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Review

Advances in the analysis of steroid hormone drugs in pharmaceuticals and environmental samples (2004–2010)☆

Sándor Görög*

Gedeon Richter Plc., P.O. Box 27, H-1475 Budapest, Hungary

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ABSTRACT

A critical review of the literature of the analysis of steroid hormone drugs is presented based on 213 publications published between 2004 and 2010. The state of the art of the assay and purity check of bulk drug materials is characterized on the basis of the principal pharmacopoeias supplemented by the literature dealing with their impurity profiling and solid state characterization. The determination of the active ingredients and impurities/degradants in pharmaceutical formulation by HPLC, other chromatographic, electrodriven, spectrophotometric and other methods is also summarized. A short section deals with the application of analytical methods in drug research. The literature of the determination of steroid hormones in environmental samples is summarized in tabulated form.

Contents

1. Introduction

Steroid hormone drugs and their (semi)synthetic analogues manufactured from steroids of plant origin or by total synthesis are among the most important groups of drug materials. Due to the decisive role of analytical chemistry in the safety of drug therapy [1] the analysis of steroid hormone drugs is an important task. The aim of this review is to give an overview on the developments in this field after 2004, a year when a review summarizing the results of the previous years was published by the present author [2].

The majority of the analytical literature of this field deals with the analytical aspects of the biosynthesis and biochemistry of steroid hormones which are present in the human and animal

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* Tel.: +36 1 4314620; fax: +36 1 4315284.
E-mail addresses: s.gorog@richter.hu, gor4745@t-online.hu

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organism (estradiol, estrone, estriol, progesterone, testosterone, hydrocortisone, etc.) and the fate of their (semi)synthetic analogues in the organism after their administration (pharmacokinetic, pharmacodynamic, metabolism studies). These are outside the scope of this review which concentrates on the analysis of bulk steroid hormone drugs, their (semi)synthetic analogues and steroid hormone-like structures (in the following *steroid hormone drugs*), the pharmaceutical formulations containing these and their determination in environmental samples. Other groups of steroid drugs (sterols, vitamins D, bile acids, cardiac glycosides) are also outside the scope of this review.

Reviews dealing with pharmaceutical, industrial, compendial aspects of steroid hormone drugs, published in the period of 2004–2010 are restricted to the above mentioned paper [2], two book chapters [3,4] and the reprint edition of one of the present author's earlier books on steroid analysis [5].

2. Bulk steroid hormone drugs

2.1. Assay

The importance of the assay methods for bulk drug materials as a quality attribute was disputed especially if non-selective methods are used (taking just the synthetic steroid hormone drug *levonorgestrel* as the example) [1,6], and this idea, suggesting the mass balance principle to be used (assay = $100 - \Sigma_{impurities}\%$) was extended to the use of the selective method high-performance liquid chromatography (HPLC) as well [7]. In spite of this, assay methods are and will certainly remain in the foreseeable future parts of the analytical protocol of bulk drug materials, among them steroid hormone drugs. The state of the art can be seen in Table 1 where the assay methods used for 121 steroid hormone drugs in the principal pharmacopoeias (European Pharmacopoeia 6 [8], United States Pharmacopoeia 33 [9] and Japanese Pharmacopoeia 15 [10]) are summarized.

As seen from the data in Table 1, selective chromatographic assay methods are preferred by *USP* [9] (75%); this means in the overwhelming majority of cases reversed-phase high-performance liquid chromatography (RP-HPLC). In the other cases non-selective methods such as titration and UV spectrophotometry are used. Moreover in the case of some old monographs for classical drugs, outdated colorimetric methods using as reagents e.g. isoniazide for 4-ene-3-oxo steroids or tetrazolium blue for the dihydroxyacetone side chain of corticosteroids are still in use. In *Ph. Eur.* [8], non-selective methods such as UV spectrophotometry (62%) and titration (17%) are mainly used with a share of only 19% for HPLC. This reflects the strategy of Ph. Eur.: the main role in ensuring the suitable quality of the drug substances is controlling the impurities rather than using a specific assay method. The approach of the *Japanese Pharmacopoeia* [10] with the share of 54% for HPLC is between the above two.

In real life drug registration agencies require stability-indicating assay methods in the case of new drug applications. In practice this is an optimized HPLC method where it is assured that the main peak is separated from all potential and real impurities and degradation products, among them degradants formed under stress-degradation conditions prescribed in an ICH guideline [11]. Pharmacopoeial methods do not always fulfil these requirements. A good example for the necessity of developing new, highly efficient methods is the case of the classical drug material *betamethasone*. The current pharmacopoeial HPLC methods are not suitable for the separation of all impurities and degradants. In a recent paper an assay method is described where making use of new achievements of stationary phase technology (after trying no less than 13 various types of columns and selecting an ACE Phenyl column, particle size 3 μm, pore size 100 Å) and a carefully optimized gradient elution system, 26 related compounds were separated from the main component and from each other and quantified [12].

Other stability indicating HPLC methods were published for the assay of *budesonide* [13], *medroxyprogesterone acetate* [14], *loteprednol etabonate* [15], *examestane* [16], *etiprednol dicloacetate* [17], *eplerenone* [18], *dutasteride* [19,20], *estradiol* [21], *betamethasone* [22] and intermediates in steroid syntheses such as *alphamethylepoxide* (16α-methyl-1,4-pregnadiene-9β,11β-oxide-17α,21-diol-3,20-dione) [23] and its 16β-methyl analogue, *betamethylepoxide* [24].

Making use of small particle size solid supports has already been mentioned. It is worth mentioning that using an ultra-high performance (UPLC) column (UPLC™ BEH C18, 1.7 μm; 50 mm × 2.1 mm) seven steroid hormone drugs (*dienogest*, *estradiol*, *ethinylestradiol*, *finasteride*, *gestodene*, *levonorgestrel* and *norethisterone acetate*) were separated within 2.5 min in the course of a cleaning validation study [25], *levonorgestrel* and *ethinylestradiol* for their determination in a contraceptive pill within 0.7 min using Ascentis Express C18 2.7 μm (50 mm × 2.1 mm), Acquity UPLC BEH C18 1.7 μm (50 mm × 2.1 mm) and Chromolith FastGradient RP-18e (50 mm × 2.0 mm) columns [26]. Important papers on the theoretical aspects and limitations of using sub-2 μm particles for the separation of steroids [27] and Design Space computer modelling for the optimization of the separation of (among others) steroids [28] were published by the same group.

Ultraviolet detection is almost exclusively used in the HPLC assay of steroid hormone drugs. Due to the α,β-unsaturated 3-oxo group in the majority of androgenic, gestogenic and corticosteroid drugs, these have a strong absorption band around 240–245 nm. As seen in Table 1, in spite of this the detector wavelength is set in the majority of cases to 254 nm, which is on the slope of this band leading to some loss in sensitivity and robustness. This anomaly certainly has historical reasons and originates from the early period of HPLC when predominantly mercury lamp-based UV detectors were only available but nothing justifies this in the era of variable wavelength and diode-array detectors.

The successful use of a circular dichroism (CD) detector has been reported in the analysis of *prasterone* and related steroids [29].

2.2. Purity check

The chromatographic estimation of steroidal impurities in bulk steroid hormone drugs is named *Related substances* test in Ph. Eur. and the Japanese Pharmacopoeia and *Organic impurities* (in some cases *Ordinary impurities*) test in the United States Pharmacopeia. As it is seen in Table 1, the USP prescribes almost exclusively HPLC for this purpose, while in Ph. Eur. and the Japanese Pharmacopoeia a thin-layer chromatographic (TLC) purity check has a remarkable share.

