Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 822 (2005) 85–90
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Application of solid phase extraction and high-performance liquid chromatography to qualitative and quantitative analysis of nucleotides and nucleosides in human cerebrospinal fluid

Joanna Czarnecka[a], Marek Cieślak[b], Komoszyński Michał[a,*]

[a] *Biochemistry Department, Nicolaus Copernicus University, 9 Gagarina St, 87-100 Toruń, Poland*
[b] *Department of Neurology, WSZ Hospital, 53 Józefa St, 87-100 Toruń, Poland*

Received 22 October 2004; accepted 15 May 2005
Available online 1 July 2005

Abstract

New method of qualitative and quantitative analysis of nucleotides in human cerebrospinal fluid (CSF), based on the combination of extraction of purines and pyrimidines to the solid phase (SPE) and high-performance liquid chromatography (HPLC), was proposed. Use of SPE and lyophilization of samples allowed for the first time to detect the presence of di- and triphosphonucleotides in human CSF. Concentration of those compounds varied from 0.003 to 5.0 μM. Differences in the nucleotide mixture composition in human CSF detected with the new method are coupled with the neurological disorders and might be a basis for an efficient diagnostic tool.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Human cerebrospinal fluid; SPE; HPLC; Nucleotides; Neurological diseases

1. Introduction

In living organisms, purine and pyrimidine nucleotides participate in numerous important metabolic processes [1]. Released outside the cell, they act as signaling molecules activating adenosine P1 and nucleotide P2 receptors [1,2]. Both classes of receptors are present in all brain structures [1]. Lately the nucleotide receptors have been also found on cerebrospinal fluid-contacting neurons [3]. In CSF, which is an integral part of the central nervous system (CNS), triphosphonucleotides have not yet been found, although prior investigations revealed a presence of adenosine-5′-monophosphate (AMP), purine nucleosides and nucleobases [4]. In CNS, adenosine-5′-triphosphate (ATP) acts via P2X receptors as a fast transmitter while adenosine-5′-diphosphate (ADP), usually operating via P2Y receptors, triggers many metabolic pathways associated with G protein [1,5,6]. The ectopurines (ATP, ADP and AMP) are also precursors of other signaling molecules [7–9]. Ectoadenosine, produced during the ectonucleotide degradation, plays an important role in neuromodulation and neuroprotection [10,11].

Recent results suggest that ectoguanosine-5′-triphosphate (ectoGTP) and ectoguanosine participate in processes of proliferation and differentiation of neuronal and glial cells [12,13]. The ectonucleotides and nucleosides present in blood are also involved in a regulation of CNS physiology, since they regulate hemostasis and blood pressure [1,14–17]. The presence of some purines and pyrimidines in CSF and occurrence of purinoceptors on the cerebrospinal fluid contacting neurons suggest that these compounds may play a signaling role also in the brain ventricular system.

So far di- and triphosphonucleotides have not been detected in CSF, probably because of their low concentration, instability of pyrophosphate bonds at low pH of solutions used for extraction of purines and pyrimidines or the presence of enzymes hydrolyzing pyrophosphate bonds [18].

* Corresponding author. Tel.: +48 56 6114520.
E-mail address: michkom@chem.uni.torun.pl (K. Michał).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.05.026

The aim of the research reported here was to develop the method of the nucleotide isolation from CSF that would preserve the pyrophosphate bonds from hydrolysis. Also the knowledge of the qualitative and quantitative composition of purines and pyrimidines in CSF might help in understanding their metabolism, as well as in explanation of their role in biochemistry, physiology and pathology of the spinal cord.

