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Isolation of a 2:1 hydrochlorothiazide–formaldehyde adduct impurity in hydrochlorothiazide drug substance by preparative chromatography and characterization by electrospray ionization LC–MS

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Abstract

Hydrochlorothiazide drug substance (19 lots) from five different manufacturers and four different countries of origin (USA, Italy, Hungary, and Croatia) were analyzed for the presence of impurities using a gradient elution chromatographic system, with acetonitrile–water as the mobile phase. Two known impurities of hydrochlorothiazide, 4-amino-6-chloro-1,3-benzenedisulfonamide and chlorothiazide, were separated, as well as a late-eluting, unknown, recurring impurity. The unknown impurity was isolated by preparative liquid chromatography followed by preparative thin-layer chromatography. It was characterized by electrospray ionization LC–MS as a 2:1 hydrochlorothiazide–formaldehyde adduct of the parent drug substance. The adduct is believed to form through the double condensation reaction of hydrochlorothiazide with excess formaldehyde during the parent compound's synthesis. The concentration of this impurity ranged from 0.02 to 1.1% (area%), and was above the 0.1% USP Other Impurities threshold in 16 of the 19 lots examined. © 2001 Published by Elsevier Science B.V. All rights reserved.

Keywords: Hydrochlorothiazide; Impurities; Adduct; Liquid chromatography; Thin-layer chromatography; Electrospray ionization mass spectrometry

1. Introduction

The quality and safety of pharmaceuticals can be significantly affected by the presence of impurities. Consequently, the testing and establishment of limits for impurities in active pharmaceutical ingredients (APIs) have become important initiatives by both federal and private organizations. Recent guidelines from the International Conference on Harmonization (ICH) have focused on thresholds for identification, qualification, and reporting of impurities [1]. In particular, the ICH requires identification of any recurring impurities at or above the 0.1% (w/w) level. In addition, the guideline requires qualification (determining the biological safety) of impurities at a level of 0.1%

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or 1 mg d^{-1}, whichever is lower, when the maximum daily dose is ≤ 2 g d^{-1} (0.05% for >2 g d^{-1}). The US Food and Drug Administration (FDA) has adopted the ICH guidelines, published them in the Federal Register [2], and issued accompanying guidance documents for the pharmaceutical industry [3–5].

For compendial materials, the United States Pharmacopeia (USP) has followed suit with the introduction of new requirements in the section entitled, Other Impurities, under Foreign Substances and Impurities, in the General Notices and Requirements section of USP 24 [6]. In this section the USP requires that the amount and identity of an impurity that is not detected by the monograph's chromatographic assay or purity test be listed in the certificate of analysis and that the total amount of impurities (monograph-detected impurities plus 'other impurities') may not exceed 2.0%. In addition, if an unlabeled, undetected impurity is present in a substance at greater than the 0.1% level, the material does not conform to the USP requirements and cannot be considered official. The USP added these requirements to recognize that changes in synthetic processes or sources of starting materials might result in impurities that were not considered during the preparation of the drug's original monograph test or assay.

A primary function of the FDA is to screen a wide range of domestic and foreign made drug substances [7] for compliance with USP specifications. Many USP monographs do not include purity tests or are limited to tests for a single impurity. Others include non-specific tests such as general TLC screening procedures that are solely based on the innovator's original synthesis. The APIs that are tested rarely fail to meet USP requirements.

The advent of analytical and preparative liquid chromatography (LC), as well as liquid chromatography–mass spectrometry (LC–MS), has demonstrated that there are impurities in many APIs that are not detected by the current USP methods. These techniques, in combination with NMR spectroscopy, have been successfully utilized in the detection and identification of impurities in a wide range of drug substances [8–14], including their application in this laboratory for the detection and structure elucidation of two major impurities in trimethoprim drug substance [15].

Hydrochlorothiazide (HCTZ) is a common diuretic that is utilized singularly or in combination with other drugs for the treatment of hypertension [16]. HCTZ typically contains chlorothiazide (CTZ) as a process impurity and 4-amino-6-chloro-1,3-benzenedisulfonamide (DSA) as a degradation product[17]. The USP monograph only controls the latter [18]. The European Pharmacopoeia [19] monograph controls both these impurities along with an additional impurity, 4-chloro-6-[[[[(6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazin-7-yl-1,1-dioxide)sulphonyl]amino]methyl]-amino]benzene-1,3-disulphonamide (an HCTZ–DSA adduct). The structures of HCTZ and these known impurities are presented in Fig. 1.

This report describes the utilization of analytical and preparative LC, preparative thin-layer chromatography (TLC), and electrospray ionization (ESI) LC–MS for the isolation and characterization of an additional, previously unknown impurity in HCTZ drug substance.

2. Experimental

2.1. Apparatus

2.1.1. Liquid chromatographic systems

Analytical. Two Shimadzu model LC-10AS pumps, a SPD-10AV UV–visible detector, a SCL-10A system controller, a SIL-10A auto-injector, a FRC-10A fraction collector, and a Class-VP data system (Shimadzu Scientific Instruments, Princeton, NJ) were used. Flow rate, 1.0 ml min^{-1}; detector wavelength, 272 nm; detector sensitivity, 4 AU V^{-1}; injection volume, 20 μl; column temperature, ambient. A column temperature of 30°C was used when comparing profiles from different manufacturers.

Preparative. Two Shimadzu model LC-8A pumps, a SPD-10AV UV–visible detector, a SCL-10A System Controller, a SIL-10A auto-injector, a FRC-10A fraction collector, and a Class-VP

data system (Shimadzu Scientific Instruments, Princeton, NJ) were used. Flow rate, 25.0 ml min^{-1}; detector wavelength, 272 nm; detector sensitivity, 1 AU V^{-1}; injection volume, 2000 μl; column temperature, ambient.

