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Estimation of impurity profiles of drugs and related materials
Part 19: Theme with variations. Identification of impurities in 3-oxosteroids☆,☆☆

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Abstract

Due to the varied reactions leading to the 3-oxo group in steroids and the reactivity of its environment, a large number of impurities related to this group are formed during the reaction steps and the degradation studies. In this paper the experiences from the authors laboratory with the 3-oxo-related impurities in 19-nor-4-ene-3-oxosteroids (norgestrel, norethisterone, nandrolone, its esters and Nestorone™) as well as corticosteroids (prednisolone, mazipredone, etc) are presented. The impurities include saturated 3-ones, 1-ene-3-ones, 5(10)-ene-3-ones, 3-deoxo and 3-ethinyl-3,5-diene derivatives, 6-ene, 8(14)-ene, 6,8(14)-diene, 6-hydroxy (α and β), 10β-hydroxy and 6-one derivatives in 4-ene-3-oxosteroids and 8(9)-ene, 9(11)-ene, 11α-hydroxy, 11-oxo and 4-ene-3-one derivatives in 11β-hydroxy-1,4-diene-3-oxosteroids. The chromatographic, spectroscopic and hyphenated techniques used in this study include TLC, GC, HPLC with diode array UV detector, GC-MS, LC-MS and NMR methods.

Keywords: Impurity profiles; 3-Oxo steroids; Nandrolone esters; Norethisterone; Norgestrel; Nestorone™; Prednisolone; Mazipredone

1. Introduction

The unusual subtitle of this paper is based on the authors proposal of an analogy between the well known genre in classical music 'Theme with Variations', and the picture presented by some drugs with their closely related impurities. For example in Bach's 'Goldberg Variations',

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Beethoven's 'Diabelli Variations' and many other pieces, the theme played on the piano is followed by several variations where the mode of presentation, the rhythm and the decoration of the theme are varied, rather than the tune itself. In an analogous manner, the general structure of the related impurities in a drug is usually the same while the variations are restricted to certain functional groups and their close neighbours.

The aim of this paper is to survey the impurities related to a single functional group, the 3-oxo group in steroid drugs. This group occurs mainly as the 4-ene-3-one or 1,4-diene-3-one moiety in androgenic, gestogenic, corticosteroid hormones and their synthetic analogues. Due to the very complex nature of the reaction steps leading to the 4-ene-3-oxo group, especially in total synthesis of the 19-nor analogues and also in the combination of microbiological and chemical reactions used for the preparation of corticosteroids with the 1,4-diene-3-one moiety, the number of variations seen as impurities related to the 3-oxo group is very high more especially because of their susceptibility to atmospheric air oxidation, particularly at position six.

This paper summarises the experience obtained in the authors laboratory in the course of the separation and identification of 3-oxo related impurities in various steroid drugs such as nandrolone (19-nortestosterone) and its esters, norethisterone, norgestrel, Nestorone™ (16-methylene-17α-acetoxy-19-norpregn-4-ene-3,20-dione, the experimental progestin drug of the Population Council, New York), prednisolone and mazipredone (the 21-deoxy-21-*N*-methylpiperazinyl analogue of prednisolone).

The analogy between 'Theme with Variations' and drug impurity profiling can be extended to include the analogy between the development of many different arrangements of a musical theme in orchestral variations, e.g. Brahms 'Variations on a theme by Haydn', and the various hyphenated combinations of instrumental techniques for solving the analytical problem. In this paper a number of hyphenated approaches to impurity profiling based on HPLC/DAD-UV, HPLC/DAD-UV/MS, GC/MS, plus GC, TLC and NMR spectroscopy are presented.

2. Experimental

2.1. Materials and reagents

All samples investigated were industrial or laboratory products of the Chemical Works of Gedeon Richter, Budapest. HPLC grade solvents and analytical grade reagents were purchased from Merck.

2.2. Instruments and methods

2.2.1. High-performance liquid chromatography and HPLC-mass spectrometry

A Hewlett-Packard model 1100 chromatograph was used with built-in diode-array UV detector.

The HPLC-MS systems were a Waters 600 M liquid chromatograph equipped with a Waters 490 UV detector attached to a Finnigan MAT 95 SQ

Table 1
Shift of λ_{max} in the UV spectra of 3-oxo-related impurities in 4-ene-3-oxo steroids[a]

Impurity	Origin	$\Delta\lambda$ nm
4-Ene-3-one	–	0
4,5-Dihydro	Birch reduction	UV-inactive
5(10)-Ene-3-one	Birch reduction/hydrolysis	UV-inactive
3-Deoxo	Birch reduction	UV-inactive
6α-Hydroxy	Oxidative degradation	−1
6β-Hydroxy	Oxidative degradation	−5
10β-Hydroxy	Oxidative degradation	−7
4-Ene-3, 6-dione	Oxidative degradation	+14
4,6-Diene-3-one	Oxidative degradation	+43
4,8(14)-Diene-3-one	Total synthesis	−5[b]
4,6,8(14)-Triene-3-one	Total synthesis/degradation	+108[c]
3-Deoxo-3-ethynyl-3,5-diene	Ethynylation	+26[d]
1-Ene-3-one	Birch reduction	−10

[a] The solvent dependent λ_{max} of the parent compound is at about 240 nm.
[b] Band broadening.
[c] Strong fluorescence (Ex: 375 nm, Em: 493 nm).
[d] Fine structure.

Table 2
Shift of λ_{max} in the UV spectra of 3-oxo-related impurities in 1,4-diene-3-oxo-11β-hydroxy steroids[a]

Impurity	Origin	$\Delta\lambda$ (nm)
11β-Hydroxy-1,4-diene-3-one	–	0
1,2-Dihydro	Incomplete dehydrogenation	0[b]
11α-Hydroxy	Fermentation	+6
11-Oxo	Fermentation	−4
11-Deoxy	Fermentation	+2
8(9)-Ene	Synthetic steps	−2
11-Deoxy-9(11)-ene	Synthetic steps	−4[c]

[a] The solvent dependent λ_{max} of the parent compound is at about 240 nm with shoulder at 265 nm.
[b] Narrow band; no shoulder.
[c] Shoulder at 270 nm.

hybrid-tandem mass spectrometer and a Hewlett-Packard 1100 LC-MSD.

2.2.2. Gas chromatography and GC-mass spectrometry

A Hewlett-Packard 5890 Series II gas chromatograph with flame-ionisation detector and a Fisons MSD 800 instrument were used.

2.2.3. Thin-layer chromatography and densitometry

TLC aluminium sheets Kieselgel 60 F254 (Merck, Order No. 5554) were used as the stationary phase. A Desaga CD-60 densitometer was used for measuring the densitograms and scan the reflection spectra.

