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Estimation of impurity profiles of drugs and related materials Part 14: The role of HPLC/diode-array UV spectroscopy in the identification of minor components (impurities, degradation products, metabolites) in various matrices [☆],[1]

S. Görög *, M. Bihari, É. Csizér, F. Dravetz, M. Gazdag, B. Herényi

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

Abstract

The possibility of the rapid identification of drug related minor components by HPLC/diode-array UV spectroscopy is demonstrated by three examples. Hydroxylated impurities (degradation products) of norgestrel (6α and β, 10β-hydroxy derivatives) were identified on the basis of their UV spectra and retention matching with the synthesized impurities. The position of the phenolic hydroxyl groups in the mono- and dihydroxylated metabolites of bisaramil was established by UV spectroscopy and retention matching with the synthesized metabolites. The discrimination between the isomeric 4-ene-3-ketone and 1-ene-3-ketone components in crude 19-nortestosterone, product of the Birch reduction of 3-methoxy-1,3,5(10)-oestratriene-17β-ol, was also based on the diode-array UV spectra.

Keywords: Impurity profiles; Diode-array UV detector; High-performance liquid chromatography; Metabolites; 19-Nortestosterone; Birch reduction; Norgestrel; Bisaramil

1. Introduction

Highly sophisticated instrumentation, such as mass spectrometers attached to a gas chromatograph or high-performance liquid chromatograph (HPLC), are inevitable tools in the identification of minor components (drugs, their impurities, degradation products, metabolites) in various matrices. The ultimate method for structure elucidation problems which cannot be solved

by mass spectrometry is NMR spectroscopy, which in turn may require the isolation (usually by preparative HPLC) of larger samples of the minor component. Since these are expensive and in the latter case very time and labour consuming procedures, it is valuable to draw as many conclusions as possible from the ultraviolet (UV) spectra easily obtainable using rapid scanning (usually diode-array) UV spectrophotometers attached as detectors to high-performance liquid chromatographs.

In addition to their general use for peak homogeneity determination [2], special and very important application fields of diode-array detectors in HPLC [2] are the identification of drugs in biological (mainly toxicological) samples by means of computerized library search [3–8] and identification of metabolites [9–12].

Although the successful application of HPLC/ diode-array UV detectors in the identification of the above mentioned minor components is restricted to those cases where the component is spectrophotometrically active and its spectrum differs sufficiently from that of the main component (parent drug) and from other small components, this technique can be successfully used in the impurity profiling of drugs. Earlier publications from the authors' laboratory describing the application of this technique include impurity profiling of norethisterone and norgestrel [14], ethinyl oestradiol [14], hexestrol [15], mestranol [15], diltiazem intermediates [16], pipecuronium bromide [17] and its intermediates [18], enalapril [17], flumecinol [19], danazol [20] and oestradiol [1].

The aim of this paper is to present further examples for the application of HPLC/diode-array UV spectrophotometry in the identification of minor components. The estimation of the position of hydroxyl groups in the oxidative degradation products of norgestrel and norethisterone and in the hydroxylated metabolites of bisaramil, as well as the discrimination between 4-ene-3-keto and 1-ene-3-keto derivatives among the byproducts of the Birch reduction of 3-methoxy-1,3,5(10)-oestratrienes, were based to a great extent on the on-line UV spectra (partly supported by the mass spectra), but avoiding the

necessity of separating the minor components by preparative HPLC for NMR investigation.

2. Experimental

2.1. Instruments

High-performance liquid chromatography

A Hewlett-Packard 1090A Series II chromatograph equipped with a built-in HP 1040 diode-array UV detector was used.

UV spectroscopy

A Varian Cary 3 double-beam instrument was used for scanning the spectra of the synthesized impurities and metabolites.

2.2. HPLC conditions

(a) Hydroxylated impurities in norgestrel and norethisterone

Column: Hypersil ODS 5 μm (Shandon). Eluent: linear gradient system. From 45:55 to 56:44 v/ v mixture of methanol and water within 40 min; 1.3 ml min^{-1}. UV detector: 240 nm.

