

High Performance Liquid Chromatography with Fluorescence Detection Methods

Subjects: [Biochemical Research Methods](#) | [Chemistry, Analytical](#) | [Chemistry, Applied](#)

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Steroids are compounds widely available in nature and synthesized for therapeutic and medical purposes. Although several analytical techniques are available for the quantification of steroids, their analysis is challenging due to their low levels and complex matrices of the samples. The efficiency and quick separation of the high performance liquid chromatography (HPLC) combined with the sensitivity, selectivity, simplicity, and cost-efficiency of fluorescence, make HPLC coupled to fluorescence detection (HPLC-FLD) an ideal tool for routine measurement and detection of steroids.

[steroids](#)[HPLC](#)[Fluorescence](#)[Quantification](#)[Clinical](#)[Environmental](#)[Food](#)[Derivatisation](#)[Sample preparation](#)

1. Introduction

Steroids are biologically active molecules that are available in many natural sources such as, animals, plants and fungi, in addition to being manufactured as therapeutics. Steroids share a common four-fused ring core consisting of 17 carbon atoms and vary according to functional groups attached to the core or side chains. Naturally secreted steroids are classified into steroid hormones (sex hormones, mineralo- and glucocorticoids, and anabolic steroids) and cholesterol. These compounds travel the bloodstream bound specifically or non-specifically to plasma proteins, such as albumin and binding globulins ^[1]. Steroids are key players in many biological, physiological, and pathological events, which are mediated after binding to their cognate tissue receptors. Most prominently, natural steroids play major roles in metabolism, signaling, immunity, reproduction, and salt-retaining activity. On the other hand, many synthetic steroids have been manufactured to mimic natural steroidal activity and have been approved to treat a wide variety of diseases including anti-inflammatory; arthritis, autoimmune disease, asthma and chronic obstructive pulmonary diseases ^{[2][3]}.

Because steroids can produce biological action at very low concentrations, analytical methods must be very sensitive and precise particularly when quantifying steroid levels in biosamples. Due to the complex nature matrices of most steroid samples, the methods should also be selective to reliably quantify target steroids and resolve them from other similar endogenous compounds and interferences, and to allow for simpler extraction and pre-treatment prior to analysis. This is especially important since sample pre-treatment may lead to the loss of analyte. Steroids possess a naturally lipophilic cyclopentanoperhydrophenanthrene core (**Figure 1**), although their

hydrophilicity is increased by reduction reactions during metabolism. Owing to this hydrophobicity, organic solvents can be used in sample preparation to extract steroids from aqueous media [4].

