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Analysis of 6*R*- and 6*S*-tetrahydrobiopterin and other pterins by reversed-phase ion-pair liquid chromatography with fluorimetric detection by post-column sodium nitrite oxidation

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ABSTRACT

A rapid and sensitive reversed-phase ion-pair liquid chromatographic system with fluorimetric detection by post-column sodium nitrite oxidation was established for measuring six pterin compounds (6*R*-5,6,7,8-tetrahydrobiopterin, 6*S*-5,6,7,8-tetrahydrobiopterin, 7,8-dihydrobiopterin, biopterin, pterin and D-neopterin). The coefficients of variation for these pterins were 0.705–3.714%, and the minimum detectable amount was *ca.* 10–20 pg at a signal-to-noise ratio of 3. A linear detector response was also verified. The concentrations of the pterin compounds in rat tissues were measured by the described method. Furthermore, by means of brain microdialysis, the output of pterin compounds from rat striatum was detected. Therefore, these results demonstrate that this system can be applied to analyses not only of various rat tissues but also of dialysates collected *in vivo*.

INTRODUCTION

Among various pterins, 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (*R*-THBP) has attracted much interest in recent years. It has been demonstrated that *R*-THBP regulates, in part, the activities of phenylalanine, tyrosine and tryptophan monooxygenases. The two latter enzymes catalyse the rate-limiting reactions in the synthesis of catecholamines and serotonin, respectively [1]. In addition, recent reports have suggested that *R*-THBP enhances dopamine and serotonin release in the rat striatum and frontal cortex by microdialytic perfusion [2,3] and systemic administration [4] of *R*-THBP. Therefore, it has been proposed that *R*-THBP regulates the release of biogenic amines as well as their biosynthesis. Decreased *R*-THBP levels in the cerebrospinal fluid (CSF) of Parkinson's [5] and Alzheimer's [6] patients have been reported. On the other hand, therapeutic effects of *R*-THBP have also been demonstrated in atypical phenylketouria (malignant hyperphenylalaninemia, PKU) [7], Parkinsonian's [8,9], depression [10] and infantile autism [11]. Therefore, *R*-THBP, neopterin and other pterins should be measured simultaneously in body fluids and tissues by a simple and rapid method, to diagnose such patients and to develop basic research.

Pterin compounds in biological samples have been measured by means of the Fukushima and Nixon method [12] of chemically converting reduced pterins by iodine (I_2) oxidation. However, this method requires a long time for sample preparation. Other direct methods have been adopted

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to determine levels of both oxidized and reduced pterins, using liquid chromatography (LC) with electrochemical detection (ED) [13,14] and/or fluorimetric detection (FD) [15,16]. Recently, Suzuki and Owada [16] reported a simple post-column method with which to determine pterins directly by means of sodium nitrite ($NaNO_2$) oxidation. However, it also requires lengthy separation times and is insufficiently sensitive. In this study, we improved the post-column $NaNO_2$ oxidation method using a reversed-phase ion-pair LC system, and demonstrated the usefulness of this method in measuring brain microdialysis samples.

EXPERIMENTAL

Chemicals

6*S*-L-*erythro*-5,6,7,8-Tetrahydrobiopterin sulphate (*S*-THBP) and 7,8-dihydrobiopterin (BH_2) were purchased from Dr. B. Schircks (Jona, Switzerland). 2-Amino-4-hydroxypteridine (pterin), 2-amino-4-hydroxy-6-(1,2-dihydropropyl)pteridine (biopterin) and D-(+)-neopterin (neopterin) were from Sigma. 6*R*-L-*erythro*-5,6,7,8-Tetrahydrobiopterin dihydrochloride (*R*-THBP), launched in 1992 for atypical phenylketouria therapy, was synthesized at Suntory.

