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Note

Quantitative high-performance liquid chromatography of bases and nucleosides in cerebral DNA of rat foetus

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Recently, the analysis of DNA constituents has progressed rapidly with the use of high-performance liquid chromatography (HPLC). The latest advances in the HPLC analysis of nucleotides, nucleosides and bases in biological fluids and tissues were reviewed by Zakaria and Brown [1]. HPLC has been applied to the determination of the relationship between modified nucleoside levels in biological fluids and the disease status in patients [2] in clinical studies and to the detection of the methylated bases in the liver DNA of animals treated with carcinogens [3]. For the brain, Heizmann et al. [4] determined the deoxyribonucleoside composition of DNA from cortex neurons of foetal and postnatal rats by HPLC after digestion with a combination of nucleic acid-degrading enzymes. There are no other reports on the analysis of DNA constituents in the developing brain. It is well known that faulty DNA metabolism in some heritable disorders or imperfect repair of environmentally induced damage to DNA can lead to an increased risk of progressive degeneration of the developing brain. This paper describes the application of reversed-phase HPLC to the quantitative analysis of base and nucleoside compositions in cerebral DNA isolated from rat foetuses.

EXPERIMENTAL

Apparatus

All studies were conducted with an Atto (Tokyo, Japan) Model SF 0709 high-speed liquid chromatograph with an Atto UV monitor II. The injection

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valve was a Rheodyne (Cotati, CA, U.S.A.) Model 7125 with a 100-μl fixed-volume sample loop. A Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} reversed-phase column (300 × 3.9 mm I.D., particle size 10 μm) was used, preceded by a Whatman (Clifton, NJ, U.S.A.) Co:Pell ODS guard column. The peaks were identified by the addition of the corresponding standard to the hydrolysates, but the standard was not added in the quantitative analysis of samples. The areas of indistinct peaks were divided vertically and all peaks were integrated by a System Instruments (Tokyo, Japan) Model 5000E integrator.

Chemicals

Adenine (Ade), cytosine (Cyt), guanine (Gua), thymine (Thy), deoxyadenosine (dAdo), deoxycytidine (dCyd), deoxyguanosine (dGuo), deoxythymidine (dThd), deoxyinosine (dIno), deoxyribonuclease (DNase I, E.C. 3.1.4.5), snake venom phosphodiesterase (PDase I, E.C. 3.1.4.1) and calf thymus DNA (Type I) were purchased from Sigma (St. Louis, MO, U.S.A.). Alkaline phosphatase (APase, grade I, E.C. 3.1.3.1) was obtained from Boehringer (Mannheim, F.R.G.). Theobromine (Thb), xanthine (Xan), acetonitrile (chromatographic grade), acetone (UV grade), hydrochloric acid (super special grade) and all other chemicals of analytical-reagent grade were obtained from Wako (Osaka, Japan).

Calibration standards

All base and nucleoside stock solutions except for those of dGuo and Gua were prepared at a concentration of 10 m*M*; dGuo stock solution was prepared at 5 m*M* by adding 0.01 *M* phosphate buffer, pH 5.65 (buffer A), and Gua was resolved in a small amount of 1 *M* sodium hydroxide and finally adjusted to 10 m*M* with buffer A. These stock solutions were diluted to each concentration accurately and used as calibration standards. The range of calibration standards used in this study was decided depending on the level in the hydrolysates. Thb as an internal standard was prepared at a concentration of 2 m*M* by adding buffer A, and 4 μl of this solution were injected together with 96 μl of a standard or sample.

