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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF BIOGENIC AMINES IN BIOLOGICAL MATERIALS AS *o*-PHTHALALDEHYDE DERIVATIVES*

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SUMMARY

A remarkably sensitive, simple and selective reversed-phase high-performance liquid chromatographic (HPLC) method has been developed, allowing, for the first time, the direct measurement of histamine, norepinephrine, octopamine, normetanephrine, dopamine, serotonin and tyramine in a single sample of plasma (2 ml), tissue (0.2 g), or urine. The biogenic amines were modified by pre-column derivatization with *o*-phthalaldehyde which stabilizes the molecules, aids in extraction, and improves HPLC detection at the nanogram level. To minimize losses during the sampling procedure a careful collection procedure was designed.

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We developed a simple sample cleanup in which the samples were thawed, neutralized with KOH, immediately derivatized, extracted into ethyl acetate (EtOAc) and then chromatographed by HPLC. The derivatives were stable in EtOAc for more then 24 h. Interfering amino acids were removed from the EtOAc by partitioning twice with Na_2HPO_4 buffer (pH 10.0). Complete separation was achieved in ca. 60—90 min on a μBondapak phenyl column using a stepwise gradient of acetonitrile and/or methanol—phosphate buffer (pH 5.1). A variable wavelength fluorometer with a 5-μl flow-cell was used (excitation 340 nm; emission 480 nm). Linearity ranged from 200 pg to 50 ng onto the column. Precision (R.S.D.) for retention times was 1% and for derivatization and injection 2.5%. Recoveries of the seven biogenic amines from plasma spiked with 25 ng/ml averaged 70%, with a relative standard deviation of 6%. Separation studies were also done using a μBondapak C_{18} column. The effects of various eluents are presented. Gas—liquid chromatography was also investigated but lacked the sensitivity achieved by HPLC. The HPLC method is used routinely for the determination of biogenic amines in plasma from pigs with malignant hyperthermia and thermally stressed bovine. Significant differences in levels of biogenic amines were noted between stressed and non-stressed animals. Data on rat brain tissue samples were compared with the trihydroxyindole method and canine heart tissue was analyzed for ventricular norepinephrine and dopamine. Application of the method to urine from normal persons and a patient with a brain tumor has been demonstrated.

INTRODUCTION

In addition to being neurotransmitters, biogenic amines function as hormones which influence and modify the secretion of a variety of other hormones, hypothalamic releasing and inhibiting factors, pituitary tropic hormones, and adrenal steroids. The abnormal secretion and/or metabolism of biogenic amines are associated with many diseases such as pheochromocytoma [1], neuroblastoma [2], schizophrenia [3], malignant hyperthermia [4], and possibly essential hypertension [5—7].

To gain more insight into the role biogenic amines play in hormonal control, and in the etiology and pathogenesis of disease, more advanced, simple, sensitive and quantitative methods are needed for bioscience research. Analytical methods are available for the analysis of biogenic amines such as the trihydroxyindole and ethylenediamine fluorescence methods [8—10], gas—liquid chromatography (GLC) [11, 12], and gas chromatography—mass spectrometry [13, 14]. These methods have in general proved inadequate for the determination of biogenic amines in the plasma due to high background levels, instability of derivatives at low concentrations, and lack of accuracy and precision. More sensitive methods include the double-isotope derivative [15] and radioenzymatic assays [16], but reproducibility, time, cost, and the small number of biogenic amines that are quantitated make these methods difficult to work with.

Initially, GLC was investigated in our laboratory because it appeared to have the potential for good separation, specificity, and sensitivity for the analysis of biogenic amines. This approach to sample analysis showed some success but ultimately was discontinued. It became evident that this approach could not easily meet the subnanogram sensitivity, efficient sample cleanup, and derivative stability; all requirements of a routine biogenic amine analysis. In addition, the cleanup procedure was not selective enough. The biogenic amines are very water soluble and in the attempt to extract them from an

aqueous sample considerable interfering material was also extracted. Finally, the use of an electron capture detector (ECD) was complicated by the fact that the ECD and the derivatizing agent (trifluoroacetic anhydride, TFAA) are incompatible and the TFAA must be vented. With all of the TFAA removed from the derivatives they tended to decompose.

At this point the development of a GLC method was discontinued and high-performance liquid chromatography (HPLC) was initiated. The prediction that a good HPLC procedure would circumvent all of the decomposition problems and also solve the extraction dilemma was to be proved correct.

The application of HPLC to the analysis of biogenic amines is a recent development. Molnár and Horváth [17] demonstrated the separation of catecholamines and their metabolites on a reversed-phase column (octadecylsilica) with aqueous, isocratic elution, and studied the effects of pH, temperature, and ionic strength on catecholamine retention time. An alternative approach, reported by Kissinger et al. [18] combines HPLC with electrochemical detection for analysis of urine and tissue but is not well-suited for plasma.

Fluorescence detection coupled with HPLC separation offered the needed specificity and sensitivity to analyze biogenic amines quantitatively at the nanogram level. To enhance the natural fluorescence of biogenic amines, derivatization reagents have been employed. The two most commonly used are fluorescamine (fluram; 4-phenylspiro[furan-2(3H), 1'-phthalan]-3,3'-dione), which reacts with primary amines [19], and *o*-phthalaldehyde (OPT; OPA; Fluoropa), which together with 2-mercapthoethanol forms a strongly fluorescent adduct (Fig. 1) that is twenty times stronger than the native compound [20, 21]. OPT has been used in HPLC for post-column [22] but not pre-column derivatization of biogenic amines (Fig. 1).

We introduced the concept of pre-column derivatization of biogenic amines with OPT to improve the separation, sensitivity, and quantitation by HPLC, and to simplify the sample cleanup. Together with reversed-phase partition chromatography and fluorometric detection we have developed for the first time a rapid, highly sensitive, and simple analytical method for measuring histamine, norepinephrine, octopamine, normetanephrine, dopamine, sero-

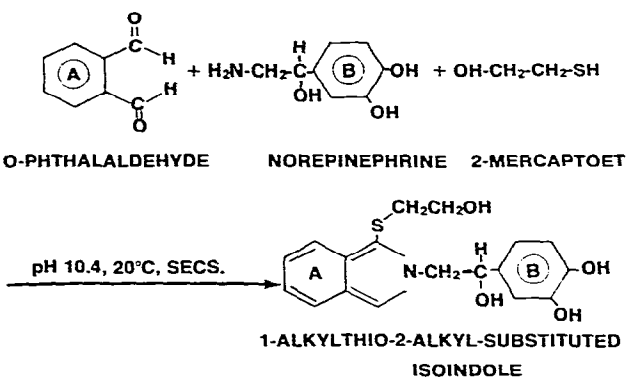


Fig. 1. Formation of isoindole fluorescent adduct from OPT and 2-mercaptoethanol [21].

tonin, and tyramine, at the nanogram level. We are using the method routinely for the chromatographic analysis of biogenic amines in plasma, tissue and urine.

