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Isolation and identification of process impurities in trimethoprim drug substance by high-performance liquid chromatography, atmospheric pressure chemical ionization liquid chromatography/mass spectrometry and nuclear magnetic resonance spectroscopy

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Abstract

Twenty-two lots of recently synthesized trimethoprim drug substance, from five different manufacturers, in three different countries of origin, China, Israel and the United States, were investigated for the presence of impurities. A liquid chromatographic system, using gradient elution, and a mobile phase consisting of 0.25% TEA/0.1% formic acid (pH 5.8)–acetonitrile, was used to separate and detect two significant, recurring impurities in trimethoprim drug substance. The two impurities were isolated by preparative liquid chromatography and identified, using a combination of liquid chromatography/mass spectroscopy and nuclear magnetic resonance, as 2,4-diamino-5-(4-ethoxy-3,5-dimethoxybenzyl) pyrimidine and 2,4-diamino-5-(3-bromo-4,5-dimethoxybenzyl) pyrimidine. These impurities were not detected by the compendial method and were present at significant levels in 17 of the lots tested. Total impurity concentrations were in the range of 0.1–2.1%.

Keywords: Trimethoprim; Impurities; Liquid chromatography; Atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy; Proton nuclear magnetic resonance spectroscopy

1. Introduction

In the past several years, there have been a number of important initiatives by both federal and industry organizations, to establish limits for the presence of impurities in active pharmaceutical ingredients. The International Conference on Harmonization (ICH), an organization devoted to the coordination of technical requirements for the registration of pharmaceuticals in the European

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Union, Japan and the United States, has recently adopted the guideline 'Impurities In New Drug Substances' [1]. This document has introduced a number of important concepts, including the requirement that recurring impurities at or above the 0.1% level be identified. It has also established thresholds for the qualification of impurities, which is the process of determining their biological safety. The guideline states that impurities should be qualified at the 0.1% level or 1 mg day^{-1}, whichever is lower, when the maximum daily dose is ≤ 2 g day^{-1} (0.05% for > 2 g day^{-1} dosage). The Food and Drug Administration (FDA) has adopted the ICH guidelines and published them in the Federal Register [2].

The most important development, however, in terms of regulatory significance, was the introduction of new requirements in the section entitled 'Other Impurities', under General Notices and Requirements, in the United States Pharmacopeia (USP) 23, 6th Supplement [3]. In this section, the USP recognizes that drug substances can be obtained from more than one process and the tests included in the monograph may not detect all impurities. The USP now requires that when an impurity is present in an official substance, and the monograph test does not detect it, the impurity should have its amount and identity, when both are known, stated in the labeling; which for drug substances, is the certificate of analysis. The USP further states that, if an unlabeled impurity at greater than the 0.1% level is present in an official substance, the substance is in violation of USP requirements. In addition, the total of monograph-detected impurities and 'other impurities' may not exceed 2.0%, unless otherwise stated in the monograph.

The FDA annually tests a wide cross-section of both foreign and domestically manufactured drug substances for compliance with USP specifications. Rarely do these materials fail to meet compendial requirements, including tests for impurities. Although the USP has clarified the requirements for impurities in drug substances, and many of the newly introduced monographs have more extensive purity tests, there remain many official substances where no purity method is available, or the method is limited to a non-specific general screening procedure or a test for a specific impurity.

A study was undertaken to develop more extensive impurity profiles of selected drug substances to determine if these materials contain significant impurities other than those detected by the USP method. The initial compound selected for this study was trimethoprim (TMP) (2,4-diamino-5-(3,4,5 trimethoxybenzyl)pyrimidine). This compound was chosen because it was available from more than one supplier, in more than one country of origin, it was amenable to analysis by liquid chromatography, and the USP thin-layer chromatographic (TLC) test is for one specific impurity, acrylonitrile 3-anilino-2-(3,4,5-trimethoxybenzyl), which it limits to 0.5%.

TMP is an anti-bacterial, folic acid antagonist used in combination with sulfamethoxazole as a treatment for urinary tract infections. Purity studies on TMP and its related compounds are limited. De Martiis et al. [4] used phase partition for impurity enrichment, followed by TLC for the determination of impurities in TMP and several other compounds. Smyth and Chabala [5] used differential pulse polarography to detect the presence of impurities in trimethoprim raw material, but no identification was attempted. Deshpande et al. [6] used TLC to test TMP for the presence of 3,4,5-trimethoxybenzaldehyde, one of the starting materials in its synthesis.

In the present study, a total of 22 lots of trimethoprim drug substance, from five different manufacturers, in three different countries of origin, China, Israel and the United States, were investigated. These compounds, recently synthesized, were collected from a variety of pharmaceutical manufacturers, through the FDA's Drug Product Surveillance Program [7]. These drug substances were initially tested by the USP method and were found to meet all specifications, including the impurity test for 3-anilino-2-(3,4,5-trimethoxybenzyl)acrylonitrile. Further examination of these compounds, using gradient HPLC, revealed two significant, recurring impurities, present individually or in combination, in 17 of the 22 lots tested. These impurities were not detected by the USP test and, in many cases, were

present at sufficiently high levels that further investigation was warranted. Identification of these compounds was accomplished using a combination of preparative chromatography, liquid chromatography–mass spectroscopy (LC/MS) and nuclear magnetic resonance (NMR).

2. Experimental

2.1. Apparatus

2.1.1. Liquid chromatographic system

Two Shimadzu model LC-10AS pumps, an SPD-10AV UV-visible detector, an SCL-10A system controller, an SIL-10A auto-injector, an FRC-10A fraction collector and a Class-VP data system (Shimadzu Scientific Instruments, Princeton, NJ) were used.

(1) Analytical: flow rate, 1.0 ml min^{-1}; detector wavelength, 272 nm; detector sensitivity, 0.5 AU V^{-1}; column temperature, ambient; injection volume, 20 μl.

(2) Preparative: flow rate, 2.0 ml min^{-1}; detector wavelength, 272 nm; detector sensitivity, 0.5 AU V^{-1}; column temperature, ambient; injection volume, 250 μl.

2.1.2. Chromatographic columns

(1) Analytical: Beckman Ultrasphere ODS (4.6 mm × 25 cm), 5 μm particle size (Beckman Instruments, Fullerton, CA).

(2) Preparative: Beckman Ultrasphere ODS (10 mm × 25 cm) 5 μm particle size (Beckman).

2.1.3. LC/MS system

(1) HPLC: two Shimadzu model LC-10AD pumps, an SPD-10AV UV-visible detector, an SCL-10A controller (Shimadzu), and a Rheodyne injector (Rheodyne, Cotati, CA) were used. Operating parameters: flow rate, 1.0 ml min^{-1}; chromatographic column, same as in Section 2.1.2, part (1); detector wavelength, 272 nm; column temperature, ambient; injection loop, 20 μl.

(2) Mass Spectrometer: Finnigan LCQ equipped with an atmospheric ionization probe (APCI). The operating parameters for on-line HPLC analyses (1 ml min^{-1}) were: vaporizer temperature, 450°C; capillary temperature, 200°C; capillary voltage, 4 V; tube lens offset voltage, 5 V; sheath gas flow rate, 80; auxiliary gas flow rate, 10; scan range 90–1200 amu. The operating parameters for sample infusion analyses were: the same as above, except the capillary temperature was 150°C; sheath gas flow rate was 40; and sample flow rate was 10 μl min^{-1}. The operating parameters for MS/MS analysis were: relative collision energy, 20%; isolation window for m/z 291 and 305, 2 amu; for m/z 339/341 bromine cluster, 10 amu; scan range, 90–400 amu.

