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# ANALYSIS OF STEROIDS

## XXXVIII*. THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY UV DETECTION FOR ESTIMATING IMPURITY PROFILES OF STEROID DRUGS

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SUMMARY

High-performance liquid chromatography (HPLC) diode-array UV-spectrophotometric detection is used for estimating impurity profiles of steroid drugs. It is shown to be a very useful first screening method for the identification of UV-active impurities and degradation products, giving a rapid answer to many questions or at least providing important initial information to complement the results obtained by other spectroscopic techniques.

In this paper the estimation of the impurity profiles of ethynyloestradiol, norgestrel, and norethisterone, and of the degradation product of RGH-1113 (3-chloro-, 6,9-difluoro-11$\beta$,16$\alpha$,17$\alpha$,21-tetrahydroxy-1,3,5-pregnatrien-20-one 16,17-acetonide 21-acetate) will be discussed.

## INTRODUCTION

As a consequence of the struggle for greater safety of drug therapy, the quality of drugs is continuously increasing. We believe that it is common practice in most pharmaceutical companies to identify and quantify all impurities with concentrations greater than 0.1% in drug formulations (and often also in production intermediates). Sometimes the identification of impurities in drugs in the range between 0.01 and 0.1% is also necessary. Since the impurity profile is a function of the synthetic route, any variations in the technological parameters, the source and quality of the starting materials, the solvents and chemicals used in the synthesis, the storage conditions, etc., may affect the impurity profiles. Therefore, the impurity profiles need to be checked frequently, and consequently, their estimation is becoming one of the most important tasks of analysts in the pharmaceutical industry. The time and labour demands of the analytical methods are very important factors, and on-line combinations of chromatographic and spectroscopic techniques are preferred.

* For Part XXXVII, see S. Görög and M. Rényei, in S. Görög (Editor), *Advances in Steroid Analysis '84*, Elsevier, Amsterdam, 1985, pp. 555–561.

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Gas chromatography–mass spectrometry (GC–MS) is an ideal tool for this purpose but without derivatization it is applicable only to volatile drugs. In applying GC–MS to low-volatile drugs, minor impurity peaks can easily be confused with decomposition products formed during the gas chromatography or with byproducts of the derivatization reaction. Liquid chromatography–mass spectrometry (LC–MS) will certainly be the method of the future, but at the present stage of development this method is not yet suitable for routine work.

The advent of rapid-scanning diode-array UV spectrophotometers as detectors for high-performance liquid chromatographs (DAD–HPLC) has created an entirely new situation. The ability of such a detector to record and store the UV spectra of impurities even at extremely low concentrations and to produce three-dimensional chromatograms and iso-absorbance plots makes it an ideal tool for gaining structural information of UV-active impurities, provided that their UV spectra differ sufficiently from that of the main component. Of course, this condition is not always fulfilled. Furthermore, the UV data will need to be confirmed by other spectroscopic techniques in the off-line mode.

With regards to the application of the DAD–HPLC technique for the solution of other problems in pharmaceutical analysis (assessment of peak homogeneity, deconvolution of overlapping peaks, metabolic profiling, etc.) we refer to a few important papers[1–3].

The objective of this paper is to demonstrate by some practical examples the usefulness of the DAD–HPLC technique for estimating the impurity profiles of some steroid drugs. Steroids may be considered to be "difficult" compounds from this point of view, because the number of UV-active chromophoric groups is very limited (mainly 4-ene- or 1,4-diene-3-keto groups or the phenolic ring A). The examples in this paper demonstrate that in spite of this the data obtained by the DAD–HPLC technique are in many cases adequate for the identification of impurities. In any case, they will be of help in more complex spectroscopic identification procedures.

## EXPERIMENTAL

A Hewlett-Packard (Waldbronn, F.R.G.) 1090 high-performance liquid chromatograph equipped with a HP-1040 diode-array detector and a HP-85B computer was used. The bandwidth was 4 nm for the chromatograms and 2 nm for the spectra. In the case of minor components, the spectra were smoothed by the computer.

