An introduction to

# Fluorescence in Biological Analysis

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## Introduction

Clinical, pharmaceutical and research biochemists are continually being stimulated into looking for more sensitive analytical methods using micro samples. Fluorescence spectroscopy is only one of the techniques that meets the requirement of high sensitivity without loss of specificity or precision; another is radioimmunoassay. More recently, the use of chemi- and bioluminescence has opened up new avenues of research and applications, primarily in the immunoassay field. The primary advantage of these new luminescence techniques is the combination of relatively simple instrumentation and excellent sensitivity. Since this review is concerned with fluorescence applications, no reference will be made to the use of chemi- and bioluminescence, which will be the subject of a future review. Other related techniques not covered in this review are flow cytometry and fluorescence microscopy.

Contrary to popular belief, fluorimetric techniques do not differ substantially from those of UV absorption spectrometry. In fact, many of the procedures are adaptations of the latter, but geared to smaller samples. The vast majority of the methods do not require sophisticated equipment and the analyst can therefore use relatively inexpensive filter fluorimeters.

Since the last review (1), several important changes have taken place which enhance the use of fluorescence. The combination of the separative power of liquid chromatography, together with the sensitivity of fluorescence has provided the analyst with a very powerful tool. The change from radioisotopes to fluorescent tags has simplified the instrumentation and at a stroke overcome the objections to the use of radionucleotides. The combination of fluorescence with immunoassay and the ever expanding use of enzymes should result in fluorimetry gaining more importance in clinical chemistry (2).

The advent of microprocessors has increased the flexibility of instruments and at the same time improved the reliability, sensitivity and precision of measurement.

Since several excellent books have been published on the theory of fluorescence, it is not intended to cover this topic, but to bring the reader up to date on the many applications of fluorescence in biological analysis.

## Amino acids

As with many of the older assays using conventional chemical extraction procedures, amino acid analysis has been greatly simplified by the use of liquid chromatography (3). The vast majority of the methods involve either pre- or post-column chemical derivatization to produce a fluorescent complex (4).

o-Phthalaldehyde, OPA, is the most widely used of these reagents, giving sensitivities in the femtomole range (270-277). OPA reacts with primary amines at alkaline pH's in the presence of certain thiols such as mercaptoethanol and ethanethiol. The reaction is essentially complete within one minute. The derivatives, which have an average quantum yield of 0.4, are unstable and decompose to non-fluorescent products. OPA does not react with proline or hydroxyproline and gives low fluorescent yields with cystein, cystin and lysine. Peptides also give a low yield due to the quenching of the isoindole group by the carboximide group. Because of the unstable nature of the derivatives, OPA is more widely used in post-column derivatization. However, since the polar amino acids are converted to hydrophobic compounds by OPA, the chromatography can be performed rapidly on reverse phase columns. Figure 1 is an example of the high speed separation of OPA-amino acids achieved using a 3 µ 3 cm reversed-phase column.

Dansyl chloride, DNS-Cl, is another popular reagent (80, 278-280, 286). However, in contrast to OPA derivatives, the emission wavelength of the DNS-amino acids varies considerably with solvent polarity. In addition, there is a tenfold increase in intensity in non-polar solvents such as hexane compared to water. The reaction of DNS-Cl is relatively slow and the majority of methods require heating in a

reaction vessel at 70 ˚C to speed up the formation of the fluorophore. The hydrolysis product of DNS-Cl is highly fluorescent and can interfere with chromatography of the more hydrophilic amino acids such as aspartic and glutamic. DNS-Cl also reacts with phenolic hydroxy groups and has been used in the determination of phenols, estrogens and drugs. In a paper, Singh and Hinze (5) investigated the micellar-enhanced fluorescence observed from OPA and DNS derivatives of amino acids.

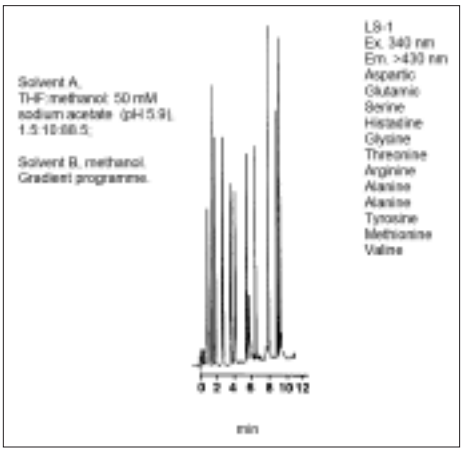



*Figure 1.* Chromatogram of amino acid standards derivatized by reaction with OPA, run on a 3 cm, 3 µ, C18 column.

Fluorescamine (fluoram) reacts rapidly with primary amines to give highly fluorescent derivatives which are stable for several hours in the dark (245, 281-285). Fluoram is predominantly used in post-column derivatization, since double peaks have been observed when the reaction mixture is injected on a column.

The halogenitrobenzfurans, NBD-Cl (207, 286) and NBD-F (287, 283), react with both primary and secondary amines to give fluorophores which are stable for one week in the dark. As with DNS-Cl derivatives, difficulties are encountered when separating the more hydrophilic compounds.

The native fluorescence of the aromatic amino acids has also been utilized. For example, the indole metabolites of tryptophan have been determined in cerebrospinal fluid and plasma after separation on a reverse phase column (6).

## Histamine

Histamine can be determined by condensation with OPA to form a highly fluorescent derivative with an excitation of 340 nm and emission of 450 nm (7-9). Histamine is extracted into n-butanol from alkalinized perchloric acid extracts of blood, re-extracted into an aqueous solution followed by condensation with OPA. Histidine produces a fluorophore with similar fluorescent properties, but is not extracted by butanol. The stability of the histamine complex was been questioned (10) with three different methods investigated. While many of the assays are on clinical samples (11-14), histamine has also been measured in cheese (15) and in wine and must (16).

## Histidine

Ambrose et al (17) developed a method for histidine in which deprotinised plasma or serum is heated with alkaline OPA for 15 min at 30 ˚C, followed by addition of a phosphoric acid reagent to stabilize the fluorophore. Gerber (18) has described a similar procedure using hydrochloric acid instead of phosphoric acid as stabilizer.

An automated procedure using 25 µL blood samples spotted onto 10 mm diameter filter paper has also been described (19).

Moriyanu and Watanabe (20) have suggested the use of fluorescamine for urinary histidine following the method of Nakamura and Pisano (21).

## Phenylalanine

The estimation of phenylalanine is one of the most commonly performed amino acid analyses. The McCaman and Robins method (22) based on the reaction of phenylalanine and ninhydrin in the presence of L-leucyl-L-alanine has undergone several modifications. Wu et al (23) reported a method that combines the accuracy of the fluorimetric method with the simplicity and convenience of collecting blood samples on filter paper. An automated screening system for phenylketonuria has been described by Hoffman et al (24).

In 1981, Neckers et al (25) introduced an LC method for plasma tyrosine, tryptophan and phenylalanine. The latter was detected by post-column derivatization with OPA while the other two amino acids were detected using their native fluorescence.

## Serotonin

The native fluorescence of serotonin was used by Ashcroft (26) for its determination in normal blood. Platelet serotonin can be estimated using the method of Vatessery et al (27). Serotonin is extracted into an ascorbic acid solution by freeze-thawing and sonication. The ascorbic acid stabilizes the extract, while ethanol is added to enhance the fluorescence which is measured in concentrated sulphuric acid with an excitation of 295 nm and an emission of 540 nm.

Serotonin can also be separated from blood using column chromatography (28) and a strong cation exchanger. Guilbault and Froehlich (29) used this procedure to measure serotonin, 5-hydroxytryptophan and 5-hydroxyindole acetic acid. Blood proteins were precipitated with trichloroacetic acid and the supernatant added to the ion exchange column. The eluant contains 5-hydroxyindole acetic acid which is estimated by adding 0.1% ascorbic acid in concentrated sulphuric acid. The 5-hydroxytryptophan is eluted from the column with saturated borate solution while the serotonin is eluted with 0.1 N sodium hydroxide.

Increasing the sensitivity by derivatization or chemical reaction is of great interest and two methods have been published using ninhydrin (30, 31). OPA will also react with serotonin (32, 33) to give a highly fluorescent product, as will 5-hydroxytryptophan (34).

