreactivity when added to fresh urine which is stored temporarily at 2-8°C prior to assay for up to two hours.

Preferably, the non-enzyme (α GST) protein is an albumin.

5 The albumin can be a mixture of an albumin and a hydrolysed albumin. A suitable hydrolysed albumin is hydrolysed gelatin. Such gelatin hydrolysates are commercially available for example from Sigma Chemicals (Code G-0262 enzymatically generated).

An especially preferred albumin (non-hydrolysed) is a serum albumin, especially bovine serum albumin (BSA).

10 A preferred mixture of a serum albumin and a hydrolysed albumin is a mixture of equal amounts (w/v) of bovine serum albumin and gelatin hydrolysate.

The concentration of non-enzyme protein is suitably in the range 5-15% w/v, more particularly of the order of 10% w/v.

15 The chelating agent is suitably an alkali metal salt of EDTA.

The stabilising medium suitably has a salt concentration in the range 4-5% w/v. Especially suitable salts or alkali metal salts, more especially sodium chloride.

20 The inclusion of a salt such as sodium chloride aids in the dissolution of albumin and/or albumin hydrolysate or other non-enzyme proteins used and which has the requisite stabilising properties.

Although not wishing to be bound by any theoretical explanation of the invention, it is considered that the salt may function by reducing assays 'backgrounds', i.e. non-specific binding.

The stabilising medium also suitably contains a protease inhibitor. A preferred protease inhibitor is a trypsin inhibitor such as aprotinin.

5 The buffer is suitably a zwitterionic buffer of the type described by N.E. Good. and S. Izawa ((1972) Methods in Enzymol., 24, Part B, 53). An especially suitable buffer is HEPES at a pH of 7.3.

The stabilising medium according to the invention can also include other additives, for example, various antimicrobial agents or preservatives.

10 Suitable preservatives include sodium azide and preservatives containing mercurothiolate also known as thiomersal or thiomerosal.

The stabilising medium according to the invention will be mixed prior to storing with a urine sample which is to be assayed for α GST following storing at -20°C at a given period of time.

15 The invention also provides a method for the quantitative determination of α GST in urine, which comprises contacting a urine sample with an insolubilised form of anti- α GST IgG, the urine sample having been pre-treated with a stabilising medium as hereinbefore defined, determining the amount of α GST bound to the anti- α GST IgG
20 by contacting the bound α GST with enzyme-labelled anti- α GST IgG and measuring the activity of the enzyme label.

The stabilising medium according to the invention enables one to achieve a sensitivity in an immunoassay for urinary α GST which
25 correlates closely with that obtained when an immunoassay is carried out on what are referred to in the art as fresh urine samples. In contrast with the situation when urine samples are stored at -20°C in the absence of the stabilising medium, there is substantially no loss of immunoreactivity following the storage of such samples in the presence of the stabilising medium according to the invention, as hereinafter
30 demonstrated in the Examples.

The preferred enzyme label is a peroxidase, more especially horseradish peroxidase.

5 Another preferred peroxidase conjugate is a biotinylated avidin (includes streptavidin)-peroxidase complex, which may be used with an antibody-biotin conjugate to amplify the enzyme assay in conventional manner. In such an enzyme assay antigen insolubilised on solid phase antibody binds to the antibody-biotin conjugate which in turn binds to the biotinylated avidin/streptavidin-peroxidase complex, whereupon the peroxidase activity is measured.

10 Further, preferably, the anti- α GST IgG-HRP-conjugate used in the enzyme immunoassay is in a liquid stable form in a stabilising medium comprising a stabilising amount of cytochrome c and a stabilising amount of serum albumin, a surfactant, a polyol and a buffer, such that the medium has a pH of the order of 6.5 and that the
15 final concentration of polyol is in the range 5-15% v/v.

The cytochrome c is preferably present in an amount of 0.02-2% weight by volume. Whereas the concentration of cytochrome c can be increased to above 2% weight by volume without substantially affecting stabilisation, decreasing the concentration below about 0.02% weight
20 by volume results in a decrease in the stabilising effect of the buffer.

By cytochrome c herein is meant a cytochrome in which there are covalent linkages between the side chains of the heme moiety and the protein.

