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High-performance liquid chromatographic measurement of cerebrospinal fluid tetrahydrobiopterin, neopterin, homovanillic acid and 5-hydroxindoleacetic acid in neurological diseases

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Abstract

Cerebrospinal fluid (CSF) samples from patients with a variety of neurological disorders were assayed to determine the concentrations of tetrahydrobiopterin (BH_4), the active cofactor of hydroxylases. Dihydroneopterin (NH_2) and neopterin (N), which are linked with BH_4 synthesis and are inflammatory biochemical markers, were also measured simultaneously in a number of patients. 5-Hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA), the main products of serotonin and dopamine breakdown, were analyzed in parallel whenever possible. As BH_4 and NH_2 are difficult to analyze owing to their instability, CSF samples were collected under special conditions to preserve the reduced BH_4 and NH_2. Liquid chromatographic assays and detection of the various substances measured also required particular precautions. BH_4 concentrations were elevated in patients with neurological disorders such as syphilis and lupus-like disease and especially in an AIDS patient with neurological complications with an increased N/BH_4 ratio.

1. Introduction

Tetrahydrobiopterin (BH_4), a reduced biopterin, is the common cofactor for phenylalanine, tyrosine and tryptophan hydroxylases, which catalyze the synthesis of the neurotransmitters dopamine, norepinephrine, epinephrine, and serotonin. It is synthesized from dihydroneopterin triphosphate [1] that breaks down into dihydroneopterin (NH_2) and its fully oxidized form neopterin (N) in biological fluids. BH_4 is also the cofactor for nitric acid synthase which produces nitric oxide, a candidate neurotransmitter [2]. In the brain, BH_4 is thought to reflect the rate of neurotransmitter synthesis, which is decreased in both the brain and cerebrospinal fluid (CSF) of patients with Parkinson's disease [3,4]. *In vitro*, NH_2 and N are released from macrophages stimulated with interferon gamma, a product of activated T cells [5]. Some authors have proposed total neopterin as a biochemical marker of central nervous system inflammation [4], and simultaneous determination of BH_4, N, and NH_2 in CSF should be useful in clinical chemistry. The assays described in the literature [6,7] were not suitable for our system and in the

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present paper we describe the modifications made.

CSF samples from patients with neurological disorders were assayed to determine these pterins, and 5-hydroxyindolcacetic acid (5-HIAA) and homovanillic acid (HVA), the main products of serotonin and dopamine breakdown were assayed in parallel. As BH_4 and NH_2 are difficult to analyze owing to their instability, CSF samples were collected under special conditions to preserve the reduced BH_4 and NH_2. Liquid chromatographic assays and detection of the various substances measured also required particular precautions, as discussed below.

2. Experimental

2.1. Reagents and chemicals

(6R)-BH_4 and NH_2 were obtained from Dr. Schircks Labs (Jona, Switzerland). 3N, 5-HIAA, and 3-methoxy-4-hydroxyphenylethyleneglycol hemipiperazate (MHPG) were from Sigma (Coger, Paris, France) and HVA from Merck (Elvetec, Nice, France).

2.2. Tetrahydrobiopterin, dihydroneopterin and neopterin assays

BH_4, NH_2, and N were assayed by high-performance liquid chromatography (HPLC). BH_4 was detected by electrochemical and N by fluorimetric detection. NH_2 was measured with the same HPLC system by fluorescence detection after electrochemical oxidation at a sufficiently high potential according to the modified method of Howells *et al.* [6]. As illustrated in Fig. 1, BH_4 was detected by an ESA Coulochem electrochemical detector (Touzart and Matignon, Paris, France); detector 1 (D1): +0.50 V (gain 10 × 99), detector 2 (D2): −0.10 V (gain 10 × 99). As NH_2 cannot be detected by fluorescence, the effluent of electrode 2 was oxidized by application of 1 V by an auxiliary cell by the electrochemical detector. Native N and N from NH_2 (separated on the analytical column) were detected by fluorescence with a highly sensitive fluorimeter (DFL, Société Grégoire Service, Cap d'Ail, France and Société Cortiula, Saint-Raphaël, France). The detector was equipped with a high-pressure mercury vapor lamp; the excitation filter selected the bands between 340 and 370 nm, and a set of high-transmission filters centered between 430 and 470 nm allowed emission selection. The arc of the lamp was stabilized to keep background noise low.

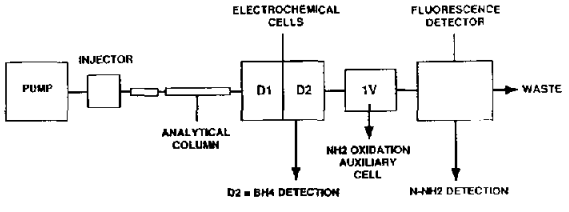

Fig. 1. Equipment configuration.