A 250 × 4 mm column was used, packed by Bio Separation Technologies (Budapest, Hungary) with LiChrosorb RP-18, 10 μm (Merck, Darmstadt, F.R.G.). The eluent was methanol–water (7:3) at a flow-rate of 1 ml/min. The concentration of the sample solution was 0.1% in the eluent. The injected volume was 25 μl.

Mass spectra were taken on a Kratos (Manchester, U.K.) MS-80/DS-55 instrument. From the impurities of ethynyloestradiol and norgestrel chemical ionization was used (reagent gas ammonia; 250 eV, 2500 μA), while for the impurities of norethisterone and the degradation product of RGH-1113 the electron impact technique was used (70 eV, 100 μA). The temperature of the ion source was between 200 and 240°C. For the IR and NMR investigations, Bruker (Karlsruhe, F.R.G.) IFS-113 and WM-250 instruments were used, respectively.

## RESULTS AND DISCUSSION

### *Sensitivity of the method*

In order to estimate the sensitivity and the concentration limits of the DAD–HPLC method, a systematic study was carried out. The spectrum of norethisterone (retention time 4.1 min) was recorded at various concentrations next to norethisterone acetate (retention time 5.6 min).

It was found that good quality spectra could be obtained without any smoothing for samples in which the concentration of norethisterone in the mixture was in excess of 0.1%. The quality of the spectra obtained for mixtures with a norethisterone content between 0.01 and 0.1% was satisfactory only if the smoothing program included in the software of the HP-85B computer was used. It should be noted that the above statements relate to the concentration and sample volume described in the experimental section. The sensitivity can be increased by increassing the concentration of the test solution and/or the injected volume.

### *Identification of 9(11)-dehydroethynyloestradiol as the impurity in ethynyloestradiol*

Fig. 1 shows the chromatogram of an ethynyloestradiol sample meeting the requirements of USP XXI. A small impurity is seen at a relative retention of 0.89. Fig. 2 shows the spectra of the main component and of the impurity. Although from the normalized spectra the intensity of the bands cannot be estimated, it is obvious that in contrast to the spectrum of ethynyloestradiol, which is characteristic of free phenols (p band as a shoulder at about 215 nm, α-band at 279 nm with shoulder at 284 nm), the spectrum of the impurity contains an intense conjugation (K) band at 261 nm and a partly overlapped α-band at about 310 nm. The latter spectrum is characteristic of α,β-unsaturated phenols, and for this reason the structure of the impurity was proposed to be dehydroethynyloestradiol. Additional evidence for this structure was obtained by mass spectrometry. Since the molecular weight of ethynyloestradiol is 296, a $(M + H)^+$ ion at $m/z$ 295 supports the dehydroethynyloestradiol structure.

The location of the double bond was established on the basis of the DAD–UV

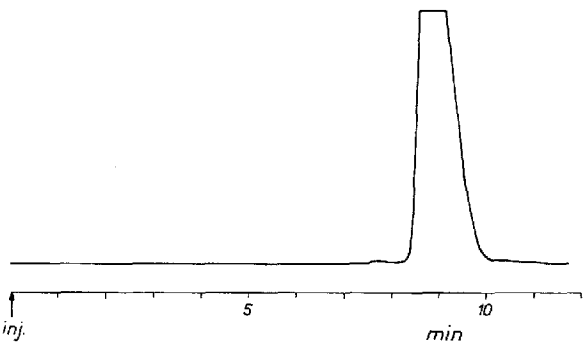



Fig. 1. Chromatogram of ethynyloestradiol. Main peak = ethynyloestradiol; impurity peak = 9(11)-dehydroethynyloestradiol. Detection at 280 nm. For chromatographic conditions, see Experimental section.

