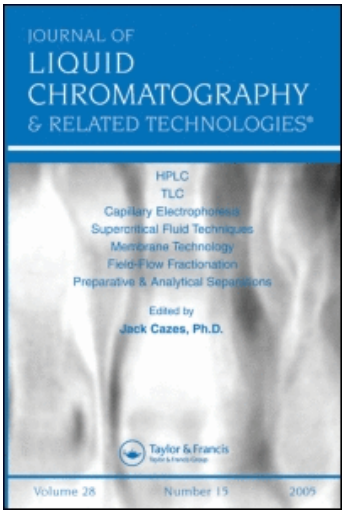

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Ultrafiltration and High-Performance Liquid Chromatographic Analysis of Seminal Carbohydrates, organic acids and Sugar Alcohols

P. Oefner[a]; G. Bonn[b]; G. Bartsch[a]
[a] Department of Urology University of Innsbruck, Innsbruck, Austrias [b] Institute of Radiochemistry University of Innsbruck, Innsbruck, Austria

To cite this Article Oefner, P. , Bonn, G. and Bartsch, G.(1985) 'Ultrafiltration and High-Performance Liquid Chromatographic Analysis of Seminal Carbohydrates, organic acids and Sugar Alcohols', Journal of Liquid Chromatography & Related Technologies, 8: 6, 1009 — 1023
To link to this Article: DOI: 10.1080/01483918508067125
URL: http://dx.doi.org/10.1080/01483918508067125

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(6), 1009-1023 (1985)

ULTRAFILTRATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SEMINAL CARBOHYDRATES, ORGANIC ACIDS AND SUGAR ALCOHOLS

P. Oefner, G. Bonn[1], and G. Bartsch

Department of Urology
University of Innsbruck
A-6020 Innsbruck, Austria

[1]*Institute of Radiochemistry*
University of Innsbruck
A-6020 Innsbruck, Austria

ABSTRACT

The efficiency of ultrafiltration and high-performance liquid chromatography (HPLC) in the analysis of the dialysable carbohydrates, organic acids and sugar alcohols in human seminal plasma is evaluated. Under standardized flow conditions an optimal separation of citric acid, inositol, fructose, sorbitol, ribose and lactic acid is achieved with a precision of ± 2% by coupling two polystyrene based strong cation-exchange resin columns in series. Proving equally efficient and reproducible, a single column allows a far more rapid routine analysis of citric acid and fructose in human seminal plasma, the system being linear within the biological range of concentrations. Moreover excellent correlation is found between enzymatic, isotachophoretic and high-performance liquid chromatographic analyses of citric acid and fructose. The efficiency of the method is further improved by means of ultrafiltration, making a simple and rapid preparation of protein-free samples possible and reducing the time of HPLC analysis by 40 minutes as compared to protein precipitation by means of methanol.

0148-3919/85/0806-1009$3.50/0

INTRODUCTION

Due to the small volume of human semen research on its biochemistry has been highly specific, the role of single known substances being assessed. On the other hand, conventional techniques, such as protein precipitation by means of organic solvents and ionic salts /1/ or enzymatic assays /2/, have shown to be too labor-intensive and time consuming. Hence, precise knowledge of the nature of infertility is still lacking. Since no curative treatment is at hand, a more extensive approach to the problem should be followed in order to obtain reliable information on a vast range of biochemical parameters. In recent years modern techniques have been developed that permit a simultaneous separation, identification and quantification of many substances in biological fluids. Ultrafiltration has been established to be an extremely reliable and convenient method for the preparation of protein-free samples /3-6/. Concerning the quantitative analysis of such structurally different compounds as alcohols, aldehydes, ketones, organic acids and carbohydrates, high performance liquid chromatography (HPLC) can be considered the most appropriate technique beside thin-layer chromatography /7-9/.

For decades citric acid and fructose have served as the most important chemical markers in assessing the functional status of male accessory organs /10-13/. Hence, the application of ultrafiltration and an isocratic HPLC system might be valuable in the rapid analysis of the dialysable portion of carbohydrates, organic acids and sugar alcohols occurring in human seminal plasma.

MATERIALS AND METHODS

Samples

Human seminal plasma was separated by low-speed centrifugation (GLC-4, Sorvall Instuments, Du Pont Company, Newtown, Conn., USA) at 1000xg for 10 minutes at room temperature.

