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Isolation and structural elucidation of an impurity of cefradine

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Abstract: An impurity of unknown identity was isolated from commercial cefradine by liquid chromatography on poly (styrene-divinylbenzene) with HOAc (0.01 M)–CH_3CN (94:6, v/v) as the mobile phase. The structure was elucidated as 4′,5′-dihydrocefradine using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). The structure was confirmed by comparison with the chromatographic retention characteristics and photodiode-array detected ultraviolet spectrum of the synthetic compound and with its infrared, NMR and MS spectra. The presence of 4′,5′-dihydrocefradine in cefradine has not been described previously.

Keywords: *Cefradine; impurity; liquid chromatography; poly(styrene-divinylbenzene); 4′,5′-dihydrocefradine.*

Introduction

We have previously reported a liquid chromatography (LC) method using poly (styrene-divinylbenzene) (PSDVB) for the assay and purity control of cefradine [1] and on a comparative study of two isocratic LC methods for the analysis of cefradine [2]. Typical LC chromatograms of commercial samples of cefradine are shown in Fig. 1. Method A is that prescribed by the European Pharmacopoeia for the assay of cefradine [3]. During those investigations, it was observed that an impurity of unknown identity (UNK) was present in all commercial samples analysed. Together with cefalexine, UNK is one of the most important impurities of cefradine. The content of UNK was found to be about 0.3% in most of the cefradine samples. This paper describes the isolation of UNK and its identification as 4′,5′-dihydrocefradine. LC on PSDVB was used for the isolation and purification of UNK from commercial cefradine. In order to facilitate the isolation a non-buffered mobile phase had to be developed. To confirm the structure of UNK, 4′,5′-dihydrocefradine was synthesized from D-3,4,5,6-tetrahydrophenylglycine and 7-ADCA. The structures of UNK, cefradine and cefalexine are shown in Fig. 2.

Experimental

Reagents, solvents and samples

7-Aminodesacetoxycephalosporanic acid (7-ADCA) was obtained from Gist-Brocades (Delft, The Netherlands). D-3,6-Dihydrophenylglycine, di-tert-butyl dicarbonate (tert-BOC), phosphoric acid 85%, acetic acid (HOAc), sodium 1-octanesulphonate (NaOS) and trifluoroacetic acid 99% were purchased from Janssen Chimica (Beerse, Belgium). Sodium acetate (NaOAc) trihydrate was from Fluka (Buchs, Switzerland). Acetonitrile 99% (Janssen Chimica) and methanol (Roland, Brussels, Belgium) were distilled from a glass apparatus. Water was distilled twice.

Commercial cefradine monohydrate (Gema Liesa, Barcelona, Spain) containing about 0.3% of UNK, as determined by LC, was used for preparative isolation of UNK.

Apparatus and operating conditions

For analytical LC, the apparatus was the same as that previously described [1, 2] and the operating conditions are shown in Fig. 1.

For preparative LC the laboratory-assembled chromatograph consisted of two LDC Milton Roy minipumps, a Model CV-6-UHPa-N60 Valco injector (Houston, TX,

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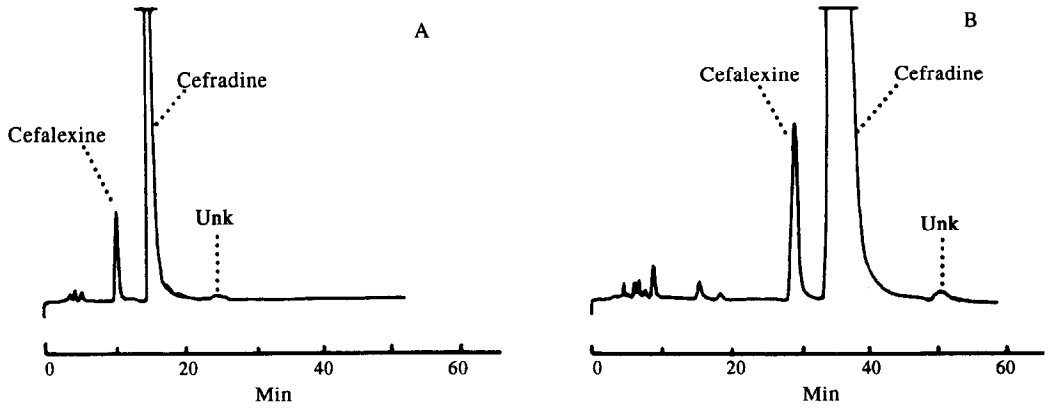

