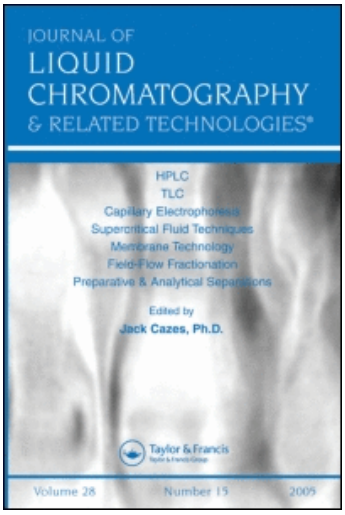

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Catecholamines by HPLC

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To cite this Article Allenmark, Stig(1982) 'Analysis of Catecholamines by HPLC', Journal of Liquid Chromatography & Related Technologies, 5: 12, 1 — 41
To link to this Article: DOI: 10.1080/01483918208082214
URL: http://dx.doi.org/10.1080/01483918208082214

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(Suppl. 1), 1-41 (1982)

ANALYSIS OF CATECHOLAMINES BY HPLC

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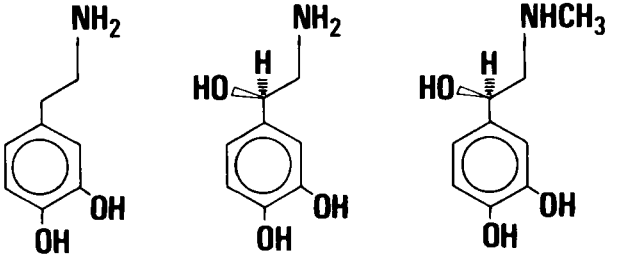
INTRODUCTION

The catecholamines (CA) constitute a class of biogenic amines which have long been known to possess hormonal activity and to be compounds biosynthesized via the tyrosine metabolic pathway. Epinephrine (adrenaline) (E), the first hormone was, in fact, isolated by Takamine as early as 1901 from the adrenal medulla (1). The chemical structures were also eluci-



2-(3,4-dihydroxyphenyl)ethylamine; dopamine (DA)

R-(-)-1-(3,4-dihydroxyphenyl)-2-aminoethanol; norepinephrine (NE)

R-(-)-1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol; epinephrine (E)

SCHEME 1. Chemical structures of the catecholamines

dated early and all shown to be derivatives of catechol. The absolute configuration of the biologically active forms of NE and E was later shown to be R (2,3).

From the chemical point of view, the catechol ring structure, because of its easy oxidative conversion, makes these compounds rather sensitive to air and light, especially in alkaline solution where they are rapidly oxidized spontaneously. The amphoteric properties of the CA's, due to the presence of amino- as well as phenolic groups, make conventional extraction procedures, i.e. from an acid phase, difficult because of the water solubility of the ionic species obtained. Ion pair or cation-exchange extraction techniques may be used, but fortunately more selective extraction methods exist, which are based on the ability of the 3,4-dihydroxyphenyl moiety to form cyclic complexes with boric acid or alumina, and which will be treated in more detail later.

The catechol ring gives rise to UV absorption maxima at 200-220 nm and 280-300 nm, respectively. Irradiation at either of these maxima gives rise to weak native fluorescence (λ_{emiss} = 310-340 nm).

The primary amino group in DA and NE is an excellent nucleophile in reactions producing highly fluorescent derivatives by the use of reagents such as fluorescamine, dansyl chloride or o-phtalaldehyde. Oxidation of the catechol ring, e.g. by the action of potassium ferricyanide and alkaline ascorbate, produces trihydroxyindole (THI) derivatives with high fluorescence quantum yields; a reaction that has been widely used for analytical purposes, but which is not well applicable to DA.

Prior to the introduction of the HPLC-methodology in the early 1970's most methods used or attempted for the estimation of CA's in biological material were based on extraction onto alumina followed

by elution and conversion to THI-derivatives and selective fluorescence measurements (4,5), column chromatographic separation prior to analysis (6-11), paper- or thin-layer chromatography (TLC) with pre- or post-chromatographic derivatization (12,13) and, last but not least, gas chromatographic (GLC) separation methods involving different types of derivatization reactions for the use of flame ionization (14,15), electron capture (16-21) or masspectrometric (20-23) detector systems. None of these methods can be said to fulfill all requirements for a good analytical procedure because they are either too complex and laborious giving low recoveries, or lack selectivity or sensitivity.

Some GLC-results from studies of biological material (24) are also, in the light of later investigations, completely erroneous and have recently been severely criticized (25). The GC-MS methodology, however, has been shown during the last years to provide the selectivity and sensitivity necessary for CA-analyses of biological specimens, and a high-resolution mass fragmentographic technique was reported recently to be a successful tool for quantitation of CA's in plasma (26).

In 1970 a radioenzymatic method, based on the enzyme-catalyzed introduction of a radiolabel (^{3}H or ^{14}C) via 3-O-methylation of the CA's and concomitant TLC-separation and use of liquid scintillation counting for quantitation was introduced (27). This method, which later has been further developed and improved (28,29), is of great importance due to its extreme sensitivity despite the disadvantages with regard to complexity, labour and cost.

A recently published review by Krstulovic (30) covers the methods other than HPLC in use for the analysis of CA's in more detail than what has been possible or appropriate to include in this introductory section.

Since HPLC-methods, generally speaking, are favourable for the separation and analysis of polar, hydrophilic compounds, it is not surprising that HPLC has made a strong impact on CA-analysis as seen from the explosive increase in the number of papers published in this field. In the following, the various factors of importance in CA-analysis by means of HPLC will be treated and a survey of existing HPLC-methodology, biomedical and related applications as well as possible future trends will be given.

CHROMATOGRAPHIC PROPERTIES OF CATECHOLAMINES. COLUMN AND MOBILE PHASE SELECTION

Numerous investigations have dealt with the retention behaviour of biogenic amines in various liquid chromatographic systems. Due to the polar nature of these compounds, particularly the CA's, it is possible to utilize quite different retention mechanisms for separation, in most cases with the use of aqueous buffer systems as the mobile phase. The various conditions that have been used are summarized in Table 1.

The division into different types of retention mechanisms given in Table 1 should not be taken too categorically and deserves a comment. It is always difficult to know whether a single type of equilibrium between the stationary and mobile phases in a column is the sole factor governing retention of a given compound. Further, many different models for the retention process in ion-pair chromatographic systems have been proposed and the borderlines between what has been called here hydrophobic interaction, dynamic ion-exchange and ion-pair reversed-phase partition are, in fact, not very clear-cut. In fact, because chromatography measures only equilibria and not kinetic properties, it is unfortunately impossible to deduce any real mechanism from chromatographic data. Nevertheless, this classification should be useful in the following discussion of the retention behaviour of CA's under various chromatographic conditions and will be explained in further detail below.

TABLE 1

Examples of Different Liquid Chromatographic Systems Used for the Separation of Catecholamines

Retention mechanism	Column type/ stationary phase		Mobile phase	Elution order (retention)	Ref.
Cation exchange	Silica-bonded phase (SO_3H) material	Nucleosil SA 10μ	Acetate/citrate buffer, pH 5.2	NE < E < DA	37
		Nucleosil SA 5μ			38,39
Hydrophobic interaction	Octadecyl silica	μBondapak C-18	0.17 M acetic acid	NE < E < DA	42
"Dynamic cation exchange"	Octadecyl silica	μBondapak C-18	0.09 M sodium octyl sulphate in citrate/ phosphate buffer	NE < E < DA	44
Ion-pair reversed phase partition	Octadecyl silica	Lichrosorb RP-18 10μ/ 1-pentanol	10-30 mM octyl sulphate in phosphate buffer pH 3.0 with 1.15% 1-pentanol	NE~E < DA	48
Ion-pair straight phase partition	0.1 M perchloric acid on spherical silica gel 10μ		tributyl phosphate-hexane (65:35)	DA < NE < E	50
	0.2 M perchloric acid/ 0.8 M sodium perchlorate on spherical silica gel 10μ		butanol - methylene chloride (40:60)	DA < E < NE	50

Cation exchange HPLC represents the most obvious and straightforward method. Retention should be controlled only by differences in net charge at the buffer pH and ionic strength used. The compounds elute therefore in order of increasing basicity. It also has the advantage that acidic and neutral compounds should elute in the void volume. It has the drawbacks, however, that it is often difficult to obtain a reasonably high operating column efficiency (say $H < 0.05$ mm), peak tailing is a frequent problem and the adsorbent may be prone to chemical degradation, especially if a slightly elevated column temperature is used. Another problem is due to the fact that the batch-to-batch variation in the properties of the ion exchanger is so great, that chromatographic retention parameters under otherwise identical conditions are not comparable unless material from one single batch is used throughout.

Most frequently strong cation-exchange materials (containing sulphonic acid groups) have been used together with purely aqueous buffer systems at pH-values between 2 and 6. Much work has been performed with the use of long glass columns (mostly in combination with electrochemical detection) dry-packed with resins such as Zipax SCX (DuPont) (31,32), Vydac SCX (The Separations Group, Hesperia, Calif.) (33-35) and Corasil CX (Waters) (36). Although adequate separation for the given purpose can generally be achieved by a proper adjustment of the mobile phase, the column efficiency obtained was for the most part very low. Despite the problems mentioned earlier, significantly better column efficiencies are obtained with the use of slurry-packed steel columns and small particle diameter (5-10μ) cation-exchange materials such as Partisil-10 SCX (33), Nucleosil SA 10μ (37) or Nucleosil SA 5μ (38,39).

