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Gel Filtration Chromatography Technique as Tool of Simple Study Seminal Plasma Proteins in Domestic Animals

Vasconcelos André Belico de*, Oliveira Jamil Silvano de, Lagares Monique de Albuquerque

[1]*University of Uberaba, Av. Nenê Sabino 1800, Uberaba, Minas Gerais, Brazil*

Abstract

Seminal fluid is the liquid component of sperm, providing a safe surrounding for spermatozoa. The seminal plasma has the feature common to many other body fluid, characterized by a high dynamic range of proteins, which visualizes physiological and biochemical processes of semen. As in other body fluids, it is convenient to distinguish in the seminal plasma between proteins and non-proteins. The molecular exclusion chromatographic technique presents important points to constitutional proteins preservation, in addition to the conventional phenomenon; witch exclusion hydrophobic interactions can provide a higher resolution in the chromatogram and do not provide non-specific interactions between protein structures. The aim was the chromatography profile to simple study of proteins seminal plasma domestic animals (Stallions, Canine, and Goat), by the technique of gel filtration chromatography. The samples (seminal plasma proteins) were chromatographed in a Superose 12 HR 10/30, equilibrated with 25 mMTris-HCl (Sigma) with 0.15 M NaCl (Sigma), pH 7.4 at room temperature in a fast performance liquid chromatography (FPLC-system), using a flow rate of 0.5 mL.min-1. There was a molecular separation of significantly different molecular weights, which makes possible the logarithmic relationship between molecular weight and the elution volume. Calibration of the gel filtration column resulted in an equation y = ax + b, where the values of a and b were 5.43 and -2.175, respectively, and the corresponding errors were 0.115 and 0.256, respectively. The experimental error was less than 5% for most of the protein molecular masses. The work showed that the gel filtration chromatography technique provided an excellent analytical repeatability, and could therefore, be a valuable tool to the study preliminary of seminal plasma.

Keywords: Stallion; Canine; Goat; Protein

Introduction

Serious chemical analysis of the seminal plasma was taken up during the present century, leading to the discovery and identification of several substances as the proteins [1]. There is increasing evidence for the multifunctional nature of proteins. It is possible that they may have different functions in the reproductive process [2,3], as biochemical markers for identification of biological properties of animal semen [4,5]. The number of distinct types of proteins normally present in the seminal plasma also varies, but most of the seminal proteins is distributed among four to ten fractions, the proportions of which vary individually and in relation to the time interval intervening between ejaculation analysis [1]. In this respect the high performance the technique of gel filtration chromatography methods is a distinct advantage as study the constituent proteins seminal plasma separating by the molecular size [6-9]. The aim was the chromatography profile to simple study of proteins seminal plasma domestic animals (Stallions, Canine, and Goat), by the technique of gel filtration chromatography, using a Superose 12 HR 10/30, in a fast performance liquid chromatography (FPLC-system).

Materials and Methods

Animals

Ejaculates of six stallions (5-20 years old) were collected using an artificial vagina (model "Hannover"), the ejaculates of six canines (2-4 year old) were collected by masturbation with a glove or bare hand, and the ejaculates of two goats (2- years old) were by electroejaculator. Semen samples were evaluated for progressive motility with a bright field microscopy (100×). Ejaculates containing a minimum of 50% of spermatozoa with progressive motility were used in the study. All samples were cooled to +5°C with a cooling rate of 1°C/min; such a rate does not induce a cold-shock effect [9-11].

Seminal plasma proteins purifications

To obtain isolated seminal plasma proteins, semen was centrifuged for 30 min at 4°C (600 g). Then, the supernatant was brought to 36% (wt/vol) saturation with ammonium sulfate adjusted to pH 2.0 with 6 mol L^{-1} HCl, stirred for 30 min, and allowed to stand at 0°C for 30 min [10]. The samples were centrifuged again (600×g, 4°C, 30 min), the supernatant and the ammonium sulfate pellet were dialyzed for 24 h up to a 8.000 dilution factor with 0.5% (by volume) aqueous acetic acid solution using exclusion membrane of 1,000 Da cut-off, and finally the sample were lyophilized, freeze-dried and stored -20°C, according to Vasconcelos et al. [11].

