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Investigation of potential degradation products of a newly synthesised β-lactam antibiotic by multi-stage liquid chromatography–electrospray mass spectrometry

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Abstract

This work describes the use of multi-stage liquid chromatography–electrospray mass spectrometry to characterise four potential degradation products associated with a suspension containing a newly synthesised β-lactam antibacterial compound. Initial identification of these degradates was obtained by analytical LC in which the mobile phase contained cetyltrimethylammonium bromide as ion-pairing agent and ammonium phosphate buffer, both of which are highly involatile making them incompatible with electrospray mass spectrometry. The use of multi-stage liquid chromatography maintained the initial LC conditions necessary for the desired separation and at the same time facilitated the use of mass spectrometry detection.

Keywords: Pharmaceutical analysis; Antibiotics; Lactams

1. Introduction

Various analytical liquid chromatography (LC) methods are routinely used to investigate the purity and stability of new drugs undergoing development [1,2]. In the course of developing such methods a number of considerations, such as the chemical and physical nature of the compound, analysis times and reproducibility of the analytical data, have to be taken into account. The design of new chromatographic methods compatible with mass spectrometry is rapidly becoming an important feature in the choice of mobile phases containing low concentration volatile buffers such as acetate or formate salts instead of the more traditional and less volatile phosphate salts [3]. However, the choice of such mass-spectrometric-friendly buffers is usually precluded by the chemical nature of the analysed compounds which require the presence of mobile-phase modifiers to achieve adequate chromatographic separation. Ion-pairing agents, such as quaternary ammonium salts and sulphonic acid salts, are commonly used for such a task. These agents are often called upon to decelerate the elution times of strongly polar components which otherwise would suffer interferences by the solvent front [4].

It is fair to say that a mobile phase containing both non-volatile buffers and ion-pairing agents represents the worst choice for on-line coupling between electrospray mass spectrometry and LC. This observation is based on two known effects: first, the non-volatile buffer results in a poor spray and consequently low sensitivity; second, the ion-pairing agents are salts containing cations/anions which compete with the analyte molecules resulting in ion chromatograms dominated by the ions of this agent

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which can mask low-level impurities and/or degradation products.

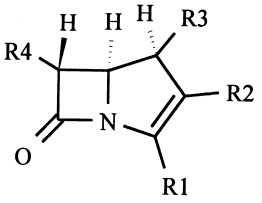
Eventual characterisation of the degradation products separated by the above LC method usually requires semipreparative LC followed by desalting and freeze-drying before performing MS and NMR analysis. It is evident that such a procedure is labour-intensive and commonly requires a large amount of the degraded material. The authors do not cite LC–MS reports on carbapenem antibiotics, however Takano et al. [5] used column switching LC to monitor B02727 carbapenem antibiotic in human plasma and urine.

In the present work an alternative method is described which makes use of multi-stage LC coupled to MS to identify degradation products separated in a chromatographic medium containing both involatile buffer and an ion-pairing agent.

2. Experimental

2.1. Materials

A constituted suspension of the compound (see below) was obtained from a batch dry-stored for 3 months at 50°C. The suspension was then stored for a further 10 days at 30°C.



Mr = 451

Methanol was supplied by Ashland (Milan, Italy), acetonitrile by Carlo Erba Reagenti (Milan, Italy), tetrahydrofuran by BDH Laboratory Supplies (Poole, UK), while water was deionised in the laboratory by a Milli-Q system (Millipore–Waters).

2.2. Instruments

The experimental arrangement used in the present work is depicted in Fig. 1, while the individual components are listed in the caption.

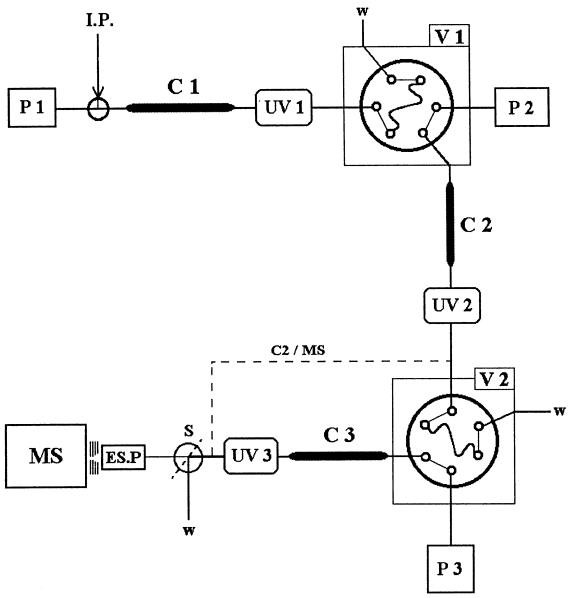


Fig. 1. Experimental arrangement. P1, P2 and P3, HPLC pumps; I.P., injection port; C1, C2 and C3, HPLC columns; UV1, UV2 and UV3, UV detectors; V1 and V2, switch valves equipped with injection loops; S, flow splitter; ESP, electrospray probe; MS, mass spectrometer; w, waste. C2/MS, temporary connection between the C2 outlet and the mass spectrometer.