The mobile phase consisted of 6.8 g/l sodium acetate and 1.05 g/l citric acid (pH 5.20) containing 20 mg/l disodium ethylenediaminetetraacetic acid (EDTA) and 150 mg/l dithioerythritol (DTE) (Sigma). The mobile phase was filtered through a 0.2-μm filter (Elvetec, Nice, France) and deaerated before and during use by continuous bubbling with dry nitrogen gas. The column was an Ultrasphere ODS 5 μm (250 × 4.6 mm I.D.) (Beckman, Gagny, France).

2.3. Homovanillic acid and 5-hydroxyindoleacetic acid assays

HVA and 5-HIAA were measured by HPLC using an ESA Coulochem electrochemical detector with the following settings: detector 1: +0.30 V, detector 2: +0.44 V. The mobile phase consisted of 6.8 g/l monopotassium phosphate buffer (pH 4.0)–methanol (77:13, v/v). HVA and 5-HIAA were separated on an Ultrasphere ODS 5 μm column (250 × 4.6 mm I.D.).

2.4. Standard solutions

Standard solutions were prepared in millimolar concentrations. To BH_4 and NH_2 solutions in 0.010 m*M* HCl 1 mg/ml diethylenetriaminepentaacetic acid (DETAPAC) (Sigma) and 1 mg/ml DTE were added. The solutions were stable for

2 months at −80°C. A stable 0.5 m*M* standard N suspension was prepared as previously described [7] and stored for 8 months at −20°C. HVA and MHPG hemipiperazate solutions were prepared in distilled water; they were stable for 2 and 1 month respectively at +4°C in the dark. 5-HIAA solution (0.5 m*M*) was prepared in 0.01 *M* HCl and was stable for 15 days at 4°C.

2.5. Patients

Patients were hospitalized in the Neurology unit for a variety of pathologies: (a) inflammatory disease: Guillain-Barré syndrome, meningoradiculitis, spinal arachnoiditis, lupus-like polyneuritis (7 patients); (b) multiple sclerosis (2 patients); (c) infectious diseases: viral meningitis, syphilis, meningomyelitis (4 patients); AIDS (2 patients), subarachnoid hemorrhage (1 patient). For ethical reasons, CSF was not obtained from healthy controls, but results were compared with concentrations assayed in other patients without neurological disease and suffering from a variety of pathologies: (a) neoplastic diseases: medulloblastoma, pulmonary and gynecological malignancies (3 patients); (b) hormonal disorders: hypopituitarism and hyperthyroidism (2 patients), (c) cerebrovascular accidents (4 patients); and (d) diffuse pyramidal syndrome and facial paralysis (2 patients).

2.6. CSF sample collection

CSF samples were collected into polypropylene tubes containing 1 mg/ml DTE and 1 mg/ml DETAPAC according to the procedure described by Howells and Hyland [7], and frozen in liquid nitrogen until analyzed (within two months).

3. Results and discussion

In order to obtain reproducible results, the chromatographic system was equilibrated overnight and the mobile phase was run to waste. Prior to injection of the samples, 150 mg/l DTE was added to the mobile phase, which was always run to waste.

Fig. 2A shows the chromatogram of a 100 n*M* BH_4 standard and Fig. 2B the chromatogram of a CSF sample (patient 12). Fig. 3A is the chromatogram of a 25 n*M* standard of N while Fig. 3B corresponds to 25 n*M* NH_2 and Fig. 3C to a CSF sample. A very small amount of the NH_2 standard (less than 4%) was in the form of N under our conditions. The NH_2 peak disappeared completely when the 1 V potential was not applied at the outlet of electrode 1 by the auxiliary cell. Fig. 4A is the chromatogram of a standard mixture of MHPG, 5-HIAA, and HVA; Fig. 4B is the chromatogram of a CSF sample in