The retention behaviour of the CA's and their metabolites on octadecyl-silica stationary phases on elution with purely aqueous buffers of varying pH and ionic strength was thoroughly investigated by Molnar and Horvath (40). Under these conditions acid

catechols are retained as well and retention is effected by hydrophobic interactions between the stationary phase and the hydrophobic part of the compound in question. The authors used phosphate buffers throughout their studies. It has been suggested that ion-pairing of the amines is the reason for the increase in retention, peak symmetry and column efficiency observed (41) on the addition of certain acids to the mobile phase. Irrespective of the mechanism responsible for this phenomenon, the effect on the retention can be substantial as shown in Table 2. It is readily seen that retention of the amines increases with increasing hydrophobicity of the added counterion.

Especially striking is the effect produced by the presence of an alkylsulphonate ion. This method of regulating retention, introduced by Knox and Jurand (43) and termed "soap chromatography", was rapidly adopted as a powerful new technique for use in HPLC of biogenic amines. The chromatographic retention mechanism can be thought of according to two extreme models, one in which an alkylsulphonate ion-pair of the amine is retained by the hydrophobic stationary phase, and another in which the alkylsulphonate covers the stationary phase by hydrophobic interaction and converts it to a cation-exchanger on which retention of the amine can take place - therefore the term "dynamic ion-exchange". The method has been successfully applied to HPLC-separations of CA's in many laboratories in recent years. A detailed investigation on the effect of different modifiers (sodium octadecyl - as well as octyl sulphate), solvent systems and stationary (C-18) phases appeared recently (45). The effect of sodium dodecyl sulphate on the retention of the CA's and other biogenic amines in the presence of some acid metabolites has also been thoroughly investigated (46).

A related technique, ion-pair reversed phase partition, was introduced by Schill (47,48) and applied to alkyl-silica supports for the separation of biogenic amines (48,49). Here, the column is coated with an organic solvent such as 1-pentanol or methylene chloride, present in a few per cent in the aqueous mobile phase containing the alkylsulphonate or other suitable counterion. Retention of the amines is sup-

TABLE 2

Effect of Various Acids on the Retention Behaviour of Catecholamines and Related Compounds. Conditions: Column: 3.9 x 300 mm μBondapak C-18 (Waters Assoc.), 0.1 M acid in water, pH 3.0. (Abbreviations: MNE:α-methylnorepinephrine, DHBA: 3,4-dihydroxybenzylamine, DOPA: L-3,4-dihydroxyphenylalanine, MD:α-methyl-DOPA, CD: carbidopa (2-hydrazino-3-(3,4-dihydroxyphenyl)-2-methyl propionic acid), MDA:α-methyldopamine, DOPAC: 3,4-dihydroxyphenylacetic acid).

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Compound	Capacity ratios, k'					
	HNO_3	H_2SO_4	CH_3CO_2H	H_3PO_4	TCA	OS*
NE	0.4	0.3	0.2	0.3	1.8	2.5
MNE	0.8	0.6	0.3	0.6	3.2	4.7
E	1.0	0.7	0.4	0.7	4.3	5.7
DHBA	1.2	0.8	0.5	0.8	5.1	7.9
DOPA	1.4	1.3	0.9	1.2	3.7	3.7
DA	2.2	1.5	0.9	1.6	9.5	15.3
MD	3.7	3.3	1.8	3.1	11.3	-
CD	5.3	4.7	2.6	4.4	-	16.3
MDA	6.2	4.2	2.4	4.3	27.7	-
DOPAC	11.2	11.2	7.4	11.2	10.3	8.4

*0.05 mM sodium octyl sulphate in 0.1 M nitric acid

posed to be governed by means of ion-pair partitioning between a stationary liquid organic phase and the aqueous mobile phase.

Ion-pair partition chromatography of CA's has also been performed in the straight-phase mode (50). In this case a silica gel column impregnated with perchloric acid/sodium perchlorate served as the

stationary phase. Elution with organic mobile phases such as tributyl phosphate - hexane (65:35) or butanol - methylene chloride (40:60) gave the elution orders shown in Table 1.

In summary, there are many successful ways to achieve a good HPLC-separation of CA's and the best choice for a given analytical purpose will depend on factors such as the detection method used, interfering compounds in the sample etc. In general, however, due to the often extremely low concentrations that have to be analyzed, chromatographic systems based on purely aqueous systems, isocratic elution and high column efficiency and selectivity should be advantageous.

METHODS FOR THE DETECTION OF CATECHOLAMINES IN COLUMN EFFLUENTS

A. UV-detection

The use of UV-detection for the analysis of CA's is strongly hampered by its relative insensitivity. The practical limit of detection under optimal conditions can be roughly estimated to 0.01 nmol (S/N = 2). For many biological samples of interest this is far from sufficient.

B. Fluorimetric detection

Fluorimetric detection has been used both with and without derivatization of the CA's. The use of fluorimetric recording of CA's and other biogenic amines utilizing their native fluorescence by excitation at 285 nm and monitoring the 340 nm emission is described in a recent paper (51). With a stopped-flow scanning technique spectra of the various peaks could also be recorded and used for identification purposes. Schüsler-van Hees et al claim that a detection limit of 10 nM (= ca 20 pg) can be obtained on detection of native fluorescence making use of λ_{exc} = 284 and λ_{emiss} = 316 nm (52). This high sensitivity seems questionable,

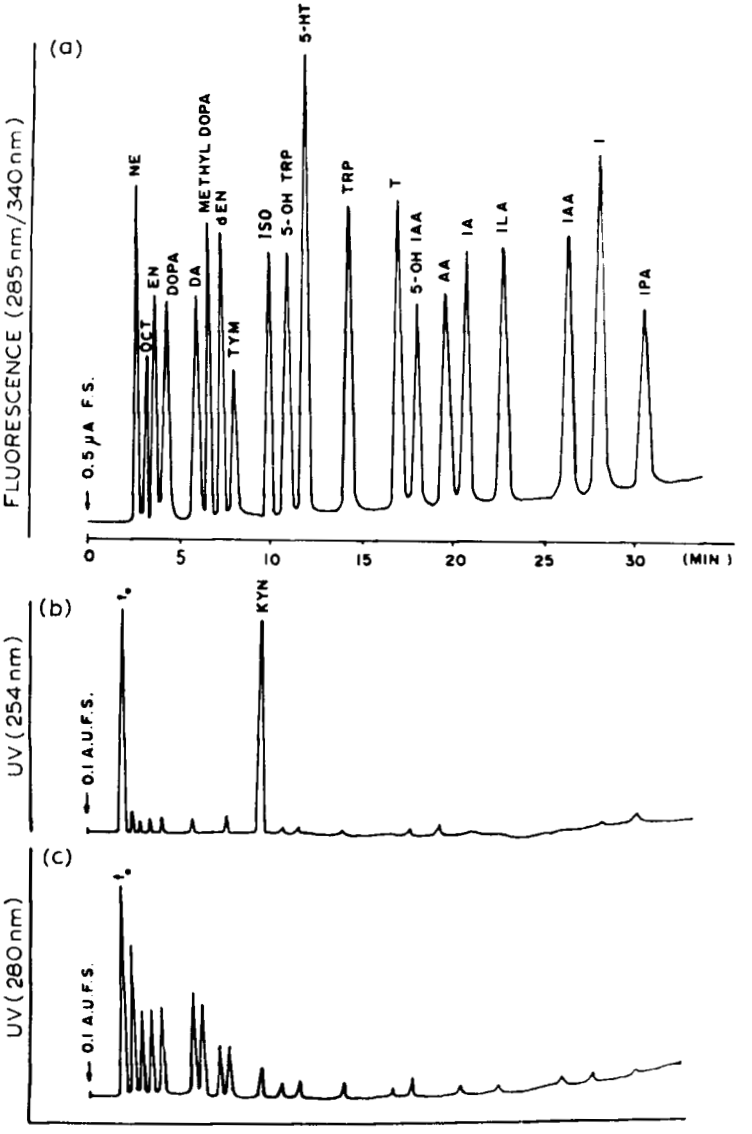


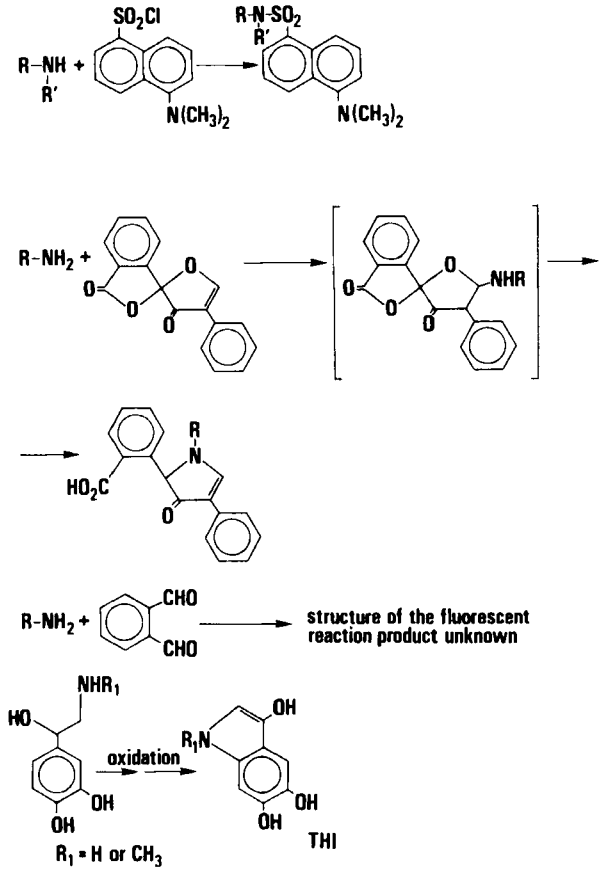
FIGURE 1. Illustration of the detection of biogenic amines in HPLC using their native fluorescence. (Reproduced from ref. 51 with the permission of the authors and the publisher.)

however, in view of other reports. Thus, Jackman et al (53) found optimal detection conditions on excitation at 200 nm and report the detection limits NE = 100 pg and DA = 300 pg.

