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Application of capillary electrophoresis in clinical chemistry: the clinical value of urinary modified nucleosides

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Abstract

Urinary modified nucleosides were determined by capillary electrophoresis using a 300 m*M* SDS–25 m*M* sodium tetraborate–50 m*M* sodium dihydrogenphosphate buffer. The nucleosides were extracted from urine by phenylboronate affinity gel chromatography. In cancer patients the levels of the modified nucleosides are generally elevated. By an artificial neural network method breast cancer patients were differentiated from normal individuals, which indicates that the modified nucleosides could be of clinical value as tumor markers.

Keywords: Nucleosides

1. Introduction

In addition to the normal ribonucleosides adenosine, guanosine, cytidine and uridine, RNA contains a number of modified nucleosides. They are formed post-transcriptionally within the polynucleotide molecule by numerous modification enzymes, in particular methyltransferases and ligases. More than 90 different modified nucleosides with great structural diversity have been described in RNA [1], most of them in tRNA, but some also in rRNA, mRNA and small nuclear RNA (snRNA). Some of the modified nucleosides occur only in one species of RNA, others in several species [1,2]. The function of the modified nucleosides within the RNA is not quite clear. It has been suggested that tRNA modification plays a structural and regulatory role [3]. By the hydrolytic action of ribonucleases and phosphatases during RNA turnover, free normal and modified nucleosides are created.

Modified nucleosides cannot be rephosphorylated and generally not be reincorporated during de novo RNA synthesis [4,5]. They circulate in the blood stream and, together with small amounts of normal nucleosides, they are excreted in the urine. Many of the modified nucleosides are not further metabolized and are virtually quantitatively excreted. Consequently, their levels are a measure for the RNA turnover [2,6,7].

It has been postulated that diseases may influence the RNA turnover and the levels of modified nucleosides. The most obvious alterations have been observed in malignant diseases. The detailed molecular basis of the increased excretion is still unclear, but in extracts of tumor tissues aberrant tRNA methyltransferases [8] and enhanced tRNA methyltransferase activities [9] have been found, leading, in part, to abnormal tRNA in tumor tissue [4], and it has been suggested that the high turnover

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of tRNA is the result of rapid degradation of aberrantly modified tRNA [8,10].

Based on the biochemical findings, modified nucleosides have been frequently proposed and partially evaluated as tumor markers. A diagnostic benefit has been suggested for a number of malignant diseases, such as leukemia, malignant lymphoma, brain tumors, nasopharyngeal cancer, small cell lung cancer, oesophagus cancer, colorectal cancer, hepatocellular carcinoma, renal cell carcinoma and breast cancer. Attempts have also been made to use modified nucleosides as diagnostic markers in patients with AIDS and in individuals at risk of developing AIDS.

As to the sample material used for the analyses, a few investigations on normal and modified nucleosides have been carried out in tumor tissue. In the majority of the studies urine and in fewer cases serum are used as samples. Because of the higher levels of modified nucleosides in urine, better analytical precision can be expected. Most of the studies on modified nucleosides are based on the urinary excretions. It has been shown [11] that it is not necessary to work with 24-h urines. When the nucleoside levels are related to creatinine, which is generally excreted in a quite constant manner within a period of 24 h, randomly collected urine samples can be used. Hereby sample collection is more convenient for the patients and the risk of sampling errors is reduced. Creatinine-related nucleoside levels in 24-h urines and randomly collected urines are identical [11].

Methodically in most of the studies the nucleosides are isolated from the urine specimen by phenylboronate affinity gel chromatography and separated by reversed-phase high-performance liquid chromatography (RP-HPLC) [12–16]. The same procedure is applied to serum nucleosides [17–21]. Because of their protein content, serum samples are ultrafiltrated prior to the nucleoside isolation on the phenylboronate columns. In addition to the RP-HPLC methods, radioimmunoassays and enzymeimmunoassays have been developed and applied for the quantification of modified nucleosides in urine and serum. Recently proton nuclear magnetic resonance (NMR) spectroscopy has been used to determine urinary-modified nucleosides [22].

