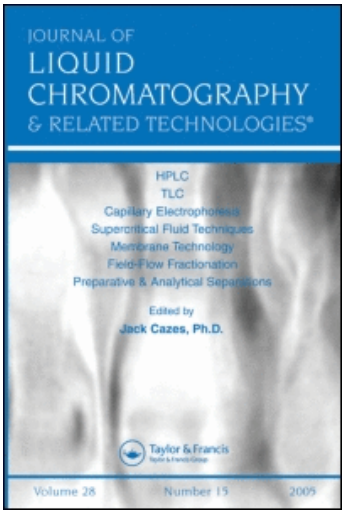

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High Speed Liquid Chromatography for In-Process Control of Sultamicillin

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Abstract: A fast HPLC method has been developed for simultaneous determination of sultamicillin and its synthesis precursors. The analytes are separated in 2.5 min by means of a Kromasil 100 C18 column (50 mm × 2.1 mm i.d., 3.5 μm) at 25°C. The mobile phase (A: 5 mM KH_2PO_4 and 20 mM KCl adjusted to pH 6.0 with H3PO4 plus 1% THF and B: acetonitrile with 1% THF) was pumped at a flow rate of 0.5 mL min^{-1} according to the fast gradient mode: 0–0.9 min, 40% B; 0.9–1.0 min, 85% B; 1.0–2.5 min, 85% B; 2.5–2.6 min, 40% B, 2.6–4.0 min, 40% B. Detection was by ultraviolet absorbance at 205 nm. The method was validated in accordance with the International Conference on Harmonisation (ICH) guidelines, good accuracy, intermediate precision ($\leq$3.8%), and linearity being observed for all compounds. This method is sensitive (limits of detection ranged between 0.1–1.1 mg l^{-1}) and selective for quantifying sultamicillin and its synthesis precursors and could be used for in-process control.

Keywords: High speed liquid chromatography, In-process control, Sultamicillin

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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, they 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, is a semisynthetic inhibitor of the β-lactamases of many gram-positive and gram-negative aerobic and anaerobic species that 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 likely in the intestinal wall) liberates equimolecular proportions of sulbactam and ampicillin into the systemic circulation. The comparability of the pharmacokinetics of ampicillin after 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. Furthermore, 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 a halomethyl ester 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 as regards 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′-dimethylcarbonyl-propen-2-yl, and several heterocyclic groups. All the methodologies previously described for the synthesis of sultamicillin suffer the same practical drawbacks, namely 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 (Figure 1), either as the free base or as the tosylate salt, giving rise to good yields. The most notable

