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Note

High-performance liquid chromatographic determination of several monoamines in brain tissue of DBA/2 mice during a single run of 20–25 minutes without prior clean-up of samples

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Norepinephrine (NE), dopamine (DA) and serotonin (5HT) control many brain functions and are important modulators of behavior. Measurements of these monoamine transmitters and their principal metabolites in brain tissue is thus critical to the progress of a number of neuroscience projects. High-performance liquid chromatography (HPLC) with amperometric detectors is now a method of choice for the determination of monoamine levels in the central nervous system, other tissues and body fluids because of its sensitivity and accuracy[1–11].

However, it has been difficult to resolve several monoamines and metabolites during a single, rapid HPLC run. Moreover, the neutral metabolite of NE, 3-methoxy-4-hydroxyphenylglycol (MHPG), has been hard to capture because it is eluted from the column quickly[1]. If measures are taken to delay its elution, other compounds of choice are also retarded and the run becomes uncomfortably long[10].

Recently, we have developed a HPLC system which surmounts these limitations[3]. It permits the simultaneous analysis of thirteen catecholamines and indoleamines during a run of less than 20 min. The thirteen compounds resolved are: NE, MHPG, DL-normetanephrine (NM), epinephrine (EPI), 3-methoxytyramine (3MT), DL-metanephrine (MN), L-β-3,4-dihydroxyphenylalanine (DOPA), DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxy-L-tryptophan (5HTP), 5-hydroxytryptamine or serotonin (5HT) and 5-hydroxyindole-3-acetic acid (5HIAA). Through ongoing development, we are adding additional compounds.

The present study was undertaken to apply this HPLC technique to the analysis of these catecholamines and indoles in discrete regions of brain tissue. We have been able to identify and resolve the majority of the compounds in various regions of mouse brain without prior clean-up of samples during a run of less than 20–25 min, depending on the chromatographic conditions. However, many of the compounds listed above are minor metabolites or precursors of monoamine transmitters

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which normally exist at very small concentrations in vertebrate brain. They are near the limits of detection for our system unless a higher sensitivity setting is selected or unless they are increased by experimental manipulations. Sporadic values obtained for these compounds are not reported.

For this initial work, we opted for a lower sensitivity setting and to concentrate on the following seven compounds: NE and its major metabolite, MHPG, DA and its metabolites, DOPAC and HVA, and serotonin and its metabolite, 5HIAA. Data are reported for the thalamus of male DBA/2 mice.

EXPERIMENTAL

Instrumentation

The HPLC system consists of a Waters (Bedford, MA, U.S.A.) 590 programmable solvent delivery pump and a refrigerated Waters Intelligent Sample Processor (WISP) connected to a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Biophase ODS 5 μm, C_{18}, 250 × 4.6 mm column with a Bioanalytical Systems Biophase ODS 5 μm, C_{18}, 30 × 4.6 mm guard column and a Bioanalytical Sytems LC4B amperometric detector with glassy carbon electrode. The integrating recorder is a Shimadzu C-R3A data processor equipped with a floppy disk drive and cathode ray tube (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.).

Chemicals

The following chemicals were purchased from Sigma (St. Louis, MO, U.S.A.): 3-methoxy-4-hydroxyphenylglycol (hemipiperazine salt) (MHPG), arterenol bitartrate (norepinephrine bitartrate) crystalline (NE), epinephrine bitartrate (EPI), L-β-3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), DL-normetanephrine hydrochloride (NM), dopamine (3-4-dihydroxyphenylethylamine HCl) (DA), DL-metanephrine hydrochloride (MN), 5-hydroxyindole-3-acetic acid (free acid) (5HIAA), 3-methoxytyramine (3-methoxy-4-hydroxyphenethylamine) HCl (3MT), 5-hydroxytryptamine hydrochloride (serotonin) (5HT), 5-hydroxytryptophan (5HTP), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) (HVA), L-(−)-isoproterenol (IP), L-cysteine free base and L-ascorbic acid. In addition perchloric acid (PCA) (70% in water) was purchased from Eastman Kodak (Rochester, NY, U.S.A.), 1-heptanesulfonic acid (HSA) was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.) and acetonitrile was purchased from J. T. Baker (Philipsburg, NJ, U.S.A.).