2.3. HPLC analysis conditions

2.3.1. Analytical HPLC

The analysis conditions were: column, Prodigy ODS2, 250×4.6 mm I.D.; 5 μm particle size (Phenomenex, USA); 45°C column temperature. Mobile phase: 25 m*M* pH 5 ammonium phosphate buffer, 5 m*M* cetyltrimethylammonium bromide (as ion-pairing agent)–acetonitrile (ACN)–MeOH–tetrahydrofuran (THF) (50:20:20:10, v/v); 1 ml/min flow-rate; 20 μl injection volume; sample concentration 0.5 mg/ml. Dissolving medium, 25 m*M* pH 5 ammonium phosphate buffer–ACN–THF (50:40:10, v/v). UV detection, 284 nm.

2.3.2. Multi-stage HPLC

The experimental conditions used to perform multi-stage LC are summarised in Table 1. The sample was dissolved in 25 m*M* pH 5 ammonium

Table 1
Experimental conditions used in multi-stage LC runs

LC Sector	Degradation product	I.P. and switch valve volumes	Column	Mobile phase
1	A, B, C, D	50 μl (I.P.)	Prodigy ODS2 (250×4.6 mm I.D.; 5 μm particle size) Phenomenex, USA	25 m*M* pH 5 NH_4PO_4, 5 m*M* cetyltrimethylammonium bromide–ACN–MeOH–THF (50:20:20:10, v/v)
2	A, B	500 μl	Spherisorb SCX (150×4.6 mm, I.D.; 5 μm particle size) Shandon, UK	50 m*M* pH 6.5 NH_4OAc–ACN (50:50, v/v)
2	C, D	1000 μl	Spherisorb SCX (150×4.6 mm I.D.; 5 μm particle size) Shandon, UK	50 m*M* pH 5 NH_4OAc–ACN (50:50, v/v)
3	A, B	200 μl	Supelcosil ABZ+Plus (150×4.6 mm I.D.; 3 μm particle size) Supelco, USA	50 m*M* pH 6.5 NH_4OAc–ACN (97:3, v/v)
3	C, D	1000 μl	Spherisorb ODS2 (250×4.6 mm I.D.; 5 μm particle size) Shandon, UK	50 m*M* pH 5 NH_4OAc–ACN–MeOH–THF (30:28:28:14, v/v)

phosphate buffer–ACN–THF (50:40:10, v/v) to obtain a final concentration of 5 mg/ml. The flow-rate was 1 ml/min and all the columns were maintained at room temperature. The UV detection was performed at 282 nm.

2.4. Mass spectrometry analysis conditions

All experiments were conducted using a single quadrupole mass spectrometer (VG Platform, Micromass, Manchester, UK) equipped with an electrospray ion source operating in positive ion mode. The LC flow eluting from the multi-stage LC system was split in the ratio of approximately 20:1 resulting in 50 μl/min effective flow into the ion source which was kept at 110°C. Nitrogen was used as both nebulising and drying gas.

3. Results and discussion

The investigated compound is known to exist as a mixture of two diastereoisomers, due to a stereogenic centre associated with the R1 moiety. Analytical liquid chromatography of the degraded solution yielded the UV trace shown in Fig. 2 which was found to be identical to that obtained at the UV1 detector in Fig. 1. The dominant peak centred at $t_R = 23.6$ min is due to the main compound (peak P), while the four secondary peaks at $t_R = 5.8$ min, 1.07% (area/area, a/a) (peak A), 6.6 min 2.47% (a/a) (peak B), 25.4 min 0.36% (a/a) (peak C) and 26.2 min 0.51% (a/a) (peak D), are attributed to potential degradation products. Considering the UV trace, these degradation products can be divided into two groups: the first refers to products A and B which have short retention times suggesting highly polar (hydrophilic) compounds, while the second group includes products C and D which elute after the main peak, indicating that both compounds are relatively lipophilic.

