Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 321–328
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

HPLC for in-process control in the production of sultamicillin

L. Laviana [a], F. Fernández-Marí [b], M. Bayod [b], D. Blanco [c,*]

[a] *MIT Fellowship at Asturpharma S.A., Silvota, Llanera, Asturias, Spain*
[b] *Asturpharma S.A., Silvota, 33192 Llanera, Asturias, Spain*
[c] *Department of Physical and Analytical Chemistry, University of Oviedo, 33006 Oviedo, Spain*

Received 25 July 2002; received in revised form 18 November 2002; accepted 18 November 2002

Abstract

A high-performance liquid chromatographic assay coupled with UV detection (215 nm) was developed for the determination of sultamicillin and its synthesis precursors. The separation of the analytes was performed on a Kromasil C_{18} column (15 cm × 4.6 mm i.d., 5 μm) at 20 °C. The mobile phase (25 mM phosphate buffer, pH 7.0 and acetonitrile 48%) was pumped at a flow rate of 1.0 ml min^{-1}. This method is sensitive (limits of detection ranged between 0.4 and 1.2 mg l^{-1}) and selective for the determination of sultamicillin and could be used for monitoring different synthetic routes.

Keywords: Sultamicillin; Process monitoring; HPLC

1. Introduction

Over the last 20 years there has been an increase in the prevalence of β-lactamase-producing strains of both Gram-positive and Gram-negative bacteria, which has restricted the usefulness of β-lactam antibiotics. Although some of the newer cephalosporins are stable to many β-lactamases, these require parenteral administration. A different approach to this problem is the administration of a β-lactamase inhibitor in combination with a β-lactam antibiotic, such as ampicillin, whose properties are already well known. The β-lactamase inhibitors clavulanic acid and sulbactam are used to extend the antimicrobial range of certain β-lactam antibiotics. Sulbactam, sodium penicillinate sulphone (CP-45, 899), is a semi-synthetic inhibitor of the β-lactamases of many Gram-positive and Gram-negative aerobic and anaerobic species which presents only weak intrinsic antibacterial activity except against *Neisseria* and related species and superior in vitro stability compared to clavulanic acid [1]. Sultamicillin is an orally absorbed double ester in which sulbactam and ampicillin are linked via a methylene group. Following absorption, first-pass hydrolysis, most probably in the intestinal wall, liberates equimolecular proportions of sulbactam and ampicillin into the systemic circulation. The comparability of the pharmacokinetics of ampicillin after

* Corresponding author. Tel.: +34-985103490; fax: +34-985103125.
E-mail address: dbg@sauron.quimica.uniovi.es (D. Blanco).

