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# Post mortem degradation of nucleosides in the brain: Comparison of human and rat brains for estimation of in vivo concentration of nucleosides

Zsolt Kovács [a], Katalin Adrienna Kékesi [b], Mátyás Bobest [c], Tamás Török [a], Nóra Szilágyi [b], Tamás Szikra [d], Zsuzsanna Szepesi [d], Rita Nyilas [d], Árpád Dobolyi [d], Miklós Palkovits [e], Gábor Juhász [d,*]

[a] *Department of Zoology, Berzsenyi Dániel College, Szombathely, Károlyi Gáspár tér 4., 9700, Hungary*
[b] *Department of Physiology and Neurobiology, Eötvös Loránd University, Budapest, Pázmány Péter sétány 1C, 1117, Hungary*
[c] *Markusovszky Hospital, Szombathely, Markusovszky Lajos u. 3., 9700, Hungary*
[d] *Research Group of Neurobiology, Hungarian Academy of Sciences-Eötvös Loránd University, Budapest, Pázmány Péter sétány 1C, 1117, Hungary*
[e] *Research Group of Neuromorphology, Hungarian Academy of Sciences-Semmelweis University, Budapest, Tűzoltó utca 58., 1094, Hungary*

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**Abstract**

There is an increasing attention paid for nucleoside metabolism and changes of nucleoside concentrations in human brain because of its pathological and physiological relevance. In order to determine the post mortem degradation of nucleosides and nucleoside metabolites, the concentrations of four nucleosides and three nucleobases were measured in rat and neurosurgical human cerebral cortical samples with 30 s to 24 h post mortem delay. Adenosine degradation coefficient (a multiplying factor for calculating concentrations of investigated substances for the living state) was 0.886 for human brain at 2 h post mortem time, while it was 1.976 for rats. Hypoxanthine, an adenosine degradation product had coefficients 0.564 for human brain and 0.812 for the rat brain. We provide data and degradation coefficients for the concentrations of adenosine, guanosine, inosine, uridine, uracil, hypoxanthine and xanthine with 2, 4, 6 and 24 h post mortem delay. We also report a method how to validate human neurosurgical brain samples in terms of sample preparation and statistical analysis.


*Keywords:* Human brain; Rat brain; In vivo nucleoside levels; Post mortem degradation of nucleoside levels; Degradation coefficients

## 1. Introduction

There is an increasing attention for the better knowledge of the physiological and pathological roles of nucleosides in the central nervous system. Some of the purine and pyrimidine nucleosides, as well as nucleotides are recognized as neuromodulators and co-transmitters in the brain (Ribeiro et al., 2003; Sawynok and Liu, 2003). Endogenous adenosine, partly as a degradation product of ATP, has a role in control of sleep and arousal, cognition and memory, neuronal damage and protection (Ribeiro et al., 2003; Noji et al., 2004). Nucleosides may also play a role in neurodegenerative diseases like Parkinson's or Alzheimer diseases (Rathbone et al., 1999; Ribeiro et al., 2003; Xu et al., 2005). Several nucleoside derivatives have been introduced in clinical applications (Nabhan et al., 2004; Borst et al., 2004) but they often induce various neurological symptoms (Sadaie et al., 2004). Therefore, determination of in vivo nucleoside levels in post mortem samples from human brains with and without neurological disorders may have diagnostic values (Noji et al., 2004).

Nucleoside metabolic pathways form a complex network (Brosh et al., 1996). It is known from experiments under hypoxia and brain ischemia that converting enzymes can make significant changes in tissue concentrations of nucleo-

* Corresponding author. Tel.: +36 1 209 0555x8110; fax: +36 1 319 6723.
*E-mail address:* gjuhasz@dec001.geobio.elte.hu (G. Juhász).

