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Article

Development and Validation of a Micellar Electrokinetic Capillary Chromatographic Method for the Assessment of Nucleosides, Potential Biomarkers, in Blood Serum

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Tumores malignos são a causa de milhões de falecimentos no mundo todos os anos, porém a detecção da doença em seu estágio inicial pode salvar vidas. Biomarcadores tumorais permitem diagnósticos mais precoces e menos invasivos, portanto são vitais no tratamento do câncer. Os nucleosídeos têm sido pesquisados como um grupo potencial de biomarcadores tumorais presentes em fluidos biológicos. Neste trabalho, um método para a análise de nucleosídeos por eletroforese capilar com detecção UV (CE-UV) em amostras de soro sanguíneo foi desenvolvido e validado de acordo com a legislação vigente no Brasil. A separação de dez nucleosídeos mais o padrão interno foi atingida em ca. 25 min. O método pode contribuir para o diagnóstico precoce e preciso de casos de câncer.

Malignant tumors are the cause of millions of deaths all over the world every year, but the detection of the disease in the first stages may save lives. Tumor biomarkers allow earlier and less invasive diagnosis, hence they are vital in the cancer treatment. Nucleosides have been investigated as a potential group of tumor biomarkers present in biological fluids. In this work, a method for the analysis of nucleosides in blood serum samples by capillary electrophoresis with UV detection (CE-UV) was developed and validated according to the current legislation in Brazil. Separation of ten nucleosides plus the internal standard was achieved in ca. 25 min. The method may contribute for earlier and more accurate diagnosis of cancer cases.

Keywords: capillary electrophoresis, nucleosides, tumor biomarkers, blood serum, micellar electrokinetic capillary chromatography

Introduction

Cancer has become a condition of great concern all over the world. World Health Organization (WHO) reports that malignant tumors were responsible for 13% of deaths throughout the world in 2008 and estimates that it will rise to 45% until 2030. One in each two human beings will develop some type of malignant tumor during lifetime, but one third of death cases could be avoided if the disease was detected and treated early, when cancer is restricted to one organ and metastasis has not initiated. However, tumors are generally asymptomatic in such phase, so a test or exam that identifies the disease in the initial stage and that is noninvasive, simple, safe and easy to be performed would be ideal.[1,2]

During carcinogenesis, morphological and histological alterations may occur inside the cells, which lead to expression of damaged genes as well as production of substances in response to the disease. Some tumor biomarkers, synthesized within cancer development, may be detectable right in the beginning of the disease, constituting an ideal system for early diagnosis.[3,4] A biomarker may be defined as a feature that is objectively measured and evaluated as an indicator of a normal or pathogenic process.[5] Most substances employed as tumor biomarkers are naturally produced by the human body,

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but their concentrations are increased during neoplasm development. The identification of biomarkers for cancer diagnoses has been widely researched due to the possibility of early detection and achievement of data about the pathology base and the stage of the disease. Different tumor biomarkers can be found for different types of cancer, and levels of the same tumor biomarker can be altered in more than one type of cancer. Tumor biomarkers can be used in conjunction with other tests, e.g., scans or biopsies, in order to assist the patient diagnosis.[6-9]

Nucleosides are excreted in human biological fluids, such as urine and blood, during normal cell activity. In the post-transcpitional stage of transfer ribonucleic acid (tRNA), the four common nucleosides (i.e., adensosine, guanosine, cytidine and uridine) are released inside the cells and are further reincorporated in nucleic acids or degraded by the action of specific enzymes. However, modified nucleosides are produced by post-transcriptional modifications in molecular structures of normal nucleosides (mainly methylation of the nitrogenous base and/or the hydroxyl group present in the sugar moiety) due to the action of modifying enzymes, such as tRNA methyltransferases. Unlike normal nucleosides, the modified molecules cannot be reincorporated into nucleic acid chains because of the absence of suitable enzymes, being eliminated in body fluids.[10,11] The highest turn-over rate of tRNA occurs in cancer cells as a consequence of the rapid degradation of nucleic acid structures, the aberrant activity of the structure modifying enzymes, and the higher division rate of affected cells. As a result, increased levels of nucleosides (both modified and non-modified) are excreted from cancer cells. Therefore, investigation of such analyte profile is of outcome importance since it may assist the early diagnosis and cancer treatment of patients, therefore increasing the survival rates. Publications have confirmed that the levels of some nucleosides are affected by tumor development, as shown in Tables 1 and 2, but the degree of such alteration in blood serum and the relation to cancer base pathologies have not been stated yet. The differences presented in published data emphasizes the necessity of further studies. Nevertheless, the practical application of nucleoside analyses as a diagnostic tool still requires improvements of methodologies, validation and expertise.[12-29]