EXPERIMENTAL

Apparatus

A Model 6000A Solvent Delivery System and U6K Universal Injector (Waters Assoc., Milford, Mass., U.S.A.) were used with the HPLC system. The fluorescence detector was a Spectrofluoro Monitor Model FS970 with a 5-μl flow-cell and selectable monochromatic excitation wavelength (Schoeffel, Westwood, N.J., U.S.A.). The recorder was a Fisher Recordall Model 5000 (Houston Instruments, Austin, Tex., U.S.A.). The HPLC columns used were μBondapak phenyl/Porasil and μBondapak C_{18}, 300 × 4 mm I.D. (Waters Assoc.) with a Co:Pell ODS pre-column (Whatman, Clifton, N.J., U.S.A.).

The temperature of the HPLC column was controlled by a constant-temperature circulating water bath Model FJ (Haake, Saddle Brook, N.J., U.S.A.) connected to an aluminum column jacket. The jacket was composed of two aluminum blocks (24 × 7 × 2.2 cm) precisely grooved to accommodate two columns and a thermometer, when bolted together. Each block had two holes (6.0 mm) drilled completely through the block, lengthwise, and fitted with Swagelok fittings and copper tubing to allow the controlled-temperature water to circulate along four sides of the columns before recycling through the bath. The aluminum column jacket blocks were specially designed and made in the University of Missouri (Columbia) Science Instrument Shop.

Peak areas, retention times, relative weight response values, and concentrations based on an internal standard were calculated by a Hewlett-Packard 3352B Laboratory Data System (Hewlett-Packard, Avondale, Pa., U.S.A.).

The samples were prepared for HPLC analysis using all-glass 12-ml PTFE faced screw-cap, round-bottom culture tubes, and graduated centrifuge tubes (KIMAX; Kimble Glass Works, Toledo, Ohio, U.S.A.).

An IEC Clinical Centrifuge Model CL (Damon/IEC Division, Needham Hts., Ma., U.S.A.), rotary shaker Model S-500 (Kraft, Mineola, N.Y.,U.S.A.), and a Corning Model 12 research pH-meter (Corning, Corning, N.Y.,U.S.A.) were used.

A special reagent-grade, Nanopure water system utilizing deionization and reverse osmosis produced all the water necessary to prepare aqueous solutions, eluents and to rinse all glassware.

Chemicals

All reagents used were of highest purity available (A.C.S. certified grade). Monobasic sodium phosphate (A.C.S. certified grade; Fisher Scientific, Fairlawn, N.J., U.S.A.), methanol, glass-distilled (Burdick & Jackson, Muskegon, Mich., U.S.A.), and acetonitrile (HPLC grade; Fisher Scientific), were used to prepare buffers.

Boric acid, 2-mercaptoethanol, and potassium hydroxide (A.C.S. certified

grade; Fisher Scientific, and OPT (Sigma, St. Louis, Mo., U.S.A.) were used in preparing the derivatization reagent.

Sodium metabisulfite, disodium ethylenediaminetetraacetate (EDTA), dibasic sodium phosphate, sodium chloride, perchloric acid (A.C.S. certified and reagent grade; Fisher Scientific), and ethyl acetate (spectrophotometric grade; Aldrich, Milwaukee, Wisc., U.S.A.) were used in the preparation of the samples.

Calibration standards

Histamine, norepinephrine, and octopamine, were purchased from Sigma, dopamine and tyramine from Aldrich and normetanephrine and serotonin from Calbiochem (San Diego, Calif., U.S.A.) and stored in methanol at 4° for daily use.

Buffer preparation

Two stock solutions of NaH_2PO_4 were prepared (25 and 50 mmole/l) in acetonitrile and/or methanol and stored at 5°. Buffers for daily use were then prepared by checking the pH and adjusting if necessary with a few drops of 1 *N* sodium hydroxide or 1 *N* hydrochloric acid. The buffer was then filtered through a 0.22-μm membrane filter and degassed.

Preparation of o-phthalaldehyde reagent

The reagent was prepared by dissolving 0.50 g of boric acid in 19 ml of water using a 50 ml beaker, adjusting the pH to 10.40 ± 0.02 with a 45 g/100 ml KOH solution, and then transferring the solution into a dark glass bottle with a PTFE-lined screw cap. Separately, 17.5 mg of OPT were dissolved in 200 μl of methanol (glass-distilled) using a 5 ml beaker. The OPT solution was added to the borate solution along with 40 μl of fresh 2-mercaptoethanol (A.C.S. reagent grade), and stored under nitrogen at 5°. The reagent was stable for seven working days.

Sample collection and storage

To minimize losses a careful sample collection was necessary. Only acid-cleaned glassware, which was exhaustively rinsed, and PTFE equipment was used in handling the samples.

Plasma. The whole blood was rapidly collected from in-dwelling catheters using a sterile 15-ml syringe which had been rinsed on the inside with sterile saline—heparin solution (100 I.U./ml). The collected blood was then emptied immediately into an ice water chilled 25-ml screw-cap glass centrifuge tube (Scientific Products, St. Louis, Mo., U.S.A.) which contained 1 ml of freshly made EDTA—metabisulfite solution (20 mg/ml EDTA, 10 mg/ml sodium metabisulfite). The tube was capped with a PTFE-lined screw-cap and inverted gently several times to mix the blood and reagent, then brought to a cold room (4°), and immediately spun for 10 min at 4000 *g* . The plasma was removed from the packed cells and measured at the same time with a 10 ml all-glass syringe with a Kel-F ever-hub and 3-inch PTFE tube (Scientific Products). Working in the cold room, the volume of plasma was recorded and made 0.4 mole/l with concentrated perchloric acid and mixed with a vortex

mixer. The tube was allowed to stand in the cold for 15 min to complete perchlorate precipitation of protein. The sample was then centrifuged at 20,000 *g* at 4° for 20 min, the supernatant was removed and immediately frozen and stored at −70° in the dark.

