Talanta 79 (2009) 1094–1099

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Estimation of postmortem interval by hypoxanthine and potassium evaluation in vitreous humor with a sequential injection system

Marieta L.C. Passos[a], Ana M. Santos[a], Ana I. Pereira[a], J. Rodrigo Santos[a], Agostinho J.C. Santos[b,c], M. Lúcia M.F.S. Saraiva[a,*], José L.F.C. Lima[a]

[a] REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal
[b] Serviço de Medicina Legal, Faculdade de Medicina, Universidade do Porto, Portugal
[c] Serviço de Patologia Forense, Instituto Nacional de Medicina Legal, I.P. – Delegação Norte, Portugal

ARTICLE INFO

Article history:
Available online 13 March 2009

Keywords:
Sequential injection analysis
Hypoxanthine
Xanthine oxidase
Potassium tubular electrode
Vitreous humor
Postmortem interval

ABSTRACT

The estimation of the time since death known as postmortem interval (PMI) is a main issue in the field of forensic science and legal medicine. In this work it is proposed a sequential injection system for the determination of hypoxanthine and potassium in the same sample of vitreous humor since the concentrations of both parameters change with PMI and the vitreous humor has been regarded as the ideal extracellular fluid for these kinds of determinations. By measuring both parameters the accuracy of estimation of PMI can be increased, and the effects of factors which influence the values in postmortem chemistry minimized.

Hypoxanthine determination is based on its oxidation to uric acid (290 nm), catalyzed by immobilized xanthine oxidase, and the quantification of potassium levels in vitreous humor was performed using a tubular potassium ion-selective electrode. With a unique analytical cycle both analytes were evaluated being potassium levels determined during the degradation of hypoxanthine in the enzymatic reactor.

Working concentration ranges between 6.04–40.00 μmol L^{-1} and 7.00×10^{-5} to 1.00×10^{-1} mmol L^{-1} were obtained, for hypoxanthine and potassium, respectively.

The method proved to be reproducible with R.S.D. <5% for hypoxanthine and <3% for potassium. Sampling rate was approximately 30 per hour for the sequential determination of both parameters being 15 and 60 determinations per hour if hypoxanthine or potassium, where evaluated independently. Statistical evaluation at the 95% confidence level showed good agreement between the results obtained, for the vitreous humor samples, with both the SIA system and the comparison batch procedures. Moreover the methodology has low environmental impact in agreement with the demands of green analytical chemistry as only 2.7 mL of chemical waste is produced during both determinations.

1. Introduction

The estimation of the time since death known as postmortem interval (PMI) is a main issue in the field of forensic science and legal medicine being one of the most difficult and challenging problem for forensic pathologists. Numerous methods have been reported for the determination of PMI by chemical means. While some studies were carried out on blood and cerebrospinal fluid (CSF), nowadays research have been performed on vitreous humor (VH) [1]. Analytical determinations in vitreous humor have advantages over those in blood and CSF. Vitreous humor is easier to collect, is isolated and well-protected anatomically, is preserved postmortem even in cases of severe head trauma and is less subject to contamination and putrefaction than blood and CSF. Additionally, in vitreous humor, chemical changes occur at a slow rate extending the period of time during which it may be used for PMI estimation purposes [2].

The postmortem increase of potassium concentrations in vitreous humor, first described by Sturner [3] is the most extensively studied parameter for estimating the PMI [1] being well known that the increasing concentration of this ion results from the energy breakdown and the related cessation of the active transport and the selective membrane permeability [4]. Several formulae have been proposed for estimating PMI on the basis of postmortem potassium levels in vitreous humor [4–11], using different kinds of methodologies, as capillary electrophoresis [4,6,8], low pressure ion chromatography [7] or flame photometry [12].

However, the correlation between potassium concentration in vitreous humor and PMI depends on several factors, related with

* Corresponding author. Tel.: +351 222078939; fax: +351 222004427.
E-mail address: lsaraiva@ff.up.pt (M.L.M.F.S. Saraiva).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.02.054

the individual and the death, that influence the accuracy of the PMI estimation [11–13].

On the other hand, several authors have looked for other biochemical vitreous humor parameters which could give increase accuracy in PMI estimation. Rogmun et al. [12] proposed the determination of hypoxanthine in vitreous humor as a biochemical method for the estimation of PMI with claimed advantages. Hypoxanthine is a vital and degradation product of adenosine nucleotide metabolism which is formed by several enzymatic reactions and then diffuses along the concentration gradient, mainly from the retina into the centre of the vitreous. Hypoxanthine determinations in vitreous humor have usually been performed using high performance liquid chromatography methods [14].

