Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 29–38
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

LC-MS and NMR determination of a dichloromethane artifact adduct, cyproheptadine chloromethochloride

Min Li *, Eric S. Ahuja, Diana M. Watkins

Regulatory and Analytical Sciences-Analytical, Merck and Co., Inc., WP82-30, P.O. Box 4, West Point, PA 19486, USA

Received 12 November 2001; received in revised form 12 August 2002; accepted 16 September 2002

Abstract

The British Pharmacopoeia (BP) monograph for cyproheptadine HCl tablets requires a 'Related substances' thin-layer chromatography (TLC) test. This test revealed an extraneous spot with an R_f of 0.1 in certain cyproheptadine HCl tablets that were under ambient retention conditions as well as those on stability programs. An investigation utilizing LC-MS, direct infusion MS, NMR, and organic synthesis has identified that the spot results from the *N*-oxide of cyproheptadine (a genuine degradate) and a co-eluting cyproheptadine–dichloromethane adduct, an artifact formed during the sample extraction step in which dichloromethane is used in the extracting solvent.

Keywords: Cyproheptadine hydrochloride; Cyproheptadine chloromethochloride; Dichloromethane adduct; LC-MS; NMR; TLC

1. Introduction

Cyproheptadine hydrochloride [4-(*5H*-dibenzo*[a,d]*cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride] (**1**, Fig. 1) is an antihistaminic and antiserotonergic agent with anticholinergic and sedative effects. It is indicated to treat a variety of allergic disorders and is also used in the symptomatic treatment of a number of other disorders such as Cushing's disease, Parkinsonism, and Nelson's syndrome and other carcinoma-related syndromes [1]. It has also been employed as an appetite stimulant [1,2] and an antiabortifacient [3]. The BP monograph for cyproheptadine tablets requires a 'Related substances' TLC test during which a mixture of 90% dichloromethane and 10% methanol is used as both the extraction solvent and mobile phase [4]. It was found that an artifact adduct (**2**, Fig. 1) was formed between cyproheptadine and dichloromethane when the BP method was applied to cyproheptadine hydrochloride (PERIACTIN) tablets. Although the possible interaction between cyproheptadine and dichloromethane was mentioned previously in the literature [5], no details were provided regarding the structure of the adduct as a result of such interaction. In the current paper, we present data from TLC, HPLC, LC-MS, ^{1}H-NMR, and high resolution MS experiments to prove the existence of such an artifact adduct and its two diastereo-

* Corresponding author. Tel.: +1-215-652-7792; fax: +1-215-652-3499.
E-mail address: min_li@merck.com (M. Li).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00599-X

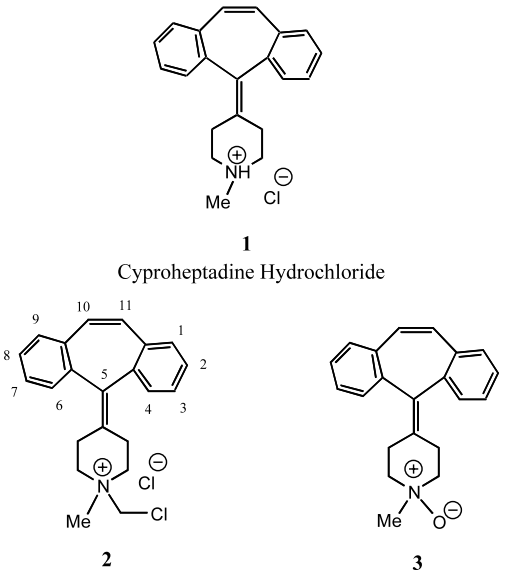


Fig. 1. Structures of cyproheptadine hydrochloride, cyproheptadine-dichloromethane, and cyproheptadine *N*-oxide.

meric forms. The adduct was found to co-elute with the *N*-oxide of cyproheptadine (a genuine degradate) and therefore it interfered with the BP test, which occasionally resulted in false failures.

2. Experimental and methods

2.1. Materials

HPLC grade acetonitrile, methanol, and dichloromethane were purchased from Fisher Scientific (USA). Trifluoroacetic acid (sequencing grade) was obtained from Pierce (Rockford, IL, USA). In-house Milli-Q water was used to prepare the aqueous components of HPLC mobile phases. TLC plates (non-fluorescent silica gel 60 and fluorescent silica gel F_{254}) were product of E. M. Sciences. *N*-Oxides of cyproheptadine [6] were obtained from in-house chemical database. PERIACTIN 4 mg tablets retained under ambient conditions as well as on stability programs (4 mg tablets at 40 °C/75% RH, 6 months) were used in this study.

2.2. Extraction and TLC of cyproheptadine HCl tablets

2.2.1. Extraction with CH_2Cl_2/MeOH

According to the BP TLC test, twenty 4 mg PERIACTIN tablets of a particular lot were ground. A part of the powder, equivalent to 50 mg cyproheptadine HCl, was weighed out and extracted with 5.0 ml of dichloromethane/methanol (9/1, v/v). The extraction mixture was filtered and a small portion of the filtrate was diluted by 1:1000 with the solvent mixture to give the 0.1% standard solution. A 10 μl aliquot of the original filtrate was applied onto a silica gel 60 TLC plate for analysis against the 0.1% standard of cyproheptadine hydrochloride.