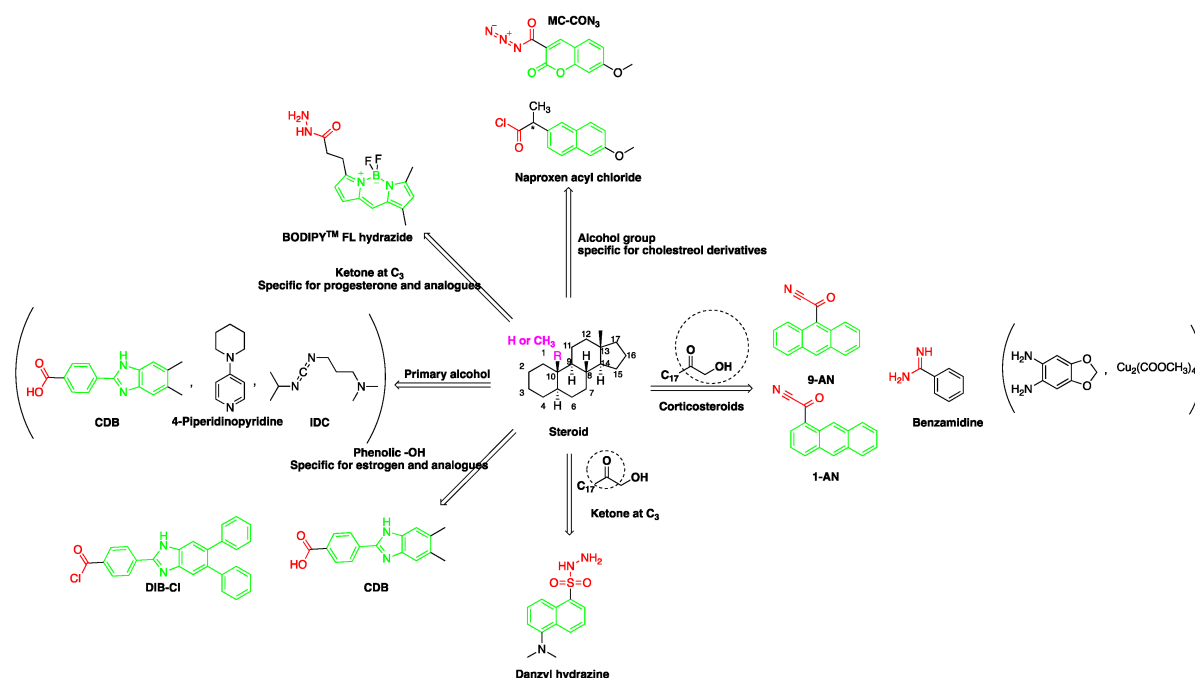


Figure 1. Representation of fluorescent derivatizing agents used in steroid analysis by the high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD).

Commonly used methods in the analysis of steroids include immuno-assays [5][6], gas chromatography-mass spectrometry (GC-MS) [7], high-performance liquid chromatography mass spectrometry (HPLC-MS or MS/MS) [4][8], capillary electrophoresis (CE) [9], and HPLC coupled with UV [10][11][12][13] or fluorescence detection (FLD) [14]. Mass spectrometry (MS) and FLD are relevant to steroid analysis due to their inherited high sensitivity detection and low detection limit. Although both usually require incorporating a derivatization step due to weak ionization of steroids in MS and lack of fluorophores, FLD is considered a simpler and low capital cost technique.

2. HPLC-FLD Methods

HPLC is commonly employed in the analysis of steroids as it provides an excellent separation and quantification tool. Regardless of the compound class, separation using the reversed phase mode is the method of choice in HPLC. Octadecyl silica (ODS or C₁₈) columns are commonly used as the stationary phase in reversed phase-HPLC. Other materials, such as C₈, C₂, phenyl, amino, and cyano phases can also be used to provide different degrees of selectivity. Selectivity depends also on the composition of the mobile phase. Methanol or acetonitrile are commonly used in combination with various percentages of water to prepare the mobile phase which can be employed in the isocratic or gradient mode. Column temperature, pH of the mobile phase and its modifiers are other parameters that can be used to optimize steroids separation. Using narrow and short columns can successfully decrease the amount of solvent needed and analysis time [4].

Prior to injecting steroid-containing samples on the HPLC, a sample preparation method is commonly employed to produce the required selectivity and sensitivity. Sample pretreatment is carried out through a process of sequential steps depending on the extracted steroid and sample type/matrix. For example, biological samples are often prepared by protein precipitation (PP) and enzyme hydrolysis in order to decrease the interferences from undesired compounds such as plasma proteins and to remove the polar groups added to steroids during metabolism. In addition to PP [9], solid phase extraction (SPE) [15][16] and liquid–liquid extraction (LLE) [17][18] are commonly used extraction techniques for the analysis of steroids. Due to the continuous need for faster and simpler sample preparation procedures, other methods such as solid phase microextraction (SPME) [19], liquid-phase microextraction (LPME) [18], turbulent flow chromatography (TFC) [20], dispersive liquid–liquid microextraction (DLLME) [18], molecularly imprinted solid-phase extraction (MISPE) [21][22], restricted access material (RAM) [4][9] are also used.