Capillary electrophoresis (CE) has been applied to pharmaceutical, industrial, environmental and biological sample analysis.[30-37] The technique presents high separation efficiency allied to low background electrolyte (BGE) and sample consumption, low analysis time and low amounts of organic waste, which allows to consider CE as a green chemistry technique of analysis. Other techniques, such as high-performance liquid chromatography (HPLC), have been used to analyze nucleosides in biological materials, but CE usually presents higher resolution power and less solvent consumption, which makes it more suitable for routine analysis.[15-29] In this work, a method for the analysis of nucleosides in blood serum samples by capillary electrophoresis with UV detection (CE-UV) was developed and validated according to Brazilian legislation. The validation steps were entirely executed in blood serum from healthy subjects, emphasizing the novelty of the method, since few works have reported the validation of this method employing the biological matrix or have presented the complete procedure used for the evaluation of the figures of merit. The combination of the developed method with the ones routinely used in clinical analysis may corroborate for more accurate diagnoses of early stage cancer and for aiding the detection of false-positive or false-negative results.

Table 1. Levels of nucleosides normally found in blood serum of healthy subjects, according to the literature data. The published values are different according to the method, technique and author

Reference	Gehrke *et al.*[27]	Xu *et al.*[13]	Djukovic *et al.*[28]	Mitchel *et al.*[29]
Technique	HPLC-UV	HPLC-UV	HPLC-tqMS	HPLC-UV
Nucleoside	Concentration / (μmol L^{-1})			
C	–	0.068 ± 0.10	0.87 ± 0.37	–
A	–	–	–	–
U	4.40-13.4	4.98 ± 1.44	12.80 ± 5.18	–
5mU	–	0.20 ± 0.098	–	–
G	0.00-0.114	0.077 ± 0.046	3.52 ± 1.68	–
X	0.016-0.167	0.16 ± 0.13	–	0.08
I	0.013-2.35	0.36 ± 0.22	9.99 ± 8.57	–

HPLC-UV and HPLC-tqMS: high-performance liquid chromatography with UV detection and with triple quadrupole mass spectrometry, respectively.

Table 2. Levels of nucleosides normally found in blood serum of cancer patients, according to the literature data. The minor amount of data justifies further studies on nucleosides in blood serum samples

Reference	Djukovic *et al.*[28]	Mitchell *et al.*[29]	Mitchell *et al.*[29]
Technique	HPLC-UV	HPLC-UV	HPLC-UV
Cancer base pathology	Esophagus adenocarcinoma	Leukemia	Lung carcinoma
Nucleoside	Concentration / (μmol L^{-1})		
C	1.58 ± 0.54	–	–
A	–	–	–
U	7.30 ± 1.65	–	–
5mU	–	–	–
G	3.65 ± 1.53	–	–
X	–	0.09	0.08
I	17.95 ± 18.29	–	–

Experimental

Reagents and solutions

All reagents used herein were of analytical purity grade. Boric acid, monohydrate monobasic sodium phosphate and sodium hydroxide were from Merck (Darmstadt, Germany); sodium dodecil sulfate (SDS) was acquired from Sigma-Aldrich (Steinheim, Germany); methanol (HPLC grade) was from Carlos Erba (Milano, Italy); hydrochloric acid was purchased from LabSynth (São Paulo, Brazil), phenylboronic acid resin Affi-Gel 601 from Bio-Rad (Hercules, USA) and acetone from Nuclear (São Paulo, Brazil). Ten nucleoside standards (cytidine: C, thymidine: T, adenosine: A, guanosine: G, 2'-deoxyadenosine: 2dA, inosine: I, 5-methyluridine: 5mU, xanthosine: X, uridine: U, and 1-methyladenosine: 1mA) and the internal standard (8-bromoguanosine: 8BrG) were acquired from Sigma-Aldrich (St. Louis, Missouri, USA) (Figure 1). All solutions were prepared with deionized water obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Ultrafiltration membranes of 3000 Da were acquired from Millipore (Bedford, MA, USA).

Nucleoside standard solutions were prepared by weighting the necessary amount of the standards and dissolving in water with the aid of ultrasound for about 20 min. The solutions were kept frozen and protected from light, and were discarded every 30 days. For method development, 0.5 mmol L^{-1} mixed solutions were employed. BGE solutions under all conditions described on the following sections were kept in refrigerator for about 3 months.

