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Isolation and identification of a new impurity in mitoxantrone hydrochloride

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Received November 27, 2008, accepted December 1, 2008

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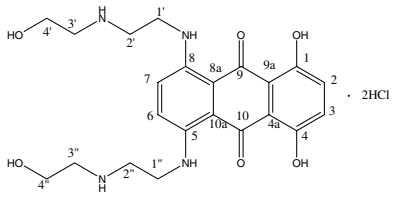
Pharmazie 64: 376–379 (2009) *doi: 10.1691/ph.2009.8351*

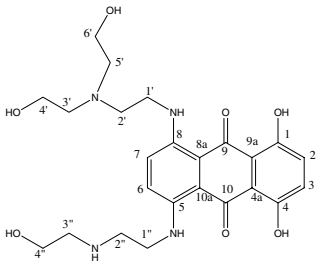
An unknown impurity in mitoxantrone hydrochloride bulk drug was detected by HPLC at levels around 0.5%. This impurity was isolated from a sample of crude mitoxantrone using preparative HPLC and was characterized as 1,4-dihydroxy-5-2-2-hydroxyethyl-amino-ethyl-amino-8-2-bis 2-hydroxyethyl-amino-ethyl-amino-9,10-anthracenedione based on its spectral data (NMR, IR and MS). The origin and structural elucidation of this impurity are discussed in the paper.

1. Introduction

Mitoxantrone hydrochloride, 1,4-dihydroxy-5,8-bis-2-(2-hydroxyethyl-amino-ethyl-amino)-9,10-anthracenedione hydrochloride, is an antineoplastic agent. It has been used extensively as a component of chemotherapeutic regimens for a number of fatal diseases, including leukemia, lymphoma, cancers of the breast and prostate, and to treat multiple sclerosis (Faulds et al. 1991; Lu et al. 2006; Ramkumar et al. 2008; Oliver et al. 2007). In the European Pharmacopoeia (European pharmacopoeia 5.0, 2005), four related substances in mitoxantrone hydrochloride are listed, impurities A, B, C and D, and a chromatographic procedure is used to determine these related substances using a column packed with phenyl silica gel.



Mitoxantrone hydrochloride



Imp-u

During the determinination of mitoxantrone hydrochloride in our laboratory, two main impurities were detected by HPLC. One was impurity A (1-amino-5, 8-dihydroxy-4-[[[2-(2-hydroxyethyl)-amino]-ethyl]-amino]-anthracene-9,10-dione), as shown in the European Pharmacopoeia (European pharmacopoeia 5.0, 2005), and the other was the unknown impurity (Imp-u). To critically control the quality of mitoxantrone hydrochloride, imp-u was isolated from a sample of crude mitoxantrone using preparative HPLC and was identified. The impurity profile of a drug substance is important for its safety assessment and is critical for monitoring the manufacturing process. Therefore, it is important to test the impurities for regulatory requirements. This paper describes the identification, isolation and characterization of an unknown impurity present in mitoxantrone hydrochloride. This new impurity has not been reported previously to the best of our knowledge.

2. Investigations and results

Several batches of mitoxantrone hydrochloride were analyzed for their impurities using the HPLC method described in Section 4.2 and the chromatogram is shown in Fig. 1. The method used was different from that described in the European Pharmacopoiea. The target imp-u and imp-A eluted at retention times of about 14.5 min and 22.6 min, respectively, while mitoxantrone hydrochloride eluted at about 15.7 min. The resolution, R, between mitoxantrone and imp-u was 2.1, and the number of theoreti-

