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An improved HPLC-DAD method for clavulanic acid quantification in fermentation broths of *Streptomyces clavuligerus*

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ABSTRACT

Clavulanic acid (CA) is an important secondary metabolite commercially produced by cultivation of *Streptomyces clavuligerus* (*Sc*). It is a potent inhibitor of bacterial β-lactamases. In this work, a specific and improved high performance liquid chromatography (HPLC) method, using a C-18 reversed phase column, diode array detector and gradient elution for CA quantification in fermentation broths of *Sc*, was developed and successfully validated. Samples were imidazole-derivatized for the purpose of creating a stable chromophore (clavulanate–imidazole). The calibration curve was linear over a typical range of CA concentration between 0.2 and 400 mg/L. The detection and quantification limits were 0.01 and 0.02 mg/L, respectively. The precision of the method was evaluated for CA spiked into production media and a recovery of 103.8%, on average, was obtained. The clavulanate–imidazole complex was not stable when the samples were not cooled during the analysis. The recovery rate was 39.3% on average. This assay was successfully tested for CA quantification in samples from *Sc* fermentation, using both, a chemically defined and a complex medium.

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1. Introduction

Clavulanic acid (CA) is produced by *Streptomyces clavuligerus* (*Sc*). It is an important metabolite that inhibits β-lactamase enzyme activity, secreted by bacteria [1–3]. CA is frequently used in antibacterial formulations, e.g. Augmentin®, for the treatment/eradication of infections caused by β-lactamase-producing bacteria [3,4].

Bioassay, spectrophotometry and high performance liquid chromatography (HPLC) methods have been developed and are widely used for CA quantification [5–10]. Early developed HPLC methods for the detection of CA applied a pre-column derivatization step, as CA has a low detection wavelength in the UV region and is poorly retained on C-18 reverse phase columns [6,11,12]. Martín and Méndez [11] and Shah et al. [12] used 1,2,4-triazole as a derivatizing reagent for the determination of CA, and CA together with sublactam in human serum and urine, respectively. Foulstone and Reading's work [6] described the use of imidazole. Later developed HPLC methods used different sample preparations without a pre-column derivatization step [13–15]. Foroutan et al. [13] and Hoizey et al. [15] reported an HPLC-UV method for the simultaneous quantification of CA and amoxicillin in human plasma, with an internal standard and a direct deproteinization of plasma with methanol, respectively. Recently, methods with high selectivity and sensitivity, such as liquid chromatography–mass spectrometry (LC–MS), have been reported for CA determination in biological samples [8–10,16,17]. Dubala et al. [8] described an LC–MS method based on solid phase extraction for the simultaneous determination of CA and cefixime in human plasma using chloramphenicol as internal standard. However, for all the above mentioned methods, that use either isocratic or gradient elution, CA is simultaneously quantified with another antibiotic, such as amoxicillin, in human samples after oral administration [8–10,15].

Aside from all these methods, also an LC–MS and LC–MS/MS method was developed for detection of CA and clavam-2-carboxylate with an imidazole pre-column derivatization step [18]. In that work, the derivatization step was crucial for the chromatographic separation of both β-lactam compounds. It also improved the detection of the ions in the MS, yet no biological samples were analyzed. Moreover, due to the absence of a specific method for CA quantification in fermentation processes, CA is often quanti-

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fied by using the Foulstone and Reading's method in isocratic mode [6]; however, the appearance of different β-lactam compounds – structurally similar to CA – during CA biosynthesis by *Sc,* creates overlapping peaks thus leading to poor efficiency of the method. The gradient elution proposed in this work substantially prevents peak interference thus improving the versatility and efficiency of the method, especially when complex matrix samples, such as fermentation broth, are used.

Here we describe a specific and improved gradient based HPLC method for CA quantification with an imidazole pre-column derivatization step, to be applied for the analysis of fermentation broths.

2. Experimental

2.1. Chemicals and preparation of standard solutions

All reagents and solvents were of analytical and HPLC grade. Potassium clavulanate vetranal, potassium dihydrogen phosphate, sodium dihydrogen phosphate monohydrate (all supplied by Sigma–Aldrich, Seelze, Germany), imidazole (Carl Roth, Karlsruhe, Germany) and methanol HPLC grade (VWR, Darmstadt, Germany) were used throughout this work. 18.2 MΩ ultra pure water was supplied by a Barnstead EASY pure II water purification system.

A Stock solution of CA was freshly prepared at 800 mg/L in a NaH_2PO_4 buffer (pH 6.8; 100 mM). The solution was stored at 3–4 °C and remained stable for at least three weeks.

2.2. Derivatization procedure

CA is poorly retained in C-18 reverse phase columns and does not produce distinctive peaks. Therefore, a derivatization procedure was implemented with the aim of creating a stable chromophore (clavulanate–imidazole) (Fig. 1). Therefore 8.25 g of imidazole were dissolved in 24 mL of distilled water; HCl (25% v/v) was used to adjust the pH to 6.8 and distilled water was added up to 40 mL as the final volume. The derivatization was performed as described by Foulstone and Reading [6], wherein 100 μL of imidazole reagent were added to 300 μL of sample and maintained in mixing block at 800 rpm, 30 °C for 30 min.

