Estimation of Impurity Profiles of Drugs and Related Materials. 12. Isolation and Identification of an Isomeric Impurity in Danazol[1]

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We report on a new isomeric impurity of danazol. This impurity designated as isodanazol was detected by reversed-phase high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Its structure was determined after separation by preparative HPLC. Mass spectrometry revealed the isomeric nature of the impurity while the UV spectrum indicated profound difference in the isoxazole moieties. The structure of the isomeric isoxazole ring in isodanazol was determined by NMR spectroscopy using COSY, HETCOR and NOE measurements. The difference between the UV spectra of danazol and isodanazol is explained on the basis of the difference between the aromaticities of their isoxazole rings supported by quantum chemical calculations. The quantitative determination of the impurity down to the 0.05% level can be performed by HPLC, gas chromatography and TLC densitometry.

KEY WORDS: danazol; isodanazol; impurity profiling; identification; isolation; HPLC; NMR spectroscopy; HPLC-UV.

INTRODUCTION

Danazol is a synthetic steroid drug with weak androgenic activity for the treatment of endometriosis, benign breast disorders, mennorhagia and hereditary angioedema. It is synthesized from ethisterone by Claisen condensation with ethyl formate followed by oxime formation and isoxazol ring closure with hydroxylamine without the isolation of the intermediates (Fig. 1) (3).

Several HPLC methods have been published for the determination of danazol and related compounds in body fluids (4,5), mixtures (6) and pharmaceutical formulations (7) but the impurities in the bulk drug have not yet been separated and identified. The United States Pharmacopoeia (8) describes a TLC purity test based on semi-quantitative visual comparison of the impurity spots after visualization with iodine vapor with reference spots of danazol. Ferenczi-Fodor et al. (9) described a validated method for the selective determination of three individual impurities in danazol based on TLC-densitometric measurement at 252 nm.

In the course of the impurity profiling of danazol based on reversed phase HPLC an interesting new compound (designated as isodanazol) was found at a level of 0.1–0.3% in addition to trivial impurities (1). The aim of this paper is to describe the detection, isolation, structure elucidation and characterization of this material.

MATERIALS AND METHODS

Materials

The danazol test samples were products of the Chemical Works of Gedeon Richter Ltd., Budapest, Hungary. After its isolation and structure elucidation, isodanazol was synthesized by Mrs. E. Tóth and Mr. J. Törley in the synthetic research laboratory of the same company.

HPLC-grade solvents (methanol, hexane, tetrahydrofuran, 2-propanol) and deuterochloroform for the NMR measurements were obtained from Merck (Darmstadt).

Instruments and Methods

Analytical HPLC measurements were conducted on a Hewlett-Packard 1090M instrument equipped with a diode array UV detector (band width 2 nm at specral scans and 4 nm at chromatographic scans). Column: LiChromosorb 10-RP-18, 250 × 4 mm (Merck/BST). Eluent: methanol – water 70:30 v/v at 1 ml/min. Temperature: ambient. The chromatograms were monitored at 240, 254 and 284 nm, which are the absorption maxima of ethisterone (starting material of the synthesis, one of the impurities), isodanazol and danazol, respectively. Injected volume: 20 µl from 0.1% solution in the eluent.

Preparative HPLC isolation was carried out using an ISCO Model 2350 pump equipped with a Rheodyne Model 7125 injector, ISCO V[4] variable wavelength UV detector and ChemResearch Chromatographic Data Management and System Controller. Column: Davisil Silica (irregular, 10 µm, 60 A), 250 × 21.2 mm (ISCO). Eluent: hexane – 2-propanol – tetrahydrofuran 95.5:4:0.5 v/v/v at 7 ml/min. Temperature: ambient. The chromatograms were monitored at 254 nm. Injected volume: 1 ml of 4% w/v sample solution in chloroform corresponding to about 40 mg steroid/cycle.

Gas chromatographic measurements were conducted on a Hewlett-Packard 5890A instrument equipped with a flame ionisation detector. Column: DB-210 fused silica capillary; 30 m × 0.25 mm × 0.25 um (J & W). Temperature: oven: 240°C, injector, FID: 280°C. Injected volume: 1 µl of 0.1% solution in chloroform.

The UV spectra were recorded using the above mentioned diode-array detector and also on a Varian DMS-200 UV spectrophotometer in ethanolic solution.