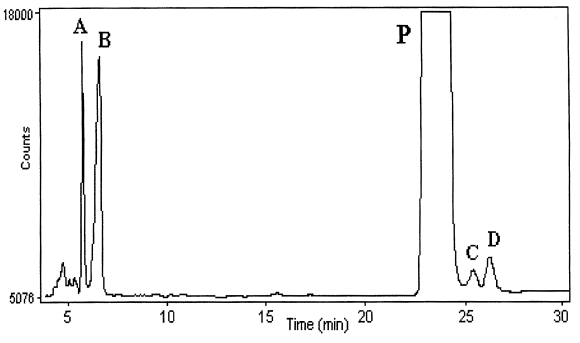


Fig. 2. UV chromatogram ($\lambda = 284$ nm) obtained by analytical liquid chromatography: peak P refers to the newly synthesised carbapenem, while peaks A, B, C, and D are attributed to its potential degradation products.

3.1. Degradation products C and D

In an attempt to reduce interference by the involatile components of the mobile phase, two ODS2 columns separated by a switch valve equipped with a loop were used to divert 1 ml from the outlet of the first column to the inlet of the second. The mobile phase used in the analytical LC method containing both ammonium phosphate and cetyltrimethylammonium bromide was used in the first column, while the mobile phase in the second column contained all the organic solvents used in the first column, the phosphate was substituted by aqueous ammonium acetate, and the elimination of the ion-pairing agent. It was assumed that switching the peak of interest from the first to the second column would result in their separation from the buffer and the ion-pairing agent, an assumption which was based on their different affinity towards the stationary phase of the second column.

The results obtained demonstrated that phosphates eluted from the second column with the solvent front while the strong interaction between the ion-pairing agent and the stationary phase resulted in an elution time starting at about 20 min and lasting for some hours making the use of MS detection not only impractical but resulted in MS spectra dominated by m/z 284 $[\text{cetyltrimethyl-NH}_4]^+$. These difficulties were overcome by inserting a third column capable of selective interaction with the cationic component of the ion-pairing agent. The column used was SCX, placed between the two ODS2 columns as indicated in Fig. 1. To obtain a mobile phase suitable for the SCX column, LC–MS measurements were performed by injecting (at IP) a reference sample with chromatographic behaviour very similar to the degradation products C and D; a representative chromatographic trace obtained in such measurements is given in Fig. 3a,b.

The UV peak corresponds to both the phosphate buffer and the reference compound, while the corresponding total ion current (TIC) chromatogram in Fig. 3b contains two peaks: the first associated with the phosphate aggregate ions together with the

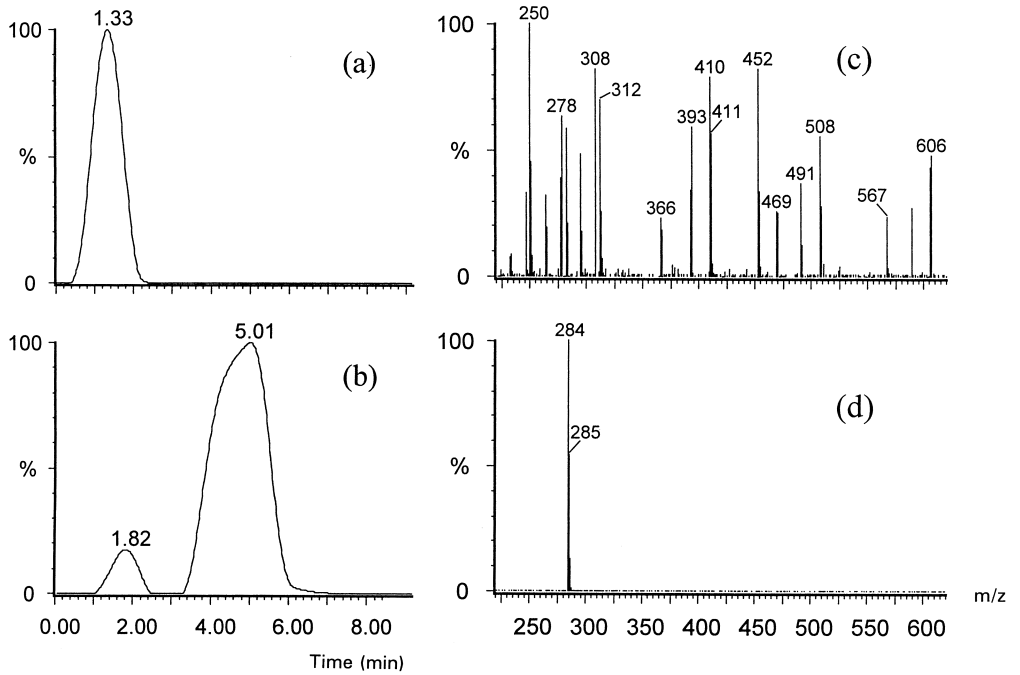


Fig. 3. (a) UV chromatogram ($\lambda = 282$ nm) and (b) TIC chromatogram of peak P obtained at the outlet of column C2 (see Fig. 1). (c,d) ES mass spectra associated with the TIC peaks at $t_R = 1.82$ and 5.0 min.

reference compound ions, while the second peak is due to the ion-pairing agent. These assignments are confirmed by the electrospray (ES) mass spectra in Fig. 3c,d.