2.3. Chromatographic instrumentation and conditions

Samples were derivatized with imidazole as described above and analyzed in an Agilent Technologies 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) operated at 30 °C with a Zorbax Eclipse XDB-C-18 chromatographic column (Agilent Technologies, Waldbronn, Germany), with a C-18 guard column (Phenomenex®, Aschaffenburg, Germany), with a flow rate of 1 mL/min, injection volume of 25 μL, an auto-sampler at 4 °C and a DAD detector. The mobile phase consisted of a KH_2PO_4 buffer (pH 3.2; 50 mM) as solvent A and HPLC grade methanol as solvent B. The gradient mode was set as follows: linear gradient from 6% to 7.6% solvent B for 8 min, linear gradient to 95% solvent B for 2 min, 95% solvent B for 2 min and linear gradient to 6% solvent B for 2 min. Solvent A was filtered through a 0.45 μL membrane filter. The clavulanate–imidazole complex was detected at a wavelength of 311 nm.

2.4. Biological sample preparation

S. clavuligerus DSM No 41826 was grown at 28 °C in a seed and production medium described by Roubos et al. [19]. The seed medium had the following composition (in g/L): glycerol 15, soy peptone 15, sodium chloride 3, calcium carbonate 1 and an initial pH-value of 6.8. Antifoam 204 (Sigma Inc., St. Louis, MO) was added at a concentration of 1:1000 v/v.

The production medium was slightly modified as follows (in g/L): glycerol 9.3, K_2HPO_4 0.8, $(NH_4)_2SO_4$ 1.26, monosodium glutamate 9.8, $FeSO_4 \times 7H_2O$ 0.18, $MgSO_4 \times 7H_2O$ 0.72, MOPS 10.5 and trace element solution 1.44 mL. The trace element solution remained (in g/L): H_2SO_4 (96%) 20.4, citrate × $1H_2O$ 50, $ZnSO_4 \times 7H_2O$ 16.75, $CuSO_4 \times 5H_2O$ 2.5, $MnCl_2 \times 4H_2O$ 1.5, H_3BO_3 2 and $Na_2MoO_4 \times 2H_2O$ 2. Antifoam 204 was added into the production medium (1:1000 v/v). During fermentation, aliquots (2 mL) were withdrawn at an interval of approx. 12 h. Then, samples were centrifuged at 15,000 rpm and 4 °C for 10 min; supernatants were used for CA quantification either immediately or stored at −20 °C until use.

For the purpose of validating the versatility of the method reported in this work, two additional culture media were evaluated. The first one had the following composition (in g/L): glycerol 15, monosodium glutamate 12, K_2HPO_4 0.8, $MgSO_4 \times 7H_2O$ 0.72 and salt solution 1 mL. The composition of salt solution was (in g/L): $MnCl_2 \times 4H_2O$ 0.001, $FeSO_4 \times 7H_2O$ 0.001, $ZnSO_4 \times 7H_2O$ 0.001. The second medium tested was the one described by Rosa et al. [20] with the following composition (in g/L): glycerol 15, bacto peptone 10, malt extract 10, yeast extract 1, K_2HPO_4 2.5, $MnCl_2 \times 4H_2O$ 0.001, $FeSO_4 \times 7H_2O$ 0.001, $ZnSO_4 \times 7H_2O$ 0.001 and MOPS buffer 21 (100 mM). All culture media were fixed at pH 6.8.

2.5. Method validation

The HPLC-DAD method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision and recovery, following the International Conference on Harmonization (ICH) guidelines [21]. For linearity validation, six CA concentrations (0.2, 2, 20, 100, 200 and 400 mg/L), were tested and a calibration curve was prepared by plotting peak area versus concentration. The LOD and LOQ were determined using the signal-to-noise (S/N) approach. For LOD the signal-to-noise ratio was 3 (S/N = 3:1) while for LOQ a signal-to-noise ratio of 10 was used (S/N = 10:1).

The precision of the method was determined by examining the intraday and interday variation. For this, six replicas of CA spiked into production medium (see biological sample preparation) at three concentration levels (400, 200 and 0.2 mg/L of CA) were analyzed, in a single day and on three consecutive days. The recovery

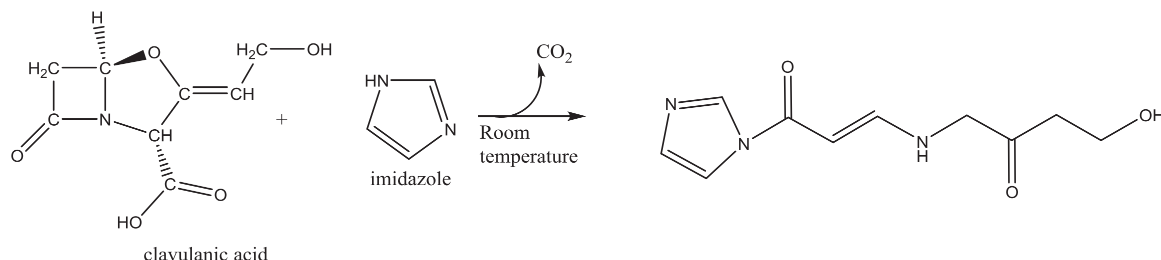

Fig. 1. Derivatization of clavulanic acid with imidazole.

