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Potassium concentration differences in the vitreous humour from the two eyes revisited by microanalysis with capillary electrophoresis

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Abstract

This paper presents a study of the variability of potassium concentrations in the vitreous humour of the two eyes of the same body at identical postmortem interval. The study was carried out by collecting microsample amounts (50 μl) of vitreous humour and by using an original method of capillary electrophoresis with indirect detection. The electrophoretic separations were carried out in a pH 4.5 running buffer composed of 5 mmol/l imidazole, 5 mmol/l 18-crown-6 ether and 6 mmol/l α-hydroxybutyric acid. Detection was by indirect UV absorption at 214 nm. Vitreous humour samples were collected from 57 medico–legal autopsies or external examinations of cases of sudden natural or violent deaths. All samples prior to analysis were diluted 1:20 with a 40 μg/ml aqueous solution of barium, the used internal standard, and finally injected by nitrogen pressure. The mean concentrations of potassium measured in the two eyes of all the cases included in the present study ranged from 4.1 to 23.5 mmol/l with the postmortem interval values varying from 7 to 144 h. A highly significant ($P<0.0001$) linear correlation was found between these two parameters as described by the equation: $y=0.1698x+2.3587$, $r=0.89$. The intra-eye variability of potassium concentrations was low with an average RSD of 3.89% (±1.83 SD) (48 eyes, five samples per eye). No statistically significant difference was found between the potassium concentrations in the two eyes of the same subject in a group of 24 cases, excepting a single case. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Forensic analysis; Potassium

1. Introduction

The postmortem rise of potassium concentrations in the vitreous humour has long since been proposed by forensic pathologists to infer the time since death and the wide research carried out in this field has been reviewed by Coe [1] and Madea and Henssge [2].

A time dependent linear rise of potassium concentrations in the vitreous humour after death, due to the postmortem release of this cation from the intracellular compartment, is well known since the 1960s. Since the early reports, a great potential of this parameter for the purpose of determining the time since death was pointed out. However, important issues have later been raised. This criticism was substantially based on the high variability of results reported by different authors using different

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sampling and sample storage procedures and different analytical techniques. Besides, an unpredictable influence on vitreous potassium has been reported to be related with modalities of death, ambient temperature, state of putrefaction (the most common postmortem transformation of the body) and pathologies altering the electrolyte equilibria, etc. All these variables have been reported to affect the slope and intercept of the correlation between potassium concentration and postmortem interval (PMI) [3,4], thus undermining the practical application of vitreous potassium to infer the PMI.

An additional problem, pointed out by Balasooriya et al. [5] and Madea et al. [6] and recently re-evaluated by Pounder et al. [7], is the claimed inconsistency of potassium concentrations in the vitreous humour from the two eyes. The reasons of this difference are still unexplained, but clearly might affect the validity of this tool for calculating the time since death.

Among the different analytical techniques for measuring potassium in the vitreous humour, flame photometry and ion selective electrodes have almost exclusively been adopted. These methods, although widely accepted in a clinical environment, are non-separative analytical techniques and consequently more prone to interferences than the more sophisticated and complex separative methods, such as ion chromatography. Selectivity towards matrix interferences is a crucial issue especially when an unusual specimen, such as cadaveric vitreous humour, is to be analysed. In fact, eye fluids, because of putrefaction and other postmortem chemical changes, may have variable composition in terms of pH, ionic strength, content of small ions and other analytes (organic acids, proteins, peptides, amines, etc.). Although sample composition variability makes, in principle, separative methods preferable to non separative techniques, instrumental complexity, column fooling by sample components and analytical costs limit the use of ion chromatography for potassium analysis in biological samples.

Capillary electrophoresis (CE) has recently been introduced in forensic analysis and its applications in this field are rapidly increasing [8,9]. In the past decade, CE has successfully been applied to the analysis of controlled drugs, explosives and gunshot residues, inks, biopolymers of forensic interest (DNA and proteins) and, last but not least, small organic and inorganic ions.

Quite recently a CE technique, known as capillary ion electrophoresis (CIE; Waters trade name CIA for capillary ion analysis), has been proposed as a new tool for the determination of potassium in the vitreous humour, showing excellent performances in terms of sensitivity, accuracy and precision [10,11].

The purpose of the present work was to re-evaluate the problem of potassium concentration inconsistencies between the two eyes with this new instrumental technique.

In particular, CE looked attractive for this purpose because of its minimum requirement of sample volume. This makes possible microsample collection of the eye fluid reducing trauma on the eye tissues, from which potassium could be released into the vitreous humour.