Tissue. Up to 1 g of tissue was homogenized in 5 ml of 0.4 mole/l perchloric acid in a glass tissue grinder submerged in an ice bath. The homogenate was transferred to a 25-ml screw-cap glass centrifuge tube and spun at 30,000 *g* at 4° for 15 min. The supernatant was removed and immediately frozen for storage at −70° in the dark.

Urine. The sample was collected in the presence of sodium metabisulfite (0.5 mg/ml urine) made 0.4 mole/l with concentrated perchloric acid and immediately frozen to −70° in the dark. Prior to analysis it was centrifuged at 5000 *g* for 15 min.

Cleanup of samples for HPLC analysis

The plasma samples were thawed in flowing water at room temperature, mixed thoroughly, and a 2-ml aliquot was pipetted into a 12-ml conical culture tube. The deproteinated sample was then adjusted to a pH of 7.0 ± 0.2 with 0.5 mole/l potassium hydroxide, and immediately derivatized with 400 μl (350 μg) of OPT at pH 10.40 ± 0.02. Two grams of sodium chloride were added to break any emulsion formed during the double extraction with 2 ml of ethyl acetate and to aid in partitioning the derivatives into the EtOAc. The sample was shaken for 1 min during each extraction and spun at 3400 *g* to separate the phases. After extraction the ethyl acetate was partitioned twice with 2 ml of 50 mmole/l dibasic sodium phosphate buffer (pH 10.0 ± 0.1) shaken for 1 min, and spun at 3400 *g*. The final ethyl acetate volume was reduced to 100 μl under a sweep of ultrapure, oxygen-free, dry nitrogen gas and stored in the cold room (4°) until analysis.

Reversed-phase HPLC separation and quantitation of biogenic amines

The ethyl acetate extract containing the derivatized biogenic amines was brought to room temperature, and 10—50 μl were injected and chromatographed with a NaH_2PO_4 buffer (pH 5.10), containing either methanol or acetonitrile for the first elution step and methanol for the second elution step. The chromatography was performed on a 300 × 4 mm I.D. μBondapak phenyl column with a flow-rate of 1.5 ml/min. The derivatives were quantitated by their fluorescence intensity at 340 nm (excitation) and at 480 nm (emission).

The areas under the peaks were integrated and the amount (ng/ml) of each biogenic amine (BA) was calculated based on an internal standard (IS) as follows:

$$\text{Amount of BA (ng/ml)} = \left[\frac{\text{area}_{BA}}{\text{area}_{IS}}\right]_{\text{sample}} \times \left[\frac{1}{\text{RWR}_{BA/IS}}\right] \times \left[\frac{\text{ng}_{IS}}{\text{ml}}\right]_{\text{sample}}$$

$$\text{where RWR}_{BA/IS} = \left[\frac{\text{area}_{BA}}{\text{ng/ml}_{BA}} \times \frac{\text{ng/ml}_{IS}}{\text{area}_{IS}}\right]_{\text{standard}}$$

The relative weight response (RWR) values for each of the biogenic amines were determined by at least ten independent analyses of calibration standards of the biogenic amines; thereafter, the RWR was determined daily. In the above expression for $RWR_{BA/IS}$, the concentration units are ng/ml (or μg/l).

Peak identification

The biogenic amines were identified on the basis of retention time by comparison with standards and also by co-chromatography of standards in different solvent systems. The biogenic amines were well resolved on the μBondapak phenyl columns, thus the chromatograms are relatively simple and identification was certain. In addition, the identity of norepinephrine and dopamine was confirmed by correlating the values from the trihydroxyindole determination and OPT—HPLC analyses of the same rat brain samples. For further confirmation of chromatographic peaks in plasma, urine and tissue samples, and the identification of unknown peaks, work is in progress to utilize high-resolution mass spectrometry. HPLC fractions are being collected, concentrated and analyzed by this technique.

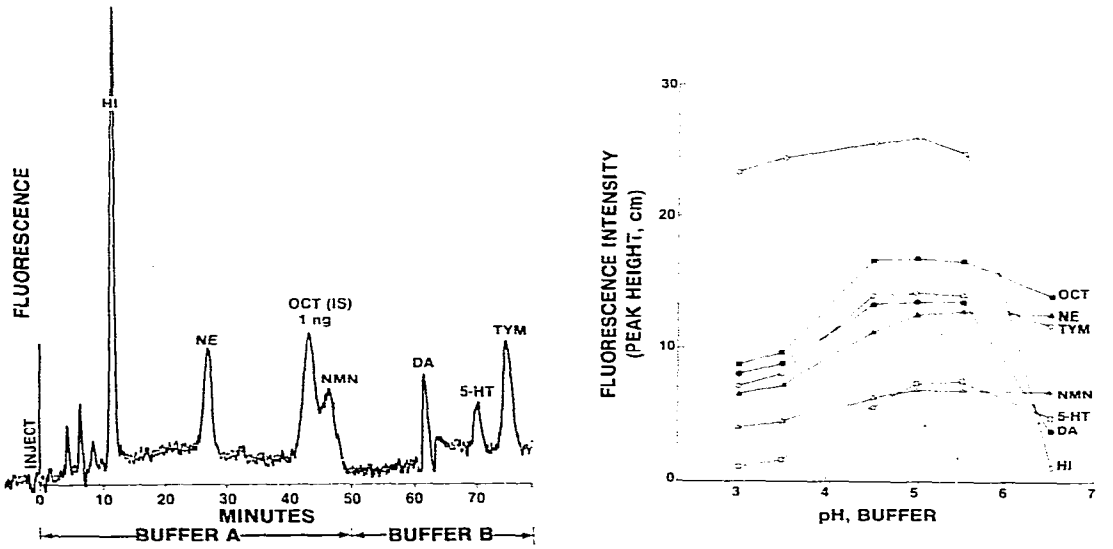
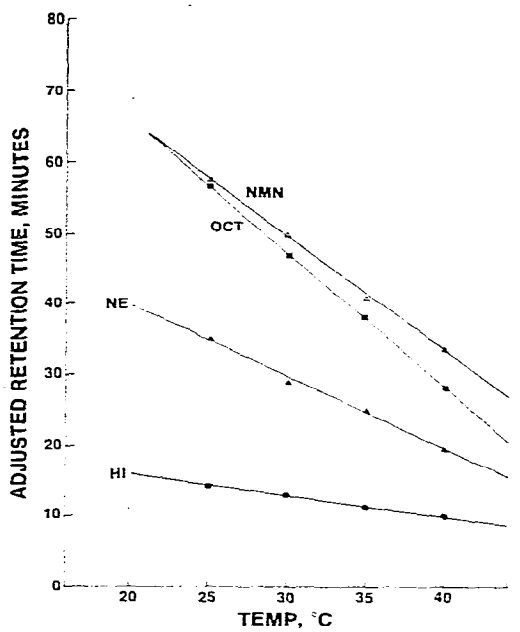