Capillary electrophoresis (CE) for the separation of standard mixtures of ribonucleosides was introduced in 1987 [23]. In the same year another group reported the separation of deoxyribonucleosides [24]. Because nucleosides are uncharged molecules, CE is applied in the mode of micellar electrokinetic capillary chromatography (MEKC). We have developed a CE method for the analysis of normal and modified nucleosides in urine combining isolation of the analytes by phenylboronate affinity gel chromatography and separation and quantification by MEKC [25–28].

2. Experimental

2.1. Urine samples

Urine samples were collected from 24 healthy individuals (11 males and 13 females, 24-h urines), 25 patients with different kinds of cancer (16 males and 9 females, randomly collected urines) and 17 patients with breast cancer (randomly collected urines). The general cancer group was comprised of unselected patients in various stages of the disease and under different therapies. The breast cancer group included women 1 day before surgery. All urine samples were collected without preservative, immediately frozen after collection and stored at −20°C. For the analysis of the nucleosides the samples were thawed at room temperature.

2.2. Chemicals

Formic acid was purchased from Riedel-de Haen (Seelze, Germany). Ammonium acetate, methanol, ammonia, sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were from Merck (Darmstadt, Germany), sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) from Sigma (Deisenhofen, Germany). Sodium dodecyl sulfate (SDS) and Affi-gel 601 were obtained from Bio-Rad (Munich, Germany). The nucleoside reference substances uridine (U), cytidine (C), adenosine (A), guanosine (G), inosine (I), xanthosine (X), pseudouridine (PseU), 5,6-dihydrouridine (DhU), 3-methyluridine (m3U), 5-methyluridine (m5U), N4-acetylcytidine (ac4C), N6-methyladenosine (m6A), 1-methylguanosine (m1G), 2-methylguanosine (m2G), 1-methylinosine (m1I)

and the internal standard 3-deazauridine (3-DzU) were purchased from Sigma.

2.3. Isolation of nucleosides from urine

The nucleosides were extracted from urine by phenylboronate affinity gel chromatography applying a modification of the method by Gehrke et al. [14] and Kuo et al. [29]. The extraction was performed on Affi-gel 601 glass columns (140×14 mm) containing 500 mg of phenylboronate gel, which possesses a specific affinity for *cis*-diol structures. After equilibration of the column with 35 ml of 0.25 *M* ammonium acetate, pH 8.5, 10 ml of centrifuged urine mixed with 0.3 ml of a 0.8 *M* aqueous internal standard (3-DzU) solution was loaded onto the column. After loading the gel was washed with 20 ml of 0.25 *M* ammonium acetate and then twice with 3 ml of methanol–water (1:1, v/v). The nucleosides were eluted with 25 ml of 0.1 *M* formic acid in methanol–water (1:1, v/v). The eluate was evaporated to dryness under vacuum at 40°C and redissolved in 1 ml of water producing an extract concentrated by a factor of 10 as compared to the original urine. Regeneration of the gel was carried out with 45 ml of 0.25 *M* ammonium acetate, pH 8.5. The same treatment was applied for standard solutions containing 15 reference nucleosides and the internal standard. Under the described conditions the gel could be used for 15 extractions.

2.4. Capillary electrophoresis

All separations were performed on a Dionex (Idstein, Germany) CE system (CES I, with an automatic constant-volume sample injection system) in uncoated capillaries of 50 μm I.D. and 500 mm or 565 mm, with a distance of 65 mm between the detection window and outlet of the column. The separations were carried out with a 300 m*M* SDS–25 m*M* sodium tetraborate–50 m*M* sodium dihydrogenphosphate buffer, adjusted to pH 6.7 with 5% hydrochloric acid. The buffer was filtered through a 0.45-μm membrane filter and degassed in an ultrasonic bath for 10 min before use. Applied voltage and current were 7.0 kV and 47–49 μA for the 500 mm capillary and 7.5 kV and 41 μA for the 565 mm capillary. After each run the capillary was rinsed for 100 s with water, followed by 100 s with 0.1 *M* sodium hydroxide, 100 s with water, and finally 120 s with buffer. Samples were introduced by gravity injection at 100 mm head height for 45 s. UV detection was performed at 260 nm and 210 nm in sequence.