2. Experimental

2.1. Reagents

For experiments we used the following reagents: ethanolamine, *n*-heptane, KCl, $HClO_4$, $P_2O_2WO_3$ and EDTA (POCh Gliwice Poland p.a. grade), $(CH_3COO)(UO_2)$ (Pliva-Lachema, Brno, Czech Republic), KH_2PO_4, K_2HPO_4, tetrabutylammonium hydrogen sulphate (TBA) and isocratic methanol (Baker Phillipsburg, USA, HPLC grade). The following nucleotides standards used as sodium salts (98–99% purity) were made by Sigma (Steinheim, Germany): adenosine-5′-triphosphate, adenosine-5′-diphosphate, adenosine-5′-monophosphate, guanosine-5′-triphosphate, guanosine-5′-diphosphate, guanosine-5′-monophosphate, cytosine-5-triphosphate, cytosine-5′-diphosphate, cytosine-5′-monoposphate, uridine-5′-triphosphate, uridine-5′-diphosphate, uridine-5′-monophosphate. Also the nucleoside and base standards as adenosine, guanosine, cytosine, xanthine, hypoxanthine were used.

2.2. Apparatus

The purines and pyrimidines were isolated from CSF by the solid phase extraction (SPE) using Supelco Preppy apparatus (Sigma–Aldrich, Europe) with Strata X column, 30 mg/1 ml (Phenomenex, Europe). After extraction, samples were lyophilized in Speed Vac SC110 apparatus (Savant). HPLC separation was performed on Waters HPLC set (Milford, MA, USA) containing: In-Line Degasser AF, 2 × 515 HPLC Pump, Waters Pump Control Module, Waters 2487 Dual λ Absorbance Detector, Waters 717 Plus Autosampler. The samples were separated on the Supelcosil LC-18-DB column, 250 mm × 4.0 mm (Sigma–Aldrich, Europe) with Shimadzu Phenomenex Security Guard (Cartridge C18 4 mm × 3 mm). Results were analyzed using Millenium 32 software by Waters Corporation (Milford, MA, USA).

2.3. Material and sample preparation

Analyses were performed for the CSF samples from patients with neurological disorders. Control group consisted of patients with rachialgia, for whom the standard analysis of CSF and medical examination indicated no neurological diseases. Immediately after the spinal puncture, CSF samples were centrifuged for 2 min at 4000 × *g*. Supernatants were frozen at −8 °C and stored at −80 °C. Before analysis, samples were melted at 0 °C and centrifuged for 2 min at 4 °C at 3000 × *g*.

2.3.1. Solid phase extraction

SPE columns were washed with 1 ml of 25 mM ethanolamine pH 8.0. Then 0.1 ml of CSF was put into each column. The obtained filtrate contained Fraction I. Subsequently the columns were washed with 1 ml 50% methanol to obtain Fraction II and then with 1 ml 100% methanol (Fraction III). All fractions were frozen and stored at −80 °C for further analysis. The yield of extraction and chromatography was determined with CTP as internal standards. Solution of CTP (1 μL) was added to 99 μL of selected samples in all analyzed series. The final concentration of internal standard in samples was 10 μM.

2.3.2. Lyophilization

Fractions II and III were lyophilized in Speed Vac apparatus. Time of lyophilization lasted about 25 h. Dried Fractions II and III were diluted in the deionised water, blended and diluted to 50 μl. All tested samples were delipidated by shaking with *n*-heptane (1:5, v/v). For further analysis 30 μl of water phase was used.

2.3.3. High-performance liquid chromatography

The HPLC column was conditioned with the phosphate buffer of pH 6.0 containing 150 mM KH_2PO_4/K_2HPO_4, 100 mM KCl and 10 mM TBA. 20 μl samples of delipidated CSF extracts were injected into the column. Chromatography was performed isocratically with a flow rate of 1 ml/min. Presence of purines and pyrimidines was detected at $\lambda = 260$ nm. Compounds (ATP, ADP, AMP, adenosine, GTP, GDP, UTP, UDP, CTP) were identified on the basis of the retention time.

2.4. Statistical analysis

Results were shown as mean ± S.D. The statistical analysis was performed using Kolgmogorov Smirnov test. Comparison between studied groups was carried on with the Mann–Whitney *U*-test or *t*-test.