2.1.4. ^1H NMR spectroscopy

A Bruker AM-400 spectrometer with frequency 400.13, giving 200.062 Hz cm^{-1} operating at 28°C, was used. A 5-mm $^1H/^{13}C$ dual probe was used under the following conditions: data point resolution, 0.492 Hz per point; decoupling frequency, 5000; pulse width, 7 μs; relaxation delay, 2 s; acquisition time, 2.032 s; and number of scans, 32 or 500.

2.1.5. Rotary evaporator

This was a Labconco with Dewar condenser (Cole–Palmer Instrument Co., Vernon Hills, IL).

2.2. Reagents

2.2.1. Solvents

These were HPLC-grade acetonitrile (Burdick & Jackson, Muskegon, MI), triethylamine (Sigma, St. Louis, MO), formic acid, 90% (Fisher Scientific, Fair Lawn, NJ) and distilled water.

2.2.2. Chemicals

Dimethyl-d_6 sulfoxide (DMSO(d_6)), isotopic purity > 99.9 atom% D, was obtained from MSD Isotopes, Merck (Rahway, NJ). Trimethoprim USP reference standard was obtained from US Pharmacopeia Convention (Rockville, MD).

2.2.3. Mobile phase: for analytical and preparative analysis

Solvent A: 0.25% triethylamine/0.1% formic acid in H_2O (pH 5.8). Solvent B: acetonitrile. Gradient: 0 min, 10% B; 10 min, 10% B; 20 min, 25% B; 30 min, 25% B; 35 min, 40% B; 45 min, 40% B; 50 min, 80% B; 60 min, 80% B.

2.2.4. Samples

All samples were obtained from supplies at various pharmaceutical companies, through the Food and Drug Administration's Drug Product Surveillance Program.

2.2.5. Sample preparation: liquid chromatography

(1) Analytical: approximately 10 mg of sample were accurately weighed and dissolved in 5 ml of CH_3CN:H_2O (50:50), and 20 μl were injected into the HPLC system. (2) Preparative: approximately 240 mg of sample was accurately weighed and dissolved in 7 ml of CH_3CN:H_2O (50:50), and 250 μl were injected into the HPLC system. Impurities were isolated by fraction collection. Solvents were removed by rotary evaporation at 40°C in vacuum. Isolates were dried for 16 h at 105°C in vacuum.

2.2.6. Sample preparation (LC/MS)

(1) Low flow infusion: residues obtained by preparatory HPLC were dissolved in 20 ml of solvents A and B (50:50) and 1 ml diluted to 20 ml in the same solvent. (2) LC/MS high flow: 10 mg TMP were dissolved in 5 ml solvents A and B (50:50).

2.2.7. Sample preparation (NMR)

Residues obtained by preparatory column HPLC were dissolved in 0.75 ml DMSO(d_6) and placed in thin-walled 5-mm NMR tubes (Wilmad glass). Samples were vortexed and degassed (dissolved air from the atmosphere was found degrading to the resolution, probably because of the paramagnetic properties of the oxygen air component). Each NMR tube was capped using a Teflon cap, and then placed in the spectrometer to obtain the NMR spectrum. Chemical shifts were referred to residual ^1H in DMSO(d_6) at 2.62 ppm. Residual moisture obscuring a certain spectrum region or interfering with signals of interest was shifted using one drop of 1 N NaOD.

3. Results and discussion

The initial step in this study was the development of a liquid chromatographic system for the analysis of TMP and possible impurities. Ion pairing, using mobile phases consisting of acetonitrile and sulfonic acid salts, produced poor peak shape, as did several combinations of acetonitrile and phosphate salts. The only mobile phase additive that produced acceptable peak shape, with minimal tailing, was triethylamine (TEA). Concentrations of TEA from 0.25 to 1.0% produced similar chromatography, but the lower concentration was chosen so that this solvent could be more readily eliminated after impurities were isolated during preparative chromatography. Formic acid was used to adjust the pH of the TEA solution to 5.8, because its volatility makes it a good choice for mobile phases that will be used for LC/MS studies.

A linear gradient, using a mobile phase of 0.25% TEA/0.1% formic acid (pH 5.8)–acetonitrile, was initially applied to three TMP drug substances from the same manufacturer in China. This analysis revealed the presence of two significant impurities (impurities I and II) in each lot, with each impurity having an area percent concentration of approximately 1%. Analysis of an additional 19 lots of TMP produced by other manufacturers in China, Israel and the United States detected impurities I or II in 14 of these lots. The only five lots free of these impurities were manufactured by one company in the United States. Chromatographic profiles of TMP from each manufacturer included in this study are presented in Fig. 1. The estimated concentrations of impurities I and II, in each lot of TMP tested, are presented in Table 1.

The next step in identifying these impurities was to scale-up the HPLC parameters so that preparative chromatography could be performed and the compounds isolated for identification by MS and NMR. For ease of transition, a semi-preparative column consisting of the same packing material, particle size and column length as the analytical column was chosen. The column differed only in internal diameter (10 vs 4.6 mm). In this way the same gradient used for profiling work could be maintained, with only slight adjustments in flow rate (2 vs 1 ml min^{-1}). A typical preparative chromatogram is presented in Fig. 2.