The advantage of the determination of impurities and degradants by *HPLC* is that this is a quantitative method. However, in the majority of monographs in pharmacopoeias the quantity of impurities is expressed *versus* the main component. This means that in the case of the determination of spectrophotometrically active impurities in a spectrophotometrically less active parent drug this may lead to serious overestimation of the impurities and *vice versa* where impurities are underestimated in the case of spectrophotometrically less active impurities in a spectrophotometrically highly active parent. A very promising new tendency to solve this problem in the pharmacopoeias is to present in the new monographs the names of the most probable impurities together with their relative retention times and relative response factors. An example for this is the monograph of *fulvestrant* in USP 33 [9] where these data for five potential impurities are presented. This approach

Table 1
Assay and related impurities tests for bulk steroid hormone drugs in European, United States and Japanese pharmacopoeias.

	Assay			Related impurities		
	Ph. Eur. 6	USP 33	Ph. Jp. 15	Ph. Eur. 6	USP 33	Ph. Jp 15
Alclometasone dipropionate	–	RP-HPLC 254 nm	–	–	TLC 254 nm	–
Amcinonide	–	RP-HPLC 254 nm	–	–	–	–
Beclomethasone dipropionate	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	–	TLC – tetrazolium
Betamethasone	UV 238.5 nm	RP-HPLC 240 nm	RP-HPLC 240 nm	RP-HPLC 254 nm	TLC – H_2SO_4	TLC 254 nm
Acetate	UV 240 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC 254 nm	–
Benzoate	–	RP-HPLC 254 nm	–	–	TLC 254 nm	–
Dipropionate	UV 240 nm	RP-HPLC 254 nm	UV 239 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Sodium phosphate	UV 241 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	Betamethasone – extraction 239 nm	TLC 254 nm
Valerate	UV 240 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Budesonide	RP-HPLC 240 nm	RP-HPLC 254 nm	–	RP-HPLC 240 nm	RP-HPLC 254 nm	–
Canrenoate, potassium	–	–	Titration, $HClO_4$	–	–	–
Chlormadinone acetete	–	–	UV 285 nm	–	–	RP-HPLC 236 nm
Clobetasol propionate	RP-HPLC 240 nm	RP-HPLC 240 nm	–	RP-HPLC 240 nm	RP-HPLC 240 nm	–
Clobetasone butyrate	UV 235 nm	–	–	RP-HPLC 241 nm	–	–
Clocortolone pivalate	–	VIS – isoniazid	–	–	TLC/elut 238 nm	–
Cortisone acetate	UV 237 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm
Cyproterone acetate	UV 282 nm	–	–	RP-HPLC 254 nm	–	–
Danazol	–	UV 285 nm	–	–	TLC – I_2	–
Desogestrel	RP-HPLC 205 nm	–	–	RP-HPLC 205 nm	–	–
Desoximetasone	–	RP-HPLC 254 nm	–	–	–	
Desoxycort(icoster)one acetate	UV 240 nm	VIS – tetrazolium	–	RP-HPLC 254 nm	–	–
Pivalate	–	RP-HPLC 254 nm	–	–	–	–
Dexamethasone	UV 238.5 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm
Acetate	UV 238.5 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Isonicotinate	Titration, $HClO_4$	–	–	RP-HPLC 240 nm	–	–
Sodium phosphate	UV 241.5 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Diflorasone diacetate	–	NP-HPLC 254 nm	–	–	NP-HPLC 254 nm	–
Drospirenone	RP-HPLC 245 nm	RP-HPLC 245 nm	–	RP-HPLC 245 nm	RP-HPLC 245 nm	–
Dydrogesterone	RP-HPLC 280	RP-HPLC 280 nm	UV 286 nm	RP-HPLC 280/385 nm	RP-HPLC 280 nm	RP-HPLC 265 nm
Estradiol	UV 238 nm NaOH	RP-HPLC 205 nm	–	RP-HPLC 280 nm	NP-HPLC 280 nm	–
Benzoate	UV 231 nm	RP-HPLC 230 nm	RP-HPLC 230 nm	RP-HPLC 230 nm	TLC-molybdate/H_2SO_4	TLC 254 nm
Cypionate	–	RP-HPLC 280 nm	–	–	–	–
Valerate	UV 280 nm	RP-HPLC 280 nm	–	RP-HPLC 220 nm	TLC – H_2SO_4	–
Estriol	UV 281 nm	UV 281 nm	RP-HPLC 280 nm	NP-HPLC 280 nm	TLC – H_2SO_4	TLC – H_2SO_4
Estrogens, conjugated	GC	GC	–	GC	GC	–
Estrogens, esterified	–	GC	–	–	GC	–
Estrone	–	RP-HPLC 280 nm	–	–	TLC – H_2SO_4	–
Estropipate	–	RP-HPLC 213 nm	–	–	RP-HPLC 213 nm	–
Ethinylestradiol	Titration ethinyl	RP-HPLC 280 nm	Titration ethinyl	RP-HPLC 280 nm	–	Estrone – colorim.
Ethynodiol diacetate	–	RP-HPLC 200 nm	–	–	RP-HPLC 200 nm	–
Finasteride	RP-HPLC 210 nm	RP-HPLC 215 nm	–	RP-HPLC 210 nm	RP-HPLC 210 nm	–
Fludrocortisone acetate	UV 238 nm	VIS – Tetrazolium	–	RP-HPLC 254 nm	TLC 254 nm	–
Flumetasone pivalate	UV 239 nm	VIS – tetrazolium	–	RP-HPLC 254 nm	TLC-H_2SO_4	–
Flunisolide	–	RP-HPLC 254 nm	–	–	TLC 254 nm	–
Fluocinolone acetonide	UV 238 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 238 nm	–	RP-HPLC 254 nm
Fluocinonide	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC – tetrazolium
Fluocortolone pivalate	UV 242 nm	–	–	RP-HPLC 243 nm	–	–
Fluorometholone	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–	–	TLC 254 nm
Fluoxymesterone	–	RP-HPLC 254 nm	NP-HPLC 254 nm	–	NP-HPLC 254 nm	TLC 254 nm
Flurandrenolide	–	RP-HPLC 240 nm	–	–	TLC 254,366 nm	–
Fluticasone propionate	RP-HPLC 239 nm	RP-HPLC 239 nm	–	RP-HPLC 239 nm	RP-HPLC 239 nm	–
Fulvestrant	–	RP-HPLC 225 nm		–	RP-HPLC 225 nm	–