Protein concentration assay was proceeded in test tube, mixing bradford reagent with protein sample (1: 0.1 mL portions, respectively) and strained with coomassie brilliant blue BG-250 [12]. Then, absorbance was measured at 595 nm by spectrophotometry (Shimadzu-160A).

Chromatography analysis

The seminal samples purifications (0.5 mL; 0.5 mL and 0.2 mL) of stallion, canine and goat, respectively were applied to a Superose 12

***Corresponding author:** Vasconcelos André Belico de, University of Uberaba, Av. Nenê Sabino 1800, Uberaba, Minas Gerais, Brazil, CEP 38061-500, Tel: +55-34-88120157; Fax: +55-34-33148910; E-mail: devasconcelos.a.b@gmail.com, andre.vasconcelos@uniube.br

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HR 10/30 column, molecular exclusion chromatography, equilibrated with 25 mM Tris-HCl (Sigma) with 0.15 M NaCl (Sigma), pH 7.4 at room temperature in a fast performance liquid chromatography (FPLC-system), using a flow rate of 0.5 mL.min^{-1} and collected fraction volume of 1.5 mL. The mobile phase was the same solution used for equilibration. The Column was calibrated according to the methods of Andrews [13]. Molecular weight markers (β-amilase 200 kDa, Alcohol desidrogenase 150 kDa, Bovine Albumin 66 kDa, Anidrase carbonic 29 kDa, Cytochrome C 12,4 kDa, Aprotinin 6,5 kDa). Molecular weight markers were like a chromatography marker. Total protein was estimated in each seminal plasma sample by spectrophotometry (Shimadzu-160A); absorbencies were read at 215 nm [14].

Statistical analysis

The linear regression between seminal plasma protein concentration, relative mass and retention time were calculated using the software (Origin version 5®).

Results and Discussion

Chromatographic calibration

Calibration results of the gel filtration column: y = ax +b; parameters (value, error): a= 5.43, 0.115; b= - 2.175, 0.256; R2= 0.973; ($p<0.001$), and exclusion volume (Vo) 7.64 min. Our results were according to Andrews methods [15] the experiment error was smaller than 5% for more in the molecular mass the protein.

Chromatography proteins

The proteins chromatography of the stallion seminal plasma were represent by 12 fractions with four different retention time peaks (t_M), according to the chromatogram (Figure 1) in six animals studied. Only four picks graphic areas were selected by showing the relative mass (M_R) increase.

The results emphasized the molecular exclusion technique, characterized by the proteins separation of high molecular weight, proceeding to low molecular weight. The proteins relative mass (M_R), the retention picks value among proteins samples of the seminal plasma were presented (Table 1). This stallion seminal plasma proteins were statistically similar in all animals ($p<0.05$), as related by Vasconcelos et al. [11].

The chromatogram of canine seminal plasma proteins (Figure 2) described several retention peaks. The relative mass were calculated according to retention time (23 min; 30.6 min; 37 min), in view of the greater proteins concentration in this range, by spectrophotometer analysis.

The retention time and molecular weight averages calculation, of each sample, related to each canine ejaculate suggests the repeatability of the technique represented by the equivalence between the results measurement (Table 2). Where molecular weights can be uncertain, the most likely values indicated often correspond to values suggested by the gel-filtration results. The technique repeatability represented among the measurements results, because there were no statistical difference between animals ($p<0.05$). This repeatability theory described by Vial and Jardy [15] defined that the equipment calibration were characterized by the use of standard proteins, according to this work, and the effective selective separation protocol of seminal plasma proteins.

Chromatographic samples analyze of goat seminal plasma were presented in five peaks (Figure 3). The results showed that proteins