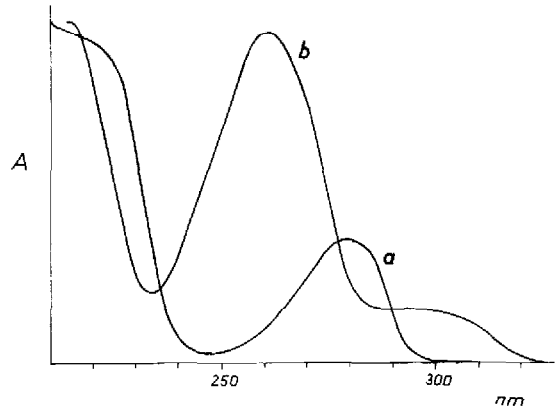
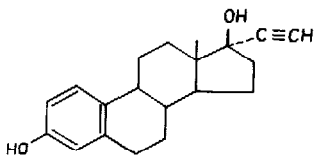
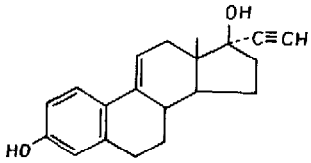



Fig. 2. DAD–UV spectra of the components separated in Fig. 1. Curve a = main peak (ethynyloestradiol), $A_{279}$ = 1.018; curve b = impurity peak [9(11)-dehydroethynyloestradiol], $A_{261}$ = 0.021.

spectrum. As seen in Table I, of the three possible isomeric α,β-unsaturated phenols, the $\Delta^8$ isomer can be excluded, because in this case the K-band appears at about 274 nm and completely overlaps with the α-band. The position of the two bands for the $\Delta^6$- and $\Delta^{9(11)}$-isomers is similar. However, distinction between the two isomers was made on the basis of the ratio of the intensities of the K- and α-bands. This value is 2.9–3.8 for the $\Delta^6$- and 5.8–6.2 for the $\Delta^{9(11)}$-isomer. Based on the 6.4 calculated from curve b, Fig. 2, it is evident that the structure of the impurity is 9(11)-dehydroethynyloestradiol [17α-ethynyl-1,3,5 (10),9(11)-oestratetraene-3,17-diol].




Ethynyloestradiol




9/11/-dehydro-ethynyloestradiol

The origin of the impurity appears to be clear. The presence of the double bond in the vicinity of the aromatic ring indicates that the oestrone used as the

TABLE I

SPECTRAL DATA OF α,β-UNSATURATED PHENOLIC STEROIDS[6,7]

| *3-Hydroxy-1,3,5(10)-triene steroid* | $\lambda_{max}$, nm | | |
|---|---|---|---|
| | *K-band* | *α-band* | $A_K/A_\alpha$ |
| 6-Dehydro | 262–272 | 302–306 | 2.9–3.8 |
| 8-Dehydro | 273–275 | – | – |
| 9(11)-Dehydro | 261–264 | 298–300 | 5.8–6.2 |
| Impurity in ethynyloestradiol | 261 | 300 | 6.4 |

