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# ISOELECTRIC FOCUSING OF HUMIC ACIDS ISOLATED FROM THE CURATIVE MUDS OF HAAPSALU BAY

The curative muds of Haapsalu Bay have long been used in the treatment of rheumatism and other chronic inflammatory diseases. Some decades ago the extracts of humic acids (HA) from the curative muds of Haapsalu Bay were found to have a similar therapeutic effect [1]. 0.01% solutions of these HA in isotonic solution are distributed under the trade mark Humisol, which is an effective injection drug against chronic inflammatory diseases [2, 3]. There are some publications in which the HA of the curative muds have been studied by gel filtration [4] and by analysing their decomposition products after acidic hydrolysis [5]. So far, however, it is unclear which compounds in Humisol are responsible for the therapeutic effect. Little is known about the isoelectric properties and the size distribution of the HAs. In our study an attempt was made to focus the HA in the pH gradient. The advantage of this method is that the HA are not disintegrated into smaller molecules, but are investigated as such. This is particularly important in our case, i. e. in analysing the HA found in the curative muds.

## Experimental

**Humic acids.** In our study we used 0.5% stock solution of HA which we obtained from the Tallinn Pharmaceutical Factory (*Tallinna Farmaatsiatehas*).

The preparation of this solution includes the following steps:
— alkaline extraction;
— separation from insoluble material by centrifugation (1800 g during 30 min);
— neutralization;
— precipitation of HA in 50% ethanol solution;
— separation of the precipitate by centrifugation;
— dissolution of the precipitate in water;
— sterilization at 100 °C.

**Isoelectric focusing (IEF).** IEF was performed in the horizontal electrophoresis unit (Vagos, Estonia) connected with the power supply 2103 (LKB, Sweden). The gel cassette (0.45×95×105 mm) was made of a glass plate and a plexiglass plate, separated by a rubber gasket and clamped together with Bulldog clamps (Myers, United Kingdom). The glass plate was covered with GelBond PAG Film (LKB, Sweden) or with 0.18 mm polyester gel support treated with 0.05% agarose solution (for agarose gel).

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Polyacrylamide gel: Monomer solution (acrylamide — 4%, N,N′ — methylenebisacrylamide — 0.16%, N,N,N′,N′ — tetramethylethylenediamine — 0.064%, ammonium persulphate — 0.044%) was pipetted into the gel cassette and the gel was polymerized during 60 min at 50 °C. The polymerized gel, which was immobilized on the GelBond support, was washed for 30 min in 300 ml distilled water and for 30 min in 300 ml 1% glycerol. Then the gel was dried with a cold air fan. The dried gel was re-swelled for 60 min in a solution which contained 5% (v/v) Ampholine 3.5—10 and 20% (w/v) glycerol.

Agarose gel: 0.4 g of Agarose EF was dissolved in 20% (w/v) glycerol solution on a boiling water bath and cooled down to 60 °C. 0.25 ml of Ampholine 3.5—10 was pipetted into the agarose solution and the volume was adjusted to 5.0 ml. The agarose solution was then pipetted into the pre-heated gel cassette. The gel was stored in the fridge overnight before the run.

**Microfiltration.** 200 nm Mifil membranes were from Himifil, Estonia, and 850 nm Synpor membranes were from Chemapol, Czechoslovakia. Microfiltration was performed under the 1 Bar pressure with stirring. The filter cake was washed several times with distilled water.

**Elemental analysis.** Carbon, hydrogen, and nitrogen were analysed with a Hewlett Packard 186 CHN analyser.

**Chemicals.** Acrylamide, N,N,N′,N′ — tetramethylethylenediamine, N,N′ — methylenebisacrylamide, and ammonium persulphate were from Reanal, Hungary. Ampholine 3.5—10 and Agarose EF 2206—222 were from LKB, Sweden. Protein Test Mixture 9 was from Serva, Germany. Coomassie Brilliant Blue R250 was from Fluka, Switzerland. All other chemicals were from Reakhim, USSR.

## Results and Discussion

An electropherogram of the IEF of humic acids is shown in Fig. 1. While the marker proteins are focused in sharp zones, the HA have only started to migrate towards the anode. Part of the HA samples even failed to enter the gel, but precipitated on the gel's surface. It can be noticed that the HA samples which were applied cathodically have migrated to the neutral pH area, while the anodically applied sample has moved even further towards the anode. This led us to the conclusion that HAs in the Humisol are in fact very large molecules with a very low isoelectric point as the soft polyacrylamide gel that we used is suitable for the IEF of proteins with the molecular weight up to 1.5 million daltons [6]. Therefore, that part of the HA sample which did not enter the gel must have considerably higher molecular weight than 1.5 million daltons.