Figure 1
Typical analytical chromatograms of cefradine (A) Hypersil ODS 5 μm, 25 × 0.46 cm column, mobile phase: HOAc (2M)–NaOAc (3.62% m/v)–MeOH–H_2O (1:17:200:782, v/v/v/v), flow rate 1 ml min^{-1}, detection 254 nm, column temperature: 30°C, amount injected: 10 μg. (B) PLRP-S 100 Å 8 μm, 25 × 0.46 cm column, mobile phase: CH_3CN–sodium 1-octanesulphonate (0.02 M)–H_3PO_4 (0.2 M)–H_2O (15:10:5:up to 100, v/v/v/v), flow rate 1 ml min^{-1}, detection 254 nm, column temperature: 55°C, amount injected: 30 μg.

USA) with a 4.5-ml loop and a Model 150 Altex Biochemical UV detector (Berkeley, CA, USA), equipped with a preparative cell and chart recorder (Ankersmit, Holland). The preparative column (25 cm × 2.1 cm i.d.) was packed in the laboratory with Bio-Gel PRP 70 Å 8 μm (Bio-Rad, Nazareth, Belgium). The mobile phase was a mixture of HOAc (0.01 M)–CH_3CN (94:6, v/v). The column temperature was 55°C. The flow rate was set at 18 ml min^{-1}. The sample was dissolved in hydrochloric acid (2 M), at a concentration of 20 mg ml^{-1}. All evaporations were carried out on a rotary evaporator, in vacuo and at room temperature (Büchi, Flawil, Switzerland) using a cooler at −10°C (Lauda, Königshofen, Germany).

Infrared Spectroscopy (IR), Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectrometry (MS)

IR spectra were recorded on a Perkin–Elmer 197 spectrophotometer (Norwalk, USA) using potassium bromide disks. ^1H and ^{13}C Fourier transform (NMR) spectra were taken with a Jeol FX 90 Q spectrometer (Tokyo, Japan), operating at 89.60 MHz (^1H) or 22.53 MHz (^{13}C), in 5-mm tubes and at a probe temperature of 30°C. Samples were dissolved in deuterated water containing deuterated trifluoroacetic acid. For ^{13}C spectra CF_3COOD was used as internal reference (set at 116.5 ppm), and for ^1H spectra sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSSA, 0 ppm) was added as internal standard. Liquid secondary ion mass spectrometry (LSIMS) was performed on a Kratos Concept 1H mass spectrometer (Manchester, UK). Samples were dissolved in glycerol.

Results and Discussion

4′,5′-Dihydrocefradine was obtained by semisynthesis as follows (Fig. 2). D-3,6-dihydrophenylglycine (1 g) was dissolved in hydrochloric acid (2 M) and reduced to D-3,4,5,6-tetrahydrophenylglycine by hydrogenation for 5 h at a pressure of 3 kg cm^{-2} using Pd/C as catalyst. This gave a mixture of three products in a ratio 3:1:1. By NMR it was established that the main component was 3,4,5,6-tetrahydrophenylglycine and the others were phenylglycine and cyclohexylglycine. The amino function was then protected with a tert-BOC-group. Tert-BOC-D-3,4,5,6-tetrahydrophenylglycine was coupled with 7-ADCA as previously described [4, 5]. The reaction product was deprotected with trifluoroacetic acid and then purified on an anion exchange column to give crude 4′,5′-dihydrocefradine using a purification method for cefradine analogues as described elsewhere [6]. The crude product was further purified by LC under the same conditions as mentioned for UNK. It is believed that 4′,5′-dihydrocefradine is formed during synthesis of cefradine due to the presence of some 3,4,5,6-tetrahydrophenylglycine in the 3,6-dihydrophenylglycine used for the synthesis of cefradine. However, in the sample of 3,6-dihydrophenylglycine we used, it was not possible to identify 3,4,5,6-tetrahydrophenylglycine by NMR.