HPLC-FLD detection can be carried out through direct or indirect methods, depending on the analyte's need for extraction or/and derivatization (**Figure 2**). Researchers seek to develop simple methods that do not require extraction or derivatization. For example, estrogens that are native fluorescents, and certain synthetic steroids, such as the anabolic steroid trenbolone, can fluoresce and, therefore, can be quantified without derivatization. Non-native steroids and non-aromatic compounds, on the other hand, show no fluorescence characteristics and must therefore be derivatized (labeled with fluorescent moieties) prior to analysis (**Figure 2**).

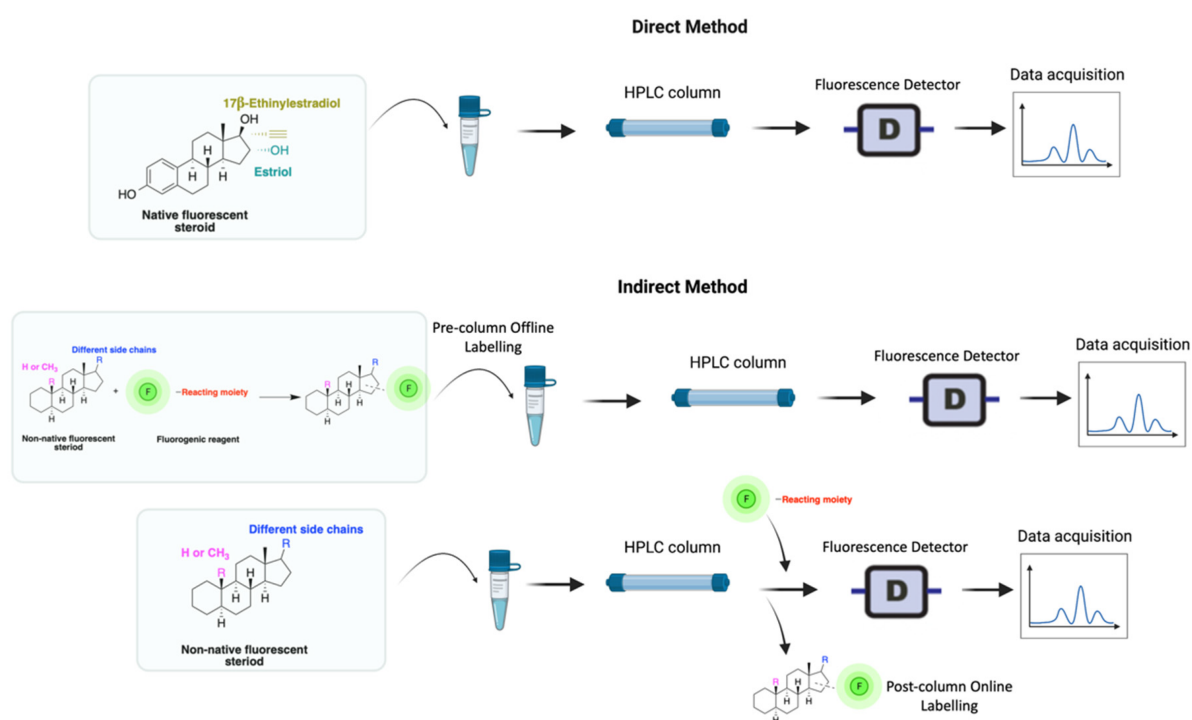
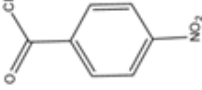
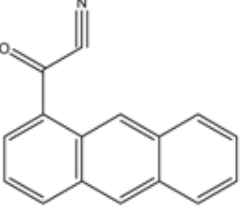
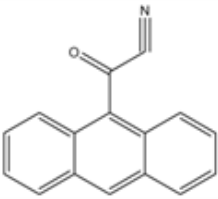


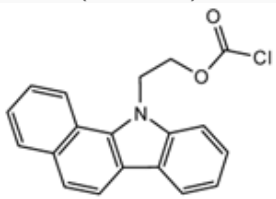
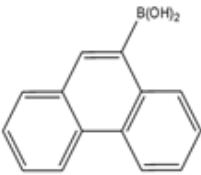
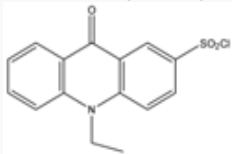
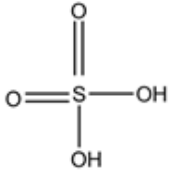
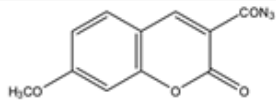
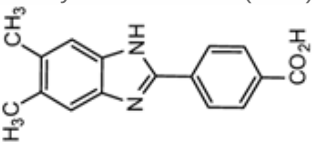
Figure 2. Derivatization using fluorophore-containing reagents for steroids analysis by HPLC-FLD.