In order to find out whether the HA particles are really formed of strong covalent bonds or are just formed of hydrogen bonds, the HA sample was run in a gel containing sodium dodecyl sulphate (SDS) according to Laemmli [7]. After the SDS-phoresis, however, there were no signs of disintegration of the HA molecules. The major part of the HA sample failed again to enter the gel. This clearly indicates that the HA in the Humisol are indeed formed of strong covalent bonds and their molecular weight is up to millions of daltons.

Fig 2. shows an electropherogram of the IEF of HA in the agarose gel. Low concentration agarose gel (0.8%) was used in order to facilitate the migration of large HA molecules. As in the previous run (Fig. 1), the HA samples have migrated towards the anode. The isoelectric point of the HA appears to be close to the acidic end of the pH gradient (pH 3.5). Part of the HA again failed to enter the gel. Although the applied voltages were quite moderate, bow-shaped holes emerged on the anodic sides of the application points (not seen in Fig. 2). It seems that the large HA which were about to enter the gel just tore apart the agarose chains at the application points, preventing the remaining sample from advancing further.

The 0.5% Humisol stock solution was also filtered through 200 nm Mifil membrane and the CHN compositions of the permeate and non-permeate were measured (Table).

**Elemental composition of the 0.5% Humisol stock solution, separated by 200 nm Mifil membrane**

| Permeability | Dry matter, % | Elemental composition, % | | |
|---|---|---|---|---|
| | | C | H | N |
| Permeate <200 nm | 29.3 | 4.1 | 0.4 | — |
| Non-permeate >200 nm | 70.7 | 42.4 | 5.6 | 3.5 |

The dry matter content was determined after drying the samples to constant weight at 105 °C. It seems that the permeate contains mainly inorganic matter and the major part of the HA remained on the membrane. The Humisol stock solution was also filtered through the 850 nm Synpor membrane and over 98% of the dry matter passed through it. The relatively narrow size distribution of the HA in the Humisol solution has been achieved obviously by removing the very large particles by centrifugation and then precipitating the large HA in 50% ethanol solution.

Considering the acquired data and the formation mechanism of HA, described by Yamamoto and Ishiwatari [8], it is hard to imagine that the curative effect of Humisol is related with some minor components in the drug. According to the pharmacopoeia [2], not more than 2 ml of sterile 0.01% Humisol can be injected per day and the possible amounts of steroids and other biologically active compounds, which a patient may get from the drug, are very small indeed. Therefore we believe that the curative effect is caused by the large HA molecules.

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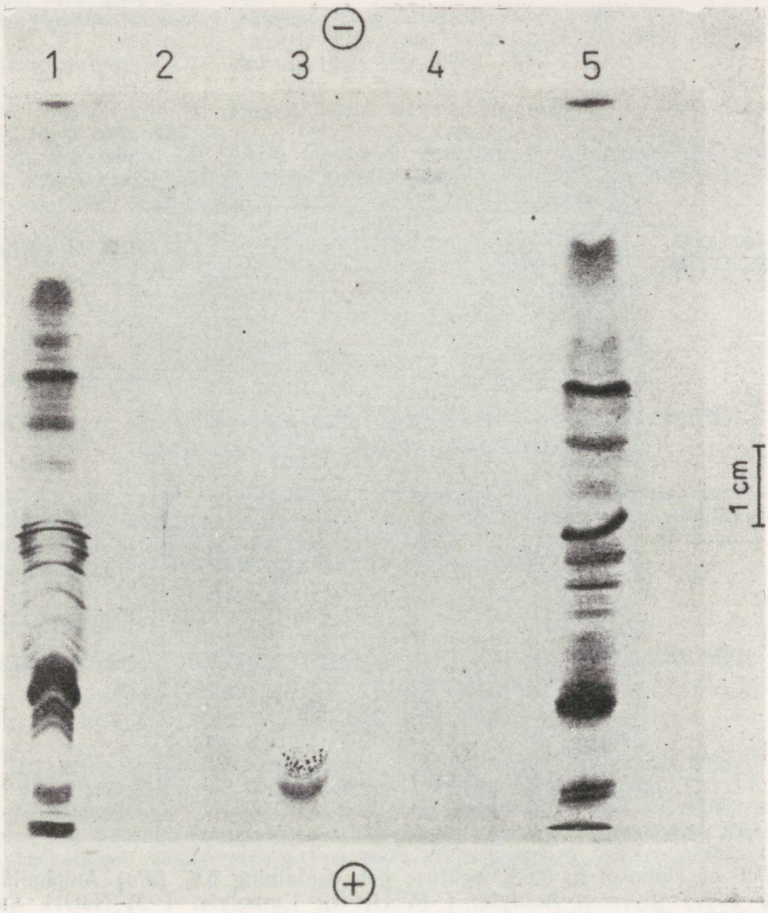