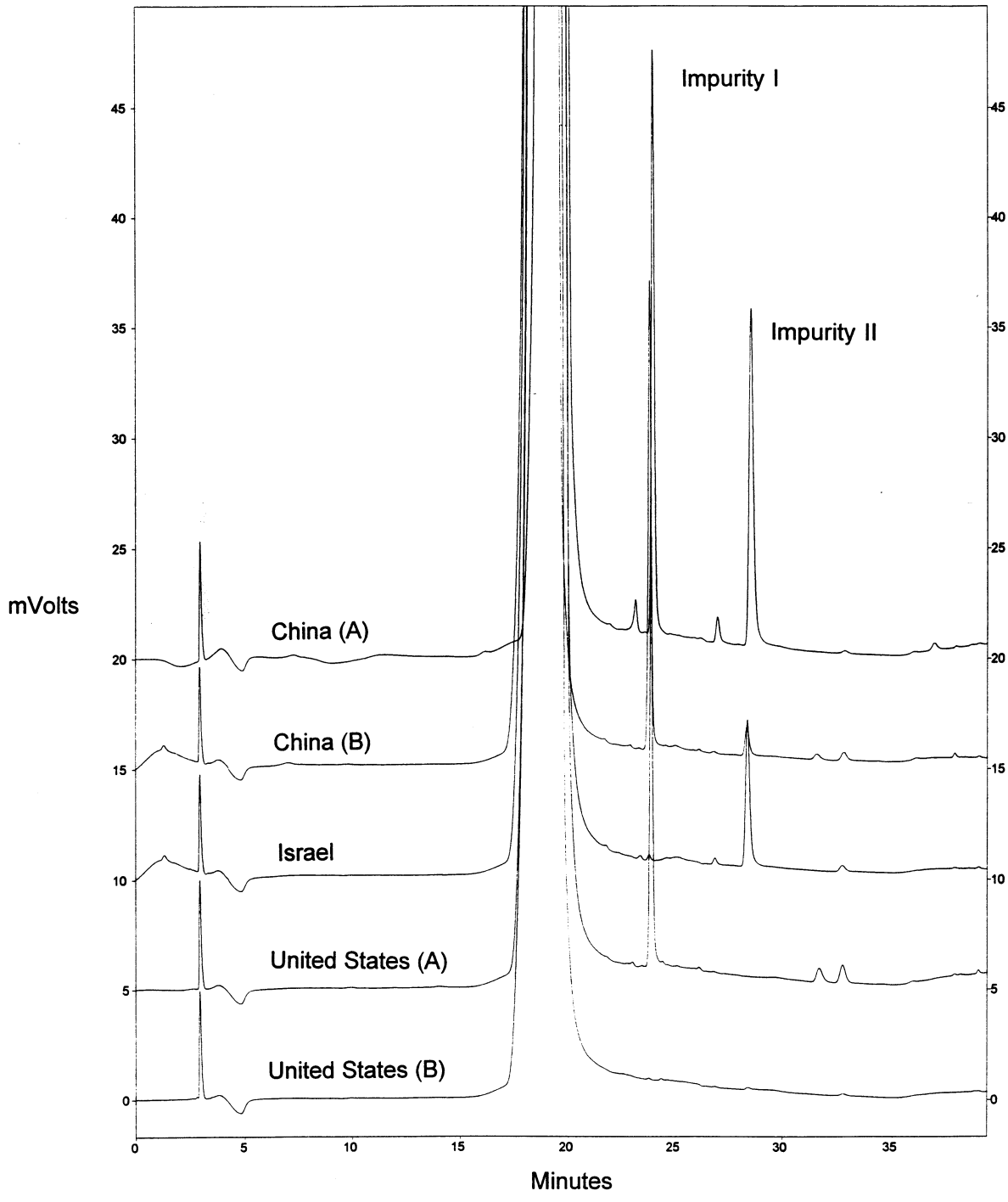

Fig. 1. HPLC profiles of TMP drug substance from various manufacturers using analytical column parameters.

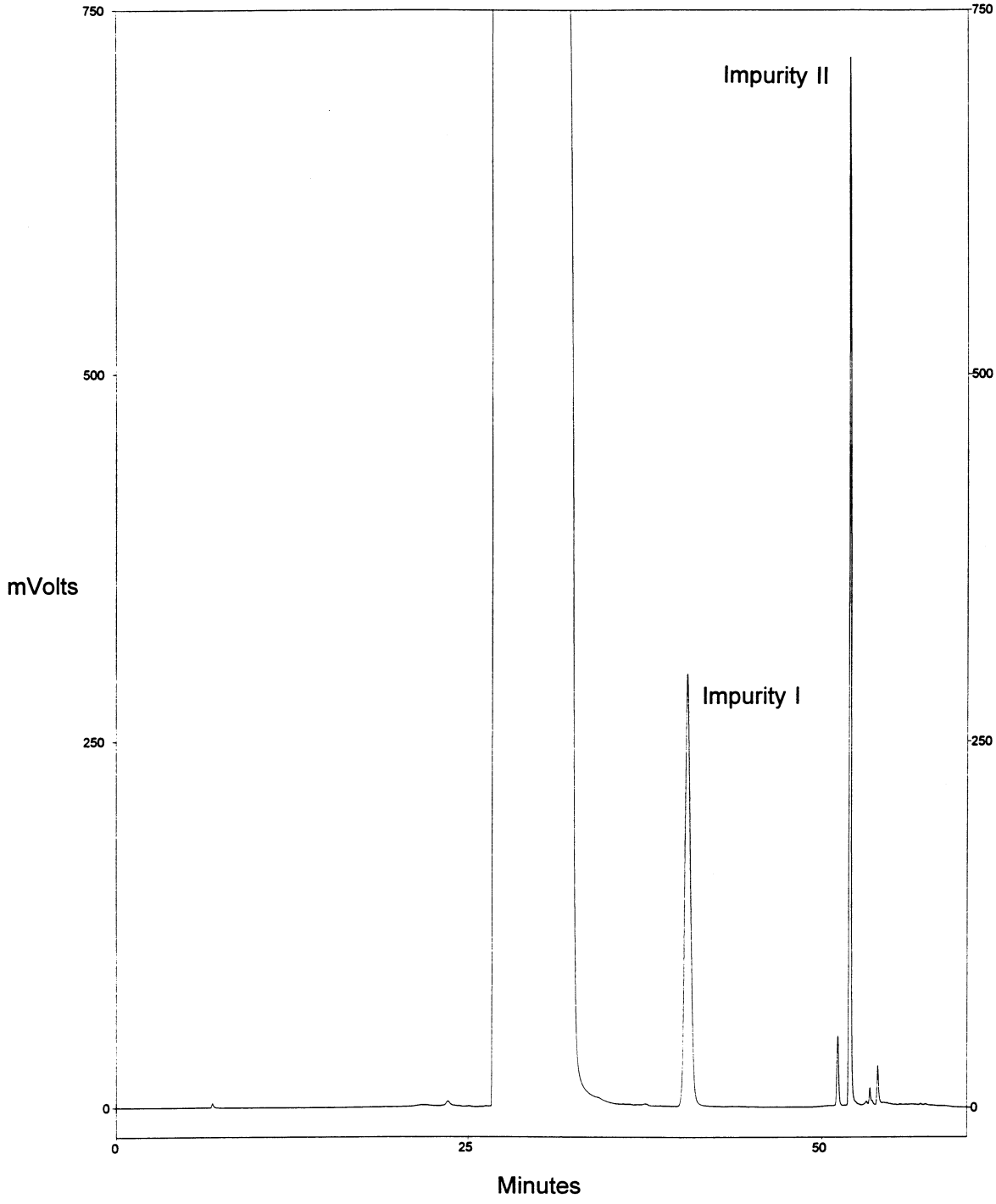

Fig. 2. Preparative chromatogram for the isolation of impurities I and II in TMP drug substance.

The amount of TMP injected for preparative work was approximately 8.5 mg, on column. This translates to approximately 85 μg of impurity isolated per injection, for impurities in the 1% range. Multiple injections (~ 20) were used so that 1–1.5 mg of impurity could be isolated for NMR analysis. For impurities present in lesser amounts, the number of injections were increased so that an equivalent amount could be isolated. Impurities were collected through an automated fraction collector, which was controlled directly by Shimadzu's Class-VP software. After collection of impurity fractions, solvents were eliminated by rotary evaporation at 40°C, under vacuum. Isolates were then dried at 105°C, under vacuum, for 16 h.

Table 1
Area percent concentrations of impurities in TMP drug substance

Manufacturer	Impurity I	Impurity II
China 'A'		
Lot 1	0.9	0.6
Lot 2	1.1	1.0
Lot 3	0.9	0.9
China 'B'		
Lot 1	1.0	ND[a]
Lot 2	1.0	ND
Lot 3	0.9	ND
Lot 4	0.9	ND
Lot 5	0.9	ND
Israel		
Lot 1	ND	0.2
Lot 2	ND	0.3
Lot 3	ND	0.2
Lot 4	ND	0.2
Lot 5	ND	0.2
Lot 6	ND	0.3
Lot 7	ND	0.1
United States 'A'		
Lot 1	0.6	ND
Lot 2	0.1	ND
United States 'B'		
Lot 1	ND	ND
Lot 2	ND	ND
Lot 3	ND	ND
Lot 4	ND	ND
Lot 5	ND	ND

[a] None detected (limit of detection 0.01%).

3.1. TMP and impurity I analysis

3.1.1. LC/MS

Portions of TMP, impurities I and II, collected by preparative HPLC, were analyzed by atmospheric pressure chemical ionization (APCI) LC/MS using the low flow (10 μl min^{-1}) sample infusion technique. Direct on-line high flow (1 ml min^{-1}) APCI LC/MS analysis was also performed on solutions of TMP drug substance containing impurities I and II. The mass spectral data obtained by both the low flow and high flow techniques were identical. The total ion current (TIC) chromatogram and the UV chromatogram are shown in Fig. 3. A peak was observed on the TIC chromatogram eluting between TMP and impurity I which was not detected on the UV chromatogram. The molecular weight of this compound was determined to be 399 but no further identification was attempted.

