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Journal of Chromatography A, 797 (1998) 311–316

JOURNAL OF CHROMATOGRAPHY A

Development of a simple liquid chromatographic method with UV and mass spectrometric detection for the separation of substances related to amoxicillin sodium

L. Valvo[a], E. Ciranni[a,*], R. Alimenti[a], S. Alimonti[a], R. Draisci[b], L. Giannetti[b], L. Lucentini[b]

[a]*Pharmaceutical Chemistry Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[b]*Veterinary Medicine Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

Abstract

The development of a selective method for the separation and identification of amoxicillin sodium-related substances is described. It is based on reversed-phase liquid chromatography followed either by UV detection (LC–UV) or by mass spectrometry (LC–MS). Mass detection was carried out by an atmospheric pressure ionization source and ionspray interface. Flow injection analyses–MS gave positive-ion mass spectra exhibiting abundant peaks due to their protonated molecules without significant fragmentation. The protonated molecules were used for selected ion monitoring LC–MS analyses. The method allowed the resolution of 13 available potential impurities from amoxicillin and from each other. Its applicability to an MS detector also permits a rapid identification of the impurities in the lack of the corresponding reference substances.

Keywords: Pharmaceutical analysis; Amoxicillin; Antibiotics

1. Introduction

Amoxicillin is a semi-synthetic penicillin widely used in clinical chemotherapy.

A large number of papers on high-performance liquid chromatographic (LC) assay of amoxicillin in biological samples [1–7] and in pharmaceuticals [8–10], as well as LC analysis of its precursors, side and degradation products [11–18] are reported in literature.

With regards to the identification of impurities, the LC methods described utilize detection systems that need impurity reference samples for their application. Due to the difficulty in the availability of some amoxicillin-related substances, we considered developing a selective LC–MS method for the resolution and identification of the amoxicillin sodium impurities indicated in the European Pharmacopoeia [19], applicable also if the corresponding reference samples are lacking. The LC–MS technique, using an atmospheric pressure ionization source (API) and an ionspray interface, performs a mild ionization process which can generate the intact protonated molecules $[M+H]^+$ of the analytes [20], thus providing the molecular mass information of the compound under investigation. A number of ionspray LC–MS methods has been developed by this research group and successfully employed in various applications such as drug analysis [21] and drug residues in foods and biological materials [22,23].

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)00952-7

Ionspray LC–MS was used in this research in order to confirm the identity of amoxicillin and its related substances chromatographed on a base-deactivated reversed-phase column and detected by LC–UV.

2. Experimental

2.1. Samples

Amoxicillin sodium (**6**) was from an injectable pharmaceutical preparation on the Italian market (SmithKline Beecham, Milan, Italy).

4-Hydroxyphenylglycine (**1**) was from Sigma (Milan, Italy). The following related substances were kindly donated by Professor J. Hoogmartens who prepared most of them in his laboratory: 6-amino-penicillanic acid (**2**); amoxilloic acid (5S) and amoxilloic acid (5R) (**3 I/II**); amoxicilloic acid (5*S*,6*R*) and amoxicilloic acid (5*R*,6*R*) (**4 I/II**); L-amoxicillin (**5**); 2-hydroxy-3-(4-hydroxy)phenyl-pyrazine (**7**); 4-hydroxyphenylglycylamoxicillin (**8**); amoxicillin (5*R*) piperazine-2′,5′-dione and amoxicillin (5*S*) piperazine-2′,5′-dione (**9 I/II**); N-pivaloyl-4-hydroxyphenyl-glycine (**10**); amoxicillin dimer (**11**).

The chemical structures of amoxicillin and related impurities are reported in Fig. 1.

