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Development and validation of urinary nucleosides and creatinine assay by capillary electrophoresis with solid phase extraction

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Abstract

For the analysis of metabolite nucleoside profiles, capillary electrophoretic (CE) methods preceded by appropriate solid phase extraction procedures have been developed. The approach has been proposed for the determination of 13 nucleosides and creatinine in human urine. A background solution composed of 100 mM borate–72 mM phosphate–160 mM SDS and a fused silica capillary of 70 cm length to detector and 50 μm i.d. were used. The methods developed were statistically validated for their linearity, trueness, precision and selectivity. Stability of the analyzed nucleoside profiles in urine during storage was checked. Validation parameters of solid phase extraction procedures for urinary nucleosides were evaluated. The developed analytical methods were employed for the analysis of 22 urine samples from healthy patients and cancer patients from the urological ward. Nucleoside profiles were compared among the subjects. It was proved that the methods proposed were suitable for a fast and reliable determination of urinary creatinine and modified nucleoside profiles, which can be further submitted for the metabonomic analysis of cancer patients.

Keywords: Urinary nucleosides; Creatinine; Metabonomics; Urogenitial tract cancer; Capillary electrophoresis

1. Introduction

In human urine, about 1000 different metabolites could be identified belonging to different classes of endogenous compounds, like amino acids, peptides, nucleosides, catecholamines, purines, etc. The variation of concentrations of characteristic metabolites in urine is specific to the physiological and pathological state of the organism as well as to individual diseases. When metabolite profiles from healthy individuals are compared with those from sick patients, the differences observed could serve as an indication or prediction of disease. One of the main goals of metabonomics is to identify metabolite profiles which could be used as diagnostic tools of various diseases [1,2]. However, because there are so many urinary components involved, measuring metabolite profiles of urine is not an easy task. As a consequence, the metabonomic analysis is often focused on smaller sets of compounds such as amino acids, catecholamines or nucleosides.

Nucleosides are primary constituents of ribonucleic acids (RNA). When RNAs are biotransformed into nucleosides, they are normally catabolized to uric acid or β-alanine. In particular cases, RNAs are transformed into modified nucleosides which cannot be reutilized and are extracted intact in urine. Therefore, it appeared reasonable and interesting to undertake measurements of urinary nucleosides in order to estimate total RNA turnover. The turnover of nucleic acids increases when cell proliferation takes place: in physiological processes, like growth and pregnancy [3] and in pathological ones, like inflammation, haemopathies and malignant diseases [4,5]. Urinary profiles of modified and normal nucleosides have been used in clinical practice as markers of leukemia [6], breast cancer [7,8], thyroid cancer [9], uterine cervical cancer [10], liver cancer [11] and rheumatoid arthritis process [12]. Profiles of urinary nucleosides could also be used in monitoring disease progress and the response of individuals to an applied therapy [8]. Nevertheless, there are no available clinical tests based on urinary nucleoside profiles as a routine diagnostic tool on groups of cancer, the lack of which could be a consequence of imperfect analytical methods used to obtain urinary nucleoside profiles.

Nucleosides in biological samples have been analyzed by the application of selective solid phase extraction (SPE) fol-

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lowed by specific separation methods. Phenylboronate gel as a SPE stationary phase, has specific affinity to *cis*-diol groups, which are present in the chemical structure of nucleosides, nucleotides and sugars. It has been successfully applied in the solid phase extraction of urinary nucleosides by Gehrke et al. and others (http://www.biorad.com) [13,14]. Different analytical techniques such as immunoassays [7,12], high performance chromatography [11,15–18] and capillary electrophoresis [8–10,19] have been employed in the separation of nucleoside and nucleotide metabolites. Nucleosides, due to their properties such as: negative electric charge in a wide range of pH, diverse molecular weight and hydrophobicity; were analyzed by capillary zone electrophoresis, micellar electrokinetic chromatography and isotachophoresis [20,21]. The advantages, such as a relatively short time of analysis, a usually high efficiency of resolution obtained and a minimal amount of sample required, make electromigration techniques especially valuable in metabonomics studies [22,23]. We also proved them valuable in the study of urinary nucleosides [24,25].

Micellar electrokinetic chromatographic (MEKC) methods with sodium dodecyl sulfate (SDS)–borate–phosphate buffers were applied to determine nucleoside metabolites by several groups of researchers. Liebich et al. used 25 mM borate–50 mM phosphate–300 mM SDS background electrolyte (BGE) (pH 6.7) [15]. Zheng et al. used the same BGE but a slightly different pH (pH 6.9) [8]. Kim et al. applied 25 mM borate–42.5 mM phosphate–200 mM SDS (pH 6.7) [10]. All the authors obtained a resolution of 13–16 nucleosides present in urine samples from cancer patients. The analyzed group of nucleosides included: pseudouridine, uridine, cytidine, methyluridine, inosine, 1-methylinosine, N^4-acethylcytidine, guanosine, 1-methylguanosine, adenosine, xanthosine, 2-methylguanosine, 6-methyladenosine and dihydrouridine.

The main goal of the present study was to develop a fast and reproducible CE method to study most of the nucleosides present in urine samples from urogenitial cancer patients and healthy controls. Our nucleoside set comprised 1-methyladenosine, which is of prognostic value in urogenitial cancer [4], and was not analyzed before by CE because of a long migration time. Moreover, the capillary electrophoretic method and solid phase extraction procedure were fully validated to assure their quality. Validation data included linearity, limits of detection, precision, trueness, recovery and stability of nucleosides in frozen urine. Therefore, an optimized CE method could be successfully applied in the analysis of nucleoside profiles in urine samples as it allows 18 different compounds to be separated and quantified.