2.2.4. NMR spectroscopy

The spectra were recorded on Varian VXR-300 or Varian ^{UNITY}INOVA-500 NMR spectrometers at 30°C in $CDCl_3$ or DMSO-d_6. Chemical shifts are given relative to δ_{TMS}. In addition to the 1D ^1H and ^{13}C NMR spectra gradient enhanced HSQC, HMBC, MQFCOSY and NOE spectra were prepared for the assignment of the signals.

2.2.5. Methods

Some data on the chromatographic methods are presented in the figure captions and the text of Section 3.

3. Results and discussion

3.1. General considerations

The general strategy used in the authors laboratory for the systematic identification of unknown impurities and degradation products in drugs has been recently described [2]. The first step is to draw as many conclusions from the HPLC/diode-array or TLC/reflection UV spectra as possible. If the information thus obtained is not sufficient, this is followed by on-line or off-line chromatographic/mass spectrometric and finally NMR spectroscopic investigations.

3.2. HPLC/diode-array UV investigations

The capabilities of UV spectroscopy as a tool with diagnostic value in the identification of related impurities are much better in the case of 4-ene-3-oxo or 1,4-diene-3-oxo steroids than with other classes of drug materials. The reason for this is that the position of their intense and characteristic $\pi-\pi^*$ band around 240 nm is very sensitive to changes in the vicinity of the unsaturated ketone group.

Tables 1 and 2 show the main types of 3-oxo-related impurities in 4-ene-, and 1,4-diene-3-oxo steroids, respectively, the reasons for their presence and the shift in nm of their UV maxima related to the parent unsaturated ketosteroid. (The reason for presenting the shift rather than the wavelength of the maximum itself is the well known fact that the latter is extremely sensitive to the dielectric constant of the solvent i.e. the HPLC eluent. For example the maximum of norethisterone in hexane and water is at 228, and 247 nm, respectively [3]).

As shown in Fig. 1, the reason for the formation of the spectrophotometrically inactive 4,5-dihydro and 3-deoxo derivatives is the over-reduction of the phenolic ring A in the Birch reduction step of the synthesis of 19-nor-4-ene-3-oxosteroids [4]. The source for 1-ene-3-oxo steroid type impurities is the irregular Birch reduction [4–6], while the presence of 5(10)-ene-3-ones indicates incomplete reaction in the hydrolysis/rearrangement step after the Birch reduction.

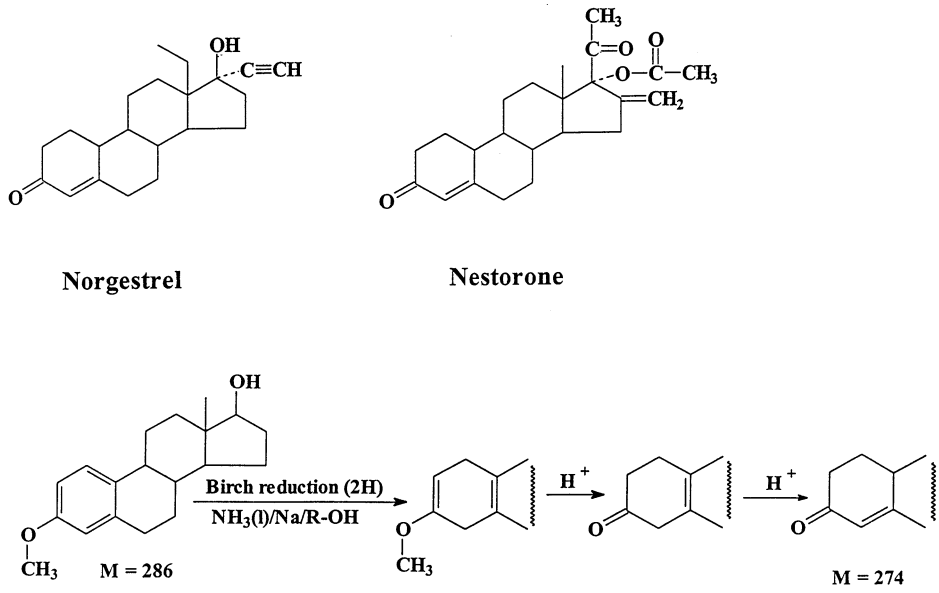


Fig. 1. Main reaction and side reactions of the Birch reduction of 3-methoxy-estra-1,3,5(10)-triene-17β-ol (estradiol-3-methyl ether) followed by hydrolysis to form 17β-hydroxy-estr-4-ene-3-one (19-nortestosterone, nandrolone).

Some impurities in Table 1 are oxidative degradation products. These can easily be separated by HPLC and the extension of the conjugation in the case of 4,6-diene-3-one and 4-ene-3,6-dione type impurities leads to very characteristic wavelength shifts. Even the minor changes due to the introduction of hydroxy groups at position 6α, β and 10β are of diagnostic value [6]. This is illustrated in Figs. 2 and 3, where the chromatogram and the diode-array UV spectra of the impurities of a crude Nestorone™ sample are depicted.

The reason for the presence of 8(14)-dehydro derivatives in almost all 19-nor steroids is a side reaction during their total synthesis [2,7]. These can easily be separated from the parent drugs by HPLC and the shift of the 4-ene-3-one band and especially the change of its shape due to the 8(14) double bond are of diagnostic value [7,8]. The strongly fluorescent 4,6,8(14)-triene-3-one derivative [9], is likely to form from the 8(14)-dehydro derivative by autooxidation. In Figs. 4 and 5 the chromatogram and the diode-array spectra of norgestrel spiked with the latter two impurities are shown.

The formation, chromatographic and spectroscopic characteristics of the unusual impurity 3-deoxo-3-ethynyl-3,5-diene have been recently described [10].

The corticosteroids which were subjects of this study (prednisolone and mazipredone [1]), contain a 11β-hydroxy group, this is why the 11β-hydroxy-1,4-diene-3-one system has been considered in Table 2 as the chromophoric system of the parent drug. In spite of the fact that the λ_{max} values of the 4-ene-3-one and 1,4-diene-3-one systems are at exactly the same wavelength, the peak shape is so much different that the diode-array UV spectrum is of diagnostic value [1,2]. Of the other derivatives (impurities) the difference between the position and shape of the bands of the 11-deoxy-9(11)-ene and the parent 11-hydroxy steroid are also of diagnostic value [1,2], and the same applies to the amazingly great difference between the position of the maxima in the spectra of the epimeric 11α and β hydroxy derivatives. The spectra are depicted in Fig. 7. Generally speaking, however, the information obtainable from the UV spectra in this case is much less important than with the previously discussed 4-ene-3-ones thus necessitating HPLC/MS investigations.