So, by measuring both potassium and hypoxanthine levels [2,12,15] the accuracy of the PMI estimation can be increased by combining estimates [2]. This is a way to minimize the effects of some factors which influence the values in postmortem chemistry.

So, the objective of this work is to combine the two determinations, so far done separately, in a unique module, and on doing so, get an expeditious way of analyzing a huge number of samples to get mathematical relationships, and also a system that can supply results whenever needed in an easier way. Consequently, and to achieve this goal, it was developed an automatic methodology, based on sequential injection analysis [16], that enables the sequential determination of both potassium and hypoxanthine in vitreous humor. This flow technique is an attractive automation tool as it allows the implementation of low cost, fast, reliable and versatile methodologies. Its computer-controlled nature assures a great operational flexibility and the establishment of distinct analytical strategies without physical reconfiguration. Moreover the multiposition selection valve, the core of the system, allows the clustering of all type of devices or solutions, which makes possible innumerous operations within the system. Regarding the batch procedures, it is also important to point out that SIA methodologies allow the execution of all the operations, without errors associated with the operator, and faster determinations, namely, than HPLC that allows, generally, about 3 hypoxanthine determinations per hour. Besides, SIA is ideally suited for multiple determinations due to its discontinuous nature providing the possibility of developing different determinations, independently. As far as we know, any flow techniques have never been used to automate these determinations (potassium and hypoxanthine) in vitreous humor. So, with this work there is an objective to reduce systematic errors and to get a prompt way to get significant statistical results to establish a mathematical relation between these parameters and PMI.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical reagent grade, high purity water (milli Q) with a specific conductance of <0.1 $\mu S\,cm^{-1}$. All chemicals were of analytical reagent grade.The sensor used for the construction of the potassium selective tubular electrode was prepared mixing 2 mg of valinomycin (Fluka), 65.8 mg of di-(2-ethylhexyl) sebacate (Fluka), 0.2 mg of tetrakis(4-chlorophenyl)boron potassium (Fluka) and 30 mg of poly(vinyl chloride) in 6 ml of tetrahydrofuran [17].

A 0.1 mol L^{-1} phosphate buffer solution was prepared by mixing adequate volumes of 0.2 mol L^{-1} solutions of NaH_2PO_4 (Merck) and of Na_2HPO_4 (Fluka) to obtain pH value of 7.5. This buffer solution was used as carrier, and also to prepare the stock and working standard solutions of hypoxanthine and in the process of immobilization of the enzymes. Hypoxanthine working standard solutions were prepared by appropriately diluting, with buffer solution, of the 1×10^{-3} mol L^{-1} stock solution of hypoxanthine (Sigma), prepared in the same buffer solution from the solid. These hypoxanthine solutions were daily fresh prepared.

Working standard solutions of potassium were prepared from a 0.1 mol L^{-1} stock solution prepared by dissolving KCl (Panreac), in water.

The used enzymes were xanthine oxidase (Sigma E.C. 1.17.3.2 from bovine milk, grade IV) and uricase (Sigma E.C. 1.7.3.3 from *Bacillus fastidiosus*).

All sample solutions were diluted and filtered, with pore size filters of 0.20 μm (Corning), before being analyzed both by the proposed automatic method and the comparison methods.

2.2. Enzymes immobilizations and column preparations

The immobilization procedure employed was similar to that reported by Peña et al. [18]. 0.125 g of aminoalkylated glass beads were incubated in 2.5 mL of a 2.5% glutaraldehyde for 1 h at room temperature, with brief nitrogen deoxygenation every 10 min for the first half hour. The activated glass beads (AGB) were then washed with water and 0.1 mol L^{-1} phosphate buffer at pH 7.5. Immobilization was then performed by adding 4 units of commercial xanthine oxidase, or 29 units of uricase to 0.125 g of the AGB. Incubation in an ice bath was done during 4 h, encompassing nitrogen deoxygenation every 10 minutes during first middle hour. After incubation the glass beads were then filtered off and washed with water and 0.1 mol L^{-1} phosphate buffer at pH 7.5, to eliminate any non-immobilized enzyme. Thereafter, 37 mg of immobilized XO beads (AGB–XO) and 80 mg of immobilized uricase beads (AGB–uricase) were packed in home-made polimethylmethacrylate (PMMA) columns with 3.3 and 3.2 cm length and internal diameters of 2.0 and 3.0 mm, respectively. Another column with the same dimensions of the AGB–XO was packed with AGB without enzyme. Two filters of 35 μm pore size (MoBiTec M2235) were placed at both ends of the reactors to entrap the glass beads. The immobilized xanthine oxidase and uricase were stored at 4 °C, in phosphate buffer at pH of 7.5 and 8.5, respectively, in a tightly closed tube. Working continuously on a daily basis, which always began with a calibration procedure, the immobilized xanthine oxidase allowed working with linearity for concentrations until 40 μmol L^{-1} during 15 days and after that it showed to be stable for about 1 month for concentrations until 20 μmol L^{-1}.