A higher sensitivity can be achieved, however, if the amines are derivatized before their fluorimetric analysis. This may be done either before the HPLC-separation as a precolumn derivatization or in a postcolumn reactor just before the detector inlet. The main disadvantage of all precolumn derivatization methods is the laborious pretreatment of the sample involving extraction with an organic solvent, a procedure which seems difficult to automatize and the effectiveness of which has to be rigorously checked by internal standardization procedures. Some common derivatization procedures are summarized in Scheme 2.

The reagents most commonly used for precolumn derivatization of the biogenic amines are o-phtalaldehyde (OPA), dansylchloride (DNS-Cl) and fluorescamine (FA). OPA-derivatives have been reported (54,55) to possess excellent stability and to be well separated on reversed phase HPLC under isocratic conditions. The HPLC separation of DNS-derivatives has been studied by Schwedt (56-58) and by Frei (59). The use of fluorescamine for precolumn derivatization prior to HPLC has been extensively investigated by Imai (60-62).

In all these derivatization methods a primary amino group in the substrate is required and they are therefore applicable only to NE and DA as far as the CA's are concerned. Imai found that the very polar nature of the derivatives obtained with fluorescamine made them migrate too fast on ordinary reversed-phase columns and therefore a glycol type gel (TSK LS 160 gel, 5 or 10 μ) was used. Schwedt, using a straight-phase HPLC-system, obtained two peaks corresponding to NE (63).



SCHEME 2. Commonly employed reactions for the transformation of catecholamines into fluorescent derivatives

In postcolumn fluorogenic derivatization methods a reactor, placed between the column outlet and the fluorimetric detector, will convert the amines to highly fluorescent reaction products. To fully utilize this principle, the reactor must have a low dead-volume to prevent band broadening but also allow for an intimate mixing of the reaction partners and a sufficiently long reaction time. Extensive studies have been carried out on three different post-column reactions, viz. the THI-, the OPA- and the FA-reactions. The last

reaction, however, which has been successfully applied to analysis of amino acids, as well as both primary and secondary amines, has not been used for post-column detection of CA's to the best of my knowledge.

The first application of the THI-method subsequent to HPLC separation of CA's was carried out by Mori and reported in a series of papers (64,65). The method was further studied by Schwedt (66) and others (67,68) whereby different modes of HPLC-separation were tried. Quite recently Yui et al (69-71) reported on a cation-exchange HPLC system allowing the determination of NE and E in amounts down to a few picograms. Unfortunately, the authors have not incorporated DA in their investigations. Okamoto et al, however, have shown that all three CA's as well as DOPA can be analyzed simultaneously by the THI method of detection (67).

Post-column derivatization with OPA is an alternative highly sensitive detection method which has the advantage that biogenic primary amines in general can be detected but the disadvantage with respect to CA-analysis that E will be excluded. Another factor of importance is that the reaction time required is much shorter (20 sec as compared to 4 min for the THI-method under optimal conditions (67)).

C. Electrochemical detection

Since the first report in 1973 by Adams and coworkers (72) on the successful use of an electrochemical flow cell as a detector component for liquid chromatography systems, there has been an explosive development within this area of research. The detection principle is illustrated in Fig. 2. The column effluent is passed over the anode surface of the detector flow cell to which any compound which is electrooxidizable at the potential chosen can lose one or more electrons and become transformed (reversibly or irreversibly) into an oxidized reaction product. For the CA's this electro-

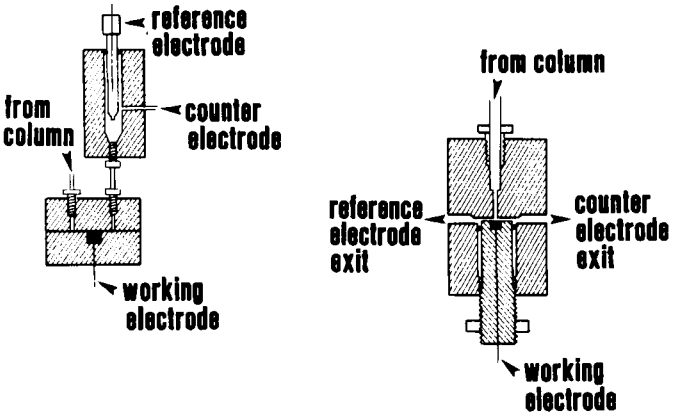


FIGURE 2. The principle for electrochemical detection in a flow cell. Thin-layer type (left), wall-jet type (right).

chemical oxidation primarily means a two electron reaction to yield an o-quinoid structure. It is important to keep in mind that all electrochemical detectors work by converting (chemically and electrochemically) a part of the compound to be analyzed to a product and consequently should be regarded as a kind of post-column reactors highly dependent upon the reaction conditions in the cell. Thus, not only the anode potential, but also the mobile phase composition, pH, ionic strength etc. are factors that greatly affect the detector response. Such influences on the electrochemical detection of CA's were thoroughly studied by Moyer and Jiang (73) and an illustration of their results is given in Fig. 3.

A widely used cell type, originally introduced by Adams et al (72), is the thin-layer flow cell housing a graphite paste anode in the bottom cell half and connected to an Ag/AgCl reference and an auxiliary electrode in a three electrode configuration. This system has been explored and further developed by Kissinger and his collaborators in a series of papers (74-77). A modification of the cell is equipped with a glassy carbon electrode material which is more resistant to organic solvents.

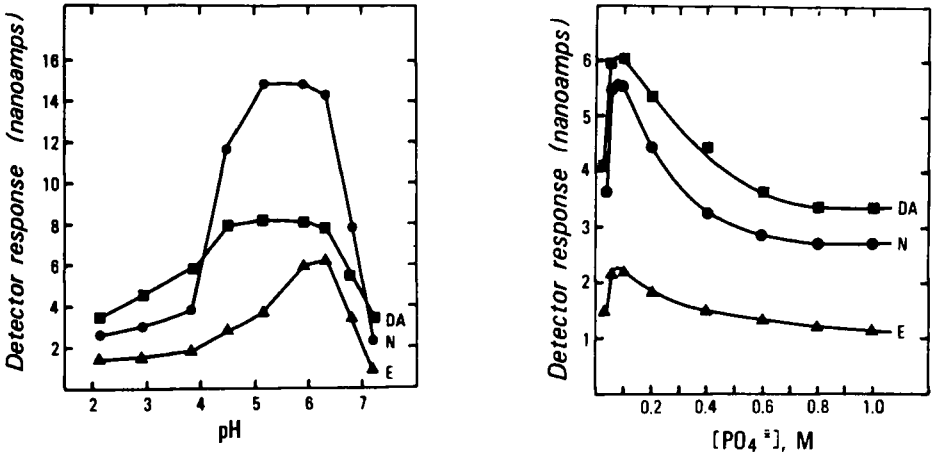


FIGURE 3. Influence of a) pH and b) ionic strength on the amperometric detector response at constant anode potential (+0.5 V vs. Ag/AgCl). (Reproduced from ref. 73 with the permission of the authors and the publisher.)

Another principle is used in the wall-jet type of detector cell (78), Here the column effluent is directed perpendicularly to the anode surface. These types of cells have been assumed to give a higher coulometric yield in general, due to the hydrodynamic properties and are usually equipped with a glassy carbon electrode. Detailed descriptions of the construction of such cells have recently appeared (78,79).

Lankelma and Poppe (80) have thoroughly investigated the requirements necessary for a high linear dynamic range (10^6), high coulometric yield and low noise level of a detector cell. Using a large vitreous carbon anode separated by a 50 μ spacer from the counter electrode they achieved a 97% coulometric yield at a flow rate of 20 ml/h.

Detector cells of the wall-jet type with a rotating disc carbon paste or vitreous carbon electrode were recently introduced by Brunt

(81-83). Despite the larger cell volume in this arrangement, the coulometric yield is highly increased in these cells due to the rotation of the electrode. This reduces the thickness of the diffusion layer, resulting in a significantly increased detector response.

A comparison of the performance of EC-detectors used with HPLC for the determination of CA's and some related compounds recently appeared (84).

D. Other methods of detection

A variant of the radioenzymatic method of CA-analysis (27-29) makes use of HPLC-separation of the enzymatically generated, radiolabelled 3-O-methyl-derivatives that are subsequently determined quantitatively by liquid scintillation counting. Thus, the technique can be regarded as a precolumn derivatization prior to HPLC. Klaniecki et al (85) used a cation-exchange column with an ammonium phosphate buffer, pH 8.5, containing 1% acetonitrile as the mobile phase for the separation and obtained an order of increasing retention: NMN < MOT < MN*. The limit of detection was given as 9, 17 and 19 pg/ml for NE, E and DA, respectively. The HPLC-separation has also been carried out with the use of reversed-phase systems (86-89). A limit of detection of ca 1 pg has been claimed (88).

A study of the detection limits for the various methods of analyzing CA's in effluents from HPLC columns was published recently (90) and the results are shown in Table 3.

---

*NMN = normetanephrine, MOT = 3-methoxytyramine, MN = metanephrine

TABLE 3

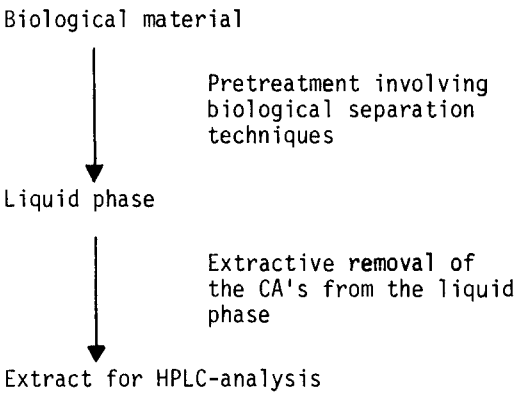
An Estimate of the Detection Limits for CA's Obtained so far in HPLC with Different Methods for Column Effluent Analysis

(Reproduced from ref. 90 with the permission of the authors and the publisher.)