25 The stabilising protein is preferably a serum albumin which is present in an amount of 0.5-2% weight by volume.

An especially suitable serum albumin is bovine serum albumin (BSA). Whereas the quantity of BSA can be increased to above 2% weight by volume without substantially affecting stabilisation as in the case of the cytochrome c component, if the concentration is decreased

below about 0.5% weight by volume the stabilising effect of the buffer is decreased.

The serum albumin can be supplemented by further stabilising protein, for example, foetal calf serum which is rich in BSA.

5 The surfactant is preferably a non-ionic surfactant selected from polyoxyethylene esters of fatty acids, polyoxyethylene sorbitan esters, polyoxyethylene alcohols, polyoxyethylene isoalcohols, polyoxyethylene ethers, polyoxyethylene esters, polyoxyethylene-*p-t*-
10 octylphenols or octylphenyl-ethylene oxide condensates, ethylene oxide condensates with fatty alcohols, polyoxyethylene nonylphenols, and mixtures of polyalkylene glycols or a mixture thereof.

Especially preferred non-ionic surfactants include: polyethylene sorbitan esters sold under the Trade Mark Tween, especially
15 polyoxyethylene sorbitan monolaurate or Tween 20, but also Tween 60 and Tween 80; polyoxyethylene ethers sold under the Trade Mark Triton, such as Triton X100, Triton X114, Triton X110E and Triton N101, and Brij; an octylphenyl-ethylene oxide condensate sold under
20 the Trade Mark Nonidet P40; ethylene oxide condensates of fatty alcohols sold under the Trade Mark Lubrol, especially Lubrol PX; and a mixture of one part by weight of polyethylene glycol and four parts
by weight of polypropylene glycol sold under the Trade Mark Synperonic F108. (Tween, Triton, Brij, Nonidet, Lubrol and Synperonic are all Trade Marks).

An especially suitable surfactant is a Synperonic F108.

25 The polyol stabilises protein-protein interactions. Suitable polyols include glucose, glycerol, mannitol, sorbitol and sucrose or a mixture thereof. An especially preferred polyol is glycerol at a final concentration of the order of 10% v/v.

An especially suitable buffer is phosphate buffered saline.

A stabilising medium for the anti- α GST IgG-HRP-conjugate can also include other additives depending on the nature of the enzyme conjugate, for example, various antimicrobial agents, preservatives and protease inhibitors, agents which stabilise protein - protein interactions, antioxidants and colouring agents which aid in identification.

A suitable antimicrobial agent is gentamicin.

Suitable preservatives include preservatives containing mercuriothiolate also known as thiomersal or thiomerosal.

A suitable protease inhibitor is, for example, a trypsin inhibitor such as aprotinin.

A suitable colouring agent is carmine dye.

The invention will be further illustrated by the following Examples.

Best Mode for Carrying Out the Invention

15 Example 1

A stabilising medium for urinary α GST with a pH of 7.3 was prepared from the following reagents:

Reagents

	HEPES	-	0.5M
20	Na ₂ EDTA	-	10 mM
	NaCl	-	4.5% (w/v)
	BSA	-	5% (w/v)
	Gelatin hydrolysate	-	5% (w/v)
	Aprotinin	-	10 μ g/ml
25	Thiomersal	-	0.01% (w/v)
	Sodium azide	-	0.05% (w/v)

All of the above components were added to 80% of the final volume of deionised water and the pH adjusted to 7.3 with NaOH. The solution was then brought to the final volume with deionised water. In the case of the addition of the BSA and the gelatin hydrolysate, it is important that these are added separately and care should be taken to ensure that the first component is completely dissolved before the second component is added.

The stabilising medium so prepared is used for stabilising urinary α GST by adding one part of the medium to four parts of urine (1/5 dilution), mixing gently and freezing the sample at -20°C or storing temporarily at $2-8^{\circ}\text{C}$ prior to assay for up to two hours.