3. Results and discussion

3.1. Chromatography and sample preparation

The presented method of separation, identification and quantification of purines and pyrimidines in CSF requires standardization of spinal puncture conditions, extraction and samples storage. After collection, all samples were frozen at −80 °C. Such low temperature inactivated 5′-nucleotidase. All steps of isolation of purines and pyrimidines from CSF and extraction to the solid phase, liophilization must be performed in temperature not higher than 4 °C. Under such

Table 1
Precision and accuracy of determination of analyzed compounds in cerebrospinal fluid

Compound ($n=4$)	Concentration measured (mean ± S.D.) (μM)	Recovery (%)	R.S.D. (%)
ATP	0.87 ± 0.011*	86.7	1.26
	1.85 ± 0.03	92.5	1.62
ADP	1.90 ± 0.025	95.0	1.32
AMP	1.96 ± 0.09	98.0	0.46
Adenosine	1.97 ± 0.008	98.5	0.42
GTP	1.83 ± 0.032	91.5	1.75
GDP	1.86 ± 0.027	93.0	1.45

Ascertained by adding 2 or * 1 μM concentration of each compounds to CSF.

conditions the efficiency of the presented method for compounds with the pyrophosphate bonds is close to 90% while for phosphoesters and nucleosides it is close to 98% (Table 1).

The preparation of samples for analysis is the major problem for the qualitative and quantitative analysis of ecto-nucleotides in tissues and body fluids. Blood and CSF contain many substances making the separation and identification of purines and pyrimidines by HPLC difficult [19]. They also contain enzymes involved in the purines and pyrimidines degradation, which are capable of changing the compound concentration and the samples composition [18,20]. Therefore, it was necessary to develop the extraction method that will not destroy labile pyrophosphate bonds and also would remove the interfering substances (e.g. proteins) from CSF.

Up to date, two sample preparation techniques were usually used for qualitative and quantitative analyses of purines and pyrimidines present in CSF [21–24]. In one of them, CSF samples were injected into a column only after preliminary centrifugation, freezing and defreezing [22–24]. The results obtained by this method have not shown the presence of nucleotides in CSF. Authors also did not describe either the column stability or the yield of such a procedure. The second technique is based on use of perchloric acid for protein removal [21]. Also in that case the presence of compounds with pyrophosphate bonds was not detected. Our experience indicates that the best are methods in which CSF samples are stored and handled at low temperature, and the nucleotide extraction from body fluids is performed with no acids used. The reasons for such requirements are the low nucleotide concentration and lability of the pyrophosphate bonds.

In preliminary effort to remove proteins from CSF, we used three solutions (all in 4 °C): 1 M $HClO_4$, 0.1 M $P_2O_2WO_3$ or 0.2 M $(CH_3COO)(UO_2)$.

The above methods of purine and pyrimidine extraction from CSF did not give the expected results, since all samples still contained proteins. Also recovery yield of purines and pyrimidines during extraction as well as HPLC separation was low and variable. Therefore, we adopted the method of solid phase extraction SPE for the sample preparation. The solutions and eluents used for conditioning of the SPE column and separation of compounds were chosen according to their low sample destruction and the highest recovery rate of the analyzed compounds.

Initially, the SPE columns were conditioned with 5% methanol according to the instructions included in the kit. This method of resin preparation had a low efficiency of the nucleotides and nucleosides extraction from CSF. In subsequent experiments, the columns were conditioned with 25 and 100 mM ethanolamine pH 6.0, 25 mM or 100 mM ethanolamine pH 8.0. Purines and pyrimidines were eluted using various concentrations of MeOH (5–100%). The best results for separation were achieved by binding of purines and pyrimidines to SPE columns preconditioned with 25 mM ethanolamine at pH 8.0 and subsequent elution with 50% and then with 100% MeOH (Table 2). The above procedure gave the best yield of purines and pyrimidines.

Using phosphate buffer pH 6.0 containing 150 mM KH_2PO_4/K_2HPO_4, 100 mM KCl and 10 mM TBA for the HPLC analysis, we were able to separate and identify at least: ATP, ADP, AMP, adenosine, GTP, GDP, UTP, UDP and CTP (Fig. 1).

