A METHOD FOR α-L-IDURONIDASE ASSAY

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1. Introduction

It is now established that α-L-iduronidase is the lysosomal hydrolase whose deficiency is responsible for the pathogenesis of Hurler and Scheie diseases [1–3].

The availability of the synthetic substrate, phenly-α-L-iduronide [4], has made possible to assay the levels of this enzyme in homogenates of various organs [5], cultured skin fibroblasts and amniotic fluids cells [6].

The methods used for the measurement of α-L-iduronidase detect the released phenol with the Folin–Ciocalteau–carbonate reagent. The interference of this protein has been reduced in various ways: by precipitating first some of the protein with the Folin–Ciocalteau reagent and adding the alkali to an aliquot of the clear supernatant [6]; or by extracting the released phenol in toluene, re-extracting it in an alkaline aqueous solution and performing the color reaction on the latter [5].

The necessity of working with low substrate concentrations and minimal volumes, the inefficient precipitation of protein with the Folin–Ciocalteau reagent, the need for microglassware, repeated extractions and occasional clarification of cloudy extracts, are factors which limit the usefulness of these methods when low levels of activity have to be measured in biological fluids or tissue extracts having a high protein content.

The method proposed here is based on the reaction of *N*,2,6-trichloro-p-benzoquinoneimine (TCBQ) with phenol, to form phenolindophenol and, upon alkalinization, a blue chromogen absorbing at 610 nm [7]. In absence of phenol, copolymerization of the reagent in alkaline medium produces a brown color whose contribution is eliminated with the use of appropriate reagents blanks.

Although minimal amounts of substrate are used, the method is not a micro-method and does not require special glassware. Moreover, it is rapid, accurate and sufficiently sensitive even in presence of large amounts of protein, such as in the assay of α-L-iduronidase of serum or fractions thereof.

2. Materials and methods

N,2,6-trichloro-*p*-benzoquinoneimine was purchased from Eastman Kodak Co., Rochester, N.Y., 14650. Four mg were dissolved in 1 ml of 95% ethanol, acidified by addition of 0.005 ml of glacial acetic acid.

2.1. *Preparation of fibroblasts homogenates and serum fractions*

Human skin fibroblasts were grown to confluent monolayers as previously described [8]. After removal of the medium and rinsing of the cell layer with cold (5°C0 0.9% NaCl twice, the cells were harvested by scraping, and homogenized twice in an all-glass tissue grinder, using 3 ml of cold 0.9% NaCl each time. The homogenates were cleared by centrifugation at 20 000 *g* for 30 min at 0°C and immediately assayed for enzyme activity and protein content [9]. The latter ranged between 85 and 400 μg protein/ml.

Human sera were stored frozen until assayed for enzyme activity. When fresh or blood bank plasma was obtained, it was clotted by adding to 100 ml 20 ml of 1% $CaCl_2$, leaving it at 37°C for 1 hr and

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removing the clot by centrifugation at 20 000 *g* for 30 min at 0°C.

An aliquot of the serum was immediately assayed for activity; the remnant was brought to 50% ammonium sulfate saturation and after 1 hr at 5°C the precipitate was collected by centrifugation at 20 000 *g* for 30 min. Both precipitate and supernatant were dialyzed against 0.9% NaCl at 5°C, to remove sulfate ions and then frozen prior to enzymic assay. Sera contained between 28 and 78 mg of protein/ml (depending on degree of serum dilution), 26% and 74% being present respectively in the ammonium sulfate precipitate and supernatant.

2.2. *Enzyme assay on fibroblasts homogenates*

The composition of the incubation mixture is as follows: 0.2 ml of pH 3.5 sodium formate buffer 2.5 M, containing 0.9% NaCl and 0.1% NaN_3; 0.1 μmole

Table 1
α-L-iduronidase activities in sera, serum fractions and extracts of cultured skin fibroblasts as measured with TCBQ reaction