0731-7085/03/$ - see front matter
PII: S0731-7085(02)00642-8

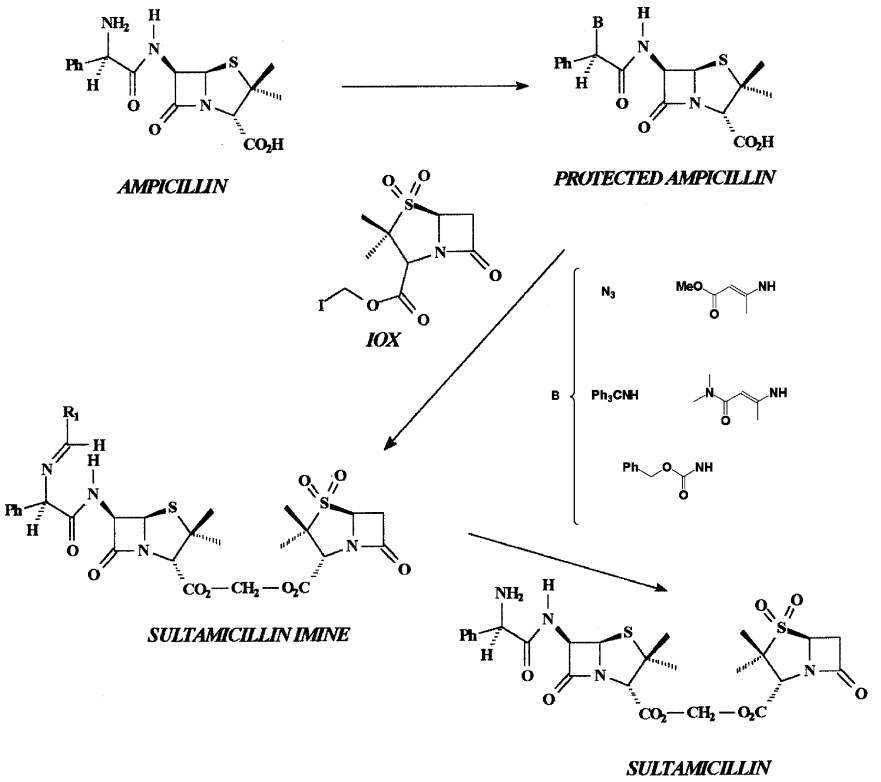

Fig. 1. Synthetic routes to sultamicillin.

administration of sultamicillin with its disposition when administered alone as determined by different studies [2] suggests that the presence of sulbactam in the body does not grossly alter the pharmacokinetics of ampicillin. In addition, the oral bioavailability of ampicillin from sultamicillin was also found to be higher than values quoted in the literature for administration of ampicillin alone [3]. Sultamicillin is therefore an efficient prodrug for sulbactam and ampicillin. Sultamicillin has been shown to be effective against infections of the respiratory tract, skin and soft tissues, and the urinary tract, as well as in obstetric and gynaecological infections [4,5].

The synthesis of sultamicillin has been previously disclosed in several patents, all of them applied by Pfizer and Leo Pharm. These patents describe the *O*-alkylation of ampicillin or *N*-protected ampicillin with an halomethylester derivative of sulbactam to yield the methanediol ester of sulbactam and ampicillin known as sultamicillin. The subsequent reported procedures for the preparation of sultamicillin have only slightly modified some steps of the original synthesis. Most significantly, the synthetic strategy has never been modified in what refers to the *N*-protection of ampicillin. Prior to our study, many different groups had been used in the protection of the amino moiety: *azido, benzyloxycarbonyl, triphenylmethyl, 1-methoxycarbonylpropen-2-yl, 1-N,N′-dimethylcarbonylpropen-2-yl* and several heterocyclic groups. All the methodologies previously described for the synthesis of sultamicillin suffer the same practical drawback, which is the relative instability of the *N*-protecting group in the coupling conditions and the instability of sultamicillin in the experimental conditions used in its synthesis. Recently, Asturpharma (Silvota, Llanera, Spain) has developed a new technology [6] for the synthesis of new intermediates in the production of sultamicillin under mild conditions (Fig. 1), either as the free base or as the tosylate salt, giving

rise to good yields. The most notable advantages introduced by the Asturpharma procedure are: (a) sultamicillin imino derivatives are much more stable than the corresponding sultamicillin enamines. This greater stability allows the isolation and purification of these derivatives to be carried out without observing any decomposition; (b) the imino derivatives have been successfully transformed into sultamicillin, either as its tosylate salt or as the free base, in a selective manner, which indisputably represents an advantage in the development of different galenic forms; (c) the elimination of residual solvents from the target is greatly simplified by the use of water in the purification procedures developed for these derivatives.