The calibration curves for purine and pyrimidine were linear over the range 0–500 pmol. The detection limit for the described method is estimated as 0.1 pmol. The method developed by us is characterized by high selectivity, since the retention times for the analyzed compounds are reproducible, and the solid phase extraction removed all the interfering compounds from the samples (Table 3). Our method has a high precision as indicated by the low R.S.D. value (Table 1).

CSF significantly differs from other tissues in the nucleotide concentration. Concentration of ATP in CSF (0.23 μM) is 4000 times lower than in cells [25]. Considering liability of nucleotides with pyrophosphate bonds, their low concentration in CSF samples as well as the sample

Table 2
Effect of SPE eluents on recovery of tri- and diphosphonucleotides

Eluents	Percent of methanol in eluents	Recovery (%)		
		ATP	ADP	CTP
	5	12 ± 6.3	21 ± 8.9	nd[a]
25 mM ethanolamine, pH 6	50	62 ± 12.3	81 ± 15.8	nd[a]
	100	58 ± 14.5	61 ± 11.3	nd[a]
	50	64.3 ± 3.5	76.5 ± 5.2	nd[a]
25 mM ethanolamine, pH 8	100	63 ± 10.4	72.1 ± 9.7	60.5 ± 9.9
	50 then 100	88.8 ± 4.8	92.5 ± 3.4	83 ± 4.2

[a] Not determined.

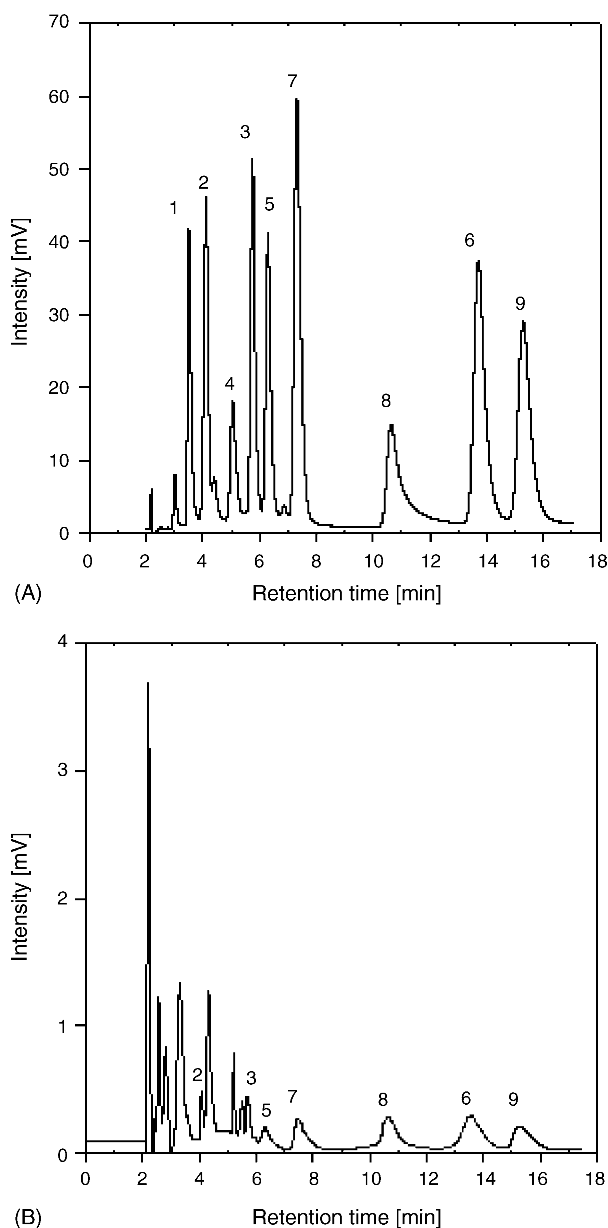

Fig. 1. HPLC separation of nucleotide and nucleoside standards (A) and rachialgia CSF sample (B) on Supelcosil LC-18-DB column (250 mm × 4.0 mm) with Shimadzu Phenomenex Security Guard (Cartridge C-18 4 mm × 3 mm) equilibrated by 150 mM buffer KH_2PO_4/K_2HPO_4 pH 6.0, with 100 mM KCl and 10 mM TBA. Twenty microlitres samples used. Separation was performed with flow 1 ml/min: 1-CTP, 2-UDP, 3-UTP, 4-GDP, 5-GTP, 6-adenosine, 7-AMP, 8-ADP, 9-ATP.