Capillary electrophoresis

Two CE-UV systems were employed for sample analyses and method development: HP3DCE for the initial steps, and CE7100 for the last optimization steps and validation (both from Agilent Technologies, Waldbroon, Germany). Fused silica capillaries with 50 μm i.d. and variable length, as described during the method development section, were bought from Agilent Technologies (California, USA) and Polymicro Technologies (Polymicro, Phoenix, USA). In the first use, capillaries were conditioned by flushing 1.0 mol L^{-1} NaOH for 30 min, followed by deionized water for 10 min and BGE for 15 min. At the beginning of the day, capillaries were prepared by flushing with 1 mol L^{-1} NaOH for 15 min, water for 10 min and BGE for 15 min. A simple conditioning procedure was applied between runs, and was constituted by 1 mol L^{-1} NaOH for 1 min, water for 1 min and BGE for 2 min. The capillaries were rinsed with water for 20 min at the end of the day and dried with flush of air for 10 min. Samples were injected under a pressure of 50 mbar for 15 s for nucleoside standards (consuming 21.6 nL of sample *per* run) and 3 s for acetone, used as electroosmotic flow (EOF) marker.

The parameters evaluated during the optimization were: BGE pH and composition, surfactant and organic modifier concentration and applied voltage.

Blood serum sample preparation

Blood serum samples for the validation step were obtained from healthy male volunteers, because the method will be applied to the analyses of samples obtained from subjects with prostate cancer. The samples were brought from the Clinics Hospital of the University of Campinas, where they were previously tested for prostate specific antigen (PSA), human chorionic gonadotropin (βHCG), alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) levels by immunoassays. Such biomolecules are tumor biomarkers commonly applied in diagnosis

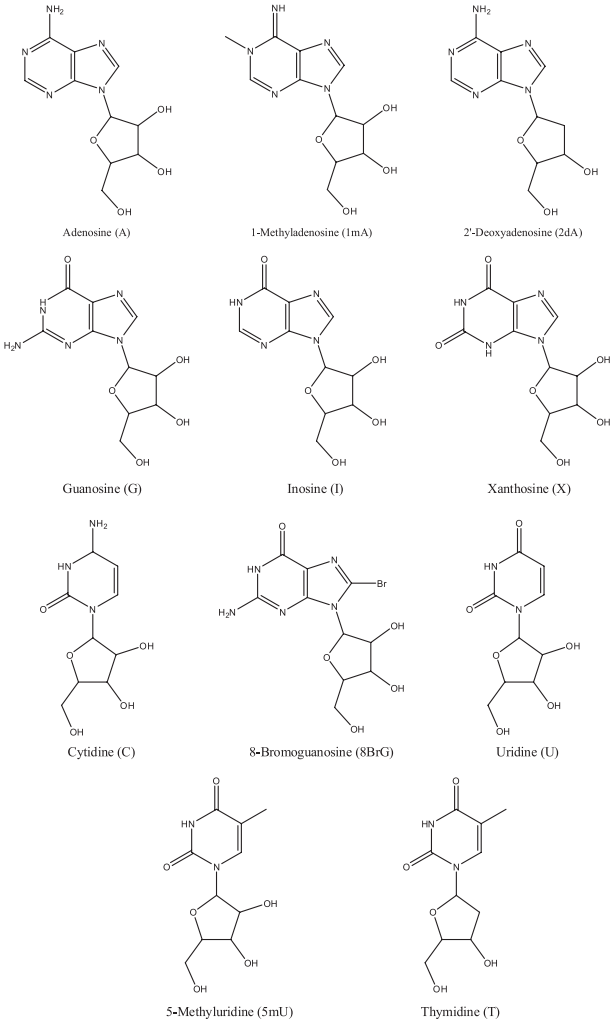

Figure 1. Chemical structures of modified and non-modified nucleoside standards and the internal standard (8BrG) investigated herein.

procedures. Therefore, the results of such assays indicated the samples were originated from healthy subjects. The Research Ethics Committee of the Medical Sciences College at the University of Campinas approved the use of biological materials for this project, which met all the provisions of Resolution 196/96 from the Brazilian Health Ministry.[38] The Committee approved without restriction the research protocol developed herein and the free enlightenment permission form was filled in by the volunteers during sampling acquisition.

The samples were extracted by an affinity procedure adapted from Davies *et al.*[39] Solid phase extraction (SPE) columns filled with 200 mg of phenylboronic acid resin Affi-Gel 601 were employed. This polyacrilamide gel presents affinity for cis-diol groups (present in most of the studied nucleosides, except 2dA and T) and high binding capacity for small molecules in basic medium. In acidic medium, however, the resin unbinds the cis-diol groups and analytes may be eluted.

