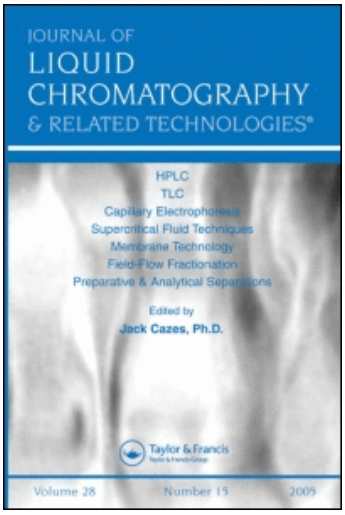

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Post-Column Reaction Detection Systems in HPLC

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POST-COLUMN REACTION DETECTION SYSTEMS IN HPLC

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ABSTRACT

Designs of post-column reaction detection systems for HPLC are discussed with respect to their effect on sensitivity and resolution on the basis of systems theory. The most promising post-column reactor types are the packed-bed reactor and the electronically desegmented liquid-segmented reactor. No experimental data on the latter are available, however.

The choice of post-column reaction detector system is greatly influenced by the reaction type. An overview of these reaction types with suggested systems are given.

For two examples, corticorsteroids and catecholamines in plasma, a comparison is made of the different detection possibilities.

INTRODUCTION

The use of on-line post-column reactions for specific and sensitive detection after separation is increasing to develop HPLC into an even more powerful analytical tool. Derivatisation should be considered if just separation and detection of native compounds will not suffice due to low signal to noise ratio or interfering peaks. Pre-column derivatisation requires a neat quantitative reaction giving stable and single well-defined reaction products

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unlike post-column derivatisation where not even a knowledge of the nature of the products is essential [1,2] as long as the reaction is reproducible and there is a difference in the detected property of the reagent and product(s) (unless an extraction is incorporated). Reactions can be performed off-line (not keeping pace with the HPLC separation) or on-line, and batch-wise or continuous. Pre-column derivatisation is usually performed batch-wise, because there is only one sample per chromatogram, and off-line since off-line reaction is subject to less time and solvent constraints. For post-column derivatisation only a fraction size of one tenth of the peak volume or less causes minimal information loss[3]. so, most often continuous reaction, which is conveniently done in an on-line mode and is easily automated for routine analyses, is chosen. The choice for off-line batch-wise pre-column or automated on-line continuous post-column derivatisation will be determined by the specifics of each case.

REACTOR DESIGN

The latest HPLC column technology imposes demands on the design of post-column reactors (PCR's) with respect to dispersion characteristics.

The influence of the PCR for a homogeneous reaction and a concentration sensitive detector on sensitivity for a component in the sample is given by:

$$\frac{I_{pm}}{I_s} = \frac{.4\ V_i\ E_M\ t_{RC}\ F_C}{AL(1 + k'(\sigma t_{icd} + \sigma t_{PCR})F_{PCR}} = \frac{.4\ V_i\ E_M}{(\sigma t_{icd} + \sigma t_{PCR})\ F_{PCR}} \quad (1)$$

in which: I_{pm} = signal height at peakmaximum in total system
I_s = signal hight for component in sample
V_i = injection volume
E_M = enhancement of signal due to derivatisation
t_{Rc} = retention time on the column
F_c = flow rate through the column

A = cross-sectional area of the column occupied by mobile phase

L = length of the column

k' = capacity factor of the component in the system

$\sigma_{t_{icd}}$ =dispersion in the chromatographic system without PCR

$\sigma_{t_{PCR}}$ =dispersion in PCR

F_{PCR}= flowrate of eluent plus reagents through PCR (excluding segmentation fluid)

The resolution in the complete system for two adjacent peaks, R_a, decreases relative to the resolution in the chromatograph without PCR, R_c:

$$R_a = \frac{R_c}{\sqrt{1 + \frac{\sigma^2_{t_{PCR}}}{\sigma^2_{t_{icd}}}}} \qquad (2)$$

From equation (1) and (2) it can be deduced that the sensitivity and (even more) the resolution will be impaired if the despersion in the PCR approaches the dispersion in the rest of the system- which for a well designed HPLC system equals the peak dispersion in the column (see below).

