EVALUATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY, GAS CHROMATOGRAPHY AND FLUOROMETRY FOR QUANTITATION OF URINARY FREE CATECHOLAMINES

by

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ABSTRACT

EVALUATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY, GAS CHROMATOGRAPHY AND FLUOROMETRY FOR QUANTITATION OF URINARY FREE CATECHOLAMINES

Eileen M. Miller

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Analytical methods for the quantitation of urinary free norepinephrine, epinephrine and dopamine are described. High pressure liquid chromatography (HPLC) with conventional ultraviolet (UV) absorbance detection at 254 nm and with electrochemical detection is investigated. Urine eluates for electrochemical detection are obtained from a two-step purification procedure, with incorporation of an internal standard, 3,4-dihydroxybenzylamine (DHBA) throughout the extraction. Rapid and simple quantitation of norepinephrine, epinephrine and dopamine from a 5-mL urine aliquot is achieved with electrochemical detection. Problems encountered with HPLC analysis are also discussed.

TMS-TFA derivatization of standard catecholamine solutions prior to gas chromatographic (GLC) analysis gives poor peak resolution. On-column acetamide derivatization of catecholamine standards utilizing gas chromatography gives better peak resolution, with potential application to urinary free catecholamine quantitation.

Comparison of precision data from traditional fluorometric estimation of norepinephrine and epinephrine with that for the HPLC method shows the latter to be more precise in most respects. Relative merits of each method are discussed, as well as ease of implementation into the clinical laboratory.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS OR REFERENCE
A. R.	Analytical reagent	--
anhydr.	Anhydrous	--
conc.	Concentrated	--
^{o}C	Degrees centigrade	--
DA	Dopamine	--
dL	Deciliter	1×10^{-1} liter
DHBA	3,4-Dihydroxybenzylamine	--
E	Epinephrine	--
EDA	Ethylenediamine	--
g	Gram	--
GLC	Gas-liquid chromatography	--
HPLC	High pressure liquid chromatography	--
hr	Hour	--
IS	Internal standard	--
L	Liter	--
mol/L	Molarity	moles per liter
mL	Milliliter	1×10^{-3} liter
mg	Milligram	1×10^{-3} gram
mmol/L	Millimolarity	mmoles per liter
µg	Microgram	1×10^{-6} gram
µL	Microliter	1×10^{-6} liter
min	Minute	--
nA	Nanoamp	1×10^{-9} amp
ng	Nanogram	1×10^{-9} gram
nm	Nanometer	1×10^{-9} meter

LIST OF SYMBOLS (CONT.)

SYMBOL	DEFINITION	UNITS OR REFERENCE
NE	Norepinephrine	--
#	Number	--
%	Percent	--
±	Plus or minus	--
Std.	Standard	--
TFA	Trifluoroacetyl	--
THI	Trihydroxyindole	--
TMS	Trimethylsilyl	--
UV	Ultraviolet	--
V	Volt	--

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CHAPTER I

INTRODUCTION

Structures and Properties

The physiologically important catecholamines are epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine. Their structures are shown in Figure 1.

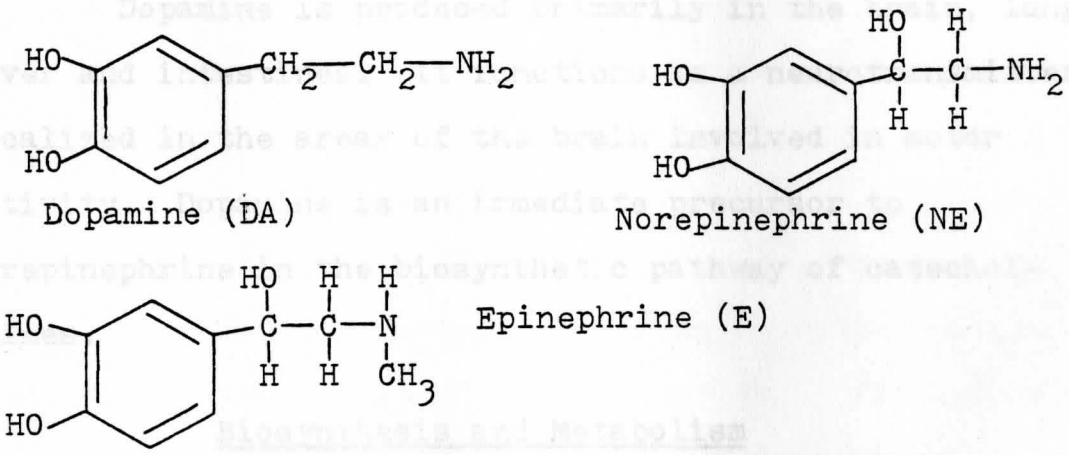


Fig. 1. Structure of catecholamines.

These compounds are characterized by the presence of a catechol nucleus. Since epinephrine has a methyl group present on the terminal amino group, it is a secondary amine. Dopamine and norepinephrine are primary amines. Catecholamines share the chemical properties of phenols, alcohols and amines.

Epinephrine is stored in the chromaffin cells of the adrenal medulla and is released in response to efferent impulses from the splanchnic nerves. Epinephrine acts as

an effective arteriolar smooth muscle constrictor or dilator and also as a metabolic agent, exerting a majority of its effects on carbohydrate metabolism.

Norepinephrine is the neurotransmitter between the sympathetic postganglionic fiber and the effector organ. This amine works principally on the circulatory system by promoting the elevation of both systolic and diastolic blood pressure. Norepinephrine plays a less significant role in carbohydrate metabolism than does epinephrine.[1]

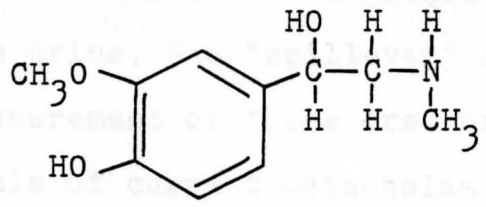
Dopamine is produced primarily in the brain, lungs, liver and intestines. It functions as a neurotransmitter localized in the areas of the brain involved in motor activity. Dopamine is an immediate precursor to norepinephrine in the biosynthetic pathway of catecholamines.

Biosynthesis and Metabolism

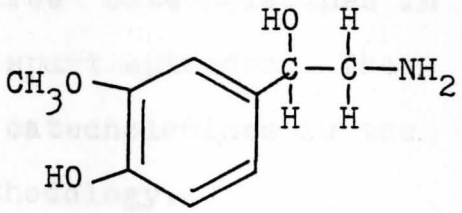
According to Galen and Gambino,[2] the major metabolic pathways of catecholamines in the body were elucidated by Armstrong and coworkers in 1957. Synthesis of catecholamines begins with the aromatic amino acid tyrosine. In the first step, tyrosine is hydroxylated in the cell mitochondrion by tyrosine hydroxylase, yielding 2-dihydroxyphenylalanine (DOPA). DOPA enters the cytoplasm where it is decarboxylated by DOPA carboxylase, producing L-dihydroxyphenylamine (dopamine). L-epinephrine is synthesized by the hydroxylation of dopamine, which occurs

in the adrenal medulla, or in the granulated vesicles of brain cells and of peripheral sympathetic nerve endings. In the final step, which occurs primarily in the adrenal medulla, phenylethanolamine-N-methyl transferase transfers a methyl group to norepinephrine from S-adenosylmethionine, yielding epinephrine.

There are normally small amounts of norepinephrine present in the blood. The basal level is about 30 ng/dL in arterial and 40 ng/dL in venous blood.[1] This continuous release is most likely the end result of tonic sympathetic activity. "Spurt" releases of catecholamines occur as a consequence of sympathetic stimulation and are followed by their rapid clearing from the blood, which is achieved by several processes. In one process, known as reuptake, released norepinephrine is again taken up by the neuron, thus terminating its physiological effects. The uptake mechanism also applies to epinephrine and dopamine, although these catecholamines are inactivated primarily in the liver by another clearing process. This process is catabolic, and permanently inactivates the catecholamines. The liver can remove up to 85% of the liberated catecholamines in one passage of blood through it, via the action of catechol-O-methyl transferase (COMT).[1] This enzyme uses S-adenosyl-methionine as the methyl donor to methylate the C-3 hydroxyl groups of epinephrine and norepinephrine, yielding metanephrine and normetanephrine, respectively. These metabolites are illustrated in Figure 2.



Metanephrine



Normetanephrine

Fig. 2. Structure of metanephrine and normetanephrine.

A certain fraction of the methylated amines are either excreted unchanged or are conjugated with sulfuric or glucuronic acid prior to excretion. Any remaining metabolites are converted to 3-methoxy-4-hydroxy-mandelic acid (vanilmandelic acid, VMA), the final end product of epinephrine and norepinephrine metabolism.

Dopamine is ultimately converted to homovanillic acid (HVA) via O-methylation and oxidative deamination. The structure of HVA is shown in Figure 3.

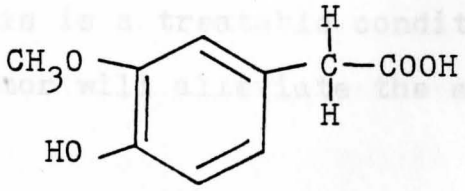


Fig. 3. Structure of homovanillic acid.

Clearing is also accomplished by the kidneys, which excrete a minute amount of unaltered of "free" catecholamines in the urine, the "spillover" during spurt episodes. The measurement of these free urinary catecholamines is the basis of current catecholamine methodology.

Clinical Significance

Decreased urinary excretion of catecholamines may occur in patients with familial dysautonomia, malnutrition or transection of the cervical spinal cord,[3] but is of little clinical interest. In contrast, elevated urinary catecholamines are clinically significant, especially in patients with hypertension. High levels of catecholamines in urine have been associated with secreting tumors of the adrenal medulla or of extra-adrenal chromaphil tissue, referred to as pheochromocytomas. Ninety percent of all pheochromocytomas develop in the adrenal medulla.[1] Symptoms of these tumors mimic those of hypertension and include pounding headache, palpitations, sweating, anxiety, pallor and hyperventilation.[1] It is important to be able to detect those hypertensive patients who have pheochromocytomas, since this is a treatable condition. Surgical removal of the tumor will alleviate the symptoms of hypertension.

The adrenal medulla tumor is characterized by increased epinephrine levels above the normal urinary excretion of 10 ± 5 µg/24 hr.[1] Measurement of the urinary

excretion of epinephrine can aid in determining the physiologic status of the adrenal medulla.

An extra-adrenal pheochromocytoma will secrete primarily norepinephrine, thereby elevating urinary levels of norepinephrine above the normal value of 40 ± 20 µg/24 hr.[1] Measurement of urinary norepinephrine can reflect the overall peripheral sympathetic activity during a relatively long period of time.

Other conditions associated with elevated epinephrine and norepinephrine secretion are malignant neuroblastomas of children, ganglioneuroblastomas and ganglioneuromas.[3] The majority of these tumors produce adequate amounts of pressor amines to cause an abnormal elevation of urinary catecholamines. Increased urinary levels are also encountered after vigorous exercise and in patients with progressive muscular dystrophy and myasthenia gravis.[3] Therefore, elevated urinary catecholamines is not an absolutely specific indication of a secreting tumor.

The normal range for urinary dopamine is reported by Bischoff and Torres[4] to be 373 ± 63 µg/24 hr. These investigators found there to be significant variation in normal dopamine excretion. Considerable increases above normal values are found in conditions of pheochromocytoma, paraganglioma and tumors of the sympathetic nervous system. Dopamine shows a moderate increase above normal in striate syndrome, phenylketonuria, manic phase/cyclothymia and in thyrotoxicosis.

CHAPTER II

HISTORICAL

Extraction

The analysis of catecholamines in urine has mainly been based on alumina adsorption followed by acid elution and estimation of the catecholamines present in the eluate. Alumina adsorption was first developed by Shaw in 1938[5] and is still widely utilized as an extraction technique for urinary catecholamines. Adsorption on alumina can be performed with a column or batch procedure (mixing the urine sample in a suspension of alumina). The column procedure was found to be the more efficient.[6]

The elution step has been studied by many investigators. Elution at pH 8.4 with 0.2 mol/L acetic acid was proposed by Lund in 1949.[7] Elution with 0.2 mol/L acetic acid was frequently used in subsequent studies.[8-12] Other eluents have been proposed, such as 0.25 mol/L sulfuric acid,[13] 0.05 mol/L perchloric acid[14] and 0.4 mol/L acetic acid.[15] Akron City Hospital (Akron, OH 44309) currently utilizes the alumina adsorption technique with 0.2 mol/L acetic acid as the eluent.[16]

There is available an alternate adsorption technique, utilizing a resin column at pH 6.5 and elution of the urinary catecholamines with 4% boric acid. This

method is based on a report by Sandhu[17] and is marketed by BioRad Laboratories (Richmond, CA 94804). The Youngstown Hospital Association (Youngstown, OH 44505) experimented with the BioRad procedure, but found the prepacked columns inconvenient and now uses the resin in glass columns.[18] BioRad claims that the resin column isolation technique has increased precision and recovery over the alumina technique, based on the premise that alumina treatment causes a significant drop in pH during the adsorption phase.[19]

Alumina adsorption is subject to many variables. These will be discussed in conjunction with the fluorometric step.