Table 3
The average retention time of analyzed purine compounds[a]

Compound	Mean retention time ± S.D.
ATP	15.29 ± 0.20
Adenosine	13.71 ± 0.17
ADP	10.64 ± 0.16
AMP	7.32 ± 0.11
GTP	6.30 ± 0.07
GDP	5.73 ± 0.02

[a] 20 μl of 25 μM purine or pyrimidine solution was injected.

liophilization during the procedure, the achieved 90% yield of our method is also sufficient (Table 1). To account for the yield loss, we used internal standards for correcting the results in all series. The observed decrease of the yield seems to results from liophilization. However, that procedure causes the increase of nucleotide concentration in a sample, what results in clear HPLC signals and simplifies the compounds identification. At the some time SPE and extraction with heptane remove proteins and lipids from the sample, what significantly improves the detection level.

Some published methods of the nucleotide analysis with the use of HPLC have even more than 100% yield [26]. Such high yield might be caused by some low molecular weight peptides or proteolipids present in samples, which are not precipitated by perchloric acid. Some peptides of low molecular weight might also dissolve during neutralization with alkali. These substances can give an intensive signal at $\lambda = 260$ nm, which can affect the method sensitivity.

Mobile phase used by us in HPLC does not contain volatile solvents such as methanol or acetonitrile. That prevents the changes of the medium composition, which in consequence shortens the time of the series analysis (in our case at least 90 injections in a series). The isocratic conditions of separation used in our method eliminate the necessity of the column re-conditioning between subsequent separations, ensure the same conditions for each separation and lower the detection level. These conditions also extend the column use, do not affect the retention time and enable the automated identification of analyzed compounds. The maximum separation time for purines and pyrimidines was 17 min. That time is longer by 30% from that reported for separation in the methanol gradient by Smoleński et al. [26] and more than three times shorter than that for gradient separation as described by Cichna et al. [27].

3.2. Clinical application

Results reported here are obtained based on the analysis of 164 human CSF samples. However, we limited the discussion to results for 68 patients—only those revealing clear symptoms of rachialgia (control group), subarachnoid haemorrhage, epilepsy, cerebral stroke or neuroinfection. We found that ATP was present in 83.5%, ADP in 90.5%, AMP in 68.2% and Ado in 85.8% of all analyzed human CSF samples. GTP was found in 76%, while GDP only in 40.5% of analyzed fluids. UTP was found in 20% and UDP in 18.2% of the investigated samples. The human CSF samples contained 0.003–4.43 μM ATP, 0.0202–4.50 μM ADP, 0.0150–4.64 μM AMP and 0.01–5.0 μM adenosine. Observed quantitative and qualitative differences in fluid composition did not correlate with either age or sex of patients. In human CSF the average aggregated concentration of adenine nucleotides and adenosine was 4.6 ± 3.8 μM. Observed ranges of standard deviation values can result from different stages of patient's disease at the moment the CSF samples were obtained. However, the approximate aggregate

Table 4
Concentration of adenine nucleotides and adenosine in the cerebrospinal fluid of patients with selected neurological diseases

