ELSEVIER

Journal of Chromatography A, 686 (1994) 213–218

JOURNAL OF CHROMATOGRAPHY A

Determination of clavam-2-carboxylate in clavulanate potassium and tablet material by liquid chromatography–tandem mass spectrometry

Christine Eckers[a,*], Kathryn A. Hutton[a], V. de Biasi[b], P.B. East[c], N.J. Haskins[a], V.W. Jacewicz[d]

[a]*Analytical Sciences Department, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts. AL6 9AR, UK*
[b]*Separation Sciences Department, SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, UK*
[c]*Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts. AL6 9AR, UK*
[d]*Chemical Development, SmithKline Beecham Pharmaceuticals, Old Powder Mills, Near Leigh, Tonbridge, Kent TN11 9AN, UK*

First received 9 June 1994; revised manuscript received 15 August 1994

Abstract

A liquid chromatography–thermospray tandem mass spectrometric (LC–MS–MS) method was developed to determine the presence of clavam-2-carboxylate in clavulanate potassium at below the 0.01% level stated in the US Pharmacopeia (USP). The USP method is for bulk chemical but it was hoped to extend the determination to formulated products. The method described here involves selected reaction monitoring of *m/z* 156 giving rise to *m/z* 114, using negative ion thermospray ionisation. The selected reaction monitoring method was highly specific for clavam-2-carboxylate and was capable of detecting clavam-2-carboxylate in clavulanate potassium at levels of 0.001% (w/w), an order of magnitude better than the sensitivity required by the USP.

1. Introduction

Clavulanic acid (Fig. 1, left) is an inhibitor of β-lactamase [1] and, as such, it is used in conjunction with penicillins sensitive to the enzyme to render them effective against β-lactamase producing bacteria.

Clavam-2-carboxylate (Fig. 1, right) may be found in clavulanic acid.

The US Pharmacopeia (USP) [2] states that clavam-2-carboxylate must not be present in clavulanate potassium at levels in excess of 0.01%. The USP method for clavam-2-carboxylate determination in clavulanate potassium chemical uses HPLC alone. The purpose of this work is to investigate the possibility of the determination of clavam-2-carboxylate in clavulanate potassium by liquid chromatog-

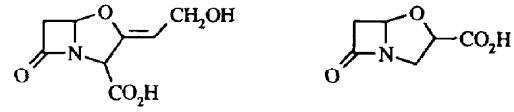


Fig. 1. Structures of clavulanic acid (left) and clavam-2-carboxylate (right).

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00781-0

raphy–tandem mass spectrometry (LC–MS–MS) with a view to increasing the sensitivity and specificity of the USP HPLC method, and to then modify the method to make it suitable for the screening of formulated tablet samples, which contain other active ingredients such as amoxycillin. The tablet excipients and other active ingredients could affect the specificity of an HPLC assay with UV detection alone, and increased sensitivity is required as clavulanate potassium is only one part of the formulated product.

2. Experimental

2.1. Chemicals

HPLC-grade methanol and glacial acetic acid (Romil, Loughborough, UK) were used. Ammonium acetate was supplied by Fisons (Loughborough, UK). The deionised water used was from a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Preparation of solutions

Clavam-2-carboxylate solutions

Clavam-2-carboxylate (10 mg) was dissolved in deionised water (100 ml) to give a 0.1 mg/ml solution that was diluted as appropriate.

Clavulanate potassium solutions

Clavulanate potassium (50 mg) was dissolved in deionised water (0.5 ml).

Spiked clavulanate potassium solutions

Clavulanate potassium (50 mg) was dissolved in a clavam-2-carboxylate solution (0.5 ml) of appropriate concentration.

Tablet material solutions

The mass of the whole tablet was recorded and the tablet was then ground to a fine powder. An amount equivalent to 20 mg clavulanate potassium was taken. A 400-μl volume of water was added. The mixture was then shaken for 1 min and centrifuged for 2 min at 11 600 *g*. The supernatant was removed and re-centrifuged for 2 min at the same rpm. The resulting supernatant was transferred to a vial for injection.

Spiked tablet solutions

These were prepared in the same way as the tablet solutions using 400 μl clavam-2-carboxylate solution of appropriate concentration to dissolve the tablet material.