Several methods for the analysis of the urinary eluates have been developed. (1) Fluorometric procedures, including the ethylenediamine (EDA) condensation and the trihydroxyindole (THI) techniques, have been documented. Differential analysis of epinephrine and norepinephrine in a single urine sample has been performed using various modifications of the THI fluorometric procedure. (2) Dihydroxyindole fluorometric methods have been described for the measurement of dopamine. (3) Gas-liquid chromatography (GLC) and (4) high pressure liquid chromatography (HPLC) procedures have been reported for the separation and quantitation of urinary catecholamines.

Fluorometric Procedures

The eluate assay has been performed by the oxidation of epinephrine and norepinephrine to their fluorescent derivatives. In the EDA condensation method, epinephrine is oxidized to adrenochrome, which condenses with one mole of ethylenediamine, eliminating 2 H_2O and 2 H. The fluorophor of epinephrine obtained by EDA condensation is shown in Figure 4.

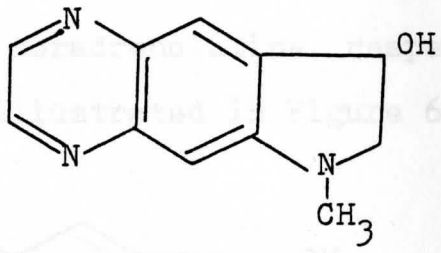


Fig. 4. Fluorophor of epinephrine from EDA condensation.

Similarly, norepinephrine is oxidized to noradrenochrome, which condenses with EDA, eliminating the side chain of norepinephrine plus 2 H. This compound then condenses with another molecule of EDA, yielding the fluorophor shown in Figure 5.

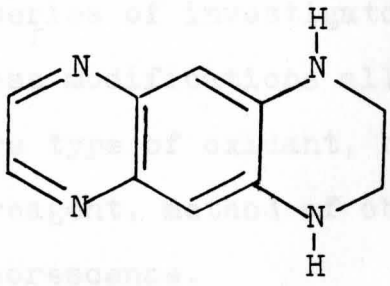


Fig. 5. Fluorophor of norepinephrine from EDA condensation.

The EDA method was studied by Manger et al.[20] in 1969 and Weil-Malherbe[21] in 1971 in an attempt to assay plasma epinephrine and norepinephrine in a single sample. It was observed that the EDA method is more sensitive than the THI method, but lacks the appropriate specificity.[12, 22-23]

In the THI reaction, epinephrine and norepinephrine are oxidized to their corresponding adrenochromes, either by potassium ferricyanide or iodine. These rearrange in alkaline solution to the fluorescent derivatives adrenolutine and noradrenolutine, respectively. Adrenolutine is illustrated in Figure 6.

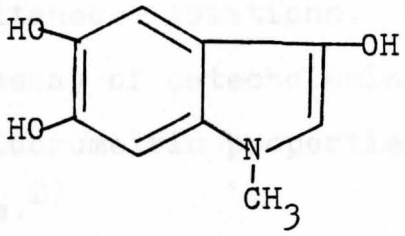


Fig. 6. Structure of adrenolutine.

The THI fluorometric procedure was studied quantitatively by Erlen[24] in 1948 and reported as a technique by Lund[7] in 1949. Variations of this method were introduced by a series of investigators,[8-9, 13, 25] along with others. These modifications all differ in detail; the main variables are type of oxidant, buffer and oxidation pH, stabilizing reagent, method of obtaining blanks and evaluation of fluorescence.

The differential analysis of urinary epinephrine and norepinephrine has received attention.[6, 10, 26] von Euler and Lishajko[10, 26] devised a manual alumina-THI method in which epinephrine and norepinephrine are estimated by the fluorescence of their lutines, using two filter sets for obtaining excitation and emission wavelengths. BioRad Laboratories market a differential procedure[19] which follows the adsorption step on ion-exchange resin described by Sandhu and Freed.[17] In this procedure, epinephrine and norepinephrine are differentiated at two different excitation/emission wavelengths. The concentration of each amine in the eluate is calculated with two simultaneous equations. The differential fluorometric assay of catecholamines is difficult owing to the similar fluorometric properties of epinephrine and norepinephrine.[27]

This difficulty can be overcome by selectively fading the fluorescence of the lutines, either by modifying the oxidation pH or the reducing agent. Weil-Malherbe[6] proposed a variation in oxidation pH. Oxidation of epinephrine is performed at pH 3 and norepinephrine is estimated with readings taken after oxidation at pH 6.

These parameters have also been investigated in several auto-analyzer fluorescence methods.[12, 27-28] Peyrin and Cottet-Emard[27] in 1973 found optimal conditions for epinephrine estimation to be oxidation at pH 2.5 with potassium ferricyanide as oxidant and alkaline ascorbate as

the reducing agent. The optimal conditions for norepinephrine estimation are oxidation at pH 6.4 with potassium ferricyanide and complete fading of epinephrine fluorescence by using a cystein, thioglycolic acid, ethanol, 5 mol/L NaOH mixture as the reducing agent. Andersson et al.[12] in 1974 investigated reducing agents and found that 4% thioglycolic acid gave satisfactory results for the selective differentiation of norepinephrine.

Methods for the fluorometric measurement of dopamine have been developed.[4, 29-30] In the method described by Bischoff and Torres[4] urinary dopamine is oxidized by iodine to a red indole derivative, converted to a 5,6-dihydroxyindole in alkaline sulfite solution and acidified to pH 5.3. The fluorescence is determined at excitation/emission wavelengths sufficiently lower than those of adrenolutine or noradrenolutine, thereby preventing interferences.

There are several problems inherent in the alumina-THI fluorometric procedure. (1) Low recoveries for internal standards are common. This is due mainly to fluorescence suppression caused by interfering urinary constituents in the eluate and column losses of the catecholamines. (2) It is difficult to distinguish adequately between epinephrine and norepinephrine in a single urinary sample, for the reasons previously discussed. (3) The fluorophors are unstable compounds. (4) Blanks tend to have nonspecific fluorescence, causing their

measurements to be high, hence decreasing stability. This problem occurs because all interfering impurities in a hydrolyzed (boiled at pH 2) urine sample are not removed in one alumina adsorption step. (5) Falsely elevated values of catecholamines may result from the presence of fluorescent vitamins or urinary metabolites of medications, including methenamine mandelate, tetracycline, quinidine and Aldomet® (methyldopa, Merck, Sharp and Dohme). Bananas or synthetic vanilla in the diet have a similar effect if total (hydrolyzed) catecholamines are measured. These interferences of dietary origin do not appear to affect the analysis of free catecholamines.

Chromatographic Methods

Recently, gas-liquid chromatography has been utilized for the measurement of urinary catecholamines and their metabolites.[31-32] Cancalon and Klingman[31] investigated the formation of trifluoroacetyl (TFA) - trimethylsilyl (TMS) derivatives of biogenic amines, including epinephrine, norepinephrine and dopamine. Separation of standard aqueous mixtures of the catecholamines was achieved with a 3% polyamide A103 Gas Chrom column (Applied Sciences Laboratories), with a detection limit in the nanomolar range.

On-column acetamide derivatization by flame ionization detection has been applied to the measurement of amphetamine concentrations in blood and urine.[41] This

method shows promise of becoming applicable to the measurement of urinary catecholamines, based on the similar structural characteristics of amphetamines and catecholamines.

More recently, reverse-phase high pressure liquid chromatography has been utilized for the estimation of catecholamines and their metabolites.[33-40, 42-43] The stationary phase in HPLC used most frequently is an octadecyl-silica surface. A polar solvent such as aqueous methanol, acetonitrile or phosphate buffer serves as the mobile phase. The chromatography is termed "reverse-phase" because the aqueous eluent is more polar than the stationary phase. Reverse-phase HPLC differs from ion-exchange chromatography in that acids and bases can be separated in one passage through the reverse-phase system. This is possible since retention depends only on the hydrophobic interactions between the nonpolar moiety of the injected biogenic amines and the nonpolar octadecyl-silica stationary phase.[33]

Mell and Gustafson[34] used reverse-phase HPLC with UV detection for the separation and quantitation of norepinephrine and dopamine in a single urine sample. The sample is treated with alumina at pH 8.4, and the catecholamines eluted with 0.2 mol/L acetic acid. The column used was an octadecyl-silica column (μBondapak C_{18}; Waters Associates Inc., Milford, MA 01757) with 0.17 mol/L acetic acid as the mobile phase. Comparative studies with the

traditional THI fluorometric procedure for norepinephrine[11] and dopamine[4] showed the HPLC method to be much more precise and less subject to drug interferences. Mell and Gustafson did not include an internal standard throughout the procedure.[34]

In 1978, Davis and coworkers[35] combined fluorescence detection with HPLC, utilizing a two-step gradient elution with methanol/phosphate buffer. A μBondapak/phenyl column (Waters Associates Inc.) was used instead of the octadecyl-silica column utilized by Mell and Gustafson.[34] These investigators derivatized tissue, plasma or urine samples prior to HPLC analysis with o-phthaladehyde and then extracted the catecholamines into ethyl acetate. The method is reported as sensitive for quantitating nanogram amounts of norepinephrine, dopamine, normetanephrine and several other recognized biogenic amines in biological samples.

Techniques have been described for the determination of catecholamines and their metabolites by HPLC with electrochemical detection.[36-40, 42-43] The main advantages are increased specificity and sensitivity of the electrochemical detector for the urinary catecholamines. The high resolution of this detection system makes it possible to include an internal standard, 3,4-dihydroxybenzylamine (DHBA) through the urine extraction procedure. The internal standard corrects for the variation in recovery that is often encountered with alumina adsorption, allowing

catecholamines to be quantitated with confidence even if recoveries are low.[37, 39-40]

An ion-pairing agent, such as heptanesulfonate, must be added to the mobile phase so that DHBA will chromatograph as a separate entity. Without the addition of an ion-pairing agent, DHBA co-elutes with epinephrine.[39] However, the use of ion-pairing surfactants decreases column lifetime and also causes problems with column stability and reproducibility.[43] The use of simple acids, such as acetic acid, nitric acid and trichloroacetic acid as ion-pairing agents has been investigated.[42] Mobile phases consisting of a simple acid have been reported to increase the lifetime of the expensive C_{18} column and also provide consistent, adequate resolution of the catecholamines.[42]

HPLC analyses have the advantage of not requiring the formation of volatile derivatives of the catecholamines prior to injection, a mandatory step in GLC procedures.

Summary

Normal ranges for urinary catecholamines vary with the method of analysis. Results obtained in representative studies for each type of method of catecholamine determination are summarized in Table 1.

TABLE 1

SUMMARY OF NORMAL RANGES FOR URINARY CATECHOLAMINES

Year	Author	Results	Method
		Total Catecholamines	
1960	Sobel and Henry[9]	68 ± 18 μg/24 hr	THI-alumina
		Free Catecholamines	
1962	Bischoff and Torres[4]	DA 373 ± 63 μg/24 hr	modified THI-alumina
1971	Weil-Malherbe[6]	E 0.22 ± 0.04 μg/L NE 0.58 ± 0.11 μg/L	EDA[a]
1974	Andersson et al.[12]	E 1.9 ± 0.4 μg/L NE 15.3 ± 2.1 μg/L	automated THI-alumina
1977	Mell and Gustafson[34]	NE 82 ± 3 μg/24 hr DA 307 ± 8 μg/24 hr	HPLC with UV detection
1979	Moyer et al.[40]	E 0.5-20 μg/24 hr NE 14-80 μg/24 hr DA 65-400 μg/24 hr	HPLC with electrochemical detection

[a] plasma levels

CHAPTER III

STATEMENT OF PROBLEM

The quantitation of total urinary catecholamines at the Youngstown Hospital Association clinical laboratory is presently performed by cation-exchange extraction, followed by estimation of the catecholamines in the eluate by the traditional trihydroxyindole reaction, with subsequent determination of fluorescence. However, the method is tedious, lacks precision, does not measure norepinephrine and epinephrine as separate entities and does not quantitate dopamine at all.

Development of a simple, reproducible method for quantitation of urinary catecholamines having good correlation with the present fluorometric method is desired. Chromatographic methods utilizing high pressure liquid chromatography and gas-liquid chromatography and requiring comparable purification steps for urine are included. The method should have the sensitivity necessary to detect catecholamines in the microgram range, with sufficient specificity so that interfering materials often present in urine samples will not affect the analysis.