The positive ion APCI spectrum of TMP (Fig. 4A) exhibited a protonated molecular ion, $[M+H]$ at m/z 291, and impurity I (Fig. 4B) exhibited a protonated molecular ion at m/z 305. The increase of 14 mass units, suggested either a carbonyl (C=O) group had replaced the bridging methylene (CH_2) group which joins the diaminopyrimidine and the trimethoxybenzyl ring in TMP, or an ethoxy (OCH_2CH_3) group had replaced one of the methoxy groups (OCH_3) on the benzyl ring.

To obtain additional structural information, APCI MS/MS analyses were performed. The MS/MS spectra of TMP (Fig. 5A) and impurity I (Fig. 5B) contained the same series of product ions, m/z 123, 230, 258, 261, 275, and 276 (Table 2), thereby indicating the two compounds were very similar structurally. However, the spectra exhibited significant differences which provided further structural information for impurity I.

Cleavage on both sides of the bridging methylene group in TMP, resulted in product ions at m/z 123, $[CH_2(C_4H_5N_4)]$, representing the methylenediaminopyrimidine portion of the molecule and m/z 181, $(OCH_3)_3C_6H_2(CH_2)$, representing the methylenetrimethoxybenzyl portion of the molecule.

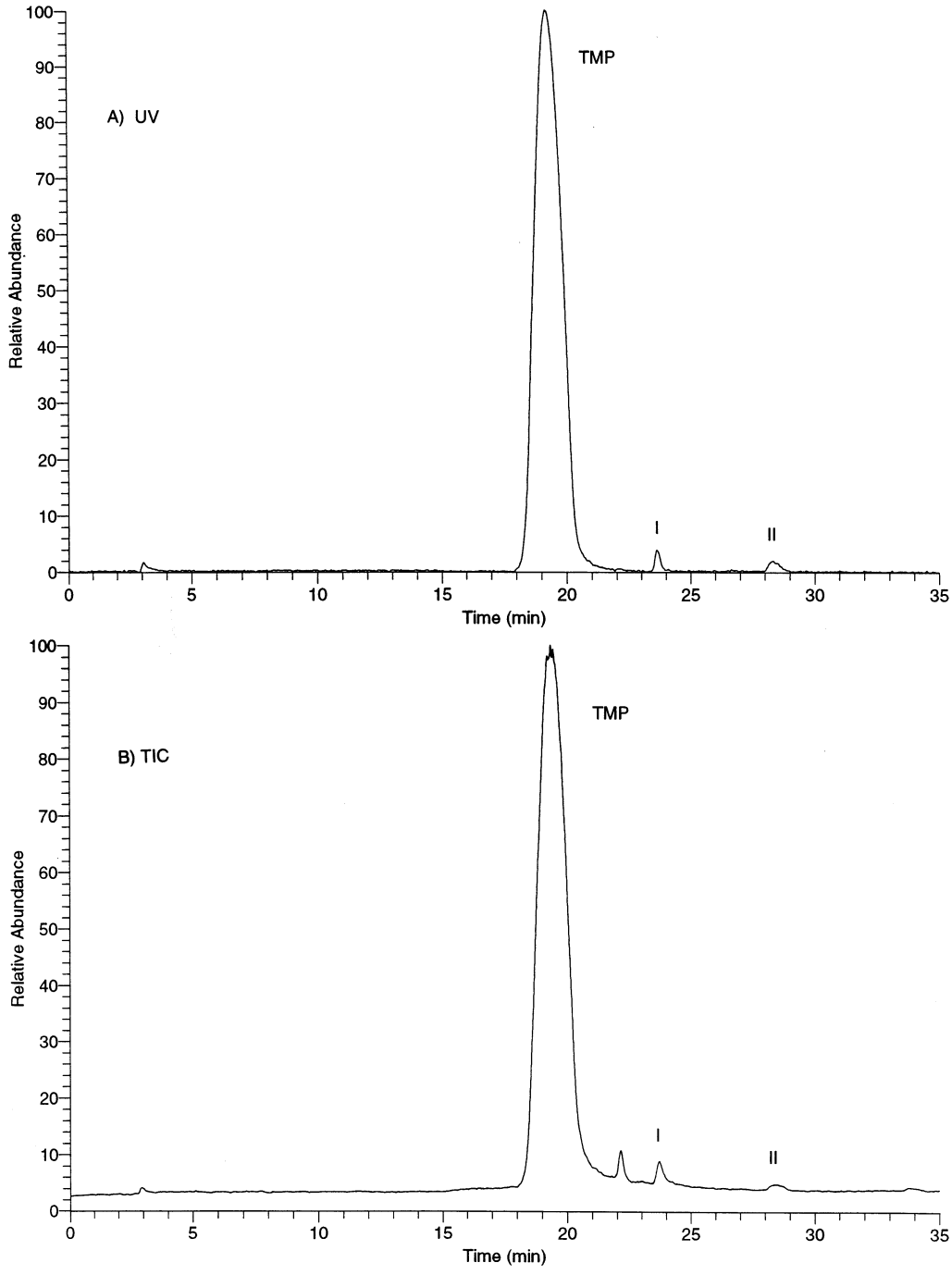

Fig. 3. LC/MS elution profile of TMP drug substance: (A) UV chromatogram; and (B) TIC chromatogram.

