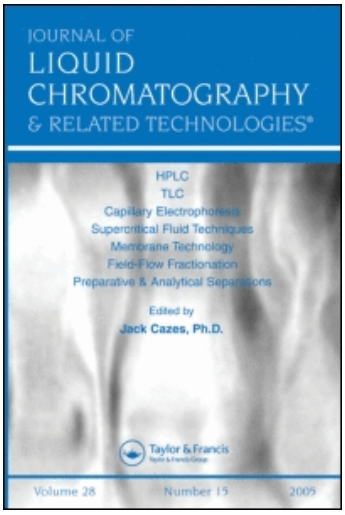

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Fluorescence Detection in High Performance Liquid Chromatography

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FLUORESCENCE DETECTION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The principles and applications of fluorescence detection and fluorescence introducing reagents and methods in HPLC are reviewed. The design and requirements for fluorescence detectors, flow cells and excitation sources and the conversion of non-fluorescent compounds into fluorescent products by pre-column and post-column derivatization reactions are discussed. For the applications the emphasis is on drug analysis, where possible in biological fluids (serum, urine, etc.). The last paragraphs are divided in a number of sections in which newly developed and some scarcely used reagents are mentioned shortly; a more complete treatment is given of the reagents and labels most frequently used in the derivatization of

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certain functional groups. In this discussion the methods of derivatization as well as the selectivity, stability, fluorescence behaviour of the reagents/labels and derivatives and the reaction conditions are included. An up-to-date survey of the applications of fluorescence detection in liquid chromatography (TABLE III, TABLE IV and TABLE V), ends this review paper.

INTRODUCTION

The various modes of chromatography belong to the most frequently applied separation methods in many laboratories. The enormous success of chromatography has been triggered by the development of chromatographic systems with a high degree of separation power, together with the introduction of sensitive detectors suitable for the in situ (TLC) or on-line column chromatographic detection and quantitation of solutes. Chromatographic methods with sensitive detection are therefore particularly well suited for the analysis of micro quantities of material in complex matrices (1-4).

The separation of solutes in liquid chromatographic (LC) systems (5) is based on differences in one or more of the following properties, adsorption onto solid surfaces, partition coefficients in systems of two immiscible fluids, interaction (in the case of ionic solutes) with surface ion-exchange sites or molecular size.

In this paper we distinguish the following modes of LC systems: chromatography on non-modified silica or other polar stationary phases (normal phase, NP), chromatography with apolar stationary phases (reversed phase, RP), ion-exchange chromatography (IE) with macromolecular organic materials containing ionogenic functional groups as stationary phase and paired-ion (PI) chromatography. PI chromatography can be executed in various ways: RP adsorption and liquid-liquid partition systems, in the NP as well as in the RP mode.

In the most commonly used RP systems the stationary phase consists of an apolar chemically modified silicagel. The mobile phase is usually a mixture of water (or buffer solution) and an organic solvent, frequently methanol or acetonitrile. For an ade-

quate behaviour of the compounds under investigation in a particular chromatographic system the type of mobile phase that has to be used may not be compatible with the kind of medium necessary for a proper fluorescence behaviour of these chemicals. E.g., for compounds in which the fluorescence transition is of the $\pi \rightarrow \pi^*$ type, the frequently used RP systems in liquid chromatographic analysis are not the systems of first choice, because the quantum yield of fluorescence of these chemicals is often low in polar solvents.

In comparison with TLC and GLC, HPLC is a young branch of chromatography. It is a still rapidly growing technique, not in the least because of continuing advances in instrumentation and column technology. Combined with high-efficiency columns, on-line fluorescence detection is one of the most powerful analytical tools today. Although fluorescence detection can be applied in every chromatographic mode (FIGURE 1) the combinations of fluorescence detection with GLC, HPTLC and TLC are rare in comparison with the combination of fluorescence detection and HPLC.

In the following paragraphs fluorescence detection, including fluorescence enhancement and derivatization techniques, is discussed in combination with HPLC. The literature on the application of fluorescence detection in TLC, HPTLC and GLC has been summarized recently by Froehlich and Wehry (6) and Hulshoff and Lingeman (7).

In HPLC no successful universal detector (8-10), comparable to the flame ionization detector in GLC, has been developed. Among the various detection modes (TABLE I) used in LC, fluorescence detection has gained a prominent position.

For many fluorescent compounds fluorescence detection offers the highest level of sensitivity obtainable in HPLC. For some compounds, however, the electrochemical detection can be even more sensitive than the fluorescence detection (11). Due to the limited number of strongly fluorescing compounds, fluorescence detection offers a high degree of selectivity. The inherent disadvantage of limited applicability can be overcome by conversion of non-fluorescent compounds into fluorescent products.

FLUORESCENCE DETECTION IN CHROMATOGRAPHY
(References 1977-1983)

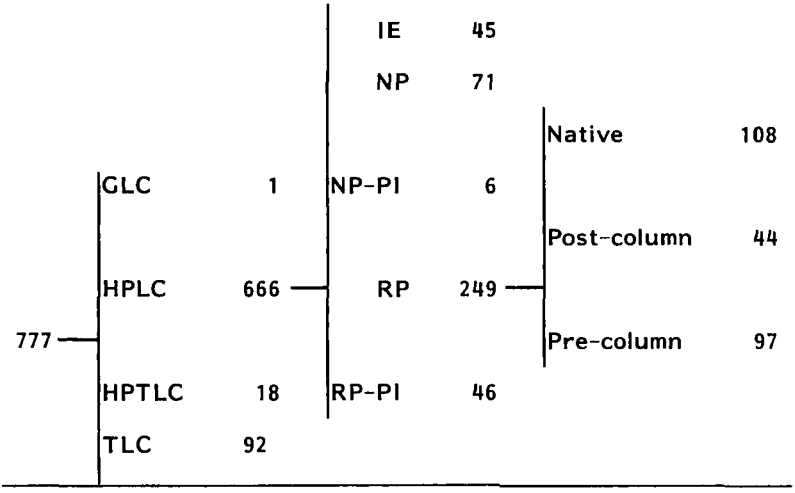

Figure 1 Reference: Hulshoff A. and Lingeman H., Fluorescence Detection in Liquid Chromatography, in "Applications of Luminescence Spectroscopy", Schulman S.G., Ed., Wiley, New York, *in press*

TABLE I
DETECTION IN LIQUID CHROMATOGRAPHY

Detection	Application Area	Sensitivity
Flame Ionization	very wide	μg range
Nuclear Magnetic Resonance	wide	μg range
Refractive Index	very wide	μg range
Absorbance(UV-Vis)	wide	ng range
Infrared	wide	ng range
Mass Spectrometry	very wide	ng range
Electron Capture	wide	ng-pg range
Atomic Absorption	narrow	pg range
Electrochemical (e.g. polarography)	variable	pg-fg range
Fluorescence	narrow	fg range

FLUORESCENCE AND INTRINSIC FLUORESCENCE SENSITIVITY

The recent upsurge of interest in fluorescence detection orientated techniques has resulted in a score of new labelling reagents and new applications of formerly described labels. In many cases, however, the fluorescence properties of the labels and the corresponding derivatives are unknown under conditions relevant for chromatography.
Without this information newly developed fluorescence labels and synthetised derivatives cannot be fully assessed to their value.

Principles of Fluorescence

Fluorescence is the emission of light accompanying the transition of an electronically excited molecule to its ground electronic state. The theoretical and practical aspects of fluorescence analysis of organic molecules are described in many textbooks (12, 13). Therefore, in this paper only a brief survey is given concerning those aspects that are important for the fluorescence labelling of organic compounds.

The excitation process of the π- or non-bonded electrons of the absorbing molecule is achieved by irradiation with light with an appropriate wavelength. The process of returning of the molecule to its ground electronic state begins with the loss of the excess vibrational energy (vibrational relaxation) to achieve the lowest excited singlet state.
For aliphatic molecules which have a high degree of vibrational freedom, the molecule returns to its ground electronic state by a subsequent vibrational relaxation process and no fluorescence will be observed.
For aromatic and highly conjugated molecules having a low degree of vibrational freedom the vibrational relaxation pathway is not effective and the molecule returns to its electronic ground state by emitting the difference in energy between the ground state and the lowest excited singlet state in the form of fluorescence light.

If all molecules arriving in the lowest excited singlet state should emit fluorescence, the observed fluorescence energy will be almost equal to the energy of the absorbed light (I_a). However, because of the several processes that are competing with each other for deactivation of the lowest excited state, the intensity of fluorescence (I_f) usually is a fraction (ϕ_f) of I_a (Equation 1).

$$I_f = \phi_f \cdot I_a \tag{1}$$

ϕ_f is called the quantum yield of fluorescence and for most compounds the value is far from unity. For quinine bisulfate in 0.1 N sulfuric acid, a drug with good fluorescence properties, the quantum yield is 0.55 . ϕ_f only approaches unity for compounds such as fluorescein or rhodamine B, but these are exceptions. For most commercial instrumentation, ϕ_f must be greater than 0.01 for fluorescence to be observable.

A combination of the Beer law (2)

$$I_a = I_0 \cdot (1 - 10^{-A.c.l}) \tag{2}$$

and Equation (1) leads to

$$I_f = \phi_f \cdot I_0 (1 - 10^{-A.c.l}) \tag{3}$$

where I_0 is the intensity of the primary light, A the molar absorptivity, c the concentration of the solute and l the path-length of light through the sample. Equation (3) indicates that I_f is not linear with the analyte concentration, but for values of A.c.l less than 0.02 Equation (3) can be simplified to

$$I_f = 2.3 \cdot \phi_f . A.c.l \tag{4}$$

This means that only at very low absorbances I_f is linear with the solute concentration.

Intrinsic Fluorescence sensitivity

In absorption studies the molar absorptivity (A) is a good quantitative parameter to establish the absorption behaviour of the compounds under investigation and, in HPLC analysis, its detection sensitivity. The quantum yield of fluorescence (ϕ_f) itself, however, is not a good parameter to quantify the fluorescence sensitivity of a compound, because it provides no information on the number of absorbed photons. The molar absorptivity (total number of absorbed photons) and the band width at half height of the emission spectrum of the drug are also important for the fluorescence sensitivity. This width at half height (BW), expressed in cm^{-1}, of the emission band controls the intensity distribution over the part of the emission spectrum seen by the detector and is therefore important for the sensitivity of the detector cell.

In 1960 Parker and Rees (14) mentioned a way of describing the fluorescence sensitivity of compounds and later on Lloyd (15) called it the intrinsic fluorescence sensitivity (IFS)(Equation 5).

$$IFS = \frac{\phi_f \cdot A}{BW} \qquad (5)$$

In order to calculate the IFS the molar absorptivity and the ϕ_f have to be known.

The band width can be calculated from the emission spectrum. The quantum yield of fluorescence can be measured with absolute (16) or relative (17) measurements. The most widely used method of determining quantum yields is by the relative method. This method is also the most suitable one for calculating the IFS, and has the advantage that no expensive fluorimetric equipment is required. The equation to calculate the quantum yield of a certain compound (18, 19) is:

$$\phi_x = \phi_r \cdot \frac{a_r \cdot E_x \cdot I_r \cdot (n^2)_x}{a_x \cdot E_r \cdot I_x \cdot (n^2)_r} \qquad (6)$$

x: compound under investigation
ϕ: quantum yield
a: absorbance of the solution
E: corrected emission intensity
r: reference compound
I: intensity of excitation light
n: average refractive index of the solution

Of course, if the same solvents and excitation wavelengths are used the I_r/I_x and $(n_x)^2/(n_r)^2$ ratios reach unity. With these prerequisites Equation 6 can be simplified to:

$$\phi_x = \phi_r \cdot \frac{a_r \cdot E_x}{a_x \cdot E_r} \qquad (7)$$

The absorbance of the solutions should not exceed the value of 0.01 to avoid inner-filter effects. E is the integrated area under the corrected emission spectrum.

In TABLE II a survey is given of the quantum yields and intrinsic fluorescence sensitivities of some derivatives of fluorescence labels. To detect 1 ng or less of the compound that has to be derivatized the IFS of the derivative with an appropriate label must be 0.5 or more. To limit the interferences (absorbance and background fluorescence) it is desirable that the excitation wavelength is higher than approximately 300 nm.

Influences on the Fluorescence Sensitivity

The presence of rigid planar aromatic rings such as benzene, naphthalene, anthracene or their heteromatic analogs pyridine, quinolone or acridine, etc., is essential for fluorescence. In general the addition of electron donating structures such as: $-NH_2$, -OH, $-OCH_3$ or $-N(CH_3)_2$ will enhance the fluorescence and the presence of electron withdrawing groups such as: $-NO_2$, -CN, -Cl, -COOH or -COH will diminish the fluorescence (20). However, there are many exceptions to these rules and it is not possible to give a complete set of rules for the influences of functional groups on the fluorescence of a molecule.

The effects of the solvent on the fluorescence are determined by the nature and degree of the interactions of the solvent with

TABLE II
QUANTUM YIELD of FLUORESCENCE and INTRINSIC FLUORESCENCE SENSITIVITY

Label	Derivatized compound	Solvent	λ (excitation)	ϕ_f	IFS	Reference
9-(Hydroxymethyl)anthracene	Benzoic acid	Methanol	365 nm	0.10	0.49	23
		Water	365 nm	0.01	0.05	23
9-(Chloromethyl)anthracene	Lauric acid	Methanol	365 nm	0.34		24
4-Hydroxymethyl-7-methoxycoumarin	Acetic acid	Methanol	323 nm	0.09	0.32	15
		Water-Methanol (9+1)	323 nm	0.43	1.57	15
4-Bromomethyl-6,7-dimethoxycoumarin	Acetic acid	Methanol	343 nm	0.43	1.14	25
		Water	343 nm	0.64	2.36	25
7-Methoxycoumarin-3-carbonyl azide	Cholesterol	Methanol-Water	335 nm	0.60		26
N,N'-Diisopropyl-O-(7-methoxy-coumarin-4-yl)methylisourea	Phenylpyruvic acid	Cyclohexane	325 nm	< 0.01		27
		Ethanol	325 nm	0.01		27
		Water-Ethanol (9+1)	325 nm	0.05		27
9,10-Diaminophenanthrene	Formic acid	Methanol	254 nm	0.69	19.7	28
		Water-Methanol (9+1)	254 nm	0.60		28
		Cyclohexane	254 nm	0.31		28
Dansyl chloride	1,2-Diaminoethane	Acetonitrile	355 nm	0.26	0.28	29
		Methanol	355 nm	0.18	0.17	29
		Water	355 nm	0.01	0.01	29
2-Dansylethyl chloroformate	Cholesterol	Methanol	342 nm	0.32	±0.5	30
		Acetonitrile-Water-Tetrahydrofuran (5+1+4)	342 nm	0.41	±0.7	30
Fluorescamine	Ethylamine	Ethanol	366 nm	0.21		31
	Leucylalanine	Water	366 nm	0.34		31

the solute molecules. Interactions which are predominantly electrostatic in nature and may be classified as dipolar or hydrogen bonding are particularly important (21). Molecules having lowest excited singlet states of the $n \rightarrow \pi^*$ type rarely fluoresce in aprotic or nonpolar solvents. However, in polar hydrogen bonding solvents such as ethanol these molecules become fluorescent. On the other hand for many of these compounds water has a quenching effect on the fluorescence. The fluorescences of hydrocarbons, $\pi \rightarrow \pi^*$ transitions of the lowest excited singlet state, are usually better in nonpolar hydrocarbon solvents. Furthermore, the use of heavy atom solvents (e.g. alkyl iodide) must be avoided, because the fluorescence is always less intense than in solvents of low molecular weight.