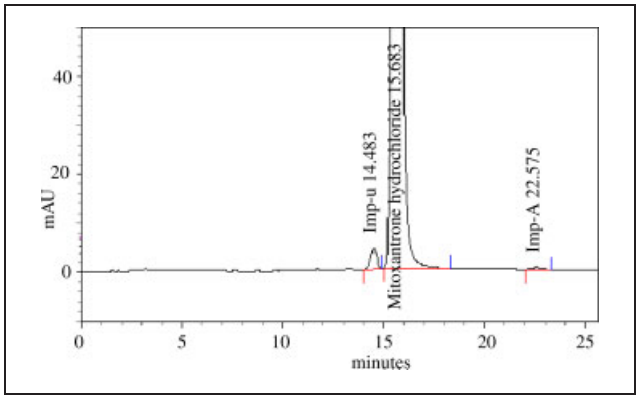


Fig. 1: HPLC chromatogram of bulk mitoxantrone hydrochloride

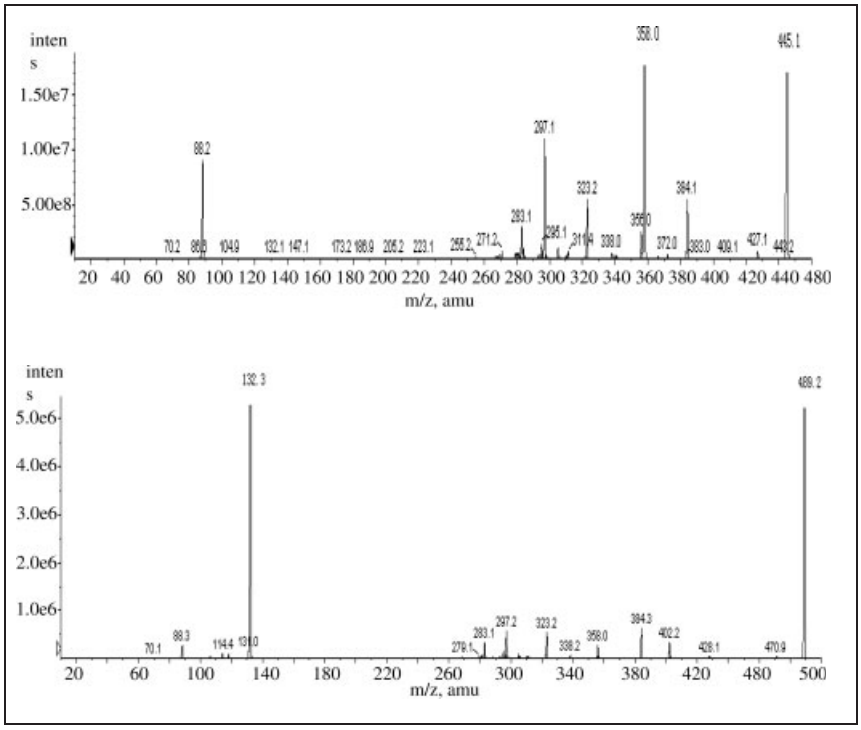


Fig. 2:
(A) ESI/MS spectrum of mitoxantrone (CE = 20eV) and (B) ESI/MS spectrum of protonated impurity (CE = 20eV)

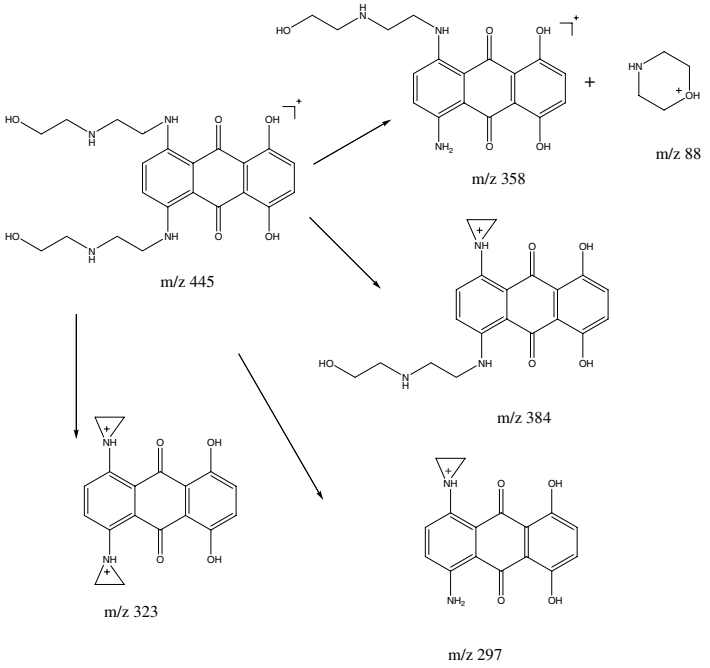
cal plates and the tailing factor for the mitoxantrone peak were 8800 and 0.99, respectively.