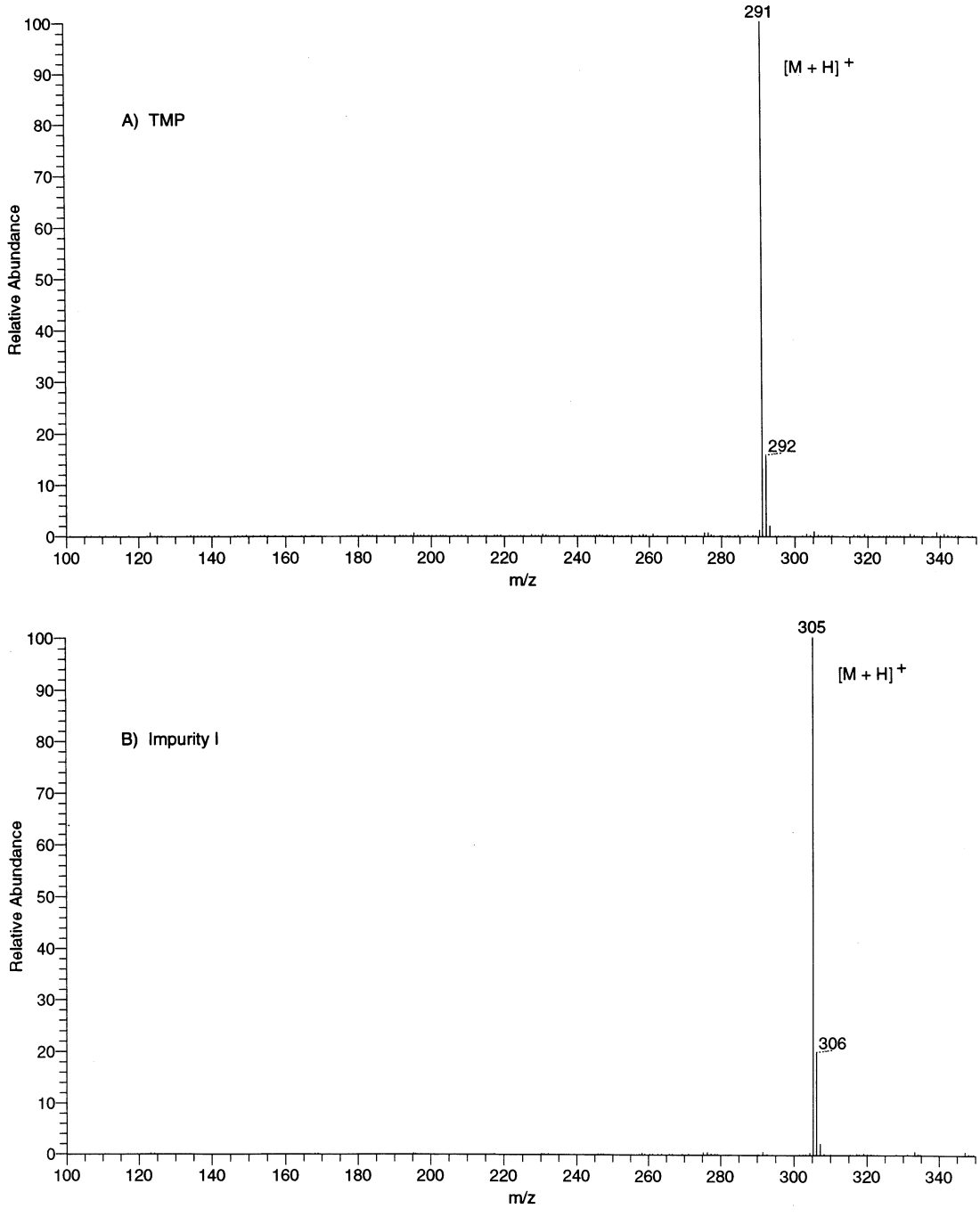

Fig. 4. APCI mass spectra of (A) TMP and (B) impurity I.

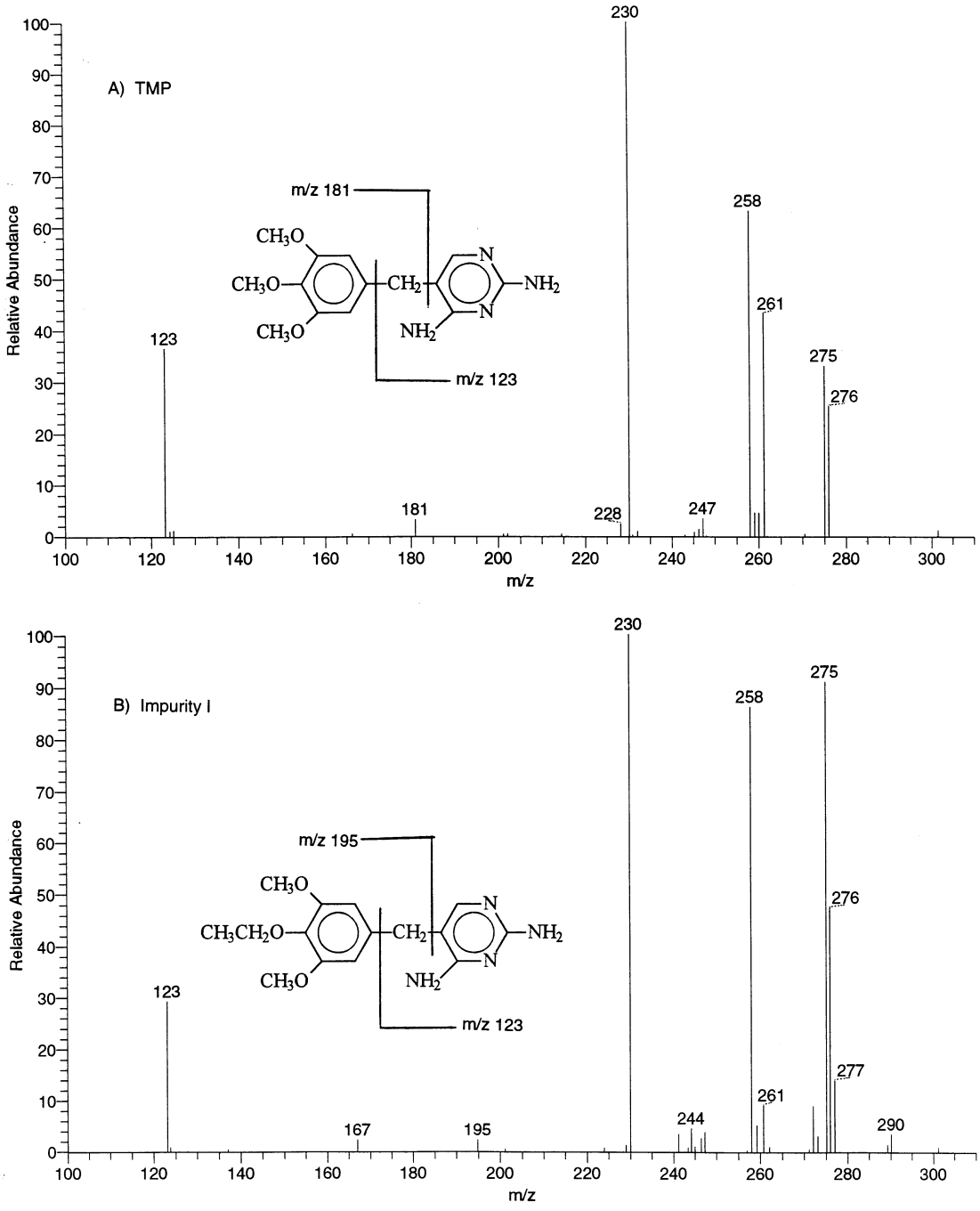

Fig. 5. APCI MS/MS spectra of (A) TMP and (B) impurity I.

The presence of m/z 123, $[CH_2(C_4H_5N_4)]$, in the spectrum of impurity I demonstrated that the methylene group remained intact on the impurity and was not replaced by a carbonyl group. The product ion m/z 181, observed for TMP, has been shifted 14 amu to m/z 195 in impurity I. The 195

ion results from cleavage on the other side of the methylene group, as in TMP; however, it now represents $(OCH_3)_2(OCH_2CH_3)C_6H_2(CH_2)$, the dimethoxy(ethoxy)benzyl portion of the molecule. Therefore, the MS/MS data indicated that the increase of 14 mass units in molecular weight for impurity I was not due to replacement of the methylene group by a carbonyl group, but due to replacement of one of the methoxy groups by an ethoxy group on the benzyl ring.

3.1.2. NMR

The NMR spectra of TMP [8] and impurity I are presented in Fig. 6A and Fig. 6B, respectively, along with assignment of signals in Table 3. The spectrum of impurity I contained a number of resonances which correlated to that of TMP; however, it differed in that the *para*-methoxy singlet, which was present at 3.73 ppm (b) for TMP, appeared to be absent. One drop of 1 N NaOD in D_2O was added to the solution of impurity I in DMSO(d_6) to shift the residual HDO. This sharpened the signals and cleared the region between 3.0 and 4.0 ppm from any residual water interference. This spectrum of impurity I confirmed the absence of the *para*-methoxy singlet and, in addition, two multiplets, a triplet at 1.29 ppm (h) and a quartet at 3.97 ppm (i), were observed. The presence of these two multiplets having the same coupling constant ($j = 7$ Hz) suggested spin–spin coupling interactions between them. This was substantiated by spin decoupling experiments. The triplet and the quartet, along with the absence of the *para*-methoxy singlet, were consistent with an ethoxy group replacing the methoxy group in the position *para* to the methylene group. The singlet at 3.63 ppm (a) represented the two protons of the bridging methylene group between the two rings. The singlet at 3.80 (c) ppm represented the six protons of the two methoxy groups *meta* to the methylene group. These protons appeared magnetically equivalent due to rotation of the ring around the single bond. The singlet at 6.62 ppm (f) was assigned to the two benzene ring protons, and the most downfield singlet at 7.59 ppm (g) was assigned to the pyrimidine ring proton. The addition of NaOD exchanged the amino hydrogens on the pyrimidine ring and therefore they were not observed in this spectrum of impurity I. They did, however, appear in the original spectrum of impurity I (before addition of NaOD) as very broad singlets at 6.18 and 6.61 ppm, respectively.