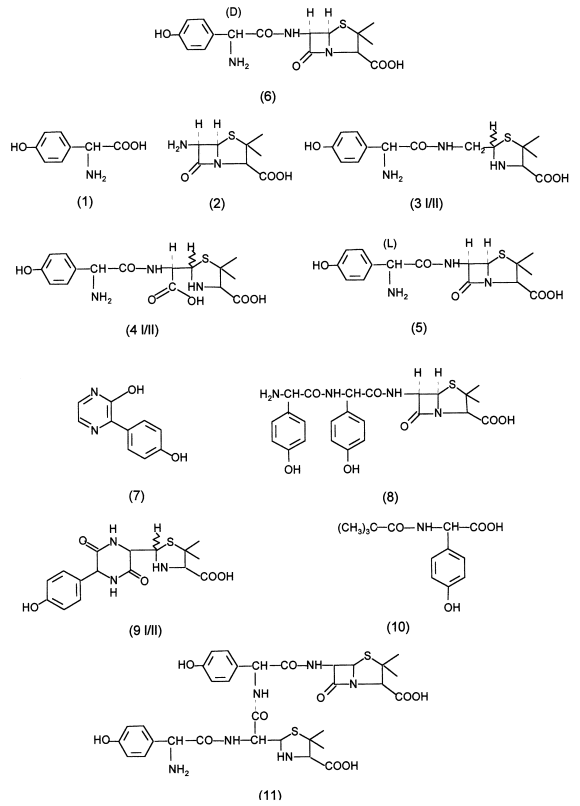

Fig. 1. Chemical structures of amoxicillin and related compounds.

2.2. Chemicals

HPLC-grade acetonitrile was obtained from Farmitalia Carlo Erba (Milan, Italy); trifluoroacetic acid was from Fluka (Buchs, Switzerland). Water was purified on a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Preparation of the solutions

Individual solutions of amoxicillin sodium and its impurities were prepared in water adjusted to pH 4 with trifluoroacetic acid at a concentration of 0.5 mg/ml. These solutions were used in both LC–UV and flow injection analysis (FIA)–MS experiments. For the LC–UV and LC–MS experiments aliquots of each individual solution were mixed together to obtain a mixture containing 180 ng of each compound in the injection volume.

2.4. LC–UV and LC–MS analysis

The analyses were performed using a high-pressure quaternary pump (HP 1050) equipped with an HP 1050 autosampler and an MS-DOS 3D workstation, all from Hewlett-Packard (Palo Alto, PA, USA). The injection volume was 5 μl for both the LC–UV and LC–MS analyses.

The chromatographic column was a 5-μm Supelcosil ABZ+plus, 150×4.6 mm I.D. (Supelco, Bellefonte, PA, USA). The mobile phases were: (A) trifluoroacetic acid (0.1%, v/v; pH 2.1)–acetonitrile (93:7, v/v); and (B) trifluoroacetic acid (0.1%, v/v; pH 2.1)–acetonitrile (80:20, v/v).

A double isocratic elution was performed as described in Table 1. The analyses were performed at room temperature at a flow-rate of 1 ml/min. For

Table 1
HPLC double isocratic programme

Time (min)	Eluent A[a]	Eluent B[b]	Description
0–13	100	0	First isocratic step
13–15	100→40	0→60	Linear gradient
15–45	40	60	Second isocratic step
45–47	40→100	60→0	Switch to initial mobile phase
47–60	100	0	Equilibration

[a]Eluent A: trifluoroacetic acid (0.1%, v/v; pH 2.1)–acetonitrile (93:7, v/v).
[b]Eluent B: trifluoroacetic acid (0.1%, v/v; pH 2.1)–acetonitrile (80:20, v/v).

the LC–MS analysis the column effluent was split to achieve a flow-rate of 40 μl/min to the mass spectrometer.

FIA–MS experiments were carried out by using a Valco valve (Valco, Houston TX, USA) with a 1-μl internal loop and a mobile phase consisting of trifluoroacetic acid (0.2%, v/v)–acetonitrile (50:50, v/v) at a flow-rate of 40 μl/min.

Detection was performed either by UV detection or by MS. UV spectral analyses were performed on an HP 1040M diode array detector (Hewlett-Packard). The monitoring wavelength was 230 nm. MS analyses were performed on a PE-Sciex API I single-quadrupole (PE-Sciex, Thornhill, Canada). The mass spectrometer was equipped with an API source and an ionspray interface set at a voltage of 5500 V; the orifice potential voltage (OR) was set at 50 V. A Macintosh IIci (Apple Computer, Cupertino, CA, USA) equipped with the standard PE-Sciex software package was used for instrument control, data acquisition and processing.

Full-scan positive ion mass spectra were acquired over the mass range m/z 100–800. For targeted analysis and maximum sensitivity, the selected-ion monitoring (SIM) mode on the protonated molecules $[M+H]^+$ of each analyte was implemented.

