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RAPID AND SENSITIVE METHOD FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PTERINS IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic (HPLC) method for the analysis of the most important urinary pterins is described. The method involves a preliminary sample oxidation to stabilize and convert pterins into their fluorescent forms and a purification by anion-exchange chromatography, followed by a short reversed-phase HPLC separation with fluorometric detection and quantitation of the different pterins. A complete HPLC analysis is accomplished in as little as 15 min. The sensitivity of the method allows the detection of as little as 20 pg of each pterin with a mean recovery greater than 99% for all pterins analysed. Reference values were obtained from 50 normal babies aged between 1 and 120 days. A significant correlation was found between urinary biopterin levels and the age of the babies ($r = 0.445$), while neopterin did not show any significant correlation with age. The "biopterin neopterin creatinine ratio" (BNCR index) was also significantly correlated with the age of the babies ($r = 0.428$). This rapid and sensitive method for pterin determination in biological fluids may be useful in the differential diagnosis of the various hyperphenylalaninemic conditions identified by neonatal mass screening programmes.

INTRODUCTION

Urinary pterin determination is receiving attention in several medical fields, including immunology, oncology, psychobiology and metabolic disorders[1–4].

One of the most important applications of this type of analysis is in the diagnosis of neonatal hyperphenylalaninemic conditions, caused by a metabolic block in the tetrahydrobiopterin biosynthetic or salvage pathway[5–7]. The diagnosis of these altered metabolic states which, if not recognized and treated early, may produce severe brain damage, requires a rapid and precise method for the determination of pterin levels in biological fluids. The establishment of an appropriate set of normal reference values is also important, since it has been demonstrated that pterin levels may vary with the age of the subject examined[6–8].

The aim of this work was: (1) to set up a rapid and sensitive method for high-performance liquid chromatographic (HPLC) analysis of urinary pterin levels

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and (2) to determine reference values for normal babies at the critical age interval between 1 and 120 days. The method may find application in the differential diagnosis of hyperphenylalaninemic conditions, detected by neonatal mass screening.

MATERIALS AND METHODS

All the reagents were of analytical grade and were used without further purification. Biopterin (B), neopterin (N), pterin (Pt) and 6-methylpterin (6-mPt) were obtained from Sigma (St. Louis, MO, U.S.A.), tetrahydrobiopterin (THB) and dihydrobiopterin (DHB) from Dr. B. Schirks (Wettsil a.A., Switzerland). To determine normal reference values, urines were collected from 50 normal babies (40 males and 10 females, age range 1–120 days), randomly selected from those attending a routine check-up visit.

Urines were collected in light-shielded containers, immediately acidified to pH 1 with 6 *M* hydrochloric acid and frozen in 1-ml aliquots at −70°C until analysed. A separate aliquot was used for a creatinine determination according to the method of Rossignol *et al.*[9].

Specimens were rapidly thawed at 40°C, and 100 μl of the internal standard (6-mPt, 28 μ*M*) were added to each aliquot. In order to measure the hydrogenated pterins THB and DHB, an oxidation to the more stable and fluorescent derivatives B and Pt was performed[11,12]. Two aliquots of the urine sample were subjected to differential oxidation in 0.1 ml of 0.5% iodine–1% potassium iodide either in alkaline solution, to convert DHB into B and THB in Pt (90 and 80% conversion, respectively), or in acidic solution, to convert both hydrogenated pterins into B (90% conversion). The difference between the amount of B derived from the two oxidative steps and the amount of B originally present in the urine sample before oxidation allows the determination of the hydrogenated pterins.

Samples were incubated in the dark at room temperature for 45 min and the reaction was stopped by the addition of 200 μl of 57 m*M* ascorbic acid. The oxidized samples were then purified by ion-exchange chromatography, using a solid-phase silica-bonded anion-exchange Bond-Elut column (Analytichem, Harbor City, CA, U.S.A.). After sample loading, the column was washed with 4 ml of water acidified to pH 4 with 1 *M* hydrochloric acid, and the analytes were eluted with 870 μl of 1 *M* potassium hydroxide–20% methanol. The analytes were then acidified by the addition of 130 μl of 6 *M* hydrochloric acid and then 20 μl were injected into a 25 cm × 0.46 cm I.D. RP-18 column (Macherey-Nagel, Duren, F.R.G.), in an 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.), equipped with a fluorometer FP-115 (Jasco, Hachioji, Tokio, Japan) or a SFM 25 spectrofluorometer (Kontron, Zurich, Switzerland) as detector. The wavelength settings were: excitation, 350 nm; emission, 410 nm.