According to systems theory the variance of the dispersion in the total system is the sum of the variances of the independent dispersions in the parts. The parts that can cause dispersion are identified in fig 1.

The dispersion due to injection, σ_{t_i}, is for a good injector less than the duration of the injection. For injection times longer than a second the dispersion can usually be reduced by on-column concentration so that $\sigma_t \lesssim 1s$.

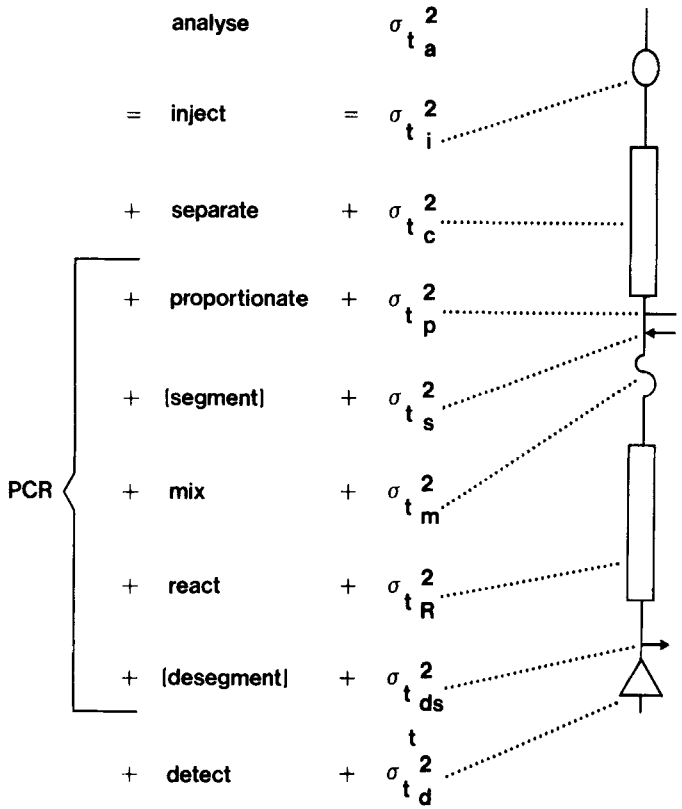

FIGURE 1

The variance of the peakdispersion in the system is the sum of the variances due to the consecutive functional units.

The dispersion in a modern UV or fluorimetric detector is less than 100 ms, but in (diffusion limited) electrochemical detection generally larger than 1s.

For 3000 theoretical plates and a retention time of 3-10 min. the column peakbroadening is σ_{t_c} = 3.3-11 s, thus:

$$\sigma^2_{t_{icd}} = \sigma^2_{t_i} + \sigma^2_{t_c} + \sigma^2_{t_d} = \sigma^2_{t_c} \qquad (3)$$

In most PCR's reagent is added and mixed with column effluent. Proportioning can be done via a tee and should not contribute to overall dispersion. Improper proportioning, however, is a source of noise, drift or variable response. A tee with 30^0 angle is capable of acting as a mixer [4]; a rotating flow mixing chamber [5] and a stirred mixing chamber [6] were documented to give better mixing. If their volume is reduced to less than a few microliter these mixers should show acceptable dispersion (a conical Kobayashi-type mixer is presently used in a commerc. PCR).

The most favorable mixing and dispersion are obtained in packed-bed (pb) mixers [7,8]. The minimum length for a pb mixer, L_m, is given by [8]:

$$L_m = 10\ d_t^2/d_p$$

in which: d_t = inside diameter of the mixer
d_p = particle diameter

e.g. a 10x3 mm tube packed with 100 μm glass beads will give adequate mixing and a (longitudinal) peakdispersion of less than .1 s (at .1 ml/min flowrate).

Segmentation may be introduced at the reagent tee and hence will not contribute to dispersion.

