Recent Advances in the Analysis of Steroid Hormones and Related Drugs

Sándor GÖRÖG

Gedeon Richter Ltd., P.O.B. 27, H-1475 Budapest, Hungary

The development during the last 15 years and the state-of-the-art in the analysis of bulk steroid hormone drugs and hormone-like structures and pharmaceutical formulations made thereof are summarized. Other steroids (sterols, bile acids, cardiac glycosides, vitamins D) as well as biological-clinical aspects and pharmacokinetic and metabolic studies are excluded from this review. The state-of-the-art is summarized based on comparisons of monographs in the latest editions of the European Pharmacopoeia, United States Pharmacopoeia and the Japanese Pharmacopoeia. This is followed by sections dealing with new developments in the methodology for the fields of spectroscopic and spectrophotometric, chromatographic, electrophoretic and hyphenated techniques as well electroanalytical methods. The review is terminated by two problem-oriented sections: examples on impurity and degradation profiling as well as enantiomeric analysis.

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1 Introduction

The aim of this paper is to give an overview of the advances during the last 15 years and of the state-of-the-art in the analysis of steroid hormone drugs and related materials. Why just 15 years? My first book on steroid hormone drug analysis was published in 1978² when this area was in transition state due to the introduction and rapid spreading of new techniques, mainly high-performance liquid chromatography (HPLC) and immunoassay methods. This was followed by another book in 1983³ dealing with wider areas (in addition to the analysis of hormone drugs other types of steroids and biological-clinical aspects). The third book, published in 1989⁴ (just 15 years ago), dealt mainly with pharmaceutical and industrial aspects. The

development in steroid hormone drug analysis during the last 15 years can be characterized by the facts that HPLC undoubtedly became the most important routinely used method and that the importance of hyphenated chromatographic-spectroscopic techniques is increasing. New techniques, mainly capillary electrophoresis (CE) and related techniques, such as micellar (or microemulsion) electrokinetic chromatography (MEKC, MEEKC), capillary electrochromatography (CEC), also attract wide interest in steroid analysis. Summarization of the results achieved with these methods will be the subject of this review.

Only hormone drugs, their semi-synthetic analogues and hormone-like structures are considered. Other groups of steroids (sterols, bile acids, cardiac glycosides, vitamins D, etc.) are not within the scope of this review. Pharmaceutical-industrial aspects are discussed, such as assay and impurity profiling of bulk drugs and drug formulations, estimating degradation profiles. Biological-clinical aspects, pharmacokinetic and metabolic studies are also not within the scope of this review. These aspects are dealt with in a book of

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E-mail: s.gorog@richter.hu

Makin *et al.*⁵ Spectroscopic studies are not discussed either, unless these are part of impurity profiling and degradation profiling studies.

2 Steroid Hormone Drugs in Pharmacopoeias

Pharmacopoeias are naturally rather conservative: new techniques appear in their monographs only if proved by many years of practice that the performance of these methods is superior to that of the currently used methods. In spite of this limitation, the state-of-the-art of methodology and requirements in official drug analysis is well reflected by monographs concerning the latest revisions of the principal pharmacopoeias.

2.1 Assay of bulk drug materials

Table 1 shows a brief summarization of the assay and related impurities tests in the monographs of bulk steroid hormones and hormone-like structures in European Pharmacopoeia 4th ed. (Ph. Eur. IV),⁶ United States Pharmacopoeia 26th ed. (USP XXVI)⁷ and the 14th edition of the Japanese Pharmacopoeia (Ph. Jp. XIV).⁸

As can be seen in Table 1, the most frequently used assay method in Ph. Eur. IV. is UV spectrophotometry at about 240 nm for steroids with strongly absorbing 4-ene-3-oxo- or 1,4-diene-3-oxo group and at about 280 for estrogens with a phenol-type ring A with its weak, but characteristic, spectra. This method is naturally non-specific: the overwhelming majority of the impurities are measured together with the main component. The predominant method in the United States Pharmacopoeia is highly specific reversed phase (in some cases normal phase) HPLC. In the overwhelming majority of cases, porous octadecylsilica support (3–10 μ m) is used for the RP and porous silica support (5–10 μ m) for the NP separations. The wavelength of the UV detector is set most often at 254 nm, which is not the maximum of the spectrum of 4-ene-3-oxo- or 1,4-diene-3-oxosteroids. This is attributable to the early period in the history of HPLC when the majority of instruments were equipped with only a mercury lamp. In the Japanese Pharmacopoeia the HPLC and UV spectrophotometric assay methods are almost equally represented. It is to be noted that UV spectrophotometry around 240 nm with limits for the specific absorbance is often part of the monographs as an identification test, even in those cases when the assay is carried out by HPLC methods.

It is interesting to note, and difficult to explain that in some instances visible spectrophotometric methods, such as a measurement around 380 nm after condensation of the unsaturated 3-oxo group with isoniazide, or an indirect measurement at 525 nm after a reaction with Tetrazolium Blue of corticosteroids with reducing side chain, is prescribed by the European and United States Pharmacopoeias; moreover, in one instance (mestranol)⁷ the assay is based on a non-stoichiometric color reaction with a methanol-sulfuric acid reagent (545 nm). The selectivity of these, rather outdated methods^{2,4,9,10} is not better than that of the spectrophotometric methods based on native absorbance measurements.

Although the overwhelming majority of steroid hormone drugs do not possess functional groups suitable for titration, in some cases this method is still used. Ethinylsteroids can be titrated with sodium hydroxide after a reaction with silver nitrate and the liberation of nitric acid. This method is used as a (non specific) assay method by Ph. Eur. and Ph. Jp. and as a "Limit of ethinyl group" by the United States Pharmacopoeia. Other titrimetric methods are restricted to the titration of

pancuronium bromide^{6,8} and stanozolol^{6,7} with acetic perchloric acid. The lactone group in oxandrolone,⁷ after opening it with sodium hydroxide and the $-O-SO_2-ONa$ group in sodium prasterone sulfate⁸ after $Na^+ \rightarrow H^+$ ion exchange, are also suitable for titrimetric determination.

Of the chromatographic methods other than HPLC, gas chromatography is only very seldom used for the assay of bulk steroid hormone drugs. The outdated and labor-consuming method of TLC separation and UV spectrophotometry after spot elution is prescribed for the assay of meprednisone, nandrolone phenylpropionate, prednisolone hemisuccinate and testosterone.⁷

2.2 Related impurities test of bulk drug materials

The test for "Related substances",⁶ also named "Chromatographic purity",⁷ "Ordinary impurities",⁷ "Related steroids"⁷ or "Other steroids",⁸ is the most important test to characterize the quality of a bulk drug material, much more important than the assay, especially if the latter is carried out by non-specific methods.

As can be seen in Table 1, with two exceptions (conjugated and esterified estrogens, where gas chromatography is used), this test is carried out in all cases by semi-quantitative TLC or quantitative (mainly but not exclusively reversed phase) HPLC methods. The proportion of HPLC tests for related impurities in bulk steroids in various pharmacopoeias is quite different: about 75% in Ph. Eur. IV, 50% in USP XXVI and 10% in Ph. Jp. XIV. It is amazing that in many cases the USP XXVI and Ph. Jp. XIV do not contain tests for related impurities (27 and 10%, respectively).

In the case of HPLC tests, a UV detector is exclusively used at the wavelength of the maximum of the main component or at 254 nm. For the thin-layer chromatographic purity test in the majority of cases, sorbent layers are used that are impregnated with dyes strongly fluorescing when irradiated at 254 nm by a mercury lamp. Since most of the steroid hormone drugs strongly absorb UV light at this wavelength, the drugs and their impurities appear as dark spots in the chromatogram suitable for their semi-quantitative estimation. These appear as "TLC 254 nm" in Table 1. In some cases the use of various visualizing reagents is necessary. Reagents containing sulfuric acid are most frequently used. "TLC- H_2SO_4 " in Table 1 means spraying with this reagent, heating and visual inspection of the plate and the use of long-wavelength UV light (366 nm). Other visualizing reagents are phosphomolybdic acid (TLC-phosph.mol.), *p*-toluenesulfonic acid, vanillin/sulfuric acid (TLC-vanillin), potassium dichromate/sulfuric acid (TLC-acid dichromate), iodine vapor (TLC- I_2) and alkaline Blue Tetrazolium (TLC-tetrazolium). With a few exceptions, both the TLC and HPLC tests express the impurities as the main component, which can be source of serious errors (under- or overestimation) if the chromophoric system or the color and/or the intensity of the TLC spot of the main component and the impurity are different.

