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Thin-layer electrophoresis of hydroxyethyl starches on a modified silica gel support

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ABSTRACT

The recent developed thin-layer electrophoresis on modified silanized silica gel was applied to the separation of hydroxyethyl starches (HES) and glycogen and of HES with different degrees of substitution. This method permits a rapid qualitative and semi-quantitative determination of HES in animal tissues such as liver, lung, heart and kidney after their disintegration with alkali and precipitation with ethanol.

INTRODUCTION

Hydroxyethyl starch (HES) is produced by hydroxyethylation of starch [1] and is applied increasingly as a blood plasma expander instead of the dextrans used so far [2]. To obtain information about its distribution and degradation in bodies it is important to determine HES concentrations in individual tissues.

Previous methods for the determination of HES are based on two principles: (a) conversion of the hydroxyethyl group attached to the starch molecules into ethyl iodide by heating the sample with concentrated hydriodic acid and determination of the ethyl iodide either by reaction with silver nitrate and bromine in acetic acid [3] or by gas chromatography [4,5]; (b) hydrolysis of the HES with acid and determination of the substituted and non-substituted glucose units by gas chromatography after derivatization either to O-trimethylsilyl ether [6,7] or to alditol acetates [8]. These methods have the disadvantage, that only the total amount of hydroxyethyl groups can be determined. In the present application, where the samples consist of a mixture of HES and glycogen, it is not possible to determine the real concentration of HES with these methods and a prior separation of the individual components has to be carried out.

Recently, a thin-layer electrophoretic method was developed for the separation of polysaccharides using silanized silica gel, the surface of which is covered with a thin film of 1-octanol [9–11]. With this stationary phase, better separations of the polysaccharides could be achieved than on chromatographic paper or cellulose acetate sheets. A further advantage lies in the possibility of revealing the separated polysaccharides with specific and sensitive sulphuric acid- and phosphoric acid-containing car-

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bohydrate reagents, which was not possible on the previously used supports such as cellulose or acetylcellulose owing to the formation of intense background coloration. This paper describes the application of this method to the separation of HES and glycogen and of HES with different degrees of substitution and its detection and determination in animal tissues.

EXPERIMENTAL

Materials

The experiments were carried out with a Bio-Rad electrophoretic cell (Type 1405) for 150 × 200 mm plates and a Bio-Rad power supply (Bio-Rad Labs., Richmond, CA, USA); thin-layer plates were prepared with equipment from Desaga (Heidelberg, Germany). The quantitative evaluation of the separated zones was carried out by the thin-layer scanner (Shimadzu, Tokyo, Japan).

Silanized silica gel for thin-layer chromatography and 1-octanol were purchased from Merck (Darmstadt, Germany), Whatman GF/C glass-fibre paper from Whatman (Maidstone, UK), 1,3-dihydroxynaphthalene (naphthoresorcinol) from Serva (Heidelberg, Germany) and polyvinylpyrrolidone K-90 (relative molecular mass M_r = 360 000 dalton) from Fluka (Buchs, Switzerland). Hydroxyethyl starches with different degrees of substitution and relative molecular mass (*e.g.*, HES 450/0.7, M_r = 450 000 dalton, degree of substitution = 0.7), non-substituted starch (degraded amylopectin) and glycogen were supplied by Laevosan (Linz, Austria).

Isolation of hydroxyethyl starches from animal tissues

The procedure is based on the method of Grossfeld [12] for the determination of starches in meat products and which was adapted for the analysis of thickening agents in the same materials by Bauer and Vali [13]. The organs were placed in 35% potassium hydroxyde solution and heated for 3 h under reflux in a boiling water-bath. The proteins and lipid compounds are degraded whereas the polysaccharides, especially the 1,4-glycans, remain unaltered by this treatment. After cooling, a sixfold excess of ethanol was added to precipitate the polysaccharides. After standing at 0–4°C overnight, the precipitate was centrifuged at 0°C and 8000 *g*. The supernatant was removed and the residue was washed three times with absolute ethanol and dissolved in 2.0 ml of the analyte buffer. An aliquot of 2–5 μl was used for electrophoresis.

For the test with albumin, a 5% aqueous solution was diluted with 0.3 *M* borate buffer (1:1, v/v) and 1.5 mg of HES 450/0.7 and 1.5 mg of glycogen were dissolved in 1 ml of this solution. With blood plasma the pure product was diluted with 0.3 *M* borate buffer (1:1, v/v) and 1.5 mg of HES 450/0.7 and 1.5 mg of HES 70/0.3 were dissolved in 1 ml.