2.1.2. Chromatographic columns

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

Preparative. Beckman Ultrasphere ODS (22.5 mm × 25 cm), 5 μm particle size (Beckman Instruments, Fullerton, CA).

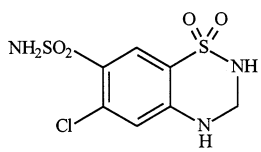
2.1.3. LC–MS system

HPLC. Two Shimadzu model LC-10AD pumps, an SPD-10AV UV–visible detector, an SCL-10A system controller, and a SIL-10A Autoinjector were used. Operating conditions: flow rate, 1.0 ml min^{-1}; detector wavelength, 272 nm; injection volume, 20 μl; column temperature, ambient. The analytical column was the same as in Section 2.1.2.

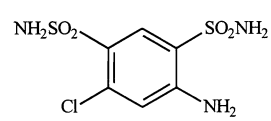
Mass spectrometer. Finnigan LCQ equipped with an electrospray ionization (ESI) probe operated in negative ion mode. The operating conditions for on-line HPLC analyses (1 ml min^{-1}) were: capillary temperature 220°C; capillary voltage, −15 V; lens offset voltage, −5 V; sheath gas flow rate, 80; aux gas flow rate, 10; spray voltage, 5.5 kV. The scan range was 80–2000 m/z. The operating conditions for MS–MS were the same as above with the relative collision energy at 35%. The isolation width was 10 m/z (to include the entire chlorine isotope cluster) and the precursor ions were selected in the center of each cluster; for HCTZ m/z 297 and for the impurity m/z 606. The scan ranges for the product ions were 80–700 and 165–800 m/z, respectively.

2.1.4. Thin layer chromatography plates

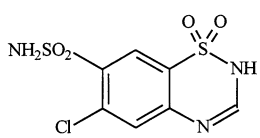
Uniplate silica gel GF TLC plates, 20 × 20 cm^2, 250 μm thickness, were purchased from Analtech (Newark, DE), and used for both analytical and preparative TLC work.



Hydrochlorothiazide (HCTZ)
(Drug substance)

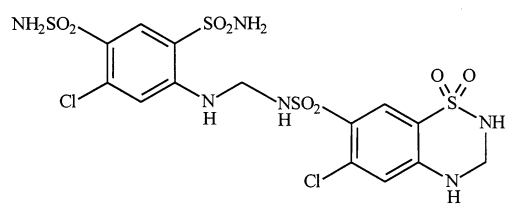


4-amino-6-chloro-1,3-benzenedisulfonamide (DSA)
(Degradation Product - USP, European Pharm. tested)



Chlorothiazide (CTZ)

(Process impurity-European Pharm. tested)



4-chloro-6-[[[[(6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazin-7-yl-1,1-dioxide)sulphonyl]amino]methyl]-amino]benzene-1,3-disulphonamide (HCTZ-DSA)

(Process impurity - European Pharm. tested)

Fig. 1. Structure of hydrochlorothiazide and its known impurities.

2.1.5. Rotary evaporator

A Buchler Digital Rotary Evaporator (Cole-Palmer Instrument Co., Vernon Hills, IL) was utilized to evaporate solvent. The condensing fluid, a mixture of ethylene glycol–water (50:50, v/v), was maintained at 0°C with the aid of a Neslab Endocal circulating bath.

2.1.6. Filters

Nuclepore® Filinert™ (PTFE) membrane filters, 13 mm, 0.45 μm porosity (Apple Scientific, Chesterland, Ohio).

2.2. Reagents

2.2.1. Solvents

HPLC grade solvents were used for mobile phases, with the exception of reagent grade toluene. Acetonitrile, dichloromethane, and toluene were purchased from Burdick & Jackson (Muskegon, MI), and acetone was purchased from Fisher Scientific (Fair Lawn, NJ).

2.2.2. Standards

The USP reference standards, hydrochlorothiazide, chlorothiazide, and 4-amino-6-chloro-1,3-benzenedisulfonamide, were obtained from the US Pharmacopeial Convention, Inc. (Rockville, MD).

2.2.3. Mobile phase

Analytical, preparative, and LC–MS analysis: Solvent A: distilled water. Solvent B: acetonitrile.

Gradient for analytical LC and LC–MS analysis: 0 min, 10% B; 5 min, 10% B; 10 min, 25% B; 20 min, 25% B; 25 min, 40% B; 30 min, 40% B; 35 min, 70% B; 45 min, 70% B; 45.1 min, 10% B.

Gradient for preparative LC: 0 min, 10% B; 16 min, 25% B; 30 min, 25% B; 30.1 min, 10% B.

2.3. Samples

Samples labeled 'Hydrochlorothiazide' were obtained from various pharmaceutical companies through the US Food and Drug Administration's Drug Product Surveillance Program. Samples were recently synthesized and were within their expiration dates at the time of testing. Samples were stored at ambient conditions and were not dried prior to testing.

2.3.1. Sample preparation: analytical and preparative LC

Analytical. 1 mg ml^{-1}. Approximately 5 mg of sample was accurately weighed and dissolved into 5 ml of acetonitrile–water (50:50, v/v), and 20 μl was analyzed with the HPLC system.

Preparative. 38 mg ml^{-1}. Approximately 490 mg of a sample containing 1% of the unknown impurity was dissolved into 13 ml of acetonitrile–water (50:50, v/v). The solution was filtered through a 0.45 μm filter, and 2000 μl was analyzed with the HPLC system. The unknown impurity was isolated by fraction collection at approximately 25–27 min. The mobile phase was removed by rotary evaporation at 35°C in vacuum, leaving isolate **I**. A typical ratio of unknown impurity concentration to HCTZ in isolate **I** was approximately 4:1.