2.3. Equipment

Chromatography

HPLC was carried out on a Hewlett-Packard (Palo Alto, CA, USA) 1090L liquid chromatograph. The separations were carried out using two 150 × 3.9 mm Waters NovaPak C_{18} (4 μm particle size) columns arranged in series. A flow-rate of 1 ml/min was used. The column was eluted with 95% 0.1 *M* ammonium acetate (pH adjusted to 4.0 with glacial acetic acid) and 5% methanol for 5 min. The clavam-2-carboxylate elutes from the column during this time. The column was then washed with 100% methanol for a further 5 min to remove any other components from the column.

A make up flow of 0.8 ml/min 0.1 *M* ammonium acetate in water–methanol (80:20) was added post column to provide a total flow of 1.8 ml/min into the MS system, which was found to be the optimum flow for this instrument. An Applied Biosystems (Warrington, UK) 400 solvent-delivery system was used for this purpose.

Mass spectrometry

A Finnigan MAT triple-stage quadrupole mass spectrometer TSQ 46 (Finnigan Instruments, San Jose, CA, USA) fitted with a modified Finnigan thermospray (TSP) ion source [3] was used to acquire data in the negative ion mode. The TSP jet temperature was 280°C and the vaporiser temperature was 115°C. The electron multiplier was operated at 2000 V for LC–MS and 3000 V for LC–MS–MS and the repeller was set to 120 V. Argon at a pressure of 1.5 mTorr (1 Torr = 133.322 Pa) and a collision energy of 12 eV was used as the collision gas for LC–MS–MS. Select-

ed reaction monitoring (SRM) was carried out on m/z 156 going to m/z 114.

Other equipment

A Micro Centaur micro centrifuge (Fisons) was used in the preparation of samples.

Modifications for the screening of tablet samples

The two NovaPak columns were replaced by a Rosil C_{18} high-load 250 × 4.6 mm column (Capital HPLC, Bathgate, UK). The elution scheme was also altered. The column was eluted with 100% 0.1 *M* ammonium acetate (pH 4.5 glacial acetic acid) for 7 min followed by washing with 100% methanol for a further 8 min.

3. Results

3.1. Determination of sensitivity

The USP states that clavam-2-carboxylate must not be present in clavulanate potassium at levels in excess of 0.01% (w/w). A 100-mg amount of clavulanate potassium is readily soluble in 1 ml water so a method capable of detecting clavam-2-carboxylate at levels of 0.001 mg/ml [equivalent to 0.001% (w/w) clavam-2-carboxylate in clavulanate potassium] was developed.

Clavulanate potassium and clavam-2-carboxylate have very similar structures and, as a result, very similar retention times. In order to achieve sufficient separation of these compounds, a number of HPLC column systems were examined. Of these, the two NovaPak C_{18} columns linked in series initially proved to be the most efficient. These were replaced by the Rosil column in the screening of the tablet samples to further improve the separation of clavam-2-carboxylate and clavulanate potassium.

Fig. 2 shows the total ion chromatogram (RIC) and spectrum for the LC–MS run after injecting an aliquot (50 μl) of standard clavam-2-carboxylate 0.1 mg/ml. The spectrum shows a base peak at m/z 156 $(M-H)^-$. An MS–MS experiment to determine the product ions of m/z 156 for standard clavam-2-carboxylate 0.01 mg/ml, was carried out. Fig. 3 shows the TIC and product ion spectrum for this experiment. One major product ion is seen at m/z 114 and another at m/z 70, probably formed by loss of ketene from the parent anion, followed by a loss of 44 mass units (i.e., loss of CO_2) from 114. However, when this method was applied to spiked clavulanate potassium samples too much interference from the clavulanate potassium was found to enable sensitivity of better than 0.01% (w/w) clavam-2-carboxylate in clavulanate potassium to be obtained. An SRM experiment, monitoring only m/z 114 ions arising from m/z 156, was used to improve the specificity of the assay.

