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Analysis of purine and pyrimidine bases, nucleosides and deoxynucleosides in brain microsamples (microdialysates and micropunches) and cerebrospinal fluid

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Abstract

A new chromatographic method is reported for the synchronous analysis of endogenous purine and pyrimidine bases, ribonucleosides, and deoxyribonucleosides in brain samples. An optimized gradient chromatography system with a cooled reversed-phase column allows the detection of these compounds in very low concentrations in microsamples (microdialysates and micropunches). Chromatographic peaks were identified via the retention times of known standards, with detection at two wavelengths, and also by electrospray tandem mass spectrometry, which permits the identification of certain compounds at extremely low concentrations. The method was tested on *in vivo* brain microdialysis samples, micropunch tissue samples and cerebrospinal fluid of rats. Extracellular concentrations of pyrimidine metabolites in brain samples and of various purine metabolites in thalamic samples are reported here first. A comparison of the results on microdialysis and cerebrospinal fluid samples suggests that the analysis of cerebrospinal fluid provides limited information on the local extracellular concentrations of these compounds. Basic dialysis experiments revealed temporarily stable baseline levels one hour after implantation of the microdialysis probes. An elevated potassium concentration in the perfusion solution caused increases in the extracellular levels of adenosine and its metabolites, and of guanosine and the pyrimidine nucleoside uridine.

An increasing body of evidence suggests the involvement of nucleotides and nucleosides in various brain functions. ATP is a properly established transmitter (Zimmermann, 1994), while adenosine is a neuromodulator in the peripheral and central nervous systems (Williams, 1990). It modulates pain sensation and sleep and regulates autonomic functions and cognition (Williams, 1990). It also protects against cell death in stroke or epilepsy (Rudolphi *et al.*, 1992; Dragunow, 1988). During the past few years, the roles of other nucleotides and nucleosides in brain functions have also been intensively investigated. Neurological side-effects of immunosuppressants (Yu *et al.*, 1981), anti-tumour (Sylvester *et al.*, 1987; Macdonald, 1991) and anti-viral agents (Rachlis and Fanning, 1993) known to act on the purine and pyrimidine metabolism enzymes drew attention to the possible roles of nucleosides and deoxynucleosides in brain cell survival. Deoxyadenosine may induce neuronal apoptosis (Wakade *et al.*, 1995), deoxycytidine may play a part in neuronal survival independently of DNA synthesis (Wallace and Johnson, 1989), guanosine may enhance nerve growth factor induced neurite outgrowth (Gysbers and Rathbone, 1996), and glial proliferation may be regulated by deoxyguanosine and deoxyadenosine (Christjanson *et al.*, 1993). Other lines of investigation have demonstrated that pyrimidine nucleotides have specific pyrimidinoceptors (Seifert and Schultz, 1989) even in cells of brain origin (Iredale *et al.*, 1992; Filippow and Brown, 1996; Nicholas *et al.*, 1996). Although some of these receptors have recently been cloned (Chang *et al.*, 1995; Communi *et al.*, 1995; Nguyen *et al.*, 1995; Webb *et al.*, 1996), their functions in the nervous system are still unknown. As extracellular nucleotides are metabolized to nucleosides in the interstitial space (Zimmermann, 1996), the measurement of nucleosides may provide information relating to the possible transmitter function of nucleotides. Additionally, nucleosides could have their own modulatory actions, as indicated by the sleep-modifying

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effect of uridine (Inoue *et al.*, 1995). Thus, the following major step towards an understanding of the functional role of nucleosides in the brain would be their *in vivo* measurement in behaving animals. However, the distributions of nucleosides and related compounds in the tissue compartments and in the extracellular space must first be established. Their measurement at low concentrations in samples such as microdialysates or microdissected brain tissue is a definite need.

The currently available chromatographic methods (Wojcik and Neff, 1982; Eells and Spector, 1983; Hagberg *et al.*, 1987; Olivares and Verdys, 1988; Mei *et al.*, 1996) are not sensitive enough and the separation of deoxynucleosides and pyrimidine nucleosides is not properly solved. In the present study we have applied a highly optimized small-bore system in which the column can be cooled for better separation. We have measured nucleosides and related compounds in cerebrospinal fluid (CSF), microdissected brain samples and brain microdialysates. In dialysis samples, we found several compounds which had previously not been detected in the extracellular space. Some basic dialysis experiments, such as baseline stability measurements, potassium (K^+)-induced release studies and a comparison of *in vivo* and *post mortem* samples, have also been performed.

Experimental procedures

Materials

Purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides were obtained from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) in at least 99% purity. All other chemicals, reagents and standards were purchased from the same company and were of analytical or HPLC grade.

