

# HPLC with electrochemical and fluorescence detection procedures for the diagnosis of inborn errors of biogenic amines and pterins

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## Abstract

The analysis of biogenic amines (BA) and pterins in cerebrospinal fluid (CSF) is essential for the early diagnosis of neurotransmission defects in the paediatric age. Our aim was to standardize previously reported HPLC procedures for the analysis of BA and pterins in CSF and to establish reference values for a paediatric population. Samples from 127 subjects (age range 11 days to 16 years; average 3.8) were analyzed by HPLC with electrochemical and fluorescence detection. Both BA (homovanilic and 5-hydroxyindoleacetic acid) and pterins (neopterin and biopterin) concentrations in CSF showed a negative correlation with age. This finding led us to stratify reference values into six groups according to age. In conclusion, analysis of BA and pterins in CSF by HPLC procedures is a useful set of tools for the diagnosis of inborn errors of metabolism of these compounds. The establishment of reference intervals may be difficult, since there is a strong correlation between BA concentrations and the age of controls and, as a result, a large number of CSF samples from control populations would be necessary for this purpose.

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## 1. Introduction

Catecholamines and serotonin are biogenic amines (BA) related with central and autonomous vegetative nervous system function. They are mainly involved in movement, sleep, thermoregulation, learning, memory, emotional behaviour, as well as other functions (Grace et al., 1998). Several inborn errors of BA metabolism have recently been established (Blau et al., 2001). The first step in the diagnosis requires the analysis of catecholamines and serotonin metabolites in cerebrospinal fluid (CSF), since blood and urine analysis of these compounds may be uninformative for the identification of these disorders (Hyland et al., 1993).

Several enzymatic defects have been related with primary BA deficiencies. Tyrosine hydroxylase deficiency (OMIM: 191290) causes a dopamine defect; it is diagnosed by low 3-ortomethylidopa (3-OMD), 3-methoxy-4-hydroxyphenylglycol (MHPG) and homovanilic acid (HVA) concentrations in CSF (Hoffmann et al., 1998). Aromatic L-amino acid decarboxylase deficiency (OMIM: 107930) causes a reduction of both dopaminergic and serotonergic metabolites; it is diagnosed by high concentrations of 5-hydroxytryptophan (5-OHtrp) and 3-OMD together with low 5-hydroxyindoleacetic acid (5-HIAA) and HVA values in CSF (Blau et al., 2001; Hoffmann et al., 1998). Dopamine β-hydroxylase deficiency (OMIM: 223360) causes norepinephrine deficiency; it is diagnosed by low plasma norepinephrine/dopamine ratio (Timmers et al., 2004). High CSF HVA with low MHPG concentrations may be observed, causing high HVA/MHPG ratio values (Blau et al.,

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2001). Furthermore, a possible tryptophan hydroxylase (EC 1.14.16.4) dysfunction causing low 5-OHtrp and 5-HIAA values has recently been reported, although no molecular identification of the primary defect is available for this disorder (Ramaekers et al., 2001).

Tetrahydrobiopterin (BH<sub>4</sub>) is the main cofactor for tryptophan, tyrosine and phenylalanine hydroxylases. Defects of this cofactor affect both serotonin and dopamine biosynthesis and are usually associated with hyperphenylalaninemia (HPA) (Blau et al., 2001). However, at least two BH<sub>4</sub> deficiencies, GTP cyclohydroxylase-1 autosomal-dominant (OMIM: 600225) and sepiapterin reductase (OMIM: 182125), may occur without HPA. The biochemical diagnosis of these disorders must be performed through the analysis of pterins (biopterin (BP) and neopterin (NP)) in CSF (Bonafé et al., 2001).

HPLC with electrochemical and fluorescence detection procedures have been applied for CSF BA and pterin analysis, respectively. These procedures are sensitive and specific, permit rapid and reproducible analysis with minimal sample preparation, and may be available in clinical laboratories (Schmidt et al., 1990). The BA and pterin analysis in CSF is important for the early diagnosis of these disorders in the paediatric age, since the potential benefits of specific treatment of some of these disorders have been demonstrated (Hyland, 2003). However, there are few reports concerning detailed analytical procedures for the diagnosis of these congenital disorders. Moreover, data concerning reference values in a paediatric population have scarcely been reported (Hyland et al., 1993; Komori et al., 1999), to our knowledge.