The 5-hydroxyindoles can also be separated by LC as shown by Graffeo and Karger (35). The native fluorescence of the compounds is measured with an excitation of 280 nm and the emission monitored between 330 and 380 nm. Improved specificity for these biogenic 5-hydroxyindoles was obtained by Hojo et al (36) by using a postcolumn reaction with perchloric acid.

## Tryptophan

As can be seen, several methods are available for measuring indoles either by monitoring the native fluorescence or by forming derivatives. In the case of tryptophan, a highly fluorescent derivative can be formed by condensation of the amino acid with formaldehyde followed by heating and oxidation with hydrogen peroxide. The derivative, called norharman, has an excitation of 360 nm and an emission of 435 nm and has been used by Williams et al (37) for the estimation of tryptophan in plasma. Park et al (38) used norharman to measure tryptophan in amino acid products. Guilbault and Froehlich (39) determined serum tryptophan by monitoring the decrease in the native fluorescence upon its reaction with the formaldehyde. The method is specific for tryptophan – other indoles such as tryptamine and serotonin do not interfere.

In a paper, Yamada (40) determined tryptophan and its metabolites in mouse brain using LC separation.

Many papers have been published on the determination of amino acid residues in peptides and proteins. These procedures are usually lengthy and involve complicated chemical reactions and separations. A simplified procedure for measuring aromatic amino acids has been introduced by Garcia-Boron (41) in which second derivative fluorescence was used for quantifying the tryptophanyl and tyrosyl residues of peptides.

## Tyrosine

The use of screening programs for phenylketonuria has increased the importance of measuring tyrosine either directly or by derivatization. 1-nitroso-2-naphthol gives a highly fluorescent product (42), with an excitation of 430 nm and an emission of 535 nm. Unreacted reagent is extracted into ethylene dichloride and the fluorophore measured in the aqueous phase. The reaction is relatively specific for tyrosine since compounds known to interfere are present only in very low concentrations in the blood of newborns.

### 5-Hydroxyindole Acetic Acid (5-HIAA)

5-HIAA is readily estimated in urine using liquid chromatography. In a procedure by Rosano (43), the 5-HIAA is extracted from acidified urine with diethyl ether. An aliquot of the organic phase is then evaporated to dryness in a stream of nitrogen and the residue taken up in the mobile phase. The latter is a mixture of methanol and water buffered at pH 5.5. Separation is achieved using a C8 reversed phase column and the 5-HIAA is detected with an excitation of 300 nm and an emission of 350 nm. Plasma 5-HIAA and indole-3-acetic acid (IAA) can also be determined by reversed phase chromatography (44) with ion pairing. Concentrations range from 0.61 to 3.52 µmol/L for IAA and from 33.0 to 102.6 µmol/L for 5-HIAA.

## Proteins and other biologically important molecular complexes

The use of fluorescence in the study of proteins and other biologically important molecular complexes is an extensive and well documented technique (45-46). Consequently, it is beyond the scope of this review to include all of the applications and the papers which have been published. The use of the technique ranges from the study of structural features at the molecular level to the use of fluorescence labeling in immunology.

Fluorescence and phosphorescence are particularly useful in physical biochemistry since they provide much information with regard to the interaction of molecular complexes with their environment. Molecular rotation and reorientation in biochemical systems can be conveniently studied as they have similar lifetimes to the excited singlet and triplet states.

Four fundamental variables can be measured. These are:

- intensity
- wavelength
- lifetime
- polarization

Two groups of molecular probes are used, depending on whether the fluorescence is intrinsic or extrinsic. The former arises from fluorescent residues in the molecule. These include, for example, aromatic amino acids such as tyrosine and tryptophan and other structures such as reduced nicotinamide adenine dinucleotide, NADH.

Extrinsic fluorescence arises from the addition of a fluorescent probe to the system under study. Dramatic changes in the emission characteristics can occur when certain labels are adsorbed or covalently bound to proteins. 1-Anilino-8-naphthalene sulphonate, ANS, fluoresces weakly in aqueous solution, but on binding to a protein such as albumin, a large increase in intensity occurs. The emission wavelength shifts to the blue together with an increase in the fluorescent lifetime (47, 48).

Table 1 lists some of the fluorescence characteristics of Aminonaphthalene Sulphonic Acid (ANS) in a variety of solvents. Other probes which have been used are DNS-Cl, fluorescamine and tetramethylrhodamine. Probes such as 4,6-diamino-2-phenylindole, DAPI (49) and dibenzimidazole (50) exhibit a specific increase in fluorescence on binding to DNA.

*Table 1.* Fluorescence parameters of aminonaphthalene sulphonic acid in various solvents.

| Solvent | Emission Wavelength (nm) | Qf | T (ns) |
|---|---|---|---|
| Water | 515 | 0.004 | 0.55 |
| MeOH | 476 | 0.216 | 6.05 |
| Octanol | 464 | 0.646 | 12.30 |
| Bound to apomyoglobin | 454 | 0.980 | 16.40 |

## Probes

The binding of small molecules to proteins is an extremely important process as the mechanism of drug action, distribution and excretion are affected by the binding. Drug-protein interactions can be investigated by monitoring the change in fluorescence which occurs on binding.

Either the intrinsic fluorescence of the protein is used or the extrinsic fluorescence of a probe which competes for the drug binding site. A third possibility is to use the fluorescence of the drug itself.

The quenching of the intrinsic fluorescence of albumin is a relatively quick and easy method of studying these interactions.

Assuming a single binding site, then the fractional quenching of the fluorescence will be linearly related to the concentration of the albumin complex. However, in the case of albumin binding bilirubin, at least four sites are involved, leading to a non-linear decrease in the albumin fluorescence. Parsons (51) has discussed the procedures used by different authors in interpreting the fluorescence data.

The study of the binding of acidic drugs is well established (52), whereas the interaction of basic drugs has received little attention, primarily due to the lack of suitable probes. However, the introduction of DAPN – a fluorescent probe derived from the basic drug propranolol – together with the use of flow injection analysis has opened up the area of analysis (53).

The use of fluorescent probes in the investigation of membrane-related phenomena has become a widespread and sophisticated technique and has been reviewed by Beddard and West (54). Membrane potentials and pH's have been measured using probes such as N-aryl naphthalene sulphonates, cyanine dyes and 9-aminoacridines.

The rotational diffusion of macromolecules in aqueous solutions has been successfully investigated using the technique of fluorescence polarization (55). Both steady state and time resolved measurements are useful, although the latter require relatively sophisticated instrumentation since fluorescence lifetimes are of the order 1-100 ns. Studies on the structure and dynamics of lipid membranes have been investigated by measuring the steady state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, DPH, (56-57). The degree of polarization depends on the rigidity of the environment. This is well illustrated by dimyristoyl lecithin which undergoes a phase transition at around 25 °C, from the gel-like rigid phase to a more crystalline phase at higher temperatures. The changes can be followed by measuring the polarization of DPH, (Figure 2). In a clinical application of the technique, fetal lung maturity has been determined by measuring the polarization of DPH in amniotic fluid (58, 59).

The mobility of membrane proteins cannot be measured by fluorescence as their rotational diffusion extends into the millisecond region. New techniques have therefore been developed using the triplet states of organic molecules which have similar lifetimes (60, 61).

Although radiative transitions from the triplet state to the ground state are not normally detected at room temperature, Murray et al (62) have successfully used the phosphorescence from erythrosin in probing the sarcoplasmic reticulum ATPase (Figure 3).

Electronic energy transfer between a donor and acceptor can be used to investigate conformational changes. In addition, since the efficiency of the energy transfer depends on the inverse 6th power of the distance between the two, the process can be used to measure the distance between various sites in a protein.

McCarville and Hauxwell (63) investigated the use of various phosphorescent compounds to probe the aromatic amino acid residues at the active sites of carbonic anhydrase.

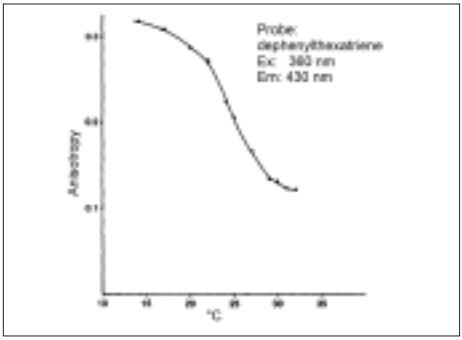



*Figure 2.* Plot of anisotropy versus temperature for dimyristyl-L-3-lecithin in 50 mM potassium chloride.