HPLC technique

The HPLC method was developed on a Pharmacia LKB SMART system by using small-bore columns. This system is able to separate 5 μl of eluent with its fraction collector for further identification of chromatographic peaks by means of mass spectrometry. The HPLC analysis method was as follows: UV absorption was detected at 254 nm with a detector sensitivity of 0.002 AUFS. Separation was performed on a Hewlett Packard Hypersyl ODS reversed-phase column (200 × 2.1 mm) filled with 5 μm C18 spherical packing material. We designed a precolumn holder and special column heads to use the original column compressor of the SMART system. Eluent A was 0.02 M formate buffer, pH = 4.45. Eluent B was 0.02 M formate buffer containing 40% acetonitrile, pH = 4.45. The gradient profile was as follows: 0% B eluent at 0 min, 0% at 2.5 min, 10% at 20 min, 30% at 25 min, 100% at 38 min. The flow rate was 300 μl/min. The column and detector temperature was 10°C. Chromatograms were evaluated by an interactive program written in Matlab for Windows.

Peak identification

Because of difficulties in the interpretation of the peaks, three independent procedures were applied for the identification and purity analysis of chromatographic peaks: (1) Comparison of retention times of chromatographic peaks of biological samples with those of external standards. The detected compounds were also added as internal standards to the biological samples and the elevations and shapes of the peaks were checked. (2) Both standard mixture samples and dialysates were analysed at 254 and 280 nm. The ratios of peak areas recorded at the two wavelengths were used for the estimation of peak purity. (3) Concentrations of compounds in microdialysis samples were measured relative to the concentrations of series of standards. The peaks were collected in separate fractions, and concentrations of candidate compounds were measured by means of electrospray tandem mass spectrometry (MS–MS). Comparison of the MS-based and HPLC-based concentration estimates also afforded a good measure of peak identification and peak purity.

The electrospray MS–MS analysis was carried out with a VG QUATTRO tandem mass spectrometer equipped with an electrospray interface (Fisons Instruments, Altrincham). Experiments were performed with the Multiple Reaction Monitoring (MRM) technique (Prinsen *et al.*, 1997). For nucleosides, the selected fragmentation results in protonated basic residues. The monitored fragmentations were optimized separately for each compound. During the measurements, the source temperature was 120°C and the capillary and cone voltages were 3.5 kV and 35 kV, respectively. For appropriate fragmentation, ions were accelerated to 40 eV and collided with argon at 7.8×10^{-4} mbar. High-purity nitrogen served as nebulizer and bath gas. The solvent (5 : 95 : 0.1 acetonitrile : water : formic acid) was delivered by a P 200 HPLC pump (Thermo Separation Products) at a rate of 100 μl/min, and 10 μl samples and standards were injected directly.

Microdialysis

Microdialysis probes were made as described earlier (Juhász *et al.*, 1989). Briefly, a hollow fibre (Travenol, cut off 5000 D, diameter 0.2 mm, length 3 mm) was adjusted into 25-gauge stainless steel tubing. Glass capillaries pulled from Jencons glass tubing were used for the inlet and outlet of the probe. These glass capillaries were guided by stainless steel tubes.

Animal experiments were carried out on the basis of local ethical rules in accordance with the Guidelines on

the Use of Living Animals in Scientific Investigations 1984. Rats (350–400 g) were anaesthetized with a 1% halothane–air mixture and then placed into a stereotaxic frame. Body temperature was measured as rectal temperature was adjusted with a heating lamp and the halothane concentration was kept between 0.5–0.8% during the collection of samples *in vivo*. The probes were placed into the VPL/PM thalamic nuclei bilaterally (A: −1.9, L: 2.5, V: −7 mm, according to the atlas of Pellegrino and Cushman, 1967). The final position of the probes was reached slowly, in not less than 20 min in order to reduce tissue damage. Perfusion was performed at 1 μl/min with artificial cerebrospinal fluid (ACSF), which contained 144 mM NACl, 3 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$ in amino acid-free, bi-distilled water, the pH being adjusted to 7.0 prior to the experiments. The ACSF was tested by HPLC in each experiment. The high-K^+ perfusion solution contained 27 mM NaCl, 120 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$. Samples of 60 μl were collected every 60 min, 50 μl of which was used for the analysis. The samples were frozen immediately and stored at −20°C. In experiments with high-K^+ solution, the first three samples were collected with the perfusion of ACSF, and a change was then made to the high-K^+-containing perfusion fluid and another two samples were collected. Finally, the rats were overdosed with a 4% halothane–air mixture and the positions of the probes in the brain were verified on coronal sections after formaldehyde fixation.

Microdissection of brain nuclei

Tissue samples containing about 1.7 mg caudate nucleus were micropunched (Palkovits, 1973) from frozen, 300 μm thick sections of rat brain within 2 min after decapitation. Samples were placed into Beckman airfuge tubes containing 20 μl eluent A. The tissue was homogenized for ten seconds using a motor-driven teflon potter whose size fitted tightly into the tube. The homogenate was treated with a 1000 W microwave beam immediately for 20 s, and then stored at −20°C. Immediately before the injection into the chromatograph, the centrifuge tubes were adjusted to a constant weight with eluent A, and centrifuged at 100,000 g for 20 min (Beckman Airfuge). 50 μl of a total of about 55 μl supernatant was then injected into the chromatograph.

