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Automated Fluorimetric Assay Procedure for Glucohydrolases Using a Routine Centrifugal Analyser Assay of Enzymes of Lysosomal Origin in Plasma, II¹⁾

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Summary: The manual fluorimetric procedure, considered as a reference method for the determination of N-acetyl- β -D-glucosaminidase, β -D-glucuronidase and β -D-galactosidase in human plasma, was automated as a routine method, using the IL Monarch centrifugal analyser. Using a liquid standard with a known enzyme content, the automated assay correlated fairly well with the reference manual method (r values very close to 1). Its analytical imprecision was much lower than that of the manual method. The automated assay of N-acetyl- β -D-glucosaminidase, β -D-glucuronidase and β -D-galactosidase gave coefficients of variation of 5.7–6.9, 3.6–5.0 and 3.8–4.2%, respectively, detection limits of 4, 2 and 1 mU/l plasma respectively, and linear responses of up to 73, 8.4 and 0.9 U/l of plasma respectively. Furthermore, the method required only small volumes of undiluted plasma (4–10 μ l). This method appears to be reliable, sensitive, simple enough for routine analyses and as cost effective as the most common routine serum enzyme assays.

Introduction

There is evidence that the determination, in body fluids, of enzymes of lysosomal origin has potential value in the diagnosis of a number of diseases (1–7). For instance, the plasma levels of lysosomal enzymes have been shown to correlate with the metabolic control of diabetes (8–12), and with the functional conditions of kidney and liver (13–14), the latter organ being implicated in the clearance of the same enzymes (15). Interestingly, the plasma determination of some lysosomal enzymes has been suggested for monitoring the functional recovery of the liver after transplantation (16).

A realistic assessment of the diagnostic potential of lysosomal enzymes requires the availability of simple, re-

liable, low cost assay procedures, suitable for routine analyses. At present, sensitive optimized and reproducible fluorimetric methods are available for the determination of lysosomal enzymes in plasma (17), including the isozymes of N-acetyl- β -D-glucosaminidase (18). Also, stable reference materials can be prepared for instrument calibration and for running of intra- and inter-laboratory quality control programmes (19). The only obstacle to the routine application of these methods is the fact that they are manual.

On these premises and considering the wide availability in clinical laboratories of the IL Monarch centrifugal analyser equipped with a fluorimetric detector, we decided to adapt the manual fluorimetric procedure for lysosomal enzyme assays to this automated instrument, using the reference calibration material prepared in our

¹⁾ Part I l.c. (19)

Tab. 1 Operational conditions for the automated assay of three enzymes of lysosomal origin using the IL Monarch

	N-Acetyl- β -D-glucosaminidase	β -D-Glucuronidase	β -D-Galactosidase
Response algorithm	Final Point	Final Point	Final Point
Results algorithm	linear	linear	linear
Loading type	Load-(crit) incubate-reload-analyse (for all enzymes)		
Reagent blank	On	On	On
Sample volume, μ l	4	7	10
Sample diluent, μ l	0	0	0
2nd reagent, μ l (buffer-substrate sol.)	55	55	55
– buffer (mmol/l)*	54	57	59
– pH	4.2	3.6	3.3
– substrate (mmol/l)	6.5	4.6	2.4
3rd–4th reagent, μ l (glycine solution)	135–40	135–40	135–40
	0.2 mol/l glycine-NaOH, 0.125 mol/l NaCl, pH 10.75 (for all enzymes)		
Temperature, $^{\circ}$ C	37	37	37
Incubation time, min	5	5	10
Delay time, s	20	20	20
Interval time, s	10	10	10
Filter 1 and 2, nm	450	450	450
Monochromator 1 and 2, nm	365	365	365

* citric acid-sodium citrate for N-acetyl- β -D-glucosaminidase and acetic acid-sodium acetate for β -D-glucuronidase and β -D-galactosidase

laboratory (19). The three enzymes²⁾ of lysosomal origin with the highest diagnostic potential, N-acetyl- β -D-glucosaminidase, β -D-glucuronidase and β -D-galactosidase, were determined in human plasma. The results showed that all three enzymes can be routinely determined on the IL Monarch centrifugal analyser, simply and reliably, and at relatively low cost.

