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Carbohydrate Polymers 54 (2003) 59–61
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

A 96-well assay for uronic acid carbazole reaction

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Received 14 February 2003; revised 2 May 2003; accepted 6 May 2003

Abstract

A sensitive and reproducible 96-well assay of uronic acid permitting a rapid processing of a number of samples with a very low consumption of reagents is described for the determination of complex uronic acid-bearing polyanions such as hyaluronic acid, chondroitin sulfate, dermatan sulfate and heparin. The sensitivity of the reaction was approx. 1 μg for glucuronic acid and 2 μg for complex polysaccharides, with a linear function of glucuronic acid concentration between 1 and 100 μg. The relative coefficient of variations ranged from 1.5 to 8.7% for the assay performed in the 96-well plate. These values were found to be lower than those obtained by the conventional procedure.

Keywords: Uronic acid; Carbazole; Glycosaminoglycans; Hyaluronic acid; Heparin; Chondroitin sulfate

The reaction of uronic acids with carbazole (Bitter & Muir, 1962) is one of the most satisfactory method for estimating polysaccharides and proteoglycans during the extraction and purification processes from various organs and tissues, quantitatively measuring glycosaminoglycans used as drugs, and determining uronic acids in chromatographic methods. At the moment, this colorimetric procedure is performed in tubes with extensive use of reagents and materials. Furthermore, this approach is very time-consuming when a great amount of samples must be processed. This modification describes an efficient 96-well assay of uronic acid permitting a rapid processing of a number of samples, with several repetitions of the same sample to better appreciate the coefficient of variation, with a very low consumption of reagents. The system was applied to the analysis of various glycosaminoglycans and excellent agreement was obtained with conventional analysis.

Hyaluronic acid from bovine trachea with an M_r of about 1,000,000 was from IBSA (Institut Biochimique SA, Lugano, Switzerland). Chondroitin sulfate from bovine trachea, and dermatan sulfate and heparin from bovine mucosa, were prepared as previously reported (Maccari & Volpi, 2002; Volpi, 1999). D-glucuronic acid lactone was from Sigma. All other reagents were analytical grade.

A serial dilution of standard or sample of 50 μl (1 mg/ml) was placed in a 96-well plate. 200 μl of a solution of 25 mM sodium tetraborate in sulfuric acid was added. The plate was heated for 10 min at 100 °C in an oven. After cooling at room temperature for 15 min, 50 μl of 0.125% carbazole in absolute ethanol were carefully added. After heating at 100 °C for 10 min in an oven and cooling at room temperature for 15 min, the plate was read in a microplate reader (BioRad, Model 550) at a wavelength of 550 nm. The carbazole reaction in tubes was performed as conventionally reported (Bitter & Muir, 1962). Any possible lid on the plate was avoid as a decrease in the reaction intensity was noted (not shown) as compared to the conventional procedure.

The photograph of Fig. 1 shows a 96-well plate in which the assay of uronic acid carbazole reaction for heparin has been performed. Fig. 2 shows the comparison of the assay performed in the 96-well plate and in tubes at increasing amounts of D-glucuronic acid and various uronic acid-composed polysaccharides. The assays of uronic acid for the various polysaccharides performed in tubes and in the 96-well plate show similar regression curves with approximately equal parameters of the equations (Fig. 2).

The sensitivity of the reaction was approximately 1 μg for glucuronic acid and 2 μg for complex polysaccharides, according to the conventional assay (Bitter & Muir, 1962) with a linear function of glucuronic acid concentration between 1 and 100 μg. The precision of the method was

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doi:10.1016/S0144-8617(03)00144-9

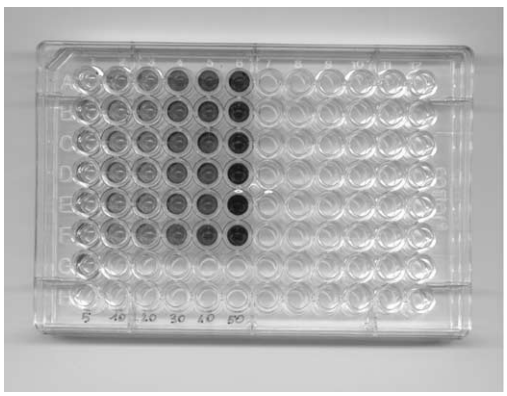

Fig. 1. The 96-well assay for uronic acid carbazole reaction of heparin. The well A1 was used as blank. Wells from B1 to G1: 5 μg of heparin; from A2 to F2: 10 μg of heparin; from A3 to F3: 20 μg of heparin; from A4 to F4: 30 μg of heparin; from A5 to F5: 40 μg of heparin; from A6 to F6: 50 μg of heparin.

determined by 10 repeated determinations. The relative coefficient of variations ranged from 1.5 to 8.7% for the assay performed in the 96-well plate. These values were found to be lower than those obtained by the conventional procedure probably due to the possibility to perform a great number of repetitions for each concentration with low consumption of reagents, materials and time.