Not only the intensity of fluorescence is influenced by the factors mentioned before but also the emission wavelength. The $n \rightarrow \pi^*$ transitions usually emit at longer wavelengths than the $\pi \rightarrow \pi^*$ transitions but for the latter the intensities of the emissions are generally higher.

The fluorescence intensity of compounds that fluoresce in aqueous media often changes with a change of pH due to the dissociation of acidic functional groups or protonation of basic functional groups associated with the aromatic portions of the fluorescing molecule (22).

The intensity of the emitted radiation is proportional to the concentration of the compound at low concentrations. The non-linearity at higher solute concentrations can be explained by inner-filter effects; e.g., re-absorption of emitted radiation by solute or solvent molecules. This is known as concentration quenching. Not only high concentrations of solute molecules will quenche the fluorescence but the fluorescence can also be diminished by high concentrations of additional molecules or ions.

The presence of high concentrations of another absorbing, but non-fluorescent component (other than the solute) in the solution can influence the sensitivity, precision and accuracy of the analysis of the solute.

An increase in temperature usually decreases the fluorescence. For most compounds a 1-2% decrease in fluorescence with 1° C increase in temperature can be expected, but in some cases a 10% decrease with 1° C rise in temperature has been reported. Some solutes decompose when they are exposed to UV or visible radiation. This also effects the precision of a quantitative measurement.

FLUORESCENCE DETECTION IN HPLC

Fluorimetry is a well-known analytical method with high selectivity and sensitivity which makes it possible to detect small quantities of many important biological compounds, e.g. pharmaceuticals, steroids and vitamins. Detection of pg amounts and in some cases even fg amounts of drugs is possible.

The fluorescence of eluted solutes can be detected on-line in a fluorimeter equipped with a flow cell; fluorescence detection at fixed wavelengths of excitation and emission is thus as simple and straight forward as the UV-absorbance detection.

In case of solvent programming and other systems in which the composition of the mobile phase changes during the analysis the fluorescence can be enhanced or diminished by changes in the physico-chemical properties of the fluorophore, changes in the interaction of the fluorophore with the eluent or by inner-filter effects. In these cases special measures are required for reliable measurements of the fluorescence.

Fluorescence Detectors

Conventional HPLC fluorescence detectors today are basically similar to fluorimeters used for off-line measurements, with exception of the low volume flow-cell (less than 20 μl). The design and use of these detectors have been described by several authors (32-36).

For the selection of excitation and emission wavelengths the detector can be equipped with filters or monochromators. Due to the se-

lectivity provided by the HPLC column, wavelength selection with filters is frequently adequate. Filter instruments also offer higher excitation and emission radiation intensities and therefore better sensitivity (35). On the other hand for the analysis of drugs in complicated matrices such as serum and urine the monochromator equipped instruments offer the possibility of further enhancement of the selectivity which is frequently essential. As a compromise instruments are developed with monochromatic excitation radiation and broad spectral band-pass of the emitted radiation by blocking or cut-off filters.

The excitation radiation source is practically always either the mercury lamp with a few narrow bands of high intensity radiation or a continuous source lamp: the deuterium lamp or the xenon lamp. The xenon lamp is at present the most frequently applied radiation source with a continuous spectrum of fairly high intensity from 260 to 660 nm.

The advantages, disadvantages and applications of the different radiation sources are described by Abbott and Tusa (37) in a recent review on optical detection and by Hulshoff and Lingeman (7). To achieve better sensitivity or to lower the noise level in detector devices, excitation by a β-radiation source (38-41) or a laser source is developed.

Laser induced fluorescence detection offers some definite potential advantages over the conventional light sources (42, 43): production of a very high photon flux (high excitation energy), improvement of the signal to noise ratios and the possibility of an accurate positioning and focussing of the beam. Improved spectral selectivity or sensitivity can be obtained by some recent developments in laser excited fluorescence detection: time-resolved fluorescence (44, 45), two photon excited fluorescence (46-48), sequentially excited fluorescence (49), solid-state fluorescence based on Shpol'skii spectroscopy (50, 51), high-temperature supersonic jet fluorescence (52, 53) or the use of second derivative spectrometry (54, 55).

Flow Cells

The volume of the flow cell must be kept small to minimize post-column band broadening. The basic need to supply the cell with sufficient excitation energy and to collect the emitted radiation requires transparancy on more than one axis of the cell. Light scattering by reflection and refraction from the walls of the flow cell is a major source of interference, particularly with laser based detectors.

Flow cells are usually constructed of narrow bore quartz tubing. Stray light interference can be effectively eliminated by detecting fluorescence with right-angle geometry from flow cells with a linear bore of the square cross section (56, 57).

In view of the advantages in the use of microcolumns in HPLC over conventional 2-5 mm I.D. columns, the development of flow cells with volumes less than 1 μl and excitation path lengths of 1 cm is of current interest. A recent development is the use of fiber optics (58, 59), where the excitation energy is guided to the cell by an optical fiber. The use of absorption-corrected fiber optics in flowing streams to reduce the inner-filter effects is presented by Ratzlaff (60).

Some potential sources of error connected with quartz flow cells can be eliminated by using the free falling drop detector (61), a windowless flow cell (62, 63) or the application of the sheath flow principle (64) to achieve an improved sensitivity or to reduce stray light effects.

Monitoring of Excitation-Emission Spectra in the HPLC Effluent

Fluorescing compounds in chromatographic effluents can be characterized by different parameters: retention time, fluorescence sensitivity and excitation and emission bands. Thus, in addition to instruments employing single excitation or emission wavelengths, detectors that are designed to scan the excitation or emission spectra of solutes eluted from an HPLC column can give a considerable amount of of qualitative and/or quantitative information. Scanning can be performed after stopping the flow (65). In principle, this is

the easiest way to obtain the excitation and/or emission spectra of fluorescing compounds in the detector zone. However, diffusion of the solute from the detection zone during scanning causes irreproducible distortions of the spectra and loss of resolution between adjacent solute bands.

The scanning of fluorescence spectra "on-the-fly" in the HPLC effluent, without altering the flow, is therefore more desirable. The possibility of rapid scanning (about 10 nm/s) has been discussed (66). Some of the spectroscopic resolution is lost by rapid scanning, but the spectra thus obtained contain usually sufficient information for identification of the solute. On-the-fly rapid scanning can also serve to determine whether a chromatographic peak results from one or more eluted compounds which will become evident by appreciable spectral changes, provided the compounds possess different fluorescent properties.

The loss of spectral resolution inherent to on-the-fly mechanical scanning of spectra can be overcome by the use of electronic array detectors (67), intensified vidicon multichannel analyzer systems (68) or the rapid scanning video fluorimeter (69-71). These detectors provide multiple excitation and emission spectra in a single (non-mechanical) scan without any need for stopping the flow eluent. Thus, three dimensional fluorescence intensity plots can be constructed at any time during elution. Strategies for analyzing data from video fluorimetric monitoring of effluents have been proposed (72).

FLUORESCENCE DETECTION OF NON-FLUORESCENT COMPOUNDS AND FLUORESCENCE ENHANCEMENT BY ELUENT MANIPULATION

Relatively few compounds show a sufficiently high quantum yield of fluorescence to allow detection at levels comparable to or lower than those obtained with UV absorption detection. The composition of the eluent (solvents, buffers and other additives, pH, ionic strength and viscosity), its oxygen content and temperature

are of paramount importance for the fluorescence detection. Moreover, weakly fluorescing or non-fluorescent compounds can be converted into fluorescing derivatives whether before or after HPLC separation.

One of the possibilities is to utilize the dependence of the emitted radiation on the environment of the fluorescing compounds by the addition of fluorescing compounds to the eluent. An HPLC detection method for lipids based on this principle is developed (73). A continuous stream of an aqueous solution of a fluorescent dye, 1-anilino-naphthalene-8-sulfonic acid (ANS), is mixed with the column effluent, giving a certain level of base-line fluorescence. The fluorescence intensity of ANS in aqueous solutions is increased in the presence of lipids. The lipids probably form micelles, into which the ANS molecules are trapped. In these hydrophobic surroundings ANS exhibits enhanced fluorescence, allowing the lipids to be detected. The addition of a solution of β-cyclodextrin to the column effluent results in a significant increase of the fluorescence of some derivatized thiols (74). The addition of small amounts of aniline to the eluents in RP chromatography makes the detector respond to fluorescent and non-fluorescent compounds (75).

The fluorescences of dansylated phenols decrease in solvents with increasing dielectric constant with a concomittant red shift of the excitation-emission spectra (76). For indoles and aromatic amino acids (77) is concluded that in mixed aqueous solvents (e.g. ethanol-water, DMSO-water) the fluorescence is enhanced in comparison to pure water.

At low pH values the fluorescence of aqueous solutions of dansyl derivatives is markedly decreased (78). Post-column acid/base manipulation can help to increase the sensitivity and/or the selectivity of the fluorescence detection system (79).

Conversion of Non-Fluorescent Compounds into Fluorescent Products

The compounds of interest can be converted into fluorescent products by a number of methods either before or after the chromatographic separation. Fluorescence labelling (i.e. the attachment

of one or more fluorophores to the molecule through covalent binding) is discussed in the next paragraph.

Many other types of derivatization reactions have been described as well. Some examples are given to demonstrate the broad range of possible reactions. Phenothiazines can be detected after post-column oxidation (80). Indomethacin yields fluorescent products by deacylation prior to HPLC analysis (81). 8-Hydroxyquinoline forms strongly fluorescing metal chelates with Mg(II) (82). Cortisol is converted into fluorescing product(s) by heating with an ethanol-sulfuric acid mixture (83) and digitalis glycosides by heating them with concentrated hydrochloric acid (84). Dimethoxyanthracene sulfonate (DAS) can serve as a fluorescent counter ion for post-column ion pair extraction of some basic drugs (85).

Another approach is using non-fluorescent reagents which are converted upon reaction with the solutes in the eluent into fluorescent products. Many compounds, such as the carbohydrates can be oxidized by cerium(IV), which is reduced to the strongly fluorescent cerium(III) (86) or ligand exchange reaction of organosulfur compounds with the non-fluorescent palladium(II)-calcein complex, resulting into the decomposition of the palladium (II)-calcein complex and the release of the fluorescent calcein (87).

Fluorescence Labelling

The recently developed fluorescent labelling reagents together with the older reagents offer the chromatographer ample choice and fluorescence labels are now available for several functional groups (TABLE III).

The selectivity of the detection system is limited by fluorescence labelling, as any other method in which a reagent reacts with functional groups. This relative loss in selectivity in favour of enhanced sensitivity can be minimized by the use of fluorescence reagents which react with one type of functional group or structural moiety only. On the other hand, the separation power of modern HPLC columns does not always require the best selectivity of the

TABLE III
FLUORESCENCE INTRODUCING LABELS AND REACTING FUNCTIONAL GROUPS

REAGENT	ABBREVIATION	FUNCTIONAL GROUP	REFERENCE
N-(9-Acridinyl)maleimide	NAM	Sulfhydryl	184, 185
(D)-(L)-1-Aminoethyl-4-dimethylaminonaphthalene	DANE (*)	Carboxyl	252, 253
4-Amino-7-nitrobenzo-2-oxa-1,3-diazole	NBD-amine	*Hydrolytic enzymes*	222
9-Aminophenanthrene		Carboxyl	263
2-Aminopropionitrile-fumarate-borate	AFB	*Carbohydrate*	290
1-Anilinonaphthylmaleimide	ANM	Sulfhydryl	180
1- and 9-Anthroylnitrile		Hydroxyl	289
p-(9-Anthroyloxy)phenacyl bromide (panacyl bromide)		see Br-Mmc	260
9-Anthryldiazomethane	ADAM	see Br-Mmc	257, 258
p-(2-Benzimidazolyl)phenylmaleimide	BIPM	Sulfhydryl	179
Benzoin		Guanidine	291, 292
p-(2-Benzoxazolyl)phenylmaleimide	BOPM	Sulfhydryl	186
Boc-aminomethyl-/Boc-aminophenylisothiocyanate		Amine	279
2-Bromoacetonaphthon (naphthacyl bromide)		see Br-Mmc	259
1-Bromoacetylpyrene		see Br-Mmc	261
4-Bromomethyl-7-acetoxycoumarin	Br-Mac	see Br-Mmc	203
4-Bromomethyl-6,7-dimethoxycoumarin	Br-Mdmc	see Br-Mmc	25
4-Bromomethyl-7-methoxycoumarin	Br-Mmc	Carboxyl, Imide, Phenol, Sulfhydryl	193, 194
N-Chlorodansylamide	NCDA	Sulfhydryl	156, 157
9-(Chloromethyl)anthracene	9-CIMA	see Br-Mmc	24
4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole	NBD-Cl	Amine (prim., second.), Phenol	207, 208
2-Cyanoacetamide		Reducing hydroxyl	284, 285
2-Dansylaminoethanol	DAE (*)	Carboxyl	254
2-Dansylethylchloroformate	Dns-ECF	see FMOCCl	30
9,10-Diaminophenanthrene	DAP	Carboxyl	28, 265
4-Diazomethyl-7-methoxycoumarin	D-Mmc	see Br-Mmc	204
5-Di-n-Butylaminonaphthalene-1-sulfonyl chloride	Bns-Cl	see Dns-Cl	142
N,N'-Dicyclohexyl-/n,N'-Diisopropyl-O-(7-methoxycoumarin)methylisourea	DCCI/DICI	Carboxyl	27, 202
4,5-Dimethoxy-1,2-diaminobenzene	DBB	Aldehyde	293, 294
N-(7-Dimethylamino-4-methyl-3-coumarinyl)-maleimide	DACM	Sulfhydryl	181
5-Dimethylaminonaphthalene-1-sulfonyl-aziridine	Dns-A	Sulfhydryl	144, 145
5-Dimethylaminonaphthalene-1-sulfonyl-cadaverine	Dns-C (*)	Carboxyl	29
5-Dimethylaminonaphthalene-1-sulfonyl chloride	Dns-Cl	Amine (prim., second.), Hydroxyl, Phenol, Sulfhydryl	122, 130
5-Dimethylaminonaphthalene-1-sulfonyl-hydrazine	Dns-H	Carbonyl	146, 153
4-Dimethylamino-1-naphthoylnitrile	DMA-NN	Hydroxyl (prim., second.)	288
4-Dimethylamino-1-naphthylisothiocyanate		Amine	278
2-Diphenylacetyl-1,3-indandione-1-hydrazone	DIH	Aldehyde	295
1,2 Diphenyl-ethylenediamine	DPE	Reducing hydroxyl	296
9-Fluorenylmethylchloroformate	FMOCCl	Amine	118
Fluoresceinisothiocyanate		Amine	276
4-Fluorobenzo-2-oxa-1,3-diazole-7-sulfonate	SBD-F	Sulfhydryl	218, 219
4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole	NBD-F	see NBD-Cl	213, 217
4-Fluoro-7-sulfamoylbenzo-2-oxa-1,3-diazole	NH_2-SBD-F	see SBD-F	220
Glycinamide		Hydroxyl	297
4-Hydrazino-7-nitrobenzo-2-oxa-1,3-diazole	NBD-H	Carbonyl	221
4'-Hydrazino-2-stilbazole	4H2S	Carbonyl	269, 270
9-(Hydroxymethyl)anthracene	HMA (*)	Carboxyl	23
4-Hydroxymethyl-7-methoxycoumarin	Hy-Mmc (*)	Carboxyl	195, 201
9-Isothiocyanatoacridine		Amine	275
7-Methoxycoumarin-3/-4-carbonyl azide	3-MCCA/4-MCCA	Hydroxyl	26
2-Methoxy-2,4-diphenyl-3(2H)-furanone	MDPF	see Flur	170, 171
4-(6-Methylbenzothiazol-2-yl)phenylisocyanate	Mbp	Amine	280, 281
(+)/(-)-2-Methyl-1,1'-binaphthalene-2'-carbonyl-nitrile		Hydroxyl	287
5-Methylphenylaminonaphthalene-1-sulfonyl chloride	Mns-Cl	see Dns-Cl	143, 121
Monobromo-trimethyl-ammoniobimane		Sulfhydryl	271, 272
1,2-Naphthoylenebenzimidazole-6-sulfonyl chloride	NBI-SO_2Cl	see Dns-Cl	273, 274
1-Naphthylamine		Caroxyl	262
2-Naphthylchloroformate	NCF	see FMOCCl	119
Naphthylisocyanate	NIC	Amine	298
o-Phenylenediamine		α-ketocarboxyl	266, 268
Phenylisothiocyanate		Amine	277
4-Phenylspirofuran-2(3H)-1'-phthalan)-3,3'-dione (fluorescamine)	Flur	Amine (prim., second.)	158, 121
o-Phthalaldehyde (o-Phthaldialdehyde)	OPA	Amine (prim., second.)	225, 231
N-(1-Pyrene)maleimide	PM	Sulfhydryl	182
N-Succinimidyl-2-naphthoxyacetate	SNA	Amine	282

(*)Derivatization in two steps: first activation of functional group that has to be derivatized followed by reaction with fluorescence introducing label

labelling reagents. Furthermore, for the chromatographer there are definite advantages in using only a few reagents, which cover a broad range of compounds with different functional groups and for which the optimum reaction conditions have been well established. Alkylating reagents (e.g. 4-methyl-7-methoxycoumarin) or acylating reagents (e.g. 5-dimethylaminonaphthalene-1-sulfonylchloride) react with several functional groups which possess an active hydrogen.

