

Bayer Award

Fructose 1,6-bisphosphatase as a marker of hepatocellular damage in liver transplantation

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Aspartate amino transferase (AST) and alanine amino transferase (ALT) are used as markers of hepatocellular damage in liver transplantation but are insensitive to acute changes. When cellular damage does occur levels remain elevated and poorly reflect further damage. Fructose 1,6-bisphosphatase (FBPase) is a key enzyme in gluconeogenesis and due to its location in the cytosol of the periportal liver cells it has been proposed as an alternative marker of hepatocellular damage. We evaluated FBPase against conventional liver enzymes in an experimental porcine transplant model.

Porcine donor livers were subjected to various periods of cold ischaemic injury, group I (n=4) 4 hours, group II (n=6) 1 hour and group III (n=4) 0 hours, post donor hepatectomy and prior to preservation by normothermic extracorporeal sanguineous machine perfusion. FBPase, AST and ALT levels were measured in perfusate samples taken from the circuit during machine preservation. FBPase was analysed enzymatically by monitoring NADPH production at 340 nm as fructose 1,6-bisphosphate is converted to fructose 6-phosphate. AST and ALT were analysed using conventional methods.

FBPase levels began to increase slowly after reperfusion in all three groups, with the lowest levels being seen in group III which remained below 100 U/L. FBPase in groups I and II rose to around 150 U/L at 6 hrs preservation. A similar pattern was seen for both AST and ALT. However, at 8 hrs preservation the FBPase levels increased in group I to 550 U/L and 750 U/L at 12 and 20 hrs preservation respectively. This was not reflected in the AST and ALT results which both increased slowly. The increase in FBPase may be related to graft failure, shown by a decline in bile production, as FBPase rose some hours after bile production fell. Therefore FBPase has the potential to be a sensitive and useful marker of liver function in transplantation.

Development of a genotyping service for the identification of butyrylcholinesterase variants

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The enzyme butyrylcholinesterase (BChE) is essential in terminating the effect of muscle relaxants, such as suxamethonium. Over 20 mutations, resulting in a decrease or loss of enzyme activity within the *BCHE*

gene, have been described. Affected individuals suffer from prolonged apnoea when given an otherwise safe dose of a muscle relaxant. The phenotypic assay is limited in that it cannot reliably distinguish between certain mutations, especially where multiple mutations are present.

DNA sequencing of two regions of the *BCHE* gene has been developed to detect the clinically significant K- and atypical variants. The method was then used to genotype 24 patients that had originally had a phenotype assigned based on current methodology. Fourteen of these patients were shown to have more than one mutation present and five of these patients were reclassified as having potential sensitivity as a result. Two patients with queried U/AK or A/K phenotypes were shown to be AK/K genotypes. The AK/K genotype is associated with increased sensitivity to muscle relaxants.

The newly developed genotyping assay will improve the current classification, which is based on phenotypic data alone and will add information about multiple mutations. Interpreting the genetic background in conjunction with measurements of the actual enzyme activity status will enhance the service providing a more detailed, individual diagnosis.

Prednisolone measurement by liquid chromatography: tandem mass spectrometry

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Prednisolone is a corticosteroid that is often used for long term suppression of the immune system; for example in asthmatics and transplant recipients. With severe side effects including osteoporosis, diabetes mellitus and adrenal suppression it is appreciated that the dose should be kept as low as possible. The monitoring of prednisolone would allow the physician to tailor the dose to individuals requirements, assess compliance and absorption and improve the outcome of these patients. We have developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the measurement of prednisolone in serum or plasma.

Samples (500 µL) and deuterated (d6) prednisolone (internal standard) were extracted using Waters Oasis® HLB columns, the methanol eluant was dried down and the extracted prednisolone was reconstituted in 50:50 mobile phases. The samples were then transferred into a 96-deep well microtitre plate, of which 20 µL was injected into the LC-MS/MS system. A Waters Atlantis® column (3.0 mm x 50 mm) was eluted with a step

gradient of 50% to 95% methanol containing 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid, at 0.5 mL/min. The column was operated at ambient temperature.