2.3. Apparatus

The SIA system (Fig. 1) consisted of a Gilson Minipuls 3 (VilliersleBel, France) peristaltic pump, equipped with a 0.90 mm i.d. Gilson PVC pumping tube and a 10-port selection valve (Valco, Vici C25-3180EMH, Houston, USA).

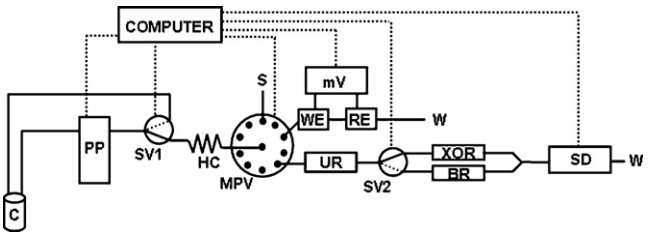

Fig. 1. SIA manifold for the sequential determinations of hypoxanthine and potassium. C, carrier (phosphate buffer 0.1 mol L^{-1} pH 7.5); PP, peristaltic pump; SV1, SV2, solenoid valves; HC, holding coil (2 m length/0.8 mm i.d.); S, sample; MPV, multiposition selection valve; WE, work electrode; RE, reference electrode; mV, decimillivoltimeter; UR, AGB–uricase reactor; XOR, AGB–XO reactor; BR, AGB reactor; SD, spectrophotometric detector (290 nm); W, waste.

Table 1
SIA analytical cycle used in the sequential determination of hypoxanthine and potassium ion.

Step	Position	Solenoid valve	Volume (μL)	Time (s)	Flow rate (mL min^{-1})	Direction	Event
1	1	Off	150	4.5	2	Aspiration	Sample (hypoxanthine)
2	3	Off/**on**	337.5	27	0.75	Propulsion	AGB reactor or **AGB–XO reactor** (hypoxanthine)
3	2	Off/**on**	75	4.5	1	Aspiration	Sample (potassium) stop period in AGB reactor or **AGB–XO reactor** (hypoxanthine)
4	4	Off/**on**	693.8	55.5	0.75	Propulsion	Detection (potassium) stop period in AGB reactor or **AGB–XO reactor** (hypoxanthine)
5	3	Off/**on**	1875	150	0.75	Propulsion	Detection (hypoxanthine)

Off (used for the sample blank); **on (used for the sample)**.

In order to guarantee reproducibility in the aspirated and propelled volumes, especially when dealing with reduced volumes, the starting position of the peristaltic pump at the beginning of each cycle was controlled. For that an NResearch 161 T031 solenoid valve (W. Caldwell, NJ, USA) (SV1, Fig. 1) and a device placed on the peristaltic pump head were also introduced in the system [19].

Another 3-way solenoid valve (SV2, Fig. 1) placed after the uricase reactor, was used to direct the fluids for the reactor with AGB without enzyme or for the reactor with AGB–XO.

All connections, including the holding coil were made with 0.8 mm i.d. PTFE tubing. The holding coil was 2 m in length, and was serpentine-shaped in configuration.

For potassium determination, the potential measurements were performed with a decimillivoltimeter micropH 2002 (Crison), a home-made tubular electrode [20] and a reference electrode AgCl/Ag from ThermoOrion (Auchtermuchty, Scotland, model 90-02, internal solution: 90-00-02; external solution: 90-00-03) coupled into the flow system through a PMMA adapter previously described [21].

For hypoxanthine determination, spectrophotometric measurements were performed with a Thermo-Spectronic (Cambridge, U.K.) Heλios γ UV–vis spectrophotometer equipped with an 80 μL Hellma flow cell (178.710 QS, Mullheim/Baden, Germany).

This system was controlled by a home-made programme written in QuickBasic language and implemented in a microcomputer equipped with an interface card (Advantech Corp., PCL 711B, San Jose, CA). Analytical signals were recorded with a Kipp & Zonen BD 111 (Delft, The Netherlands) strip chart recorder or acquired via computer.

2.4. Sequential injection procedure

The analytical cycle (Table 1) described here, basically consists of five steps. As the determination of hypoxanthine was based on the quantification of uric acid produced during the oxidation of hypoxantine by xanthine oxidase, it was necessary to perform a sample blank since the samples had uric acid in their composition, before the reaction.