Additionally, another CE method for the assay of urinary creatinine levels was developed. Creatinine is a compound whose concentration strictly corresponds to urine dilution and therefore is included in the calculation and comparison of nucleoside levels. The CE method for the determination of creatinine levels employs the same analytical conditions as for nucleosides with some minor changes in operation conditions. This makes the complete assay of urinary nucleoside profiles feasible consisting of the analysis of nucleosides and the following analysis of creatinine in one capillary run-by-run. Based on the developed procedure, urine samples could be effectively examined and reliable profiles of nucleosides could be obtained.

2. Experimental

2.1. Chemicals and preparation of standard solutions

Reference standards for 13 nucleosides, *i.e.*, uridine, pseudouridine, cytidine, 5-methyluridine, inosine, N^4-acetylcytidine, guanosine, adenosine, N^2,N^2-dimethylguanosine, N^6-methyladenosine, N^1-methyladenosine, xanthosine, and 8-bromoguanosine (internal standard) and creatinine were purchased from Sigma–Aldrich (St Louis, MO, USA) Phosphoric acid, borax (sodium tetraborate decahydrate), sodium dodecyl sulfate 98.5% and ammonium acetate were from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Formic acid came from Lancaster Synthesis UK (Newgate, Lancashire, UK). Sodium hydroxide, methanol and ammonia were obtained from POCH (Gliwice, Poland). The Affi-gel 601, used as the stationary phase for the extraction of nucleosides from urine, was purchased from Bio-Rad (Hercules, CA, USA). Reversed osmosed deionised water for the preparation of the standard solution, the background electrolyte and other solutions were from MiliQ-Plus system (Millipore, Vienna, Austria).

The 10 mM stock solutions of all standards were prepared in deionised water (except the stock solution of guanosine, which was prepared in 0.1M NaOH), and kept frozen at −34 °C. The working standard solutions were prepared by dilution of the stock solutions with deionised water to concentrations in the range of 5–5000 μM.

2.2. Urine samples

Spontaneous urine samples from 12 healthy adults and 10 cancer patients from the Department of Urology, the Medical University of Gdańsk, Gdańsk, Poland were collected after their informed consents. The studies were performed in accordance with the principles embodied in the Declaration of Helsinki. The group of healthy controls (5 women, 7 men) consisted of people who were not undergoing medication at the time of sample collection and whose condition was proved by medical examination. The group of cancer patients (5 women, 5 men) included people with a medical diagnosis of kidney, prostate and bladder cancer. The cancer patients were undergoing chemotherapy or radiotherapy treatment but neoplastic changes existed at the time of collection. After urine collection, the samples were frozen immediately and stored at −34 °C. Directly before the analysis, the samples were thawed at room temperature.

2.3. Instrumentation

The CE experiments were carried out on a Beckman Coulter P/ACE MDQ (Beckman Instruments, Fullerton, CA, USA) fitted with a diode array UV-absorbance detector (190–600 nm), a temperature-controlled capillary compartment (liquid cooled) and a temperature-controlled autosampler (air cooled). Elec-

trophoretic data were acquired and analysed by 32 Karat software.

Separations were performed in fused silica capillaries (50 μm, i.d.) (Microsolv CE, Microsolv Technology Corporation, Eatontown, NJ, USA). New capillaries were conditioned for 10 min at 30° C with deionised water, followed by 1 M NaOH for 10 min, 0.1 M NaOH for 20 min, deionised water for 20 min and BGE for 60 min using pressure 20 psi (1 psi = 6894.76 Pa). Then, the capillaries were conditioned with high voltage: 10 kV for 20 min, followed by 25 kV for 20 min. Each capillary was washed every morning with deionised water for 5 min and BGE for 20 min, and immediately before each analysis with 0.1 M NaOH for 2 min and BGE for 3 min, also using a pressure of 20 psi.

Solid phase extractions were performed on a vacuum manifold column processor (J.T. Baker, Griesheim, Germany). Eluates obtained during the extraction procedure were lyophilized in a Christ freeze dryer Alpha 1-2LD (Martin Christ, Osterode am Harz, Germany).

2.4. Capillary electrophoretic conditions

The running buffer for the analysis of creatinine and nucleosides was composed of 100 mM sodium borate, 72.5 mM phosphoric acid and 160 mM SDS. The pH was adjusted to 6.7 with 1 M NaOH before the addition of SDS. Buffer solutions were filtered before use through a 0.45 μm Minisart filter (Sartorius, Gottingen, UK).

Optimum separations for nucleosides and creatinine were obtained using an 80 cm capillary (70 cm effective length), 25 kV applied voltage (observed current was 78–83 μA) with 0.1 psi pressure applied during electrophoretic run and 30 °C maintained temperature of the capillary. Samples were introduced to the capillary in the pressure injection mode for 5 s at 0.5 psi for the analysis of nucleosides, and for 10 s at 0.5 psi for the analysis of creatinine. For the analysis of creatinine, the time of separation was 12 min and detection was carried out at 234 nm. During the analysis of nucleosides, the time of separation was 25 min and detection was performed at 214 and 254 nm.

2.5. Sample preparation and extraction conditions

Each urine sample was used for the analyses of both creatinine and nucleosides. The process was composed of several steps and is briefly illustrated in Fig. 1.

