Journal of Chromatography, 431 (1988) 317–325
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4303

ANALYSIS OF THE AQUEOUS PHASE OF HUMAN CERVICAL MUCUS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ISOTACHOPHORESIS

M.M. GLADDINES*, M.T. ACKERMANS and F.M. EVERAERTS

Laboratory of Instrumental Analysis, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven (The Netherlands)

and

P.J.Q. VAN DER LINDEN, H.L. VADER and M.A.H.M. WIEGERINCK

Departments of Obstetrics/Gynaecology and Clinical Chemistry, St. Joseph Hospital Eindhoven, Aalsterweg 259, 5644 RC Eindhoven (The Netherlands)

(First received March 22nd, 1988; revised manuscript received May 2nd, 1988)

SUMMARY

The aqueous phase of human cervical mucus was analysed by reversed-phase high-performance liquid chromatography (HPLC) and capillary isotachophoresis (ITP). With HPLC, seventeen ultraviolet-absorbing and eight fluorescent components and with ITP five anionic and four cationic components could be determined. The sample pre-treatment consisted of a simple ultrafiltration. Ten samples from fertile women and eleven samples from infertile women were analysed. In six samples from the infertile group higher median concentrations of several components were found. This may be an indication of disturbances in the biochemical processes of the cervical mucus of woman with fertility problems.

INTRODUCTION

Human cervical mucus consists of a phase composed of glycoproteins and an aqueous phase. The glycoprotein phase is viscous and forms a matrix, in which the aqueous phase is dispersed. Both phases play an important role in the functioning of cervical mucus [1]. An important function of cervical mucus is the regulation of the access of the spermatozoa to the uterus. The mucus takes care of the carriage, feeding and protection of the spermatozoa. During the menstrual cyclus the percentage of the aqueous phase fluctuates. At the time of the ovulation it is approximately 98% but shortly after ovulation it decreases to 90% and the mucus becomes more viscous so that the penetration of the spermatozoa through

0378-4347/88/$03.50

the mucus is hampered. These changes in the properties of the mucus are controlled by hormones. Oestrogens stimulate the excretion of mucus with a high water content whereas progesterone stimulates the excretion of viscous mucus.

Abnormalities of the cervical mucus can be an important cause of infertility. Much research has been carried out on the structure and the functioning of the viscous phase, the glycoproteins. Through cross-linking of the glycoprotein side-chains a network is formed. Depending on the phase in the cycle, the density of this network varies. Wolf et al. [2] studied the viscoelasticity of the mucus during different phases of the menstrual cycle and Yurewicz and Moghissi [3] and Carlstedt et al. [4] studied the saccharide side-chains and the amino acid composition of the proteins.

Less research has been carried out on the composition of the aqueous phase. Kopito et al. [1] examined the electrolyte composition of this phase, determining sodium, potassium, calcium, magnesium, zinc and copper. Not only electrolytes but also lipids, trace metals, enzymes and prostaglandins have been determined [5]. Some of these components are important for the structure and functioning of the mucus. For example electrolytes may influence the intermolecular or intramolecular interactions between glycoproteins and through this the viscoelasticity and the density of the mucus.

Not all components of cervical mucus relating to infertility have been investigated. We have examined the aqueous phase of the preovulatory cervical mucus of fertile women and women with unexplained causes of infertility. These infertile women have normal ovulatory cycles, normal patent tubes, normal sperm–mucus interaction in the post-coitum test, normal sperm counts and sperm mobility and regular intercourse.

The aqueous phase was analysed by high-performance liquid chromatography (HPLC) and capillary isotachophoresis (ITP). These are powerful techniques that give complementary information. Components such as nucleosides, purines, amino acids and other low-molecular-mass UV-absorbing or fluorescent components can be determined by HPLC and ionic components by ITP. Automated HPLC equipment can process approximately 75 samples per week. The ITP analysis is not automated, but in two separate 20-min runs anionic and cationic components can be analysed.

In this study we compared the HPLC and ITP profiles of cervical mucus from ten fertile women and eleven women with fertility problems.