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sides under low oxygen level or even post mortem. Hypoxanthine and xanthine, as nucleoside degradation end products are metabolised from adenosine, inosine and guanosine (Barsotti and Ipata, 2004), like uracil from uridine (Spector, 1985). The directions of chemical reactions responsible for degradation have been established (Barsotti and Ipata, 2004) but the kinetics of the degradation is not fully understood in experimental hypoxia, and it is poorly investigated post mortem. Hypoxia experiments have been done only on animals but the metabolic rates of nucleosides are hardly comparable to those in humans indicating an urging need for nucleoside degradation studies in human brains. In the present study, we report on the first comparative study on post mortem degradation velocity of nucleosides and their metabolites in order to establish correction factors for estimating the in vivo concentration of nucleosides and nucleoside metabolites in the human, as well as in rat brains. In general, we provide a standard approach to estimate concentrations of rapidly degrading compounds from human brain bank samples.

## 2. Materials and methods

Human brain samples were collected in agreement with the Ethical Rules for Using Human Tissues for Medical Research in Hungary (HM 34/1999). Experiments on animals were carried out on the basis of local ethical rules in accordance with the Use Animals in Scientific Procedures Act 1986 and associated guidelines, the European Communities Council Directive 24 November 1986 (86/609/EEC). All efforts were made to eliminate animal suffering and to reduce the number of animals.

### *2.1. Chemicals and materials*

Nucleoside standards for peak identification (uracil, hypoxanthine, xanthine, uridine, inosine, guanosine and adenosine) and other chemicals, reagents, standards, etc. were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) and Merck Co. (Darmstadt, Germany) in 99% purity, analytical or HPLC grade.

### *2.2. Determination of nucleoside concentrations by HPLC*

A recently modified HPLC method for analysis of nucleosides from rat and human brain samples was used (Dobolyi et al., 1998). Using diode array detector with a cooled column system allows better selectivity than other HPLC methods. This method has been proved to be sensitive and selective enough for the measurement of uracil, hypoxanthine, xanthine, uridine, inosine, guanosine, adenosine, thymidine and deoxynucleosides in milligrams of brain tissue. Nucleosides were measured by HP 1100 series gradient chromatograph with diode array detector. The separation was performed on a HP Hypersil ODS C18, 2.1 mm × 200 mm analytical column and a 2.1 mm × 20 mm guard column. The flow rate was 300 μl/min. Eluent A was 0.02 M formiate buffer containing 0.55% (v/v) acetonitrile, pH 4.45 and eluent B was 0.02 M formiate buffer containing 40% (v/v) acetonitrile, pH 4.45. The gradient profile was as follows: 0% B at 0–10 min, 10% B at 22 min and 100% B at 30 min. The column temperature was 10 °C. The injection volume was 10 μl. The diode array detector was adjusted so that it measured at 254 nm (reference wave length 360 nm) and 280 nm (reference wave length 450 nm). Using automatic integrator function of HP Chemstation software, chromatograms were evaluated and only those peaks were measured manually which were under the limit of automatic integration.

### *2.3. Sample collection and sample preparation for nucleoside analysis*

Human brain tissue samples were collected in the surgical room. Single undamaged sample was removed from each of the three brains (two males and one female, median age 61 ± 7.5 [S.E.M.] years) from frontal, temporal and occipital cortices, respectively.

In all three cases, glioblastoma removal was done in Markusovszky Hospital, Szombathely, Hungary. Tissue removal was done under the usual brain surgery conditions. The shortest post mortem time sample was in room temperature only for less than 30 s including the following sample preparation steps. Therefore, it matches the conditions of animal tissue sample collection and called as 30 s sample. Because of the very limited sample size, samples were divided into five fractions. One fraction was immediately frozen to −70 °C. The other four fractions were kept at room temperature (+25 °C) for 2, 4, 6 and 24 h, respectively, then frozen to −70 °C. The selected time periods cover the most frequently post mortem delay of brains collected by most of the brain banks.

A 1–1.5 mg piece of each brain sample was placed into Eppendorf tube containing 20 μl eluent A. The wet tissue weight was measured at 0.01 mg accuracy. Tissue samples were homogenized with a Teflon potter homogenizer for 10 s. The potter speed was 6000 rpm. The homogenized samples were treated with 1000 W microwave beam for 10 s. Then, the samples were centrifuged at 12,000 rpm for 20 min by Eppendorf centrifuge. Ten microliter supernatants were stored for no more than 1 week at −20 °C then they were analyzed by HPLC.