In the case of creatinine analysis, the urine samples were diluted 20-fold in deionised water, mixed, centrifuged and subjected to analysis in a CE system.

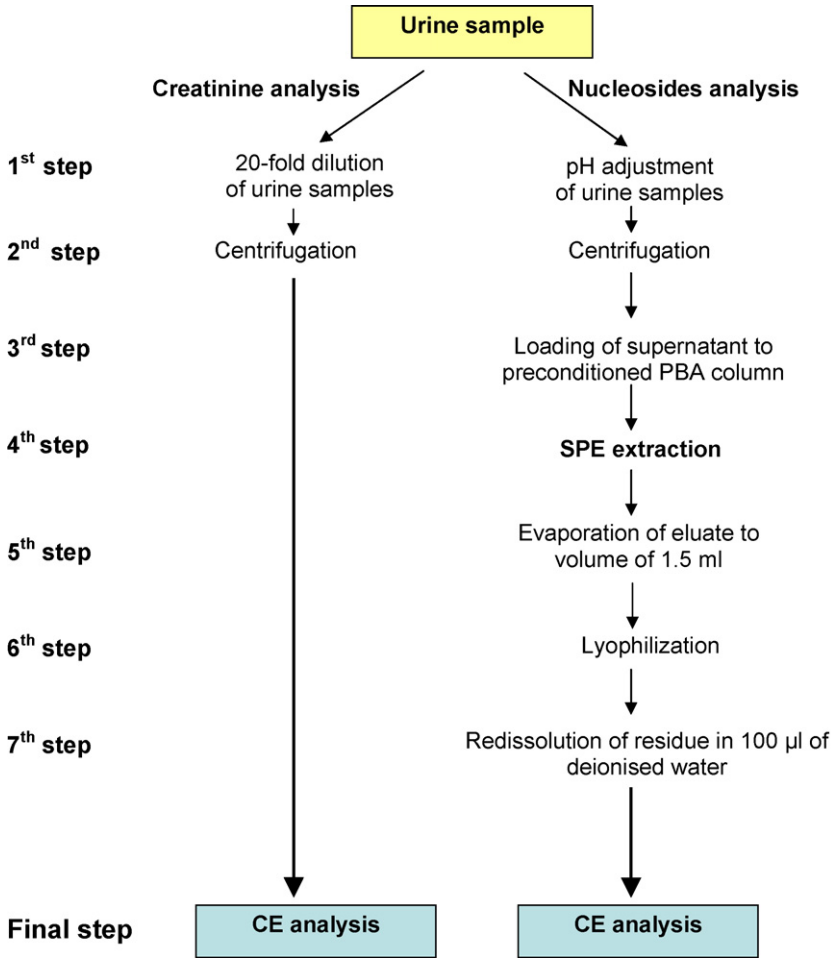

Fig. 1. Schematic overview of the preparation of urine samples for capillary electrophoretic analysis.

For the analysis of nucleosides, 25% ammonia was added to urine samples to adjust pH to the range from 8.2 to 8.6. Next, urine samples were mixed and centrifuged. After centrifugation, 2 ml of the supernatant was loaded on a preconditioned PBA column, together with a constant volume of the internal standard (100 μl of 150 μM internal standard solution). The PBA column consisted of a luer-tipped polypropylene SPE tube (3 ml) purchased from Supelco (Bellefonte, PA, USA), packed with stationary phase material Affi-gel 601 (200 mg). Before first use, the gel was allowed to swell for 5 min in 3 ml of water and after that, was alternately washed 10 times with methanol and water (0.5 ml each time). Before the application of supernatant, the column was equilibrated by washing sequentially with 0.1 M formic acid in 50% methanol and 0.25 M ammonium acetate (pH 8.6), as described by Gehrke et al. [13]. After loading the sample on the PBA column, it was washed with 0.5 ml of 0.25 mM ammonium acetate and the column was left to stand for 10 min. Then, the column was rinsed with 4 ml of 0.25 mM ammonium acetate and 0.3 ml 50% methanol (two times). Between every rinse, a 3 min interval was applied. Then, 0.5 ml 0.1 M formic acid in 50% methanol was introduced to the SPE column to replace 50% methanol and again a 3 min interval was adopted to prepare the column for the elution of nucleosides. At the end, the PBA column was eluted with 3 ml of 0.1 M formic acid in 50% methanol. Methanol from the eluate was evaporated under a stream of nitrogen at 36 °C in a thermoblock and the final volume of *ca*. 1.5 ml was lyophilized. The residue was dissolved in 100 μl of deionised water and injected into the capillary. The obtained aliquot was concentrated by the factor of 20. The PBA column was reconditioned and used in SPE extraction 12 times without loss of recovery performance.

2.6. Validation of analytical methods

The concentrations used during the studies were based on the expected range during analysis of urine samples. The following concentrations in water were used to construct the calibration curves: 0, 5, 10, 25, 50, 100, 250, 500 μM for cytidine, 5-methyluridine, guanosine, adenosine, N^2,N^2-dimethylguanosine, N^6-methyladenosine, xanthosine; 0, 10, 25, 50, 100, 250, 500, 1000 μM for N^1-methyladenosine, uridine, inosine, N^4-acetylcytidine; 10, 25, 50, 100, 500, 1000, 2000 μM for creatinine; and 0, 40, 100, 200, 400, 1000, 2000, 5000 μM for pseudouridine.