Further LC–MS measurements were performed using the reference compound, which allowed optimisation of the chromatographic conditions in C3. In one of these runs the reference component was injected at IP, switched to the second column and the only peak observed at UV2 was collected in a 1000 μl loop and diverted into the third column. The mobile phase in the third column was similar to that used in column 1 except that it did not contain the ion-pairing agent, while the phosphate buffer was substituted with acetate. The results indicated that adjustment of the organic–aqueous solvent ratio in favour of the former was the most influential factor allowing a faster elution of the injected standard and consequently a narrower peak.

The resulting UV and TIC traces are shown in Fig. 4a,b while the spectrum associated with the TIC peak at $t_R = 5.22$ min is shown in Fig. 4c. The spectrum contains a number of ions including the protonated β-lactam antibacterial m/z 452 $[P+H]^+$, m/z 469 $[P+NH_4]^+$, and a number of fragments, including 366, 308, 282, 250 and 196.

3.2. Characterisation of degradation products C and D

Injecting the degraded suspension at IP and switching the UV1 peak ($t_R = 25.4$ min) to the second and then to the third column and monitoring the UV and TIC signals at the final LC outlet the traces reported in Fig. 5a,b were obtained.

The UV chromatogram contains a single peak associated with the degradation product peak C (the only compound containing chromophoric groups), while the TIC chromatogram contains two peaks at the indicated retention times. The two TIC peaks at $t_R = 2.30$ and 6.92 min are assigned to the phosphate buffer, known not to absorb at the used wavelength, and the degradation product peak C.

The ES spectrum associated with the latter peak (Fig. 5c) shows a number of intense ions at the indicated mass-to-charge ratios. Considering the structure of the precursor (parent) product and the analysis conditions, a number of these ions can be

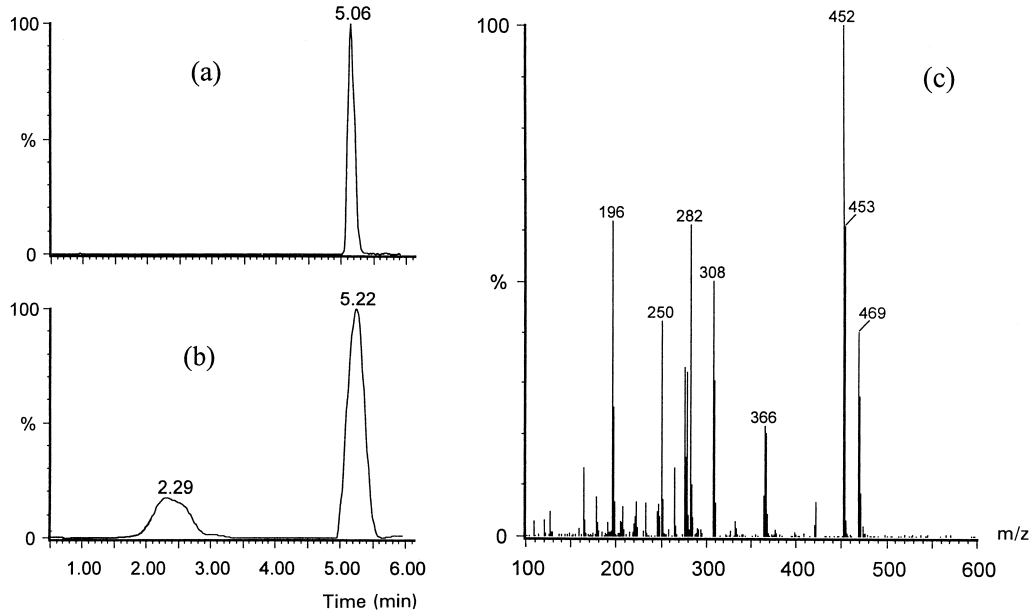


Fig. 4. (a) UV chromatogram ($\lambda = 282$ nm) and (b) TIC chromatogram of peak P obtained at the outlet of column C3 (see Fig. 1). (c) ES mass spectrum associated with the TIC peak at $t_R = 5.22$ min.