Monitoring the concentrations of β-lactam antibiotics and their precursors is required for the optimisation of their production. This allows the identification of the bottlenecks in their synthesis and is also an essential parameter in their scale-up process. During production, the concentrations of the key components are usually monitored on-line and the information used for process control. Therefore, the time lag of the analysis caused by sampling and preconditioning must be short enough to detect the actual state of the reaction. High-performance liquid chromatography (HPLC) methods have been developed for the determination of ampicillin and other penicillins in biological fluids [7–9]. The chromatographic determination of metampicillin and its metabolite ampicillin in biological fluids has been reported previously [10]. This work reported the instability of metampicillin at acidic pH. The chromatographic separation of sultamicillin using an acidic mobile phase has also been reported [11,12]. As imines are readily hydrolysed at acidic pH, this method could not be employed for the on-line monitorisation of Asturpharma's sultamicillin synthesis.

The purpose of the present study was to develop a rapid, specific and sensitive HPLC method for the analysis of sultamicillin and its precursors. Furthermore, the simultaneous analysis of the potential impurities and degradation products was also required. This article describes the advantages obtained from in-process control of sultamicillin by reversed-phase high-performance liquid chromatography (RP-HPLC). The power to carry out thorough in-process control of samples taken during the course of the entire process is highlighted.

2. Experimental

2.1. Reference compounds

Sultamicillin, iodomethylsulbactam (IOX), ampicillin imine, sultamicillin imine, sultamicillin acetonolide and sultamicillin enamine were kindly supplied by Asturpharma. Benzaldehyde and dimethylformamide were purchased from Panreac (Barcelona, Spain).

2.2. Reagents

Acetonitrile and methanol (Panreac) were of HPLC grade. Phosphoric acid and potassium hydroxide were obtained from Merck (Darmstad, Germany). Phosphate buffer (ACS quality) was purchased from Panreac. The water used in the mobile phase preparation was first distilled and then deionized in a Milli-Q apparatus from Millipore (Bedford, MA).

2.3. Standard and sample preparation

Standard solutions of sultamicillin and related compounds were prepared at a concentration of 1 mg ml^{-1} by dissolving the appropriate amount of the pure drug in the sample solvent. The sample solvent was 25 mM phosphate buffer (pH 7.0) and acetonitrile (70%). Dilutions of the 1 mg ml^{-1} standards were used to make the suitable working solutions of the drugs.

Samples for process control of the different reaction steps were accurately pipetted (10 μl) and diluted to 4 ml with sample solvent. The mixture was shaken then for 1 min in an ultrasonic bath and subsequently injected.

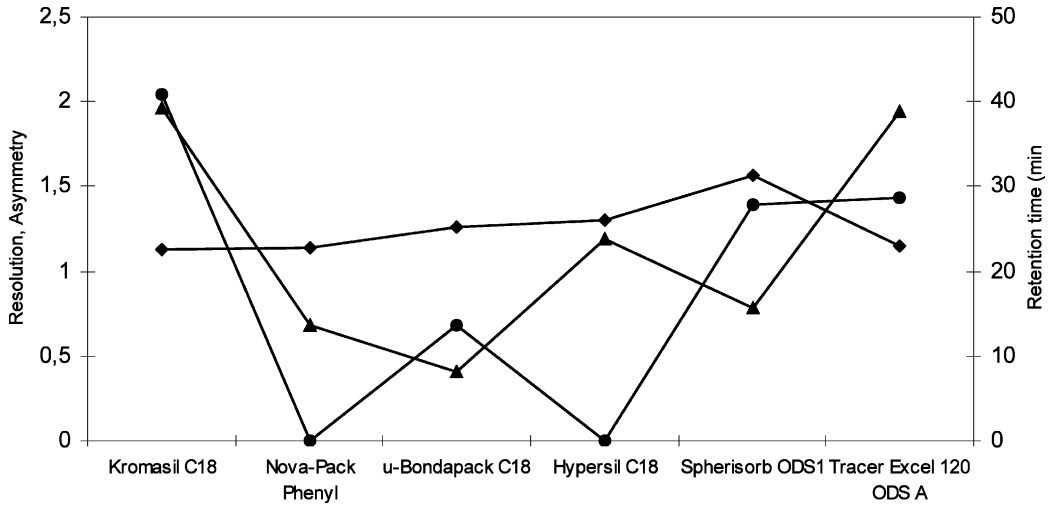

Fig. 2. Effect of stationary phase type on (●) the minimum resolution, (◆) the asymmetry factor of sultamicillin and (▲) the analysis time.

