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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COLUMN SWITCHING FOR THE ANALYSIS OF BIOGENIC AMINE METABOLITES AND PTERINS*

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SUMMARY

An automatic high-performance liquid chromatographic system with facilities for column switching is described which involves minimal pre-treatment of biological samples, separates complex mixtures of compounds in a short period of time and uses fluorimetric or amperometric detection. The system has been applied to the analysis of oxidized pterins in urine and reduced pterins in cerebrospinal fluid and rat brain fractions (*R*- and *S*-enantiomers of tetrahydrobiopterin resolved). The system can also be used for the analysis of most of the dopamine and serotonin metabolites in cerebrospinal fluid and brain fractions from norepinephrine to serotonin.

INTRODUCTION

The metabolism of the biogenic amine neurotransmitters dopamine and serotonin is essential for the normal functioning of the central nervous system. Biosynthesis of both neurotransmitters is dependent on tetrahydrobiopterin (BH_4), a cofactor of tyrosine-3-hydroxylase and tryptophan-5-hydroxylase. The deleterious effect of a deficiency of this cofactor on human health has been recognized only recently by the detection of rare patients suffering from atypical phenylketonuria due to BH_4 deficiency[1]. Since then, growing interest has been focused on the metabolism and analysis of pterins[2,3].

Most investigators engaged in the analysis of biogenic amines and/or pterins are using advanced methods which, however, are applicable only to a few of the many compounds of dopamine and serotonin metabolism. Because some of the metabolites can influence each other by feedback regulation, etc., and because of the minute amounts of material often available, a method is needed by which all important metabolites involved can be analysed simultaneously in the same sample. Biological materials contain thousands of compounds that might seriously interfere with the analysis of these metabolites. Such interferences can be reduced by suitable pre-

* Dedicated to Prof. Dr. H.-Ch. Curtius on the occasion of his 60th birthday.

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treatment of the sample. However, some of the compounds of interest will be lost during the pre-treatment.

It was our purpose to investigate whether high-performance liquid chromatographic (HPLC) column switching techniques can be employed to minimize the pre-treatment of biological samples for the simultaneous analysis of as many as possible of the metabolites of dopamine and serotonin and of pterins. This paper, although preliminary, shows that column switching can be of advantage in the HPLC of biological materials.

EXPERIMENTAL

Chemicals

Pterins were obtained from Dr. B. Schircks, Wettswil a.A., Switzerland, except 6-cyclopropylpterin (synthesized by us; to be published), isoxanthopterin, pterin, pterin-6-carbonic acid, xanthopterin (Fluka, Buchs, Switzerland) and 6-phenylpterin (a gift from Dr. B. Levine, National Institutes of Health, Bethesda, MD, U.S.A.). Biogenic amines and metabolites were obtained from Sigma, St. Louis, MO, U.S.A.). LiChrosorb RP-8 (5 μm) and manganese dioxide was purchased from Merck (Darmstadt, F.R.G.), and MOS Hypersil (3 μm) from Shandon (Runcorn, U.K.).

Abbreviations

B = L-*erythro*-Biopterin [6-(1′,2′-dihydroxypropyl)pterin]; *threo*-B = *threo*-biopterin; BH_4 = L-*erythro*-5,6,7,8-tetrahydrobiopterin; (*R*)BH_4 = (6*R*)-BH_4; (*S*)BH_4 = (6*S*)-BH_4; DA = dopamine; 6,7DMP = 6,7-dimethylpterin; DOPA = 3,4-dihydroxyphenylalanine; DOPAC = 3,4-dihydroxyphenylacetic acid; DTE = dithioerythritol; E = epinephrine; 5HIAA = 5-hydroxyindoleacetic acid; 6HMP = 6-hydroxymethylpterin; 5HT = 5-hydroxytyramine (serotonin); 5HTP = 5-hydroxytryptophan; HVA = homovanillic acid; Ix = isoxanthopterin (7-hydroxypterin); Ix6C = isoxanthopterin-6-carbonic acid; M = monapterin (*threo*-neopterin); MHPG = 3-methoxy-4-hydroxyphenylglycol; 6MIx = 6-methylisoxanthopterin; 6MP = 6-methylpterin; $6MPH_4$ = 6-methyl-5,6,7,8-tetrahydropterin; N = D-*erythro*-neopterin [6-(1′,2′,3′-trihydroxypropyl)pterin]; NE = norepinephrine; P = pterin (2-amino-4-hydroxypteridine); 6ΔP = 6-cyclopropylpterin; $6\Delta PH_4$ = 6-cyclopropyl-5,6,7,8-tetrahydropterin; P6C = pterin-6-carbonic acid; P7C = pterin-7-carbonic acid; 6PP = 6-phenylpterin.