2.5. Identification and quantification of nucleosides

The peaks in the electropherograms were identified by comparing migration times of the unknown components with those of the standard nucleosides eluted under the same conditions, and by spiking the urine sample with standard solution. For the calibration, three different volumes of an aqueous standard stock solution containing the 15 nucleosides, to each of which was added 0.3 ml of the internal standard solution, were treated separately on the phenylboronate affinity gel. With these three standard samples, calibration curves were established at 260 nm and 210 nm for each of the 15 nucleosides. The nucleoside concentrations in the standard stock solution were 1280 μ*M* for PseU, 320 μ*M* for DhU, 64 μ*M* for m1G and m1I, 32 μ*M* for U, ac4C and m2G, 16 μ*M* for C, A, G, I, m3U and m6A, 8 μ*M* for m5U, and 3.2 μ*M* for X. Based on the calibration curves, the concentrations of the nucleosides in urine could be calculated. The concentrations were then transformed into nmol/μmol creatinine. Urinary creatinine levels were determined by colorimetry using the conventional reaction between creatinine and picric acid [30].

3. Results and discussion

3.1. Optimization of operation conditions

The quality of the electrophoretic separation and the time required for this separation depend on a number of analytical parameters, in particular on the voltage applied, the length of the column, the composition of the buffer and the sample size introduced.

Voltage control in CE offers an important parameter affecting analyte migration time and resolution efficiency. As voltage is increased, migration ve-

locity increases, and therefore migration time decreases. Consequently, analysis time is shortened by increasing the voltage. Furthermore, resolution increases as a function of voltage up to a certain point under a given set of other analytical conditions. Above this point resolution efficiency may diminish. The voltage suitable for the separation is often determined on the basis of a ''critical pair of substances'', that is a pair of substances which is difficult to separate. Considering the pair ac4C/G, the optimal voltage was found to be 7.0 kV when using the 500 mm column and 7.5 kV when using the 565 mm column. The currents were 47–49 μA and 41 μA, respectively. Shortening the length of the capillary from 565 mm to 500 mm reduced the analysis time by about 10%.

The composition of the buffer, e.g., components, ionic strength, pH value, amount of SDS, influences the separation. Because at pH values near neutral the nucleoside molecules are uncharged, CE has to be performed in the mode of MEKC. Micelles are formed by adding SDS. The concentration of SDS in the buffer has a strong effect on the migration order, the resolution and the analysis time. Pyrimidine nucleosides migrated in front of purine nucleosides and normal nucleosides in front of their corresponding methylated analogs. No sufficient separation was obtained with 100 m*M* SDS. Even though, using a buffer with a 200 m*M* SDS concentration, the nucleosides in the standard solution could be separated, the optimal concentration was 300 m*M* SDS, because in the urine samples interferences from other endogenous components were reduced at the higher SDS concentration. At none of the SDS concentrations m3U and m5U could be separated. The higher SDS concentration results in a longer analysis time, about 45 min for the 565 mm capillary and about 40 min for the 500 mm capillary.

The time applied in gravity injection defines the volume of sample which is introduced into the capillary. The increase in peak area is linear with injection time. However, when injection time is too long, the separation efficiency is reduced. At 100 mm head height, no obvious loss in resolution was observed using an injection time of 60 s or less. A loading time of more than 100 s led to a dramatic reduction of peak resolution. A 45 s load time (sample volume 10–15 nl) was found to be optimal.

Nucleosides are usually monitored by UV detection at a wave length between 254 nm and 280 nm. In our work we chose 260 nm. However, DhU does not absorb to any significant degree above 240 nm and has its absorption maximum around 210 nm [2]. DhU should be included in the profile analysis of normal and modified nucleosides, because it is considered to be a good marker for the tRNA turnover [2]. Therefore detection at 260 nm and 210 nm were run in sequence.

Fig. 1 shows typical electropherograms of the nucleoside standard mixture, Fig. 2 those of the normal and modified nucleosides in urine of a healthy individual. The electropherograms were run under the described optimized analytical conditions.