LC equipment

A schematic diagram of the LC system (Shimadzu, Japan) is shown in Fig. 1. It consisted of two pumps (LC-6A), a system controller (SCL-6B), an autoinjector (SIL-6B), a Cosmosil 5C18 reversed-phase column (250 × 4.6 mm I.D., Nacalai Tesque, Japan) with a Cosmosil 5C18 guard column (50 × 4.6 mm I.D., Nacalai Tesque) coupled with a fluorimetric detector (RF-535). The excitation wavelength was 350 nm, and the emission wavelength was 440 nm. The temperature of the column and reaction coil were set at 40°C and 80°C, respectively, using two column ovens (CTO-6A, Shimadzu). The concentration of pterins was determined by calculating the areas under the curves using an integrator (C-R4AX, Shimadzu).

Mobile phase

The mobile phase was 0.1 *M* sodium phosphate buffer (pH 3.0) containing 5% (v/v) methanol, 3 m*M* sodium octylsulphate, 0.1 m*M* disodium EDTA and 0.1 m*M* ascorbic acid (to prevent oxidation). The flow-rate was maintained at 1.0 ml/min. The mobile phase was filtered through a 0.45-μm membrane filter (Millipore) and then degassed under vacuum before use.

Oxidation of reduced pterins

Reduced pterins were oxidized according to the method of Suzuki and Owada [16] with some modifications. After separating the pterins on the reversed-phase column, 7,8-dihydrobiopterin (BH_2), *R*- and *S*-THBP, which are not naturally fluorescent, were oxidized to their fluorescent compounds by $NaNO_2$ (5 m*M*, flow-rate, 1.0 ml/min) in the reaction coil (80°C).

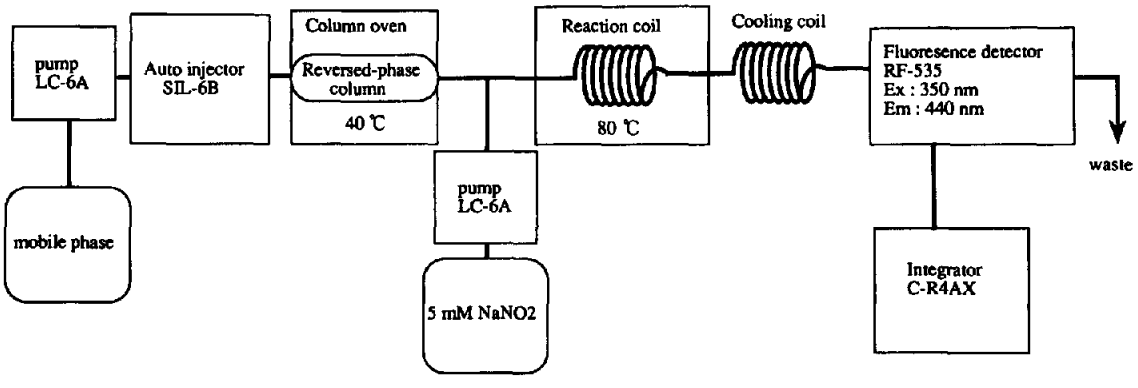

Fig. 1. Post-column LC–FD system.

Preparation of standard solutions

Standard solutions of *R*-THBP, *S*-THBP, BH_2, biopterin, pterin and neopterin (1.0 mg/ml each) were prepared in 0.5 *M* perchloric acid (PCA) containing 0.1 m*M* disodium EDTA and 0.1 m*M* $Na_2S_2O_3$.

Animal studies

Fischer-344 male rats (8 weeks of age, body weight 190–210 g, supplied by Charles River Japan) were used. After the rats had been decapitated, the kidney (one side), a part of the liver and the brain were quickly removed. The brain was dissected on an ice-cold glass plate into six regions, *i.e.* cortex, striatum, hippocampus, diencephalon, cerebellum and brainstem, as described by Glowinski and Iversen [17]. The tissues were homogenized in 1.0–3.0 ml of 0.5 *M* PCA containing 0.1 m*M* disodium EDTA and 0.1 m*M* $Na_2S_2O_3$. After centrifugation (1700 *g*, 15 min), the supernatant was filtered through a 0.45-μm membrane filter and then directly injected into the LC system in 10–100 μl volumes. The samples were prepared and the pterins were assayed within 24 h.