Fig. 1. IEF of Humisol in 4% polyacrylamide gel, containing 5% (v/v) Ampholine 3.5—10 and 20% (w/v) glycerol. Anolyte: 1 M $H_3PO_4$; Catholyte: 1 M NaOH. Samples of 2×5 µl 2.5% Serva Protein Test Mixture 9 were applied diagonally (track 1 anodically and track 5 cathodically); 10 µl 0.5% Humisol stock solution was applied anodically (track 3), and 2×5 µl cathodically (tracks 2, 4). Running conditions: 400 V, 800 V, 1000 V, 1500 V for 30 min each. Staining: Coomassie Brilliant Blue R 250 according to Altland and Rossmann [9].

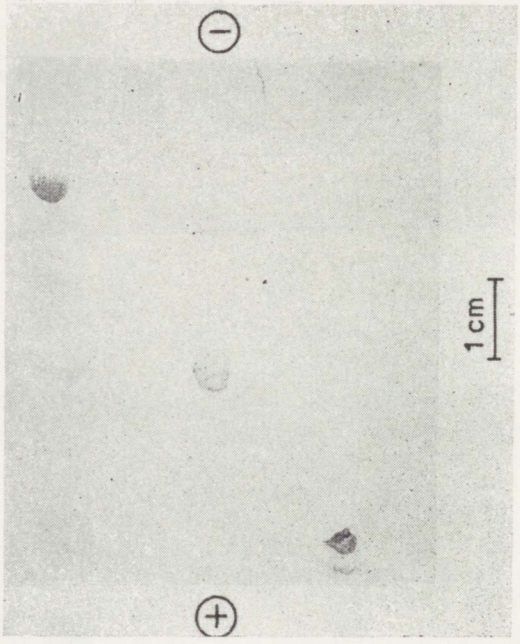


Fig. 2. IEF of Humisol in 0.8% agarose gel, containing 5% (v/v) Ampholine 3.5—10 and 20% (w/v) glycerol. Anolyte: 1 M $H_3PO_4$; Catholyte: 1 M NaOH. Samples of 3×5 µl 0.5% Humisol stock solution were applied diagonally. Running conditions: 80 V, 160 V, 180 V for 30 min each. Staining: The gel was fixed in 20% (w/v) trichloroacetic acid for 30 min, washed in tap water for 30 min, and stained with 0.1% Coomassie Brilliant Blue R 250 for 30 min according to [9].

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### HAAPSALU LAHE RAVIMUDAST ERALDATUD HUMIINHAPETE ISOELEKTRILINE FOOKUSTAMINE


Haapsalu lahe ravimudast eraldatud humiinhapete (HA) isoelektrilise fookustamise tulemuste põhjal võib järeldada, et HA-d on väga madala isoelektrilise punktiga ($pI \sim 3{,}5$) ühendid, mille molekulmass ulatub miljonitesse daltonitesse. HA-de suurele molekulmassile viitavad ka mikrofiltratsiooni ja SDS-foreesi tulemused.



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### ИЗОЭЛЕКТРИЧЕСКОЕ ФОКУСИРОВАНИЕ ГУМИНОВЫХ КИСЛОТ, ВЫДЕЛЕННЫХ ИЗ ЛЕЧЕБНЫХ ГРЯЗЕЙ ХААПСАЛУСКОГО ЗАЛИВА


Исходя из результатов изоэлектрического фокусирования гуминовых кислот (ГК), выделенных из лечебных грязей Хаапсалуского залива, сделан вывод, что ГК представляют собой соединения с очень низкой изоэлектрической точкой ($pI \sim 3{,}5$) и большой молекулярной массой. Тот факт, что последняя достигает нескольких миллионов дальтон, подтверждают также результаты микрофильтрации и СДС-фореза.