Sample preparation

Cerebral DNA was isolated from rat foetuses on gestational day 21 using the methods of Lφvtrup-Rein and McEwen [5] and Marmur [6] with minor modifications. The acidic and enzymatic hydrolysis of cerebral DNA were performed by the methods of Sharma and Yamamoto [7] and Breter et al. [8] with minor modifications. A 0.5-mg amount of freeze-dried DNA was hydrolysed in 0.5 ml of 6 *M* hydrochloric acid for 3 h at 100°C for base analysis and in 1.0 ml of an enzyme mixture (DNase I, 100 U/ml; PDase I, 0.0019 U/ml; APase, 0.05 U/ml) for 4 h at 37°C for nucleoside analysis. Enzymatic hydrolysates were precipitated with 5 vols. of acetone and centrifuged at 7700 *g* for 10 min, and the supernatants were removed. Both hydrolysates were evaporated to dryness under a stream of nitrogen at 60°C, and the residues were dissolved in 0.5 ml of buffer A for the acidic hydrolysate and 1.0 ml of buffer A for the enzymatic hydrolysate and then filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A.).

Chromatographic conditions

A 100-μl volume of a sample or standard mixture was injected and eluted with a linear gradient of acetonitrile to 10% in buffer A after 30 min. The flow-rate was set at 1.0 ml/min and the column effluent was monitored at 254 nm.

TABLE I

PRECISION OF THE ASSAY FOR THE ACIDIC HYDROLYSATE OF FOETAL CEREBRAL DNA

Compound	Concentration (nmol/ml)	Mean ± S.D.* (nmol/ml)	C.V.** (%)	Recovery (%)
Cytosine	31.25	36 ± 3	8.1	113.6
	62.5	64 ± 4	6.1	102.0
	125	132 ± 5	4.0	105.4
	250	257 ± 5	2.1	103.0
Thymine	37.5	38 ± 3	7.4	101.3
	75	72 ± 6	8.2	96.0
	150	145 ± 5	3.5	96.3
	300	292 ± 12	4.0	97.2
Guanine	31.25	45 ± 3	6.4	145.3
	62.5	71 ± 5	6.4	113.2
	125	131 ± 14	10.6	104.8
	250	267 ± 6	2.1	106.9
Adenine	37.5	38 ± 2	5.3	101.3
	75	71 ± 5	6.7	94.0
	150	153 ± 5	3.3	101.7
	300	305 ± 8	2.7	101.7

*Each value represents the mean ± S.D. for four samples assayed at each compound concentration.

**C.V. denotes coefficient of variation.

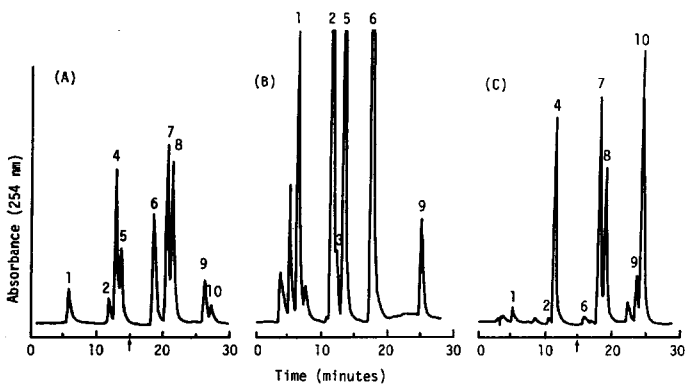

Fig. 1. Chromatograms of bases and nucleosides (A) in the standard mixture, (B) in the acidic hydrolysate and (C) the enzymatic hydrolysate of cerebral DNA. The chromatographic conditions are given in the text. Peaks: 1 = Cyt; 2 = Gua; 3 = Xan; 4 = dCyd; 5 = Thy; 6 = Ade; 7 = dGuo; 8 = dThd; 9 = Thb; 10 = dAdo. The arrows show the time of switching the sensitivity range from 0.05 to 0.10 a.u.f.s.

The sensitivity range used was usually 0.05 a.u.f.s. and switched to 0.10 a.u.f.s. after 15 min in the separation of the enzymatic hydrolysate. Chromatography was carried out at ambient temperature.