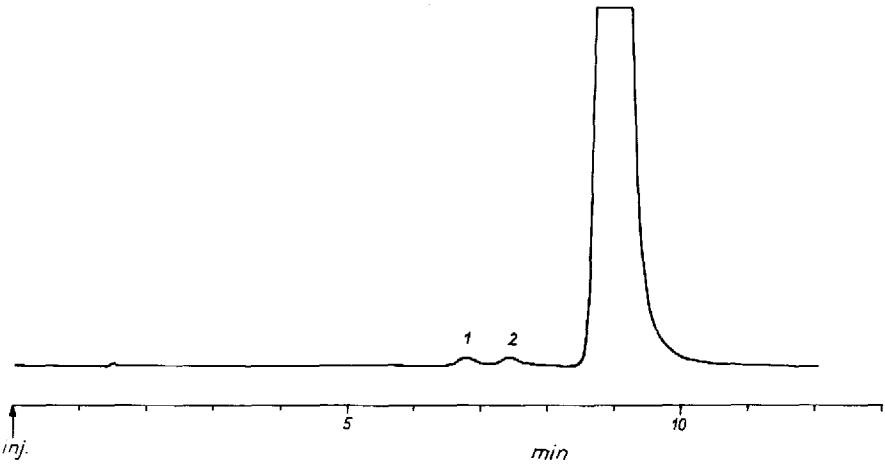



Fig. 3. Chromatogram of norgestrel. Main peak = norgestrel; impurity 1 = norethisterone; impurity 2 = 8(14)-dehydronorgestrel. Detection at 240 nm. For chromatographic conditions, see Experimental section.

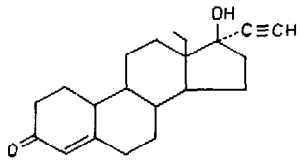
starting material in the ethynylation step of the synthesis was of total synthetic origin. The 8-dehydro derivative is an intermediate in this synthesis[4] and the $\Delta^8$-double bond of these derivatives easily isomerizes to the $\Delta^{9(11)}$-position[5].

*Identification of 8(14)-dehydronorgestrel as the impurity in norgestrel*

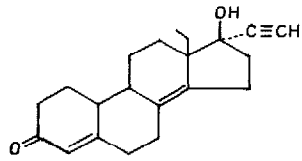
In this section, the detection and identification of an unsaturated impurity is described in another 19-norsteroid. This task was much more difficult than the preceeding example, since in this case the double bond of the impurity was not in conjugation with the chromophoric system of the drug.

Fig. 3 shows the chromatogram of a norgestrel sample. The peak of the unknown impurity in the chromatogram is peak number 2. Peak 1 was identified by retention time matching with the reference substance as norethisterone, the 13-methyl analog of norgestrel.

The on-line spectra of norgestrel and of the unknown impurity, obtained from the diode-array detector, are shown in Fig. 4. Curve a, the spectrum of norgestrel, corresponds to the wellknown spectrum of 4-ene-3-ketosteroids[6,7]. The slight bathochromic shift of about 2 nm, as compared with the spectrum in alcoholic media, is due to the presence of 30% water in the eluent[8]. Curve b shows that the 4-ene-3-keto group is also present in the unknown impurity, but the spectrum is to some extent distorted compared with that of the main component. The differences are a slight but




Norgestrel




8/14/dehydro-norgestrel

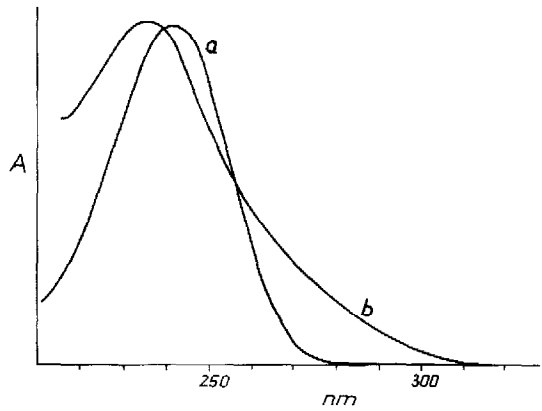



Fig. 4. DAD–UV spectra of the components separated in Fig. 3. Curve a = main peak (norgestrel), $A_{242}$ = 1.951; impurity 1 (norethisterone); curve b = impurity 2 [8(14)-dehydronorgestrel], $A_{238}$ = 0.013.

marked hypsochromic shift of the maximum and a broadening of the peak. These effects are characteristic of the 8(14)-dehydro derivatives of 4-ene-3-ketosteroids, as shown by Herrmann and Hoyer[9]. In ref. 9 maxima were observed between 231 and 236 nm (in alcoholic media) for a number of 3-keto-4,8(14)-dienes. This unusually strong effect of a remote double bond on the 4-ene-3-keto system has been explained by "through-space" interaction of the two C=C double bonds, which are in the favourable parallel position for such an interaction. Because of this effect, the change in the molecular structure was sufficient for identifying the impurity. The structure was later supported by the mass spectrum and, finally confirmed with the aid of reference material synthesized according to Johns[10].

