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REVIEW

NVESTIGATIONS OF CATECHOLAMINE METABOLISM USING HIGH-ERFORMANCE LIQUID CHROMATOGRAPHY

ANALYTICAL METHODOLOGY AND CLINICAL APPLICATIONS

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CONTENTS

1. INTRODUCTION

The three principal catecholamines, norepinephrine (NE), epinephrine (E),

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and dopamine (DA) perform a number of important neuronal and hormonal functions in mammalian systems.

Measurements of the levels of the parent amines, their acidic and neutral metabolites, as well as the activities of related biosynthetic enzymes, have been widely used as diagnostic and prognostic tools for a variety of disease states, among them: hypertension [1], Parkinson's disease [2], affective disorders [3], heart disease [4], neural crest tumors [5–9], hyper- and hypothyroidism [10], muscular dystrophy [11], and familial dysautonomia [12].

The degradation of catecholamines in the body takes place via two reactions: the O-methylation at the 3-position of the catechol group and oxidative deamination of the alkylamine side chain. The two enzymes responsible for the above reactions, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) are widely distributed throughout the body; the highest COMT activity is found in the liver and that of MAO in the brain and the heart. The catabolites of greatest clinical utility are: vanillylmandelic acid (VMA), homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and the metanephrines (normetanephrine, NMN; metanephrine, MN; 3-methoxytyramine, 3-MT).

Catecholamines and their metabolites are found in physiological fluids in two forms: free and as conjugates of sulfuric and glucuronic acids. Conjugation is catalyzed by the transferase enzymes found primarily in the liver and, to a smaller extent, in the brain. The ratio of the free and conjugated forms varies with the type of physiological sample and in some cases the conjugated form predominates by far. For example, MHPG, the principal metabolite of NE in mammals, appears as the free, non-conjugated molecule and as the sulfate conjugate (MHPG-SO_4) and β-conjugate of glucuronic acid (MHPG-Glu). The conjugated forms predominate in the urine and it has been postulated that, while the β-glucuronide form results from the metabolism of systemic NE, the sulfate form reflects the central NE metabolism.

Because of the varying physiological distribution of catecholamines and their metabolites, the clinical interpretation of data must be done carefully. Since urinary catecholamine metabolites reflect both the central and peripheral metabolism, extreme caution must be exercised in relating the fluid composition to a specific area of origin. Because of this, the diagnosis of certain disease states on the basis of altered urinary profiles of catabolites is possible only when gross changes in metabolism exist. Conversely, plasma catecholamines contain useful information concerning the temporary response of the sympathetic nervous system to stress or disease. Except for the direct analysis of brain tissue, only the cerebrospinal fluid (CSF) analysis provides information which is not clouded by peripheral contributions.

The complexity of biological matrices and the extremely low levels of endogenous compounds require the use of an efficient separation technique and sensitive detection devices. High-performance liquid chromatography (HPLC) with fluorometric and/or thin-layer electrochemical (EC) detection is rapidly becoming the analytical method of choice for the assessment of these neurologically important substances in body fluids and tissue samples.

This review will examine the liquid chromatographic methodology and

current examples of the diagnostic use of this technique in clinical investigations of catecholamine metabolism.

2. SAMPLE HANDLING AND STORAGE

Because of the relatively low levels of catecholamines and their metabolites in various physiological fluids, extreme care must be exercised during sample collection, storage and processing in order to avoid contamination of specimens and/or losses due to decomposition. Thus, all glassware used in handling the samples must be scrupulously washed and acid-cleaned. Due to the known instability of catecholamines in basic media, samples must be acidified prior to storage. In addition, antioxidants such as ascorbate, ethylene-bis(nitrilo)-tetraacetic acid (EDTA), ethylene-bis(oxyethylene-nitrilo)tetraacetic acid (EGTA), sodium bisulfite and thioglycolate are added in order to maintain the compounds in their reduced form. This is particularly important for EC detection since catecholamines are generally monitored in the oxidative mode. Internal standard(s) can be added at this stage to correct for losses which may incur during prolonged storage. Removal of proteins can be achieved by centrifugation of acidified samples.

Protein-free specimens or extracts can be safely stored for several months at —70°C to —80°C [13,14]. If, however, samples are to be analyzed within one week of collection, freezing at —20°C will suffice [15].

Since each type of physiological sample requires different pretreatment, a detailed discussion of the preparation of extracts of biological samples is beyond the scope of this review and it can be found elsewhere [9,16—22].

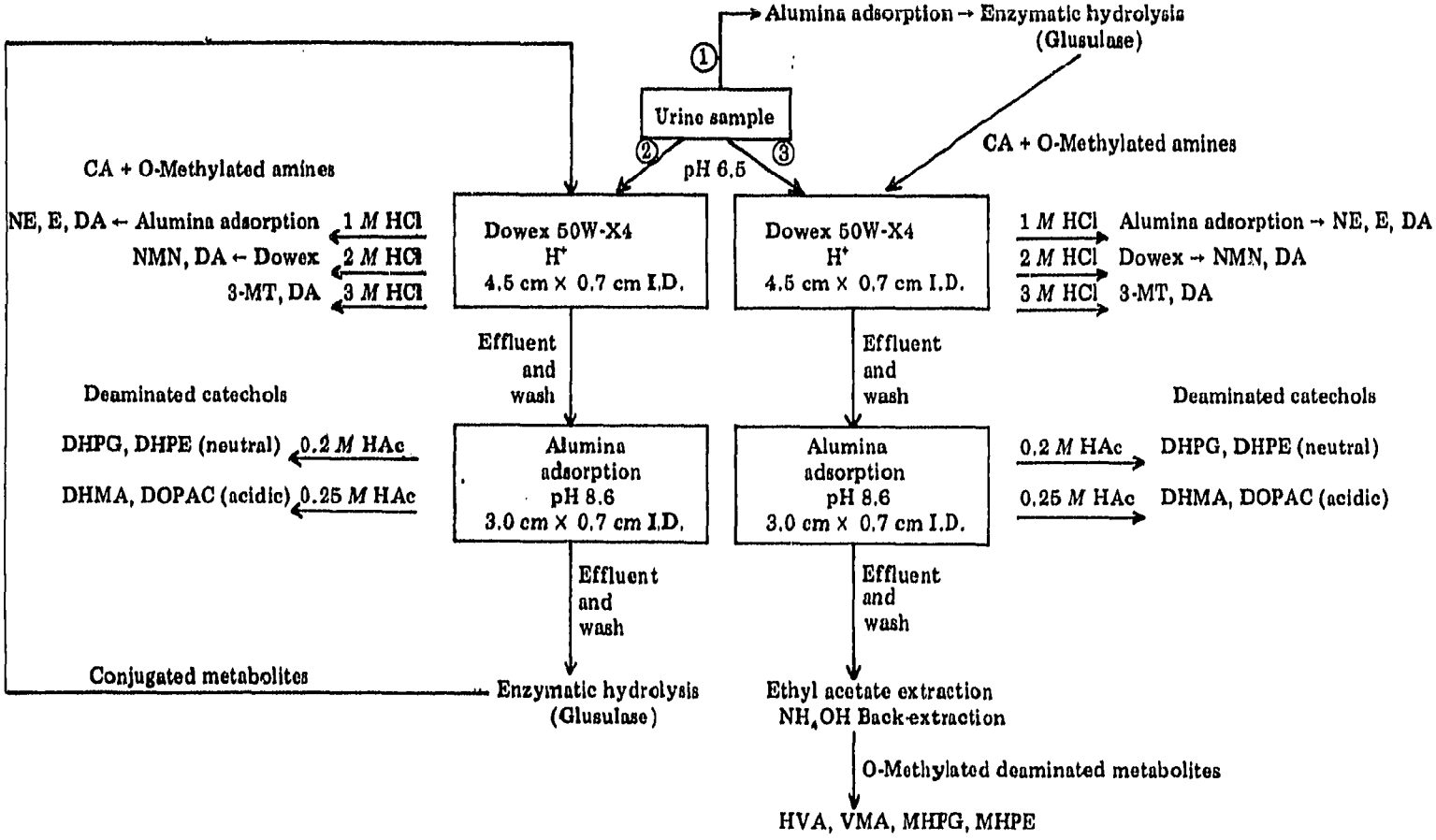
2.1. Post-mortem changes of tissue samples

The determination of catecholamines in tissue samples is often plagued with the problem of post-mortem changes. The extent of these changes depends upon the time elapsed between death and the sample preparation [23, 24], as well as the temperature at which samples were stored prior to processing [25, 26]. While the latter condition can be controlled, the first one will inevitably pose problems in investigations of catecholamines in human brain. Dopamine levels are known to be affected by both factors [25]. However, the rate of decline is reduced if the brain is kept in situ [24, 27] at lower temperature [25]. The reduction in DA levels results in concomitant increase in 3-methoxytyramine (3-MT). This reaction is favored by decreased activity of monoamine oxidase (MAO), by a reduction in tissue pO_2, and by maintained activity of catechol-O-methyl transferase (COMT). However, these problems can be circumvented by destroying the enzymes by means of microwave irradiation [28]. However, less problems are encountered when working with brains of experimental animals since homogenization of tissues in acid or microwave irradiation performed immediately after decapitation minimizes the post-mortem changes [29].

The levels of deaminated metabolites are less affected by the above-mentioned factors. However, prolonged exposure of dissected samples to oxygen is known to increase the levels of 3,4-dihydroxyphenylacetic acid (DOPAC)

TABLE 1

GENERAL PROCEDURE FOR ISOLATION OF CATECHOLAMINES AND MAJOR METABOLITES FROM PHYSIOLOGICAL SAMPLES*



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[30] and HVA [24] due to the higher activity of MAO. In conclusion, assessment of catecholamine levels in samples of human brain requires storage of the body at 4°C and immediate homogenization after dissection. The analysis of tissues from experimental animals is less affected by the post-mortem changes, since dissection and homogenization can be carried out immediately after decapitation.

3. SAMPLE FRACTIONATION SCHEMES FOR CATECHOLAMINES AND RELATED COMPOUNDS

Physiological fluids represent a complex mixture of catecholamines and their major and minor metabolites superimposed upon a background of other potentially interfering compounds present in considerably higher amounts. In addition, compounds involved in the catecholamine metabolic pathways differ significantly among themselves in their chemical, physical and spectroscopic properties. Since in many studies of catecholamine turnover, the measurement of the acidic, neutral or basic metabolites is of greater interest than the monitoring of parent amines or activities of relevant enzymes, specific isolation procedures have been developed for the assessment of isolated compounds or groups of compounds. In spite of the complexity of the problem, it is possible to incorporate all separation schemes for individual compounds or groups of compounds into a general, integrated procedure which will enable sequential analysis of most compounds involved in catecholamine metabolism [31]. This general procedure is outlined in Table 1. The recovery of all the compounds included in this scheme was reported to be in the range of 40—80% [31].

4. METHODS OF ANALYSIS FOR CATECHOLAMINES AND RELATED COMPOUNDS

The amount of attention currently focused on the role of catecholamines in a number of psychiatric and neurological disorders, has prompted the development of a wide number of analytical methods, despite the many difficulties associated with the measurement of the minute amounts of these compounds and some of their metabolites in complex biological matrices. The assessment of quantitative levels requires the use of analytical techniques of high sensitivity and selectivity. Several of them fulfill this requirement with varying success and degree of facility: radioenzymatic procedures [32], the double-isotope derivative analyses [33], gas chromatography alone [34] or coupled with mass spectrometry [35, 36], and more recently, HPLC with fluorometric [37—40] or EC detection [8, 9, 21, 41—48].

