Purification and Identification of an Impurity in Bulk Hydrochlorothiazide

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ABSTRACT: Hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide) (HCTZ) **1** is a widely used diuretic and anti-hypertensive. Recently, the Pharmeuropa recognized a new impurity initially thought to be an HCTZ dimer **6**, which consists of the active drug (HCTZ) linked via the former β-ring methylene to a known degradate, 5-chloro-2,4-disulfamylaniline **2**. In an effort to meet a new requirement, an analytical high-pressure liquid chromatography method was developed that was selective and sensitive to the subject impurity. The impurity was concentrated and purified using a combination of solid phase extraction and reverse-phase high-pressure liquid chromatography. Subsequently, the impurity has been identified as a specific HCTZ-CH_2-HCTZ isomer utilizing a variety of analytical techniques, including hydrolysis, ultraviolet spectroscopy, liquid chromatography/mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The data resulting from the application of these analytical techniques confirm the identity of the impurity as a methylene bridged pair of HCTZ molecules; however, a total of six possible isomers **7a–f** exist because of the presence of three reactive amines/sulfonamides on each HCTZ molecule. One unique molecular structure (4-[{6-chloro-3,4,-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide}-methyl]-chloro-3-hydro-H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide) **7f** was identified using two-dimensional COSY, NOESY, and TOCSY ^{1}H NMR experiments. © 2001 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 90:1800–1809, 2001
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INTRODUCTION

Hydrochlorothiazide (HCTZ) **1** is a common diuretic used for the treatment of hypertension. HCTZ affects the distal renal tubular mechanism of electrolyte reabsorption for diuretic efficacy and increases excretion of sodium and chloride in approximately equivalent amounts. The mechanism of this anti-hypertensive effect is unknown but may be related to the excretion and redistribution of body sodium. HCTZ is produced by the combination of 5-chloro-2,4-disulfamylaniline (DSA) **2** and formaldehyde in aqueous alkaline solution (Fig. 1). Formaldehyde first reacts with the more nucleophilic aryl amine to give the hemi-animal **3**. The newly formed hemi-animal is unstable and quickly loses water to form the imine **4**. The imine then reacts with the neighboring sulfonamide to give HCTZ.

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Figure 1. Synthesis of HCTZ from DSA and the known process impurity, CTZ.

The impurity and degradation profiles of a drug substance are critical to its safety assessment and the optimization of the manufacturing process. The International Conference on Harmonization (ICH) has established a guideline that states that all reoccurring impurities and degradates at or above the 0.05% level in drug substance should be identified. Chlorothiazide (CTZ) **5** and DSA are well-recognized process impurities that are formed during the synthesis of HCTZ. Furthermore, the degradation of HCTZ in aqueous solution has been previously studied by Mollica et al.[1,2] and Yamana et al.[3] HCTZ was found to degrade to DSA and formaldehyde via hydrolysis. Mollica et al. studied the pH-rate profile of the hydrolysis of HCTZ at 60°C and found a bell-shaped curve plot of log K_1 versus pH with maximum at about pH 7.2 and minimum at pH 2.2 to 3. The impurity and solid-state stability profiles of HCTZ have also been previously studied. Deppeler[4] found that solid forms of HCTZ could be stored at ambient temperature for 5 years without evidence of degradation. Stress testing of HCTZ,[5] either 3 months at room temperature (10 mg HCTZ/mL methanol) or 4 days at 100°C (dry powder HCTZ), yielded only the degradate DSA. However, an additional unknown degradate was detected after 16 h in either 0.1 N HCl or 0.1 N NaOH solution.[5] Thomas et al.[6] reported analysis of bulk HCTZ by micellar electrokinetic capillary chromatography, and found three unknown peaks, which were not identified in their work.

In 1995, the Pharmeuropa[7] submitted a proposed monograph including high-pressure liquid chromatography (HPLC) method to replace the current thin-layer chromatography related substances test in the HCTZ monograph. This method identifies DSA, CTZ, and an additional more-retained impurity compared with HCTZ. The Pharmeuropa has proposed that this impurity is a dimer of HCTZ (DSA-CH_2-HCTZ), namely, 5-chloro-1-{[6-chloro-3,4-dihydro-2H-1,2,4-benzothiadizine-7-sulfonamide-1,1-dioxide(methyl)]-amino}-benzene-2,4-disulfonamide **6** (Fig. 2). Concurrently, a reverse-phase HPLC method was developed in our laboratory to characterize the impurity profile for HCTZ. Samples of bulk HCTZ from three suppliers were analyzed by the subject method. Similar impurity profiles were observed for samples from three different sources. In addition to CTZ and DSA, one impurity peak was observed at 0.2–0.4% of HCTZ levels (by area) and several impurity peaks were observed at less than 0.05% of HCTZ active level. The impurity peak was concentrated by solid phase extraction (SPE) and further purified by reverse-phase HPLC. The structure of this molecule (Fig. 2) has been determined to be an HCTZ-CH_2-HCTZ isomer (4-[{6-chloro-3,4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide-1,1-dioxide}-methyl]-6-chloro-3-hydro-H-1,2,4-benzothiadiazine-7-sulfonamide-1,1,dioxide) **7f**. The molecular structure of the impurity was elucidated using hydrolysis, ultraviolet (UV) spectroscopy, liquid