The HPLC operating conditions were: eluent, water–methanol (97:3, v/v); oven temperature, 35°C; flow-rate, 0.7 ml/min. From 1.5 to 3 min after sample injection, a linear gradient was applied starting from 3 to 12% methanol. The quantitation of the analytes was performed according to the internal standard method.

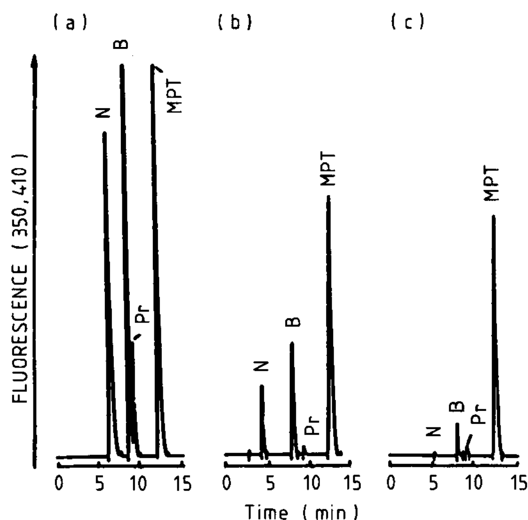

Fig. 1. Three typical chromatograms for (a) a standard mixture of B, N, Pt and 6-mPt, (b) a urine sample treated with acidic oxidation and (c) the same urine sample treated by alkaline oxidation.

RESULTS

Under our experimental conditions, a good separation between N, B, Pt and 6-mPt was achieved in 15 min (Fig. 1). The sensitivity of the method allowed the detection of as little as 20 pg of each pterin in an injection volume of 20 μl, with a linear dose–response curve over the range of 0.02–60 ng of pterins.

The recovery of the method was assessed by adding different quantities of each pterin to a known amount of urine. Fig. 2 shows the correlation between observed and expected values for the pterins indicated. The correlation cooefficient was highly significant ($r = 0.9994$) with a mean recovery of 101.4% for P, 99.6% for N and 99.5% for B.

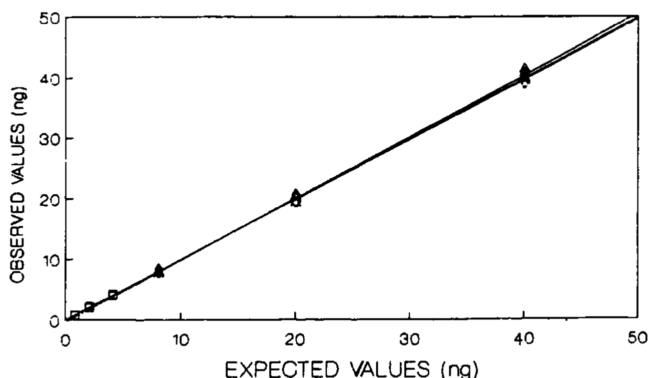

Fig. 2. Correlation between observed and expected values for the different pterins obtained in the analysis of an urine sample containing known amounts of each pterin. □, Pterin; △, neopterin; ◇, biopterin.

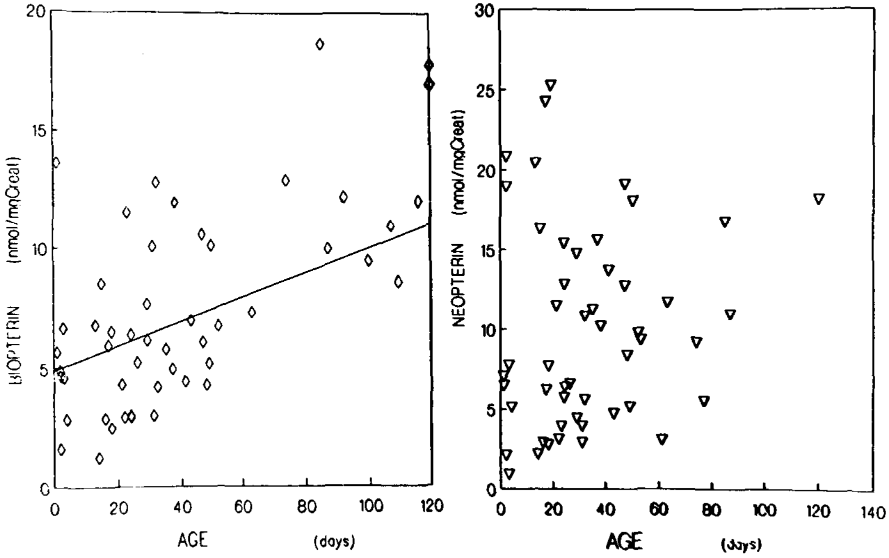

Fig. 3. Reference values for pterin urinary excretion: correlation between levels (nmol/mg creatinine) of N and B with the age of 50 normal babies. The urinary levels of B are significantly correlated with the age ($r = 0.445$ for $p < 0.0012$); the correlation between N and the age is not significant.