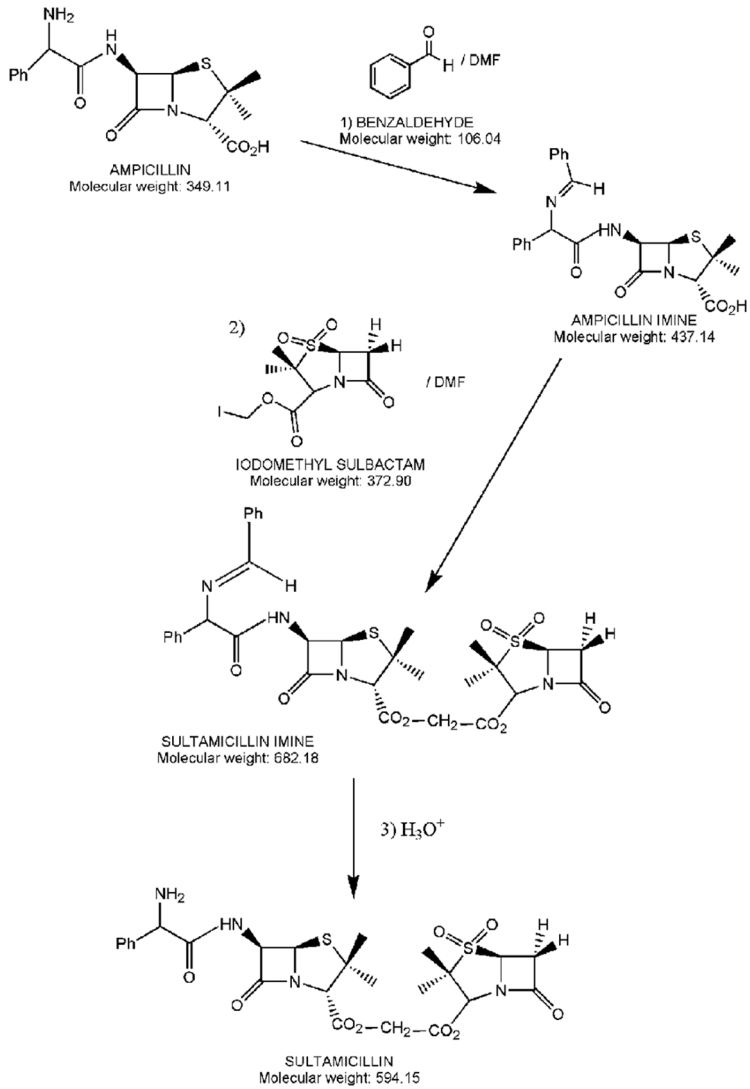

Figure 1. Synthetic route to sultamicillin patented by Asturpharma S.A. Spanish patent: ES21616002.

advantages introduced by Asturpharma's 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 removal 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 in order to optimise their production. This allows identification of the bottlenecks in their synthesis and is also an essential parameter in their scaleup process. During production, the concentrations of the key components are normally monitored online 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.

Ampicillin and sulbactam determination has been performed, both in biological fluids and pharmaceuticals by different techniques, such as flow injection analysis,[7,8] polarography,[9,10] and spectrophotometry,[11–13] although HPLC[14–25] has chiefly been used. Furthermore, sultamicillin analysis has primarily been carried out in pharmaceutical formulations by HPLC,[26–31] according to recommendations by pharmacopoeias. In addition, two spectrophotometric sultamicillin determinations[32,33] were recently published in the literature. The chromatographic separation of sultamicillin using an acidic mobile phase has also been reported. However, as imines are readily hydrolysed at acidic pH, this method could not be employed for the online monitoring of Asturpharma's sultamicillin synthesis.

There are no published reports to date concerning the simultaneous analysis of sultamicillin, its synthesis intermediates and potential impurities in a short analysis time. Only one article concerning the in-process control of sultamicillin has been published,[34] but it makes use of classical HPLC.

In its conventional format, high speed liquid chromatography employs columns of standard diameter (4.6 mm) measuring up to 10 cm in length, generally with stationary phases of 3 μm particle size, working at a mobile phase flow rate higher than 2 mL min^{-1}. In our opinion, the diameter of these columns should be smaller than 3.2 mm, as such columns allow the flow rate to be reduced up to approximately fivefold. Related microbore columns increase the effectiveness, sensibility, and precision of the chromatographic methods.

In order to improve resolution, modern stationary phases of fine granulometry (2 or 1.5 μm, porous or not porous) could be used, although their current price limits their applications. Another alternative might be the use of monolithic stationary phases, characterized by high porosity and permeability. The reduction in analysis time is achieved by increasing the flow rate up to 9 times the usual in a conventional column, although high solvent consumption and the price of the columns are important drawbacks. Furthermore, these monolithic columns may generate more peak asymmetry on basic compounds than good classical full endcapped columns.[35]

When the required chromatographic effectiveness is not very high, a short microbore column with conventional 3 μm stationary phases is the choice, due to the fact that column and operation price are cheap and the instrumental setup is also relatively simple and economical. Basically, modifications involve the injection system (maximum volume $\leq 3\,\mu L$), the detector cell dead volume ($\leq 3\mu L$), the connecting capillary tubes (length ≤ 80 cm, inner diameter $\leq 170\,\mu m$) and, when the injection valve turns (manual or automatically) slowly, it is convenient to install a bypass that avoids pressure pulses that could rapidly deteriorate short columns.[36,37]

The aim of the present research work is to develop a rapid and reliable liquid chromatographic method for the analysis of sultamicillin and its precursors in order to enhance laboratory productivity. Furthermore, simultaneous analysis of the potential impurities and degradation products was also required. The validation parameters stated by ICH guidelines[38,39] were taken into consideration. This article describes the advantages obtained from in-process control of sultamicillin by high speed liquid chromatography (HSLC).