Fig. 2. Reversed-phase HPLC separation of seven biogenic amine standards. Sample: 1.0 μl standards, 500 pg each; column: μBondapak phenyl (300 × 4 mm); buffer: 0.05 mole/l NaH_2PO_4 (pH 5.10) with 320 ml of methanol per l for first elution step (A) and 450 ml of methanol per l for second elution step (B); flow-rate: 1.5 ml/min; detector: Schoeffel FS970, 0.10 μA full-scale, excitation 340 nm, emission 480 nm; temperature: 30°. The internal standard (IS) is octopamine. Peaks: HI, histamine; NE, norepinephrine; OCT, octopamine; NMN, normetanephrine; DA, dopamine; 5-HT, serotonin; TYM, tyramine.

Fig. 3. Effect of eluent pH on fluorescence intensity. This figure demonstrates that maximum fluorescence intensity is obtained at pH 5.0. All other conditions and abbreviations as in Fig. 2.



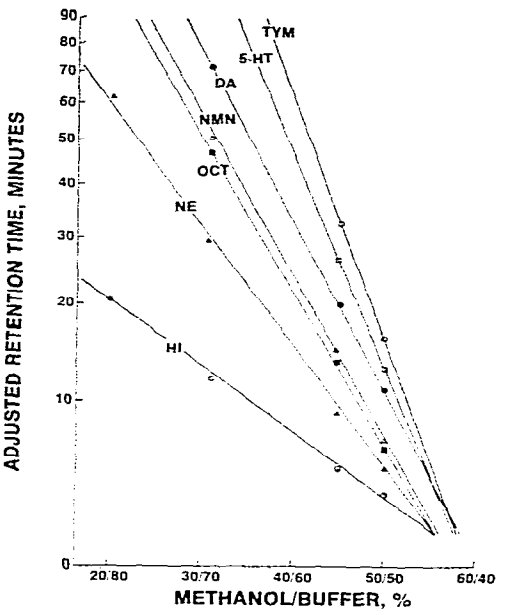


Fig. 4. Effect of column temperature on adjusted retention time. Sample: 10 μl standards, 10 ng each; buffer: 0.05 mole/l NaH_2PO_4 (pH 5.10) with 320 ml of methanol added per liter. All other conditions and abbreviations as in Fig. 2.

Fig. 5. Effect of eluent methanol concentration on adjusted retention time. Abbreviations and all conditions, except methanol concentration of buffers, are the same as in Fig. 2.

TABLE I

RELATIVE WEIGHT RESPONSE OF BIOGENIC AMINES IN HPLC ANALYSIS

Eluent is 50 mmole/l NaH_2PO_4 buffer (pH 5.10) containing 32% methanol for the first elution step and 45% methanol for the second elution step.

Biogenic amine	Retention time (min)*	RWR**
Histamine	13.21	1.676
Norepinephrine	30.41	0.904
Normetanephrine	51.76	0.859
Dopamine	69.33	0.749
Serotonin	77.46	0.437
Tyramine	82.97	1.058
Octopamine (IS)	48.13	1.000

*Uncorrected for void volume (3.0 ml). Pumping rate, 1.5 ml/min.

**RWR compared to IS, octopamine = 1.000. Each value is an average of 10 independent analyses with a relative standard deviation of 2%.

RESULTS AND DISCUSSION

Reversed-phase HPLC analysis of biogenic amines

Optimization of chromatography. Fig. 2 shows the two-step isocratic separation of a standard solution containing 500 pg each of seven biogenic amines in less than 80 min using a μBondapak phenyl column. An internal standard, octopamine, was used to facilitate accurate quantitation of the biogenic amines in plasma. Other internal standards are being investigated for urine because octopamine is indigenous to the sample matrix. The eluent used for each separation is outlined in the legend for the chromatograms. A 300 × 4 mm I.D. μBondapak phenyl column with a 70 × 2 mm I.D. Co:Pell ODS pre-column (Whatman) hand packed with octadecyl-silica particles was used, and a flow-rate of 1.5 ml/min was maintained. The conditions of eluent pH, column temperature, and eluent methanol concentration on separation and fluorescence intensity had been systematically studied to find the optimal conditions (Figs. 3—5).

Minimum detection limit. The high efficiency of the HPLC separation combined with the enhanced sensitivity of fluorescence detection allows an extremely low detection of about 100 pg for all seven of the biogenic amines of a standard mixture, shown in Fig. 2.

Retention times and RWR

The retention times and RWR, compared to the internal standard octopamine, for six biogenic amine derivatives are presented in Table I. The relative weight response, $RWR_{BA/IS}$ values are given for comparative purposes and must be determined in each laboratory. The RWR values represent averages of ten independent analyses with an R.S.D. of less than 2%. The RWR values remained constant over a six-month period.

Stability of OPT derivatives

Fig. 6 demonstrates the stability of the OPT derivative in the ethyl acetate extract (stored at 4°) over time. The good stability of the adduct enables one to prepare samples in advance and to use an auto-injection system for chromatographic analyses.

Precision of HPLC analysis

Biogenic amine standards added to plasma were analyzed with good precision at concentrations comparable to those in samples of tissue and biological fluids (Table II). Repeated injections of 5—10 ng each of the seven biogenic amine derivatives gave an average R.S.D. of less than 3%. The retention time was not affected by the sample matrix, thus excellent precision of retention times for five biogenic amines was obtained in routine analysis over a four-day period for eight different plasma samples (Table III). The R.S.D. ranged from 0.9 to 2.8%. Table IV demonstrates the precision obtained from eight independent analyses of a pooled plasma sample on four different days for an average R.S.D. of 4.3%.