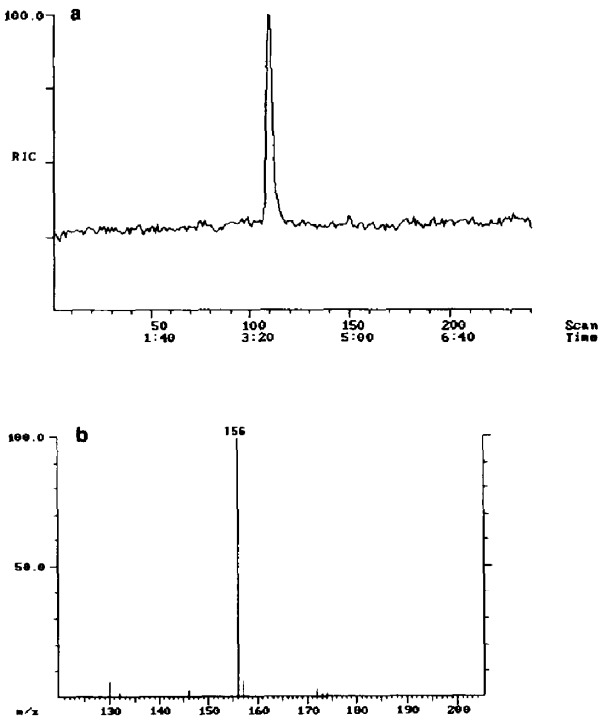
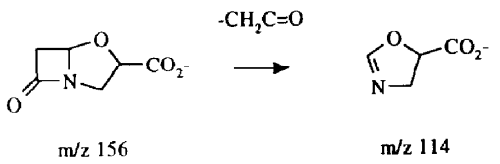


Fig. 2. (a) LC–MS chromatogram for standard clavam-2-carboxylate 0.1 mg/ml; time in min:s. (b) Spectrum for standard clavam-2-carboxylate 0.1 mg/ml.



m/z 156 m/z 114

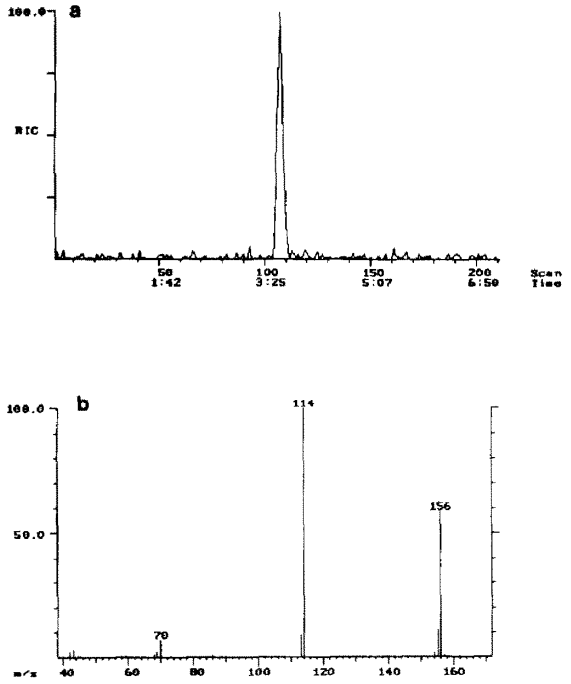


Fig. 3. (a) LC–MS–MS chromatogram for standard clavam-2-carboxylate 0.01 mg/ml; time in min:s. (b) Product ion spectrum for LC–MS–MS of standard clavam-2-carboxylate 0.01 mg/ml.

Fig. 4 shows the chromatogram obtained for an approximately 0.001% spike of clavam-2-carboxylate in clavulanate potassium. A peak for clavam-2-carboxylate can clearly be seen maximising at around scan 150. The sensitivity obtained is an order of magnitude better than that required by the USP limit.

When this method was applied to tablet material, the clavulanate potassium and various other components of the tablet were found to rapidly contaminate the source of the instrument. Accordingly, the HPLC procedure was modified to increase the separation of clavam-2-carboxylate and clavulanate potassium in order to facilitate the diversion of the eluent containing clavulanate potassium and other components away from the mass spectrometer. Fig. 5 shows the SRM LC–MS–MS chromatogram obtained for an approximately 0.0025% spike of clavam-2-carboxylate in a tablet sample.