Side reactions

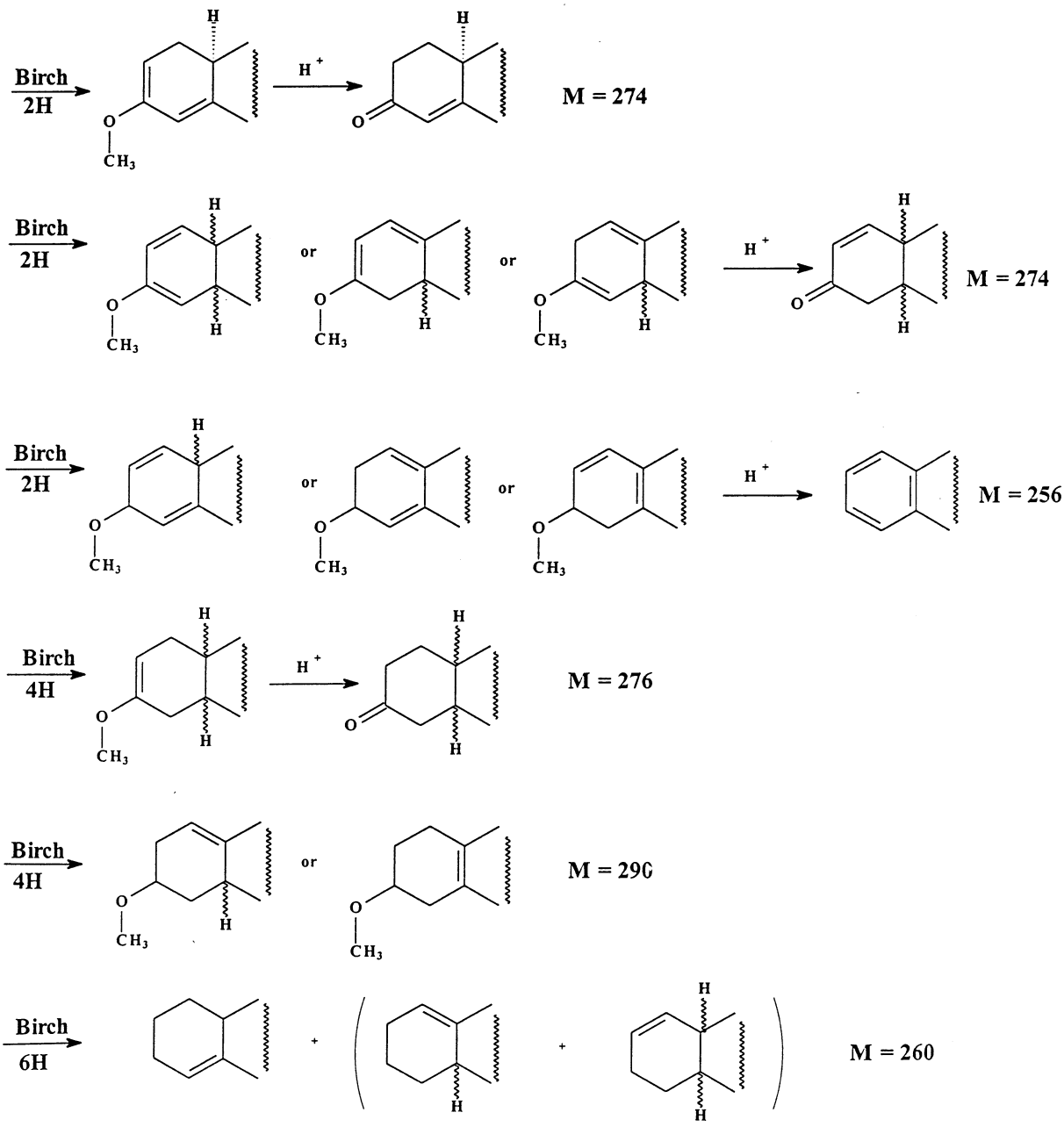


Fig. 1. (*Continued*)