The use of other methods, such as the extraction of free corticosteroids from the solution of their water soluble esters, or elution of TLC spots followed by UV spectrophotometric measurement or gas chromatography, is restricted to a few cases.

2.3 Assay of steroid hormone formulations

In this section the state-of-the-art in the field of determining the active ingredient content of steroid hormone formulations is based mainly on USP XXVI, since the European Pharmacopoeia does not contain monographs for formulations

Table 1 Assay and related impurities tests in European, USP and Japanese pharmacopoeias

	Assay			Related impurity		
	Ph. Eur. 4	USP XXVI	Ph.Jp. XIV	Ph. Eur. 4	USP XXVI	Ph. Jp XIV
Alclometasone dipropionate	—	RP-HPLC 254 nm	—	—	TLC 254 nm	—
Amcinonide	—	RP-HPLC 254 nm	—	—	—	—
Beclomethasone dipropionate	VIS-tetrazolium	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	TLC-H ₂ SO ₄	TLC-H ₂ SO 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 240nm	—	—	RP-HPLC 240 nm	—	—
Chlormadinone acetate	—	—	UV 285 nm	—	—	RP-HPLC 236nm
Clobetasol propionate	—	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	—
Cyproterone acetate	UV 282 nm	—	—	RP-HPLC 254 nm	—	—
Danazol	—	UV 285 nm	—	—	TLC-I ₂	—
Desoxycort(icoster) one acetate	UV 240 nm	VIS-tetrazolium	—	RP-HPLC 254 nm	—	—
-pivalate	—	RP-HPLC 254 nm	—	—	—	—
Desoximetasone	—	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	TLC 254 nm
-acetate	UV 238.5 nm	RP-HPLC 254 nm	—	RP-HPLC 254 nm	RP-HPLC 254 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	—
Drostanolone propionate	—	—	GC	—	—	TLC-vanillin
Dydrogesterone	—	RP-HPLC 280 nm	—	—	RP-HPLC 280 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	—	TLC 254 nm
-cypionate	—	RP-HPLC 280 nm	—	—	—	—
-valerate	UV 280 nm	RP-HPLC 280 nm	—	RP-HPLC 220 nm	TLC-H ₂ SO ₄	—
Estril	UV 281 nm	UV 281 nm	RP-HPLC 280 nm	NP-HPLC 254 nm	TLC-H ₂ SO ₄	TLC-H ₂ SO ₄
Estrogens, conjugated	GC	GC	—	GC	GC	—
Estrogens, esterified	—	GC	—	—	GC	—
Estrone	—	RP-HPLC 280 nm	—	—	TLC-H ₂ SO 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.
Ethinodiol 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 ₂ SO 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 254nm
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

Continued

Fluoxymesterone	—	RP-HPLC 254 nm	NP-HPLC 254 nm	—	RP-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	—	—
Halcinonide	—	UV 239 nm	—	—	TLC/elut. 239 nm	—
Hydrocortisone	UV 241.5 nm	NP-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 (hemi)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 254nm
-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 ₂ SO ₄	—
Isoflupredone acetate	—	NP-HPLC 254 nm	—	—	RP-HPLC 254 nm	—
Levonorgestrel	Titration ethinyl	UV 241 nm	—	TLC-phos.mol.	TLC-phos.mol.	—
Lynestrenol	Titration ethinyl	—	—	TLC-H ₂ SO ₄	—	—
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 ₂ SO ₄
Meprednisone	—	TLC 238 nm extr.	—	—	—	—
Mesterolone	RP-HPLC 254 nm	—	—	RP-HPLC 200 nm TLC- <i>p</i> -toluene-sulfonic acid	—	—
Mestranol	Titration ethinyl	VIS-H ₂ SO ₄	UV 279 nm	TLC-H ₂ SO ₄	—	TLC-H ₂ SO 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	TLC 254 nm	RP-HPLC 254 nm	TLC-tetrazolium
-acetate	UV 243 nm	—	—	RP-HPLC 254 nm	—	—
-hydrogen succinate	UV 243 nm	—	—	RP-HPLC 254 nm	—	—
Methyltestosterone	UV 241 nm	RP-HPLC 241 nm	UV 241 nm	TLC 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	—	RP-HPLC 240 nm	—	—	NP-HPLC 238 nm	—
Nandrolone phenylpropionate	—	TLC 239 nm extr.	—	—	—	—
Norethisterone (norethindrone)	Titration ethinyl	UV 240 nm	Titration ethinyl	TLC-H ₂ SO ₄	TLC-H ₂ SO ₄	—
-acetate	Titration ethinyl	UV 240 nm	Titration ethinyl	RP-HPLC 254 nm	—	RP-HPLC 254nm
Norethynodrel	—	UV 240 nm-HCl	—	—	TLC-H ₂ SO ₄	—
Norgestimate	—	RP-HPLC 244nm	—	—	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	—	Titration lactone	—	—	TLC-H ₂ SO ₄	—
Oxymetholone	—	TLC 315 nm extr.	—	—	—	—
Pancuronium bromide	Titration HClO ₄	—	Titration HClO ₄	TLC-I ₂	—	TLC-NaNO ₂ +BiI ₃
Prasterone sodium sulfate	—	—	Ion-exchange/ Titration NaOH	—	—	TLC-H ₂ SO ₄
Prednicarbate	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	TLC 254,366 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
-hemisuccinate	—	TLC 243 nm extr.	—	—	—	—
-pivalate	UV 243 nm	—	—	RP-HPLC 254 nm	—	—
-sodium	UV 247 nm	UV 241 nm	—	RP-HPLC 254 nm	Prednisolone	—

Continued

phosphate		enzyme, extract.			extraction 241 nm	
-sodium	—	VIS-tetrazolium	RP-HPLC 254 nm	—	—	—
succinate						
-succinate	—	—	RP-HPLC 242 nm	—	—	TLC 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
Rimexolone	—	RP-HPLC 242 nm	—	—	RP-HPLC 242 nm	—
Spironolactone	UV 238 nm	RP-HPLC 254 nm	UV 238 nm	RP-HPLC 254/283 nm	TLC-H ₂ SO ₄	TLC-H ₂ SO ₄
Stanozolol	Titration HClO ₄	Titration HClO ₄	—	TLC-H ₂ SO ₄	TLC-H ₂ SO ₄	—
Testolactone	—	VIS-isoniazid	—	—	TLC 254 nm + acid dichromate	—
Testosterone	UV 241 nm	TLC 239 nm extr.	—	TLC 254 nm	—	—
-cypionate	—	GC	—	—	—	—
-enantate	UV 241 nm	VIS-isoniazid	UV 241 nm	TLC-H ₂ SO ₄	TLC- <i>p</i> -toluene sulfonic acid	—
-propionate	UV 241 nm	VIS-isoniazid	UV 241 nm	RP-HPLC 240 nm	—	TLC 254 nm
Trenbolone acetate	—	RP-HPLC 344 nm	—	TLC 254 nm	TLC-phos.mol. RP-HPLC 344 nm	—
Triamcinolone	UV 238 nm	RP-HPLC 254 nm	RP-HPLC 254nm	RP-HPLC 238 nm	—	—
-acetanide	UV 238.5 nm	RP-HPLC 254 nm	RP-HPLC 254nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
-diacetate	—	RP-HPLC 254 nm	—	—	—	—
-hexacetanide	UV 238 nm	RP-HPLC 254 nm	—	RP-HPLC 254 nm	RP-HPLC 254 nm	—

and Pharm. Jp. XIV contains only a few (the formulations in Pharm. Jp. XIV are indicated by *cursive* letters).

