Kinetic Enzyme Assay

Enzymes are complex naturally occurring proteins that catalyze many biological reactions. Most enzymes are inside the cells and function there while only few in small quantities are present in plasma and other body fluids. What we estimate are mostly these intracellular enzymes which enter the blood/ body fluids due to diffusion from the aging cells(in smaller quantities) under normal conditions but from the damaged cells the enzymes pass in to the blood in much larger quantities.

Concentration of enzymes in the blood / plasma remain fairly constant under normal conditions but gets significantly altered when:

1) Change of synthesis of enzymes within the cells

2) Change in the size of enzyme forming tissue

3) Cellular damage to the enzyme forming tissue

4) Alteration in the rate of inactivation and disposal of the enzyme and

5) Obstruction to the disposal pathway of the excretion.

Most enzymes are quite specific in their action catalyzing a definite type of chemical reaction or act on a particular compound / substrate and so these have been named after the type of substrate on which these are acting e.g. Lipase, Amylase, Phosphatase etc. Concentration of enzymes are very low in plasma or body fluid and could-not be directly measured like Glucose, Protein etc. Therefore the enzymes are indirectly measured by their catalytic activity which is proportional to their concentration.

Enzymes can be estimated in two different ways:

1) End point assay in which sample is incubated with the buffered substrate for a fixed period of time & at the end the reaction it is stopped and the amount of product formed or substrate used is estimated.

2) Rate analysis or Kinetic assay where such changes are measured at short intervals or are continuously monitored.This technique has advantages over the end point assays:

Parameters	Kinetic assay	End point assay
Range of measurable activity	High	Limited
Reaction Monitoring	5 minutes	Longer incubation period
Detection of high enzyme activity	Within 3 minutes	More than 30 minutes

Standard Not required Essential

Thus kinetic assay technique has distinct advantages & the Principal is: If the concentration of the substrate (S) is sufficiently high in comparison to enzyme (E) then rate of reaction will be proportional to the concentration of enzyme. Thus the amount of product (P) formed in a given period of time would be proportional to the amount of active enzyme present, with all other factors remaining constant.

E + S --- ES --- E +P

The result of enzyme determination is expressed in terms of product (P) formed per unit of time under specified conditions for a given amount of sample. Thus International Unit (IU) has been adopted by International Union of Pure & Applied Chemistry to standardize the Unit system. One Unit of enzyme is that amount which will catalize the transformation of 1 Micromol of substrate per minute or number of micromol of product formed per minute by the enzyme present in 1 li ter of serum i.e. IU / L.

In practice the reaction rate is not constant with time. There is a initial (i) lag phase (with very little change per unit of time), then comes (ii) linear phase of constant change per unit of time and then finaly (iii) phase of substrate exhaustion with very little change. In order to obtain the best result we should have a long Linear phase and assay should be stopped before Substrate exhaution takes place.

Many of the enzyme system involve the conversion of Nicotinamide Adinine Dinucleotide (NAD) to its reduced form NADH or vice-versa. The reduced form NADH has much greater abosrption at 340 UV range and consequently reaction can be followed by measuring change in absorption per minute of time.Without going into further intricasies of the enzyme chemical reactions it can be simplified that the change in absorbance per minute when multiplied by the Factor (Given in the literature of the manufactures of the kits) gives the enzyme activity in IU /L. Factor can also be calculated & is similar to that of End point assays and this can be detected with help of known calibirators / controls, if required:

$$\text{Factor} = \frac{\text{Value of the known Standard or Calibirator}}{\text{Change in Absorbance of enzyme activity in one min.}} \quad \& \quad \text{Enz.U/L} = \text{Absorb./ min X Factor}$$

Enzymes are active only over a limited range of pH. But this optimum pH may vary some times with the type of substrate

e.g.: Alk. Phosphatase - Substrate B-Glycerophospahte - pH 8.6
Phenyl phosphate 9.8

Alkaline phosphatase assay does not include either NADH or NADPH. Here the rate of formation of Nitrophenol i.e the rate of increase (Ascending reaction) in absorbance at 405 nm is measured. Whereas in AST assay NADH and excess of MDH and LDH are added where NADH is converted into NAD+ and the rate of decrease (Descending reaction) in absorbance becomes the parameter for the AST activity.

Aspartate +Oxogluterate -------- Glutamate +Oxaloacetate

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Oxaloacetate + NADH+ H --------Maleate + NAD+

Some practical tips are being given so as to get better results with economy:

1) Incubate the reagents dispensed in quantities required for your assay at 37 degree for 1-2 minutes to avoid strain on the analyzer and keep back the kit in the freeze immediately to preserve its potency.

2) After mixing the sample to reagent feed it immediately to the analyzer so as to observe the Initial Absorbance which must fall with in the range as given in the table below. If not, this shows poor quality of the reagent and it should be avoided. Good manufacturers usually mention this figure.

3) Those instrument which displays the absorbance and continue to display the changing abosorbance with the chemical reaction (Enzyme + Substrate) are considered superior for kinetic assays. It can monitor the rate of reaction fast or slow depending on the high or low cons of enzyme and also help in detecting substrate exhaustion along with timings.

4) If we can anticipate higher values of the enzyme (Beyond the linearity range) we can predilute the sample 1 in 5 or 1 in 10 then run the test so as to avoid repetition e.g An Icteric sample may have higher values of ALT /AST / ALP.

5) We can alter the parameters of the kinetic assay with in the reasonable limits: Initial delay time can be reduced if we have preincubated the reagent as described in the para-1 above. Likewise the number of reading and the timings in linear reaction phase can be altered e.g. from 3 mins. to 2 mins., if you fear substrate exhaustion, while still maintaining the accuracy.

6) All the Descending reactions (where there is continuing fall in absorbance as in ALT, AST, LDH) and those with Ascending reaction as described in the table below) are better monitored in the analyzers as described in para-3 above.

7) Most of the enzymes are tested at 37 degree as IU of enzyme is defined at this temperature and India , being a tropical country, it is best suited. We should stick to uniform parameters so to make the results comparable for inter laboratory evaluation.

Chemistry	Methodology	Filter	Reagent / Sampl Ratio	Reaction	Initial Absorbance
Acid phosphatase	Alp.Nepthyl Phos.	405	50 /500	Ascending	< 0.3
Alk. Phosphatase	PNP DEA Kinetic	405	10 / 500	Ascending	< 0.9
Amylase	CNP	405	10 / 500	Ascending	< 0.35
C.K. (Nac)	Mod. IFCC	340	20 / 500	Ascending	< 0.6
C.K (M.B)	Immunoinhibition	340	25 / 500	Ascending	< 0.6
ALT (SGOT)	U.V Kin. IFCC	340	50 / 500	Descending	> 1.2
AST (SGPT)	-do-	340	-do-	-do-	> 1.2
Gamma G.T	Carboxy p-nitroanilid	405	25 / 500	Ascending	< 0.9
L D H	Pyruv +NADH-NAD	340	10 / 500	Descending	> 0.9
Lipase (FixT Kin)	Turbidom-Triolein	340	20 /500	Descending	> 1.2

What has been described and discussed above are based on the available ready to use kits and the semiautoanalyzers, and perhaps most of our laboratories are doing the same. However variations may occur and so the parameters are suitably altered and adjusted to our individual needs.

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