So, the total peakdispersion in the PCR simplifies to:

$$\sigma^2_{PCR} = \sigma^2_{t_R} + \sigma^2_{t_{ds}} \qquad (4)$$

There are three types of PCR design: the capillary (cap), the pb and the segmented flow (sf) reactor (see fig. 2). Dispersion in the reactor proper for cap reactors can be described by [8]:

$$\sigma^2_{t_R} = .04/D_m \left(\frac{\eta . F_{PCR}}{\Delta P}\right)^{1/3} t_R^{4/3} \quad \text{if } \varkappa > 1 \qquad (5A)$$

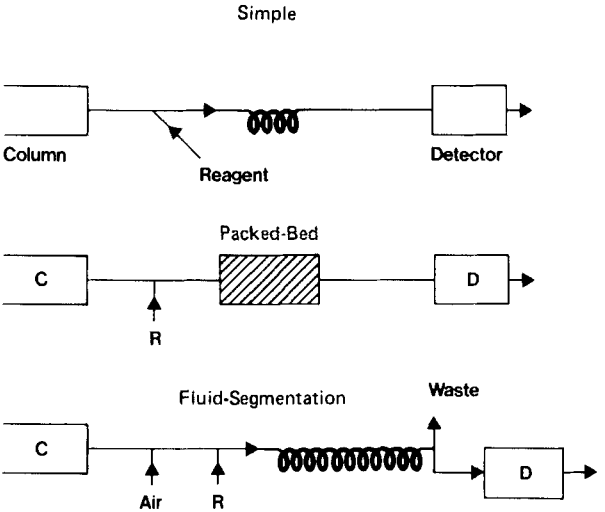

FIGURE 2

Post-column reaction detector designs.
a capillary PCR
b packed-bed PCR
c segmented flow PCR

$$\sigma^2_{t_R} = .004/D_m \left(\frac{\eta . F_{PCR}}{\Delta P}\right)^{1/3} t_R^{4/3} \quad \text{if } \kappa < .1 \quad (5B)$$

$$\sigma^2_{t_R} = .23 \left(\frac{\eta^{13} . F_{PCR}^{2} d_c^{6}}{D_m^{12} \Delta P \rho}\right)^{1/18} t_R^{25/18} \quad \text{if } .1 \leq \kappa \leq 1 \quad (5C)$$

in which [9]:

$$\kappa = 4.8 \left(\frac{d_t\, d_c\, \eta\, Dm}{\rho^3 F_{PCR}^2}\right)^{1/3} \quad (5D)$$

η = dynamic viscosity of the reaction solvent
D_m = diffusion coefficient of the analyte
Δp = pressure drop over the reactor

t_R = reaction time
d_c = diameter of the coil of reaction capillary
ρ = density of the reaction solvent

For pb reactors of non-porous packing material the dispersion is [8]:

$$\sigma^2_{tR} = 40 \lambda \frac{\eta^{1/2}}{\Delta P^{1/2}} t_R^{3/2} \qquad (6)$$

if A_r 100 d_p^2 and d_p D_m/u.

in which: λ = dispersion factor; λ=3 for a well packed reactor
A_r = cross-sectional area of the reactor
u = linear velocity in the reactor

A semi empirical equation was derived for sf reactors in which a gas is used for segmentation [10]:

$$\sigma^2_{t_R} = \left(\frac{538\, d_t^{2/3} \cdot \varkappa \cdot \eta^{5/3}}{D_m} + 1/n \right) \left(\frac{2.35\, \varkappa t_R}{d_t^{4/3}} \right) \qquad (7A)$$

in which: $$\varkappa = \frac{(F_{PCR} + .92\, d_t^3\, n)^{5/3}\, \eta^{2/3}}{\gamma^{2/3}\, F_{PCR}}$$

γ = surface tension
n = segmentation frequency
D'_m = mass transfer coefficient=$4 \times 10^{-4}\, \eta^{-1.67} D_m$

Unfortunately a like equation has not been derived yet for solvent segmented reactors.

Using equations 5, 6 and 7 a good idea of the relative performance of the repective types of reactors is obtained. Examples are given in fig 3, showing the dispersion as a function of reaction time at several flowrates and a wide range of other experimental conditions.