3. Results and discussion

3.1. LC–UV analysis

UV detection was utilized to develop and optimize the chromatographic method. Injections of amoxicillin sodium and its related substances, both one at a time and in mixture, were made to evaluate the capacity of the method in separating all the potential impurities from each other and from amoxicillin. Fig. 2 shows the resolution of 13 potential impurities from amoxicillin. The k values are reported in Table 2.

The method was successively tested by MS detection to verify if it was suitable for the appointed purpose.

3.2. LC–MS analysis

The ionspray MS experiments were firstly carried out by FIA on the individual solutions of the amoxicillin-related substances, as this technique is

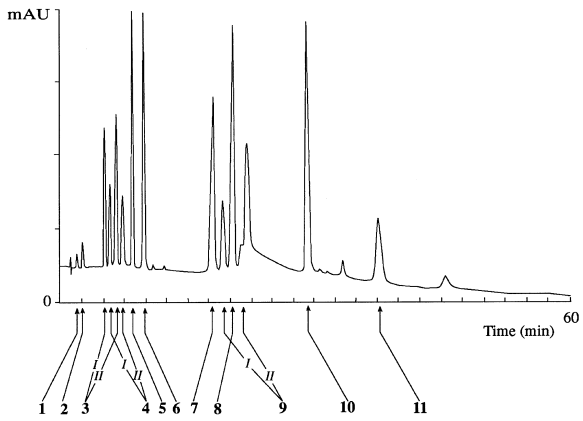

Fig. 2. Chromatographic profile of LC–UV analysis of a solution of amoxicillin-related substances (0.036 mg/ml). Peaks: (**1**) 4-hydroxyphenylglycine; (**2**) 6-aminopenicillanic acid; (**3 I/II**) amoxilloic acid (5*S*) and amoxilloic acid (5*R*); (**4 I/II**) amoxicilloic acid (5*S*,6*R*) and amoxicilloic acid (5*R*,6*R*); (**5**) L-amoxicillin; (**6**) amoxicillin; (**7**) 2-hydroxy-3-(4-hydroxy)-phenylpyrazine; (**8**) 4-hydroxyphenylglycylamoxicillin; (**9 I/II**) amoxicillin (5*R*) piperazine-2′,5′-dione and amoxicillin (5*S*) piperazine-2′,5′-dione; (**10**) N-pivaloyl-4-hydroxyphenylglycine; (**11**) amoxicillin dimer. LC–UV conditions as described in Section 2.