EXPERIMENTAL

Sample collection

Cervical mucus was aspirated with a sterile, dry tuberculine syringe from the cervical canal of women in the preovulatory phase of the menstrual cycle as assessed by ultrasound follicle imaging. Contamination with vaginal secretions was avoided. The syringes with aspirated mucus were kept at $-20\,^{\circ}C$ until processed.

Sample pre-treatment

The sample was removed from the syringe by adding a known amount of water and transferred to a plastic ultrafiltration unit (Amicon Centrifree micropartition system; Amicon, Danvers, MA, U.S.A.) that had been weighed. After adding the sample the filter unit was weighed again. From these data the amount of cervical mucus was calculated.

The concentration of a component is expressed as relative peak height (HPLC) or zone length (ITP) per gram of cervical mucus.

The aqueous phase was separated from the glycoprotein phase by ultrafiltration by centrifuging the samples in the filter units in a centrifuge at an angle of 45° for 30 min at 2000 *g*.

Isotachophoresis

The ITP analyses were carried out with laboratory-made equipment as described by Everaerts et al. [6]. The separation compartment consisted of a PTFE capillary of length ca. 200 mm and I.D. 0.2 mm. The driving current, delivered by a modified high-voltage supply (Brandenburg, Thornton Heath, U.K.), was 30 μA for anions and 60 μA for cations. For detection an a.c. conductivity detector was used. Signal processing was achieved with and IBM-XT computer (IBM, Boca Raton, FL, U.S.A.) equipped with and ADC Labmaster (Scientific Solutions, Solon, OH, U.S.A.). A laboratory-produced data analaysis program was used [7]. This program, written in Turbo Pascal (Borland International, Scotts Valley, CA, U.S.A.) calculates the length (the quantitative information) and height (the qualitative information) of each zone.

The operational systems for the analysis of anions and cations are listed in

TABLE I

OPERATIONAL SYSTEMS FOR ISOTACHOPHORETIC ANALYSIS OF ANIONS AND CATIONS

Ions	Parameter	
Anions	Leading ion	Cl^-
	Concentration	0.01 *M*
	Counter ion	Histidine (Merck, Darmstadt, F.R.G.)
	pH	6.0
	Additive	0.2% Hydroxyethylcellulose (Polysciences, Warrington, PA, U.S.A.)
	Terminating ion	2-Morpholinoethanesulphonic acid (Sigma, St. Louis, MO, U.S.A.)
	Concentration	0.005 *M*
Cations	Leading ion	Cs^+ (from cesium carbonate) (Merck)
	Concentration	0.025 *M*
	Counter ion	Hydroxyisobutyric acid (Merck)
	pH	4.5
	Additives	0.05% Poly(vinyl alcohol) (Hoechst, Frankfurt, F.R.G.)
		3.73 m*M* 18-crown-6 (Merck)
	Terminating ion	H^+
	Counter ion	Acetate
	Concentration	0.005 *M*

Table I. Ultrafiltered samples were injected without further sample pretreatment. The injection volume was 0.2 μl.

High-performance liquid chromatography

After the ITP analyses the original sample was diluted 1:1 with a solution containing 0.027 mg per 100 ml of naphthalenesulphonic acid (Fluka, Buchs, Switzerland), which served as an internal standard. The HPLC equipment [8] will be described briefly. The column (250 mm×4.6 mm I.D.) was filled with 5-μm C_8-modified silica (prepacked, Ultrasphere Octyl; Altex, Berkeley, CA, U.S.A.). The guard column (30 mm×2 mm I.D.) was packed with 10-μm Ultrasphere Octyl. A solvent gradient between aqueous ammonium formate buffer (0.05 mol/l, pH 4) and methanol (Fisons, Loughborough, U.K.) was used for the analysis. The equipment consisted of two 100 A piston pumps, a Model 421 solvent controller, a Model 160 fixed-wavelength UV absorbance detector (254 nm, 0.025 a.u.f.s. sensitivity) and a Model 500 autosampler (all from Beckman, Berkeley, CA, U.S.A.). The fluorescence detector was a Shimadzu (Tokyo, Japan) Model RF-530. The excitation wavelength was 280 nm and the emission wavelength was 340 nm. Automated data processing was carried out with an IBM-XT computer with Nelson software (Nelson Analytical, Curpertino, CA, U.S.A.).

