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ISOLATION OF BRAIN MONOAMINES BY HIGH-PERFORMANCE REVERSED PHASE LIQUID CHROMATOGRAPHY

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The development of high-performance liquid chromatography (HPLC), together with the introduction of electrochemical detectors (ED), has made the procedure of determination of monoamines and their precursors and metabolites in brain samples weighing only a few milligrams universally available. Early studies [8, 9] were based on cation-exchange chromatography with glass or metal columns 300-1000 mm long. The effectiveness of these columns for catecholamines was shown to be relatively low. Columns packed with a reversed phase, in combination with a mobile phase containing an ion-pair agent, were found to be much more suitable [2, 10].

To analyze monoamines the writers have used a microcolumn version of HPLC which has several advantages, especially when tissue samples of small size are used [1]. The internal diameter of the stainless steel columns was 2 mm and their length 60 or 120 mm. The eluant was supplied in some experiments by a "Du Pont 8800" pump (USA), in others by means of a pump of syringe type with electromechanical drive from an MSFP-3 microspectrophotometer (made by Novosibirsk Institute of Organic Chemistry, Siberian Branch, Academy of Sciences of the USSR). The sample was introduced either through a "Rheodyne 7124" injector (USA) or through the sample introducing unit of the "Milikhrom" chromatograph.

Concentrations of monoamines in the peaks were measured by means of an ED with glass-carbon working surface of the cell. The cell was designed and made on the basis of drafts and from materials described in the literature [4, 5, 10]. The electronic circuit of the instrument is similar to that given by Keller et al.[3], with minor modifications. The glass-carbon plate was cut out of chemical crucible glass of Soviet manufacture.

In this paper we examine the questions of packing the columns with sorbent, choice of stationary and mobile phases, preparation of specimens and standards, and reduction of the noise level during chromatography of monoamines in brain samples weighing a few milligrams.

Stationary Phase

We compared the properties of four types of sorbents: Lichrosorb RP_{18} (from Merck, West Germany), Silasorb C_{18} and C_8 (from Lachema, Czechoslovakia), and Nucleosil C_8 (Macherey, Nagel, West Germany). All sorbents had a particle diameter of 5 μ. Analysis showed that for catecholamines Lichrosorb RP_{18} and Silasorb C_{18} have approximately identical properties for use with catecholamines. Under selected conditions of

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chromatography, without the use of an ion-pair agent, they gave fair revolution (height of theoretical plate between 6 and 15 diameters of sorbent particles). The spherical sorbent Nucleosil was 1.5 times more effective for catecholamines and had a column life 2-5 times longer. The sorbent Silasorb C_8 occupies an intermediate position as regards quality of separation of amines. The reason for differences in the properties of the sorbents is probably a difference in the technologies of production of the silica gels and their modification.

Column Packing

A 1% or 2% suspension of the sorbent in acetone was poured into a special high-pressure cylinder 500 mm long and 9 mm in diameter, to the top part of which a well washed column was fixed by means of a connecting nut. The quantity of suspension poured in was chosen so that the weight of sorbent in it was 10% greater than the weight of sorbent introduced into the column (with a density of about 1). Acetone was pumped through the cylinder and column from below upward with an MMC pump (Czechoslovakia) at the rate of 0.5-3 ml/min under pressure control. When the pressure reached about 200 atm the speed was chosen to be sufficient to maintain this pressure. Altogether 60 ml of acetone was pumped through. Next, 30 ml of methanol was pumped through under a pressure of 300-350 atm. The column was washed under the same pressure with a mixture of water and methanol (1:1) and the procedure ended with equilibration of the column with the mobile phase, i.e., pumping through the eluant for 1-2 h at the rate of 0.1-0.2 ml/min.

Mobile Phase

Acetate-citrate buffer, used as the mobile phase, was made up as follows: 0.1 mole of sodium acetate was dissolved in 700 ml of bidistilled water, and 100 ml methanol and 50 ml acetonitrile were added, followed by 2-4 mmoles of sodium octylsulfonate and 0.05 mmole EDTA. The pH was adjusted to 4.8 with citric acid and bidistilled water was added up to 1 liter. All reagents were of the highest grade of purity. After preparation of the eluant it was filtered through Millipore (USA) or Synpor (Czechoslovakia) filters with pore size of 0.3-0.45 μ. The day volume of eluant was degassed in vacuo.

Comparison of the properties of the eluant with the widely used phosphate buffer showed that both have their advantages and disadvantages. Acetate-citrate buffer has a much lower ED noise level. Sensitivity during working with this buffer is almost three times higher. However, acetate buffer cannot be used at pH values below 4.2, because the pK_a for acetic acid is 4.75. This makes it difficult to determine some of the products of catecholamine metabolism. In our case, however, these limitations could be disregarded in favor of the higher sensitivity. The ion-pair agent, sodium octylsulfonate, was synthesized and purified by the method in [7].

