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Determination of anions in human and animal tear fluid and blood serum by ion chromatography

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Abstract

An important factor contributing to the development of ion chromatography (IC) has been the need for repetitive analyses of samples with high ionic contents and samples available in microvolumes. IC was selected for the determination of Cl^-, NO_3^-, SO_4^{2-} and PO_4^{3-} anions in tear fluid and serum from ten human volunteers of both sexes, seven young-adult black vultures (*Coragyps atratus*) and three young-adult chickens (*Gallus gallus domesticus*). The samples were analysed on a Dionex Model 2000i/SP ion-exchange chromatograph equipped with an anion guard column (*Dionex* IonPac AG4A), anion separator column (*Dionex* IonPac AS4A), suppressor column (*Dionex* AMMS-II) and a conductivity detector. The flow-rate of the mobile phase, 1.7 m*M* $NaHCO_3$–1.8 m*M* Na_2CO_3 was set at 2.0 ml/min. The R.S.D. was calculated to be less than 1.5% for all anions. In the human, black vulture and chicken serum samples, the NO_3^-, PO_4^{3-} and SO_4^{2-} anion contents were higher than in tears; for Cl^- the reverse was found. No correlation was found amongst the anion concentrations present in the tear fluid and blood serum in all samples ($p > 0.05$). With no sample treatment, column maintenance was required.

1. Introduction

Chemical equilibrium in the animal body is achieved through fluid, electrolyte and acid–base balance, all responsible for its dynamic condition. Extracellular fluids are continuously mixed by the circulatory system and fluid interchange takes place between capillaries and interstitial space. This chemical exchange reflect the normal volume, distribution, composition and pH of body fluids. These fluids constitute both the external and internal environments of the cell, serving various important functions. They are composed of water and numerous solutes, comprising electrolytes and non-electrolytes.

Fluid and electrolytes contained in the intracellular and extracellular space have distinct electrolyte patterns. In the intracellular fluid [1], potassium is the major cation and phosphate and proteins are the major anions. In the extracellular fluid, sodium is the major cation and chloride the major anion. An important difference amongst the components in the intravascular and interstitial fluid is the greater concentration of negatively charged proteins in the intravascular fluid. Blood serum serves as an essential electrolyte source for other body fluids, reflecting its general composition in all extracellular fluids.

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As body fluids are regulated by the same mechanism, balance disruption in one of the compartments induces a corresponding alteration in the electrolyte concentration in other body spaces and fluids, including serum and lachrymal secretions [2]. Tear fluid may serve as a biological source of reference electrolyte levels, when available. The ionic content of lachrymal fluid, amongst other elements, is significantly responsible for tear osmolarity. The cornea, conjunctiva and the integrity of other ocular tissues depend on this biological lubricant. Additionally, a number of tear constituents bear a relationship to elements present in blood serum. Its composition includes proteins, lipids, metabolites, ions and excretable drugs [3]. Potassium, sodium and chloride ions have been reported to be present in higher concentrations than in plasma. No studies on the anion content of tear fluid could be found in the literature. The average tear pH is 7.35 and the osmolarity of tear films varies between 295 and 309 mOsm/l [4].

In plasma, the chloride concentration has been reported to be between 97 and 103 mmol/l [5] and in tear fluid between 100 and 138 mmol/l [6–8]. The levels of phosphorus and calcium are internally related as both metabolisms are regulated by the same mechanisms.

Higher animals, such as bovines, may utilize ammonia as a nitrogen source for the synthesis of non-essential amino acids, but are unable to use nitrate [9], nitrite or gaseous nitrogen. The presence of nitrate in serum and tear fluid may be considered as an indicator of blood urea-nitrogen linked to homeostatic balance [10].

The sulfate anion present in plasma, tears and other body fluids may be difficult to associate with particular metabolic functions or homeostatic balance. Its presence may be related to sulfur amino acids and their metabolic pathway.

Vultures exhibit particular feeding habits where severe chemical and biological contamination may be present, without apparently affecting their overall healthy status. Eye involvement during their feeding process does not result in the development of ocular diseases. Lachrymal secretion may play an important role in a non-specific protective mechanism and amongst the tear constituents, the anion content has not been reported.

The study of certain serum cations by ion chromatography (IC) [11] and capillary zone electrophoresis [12] has been reported. In this work, the basal chloride, nitrate, phosphate and sulfate anion contents in tears and serum samples from humans, black vultures and chickens were determined by IC. The values obtained were analysed and general relationships were determined. Chickens were included in this study to establish comparable anion values in samples from a well documented source.