2.4. Chromatography

Method development was performed on two different chromatographic systems. The first system consisted of two LC-10ADvp chromatographic pumps, a SIL-10ADvp autoinjector and a SPD-M10Avp diode array detector which were all coupled by a programmable system controller SCL-10Avp (Shimadzu, Columbia, MD). The second system consisted of two Kontron Model 522 chromatographic pumps and a Kontron Model 540 diode array detector, which were all coupled by the Kromasystem 2000 system controller. The injection of the sample was performed with a Rheodyne 7725 manual injector fitted with a 20-μl injection loop (Rheodyne, Cotati, CA). Detection took place at 215 nm. When sultamicillin and benzaldehyde were seriously overlapped, as a consequence of high concentrations of sultamicillin, the quantification of benzaldehyde could be carried out at 245 nm.

Analisis Vínicos model MFE-01 column ovens were used on both systems to set the column temperature at 20 °C.

The following chromatographic columns were employed in the optimisation of this analytical method: a Spherisorb S5W silica (30 × 0.46 cm) normal-phase column (Waters Assoc., Milford, MA) and several reversed-phase columns (150 × 0.45 cm, 5 μm): Kromasil C_{18} (Analisis Vínicos, Tomelloso, Spain), Nova-pack Phenyl, μ-Bondapack C_{18}, Hypersil C_{18} (Waters Assoc.), Spherisorb ODS1 (Analisis Vínicos), and Tracer Excel 120 ODS-A (Teknokroma, Barcelona, Spain). Two mobile phases were used: phosphate buffer (pH 7.0; 25 mM) (A), and acetonitrile (B). The mobile phase used in the isocratic mode was a mixture of 52% A and 48% B. The gradient run conditions were programmed as follows: 0–10 min 48% B, 10–18 min 70% B, 18–21 min 70% B, 21–24 min 48% B. The final solution was filtered through a 0.45 μm Nylon pore filter (Analisis Vínicos) and degassed prior to use. The mobile phase was pumped at a flow rate of 1.0 ml min^{-1}.

3. Results and discussion

In according with the nature and instability of sultamicillin and especially the imines, normal-phase chromatography (NPC) seemed to be an adequate technique for the analysis of this kind of product. Consequently, the initial experiments were performed by NPC with a Spherisorb S5W silica column. At this point, several mobile phases were tested. A mixture of acetonitrile:2-propanol:-Hexane (1:2:8, v/v/v) gave the best results. Under these conditions, the imines showed excellent