Radioenzymatic procedures, which employ the specific radiolabeling of the catechol moiety with 5-adenosylmethionine and catechoi-O-methyltransferase (COMT), possess the requisite sensitivity and specificity. However, they require multiple sample handling steps, enzyme preparations and labelled compounds which makes them tedious, time-consuming and expensive for routine analysis. In addition, the enzymatic reaction may be inhibited by the sample components and compounds which are structurally related to catecholamines

can be a potential source of error. The double-isotope derivative methods are also plagued with similar problems.

Earlier gas chromatographic methods employing the electron-capture detector have not achieved wide popularity due to the problems associated with derivatization of complex matrices and the vagaries of the electron-capture detector. In this procedure catecholamines and their metabolites are treated with halogenated anhydrides and the resulting volatile derivatives possess excellent electron-capturing properties [49]. Although the absolute sensitivity of this detector is high, the usable sensitivity is usually decreased due to the sample background arising from the derivatization reagents and contaminants from the sample matrix and solvents. More recently, the tandem operation of gas chromatography and mass spectrometry has emerged as an extremely sensitive method, in spite of the problems associated with the derivatization, high-cost instrumentation and the need for a high level of technical expertise. However, the mass spectrometer, which is considered as a highly specialized detector for gas chromatography, offers three distinct advantages:

(a) the sample does not have to be highly purified when only certain molecular ions are of interest;

(b) deuterated reference compounds, which behave identically as their non-deuterated analogues, can be used and thus high precision can be achieved;

(c) with halogenated anhydrides as derivatizing reagents, excellent fragmentation patterns with prominent molecular ions are obtained.

4.1. Liquid chromatographic methods

HPLC, particularly in its reversed-phase (RP) mode, offers another approach for circumventing the problems commonly associated with other methods of analysis. This technique is ideally suited for the determination of thermally labile biological molecules since it affords high resolution and rapid analyses without prior derivatization. However, the use of this technique could be extended to the investigations of catecholamines only after the introduction of highly sensitive fluorometric and EC detectors.

Several modes of HPLC have been used for the analysis of catecholamines and their metabolites: ion-exchange, reversed-phase and reversed-phase with ion-pairing (RP—IP). More recent examples of the use of these methods in various applications are outlined in Table 2.

Although the development of chemically-bonded microparticulate ion-exchange materials has resulted in improved column stability, increased efficiency and faster analyses, the success of this mode in catecholamine research has been overshadowed by the increasingly popular reversed phases. Therefore, the ion-exchange mode will be discussed briefly.

Generally speaking, weak cation exchangers have been used for the analysis of the amines, and anion exchangers for the acidic metabolites. The ion-exchange packings suffer from several drawbacks: columns are less efficient, less reproducible and stable than the reversed-phase columns and because of the limited number of packings available, the choice of selectivities is rather limited. In addition, neither the cation- nor anion-exchange mode can afford simultaneous separation of a wide range of neurochemical substances.

TYPICAL EXAMPLES OF SOME HPLC SEPARATIONS OF CATECHOLAMINES AND METABOLITES

Sample	Sample preparation	Compounds analyzed	LC mode	Column	Mobile phase	Detection	References
Plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP—IP	μBondapak C_{18}	6.8 g sodium acetate, 100 mg EDTA, 1 g heptane-sulfonic acid, 70 ml acetonitrile per liter; pH 4.8	EC, +0.72 V	33
Plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP—IP	Ultrasphere ODS	0.1 *M* sodium acetate, 0.02 *M* citric acid, 100 mg/l sodium octyl sulfate, 50 mg/l EDTA, 10% methanol	EC, +0.60 V	34
Rat brain homogenate	Homogenization in $HClO_4$	NE, E, DHBA (IS), DA	CE	Vydac SC	Citrate—acetate buffer	EC, +0.50 V	49
Rabbit brain homogenate	Homogenization in $HClO_4$; alumina adsorption; heptanol—$HClO_4$ extraction	NE, E, DA, 5-HT	RP	Micropak MCH 10 ODS	0.1 *M* perchloric acid—acetonitrile (99:1)	Fluorescence, 200 nm excitation/ 320—400 nm emission	26
Plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP	Strong cation-exchange	0.008 *M* citric acid, 0.012 *M* sodium acetate, 0.01 *M* EDTA; pH 5.2	EC, +0.50 V	35
Rat brain homogenate	Sonication in $HClO_4$	NE, DHBA(IS) DA, AA, DHPG, MHPG, DBA, NMN, DOPAC	RP—IP	Ultrasphere ion-pair C_{18}	1 vol. methanol + 9 vol. 0.1 *M* KH_2PO_4 (pH 3.0), 0.2 m*M* sodium octyl-sulfonate, 0.1 m*M* EDTA	EC, +0.72 V	41

(Continued on p. 8)

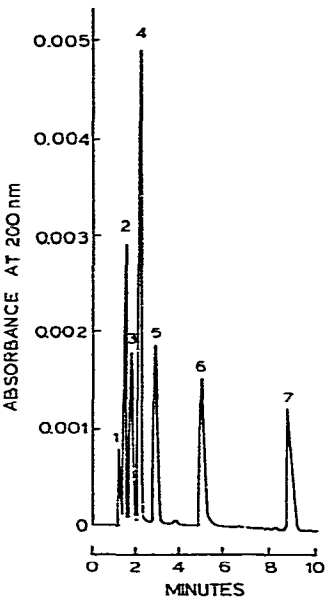
TABLE 2 (continued)

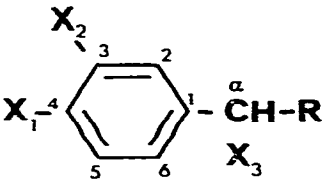
Sample	Sample preparation	Compounds analyzed	LC mode	Column	Mobile phase	Detection	References
Human plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP–IP	Ultrasphere ODS	0.0347 *M* KH_2PO_4, 0.030 *M* citric acid, 3.0 m*M* sodium octyl sulfate + 14% (v/v) methanol	EC, +0.50 V	8
Tissues, red blood cells	Homogenization in 0.15 *M* KCl, incubation with DA	COMT	RP–IP	Chromegabond, MC-18	Phosphate–citrate buffer (pH 4.4) + 50 mg sodium octyl sulfate + 90 ml methanol per liter	EC, +0.85 V	50
Serum	Incubation with DA; alumina adsorption	DβH	RP–IP	E.M. Labs. RP-18	Phosphate–citrate buffer (0.15 *M*, pH 4.4) + 23 mg sodium octyl sulfate + 35 ml methanol per liter	EC, +0.75 V	51
Rat brain homogenate	Sonication in $HClO_4$	TRP, 5-HT, 5-HIAA, HVA, 5-HTOL, MEL	RP	μBondapak C_{18}	(1) 88% 0.01 *M* sodium acetate (pH 4.25)–12% methanol (2) 85% 0.01 *M* sodium acetate (pH 4.50)–15% methanol (3) 65% 0.01 *M* sodium acetate (pH 4.25)–35% methanol	EC, +0.70 V Fluorescence, 254 nm excitation/ 360 nm emission	52
Mouse brain homogenate	Homogenization in HCl; butanol–heptane extraction	DA, DHBA (IS) 5-HT	SCE	Zipax SCX	Citrate–acetate buffer (pH 5.1)	EC, +0.60 V	53

Rat brain striatum homogenate	Homogenization in TCA	DA[1], HVA[2], DOPAC[2]	SCX[1] RP[2]	Zipax SCX[1] Zorbax ODS[2]	[1]Citrate—acetate buffer, pH 5.2 [2]Citrate—acetate buffer + 10% methanol	[1]EC, +0.61 V	54
Mouse brain homogenate, mouse brain perfusate	Homogenization in $HClO_4$; ethyl acetate extraction	MHPG	RP	Rheodyne RP-2 μBondapak C_{18} LiChrosorb	10 m*M* potassium phosphate buffer (pH 7.0)	EC, +0.80 V	55
Human CSF	Ethyl acetate extraction	MHPG (free) MHPG·SO_4	RP	μBondapak C_{18}	Low-strength, 0.1 *M* KH_2PO_4, pH 2.50; high-strength, methanol—water (3:2, v/v); gradient, linear from 0—60% of high-strength eluent in 45 min	EC, +0.70 V	42
Mouse brain homogenate	Homogenization in $HClO_4$; diethyl ether extraction	DOPAC, HVA, VA	RP	μBondapak C_{18}	0.05 *M* sodium acetate buffer (pH 5.0)	EC, + 0.70 V	56
Human urine	Alumina adsorption; heating in alkaline buffer	NE, E, DA, D	SCE	Zipax SCX	50 m*M* NaH_2PO_4 + 50 ml acetonitrile per liter	Fluorescence 400 nm excitation/ 490 nm emission	57
Human urine	Alumina adsorption; THI reaction	NE, E, α-MD, ISO, DA, D, DOPAC, DOMA	RP—IP	Partisil PXS 10/25 ODS	1% acetic acid, 0.0001% sodium dodecyl sulfate, 10% methanol	Fluorescence	58
Human urine	Ion-exchange, alumina adsorption	CA and minor and major metabolites	RP—IP	μBondapak C_{18}	See article	Liquid scintillation	31 59
Animal serum	Incubation with L-DOPA; amberlite CG-50 elution	L-amino acid decarboxylase	RP	Yanapak ODS-T	0.01 *M* KH_2PO_4, pH 3.2	EC	59

4.1.1. Reversed-phase liquid chromatography

As evidenced by the ever growing number of publications, the reversed-phase mode of HPLC is experiencing a tremendous increase in popularity due to the high column stability, efficiency and speed of analysis, minimal reequilibration time in gradient elution, and the ability to separate a wide range of compounds through the use of secondary equilibria [60]. The great potential of this technique for catecholamine analysis was first demonstrated by Molnár and Horváth in 1976 [61]. By exploiting the hydrophobic interactions, the authors have separated a mixture of the amines and some metabolites in a single chromatographic run, using isocratic elution with a neat aqueous phosphate buffer (Fig. 1). Since most catecholamine metabolites possess a wide range of polarities, a relatively high carbon content of the stationary phase is necessary for sufficient retention. Molnár and Horváth [62] have also investigated the effects of various ring and side-chain constituents (Fig. 2) on the capacity factors of most catecholamines and related compounds. The results are shown in Table 3. The extent of retention of these compounds on reversed-phase columns is affected significantly by the eluent pH. At low pH values (approximately 2), the amino groups are fully protonated while the dissociation of the carboxyl groups is suppressed [61]. The effects of pH on the extent of retention of the amines and their acidic metabolites are shown in





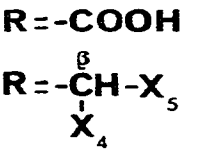


Fig. 1. Separation of some catecholamines and related compounds. Chromatographic conditions: column, 5 μm LiChrosorb RP-18; eluent, 0.1 *M* phosphate buffer, pH 2.1; flow-rate, 2.0 ml/min; temperature, 70°C; inlet pressure, 160 bar. Peaks: 1, norepinephrine; 2, epinephrine; 3, dopamine; 4, tyramine; 5, phenylethanolamine; 6, phenylethylamine; 7, dimethyldopamine. (Reproduced from ref. 62 with permission.)

Fig. 2. Generalized structural formulae for catecholamines and metabolites. (Reproduced from ref. 62 with permission.)