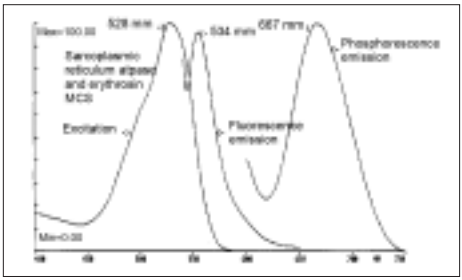



*Figure 3.* Room temperature phosphorescence and fluorescence of sarcoplasmic reticulim ATPase labeled with erythosin isothiocyanate measured under anaerobic conditions on the Model LS-55.

Luk (64) used trivalent lanthanides such as terbium to measure the distance between the metal binding sites of transferrin. Measurements were made at room temperature and investigations have shown that energy transfer occurs between the tyrosine/tryptophan residues and the terbium. The use of lanthanides as probes in biological systems has been reviewed by deJersey et al (65).

## Immunoassay

The application of fluorescence in immunology has expanded rapidly in the last few years and several reviews are available (66-70). Both homogeneous and heterogeneous fluorescence immunoassays have been developed for a wide variety of analytes. Homogeneous assays show sensitivities in the range $10^{-10}$ mol/L, whereas heterogeneous assays, because of their better discrimination of background interferences, show sensitivities in the range $10^{-12} - 10^{-15}$ mol/L. Many different approaches have been made in the use of fluorescence and the more common of these are summarized in Table 2.

*Table 2.* Fluoroimmunoassay Methods.

| Analyte | Assay Type | Assay Range | Label | Ref |
|---|---|---|---|---|
| Digozin | FETI | 0.5-5.0 ng/mL | b-Phycoerythrin | 247 |
| HCG | TRIFA | 0.7-350 IU/L | Europium | 244 |
| Phenobarbital | SRFPIA | 0.5-40 µg/mL | Fluorescein | 240 |
| Progesterone | DAFIA | 0.3-90 nmol/L | Fluorescein | 245 |
| Quinidine | FPIA | 0.5-8 µg/mL | Fluorescein | 243 |
| Serum IgE | FEIA | 1-60 IU/mL | Umbelliferone | 256 |
| Serum IgE | SPFIA | 2.5-200 IU/mL | Fluorescein | 242 |
| Theophylline | SLIFA | 0.3-40 µg/mL | Umbelliferone | 241 |

FETI Fluorescence energy transfer immunoassay
TRIFA Time-resolved immunofluorescence assay
SRFPIA Single-reagent fluorescence polarization immunoassay
DAFIA Double antibody fluorescence immunoassay
FPIA Fluorescence polarization immunoassay
FEIA Fluorescence enzyme immunoassay
SPFIA Solid phase fluorescence immunoassay
SLIFA Substrate-labeled fluorescence immunoassay

The use of polarization FIA is perhaps the most popular technique and Dandliker et al (71) have reviewed the principles and experimental procedures involved in these assays. Antigens of low molecular weight are labeled with a fluorophore such as fluorescein. In aqueous solutions, the complexes rotate relatively quickly and the fluorescence emission is almost totally depolarized. On complexing with an antibody, the molecular size is considerably increased and the rotation slowed, giving a high degree of polarization. In a competitive situation between labeled and unlabeled antigen, the degree of polarization is proportional to the bound-labeled antigen. For example, Figure 4 shows the standard curve obtained for the drug flecainide using the single-reagent polarization FIA as described in reference (240).

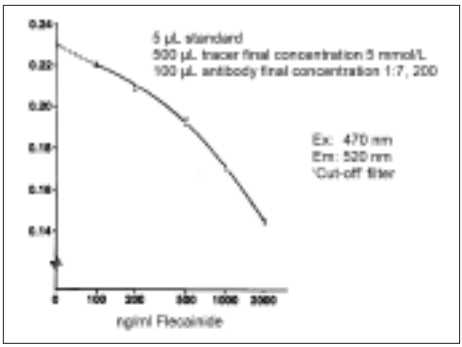



*Figure 4.* Polarization fluorescence immunoassay curve for the drug flecainide, using the Model LS-55.

The measurement of larger antigens such as proteins has been made possible by the use of fluorescence energy transfer. The technique, reviewed by Ullman and Khanna (72), involves labeling the antigen with fluorescein, the donor, and the antibody with rhodamine, the acceptor. On binding, the emission from the fluorescein, excited at the appropriate wavelength, is quenched through energy transfer to the rhodamine.

Many of the FIA methods use fluorescein as the label since it has a high quantum yield (good sensitivity) and is relatively simple to attach to antigens. However, the label does suffer from several disadvantages. The Stokes shift between the excitation and emission is relatively small, 30 nm, leading to light scattering interference if high quality optics are not used. In addition, high levels of serum bilirubin can interfere, since its luminescence characteristics are similar.

To avoid some of the problems associated with fluorescein, other labels have been used, including umbelliferone (241) and β-phycoerythrin (247).

More recently, chelates of rare earth such as europium (244) and terbium (73) have been used. The primary advantages of these labels are the large Stoke shifts and comparatively long emission lifetimes, 0.1-1.0 ms. With suitable electronic gating and a pulsed source it is possible to discriminate between the non-specific background fluorescence and that from the label.

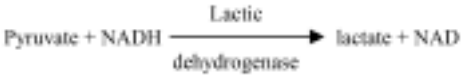
## Enzymes

Since certain enzymes are capable of catalyzing specific reactions with particular substrates, they are of great use in chemical analysis. When combined with the sensitivity of fluorescence spectroscopy several advantages accrue, such as shorter incubation times and smaller sample and reagent volumes. Lowery and Passenneau (74) have published a book giving information on all aspects of enzyme analysis from whole organs to single cells, providing an excellent grounding on the use of fluorescence in enzyme analysis.

The majority of the assays are based on the fluorescence of the reduced form of pyridine nucleotides, for example, NADH and NADPH and not on synthetic fluorogenic substrates, such as α-naphthol phosphate (75).

Assays involving the fluorescence of NADH and NADPH, are based either upon the forward reaction when they are the product or on the reverse reaction in which they are oxidized to NAD and NADP. The assays are either carried out by a timed method in which the fluorescence, after a fixed time, is measured or by a kinetic method in which the rate of increase or decrease of the fluorescence is measured. The excitation and emission wavelengths commonly used are 340 nm and 450 nm respectively.

In the determination of pyruvate, for example,




the concentration of pyruvate can be determined by measuring the rate of decrease in the fluorescence of NADH for fixed concentrations of lactate dehydrogenase, LDH. For the equilibrium method, a large excess of enzyme over pyruvate is used to ensure a relatively complete reaction and the difference between the signal at the beginning and the end is proportional to the concentration of pyruvate (76).

Generally, the kinetic method is preferred. It is faster and can be used for the determination of substrates, activator and inhibitor, whereas the equilibrium method is only useful for substrate determinations.

The potential sensitivity of enzyme assays can be increased by a procedure known as enzymatic cycling. In this system, several enzymes are used which catalyze interrelated reactions. For example, in the following assay $10^{-13}$ M of ADP can be determined:


The first two reactions are carried out at 37 ˚C for one hour, after which the reactions are stopped by heating at 100 ˚C for two minutes. The third reaction is started, after cooling, by adding glucose-6-phosphate dehydrogenase and incubating at room temperature for 15 minutes. ADP concentration is proportional to the NADH fluorescence.

The use of synthetic substrates in enzymology, has been expanded to cover a wide range of reactions. The principle of the assays is analogous to those of the chromogenic assays in that the product of the hydrolysis has different spectral characteristics from those of the substrate. Either the product fluoresces while the substrate does not, or the fluorescence of the product is different.

Early work was based on α-naphthol because of its prior use in clinical enzymology and several assays have been published for acid (77) and alkaline phosphatase (78). At pH 5.2, acid phosphatase hydrolyzes α-naphthylphosphate to α-naphthol and inorganic phosphate. After 30 minutes at 37 ˚C, the reaction is stopped by the addition of sodium hydroxide and the fluorescence of the α-naphthol measured directly, at an excitation of 340 nm and an emission of 460 nm. At these wavelengths, the substrate does not interfere, since its emission is at 360 nm with an excitation at 295 nm.