RESULTS AND DISCUSSION

The separation of the eight standard bases and nucleosides is shown in Fig. 1A. Fig. 1B and C show chromatograms of acidic and enzymatic hydrolysates

TABLE II

PRECISION OF THE ASSAY FOR THE ENZYMATIC HYDROLYSATE OF FOETAL CEREBRAL DNA

Compound	Concentration (nmol/ml)	Mean ± S.D.* (nmol/ml)	C.V.** (%)	Recovery (%)
Cytosine	3.75	—	—	—
	7.5	7.6 ± 1.6	2.1	101.7
	15	15.3 ± 0.5	3.3	101.7
	30	30.3 ± 0.5	1.7	100.8
	60	60.0 ± 2.3	3.8	100.0
Guanine	1.25	1.1 ± 0.3	25.5	90.0
	2.5	2.3 ± 0.3	14.9	89.0
	5	5.9 ± 0.3	5.1	117.5
	10	9.4 ± 1.3	14.0	93.5
Deoxycytidine	37.5	38 ± 3	6.8	101.3
	75	71 ± 4	6.1	94.7
	150	147 ± 3	2.1	97.7
	300	298 ± 14	4.8	99.3
Deoxythymidine	50	53 ± 11	20.7	106.0
	100	95 ± 7	7.9	95.0
	200	187 ± 6	3.2	93.5
	400	381 ± 17	4.4	95.2
Adenine	3.75	3.7 ± 0.3	6.9	97.4
	7.5	6.8 ± 0.2	2.4	89.7
	15	13.9 ± 0.6	4.0	92.4
	30	28.8 ± 1.2	4.3	95.8
	60	—	—	—
Deoxyguanosine	37.5	44 ± 7	16.0	117.4
	75	78 ± 5	6.1	104.0
	150	161 ± 6	3.7	107.0
	300	329 ± 22	6.6	109.5
Deoxyadenosine	50	50 ± 3	6.9	100.0
	100	95 ± 6	6.3	94.5
	200	195 ± 4	2.1	97.5
	400	391 ± 32	8.1	97.7

*Each value represents the mean ± S.D. for four samples assayed at each compound concentration.

**C.V. denotes coefficient of variation.

of cerebral DNA, respectively. Heizmann et al. [4] assayed dAdo as dIno with addition of excess of adenosine deaminase because the commercial preparation of APase contained small amounts of adenosine deaminase, which converted dAdo to dIno. In our experiments, the peak of dIno would be virtually negligible because the APase level was very low (0.05 U/ml).

In order to determine the linearity and limits of detection of the present quantitative HPLC method, we made three non-consecutive injections of each of the base and nucleoside mixtures. The responses for bases and nucleosides were found to be linear, with correlation coefficients of 0.997—1.000. The limits of detection in the present analysis were 2.5 nmol/ml for Gua, 7.5 nmol/ml for Ade, 15 nmol/ml for Cyt, 75 nmol/ml for Thy, dCyd and dGuo and 100 nmol/ml for dThd and dAdo. These values for bases and nucleosides were slightly higher than the reported limits [9] because of the detection of some small modified peaks.

The coefficients of variation (C.V.) and recoveries of standard mixtures added to acidic and enzymatic hydrolysates of prepared foetal cerebral DNA are shown in Tables I and II. Base and nucleoside levels showed good precision, C.V. of 2.1—10.6% and recoveries of 89.7—113.6%, except for Gua in the enzymatic hydrolysate and at the lowest concentrations of Gua, dGuo and dThd. These unauthentic data were partly due to the low level of Gua, the interference of Xan with the Gua peak and the indistinct separation of dGuo and dThd. Consequently, it is possible to determine the base and nucleoside levels of a sample within the authentic range. Hence the present method has the advantage that all DNA bases and nucleosides can be identified and determined under the same HPLC conditions, although the limits of detection are not so low. It is thought that this method is useful for clarifying the quantitative and qualitative changes of DNA base and nucleoside compositions in the developing brain of animal models for brain dysfunction.

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