Method	Limits of detection (pg)		
	DA	NE	E
1. Electrochemical	25	25	25
2. Ultraviolet	1250	1400	1200
3. Natural fluorescence	300	300	300
4. OPA post-column derivatization	130	75	-
5. THI	800	1	1

TECHNIQUES FOR THE ISOLATION OF CATECHOLAMINES FROM A BIOLOGICAL MATRIX

The preparation of a sample from a biological source as a homogeneous solution of the species of interest and suitable for analysis by HPLC, represents an advanced bioanalytical problem of great chemical complexity at least of quantitative estimates are to be made. Each biological matrix offers different problems and a variety of methods for pretreatment of the biological material has been worked out. Therefore, the selection of a suitable method for processing the original material for CA-analysis by HPLC will be entirely dependent on the nature of the biological source. A general representation of the isolation techniques used is given below.



The complexity of this sample preparation procedure will also depend on the selectivity of the HPLC system used for analysis. For example, it was shown recently that rat brain endogenous biogenic amines could be analyzed, *in vivo*, by HPLC with a C-18 reversed-phase column and electrochemical detection without any "work-up" at all of the perfusate from the brain (91). Usually, however, an HPLC method for quantitative analysis of CA's cannot be applied, unless an extensive pre-purification of the biological material has been carried out. In the following some different methods used to achieve this will be briefly discussed.

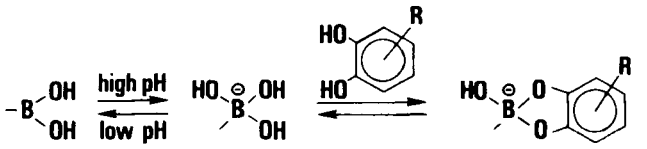
While the pretreatment of the biological material only has the purpose of giving a liquid phase suitable for an effective liquid-solid extraction of the CA's, the purpose of the extraction step is two-fold: 1) to isolate the CA's as selectively as possible, and 2) to obtain a preconcentration to a small volume suitable for injection into the chromatograph.

Of the liquid-solid extraction methods, viz. the use of a cation-exchanger, either in the column (92) or in the batch (93) mode, the use of acid-washed alumina (36) according to the method originally developed by Anton and Sayre (4,5), or the adsorption to a boric acid affinity gel (94-96), only the latter two techniques are

selective for the CA's in the sense that adsorption to the solid phase is governed by the presence of the o-dihydroxyphenyl functional group. The adsorption process, based on the reversible formation of cyclic boronates or aluminates, is outlined below. Accordingly, it is essential that acidic alumina (i.e. aluminium oxide that has been treated with a strong mineral acid) is used for this process.

In their exhaustive investigation on CA isolation via adsorption onto alumina Anton and Sayre found an optimal recovery at pH 8.6 (4), which was interpreted as the result of a competition between an increased stability at lower pH and a better adsorption at higher pH. Correct pH-adjustment is therefore an important factor in the alumina extraction method. In most cases it is also essential to add an antioxidant (usually sodium metabisulphite or ascorbic acid) and EDTA to protect the CA's during the work at this relatively high pH. Elution from the adsorbent has been effected by means of various mineral and organic acids (hydrochloric, perchloric or acetic acid). Acetic acid has the advantage that the extract may be easily concentrated by evaporation. A detailed description of the alumina extraction method as applied to brain tissue and urine with the aid of a centrifugal microfiltration technique giving almost 100% recovery with as little as 100 µl of eluent has recently been published (97).

The boric acid gel method for CA isolation has been applied to urine (96) as well as plasma (98) with excellent recoveries (81±5%) on elution from a column with 1.3 M acetic acid in methanol (98). It



SCHEME 3. Chemistry of the reversible adsorption of catecholamines to a boric acid affinity gel

was suggested that this isolation technique should be superior because pH adjustment before adsorption seems to be much less critical and a lower pH (7.5) can be used. A boronic acid substituted microparticulate silica adsorbent for potential use in high performance liquid affinity chromatography (HPLAC) of nucleosides, nucleotides and carbohydrates has recently been synthesized by Mosbach and collaborators (99).

With the rapid development of column switching techniques in HPLC it seems very likely that extraction and preconcentration of CA's from biological material can be performed "on line" with the use of an extractive HPLC-precolumn of the boronic acid type prior to the analytical column, using a column switching technique similar to the one described by Davis and Kissinger for the determination of serotonin in plasma (100).

QUANTITATIVE DETERMINATION OF CATECHOLAMINES IN BIOLOGICAL MATERIAL BY HPLC

A prerequisite for a quantitative analysis is the knowledge of two factors for each individual compound to be quantitated: a) the recovery from the biological source and b) the detector response factor. This latter factor is most often readily determined from injections of mixtures of the compounds in varying known concentrations and plotting the detector response in arbitrary units as a function of the concentration of each individual compound. A good detector system should give a linear concentration dependence which does not change with time. The recovery problem is usually solved by use of the internal standard technique and is often based on the assumption that the recovery of the internal standard and of all the compounds to be quantitated is the same. Neglection of differences in recovery may be acceptable only if the molecular structures and chemical behaviour are very similar, a fact which in HPLC may often cause problems with respect to resolution.

Recovery data for the individual CA's and the internal standard used are, however, readily obtained experimentally from extractions

of samples of known concentration. In many cases when samples of low concentration are analyzed, inter-assay recovery variations are found to be greater than the intra-assay differences between the individual compounds. Very useful, non-endogenous internal standards have been found in 3,4-dihydroxybenzylamine (DHBA), α-methyldopamine (MDA) and isoproterenol (N-isopropylnorepinephrine) (IP).

The validation of a method used for quantitative analysis is usually carried out in terms of relative standard deviation (RSD) or coefficient of variation (CV) for a series of repeated analyses on the same object which will give the over-all precision of the method. Large systematic errors may produce inaccurate results, however, even though the precision of the method is high. Therefore, it is very valuable if the same material can be repeatedly analyzed by means of two or more quite different methods.

A high sensitivity of a method, i.e. a low limit of detection, does not imply a high precision or accuracy. A less sensitive method may give more precise and accurate results over a broad concentration range, provided this lies far enough away from the detection limit. The situation may be roughly visualized as depicted in Fig. 4.

It is definitely true that HPLC in combination with a sufficiently sensitive and stable detector system is the tool of choice for quantitation of small amounts of CA's from biological material even compared to GC/MS. However, some aspects of the chromatographic system design for work at the "quantitative trace analysis" level should be noted.

A common problem in trace analysis is to reduce the background noise level as much as possible in order to reduce the detection limit at a given signal-to-noise ratio. A high background in itself can be acceptable as long as it is stable. In general, however, lowering of the background will also reduce the noise level. Therefore, in order to make full use of the most sensitive detector systems for HPLC,

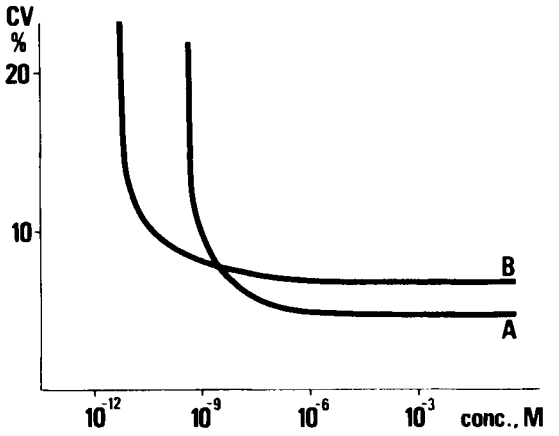


FIGURE 4. The coefficient of variation as a function of the analyzed concentration for two different methods A and B.

the fluorimetric and the electrochemical, very great demands are placed on the chromatographic system with respect to solvent purity, constant flow rate, stability of the stationary phase, etc. Therefore, from our own experience, it is advisable to use specially purified water (in case of electrochemical detection prepared by distillation from alkaline permanganate) for all aqueous solutions, including the mobile phase, to remove flow rate fluctuations from the pump by an external pulse dampening device, to use a mains voltage stabilizer, if an electrochemical detector is used, to reduce external field influences by means of a Faraday cage, and finally, to stabilize the whole system by continuous operation, i.e. being kept in a recycling mode with the detector on over-night.

SOME APPLICATIONS OF THE HPLC ANALYSIS METHOD TO PROBLEMS WITHIN BIOLOGY AND MEDICINE

A. Determination of catecholamine concentrations in plasma or serum

During the last few years considerable progress has been made in the field of plasma CA analysis and the "normal" levels in

humans have been more definitely ascertained, partly due to the demonstration of a good correlation between HPLC methods and radioenzymatic methods for NE and E (37,39,101). Goldstein et al (101) found the following normal plasma levels: NE = 311±53 (300±65) pg/ml and E = 57±18) (52±38) pg/ml, where the values within parentheses are those obtained by the radioenzymatic method. The result agrees very nicely with the value for NE = 342±30 reported by Davis and Kissinger (92). The latter, likewise using C-18 reversed-phase columns modified by the use of anionic surfactants, found difficulties in chromatographic overlap with uric acid which co-extracts with the CA's in the isolation procedure, a problem not mentioned by Goldstein et al. The values reported by Hjemdahl et al (37) and obtained in our laboratory (39), both with the use of cation-exchange HPLC and amperometric detection, are only slightly different: NE = 252 (254) and E = 75 (73) pg/ml (values within parentheses are from ref.39). The agreement with other HPLC-methods is, however, poorer. The present situation is summarized in Table 4. Figs. 5 and 6 are recent, illustrative examples of the appearance of chromatograms obtained with fluorimetric and electrochemical detection techniques, respectively.