In cases where HPLC and/or LC-MS analyses were to be performed, approximately 0.1 ml of the original filtered CH_2Cl_2/MeOH extraction solution was transferred to a 2 ml HPLC vial. After the filtrate was blown to dryness with compressed air, 0.2 ml of EtOH was added to dissolve the residue. One ml of a diluent [2.5 N HCl, ethanol, and water in the ratio of 1/5/19 (v/v/v)] was then added and the resulting mixture was filtered through a 0.45 μm PTFE filter unit for the HPLC (Section 2.3) and/or LC-MS (Section 2.4) analyses.

For the extraction of a 40 °C/75%RH stability lot[1], the remaining filtered CH_2Cl_2/MeOH extraction solution ($\sim$4 ml) was concentrated, applied onto a fluorescent silica gel 60 F_{254} TLC plate, and developed with a mixture of dichloromethane/methanol (9/1, v/v). The band around R_f 0.1 was scraped off the plate and then extracted with EtOH. The mixture was filtered and analyzed with low and then high resolution MS.

2.2.2. Extraction with a diluent consisting of 2.5 N HCl, ethanol, and water (1/5/19, v/v/v)

Part of the above ground tablets were extracted with a diluent of 2.5 N HCl, ethanol, and water (1/

[1] May be referred to hereafter as 'the stability lot' or the 'stability tablets'.

Table 1
HPLC conditions

Time (min)	0.1% TFA in water	Acetonitrile
0	81%	19%
10	50%	50%
15	50%	50%
25	81%	19%

Flow rate = 1.5 min/min. Run time = 20 min. Detection was made at either 250 nm or by scanning from 220 to 400 nm. Gradient was generated by linear mode and all runs were performed under ambient temperature.

5/19, v/v/v), and the filtrate was analyzed with the HPLC method described in Section 2.3.

2.3. HPLC analyses

HPLC analyses were conducted on a Thermo Separation Products (TSP) HPLC system

Table 2
Gradient of LC in LC-MS experiments

Time (min)	0.1% TFA in water	Acetonitrile	Flow rate
0	81%	19%	1.0 ml/min
15	50%	50%	1.0 ml/min
22.5	50%	50%	1.0 ml/min
30	81%	19%	1.0 ml/min

Gradient was generated by linear mode and all runs were performed under ambient temperature.

equipped with a Zorbax SB-C8, 15 cm × 4.6 mm column (80 Å, 5 μm) and a TSP UV3000 UV-visible absorbance detector. Other HPLC parameters are listed in Table 1.

2.4. LC-MS and MS analyses

The LC-MS analyses were performed with a TSP HPLC system interfaced to a Finnegan LCQ

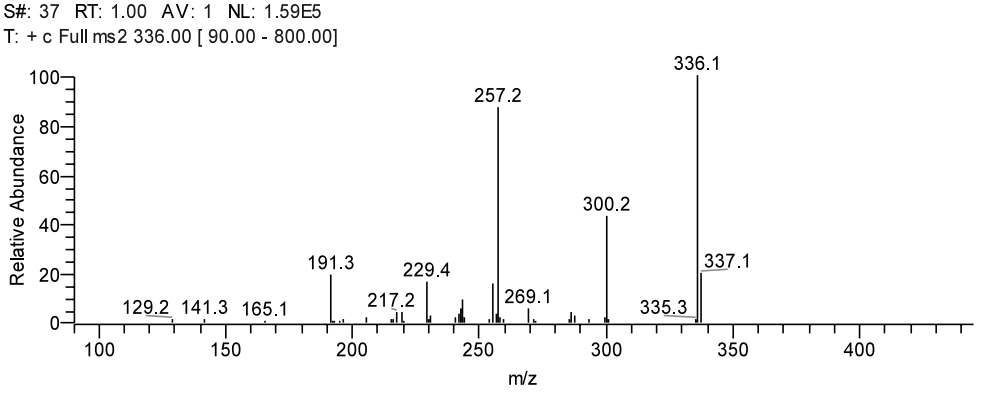
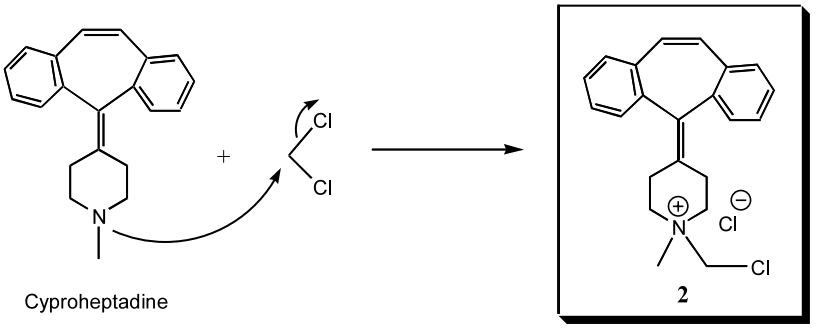
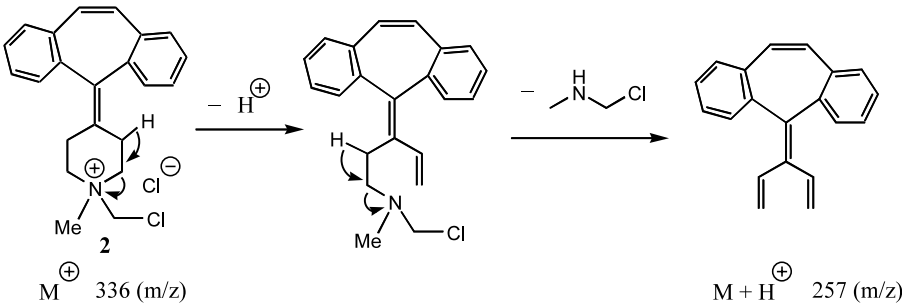