A great variety of *sample-preparation* methods are used to assay the formulations. Solid dosage forms (*tablets, etc.*) are either disintegrated with water, followed by dilution with methanol, acetonitrile or the mobile phase of the RP-HPLC system, or extraction of the aqueous phase with chloroform. More common is direct extraction mainly with methanol. Direct extraction with methanol, chloroform, acetonitrile, *etc.* is the most widely used sample-preparation method for *creams*, while in addition to these, extraction with hexane, heptane, i-octane followed by extraction of the apolar phase with mixtures of water and methanol, acetonitrile, *etc.* is also used for *ointments*. Aqueous *solutions* are usually diluted with water or the mobile phase of the RP-HPLC method, or extracted with chloroform, ethyl acetate *etc.* Aqueous *suspensions* are homogenized by simple dilution with methanol, 2-propanol or the mobile phase of the RP-HPLC system. Oily injections are either simply diluted, mainly with chloroform or diluted with hexane, *etc.*, followed by extraction with polar solvents or solvent mixtures.

The predominant method for the assay is reversed phase HPLC with UV detection, either at 254 nm or at the maximum of the UV spectra of the active ingredients. This method is used among others for the assay of acemetasone dipropionate and amcinonide cream and ointment, betamethasone cream and tablets, betamethasone benzoate gel, betamethasone dipropionate cream, ointment, lotion and topical aerosol, betamethasone sodium phosphate injection, betamethasone sodium phosphate and acetate injectable suspension, betamethasone valerate cream, ointment and lotion, clobetasol propionate cream, ointment and topical solution, cortisone acetate injectable suspension, danazol capsules, desoxycorticosterone pivalate injectable suspension, desoxymetasone cream and ointment, dexamethasone injection, ophthalmic suspension, oral solution elixir and tablets, dexamethasone acetate injectable suspension, dexamethasone sodium phosphate cream, injection, ophthalmic ointment and ophthalmic solution, dydrogesterone tablets, estradiol tablets and vaginal cream, *estradiol benzoate injectable suspension*,

estradiol cypionate and valerate injection, *estriol tablets*, estrone injectable suspension, estropipate tablets and vaginal cream, ethynodiol diacetate and mestranol tablets, ethynodiol diacetate and ethinylestradiol tablets, finasteride tablets, fludrocortisone acetate tablets, flunisolide nasal solution, fluocinolone acetanide cream, ointment and topical solution, fluocinonide cream, ointment, gel and topical solution, fluorometholone ophthalmic suspension, fluoxymesterone tablets, flurandrenolide cream, lotion, tape, halcinonide cream and ointment, hydrocortisone cream, ointment, gel, lotion, rectal suspension, hydrocortisone butyrate cream, hydrocortisone valerate cream and ointment, isofluprednone acetate injectable suspension, levonorgestrel and ethinylestradiol tablets, medroxyprogesterone acetate tablets, megestrol acetate oral suspension and tablets, methylprednisolone tablets, methylprednisolone acetate injectable suspension, mometasone furoate cream, ointment and topical solution, nandrolone decanoate injection, norethindrone and ethinylestradiol tablets, norethindrone and mestranol tablets, norgestrel and ethinylestradiol tablets, prednisolone oral suspension and syrup, *prednisolone tablets*, prednisolone acetate injectable suspension and ophthalmic suspension, prednisone oral solution, injectable suspension and tablets, progesterone injection, injectable suspension and vaginal suppositories, rimexolone ophthalmic suspension, spironolactone tablets, spironolactone and hydrochlorothiazide tablets, triamcinolone tablets, triamcinolone acetanide cream, ointment, topical aerosol, lotion and injectable suspension, triamcinolone diacetate oral suspension, syrup and injectable suspension and triamcinolone hexacetanide injectable suspension.

Normal-phase HPLC is used for the assay of cortisone acetate tablets, diflorasone cream, drostanolone propionate ointment, fluorometholone cream, hydrocortisone tablets, hydrocortisone acetate cream, ointment and lotion, medroxyprogesterone acetate injectable suspension, prednisolone tablets and prednisolone tebutate injectable suspension.

Of the other chromatographic methods, gas chromatography is used for the assay of *drostanolone propionate* injection, esterified and conjugated estrogen tablets, mibolerone oral solution, oxandrolone tablets and testosterone cypionate injection. TLC separation, extraction followed by Tetrazolium

Blue colorimetry is the assay method for betamethasone syrup and methylprednisolone acetate cream, while column chromatographic separation followed by Tetrazolium Blue colorimetry or iron-phenol colorimetry is used for the assay of dexamethasone gel and estradiol pellets and injectable suspension, respectively.

The above-discussed chromatographic methods are selective and stability indicating. The same cannot be said for the non-chromatographic methods. UV spectrophotometric assay based on natural absorption is carried out for dexamethasone sodium phosphate inhalation aerosol, *dydrogesterone* tablets, *methyltestosterone* tablets and capsules, oxymetholone tablets, prednisolone sodium phosphate injection and ophthalmic solution (after enzymatic hydrolysis and extraction), progesterone in intrauterine contraceptive system, stanozolol tablets and testosterone injectable suspension. A UV spectrophotometry is used in some other cases as well in the content uniformity and dissolution tests of tablet formulations. The selectivity of some colorimetric methods also used for the assay of steroid hormone formulations is not better either.^{2-4,9,10} For example, reactions based on the condensation of the unsaturated 3-oxo group with isoniazid (clocortolone pivalate cream, hydrocortisone caproate injection, nandrolone phenylpropionate injection, norethindrone tablets, norgestrel tablets, testolactone tablets, *testosterone enanthate* injection and *testosterone propionate* injection) or 4-aminoantipyrine (flumethasone pivalate cream) measure the less vulnerable part of the molecules. The reactions of the phenol-type ring A based on diazo-coupling (*estradiol benzoate* injection) or on the non-stoichiometric reaction with the sulfuric acid-methanol reagent (*ethinylestradiol* tablets) are not stability-indicating either. The situation is somewhat better with the color reactions of corticosteroids with sensitive, reducing side chains at position 17. Most of their degradation products do not react with the alkaline Tetrazolium Blue (desoxycorticosterone acetate injection and pellets, dexamethasone topical aerosol, hydrocortisone injectable suspension and hydrocortisone acetate ophthalmic ointment and suspension, injectable suspension) or phenylhydrazine-sulfuric acid reagents (hydrocortisone sodium phosphate injection and prednisolone cream).

It is amazing that an extremely outdated and labor-extensive method is used by USP XXVI for the assay of estrone oily injection. The procedure includes three extraction steps (consuming 50 ml of hexane, 50 ml of benzene (!) and 95 ml of chloroform), two evaporations to dryness, formation of hydrazone derivative with trimethylacetylhydrazide ammonium chloride and its decomposition. Finally the weight of estrone is measured!