The peakdispersion due to desegmentation (debubbling or phase separation) in sf reactors should be taken into account

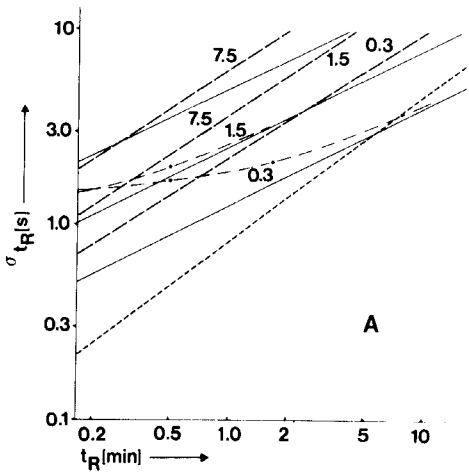

FIGURE 3

The peakdispersion in packed-bed(- - - -),capillary(— —), and segmented-flow(—•—) reactors.
Experimental conditions:
$\Delta p \leqslant 100$ bar; $d_t \geqslant 0.01$ cm(cap,pb)or $0.05 \leqslant d_t \leqslant 0.4$(sf); $d_c/d_t \geqslant 5$; $d_p \geqslant 5\mu m$; $K_o = 2.10^{-3}$; $\varepsilon_p = 0.4$; $\lambda = 3$; $n \leqslant 5$.

	A	B	C
D_m (x10^5 cm/s)	0.3	0.3	3
η (x100 g/cm.s)	1.5	0.4	0.4
ρ (g/cm^3)	0.8	1.4	0.8

The numbers next to the curves gave the respective PCR flowrates (in ml/min).

when comparing PCR's (see eq. 4). Recently it was found that peakdispersion in solvent segmented reactos may be caused mainly by (physical) desegmentation [11]. Over a limited range of flowrates: $\sigma_{v,ds} = 12 \pm 1$ µl for a miniaturized phase separator. This was confirmed for gas desegmentation [12].

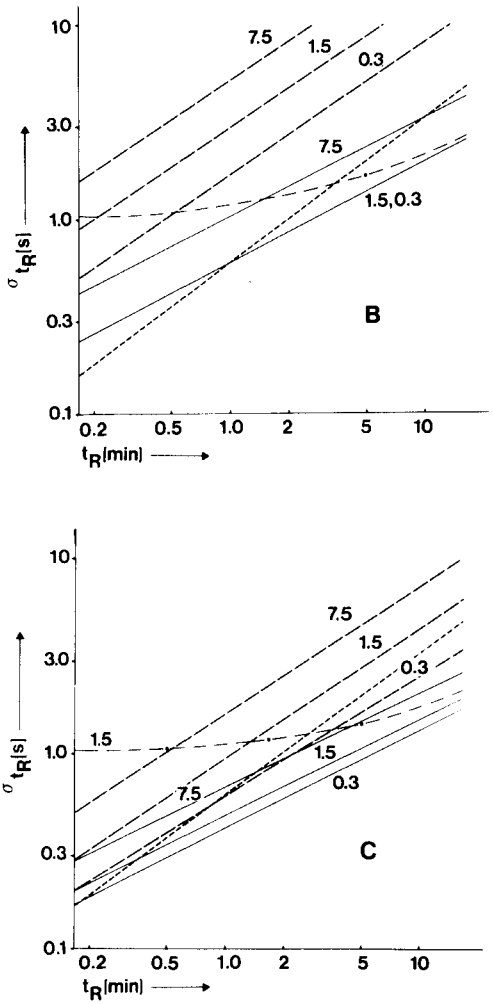

FIGURE 3B&C

An alternative to physical desegmentation is modification of the detector in order to measure only the segments of interest [13,14]. Segment volumes of ca 1 µl seem within the capabilities of present equipment.

COMPARISON

Pb PCR's give less dispersion than cap or sf PCR's at high flowrates and viscosities of the reaction solvent (c.f. fig 3). The only pb PCR's demonstrated [8,13], however, were not suitable for aggressive solvents (e.g. extreme pH).

Cap reactors are commercially available and simpler than pb reactors. They should only be used when dispersion is not an issue (i.e. for short reaction times).

Sf reactors show better dispersion performance than cap PCR's except for very short reaction times ($<$30 s) when physical de-segmentation is applied (broken lines in figs 3).

At reaction times larger than 120 s and low viscosity sf reactors should be preferred even over pb reactors for their commercial availability and versatility.