Imp-u was isolated from mitoxantrone hydrochloride bulk drug collected during the synthesis of mitoxantrone, the impurity being present at about the 3% level. It was isolated by preparative HPLC as described in Section 4.3. The compound was obtained as a dark blue powder.

The molecular formula of imp-u was determined to be $C_{24}H_{32}N_4O_7$ based on time of flight mass spectroscopy (TOF-MS) data ($(M + H)^+$ m/z 489.23). The molecular weight of mitoxantrone was 444.48, which is just 44 aum less than that of imp-u.

The product ion spectrum of protonated Mitoxantrone is shown in Fig. 2(A). The ESIMS spectrum of protonated Imp-u is shown in Fig. 2(B), and imp-u has the same dissociation products at m/z 384, m/z 358, m/z 323, m/z 297 and m/z88 as those of mitoxantrone. The two dissociation spectra exhibit a high degree of similarity. The presence of the peak at m/z 132 in imp-u is one significant difference between the two spectra. The probable fragmentation pattern of mitoxantrone and imp-u are shown in Schemes 1 and 2. Due to the fact that the impurities are usually process-related compounds, they are most probably structurally similar to the synthesized target drugs (Xu et al.

Scheme 1 Fragmentation pattern of mitoxantrone



Scheme 2 Fragmentation pattern of imp-u

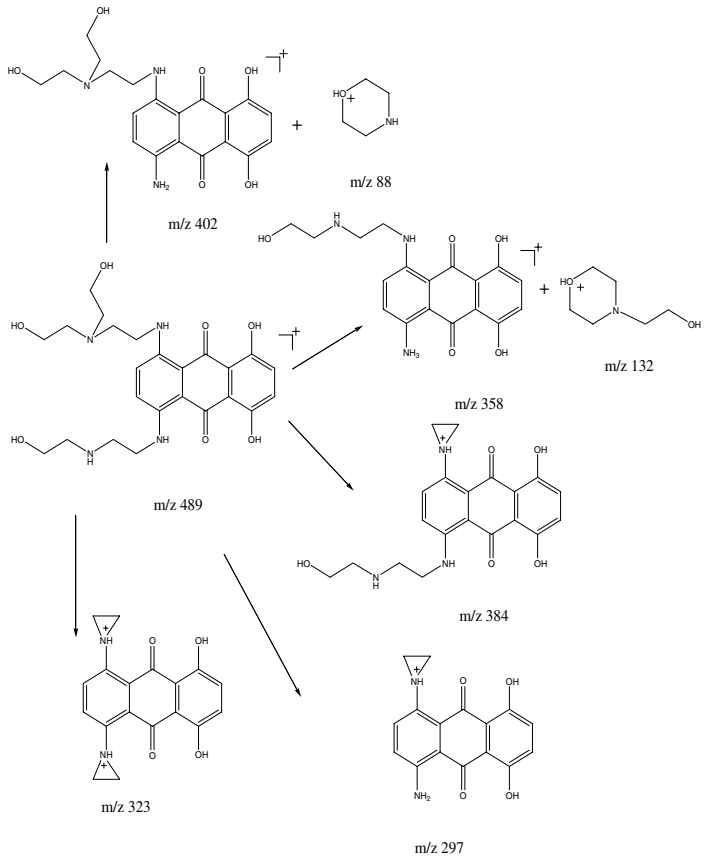


Table: NMR data of mitoxantrone and imp-u(DMSO-d6)

Position[a]	Mitoxantrone hydrochloride			Imp-u		
	δH	δC	DEPT	δH	δC	DEPT
1	–	154.7	–		154.9	–
2	7.54, d	125.2	CH	7.49, d	126.0	CH
3	7.54, d	125.2	CH	7.48, d	126.2	CH
4	–	154.7	–		154.9	–
5	–	146.2	–		147.3	–
6	7.11, d	125.0	CH	7.46, d	124.4	CH
7	7.11, d	125.0	CH	7.44, d	124.5	CH
8	–	146.2	–		147.5	–
9	–	184.6	–	–	183.4	–
10	–	184.6	–	–	183.3	–
4a	–	114.8	–	–	115.5	–
8a	–	108.7	–	–	107.5	–
9a	–	114.8	–	–	115.5	–
10a	–	108.7	–	–	107.5	–
1′	3.72, m	46.2	CH_2	3.48, m	42.7	CH_2
2′	3.08, m	38.7	CH_2	2.85, m	54.2	CH_2
3′	3.18, m	49.5	CH_2	2.86, m	56.9	CH_2
4′	3.85, m	56.6	CH_2	3.50, m	59.9	CH_2
1″	3.72, m	46.2	CH_2	3.39, m	48.5	CH_2
2″	3.08, m	38.7	CH_2	2.87, m	41.1	CH_2
3″	3.18, m	49.5	CH_2	2.84, m	51.6	CH_2
4″	3.85, m	56.6	CH_2	3.53, m	60.6	CH_2
5′	–	–	–	2.83, m	56.9	CH_2
6′	–	–	–	3.52, m	59.9	CH_2