3 Spectroscopic and Spectrophotometric Methods

3-1 Ultraviolet-visible spectrophotometry

As shown in the previous sections, in spite of its poor selectivity UV spectrophotometry is widely used for the identification and assay of bulk steroid drugs. The assay is carried out either using a reference standard material or by determining and describing the value of the specific absorbance. The validation of the determination of the latter by interlaboratory study preceded by recrystallization and checking the purity of the test sample by phase solubility analysis, HPLC and differential scanning calorimetry was described on the example of triamcinolone.¹¹ Problems to be solved for the application of this method to the assay of drug formulations is the elimination of matrix effects and simultaneous

determination of the active ingredients of two- or multicomponent formulations. In simple cases improved extraction procedure such as solid phase extraction in the case of the determination of hydrocortisone in ointments¹² is sufficient. Dual-wavelength measurements can also give satisfactory results. For example, the release of hydrocortisone acetate from microcapsules in aqueous suspension was monitored at 247.6 nm with 277 nm as the reference wavelength.¹³ The classical dual-wavelength spectrophotometry was successfully used for the simultaneous determination of spironolactone and hydrochlorothiazide with their overlapping spectra in a tablet formulation.¹⁴ The performance of the method can be improved by multiwavelength measurements and chemometric methods. Partial least-squares regression analysis was applied to other diuretic preparations, such as spironolactone-althiazide¹⁵ and spironolactone-chlorthalidone¹⁶ formulations. The much more delicate problem of the assay of the low-dosed levonorgestrel-ethinylestradiol¹⁷ and gestodene-ethinylestradiol¹⁸ oral contraceptive tablets was also solved by the partial least-squares regression analysis. Even three-component drug formulations were successfully analyzed by this and other chemometric methods, such as the principal component regression method. One example is the mixture of hydrocortisone, nystatin and oxytetracycline.¹⁹ The accuracy and precision of the determination of even as low a quantity as 2 mg/vial dexamethasone in the presence of 250 and 10 mg of vitamins B₆ and B₁₂, respectively, in an injection formulation were good.²⁰ Other 3-component formulations containing low quantities of dexamethasone resolved by partial least-squares regression analysis and other chemometric methods were Polymyxin B, trimethoprim, dexamethasone²¹ and chlorpheniramine, naphazoline and dexamethasone.²² In the latter case, the use of artificial neural network analysis was necessary for the determination of dexamethasone due to the non-linearity caused by interactions among the components.

Derivative UV spectrophotometry¹⁰ was also successfully applied to the analysis of steroid hormone formulations. In simple cases (single-component formulations) only the spectral background caused by the excipients should be eliminated. For this purpose first-derivative spectra are usually sufficient. An example for this is the determination of triamcinolone acetonide in an ointment.²³ The same technique using the "zero-crossing" method was found to be suitable for the simultaneous determination of the components of dual-component drug formulations such as creams and tablets containing triamcinolone acetonide and terbinafine hydrochloride²⁴ and oral contraceptives containing ethinylestradiol and levonorgestrel^{25,26} as well as ethinylestradiol and gestodene.²⁷ A zero-crossing second-derivative method was used for the determination of fluorometholone and tetrahydrozoline hydrochloride²⁸ as well as estradiol and medroxyprogesterone acetate²⁹ in formulations. Another possibility to improve the selectivity of the method is the use of "ratio spectrum (zero-cross) derivative spectroscopic" method. Examples for this are the determination of spironolactone and hydrochlorothiazide,¹⁴ hydrocortisone, nystatin and oxytetracycline,¹⁹ cyproterone acetate and estradiol valerate³⁰ in pharmaceutical formulations. The performance of various multivariate chemometric and derivative spectrophotometric methods is compared for examples of formulations containing hydrocortisone and Zn-Bacitracin³¹ as well as dexamethasone and polymyxin B.³² An interesting feature of another method for the assay of spironolactone and hydrochlorothiazide, based on ratio spectrum derivative spectroscopy, making use of a rapid-scanning diode-array spectrophotometer, is that it is adopted to

flow-injection analysis.³³

The importance of spectrophotometric/colorimetric methods based on chemical reactions has dramatically decreased in the last years both in the analysis of bulk steroid drugs and formulations made thereof. As can be seen in Table 1 and in the section "Assay of steroid hormone formulations" some of the classical reactions (*e.g.* condensation with isoniazid and phenylhydrazine, redox reaction with tetrazolium reagents) are still in use in some instances. Many other reactions were also described.^{2-4,9,10} Further research in this field can be considered to be unnecessary: publications of this kind published in the last 15 years are not referred to in this review unless these are particularly interesting. This is the case with a paper where Friedel-Crafts acetylation of steroids followed by taking the UV spectrum enables interesting discrimination to be made between various isomeric derivatives.³⁴ Kinetic variants of the classical Blue Tetrazolium and phenylhydrazine³⁵ and isoniazid³⁶ reactions and their application to the analysis of corticosteroids in various pharmaceuticals are also worth mentioning.

3-2 Fluorimetry

The native fluorescence of estrogens enables their direct fluorimetric determination, *e.g.* the determination without a preliminary separation of estradiol valerate in an injection formulation in the presence of hydroxyprogesterone caproate (Ex: 295 nm/Em: 308 nm).³⁷ However, the main application field of this is their sensitive HPLC detection.

Measurements based on fluorescence induced by non-stoichiometric reactions with various strongly acidic reagents^{3,4,9} have almost completely lost their importance in modern pharmaceutical analysis. However, as seen in the sections dealing with pharmacopoeias, methanol-sulfuric acid is still an important spray reagent in the thin-layer chromatographic analysis of steroid drugs. An interesting, highly specific and sensitive FIA method for the determination of fluticasone propionate is based on splitting with sodium hydroxide of its $-\text{CO}-\text{S}-\text{CH}_2\text{F}$ side chain to form fluoromethylthiol which is further reacted with glycine and *o*-phthalaldehyde. The strongly fluorescent isoindole derivative is formed in the reaction coil of the FIA apparatus.³⁸

3-3 Infrared and Raman spectroscopy

Infrared (IR) spectroscopy is the most generally used method for the identification of bulk steroid drugs in modern pharmacopoeias.⁶⁻⁸ In addition to this, IR spectroscopy, especially Fourier-transform IR (FTIR) is one of the most important methods, together with X-ray powder diffractometry, differential scanning calorimetry (DSC), thermogravimetry (TGA) and thermomicroscopy to characterize the solvates and polymorphic modifications frequently occurring among steroid hormone drugs.³⁹ One of the most characteristic examples is spironolactone, where in the early literature several polymorphs and solvates were described. Conventional IR spectrometry using fused KBr discs is not suitable for their differentiation due to the easy transformation of the polymorphs under pressure. On the basis of diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and Fourier transform Raman spectroscopic investigations supported by DSC and TGA measurements⁴⁰ four different polymorphic forms⁴¹ and five solvates⁴² were identified and characterized. FT-IR studies combined with all of the above mentioned solid-phase techniques revealed the existence of three polymorphs of flucinolone acetonide⁴³ as well as two polymorphs and a hemihydrate of flunisolide.⁴⁴

The DRIFTS method is also suitable for the rapid verification

of identity, as well as the content of various drugs, among others finasteride in their solid formulations.⁴⁵ TG/FT-IR studies supported by X-ray diffraction and DSC revealed the mechanism of the decomposition of prednisolone hemisuccinate in a freeze-dried formulation.⁴⁶

Near-infrared reflectance spectroscopy has become a versatile tool in drug analysis and in-process control in drug production. It was found to be suitable to solve as delicate a problem as the simultaneous determination of norethisterone and ethinylestradiol in oral contraceptive tablets.⁴⁷

3-4 Mass spectrometry

In the majority of cases, mass spectrometry is applied in pharmaceutical steroid analysis associated with various chromatographic techniques. Examples for these will be presented in some of the subsequent sections. Here, only one direct application is mentioned. Time-of-flight secondary ion mass spectrometry was successfully applied for the characterization and imaging of a controlled-release drug delivery system containing prednisolone sodium metasulfobenzoate. Cross sections of the device containing a multi-polymer-layer system were probed for the distribution of the active ingredient.⁴⁸

3-5 NMR spectroscopy

One of the relatively rare direct applications of NMR spectroscopy in pharmaceutical steroid analysis is its use for the rapid screening and compositional analysis of steroid cocktails and veterinary drug formulations illegally used to livestock. Stanazolol, fluoxymesterone, methylboldenone, norethandrolone, clobestol acetate, testosterone and its cypionate and decanoate and other steroids were identified directly or after HPLC fractionation with the aid of a ¹H-NMR database.⁴⁹ When using interrupted decoupling, solid state ¹³C-NMR is suitable not only for detecting low-dose active ingredients (among others prednisolone) in solid dosage forms, but also for discrimination between the two polymorphic forms of the latter.⁵⁰ Further analytical applications of NMR spectroscopy are discussed in the section dealing with HPLC.

In addition to analytical applications, NMR spectroscopy is a good tool for studying the mechanism of interactions taking place between the analyte and stationary phase. As an example, the transferred NOESY study of the interactions between various steroids and calix[8]arene-based stationary phases is mentioned.⁵¹ Similar studies related to chiral interactions are shown in the last section of this review.