B. Studies on brain tissue

Because the two primary amines DA and NE act as neurotransmittors, HPLC has come to play a dominant role as an analytical tool for brain tissue investigations. ECD is ideal for this purpose and the majority of papers published in this field during the last few years are based on the Adams-Kissinger technique first published in 1974 involving alumina extraction (31). In 1978 Kissinger and coworkers improved the resolution and over-all sensitivity of the method by the application of "dynamic ion-exchange" on a 10 μ C-18 reversed-phase column (103) and this technique has since been widely adopted in neurochemical research (104) and further modified to permit the simultaneous determination of E and DOPAC (3,4-dihydroxyphenyl-

TABLE 4

A Comparison of "Normal" Plasma Catecholamine Levels Found by Different Methods.
Abbreviations: ECD= electrochemical detection, GC-MS = gas chromatography - mass spectrometry, FD = fluorimetric detection, RE = radioenzymatic precolumn derivatization

Method	CA concentration (pg/ml)			
	NE	E	DA	Ref.
HPLC-ECD	311	57	-	101
HPLC-ECD	342	-	-	92
HPLC-ECD	252	75	34	37
HPLC-ECD	292	81	29	102
HPLC-ECD	254	73	60	39
GC-MS	297	-	-	98
HPLC-FD	185	32	-	70
RE-HPLC	355	61	-	89
RE-HPLC	182	87	33	85

acetic acid) as well (105,106). Recent papers describe a technique in which the alumina extraction step has been omitted (91,107,108).

A very good chromatographic resolution and a high sensitivity was obtained by Wagner et al (109) who were able to separate and quantitate the CA's and DOPA, DOPAC, as well as two aromatic amino acid decarboxylase inhibitors α-monofluoro- and α-difluoromethyl-DOPA, in brain and other tissue. The authors used a C-18 column with an 0.02 M citrate-phosphate buffer pH 3.2 modified with ca. 13% of methanol and 2.5 mM octane-sulphonic acid. Detection was achieved amperometrically by a wax-impregnated carbon paste anode at a working potential of +0.90 V.

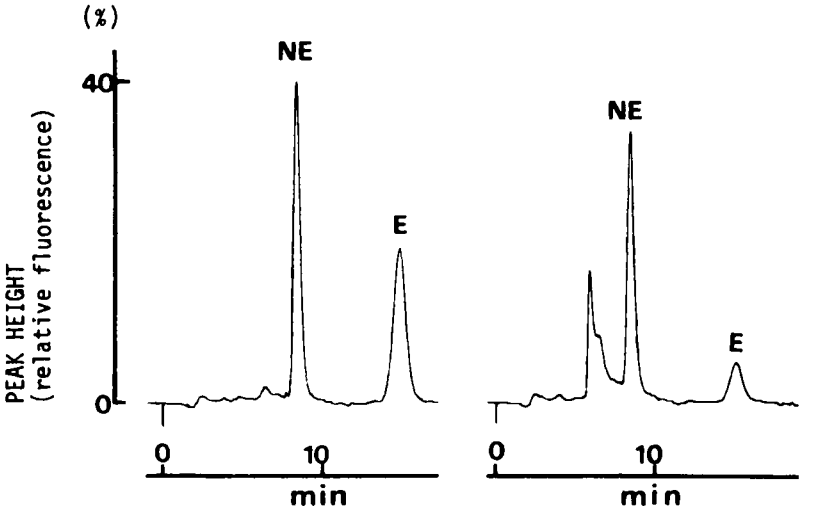


FIGURE 5. Chromatograms of CA standards (200 pg each of NE and E) (left), and an extract obtained from 1 ml of a human plasma sample (right). Conditions: glass column 2.1x1000 mm with Zipax-SCX, mobile phase 0.03 M Na_2HPO_4 containing 6% (w/v) of acetonitrile, flow rate 0.8 ml/min, post-column THI-reaction, fluorimetric detection 403/510 nm.

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A minireview covering applications of liquid chromatographic-fluorimetric systems in neurochemistry appeared recently (110).

C. Enzyme kinetic studies via catecholamine analysis by HPLC

Highly sensitive HPLC-methods of CA-analysis are ideally suited for the monitoring of certain enzyme activities related to CA production or transformation reactions. Thus, such methods have greatly facilitated the study of enzymes involved in the tyrosine metabolic pathway and continue to grow in importance. As examples may be given the assay of tyrosine hydroxylase (TH) by the use of ECD described by Blank and Pike (111) and by Nagatsu et al (112),

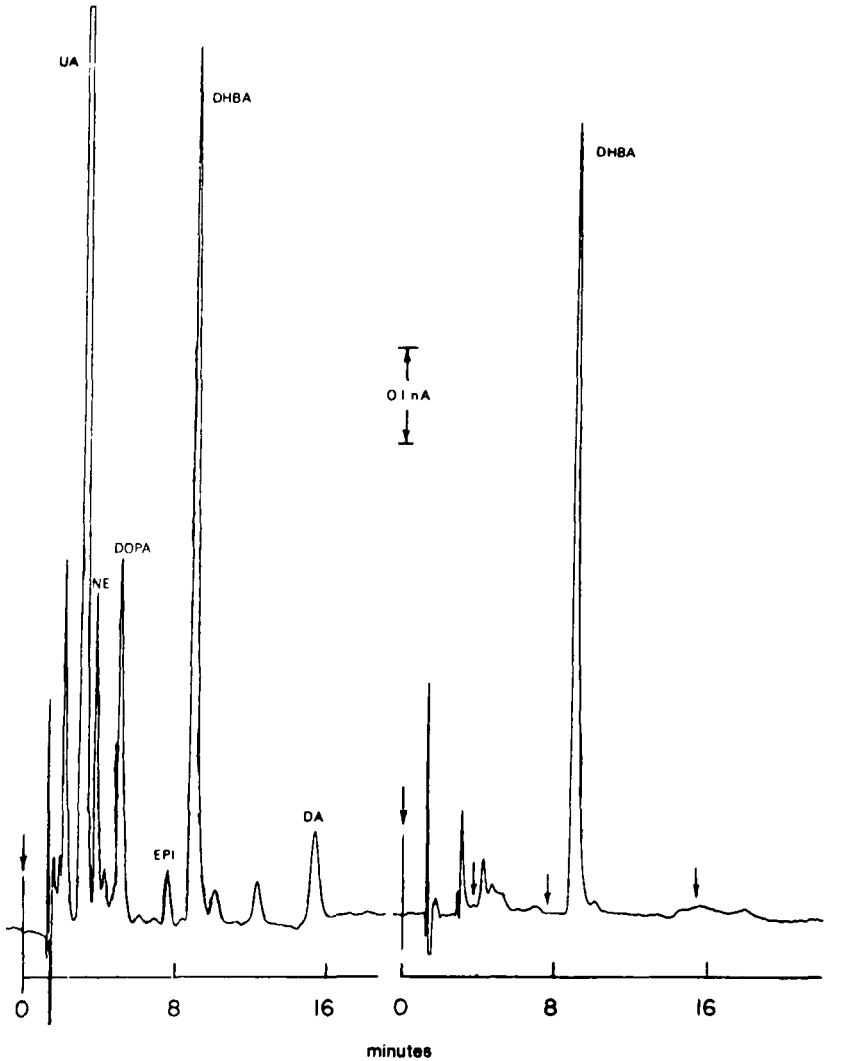

FIGURE 6. Chromatograms of an extract obtained from 3 ml of a human plasma (left) and a reagent blank (right). Conditions: steel column 4.6x250 mm with Biophase ODS 5μ, mobile phase 0.15 M monochloroacetate buffer pH 3.10 with 1.0 mM EDTA and 25 mg/lit of sodium octyl sulphate, flow rate 1.3 ml/min, amperometric detection at +0.75 V, carbon paste electrode. Plasma concentrations: NE 415, E 201 pg/ml.

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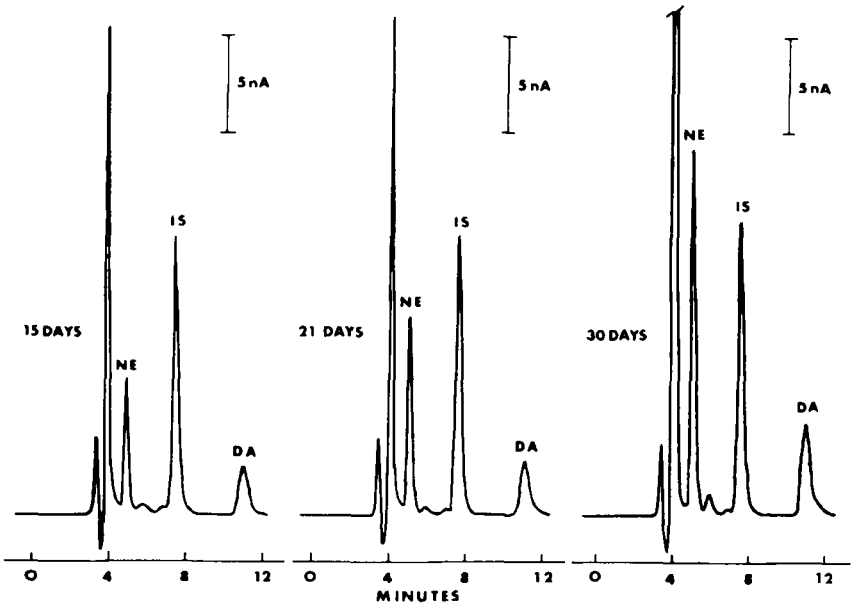

FIGURE 7. Illustration of the increase in neurotransmittor (NE,DA) concentrations with the development of the rat brain hypothalamus.
Conditions: steel column 3.9x300 mm with Bondapak C-18, mobile phase 0.1 M citrate/phosphate buffer, 0.3 mM in sodium octyl sulphate, flow rate 1.0 ml/min, amperometric detection at +0.72 V, carbon paste electrode.
(Reproduced from J. Neurochem. 31, 1461 (1978)(ref. 103) with the permission of the authors and the copyright owner, the International Society for Neurochemistry.)

assays of aromatic amino acid decarboxylase involving UV (113) or amperometric (114,115) detection, the monitoring of tyrosine decarboxylase activity as an enzyme kinetic method for the determination of pyridoxal-5'-phosphate in plasma (116,117), studies of catechol O-methyl- (COMT) and N-methyl-transferase (phenylethanolamine-N-methyltransferase, PNMT) activities (118-120) and assays for dopamine-beta-hydroxylase (121,122).