TABLE 3

STRUCTURES AND CAPACITY FACTORS, k', FOR CATECHOLAMINES AND METABOLITES*

Symbol	Name	Structure				Capacity factor k'
		X_1	X_2	X_3	R	
Amines						
NE	Norepinephrine	OH	OH	OH	CH_2NH_2	0.145
OCT	Octopamine	OH	H	OH	CH_2NH_2	0.26
E	Epinephrine	OH	OH	OH	CH_2NHCH_3	0.28
NMN	Normetanephrine	OH	OCH_3	OH	CH_2NH_2	0.48
SYN	Synephrine	OH	H	OH	$CH(CH_3)NH_2$	0.51
DA	Dopamine	OH	OH	H	CH_2NH_2	0.56
MN	Metanephrine	OH	OCH_3	OH	CH_2NHCH_3	0.93
T	Tyramine	OH	H	H	CH_2NH_2	0.96
PEOA	Phenylethanolamine	H	H	OH	CH_2NH_2	1.03
3MDA	3-O-Methyldopamine	OH	OCH_3	H	CH_2NH_2	1.86
PEA	Phenylethylamine	H	H	H	CH_2NH_2	4.06
NIE	Norisoephedrine	H	H	NH_2	$CH(OH)CH_3$	5.17
EPH	Ephedrine	H	H	OH	$CH(CH_3)NHCH_3$	6.76
DMDA	Dimethyldopamine	OCH_3	OCH_3	H	CH_2NH_2	9.09
Acids						
DOMA	Dihydroxymandelic	OH	OH	OH	COOH	0.51
POMA	*p*-Hydroxymandelic	OH	H	OH	COOH	0.87
MOMA	*m*-Hydroxymandelic	H	OH	OH	COOH	1.66
VMA	Vanillylmandelic	OH	OCH_3	OH	COOH	1.69
DOBA**	3,4-Dihydroxybenzoic	OH	OH	—	—	2.66
DOPAC	Dihydroxyphenylacetic	OH	OH	H	COOH	4.42
MA	Mandelic	H	H	OH	COOH	5.57
POPAC	*p*-Hydroxyphenylacetic	OH	H	H	COOH	7.57
VA**	Vanillic	OH	OCH_3	—	—	9.13
HVA	Homovanillic	OH	OCH_3	H	COOH	14.67
PAC	Phenylacetic	H	H	H	COOH	47.80

*Reproduced from ref. 62 with permission.
**The general formula in Fig. 2 is not applicable to the structures of these compounds.

Fig. 3a and b, respectively. It is evident that by suppressing the degree of dissociation, the interaction between the solute molecules and the hydrocarbonaceous stationary phase is enhanced due to increased solute hydrophobicity. Thus, the acidic metabolites are retained longer at lower pH values, while the opposite is true for the amines [61]. The effect of the ionic strength of the medium on the extent of retardation is less pronounced than that of the pH (Fig. 4). This indicates that, under the chromatographic conditions used, the hydrophobic interactions are more important than the coulombic forces.

The effect of temperature on retention of catecholamines and related compounds is illustrated with the Van 't Hoff plots (Fig. 5). The k' values are approximately halved by a 30—40°C increase in temperature. However, the relative retention is not changed significantly.

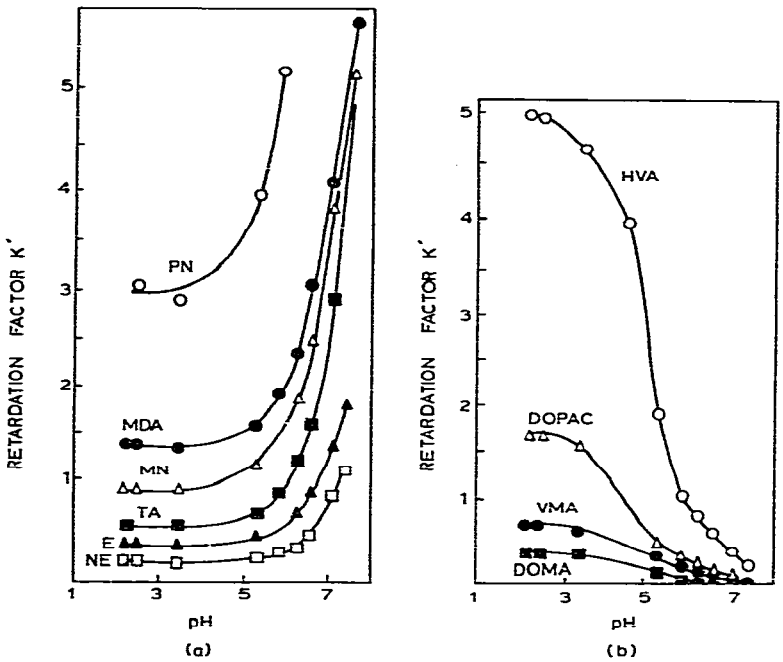


Fig. 3. (a) Effect of pH on the retention of catecholamines. Peaks: E, epinephrine; MN, metanephrine; NE, norepinephrine; MDA, 3-O-methyldopamine; PN, paranephrine; TA, tyramine. (b) Effect of pH on the retention of acidic metabolites. Peaks: DOMA, 3,4-dihydroxymandelic acid; DOPAC, 3,4-dihydroxyphenyl acetic acid; HVA, homovanillic acid; VMA, vanillylmandelic acid. Chromatographic conditions: column, Partisil 10/25 ODS; eluent, 0.1 *M* phosphate buffer; flow-rate, 1.0 ml/min; inlet pressure, 100 atm; temperature, 25°C. (Reproduced from ref. 61 with permission.)

4.1.1.1. Ion-pair reversed-phase chromatography. From the pK_a values for the three major catecholamines it is evident that they are protonated in the pH range commonly employed with the reversed phases [2—7]. The existence of charged moieties results in diminished retention; this is particularly evident in the case of NE which elutes near the void volume, as shown in Fig. 6A. The extent of retention of NE is not improved by changing the eluent pH from 2 to 7.5. In addition, all metabolites with the pK_a values below the pH of the eluent would be present mostly in their dissociated form and would thus elute with minimal retention. Increase in the ionic strength of the eluent upon addition of citrate ions improves the peak shapes by facilitating the protonic equilibria with no effect on retention (Fig. 6B). Therefore, although the reversed-phase mode offers higher efficiency and faster analyses, it still exhibits a discriminatory effect with respect to a wider range of catecholamine metabolites.

The retention of basic solutes such as catecholamines can be augmented by increasing the eluent pH. However, this is undesirable for the following reasons:

(a) catecholamines are notoriously unstable at elevated pH values;

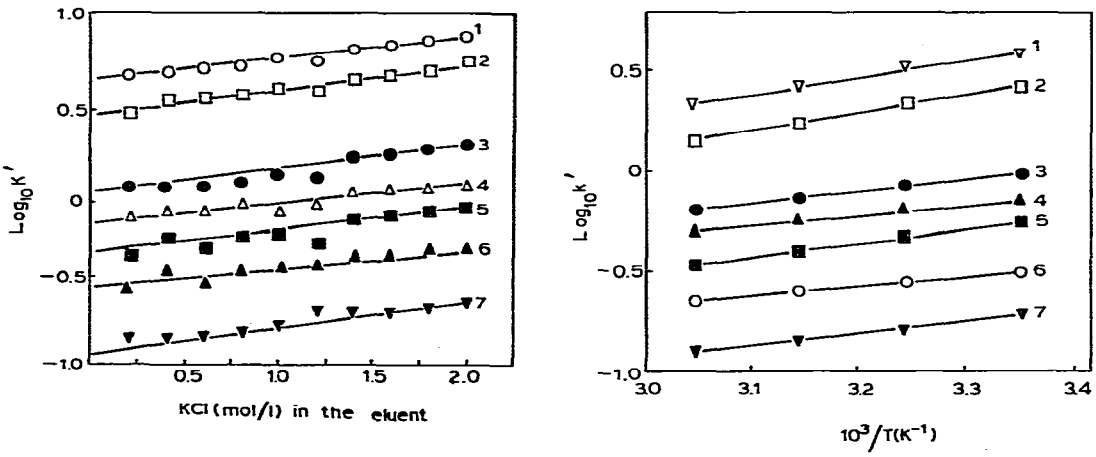


Fig. 4. Effect of ionic strength on the retardation factor; the concentration of KCl in a 50 m*M* KH_2PO_4 solution was varied. Peaks: 1, paramephrine; 2, homovanillic acid; 3, 3-O-methyldopamine; 4, 3,4-dihydroxyphenylacetic acid; 5, tyramine; 6, tyrosine; 7, DOPA. Chromatographic conditions as in Fig. 3. (Reproduced from ref. 61 with permission.)

Fig. 5. Effect of temperature on retardation factor. Peaks: 1, homovanillic alcohol; 2, homovanillic acid; 3, 3-O-methyldopamine; 4, metanephrine; 5, tyramine; 6, dopamine; 7, vanillylmandelic acid. Eluent, 0.05 *M* KH_2PO_4; all other chromatographic conditions as in Fig. 3. (Reproduced from ref. 61 with permission.)

(b) at the same pH (basic), the acidic metabolites can not be efficiently separated (they would elute near the void volume);

(c) with the currently available silica-based reversed phases, dissolution of the support takes place in alkaline media.

The retention of charged solutes can be altered dramatically upon addition of ionic surfactants (ion-pairing reagents, ion-association reagents) to the mobile phase. This technique, which combines the best features of reversed phase (high resolution, high column stability) and ion exchange (improved separation), is finding widespread acceptance under a multitude of names, the most common one being ion-pairing (IP). By exploiting the interplay between the electrostatic and hydrophobic forces, the capacity factors of basic solutes can be greatly modulated by addition of anionic surfactants and those of acidic metabolites can be altered upon addition of cation-pairing reagents. Because of the experimental difficulties associated with obtaining umambiguous physicochemical evidence, the underlying thermodynamic equilibria are a subject of considerable controversy. The retention is believed to occur by dynamic ion exchange [63], ion-pair formation in the mobile phase [64], or the dynamic complex exchange [65].

The main equilibria involved in ion-pair chromatography of catecholamines are the following [8]:

(a) ion-pair formation in the mobile phase with the anion-pairing reagent, L^-

$$CA^+_m + L^-_m \leftrightarrows (CA^+L^-)_m \quad (1)$$

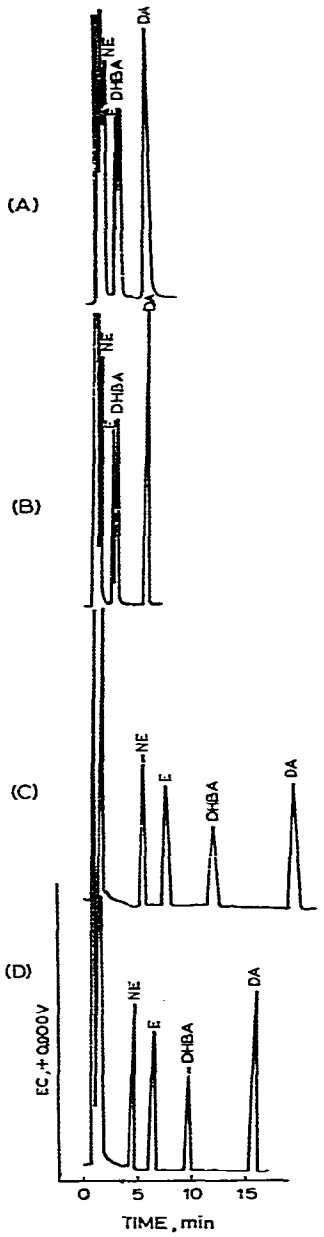


Fig. 6. Separation of a synthetic mixture of norepinephrine (NE), epinephrine (E), 3,4-dihydroxybenzylamine (DHBA) and dopamine (DA). Chromatographic conditions: column, Ultrasphere ODS (15 cm × 4.6 mm I.D.), 5 μm average particle size; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at +0.500 V vs. Ag/AgCl; detector sensitivity, 7.5 nA full scale; eluent, (A) 0.0347 *M* KH_2PO_4, pH 4.85; (B) 0.0347 *M* KH_2PO_4, 0.030 *M* citric acid, pH 4.85; (C) 0.0347 *M* KH_2PO_4, 3.0 m*M* sodium octyl sulfate, 14% methanol (v/v), pH 4.85; (D) 0.0347 *M* KH_2PO_4, 3.0 m*M* sodium octyl sulfate, 0.030 *M* citric acid, 14% methanol (v/v), pH 4.85. (Reproduced from ref. 8 with permission.)