A way of increasing reaction time without appreciable peak dispersion is storage of the reaction mixture in a holding reactor [16] (see fig 4). A part of the chromatogram can be stored in each

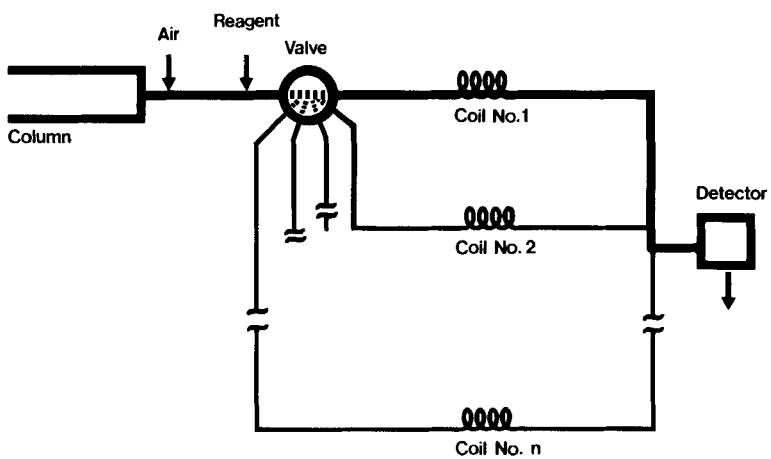

FIGURE 4

PCR for extended time and/or multiple reactions.

reactor via a switching valve, increasing the reaction time. This method proposed for sf but should also work for pb reactors[17].

Flow-splitting to obtain a low flowrate and increased reaction time without changing the volume of the reactor chould be used too, but with a loss in sensitivity proportional to the split ratio.

The versatility of sf and pb reactors can be seen clearly in fig 4: except for extended reaction times this PCR can also be used to apply different reactions with multiple reactiontimes to consecutive parts of the chromatogram without sacrificing resolution.

REACTION TYPES

Specific reactions for post-column derivatisation have been extensively reviewed [18, 19] and will not be summarized here. General PCR considerations with respect to reaction conditions are:

1. A high reagent concentration is needed to keep the dilution factor and the reaction time as small as possible.
2. Since the reaction rate increases usually 2-3 fold per 10°C, the highest reactiontemperature that the solvents permit will be used unless the analytes or products are unstable or side reactions are limiting.
3. The reaction solvent should have a high boiling point, low viscosity and should not slow the reaction or quench the signal of the product. Impurities in the solvent are known to determine the detection limit in fluormetric detection.

Most applied derivatisations are homogeneous (pseudo) first order reactions. They can be performed to create e.g. a chromophore [20], fluorophore[21], electrochemically active compound [22], luminescence or precipitation.

Post-column redox reactions based on an indicator technic e.g. carboxylic acids with nitrophenol or phenols and carbohydrates with Ce, will likely lose importance to electro-chemical detection. In the case of thioridazines the analyte products are

specifically detected and not the reagent products, giving additional selectivity not matched by electrochemical detection[23].

Efficient chemiluminescence can be obtained in several ways (see figure 5):

1. When a reaction can be made to produce a luminol oxidant like H_2O_2.
2. When a fluorophore can be produced that reacts in high yield with peroxyoxalate.
3. When a metal ion is generated that catalizes the reaction of luminol with H_2O_2 (e.g. detection limit CoII: 10 pg [24]).

A post-column <u>precipitation</u> reaction with nephelometric detection has been used with triglycerides by changing the polarity of the mobile phase by addition of ammonium sulfate [25]. The specificity, sensitivity and linearity of this method is likely much better when a real reaction with formation of an insoluble product is performed [25].

Some reactions do not even need addition of reagents. Energy in the form of heat or photons or the permanent presence of a

a oxidant + luminol $\xrightarrow{M^{n+}}$ luminescence

b H_2O_2 + TCPO $\Longrightarrow$ peroxyoxalate

peroxyoxalate + fuorophore $\Longrightarrow$ luminescence

oxidant = H_2O_2, OCl^-, I_2, MnO_4^-
luminol = 5-amino-2,3dihydrophtalazine-1,4dione
TCPO = bis-trichlorophenyloxalate
fluorophore: e.g. perylene

FIGURE 5

Chemiluminescence reactions

catalyst in the reactor may be sufficient. Solid phase reactors, in which the reagent is immobilized in a reactor have a definite drawback: depending on the analyte to immobilized reagent ratio the reagent is depleted with time and the reactor will have to be reloaded.