Our aim was to standardize previously reported HPLC procedures for the analysis of BA and pterins in CSF and to establish reference values for a paediatric population.

## 2. Materials and methods

### 2.1. Subjects

Reference values were established in 127 subjects (age range 11 days to 16 years; average 3.83; sex: 71 males and 56 females) whose CSF samples were submitted to our laboratory under suspicion of viral or bacterial meningitis, encephalitis, or other neurological conditions of non-metabolic origin. Exclusion criteria were: disturbance of the biogenic amines or pterin metabolism, inborn errors of intermediary and energy metabolism, movement disorders and neuroimaging abnormalities, and diagnosis of viral or bacterial meningitis or other infectious diseases. One patient with confirmed tyrosine hydroxylase deficiency and one case with GTP cyclohydroxylase deficiency were also studied. Samples from patients were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. The ethical committee of the Hospital Sant Joan de Déu approved the study.

### 2.2. Sample preparation

CSF samples were collected following a previously reported protocol (Hyland et al., 1999), with the samples taken between 8:00 and 10:00 a.m. The first ml of CSF was collected for determination of routine biochemical analysis, and the second ml was used for BA (0.5 ml) and pterin analysis (0.5 ml, protected from light). Samples were immediately stored at  $-70^{\circ}\text{C}$  until the moment of the analysis. Haematic samples were centrifuged immediately and the clear CSF supernatant was stored at  $-70^{\circ}\text{C}$ .

*Biogenic amine HPLC analysis:* CSF samples were diluted 1:2 in the chromatographic mobile phase, centrifuged at  $1500 \times g$  (10 min), and filtered through  $0.22 \mu\text{m}$  nylon filters (Millipore: SLGVR04NL, Bedford, MA, USA). The  $30 \mu\text{l}$  were injected onto the HPLC.

*Pterin HPLC analysis:* For the oxidation of reduced pterins to biopterin (Blau et al., 2003),  $200 \mu\text{l}$  of CSF was mixed in an Ultrafree-Mc filter (Millipore) with  $20 \mu\text{l}$  of 1 mol/l HCL and 1 mg of manganese dioxide (Merck, 1.05957.1000, Darmstadt, Germany). Samples were shaken for 10 min at room temperature and centrifuged at  $12,000 \times g$  (10 min). The  $20 \mu\text{l}$  of the clear supernatant was injected onto the HPLC.

### 2.3. Standards

3-OMD (Sigma Chemical Co., M-4255, St Louis, USA), MHPG (Sigma, H-8759), 5-OHtrp (Sigma, H-9772), 5-HIAA (Sigma, H-2255) and HVA (Sigma, H-1252) calibrators were diluted in buffer citrate–acetate (0.1 mol/l, pH 4) to attain a final concentration of 78, 74, 76, 72 and 73 nmol/l, respectively. NP (Schircks Laboratories, ref.: 11.325, Jona, Switzerland) and BP (Schircks Laboratories, ref.: 11.203) calibrators were diluted in HCl 0.1 mol/l to attain a final concentration of 37.5 and 52 nmol/l, respectively.

### 2.4. Chromatographic conditions

Biogenic amines (3-OMD, MHPG, 5-OHtrp, 5-HIAA and HVA) were analysed by ion pair HPLC (Serie 200, Perkin Elmer, Norwalk, CT, USA) with electrochemical detection (Coulchem II, ESA, Chelmsford, MA, USA), following a modified procedure (Mena et al., 1984). Briefly, the mobile phase consisted of citrate–acetate buffer (0.1 mol/l, pH 4), plus 1.2 mmol/l EDTA and 1.2 mmol/l 1-heptanosulphonic acid (Sigma, H-8901) mixed with methanol (91/9 v/v). Electrochemical conditions were: analytical cell +400 mV, sensitivity 200 nA. Biogenic amines were separated in a nucleosil C-18 column (250 mm  $\times$  5 mm;  $5 \mu\text{m}$  particle size; Teknokroma, Barcelona, Spain). Chromatographic data were processed with the Turbochrom Navigator program (Perkin Elmer). Flow rate was 1.2 ml/min and injection volume  $30 \mu\text{l}$ .