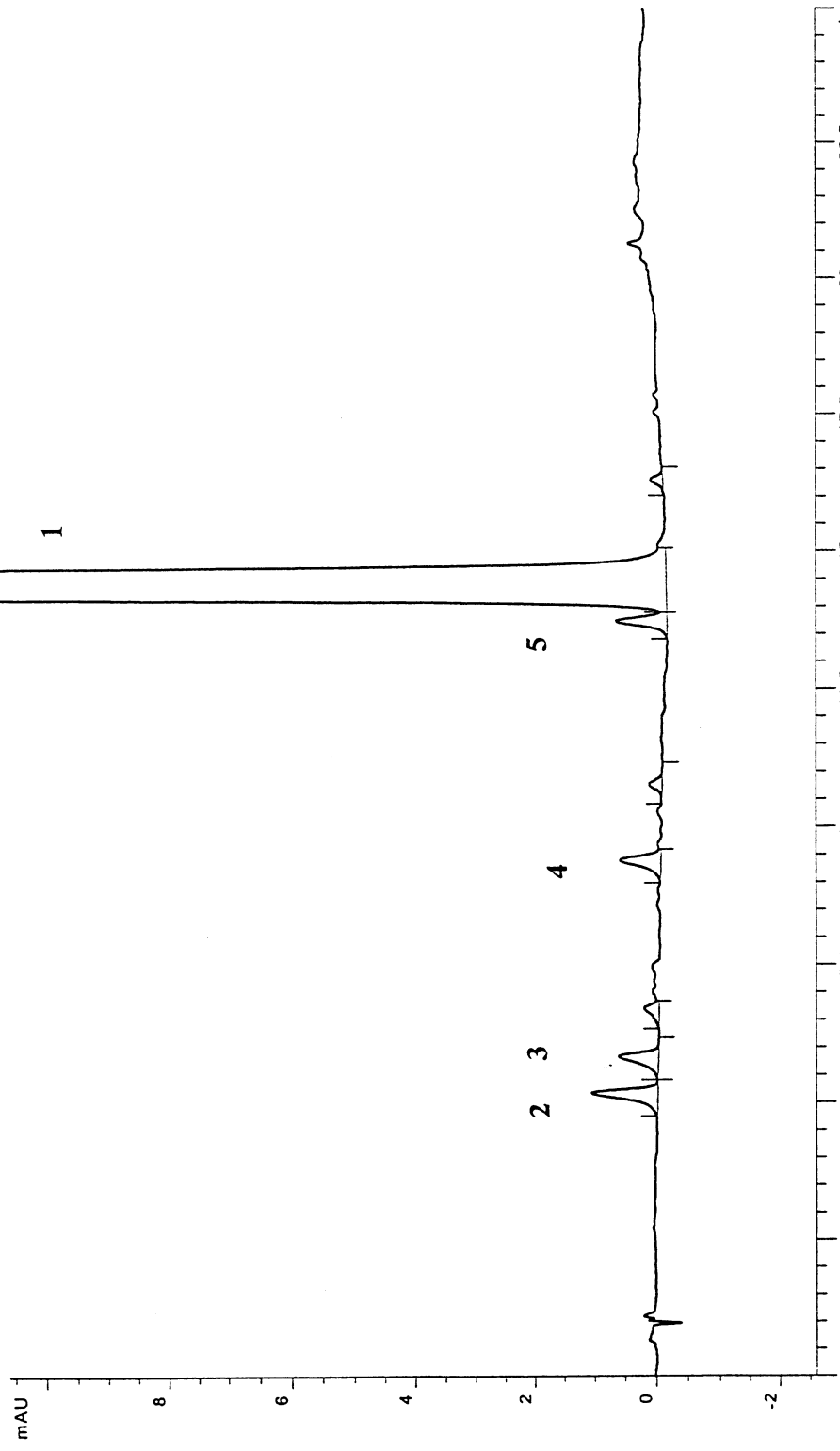


Fig. 2. HPLC separation of Nestorone™ (peak 1) and its impurities, 6α-hydroxy (2), 6β-hydroxy (3), 6-oxo (4), 6-ene (5) derivatives. Column, Waters Nova-Pack C-18, 4 μm, 150 × 3.9 mm. Gradient elution. A, acetonitrile-water 1:9 v/v, B, acetonitrile. Linear gradient, 0 min 25% B, 14 min 45% B, 20 min 75% B. Flow rate, 1 ml min^{-1}. UV detector, 254 nm. Temperature ambient.

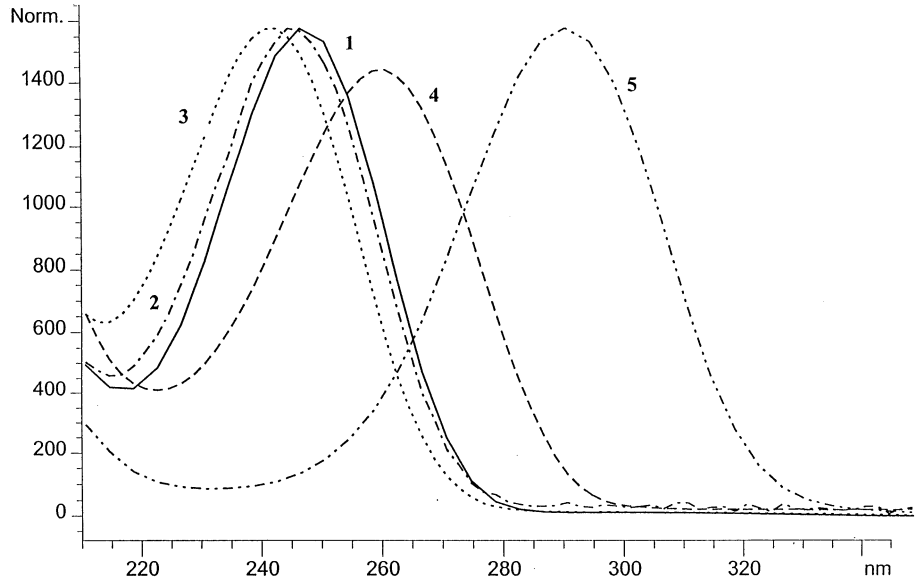


Fig. 3. Diode-array UV spectra of Nestorone™ and its impurities. For the Key see Fig. 2.

3.3. HPLC/UV/MS investigations

The chromatograms of a prednisolone sample simultaneously scanned by diode-array UV (a) and the mass spectrometric (b) detectors are shown in Fig. 6. The molecular masses obtained from the mass spectra together with the UV spectra Fig. 7, furnished sufficient evidence to support the structures presented in Fig. 8. The molecular masses are shown in Fig. 6(b). It is to be noted that the 1,2-dihydro derivative (hydrocortisone) is not sufficiently separated from prednisolone in the given HPLC system, it was, however, easily recognisable in the course of the peak purity tests.

3.4. Gas chromatographic and GC/MS investigations

As a consequence of their highly polar character and the instability of the side chain at C-17, gas chromatography is not suitable for impurity profiling of corticosteroids [11,12]. Although at high temperature the 17α-ethynyl-17β-hydroxy system is also rather unstable, under optimised conditions gas chromatography is suitable for the solution of delicate problems in the field of impurity profiling of 19-nor-4-ene-3-one type gestogenic hormone analogues such as the separation of traces of the epimeric 17β-ethynyl-17α-hydroxy derivative [13,14], and of the 8(14)-ene derivative, mentioned in Section 3.2.

The most important application of gas chromatography and GC/MS in this field was the detection, identification and quantitation of the spectrophotometrically inactive impurities in Table 1 originating from the Birch reduction of 3-methoxy-1,3,5(10)-trienes which is the key step in the synthesis of 19-nor-4-ene-3-oxo steroids [4]. Fig. 9 shows the GC/MS scan of a crude 19-nortestosterone (17β-hydroxy-estr-4-ene-3-one) sample prepared by Birch reduction from estradiol-3-methyl ether (peak 20; $M = 286$) (see the main reaction in Fig. 1). The following conclusions could be drawn from Fig. 9. In addition to the main component (peak 1) several minor components have the molecular mass of 274 (peaks 15–19, 21). These are the 5(10)-ene-3-one isomer (peak 16) and the stereoisomers of the main component and of the 1-ene-3-one isomer [6]. The formation of these is shown in Fig. 1. The reason for the formation of the unsubstituted 1,3,5(10)-triene is the addition of two hydrogens at posi-