So, following the aspiration of 150 μL of sample of vitreous humor the flow was reversed and sent (step 2) to the uricase-AGB reactor. Then, for the sample blank, the sample zone was sent through the AGB reactor with the solenoid off. To obtain the sample analytical signal, and after activation of the solenoid valve, the flow was diverged to AGB–XO reactor. In these reactors the flow stopped, during 60 s (steps 3 and 4), to increase the time of contact between the sample and the xanthine oxidase enzyme. This waiting time, was tapped for the potassium determination. It was aspirated 75 μL of sample for the holding coil (step 3) and after that it was propelled to the potentiometric detector (step 4). After that the detection of hypoxanthine was done, propelling the fluids to the spectrophotometer (step 5), where the uric acid was detected at 290 nm.

So, in each analytical cycle, two peaks were obtained; the first corresponding to the quantification of potassium, and the second referring to the evaluation of hypoxanthine. The increases of signals are proportional to potassium and hypoxanthine levels in the sample.

2.5. Comparison methods

To assess the accuracy of the results obtained by the developed SIA system, the samples were also analyzed by comparison methods. For the determination of potassium ion it was used a flame photometer (Jenway, East Norwalk, USA), where the samples were introduced and for hypoxanthine determinations it was used the HPLC [22]. In this methodology it was used a mobile phase of phosphate buffer and methanol in gradient mode.

3. Results and discussion

The determination of hypoxanthine concentration was based on the oxidation of the hypoxanthine catalyzed by xanthine oxidase to uric acid [23] which was detected spectrophotometrically at 290 nm. The quantification of potassium levels in vitreous humor was performed using a tubular potassium ion-selective electrode.These two determinations were implemented in a SIA system that due to its *modus operandi* made possible their evaluation within the same analytical cycle. The influence of the physical and chemical parameters involved in the respective analytical signals was investigated and the choice of the optimum conditions respected the objective of doing the two determinations sequentially. Thereafter, the designed SIA system was applied in the analysis of vitreous humor samples.

3.1. Enzymatic reactors

In the developed system, it was incorporated two reactors with immobilized enzymes in activated glass beads. One of them had immobilized xanthine oxidase, responsible for the oxidation of hypoxanthine and the other one had immobilized uricase. It was used immobilized enzymes since, in general, when compared their utilization with enzymes in solution, they show some advantages including great productivity, automation and continuous processing, precise control of the extent of the reaction, easy product recover and the enzyme does not contaminate the final product [24].

There were tested different quantities (between 2 and 4 units) of xanthine oxidase in the immobilization procedure. In spite of obtaining similar analytical signals with lower values it was chosen to proceed with the higher one (4 units) because it, counterbalanced some inactivation, enabling to obtain the same linear range for longer time. The internal diameter of the reactor was set in 2.0 mm. Smaller ones tend to give some flow pressure and with larger ones dispersion was observed.

Uric acid is a component of vitreous humor and also the product of hypoxanthine oxidation by xanthine oxidase. So, it was necessary to use uricase to reduce the quantity of uric acid in the samples before the reaction, to turn the detection possible. In order to achieve it, the sample flowed through the reactor with immobi-

lized uricase, before going to the reactor with immobilized xanthine oxidase. Uricase had to be used immobilized and not in solution because otherwise it would act not only in the endogenous uric acid but also in the uric acid produced in the reaction. Since the uric acid detected in the spectrophotometer was an addition of the remained uric acid from the sample and the uric acid from the oxidation of hypoxanthine by xanthine oxidase, it was necessary to perform sample blanks. For that, it was used another column with the same dimensions that was used for the xanthine oxidase immobilized in AGB, but filled only with AGB. With this column it was obtained the same degree of dispersion for samples flowing either through AGB or the reactor with immobilized xanthine oxidase in AGB. The differences between sample signals and blank sample signals gave the signal of uric acid resulted from the oxidation of hypoxanthine.

3.2. SIA system optimization

Regarding the usual importance of the pH value for the activity of the enzymes it was tested its effect in the range of 7.5–9, using 0.1 mol L^{-1} Tris–HCl and 0.1 mol L^{-1} phosphate buffers. It was observed that with a carrier solution of phosphate buffer with pH of 7.5 there was an increase in the activity of xanthine oxidase and the sensitivity was 20% higher than with a pH of 9. So, this was used for further studies. Stock and working standard solutions of hypoxanthine were also prepared with phosphate buffer solution of pH 7.5 to ensure maximum stability of the immobilized xanthine oxidase.

The 0.1 mol L^{-1} phosphate buffer solution with a pH of 7.5 was also studied as carrier for the potentiometric determinations of potassium, in order to perform both analyte determinations using the same carrier solutions. Preliminary results obtained with the carrier composition referred, shown an efficient ionic strength adjustment and pH buffering together with a stable baseline which did not justified supplementary addition of the primary ion in the carrier solution. So, for all these reasons and once both determinations should be done in the same system, and consequently with the same carrier, it was used the 0.1 mol L^{-1} phosphate buffer solution with pH 7.5 as carrier.