In assessment of the intra- and inter-day precision, aqueous solutions and pooled urine spiked with nucleosides and creatinine were repeatedly analyzed. Between day validation was calculated for three concentrations (100%, 150% and 50% mean concentration of each compound in urine multiplied by a factor of 20 for nucleosides and by a factor 0.05 for creatinine) and one pooled urine sample spiked with nucleosides or creatinine corresponding to 100% mean concentration in urine, also multiplied by a factor 20 or 0.05. The mean concentrations of each compound were: 2500 μM for pseudouridine, 75 μM for uridine, 200 μM for 5-methyluridine, 30 μM for cytidine, 100 μM for N^4-acetylcytidine, 85 μM for guanosine, 120 μM for N^2,N^2-dimethylguanosine, 100 μM for inosine, 60 μM for xanthosine, 40 μM for adenosine, 30 μM for N^6-methyladenosine, 500 μM for N^1-methyladenosine and 600 μM for creatinine.

Trueness of the method was investigated on five concentrations of the analyzed compounds, on the same samples as used in the construction of calibration curves. The detection limit was calculated on the basis of standard deviation of results for blank samples and calibration curve parameters. Selectivity of the assay was determined by chemometric tools such as multivariate curve resolution-alternating least squares (MCR-ALS) applied to electrophoretic peaks from urine samples [26]. The stability of nucleosides in urine, stored at −24 °C for 3 months, has also been monitored.

3. Results

3.1. Optimization of the MEKC method for analysis of nucleosides

Urinary nucleosides were analyzed by several capillary electrophoretic methods but the most common are those with BGE containing sodium borate, sodium phosphate and relatively high concentrations (250–300 mM) of SDS, with pH in the range of 6.7–6.9 [8,10,15,19]. During our studies, BGE consisting of 25 mM borate–42.5 mM phosphate–300 mM SDS developed by Kim et al. [10] was used as a starting BGE for method optimization.

Finally, after several modifications, a background electrolyte containing 100 mM borate–72.5 mM phosphate–160 mM SDS appeared to possess the best separation properties. Additionally, other capillary electrophoretic conditions have been changed, namely the length of the capillary (70 cm – length to detector, 80 cm – length of the whole capillary), the applied voltage – 25 kV and the temperature during separation – 30 °C. Another improvement was the application of additional pressure during the electrophoretic run (0.1 psi) for the reduction of analysis time.

A shorter time of analysis for a similar set of compounds and very good reproducibility of results (see Section 3.2) were achieved in comparison to the previously-described methods. This is an effect of:

- Involvement of 0.1 psi during the analytical run and BGE with a higher concentration of phosphoric acid anions (72.5 mM), which increases the background solution conductivity (the current observed during electrophoretic analysis was 75–85 μA) and by doing so, increases the migration of compounds in the electric field, resulting in a decrease in the analysis time to 25 min (Fig. 2).
- Application of BGE with a higher content of borate anions (100 mM), which creates complexes with diols such as nucleosides, which introduces an additional mechanism for the separation of nucleosides besides the electric field and pseudostationary phase (SDS), resulting in high efficiency of the resolution of analyzed compounds.
- Application of BGE with a concentration of 160 mM of SDS assures a higher stability of the prepared BGE at

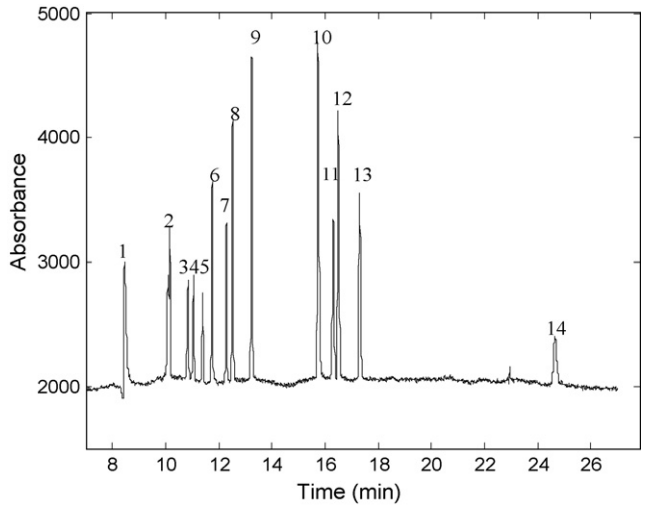

Fig. 2. Typical electropherogram of standard modified nucleosides in a water sample. Peaks: 1 – EOF; 2 – pseudouridine; 3 – uridine; 4 – cytidine; 5 – 5-methyluridine; 6 – inosine; 7 – N^4-acetylcytidine; 8 – guanosine; 9 – adenosine; 10 – N^2,N^2-dimethylguanosine; 11 – 6-methyladenosine; 12 – xanthosine; 13 – I.S. (8-bromoguanosine); 14 – 1-methyladenosine.

room temperature and fewer problems in cleaning the capillary and electrodes after analysis. It improves method reproducibility.

Peaks of nucleoside standards were identified on the basis of their migration time and spiking. In the case of urine samples, peak identity was confirmed by chemometric analysis as described below in Section 3.6.

In the SPE method, significant changes included the introduction of an elapse time (3 min) between every rinse of the column after sample application and a change in the amount of water (100 μl) used for redissolution of freeze-dried compounds at the end of the analytical procedure. The elapsed time improves the selectivity of impurity and nucleoside elution. Redissolution of the residue in 100 μl enhances the recovery of compounds from sample walls.