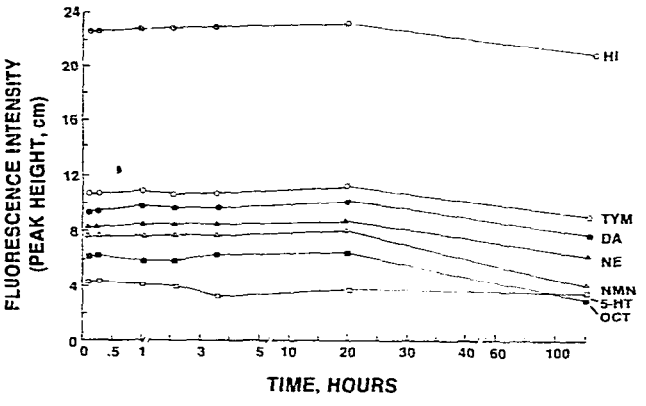


Fig. 6. Stability of biogenic amine OPT derivatives in ethyl acetate at 4°. Abbreviations and all conditions as in Fig. 2.

TABLE II

PRECISION OF RECOVERY FOR BIOGENIC AMINES BY THE EXTERNAL STANDARD METHOD

Each value is the mean of eight or more analyses.

Biogenic amine	Biogenic amines added (ng/ml)*		* Average R.S.D. (%)
	25.0	50.0	
Histamine	54.3**	56.4**	
R.S.D. (%)	5.2	4.0	4.6
Norepinephrine	67.0	69.5	
R.S.D. (%)	5.3	4.1	4.7
Normetanephrine	77.8	75.0	
R.S.D.(%)	3.7	3.2	3.5
Dopamine	75.3	78.0	
R.S.D. (%)	8.6	8.5	8.6
5-Hydroxytryptamine	76.0	74.1	
R.S.D. (%)	7.4	6.2	6.8
Tyramine	68.5	69.0	
R.S.D. (%)	8.6	7.4	8.0

*The standards were added to bovine plasma prior to sample cleanup and HPLC analysis.
**Percentage recovery of biogenic amines added prior to sample cleanup.

Linearity

All seven biogenic amines responded linearly at concentrations ranging from 200 pg to 15 ng injected onto the column (Fig. 7), thus providing a more than adequate range for the analysis of biogenic amine levels found in biological materials.

TABLE III

PRECISION OF RETENTION TIMES OF BIOGENIC AMINE ANALYSIS IN BOVINE PLASMA

Eluent was 32% methanol in 50 mmole/l NaH_2PO_4 buffer for the first elution step, then 45% methanol in 50 mmole/l NaH_2PO_4 buffer (pH 5.1).

	Retention time (min)					
	HI**	NE	OCT (IS)	DA	5-HT	TYM
$\bar{x}$	13.5	30.5	48.7	70.2	81.6	86.2
σ	0.34	0.85	0.58	1.0	0.73	0.86
R.S.D. (%)	2.5	2.8	1.2	1.5	0.9	1.0

*$\bar{x}$ is the average of eight independent analyses.
**Abbreviations of the amines: HI, histamine; NE, norepinephrine; OCT, octopamine; DA, dopamine; 5-HT, 5-hydroxytryptamine; TYM, tyramine.

TABLE IV

PRECISION OF HPLC ANALYSIS FOR BIOGENIC AMINES IN BOVINE PLASMA

Each value is the average of eight independent runs on four different days from a pooled bovine plasma sample.

Biogenic amines	Mean ± σ (ng/ml)	R.S.D. (%)
Histamine	28.5 ± 0.94	3.3
Norepinephrine	2.4 ± 0.08	3.3
Dopamine	1.2 ± 0.05	4.2
Serotonin	14.5 ± 0.90	6.2
Tyramine*	28.5 ± 1.31	4.6

*An unknown peak co-eluted with tyramine and both were integrated together.

Recovery of biogenic amines added to pooled bovine plasma

The recoveries of biogenic amines added to eight 2-ml pooled bovine plasma samples prepared on four different days are shown in Table V. Recoveries were computed by comparing the spiked value to eight 2-ml samples of pooled bovine plasma to which no biogenic amines were added. The difference was calculated as the recovery. Each of the seven standard biogenic amines was added at a level of 25 ng per ml of plasma. The recoveries ranged from 55 to 78% with an R.S.D. of ca. 6%.

Chromatograms of a standard mixture of seven biogenic amines, of a plasma sample, and of an identical plasma sample to which biogenic amine standards were added before analysis, are shown in Fig. 2, 8, and 9, respectively.

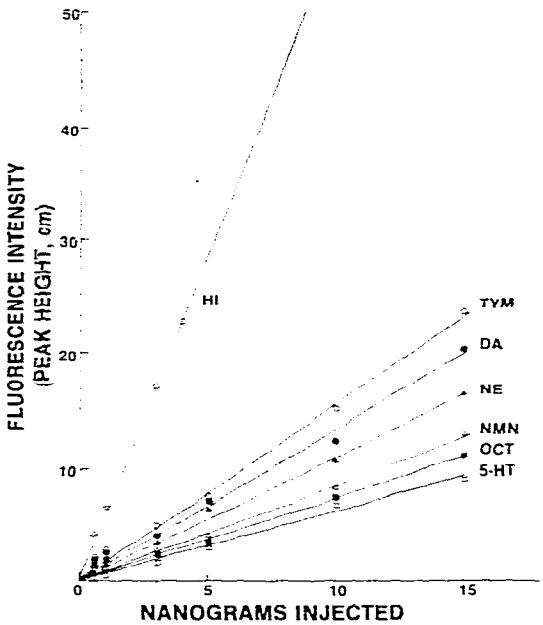


Fig. 7. Linearity of detection for seven biogenic amine standards. Sample: 1.0—15 μl standards, 0.2—15 ng each. All other conditions and abbreviations as in Fig. 2.

TABLE V

ANALYTICAL RECOVERY OF BIOGENIC AMINES ADDED TO POOLED BOVINE PLASMA

Each value is the average of eight independent runs on four different days.

Biogenic amine	Amine (ng/ml)			Average recovery (%)
	Plasma	Plasma + spike*	Spike found	
Histamine	30.0	55.0	13.6	54.4
Norepinephrine	2.0	27.0	16.8	67.2
Normetanephrine	<0.2	25.0	19.4**	77.6**
Octopamine	<0.2	25.0	15.8	63.2
Dopamine	1.0	26.0	18.8	75.2
Serotonin	15.0	40.0	19.0	76.0
Tyramine	30.0***	55.0***	17.0	68.0

*Spike added at a level of 25 ng/ml.
**Average recovery = (19.4 ng found)/(25 ng added) × 100 = 77.6%.
***An unknown peak eluted with tyramine and they were integrated together.