EXPERIMENTAL

Reagents and Standards

Sultamicillin, ampicillin, ampicillin imine, iodomethyl sulbactam, and sultamicillin imine were kindly supplied by Asturpharma (Silvota-Llanera, Spain). Benzaldehyde, potassium dihydrogen phosphate and ammonium acetate were obtained from Sigma Chemical Co. (St. Louis, MO, USA), potassium hydroxide was purchased from Merck (Darmstadt, Germany), acetic acid was obtained from Panreac (Barcelona, Spain), potassium chloride was purchased from Merck (Darmstadt, Germany), and HPLC gradient quality acetonitrile and tetrahydrofurane were obtained from Romil (Loughborough, UK). Milli-Q water (Millipore, Milford, MA, USA) was used throughout. All other chemicals and solvents were of analytical reagent or HPLC grade.

Standard Solutions

Standard solutions of sultamicillin and related compounds were prepared by dissolving the appropriate amount of the pure drug in acetonitrile at a concentration of 60, 300, and 600 mg l^{-1} for benzaldehyde, sultamicillin, and iodomethyl sulbactam, respectively. All solutions proved to be stable for more than 3 hours, using acetonitrile as solvent. Significant peak broadening was not observed as a result of a stronger sample solvent than the mobile phase. Dilutions of the standards were used to make the appropriate working solutions of the drugs in acetonitrile. The solutions were sonicated in an ultrasonic bath for 1 min and filtered through a 0.22 μm PVDF syringe filter (Lida, Kenosha, WI, USA). The resulting filtered solution was placed in an HPLC vial.

Apparatus and Conditions

HPLC analyses were performed on a Shimadzu HPLC system (Duisburg, Germany) equipped with two LC-10AD pumps, a UV-Vis SPD-M10AD photodiode array detector with a 2.5 μL flow cell and 80 ms response time, a SIL-10AD automatic injector, and DGU-14A degas on-line and 60 cm connecting tubes with an internal diameter of 127 μm. The column employed was a Kromasil 100 C_{18} (50 mm × 2.1 mm i.d., 3.5 μm) (Teknokroma, Barcelona, Spain). An ODS guard column was used to protect the analytical column. Before use, the mobile phase was vacuum filtered through a 0.22 mm nylon membrane filter (Lida, Kenosha, WI, USA). Chromatographic experiments were carried out at 25°C. The binary gradient used at a flow rate of 0.5 mL min^{-1} was as follows: 0–0.9 min, 40% B; 0.9–1.0 min, 85% B; 1.0–2.5 min, 85% B; 2.5–2.6 min, 40% B, 2.6–4.0 min, 40% B, where solvent A was water with 5 mM potassium dihydrogen phosphate and 20 mM potassium chloride adjusted to pH 6.0 with phosphoric acid plus 1% THF and solvent B was acetonitrile with 1% THF. The sample injection volume was 1 mL.

The chromatographic method developed using the aforementioned equipment was transferred to an HPLC-MS system to take mass spectra analysis. Analysis was also performed on an HPLC/MSD Agilent 1100 Series system (Agilent Technologies Inc., USA), consisting of a G1322A degasser, a G1311A quaternary pump, a G1328A manual injector, a G1316A column thermostat, and a G1946B detector equipped with ESI and APCI sources, with a single quadrupole analyser. The APCI conditions were as follows: fragmentor voltage, 120 V; drying gas, 6.0 mL $min^{-1;}$ drying gas temperature, 350°C; vaporizer temperature, 325°C; nebulizer pressure, 40 psi; capillary voltage, 3.0 kV; corona current, 4 μA; positive polarity.

Identification of the compounds was performed by means of their retention time and UV or MS spectra. Quantification was carried out at 205 nm by the external standard method. All measurements were made using Shidmadzu CLASS-VP Version 5.032 software. The asymmetry factor (As), which is the width from the frontside and the backside of the peak to the apex, was calculated at 10% of the peak height. All results were the mean of at least triplicate injections.

RESULTS AND DISCUSSION

Optimization of the Chromatographic System

It is widely recognized that conventional HPLC methods may be too time consuming for certain applications such as process control.[40] This is the case of the recommended methods for the analysis of sultamicillin and related compounds, which need an analysis time greater than 15 min. For this reason, an alternative should be considered to reduce analysis time, increase laboratory productivity with a reduced number of HPLC instruments, and to reduce operation costs.