Lowering the Noise Level

Since contamination of the eluant with metals considerably increases detector noise and limits the sensitivity of the method, reduction of contact between eluant and metal of the chromatograph is very important. It is particularly important for microcolumn chromatography, in which the rate of flow of the eluant along metal channels is slow. To reduce noise, the eluant was allowed to stand in the apparatus for 10-15 h, and was then poured out with the column disconnected. About once a week the system also was washed out with buffer with a high EDTA concentration (10 mM).

When push-pull pumps (Du Pont 8800 or MMC) were used to supply the eluant, a gas damper was fitted along the path of the eluant in order to suppress the pulsations to which the ED is highly sensitive. In its design the damper consisted of a high-pressure tube in which a bubble of argon or air was compressed to the working pressure of the eluant.

Preparation of Specimen

The rats were decapitated, the brain removed in the course of about 1 min, and immersed for 2-5 sec in liquid nitrogen. The solidified tissue was thawed to a state suitable for preparation of slabs 1.5-2 mm thick, cut in the frontal plane. The brain area of interest, identified by the atlas [6], was cut out of the slabs with a tube 1.8 mm in diameter with sharpened edges. The specimen was weighed and homogenized on an ice bath in 0.1 M perchloric acid, by means of a UDTN-1 or MSE (England) ultrasonic homogenizer. The homogenate was centrifuged for 15-20 min at 11,000 rpm on a K-24 centrifuge (East Germany). The supernatant was drawn off and 10-20 μl of it was introduced directly into the chromatograph. In the next experiments, to increase the column life, the supernatant was additionally filtered by centrifugation through a conical filter, packed with GFC glass filter material (Whatman, England) by the method in [10].

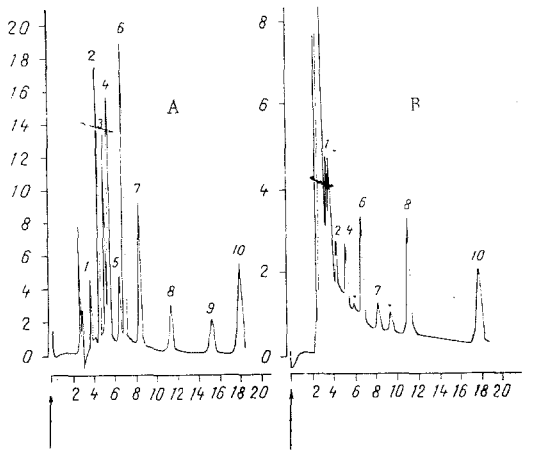

Fig. 1. Chromatograms of mixture of standards (A) and tissue sample from amygdaloid complex weighing 9 mg (B). Abscissa, time (in min); ordinate, response of detector (in nA). 1) DOPA; 2) dihydroxyphenylacetic acid; 3) 5-hydroxytryptophan; 4) noradrenalin; 5) homovanillic acid; 6) 5-hydroxyindoleacetic acid; 7) dopamine; 8) isoproterenol (internal standard); 9) 3-methoxytyramine; 10) serotonin, unidentified peaks marked by asterisk. Arrow indicates application of sample. Standards of homovanillic acid and isoproterenol were used in concentrations of $400 \cdot 10^{-9}$ and $300 \cdot 10^{-9}$ g/ml respectively, others in a concentration of $200 \cdot 10^{-9}$ g/ml.

Standards

Concentrated solutions of standards were prepared in the proportion of 1 mg of base to 1 ml of 0.1 M $HClO_4$ and kept at −70°C for up to 6 months. Solutions of working concentrations (200 ng/ml) were prepared every 2-3 days and kept at 2-4°C. Isopropylnoradrenalin, a synthetic preparation with the trade name of "Izadrin" (isoproterenol) was used as the internal standard. The retention time of this substance is not the same as that of any of the substances in which we are interested, and it is therefore very convenient for use to calibrate chromatograms.

The concentration of a substance in the sample was calculated by the equation:

$$K^{s}_{sa} = \frac{H^{s}_{sa} \cdot H^{st}_{st}}{H^{s}_{st} \cdot H^{st}_{sa}} \cdot C^{s}_{st}$$

where C^{s}_{sa} and C^{s}_{st} denote concentrations of the test substance in the sample and standards respectively; H^{s}_{sa} and H^{s}_{st} the heights of the peaks of the substance in the sample and standards; H^{st}_{st} and H^{st}_{sa} the heights of the peaks of the internal standard in the standards and sample respectively. The change from C^{s}_{sa} to the concentration of the substance in the brain sample presents no difficulty.

Trials of the system in use showed that resolution of the chromatograph is sufficient for isolating 8-10 peaks of monoamines and their precursors and metabolites (Fig. 1). The linearity of the measurements is not below 5% within the concentration range 500-50,000 pg/ml. Reproducibility is about 3-5% of deviation of amplitude. Columns 120 mm long and with a volume of 400 μl can be used to analyze about 600 samples requiring minimal preparation.

Microcolumn HPLC was thus found to be substantially more suitable for brain catecholamines than the ordinary method using columns 4.6 mm in diameter and 25 cm long from the standpoint of economy of sorbent and also of reagents and time required to prepare the eluant and samples.

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