Determination of rat cerebrospinal fluid concentrations of adenosine, inosine, hypoxanthine, xanthine and uric acid by high performance liquid chromatography

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Abstract—Isocratic reverse-phase high performance liquid chromatography techniques were developed to resolve and quantitate the purine nucleosides adenosine (Ado) and inosine (Ino) and their metabolites hypoxanthine (Hyp), xanthine (Xan), and uric acid (UA) in the cerebrospinal fluid of the rat. The moving phase composition for resolving hypoxanthine, xanthine and uric acid was a 0·22 M, pH 5·8 phosphate buffer. The moving phase composition for resolving adenosine and inosine was a 0·22 M, pH 6·8 phosphate buffer, 7% methanol (v/v) and 2·5 mM tetrabutylammonium phosphate. The observed cerebrospinal fluid concentrations in the rat were: Ado = 35 ± 9 nM (s.e.m.), Ino = 359 ± 85 nM, Hyp = 243 ± 77 nM, Xan = 1340 ± 423 nM and UA = 6130 ± 678 nM.

Our laboratory has been investigating the postulated adenosinergic regulation of cerebral blood flow (Berne et al 1974; Winn et al 1981; Phillis et al 1984) by studying the release of adenosine and its metabolites in rat cerebral cortex. Adenosine (Ado), inosine (Ino), hypoxanthine (Hyp), xanthine (Xan) and uric acid (UA) are the products of purine nucleotide catabolism, with uric acid being the principal endproduct in primates and the next to last metabolite in other mammals, including the rat, in which uric acid is further oxidized to allantoin. Cerebrospinal fluid (CSF) provides a convenient source of brain perfusate samples, which have equilibrated with cerebral interstitial fluid, in which to monitor the levels of extracellular adenosine and its metabolites. CSF concentrations of inosine, hypoxanthine and xanthine have been demonstrated to have clinical value as a measure of human foetal hypoxia (Harkness & Lund 1983).

Rat CSF concentration for adenosine and its metabolites were measured to provide basal values with which follow-up studies on the effects of hypoxia could be compared. The most striking aspect of our results is that the concentrations of uric acid were an order of magnitude higher than the other purine metabolites in rat CSF. This is presumably due to the accumulation of uric acid as a product of the purine catabolic chain. Two separate, moving phase high performance liquid chromatographic systems were developed for this study; one of which was capable of resolving hypoxanthine, xanthine and uric acid and the other of resolving adenosine and inosine. These systems were derived, with modifications, from earlier methods used to study nucleosides in other biological systems (Simmonds & Harkness 1981; Ingebretsen et al 1982).

Materials and methods

Animal preparation. Adult male Sprague-Dawley rats were anaesthetized with halothane and, after tracheal cannulation, were maintained under anaesthesia using methoxyflurane in air. The occipital bone was exposed surgically and a small hole was made in the atlanto-occipital membrane through which a PE-50 tube was inserted into the cisterna magna. CSF was allowed to drip from the tube into a series of sequentially numbered polypropylene microvials at room temperature. The average CSF flow rate for all of the animals used was 3·0 ± 0·27 µL min⁻¹ (n = 7).

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HPLC procedures. Individual 100 µM stock standard solutions of adenosine, inosine, hypoxanthine, xanthine and uric acid were prepared from weighed reference chemicals (Sigma Chemical Co.), volumetrically diluted in HPLC grade water (J. T. Baker). The pH of the hypoxanthine and xanthine standard solutions was adjusted to pH 8·0 with 0·2 M KOH to ensure complete dissolution of these substances. All HPLC injections were performed using Hamilton syringes (10, 25 or 100 µL).

Moving phase buffers were prepared from 0·22 M solutions of reagent grade monobasic potassium phosphate (KH₂PO₄) and dibasic sodium phosphate (Na₂HPO₄). These solutions were mixed in a volume ratio of either 901 : 99 monobasic to dibasic phosphate to produce a buffer solution of pH 5·8 for use in resolving hypoxanthine, xanthine and uric acid or in a ratio of 446 : 554 monobasic to dibasic phosphate to produce a buffer of pH 6·8 for use in resolving adenosine and inosine.