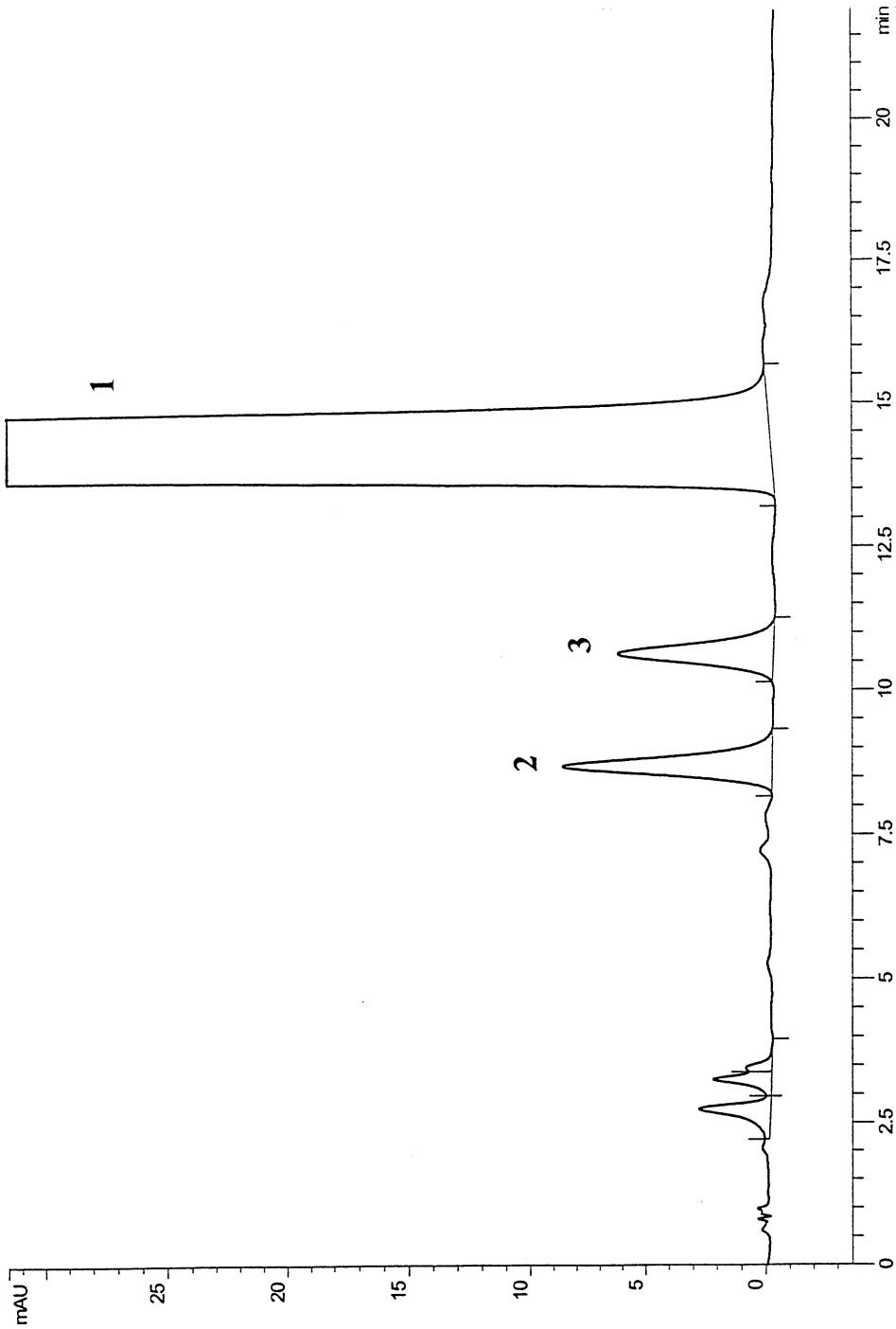


Fig. 4. Chromatogram of norgestrel (peak 1) spiked with 6,8(14)-dehydronorgestrel (2) and 8(14)-dehydronorgestrel (3) (0.5% each) Column, Hewlett-Packard Spherisorb ODS 2, 5 μm, 125 × 4.0 mm. Eluent, acetonitrile-methanol-water 27:18:55 v/v/v. Flow rate, 1 ml min^{-1}. UV detector, 240 nm (reference 360 nm; for peak (2) 355 nm; reference 240 nm). $t = 40°C$.

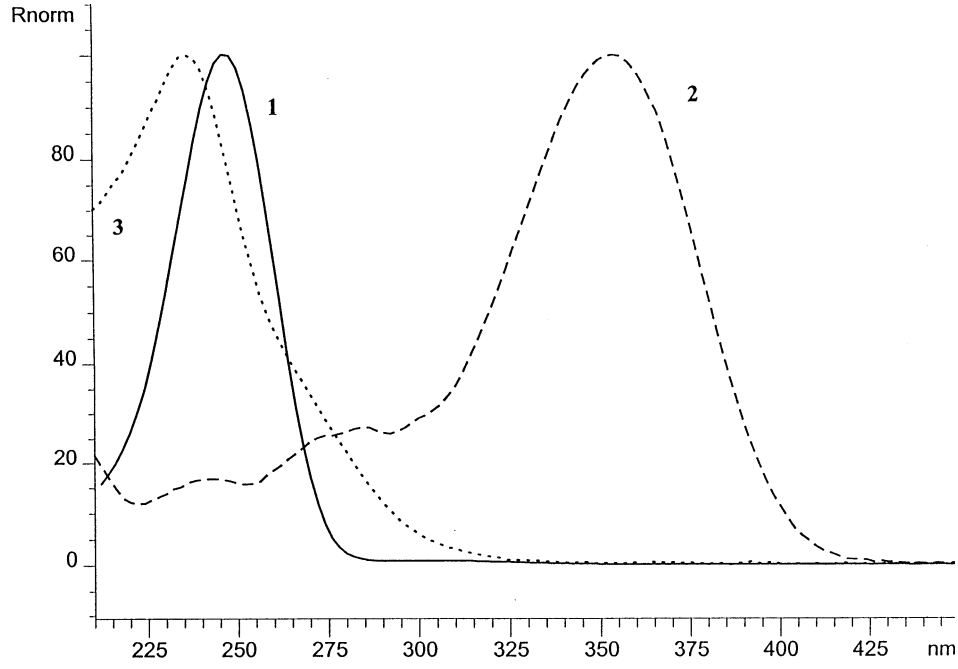


Fig. 5. Diode array UV spectra of the steroids separated in Fig. 4, norgestrel (1), 6,8(14)-dehydronorgestrel (2) and 8(14)-dehydronorgestrel (3).

tions 3,10 or 2,3 or 3,4 followed by acid-catalysed elimination of methanol (peak 8; $M = 256$). Of the other impurities in Figs. 1 and 9, the following products of various types of over-reduction (addition of 4 or 6 hydrogens), have been identified on the basis of the mass spectra from the GC/MS scan and NMR spectra of samples obtained from the crude reduction product by preparative HPLC: (a) stereoisomeric 4,5-dihydro-3-oxo derivatives (peaks 13 and 14; $M = 276$); (b) 3-methoxy-1(10)-ene or 3-methoxy-5(10)-ene derivative (peak 11; $M = 290$); and (c) 3-deoxo derivatives: 4-ene (peak 5) accompanied by 1-ene and 1(10)-ene and minor isomers (peaks 2,3,5,6) ($M = 260$). Other minor impurities with molecular masses of 258 (peaks 7 and 12) and 284 (peak 9) contain a further double bond in ring B or C which originates from the total synthetic steps prior to the Birch reduction. Fig. 9

It is worth mentioning that the 3-deoxo derivative in norgestrel originating from the above mentioned by-product of the Birch reduction was detected and identified by GC/MS [10].