Ideally the following conditions are fulfilled in a fluorescence labelling procedure:

- the absorption transition of the lowest energy of the introduced fluorophore is intensive (large molar absorptivity);
- the fluorophore does not contain structural moieties or functional groups which increase the rates of radiationless transitions (large quantum yields);
- the reagent solution used for the derivatization reaction is stable for prolonged periods;
- the fluorogenic reagent and its degradation products formed during the reaction are non-fluorescent or they are well separable from the derivatized compound of interest;
- the compound of interest is rapidly and quantitatively derivatized under mild conditions, yielding a single product;
- the derivatives are stable and possess favourable chromatographic properties;
- the formation of relatively non-polar derivatives is advantageous to achieve a successful isolation and concentration by extraction with organic solvents;
- the reagent is non-toxic.

When developing a derivatization procedure for a certain compound the influence of all reaction conditions (choice of solvent, concentrations of reactants and catalyst(s), temperature and reaction time) must be thoroughly investigated to obtain optimal reaction conditions.

The identity of the reaction product(s) must be established and the fluorescence properties of the derivative(s) in several HPLC eluents determined. The reproducibility of the labelling procedure

should be thoroughly tested, especially if the yield of the reaction is less than 100%.

Pre-Column Derivatization

Pre-column derivatization is the most widely used derivatization technique with many advantages and only a few disadvantages (1, 88-91). Because the derivatization is performed prior to the chromatographic separation the major advantages are:

- no restrictions on the reaction kinetics, provided that the reaction goes to completion within a reasonable time, yielding one derivatization product for the compound in question without the formation of side products;
- a free choice in varying the conditions in order to optimize the reaction time and reaction yield;
- the solvent in which the pre-column reaction takes place need not be compatible with the mobile phase of the HPLC system;
- side products formed during the derivatization which might quench the fluorescence of the derivative will be separable, either on the HPLC column or by a pre-chromatographic clean-up step.

The main disadvantage of the pre-column derivatization is sometimes the formation of side products which cause troubles in the chromatographic analysis or in the reproducibility of the derivatization reaction.

One of the most frequently encountered problems in derivatization procedures is the occurrence of interfering peaks in the chromatograms, due to degradation products or impurities of reagents and/or solvents.

Great care should therefore be taken in checking the purity of the reagents and solvents - the analysis of control samples (reagents blanks) is essential - and in storing them under suitable conditions. Impurities in solvents causing interferences can be hard to identify and to remove.

Ideally the components of the derivatization mixture do not cause changes in the chromatographic system through chemical re-

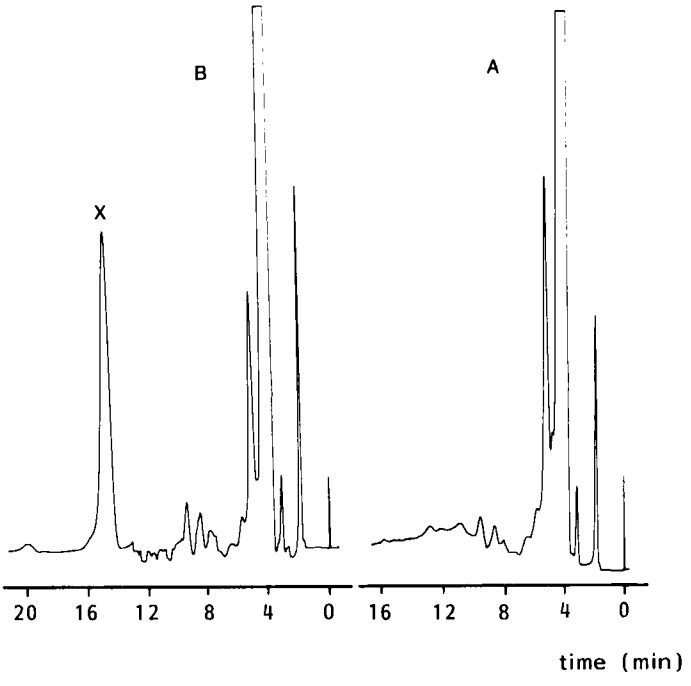

Figure 2

Chromatograms obtained after derivatization of ibuprofen with 9-(Hydroxymethyl)anthracene after extraction from plasma (B) and from the reagent blank (A). Peak x is 5 nmol ibuprofen derivative. Column, LiChrosorb RP 18 (10 μm); eluent, methanol-water (9+1); excitation wavelength, 365 nm; emission wavelength, 415 nm; flow 1 ml/min; room temperature. (Reference 23)

actions, precipitation or demixing of the eluent upon injection or otherwise, and do not interfere with the detection of the derivatized compounds. If these conditions are fulfilled, aliquots of the derivatization mixture can be directly injected into the chromatographic system (Figure 2). However, it is frequently necessary to remove the excess reagent or the solvent or other components of the reaction mixtures before HPLC analysis.

Evaporation of the reagent mixture under a stream of dry nitrogen, either at room temperature or under heating, is a simple and convenient way of removing the solvents, and if volatile, the

reagent. This procedure is also a concentration step. Pre-column clean-up of the derivatization can also be achieved by liquid-liquid extraction or other pre-column separation steps. Excess reagent is sometimes removed by allowing it to react with an excess of another compound; neither that compound nor its derivative must interfere with the analysis.

Although automated post-column labelling is more frequently used, automated pre-column labelling methods are described (92, 93).

Post-Column Derivatization; Reaction Detectors

Considerable efforts have been put into the development of on-line post-column derivatization methods and reaction detectors. A number of reviews on these topics have been published recently (94-100).

Post-column reactions take place in a part of the HPLC system, called the reactor, between the column and the detector. Post-column derivatization techniques have some important advantages in comparison with pre-column methods:

- the reaction must be reproducible without the necessity of the formation of only one derivative;
- the underivatized compounds are separated and eluted from the column and can therefore be detected with other, non-destructive, detection methods before being derivatized.

Obvious disadvantages of post-column derivatization methods are:

- the restricted freedom in the choice of the reaction conditions;
- band broadening in the reactor with a resulting loss of chromatographic resolution;
- the possible interference by excess reagent or reagent degradation products in the detection of the derivatized compounds;
- the need for instrument modifications to suit the post-column reaction.

Reactors consist of one or more units (made of inert material), depending on the chemical operations to be carried out. Low press-

ure peristaltic pumps and low volume mixing tees are used to add reagent solutions, solvents or air to the column effluent.

Three different types of post-column reactors have been described. These are tubular or capillary reactors with a non-segmented reaction medium, bed reactors and segmented stream reactors. The design of post-column reaction detectors and the theory concerning the effects influencing the sensitivity and resolution in these systems are discussed in recent reviews by Van der Wal (101) and Hulshoff and Lingeman (7).

The tubular or capillary reactor is the simplest reactor to handle and is well-suited for fast reactions. This reactor is to be preferred over the other reactor types only for reactions which take place in a few seconds (the reaction of primary amines with fluorescamine). Coiling of the capillary and diminishing of the internal diameter of the capillaries helps to minimize band broadening.

Bed reactors are columns filled with small glass beads to provide an extra surface area for the reaction to proceed. The particle size of the glass beads is important and should be small to avoid excessive band broadening. The bed reactor is considered to be a suitable device for reaction times up to 4 minutes.

In the flow segmented systems air bubbles or solvent plugs which are immiscible with the mobile phase, are introduced into the column effluent. The segmentation of the flow serves to reduce band broadening in case of comparatively long residence times of the solutes in the reactor system. Slow on-line post-column reactions are then still feasible.

However, the choice of reactor type does not only depend on the reaction time. Extreme reaction conditions, e.g. high temperatures or the use of agressive reagents, limit the freedom of reactor design. Solvent-segmented systems must be used in case of extraction detection systems. Packed bed reactors are particularly well suited for reactions taking place at solid-liquid interfaces as illustrated by the immobilized enzyme bed reactor (102). The beads of this packed-bed reactor are coated with an enzyme, which interacts with the solute(s) in the column effluent.

Excess reagent frequently interferes with the fluorescence signal of the derivatized compound. If the difference between the partition coefficients of the derivative and the reagent is sufficiently large, separation by liquid-liquid extraction prior to detection is possible. In such an extraction detector the eluent flow is segmented with non-miscible solvent plugs into which derivative or reagent partition; the plugs also serve to diminish band broadening (103).

Post-column photochemical reactors have been applied to improve the fluorescence detection properties of selected groups of compounds (104, 105). The simplest way of achieving this is by UV irradiation of the HPLC effluent flowing through a reactor coil with good UV transparancy (e.g. teflon). The tubular reactor system can be coiled around the UV source. The selectivity of this detection principle is very high, because interference can only be caused by compounds that co-elute with the solute of interest and undergo a fluorescent derivative producing photochemical reaction as well. Furthermore, photochemical reagents added to the eluent can increase the applicability of this type of reaction detector. For instance, aldehydes, aliphatic alcohols and ethers can be detected by the addition of a sensitizer, anthraquinone-2,6-disulfonate, to the mobile phase of an RP HPLC system (106, 107). This reagent is reduced by the aliphatic oxygen containing compounds in a photochemical reaction resulting in the formation of the highly fluorescent hydroquinone.

FLUORESCENCE INTRODUCING REAGENTS AND METHODS

A great number of drugs in biological materials has been determined with high sensitivity using HPLC-fluorescence methods as can be seen in the TABLES IV and V. However, it is not always possible to obtain satisfactory results in the determination of compounds in complex biological materials, due to the influences of several external factors.

TABLE IV
(BIO-)ANALYTICAL APPLICATIONS OF LIQUID CHROMATOGRAPHY WITH NATIVE FLUORESCENCE DETECTION

I. ACIDIC COMPOUNDS

Compound(s)	Sample			Clean-up	HPLC	Reference
I-a Carboxylic acids						
Bromo-Lasalocid	plasma	1	ml	b	RP	299
Carprofen	plasma	1	ml	b	NP	300
Gentisic acid	urine			-	RP-PI	301
Gibberellins					RP	302
Hippuric acid	urine	1	ml	a	NP-PI	303
Ibuprofen	plasma	1	ml	a	RP	304
3-Indolacetic acid	urine				IE	305
	urine	.05	ml	b	RP	306
	urine	.2	ml	-	RP-PI	307
Indomethacin	urine	.4	ml	b	RP	308
Isoxepac	plasma	1	ml	b	RP	309
Lonazolac	serum	.2	ml	c	RP	310
Naproxen	plasma	.01	ml	-	RP	311, (312)
Salicylic acid	plasma	.01	ml	-	RP	311
	urine			-	RP-PI	301
Vanillylmandelic acid	urine	5	ml	d	RP-PI	313
I-b Phenolic compounds						
Alkylphenols					NP/RP	314
Capsaicin	blood			a	RP	315
	tissue			a	RP	315
o-Cresol	urine	10	ml	d	NP-PI	303
Meptazinol	plasma	1	ml	b	RP	316
1-Naphthol	blood	.25	ml	b	RP	317
Phenols					RP	318, 319
Salicylamide	plasma	1	ml	-	RP	320
I-c Miscellaneous						
Atracurium besylate	plasma	2	ml	d	IE	321
Porphyrins	fluids				RP	322
	urine	.025	ml	-	RP	323, 324
	urine	10	ml	b/c	RP	325, 326
	blood	.1	ml	a	RP-PI	327, (328)

II. AMPHOTERIC COMPOUNDS

Compound(s)	Sample			Clean-up	HPLC	Reference
II-a Amino acids						
Hydroxyphenylalanine					RP	329
Phenylalanine	plasma	2	ml	a	RP	330
Tryptophan	brain			a	IE	331, 332
	plasma	.02	ml	a	RP	333, (334)
	urine	.02	ml	a	RP	333
					RP (PI)	335, 336
m-Tyrosine	plasma	2	ml	a	RP	330
II-b Miscellaneous						
Aminobenzoic acid	plasma	.1	ml	a	RP-PI	337
Aminosalicylic acid	plasma	1	ml	a	NP-PI	338
	urine	1	ml	a	NP-PI	338
	plasma	.1	ml	a	RP-PI	337, 338
Bumetanide	plasma	.2	ml	a/b	RP	339, 340
	urine	.2	ml	a/b	RP	339, 340
Furosemide	plasma	.5	ml	d	RP	341
	urine	.5	ml	d	RP	341
	plasma	.2	ml	a/b	RP	342, 343
	urine	.5	ml	a/b	RP	342, 343
Glyburide	serum	1	ml	b	RP	344

(continued)