The formation of the observed impurity can be explained from knowing the synthetic route of norgestrel. An important step in this synthesis is the hydrogenation of the $\Delta^{14}$-double bond of 13$\beta$-ethyl-3-methoxy-1,3,5(10),8,14-gonapentaen-17$\beta$-ol. As a result of a side reaction (1,4-addition of hydrogen), the 1,3,5(10),8(14)-tetraene is formed and this is probably the precursor of the 8(14)-dehydronorgestrel.

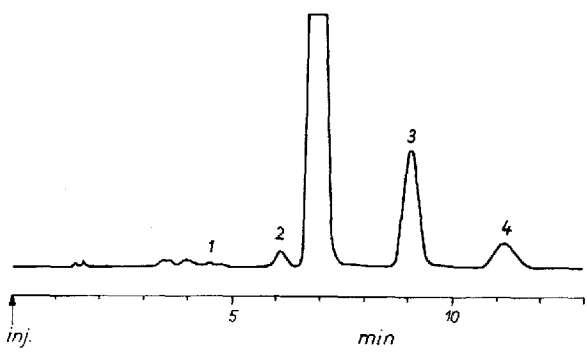



Fig. 5. Chromatogram of norethisterone (mother liquor product). Main peak = norethisterone; impurity 1 = 17$\alpha$-ethynyl-17-hydroxy-4-oestrene-3,6-dione; impurity 2 = 4-oestrene-3,17-dione; impurity 3 = 17$\beta$-ethynyl-17$\alpha$-hydroxy-4-oestren-3-one; impurity 4 = dimeric product (see formula); detection at 240 nm. For chromatographic conditions, see Experimental section.

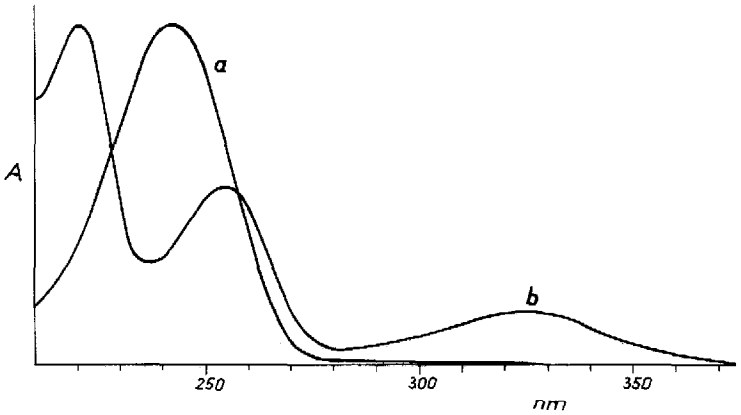
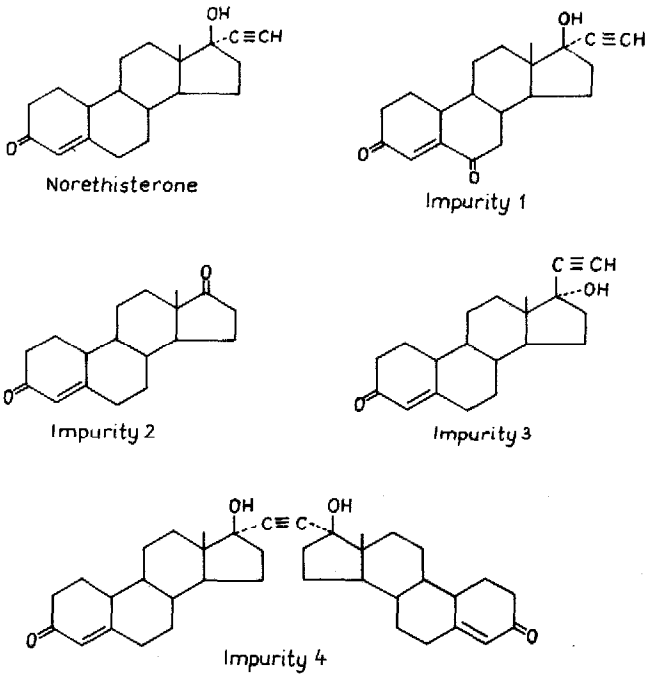