The retention times were 2.75 min for prednisolone and 2.72 min for d6 prednisolone. Cycle time was 5 min. The transitions used were m/z 361.3>147.1 for prednisolone and m/z 367.2>150.3 for d6 prednisolone; monitored using a Quattro micromass spectrometer. The between-batch precision of the method was 8%, 4% and 5% at concentrations of 75 $\mu\text{g/L}$, 375 $\mu\text{g/L}$ and 750 $\mu\text{g/L}$ respectively. The within batch precision was <8% for the same concentrations. The lower limit of detection was 25 $\mu\text{g/L}$ and the assay was linear to 4000 $\mu\text{g/L}$. There was negligible suppression of ionisation.

We have developed a robust assay for the measurement of prednisolone. This should allow easy monitoring of treatment in many patient groups, optimising prednisolone therapy.

Identifying the sequence elements important for mitochondrial targeting of coproporphyrinogen oxidase

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Hereditary Coproporphyrria (HCP) is an autosomal dominant disorder of haem synthesis due to deficient Coproporphyrinogen Oxidase (CPO) activity. CPO is the sixth enzyme of the haem biosynthetic pathway and is located within the inter-membrane space of mitochondria where it catalyses the oxidation of coproporphyrinogen III to protoporphyrinogen IX. CPO is nuclear encoded and synthesised on cytosolic ribosomes as a pre-protein that contains a presequence of 110 amino acids at the amino terminus. The aim of this study was to identify the sequence elements necessary to target CPO to mitochondria. We have fused human CPOs containing N-terminal and C-terminal deletions, to the amino terminus of yellow fluorescent protein (YFP) and have used these constructs to investigate the mitochondrial import of CPO in human cells. Constructs were transfected by lipofection into HeLa cells and their cellular location imaged by fluorescence and confocal microscopy. Inspection of the CPO presequence predicts a bipartite structure with dual targeting and sorting information: a matrix-targeting signal consisting of a positively charged region (residues 1-69) followed by an extended

hydrophobic sorting region (residues 70-103) that directs the protein to the inter-membrane space. Here we have shown that the residues 1-69 contain all the information necessary to target YFP to the mitochondria. We are currently investigating the role of the hydrophobic region in localising CPO to the inter-membrane space. This and other findings will help identify some of the molecular defects causing Hereditary Coproporphyrria

Exonic deletions in an 8-year-old boy with erythropoietic protoporphyria

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Erythropoietic protoporphyria (EPP) normally presents in childhood with cutaneous photosensitivity caused by the build up of protoporphyrin in the skin as a result of deficient ferrochelatase activity. Evidence suggests that clinical expression of classical EPP requires the coinheritance of a severe ferrochelatase mutation, in trans to the commonly occurring polymorphism, IVS3-48C, which is associated with low enzyme activity.

An 8-year-old boy presented with photosensitivity. A diagnosis of EPP was made following demonstration of increased concentrations of protoporphyrin in erythrocytes and plasma. A referral was made for mutation detection with a view to genetic counselling.

All eleven exons and flanking intronic regions of the ferrochelatase gene were analysed by bi-directional sequencing but no mutation was identified. Samples from the proband and parents were analysed for the IVS3-48C low expression polymorphism. The haplotypes of the parents were inconsistent with the proband's sample, suggesting the inheritance of a partial gene deletion or non-paternity.

Deletion studies were undertaken using gene dosage analysis. Exons 2-11 of the ferrochelatase gene were amplified by PCR in a multiplex reaction, with incorporation of a fluorescent label and subsequent analysis by gene scanning. Gene dosage was determined by comparison with internal controls. Deletion of exons 3 and 4 in the father and the proband was identified by this method. These results explain the clinical expression of EPP in the proband who, in addition to the deletion, carries the low expression polymorphism. This is in contrast to the father who, although carrying the deletion, is unaffected due to the absence of the low expression polymorphism.