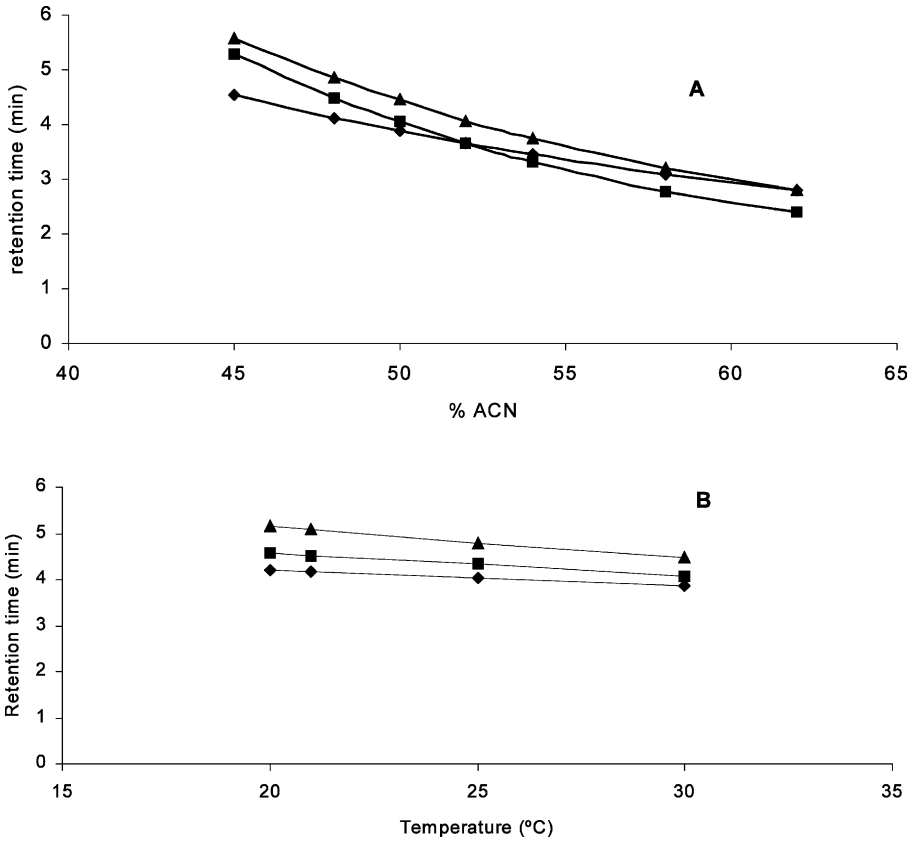

Fig. 3. Influence on retention time with varying (A) acetonitrile content and (B) column temperature. (◆) Benzaldehyde; (■) sultamicillin; (▲) iodomethylsulbactam.

stability, but their selectivity was poor because the sultamicillin imine and the ampicillin imine were partially overlapped. Moreover, sultamicillin presented an inappropriate peak profile. Consequently, reversed-phase chromatography appeared to be a good alternative. The development of reversed-phase chromatographic methods for the simultaneous determination of metampicillin and ampicillin has been reported in Ref. [10]. In this study, the instability of metampicillin (an imine) is shown to be an important drawback. What is more, it was reported that the hydrolysis of metampicillin to ampicillin may be limited by the use of neutral pH (7.0). In our case, the hydrolysis of ampicillin and sultamicillin imines was tested at acidic pH. Therefore, pH 7.0 was selected as the optimum value to develop a new reversed-phase chromatographic method for the analysis of sultamicillin and related substances. Different types of reversed-phase materials and mobile phase mixtures were tested during method development. The stationary phase, in particular, was found to have a great influence on the retention time, resolution and peak shape (Fig. 2). The experiments showed that Nova-pack phenyl, μ-Bondapack C_{18}, and Hypersil C_{18} were not chosen due to their inappropriate resolution (calculated at half-height) ($Rs < 1$). The Spherisorb ODS1 material showed an acceptable resolution for sultamicillin ($Rs > 1$) but its peak symmetry was unacceptable ($As > 1.5$). The best results were obtained with the Kromasil C_{18} and Tracer Excel 120 ODS-A columns. Since the Tracer Excel 120 ODS-A column is more expen-