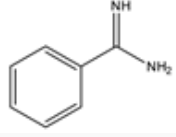
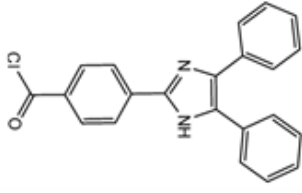
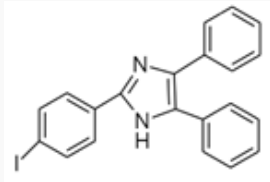
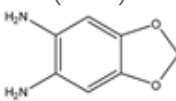
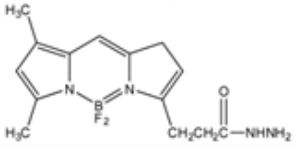
A good derivatization procedure should be simple with high yield and minimum side products and is performed under mild conditions to avoid decomposition of the target steroid [4]. Derivatization enhances the detection of steroids and can be done by either pre-column offline labeling or post-column online labeling (**Figure 2**), which

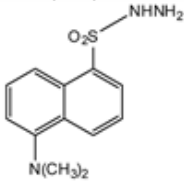
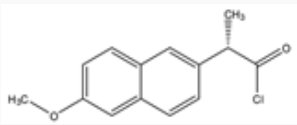
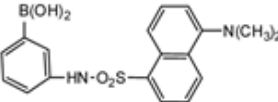
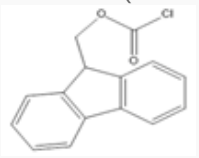
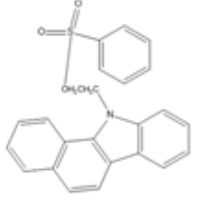
ideally must be rapid. These procedures can, however, be accelerated by modifying the reaction's temperature and pH or incorporating a catalyst. **Figure 1** is a representation for the most common fluorescent derivatizing pathways and their targets in steroid analysis. There are several classes of fluorescent derivatizing agents including anthracenes, coumarins and phenanthrenes. The derivatization process depends on functionalization of specific groups that are targeted in steroids structure as well as the type of derivatization agent used. For example, steroids with primary alcohol moiety, e.g., cortisone, can be targeted with a fluorescent agent in a selective reaction thus differentiating them from steroids containing secondary and tertiary alcohol groups such as prednisolone. Similarly, other steroids, such as estrogens, have specific phenolic groups, which can also be targeted by other agents. The various derivatization procedures reported in the literature used for steroid detection by HPLC-FLD including the reagents employed and conditions used in each procedure are summarized in **Table 1**.

Table 1. Derivatization reagents and conditions used in steroid quantification by HPLC-FLD.

