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Hemoglobin precipitation greatly improves 4-methylumbelliferone-based diagnostic assays for lysosomal storage diseases in dried blood spots

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ABSTRACT

Derivatives of 4-methylumbelliferone (4MU) are favorite substrates for the measurement of lysosomal enzyme activities in a wide variety of cell and tissue specimens. Hydrolysis of these artificial substrates at acidic pH leads to the formation of 4-methylumbelliferone, which is highly fluorescent at a pH above 10. When used for the assay of enzyme activities in dried blood spots the light emission signal can be very low due to the small sample size so that the patient and control ranges are not widely separated. We have investigated the hypothesis that quenching of the fluorescence by hemoglobin leads to appreciable loss of signal and we show that the precipitation of hemoglobin with trichloroacetic acid prior to the measurement of 4-methylumbelliferone increases the height of the output signal up to eight fold. The modified method provides a clear separation of patients' and controls' ranges for ten different lysosomal enzyme assays in dried blood spots, and approaches the conventional leukocyte assays in outcome quality.

1. Introduction

For many years sugar derivatives of 4-methylumbelliferone (4MU) have been favorite substrates for the measurement of lysosomal enzyme activities in a wide variety of cell and tissue specimens. Hydrolysis of these artificial substrates at acidic pH leads to the formation of 4-methylumbelliferone, which is strongly fluorescent at a pH above 10 [1–3]. The optimal excitation wavelength is 365 nm, and the emission is optimal at 448 nm. Thanks to the sensitivity of optical systems and electronic sensors for the light emission signal, 4MU-based substrates are ideally suited for the measurement of lysosomal enzyme activities in samples of small size even down to the level of single cells [4]. This virtue has been successfully employed for the measurement of lysosomal enzyme activities in dried blood spots on Guthrie cards [5–12]. The easiness of sample transport is a clear advantage of the blood spot analysis over other routine diagnostic procedures. In addition, blood spot technology enables high throughput analysis and newborn screening for lysosomal storage diseases [13–19].

Several reports have demonstrated the feasibility of lysosomal enzyme assays in blood spots using 4MU-based substrates for both diagnostic purposes as well as for newborn screening. However, not all assays are fully satisfactory in that the activities of many lysosomal enzymes in blood spots are extremely low so that long incubation times are required and subtle differences between low *versus* no enzyme activity are lost. In addition, it is not uncommon that the patients' samples generate lower signals than the substrate blank, which usually is a punch from an empty circle on the Guthrie card [20–23]. In particular cases, the diagnostic outcome can be challenged by poor separation of the patients' and controls' ranges [5,9,20–23].

We have investigated the hypothesis that quenching of the fluorescence by hemoglobin leads to appreciable loss of signal, and we have demonstrated that the sensitivity of the procedure is strongly enhanced by precipitation of hemoglobin prior to measuring the 4-methylumbelliferone.

2. Materials and methods

2.1. Blood spot collection

Blood spots were prepared from heparin-blood samples that were sent to our laboratory for diagnostic testing. For the purpose of this study we only used blood spots from patients who were positively diagnosed on the basis of enzyme deficiency in leukocytes or cultured fibroblasts. The patient population included infants, adolescents and adults.

The blood was applied with a pipet on Whatman 903 filter paper (Guthrie cards as used for newborn screening) from the middle of the circles till the circles were completely filled (approximately 60 μl). The spots were dried for at least 17 h (overnight) and the cards were

