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The use of fluorescent probes in pharmaceutical analysis*

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Abstract: Even though many pharmaceuticals show native fluorescence, there is also an important group of compounds which is not fluorescent. A main object of studies is to make them fluorescent, principally by using fluorescent probes through derivatisation reactions. An account of the fluorescent probes more widely used for the determination of drugs and related compounds is presented in this review paper. A wide variety of fluorescent probes is described on the basis of their ability to react specifically with various functional groups. Attention is focused on derivatisation reactions used in spectrofluorimetry and chromatographic techniques (HPLC, TLC) with fluorimetric detection. The review covers only those fluorescent probes whose use involves a chemical reaction with the analyte, and not those methods that involve physico-chemical interactions such as sensitised or charge transfer processes.

Many of these derivatisation reactions have been widely used in the detection of primary and secondary amines. Reagents such as dansyl chloride, fluorescamine, *o*-phtalaldehyde are very well known. Other reagents have also been developed for other functional groups, for example dansyl hydrazine for compounds with a carbonyl function or 4-bromomethyl-7-methoxy-coumarin for acidic compounds. Acid chlorides such as dansyl chloride may also react with different functional groups carrying active hydrogens as do phenols.

The use and development of new fluorescent probes in pharmaceutical analysis is a subject for further studies.

Keywords: *Luminescence spectrometry; fluorescent probes; pharmaceuticals; derivatisation reactions.*

Introduction

Since some pharmacologically active compounds are administered in very small doses, suitable analytical methods are required for pharmacokinetic studies. One of these methods is spectrofluorimetry. It plays an important role in the field of biomedical analysis. Furthermore, the association of luminescent detection in tandem processes with, for example, flow injection analysis or liquid chromatographic techniques, has yet other analytical benefits. Besides, the use of fluorescent labels in fluorescence

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polarisation immunoassay and in general, fluoroimmunoassay presents several advantages in pharmaceutical analysis over the use of radioactive labels in radioimmunoassay.

Many compounds with pharmacological activity may present native fluorescent emission, but many others are not fluorescent or may even quench the fluorescence of the fluorescent ones. For this reason, the preparation of new compounds which are able to develop fluorescence from non-fluorescent ones is an important subject of studies. Hence, the use of compounds which enhance the quantum yield of weakly fluorescent molecules should present obvious analytical advantages.

Fluorescence can be induced in non-fluorescent compounds by different physical and chemical treatments. Among them, derivatisation reactions, i.e. formation of fluorescent derivatives by the use of fluorescent probes, will be considered.

Derivatisation

Advantages and limitations

The use of fluorescent probes in derivatisation reactions has several advantages. First of all, detectability may be improved by increasing sensitivity, transforming the compound object of analysis into another compound which is able to produce a greater fluorescent response. Also selectivity of the analysis can be enhanced by altering some of the physical or chemical characteristics of the basic compound. Another advantage of derivatisation is its role in stabilising the analytes, especially those which may be volatile or reactive. Finally, it is also useful for confirmatory purposes.

However, there are also certain limitations which could be more or less important depending on the application. First of all, derivatisation involves an additional step in the analytical process. In addition, due to the differences of the sample matrix, reaction yields may vary and thus lead to error.

Main Characteristics of Fluorescent Probes

The selection of the fluorescent probe is basic to optimisation of the assay. The ideal fluorogenic probe should not show native fluorescence; the emission should be exhibited only after reaction between the probe and the analyte. However, many of the commercially available fluorescent probes display emission even before reacting. In any case, the fluorogenic probe must be able to show fluorescent emission once bound to the unknown non-fluorescent compound. The following characteristics are necessary: a different emission from the reaction medium as well as a high fluorescence quantum yield. The reaction must be versatile, rapid and preferably should not require forcing conditions; the reaction products must be stable in the medium.

Derivatisation reactions are often used to analyse a great variety of pharmacologically interesting compounds. The probe should be selected according to a suitable functional group of the assayed drug.

Some methods are based on the increase of the molecular rigidity and thus an increase of the emission intensity.