TABLE IV *(continued)*

Compound(s)	Sample			Clean-up	HPLC	Reference
Metolazone	plasma	2	ml	d	RP	345
	urine	.5	ml	b	RP	345
Piretanide	plasma	.5	ml	d	RP	341
	urine	.5	ml	d	RP	341
Sulfapyridine	plasma			a	RP-PI	346
Sulfasalazine	plasma	.5	ml	a	RP-PI	346, 347
Sulpiride	serum	4	ml	b	RP	348
	urine	1	ml	b	RP	348
III. BASIC COMPOUNDS						
Compound(s)	Sample			Clean-up	HPLC	Reference
III-a Amines						
Alizapride	plasma	1	ml	a	RP	349
Amiloride	plasma	.1	ml	b	RP	350
	urine	.05	ml	b	RP	350
Amines					NP	351, 352
Apomorphine	plasma	1	ml	d	RP	353
Aptazapine	plasma	.5	ml	b	RP	354
Carbamazepine	plasma	.2	ml	a	RP	355
	urine			a	RP	355
Chloroquine	plasma	1	ml	c/d	NP	356, 357
	urine	.1	ml	d	NP	356
Chlorpromazine					NP	358
Citalopram	plasma	1	ml	b	RP	359,360
	urine	.2	ml	b	RP	360
Codeine	plasma	2	ml	b	RP	361
Desipramine	plasma	2	ml	b	RP	362
Dihydroergot alkaloids	plasma	5	ml	b	NP	363
	plasma	1	ml	b	RP	364
Ergometrine					IE	365
					RP-PI	365
Ergot alkaloids	plasma	3	ml	d	RP	366
Flecainide	plasma	.05	ml	b	NP	367
	blood	1	ml	a	RP	368
	plasma	1	ml	b	RP	369
Fostedil	plasma			c	RP	370
Glaucine	fluids	1	ml	d	IE	371
	plasma	.1	ml	b	NP	372
	urine	.2	ml	b	NP	372
Harmane alkaloids					RP	373
Imipramine	plasma	2	ml	b/d	RP	362, 374
Ketanserin	plasma	1	ml	b/d	RP	375, 376
	urine	.1	ml	b	RP	375
LSD					IE	377
	urine			d	RP	378, 379
Mexiletine	plasma	.05	ml	b	NP	367
Mianserin	plasma	.5	ml	b	RP	354
Nafazatron	plasma	1	ml	b	NP	380
	plasma	1	ml	d	RP	381
Nafimidone	plasma	.05	ml	d	RP-PI	382
Norverapamil	plasma	.1	ml	b	NP	352
Ochratoxin					RP-PI	383
Opiates					RP	384
Oxytocin					IE	365
					RP-PI	365
Pimozide	plasma	1	ml	d	RP	385
Pipotiazine	plasma	2	ml	b	NP	386
	urine	2	ml	b	NP	386
Prajmaline	plasma	1	ml	c	NP-PI	387

(continued)

TABLE IV *(continued)*

Compound(s)	Sample			Clean-up	HPLC	Reference
Prazosin	fluids	1	ml	d	IE	371
	plasma	.2	ml	a	RP	355, 388
	plasma	1	ml	b	RP-PI	389, 390
Pyrimethamine	plasma	.5	ml	b	NP	391
Quinidine	plasma	.5	ml	a/d	RP	392, 393
	urine			a	RP	394
	serum	1	ml	d	RP-PI	395
Ro 11-2465	plasma	1	ml	b	RP	396
Ro 12-6995	plasma	1	ml	b	NP	397
Sulmazole	fluids	.05	ml	c	RP	398
Thiabendazole	serum	.05	ml	a	RP	399
Thiazides	urine	1	ml	-	RP	400
Tiodazosin	plasma	.2	ml	a	RP	388
Tricyclic antidepressants	plasma	1	ml	b	RP	401
Trimipramine	plasma	2	ml	b	RP	362
UK-33274	plasma	1	ml	b	RP-PI	402
Verapamil	fluids	.1-1	ml	d	IE	403
	plasma	.1	ml	a/b	RP	336, 404
	plasma	1	ml	b	RP-PI	405
III-b Aryloxypropanolamines						
Acebutolol					NP	406
	plasma	1	ml	d	RP	407
	urine	.05	ml	d	RP	407
Alprenolol	plasma	1	ml	d	RP	408, 409
Atenolol	plasma	1	ml	d	RP-PI	410
Bunitrolol	plasma	1	ml	b	RP	411
	urine	1	ml	b	RP	411
Celiprolol	plasma	1	ml	d	RP	407
	urine	.05	ml	d	RP	407
FM-24	plasma	2	ml	d	RP	374
Indenolol	plasma			b	RP	412
	urine	2	ml	b	RP	412
Labetalol	plasma	1	ml	b	RP-PI	413, 414
Metoprolol	plasma				NP	415
	plasma	1	ml	d	RP	408
	plasma	1	ml	b/d	RP (PI)	409, 416
	urine	1	ml	b	RP (PI)	416
Nadolol	fluids	1	ml	d	IE	371
Penbutolol	plasma	1	ml	b	RP	417
	urine	1	ml	b	RP	417
	plasma	1	ml	b	RP-PI	418
	urine	1	ml	b	RP-PI	418
Pindolol	plasma	2	ml	d	RP	419
Prenalterol	plasma	2	ml	d	RP	419
Pronethalol	plasma	1-2	ml	d	RP	420, 421
	urine				RP	422
Propranolol	fluids	1	ml	d	IE	371
	plasma	1-2	ml	a-d	RP	423, 424
	urine	1	ml	b	RP	411, 424
	plasma	1	ml	d	RP-PI	425, 426
Sotalol	plasma	2	ml	d	RP	427
	urine	2	ml	d	RP	427
Xamoterol	plasma	1	ml	c	RP	428
	urine	.1	ml	c	RP	428
III-c Phenylethylamines						
Catecholamines					IE	429
	serum	.01	ml	a	NP	430
	brain				RP	431, 432
	urine	.1	ml	c	RP	433
	urine			c	RP-PI	434, (435)
L-dopa					IE	436

(continued)

TABLE IV *(continued)*

Compound(s)	Sample		Clean-up	HPLC	References
Indoles	serum	.01 ml	a	NP	430
	urine	10 ml	a	RP	437, 438
Indoramin	plasma	1 ml	b	RP	439
Metanephrines	urine	10 ml	d	RP-PI	440
Serotonin	brain		d	IE	331, 332
			a	RP	441
	plasma	2 ml	a/d	RP	333, 419
	tissue		a/d	RP	334, 442
Terbutaline				RP-PI	435

IV. NEUTRAL COMPOUNDS

Compound(s)	Sample		Clean-up	HPLC	Reference
Aflatoxins	urine		b	NP	443, (444)
	serum	1 ml	b	RP	445, (446)
Alkylphenol ethoxylates				NP	447
Napropamide	blood	.25 ml	b	RP	317
Psoralene	plasma	1 ml	b	RP	448

V. VARIOUS GROUPS OF DRUGS

Compound(s)	Sample		Clean-up	HPLC	Reference
V-b Cytostatics					
ADCA	fluids			RP	449
Antitumor antibiotics	(review)				450
Anthracyclines	plasma	1 ml	d	RP	451
	urine		-	RP	451, 452
Aclacinomycin	plasma	1 ml	b	RP	453
Adriamycin, Daunorubicin	plasma		a	NP	454
	plasma	.1 ml	b	NP (PI)	455
	urine		b	RP	456, (457)
	plasma	.1 ml	a/d	RP	458, 459
Carminomycin	serum	2 ml	b	RP	460
4-Demethoxydaunorubicin	plasma	1 ml	d	RP	461
4'-Epidoxyrubicin	plasma	.6 ml	a	RP	452
	plasma	2 ml	d	RP	462
	urine		-	RP	452
BD 40	plasma	1 ml	b	RP-PI	450
	urine	2 ml	b	RP-PI	450
Bisantrene	fluids		c	RP	463
Ellipticine	blood	.5 g	b	RP	464
Etoposide (VP-16)	plasma	1 ml	b	RP	465, (466)
Harringtonine	plasma	1 ml	a	RP	467
Melphalan	plasma	1-3 ml	d	RP	468, 469
Teniposide (VM-26)	plasma	1 ml	b	RP	465, (466)
V-c Steroids					
Estriol	plasma	.5 ml		RP	470
	urine	2 ml	b	RP	471
Estrogen				NP	472
Ethinylestradiol				RP	473
Mestranol				NP/RP	474
Zearalenol, Zearalenone				NP	475
	plasma	2 ml	d	RP	476

(continued)

TABLE IV *(continued)*

Compound(s)	Sample			Clean-up	HPLC	Reference
V-d Vitamins						
Menaquinones					NP/RP	477
Riboflavin	urine	.01	ml	-	RP	478, (479)
Tocopherols	plasma	.05	ml	a	NP	480
	liver	.8	g	b	RP	481
	plasma	.05	ml	a/b	RP	482, 483
Vitamin A	serum	.5	ml	b	NP	484
Vitamin B2, B3, B6					IE	485
Vitamin B2	blood	1	ml	a	RP	486
Vitamin B6	blood	1	ml	a	NP	487
	tissue	1	g	a	RP	488
Vitamin E					NP	489

Derivatization with apolar reagents, shown in TABLE III, involves the use of organic solvents. The application of these organic solvents is not directly compatible with aqueous biological materials. Therefore, the water-soluble derivatization reagents deserve more attention in order to solve this problem.

As mentioned before the ideal fluorescence derivative is rapidly formed under mild conditions, preferably at room temperature without significant formation of side products. Not all derivatization reactions utilized in characterizing organic compounds meet these requirements and modifications of the reaction conditions without losing the fluorescence sensitivity in HPLC analysis are often necessary. The parameters influencing the reaction rates are: concentration and stoichiometry (108), temperature (109), solvent (110, 111) and substituent effects (112, 113). These factors are discussed in a review of Morozowich and Cho (114) as an aid in the development of optimal reaction conditions.

Fluorescence Introducing Reagents

The most frequently used fluorescence introducing reagents are surveyed in the next paragraphs. In the discussion the following aspects will be reviewed: selectivity for different functional

TABLE V
(BIO-)ANALYTICAL APPLICATIONS OF LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DERIVATIZATION

I. ACIDIC COMPOUNDS

Compound(s)	Sample		Clean-up	HPLC	Fluorescence method	Reference
I-a Carboxylic acids						
Arachidonic acid	plasma	.1 ml		RP	Pre - 9-Aminophenanthrene	490
Bile (acids)	serum	.1 ml	c	RP	Pre - 1-Anthroylnitrile	292
	bile	.01 ml	d	RP	Pre - Bromoacetylpyrene	261
	serum	.1 ml	d	RP	Pre - Bromoacetylpyrene	261
	serum	.1 ml	c/d	RP	Pre - Dns-H	152, 491
Caprylic	plasma	.5 ml	d	RP	Pre - Br-Mmc	492
Carboxylic	plasma/urine		b	RP	Pre - HMA	23
				NP	Pre - DANE	252
				RP	Pre - Br-Mac	203
				RP	Pre - Br-Mdmc	25
				RP	Pre - Br-Mmc	493
				RP	Pre - D-Mmc	204
Dicarboxylic				RP	Pre - Br-Mmc	493, 190
Eicosapentaenoic	plasma	.1 ml		RP	Pre - 9-Aminophenanthrene	490
Fatty				RP	Pre - ADAM	257, 258
	serum	.5 ml	c	RP	Pre - 9-Aminophenanthrene	263
	serum	1 ml	b	RP	Pre - Br-Mmc	494, (191)
				RP	Pre - 9-CIMA	24
				RP	Pre - DAP	28
				RP	Pre - Naphthacylbromide	259
Gibberellins				NP/RP	Pre - Br-Mmc	193
p-Hydroxybenzoic	urine	1 ml	d	NP	Pre - Dns-Cl	129
Hydroxy substituted carboxylic	urine			IE	Post- Oxidation	495
Ibuprofen	plasma	.15 ml	b	RP	Pre - HMA	23
Indomethacin	plasma	1 ml	a	RP	Post- Hydrolysis	496
	urine	.3 ml	a	RP	Post- Hydrolysis	496
	plasma	.1 ml	b	RP	Pre - Deacylation	81
	urine	.5 ml	b	RP	Pre - Deacylation	81
α-Ketocarboxylic				RP	Pre - o-Phenylenediamine	266, 497
	plasma	.05 ml	d	RP-PI	Pre - o-Phenylenediamine	498
Naproxen	serum	.02 ml	d	NP	Pre - DANE	253
Oxalic				RP	Pre - ADAM	499
Phenylpyruvic	serum	.2 ml	d	RP	Pre - 4H2S	270
	urine	.2 ml	d	RP	Pre - 4H2S	270
Prostaglandins				GPC	Pre - ADAM	500
				NP	Pre - Br-Mmc	501
	seminal fluid		b	RP	Pre - Br-Mac	203
				RP	Pre - Panacylbromide	260
Uronic				IE	Post- 2-Cyanoacetamide	284
Valproic	plasma	.5 ml	d	RP	Pre - Br-Mmc	492
Vanillylmandelic	urine	1 ml	d	NP	Pre - Dns-Cl	129
I-b Phenolic compounds						
Cannabinoid derivatives	urine	10 ml	b	NP	Post- Irradiation	502
				RP	Pre - Dns-Cl	140
8-Hydroxyquinoline				IE	Post- Metalchelates	82
Phenols				RP	Post- Oxidation	503
	urine	5 ml	b	NP	Pre - Dns-Cl	123
Warfarine	plasma/urine		a	NP	Post- Acid/Base manipulation	79
I-c Miscellaneous acidic compounds						
Barbiturates	serum	1 ml	b	RP	Pre - Br-Mmc	195
	blood	.02 ml	b	RP	Pre - Dns-Cl	504
Captopril	plasma	1 ml	b	RP	Pre - PM	187
Cysteine derivatives	plasma	1 ml	d	RP	Pre - NAM	185
	urine	5 ml	d	RP-PI	Pre - DACM/PM	188
	plasma	.2 ml	a	RP-PI	Pre - OPA	505, (506)
Glutathione				RP-PI	Post- NCDA	156
	blood/tissue			RP	Pre - NAM	183, (507)
S-Sulfocysteine	urine	1 ml	c	IE	Pre - Dns-Cl	508
Thiol derivatives				IE	Post- Ligand-exchange	87
				IE	Post- OPA	250
				IE/RP	Pre - Monobrobimane	271, 272
				RP	Pre - Dns-A	144
	blood/tissue			RP	Pre - NAM	183, (184)
				RP	Pre - OPA	509
	plasma	.5 ml	a	RP	Pre - SBD-F	219
				RP	Pre - NH_2-SBD-F	220

II. AMPHOTERIC COMPOUNDS

Compound(s)	Sample		Clean-up	HPLC	Fluorescence method	Reference
II-a Amino acids (peptides, proteins)						
				IE	Post- Flur	510
				IE	Post- NBD-Cl	210
				IE	Post- Ninhydrin	511
				IE	Post- OPA	512, 513
				IE	Pre - OPA	514, 515
				IE	Pre - Pyridoxal	516
				NP	Post- Flur	178
				NP	Pre - Dns-Cl	517
				RP	Post- Flur	518, 519
				RP	Post- OPA	247, 520
				RP	Post- Phenylisothiocyanates	279
	serum	1 ml	a	RP	Pre - Dns-Cl	138, (152)
				RP	Pre - Flur	173
				RP	Pre - FMOCCl	522
				RP	Pre - NBD-F	216
				RP	Pre - NBD-OCH_3	208
				RP	Pre - NCDA	157
				RP	Pre - OPA	523, 524
	plasma	.01 ml	a	RP	Pre - OPA	525, 526
	urine	.02 ml	a	RP	Pre - OPA	236, 527

(continued)