s, singlet; d, doublet; m, multiplet, brs, broad singlet
a, Refer to chemical structures for numbering of mitoxantrone hydrochloride and Imp-u

2007). Meanwhile, the UV absorption maxima of both mitoxantrone and imp-u were 289 nm (both dissolved in methanol for UV scan), which indicated that imp-u had no characteristic absorption band different from mitoxantrone. This means that imp-u has the same structural unit as mitoxantrone, and only differs in one unknown group, which is of 44 aum. Comparing their molecular formulae, the unknown group of imp-u is C_2H_5O and the possible composition could be $-CH_2CH_2OH$ or $-CH_2OCH_3$. Its detailed structure could be confirmed by ^1H NMR and ^{13}C NMR experiments. Based on mass spectral information, the tentative structure of the new impurity was as proposed above.

The NMR spectra of both mitoxantrone and imp-u were determined for comparison. The ^1H NMR, ^{13}C NMR and DEPT data of mitoxantrone and imp-u are listed in the table. Due to the completely symmetrical structure of mitoxantrone, only half the number of carbon atoms could be observed in ^{13}C NMR, that is eleven. However, in the ^{13}C NMR of imp-u, the number of carbon atoms was twenty-one, three less than the actual number, indicating a partly symmetrical structure for the impurity. There was no corresponding change in the chemical shift of the anthraquinone ring which confirmed the presence of the anthraquinone ring in imp-u. The signal at δc 154.7 in mitoxantrone corresponds to quaternary carbon at the 1th and 4th positions, due to the effect of hydroxyl groups. Therefore, the signal at δc 154.9 in imp-u could be reasonably regarded as indicating quaternary carbon at the 1th and 4th positions of the anthraquinone ring. The signals at δc 59.9

and 56.9, two relatively strong spectral lines, confirmed as methylene carbon by DEPT135, were regarded as coinciding carbon atom signals. The signal at δc 59.9 represents the methylene carbon at the 4th and 6th positions, where the two carbons are attached to an electronegative atom, oxygen, with induced effect; and δc 56.9 represents the methylene carbon the at 3th and 5th positions respectively. So the composition of the unknown group could be $-CH_2CH_2OH$. The chemical shift values of the other methylenes were all nearly 3 ppm higher than those of mitoxantrone, which also indicated the presence of an electronegative $-CH_2CH_2OH$ group. The significant information about the partly symmetrical structure further confirmed that the $-CH_2CH_2OH$ group was bonded to the nitrogen atom. Furthermore, four methine carbons were confirmed by DEPT90, which also suggested the $-CH_2CH_2OH$ group was not bonded to the anthraquinone ring. In H-D exchange NMR, signals at δ_H13.55, δ_H10.64, and δ_H3.54 disappeared, indicating the presence of reactive hydrogen (–OH, –NH). The ^{1}H NMR spectrum of the impurity was slightly different from that of mitoxantrone. In the imp-u ^{1}H NMR, a signal at δ_H 4.37 corresponds to the three hydroxyl groups in $-CH_2OH$ groups and δ_H 13.55 corresponds to the phenolic hydroxyl group. A multiplet at δ_H 10.64 corresponds to –NH– connected on the anthraquinone ring and the signal at δ_H 3.5 corresponds to the secondary amine in the carbonchain. On the basis of the NMR spectroscopy data, the structure of imp-u was assigned as 1,4-dihydroxy-5-2-2-hydroxyethyl-amino-ethyl-amino-8-2-bis-2-hydroxyethyl-amino]ethyl-amino-9,10-anthracenedione, which is shown above.