Fig. 6. DAD–UV spectra of the components separated in Fig. 5. Curve a = main peak (norethisterone), $A_{242}$ = 1.912; impurities 2, 3 and 4, $A_{242}$ = 0.032, 0.212 and 0.038 as well as the minor unidentified impurities. Curve b = impurity 1 (17α-ethynyl-17-hydroxy-4-oestrene-3,6-dione), $A_{252}$ = 0.013.

*Identification of impurities in norethisterone*

Figs. 5 and 6 show the chromatogram of a mother liquor product of norethisterone and the spectra of the components recorded by the diode-array detector, respectively. All peaks except peak 1 showed the spectral characteristics of the 4-ene-3-ketones. The spectrum of impurity 1 is characteristic of the 4-ene-3,6-dione system[6,7], the presence of which is not surprising since the autoxidation of 4-ene-3-



ketones is known to take place at the 6-position and, hence, the impurity (17α-ethynyl-17-hydroxy-4-oestrene,3,6-dione) can be considered to be an oxidative degradation product of norethisterone. It is highly probable that the precursors of this oxidation product, 6α- and 6β-hydroxy-norethisterone are among the unidentified minor components appearing in the chromatogram between 3 and 5 min.

4-Oestrene-3,17-dione (impurity 2), which is an intermediate of the synthesis, was identified by retention time matching using the reference material.

Elucidating the structure of impurities 3 and 4 was more problematic. On the basis of the electron impact mass spectrum of impurity 4 (with a molecular ion peak at $m/z$ 570 and further peaks at 552, 298, and 272) and from the absence of the $\nu_{\equiv C\text{-}H}$ band in the IR spectrum, a dimeric structure could be deduced. The only conclusion obtained from the mass and infrared spectra of impurity 3 was that it was an isomer of norethisterone. For an exact structure elucidation, preparative HPLC separation was necessary to produce enough material for a high-resolution NMR investigation. The difference between the NMR spectra of norethisterone and impurity 3 was that for the former the signals of the acetylenic proton and the angular methyl group appear at 2.58 and 0.91 ppm, whereas for impurity 3 the same signals are shifted to 2.51 and 0.95 ppm, respectively. This indicated the "iso-norethisterone" structure, characterised by the 17β-ethynyl-17α-hydroxy configuration. It is evident that both impurities 3 and 4 are by-products of the ethynylation step of the synthesis.

*Investigation of the oxidative degradation of RGH-1113*

This section is intended to demonstrate that the HPLC–DAD is also useful for establishing of the degradation pathways of drugs. In the course of the stability studies of the experimental corticosteroid drug RGH-1113[11] the use of HPLC has been described[12]. Here we report on a re-investigation using the HPLC–DAD technique.

It has been found that, upon exposing the bulk drug material to heat in the presence of atmospheric oxygen, degradation takes place. Fig. 7 shows the DAD–