Serum samples were centrifuged in ultrafiltration membranes for 1 h 30 min at 5000 rpm and 5 °C. The SPE column was equilibrated with 20 mL of 0.250 mol L^{-1} ammonium acetate solution at pH 8.80. An aliquot of 1 mL of the sample was added, followed by 4 mL of 0.250 mol L^{-1} ammonium acetate pH 8.80. Nucleosides were eluted by employing 4 mL of 0.100 mol L^{-1} formic acid solution. Eluate was evaporated under nitrogen flow to half the volume and lyophilized for 24 h. The sample was ressuspended in 1 mL of deionized water and analyzed by CE-UV, showing the method sensitivity, since 21.6 nL of sample were required for each CE run.

Validation

All validation parameters were evaluated in standard solutions prepared in the biological matrix, i.e., blood serum samples from healthy male volunteers. Method validation was carried out in agreement with the parameters specified by the Brazilian National Health Surveillance Agency (ANVISA) for bioanalytical methods, i.e., limits of detection and quantification, linearity, precision and accuracy, which must be followed in the country.[40] Analytical curves were elaborated by the internal standard method, in which the peak areas were divided by the internal standard peak area.

All assays were performed in triplicate in the biological matrix, employing a pool composed by blood serum from 10 subjects spiked with standard solutions before extraction. The serum was centrifuged in ultrafiltration membranes and the filtrate was spiked with the desired amount of 1.0 mmol L^{-1} standard solution, according to the required concentration. The resulting solution was extracted as earlier described and injected.

Results and Discussion

Method development

Aiming at the best separation of nucleoside standards, several electrophoretic parameters were evaluated. For assessment of the best conditions, electrophoretic mobility and resolution graphs against each evaluated parameter were constructed. Peaks were identified by UV absorption spectra and migration times.

BGE pH and composition were first evaluated. A good compromise between separation and analysis time was observed for borate buffer at pH 9.20 (Figure 2a), in which the maximum difference among evaluated analytes mobilities was noticed. BGE concentration was evaluated employing borate buffer solutions from 20 to

100 mmol L^{-1} at pH 9.20. The best separation and a suitable analysis time were observed with 20 mmol L^{-1} borate buffer due to higher EOF velocity under more diluted BGEs (Figure 2b). However, capillary zone electrophoresis (CZE) mode was not considered adequate for analytes separation since peak resolutions did not reach the electropherogram baseline.

Therefore, SDS was added to BGE within the range of 10 to 350 mmol L^{-1} to evaluate micellar electrokinetic capillary chromatography (MEKC) suitability for