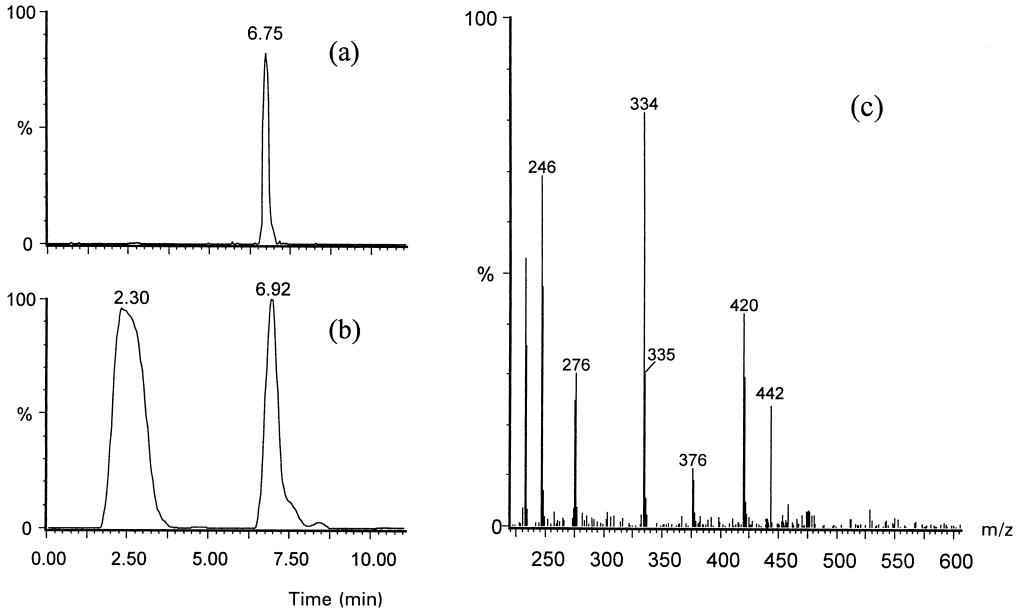
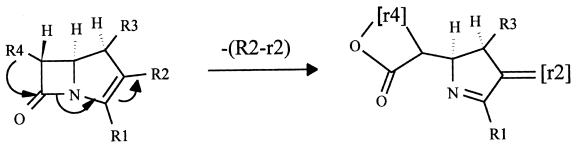


Fig. 5. (a) UV chromatogram ($\lambda = 282$ nm) and (b) TIC chromatogram of a degraded solution obtained at the outlet of column C3. (c) ES mass spectrum associated with the TIC peak at $t_R = 6.92$ min.

reasonably assigned: m/z 420 $[C+H]^+$, m/z 442 $[C+Na]^+$ and a dominant fragment at m/z 334 $[C-R3]^+$.

Injecting the suspension at IP and switching the UV1 peak $t_R = 26.2$ min into the second and then into the third column and monitoring the UV and TIC signals at the final LC outlet resulted in the chromatograms in Fig. 6a,b. The TIC peak at $t_R = 2.34$ min is associated with the phosphate buffer, while the peak at $t_R = 7.00$ min is attributed to degradation product D. The ES mass spectrum associated with this peak (Fig. 6c) resembles the spectrum shown in Fig. 5c which implies that the two degradation products C and D are isomeric structures, the proposed structures of which are given below together with the possible route of formation of such structures.



3.3. Degradation products A and B

The use of ODS2 as the third column together with the same mobile phase employed to analyse degradation products C and D failed to separate A and B from the solvent front containing the phosphate buffer. In an attempt to obtain chromatographic conditions suitable for the separation of these components from the solvent front, a number of LC measurements were performed on two reference compounds derived from forced degradation of the product P.

To separate these reference compounds from the phosphate peak, various experimental conditions were tried, including: (i) the use of ODS2 as a third column together with a slower mobile phase including ammonium acetate–ACN (90:10); (ii) the use of a strong anion exchanger as a third column combined with ACN–ammonium acetate mobile phase; and (iii) the use of an ABZ+Plus ODS column combined with a highly aqueous mobile phase (ammonium acetate–ACN, 97:3). The latter combination of stationary and mobile phases gave the desired results both as chromatographic separation of the peaks of interest and high quality mass spectrometry data.

The UV and TIC chromatograms associated with the first reference compound are given in Fig. 7a,b. The TIC chromatogram in Fig. 7b exhibits two