Rat brain samples were collected from Wistar rats (body weights 250 ± 15 g, $n = 3$). Rats were anaesthetized in urethane (1000 mg/kg i.p.). The brains were removed from the skull, and samples from the frontal and parietal cortex (one sample from each side, 5–7 mg wet weight (w.w.) of each) were dissected within 30 s (called as 30 s samples). Samples were divided into five fractions: one fraction (1–1.5 mg) was frozen immediately at −70 °C, the other fractions were kept at room temperature for 2, 4, 6 and 24 h. Then, the tissue sam-

ples were prepared for HPLC analysis by the same method as human brain samples.

### 2.4. Calculation of nucleoside content and data analysis

Data were calculated for 1 mg wet weight. The ratios of concentrations obtained in the 30 s and nucleoside levels in 2, 4, 6 and 24 h post mortem samples were calculated and called as degradation or back-extrapolation coefficients. The differences in concentrations of nucleosides obtained at different post mortem times were compared to the 30 s sample data by Student's *t*-test for correlated samples. Changes were regarded significant at $p < 0.05$.

## 3. Results

In the human and rat brain tissue samples, concentrations of four nucleosides and their degradation products (uridine, inosine, guanosine, adenosine, uracil, hypoxanthine and xanthine) have been measured (Table 1). The concentration of uracil, hypoxanthine and adenosine are lower in the 30 s human brain samples (6.4, 6.6 and 5.1 times, respectively) as compared to those in rat brain. Levels of xanthine, uridine and inosine are slightly higher in rat (1.9, 2.1 and 1.6 times, respectively) than in human brain, while the guanosine level is 1.9 times higher in human brain.

In human cortical samples, the concentrations of hypoxanthine, xanthine and uridine changed significantly by the end of the 2nd post mortem hour (Table 1). Levels of uracil and inosine changed significantly at the 4th post mortem hour, while the adenosine concentrations showed the highest changes at the 24th post mortem hour. Guanosine did not changed significantly along the investigated period of time. In function of time, concentration of inosine decreased, the concentrations of uracil, hypoxanthine and xanthine increased continuously (Fig. 1). Levels of uridine, guanosine and adenosine in the human cortical samples showed fluctuations within the 2–24 h post mortem periods.

Based on data obtained from 2–24 h post mortem samples, concentration of inosine, guanosine and adenosine reached a minimum level while the level of uracil, hypoxanthine, xanthine and uridine reached a maximum level after 24 post mortem hours in the human brain (Table 1).

However, guanosine and adenosine levels were the lowest at the 4th and 2nd post mortem hours, while hypoxanthine was the highest in the 6th hour samples of the rat brain.

Concentration of hypoxanthine declined at the 6th post mortem hour in the rat, while it increased in the human brain by that time. Approximately, parallel changes of uri-

Table 1
Concentrations and degradation coefficients of nucleosides in the human and rat brain