Ultrafiltration

Seminal plasma was deproteinized by ultrafiltration, using the disposable Amicon micropartition system Centrifree (Amicon Corp., Danvers, MA, USA). The Centrifree system is an all-plastic device consisting of a single anisotropic, hydrophilic YMT membrane and an o-ring sealed between a sample reservoir, which is capped to prevent sample evaporation and pH change due to a loss of CO_2, and a support base, to which a removable filtrate collection cup is attached. The YMT membrane contains trace amounts of glycerin (2µL) and sodium azide (9 µg) as preservatives. 400-600 µL of seminal plasma were loaded into the sample reservoir and centrifuged in an angle-head rotor (Runne,Heidelberg,FRG) at 1500xg for 20 minutes at a temperature of 20 °C. Ultrafiltrates were stored at -30 °C.

HPLC

The liquid chromatographic system consisted of a pump (Beckman,Model 112, Beckman Inc.,Berkeley,Ca,USA), a sample injection valve (Beckman,Model 210) with a 20 µL loop, a differential refractive index detector (Altex-Beckman), a Shimadzu column oven unit CTO-2A and a Shimadzu C-R2A-X (Shimadzu Corpor., Kyoto,Japan) integration system. The stationary phase material consisted of pre-packed Aminex HPX 87H strong cation-exchange resin columns (300x7.8 mm I.D.,Bio-RAD), fitted with an ion exclusion micro-guard refill cartridge (Bio-RAD Labs., Richmond, CA, USA). The eluent was sulphuric acid (Merck, Darmstadt, FRG). All reference substances were of analytical grade and purchased from Fluka (Buchs, Switzerland) and Sigma (St.Louis, MO,USA). Prior to HPLC analysis all ultrafiltrates were diluted 1:1 with water distilled twice.

Isotachophoresis

The isotachophoretic analyses /14/ were carried out on an LKB 2127 Tachophor (LKB-Produkter AB,Bromma 1,Sweden), equipped with a 230

mm Teflon capillary with an internal diameter of 0.5 mm. The leading electrolyte system was 0.0015 M of hydrochloric acid (Suprapur, Merck,Darmstadt,FRG) titrated to pH 2.90 with glycylglycine (Merck, Darmstadt, FRG). The terminating electrolyte was 0.039 M of caproic acid (Merck, Darmstadt, FRG). Triton-X-100 (Serva, Heidelberg, FRG) was added to the leading and terminating ions to sharpen the zone boundaries by depressing electroendosmosis. The samples were injected by means of 10 µl Hamilton syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) through the inlet menbrane into the leading electrolyte. Separations were started with a current of 150 µA, which was reduced to 50 µA shortly before detection of the separated zones on account of their conductivity and UV absorption at 254 nm.

Enzymatic determination of fructose

The glucose/fructose test provided by Boehringer Mannheim (No.139106, Boehringer Mannheim GmbH, Mannheim, FRG) was used for the enzymatic determination of fructose in ultrafiltered human seminal plasma.

Enzymatic peak-shift

Converting fructose to fructose-6-phosphate, 100 µl of ultrafiltered human seminal plasma, diluted 1:9 with water distilled twice, were incubated in a reaction mixture, consisting of triethanolamine buffer (pH 7.6), 210 units of hexokinase, 15.8 mM of adenosine-5-triphosphate and magnesium sulphate, at a temperature of 37 °C for 90 minutes.

RESULTS AND DISCUSSION

Fig.1 shows the HPLC analysis of a mixture of organic acids, sugar alcohols and carbohydrates which usually occur in human

