SIMULTANEOUS DETERMINATION OF DOPAMINE, SEROTONIN AND THEIR METABOLITES IN THE RAT BRAIN BY HPLC METHOD WITH COULOMETRIC DETECTION

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A rapid and sensitive method for simultaneous determination of 3-hydroxytyramine (dopamine), 5-hydroxytryptamine (serotonin) and their metabolites – 3,4-dihydroxyphenylacetic acid, 3-methoxytyramine, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and 5-hydroxyindole-3-acetic acid in the rat brain was developed. Brain samples with the internal standard and heparin were deproteinized by perchloric acid with ethylenediaminetetraacetic acid disodium salt and sodium sulfite. Following homogenization, centrifugation and filtration, the supernatant was directly injected into a reversed-phase HPLC system with coulometric detector. The response of the detected substances was linear in the range 12–700 ng/g of cerebellum homogenate (24–1 400 pg on column). Total recovery of the method was higher than 95%. The method was used for the determination of catecholamines and their metabolites in the chosen part of rat brain following the inhalation exposure to sarin (organophosphate).

Key words: Chromatography; HPLC; Coulometry; Dopamine; Serotonin; Sarin; Brain; Acetylcholinesterase; Electrochemistry; Organophosphates.

HPLC methods with electrochemical detection are believed to be the best for the determination of substances with indole moiety in their chemical structure including catecholamines[1–3]. The use of the electrochemical detector with series of three coulometric electrodes, working in the oxidation-reduction mode, markedly increases the sensitivity and selectivity of this method in comparison with classic amperometry and UV detection[1–5].

This method was used to evaluate an influence of highly toxic organophosphate (OP) sarin on some catecholamines in a part of the rat brain. The irreversible inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) with subsequent accumulation of acetycholine in the cholinergic synapses and overstimulation of the cholinergic nervous system is believed to be the major mechanism of its toxic effects[6–7]. Nevertheless, there are other toxic non-cholinergic effects of organophosphates, such as mutagenic, immuno-

toxic, hepatotoxic and stressogenic effects[8]. A significant increase in the activity of hypothalamo-pituitary-adrenergic as well as sympatho-adrenergic systems following severe poisonings with OP compounds was clearly documented[9,10]. However, very little is known about the long-term stressogenic effects of low-level exposure to OP compounds including the influence on catecholamines in the central nervous system. Therefore, HPLC methods with coulometric detection was used to demonstrate possible effects of low-level sarin exposure on catecholamine levels in the rat brain.

EXPERIMENTAL

Chromatography

The chromatographic system consisted of SpectraSERIES model P200 gradient pump (Spectra-Physics, Fremont (CA), U.S.A.), model 7125 high-pressure injection valve fitted with a 10 µl sample loop (Rheodyne, Cotati (CA), U.S.A.), an analytical stainless steel column Purospher RP-18E (5 µm), 124 × 4 mm (i.d.) (Merck) with a pre-column Purospher RP-18 (5 µm), 4 × 4 mm (i.d.) (Merck), Coulochem II Multi-Electrode detector (ESA, Bedford (MA), U.S.A.) with three electrodes – model 5011, high-sensitive analytical cell consisting of two working electrodes (an enhanced response amperometric electrode is coupled with a coulometric electrode in stainless steel body) and model 5021, single porous grafite electrode, is placed immediately before the analytical cell to provide additional selectivity.

The elution profiles were integrated and recorded using a Chromatographic Station for Windows, version 1.6 (Data Apex, s.r.o., Prague, Czech Republic) (CSW).

The mobile phase consisted of 50 mmol/l sodium phosphate, 50 mmol/l sodium acetate, 0.6 mmol/l sodium octanesulfonate, 0.6 mmol/l EDTA and 9 vol.% acetonitrile. The final pH adjusted to 3.1 with concentrated (850 g/l) orthophosphoric acid and filtered under vacuum through a 0.45 µm HV filter (Millipore, Bedford (MA), U.S.A.). The flow rate was 0.5 ml/min. The column was equilibrated with the mobile phase at least 10 h before use. The first electrode in the detector cell was set at a potential of +50 mV (screen electrode) and the second electrode was set at –350 mV. The conditioning cell potential was +350 mV (ref.[3]). Output was monitored from the second electrode. Full-scale sensitivity was 50 nA.

Chemicals

All used chemicals were of analytical or HPLC grade. Dopamine hydrochloride (DA), serotonin creatinine sulfate (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine hydrochloride (3-MT), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and the internal standard *N*-isopropyl-DL-noradrenaline hydrochloride (isoproterenol, ISTD) were purchased from Fluka; acetonitrile, phosphoric acid, perchloric acid (PCA), ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), ethylenediaminetetraacetic acid (EDTA) and sodium sulfite from Merck; heparin (25 000 IU/ml) from Léčiva (Prague, Czech Republic). Water was purified by reverse osmosis and filtration (apparatus Aqua 50, Goro, s.r.o., Czech Republic).