Cerebrospinal fluid

CSF (30 μl) was obtained from the cisterna magna in ketamine-anaesthetized rats, centrifuged at 1500 g for 10 min to remove any cells, then maintained at −20°C, and 25 μl aliquots were injected into the chromatograph.

Statistics

All the statistical tests were performed with Statistica for Windows 5.0 (StatSoft Inc.). The stable baselines of different substances in control microdialysis experiments were tested with repeated measures ANOVA, or with the Friedmann (nonparametric) ANOVA procedure when the data violated the homogeneity and sphericity assumptions (Levene's test for homogeneity of variances and Mauchly's test for sphericity). The data obtained by perfusing the high-K^+ concentration and *post mortem* data were compared with the baseline by Student's *t*-test for dependent samples. The changes were considered significant when the calculated *P* value was less than 0.05.

Results

HPLC method

The optimized HPLC method allowed the separation of many endogenous nucleoside compounds in less than 25 min as indicated in the standard chromatogram in Fig. 1 and in Table 1. The extremely high sensitivity of the method permitted the measurement of these compounds even in diluted microdialysis samples (Fig. 2, Table 1). Among the compounds, the concentrations of uridine, 2′-deoxyuridine, uracil, cytidine, 2′-deoxycytidine, thymidine, thymine, 2′-deoxyadenosine and 2′-deoxyguanosine in the extracellular space of the brain had not been determined before this study.

The sensitivity of the method is reflected in the low detection limits, which were found to lie in the range 0.1–1 pmol/50 μl for all the measured compounds, as seen in Table 1. The reproducibility of the method was tested by injecting the same standard solution 10×. The variation did not exceed 2% of the peak area and the concentration vs peak area function was linear between 0.1–50 pmol/50 μl. Even after storage at −20°C for 2 weeks, the reproducibility of the technique was more than 95%.

To demonstrate the efficiency of the new method, a comparison was made between extracellular space, CSF and tissue sample concentrations. The concentrations of pyrimidines were higher in the CSF than in the thalamic extracellular space, while the concentrations of most purines were lower in the CSF (Table 3). Tissue samples from the caudate nucleus contained extremely high concentrations (about 500 μM) of adenosine, inosine, hypoxanthine and xanthine. The concentrations of uridine and guanosine were of the order of 100 μM, while those of deoxynucleoside compounds were similar to those in the interstitial fluid (0.5–5 μM).

Peak identification and peak purity analysis

Endogenous nucleosides and their metabolites were tested to establish whether they peaks correspond to peaks in microdialysis samples. Standards were co-injected with the sample and the peaks which were elevated without change in shape were analysed further for

