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Preparation of a Stable Liquid Material for Calibration and Quality Control for Lysosomal Enzymes in Plasma

Assay of Enzymes of Lysosomal Origin in Plasma, I.[1])

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Summary: Several lysosomal enzymes present in human plasma (N-acetyl-β-glucosaminidase, β-glucuronidase, β-galactosidase, α-galactosidase, α-*L*-fucosidase, α-mannosidase, β-glucosidase) were maintained in a fully active state for at least 8 months by the addition of ethylene glycol (300 g/l final concentration) to freshly prepared plasma and storage at −20 °C.

Pools of human plasma from healthy humans, stabilized and stored as above, and containing a low, medium or high content of the above enzymes, were used to establish the analytical imprecision (within-run, day-to-day and total imprecision) of the fluorimetric assay. Ten replicates in ten different analytical series, covering a period of two months, were performed. The total imprecision (expressed as coefficient of variation) was in general lower than 10%; in a few cases, particularly plasma samples with a low enzyme content, the total imprecision was 18%. The isozymes A, B, I_1, and I_2 of N-acetyl-β-glucosaminidase displayed the same stability upon storage as the unfractionated enzyme.

It is concluded that pools of human plasma containing known amounts of lysosomal enzymes, stabilized by the addition of 300 g/l ethylene glycol and stored at −20 °C, are suitable liquid materials for calibration and quality control for the assay of the same enzymes.

Introduction

There is increasing evidence that lysosomal enzymes[2]) in blood serum or plasma have a diagnostic significance not only for some of the rare syndromes derived from inborn lysosomal pathology (1), but also for a number of acquired diseases (2, 3), including diabetes (4, 5). However, due mainly to methodological problems, lysosomal enzymes are not currently measured

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[2]) Enzymes:
N-Acetyl-β-glucosaminidase:
N-acetyl-β-*D*-glucosaminide N-acetylglucosaminohydrolase EC 3.2.1.30
β-Galactosidase:
β-*D*-galactoside galactohydrolase EC 3.2.1.23
β-Glucuronidase:
β-*D*-glucuronoside glucuronosohydrolase EC 3.2.1.31
α-Mannosidase:
α-*D*-mannoside mannohydrolase EC 3.2.1.24
α-*L*-Fucosidase:
α-*L*-fucoside fucohydrolase EC 3.2.1.51
β-Glucosidase:
β-*D*-glucoside glucohydrolase EC 3.2.1.21
α-Glucosidase:
α-*D*-glucoside glucohydrolase EC 3.2.1.20
α-Galactosidase:
α-*D*-galactoside galactohydrolase EC 3.2.1.22

in the clinical investigation of blood enzymes. Colorimetric and fluorimetric procedures for the assay of lysosomal enzymes in serum or plasma are actually available. They are reliable, and in the fluorimetric assays are also highly sensitive (6, 7). However, all these methods are manual and not always easily adopted for routine analyses. Moreover, stable materials for calibration, and for running of intra-laboratory and inter-laboratory quality control programmes, are lacking.

The present work was undertaken with the aim of solving some of these difficulties.

The work was carried out on N-acetyl-β-glucosaminidase, β-glucuronidase, β-galactosidase, α-*L*-fucosidase, α-mannosidase, α-galactosidase, α-glucosidase and β-glucosidase, which are the lysosomal enzymes (glycohydrolases) with the highest diagnostic potential in acquired diseases. The isozymes A, B, I_1 and I_2 of N-acetyl-β-glucosaminidase, known to be of diagnostic interest (8), were also considered.

Materials and Methods

Chemicals

Commercial chemicals were of the highest purity available and water was freshly distilled in an all glass still before use. 4-Methylumbelliferone, purchased from Fluka GmbH (Buchs, Switzerland) was three times recrystallized from ethanol; 4-methylumbelliferyl-glycosides, to be used as substrates for the individual glycohydrolase, were purchased from Melford (Suffolk, U. K.), ethylene glycol, polyglycols and glycerol from Sigma Chem. Co. (St. Louis, Mo, USA), polybuffer exchanger PBE-94 and "polybuffer 74"-HCl, pH 4.0 from Pharmacia Fine Chemicals (Uppsala, Sweden).