TABLE V *(continued)*

Compound(s)	Sample		Clean-up	HPLC	Fluorescence method	Reference
	urine		d	RP-PI	Post- Flur	528
				RP-PI	Post- OPA	529
				RP-PI	Pre - Dns-Cl	530
				RP-PI	Pre - Flur	172
				RP-PI	Pre - OPA	531
Aminophospholipids				NP	Pre - SNA	283
Asparagine				RP	Pre - OPA	532
γ-Carboxylglutamic acid	urine		b	IE	Pre - OPA	533
Glutamine				RP	Pre - OPA	532
Hydroxyproline				IE	Post- NBD-Cl	210
	serum	.1 ml	a	IE	Post- OPA	534
				RP	Pre - NBD-Cl	207
5-Hydroxytryptophan	plasma	.75 ml	d	IE	Post- OPA	535
Methylhistidine				RP	Pre - OPA	536
Norleucine	serum	.01 ml	a	RP	Pre - Dns-Cl	537
Phenylalanine	serum	.01 ml	a	RP	Pre - Dns-Cl	137
Pipecolic acid	brain			RP	Pre - Dns-Cl	538
Proline				IE	Post- NBD-Cl	210
Taurine				RP	Pre - Dns-Cl	539
				RP	Pre - OPA	240, 540
Trimethyllysine	plasma		d	RP	Post- OPA	541
	tissue		d	RP	Post- OPA	541
Tryptophan	plasma/tissue		a	RP	Pre - Oxidation	542
II-b Miscellaneous amphoteric compounds						
4-Aminobutyric acid	csf		a	IE	Post- OPA	543
				RP	Pre - Dns-Cl	544
				RP-PI	Pre - OPA	531
ε-Aminocaproic acid	serum	.01 ml	a	RP	Pre - Flur	545
Aminosalicylic acid	plasma	.5 ml	b	RP	Pre - Acetylation	546
	urine	.5 ml	b	RP	Pre - Acetylation	546
Baclofen	plasma	.5 ml	c	RP	Pre - OPA	547
	urine	.5 ml	c	RP	Pre - OPA	547
Sulfapyridine	saliva	1 ml		RP-PI	Post- Flur	548
γ-Vinyl-γ-aminobutyric acid	plasma	.5 ml	a	IE	Post- OPA	549
	urine	1 ml	a	IE	Post- OPA	549
YM-09538	plasma	1 ml	b	NP	Pre - Bns-Cl	142

III. BASIC COMPOUNDS

Compound(s)	Sample		Clean-up	HPLC	Fluorescence method	Reference
III-a Amines						
Aliphatic amines				RP	Pre - NBF-SO_2Cl	274
Amines				RP	Post- DAS	103
				RP	Pre - Mbp	281
	blood			RP	Pre - NBD-F	214
Amino-flunitrazepam	plasma	.5 ml	d	RP	Pre - Flur	175
Antihistamine derivatives				RP	Pre - NCF	119
Atropine				NP	Post- DAS	550
Bestatin	serum	.02 ml	-	RP	Pre - Oxidation + DBB	293
Biopterins				IE	Post- Oxidation	551
Bradykinin				RP	Pre - Flur	552
Bromopheniramine	urine	1 ml	c	RP	Post- DAS	553
Cephaeline				NP	Pre - Dns-Cl	124
Chloropheniramine	urine	1 ml	c	RP	Post- DAS	553
Clobazam	serum			RP	Post- Irradiation	105
Clovoxamine				RP	Post- Dns-Cl	122
CV-1808	serum	2 ml	d	NP	Pre - Propionic anhydride	554
	urine	1 ml	d	NP	Pre - Propionic anhydride	554
Cystamine				IE	Post- OPA	555
Cystine	serum	.1 ml	a	RP	Pre - OPA	229
	urine	.1 ml	a	RP	Pre - OPA	229
Demoxepam	serum/urine		a	RP	Post- Irradiation	556
Digitalis glycosides				RP	Post- Dehydroascorbic acid	84
Dihydromorphine	urine	5 ml	b	NP	Pre - Oxidation	557
Emetine				NP	Pre - Dns-Cl	124
	plasma	9 ml	b	RP-PI	Pre - Oxidation	558
Endralazine	plasma		b	RP	Pre - Formic acid	559
Ergot alkaloids	urine			RP	Post- Irradiation	560
Ergotamine				NP	Post- DAS	550
Fenbendazole	serum			RP	Post- Irradiation	105
Fluvoxamine				RP	Post- Dns-Cl	122
Guanadrel				RP	Pre - Acetylacetone	561
Guanidino compounds	serum	.05 ml	-	IE	Post- Benzoin	562
	urine	.1 ml	-	IE	Post- Benzoin	562
	plasma	.2 ml	a	IE	Post- Ninhydrin	563
				RP	Post- Benzoin	564
				RP-PI	Post- Phenanthrenequinone	565
Heptaminol	plasma	.1 ml	a/b	RP	Pre - OPA	566, 567
	urine	.01 ml	-	RP	Pre - OPA	566
Hexosamines	urine	50 ml	-	IE	Post- 2-Cyanoacetamide	568
				IE	Post- 2,4-Pentanedione-formaldehyde	569
Histamine	urine	1 ml	d	RP	Pre - OPA	570
	plasma	.5 ml	d	RP	Pre - OPA	571
	blood		c	RP-PI	Post- OPA	572
	tissue		a	RP-PI	Pre - OPA	573
	urine	1 ml	c/d	RP-PI	Pre - OPA	574
Hydroxyatrazine				NP	Post- DAS	550
				RP	Pre - DAS	85
Leucinol	plasma	.1 ml	a	RP	Pre - OPA	566
	urine	.01 ml	-	RP	Pre - OPA	566
Maprotiline	plasma	.5 ml	d	RP	Pre - Dns-Cl	130
Metapramine	urine	.5 ml	b	RP	Pre - Dns-Cl	130
N-Methylcarbamates				IE	Post- OPA	575
Methadone	plasma	1 ml	d	RP	Post- DAS	576

(continued)

TABLE V *(continued)*

Compound(s)	Sample		Clean-up	HPLC	Fluorescence method	Reference
Methadone	serum	.1 ml		RP	Pre - Dns-Cl	577
Monosodium glutamate				RP	Pre - Dns-Cl	578
Morphine				NP	Pre - Dns-Cl	124
	urine	5 ml	b	NP	Pre - Oxidation	557
	blood	10 ml	d	RP	Post- Oxidation	579
	urine	1 ml	b	RP	Post- Oxidation	579
Nalorphine	blood	10 ml	d	RP	Post- Oxidation	579
	urine	1 ml	b	RP	Post- Oxidation	579
Nitrosaminen				NP	Pre - NBD-Cl	580
Perhexiline maleaat	plasma	1 ml	b	RP	Post- Dns-Cl	581
Phenothiazines	fluids		a	RP	Post- Irradiation	556
Phenylpropanolamine	urine	2 ml	-	RP	Post- OPA	582
Polyamines	plasma		d	IE	Post- OPA	583
	urine	1 ml	-/b	IE	Post- OPA	584, 585
	urine		c	NP	Pre - Dns-Cl	586
	urine	.2 ml		RP	Pre - Dns-Cl	126, 587
	serum	.5 ml	d	RP	Pre - Flur	160, 588
	urine	.5 ml	d	RP	Pre - Flur	160
				RP	Pre - OPA	589
	fluids		d	RP-PI	Pre - Dns-Cl	127
				RP-PI	Pre - Flur	590
				RP-PI	Post- OPA	591, 592
Primary amines				IE	Post- Flur	593
				IE	Pre - FMOCCl	118
				IE	Pre - NBD-F	595
				RP	Post- Dns-Cl	122
				RP	Post- OPA	247
				RP	Pre - MDPF	596
Purines				RP	Post- pH manipulation	597
Pyrimidines				RP	Post- pH manipulation	597
Rauwolfia alkaloids				RP	Post- p-Toluenesulfonic acid	598
Reserpine				RP	Post- Oxidation	599
	plasma	2 ml	b	RP-PI	Pre - Oxidation	600, 601
Secondary amines				IE	Post- OPA	233
				IE	Pre - FMOCCl	118
				IE	Pre - NBD-F	595
				RP	Post- Dns-Cl	122
				RP	Pre - MDPF	596
Secoverine	serum	1 ml	c	NP	Post- DAS	602
Tamoxifen	serum	.01 ml	a	NP/RP	Post- Irradiation	603
	plasma	1 ml	c	RP	Post- Irradiation	604
Tertiary amines	urine	1 ml	c	RP	Post- DAS	85, 553
				RP	Pre - NCF	119
Thioridazine	plasma	1 ml	d	NP	Post- Oxidation	80, (605)
Tocainide	plasma	.05 ml	d	NP	Pre - Dns-Cl	133
	plasma	.5 ml	a	RP	Pre - Flur	163
Trimetazidine	plasma	2 ml	b	NP	Pre - Dns-Cl	136
WR 2721	plasma	.09 ml	-	RP-PI	Pre - Flur	606
(Hypo)Xanthine	serum	.5 ml	a	RP	Post- Enzyme reactor	607
III-c Phenylethylamines						
Amphetamine	fluids			NP/RP	Various	608
	urine	.5 ml	b	RP	Pre - OPA	609
Catecholamines				various	Various	610, 611
				IE	Post- Glycylglycine	612
				IE	Post- OPA	613, 614
				IE/RP	Post- Aethylenediamine	615
				NP	Pre - Flur	166
				NP/RP	Pre - Dns-Cl	125
				RP	Post- OPA	613
	tissue	1 g	c	RP	Pre - Dns-Cl	616
				RP	Pre - DPE	296
	plasma	2 ml	a	RP	Pre - OPA	617
	urine		b	RP	Pre - OPA	618
	plasma	2 ml	b	RP-PI	Post- Oxidation	619
	urine	.5 ml	d	RP-PI	Post- Oxidation	619
Ephedrine				NP	Pre - Dns-Cl	124
(Nor)Epinephrine	urine	5 ml	c	IE	Post- Heating	620, (621)
	urine	5 ml	-	RP	Post- Oxidation	622
5-Hydroxyindoles				RP	Post- $HClO_4$	623
Indoles	fluids			IE	Post- OPA	624
Isoproterenol	plasma	5 ml	a	IE	Post- Oxidation	625
	urine	10 ml	a	IE	Post- Oxidation	625
Metanephrines	urine	5 ml		IE	Post- Oxidation	626
5-Methoxyindoles				RP	Post- $HClO_4$	623
D-Norpseudoephedrine	plasma	1 ml	b	NP	Pre - Dns-Cl	627
Phenylethanolamine				RP-PI	Post- Trihydroxyindole	628
Serotonin	plasma	1 ml	b	RP	Pre - Dns-Cl	627

IV. NEUTRAL COMPOUNDS

Compound(s)	Samples		Clean-up	HPLC	Fluorescence method	Reference
Aflatoxins				RP	Post- Iodine	629
				RP	Pre - Trichloroacetic acid	630
Alcohol ethoxylates				RP	Pre - 1-Anthroylnitrile	987
Aldehydes				RP	Pre - DIH	295
				RP	Pre - Dimethylcyclohexanedione	631
Aldoses				IE	Post- 2-Cyanoacetamide	632
Avermectins	plasma	1 ml	d	RP	Pre - Acetic anhydride	633
Carbohydrates				IE	Post- Ethylenediamine	634
				IE/RP	Post- AFB	290
				RP	Post- Photoreduction	197
	urine			RP	Post- Tetrazolium Blue	635
Carbonyl compounds				RP	Pre - NBD-H	221
Cardiac glycosides				RP	Post- Photoreduction	106
Cholesterol				RP	Pre - D-Mmc	204
Ethers				RP	Post- Photoreduction	107

(continued)

TABLE V *(continued)*

Compound(s)	Sample	Clean-up	HPLC	Fluorescence method	Reference
Hydroxyl groups		NP	Pre -	Binaphthalene-carbonyl-nitriles	287
		NP	Pre -	DMA-NN	288
		RP	Post-	Photoreduction	107
		RP	Pre -	Anthroylnitrile	289
		RP	Pre -	D-Mmc	204
		RP	Pre -	Dns-ECF	30
		RP	Pre -	MCCA	26
Monosaccharides		IE	Pre -	2-Cyanoacetamide	632
Naphthylurethanes		RP	Pre -	*Dns*-H	636
Neutral sugars		NP/RP	Pre -	NIC	298
Reducing sugars		RP	Pre -	Dns-H	153
		NP	Pre -	Dns-H	155
		RP	Post-	2-Cyanoacetamide	637
		RP	Pre -	Dns-H	154

V. VARIOUS GROUPS OF COMPOUNDS

Compound(s)	Sample	Clean-up	HPLC	Fluorescence methods	Reference
V-a Antibiotics (without antineoplastic antibiotics					
Amikacin	serum .5 ml	c	IE + RP	Pre - OPA	638
6-Aminopenicillanic acid			RP	Pre - Flur	639
Amoxicillin	plasma .5 ml	a+b	RP	Pre - Degradation	640
Ampicillin	plasma .5 ml	a+b	RP	Pre - Degradation	640
Astromicin	serum .5 ml	c	RP	Post- OPA	641
Cephadrine	plasma .5 ml	a+b	RP	Pre - Degradation	640
Cephadrine }	serum .2 ml	a	RP	Post- Flur	642
Cephatrizine }	urine .2 ml	a	RP	Post- Flur	642
Erythromycin	serum 1 ml	b	RP	Post- Tinopal	643
Gentamicin	plasma .1 ml	a	IE	Pre - Flur	167
	urine .05 ml	a	IE	Pre - Flur	167
	serum .5 ml	c	RP	Pre - OPA	244, 644
Kanamycin			IE	Post- OPA	645
B-Lactam antibiotics			RP	Post- OPA	646
Micronomicin	serum .5 ml	c	RP-PI	Pre - OPA	641
Netilmicin	serum		RP	Pre - OPA	646
	plasma	-	RP-PI	Post- OPA	647
	serum .4 ml	c	RP-PI	Post OPA	641
D-Penicillamine	plasma 1 ml	a	RP	Pre - BOPM	186
	serum .5 ml	a	RP	Pre - Dns-H	145
Penicillin G			RP	Pre - Flur	639
Penicillin V			RP	Post- OPA	648
Sisomicin	serum .4 ml	c	RP-PI	Post- OPA	641
Spectinomycin			RP-PI	Post- OPA	649
Tobramycin	serum	a	RP	Post- OPA	650
	serum		RP	Pre - OPA	644
V-b Cytostatics					
5-Fluorouracil			RP	Pre - Br-Mmc	194
6-Mercaptopurine	plasma 1 ml	d	RP-PI	Post- Oxidation	651
Methotrexate	plasma 1 ml	a	RP	Pre - Oxidation	652
Tamoxifen	serum .01 ml	a	NP/RP	Post- Irradiation	603
	plasma 1 ml	c	RP	Post- Irradiation	604
	plasma	b	RP-PI	Pre - Irradiation	653
V-c Steroids					
Anabolic agents	fluids	b	RP	Pre - Dns-A	131
Clomiphene	plasma 3 ml	d	NP	Post- Irradiation	654
Cortisol	serum .1 ml	a	NP	Pre - 9-Anthroylnitrile	655
	plasma .5 ml	a	NP	Pre - Dns-H	147
	urine 1 ml	a	NP	Pre - Dns-H	147
			RP	Pre - Anthroylnitriles	289
	serum .05 ml	a	RP	Pre - H_2SO_4	83
	urine .5 ml	b	RP	Pre - H_2SO_4	656
Cortisone	serum .1 ml	b	NP	Pre - 9-Anthroylnitrile	655
Corticosteroids	urine 2 ml	b	RP	Post- Glycinamide	657
Diethylstilbestrol	urine 10 ml	b/c	RP	Post- Irradiation	658
Hydrocortisone	plasma 1 ml	b	NP	Pre - Dns-H	659
			RP	Post- Photoreduction	197
6β-Hydroxycortisol	urine .5 ml	d	NP	Pre - 9-Anthrovlnitrile	660
17-Hydroxycorticosteroids	urine .5 ml	d	NP	Pre - Dns-H	150
	urine 2 ml	b	RP	Post- Benzamidine	661
Ketosteroids			NP	Post- Isoniazide + $AlCl_3$	662
	plasma .5 ml	b	NP	Pre - Dns-H	148
	urine 1 ml	b	NP	Pre - Dns-H	148
17-Oxosteroids	plasma .2 ml	c	NP/RP	Pre - Dns-H	151
	urine .5 ml	c	NP/RP	Pre - Dns-H	151
Prednisolone	serum .1 ml	b	NP	Pre - 9-Anthroylnitrile	655
Steroids			RP	Various	663
V-d Vitamins					
Biotin			RP	Pre - Br-Mmc	664
Menaquinones }	serum .5 ml	d	RP	Post- Irradiation/Reduction	665
Phylloquinones }					
Thiamin	blood 1 ml	a	RP	Post- Oxidation	666, (667)
	tissue 1 g	a	RP	Pre - Oxidation	668
	fluids 2 ml	a	RP-PI	Post- Oxidation	669
Vitamin C	blood 1 ml	d	NP	Pre - o-Phenylenediamine	670
Vitamin K_1	plasma 1 ml	d	NP	Post- Reduction	671
	serum	d	RP	Post- Reduction	672

groups; properties of the formed derivative, more specifically the detection limit in the used HPLC system; stability of the reagents, labels and derivatives under the appropriate analytical conditions; procedure and mechanism of the derivatization reaction; chromatographic behaviour of the derivatives, regarding the choice of the HPLC system and the possibility to use the derivatization reaction as a pre-column or post-column step in the LC analysis.