A VG Trio-2 spectrometer was used for taking the mass spectra of danazol and the chromatographically isolated impurity.

The ^{1}H- and ^{13}C-NMR spectra were recorded using Varian XLAA-400 and VXR-300 instruments with $CDCl_3$/TMS as the solvent/Reference.

RESULTS AND DISCUSSION

Detection of Isodanazol

Isodanazol was detected as an impurity in danazol by

[1] For Part 11. see reference 1. This paper is Part 47. in the series of papers "Analysis of Steroids." For Part 46. see reference 2.

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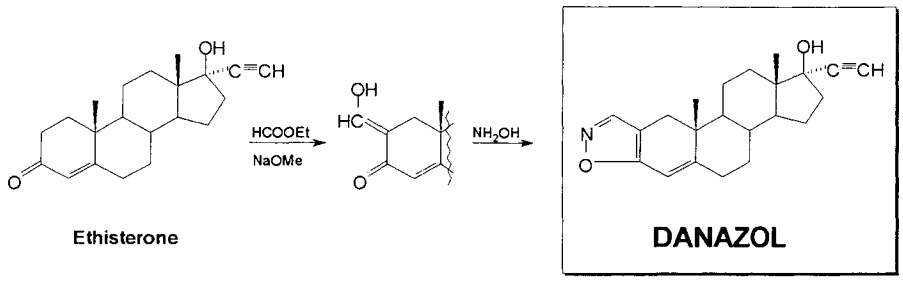


Fig. 1. Reaction scheme of the preparation of danazol.

thin-layer chromatography and high-performance liquid chromatography. The R_f values of danazol and isodanazol in the TLC system of USP XXII (8) are 0.29 and 0.33, respectively, while in the system of Ferenczi-Fodor et al. (9) 0.47 and 0.58.

In the reversed phase HPLC system described in the experimental section isodanazol and danazol are eluted at 11.5 and 15.9 min, respectively (Fig. 2). Monitoring the chromatogram at 286 nm (absorption maximum of the main component), neither isodanazol nor ethisterone (starting material of the synthesis eluted at 7.3 min) can be detected since this wavelength is beyond the cut-off wavelengths of their spectra. At shorter wavelengths, however, (at 240 and 254 nm) both impurities can easily be detected.

Danazol was found to slightly decompose under gas chromatographic conditions; its GC-MS analysis in biological samples is carried out after derivatization (10). The isomeric impurity, however, can be chromatographed without decomposition and is easily detectable in danazol, the retention times being 29.6 min for danazol and 30.7 min for isodanazol in the system described in the experimental section.

Isolation of Isodanazol

Although the diode-array UV spectra of danazol (λ_{max} 286 nm) and isodanazol (λ_{max} 227 and 254 nm) were found to be considerably different, it was impossible to suggest a structure for the impurity on the basis of these spectra and therefore, the isolation of the impurity by preparative HPLC was necessary for structure elucidation. In order to avoid the difficulties of the isolation of the separated minor component from the aqueous eluent of the analytical reversed phase HPLC system, a normal phase separation system described in the experimental section was developed; (retention times of isodanazol and danazol: 35 and 41 min, respectively).

The isolation of the impurity was performed in two steps. Starting from a crude danazol sample containing about 1.1% isodanazol 31 injections were made yielding 16 mg of crude isodanazol (81% purity). This was further purified in the same system. This second step of the purification yielded 11 mg of isodanazol (purity 98%) which was sufficient for the spectroscopic investigations.

Structure Elucidation of Isodanazol

The identical molecule peaks at m/z 337 in the mass spectra of danazol and the impurity and the identity of the main fragments indicate that the impurity is the positional isomer of danazol. (The peaks of danazol with their relative intensities are 337(100), 270(23), 254(9), 173(54), 149(63), 132(48), 121(43), 105(42), 91(99), 77(68), 67(61), 53(94), while the same values for isodanazol are 337(59), 270(58), 254(32), 173(37), 149(100), 133(28), 121(63), 105(39), 91(52), 77(67), 67(51), 53(46). The characteristic peaks at m/z 270 and 254 indicate the presence of the 17-ethynyl-17-hydroxy group and hence it could be concluded that the difference between the two molecules is at the other end of the molecule, the isoxazole moiety.