Analysis of Fluorescent Probes for Different Functional Groups

Amines

Amines are perhaps the most abundant functional group of all drugs and related compounds. There are numerous specific methods for their fluorimetric assay.

Some of them, described in the literature, include the following. For instance, to determine primary aliphatic amines, such as amphetamines, aromatic aldehydes are used as fluorescent labels [1], leading to the formation of Schiff bases between carbonylic compounds and amines. Examples are fluorene-2-carboxaldehyde and pyrene-1-carboxaldehyde. The products of these reactions are thus highly fluorescent owing to the great aromaticity of these probes.

By means of a similar reaction, ninhydrin [2] has been widely used to determine amines, amino acids, amino sugars and peptides at a sensibility of nm levels (λ_{ex} = 385 nm, λ_{em} = 450–495 nm).

Fluorescamine is another fluorescent probe (Fig. 1) that also reacts almost instantaneously with primary amines to form fluorescent pyrrolinones. With secondary amines, it may also form non-fluorescent aminoenones, which can adequately be transformed into a fluorescent pyrrolinone by reaction with primary amines [3]. Fluorescamine is therefore a highly sensitive fluorescent probe which may directly react with amines, whether aliphatic or aromatic [4, 5] to form fluorophores of high intrinsic fluorescence. It is greatly used in a wide variety of pharmaceutical analyses of compounds containing amino groups, through reaction with fluorescamine in solution. The analysis of the aminoglycosidic antibiotic actinomycin by HPLC with fluorimetric detection uses fluorescamine as derivatising agent [6]. Klein [7] also described a method for the determination of amphetamines in either pharmaceutical products or biological fluids by fluorimetric labeling.

For the determination of procainamide hydrochloride in pharmaceutical products Tan and Beiser [8] developed a fluorimetric method quicker, easier and more sensitive than the official methods. The procedure is based on the reaction with fluorescamine in aqueous solution at pH 7.5, to form a fluorescent compound (λ_{ex} = 400 nm, λ_{cm} = 485 nm). Linearity has also been observed at ng levels, the product being stable for at least 2 h. The method is accurate and reproducible and has therefore been applied with success in pharmaceutical dosage forms.

Fabre [9] has also used fluorescamine to determine cephradine and other cephalosporins in different commercial formulations.

To evaluate *n*-alkylamines and di-*n*-alkylamines at nmol levels an analogue of fluorescamine, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) [10] has been used as a

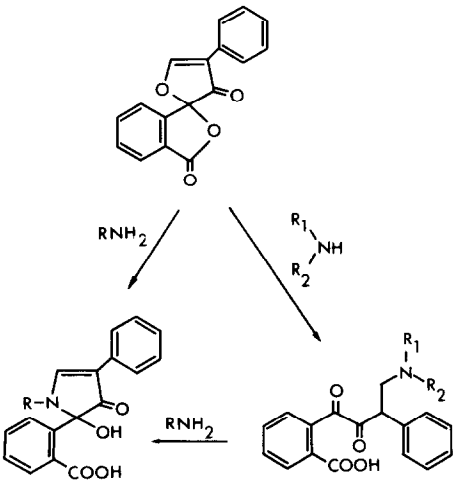

Figure 1
Fluorescamine reaction with primary amines to give pyrrolinones (fluorescent) and with secondary amines to give aminoenones (non-fluorescent).

fluorescent probe. This method is basically useful to distinguish primary and secondary amines in unknown samples.

Another fluorescent probe is OPA (*o*-phthalaldehyde). It was the first used to analyse amino acids quantitatively [11] as a more sensitive test than ninhydrin reaction was needed in pharmaceutical analysis for amine groups. OPA reacts with primary amines (Fig. 2) in alkaline medium in the presence of strong reducing agents such as mercaptoethanol, to form highly fluorescent compounds. The optimal wavelengths are λ_{ex} = 340 nm and λ_{em} = 455 nm. The reaction is very sensitive as nmol levels can be determined and the fluorescence can easily be measured 2 min after mixing the reagents, without requiring previous heating.