For the determination of HES in animal tissues, rabbits had been treated with HES injections and killed after certain periods of time. Their organs were kept at −70°C until analysis. After thawing and weighing the organs, the polysaccharide were isolated according to the procedure described above.

Electrophoresis on the silanized silica gel–1-octanol support

The idea for this system was to develop a support for electrophoretic separations on which the adsorption and molecular sieve effect and the electroendoosmosis are minimal and on which the separated substances can be revealed with agressive reagents (*e.g.*, those containing sulphuric or phosphoric acid) as on normal thin-layer chromatograms. This was achieved with silanized silica gel, the surface of which was covered with a thin film of 1-octanol [9]. The latter compound can be removed from the plate after the run by evaporation and the separated substances can be detected on the dry silica gel layer as usual by spraying with the respective detection reagent and additional heating if necessary.

For its preparation, silanized silica gel is suspended in a low-boiling organic solvent (*e.g.*, methyl acetate, dichloromethane) containing 1-octanol. After stirring for *ca.* 30 min to obtain a uniform impregnation of the particles with the solution, the solvent is evaporated *in vacuo* until a lump-free powder is obtained. For impregnation with buffer, the dry powder is placed in a mortar and the buffer solution used for electrophoresis is added stepwise under vigorous stirring with the pestle. A small separate part of this buffer contains 2% of polyvinylpyrrolidone as binding agent for the dry layer ready for detection after the electrophoresis. The particles are

wetted and the homogeneous slurry obtained is spread on 200 × 200 mm glass plates (layer thickness 0.3 mm). Immediately after coating, the plates are transferred to a desiccator with a humid atmosphere. They can be used after a storage time of 3 h; this times should not exceed 48 h because after that period the loss of water from the layer becomes too high.

The silanized silica gel–1-octanol system prepared in this manner has a structure like a very weak gel. The 1-octanol liquid phase is attached strongly to the surface of the silanized silica gel particles even after their suspension in the aqueous buffer and no leaching out into the surrounding aqueous phase was observed. It is possible that this weak gel structure is formed by hydrogen bonds between the OH groups of the 1-octanol and the water molecules while the strong adsorption of 1-octanol itself is due to a hydrophobic interaction between the hydrocarbon part of the latter molecules with the hydrophobic groups on the surface of the silanized silica gel. The electroendoosmosis is also strongly diminished (see the low mobility of ω-hydroxymethylfurfurol in the present electropherograms) and this effect can be explained by coverage of the charges on the silica gel surface by the 1-octanol phase.

Conversely, when normal unsilanized silica gel is used for the preparation of this system, the 1-octanol is detached from the surface of the particles immediately after wetting with the aqueous buffer with the formation of an organic phase in the liquid. The adhesion forces between 1-octanol and the silica gel surface are too low in that event to maintain further adsorption.

The electrophoresis has to be carried out under very moist conditions and substantial evaporation of water from the surface of the layer diminishes the electrophoretic mobilities of the samples.

For the run, 10–15 mm long groves are made with a tip of the needle in the layer and the samples, dissolved in the running buffer, are deposited in the groves. After the electrophoresis the plate is dried at 90°C for 30 min to evaporate the 1-octanol and water. The remaining dry, inert silica gel layer can then be treated with detection reagents is the same way as a normal thin-layer plate.

The exact procedures for preparing the support to be spread on glass plates, electrophoresis and detection were described in detail in previous papers [9–11].

In subsequent experiments the electrophoretic runs were performed under the following conditions: buffer, 0.3 *M* borate (pH 10); current 200 V; time, 120–240 min; temperature, 22–28°C; detection reagent, naphthoresorcinol–sulphuric acid according to Scherz [9].

RESULTS AND DISCUSSION

Separation of standard compounds

Fig. 1 shows the electropherogram of HES with different degrees of substitution but equal average relative molecular mass (M_r = 70 000 dalton) together with unsubstituted starch (M_r = 70 000 dalton) and glycogen. The results from the electrophoretic runs with HES compounds with the same degree of substitution but different relative molecular masses show no significant differences in their mobilities. These results demonstrate that only the degree of substitution is important for the electrophoretic mobilities of these starches on this stationary phase. The higher the degree of substitution, the lower is the formation of charged complexes with