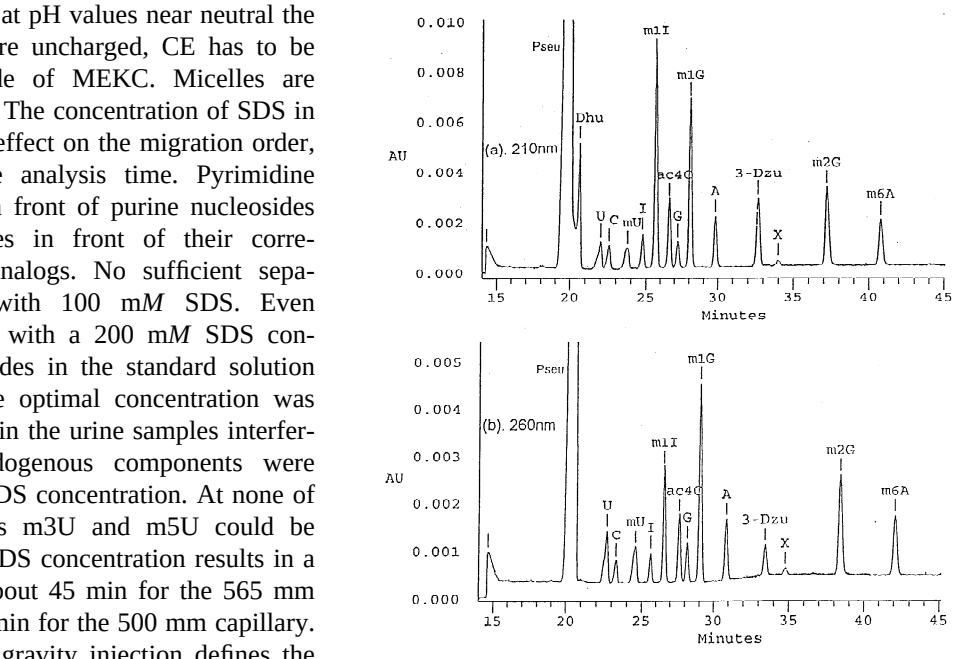

Fig. 1. Electropherograms obtained from an aqueous solution of standard nucleosides. Capillary: 565 mm (500 mm to detection window)×50 μm I.D.; applied voltage: 7.5 kV; current: 41 μA; buffer: 300 m*M* SDS–25 m*M* sodium borate–50 m*M* sodium dihydrogenphosphate; detection: UV at (a) 210 nm, (b) 260 nm; peak identifications: PseU=pseudouridine, DhU=5,6-dihydrouridine, U=uridine, C=cytosine, mU=3-methyluridine+5-methyluridine, I=inosine, m1I=1-methylinosine, ac4C=N4-acetylcytidine, G=guanosine, m1G=1-methylguanosine, A=adenosine, 3-DzU=3-deazauridine (internal standard), X=xanthosine, m2G=2-methylguanosine, m6A=N6-methyladenosine.

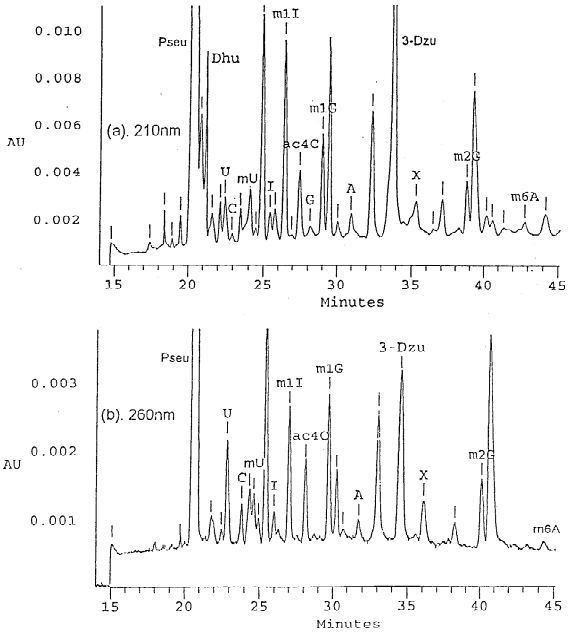

Fig. 2. Electropherograms obtained from urinary nucleosides in a healthy individual. (a) Detection at 210 nm; (b) detection at 260 nm; other conditions as in Fig. 1.

3.2. Analytical characteristics

The calibration curves are characterized by a linear correlation between the nucleoside concentrations and the peak areas. Typical equations for the calibration lines have been published [25]. The correlation coefficients were better than 0.99 for most of the nucleosides, when determined for standard nucleoside concentrations comparable to those of the urinary nucleosides. Only for X and m6A the correlation coefficients were 0.96 and 0.98, respectively.