Pterins (BP and NP) were analysed by reverse phase HPLC with fluorescence detection (Waters, MA, USA), following a previously reported procedure (Blau et al., 1992). Briefly,

Table 1

Within-run and between-run imprecision data and analytical range of BA and pterins measured by HPLC with electrochemical and fluorescence detection

	CV1 <sup>a</sup>	CV2 <sup>a</sup>	CV3 <sup>b</sup>	Analytical range
3-OMD	10.7% (57)	8.2% (42)	12.5% (24.4)	2.4–550
MHPG	8.0% (73)	6.0% (34)	12% (27.5)	3.0–500
5-OHTrp	8.5% (15)	6.5% (6.9)	9.3% (5.3)	1.3–500
5-HIAA	3.5% (849)	4.9% (244)	7.3% (170.8)	4.6–3000
HVA	2.5% (1263)	2.0% (288)	3.2% (757.6)	9.0–3000
NP	2.4% (36)	2.5% (14.7)	2.4% (37.5)	0.6–1500
BP	1.5% (42)	1.1% (23.2)	2.3% (51.2)	0.8–1500

Data representing coefficients of variation are expressed as percentages (average concentration in nmol/l).

<sup>a</sup> Within-run coefficients of variation at two different concentrations (in parenthesis).<sup>b</sup> Between-run coefficient of variation.

the mobile phase consisted of 1 μmol/l potassium phosphate (Sigma, ref.: P-5379) plus methanol (91/9 v/v). Excitation was 350 nm and emission 450 nm. BP and NP were separated in a nucleosil C-18 column (250 mm × 5 mm; 5 μm particle size; Teknokroma) in conjunction with an ODS guard column (20 mm × 4 mm; Teknochroma). Chromatographic data were processed with Breeze GP Software (Waters). Flow rate was 1 ml/min and injection volume 20 μl.

### 2.5. Statistical analysis

Kolmogorov–Smirnov test was applied for data distribution. Since data followed a Gaussian distribution, Pearson test was applied to search for correlation among the different variables in the study. Levene and Student's *t*-tests were applied to compare biogenic amine and pterin results between the different age groups. Statistical calculations were performed with the SPSS.11.0 program.

### 3. Results

Within-run and between-run imprecision data and analytical interval for BA and pterin metabolites are reported in Table 1. Recovery was evaluated by adding, 3-OMD, MHPG, 5-OHTrp, 5-HIAA, HVA, NP and BP to a CSF sample to obtain final concentrations of 216, 124, 70, 494, 316, 25.1 and 27.9 nmol/l, respectively. Samples obtained were analysed in triplicate, and the range of the mean recoveries was 96–104%.

In the whole group of controls, a negative correlation was observed between all the variables and age (3-OMD:  $r = -0.344$ ,  $P < 0.0001$ ; MHPG:  $r = -0.410$ ,  $P < 0.0001$ ; 5-OHTrp:  $r = -0.204$ ,  $P = 0.029$ ; 5-HIAA:  $r = -0.587$ ,  $P < 0.0001$ ; HVA:  $r = -0.654$ ,  $P < 0.0001$ ; NP:  $r = -0.231$ ,  $P = 0.030$  and BP:  $r = -0.222$ ,  $P = 0.034$ ). Conversely, HVA/5-HIAA ratio showed a positive correlation with age ( $r = 0.354$ ,  $P < 0.0001$ ). Significantly positive correlation was observed between HVA and HIAA ( $r = 0.871$ ,  $P < 0.0001$ ), HVA and MHPG ( $r = 0.518$ ,  $P < 0.0001$ ), HVA and BP ( $r = 0.412$ ,  $P <$

Table 2

Reference values of BA and pterins in a paediatric population, and results from tyrosine hydroxylase (TH) and GTP cyclohydroxylase-I (GTP-CH) deficiency patients