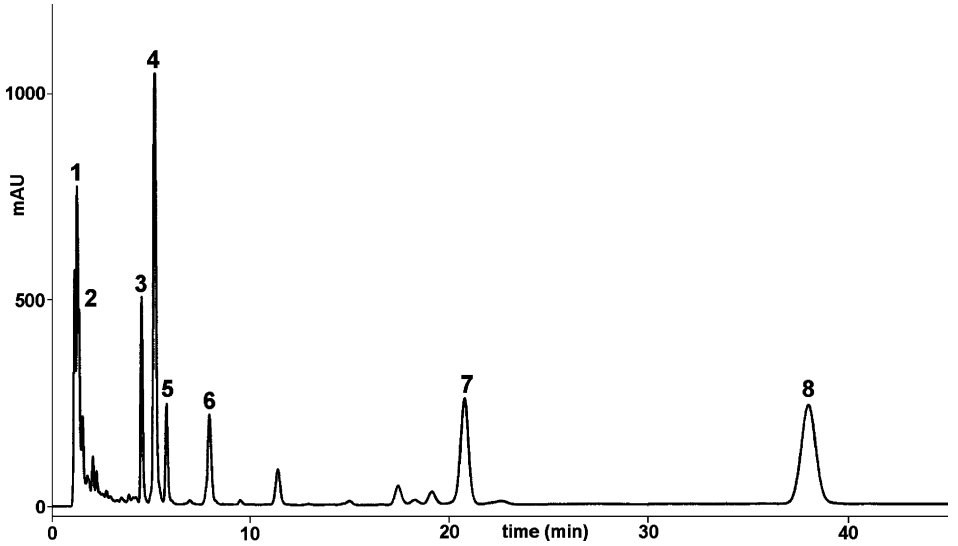

Fig. 4. Chromatogram of sultamicillin, its synthesis intermediates and degradation products obtained in the isocratic mode. Column, Kromasil C_{18}, 150 × 4.5 mm i.d., 5 μm. Flow rate, 1 ml min^{-1}. Mobile phase, 25 mM phosphate buffer (pH 7.0) and acetonitrile (48%). Detection at 215 nm. Peak identification: (1) dimethylformamide (reaction solvent); (2) ampicillin imine; (3) benzaldehyde; (4) sultamicillin; (5) iodomethylsulbactam; (6) sultamicillin acetonolide; (7) sultamicillin enamine; (8) sultamicillin imine.

sive, the Kromasil C_{18} packing material was selected as it provides an excellent peak symmetry and selectivity.

Once Kromasil C_{18} was selected as the stationary phase, the ensuing experiments were performed in order to establish the best chromatographic conditions for the analysis of sultamicillin and related substances. Subsequently, the nature and percentage of the organic modifier were evaluated. Methanol was discarded as organic modifier because of its nucleophilicity, as penicillins can undergo methanolysis with prolonged exposure to this solvent. The chromatographic behaviour of sultamicillin, iodomethylsulbactam and benzaldehyde with different percentages of acetonitrile is shown in Fig. 3A. From this data, the concentration of the acetonitrile was set to 48% to achieve the optimal separation of all the analytes. Temperature is another essential chromatographic variable in sultamicillin analysis and was studied in the range of 20–30 °C (Fig. 3B). According to the experiments, the temperature of the column was set at 20 °C. Fig. 4 shows the typical chromatogram of a mixture of standard substances. The retention times of sultamicillin and its related compounds are 1.93, 4.44, 4.92, 5.62, 7.79, 20.81 and 38.00 min for ampicillin imine, benzaldehyde, sultamicillin, iodomethylsulbactam, sultamicillin acetonolide, sultamicillin enamine and sultamicillin imine. As the analysis time is too long, gradient elution is required for the analysis of the enamine and the imine of sultami-

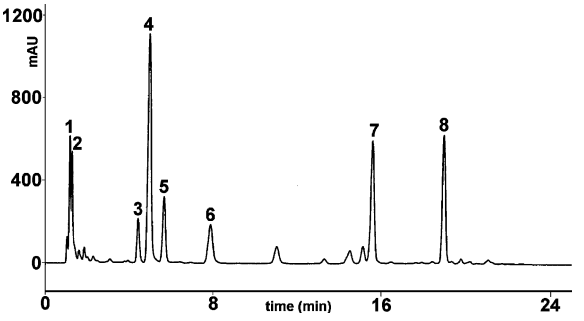

Fig. 5. Chromatogram of standard mixture using gradient elution. Mobile phase, solvent A 25 mM phosphate buffer (pH 7.0), solvent B acetonitrile. Gradient conditions 0–10 min 48% B, 10–18 min 70% B, 18–21 min 70% B, 21–24 min 48% B. Other conditions as in Fig. 4. Peak identification: (1) dimethylformamide (reaction solvent); (2) ampicillin imine; (3) benzaldehyde (157 mg l^{-1}); (4) sultamicillin (340 mg l^{-1}); (5) iodomethylsulbactam (450 mg l^{-1}); (6) sultamicillin acetonolide (500 mg l^{-1}); (7) sultamicillin enamine (620 mg l^{-1}); (8) sultamicillin imine (600 mg l^{-1}).