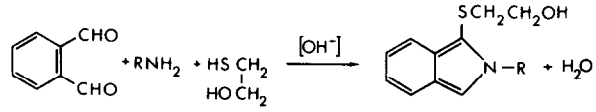

Figure 2
OPA reaction with amines in the presence of stabilising agents (mercaptoethanol).

OPA can also be used to analyse secondary amines after conversion to primary amines with oxidising agents such as sodium hypochlorite [12, 13] and chloramine T [14].

There are many different procedures described using OPA for drug analysis, for example, dihydroergot alkaloids [15] in plasma. In 1982 D'Souza and Ogilvie [16] described the use of OPA to evaluate gentamycin in plasma and urine. Quantitative analysis of other antibiotics in plasma such as kanamycin [17] have also been developed. However, for cephalosporins, detection with fluorescamine is between 10 and 20 times more sensitive than with OPA [9].

Recently OPA has also been used to determine 5-aminolevulinic acid (ALA) [18] a very important metabolite of tetrapyrrole biosynthesis found in body fluids. Also this reagent has been employed for the analysis of phytohormones, such as indol-3-yl acetic acid (IAA) [19].

Another fluorescent probe is dansyl chloride (5-dimethylamino naphthalene-1-sulphonyl chloride or DNS-Cl). Its reaction with primary and secondary amines gives rise to highly fluorescent derivatives. However, this reaction is relatively slow at room temperature (30–40 min), so heating is sometimes required. The excitation wavelength maximum is around 350 nm, with a fluorescent emission between 450 and 480 nm depending on the type of the solvent used. Some alkaloids [20, 21], such as morphine, codeine, emetine and cephaeline can be determined at the range of 1–10 ng in complex pharmaceutical dosage forms. Meanwhile other drugs, for instance tocainide [22], cannabinoids [23] and barbiturates [24] have also been assayed without difficulty using dansyl chloride. For example, barbiturate concentration levels of 1 ppm can be measured in 20 μl blood samples.

There is also another fluorescent probe analogue of DNS-Cl but with a butyl group in its structure instead of methyl, 5-di-*N*-butylaminonaphthalene-1-sulphonyl chloride, (Bansyl chloride, BNS-Cl) [25], with a reaction mechanism similar to DNS-Cl.

Furthermore, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [26, 27], is also a very useful fluorescent probe for both primary and secondary aliphatic amine group, λ_{ex} = 480 nm, λ_{em} = 530 nm. For amphetamine and its analogues, spectrofluorimetric analysis has been described [28] after reaction with NBD-Cl, thus determining amphetamine in urine.

Concerning NBD-F, Imai [29], in 1981, stated that it seemed to be more reactive than other halogeno-NBDs (Cl or Br). So with NBD-F, higher fluorescent intensities can be obtained and the sensitivity of the analytical method can be relatively increased.

Steward and Lotti [30, 31] developed a spectrofluorimetric method for amphetamine analysis based on the interaction between aliphatic amines and 3-carboxy-7-hydroxy-coumarin. The coumarin-amine salts produced are highly fluorescent. This is a very interesting method that allows the determination of a series of amphetamines such as amphetamine sulphate, methamphetamine hydrochloride, benzophetamine hydrochloride, chlorphentermine hydrochloride, methylphenidate hydrochloride and phendimetrazine tartrate in the presence of other drugs also present in pharmacological dosage forms.

The antidepressant amines amitriptyline and protriptyline may also react with 9,10-dimethoxyanthracene-2-sodium sulphonate to form fluorescent derivatives [32].

Recently, Sinsheimer [33] has proposed a very specific and sensitive method (ng levels being determined), to analyse penicillins. A non-acidic fluorescent label, 9-isothiocyanatoacridine reacts with the secondary amine functional group of the penicilloic acid produced by hydrolysis of penicillin with a base or β-lactamase.

There are also many other fluorogenic reagents used to label biogenic amines such as 2,2-naphthalene-dialdehyde [34], sulphoindonyl chloride [35], 7,7,8,8-tetracyanoquinodimethane [36], naphthylisocyanate [37] and formaldehyde [38].

Quinolizinium salts (Fig. 3) have been proposed [39] for the determination of catecholamines, ephedrine and bezocaine and other amines.