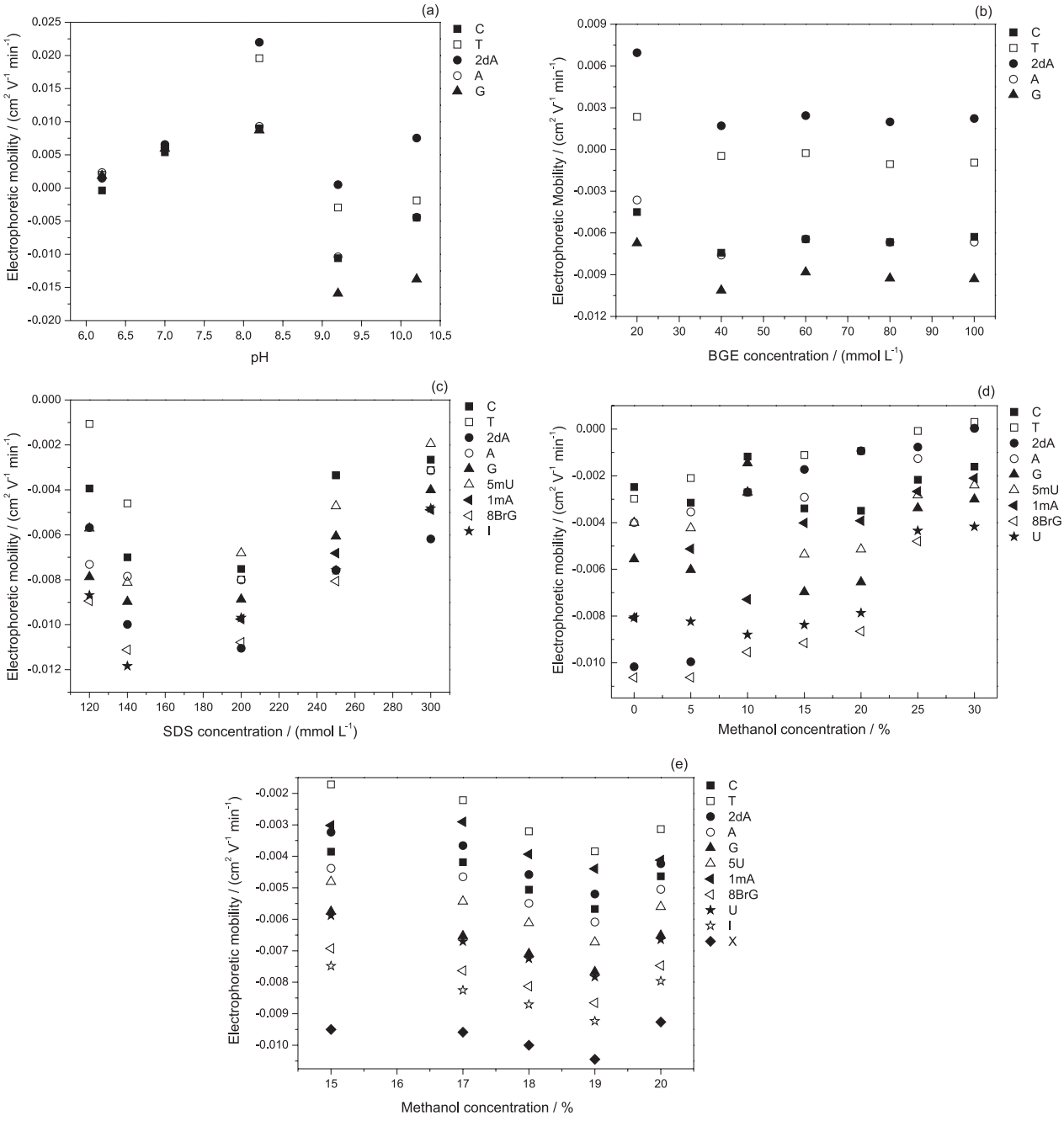

Figure 2. Optimization of CE parameters for the separation of the nucleoside standards, as follows: (a) electrophoretic mobility as a function of BGE pH and composition. Analysis conditions: 20 mmol L^{-1} phosphate buffer at pH 6.20 and 7.00; and 20 mmol L^{-1} borate buffer at pH 8.20, 9.20 and 10.20; total capillary length (L_T): 62 cm, effective capillary length (L_{eff}): 56 cm and inner capillary diameter (i.d.): 50 µm; 27 °C; 17 kV; 0.5 mmol L^{-1} solutions of the nucleoside standards T, C, A, 2dA and G; (b) electrophoretic mobility as a function of BGE concentration. Analysis conditions were the same as in (a), except for L_T: 60 cm, L_{eff}: 52 cm and BGE borate at pH 9.20; (c) electrophoretic mobility as a function of SDS concentration. Analysis conditions were the same as in (b) except for 20 mmol L^{-1} BGE borate at pH 9.20, 20 °C and 0.5 mmol L^{-1} solutions of the nucleoside standards T, C, A, 2dA, G, 1mA, 5mU, U plus the internal standard 8BrG; (d) electrophoretic mobility as a function of methanol concentration. Analysis conditions were the same as in (c) except for 20 mmol L^{-1} BGE borate, 300 mmol L^{-1} SDS at pH 9.20; and (e) electrophoretic mobility as a function of methanol concentration. Analysis conditions were the same as in (d) except for 20 mmol L^{-1} BGE borate, 260 mmol L^{-1} SDS at pH 9.00.

adequate separation of the analytes. It was noticed that the resolution between peaks was improved with higher SDS concentrations. The best separation was obtained with 20 mmol L^{-1} borate buffer at pH 9.20 and 300 mmol L^{-1} SDS. Nevertheless, under this condition, co-migration of C and 1mA, and 2dA and 8BrG (internal standard) pairs was observed (Figure 2c), leading to the investigation of organic modifier addition to BGE. Methanol concentrations varying from 0 to 30% were evaluated. The best separations were observed with 15 and 25% methanol, but the former was chosen based on a shorter analysis time (Figure 2d) and integrity of SDS micellar structures in such lower organic solvent concentration. In fact, nucleosides present very similar structure, which differs slightly in charge to radius ratio. However, hydrophobicity differences are more prominent, justifying the best separations obtained with surfactant and organic modifier addition to the BGE. Finally, the applied voltage was evaluated between 10 and 30 kV, with the construction of Ohm's curve, which showed that Joule effect was observed in values higher than 25 kV (data not presented). Voltage did not affect the separation, but the analysis time. For this reason, the value of 25 kV was applied in all subsequent analysis aiming at smaller analysis time and avoiding the Joule effect.