2. Experimental

2.1. Sample collection

Tears

Tear samples were obtained from the lower parpebral sac and internal canthus of seven adult black vultures (*Coragyps atratus*) with an average mass of 1.5 kg ± 300 g. Tear samples from individual specimens ranged from 10 to 100 μl and were pooled until a working volume of at least 150 μl was obtained. A similar procedure was applied for the collection of the human and chicken samples. The black vulture specimens were captured in a nearby rural area (Palmarejo, Estado Zulia, Venezuela) between June 1993 and February 1994. For comparison purposes, three domestic chickens (*Gallus gallus domesticus*) were included in the study, with an average mass of 2.15 kg ± 200 g. The specimens were acquired locally.

Ten human adults (five males and five females, all volunteers) participated in the investigation. Their mean age was 20 ± 3 years. Tear samples were collected as indicated previously.

Tear volumes of up to 150 μl per individual (animal and human) were collected with a blood dilution pipette (Thomas Type, 120 mm). Prior to use, the pipettes were washed with non-ionic soap and rinsed with doubly distilled water, 95% (v/v) ethanol, acetone, distilled water and finally deionized water.

Serum

Blood samples were obtained from the brachial or marginal wing vein of seven healthy adult black vultures and three healthy chickens, using a venous infusion set with a 22 XG needle. A maximum of 2.0 ml of whole blood was collected from each bird and dispensed into glass test-tubes. Each sample was allowed to coagulate for 20 min at room temperature and was then centrifuged in a Hettich Model EBA 35 centrifuge at 2000 rpm for 20 min. The serum aliquots were kept refrigerated at 4°C until needed. The serum samples were processed within ten consecutive days.

Human blood samples were obtained by venipuncture of the anticubital vein from healthy young adult volunteers (five males and five females) aged 20 ± 3 years. Whole blood was collected with a 6-ml syringe, coupled to a 22 XG needle. The volume collected ranged from 4 to 5 ml. The serum was processed as indicated previously.

2.2. Sample preparation

Tears

During collection, tear aliquots were maintained at 4°C and they were subsequently refrigerated at −70°C in 1.5-ml Eppendorff capped vials until processed. Prior to analysis, the samples were allowed to thaw and maintained under refrigeration at 4°C. Then they were centrifuged at 13500 rpm for 10 min in an Eppendorff Model 5412 centrifuge. The samples were diluted 100-fold in deionized water and filtered immediately before chromatography.

Serum

Serum samples were allowed to reach room temperature (28°C), before analysis. A 100-fold dilution was prepared with deionized water and filtered before chromatography.

2.3. Reagents, apparatus and conditions

Reagents

All reagents were filtered at least twice through a 0.22-μm Millipore membrane filter and were of the highest purity. Deionized water was used for dilution. Calibration standards were prepared by serial dilutions of stock solutions containing 28.2 mmol/l Cl^-, 71.4 mmol/l N-NO_3^-, 32.3 mmol/l P-PO_4^{3-} and 10.4 mmol/l SO_4^{2-}.

Apparatus

Samples were analysed on a Dionex Model 2000i/SP ion chromatograph equipped with a (Dionex AG4A) anionic pre-column, a (Dionex AS4A) anionic column separator, a (Dionex AMMs-II) suppressor column, a Dionex Conductivity Detector II CDM conductivity detector and a (Dionex 4400) integrator.

Chromatographic conditions

Standards and samples were membrane filtered before injection into the chromatograph. The analytical run time was 10 min, which allowed resolution of the peaks. External standardization was used, with recalibration following every ten samples and column washing with deionized water. Tetrabutylammonium hydroxide was used occasionally to prevent column poisoning. The mobile phase (flow-rate 2.0 ml/min) was 1.7 m*M* $NaHCO_3$–1.8 m*M* Na_2CO_3 and the regenerating solution was 12.5 m*M* H_2SO_4. The injection volume, conductivity sensitivity and chart speed were 100 μl, 30 μs and 0.5 cm/s, respectively. The integrator attenuation range was from 0.5 to 4096.

3. Results and discussion

3.1. Ion chromatography

Typical human, vulture and chicken serum anion chromatograms are illustrated in Fig. 1. The corresponding tear chromatograms are represented in Fig. 2. The detection limits were $Cl^- = 2.7$ μg/l, $NO_3^- = 5.6$ μg/l, $PO_4^{3-} = 5.0$ μg/l and $SO_4^{2-} = 3.318$ μg/l. The R.S.D. for all anions was less than 1.5% ($n = 18$).