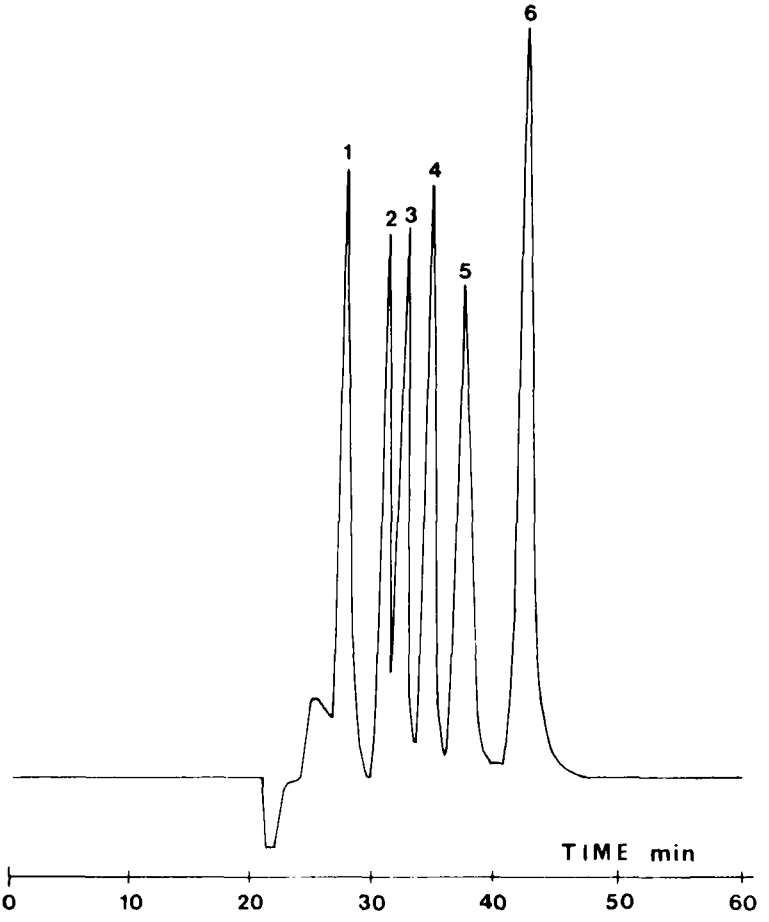

FIG.1 Separation of a standard mixture by isocratic HPLC; conditions: 2 x HPX 87H; mobile phase :0.01N H_2SO_4; flow rate:0.6 mL/min;column temp.: 40°C; detection: RI; injection loop: 20 µL
Peaks: 1...citric acid, 2...inositol, 3...fructose 4...sorbitol, 5...ribose, 6...lactic acid

seminal plasma. The mixture contained citric acid, inositol, fructose, sorbitol, ribose and lactic acid. By using two columns of polystyrene based strong cation-exchange resin as stationary phase and 0.01 N of sulphuric acid as mobile phase, optimal resolution was achieved at a column temperature of 40 °C and a flow rate of 0.6 mL/min.