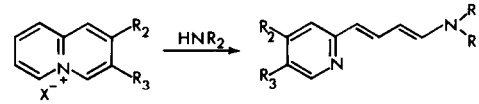

Figure 3
Reaction between quinolizinium salts and nucleophiles (amines).

Carbonylic compounds

For organic compounds with a carbonyl group, there are several fluorescent probes which may give rise to fluorescent products such as salicyloylhydrazide [40], 2-diphenylacetyl-1,3-indandione-1-hydrazone [41] and dansyl hydrazine [42] among others.

To determine α-oxoacids such as pyruvic acid in blood, a specific reagent, 4′-hydrazino-2-stilbazole [43], has proved to be highly selective. It usually forms hydrazones with strong fluorescent properties.

For aromatic aldehydes 2,2′-dithiobis(1-amino-naphthalene) (DTAN), a very specific reagent especially for *p*-hydroxybenzaldehyde has been selected [44]. DTAN has thus been satisfactorily employed to verify fluorimetric assays of cinnamic aldehyde, and for similar purposes 1,2-diaminonaphthalene [45] has been used.

However, as some of the above mentioned reagents could present native fluorescence themselves, a non-fluorescent reagent, *N*-methylnicotinamide chloride, has been recently proposed [46] for the determination of carbonylic compounds. A first step of this reaction is the reaction of the *N*-alkylpyridinium compounds with ketones in an alkaline medium followed by heating in an excess of acid to form a fluorophore with an intense fluorescence.

This fluorogenic reaction is also used for the determination of testosterone, progesterone and androsterone at nmol levels.

For Δ^4-3-ketosteroids, isonicotinyl hydrazine is also used [47] and for strogens in urine the Kober reaction, (i.e. hydroquinone in concentrated sulphuric acid).

Finally for α-dicarbonylic compounds, such as glyoxal and biacetyl, the fluorescent probe 3,4-diaminoanisole [48] is used forming the strongly fluorescent product 2,3-dimethyl-6-methoxyquinoxaline.

Carboxylic acids

Next to be considered are monocarboxylic acids. 4-Bromomethyl-7-methoxycoumarin (Br-Mmc) (Fig. 4) is used in this case, but this reaction presents several disadvantages [49]. It requires long reaction times, reflux temperatures and presence of an excess of the reagent besides working in total darkness. Nevertheless, this reaction may be advantageously modified by adding catalysts such as dibenzo-18-crown-6. This reaction is also used for fatty acids, gibberellins, valproic acid and acidic drugs which give rise to fluorescent esters with Br-Mmc.

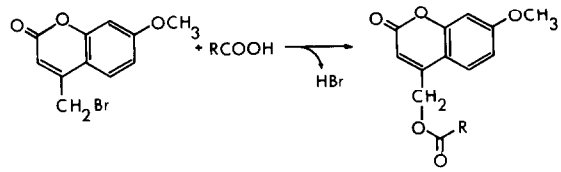

Figure 4
4-Bromomethyl-7-methoxycoumarin as fluorescent probe for monocarboxylic acids.

Hydroxy compounds

There are a great number of fluorescent reagents available for acids, amines and even carbonylic compounds. However, there are fewer reagents for hydroxy compounds. One outstanding exception is 1-ethoxy-4-(dichloro-s-triazinyl)naphthlene [50].

Recently, a new fluorescent probe for hydroxy compounds has been assayed to evaluate steroid metabolites and prostaglandins in human blood or urine. It is the 7-[(chlorocarbonyl) methoxy]-4-methylcoumarin [51].

For 1,2, 1,3 or 1,4 diols, the derivatising agent phenanthrene boronic acid is usually employed [52].

Primary or secondary, phenolic groups can be derivatised as readily as amines with dansyl-Cl the same as with other acid halides such as NBD-F, which can react with a series of functional groups carrying active hydrogen [53].