CHAPTER IV

MATERIALS AND APPARATUS

Materials

Following is a list of chemicals used in this research:

Name	Grade	Manufacturer
glacial acetic acid	A. R.	Mallinckrodt
hydrochloric acid	A. R.	Mallinckrodt
sulfuric acid	A. R.	Mallinckrodt
ammonium sulfate	A. R.	J. T. Baker
sodium phosphate dibasic	A. R.	Fisher Scientific
sodium phosphate monobasic	A. R.	J. T. Baker
potassium phosphate monobasic	A. R.	J. T. Baker
disodium ethylenediamine tetraacetate (EDTA)	A. R.	Fisher Scientific
1-heptanesulfonic acid sodium salt	A. R.	Eastman Kodak
citric acid	A. R.	J. T. Baker
methanol		A. C. S.
sodium metabisulfite	A. R.	Fisher Scientific
TRIS (buffer)		J. T. Baker
aluminum oxide, activated 80-325 mesh	chromatographic grade	Matheson, Coleman, Bell
sodium hydroxide	A. R.	J. T. Baker

potassium ferricyanide	A. R.	Baker and Adamson
cupric acetate	A. R.	Fisher Scientific
2-mercaptoethanol	A. R.	Eastman Kodak
formic acid, 90%	A. R.	Fisher Scientific
acetic anhydride	A. R.	Mallinckrodt
methylene chloride	A. R.	Eastman Kodak
pyridine	A. R.	J. T. Baker
MSTFA (N-methyl-N-TMS-trifluoroacetamide)		Pierce Chemical Co.
BSTFA (N,O,bis-(trimethylsilyl)-trifluoroacetamide)		Pierce Chemical Co.
L-epinephrine		Sigma Chemical Co.
L-arterenol, free base (norepinephrine)		Sigma Chemical Co.
3-hydroxy-tyramine·HCl (dopamine)		Sigma Chemical Co.
3,4-dihydroxybenzylamine hydrobromide, 98%		Aldrich Chemical Co.
BioRad Cation Exchange Columns cat. no. 1892202		BioRad Laboratories Richmond, CA 94804

Apparatus

High Pressure Liquid Chromatography

All sample analyses are performed using a Model 204 Liquid Chromatograph with a Model 6000A Solvent Delivery System and a U6K Universal Injector (Waters Associates Inc., Milford, MA 01757). A Model 440 Ultraviolet (UV) Absorbance Detector (Waters Associates Inc.) is employed with absorbance recorded at 254 nm with a Model 252A strip-

chart recorder (Linear Instruments Corp., Costa Mesa, CA 92626). A PAR Model 170 Electrochemical System (Princeton Applied Research, Princeton, NJ 08540) is used in conjunction with LC-12 Electrochemical Detector accessories (Bioanalytical Systems Inc., West Lafayette, IN 47906). This package includes a TL-3 plexiglass flow-through, thin-layer detector cell, packed with CP-0, carbon paste of graphite/mineral oil composition and a RC-1 combination reference and auxilary electrode compartment. The reference electrode is an RE-1, Ag/AgCl electrode, and the auxilary electrode is a stainless steel tube. These electrodes, in combination with the CP-0 packed working electrode, comprise the three electrode system. Measurements are taken by controlling the potential between the working and auxilary electrodes so that the potential of the working electrode is +0.500 volts versus the Ag/AgCl reference electrode, while monitoring the current passing between the working electrode and the auxilary electrode. A reverse-phase C_{18} column (μBondapak C_{18}; Waters Associates, Inc.) is used throughout.

Gas-Liquid Chromatography

All sample analyses are performed using a Bendix Toxichron Gas Chromatograph (Scientific Products, McGraw Park, IL 60085) equipped with a Hewlett-Packard Model 3380 Integrator (Mountainview, CA 94043). The Bendix Gas Chromatograph operates with dual flame ionization

detectors, with one side containing a tube-shaped glass column filled with 3% SP-2401 DB on 100-120 Supelcoport (Supelco, Inc., Bellefonte, PA 16823). The column is conditioned at 250 °C for 24 hours with a nitrogen flow rate of 25 mL/min, prior to use.

Fluorometry

All samples are analyzed with a Turner Model 430 Spectrofluorometer (G. K. Turner Associates, Palo Alto, CA 94303).

Additional Apparatus

centrifuge GLC-1 (General Laboratory Centrifuge)

rotovapor (Büchi Laboratory Techniques Ltd., Flawil, Switzerland)

analytical balance (Sartorius, division of Brinkmann Instruments, Westbury, NY 11590)

vortex mixer (Scientific Industries Inc., Bohemia, NY 11716)

CHAPTER V

EXPERIMENTAL

High Pressure Liquid Chromatography

Reagents

Preparation of reagents for HPLC analysis is described below.

hydrochloric acid, 6 mol/L	Add 500 mL of conc. HCl to distilled water and dilute to 1 L.
hydrochloric acid, 0.1 mol/L	Add 8.3 mL of conc. HCl to distilled water and dilute to 1 L.
ammonium sulfate, 2 mol/L	Dissolve 264 g $(NH_4)_2SO_4$ in distilled water and dilute to 1 L.
sulfuric acid, 0.7 mol/L	Add 40 mL of conc. H_2SO_4 to distilled water and dilute to 1 L.
phosphate buffer, pH 7.0	Dissolve 4.32 g Na_2HPO_4, 1.18 g KH_2PO_4 and 10.0 g EDTA in distilled water and dilute to 1 L.
Tris buffer, 3 mol/L, pH 8.6	Dissolve 363 g TRIS in distilled water and dilute to 1 L. Adjust to pH 8.6 with 6 mol/L HCl.
acetic acid, 0.05 mol/L with 5 mmol/L sodium metabisulfite	Add 2.9 mL glacial acetic acid to distilled water containing 0.95 g $Na_2S_2O_5$ and dilute to 1 L.

aluminum oxide, prepared by the method of Crout.[11]

stock DHBA solution, 1 mg/mL	Dissolve 100 mg DHBA in 0.1 mol/L HCl and dilute to 100 mL.
dilute DHBA solution, 10 μg/mL	Dilute 1 mL of stock DHBA solution to 100 mL with 0.1 mol/L HCl.
stock NE solution, 1 mg/mL	Dissolve 100 mg L-arterenol in 0.1 mol/L HCl and dilute to 100 mL.
stock E solution, 1 mg/mL	Dissolve 100 mg L-epinephrine in 0.1 mol/L HCl and dilute to 100 mL.
stock DA solution, 1 mg/mL	Dissolve 123 mg 3-methoxy-tyramine·HCl in 0.1 mol/L HCl and dilute to 100 mL.
standard mixture, 10 μg/mL	Dilute 1 mL of each stock solution (NE, E, DA, DHBA) to 100 mL with 0.1 mol/L HCl.
urine pool	Urine is collected from healthy individuals and acidified to pH 2-3 with 6 mol/L HCl.

Sample Collection

A 24-hour urine specimen is collected and preserved during the collection period with 15 mL of 6 mol/L HCl. The volume is measured using a 2000-mL graduated cylinder. Specimens not immediately analyzed are stored in glass amber bottles at 4 °C and are stable for several months.

Sample Extraction

This procedure has two steps. The cation-exchange step is adapted from the procedure of Riggin and Kissinger.[37] A 5-mL aliquot of the 24-hr urine specimen is pipetted into a 50-mL beaker. 50 µL of the 10 µg/mL DHBA internal standard solution is added to the urine, followed by 15 mL of phosphate buffer, pH 7.0. The entire mixture is poured onto a BioRad cation-exchange column and allowed to drain completely. The resin is washed with 10 mL of distilled water, allowed to drain completely, then is washed with 1.5 mL of 0.7 mol/L H_2SO_4. The catecholamines are eluted with 4 mL of 2 mol/L $(NH_4)_2SO_4$ into a 10-mL beaker. Immediately prior to the alumina adsorption step, 500 µL of 3 mol/L Tris buffer, pH 8.6, is added to the eluate.

The alumina adsorption step is a modification of the procedure of Moyer *et al.*[40] The eluate from the cation-exchange step containing 500 µL of Tris buffer, pH 8.6, is pipetted onto a BioRad column containing 0.5 g of alumina. When the meniscus of the solution reaches the top of the alumina, the column is washed with two 3-mL portions of distilled water. The alumina must never go dry. The catecholamines are eluted with 5 mL of 0.05 mol/L acetic acid containing 5 mmol/L $Na_2S_2O_5$ into a 50-mL round-bottom flask. The acetic acid is evaporated to dryness using a rotovapor and the dried catecholamines are taken up in 1 mL of mobile phase for HPLC analysis.

A series of urine pool aliquots is analyzed by the method described to establish the range of acceptable values. Thereafter, at least one aliquot of the urine pool is analyzed with each group of urine specimens, to insure day-to-day reproducibility. Also, one aliquot of the standard mixture containing equal amounts of the three catecholamines plus the internal standard, DHBA, is analyzed with each batch run, so that relative recovery rates can be determined and applied to the quantitation of norepinephrine, epinephrine and dopamine in each sample extracted that day.

HPLC Analysis

The reverse-phase column (Waters Associates Inc.) is operated at a flow rate of 1.5 mL/min with absorbance monitored at 254 nm. With electrochemical detection, the detector potential is held at +0.500 V for all analyses, and the C_{18} column is operated at a flow rate of 2.0 mL/min. Injection volumes vary from 5 to 85 µL.

Calculation

$$\frac{\text{peak height of catecholamine}}{\text{peak height of IS}} \times \frac{\text{amount of IS added (µg)}}{\text{volume of urine analyzed (mL)}}$$

$$\times \text{ factor } \times \text{ 24-hr volume (mL)} = \text{µg/24 hr.}$$

where factor $= \dfrac{\text{peak height of IS}}{\text{peak height of catecholamine}}$, for the standard mixture analyzed by the method described.

Modifications

Several mobile phases were tested. (1) 0.17 mol/L acetic acid - add 9.8 mL of glacial acetic acid to distilled water and dilute to 1 L. (2) 0.1 mol/L phosphate-citrate buffer, pH 4.0 - dissolve 21.0 g of citric acid in distilled water and dilute to 1 L. Dissolve 14.2 g of Na_2HPO_4 in distilled water and dilute to 1 L. Adjust the pH of 300 mL of the citric acid solution to 4.0 by the addition of the Na_2HPO_4 solution (ca. 400 mL). Dissolve 440 mg of heptanesulfonate (2 mmoles) in each liter of solvent. (3) 70 mmol/L sodium phosphate/methanol (95/5), pH 4.8 - dissolve 9.66 g of NaH_2PO_4 in distilled water and dilute to 1 L. Dissolve 440 mg of heptanesulfonate and 32.7 mg of EDTA (0.1 mmole) in each liter of the 95/5 solvent mixture. Methanol is distilled before use. (4) 0.1 mol/L trichloroacetic acid, pH 3.0 - dissolve 16.3 g trichloroacetic acid in distilled water and dilute to 1 L. Adjust to pH 3.0 with 15 mol/L sodium hydroxide. All solvents are boiled with slow stirring and immediately cooled in order to degas them. After addition of the salts, solvents are filtered through a Millipore filter (Millipore Corp., Bedford, MA 01730).

The column is flushed out daily with methanol for at least 20 minutes. The electrochemical cell is disconnected prior to flushing and stored in distilled water when not in use. To remove endogenous metal ions in the stainless steel system which may interfere with detector

performance, the system is passivated twice with 200 mL of 6 mol/L nitric acid, followed by 500 mL of distilled water. The column, UV and electrochemical cells are bypassed during this process. Afterwards, 5 liters of 1 mol/L NaH_2PO_4, pH 7.0, containing 1 mmol/L EDTA is pumped through the system, including the column and detector cells.[40]

Gas-Liquid Chromatography

Reagents

acetone, distilled before use
methanol, distilled before use
methylene chloride, distilled before use
pyridine
MSTFA
BSTFA
acetic anhydride, distilled before use

Sample Derivatization

Two derivatization procedures are presented. The first is based on a modification of the method of Cancalon and Klingman.[31] 100 µL each of the stock 1 mg/mL solutions of E, NE and DA is placed in a 15-mL concical centrifuge tube and evaporated to dryness under a stream of nitrogen. 50 µL of methylene chloride and 25 µL of MSTFA are added to the dried amines and the solution is mixed on a vortex mixer. The mixture is then heated for 14 minutes at 95 °C in a steam bath, cooled quickly under water and the excess

solvent removed under a stream of nitrogen. 50 µL of pyridine and 50 µL of BSTFA are added to the dried mixture and the solution is mixed on a vortex mixer. The tube is sealed with parafilm and heated for 25 minutes in a boiling water bath. Excess solvent is again removed under a stream of nitrogen, the tube sealed with parafilm and placed on ice. The derivatives are taken up in 10 µL of acetone for subsequent GLC analysis.