A similar reaction occurs using alkaline phosphatase at pH 9.0. However, a kinetic approach may be used since the α-naphthol is fluorescent at this pH.

Thus, the absolute fluorescence is unimportant and compounds such as salycilate do not interfere since their fluorescence does not alter with time.

The use of α-naphthol has been superseded by more sensitive fluorimetric methods based on, for example 3-hydroxy-2-naphthilides (79) and derivatives of 7-hydroxycoumarins (75). The use of 4-methyl-7-hydroxycoumarin is well suited to filter instruments since the fluorescence of the anion has an excitation of 360 nm with an emission of 450 nm.

A wide range of biological materials have been examined using a number of enzymes such as esterases, galactosidases, glucosidases, phosphatases and aryl sulphatases. For a more comprehensive list, the reader is referred to Leaback (75).

Uete et al (81) evaluated a method for the estimation of leucine aminopeptidase activity with L-leucyl-β-naphthylamide and found good

correlation with a colorimetric method. The β-naphthylamide liberated is excited at 340 nm and fluoresces at 400 nm. Automated assays have been described and a wide range of β-naphthylamide derivatives have been prepared for the estimation of aminopeptidase activities.

Sphingomyelinase was determined by Gatt et al (82) after the synthesis of a fluorescent derivative of sphyngomyelin (N-acyl-sphyngomyelin phosphocoline). Enzymatic hydrolysis of the derivative yields a fluorescent compound which is selectively extracted into heptane. Among other assays which have been published are spermidine synthase (83); arginase activity in dried blood spots (84); angiotensin converting enzyme (85); ornithine decarboxylase (86); β-glucoronidase (87); galactose in blood (88); kinase activity in rat tissue (89); and the detection of trypsin-like proteases and pancreatic elastses (90).

Because of the sensitivity involved with the various substrates, great care is necessary in keeping solutions free from contamination by microorganisms, otherwise unacceptably high blanks occur. Also, the specificity of the reaction should be carefully monitored when, for example, non-specific enzymes such as esterases are used.

## Biogenic amines and metabolites

### Catecholamines

The determination of catecholamines has generated much interest with the latest publications concentrating on obtaining lower and lower detection limits. In the clinical laboratory, the examination of urinary levels is fairly uncomplicated. On the other hand, plasma analysis is considerably more difficult because of the much lower levels.

Several fluorometric methods have been published and they can be classified into three separate groups. These are based either on the native fluorescence, an ethylene diamine condensation and the trihydroxyindole reaction.

- Ethylenediamine condensation involves oxidizing the amines to adrenochrome with ferricyanide followed by condensation with ethylenediamine (91). The fluorophore is measured at an excitation of 420 nm with the emission measured at 490 nm and 520 nm. In a paper (92), 1,2-diphenyl-ethyl-enediamine has been shown to react with catechol compounds giving sensitivities as low as 15-20 pmol/mL.
- The trihydroxyindole reaction involves oxidizing the amines with either iodine or ferricyanide followed by the addition of an alkali containing an antioxidant. The alkali causes the rearrangement of the oxidized compounds to the highly fluorescent 3,5, 6-trihydroxyindole (93-94). This procedure is the most widely used and is the most sensitive of the three methods.

In either procedure, internal standards are used as calibration markers to correct for any non-specific fluorescence. Various attempts have been made to improve the accuracy of plasma determinations. Valori (95) used a 10% solution of 2,3-dimercapto ethanol in 25% formaldehyde, while Merrils (96) and McCullough (97) used thioglycollic acid to stabilize the fluorescence.

Urinary norepinephrine, epinephrine and dopamine were determined by Oka et al (98) by first separating the compounds by boric acid gel chromatography. The amines were then oxidized at two different pH's using a modified trihydroxyindole reaction and the fluorescence measured at three different wavelengths.

Molnar and Horvath (99) demonstrated the separation of the amines and their metabolites using a reversed phase column and opened up a new avenue of investigation. Davis (100) derivatized deprotinized plasma samples with OPA followed by an ethylacetate extraction of the derivatives. After washing the organic layer with buffer, the former was reduced in volume and injected onto a reversed phase column and the fluorescence detected at an excitation of 340 nm with an emission of 490 nm.

Yui et al (101) combined liquid chromatographic separation with a continuous flow system using the trihydroxyindole reaction to determine norepinephrine and epinephrine in human plasma. A simplified procedure for the quantitation of total metanephrines in urine was described by Christenson et al (102).

The amines are removed from urine using a weakly acidic ion-exchange column and eluted using dilute formic acid. The amines are then oxidized to the trihydroxyindole derivatives using iodine. Wu (103) extended the latter to include the injection of the oxidation products onto a reversed phase column.

An alkaline-ascorbate solution was mixed with the column eluate and the fluorescence monitored at an excitation of 400 nm with an emission of 520 nm.

The native fluorescence of the catecholamines, excitation 275 nm, emission 318 nm, can also be used after their separation on a reversed phase column.

### Homovanillic acid

Homovanillic acid (3-methoxy-4-hydroxyphenyl acetic acid) is the major urinary metabolite of dopamine. Elevated levels of the compound are found in patients with neuroblastomas. Rosano et al (104) determined the compound in urine following a liquid chromatographic separation on a reverse phase column. The homovanillic acid was detected by reacting the column effluent with potassium ferricyanide to produce a fluorophore with an excitation of 320 nm and an emission of 420 nm.

## Glucose and related metabolites

### Glucose

The first papers to appear on the fluorimetric determination of blood glucose were based on the 5-hydroxytetralone reaction to give a fluorescent product, naphthenone, which has an excitation of 470 nm and an emission of 550 nm.

Bourne (105) modified these earlier procedures so that 2 µL of whole blood could be used. Proteins are precipitated with TCA, and the reagent, dissolved in concentrated sulphuric acid, added to an aliquot of the filtrate. The mixture is heated on a boiling water bath for 30 minutes and, after cooling, the solution is diluted with water and measured.

Phillips and Elevitch (106) published a method based on the peroxidase glucose oxidase reaction as used in the colorimetric procedures. The hydrogen peroxide produced oxidizes homovanillic acid to a highly fluorescent product, excitation 319 nm, emission 445 nm, which is proportional to the glucose concentration. This procedure has been automated by Seiter et al (107) with 60 samples running an hour. Lloyd et al (76) estimated blood glucose using a continuous flow enzymatic method based on the following reaction:


Peroxidase galactose has been determined in blood and plasma using a continuous flow system and two coupled enzyme systems (108):


The p-hydroxyphenylacetic acid is used in preference to homovanillic acid for improved sensitivity.

Amino sugars have been determined by Chen (109) and saccharides by Mopper et al (110) by reacting the sugars with ethylenediamine. Mopper et al (111) used dansyl hydrazine as a pre-column fluorimetric reagent for reducing sugars. Detection limits were 2-5 pmol per injection.

### Lactic Acid

Lactic acid is determined by an enzymatic procedure using lactate dehydrogenase to convert lactic acid to pyruvic acid with the corresponding reduction of NAD to NADH (112). Semicarbazide is often added to fix the pyruvate. Using the direct assay, $0.2 \times 10^{-9}$ moles of lactic acid can be estimated, but with cycling, as little as $10^{-12}$ moles have been estimated.

### Oxalic Acid

Zarembski et al (113) described a fluorimetric method for determining oxalic acid in blood based on the following procedure. Whole blood is deprotinized with calcium formate and the acid extracted into tri-n-butylphosphate followed by co-precipitation with calcium sulphate. The acid is then reduced to glyoxylic acid with nascent hydrogen and coupled with resorcinol to form a fluorescent compound, excitation 490 nm, emission 530 nm. Good agreement was found with colorimetric methods. Devoto et al (114) published a procedure based on the quenching effect of oxalate on the fluorescence of the zirconium-flavanol chelate. Urinary oxalic acid has been determined by Meola et al (115) based on the reduction of cerium (IV) to the highly fluorescent cerium (III) by oxalic acid.

### Pyruvic Acid

Pyruvic acid is determined by using the reverse reaction to the assay of lactic acid, that is, measuring a loss in NADH.