Afterwards, it was evaluated the optimum sample volume for potassium determination till 150 μL. It was necessary to maintain the dispersion of the sample plug in the carrier low before reaching the tubular detector. It was chosen the volume 75 μL, because with lower volumes the dispersion effect was too high impairing the determination of potassium in samples with lower concentrations.

For hypoxanthine determination a volume of 150 μL was established, after being tested volumes between 50 and 200 μL. While for volumes below 150 μL the sample amount became insufficient and sensitivity decreased, with higher volumes the analytical signals decreased, probably due to the inhibition of the xanthine oxidase by an excess of substrate [25].

The response to temperature by enzymes was evaluated from room temperature to 35 °C. At 35 °C the sensitivity was 58% lower than for the room temperature that was used for further studies.

The obtained linear calibration curve depended on the contact time between the hypoxanthine and the immobilized xanthine oxidase. Different reaction times are obtained by controlling the flow rate or by adopting a stopped flow strategy. So, it was checked the effect of the propelling flow rate between 0.5 and 1 mL min^{-1}. It was verified that with the increase of flow rate the obtained sensitivity decrease. From 0.5 to 1 mL min^{-1} it was observed a 30% of decrease in sensitivity. Stopped flow periods were also evaluated and an increase of signal was observed till 60 s. Regarding these results, and also the fact that during the stopped flow period it was possible to perform the potassium determination, and so the sampling rate was not affected, the flow rate of 0.75 mL min^{-1} and a 60 s of stopped flow was chosen for subsequent work.

For potassium determination, it was also studied the optimum propelling flow rate between 0.5 and 1 mL min^{-1}. In this case, it was also chosen the flow rate of 0.75 mL min^{-1}, since it allowed the necessary sample dispersion, to get a large linear concentration range with Nernstinean behavior.

The defined conditions allowed a sampling rate of approximately 15 and 60 determinations per hour, for hypoxanthine and potassium ion, respectively, when the determinations were considered independently. However, if it was considered that the system is used for the both sequentially determinations, the sampling rate would be approximately 30 determinations per hour.

3.3. Analysis of vitreous humor samples

After system optimization, calibration curves, with 95% confidence limits for the intercept and slope, the linear working range, detection limits, sampling rate and repeatability (Table 2) were evaluated for hypoxanthine and potassium ion determinations.

Detection limits were calculated as the concentrations corresponding to the intercept value of three times $S_{y/x}$, [26] or of the extrapolated linear mid-range and final low concentration level segment of the calibration plot [27], for hypoxanthine and potassium ion determinations, respectively.

Sample throughput was calculated by adding the time necessary to perform each step of the protocol sequence, including aspiration of solutions to the holding coil, propulsion to the detector and stopped flow. Repeatability was estimated by calculating the relative standard deviation (R.S.D.%) from ten consecutive sample injections of different concentrations.

To evaluate the accuracy of the system developed some samples were analyzed (Table 3) according to the SIA method and their comparison batch methods (high performance liquid chromatography for hypoxanthine [22] and flame photometry for potassium).

Linear relationships (Fig. 2), described by the equation $C_{SIA} = (0.951 \pm 0.078)C_{HPLC} + (2.9 \pm 7.9)$ for the hypoxanthine and $C_{SIA} = (1.001 \pm 0.092)C_{FP} - (0.35 \pm 0.84)$ for potassium, were established. C_{SIA} and C_{HPLC} or C_{FP} correspond to the sequential injection and comparison procedures results, respectively, with 95% confi-

Table 2
Figures of merit of SIA system.

	Hypoxanthine	Potassium
Regression equation	AU = $(3.30 \pm 0.26) \times 10^{-3}$Conc. (μmol L^{-1}) + $(3.7 \pm 6.5) \times 10^{-3}$	E (mV) = (59.6 ± 2.2)Conc. (mmol L^{-1}) + (280.2 ± 6.7)
R^2	0.998	0.999
Detection limit	1.02 μmol L^{-1}	5.00×10^{-5} mmol L^{-1}
Linear response range	6.04–40.00 μmol L^{-1}	7.00×10^{-5} to 1.00×10^{-1} mmol L^{-1}
R.S.D.% (sample conc.)	4.03% (22.95 μmol L^{-1})	2.58% (0.519 mmol L^{-1})
R.S.D.% (sample conc.)	4.84% (31.08 μmol L^{-1})	1.05% (13.1 mmol L^{-1})
Determ. frequency (as single determination) (determ. h^{-1})	15	60
Determ. frequency (as sequential determinations) (determ. h^{-1})	30	

Table 3
Results obtained by the proposed SIA methodology and the comparison methodologies for the determination of hypoxanthine and potassium in vitreous humor samples and the respective relative deviation percentages (RD, %).