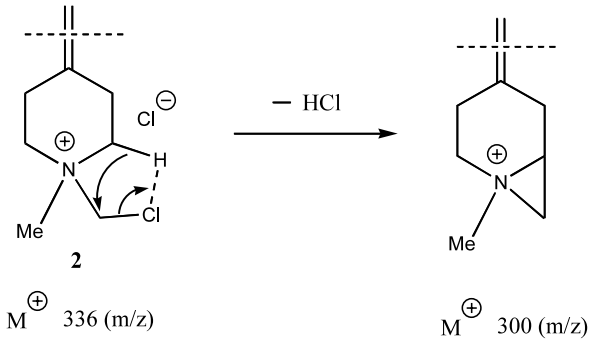
Fig. 2. MS/MS spectrum of the m/z 336 peak, indicative of the cyproheptadine-dichloromethane adduct.



Scheme 1. Formation of the cyproheptadine-dichloromethane adduct (**2**).



Scheme 2. Proposed mechanism for the formation of the m/z 257 fragment from the cyproheptadine-dichloromethane adduct (m/z 336).



Scheme 3. Proposed mechanism for the formation of the m/z 300 fragment from the cyproheptadine-dichloromethane adduct (m/z 336). The tricyclic ring is omitted in the drawing for clarity.

mass spectrometer. Initial experiments were performed in both electrospray ionization (ESI)-positive and negative ion modes and atmospheric pressure chemical ionization (APci)-positive ion mode. The ionization efficiency of cyproheptadine and related compounds was best in ESI^+. A flow rate of 1.0 ml/min directly in source (no-post column split) was used. The source temperature was set at 450 °C and the heated capillary set to 175 °C. The LCQ instrument was auto-tuned on the cyproheptadine peak with its $[M+H]^+$ at m/z 288. In some cases, the cyproheptadine peak was diverted to waste prior to entering the source. This aided in the identification of the two low-level components, cyproheptadine *N*-oxide and the cyproheptadine-dichloromethane adduct, which elute immediately following cyproheptadine. The LC gradient is listed in Table 2.

MS analyses were also performed on the LCQ using direct infusion. Flow rates between 3.0 and 30.0 μl/min were typically utilized in these experiments.

2.5. High resolution mass spectrometry

High-resolution molecular weight measurements were performed on a Bruker BioApex 3T FT/ICR high resolution mass spectrometer with an electrospray ionization method.

2.6. Synthesis of cyproheptadine chloromethochloride

Cyproheptadine HCl (50 mg, 0.14 mmol) was neutralized with a 5% NaOH solution and the resulting mixture was extracted with ether. The organic phase was then dried (K_2CO_3) and evaporated to dryness. The residue was dissolved in 20 ml of CH_2Cl_2/MeOH (9/1, v/v) and the resulting solution was refluxed overnight. The reaction solution was allowed to cool to room temperature. The solution was then concentrated and applied onto a fluorescent silica gel 60 F_{254} plate (20 × 20 cm) for separation. The plate was developed with a mixture of CH_2Cl_2/MeOH (9/1, v/v) and, after drying, the slower moving band ($R_f \sim 0.1$) was collected by scraping. The scraped silica gel was extracted with EtOH and the mixture was filtered.