## Lipids

### Bile Acids

Serum 3-hydroxy bile acids are determined by measuring the increase in fluorescence of NADH formed between the reaction of the bile acids and NAD in the presence of 3-α-hydroxysteroid dehydrogenase (116). The normal range found in serum varies from 0.3-8.3 µM/L. An Amberlite®, XAD-2 ion exchange column was used to extract the bile acids from serum and, after washing with water, the acids were eluted with methanol. The latter was evaporated to dryness and the residue dissolved in a glycine buffer for enzymic assay. The sensitivity of the assay was increased by linking it to a resazurin-diopharase system (117). Serum proteins interfere with the assay but can be overcome by adding bovine serum albumin to the reagent (118).

Kawasaki (119) reported a liquid chromatographic method. Immobilized 3-α-hydroxysteroid dehydrogenase was used to convert the bile acids to the oxo derivatives which were labeled with dansyl hydrazine. The derivatives were then injected on a reverse phase column.

### Cholesterol

The fluorimetric estimation of cholesterol is based on the well known Liebermann-Bouchard reaction in which cholesterol is reacted with chloroform and acetic anhydride in concentrated sulphuric acid. Albers and Lowery (120) found that the colored product was fluorescent. McDougall and Farmer (121) used this procedure to determine cholesterol in 20 µL plasma samples. The fluorescence excitation is at 546 nm with an emission at 570 nm. Using this procedure, other steroids contribute up to an equivalent of 3 mg% cholesterol.

Fruchant et al (122) describe a method based on the enzymatic conversion of cholesterol to 4-cholestane and hydrogen peroxide using cholesterol esterase. The hydrogen peroxide oxidizes methanol in the presence of catalase to give formaldehyde. The latter is reacted with ammonia and acetylacetone to give the highly fluorescent 3,5,diacetyl-l-4-dehydrolutidine, excitation 405 nm, emission 500 nm.

### Triglycerides

The estimation of triglycerides is generally based on the measurement of glycerol formed after saponificaton. Mendleshon and Antonis (123) heated glycerol with o-aminophenol in the presence of sulphuric acid to form the 8-hydroxyquinoline. The latter was then complexed with magnesium to give a fluorescent derivative.

Other methods are based on the oxidation of glycerol to formaldehyde. A Hantzsch condensation reaction between ammonia, acetylacetone and the formaldehyde gives a fluorescent lutidine product. Kessler and Lederer (124) developed an automated procedure based on this method which was improved upon by Noble et al (125). Isopropanol extracts of 500 µL serum samples are treated with a zeolite mixture to remove phospholipids, glucose and bilirubin. An automated flow system carries out the saponification of the triglycerides to glycerol, oxidation to formaldehyde and the formation of the fluorescent product. A comparison between this procedure and five enzymatic kits was made by Walker et al (126).

An enzymatic procedure described by Winartasaputra et al (127) involves a coupled reaction as follows:

*Triglycerides*


The formation of resorufin is measured by monitoring the fluorescence at an excitation of 548 nm and an emission of 580 nm.

### Phospholipids

The measurement of the lecithin-sphingomyelin ratio as an indicator of fetal lung maturity has already been mentioned. The phospholipids are probed using a polarization method with DPH. Egberts et al (128) proposed a method based on measuring the difference in fluorescence of DPH added to amniotic fluid before and after micropore filtration. The fluorescence intensity varies linearly with phospholipid concentration over the range 3-160 µmol/L with good agreement with the polarization method.

## Porphyrins and related structures

### Porphyrins

Porphyrins are among the most fluorescent compounds in nature, emitting in the red and infrared regions. They consist of a tetrameric structure and differ with respect to the substituent groups on the carbon atoms of the pyrrole rings. Of the porphyrins that are of interest to the clinical chemist, only coproporphyrin, uroporphyrin and porphobilinogen are routinely determined by fluorimetry and are of interest for monitoring porphyrins. Zn-protoporphyrin was also of interest in cases of blood lead poisoning.

Coproporphyrin is usually predominant in urine and the separation from the uroporphyrins is performed using the following procedure. Fresh urine is shaken with iodine to convert non-fluorescent coproporphyrins to fluorescent forms, after which they are extracted with ethylacetate. They are back extracted into dilute hydrochloric acid for measurement, excitation 405 nm, emission 595 nm.

Preformed uroporphyrins are determined in the aqueous phase by stirring with alumina, washing the particles with sodium acetate solution and eluting the uroporphyrins with dilute hydrochloric acid. Porphobilinogen is converted to uroporphyrin by acidifying urine with acetic acid and heating in a boiling water bath for 30 minutes.

Solvent extraction was replaced by Sobel et al (129) with an ion exchange column. The coproporphyrin was separated from uroporphyrin by means of eluants of differing pH and polarity.

The use of liquid chromatography has considerably simplified the analysis of both urinary and plasma porphyrins. The former are separated initially by ion exchange chromatography followed by injection on a reverse phase column using an acidic methanol-water eluant (130). As little as 1 ng of eluted porphyrins can be detected. Plasma porphyrins have been estimated by methylating prior to injection (131).

Studies in lead poisoning have shown that the predominant erythrocyte porphyrin is Zn-protoporphyrin which has an excitation at 424 nm and an emission at 594 nm. Since the fluorescence characteristics of this compound are different from other porphyrins, as shown in Figure 5, a simple assay can be constructed to differentiate lead intoxication from iron deficiency anemia (132).

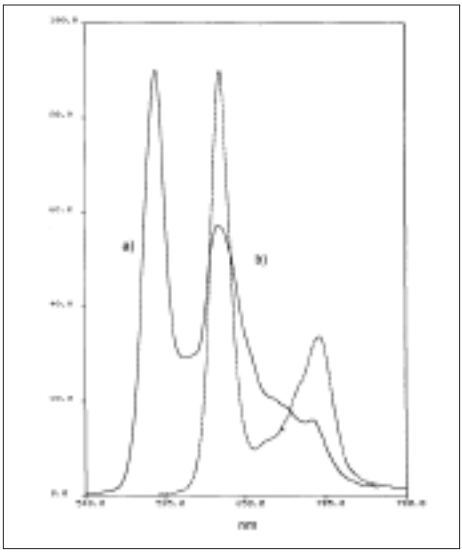



*Figure 5.* Fluorescence emission spectra of a) zinc protoporphyrin and b) protoporphyrin.

Blumberg et al (133) determined Zn-protoporphyrin using "front surface" fluorescence which allows the direct determination of the compound in blood. Schifman and Finley (134) compared the assay with an extraction procedure and found good correlation except for a constant error associated with the time of measurement. Suga and Koch (135) determined Zn-protoporphyrin against blood lead and found correlation down to 40 µg/dL. Reverse phase liquid chromatography has been used by Gotelli et al (136) to estimate Zn-protoporphyrin IX, protoporphyrin IX and coproporphyrin in whole blood.

## Bilirubin

Roth (137) developed a simple and sensitive assay for total bilirubin in serum. 50 µL of the latter are mixed with phosphoric acid and allowed to stand for one minute. Following dilution with water, the fluorescence is measured at an excitation of 435 nm with an emission of 500 nm. The presence of albumin is necessary for the intensification of the fluorescence when the bilirubin concentration exceeds 110 mg/L of serum.

Uete (138) modified Roth's procedure by carrying out the reaction at 0 ˚C. 10 µL of plasma are added to 40 µL of 4% bovine albumin and mixed with 600 µL of 85% phosphoric acid solution. After 3 minutes at 0 ˚C, 3 mL of water are added and the fluorescence measured.

## Urea

The fluorimetric determination of blood urea is derived from the colorimetric method using diacetylmonoxime. McClesky (139) found that the product had an emission at 415 nm when excited at 380 nm. Blood proteins are precipitated with trichloroacetic acid and the filtrate heated on a boiling water bath with diacetylmonoxime and concentrated sulphuric acid. After cooling, the fluorescence is measured directly. Rho (140) found that the fluorescence intensity was not proportional to the concentration of urea at the wavelengths used and suggested measuring the emission at 525 nm.