2.3.2. Sample preparation: analytical and preparative TLC

1. Solvent system for analytical and preparative TLC. Dichloromethane–acetonitrile–acetone (4:1:1, v/v/v).
2. Tank preparation. An $11 \times 11\frac{1}{2} \times 3\frac{3}{4}$ in.^{3} tank lined with chromatography paper was equilibrated with the solvent system for 60 min prior to use.
3. Analytical TLC. Solutions of USP hydrochlorothiazide, chlorothiazide, and isolate **I**, approximately 1 mg ml^{-1} in acetone, were spotted (10 μl) on a TLC plate (5×20 cm^2), and the plate was developed. The following results were obtained: HCTZ, one spot at $R_f = 0.5$; CTZ, one spot at $R_f = 0.28$; isolate **I**, spots at $R_f = 0.5$ (HCTZ) and 0.36 (unknown impurity).
4. Preparative TLC. Five preparations of isolate **I** were pooled, dissolved in 1 ml of acetone, and filtered. The entire sample was carefully spotted along a TLC plate (20×20 cm^2) and developed. The unknown impurity band, isolate **II** ($R_f = 0.36$) was scraped off the plate and extracted in acetonitrile. This solution was

filtered and used for ESI LC–MS and ^{1}H NMR analysis.

3. Results and discussion

In this study, a total of 19 lots of hydrochlorothiazide drug substance from five different manufacturers and four different countries of origin (USA, Italy, Hungary, and Croatia) were examined using the USP impurity test for 4-amino-6-chloro-1,3-benzenedisulfonamide (DSA) [18]. Each material conformed to USP specifications. These materials were then reexamined by gradient elution LC using a linear gradient of acetonitrile–water (10–70% CH_3CN, v/v). Typical HPLC profiles of these materials along with their countries of origin are presented in Fig. 2. In addition to minor amounts ($<0.1\%$) of DSA (retention time ~ 8 min) and CTZ (retention time ~ 9 min), a significant late-eluting peak was detected at a retention time of approximately 23 min. Although the impurity profiles differ in some respects, all of the lots examined contained this late-eluting impurity. Neither the USP nor the European Pharmacopoeia explicitly controls the content of this impurity in their monographs.

The concentration of this impurity (by area percent) ranged from 0.02 to 1.1%. In 16 of 19 lots this was above the 0.1% threshold described in Other Impurities in USP 24 [6]. Two of the five manufacturers listed the amount of an 'unknown impurity' in their certificate of analysis that was approximately the same level as the above impurity. Table 1 shows a summary of the area percent concentration of the two known impurities of HCTZ (DSA and CTZ), and of the unknown impurity identified in this study, in each of the nineteen lots of HCTZ drug substance analyzed.

3.1. On-line LC–MS and LC–MS–MS of hydrochlorothiazide and the unknown impurity

The on-line, negative ion ESI spectra of HCTZ and the unknown impurity are presented in Fig. 3. The spectrum of HCTZ (MW 297, $C_7H_8N_3O_4S_2Cl$) (Fig. 3a) exhibits a one-chlorine $[M-1]$ molecular ion cluster at m/z 296/298, and a second small one-chlorine fragment ion isotope cluster at m/z 269/271 $[(M-1)-HCN]$. The two-chlorine isotope cluster at m/z 593/595/597, $[2M-1]$ is consistent with an HCTZ–HCTZ complex, $C_{14}H_{16}N_6O_8S_4Cl_2$, MW = 594. The formation of in-source 2M complex ions is some-

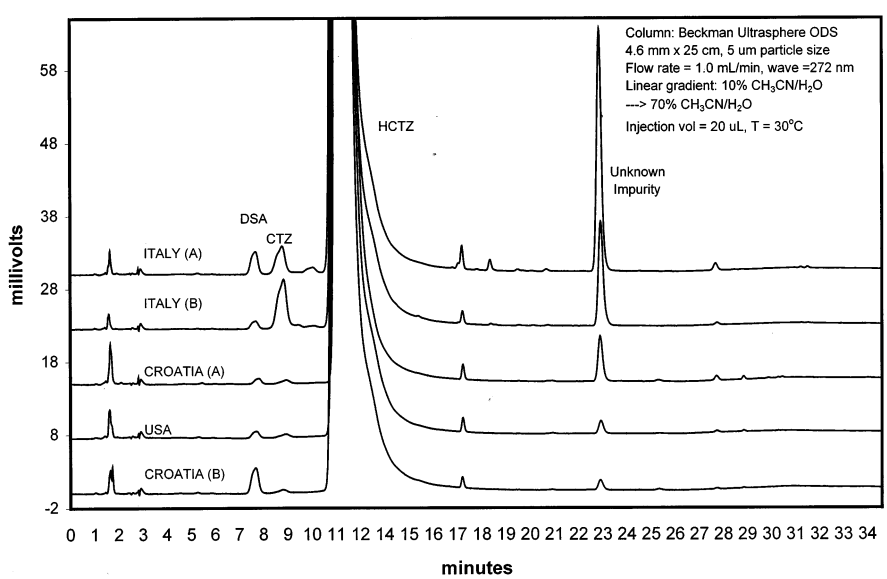


Fig. 2. HPLC profiles of hydrochlorothiazide drug substance from various manufacturers.