Based on a combination of the MS and NMR data, impurity I was identified as 2,4-diamino-5-(4-ethoxy-3,5-dimethoxybenzyl) pyrimidine.

Table 2
MS/MS product ion assignment for TMP and impurity I

TMP	m/z	Impurity I
—	305	M
M	291	—
—	290	$M-(CH_3)$
$M-(CH_3)$	276	$M-(CH_2CH_3)$
$276-H$	275	$276-H$ plus $M-(OCH_3)+H$
$M-(OCH_3)+H$	261	$M-(OCH_2CH_3)+H$
$M-2(OCH_3)+H$	230	$M-(OCH_3+OCH_2CH_3)+H$
—	195	$(OCH_3)_2(OCH_2CH_3)C_6H_2(CH_2)$
$(OCH_3)_3C_6H_2(CH_2)$	181	—
$CH_2(C_4H_5N_4)$	123	$CH_2(C_4H_5N_4)$

$M = [M+H]^+$

3.2. TMP and impurity II analysis

3.2.1. LC/MS

The positive ion APCI spectrum of impurity II (Fig. 7A) exhibited a one-bromine protonated molecular ion isotope cluster at m/z 339/341. The increase in molecular weight of 48 mass units (trimethoprim = 290) along with the one-bromine isotope cluster, suggested a bromine atom (79 amu) substitution for one of the methoxy groups (31 amu) on the benzyl ring.

The positive ion APCI MS/MS spectrum of the protonated molecular ion cluster, 339/341 (Fig. 7B), produced a one-bromine product ion cluster at m/z 324/326, $[M+H]-CH_3$, and non-brominated fragment ions at m/z 244, [m/z 324-HBr], m/z 216, and m/z 123. The non-brominated product ion at m/z 123, which again results from cleavage on one side of the bridging methylene group, represents $[CH_2(C_4H_5N_4)]$, the methylene diaminopyrimidine portion of the molecule, indicating that the bromine atom is on the benzyl ring portion of the molecule. This was confirmed by the presence

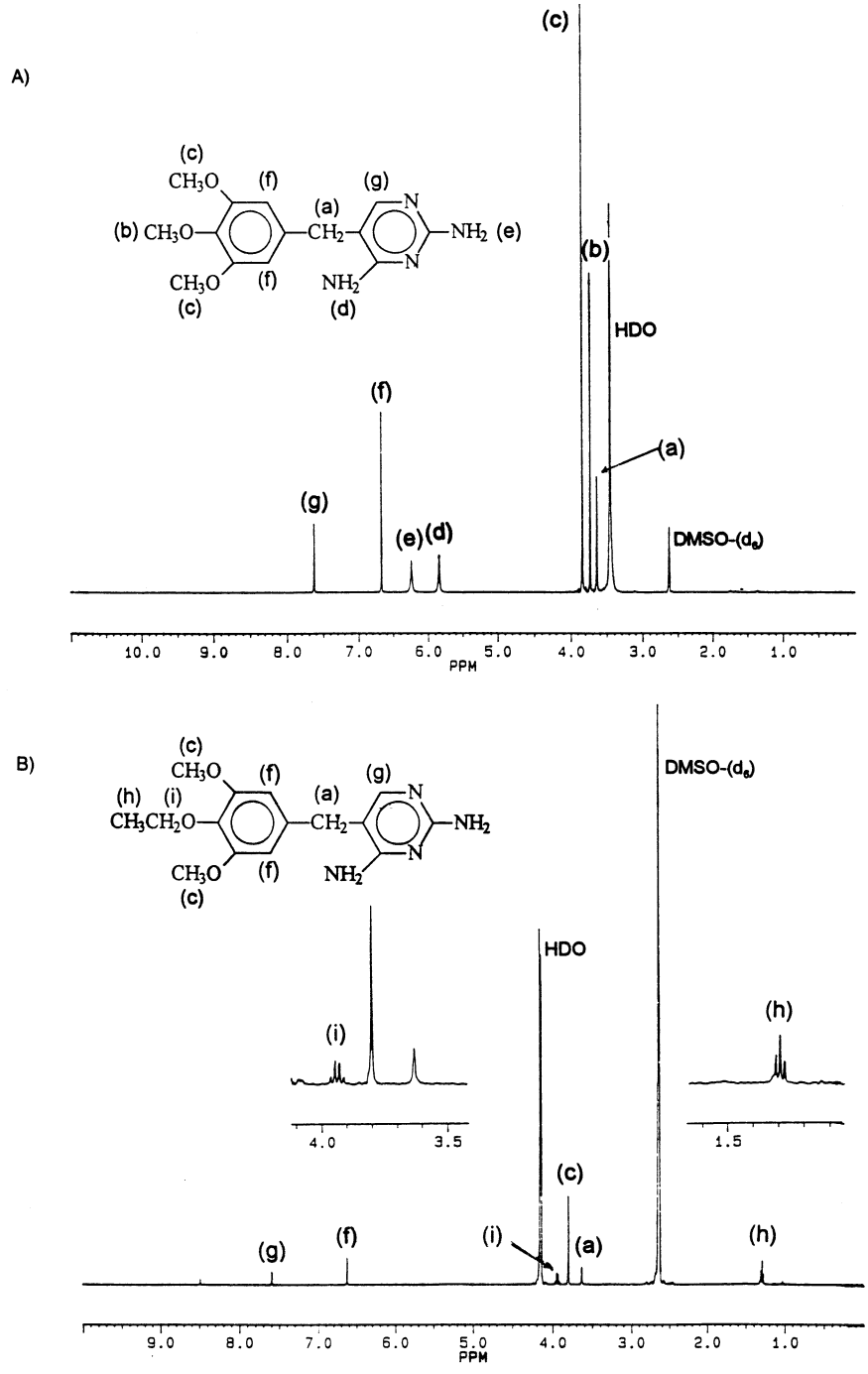

Fig. 6. ^{1}H NMR spectra of (A) TMP and (B) impurity I.