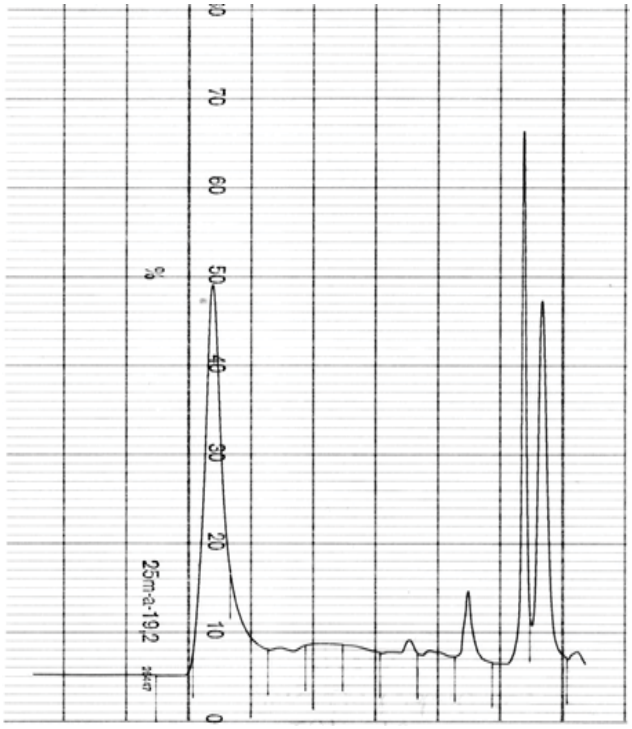

Figure 1: Stallion seminal plasma proteins by chromatography analysis. Superose 12 Column HR 10/30 (FPLC system; 0.025M Tris-HCl buffer pH 7.4; flow rate 0.5 ml/min, sensibilitiy 0.5).

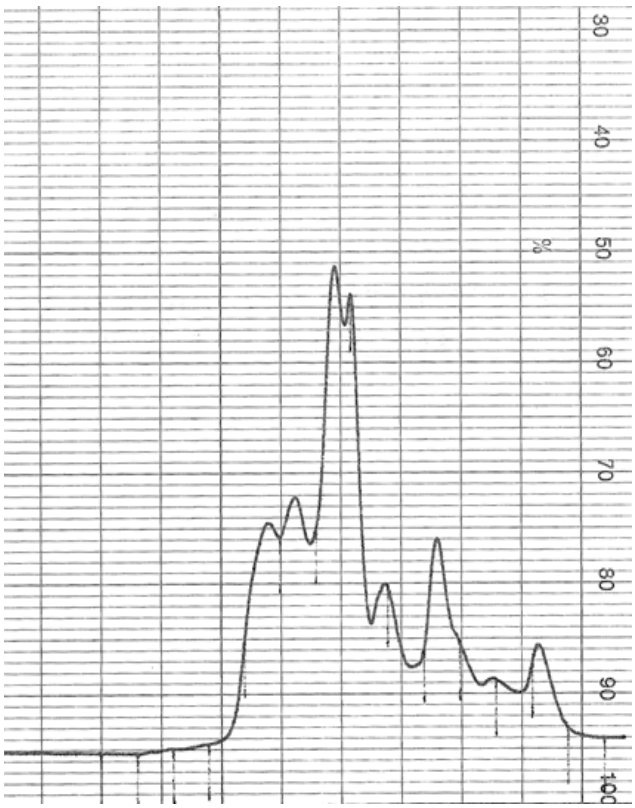

Figure 2: Canine seminal plasma proteins by Chromatography analysis. Superose 12 Column HR 10/30 (FPLC system; 0.025M Tris-HCl buffer pH7.6; flow rate 0.5 ml/min, sensibilitiy 0.5).

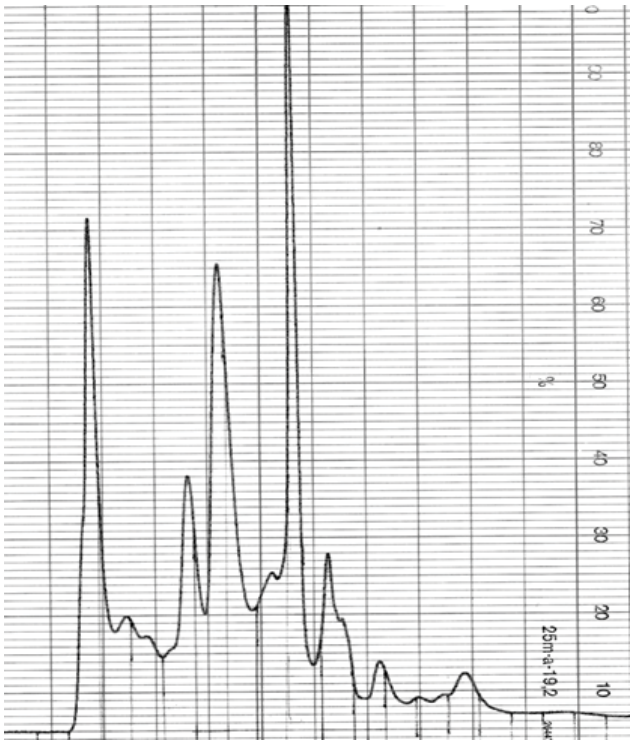

Figure 3: Goat seminal plasma proteins by chromatography analysis. Superose 12 Column HR 10/30 (FPLC system; 0.025M Tris-HCl buffer pH7.6; flow rate 0.5 ml/min, sensibilitiy 0.5).