Analysis of biogenic amines in plasma, tissue and urine

In addition, the previously described method has been applied to the routine analysis of plasma samples from control pigs and pigs afflicted with malignant hyperthermia [23]. Chromatograms illustrating the difference in biogenic

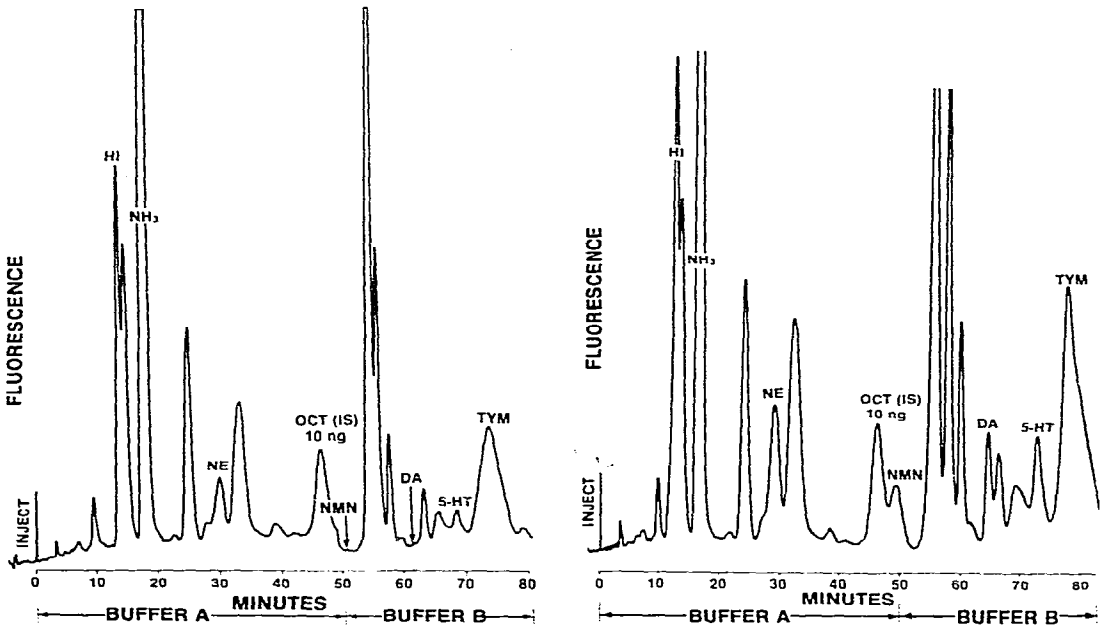


Fig. 8. Reversed-phase HPLC separation of biogenic amines in bovine plasma. Sample: 10 μl, equivalent to 0.2 ml plasma. All other conditions and abbreviations as in Fig. 2.

Fig. 9. Reversed-phase HPLC separation of biogenic amines in bovine plasma with six biogenic amines added. Mixture of biogenic amines shown in Fig. 2 added to bovine plasma shown in Fig. 8 to obtain sample for analysis shown in this figure. Sample: 10 μl, equivalent to 0.2 ml plasma with 5 ng of each biogenic amine. All other conditions and abbreviations as in Fig. 2.

amine levels between control and malignant hyperthermic pigs are shown in Figs. 10 and 11.

Furthermore, the HPLC method has also been applied to quantitating biogenic amines in rat brain tissue (Fig. 12), dog ventricle (Fig. 13), human control urine (Fig. 14), and urine from a patient with a brain tumor (Fig. 15). Note the large difference in the peak heights of the urine sample from the patient with a brain tumor (Fig. 15) and of the control (Fig. 14). Both samples were from 24-h total collections ranging from 1100—1200 ml of urine.

A comparison of the OPT—HPLC method with the trihydroxyindole procedure

Samples of five whole rat brains were analyzed for norepinephrine and dopamine by both the trihydroxyindole and OPT—HPLC procedures. The mean value ± S.E. obtained for the trihydroxyindole procedure was 0.44 ± 0.06 μg/g (range, 0.33—0.60 μg/g) for norepinephrine and 0.89 ± 0.01 μg/g (range 0.86—0.92 μg/g) for dopamine. The mean value ± S.E. determined by the HPLC method was 0.6 ± 0.09 μg/g (range 0.54—0.93 μg/g) for norepinephrine, and 1.02 ± 0.12 μg/g (range 0.67—1.2 μg/g) for dopamine. When the values for norepinephrine and dopamine obtained by both methods were analyzed by linear-regression statistics the slope of the linear least-squares line was 1.02,