Materials and Methods

Chemicals and other products

Commercial chemicals were of the highest purity available. The water routinely used was freshly redistilled in a glass apparatus. 4-Methylumbelliferone, purchased from Fluka GmbH (Buchs, Switzerland), was recrystallized three times from ethanol; 4-methylumbelliferyl-glycosides, used as substrates for the individual glycohydrolases, were purchased from Melford (Suffolk, England); ethylene glycol, haemoglobin and bilirubin from Sigma Chem. Co (St. Louis, Mo, USA); intralipid from Cutter Labs. Inc. (Berkeley, CA 94710).

Blood sampling and preparation of plasma specimens

Plasma was prepared from blood treated with sodium citrate (final concentration: 11 mmol/l), which is known not to affect lysosomal enzyme activities (17). Ethylene glycol was added (300 g/l) to the

plasma samples immediately after collection (19). The samples were then stored at -20°C until assayed.

Blood was withdrawn from two groups of individuals. The first group provided plasma to be used for studying the analytical characteristics of the method (total imprecision, sensitivity) and for evaluating possible interferences. These individuals, who were informed of the purpose of the investigation, were healthy adults, both males and females, aged 25–55 years, mostly volunteer blood donors. Some of them were women in the final trimester of pregnancy. In all cases blood was withdrawn immediately before the control laboratory analyses, and the common blood analytes were found to be within normal ranges. Care was taken to check the absence of antibodies indicative of particular infectious diseases, like AIDS and hepatitis, in all the specimens collected. The plasma specimens were divided into three pools:

pool A, carrying high lysosomal enzyme activities, obtained by pooling plasma from the pregnant women, who are known to have elevated plasma levels of these enzymes (20);

pool B, carrying medium levels of lysosomal enzyme activities, obtained by pooling plasma from normal individuals;

pool C, carrying low levels of lysosomal enzyme activities, obtained by dilution 1+1 pool B with a sample of the same pool B in which lysosomal enzymes had been inactivated by heating at 56°C for 5 hours (O pool) (19).

Owing to the extremely low, if any, enzyme activities in the "O pool", the same pool was also utilized to study the sensitivity of the method.

A different plasma pool containing intermediate activity was prepared for use as a "standard" for the automated instrument. The enzyme levels of this standard were established by the manual method.

The second group of individuals provided the plasma specimens used to compare the routine automated method with the manual method. This group comprised 60 randomly chosen individuals who entered the Laboratory of Clinical Chemistry and Microbiology of the Bassini Hospital for scheduled haemato-chemical tests.

²⁾ Enzymes:

N-acetyl- β -D-glucosaminidase:

N-acetyl- β -D-glucosaminide N-acetylglucosaminohydrolase, EC 3.2.1.30

β -D-galactosidase:

β -D-galactoside galactohydrolase EC 3.2.1.23

β -D-glucuronidase:

β -D-glucuronoside glucuronosohydrolase EC 3.2.1.31

Linearity of the automated method

To assess the linearity of the automated method, we studied samples made from various proportions of serum pools with extreme levels of lysosomal enzyme activities. The expected (theoretical) values were calculated from the corresponding values of the original pools. Each determination was performed in triplicate.

Imprecision of the automated method

The analytical imprecision of the automated method was studied using plasma pools A, B and C. Two replicate analyses were performed for each enzyme in twenty different analytical series, covering a period of three months, according to the model of Krouwer (21) and NCCLS recommendations (22).

Fluorimetric assay of lysosomal enzymes on the "IL Monarch" centrifugal analyser

The following enzymes of lysosomal origin were assayed fluorimetrically: N-acetyl- β -D-glucosaminidase, β -D-glucuronidase, β -D-galactosidase, using the corresponding 4-methylumbelliferyl glycosides as substrates. The manual assay procedure for these enzymes, provisionally adopted as the reference method, was as described (17).