3.5. Thin-layer chromatographic investigations

Thin-layer chromatography is suitable for the separation and quantitation by densitometry of almost all impurities in Tables 1 and 2, and can play an important role in their identification and structure elucidation. For example the 4,5-dihydro derivative as an impurity in 19-nortestosterone (nandrolone) decanoate was separated from the main component using 7:3 v/v mixture of hexane and acetone as the mobile phase; (R_f values of the impurity and the main component are 0.66, and 0.53, respectively). The impurity was identified by mass spectrometry after spot elution. Other examples are the separation of epimeric 6-hydroxy derivatives of norethisterone and norgestrel. The R_f values in a 80:20 v/v mixture of chloroform and acetone as the mobile phase were 0.74 for norethisterone and 0.36 and 0.40 for the 6α-, and 6β-hydroxy derivatives, respectively. For the separation of the same impurities in norgestrel a 85:15 v/v mixture of the same solvents was used. (R_f values, 0.78, 0.43 and 0.46). Fig. 10 shows the

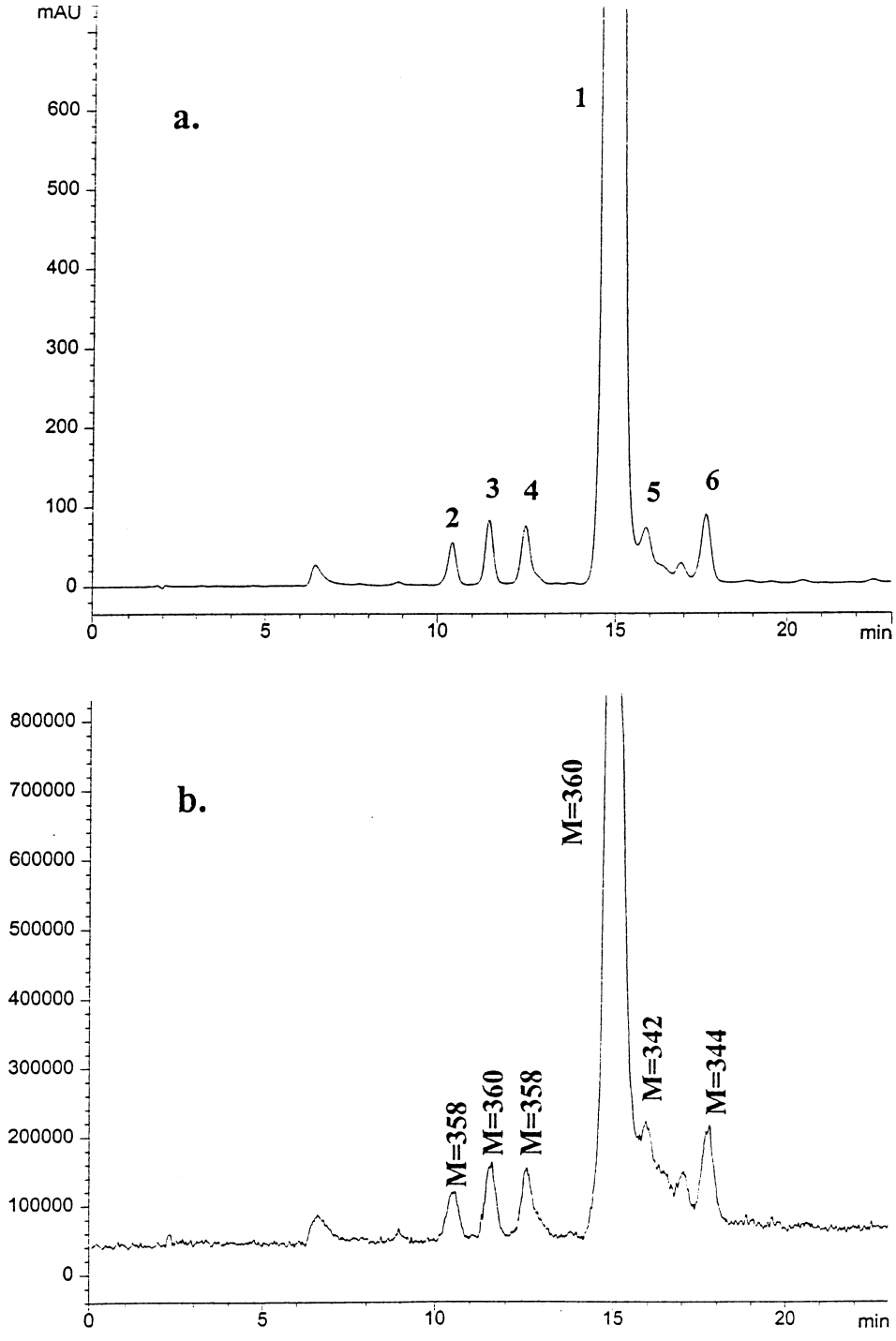


Fig. 6. HPLC separation of prednisolone and its impurities. For the Key see the numbering of the formulae in Fig. 8 (a) UV scan. (b) MS scan with the molecular masses. Column, Merck LiChrospher 100 RP-18, 5 μm, 125 × 4.0 mm. Gradient elution. A, 0.1 M aqueous ammonium acetate-water-methanol 10:80:10 v/v/v, B, 0.1 M aqueous ammonium acetate-methanol 10:90 v/v. Linear gradient, 0 min 40% B, 40 min 80% B. Flow rate, 0.5 ml min^{-1}. UV detector, 240 nm. MS parameters, electrospray positive ionisation mode, drying gas temperature 200°C, flow rate, 10 l min^{-1}, nebuliser pressure, 172 kPa, capillary voltage, 3500 V, TIC scan.

