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ANALYSIS OF THE PROTEINS IN SWEAT AND URINE BY AGAROSE-GEL ISOTACHOPHORESIS

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SUMMARY

A sensitive and practical method is described for the analysis of the proteins contained in human sweat and urine which does not require pre-concentration of the sample. Technical details are provided of the agarose-gel isotachophoresis and the proteinograms of normal and pathological urine samples, as well as proteinograms of human sweat. The method can also be applied as an electro-concentration system in a field-strength gradient. By means of this electro-concentration system, and in combination with immunodiffusion against monospecific antisera, a detection limit of albumin of 50 ng/ml has been obtained.

INTRODUCTION

Unconcentrated human sweat and urine contain trace amounts of proteins which are difficult to analyse by means of electrophoresis, where the diffusion forces constantly counteract the separation power of the method. Even in disc electrophoresis[1,2] the sharpness of the fraction is partially disrupted by diffusion effects. For this reason an analytical method such as free isotachophoresis, which combines a concentration power with an antidiffusion force created by ionophoresis in the potential gradient, is of great interest[3,4].

This paper describes technical details of the agarose-gel isotachophoresis of diluted biological fluids without pre-concentration. The selectivity and the reproducibility of the protein pattern are discussed.

MATERIALS

Samples

For feasibility studies, solutions (1 mg/ml) of human albumin and human transferrin (Behringwerke, Marburg, G.F.R.) were analysed. "Sauna" sweat was collected from healthy volunteers at the Zeepreventorium, Den Haan. The individual

samples were stored at 4°. Urine samples (Department of Nephrology, St. Janshospitaal, Brugge, Belgium) exhibiting a pattern of glomerular, tubular and mixed proteinuria were selected by routine agarose-gel electrophoresis. When spacer molecules were required, ampholines (pH 6–8) (LKB, Stockholm, Sweden) were added to the samples before isotachophoresis[5,6].

Antisera

Commercial rabbit antisera (Behringwerke) against human albumin, total human serum, human α-lipoprotein, human β-lipoprotein, human transferrin and human Ig were used. An antiserum against sweat lipoproteins was raised in the rabbit at our laboratory.

METHODS

Isotachophoresis

Isotachophoresis was carried out with tap-water cooling on an LKB Type 2117 electrophoresis system. Glass plates (1.5 × 11 cm or 3 × 11 cm) were covered with agarose gel containing the leading electrolyte. For clinical purposes (analysis of sweat and urine), 0.018 *M* orthophosphoric acid adjusted with tris(hydroxymethyl)aminomethane (Tris) to pH 5.5 was used as the leading electrolyte at the anode. After gelification, one third of the leading gel was cut off and replaced by the cathodic agarose gel containing a terminating electrolyte solution of 0.04 *M* glycine adjusted by use of Tris to pH 8.6. In some experiments 0.014 *M* orthophosphoric acid (adjusted with Tris to pH 7) and 0.028 *M* glycine (adjusted with Tris to pH 8.6) were used as leader and terminator. De-ionized and twice distilled water was used in all of the solutions. Connection of the gel with the corresponding electrolyte vessels was by means of paper wicks.

The protein samples, with or without spacer molecules, were applied to a filter strip (3 × 1.5 cm) and placed on top of the terminator gel. A constant current of 5 mA was applied under a voltage of 300 V. After 2 h, at the end of the run, the voltage rose to 400 V depending on the concentration, pH and mobility of the ions. Coomassie brilliant blue staining and destaining was carried out before drying of the gel[7]. For Sudan Black B and PAS (periodic acid Schiff) staining, the gels were first fixed in ethanol–acetic acid–water (10:1:9) and stained after drying.

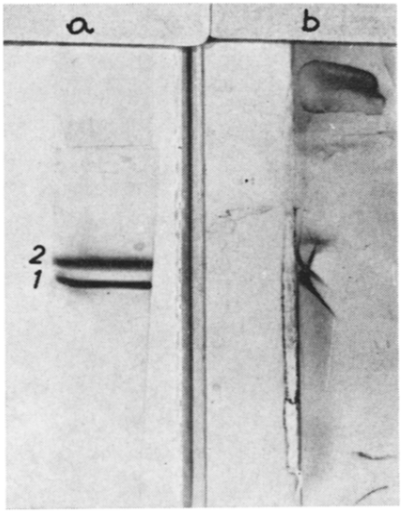
Immunological analysis

For gel immunoisotachophoresis, samples were separated in the presence of 2 μl of ampholine. Antiserum was applied in a slot cut out along the migration path. For immunological analysis of the leader–terminator boundary, antiserum was applied in holes punched in the gel at 5 mm from the boundary. After immunodiffusion for 24 h, the gels were washed, dried and stained as described earlier[8].