Gestodene	RP-HPLC 254 nm	–	–	RP-HPLC 205/254 nm	–	–
Halcinonide	–	UV 239 nm	–	–	TLC/elut. 239 nm	–
Hydrocortisone	UV 241.5 nm	RP-HPLC 254 nm	NP-HPLC 254 nm	RP-HPLC 254 nm	NP-HPLC 254 nm	TLC 254 nm
Acetate	UV 241.5 nm	NP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Butyrate	–	RP-HPLC 254 nm	UV 241 nm	–	RP-HPLC 254 nm	TLC – tetrazolium
Hydrogen succinate	UV 241.5 nm	NP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Sodium phosphate	–	Enzyme, extraction 239 nm	RP-HPLC 254 nm	–	Hydrocortisone, extraction 239 nm	RP-HPLC 254 nm
Sodium succinate	–	VIS – tetrazolium	UV 240 nm	–	–	TLC 254 nm
For injection	–	NP-HPLC 254 nm	–	–	NP-HPLC 254 nm	–
Succinate	–	–	RP-HPLC 254 nm	–	–	TLC 254 nm
Valerate	–	RP-HPLC 254 nm	–	–	–	–
Hydroxyprogesterone caproate	–	UV 240 nm	–	–	TLC – H_2SO_4	–
Isoflupredone acetate	–	NP-HPLC 254 nm	–	–	RP-HPLC 254 nm	–
Levonorgestrel	Titration ethinyl	UV 241 nm	–	TLC – P.molybd.	TLC – P.molyb.	–
Medroxyprogesterone acetate	UV 241 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Megestrol acetate	UV 287 nm	RP-HPLC 280 nm	–	RP-HPLC 254 nm	–	–
Mepitiostane	–	–	RP-HPLC 265 nm	–	–	TLC – H_2SO_4
Meprednisone	–	TLC extr. 238 nm.	–	–	–	–
Mesterolone	RP-HPLC 200 nm	–	–	RP-HPLC 200 nm	–	–
Mestranol	Titration ethinyl	H_2SO_4 545 nm	UV 279 nm	TLC – H_2SO_4	–	TLC – H_2SO_4
Metenolone acetate	–	–	UV 242 nm	–	–	TLC 254 nm
Metenolone enanthate	–	–	UV 242 nm	–	–	TLC 254 nm
Methylprednisolone	UV 243 nm	NP-HPLC 254 nm	UV 243 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Acetate	UV 243 nm	NP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Hydrogen succinate	UV 243 nm	NP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm
Sodium succinate	–	VIS – tetrazolium	–	–	–	–
Sodium succinate for injection	–	NP-HPLC 254 nm	–	–	NP-HPLC 254 nm	–
Methyltestosterone	UV 241 nm	RP-HPLC 241 nm	RP-HPLC 241 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Mibolerone	–	RP-HPLC 254 nm	–	–	–	–
Mometasone furoate	UV 249 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC 254 nm	–
Nandrolone decanoate	UV 240 nm	RP-HPLC 240 nm	–	RP-HPLC 254 nm	NP-HPLC 238 nm	–
Nandrolone phenylpropionate	–	TLC extr. 239 nm	–	–	–	–
Nomegestrol acetate	UV 287 nm	–		RP-HPLC 245/290 nm		
Norethisterone (norethindrone)	Titration ethinyl	UV 240 nm	Titration ethinyl	RP-HPLC 210/254 nm	RP-HPLC 254 nm	–
Acetate	Titration ethinyl	UV 240 nm	Titration ethinyl	RP-HPLC 210, 254 nm	TLC – H_2SO_4	RP-HPLC 254 nm
Norgestimate	Titration ethinyl	RP-HPLC 244 nm	–	RP-HPLC 244 nm	RP-HPLC 244 nm NP-HPLC 210 nm	–
Norgestrel	Titration ethinyl	UV 241 nm	Titration ethinyl	TLC – phos.mol.	TLC – phos.mol.	TLC 254 nm
Oxandrolone	–	RP-HPLC 210 nm	–	–	RP-HPLC 210 nm	–
Oxymetholone	–	TLC extr. 315 nm	UV 315 nm	–	–	TLC – vanilline/H_2SO_4
Pancuronium bromide	Titration $HClO_4$	Titration $HClO_4$	Titration $HClO_4$	TLC – $NaNO_2/BiI_4^-$	TLC – $NaNO_2/BiI_4^-$	TLC – $NaNO_2/BiI_4^-$
Paramethasone acetate	–	TLC extr. 242 nm	–	–	–	–
Prasterone sodium sulfate	–	–	Ion-exchange/titration NaOH	–	–	TLC – H_2SO_4
Prednicarbate	RP-HPLC 243 nm	RP-HPLC 243 nm	–	RP-HPLC 243 nm	RP-HPLC 243 nm	–
Prednisolone	UV 243.5 nm	NP-HPLC 254 nm	RP-HPLC 247 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Acetate	UV 243 nm	NP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	NP-HPLC 254 nm	TLC 254 nm
Hydrogen succinate	–	TLC extr. 243 nm	RP-HPLC 242 nm	–	–	TLC 254 nm
Pivalate	UV 243 nm	–	–	RP-HPLC 254 nm	–	–
Sodium phosphate	UV 247 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Sodium succinate	–	VIS – tetrazolium	RP-HPLC 254 nm	–	–	–
Tebutate	–	NP-HPLC 254 nm	–	–	–	–
Prednisone	UV 238 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	NP-HPLC 254 nm	–
Progesterone	UV 241 nm	RP-HPLC 254 nm	UV 241 nm	RP-HPLC 241 nm	–	TLC 254 nm

Table 1 (*Continued*)

	Assay			Related impurities		
	Ph. Eur. 6	USP 33	Ph. Jp. 15	Ph. Eur. 6	USP 33	Ph. Jp 15
Rimexolone	–	RP-HPLC 242 nm	–	–	RP-HPLC 242 nm	–
Spironolactone	UV 238 nm	RP-HPLC 230 nm	UV 238 nm	RP-HPLC 254/283 nm	TLC – H_2SO_4	TLC – H_2SO_4
Stanozolol	Titration $HClO_4$	Titration $HClO_4$	–	TLC – H_2SO_4	TLC – H_2SO_4	–
Testolactone	–	VIS – isoniazid	–	–	TLC 254 nm + acid dichromate	
Testosterone	UV 241 nm	TLC extr. 239 nm	–	RP-HPLC 254 nm	–	–
Cypionate	–	GC	–	–	–	–
Decanoate	RP-HPLC 240 nm			RP-HPLC 240 nm		
Enantate	UV 241 nm	VIS – isoniazid	UV 241 nm	TLC – H_2SO_4	TLC – p-toluene sulfonic acid	–
				RP-HPLC 240 nm		
Isocaproate	RP-HPLC 240 nm	–	–	RP-HPLC 240 nm	–	–
Propionate	UV 240 nm	VIS – isoniazid	UV 241 nm	RP-HPLC 254 nm	–	RP-HPLC 241 nm
Tibolone	Titration ethinyl	–	–	RP-HPLC 205 nm	–	–
Trenbolone acetate	–	RP-HPLC 229 nm	–	–	RP-HPLC 229 nm	–
Triamcinolone	UV 238 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 238 nm	–	–
Acetonide	UV 238.5 nm	RP-HPLC 254 nm	RP-HPLC 240 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Diacetate	–	RP-HPLC 254 nm	–	–	–	–
Hexacetonide	UV 238 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Vecuronium bromide	Titration $HClO_4$	–	–	RP-HPLC 210 nm	–	–

can be found in the papers already mentioned in connection with the stability-indicating assays [13–24].

In spite of the fact that in possession of modern densitometers *TLC* can be used for the quantitative determination of impurities and degradants as demonstrated on the examples of *ethinylestradiol*, *guggulsterone* and *pipecuronium bromide* in a review [30], TLC is used in the pharmacopoeias as a semi-quantitative limit test based on visual inspection of the plates. Another example for the successful use of TLC-densitometry is the investigation of *ethinylestradiol* and *levonorgestrel* [31]. The optimization of the separation of *androstanolone* isomers by the "simplex" and "prisma" methods [32], the application of temperature-controlled micro-TLC for separation and quantification of *testosterone* and its derivatives [33] and the comparison of the performance of TLC and HPLC using estrogens as the model compounds [34] are also worth mentioning.

A non-chromatographic method, *differential scanning calorimetry* (*DSC*) is known to be able to characterize the purity of drug substances. In the course of a study involving 16 pharmaceutical reference standards, among them two steroids, very good agreement was found between the results of HPLC and DSC purity tests: for ethinylestradiol HPLC 100.0%, DSC 99.9% and for betamethasone dipropionate both methods gave 99.2% [35].

2.3. Impurity profiling (structure elucidation of impurities and degradation products)

As already mentioned, the contribution of impurity profiling (detection, identification and quantitative determination of impurities and degradation products) is much more important in assuring the safety of drug therapy than assaying bulk drug materials [36].

After their detection usually by HPLC and/or TLC the structures of the impurities and degradation products are determined by on-line or off-line coupling of chromatographic and spectroscopic techniques. Of the spectroscopic techniques, ultraviolet (UV) spectra are the easiest to obtain by on-line HPLC-diode array UV (HPLC/DAD-UV). However, the information thus obtained is of value in advantageous cases only. The most generally used method in elucidating the structures of drug impurities is mass spectrometry (MS) coupled with HPLC (HPLC/MS). In spite of the commercial availability of coupled on-line HPLC/NMR, moreover HPLC/NMR/MS instruments, nuclear magnetic resonance spectroscopy (NMR) is usually used off-line. New possibilities and some advantages of this approach are discussed in [37].