3-6 Chemiluminometry

Although the main application field of chemiluminescence in steroid analysis is chemiluminescence immunoassay in biological-clinical analysis, there are also a few direct applications in pharmaceutical analysis. The sensitising effect of corticosteroids on the chemoluminogenic reaction between sulfite and cerium(IV) was exploited for their flow-injection (FIA) determination in pharmaceutical formulations.^{52,53} A luminol chemiluminescence determination for the same purpose is also described.⁵⁴

3-7 Circular dichroism (CD) spectropolarimetry

The main application field of CD spectroscopy is structural analysis but this technique can be successfully used also for analytical purposes. The early literature of the applications to steroid hormone analysis was reviewed.⁵⁵ Of the recent applications, two are mentioned here. The profound difference between the ellipticities of ethisterone and its 5-ene isomer

enables their selective determination (ethisterone at 348 nm at its negative maximum of the CD spectrum) and the isomer at 296 nm at the positive maximum of the CD curve. When the simultaneous determination was carried out by HPLC, the spectropolarimeter could act as a CD-detector.⁵⁶ The applicability of the CD method can be enhanced by the use of preliminary chemical reactions. A difference CD method was developed for the determination of 4-ene-3-oxo steroids based on oxime formation making use of the large difference between the ellipticities before and after oximation. This method was found to be suitable for the determination to as low a concentration as 0.02% of levonorgestrel acetate impurity in norgestimate.⁵⁷

4 Chromatographic and Hyphenated Methods

4.1 Planar chromatography

In contrast to the pharmacopoeias where TLC is used only as a semi-quantitative limit test for purity check of bulk steroid hormone drugs, in the literature of the last 15 years almost exclusively quantitative densitometric methods are described for the purity check and also for the assay of steroid hormone formulations. Typical papers describing the assay of formulations are *e.g.*, the determination of betamethasone valerate in various creams together with other active ingredients. Conventional silica gel 60 F254 plates were used; (see section "Related impurities test of bulk drug materials"). The densitograms were scanned in the reflectance mode at wavelengths which correspond to the isobestic point of the two components: 228 nm for betamethasone-clotrimazole cream,⁵⁸ 247 nm for betamethasone-clioquinol cream⁵⁹ and 233 nm for betamethasone-miconazole nitrate cream.⁶⁰ The determination of the six components of conjugated estrogens in raw materials and tablet formulations was carried out after hydrolysis, extraction, followed by TLC separation and detection at 280 nm.⁶¹

If carefully validated, the quantitative TLC densitometric purity test is equivalent to the HPLC purity test. The principles and practice of validation were described taking danazol⁶² and estradiol benzoate⁶³ as examples (scanning at 252 and 229 nm, respectively).

The popularity of high-performance thin-layer chromatography (HPTLC) is continuously increasing: in the majority of papers published in the last 15 years the use of this method is reported. The advantages of this technique are clear: due mainly to the smaller particle size (about 4 μm) and narrow particle-size distribution of the stationary phases, the separation efficiency increases, enabling well-separated and well-quantifiable spots to be achieved with smaller plates, shorter running distance and shorter running time. Some of the important parameters (retention behavior, separation efficiency, selectivity) of TLC and HPTLC were compared using steroids as the model compounds.⁶⁴ In the majority of cases, silica gel 60 F254 plates were used for the assay of steroid preparations. Some of the investigated formulations are as follows (the wavelength of the densitometric scan in parentheses): finasteride tablets (228 nm),⁶⁵ betamethasone tablets (245 nm),⁶⁶ mometasone furoate topical preparations (260 nm),⁶⁷ cyproterone acetate-ethinylestradiol tablets (284 nm)⁶⁸ or ethinylestradiol (220 nm) and cyproterone acetate (284 nm),⁶⁹ dexamethasone and xylometazoline in nasal drops (240 nm),⁷⁰ dexamethasone sodium phosphate-neomycin sulfate solution (246 nm),⁷¹ prednisolone acetate in pharmaceutical formulations (243 nm; stepwise gradient).⁷² The basis for the assay of

hydrocortisone, hydrocortisone acetate and clioquinol containing ointment and cream preparations was fluorescence quenching at 254 nm.⁷³ In the case of estradiol tablets (284 nm), the HPTLC method was suitable not only for the assay but also for the detection of degradants formed under stress conditions.⁷⁴

Reversed-phase (ion pair) TLC⁷⁴ and HPTLC⁷⁵ systems were used for the assay of corticosteroid sodium phosphate salts in parenteral preparations and ear drops⁷⁵ and various steroids,⁷⁶ respectively.

Overpressured thin-layer chromatography (OPLC) is a variant of HPTLC in which the sorbent layer is covered with a pressurized elastic plate thus eliminating the vapor phase. Due to the forced flow of the eluent delivered by an automatic pump its migration velocity is constant and the migration distance can be increased up to 18 cm thus enabling excellent separations and improved quantifiability to be achieved. As a result of these factors, OPLC is eminently suitable for the separation of impurities and degradants in bulk drugs and their formulations. Examples for the application of OPLC to steroid hormone drugs are the purity test of norgestrel and levonorgestrel (visualization by fluorescence quenching at 254 nm, phosphomolybdic acid or sulfuric acid),⁷⁷ allylestrenol drug substance and tablet (sulfuric acid),⁷⁸ nandrolone (sulfuric acid; comparison of the impurity profile with those obtained by TLC, HPLC and GC),⁷⁹ norethisterone drug substance and tablet (sulfuric acid).⁸⁰ OPLC was successfully used in in-process-monitoring⁸¹ and cleaning validation of equipment⁸² in the course of the production of bulk steroids and steroid formulations.

The usefulness of further innovations, such as temperature-controlled programmed TLC,⁸³ programmed multiple development TLC with a new modification of the horizontal sandwich chamber⁸⁴ and ultra-thin-layer chromatography (UTLC) where the granular adsorbents are replaced by a monolithic structure based on a silica-gel matrix has been demonstrated by using, among others, the separation of steroid mixtures.⁸⁵ An interesting study dealing with the reasons for and the elimination of the irreversible adsorption of some steroids during multiple development TLC merits mentioning.⁸⁶

A tandem TLC-HPLC method is described for screening cosmetic products for the presence of undeclared synthetic corticosteroids.⁸⁷ Chiral selectors improved the separation of diastereomeric steroid pairs, *e.g.*, the addition of β -cyclodextrin to the mobile phase in the case of the separation of R(+)- and R(-)-budenoside on cellulose-coated HPTLC plates,⁸⁸ and impregnation of the silica gel TLC plate with *d*-camphorsulfonic acid/copper(II) acetate for the separation of the diastereomeric 21-ester derivatives of prednisolone formed with racemic tetrahydrophthalic acid.⁸⁹

Some papers deal with the possibilities of applying only seldom-used stationary phases *e.g.*, Florisil, derivatized reversed-phase systems for steroid separations and with the elucidation of the separation mechanism.^{90,91}

4.2 Gas chromatography (GC) and GC-MS

As already mentioned earlier in this review, and as seen in Table 1, gas chromatography does not play a very important role in the analysis of steroid hormone drugs. The reasons for this are their low volatility and (especially in the case of corticosteroids) thermal instability. In the case of sufficiently stable steroids it is a good method of choice for the assay of among others methyltestosterone (after dimethylethyl- or dimethylisopropylsilylation)⁹² and cyproterone acetate-ethinylestradiol⁹³ tablets.

GC-MS is an ideal tool for screening purposes. As many as

134 underivatized drugs (among them 10 steroids) were included in a study to screen ethnic patent medicines available in health-food stores and ethnic markets. It is to be noted that two of the ten (dexamethasone and prednisolone) could not be detected by GC-chemical ionization MS, probably due to their vulnerable 17-side chain.⁹⁴ One possibility to enhance the applicability of GC and GC-MS to some of the thermally labile compounds is "supersonic GC-MS" using shorter capillaries with lower film thickness, an increased carrier gas flow rate and a decreased temperature. This enables several steroids (among them corticosterone with a vulnerable side chain) to be determined.⁹⁵ Another screening study using GC-MS aimed at detecting anabolic steroids⁹⁶ and prohormones⁹⁷ in nutritional supplements.