The automated procedure was developed by adapting the assay conditions of the manual method to the random access centrifugal analyser IL Monarch (Instrumentation Laboratory, Lexington, MA, USA). For fluorimetric analysis this instrument uses disposable reaction rotors made of UV-transmitting plastic material. The basic operating conditions were those suggested by the manufacturer. The plasma specimens were thawed immediately before use, brought to room temperature and employed as such without dilution. The nature, pH and concentration of the buffer, substrate molarity and the time of incubation at 37 °C were chosen to give the optimal values for each enzyme activity, and they were found to be the same as those already established for the manual method (17). Details of the conditions used to measure lysosomal enzyme activities on the IL Monarch centrifugal analyser are given in table 1. Regular incubation mixtures containing physiological saline in place of plasma were used as controls (blanks).

All enzyme activities were expressed as units (U, μ moles hydrolysed substrate, min^{-1}) per litre.

Interference in the determination of plasma lysosomal enzymes by the routine automated method

For this purpose the plasma was added with increasing amounts of haemolysate (prepared from human erythrocytes), lipids (as Intralipid) and bilirubin, following the indication of Glick et al. (23). The

Fig. 1 Linearity of the automated method for the determination of N-acetyl- β -D-glucosaminidase (a), β -D-glucuronidase (b) and

β -D-galactosidase (c) in human plasma. For details see the experimental section.

Tab. 2 Estimation of the imprecision of the automated method for the assay of lysosomal enzymes in plasma.

Enzyme	Plasma pool	Automated method				Manual method
		Enzyme activity (mU/l) Mean	Coefficient of variation (%)			Coefficient of variation (%) Total
			Within-run	Day to day	Total	
N-Acetyl- β -D-glucosaminidase	A	42100	3.9	4.2	5.7	5.2
	B	28000	3.7	4.7	5.9	5.5
	C	8200	3.6	5.9	6.9	9.5
β -D-Glucuronidase	A	4700	3.2	0.5	3.6	4.2
	B	2400	3.0	2.0	4.1	4.4
	C	1100	4.5	2.2	5.0	11.9
β -D-Galactosidase	A	700	3.5	0.2	3.8	9.3
	B	300	3.5	1.1	4.1	14.7
	C	140	3.6	1.2	4.2	17.5

haemoglobin content in haemolysate was quantified by measuring the cyanomethaemoglobin derivative (24).

Statistical analysis

Parametric techniques of analysis were employed. Regression analysis and correlation coefficients were calculated following the *Snedecor & Cochran* indications (25) for linearity study and with the standard model of *Denning* (26) for comparison of methods study. The estimation of total imprecision by analysis of variance was performed following the model of *Krouwer* (21), and NCCLS recommendations (22).

Results

Application of the manual method to a routine centrifugal autoanalyser, namely the IL Monarch, presented no particular difficulties, provided the manufacturer's instructions were rigorously followed. The only discrepant finding was the instrument's response to the liberated fluorophore, 4-methylumbelliferone, which was about three-fold lower with the autoanalyser than with the spectrofluorimeter employed in the manual method. This difference, which remained unchanged after careful calibration of the autoanalyser with 4-methylumbelliferone solutions of known concentration, was found to be related to the plastic cuvettes used in the centrifugal analyser. However, when the stable liquid material was used as an analytical internal standard (see later), the method sensitivity remained practically unaffected.

The detection limits, calculated as the minimal enzyme activity detectable above the mean plus three standard deviations measured daily for 15 days in triplicate, were 4.0, 2.0, 1.0 mU/l for N-acetyl- β -D-glucosaminidase, β -D-glucuronidase and β -D-galactosidase, respectively.