The precision of the method was assessed by processing 60 replicates of the same sample in three separate assays, resulting in a mean intra-assay coefficient of variation (C.V.) of 3.75% and a mean inter-assay C.V. of 4.3%.

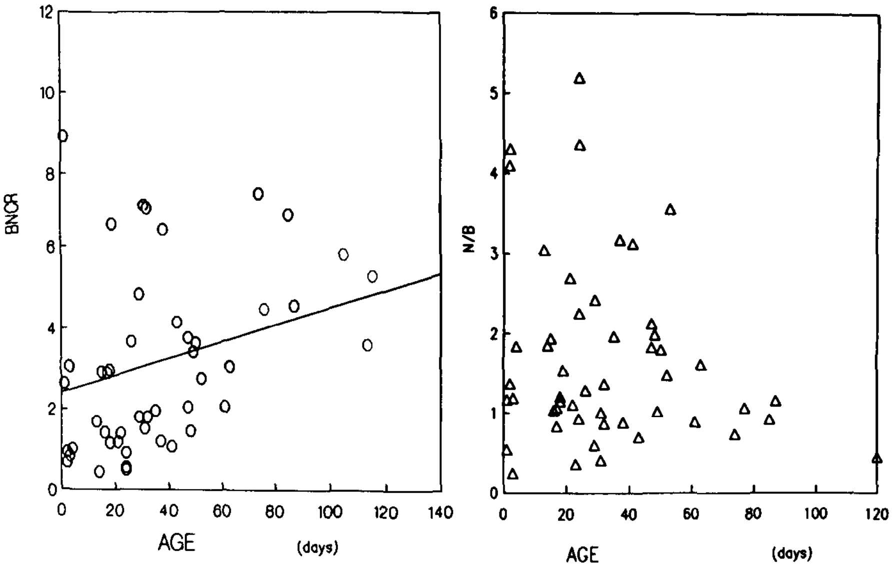

Fig. 4. Reference values for pterin urinary excretion: correlation between BNCR {[B/(N + B)] × (B/creatinine) · 10^5} and N/B with the age of 50 normal babies. BNCR is significantly correlated with age ($r = 0.428$ for $p < 0.002$); the correlation between N/B and age is not significant.

The method was also used in a preliminary field trial to establish urinary excretion levels of B and N in a group of 50 normal babies with ages ranging between 1 and 120 days. In Fig. 3 is shown the correlation between the age and urinary levels, expressed as nmol/mg creatinine. The levels of B were significantly correlated with the age of the babies ($r = 0.445$ for $p < 0.0012$) while the correlation between the levels of N and the age was not significant. The "biopterin neopterin creatinine ratio"[10] {BNCR index = $[B/(N + B)] \cdot (B/\text{creatinine}) \cdot 10^5$}, and the ratio between N and B (N/B), generally considered the best informative biochemical indices for the discrimination between phenylketonuria and the variant forms of hyperphenylalaninemia[10], were also determined for each sample on the basis of the excretion values of N and B. Fig. 4 shows the correlation between the BNCR index or the ratio N/B and the age of the babies: BNCR was significantly correlated with the age ($r = 0.427$ for $p < 0.002$), while N/B did not show any significant correlation.

DISCUSSION

In almost all the methods so far described the extraction of pterins from urine is accomplished by using ion-exchange chromatographic procedures[11–13]. On the contrary, the methods described for HPLC analysis of extracted samples are different, being based on either ion-exchange[14,15] or reversed-phase (RP) chromatography[13,16] with either fluorometric[13,14] or electrochemical detection[17]. Most of the methods so far described are also time consuming, requiring several purification steps, followed by an HPLC analysis that lasts *ca.* 20 min with RP columns[13] or even 30 min when ion-exchange columns are used[14].

The method described here allows HPLC analysis of the most important pterins (N and B) and of the hydrogenated forms THB and DHB in only 15 min, preceded by an extraction and purification step, taking *ca.* 10 min. The recovery, accuracy, precision and sensitivity of the method are in the range of other methods so far described[13–15].