Subject		Sample	Units[a] of α-L-iduronidase activity	
Normal	(J.R.)	Fibroblasts	18.03	
	(N.B.)		31.37	(23.9)[b]
	(S.M.)		22.33	
Hurler	(C.R.)		1.03	
	(D.C.)		0	(0.343)[b]
Scheie	(B.R.)		0	
Hunter	(W.P.)		22.97	
	(T.K.)		15.50	(18.5)[b]
Sanfilippo A	(B.J.)		18.23	
Sanfilippo B	(M. K.)		17.20	
Normal	(S.R.)	Serum	0.00410	
	(T.L.)		0.00900[c]	
	(T.L.)		0.01050[c]	
	(S.J.)		0.01381	
	(T.D.)		0.02429	
	(F.G.)		0.01291	
	(D.K.)		0.02176	(0.0167)[b]
	(F.H.)		0.01733	
	(N.Y.)		0.02057	
	Pooled sample from blood bank		0.03291	
	Pooled sample from blood bank		0.01600[d]	
	50% $(NH_4)_2SO_4$ precipitate		0.01740[d]	
	supernatant		0.01410[d]	
Hurler	(P.C.)	Serum	0	
	(S.R.)		0.00070	(0.0003)[b]
Scheie	(B.R.)		0.00030	
Hurler heteroxygote	(Mrs. S.)		0.0020	(0.0051)[b]
Scheie heterozygote	(Mrs. B.)		0.0082	

[a] 1 unit of activity = 1 nmole of phenol released at 23°C per mg protein per hr.
[b] Average.
[c] Same sample assayed on separate days.
[d] 420 ml of serum containing 12 g of protein and 191 units of α-L-iduronidase was fractionated and units of enzyme activity recovered in 50% $(NH_4)_2SO_4$ precipitate and supernatant were 57 and 138, respectively.

phenyl-α-L-iduronide in 0.1 ml of 0.9% NaCl; 0.5 ml of fibroblasts homogenate and enough 0.9% NaCl to 1.0 ml final volume. In the substrate control 0.9% NaCl replaces the cells homogenate, while in the enzyme control it replaces the substrate. A saline control, in which both cells homogenate and substrate are replaced by 0.9% NaCl is also prepared. After incubation for 3 hr at 23°C, to each tube is added 0.15 ml of TCBQ reagent and 0.75 ml of 3 M NH_4OH. After 10 min at 23°C, the absorbance is read at 610 nm against water. With this technique, substrate controls do not exhibit any color when compared to saline controls and may be omitted for reasons of economy.

The data given in table 1 are based on net absorbancies obtained by subtracting the values of the enzyme control from that of the corresponding complete incubation mixture. The corresponding amounts of phenol liberated are computed from line D of fig. 1.

2.3. *Enzyme assay on serum or its fractions*

Incubation mixtures for enzyme assay and for substrate, enzyme and saline controls are prepared as given in the preceding paragraph, replacing cells homogenate with 0.5 ml of serum or serum fractions.

After 21 hr of incubation at 23°C, the content of each tube is diluted to 2 ml with 0.9% NaCl and the tubes are placed in boiling water for 30 min to precipitate the protein. After centrifugation at 9750 *g* for 20 min at 23°C, the color reaction is performed on 1 ml of clear supernatant, as described above. The results shown in table 1 are based on net absorbancies calculated by subtracting from the absorbance of the complete incubation mixture those of the enzyme control, of the substrate control and of the saline control. Since during the heating step a slight but consistent hydrolysis of the substrate takes place, a substrate control is necessary. The amounts of phenol liberated are computed from line E of fig. 1.

3. Results and discussion

The indicated amounts of TCBQ and NH_4OH are critical, since their increase or decrease leads to a lesser color yield in presence of 0.1 μmole phenol. Because of the limited availability of the substrate, the concentration of 0.1 μmole previously suggested [6] was used in our experiments. With fibroblasts extracts, containing a maximum of 400 μg protein/ml, a 3 hr incubation period is sufficient to obtain reliable levels of α-L-iduronidase activity, while with serum or serum fractions, 0.5 ml sample and 21 hr incubation period are necessary. Fig. 1 shows the calibration lines obtained with various amounts of phenol. Line A was obtained with the Folin–Ciocalteau reagent, as described [6], the absorbancies being read at 660 nm. Addition of