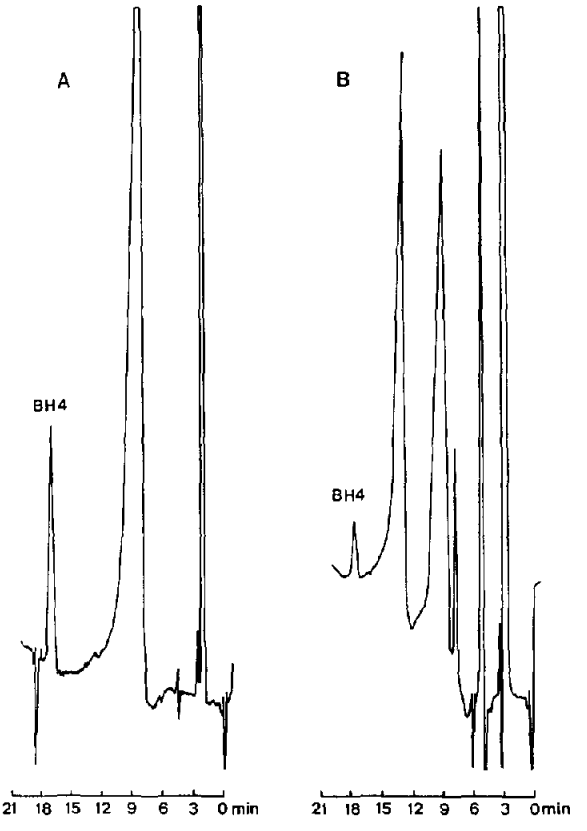

Fig. 2. Chromatograms of (A) 50 μl of a 100 n*M* standard solution of BH_4; (B) 50 μl of a CSF sample (patient P12). Mobile phase: 6.8 g/l sodium acetate and 1.05 g/l citric acid supplemented with 20 mg/l disodium EDTA and 150 mg/l DTE, pH 5.2. Ultrasphere ODS 5 μm column (250 × 4 mm I.D.); detector 1 = +0.5 V, detector 2 = −0.10 V.

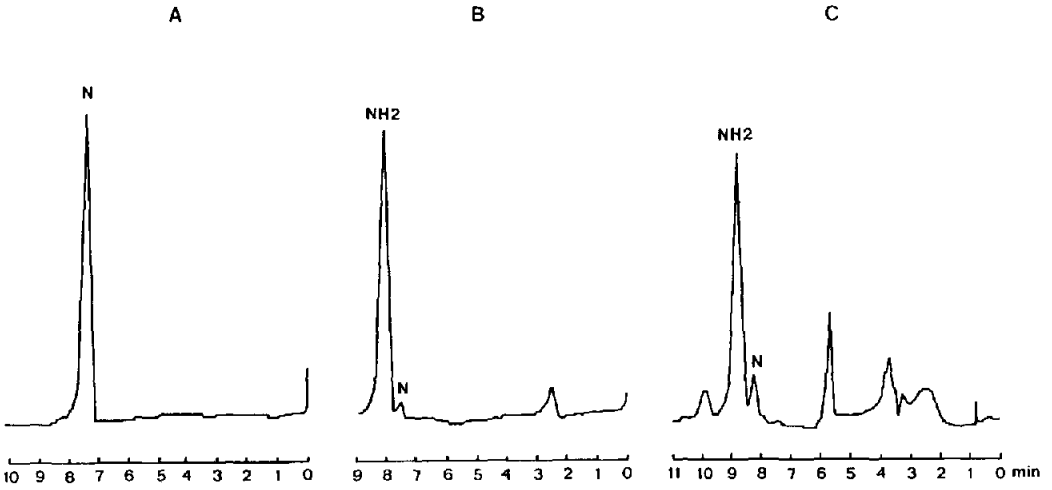

Fig. 3. Chromatograms of (A) 50 μl of a 25 nM standard solution of N; (B) 50 μl of a 25 nM standard solution of NH_2; (C) 50 μl of a CSF sample (patient P12).

which 5-HIAA and HVA were assayed. MHPG, a norepinephrine metabolite, could not always be separated from the other chromatographic peaks in CSF samples. As peak heights are strongly influenced by the pH, chromatography must be performed with a mobile phase that can not be recycled, and calibration must be repeated. Under these conditions, the intra-assay variation was 9% and the interassay variation was 12%, calculated with a 50 nM concentration.