As the separation of sultamicillin, its precursors and byproducts does not seem to constitute a very complicated separation problem requiring many theoretical plates, we chose the most economical alternative employing fast liquid chromatography, i.e., narrowbore short columns (50 mm × 2 mm i.d.) packed with 3–3.5 μm octadecylsilane stationary phases. In comparison with other HPLC methods for sultamicillin analysis, which use conventional columns with lengths ranging between 150 and 250 mm, an inner diameter of 4.6 mm and a particle size of 5 μm, the use of short narrowbore columns increases mass sensitivity at least fourfold and reduces the flow rate approximately fivefold, while also increasing the effectiveness per unit of time.

As some analytes are polar in nature, we tested several full end-capped stationary phases in order to obtain the best chromatographic shape considering asymmetry and resolution. Good results were obtained with both Kromasil 100 C_{18} and Tracer Excel 120 ODS-A columns. However, the former was eventually chosen because of its lower price. A particular advantage of high speed columns with low void volumes is the reduced time for re-equilibration after changing the mobile phase. Several column volumes can thus be passed through a column in 1–2 minutes and the column is then ready for the subsequent analysis, ideally to gradient mode. At the same time, the minimum of the van Deempter curve is displaced to a higher flow rate.

The assembly of these high speed columns in a conventional HPLC instrument requires an injection system capable of injecting volumes of

below 3 μL. Furthermore, the use of smaller particle columns, with their inherently higher efficiencies and lower void volumes, imposes additional constraints on the design and construction of injection valves, as extra column band broadening must be minimized if the potential of these columns is to be fully exploited. Therefore, the injector bypass must be constructed so as to eliminate pressure pulses while not substantially contributing additional extra column band broadening. When properly designed and constructed, the injector bypass extends the lifetime of a high speed column from an intolerably short period to a reasonably long one.[39] An injector bypass was constructed using two T-joints, the appropriate capillary tubes and fittings to obtain a division rate of 2.73. The flow cell volume and response time of the detector should be lower than 3 μL and 100 ms, respectively. A 2.5 μL flow cell and 80 ms response time were chosen. The different components of equipment were connected using capillary tubes of 60 cm overall length and 127 μm internal diameter to minimize instrumental bandwidth (30 μL), thus improving the effectiveness of the separation per unit of time.

Optimization of Chromatographic Conditions

The main chromatographic conditions to be optimized for the development of the fast RP-HPLC method were: pH, ionic strength, percentage organic modifier, temperature, and flow rate.

As can be seen in Figure 2, the retention of most of the compounds does not significantly change in the studied range of pH (5.0–7.5), except sultamicillin, which shows a basic behaviour with various changes in the elution order. Hence, it is not advisable to select values of pH close to the crossing points. Although the best separation is obtained at pH 6.0, it is still not complete, seeing that the resolution of iodomethyl sulbactam is lower than 1.5.

The effect of the ionic strength on the capacity factor, tested between 5 and 25 mM, is small, showing the highest resolution for the critical separation peaks (sultamicillin-iodomethyl sulbactam) at 25 mM, with a resolution of 1.3. In consequence, the composition of the mobile phase was also studied more thoroughly in order to achieve complete separation, changing the selectivity without significantly altering the elution strength by adding THF. As can be seen in Figure 3, complete separation is achieved at 1% THF.

Under these conditions, with an isocratic method at 40% acetonitrile, the analysis time is quite time consuming: about 44 minutes when all analytes are considered. Therefore, a gradient mode is required to reduce the analysis time. Before that, however, the solubility of the buffer salt should be taken into account, due to the fact that phosphate salts are