The analysis started with 0% methanol increased linearity to 60% within 45 min. After the analysis the concentration of methanol was increased to 100% to regenerate the column, then decreased to 0% for the next analysis. The flow-rate was 1.0 ml/min. One analysis, including regeneration, took 1.5 h.

The components were quantified by measuring the ratio of the peak height to that of the internal standard.

RESULTS

Fig. 1 shows typical isotachopherograms of two samples of anions, one sample from the fertile group and one from the infertile group. The differences between the two samples, especially zone 3, are remarkable. The zone between zones 1 and 2 in Fig. 1B were found only in this sample and it was not quantified. The zones indicated with numbers were measured and the median and range for each group were calculated (Table II). It is seen that the ranges are fairly large. Possible explanations are differences in the time interval until ovulation between mucus samples, differences in mucus composition from differences in ovarian activity, inter-individual differences or a factor in cervical mucus related to a disturbed metabolism.

Zone 3 has been identified as lactate. Zone 3 did not absorb in the UV region and had both in the mentioned pH 6 system and in a pH 3 system the same relative step height as lactate. The concentration of lactate in the sample was also determined using a specific enzymatic reaction. With lactate dehydrogenase lactate was converted into pyruvate simultaneously with the conversion of NAD^+ into NADH. The concentration of NADH was determined by measuring the absorbance at 340 nm. The concentration of lactate in the aqueous sample determined by ITP and by the enzymatic method were 10 and 11.6 mmol/l, respectively.

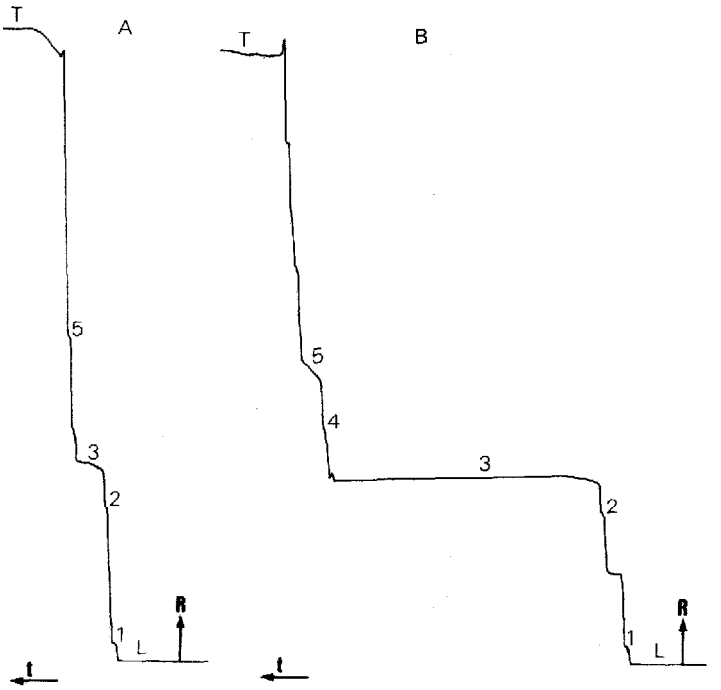

Fig. 1. (A) Isotachopherogram of the separation of anions of a sample taken from the fertile group. The operational system is listed in Table I. The numbers of the zones correspond to the numbers listed in Table II. Zone 3 has been identified as lactate. R = Resistance; t = time. (B) The same analysis for a sample originating from the infertile group.