Of the impurity and degradation profiling studies published in the recent years those dealing with *betamethasone* and its esters merit emphasizing. In the course of these studies on-line HPLC/UV and HPLC/MS(MS) with off-line MS and NMR after semi-preparative HPLC separation were used [38–43]. The main results were the identification of the epimeric 16α-methyl derivative (dexamethasone-17-valerate) as a process impurity in *betamethasone-17-valerate* [38], the identification of 4 diastereomeric 17-deoxy-20-hydroxy-21-oic acid-type solid-phase degradation products of *betamethasone sodium phosphate* [39] (see Fig. 1), identification of *Z*- and *E*-isomers of 20-hydroxy-17(20)-en-21-aldehyde degradation products and elucidation of the mechanism of their formation under acidic and basic stress conditions [40,41] (see Fig. 1). Three isomeric D-homoannular ring expansion degradants of non-crystalline *betamethasone sodium phosphate* were also identified [42]. The investigation of the photochemical degradation pathways under the effect of UVB radiation is especially important for topically used corticosteroids. As shown in Fig. 1, photoproducts with cleaved and rearranged ring A were identified in various betamethasone esters by two groups in the same year [43,44]. Similar photoproducts were found in the course of a study with *loteprednol etabonate* [45], while in the case of *hydrocor-*

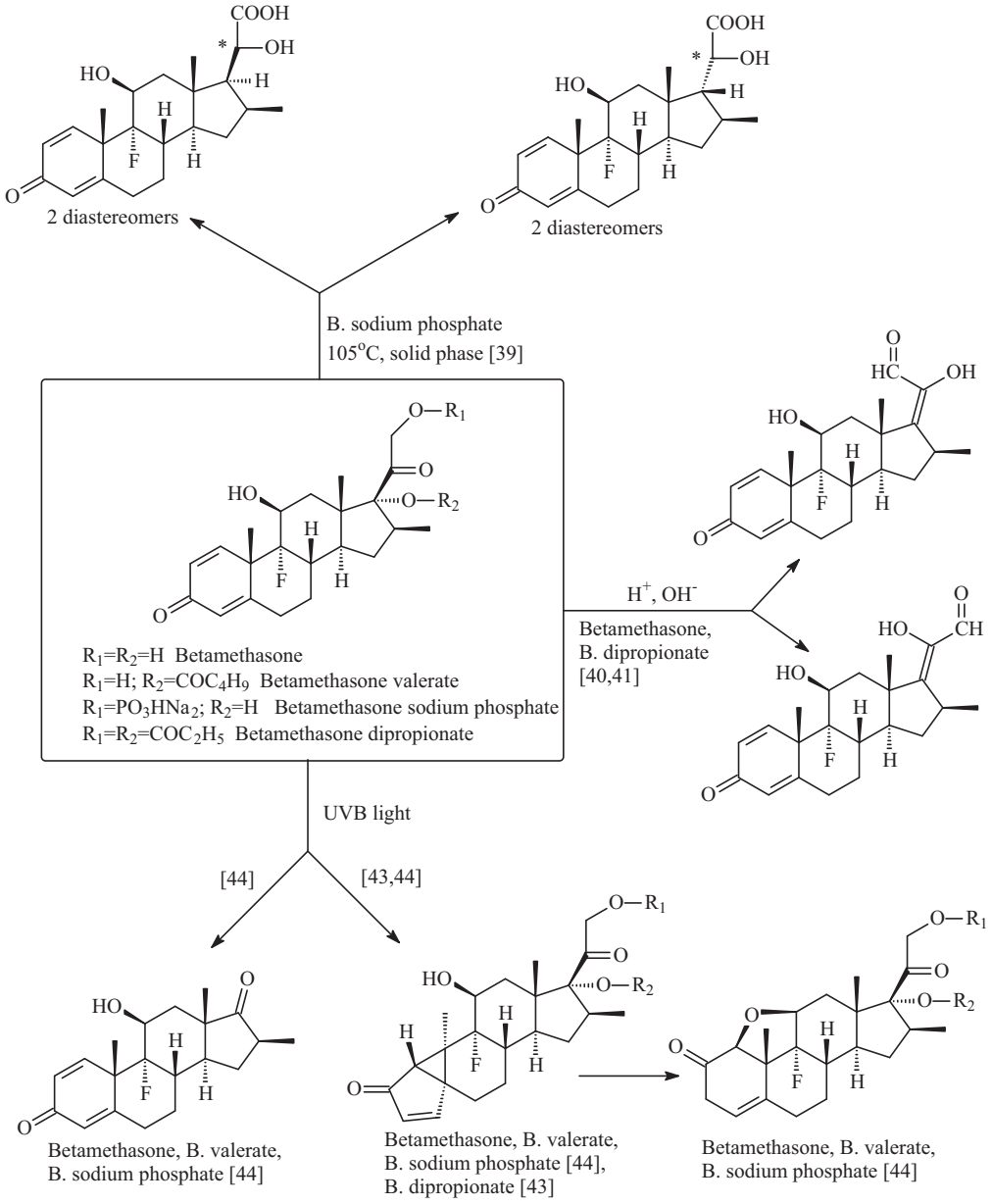

Fig. 1. Degradation pathways of betamethasone and its ester derivatives [38–42].

tisone acetate the photodegradation affects the 17-side chain and ring D [46].

Further studies, elucidating the structures of impurities in *budesonide* [47,48], *spironolactone* [49,50], *megestrol acetate* [51], *flurogestone acetate* [52], *dutasteride* [53] and degradation products in *mometasone furoate* [54] using methodological approach as outlined above are also worth mentioning.

Several studies deal with the impurities forming in the course of sterilization by irradiation with gamma or beta rays or by high-energy electron beams. The effect of the latter on various corticosteroids is described in [55].

2.4. Solid phase characterization

The characterization of pharmaceutical solids with physicochemical and analytical methods has become in recent years one of the most important tasks in pharmaceutical R&D and quality control. The reason for this is that by means of the proper selection and control of polymorphic forms (frequently occurring among steroids), amorphous form, solvates and particle size, the technology of solid dosage forms can be optimized to obtain good solubility, dissolution rate and thus optimal bioavailability. In addition, the discovery and characterization of new polymorphic form with improved technological properties can be a successful means for originators of new drug materials to extend the period of patent protection. On the other hand, generic manufacturers need the physicochemical and analytical methods to prove that the generic product is pharmaceutically equivalent and bioequivalent, and therefore, therapeutically equivalent to that of the originator. The armoury of analytical techniques includes spectroscopic methods, mainly infrared (IR), Raman and increasingly solid phase nuclear magnetic resonance (NMR) spectroscopy, X-ray powder diffraction (XRPD) and thermal methods, mainly DSC and thermomicroscopy.

An example for the complex application of various techniques is the determination of amorphous proportion of thermodynamically metastable *ciclesonide* which possesses a higher dissolution rate and apparent solubility than its crystalline counterpart. DSC was found more accurate and sensitive than FT-Raman spec-

troscopy [56]. *Beclomethasone dipropionate* clathrate formation with trichloromonofluoromethane for being used in a suspension metered dose inhaler was characterized by XRPD, X-ray photoelectron spectroscopy, scanning electron microscopy and DSC [57]. The desolvation of *cortisone acetate* hydrates and tetrahydrofuran solvate was followed and characterized by XRPD and DSC [58].

The use of magic-angle spinning ^{13}C NMR spectra provides a complementary tool to X-ray diffraction data in crystallographic space group assignment as demonstrated in the example of (among others) *cortisone acetate* [59], various modifications and solvates of *androsterone* and *beclomethasone dipropionate* [60] as well as *trans-dehydroandrosterone*, *hydrocortisone*, *prednisolone*, *prednisone* and *estradiol* [61]. The joint application of NMR, XRPD and DSC is described for the study of polymorphs and solvates of *finasteride* [62] and *diflorasone diacetate* [63].

In addition to its use for the characterization of pure polymorphs, solvates and their mixtures, the above listed methods were successfully applied in the characterization of steroids in drug formulations, e.g. solid-phase NMR in the case of *prednisolone* in controlled porosity osmotic pump pellets in the presence of excipients [64]. The polymorphic status of *progesterone* in a polyisoprene matrix for intravaginal drug delivery was measured by FT-IR and XRPD [65].