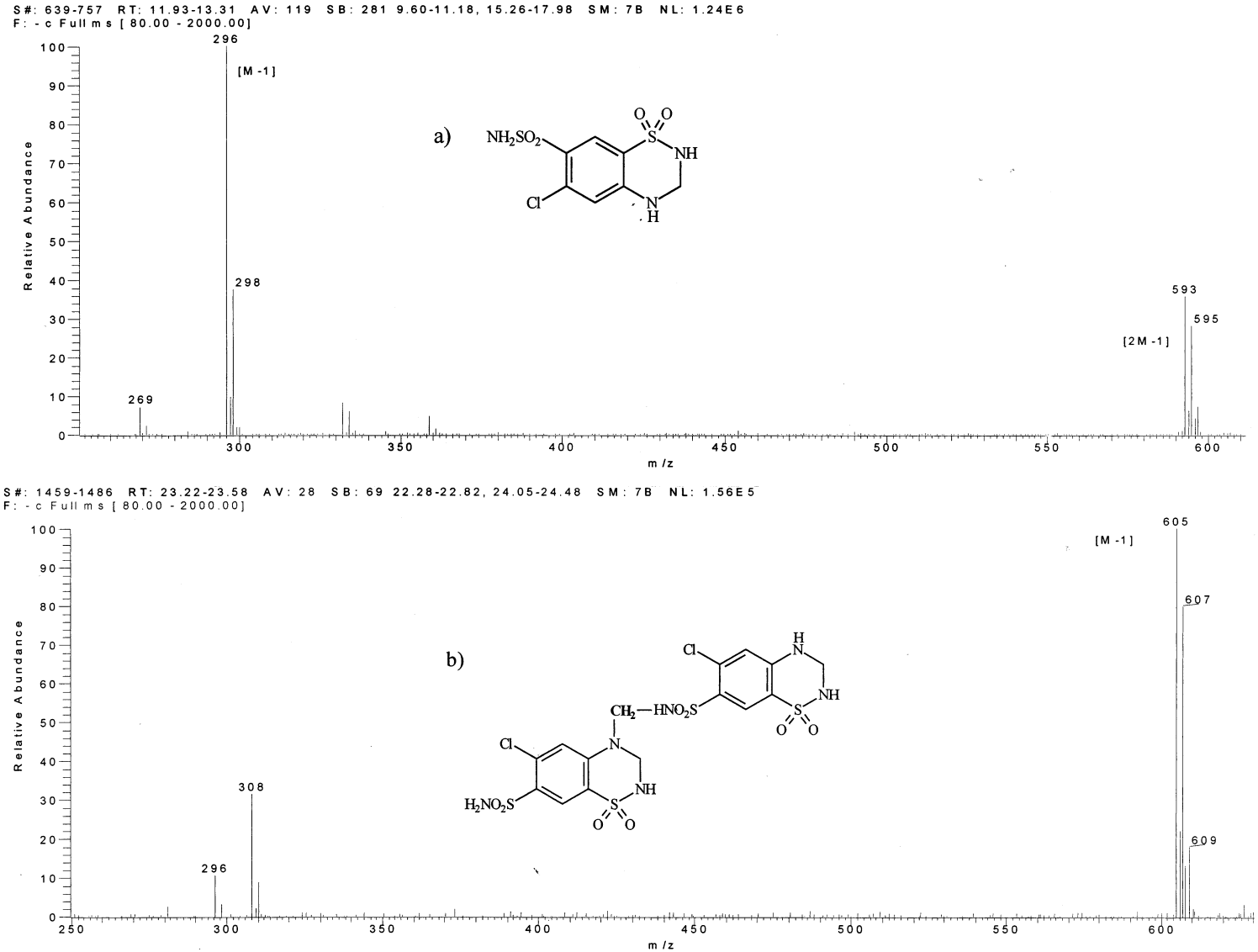


Fig. 3. Negative ion ESI LC–MS of: (a) hydrochlorothiazide and (b) unknown impurity.

times observed in ESI spectra. This HCTZ–HCTZ complex was only detected in the mass spectrometer and was not observed in any of the HPLC chromatograms of HCTZ, thereby supporting the fact that it was formed by an in-source reaction.

The negative ion ESI spectrum of the unknown impurity (Fig. 3b) exhibits a two-chlorine [M − 1] molecular ion isotope cluster at m/z 605/607/609, and two significant one-chlorine fragment ions at m/z 308/310 and 296/298. The [M − 1] ion cluster, along with the presence of a fragment ion at m/z 296, suggested an adduct ion consisting of two monomer units of HCTZ (HCTZ − H) bridged by a methylene (CH_2) group, consistent with an elemental composition of $C_{15}H_{16}N_6Cl_2O_8S_4$ (MW = 606). The in-source HCTZ–HCTZ complex, which was detected in the spectrum of HCTZ, was not observed in the spectrum of the impurity, further indicating that it is intrinsic to an in-source reaction of HCTZ, and not related to the chemically formed adduct.

Further structural information about the impurity was obtained by on-line, negative ion ESI LC–MS–MS analysis (Fig. 4). The MS–MS spectrum of HCTZ (Fig. 4a) exhibits one-chlorine isotope cluster product ions at m/z 269/271 [(M − 1) − HCN], and m/z 205/207 [m/z 269 − SO_2]. The MS–MS spectrum of the impurity (Fig. 4b) exhibits two major product ions at m/z 308/310 [((HCTZ − 1):CH_2) − 2H] and m/z 296/298 [HCTZ − 1]. The two product ions suggest that the fragmentation of the adduct occurs between the first monomer unit containing the bridging methylene group and the second monomer unit. The MS^3 spectra of the product ions m/z 296 and 308 exhibit a similar fragmentation pattern as observed for HCTZ: loss of 27 mass units (HCN) followed by the loss of 64 mass units (SO_2).