## Uric Acid

The determination of uric acid is similar to that of the glucose procedure in that homovanillic acid is oxidized in the presence of hydrogen peroxide to a fluorescent derivative (141). In borate buffer at pH 9.0, uric acid reacts with uricase to form hydrogen peroxide and other degradation products. After allowing sufficient time for the quantitative formation of hydrogen peroxide, homovanillic acid and peroxidase are added. The relative fluorescence after a fixed time interval is proportional to the uric acid concentration. When uricase and peroxidase are used in the same system at

the same time, there is competition between the uric acid and homovanillic acid towards the hydrogen peroxide, hence the need for the two separate stages of analysis.

## Steroids

### Cortisol

A great number of fluorimetric procedures have been developed for plasma cortisol, primarily based on the work of Sweat (142) and Mattingly (143).

The methods utilize the fact that cortisol is present in plasma in a much higher concentration than other 11-hydroxycorticosteroids and is extracted into an organic solvent such as dichloromethane. The extract is added to ethanolic sulphuric acid to develop the fluorescence with an excitation of 470 nm and an emission of 525 nm. The methods vary according to the type of extraction procedure and the timing of the fluorescence measurement. The original Mattingly procedure was improved upon by Meyer and Blanchard (144) by pre-alkalinization of the plasma sample. This eliminates non-cortisol fluorescence (due to phenolic steroids ) without detracting from the assay. The fluorescence must be measured within a fixed time since the signal deteriorates slowly. Free urinary cortisol has been measured by Ratliff and Hall (145) by removing the hydroxycorticosteroids with dichloromethane and then washing the organic layer with sodium hydroxide solution to remove the phenolic steroids. Burke (146) has reviewed the analysis of urinary cortisol giving 33 references.

The majority of cortisol assays are not specific in that corticosterone is also measured. Clark and Rubin (147) developed a method which differentiates between the two. Only the cortisol derivative of a metaperiodate oxidation of both compounds gives an alcoholicsulphuric acid induced fluorescence.

Liquid chromatography has been used by several workers to improve the specificity for corticosteroid analysis. Kawasaki and Maeda (148, 149) determined plasma and urinary cortisol and other 17-hydroxycorticosteroids using dansyl hydrazine derivatives.

Goto and Nambara (150) used anthroyl derivatives while Gotelli et al (151) utilized the acid induced fluorescence. Methylene chloride is used to extract the cortisol from serum and then ethanolic sulphuric acid is added to the organic extract. After shaking, the organic layer is discarded, the aqueous layer heated at 70 ˚C for two minutes, an internal standard added and an aliquot injected on a reverse phase column. Fluorescence detection is at 360 nm excitation with an emission of 488 nm.

### Estrogens

The three estrogens of clinical interest are estriol, estradiol and esterone, which are generally determined using the Kober-Ittrich procedure as described by Brown et al (152) and others (153-156). Kober observed the fluorescence and color changes which occur on heating estrogens in sulphuric acid solutions (157) and Ittrich discovered that these products could be extracted into tetrachloroethane containing p-nitrophenol, leaving the non-specific fluorophores in the acid phase (158).

The procedure of Brown can also be used to determine the total estrogen content of the urine of non-pregnant women. The estrogens are released from the conjugates by acid hydrolysis and then extracted with ether. The latter is washed with sodium carbonate solution to remove neutral and strongly acidic compounds and the estrogens reextracted with ether which is then evaporated to dryness. Kober reagent is added to the residue and after heating on a boiling water bath, the sample tubes are stored on ice. Ittrich reagent is added to a special fluorimeter cell and a diluted solution of the acid phase added to it. After vigorous shaking, the tubes are centrifuged and the fluorescence of the lower layer measured immediately at two pairs of excitation and emission wavelengths, 490 nm and 520 nm; 536 nm and 565 nm. The first set of wavelengths is used to correct for any non-specific fluorescence due to impurities. The procedure has been adapted for automated flow systems to determine estrogens throughout pregnancy (159, 160).

Various methods have been employed to improve the selectivity of the assay. These include automatic solvent extraction in tubes with cyclohexane-ethyl acetate after enzymatic hydrolysis, followed by anion exchange chromatography (161). The use of liquid chromatography has speeded up the analysis and has enabled the use of relatively small sample volumes. Pre-column derivatization with DNS-Cl has been described by Schmidt et al (162) while Taylor et al (163) measured the native fluorescence of estriol excited at 220 nm. The urine is generally hydrolyzed with β-glucuronidase prior to extraction of the free estriol. Bowers and Johnson (164) used an on-line hydrolysis with immobilized enzymes.

### Anabolic Agents

Despite the fact that it is a known carcinogen, diethylstilbesterol, DES, is used for both human and animal applications because of its estrogenic activity. Although it has been banned from use in animal feeds in many countries, its efficiency in promoting weight gain ensures its illegal use. Residues of DES in animal tissues can be as low as the ppb range. A combination of thin-layer chromatography and sulphuric acid-induced fluorescence (165) has been used for determining nanogram levels of DNS.

In a novel approach, Rhys Williams et al (166) utilized the photoconversion of DES to a highly fluorescent phenanthrene derivative as a means of detecting DES in urine. After a simple extraction procedure, the DES is separated from interfering substances by liquid chromatography and the derivative detected at an excitation of 280 nm and an emission of 390 nm.

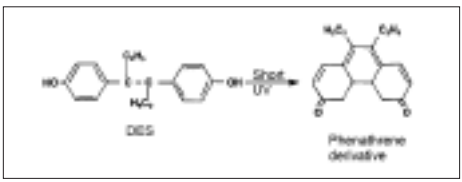



*Figure 6.* Photoconversion of DES to phenarthrene derivative.

Anabolic agents of the resorcyclic acid lactones and stilbene groups can be determined in animal tissues and fluids by pre-column derivatization with DNS-Cl (167). The reaction and chromatography time is less than 1 hour.

## Vitamins

Vitamins are basic to human health and as such their estimation is of interest to those involved in biochemistry, food and pharmaceutical sciences. Many of the vitamins exhibit a native fluorescence or can be easily modified to produce a fluorescent signal (168). Liquid chromatography offers a considerable improvement in analysis compared to the classical chemical procedures and provides the capability for the simultaneous determination of both water- and fat-soluble vitamins (169).

### Vitamin A

Vitamin A occurs in nature in three or more forms, either as free alcohols or as esters. They are soluble in fats, insoluble in water and are easily oxidized. Several methods are available for their determination, with the analysis of serum vitamin A first published by Kahan (170) in 1960.

Following a cyclohexane-ethanol extraction procedure, the fluorescence of the organic layer was measured at an excitation of 345 nm and an emission of 490 nm. By also measuring the organic layer at an excitation of 295 nm and an emission of 340 nm, Hansen and Warwick (171) were able to determine vitamins A and E simultaneously.

Papers from Thompson et al (172) and Kahan (173) indicated that these simple procedures gave erroneous values for vitamin A because of the interference from phytofluene. The latter has an emission spectrum which closely overlaps that of retinol. However, the excitation spectrum differs substantially and so it is possible to calculate the proportion of the two compounds in a mixture by measuring the fluorescence at two different excitation wavelengths. Analysis of blood using the correction procedure gave values which correlated well with the antimony trichloride method.

## Vitamin B1

Vitamin B1 or thiamine occurs in plants in the free form and in animals as the pyrophosphate. The vitamin is soluble in water and is easily decomposed by oxidizing agents and by UV light. The most commonly used fluorimetric method is based on the thiochrome procedure which involves oxidation of the thiamine with ferricyanide to form the fluorescent thiochrome. Other oxidizing reagents, such as cyanogen bromide and mercuric oxide, have been used.

The method of analysis given by AOAC can be used for almost any type of sample and basically is as follows:

1. Extraction with hydrochloric acid
2. Enzyme hydrolysis if pyrophosphate is to be measured
3. Purification with an ion exchange column if appreciable quantities of basic substances are present
4. Oxidation with alkaline potassium ferricyanide
5. Extraction with isobutanol
6. Measure against a standard treated in a similar manner

Blanks are prepared by substituting the oxidation solution with sodium hydroxide solution.

Blood analysis involves a hydrolysis procedure with either dilute acid or with diastase (174). No purification by an ion exchange column is necessary.

The fluorescence is measured with an excitation of 366 nm and an emission of 430 nm. Thiamine is also determined in urine using the method of Leveille (175) or automated using the method of Pelletier and Madere (176) and Muiruri et al (177).