Disease	Nucleotide and adenosine concentration (μM)				Aggregate[a] concentration of adenine nucleotides and adenosine (μM)
	ATP	ADP	AMP	Adenosine	
Rachialgia (control group) ($n=31$)	0.23 ± 0.19 (5.2%)	1.10 ± 0.60 (25.0%)	2.24 ± 1.64 (51.1%)	0.82 ± 0.79 (18.7%)	4.39 ± 0.8 (100%)
Subarachnoid hemorrhage ($n=5$)	0.80 ± 0.63 (16.9%)	0.91 ± 0.46 (19.3%)	0.86 ± 1.0 (18.2%)	2.15 ± 2.09 (45.6%)	4.72 ± 0.8 (100%)
Epilepsy ($n=9$)	1.08 ± 0.77 (27.4%)	0.63 ± 0.37 (28.1%)	1.05 ± 0.33 (26.6%)	0.71 ± 0.56 (18.0%)	3.4 ± 0.51 (100%)
Cerebral stroke ($n=9$)	0.26 ± 0.11 (29.3%)	1.86 ± 1.67 (66.7%)	0.0 (0.0%)	0.67 ± 0.58 (24.0%)	2.79 ± 0.59 (100%)
Neuroinfection ($n=14$)	1.09 ± 0.76 (24.8%)	1.62 ± 0.96 (37.0%)	0.97 ± 0.66 (22.0%)	0.71 ± 0.43 (16.2%)	4.39 ± 0.70 (100%)

[a] Aggregate concentration of adenine nucleotides and adenosine means a sum of [ATP] + [ADP] + [adenosine].

concentration of adenine nucleotides and adenosine in most of analyzed patient groups can indicate the correctness of the reported method (Table 4).

3.2.1. *Concentration of adenine nucleotides and adenosine in CSF of patients with different CNS diseases*

According to Polish low we could not collect the samples from healthy volunteers. Therefore, the control group consisted of CSF derived from patients with rachialgia without neurological disorders. The concentration of AMP and adenosine in CSF of that group of patients was close to the levels for healthy children as reported by Rodriguez-Nunez et al. [28]. Concentration of ATP in CSF of rachialgia group was at least two times lower compared to that found in other investigated groups (Table 4).

In most analyzed diseases (4/5) the aggregated concentration of adenine nucleotides and adenosine in CSF varied from 3.4 to 4.4 μM. Only in cerebral stroke the 60% decrease was found compared to the control group. The CSF samples of all analyzed groups contained ATP, ADP and adenosine. However, concentration of analyzed purines was different in these groups (Table 4). The CSF of patients with the cerebral stroke did not contain AMP.

The CSF samples of patients with subarachnoid hemorrhage revealed the substantial increase in concentration (more than 2.5 times) of adenosine, being the neuroprotective agent, and simultaneous decrease in concentration of AMP (precursor of ectoadenosine), when compared to the control group [29].

In case of cerebral stroke patients, the ADP concentration was almost two times higher then in the control group and also higher compared to the other groups. It may result from damage of vessels and cerebral cells, and release of that nucleotide from α-granules during the platelet aggregation [15,17].

Changes in the concentration of analyzed compounds can correlate with the extent of the CNS tissue damage resulting from a disease process, or with changes in the activity of ectoenzymes present on the surface of cells contacting the spinal fluid. These changes can also result from the different activity levels of exoenzymes that degrade purines and pyrimidines in CSF. Earlier investigation of Cruz Portela et al. revealed the presence of exoenzymes hydrolyzing di- and triphosphonucleotides in rat CSF [18]. We also detected an activity of exonucleotidases in human CSF.

The obtained results reveal differences in the average concentration of nucleosides and nucleotides between investigated groups of diseases. However, only few of these differences were statistically significant. We have found the statistically significant differences in the AMP concentration between CSF of patient with cerebral stroke and those of the control group, those affected by neuroinfection ($p<0.01$), subarachnoid hemorrhage and epilepsy ($p<0.05$). Above results suggested that AMP is a key compound in metabolism of CSF ectopurines. Also statistically significant increase of the ATP concentration ($p<0.01$) in CSF of patients with epilepsy, compared to the control group, was observed. The statistically significant increase of ATP ($p<0.05$) and decrease of AMP concentration ($p<0.05$) in CSF of patients with neuroinfection compared to the control group was also found. The CSF of patients with neuroinfection was characterized by the negative correlation between ADP and AMP ($p<0.05$, $r=-0.86$) or adenosine ($r=-0.61$, $p<0.05$) concentration. Detection of statistically significant differences in concentration of nucleotides and nucleosides in CSF of patients from different groups suggests the applicability of quantitative and qualitative analysis of purines in CSF for diagnosis of some CNS diseases.