Figure 2. Proposed Structures of DSA-CH_2-HCTZ and HCTZ-CH_2-HCTZ.

chromatography/mass spectrometry (LC/MS), and $^1H/^{13}C$ nuclear magnetic resonance (NMR) spectroscopy. The HCTZ-CH_2-HCTZ structure **7f** is different from the DSA-CH_2-HCTZ **6** structure that Pharmeuropa proposed in 1995. European Pharmacopoeia (EP) changed the structure from DSA-CH_2-HCTZ to HCTZ-CH_2-HCTZ in the 2000 supplement after this work was presented.[8]

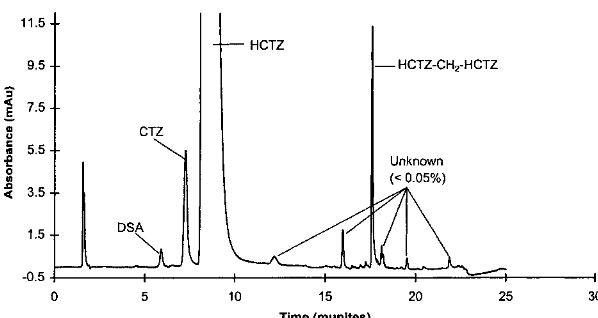

Figure 3. Chromatogram of a typical HCTZ sample using the HPLC analysis conditions specified in Experimental Section.

EXPERIMENTAL SECTION

Materials

Bulk HCTZ was obtained from Merck Manufacturing Division (Albany, GA), Sigma (St. Louis, MO), and Lancaster Synthesis, Inc. (Windham, NH). Acetonitrile and sodium phosphate monobasic were obtained from Fisher Scientific (Fair Lawn, NJ). Dimethyl sulfoxide-d_6 was obtained from Cambridge Isotopes Laboratory (Andover, MA). Solid phase extraction C18 cartridges were obtained from J. T. Baker, Inc. (Phillipsburg, NJ). Formic acid was obtained from EM Science (Gibbstown, NJ). All chemicals and reagents were used as received.

Instrumentation

HPLC analysis and purification were performed on a Thermoseparations Products (TSP) HPLC system equipped with a P4000 pump, an AS3500 injector, and a UV3000 detector interfaced via an SN4000 controller to an IBM PC equipped with PC1000 software. UV spectra were obtained by using the UV3000 detector on a TSP HPLC. LC/MS data were obtained on a FISONS VG Platform mass spectrometer interfaced to a Hewlett-Packard 1050 HPLC. ^1H and ^{13}C NMR spectra were acquired on a Bruker DMX 300 MHz NMR spectrometer. Proton chemical shifts were referenced relative to tetramethylsilane.

HPLC Analysis

Various sources of HCTZ were analyzed on an HPLC system equipped with a Phenomenex Nucleosil 5 C18 column (150×4.6 mm). The method of analysis incorporated a mobile phase which was composed of acetonitrile and aqueous 0.1% formic acid. The mobile phase was run with a flow rate of 1 mL/min under ambient conditions. A linear gradient was applied to the mobile phase with the following conditions: initiated with 10% acetonitrile for 10 min and then increased to 86% acetonitrile over 10 min, returned to 10% acetonitrile over 1 min, and maintained for another 4 min. Samples were dissolved in 10% acetonitrile/90% 20 mM NaH_2PO_4 (pH = 2.5). Typically, 20 μL of a 0.5 mg/mL solution of sample were injected onto the column and peaks were detected by UV absorbance at a wavelength of 280 nm. The retention times of DSA, CTZ, HCTZ, and the proposed HCTZ-CH_2-HCTZ impurity were 5.9, 7.1, 8.5, and 17.5 min, respectively (Fig. 3).

SPE Sample

One gram of HCTZ was dissolved in a mixture of 5 mL of 20 mM NaH_2PO_4 (pH = 2.5) and 30 mL of acetonitrile, then diluted to 300 mL with 20 mM NaH_2PO_4 (pH = 2.5). A 30-mL aliquot of this sample was loaded onto an SPE C18 cartridge which was previously conditioned with 2 mL of acetonitrile and 2 mL of water. The cartridge was then washed with 15 mL of water and 3 mL of 20% acetonitrile/80% water. The sample was eluted from the cartridge with 1 mL of acetonitrile and the eluent was diluted to 10 mL with water. The concentration process was repeated until most of the HCTZ was removed upon subsequent HPLC analysis.