To study microdialysis in freely moving rats, a guide cannula (CMA12, Carnegie Medicin) was implanted sterotaxically into the right striatum. The coordinates were: anterior from the bregma, −0.2 mm; lateral to the midline, 3.0 mm; horizontal below the skull surface, 4.5 mm (according to the brain atlas of Paxinos and Watson [18]). Animals were allowed 1 day to recover prior to intrastriatal microdialysis. The microdialysis probe (CMA12, Carnegie Medicin), covered by a dialysis membrane 3.0 mm in length and 0.5 mm in diameter, was perfused with Ringer's solution (Na^+, 147; K^+, 4; Ca^{2+}, 4.5; Cl^-, 155.5 mequiv., pH 6.4) at 2.0 μl/min using a microinjection pump (CMA100, Carnegie Medicin). The perfusates were collected every 30 min into 10 μl of 0.5 *M* PCA, 0.1 m*M* disodium EDTA and 0.1 m*M* $Na_2S_2O_3$. After the microdialysis study had been completed, the site of the dialysis probe was verified histologically.

RESULTS AND DISCUSSION

Measurement of pterins

Fig. 2A shows an LC–FD chromatogram obtained after injecting a standard mixture containing 500 pg of *R*-THBP, *S*-THBP, BH_2, biopterin, pterin and neopterin. These pterins were completely baseline-separated. The chromatograms obtained after injecting the diencephalon and liver are shown in Fig. 2B and C. In this LC–FD system, six different pterins were separated and measured, including *S*-THBP and neopterin. However, neither *S*-THBP nor neopterin was detectable in rat tissues. These results were consistent with previous studies, in which *S*-THBP could not be detected in biological materials [14] and neopterin was not normally present in significant amounts in rat tissue [19]. According to the report of Duch *et al.* [20], the neopterin level was very low in rat tissues, but relatively high in monkey and human tissues and fluids [20].

$NaNO_2$ concentration and reaction coil temperature

The oxidation conditions that influenced the quantitation of the reduced pterin compounds were examined. To determine the optimum concentration of $NaNO_2$ to oxidize the reduced pterin compounds, we examined four different concentrations (2, 5, 10, 20 m*M*) of $NaNO_2$ solution. $NaNO_2$ had no influence on neopterin, biopterin and pterin measurements, because these compounds fluoresce naturally. The BH_2 peak was virtually unchanged by any concentration of $NaNO_2$ solution (2–20 m*M*). However, the *R*- and *S*-THBP peaks were the highest when the concentration of $NaNO_2$ was 5 m*M* (Fig. 3). Increasing the concentration of $NaNO_2$ to 10 or 20 m*M* was found to slightly decrease *R*- and *S*-THBP peaks (data not shown).

Fig. 4 shows the effects of the reaction coil temperature on the oxidation of the reduced pterins. The peak areas of neopterin, biopterin and pterin were not affected by temperature. The reduced pterin, BH_2, was oxidized by $NaNO_2$ even at room temperature (23°C). However, it was found that the oxidation of both *R*- and *S*-THBP re-