| Post m. time | | Nucleosides and their metabolites | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Ura | Hyp | Xn | Urd | Ino | Guo | Ado |
| | | Human brain | | | | | | |
| 30 s | *c* | 4.03 ± 0.69 | 30.27 ± 2.51 | 26.57 ± 2.54 | 12.03 ± 1.00 | 93.46 ± 10.10 | 12.87 ± 1.73 | 9.81 ± 1.31 |
| 2 h | *c* | 4.17 ± 0.36 | 53.63 ± 2.34** | 39.15 ± 2.36** | 17.08 ± 0.66** | 76.98 ± 4.24 | 11.81 ± 0.96 | 11.07 ± 1.47 |
| | dc | 0.966 | 0.564 | 0.679 | 0.704 | 1.214 | 1.089 | 0.886 |
| 4 h | *c* | 7.20 ± 0.35** | 60.00 ± 2.40** | 42.51 ± 2.08** | 15.79 ± 0.79** | 69.13 ± 5.01* | 12.53 ± 1.17 | 7.36 ± 0.86 |
| | dc | 0.559 | 0.505 | 0.625 | 0.762 | 1.352 | 1.027 | 1.333 |
| 6 h | *c* | 10.74 ± 1.03** | 85.82 ± 3.81** | 62.52 ± 2.63** | 18.93 ± 0.69** | 53.25 ± 3.44** | 11.68 ± 0.96 | 7.95 ± 1.50 |
| | dc | 0.375 | 0.353 | 0.425 | 0.635 | 1.755 | 1.102 | 1.234 |
| 24 h | *c* | 27.42 ± 1.36** | 106.52 ± 4.81** | 110.78 ± 5.34** | 23.53 ± 1.28** | 31.69 ± 2.23** | 9.20 ± 0.74 | 5.22 ± 0.49** |
| | dc | 0.147 | 0.284 | 0.239 | 0.511 | 2.949 | 1.399 | 1.879 |
| | | Rat brain | | | | | | |
| 30 s | *c* | 25.90 ± 3.26 | 199.72 ± 19.80 | 50.53 ± 4.92 | 25.13 ± 1.78 | 148.66 ± 16.41 | 6.67 ± 0.88 | 50.02 ± 7.39 |
| 2 h | *c* | 29.04 ± 2.99 | 246.11 ± 19.11 | 54.68 ± 4.59 | 17.26 ± 1.36** | 104.76 ± 11.49* | 4.06 ± 0.22* | 25.31 ± 2.64* |
| | dc | 0.892 | 0.812 | 0.924 | 1.456 | 1.419 | 1.643 | 1.976 |
| 4 h | *c* | 61.10 ± 1.39** | 310.27 ± 7.64** | 110.80 ± 2.46** | 16.85 ± 0.13** | 17.10 ± 9.38** | 1.56 ± 0.41** | 53.70 ± 0.98 |
| | dc | 0.424 | 0.644 | 0.456 | 1.491 | 8.694 | 4.276 | 0.932 |
| 6 h | *c* | 64.30 ± 1.51** | 325.32 ± 10.02** | 126.42 ± 4.28** | 18.62 ± 0.16** | 19.87 ± 8.12** | 2.19 ± 0.16** | 46.70 ± 1.88 |
| | dc | 0.403 | 0.614 | 0.399 | 1.349 | 7.482 | 3.046 | 1.071 |
| 24 h | *c* | 72.08 ± 2.53** | 267.99 ± 2.60** | 158.60 ± 8.44** | 25.72 ± 0.45 | 7.99 ± 7.39** | 1.88 ± 0.23** | 27.47 ± 0.91** |
| | dc | 0.359 | 0.745 | 0.319 | 0.977 | 18.606 | 3.548 | 1.821 |

*Abbreviations*: post m., post mortem; 30 s, 30 s post mortem time; h, hour; *c*, concentration of nucleosides (pmol/mg w.w.); dc, degradation coefficients; Ura, uracil; Hyp, hypoxanthine; Xn, xanthine; Urd, uridine; Ino, inosine; Guo, guanosine; Ado, adenosine.

* $p < 0.05$.