Photochemical reaction was applied for increase as well as extinction of signal in a UV detector [26], fluorimeter[26,27,28] and conductivity detector [29] with differential detection to enhance selectivity and sensitivity. Although the application of quartz or teflon pb reactors for photochemical reaction may be feasible [28], it has not yet been demonstrated. A class of catalytic PCR's of growing importance is enzymatic reactors. While their selectivity makes them attractive, temperature, pH and inhibitor/activitor sensitivity are not favorable. Most enzyme catalysed reactions have a temperature and pH otimum resp. slightly above and at the in vivo temperature and pH. Organic solvents like those used in HPLC tend to decrease the enzymatic activity. The pH optimum neccessitates buffering and limits sensitivity and linearity for pH-indicator aided detection of oxidoreductases [30].

Often enzymatic reactions obey Michaelis-Menton kinetics. The rate of product formation is given by:

$$\frac{d[P]}{dt} = \frac{k[E_o][S]}{K_m + [S]} \qquad (8)$$

in which:
- k = specific activity
- $[P]$ = product concentration
- $[E_0]$ = enzyme concentration
- $[S]$ = substrate concentration
- K_m = Michaelis constant

Two types of enzymatic reactors exist for:

Enzyme Analysis

Essential is that the PCR does not adsorb the analytes. For enzyme detection the substrate (reagent) concentration will

be as high as possible: $[S] \gg K_m$, so

$$t_R = [P]/\ k.\ [E_0] \qquad (9)$$

According the eq. 9 the detection limit is proportional to the reaction time. For example, LDH isoenzymes ($k > 100\ s^{-1}$) can be determined with NAD as substrate and UV detection with a detection limit of less than 1 nM per second of reaction time. Another application is the measurement of insecticide residues by their cholinesterase inhibiting effect [31].

Substrate Analysis

The price of enzymes and simplicity of design make it desirable to immobilize enzymes in the reactor. The stability of these reactors is good ("lifetime" larger than fifty days) but the bound enzymes have usually much less activity and a larger K_m than the free enzyme. When $K_m \gg [S]$ then from eq 8:

$$t_R = \frac{K_m}{k[E_0]} \ln \frac{[S] - [P]}{[S]}$$

For short reaction times a high enzyme concentration is required, therefore, pb reactors are preferred over cap reactors by virtue of their larger surface area. For example, bile acids have been detected by 3-hydroxysteroiddehydrogenase with $t_R < 6$ s and a detection limit of 10 ng [32] (see fig 6).

The sf reactor is the only PCR that employes multi (mobile) phases. This has been successfully utilized in the extraction of ion-pair complexes [33] and for separation of reagents and products [34]. The phase ratio and composition of the phases have to be carefully optimized. The distribution of solvents of intermediate polarity (e.g. the organic modifier in reverse phase separations) over the phases is often problematic. The combination of neat aqueous mobile phases and polar bonded stationary phases in HPLC may offer a solution [35].

An important aspect of the multi phase reactor is that reactions can be performed in liquid bubbles interspaced by an