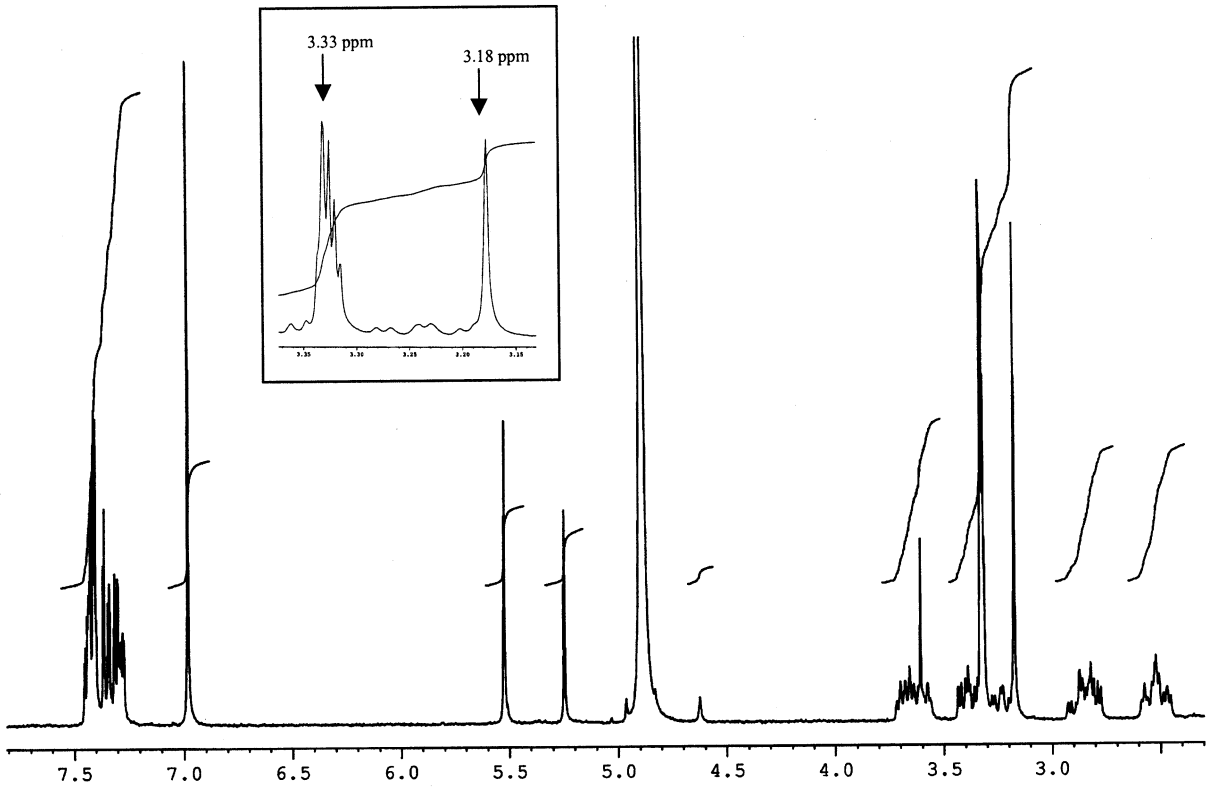


Fig. 3. ^{1}H-NMR spectrum of the synthesized adduct, **2**, in CD_3OD. The inset shows the expanded region of 3.15 to 3.35 ppm that includes the two NCH_3 signals.

The filtrate was then evaporated in vacuo and the residue (~ 5 mg) was dissolved in 1 ml of CD_3OD for ^{1}H-NMR analysis. Aliquots of the NMR solution were further diluted with large volumes of methanol for analysis by HPLC and MS.

2.7. ^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H-NMR)

^{1}H-NMR spectra were acquired using a Bruker DMX 300 MHz NMR spectrometer. Residual ^{1}H

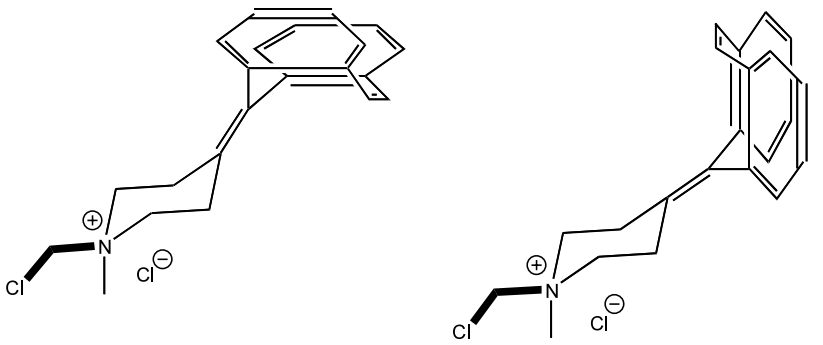


Fig. 4. The putative conformations of the two isomers of cyproheptadine-dichloromethane.

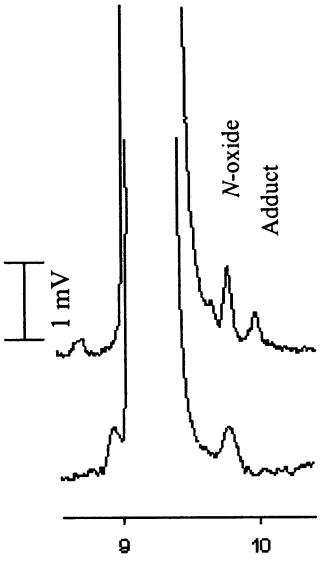


Fig. 5. Upper curve: HPLC analysis of the dichloromethane / methanol (9/1, v/v) extraction solution of the cyproheptadine HCl tablets. Lower curve: HPLC analysis of the 2.5 N HCl/ ethanol /water (1/5/19, v/v/v) extraction solution of the cyproheptadine HCl tablets. The detailed extraction procedures and HPLC conditions are outlined in the Experimental Section.

signal of the solvent was used as the internal reference in determining the chemical shift values (δ).