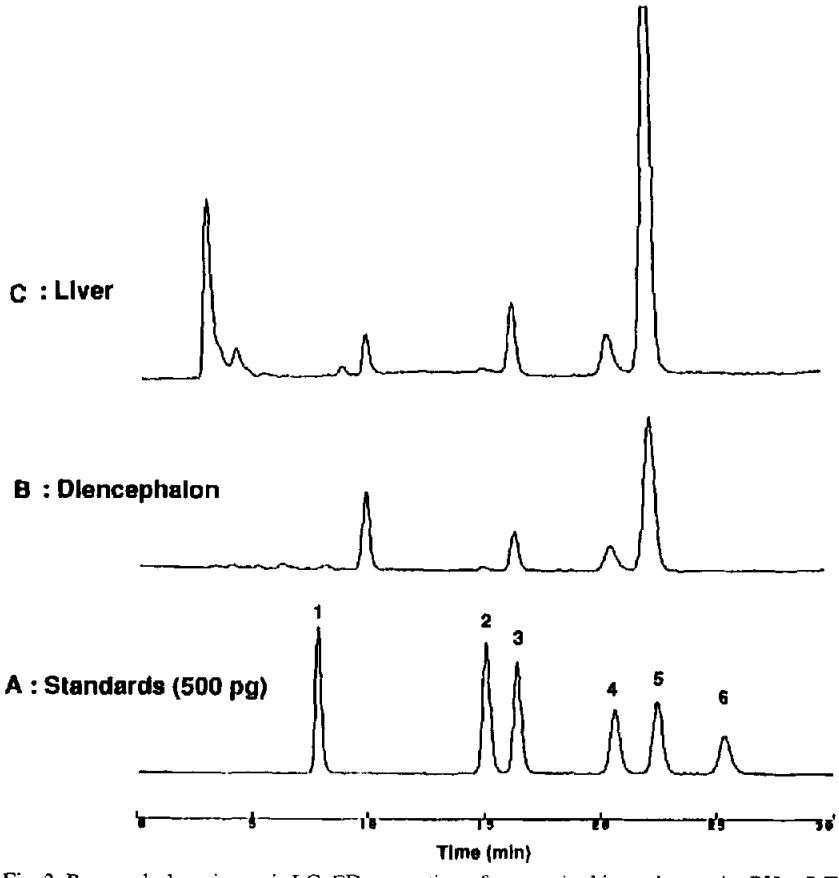

Fig. 2. Reversed-phase ion-pair LC–FD separation of neopterin, biopterin, pterin, BH_2, *R*-THBP and *S*-THBP: (A) standard mixture (500 pg each); (B) rat diencephalon (50 μl injected); (C) rat liver (10 μl injected). Peaks: 1 = neopterin; 2 = biopterin; 3 = pterin; 4 = dihydrobiopterin (BH_2); 5 = 6*R*-tetrahydrobiopterin (*R*-THBP); 6 = 6*S*-tetrahydrobiopterin (*S*-THBP).