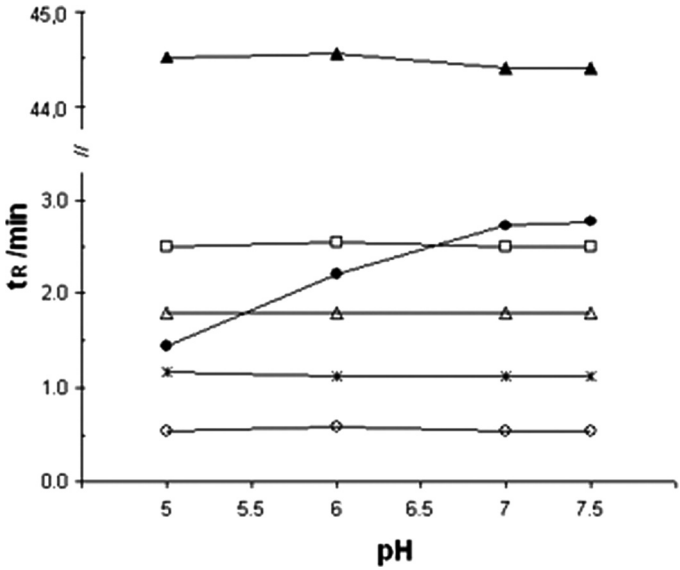

Figure 2. Effect of the mobile phase pH on the retention of sultamicillin and related compounds. Column: 50 mm × 2.1 mm i.d., 3.5 µm, Kromasil 100 C_{18}. Mobile phase: 25 mM KH_2PO_4, adjusted to pH with 1 M KOH, and acetonitrile (40%). Temperature: 30°C. Flow rate: 0.2 mL min^{-1}. Detection at 205 nm. Injected volume: 2 µL. Response time: 80 ms. Peak identification: (○) ampicillin; (×) ampicillin imine; (◇) benzaldehyde; (●) sultamicillin; (□) iodomethyl sulbactam, (▲) sultamicillin imine.

not very soluble in apolar mobile phases. In this study, 25 mM phosphate is only soluble up to 65% of the organic modifier. Hence, it is necessary to encounter an alternative buffer salt in order to achieve higher organic percentages with transparency and solubility at 25 mM without altering selectivity. Potassium dihydrogen phosphate (5 mM) plus 20 mM KCl was found to be appropriate, enabling an analysis time of less than three minutes to be obtained (Figure 4).

Likewise, the effect of temperature on resolution and analysis time is irrelevant up to 30°C. Above this temperature, the resolution falls below 1.5. Accordingly, 25°C was chosen to maintain a good resolution.

The Kromasil 100 C_{18} column shows similar efficiency working with flow rates of between 0.2 and 0.5 mL min^{-1}. Thus, a flow rate of 0.5 mL min^{-1} was chosen with the aim of shortening the analysis time. The pressure drop does not exceed 175 bar. The low flow rate employed is markedly lower than the one used in conventional HPLC methods (about 1 mL min^{-1}), thus notably reducing solvent consumption.

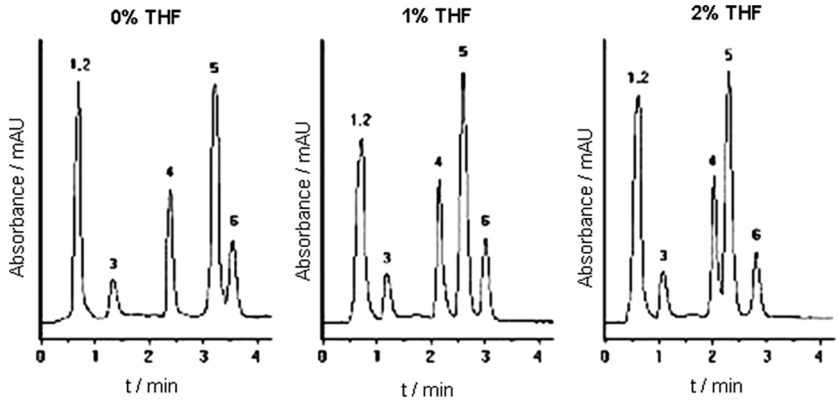

Figure 3. Effect of mobile phase selectivity on the separation of sultamicillin and related compounds. Column: 50 mm × 2.1 mm i.d., 3.5 µm, Kromasil 100 C_{18}. Mobile phase: A/B (60:40); solvent A, 25 mM KH_2PO_4 adjusted to pH 6.0 with 1 M KOH plus % THF; solvent B, acetonitrile plus % THF. Temperature: 25°C. Flow rate: 0.2 mL min^{-1}. Detection at 205 nm. Injected volume: 1 µL. Response time: 80 ms. Peak identification: (1) dimethylformamide; (2) ampicillin; (3) ampicillin imine; (4) benzaldehyde; (5) sultamicillin; (6) iodomethyl sulbactam.