Similarly, the only conclusion we were able to draw from the diode-array UV spectra already mentioned earlier was that there were considerable differences in the chromophoric α,β-unsaturated isoxazole moieties of the molecules.

The structural identification of the impurity was accomplished by detailed NMR investigations. This required full ^1H and ^{13}C assignments for danazol itself (Table I). Some signal assignments were straightforward on the basis of their characteristic chemical shifts and multiplicities (as e.g. in the case of the acetylene and olefinic protons and carbons). Other hydrogen-bearing carbons and the respective protons were identified by tracing the ^{13}C-^1H and ^1H-^1H coupling

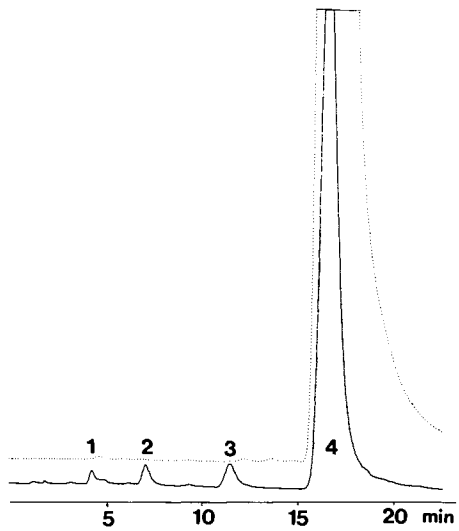


Fig. 2. High-performance liquid chromatogram of a danazol sample. See "Instruments and methods" for chromatographic details.

Key: 1. Unknown impurity
2. Ethisterone (0.04%)
3. Isodanazol (0.13%)
4. Danazol
Full line: monitoring at 240nm
Dotted line: monitoring at 286nm.

Table I. Chemical shift data of danazol and isodanazol

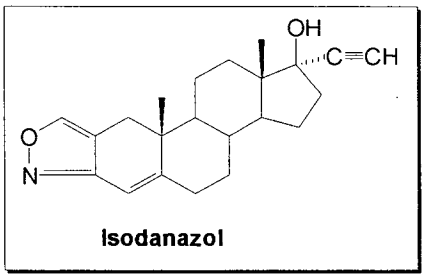
Carbon Site	DANAZOL		ISODANAZOL	
	^{1}H NMR δ(ppm)	^{13}C NMR δ(ppm)	^{1}H NMR δ(ppm)	^{13}C NMR δ(ppm)
C-1	2.50 (d *br*[a]) & 2.73 (d)	33.4	2.38 (d *br*[a]) & 2.88 (d)	31.8
C-2		107.6		112.2
C-3		164.9		157.4
C-4	6.18 (*m*)[a]	108.7	6.30 (*m*)[a]	109.8
C-5		154.4		156.3
C-6	2.39 (m) & 2.43 (m)	32.2	2.42 (m) & 2.44 (m)	32.7
C-7	1.06 (m) & 1.79 (m)	30.7	1.05 (m) & 1.80 (m)	30.8
C-8	1.50 (m)	37.0	1.50 (m)	36.9
C-9	1.19 (m)	53.9	1.14 (m)	53.2
C-10		41.0		40.2
C-11	1.46 (m) & 1.63 (m)	21.2	1.49 (m) & 1.67 (m)	21.3
C-12	1.66 (m) & 1.74 (m)	32.6	1.66 (m) & 1.75 (m)	32.6
C-13		46.7		46.7
C-14	1.51 (m)	49.8	1.50 (m)	49.9
C-15	1.38 (m) & 1.75 (m)	23.2	1.37 (m) & 1.74 (m)	23.2
C-16	2.01 (ddd) & 2.31 (ddd)	38.9	2.01 (ddd) & 2.31 (ddd)	38.9
C-17		79.7		79.7
C-18	0.90 (*d*)[a]	12.7	0.90 (*d*)[a]	12.7
C-19	1.02 (*d*)[a]	18.8	1.01 (*d*)[a]	19.1
-C≡		87.4		87.4
≡CH	2.58 (s)	74.1	2.58 (s)	74.1
CH′	8.00 (*m*)[a]	148.6	7.99 (*dt*)[a]	151.4
OH	2.06 (s)		2.06 (s)	

[a]Long-range coupling (J < 2 Hz).

network of the system via two-dimensional (2D) homo- and heterocorrelation experiments (COSY and HETCOR). The quaternary carbons were assigned unambiguously from 2D long-range heterocorrelation measurements (FLOCK).