The limits of detection, defined as the sample concentration that produces a peak with a height three times the level of the baseline noise, varied between 1.7 μM and 9.2 μM for the different nucleosides and were similar for detection at 260 nm and 210 nm. Only for PseU the limit of detection was higher by a factor of 10. The low micromolar detection limits are suitable for the analysis of the nucleosides in urine.

The reproducibility of the migration times were very good. For standard samples the relative standard deviations (RSDs) were less than 1% for migration times and less than 0.5% for migration times relative to the internal standard. The RSDs of the peak areas as determined by analysis of nucleoside standard solutions were between 2 and 6%. The reproducibility of peak areas relative to the area of the internal standard was similar.

Table 1 shows the precision of the method when applied to the analysis of urine samples. Nine 10-ml aliquots were obtained from a randomly collected urine sample and extracted and analyzed under identical conditions. The RSDs were determined to

Table 1
Precision of the quantitative determination of normal and modified nucleosides in urine by CE

Component	Concentration (nmol/μmol creatinine)	Standard deviation (nmol/μmol creatinine)	Relative standard deviation (%)
PseU	17.21	0.960	5.59
U	0.41	0.032	7.77
C	0.10	0.017	16.80
I	0.25	0.017	6.76
m1I	0.98	0.062	6.35
ac4C	0.58	0.040	7.01
G	0.05	0.006	11.81
m1G	0.64	0.036	5.61
A	0.38	0.022	5.80
X	0.25	0.038	14.96
m2G	0.26	0.020	7.66
m6A	0.04	0.013	33.10

be between 5.6% and 16.8%, except for m6A with an RSD of 33%.

The CE method is a complementary technique to RP-HPLC and offers several advantages over RP-HPLC in the analysis of urinary nucleosides. CE uses uncoated capillary columns, which are cheaper and require less maintenance than RP-HPLC columns. The CE capillaries usually have a longer lifetime than RP-HPLC columns. For the separations in this study the column could be used more than 500 times. Whereas RP-HPLC consumes considerable amounts of solvents as the mobile carrier, CE uses the electrical field for separation and does not consume solvents. A further reduction in the costs of consumables results from a better degree of miniaturization of CE as compared to RP-HPLC. The CE method for the determination of normal and modified nucleosides is suitable for the analysis of large series of urine samples and is applicable in the clinical laboratory.

3.3. Urinary nucleoside excretion in healthy adults and in cancer patients and relevance of modified nucleosides as tumor markers

The reference values for the urinary excretion of normal and modified nucleosides in healthy individuals determined with the CE method, are summarized in Table 2. Because the values found in this study and the values reported by other investigators [4,6,7] show very little variance, it can be concluded that the excretion of nucleosides in urine is very constant in healthy adult persons.

Table 2
Mean levels of normal and modified nucleosides in urine of healthy individuals ($n=24$)

Component	Concentration (nmol/μmol creatinine)	Standard deviation (nmol/μmol creatinine)
PseU	25.32	10.320
DhU	4.25	1.105
U	0.47	0.190
C	0.07	0.095
mU	0.09	0.123
I	0.14	0.100
m1l	1.27	0.457
ac4C	0.60	0.384
G	0.01	0.021
m1G	0.82	0.298
A	0.18	0.172
X	0.45	0.260
m2G	0.39	0.197
m6A	0.01	0.023

In the group of patients with different kinds of cancer the levels of nucleosides in urine were generally elevated, and the increase of modified nucleosides was more pronounced than that of normal nucleosides (Fig. 3). When a significant elevation of nucleosides is defined as a value higher than the average plus two standard deviations determined in normal urine, the concentrations of PseU, m1I, ac4C, m1G and m2G in urine of patients with cancer were elevated significantly. These five modified nucleosides include PseU, which has been reported in the literature many times as being increased in urine of cancer patients, e.g., in patients with leukemia,, malignant lymphoma, nasopharyngeal cancer, small cell lung cancer, oesophagus cancer, colorectal cancer and breast cancer. In some of these reports, an increase of other modified nucleosides has also been described in addition to the elevation of PseU, including m1I, ac4C, m1G and m2G.

