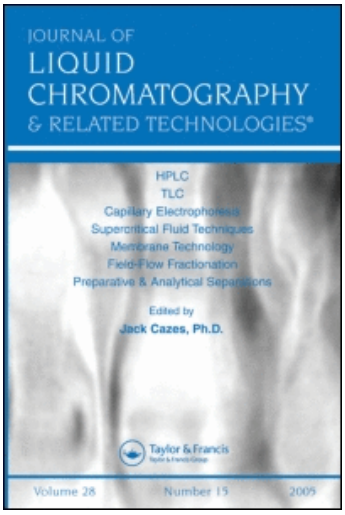

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Martín, J. and Méndez, R.(1988) 'High-Performance Liquid Chromatographic Determination of Clavulanic Acid in Human Serum and Urine Using a Pre-Column Reaction with 1, 2, 4-Triazole', Journal of Liquid Chromatography & Related Technologies, 11: 8, 1697 — 1705
To link to this Article: DOI: 10.1080/01483918808076730
URL: http://dx.doi.org/10.1080/01483918808076730

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(8), 1697-1705 (1988)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLAVULANIC ACID IN HUMAN SERUM AND URINE USING A PRE-COLUMN REACTION WITH 1,2,4-TRIAZOLE

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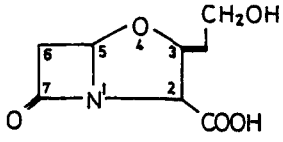
ABSTRACT

A reversed-phase high-performance liquid chromatographic (RP-HPLC) assay for the determination of clavulanic acid in serum and urine is described. The clavulanic acid was assayed by reacting the sample with 1,2,4-triazole reagent which rapidly produces a derivative that has its UV absorption maximum at 317 nm. The resulting product was separated in a RP-18 column (μ-Bondapak, 10 μm) and detected at 313 nm. The method was applied to assays of clavulanate in serum and urine, and is reproducible with a lower limit of quantitation of 0.1 μg/ml.

INTRODUCTION

An important mechanism of bacterial resistance to the β-lactam antibiotics is the destruction of penicillins and cephalosporins by the action of β-lactamase enzymes (1). Various substances have been described in the past as having inhibitory activity against some of

these enzymes (2). However clavulanic acid (I), produced by Streptomyces clavuligerus (3), is a new type of compound which can inhibit a wide range of β-lactamases and can more effectively potentiate the activity of penicillins against bacteria which owe their resistance to β-lactamase prodution. The compound is usually used in the form of its potassium salt.



(I)

Clavulanic acid may be detected by using a microbial assay method (4,5). It may also be determined biochemically by using its enzyme inhibitory properties (6,7) and spectrophotometric titration of the reaction product with imidazole (8). High-performance liquid chromatography (HPLC) has been applied to the assay of clavulanate in body fluids (9,10).

High-performance liquid chromatography offered the best potential for quantitating the drug after separating and differentiating it from undesirable substances such as oligomers, degradation products, and residual contaminants.

MATERIAL AND METHODS

Materials and Reagents

The commercial potassium clavulanate used throughout the study was kindly donated by Antibióticos S,A. (León, Spain).

1,2,4-Triazole puriss p.a. (Aldrich Chemical Co., Milwankee, Wis) was recrystallized from toluene, the precipitate being filtered and washed with diethyl ether and vacuum-dried in a desiccator overnight. Other chemicals of analytical-reagent grade and methanol of HPLC grade were purchased from Carlo Erba (Milán, Ttaly) and used as received.

All the water used was purified by the Milli-Q-Reagent-Grade water system (Millipore Bedford, MA, U.S.A.).

1,2,4-Triazole reagent solution : Prepared by dissolving 13.81 g of 1,2,4-triazole in 70 ml of water and adjusting the pH to 9.00 ± 0.05 by the addition of 4 M sodium hydroxide solution. This was then diluted to 100 ml.

Instruments.

The HPLC system consisted of a Consta Metric pump II G (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a model 441 absorbance detector with a fixed wavelength of 313 nm (Waters Assoc., Milford, MA, U.S.A.). Samples were loaded on to the column via a Rheodyne Model 7210 loop injector. Separation was accomplished on a μ-Bondapak C18 column (3.9 mm x 30 cm) and the quantitation was based on integration of peak areas using a Varian computing integrator (Model 4290, Palo Alto, Calif.).