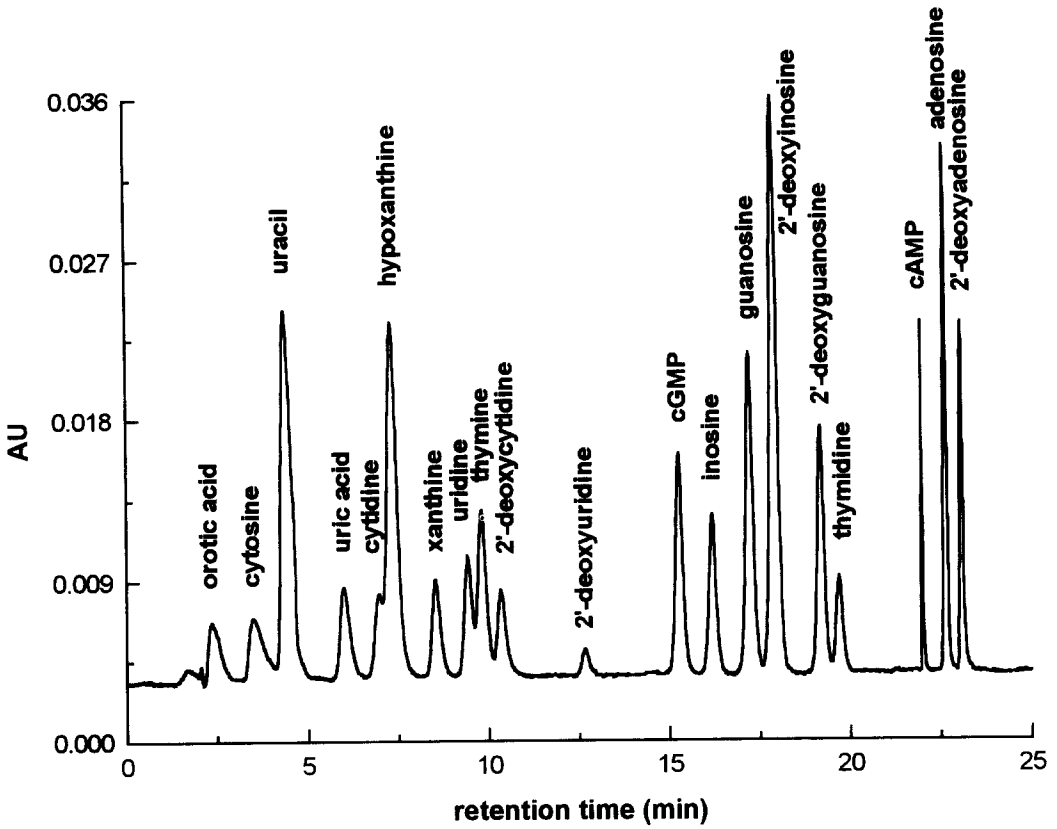

Fig. 1. Elution profile of a standard mixture of purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides, using the chromatographic method described in the Experimental Procedures. The concentrations of standards are about 25–50 pmol/50 μl.

peak purity. The comparison of ratios of sample peaks at two different wavelengths and ratios of standard peaks at two different wavelengths demonstrated that the peaks of compounds seen in Table 2 are pure. The one notable exception was the uracil peak, which was indicated not to be pure. Thus, we consider these peaks are clean, except for uracil. Compounds with primary amino groups which possess sufficiently low detection limits in mass spectrometry were analysed for peak purity with that method as well. A peak was suggested to be not pure when a zero or lower concentration of the particular compound in the collected fraction of the peak was found by mass spectrometry relative to UV chromatography. Each mass spectrometrically analysed compound possessed a pure chromatographic peak (Table 2).

Microdialysis experiments

Since the concentrations of several compounds investigated here had not previously been measured in the extracellular space of the brain, experiments were performed to investigate the dependence of the concentrations of purines and pyrimidines on experimental conditions. First, the rates of spontaneous changes in the concentrations of compounds were determined in microdialysis experiments. The concentrations of adenosine, inosine, hypoxanthine, xanthine, uridine and guanosine were high immediately after the implantation of the probes, but then dropped rapidly, the concentrations of deoxyribonucleosides and uracil showed no initial increase, and the uric acid level was low immediately after the implantation. There were no changes in concentration of any compound following the second hour of perfusion (Fig. 3).

To obtain information on the intracellular–extracellular concentration ratios of purines and pyrimidines, *post mortem* samples were measured (Fig. 4). In these samples, the concentrations of adenosine, its metabolites inosine and hypoxanthine, and also guanosine, uridine and its metabolite uracil were elevated, while the levels of 2′-deoxyuridine, 2′-deoxycytidine and thymidine were decreased. When high-K^+ (120 mM)-containing ACSF was applied in the microdialysis probe (Fig. 5), the concentrations of the above substances showed alterations similar to those in *post mortem* samples, but as concerns deoxynucleoside compounds, only the decrease in the 2′-deoxyuridine level was significant. It is also noteworthy that compounds with increased levels in *post mortem* and high-K^+ samples were those whose concentrations rose during the first hour following implantation (Fig. 3).

Table 1
Detection limits (signal to noise ratio, S/N = 3), concentrations in perfusate and estimated extracellular thalamic (ventral posterolateral (VPL) and ventral posteromedial (VPM) nuclei) concentrations determined with the new method. Detection limits were determined via series of dilutions of standard mixtures. Perfusate concentrations are averages of the data on 13 rats. On the basis of previous results (Juhász *et al.*, 1989), we assumed a relative recovery of 0.30 when estimating extracellular concentrations

	Detection limit pmol/50 μl	nM	Concentration of perfusate (nM)	Estimated extracellular concentration in VPL/VPM (μM)
Purine bases				
adenine	5.1	102	n.d.	n.d.
guanine	3.0	60	n.d.	n.d.
hypoxanthine	0.8	16	1344±75	4.48±0.25
xanthine	1.1	22	351±24	1.17±0.08
uric acid	2.3	46	345±38	1.15±0.13
Purine nucleosides				
adenosine	0.1	2	285±8	0.95±0.04
guanosine	0.2	4	51±7	0.17±0.02
inosine	0.3	6	156±10	0.52±0.03
Purine deoxynucleosides				
2′-deoxyadenosine	0.2	4	51±11	0.17±0.04
2′-deoxyguanosine	0.2	4	3±0.4*	0.01±0.001
2′-deoxyinosine	0.2	4	n.d.	n.d.
Cyclic nucleotides				
cAMP	0.4	8	n.d.	n.d.
cGMP	0.5	10	n.d.	n.d.
Pyrimidine bases				
thymine	0.8	16	15±1.3*	0.05±0.004
uracil	1.0	20	367±66	1.22±0.22
cytosine	2.1	42	n.d.	n.d.
orotic acid	1.5	30	n.d.	n.d.
Pyrimidine nucleosides				
uridine	0.5	10	228±12	0.76±0.04
cytidine	1.3	26	35±3	0.11±0.01
Pyrimidine deoxynucleosides				
thymidine	0.3	6	75±6	0.25±0.02
2′-deoxyuridine	0.8	16	318±16	1.06±0.05
2′-deoxycytidine	0.7	14	324±18	1.08±0.06

* Extracellular concentrations were estimated with a higher sample volume.