3.2. Validation of MEKC method for analysis of nucleosides

Method validation is the last step in method development and is carried out to ensure its quality. Because our method is going to be used in the analysis of numerous nucleoside profiles in biological samples, we decided to validate MEKC and SPE methods separately. We have checked if validation parameters of the MEKC method allow its use not only in the analysis of urinary nucleosides but also nucleosides present in other biofluids. On the other hand, separate validation of SPE procedure also ensures its quality when we would like to use it with other separation methods, *e.g.* high performance liquid chromatography (HPLC).

The linearity of the analytical method is its ability (within a given range) to obtain results which are directly proportional to the concentration of the analytes in the sample. It was tested by analyzing eight stock solutions in the concentration range of 5–1000 μM for most nucleosides as described in Section 2.6. The data were subjected to linear regression analysis in order to achieve the appropriate calibration factors. The regression parameters such as linearity range, slope, intercept, standard error and correlation coefficients (r) are presented in Table 1.

The limits of detection (LOD) were determined at 254 nm and calculated as described in Section 2.6. They were below 1 μM for most investigated compounds which confirmed the suitability of the developed method for the analysis of biological samples. The values of LOD are also presented in Table 1 and are expressed in μM and μg/ml.

The precision of the method was evaluated by means of area and migration time reproducibility in four samples as described in Section 2.6. In comparison to previously-reported methods [8,10,15,19], in our method, a lower concentration of SDS was applied, which improved the migration time precision between runs within and between the days of analysis. This is also due to appropriate capillary conditioning. The intra-day (each sample analyzed six times one-by-one) and inter-day (three consecutive days, three different BGEs, each sample analyzed six times each day) precision of the assay was tested. The calculations are presented in Tables 2 and 3 as relative standard deviations

Table 1
Linearity parameters: linear range, slope, limit of confidence for slope, intercept, limit of confidence for intercept, standard error, correlation coefficients and limit of detection (LOD) for modified nucleosides and creatinine

Compound	Linear range (μM)	Slope (μM/μA)	Limit of confidence for slope $p = 95\%$	Intercept (μM)	Limit of confidence for intercept $p = 95\%$	Standard error	r	LOD (μM)	LOD (μg/ml)
Pseudouridine	20–5000	0.0445	0.0419–0.0471	33.83	−15.51–83.17	40.7	0.9987	1.12	0.27
Uridine	10–500	0.0468	0.0436–0.0501	9.21	−5.21–23.62	11.91	0.9982	0.17	0.04
5-Methyluridine	5–500	0.0356	0.0330–0.0383	4.11	−4.07–12.28	5.59	0.9986	0.98	0.28
Cytidine	5–500	0.0564	0.0527–0.0601	3.09	−10.92–17.11	11.38	0.9984	0.5	0.12
N^4-Acetylcytidine	10–500	0.0425	0.0388–0.0462	5.34	−4.13–14.81	6.53	0.9981	2.26	0.58
Inosine	10–500	0.0405	0.0378–0.0432	−7.71	−22.35–6.95	11.53	0.9984	0.61	0.16
Guanosine	5–500	0.0198	0.0181–0.0216	4.79	−4.64–14.22	6.47	0.9981	0.55	0.16
N^2,N^2-Dimethylguanosine	5–500	0.0110	0.0101–0.0119	4.99	−3.88–13.86	6.10	0.9983	0.93	0.29
Xanthosine	5–500	0.0321	0.0302–0.0339	−1.69	−14.78–10.79	10.00	0.9988	0.41	0.12
Adenosine	5–500	0.0249	0.0237–0.0261	−4.71	−15.22–5.81	8.35	0.9992	0.78	0.21
1-Methyladenosine	10–500	0.0403	0.0370–0.435	−1.68	−22.37–19.02	9.96	0.9990	0.65	0.18
6-Methyladenosine	5–500	0.0179	0.0165–0.0193	4.74	−4.06–13.53	6.04	0.9983	0.93	0.26
Creatinine	10–2000	0.0250	0.0238–0.0262	6.30	−16.03–28.62	15.70	0.9994	0.85	0.09

Table 2
Migration time precision of standard nucleosides and creatinine in water and urine samples expressed as relative standard deviation R.S.D. (%)

Compound/Sample	Intra-day repeatability				Inter-day repeatability			
	100%[a] in water	150%[a] in water	50%[a] in water	100%[a] in urine	100%[a] in water	150%[a] in water	50%[a] in water	100%[a] in urine
Pseudouridine	0.14	0.17	0.16	0.42	1.31	1.27	0.89	0.90
Uridine	0.3	0.32	0.10	0.26	1.39	1.07	1.04	1.14
5-Methyluridine	0.16	0.18	0.09	0.46	1.44	1.34	0.87	0.97
Cytidine	0.32	0.31	0.11	0.30	1.57	1.17	1.19	1.2
N^4-Acetylcytidine	0.16	0.17	0.11	0.50	1.34	1.21	0.76	0.81
Inosine	0.38	0.34	0.10	0.32	1.46	1.13	0.92	1.23
Guanosine	0.19	0.22	0.11	0.54	1.48	1.10	0.65	0.92
N^2,N^2-Dimethylguanosine	0.25	0.28	0.11	0.67	2.48	1.61	1.58	1.02
Xanthosine	0.48	0.62	0.13	0.47	1.80	1.26	1.37	1.23
Adenosine	0.53	0.41	0.14	0.35	0.99	2.10	1.72	1.41
1-Methyladenosine	0.76	0.62	0.26	0.75	5.51	5.25	4.62	5.94
6-Methyladenosine	0.26	0.29	0.12	0.69	1.93	1.73	1.71	1.08
Creatinine	0.14	0.13	0.12	0.15	0.15	0.71	0.40	0.26

For the intra-day repeatability, the number of replicates in each case was 6 ($n = 6$), for inter-day precision, the number of replicates was 18 ($n = 18$) run within three consecutive days.
[a] percent mean concentration of each compound in 22 analyzed water or urine samples.