Table 1
Analytical characteristics of the chromatographic method

	Limit of detection (mg l^{-1})	Recovery (%)
Benzaldehyde	0.4	97–105
Sultamicillin	0.2	98–102
Iodomethylsulbactam	1.2	96–101

iodomethylsulbactam. The calibration graphs for these substances fitted the equation of a straight line through the origin well; the correlation coefficients being greater than 0.999.

The assay precision (RSD) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value.

Intra-day precision was estimated from ten

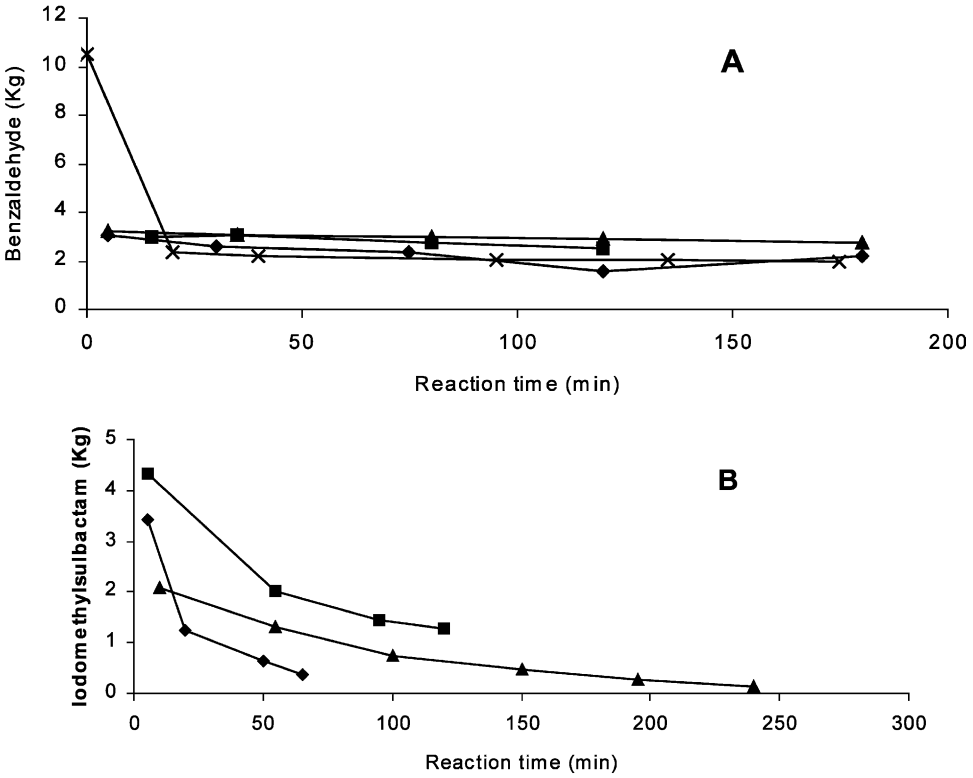

Fig. 6. In-process control of sultamicillin synthesis: (A) ampicillin protection; (B) iodomethylsulbactam coupling. (◆) Batch 1/01; (■) Batch 2/01; (▲) Batch 3/01; (X) Batch 4/01.

cillin. Fig. 5 presents the chromatogram of a mixture of standards using gradient elution.