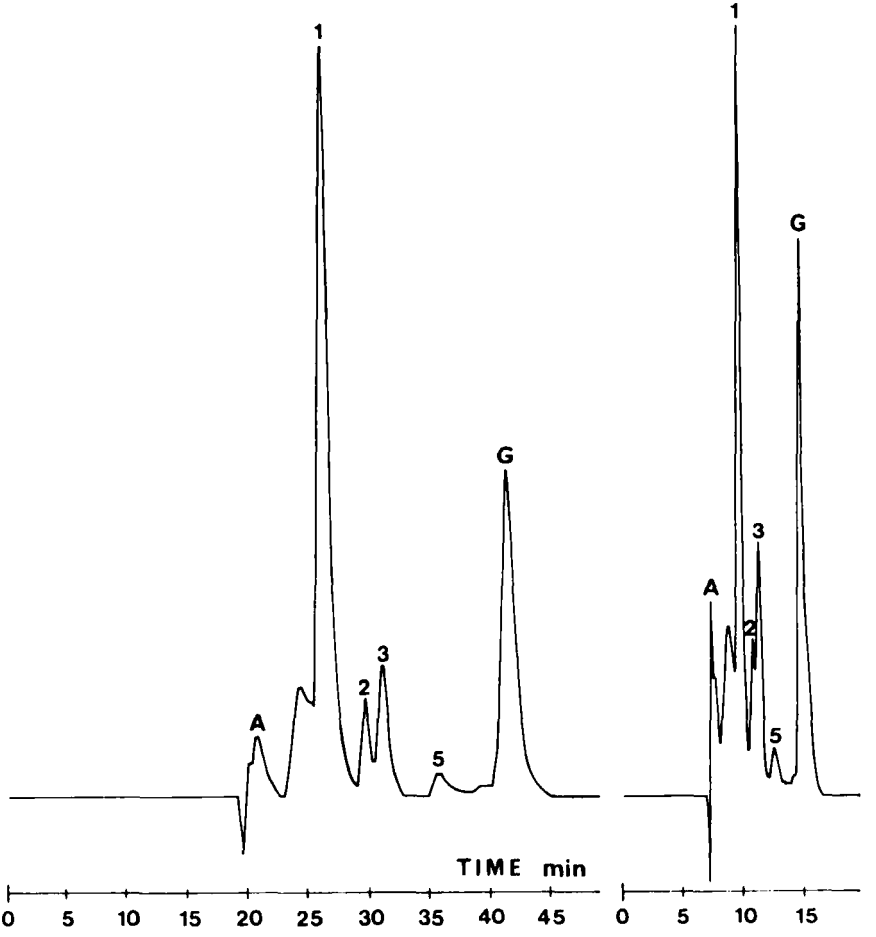

FIG.2 Comparison of chromatograms of human seminal plasma
A... 2 x HPX 87H; conditions: see FIG.1
B... 1 x HPX 87H; conditions: flow rate:0.6 ml/min; column temp.:40 °C; detection: RI
Peaks: A...choline and phosphate esters, 1...citric acid, 2...inositol, 3...fructose, 5...ribose, G... glycerin

Investigations revealed a detection limit of 0.05 mg/mL /8/; standard calibration curves of citric acid, inositol, fructose and lactic acid showed linearity over their biological ranges of concentrations. Checks of the reproducibility of peak area and height, which were carried out by multiple determinations with five different samples of human seminal plasma, yielded a precision of ± 2%. Under the same flow conditions the use of a single column did not only prove equally efficient and reproducible, but also far more rapid in the routine analysis of citric acid and fructose (Fig.2). Moreover a linear relationship between isotachophoretic and high — performance liquid chromatographic analyses of citric acid was observed in human seminal plasma (Fig.3).

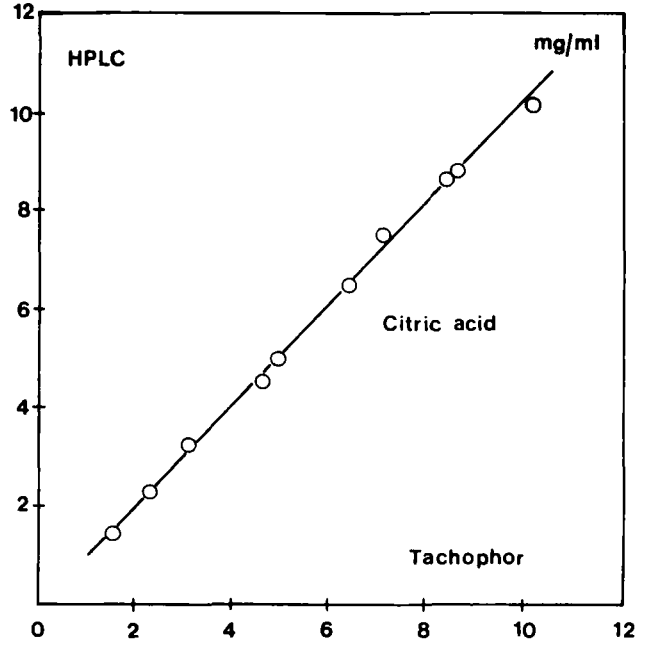

FIG.3 Relationship between the isotachophoretic and the high-performance liquid chromatographic analysis of citric acid in human seminal plasma