Glycosaminoglycans, such as those tested in this study—hyaluronic acid, chondroitin sulfate, dermatan sulfate and heparin—are produced by extraction and purification from different animal tissues, and have several fundamental biological activities, as well as pharmacological properties, making them important drugs for use in clinical and pharmaceutical fields (Lane & Lindahl, 1989; Ofosu, Danishefsky, & Hirsh, 1989;). As a consequence, the uronic acid assay is largely used during the isolation

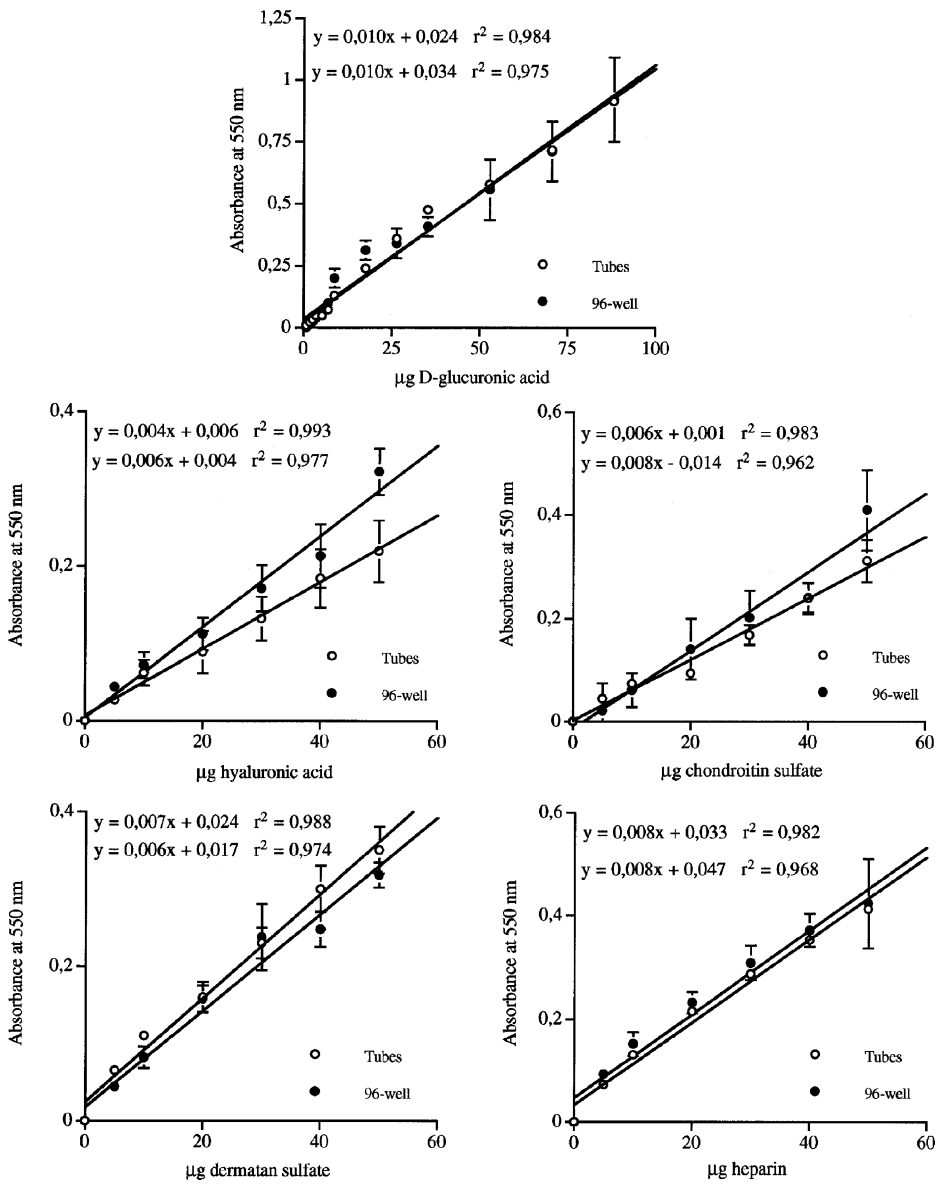

Fig. 2. Calibration curves of the uronic acid reaction for glucuronic acid standard and several complex polysaccharides performed in the 96-well plate and by the conventional procedure in tubes. The equations and the coefficient of correlations are reported.

and purification processes, to evaluate the purity of preparations, to eventually monitor a chemical or enzymatic process of modification of their structure to produce new and more active derivatives (Linhardt, 1992). The 96-well assay for uronic acid carbazole reaction described here and applied to complex uronic acid-bearing polysaccharides, has the advantage of considerably lower consumption of reagents, materials and time, with a greater reproducibility of results.

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