3.2. Calibration lines

Calibration lines were prepared for clavam-2-carboxylate in clavulanate potassium (Fig. 6) and in tablet samples (Fig. 7), prepared by obtaining the SRM LC–MS–MS traces for solutions spiked with varying quantities of clavam-2-carboxylate and calculating the area of the peak produced. (The peak areas were calculated using the SuperIncos data system attached to the instrument.) Linear regression was used to calculate the equation of the straight line.

The calibration lines show that the relationship between the amount of clavam-2-carboxylate added to the sample and the instrument response is linear. The equation of the straight line for the

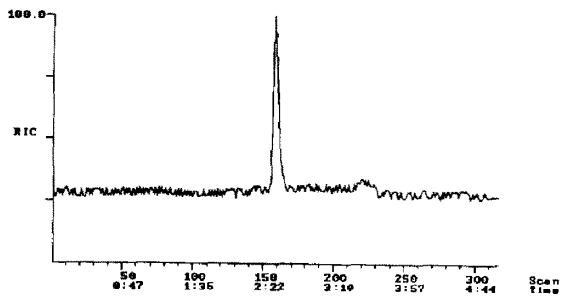


Fig. 4. SRM LC–MS–MS chromatogram for 0.001% spike of clavam-2-carboxylate in clavulanate potassium. (The apparent retention time for the clavam-2-carboxylate peak differs between Figs. 3 and 4 because of the different scan parameters.) Time in min:s.

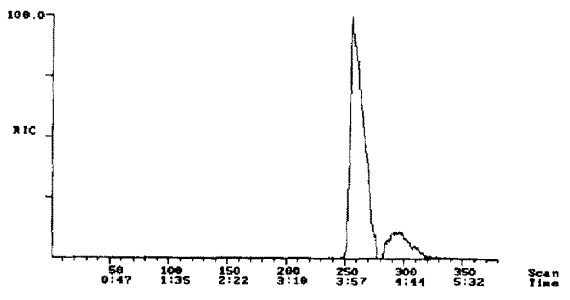


Fig. 5. SRM LC–MS–MS chromatogram for 0.0025% spike of clavam-2-carboxylate in a tablet sample. (The retention time for clavam-2-carboxylate in Figs. 4 and 5 differs as the column and elution scheme were altered for the screening of tablet samples.) Time in min:s.

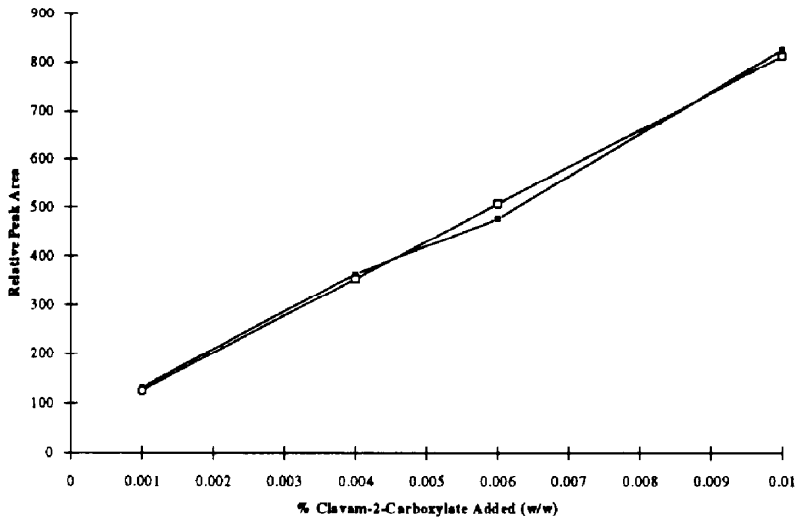


Fig. 6. Calibration line for clavam-2-carboxylate in clavulanate potassium. ■ = Experimental; □ = calculated.

calibration line for the clavulanate potassium sample can be extrapolated in order to determine the clavam-2-carboxylate content of the clavulanate potassium sample used. In this case the sample could be estimated to contain 0.0005% clavam-2-carboxylate.