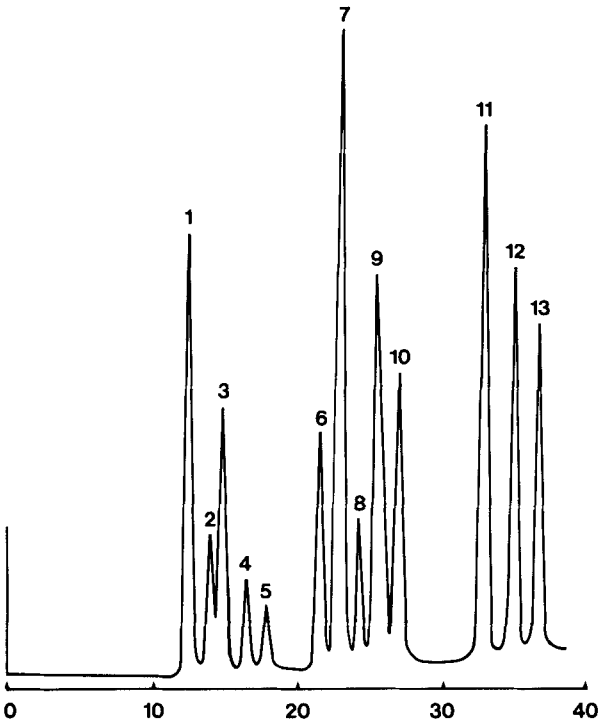

FIGURE 6

Analysis of bile acid standards.
Identity of the peaks:1 ursodeoxyC;2 C;3 glycoursodeoxyC; 4 glycoC;5 tauroC;6 chenodeoxyC; 7 deoxyC;8 glycochenodeoxyC;9 glycodeoxyC, taurochenodeoxyC;10 taurodeoxyC;11 lithoC;12 glycolithoC;13 taurolithoC. C=cholic acid. (From ref. 32, reproduced with permission of The Chemical Society of Japan.)

immiscible solvent-that may contain reagent or not-wetting the tube wall. In this case adsorption of the sample to the tube material is prevented and dispersion should be minimized.

The multiphase reactor opens possibilities for coupling of HPLC with mass spectrometry via ion-pair extraction [36], with

infra-red detection, flame ionisation detection or with HPLC in another mode.

TWO EXAMPLES

Corticosteroids

Here the analyte (e.g. cortisol) is a complex molecule with many fuctional groups and the analyst is faced with a choice (see fig 7). The Δ4-3 keto chromophore has ε_M=16000 at 240 nm[37]. The Zimmermann (Z) reaction attacks the 3- and 20-keto group[38] but also many other ketones. For the analysis of complex mixtures these detection methods may not be specific enough-UV detection at 240 nm is in ca 20% of the samples unfit for determining cortisol in urine [39].

Reaction with isonicotinoylhydrazine (INH) on the Δ4-3 keto group [40] or the multi-stage Porter-Silber (PS) reaction with phenylhydrazine on the 17-hydroxy (= keto) group[41] are more specific. The fastest and most sensitive reaction for corticosteriods is the Blue Tetrazolium (BT) reaction on the α-ketol chain [42]. Reducing sugars also react with BT, but do not interfere with corticosteroids when HPLC is applied. The INH and PS reaction do not allow water in the reactionsolvent, with the BT reaction less than 20% water is optimum. As for bile acids these endogenous corticosteroids could be enzymatically

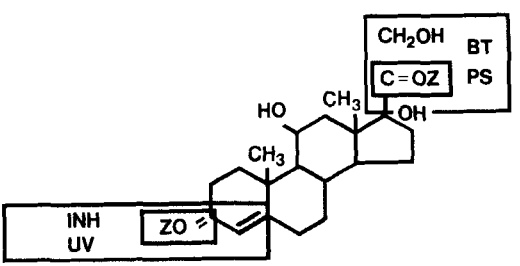

FIGURE 7

Chemical structure and reactive sites of cortisol.

detected. The convenience, sensitivity and selectivity of the BT reaction apparently has prompted no attempts to do so.

Reaction conditions for corticosteroids are summarized in Table I.

Catecholamines

Catecholamines are even more extensively studied and offer therefore greater choice in detection methods (see table II).

TABLE I

DETECTION OF CORTICOSTEROIDS

Method	λ (nm)	t_R(s)	T(^{0}C)	det.lim.	Specific	Ref.
UV	240	0	25	5 ng	-	37
Z	520	10	50		-	38
INH	370/450	55	25	7 ng	+	40
PS	420	960	60	20 ng	+	41
BT	525	20	55	1 ng	+	42
Enzy.			40		+	

TABLE II

DETECTION LIMITS FOR CATECHOLAMINES (pg)

Method	NE	E	DA	t_R	ref.
UV	10^3	10^3	10^3	0	43
Fluor.	300	300	300	0	43
Electrochem.	25	25	25	0	43
OPA	75	-	130	9	43
Borate	250	250	-	90	44
THI	1	1	800	230	43
Fl-lumin.	10	-	10	10	5
PABA	-	-	20	?	45