The in-process control of sultamicillin synthesis only required the calibration of benzaldehyde, sultamicillin and iodomethylsulbactam. Calibration curves were constructed from eight different concentrations. Each concentration sample was injected three times. The linearity range was among the quantification limit and at least up to 500 mg l^{-1} for benzaldehyde, sultamicillin and replicates of a standard mixture sample (RSD < 2%).

Inter-day precision was estimated from the analysis of freshly prepared standard mixture samples on three separate days (RSD < 4%). Acceptable precision was obtained for all preparations.

The limits of detection (LOD) at 215 nm (Table 1), defined as the lowest concentration of the analyte that can be clearly detected above the

baseline signal, is estimated as three times the signal-to-noise ratio; following Winefordner and Long [13].

The determination of percentage recovery (Table 1) was calculated by comparing the absolute response of the processed (recovered) substances to the absolute response of the external standards. The recoveries were between 95 and 105%, testifying to the accuracy of the proposed method.

A well-functioning process monitoring system is necessary for the optimisation of synthetic and purification processes and to maintain the conditions at the optimum level required to secure production of high purity sultamicillin with maximum yield. On the basis of our experience, the desired yield and purity can only be reached through improved, expanded analytical control. In Fig. 6, monitoring the benzaldehyde concentration can be seen to be a way of controlling the first synthetic step. When the first reaction has finished, the monitoring of the iodomethylsulbactam concentration was used to optimise the next critical step of the synthesis. In situ reaction analysis using RP-HPLC is shown to provide real-time monitoring of the different reagents and products.

4. Conclusions

An HPLC method was developed for the simultaneous determination of sultamicillin and potential impurities and degradation products. Furthermore, the chromatographic method was demonstrated to be useful for the in-process control of sultamicillin synthesis at an industrial scale. The advantages of monitoring the process of manufacturing sultamicillin by RP-HPLC were highlighted. The described method yielded good recoveries and precision. In addition, the LOD were in the range of low mg l^{-1}.

Acknowledgements

This work was funded by the Spanish Ministerio de Ciencia y Tecnología (Acciones MIT).

References

[1] A.R. English, J.A. Retsema, A.E. Girand, J.E. Lynch, E. Barth, Antimicrob. Agents Chemother. 14 (1978) 414–419.
[2] H.J. Rogers, I.D. Bradbrook, P.J. Morrison, R.G. Spector, D.A. Cox, L.J. Lees, Antimicrob. Agents Chemother. 11 (1983) 435–445.
[3] G.P. Lewis, W.J. Jusko, Clin. Pharmacol. Ther. 18 (1975) 475–484.
[4] H.A. Friedel, D.M. Campoli-Richards, K.L. Goa, Drugs 37 (1989) 491–522.
[5] D.M. Campoli-Richards, R.N. Brogden, Drugs 33 (1987) 577–609.
[6] C. del Pozo, E. Alonso, F. López-Ortiz, F. Fernández-Marí, M. Bayod, J. González, Tetrahedron 57 (2001) 6209–6214.
[7] T. Uno, M. Masada, K. Yamaoka, T. Nakagawa, Chem. Pharm. Bull. 29 (1981) 1957–1968.
[8] R.A. Blum, R.K. Kohli, N.J. Harrison, J.J. Schentag, Antimicrob. Agents Chemother. 33 (1989) 1470–1476.
[9] F. Salto, J.G. Prieto, M.T. Alemany, J. Pharm. Sci. 69 (1980) 501–506.
[10] H. Lee, J.S. Lee, H.S. Lee, J. Chromatogr. B 664 (1995) 335–340.
[11] A.A. El-Shanawani, Acta Poloniae Pharmaceutica-Drug Res. 55 (1998) 9–14.
[12] A.P. Argekar, S.S. Kunjir, J. Pharm. Biomed. Anal. 15 (1996) 423–427.
[13] J.D. Winefordner, G.L. Long, Anal. Chem. A 55 (1983) 712A–724A.