Followed by reversible binding of the ion-pair to the hydrocarbonaceous surface:

$$(CA^+L^-)_m \leftrightarrows (CA^+L^-)_s \quad (2)$$

where the subscripts m and s refer to the mobile phase and stationary phase, respectively;

(b) dynamic ion exchange in which the solute molecule forms a complex with the ligand already adsorbed on the nonpolar surface:

$$CA^+_m + L^-_s \leftrightarrows (CA^+L^-)_s \quad (3)$$

(c) dynamic complex exchange in which a metathetical exchange occurs between the ion-pair formed in the mobile phase and the ion-pairing reagent bound to the column:

$$(CA^+L^-)_m + L^-_s \leftrightarrows (CA^+L^-)_s + L^-_m \quad (4)$$

It is important to remember that each model represents a limiting case and "the retention process is not expected to follow any of them over a wide range of chromatographic conditions" [65].

Regardless of the exact type of thermodynamic equilibrium responsible for retention, it is mandatory that the solute molecules be charged for the interaction with the ion-pairing reagent to occur.

The dramatic effect of the ion-pairing reagent, octyl sulfate, on retention of catecholamines is shown in Fig. 6C; the chemical nature and concentration of the pairing reagent are important in determining the extent of retention. However, high concentrations and excessively long chain lengths of the pairing reagent must be avoided since they give rise to excessively long retention and long equilibration times. Octyl sulfate gives excellent selectivity with minimal equilibration time (20 min). However, under the conditions shown in Fig. 6C, DA exhibits excessively long retention which gives rise to considerable peak broadening. This is prohibitive, for example, for the analysis of the free levels of trace amines in plasma samples from normal subjects. The k' values, however, can be adjusted by addition of an organic modifier, such as methanol, to the mobile phase (Fig. 6C). Furthermore, the presence of citrate ions in the mobile phase exerts a pronounced effect on retention (Fig. 6D). This effect is not due to the change in ionic strength, since the same was not observed when citrate was replaced with an equivalent amount of KCl. From the behavior of catecholamines under the chromatographic conditions shown in Fig. 6A, B, C and D, it can be assumed that the ion-pairing takes place on the column surface covered with octyl sulfate. The citrate ions in the mobile phase lower the retention of catecholamines by exerting an electrostatic attraction on the "pairs".

5. DETECTION AND CLINICAL APPLICATIONS

With the increasing ability to achieve efficient and rapid separations of catecholamines and their metabolites, the central problem in biomedical and biochemical investigations becomes the ability to monitor the minute amounts of these compounds with detection systems of sufficient sensitivity and com-

patibility with the chromatographic system. Only two detectors, the fluorometric and the electrochemical, fulfil these requirements successfully. The relative advantages and disadvantages of both detectors will be discussed in greater detail and illustrated with examples of clinical applications.

5.1. Fluorometric detection

Prior to the advent of the EC detector, fluorometric detection was the only method capable of monitoring picomole amounts of catecholamines and their metabolites. Since only relatively few compounds fluoresce naturally, the use of this detection affords yet another level of selectivity to the analytical system [38]. In addition, each group of fluorescent molecules exhibits different excitation and emission spectra: catecholamines have excitation maxima between 200—220 nm and 280—300 nm and an emission maximum at 310—330 nm, while the indolic compounds exhibit the excitation maxima at 210—220 nm and 270—280 nm and an emission maximum at approximately 360 nm. With the currently available sources of UV radiation, efficient columns, and in view of the molar absorptivities and quantum efficiencies of underivatized catecholamines and indoles, the detection limits are 100—500 pg and 5—25 pg, respectively. Even without the future improvements in HPLC design, considerably lower detection limits can be achieved with the fluorescent derivatives of catecholamines and indoles. The most commonly employed fluorogenic reagents are: fluorescamine, dansyl chloride, *o*-phthalaldehyde and ethylenediamine. Another frequently employed derivatization method, the trihydroxyindole (THI), involves catechol oxidation and alkaline rearrangement.

Fluorogenic reactions can be carried out in the following ways:

(a) pre-column: off-line: the reaction is carried out separately before the chromatography; on-line: the reaction is carried out between the injector and the column inlet;

(b) post-column: off-line: derivatization is carried out on collected fractions, after the chromatography; on-line: derivatization is carried out in a specifically designed reactor cell placed between the column outlet and the detector.

Generally speaking, pre-column, off-line derivatization is often used since it does not pose any restrictions on the mobile phase composition, duration and temperature of reaction and product stability, which is not the case with the on-line derivatization. Post-column, on-line derivatization is carried out on separated chromatographic bands. Therefore, the mobile phase composition must be compatible with the reaction medium. In addition, the reaction detector must be carefully designed in order to minimize dispersion.

5.1.1. Selected clinical applications

Measurements of native fluorescence usually do not afford sufficient sensitivity for the analysis of picomole amounts of catecholamines. Enhanced sensitivity can be achieved with THI or *o*-phthalaldehyde (OPA) derivatizations. The RPLC separations of the OPA and THI derivatives of catecholamines detected fluorometrically are illustrated with the extract of a sample of control urine and a purified sample of human plasma shown in Figs. 7 and 8, respectively.

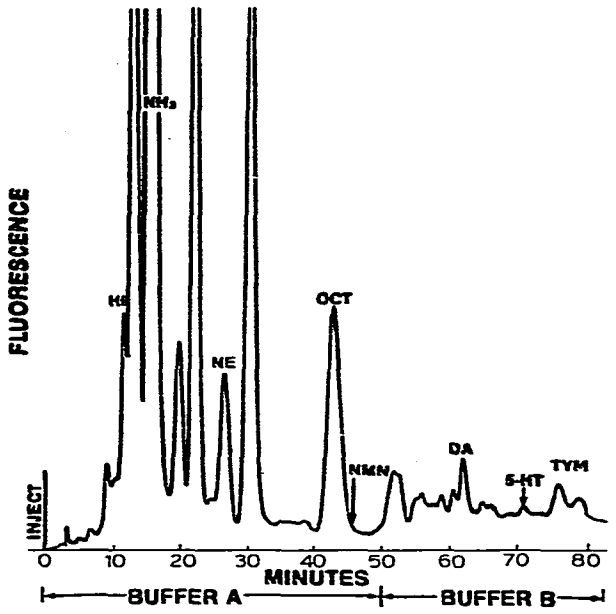


Fig. 7. RPLC separation of OPA derivatives of biogenic amines in control urine. Chromatographic conditions: sample, 15 μl, equivalent to 15 μl urine; column, μBondapak phenyl (300 mm × 4 mm I.D.); buffer, 0.05 *M* NaH_2PO_4, pH 5.10 with 320 ml of methanol added per l for first elution step (A) and 450 ml of methanol added per l for second elution step (B); flow-rate, 1.5 ml/min; detector, Schoeffel FS 970, 0.10 μA full scale, excitation 340 nm, emission 480 nm; temperature, 30°C. The internal standard (IS) is octopamine (OCT). Peaks: HI, histamine; NE, norepinephrine; NMN, normetanephrine; DA, dopamine; 5-HT, serotonin; TYM, tyramine. (Reproduced from ref. 13 with permission.)

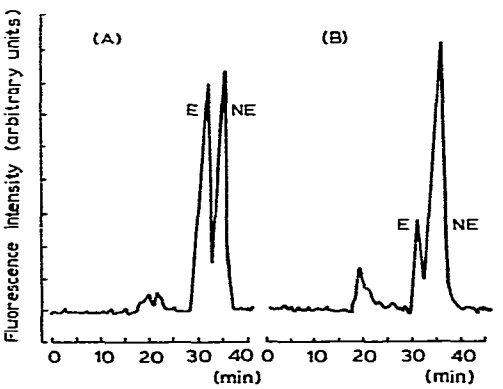


Fig. 8. Elution pattern of fluorometrically detected THI derivatives of epinephrine (E) and norepinephrine (NE). (A) Standard sample of E (0.5 ng) and NE (1.0 ng); (B) plasma sample. Chromatographic conditions: column, Amberlite IRC-50; eluent, succinic acid—boric acid—EDTA (pH 5.4); flow-rate, 0.7 ml/min; temperature, 42°C; detection, excitation 405 nm, emission 500 nm. (Reproduced from ref. 66 with permission.)

Under the chromatographic conditions used, the detection limits for the OPA derivatives were approximately 50—100 pg. It is evident, therefore, that this type of derivatization is not suitable for the analysis of catecholamines in samples of human plasma. However, the post-column THI derivatization shown in Fig. 8 allowed the detection of 20 pg of NE and DA, and 40 pg of NE. Thus, with the latter method it was possible to determine the catecholamine levels in 1—2 ml of human plasma purified by cation-exchange chromatography. It should be pointed out that both methods illustrated with Figs. 7 and 8 could be further improved by reducing the retention volumes and optimizing the method of detection. Since the levels of metabolites often excede those of the parent amines, direct monitoring of native fluorescence can be used for the assessment of metabolite levels in various physiological fluids [38,67]. The comparative features of fluorometric and amperometric detection of the catechol and indolic compounds are illustrated with the analysis of human lumbar cerebrospinal fluid (Fig. 9A and B). Under the conditions used, the phenolic indole (5-HIAA) gives approximately the same

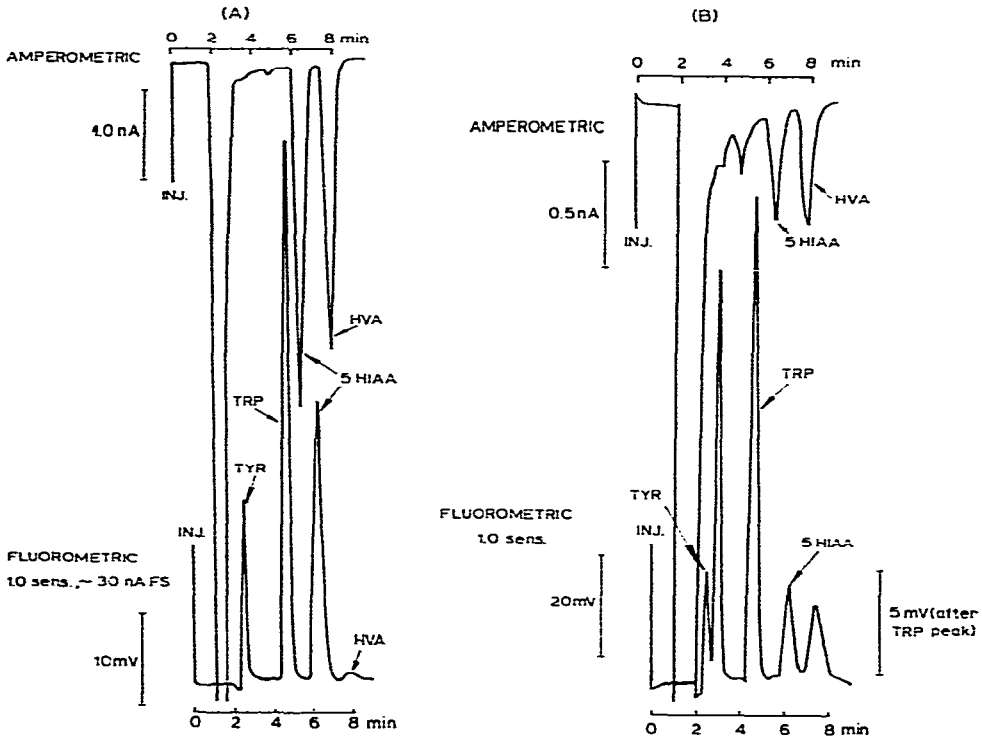


Fig. 9. (A) Chromatogram of reference compounds: 25 ng tyrosine (TYR), 5 ng tryptophan (TRP), 5 ng 5-hydroxyindole-3-acetic acid (5-HIAA), 10 ng homovanillic acid (HVA). TYR was not usually separated from the amperometric solvent front. (B) Chromatogram of a 20-μl unprocessed lumbar CSF sample. Concentrations: TYR, 1.04 mg/ml; TRP, 396 ng/ml; 5-HIAA, 32.6 ng/ml; HVA, 89.7 ng/ml. The fluorometric peaks immediately following the TYR and 5-HIAA peaks are unidentified. Chromatographic conditions: column, μBondapak C_{18}; eluent, 300 ml methanol + 1700 ml 0.01 *M* sodium acetate, pH 4.0; flow-rate, 1.5 ml/min; temperature, ambient; detection, EC, +0.8 V vs. Ag/AgCl; fluorescence, 254 nm excitation, 360 nm emission. (Reproduced from ref. 67 with permission.)