IC has previously been compared with potentiometric methods in a reference laboratory

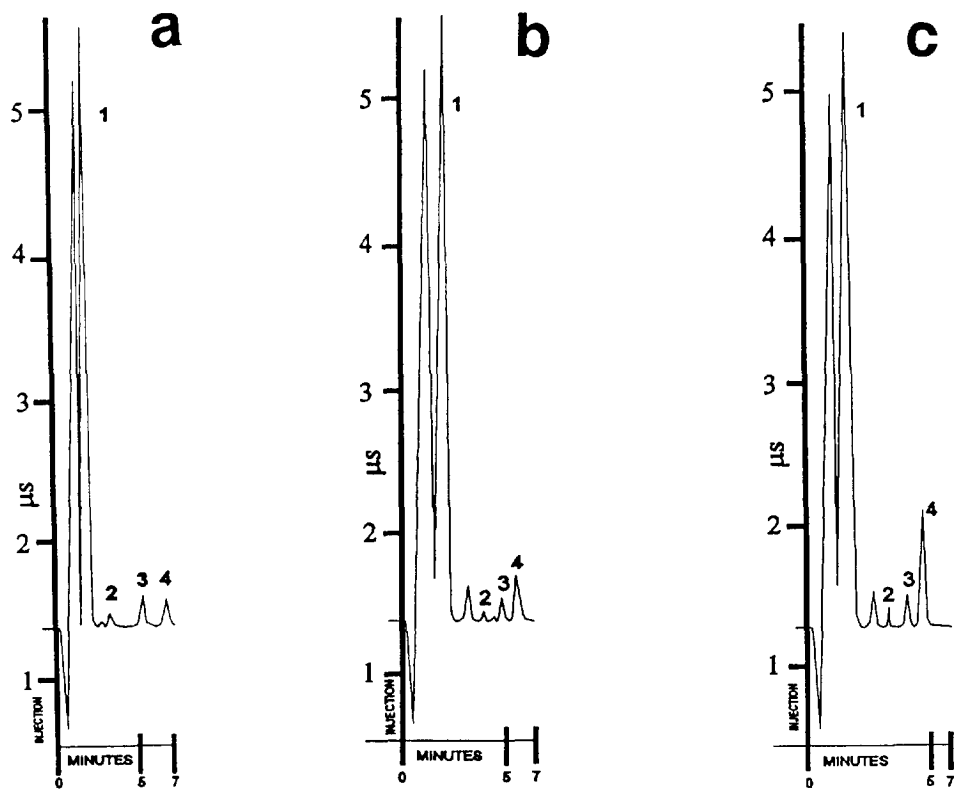

Fig. 1. (a) Chromatogram of human serum sample. Peaks: 1 = Cl (44.86 mmol/l); 2 = NO_3^- (0.17 mmol/l); 3 = PO_4^{3-} (2.04 mmol/l); 4 = SO_4^{2-} (0.54 mmol/l). Chart range: 65 μs FS. (b) Chromatogram of black vulture serum sample. Peaks: 1 = Cl^- (42.96 mmol/l); 2 = NO_3^- (0.40 mmol/l); 3 = PO_4^{3-} (1.37 mmol/l); 4 = SO_4^{2-} (0.66 mmol/l). Chart range: 65 μs FS. (c) Chromatogram of chicken serum sample. Peaks: 1 = Cl (40.35 mmol/l); 2 = NO_3^- (0.20 mmol/l); 3 = PO_4^{3-} (1.88 mmol/l); 4 = SO_4^{2-} (2.00 mmol/l). Chart range: 65 μs FS.

using an ion-selective electrode [13]. The results obtained in that study were in good agreement with those obtained by IC.

3.2. Mean anion concentrations in human serum and tear samples

The mean nitrate (0.14 mmol/l), phosphate (0.22 mmol/l) and sulfate (0.39 mmol/l) anion concentrations obtained were 26, 85 and 26% lower, respectively, in the human tear samples than in serum (0.19, 1.42 and 0.53 mmol/l, respectively). The opposite effect was observed for chloride ion (Tables 1 and 2). The high levels of chloride ion in the tear fluid, with a maximum value of 104.81 mmol/l, were in agreement with the mean reported concentration of 100 mmol/l [14].

3.3. Mean anion concentrations in vulture serum and tear samples

Nitrate (0.29 mmol/l), phosphate (1.27 mmol/l) and sulfate (0.75 mmol/l) concentrations determined in black vulture serum (Table 1) were 62, 9 and 11%, higher, respectively, than in tear samples (Table 2). The overall mean chloride ion concentration determined in the tear samples (82.59 mmol/l) was 47% higher than in serum (43.71 mmol/l). This chloride concentration pattern was constant throughout the values obtained, from all samples studied except for chicken.