The second method, on-column acetamide derivatization, is based on a procedure for the on-column derivatization of amphetamines with acetic anhydride by flame ionization detection.[41] The standards are not extracted into chloroform prior to derivatization, since the detector temperature of the gas chromatograph utilized could not be brought above 275 °C. A detector temperature of at least 300 °C is necessary to burn off chloroform. 3 µL of the aqueous catecholamine standard is aspirated into a 10 µL Hamilton syringe (Hamilton Co., Reno, NE 89510), followed by 1.5 µL of acetic anhydride. This mixture is slowly injected into the column injection port of the Bendix gas chromatograph. Derivatization is performed using aqueous catecholamine standards only.

GLC Analysis

Optimum operating conditions for the TMS-TFA derivatives are as follows: detector temperature, 275 °C; injector temperature, 250 °C; column temperature program:

170 to 240 °C at a rate of 5 °C/min; nitrogen flow rate, 22 mL/min; and hydrogen flow rate, 40 mL/min. Injection volumes are 1 μL.

Optimum operating conditions for the acetamide derivatives are as follows: detector temperature, 275 °C; injector temperature, 250 °C; column temperature program: 190 to 240 °C at a rate of 5 °C/min; nitrogen flow rate, 28 mL/min; and hydrogen flow rate, 40 mL/min.

Fluorometry

Reagents

aluminum oxide, prepared by the method of Crout.[11]

sodium hydroxide, 10 mol/L	Dissolve 400 g NaOH in distilled water and dilute to 1 L.
sodium hydroxide, 5 mol/L	Dilute 500 mL of 10 mol/L NaOH to 1 L with distilled water.
sodium hydroxide, 1 mol/L	Dissolve 40 g NaOH in distilled water and dilute to 1 L.
ammonium acetate, 0.1 mol/L	Dissolve 3.86 g $NH_4(C_2H_3O_2)$ in distilled water and dilute to 500 mL.
ammonium hydroxide, 1 mol/L	Add 7.0 mL of conc. NH_4OH to distilled water and dilute to 100 mL.
EDTA, 10%	Dissolve 100 g Na_2-EDTA in distilled water and dilute to 1 L.

acetic acid, 0.2 mol/L		Add 11.5 mL of glacial acetic acid to distilled water and dilute to 1 L.
hydrochloric acid, 0.01 mol/L		Add 0.8 mL of conc. HCl to distilled water and dilute to 1 L.
formic acid, 1 mol/L		Add 43.5 mL of 90% formic acid to distilled water and dilute to 1 L.
acetic acid, 10 mol/L		Add 575 mL of glacial acetic acid to distilled water and dilute to 1 L.
cupric acetate, 0.2%		Dissolve 0.2 g $Cu(C_2H_3O_2)\cdot H_2O$ in distilled water and dilute to 100 mL.
potassium ferricyanide, 0.25%		Dissolve 0.25 g $K_3Fe(CN)_6$ in distilled water and dilute to 100 mL. Stable for 1 month at 4 °C.
sodium sulfite, 20%		Dissolve 200 g Na_2SO_3 (anhydr.) in distilled water and dilute to 1 L.
β-mercaptoethanol, 1% (v/v) in 20% sodium sulfite		Prepare fresh.
5 mol/L and 10 mol/L mercaptoethanol reagents		Mix equal volumes of 5 or 10 mol/L NaOH and 1% B-mercaptoethanol in 20% Na_2SO_3, immediately before use.
working standards	1 μg/mL NE	Dilute 100 μL of stock NE solution (1 mg/mL) to 100 mL with 0.01 mol/L HCl. Stable one week at 4 °C.
	1 μg/mL E	Dilute 100 μL of stock E solution to 100 mL with 0.01 mol/L HCl. Stable one week at 4 °C.

All glassware is rinsed in dilute nitric acid prior to reagent preparation.

Sample Extraction

Alumina extraction is according to the method of Weil-Malherbe.[6] 0.7 g of alumina is suspended in 10 mL of 0.1 mol/L $NH_4(C_2H_3O_2)$ and adjusted to pH 8.4 with 1 mol/L NH_4OH and 1 mol/L NaOH. The alumina is poured into a recycled empty BioRad cation-exchange column. A 25-mL aliquot of a 24-hr urine specimen is pipetted into a 50-mL beaker. 0.5 mL of 10% EDTA is added and the urine adjusted to pH 8.4 with 5 mol/L NaOH and finally with 1 mol/L NaOH. The urine is filtered through Whatman #41 filter paper and poured onto the alumina column. When the meniscus reaches the top of the adsorbent, the column is washed with 10 mL of distilled water and then eluted with 5 mL of 0.2 mol/L acetic acid, followed by 5 mL of distilled water.

Fluorescence Development

This procedure is based on the method of Weil-Malherbe for the differential analysis of epinephrine and norepinephrine.[21] Glassware rinsed in dilute nitric acid is used throughout.

The acetic acid eluate is diluted to 13.0 mL. For epinephrine determination, 4.5 mL of the eluate is adjusted to pH 2.85 with 1 mol/L formic acid, then diluted to 5.4 mL. 1.2-mL aliquots are pipetted into each of 4 test tubes

labeled a, b, c and d. Table 2 illustrates the order of addition of reagents.

TABLE 2

REAGENT ADDITION FOR FLUOROMETRIC REACTION

Reagent	Tube (mL) a	b	c	d
Epinephrine std. (1 µg/mL)	--	0.1	--	--
Norepinephrine std. (1 µg/mL)	--	--	0.1	--
0.01 mol/L HCl	0.1	--	--	0.1
0.2% cupric acetate	0.1	0.1	0.1	--
0.25% potassium ferricyanide	0.1	0.1	0.1	--
Wait 5 minutes. While waiting, mix the 10 mol/L NaOH-mercaptoethanol reagent.				
10 mol/L NaOH-mercaptoethanol	0.3	0.3	0.3	--
Wait 4 minutes.				
10 mol/L acetic acid	0.3	0.3	0.3	--

The reagents are then added in reverse order to the blank, tube d, i. e., acetic acid, followed by NaOH-mercaptoethanol reagent, ferricyanide and cupric acetate. All tubes should be vortexed for at least 10 seconds upon addition of each reagent. Samples must be filtered prior to reading of fluorescence.

For norepinephrine determination, 7.5 mL of the diluted eluate is adjusted to pH 6.0 with 5 mol/L NaOH and finally with 1 mol/L NaOH, then diluted to 9.0 mL. 1.2-mL

aliquots are pipetted into each of 4 test tubes labeled a, b, c and d. The addition of reagents is the same as for epinephrine, except that the addition of cupric acetate is omitted and 5 mol/L NaOH-mercaptoethanol reagent is added instead of 10 mol/L NaOH-mercaptoethanol reagent.

Fluorometric Analysis

Epinephrine fluorescence is determined at an excitation wavelength of 415 nm and an emission wavelength of 500 nm. Fluorescence due to norepinephrine is read at 395 nm and 475 nm, excitation and emission wavelengths, respectively.

Contribution to the fluorescence by norepinephrine in the epinephrine determination is negligible at pH 2.85. Similarly, error due to the presence of epinephrine in the norepinephrine determination at pH 6.0 may be neglected.

Calculation

$$\frac{\text{reading of sample - reading of blank}}{\text{reading of (sample + IS) - reading of sample}} \times \frac{\text{amount of IS added (}\mu\text{g)}}{\text{volume of urine analyzed (mL)}} \times \frac{\text{volume of eluate (mL)}}{\text{volume of eluate analyzed (mL)}} \times \text{24-hr volume (mL)} = \mu\text{g/24 hr.}$$

CHAPTER VI

RESULTS

High Pressure Liquid Chromatography

UV Absorbance Detection

Figure 7 shows the chromatogram obtained for a standard solution containing 100 μg/mL each of epinephrine (E), norepinephrine (NE), 3,4-dihydroxybenzylamine (DHBA) and dopamine (DA), using UV absorbance detection at 254 nm. The elution order is determined by the polarity of the compounds, the most polar catecholamine being eluted first. Norepinephrine is eluted first, followed by epinephrine, the internal standard and finally, dopamine. The mobile phase, 0.17 mol/L acetic acid, acts as an ion-pair in itself to effect separation between epinephrine and the internal standard.

The limit of detection for each catecholamine using UV absorbance detection at 254 nm is approximately 50 ng. The expected concentrations for norepinephrine, epinephrine and dopamine in the alumina column eluate prepared by the method of Crout[11] from over 100 mL of urine are 6 μg, 2 μg and 50 μg, respectively. It is somewhat unfeasible to detect norepinephrine and epinephrine with this detection system. Dopamine, however, can be detected, due to its higher concentration in normal urine samples.

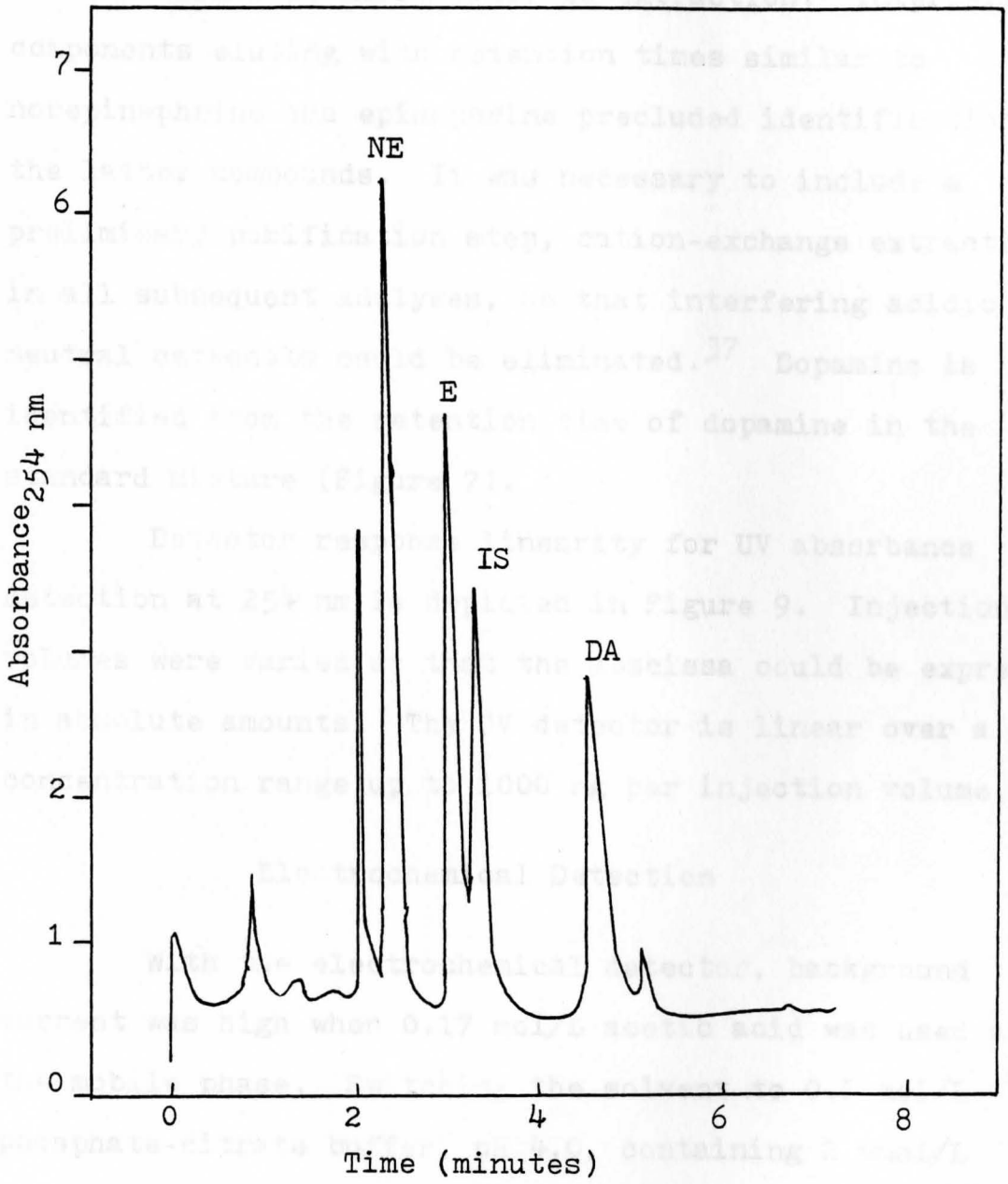


Fig. 7. Chromatogram of a standard mixture containing 100 µg/mL each of NE, E, DHBA and DA. conditions: column µBondapak C_{18}; eluent 0.17 mol/L acetic acid; flow rate, 1.5 mL/min; injection vol., 10 µL.

Figure 8 illustrates a representative chromatogram for a urine specimen extracted according to the alumina batch-column method described by Crout.[11] A 150-mL aliquot of a 24-hour urine speciman was analyzed, without addition of an internal standard prior to extraction. Interfering components eluting with retention times similar to norepinephrine and epinephrine precluded identification of the latter compounds. It was necessary to include a preliminary purification step, cation-exchange extraction, in all subsequent analyses, so that interfering acidic and neutral catechols could be eliminated.[37] Dopamine is identified from the retention time of dopamine in the standard mixture (Figure 7).