Complete signal assignments for the impurity (isodanazol) were carried out as described above for danazol (Table I). The NMR data of isodanazol show that the number of protons and carbons, as well as the number of carbons with the same degree of substitutions, is identical in both compounds. A comparison of the ^{13}C chemical shifts of the two compounds (Table I) indicates that significant shift differences occur only for C-2 and C-3. This in turn suggests that the structural difference in question must involve the isoxazole ring. In addition, in ^{1}H-^{1}H NOE difference experiments irradiation of the olefinic isoxazole proton gave an enhancement only on the H_β-1 proton (ca. 1%) in isodanazol as well as in danazol. Likewise, H-4 gave an NOE connection to H_α-6 (ca. 6%) in both compounds.

All these pieces of evidence strongly suggest the structure of isodanazol as shown:



Isodanazol

Proof for the Structure of Isodanazol

The structure proposed on the basis of the above described spectroscopic investigations was proved by synthesis. Isodanazol was synthesized from ethisterone in a similar way as shown for danazol (Fig. 1) the difference being that the enolic hydroxyl group in the reaction product of the Claisen condensation is protected as the isopropyl ether prior to the addition of hydroxylamine thus ensuring that oximation takes place at the 3-keto group rather than at the 2-formyl group which is the case in the synthesis of danazol. The ring closure takes place after the removal of the protecting group. The full identity of the spectra and chromatographic retention data of the impurity and the synthesized material furnished convincing evidence for the above described structure of isodanazol.

Interpretation of the Ultraviolet Spectra of Danazol and Isodanazol

Fig. 3 shows the ultraviolet spectra of danazol and isodanazol which are in good agreement with those obtained by the diode-array detector. Taking into consideration that both compounds are α,β-unsaturated isoxazole derivatives the differences between the spectra are surprising. Isoxazol derivatives, however, are known to be considerably less aromatic than other five-membered heterocycles (11). Hence of the α,β-unsaturated derivatives danazol can be considered to be a linearly conjugated triene while the triene system in isodanazole is cross-conjugated explaining its much lower wavelength absorption maximum.

In order to further rationalize the differences in the ex-

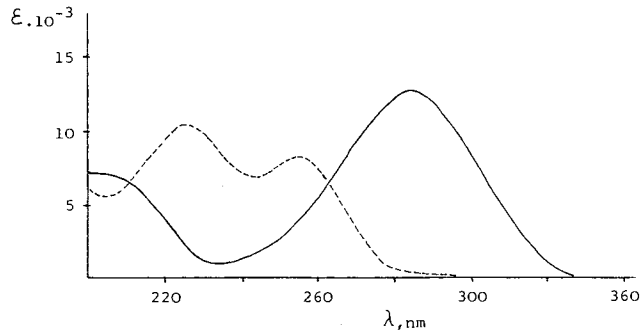


Fig. 3. Ultraviolet spectra of danazol (full line) and isodanazol (dotted line). Solvent: 95% ethanol.

citation energies of the danazol and isodanazol molecules, quantum chemical calculations were performed for model molecules. These were derived from the danazol and isodanazol structures by omitting rings C and D and saturating the dangling bonds at C-8 and C-9 with hydrogens. The geometries of the model molecules were fully optimized in their ground states using the AM1 Hamiltonian (12). Then the energies of the first excited singlet states were also calculated in the geometries obtained for the ground states. The differences in the energies are 4.02 eV (309 nm) for the danazol and 5.00 eV (248 nm) for the isodanazol model. These are in qualitative agreement with the observed experimental excitation energies of the danazol (286 nm) and isodanazol molecules (254 nm).

Quantification of Isodanazol as an Impurity in Danazol

The paper of Ferenczi-Fodor et al. (9) describes the quantitative thin-layer densitometric determination of isodanazol impurity (designated as Impurity III) in danazol. Linearity and recovery studies have shown that the HPLC (monitoring at 254 nm) method described in the experimental section is also suitable for the determination of the same down to the 0.05% level. For example, an isodanazol content of 0.111 ± 0.010% (n = 6) was found for a danazol sample spiked with 0.107% of isodanazol.

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