HPLC Purification

The HCTZ-CH_2-HCTZ sample, preconcentrated by the SPE C18 cartridge, was eluted through a Phenomenex Nucleosil 5 C18 column (150×4.6 mm) using a linear gradient with acetonitrile and aqueous 0.1% formic acid. The mobile phase was run with a flow rate of 1 mL/min under ambient conditions. The method utilized a linear gradient from 30% acetonitrile to 90% acetonitrile

from 0 to 10 min, a linear gradient from 90% acetonitrile to 30% acetonitrile from 10 to 11 min, and was maintained at 30% acetonitrile from 11 to 15 min. The sample was detected by UV absorbance at a wavelength at 280 nm. The retention times of DSA, HCTZ, and HCTZ-CH_2-HCTZ were 2.8, 3.0, and 4.9 min, respectively (Fig. 4). Fractions of HCTZ-CH_2-HCTZ were collected between 4.7–5.2 min. A total of approximately 5 mL was collected. The eluent was diluted to 50 mL with water and loaded onto a SPE C18 cartridge that was previously conditioned with 2 mL of acetonitrile and 2 mL of water. The sample-loaded cartridge was washed with 20 mL of water and then 3 mL of a 20% acetonitrile/80% water solution. To minimize water in the sample, the cartridge was dried for 2 h under vacuum. The sample was then eluted from the cartridge into an NMR tube using 1 mL of acetonitrile. The acetonitrile was evaporated to dryness under a nitrogen stream. Finally, the sample in the NMR tube was further dried under vacuum at room temperature for 2 h. The sample was reinjected under Figure 3 conditions and the peak eluted at the HCTZ-CH_2-HCTZ position.

UV Spectrum

UV spectra of HCTZ-CH_2-HCTZ were obtained online using the HPLC purification method. The peaks at 3.0 min (HCTZ) and 4.7 min (HCTZ-CH_2-HCTZ) were scanned from 200 to 350 nm on a UV3000 detector. The HCTZ-CH_2-HCTZ impurity was found to exhibit wavelength maxima at 223, 273, and 316 nm. For HCTZ, the wavelength maxima are 223, 272, and 320 nm.

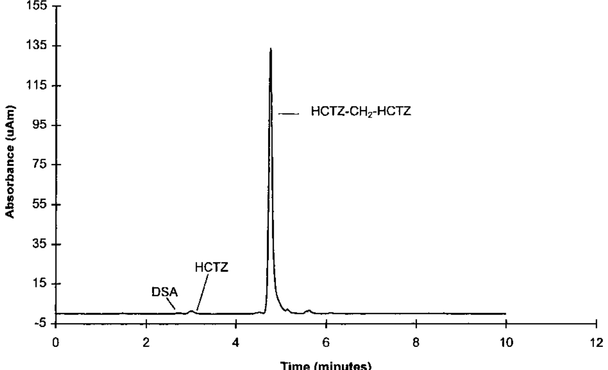

Figure 4. Chromatogram of HCTZ-CH_2-HCTZ after purifications analyzed using the purification HPLC conditions.

NMR Analysis

Dimethyl sulfoxide-d_6, 0.6 mL, was added to an NMR tube containing the dried sample to dissolve the HCTZ-CH_2-HCTZ impurity and then one drop of 0.1 N HCl was added. Each sample was mixed well before acquiring NMR spectra.

HCTZ

^{1}H NMR (ppm) (dimethyl sulfoxide-d_6 + one drop of 0.1 N HCl) (Table 1): 8.02 (1 H, s, N4), 7.98 (1 H, s, C8), 7.97 (1 H, t, $J = 8.3$ Hz, N2), 7.51 (2 H, s, C7-SO_2NH_2), 6.98 (1 H, s, C5), and 4.71 (2 H, d, $J = 8.3$ Hz, C3). ^{13}C NMR (ppm) (acetone-d_6)[4]: 147.7 (arom. C4′), 135.9 (arom. C7), 129.6 (arom. C6), 127.3 (arom. C8), 120.8 (arom. C8′), 118.9 (arom. C5), and 55.9 (C3).