Particle size distribution also belongs to the solid phase characteristics of e.g. corticosteroids used in pressurized metered dose inhalers. The determination of this by laser diffraction analysis was part of a comparative evaluation study of different inhalers using *fluticasone*, *budesonide*, *beclomethasone* and *ciclesonide* as the model compounds [66].

3. Steroid hormone formulations

3.1. Assay of formulations and determination of impurities/degradants

3.1.1. HPLC methods

Unlike in the case of bulk steroid hormone drugs the importance of assay as a quality control attribute of their drug formulations is naturally unquestionable and the stability indicating nature of the assay method is obligatory. This is characteristic of the monographs of USP 33 [9]. (Ph. Eur. does not contain monographs for steroid hormone formulations and the Japanese Pharmacopoeia contains only a limited number.) The share of stability-indicating chromatographic assay methods in USP is over 80% (65% RP-, 11.5% NP-HPLC and 3% GC methods).

Several papers have been published dealing with the determination by HPLC of the active ingredients (and in the majority of cases also impurities and degradation products) of steroid hormone formulations. These stability-indicating methods are almost exclusively RP-HPLC methods with UV detection. The determination of *betamethasone* [67], *betamethasone dipropionate* [68], *budesonide* [69], *clobetasol propionate* [70], *deflazacort* [71], *dexamethasone* [71–74], *dexamethasone acetate* [75], *dexamethasone sodium phosphate* [72,76], *diflucortolone valerate* [77], *dutasteride* [19], *eplerenone* [18], *estradiol* [21,78,79], *ethinylestradiol* [80], *fluocinolone acetonide* [81], *finasteride* [82], *fluticasone propionate* [83], *gestodene* [80], *medroxyprogesterone acetate* [14], *meprednisone* [84], *mometasone furoate* [85], *testosterone propionate* and *cypionate* [86] and *triamcinolone acetonide* [87] in various solid, semi-solid and liquid formulations have been described. The on-line assay of *beclomethasone propionate* pressurized metered dose inhalers [88] and the determination of the solubility of *beclomethasone propionate* [89] and *budesonide* [90] in the same device are also worth mentioning.

When necessary, the RP-HPLC system was modified by additives. For example, ion-pairing reagent (octanesulfonic acid) was added to the eluent for the determination of the quaternary ammonium derivative *pancuronium bromide* [91] and hydroxypropyl-β-cyclodextrin for the separation of *norgestrel* enantiomers [92]. A special branch of HPLC, *micellar liquid chromatography* where micelle forming agents such as in this case cetyl trimethyl ammonium bromide are added to the eluent was applied to the determination of *betamethasone* and *dexamethasone* in tablets and in a cocktail containing both drugs [93].

The impact of the development in solid support technology on the HPLC analysis of steroid hormone drugs was already shortly discussed in Section 2.1. Further theoretical and practical aspects [94] and application to formulations are the comparison of porous, fused-core and monolithic C18 columns for the determination of *betamethasone valerate* and its degradants in an ointment [95] and *estradiol* in a gel formulation [96]. Monoliths were also used for the assay of *triamcinolone*, *prednisolone* and *dexamethasone* in tablets [97] as well *betamethasone* [98] in eye drops together with chloramphenicol.

UV detection is almost exclusively used in the HPLC systems for the analysis of steroid hormone formulations. The high sensitivity of the *fluorimetric detector* can be useful in the determination of low-dose formulations containing estrogens possessing native fluorescence such as *ethinylestradiol* tablets and *desogestrel + ethinylestradiol* tablets in USP 33 with excitation at 285 nm and emission at 310 nm [9].

Mass spectrometric detection is mainly used in the bioanalysis of steroid hormone derivatives. Some of the rare applications in pharmaceutical analysis are the simultaneous determination of *ethinylestradiol*, *gestodene*, *levonorgestrel*, *cyproterone acetate* and *desogestrel* in contraceptives and river water samples using electrospray and atmospheric pressure chemical ionization detectors (ESI-MS and APCI-MS) [99], the determination of *triamcinolone* in cosmetics using ESI-MS [100] as well as the determination of *fluticasone propionate* in nasal spray [101] and 19-norandrostenedione impurity in *norethisterone* tablets by HPLC-MS/MS [102].

The high sensitivity of electrochemical detectors provides higher sensitivity than UV detection for spectrophotometrically poorly active steroids such as the quaternary ammonium neuromuscular blocking agents. Amperometric detection was used for the determination of *rocuronium bromide* and eight impurities in a solution formulation [103], while *pipecuronium bromide* and four impurities were determined using a coulometric array detector [104].

3.1.2. Other chromatographic methods

Although the main application field of thin-layer chromatography in the analysis of steroid hormone drugs is the purity check of bulk materials, *TLC-densitometry* enables the use of this technique for the assay of formulations as well but this method cannot be regarded to be a real alternative to HPLC as shown by the low number of publications, e.g. the stability-indicating assay of *dutasteride* tablets [105]. It is amazing that the old-fashioned version of the TLC-based assay, i.e. spot elution followed by UV spectrophotometric or tetrazolium blue colorimetric measurement ("Single steroid assay") is still official for a few formulations in the United States Pharmacopoeia [9].

Gas chromatography (GC) played an important role in both biomedical and pharmaceutical steroid analysis before the introduction and widespread use of HPLC. This technique has retained some of its importance in the biomedical field (especially in the control of doping with anabolic steroids) but has almost completely disappeared from pharmaceutical product analysis. This can be characterized by figures from USP 33 [9]: in contrast to 125 monographs for steroid hormone formulations where HPLC

Table 2
Determination of steroid hormone drugs in environmental samples.