Discussion

Methodological considerations

With the Pharmacia LKB SMART system, the present method for the analysis of purines and pyrimidines in brain samples is about 20× more sensitive and selective than the previously published techniques (Wojcik and Neff, 1982; Eells and Spector, 1983; Hagberg *et al.*, 1987; Olivares and Verdys, 1988; Mei *et al.*, 1996). The chromatography set-up applied is highly optimized for small-bore gradient chromatography involving extremely short fluid paths, with syringe type, pulsation-free pump and an inbuilt on-column UV detector. Besides the sensitivity, the selectivity of the HPLC method was also improved. The column cooling markedly improved the separation by increasing the affinity of compounds for the reversed phase. This effect was especially important in the separation of the most hydrophilic compounds with low retention times. On the other hand, the cooling decreased the band-broadening effect of diffusion, thereby contributing to the detectability of the most hydrophobic compounds with high retention times.

Application of the method

The very high-sensitivity analysis method is crucial in work with microdialysis samples because the compounds

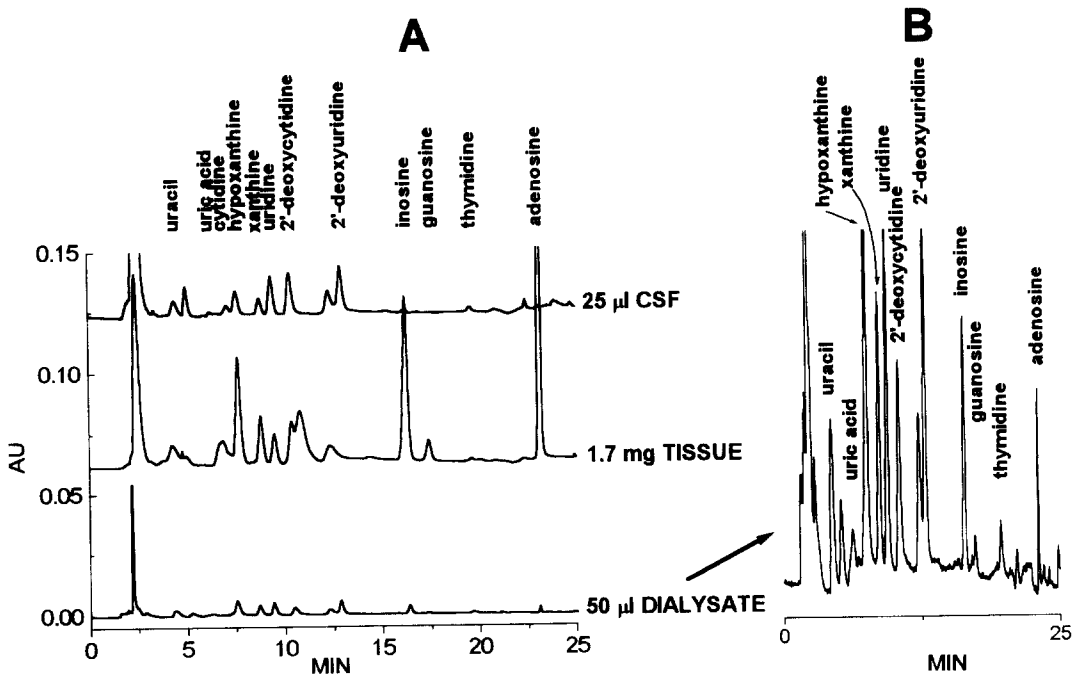

Fig. 2. A: Chromatograms of 50 µl cerebrospinal fluid, 1.7 mg tissue from the microdissected, caudate nucleus and 50 µl microdialysis sample, obtained with the chromatographic method described in the Experimental Procedures. B: The same chromatogram of the dialysate as in A, but on an enlarged y scale, demonstrating the applicability of the method with even small amounts of diluted samples.