response with both detection methods while amperometric detection is more sensitive for the catechol HVA. The use of fluorometry is generally advantageous for the determination of indolic compounds. An additional advantage of the fluorometric detection in this application is the virtual absence of solvent front, which is pronounced in the amperometric tracing, and arises from ascorbic acid present in the CSF sample and the synthetic mixture of reference compounds [67]. Since biological matrices contain many compounds, the elucidation of identity of chromatographic bands is a step of paramount importance. Quite often, peak assignments are based only on retention times and co-chromatography with the reference compounds. This is by no means sufficient, even with the most selective sample purification and detection. Every detection device offers different possibilities for on-line characterization. With fluorescence monitoring, which is highly selective for the limited number of naturally fluorescing molecules in physiological samples, excitation and/or emission spectra of the solutes can be obtained and compared with those of the reference compounds [37]. Since each compound or a class of closely related compounds exhibits distinctly different absorption and/or emission characteristics, further proof of peak identity can be obtained. The usefulness of the excitation spectra is illustrated with the analysis of catecholamines in a sample of rat brain homogenate (Fig. 10).

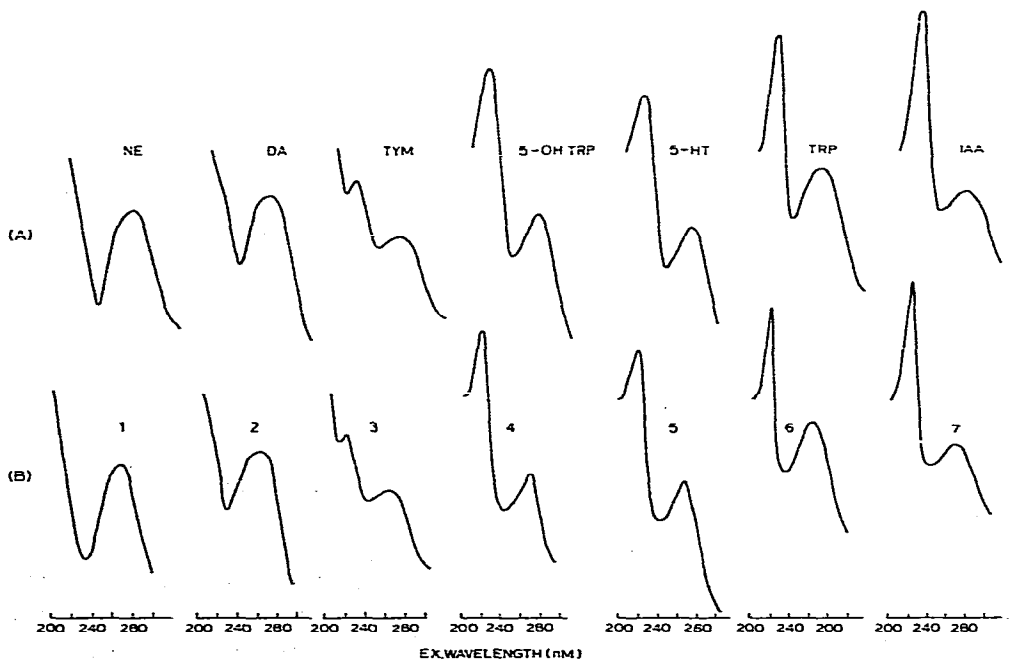


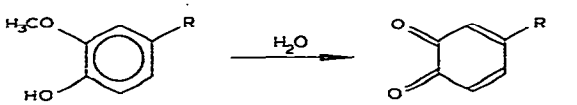
Fig. 10. Comparison of corrected stopped-flow excitation spectra for (A) some reference compounds and (B) chromatographic peaks in a rat brain extract. Scanning rate, 100 nm/min; range, 0.1 μA full scale; chromatographic conditions: column, μBondapak C_{18}; eluents, (low-strength) 0.02 *M* KH_2PO_4, pH 3.7; (high-strength) methanol—water (3:2, v/v); gradient, linear from 0% to 100% of the high-strength eluent in 35 min; flow-rate, 1.5 ml/min; temperature, ambient. (Reproduced from ref. 37 with permission.)

These spectra were obtained by the stopped-flow method, whereby the desired solute is arrested in the detector cell under the stopped-flow conditions and the corrected spectrum is obtained by automatic subtraction of the spectral background arising from the mobile phase, flow cells and the photomultiplier. The marked similarity between the spectra of the sample peaks and those of the reference compounds confirms the identity of the endogenous compounds.

5.2. Electrochemical detection

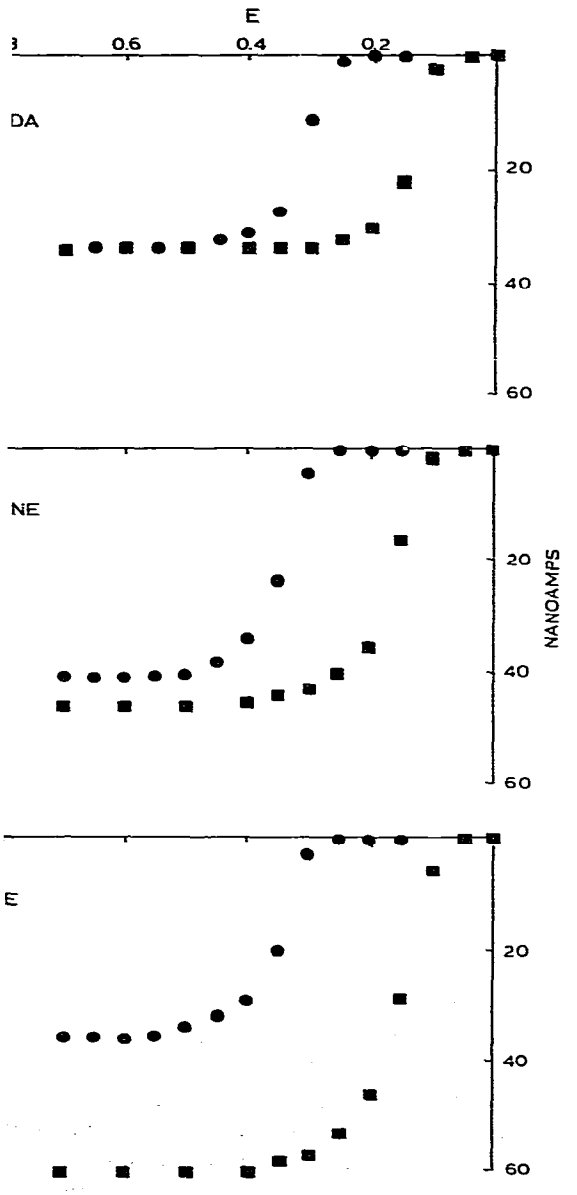
The use of thin-layer electrochemical reactions is becoming increasingly popular for the determinations of substances of neurochemical interest such as phenols and indoles [43] and related enzymes. The electrochemical reaction(s) involve(s) a direct conversion of chemical information into an electrical signal without the need for intermediate magnetic or optical carriers. Thus catecholamines (a) and vanil compounds (b) can be determined analytically by oxidation to the corresponding orthoquinones at the surface of a graphite electrode, according to the following raction:

(a)  $+ 2H^+ + 2e^-$ (5)

(b)  $+ 2H^+ + ROH + 2e^-$ (6)

The instantaneous anodic current is directly proportional to the number of solute molecules in contact with the interface per unit time. The physicochemical process occurring at the electrode surface poses certain requirements on the nature and properties of the solute and mobile phase: the compounds must be electroactive under the conditions used and the mobile phase must have sufficient electrical conductivity (optimal ionic strength > 0.05 *M*). The latter parameter helps to minimize the internal *iR* drop (where i = current and R = resistance), and to control the electrode potential.

The electrochemical behavior of catecholamines and related compounds is affected by the pH of the medium, as expected from eqns. 5 and 6. According to the Nernst equation, a 60-mV shift in half-wave potential is expected for fast reactions in which the number of electrons transferred across the electrode—solution interface equals the number of protons (lost or gained). This is illustrated with the change in limiting current for NE, E and DA upon change in pH from 3.75 to 7.28 (Fig. 11). At low pH the oxidation of the catechol takes place according to eqn. 5. In the basic range, however, the free base of the alkylamine will attack the aromatic nucleus and the product of the reaction, indoline, will subsequently be oxidized (Fig. 12). If the reaction is fast compared to the residence time of the molecule in the detector cell, a double response will be obtained due to the overall transfer of $4e^-$. Since the extent of this effect depends upon the rate of nucleophilic addition,



ig. 11. Hydrodynamic voltammograms obtained from chromatograms of dopamine (DA), orepinephrine (NE), and epinephrine (E) at pH 3.75 (•) and pH 7.28 (▪). Chromatographic conditions: stationary phase; 15 cm × 4.6 mm I.D., Merck C_2 reversed-phase packing; mobile phase, acetate buffer (0.04 *M*, pH 3.75), 0.2 m*M* octyl sulfate; mobile phase low-rate, 0.40 ml/min; temperature, ambient; amount injected, 20 ng of each catecholmine. Mixing conditions: mixing phase, •, same as mobile phase, ▪, 0.5 *M* Na_2PO_4; mixing hase flow-rate, 0.13 ml/min; reaction coil residence time, 8.1 sec; final pH •, pH 3.75, , pH 7.28. (Reproduced from ref. 68 with permission.)