For the examination of pre-column reaction conditions, we used a Bausch-Lomb Spectronic 2000 spectrophotometer. The serum samples were ultrafiltered using a MPS-1 micropartition system (Amicon Ltd. Woking, Surrey, England).

Chromatographic procedure.

The mobile phase consisted of methanol and aqueous 0.03 M phosphate buffer (pH 7.0), (20:80, v/v). A pre-column (3 cm x 4.6 mm I.D.) packed with the same packing materials was used to guard the main column. The flow-rate was set at 2.0 ml/min and 50 μl samples were injected.

All chromatographic operations were carried out under ambient conditions.

Pre-column derivatisation procedure and analysis.

1. Serum Samples : To a 0.5 ml of serum sample was added 0.2 ml of 0.2 M phosphate buffer, pH 7.0, and mixing was carried out on a vortex mixer for 10 sec. The mixture was ultrafiltered using Amicon YMT membranes at 5°C at 1500 g for 15 min. To a 0.25 ml of the ultrafiltrate were added 0.25 ml of 2 M 1,2,4-triazole reagent (pH 9.0) and a 50 μl portion of the mixture, after a 5 min reaction period at 30°C, it was loaded into the HPLC column.

2. Urine Samples : 0.5 ml of urine were transferred into a 5-ml glass tube, 4.5 ml of water added and mixed vigorously for 20 sec; 1 ml of this solution was filtered through an assembly consisting of a 0.45-μm-pore filter (Millex-HA; Millipore Corp., Bedford, Mass) attached to a 5 ml syringe. The filtrate was treated and analysed according to the same procedures as those for serum samples.

3. Quantitation : Standard solutions were prepared by dissolving known amounts of clavulanate in control serum to make six different concentrations between 0.1 - 10 μg/ml and treated in the same manner as described above. A calibration graph of peak area versus clavulanate concentration was constructed. The calibration graphs for urine samples, concentrations between 1 - 50 μg/ml, were constructed according to the same procedures as those for serum samples.

RESULTS AND DISCUSSION

The ultraviolet spectrum of the reaction product of 1,2,4-triazole with clavulanate, run against the 1,2,4-triazole-water as reference solution, shows a maximum only at 317 nm. This product is relatively stable at room temperature under. HPLC examination it revealed a single peak.

The structural investigations of this reaction product will be discussed elsewhere in the near future.

Pre-column reaction conditions.

The factors affecting the pre-column reaction such as pH and concentrations of 1,2,4-triazole solution and reaction temperature were examined in aqueous solutions by plotting the absorbance at 317 nm against the reaction time.

1. Effect of pH : Fig.1 shows the effect of pH (pH 8.0, 9.0 and 10.0) on the formation of the derivatised product for the reaction of clavulanate with 2 M 1,2,4-triazole reagent at 30°C. As one can see, when the pH of the reaction solution was high the reaction was faster, but the reaction product was less stable at pH 10.0 than at lower pH values. At pH 10.0 the reaction led to the maximum absorbance at 2 min, but a decrease in absorbance was observed as the reaction proceeded.

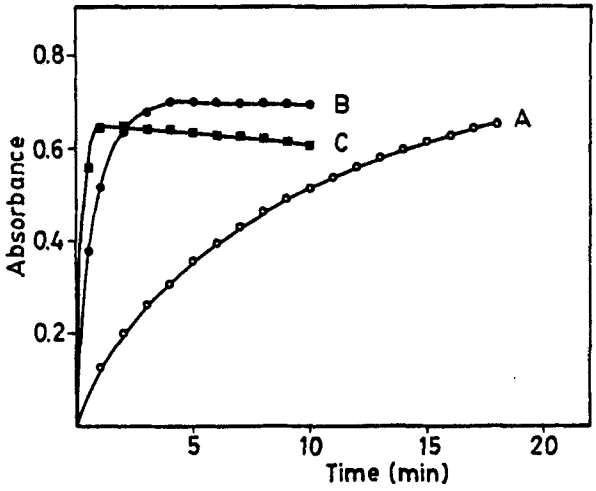


FIGURE 1. Effect of pH on the formation of the clavulanate chromophore at 217 nm using 2 M 1,2,4-triazole reagent at 30°C. Sample solution contained 13 μg/ml potassium clavulanate. To a 1 ml of the clavulanate aqueous solution was added 1 ml of 1,2,4-triazole reagent. A, pH 8.0; B, pH 9.0; and C, pH 10.0.