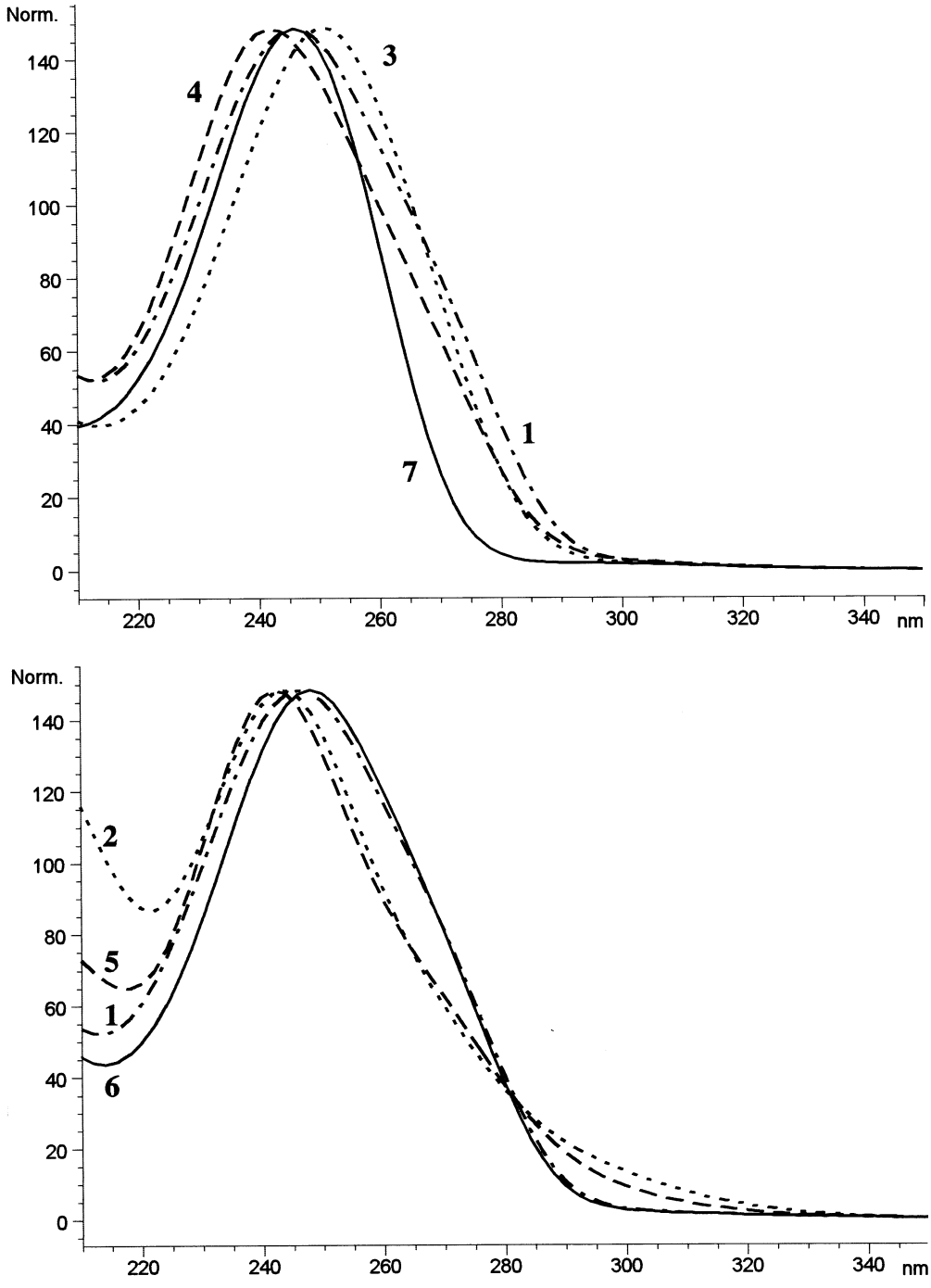
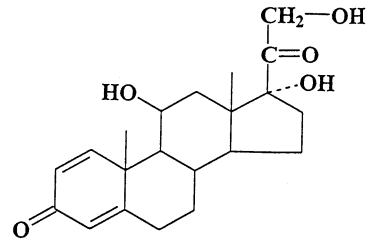


Fig. 7. Diode-array UV spectra of prednisolone and its impurities. For the Key see Fig. 8.



Prednisolone (1)

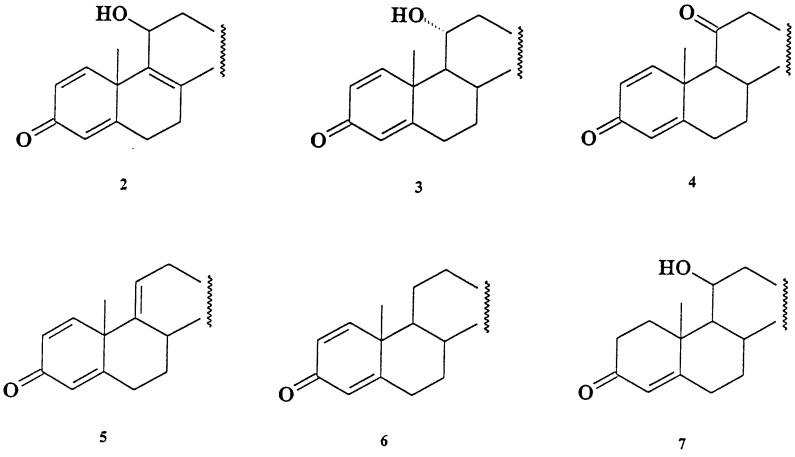


Fig. 8. Structures of prednisolone and its impurities.

reflection UV spectra of norgestrel and its separated epimeric 6-hydroxy impurities. As it is seen, the characteristic difference between their maxima shown in Table 1 and Fig. 3 is observable in the reflection mode, too.

3.6. NMR spectroscopic investigations

The ultimate method for the identification of impurities in drugs mainly after preparative HPLC separation (or by using the recently introduced on-line instrument) is NMR spectroscopy [2,15]. Some characteristic NMR features of 4-ene-3-oxo steroids and their derivatives which are of diagnostic value in the identification of the impurities are as follows.

A quick information on the structure of the isolated impurities is obtainable by investigating their ^{1}H-NMR spectrum in the range downfield of 3 ppm. The lack of signals is an indication of the absence of a hydrogen atom attached to a C=C double bond and a hydroxy, alkyloxy or acyloxy group. If the latter are present, the coupling pattern of the protons on the carbon atom adjacent to the oxygen furnishes evidence regarding its axial or equatorial orientation. The β configuration of the hydroxy groups in 6β-hydroxy and 10β-hydroxy derivatives is easily detectable by the change of the multiplicity of the signal of the 4-H proton from dd to d ($^{4}J = 1-2$ Hz) due to the lack of long range coupling. The ^{13}C-NMR spectra furnish information also on non-protonated carbon atoms (double bonds at ring junctions, conjugated or non-conjugated carbonyl group, etc). Due to the rigid steroid skeleton the spectra are suitable for the investigation of stereochemical problems, too. In the case of