HPLC equipment

The following equipment was used: analytical column (250 × 4.9 mm I.D.), Spherisorb S5 ODS (Kontron, Zurich, Switzerland), 40- and 120-mm empty columns (Knauer, Berlin, F.R.G.); three Model 410 pumps with pulse damping device, mixing chamber, Model 200 programmer, one RE 5302 three-way valve with RE 5300 pneumatic device (Kontron, Zurich, Switzerland); WISP 710 automatic sampler (Waters Assoc., Milford, MA, U.S.A.), four Valco AH-CV-6-UH Pa-N60 six-port valves (Valco Instruments, Houston, TX, U.S.A.); Model 650-10S fluorimeter (Perkin-Elmer, Oak Brook, IL, U.S.A.) with settings 350/450 nm, each slit 10 nm, range 0.3, response normal (2 sec); LC 4A amperometric detector with TL 5A glassy carbon flow cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and HP 3390 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

External standard pterin mixture, blue fluorescing

Biopterin, monapterin, neopterin and pterin, each 0.5 mg/l, and isoxanthopterin, 0.2 mg/l, in 0.1 *M* hydrochloric acid were used. For trials with cofactor analogues, 6-cyclopropylpterin and 6-methylpterin, each 0.5 mg/l in 0.1 *M* hydrochloric acid, were used.

Stock solution of tetrahydrobiopterin and other reduced pterins

Tetrahydrobiopterin dihydrochloride, 0.1 g/l, and dithiothreitol, 1 g/l in deaerated 0.1 *M* hydrochloric acid, were stored frozen in small vials under inert gas. Just before use, the stock solution was diluted with deaerated 0.1 *M* hydrochloric acid.

Stock solutions of biogenic amines and metabolites

Solutions of 5-hydroxyindoleacetic acid, methoxyhydroxyphenylglycol and serotonin, each 100 mg/l in water, plus 50 mg/l dithiothreitol were prepared, together with dihydroxyphenylacetic acid, DOPA, dopamine, epinephrine, homovanillic acid and norepinephrine, each 100 mg/l in 0.1 *M* hydrochloric acid, plus 50 mg/l DTE.

Working standard solutions were prepared by 1:100 dilution of the stock solutions and mixing equal volumes.

Solvents for HPLC

The following solvents were used (all proportions v/v): A, methanol–water (3:97); B, isopropanol–methanol–acetic acid (49:49:2); C, isopropanol–methanol–water (1:1:8); D, 6.6 m*M* Na_2HPO_4–13.3 m*M* citric acid–0.06 m*M* Na_2EDTA–1.4 m*M* octanesulphonic acid–10% methanol (pH 3.3); after degassing *in vacuo*, crystalline DTE was added to give a concentration of 0.16 m*M*; E, same as D, but 10% isopropanol was used instead of methanol; F, 20 m*M* KH_2PO_4–0.85 m*M* octanesulphonic acid–0.1 m*M* Na_2EDTA–1% methanol, pH adjusted to 3.0 with 0.1 *M* perchloric acid; G, same as F, but containing 6.5% methanol.

Oxidation of reduced pterins

For stabilization, to simplify the analytical system or to permit specific and sensitive fluorimetric analysis, reduced pterins can be oxidized, as described for urine in the following: 500 µl of urine, preferably fresh (when frozen, the specimen was thawed by microwaves and mixed by ultrasonication), was acidified to pH 1–1.5 with 6 *M* hydrochloric acid (usually 20 µl were sufficient). Manganese dioxide (10 mg) was added and the mixture was shaken gently for 5 min, then 5 ml of water were added (or only 2 ml if the urinary creatinine level was less than 2 mmol/l) and the suspension was centrifuged at 2000 *g* for 5 min. Of the clear supernatant, 200 µl were transferred without delay into a vial for HPLC. This solution was stable for 1 month at 4°C when protected from light.