Groups	3-OMD	MHPG	5-OHTrp	5-HIAA	HVA	HVA/HIAA	NP	BP
A ( $n = 11$ )	24–148	44–106	6.0–24.0	428–1122	658–1434	0.76–1.67	12–64	22–70
0–30 days	85 (37)	76 (18)	12.6 (5.9)	766 (197)	955 (236)	1.28 (0.24)	35 (16)	39 (13)
B ( $n = 22$ )	20–162	30–124	2.7–26.0	217–1142	354–1328	1.16–2.4	9–34	12–44
1–5 months	56 (33)	58 (25)	8.8 (4.9)	514 (231)	757 (231)	1.58 (0.37)	19 (6.9)	30 (8.5)
C ( $n = 38$ )	4–50	20–80	1.6–15.0	170–490	344–906	1.11–3.48	8–43	8–54
6 months–2 years	27 (12)	41 (14)	6.7 (3.1)	305 (78)	579 (131)	1.99 (0.61)	18 (8.2)	23 (11)
D ( $n = 24$ )	3–64	22–54	4.0–23.0	106–316	304–658	1.92–3.44	7–55	10–52
3–6 years	26 (17)	35 (9)	8.8 (4.7)	201 (53)	478 (90)	2.45 (0.48)	18 (11)	24 (12)
E ( $n = 20$ )	5–60	13–68	1.6–16.0	87–366	202–596	1.20–3.45	10–46	8.2–68
7–10 years	26 (17)	38 (15)	6.2 (4.5)	184 (98)	383 (174)	2.15 (0.62)	17 (8.7)	23 (17)
F ( $n = 12$ )	3–54	11–46	2.4–12	63–185	156–410	1.44–3.17	10–24	13.7–36
11–16 years	23 (18)	31 (9)	6.5 (2.9)	121 (42)	269 (73)	2.3 (0.49)	15 (5.3)	22 (9.3)
TH (4 years)	1.7	n.d. <sup>a</sup>	4.6	177	15	0.08	14	31
GTP-CH (1 year)	25	25	3.0	236	268	1.13	2	8

Results were expressed in nmol/l as range, average (S.D.). Student's *t*-test: group A vs. B: 5-HIAA,  $P = 0.004$ ; HVA,  $P = 0.028$ . Group B vs. C: 5-HIAA,  $P < 0.0001$ ; HVA,  $P = 0.002$ . Group C vs. D: 5-HIAA,  $P < 0.0001$ ; HVA,  $P = 0.001$ . Group D vs. E: 5-HIAA,  $P = 0.401$ ; HVA,  $P = 0.012$ . Group E vs. F: 5-HIAA,  $P = 0.014$ ; HVA,  $P = 0.017$ .

<sup>a</sup> Not detectable.

0.0001), HIAA and BP ( $r = 0.430$ ,  $P < 0.0001$ ) and NP and BP concentrations ( $r = 0.352$ ,  $P = 0.001$ ).

Reference values for our paediatric population were stratified according to age in six different groups (Table 2 and Fig. 1). For 5-HIAA and HVA CSF concentrations, Student's  $t$ -test showed significantly different values when comparing the six age groups (Table 2). Significantly higher NP and BP values were only observed when comparing the 0–30 days group with the 1–5 months old group ( $P = 0.006$  and  $P = 0.046$ , respectively). No differences were observed when comparing HVA/MHPG ratio among the different age groups. Therefore, a unique reference range was established (average 11.7; standard deviation 4.1; range 5.1–21.7).

Patient results are reported in Table 2 and chromatograms of BA calibrator mixture, controls and the tyrosine hydroxylase deficient patient in Fig. 2. A chromatogram of a pterin calibrator mixture is reported in Fig. 3. The patient with tyrosine hydroxylase deficiency showed clearly