variability were seen between the two goats evaluated and the five picks were maintained. This variability can occur since the proteins may have different structural characteristics as a free carbon [15]. However, this technique described a good correlation between relative mass (M_R) and gel-filtration behavior.

The similarity among the chromatographic profiles obtained from different animals was evaluated through the comparison of the retention time of the signs chromatographically observed and quantified in the Table 3. The related in work with gel exclusion chromatography on G-200 Shephadex performed by Mann [1] showed that two of the basic proteins were purified to apparent homogeneity.

During the analysis of a new sample, it is possible that not all frequently detected components will be. The failure of their detection indicates that there is a variation in the identifications process concerning mainly technical aspects, like spot excision, digestion, peptide recovery, mass spectra acquisition, and identity assignment [16]. This technique of gel filtration chromatography can be a tool for studying the initial fraction seminal plasma proteins.

A proteomic analysis involves proteins separation and proteins identification as well as characterization of the post-translational modifications. The limitations in a proteomic analysis are of two kinds: (i) those related to the composition of the proteome to be analyzed, mainly concerning protein expression levels and (ii) limitations of the analytical methods [14]. The chromatographic methods can reduce the complexity of the proteins mixtures because of different binding's principles, and every approach adds a unique resolving power. The standardization using known proteins enable one reason for study area in chromatogram and define a relative protein samples mass present in

Stallion	w_b 1		w_b 2		w_b 3		w_b 4	
	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)
1	14.7	295120.9	34.8	13803.8	38.7	7585.8	40.7	5495.4
2	15.1	275422.9	34.5	14454.4	38.9	7244.4	40.9	5370.3
3	15.2	275422.9	35.0	13182.6	39.0	7244.4	40.0	6165.9
4	14.9	288403.2	35.7	12022.6	39.7	6456.5	40.7	5495.4
5	14.6	301995.2	35.1	13182.6	39.6	6606.9	40.6	5623.4
6	14.9	288403.2	34.9	13489.8	39.1	7079.5	40.1	6025.9

Data the same column were not statistically different ($p<0.05$).

Table 1: Values of retention time (t_M) and relative mass (M_R) in the stallion seminal plasma proteins after chromatography of molecular exclusion.

Canine	w_b 1		w_b 2		w_b 3	
	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)
1	23.2	81283.1	30.6	26302.7	37.6	8912.5
2	23.2	81283.1	30.6	26302.7	37.7	8709.6
3	23.1	81283.1	30.8	25118.9	37.5	8912.5
4	23.1	81283.1	30.7	25703.9	37.8	8709.6
5	23.2	81283.1	30.9	24547.1	37.3	9332.5
6	23.2	81283.1	30.6	26302.7	37.7	8709.6

Data the same column were not statistically different ($p<0.05$).

Table 2: Values of the estimate of total protein and of retention times (t_M) of the samples of canine seminal plasma.

Goat	w_b 1		w_b 2		w_b 3		w_b 4		w_b 5	
	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)	(t_M) (min)	M_R (Da)
1	15.5	251188.9	24.5	63095.7	27.9	39810.7	34.6	12589.3	43.9	3162.3
2	15.9	251188.6	24.7	63095.7	28.2	39810.7	35.1	12589.3	43.7	3162.3

Data the same column were not statistically different ($p<0.05$).

Table 3: Results of time retention (t_M) and values of the estimate of relative mass (M_R) of goat seminal plasma samples.

biological fluids. This technique allows that these molecules interaction with the column matrix do not occur [17,18], concluded that the physico-chemical properties of the technique does not interfere with the protein structure sample, allowing to monitor the proteins variability presented in the plasma seminal of individuals and species studied.

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