Moreover, a short review will be presented of some recently developed fluorescence derivatization reagents and methods.

Chloroformates

Chloroformates (ROCOCl) are a class of compounds known for their reactivity with amines and alcoholic functions (115). This reactivity is often used for the protection of these functions and to obtain derivatives that are suitable for chromatographic analysis, both GLC (116, 117) as well as HPLC.

Recently the method has been adapted to a suitable one for the analysis of compounds with detection problems in chromatographic systems. This led to the introduction of groups with strong absorption and fluorescence properties (30, 118, 119). Efforts have been made in modifying the reagents as well as the reaction conditions to develop more selective methods for the various functional groups with strong fluorescing properties in order to achieve the lowering of the detection limits of these compounds.

Fluorescence labelling of amines and alcohols is usually performed with three reagents from this group: 9-*fluorenylmethylchloroformate* (FMOCCl) (118), 2-*naphthylchloroformate* (NCF) (119) and 2-*dansylethylchloroformate* (Dns-ECF) (30).

The general reaction pattern for primary and secondary amines and alcohols is presented in Figure 3, while for tertiary amines, instead of HCl, R^4Cl is formed after dealkylating the tertiary amine.

The procedures for labelling with FMOCCl and Dns-ECF are basically identical. Both reactions are performed at room temperature in, respectively, acetone and dichloromethane with sodium

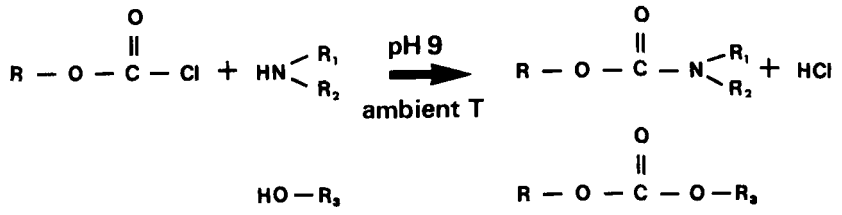

Fig. 3: Mechanism of the fluorescence labelling with chloroformates.

borate or pyridine to establish a pH around 9. Under these circumstances FMOCCl reacts with primary and secondary amines, while Dns-ECF primarily reacts with alcoholic functions. Due to the rather drastical changes in the reaction conditions (keeping the reaction mixture in benzene at 100° C in a closed conical vial for 1 hour) Guebitz et al. (119) achieved the derivatization of tertiary amines.

Analysis of the reaction mixture reveals a good reproducibility with derivatization yields varying from 80-100%. The reagents are sensitive to moisture but can be kept stable in a cool dry place. The stability of the derivatives is sufficient, indicated by a fluorescence stability of about 24 hours.

In all cases the derivatization is carried out as a pre-column method. HPLC analysis is performed using either RP chromatography with methanol/acetonitrile-water-tetrahydrofuran mixtures as eluents or anion exchange chromatography with 0.1 M fosfate (pH 6.0) with 25% acetonitrile as eluent. The detection limits range from 0.5 ng (119) to 50 pg (30), due to the high quantum yields of fluorescence, usually in the range of 0.3-0.4.

Dansyl Reagents

Dansylation is probably the most widely used derivatization technique for the introduction of a fluorophore into weakly or non-fluorescent compounds in order to permit more selectivity and sens-

itivity in the detection of these compounds after HPLC analysis. The dansyl (5-*dimethylaminonaphthalene*-1-*sulfonyl*) group reacts in a simple and rapid manner with a variety of functions such as primary and secondary amines, hydroxylic and phenolic functions, thioles and, dependent on the nature of the reagent, carboxylic functions. The derivatization reagent in most cases is dansyl chloride (Dns-Cl). The resulting derivative shows highly fluorescent properties and, after HPLC separation, enables the detection to a low limit. This procedure permits the analysis of a great number of active compounds in biological substrates, whereas the clean-up steps are relatively simple. Lawrence and Frei (88, 120) as well as Seiler and Demisch (121) reviewed extensively this derivatization technique.

Ever since attempts have been made to optimize the technique and to use other dansyl derivatives in order to develop methods for the selective determination of various functional groups. This led to the development of derivatives like *bansyl chloride* (Bns-Cl), *mansyl chloride* (Mns-Cl), *dansyl hydrazine* (Dns-H) and *dansyl aziridine* (Dns-A), while also a related compound N-*chloro*-5-*dimethylaminonaphthalene*-1-*sulfonamide* (NCDA) is used for the derivatization of several functions.

A short review of the various reagents and their uses is given below.

Dansyl chloride (Dns-Cl)

The most widely used dansylation reagent is Dns-Cl. This compound reacts easily with primary and secondary amino groups, hydroxylic functions of both phenolic and alcoholic nature, and thioles. During the dansylation both the acetylation reaction and the hydrolysis of the reagent occur competitively (122) (Figure 4).

The reaction is performed either directly in the medium in which the analysis has to be carried out (solutions in water, serum, blood, urine samples) (123-131) or after extraction from the sample using different organic solvents (132-135). The reaction is perform-

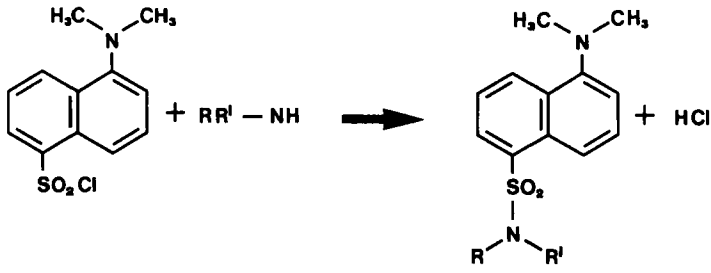

Fig. 4: Mechanism of the fluorescence labelling with Dns- Cl .

ed in alkaline solution, in the presence of sodium carbonate or trimethylamine. Reaction times vary from 24 hours to 30 minutes, depending on the conditions. In general, the reaction time is shorter when the labelling is carried out in non-aqueous media. The derivatization yield also depends on the conditions, especially the presence of water and the pH (125). Despite the fact, that not always a maximum yield is obtained, the reproducibility is good (124, 126, 127, 136). The method is generally used as a pre-column derivatization technique, although also post-column dansylations have been investigated (122). HPLC analysis of the dansyl derivatives usually is performed using RP techniques, although also silicagel (137) is tried. Usually, when chemically bonded stationary phases are used, the eluents consist of mixtures of methanol-water or acetonitrile-water.

Most authors mention the fluorescence of the derivatives to be intense. The quantum yield of, for example, dansylcadaverine is about 0.18 (29). The sensitivity of the method is good with detection limits in the sub ng or pg regions.

As mentioned earlier the reagent is subject to hydrolysis but the derivatives are relatively stable. The method is used to analyse amines and amino acids (TABLES IV and V), more specifically in order to obtain chromatographic separation between the enantiomers (128). Several amino acids give complex derivatives and fluorescent by-products (138, 139). Furthermore, the method is applied in the analysis of barbiturates (132), phenols (129, 132) and cate-

cholamines (TABLE V) as both phenol and amino derivatives as well as in the analysis of natural products such as cannabinnoids (140), estrogens and anabolic agents (131, 141) and alkaloids (TABLE V).

Bansyl chloride (Bns-Cl)

This reagent only differs from Dns-Cl with respect to the 5-dimethylamino function which is replaced by a dibutylamino group. The resulting compound is more lipophilic in nature so that extraction from aqueous media should be simple and complete. The fluorescence properties of the derivatives are similar to those of the dansylation products, while the method for derivatization is also the same. The detection limit with this method is also in the ng region (142).

Mansyl chloride (Mns-Cl)

Another modification of the dansyl function led to the introduction of Mns-Cl, where the 5-dimethylamino group has been replaced by a 5-methylphenylamino function (143), resulting in a more intensely fluorescing class of derivatives. The derivatization procedures are similar to those of Dns-Cl and Bns-Cl but the resulting derivatives show emission maxima at a shorter wavelength (121).

Dansylaziridine (Dns-A)

Replacement of the chlorine in Dns-Cl by an aziridine function led to the introduction of Dns-A as fluorescence label. This compound reacts selectively with sulfhydryl groups whereby the aziridine ring is opened. This requires a strongly nucleophile functional group (144) (Figure 5). Other functions, having weaker nucleophilic properties, like phenols, amines and alcohols do not react.

The reaction is performed in fosfate buffer pH 8.2 at 60° C and completed after 1 hour. No further isolation of the resulting derivative is necessary. HPLC analysis is performed using RP-18 material as stationary phase and mixtures of acetonitrile-fosfate

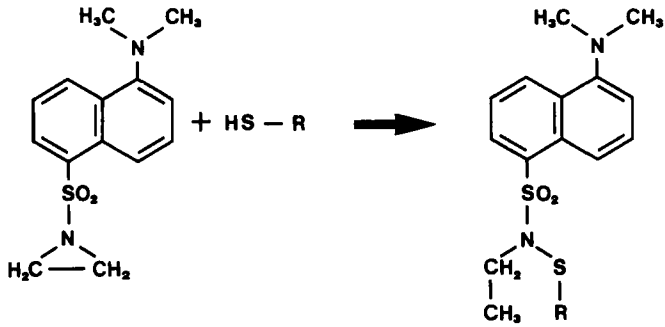

Fig. 5: Mechanism of the fluorescence labelling with Dns-A.

buffer pH 8.2 as eluents (144, 145). Amino acids containing sulfhydryl groups (144) as well as other thiols like penicillamine (145) can be analyzed with this method.

Dansylhydrazine (Dns-H)

The introduction of a hydrazine function in the dansyl label yields a reagent with specific affinity to carbonyl groups, especially in the ketosteroids (146-152) and sugars (153-155). The hydrazine function reacts with the carbonyl function to form highly fluorescent dansyl hydrazones that can be subjected to HPLC analysis. Both NP chromatography on silica columns, using dichloromethane-ethanol-water mixtures as well as RP procedures, using acetonitrile-water, sometimes containing low concentrations of acetic acid, are used.

The derivatization of the steroids is carried out in the presence of trichloroacetic acid. Both polar (ethanol-water) and non-polar (benzene) media are suitable. The method is also suitable for the determination of bile acids after oxidation of the 3-hydroxyl group to a 3-oxo function (152).

The HPLC analysis of the hydrazones must be performed within 2 hours due to the limited stability of the compounds. The derivatization yields with the various sugars differ greatly (154), requir-

ing the method to be adapted for individual sugars. Interferences can also be expected from other carbonyl compounds like ketones and aldehydes. The excess of reagent should be adapted to these interferences.

N-*Chlorodansylamide* (NCDA)

Dansylamide is a highly fluorescent dansyl derivative, whose fluorescence however, is quenched completely with sodium hypochlorite to form NCDA. This compound reacts with sulfhydryl groups, organic sulfides and peptides to form dansylamide. The fluorescence of the reaction product is proportional to the concentration of the reacting compound (156) (Figure 6).

The observation that NCDA shows fluorescence after treatment with amino acids (156, 157) indicates, that these compounds also have potency to dechlorinate NCDA. Nevertheless the fluorescence intensity is much higher for compounds containing sulfhydryl groups showing that the reaction with other groups is far from complete.

After RP HPLC separation of the compounds the effluent is subjected to post-column reaction with NCDA and the fluorescence of the resulting dansylamide is measured and used for the assay of the compounds.

FLUORESCAMINE and Related Compounds

The non-fluorescent *fluorescamine*, 4-*phenylspiro*[*furan*-2(3H)-1'-*phthalan*]-3,3'-*dione*, reacts with nucleophilic functional groups (alcohols, primary and secondary amines and water) but it only forms highly fluorescent derivatives with primary amines, including amino acids, diamines (158) and polyamines (88, 121). It is routinely used for the post-column derivatization of amino acids (159), but also for pre-column derivatizations (160). The reagent has been developed as a result of studies of the structure of fluorescent derivatives formed with ninhydrin (161).

The reaction of fluorescamine with primary amines for a pre-column derivatization (Figure 7), proceeds at pH 9 at room temper-

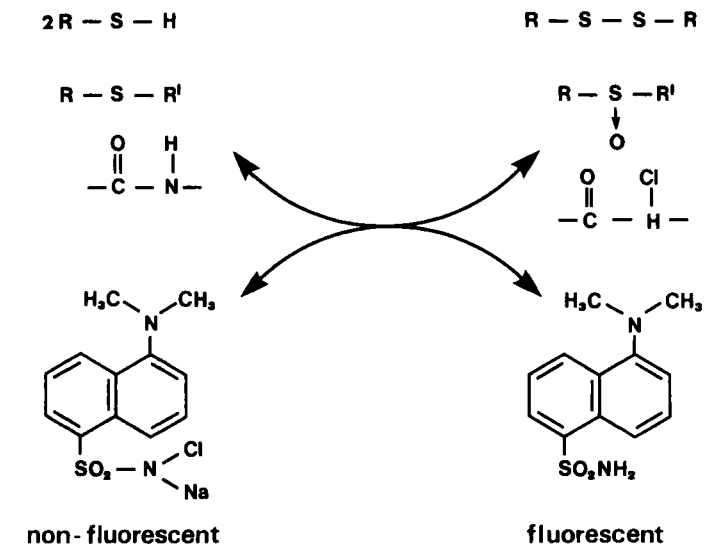

Fig. 6: Mechanism of the reactions with NCDA.

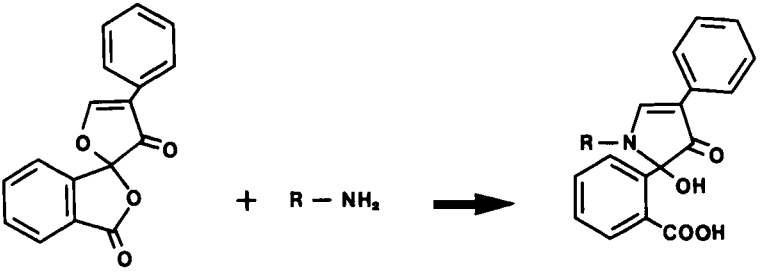

Fig. 7 : Mechanism of the fluorescence labelling with fluorescamine.

ature, with a reaction time of about one second. The excess reagent is hydrolyzed to non-reactive, non-fluorescent, water-soluble products with a reaction time of several seconds (31, 162).

The derivatization of the majority of the primary amines is performed by dilution of an aqueous solution of the amine with borate or fosfate buffer (pH 7.5-9) and addition of a fluorescamine solution in ethanol, acetonitrile or acetone. The derivatives are separated

on, for example, RP HPLC systems (TABLE V). The derivatization yield is 80 to 95 percent with good reproducibility.

