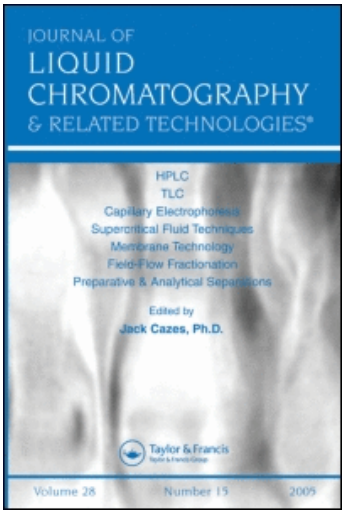

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DETERMINATION OF 13 CATECHOLAMINES, INDOLEAMINES, METABOLITES AND PRECURSORS IN LESS THAN 20 MINUTES DURING A SINGLE HPLC RUN

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ABSTRACT

Thirteen catecholamines and indoleamines are rapidly separated during a single HPLC run. They include epinephrine, norepinephrine, dopamine, serotonin, the major metabolites of these compounds and their immediate precursors. The technique was developed in order to analyze several biogenic amines in brain tissues simultaneously but the methodology has universal application. The separation is based on a fine balance between organic solvent, ion pairing reagent, buffers, pH, choice of column and flow rate.

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INTRODUCTION

High performance liquid chromatography (HPLC) with electrochemical detection (EC) is now the method of choice for determining catecholamines (CA's) and indoleamines (IA's) in many body fluids and tissues (1-10). With this technique, detection of these monoamines can be achieved in the nanogram and picogram range with separation of most desired compounds. But it has been difficult to measure all the monoamines one wishes to identify during a single, brief HPLC run. This has necessitated multiple determinations which not only consume time and money but hinder precise comparisons of experimental data under identical conditions.

In particular, 3-methoxy-4-hydroxyphenylglycol (MHPG), the major metabolite of norepinephrine (NE), has been the stumbling block. This neutral compound typically elutes first and if the solvent front is large, as in tissues extracted with perchloric acid (PCA), like the brain, MHPG is obscured (5). If measures are taken to delay the exit of MHPG, the remaining compounds are also delayed and the run becomes uncomfortably long (6).

In the present studies we have solved this problem by using a different composition of mobile phase, a longer 5μ column and a lower pH than are commonly employed. This simple HPLC method permits the simultaneous analysis of 13 of the most important monoamines, metabolites and precursors during a single 20 minute run. The method has wide application but is particularly useful for brain tissue since MHPG can be separated from the solvent front and the PCA extraction medium (10).

MATERIALS AND METHODS

The following chemicals are purchased from Sigma (St. Louis, MO):

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-hydroxy-L-tryptophan .....(5HTP)
5-hydroxyindole-3-acetic acid (free acid) .....(5HIAA)
3-methoxytyramine (3-methoxy-4-hydroxyphenethylamine) HCl....(3MT)
5-hydroxytryptamine hydrochloride (serotonin) .....(5HT)
4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) ....(HVA)
3,4-dihydroxybenzylamine hydrobromide .....(DHBA)
6-hydroxydopamine hydrochloride .....(6HDA)
deoxyepinephrine hydrochloride (epinine, N-methyldopamine) ...(EN)
5-hydroxyindole .....(5HI)
L-(-)-isoproterenol .....(IP)
3-hydroxy-4-methoxyphenethylamine hydrochloride .....(MDA)
N-methyl-5-hydroxytryptamine oxalate salt .....(M5HT)
L-cysteine free base and L-ascorbic acid.

In addition, PCA (70% in H_2O) is purchased from Eastman Kodak Co., Rochester, N.Y., 1-heptanesulfonic acid (HSA) is purchased from Fisher Scientific Co., Fairlawn, N.J. and acetonitrile (CH_3CN) is purchased from J.T. Baker Chemical Co., Philipsburg, New Jersey.

Apparatus

The HPLC system consists of a Waters (Medford, Mass.) 590 programmable solvent delivery pump and refrigerated WISP automatic injector connected to a Bioanalytical Systems (BAS, Lafayette, Ind.) Biophase ODS 5μ, C-18, 250 x 4.6 mm column with a BAS Biophase ODS 5μ, C-18, 30 x 4.6 mm guard column and a BAS LC4B amperometric detector with glassy carbon electrode. The intergrating recorder is a Shimadzu C-3A data processor equipped with a floppy disk drive and cathode ray tube (Shimadzu Scientific Instruments, Inc., Columbia, MD).

Chromatography

The mobile phase is a mixture of 0.10 M citric acid, 0.06% diethylamine, 0.05mM Na_2EDTA, 225 mg/ml HSA and 3% CH_3CN at pH 2.55. These components are dissolved in deionized water (<1 megohm resistance), then filtered through a 47mm, 0.2μ filter (Rainin Instrument Co., Woburn, Mass.). The solvent is sparged with helium gas to deaerate it. All separations are performed isocratically at a flow rate of 1.5 ml/min at room temperature and a detector setting of 0.85 mV.

Standards

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

RESULTS AND DISCUSSION

Fig. 1 demonstrates the resolution of all 13 compounds studied. The exact retention times are listed in Table 1. The rapid, simultaneous separation of so many monoamines during a single, 20 minute run puts considerable demands on the chromatographic system. The most critical adjustments concern the mobile phase. In order to separate this mixture of acidic, neutral and basic substances, a fine balance must be obtained between ion-pairing reagent, organic solvent and pH. The principle ingredients are HSA and CH_3CN. The addition of HSA tends to spread out the compounds while CH_3CN tends to contract the chromatogram. But since the individual monoamines react differently to both HSA and CH_3CN, a great deal of trial and error was initially required to achieve the proper ratio. The ionic strength of the citrate buffer also helps with the separation by enhancing retention (1).