While cortisol normal values in plasma are 0.3-3 ng/ml, the range for epinephrine (E) is 10-80 pg/ml, norepinephrine (NE) is 100-600 pg/ml and dopamine (DA) is 10-150 pg/ml[46]. For accurate determination of normal plasma levels of catecholamines UV detection, fluorimetry and a borate complexation technic are not sensitive enough. O-phtalaldehyde (OPA) and fluorescamine (Fl) do also react with all other primary amines in the plasma but not with the secondary amine E. Even if this is overcome by a preceding demethylation of E,OPA sensitivity will be marginal and Fl fluorophores will have to be enhanced by chemiluminescence [5]. The Trihydroxyindole (THI) method[43], is sensitive for NE and E, selective and fast (t_R < 4 min). It is a multistage reaction involving oxidation to adrenochrome and alkaline rearrangement to fluorescent trihydroxyindoles. Usually ascorbic acid is added which prevents oxidation of the fluorophore. The detection limit for DA in plasma is not low enough but NE and E can be determined with relatively little sample preparation (see fig. 8). A complimentary specific and sensitive method for DA in plasma was reported[45]. Electrochemical detection is well suited for detection of the higher concentrations of catecholamines in urine after multicolumn separation [47].

PRESENT AND FUTURE

At commonly used HPLC flowrates commerical systems of the cap PCR type having a volume of 0.3-2 ml will cause an additional peakbroadening of 0.5-4 s, which is for most analyses acceptable. For longer reaction times or extractions sf has to be used. Peak dispersion in these PCR's was shown to be 1-10 s [11,34].

Problems arise at the application of PCR's for high speed separations[48,47] since peakwidths leaving the HPLC column are of the order of 0.5-2 s. From figs 3 it is clear that for high speed HPLC only sf reactors with electronic debubbling or pb reactors and very rapid reaction should be used. At the

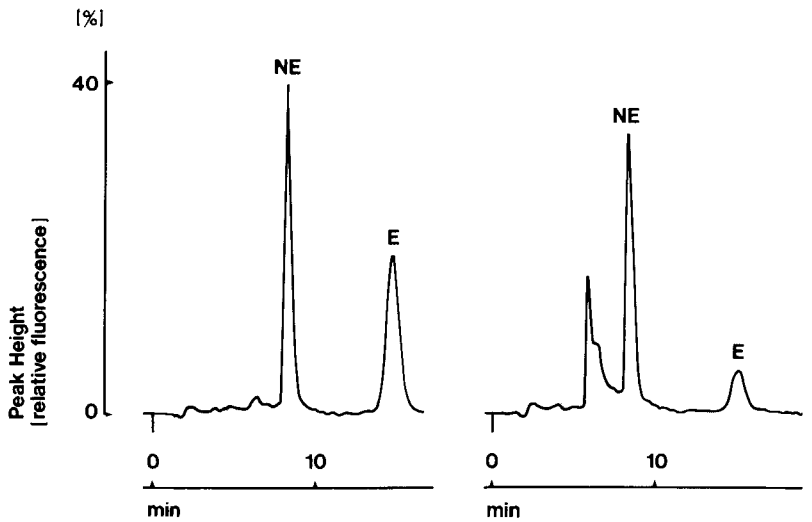

FIGURE 8

Catecholamine analysis in human plasma.
Chromatogram of standards (200 pg each of norepinephrine and epinephrine) (left) and of 1 ml of human plasma (right). (From ref. 50, reproduced with permission of The American Association for Clinical Chemistry.)

flowrates optimal for microbore and capillary HPLC columns no data are available and extrapolation seems futile. It is therefore, imperative that efficient pb reactors are developed that do not dissolve or corrode at extreme pH (e.g. made of titanium).

The type of catalytic PCR in which an analyte (or analyte product) is acting as a catalyst holds the promise that with increasing possibilities of extending the reaction time in PCR's the detection limits of the analytes will be proportionally decreased.

The application of new derivatives, different immobilized enzymes and electrochemical detection is expected to increase dramatically in the near future.

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