The quantum yields of the amine derivatives in ethanol are normally in the range of 0.2-0.34 (31) and the fluorescence efficiency is constant in the pH region of 7.5-10 (163), but is depending on the nature of the solvent in which the fluorescence is measured. The method is useful for samples containing 5 ng of, for example, catecholamines (164, 165). Rapid addition and mixing is essential because the fluorescence intensity decreases rapidly at room-temperature (163). The use of acetone as solvent for fluorescamine led to the formation of the Schiff base. For a quantitative reaction the molar excess of fluorescamine must be 10-40 (167) and the acetone or acetonitrile concentration of the mixture must be at least 30 percent.

Secondary amines do not yield fluorescence products with fluorescamine, but Weigele et al. (168) have shown that secondary amines can be converted to detectable primary amines by N-chlorosuccinimide. Sterling and Haney (169) have shown that conditions can be found for the selective reaction of aromatic amines with fluorescamine in the presence of aliphatic amines.

Closely related in structure and reactivity to fluorescamine is 2-*methoxy*-2,4-*diphenyl*-3(2 H)-*furanone* (MDPF). Both primary and secondary amines react with MDPF in acetonitrile (170, 171). The primary amines produce fluorescent pyrrolinones and the secondary amines form the non-fluorescent aminodienones, which can be converted to fluorescent products with ethanolamine. The derivatives are separated with RP chromatography. The excess of the reagent can be easily hydrolyzed with water and the detection limit of simple alkylamines is 3 pmol. The stability of the derivatives is sufficient for LC analysis.

The use of fluorescamine as a pre-column derivatization reagent for amino acids has been investigated (172-174) and these investigators found that HPLC of the derivatives gave two peaks, due to an equilibrium reaction involving lactone formation between the free carboxylic acid group and a proximal hydroxyl group.

The pre-column derivatization is used for the analysis of drugs in biological fluids. Sumirtapura (175) described the pre-column derivatization of 7-aminoflunitrazepam directly in the mobile phase of an RP HPLC system.

The main application of fluorescamine is the post-column derivatization after IE separations (176, 177). In an amino acid analyzer the HPLC column effluent is buffered and then fluorescamine in ethanol or acetone is added. The sensitivity of this type of analysis is about 5 ng (178).

Maleimides

Sulfhydryl groups react selectively with maleimide derivatives to form adducts, a phenomenon recognized in the literature for a long time (179-182). The reaction mechanism is shown in Figure 8 a. These adducts are subject to rearrangement reactions. The rearrangement of the reaction product of the cysteine ethyl ester derivative is presented in Figure 8 b.

The use of maleimide derivatives with potential fluorophores as substituent led to the synthesis of a class of fluorescent thioles suitable for the analysis of these compounds with good selectivity and sensitivity. Several fluorogenic groups were introduced for this purpose, such as the p-(2-*benzimidazolyl*)*phenyl* (BIPM)(179), the 1-*anilinonaphthyl* (ANM)(180), the 7-*dimethylamino*-4-*methylcoumarinyl* (DACM)(181), the 1-*pyrene* (PM)(182), the 9-*acridinyl* (NAM) (183-185) and the p-(2-*benzoxazolyl*)*phenyl* (BOPM)(186) functions, each leading to the occurrence of fluorescent sulfhydryl derivatives with different maxima of excitation and emission. Thus it is possible to adapt the method for compounds with native absorptivities and to increase the selectivity and sensitivity. In all cases the maleimide reagents themselves do not show a significant fluorescence while the resulting derivatives generally have high quantum yields (179-181), be it that these quantum yields are highly dependent on the solvent.

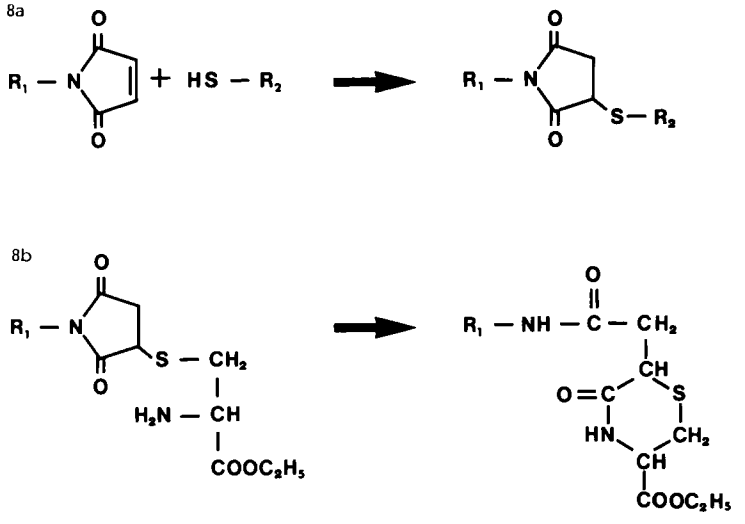

Fig. 8a: Mechanism of the fluorescence labelling with maleimides.

b: Rearrangement of the derivatized cysteine ethyl ester.

Most publications on the analysis of thiols with this method use pre-column derivatization techniques and separation of the derivatives by RP chromatography with water-methanol or methanol-buffer mixtures (183, 185, 187, 188). The derivatization is performed directly in the biological fluid (i.e. plasma, serum, or urine), using a 5-10-fold excess of the appropriate reagent. The sample generally is subjected directly to LC after dilution with the mobile phase. The sensitivity is in the order of 10-100 pmol/ml.

A post-column derivatization method for the fluorimetric analysis of thiols is also described (184). After separation of the compounds on an RP system derivatization is performed with NAM. The sensitivity and reproducibility are similar to the pre-column methods.

Methoxycoumarin Derivatives

At the moment a great number of methoxycoumarin derivatives is used for the fluorescence labelling of acidic functions. The most

widely used reagent 4-*bromomethyl*-7-*methoxycoumarin* (Br-Mmc), is introduced by Duenges (189) and is used for the derivatization of dicarboxylic acids (190), fatty acids (191, 192), gibberellins (193), imides (194, 195), α-keto carboxylic acids (190), different types of organic acids (196) and phenols (196).

The general procedure is simple. The derivatives are prepared by adding 5.0 mg Br-Mmc, 1.0 mg crown-ether and 25 mg K_2CO_3 to a solution containing 0.5 mg of the acid in 20 ml aceton. The mixture is refluxed for 30 min at 70° C and if necessary the excess Br-Mmc is treated with n-valeric acid after completion of the reaction (194). The reaction scheme for the reaction of Br-Mmc with a carboxylic acid is presented in Figure 9.

The crown-ether is used for acceleration of the derivatization reaction in the form of a phase-transfer catalysis (197). The crown-ether influences the solvation of the cations and therefore activates the anions (192, 198). The reaction rate is dependent on the kind of base that is used. With KOH the reaction is faster than with K_2CO_3 and triethylamine (192). Moreover, the solvent has to be aprotic (199).

Br-Mmc is rather unstable: it hydrolyses in the presence of water and also Br-Mmc has to be protected from light and whenever the derivatization is carried out in the dark the derivatization yield is 100% (200). Contrary to the esters, Br-Mmc itself shows little fluorescence. In most cases LC is performed with RP columns, although NP chromatography is described (913). The detection limit for fatty acids is 7 pmol (191).

Instead of Br-Mmc a number of other methoxycoumarin derivatives have been developed to improve the stability of the reagent, to change the reactivity towards other functional groups with reactive protons or to enhance the fluorescence intensity of the derivatives.

4-*Hydroxymethyl*-7-*methoxycoumarin* (Hy-Mmc) is a coumarin derivative which reacts with carboxylic acids under the formation of the ester derivatives(195). The fluorescence characteristics of Hy-Mmc derivatives of fatty acids are established by Lloyd (15). The

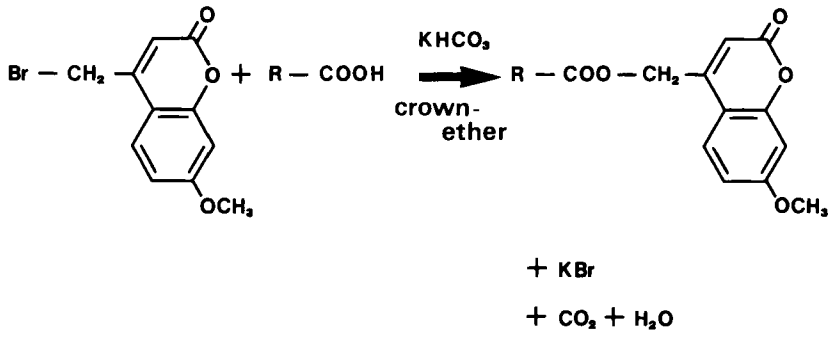

Fig. 9: Mechanism of the fluorescence labelling with Br-Mmc.

ϕ_f values of the esters in methanol are less than 0.1 but with the addition of water to the solvent the values rise to 0.4. In non-hydrogen bonding solvents the quantum yields are less than 0.02.

If Hy-Mmc is used in the presence of diethyl-azocarboxylate and triphenylphosphine (201) the reaction with carboxylic acids to form the corresponding esters is accelerated.
Hy-Mmc possesses excellent storage properties and is stable for one year, even in solution.

N,N'-*Dicyclohexyl-* and N,N'-*Diisopropyl*-O-(7-*methoxycoumarin*-4-*yl*)*methyl-isourea* (DCCI, DICI) are developed in succession of Hy-Mmc as labelling agents for carboxylic acids (27, 202). DCCI reacts readily in benzene or dioxane at 80° C without a catalyst (202). The products that are obtained are the same as those labelled with Br-Mmc.
α-Keto carboxylic acids react with DCCI or DICI in the presence of N,N'-dimethylhydrazine in acetonitrile yielding the corresponding esters. The detection limit for phenylpyruvic acid (27) is 10 pmol/ml.

4-*Bromomethyl*-6,7-*dimethoxycoumarin* (Br-Mdmc) (25) is developed as reagent to achieve a better quantum yield of fluorescence than the corresponding 7-methoxycoumarin esters. The ϕ_f (0.64 in

water) is not affected by the number of carbon atoms in the fatty acid contrary to Mmc esters. Furthermore, the emission is only slightly affected by pH, ionic strength and electrolyte changes of the solvent.

Pre-column derivatization with 4-*bromomethyl*-7-*acetoxycoumarin* (Br-Mac) has some advantages over the use of Br-Mmc and Br-Mdmc. The Br-Mac derivative is separated with RP chromatography and in a post-column system the derivative is hydrolyzed in alkaline media to a fluorophore which is the same for every carboxylic acid (203). Gradient elution systems can be effectively used in combination with this method since the composition of the mobile phase does not effect the quantum yield of the fluorescent hydrolysate. With this system the detection limit of fatty acids is in the fmol range.

7-*Methoxycoumarin*-3-(and -4)-*carbonyl azides* (3-MCCA, 4-MCCA) are synthesized as labelling reagents for hydroxyl functions (26). The reactions with primary and secondary alcohols in dichloromethane yield the corresponding coumarin carbamic esters. The esters are separated on an RP system (mobile phase: water-methanol or water-chloroform).
The 3-MCCA derivatives show more intense fluorescence than the 4-MCCA derivatives. The detection limit for cholesterol labelled with 3-MCCA is 50 fg/ 100 µl.

4-*Diazomethyl*-7-*methoxycoumarin* (D-Mmc) is another reagent for alcohols and carboxylic acids (204). D-Mmc is practically non-fluorescent in solution and possesses excellent storage properties. D-Mmc reacts with alcohols in dichloromethethane at room temperature in the presence of HBF_4 as a catalyst and yields the corresponding fluorescent ether. With carboxylic acid it reacts in acetonitrile on heating. The derivatives are separated with an RP system with a mixture of acetonitrile-tetrahydrofuran-water as the mobile phase.

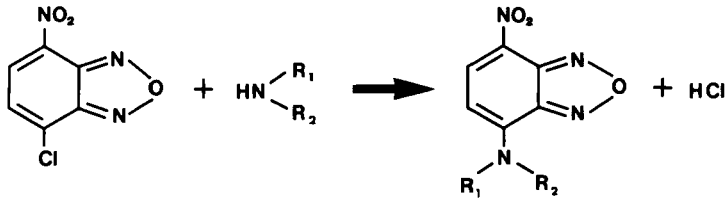

Fig. 10: Mechanism of the fluorescence labelling with NBD-Cl.

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and *Related Compounds*

In 1968 Ghosh and Whitehouse (205) introduced NBD-Cl as a new fluorogenic reagent for primary and secondary amines, including amino acids. Ever since this reagent has been widely used for the analysis of compounds containing these functions.

The general reaction, in this case with a secondary amine (Figure 10) can be performed in aqueous as well as in organic medium. The optimum pH is in the range 8-9. The reagent itself is non-fluoresent, while the resulting derivatives show intense fluorescence properties.

Since the publication of reviews by Seiler and Demish (121) and Seiler (206) a number of investigations in this field have been published. Ahnoff et al. (207, 208) suggest that, in methanol containing media, NBD-Cl partly solvolyzes to form NBD-OCH_3 which reacts with the amino function yielding the fluorescent derivative. Moreover, non-polar 7-nitro-4-benzofurazanyl ethers react faster than NBD-Cl, while polar ethers show lower reactivity but due to increased solubility in water, do not require the presence of an organic solvent. Phenolic and sulfhydryl groups react less readily, indicating the selectivity of the reaction for amino functions. A disadvantage of the method is that both the reagent and the derivatives have a limited stability.

Many applications of NBD-Cl derivatization for the determination of amino acids, peptides and metabolites in biological media

have been published, either with pre-column (207, 211, 212) or post-column (209, 210) derivatization. Generally, using pre-column methods, separation is obtained using RP HPLC, while prior to post-column derivatization IE HPLC is applied. The detection limits for amines are, in general, in the pmol region.

Several authors investigated modifications of the NBD-Cl derivatization in order to reduce the reaction time and to increase the derivatization yield. Imai and Watanabe (213) introduced 4-*fluoro*-7-*nitrobenzo*-2-*oxa*-1,3-*diazole* (NBD-F), which is more reactive than NBD-Cl. With this reagent the detection limits for amines are in the same region as with NBD-Cl. A number of applications of NBD-F have been published, either with pre-column (214-216) or post-column (217) derivatization. The post-column method could also be modified to make it suitable for the analysis of thiols, although the sensitivity is rather poor.

Attempts to improve the selectivity towards thiols led to the introduction of the anion 4-*fluorobenzo*-2-*oxa*-1,3-*diazole*-7-*sulfonate* (SBD-F) as a derivatization reagent for sulfhydryl groups (218). The selectivity for thiols is indicated by the fact that no derivatization occurs with alanine and proline. Using the pre-column method separation of the fluorescent derivatives could be obtained with RP HPLC. Detection limits are in the pmol region (219). The same sensitivity could be obtained with 4-*fluoro*-7-*sulfamoylbenzo*-2-*oxa*-1,3-*diazole* (NH_2-SBD-F)(220).

Introduction of a hydrazine function led to the possibility of derivatizing carbonyl compounds with 4-*hydrazino*-7-*nitrobenzo*-2-*oxa*-1,3-*diazole* (NBD-H)(221). Both aldehydes and ketones react, although the latter show a considerably lower reactivity in comparison to the former, resulting in much lower reaction times. The hydrazones formed can be separated either on silicagel or RP-8 columns. The derivatization yields for both aldehydes and ketones is 99%.

The most recent development in the technique is the application of derivatives of the fluorogenic amine 4-*amino*-7-*nitrobenzo*-2-*oxa*-1,3-*diazole* (NBD-amine) as substrate for the determination

of hydrolytic enzymes like chymotrypsin (222). Enzymatic hydrolysis of the non-fluorescent derivative yields NBD-amine, which is highly fluorescent. The fluorescence intensity can serve as a measure for the enzymatic activity.

o-*Phthalaldehyde*

o-Phthalaldehyde (OPA) is a reagent with no native fluorescence. It is developed for primary amino functions, such as amino acids and polyamines (223, 224).