(b) Hydroxylated metabolites of bisaramil

Column: 150 × 3.9 mm Nova-Pak C-18 4 μm (Waters). Eluent: (0.1 M aqueous trifluororacetic acid/ammonium trifluoroacetate pH 3.5)–acetonitrile (73:27, v/v); 1 ml min^{-1}. UV detector: 246 nm.

(c) Isomeric impurities in nortesterone

Column: 250 × 4 mm LiChrosorb SI-60 10 μm (Merck). Eluent: n-hexane–2-propanol (90:10, v/ v); 1 ml min^{-1}. UV detector: 240 nm.

2.3. Materials and reagents

All samples investigated were industrial or laboratory products of the Chemical Works of Gedeon Richter Ltd., Budapest. The hydroxylated norgestrel impurities and bisaramil metabolites were synthesized by Dr. Z. Tuba and Mr. Gy. Visky, respectively. HPLC grade solvents were used.

3. Results and discussion

3.1. Hydroxylated impurities in norgestrel and norethisterone

In the course of earlier studies aiming at impurity profiling the synthetic progestogenes norethisterone and norgestrel, several interesting structures were found in the authors' laboratory such as epimeric 17α-hydroxy-17β-ethinyl derivative [14,15], "dimeric" ethinyl derivative [14], and 8(14)-dehydro derivatives [14]. (In the latter case, diode-array UV spectrophotometry played an important role in the identification).
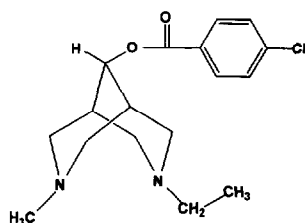
Bisaramil

During these studies, no attention was paid to very polar impurities (eluting in the 0.3–0.5 relative retention time range relative to the main components) since their individual quantities did

not exceed the 0.1% level. As a consequence of the increasing demands regarding the purity of bulk drugs, quite recently we became interested in the structure of these impurities. On the basis of their high polarity and the well known oxidizibility of 4-ene-3-keto steroids, it was supposed that these impurities are autoxidation products of the drugs or originate from the autoxidation products of the intermediates in their synthesis and contain hydroxyl groups in the vicinity of the 4-ene-3-keto group, presumably at the 6 (α and β) and 10β positions.

Table 1 contains UV absorption maxima of hydroxylated 4-ene-3-keto steroids taken from the literature [21]. It can be seen that the slight but characteristic hypsochromic shifts are caused by the hydroxyl groups at various positions and configurations. Table 1 also contains the UV maxima of the three polar impurities of norgestrel; the spectra can be seen in Fig. 1. As is seen, the found UV maxima are in agreement with the proposed three structures, but no discrimination is possible on this basis between the 6β and 10β-OH derivatives. At this point the three compounds were synthesized. The mixture of the two 6-hydroxy epimers was prepared by oxidation of the 3-ethoxy-3,5-diene derivative of norgestrel [22]. Separation of the epimers by column chromatography yielded predominantly the 6β-hy-

Table 1
Estimation of the position of hydroxyl groups in the polar impurities of norgestrel on the basis of the hypsochromic shifts of their UV maxima

	Hypsochromic shift effected by hydroxyl groups adjacent to the 4-ene-3-keto group (nm)[a]	UV maxima of norgestrel and its polar impurities determined by the diode-array UV detector (nm)[b]		
		Norgestrel	Impurities at various relative retention times (relative to norgestrel)[c]		
			0.21	0.23	0.34
–	0	246			
6α-OH	1–2		245		
6β-OH	5–6			241	
10β-OH	4–7				239

[a] See Ref. [21].

[b] For the HPLC system see Experimental. The unusually high value for norgestrel is due to the high water content in the eluent [27].