RESULTS

Isotachophoretic protein patterns

Selectivity and reproducibility of the method. 500 μl of a solution of albumin transferrin containing 10 μg of each protein and 2 μl of ampholine was analysed at



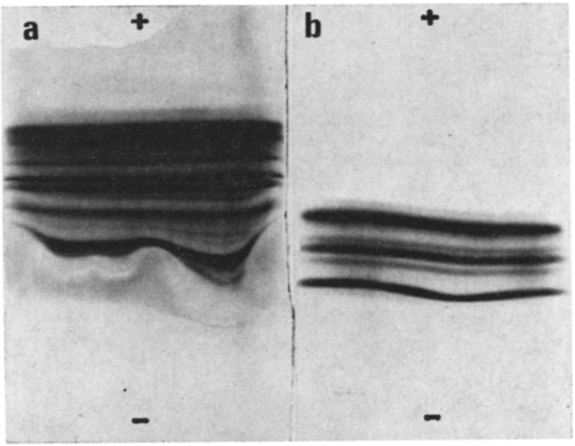


Fig. 1. Isotachophoresis (a) and immunoisotachophoresis (b) in agarose gel of solutions of albumin (1) and transferrin (2).

Fig. 2. Isotachophoresis of a proteinuria sample (250 μl) without pre-concentration. (a) Leading buffer, Tris–0.018 *M* H_3PO_4 (pH 5.5); terminating buffer, Tris–0.04 *M* glycine (pH 8.6). (b) Same sample as in a: leading buffer, Tris–0.014 *M* H_3PO_4 (pH 7); terminating buffer, Tris–0.028 *M* glycine (pH 8.8).

several pH values and electrolyte concentrations. Optimal separation for both proteins was obtained at a leader-electrolyte concentration of 0.014 *M* orthophosphoric acid adjusted by use of Tris to pH 7 and at a terminator concentration of 0.028 *M* glycine adjusted by Tris to pH 8.6 (Fig. 1a). The purity of the protein fractions was demonstrated by immunoisotachophoresis and two crossed well-separated immunoprecipitation lines were obtained (Fig. 1b).

The selectivity of the method is dependent on the composition of the leading electrolyte as demonstrated by a comparison of the protein pattern of a nephrotic urine sample at pH 5.5 and pH 7 (Fig. 2). Previous studies have shown that the protein patterns of human urine and human sweat stored at 4° in the presence of thymol are reproducible over a period of 6 months[5,8,9].

Proteinogram of human sweat. The protein pattern of unconcentrated human sweat is shown in Fig. 3a. The pattern shows three main protein bands next to several smaller fractions. The major band corresponds to albumin, as was demonstrated by immunological techniques. The second band corresponds to the inter-α band detected by electrophoresis of human sweat after a 200-fold concentration[9–11]. The third band contains transferrin and fast-migrating γ-globulins, as confirmed by immunoisotachophoresis with specific antisera. Moreover, as shown in Fig. 3b, three lipophilic fractions appeared after staining with Sudan Black B. The presence of α- as well as of β-lipoproteins was established, but the major component is a lipid protein fraction having plasma-albumin mobility. The concentration of this native fraction is season dependent[9]. After staining for polysaccharides by Schiff's method, two glycoprotein lines were demonstrated as shown in Fig. 3c.

Proteinogram of urine. The proteinograms of healthy fasting patients at rest

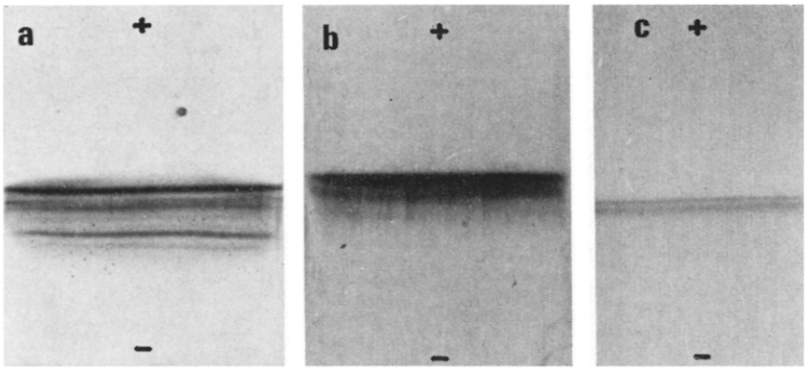


Fig. 3. Isotachophoresis of normal human sweat (500 μl). With Coomassie brilliant blue (a), Sudan Black B (b) and periodic acid Schiff staining (c).

showed only trace amounts of albumin. Samples from patients with orthostatic albuminuria showed, in addition to the presence of albumin, some α- and β-proteins, which were not visible after classical zone electrophoresis without pre-concentration[4]. Urine samples from patients with kidney disease were analysed for classification according to the type of proteinuria.