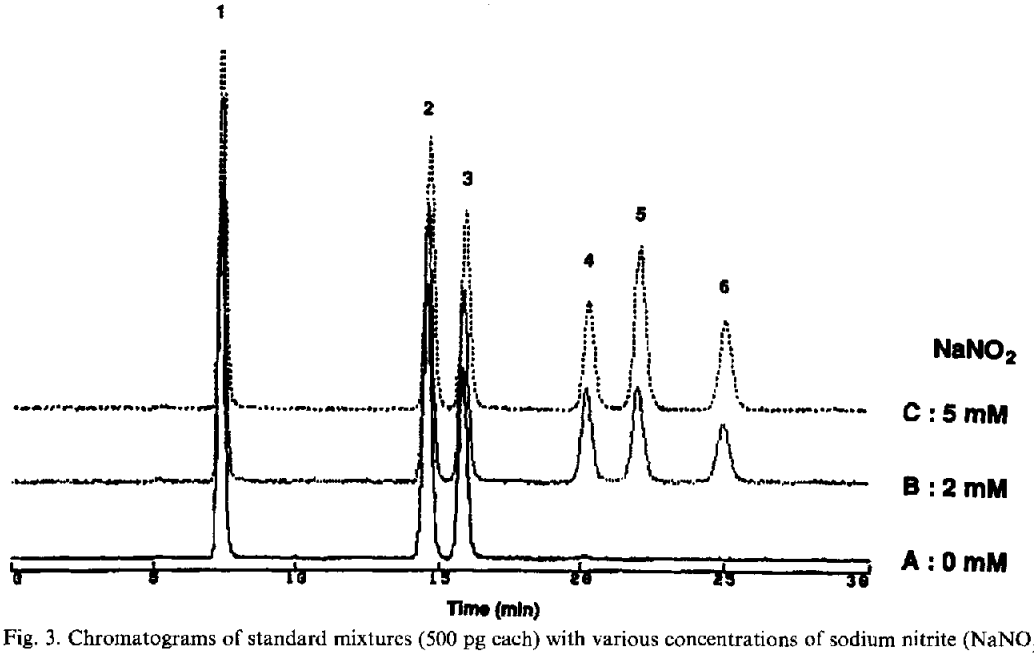

Fig. 3. Chromatograms of standard mixtures (500 pg each) with various concentrations of sodium nitrite ($NaNO_2$): (A) 0 m*M*; (B) 2 m*M*; (C) 5 m*M*. The flow-rate of the $NaNO_2$ solution was 1.0 ml/min, and the reaction coil temperature was maintained at 80°C. Peak identities as in Fig. 2.

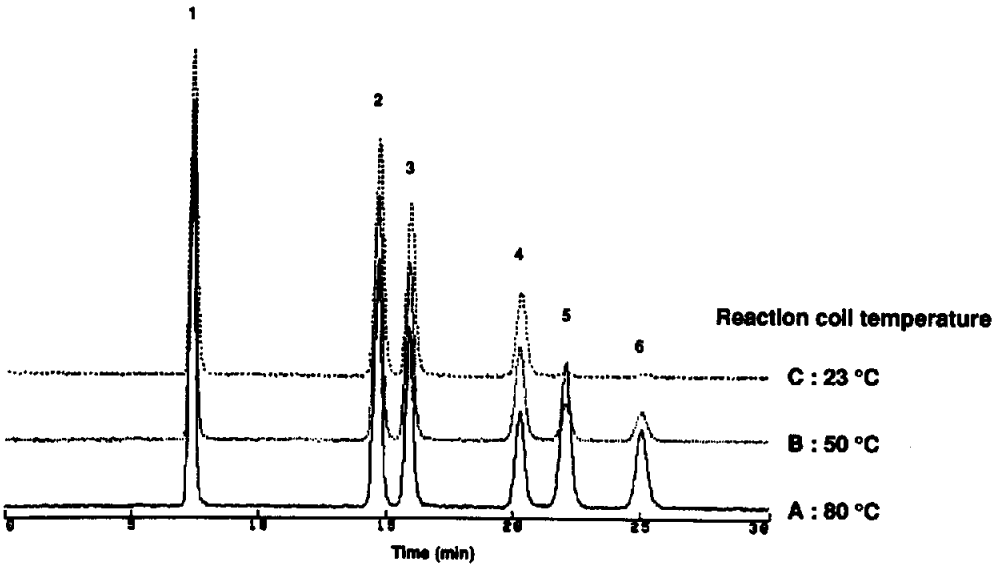

Fig. 4. Chromatograms of standard mixtures (500 pg each) at different reaction coil temperatures: (A) 80°C; (B) 50°C; (C) 23°C. The flow-rate and $NaNO_2$ concentration were 1.0 ml/min and 5 m*M*, respectively. Peak identities as in Fig. 2.

quires high temperatures. These results demonstrate that severe conditions are necessary to oxidize *R*- and *S*-THBP as compared with those for BH_2.