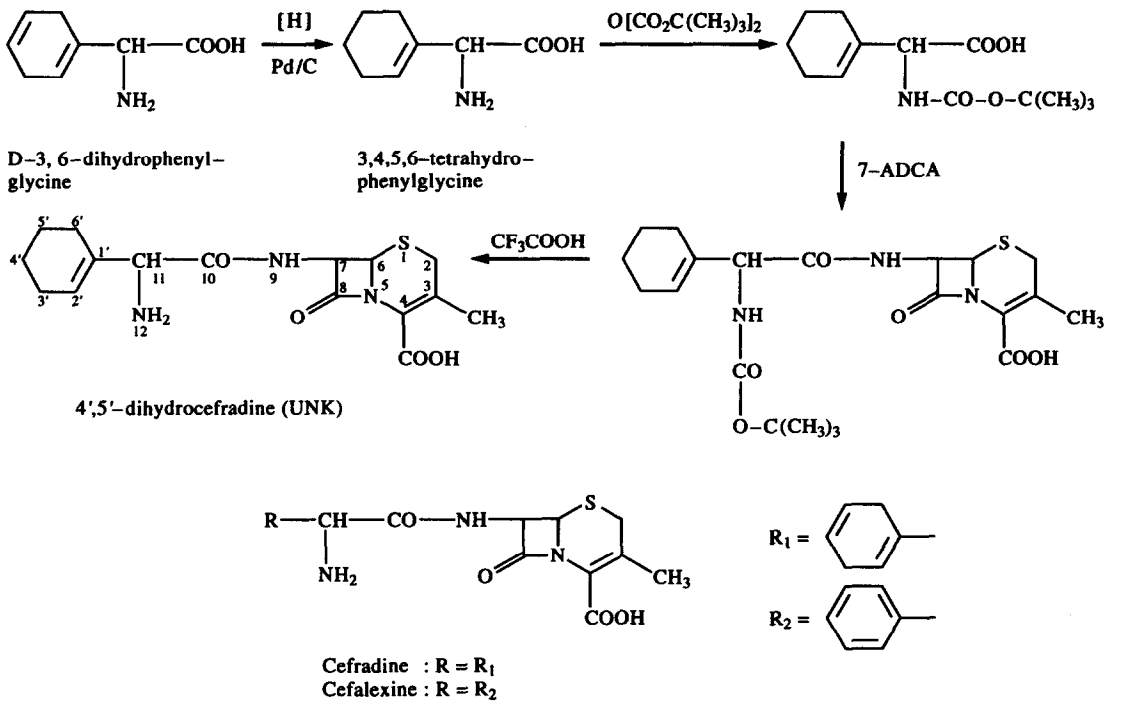

Figure 2
Scheme of the semisynthesis of 4',5'-dihydrocefradine and the structure of cefradine and cefalexine.