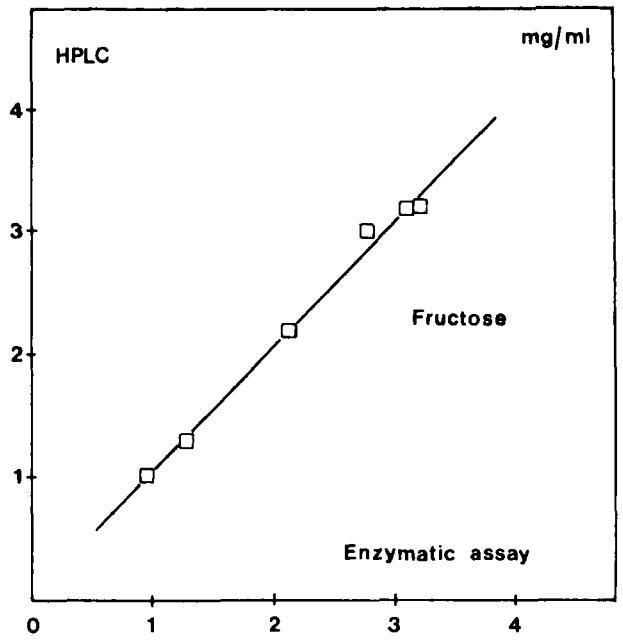

FIG.4 Relationship between the enzymatic and the HPLC-analysis of fructose in ultrafiltrated human seminal plasma

A comparison of the enzymatic assay of fructose and the high performance liquid chromatographic results also showed a good correlation (Fig.4). Besides, the enzymatic conversion of fructose to fructose-6-phosphate verified the identity of fructose and the specifity of its analysis.

Prior to HPLC analysis the Amicon microparti tion system Centrifree permitted a convenient deproteinization of seminal plasma under standardized conditions. The method was designed for the rapid preparation of ultrafiltrates and is based on low-speed centrifugation through a nonabsorptive filter with a high degree of protein retention. In contrast to equilibrium dialysis, protein-free filtrates can be obtained in a few minutes without dilution. Regarding the HPLC analysis of the dialysable portion of

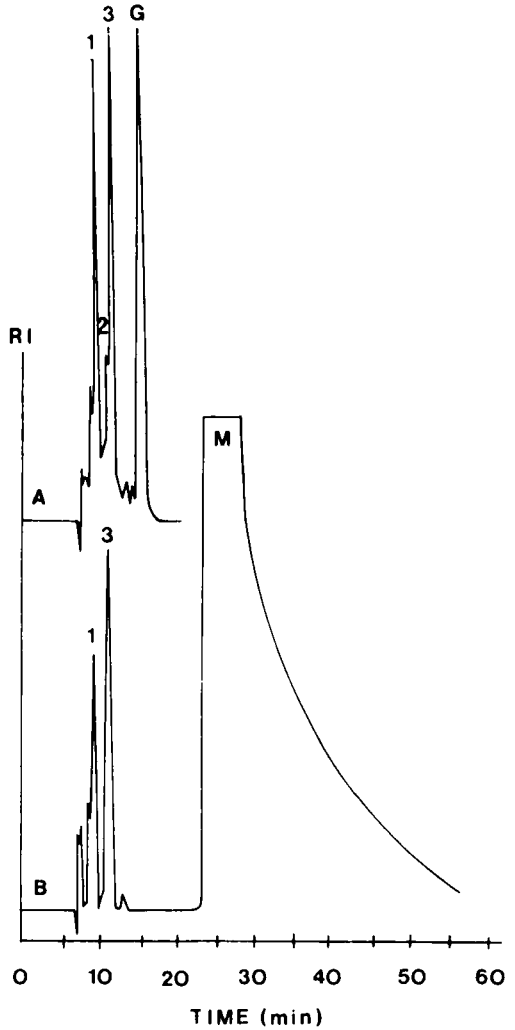

FIG.5 Comparison of chromatograms of human seminal plasma
(A) after ultrafiltration
(B) after methanol extraction
Conditions: see FIG.2 B
1...citric acid,2...inositol,3...fructose,G...glycerin,
M...methanol