Table 1
Area percent concentration of impurities [a] in hydrochlorothiazide drug substance

Manufacturer	DSA	CTZ	Unknown impurity	Total impurities
Italy				
Lot 1	0.02	0.03	0.1	0.2
Lot 2	0.06	0.09	0.4	0.6
Lot 3	0.03	0.06	0.2	0.3
Lot 4	0.03	0.08	0.1	0.2
Lot 5	0.04	0.05	0.2	0.4
Lot 6	0.02	0.2	0.2	0.4
Lot 7	0.02	0.06	0.1	0.2
Lot 8	0.01	0.01	0.1	0.2
Lot 9	0.04	0.03	0.5	0.7
Lot 10	0.05	0.04	0.5	0.6
Lot 11	0.04	0.05	1.1	1.4
Lot 12	0.04	0.03	0.2	0.3
Lot 13	0.08	0.06	0.2	0.3
Lot 14	0.1	0.04	0.2	0.7
Croatia				
Lot 1	0.05	0.01	0.02	0.1
Lot 2	0.02	0.01	0.1	0.2
Hungary	0.02	0.03	0.4	0.6
USA'A'	0.07	0.01	0.02	0.1
USA'B'	0.06	0.01	0.04	0.4
USP reference (Lot H)	0.09	0.05	0.3	0.8

[a] Limit of detection 0.01%, DSA = 4-amino-6-chloro-1,3-benzenedisulfonamide, CTZ = chlorothiazide.

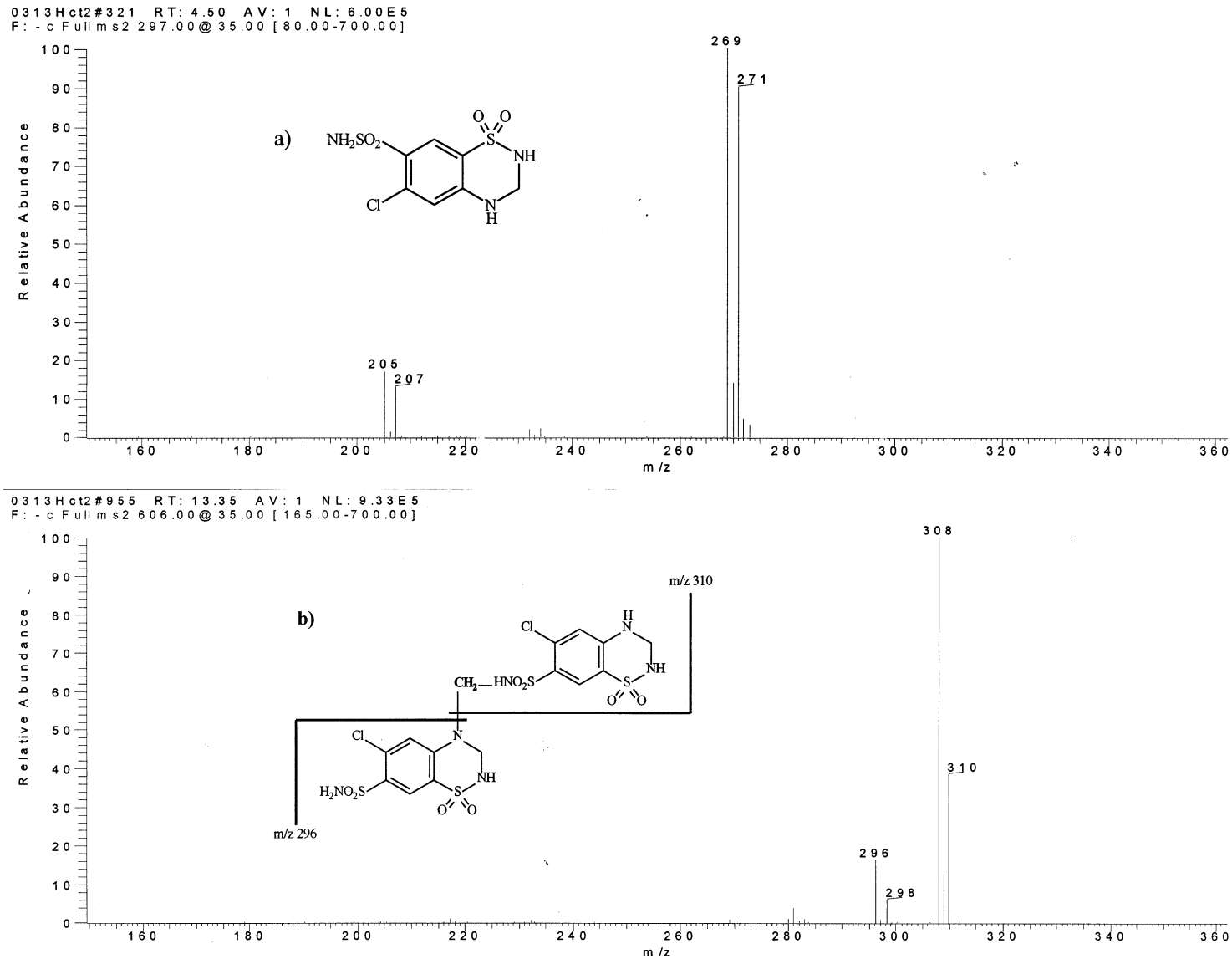


Fig. 4. Negative ion ESI LC–MS–MS of: (a) hydrochlorothiazide and (b) unknown impurity.