HCTZ-CH_2-HCTZ

^{1}H NMR (ppm) (dimethyl sulfoxide-d_6 + one drop of 0.1 N HCl) (Fig. 5 Table 1): 8.86 (1 H, t, $J = 5.9$ Hz, C7′– SO_2NH–), 8.36 (1 H, t, $J = 8.3$ Hz, N2), 8.19 (1 H, s, N4′), 8.04 (1 H, s, C8), 8.03 (1 H, s, C8′), 7.97 (1 H, t, J=8.1 Hz, N2′), 7.61 (2 H, s, C7–SO_2NH_2), 7.33 (1 H, s, C5), 7.00 (1 H, s, C5′), 4.81 (2 H, d, $J = 8.3$ Hz, C3), and 4.73 (4 H, d, $J = 6.5$ Hz, C4–7′ and C3′). ^{13}C NMR (ppm) (dimethyl sulfoxide-d_6 + one drop of 0.1 N HCl): 148.19 (arom. C–NH–R), 145.22 (arom. C–NH–R), 135.88 (arom. C–SO_2NH_2), 135.35 (arom. C–SO_2NH–), 130.65 (arom. C–Cl), 128.00 (arom. C–Cl), 126.19 (arom. C–H), 124.74 (arom. C–H), 121.50 (arom. C–SO_2NH–ring), 119.49 (arom. C–SO_2NH–ring), 118.24 (arom. C–H), 117.73 (arom. C–H), 61.96 (N–CH_2–N), 58.06 (N–CH_2–N), and 55.20 (N–CH_2–N).

LC/MS Analysis

The HPLC assay for HPLC purification was used to perform subsequent LC/MS analyses. Samples used for NMR analysis were injected on the LC/MS and analyzed using electrospray ionization with a source temperature of 80°C and a sample cone voltage of 60 eV. Positive ion spectra were acquired over the mass range (m/z) from 150 to 700 amu. For the HCTZ-CH_2-HCTZ peak at a retention time of about 5 min (Fig. 4), a protonated molecular ion $[M+H]^+$ of m/z 607 was obtained with several fragment ions at m/z 310, 298, and 281. Under the same conditions, HCTZ (retention time about 3 min) gave the molecular ion $[M+H]^+$ at m/z 298 and one fragment ion at m/z 281 which

Table 1. Assignments of ^{1}H NMR Resonances for HCTZ[a] and HCTZ-CH_2-HCTZ in Acidified DMSO-d_6

Resonance (ppm)	Multiplicity[b]	Magnitude	Assignment	Functional Group
HCTZ				
4.71	d (8.3 Hz)	2 H	C3	Methylene ring
6.98	s	1 H	C5	Ar-H
7.51	s (broad)	2 H	C7−SO_2−NH_2	1° Sulfonamide
7.97	t (8.3 Hz)	1 H	N2	2° Sulfonamide
7.98	s	1 H	C8	Ar-H
8.02	s (broad)	1 H	N4	Aniline
HCTZ−CH_2−HCTZ				
4.73	d (6.5 Hz)	4 H	C3′ and C4−7′	Methylene ring and bridge
4.81	d (8.3 Hz)	2 H	C3	Methylene ring
7.00	s	1 H	C5′	Ar-H
7.33	s	1 H	C5	Ar-H
7.61	s (broad)	2 H	C7−SO_2−NH_2	1° Sulfonamide
7.97	t (8.1 Hz)	1 H	N2′	2° Sulfonamide
8.03	s	1 H	C8′	Ar-H
8.04	s	1 H	C8	Ar-H
8.19	s (broad)	1 H	N4′	Aniline
8.36	t (8.3 Hz)	1 H	N2	2° Sulfonamide
8.86	t (5.9 Hz)	1 H	C7′-SO_2-NH-	2° Sulfonamide

[a] The ^{1}H NMR assignmets for HCTZ were correlated from Ref. 4.
[b] s = singlet, d = doublet, t = triplet.

is $[M+H-NH_3]^+$. The negative ion mass spectrum gave a deprotonated molecular ion $[M-H]^-$ at m/z 605 and no fragment ions were formed.

Hydrolysis Analysis

Fractions of HCTZ-CH_2-HCTZ (retention time, 4.7 min) were collected using the HPLC conditions for purification. The eluent was diluted with 20 mM NaH_2PO_4 at pH 9, pH 7, and pH 5.5. The samples were analyzed at 0 and 60 h using the HPLC purification method conditions. HCTZ was the only product observed in all three pH buffer solutions examined.

RESULTS AND DISCUSSION

The UV, IR, ^{1}H and ^{13}C NMR, and MS spectra for HCTZ have been documented in *Analytical Profiles of Drug Substances*.[4] The UV spectrum of the impurity revealed absorption maxima at 223, 273, and 316 nm which are comparable to the absorption maxima of HCTZ (223, 272, and 320 nm). The similarity of the two UV spectra suggests that the conjugation of the impurity is not significantly altered from that of HCTZ. However, it is expected that all of the known degradates/impurities (DSA, CTZ, etc.) are also similarly conjugated as HCTZ and thus UV analysis alone still leaves open several potential molecular combinations to yield the resultant impurity. Therefore, the impurity was isolated by preparative HPLC to allow the application of further chemical and spectroscopic studies. The initial study involved hydrolysis stress testing of the isolated impurity. After 60 h in 20 mM NaH_2PO_4 buffer at pH 9, pH 7, and pH 5.5, the only hydrolysis product observed was HCTZ. Furthermore, the combined amount of HCTZ