As shown in figure 1, linearity was assured within a relatively wide range of enzyme concentrations (up to 73, 8.4 and 0.9 U/l of plasma for N-acetyl- β -D-glucosaminidase, β -D-glucuronidase and β -D-galactosidase, respectively). The correlation coefficients were very high ($r = 0.999$) for all enzymes and no significant differences in the internal consistency of the linear regression were detectable.

With the plasma pools of low, medium and high enzyme levels, the automated method showed low and acceptable coefficients of variation: from 5.7 to 6.9 for N-acetyl- β -D-glucosaminidase, from 3.6 to 5.0 for β -D-glucuronidase and from 3.8 to 4.2 for β -D-galactosidase, the lower values applying to the plasma pool with the highest enzyme activity (tab. 2). Moreover, and as expected, the degree of imprecision decreased with increasing the enzyme concentration in the sample. It is noteworthy that the degree of imprecision was much lower with the automated method than with the manual one (19).

The data in figure 2 on the effects of potential interferents show that haemoglobin did not interfere in the determination of these lysosomal enzymes, bilirubin exhibited a very modest (-8%) inhibition effect on the enzyme determination, while lipids (as intralipid) caused an increase of β -D-galactosidase (40% at 10 g/l), without affecting the determination of the other enzymes.

As shown in figure 3 the correlation between the automated and the manual method, for plasma from 60 individuals, was good for all the considered enzymes. The response of the automated method appeared to be markedly lower than that of the manual one, especially for β -D-galactosidase, and the r values, although good, were further from unity for N-acetyl- β -D-glucosaminidase and β -D-glucuronidase. However, the use of the stable liquid material as analytical standard material made the results of the automated method practically identical to those of the manual one for all enzymes, and increased

Fig. 2. Interference by haemoglobin, bilirubin and intralipid in the determination of plasma N-acetyl- β -D-glucosaminidase ●, β -D-glucuronidase ■ and β -D-galactosidase × by the automated procedure.

Fig. 3 Comparison of the automated with the manual method using or not using the enzyme reference material as standard. Number of subjects: 60.
 x without the use of the enzyme reference material.
 ■ with the use of the enzyme reference material.

a) N-acetyl- β -D-glucosaminidase
 x: $r = 0.91$, $y = -3.8 + 0.86x$ ■: $r = 0.97$, $y = -0.28 + 0.98x$
 b) β -D-glucuronidase
 x: $r = 0.88$, $y = 0.06 + 0.5x$ ■: $r = 0.99$, $y = 0.087 + 0.94x$
 c) β -D-galactosidase
 x: $r = 0.96$, $y = 0.02 + 0.43x$ ■: $r = 0.96$, $y = 0.02 + 0.98x$

the r values to almost unity (0.97, 0.99 and 0.96 for N-acetyl- β -D-glucosaminidase, β -D-glucuronidase and β -D-galactosidase, respectively). Thus, the use of this material standard is recommended, together with the 4-methylumbelliferone solution of known concentration for routine instrument calibration.

Discussion

The introduction into the clinical laboratory market of routine autoanalysers suitable for fluorimetric assays, like the IL Monarch centrifugal analyser, prompted us to use this instrument for the optimized fluorimetric assay of lysosomal enzymes, which are already available as manual methods.

The results showed that the automated procedure presented here, using a stable liquid material as internal standard, is as sensitive as the manual reference method, but more precise than the latter, with coefficients of variation in the range of 3.6–6.9, which are very low for

enzyme assays. The automated method requires small amounts of undiluted plasma (4–10 μ l), covers a sufficiently wide range of enzyme concentration and is as simple and as cost effective as most routine serum enzyme assays. The availability of this method and of liquid stable materials for use as reference standards and for instrument calibration will enable large scale screening programmes on selected pathologies, as well as facilitating intra- and inter-laboratory quality control programmes. On this basis, the determination of selected enzymes of lysosomal origin in plasma might gain sufficient analytical reliability and diagnostic utility to be included in current clinical enzymology.

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