Kimura et al (178) used liquid chromatography to determine total blood thiamine. Thiamine esters were converted to free thiamine using takadiastase, while the column eluent was mixed with alkaline potassium ferricyanide.

## Vitamin B2

Vitamin B2 or riboflavin occurs in nature either free or as a component of coenzymes. It is soluble in water, stable in acid solutions, but is light sensitive. Vitamin B2 has a native fluorescence with an excitation of 440 nm and an emission of 513 nm.

Reduction with dithionite or sodium hydrosulphite converts riboflavin to the leuco form which is non-fluorescent, thus enabling the determination of blank solutions. When riboflavin in an alkaline solution is irradiated with UV light, the more highly fluorescent lumiflavin is produced.

Liquid chromatography has been applied to the determination of the vitamin by many workers. Rhys Williams and Slavin (179) reported riboflavin in urine and grape juice, while Gatautis and Naito (180) described the determination of riboflavin in urine.

## Vitamin B6

Vitamin B6 occurs in nature in a variety of forms, although in animals it is chiefly found as pyridoxal, PL, and pyridoxamine. The active form of the vitamin is pyridoxal phosphate, PLP, and the predominant metabolite in urine is 4-pyridoxic acid. Several methods are available for the determination of the vitamins, including those which are based on the formation of a fluorescent lactone (181).


Reddy et al (182) improved the method of Fuijita for determining 4-pyridoxic acid in urine by using ion exchange chromatography on Dowex 1 and 50 to remove interfering compounds.

Schrijver et al (183) and Gregory (184) have shown that a stable semicarbazide can be formed between PL and PLP. Ubbink (185) used the reaction together with the addition of an internal standard of 6-methyl-2-pyridine

carboxaldehyde for measuring PL and PLP in plasma samples. The semicarbazide was added in a post-column reaction and the fluorescence measured at an excitation of 367 nm and an emission of 478 nm.

The native fluorescence of the vitamin B6 vitamers has been used by Durko (186) in the LC determination of blood B6.

## Vitamin C

Vitamin C occurs in nature in a reduced form – ascorbic acid. The most widely used fluorimetric method is based on the condensation of o-phenylenediamine with the dehydro form to give the highly fluorescent quinoxaline (187). The excitation is at 350 nm and the emission at 430 nm. Ascorbic acid is oxidized to the dehydro form with activated carbon and the total anti-scorbutic activity measured. Blanks are prepared by forming a boric acid-dehydro complex before the addition of o-phenylenediamine. The method shows a high degree of specificity and can be used in the presence of a wide range of reducing substances.

The quinoxaline derivative has been formed by pre-column derivatization and used in liquid chromatographic analysis of the vitamin (188).

## Vitamin E

Vitamin E consists of a group of compounds known as the tocopherols. They are soluble in organic solvents and, in general, the esters are more stable than the free form. Their determination can be made either by measuring the native fluorescence at an excitation of 295 nm and an emission of 330 nm, or by measuring the fluorescence of the phenazine derivative at an excitation of 370 nm, emission 450 nm.

As with many of the traditional methods of analysis, liquid chromatography has considerably improved the determination of the tocopherols. The sensitivity is sufficient to permit their detection in platelets (189) and in erythrocytes (190). The simultaneous determination of serum vitamins A and E by liquid chromatography has been described using UV absorption detection with the wavelength set at 292 nm or 250 nm. As the excitation and fluorescence wavelengths differ considerably, as shown in Figure 7, the fluorescence detector must be set at a compromise pair of wavelengths. By using a detector which can automatically change wavelengths with respect to retention times, the analysis is optimized for specificity and sensitivity. Normal phase chromatography provides compatibility with the extraction procedure and improves the sensitivity compared with reverse phase high polarity solvents.

The effect of changing wavelengths through the chromatogram is shown in shown in Figure 8. The first two chromatograms are

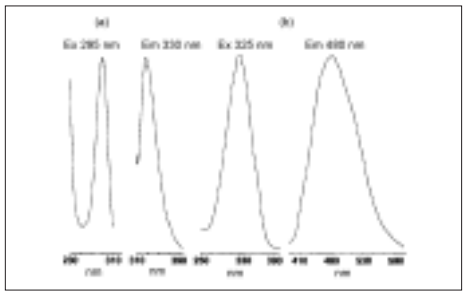


*Figure 7.* Excitation and emission spectra of a) vitamin E and b) vitamin A.

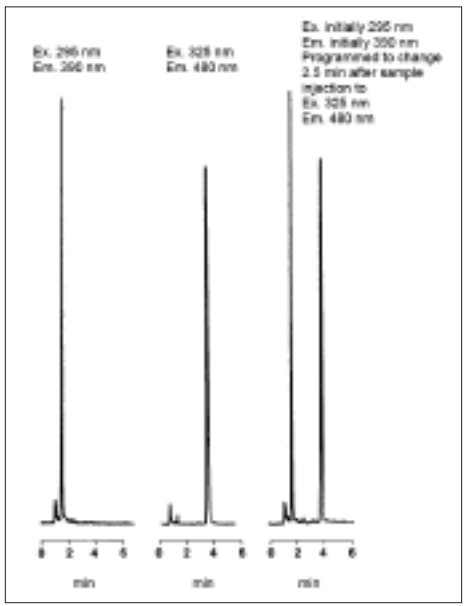



*Figure 8.* Chromatograms of serum extracts run on an HS-3 silica column using the Model LS-55.

of a serum monitored with the appropriate wavelengths of each vitamin. The third chromatogram is of the same sample but with the detection initially set at an excitation of 295 nm, emission 340 nm and after 2.5 minutes changed to 325 nm, 480 nm respectively.

## Drugs and pharmaceutical products

In addition to the drugs of abuse, there have been included in this section a number of pharmaceutical products for which fluorimetric methods exist. Drug detection and analysis usually involve an extraction procedure as a wide range of sample matrices are found. For example, LSD is sometimes taken on sugar cubes and cannabis resin found on vegetable matter. Liquid samples are predominantly body fluids such as blood or urine.

Many of the assays first published in the sixties and seventies (192-196) have been modified to include liquid chromatography and the use of derivatizing reagents has contributed significantly to the number of compounds detected by fluorescence as shown in Table 3. In addition, photochemical derivatization has provided the analyst with a highly sensitive and specific means of drug identification, for example, cannabinol (197), chlordiazepoxide (198) and clomiphene (199).

The use of fluorescence immunoassay, FIA, for drug analysis has been reviewed by Jolley (200) and by Quattrone and Putnam (201). In particular, therapeutic drug monitoring by FIA is a fast growing technique and is now routine in many clinical laboratories.

*Table 3.* Analysis of pharmaceutical compounds by fluorescence spectroscopy.

| Compound | Label | Ex. nm | Em. nm | Sensitivity | Ref. |
|---|---|---|---|---|---|
| E-Aminocaproic acid | Fluoram | 390 | 490 | 10 pg | 248 |
| Amikicin | OPA | 340 | 450 | 2 mg/L | 249 |
| Carazol | Native | 285 | 344 | $10^{-8}$ M | 250 |
| Carminomycin | Native | 470 | 550 | 2 nm/mL | 251 |
| Chlordiazepoxide | Fluoram | 390 | 486 | .25 µg/mL | 252 |
| Clomiphene | Photo | 257 | 367 | 1 ng/mL | 199 |
| Digoxin | Acetic acid | 345 | 435 | .5 µLg/mL | 253 |
| Diphenylhydantoin | Hydrolysis | 360 | 490 | 50 µg/mL | 254 |
| Fluorocytosine | Native | 300 | 370 | .5 µg/mL | 255 |
| Fluorosemide | Native | 345 | 417 | .09 µg/mL | 256 |
| Griseofulvin | Native | 310 | 430 | .05 µg/mL | 257 |
| Isoniazid | Oxidation | 325 | 400 | .5 µg/mL | 258 |
| Labalol | Native | 340 | 415 | 1 ng/mL | 259 |
| Nadolol | Native | 265 | 305 | 10 µg/mL | 260 |
| Nitrazepam | OPA | 348 | 480 | .01 µg/mL | 261 |
| Phenothiazines | Native | 320 | 400 | .5 µg/mL | 262 |
| Phenylethylamine | Ninhydrin | 390 | 495 | 16 µg/day | 263 |
| Propranolol | Native | 205 | > 340 | 1 ng/mL | 264 |
| Propoxyphene | NBD-Cl | 468 | 517 | .02 µg/mL | 265 |
| Tamoxifen | Photo | 220 | 360 | 2 ng/mL | 266 |
| Tobramicin | OPA | 340 | 450 | 1 mg/L | 267 |
| Trimetazidine | DNS-Cl | 252 | > 370 | 1 ng/mL | 268 |
| Verapamil | Native | 275 | 310 | .1 µg/mL | 269 |

Reviews on the use of fluorescence in forensic analysis have been written by Gibson (202), Jones (203) and by King (204).