3.2.2. *Concentration of guanine nucleotides in CSF of patients with different CNS diseases*

The guanine derivatives (ectoguanosine and ectoGTP) were found in the rat brain and synapses [12,13]. The source of both compounds can be either neurons or glia cells [12,13]. GTP was also found in synaptic vesicles [12]. Moreover, in the neuron cell membranes the guanosine-like receptor was found. That "pseudoreceptor" triggered the G-protein-coupled metabolic pathway [30]. Above facts strongly suggest that guanine may play a role of the signaling molecule in CNS [12,13,31].

The concentration of guanine derivatives in most of CSF samples was higher then the concentration of ATP and ADP. In the majority of analyzed patient groups the amount of GDP was higher then that of GTP (Table 5). The aggregate concentration of above nucleotides in CSF of patients with the epilepsy was lower then in samples from other

Table 5
Concentration of GTP and GDP in the cerebrospinal fluid of patients with selected neurological diseases

Disease	Nucleotide concentration (μM)	
	GTP	GDP
Rachialgia (control group) ($n = 31$)	1.59 ± 1.42	2.11 ± 1.93
Subarachnoid hemorrhage ($n = 5$)	2.80 ± 1.80	4.05 ± 0.03
Epilepsy ($n = 9$)	2.08 ± 1.91	0.99 ± 1.32
Cerebral stroke ($n = 9$)	1.30 ± 1.23	2.55 ± 1.62
Neuroinfection ($n = 14$)	1.92 ± 1.53	2.59 ± 1.77

groups. The CSF of patients with subarachnoid hemorrhage has the highest concentration of GTP. In cerebral stroke, the amount of GDP in CSF was two times higher than that of GTP.

At this stage of investigation, the complete evaluation of obtained results for the spinal cord physiology and biochemistry is impossible because of temporary lack of information about the presence of guanine nucleotides in CSF. However, many authors suggest, that guanosine regulates such important processes as exocytosis and re-uptake of glutamate, activates the neuron grows, proliferation and differentiation of astrocytes [12,13]. That compound also stimulates the synthesis and secretion of trophic factors [12,13]. Therefore, the guanine nucleotides present in human CSF can modulate some physiological processes ongoing in human ventricular system, e.g. they may regulate release of ATP and adenosine or selectivity of the brain/CSF barrier [12,13].

4. Conclusions

The developed method is designed to allow the analysis of small amounts of unstable tri- and diphosphonucleotides in the physiological fluids. The yield and precision achieved in our method is sufficient for detection of changes of the nucleotide levels in CSF of patients with different neurological disorders. The proposed method enables detection of quantitative and qualitative differences of purines and pyrimidines concentration in CSF and may be useful for the diagnostics of different neurological diseases.