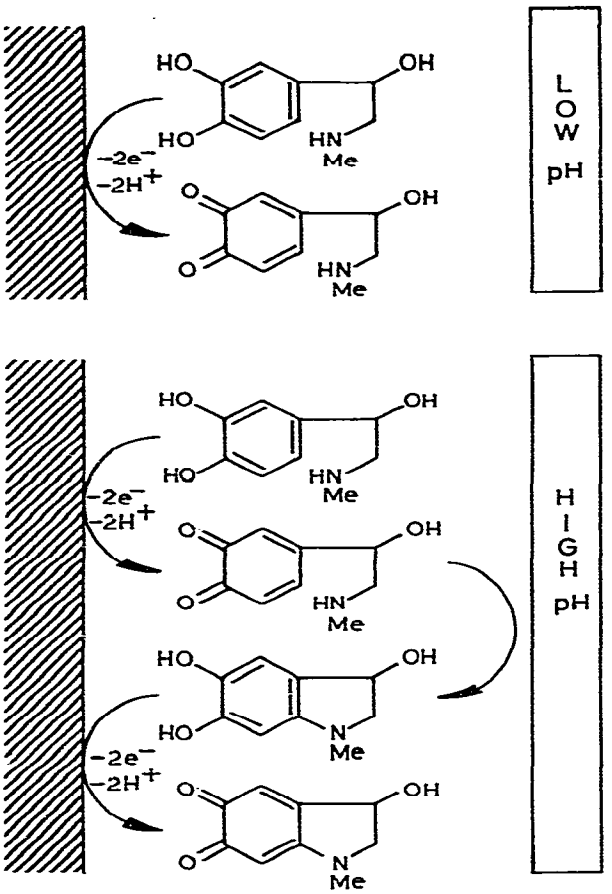


Fig. 12. Mechanisms for the electrochemical oxidation of epinephrine. (Reproduced from ref. 68 with permission.)

the response of NE and DA will not be increased significantly at pH 7.28. It is important to realize that like any other LC detector, the EC detector also suffers from some shortcomings. Although it is possible to vary the applied potential within the useful range of the working electrode material (Fig. 13A and B), the resulting molecular specificity is not adequate when working with complex physiological matrices. Therefore, in order to increase the detection specificity, an isolation step must be incorporated into the analytical procedure.

5.2.1. Selected clinical applications

Since a complete survey of the LC—EC applications in biomedical and biochemical research is beyond the scope of this review, only some examples will be used to demonstrate the tremendous utility of this technique. The determination of plasma catecholamines under standardized conditions is of considerable importance for clinical investigations of disorders such as hypertension, neural crest tumors, neurological disorders such as depression, schizophrenia, Parkinson's disease and many others. If the method is to be used as

a diagnostic tool in clinical applications, prior to the determination of cut-off values, it is necessary to establish the baseline levels in control subjects. As stated previously, many variables must be clearly specified in catecholamine investigations [8].

After the normal levels have been established, it is possible to use this technique to monitor certain disease states, such as pheochromocytoma [9,42].

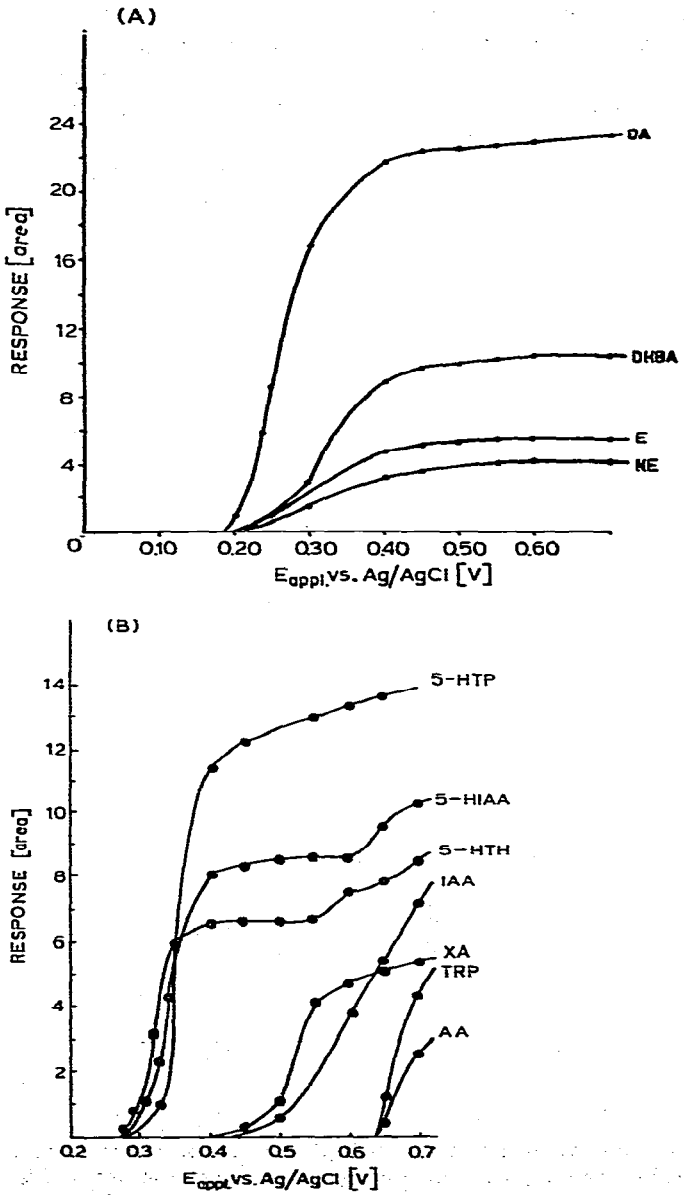


Fig. 13. (A) Voltammograms of the three principal catecholamines and internal standard (DHBA). (Reproduced from ref. 8 with permission.) (B) Voltammograms of some tryptophan metabolites. (Reproduced from ref. 69 with permission.)

Pheochromocytoma is a tumor of the sympathetic nervous system, derived from primitive neural crest tissue. This catecholamine-secreting lesion is a rare cause of hypertension and is fatal if undetected. Since these tumors exhibit tremendous variability in their clinical presentation and have all the symptoms of essential hypertension, a reliable diagnosis is possible only on the basis of altered patterns of catecholamine metabolism. Fig. 14 illustrates the catecholamine levels in the alumina extracts of plasma samples from the same patient before (A, B) and after (C) surgical removal of the tumor. It is evident that this tumor was predominantly NE-secreting. Prior to the final assignment of peak identities and subsequent quantitation, it is mandatory to characterize chromatographic bands by methods which rely on properties other than the retention behavior. It can not be overemphasized that the agreement between the retention times of the peak on the chromatogram and the reference compound is not a proof of identity but merely an indication

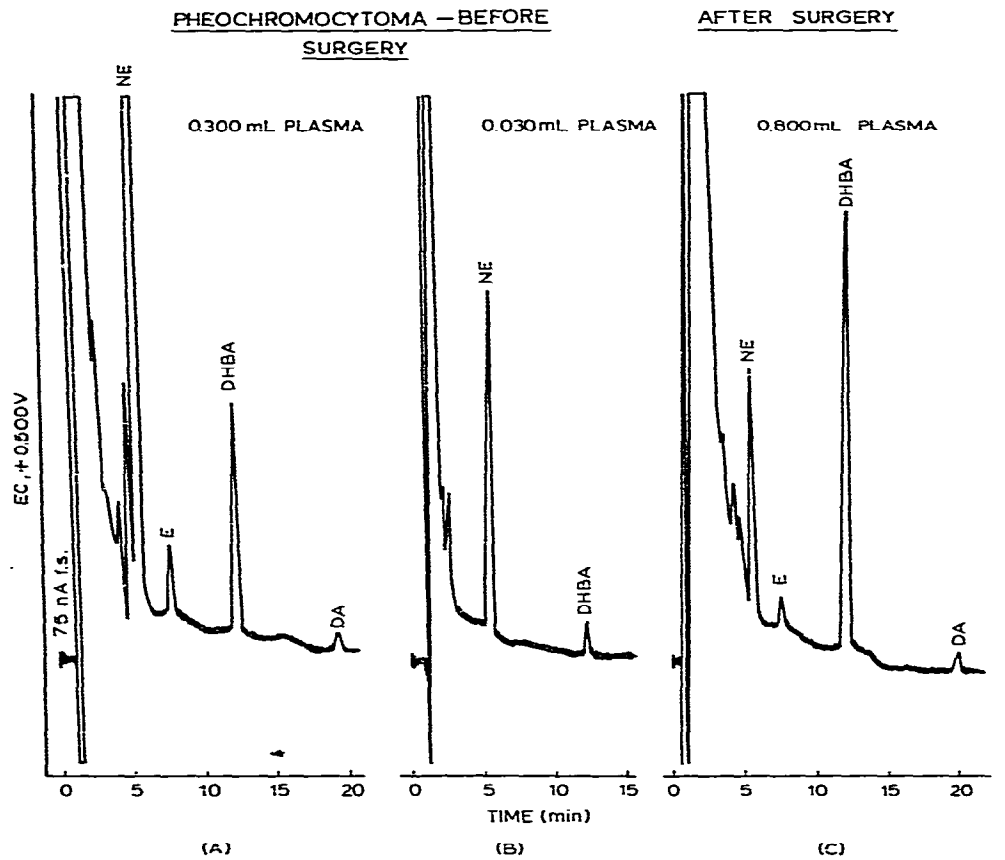


Fig. 14. Chromatograms of plasma samples from a patient with pheochromocytoma, before (A, B) and after (C) surgical removal of the tumor. Chromatographic conditions as in Fig. 6D; volumes of extract injected in (A), (B), and (C), 100 μl; volumes indicated in the figure refer to volumes of plasma extracted by alumina adsorption. Catecholamine levels, (A, B) NE, 14.5 ng/ml; E, 180 pg/ml; DA, 85 pg/ml; (C) NE, 350 pg/ml; E, 25 pg/ml; DA, 40 pg/ml; DHBA is the internal standard. (Reproduced from ref. 8 with permission.)

of it. Amperometric detection offers an attractive possibility for on-line characterization of electroactive solutes by comparison of hydrodynamic voltammograms of the sample component and the reference compound [8, 70]. By making replicate injections of the sample and recording the response (current) at several potentials, the ratios of the current at any given potential to that of the maximal response can be computed. These relative current ratios (ϕ) can be plotted as a function of the applied potential, and the resulting curves for the sample peak and the reference compound compared. Fig. 15 illustrates the use of hydrodynamic voltammograms for characterization of NE in the plasma sample shown in Fig. 14. Careful confirmation of peak identities is essential for ensuring that the right peaks are quantified and that there are no impurities coeluting with the compound of interest. The quantitative data for catecholamines and some metabolites in plasma and urine samples from control normotensive subjects, hypertensive patients and those with pheochromocytoma are given in Table 4. Significant differences in NE levels among the three groups of subjects illustrate the usefulness of these compounds as biochemical determinants of certain disease states.

The aberrations in the metabolic pathways of catecholamines in certain disease states manifest themselves in characteristic urinary excretion patterns of their catabolites (Table 4). Since the levels of VMA, HVA, MHPG and total metanephrines (NMN, MN) are considerably higher than those of the

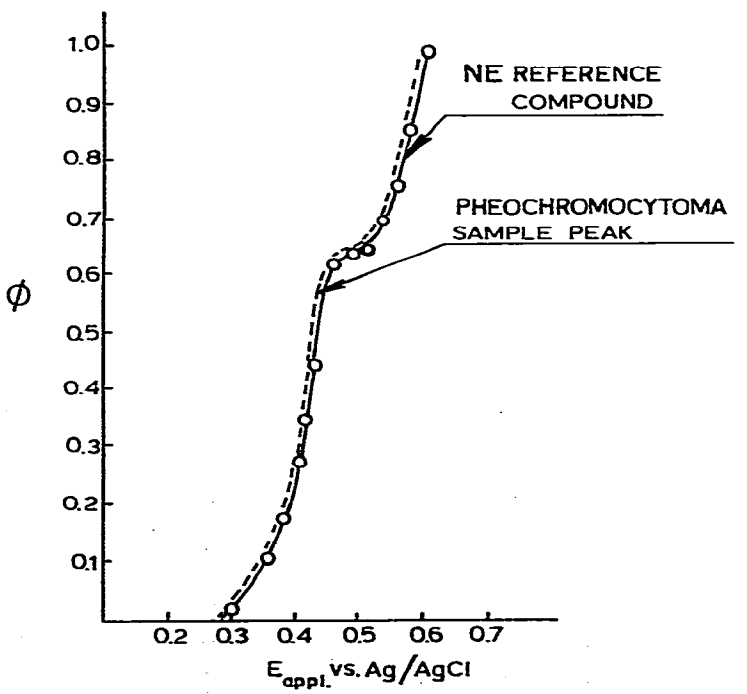


Fig. 15. Hydrodynamic voltammograms for the NE reference compound and the peak in the sample from a patient with pheochromocytoma shown in Fig. 14A; X-axis, oxidation potential vs. Ag/AgCl; Y-axis, ratio of response (current) at a particular potential to the maximal response. (Reproduced from ref. 8 with permission.)