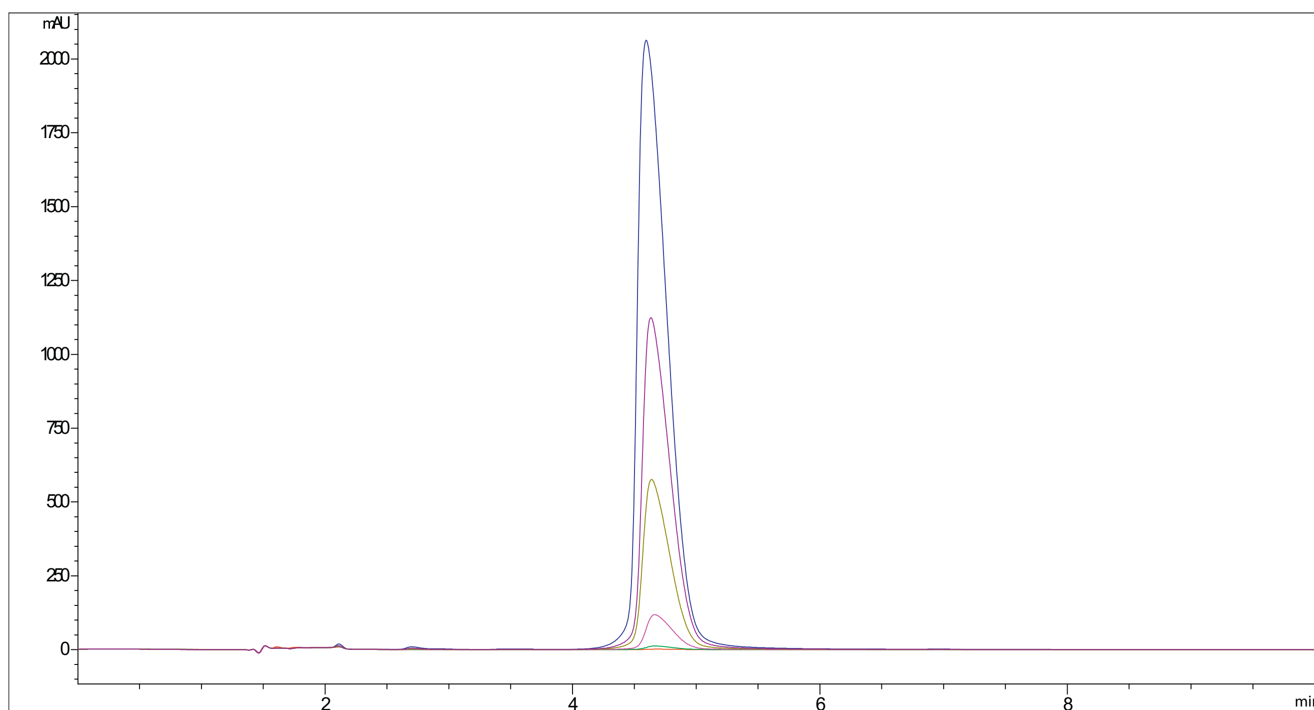

Fig. 2. Representative chromatograms of the clavulanate–imidazole complex, at six different clavulanic acid concentrations (0.2, 2, 20, 100, 200 and 400 mg/L).