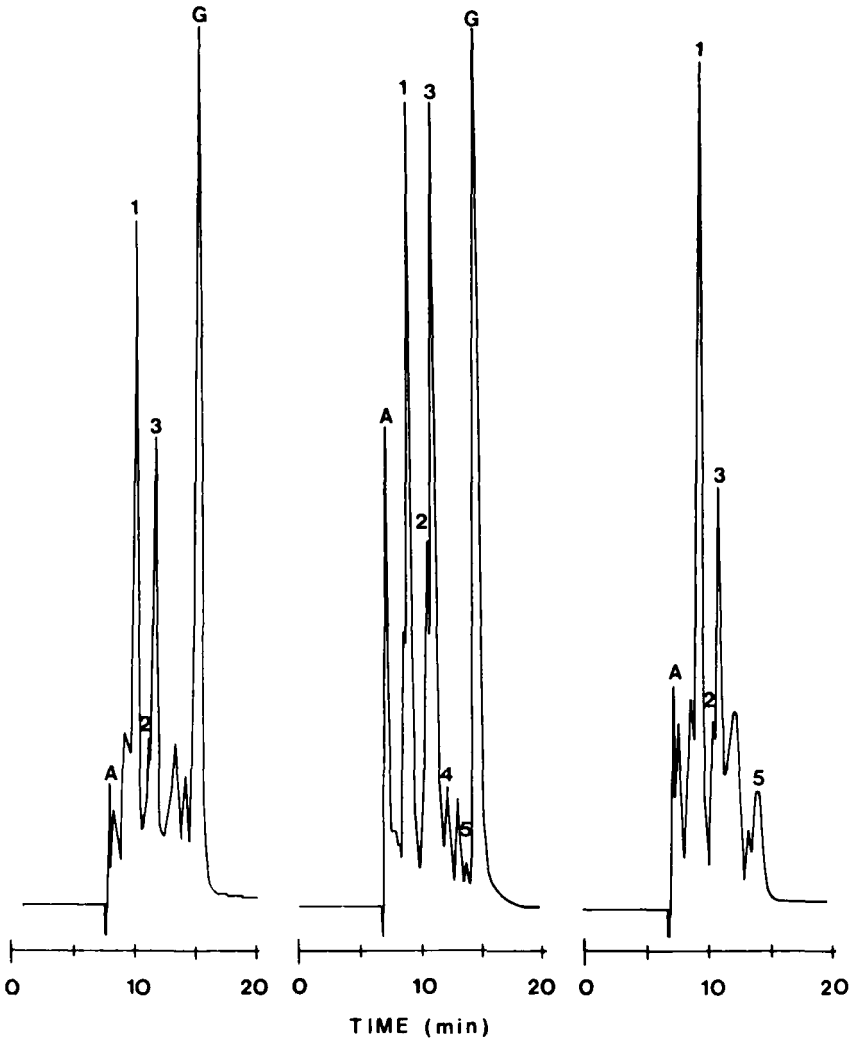

Fig.6 HPLC analyses of ultrafiltered human seminal plasma with variation of analytical and experimental conditions
(A) under optimized conditions, see Fig.1
(B) at a column temperature of 60 °C
(C) under optimized conditions, after having rinsed away the filter preservatives by means of deionized water
A...choline and phosphate esters, 1...citric acid, 2...inositol, 3...fructose, 4...ribose, 5...lactic acid, G...glycerin