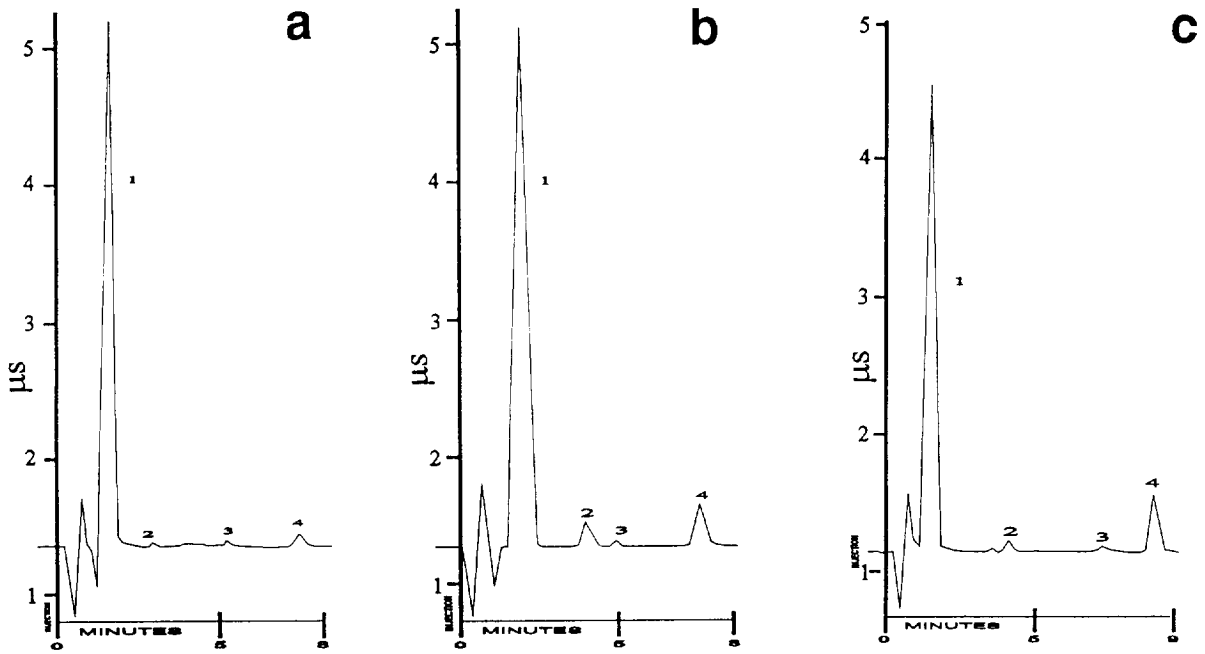

Fig. 2. (a) Chromatogram of human tear sample. Peaks: 1 = Cl⁻ (104.81 mmol/l); 2 = NO_3^- (0.13 mmol/l); 3 = PO_4^{3-} (0.52 mmol/l); 4 = SO_4^{2-} (0.17 mmol/l). Chart range: 65 µs FS. (b) Chromatogram of black vulture tear sample. Peaks: 1 = Cl^- (107.01 mmol/l); 2 = NO_3^- (0.16 mmol/l); 3 = PO_4^{3-} (0.84 mmol/l); 4 = SO_4^{2-} (0.45 mmol/l). Chart range: 65 µs FS. (c) Chromatogram of chicken tear sample. Peaks: 1 = Cl⁻ (186.30 mmol/l); 2 = NO_3^- (0.67 mmol/l); 3 = PO_4^{3-} (1.08 mmol/l); 4 = SO_4^{2-} (3.15 mmol/l). Chart range: 65 µs FS.

3.4. Mean anion concentrations in chicken serum and tear samples

Nitrate (0.20 mmol/l), phosphate (1.88 mmol/l) and sulfate (2.00 mmol/l) concentrations determined in chicken serum (Table 1) were 41, 2 and 61% lower, respectively, than in tear samples (Table 2). The overall mean chloride ion concentration determined in the tear samples (33.69 mmol/l) was 17% lower than in serum

Table 1
Comparative anion concentrations (mmol/l) present in human, black vulture and chicken serum samples

Anion	N	Nature	Mean ± S.D.	Maximum value	Minimum value
Cl^-	10	Human	37.75 × 6.20	47.48	25.53
	7	Vulture	43.71 ± 4.27	50.16	35.07
	3	Chicken	40.35 ± 2.61	42.57	36.62
NO_3^-	10	Human	0.19 ± 0.07	0.29	0.11
	7	Vulture	0.29 ± 0.16	0.45	0.00
	3	Chicken	0.20 ± 0.02	0.21	0.16
PO_4^{3-}	10	Human	1.42 ± 0.46	2.27	0.63
	7	Vulture	1.27 ± 0.36	1.84	0.49
	3	Chicken	1.88 ± 0.25	2.20	1.50
SO_4^{2-}	10	Human	0.53 ± 0.16	0.78	0.14
	7	Vulture	0.75 ± 0.38	1.39	0.21
	3	Chicken	2.00 ± 0.28	2.18	1.89

