AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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α–Mannosidosis

Detection of the Bovine Heterozygote

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Contents

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1. Introduction

α-Mannosidosis is a lethal lysosomal storage disease of cattle that is inherited in an autosomal recessive manner. Clinical expression varies from perinatal death to mild locomotor dysfunction in weaners. There are no consistent gross abnormalities evident on necropsy. Microscopic examination reveals cytoplasmic vacuolation of neurones, of epithelial cells of the proximal tubules in the kidney and of epithelial cells of the pancreas and the thyroid. There is a profound deficiency of α-mannosidase activity in plasma and leucocytes. The defect is caused by a deficiency of acidic α-mannosidase that results in an accumulation of its oligosaccharide rich substrate in lysosomes (Hocking *et al.*, 1972). The disease has been recorded in Angus, Murray Grey and Galloway calves (Whittem and Walker, 1957; Healy and Cole, 1976; Borland *et al.*, 1984). Heterozygotes can be identified by demonstration of a partial deficiency of α-mannosidase activity in plasma or leucocytes (Jolly *et al.*, 1973; Healy, 1981).

The plasma test may be used as a screening test. Subjects whose plasma activity is < 22 U/L should be investigated with the granulocyte test.

2. Principle of the Method

Plasma or extracts of granulocytes are incubated with appropriate chromogenic substrates in specific buffers. The reactions are terminated by addition of a basic buffer. The concentration of p-nitrophenol or nitrocatechol is determined by spectrophotometry. Activities are expressed as U/L, where one U is the amount of enzyme activity that produces 1 µmole of product per minute.

Granulocytes are isolated from blood collected in EDTA tubes by flash lysis of erythrocytes with distilled water. Isotonicity is restored by addition of concentrated saline, and the cells pelleted by centrifugation. Erythrocytes remaining in the pellet are lysed by resuspension in 0.1 mol/L ammonium chloride (NH_4Cl), then the granulocytes pelleted by centrifugation and washed in saline. The granulocytes are then resuspended in triton/saline, decanted into a microfuge tube and frozen. On the day of analyses the preparation is thawed, centrifuged and the supernatant retained for analysis of α-mannosidase (α-M), hexosaminidase (Hex) and arylsulfatase (Ars) activities.

Activities of Hex and Ars in granulocytes are not significantly different between animals homozygous normal or heterozygous for α-mannosidosis. Linear positive relationships exist between the activity of each of the three enzymes, but not for protein concentration, and the proportion of eosinophils in granulocyte preparations (Healy, 1983). In comparison with homozygous normal animals granulocyte α-M activity in heterozygotes is low relative to activity of Hex and Ars.

3. Materials Required for the Assays

3.1. Sample

Blood (20 mL) is collected using a large bore needle, 18 G or larger, into a vessel containing 1.5 mg of sodium or potassium ethylenediamminetetraacetic acid (EDTA) per mL of blood. Evacuated tubes are ideal providing the sample is collected with a rapid flow of blood. The sample must arrive at the laboratory within 24 hours of collection and be transported in cool insulated containers.

3.2. Chemicals

3.2.1. Analytical Reagent Grade Chemicals

Ammonium chloride (NH_4Cl), citric acid ($C_6H_8O_7$), disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), glacial acetic acid (CH_3CO_2H), glycine ($C_3H_7O_3$), sodium acetate (CH_3CO_2Na), sodium chloride, sodium hydroxide (NaOH), Triton X100 and zinc acetate [$(CH_3CO_2)_2.Zn.2H_2O$].

3.2.2. Substrates

p-Nitrophenyl-α-D mannopyranoside: Sigma N2127

p-Nitrophenyl N-acetyl-β-D glucopyranoside: Sigma N9376

2-Hydroxy-5-nitrophenylsulfate: Sigma N7251

3.2.3. Standards

p-Nitrophenol: Sigma 104.8

4-Nitrocatechol: Sigma N7126

3.3. Reagents

3.3.1. Saline

Make up a 0.154 mmol/L solution of NaCl (i.e. 9 g NaCl per litre of distilled water.

3.3.2. Triton Saline

Add 2 mL of Triton X100 to 1 L of saline (0.2%)

3.3.3. Ammonium Chloride

Dissolve 5.53 g of ammonium chloride in 1 L of distilled water (0.1 mol/L).

3.3.4. Concentrated Saline

Dissolve 45 g of NaCl in 1 L of distilled water (0.77 mol/L).

3.3.5. α-Mannosidase and Hexosaminidase Standard

Add 27.8 mg p-nitrophenol per 100 mL of distilled water (2 mmol/L).

3.3.6. Arylsulfatase Standard

Add 31.02 mg nitrocatechol per 100 mL of distilled water (2 mmol/L).

3.3.7. α-Mannosidase Buffer

0.1 mol/L acetate, pH 3.7

(a) Add 11.55 mL of glacial acetic acid to 1 L of distilled water.
(b) Add 16.4 g of anhydrous sodium acetate to 1 L of distilled water.