Detector response linearity for UV absorbance detection at 254 nm is depicted in Figure 9. Injection volumes were varied so that the abscissa could be expressed in absolute amounts. The UV detector is linear over a concentration range up to 1000 ng per injection volume.

Electrochemical Detection

With the electrochemical detector, background current was high when 0.17 mol/L acetic acid was used as the mobile phase. Switching the solvent to 0.1 mol/L phosphate-citrate buffer, pH 4.0, containing 2 mmol/L heptanesulfonate allowed the current range to be brought to the 5 nA range, necessary for the quantitation of all catecholamines in a single 5-mL urine aliquot.

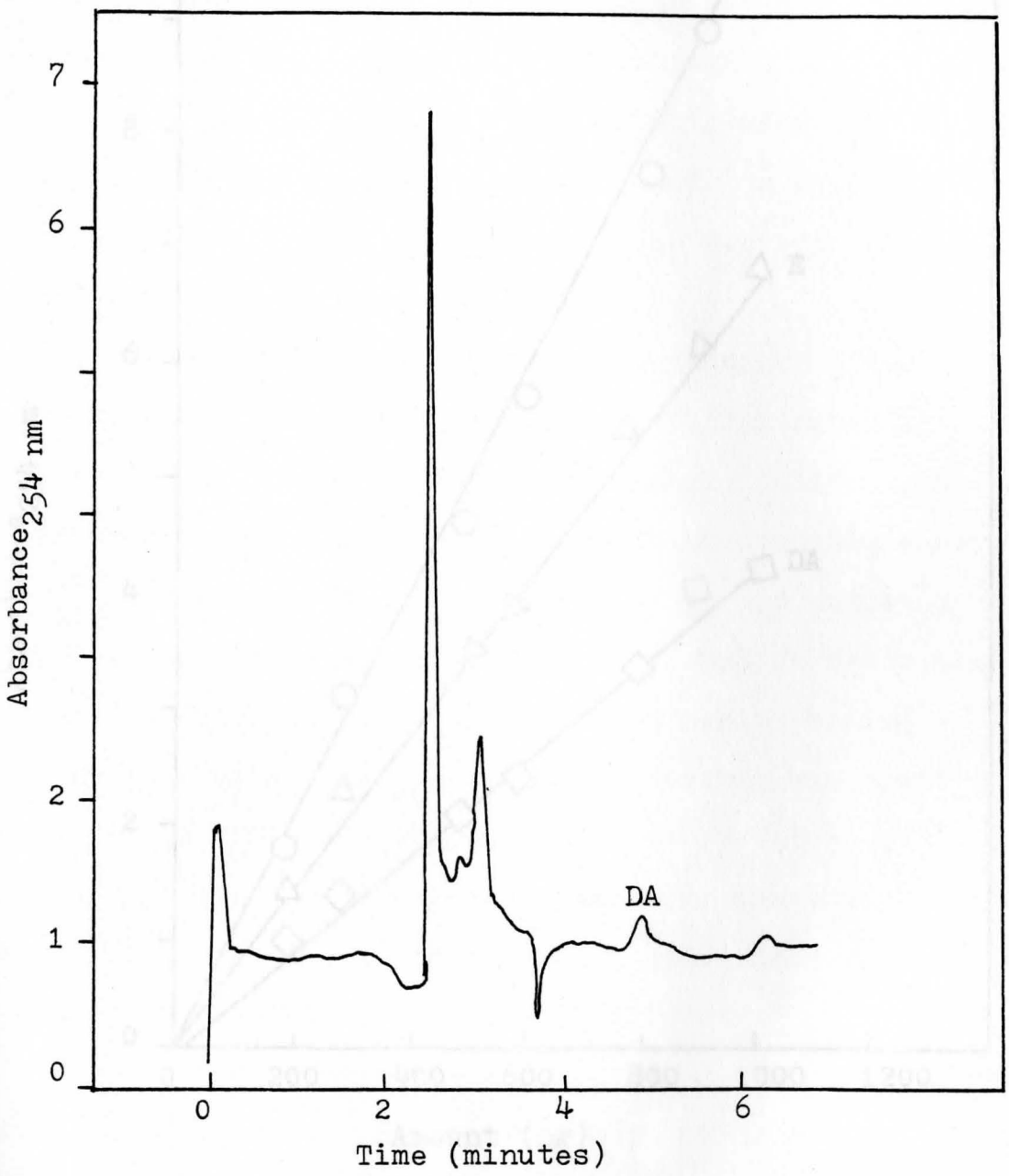


Fig. 8. Chromatogram of a 150-mL aliquot of a 24-hr urine after alumina extraction by the method of Crout. conditions: column μBondapak C18; eluent 0.17 mol/L acetic acid; flow rate, 1.5 mL/min; injection vol. 50 μL.

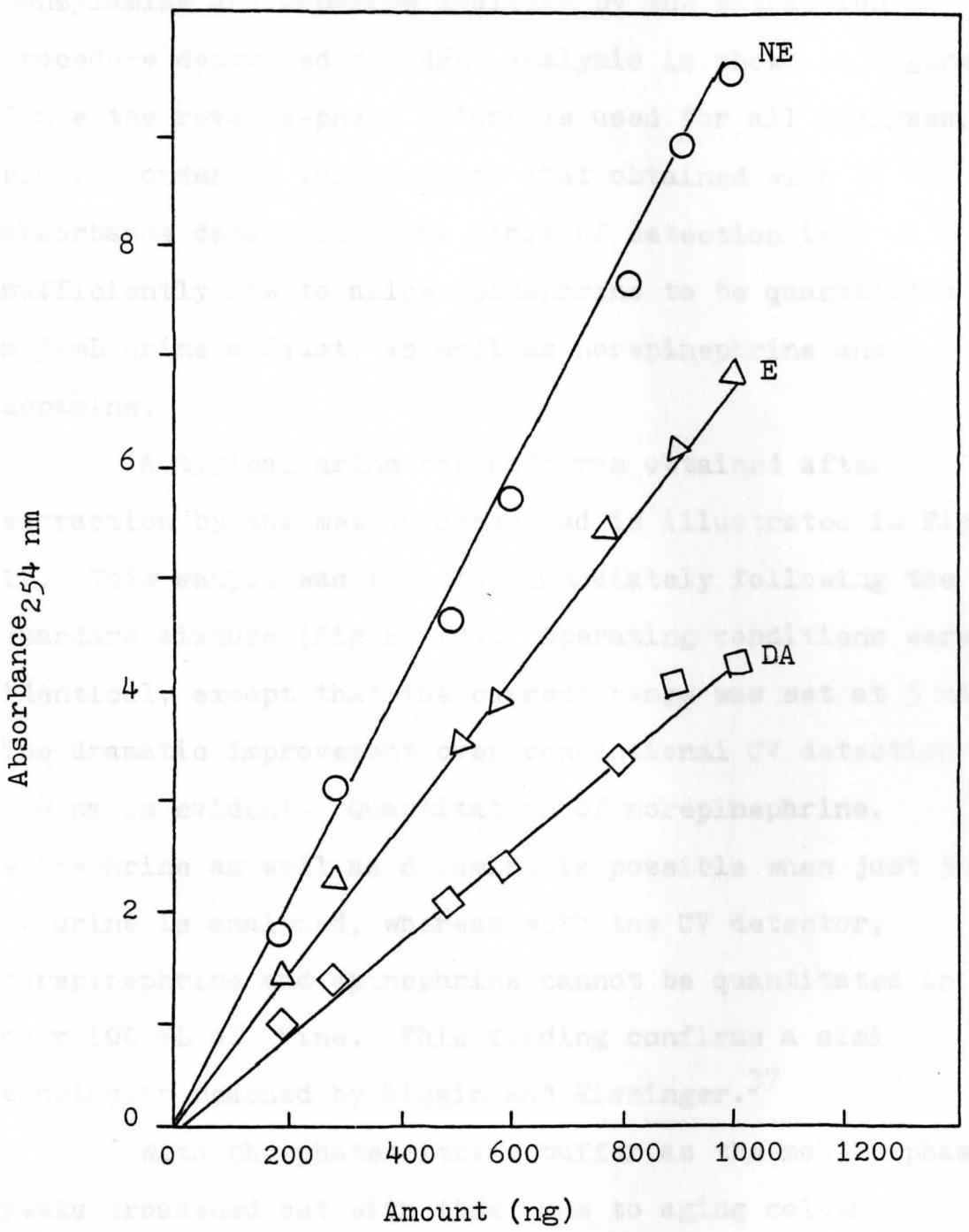


Fig. 9. Peak height (absorbance) versus catecholamine amount (ng) for UV absorbance detection at 254 nm.

The chromatogram of a standard mixture containing 10 μg/mL each of norepinephrine, epinephrine, 3,4-dihydroxybenzylamine and dopamine analyzed by the extraction procedure described for HPLC analysis is shown in Figure 10. Since the reverse-phase column is used for all analyses, elution order is identical to that obtained with UV absorbance detection. The limit of detection is sufficiently low to allow epinephrine to be quantitated in a 5-mL urine aliquot, as well as norepinephrine and dopamine.

A typical urine chromatogram obtained after extraction by the method described is illustrated in Figure 11. This sample was injected immediately following the standard mixture (Figure 10). Operating conditions were identical, except that the current range was set at 5 nA. The dramatic improvement over conventional UV detection at 254 nm is evident. Quantitation of norepinephrine, epinephrine as well as dopamine is possible when just 5 mL of urine is analyzed, whereas with the UV detector, norepinephrine and epinephrine cannot be quantitated in over 100 mL of urine. This finding confirms a similar conclusion reached by Riggin and Kissinger.[37]

With phosphate-citrate buffer as the mobile phase, peaks broadened out with time, due to aging column conditions. In an effort to restore adequate peak integrity, the solvent was switched to 70 mmol/L sodium phosphate/methanol (95/5), pH 4.8, containing 2 mmol/L

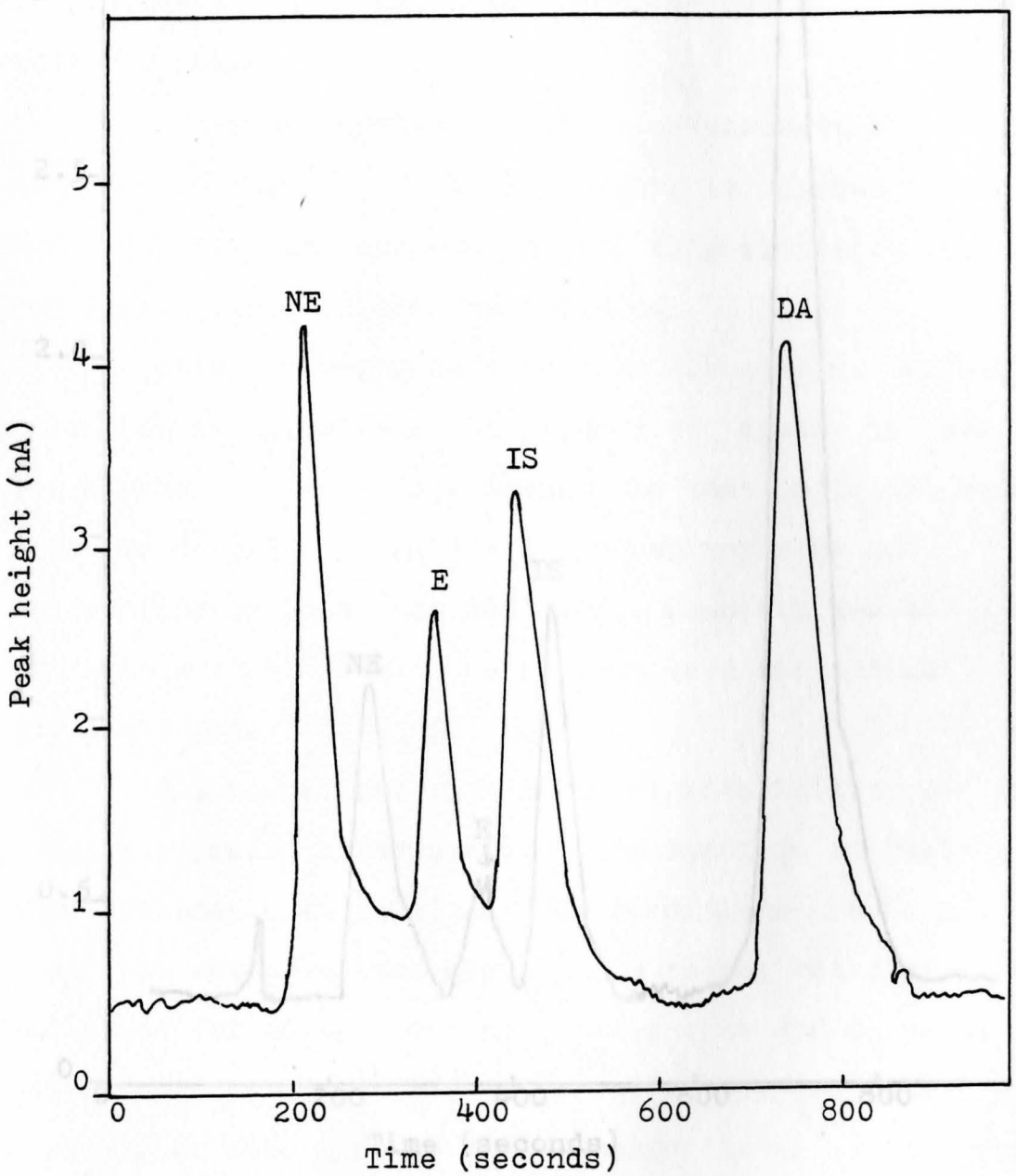


Fig. 10. Chromatogram of a standard mixture containing 10 µg/mL each of NE, E, DHBA and DA after cation-exchange and alumina extraction.

conditions: column µBondapak C18; eluent, phosphate citrate buffer; potential, +0.500 V; injection vol., 7 µL.