TABLE II

MEDIAN CONCENTRATIONS (EXPRESSED AS ZONE LENGTH PER GRAM OF MUCUS) AND RANGES OF THE COMPONENTS MEASURED BY CAPILLARY ISOTACHOPHORESIS

Ions	No.	Concentration				Component
		Fertile group		Infertile group		
		Median	Range	Median	Range	
Anions	1	25.1	7.6- 81.0	49.5	6.9- 1524.5	
	2	15.0	0- 158.8	81.1	4.7- 2214.2	
	3	543.6	154.6-1405.0	1425.7	145.0-15272.3	Lactate
	4	12.6	0- 108.9	63.2	7.2- 371.2	
	5	38.9	9.4- 128.5	133.1	25.7- 1324.3	
Cations	6	134.2	71.2- 640.0	76.3	0- 330.6	Potassium
	7	2228.6	454.7-3484.85	2385.1	774.6- 5760.8	Sodium
	8	34.8	10.0- 108.0	44.9	10.2- 288.9	Calcium
	9	36.5	11.1- 104.7	34.3	0- 54.9	Magnesium

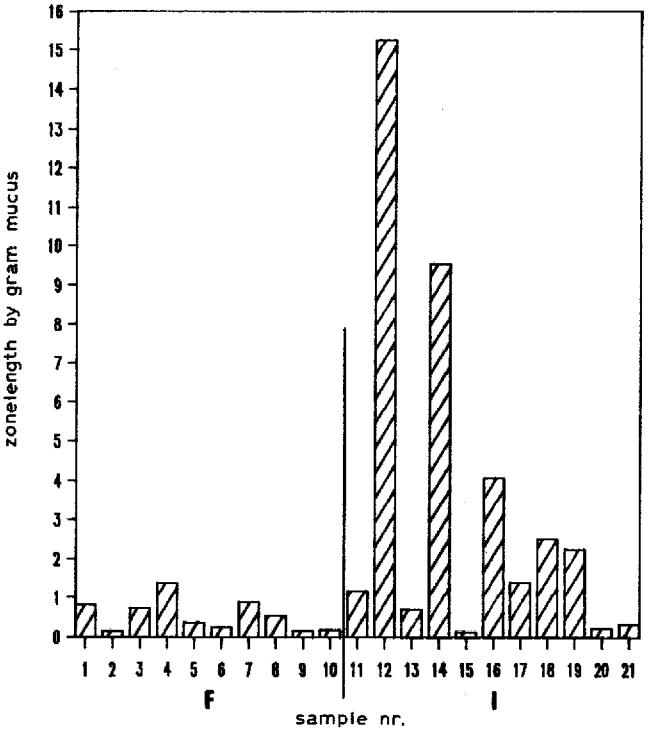

Fig. 2. Distribution of lactate concentration, in relative units, expressed as zone length (ITP quantitative parameter) per gram of mucus. Abscissa: sample numbers. F = fertile group; I = infertile group.

Fig. 2 shows the concentration of lactate for each sample. It is seen that the concentration of lactate of samples 12, 14, 16, 17, 18 and 19 of the infertile group is clearly higher than that of lactate in samples originating from the fertile group. Lactate is an end product of the carbohydrate metabolism. It has been reported that with cows an abnormal carbohydrate metabolism in the mucus may be an important cause for infertility [9]. Especially glucose, fructose, sorbitol, lactate and glucuronate are important components in this metabolism [9,10]. With ITP, however, only lactate can be determined. It was expected that with ITP glucuronate could also be determined but the concentration of glucuronate in preovulatory cervical mucus was below the detection limit. We are currently investigating the use of gas chromatography for the analysis of non-ionic carbohydrate components and glucuronate. Fig. 2 shows that lactate may be an indicator of carbohydrate metabolism.

In Table II the median concentrations of K^+, Na^+, Ca^{2+} and Mg^{2+} are also given.

In Fig. 3 chromatograms of a sample from the fertile group and of a sample from the infertile group are compared. Several peaks have been identified (Table III). These components belong to the following classes of substances: nucleosides (pseudo-uridine), purines (uric acid, hypoxanthine) and amino acids (tryptophan, tyrosine). The numbered peaks have been quantified and Table III shows