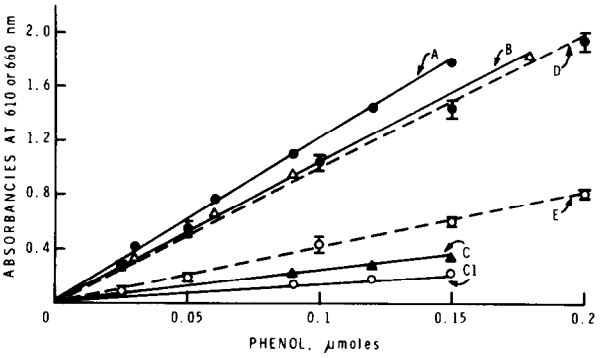

Fig. 1. Absorbancies of increasing amounts of phenol detected with different colorimetric methods. Line A was obtained with the Folin-Ciocalteau-carbonate method, according to Hall and Neufeld [6] in absence of protein; line B with the same method but in presence of 0.04 ml of normal human serum; line C with the following modification of the method, in order to accomodate 0.5 ml of serum; 0.15 ml of pH 3.5, 0.4 M formate buffer, 0.5 ml serum, 0.1 ml of 0.9% NaCl containing phenol and 0.83 ml of undiluted (2 N) Folin reagent. Protein precipitate was removed by centrifugation; to 1.0 ml of supernatant 1.6 ml of 24% Na_2CO_3 was added. After 20 min at 37°C, solutions were slightly turbid and absorbancies could not be measured with reliability. Line Cl was obtained with more dilute reagents, in order to avoid salt precipitation: 0.15 ml of pH 3.5, 0.4 M formate buffer, 0.5 ml serum, 0.1 ml of 0.9% NaCl containing phenol and 2.5 ml of 0.66 N Folin reagents. After removal of protein precipitate, 2.5 ml of clear supernatant and 3.8 ml of 12% Na_2CO_3 were used to develop the color. Absorbancies for the above lines were read at 660 nm.

Line D was obtained with the TCBQ method, in absence of protein or in presence of 200 μg protein from fibroblasts homogenates, while line E was obtained with the same method in presence of 0.5 ml of serum. After precipitation of protein by heat denaturation, 1 ml of supernatant was used for color development. Absorbancies were read at 610 nm. Vertical bars on lines D and E represent standard deviations of recorded absorbancies. See text for additional details.

0.04 ml of normal human serum depressed slightly the color yield (line B). When the method was modified in order to accomodate 0.5 ml of serum, the volumes and concentrations of the various reagents had to be changed in order to obtain satisfactory precipitation of protein and sufficient volume of clear supernatant, while keeping the various salts in solution. Under those conditions, lines C and Cl were obtained.

Line D represents the absorbancies, read at 610 nm, of increasing amounts of phenol in absence of protein or in presence of 200 μg of fibroblasts protein, when the color was developed with TCBQ and NH_4OH; line E was obtained with the same reaction, using 0.5 ml of serum or serum fractions and 1 ml of clear supernatant, representing 50% of the volume prior to protein precipitation by boiling.

Levels of α-L-iduronidase activity found in normal serum and its fractions, in serum of various patients with mucopolysaccharidoses and of individuals heterozygous for the Hurler and Scheie gene, or in their fibroblasts homogenates are given in table 1.

Normal human serum shows an average of 16.7×10^{-3} units of enzyme activity and the activity may be quantitatively recovered between precipitate and supernatant obtained at 50% ammonium sulfate precipitation.

Levels of activity in normal serum show considerable individual variability, but a good agreement among assays performed at different times on the same sample. Nevertheless, they reveal a significant difference with values obtained with sera of Hurler or Scheie patients (0.3×10^{-3} units) or of heterozygotes for those traits (5.1×10^{-3} units). Thus, the proposed technique represents a fast method for detecting α-L-iduronidase deficiencies or heterozyous states, prior to culture of skin fibroblasts or harvesting of urinary enzymes.

The enzymic activities detectable in homogenates of cultured fibroblasts are also indicated in table 1. While fibroblasts of normal individuals and of patients affected by mucopolysaccharidoses other than Hurler and Scheie have values averaging 21 units, those of Hurler and Scheie patients are either undetectable or extremely low.

These values agree well with those obtained previously with the Folin-Ciocalteau reagent [6] and in this particular application the two methods are similar in sensitivity, although they differ in the length of incubation and in the need for protein precipitation. A distinct advantage of the method with the TCBQ reagent is the possibility of performing it in presence of large amounts of protein, making it suitable for serum assays. The latter techniques represent increasingly popular screening procedures for enzymic defects [10–12] because of their relative ease, rapidity and economy.

The value of the proposed procedure in the analyses of amniotic cells homogenates will be evaluated as the cells will become available to us.

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