TABLE 4

QUANTITATIVE DATA FOR CATECHOLAMINES AND SOME METABOLITES DETERMINED BY HPLC*

	NE (pg/ml)	E (pg/ml)	DA (pg/ml)	VMA** (μg/mg CRE)	MHPG** free (ng/mg CRE)	MHPG** conjugated (ng/mg CRE)	NMN** (μg/mg CRE)	MN** (μg/mg CRE)
Control subjects (normotensive)	100–600 $n = 20$	10–80 $n = 20$	10–150 $n = 20$	0.5–3.5 $n = 15$	0.02–0.1 $n = 15$	0.4–0.6 $n = 15$	0.09–0.21 $n = 7$	0.01–0.09 $n = 7$
Control subjects under stress	900–1200 $n = 2$	10–70 $n = 2$	10–50 $n = 2$	—	—	—	—	—
Essential hypertension	1600–1800 $n = 3$	40–80 $n = 3$	200–250 $n = 3$	—	—	—	—	—
Pheochromocytoma	4000–14500 $n = 3$	80–250 $n = 3$	65–470 $n = 3$	4.5–50.0 $n = 15$	0.07–1.15 $n = 15$	2.4–15.9 $n = 15$	1.5 –27.5 $n = 6$	0.10–1.60 $n = 6$
Neuroblastoma	—	—		5.0–21.0 $n = 5$	0.09–1.50 $n = 5$	2.4–16.0 $n = 5$		
Ganglioneuroma	—	—		—			4.0 $n = 1$	0.79 $n = 1$

* Results reproduced from refs. 8, 9, 42, 48 and 71.

** In urine (referred to creatinine, CRE); all other values pertain to plasma.

parent amines, the determination of these metabolites is an easier analytical problem.

The clinical usefulness of VMA and MHPG as a diagnostic tool for pheochromocytoma is evident from the elevated levels of both metabolites (Fig. 16B) compared to those of the control subject (Fig. 16A). Total metanephrines have also been used as a clinical index of neural crest tumors (Table 4).

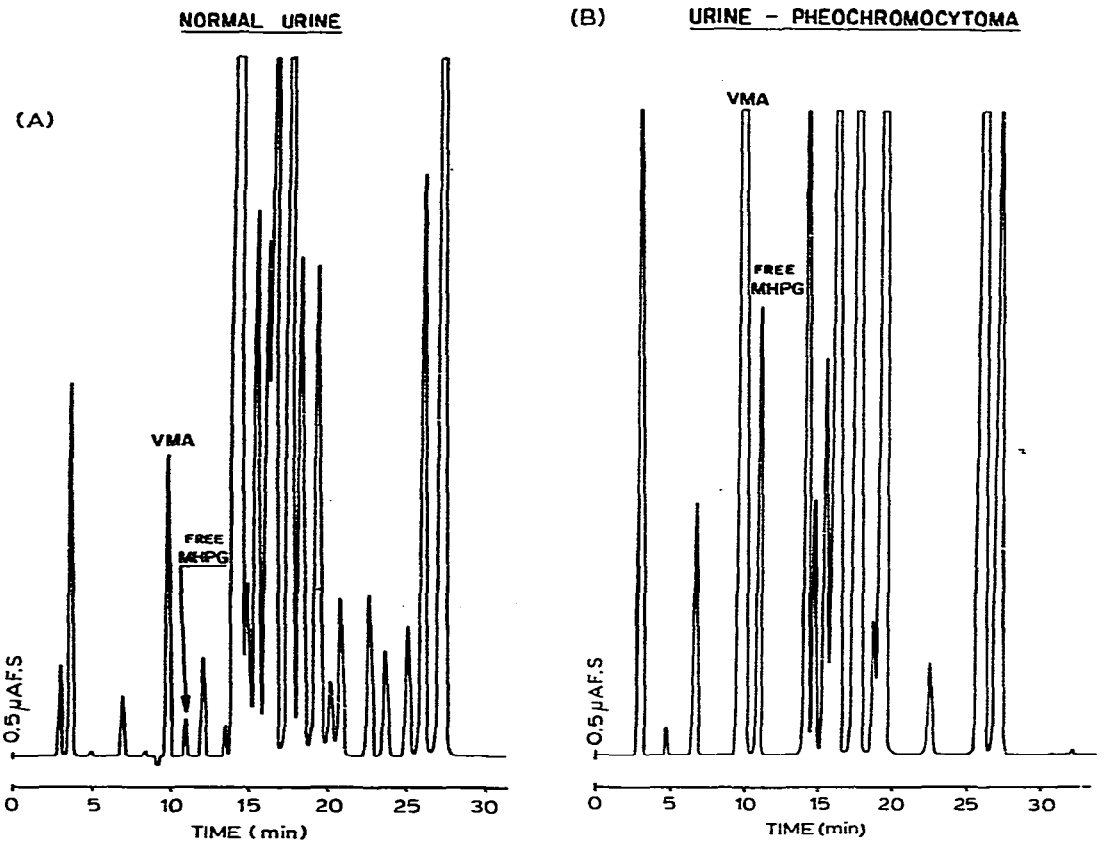


Fig. 16. (A) Chromatogram of the ethyl acetate extract of a urine sample from a healthy subject. Volume injected, 5 μl (15 μl urine); column, μBondapak C_{18}; eluents, (low-strength) 0.1 *M* KH_2PO_4, pH 2.50, (high-strength) acetonitrile—water (3:2, v/v); gradient, linear from 0% to 60% of the high-strength eluent in 45 min; flow-rate, 1.4 ml/min; temperature, ambient; detection, EC, + 1.00 V vs. Ag/AgCl. (B) Chromatogram of the ethyl acetate extract of a urine sample from a patient with clinically diagnosed pheochromocytoma. Chromatographic conditions as same as in (A). (Reproduced from ref. 9 with permission.)

Fig. 17 illustrates the enormously elevated levels of metanephrines in the urine sample from a patient with pheochromocytoma (B) compared to the control subject (A). However, urinary metabolite levels reflect both the CNS and peripheral metabolism. Therefore, the least invasive method for the assessment of monoamine metabolism in the CNS is the analysis of the CSF levels of catecholamines or their metabolites. Fig. 18 illustrates the concurrent

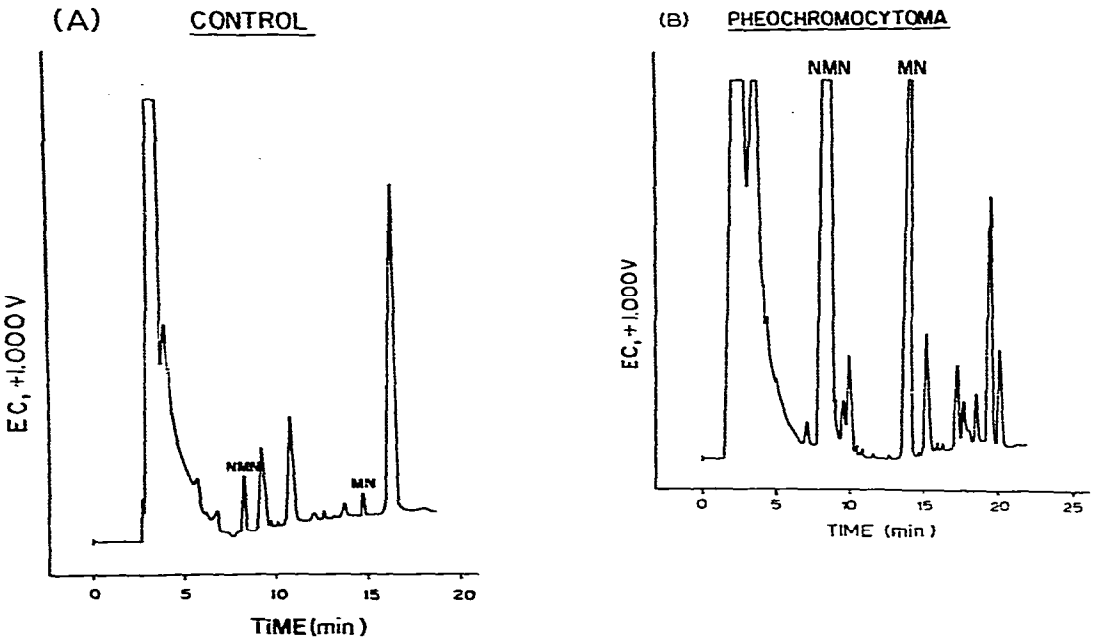
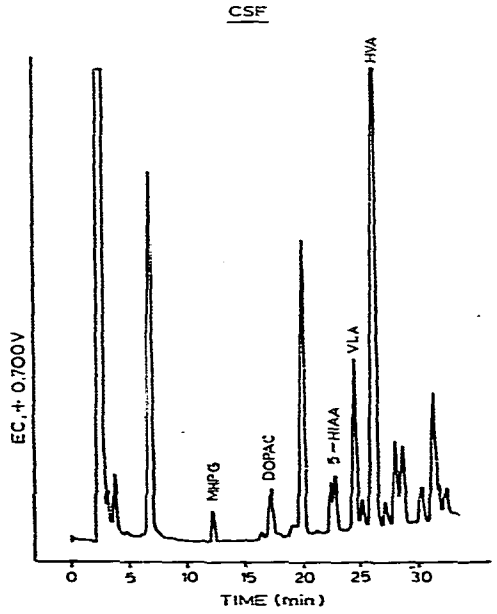


Fig. 17. (A) Chromatogram of the ammonia eluate from urine specimen of a control subject. Volume injected, 20 μl. Chromatographic conditions: column, Ultrasphere ODS; eluents, (low-strength) 0.1 *M* KH_2PO_4, pH 4.56, (high-strength) methanol—water (3:2, v/v); gradient, linear from 0% to 60% in 35 min; flow-rate, 1.0 ml/min; temperature, ambient; detection, EC, + 1.00 V vs. Ag/AgCl; sensitivity, 50 nA; attenuation, × 256. (B) Chromatogram of ammonia eluate from urine specimen of a patient with pheochromocytoma. Volume injected, 15 μl. Chromatographic conditons same as in (A). (Reproduced from ref. 7 with permission.)