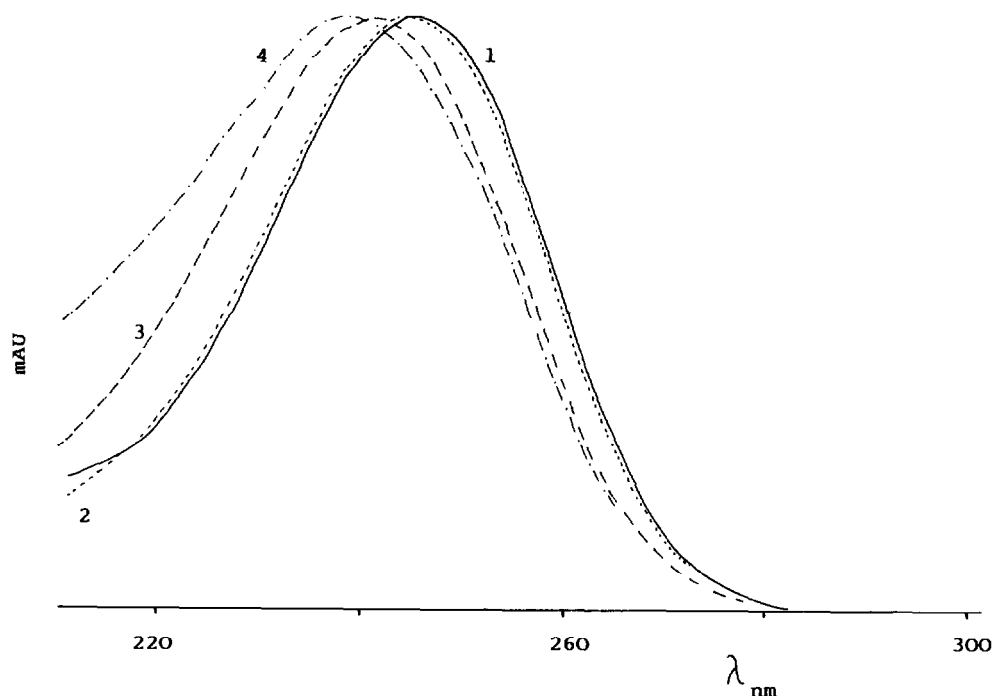[c] t_R (norgestrel) = 28.9 min.

Fig. 1. Ultraviolet spectra of norgestrel and its hydroxylated impurities recorded by the diode-array detector. See Experimental for the HPLC system. Key: (1) norgestrel; (2) 6α-hydroxynorgestrel; (3) 6β-hydroxynorgestrel; (4) 10β-hydroxynorgestrel.

droxy derivative and some of the epimeric 6α-hy-droxy derivative. 10β-hydroxynorgestrel was prepared by ethinylation of 18-ethyl-10β-hydrox-ygona-4-ene-3,17-dione. The synthesized impuri-ties were characterized by spectroscopic methods (UV, IR, NMR, MS). Retention matching in the above HPLC system and several TLC systems [23] revealed that they really represent the three polar impurities in norgestrel as shown in Table 1.

Exactly the same results were obtained for the polar impurities in norethisterone.

3.2. Hydroxylated metabolites of bisaramil

The detailed description of the metabolism of the experimental antiarrhytmic drug bisaramil is not the subject of this paper; it will be published elsewhere [24]. Here, we report only on the struc-ture elucidation of two compounds in the polar fraction of rat urinary metabolites, since this is a good example for demonstrating the potential of

diode-array UV spectroscopy in metabolic studies.