References

[1] V. Ralevic, G. Burnstock, Am. J. Pharmacol. Exp. Ther. 50 (1998) 413.
[2] M.P. Abbracchio, G. Burnstock, Pharmacol. Ther. 64 (1994) 445.
[3] M.E. Stoeckel, S. Uhl-Bronner, S. Hugel, P. Veinante, M.-J. Klein, M.-J. Freund Mercier, R. Schlichter, J. Comp. Neurol. 457 (2003) 159.
[4] A. Rodrigues-Nunez, F. Camina, S. Lojo, S. Rodrigues-Segade, M. Castro-Gago, Acta Paediatr. 82 (1993) 849.
[5] F. Kukulski, M. Komoszyński, Post Biol. Kom. 28 (2001) 33.
[6] C. El-Moatassim, J. Dornand, J.C. Mani, Biochim. Biophys. Acta 1134 (1992) 31.
[7] H. Zimmermann, Drug Dev. Res. 39 (1996) 337.
[8] F. Kukulski, M. Komoszyński, Post Biol. Kom. 29 (2002) 449.
[9] F. Kukulski, M. Komoszynski, Eur. J. Biochem. 270 (2003) 3447.
[10] J.M. Brundege, T.V. Dunwiddie, Adv. Pharmacol. 39 (1997) 353.
[11] J. Wardas, Pol. J. Pharmacol. 54 (2002) 313.
[12] M. Rathbone, P. Middlemiss, J. Gysbers, C. Andrew, M. Herman, J. Reed, R. Ciccarelli, P. Iorio, F. Caciagli, Prog. Neurobiol. 59 (1999) 663.
[13] R. Ciccarelli, P. Ballerini, G. Sabatino, M.P. Rathbone, M. D'Onofrio, F. Caciagli, P. Iorio, Int. J. Dev. Neurosci. 19 (2001) 359.
[14] K. Enjyoji, J. Sevigny, Y. Lin, P. Frenette, P. Christie, J. Schulte Am Esch, M. Imai, J. Eelnerg, H. Rayburn, M. Lech, D. Beeler, E. Csizamadia, D. Wagner, S. Robson, R. Rosenberg, Nat. Med. 5 (1999) 1010.
[15] M. Cattaneo, Ch. Gachett, Haematologica 86 (2001) 346.
[16] E. Kaczmarek, K. Koziak, J. Seǐvigny, J. Siegel, J. Anrather, A. Beaudoin, F. Bach, S. Robson, J. Biol. Chem. 271 (1996) 33116.
[17] J. Łęcka, M. Komoszyński, Prog. Med. Res. 1 (2003).
[18] L.V. Cruz Portela, J.P. Oses, A.L. Silvera, A.P. Schmidt, D.R. Lara, A.M. Oliveira Bastini, G. Ramirez, L. Vinade, J.J. Freitas Sarkis, D.O. Souza, Brain Res. 1 (2002) 74.
[19] P. Lewaczuk, M. Mantur, Płyn mózgowo-rdzeniowy. Badanie i interpretacja wyników, Wydawnictwo Ekonomia i środowisko, Białystok, 2002.
[20] J.P. Oses, C.M. Cardoso, R.A. Germano, I.B. Kirst, B. Rucker, C.R. Furstenau, M.R. Wink, C.D. Bonan, A.M. Battastini, J.J. Sarkis, Life Sci. 74 (2004) 3275.
[21] G.P. Gerrits, A. Haagen, De Aubreu, L. Monnens, F. Gabreels, F. Trijbels, A. Theeuwes, J. Baal, Clin. Chem. 34 (1988) 1439.
[22] H. Holst, A. Sollevi, Acta Neurochirurgica 77 (1985) 52.
[23] L. Kuracka, T. Kalnowicowa, B. Liska, P. Turcani, Clin. Chem. 42 (5) (1996) 756.
[24] H. Schmidt, W.G. Siems, T. Grune, E.L. Grauel, J. Perinat. Med. 23 (1995) 167.
[25] F. Gribble, G. Loussouarn, S. Tucker, C. Zhao, C. Nichols, F. Ashcroft, J. Biol. Chem. 275 (2000) 30046.
[26] R.T. Smoleński, D.R. Lachno, S.J. Ledingham, M.H. Yacoub, J. Chromatogr. 527 (1990) 414.
[27] M. Cichna, H. Daxecker, M. Raab, Anal. Chim. Acta 481 (2003) 245.
[28] A. Rodriguez-Nunez, E. Cid, J. Rodriguez-Garcia, F. Camina, S. Lojo, S. Rodrigues-Segade, M. Castro-Gago, Brain Dev. 25 (2003) 102.
[29] T. Obata, Life Sci. 71 (2002) 2083.
[30] U. Traversa, G. Bombi, E. Camaioni, A. Macchiarulo, G. Costantino, C. Palmieri, F. Caciagli, R. Pellicciari, Bioorg. Med. Chem. 11 (2003) 5417.
[31] M. dos Santos Frizzo, F. Soares, L. Dall'Onder, D. Lara, R. Swanson, D. Souza, Brain Res. 972 (2003) 84.