Table 2
Peak identification and verification of peak purity by measurement at two different wavelengths and by mass spectrometry. Estimation of concentrations of fractions by chromatography was performed according to dilutions of perfusates during peak collection. Measurements of concentrations of fractions by mass spectrometry was performed as described in the Experimental Procedures

	Ratio of peak areas at two different wavelengths		Concentrations (µM) of collected fractions estimated by	
	Standard ± SEM (254/280)	Sample ± SEM (254/280)	HPLC	MS
Purines				
adenosine	7.51 ± 0.65	7.26 ± 0.68	0.095	0.069
inosine	8.34 ± 0.69	7.88 ± 1.01	0.052	0.042
hypoxanthine	16.59 ± 3.48	14.26 ± 1.69	0.448	0.63
xanthine	1.72 ± 0.14	1.70 ± 0.11	0.117	0.12
uric acid	0.42 ± 0.10	0.36 ± 0.06	0.115	**
guanosine	2.06 ± 0.12	2.15 ± 0.3	0.017	0.016
2′-deoxyadenosine	7.58 + 0.51	*	0.017	0.015
Pyrimidines				
uracil	8.52 ± 8.57	11.45 ± 3.22	0.122	**
uridine	3.36 ± 0.26	3.40 ± 0.24	0.076	**
cytidine	0.57 ± 0.13	0.54 ± 0.06	0.011**	
2-deoxycytidine	0.53 ± 0.03	0.53 ± 0.04	0.108	0.124
2′-deoxyuridine	3.08 ± 0.20	3.21 ± 0.22	0.106	**
thymidine	1.40 ± 0.11	1.36 ± 0.16	0.025**	

* Not detectable at 280 nm at appropriate concentration.
** Not detectable with MS at appropriate concentrations.

Table 3
Comparison of extracellular concentrations of thalamus with cerebrospinal fluid results and where data are available, with previously reported striatal data. The thalamus and cerebrospinal fluid data are the average of data on 13 and 7 animals, respectively

	Cerebrospinal fluid (μM)	Thalamic extracellular space (μM)	Striatal extracellular space* (μM)
Purines			
adenosine	0.09±0.01	0.95±0.04	1.92±0.35
inosine	0.14±0.005	0.52±0.03	1.50±0.17
hypoxanthine	3.78±0.27	4.48±0.25	2.82±-0.43
xanthine	1.31±.0.08	1.17±0.08	3.95±0.47
uric acid	0.96±0.06	1.15±0.13	
guanosine	0.02±0.004	0.17±0.02	
2′-deoxyadenosine	0.008±0.002	0.17±0.04	
Pyrimidines			
uracil	2.66±0.27	1.22±0.22	
uridine	3.83±0.40	0.76±0.04	
cytidine	0.51±0.05	0.11±0.01	
2′-deoxycytidine	7.35±0.43	1.08±0.06	
2′-deoxyuridine	4.82±0.19	1.06±0.05	
thymidine	0.76±0.02	0.25±0.02	

* Hagberg *et al.*, 1987.

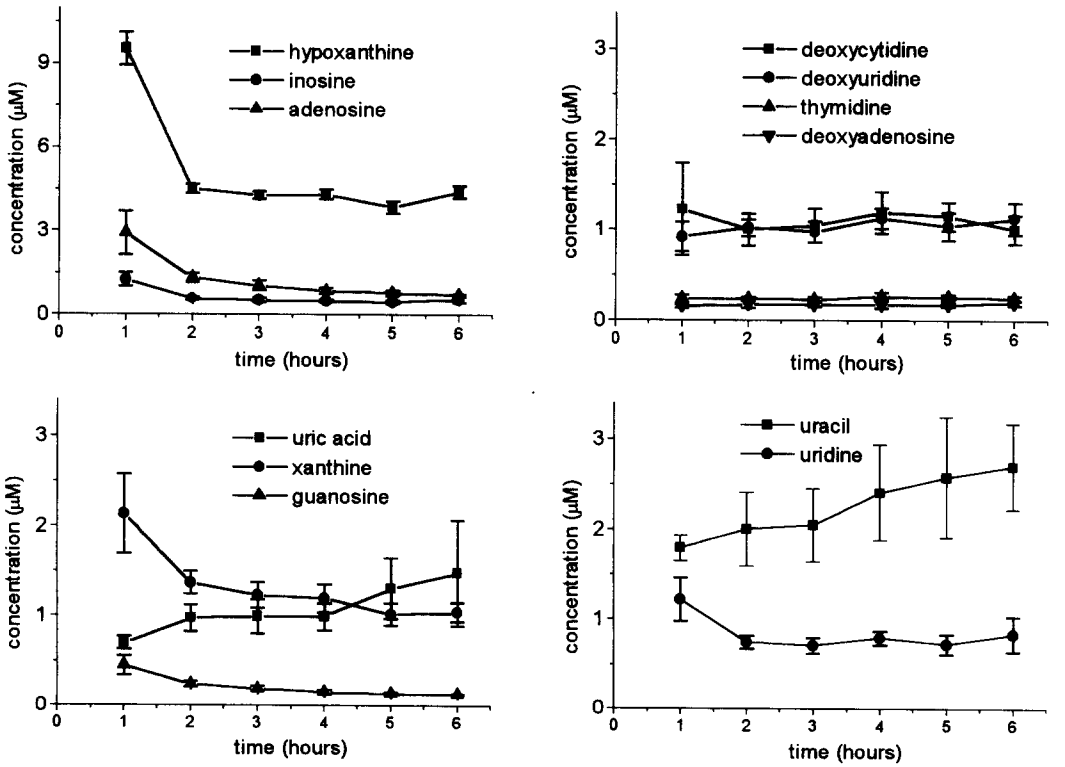

Fig. 3. Rates of change in concentrations in control acute microdialysis experiments measured in 13 rats. The end of implantation was considered to be 0 h and the calculated extracellular thalamic concentration for the first hour is indicated at 1 h, and so on. The compounds are grouped as adenosine and its direct metabolites, other purines, deoxyribonucleosides and pyrimidines. The Y axis time scale for adenosine and related metabolites is different from that for the others.