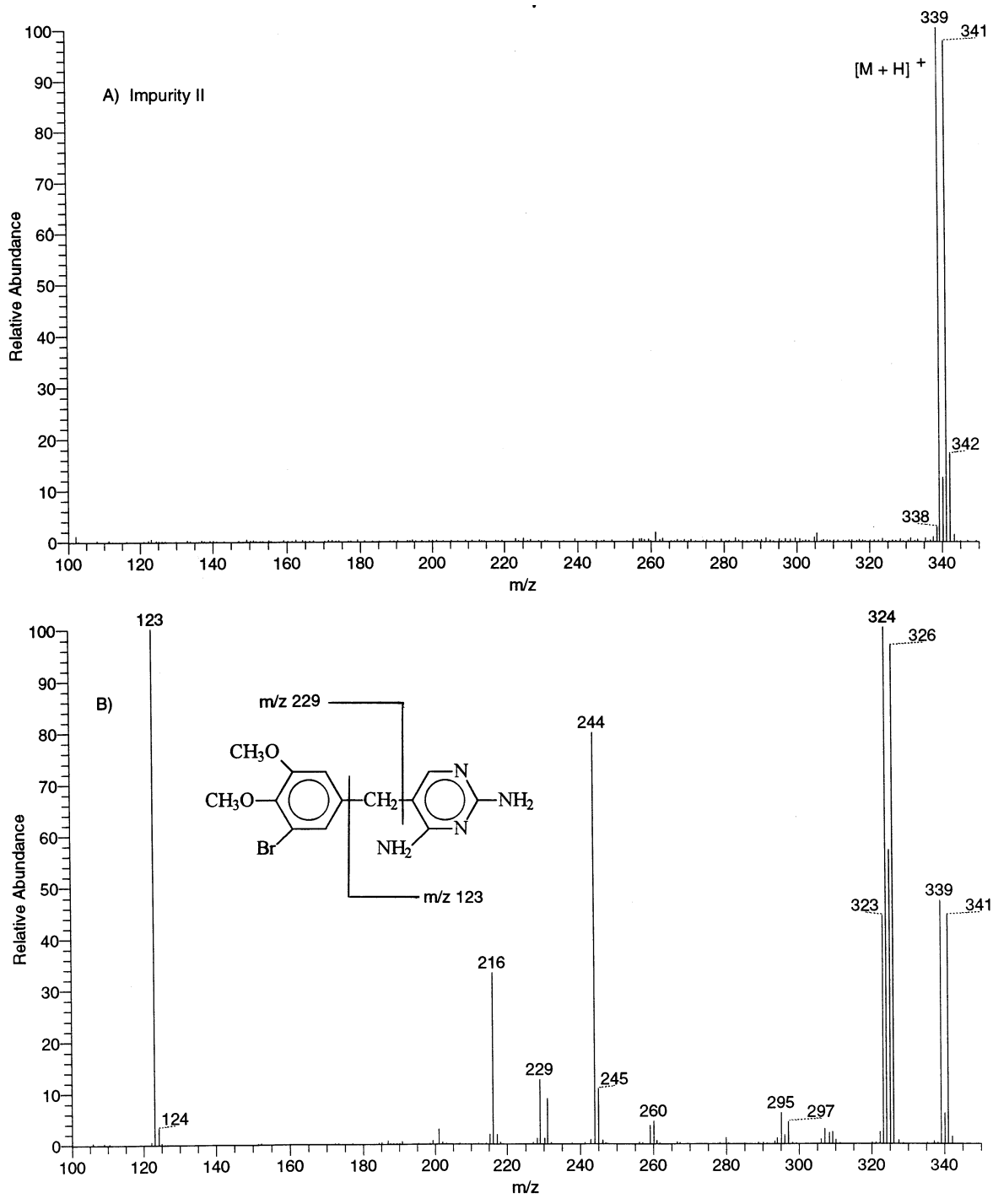

Fig. 7. Positive ion mass spectra of impurity II: (A) APCI and (B) APCI MS/MS.

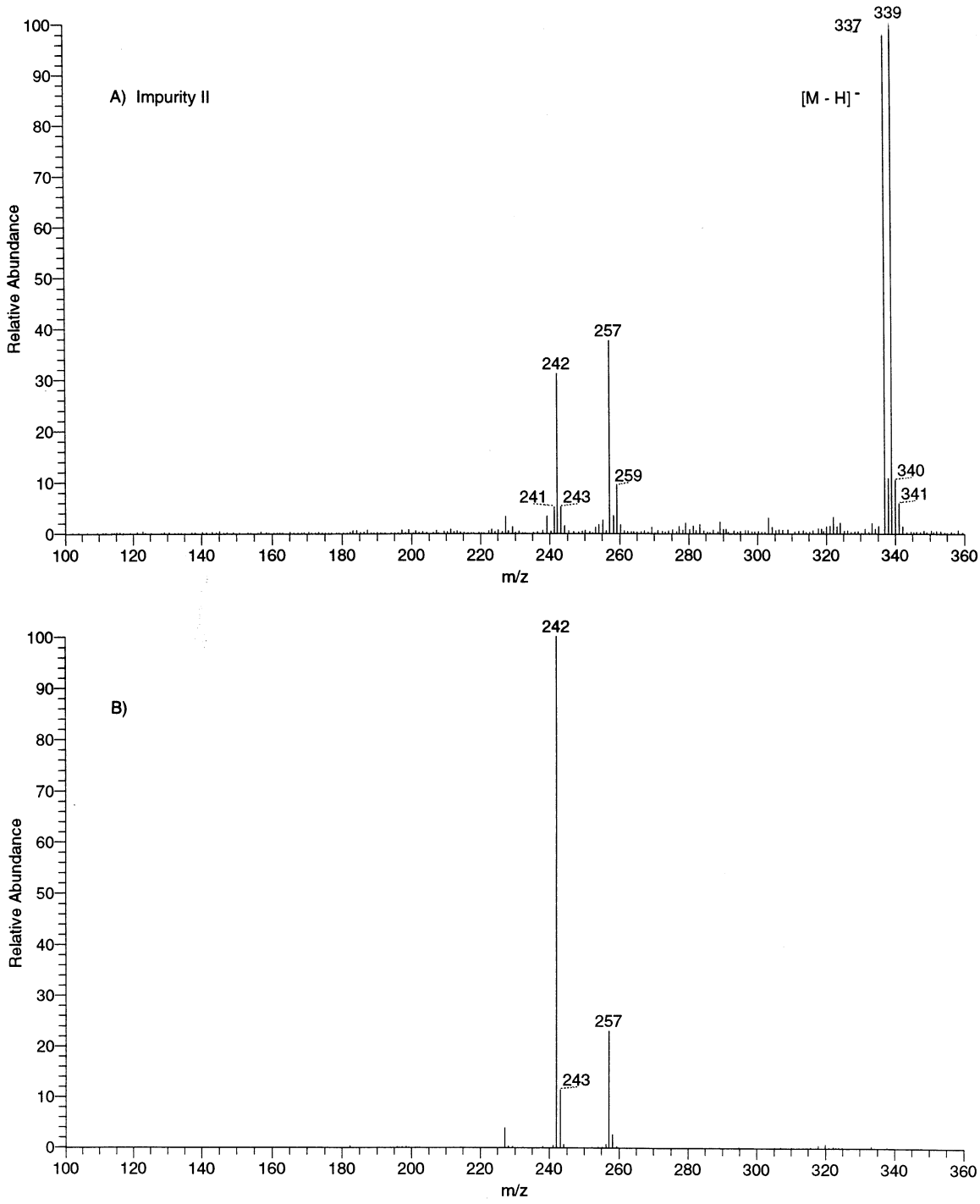

Fig. 8. Negative ion mass spectra of impurity II: (A) APCI and (B) APCI MS/MS.

Table 3
Chemical shift data and assignments for TMP, impurities I[a] and II

R	TMP			Impurity I			Impurity II		
	δ (ppm)	M	No. of Hs	δ (ppm)	M	No. of Hs	δ (ppm)	M	No. of Hs
a	3.64	S	2	3.63	S	2	3.69	S	2
b	3.73	S	3	—	—	—	3.81	S	3
c	3.86	BS	6	3.80	S	6	3.91	S	3
d	5.84	BS	2	—	—	—	6.19	BS	2
e	6.24	S	2	—	—	—	6.59	BS	2
f	6.67	S	2	6.62	S	2	7.10	S	1
f[1]	—	—	—	—	—	—	7.11	S	1
g	7.63	S	1	7.59	S	1	7.67	S	1
h	—	—	—	1.29	T ($J=7$)	3	—	—	—
i	—	—	—	3.94	Q ($J=7$)	2	—	—	—

R, resonance; S, singlet; T, triplet; M, multiplicity; BS, broad singlet; Q, quartet.
[a] Impurity I in DMSO+NaOD.

of a one-bromine isotope cluster product ion at m/z 229/231, $Br(OCH_3)_2(C_6H_2)CH_2$, which resulted from cleavage on the other side of the bridging methylene group.