2. Materials and methods

2.1. Standards and chemicals

Imidazole (99% pure) was obtained from Sigma (St. Louis, MO, USA), 18-crown-6 ether (99% pure) and α-hydroxybutyric acid (HIBA) (99% pure) from Aldrich (Milan, Italy). Standard solutions of K^+, Ba^{2+}, NH^{4+}, Na^+ and Ca^{2+} were prepared from AnalaR salts (KNO_2, $BaCl_2$, NH_4NO_3, NaCl and $CaCl_2$, respectively) (Merck, Darmstadt, Germany). K^+ standard solutions were checked versus reference standards for clinical chemistry analysers from Boheringer Mannheim (Mannheim, Germany) containing, respectively, 6.0 and 3.5 mmol/l of K^+. Water used for the preparation of the buffers and for sample dilution was of HPLC grade (Carlo Erba, Milan, Italy). Buffer and rinsing solutions were filtered and degassed under vacuum before use through a membrane filter of 0.22 μm (Millipore, Vimodrone, Italy).

2.2. Instrumentation and analytical conditions

A P/ACE 5500 automated capillary electropherograph (Beckman, Fullerton, CA, USA) equipped with a filter UV absorbance detector was used throughout the present study. Untreated fused-silica

capillaries (50 cm effective length×75 μm I.D., Beckman) were used with a detection window of 100×200 μm. Beckman P/ACE Station (version 1.0) was used for instrument control, data acquisition and processing.

Analytical conditions and method validation were described in detail in a previous paper [11]. In brief, the electrophoretic separations were carried out in a pH 4.5 running buffer composed of 5 mmol/l imidazole, 5 mmol/l 18-crown-6 ether and 6 mmol/l HIBA. The buffer pH was adjusted with 1 *M* acetic acid. Electrophoretic runs were performed by applying a constant voltage of 500 V/cm at 25°C. Detection was by indirect UV absorption at 214 nm. The samples were injected by nitrogen pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) application for 10 s at the anodic end of the capillary. Between two consecutive runs, the capillary was washed with water (1 min) and then with the electrolyte buffer (4 min).

Quantification was carried out on the basis of peak areas by using the internal standard method (barium).

Statistical analysis of data was done by using descriptive statistics and the Student *t*-test.

2.3. Sample collection and preparation

Vitreous humour samples were collected from 57 medico–legal autopsies or external examinations of cases of sudden natural or violent deaths, in which the time of death was exactly known. All the subjects studied were adults and the time elapsed since death ranged from 7 to 144 h. The causes of death were different and included infarction, pulmonary embolism, carbon monoxide intoxication, traffic accidents, drowning, gunshot injuries, hanging, suicidal precipitation, drug overdosing and sharp force injuries.

In a first group of 24 bodies (group A), five samples of vitreous humour of about 50 μl each were collected at the same time by needle puncture (25 G) and gentle sucking from the posterior chamber of each eye with plastic syringe (insulin type). In a second group of 33 bodies (group B) single sampling of vitreous humour from each eye (about 50 μl) was carried out.

All samples prior to analysis were diluted 1:20 with a 40 μg/ml aqueous solution of barium, the used internal standard (I.S.), and finally injected.

Samples were stored in plastic vials frozen at −20°C until analysis, which was performed within few days from sample collection.

3. Results

The analytical method used in the present study was described and validated in a previous paper to which the readers are referred for details [11]. In brief, analysis is based on the capillary electrophoretic separation of the cations present in the sample with ''indirect UV detection'', using imidazole as the UV absorbing additive and HIBA and 18-crown-6 ether to optimize the separation of potassium from sodium and ammonium ions. Fig. 1 shows a typical electropherogram from a real sample of vitreous humour. It is worth noting that other cations of the sample, even if present in large excess, such as sodium, are baseline separated from potassium and from the I.S. Also ammonium, the presence of which is dependent on putrefaction, is completely resolved from potassium.

The mean concentrations of potassium measured in the two eyes of all the cases included in the present study ranged from 4.1 to 23.5 mmol/l with the PMI values varying from 7 to 144 h.

A highly significant ($P<0.0001$) linear correlation was found between these two parameters. It is described by the following equation: $y=0.1698x+2.3587$ (x=time in hours; $y=K^+$ concentration, mmol/l) with an $r=0.89$, which is in substantial agreement with the reports in the literature [1,2].

In order to evaluate correctly the concentration differences between the two eyes, we have preliminarily studied the analytical variability and the variability of microsamples collected from a single eye.

Analytical variability was investigated by repeated analyses ($n=12$) on samples from three different eyes, with mean potassium concentrations of 7.54, 7.86 and 15.59 mmol/l; the respective relative standard deviations (RSDs) were 2.93, 3.58 and 3.10%.

The variability between different samples collected from the same eye at the same PMI was carefully studied because of the reported intra-ocular local differences in potassium concentration [12].