2. Effect of 1,2,4-triazole concentration : The effect of the change in the 1,2,4-triazole concentration between 0.5 and 2 M (final concentration) at pH 9.0 and 30°C is shown in fig. 2. As can be seen the rate of formation of the derivatised product was determined by the 1,2,4-triazole concentration. These results show that the absorbance obtained by the reaction with 1,2,4-triazole at 1 M concentration was stronger than that at concentration of 0.5 or 2 M. The reaction product is slightly less stable at 2 M concentration than at lower concentrations.

3. Effect of temperature : The effect of reaction temperature (30 - 40°C) was not as great as that of pH and 1,2,4-triazole concentration. Fig. 3 shows that the initial rate increases slightly with temperature and that the reaction product is unstable at high temperatures. In accordance with these results we selected the pre-column reaction conditions. These conditions for routine use

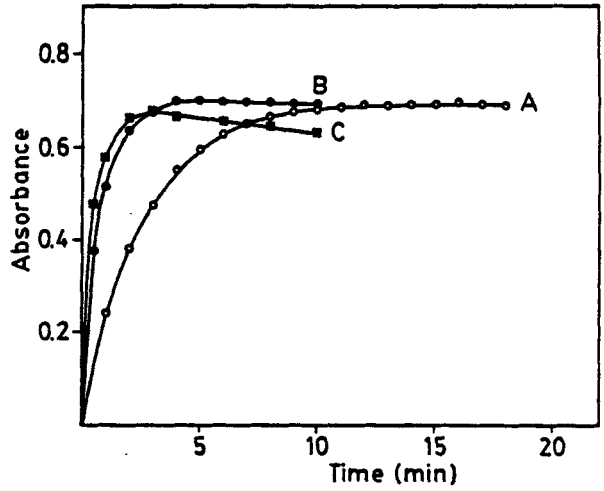


FIGURE 2. Effect of 1,2,4-triazole concentration of the formation of the clavulanate chromophore at 317 nm (pH 9.0, 30°C). Sample solution contained 13 μg/ml potassium clavulanate. To a 1 ml of the clavulanate aqueous solution was added 1 ml of 1,2,4-triazole reagent.
A, 0.5 M; B, 1 M; and C, 2M 1,2,4-triazole (final concentration).

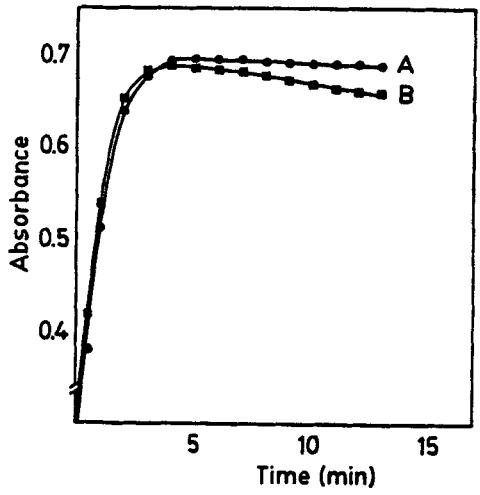


FIGURE 3. Effect of temperature on the formation of the clavulanate chromophore at 317 nm using 2 M 1,2,4-triazole reagent at pH 9.0. Sample solution contained 13 μg/ml potassium clavulanate. To a 1 ml of the clavulanate aqueous solution was added 1 ml of 1,2,4-triazole reagent.
A, 30°C and B, 40°C.