The negative ion APCI spectrum of impurity II (Fig. 8A) exhibited a one-bromine molecular ion isotope cluster at m/z 337/339, $[M-H]$. Minor fragment ions were observed at m/z 257 and 242. The negative ion APCI MS/MS spectrum of m/z 337/339 (Fig. 8B) produced the same two product ions, m/z 257 and 242. The negative ion data did not produce any additional structural information other than to confirm the presence of bromine on the molecule.

The APCI MS/MS data indicated that impurity II was a brominated dimethoxy analog of trimethoprim.

3.2.2. NMR

The NMR spectrum for impurity II (Fig. 9) exhibited similar features to that of both TMP and impurity I (Fig. 5), again suggesting structural similarity (Table 3). The upfield singlet at 3.69 ppm (a) was assigned to the two protons of the bridging methylene group between the two rings. The broad singlets at 6.19 (d) and 6.59 ppm (e) represented the protons of the two amino groups, with the downfield signal (e) assigned to the amino group attached to the carbon located between the two nitrogens in the pyrimidine ring. The remaining hydrogen on the pyrimidine ring also shifted slightly downfield to 7.67 ppm (g) and appears as a sharp singlet.

The major difference in the spectrum of impurity II resulted from the changes in the trisubstituted benzyl portion of the molecule. The spectrum of TMP (Fig. 6A) exhibited two resonances at 3.73 (b) and 3.86 ppm (c), representing the *para*- and *meta*-methoxy groups, respectively. Integration revealed that the *para*-methoxy signal represented three protons while the *meta*-methoxy signal represented six protons. Impurity II (Fig. 9) exhibited the corresponding signals for the *para*- and *meta*-methoxy groups shifted slightly downfield at 3.81 (b) and 3.91 (c), respectively. However, integration revealed that the *para* and *meta* signals now represented three protons each, thereby indicating one of the *meta*-methoxy groups was replaced. The addition of bromine as a replacement for one of the *meta* substituents was consistent with this data. The presence of bromine in the *meta* position breaks the symmetry around the benzyl–methylene C–C single bond and gives rise to two non-equivalent benzyl ring hydrogens at 7.10 (f) and 7.11 (f[1]) ppm, each representing one proton. The signal at 7.11 ppm was assigned to the proton *ortho* to the electron-withdrawing bromine. The corresponding two protons in TMP were equivalent and appeared as a singlet at 6.67 ppm.

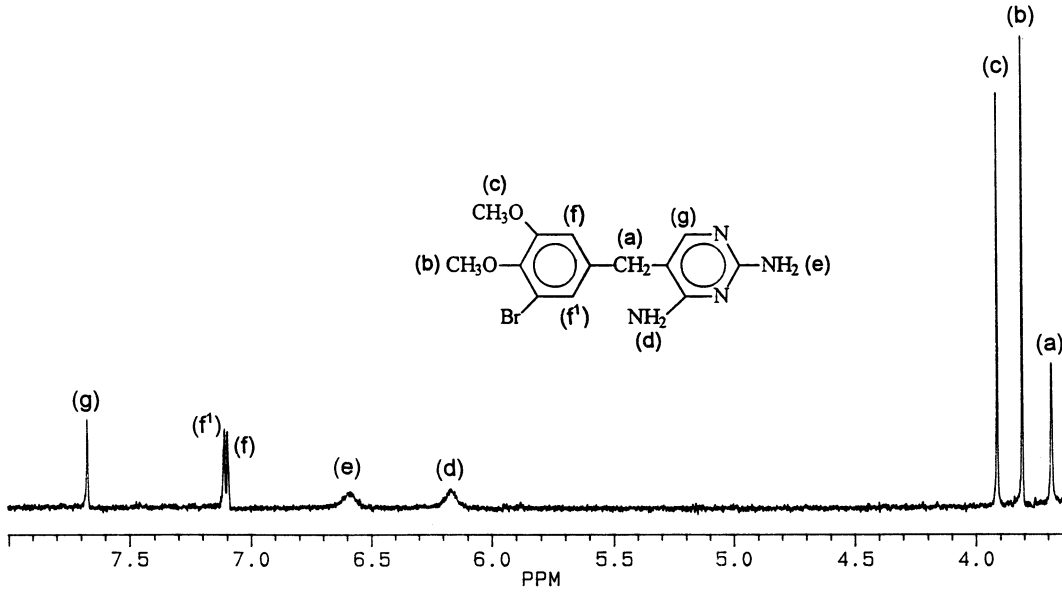

Fig. 9. ^{1}H NMR spectrum of impurity II.

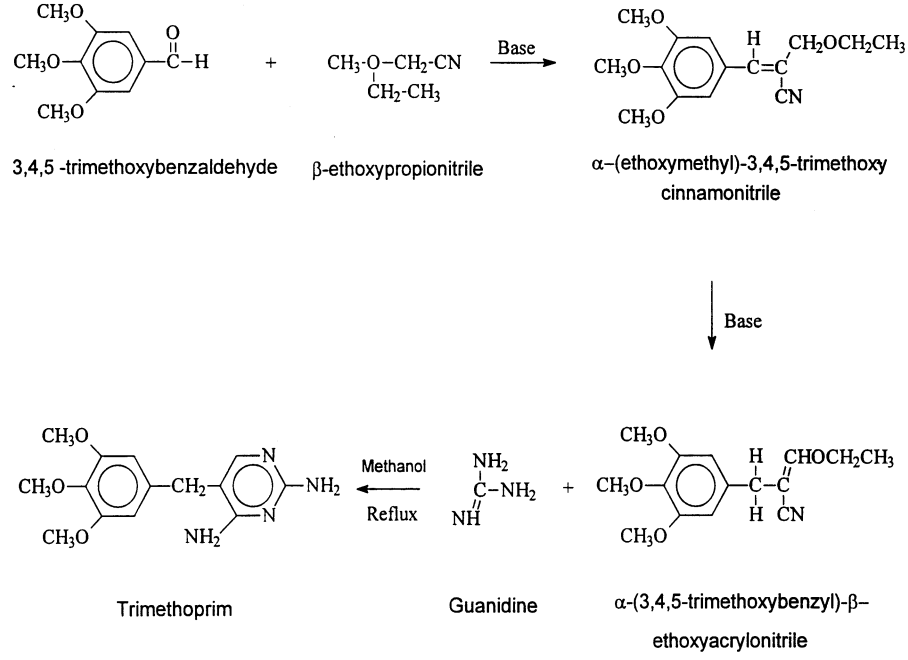

Fig. 10. Synthesis of TMP.

Based on a combination of the MS and NMR data, impurity II was identified as 2,4-diamino-5-(3-bromo-4,5-dimethoxybenzyl) pyrimidine. This compound was included as a possible impurity in TMP in the study conducted by De Martiis et al. [4]. The authors suggested this bromo analog was due to the presence of a bromodimethoxy benzaldehyde impurity in the trimethoxy benzalde-

hyde starting material. One of the synthetic processes incorporating this starting material is shown in Fig. 10 [9].

4. Conclusions

The combined use of gradient and preparative liquid chromatography, LC/MS and NMR has resulted in the identification of two important impurities, 2,4-diamino-5-(4-ethoxy-3,5-dimethoxybenzyl) pyrimidine and 2,4-diamino-5-(3-bromo-4,5-dimethoxybenzyl) pyrimidine, in trimethoprim drug substance. These compounds were present, either singularly or in combination, in 17 of 22 lots, produced by five manufacturers in three different countries. The total concentration of these impurities ranged from 0.1 to 2.1%. These data clearly demonstrate that the purity and quality of active pharmaceutical ingredients can vary significantly from one manufacturer to another. The fact that each of the lots included in this study met compendial requirements, despite several of these materials containing impurities between 1 and 2%, emphasizes the need for a more comprehensive purity test to analyze this compound.

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