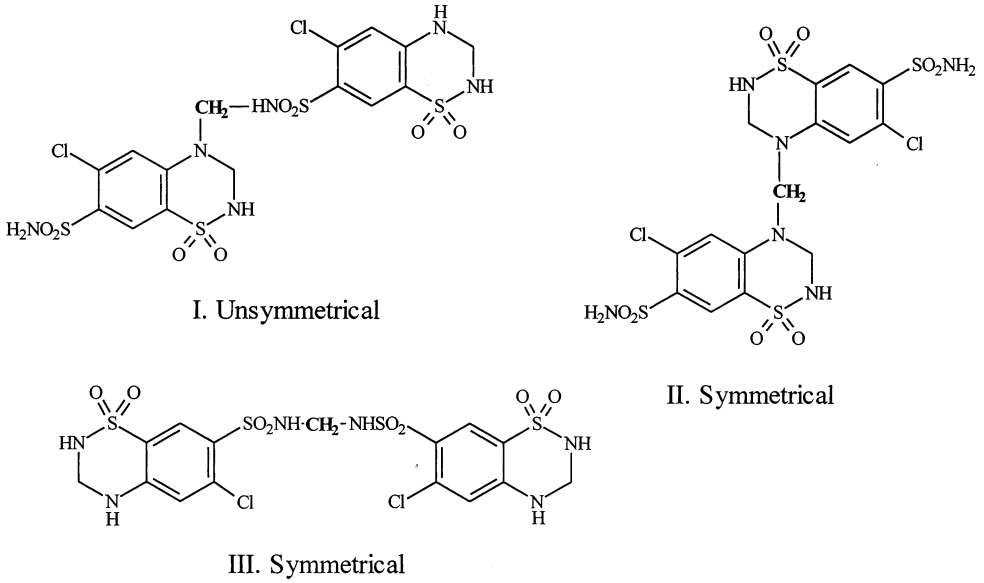


Fig. 5. Proposed structures of the adduct impurity of hydrochlorothiazide.

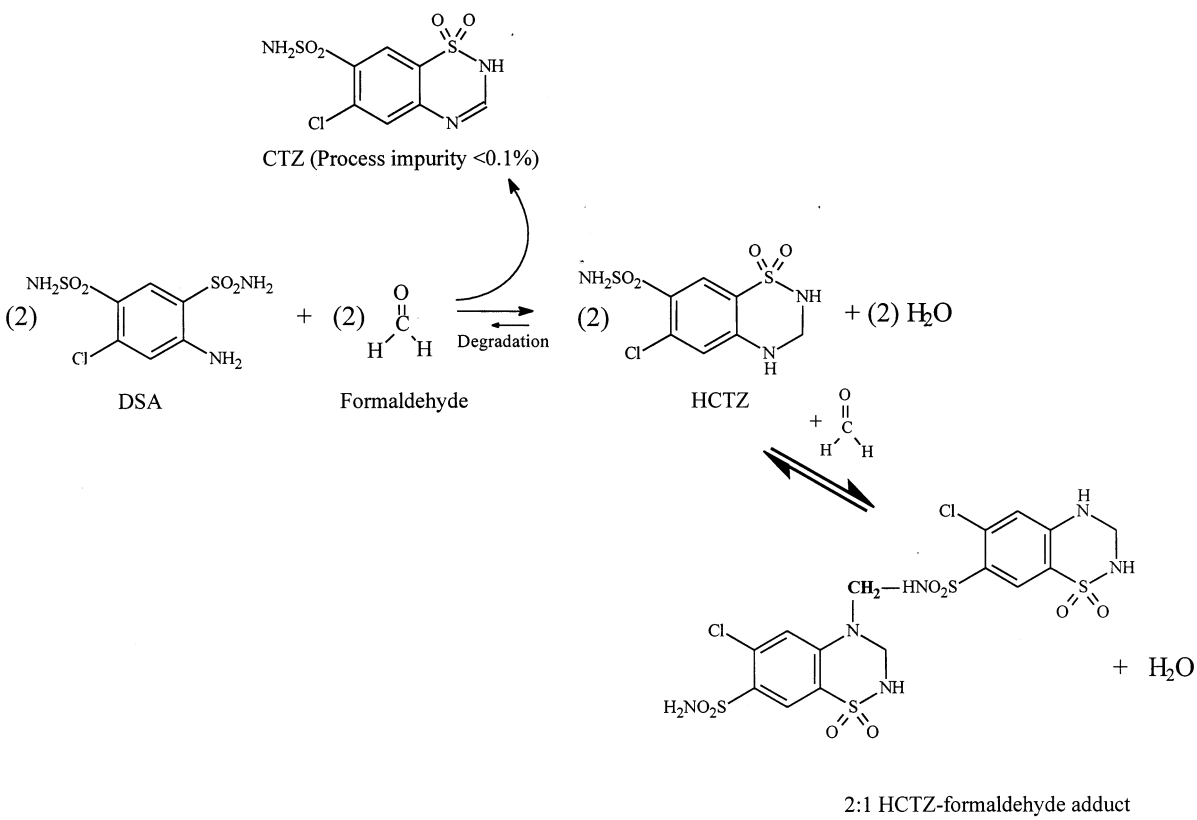


Fig. 6. Proposed synthetic and degradation pathway for hydrochlorothiazide.