4.3 Supercritical fluid chromatography (SFC)

Although the problem of thermal instability in GC can be solved by using supercritical fluid chromatography, this technique seems to have attracted little interest in steroid analysis. Several steroids were separated by SFC with light-scattering detection.^{98,99} The sensitivity of the laser light scattering detector is similar to that of the UV detector with the advantage that UV-inactive steroids can also be detected with the same sensitivity.⁹⁹ Examples for the use of chiral SFC will be shown in the section "Enantiomeric separations".

Supercritical fluid extraction can be a useful alternative to classical extractions as demonstrated on the examples of megestrol acetate,¹⁰⁰ medroxyprogesterone acetate¹⁰¹ and cyproterone acetate¹⁰¹ from tablet matrices.

4.4 High-performance liquid chromatography (HPLC) and HPLC-MS

HPLC as the most important and most generally used method in pharmaceutical steroid analysis is described and characterized in the chapter "Steroid hormone drugs in pharmacopoeias", where the assay and purity test for several bulk drugs is presented in Table 1 followed by listing several formulations assayed by HPLC. Some further examples are briefly summarized here, taken from the literature where many "pharmacopoeial-like" reversed-phase HPLC assays for formulations are described (the abbreviation "St" in parentheses relate to stability assays). Allylestrenol- α -tocopherol tablets,¹⁰² beclomethasone dipropionate inhalers¹⁰³ and other formulations (St),¹⁰⁴ betamethasone, its acetate and other corticosteroids in ointments,^{105,106} betamethasone and dexamethasone (delicate separation problem!),^{107,108} betamethasone-diclofenac sodium-cyanocobalamin tablet,¹⁰⁹ cyproterone acetate tablet (St),¹¹⁰ dexamethasone-xylometazoline nasal drops,¹¹¹ dexamethasone-retinol palmitate ointment,¹¹² dexamethasone-trimethoprim liquid formulations,¹¹³ dexamethasone acetate-prednisolone tablet,¹¹⁴ estradiol-levonorgestrel transdermal drug delivery formulation (St),¹¹⁵ estradiol valerate-medroxyprogesterone acetate tablet,¹¹⁶ ethinylestradiol-norgestrel tablet,¹¹⁴ ethinylestradiol-levonorgestrel and ethinylestradiol-gestodene tablets,¹¹⁷ finasteride tablet (St),¹¹⁸ flucortolone pivalate and hexanoate suppositories,¹¹⁹ hydrocortisone-oxytetracycline-nystatin in pharmaceutical preparations,¹²⁰ hydrocortisone acetate and hemisuccinate-lidocaine in pharmaceutical preparations,¹²¹ hydrocortisone acetate-methyl- and propylparaben topical cream (St),¹²² hydrocortisone sodium phosphate gel (St),¹²³ nestorone implants (St),¹²⁴ pancuronium bromide injection (St),¹²⁵ prednisolone acetate-tetrahydrozoline hydrochloride-ofloxacin ophthalmic preparations,¹²⁶ prednisolone sodium phosphate implantable infusion pump (St),¹²⁷ progesterone in

micellar systems and other formulations,¹²⁸ triamcinolone, its acetate and acetonide and several other corticosteroids (St),¹²⁹ triamcinolone acetonide dermatological patches,¹³⁰ triamcinolone acetonide, methyl- and propylparaben topical cream (St).¹³¹

Carefully validated stability-indicating HPLC methods were used for the determination of impurities and degradants in bulk pipercuronium bromide,¹³² budesonide,¹³³ aqueous spirinolactone- β -cyclodextrin solutions,¹³⁴ and for the photodegradation kinetic study of danazol.¹³⁵

HPLC is eminently suitable for *screening* purposes, *e.g.* RP-HPLC for anabolic steroids,¹³⁶ corticosteroids in topical pharmaceuticals using diode-array UV detector,¹³⁷ toxicological analysis of steroids and other compounds with diode-array UV detector,¹³⁸ NP- and RP-HPLC for corticosteroids.¹³⁹ Especially useful is the coupling of HPLC with mass spectrometry: a HPLC/time-of-flight MS method was developed for screening anabolic steroids in illegal cocktails,¹⁴⁰ while for their determination in oily formulations RP-HPLC separation followed by off-line GC-MS analysis was used.¹⁴¹ HPLC-MS is the most suitable method for the rapid screening of high-throughput drug mixtures.¹⁴² Capillary HPLC-MALDI-TOF-MS after post-column derivatization with 2,4-dinitrophenylhydrazine was used for the screening of combinatorial libraries with 7 corticosteroids as model compounds.¹⁴³ HPLC-MS was also used for the rapid identification of the products of biotechnological transformations. The model for this study was the hydroxylation of progesterone to 9 α -hydroxyprogesterone.¹⁴⁴ HPLC-UV was used as the in-process-control method to follow this reaction at the industrial scale.¹⁴⁵

On-line HPLC-NMR—although only few data are available for its use in pharmaceutical steroid analysis—is certainly the method of the future even in this field. For example, using this technique estradiol and norethisterone acetate were detected in a galenic emulsion.¹⁴⁶ Further examples for the use of HPLC coupled with spectroscopic methods are presented in the section "Impurity and degradation profiling of steroid drugs".

In addition to the above discussed use of HPLC for the analytical investigation of bulk steroids and steroid formulations it is a useful tool also in the hands of pharmaceutical technologists to investigate *drug release* from various formulations and devices for controlled drug delivery. Examples are the release of prednisolone from chitosan gel beads¹⁴⁷ or from chitosan-gelatin sponges,¹⁴⁸ estradiol and its 3-acetate from intravaginal rings¹⁴⁹ and triamcinolone acetonide from topical dosage forms.¹⁵⁰

As mentioned earlier, in the majority of cases reversed-phase HPLC is used, mainly C18 columns with unbuffered binary or ternary mixtures of water with methanol, acetonitrile or tetrahydrofuran. Some innovations regarding *stationary phases* are as follows. Monolithic columns were found useful tools for the ultrafast separations of steroids,¹⁵¹ among others ingredients of a topical cream containing triamcinolone acetonide and preservatives.¹⁵² Ultrafast analysis is also achievable by decreasing the column length with simultaneous optimization of the flow rate and gradient profile (ballistic gradients).¹⁵³ Temperature-responsive stationary phases showing hydrophilic properties at lower while hydrophobic properties at higher temperatures were prepared by covalently binding poly(*N*-isopropylacrylamide) to aminopropylsilica. This is a promising tool for the temperature-controlled separation of steroids.¹⁵⁴⁻¹⁵⁶ Excellent separation of the epimers of estradiol was achieved by modifying the surface of silica gel with the covalent binding of cholesterol.¹⁵⁷ Molecularly imprinted artificial receptors as

HPLC stationary phases were prepared among others for the screening of corticosteroids¹⁵⁸ and estrogens.¹⁵⁹ Porous graphitic stationary phases were also used for the separation of corticosteroids especially if a voltage is applied to the column, while making use of its electric conductivity.¹⁶⁰ This technique, termed "electrochemically modulated liquid chromatography", was successfully coupled on-line with electrospray MS.¹⁶¹

Several papers have been published dealing with the *mobile phase* selection and optimization in the HPLC analysis of steroid hormone drugs. The selectivity of the separation in RP-HPLC was improved by using ternary systems containing methyl or ethyl acetate as demonstrated by the separation of 9 α -fluoroprednisolone acetate from its impurities.¹⁶² Using 2,2,2-trifluoroethanol as a mobile phase component in ternary systems together with perfluorinated RP-HPLC stationary phases unique selectivity was attained explicable by the strong adsorption of the co-solvent on the surface of the stationary phase.¹⁶³ Another fluorinated solvent, ethoxynonafluorobutane was introduced as an environmentally friendly and highly selective solvent in the NP-HPLC analysis of several compounds, among others steroids.¹⁶⁴ Cyclodextrins are known to be excellent chiral selectors in enantiomeric analysis (see section "Enantiomeric analysis"). The effect of β -cyclodextrin on the retention of steroids and their separation in general¹⁶⁵ and the effect of temperature on these characteristics¹⁶⁶ were thoroughly studied. Chromatographic data-topological index dependence of steroids in RP-HPLC and RP-TLC are also worth mentioning.¹⁶⁷