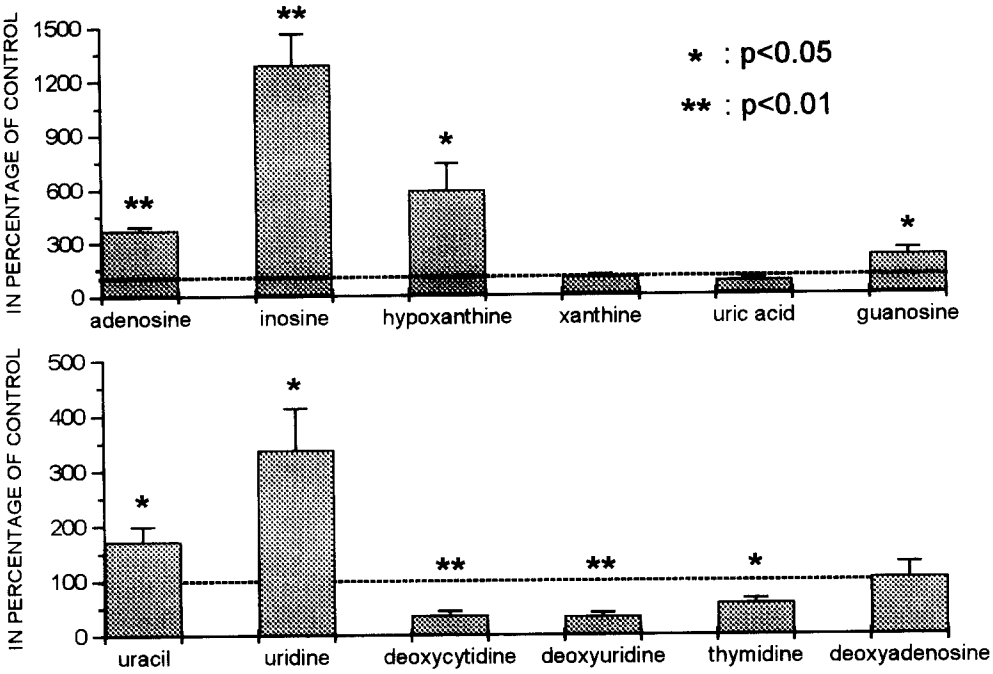

Fig. 4. *Post mortem* concentration changes measured in the first hour after overdosing halothane. 100% represents the average of the *pre mortem* data. Values are averages ±SEM of data on 5 animals.

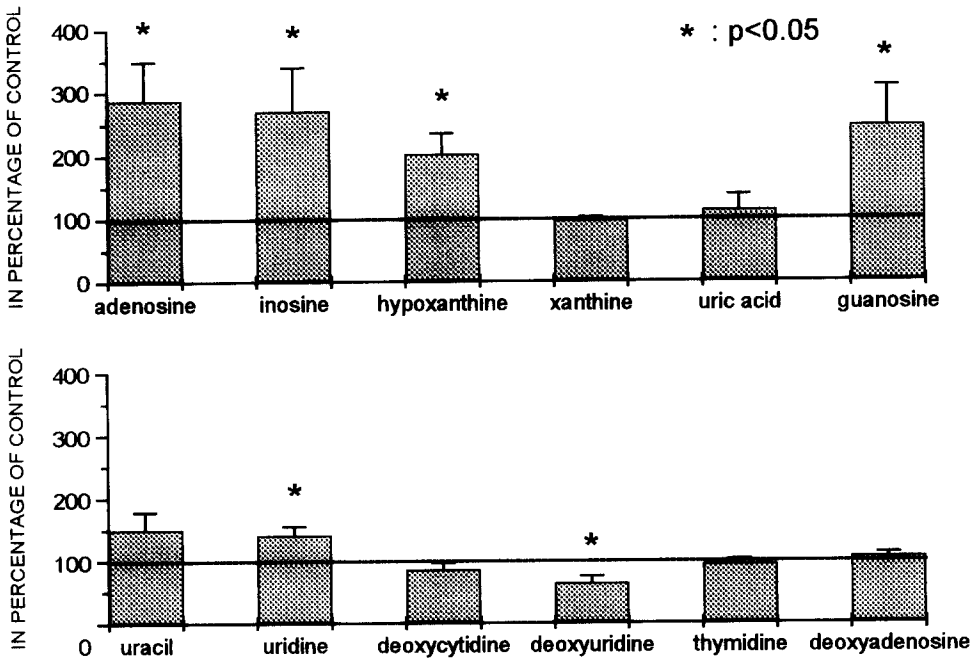

Fig. 5. The effect of perfusing 120 mM K^+-containing ACSF through the probe. Data obtained after perfusion with 120 mM K+ are expressed as percentages of baseline levels. Baseline levels are calculated as the averages for the samples 2 and 3 h after implantation of the probe. Values are averages ±SEM of data on 4 animals.