Homogenization of brain tissue

Brain tissue (1 part) was homogenized at 0°C in 4 volumes of 0.1 *M* hydrochloric acid containing ascorbic acid (100 mg/l), centrifuged at 18,000 rpm (40,000 *g*) for 20 min. The supernatant was used for HPLC.

Deproteinization of cerebrospinal fluid

Aqueous 30% (w/v) trichloroacetic acid (20 µl) was added to 100 µl of cere-

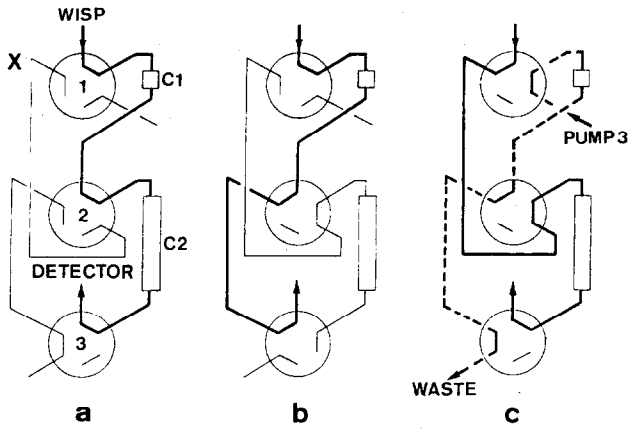

Fig. 1. Connections and positions of the three high-pressure valves used for column-switching HPLC of oxidized pterins. Status (a), 0–2 min, injection of sample with columns C1 and C2 in series. Status (b), 2–12 min, gradient elution of slow-moving compounds from C1 into detector. Status (c), 12–32 min, elution of faster moving compounds from C2 into detector; simultaneously, cleaning of C1.

brospinal fluid, kept at 0°C for 10 min and centrifuged at 10,000 *g* in an Eppendorf centrifuge for 2 min. The supernatant was used immediately for HPLC.

HPLC of oxidized pterins (blue fluorescing)

Pumps 1 and 2 delivered solvents A and B into a mixing chamber and the autosampler. The sample was injected through high-pressure valve 1 on to pre-column C1 (40 × 4.6 mm I.D., 5 μm, LiChrosorb RP-8) connected in series through high-pressure valve 2 with the analytical column C2 (250 × 4.9 mm I.D., Sperisorb S5 ODS). The eluent flowed through high-pressure valve 3 into the fluorimetric detector. Both columns C1 and C2 were equilibrated with solvent A (see above). The faster moving compounds up to and including pterin were chromatographed with solvent A into C2 (Fig. 1, status a). After 2 min, before any of the compounds were able to elute from the analytical column, C2 was disconnected and stopped temporarily by switching valves 2 and 3 (Fig. 1, status b). The slow-moving compounds were then eluted from the pre-column C1 into the detector using a solvent gradient (5–20% B within 5 min). Thus, the slow-moving compounds appeared on the chromatogram first. Thereafter, the pre-column was disconnected and thoroughly rinsed twice with solvents B and C, delivered through low-pressure valve 4 by pump 3 (Fig. 1, status c). During this time, the compounds temporarily trapped within the analytical column were eluted with solvents A and B. After elution of pterin, both columns were connected in series and equilibrated again. For details of the programme, see Table I.

HPLC of reduced pterins

HPLC of reduced pterins was performed on pre-column C1 and analytical column C2 (see above(with isocratic elution using solvent D (for BH_4 and 6-methyl-PH_4) and E (for 6-cyclopropyl-PH_4), respectively. The solvents were modifications of that described by Bräutigam *et al.*[4]. The pterins were detected amperometrically

TABLE I

PROGRAMME FOR HPLC OF OXIDIZED PTERINS WITH COLUMN SWITCHING

The auxiliary functions (flags) Aux 1–3 switch the three high-pressure valves (see Fig. 1). Aux 4 activates pump 3 operating at a flow-rate of 1 ml/min. Aux 5 switches on valve 4 for solvent C (at off-position solvent B is running, as long as pump 3 is activated).