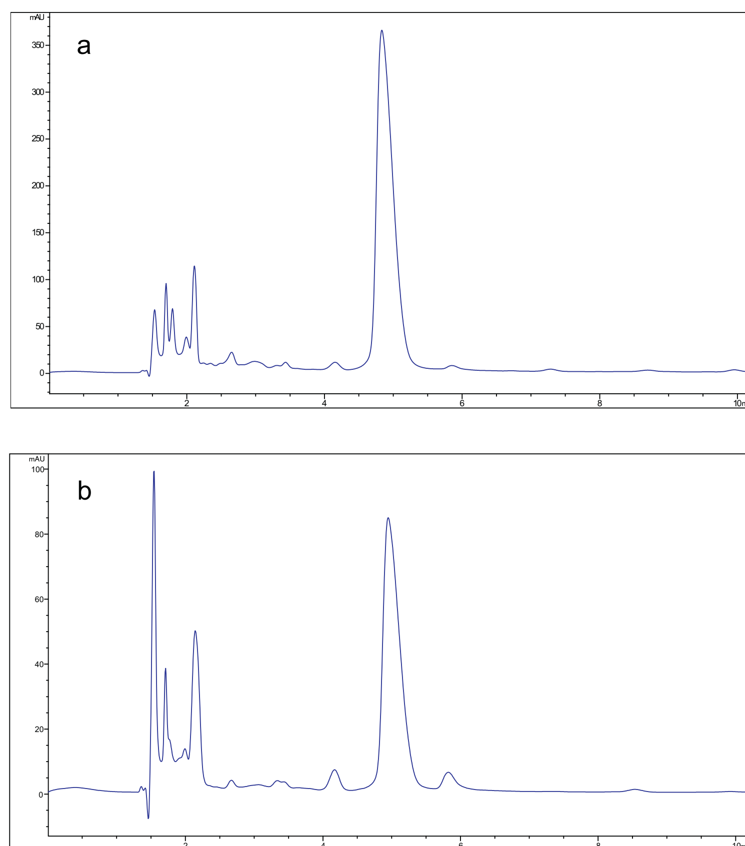

Fig. 3. Representative chromatograms of the clavulanate–imidazole complex taken from fermentation samples of *Streptomyces clavuligerus;* (a) using a complex medium; (b) using a chemically defined medium. *Note*: The complex medium used was the seed medium described by Rosa et al. [20].