Chromatographic conditions

The mobile phase was a mixture of 0.10 *M* citric acid, 0.06% diethylamine, 0.05 m*M* Na_2EDTA, 225 ml/l HSA and 3% acetonitrile at pH 2.55. These components were dissolved in deionized water (< 1 MΩ resistance), then filtered through a 47-mm, 0.2-μm filter (Rainin Instrument, Woburn, MA, U.S.A.). The solvent was sparged with helium gas to deaerate it. All separations were performed isocratically at a flow-rate of 1.5 ml/min at room temperature, a detector setting of 0.85 V and a sensitivity setting of 5 nA.

Standards

Fresh standard compounds were ordered in brown bottles and were kept in a dessicator in the freezing compartment. The stock solutions were prepared in deionized water at a concentration of 1 mg/ml then diluted serially with 5% PCA to give the working solution of 20 ng/ml. Aliquots of 50 μl of this were injected into the HPLC system from the refrigerated sample compartment.

Tissue preparation

Brain tissue was obtained from 5–7 weeks old DBA/2 male mice. They were killed by cervical dislocation and the brains were rapidly removed and frozen in liquid nitrogen. Dissections were carried out over liquid nitrogen on a frosted plate. The thalamus was obtained from appropriate coronal sections. The tissues were then weighed and homogenized (Tekmar Tissumizer, Cincinnati, OH, U.S.A.). The extractions were performed in 5% PCA containing the internal standard (20 ng/ml IP) and 1% cysteine. Then the homogenate was centrifuged through Isolab (Norton, OH, U.S.A.) QS-GS filter columns. Aliquots of 50 μl of this material were then injected into the HPLC system. The brains from eight animals were analysed.

RESULTS AND DISCUSSION

The standard chromatogram is illustrated in Fig. 1 and a brain tissue chro-

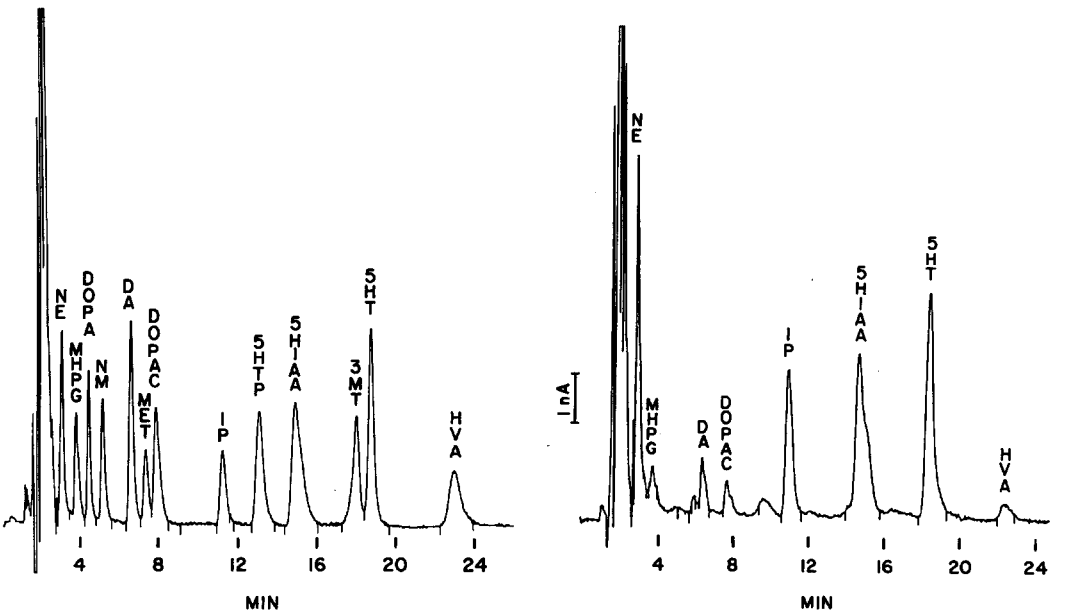

Fig. 1. Chromatogram of standard compounds showing elution times in minutes. The chromatographic conditions and abbreviations are given in the Experimental section.

Fig. 2. Representative chromatogram of thalamic tissue from the brain of a DBA/2 mouse. The elution times of endogenous monoamines are shown in minutes along with the internal standard, (20 ng/ml IP). The chromatographic conditions and abbreviations are found in the Experimental section.