Time (min)	*Function*	*Duration (min)*	*Remarks*
0	0% solvent B; flow 1.3 ml/min		Solvent A is running
2	Aux 2	30	Disconnects column 2
	Aux 3	10	Connects column 1 with detector for 10 min
2.1	5% solvent B		
4.0	20% solvent B	5	5–20% B within 5 min
9.0	0% solvent B; flow 2 ml/min		
12.0	Aux 1	20	Connects column 2 with detector for 20 min
	Aux 4	20	Starts pump 3 for 20 min (1 ml/min)
	Aux 5	5	Rinses column 1 with solvent C for 5 min
	Flow 1.2 ml/min		
19.0	20% solvent B	10	0–20% within 10 min
22.0	Aux 5	5	Rinses column 1 with solvent C for 5 min
29.0	100% solvent B		
32.0	0% solvent B		Equilibrates with solvent A
53.0	Aux 1	1	Rinses capillary X (Fig. 1) with solvent A
55.0	Flow 1.3 ml/min		Next sample injected

at 250 mV, range 0.2 nA. The flow-rate was 1.2 ml/min and the run time was 20 min for solvent D and 15 min for E. The autosampler was placed in a cooled room at 4°C. After 20–30 injections, the columns were rinsed with methanol–isopropanol (1:1, v/v).

HPLC of biogenic amines and metabolites

Three pumps were needed, pumps 1 and 2 delivering solvents F and G (see above) into a mixing chamber and the autosampler. Pump 3 delivered solvent F for rinsing the two short columns C1 and C3 (40 × 4.6 mm I.D., 3 μm MOS Hypersil). The positions and connections of the four high-pressure valves used for column switching are shown in Fig. 2 (only three valves were necessary for the following programme; however, see below). Before injection, all columns, including the analytical column C2 (250 × 4.9 mm I.D., Spherisorb S5 ODS), were equilibrated with solvent F. For injection of the sample, columns C1 and C2 were connected in series (Fig. 2, status a). Four minutes later, columns C1 and C3 were connected in series to the amperometric detector for elution and further separation of the slow-moving compounds such as 5-HIAA, DA, HVA and 5HT (Fig. 2, status b). The last com-

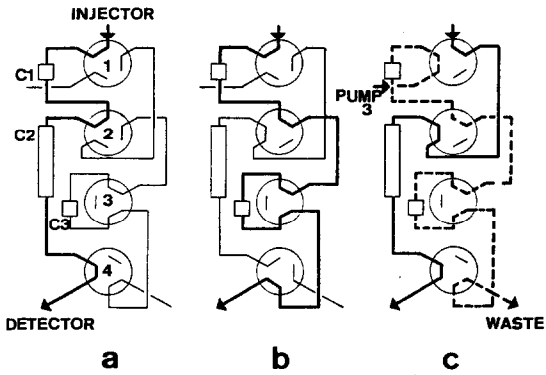

Fig. 2. Connections and positions of high-pressure valves used for column-switching HPLC of biogenic amines and metabolites. Status (a), 0–4 min, injection of sample with columns C1 and C2 in series. Status (b), 4–28 min, short columns C1 and C3 in series, elution of slow-moving compounds using a gradient. Status (c), 28–48 min, elution of faster moving compounds from C2; simultaneously, re-equilibration of columns C1 and C3.

pound was eluted by a step gradient. Thereafter, the fast-moving compounds such as NE, MHPG, DOPA, DOPAC and E, which were temporarily trapped within column C2, were eluted isocratically by solvent F (Fig. 2, status c). Simultaneously, columns C1 and C3 were re-equilibrated by pump 3 and solvent F. Details of the programme (run time 50 min) are shown in Table II.

TABLE II

PROGRAMME FOR HPLC OF BIOGENIC AMINES AND METABOLITES USING COLUMN SWITCHING

The auxiliary functions (Aux 1–4) switch the high-pressure valves 1–4 (see Fig. 2). Aux 5 activates pump 3. While switching the valves, the solvent flow was always stopped to reduce drastic pressure changes.