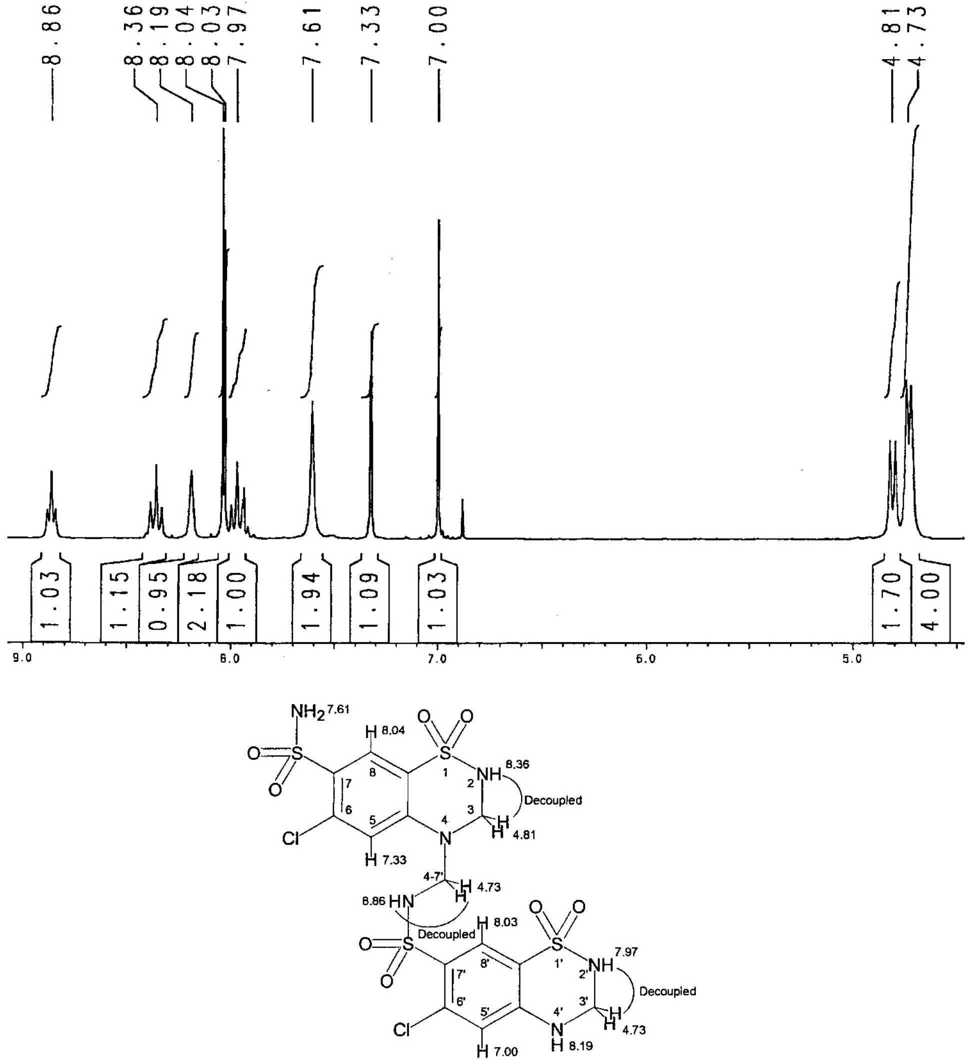

Figure 5. ^{1}H NMR of HCTZ-CH_2-HCTZ in acidified DMSO-d_6.

and the impurity at 60 h was equivalent to the initial amount of the impurity. Mollica et al.[2] proposed that, during the hydrolysis of the methylene in the HCTZ ring, the intermediate imine and hydroxymethylamine are not stable and would further hydrolyze; therefore, the only hydrolysis product observed was DSA. It is hypothesized that the impurity would follow a similar hydrolysis pathway as that of HCTZ; however, the bridging methylene in HCTZ-CH_2-HCTZ would be more susceptible toward hydrolysis than the methylene contained in the HCTZ ring. Therefore, limited hydrolysis of HCTZ-CH_2-HCTZ should result in only the production of HCTZ.

LC/MS analysis of the isolated impurity gave a protonated molecular ion at m/z 607, with a characteristic isotope pattern, indicating the presence of two chlorine atoms in the molecule. The measured molecular weight is 12 amu greater than two molecules of HCTZ and is consistent with two molecules of HCTZ bridged by a methylene group. The base peak in the spectrum at m/z 310 shows an isotope pattern characteristic of a single chlorine and was assigned to $[HCTZ+CH_2]^+$. An ion at m/z 298 corresponds to the $[M+H]^+$ ion of HCTZ, and a signal at m/z 281 is due to loss of NH_3 from the m/z 298 ion. The partial structure of the impurity was further elucidated using NMR spectroscopy specifically through resonance multiplicity, selective decoupling, and by comparison with the spectrum of the parent compound, HCTZ. One-dimensional ^{1}H NMR and ^{13}C NMR analyses reveal that the impurity has three unique methylene groups (4.81 ppm; d, 2H and 4.73 ppm; d, 4H in ^{1}H

NMR (Fig. 5); and 61.96, 58.06, and 55.20 ppm in ^{13}C NMR), further substantiating that the impurity is truly an HCTZ-CH_2-HCTZ isomer.