(R.S.D.) (%). The inter-run precision of the migration time and the peak area for the internal standard were also evaluated and were 0.24% and 2.22%, respectively.

Trueness of the method for within-day variability is presented in Table 4 and is at a satisfactory level.

3.3. Extraction efficiency

In our work, recovery pertains to the extraction efficiency of the optimized method within the limits of variability. The recoveries of nucleosides were measured by assaying pooled urine samples with an added standard mixture. Recoveries were performed for urine samples fortified with two concentrations of nucleosides: 10 and 100 nmol/ml. The average recoveries for 13 nucleosides were for 100 nmol/ml (93.63%, R.S.D. 7.84%, $n = 12$) and for 10 nmol/ml (112.15%, R.S.D. 15.75%, $n = 15$), respectively. The results for all the analytes were consistent, precise and reproducible.

3.4. Stability studies

The stability of nucleosides in urine samples after one, two and three months storage at −24 °C was evaluated. According to [27,28], there is high probability that most nucleosides degrade in solutions at pH above 5.0. Thus, pH adjustment of urine samples is essential to limit the degradation process.

The urine samples with pH 5.2, 6.7 and 7.4 were divided into two sets. One set of urine samples was adjusted to pH 4.0 with

Table 3
Peak area precision of standard nucleosides and creatinine in water and urine samples expressed as relative standard deviation R.S.D. (%)

Compound	Intra-day repeatability				Inter-day repeatability			
	100%[a] in water	150%[a] in water	50%[a] in water	100%[a] in urine	100%[a] in water	150%[a] in water	50%[a] in water	100%[a] in urine
Pseudouridine	1.49	3.78	5.93	5.96	10.39	8.81	12.57	11.76
Uridine	1.86	4.61	8.96	6.73	9.30	6.79	9.33	26.40
5-Methyluridine	1.90	3.11	5.67	6.30	9.99	8.38	12.56	14.06
Cytidine	2.77	5.53	7.16	5.42	9.23	9.23	8.57	11.60
N^4-Acetylcytidine	4.06	4.43	7.47	9.50	8.22	6.10	12.68	12.69
Inosine	2.69	4.80	5.71	6.17	8.26	7.57	9.06	13.15
Guanosine	1.88	3.46	4.15	6.29	10.92	8.73	12.53	12.52
N^2,N^2-Dimethylguanosine	5.18	4.13	6.81	5.70	11.58	9.95	13.78	10.65
Xanthosine	4.69	6.60	5.92[b]	6.45	11.90	8.13	14.13	12.94
Adenosine	2.45	4.37	6.77	6.68	10.92	7.27	8.68	19.74
1-Methyladenosine	5.04	6.42	6.36	6.50	12.27	7.68	8.71	6.49
6-Methyladenosine	4.32	3.93	10.41[b]	8.78	13.75	8.98	15.02	20.50
Creatinine	3.71	3.78	3.95	7.23	8.01	6.94	7.62	10.04

For the intra-day repeatability, the number of replicates in each case was 6 ($n = 6$), for inter-day precision, the number of replicates was 18 ($n = 18$) run within three consecutive days.
[a] percent mean concentration of each compound in 22 analyzed water or urine samples.
[b] R.S.D. (%) calculated for five replicates ($n = 5$).

Table 4
Trueness of nucleoside analysis expressed as mean percentage recovery of amount added to 1 ml sample

Compound	A	B	C	D	E
Pseudouridine	115.60 (107.96–123.24)	101.20 (87.56–114.84)	85.42 (80.46–90.38)	95.40 (83.76–107.04)	101.43 (93.13–109.73)
Uridine	117.08 (105.42–128.49)	90.80 (82.74–98.86)	97.75 (83.31–112.19)	91.37 (86.99–95.75)	101.97 (96.01–107.93)
5-Methyluridine	94.75 (84.73–104.77)	103.84 (94.36–113.32)	96.94 (83.3–108.58)	88.97 (81.55–96.39)	101.48 (96.12–106.84)
Cytidine	103.6 (96.8–110.4)	95.72 (90.58–100.86)	108.86 (94.22–123.5)	91.24 (85.56–96.92)	101.59 (86.15–117.03)
N^4-Acetylcytidine	124.80 (117.52–132.08)	105.88 (97–114.76)	96.06 (78.78–113.34)	90.72 (85.58–95.86)	101.31 (95.31–107.31)
Inosine	87.70 (77.46–97.94)	93.30 (81.34–105.26)	120.39 (102.09–138.69)	103.19 (94.29–112.09)	98.25 (93.01–103.49)
Guanosine	98.74 (86.4–111.08)	105.48 (96.24–114.72)	96.31 (90.49–102.13)	89.16 (76.52–101.8)	101.48 (95.7–107.26)
N^2,N^2-Dimethylguanosine	124.91 (114.83–134.99)	107.59 (99.81–115.37)	96.29 (79.27–113.31)	89.74 (83.88–95.6)	101.40 (88.44–114.36)
Xanthosine	99.68 (95.84–103.52)	94.29 (78.35–110.23)	117.68 (102.5–132.86)	94.86 (85.74–103.98)	100.04 (94.18–105.9)
Adenosine	85.85 (73.79–97.91)	93.23 (72.43–114.03)	114.71 (97.45–131.97)	102.48 (96.72–108.24)	98.78 (94.34–103.22)
1-Methyladenosine	93.88 (68.76–119)	93.03 (75.17–110.89)	97.11 (78.89–115.33)	105.99 (95.13–116.85)	98.58 (80.9–116.26)
6-Methyladenosine	128.46 (105.84–151.08)	105.64 (99.7–111.58)	95.74 (80.86–110.62)	90.03 (85.21–94.85)	101.39 (88.85–113.93)