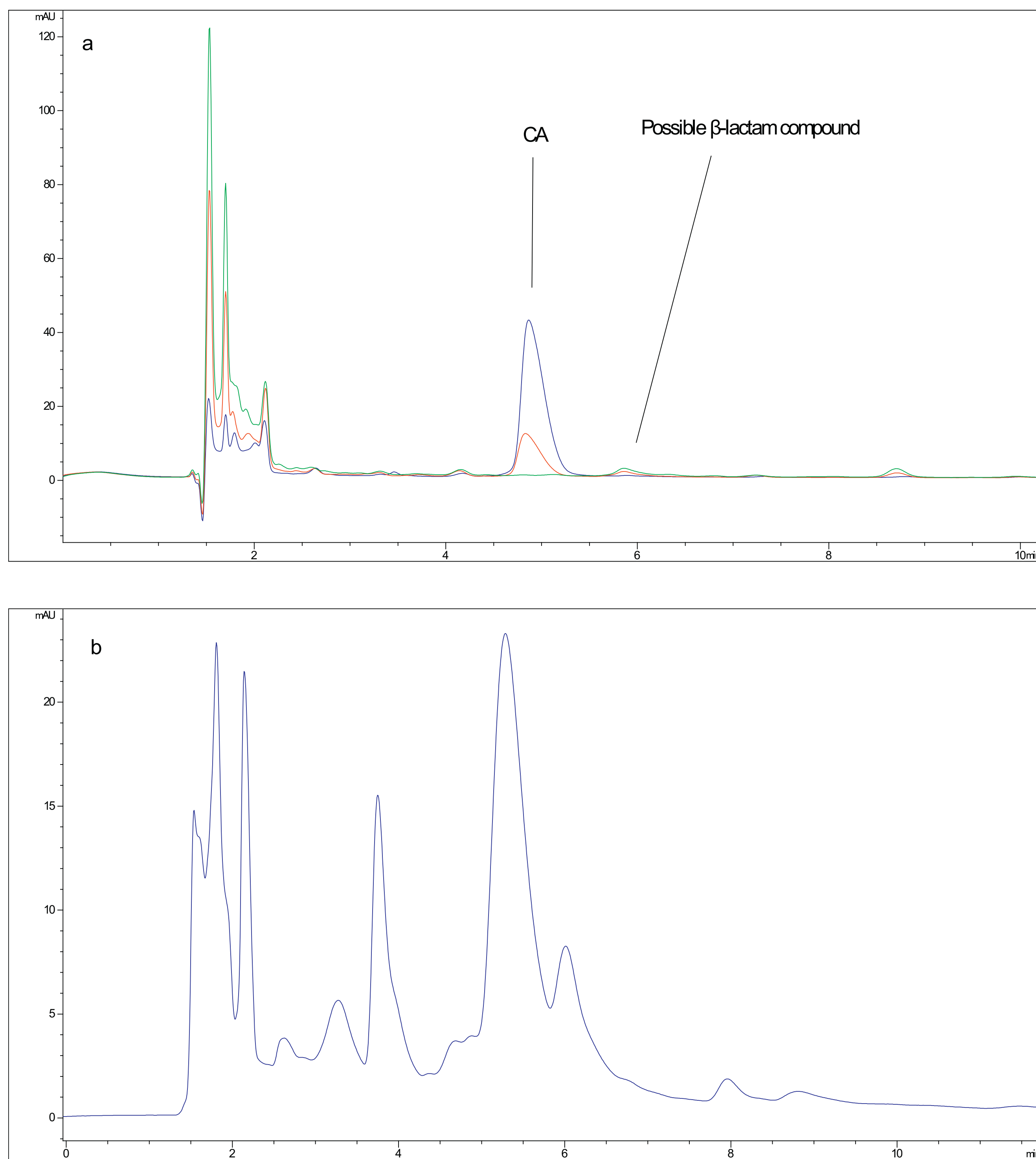

Fig. 4. Differences in clavulanic acid peak resolution acquired while using either isocratic or gradient approaches. (a) Representative chromatograms for fermentation samples wherein clavulanic acid was degraded and/or converted into another unknown compound. Color Code: The sequence of samples is: blue, red and green at 0, 29 and 53 h of fermentation time, respectively. Here, clavulanic acid was produced in a complex medium; then, this broth was used as inoculum in a production medium with the following composition (in g/L): glycerol 15, monosodium glutamate 12, K_2HPO_4 0.8, $MgSO_4 \times 7H_2O$ 0.72, salt solution 1 mL: $MnCl_2 \times 4H_2O$ 0.001, $FeSO_4 \times 7H_2O$ 0.001, $ZnSO_4 \times 7H_2O$ 0.001. (b) Poor separation of the clavulanic acid peak in isocratic mode. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the method was examined by comparison of the areas below the curves of a known amount of CA, spiked to the production medium and its equivalent areas in the standard, determined at three concentration levels (400, 200 and 0.2 mg/L of CA).

3. Results and discussion

3.1. Chromatography

Representative chromatograms for CA were found at a retention time of 4.65 min for the standard solution (Fig. 2). A CA peak

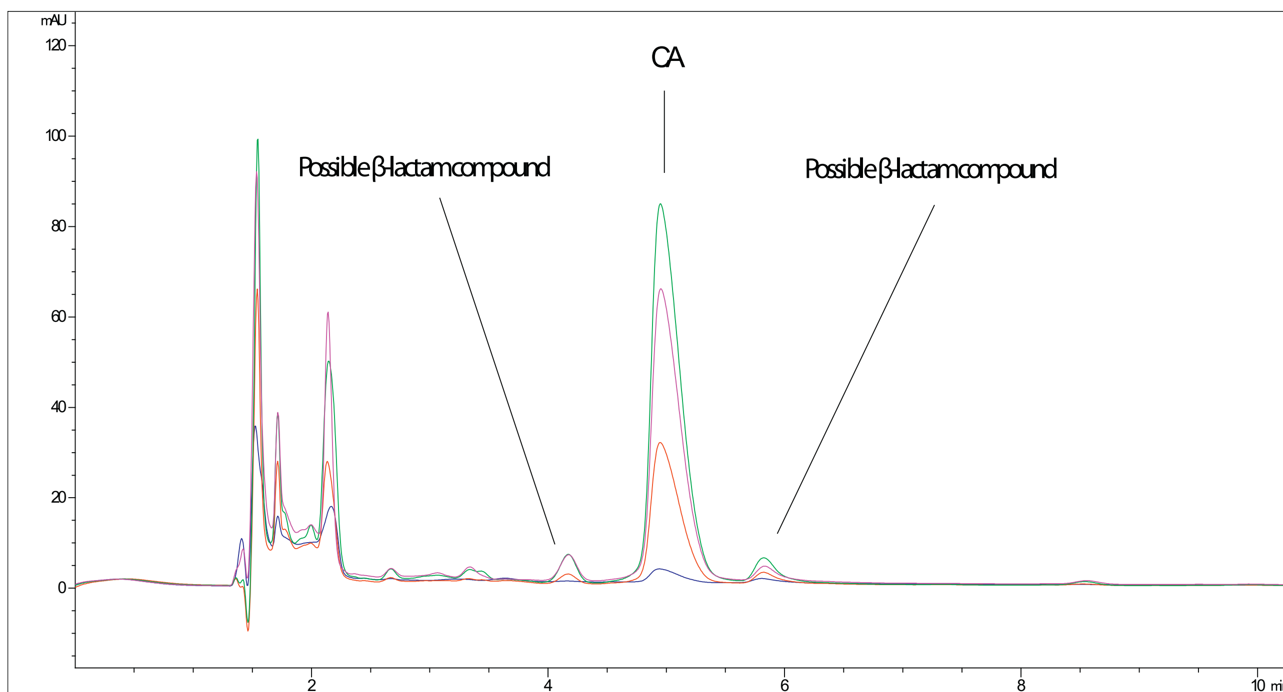

Fig. 5. Chromatographic profiles for clavulanic acid during *Streptomyces clavuligerus* cultivation. Color Code: The sequence of samples is: blue, red, green and pink at 0, 24.4, 36 and 57 h of fermentation time, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Quality parameters for low, medium and high CA concentrations in a fermentative medium.

Linearity				CA concentration added into	Precision RSD (%)		Recovery	
Equation	Range (mg/L)	LOD (mg/L)	LOQ (mg/L)	the production medium (mg/L)	Intraday (n = 6)	Interday (n = 18)	Mean (%)	RSD (%)
$y = 86.73x + 155.86$	0.2–400	0.01	0.02	0.2	4.7	5.2	94.7	4.5
				200	1.9	8.5	100.2	9.5
				400	2.3	7.8	116.6	7.9

Table 2
Comparison between the HPLC-DAD method and other quantitative methods reported in literature.