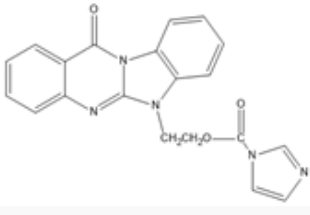
Reagent	Target Steroid	Sample Preparation and Derivatization Conditions	Reference
<p><i>p</i>-Nitrobenzoyl chloride</p> 	Estrogens	Sample was extracted by SPE C ₁₈ . Residue reacted with <i>p</i> -nitrobenzoyl chloride at 25 °C, 30 min	Mao et al. 2004 [23]
<p>1-Anthroyl nitrile (1-AN)</p> 	18-Oxygenated corticosteroids, 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol Pregnenolone and C21 steroids; especially corticoids	Sample was extracted by SPE or LLE with a mixture of diethyl ether and dichloromethane. Extract reacted with 1-anthroyl nitrile in acetonitrile at room temperature for 10 min.	Kurosawa et al. 1995 [24] Shimada et al. 1996 [25] Shimada et al. 1991 [26]
<p>9-Anthroyl nitrile (9-AN)</p> 	Glucocorticoids; F and E, Hydroxysteroids Corticosteroids	Sample was extracted by LLE or SPE. Extracts reacted with 9-AN in a mixture of quinuclidine and triethylamine for 30 min at RT.	Glowka et al. 2009 [27] Goto et al. 1983 [28] Shibata et al. 1997 [29] Haegele et al. 1991 [30] Kosicka et al. 2018 [31] Neufeld et al. 1998 [32] Shimada et al. 1991 [26]
2-(11H-Benzo[a]carbazole-11-yl) ethyl carbonochloridate	E1, E2, E3, BPA, NP, OP	Sample was extracted by DLLME. Extracts were added to BCEC-Cl in a NaHCO ₃ buffer at pH 10; it	Wu et al. 2015 [33]

Reagent	Target Steroid	Sample Preparation and Derivatization Conditions	Reference
(BCEC-Cl) 		was shaken for 10 s, and then allowed to stand for 14 min at 43 °C.	
9-Phenanthrene boronic acid 	Brassinosteroids	Sample was extracted by LLE. Extracts reacted with 9-phenanthrene-boronic acid in a mixture of pyridine and acetonitrile for 10 min at 70 °C.	Gamoh and Takatsuto. 1989 [34]
10-Ethyl-acridone-2-sulfonyl chloride (EASC) 	Estrogens and Biogenic Amines (aliphatic amines)	Sample was extracted by LLE or SPE. Extracts reacted with EASC in anhydrous acetonitrile and NaHCO ₃ buffer pH (10.2), for 5 min at 60 °C.	Zhang et al. 2012 [35]
Sulfuric acid 	F and Corticosterone	Sample was extracted by SPE or LLE. Extracts reacted with sulfuric acid in ethanol; it was cooled in crushed ice for 15 min in the dark.	Nozaki et al. 1991 [36] Nozaki et al. 1992 [37] Sudo et al. 1990 [38] Gao et al. 2010 [39]
7-Methoxycoumarin-3-carbonyl azide (MC-CON ₃) 	7α-hydroxycholesterol	Sample was extracted by LLE and purified by normal-phase SPE. Extracts reacted with MC-CON ₃ in ethyl acetate (AcOEt) for 40 min at 140 °C.	Saisho et al. 1998 [40]
2-(4-Carboxyphenyl)-5,6-dimethylbenzimidazole(CDB) 	Corticosteroids, BPA and alkylphenols	Sample was extracted by LLE. 0.02% w/v CDB solution, 2.0% w/v 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC) solution, and 0.01% w/v (10 mg/mL) 4-piperidinopyridine, for 60 min at 40 °C.	Katayama et al. 1991 [41] Katayama et al. 1992 [41] Katayama et al. 2001 [42]