Many studies on modified nucleosides in cancer patients demonstrate that in malignant diseases not only one of the nucleosides is elevated, but always several. In such a multi-component alteration of the nucleoside levels a pattern recognition method could reveal more information on differences between healthy individuals and cancer patients than the evaluation of single components. The artificial neural network (ANN) method was applied for evaluation of the nucleoside levels in the group of patients with different kinds of cancer. Dividing the data from both healthy individuals and cancer patients in a training set and a predicting set, the ANN method resulted in a good classification of the healthy individuals and the cancer patients of the predicting set in two clusters. The recognition rate was 85% [28].

Also in the group of the patients with breast cancer the modified nucleoside levels in urine were elevated. Applying the factor analysis method for classification, a clear cut differentiation of the breast cancer group and the healthy individuals in two clusters without overlapping was obtained.

It cannot be conclusively answered which diagnostic sensitivity and specificity the modified nu-

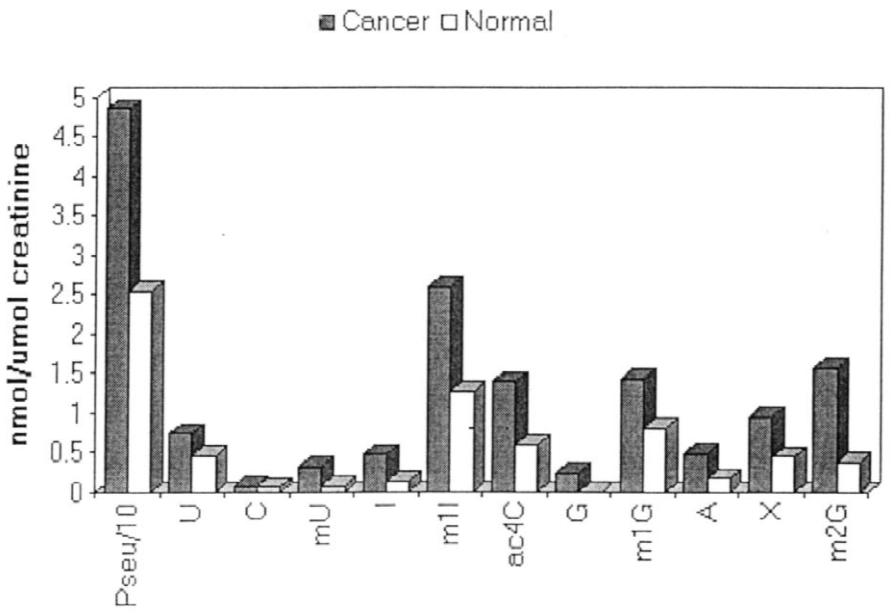

Fig. 3. Mean levels (nmol/μmol creatinine) of normal and modified nucleosides in urine of healthy individuals (n=24) and patients with cancer (n=25).

cleosides have and in which way they are superior to presently used tumor markers. In the study on the women with breast cancer the modified nucleosides had a higher diagnostic sensitivity than carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3), which are conventionally used as tumor markers for breast cancer. By factor analysis all 17 patients were classified correctly on the basis of the modified nucleosides, whereas only three patients had abnormal CEA and CA 15-3 levels in serum. This is of particular interest, because at present no satisfactory tumor marker is available for breast cancer. Not enough investigations have been carried out concerning the diagnostic specificity. In order to use the modified nucleosides as tumor markers, it has to be clarified how their levels are affected by benign diseases of the same or another organ and by other influences. For PseU which is increased for instance in small cell lung cancer, it has been shown, that in several pulmonary infectious diseases the urinary excretion of PseU is normal. On the other hand modified nucleosides including PseU were elevated in patients who had undergone major surgery because of various non-malignant diseases [22]. The elevation originates from an increased whole-body RNA turnover in conjunction with surgical stress and continued catabolism. Because the whole-body RNA turnover correlates quite well with the protein turnover, attention has to be paid to alterations of the modified nucleosides under various catabolic conditions other than that occurring in malignancies, e.g., malnutrition, cardiac cachexia, endocrine abnormalities, alcoholism, infections and stress.

4. Conclusion

The described CE method for the quantification of modified nucleosides is suitable for analyzing large series of urine samples and for carrying out clinical studies on the validity of the nucleosides as tumor markers.

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