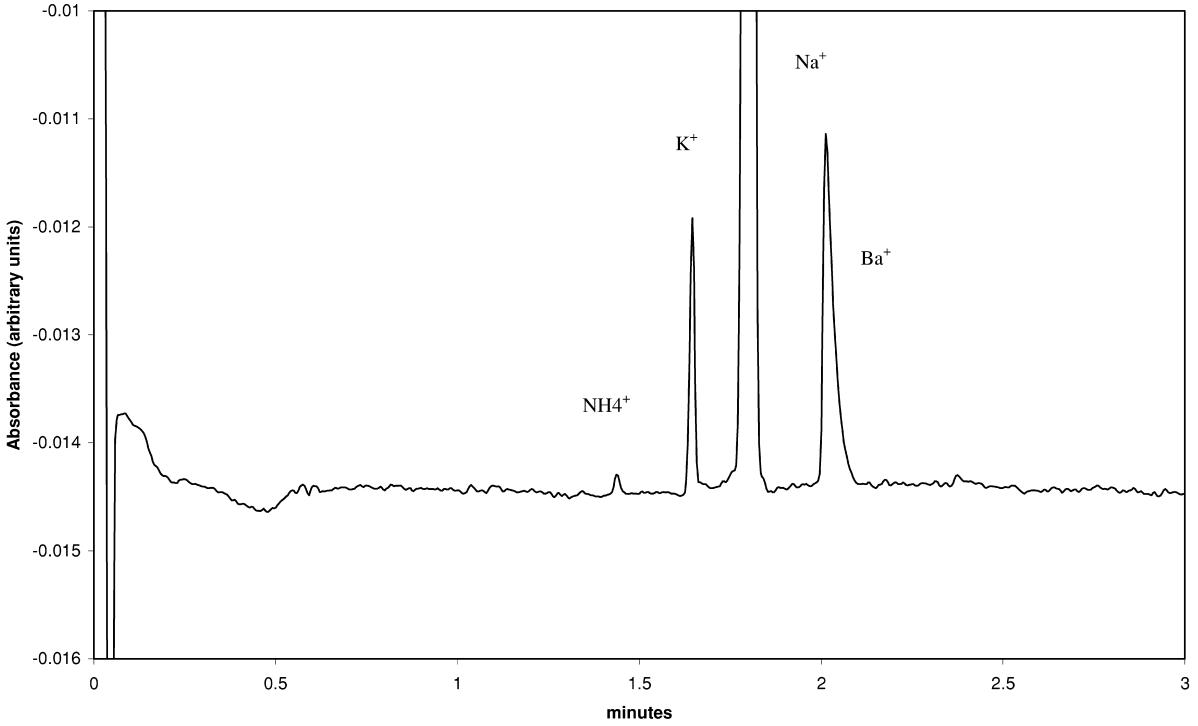

Fig. 1. Electropherogram from a sample of vitreous humour: K^+=7.71 mmol/l. For analytical conditions, see text.

This evaluation was carried out on group A (n=24), in which the both eyes of each body were subjected to multiple sampling (n=5) of 50 μl of vitreous humour. The intra-eye variability of concentration in the 48 eyes (24×2), expressed as RSDs, was comprised between 1.39 and 7.77% with an average of 3.89% (±1.83 SD).

The RSD values of multiple microsampling are clearly similar to analytical variability, thus excluding that the collection of microsample amounts of vitreous humour may affect the significance of the assay of potassium concentrations.

The statistical difference between the potassium concentrations in the two eyes of the same subject was evaluated in the 24 cases of group A, in which multiple microsampling of vitreous humour had been carried out. The study was performed by comparing with the Student t-test the potassium concentrations measured in the five samples collected from each eye of bodies at the same PMI. No statistically significant differences were found, excepting one case, in which the difference of potassium concentration in the two eyes was 0.54 mmol/l ($0.01>P>0.001$).

The real effect of between-eye differences on the estimation of PMI was studied in the whole casework (group A+group B, n=57) by calculating the difference of PMI values obtained from potassium concentration in each eye. The results expressed in absolute values were the following: mean PMI difference=1.89 h (±1.51 SD) with a range from 0.11 to 5.59 h; as percent of the mean PMI the mean difference was 4.79% (±3.68 SD) with a range from 0.12 to 13.9%. Fig. 2 depicts the distribution of the percent PMI differences showing no correlation with PMI. In addition it appears as only in four out of 57 cases the PMI difference exceeds 10%.