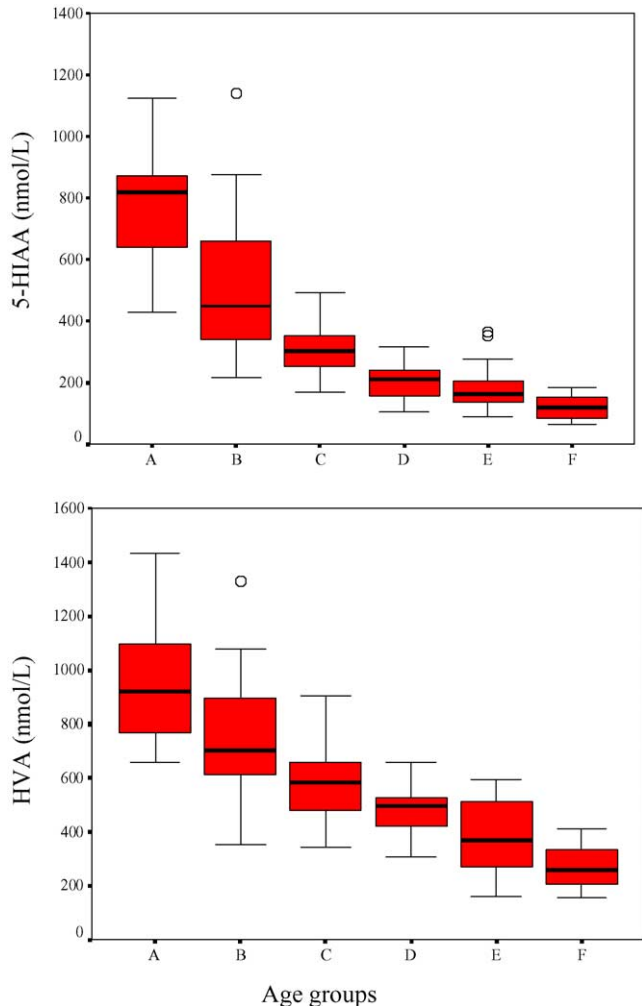


Fig. 1. Box plot representation of HVA and 5-HIAA concentrations in CSF samples from a paediatric population. The length of the boxes indicates the interquartile space (P25–P75); the horizontal line into the box represents the median (P50) and the whiskers indicate the adjacent values. The circles indicate the outliers.

decreased HVA concentrations, while the patient with GTP-cyclohydroxylase-1 deficiency showed low values for both BP and NP.

#### 4. Discussion

HPLC with electrochemical and fluorescence detection offers enough sensitivity and quality for the analysis of BA and pterins in CSF in order to diagnose inborn errors of metabolism of these compounds (Schmidt et al., 1990). To date several HPLC procedures have been published for BA (Mena et al., 1984; Schmidt et al., 1990; Yi et al., 1994) and pterin analysis (Blau et al., 1992), with slight differences among them. We present here two modified HPLC procedures for BA and pterin analysis, as well as our reference values for a paediatric population.

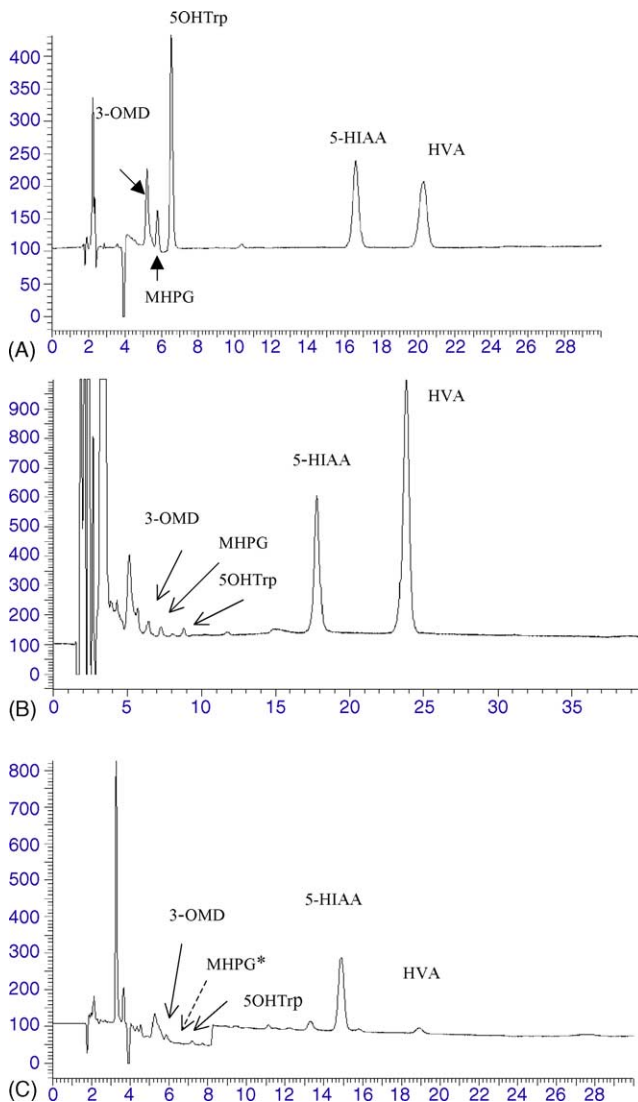


Fig. 2. (A) Standard mixture; (B) healthy control; (C) patient with tyrosine hydroxylase deficiency (\* MHPG was not detectable).

