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## Note

# Simultaneous quantitative high-performance liquid chromatographic determination of cyclic adenosine 3′,5′-monophosphate and adenosine in mouse brain after microwave irradiation: application to isoprenaline effect in vivo

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Cyclic adenosine 3′,5′-monophosphate (cAMP) is a second messenger that mediates cellular response to receptor stimulation [1]. Adenosine (A) is described as a neuromodulator [2,3]. Several methods for the determination of cAMP content in biological samples use high-performance liquid chromatography (HPLC) with UV [4], radiolabelled [5] or fluorescent derivative (etheno-cAMP) [6] detection. Other HPLC methods have been reported for the determination of A in biological samples with UV [7,8] or a fluorescent derivative (etheno-A) [9,10] or radiolabelled [5] detection. Since there are interactions between cAMP and A metabolism [2,3,11], we were interested in developing a simultaneous, quantitative determination of these two compounds from experiments in vivo.

The HPLC–UV method reported here provides a rapid and simple procedure for the simultaneous quantitation of cAMP and A in mouse brain after microwave irradiation, since it has been shown to be one of the best techniques to minimize post-mortem changes of A and cAMP levels [10,12]. We have used the isoprenaline $\beta$ agonist administration to mice, which has been shown to influence the brain level of cAMP in rats [13], to study the concomitant variations of the concentration of A in the brain.

## EXPERIMENTAL

### *High-performance liquid chromatographic system*

An M590 solvent-delivery system with a U6K injector, a Nova-Pak ($C_{18}$; 4 $\mu$m) column (15 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.), a UV de-

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tector (Philips) set at 254 nm and 0.01 a.u.f.s., and a dual-trace recorder (10 and 200 mV) were used. The mobile phase consisted of 20 m*M* $KH_2PO_4$ and 9% (v/v) methanol. The pH was adjusted to 3.5 by addition of an 85% $H_3PO_4$ solution, and the mobile phase was then filtered and degassed under vacuum through a 0.45-$\mu$m Millipore membrane filter. The HPLC flow-rate was set at 0.5 ml/min. The standard compounds tested were cAMP (Sigma, St. Louis, MO, U.S.A.), A (Sigma) and inosine (Sigma); they were dissolved in the mobile phase at a concentration of 1 $\mu$mol/l and filtered through a 0.45-$\mu$m filter. The injection volume was 25 $\mu$l. It was found important to condition a new Nova-Pak column with acetonitrile (20 column volumes) to obtain the separations shown in Fig. 1. cAMP and A were identified in biological samples during HPLC analysis on the basis of their retention times and co-chromatography with reference compounds; the identity of the cAMP and A peaks was confirmed by the enzymic peak shift following treatment with cyclic nucleotide phosphodiesterase (EC 3.1.4.17) (Boehringer Mannheim, Mannheim, F.R.G.) and adenosine deaminase (EC 3.5.4.4) (Sigma), respectively. The pH of the brain extract solution was adjusted to between 7.0 and 7.5, and an aliquot (20 $\mu$l) of the enzyme solution as obtained from

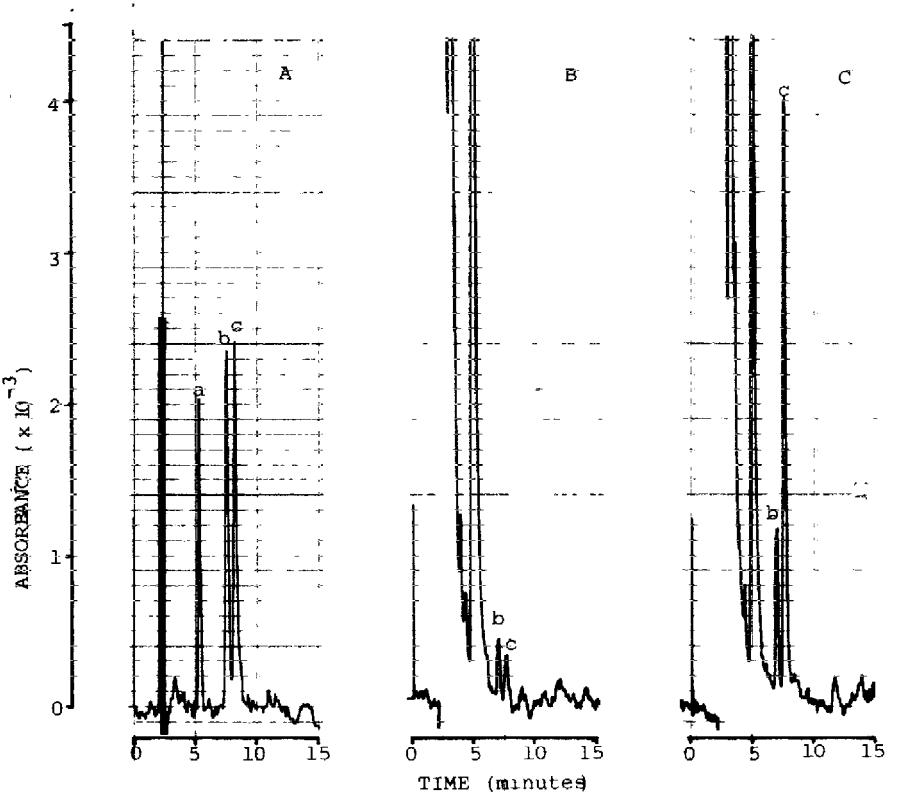


Fig. 1. (A) Chromatogram of the standard 1 $\mu$*M* inosine (a), cAMP (b) and adenosine (c) in a 25-$\mu$l injection (B) Chromatogram of a control mouse brain extract. (C) Chromatogram of an isoprenaline-treated (400 mg/kg, intraperitoneally, 10 min before sacrifice) mouse brain extract Experimental procedure described in text.

the manufacturer was directly added to the brain extract (100 μl). Incubation (15 min; 25°C) was stopped by heating in boiling water for 30 s.