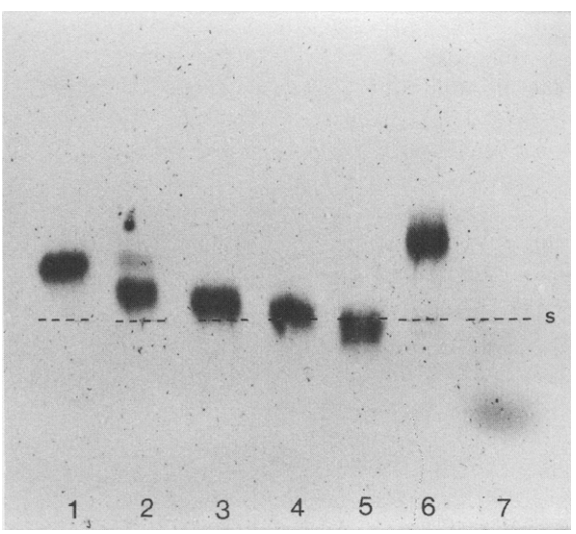

Fig. 1. Electropherogram of hydroxyethyl starches, unsubstituted starch and glycogen. Electrophoresis: 0.3 *M* borate (pH 10.0); 220 V; 240 min; 22°C. 1 = unsubstituted starch; 2 = HES 70/0.3; 3 = HES 70/0.4; 4 = HES 70/0.5; 5 = HES 70/0.7; 6 = glycogen; 7 = ω-hydroxymethylfurfurol. 1–7 dissolved in 0.3 *M* borate buffer (5 mg/ml).

borate ions, which are responsible for the movement in the electric field. The relative molecular mass has no significant influence, *i.e.*, with this material the molecular sieve effect does not occur, in contrast to other materials such as polyacrylamide gel.

Effect of temperature on thin-layer electrophoresis of HES

The temperature of the thin-layer plates exerts a significant influence on the thin-layer electrophoretic separation. By enhancement of the temperature, zone sharpening occurs with a decrease in the individual mobilities and the electroendosmosis is markedly reduced. The optimum temperature was found to be 25–26°C, which was used in subsequent experiments. Fig. 2 shows an optimized separation of the individual components of a mixture of glycogen and HES with of different degrees of substitution.

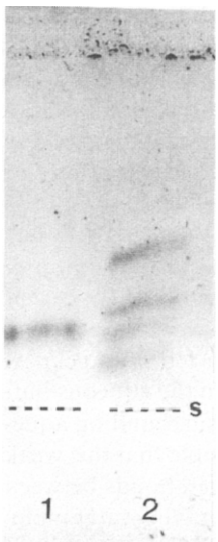

Fig. 2. Effect of temperature of the thin-layer electrophoresis of HES; electropherogram at the optimum temperature for separation. Electrophoresis: 0.3 *M* borate buffer (pH 10); 220 V; 240 min; 26°C. 1 = HES 200/0.5 (1 mg/ml); 2 = HES 70/0.3, HES 200/0.5, HES 450/0.7 and glycogen (0.35 mg/ml each). 1 and 2 dissolved in 0.3 *M* borate buffer.

Quantitative evaluation of thin-layer electropherograms

Solutions of HES 200/0.5 in 0.3 *M* borate buffer were prepared with different concentrations. The

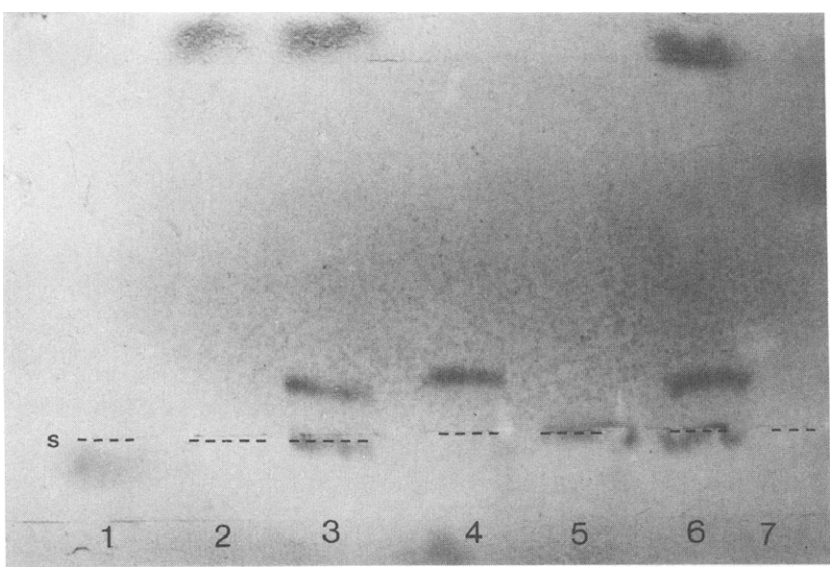

Fig. 3. Detection of HES in human albumin. Electrophoresis: 0.3 *M* borate buffer (pH 10); 180 V; 180 min; 28°C. 1 = ω-hydroxymethylfurfurol; 2 = 5% aqueous solution of human albumin–0.3 *M* borate buffer (pH 10) (1:1, v/v); 3 = HES 450/0.7 (1.5 mg/ml) and glycogen (1.5 mg/ml) dissolved in 5% aqueous solution of human albumin–0.3 *M* borate buffer (pH 10) (1:1, v/v); 4 = glycogen (1.5 mg/ml); 5 = HES 450/0.7 (1.5 mg/ml); 6 = 3; 7 = 3-hydroxyethylglucose (6 mg/ml). 1, 4, 5 and 7 dissolved in 0.3 *M* borate buffer.