3. Results and discussion

3.1. Isolation of cyproheptadine chloromethochloride (2, the adduct) and its identification by MS

Analyzed by the BP TLC test for 'Related substances', the PERIACTIN tablets withdrawn from ambient retention and stability programs both showed a slow-moving spot with an R_f of ~ 0.1, in addition to the spot of the active ingredient that occurred at R_f 0.3. On one occasion, tablets from a 40 °C/75% RH stability program displayed the R_f 0.1 spot with an intensity that appeared to exceed that of the 0.1% reference standard, causing the tablets not to pass the requirement for 'Related substances' as described in the BP test. In this case, approximately 4 ml of the remaining tablet extraction solution was concentrated and then applied onto a fluorescent silica gel 60 F_{254} plate for a 'semi-preparatory' scale separation. The band around R_f 0.1 was scraped off and extracted with ethanol. HPLC analysis of the ethanol solution showed a single peak whose UV spectrum is identical to that of cyproheptadine but its retention time is slightly longer than that of the latter (data not shown). ESI-MS analysis of the same solution indicated a predominant peak that occurred at m/z 336. Moreover, the [M+2] peak (338) had an unusually high intensity (32% of the 336 peak), indicative of the presence of one chlorine atom. MS/MS analysis of the m/z 336 peak gave rise to a major fragment peak at m/z 257 in addition to several minor ones (Fig. 2). High resolution MS determination of the m/z 336 peak provided an accurate mass of 336.1520, which corresponds to a formula of $C_{22}H_{23}NCl$ or $C_{21}H_{21}N$ (cyproheptadine)+CH_2Cl. The most probable product with such a formula would be an adduct (**2**) formed between cyproheptadine and dichloromethane as illustrated in Scheme 1. The MS/MS result can now be reasonably explained: the m/z 257 peak apparently stemmed from the fragment that lost the CH_3NCH_2Cl moiety in a two-step elimination process (Scheme 2), while the m/z 300 peak appeared to result from the elimination of HCl as suggested in Scheme 3.

TLC studies of the *N*-oxides of cyproheptadine (**3**) indicated that both isomers co-eluted with the dichloromethane adduct (**2**) under the BP TLC conditions. When the silica gel 60 plate (as required by the BP test) was replaced by the fluorescent silica gel 60 F_{254} plate, the *N*-oxides moved slightly ahead of the adduct. This explains the fact that only the adduct was isolated in the above 'semi-preparatory' separation (with the fluorescent plate) of the tablet extraction from the stability lot.

3.2. Synthesis of cyproheptadine chloromethochloride (2) and its characterization by HPLC, MS and NMR

Such a cyproheptadine-dichloromethane adduct (**2**) was previously mentioned in the literature by Beckett and Ali [5]. Nevertheless, no detailed