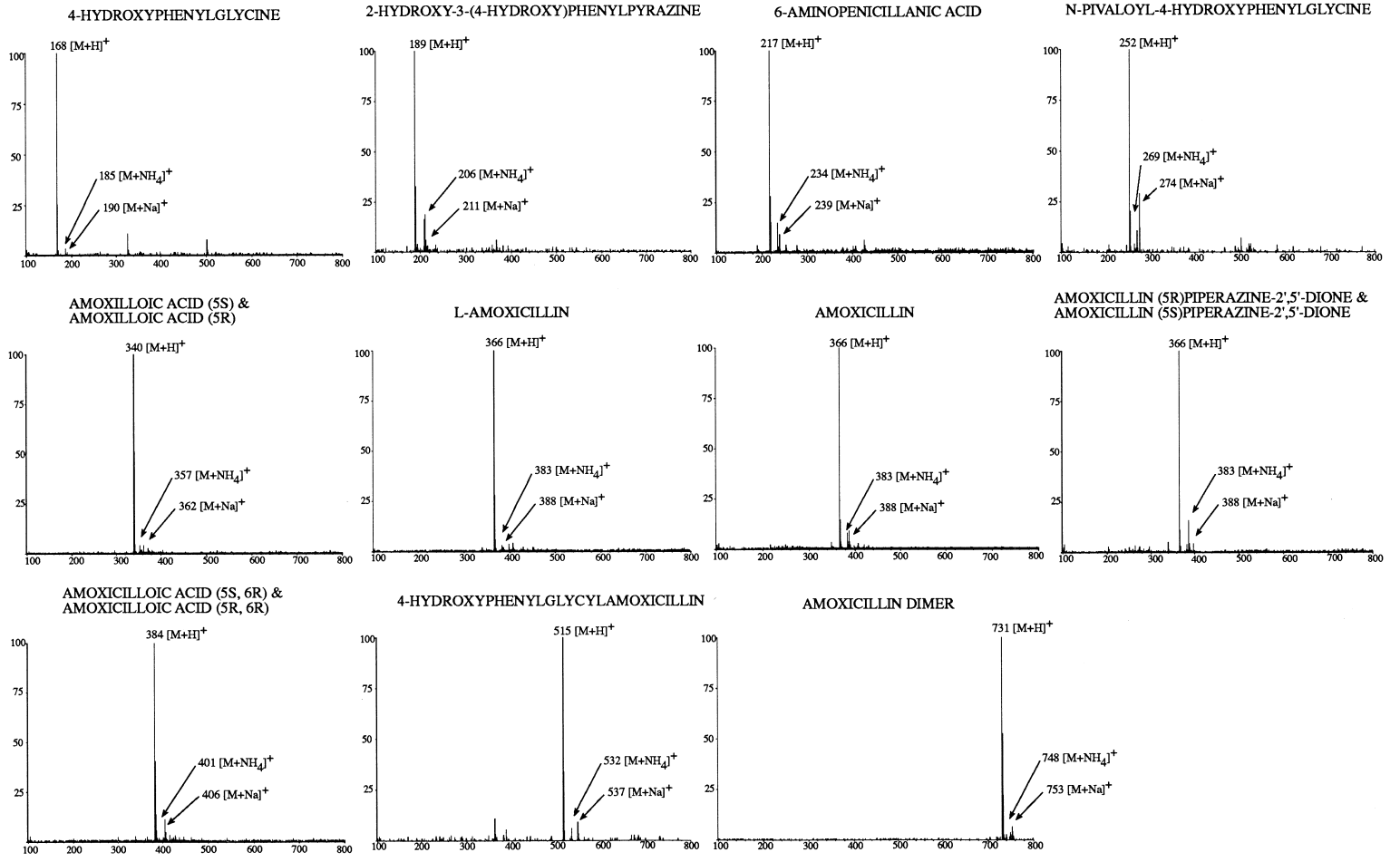

Fig. 3. Full-scan (m/z 100–800) positive ion mass spectra of solutions (0.5 mg/ml) of amoxicillin and its related substances. FIA–MS conditions as described in Section 2.

Table 2
Retention times (t_R) and k values for amoxicillin and its related compounds

Compound no.	t_R (min)	k[a]
1	2.04	0.34
2	2.42	0.66
3 I/II	5.34/6.74	2.51/3.43
4 I/II	6.01/7.52	2.95/3.95
5	8.64	4.68
6	9.99	5.57
7	17.81	10.72
8	20.12	12.24
9 I/II	19.31/22.23	11.70/13.62
10	29.65	18.51
11	37.72	23.82

[a]The hold up time (t_0) was obtained by six replicated injections of uracil (25 μg/ml).

commonly used in order to select the appropriate ions for SIM LC–MS experiments.

Fig. 3 shows the FIA positive full-scan (m/z 100–800) ionspray mass spectra of amoxicillin and its related substances. All these mass spectra were dominated by the protonated molecules $[M+H]^+$ of the analytes: at m/z 168 for 4-hydroxyphenylglycine; at m/z 189 for 2-hydroxy-3-(4-hydroxy)-phenylpyrazine; at m/z 217 for 6-aminopenicillanic acid; at m/z 252 for N-pivaloyl-4-hydroxy-phenylglycine; at m/z 340 for amoxilloic acid (5*S*) and amoxilloic acid (5*R*); at m/z 366 for L-amoxicillin, amoxicillin and amoxicillin (5*R*) piperazine-2′,5′-dione and amoxicillin (5*S*) piperazine-2′,5′-dione; at m/z 384 for amoxicilloic acid (5*S*,6*R*) and amoxicilloic acid (5*R*,6*R*); at m/z 515 for 4-hydroxy-phenylglycylamoxicillin; and at m/z 731 for amoxicillin dimer. Ammonium adducts $[M+NH_4]^+$ and sodium adducts $[M+Na]^+$, were also observed in all the spectra, although their relative abundances were always less than 30% of the respective base peaks.

The abundant protonated molecules were therefore considered suitable for the SIM purposes.

The LC–MS analysis was then carried out under the same chromatographic conditions as for the LC–UV analysis.