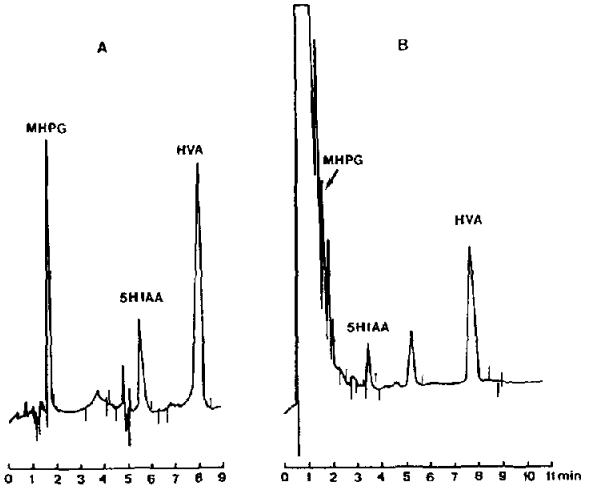

Fig. 4. Chromatograms of (A) 100 μl of a standard solution of HVA (40 nM), 5-HIAA (100 nM), and MHPG (60 nM); (B) 50 μl of a CSF sample: HVA = 87 nM; 5-HIAA = 60 mM. Mobile phase 6.8 g/l monopotassium phosphate (pH 4.0) and methanol (77:13); Ultrasphere ODS 5 μm column (250 × 4.6 mm I.D.); detector 1 = +0.30 V, detector 2 = + 0.44 V. Gain 1000.

The results are summarized in Tables 1 and 2. In the group without any neurological disorder, the two patients with a diffuse pyramidal syndrome and facial paralysis had low BH_4 concentrations (35 and 16.5 nmol/l) and low N values (1.5 and 1.7 nmol/l); their N/BH_4 ratios were also low (0.04 and 0.1 nmol/l). Similar results were obtained for the three patients with neoplastic diseases. The N/BH_4 ratio was elevated in some of the patients who had a cerebrovascular accident. In the literature, normal BH_4 values in CSF range from 18 to 24 nmol/l [9] and the normal values for N are below 3 nmol/l [10].

In inflammatory neurological diseases, BH_4 was sometimes increased and the N/BH_4 ratio remained low. BH_4, N, and NH_2 were all low in the two patients with multiple sclerosis; this concurs with the low values previously reported for patients in remission [11], which was the case for our patients. N was considerably elevated in meningomyelitis; a dramatic increase was also observed in an AIDS patient with neurological complications. N is considered a marker of neurological diseases in CSF, and a marker of neurological complication in AIDS [10].

Table 1
CSF values in patients without neurological disease

Patient[a]	Sex[b]	Age (years)	CSF level (nmol/l)					
			BH_4	N	NH_2	N/BH_4	5-HIAA	HVA
P1	F	32	17	1.7	22	0.10	180	143
P2	M	51	35	1.5	15	0.04		
P3	M	56	43	2.1	80	0.05		
P4	F	39	49	2.3	15	0.05		
P5	F	25	34	1.3	36	0.04	230	415
P6	M	43	37	3.1		0.08		
P7	M	74	29	4.2	24	0.14		
P8	M	44	33	5	42	0.15		

[a] P1 = headaches; P2 = diffuse pyramidal syndrome; P3 = cerebral arteritis; P4 = gynecologic neoplasm; P5 = medulloblastoma; P6 = pulmonary neoplasm; P7 = hypopituitarism; P8 = hyperthyroidism.
[b] F = female; M = male.

Table 2
CSF values in patients with neurological diseases

Patient[a]	Sex	Age (years)	CSF level (nmol/l)					
			BH_4	N	NH_2	N/BH_4	5-HIAA	HVA
P1	F	51	63	4.4	75	0.07		
P2	F	58	31	1.6	44	0.05		
P3	M	72	75	4.7		0.06	143	250
P4	M	80	25	2.2		0.09	149	169
P5	M	69	12	1.1	22	0.09		
P6	M	18	30	3.6	18	0.12	90	230
P7	F	25	46	2.2		0.04	109	330
P8	M	29	41	2.1		0.05		
P9	M	29	74	4.8	24	0.06	49	154
P10	M	45	12	1.1	22	0.09		
P11	F	50	20	2.3	49	0.01	160	77
P12	M	54	22	4.7	29	0.21	200	346
P13	M	59	21	4.1	23	0.19		
P14	F	77	31	6	37	0.09	109	210
P15	F	31	34	5.2	37	0.15		
P16	M	23	16	3	12	0.19		
P17	F	31	32	2.5	16	0.08	99	160
P18	F	69	7.5	19	66	2.5	230	140
P19	M	63	21	3.8	21	0.18		
P20	F	35	53	24	90	0.45		
P21	M	35	25	2.5	50	0.08	49	130
P22	M	31	59	102	268	1.73	200	346

[a] Inflammatory diseases: P1 = syphilis; P2 = syphilis; P3 = meningoradiculitis; P4 = myelinic neuropathy; P5 = polyneuritis; P6 = Guillain-Barré syndrome; P7 = demyelinization; P8 = postanorexic encephalopathy; P9 = lupus-like disease; P10, P11 = alcoholic patients; P12, P13, P14, P15 = cerebrovascular diseases; P16, P17 = multiple sclerosis; P18 = subarachnoid hemorrhage; P19 = viral meningitis; P20 = meningomyelitis; P21 = AIDS but normal CD4/CD8 ratio indicating sufficient cellular immunity; P22 = AIDS with neurologic involvement.