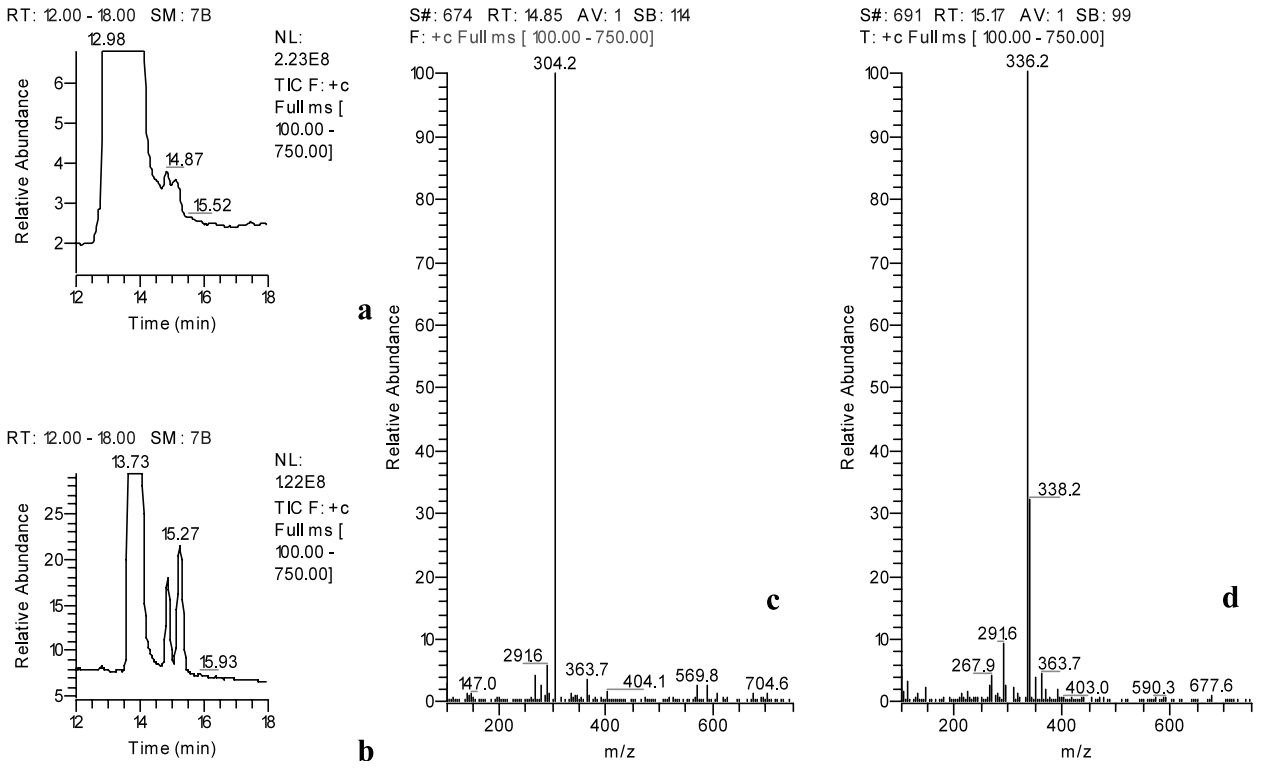


Fig. 6. (**a**) LC-MS analysis of PERIACTIN stability tablets extracted with dichloromethane/methanol (9/1, v/v). The total ion chromatogram (TIC) shows two peaks eluting after the active. The corresponding mass spectra are shown in panel **c** (m/z 304, *N*-oxide, **3**) and **d** (m/z 336, the adduct, **2**). (**b**) TIC from LC-MS analysis of PERIACTIN stability tablet extraction solution (with dichloromethane/ methanol, 9/1, v/v) spiked with authentic samples of *N*-oxide and the adduct (**2**).

information or analytical data, except TLC evidence, was given regarding the structure of the adduct. In order to completely characterize its structure, the compound was synthesized by refluxing cyproheptadine in dichloromethane. The product was purified through semi-preparatory TLC. HPLC spiking and ESI-MS experiments showed that the synthetic compound was identical to the one isolated from the tablets. ^{1}H-NMR of the product indicated that the CH_2Cl moiety existed in two distinctive conformations in a ratio of 60/40 as evidenced by the occurrence of the two CH_2Cl signals at 5.53 and 5.25 ppm, respectively (Fig. 3). It is not known whether such a ratio remains the same for the adduct that was formed during the actual extraction of the tablets, due to the minute, isolated amount that was not enough for NMR determination. Interestingly, the signals for the two protons on the seven-membered ring (H_{10} and H_{11}, Fig. 1) were the same for the two conformations (6.99 ppm). This phenomenon is similar to what was observed with the two diastereomers (α- and β-isomers) of cyproheptadine *N*-oxide: the methyl group on the nitrogen had chemical shifts of 3.12 and 3.28 for the α- and β-isomers, respectively, while the H_{10} and H_{11} signals occurred at the same place (6.88 ppm) [6]. It was further revealed that the methyl group occupied the equatorial position in both isomers. In the present case, however, the larger CH_2Cl moiety (vs. the CH_3) would probably orient the same ways as does the methyl group in the α- and β-isomers of the *N*-oxide (Fig. 4), which would leave the methyl group in the axial position. Although the parent cyproheptadine could adopt a total of four conformations [7,8], the *N*-oxide existed only in two conformations (the α- and β-isomers) [6]. This two conformation/isomer sce-

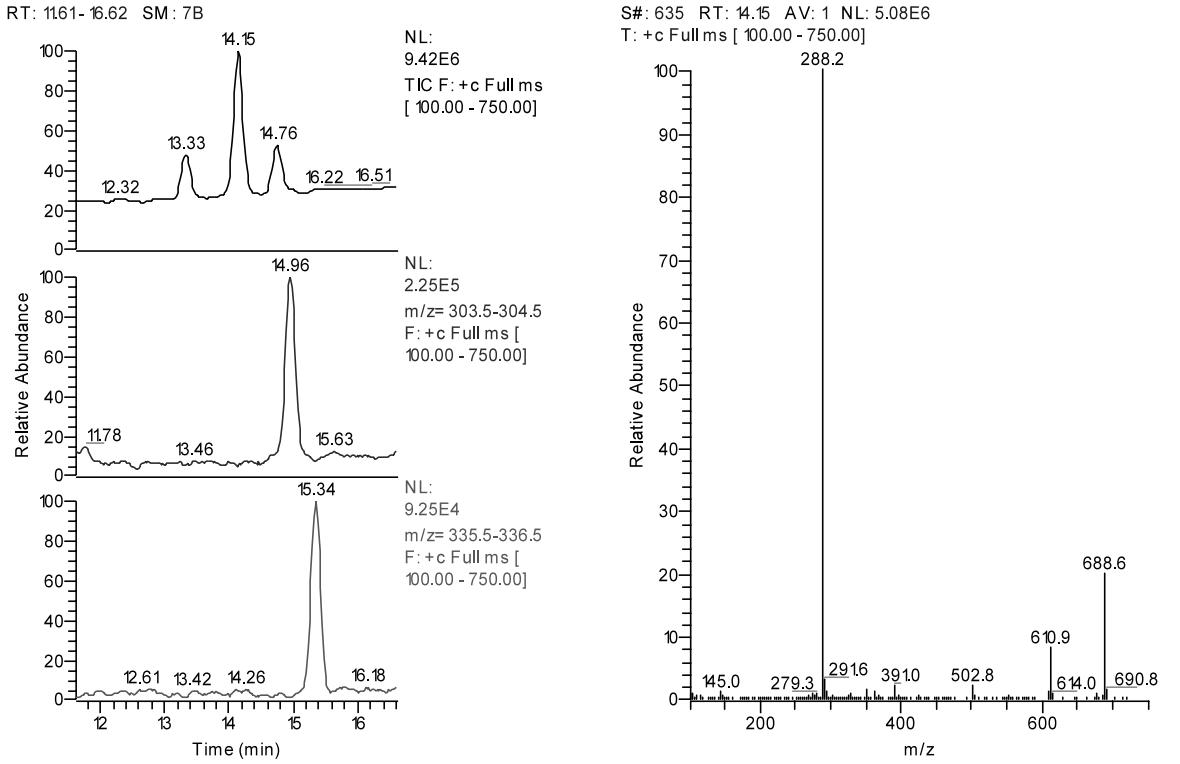


Fig. 7. Left panel: LC-MS analysis of the scraped TLC spot at $R_f \sim 0.1$. The peaks to the immediate left (13.33 min) and right (14.76 min) of the active (14.15 min) are due to silicates. Right panel: mass spectrum of the 14.15 min peak, which shows the $[M+H]^+$ peak of cyproheptadine (m/z 288).