The high surfactant concentration caused problems like capillary clogging and breakage, as well as formation of bubbles and salt deposition inside the equipment due to high SDS concentration. The system was not able to maintain a stable current throughout successive runs, therefore poor reproducibility was frequently observed. The capillaries needed to be changed often and instrument maintenance was necessary at least once a week. The overall procedure could take up to half a day and the constant changes of capillary represented an additional cost. Therefore, after method optimization, this parameter was reevaluated aiming at a better compromise between separation, analysis time and equipment maintenance reduction. SDS concentration was reinvestigated within the concentration range of 240 to 300 mmol L^{-1}. Electrophoretic mobilities did not vary significantly with lower SDS concentrations, but suitable resolutions and efficiencies were found with 260 mmol L^{-1}. Thus, this concentration was employed in the subsequent analyses. Likewise, methanol concentrations were reinvestigated after SDS reevaluation on a fine tuning (Figure 2e). The addition of 17% methanol increased the resolution of all studied nucleosides, except G and U, which maintained almost the same values for all concentrations.

Figure 3 shows an electropherogram obtained under the optimized analysis conditions. Total analysis time was less than 25 min, the peak efficiencies were within a range of 4.1×10^4 (for X) to 3.0×10^5 (for 1mA) theoretical plates, and the minimum resolution was 1.1 (between G and U). Resolution values obtained by the optimized method are presented in Table 3.

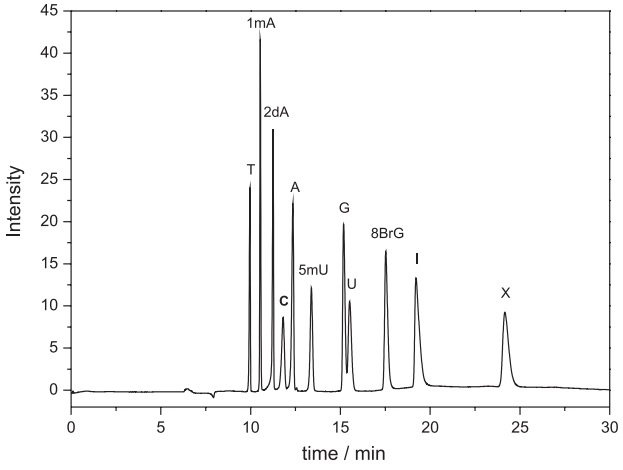

Figure 3. Electropherogram obtained under optimized analysis conditions, as follows: BGE composed by 20 mmol L^{-1} borate at pH 9.20, 260 mmol L^{-1} SDS and 17% methanol; 25 kV; 20 °C; L_t: 60 cm, L_{eff}: 52 cm, i.d.: 50 µm; 0.500 mmol L^{-1} solution of nucleoside standards.

Table 3. Observed resolutions in the optimized method of analysis

Pair of nucleosides	Resolution
T - 2dA	10.5
2dA - 1mA	9.4
1mA - C	5.1
C - A	2.9
A - 5mU	8.9
5-mU - G	13.8
G - U	1.1
U - 8BrG	9.0
8BrG - I	8.6
I - X	14.4

Method validation

The first step on method validation was to observe the method selectivity by analyzing a blank sample. No peaks were observed with the same migration time of the analytes (data not presented). Therefore, the method was considered selective for the target analytes. The nucleosides 2dA and T were not extracted since both of them do not present the binding cis-diol affinity group. Consequently, they could not be retained by the resin. For this reason, they were not validated.

The method detectability was evaluated by the limit of detection (LOD) and limit of quantification (LOQ). LOD was determined by a signal to noise ratio of 3:1, while LOQ

Table 4. Experimental limits of detection (LOD) compared with literature data. The smallest value for each analyte is underlined

Nucleoside	Developed method	Jiang *et al.*[25]	Zheng *et al.*[19]	Liebich *et al.*[14]	Szymanska *et al.*[20]	Helboe *et al* [26]
	LOD / (μmol L^{-1})					
1mA	<u>0.30</u>	0.76	N.A.	N.A.	N.A.	N.A.
C	1.2	1.67	4.0	3.1	<u>0.5</u>	4.1
5mU	<u>0.60</u>	N.A.	3.5	N.A.	0.98	N.A.
G	<u>0.30</u>	0.77	5.6	2.6	0.55	3.5
A	<u>0.60</u>	1.09	6.4	2.0	0.78	3.7
U	1.5	1.06	5.0	3.5	<u>0.17</u>	4.1
I	1.1	<u>0.56</u>	3.1	2.5	0.61	3.7
X	2.1	0.78	10	9.2	<u>0.41</u>	N.A.