Method	LOD (mg/L)	Applicability	Interference
Spectrophotometric assay [5]	2	CA quantification in biological samples.	CA and other β-lactam compounds give strongly absorbing products under the assay condition
HPLC-UV (isocratic) [6]	0.1	Applied for Augmentin quantification in body fluids. Currently, the method is also used for CA quantification in biological samples	The appearance of additional β-lactam compounds during CA production by *Sc*, creates overlapping peaks causing poor/low efficiency
HPLC-UV (gradient) [15]	0.08	Applied to a pilot pharmacokinetic study in healthy volunteers after a single-oral administration of amoxicillin/CA combination (500/125 mg)	CA stability is reduced in methanol
LC–MS [10]	0.02	Applied to the analysis of amoxicillin and CA in human plasma. The method was tested in clinical studies	The internal standard used is not structurally related to CA
LC–MS [8]	0.1 (LOQ)	Applied for the analysis of plasma obtained after oral administration of cefixime/CA combination (200/125 mg)	The internal standard used is not structurally related to CA
This method, HPLC-DAD (gradient)	0.01	CA quantification in samples from *Sc* fermentation broths	None

was clearly resolved and no interfering peaks were observed neither using a chemically defined medium nor a complex medium (Fig. 3). Complex media and chemically defined media are used in microbial cultures for the production of larger CA titers; yet, after certain fermentation time has elapsed, CA is either degraded and/or converted into another unknown compound [19,22]. Also, in our case, possible additional β-lactam compounds are produced having within their chemical structure *3S, 5S* stereochemistry (Fig. 4a). These compounds are not properly separated in isocratic mode (Fig. 4b).

In this work, a CA peak was observed at a retention time of 4.95 min in samples from fermentation broths of *Sc* (Fig. 5). Besides, two additional close peaks were found, presumably also β-lactam compounds. These peaks did not overlap with the CA peak, probably due to peak capacity which is higher in gradient than in the isocratic mode [23]. As a result, a good separation of all peaks present in complex samples was obtained compared to the prior isocratic elution method where the peaks interfered with each other. The gradient elution configuration proposed in this paper avoids such an undesirable circumstance, providing versatility to the method.

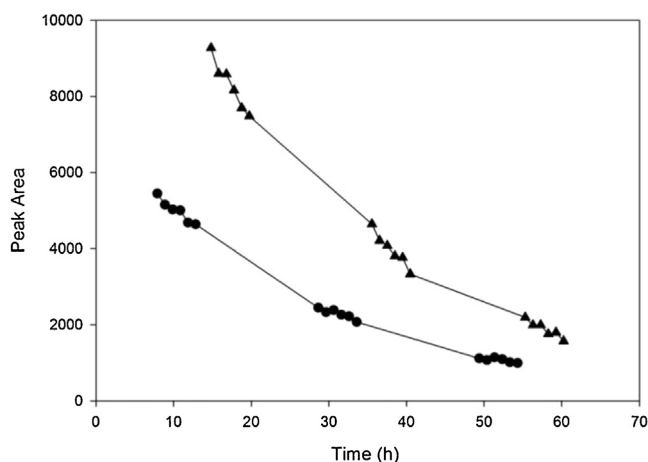

Fig. 6. Peak area profiles of clavulanate–imidazole complex at 200 (-●-) and 400 (-▲-) mg/L of clavulanic acid, on three consecutive days.

This is especially appropriate for samples of complex matrices such as fermentation broths (Fig. 5).

3.2. Method validation

3.2.1. Linearity, limit of detection and limit of quantification

In order to determine the linearity of the method, six points (0.2, 2, 20, 100, 200 and 400 mg/L for CA) were processed separately and analyzed in triplicate. The regression analysis was determined by the least squares method and the regression estimates were evaluated in a confidence interval of 95%. For CA quantification, the calibration curve was linear in a CA concentration range of 0.2 to 400 mg/L. A good linear relationship was found, as described by the following linear equation:, where y is the peak area of CA and x is the CA concentration in mg/L; the standard typical errors are denoted in parenthesis. The relative standard deviation (RSD) of (y/x) was ≤4.3% for the analysis (n = 18) in 6 levels of standard solution of CA.

The LOD and LOQ were 0.01 and 0.02 mg/L, respectively. The summary of quality parameters is presented in Table 1.

3.2.2. Precision and recovery

The intraday precision at the different levels of CA concentration showed a %RSD between 1.9% and 4.7% while the interday precision had a %RSD between 5.2% and 8.5%. These results are within the ICH criteria, where the %RSD should be lower than 20% and 15% for the low and high concentrations, respectively [21]. The mean recovery of the CA spiked into the production medium was between 94.7% and 116.6%. Besides, the recovery percentages had a %RSD in a range of 4.5% and 9.5%. Bearing in mind all the above-mentioned results one can conclude that the method is reliable, including precision and accuracy.