Steroid	Sample	Sample pre-treatment	Method	LOD/LOQ	Ref.
E1, E2, E3, EE2, E1-sulfate	Sewage	SPE/tC18 + PLgel column	HPLC–(ESI-MS/MS)	*LOD* E1 and sulfate: 0.1; E2, E3, EE2: 0.2 ng/l	[147]
E1, E2, E3, EE2	Natural water	SPE/poly(divinylbenzene-*co*-methacrylic acid) coated Fe_3O_4 magnetic microspheres	HPLC–(ESI-MS/MS)	*LOD* < 10 ng/l	[148]
E1, E2, E3, EE2 and conjugates	Sediment	Microwave-assisted extraction, SPE/Oasis WAX	HPLC–(ion trap-MS/MS)	*LOD* 1 ng/g	[149]
E1, E2, E3, EE2 and conjugates	Natural water, sewage	SPE/Oasis HLB + Florisil	HPLC–(ESI-MS) Bioassay/MELN test	*LOQ* 0.4–1.2 ng/l	[150,151]
E1, E2, E3, EE2 and conjugates	Natural water	SPE/PLPR cartridge	HPLC–(ESI-MS/MS)	*LOQ* E1 sulfate: 0.02; others: 0.2–1.0 ng/l	[152]
E1, E2, E3, EE2 and conjugates	Sewage sludge	Pressurized liquid extraction	HPLC–(ESI-MS/MS)	*LOQ* 0.25–375 ng/g	[153]
E1, E2, EE2	River sediment	Microwave-assisted extraction, SPE/Strata X-AW + silica cartridges	HPLC–(TOF-MS) or HPLC–(ESI-MS/MS)	*LOD* E1: 15, E2: 30, EE2: 40 ng/l	[154]
E2	Natural water	SPE/molecularly imprinted polymer	HPLC–MS, HPLC–UV	*LOQ* 5.4 ng/l	[155]
E1, E2, E3, EE2	Sea water	SPE/Oasis HLB + silica cartridges	HPLC–(ESI-MS/MS)	*LOD* E1: 0.02; E2: 0.3; E3: 1.0; EE2: 0.45 ng/l	[156]
E1, E2, E3, EE2	Natural water, sewage	Solid-phase microextraction (SPME)	HPLC–(ESI-MS/MS)	*LOD* E1: 2.7; E2: 7.4; E3: 11.7; EE2: 10.5 ng/l	[157]
E1, E2, E3, EE2	Natural water	SPE/C18 + Florisil + NH_2-SPE cartridges	HPLC–(ESI-MS)	*LOD* E1, E2, EE2: 0.1; E3: 0.2 ng/l	[158]
E1, E2, E3 and conjugates	Natural water	SPE/Oasis HLB cartridge	Column switching HILIC-RP-HPLC–(ion trap-MS/MS)	*LOD* E1, E2, E3: 0.038; conjugates: up to 6.9 ng/l	[159]
E1 and sulfate, E2, E3, EE2	Sewage sludge	Multi Reax solvent extraction, SPE/silica + GPC + ion exchange cartridges	HPLC–(ESI-MS/MS)	*LOD* E1: 2.1; E2: 4.9; E3: 4.5; EE2: 5.3, E1 sulfate: 2.6 ng/g.	[160]
E1, E2, E3, EE2	Natural water	SPE/Oasis HLB + Florisil + derivatization with 12-(difluoro-1,3,5-triazinyl)-benz[f]isoindolo[1,2b][1,3]benzothiazolidine	HPLC–(ion trap-MS/MS)	*LOQ* E1: 0.23; E2: 0.26; E3: 0.95; EE2: 0.22 ng/l	[161]
E1, E2, E3, EE2	Sewage sludge, sediment	SPE/GPC SX-3 + C18 cartridges	HPLC–(ESI-MS), GC–MS	–	[162]
E1, E2, E3, EE2	Natural water, sewage	SPE/Oasis HLB cartridge	HPLC–(ESI-MS/MS)	*LOD* E1: 0.1; E2: 0.2; E3: 0.3; EE2: 0.4 ng/l	[163]
E1, E2, E3, EE2	Natural water, sewage	SPE/Oasis HLB cartridge	HPLC–(dual-polarity-ESI-ion trap-MS/MS)	*LOQ* E1, E2, E3: 4.0; EE2: 2.0 ng/l	[164]
E1, E2, EE2	Natural water, sewage	SPE/Oasis HLB cartridge + derivatization with dansyl chloride	HPLC–(ESI-MS/MS)	*LOD* E1, E2: 0.4; EE2: 0.7 ng/l	[165]
E1,E2,EE2	Natural water, sewage	SPE/C18 cartridge	HPLC–(ESI-MS/MS)	*LOQ* 0.1–0.4 ng/l	[166]
E1, E2, EE2	Natural water, sewage	SPE/Lichrolut EN	HPLC–(ESI-MS/MS)	*LOQ* E1: 1.5; E2: 5.2; EE2: 4.6 ng/l	[167]
E1, E2, E3, EE2	Natural water, sewage	SPE/C18 + Sep-Pak aminopropyl cartridges	UPLC–(Q-TOF-MS/MS) ELISA	~2.5 ng/l	[168]
E1, E2, E3, EE2, T, epiT, AD, DHT, Et, AN, epiAN, P, Dros, Lev, Nor, Med, Meg, etc.	Natural water	SPE/Strata C18 cartridge	HPLC–(ESI-MS/MS)	*LOD* E1: 0.01; E2: 0.01; E3: 0.03; EE2: 0.20; T: 0.01; DHT: 1.94; P: 0.01 ng/l, etc.	[169,170]
E1, E2, E2 benzoate, E3, EE2, P, Lev, Med, Nor, Nor acetate, Cy, Meg, Hy-C, Ch	Natural water, sewage	Solid phase disk extraction/C18	UPLC–(ESI-MS/MS)	0.5–3.4 ng/l	[171]
E1, E2, AD, AN, epiAN, Hy-C, Med, Meg, P, T, Cor, Dex, Hyd, Pr, Prd	Natural water	SPE/Oasis HLB + silica or Florisil cartridges	HPLC–(ESI-MS/MS)	*LOD* 0.008–0.5 ng/l	[172]

Table 2 (*Continued*)

Steroid	Sample	Sample pre-treatment	Method	LOD/LOQ	Ref.
Cor, Hyd, B, Pr, Prd, Bu, Dex, Flun, Fluo, Tr + acetonide	Sewage	SPE/Oasis MCX cation exchanger cartridge	HPLC–(ESI-MS/MS)	e.g. Fluo: 0.3 ng/l determined	[173]
Hyd, Pre, Tr + acetonide, Dex, Flum, Bol, MT, P, Nor	Natural water	SPE/Oasis HLB cartridge	HPLC–(APCI-MS)	*LOD* 0.01–021 ng/l	[174]
E1	Natural water	SPE/molecularly imprinted polymer	HPLC–UV	*LOD* 5.7 ng/l	[175]
E1,E2,EE2	Natural water	Electrospun Nylon6 nanofibrous membrane	HPLC–UV	*LOD* E1: 0.17; E2: 0.05; EE2: 0.08 ng/l	[176]
E1, E2, E3	Natural water	SPE/C18 cartridge	HPLC–UV	*LOD* 0.98–78 ng/l	[177]
E1, E2, E3, EE2	Natural water	SPE/Polymeric Strata-X cartridge	HPLC–UV	*LOD* 41–160 ng/l	[178]
E1, E2	Natural water	Dispersive liquid–liquid microextraction/methanol, tetrachloroethane	HPLC–UV	*LOD* E1: 0.2; E2: 0.1 ng/l	[179]
E1, E2, EE2, Mes, P, Lev, Nor	Natural water	Stir bar/polydimethysiloxane	HPLC–UV	*LOQ* E1: 75; E2: 150; EE2: 300; P: 150; Nor: 150 μg/l	[180]
Med acetate	Natural water	Dispersive liquid–liquid microextraction	HPLC–UV	*LOD* 200 ng/l	[181]
Pr, Dex	Natural water	SPE/Oasis HLB cartridge	UHPLC–UV	*LOQ* Pr: 240; Dex: 260 ng/l	[182]
E2, E3, EE2	Natural water, sewage	SPME/poly(acrylamide-vinylpyridine-*N*,*N*′-methylenebisacrylamide) monolith	HPLC-fluorescence detector	*LOQ* E2: 490; E3: 90; EE2: 2000 ng/l	[183]
E1, E2, EE2	Natural water, sewage	SPE/SDS hemimicelle coated alumina	HPLC-fluorescence detector	*LOD* 20–10 ng/l	[184]
E1, E2, E3, EE2, Mes	Natural water, sewage	SPE/Oasis HLB cartridge + silylation/MSTFA	GC–MS, GC–MS/MS	*LOQ* E1: 1; E2: 2; E3: 3; EE2: 3; Mes: 3 ng/l	[185]
E1, E2, EE2	Natural water, sewage	SPE/Oasis HLB cartridge + silylation/BSTFA–pyridine	GC–MS	*LOD* E1, E2: 0.005; EE2: 0.05 ng/l (natural water). E1: 1; E2: 0.5; EE2: 2.5 ng/l (sewage)	[186]
E1, E2	Sewage	SPE/Oasis MAX + penatafluoropropionylation	GC–MS	*LOD* 1 ng/l	[187]
E1, 16α-OH-E1, E2, EE2	Sediment	Microwave-assisted extraction + SPE/sodium sulfate + silica cartridge + silylation/BSTFA–TMCS–pyridine	GC–MS	*LOQ* El: 0.9; 16α-OH-E1: 0.5; E2: 0.9; EE2: 1.4 ng/g	[188]
E1, 16α-OH-E1, E2, EE2	Natural water, seawater	SPE/Oasis HLB cartridge + silylation/BSTFA–TMCS	GC–MS	*LOQ* El: 5.6; 16α-OH-E1: 1.0; E2: 11.2; EE2: 2.6 ng/l	[189,190]
E1, E2, EE2	Natural water, sewage	SPE/C18	GC–MS, large volume injection, programmable temperature vaporizer	*LOD* E1: 0.041; E2: 0.046; EE2: 0.031 ng/l	[191]
E2	Natural water	Stir bar/polydimethysiloxane + acetylation + in-tube silylation/BSTFA	Thermal desorption GC–MS	*LOQ* 0.002 ng/l	[192]
E1, E2, E3, EE2	Activated sludge	Ultrasonic liquid extraction + SPE/Oasis HLB + (Al_2O_3)/silica cartridges + silylation/BSTFA–TMCS–pyridine	GC–MS	*LOQ* E1: 1.2; E2: 0.8; E3: 2.3; EE2: 4.0 ng/l	[193]
E1, E2, EE2, Mes	Natural water, sewage	SPME/polymer-coated fibers + on-fiber silylation/MSTFA	GC–MS/MS	*LOQ* E1: 1.0; E2: 0.7; EE2: 3.0; Mes: 3.0	[194]
E1, E2, EE2	Natural water	Solid phase disk extraction/C18 + silica + aminopropyl silica cartridges	GC–MS/MS	*LOQ* 0.25 ng/l	[195]