In brackets, limits of confidence for mean recovery ($p = 95\%$) are included. The number of replicates in each case was 6 ($n = 6$). A, B, C, D, E correspond to 25, 50, 100, 250, 500 nmol added to 1 ml sample, respectively, for uridine, cytidine, inosine, xanthosine, adenosine and 1-methyladenosine, 100, 200, 400, 1000, 2000 nmol for pseudouridine and 10, 25, 50, 100, 250 nmol for other nucleosides analysed.

concentrated formic acid and frozen. The second, unadjusted set of urine samples, was frozen immediately. They were analyzed after 1, 2 and 3 months of storage and the results of the assay were compared with the urine samples not subjected to storage. There was no significant or relevant change (no more than 10%) in nucleoside concentrations between samples with an adjusted pH in a different range of pH. Therefore, there is no need for special urine pH adjustment before freezing and storage.

3.5. Optimization and validation of MEKC method for the analysis of creatinine

Creatinine could be analyzed by several analytical methods including capillary electrophoretic procedures [29–31]. The simplest solution in this study appeared to be the analysis of creatinine in the same conditions as nucleosides and this idea appeared to be easy to employ. Creatinine could easily be measured in urine with the application of the nucleosides' method with only a change of time of sample injection (10 s) and detection wavelength (234 nm). The migration time of creatinine under these conditions was *ca.* 10.1 min. The quantification at 234 nm is appropriate for creatinine, which has its second absorbance maximum at this wavelength. The capillary electrophoretic method was compared and verified with a colorimetric method based on the Jaffe reaction (absorbance at 490 nm). The accuracy of the CE method in comparison with the colorimetric one was 109.67% with R.S.D. 6.35% for 20 urine samples. The trueness of the assay for creatinine in aqueous solutions was 92.00% with R.S.D. 1.32% for three different concentrations (1 mM, 100 μM and 25 μM).

Other validation parameters like linearity, LOD and migration time and area precision are presented in Tables 1–3.

3.6. Application of the developed methods in the analysis of biological samples

In order to demonstrate the applicability of the validated procedure, it was applied to 22 urine samples collected from healthy and cancer patients. Samples were extracted by SPE

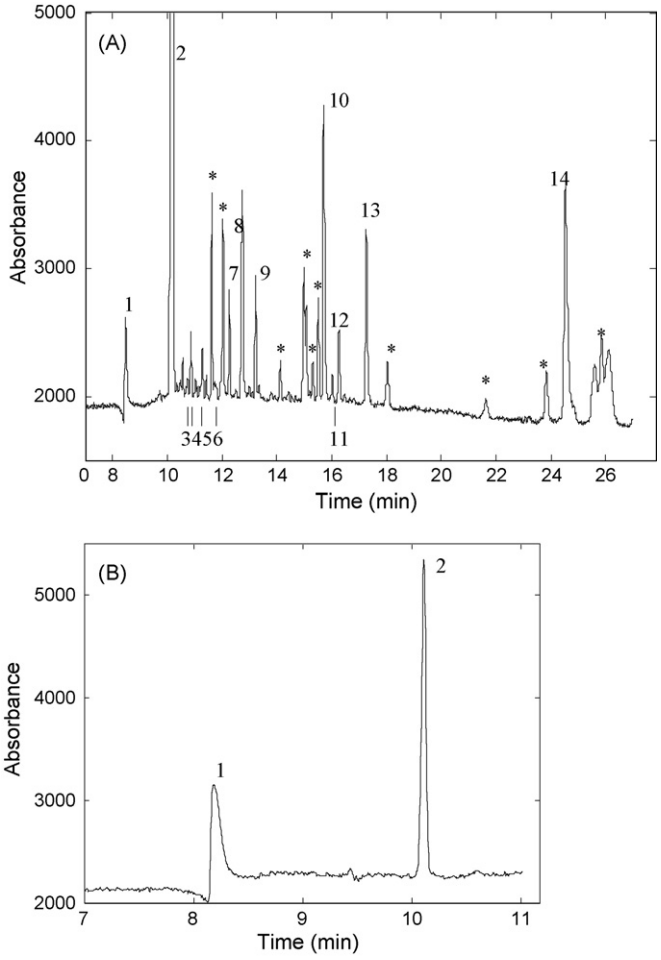

Fig. 3. Typical electropherograms: (A) Modified nucleoside analysis in a urine sample; peaks: 1 – EOF; 2 – pseudouridine; 3 – uridine; 4 – cytidine; 5 – 5-methyluridine; 6 – inosine; 7 – N^4-acetylcytidine; 8 – guanosine; 9 – adenosine; 10 – N^2,N^2-dimethylguanosine; 11 – 6-methyladenosine; 12 – xanthosine; 13 – I.S. (8-bromoguanosine); 14 – 1-methyladenosine; * – unidentified peaks; (B) of creatinine analysis in a urine sample; peaks: 1– EOF; 2 – creatinine.