3.2.3. Clavulanate–imidazole complex stability

Owing to an experimentally observed CA degradation during fermentation processes [19], we wanted to further explore the clavulanate–imidazole complex stability, during the quantification step. For this, the same levels and equal number of replicas as in Section 3.2.2 were evaluated. A fresh 800 mg/L CA stock solution was prepared at in NaH_2PO_4 buffer (pH 6.8; 100 mM) and kept at room temperature for a day. Next, the solution was stored at 3–4 °C for a week. Afterwards, aliquots of this solution were subjected to sample preparation procedures as in Section 2.2. The clavulanate–imidazole complex was quantified by HPLC-DAD as described in Section 2.3, during three consecutive days, using an uncooled auto-sampler carousel.

The clavulanate–imidazole complex was shown to be unstable; a clear reduction of the peak area was observed (Fig. 6). The intraday stability had a %RSD, between 4.6% and 7.9%, while the interday stability showed a %RSD between 4.4% and 61%. The recovery of the CA spiked into the production medium was between 88.6% and 13.6%. Likewise, the CA stock solution was unstable at room temperature, since the peak areas at medium and high CA concentration levels were considerably lower than those compared with their equivalent areas, when the solution was freshly prepared. A decline in the CA concentration of 46.9% every 20.77 h, on average, was observed at 200 and 400 mg/L of CA (Fig. 6). The half-life times, $t_{1/2}$ at low and high CA concentrations were 17.29 h and 19.58 h at 200 and 400 mg/L of CA, respectively; thus, the rate of clavulanate–imidazole complex degradation appeared rather constant.

These results indicate that for an accurate quantification of CA, the clavulanate–imidazole complex samples must remain cooled at all time, during its quantification process.

3.2.4. Features of the improved method

The clavam pathway produces structurally similar compounds whose main difference relays on their stereochemistry [3]. CA, with a stereochemistry (*3R, 5R*), is a potent inhibitor of β-lactamase enzymes secreted by a wide spectrum of Gram-positive and Gram-negative bacteria; in contrast, a fraction of the clavam pathway, the so-called 5S clavam, synthesizes compounds – without β-lactamase inhibitory activity – which have a *5S* stereochemistry. Interestingly, while CA acquires a high concentration, the 5S clavam pathway decreases its metabolic flux, and vice-versa.

Due to an unbalanced production of compounds with stereochemistry *R* and *S*, and because of the demonstrated CA instability, the isocratic method is not able to properly separate the diverse clavam compounds. In contrast, in this work, the proposed gradient elution configuration avoids overlapping of new peaks with the CA peak, a clear advantage over the isocratic mode (see Fig. 4a,b).

Table 2 summarizes and compares five different HPLC methods, considering LOD and interference. In addition to a lower LOD, our method provides versatility in using either chemically defined media or complex media, during a fermentation process. Furthermore, our method avoids overlapping peaks when β-lactam compounds are produced, as it is observed during CA production.

4. Conclusion

HPLC methods for simultaneous quantification of CA and other antibiotics, using either isocratic or gradient elution, have been widely used. To the best of our knowledge, gradient elution methods for CA quantification in samples from *Sc* fermentation, both, using a chemically defined medium and a complex medium with a C-18 reversed phase column and DAD, have not been reported. This work demonstrates its applicability to resolve clavam overlapping peaks. The gradient elution configuration proposed in this work avoids overlapping of new peaks (possibly β-lactam compounds with *3S, 5S* stereochemistry) with the CA peak, a case commonly found in isocratic elution. This method was proven to be precise and accurate for the working range. The method was also tested using different complex and chemically defined media, and in each case, the CA peak was clearly resolved without interferences and/or overlapping peaks. Additionally, we demonstrate that due to its instability, the clavulanate–imidazole complex samples must remain cooled at all times during its quantification to obtain reliable data.