Preliminary experiments using analytical LC indicated that UNK is not a degradation product. UNK was not formed upon degradation of cefradine by heating and by treatment with acid and it disappeared when the sample was treated with alkali. Therefore UNK could not be prepared by degradation but had to be separated by LC from commercial cefradine containing only about 0.3% of UNK. Attempts to prepare samples enriched in UNK by recrystallization were unsuccessful. Attempts to separate UNK from cefradine or to enrich the content of UNK in the sample by using silica gel chromatography were also unsuccessful. The reversed-phase LC methods shown in Fig. 1 were not suitable for preparative work since the mobile phases are not completely volatile. Therefore a volatile mobile phase was developed which was able to separate UNK from all other peaks. HOAc (0.01 M)–CH_3CN (94:6) was found to give suitable separation on PSDVB stationary phase. PSDVB stationary phase was preferred over silica-based reversed-phases for its better stability. UNK was obtained in the crude form by preparative LC on PSDVB (25 × 2.1 cm i.d.). Commercial cefradine (0.1 g) was dissolved in a mixture of 1 ml of 2 M HCl and 4 ml of water and injected. After evaporation of the collected fractions, UNK was purified further using the analytical PSDVB column (25 × 0.46 cm i.d.) and the volatile mobile phase.

The identity of UNK and synthesized 4',5'-dihydrocefradine was established by a combination of chromatographic and spectroscopic methods. In the two analytical LC systems, synthesized 4',5'-dihydrocefradine, added to the commercial sample, coeluted with the UNK. The UV spectra obtained by photodiode array detection during LC were identical. This UV spectrum was similar to that obtained from cefradine. The IR spectra were identical (KBr): 3600-2500 cm^{-1} (–COOH, $-NH_2$, amide), 1760 cm^{-1} (β-lactam), 1670 cm^{-1} (amide I), 1510 cm^{-1} (amide II). The LSIMS spectra were also identical: M + Na^+ *m/z* 374, M + H^+ *m/z* 352 indicated that UNK is a dihydrocefradine. The fragment ion at *m/z* 158 revealed that the heterocyclic part of UNK is the same as that of cefradine. The fragment ions at *m/z* 178 and 110 pointed to the presence of a tetrahydrophenylglycyl side chain [7] (Fig. 3). Comparing the NMR spectrum with that of cefradine, it was found that the supplementary hydrogens are located on the 4',5' positions of the side chain (in particular by using comparative integrals of vinylic and alicyclic 3'–6' resonances), so the structure of UNK was

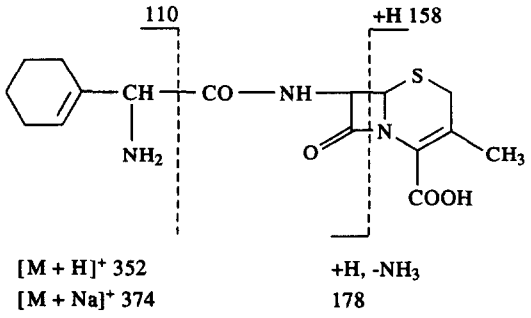

Figure 3
Fragmentation of UNK in liquid secondary ion mass spectrometry.

determined as 4′,5′-dihydrocefradine. The ^{1}H NMR spectral data are: δ (ppm) 1.54(4H, br, 4′-H_2, 5′-H_2), 1.94(4H, br, 3′-H_2, 6′-H_2), 2.08(3H, s, 3-CH_3), 3.41(2H, AB pattern, J = 18 Hz, 2-H_2), 4.53(1H, s, 11-H), 5.06(1H, d, J = 4.4 Hz, 6-H), 5.62(1H, d, J = 4.6 Hz, 7-H), 6.09(1H, br, 2′-H). The ^{13}C NMR spectral data are: δ (ppm) 20.0(CH_3), 21.8(C-5′), 22.5(C-4′), 24.3(C-3′), 25.8(C-6′), 30.6(C-2), 58.2, 59.4, 60.1 (C-6, C-7, C-11), 122.9(C-4), 129.6(C-1′), 135.6(C-2′), 138.6(C-3), 165.9(C-8 and COOH), 169.9(C-10) (cf. NMR spectral data for cefradine [8, 9]).

Conclusions

4′,5′-Dihydrocefradine has not been previously described, but it is present in all commercial samples of cefradine examined and even in official reference substances of cefradine.

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