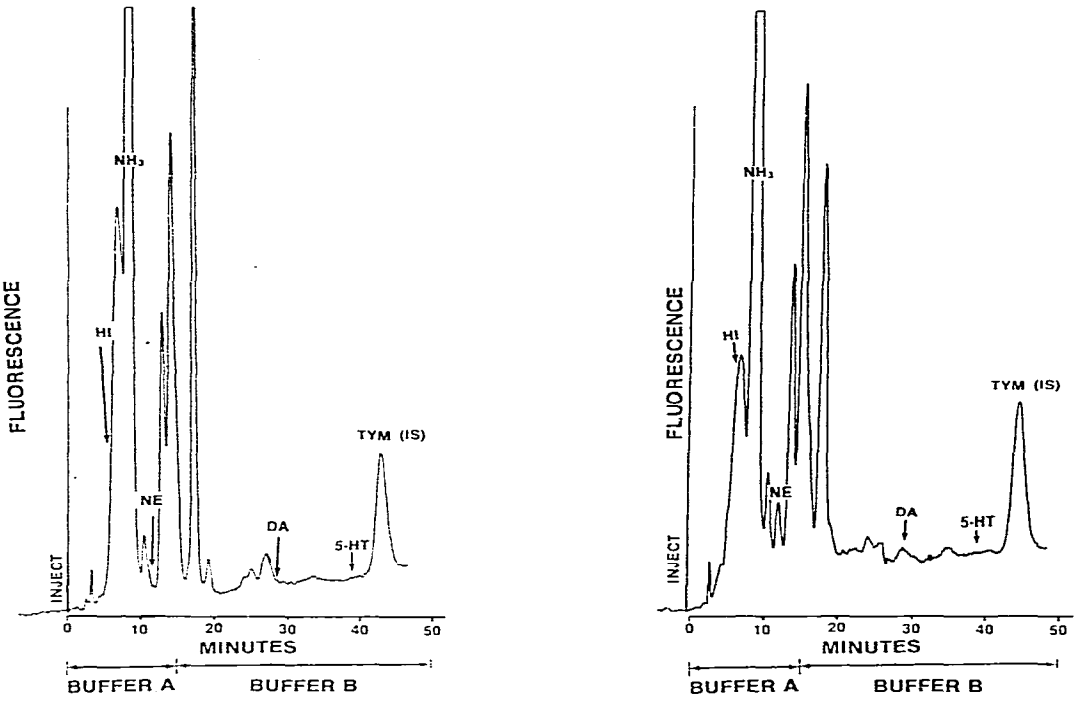


Fig. 10. Reversed-phase HPLC separation of biogenic amines in plasma from a control pig under halothane anesthesia. Sample: 10 μl, equivalent to 0.20 ml plasma; column: μBondapak phenyl (300 × 4 mm); buffer: 0.025 mole/l NaH_2PO_4 (pH 5.10), with 250 ml acetonitrile added per liter for first elution step (A) and 450 ml of methanol added per liter for second elution step (B); flow-rate: 1.5 ml/min; detector: Schoeffel FS970, 0.10 μA full-scale, excitation 340 nm, emission 480 nm; temperature: 26°. IS is tyramine because no indigenous tyramine was detected in the pig plasma. Abbreviations as in Fig. 2.

Fig. 11. Reversed-phase HPLC separation of biogenic amines in plasma from a malignant hyperthermic pig under halothane anesthesia. Sample: 10 μl, equivalent to 0.20 ml plasma. All other conditions as in Fig. 10. Abbreviations as in Fig. 2.

the intercept was 0.15, and the coefficient of correlation was 0.7. The low correlation may be due to the poor precision and recovery from the cation-exchange resin cleanup prior to the trihydroxyindole analysis.

Eluent composition and separation of biogenic amines

Prior to our separation studies on the μBondapak phenyl column, extensive research was done to separate the biogenic amines by isocratic elution using a μBondapak C_{18} column.

The effect of the organic solvent in the eluent mixture on the separation is demonstrated in Figs. 16 and 17. We found that under optimal temperature conditions using only a methanol and phosphate buffer mixture, an adequate separation appeared possible (Fig. 16). Norepinephrine seemed to be identified correctly by co-injection and dopamine co-eluted with a larger peak. By adding N,N-dimethylformamide (5%, v/v) to the eluent (methanol—phosphate

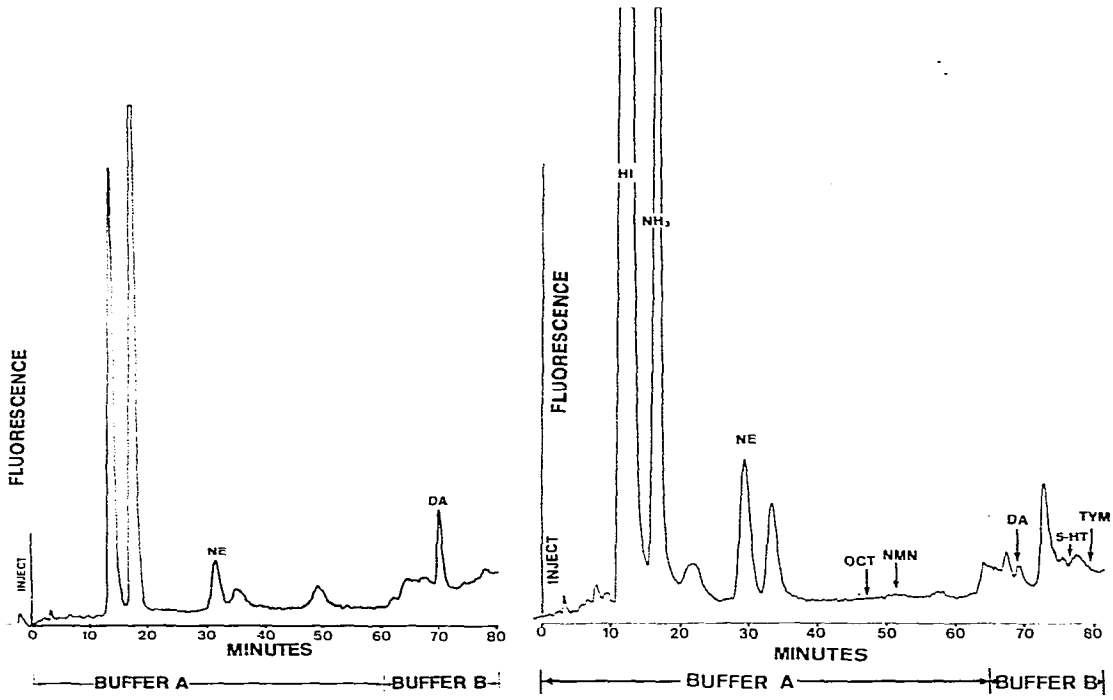


Fig. 12. Reversed-phase HPLC separation of biogenic amines in rat brain tissue after Bio-Rex 70 cleanup. Sample: 10 μl, equivalent to 12.5 mg whole brain. Samples were quantitated by external standard. The first elution step (A) consisted of 300 ml of methanol per liter. All other conditions and abbreviations as in Fig. 2.

Fig. 13. Reversed-phase HPLC separation of biogenic amines in dog heart tissue. Sample: 10 μl, equivalent to 15.1 mg heart. All other conditions as in Fig. 12. Abbreviations as in Fig. 2.

buffer) it was found, based on co-injection of a norepinephrine standard, that the previously symmetrical norepinephrine peak was not homogeneous (Fig. 17). Similarly, an improvement of the selectivity for norepinephrine was observed when a mixture of 40% (v/v) methanol 10% (v/v) acetonitrile in 0.05 *M* phosphate was used as the eluent.

Since norepinephrine, normetanephrine, dopamine and 5-hydroxytryptamine were not retained sufficiently on the C_{18} column for a good separation from interfering primary amines, we investigated the more selective and polar reversed-phase μBondapak phenyl column. This column proved to be far superior in efficiently and selectively separating the biogenic amines in various biological materials, after studying the effects of eluent composition and temperature.

Our work has shown that the following molecules are removed by the cleanup or do not chromatograph under the present experimental conditions: polyamines, N-acetylserotonin, melatonin, amino acids, *p*-methoxybenzylamine, 5-methoxytryptamine, 3-methoxytyramine, 6-hydroxydopamine, DOPA, phenylethylamine and 3-methoxy-DOPA.