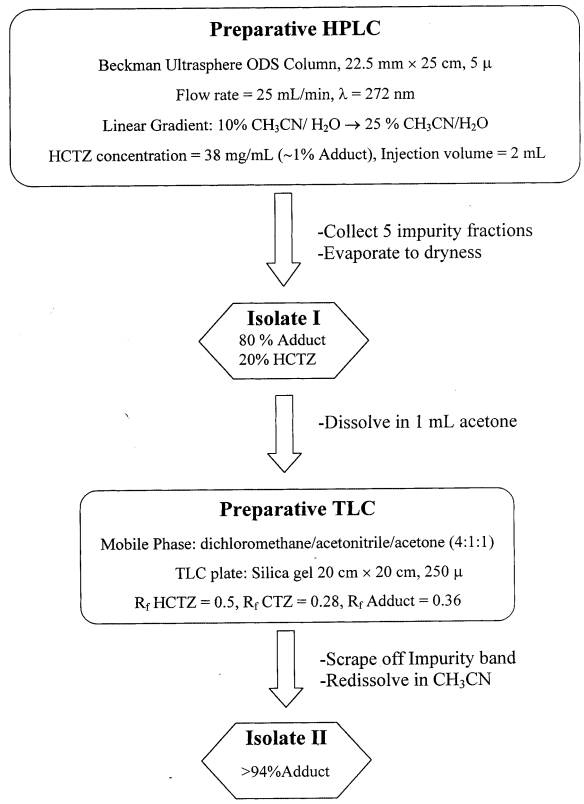
The LC–MS and LC–MS–MS mass spectral data support characterizing the unknown impurity as a 2:1 HCTZ–formaldehyde adduct consisting of two HCTZ units (minus H) bridged by a methylene group. The three possible structures of the impurity are presented in Fig. 5. The mass spectral data does not address whether the impurity is the unsymmetrical adduct (structure **I**) or the symmetrical adduct (structures **II** or **III**).

The occurrence of this impurity in all of the tested lots suggests a similar synthetic or degradation pathway for HCTZ. HCTZ is synthesized by the cyclization reaction of DSA with formaldehyde through double condensation [20]. HCTZ is also known to degrade in aqueous media via hydrolysis to form DSA and formaldehyde [21,22]. It is not surprising, therefore, that HCTZ may undergo an additional double condensation reaction in the presence of formaldehyde to form a 2:1 HCTZ–formaldehyde adduct. A proposed synthetic and degradation pathway for HCTZ is summarized in Fig. 6.

The formation of an adduct impurity in a drug substance, through the condensation reaction of two monomers, is not unprecedented. Recently, dimeric degradation products in stressed tablets of losartan have been identified by LC–MS and LC–MS–MS [14]. Other reports have described adduct contaminants in the dietary supplements L-tryptophan [23] and melatonin [24]. These studies used LC–MS and LC–MS–MS to identify adducts that formed through the condensation reactions of L-tryptophan and melatonin with acetaldehyde and formaldehyde, respectively. Although only present in approximately 0.05–0.1% of the parent compound, the tryptophan adduct has been strongly implicated in the outbreak of eosinophilia–myalgia syndrome (EMS) epidemic of 1989, in which the consumption of the contaminated dietary supplement resulted in at least 28 deaths and severely affected over 1500 people [25].

3.2. Isolation of the adduct impurity

Isolation of the adduct impurity by a combination of preparative LC and TLC is illustrated in Scheme 1. In the preparative LC procedure, the corresponding analytical conditions were scaled up for the preparative run (column diameter: 22.5 mm, flow rate: 25 ml min^{-1}). A typical preparative HPLC chromatogram of hydrochlorothiazide with approximately 1% adduct impurity content is presented in Fig. 7.



Scheme 1.

The impurity fraction was collected, and the solvents were eliminated by rotary evaporation at 35°C, leaving isolate **I**. Isolate **I** typically contained the adduct impurity and hydrochlorothiazide in a 4:1 ratio. Separation of the pure impurity from isolate **I** was accomplished by preparative TLC in aprotic solvents in order to preclude hydrolysis of the impurity. The product of the preparative TLC procedure, isolate **II**, was analyzed further to establish purity.

The negative ion LC–MS chromatogram of isolate **II** is presented in Fig. 8. The impurity

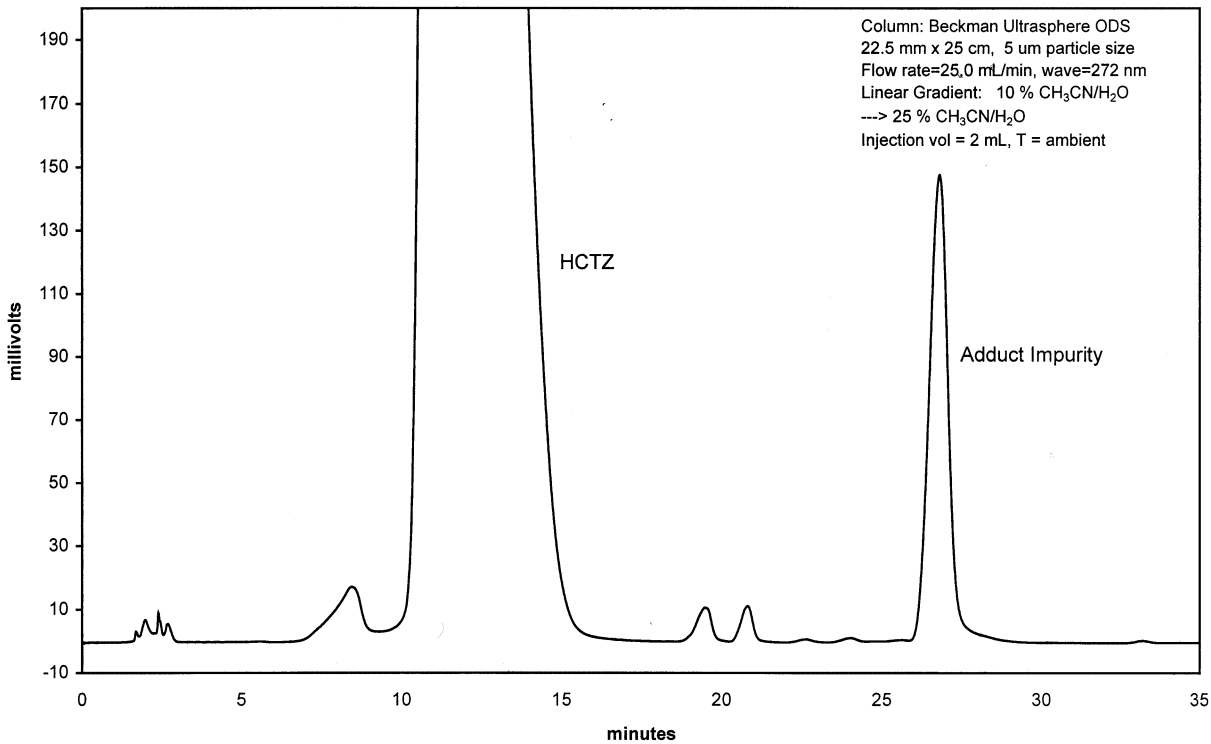


Fig. 7. Preparative HPLC chromatogram of hydrochlorothiazide with approximately 1% adduct impurity.