Preparation of Standard Solutions

Spare standard solutions of DA, 5-HT, DOPAC, 3-MT, HVA, 5-HIAA and ISTD (each 0.3 mg/ml) were prepared in 0.1 mol/l $HClO_4$. The solutions were stored at –70 °C and freshly prepared every four weeks. Working mixture standards (150–705 ng/ml) were prepared on the day of assay by dilution of stock standard solutions with 0.1 mol/l $HClO_4$ containing 0.3 mmol/l Na_2EDTA and 0.5 mmol/l Na_2SO_3 (ref.[2]).

Standard Curve

An amount of 0.1 ml of working mixture standard (containing DA, 5-HT, DOPAC, 3-MT, HVA, 5-HIAA and ISTD) was added to an 0.9 g aliquot of pooled cerebellar homogenates[11] in glass centrifugal test-tubes with 3.5 ml 0.4 mol/l perchloric acid (containing 2 mmol/l sodium sulfate and 1.2 mmol/l Na_2EDTA) and 0.7 ml heparin (further elaboration see below). The levels of endogenous compounds contained in aliquots of the same homogenates without these added exogenous compounds were less than a detection limit.

Preparation of Samples

Removed rat brain was immediately frozen with liquid nitrogen and stored in a freezing box in –70 °C for further preparation. The chosen part of rat brain (frontal slice – anteroposterior distance interaural bregma 1.2 mm)[12] was weighed (*ca* 0.9 g) and placed into glass centrifugal test-tubes with 3.5 ml 0.4 mol/l perchloric acid (containing 2 mmol/l Na_2SO_4 and 1.2 mmol/l Na_2EDTA), 0.7 ml heparin[2] and 0.1 ml (705 ng) of the internal standard. Following the homogenization with Ultraturax, the reaction mixture was centrifuged at 4 000 G for 10 min and filtered. The supernatant was injected into the HPLC system in a volume of 10 μl.

The concentrations of DA, 5-HT, DOPAC, 3-MT, HVA and 5-HIAA were determined by comparing the peak area ratios of DA : ISTD, 5-HT : ISTD, DOPAC : ISTD, 3-MT : ISTD, HVA : ISTD, 5-HIAA : ISTD to those of linear standard curves.

Exposure Experiments

Male albino SPF rats weighing 180–220 g, purchased from VÚFB (Konárovice, Czech Republic) were divided into groups of ten animals and exposed to low concentrations of sarin (Military Technical Institute 072, Zemianské Kostoľany, Slovak Republic) in the inhalation chamber for 60 min. Three levels of sarin were used for the inhalation exposure of rats:

– level 1 resulting in no clinical symptoms and erythrocyte AChE inhibition lower than 20% following a 60 min exposure (0.8 μg/l)

– level 2 resulting in no clinical symptoms but a moderate erythrocyte AChE inhibition (about 20%) following a 60 min exposure (1.25 μg/l). This level was used for a single (2S) or repeated (2R) exposure (three times per week)

– level 3 resulting in mild clinical symptoms such as salivation and miosis without convulsions and an erythrocyte AChE inhibition of 40–50% following a 60 min exposure (2.5 μg/l).

Twelve months after exposure to sarin, the rats were killed by exsanguination in total anaesthesia, their brains were removed and immediately frozen with liquid nitrogen. The experimental animals were handled under supervision of the Ethics Committee of the Medical Faculty of Charles University and the Military Medical Academy in Hradec Králové.

Data Analysis

The experimental data were compared with the control values obtained from the rats exposed to pure air instead of sarin. The statistical significance was determined using Student's *t*-test, the differences being considered significant when $P < 0.05$ (ref.[13]).

RESULTS AND DISCUSSION

Typical chromatograms of standards DA, DOPAC, 3-MT, 5-HT, 5-HIAA, HVA and ISTD in perchloric acid and chromatograms of the same substances present in the chosen part of rat brain are shown in Fig. 1.

A linear response to each standard was found in the range 12–700 ng/g cerebellar homogenate (24–1 400 pg on column) ($r > 0.992$, $n = 6$). The sensitivity limit (as defined by peak area = 3 × baseline noise) was 24 pg on the column. The withing-day coefficient of variation at 20 ng/g cerebellar ho-