nario is apparently also true for the adduct, as supported by the aforementioned NMR evidence [9]. The observation of only two conformations is due to the fact that one particular group (such as the methyl in *N*-oxide or the chloromethyl in the adduct) predominantly occupied only the equatorial position. When the adduct was analyzed by HPLC, only a single peak was detected. Likewise, under the same HPLC conditions, both isomers of the *N*-oxide co-eluted.

3.3. HPLC and LC-MS analysis of TLC extraction solutions and LC-MS analysis of the TLC spot at R_f 0.1

The extraction solution (with CH_2Cl_2/MeOH) of the stability lot was also directly analyzed by HPLC and LC-MS. Two extra peaks were found to elute immediately after cyproheptadine in the chromatograms of both the HPLC and LC-MS (Figs. 5 and 6). These two peaks were identified as the *N*-oxide (**3**, both isomers co-eluted) and the dichloromethane adduct (**2**), respectively, based on the spiking of the authentic samples as well as the molecular weights of the two peaks (MW 303 and 336, respectively). Since the formation of the adduct requires CH_2Cl_2, HPLC analysis of the tablet extraction of the stability lot with the EtOH-containing diluent showed only the *N*-oxide peak (Fig. 5).

To further confirm that the R_f 0.1 spot really stems from the *N*-oxide and the adduct, the above CH_2Cl_2/MeOH extraction solution of the stability lot was also analyzed by the BP TLC method but without going through the final staining step. Thus, aliquots (10 μl) of the extraction solution were applied onto a silica gel 60 TLC plate in triplicate and developed with CH_2Cl_2/MeOH (9/1, v/v). The regions inclusive of the R_f 0.1 spots were collected by scraping. The silica gel collected was

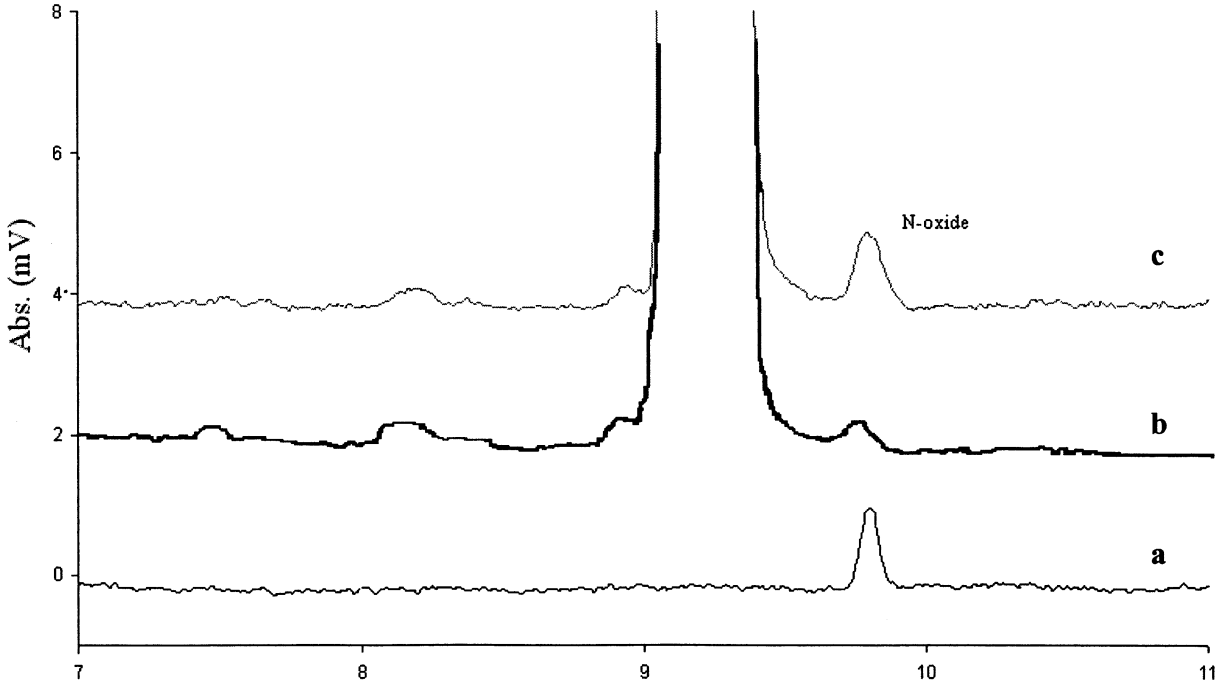


Fig. 8. Recovery study: trace **a**, a 0.1% LOQ solution; **trace b**, a PERIACTIN sample preparation solution of the stability lot (prepared according to Section 2.2.2) which contains 0.027% of *N*-oxide; **trace c**, the PERIACTIN sample preparation solution spiked with 0.1% of cyproheptadine *N*-oxide.