The IR spectra showed the presence of benzene rings (1458, 1572 cm^{-1}), carbonyl (1641 cm^{-1}), and conjugated hydroxyl (3388 cm^{-1}).

3. Experimental

3.1. Samples

The samples of mitoxantrone hydrochloride investigated were prepared in the SH pharmaceutical factory research centre. The impurity A reference substance was purchased from the USA. Reagents used for analysis, i.e., ammonium acetate (HPLC grade), trifluoroacetic acid (HPLC grade), glacial acetic acid (HPLC grade), methanol (HPLC grade) and acetonitrile (HPLC grade), were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water used was double distilled and all other chemicals used were of analytical grade.

3.2. High performance liquid chromatography (analytical)

Analytical HPLC was performed on a Shimadzu LC 10AT series consisting of an LC-10AT VP pump, an SPD-10A VP UV detector, an SPD-M10A DAD detector, an SCL-10A VP system controller, and a CTO-10AS column temperature controller. The data were recorded using Class-VP™ software, version 5.03 (all from Shimadzu, Nakagyo-ku, Kyoto, Japan). The analysis was carried out on a Kromasil ODS column, 150 mm long, 4.6 mm i.d., and 5 μm particle diameter. The mobile phase consisted of H_2O–MeCN– glacial acetic acid (70 : 30 : 0.65, v/v/v), with sodium heptanesulfonate solution 0.45 g. UV detection was at 254 nm. Column temperature was maintained at 35 °C. Data acquisition time was 30 min.

3.3. Isolation of imp-u

A Shimadzu LC-6AD liquid chromatograph equipped with a SPD-10 A VP, UV-Vis detector (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) was used. A Shim-pack Prep.ODS(H)KIT C-18 (250 mm long × 20 mm i.d.) preparative column packed with 10 μm particle size was employed to isolate impurities. The mobile phase consisted of 0.5% trifluoroacetic acid-methanol (50 : 50, v/v). Flow rate was 5 ml/min and detection was carried out at 254 nm. Using the analytical HPLC method mentioned above for monitoring, fractions of >95% were pooled together. Data acquisition time was 80 min.

3.4. MS analysis

TOFMS analysis was carried out using an Agilent 6210 TOFMS spectrometer coupled with an Agilent 1200 HPLC. The mass spectrum of the isolated new impurity was acquired in positive spray ionization (ESI^+) mode. MS capillary voltage was set at 3500 V. Nitrogen was used as the drying gas at a flow rate of 11 ml/min and 350 V the gas temperature was 350 °C. The nebulizer gas was at a back-pressure of 20 psi and the fragmentator voltage was set at 150 V. The mass detected was 488.23.

ESIMS analysis was carried out using a Perkin-Elmer triple quadruple mass spectrometer (API3200 LC/MS/MS system). The isolated impurity and mitoxantrone were dissolved (about 0.05 mg/ml) in methanol and infused into the ion source by a syringe pump at the rate of 10 μl/min. The triple quadrupole mass spectrometer equipped with an electrospray source using a crossflow counter electrode run in positive mode (ESI^+), was set up in multiple reaction monitoring (MRM) mode. Ironspray voltage, declustering potential, and source temperature were 5.0 kV, 25V, and 50 °C, respectively. The dwell time was set at 0.5 s, the nebulizing gas and heater gas pressures were 20 psi and 30 psi respectively. The collision energy increased progressively from 20 V, 30 V, and 40 V to 50 V for both imp-u and mitoxantrone hydrochloride.

3.5. NMR spectroscopy

Nuclear magnetic resonance spectra were recorded on a Bruker–Avance 600 MHz instrument, using DMSO-d6 solution with tetramethylsilane (TMS) as the internal standard. Splitting patterns are designated as follows: s, single; d, doublet; m, multiplet; br, broad.

3.6. Infra-red spectroscopy

IR spectra were recorded in the solid state as KBr dispersions using a VECTOR 22 instrument. The wave number ranged from 400 to 4000 cm^{-1}.

Acknowledgements: This research was partly supported by the laboratory for the control of drugs in SiChuan Province and the analysis and testing center of SiChuan University. The authors gratefully acknowledge their cooperation.

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