The chromatographic system consisted of Waters Associates: M6000A pump, U6K injector, RCM-100 radial compression module with Resolve C18 5 µm stationary phase cartridge, 450 absorbance detector set at a detection wavelength of 260 nm and a Spectra-Physics 4270 integrator. The CSF samples collected from the animals were centrifuged (1200 g, 5 min, 4°C) and the supernatants injected directly into the HPLC system without extraction or deproteination. To protect the system from clogging with particulate matter, a Waters In-Line filter with 0·2 µm pore size and a Waters Guard-Pak with Resolve C18 precolumn insert were incorporated into the HPLC system upstream of the stationary phase column.

The two separate moving phase compositions were: 0·22 M pH 5·8 phosphate buffer alone, to resolve hypoxanthine, xanthine and uric acid and 0·22 mM, pH 6·8 phosphate buffer, 7% (v/v) methanol (J.T. Baker) and 2·5 M tetrabutylammonium phosphate (Waters PIC-A) to resolve adenosine and inosine. Both moving phases were degassed by filtration under vacuum (Millipore type HA) before use. Individual standard curves for adenosine, inosine, hypoxanthine, xanthine and uric acid were constructed using known amounts of standard versus the observed chromatographic peak areas or heights. Injection volumes were 50 µL of CSF for hypoxanthine, xanthine and uric acid determinations and 100 µL for adenosine and inosine measurements. Substance identification was by retention time. Confirmation of substance identity was obtained by enzymatic degradation using adenosine deaminase, nucleoside phosphorylase, xanthine oxidase or uricase (Sigma Chemical Co.; Hartwick et al 1979). The minimum detectable amount of adenosine and inosine was 1·0 pmol which with a 100 µL injection volume corresponds to a minimum detection concentration of 10 nM. The minimum detectable concentration of hypoxanthine, xanthine or uric acid was 100 nM.

Results

The chromatograms, Figs 1, 2 illustrate the nearly baseline resolution of the substances of interest achieved using the HPLC parameters described in the procedures section. Fig. 1A, B shows the resolution of hypoxanthine, xanthine and uric acid in an aqueous standard mixture and in a rat CSF-sample. Fig. 2A, B shows the
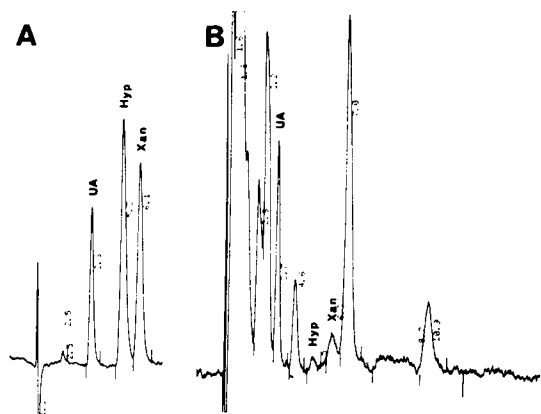
FIG. 1. HPLC chromatograms of a hypoxanthine, xanthine and uric acid aqueous standard mixture (A) and of a rat cerebrospinal fluid sample (B). Moving phase composition was 0·22 M, pH 5·8 phosphate buffer with a flow rate of 2·0 mL min⁻¹.
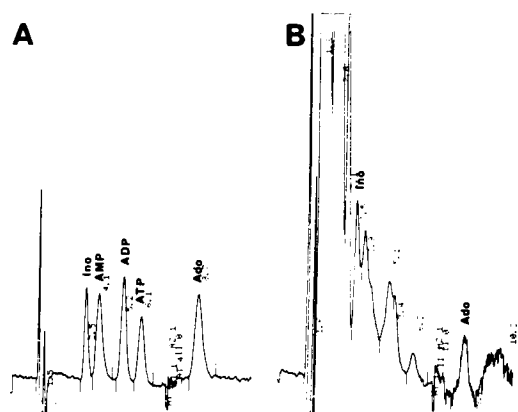

FIG. 2. HPLC chromatograms of an inosine, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate and adenosine aqueous standard mixture (A) and of a rat cerebrospinal fluid sample (B). Moving phase composition was 0·22 M, pH 6·8 phosphate buffer with 7% (v/v) methanol and 2·5 mM tetrabutylammonium phosphate with a flow rate of 2·0 mL min⁻¹. The recorder amplification was increased ×4 before the elution of adenosine in B.

resolution of adenosine and inosine in an aqueous standard solution and in a rat CSF sample.