Example 2

A stabilising medium for anti- α GST IgG-HRP-conjugate with a pH of 6.5 was prepared from the following reagents:

15	<u>Reagent</u>	<u>Quantity</u>
	NaCl	8.000 g
	NaH ₂ PO ₄ .2H ₂ O	0.260 g
	Na ₂ H ₂ PO ₄ .2H ₂ O	1.425 g
	Cytochrome c	0.250 g
20	Synperonic F108	10.000 g
	BSA	10.000 g
	Foetal calf serum	25.000 ml
	Thiomersal	0.100 g
	Gentamicin	0.100 g
25	Carmine dye	0.930 g
	Concentrated HCl (to adjust pH)	variable
	Deionised water	variable
	Made up to 1000 ml with deionised water	

700 ml of deionised water was added to a glass container. To this was added the NaCl, NaH₂PO₄.2H₂O, Na₂H₂PO₄.2H₂O and thiomersal with stirring until dissolution of the reagents occurred. The Synperonic F108 was then added to the solution following by stirring
5 until the surfactant dissolved. The pH of the solution was then checked and adjusted to pH 6.5 with 5M HCl. The BSA was then added to the solution and allowed to dissolve. To this was then added the foetal calf serum with further stirring until dissolution occurred. The gentamicin, cytochrome c and carmine dye were then added with further stirring
10 until dissolution occurred. The pH was rechecked and adjusted as necessary to pH 6.5. The final volume was adjusted to 1000 ml and the buffer was filtered through a 0.2 µm filter ready for storage. In use nine parts of the buffer are added to one part glycerol.

Example 3

15 The stability of the anti-αGST IgG-HRP conjugate currently provided in lyophilised form in an enzyme immunoassay kit marketed by Biotrin International Limited under the trade mark Nephkit was investigated in the stabilising medium prepared in Example 2. The Nephkit assay provides a method for the quantitative determination of
20 αGST in urine and can be indicative of proximal tubule damage in the kidney.

100% stability of a 10 x concentrate was obtained when the conjugate was stored for twenty four hours at room temperature in the stabilising medium prepared in Example 2.

25

Example 4

Utility of the stabilising medium of Example 1 for stabilising urinary αGST during storage at -20°C

30 Nine urine samples (male and female) were obtained and assayed immediately for αGST using the enzyme immunoassay kit marketed by Biotrin International Limited, Mount Merrion, County Dublin, Ireland,

under the trade mark Nephkit. The results are shown in column 2 in Table 1.

The samples were then split and the stabilising medium prepared in Example 1 added to one lot (-20°C (SM)) and not to the other
5 (-20°C).

The samples were retained at -20°C for three days after which they were assayed using the Nephkit assay.

It was found that the values obtained for the samples stored at -20°C in the stabilising medium correlated closely with the original
10 4°C (fresh) data. However, examples frozen without the stabilising medium all exhibited a diminution in α GST immunoreactivity. As indicated above this loss of immunoreactivity is most likely due to freeze-thawed induced denaturation.

Table 1

Sample	Storage Temperature		
	4°C	-20°C [α GST] ng/ml	-20°C (SM)
1	2.62	0.58	3.01
2	2.63	0.81	2.93
3	0.44	0.00	0.71
4	8.54	3.64	8.43
5	1.40	0.43	2.15
6	3.30	0.95	4.43
7	4.12	0.93	4.29
8	1.80	0.43	2.13
9	4.31	0.50	4.88

Example 5Stabilisation of urinary α GST-spiked samples

5 Two urine samples, A and B, were each spiked to five different levels of α GST (10, 25, 50, 100 and 500 ng/ml) and then stored in the presence of the stabilising medium of Example 1 for two days at 4°C and -20°C. The results are shown in Tables 2 and 3.

It will be observed from Tables 2 and 3 that the presence of the stabilising medium protects α GST against loss of immunological activity which facilitates the detection in the enzyme immunoassay used.

10

Table 2

Storage Temperature		
Sample	4°C [α GST] ng/ml	-20°C(SM)
A: 500	191.1	507.8
A: 100	57.22	103.2
A: 50	29.48	47.11
A: 25	15.35	25.9
A: 10	7.71	11.82

Table 3

Storage Temperature		
Sample	4°C [αGST] ng/ml	-20°C(SM)
B: 500	49.30	467.8
B: 100	37.30	88.95
B: 50	17.52	45.23
B: 25	8.63	21.12
B: 10	3.40	8.26

Example 6

Two samples, denoted C 200 and D 200, were prepared containing 200 ng/ml αGST and the stabilising medium of Example 1 was added to each sample.