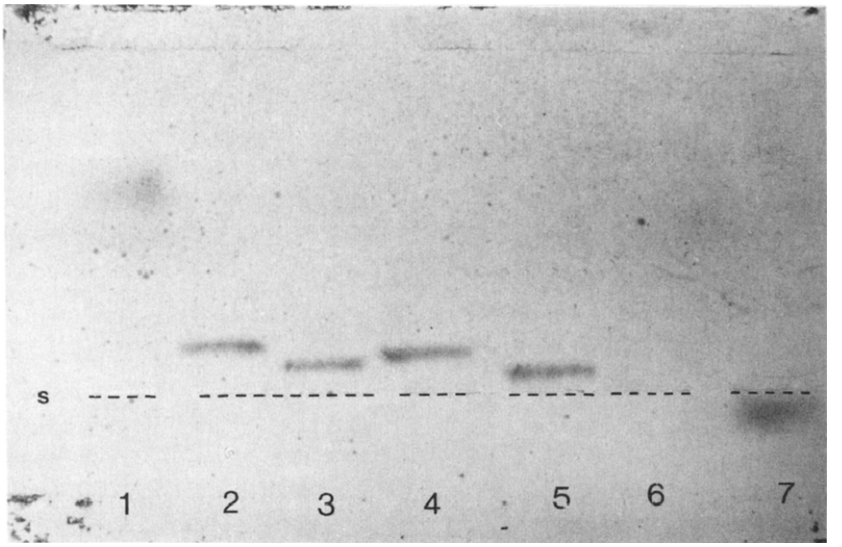

Fig. 4. Detection of HES in blood plasma. Electrophoresis: 0.3 *M* borate buffer (pH 10); 220 V; 120 min; 26°C. 1 = 3-hydroxyethylglucose (6 mg/ml); 2 = HES 70/0.3 (1.5 mg/ml); 3 = HES 450/0.7 (1.5 mg/ml); 4 = HES 70/0.3 dissolved in blood plasma–0.3 *M* borate buffer (pH 10) (1:1, v/v) (1.5 mg/ml); 5 = HES 450/0.7 (1.5 mg/ml) dissolved in blood plasma–0.3 *M* borate buffer (pH 10) (1:1, v/v); 6 = blood plasma–0.3 *M* borate buffer (pH 10) 1:1, v/v); 7 = ω-hydroxymethylfurfurol. 1, 2, 3 and 7 dissolved in 0.3 *M* borate buffer.

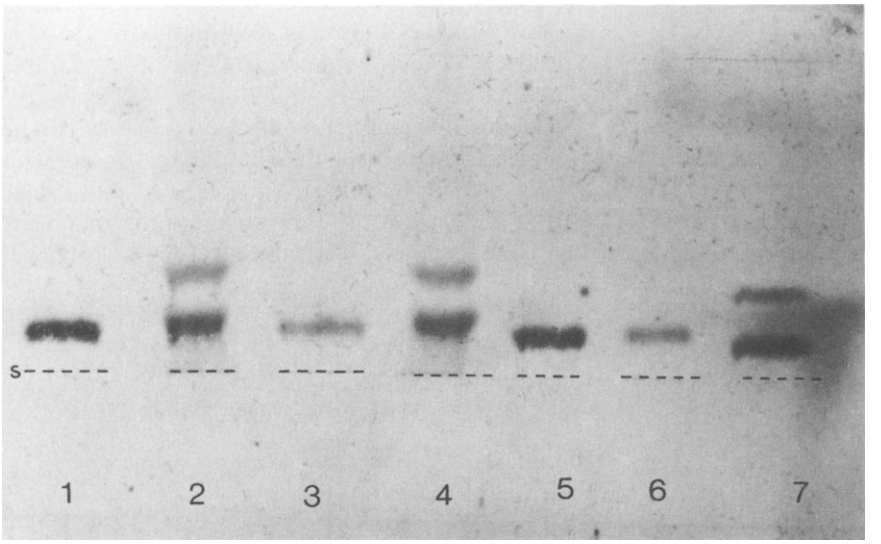

Fig. 5. Detection of HES in rabbit heart. Electrophoresis: 0.3 *M* borate buffer (pH 10.0); 180 V; 120 min; 25°C. 1 = HES 450/0.7 (3 mg/ml); 2 = HES isolated from rabbit heart; 3 = HES 450/0.7 (0.5 mg/ml); 4 = 2; 5 = 1; 6 = 3; 7 = HES 450/0.7 (3 mg/ml) and glycogen (0.5 mg/ml). 1–7 dissolved in 0.3 *M* borate buffer.