Blood sampling and preparation of plasma pools

Plasma was prepared, under standard conditions, from blood treated with sodium citrate (final concentration, 11 mmol/l, which is known not to affect lysosomal enzymes activities (7)). Subjects were male and female healthy adults, aged 25–55 years; most were volunteer blood donors, and all gave informed consent. Some were women in the final trimester of pregnancy which is characterized by a very high content of lysosomal enzymes in the plasma (9). In all cases blood was withdrawn for control laboratory analyses, and the common blood analytes were within normal ranges.

Tab. 1. Assay of lysosomal enzymes in plasma stabilized by the addition of ethylene glycol (300 g/l, final concentration) and stored at −20 °C. Analytical imprecision was estimated by analysis of variance. Ten replicates were performed of each determination.
(A), (B), (C) = stabilized plasma pools containing high, intermediate and low levels of enzyme activity, respectively.

Enzyme		Enzyme activity in plasma (mU/l)	Coefficient of variation (%)		
		Mean	Within-run	Day-to-day	Total
N-Acetyl-β-glucosaminidase	(A)	41830	1.63	4.85	5.22
	(B)	17621	1.72	5.25	5.53
	(C)	8122	2.76	7.65	9.53
β-Glucuronidase	(A)	4869	2.45	2.71	4.21
	(B)	1923	3.03	3.23	4.44
	(C)	986	4.23	8.42	11.89
β-Galactosidase	(A)	631	3.21	8.45	9.27
	(B)	285	5.18	13.87	14.74
	(C)	112	9.76	14.67	17.53
α-*L*-Fucosidase	(A)	11223	1.05	2.86	3.42
	(B)	5142	1.22	3.73	3.92
	(C)	2471	2.56	6.25	8.41
α-Galactosidase	(A)	279	2.13	3.52	4.32
	(B)	141	2.50	4.68	5.31
	(C)	94	4.06	7.09	8.51
β-Glucosidase	(A)	9.35	6.10	6.19	8.87
	(B)	4.71	8.56	12.65	14.91
	(C)	2.67	9.95	15.31	17.05
α-Mannosidase	(A)	1064	2.18	3.15	4.09
	(B)	429	2.48	4.53	5.17
	(C)	234	4.63	7.85	8.27
α-Glucosidase	(A)	153.5	2.41	6.81	8.06
	(B)	71.5	3.69	12.58	13.11
	(C)	34.2	6.54	15.17	18.13

Preparation of a stable liquid material for lysosomal enzyme assays

Plasma specimens were divided into three pools:

pool A, containing high lysosomal enzyme activities, obtained by pooling sera from pregnant women in the third trimester;

pool B, containing medium lysosomal enzyme activities, obtained by pooling plasma from normal individuals;

pool C, containing low levels of lysosomal enzyme activities, obtained by diluting pool B 1 : 2 (1 + 1, by vol.) with the same pool, which had been heat-treated (56 °C, 3 hours) to completely inactivate lysosomal enzymes.

Plasma pools A, B, C, were supplemented with several compounds (glycerol, ethylene glycol and a variety of polyglycols) known to stabilize enzymes or other analytes, carefully mixed by gentle stirring at 4 °C for 30 min, then divided into 1 ml glass ampules, tightly closed and stored at −20 °C.

Determination of lysosomal enzymes

The lysosomal enzymes were determined fluorimetrically by the manual optimized method previously described (7). Using plasma pool A, the isozymes of N-acetyl-β-glucosaminidase were separated and quantified by an automated method consisting of chromatofocusing on a polybuffer exchanger PBE-94 column, with flowthrough fluorimetric determination of activity and computer assisted quantification (10).

All enzyme activities were expressed as Units (U) per litre (l).

Statistical analysis

Regression analysis and *Student*'s t test were calculated as described by *Snedecor & Cochran* (11). The estimation of total imprecision by analysis of variance was performed according to the model of *Krouwer*, and NCCLS recommendations (12, 13).