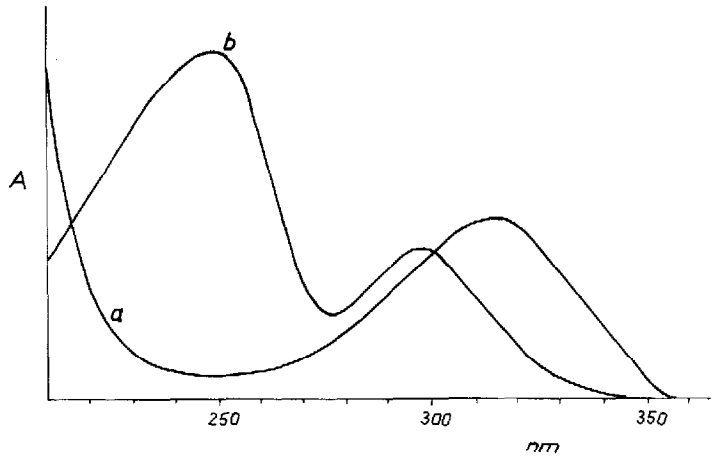
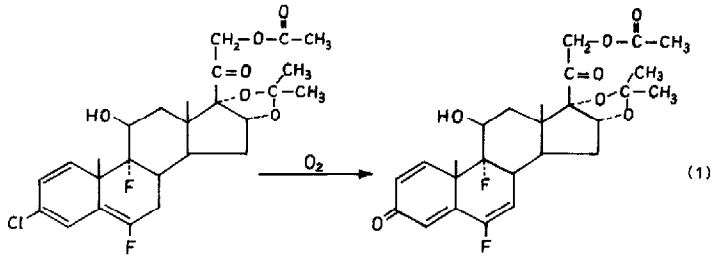



Fig. 7. DAD–UV spectra of RGH-1113 (curve a, $A_{310} = 0.870$) and its main degradation product (curve b, $A_{249} = 0.081$).

UV spectra of the unchanged drug, eluted at 21.3 min (curve a) and of the main degradation product (retention time 4.8 min; curve b). The spectrum of the degradation product with the maxima at 248 and 300 nm is that of a typical 1,4,6-triene-3-ketone[6,7] and, hence, the structure given in reaction equation 1 was proposed for the degradation product.




(1)

This structure was confirmed by mass spectrometry. The spectrum clearly showed the loss of the chlorine and the uptake of one oxygen (molecular ion peak at *m/z* 492). In addition, the fragmentation pattern of the side chain and of the D ring is the same as that of the parent material[12], indicating that only the A ring is affected by the degradation. Another piece of evidence for the 1,4,6-triene-3-one structure is that after the eluent fraction containing the degradation product was treated with sodium borohydride[13], its UV spectrum showed a maximum at 242 nm with shoulders at 237 and 248 nm, characteristic of the 4,6-diene system.

It is worth mentioning that the DAD–UV spectra of the other degradation products with maxima in the range of 235–245 nm also indicates the disappearance of the 1,3,5-triene system and the formation of various unsaturated ketone derivatives.

## CONCLUSIONS

The DAD–HPLC technique provides a very simple and rapid method for obtaining good quality UV spectra during chromatography run. Therefore it is highly advisable to begin the investigation of the impurity profile with the HPLC–DAD system. Of course, this should be regarded only as a first screening procedure, which in fortunate cases will furnish useful data, from which the structure of impurities can be established. In less fortunate cases, the DAD–HPLC data provide information that is complementary to the results of other spectroscopic techniques. As the second step of the procedure, mass spectrometric investigation of the HPLC fractions is advisable. Even if on-line LC–MS instrumentation is not available, the high sensitivity of the MS technique enables analysis in the off-line mode. Infrared spectroscopy (IR) can also be useful, if a very sensitive Fourier transform (FT) IR instrument is available. With the aid of all these techniques the structure of even minor impurities can be determined in the overwhelming majority of cases. If, however, the information obtained from the techniques discussed above is not sufficient, then the final answer may be obtained by NMR spectroscopy. In the case of major impurities (or

if a mother liquor sample which is richer in the impurity than in the bulk drug material is available), samples collected from a few HPLC runs on an analytical column will suffice to obtain a good quality spectrum, provided that a very sensitive FT-NMR instrument is available. However, it is more convenient to collect the sample for the NMR investigation by preparative HPLC.

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