Reagent	Target Steroid	Sample Preparation and Derivatization Conditions	Reference
Benzamidine 	17-hydroxycorticosteroids	Sample was extracted by LLE. Extracts reacted with benzamidine in a basic medium of sodium hydroxide solution, with a mixture of propanol and water for 5 min at 95 °C.	Seki et al. 1984 [43]
4-(4,5-Diphenyl-1 <i>H</i> -imidazol-2-yl) benzoyl chloride (DIB-Cl) 	BPA	Sample was extracted by LLE or SPE. Extracts reacted with DIB-Cl in a mixture of acetonitrile and triethylamine for 20 min at RT.	Sun et al. 2004 [44] Kuroda et al. 2003 [45] Sun et al. 2002 [46]
4-(4,5-Diphenyl-1 <i>H</i> -imidazol-2-yl) iodobenzene (DIB-I) 	EE	Sample was extracted by the SPE disc method (C ₁₈ SPE disk). A total of 50 µL of the extracts reacted with 50 µL of DIB-I (3.0 mM), in a mixture of 50 µL of solution containing 0.2 mM of PdCl ₂ and 0.3 mM of CuI, and 50 µL of DIPEA (3.0 mM). The vial contents were deoxygenated by N ₂ purge for 10 sec, heated at 100 °C for 40 min, cooled, then filtered through a 0.45-µm membrane filter before injection into the HPLC-FLD system.	Ali et al. 2020 [47]
1,2-Diamino-4,5-methylenedioxybenzene (DMB) 	α-Dicarbonyl compounds; prednisolone, PN and 21-hydroxycorticosteroids	Sample was extracted by LLE. Extracts reacted with DMB for 40 min at 60 °C.	Yamaguchi et al. 1991 [48] Yoshitake et al. 1989 [49] Yamaguchi et al. 1989 [50]
2-(4-Carboxyphenyl)-5,6-dimethylbenzimidazole (BODIPY FL hydrazide) 	Aldehydes and ketones; progesterone, 17-hydroxyprogesterone, and other 3-keto steroids.	Sample was extracted by LLE. Extracts reacted with BODIPY FL hydrazide in ethanol for 15 h at RT.	Katayama et al. 1998 [51]

Reagent	Target Steroid	Sample Preparation and Derivatization Conditions	Reference
<p>Dansyl hydrazine</p> 	<p>F, Butane acid-(5-androsten-17-one-3beta-ol)-diester (A1998)^[52], alfaxalone and pregnanolone ^[53]</p>	<p>Sample was extracted by LLE. Extracts reacted with dansyl hydrazine in organic solvent for 30 min at RT in acidic medium.</p>	<p>Kawasaki et al. 1979 ^[54] Visser et al. 2000 ^[52] Peng et al. 2007 ^[53]</p>
<p>Naproxen acyl chloride in toluene</p> 	<p>Cholesterol and sitosterol</p>	<p>Sample was extracted by LLE. Extracts reacted with naproxen acyl chloride in toluene, while shaking for 1.5 h at 90 °C. Diethylamine in toluene was then added to inactivate the excess naproxen acyl chloride, while shaking for 5 min at 30 °C.</p>	<p>Lin et al. 2007 ^[19]</p>
<p>Dansylaminophenylboronic acid</p> 	<p>Brassinolide and castasterone</p>	<p>Sample was extracted by LLE. Extracts reacted with dansylaminophenylboronic acid in a mixture of pyridine and acetonitrile for 20 min at 70 °C.</p>	<p>Motegi et al. 1994 ^[55]</p>
<p>9-Fluorenylmethyl chloroformate (Fmoc-Cl)</p> 	<p>E1, E2, E3, BPA, NP, OP</p>	<p>Samples were extracted by MSPE. Extracts were reacted with Fmoc-Cl in NaHCO₃ (pH = 10.5) at 60 °C for 10 min, and then added to a mixture of aqueous acetic acid and acetonitrile. The mixture was cooled to RT.</p>	<p>Qianyu Li et al. 2018 ^[56]</p>
<p>2-(11H-Benzo[a]carbazole-11-yl)-ethyl-4-methylbenzenesulfonate (BCETS)</p> 	<p>FFA</p>	<p>The samples were extracted by supercritical CO₂ and organic solvent extraction. The extracted samples were derivatized in BCETS in a solution of K₂CO₃ at 84 °C; then the mixture was cooled down to RT and diluted with DMF.</p>	<p>Li et al. 2011 ^[57]</p>
<p>Benzimidazo[2, 1-b]quinazoline-12(6H)-one-5-</p>	<p>OP, NP, TBP, BPA, E1, E2, E3</p>	<p>The samples were extracted by LLE. BQEIC in a solvent of DMAP at 80 °C for 60 min. Finally, the mixture</p>	<p>Liu et al. 2018 ^[58]</p>

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Reagent	Target Steroid	Sample Preparation and Derivatization Conditions	Reference
ethylimidazole ester (BQEIC) 		was cooled to RT and diluted with acetonitrile.	

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