Results and Discussion

All lysosomal enzymes in serum or plasma are known to gradually loose their activity upon storage, even at −20 °C (7). In pilot experiments, several substances (glycerol, polyglycols, ethylene glycol), which behave as enzyme stabilizers, were added to plasma and the content of the lysosomal enzymes was monitored after storage at different temperatures and various periods of time. The most encouraging results were obtained by the addition of ethylene glycol (final concentration of 300 g/l) and storage of plasma at −20 °C. Ethylene glycol proved not to affect per se the assay method of any of the lysosomal enzymes studied. The activity values of the measured lysosomal enzymes in a pool of high enzyme content (pool A) after different periods (from 0 to 24 months) of storage at −20 °C are represented in figure 1. The same figure also shows

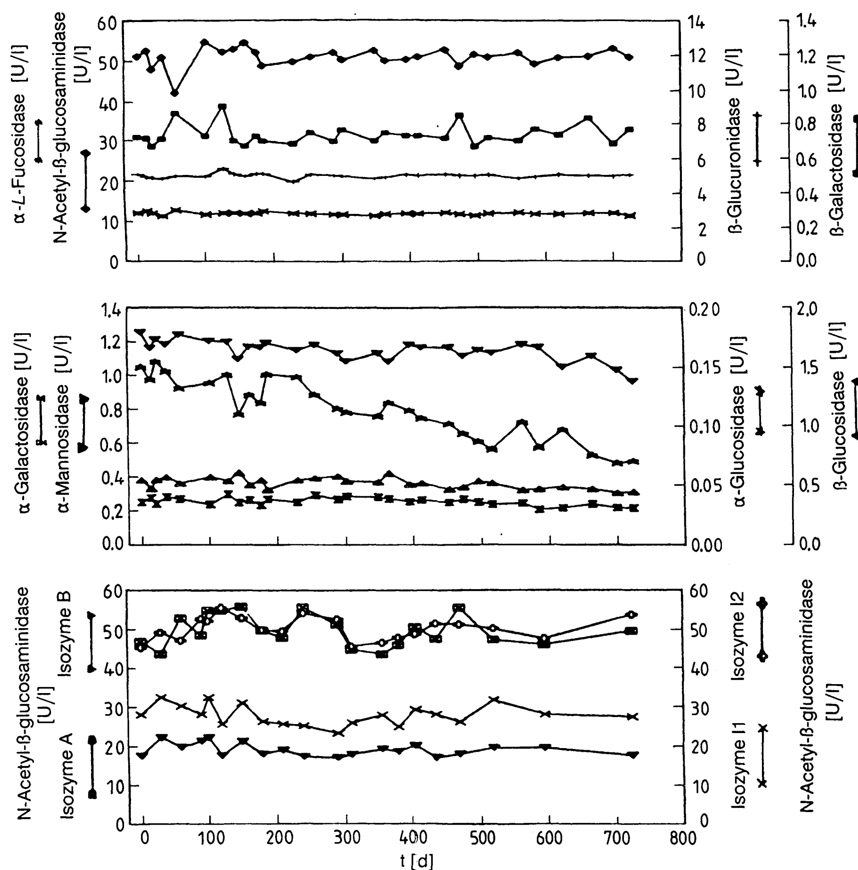

Fig. 1. Stability of lysosomal enzymes in human plasma stored at −20 °C in the presence of 300 g/l (final concentration) ethylene glycol. The different enzymes were determined in plasma pool A, which contained high enzyme levels. Each value is the mean of 5 determinations.

the behaviour upon storage of isozymes A, B, I_1 and I_2 of N-acetyl-β-glucosaminidase in the same plasma pool. Linear regression analysis of these data showed that no statistically significant change of activity occurred:

(a) up to 2 years for N-acetyl-β-glucosaminidase and its isozymes, β-glucuronidase, β-galactosidase and α-*L*-fucosidase;

(b) up to 15 months for α-galactosidase;

(c) up to 12 months for β-glucosidase;

(d) up to 8 months for α-mannosidase;

(e) up to 3 months for α-glucosidase.

The same degree of stability of the analysed enzymes upon storage at −20 °C was observed for plasma pools B and C (tab. 1). The reason for the lower stability of α-glucosidase during storage (matrix problems; necessity of special stabilizers, etc) is currently under investigation.