E1, E2, EE2	Sediment	Extraction/hexane–acetone + SPE/Oasis HLB	GC–GC–TOF-MS	*LOD* E1: 2.5; E2: 2.5; EE2: 0.4 ng/g	[196]
E1, EE2	Natural water	Silylation/BSTFA–TMCS–pyridine	GC–MS	*LOD* 0.05 ng/l	[197]
E1,E2,EE2	Natural water, sewage	SPME/polypropylene hollow fiber coated with di-OH-polymethylmethacrylate + silylation/MSTFA	GC–MS	*LOQ* E1: 2.3; E2: 2.7; EE2: 0.3 ng/l	[198]
E1, E2, EE2	Natural water, sewage	SPME/polypropylene hollow fiber membrane coated with dihexylether–trioctylphosphine oxide	GC–MS	*LOQ* E1: 12; E2: 5.5; EE2: 30 ng/l	[199]
E1, E2, T, DHEA, Preg	Natural water	SPME/polyacrylate fiber + on-fiber silylation/BSTFA	GC–MS	*LOQ* E1: 57; E2: 22; T: 393; DHEA: 170; Preg: 211 ng/l	[200,201]
E1, E2, E3, EE2, Mes, P, Lev, Nor	Natural water, sewage	Oasis HLB + Env+ cartridges	GC–MS	*LOD* estrogens: 0.3–8.0; progestogens: 1.0–8.0 ng/l	[202]
E1, E2, E3, EE2, P, T	Sewage	On-line continuous liquid–liquid extraction/CH_2Cl_2	GC–MS	*LOD* E1: 15; E2: 40; E3: 250; EE2: 25; P: 25; T: 15 ng/l	[203]
E1, E2, E3, EE2, P, Lev, Nor, Med	Sewage	SPE/Strata X polymeric cartridge	Laser diode thermal desorption APCI-MS	*LOD* 13–43 ng/l	[204]
EE2	Sewage	SPE/C18 cartridge	MEKC/amperometric detector	*LOD* 3.9 μg/l	[205]
E2	Natural water	SPE/molecularly imprinted polymer	Flow injection fluorimetry	*LOD* 1.12 μg/l	[206]
EE2	Natural water	SPE/molecularly imprinted polymer	HPLC–UV	*LOD* 27 μg/l	[207]
E2	Natural water, sewage	On-line SPE/C18 cartridge	Chemiluminescence immunoassay	*LOD* 1.5 ng/l	[208]
E1, E2, E3	Natural water	SPE/multiwall carbon nanotube	ELISA	*LOD* E1:40 (70); E2:40; E3: 200 ng/l	[209]
E2	Natural water	–	Electrochemical ELISA	*LOD* 21 ng/l	[210]
Lev	Sewage	SPE/C18 cartridge	ELISA	*LOD* 70 ng/l	[211]
Total estrogens	Natural water	–	Estrogen receptor fluorescence-based binding inhibition assay	*LOD* 0.139 nM (E2 equivalents)	[212]
Total estrogens	Natural water	–	Surface plasmon resonance assay based on estrogen receptor dimerization	*LOD* 5 nM (E2 equivalents)	[213]

Abbreviations: SPE: solid phase extraction; BSTFA: *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; TMCS: trimethylchlorosilane; MSTFA: *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; ELISA: enzyme linked immunosorbent assay. Steroids: E1: estrone; E2: 17β-estradiol; E3: estriol; EE2: ethinylestradiol; AD: androstenedione; AN: androsterone; epiAN: epiandrosterone; B: betamethasone; Bol: boldenone; Bu: budesonide; Ch: chlormadinone acetate; Cor: cortisone; Cy: cyproterone acetate; Dex: dexamethasone; DHEA: dehydroepiandrosterone acetate; DHT: dihydrotestosterone; Dros: drospirenone; Et: Etiocholanolone; Flum: flumethasone; Flun: flunisolide; Fluo: fluoninolone acetonide; Ges: gestodene; Hy-C: hydroxyprogesterone caproate; Hyd: hydrocortisone; Lev: levonorgestrel; Med: medroxyprogesterone; Meg: megestrol; Mes: mestranol; MT: methyltestosterone; Nor: norethisterone; P: progesterone; Pr: prednisolone; Prd: prednisone; Preg: pregnenolone; T: testosterone; epiT: epitestosterone; Tr: triamcinolone.

methods are prescribed for their assay, GC is used for 5 classical formulations only, such as for conjugated and esterified estrogens. One of the main drawbacks of GC in the analysis of steroids is that it usually requires derivatization to improve their volatility and thermal stability. This was the case in one of the rare new applications of GC where the determination of *finasteride* in tablets is described after trimethylsilylation with *N,O*-bis(triethylsilyl)trifluoroacetamide–trimethylchlorosilane reagent [106]. Optimization of the trimethylsilylation by chemometric methods for anabolic agents merits mentioning [107]. However, derivatization is not necessary in all cases: a gas chromatographic alternative without derivatization of the pharmacopoeial HPLC separation of the epimers [8,9] of *budesonide* is also described [108]. Some further applications of GC to environmental samples are mentioned in Section 5 and Table 2.

3.1.3. Electrodriven methods

The overwhelming majority of steroid hormone and hormone analogue drugs are neutral, uncharged molecules and for this reason *capillary electrophoresis* does not play an important role in their analysis. In contrast to this, CE-based, electrodriven chromatographic techniques such as *micellar electrokinetic chromatography* (*MEKC*) and *microemulsion electrokinetic chromatography* (*MEEKC*) have attracted interest among analysts dealing with steroid hormone drugs from the beginnings of these techniques. As some recent publications show, this tendency continues up to the present time.

Examples for the use of *MEKC* are a stability-indicating method for the determination of *fluticasone propionate* in a nasal spray [109] and the determination of *dexamethasone* in cosmetics [110]. In both methods, sodium dodecyl sulfate (SDS) was the micelle forming agent, while sodium cholate and sodium deoxycholate were used in the determination of *cortisone*, *prednisolone*, *methylprednisolone*, *prednisone*, *betamethasone* and *triamcinolone* in various pharmaceutical formulations [111]. Experimental design was used for the optimization of the separation of *budesonide* epimers and its four impurities in a MEKC method where a mixed micelle forming agent sodium cholate + 3-(*N,N*-dimethylmyristylammonio)propanesulfonate was used [112].

The *MEEKC* method was adopted for the determination of among others *beclomethasone dipropionate* in poly(lactic acid) nanoparticles using for the separation 1-hexanol–1-octanol–SDS microemulsion [113]. In the course of a systematic optimization study a heptane–1-butanol–Brij 76–sodium taurodeoxycholate microemulsion in a phosphate buffer containing β-cyclodextrin was found suitable for the separation of the components of the mixture of *cortisone*, *hydrocortisone*, *prednisolone* and their acetates and this method was used for the purity check of these drugs [114]. In other studies for the separation of corticosteroids the microemulsion contained 1-butanol, SDS and diethyl L tartrate [115] and for estrogens octane–1-butanol–sodium bis(2-ethylhexyl)sulfosuccinate–sodium 3-cyclohexylamino-2-hydroxy-1-propane sulfonate [116].