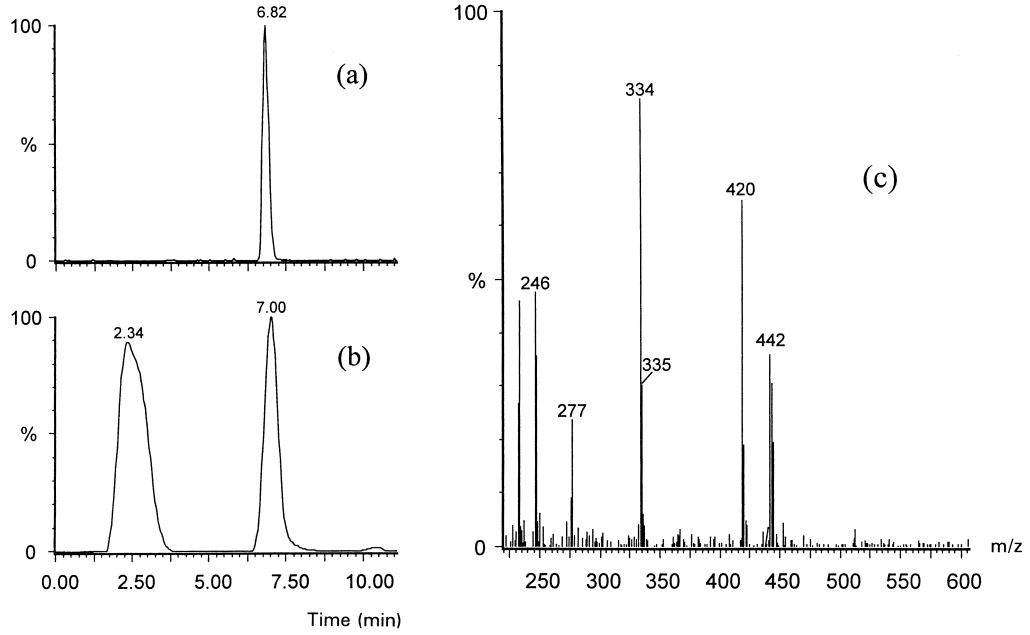


Fig. 6. (a) UV chromatogram ($\lambda = 282$ nm) and (b) TIC chromatogram of a degraded solution obtained at the outlet of column C3. (c) ES mass spectrum associated with the TIC peak at $t_R = 7.0$ min.

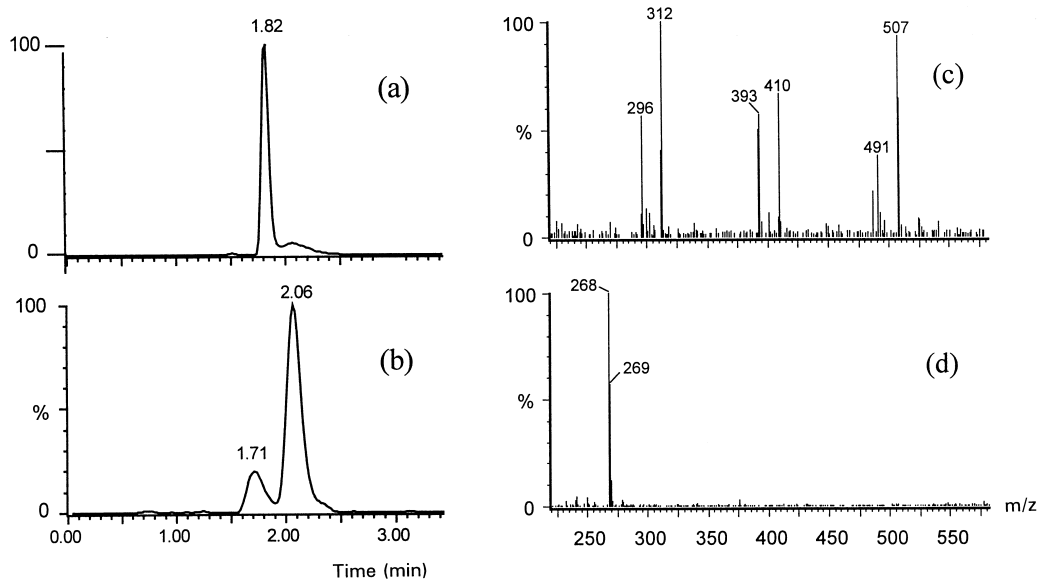


Fig. 7. (a) UV chromatogram ($\lambda = 282$ nm) and (b) TIC chromatogram of a reference compound obtained at the outlet of column C3. ES mass spectra associated with the TIC peaks, $t_R = 1.71$ min (c) and $t_R = 2.06$ min (d).

distinct peaks which yielded the ES mass spectra in Fig. 7c,d. The top spectrum contains a series of phosphate aggregate ions, while the lower spectrum contains a single peak at m/z 268 $[MH–Na+H]^+$. Similar chromatographic and mass spectrometry data were obtained for the second reference compound (Fig. 8). The ES mass spectrum associated with the TIC peak at $t_R=2.06$ contains the ions m/z 304 $[M+H]^+$, m/z 282 $[MH–Na+H]^+$, m/z 250 $[MH–Na–OCH_3]^+$ as well as m/z 299 $[MH–Na+NH_4]^+$.