The analytical imprecision of the manual assays for plasma lysosomal enzymes (the isozymes of N-acetyl-β-glucosaminidase were not included in this part of the study) was determined using the three plasma pools A, B, C over a period of two months, and expressed as the coefficient of variation. Ten replicate analyses were performed for each enzyme in ten different analytical series, covering a period of two months, according to the model of *Krouwer*, and NCCLS recommendations (12, 13). The analytical imprecision for all the studied enzymes was higher in day-to-day than within-run determinations, and it decreased with increased enzyme catalytic concentration. In general, the analytical imprecision of the assay methods was acceptable (with a total coefficient of variation below 10%) for all determinations using plasma pool A, which contained the highest concentrations of lysosomal enzymes. An acceptable degree of imprecision was found with plasma pools B and C (intermediate and low enzyme levels) for the assay of N-acetyl-β-glucosaminidase, α-*L*-fucosidase, α-galactosidase and α-mannosidase. Coefficients of variation above 10% (and up to 18%) were shown by the assay of β-glucuronidase (plasma pool C), β-galactosidase, β-glucosidase and α-glucosidase (plasma pool B).

In conclusion, human plasma, supplemented with 300 g/l ethylene glycol and stored at −20 °C, fulfils the requirements for a stable (for at least 8 months) liquid material suitable for calibration and quality control purposes in the plasma assay of several glycohydrolases of lysosomal origin.

References

1. Glew, R. H., Basu, A., Prence, E. M. & Remale, A. T. (1985) Biology disease — Lysosomal storage diseases. Lab. Invest. *53*, 250–269.
2. Calvo, P., Barba, J. L. & Cabezas, J. A. (1982) Serum β-glucuronidase, α-glucosidase, β-glucosidase, α-fucosidase, α-galactosidase levels in acute viral hepatitis, pancreatitis, myocardial infarction and breast cancer. Clin. Chim. Acta *11*, 15–19.
3. Reglero, A., Carrettero, M. I. & Cabezas, J. A. (1980) Increased serum α-L-fucosidase and β-D-N-acetylglucosaminidase activities in diabetic, cirrhotic and gastric cancer patients. Clin. Chim. Acta *103*, 155–158.
4. Kohler, E., Sheeth, K. J. & Good, T. A. (1981) Plasma acidic glycohydrolases in insulin-dependent diabetes mellitus. Acta Diabetol. Lat. *18*, 243–250.
5. Goi, G., Fabi, A., Lorenzi, R., Lombardo, A., Tettamanti, G., Burlina, A. B., Pinelli, L. & Gaburro, D. (1986) Serum enzymes of lysosomal origin as indicators of the metabolic control in diabetes: comparison with glycated hemoglobin and albumin. Acta Diabetol. Lat. *23*, 117–125.
6. Tettamanti, G., Lombardo, A., Marchesini, S., Giuliani, A., Berra, B., Di Donato, S., Rimoldi, M. & Bertagnolio, B. (1977) Lipid storage diseases: Biochemical diagnosis. Biochem. Exp. Biology *13*, 45–60.
7. Lombardo, A., Caimi, L., Marchesini, S., Goi, G. & Tettamanti, G. (1980) Enzymes of lysosomal origin in human plasma and serum; assay conditions and parameters influencing the assay. Clin. Chim. Acta *108*, 337–346.
8. Mahuran, D., Novak, A. & Lowden, J. A. (1985) The lysosomal hexosaminidase isozymes. In: *Isozymes: Current topics in biological and medical research*, Vol. 12, New York: Alan R. Liss, Inc., pp. 229–288.
9. Lombardo, A., Goi, G., Pistolesi, E., Rocca, E., Agosti, S., Fabi, A., Giuliani, A., Burlina, A. B. & Tettamanti, G. (1984) Behaviour of several enzymes of lysosomal origin in human plasma during pregnancy. Clin. Chim. Acta *143*, 253–264.
10. Goi, G., Fabi, A., Lombardo, A., Bairati, C., Burlina, A. B., Agosti, S., Serio, C., Tettamanti, G. (1989) The lysosomal β-D-N-acetylglucosaminidase isozymes in human plasma during pregnancy: separation and quantification by a simple automated procedure. Clin. Chim. Acta *179*, 327–340.
11. Snedecor, G. W. & Cochran, W. G. (1967) *Statistical Methods*. Ames: The Yowa State University Press.
12. Krouwer, J. S. (1981) Observations on comparisons of within-run and day-to-day precision. Clin. Chem. *27*, 202.
13. NCCLS Document EP 10-T (1989) Preliminary evaluation of Clinical Chemistry methods; vol. 9.

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