In almost all papers dealing with the application of MEKC and MEEKC to pharmaceutical (among others steroid hormone analytical) problems, the equivalence of the performance of these methods with that of HPLC is emphasized. In spite of this, no breakthrough is observable: these methods do not yet seem to be real competitors of HPLC in official, routine analysis.

3.1.4. Other methods

Non-selective *UV spectrophotometric or colorimetric* methods are still in use in USP for the assay of 18% of steroid hormone formulations [9]. The relatively most frequently used reagents are isoniazide for some 4-ene-3-oxosteroids, blue tetrazolium or phenylhydrazine-sulfuric acid for the dihydroxyacetone side chain of corticosteroids.

Due to the non-selectivity of assay methods based on natural absorption, new methods based on this are not stability-indicating and are for this reason of very limited importance even if chemometric methods support the simultaneous determination of the ingredients of two-component formulations such as a table containing *finasteride* and tamsulosin [117]. An interesting application of this approach was the estimation of the kinetics of *in vitro* solvolysis of the prodrug *fluocinolone acetonide 21-(2-phenoxypropionate)* [118]. Flow injection analysis (FIA) based on natural absorption greatly improves the speed of the analysis but naturally not its selectivity as shown for *deflazacort* tablets [119]. Spectrophotometrically poorly active steroids such as *finasteride* in tablets can be determined using colour reactions (in this case the classical extractive colorimetric reaction with bromophenol blue or related dyes) [120]. Of course the selectivity and stability indicating nature of these methods are not any better than those based on natural absorption.

The limitations discussed above in connection with the UV spectrophotometric-colorimetric assay of steroid hormone formulations apply to the *fluorimetric* methods as well, such as e.g. the assay of tablets containing *dexamethasone*, dexchlorpheniramine and fluphenazine based on native fluorescence and ratio of the second derivative of the emission spectra [121] or the assay of *prednisolone* and *triamcinolone acetonide* in tablets, ointment and suspensions based on photochemically induced fluorescence [122].

The still official *IR spectroscopic* assay in USP 33 (for *paramethasone acetate* tablets) [9] can be regarded as a curiosity only. At the same time, *FT-Raman spectroscopy* in conjunction with chemometric methods offers the advantage of being suitable to analyse dosage forms without extraction and sample preparation e.g. for the assay of *prednisolone* tablets [123] and *medroxyprogesterone acetate* suspension [124]. Raman imaging microspectroscopy was found to be a useful tool not only for the identification but also for particle size and particle size distribution measurement of a *beclomethasone dipropionate* aqueous suspension nasal spray formulation [125].

Electroanalytical methods have never played an important role in steroid hormone analysis. These are non-selective and non stability-indicating methods. For this reason and since mercury is considered to be a health hazard, this importance has recently further decreased. In spite of this, several papers have been published up to the present time based on dropping [126] or hanging [127] mercury electrodes. Two of these are mentioned here: the voltammetric determination of *finasteride* in tablets [126] and the assay of *danazol* capsules by square-wave adsorptive stripping voltammetry [127]. A more up-to-date approach is the development of drug-selective electrodes, e.g. a modified carbon paste-based electrode for the selective potentiometric determination of *estradiol valerate* in formulations [128].

3.2. Steroid hormones in counterfeit products

The struggle against pharmaceutical counterfeiting by analytical means is becoming an important task in pharmaceutical analysis. For reviews including steroid analytical aspects see [129,130].

A HPLC screening system for tablets containing various steroid hormone drugs was based on $\log k$ values of the active ingredients at different water-methanol ratios in the eluent and the assay of the tablets [131]. Another HPLC screening method with DAD-UV and ESI-MS detection was developed for the detection of not allowed steroids (*estrone*, *progesterone*, *spironolactone*, *canrenone*, *hydrocortisone* and *triamcinolone acetonide*) in cosmetic products [132]. Illegal *betamethasone valerate* [133] and *clobetasol propionate* [134] were identified in various cosmetic creams by the combined application of TLC and HPLC.

4. Steroid analysis in drug research

Of the analytical fields discussed in the previous part of this review impurity profiling (see Section 2.3) plays an important role in the development and characterization of new drug materials. There are, however, other fields as well where analytical measurements contribute to drug R&D. One of these fields is the characterization of the lipophilicity of the (potential) drugs by determining their *1-octanol-water partition coefficient*. The log *P* value is an important parameter for the prediction of the bioavailability of drug candidates. Although the fundamentals of the determination of log *P* on the basis of RP-HPLC retention factors as an advantageous alternative of the classical shake flask method have been laid down earlier [135 and its author's previous publications], there are many publications up to the present time describing the refinement of this technique and its application to new groups of drug compounds, among them steroids. The determination of log *P* of steroid hormone drugs is based on its linear relationship with the CHI (Chromatography Hydrophobic Index, the percentage of the organic modifier required to obtain equal distribution of the analyte between the mobile and stationary phases) in isocratic and gradient HPLC using acetonitrile [135] or methanol [136] as the organic modifiers. The correlation for (among others) steroids could be further improved by adding 1-octanol to the mobile phase using Amide-C16 RP-HPLC [137] or C18 [138] stationary phases. The comparison of the capabilities of RP-HPLC and RP-TLC was demonstrated on the example of cortisone derivatives [139]. A theoretical study where the interaction between steroids and an RP-HPLC stationary phase modified with cholesteryl-undecanoate was investigated is worth mentioning. The hydrophobic binding of the steroids was characterized by NMR imaging [140].

Solubility is another important characteristic of drug candidates influencing their bioavailability. A high-throughput ^{1}H NMR method was proposed for the determination of the solubility of potential drugs, among them steroid hormones as an alternative to the generally used HPLC-UV method [141].

The interaction of drugs with various *cyclodextrins* and derivatives is of great importance especially in pharmaceutical technology. In addition to the standard methods to quantitatively characterize this interaction of various steroid hormone drugs with cyclodextrins, the change of their electrophoretic mobilities was successfully used [142].

5. Determination of steroid hormone drugs in environmental samples

Natural and (semi)synthetic steroid hormones and hormone analogue drugs, first of all estrogens are endocrine disruptors. For this reason and due to other toxic effects their presence in the environment is a health hazard. This is why the determination of steroid hormone drugs, their metabolites and transformation products in the aquatic environment (mainly sewage, rivers, lakes, sea) and water-related samples (sludge, sediment) is one of the most important tasks in modern steroid hormone analysis. In Table 2 some examples are presented representing the state of the art of the analytical methodology for the determination of steroid hormone drugs in the above listed samples. Of the several reviews published on the determination of drugs in the environment three papers published after 2004 concentrating on steroids are mentioned [143–145]. The analytical aspects of the transformation of steroid hormones in sewage by microbiological, oxidative and photochemical treatment are also a challenging field but this is outside the scope of this paper; for a review see [146]. It is to be mentioned that in many studies summarized in Table 2 the determination of non-steroidal (synthetic) estrogens such as diethylstilbestrol, other endocrine disruptors, mainly degradation products of nonionic surfactants, octylphenol and nonylphenol, as well as other, widely used drugs were also included. These are not listed in Table 2.

As it is seen in Table 2, the two main requirements, i.e. high selectivity and high sensitivity is achieved in the overwhelming majority of cases by the combined use of HPLC or (to a lesser extent) GC with MS(MS). The usual way of sample pre-treatment – sample enrichment is extraction by an SPE cartridge which is often followed by fractionation using another column/cartridge. For the GC analysis usually a derivatization step is also necessary. It is also seen in Table 2 that the achievable sensitivity can be characterized by LOQs in the ranges of 0.1 or 1 ng/l or sometimes even lower. The selectivity of the less generally used immunoanalytical methods is excellent while their LOQ is inferior to the MS-related methods but these do not necessarily require a solid phase extraction step. The limited selectivity can be an advantage if the aim of the analysis is the determination of the total estrogenicity of the water sample rather than the determination of individual steroidal and non-steroidal compounds binding to fluorescence labelled estrogen receptor α [212].

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