The HCTZ-CH_2-HCTZ impurity is likely a by-product of the synthesis, where formaldehyde and DSA are combined in aqueous alkaline solution to produce HCTZ (Fig. 1). However, HCTZ has three additional active amine/sulfonamide groups (N2, N4, and C7-SO_2NH_2) that can also react with a second equivalent of formaldehyde via nucleophilic addition. The resultant intermediate can subsequently react with an additional amine/ sulfonamide group on another HCTZ molecule. Because there are three amine/sulfonamide groups on each HCTZ molecule, the possibility exists then for six structural isomers (Fig. 6) to potentially form through this reaction mechanism. Structures **7a**, **7b**, and **7c** are symmetric and there fore the ^{1}H NMR of these three structures should give a spectrum very similar to HCTZ. However, the ^{1}H NMR spectrum of HCTZ-CH_2-HCTZ (Fig. 5) is not similar to that of HCTZ and suggests that the structure of HCTZ-CH_2-HCTZ is either structure **7d**, **7e**, or **7f** all which have asymmetric substitutions about the bridging methylene group. The ^{1}H NMR shows only one broad resonance integrating to 2H, which is characteristic of the primary sulfonamide, thus eliminating **7d**, which has two primary sulfonamides. The ^{1}H NMR also shows only one broad resonance integrating to 1H, which is characteristic of the aniline proton, thus eliminating **7e**, which has two aniline protons. The three triplets in the ^{1}H NMR are characteristic of the secondary sulfonamide group being split by a neighboring methylene group. Only compound **7f** has three such secondary sulfonamide groups. Mechanistically, the most likely structure of the HCTZ-CH_2-HCTZ isomer would be compound **7f**. It is likely that the first step in the formation reaction would involve nucleophilic addition of an HCTZ amine to formaldehyde and would proceed through the aniline moiety, which is the strongest nucleophile on HCTZ, to form a hemi-animal.[9] The newly formed hemi-animal is unstable, and can then react with the more acidic and least sterically hindered primary sulfonamide on another molecule of HCTZ to yield the HCTZ-CH_2-HCTZ isomer (Fig. 7).

Compound	Structure	Bridge	# of Protons by Functional Group: Aniline	Sulfonamide 1°	Sulfonamide 2°
7a		2-2	2	4	0
7b		4-4	0	4	2
7c		7-7	2	0	4
7d		2-4	1	4	1
7e		2-7	2	2	2
7f		4-7	1	2	3

Figure 6. Six potential structures of HCTZ-CH_2-HCTZ.

To further elucidate the proposed structure, the ^{1}H NMR of HCTZ in dimethyl sulfoxide-d_6 with added hydrochloric acid was acquired as a point of reference, using two-dimensional correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY), and total correlation spectroscopy (TOCSY) ^{1}H NMR. The COSY experiment shows that the methylene resonance at 4.71 ppm (d, 2H, C3) is directly correlated to the resonances at 7.97 ppm (t, 1H) and 8.02 ppm (br, 1H). Furthermore, in the NOESY ^{1}H NMR spectra for HCTZ, cross peaks are observed between the resonance at 6.98 ppm (C5) and the resonance at 8.02 ppm. The combination of these two results shows that the resonance at 8.02 ppm must be that of the aniline proton (N4) which is not strongly coupled with the methylene and resonates as a broad singlet. Also, the peak at 7.97 ppm is that of the secondary sulfonamide (N2) which couples strongly with the methylene and thus resonates as a triplet. The complete ^{1}H

Figure 7. Proposed reaction mechanism for the formation of HCTZ-CH_2-HCTZ in the presence of excess formaldehyde.