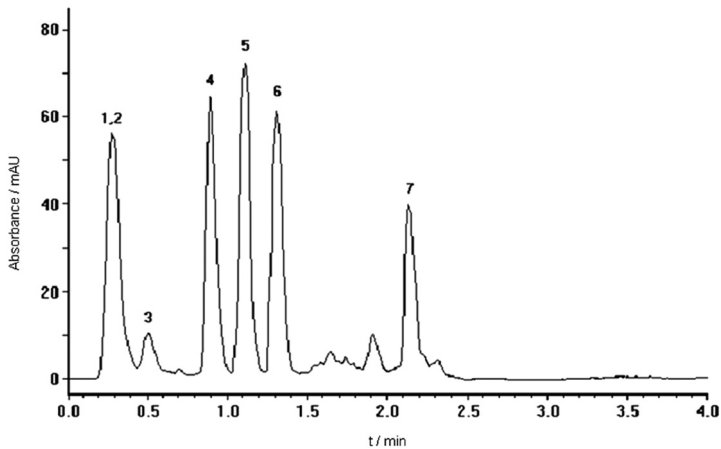

Figure 4. Chromatogram of sultamicillin, its synthesis intermediates and degradation products obtained in the gradient mode. Column: 50 mm × 2.1 mm i.d., 3.5 µm, Kromasil 100 C_{18}. Mobile phase: solvent A, 5 mM KH2PO4 and 20 mM KCl adjusted to pH 6.0 with 1 M KOH plus 1% THF; solvent B, acetonitrile plus 1% THF. Gradient conditions: 0–0.9 min, 40% B; 0.9–1.0 min, 85% B; 1.0–2.5 min, 85% B; 2.5–2.6 min, 40% B; 2.6–4.0 min, 40% B. Temperature: 25°C. Flow rate: 0.5 mL min^{-1}. Detection at 205 nm. Injected volume: 1 µL. Response time: 80 ms. Peak identification: (1) dimethylformamide; (2) ampicillin; (3) ampicillin imine; (4) benzaldehyde; (5) sultamicillin; (6) iodomethyl sulbactam; (7) sultamicillin imine.

The appropriate in-process control of sultamicillin synthesis, according to Asturpharma's route, only requires the monitoring of benzaldehyde, sultamicillin, and iodomethyl sulbactam.

The optimized gradient program begins with 40% of organic modifier and increases quickly to 85% in 1 min, as reported in the Apparatus and Conditions section. Under these conditions, analysis can be carried out in 2.5 min. As can be seen in Figure 4, which shows the chromatogram obtained in accordance with these optimized conditions, adequate resolution (>1.5) and asymmetry (<1.5) were obtained in a short analysis time. Comparing this new method with the one currently used in the pharmaceutical industry,[36] chromatography separation time has been reduced from 24 min to less than 3 min, approximately.

The high speed HPLC method was adapted to mass spectrometry detection with the aim of obtaining more information about sultamicillin synthesis thanks to the study of some unknown impurities that appeared around 1.5–2.0 minutes (See Figure 4). This is why the original gradient mode was slightly modified so as to better separate these unknown peaks, as is shown in Figure 5, where an unknown substance appears after about 3.7 min.