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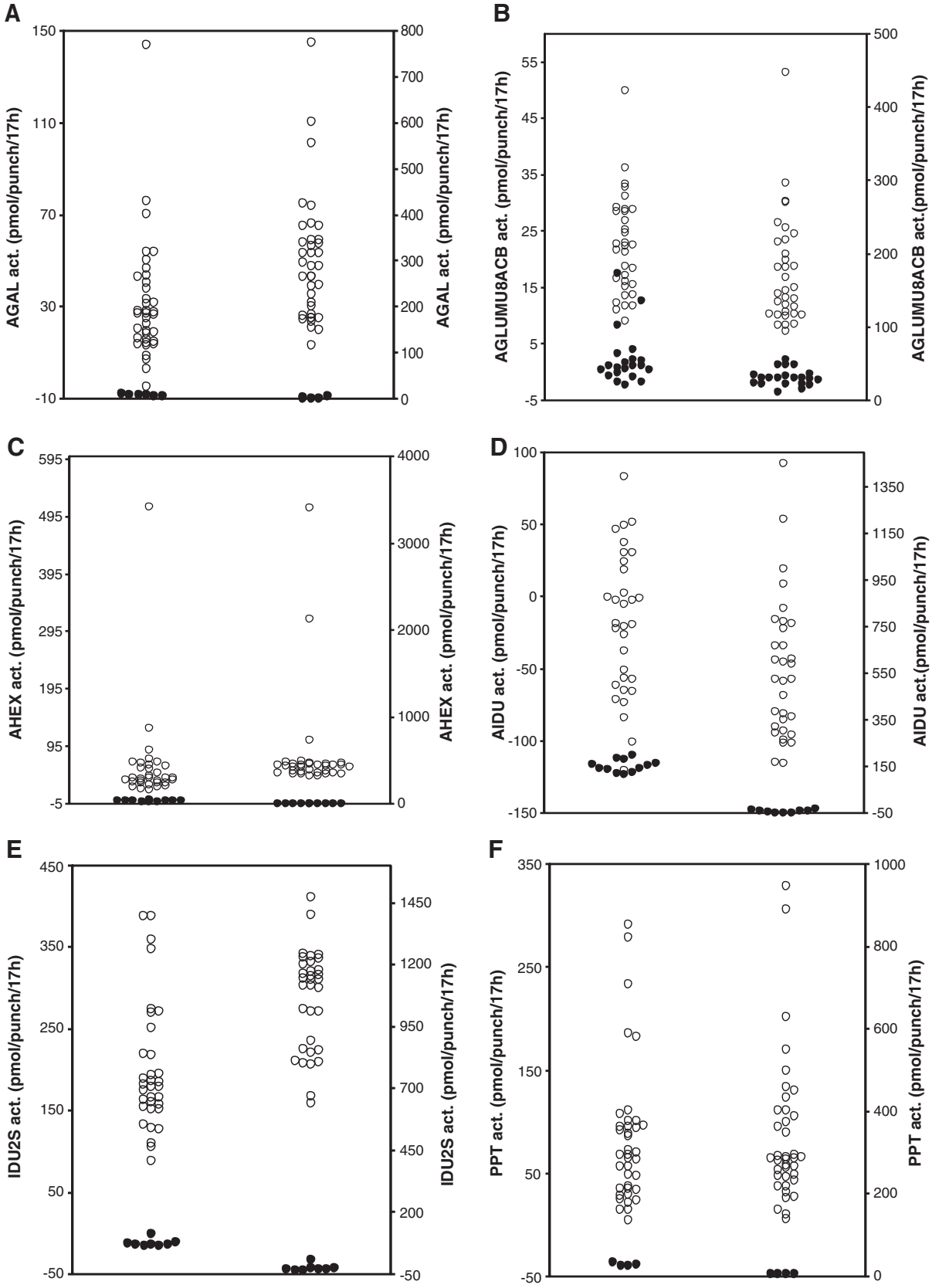

Fig. 1. Lysosomal enzyme activities in bloodspots with and with haemoglobin precipitation. The panels A–F show representative experiments in which the activities of six different lysosomal enzyme activities were measured in bloodspots from healthy individuals (open circles) and patients with selected lysosomal enzyme deficiencies (closed circles). A: α-galactosidase A activity (AGAL, Fabry disease); B: α-glucosidase activity (AGLU, Pompe disease); C: α-*N*-acetylglucosaminidase activity (AHEX, Sanfilippo B disease); D: α-L-iduronidase activity (AIDU, Hurler disease); E: iduronate-2-sulfatase activity (IDU2S, Hunter disease); F: palmitoyl-protein thioesterase 1 activity (PPT). Note: in each panel the activities as measured with (right scale) and without (left scale) TCA precipitation of haemoglobin are plotted on a different scale (*y*-axis).