The mean rat CSF purine concentrations determined and shown in Table 1 are: Ado = 35±9 nM, In. = 359±85 nM, Hyp =

Table 1. Adenosine, inosine, hypoxanthine, xanthine and uric acid concentrations in rat cerebrospinal fluid collected from the cisterna magna.

Purine	No. of Samples	Range (nM)	Mean ± s.e.m. (nM)
Adenosine	19	10–120	35±9
Inosine	18	10–1100	359±85
Hypoxanthine	10	100–840	243±77
Xanthine	10	100–3600	1340±423
Uric acid	10	2600–8800	6130±678

243±77 nM, Xan = 1340±423 nM and UA = 6130±678 nM. To verify that adenosine was not being catabolized by endogenous degradation enzymes in the CSF, exogenous adenosine was added to aliquots of pooled CSF and incubated for 5, 10, 20 or 40 min at 37°C followed by injection into the HPLC. There was no appreciable decrease in adenosine concentration in any of these samples, indicating that there was no adenosine deaminase or other metabolizing enzymes present in the CSF.

Discussion

The values for rat CSF adenosine and its metabolites observed in this study are comparable with other published mammalian CSF concentrations: rabbit CSF Ado = 100 nM (Eells & Spector 1983); piglet CSF Ado = 40 nM and Ino = 1·05 μM (Park et al 1987); human CSF Ado = 40 nM (Sollevi 1986); human CSF Ino = 200 nM, human CSF Hyp = 1800 nM; human CSF Xan = 1700 nM (Harkness & Lund 1983) and dog CSF UA = 9500 nM (Kim et al 1987).

It was interesting to find that xanthine and uric acid represented the bulk of purine nucleotide derivatives present in CSF. The formation of these compounds is catalysed by the enzyme xanthine oxidase which promotes the conversion of hypoxanthine to xanthine, and xanthine to uric acid. This finding was unexpected in that xanthine oxidase activity is reportedly present in relatively low amounts in brain (Parks & Granger 1986), although higher levels of activity have been found in brain capillaries (Betz 1985). The results suggest that uric acid may be the form in which adenosine is removed from the brain.

The reactions catalysed by xanthine oxidase could be of importance during cerebral ischaemia, when purine nucleotides are metabolized and hypoxanthine and xanthine accumulate. With reperfusion, molecular oxygen and substrate can react with xanthine oxidase to form superoxide anions and hydrogen peroxide, both of which have been implicated in the tissue damage associated with various pathological conditions, including cerebral ischaemia (Chan et al 1982; Demopoulos et al 1982). Increases in uric acid levels in the rat brain following experimentally induced ischaemia have been documented (Kanemitsu et al 1986). The data presented in this report would be in agreement with the suggestion that sufficient xanthine oxidase activity is available in the brain to metabolize hypoxanthine to xanthine and uric acid and to thus contribute to oxygen-free radical formation during cerebral ischaemia.

In summary, HPLC systems have been developed for the separations of adenosine and its metabolites in rat cerebrospinal fluid. Xanthine and uric acid were present in the highest concentrations, with lower levels of adenosine, inosine and hypoxanthine.

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The emetic activity of centrally administered cisplatin in cats and its antagonism by zacopride

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Abstract—Cisplatin administered by either the intravenous (i.v.) or intra-cerebroventricular (i.c.v.) route produced emesis in cats. The average time to onset of emesis was decreased significantly (4·0 min versus 100·6 min) when cisplatin was administered i.c.v. Zacopride administered either i.c.v. (0·02 mg) or i.v. (0·1 mg kg^{-1}) completely blocked the emesis due to cisplatin given by either route. Their data show that cisplatin possesses a central emetic component and that this is blocked by zacopride.