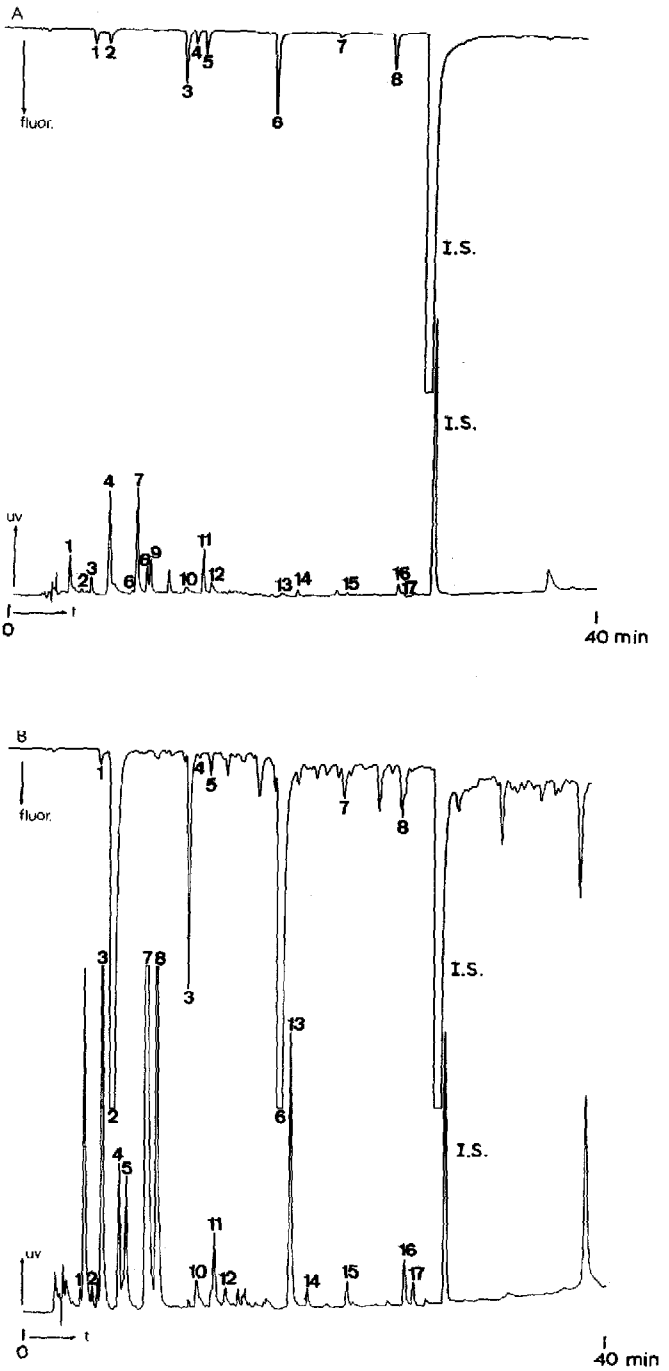

Fig. 3. (A) Chromatograms of the HPLC analysis of a sample from a fertile woman. The numbered peaks have been quantified in Table III. UV, 0.025 a.u.f.s.; fluorescence, arbitrary units. I.S. = internal standard. Stationary phase: reversed-phase (C_8, 5-μm particles). Mobile phase: gradient of 0.05 *M* ammonium formate buffer (pH 4) and methanol (0–60% methanol in 45 min). (B) Chromatogram of a sample taken from the infertile group.

TABLE III

MEDIAN CONCENTRATIONS (EXPRESSED AS RELATIVE PEAK HEIGHT PER GRAM OF MUCUS) AND RANGES OF THE COMPONENTS MEASURED BY HPLC