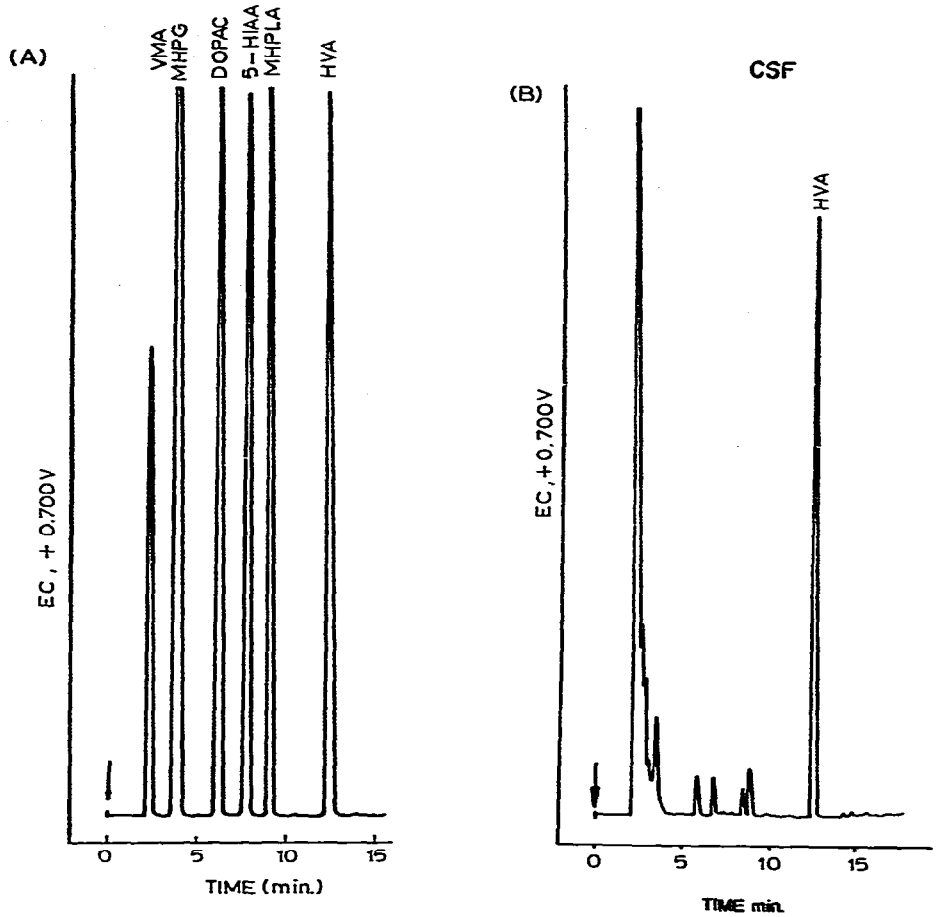


Fig. 19. Chromatogram of synthetic mixture of reference compounds analyzed under isocratic conditions. Chromatographic conditions: column, Ultrasphere ODS (5 μm average particle size); eluent, 25% methanol in 0.08 *M* KH_2PO_4, pH 2.50; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at + 0.700 V vs. Ag/AgCl; detector sensitivity, 75 nA full scale. (B) Chromatogram of the ethyl acetate extract of a CSF sample under isocratic conditions. Chromatographic conditions same as in (A). Volume of the extract injected: 60 μl (180 μl of CSF). (Reproduced from ref. 72 with permission.)

separation of several acidic components of the ethyl acetate extract of an unselected diagnostic specimen of human lumbar CSF. Because of the differences in acidities of various metabolites under study, a gradient elution mode of reversed phase was used. In diseases which involve a derangement in only

Fig. 18. Chromatogram of the ethyl acetate extract of a sample of human lumbar CSF. Volume of the extract injected, 60 μl (180 μl of CSF). Chromatographic conditions: column, Ultrasphere ODS (5 μm average particle size); eluents, (low-strength) 0.10 *M* KH_2PO_4, pH 2.50, (high-strength) methanol—water (3:2, v/v); gradient, linear from 0% to 100% of the high-strength eluent in 35 min; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at + 0.700 V vs. Ag/AgCl; sensitivity, 75 nA full scale. (Reproduced from ref. 72 with permission.)

one neurochemical substance, it is often desirable to monitor one specific compound. For example, in Parkinson's disease, the DA metabolism is of interest and, thus, a rapid, isocratic analysis of HVA is needed. The separation of a synthetic mixture of acidic metabolites, optimized for HVA, is shown in Fig. 19A. The same analysis was applied to the determination of HVA in a CSF sample and a representative chromatogram is shown in Fig. 19B.

The determination of catecholamine levels in the brain using reversed phase with EC detection has been the focus of many investigations [52,74]. The reversed-phase ion-pair separation of a synthetic mixture of reference compounds, and the components of an alumina extract of a rat brain are shown in Fig. 20A and B, respectively. Under the chromatographic conditions used, ascorbic acid and dihydroxyphenylglycol are well resolved from NE and the total analysis time is approximately 10 min. EDTA was reported to be an essential component of the mobile phase, since it decreases dramatically the spread of the solvent front, presumably due to complexation of metal ions leached by the acid from the syringe needle, the injector and the pumping system [41].

The combination of liquid chromatography with electrochemistry is rapidly becoming a method of choice for the determination of catecholamine biosynthetic enzymes, such as COMT [22,50], dopamine-β-hydroxylase [50],

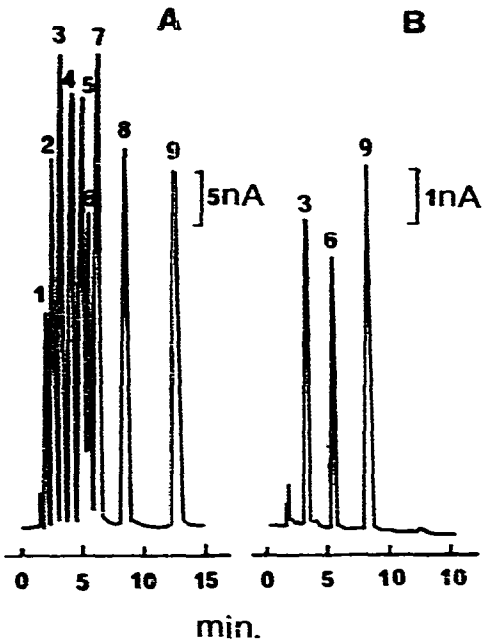
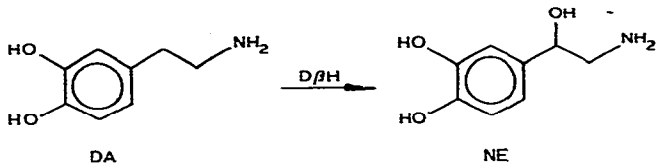


Fig. 20. Chromatogram of reference compounds (A) and of catecholamines from adult rat whole brain with DHBA added as internal standard (B). Chromatographic conditions: column, Ultrasphere ion-pair C_{18}; eluent, 0.1 *M* KH_2PO_4 (pH 3.0)—methanol (9:1), 0.2 m*M* sodium octyl sulfate, 0.1 m*M* EDTA; flow-rate, 1.5 ml/min; temperature, ambient; detection, EC, +0.72 V vs. Ag/AgCl. Peaks: 1, ascorbic acid; 2, dihydroxyphenylglycol; 3, norepinephrine; 4, epinephrine; 5, hydroxymethoxyphenylglycol; 6, dihydroxybenzylamine; 7, normetanephrine; 8, dopamine; 9, dihydroxyphenylacetic acid. (Reproduced from ref. 41 with permission.)

aromatic L-amino acid decarboxylase [59], DOPA decarboxylase [73], etc. The utility of this approach will be illustrated with one example. Dopamine-β-hydroxylase (DβH), which is found in the adrenal medulla and the storage vesicles of noradrenergic neurons, is involved in the terminal step in the biosynthetic pathway of NE:



(7)

The general approach to the assay for both DβH and COMT is shown in Fig. 21; the sample is incubated with the enzyme substrate, DA, the reaction stopped with an acidic protein precipitant, and the product, NE, isolated by alumina adsorption. The substrate, DA, must be added in large excess in order to ensure zero order kinetics and the enzymatically generated NE is measured. In order to avoid saturation of the detector and overloading of the column with a large amount of unreacted DA, Davis and Kissinger [51] have used a novel approach, split column chromatography. Two columns were used with a valve between them, and NE was allowed to pass through both columns. DA was arrested on the first column by switching the valve and shunt to waste. This method is possible only if the two compounds are well separated, which can be achieved by careful selection of the mobile phase and the columns.

An example of the use of this technique for the determination of DβH activity in human serum is shown in Fig. 22.

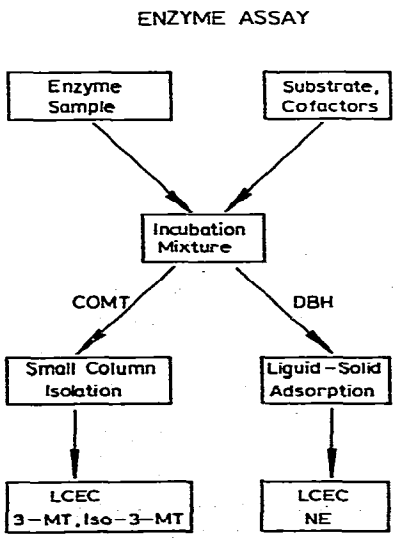


Fig. 21. General approach for the determinations of activities of COMT and DβH. (Reproduced from ref. 12 with permission.)

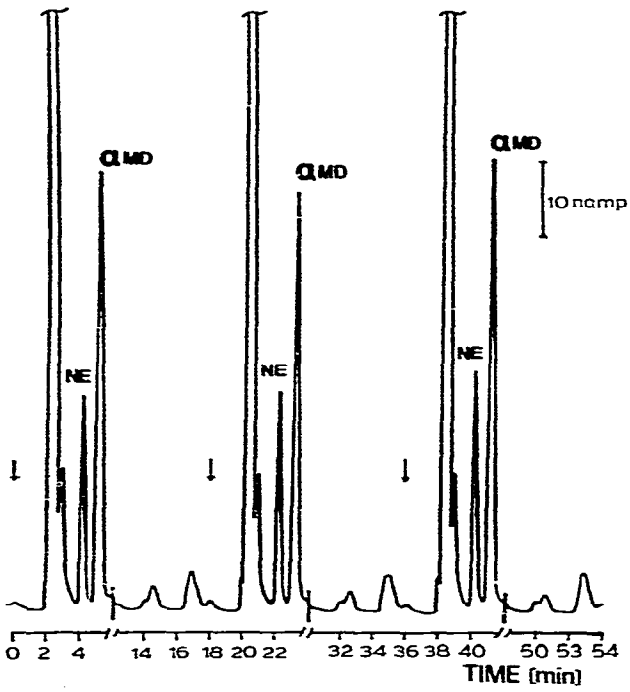


Fig. 22. Chromatograms illustrating the within-run reproducibility for the determination of serum DβH activity. The product, NE, is easily separated from the internal standard, α-methyldopa (αMD). The break in the time axis indicates where the column switching was employed. Chromatographic conditions: column, two RP-18; mobile phase, phosphate—citrate buffer (pH 4.4) with 23 mg of sodium octyl sulphate and 35 ml of methanol added to each liter of buffer; flow-rate, 1.3 ml/min; detection, EC, +0.750 V vs. Ag/AgCl. (Reproduced from ref. 51 with permission.)

6. SUMMARY

High-performance liquid chromatography, particularly in its reversed-phase mode, coupled with electrochemical or fluorometric detection, is becoming increasingly popular as an analytical tool for metabolic profiling of substances of neurochemical interest, such as catecholamines and their metabolites. During the last decade, a continued effort has been made to improve and simplify the analytical methodology for routine use in clinical laboratories where this technique is tremendously needed. New developments in column technology, reliable detectors, simplified sample cleanup procedures, and particularly better understanding of the complex physicochemical phenomena underlying the operation of electrochemical detection, have resulted in a steady and encouraging progress.

The purpose of this review was to describe the current analytical methodology and recent applications of HPLC in the field of catecholamine metabolism. Although this discussion is by no means detailed and complete, it, at least, hints at the impact of this technique on biochemical investigations and its future potential in clinical laboratories.

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