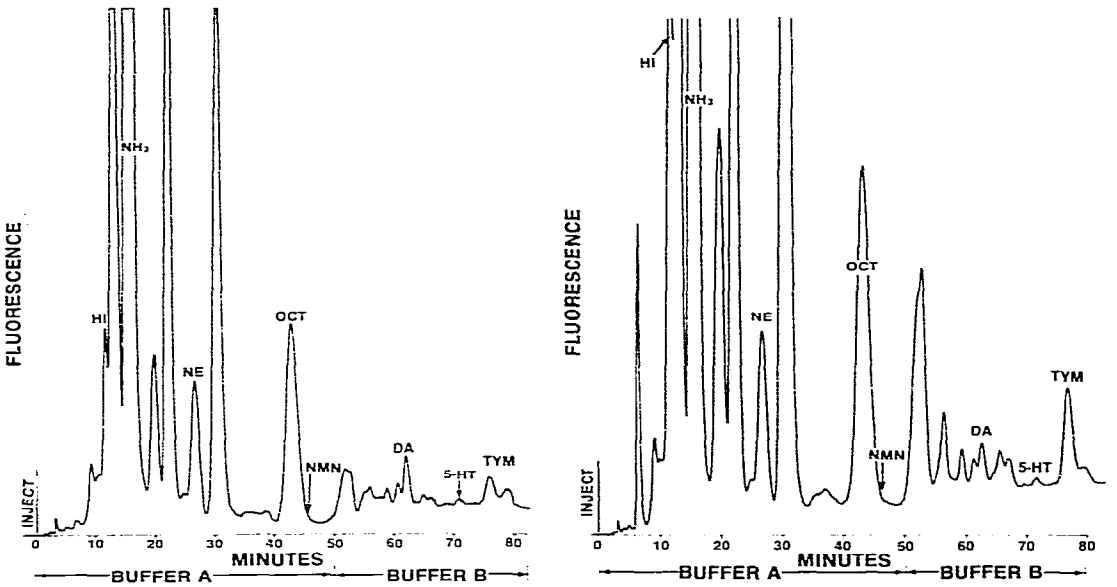


Fig. 14. Reversed-phase HPLC separation of biogenic amines in control urine. Sample: 15 μl, equivalent to 15 μl urine. All other conditions and abbreviations as in Fig. 2 except that no IS was used.

Fig. 15. Reversed-phase HPLC separation of biogenic amines in urine of brain tumor patient. Sample: 15 μl, equivalent to 15 μl urine. All other conditions and abbreviations as in Fig. 2 except that no IS was used.

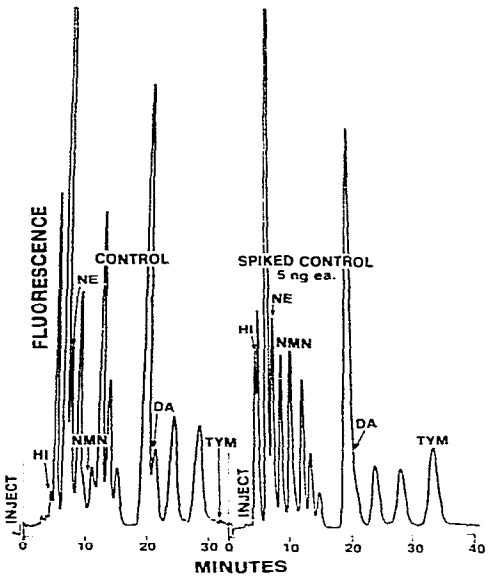
Experiments are underway to collect the major unknowns that are chromatographed and identify them by high-resolution mass spectrometry. These unknowns change in response to experimental treatments and disease and thus may indicate important biochemical relationships.

CONCLUSIONS

The reversed-phase partition mode of HPLC with fluorescence detection and pre-column derivatization with OPT for separation provides a rapid, highly sensitive, simple and quantitative method for the simultaneous analysis of many biogenic amines at the subnanogram level.

The pre-column derivatization with OPT proved very advantageous by stabilizing the labile molecules, aiding in their extraction with organic solvent and allowing for sensitive HPLC fluorescence detection at the subnanogram level. The classical aluminum oxide or weak cation-exchange resin cleanup methods can also be used (Fig. 12) if only two to three biogenic amines need quantitating and if the recoveries are adequate at physiological levels.

The use of a two-step gradient elution, with the μBondapak phenyl column decreased analysis time since the more strongly retained compounds eluted earlier than by isocratic elution. In addition, the more polar phenyl column efficiently separated the biogenic amines from the sample matrix, while the



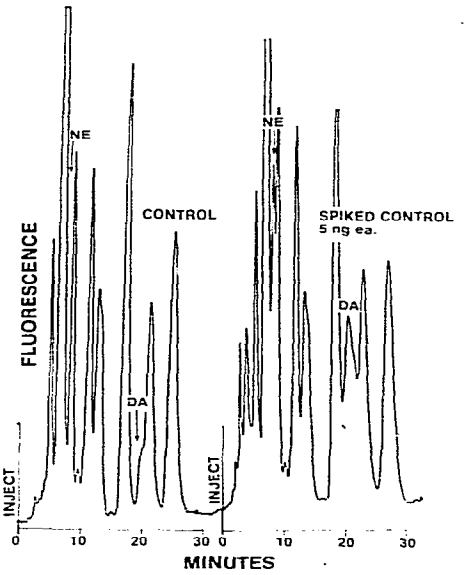


Fig. 16. Effect of eluent composition on separation of biogenic amines in plasma. Sample: 5 µl, equivalent to 0.05 ml plasma; column: µBondapak C_{18} (300 × 4 mm); eluent: 0.05 mole/l NaH_2PO_4 (pH 5.10) with 480 ml of methanol per l; flow-rate: 1.5 ml/min; temperature: 35°. Abbreviations as in Fig. 2.

Fig. 17. Effect of eluent composition on separation of biogenic amines in plasma. All conditions as in Fig. 16 except eluent, made with 450 ml of methanol and 50 ml of DMF per l; and temperature: 25°. Abbreviations as in Fig. 2.

less-selective C_{18} column did not. Even complex mixtures of organic solvents and buffers did not satisfactorily improve the separation on the C_{18} column.

This HPLC chromatographic method is now used routinely in our laboratory. Instead of quantitating only two or three biogenic amines in a single sample we are able to measure seven biogenic amines in one analysis. In addition, eight to ten well-separated yet unidentified primary amines appear in the chromatograms, which could have physiological significance. The method provides a powerful research and clinical tool for studying various diseased states in both man and animals.

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