Fig. 4 shows the total ion current (TIC) and extracted ion current profiles of the ionspray SIM LC–MS analysis of the mixture of amoxicillin and its related substances, with the mass spectrometer operating in positive-ion mode. The LC–MS chromatographic profiles corresponded well to the LC–UV chromatogram and the confirmation of the peak identities was therefore readily obtained on the basis of the known retention times and specific ion signals, corresponding to the protonated molecule of each analyte, for 4-hydroxyphenylglycine (**1**), 4-hydroxy-phenylglycylamoxicillin (**8**), 2-hydroxy-3-(4-hydroxy)phenylpyrazine (**7**), N-pivaloyl-4-hydroxy-phenylglycine (**10**), 6-aminopenicillanic acid (**2**), L-

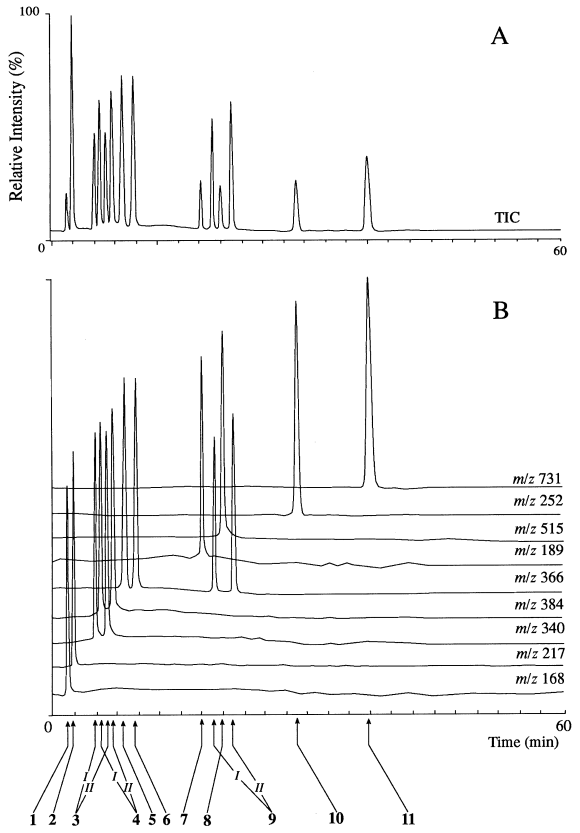

Fig. 4. Total ion current profile (A) and extracted ion current profiles (B) of an ionspray SIM LC–MS analysis of solutions of amoxicillin and its related substances (0.036 mg/ml). Peaks: (**1**) 4-hydroxyphenylglycine (m/z 168); (**2**) 6-aminopenicillanic acid (m/z 217); (**3 I/II**) amoxilloic acid (5*S*) and amoxilloic acid (5*R*) (m/z 340); (**4 I/II**) amoxicilloic acid (5*S*,6*R*) and amoxicilloic acid (5*R*,6*R*) (m/z 384); (**5**) L-amoxicillin (m/z 366); (**6**) amoxicillin (m/z 366); (**7**) 2-hydroxy-3-(4-hydroxy)phenylpyrazine (m/z 189); (**8**) 4-hydroxyphenylglycylamoxicillin (m/z 515); (**9 I/II**) amoxicillin (5R) piperazine-2′,5′-dione and amoxicillin (5*S*) piperazine-2′,5′-dione (m/z 366); (**10**) N-pivaloyl-4-hydroxy-phenylglycine (m/z 252); (**11**) amoxicillin dimer (m/z 731). SIM LC–MS conditions as described in Section 2.

amoxicillin (**5**), amoxicillin (**6**), and amoxicillin dimer (**11**). On the other hand, no definite assignment of peaks **I** and **II** to the corresponding stereospecific structure was possible for three couples of stereoisomers under investigation, namely amoxilloic acid (5*S*) and amoxilloic acid (5*R*) (**3 I/II**), amoxicilloic acid (5*S*,6*R*) and amoxicilloic acid (5*R*,6*R*) (**4 I/II**) and amoxicillin (5*R*) piperazine-2′,5′-dione and amoxicillin (5*S*) piperazine-2′,5′-dione (**9 I/II**), due to the unavailability of the individual stereoisomers as pure compounds.

4. Conclusions

The proposed LC method proved to be selective for the separation of the available impurities from amoxicillin and from each other. It is suitable for either UV and MS detection, thus also allowing a rapid identification of the impurities in the lack of the corresponding reference substances. Furthermore, it could be applied to the analysis of pharmaceutical preparations for the identification of the impurities limited by the European Pharmacopoeia monograph.

Acknowledgements

We thank Professor J. Hoogmartens for kindly supplying amoxicillin-related compounds.

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