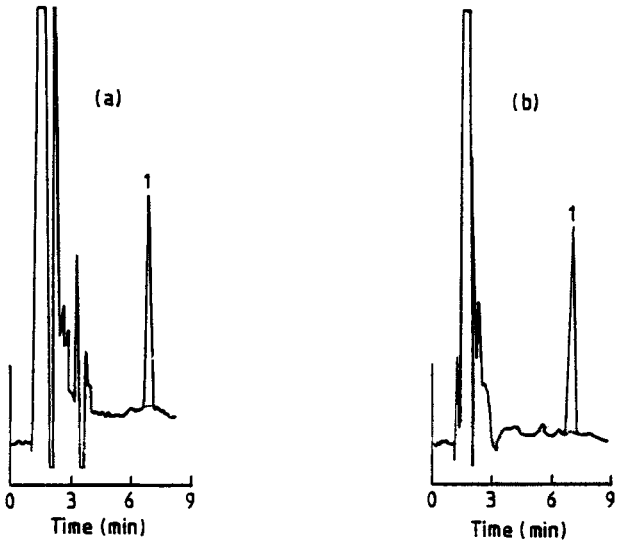


FIGURE 4. Chromatograms of clavulanate chromophore (peak 1). (a) The clavulanate in control serum (2 μg/ml) was ultrafiltered, (b) the clavulanate in control urine (20 μg/ml) was filtered and diluted 10-fold, and each sample reacted with 2 M 1,2,4-triazole reagent at pH 9.0 for 5 min at 30°C. See material and methods for chromatographic conditions. Dotted lines indicate serum and urine blanks.

were 2 M 1,2,4-triazole reagent adjusted to pH 9.0 at 30°C for 5 min. Similar results were also obtained for the reaction of clavulanate with 1,2,4-triazole in serum and urine samples, so the same reaction conditions were adopted. The reaction product was stable for at least 1 h at 20°C.

HPLC assay of derivatised clavulanate.

Fig. 4 (a),(b) show chromatograms of blank serum and urine, and of serum and urine samples. The serum and urine samples spiked with 2 μg/ml and 20 μg/ml were treated according to the pre-column reaction conditions, and 50 μl of the treated samples were injected into the HPLC system. The analysis conditions are described in Material and Methods. The derivatised product is completely separated from the

TABLE 1

Recovery of Clavulanate from Serum and Urine Samples.

	Sample	
	Serum	Urine
Concentration (μg/ml)	5.0	50.0
Recovery (%)	86.4	93.8
Coefficient of variation (%) (n=5)	3.15	2.08

normal components of serum and urine. In the mobile phase used - methanol /aqueous 0.03 M phosphate buffer, pH 7.0, (20:80,v/v) - the derivatised product was relatively stable and was eluted within 7 min after injection.

1. Linearity : Linearity was checked by measuring six different concentrations in the range 0.1 - 10 μg/ml for serum assay and 1 - 50 μg/ml for urine assay. There was a good linear relationship between the peak area ratio and the concentration in serum and diluted urine samples, with regression analysis of the data revealing a correlation coefficient of 0.99 (or more).

2. Sensitivity : We estimated that the limit of accurate determination was 0.1 μg/ml with a 50 μl injection.

3. Recovery and precision : Table 1 shows the total recovery of clavulanate from spiked serum and urine samples, and the coefficients of variation.

The precision for the assays was determined by analyzing serum and urine samples containing different concentrations of clavulanate. Table 2 shows the within- and between-run precisions for the assays of clavulanate in serum and urine.

CONCLUSION

The reaction of the 1,2,4-triazole reagent with clavulanic acid yielded a product having its UV absorption maximum at 317 nm.

TABLE 2

Precision of Clavulanate Assay in Serum and Urine Samples. Control Serum and Urine were spiked with 2.0 and 20.0 μg/ml, repectively.

	Sample	
	Serum	Urine
Within-run coefficient of variation (%)(n=5)	1.64	1.25
Between-run coefficient of variation (%)(n=3)	4.79	3.48

An HPLC procedure assay of clavulanate in serum and urine using the above reaction for pre-column derivatisation were developed. This HPLC method is specific for intact clavulanate and under the routine conditions used for derivatisation, penicillins, penicilloic acids and clavulanic acid degradation product do not interfere. The proposed method will be useful for the determination of clavulanic acid.

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