## Amphetamines

The continuing use of amphetamine and its derivatives has stimulated many papers on their identification. Mehta et al (205) reviewed five methods for determining amphetamines in urine. Wacksmuth et al (206) reacted amphetamine with acetylacetone and formaldehyde to form the 1,4-dihydrolutidine, with an excitation of 395 nm and an emission of 475 nm. Fluorescamine (207) reacts with amphetamine giving a detection limit of 5 x $10^{-11}$ g. Van Hoof and Heyndrickx (208) used NDB-Cl for the TLC-spectrofluorimetric analysis.

## Barbiturates

The UV absorption methods published for barbiturates do not have the required sensitivity for the therapeutic levels of the drugs. Udenfriend (209) suggested a simple extraction procedure be used to improve the sensitivity and many papers have been published using this technique.

King (210) discussed the effect of chemical structure on the luminescence characteristics of thiobarbiturates. The latter may be distinguished from their oxygen analogs by their spectra. The thio forms have an excitation of 315 nm and an emission of 530 nm, while the oxy forms have an excitation of 265 nm and an emission of 440 nm. Hsiung (211) used rapid fiberglass TLC to separate the barbiturates. The spots were scraped off and dissolved in dilute sodium hydroxide solution for measurement. Dunges (212) used liquid chromatography for the separation of the DNS-Cl derivatives.

## LSD

Lysergic acid diethylamide has an intense native fluorescence in dilute acid with an excitation of 315 nm and an emission of 445 nm. After extraction from an alkaline-sodium chloride saturated plasma or urine sample with hexane, the LSD is back extracted into the dilute acid for measurement (213, 214).

## Morphine

Several procedures exist for estimating morphine in urine. One of them involves the formation of a fluorophore with an excitation of 395 nm and an emission of 424 nm after treating the sample with concentrated sulphuric acid. Potassium ferricyanide is used in another procedure to form the fluorescent pseudomorphine, which, when used in an automated assay, can give a detection limit of 6 µg/mL in urine (215). Two procedures have been released using liquid chromatography to separate morphine and other opiates. Jane (216) and Glassel (217) used the native fluorescence while Nelson (218) used post-column derivatization. In a method to determine codeine in plasma (219), hexane and dichloromethane are used to extract the drug from an alkaline sample. After washing with dilute alkali, the organic layer is evaporated to dryness, reconstituted with water-methanol and injected on a reverse phase column. The native fluorescence of the codeine is measured at an excitation of 213 nm with an emission of 350 nm.

## Quinine and Quinidine

Quinine and its stereoisomer, quinidine, have been determined by fluorescence for many years. Quinine is frequently used as an adulterant of morphine and heroin and therefore its detection could be used as a screening procedure for these drugs (220). Broussard (221) determined quinidine in serum by extracting an alkaline sample with benzene followed by back extraction of the organic layer into dilute sulphuric acid. The fluorescence excitation is at 360 nm with an emission of 450 nm. Because of the objections raised against benzene, a possible carcinogen, Horvitz (222) suggested the use of toluene as a substitute.

A liquid chromatography assay for quinine and its metabolites in biological fluids has been described by Rakhit et al (223). Proteins are precipitated by acetonitrile, the supernatant evaporated to dryness and the reconstituted residue separated on a reverse phase column. As little as 1 µg/L can be quantitated.

### Salicylate

Salicylic acid and its conjugate are intensely fluorescent in alkaline solutions with an excitation of 319 nm and an emission of 408 nm. This property has been used to determine salicylates directly in tungstic acid filtrates of serum or plasma (224).

## Cations

### Calcium

Calcium is determined fluorimetrically in one of two ways: either by titration with EGTA using calcein as indicator (225, 226), or by direct estimation using Calcein as the reagent. The latter technique is more common and was introduced by Wallack et al (227, 228) and Keeper and Hercules (229).

In alkaline conditions, calcein complexes with calcium to form fluorophore with an excitation of 450 nm and emission of 520 nm.

In a typical procedure, 50 µL samples are diluted with 4 mL of water mixed with 1 mL of reagent and incubated at room temperature for ten minutes. The calibration curve is S-shaped with a central linear portion. Other cations are reported not to interfere, but bilirubin levels above 2 µg/mL do. An automated procedure was introduced by Toffalleti et (230, 231) for measuring dialysable calcium. Day to day precision was 2-3% with a sample volume of 250 µL.

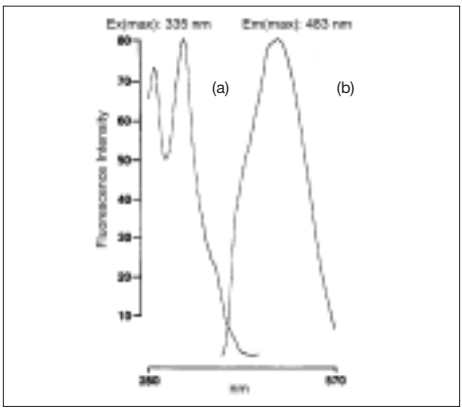



*Figure 9.* Fluorescence excitation a) and emission b) spectra of a quin-2/calcium complex.

The measurement of intracellular ion concentrations has been reviewed by Tsien (232). Intracellular free calcium has been monitored using a new fluorescent indicator called quin-2 (233, 234).

Quin-2 has a high affinity for calcium with very low affinity for magnesium. A large change in the absorbance and fluorescence intensity occurs on binding. The non-polar acetoxymethyl ester, quin 2/AM, is used as a means of crossing the plasma membrane of cells. The ester is hydrolyzed intracellularly back to the polycarboxylic anion which then binds with the cytoplasmic free calcium. Excitation of the complex is at 339 nm with an emission of 492 nm. The calibration curve is linear from 10 nM to 100 µM calcium.

A new family of highly fluorescent indicators have been introduced based on combining the 8-coordinate tetracarboxylate chelating site with a stilbene chromophore. These indicators called fura-2 and indo-1 reportedly give up to 30-fold increase in fluorescence over quin-2 on binding to $Ca^{2+}$ and the complexes have better selectivity against $Mg^{2+}$ and heavy metals.

A similar fluorescence indicator, quene-1, which is related to quin-2, has been introduced and is used to monitor intracellular pH. The dye shows a 30-fold increase in fluorescence between pH 5 and 9 with a pKa of 7.3.

### Magnesium

Procedures for determining magnesium in blood and urine are based on the work of Schachter (235) using 8-hydroxyquinoline-5-sulphonate as the complexing reagent. The fluorescence of the complex is minimal below pH 5 and high at pH 6.5. The excitation is at 374 nm with an emission at 505 nm. Other cations such as calcium and zinc also form fluorescent complexes which are not pH dependent. Thus, the procedure is based on measuring the difference in fluorescence between two solutions, one at pH 3.5 and the other at pH 6.5. Pesce and Bodourian (236) have automated this method using a centrifugal analyzer in which the detection limit quoted is 1 mg/L.

### Selenium

Lalonde et al (237) estimated selenium in urine following wet digestion with a mixture of nitric and perchloric acids. The selenate formed in this reaction is converted to the selenite with dilute hydrochloric acid. Diaminonaphthalene, DAN, is added and the selenite-DAN complex extracted with cyclohexane prior to measurement at an excitation of 360 nm, emission 520 nm. A single test-tube procedure has been proposed by Alfthan (238) which simplifies sample digestion and complexation of the selenium with DAN. The whole of the acid digestion and complexation is carried out in the same test-tube and the cyclohexane extract transferred to a cuvette for measurement. The sensitivity of the method is sufficient to enable the determination of selenium with very small samples, ≈10 mg, with good precision and accuracy. A critical re-appraisal of the fluorimetric methods for determining selenium in biological materials has been made by Tee-Siaw (239).

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