** $p < 0.005$.

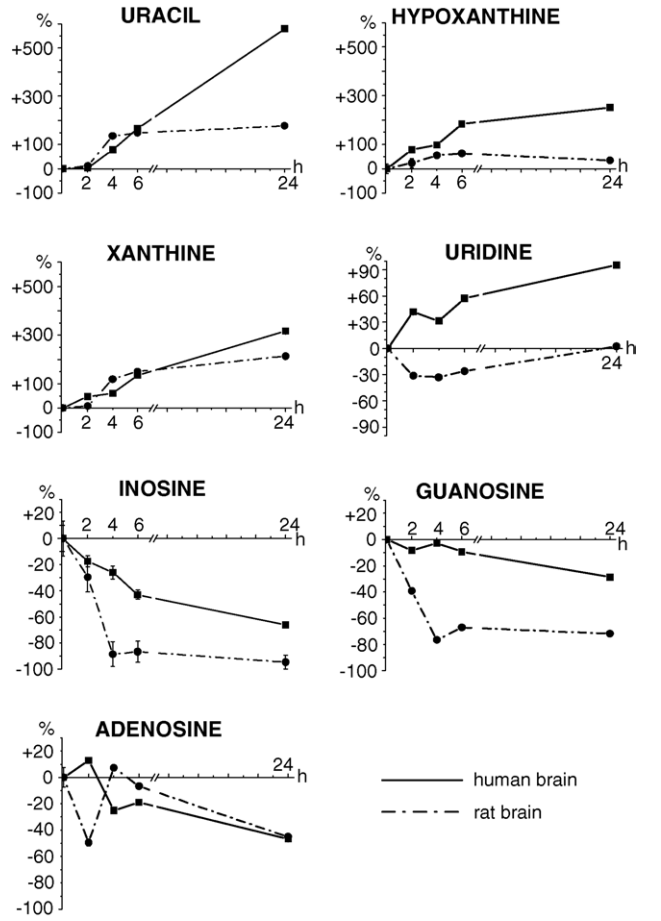


Fig. 1. Degradation kinetics of nucleosides in the human and rat brain between 30 s and 24 post mortem hours. The *X*-axis indicates the post mortem hours (h). Change of nucleoside concentration ("+" up and "−" down) are given as percentage of control ("0" means the control 30 s sample nucleoside level).

dine and uracil concentration were observed between 2nd and 24th post mortem hours in human versus rat samples, however, the changes were opposite to each other between the 2nd and 4th post mortem hours. In the rat samples (Table 1; Fig. 1), concentrations of uridine, inosine, guanosine and adenosine changed significantly from the 2nd, uracil, hypoxanthine and xanthine from the 4th post mortem hour significantly. Concentrations of uracil and xanthine increased continuously from the 2nd to the 24th post mortem hour.

The comparison of degradation coefficients for human and rat brain (Table 1) demonstrates differences in degradation kinetics between the two species. As a general rule, alterations in the post mortem concentrations of uracil, hypoxanthine, xanthine and inosine seemed to be the largest. Inosine levels decreased with post mortem time while the level of other nucleosides increased. Thus, the degradation coefficients were found relatively small for uracil, hypoxanthine and xanthine, while high for inosine.

## 4. Discussion

In the present study, we compared the degradation of four nucleosides and three nucleobases in rat and human brain tissue samples as a function of post mortem time. Nucleoside and nucleobase concentrations we obtained (Table 1), are in agreement with the data available in the literature in spite of considerable differences in sample treatment methods (Hagberg et al., 1987; Kanemitsu et al., 1988; Pillwein et al., 1990; Mascia et al., 1999). We used microwave treatment to block metabolising enzymes because it is a reliable method to immediately stop enzyme activities (Butcher et al., 1976; Lenox et al., 1977; Miller et al., 1990). We either stored microdissected brain samples at −70 °C before microwaving or microwave treated homogenates at −20 °C, which result in no significant alterations in nucleoside concentrations (Iriyama et al., 1986; Dobolyi et al., 1998).

The degradation of nucleotides during dissection is a concern because hypoxia could lead to significant changes in nucleoside concentrations. Therefore, we kept a 30 s interval between brain removal and sample dissection in case of rats. Human samples were taken from alive subjects during brain surgery but in this case also less than 30 s time was spent to take the samples out from the surgery area and divide the samples into separate pieces. Still, the human samples are heterogeneous concerning duration and extent of hypoxia before dissection because of the heterogeneity of surgery procedures applied for different patients. These differences in sample collection may contribute to the differences in the nucleoside and nucleobase concentrations between human and rat, however, it has to be emphasized that there is no more accurate method for sample collection in surgery rooms than those we applied. The less than 30 s time for treating human samples at room temperature makes the degradation of rat and human tissue highly comparable. In addition, the differences in rat and human data may also be caused by metabolic rate differences between the species. Nucleoside metabolism velocity is different in the two species (Nagata et al., 1984; Fastbom et al., 1987; Yamamoto et al., 1987). However, our comparison of nucleoside degradation (Fig. 1; Table 1) suggests that differences between such metabolically so different species as rats and human, is smaller than expected on the basis of velocity of metabolism in the two species.