ECD is particularly favourable for reactions in which the enzymatically generated product is more prone to electrooxidation than the substrate itself. This situation is present in the assay of TH. The determination of estrogen-2-hydroxylase activity by HPLC-ECD is another recently published example of an application of this technique to a reaction by which a catechol structure is enzymatically generated (123).

D. Applications to problems within clinical diagnoses and related areas

Metabolic disorders, either inherited or produced by acute disease, are always in some way reflected in an abnormal metabolite pattern, if an appropriate body fluid (such as urine) can be analyzed for that purpose. Because CA's are involved in many metabolic transformations, their analysis in body fluids is of great value for the clinical diagnosis of a variety of disease states. Particularly, neural crest tumors such as neuroblastoma, pheochromocytoma and ganglioneuroma are all associated with an abnormal secretion of CA's. Thus, highly increased levels of plasma CA's, especially NE, may be direct evidence for a suspected pheochromocytoma as illustrated in Fig. 8. A rapid HPLC-method whereby the extraction step has been omitted, suitable for fast diagnosis of the disease has been described recently (124).

The increased CA production is accompanied by an increased catabolic deactivation via COMT-mediated 3-O-methylation and subsequent oxidation. The respective end-products, homovanillic acid (HVA), vanillylmandelic acid (VMA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), are conjugated and excreted by the urine. Therefore, the profiling of these acid metabolites in urine is also of great diagnostic value. Recently, Krstulovic et al (125) demonstrated the clinical use of the analysis of urinary levels of VMA and MHPG by means of reversed-phase HPLC with ECD or UV-detection.

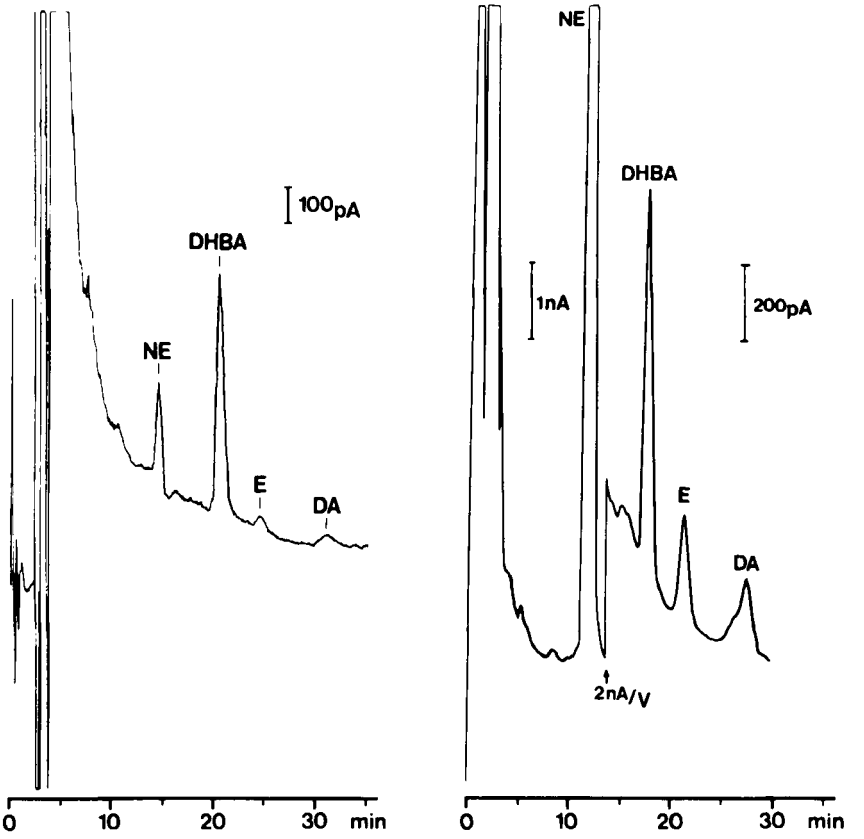

FIGURE 8. Chromatograms showing the difference in human plasma CA concentrations of a normal control subject (left) and a patient with a pheochromocytoma (right). Conditions: steel column 4.6x150 mm with Nucleosil SA 5µ, mobile phase 0.04 M/0.02 M acetate/citrate buffer pH 5.2, flow rate 0.60 ml/min, amperometric detection at +0.65 V, carbon paste electrode.

(Results from the author's laboratory)

A semi-automated HPLC-method with fluorimetric detection of THI's derived from NE and E was used by Nelson and Carruthers for tumor diagnoses via routine urine analyses (68).

In the serum of patients with a malignant melanoma, S-cysteinyl-L-DOPA compounds, especially 5-S-cysteinyl-L-DOPA which is the most

important pheomelanin precursor, are present in highly increased amounts and the reversed-phase HPLC separation and quantitation of these L-DOPA metabolites from serum and urine as well as their diagnostic value have been studied in detail by Hansson et al (126,127). It was found that the amount of 5-S-cysteinyl-L-DOPA correlated well with the degree of dissemination of the tumor.

A recent paper describes a study of the metabolization of the hypotensive drug α-methyl-DOPA (MD) to α-methylnorepinephrine (MNE) (128). While the basal plasma concentration of MNE in patients on MD therapy was too low to be determined, it rose to levels in the range 90 - 350 pg/ml after exercise. The authors contribute this to a release of the metabolite from the sympathetic nerve endings.

The two existent sulfoconjugates of DA, the 3-O- and 4-O-sulphate, respectively, were analyzed successfully in urine from Parkinsonian patients undergoing L-DOPA therapy (129). In this case the urine sample was pretreated by means of successive passage through small columns of cation and anion exchange resins, in order to isolate and concentrate the conjugates before injection onto a silica gel column. The mobile phase consisted of acetonitrile - 3% aqueous ammonia (85:15) of pH 10.0. UV-detection at 277 nM was used. The time course of the excretion of these conjugates, as well as the 3-O/4-O-ratio, was readily followed by this method.

E. Miscellaneous biomedical applications

The application of HPLC as an analytical tool for CA studies has attracted interest in many different biomedical areas. The technique is being rapidly explored, especially for investigations on biosynthesis and metabolism. The possible biotransformation of CA's into tetrahydroisoquinoline alkaloids via a Pictet-Spengler reaction as a cause of alcohol addiction has been studied by different groups (33,

130,131). Stout et al used HPLC combined with UV and radio-isotope detection for the investigation of dopamine metabolites obtained in extracts from cultivated neuroblastoma cells (132). Hypothalamic CA biosynthesis in vitro has been studied recently (133) using HPLC and electrochemical detection. Plotsky et al, in connection with research on inhibition of prolactin inhibition, determined DA in hypophysial stalk blood (134) with the same technique. Amperometric detection has also been used for the analysis of α-methyl-DOPA and its principal biotransformation products in serum (135). Identification of 4-O-methyldopamine in tissue as an L-DOPA metabolite was elegantly carried out by Bidard and Cronenberger (136) using HPLC and detection by UV, fluorimetry and tritium counting. A very nice example of how drug effects on plasma CA levels can be visualized by HPLC is given in a recent paper by Watson (137) and shown in Fig. 9. The effect of the α adrenergic antagonist clonidine, a hypotensive drug, on the CA concentrations in plasma is evident from the chromatograms and quite dramatic.

FUTURE TRENDS: POSSIBLE FURTHER IMPROVEMENTS AND NEW APPLICATIONS

At present, the majority of CA-analyses carried out by HPLC, include a laborious and time-consuming pre-purification from the biological material. However, using a dual column technique it should be quite possible to adsorb the CA's on a high affinity precolumn, wash out most other material and subsequently release the CA's by a solvent change with transfer to the analytical column, by means of a switching valve. Such multicolumn isolation and preconcentration techniques have recently come into use for similar analytical purposes (100) and have the advantage of making the analytical process faster and easier to control by a microcomputer (138). One should also, as pointed out by Kissinger (139), expect an improvement in the performance of the various HPLC sorbents available, towards higher reproducibility and column efficiency.

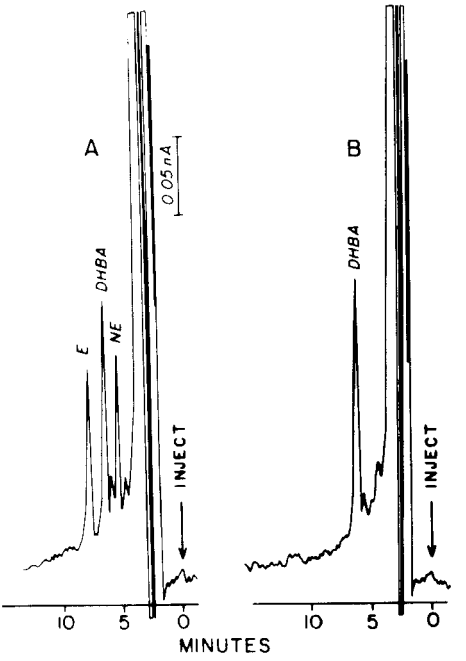

FIGURE 9. The effect of the hypotensive drug clonidine on dog plasma CA levels. Chromatograms of a dog plasma extract immediately before (left) and 15 min after the intravenous administration of clonidine (20 mg/kg)(right). Conditions: steel column 4.6x250 mm with Partisil SCX 10μ, mobile phase 0.013 M/0.008 M acetate/citrate buffer pH 5.2 with 10 μM EDTA, flow rate 1.0 ml/min, amperometric detection at +0.50 V, carbon paste electrode.