Hypoxanthine (μmol L^{-1})		RD (%)	Potassium (mmol L^{-1})		RD (%)
SIA	HPLC		SIA	FP	
25.67 ± 2.55	25.42 ± 1.02	+0.97	8.04 ± 0.16	8.38 ± 0.27	−4.08
53.28 ± 0.32	50.25 ± 1.21	+6.03	7.79 ± 0.14	7.91 ± 0.38	−1.47
54.85 ± 2.54	53.61 ± 0.95	+2.32	9.02 ± 0.15	8.89 ± 0.30	+1.46
133.36 ± 3.35	140.60 ± 3.82	−5.15	6.17 ± 0.01	6.68 ± 0.37	−7.58
129.83 ± 3.53	139.56 ± 4.22	−6.97	8.81 ± 0.21	9.26 ± 0.81	−4.87
119.95 ± 2.86	114.54 ± 2.16	+4.73	13.14 ± 0.20	13.14 ± 1.71	−0.02
60.20 ± 14.19	61.84 ± 1.36	−2.65	12.53 ± 0.10	13.31 ± 0.25	−5.87
111.89 ± 8.27	119.35 ± 1.55	−6.26	6.62 ± 0.02	6.94 ± 0.10	−4.71
157.09 ± 4.36	156.11 ± 0.86	+0.63	6.82 ± 0.27	7.40 ± 0.06	−7.90
62.08 ± 2.37	62.85 ± 0.27	−1.23	6.52 ± 0.11	6.93 ± 3.05	−5.96

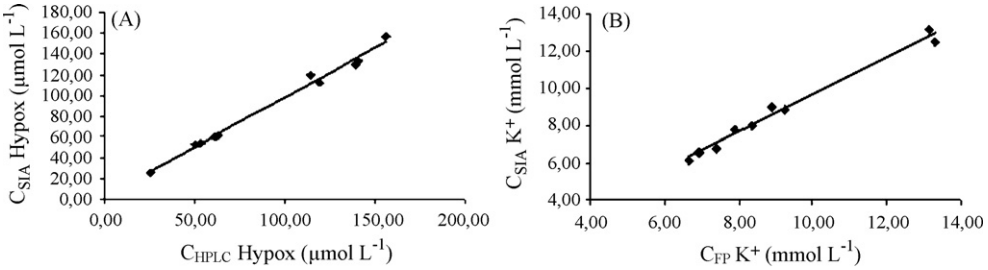

Fig. 2. Correlation between SIA method and their comparison batch methods (high performance liquid chromatography for hypoxanthine (A) and flame photometry for potassium (B)).

dence limits for the intercept and slope. From these figures it is evident that the estimated intercept and slope values do not differ significantly from 0 and 1, respectively [26].

The agreement between both methods was also evaluated, using the *t*-test, carried out as a bilateral coupled test [26]. The tabulated *t* value of 2.31 when compared with the calculated *t* values of −1.02 and −3.88 (for hypoxanthine and potassium, respectively) show the absence of statistical differences for those results obtained by the methodologies at the 95% confidence level.

The developed analytical methodology was then applied to samples of vitreous humor from subjects with known PMIs. The obtained results confirmed the strong correlation between vitreous hypoxanthine and vitreous potassium levels (Fig. 3) as it was reported before by Rogmun et al. [12].

The vitreous samples of both eyes taken at the same post-mortem time indicate a higher correspondence for hypoxanthine ($R = 0.82544$) than for potassium values ($R = 0.67814$) (Fig. 4).

In any case, the concentrations of both hypoxanthine and potassium in vitreous humor increase linearly with time after death, results that complied with the literature, and confirm once more the validity of the SIA methodology. So, simple linear regressions could be performed on the data collected, and equations for estimation of PMI constructed. The obtained equation based on hypoxanthine concentrations was C_{Hypox} (μmol L^{-1}) = 7.12PMI

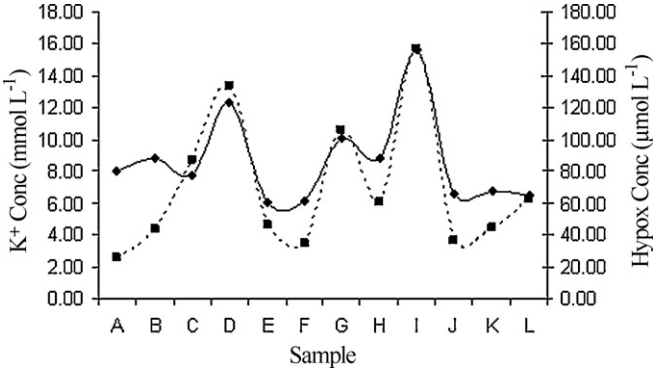

Fig. 3. Correlation between hypoxanthine and potassium concentrations in different samples of vitreous humor. —♦—, potassium ion concentrations; - -■ - -, hypoxanthine concentrations.