Two major metabolites were detected eluting at 9.3 and 11.6 min, respectively, in the HPLC sys-tem described in the Experimental section. Ther-mospray LC/MS investigation gave molecular masses of 338 and 354 for the first and second peaks, respectively. Since the moelcular mass of unreacted bisaramil is 322, this means that the metabolite eluting at 9.3 min is likely to be a
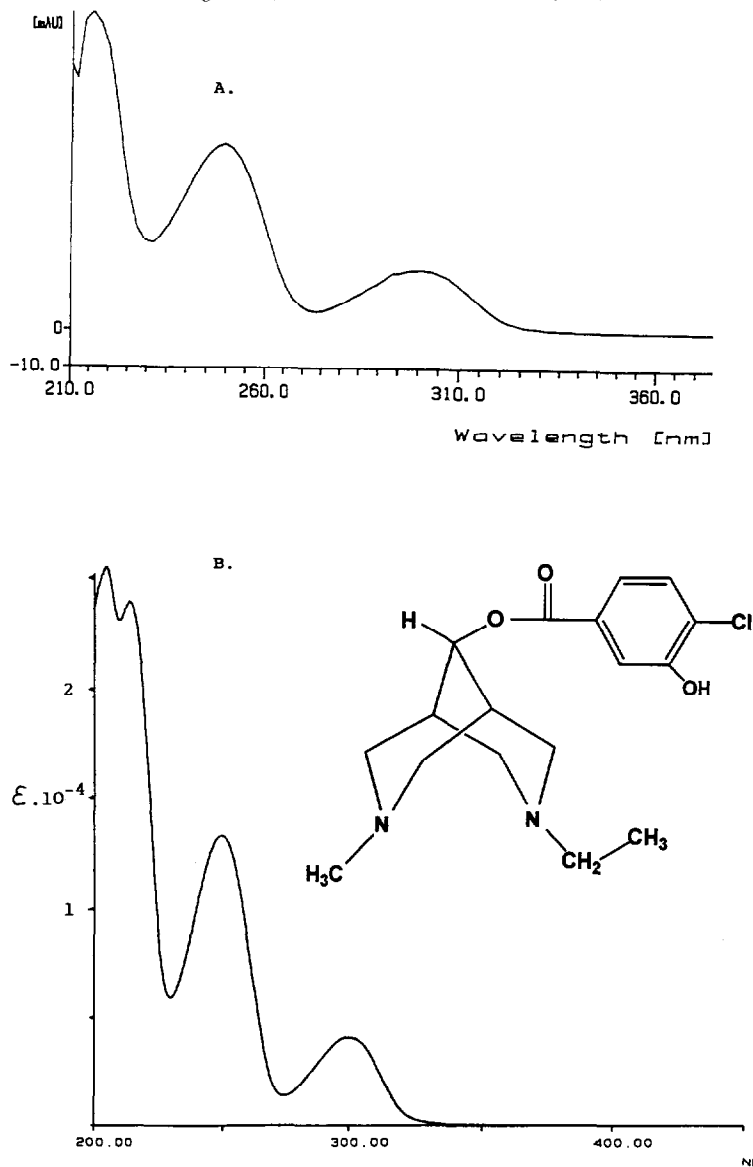

$R = CH_3$	Norethisterone
C_2H_5	Norgestrel
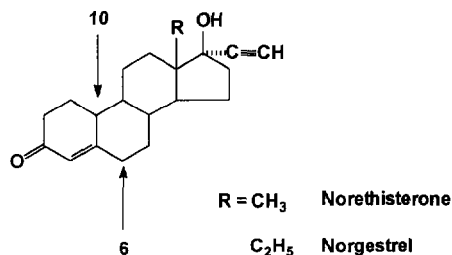
Fig. 2. Ultraviolet spectra of the metabolite of bisaramil eluting at 9.3 min in the HPLC system described in Experimental. (A) HPLC/diode-array UV spectrum. (B) Spectrum of the synthesized metabolite. Solvent: 9:1 v/v mixture of water and ethanol.

monohydroxy and the other at 11.6 min a dihydroxy derivative. The large differences between the diode-array UV spectra of the metabolites (see Figs. 2 and 3) and bisaramil (λ_{max} = 242 and 284 nm; ε = 17 500 and 700) indicate that the hydroxylation took place at the aromatic ring. Of course, mass spectrometry does not provide information regarding the position of the hydroxyl groups. To avoid the necessity of separating

larger quantities of the metabolites for NMR studies we tried to solve the problem on the basis of their UV spectra. Another question to be answered was why the dihydroxy derivative eluted after the monohydroxy metabolite in the reversed-phase HPLC system.