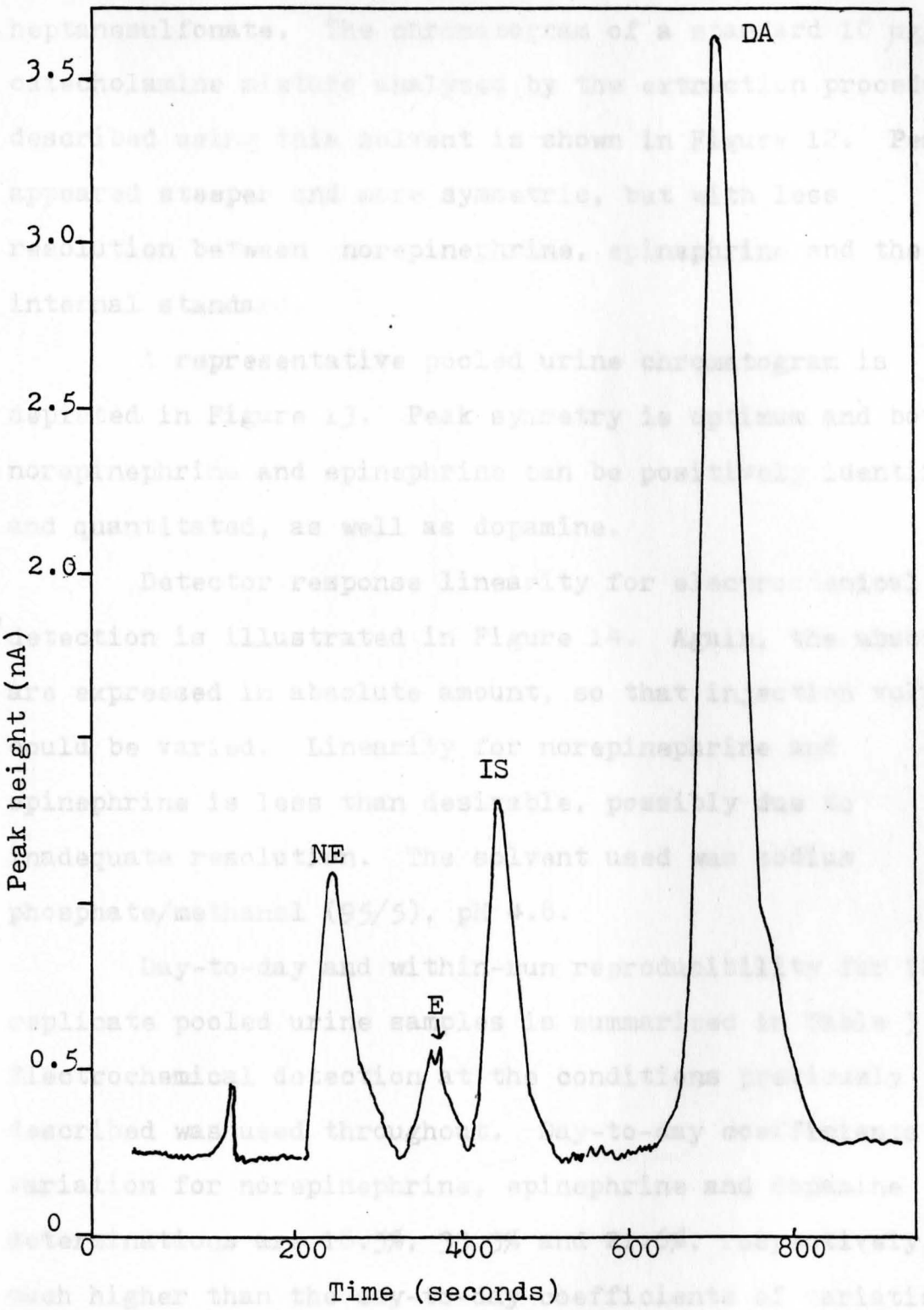


Fig. 11. Chromatogram of a 5-mL aliquot of a 24-hr urine after cation-exchange and alumina extraction. injection vol., 50 µL; quantitation: NE 64 µg/24 hr; E 39 µg/24 hr; DA 267 µg/24 hr.

heptanesulfonate. The chromatogram of a standard 10 μg/mL catecholamine mixture analyzed by the extraction procedure described using this solvent is shown in Figure 12. Peaks appeared steeper and more symmetric, but with less resolution between norepinephrine, epinephrine and the internal standard.

A representative pooled urine chromatogram is depicted in Figure 13. Peak symmetry is optimum and both norepinephrine and epinephrine can be positively identified and quantitated, as well as dopamine.

Detector response linearity for electrochemical detection is illustrated in Figure 14. Again, the abscissa are expressed in absolute amount, so that injection volume could be varied. Linearity for norepinephrine and epinephrine is less than desirable, possibly due to inadequate resolution. The solvent used was sodium phosphate/methanol (95/5), pH 4.8.

Day-to-day and within-run reproducibility for 12 replicate pooled urine samples is summarized in Table 3. Electrochemical detection at the conditions previously described was used throughout. Day-to-day coefficients of variation for norepinephrine, epinephrine and dopamine determinations are 18.5%, 33.3% and 21.6%, respectively, much higher than the day-to-day coefficients of variation previously reported by Mell and Gustafson for norepinephrine and dopamine.[34] Within-run coefficients are 8.5% and 4.7% for norepinephrine and dopamine, compared to 4.7% and 3.5%

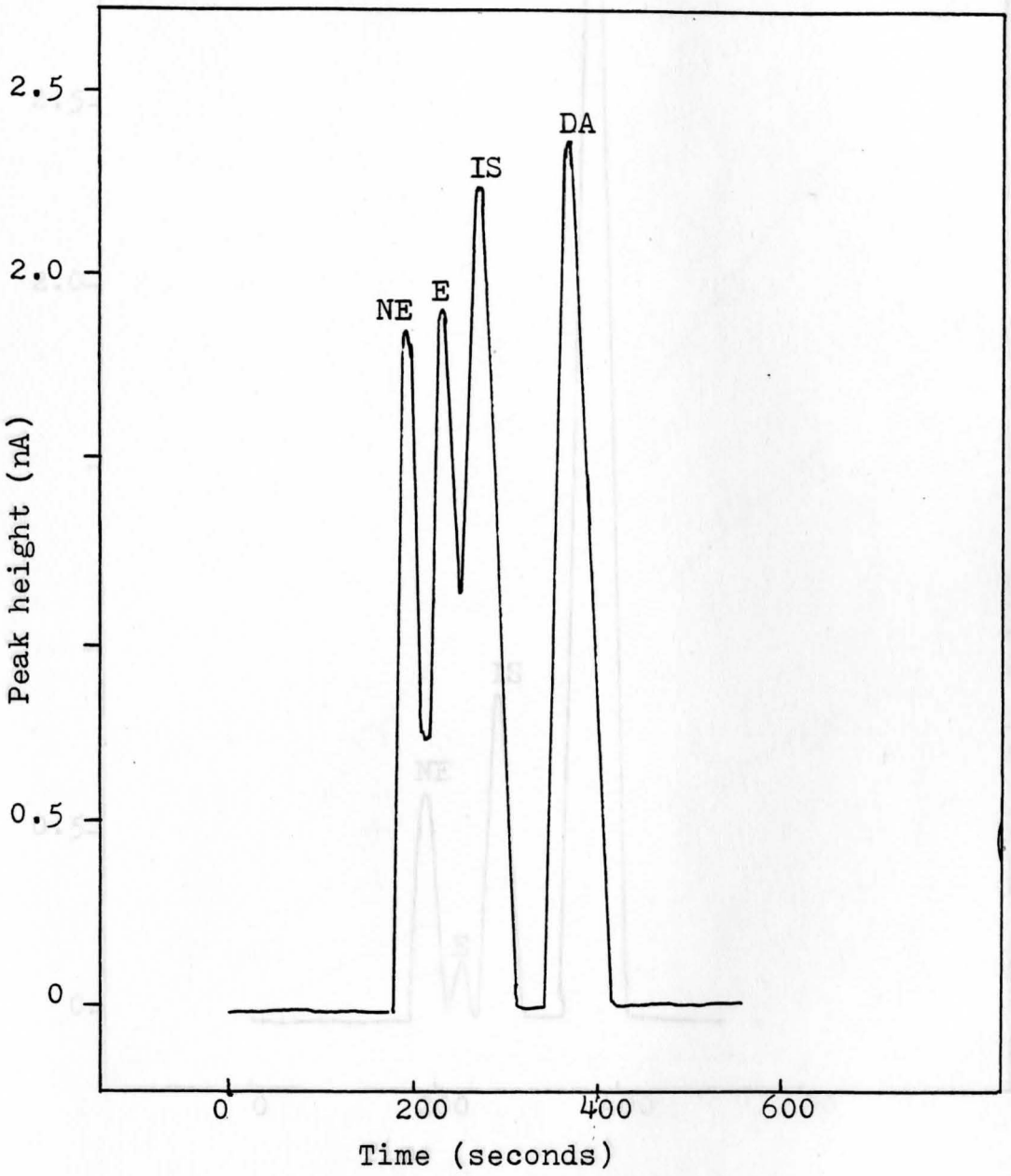


Fig. 12. Chromatogram of a standard mixture containing 10 µg/mL each of NE, E, DHBA and DA after cation-exchange and alumina extraction.
conditions: column µBondapak C_{18}; eluent phosphate/methanol; potential, +0.500 V; injection vol., 5 µL.