The use of *micellar liquid chromatography*, a special branch of RP-HPLC where the eluent contains very low concentration of organic modifiers (mainly 1-pentanol and acetonitrile) and about 0.1 M sodium dodecyl sulfate (SDS) as the micelle forming agent was applied to steroid analysis by Spanish groups.¹⁶⁸⁻¹⁷⁶ This technique enables the relatively rapid analysis of derivatives with wide range of polarity with minimum need for sample preparation. In addition to retention-structure relationship studies¹⁶⁹ and screening of steroids,¹⁷⁰⁻¹⁷² the method was successfully used for the determination of spironolactone,¹⁶⁸ corticosteroids,^{173,176} methyltestosterone,¹⁷⁴ anabolic steroids¹⁷⁶ and danazol¹⁷⁵ in various formulations.

Although the principles and practice of *mobile phase optimization* in HPLC were already set up in the early period of the history of HPLC, further studies are time to time made refining of the optimization with steroids and other compounds as the models. These include the description of a general quantitative relationship for column selectivity in RP-HPLC and the study of the effect of the change in conditions,¹⁷⁷ the use of experimental design for spherical coordinate representations of solvent composition in RP-HPLC,¹⁷⁸ prediction of the RP-HPLC retention of steroids using solvatochromic parameters,¹⁷⁹ the use of 2³ factorial design and computer simulation,¹⁸⁰ triangle optimization for the separation of finasteride and related compounds,¹⁸¹ the effect of the pH of the mobile phase for the separation of ionisable steroids,¹⁸² the effect of column overloading on the separation of mometasone furoate and clotrimazole at widely different concentrations.¹⁸³

The first example for the improvements in the selectivity of the UV *detection* in the HPLC analysis of steroids is the detection of co-eluting species in *e.g.*, danazol by subtracting the up-slope and down-slope diode-array spectra from the apex spectrum.¹⁸⁴ HPLC-MS is an excellent tool for estimating minor components under overlapping peaks, *e.g.*, the identification and quantitation of as low as 0.01% prednisolone in hydrocortisone.¹⁸⁵ On-line post-column photochemical derivatization followed by electrochemical detection for the determination of spironolactone-hydrochlorothiazide tablets¹⁸⁶

or diode-array UV detection for various drugs including corticosteroids in creams¹⁸⁷ can be considered to be curiosities. Evaporative light scattering detector can be of importance in the case of UV-inactive steroids such as pancuronium bromide and related compounds.¹⁸⁸ The native fluorescence of ethinylestradiol (ex: 285 nm/em: 310 nm) is used in the fluorimetric detection of the USP dissolution test of levonorgestrel-ethinylestradiol tablets⁷ while terbium(III)-sensitized fluorescence (ex: 245 nm/em: 545 nm) detection was described for various steroids in the course of their SDS micellar HPLC determination.¹⁷⁰

In addition to its analytical application, HPLC is a useful tool in other fields of steroid drug research, too. For example HPLC was used for the determination of apparent association constants of steroid-cyclodextrin inclusion complexes,¹⁸⁹ lipophilicity profiling,¹⁹⁰ for studying drug-membrane interactions¹⁹¹ and high-throughput log *P* measurements.¹⁹² Biopartitioning micellar chromatography with polyoxyethylene (23) lauryl ether (Brij35) as the micelle forming agent was used for the prediction of human drug absorption.¹⁹³ Calculations based on linear solvation energy relationship were found to be useful to find "open windows" for internal standards in RP-HPLC chromatograms of various compounds, among others steroids¹⁹⁴ and these calculations were used to find suitable internal standard for the determination of estradiol and levonorgestrel in transdermal drug delivery formulations.¹⁹⁵

5 Electrophoretic and Related Methods

5.1 Capillary electrophoresis (CE)

Being neutral compounds the overwhelming majority of steroid hormone drugs cannot be analyzed by direct CE. This technique is restricted to ionizable derivatives. For example, the simultaneous determination of hydrocortisone 21-hemisuccinate, oxytetracycline and nystatin in pharmaceutical preparations was carried out at pH 11.¹⁹⁶ Covalent derivatization with electrically charged reagents, *e.g.* ketosteroids with Girard P or T reagents is a solution for this problem: the Girard hydrazones of 4-ene-3-oxo- and 17-oxosteroids can easily be separated and quantified.¹⁹⁷

Capillary electrophoresis can be a useful tool for the preconcentration of different classes of steroids, making use of electroosmotic flow and capillaries partially filled with various reagents, *e.g.*, borate for the complexation of steroids with proximal hydroxyls, high pH buffers for weakly acidic estrogens, micelle forming agents for hydrophobic steroids.¹⁹⁸

5.2 Micellar electrokinetic chromatography (MEKC)

MEKC is an ideal tool for the separation and quantification of lipophilic steroid drugs which cannot be directly analyzed by CE: these (mainly corticosteroids) were among the first models investigated after the invention of this new technique at the mid 1980s.¹⁹⁹⁻²⁰¹ Since then, great many papers have been published on this topic. Based on these, it is predictable that due to the minimum requirements of sample pretreatment, low costs and high speed MEKC can be a real alternative to HPLC in steroid analysis as demonstrated in a comparative study using betamethasone dipropionate, clotrimazole and their related substances as the model compounds.²⁰²

Some of the several new applications of MEKC to pharmaceutical steroid analysis are listed here: the determination of ethinylestradiol and levonorgestrel²⁰³ or gestodene²⁰⁴ in oral contraceptives, hydrocortisone and its acetate together with various antibiotics,²⁰⁵ prednisolone, Zn-

bacitracin and phenylephrine in ointments and ocular drops,²⁰⁶ prednisolone acetate, sulfacetamide and phenylephrine in local pharmaceutical preparations,²⁰⁷ dexamethasone, trimethoprim and polymyxin B in various formulations,²⁰⁸ betamethasone in dissolution tests.²⁰⁹

In the above-listed studies (and the majority of those not mentioned here) sodium dodecyl sulfate (SDS) was used as micelle forming agent in the background electrolyte. In a few cases, bile salts were also used.^{199,209} A comparison of the two systems using corticosteroids as the model compounds is also described.²¹⁰ High concentrations of organic solvents (up to 50% acetonitrile or methanol) in the background electrolyte containing SDS enables ionic (benzalkonium chloride) and non-ionic (beclomethasone dipropionate, 2-phenylethanol) compounds to be determined within one electrokinetic run.²¹¹ Using a related method, termed hydrophobic interaction electrokinetic chromatography seven active ingredients of an ointment (among them hydrocortisone) were separated and quantitated. The migration behavior of the hydrophobic and ionic constituents was influenced by the concentration of tetradecyl ammonium bromide and ammonium chloride, respectively.²¹² On-line sample preconcentration (370-fold improvement in detector response) was achieved for steroids by sweeping with anionic-zwitterionic micelles.²¹³

Chiral aspects of MEKC are shown in the section "Enantiomeric analysis".