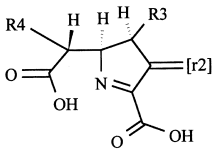
3.4. Characterisation of degradation products A and B

Injecting the degraded suspension at IP and switching the UV1 peak eluting at $t_R=5.8$ min into the second and then into the third column and monitoring the UV and MS signals at the outlet of column 3 yielded the UV and TIC chromatograms shown in Fig. 9a,b.

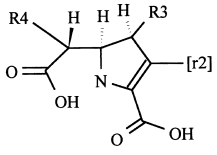
The first TIC peak at 1.66 min yielded a typical phosphate mass spectrum, while the second TIC peak at 2.06 min yielded the ES mass spectrum shown in Fig. 9c. The dominant signal at m/z 268 is attributed to the protonated molecule of degradation product A.

Injecting the same sample at IP and switching the UV1 peak eluting at $t_R=6.6$ min into the second and then into the third column and monitoring the UV and MS signals at the outlet of column 3 resulted in the UV and TIC chromatograms shown in Fig. 10a,b.

The TIC chromatogram contains two peaks at $t_R=1.71$ and 2.06 min, which are attributed to the phosphate buffer and degradation product B. The first TIC peak yielded a mass spectrum dominated by phosphate aggregate ions, while the second peak yielded the ES mass spectrum shown in Fig. 10c. This spectrum contains the same ion which was observed in Fig. 9c which implies that products A and B have the isomeric structures proposed below.



Degradation product peak B



Degradation product peak A

4. Conclusions

Recent pharmaceutical applications of hyphenated

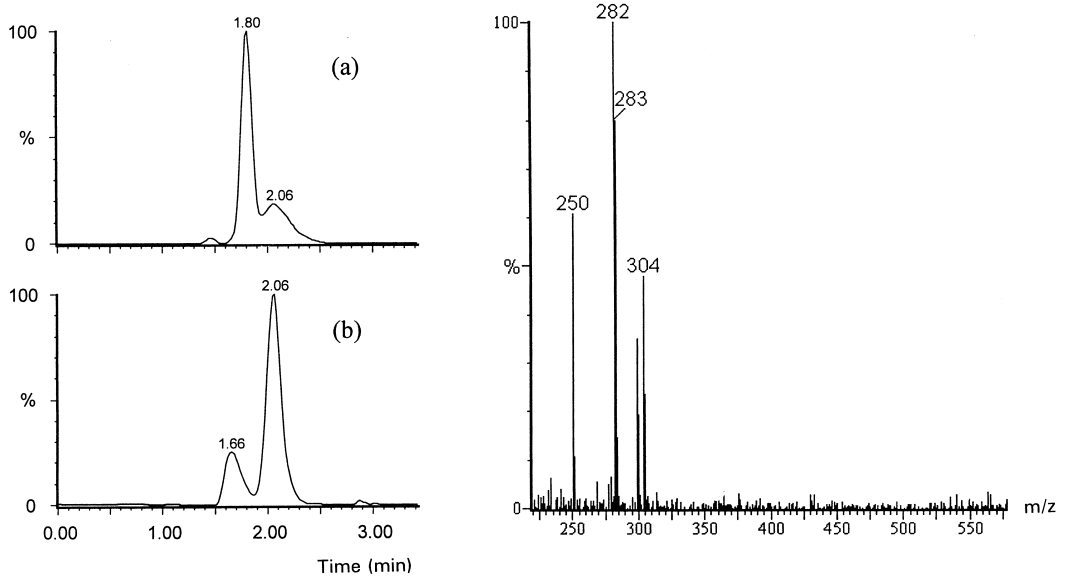


Fig. 8. (a) UV chromatogram ($\lambda=282$ nm) and (b) TIC chromatogram of a second reference compound obtained at the outlet of column C3. ES mass spectra associated with the TIC peaks, $t_R=1.66$ min (c) and $t_R=2.06$ min (d).