Precision of the assay

The detector response for pterin compounds was found to be linear in the range for 20 pg to 5 ng. The correlation coefficients for pterin compounds were 0.993–1.000. With the rat brain samples, the limits of detection for biopterin and pterin were *ca.* 10 pg, and those for BH_2 and *R*-THBP were *ca.* 20 pg at a signal-to-noise-ratio (S/N) of 3. The within-assay coefficients of variation (C.V.) for the peak area of pterin compounds were 0.705–3.714% (calculated from the

TABLE I

DISTRIBUTION OF PTERIN COMPOUNDS IN VARIOUS BRAIN REGIONS, THE KIDNEY AND THE LIVER OF YOUNG ADULT (EIGHT WEEKS OF AGE) FISCHER-344 RATS

Tissue	Concentration (mean ± S.D., n = 3) (ng/g tissue)					
	Neopterin	Bioterin	Pterin	BH_2	*R*-THBP	*S*-THBP
Cortex	N.D.[a]	0.55 ± 0.37	10.37 ± 0.32	16.41 ± 0.67	62.89 ± 2.32	N.D.
Hippocampus	N.D.	3.76 ± 0.50	69.14 ± 6.84	50.98 ± 2.03	102.81 ± 4.86	N.D.
Striatum	N.D.	6.15 ± 0.47	60.07 ± 2.20	104.01 ± 9.56	695.86 ± 98.40	N.D.
Diencephalon	N.D.	1.32 ± 0.30	32.45 ± 2.12	31.23 ± 1.60	176.75 ± 6.81	N.D.
Cerebellum	N.D.	0.72 ± 0.24	61.17 ± 14.57	21.89 ± 0.40	89.30 ± 8.94	N.D.
Brainstem	N.D.	1.79 ± 0.17	45.68 ± 6.82	32.76 ± 0.95	110.00 ± 3.59	N.D.
Kidney	N.D.	1.33 ± 0.31	90.56 ± 16.04	101.79 ± 30.87	235.76 ± 22.91	N.D.
Liver	N.D.	16.67 ± 2.37	357.03 ± 46.65	261.66 ± 21.00	2883.22 ± 172.37	N.D.

[a] N.D. = not detectable.

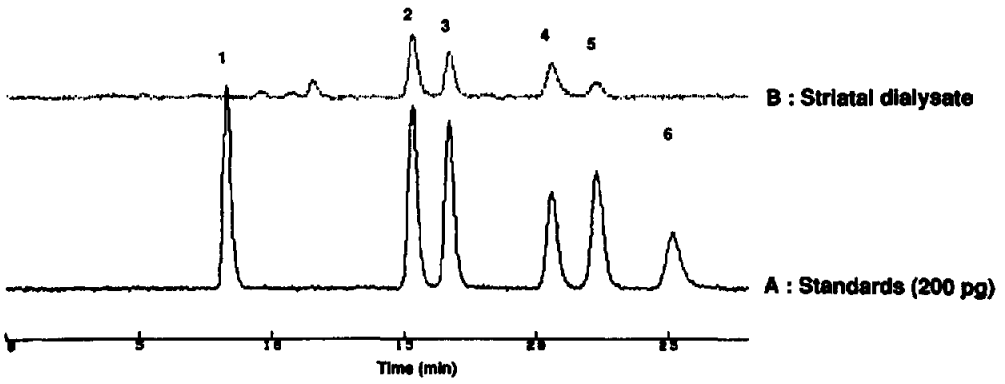

Fig. 5. Reversed-phase ion-pair LC–FD separation of pterin compounds: (A) standard mixture (200 pg each); (B) rat striatal dialysate (50 μl injected). The perfusate was collected at 2.0 μl/min every 30 min in a small tube containing 10 μl of 0.5 *M* PCA, 0.1 m*M* disodium EDTA, 0.1 m*M* $Na_2S_2O_3$ and 6 m*M* ascorbic acid. A dialysis probe was implanted into the striatum of a freely moving rat. Peak identities as in Fig. 2.

peak area for standard solution injections, $n = 6$). Analytical recoveries of pterin compounds added to rat striatal homogenates ranged from 84.7% to 99.6%.