5.3 Microemulsion electrokinetic chromatography (MEEKC)

Although in an early study better separation characteristics were attained for steroids with MEEKC than with the related technique MEKC, this method does not seem to have been used as extensively in the practice as MEKC. In this study SDS or glycodeoxycholate were used as the micelle forming agent in MEKC and various higher alcohols, *n*-hexane, cyclohexane, *etc.* with SDS as the emulsion-forming solvents.²¹⁴ In some further studies aiming at comparing the selectivities of the two techniques among many other compounds some steroids were also included.²¹⁵⁻²¹⁷ A good correlation was found between the retention characteristics of betamethasone and its esters and the respective log P_{ow} values;²¹⁸ MEEKC is a good tool for the rapid estimation of log P_{ow} values of a wide range of compounds, among them steroid hormone drugs.²¹⁹

5.4 Capillary electrochromatography (CEC) and CEC-MS

CEC, which can be considered to be the combination of RP-HPLC and CE, has been successfully applied to separation and quantitation of steroid drugs. The performance of HPLC, MEKC and CEC was compared using six steroids (androst-4-ene-3,17-dione, testosterone, norethisterone, progesterone and the 17-OH and 20-OH derivative of the latter). Higher peak efficiencies were obtainable by MEKC and CEC.²²⁰ With an in-house packing method of capillaries up to 190.000 plates per meter was achieved.²²¹ High-temperature CEC and temperature programming resulted in a considerable reduction of the separation run time of steroids, and the latter may be a good alternative to solvent programming.²²² Usually ODS phases are used for steroid separations but the successful use of macroporous, spherical polystyrene-divinylbenzene stationary phase was also described.²²³ Of the practical applications the rapid analysis of norgestimate and its degradants,²²⁴ tipredane and related impurities²²⁵ and fluticasone propionate and related impurities²²⁶ is mentioned. An example for chiral CEC is presented in the section "Enantiomeric analysis".

The very promising possibilities of the new technique CEC-

MS have been reviewed with several examples from steroid drugs.²²⁷ The identification of impurities in fluticasone propionate²²⁸ and the combination of CEC with nanospray MS with detection limits of 50 fg for corticosteroids merit special attention.²²⁹

6 Electroanalytical Methods

A review of electroanalytical methods in steroid analysis was published in 1989.²³⁰ These methods have never played very important role in this field but sufficiently sensitive and selective methods are often published up to the present time mainly for the assay of formulations. In the majority of cases differential pulse polarography, based on the reduction of the 4-ene-3-oxo group is used, for *e.g.* spironolactone,²³¹ beclomethasone dipropionate,²³² dexamethasone sodium phosphate²³³ and betamethasone valerate.²³⁴ Gestodene was determined in oral contraceptives by square-wave voltammetry and adsorptive stripping techniques.²³⁵

A direct potentiometric method was developed for muscle relaxant quaternary ammonium compounds, among them pancuronium bromide, based on ion-selective membrane electrodes prepared from ion pairs with tetraphenylborate or dipicrylamine as the counter ions in a PVC matrix.²³⁶

7 Selected Analytical Tasks

7.1 Impurity and degradation profiling of steroid drugs^{237,238}

Impurity and degradation profiling *i.e.* detection, identification/structure elucidation and quantitative determination of impurities and degradants usually require the complex application of one or two separation method and at least 2–3 spectroscopic techniques (usually UV, MS, NMR) either off-line but preferably on-line.

Of the innumerable studies, a few resulting in interesting structures are listed here beginning with corticosteroids. Six synthesis-related impurities were identified in prednisolone.²³⁹ Several synthesis-related impurities^{240,241} and degradants^{242,243} (mainly products of intramolecular oxido-reduction reactions) were identified in the course of the stress stability test of mazipredone (21-deoxy-21-*N*-methylpiperazinyl derivative of prednisolone). Among minor impurities the 9 α -bromo analogue of the parent drug was found as the main impurity in triamcinolone acetonide.²⁴⁴ Of the other corticosteroids several synthesis-related impurities and degradants were identified in dexamethasone.^{245,246} Of these, the 17 β -carboxy-17 α -formyloxy derivative forming under storage conditions *via* a mixed anhydride is especially interesting.²⁴⁶ The structure elucidation of impurities in fluticasone propionate (among others an interesting –S–S– bridged dimer) is a good example for the application of new techniques such as CEC-MS²²⁸ and on-line HPLC-NMR.²⁴⁷ The light-induced degradation of methylprednisolone suleptamate lead to interesting, unpredictable structures.²⁴⁸ Of the (contraceptive) gestogens (norethisterone, norgestrel) by-products of their ethinylation step,^{240,249} oxidative degradation products of these²⁵⁰ and of nestorone²³⁹ are described. *Z* and *E* isomers of the 17-(3'-acetoxy-2'-butenoate)^{251,252} and 17-(3-oxo-butenate)¹ identified in ethynodiol diacetate are by-products of the acetylation of the 17-OH group. Impurities found in allylestrenol originate from its total synthesis.²⁴⁰

Two estrogens are also worth mentioning. In the course of the impurity profiling of estradiol HPLC-UV played predominant

role,²⁵³ while HPLC-NMR and HPLC-MS were necessary to determine the structures of five most interesting structures (a hydroperoxide and four different dimers) in the course of oxidative and light-stress studies of ethinylestradiol.²⁵⁴

Of the other hormonal drugs, several impurities originating from the Birch reduction step were identified in norethisterone,^{239,250} an isomeric impurity in danazol²⁵⁵ and several oxidative degradation products in tipredane.^{225,256} Of the non-hormonal steroid drugs pipercuronium bromide is mentioned. An oxidative degradation product^{243,251} and synthesis-related impurities were identified and quantified by NP-HPLC,¹³² NP-ion-pairing HPLC²⁵⁷ and the oxidative degradant also by quantitative NMR spectroscopic measurement.²⁵¹

7.2 Enantiomeric analysis

Although steroid hormone drugs usually contain 6–7 chiral centers, their configuration is fixed in natural steroids and the semi-synthetic drugs prepared from them. For this reason enantiomeric analysis is not necessary in the overwhelming majority of cases. The only important exception is the total synthetically prepared norgestrel, which is mainly administered as enantiomerically pure levonorgestrel, and also (at a decreasing extent) as the racemate. Several papers have been published on the separation of the enantiomers of norgestrel. Only a few of these deal with the determination of the enantiomeric purity of levonorgestrel. In the first validated method RP-HPLC with γ -cyclodextrin in the mobile phase was used and a limit of quantitation of 0.1% dextronorgestrel was achieved.²⁵⁸ (With β -cyclodextrin fairly good separation is achievable at 0°C only.)²⁵⁹ Using α_1 -acid glycoprotein (Chiral AGP) column for the HPLC separation good separation was obtained,^{260,261} but the limit of quantitation was inferior (0.6%).²⁶⁰

Norgestrel is an excellent model compound for testing the separation efficiency of various enantioselective chromatographic and related techniques. For this reason many papers contain data for norgestrel, usually among many other model compounds. Acceptable to excellent separation was achieved using Chiralcel-OJ,²⁶² cellulose tris(3,5-dichlorophenylcarbamate),²⁶¹ amylose tris(3,5-dimethylphenylcarbamate),^{263,264} and covalently bonded β -cyclodextrin,^{265–267} even bonded to a monolithic silica column.²⁶⁷ SFC using immobilized polysiloxane-anchored permethyl- β -cyclodextrin (Chirasil-Dex)²⁶⁸ and teicoplanin or its aglycone (Chirobiotic T or TAG)²⁶⁹ was also used to separate the enantiomers of norgestrel. The above-mentioned cellulose tris(3,5-dichlorophenylcarbamate) and amylose tris(3,5-dimethylphenylcarbamate) were successfully used for CEC separation of the enantiomers.^{270,271} A comparison of the latter in the HPLC and CEC modes revealed significant advantages of CEC.²⁷¹ This chiral selector was successfully used also for the HPLC separation of as many as 26 steroid enantiomeric pairs including estradiol, estrone, ethinylestradiol, nandrolone, etc.^{263,264}

¹H-NMR was found to be an excellent tool for modelling interactions between norgestrel enantiomers and cyclodextrins, thus creating the possibility to find a correlation between the NMR and RP-HPLC data.²⁷² In addition to this, NMR spectroscopy using γ -cyclodextrin²⁷³ or the lanthanide shift reagent praseodymium tris[3-heptafluoropropyl-hydroxymethylene-(+)-camphorate]²⁶⁰ was found to be suitable for estimating the enantiomeric purity of levonorgestrel without separation. The limit of detection is somewhat higher than that achievable by HPLC.

8 References

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