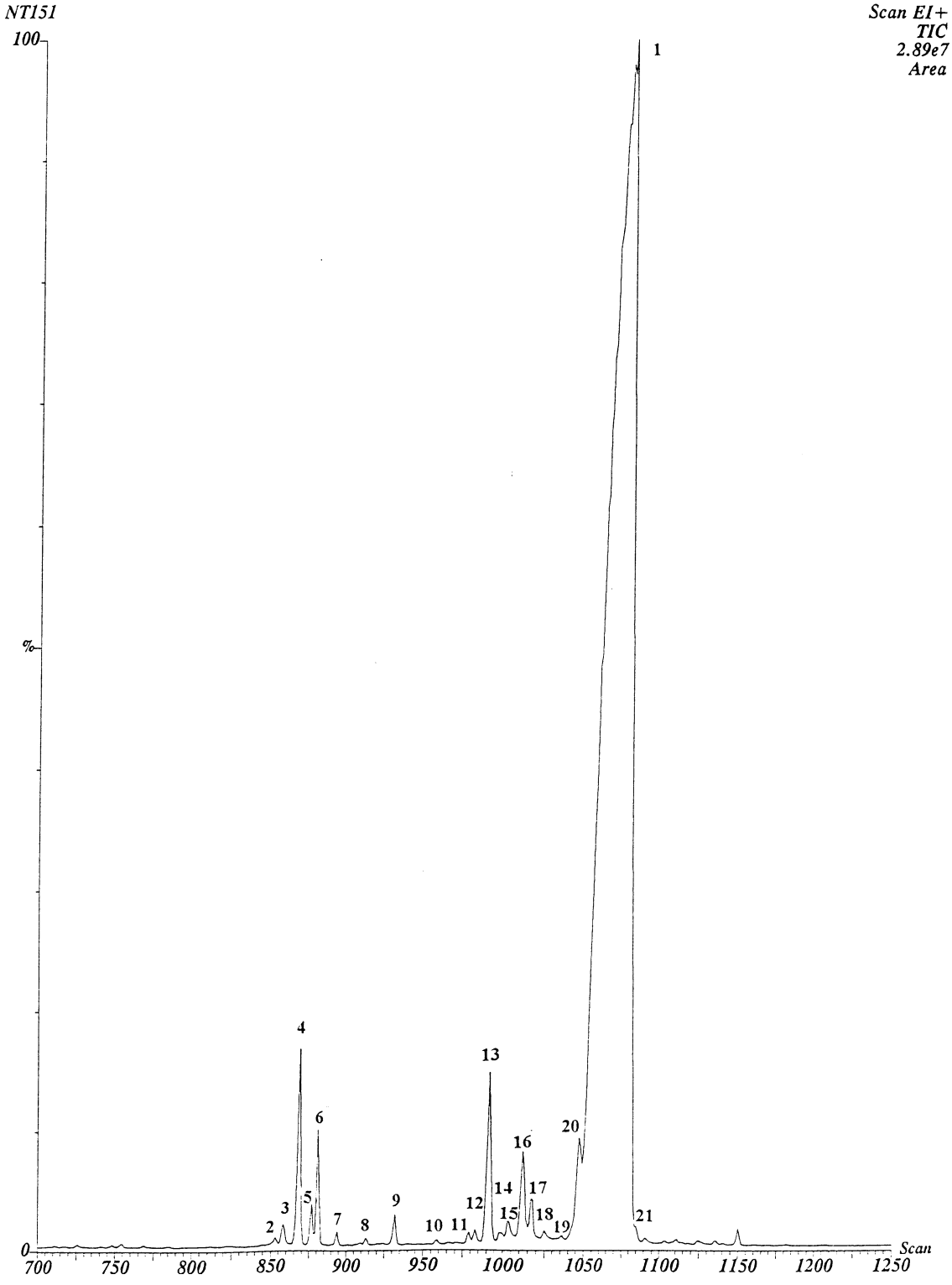


Fig. 9. GC/MS scan of crude 19-nortestosterone. Column, J and W DB-5 30m × 0.25 mm × 0.25 μm. Temperature, injector 260°C, oven 40°C 1 min, 10°C min^{-1} up to 260°C. Injection, 1 μg crude19-nortestosterone; splitless. Ionisation, EI, 70 eV, TIC.For the Key see the text of Section 3.4. Scan 1000 is at 18.63 min.

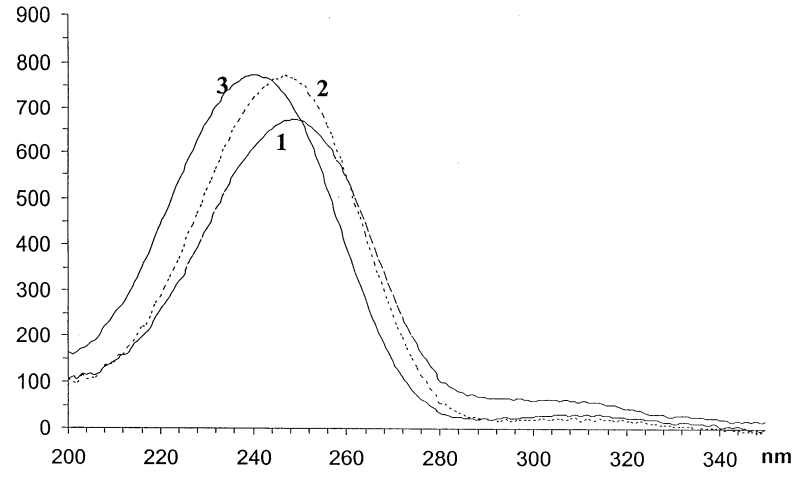


Fig. 10. Reflection spectra of norgestrel (1), 6α-hydroxynorgestrel (2) and 6β-hydroxynorgestrel (3) after TLC separation. For the TLC conditions and R_f values see Section 2.2.3, and Section 3.5, respectively.

4,5-dihydro impurities of 4-ene-3-oxo steroids the 5α and 5β isomers are easily distinguishable on the basis of the chemical shifts of the methine carbon atoms (C-5, C-8, C-9, C-10). Due to the steric interactions (e.g. C-2,4 and C-9) an upfield shift of the methine carbon signals is observable in the case of the 5β-isomer. For example in the case of 4,5-dihydronortestosterone the signals are shifted from 47.9, 45.8, 43.7 and 41.5 ppm to 41.5, 39.8, 38.5 and 38.3 ppm relative to the 5α isomer.

As an example the NMR assignment of 6α-hydroxy-norethisterone (17α-ethynyl-6α,17-dihydroxy-estr-4-ene-3-one) is presented as follows. ^{1}H NMR [DMSO-d_6(TMS)] [δ(ppm)]: 1.54 and 2.16 (2H, m, H-1); 2.15–2.28 (2H, m, H-2); 6.00 (1H, dd, H-4); 4.07 (1H, m, H-6); 0.96 and 1.98 (2H, m, H-7); 1.40 (1H, m, H-8); 0.77 (1H, m, H-9); 2.14 (1H, m, H-10); 1.19 and 1.81 (2H, m, H-11); 1.27 and 1.61 (2H, m, H-12); 1.44 (1H, m, H-14); 1.28 and 1.56 ((2H, m, H-15); 1.84 and 2.07 (2H, m, H-16); 0.78 (3H, s, H-18); 3.28 (1H, s, ≡CH); 5.21 (1H, d, 6-OH); 5.28 (1H, s, 17-OH)

^{13}C NMR [DMSO-d_6(TMS)] [δ(ppm)]: 25.6 (C-1); 35.5 (C-2); 198.3 (C-3); 119.6 (C-4); 169.0 (C-5); 69.5 (C-6); 40.33 (C-7); 38.1 (C-8); 48.7 (C-9); 40.27 (C-10); 25.5 (C-11); 32.2 (C-12); 46.5 (C-13); 48.5 (C-14); 22.5 (C-15); 38.6 (C-16); 77.9 (C-17); 12.6 (C-18); 88.7 (-C≡); 75.0 (≡CH)

Some characteristic NMR signals of the epimeric 6β-hydroxy-norethisterone:^{1}H 5.74 (1H, d, H-4); 4.18 (1H, m, H-6) and ^{13}C 123.7 (C-4); 166.4 (C-5); 69.9 (C-6); 33.7 (C-8); 48.7 (C-9); 37.5 (C-10)

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