On the basis of the comparison of the diode-array UV spectrum of the monohydroxy derivative with spectra of various isomeric chlorohy-
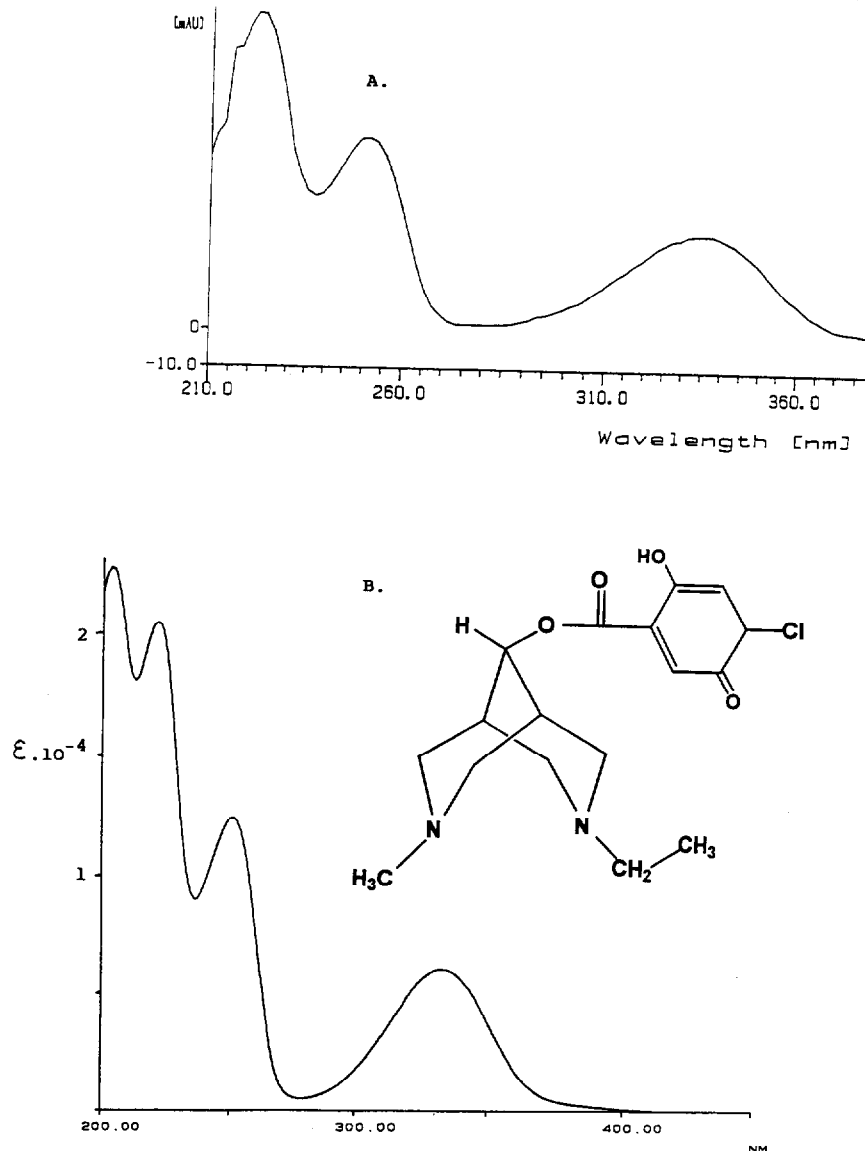
Fig. 3. Ultraviolet spectra of the metabolite of bisaramil eluting at 11.6 min in the HPLC system described in Experimental. (A) HPLC/diode-array UV spectrum. (B) Spectrum of the synthesized metabolite. Solvent: 9:1 v/v mixture of water and ethanol.

droxybenzoic ester derivatives, the likely location of the hydroxyl group was found to be position 3. (The long-wavelength maximum of the isomeric 2-hydroxy derivative would be higher by about 5 nm.) The 3-hydroxy derivative of bisaramil was then synthesized and the supposition proved by retention matching and the comparison of the UV spectra (see Fig. 2).