TABLE I

HPLC VALUES [pg/mg (ORIGINAL TISSUE) ± S.E.] FOR MONOAMINES IN THE THALAMUS OF DBA/2 MICE

n = 8. See Experimental for abbreviations. ND = not detected.

Monoamine	*pg/mg original tissue*
NE	250 ± 17.5
MHPG	22.5 ± 5
DA	45 ± 5
DOPAC	27.5 ± 2.5
HVA	25 ± 2.5
3MT	ND
5HT	218 ± 12.5
5HIAA	183 ± 10

matogram for the thalamus is illustrated in Fig. 2 as a representative case. The mean monoamine levels ± standard error (S.E.) obtained from this brain region are given in Table I. The significance of these results is discussed below.

The rapid separation of so many acid, neutral and basic compounds, with varying polarities, is demanding on the chromatographic system. The principal factor responsible for this resolution is the constitution of the mobile phase. The ionic strength of the buffer, the concentration of ion-pairing reagent and organic solvent as well as pH were all varied to obtain optimal separation. As a result, the mobile phase is quite rich in solutes and rather acidic. The ionic strength of the citrate buffer enhanced retention as, in general, did HSA, thus permitting adequate separation of peaks and freedom from the solvent front. The high concentration of acetonitrile dramatically shortened the chromatogram to keep it within reasonable time constraints. The low pH also enhanced separation of certain compounds.

The reversed-phase ODS column is a powerful one for catecholamine separations and the 25-cm one used here is high in theoretical plates. However, we have found that individual columns of this manufacturer (Bioanalytical Systems) may sometimes produce a larger front than desired. This can be mitigated to some extent by changing to a Supelco (Bellafonte, PA, U.S.A.) 15-cm, 3-μm ODS column which has higher resolution properties. Minor adjustments in concentration of acetonitrile (3–4%) and HSA (175–225 mg/l) or in pH (2.45–2.65) may then be required to fine-tune the system. One should begin first with a simple pH change in increments of 0.05. Once a perfect separation is effected, one can expect 3–6 months of column life despite heavy use, the low pH and the syrupy mobile phase. But the column should be washed daily in deionized water for 1 h and stored in 30% methanol when not in use (always preceded and followed by a water wash). At the point in time when the column appears to decay, it can sometimes be reconstituted by passing 50% isopropanol through it overnight.

To prevent breakdown of standards and sample compounds or to identify them better, we take additional precautions. We use cysteine as an antoxidant to circumvent the use of ascorbate which produces a larger front (Bioanalytical Systems, personal communication). We also refrigerate the sample compartment of the automatic injector. Diethylamine helps prevent tailing of peaks and EDTA is used to

chelate heavy metallic ions such as Ca^{2+}. It is not possible to use the optimal potential for each compound but the compromise chosen was 0.85 V. This provides much greater sensitivity for MHPG, for example, while still maintaining high sensitivity for other catechols.

We have tested a number of internal standards to use with the present chromatogram. The results are reported elsewhere[2]. IP is the standard of choice for catecholamines and if a double internal standard is desired, a second one for the indoles, 5-hydroxyindole can be added. It elutes in mid-run near IP yet is completely separated from IP and the other compounds.

In the present study, we report recovery of seven monoamines from the thalamus of DBA/2 mice (Table I). The values are given in pg/mg original tissue ± standard error (S.E.). Please note the recovery in the low picogram range for several compounds including MHPG. The values are similar to those obtained by other methods (see refs. 1–11). Quality control was carried out by mass spectrometry and by other HPLC methods to determine purity of those peaks which we thought could possibly conflict with other compounds. Depending on the tissue, the front may be larger or smaller. If it tends to conflict with NE, the run can be prolonged by adding more HSA or decreasing the amount of acetonitrile. Better still, the brain tissue can be extracted using an acetate buffer containing ascorbate oxidase[2] instead of the PCA extraction reported here. Refinement using this latter technique and the recovery of additional monoamines in other brain tissues will be reported elsewhere.

CONCLUSIONS

This HPLC technique permits the rapid determination of several catecholamines and indoleamines, including metabolites and precursors during a single run. It is valuable for brain tissue analysis for the following reasons: It measures most desired monoamines found in the central nervous system, it permits MHPG to be determined along with the other compounds, it requires no prior cleanup of samples, it generates a vast amount of data in a short period of time and it allows for simultaneous comparisons of several neurotransmitters and metabolites in the same tissues under identical experimental conditions.

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