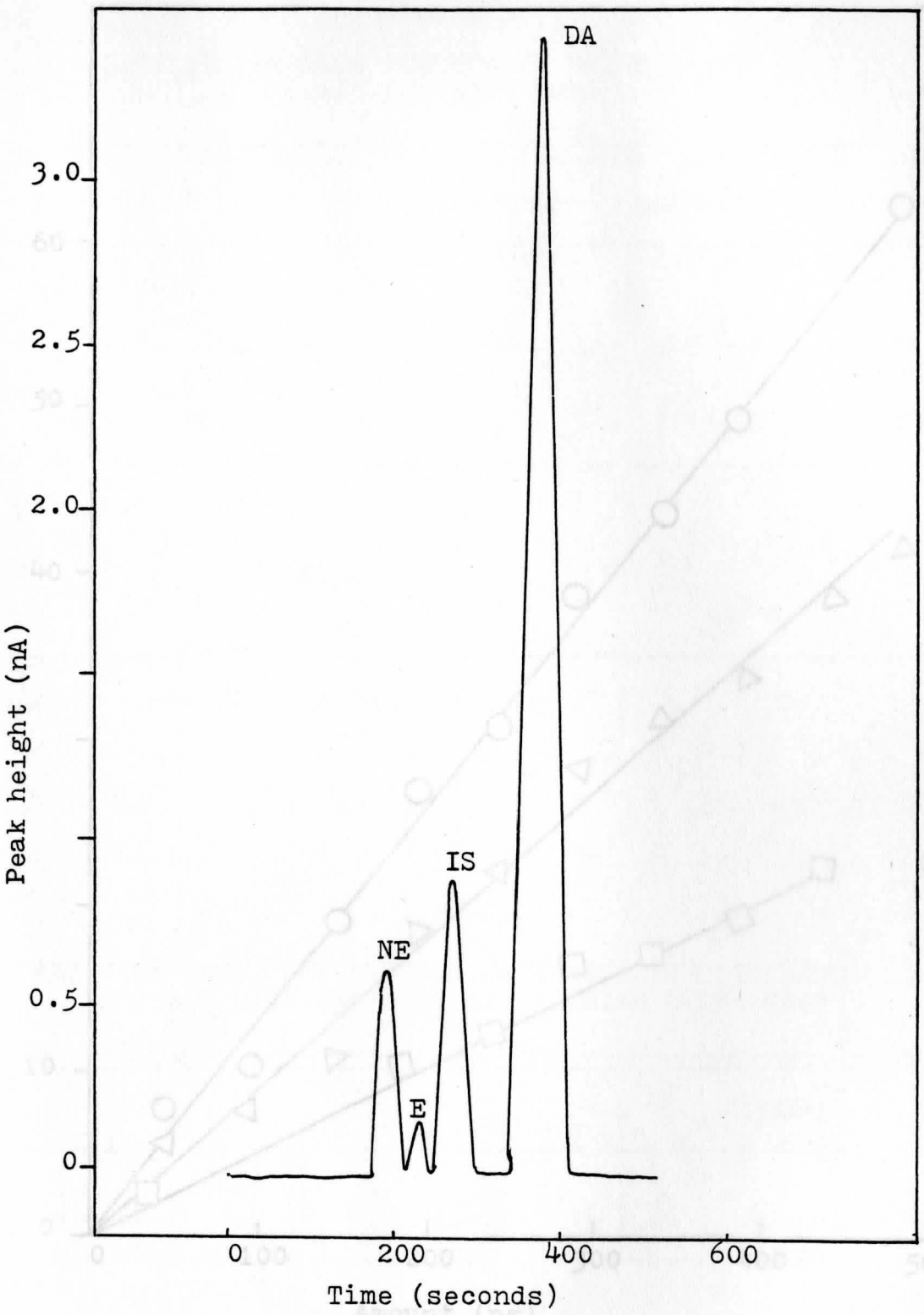


Fig. 13. Chromatogram of a 5-mL aliquot of a pooled urine specimen after cation-exchange and alumina extraction.
injection vol., 40 µL; quantitation: NE 107 µg/L; E 29 µg/L; DA 553 µg/L.

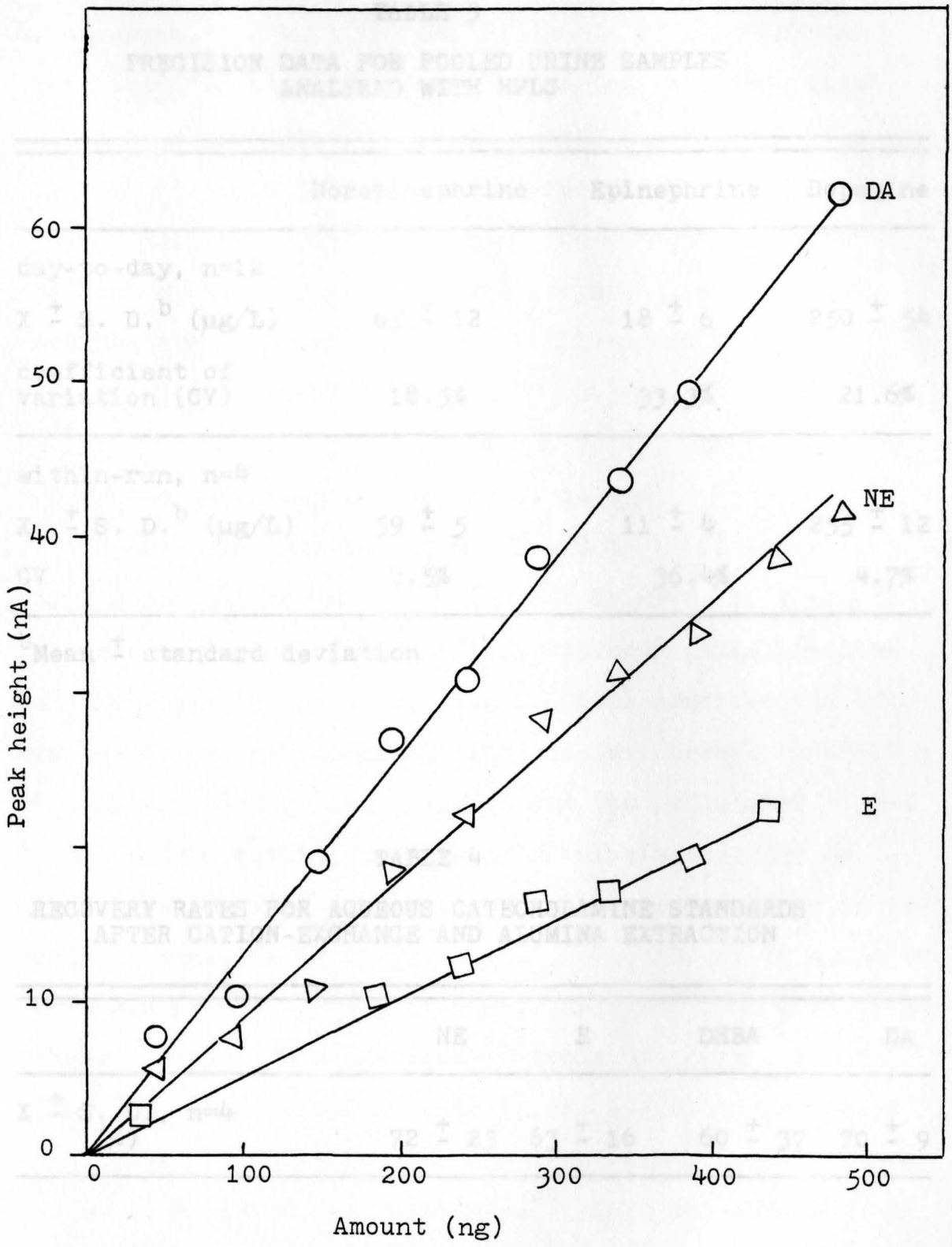


Fig. 14. Peak height (nA) versus catecholamine amount (ng) for electrochemical detection.

TABLE 3

PRECISION DATA FOR POOLED URINE SAMPLES ANALYZED WITH HPLC

	Norepinephrine	Epinephrine	Dopamine
day-to-day, n=12			
X ± S. D.[b] (µg/L)	65 ± 12	18 ± 6	250 ± 54
coefficient of variation (CV)	18.5%	33.3%	21.6%
within-run, n=4			
X ± S. D.[b] (µg/L)	59 ± 5	11 ± 4	255 ± 12
CV	8.5%	36.4%	4.7%

[b]Mean ± standard deviation

TABLE 4

RECOVERY RATES FOR AQUEOUS CATECHOLAMINE STANDARDS AFTER CATION-EXCHANGE AND ALUMINA EXTRACTION

	NE	E	DHBA	DA
X ± S. D., n=4 (%)	72 ± 25	63 ± 16	60 ± 37	70 ± 9

as determined by Mell and Gustafson.[34] The within-run coefficient of variation for epinephrine is 36.4%, which should be much lower for a HPLC method incorporating an internal standard throughout the procedure.

Recovery rates for aqueous catecholamine mixtures containing equal amounts of norepinephrine, epinephrine, internal standard and dopamine are listed in Table 4. Mean recoveries $\pm$ standard deviation (S. D.) are 72 $\pm$ 25 for NE, 63 $\pm$ 16 for E, 60 $\pm$ 37 for DHBA and 70 $\pm$ 9 for DA. Recoveries were seen to vary by up to 45%.

The quantitation of NE, E and DA in 17 urine samples as well as in Ortho® Control Urine II is presented in Table 5. Values for total (hydrolyzed) catecholamines as determined by the Youngstown Hospital Association, if available, are given for patient samples; however, direct correlation between these values and the calculated values from the HPLC method for free catecholamines cannot be made. The range of excretion of free catecholamines in the subjects analyzed is 23-176 µg/24 hr for NE, 11-79 µg/24 hr for E and 64-544 µg/24 hr for DA, excluding abnormally high values. These are summarized in Table 6. Normal adult values of urinary free catecholamines (µg/24 hr) reported in a recent HPLC method with electrochemical detection are NE 14-80, E 0.5-20 and DA 65-400.[40] Only the dopamine values show good correlation.

TABLE 5

URINARY FREE CATECHOLAMINE LEVELS FOR 17 24-HR URINE SAMPLES ANALYZED BY HPLC

Subject	Norepinephrine (μg/24 hr)	Epinephrine (μg/24 hr)	Dopamine (μg/24 hr)
EM	83	42	544
PI	37	18	104
DM	64	39	267
SS	73	35	290
LA	26	11	133
Patient 1 (97)[c]	95	79	83
Patient 2 (124)[c]	41	33	64
Patient 3 (29)[c]	23	280	122
Patient 4 (71)[c]	56	36	197
Patient 5 (249)[c]	61	34	271
Patient 6 (84)[c]	89	26	99
Patient 7 (100)[c]	91	60	281
Patient 8 (94)[c]	176	44	305
Patient 9 (125)[c]	67	33	1919
Patient 10 (274)[c]	113	20	347
Patient 11 (261)[c]	103	22	207
Patient 12	88	25	219
Ortho® II (40)[d]	80	23	40

[c]Total catecholamines in μg/24 hr analyzed by the Youngstown Hospital Association fluorometric procedure.

[d]Free catecholamines in μg/L analyzed by BioRad Laboratories.

TABLE 6

EXCRETION RATES FOR FREE CATECHOLAMINES
FOR THE SUBJECTS ANALYZED

	Range, in μg/24 hr
Norepinephrine	23-176
Epinephrine	11-79
Dopamine	64-544

TABLE 7

PRECISION DATA FOR POOLED URINE SAMPLES
ANALYZED FLUOROMETRICALLY

	Norepinephrine	Epinephrine
day-to-day, n=10		
X ± S. D. (μg/L)	34 ± 11	14 ± 7
CV	32.3%	50.0%
within-run, n=5		
X ± S. D. (μg/L)	38 ± 14	14 ± 4
CV	36.8%	28.6%

Gas-Liquid Chromatography

The chromatogram of TFA-TMS derivatives of epinephrine, norepinephrine and dopamine is presented in Figure 15. According to Cancalon and Klingman,[31] the order of elution is epinephrine, dopamine and norepinephrine, but this could not be confirmed since retention times of the individual catecholamines did not coincide with those obtained with the standard mixture. There is no apparent explanation for this occurence.

The chromatogram of a standard mixture of norepinephrine, epinephrine and dopamine after on-column acetamide derivatization is illustrated in Figure 16. Attempts at derivatizing the individual catecholamines were unsuccessful, so peak identity is unknown. The peaks seen after acetamide derivatization had improved integrity and resolution over the peaks obtained after BSTFA-MSTFA derivatization.

Fluorometry

Precision data for free norepinephrine and epinephrine obtained for 10 replicate pooled urine samples using a documented differential trihydroxyindole fluorometric procedure[21] are presented in Table 7. Day-to-day coefficients of variation are 50.0% and 32.3%, for epinephrine and norepinephrine, respectively, considerably higher than the values obtained with the HPLC method (33.3% and 18.5%, respectively). Results are similar when