As is seen in Fig. 3, the spectrum of the dihydroxy derivative with its long-wavelength maximum at 335 nm is characteristic of a tautomeric conjugated ketone derivative of hydroxybenzoic acid rather than a diphenolic aromatic derivative. Investigation of the spectra of the isomeric 2,3-, 2,5-, 2,6- and 3,5-dihydroxybenzoic acid derivatives has shown that the above mentioned tautomeric derivative exists only in the case of the
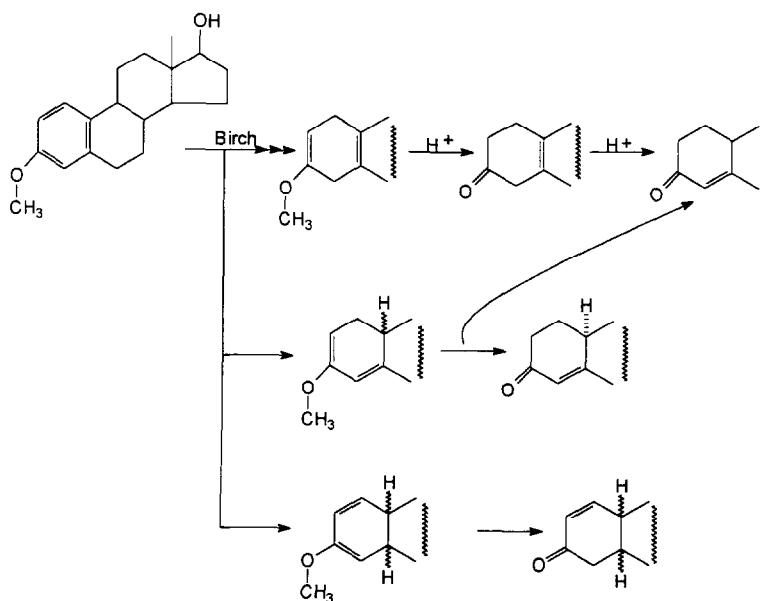
Equation 1.

2,5-dihydroxy derivative. After the synthesis of this, the identity of the UV spectra and the successful retention matching furnished evidence to prove this supposition, too (see Fig. 3). The anomalous chromatographic behaviour of this derivative can be explained on the basis of its structure. One of the hydroxyl groups is present as a tautometric ketone and the other (as shown by the infrared spectrum of the synthesized material) forms strong hydrogen bonds with the ester carbonyl group. This is the reason why this metabolite is eluted after the monohydroxy derivative in the reversed-phase HPLC system.

3.3. Discrimination between 4-ene3-keto- and 1-ene-3-ketosteroids among the side products of the Birch reduction of 1,3,5(10)-oestratriene-3,17β-diol 3-methyl ether

The Birch reduction of 3-methoxy-1,3,5(10)-oestratriene derivatives (reduction with sodium in liquid ammonia in the presence of alcohols) is the key reaction of the preparation of 19-norsteroids [25]. For example, oestradiol-3-methyl ether is converted in such a way to its 1,4-dihydro derivative, which is hydrolyzed with hydrochloric acid

to 19-nortestosterone (see Eq. 1).

The side reactions of the Birch reduction were recently studied in the authors' laboratory by investigating the impurity profile of crude 19-nortestosterone [26]. The details of the results of this study obtained mainly by GC/MS and NMR spectroscopy of fractions separated by preparative HPLC will be published elsewhere. Here, we report only on the HPLC investigation of crude 19-nortestosterone, where the diode-array detector enabled the discrimination between 4-ene-3-ketones and 1-ene-3-ketones.

In the normal phase HPLC system described in the Experimental section, 19-nortestosterone (17β-hydroxy-4-oestrene-3-one) is eluted at 11.63 min. The maximum of the spectrum scanned with the diode-array detector is at 236 nm. (The hypsochromic shift relative to the well known 240–241 nm maximum of 4-ene-3-ketosteroids in alcoholic medium is due to the apolar eluent [27].) There are two further peaks in the chromatogram of crude nortestoerone eluting at 13.35 (236 nm) and 18.08 min (238 nm) characteristic of the 4-ene-3-keto system, presumably two of the several possible stereoisomers of nortestosterone (detected by GC/MS), e.g. 10α-19-nortestosterone [28] originating from a side

reaction of the Birch reduction and 9β,10α-19-nortestosterone [29] originating from a side reaction in one of the previous steps of the total synthesis (see Eq. (1)).

Two impurities eluting at 8.35 and 10.16 min, respectively, have absorption maxima at 226 nm. This wavelength is characteristic of 1-ene-3-keto-steroids [21], which also originate from the side reactions of Birch reaction and can be present in four stereoisometric forms (5α,10α; 5α,10β; 5β,10α; 5β,10β) (see Eq. (1)).

In this example, the possibilities of the diode-array UV spectra are restricted to differentiation between 4-ene-3-keto- and 1-ene-3-ketosteroids: the stereochemistry of the individual impurities has to be established by NMR spectroscopy after their isolation by preparative HPLC.

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