Type	No.	Concentration				Components
		Fertile group		Infertile group		
		Median	Range	Median	Range	
UV-absorbing components	1	10.9	2.5– 31.4	9.2	2.2– 28.2	Creatinine
	2	2.4	1.2– 11.5	4.1	1.3– 27.8	
	3	10.9	0– 27.7	18.1	0.7–211.8	Pseudouridine
	4	25.9	4.8– 75.6	19.2	0–153.4	Uric acid
	5	1.4	0– 4.1	2.2	0– 16.0	
	6	3.5	0– 20.3	0.0	0– 23.1	
	7	88.7	0–188.2	55.6	1.9–408.7	Hypoxanthine
	8	4.9	0– 19.6	5.9	1.4–140.1	
	9	10.3	1.0– 67.1	13.3	0–253.6	
	10	6.3	0– 25.2	5.1	1.2– 42.1	
	11	5.3	0.3–110.8	8.4	3.8– 70.3	
	12	3.3	0– 32.4	3.1	0.5– 38.0	*p*-Hydroxyhippuric acid
	13	3.2	0– 23.8	8.9	0– 45.4	Hippuric acid
	14	3.0	0.7– 17.1	3.5	0– 22.5	
	15	2.4	0– 9.3	4.0	0– 45.2	
	16	5.0	0.6– 44.9	9.1	2.9– 64.5	
	17	1.6	0– 20.6	3.6	0– 42.7	
Fluorescent components	1	0.1	0– 0.3	0.1	0– 0.7	
	2	0.2	0– 1.3	0.8	0– 4.7	Tyrosine
	3	0.3	0.1– 0.8	0.3	0.1– 0.8	
	4	0.0	0– 1.2	0.0	0– 2.7	
	5	0.1	0– 0.5	0.1	0.1– 1.2	
	6	1.0	0.2– 9.6	3.3	0.1– 18.1	Tryptophan
	7	0.0	0– 0.1	0.0	0– 0.2	
	8	0.2	0.1– 0.8	0.2	0.1– 1.8	

the median and range for each component. The median concentrations of several components are clearly higher in the infertile group than in the fertile group.

Six of the eleven infertile samples have values for several components that differ from those for the fertile samples, e.g., lactate. It must be stressed that women were placed in the infertile group when no causes had been found for their infertility. For some of the infertile group a possible explanation might be a disturbed metabolism in the cervical mucus. For others causes other than disturbances in the cervical mucus or endometrium are possible.

CONCLUSIONS

Human cervical mucus is a complex matrix in which many components of different size and structure can be found. Cervical mucus plays a role in fertility but not all disturbances at the level of cervical mucus have been clarified. The HPLC

and capillary ITP techniques presented have been found to be suitable for the analysis of the aqueous phase of human cervical mucus. These techniques provide information about substances with relatively low molecular masses, related to carbohydrate, nucleotide, nucleoside and amino acid metabolism.

Altogether, 34 compounds have been quantified: seventeen UV-absorbing and eight fluorescent components, five anionic and four cationic components. These components were determined in ten samples from fertile women and eleven samples from women with fertility problems. In both groups the median and ranges were calculated for each component. The median concentrations of several components were higher in the infertile group than in the fertile group. Six of the eleven samples from the infertile group have different values for several components compared with the fertile group. These differences may indicate disturbances in the biochemical processes in the cervical mucus. The high concentration of lactate in the infertile group, for example, may be an indication of a disturbance in glycolysis.

For the investigation of other biochemical pathways it will be necessary to identify more components and to analyse more samples. Especially the variation in the concentrations of several components during the menstrual cycle should be investigated.

REFERENCES

1 L.E. Kopito, H.J. Kosasky, S.H. Sturgis, B.L. Lieberman and H. Shwachman, Fertil. Steril., 24 (1973) 499.
2 D.P. Wolf, J.E. Sokoloski and M. Litt, Biochim. Biophys. Acta, 630 (1980) 545.
3 E.C. Yurewicz and K.S. Moghissi, J. Biol. Chem., 256 (1981) 11895.
4 I. Carlstedt, H. Lindgren, J.K. Sheehan, U. Ulmsten and L. Wingerup, Biochem. J., 211 (1983) 13.
5 B. Daunter, Scanning Electron Microsc., (1983) 343.
6 F.M. Everaerts, J.L. Beckers and Th.P.E.M. Verheggen, Isotachophoresis — Theory, Instrumentation and Applications, Journal of Chromatography Library, Vol. 6, Elsevier, Amsterdam, Oxford, New York, 1976.
7 M.M. Gladdines, A.A.G. Lemmens, B.J. Wanders and F.M. Everaerts, in preparation.
8 A.C. Schoots, H.R. Homan, M.M. Gladdines, C.A. Cramers, R. de Smet and S.M.G. Ringoir, Clin. Chim. Acta, 146 (1985) 37.
9 C.J.G. van der Horst, Cytobios, 45 (1986) 85.
10 T. Suga, Jpn. Agric. Res. Q., 9 (1975) 225.