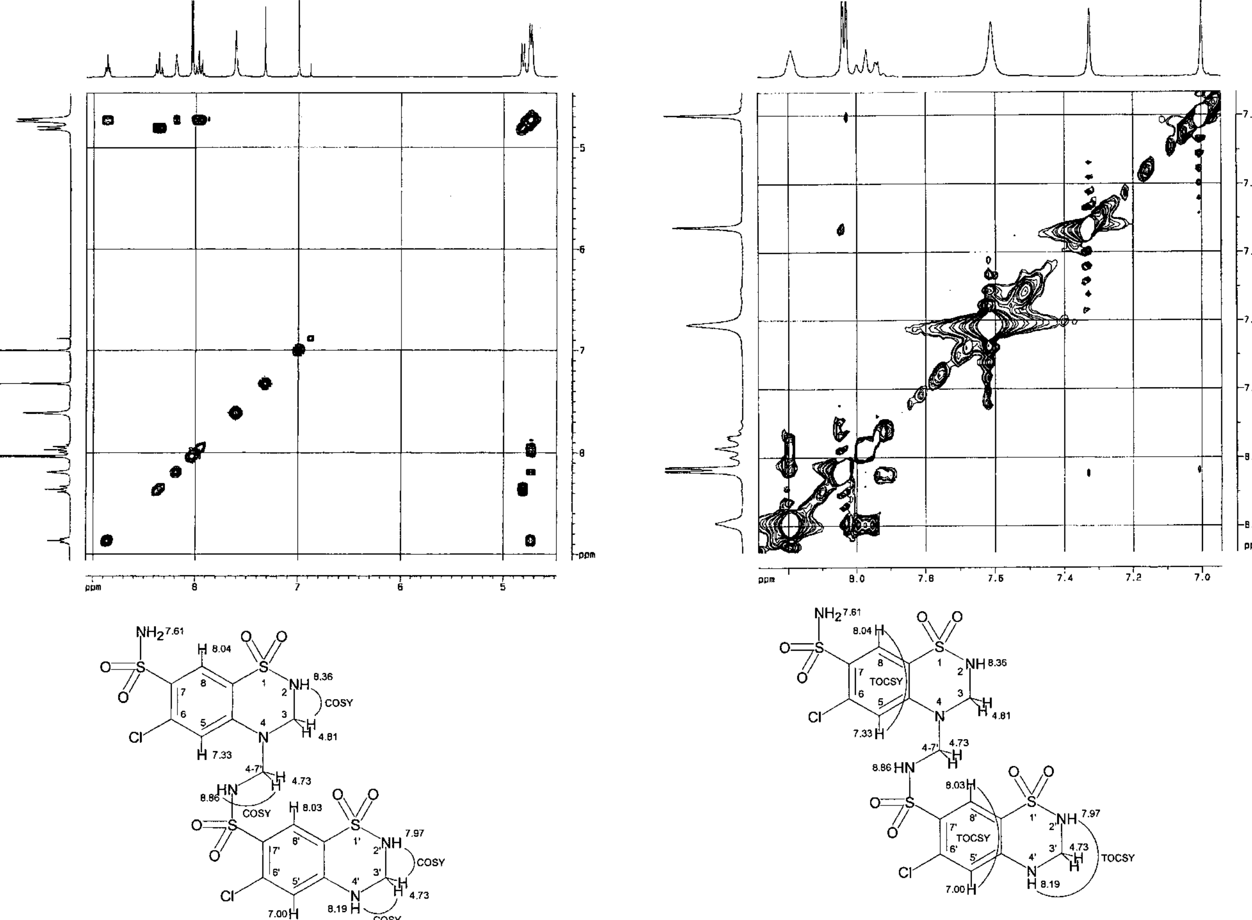

Figure 8. Two dimensional COSY ^1H NMR of HCTZ-CH_2-HCTZ in acidified DMSO-d_6.

Figure 9. Two-dimensional TOCSY ^1H NMR of HCTZ-CH_2-HCTZ in acidified DMSO-d_6.

NMR results for HCTZ are summarized in Table 1. Upon analysis of the ^1H NMR of HCTZ-CH_2-HCTZ, taken in an identical solvent matrix, there are three resonances at 8.86, 8.36, and 7.97 ppm which are 1H and triplet, similar to that of the secondary sulfonamide (N2) in HCTZ. The resonances at 7.61 ppm (br, 2H) and 8.19 ppm (br, 1H) have the same pattern as the primary sulfonamide (C7-SO_2NH_2) and the aniline (N4) in the ^1H NMR of HCTZ, respectively. Of the six possible HCTZ-CH_2-HCTZ isomers (Fig. 6), the only one that contains one aniline proton, one primary sulfonamide group, and three secondary sulfonamides, is isomer **7f**.

As a final confirmation of the proposed structure **7f**, decoupling experiments, COSY, NOESY, and TOCSY ^1H NMR of the impurity were studied in the identical solvent matrix. The COSY ^1H NMR spectrum (Fig. 8) of HCTZ-CH_2-HCTZ reveals a correlation between the methylene resonance at 4.81 ppm (d, 2H) and 8.36 ppm (t, 1H) as well as between the methylenes at 4.73 ppm (d, 4H), 7.97 ppm (t, 1H), 8.19 ppm (br, 1H), and 8.86 ppm (t, 1H). These results were confirmed by a selected decoupling experiment (Fig. 5) and indicate that the peaks that have a correlation are adjoining each other. The methylene at C3′ must be one of the resonances at 4.73 ppm because it correlated to two different protons (N2′ and N4′). Drawing analogy to the ^1H NMR analysis of HCTZ (Table 1), the resonances at 8.04 ppm (s, 1H) and 8.03 ppm (s, 1H) are assigned to C8 and C8′, respectively, which is later confirmed by the NOESY experiment. Also, drawing analogy to the ^1H NMR analysis of HCTZ, the resonances at 7.33 ppm (s, 1H) and 7.00 ppm (s, 1H) are assigned to C5 and C5′, respectively. The TOCSY ^1H NMR spectrum (Fig. 9) of HCTZ-CH_2-HCTZ reveals a correlation between the resonances at 7.00 ppm (s, 1H, C5′) and 8.03 ppm (s, 1H, C8′) as well as between 7.33 ppm (s, 1H, C5) and 8.04 ppm (s, 1H, C8).