The biochemical pathways for degradation have been previously established (Zimmermann, 1992; Brosh et al., 1996; Barsotti and Ipata, 2004). It is also known that the activity of nucleotide metabolic enzymes is still detectable in the brain for hours after death (Phillips and Newsholme, 1979; Nagata et al., 1984). Nucleoside concentrations reflect the availability and activity of nucleoside converting enzymes in post mortem brain samples. Consequently, various changes in both extracellular and intracellular nucleoside concentrations were observed in post mortem samples (Hagberg et al., 1987; Dobolyi et al., 1998). The degradation process is very fast in the first 5–10 min of hypoxia (Hagberg et al., 1987; Pissarek et al., 1998). ATP is degraded to adenosine, inosine,

hypoxanthine and xanthine by adenylate kinase, 5′ nucleotidase, adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase while GTP via guanosine to xanthine by guanase during brain ischemia increasing their levels 4–33 times (Kleihues et al., 1974; Hagberg et al., 1987; Barsotti and Ipata, 2004). In addition, extracellular nucleotidases may also contribute to the nucleoside concentrations (Zimmermann, 1996). Since these enzymes are markedly different than the enzymes of the intracellular nucleotide metabolism, they could result in different concentrations over time. However, such differences are not likely to remain between compartments of the post mortem brain because nucleoside transporters would equilibrate the nucleosides and nucleobases. As a combined result of these enzyme activities, a fast degradation of nucleosides takes place between 0 and 15 post mortem minutes. Hypoxanthine, xanthine and uracil concentrations correlated with the extensive decrease in adenosine, inosine and uridine levels between 30 s and 15 min post mortem time in the rat brain (Fig. 1; Table 1). Hypoxanthine and xanthine formation coincided with an extensive decrease in inosine and to a lesser degree in adenosine and guanosine content between 30 s and 24 h post mortem times both human and rat brain (Fig. 1; Table 1). However, degradation of nucleosides between 15 min and 24 post mortem hours (Fig. 1) is difficult to explain at the actual state of knowledge about post mortem changes in enzyme activity. There is no systematic study available about functioning of nucleoside metabolising enzymes under post mortem conditions. Therefore, explaining, e.g. the fluctuation of adenosine and uridine concentration in the human and rat samples or diminution of hypoxanthine level in the human samples between 2–24 and 6–24 post mortem hours (Fig. 1) is not straightforward. However, for uridine, the fluctuation could be attributed to uridine phosphorylase acting catabolically first and then anabolically (Mascia et al., 1999; Mascia and Ipata, 2001). Notwithstanding the underlying mechanisms, the continuous degradation of nucleosides under post mortem conditions observed both in human and rat brain tissue samples raise a need for determination of degradation coefficients of nucleosides to estimate in vivo nucleoside concentrations from data revealed from post mortem brain samples. In fact, these concentrations may not be real in vivo nucleoside levels because methodological problems, e.g. more then 0 s elapsing between removing brain sample and microwaving. In our experiments, fresh samples were removed and dissected within about 30 s post mortem time. However, to the best of our knowledge, our results are one of the most exact investigation of in vivo nucleoside levels from post mortem human and rat brain samples at the moment, in turn, our estimated in vivo concentrations are the closest to the real in vivo data about nucleoside concentrations in the human brain.

Our data reported here opened the application of human brain bank materials for studying the roles of nucleosides in human diseases giving reliable coefficients for estimation of in vivo nucleoside concentrations. Since surgical human brain samples are restricted, most brain samples are available for research only following various times after death. The use of these samples for measuring nucleosides and nucleobases has been hindered by the post mortem time-dependent concentration changes of these substances. Our method allows the comparison of nucleoside concentrations obtained at various times after death thus providing an important method to eliminate variances in these measurements. In addition, we also promoted the use of animal models allowing more sophisticated experimental manipulations for understanding pathomechanisms of brain diseases since the comparison of metabolic degradation of nucleosides in rats and humans allows animal experimentation to research on pathomechanisms of human brain diseases. Finally, our studies provided a model for validation of brain bank tissue sample materials in research on mechanisms of brain diseases.

In conclusion, our method opens a possibility to use degradation coefficients to study nucleoside metabolism in the human and rat brain on the basis of post mortem samples from human brain bank and 30 s brain samples from rats. We also provide here correction factors for in vivo estimation of nucleosides and we compare the degradation in two different species as rats and humans giving a model for application of animal experiments for estimation of post mortem degradation processes in human brain.

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