*Animals*

Male mice (30–35 g) were obtained from Iffa Credo (Saint-Germain sur l'Arbresle, France). Before use, they were kept for one week in standard laboratory conditions. Isoprenaline (Sigma) was administered (intraperitoneally, 0.1 ml per 10 g body weight) after dissolution in saline, 10 min before sacrifice.

*High-power focused microwave sacrifice*

The mouse was first immobilized in a glass cylinder inserted into the microwave waveguide. The head was irradiated at 2450 MHz for 0.8 s using 3.5 kW forward power. Following irradiation, the head was cooled in liquid nitrogen and the brain was carefully taken out and immediately frozen (−70°C).

*Sample preparation*

Brains were weighed, homogenized [5] with an Ultra-Turrax in 1.8 ml of 0.1 *M* NaOH, and then kept for 30 min at room temperature before addition of 0.6 ml of 0.15 *M* $ZnSO_4$. Samples were then centrifuged at 10 000 *g* for 20 min at 4°C, the supernatants were filtered through a 0.45-μm filter and stored frozen (−70°C). A 25-μl aliquot of each sample was injected into the HPLC system.

## RESULTS

*HPLC elution profiles*

Fig. 1A shows a typical chromatogram after injection (25 μl) of a mixed solution of standard compounds (1 μmol/l each). The retention times are: inosine (peak a), 5.2 min; cAMP (peak b), 7.5 min; A (peak c), 8.1 min. For the three

TABLE I

ADENOSINE AND cAMP CONCENTRATIONS IN MOUSE BRAIN 10 MIN AFTER INTRAPERITONEAL ISOPRENALINE ADMINISTRATION

Values represent the mean ± S.E.M. ($\sqrt{s^2/n}$, where $s^2$ is the variance of the mean and $n$ the number of animals).

| Treatment | *n* | Concentration (nmol/g wet brain tissue) | |
|---|---|---|---|
| | | Adenosine | cAMP |
| Saline | 7 | 1.3 ± 0.3 | 1.25 ± 0.3 |
| Isoprenaline | | | |
| 100 mg/kg | 5 | 3.7 ± 1.3 | 2.0 ± 0.15 |
| 200 mg/kg | 6 | 6.0 ± 2.4 | 2.4 ± 0.35[a] |
| 400 mg/kg | 6 | 24.2 ± 9.6[a] | 4.2 ± 0.8[b] |

[a]Significantly different from saline-injected animals (Student's *t*-test, $p < 0.05$).
[b]Significantly different from saline-injected animals (Student's *t*-test, $p < 0.01$).

compounds, the response curves were linear from 0.25 to 25 μmol/l for a 25-μl injection volume. Fig. 1B shows a chromatogram obtained from a 25-μl injection of a control mouse brain extract; cAMP (peak b) and A (peak c) are well resolved, but inosine cannot be determined because it is not resolved from unknown compounds. Fig. 1C shows a chromatogram of an isoprenaline-treated mouse brain extract; the peak height of cAMP and A are clearly augmented. Values are given in Table I, where it is shown that isoprenaline, 10 min after its intraperitoneal administration to mice, causes a significant increase of brain cAMP and A.

## DISCUSSION

The method reported here provides a simple and rapid procedure to study variations of cAMP and A levels in mouse brain after in vivo administration of pharmacological agents. This paper describes the simultaneous determination with UV detection of cAMP and A levels in mouse brain extracts after microwave irradiation. The use of UV detection of cAMP and A circumvents the need for the synthesis of fluorescent derivatives, and the results are directly given with the HPLC profile, whereas with the use of radioactively labelled compounds it might be necessary to collect the eluted fractions containing the compound to be quantified. UV detection is convenient for laboratories that are not equipped to work with labelled compounds. Nevertheless, because the lowest limit of detection for the two compounds is 2.5 pmol per 25-μl injection from a 1 nmol/l solution, UV detection is less sensitive than fluorescence detection of etheno-A (0.5 pmol per 2 ml [9] and 0.1 pmol per 20 μl [10]) and than radioimmunoassay of cAMP (0.1 pmol per regular assay and 3 fmol per acetylated assay [13]). However, the UV detector used here does not allow a better sensitivity than 0.01 a.u.f.s., and it should be possible with a better one to improve sensitivity up to 0.001 a.u.f.s., making this method as sensitive as the one using fluorescence detection. The concentration of cAMP in control mouse brain, as determined by the procedure described here, is in good agreement with reported values [12].

Kant et al. [13] reported that the cAMP brain level response either (a) results from the stimulation of β-adrenergic receptors located in the brain capillaries because only a small percentage of administered isoprenaline is taken up by the brain, or (b) is caused indirectly by the isoprenaline-induced peripheral effects. We confirm here that intraperitoneal administration of isoprenaline increases the cAMP brain level, and it is interesting to observe the simultaneous increase of the A level. A can be formed from ATP or cAMP via AMP dephosphorylation [3,14] or derived from hydrolysis of S-adenosylhomocysteine [14]. A is also produced in ischaemic tissue (heart [14], brain [15]). These results, however, do not explain the increase in the level of A, which will require further investigation.

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