The requirement of a proper set of reference values is important in the analysis of urinary pterins, since an age-dependent variation of urinary pterin excretion has been reported[18,19]. Since most blocks in pterin metabolism become evident during the first months of postnatal life, resulting in hyperphenylalaninemic conditions that are usually detected by means of neonatal screening programme, we applied this method to the determination of reference values in this critical age period. To our knowledge, reference values for this age interval have been extensively reported only by Dhondt *et al.*[18]. Our results are in good agreement with that report in terms of absolute values. The progressive increase with age of urinary B levels is in accordance with the results reported by Dhondt *et al.*[18] and by Niederwieser *et al.*[19]; moreover the lack of correlation between urinary N levels and age, in the first trimester of postnatal life, is not in contrast with the above mentioned reports that showed a decrease in urinary N levels starting only after the twelfth month of neonatal life. Our findings confirm the hypothesis that the age-dependent decline of N and the increase of B in children may be attributed to progressive maturation of the biopterin synthetic pathway, mainly of dihydrobiopterin synthetase (DHBS), in the first 3 months of life[10].

For these reasons, the variation in urinary pterin levels should be seriously considered when studying babies of this age.

In conclusion, we have described a rapid, sensitive and precise method for urinary pterin determination that can be applied to the differential diagnosis of the various hyperphenylalaninemic conditions together with mass neonatal screening for the metabolic disorder.

REFERENCES

1 A. M. G. Farci, F. Cabras, G. Cappai, L. Chessa, I. Pillosu, G. Tuveri, A. Lovisello, E. Atzeni, A. Mathieu, P. Usai, R. Laconi, A. Balestrieri, V. L. Garau, M. Tocco and D. Casula, in H. Wachter, H. Ch. Curtius and W. Pfeiderer (Editors), *Biochemical and Clinical Aspects of Pteridines*, Vol. 5, Walter de Gruyter, Berlin, New York, 1987, p. 193.
2 D. Schonitzer, M. Honlinger, D. Fuchs, A. Hausen, G. Reibnegger, E. R. Werner and H. Watcher, in H. Wachter, H. Ch. Curtius and W. Pfeiderer (Editors), *Biochemical and Clinical Aspects of Pteridines*, Vol. 5, Walter de Gruyter, Berlin, New York, 1987, p. 331.
3 S. A. Jones and J. A. Blair, in H. Wachter, H. Ch. Curtius and W. Pfeiderer (Editors), *Biochemical and Clinical Aspects of Pteridines*, Walter de Gruyter, Vol. 5, Berlin, New York, 1987, p. 365.
4 B. Stea, R. M. Halpern, B. C. Halpern and R. A. Smith, *Clin. Chim. Acta*, 113 (1981) 231.
5 S. Kaufman, *J. Pediatr.*, 109 (1986) 572.
6 R. Matalon, *J. Pediatr.*, 104 (1984) 579.
7 H. Ch. Curtius, M. Hausermann, A. Niederwieser and S. Ghisla, in H. Wachter, H. Ch. Curtius and W. Pfeiderer (Editors), *Biochemical and Clinical Aspects of Pteridines*, Vol. 1, Walter de Gruyter, Berlin, New York, 1982, p. 27.
8 J. L. Dhondt, *J. Pediatr.*, 104 (1984) 501.
9 B. Rossignol, D. Rossignol and C. Petitclerc, *Clin. Biochem.*, 17 (1987) 203.
10 A. Niederwieser, H. Ch. Curtius, R. Gitzelmann, A. Otten, K. Baerlocher, B. Blehova, S. Berlow, H. Grobe, F. Rey, J. Schaub, S. Scheibenreiter, H. Schmidt and M. Viscontini, *Helv. Paediatr. Acta*, 35 (1980) 335.
11 B. Stea, R. M. Halpern, B. C. Halpern and R. A. Smith, *J. Chromatogr.*, 188 (1980) 363.
12 T. Fukushima and J. C. Nixon, *Anal. Biochem.*, 102 (1980) 176.
13 A. Niederwieser, W. Staudenmann and E. Wetzel, *Biochemical and Clinical Aspects of Pteridines*, Vol. 1, Walter de Gruyter, Berlin, New York, 1982, p. 81.
14 B. Stea, R. M. Halpern and R. A. Smith, *J. Chromatogr.*, 168 (1979) 385.
15 J. L. Dhondt, C. Largillicre, P. Ardouin, J. P. Farriaux and M. Dautrevaux, *Clin. Chim. Acta*, 110 (1981) 58.
16 H. Rokos, K. Rokos, H. Frisius and H. J. Kirstaedter, *Clin. Chim. Acta*, 105 (1980) 275.
17 A. Niederwieser, W. Staudenmann and E. Wetzel, *J. Chromatogr.*, 290 (1984) 237.
18 J. L. Dhondt, P. Ardouin, J. M. Hayte and J. P. Farriaux, *Clin. Chim. Acta*, 116 (1981) 143.
19 A. Hausen, D. Fuchs, K. König and H. Wachter, *J. Chromatogr.*, 227 (1982) 61.