N.A.: not analyzed.

was determined by a 10:1 ratio. The obtained values are smaller than those normally found for nucleoside analyses in urine samples for most of the analytes. Table 4 shows LOQs and LODs for the developed method and compares them with the literature data.

The results presented in Table 4 emphasize that, except for uridine, inosine, xantosine and cytidine, the LODs obtained by the developed method were smaller than those reported in the literature, although such analytes have shown smaller LODs than some of the cited works. However, the analyses presented herein were performed on biological matrix (blood serum) spiked with standard solutions, whereas the values in most published works were obtained with aqueous solutions of the standards, a condition that completely despises any possible matrix effect and reduces baseline noise, which improves significantly LOD values. That is the case of the literature values for LOD presented in Table 4.[14,20,25,26] The cleaner matrix justifies the smaller LOD value obtained in some cases, but it can cause mistakes during real sample analysis. Even if biological matrix is applied, most of the methods used urine, which may lead to differences in the LOD values due to matrix effect. The differences in the techniques must also be considered since most of the published methods employed HPLC and MS techniques.

The determinations of linearity and linear range were performed in triplicates through the elaboration of analytical curves with five points each, equally distributed within the linear range. Such determinations were also carried out with blood serum spiked with standard solutions, therefore matrix effects are considered on the curves. Correlation coefficients are acceptable for values higher than 0.99. The data obtained from the analytical curves are presented in Table 5 and Figure 4.

The method precision was evaluated within the same day (intra-run) and alternate days (inter-run) through 3 concentration levels, namely: minimum (the minimum concentration within the linear range), medium and maximum (the highest concentration within the linear range). The obtained results were expressed as relative standard deviation (RSD) and, according to the followed legislation, values higher than 15% should not be accepted for medium and maximum levels. For the minimum level, the RSD limit was 20%. Results presented in Table 6 show

Table 5. Analytical curves and linear regression coefficients (R) obtained by internal standard method

Nucleoside	Sensibility / (mAU μmol L^{-1})	Intercept	R	Linear range / (μmol L^{-1})
1mA	0.07080	0.05255	0.9984	2-10
C	0.04631	0.7880	0.9954	40-120
5mU	0.05662	0.2208	0.9971	8-16
G	0.07081	0.3141	0.9963	8-16
A	0.1200	–0.1238	0.9959	5-13
U	0.09131	–0.2580	0.9989	11-19
I	0.1046	–0.05544	0.9962	7-15
X	0.2232	–0.4810	0.9980	7-15

Analytical curves follow the equation: $y = ax + b$, where a is the curve slope (sensibility), b is the curve intercept with y axis, y is the peak area divided by the internal standard peak area, and x is the analyte concentration.

Table 6. Inter and intra-run precision of the method expressed as relative standard deviation (RSD) values

Nucleoside	Intra-run precision / % (Concentration / (μmol L^{-1}))			Inter-run precision / % (Concentration / (μmol L^{-1}))		
	Minimum	Medium	Maximum	Minimum	Medium	Maximum
1mA	16 (2.0)	10 (6.0)	10 (10.0)	16 (2.0)	2 (6.0)	13 (10.0)
C	10 (40.0)	12 (80.0)	2 (120.0)	22 (40.0)	1 (80.0)	5 (120.0)
5mU	4 (8.0)	6 (12.0)	2 (16.0)	14 (8.0)	3 (12.0)	8 (16.0)
G	6 (8.0)	8 (12.0)	3 (16.0)	16 (8.0)	6 (12.0)	5 (16.0)
A	13 (5.0)	9 (9.0)	5 (13.0)	7 (5.0)	7 (9.0)	5 (13.0)
U	17 (11.0)	7 (14.5)	12 (19.0)	9 (11.0)	6 (14.5)	12 (19.0)
I	18 (7.0)	15 (11.0)	15 (15.0)	10 (7.0)	5 (11.0)	12 (15.0)
X	1 (7.0)	13 (11.0)	7 (15.0)	16 (7.0)	16 (11.0)	10 (15.0)