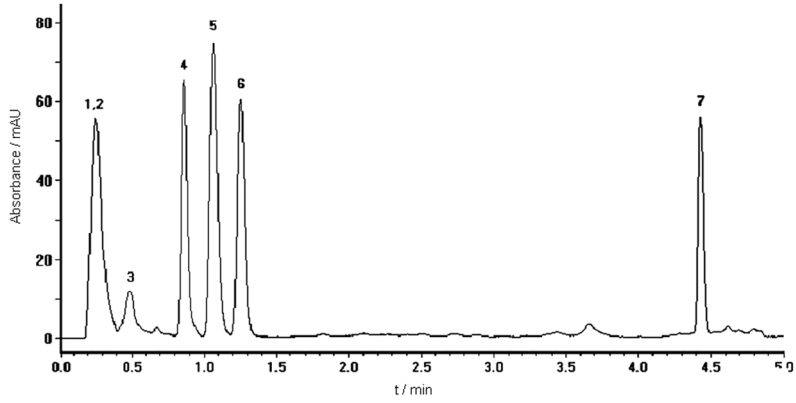

Figure 5. Chromatogram of sultamicillin, its synthesis intermediates and degradation products obtained in the gradient mode. Column: 50 mm × 2.1 mm i.d., 3.5 μm, Kromasil 100 C_{18}. Mobile phase: solvent A, 5 mM KH_2PO_4 and 20 mM KCl adjusted to pH 6.0 with 1 M KOH plus 1% THF; solvent B, acetonitrile plus 1% THF. Gradient conditions: 0–1.0 min, 40% B; 1.0–1.6 min, 50% B; 1.6–3.4 min, 50% B; 3.4–3.6 min, 60% B; 3.6–3.7 min, 85% B; 3.7–5.0 min, 85% B; 5.0–5.1 min, 40% B; 5.1–6.5 min, 40% B. Temperature: 25°C. Flow rate: 0.5 mL min^{-1}. Detection at 205 nm. Injected volume: 1 μL. Response time: 80 ms. Peak identification: (1) dimethylformamide; (2) ampicillin; (3) ampicillin imine; (4) benzaldehyde; (5) sultamicillin; (6) iodomethyl sulbactam; (7) sultamicillin imine.

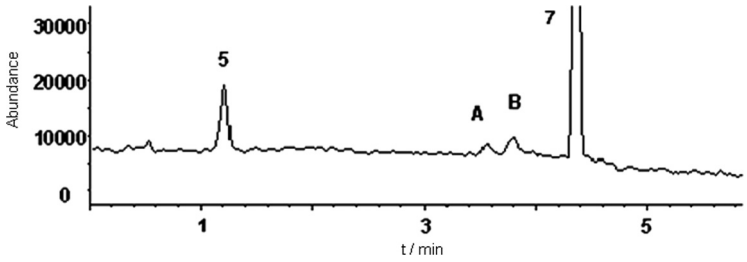

Figure 6. Chromatogram obtained in the HPLC-MS equipment for the separation of sultamicillin and related compounds. Column: 50 mm × 2.1 mm i.d., 3.5 μm, Kromasil 100 C_{18}. Mobile phase: solvent A, 25 mM AcONH4 adjusted to pH 6.0 with 1 M AcOH plus 1% THF; solvent B, acetonitrile plus 1% THF. Gradient conditions: 0 min, 40% B; 0–0.6 min, 50% B; 0.6–2.4 min, 50% B; 2.4–2.6 min, 60% B; 2.6–2.7 min, 85% B; 2.7–4.0 min, 85% B; 4.0–4.1 min, 40% B; 4.1–5.5 min, 40% B. Temperature: 25°C. Flow rate: 0.5 mL min^{-1}. Detection at 205 nm. Injected volume: 1 μL. Response time: 100 ms. The APCI conditions: fragmentor voltage, 120 V; drying gas, 6.0 mL min^{-1}; drying gas temperature, 350°C; vaporizer temperature, 325°C; nebulizer pressure, 40 psi; capillary voltage, 3.0 kV; corona current, 4 μA; positive polarity. Peak identification: (5) sultamicillin; (A and B) isomeric forms of sultamicillin imine; (7) sultamicillin imine.

The only modification needed was the substitution of the buffer salt by ammonium acetate, which is compatible with the ionization source and does not alter the chromatographic separation. As can be seen in Figure 6, a different ionisation efficacy was obtained for all analytes, but it was enough to monitor two unknown impurities together with sultamicillin and its imine. Mass spectra of these peaks show that the unknown peaks A and B are isomeric forms of the sultamicillin imine.

Validation

Calibration curves were constructed via the analysis of triplicates of seven points in the range from 1.2 to 60 mg l^{-1} of benzaldehyde, from 6 to 300 mg l^{-1} of sultamicillin, and 12 to 600 mg l^{-1} of iodomethyl sulbactam. The calibration curves for all the compounds showed a good fitting to a linear model between the peak areas and analyte concentrations, with regression coefficients >0.999 in all cases. The linearity of the calibration curves was also checked with two different statistical tests: linearity and proportionality. For the former test, the linearity of the method was confirmed by showing that the response factor RSD and slope RSD values were lower than 5% and 2%, respectively. The values obtained from the Fisher test (Analysis of Variance, ANOVA) were always lower than

Table 1. Calibration parameters for linearity

		Specification	Benzaldehyde 1.2–60 (mg L^{-1})	Sultamicillin 6–300 (mg L^{-1})	Iodomethyl sulbactam 12–600 (mg L^{-1})
Correlation coefficient		≥0.997	0.9999	0.9998	0.9997
Standard error			5.3	7.1	7.8
Linearity test	Response factor RSD	≤5%	4.7	4.5	4.9
	Slope		22.46	4.17	1.581
	Slope SD		0.06	0.02	0.009
	Slope RSD	≤2%	0.3	0.4	0.5
	Confidence interval	0 not included	22.33–22.59	4.14–4.20	1.56–1.60
	Experimental t	texp > ttab*	369.720	263.565	184.407
	ANOVA	Fexp < Ftab*	1.218	0.553	0.555
Proportionality test	Intercept		2.4	5.3	0.8
	Intercept SD		1.7	2.7	2.8
	Confidence interval	0 included	−1.2–6.0	−0.3–10.9	−5.1–6.7
	Experimental t	texp < ttab	1.409	1.978	0.297

*ttab = 2.093 and Ftab = 2.958.