Table 3
Summary of the recovery study (results of Fig. 8)

Sample injected	Area of *N*-oxide	Recovery
0.1% *N*-oxide solution	5458	–
Stability tablets	1463	–
Stability tablets spiked with 0.1% *N*-oxide	6995	6995/(5458 + 1463) = 101.1%

extracted with a minimal amount of EtOH and the mixture filtered. LC-MS analysis of the filtrate indicated that the *N*-oxide and the adduct were present through the use of single ion trace at m/z 303 and m/z 336, respectively (Fig. 7). The peaks of 13.33 and 14.76 min stemmed from silica gel-related material as the peaks were also seen in the extraction of blank silica gel. A trace amount of the active was also observed at 14.15 min, which was apparently due to the tailing of the active on the TLC plate.

3.4. Recovery of the spiked N-oxide and estimate of the endogenous N-oxide in the stability tablets

In order to estimate the amount of the endogenous *N*-oxide in the stability tablets, tests of the recovery of the cyproheptadine *N*-oxide at 0.1% level were conducted. Thus, an *N*-oxide solution consisting of equal amounts of the α- and β-isomers was prepared. A small aliquot of this solution was spiked into the tablet extraction solution in such a way that the spiked *N*-oxide was at 0.1% level (total of two isomers). Results of the subsequent HPLC analyses indicated a recovery of 101% for the spiked *N*-oxide (Fig. 8 and Table 3). The amount of the endogenous *N*-oxide

was estimated at 0.027% by a comparison of the corresponding areas shown in Table 3 (1463 vs. 5458).

4. Conclusion

The results described above clearly demonstrate that the $R_f \sim 0.1$ spot in the BP TLC test of the cyproheptadine hydrochloride stability tablets consists of two components: 1) the *N*-oxide degradate of cyproheptadine, and 2) the adduct of cyproheptadine and dichloromethane. The *N*-oxide is a true degradate of the cyproheptadine [10], while the cyproheptadine-dichloromethane adduct is an artifact that was formed during the sample preparation procedure. The co-elution of these two substances on the TLC plate has led to false failing results occasionally, in which cases the co-eluted spot is more intense than the 0.1% standard. If the TLC method were selective for the two substances, the intensity of each individual spot would be lower than the 0.1% level in these cases. The results presented here will help support efforts to petition the BP to modify (with new TLC conditions) or replace (with an HPLC method) the 'Related substances' method, such that the potential for false failure be eliminated.

Acknowledgements

We thank Charles W. Ross of Merck Research Laboratories for assistance with the high-resolution mass spectrometric determinations.

References

[1] J.E.F. Reynolds (Ed.), Martindale: The Extra Pharmacopoeia, 28th edition, The Pharmaceutical Press, London, 1982.
[2] J.N. Stiel, G.W. Liddle, W.W. Lacy, Metabolism 19 (1970) 192–200.
[3] E. Sadovosky, Y. Pfeifer, W.A. Polishuk, F.G. Sulman, Israel J. Med. Sci. 8 (1972) 623.
[4] British Pharmacopoeia, vol. II, British Pharmacopoeia Commission, 2000, London, 2000, pp. 1870–1871.
[5] A.H. Beckett, H.M. Ali, J. Chromatogr. 177 (1979) 255–262.
[6] M.E. Christy, P.S. Anderson, B.H. Arison, D.W. Cochran, E.L. Engelhardt, J. Org. Chem. 42 (1977) 378–379.
[7] M. Sadek, D.J. Craik, J.G. Hall, P.R. Andrews, J. Med. Chem. 33 (1990) 1098–1107.
[8] B. Birknes, Acta Cryst. B33 (1977) 687–691.
[9] In addition to the signals of H_{10}, H_{11}, and CH_2Cl, the NCH_3 signals of the adduct appear to be resolved as well; they occur at 3.18 and 3.33 ppm, respectively, corresponding to presumably the two conformations. The problem is that the 3.33 ppm peak is buried in the residual $C\underline{H}D_2OD$ signal of the NMR solvent (Fig. 3), which nevertheless can be clearly seen by the expansion of the region from 3.15 to 3.35 ppm (inset of Fig. 3). All the remaining signals are not well resolved, so that it could not be determined whether there are two separate sets of signals for the two conformations of the adduct. The observation that the H_{10} and H_{11} signals had the same chemical shift in the two conformations of the *N*-oxide [6] as well as the adduct can probably be attributed to the fact that H_{10} and H_{11} are at a relatively great distance from the piperidine nitrogen and its immediate surrounding groups. The two conformations can be superimposed with each other completely if the nitrogen and its immediate surrounding groups are excluded from consideration.
[10] H.B. Hucker, A.J. Balletto, S.C. Stauffer, A.G. Zacchei, B.H. Arison, Cyproheptadine N-oxide is also a metabolite of cyproheptadine, Drug Metabolism Disposition 2 (1974) 406–415.