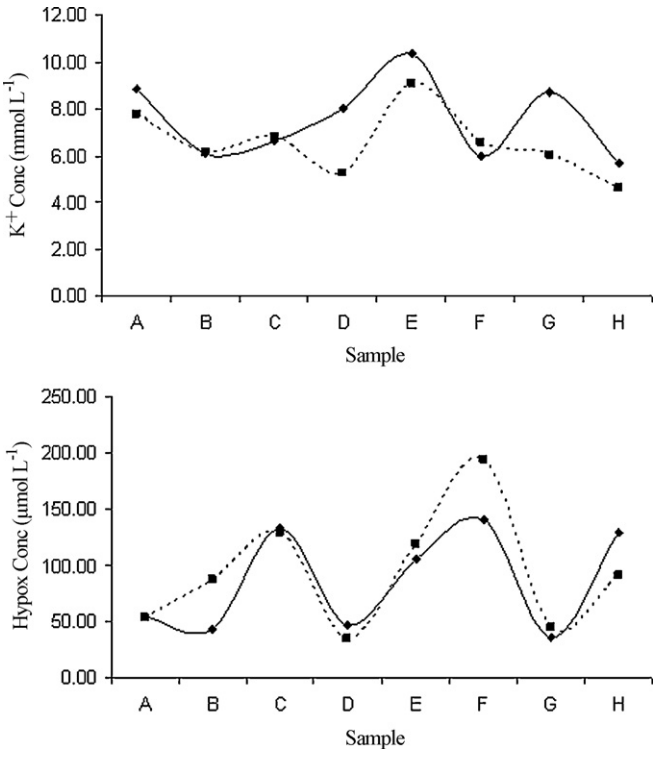

Fig. 4. Correlations between hypoxanthine and potassium concentrations determined in samples of vitreous humor of right and left eyes. —♦—, right eye; - -■ - -, left eye.

(h) + 31.49, while the correlation between potassium ion concentrations in vitreous humor and PMI was expressed by the equation C_{K^+} (mmol L^{-1}) = 0.19PMI (h) + 6.23. Some studies [1,10,15] point out that a more precise estimation of PMI could be obtained if potassium and hypoxanthine concentrations were used as independent variables. By changing the variables, other formulas can then be proposed as PMI (h) = 0.14C_{Hypox} (μmolL^{-1}) − 4.42 and PMI (h) = 5.36C_{K^+} (mmol L^{-1}) − 33.41. As it was reported by James et al. [2] and by Rogmun et al. [12], hypoxanthine levels were less scatter (R^2 = 0.238) than potassium ion (R^2 = 0.222), suggesting that measurements of vitreous hypoxanthine may be of great benefit in determining PMI. However, as it was reported by Madea [1] the correlation of the potassium values with the time since death was found to be much stronger than the hypoxanthine values. The hypoxanthine concentration increased 7.12 μmol L^{-1} in each hour after death while potassium concentration increased 0.19 mmol L^{-1}. These contradictory facts confirmed that in the estimation of PMI errors are minimized by the conjugation of the determination in vitreous humor of the both parameters (hypoxanthine and potassium).

4. Conclusions

For the first time, it was developed and implemented a flow-based automatic methodology, for the sequential determination of both hypoxanthine and potassium in vitreous humor samples.

Relative to the existing batch methodologies, it is to be emphasised that the automatic SIA methodology here presented, allows within only one system and with only one analytical cycle, two determinations to be carried out in the same sample. While potassium was being evaluated in one branch port of the selection valve hypoxanthine was being oxidized in another in the enzymatic reactor. For the hypoxanthine determination it was used two immobilized enzymes, so this system offers a great deal of advantages over the conventional methods that used soluble enzymes, such as the reduction of cost per analysis, increase the speed, the reliability and accuracy. Other advantages over conventional methods were the simple sample pre-treatment. While in some other methods, treatments like centrifugation, heating or enzymatic digestion are necessary, in the proposed method only a filtration and a dilution are required.

The quantification of potassium levels in vitreous humor using a tubular potassium ion-selective electrode incorporated in the flow manifold, showed good tolerance to the matrix constituents as the detector could be used for several months without losing its intrinsic response characteristics.