4. Discussion and conclusions

The present study shows that no statistically

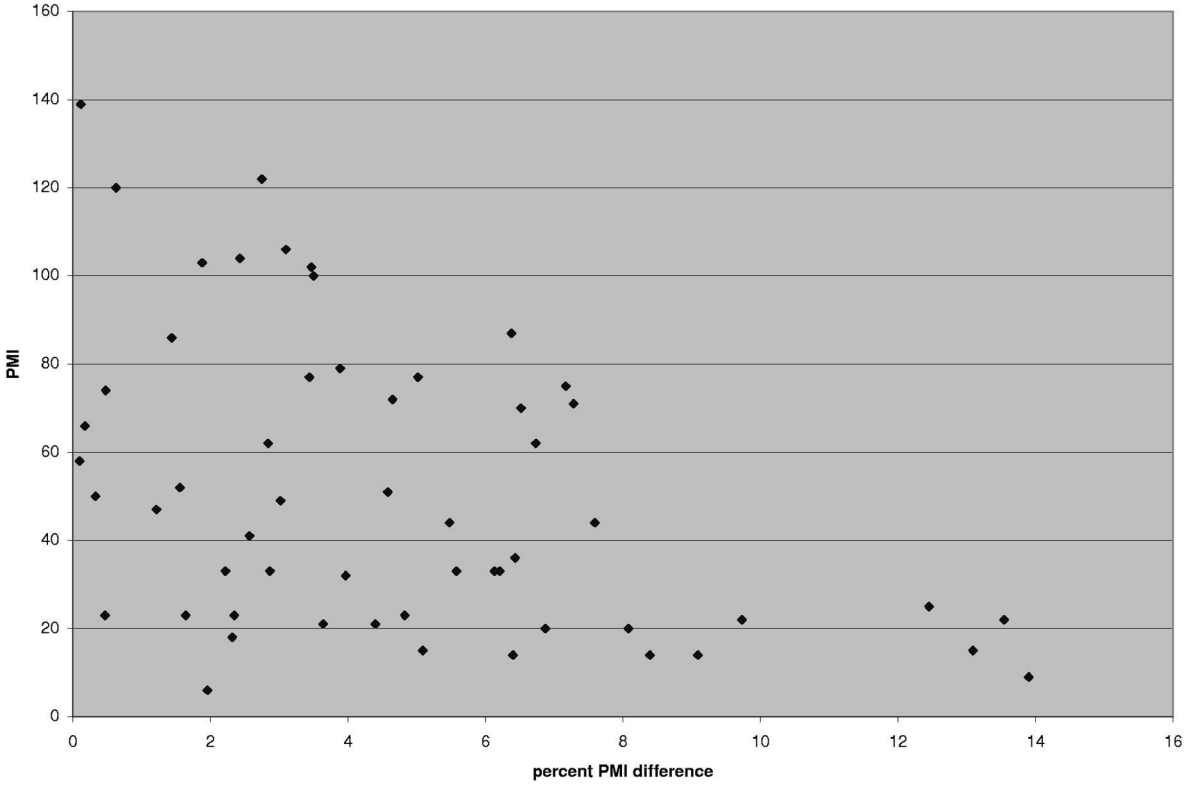

Fig. 2. Plot of percent PMI difference vs. PMI (h).

significant differences in potassium concentrations are present between the two eyes of a body at the same PMI, irrespectively of the time elapsed since death and the cause of death. The only case (out of 24) in which this difference was significant ($0.01 > P > 0.001$), could be explained by a traumatic damage involving the ocular tissue of one eye. On the other hand, in this case the difference was less than 10% of the mean potassium concentration (6.69 mmol/l). Our findings are not consistent with the reports from Pounder et al. [7], Madea et al. [6] and Balasooriya et al. [5], who found relevant differences between the two eyes.

To explain these different findings, we have to take into account that in the present paper we have adopted a microsampling technique, collecting only 50 μl of vitreous humour, whereas in most of the previous studies millilitre volumes of ocular fluid were collected according to the suggestions by Coe [12], requiring the suction of all the available vitreous humour. This procedure was intended to overcome the problem of the reported local differences of potassium concentrations in the different sites of the posterior chamber of the eye. However, in our opinion, it may cause a pressure stress on the cells of the eye tissues depending on the manual operation of sample suction. Thus, an artefactual release of potassium in the vitreous humour can easily be hypothesised, which could explain the observed inter-eye differences.

The variability of potassium determination from multiple microsampling was comparable to analytical variability of the used method. Hence, the present study shows that even 50 μl amounts of vitreous humour are representative of the potassium concentration in the entire volume, allowing the use of microsamples for this analysis.

Last but not least we have calculated the PMIs

corresponding to the potassium concentrations of the two eyes of each subject, in order to evaluate the real impact of concentration differences (although not statistically significant) on the estimation of the time since death.

The mean PMI difference was limited in both absolute and relative terms (1.89 h and 4.79%, respectively), also taking into account that the highest observed difference was 13.90% (moreover, this difference is to be halved if we refer to the deviation from the mean of the two levels, as several authors do). Thus, the observed between eye inconsistencies do not induce discrepancies in PMI calculation relevant in a real forensic environment.

In conclusion, our findings support the validity of using potassium determination in vitreous humour to infer the PMI and point out the advantage of using microsampling techniques coupled to CE.

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