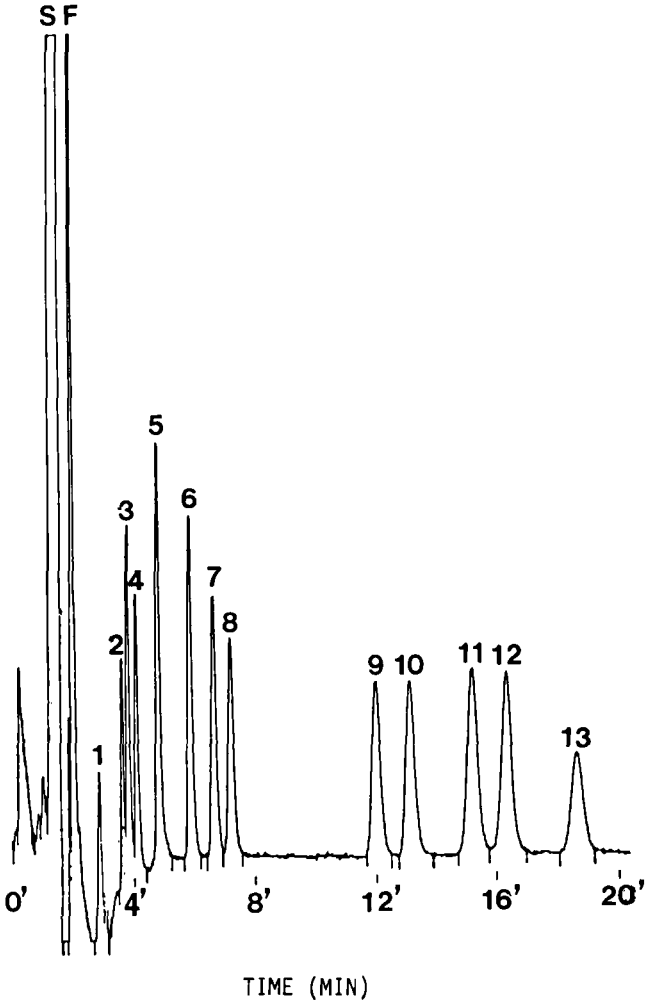

Fig. 1. Chromatogram containing 13 monoamines: 1=NE, 2=EPI, 3=MHPG, 4=DOPA, 5=NM, 6=DA, 7=DOPAC, 8=MN, 9=5HTP, 10=5HIAA, 11=3MT, 12=5HT and 13=HVA; SF=solvent front. Peak integration marks descend as small vertical lines from the baseline.

When all the compounds are reasonably well separated, fine adjustments in pH are then required to produce optimal separation.

If run time is not important, the flow rate may be decreased to produce wider separation of the initial three peaks. This is only needed if very large quantities of these compounds are being analyzed. In the brain, the levels of EPI, MHPG and DOPA are smaller than those corresponding to standard peaks under most conditions. Indeed, EPI is essentially missing in most regions of the vertebrate brain (1,3) and for the majority of central analyses can be omitted. However, if the run is lengthened, some band broadening of the later eluting peaks will occur.

To prevent breakdown of standards and sample, additional precautions are taken. We use cysteine as an antoxidant because it produces a narrower peak at the beginning of the run than does ascorbate (Fig. 1). We also refrigerate the sample compartment of the automatic injector. These measures reduce the lability of unstable compounds, particularly the indoleamines and 3MT.

TABLE 1

Retention Times For Monamines

MONOAMINE	RETENTION TIME (Min).
1 NE	2.91
2 EPI	3.65
3 MHPG	3.85
4 DOPA	4.13
5 NM	4.84
6 DA	5.89
7 DOPAC	6.67
8 MN	7.22
9 5HTP	12.04
10 5HIAA	13.15
11 3MT	15.19
12 5HT	16.33
13 HVA	18.67

For simplicity, Fig. 1 is illustrated without internal standards, but we have tested the suitability of a number of possible compounds as internal standards for the present study. The elution times are listed in Table 2.

The ideal choices are IP for CA's and 5 HI for IA's. They both fit in the void between 8 and 12 minutes without conflicting with one another or any other compounds. They can be used as double internal standards if desired. Another advantage is that they elute at mid-run. If this slot is desired for other compounds of choice which may prove to elute here, MDA or M-5HT, which elute last of all, can be used as internal standard. The remaining compounds conflict with the elution times of monoamines of interest (compare with Table 1).

In order to calibrate so many standards and efficiently analyze 13 monoamines, including several repetitions each day, a sophisticated recorder and data processor is invaluable. The one chosen for the present work will permit storage of chromatograms for future analysis, has several standard calibration programs and will calculate the concentration of the analytes per ml or mg tissue automatically, then statistically analyze the data.

TABLE 2

Retention Times For Internal Standards

INTERNAL STANDARD	RETENTION TIME (Min).
DHBA	4.58
6OHDA	4.65
EN	8.26
5HI	9.76
IP	11.02
MDA	23.07
M5HT	23.32

The extraction procedure for mouse brain, along with the monoamine and metabolite levels achieved for various brain regions, will be reported elsewhere (10). The sensitivity for all compounds is in the picogram range (≅ 1 pg/mg).

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