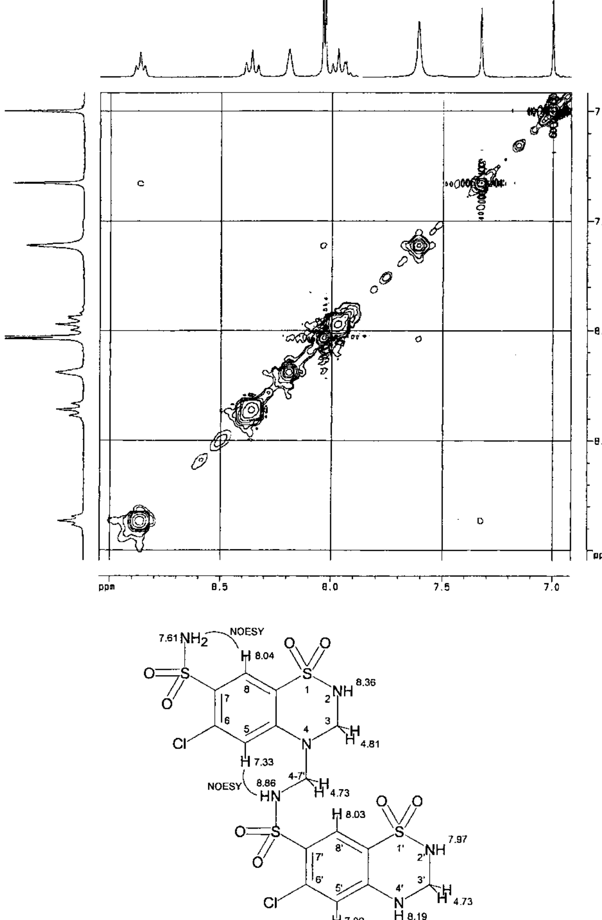

Figure 10. Two dimensional NOESY ^{1}H NMR of HCTZ-CH_2-HCTZ in acidified DMSO-d_6.

There is a strong resonance correlation between 7.97 ppm (t, $J = 8.1$ Hz, 1H) and 8.19 ppm (br, 1H). Drawing from the ^{1}H NMR of HCTZ, the broad singlet of 1H at 8.19 ppm in HCTZ-CH_2-HCTZ belongs to the aniline proton (N4′). The correlation of this resonance to the one at 7.97 ppm, indicates that the resonance at 7.97 ppm must be N2′.

The NOESY ^{1}H NMR spectrum (Fig. 10) of HCTZ-CH_2-HCTZ shows a correlation between 7.61 ppm (s, 2H, C7-SO_2NH_2), the primary sulfonamide, and 8.04 ppm (s, 1 H), confirming the assignment of C8. By elimination, the resonance at 8.03 ppm (s, 1H) is C8′, and thus, because of the TOCSY, the assignments of C5 and C5′ are 7.33 (s, 1H) and 7.00 (s, 1H) ppm, respectively. The spectrum also indicates that the resonance at 8.86 ppm (t, 1H) is C7′−SO_2NH−due to its correlation with the resonance at 7.33 ppm (C5). With the resonance at 8.86 ppm (t, 1H) being C7′−SO_2NH−, it can be concluded from the COSY data that the remaining resonance at 4.73 ppm (d, 2H) must belong to the bridging methylene, C4–7′. Therefore, because of the COSY data, the resonance at 4.81 (d, 2H) must be C3 and the resonance at 8.36 (t, 1H) must be N2.

In summary, an HCTZ-CH_2-HCTZ isomer (4-[{6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide}-methyl]-6-chloro-3-hydro-H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide) has been structurally characterized using hydrolysis, UV, LC/MS, and NMR experimental data. Two-dimensional COSY, NOESY, and TOCSY ^{1}H NMR spectral analyses were required to confirm a unique HCTZ-CH_2-HCTZ structure out of six possible isomers. The identification of this HCTZ-CH_2-HCTZ impurity allows one to speculate on a possible reaction mechanisms for its formation, and can lead to the minimization of this impurity in the drug substance.

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