The SIA methodology developed is in good agreement with the current Green Chemistry recommendations as it involves the use of reusable reagents in solid supports, and a chemical waste of only 2.7 mL per both determinations.

This developed system provided reliable results, similar to the comparison methods, and showed to be a prompt way of analyzing a large numbers of samples and consequently to supply with significant statistical results to establish a mathematical relation between hypoxanthine and potassium concentrations and PMI, being a good alternative to be used in forensic science.

Acknowledgements

Marieta L.C. Passos thanks the Fundação para a Ciência e Tecnologia and FSE (III Quadro Comunitário) for the Ph.D. grant (SFRH/BD/22752/2005).

References

[1] B. Madea, Forensic Sci. Int. 151 (2005) 139.
[2] R.A. James, P.A. Hoadley, B.G. Sampson, Am. J. Forensic Med. Pathol. 18 (1997) 158.
[3] W.Q. Sturner, Lancet I (1963) 807.
[4] F. Tagliaro, G. Manetto, F. Cittadini, D. Marchetti, F. Bortolotti, M. Marigo, J. Chromatogr. B 733 (1999) 273.
[5] R.J. Stephens, R.G. Richards, J. Forensic Sci. 32 (1987) 503.
[6] K.E. Ferslew, A.N. Hagardorn, M.T. Harrison, W.F. McCormick, Electrophoresis 19 (1998) 6.
[7] B. Zhou, L. Zhang, G. Zhang, X. Zhang, X. Jiang, J. Chromatogr. B 852 (2007) 278.
[8] G. Bocaz-Beneventi, F. Tagliaro, F. Bortolotti, G. Manetto, J. Havel, Int. J. Legal Med. 116 (2002) 5.
[9] N. Lange, S. Swearer, W.Q. Sturner, Forensic Sci. Int. 66 (1994) 159.
[10] J.I. Munoz, J.M. Suárez-Peñaranda, X.L. Otero, M.S. Rodríguez-Calvo, E. Costas, X. Miguéns, L. Concheiro, J. Forensic Sci. 46 (2001) 209.
[11] Myo-Thaik-Oo, E. Tanaka, H. Oikawa, K. Aita, K. Tanno, K. Yamazaki, S. Misawa, J. Clin. Forensic Med. 9 (2002) 70.
[12] T.O. Rogmun, S. Hauge, S. Oyasaeter, O.D. Saugstad, Forensic Sci. Int. 51 (1991) 139.
[13] B. Madea, A. Rödig, Forensic Sci. Int. 164 (2006) 87.
[14] B. Madea, H. Käferstein, N. Hermann, G. Sticht, Forensic Sci. Int. 65 (1994) 19.
[15] J.I.M. Barús, J.M. Suárez-Peñaranda, X.L. Otero, M.S. Rodríguez-Calvo, E. Costas, X. Miguéns, L. Concheiro, Forensic Sci. Int. 125 (2002) 67.
[16] J. Ruzicka, G. Marshall, Anal. Chim. Acta 237 (1990) 329.
[17] R. Perez-Olmos, A. Rios, R.A.S. Lapa, J.L.F.C. Lima, Fresenius J. Anal. Chem. 360 (1998) 659.
[18] R.M. Peña, J.L.F.C. Lima, M.L.M.F.S. Saraiva, Anal. Chim. Acta 514 (2004) 37.
[19] M.L.C. Passos, M.L.M.F.S. Saraiva, J.L.F.C. Lima, M.G.A. Korn, J. Bras. Chem. Soc. 19 (2008) 563.
[20] J.L.F.C. Lima, M.C.B.M. Montenegro, Mikrochim. Acta 131 (1999) 187.
[21] S. Alegret, J. Alonso, J. Bartroli, A.A.S.C. Machado, J.L.F.C. Lima, J.M. Paulis, Quim. Anal. 6 (1987) 278.
[22] E.J.C.M. Coolen, I.C.W. Arts, E.L.R. Swennen, A. Bast, M.A.C. Stuart, P.C. Dagnelie, J. Chromatogr. B 864 (2008) 43.
[23] G. Hussain, M. Yaqoob, A. Nabi, M. Masoom, J. Chem. Soc. Pak. 17 (1995) 109.
[24] S. Nam, M.K. Walsh, J. Food Biochem. 29 (2005) 1.
[25] M.M. Jezewska, Eur. J. Biochem. 36 (1973) 385.
[26] J.C. Miller, J.N. Miller, Estadística para Química Analítica, 2nd ed., Addison-Wesley Ibroamerican, S.A., Wilmington, 1993.
[27] International Union of Pure Applied Chemistry, Compendium of Analytical Nomenclature Definitive Rules 1997, 3rd ed., Blackwell Science, 1998.