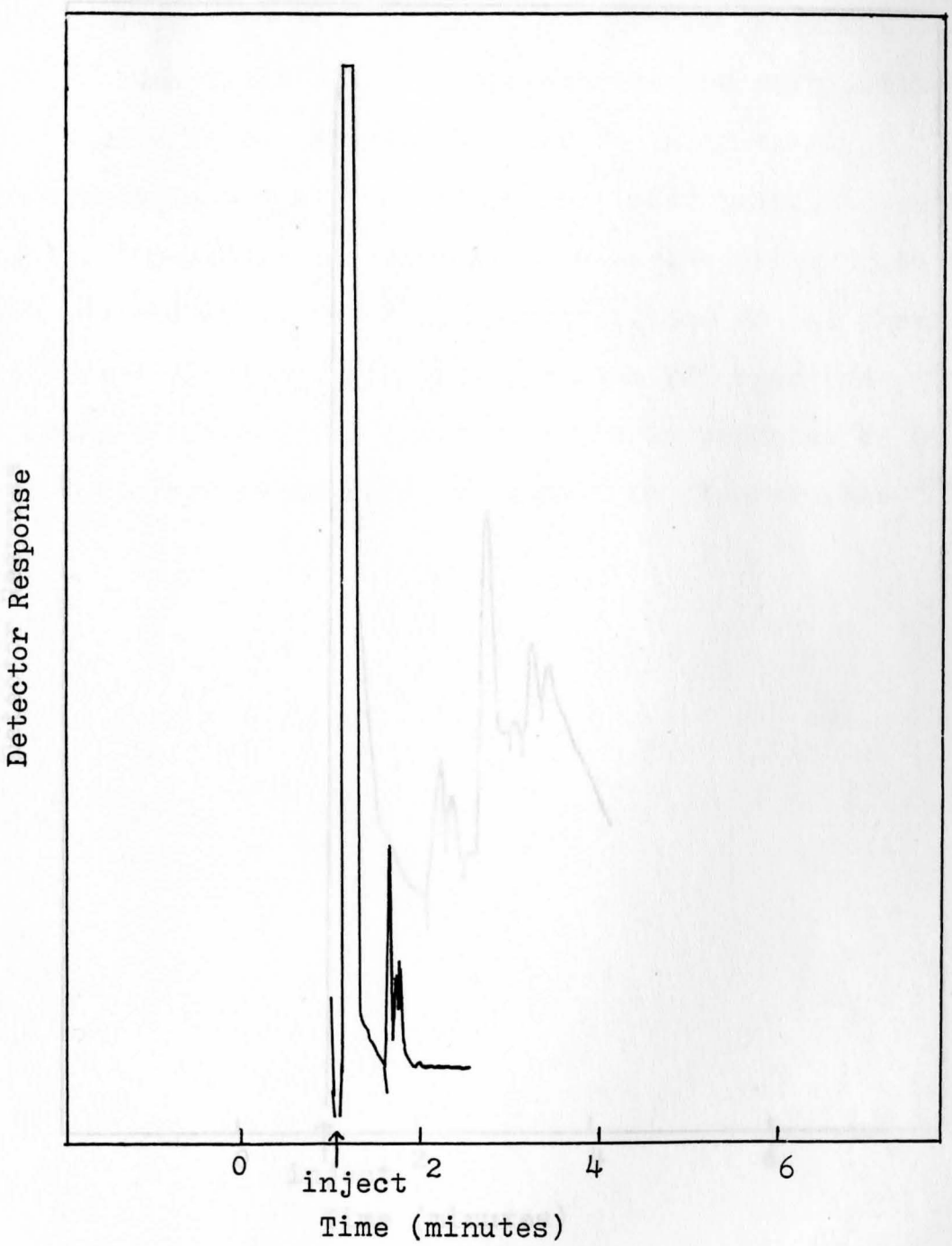


Fig. 15. Chromatogram of a standard mixture containing 100 µg each of NE, E and DA after TFA-TMS derivatization.
injection vol., 1 µL.

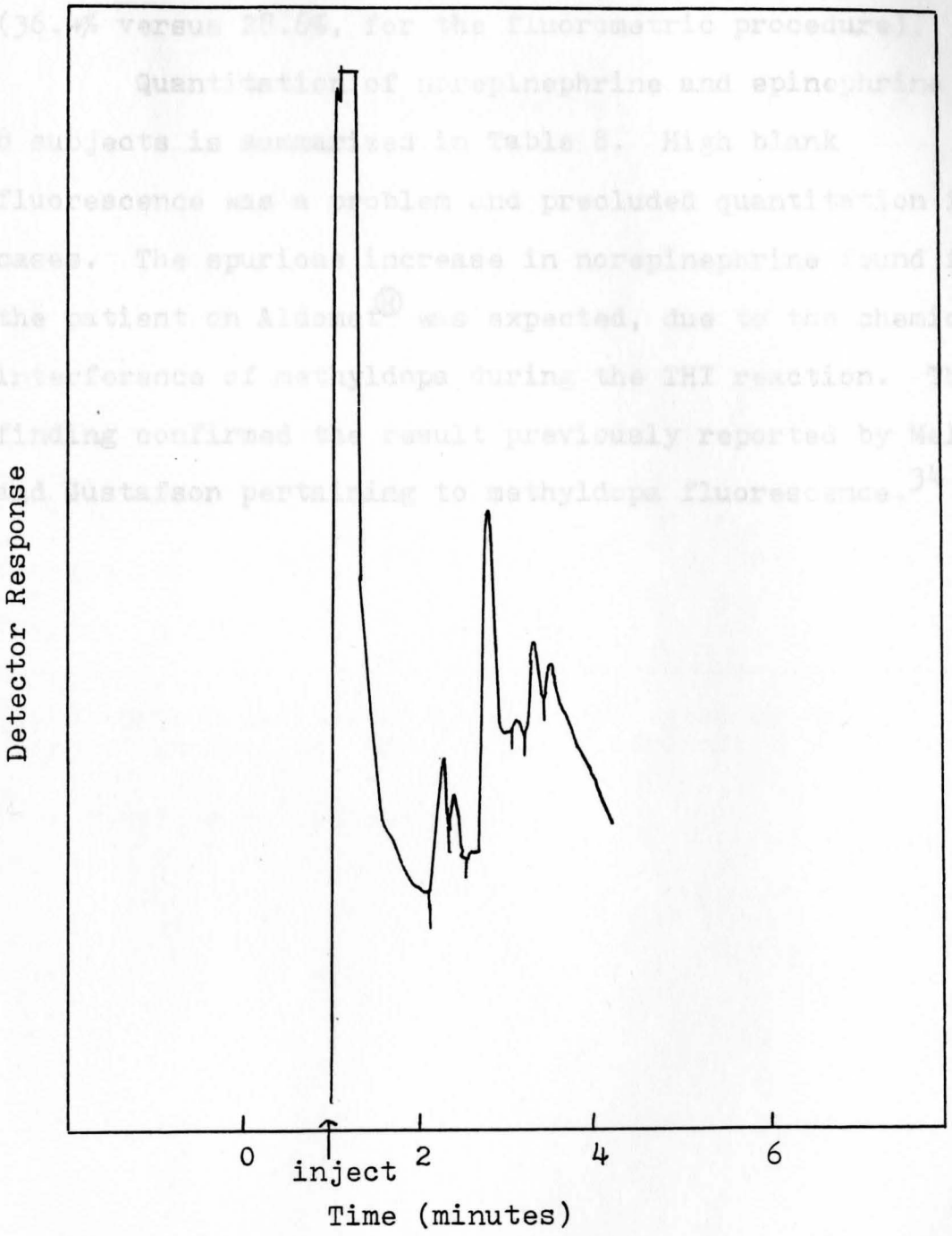


Fig. 16. Chromatogram of a standard mixture containing 100 µg each of NE, E and DA after on-column acetamide derivatization.
injection vol., 3 µL.

within-run coefficients of variation for the two methods are compared for norepinephrine, but the coefficient of variation for epinephrine is higher for the HPLC method (36.4% versus 28.6%, for the fluorometric procedure).

Quantitation of norepinephrine and epinephrine in 8 subjects is summarized in Table 8. High blank fluorescence was a problem and precluded quantitation in 3 cases. The spurious increase in norepinephrine found for the patient on Aldomet® was expected, due to the chemical interference of methyldopa during the THI reaction. This finding confirmed the result previously reported by Mell and Gustafson pertaining to methyldopa fluorescence.[34]

TABLE 8

URINARY FREE CATECHOLAMINE LEVELS FOR
8 24-HR URINE SAMPLES ANALYZED FLUOROMETRICALLY

Subject	(μg/24 hr) Norepinephrine	Epinephrine
EM	0[f]	5
LA	9	0[f]
PI	5	0[f]
SS	12	4
DM	23	9
Patient 13 (1294)[e] (put on Aldomet)	563	32
Patient 14 (115)[e]	16	73
Patient 15 (272)[e]	40	15

[e]Total catecholamines in μg/24 hr analyzed by the Youngstown Hospital Association fluorometric procedure.

[f]Zero implies not detected.

CHAPTER VII

DISCUSSION

High Pressure Liquid Chromatography

HPLC with electrochemical detection offers the necessary sensitivity for the quantitation of urinary catecholamines. However, the problems encountered with this system should be mentioned. Reverse-phase columns are highly susceptible to loss of capacity with time, using aqueous mobile phases.[42] Trace organics are strongly retained on these columns and interfere with the established equilibrium between the solid phase and the mobile phase. Flushing the column with methanol partially washes off the strongly adsorbed organic compounds, but cannot totally eliminate them. Switching the solvent to 0.1 mol/L trichloroacetic acid, pH 3.0, as recommended by Asmus and Freed[42] for the regeneration of an older column, helped to restore lost resolution, but produced too high a background current to be compatible with the electrochemical detector set at or below 50 nA full scale. 0.1 mol/L trichloroacetic acid was thereafter used only for flushing purposes.

Problems with column reproducibility and stability occurred as a result of adding heptanesulfonate, the paired-ion of choice to the mobile phases used. Quantitation of

norepinephrine, epinephrine and dopamine in pooled urine specimens showed high variability, possibly due to the constant, gradual deterioration in column conditions that occurs when heptanesulfonate is used as the ion-pairing agent. 0.17 mol/L acetic acid, used initially, effected adequate separation between epinephrine and DHBA with UV detection at 254 nm without the disadvantage of decreasing column lifetime. In addition, this solvent is inexpensive and simple to prepare, but cannot be feasibly utilized with electrochemical detection, due to the high background current it produces.

The carbon paste used in the electrochemical detector cell became contaminated quite frequently, as evidenced by increasing background current with time, and had to be changed weekly.

Under ideal conditions, quantitation of urinary free catecholamines is rapid and simple, with all components eluting in less than 8 minutes. All illustrated chromatograms are examples of those obtained from sample injections made under proper operating conditions. Many inferior chromatograms were also obtained and it was extremely difficult to achieve reproducibility with this instrumental method.

The clinical hospital laboratory must consider these drawbacks inherent in the HPLC system. Care must be taken to introduce only highly pure, chromatographic-grade mobile phases into the column, perferably commercially

prepared solvents. This instrument should be handled only by skilled technologists, trained in its operation and knowledgeable in troubleshooting techniques.

Due to their low 24-hour urinary concentrations, norepinephrine and epinephrine were most directly affected by inconsistencies in the instrument conditions. Dopamine, however, due to its higher urinary levels, could usually be quantitated from the urine samples analyzed, provided the internal standard peak is adequately symmetric to allow peak height measurement.

HPLC with electrochemical detection offers potential for the quantitation of urinary free dopamine in the clinical laboratory. Dopamine is not normally estimated fluorometrically in this setting, as are total catecholamines (norepinephrine and epinephrine). Implementation of this method would provide the clinical laboratory with an efficient means of detecting high dopamine values in the presence of normal total catecholamines, as well as detecting low dopamine levels, which have been implicated in Parkinson's disease. The ability to estimate dopamine as an entity separate from epinephrine and norepinephrine would be beneficial to the assessment of a variety of biochemical disorders. In addition, the fact that HPLC is less subject to drug interferences supports its application to the clinical laboratory.

Gas-Liquid Chromatography

BSTFA-MSTFA derivatization is lengthy, tedious, gives poor peak resolution and is subject to many interferences. It is not the method of choice for analysis of catecholamines by GLC.

On-column acetamide derivatization shows more promise. The method is rapid, simple and provides good peak integrity with few interferences. Potentially, urinary catecholamines could be quantitated in an alumina column eluate, without further extraction, by aspirating an aliquot into a syringe, followed with acetic anhydride and injecting the mixture onto a gas chromatographic column.

An internal standard, methoxyphenamine hydrochloride, employed in the procedure reported for amphetamine analysis,[41] could be incorporated into the alumina extraction step and utilized to correct for recovery losses. No attempts were made to include the internal standard in the catecholamine solutions analyzed, since positive peak identity of the catecholamines themselves should be established first. On-column acetamide derivatization has not been applied to catecholamines prior to this investigation, so the method described is developmental at best.

Limited documentation relevant to the preparation of volatile derivatives from norepinephrine, epinephrine and dopamine prior to gas chromatographic analysis will hinder the implementation of this instrumental technique as

a useful method in the clinical laboratory.

Fluorometry

Differential analysis of norepinephrine and epinephrine by the method described, like all fluorometric procedures for catecholamine estimation, is tedious and subject to drug and dietary interferences. Total time for preparation of the fluorescent derivatives is approximately 30 minutes, but reading of fluorescence requires only a few minutes. The advantages of this method are its extensive documentation and its reliance on a relatively dependable and durable laboratory instrument, the fluorometer.

The two-column purification step for catecholamine extraction from urine described in this investigation is impractical for the clinical laboratory. However, HPLC analysis necessitates this procedure, if epinephrine and norepinephrine are to be quantitated. Therefore, until HPLC methods are further refined and simplified, fluorometry for norepinephrine and epinephrine estimation remains the most feasible alternative. Dopamine, in contrast, can be easily quantitated by HPLC analysis after a single alumina extraction step, an attractive prospect for the clinical laboratory.

CHAPTER VIII

CONCLUSIONS

From this investigation, the following conclusions can be drawn: (1) High pressure liquid chromatography cannot be readily implemented into the routine clinical laboratory until its ease of operation and ability to maintain day-to-day precision are better established. (2) Gas-liquid chromatographic application to the analysis of urinary catecholamines is presently in the developmental stage. Much more experimentation is required before this instrumental method can be considered as a probable candidate for catecholamine quantitation in the clinical laboratory. (3) Fluorometric analysis, despite its drawbacks, is currently the most dependable means of quantitating urinary catecholamines in the clinical laboratory, and will remain as such until other instrumental methods are further refined and simplified.

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