are diluted even relative to the extracellular space (Benveniste and Huttemeier, 1990). On the other hand, the microdialysis technique provides a unique way to measure local extracellular concentrations. Furthermore, dialysis samples are relatively clean and can easily be analysed by HPLC, since the samples can be injected directly into the chromatograph without any clean-up procedure (Benveniste and Huttemeier, 1990). The analy-

sis of purine bases and purine ribonucleosides in *in vivo* brain microdialysis samples has become a widespread technique during the past decade, and has helped towards an understanding of the roles of adenosine and some other purines in physiological and pathological brain processes, such as the regulation of transmitter release, blood supply and hypoxia of various brain areas (Hagberg *et al.*, 1987; Wang *et al.*, 1992; Kaku *et al.*, 1994). Since the involvement of other nucleosides in brain functions came into the attention, there has been a definite need for the measurement of other purine and pyrimidine nucleosides and deoxynucleosides in restricted brain regions *in vivo*. The simultaneous measurement of these compounds can be particularly important, for they are often converted into each other, and their enzymes and transporters are often not entirely specific (Carrera *et al.*, 1994; Kraupp and Marz, 1994; Staub *et al.*, 1994). The very low detection limits (Table 1) and high reproducibility make the new method well suited for measurements on small amounts of biological samples and/or diluted samples such as microdialysates and micropunches. With the microdialysis technique, we first report the interstitial concentrations of many deoxynucleosides and pyrimidine nucleosides in the brain, *viz.* uridine, 2′-deoxyuridine, uracil, cytidine, 2′-deoxycytidine, thymidine, thymine, 2′-deoxyadenosine and 2′-deoxyguanosine. Some other compounds, the purines, have already been detected in microdialysis samples in the striatum but not in the thalamus, (Ballarin *et al.*, 1987; Hagberg *et al.*, 1987). The concentrations are of the same order of magnitude and the slight discrepancies might arise from regional differences. There is a difference between the two areas in adenosine receptor distribution; for example, A_2 receptors are abundant in the striatum, but are absent from the thalamus (Dixon *et al.*, 1996).

The marked difference between the interstitial space and the CSF nucleoside compositions (Table 3) indicates that little information can be obtained concerning the local extracellular concentrations of these compounds by measuring them in the CSF. High concentrations of pyrimidine nucleosides and deoxynucleosides in the CSF may be a consequence of their significant active transport through the choroid plexus (Spector, 1989). Conversely, purines which reach the brain cells predominantly through the blood–brain barrier (Spector, 1989), exhibit lower levels in the CSF than in the interstitial space. Theoretically, the higher purine concentrations in the extracellular space as compared to the CSF could be a consequence of tissue damage caused by the insertion of microdialysis probe. However, it is not likely because the major drop in extracellular space purine concentrations is over by the second hour after the introduction of probes as discussed below. Furthermore uridine, a compound showing elevated level after the insertion of probe, had a higher concentration in CSF as compared to extracellular space.

Microdialysis experiments

In acute microdialysis experiments in rats, the concentrations of the examined compounds in the VPL/VPM nuclei of the thalamus were measured 7 h after the implantation of microdialysis probes for seven hours. Elevated concentrations of many compounds reported in the first hour (Fig. 3) are presumably a consequence of the tissue damage (Benveniste and Huttemeier, 1990). From the second hour after implantation, the baseline was stabilized for each compound. Thus, the concentrations of substances in the interstitial space and also the changes in the concentrations following particular treatments can be determined from this period.

The effect of depolarization of the neurones on the nucleoside concentration was tested by perfusing 120 mM K^+-containing solution in acute experiments. This K^+ concentration in the perfusate results in a concentration of at least about 30–35 mM in the region around the probe, which is high enough to depolarize the cells. The elevated levels of adenosine and its metabolises can be explained by its known neuromodulator activity (Williams, 1990; Pazzagli *et al.*, 1993) however, increased levels of guanosine and uridine were also observed in our experiment (Fig. 5). Although metabolic effects provide an explanation of this result, it could also be consistent with a possible neuromodulatory role of some of the examined compounds. The same compounds were released *post mortem* (Fig. 4) and they exhibited high tissue concentrations, suggesting the presence of large intracellular pools.

In conclusion, a new chromatographic method was worked out for the synchronous analysis of endogenous purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides in brain microsamples. Animal experiments demonstrating the applicability and consequence of the method are also reported.

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