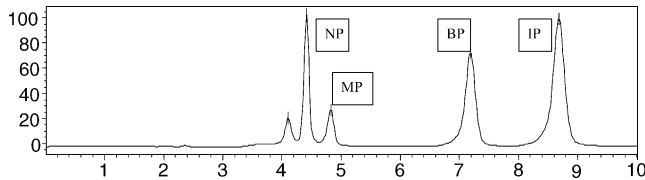


Fig. 3. A chromatogram of pterin separation of a standard mixture containing neopterin (NP), monapterin (MP), biopterin (BP) and isoxanthopterin (IP).

To obtain a better chromatographic separation for BA analysis, we modified the composition of the mobile phase (Mena et al., 1984) adding an ion pair reagent, in accordance with a previously reported procedure (Schmidt et al., 1990). We also changed the pH value (4 instead of 3.5) and increased the percentage of methanol up to 9%, with all BA separated in less than 25 min (Fig. 2). Regarding electrochemical detection, hydrodynamic voltammograms showed a maximum oxidation of the five compounds analysed at +400 mV (data not shown). This low potential precluded the presence of interfering compounds, which may be oxidized at higher oxidation potentials. Under these conditions of analysis, imprecision (evaluated as coefficient of variation) and accuracy results were acceptable for all of the BA analysed.

For NP and BP analysis, both molecules were oxidised in acidic conditions and detected by native fluorescence, as previously reported (Blau et al., 1992). All pterins analysed were separated in less than 10 min (Fig. 3), and imprecision and accuracy results were also acceptable for this procedure.

One of the more important troubles in BA analysis is CSF sampling, since there is a rostrocaudal gradient concentration of BA (Hyland, 2003). Although it would be advisable to use higher CSF fractions, especially in elder patients, we always used the second ml for BA analysis in both patients and controls, mainly for ethical and technical reasons. In fact, BA quantification necessitates that the same fraction of CSF be used for each metabolite analysis and that the values obtained be compared to reference ranges established using the same collection criteria (Hyland, 2003).

There are few reports regarding reference values in paediatric populations, probably due to the difficulties involved in selection of control populations and CSF sampling. Moreover, the number of subjects studied for reference values establishment was limited (75 in reference from Hyland et al., 1993; 56 in reference from Komori et al., 1999). Higher 5-HIAA and HVA CSF concentrations have been observed in the first months of life. This finding has been explained by the presence of large amounts of the key enzymes regulating both metabolic pathways during the mitosis, neurogenesis, migration and network formation of dopamine and serotonin neurons in foetus brain (Sladek et al., 1995). In agreement with previous studies (Hyland et al., 1993; Komori et al., 1999), we also observed a negative association between all BA and the age of controls. This finding led us to stratify reference values into six groups according to age. We observed significantly higher 5-HIAA and HVA values in controls younger than 1

month compared with controls of 2–5 months (Table 2), in contrast to previously reported reference values (Hyland et al., 1993; Komori et al., 1999), which included larger age-intervals for the reference groups. It is likely that the different number of controls studied and the age-distribution of them may explain these differences. Furthermore, according to our results, HVA/5-HIAA ratio was lower in controls younger than 1 month than in the other age groups, indicating a relative increment of serotonin metabolites compared with dopamine compounds. This finding might also be a consequence of adaptive changes of neurotransmitter metabolites in the newborn period. The age-related changes were also observed for pterins, although this effect seems especially important in the first months of life, with NP and BP values remaining similar from 5 months to 16 years.

Concerning the diagnosis of primary BA and pterin metabolism defects, we diagnosed tyrosine hydroxylase and GTP cyclohydroxylase-1 deficiencies. Both defects were confirmed by molecular analysis, which detected the presence of mutations at the TH1 and GCH1 genes (unpublished results). Furthermore, a significant positive correlation between HVA and MHPG concentrations was observed, suggesting that the diagnosis of dopamine  $\beta$ -hydroxylase deficiency would be possible (high HVA/MHPG ratio). Therefore, this procedure would seem to be useful for diagnosis of inborn errors of metabolism of BA and pterins.

In conclusion, analysis of BA and pterins in CSF by HPLC procedures is a useful set of tools for the diagnosis of inborn errors of metabolism of these compounds. The establishment of reference intervals may be difficult, since there is a strong correlation between BA concentrations and the age of controls and, as a result, a large number of CSF samples from control populations would be necessary for this purpose. In addition to the age, a clear association between 5-HIAA and HVA concentrations was demonstrated.

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