Time (min)	*Function*	*Duration (min)*	*Remarks*
0	0% solvent G; flow 1.5 ml/min		Solvent F is running
3.9	Flow 0 ml/min		
4.0	Aux 2	44	Disconnects column 2, connects C1 with C3
	Aux 4	24	Connects C3 with detector
4.1	Flow 1.5 ml/min		
14.0	100% solvent G		
24.0	0% solvent G		
27.9	Flow 0 ml/min		
28.0	Aux 1	20	Connects C2 with detector
	Aux 5	19.9	Pump 3 is activated for re-equilibration of C1 and C3 with solvent F
47.9	Flow 0 ml/min		
48.1	Flow 1.5 ml/min		
50.0			Next sample injected

Valve 3 can be used to separate a pair of compounds unresolved on column C1 on a further column (C3) filled with a different adsorbent. For example, dopamine and 5-hydroxytryptophan cannot be separated on C1. However, they can be separated on a short column of 3 μm Hypersil ODS. In this instance, at time 4 min, just before this pair of compounds elute from C1, valve 3 is switched as long as necessary to transfer both on to C3 (*ca.* 1.5 min). At time 28 min, valve 3 is switched again (Fig. 2, status b) to elute dopamine followed by 5-hydroxytryptophan. Then the programme (Table II) is continued with elution of the compounds from C2.

Cleaning of HPLC columns

When a high back-pressure was built up or chromatographic resolution was lost, the column was cleaned by rinsing sequentially as follows: 20–50 ml of solvent B, 70–100 ml of chloroform, 20–50 ml of solvent B, 100-ml of 1 *M* pyridinium acetate (pH 5) (concentration based on pyridine), *ca.* 500 ml of solvent C (overnight), 50–70 ml of 5% acetic acid, equilibration with solvent A. A "dead volume" at the top of the column (shoulder and broadening of all peaks) was corrected by filling up with the corresponding adsorbent material.

Cleaning of the analytical column became necessary only after *ca.* 200–300 injections of urine samples. After cleaning by the procedure outlined above, the same column could be used further. For safety reasons, the pre-column was replaced after about 100 injections.

RESULTS AND DISCUSSION

HPLC of oxidized, blue-fluorescing pterins

The oxidation conditions controlled with respect to the amount of oxidizing

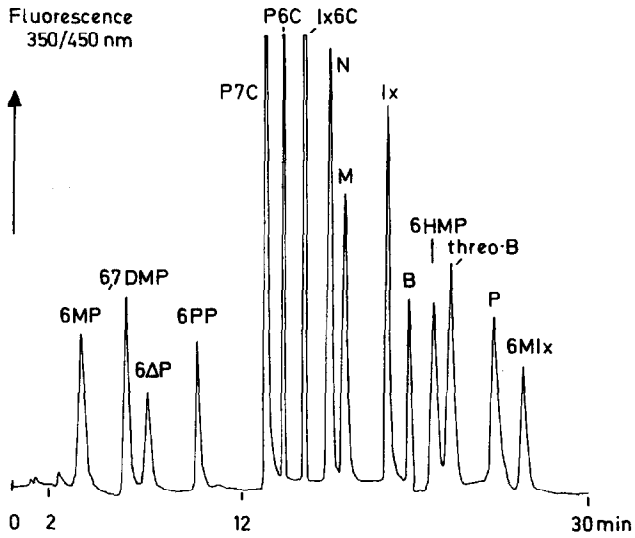

Fig. 3. HPLC of a mixture of oxidized pterins using the column-switching program in Table I. Between 2 and 12 min the slow-moving compounds were eluted first from the short column C1. The amounts injected varied in this mixture from 5 to 20 ng.