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As far as detector systems are concerned, developments in the construction of electrochemical flow-cells, resulting in a still higher sensitivity, are to be expected. Amperometric detection with rotating disc electrodes has recently given very promising results for brain tissue analysis (140). Further, experiments with dual working electrodes (34) have demonstrated the possibility of distinguishing between chromatographic peaks origi-

nating from (quasi-)reversible (shown by catechol derivatives) and irreversible electrochemical reactions, respectively.

SUMMARY AND CONCLUSIONS

Very great progress has been made during the last ten years in the field of analysis of catecholamines by HPLC. The improvements in column efficiency, resulting from better bonded-phase packing materials and general column technology, have aided in increased resolution and detectability. Reversed-phase or cation-exchange microparticulate (10 or 5μ) columns with aqueous mobile phases and isocratic elution are at present the most successful chromatographic systems; the column type preferred being dependent on the purity of the sample to be injected and the type of detector system used. Very low detection limits are obtained with the use of electrochemical detectors or of fluorimetric detectors combined with postcolumn transformation of the catecholamines to trihydroxyindole (THI) or o-phtalaldehyde (OPA) derivatives. However, only EC detectors are capable of being almost equally sensitive to all catecholamines; by the THI-fluorimetric method DA is severely discriminated, whereas the OPA-technique totally excludes E. For the determination of catecholamines in biological material containing only trace amounts, such as plasma or serum, detection limits (S/N = 2) of 1 - 5 pg have been obtained recently with the use of fluorimetric (THI) and electrochemical detection, respectively. Such highly sensitive HPLC-systems are increasingly being used within biology and medicine for catecholamine analysis of diverse biological material and are successfully competing with the radioenzymatic procedures for plasma and for serum. Further developments in the field would probably be possible by the application of a column-switching technique permitting pre-column extraction and sample enrichment as well as by improvements in detector constructions, particularly the electrochemical flow-cells.

ACKNOWLEDGEMENTS

The valuable contributions of Mrs. L. Hedman to the results from our laboratory presented in this paper and of Prof. D.H. Lewis to the final linguistic editing are gratefully acknowledged. The author is also indebted to Linköping University for financial support.

REFERENCES

Introduction

1. Takamine, J., J. Soc. Chem. Ind. 20, 746 (1901).
2. Pratesi, P., La Manna, A., Campiglio, A. and Ghislandi, V., J. Chem. Soc. 1958, 2069.
3. Pratesi, P., La Manna, A., Campiglio, A. and Ghislandi, V., J. Chem. Soc. 1959, 4062.
4. Anton, A.H. and Sayre, D.F., J. Pharmacol. Exp. Ther. 138, 360 (1962).
5. Anton, A.H. and Sayre, D.F., J. Pharmacol. Exp. Ther. 145, 326 (1964).
6. Kirshner, N. and Goodball, M., J. Biol. Chem. 226, 207 (1957).
7. Bertler, A., Carlsson, A. and Rosengren, E., Acta Physiol. Scand. 44, 273 (1958).
8. Mattok, G.L. and Wilson, D.L., Anal. Biochem. 11, 575 (1965).
9. Minard, F.N. and Grant, D.S., Biochem. Med. 6, 46 (1972).
10. Seki, T. and Wada, H., J. Chromatogr. 114, 227 (1975).
11. Seki, T., J. Chromatogr. 124, 411 (1976).
12. Breakefield, X.O., J. Neurochem. 25, 877 (1975).
13. Imai, K., Böhler, P., Stein, S. and Udenfriend, S., Arch. Biochem. Biophys. 161, 161 (1974).
14. Horning, M.G., Moss, A.M. and Horning, E.C., Biochim. Biophys. Acta 148, 597 (1967).

15. Moffat, A.C. and Horning, E.C., Biochim. Biophys. Acta 222, 248 (1970).

16. Horning, M.G., Moss, A.M., Boucher, E.A. and Horning, E.C., Anal. Lett. 1, 311 (1968).

17. Moffat, A.C., Horning, E.C., Matin, S.B. and Rowland, M., J. Chromatogr. 66, 255 (1972).

18. Wong, K.P., Ruthven, C.R.J. and Sandler, M., Clin. Chim. Acta 47, 215 (1973).

19. Imai, K., Wang, M.-T., Yoshive, S. and Tamura, Z., Clin. Chim. Acta 43, 145 (1973).

20. Wang, M.T., Imai, K., Yoshioka, M. and Tamura, Z., Clin. Chim. Acta 63, 13 (1975).

21. Lhuguenot, J.-C. and Maume, B.F., J. Chrom. Sci. 12, 411 (1974).

22. Anthony, G.M., Brooks, C.J.W. and Middleditch, B.S., J. Pharm. Pharmacol. 22 (3), 205 (1970).

23. Curtius, H.C., Wolfensberger, M., Steinmann, B., Redweik, U. and Siegfried, J., J. Chromatogr. 99, 529 (1974).

24. Lovelady, H.G. and Foster, L.L., J. Chromatogr. 108, 43 (1975).

25. Boutagy, J. and Benedict, C., J. Chromatogr. 151, 263 (1978).

26. Jacob, K., Vogt, W., Knedel, M. and Schwertfeger, G., J. Chromatogr. 146, 221 (1978).

27. Engelman, K. and Portnoy, B., Circ. Res. 26, 53 (1970).

28. Passon, P.G. and Peuler, J.D., Anal. Biochem. 51, 618 (1973).

29. Da Prada, M. and Zürcher, G., Life Sci. 19, 1161 (1976).

30. Krstulovic, A.M., Adv. Chromatogr. 17, 279 (1979).

Chromatographic properties of catecholamines, column and mobile phase selection

31. Refshauge, C., Kissinger, P.T., Dreiling, R., Blank, L., Freeman, R. and Adams, R.N., Life Sci. 14, 311 (1974).

32. Hashimoto, H. and Maruyama, Y., J. Chromatogr. 152, 387 (1978).

33. McMurtrey, K.D., Cashaw, J.L. and Davis, V.E., J. Liquid Chromatogr. 3, 663 (1980).

34. Fenn, R.J., Siggia, S. and Curran, D.J., Anal. Chem. 50, 1067 (1978).

35. Hallman, H., Farnebo, L.-O., Hamberger, B. and Jonsson, G., Life Sci. 23, 1049 (1978).

36. Keller, R., Oke, A., Mefford, I. and Adams, R.N., Life Sci. 19, 995 (1976).

37. Hjemdahl, P., Daleskog, M. and Kahan, T., Life Sci. 25, 131 (1979).

38. Allenmark, S. and Hedman, L., J. Liquid Chromatogr. 2, 277 (1979).

39. Allenmark, S., Hedman, L. and Söderberg, A., Microchem. J. 25, 567 (1980).

40. Molnar, I. and Horvath, C., Clin. Chem. 22, 1497 (1976).

41. Asmus, P.A. and Freed, C.R., J. Chromatogr. 169, 303 (1979).

42. Mell, L.D. and Gustafson, A.B., Clin. Chem. 23, 473 (1977).

43. Knox, J.H. and Jurand, J., J. Chromatogr. 125, 89 (1976).

44. Riggin, R.M. and Kissinger, P.T., Anal. Chem. 49, 2109 (1977).

45. Scratchley, G.A., Masoud, A.N., Stohs, S.J. and Wingard, D.W., J. Chromatogr. 169, 313 (1979).

46. Crombeen, J.P., Kraak, J.C. and Poppe, H., J. Chromatogr. 167, 219 (1978).

47. Schill, G. in Ion Exchange and Solvent Extraction, Marinsky, J.A. and Marcus, Y., eds., vol 6, p.1. Marcel Dekker, New York, 1974.

48. Johansson, I.M., Wahlund, K.G. and Schill, G., J. Chromatogr. 149, 281 (1978).

49. Johansson, I.M., J. Liquid Chromatogr.,in press.

50. Persson, B.A. and Karger, B.L., J. Chromatogr. Sci. 12, 521 (1974).

Methods for the detection of catecholamines in column effluents

51. Krstulovic, A.M. and Powell, A.M., J. Chromatogr. 171, 345 (1979).

52. Schüsler-van Hees, M.T.I.W. and Beijersbergen van Henegouwen, G.M.J., J. Chromatogr. 196, 101 (1980).

53. Jackman, G.P., Carson, V.J., Bobik, A. and Skews, H., J. Chromatogr. 182, 277 (1980).

54. Davis, T.P., Gehrke, C.W., Gehrke Jr., C.W., Cunningham, T.D., Kuo, K.C., Gerhardt, K.O., Johnson, H.D. and Williams, C.H., Clin. Chem. 24, 1317 (1978).