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References

[1] A. Brown, D. Butterworth, M. Cole, G. Hanscomb, J. Hood, C. Reading, et al., Naturally occurring β-lactamase inhibitors with actibacterial activity, J. Antibiot. (Tokyo) 29 (1976) 668–669.
[2] L.I. Llarrull, S.A. Testero, J.F. Fisher, S. Mobashery, The future of the β-lactams, Curr. Opin. Microbiol. 13 (2010) 551–557, http://dx.doi.org/10.1016/j.mib.2010.09.008.
[3] G. Ozcengiz, A.L. Demain, Recent advances in the biosynthesis of penicillins, cephalosporins and clavams and its regulation, Biotechnol. Adv. 31 (2013) 287––311, http://dx.doi.org/10.1016/j.biotechadv.2012.12.001.
[4] R.P. Elander, Industrial production of β-lactam antibiotics, Appl. Microbiol. Biotechnol. 61 (2003) 385––392, http://dx.doi.org/10.1007/s00253-003-1274-y.
[5] A.E. Bird, J.M. Bellis, B.C. Gasson, Spectrophotometric assay of clavulanic acid by reaction with imidazole, Analyst 107 (1982) 1241–1245.
[6] M. Foulstone, C. Reading, Assay of amoxicillin and clavulanic acid, the components of Augmentin, in biological fluids with high-performance liquid chromatography, Antimicrob. Agents Chemother. 22 (1982) 753–762.
[7] P. Liras, J.F. Martín, Assay methods for detection and quantification of antimicrobial metabolites produced by *Streptomyces clavuligerus*, Method Biotechnol. 18 (2005) 149–163.
[8] A. Dubala, J.S.K. Nagarajan, C.S. Vimal, R. George, Simultaneous liquid chromatography–mass spectrometry quantification of cefixime and clavulanic acid in human plasma, J. Chromatogr. Sci. 53 (2015) 694––701, http://dx.doi.org/10.1093/chromsci/bmu106.
[9] F. Meng, X. Chen, Y. Zeng, D. Zhong, Sensitive liquid chromatography–tandem mass spectrometry method for the determination of cefixime in human plasma: application to a pharmacokinetic study, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 819 (2005) 277––282, http://dx.doi.org/10.1016/j.jchromb.2005.02.015.
[10] K.-H. Yoon, S.-Y. Lee, W. Kim, J.-S. Park, H.-J. Kim, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC–ESI mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 813 (2004) 121–127, http://dx.doi.org/10.1016/j.jchromb.2004.09.018.
[11] J. Martín, R. Méndez, High-performance liquid chromatographic determination of clavulanic acid in human serum and urine using a pre-column reaction with 1,2,4-triazole, J. Liq. Chromatogr. 11 (1988) 1697––1705, http://dx.doi.org/10.1080/01483918808076730.
[12] A.J. Shah, M.W. Adlard, J.D. Stride, A sensitive assay for clavulanic acid and sulbactam in biological fluids by high-performance liquid chromatography and precolumn derivatization, J. Pharm. Biomed. Anal. 8 (1990) 437––443.
[13] S.M. Foroutan, A. Zarghi, A. Shafaati, A. Khoddam, H. Movahed, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by isocratic reversed-phase HPLC using UV detection, J. Pharm. Biomed. Anal. 45 (2007) 531––534, http://dx.doi.org/10.1016/j.jpba.2007.06.019.
[14] T. Tsou, J. Wu, C. Young, T. Wang, Simultaneous determination of amoxycillin and clavulanic acid in pharmaceutical products by HPLC with β-cyclodextrin stationary phase, J. Pharm. Biomed. Anal. 15 (1997) 1197–1205.
[15] G. Hoizey, D. Lamiable, C. Frances, T. Trenque, M. Kaltenbach, J. Denis, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC with UV detection, J. Pharm. Biomed. Anal. 30 (2002) 661––666.
[16] T. Reyns, S. De Baere, S. Croubels, P. De Backer, Determination of clavulanic acid in calf plasma by liquid chromatography tandem mass spectrometry, J. Mass Spectrom. 41 (2006) 1414––1420, 10.1002/jms.
[17] A. Dubala, J.S.K. Nagarajan, C.S. Vimal, R. George, Simultaneous quantification of cefpodoxime proxetil and clavulanic acid in human plasma by LC–MS using solid phase extraction with application to pharmacokinetic studies, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 921–922 (2013) 49–55, http://dx.doi.org/10.1016/j.jchromb.2013.01.018.
[18] C. Eckers, R. Chalkleya, N. Haskinsa, J. Edwardsb, S. Elsonc, Investigation into the use of derivatization with imidazole for the detection of clavam compounds by Liquid Chromatography–Mass Spectrometry, Anal. Commun. 33 (1996) 215–218.
[19] J.A. Roubos, P. Krabben, W.T.A.M. De Laat, J.J. Heijnen, Clavulanic acid degradation in *Streptomyces clavuligerus* fed-batch cultivations, Biotechnol. Prog. 18 (2002) 451–457.
[20] J.C. Rosa, A. Baptista Neto, C.O. Hokka, A.C. Badino, Influence of dissolved oxygen and shear conditions on clavulanic acid production by *Streptomyces clavuligerus*, Bioprocess Biosyst. Eng. 27 (2005) 99––104, http://dx.doi.org/10.1007/s00449-004-0386-9.
[21] ICH Expert Working Group, Validation of analytical procedures: text and methodology Q2(R1), in: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, 2005.
[22] W.-D. Deckwer, A.F. Mayer, Simultaneous production and decomposition of clavulanic acid during *Streptomyces clavuligerus* cultivations, Appl. Microbiol. Biotechnol. 45 (1996) 41–46.
[23] A.P. Schellinger, P.W. Carr, Isocratic and gradient elution chromatography: a comparison in terms of speed, retention reproducibility and quantitation, J. Chromatogr. A 1109 (2006) 253–266, http://dx.doi.org/10.1016/j.chroma.2006.01.047.