The calibration line for the tablet sample crosses the y axis at a point below the origin. As a result of this, the amount of clavam-2-carboxylate in the tablet sample used in the spiked solutions cannot be estimated. The fact that the calibration line has a negative intercept may be due to a compound co-eluting with the clavam-2-carboxylate which is suppressing its ionisation. The amount of suppression is independent of the amount of clavam-2-carboxylate present.

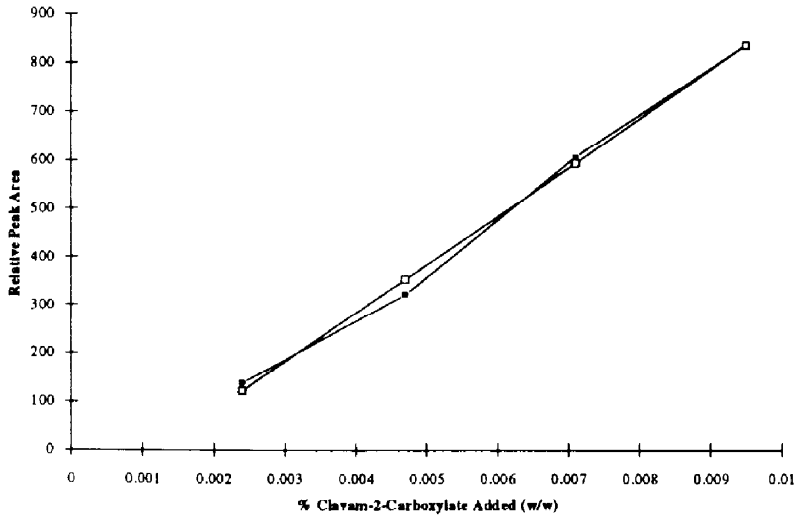


Fig. 7. Calibration line for clavam-2-carboxylate in tablet samples. (The straight lines seen in Figs. 6 and 7 are calculated using linear regression.) ■ = Experimental; □ = calculated.

4. Discussion

This method is capable of detecting clavam-2-carboxylate in clavulanate potassium and formulated tablets at levels lower than that specified in the USP. However, this method is very labour intensive in terms of instrument maintenance. Clavam-2-carboxylate and clavulanate potassium have very close retention times, and it is not possible to divert all the clavulanate potassium away from the mass spectrometer. This means that only about five sample solutions may be run before the source of the instrument becomes contaminated, due to the clavulanate potassium, resulting in a drop in sensitivity. It was for this reason that only four solutions were used in the preparation of the calibration lines and, as a result of this, the calibration lines will only suffice to show that the relationship between clavam-2-carboxylate concentration and instrument response (peak area) is linear.

The Rosil column improved the separation of clavam-2-carboxylate and clavulanate potassium and facilitated the diversion of the acid away from the mass spectrometer thus reducing the amount of instrument maintenance. However, clavulanate potassium is not the major component of the tablet samples and in order to achieve the required concentration, a large amount of tablet material must be dissolved in a small amount of water (approximately 200 mg/400 μl). This results in a highly concentrated solution being injected into the system which, despite efforts to divert all but the eluent containing clavam-2-carboxylate away from the mass spectrometer, quickly contaminates the source and reduces the sensitivity. Clavam-2-carboxylate and clavulanate potassium are both very soluble in water, therefore the extraction efficiency should be very high, however, this has not been investigated.

In order to use LC–MS for accurate quantification of clavam-2-carboxylate concentration in clavulanate potassium, an internal standard would have to be chosen and incorporated into the sample material, to overcome any effects on the linearity of response as the instrument becomes contaminated.

5. Conclusions

This method has been shown to meet the specificity and sensitivity requirements for the determination of the presence of clavam-2-carboxylate in clavulanate potassium and tablet samples at levels below that of the USP limit. The method is very labour intensive and is not recommended to replace the USP method; however, it does give the added specificity allowing clavam-2-carboxylate to be determined in formulated products.

References

[1] A.E. Bird, J.M. Bellis and B.C. Gasson, *Analyst*, 107 (1982) 1241.
[2] *US Pharmacopeia*, XXII, The US Pharmacopeial Convention, Rockville, MD, 1990, p. 316.
[3] P.B. East, C. Eckers, N.J. Haskins, J.F. Hare and J. James, *Rapid Commun. Mass Spectrom.*, 6 (1992) 179.