Mix 450 mL of (a) with 50 mL of (b).
Check pH. If necessary, adjust with either (a) or (b) and make to 1 L with distilled water.

3.3.8. Arylsulfatase Buffer

Acetate (0.1 mol/L) , pH 5.15. Mix 126 mL of (a) with 374 mL of (b). Check pH. If necessary adjust with either (a) or (b), and make to 1 L with distilled water.

3.3.9. Hexosaminidase buffer citrate/phosphate, pH 4.35

(c) Add 21.01 g of citric acid (1 H_2O) to 1 L of distilled water.
(d) Add 71.7 g of disodium hydrogen phosphate to 1 L of distilled water.

Mix 282 mL of (c) with 212 mL of (d). Check pH. If necessary adjust with either (c) or (d), and make to 1 L with distilled water.

3.3.10. Zinc buffer (12 mmol/L)

3.3.10.1. Stock

Add 21.95 g of zinc acetate to 100 mL with distilled water.

3.3.10.2. Working

Add 1.2 mL of stock zinc per 100 mL of α-mannosidase acetate buffer.

3.3.11. α-Mannosidase Substrate (5 mmol/L)

Dissolve 1.502 mg of p-nitrophenyl-α–D-mannopyranoside/mL of α-M acetate buffer using a stirring bar and a little heat. Store frozen.

3.3.12. Hexosaminidase Substrate (2.5 mmol/L)

Dissolve 0.856 mg of p-nitrophenyl N-acetyl-β–D-glucopyranoside/mL of Hex buffer using a stirring bar and a little heat. Store frozen.

3.3.13. Arylsulfatase Substrate (10 mM)

Dissolve 2.732 mg of 2-hydroxy-5-nitrophenyl sulfate/mL of arylsulfatase acetate buffer. Store frozen.

3.3.14. Glycine Buffer

Add 15.01 g of glycine in 600 mL of water and adjust pH to 10.7 with 5 mol/L sodium hydroxide and make to 1 L with distilled water.

3.3.15. Sodium Hydroxide

Dissolve 8 g of sodium hydroxide in 1 L of distilled water (0.2 mol/L).

4. Analytical Procedures

4.1. Plasma α-Mannosidase Assay

(a) Thaw samples, controls and α-mannosidase substrate. Invert several times to mix.
(b) Label test tubes in duplicate:
Sb for substrate blanks
St for standard
Nb, N1, N2 for each control and unknown.
(c) Add 50 µL saline to Sb tubes
50 µL α-M/Hex standard to St tubes
50 µL Control/unknowns to N tubes
(d) Add 100 µL of zinc buffer to all tubes. Mix by shaking and place in rack in water bath at 37°C, and start timer.
(e) At five minutes add 400 µL of α-M acetate buffer to Nb tubes (plasma blanks).
(f) At precisely 10 min add 400 µL of α-M substrate to all remaining tubes. Mix by gently shaking the rack.
(g) At precisely 70 min (i.e. 60 min after adding substrate) terminate the reaction by adding 4 mL of glycine buffer to each tube; vortex briefly (10 s).
(h) Set wavelength of spectrophotometer at 405 nm. Zero instrument on water and read in following order: Sb, St, then Nb, N1 and N2 for each control and unknown.

4.2. Isolation of Granulocytes

(a) Centrifuge 2x 10 mL portions of blood at 1500 *g* for 15 min. Harvest 1–2 mL of plasma (from top of the tube) and either estimate plasma α-M activity immediately, or store at -20°C.
(b) Remove and discard remaining plasma and buffy coat. Granulocytes remain in the upper 20% of the red cell column.
(c) Add 2 mL saline to each tube and resuspend packed cells. Transfer contents of both tubes to a common 50 or 100 mL centrifuge tube. Rinse one tube with a further 2 mL saline, transfer to the other tube, then add rinsings to the 50 or 100 mL tube.
(d) To the 50 mL or 100 mL tube add 20 mL of distilled water and mix. 30 s later add 5 mL of 0.77 mol/L sodium chloride and mix.
(e) Centrifuge at 400 *g* for 10 min then discard supernatant.
(f) Suspend pellet in 4 mL of 0.1 mol/L ammonium chloride. Mix well and transfer to a 15 mL centrifuge tube.
(g) Centrifuge 400 *g* for 10 min then discard supernatant — *do not delay.*
(h) Suspend pellet in 4 mL of saline and centrifuge at 100 *g* for five minutes. Discard supernatant and drain the pellet by inversion of the tube for one to two minutes.
(i) Suspend cells in 8 mL of saline. Determine optical density (OD) at 700 nm. Centrifuge at 400 *g* for 10 min.
(j) Resuspend cells in triton/saline:
If OD at 700 nm = 0.25 use 250 µL
If OD at 700 nm = 0.5 use 500 µL
If OD at 700 nm = 1.0 use 1000 µL
Freeze at -20°C.