Each of the two samples was then split and the resulting split samples in each case were stored at 4°C and -20°C.

After storage for two days each of the samples was assayed at different dilutions (1/40 - 1/320) in assay diluent using the Nephkit assay procedure referred to in Example 4.

The results are shown in Tables 4 and 5.

It will be apparent from the data set out in Tables 4 and 5 that sample dilution does not affect α GST determination and that storage at -20°C is surprisingly superior to storage at 4°C .

Table 4

Storage Temperature		
Sample	4°C [α GST] ng/ml	-20°C (SM)
C 200 (1/40)	106	175
(1/80)	109	185
(1/160)	104	188
(1/320)	111	180

5

Table 5

Storage Temperature		
Sample	4°C [α GST] ng/ml	-20°C (SM)
D 200 (1/40)	141	183
(1/80)	141	183
(1/160)	134	180
(1/320)	128	187

Claims:-

- 5 1. A stabilising medium for α GST in urine, which comprises a stabilising amount of a non-enzyme protein, a chelating agent and a buffer, such that the medium has a pH in the range 7.0-7.5, and the medium being effective to prevent loss of α GST immunological activity.
2. A stabilising medium according to Claim 1, wherein the non-enzyme protein is an albumin.
- 10 3. A stabilising medium according to Claim 1 or 2, wherein the non-enzyme protein is a mixture of an albumin and a hydrolysed albumin.
4. A stabilising medium according to Claim 3, wherein the mixture is a mixture of a serum albumin and a hydrolysed albumin.
- 15 5. A stabilising medium according to Claim 3 or 4, wherein the mixture is a mixture of equal amounts (w/v) of bovine serum albumin and gelatin hydrolysate.
6. A stabilising medium according to any preceding claim, wherein the concentration of non-enzyme protein is of the order of 10% w/v.
- 20 7. A stabilising medium according to any preceding claim, wherein the chelating agent is an alkali metal salt of EDTA.
8. A stabilising medium according to any preceding claim, which has a salt concentration in the range 4-5% w/v.
- 25 9. A stabilising medium according to Claim 8, wherein the salt is an alkali metal salt.

10. A stabilising medium according to Claim 8 or 9, wherein the salt is sodium chloride.

11. A stabilising medium according to any preceding claim, which includes a protease inhibitor.

5 12. A stabilising medium according to any preceding claim, wherein the buffer is a zwitterion buffer.

13. A stabilising medium according to Claim 12, wherein the buffer is HEPES.

10 14. A stabilising medium according to any preceding claim, wherein the buffer has a pH of 7.3.

15 15. A method for the quantitative determination of α GST in urine, which comprises contacting a urine sample with an insolubilised form of anti- α GST IgG, the urine sample having been pre-treated with a stabilising medium according to any one of Claims 1-14, determining the amount of α GST bound to the anti- α GST IgG by contacting the bound α GST with enzyme-labelled anti- α GST IgG and measuring the activity of the enzyme label.

16. A method according to Claim 15, wherein the enzyme label is a peroxidase.

20 17. A method according to Claim 15 or 16, wherein the peroxidase is horseradish peroxidase.

25 18. A method according to Claim 17, wherein the anti- α GST IgG-HRP-conjugate is in a liquid stable form in a stabilising medium comprising a stabilising amount of cytochrome c and a stabilising amount of serum albumin, a surfactant, a polyol and a buffer, such that the medium has a pH of the order of 6.5 and that the final concentration of polyol is in the range 5-15% v/v.

INTERNATIONAL SEARCH REPORT

Intern: 1 Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/573 C12N9/10 C07K16/40 G01N33/53 G01N33/531

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO-A-93 22452 (C.G. KILTY ET AL.) 11 November 1993 see the whole document ---	1-18
A	PATENT ABSTRACTS OF JAPAN vol. 16 no. 189 (P-1348) [5232] ,8 May 1992 & JP,A,04 025763 (MARUKO SEIYAKU KK) 29 January 1992, see the whole document -----	1-18

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Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9322452	11-11-93	EP-A- 0640145 US-A- 5217868	01-03-95 08-06-93