Table 2. Analytical characteristics of the chromatographic method

	Specification (%)	Benzaldehyde	Sultamicillin	Iodomethyl sulbactam
Instrumental repeatability	≤2	1.9	1.7	1.6
Intermediate precision	≤5	3.3	3.8	3.7
Limit of detection ($mg\ l^{-1}$)		0.1	1.1	1.1
Limit of quantification ($mg\ l^{-1}$)		0.2	1.5	1.6

the tabulated ones ($\alpha = 0.05$). Finally, the slopes of the linear calibration curves were statistically different from 0 ($texp > ttab$, $\alpha = 0.05$). In the proportionality test, it was demonstrated that the intercept was not statistically different from 0, as the confidence limits include zero and the calculated Student's t values were always lower than the tabulated values for the same level of significance. This indicates the absence of systematic error, linearity thus being demonstrated.[41] The calibration data are summarized in Table 1.

The precision of the method was assessed by expressing the relative standard deviation of several repeated measurements (Table 2). Instrumental repeatability was estimated from six replicates at three concentrations, low, medium, and high level within the linear range: 6, 30, and 60 $mg\,l^{-1}$ for benzaldehyde, 30, 150, and 300 $mg\,l^{-1}$ for sultamicillin, and 60, 300, and 600 $mg\,l^{-1}$ for iodomethyl sulbactam, obtaining values (1.6–1.9%) below the acceptance criterion (≤2%). The estimation of repeatability was performed during three hours. The compounds were stable and showed no significant difference in the peak area after this time. Intermediate

Table 3. Validation parameters for accuracy

		Compounds								
		Benzaldehyde			Sultamicillin			Iodomethyl Sulbactam		
Range ($mg\ l^{-1}$)	Specification	6	30	60	30	150	300	60	300	600
Recovery (%)	97–103	98.4	98.3	101.4	101.4	101.8	99.3	100.6	101.1	100.0
texp	texp < ttab*	1.0338			2.0095			2.1766		
Gexp	Gexp < Gtab*	0.7130			0.6928			0.4650		

*ttab = 2.3060 and Gtab = 0.8709.

precision was determined by comparing the results obtained from the analysis of freshly prepared samples on two separate days. The results, ranging between 3.3% and 3.8%, were also below the acceptance criterion ($\leq 5\%$). Hence, acceptable precision was obtained for all preparations.

The detection and quantification limits are shown in the aforementioned Table 2. These were determined by ten repeated measures of the blank, followed by the preparation of calibration curves (peak height versus concentration) in the same range as the one mentioned above for each analyte. Detection and quantification limits were within the range of 0.1–1.1 mg l^{-1} and 0.2–1.6 mg l^{-1}, respectively.

Recovery experiments were performed to study the accuracy of the method. A mixture of known concentrations of these substances was prepared and analyzed at low, medium, and high calibration ranges by this method on the same day. All analyses were carried out in triplicate. The average recoveries obtained ranged between 98.3% and 101.8%. It was also proven, by means realization of a Student's t test ($texp < ttab$, $\alpha = 0.05$), that a significant difference does not exist between the average recovery and 100%. Furthermore, carrying out Cochran's G test ($Gexp < Gtab$, $\alpha = 0.05$), it was demonstrated that the factor concentration does not influence the variability of the results. All these results thus testify to the accuracy of the proposed method (Table 3).

CONCLUSIONS

This newly developed high speed chromatographic method has been found to be effective for rapid in-process control of sultamicillin synthesis. Compared to a conventional column, separation was performed in approximately 70% less analysis time, thus dramatically reducing solvent consumption and cost per analysis. High speed columns can be satisfactorily assembled to a conventional HPLC instrument with few modifications. They offer great potential for replacing larger conventional columns for in-process and quality control in the pharmaceutical industry.

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