4.3. Granulocyte Assays

Granulocyte preparation is thawed in a 37°C water bath, mixed on a vortex mixer for 10 s and then centrifuged in a microfuge at 13 000 rpm for five minutes. The supernatant is carefully decanted into a clean tube and assayed for α-M, Hex and Ars activities.

4.4. α-Mannosidase Assay

(a) Thaw α-M substrate. Invert several times to ensure mixing.
(b) Label test tubes in duplicate:
Sb for substrate blanks
St for standard
N1, N2 for each control and unknown
(c) Add 50 µL saline to Sb tubes
50 µL α-M/Hex standard to St tubes
50 µL Control/unknowns to N tubes
(d) Add 100 µL of zinc buffer to all tubes, mix by shaking and place in rack in water bath at 37°C, and start timer.
(e) At precisely 10 min, add 400 µL of α-M substrate to all tubes. Mix by gently shaking the rack.
(f) At precisely 70 min (i.e. 60 min after adding substrate) terminate the reaction by adding 4 mL of glycine buffer to each tube. Vortex.
(g) Set wavelength of spectrophotometer at 405 nm, zero instrument on water and read in following order: Sb, St, then N1 and N2 for each control and unknown.

4.5. Hexosaminidase Assay

(a) Thaw Hex substrate. Invert several times to ensure mixing.
(b) Label test tubes in duplicate
Sb for substrate blanks
St for standard
N1, N2 for each control and unknown
(c) Add 25 µL saline to Sb tubes
25 µL α-M/Hex standard to St tubes
25 µL Control/unknowns to N tubes
(d) Add 250 µL of Hex buffer/substrate to all tubes, mix by shaking and place in rack in water bath at 37°C and start timer.
(e) At precisely 30 min, terminate the reaction by adding 4 mL of glycine buffer to each tube. Vortex briefly (10 s).
(f) Set wavelength of spectrophotometer at 405 nm, zero instrument on water and read in following order: Sb, St, then N1 and N2 for each control and unknown.

4.6. Arylsulfatase Assay

(a) Thaw Ars substrate. Invert several times to ensure mixing.
(b) Label test tubes in duplicate:
Sb for substrate blanks
St for standard
N1, N2 for each control and unknown.
(c) Add 25 µL saline to Sb tubes
25 µL Ars standard to St tubes
25 µL Control/unknowns to N tubes.
(d) Add 250 µL of Ars substrate to all tubes, mix by shaking and place in rack in water bath at 37°C and start timer.
(e) At precisely 60 min terminate the reaction by adding 4 mL of 0.2 mol/L sodium hydroxide to each tube. Vortex briefly (10 s).
(f) Set wavelength of spectrophotometer at 515 nm, zero instrument on water and read in following order, Sb, St, then N1 and N2 for each control and unknown.

5. Calculations

5.1. Plasma α-Mannosidase Assay

D OD = [(N1+N2)/2] - (Nb + Sb)
Factor = 33.3/(mean St - mean Sb)
Activity U/L = D OD * Factor

5.2. Granulocyte assay — α-Mannosidase

D OD = ((N1+N2)/2) - Sb
Factor = 33.3/(mean St - mean Sb)
Activity U/L = D OD * Factor

5.3. Granulocyte assay — Hexaminidase

D OD = [(N1+N2)/2] - Sb
Factor = 66.7/(mean St - mean Sb)
Activity U/L = D OD * Factor

5.4. Granulocyte assay — Arylsulfatase

D OD = [(N1+N2)/2] - Sb
Factor = 33.3/(mean St - mean Sb)
Activity U/L = D OD * Factor

6. Interpretation of Results

6.1. Plasma α-Mannosidase Activity

If plasma activity is >22 U/L the subject can be considered homozygous normal.

With large peer groups (20 animals) calculate the mean, note animals with activities less than 60% of the mean, then recalculate the mean excluding these animals. All animals with activities less than 70% of the recalculated mean should be considered presumptive heterozygotes and retested using the granulocyte test.

6.2. Granulocyte Assays

(a) Ratio 1 = α-M*100/Hex
(b) Ratio 2 = α-M*40/Ars
(c) If ratio 1 is >16 the result is consistent with the animal being homozygous normal.
(d) If ratio 1 is <6 the result is consistent with the animal being heterozygous.
(e) If ratio 1 is >6 but <16 ratio 2 should be examined.
(f) If ratio 2 is >12 then result is consistent with animal being homozygous normal.

(g) If ratio 2 is <7 then result is consistent with animal being heterozygous.
(h) If ratio 2 is between 7 and 12, the result is inconclusive and resample the animal.

7. Notes on the Assay

(a) α-Mannosidase activity is stable for six months in plasma frozen at -20°C.
(b) α-Mannosidase activity declines with time in frozen granulocyte preparations.
(i) Granulocyte preparations should be analysed within one week of preparation.
(ii) Quality control samples should be collected from a known homozygous normal and a known heterozygote every month.
(c) Frequently inconclusive results are obtained when testing very young calves. Usually their genotype can be established if they are tested after they reach six months of age.

8. References

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