content is raised by the preparative TLC procedure (isolate **I**, 80% adduct → isolate **II**, 94% adduct), but some HCTZ remains due to hydrolysis of the impurity in the aqueous portion of the mobile phase. In addition to mass spectral confirmation, the purpose of the isolation procedure was to obtain further structural information of the impurity by NMR. However, preliminary ^{1}H NMR data of isolate **II** proved inconclusive due to the presence of numerous broadened signals with undefined coupling.

4. Conclusions

The combination of gradient elution analytical and preparative LC, preparative thin-layer chromatography (TLC), and negative-ion electrospray ionization LC–MS and LC–MS–MS has resulted in the isolation and characterization of a 2:1 HCTZ–formaldehyde adduct impurity of hydrochlorothiazide. The impurity structure consists of two hydrochlorothiazide units (minus H) bridged by a methylene group, although the position of the methylene link between the monomer units is not known at this time. It is believed that the impurity forms during the synthesis of the parent compound through the double condensation reaction of hydrochlorothiazide with excess formaldehyde. This adduct impurity was detected using gradient elution HPLC in 19 lots of hydrochlorothiazide drug substance sampled from five different manufacturers and four different countries of origin. The concentration of this impurity ranged from 0.02 to 1.1%, and exceeded the 0.1% USP Other Impurities threshold in 16 of the 19 lots examined. This adduct impurity is not detected by the USP monograph impurity test. Preliminary work has shown that the gradient system used in this study may also be used to examine the impurity profiles of other thiazide drug substances, including hydroflumethiazide,

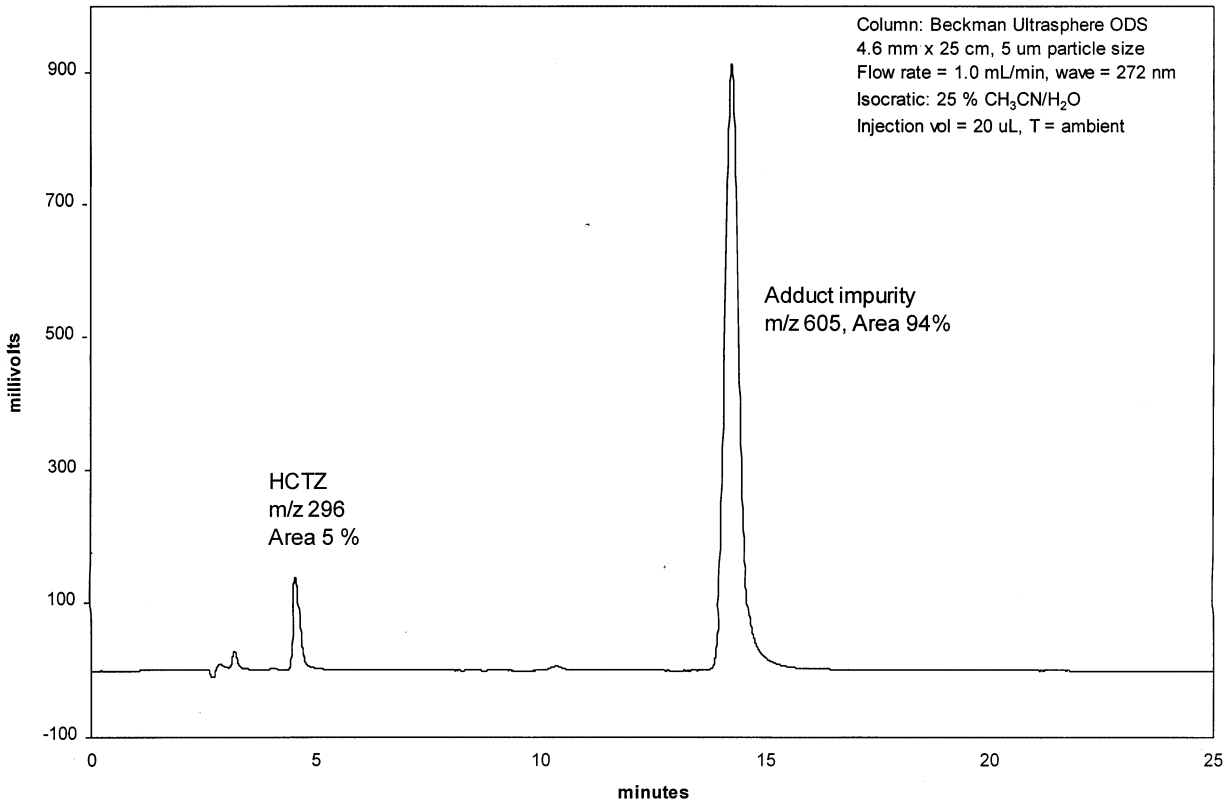


Fig. 8. Negative ion ESI LC–MS chromatogram of isolate **II**.

which has been shown to contain a similar adduct impurity. Future work will focus on developing a single method for all thiazide drug substances and their impurities.

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