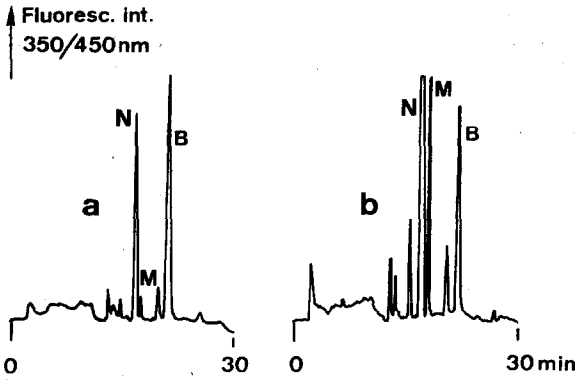

Fig. 4. HPLC of urinary pterins (oxidized) from a healthy control and a patients with AIDS syndrome. Note the dramatically increased neopterin (N) and monapterin (M) and normal biopterin concentrations. In native urine, most of the neopterin was present as 7,8-dihydroneopterin. The column-switching programme in Table I was used.

reagent added, reaction time and pH, and the reproducibility of the whole procedure (coefficient of variation better than ±4%) were all satisfactory and have been described in detail elsewhere[5]. The recovery of reduced pterins added to urine depended on the species and the amount, and ranged from 90% (dihydroneopterin) to 97% (tetrahydrobiopterin). The limit of detection of biopterin (at 350/450 nm, slits 10/20, range 3) was 50 pg per injection at a signal-to-noise ratio of 5:1.

A chromatogram of a standard mixture is shown in Fig. 3. Even non-polar pterins such as 6-phenylpterin eluted as sharp peaks between 2 and 12 min. A chromatogram of urinary pterins from a patient with aquired immune deficiency syndrome (AIDS) is shown in Fig. 4. This technique has been used successfully for 2 years for screening of BH_4 deficiency[6,7] and a new variant of hyperphenylalaninaemia, GTP cyclohydrolase I deficiency, was detected[8].

HPLC of reduced pterins

5,6,7,8-Tetrahydropterins are very labile against oxygen. Dilute solutions can be stabilized by thiol additives such as DTE. Tetrahydropterins can be detected amperometrically at 250 mV where the response of many other oxidable compounds is still low[9]. Chromatograms of reduced pterins are shown in Fig. 5. Synthetic BH_4 is a *ca.* 2:1 mixture of the 6*R* and 6*S* enantiomers. Both were separated by HPLC[4,5,9,10], the 6*R* enantiomer moving faster. In biological materials only the 6*R* enantiomer could be found. The method was applied to *in vivo* studies of penetration of BH_4 and cofactor analogues such as 6-methyltetrahydropterin ($6MPH_4$) and 6-cyclopropyltetrahydropterin ($6PH_4$) into rat brain. By difference measurements it was shown that most of the pterins were present as tetrahydro compounds[11]. The method was also applied for measurement of BH_4 in human milk and for purity control of BH_4 preparations.

HPLC of biogenic amines and metabolites

The separation of catecholamines, serotonin and their metabolites detected amperometrically is shown in Fig. 6. Similarly to the HPLC method for oxidized