55. Mell, L.D., Dasler, A.R. and Gustafson, A.B., J. Liquid Chromatogr. 1, 261 (1978).

56. Schwedt, G. and Bussemas, H.H., Chromatographia 9, 17 (1976).

57. Schwedt, G. and Bussemas, H.H., Z. Anal. Chem. 283, 23 (1977).

58. Schwedt, G. and Bussemas, H.H., Z. Anal. Chem. 285, 381 (1977).

59. Frei, R.W., Thomas, M. and Frei, I., J. Liquid Chromatogr. 1, 443 (1978).

60. Imai, K., J. Chromatogr. 105, 135 (1975).

61. Imai, K., Tsukanoto, M. and Tamura, K., J. Chromatogr. 137, 357 (1977).

62. Imai, K. and Zamura, Z., Clin. Chim. Acta 85, 1 (1978).

63. Schwedt, G., J. Chromatogr. 118, 429 (1976).

64. Mori, K., Jap. J. Ind. Health 16, 490, 494 (1974).

65. Mori, K., Jap. J. Ind. Health 17, 116, 170 (1975).

66. Schwedt, G., J. Chromatogr. 143, 463 (1977).

67. Okamoto, K.-I., Ishida, Y. and Asai, K., J. Chromatogr. 167, 205 (1978).

68. Nelson, L.M. and Carruthers, M., J. Chromatogr. 183, 295 (1980).

69. Yui, Y., Kimura, M., Itokawa, Y. and Kawai, C., J. Chromatogr. 177, 376 (1979).

70. Yui, Y., Fujita, T., Yamamoto, T., Itokawa, Y. and Kawai, C., Clin. Chem. 26, 194 (1980).

71. Yui, Y., Itokawa, Y. and Kawai, C., Anal. Biochem. 108, 11 (1980).

72. Kissinger, P.T., Refshauge, C.J., Dreiling, R. and Adams, R.N., Anal. Lett. 6, 465 (1973).

73. Moyer, T.P, and Jiang, N.-S., J. Chromatogr. 153, 365 (1978).

74. Kissinger, P.T., Felice, L.J., Riggin, R.M., Pachla, L.A. and Wenke, D.C., Clin. Chem. 20, 992 (1974).

75. Shoup, R.E. and Kissinger, P.T., Chem. Instrum. 7, 171 (1977).

76. Kissinger, P.T., Anal. Chem. 49, 447A (1978).

77. Kissinger, P.T., Bruntlett, C.S., Bratin, K. and Rice, J.R., Proc. 9th Materials Research Symposium "Trace Organic Analysis", National Bureau of Standards Special Publication 519, 705 (1979).

78. Fleet, B. and Little, C.J., J. Chromatogr. Sci 12, 747 (1974).

79. White, M.W., J. Chromatogr. 178, 229 (1979).

80. Lankelma, J. and Poppe, H., J. Chromatogr. 125, 375 (1976).

81. Brunt, K., Thesis, Groningen, The Netherlands, 1980.

82. Oosterhuis, B., Brunt, K., Westerink, B.H.C. and Doornbos, D.A., Anal. Chem. 52, 203 (1980).

83. Brunt, K., Bruins, C.H.P., Doornbos, D.A. and Oosterhuis, B., Anal. Chim. Acta 114, 257 (1980).

84. Humphrey, D.W., Goldman, M.E., Wilcox, R.E., Erickson, C.K. and Smith, R.V., Michrochem. J. 25, 186 (1980).

85. Klaniecki, T.S., Corder, C.N., McDonald Jr., R.H. and Feldman, J.A., J. Lab. Clin. Med. 90, 604 (1977).

86. Eriksson, B.-M., Andersson, I., Borg, K.-O. and Persson, B.A., Acta Pharm. Suec. 14, 451 (1977).

87. Harapat, S. and Rubin, P., J. Chromatogr. 163, 77 (1979).

88. Müller, T., Hofschuster, E., Kuss, H.-J. and Welter, D., J. Neural. Transm. 45, 219 (1979).

89. Endert, E., Clin. Chim. Acta 96, 233 (1979).

90. Yui, Y. and Kawai, C., J. Chromatogr. 206, 586 (1981).

Techniques for the isolation of catecholamines from a biological matrix

91. Loullis, C.C., Hingtgen, J.N., Shea, P.A. and Aprison, M.H., Pharmacol. Biochem. Behav. 12, 959 (1980).

92. Davis, G.C., Kissinger, P.T. and Shoup, R.E., Anal. Chem. 53, 156 (1981).

93. Persson, B.-A. and Lagerström, P.-O., J. Chromatogr. 122, 305 (1976).

94. Higa, S., Suzuki, T., Hyashi, A., Tsuge, I. and Yamamura, Y., Anal. Biochem. 77, 18 (1977).

95. Gelijkens, C.F. and De Leenheer, A.P., J. Chromatogr. 183, 78 (1980).

96. Moyer, T.P., Jiang, N.S., Tyce, G.M. and Sheps, S.G., Clin. Chem. 25, 256 (1979).

97. Hahn, Z., J. Biochem. Biophys. Methods 2, 163 (1980).

98. Yoshida J.-I., Yoshino, K., Matsunaga, T., Higa, S., Suzuki, T., Hayashi, A. and Yamamura, Y., Biomed. Mass Spectrom. 7, 396 (1980).

99. Glad, M., Ohlson, S., Hansson, L., Månsson, M.-O. and Mosbach, K., J. Chromatogr. 200, 254 (1980).

100. Koch, D.D. and Kissinger, P.T., Anal. Chem. 52, 27 (1980).

Some applications of the HPLC analysis method to problems within biology and medicine

101. Goldstein, S., Feuerstein, G., Izzo, Jr., J.L., Kopin, I.J. and Keiser, H.R., Life Sci. 28, 467 (1981).

102. Mefford, I., Ward, M.M., Miles, L., Taylor, B., Chesney, M.A., Keegan, D.L. and Barchas, J.D., Life Sci. 28, 477 (1981).

103. Felice, L.J., Felice, J.D. and Kissinger, P.T., J. Neurochem. 31, 1461 (1968).

104. Hegstrand, L.R. and Eichelman, B., J. Chromatogr. 222, 107 (1981).

105. Maruyama, Y., Oshima, T. and Nakajima, E., Life Sci. 26, 1115 (1980).

106. Mefford, I., Gilberg, M. and Barchas, J.D., Anal. Biochem. 104, 469 (1980).

107. Anderson, G.M., Batter, D.K., Young, J.G., Shaywitz, B.A. and Cohen, D.J., J. Chromatogr. 181, 453 (1980).

108. Magnusson, O., Nilsson, L.B. and Westerlund, D., J. Chromatogr. 221, 237 (1980).

109. Wagner, J., Palfreyman, M. and Zraika, M., J. Chromatogr. 164, 41 (1979).

110. Anderson, G.M. and Young, J.G., Life Sci 28, 507 (1981).

111. Blank, C.L. and Pike, R., Life Sci. 18, 859 (1976).

112. Nagatsu, T., Oka, K. and Kato, T., J. Chromatogr. 163, 247 (1979).

113. D'Erme, M., Rosei, M.A., Fiori, A. and Di Stazio, G., Anal. Biochem. 104, 59 (1980).

114. Nagatsu, T., Yamamoto, T. and Kato, T., Anal. Biochem. 100, 160 (1979).

115. Rahman, M.K., Nagatsu, T. and Kato, T., Life Sci. 28, 485 (1981).

116. Allenmark, S., Hjelm, E. and Larsson-Cohn, U., J. Chromatogr. 146, 485 (1978).

117. Allenmark, S. and Hjelm, E., Current Separations (Bioanal. Syst. Inc.) 1, 3 (1979).

118. Borchardt, R.T., Hegazi, M.F. and Schowen, R.L., J. Chromatogr. 152, 255 (1978).

119. Shoup, R.E., Davis, G.C. and Kissinger, P.T., Anal. Chem. 52, 483 (1980).

120. Borchardt, R.T., Vinchek, W.C. and Grunewald, G.L., Anal. Biochem. 82, 149 (1977).

121. Davis, G.C. and Kissinger, P.T., Anal. Chem. 51, 1960 (1979).

122. Allenmark, S. and Qureshi, G.A., J. Chromatogr. 223, 188 (1981).

123. Allenmark, S., Hjelm, E., Hammar, M. and Å:son Berg, A. J. Biochem. Biophys. Methods 4, 209 (1981).

124. Devynck, M.A., Elghozi, J.L., Le Quan-Bui, K.H. and Meyer, P., Nouv. Presse Med. 9, 2061 (1980).

125. Krstulovic, A.M., Zakaria, M., Lohse, K. and Bertani-Dziedzic, L., J. Chromatogr. 186, 733 (1979).

126. Hansson, C., Edholm, L.-E., Agrup, G., Rorsman, H., Rosengren, A.-M. and Rosengren, E., Clin. Chim. Acta 88, 419 (1978).

127. Hansson, C., Agrup, G., Rorsman, H., Rosengren, A.-M. and Rosengren E., J. Chromatogr. 162, 7 (1979).

128. Jenner, D.A., Brown, M.J. and Lhoste, F.J.M., J. Chromatogr. 224, 507 (1981).

129. Arakawa, Y., Imai, K. and Tamura, Z., J. Chromatogr. 162, 311 (1979).

130. Riggin, R.M. and Kissinger, P.T., Anal. Chem. 49, 530 (1977).

131. Kenyhercz, T.M. and Kissinger, P.T., J. Pharmaceut. Sci. 67, 112 (1978).

132. Stout, R.W., Michelot, R.J., Molnar, I., Horvath, C. and Coward, J.K., Anal. Biochem. 76, 330 (1976).

133. Sundberg, D.K., Bennett, B., Wendel, O.T. and Morris, M., Res. Commun. Chem. Pathol. Pharmacol. 29, 599 (1980).

134. Plotsky, P.M., Gibbs, D.M. and Neill, J.D., Endocrinology 102, 1887 (1978).

135. Cooper, M.J., O'Dea, R.F. and Mirkin, B.L., J. Chromatogr. 162, 601 (1979).

136. Bidard, J.-N. and Cronenberger, L., J. Chromatogr. 164, 139 (1979).

137. Watson, E., Life Sci. 28, 493 (1981).

Future trends: Possible further improvements and new applications

138. Willmott, F.W., Mackenzie, I. and Dolphin, R.J., J. Chromatogr. 167, 31 (1978).

139. Kissinger, P.T., Bruntlett, C.S. and Shoup, R.E., Life Sci. 28, 455 (1981).

140. Westerink, B.H.C. and Mulder, T.B.A., J. Neurochem. 36, 1449 (1981).