Table 5
Comparison of levels of nucleosides (μM nucleoside/mM creatinine) in urine from healthy subjects evaluated by different analytical methods

Compound/Sample	Capillary electrophoretic methods				Other methods			
	Our method UV detection $n=12$	Zheng et al. [8] UV detection $n=41$	Kim et al. [10] UV detection $n=10$	Liebich et al. [19] UV detection $n=24$	Lee et al. [16] HPLC/MS $n=4$	Liebich et al. [17] HPLC/UV $n=29$	Vidotto et al. [18] HPLC/UV $n=25$	Tebib et al. [12] immuno-enzymatic $n=27$
Pseudouridine	18.56 (±4.95)	19.25 (±5.82)	14.80 (±3.36)	25.32 (±10.32)	13.47 (±5.16)	25.52 (±4.82)	18.80 (±35.1)	42.3 (±10)
Uridine	0.32 (±0.18)	0.35 (±0.14)	0.23 (±0.11)	0.47 (±0.19)	0.99 (±0.36)	0.21 (±0.08)	–	–
5-Methyluridine	0.08 (±0.04)	–	0.14 (±0.04)	–	–	–	–	–
Cytidine	0.014 (±0.01)	0.33 (±0.21)	0.1 (±0.05)	0.07 (±0.09)	0.78 (±0.83)	0.07 (±0.05)	–	–
N^4-Acetylcytidine	0.59 (±0.23)	0.53 (± 0.18)	0.23 (±0.16)	–	–	–	–	1.6 (±0.6)
Inosine	0.32 (±0.18)	0.35 (±0.19)	0.09 (±0.07)	0.14 (±0.1)	–	0.30 (±0.16)	8.8 (±11.6)	–
Guanosine	0.68 (±0.23)	0.12 (±0.08)	–	0.01 (±0.021)	0.13 (±0.1)	0.06 (±0.02)	10.7 (±10)	–
N^2,N^2-Dimethylguanosine	1.02 (±0.33)	–	1.74 (±0.60)	–	0.36 (±0.14)	–	–	–
Xanthosine	0.24 (±0.09)	0.86 (±0.38)	0.62 (±0.25)	–	–	0.59 (±0.30)	–	–
Adenosine	0.29 (±0.14)	0.44 (±0.17)	0.21 (±0.08)	0.18 (±0.17)	0.51 (±0.42)	0.21 (±0.13)	4.7 (±7)	–
1-Methyladenosine	2.02 (±0.74)	–	–	–	5.96 (±6.02)	–	–	2.9 (±1.8)
6-Methyladenosine	0.08 (±0.06)	0.09 (±0.11)	0.05 (±0.05)	0.01 (±0.023)	–	–	–	–

and analyzed by MEKC as described above. The typical electropherogram of a urine sample is shown in Fig. 3A.

In order to assess the selectivity of the method and verify the identity of peaks in urine samples, we applied MCR-ALS to electrophoretic profiles acquired at 190–300 nm wavelengths by a diode array UV-absorbance detector. We have used spectra of pure standards as initial estimates in MCR-ALS and the collected multiwavelength electrophoretic profiles were analyzed instantly by matrix augumentation [26]. By this method the presence of analyzed nucleosides in urine profiles were confirmed.

In Fig. 3A, the peaks corresponding to 12 assayed nucleosides are displayed and a few peaks of unidentified substances present both in healthy and cancer patients' urine. The mean concentrations of nucleosides in 22 urine samples were calculated on the basis of calibration equations. They were expressed in μM nucleoside/mM creatinine and compared with values in the bibliography (Table 5). In Table 5, we have compared the levels of 12 nucleosides measured by seven different electrophoretic, chromatographic and immunoassay methods, with the developed one. It could be seen that levels of nucleosides in urine from healthy subjects could vary between analytical methods but the levels assessed by our analytical method strictly correspond to levels obtained by other authors [8,10,16–19]. Therefore, we could recommend our method in the analysis of those nucleosides. In the case of other peaks observed, whose identity is not known, we could consider the use of the untargeted analysis of obtained nucleoside profiles by the chemometric evaluation of data [24,25]. Untargeted analysis has shown a trend of discrimination of more numerous groups of cancer and healthy subjects.

In the case of creatinine assay, the analysis of biological samples by developed analytical procedure also confirmed its reliability and analytical value. The typical electropherogram of creatinine analysis in urine samples is given in Fig. 3B and presents only one peak corresponding to creatinine, due to the selective absorption of this compound at 234 nm.

4. Conclusions

The developed new procedure of sample preparation, involving solid phase extraction with subsequent CE analyses, was appropriate for the study of nucleoside urinary profiles within a reasonably short time (25 min of electrophoretic assay). The method was successfully verified by means of trueness, recovery, linearity and precision. Subsequently, analyses of 22 urine samples from healthy controls and cancer patients from the urological ward were done. Levels of nucleosides for healthy subjects compared with data from literature prove the applicability and reliability of the developed analytical procedure. Additionally, the method was modified and employed in the assay of urine creatinine levels, with good results. The application of the developed analytical procedure allows analysis of profiles of nucleosides and creatinine in urine with similar analytical conditions and hence, comparison of nucleoside profiles from different subjects instantly. An extension of the developed method to a greater number of cancer patients will enable the

examination of the method's suitability as a clinical tool in tumor diagnosis.

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