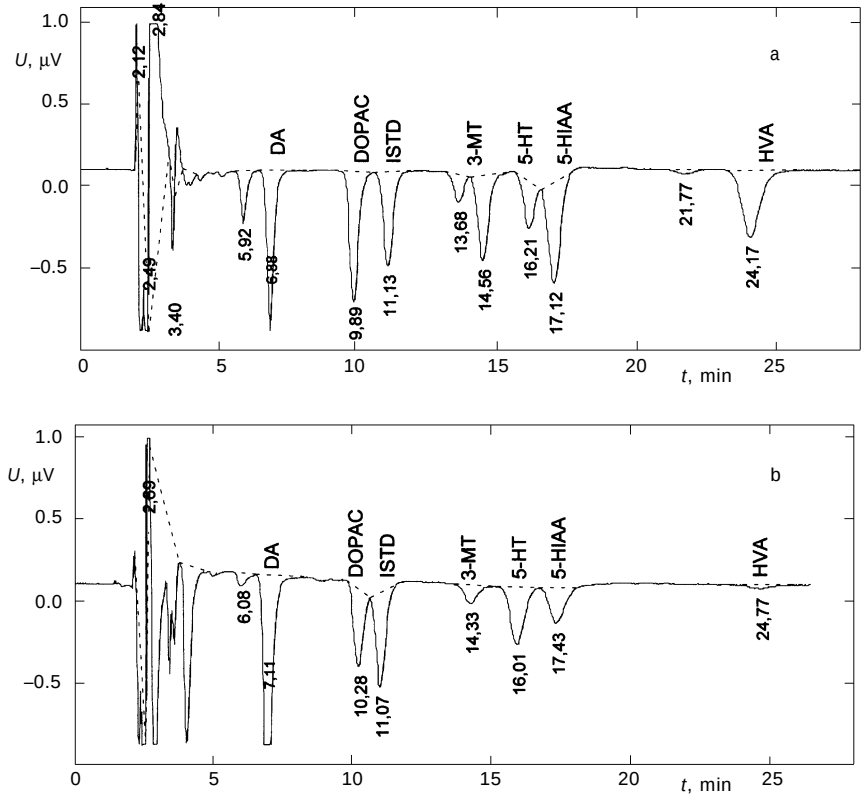

FIG. 1

Chromatogram of standards (a, 15 ng of each substance injected) and of rat brain extract (b)

mogenate was <5% (n = 5). The between-day coefficient of variation was <7% (n = 5). The analytical recovery for each substance was calculated by comparison of its concentration found in a cerebellum homogenate sample with an added known amount of the detected substance with its concentration in a sample containing the same amount of substance in perchloric acid in the same volume. The relative recoveries of 350 ng/ml DA, DOPAC, 3-MT, 5-HT, 5-HIAA, HVA and ISTD from cerebellar homogenate was 100 ± 1%, 100 ± 1.2%, 100 ± 1%, 96 ± 1%, 95 ± 0.2%, 100 ± 0.2% and 99 ± 0.8%, respectively (mean ± standard deviation, n = 8).

A comparison of control and experimental values of both tested catecholamines (5-HT, DA) as well as of their metabolites in the chosen part of rat brain in twelve months after the low-level sarin inhalation exposure is demonstrated in Fig. 2. The values for both catecholamines (5-HT, DA) significantly increased after sarin exposure regardless of its dose (P < 0.05). On the other hand, the changes in their metabolite levels are negligible with the exception of 5-HIAA which also significantly increased using the same doses of sarin (Fig. 2).

Our results confirm that not only high doses of OP (refs[14,15]) but also low, clinically asymptomatic doses of OP such as sarin can stimulate the central catecholaminergic system. Thus, besides stressogenic markers describing an

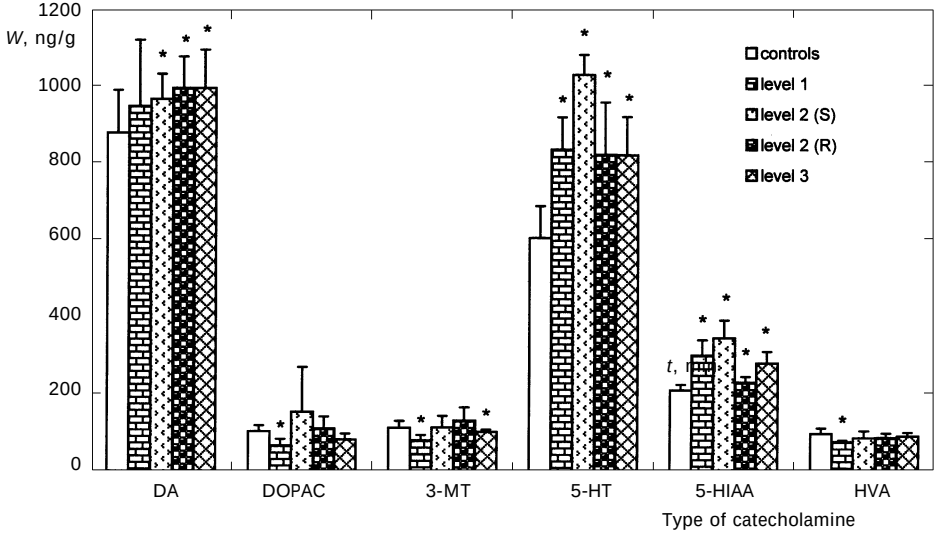

FIG. 2

The changes in serotonin, dopamine and their metabolite levels (mean content ± standard deviation) in rat brain twelve months after exposure to low dose of sarin. Significant at P < 0.05

increased activity of the hypothalamo-pituitary-adrenergic system, central catecholamines such as serotonin and dopamine describing an increased activity of the central catecholaminergic system are also sufficiently sensitive to demonstrate small but significant long-term stressogenic effects of low level exposure to OP.

In addition, the new HPLC method using electrochemical detector Coulochem II was found to be sufficiently sensitive to detect very small changes in brain levels of catecholamines and their metabolites following exposure to asymptomatic levels of OP such as sarin.

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