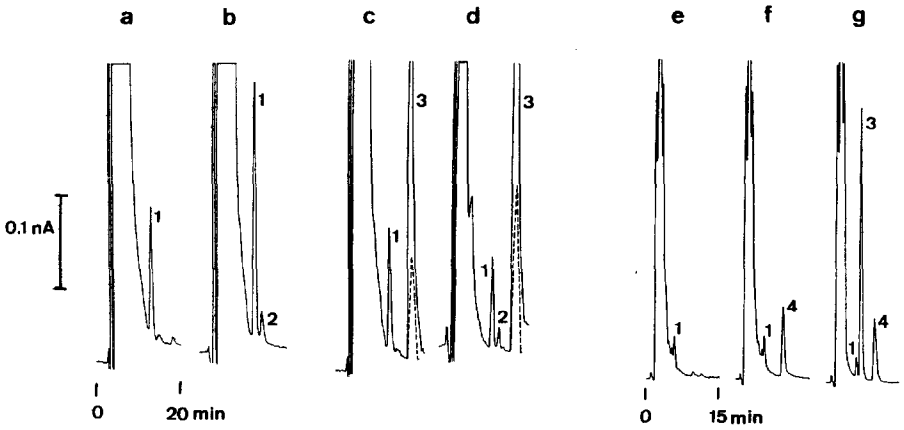

Fig. 5. HPLC of reduced pterins in rat brain (cerebellum); 30 μl $\approx$ 6 mg of tissue. (a) Control; (b) after BH_4, 50 mg/kg i.p.; (c) after $6MPH_4$, 59 mg/kg; (d) standard, 1.8 pmol(*R*)-BH_4 plus 31 pmol $6MPH_4$; (e) control, 10 μl $\approx$ 2 μg of tissue; (f) after $6PH_4$, 50 mg/kg i.p.; (g) standard, 0.3 pmol BH_4, 5.3 pmol $6MPH_4$ and 2.3 pmol $6PH_4$. (a)–(d) were chromatographed with solvent D, (e)–(g) with solvent E. 1 = (*R*)-BH_4; 2 = (*S*)-BH_4; 3 = $6MPH_4$; 4 = $6PH_4$. Dashed lines: detector range 1.

pterins, the slow-moving compounds appeared first on the chromatogram. The second short column (C3) became necessary to separate 5HIAA from the front peak appearing in biological materials. Serotonin, which binds strongly even to MOS Hypersil, could be eluted in a reasonable time and with a good peak shape only with the aid of a gradient. Because the amperometer detector is sensitive to concentration changes of the mobile phase, an increase in the base signal (depending on the sen-

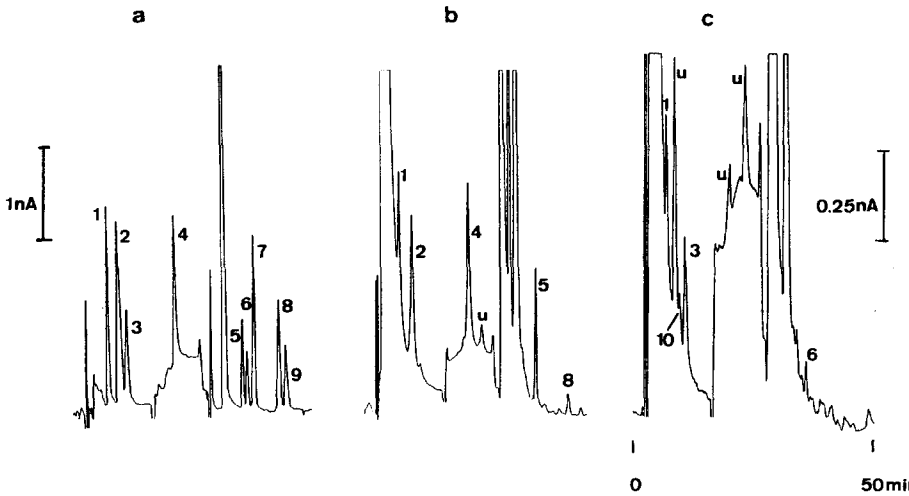

Fig. 6. HPLC of biogenic amines and metabolites with amperiometric detection at 700 mV. (a) Standard mixture, 20–30 pmol each, in 20 μl injected; (b) whole rat brain homogenate, 20 μl injected, equivalent to 4 mg of tissue; (c) deproteinized cerebrospinal fluid, 25 μl injected. Range settings were 2 nA (a and b) and 0.5 nA (c). 1 = 5HIAA; 2 = DA, 3 = HVA; 4 = 5HT; 5 = NE; 6 = MHPG; 7 = DOPA; 8 = DOPAC; 9 = E; 10 = 5HTP; u = unidentified.

sitivity) had to be tolerated. Using modern integrators, this increase could be compensated fully. The fast-moving compounds were eluted thereafter from the longer C2 column (Spherisorb ODS C-18). Even norepinephrine was separated from the large front peak. Because of the higher sensitivity required, this method is less suitable for measurement of the metabolites in CSF (Fig. 6).

Although these results are preliminary and extended studies are necessary, it can be concluded that column switching techniques are of advantage in the analysis of biological materials. Sample pre-treatment can be reduced or even omitted without rapid deterioration of the analytical column; only the short pre-column has to be replaced from time to time. A combination of weak and strongly absorbing chromatographic materials is of special advantage when complex mixtures have to be analysed and no or only moderate gradients can be used, as with amperometric detection. Unresolved pairs or groups of compounds can be automatically cut out and re-chromatographed on a column better suited for this purpose.

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