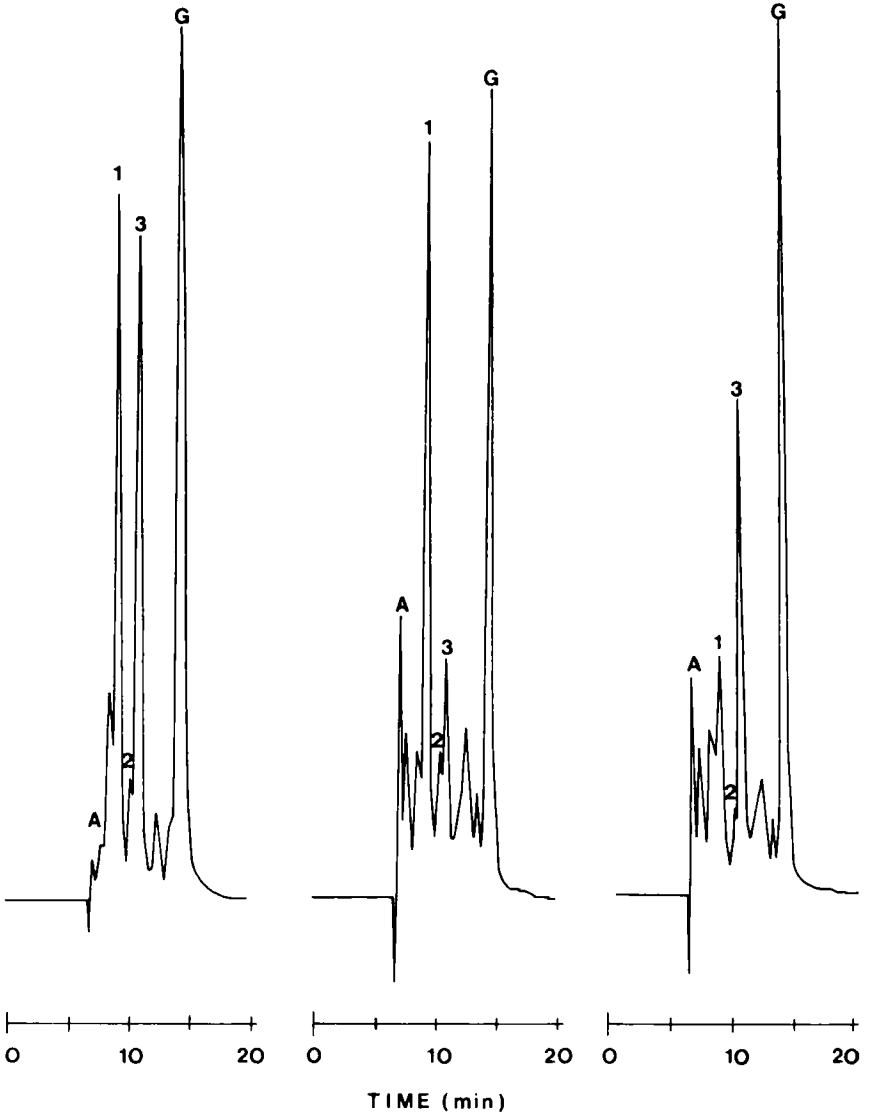

FIG.7 Chromatograms of different samples of ultrafiltered human seminal plasma;A...choline and phosphate esters,1...citric acid, 2...inositol, 3...fructose, G...glycerin
Conditions: see Fig.2 B

seminal carbohydrates, organic acids and sugar alcohols, ultrafiltration reduced the time of analysis by 40 minutes as compared to protein precipitation by means of methanol (Figs.5A and 5B).

The use of a fixed angle rotor is recommended since it provides polarization control by accumulation of macromolecules at the edge of the filter. When using an angle-head rotor flow rates are approximately 2-3 times higher than those obtained by means of a swinging-bucket rotor at the same transmembrane pressure.Thus 100-200 µL of ultrafiltrate can be gained from 400 µL of seminal plasma at 1000-2000xg in 20 minutes. The reproducibility of ultrafiltration amounted to 3%.

Since the YMT membrane in the device contains glycerin and sodium azide as preservatives, these substances may interfere with the HPLC analysis of the examined substances on the HPX 87H column. Concerning the analysis of seminal plasma under standardized flow conditions, glycerin masks the lactic acid peak (Fig.6A), which can be revealed either by increasing the column temperature to 60 °C (Fig.6B) and thus reducing the resolution of inositol, fructose and sorbitol, or by rinsing away the preservatives by centrifugation of 3 mL of buffer or deionized water through the device (Fig.6C). To avoid a consequent dilution error caused by entrapped fluid after rinsing, the first aliquot of ultrafiltrate (~10 µL) should be discarded. Since the accurate evaluation of citric acid and fructose concentrations was considered far more important within the scope of this study, the flow temperature was maintained at 40 °C. For the purpose of metabolic studies or the analysis of oviduct secretions quantification of lactic acid might be valuable /15-17/.

HPLC chromatograms of different semen samples are shown in Fig.7. The first two peaks (A) contain compounds such as choline and phosphate esters. Citric acid (1) serves as an important marker in the diagnosis of prostatitis /18/. Being essential for the survival and growth of cultured strains of mammalian cells /19,20/, inositol (2) may act as a reserve carbohydrate. Fructose

(3) has always been associated mainly with nutrition /1/. Besides fructose serves as an important parameter in evaluating the functional status of the seminal vesicles /12/. Only minor concentrations of ribose (4) have been seen so far. Quantitative studies performed on seminal plasma samples of 30 patients revealed a high biological variation of citric acid and fructose concentrations (7-63 mmol/L and 3.1-27.4 mmol/L respectively).