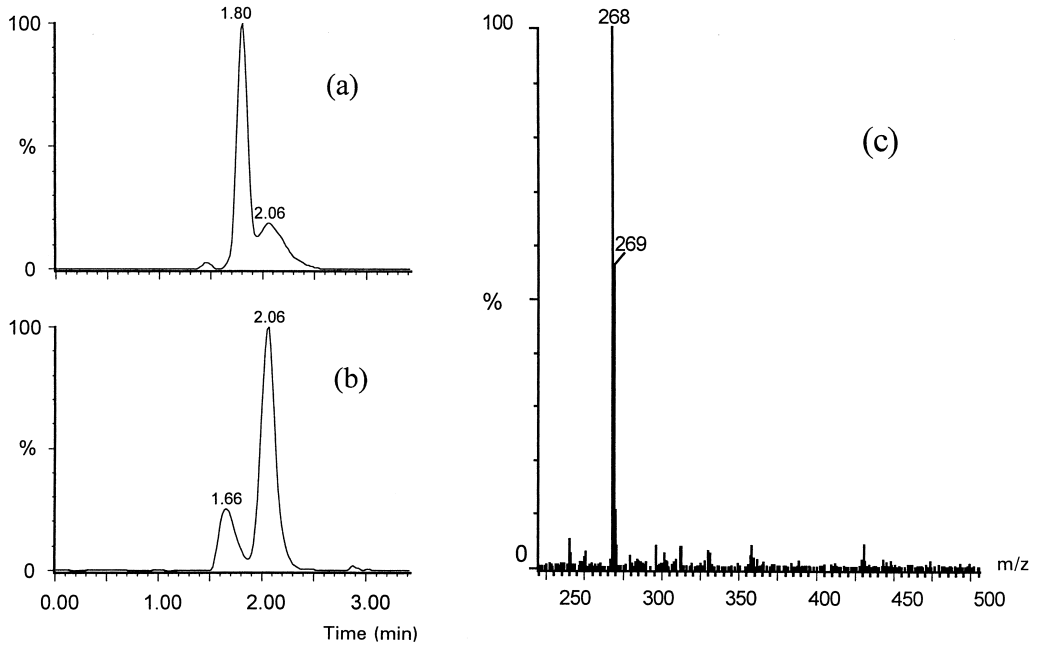


Fig. 9. (a) UV chromatogram (λ=282 nm) and (b) TIC chromatogram of the degraded solution obtained at the outlet of column C3. (c) ES mass spectrum associated with the TIC peak at t_R=2.06 min.

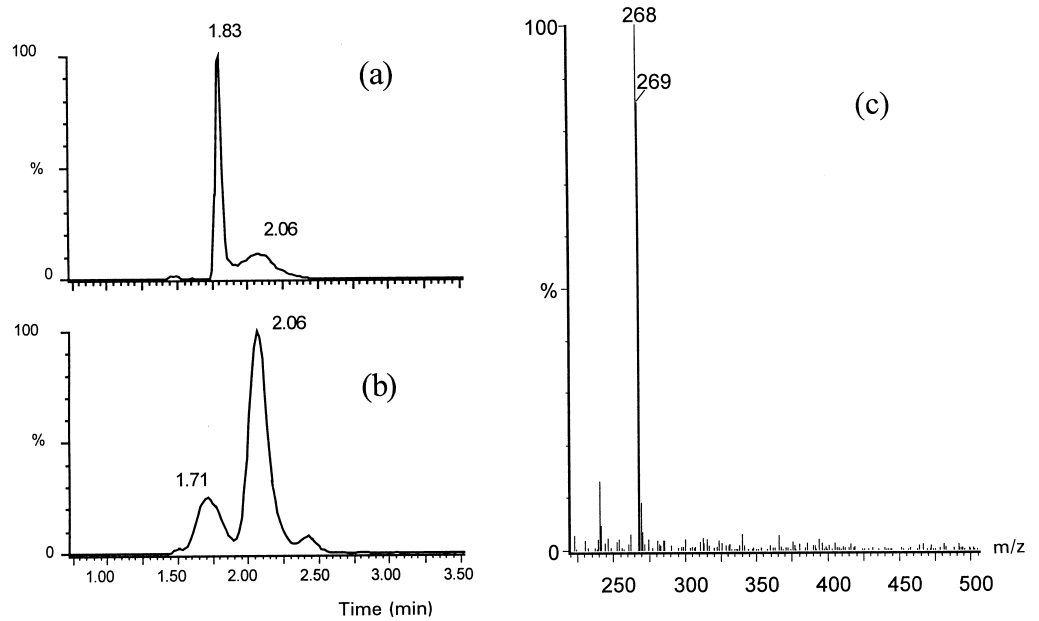


Fig. 10. (a) UV chromatogram (λ=282 nm) and (b) TIC chromatogram of the degraded solution obtained at the outlet of column C3. (c) ES mass spectrum associated with the TIC peak at t_R=2.06 min.

techniques (chromatography, electrophoresis–mass spectrometry) have underlined the need for mobile phases and electrophoretic buffers compatible with on-line mass spectrometry. Recent years have witnessed substantial advances towards such a goal, however there remains a wide range of pharmaceutical products and their potential degradates which require the use of involatile buffers and/or ion-pairing agents for their efficient chromatographic separation. The present work demonstrated that the use of multi-stage LC allowed on-line mass spectrometry detection of components separated by chromatography in which phosphates buffer and ion-pairing agent were used. It is fair to deduce that the described experimental arrangement makes full use of the sensitivity and specificity of mass spectrometry and yet it does not compromise the chromatographic separation of the analytical method.

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