A glomerular proteinuria is shown in Fig. 4a with albumin as the major and transferrin as a minor fraction. A pattern of tubular proteinuria (Fig. 4b) had nine well-defined protein fractions from which prealbumin, albumin, transferrin and γ-globulins were identified by immunoisotachophoresis. Several samples of proteinuria belonged to an intermediate type characterized by a high number of protein fractions having relatively high albumin concentrations (Fig. 4c).

Protein concentration at the leader–terminator boundary

Isotachophoresis without spacer molecules concentrates all of the proteins present in the sample into one band at the leader–terminator boundary. For testing the sensitivity of the method, a very dilute sample of albumin was analysed. By means of immunological analysis after isotachophoresis, we were able to detect the presence

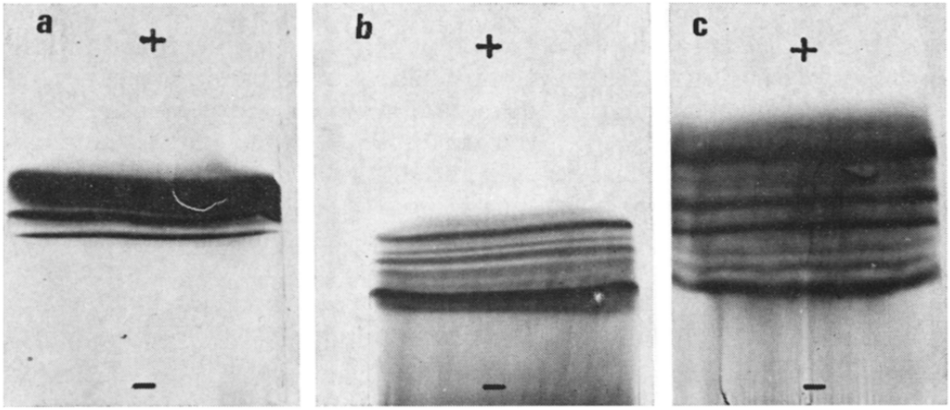


Fig. 4. The proteinuria patterns: glomerular (a), tubular (b) and mixed (c).

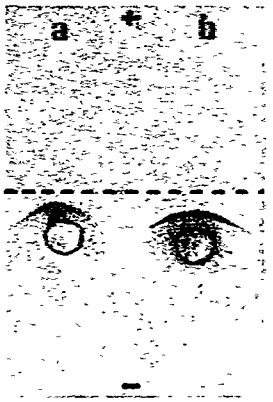


Fig. 5. The immunological analysis of the leader–terminator boundary of human sweat (500 μl) using 5 μl (a) and 10 μl (b) of rabbit anti-sweat lipoprotein.

of 24 ng of albumin which had been previously diluted with 500 μl of water. This concentration procedure was also applied to the detection of proteins and lipoproteins in human sweat (Fig. 5). We were able to demonstrate the presence of α- and β-lipoproteins in sweat collected on filter paper[5]. The same method can also be applied to the detection of trace amounts of albumin on fatty hair and in tears and to the detection of immunoglobulins in nasal secretions, all without previous concentration.

DISCUSSION

By means of agarose-gel isotachophoresis, proteins present in very low concentrations in different biological fluids can be separated. The fractionation will be a function of the experimental conditions of pH and ionic strength, of the nature of the spacer used and of the mobility of the leading and terminating ions[12].

The isotachophoretic urine pattern shows all of the proteins possessing a mobility intermediate between those of the leading and terminating ions. The observed patterns correlate well with those obtained by classical electrophoretic techniques after concentration. As the mobility of glycine is nearly the same as that of the γ-globulins, the described conditions cannot be used for differentiation of γ-globulins. ε-Aminocaproic acid as terminating ion, having a mobility lower than that of glycine, would give better results.

The high concentration power of agarose-gel isotachophoresis is an important advance in the analysis of proteins in urine and sweat. Owing to the high sensitivity of the technique, new ways are made available for further study of the pathology of the kidney, and this technique could be of interest in the analysis of urine from newborn babies.

The applicability of the described method to biological fluids containing very low protein concentrations is shown by the proteinogram of 500 μl of human sweat. Since normal electrophoretic techniques require a 200-fold pre-concentration of the sample before any result can be observed, most published studies describe results obtained on pooled samples of human sweat which were pre-concentrated before analysis. The introduction of the isotachophoretic agarose technique enables the

analysis of protein and glycoprotein fractions in individual samples. The presence of a lipoprotein which does not precipitate anti-α and anti-β plasma lipoproteins in human sweat is also of importance in the study of the function of sweat glands in the metabolism of human skin. Immunoisotachophoresis and the immunological analysis of the proteins concentrated at the leader–terminator boundary are helpful tools in the identification of dilute protein solutions.

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