Table 2
Comparative anion concentrations (mmol/l) present in human, black vulture and chicken lachrymal secretion samples

Anion	N	Nature	Mean ± S.D.	Maximum value	Minimum value
Cl^-	10	Human	68.71 ± 17.93	104.81	50.22
	7	Vulture	82.59 ± 19.85	108.76	55.45
	3	Chicken	33.69 ± 2.91	37.91	30.35
NO_3^-	10	Human	0.14 ± 0.13	0.40	0.01
	7	Vulture	0.11 ± 0.08	0.27	0.00
	3	Chicken	0.34 ± 0.09	0.45	0.19
PO_4^{3-}	10	Human	0.22 ± 0.37	1.17	ND[a]
	7	Vulture	1.15 ± 0.61	2.72	0.77
	3	Chicken	1.91 ± 0.76	2.63	0.71
SO_4^{2-}	10	Human	0.39 ± 0.30	1.18	0.06
	7	Vulture	0.67 ± 0.41	1.37	0.25
	3	Chicken	0.79 ± 0.32	1.07	0.36

[a]Not detectable.

(40.35 mmol/l). This chloride concentration value pattern was the opposite to that for the other samples studied.

3.5. Comparative anion concentrations among human, vulture and chicken serum samples

Vulture blood serum chloride (43.71 mmol/l) and nitrate (0.29 mmol/l) concentrations were 14 and 34% higher, respectively, than the human values and 8 and 31% higher, respectively, than the chicken values. Direct comparison of mean phosphate concentrations in the chicken serum samples yielded a 24% higher value than the human value and 32% higher than the vulture value (Table 1). The sulfate anion concentration in chicken serum (2.00 mmol/l) was 63% than that in vulture serum and 74% higher than that in human serum.

3.6. Comparative anion concentrations among human, vulture and chicken tear samples

The mean chloride content determined in the vulture tear samples (82.59 mmol/l) was 17% higher than the human value (68.71 mmol/l) and 59% higher than the chicken value. Nitrate (0.34 mmol/l) and phosphate (1.91 mmol/l) values in the chicken sample were 59 and 90% higher, respectively, than the human values and 68 and 40% higher, respectively, than the vulture values (Table 2). Comparative analysis of sulfate mean values among the tear samples analysed yielded differences of 51% (0.79 mmol/l) higher for chicken than for human (0.39 mmol/l) and 15% higher than for vulture (0.67 mmol/l).

3.7. Correlations

No correlation was found ($p > 0.05$) amongst the anion concentrations present in the tear fluid and those in blood serum for all the samples analysed under the present conditions. The chloride and nitrite values in vulture samples were higher than in the other samples analysed. The chicken phosphate and sulfate values were higher than those in the other samples studied. High chloride levels present in human serum corresponded to high levels of phosphate ($r = 0.795$; $p < 0.01$) and high concentrations of nitrate corresponded to high contents of sulfate ($r = 0.726$; $p < 0.05$). No correlation was obtained amongst the individual values obtained for human, vulture and chicken tear fluid and serum ($p > 0.05$). It must be stated that the anion contents of the samples analysed in this investigation are pertinent to the population studied. Further generalization of these data should include a larger population sample and particular pathophysiological variables if reference concentrations are to be considered. The higher chloride anion concentration obtained in all the tear

samples studied than in serum samples may be due to its relevant function in maintaining tear osmolarity.

4. Conclusions

Ion chromatography was used for the quantitative study of the anion contents of two important biological extracellular fluids in humans and birds. Analysis of the data obtained indicate a high level of all anions studied in the bird specimens when compared with the human values. The chloride content considerably higher in tear fluid than in serum for all samples. For all samples, no correlation was found amongst anion concentrations present in the tear fluid and in serum samples. High chloride contents present in human serum corresponded to high levels of phosphate and high concentrations of nitrate corresponded to high levels of sulfate. No correlation was obtained amongst the studied ions in human and vulture tear fluid.

Ion chromatography is a relatively simple and reproducible procedure allowing repetitive ion analyses of large numbers of samples. Ion concentrations in biological samples, available only in critical volumes, can be readily studied. Column washing was required after repetitive substance analyses. Sample membrane filtration was a critical factor for keeping column fouling to the minimum.

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