McCarthy & Borison (1984) demonstrated in cats that the emesis due to i.v. cisplatin could be prevented by lesion of the area postrema. Alphin et al (1986a) recently proposed that the activity of metoclopramide and dazopride against cisplatin-induced emesis was due to their gastrokinetic properties. These results in addition to those obtained by Akwari (1983) suggest that cisplatin possesses a peripheral mechanism in inducing emesis. Zacopride is a potent gastrokinetic and antiemetic agent devoid of dopamine blocking properties (Smith et al 1986; Alphin et al 1986b). The present study will show that, in cats, cisplatin produces emesis when administered i.c.v. and that zacopride administered i.c.v. or i.v. blocks the emetic response.

Materials and methods

Adult mongrel cats, of either sex, between 2·5 and 3·0 kg, were used.

In Study 1, cats were injected i.v. with cisplatin (7·5 mg kg^{-1}) and observed for 5 h for the time to onset and number of emetic episodes (expulsion of vomitus). In Study 2, cats were anaesthetized with halothane. A 25-gauge stainless-steel cannula was placed into the 4th cerebral ventricle using a David Kopf stereotaxic apparatus. The animals were allowed to recover for 24 h. After recovery each animal was dosed with cisplatin (0·3 mg in 0·1 mL) or saline (0·1 mL) into the 4th ventricle. Each animal was then observed for 5 h for time to onset and number of emetic episodes. In Study 3, cats were dosed either i.v. (7·5 mg kg^{-1}) or i.c.v. (0·3 mg) with cisplatin. In those animals dosed i.v. with cisplatin, zacopride (0·02 mg in 0·1 mL) was administered i.c.v. immediately after the first emetic episode. In those animals dosed i.c.v. with cisplatin, zacopride (0·1 mg kg^{-1}) was administered i.v. 15 min before the cisplatin dose.

For the studies with cannulas placed in the 4th ventricle, the location of the cannula was confirmed by passing a 1 MV current through the cannula for 30 s after the animals were killed by

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lethal injection. The brains were removed and placed in a potassium ferricyanide solution. The cannula track was stained blue by this procedure and was readily visualized upon dissection of the brain.

Cisplatin was obtained from Sigma Chemical Company and prepared for injection by adding 70°C deionized water to volume. The resulting solutions (3 mg mL^{-1} for i.c.v. and 7·5 mg mL^{-1} for i.v.) were maintained at 40°C until administration. Solutions were freshly prepared, immediately before use. Zacopride, A. H. Robins, Company, Inc., was prepared for injection by adding deionized water to volume. The resulting solutions (0·2 mg mL^{-1} for i.c.v. and i.v.) were prepared immediately before use.

Results

Table 1 gives the results seen when cisplatin was administered alone either i.v. or i.c.v. Cisplatin given i.v. produced emesis with a relatively long time to onset when compared with cisplatin given i.c.v. Saline given either i.v. or i.c.v. did not produce emesis.

When zacopride was administered i.c.v. immediately following the onset of emesis from i.v. cisplatin, no further emesis occurred. When zacopride was administered i.v. 15 min before i.c.v. cisplatin, no emesis occurred (Table 2).

Discussion

The mechanism of action for emesis produced by cisplatin is unclear. After intravenous or intraperitoneal injection, high levels of cisplatin are found in the gastrointestinal tract, and cisplatin is known to produce marked gastrointestinal effects in addition to nausea and vomiting (Pretorius et al 1981). Akwari (1983) as well as Alphin et al (1986a) suggest that emesis due to cisplatin is caused by a peripheral action, specifically an action on the gastrointestinal tract. McCarthy & Borison (1984) found that cats with lesions of the area postrema, the site of the chemoreceptor trigger zone, failed to develop emesis after i.v. cisplatin (7·5 mg kg^{-1}). This finding suggests a central site for the emetogenic activity of cisplatin. The finding in this study that emesis occurs only after a considerable time delay following i.v. injection and immediately after i.c.v. injection would seem consistent with the concept of a direct gastrointestinal effect. Cisplatin does not readily cross the blood brain barrier, but does readily accumulate in the intestinal tract (Rosenberg 1985). The delay in onset of emetic effects after i.v. administration could reflect the time required to reach a central site of action. That site may be located in the area postrema. Our findings as well as those of McCarthy & Borison (1984) would seem to support the