Thiols

To determine thiol groups other reagents such as dansylaziridines [54], bimanes [55] and maleimides [56, 57], (Fig. 5) have been used. However, dansylaziridine shows native fluorescence itself with a large interfering peak. With respect to the various *N*-substituted maleimides, they usually produce multiple fluorescent products, due to the hydrolysis of the initial fluorophores. But in the case of bimanes, there seem to be non-fluorescent compounds that may react slowly and non-enzymically with thiol groups, which is the case for monobromobimane (mBrB) amongst others. It has thus proved to be useful for evaluation of glutathione (GSH) [58], with which a fluorescent conjugate is formed in the presence of an excess of unreacted reagent.

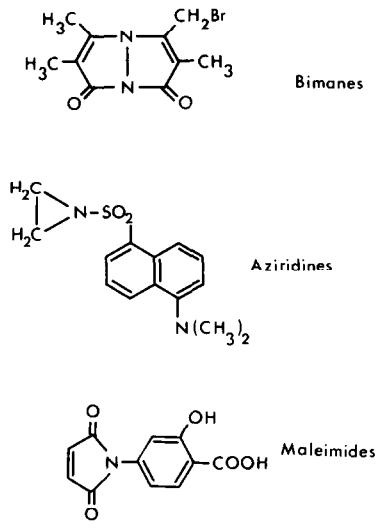

Figure 5
Chemical structures of some thiol fluorescent probes.

However, Imai is of the opinion that bimanes are not a very selective type of fluorogenic reagent for thiol groups because of their tendency to react with alcohols, phenols and amine groups.

The already mentioned reagents NBD-Cl and NBD-F are generally known as amine fluorogenic reagents, but they can also be useful in determining thiol groups in biological samples [59].

The new probe 4-(aminosulphonyl)-7-fluoro-2,1,3,-benzoxadiazole, (ABD-F) [60], seems to be the ideal fluorogenic reagent, very useful in determination of thiol groups of low molecular weight, reacting specifically with thiols to form fluorescent adducts. This fluorogenic label has been described in the literature for determinations of both glutathione and cysteine as well as the antihypertensive agent captopril [61].

Moreover, different quinolizinium salts may react with thiol groups in appropriate conditions.

Inorganics

Some inorganics have also been used to generate fluorescence in pharmaceutical compounds.

These methods are based on different types of reactions, such as substitution, redox, the production of binary or ternary complexes and catalytic or enzymatic reactions.

For example, the chelation of different organic analytes with certain metal ions gives rise to fluorescent chelates which are very useful in fluorescence analysis. Tetracycline–magnesium chelates are very fluorescent and have already been employed, amongst other tetracycline–metal complexes, in drug analysis.

In 1961, Kohn [62] developed a fluorimetric method to analyse tetracyclines based on the production of a calcium–barbital–tetracycline complex. This method eventually led to another one capable of determining oxytetracycline and other tetracyclines in biological media, by forming a magnesium–oxytetracycline fluorescent chelate [63].

In the same way, tetracyclines have also been determined in blood serum [64] by fluorimetric assays after incubation of the serum with beryllium ions and measurement of the fluorescence produced by the antibiotic–beryllium complex at 460 nm.

Cerium (IV) has also been widely employed as fluorogenic reagent, as it can be easily reduced to cerium (III) in the presence of either phenols, carbohydrates, carboxylic acids or other oxidisable organics. Cerium (III) is very fluorescent (λ_{ex} = 260 nm, λ_{em} = 350 nm), so this method is very useful in determination of various pharmacological compounds such as analgesics of the morphine series, pyrazolones, salicylic acid derivatives, phenothiazines, local anaesthetics, sulphonamides, purines, or different organic acids [65]. It has also proved to be useful in evaluation of reducing sugars in urine and blood serum [66].

Thiazolic compounds

There are drugs that present in their structure a thiazolic ring, for example, sulphathiazole, tazolol, sudoxicam, thiamine etc. [67]. Most of them do not present native fluorescence, but they can easily be reduced in the presence of zinc and acidic hydrolysis, producing with *p*-phenylenediamine, an intermediate compound which is oxidised with ammonium iron (III) sulphate, to finally produce the fluorescent product thionine.

This method has satisfactorily been applied in the analysis of thiopeptin, a peptide antibiotic used to increase the growth of farm animals when added to their feed.