In subarachnoid hemorrhage, N and NH_2 were elevated while BH_4 was very low, resulting in a high N/BH_4 ratio. Increased concentrations of N in CSF have previously been reported in both subarachnoid hemorrhage and patients with cerebral ischemia [12], as observed in some of our patients with cerebrovascular accidents. The N/BH_4 ratio (Table 2) reflects the variations in the two parameters [13].

The HVA and 5-HIAA concentrations found in this study were compatible with the normal values reported in the literature [14] (5-HIAA 104 ± 27 nmol/l; HVA 230 ± 35 nmol/l). One patient with inflammatory disease (polyneuritis) exhibited decreased HVA and 5-HIAA concentrations, but this was probably due to alcohol-related deficiencies. HVA and 5-HIAA concentrations were higher in infectious diseases, meningomyelitis, and AIDS with neurological complications.

3.1. Correlations

As expected, a highly significant correlation was observed between N and NH_2 ($p < 0.001$) using a simple regression. NH_2 was also correlated with BH_4 ($p = 0.04$); surprisingly, this was not the case for N and BH_4 (not significant). Despite the low number of samples tested, HVA (the product of dopamine breakdown) was weakly correlated with BH_4 ($p = 0.06$), the cofactor of tyrosine hydroxylase in dopamine biosynthesis, and with N ($p = 0.05$). HVA was also correlated with 5-HIAA (the product of serotonin breakdown) ($p = 0.04$). This might reflect the fact that both dopamine and serotonin are correlated with BH_4 concentrations in the neurological disorders investigated.

4. Conclusions

The reduced pterins BH_4 and NH_2 can be measured simultaneously with N when the samples are assayed in a reducing medium. Increased concentrations of BH_4 may be accompanied by both high levels of N and NH_2 and increased amounts of HVA and 5-HIAA reflecting the rate of dopaminergic and serotoninergic activation.

In this study, the BH_4 concentrations in patients with neurological disorders were higher than the normal values reported in the literature. The highest concentrations were observed in patients with syphilis, lupus-like disease, and meningoradiculitis. An AIDS patient with neurological complications also showed a dramatic elevation in BH_4 and an increased N/BH_4 ratio which may reflect neurological complication.

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6. References

[1] T. Nagatsu, S. Matsura and T. Sugimoto, *Med. Res. Rev.*, 9 (1989) 25–44.
[2] G. Werner-Felmayer, E.R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, K. Schmidt, G. Weiss and H. Wachter, *J. Biol. Chem.*, 268 (1993) 1842–1846.
[3] T. Yamaguchi, T. Nagatsu, T. Sugimoto, S. Matsuura, T. Kondo, R. Iizuka and H. Narakashi, *Science*, 219 (1983) 75–81.
[4] Y. Furukawa, K. Nishi, T. Kondo, K. Tanabe and Y. Mizuno, *J. Neurol. Sci.*, 111 (1992) 65–72.
[5] C. Huber, D. Fuchs and A. Niederwieser, *Klin. Wochenschrift*, 62 (1984) 103–113.
[6] D.W. Howells, I. Smith and K. Hyland, *J. Chromatogr.*, 381 (1986) 285–294.
[7] D.W. Howells and K. Hyland, *Clin. Chim. Acta*, 167 (1987) 23–30.
[8] M. Candito, C. Cavenel, J. Gugenheim, J. Mouiel, F. Parisot, Y. Jacomet, P. Sudaka and P. Chambon, *J. Chromatogr.*, 614 (1993) 164–168.
[9] A.C. Williams, R.A. Levine, T.N. Chase, W. Lovenberg and D.B. Calne, *J. Neurol. Neurosurg. Psychiatry*, 43 (1980) 735–738.
[10] D.E. Griffin, J.C. McArthur and D.R. Cornblath, *Neurology*, 41 (1991) 69–74.
[11] S. Fredrikson, H. Link and P. Eneroth, *Acta Neurol. Scand.*, 75 (1987) 352–355.
[12] T. Mathiesen, D. Fuchs, H. Wachter and H. Von Holst, *J. Neurosurg.*, 73 (1990) 69–71.
[13] K.I. Fujishiro, M. Hagihara, A. Takahashi and T. Nagatsu, *Biomed. Metabol. Biol.*, 44 (1990) 97–100.
[14] M. Scheinin, W.O. Chang, K.L. Kirk and M. Linnoila, *Anal. Biochem.*, 131 (1983) 246–253.