OPA is used in an aqueous reducing medium, e.g. 2-mercaptoethanol, 3-mercapto-1-propanol (225) or ethanethiol (161, 226) buffered with borate around pH 10 and with primary amino functions a fluorescent isoindole is formed (Figure 11). However, some derivatives of amino acids have little or no fluorescence activity (227-229). OPA is used in pre-column as well as in post-column derivativatizations. The detection sensitivity with this reagent is greater than with fluorescamine and the reaction is completed within 1-2 minutes at room temperature.

Secondary amines do not react directly with OPA, but this limitation can be overcome with chloramine T (230), N-chlorosuccinimide (168, 231) or sodium hypochlorite (232, 233). If NaOCl is used for the conversion of secondary amines into primary amines the detectability of the latter is decreased by side reactions caused by the excess of NaOCl (234).

Both the compound itself and the reagent solution are stable. However, a significant limitation of the pre-column derivatization is the instability of the derivatives (235, 236). This instability is caused by slow, spontaneous intermolecular rearrangements (237). The stability of the derivative is influenced by the structure of the thiol (238) and the excess of OPA (225, 239). The fluorescence properties also depend on the structures of the thiol and the primary amine (225, 239). 2-Ethanethiol and 3-mercapto-1-propanol are used instead of 2-mercaptoethanol because they are more stable in the above reaction than the latter (238). The derivatization with ethan-

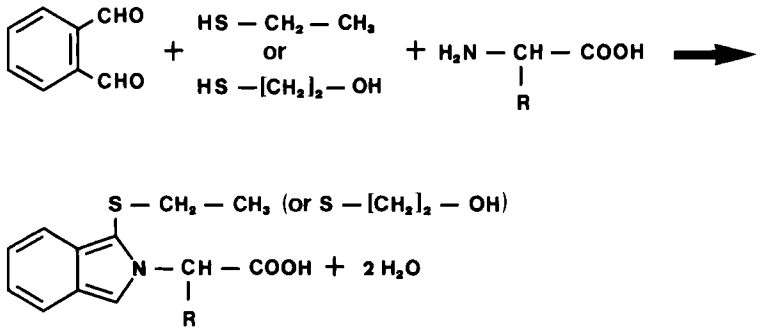

Fig. 11: Mechanism of the fluorescence labelling with OPA.

ethiol, however, is somewhat slower but still 100% conversion of the amino acid is achieved (240). When the ethanethiol derivative is prepared in an aqueous buffer and then transferred to a 95% ethanol solution, the fluorescence increases with 60-70 percent in contrast with the 1-6 percent increase for the 2-mercaptoethanol derivative (238, 242). Since the reaction time of the derivatization reaction and the stability of the derivatives influence the fluorescence intensity of the products during HPLC analysis, a standardized procedure for derivatization and chromatography is necessary (235).

After pre-column derivatization of amines with OPA the derivatization mixture is diluted and injected into an RP HPLC system (240-243). For a derivatization yield of 100% a 200-fold excess of the reagent is necessary. The analysis of primary amines in biological fluids after pre-column derivatization with OPA is described with detection limits of 1-10 ng and recoveries of 98% after extraction of urine or plasma (235, 244, 245).

For post-column derivatizations, impurities in the reagent and mobile phases must be rigorously avoided because of their contribution to the background fluorescence (246). A post-column RP system for the analysis of amino acids and primary amines is developed by Kucera and Umagat (247). For the analysis of netilmicin a post-column RP-PI system is described (248). Some investigators have developed post-column systems for the simultaneous analysis

of all amino and imino acids (primary and secondary amines) in an amino acid analyzer. The methods are based on the reaction of the imino acids with dilute NaOCl at high pH and the derivatives are separated with IE chromatography (249). Himuro and co-workers, instead of the usual NaOCl-OPA reagent, used an NaOCl-OPA-TDE reagent, and studied the influence of 2,2'-thiodiethanol (TDE) on the fluorescence suppressing effects of the NaOCl excess (234). With an amino acid analyzer ng amounts can be analyzed (249).

Nakamura and Tamura (250) described a post-column derivatization system for thiols. The derivatives are formed with taurine as the primary amine and are separated with an IE system. Instead of taurine, n-propylamine is also used (251).

Fluorescence Introducing Reagents after Activation of Reactive Functions

In the previous paragraphs methods have been discussed to convert non- or weakly fluorescent compounds into fluorescent derivatives. The disadvantage of these methods is that the choice of labels is limited because it requires a good reactivity of both the functional group and the label.

Another possibility is the use of indirect fluorescence introducing methods. With these methods either the compound to be analyzed or the fluorescence introducing label is activated before derivatization. If the label is first activated the advantage is that functional groups that do not react spontaneously under these circumstances, will react more easily (27). Activation of the solute, followed by reaction with the fluorescence reagent, offers the possibility to use different fluorescence reagents. By the choice of the activator only the reacting functions of the fluorescence label and the compound are committed but not the structure of the label.

For the derivatization of carboxylic acids a few methods have been described. The general reaction of a carboxylic acid group with an alcohol or amine only yields a considerable amount of the

resulting ester or amide after activation of either the carboxylic acid function or the hydroxylic/amine function prior to coupling.

N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (DAC) is a water-soluble representant of the carbodiimides suitable for activation of the carboxylic acid function (252). This activation is relatively slow, but can be accelerated by the addition of 1-hydroxybenzotriazole as a catalyst (253). The activated carboxylic acid reacts with alcohols (23) and primary amines (253). The yields are usually high. If, with this method, *9-(hydroxymethyl)anthracene* (HMA) is used as fluorescence label for benzoic acid the detection limit is 100 fmol after RP separation (23). *Aminoethyl-4-dimethylaminonaphthalene* (DANE) is a chiral derivatization reagent and both the D- and L-forms are used. With enantiomeric carboxylic acids the diastereomeric amides are formed (252). Separation of these diastereomers is possible with NP HPLC. The method is applied for the analysis of naproxen in serum (253) with a detection limit of 100 pg.

Carboxylic acids can also be activated by *N,N'-carbonyldiimidazole* (CDI) and the activated function reacts with a fluorescent alcohol, i.e. HMA to form the corresponding ester (23).

Benzoic acid reacts in the presence of *diethylazodicarboxylate* (DA) and *triphenylphosphine* (TPP) in tetrahydrofuran with *2-dansylaminoethanol* to form 2-dansylaminoethyl benzoate (254) or with Hy-Mmc (201) to the corresponding ester.

Activation of the carboxylic acid is also possible with *2-bromo-1-methylpyridinium iodide* (BMP). After activation with BMP the carboxylic function reacts either with a fluorescent alcohol (23) or with a fluorescent amine (29)(Figure 12). Separation of the esters can be achieved on an RP system, while HPLC of the amides requires a more complex system (255). The method is applied for the analysis of ibuprofen and glucuronides in plasma and urine samples.

New Fluorescence Derivatization Reagents

For the fluorescence labelling of potentially acidic functions a number of alkylation reagents have been developed, e.g. *diazoal-*

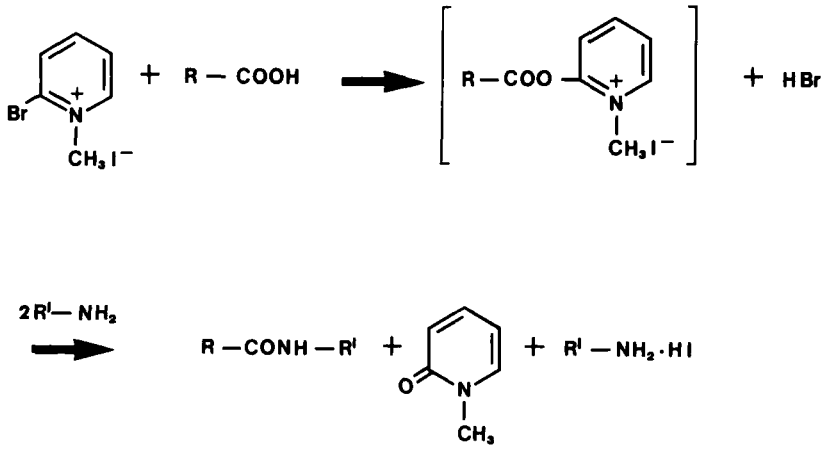

Fig. 12: Mechanism of fluorescence labelling after activation with BMP.

kane derivatives (256-258), 4-*bromomethyl-7-methoxycoumarin* derivatives (see before), *naphthacyl bromide* (259), *panacyl bromide* (260), 1-*bromoacetylpyrene* (261) and 9-(*chloromethyl*)*anthracene* (24). The major disadvantage of these labels is their lack of selectivity. In most cases these reagents react with all functions with acidic protons (e.g. carboxyl, hydroxyl, phenol, thiol or imide) in a more or less quantitative way.

The derivatization with all these alkylation reactions is executed in the same way; deprotonation of the acidic function with a base and reaction of the anion, with or without catalysis, with the alkylation reagent in relatively polar solvents.

Diazoalkane labelling is advantageous over the other methods because of the high reaction rates. A disadvantage, however, is the lack of stability of most of these reagents.

Primary amines such as 1-*naphthylamine* (262) and 9-*aminophenanthrene* (263) can be used in the fluorescence labelling of the acid chlorides of fatty acids (264). In the analysis of unsaturated fatty acids oxalyl chloride is preferred over thionyl chloride for its higher derivatization yield. The detection limit of the esters is

about 10 pmol and the method can be applied in biological fluids (263).

Instead of 9-aminophenanthrene Lloyd (28) used 9,10-*diamino-phenanthrene* (DAP) for the fluorescence labelling of fatty acids. The acids are condensed in chloroform, in the presence of methyl polyfosfate, with DAP. The method is described as a pre-column derivatization before RP chromatography. The polyfosfate is used because of the poor solubility of DAP (265). This solubility problem and the possibility of fosforylation of other functional groups that are present in the sample are the main disadvantages of the method. The detection limit for fatty acids is about 50 fmol.

α-Keto carboxylic acids are derivatized with o-*phenylenediamine* to 2-quinoxalinol derivatives (266, 267). The method is applied for the analysis of pyruvic acid (268). The detection limit is less than 1 nmol/50 μl keto carboxylic acid.

4'-*Hydrazino-2-stilbazole* (4H2S) is a fluorescent reagent selective for carbonyl functions and especially for α-keto acids (269). These acids (270) are converted to hydrazone derivatives with 4H-2S. The detection limit for phenylpyruvic acid in plasma or urine is 30 pmol/200 μl.

For thiols a number of selective fluorescence labels have been developed including the monobrobimanes which have little native fluorescence. One of them, *monobromo-trimethyl-ammoniobimane* (271, 272), reacts quantitatively with thiols. After IE chromatography 1 pmol of the derivatives can be detected. The rapid hydrolysis of the reagent and the photodecomposition of the derivatives can limit the accuracy of the quantitative measurement.

1,2-*Naphthoylenebenzimidazole-6-sulfonylchloride* (1,2-NBI-SO_2Cl) is used as fluorescent reagent for the derivatization of aliphatic amines prior to RP chromatography (273, 274). The reaction is quantitative but during the derivatization some side

products are formed, which can influence the chromatographic analysis. The reagent is stable for relatively long periods and 1 pg of the aliphatic primary or secondary amine can be detected. The reagent is comparable to dansyl chloride but its sensitivity is better.

Isocyanates and isothiocyanates react with primary amines to give urea and thiourea derivatives, respectively. Isocyanates however, react quite readily with water and alcohols to give urethanes. The isothiocyanates are less reactive concerning the reaction with water or alcohols.

The most recent examples of this group of reagents are: 9-*isothiocyanatoacridine* (275), *fluoresceinisothiocyanate* (276), *phenylisothiocyanate* (277), 4-*dimethylamino*-1-*naphthylisothiocyanate* (278), *boc-aminophenyl*- and *boc-aminomethylphenylisothiocyanate* (279) and 4-(6-*methylbenzothiazol*-2-*yl*)*phenylisocyanate* (Mbp) synthetized to improve the fluorescence sensitivity in comparison to phenylisocyanate (280, 281).

N-*Succinimidyl*-2-*naphthoxyacetate* (SNA) is used for the derivatization of amino acids (282) and some phospholipids (283). The detection limit for the phospholipid analysis is 2 pmol.

2-*Cyanoacetamide* reacts with reducing compounds, such as carbohydrates and polyphenols which leads to the formation of fluorescent condensation products (284). The reaction is selective and only high concentrations of aldehydes or phenols may react to some extent. The derivatization can be used either in the pre-column (285) or in the post-column (284) mode. In both cases IE chromatography is used. The detection limit for catecholamines is 5 pmol (286).

Hydroxylic compounds can be derivatized with labelling reagents, containing nitrile functions, such as (+)- and (-)-2-*methyl*-1,1'-*binaphthalene*-2'-*carbonylnitrile* for the analysis of enantiomers

(287), 4-*dimethylamino*-1-*naphthoylnitrile* (DMA-nn)(288) for primary and secondary hydroxyl functions as well as 1- and 9-*anthroyl nitrile* (289).

APPLICATIONS

An up-to-date survey of the many applications of fluorescence detection in HPLC is presented in the Tables III, IV and V, with the emphasis on the quantitative determination of drugs and drug metabolites in biological fluids. The drugs mentioned in the Tables IV and V are divided in five main groups. Four of these groups are: acidic, amphoteric, basic and neutral compounds. Antibiotics, cytostatic agents, steroids and vitamins are dealt with in a separate group (V). Each group is subdivided into one or more structurally related subgroups and/or a number of miscellaneous compounds.

In Table III an overview is given of the fluorescence introducing reagents, including their abbreviations, mentioned in this review. In Table IV those compounds are listed of which the native fluorescences are used in combination with HPLC and Table V contains a list of drugs that have been analyzed with fluorescence detection and HPLC.

In the first column of the Tables IV and V the name of the product is mentioned, for which the procedure referred to, is originally designed. In some cases the internal standard and/or other drugs mentioned in the original publication are also reported.

"Sample" (second column of Tables IV and V) refers to the type of biological fluid (blood, urine, etc.) or tissue to which the method may be applicable as well as the volume required per analysis. In the cases where the method is applied in several biological media the term "fluids" is introduced.

In the third column the clean-up procedure used in the analysis, prior to chromatographic separation, is denoted. The various clean-up procedures are classified according to type and number of clean-up steps in the pre-chromatographic sample treatment. Simple

"washings" of samples or extracts, in which the analyte is not transferred to another phase, is not considered a clean-up step here. The meaning of the symbols is as follows:

-, no clean-up step is used in the pre-chromatographic sample treatment;

a, the only clean-up step is some form of protein removal;

b, a single extraction step;

c, a single clean-up step other than liquid-liquid extraction, e.g. by TLC or column LC;

d, more than one clean-up step, as is the case in liquid-liquid extraction of the analyte from the sample with an organic solvent and back extraction into an aqueous phase.

In the fourth column is mentioned which type of HPLC is used: chromatography with polar stationary phases (NP), chromatography with apolar stationary phases (RP), ion-exchange chromatography (IE) and paired-ion chromatography (PI).

In the fifth column of Table V is mentioned whether the fluorescence is measured after pre-column (Pre-) or post-column (Post-) derivatization. The name of the abbreviation of the derivatization reagent (Table III) is also given in this column.

The last column gives the reference to the literature, in Table IV as well as in Table V. The references between brackets describe the analysis of the drug mentioned in the same line with the discussed derivatization and chromatographic method, however, the determination is not performed in a biological sample.

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