Concentration of pterins in rat tissues

The concentrations of pterins in various tissues are shown in Table I. As previously reported by Baker *et al.* [21], the major pterin in rat tissues was *R*-THBP. The highest level of *R*-THBP was observed in the liver. In the rat brain, the level of *R*-THBP was the highest in the striatum, followed by the diencephalon, brainstem, hippocampus, cerebellum and cortex; the levels of BH_2 and pterin were rather high in the hippocampus and striatum. As reported previously, neopterin

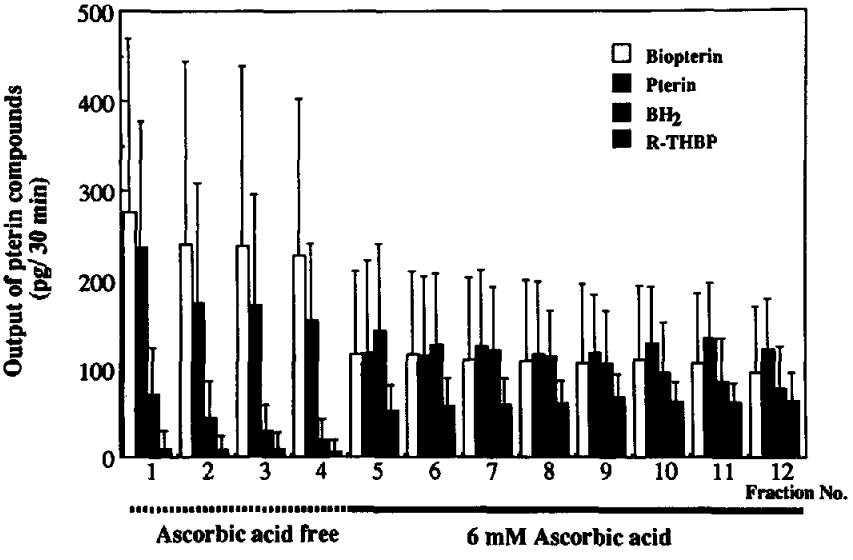

Fig. 6. Effect of ascorbic acid added to a sample of pterin compounds from rat striatal dialysates. The perfusates were collected every 30 min in a small tube containing 10 μl of 0.5 *M* PCA, 0.1 m*M* disodium EDTA, 0.1 m*M* $Na_2S_2O_3$ without (dotted line) or with (solid line) 6 m*M* ascorbic acid. The final concentrations of PCA and ascorbic acid were 0.07 *M* and 0.86 m*M*, respectively. Each column represents the mean ± S.D. (pg/30 min, $n = 6$).

and *S*-THBP were not detected in any rat tissues. The level of biopterin in all tissues examined was very low compared with those of other pterin compounds.

Pterin compound levels in rat striatal dialysates

A representative chromatogram obtained after injection of a dialysate from rat striatum is shown in Fig. 5B. Small amounts of *R*-THBP, BH_2, biopterin and pterin were detected in intrastriatal dialysate, but neopterin and *S*-THBP were not. Fig. 6 shows the effect of ascorbic acid on the level of pterin compounds in striatal dialysates. When 10 μl of 0.5 *M* PCA containing 0.1 m*M* disodium EDTA and 0.1 m*M* $Na_2S_2O_3$ were added to a sampling tube, biopterin, pterin and a little BH_2 and *R*-THBP were detected. After the addition of 6 m*M* ascorbic acid to 10 μl of 0.5 *M* PCA solution, the biopterin and pterin peaks decreased and those of BH_2 and *R*-THBP markedly increased. These results indicate that ascorbic acid (final concentration, 0.86 m*M*) prevents the oxidation of reduced pterins in a sampling tube. Since these results are preliminary, further studies are required to clarify the optimal conditions for preventing oxidation during microdialysis.

CONCLUSION

A sensitive and simple method for measuring of pterin compounds was established by means of reversed-phase ion-pair LC–FD. This method will be applicable to the assay of various biological samples, even when the volume is small. Furthermore, microdialysis in combination with this sensitive method can measure the levels of endogenous intrastiatal pterin compounds. Therefore, this method will be useful in clinical diagnosis and for basic studies of pterins.

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