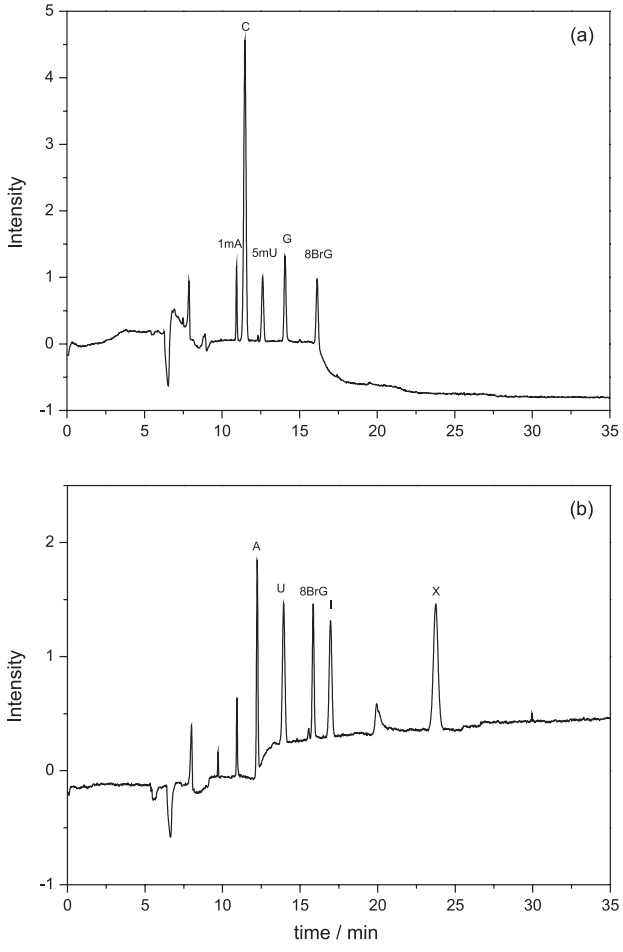

Figure 4. Electropherograms of the analyzed sample with analytes concentration referent to the middle point of the analytical curves: (a) 1mA, C, 5mU, G and 8BrG (internal standard); and (b) A, U, I, X and 8BrG. The conditions of analysis are the same presented in Figure 3, except for nucleosides concentration.

that, except for cytidine in minimum concentration and xanthosine in medium concentration in inter-run precision, all values were acceptable.

Accuracy was evaluated by recovery of the analytes after extraction in three levels of concentration that covered the linearity range (low, medium and high). Recovery calculation consisted on the ratio between the corrected peak area of samples fortified with the standards previously to the extraction and extracted samples fortified with standards, both in the spiked biological matrix (Table 7). The Brazilian legislation does not dictate specific values for recovery, but it states that percentages at about 100% are desirable. However, smaller values are admitted as long as the methodology is precise. The method accuracy varied from 87 to 117% and was considered satisfactory since the method is precise according to the followed legislation.

Table 7. Accuracy of the method expressed as recovery values

Nucleoside	Recovery / % (Concentration / (μmol L^{-1}))		
	Minimum	Medium	Maximum
1mA	117 (1.0)	90 (5.0)	102 (10.0)
C	98 (4.0)	93 (62.0)	95 (120.0)
5mU	92 (2.0)	93 (9.0)	89 (16.0)
G	99 (1.0)	104 (8.5)	95 (16.0)
A	87 (2.0)	111 (7.5)	107 (13.0)
U	95 (5.0)	104 (12.0)	113 (19.0)
I	91 (3.5)	100 (9.3)	97 (15.0)
X	105 (7.0)	91 (11.0)	92 (15.0)

Since the developed method is confident for quantification of analytes according to bioanalytical method validation, the next steps involve the analyses of real samples from different cancer base pathologies. Such samples will be firstly analyzed by immunoassays in order to guide the evaluation of the results obtained by CE-UV.

Conclusions

The developed method is efficient, fast, has good resolution and presents low sample and BGE consumption. The described CE analysis is suitable for cancer screening within 25 min with baseline resolution. The sample preparation eliminates possible interfering compounds from the matrix as a clean-up step and the 1 mL volume described could be reduced for routine analysis. A total of 10 nucleosides plus internal standard were separated and 8 of them were validated for blood serum analysis. The figures of merit evaluated during the validation show that the bioanalytical method is reliable for quantification of analytes in biological real samples, according to Brazilian regulations. Therefore, it has suitable detectability, precision, accuracy and linearity within the working concentration range. The methodology is appropriate for the analysis of modified and non-modified nucleosides in blood serum samples. The next step consists of the analyses of samples from healthy and prostate cancer subjects, followed by a chemometric study in order to characterize the potentiality of modified and non-modified nucleosides as tumor biomarkers. In the near future, the application of this method in clinical diagnosis, allied to well-established methods, may contribute for earlier and more accurate cancer detection and the recognition of false-positive results.

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