CONCLUSION

Ultrafiltration has proven to be superior to conventional precipitation methods in the preparation of protein-free samples. The development of a simple isocratic HPLC system permits a simultaneous separation, identification and quantification of seminal carbohydrates, organic acids and sugar alcohols within 16 minutes. HPLC might be a useful diagnostic aid in men presenting with infertility and urogenital disease.

ACKNOWLEDGMENTS

The authors thank Mrs.Evelyn Monz and Agnes Tautermann for technical assistance.This work was supported by grant no.P-4030, awarded by "Fonds zur Foerderung der wissenschaftlichen Forschung", Vienna, Austria.

REFERENCES

1. Blanchard,J., Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high performance liquid chromatographic analysis. J.Chromatogr., 226,455-460 (1981)

2. Bergmeyer, H.U., Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim (1974)

3. Sophianopoulos, J.A., Durham, S.J., Sophianopoulos, A.J., Ragsdale, H.L., Cropper, W.P., Ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out. Arch.Bioch.Bioph., 187, 132-137 (1978)

4. D Costa, M., Pei-Tak Cheng, Ultrafiltrable calcium and magnesium in ultrafiltrates of serum prepared with the Amicon MPS-1 system. Clin.Chem., 29 (3), 519-522 (1983)

5. Vlahos,I.,Mac Mahon,W.,Sgoutas,D.,Bowers,W.,Thompson,J.,Trawik W., An improved ultrafiltration method for determining free testosterone in serum, Clin.Chem.,28(11),2286 (1982)

6. Green,P.J., Yucis,M.J., Free-testosterone determination by ultrafiltration and comparison with dialysis. Clin.Chem., 28(5),1237 (1982)

7. Pecina,R., Bonn,G., Burtscher,E., Bobleter,O., High performance liquid chromatographic elution behaviour of alcohols, aldehydes, ketones, organic acids and carbohydrates on a strong cation exchange stationary phase, J.Chromatogr.287/2,245 (1984)

8. Binder,H., Separation of monosaccharides by high performance liquid chromatography - comparison of UV- and RI-detection, J.Chromatogr., 189,414 (1980)

9. Guerrant,G.O.,Lambert,M.A.,Moss,C.W., Analysis of short-chain acids from anaerobic bacteria by high performance liquid chromatography. J.Clin.Microbiol.,16(2),355-360 (1982)

10. Mann,T., Lutwak-Mann,C., Male reproductive function and semen. Themes and trends in physiology, biochemistry and investigative andrology, Springer (1981)

11. Kuehnau,J., Nowakowski, H., Untersuchungen ueber Ejakulatzitronensaeure beim Menschen, Endokrinologie, 40,1 (1960)

12. Eliasson,R., Correlation between the sperm density, morphology and motility and the secretory function of the accessory, genital glands, Andrologie,2,165 (1970)

13. Homonnai,Z.T., Paz,G., Weiss,J.N., David,M.P., Quality of semen obtained from 627 fertile men. Int.J.Androl., 3,217-228 (1980)

14. Oefner,P.J., Bonn,G., Bartsch,G., Isotachophoretic analysis of organic acids in human seminal plasma. Fresenius Z.Anal.Chem. (in press).

15. Jutte,N., Grottegoed,J.A., van der Molen,H.J., Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. J.Reprod.Fert.,62,399-405 (1981)

16. Mita,M., Hall,P.F., Metabolism of round spermatids from rats:lactate as the preferred substrate. Biol.Reprod., 26,445-455 (1982)

17. Holmdahl,T., Mastroianni,L., Continuous collection of rabbit oviduct secretions at low temperature. Fert.& Steril., 16(5),-587-594 (1965)

18. Perez-Plaza,M., Padron,R.S., Mas,J., Peralta,H., Semen analyses in men with asymptomatic genital gonorrhoea. Int.-J.Androl.,5,6-10 (1982)

19. Eagle,H.,Oyama,V.I.,Levy,M.,Freeman,A.E.F., myo-Inositol as an essential growth factor for normal and malignant human cells in tissue culture. J.Biol.Chem.,226,191-205 (1957)

20. Eagle,H.,Agranoff,B.W.,Snell,E.E., The biosynthesis of meso-inositol by cultured mammalian cells, and the parabiotic growth of inositol-dependent and inositol-independent strains. J.Biol.Chem.,235(7),1891-1893 (1960)