Miscellaneous

Finally, different types of acidic dyes [68] have been used as fluorescent probes in analyses of various basic drugs. The probe tetrabromofluorescein has been employed in atropine assay, measuring the fluorescent intensity of the complex formed at λ_{ex} = 365 nm, λ_{em} = 556 nm. For d-tubocurarine and diphenhydramine, the dyes rose bengal and Tinopal GS have respectively been used.

To evaluate drugs, both pre-column and post-column derivatisation in HPLC with fluorimetric detection are widely employed. Practically all the above mentioned examples of fluorescent reactions have their own applications.

The compounds used as fluorogenic reagents are therefore not only useful in the fluorimetric analysis of various drugs, but they may also permit the characterisation of several compounds that form part of a complex mixture using HPLC and fluorimetric detection. Therefore, pre-column or post-column derivatisation imply greater selectivity and sensitivity.

Cycloserine (D-4-amino-3-isoxazolidone) [69] for example, is an antibiotic of broad spectrum which works by inhibiting the incorporation of alanine into the bacterial cell wall. Clinically, cycloserine together with other antibiotics, is primarily used to treat various forms of tuberculosis.

There are several methods for quantitation of cycloserine in pharmaceutical preparations. One of these is the ion-pair reversed-phase HPLC assay, recently developed to determine cycloserine by post-column derivatisation with OPA and fluorimetric detection.

FUDR (5-fluoro-2′-deoxyuridine) is widely used to treat a variety of human carcinomas. In comparison with radioimmunoassay the fluorimetric method used to determine FUDR, based on a pre-column derivatisation with 4-bromomethyl-7-methoxy-coumarin (Br-Mmc), proved to be more advantageous as an increased sensitivity was observed [70].

Baclofen (4-amino-3-*p*-chlorophenyl butyric acid) [71] is a skeletal muscle relaxant used in the treatment of spastic disorders. It is clinically administered as a racemic mixture. However the stereoselective analysis of a mixture of enantiomers is very

difficult. Separation is possible with HPLC using either chiral mobile or stationary phases, but derivatisation with chiral reagents is also feasible. In this case, the assay for racemic baclofen is based on derivatisation with OPA in the presence of thiol compounds.

Another use of fluorimetry is in immunoassay techniques. Fluorescence polarisation immunoassay is often the selected method in therapeutic drug monitoring to analyse various pharmacological series, such as antibiotics [72, 73], analeptics [74], antiepileptics [75, 76], hormones [77] (Table 1). This list increases periodically and some of these compounds also have a great importance in clinical biochemistry.

Phosphorimetry

Compared to fluorimetry, phosphorimetry is a less used luminescent technique due to the problems that liquid nitrogen implies. Room temperature phosphorescence (RTP) was primarily only used to assay natural phosphorescent drugs such as caffein, acetylsalicylic acid, or propanolol. Fortunately, it is now possible to detect phosphorescence at room temperature and so derivatisation reactions have been described for this technique. Tobramycin, procainamide and *p*-aminobenzoic acid can therefore be determined after fluorescamine reaction and RTP detection in the ng range [78].

Table 1
Therapeutic drug monitoring using fluorescence polarisation immunoassay

Fluorescence polarisation immunoassay	
A. Analeptics	E. Antineoplasitcs
1. Theophilline	1. Methotrexate
B. Antiarrythmics	F. Cardiotonics
1. Lidocaine	1. Digitoxin
2. *N*-Acetylprocainamide	2. Digoxin
3. Procainamide	
4. Quinidine	G. Hormones
C. Antibiotics	1. Cortisol
1. Amikacin	2. Estriol
2. Dibekacin	3. Thyrosine
3. Gentamycin	
4. Kanamycin	H. Various
5. Streptomycin	1. Cocaine (metabolite)
6. Tobramycin	2. Salicylate
7. Vancomycin	3. Opium alkaloids
D. Antiepileptics	I. Clinical biochemistry
1. Valproic acid	1. Cholesterol
2. Carbamazepine	2. Ethanol
3. Ethosuxide	3. Glucose
4. Phenobarbital	4. Urea
5. Phenitoin	5. Uric acid
6. Primidone	

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