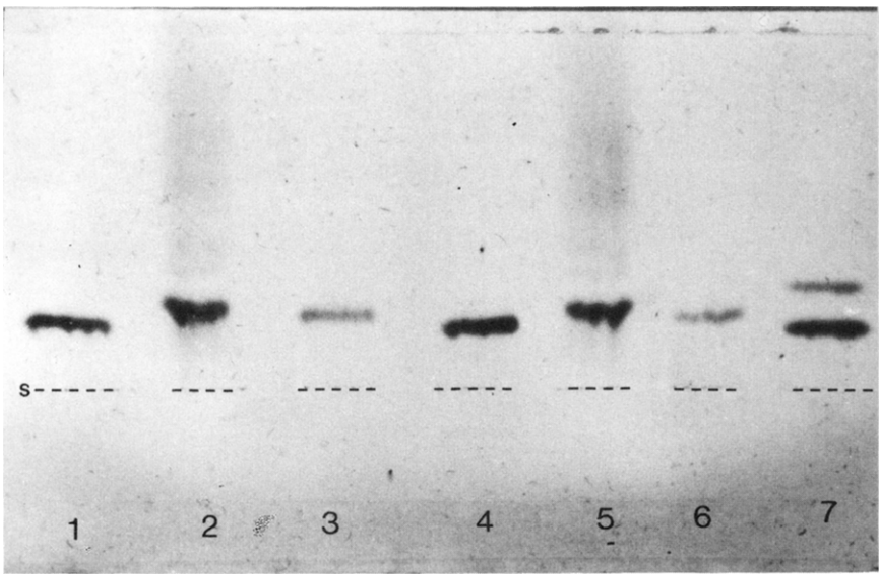

Fig. 6. Detection of HES in rabbit lung. Electrophoresis: 0.3 *M* borate buffer (pH 10); 180 V; 120 min; 25°C. 1 = HES 450/0.7 (3 mg/ml); 2 = HES isolated from rabbit lung; 3 = HES (0.5 mg/ml); 4 = 1; 5 = 2; 6 = 3; 7 = HES 450/0.7 (3.0 mg/ml) and glycogen (0.5 mg/ml). 1–7 dissolved in 0.3 *M* borate buffer.

scanning of their detected zones on the electropherograms with a thin-layer scanner with a linearization program showed a good linear correlation between concentrations and integration values in the range 0.25–3.0 mg/ml HES.

Application of the method to physiological samples

HES–albumin and HES–blood plasma. The electropherograms in Figs. 3 and 4 show that in all instances the HES compounds appear as sharp zones. The large excess of proteins in the albumin and blood plasma samples does not influence either the shape of the zones or the colour intensities in comparison with the pure compounds. Additionally, the albumin electropherograms show in the upper region deep blue zones of D-glucose, which was present as a stabilizing agent in the albumin solution.

Animal tissues. To demonstrate the usefulness of this method for the determination of HES in animal tissues, a weighed amount of HES was added to an HES-free pig liver sample and the isolation was carried out as described. Simultaneously, the same amount of HES without liver tissue was treated in the same way. The isolated polysaccharides were dissolved in 0.3 *M* borate buffer and the electrophoresis was carried out with aliquots of the solutions. The detected sample zones and those of pure HES (two different concentrations) on the electropherograms were scanned and the HES concentrations were calculated from the corresponding calibration graphs. For the amount of HES in the liver sample a recovery of 87.5% relative to the liver-free sample was obtained.

The method was also applied to several tissue samples from rabbits that had been treated with HES, such as the spleen, kidney, heart and lungs. Figs. 5 and 6 show the electropherograms of a heart and a lung sample. The concentrations of HES in

TABLE I

AMOUNTS OF HES IN DIFFERENT RABBIT ORGANS

Mean values of two different determinations.

Organ	Fresh weight of organ (g)	HES per organ (mg)
Lung	6.66	1.7
Heart	5.12	0.55
Kidney	12.7	0.95
Spleen	2.3	0.41

the organs were determined by scanning the detected zones. The results of a few determinations are given in Table I.

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