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Determination of amoxicillin in plasma samples by capillary electrophoresis

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Abstract

We have developed a capillary zone electrophoresis (CZE) method for determining amoxicillin in animal plasma samples. Sample clean-up involved solid-phase extraction onto Sep-Pak C_{18} cartridges followed by elution with water–methanol (85:15). This paper describes two different techniques to increase the sensitivity of the CZE method: field-amplified sample injection (FASI) and electrokinetic injection. We have enhanced the detection limit to 280 $\mu g\ l^{-1}$ by the FASI technique.

Keywords: Solid-phase extraction; Injection methods; Amoxicillin; Antibiotics

1. Introduction

Many veterinary drugs are used to treat food-producing animals. Two commonly used types of antibiotics are sulphonamides and β-lactams. β-Lactam antibiotics are two types of compounds: penicillins and cephalosporins. Amoxicillin is one penicillin widely used in clinical practice as a bactericidal against many gram-positive and gram-negative microorganisms. One of the most difficult problems in pharmaceutical analysis is to separate and quantitatively determine penicillins, their degradation products and impurities. The iodometric method for determining amoxicillin in bulk drug substance is official in the Code of Federal Regulations [1]. Due to the lack of specificity of the official methods, others have been developed for determining amoxicillin in bulk drug substance and in biological fluids, as high-performance liquid chromatography (HPLC) [1–15] and capillary electrophoresis [16–29] techniques.

In the literature there have been several studies of amoxicillin by HPLC using UV detection. Some papers [1–3] have only been concerned with separating, identifying and quantifying its major impurities and decomposition products in these bulk drugs and oral dosage forms. Others [4–10], however, were concerned with determining amoxicillin in biological fluids using different kinds of pretreatment to obtain samples without interference and in a suitable medium.

Adamovics [11] suggested a method for injecting plasma samples directly. Unfortunately it was suitable only for plasma concentrations of between 20 and 100 $mg\ l^{-1}$, and this is too high for most applications. Tyczkowska and Aronson [12] injected

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plasma samples after filtration through a 30 000 molecular mass cut-off filter to prevent the column from deteriorating. Since amoxicillin is insoluble in most organic solvents, currently available methods of measuring amoxicillin in biological fluids are usually based on protein precipitation by acid or organic solvents. These assays also tend to utilise UV detection [13], through postcolumn reactions and derivatization enhance assay sensitivity [9,10,14,15].

Solid-phase extraction (SPE) has gained popularity over the years in preparing samples for a wide range of analytes in complex matrices thanks to its better selectivity, simple operation, and lower consumption of solvents. However, there have been only a few reports of SPE methods of extracting amoxicillin by C_{18} cartridges from human plasma [4,6,7] and urine [5] or of systems which use SPE for other β-lactam antibiotics [13,14]. Some authors have used tetrabutyl-ammonium bromide to form an ion-pair with the acid group of amoxicillin. In these cases, the recovery of amoxicillin from plasma was greater than 80%, but other authors [6–8] have employed SPE without adding an ion-pair reagent. In these cases the recovery of amoxicillin changes from study to study, and values are between 60 and 90%.

When amoxicillin in plasma was analysed by HPLC and after on-line oxidation with an electrochemical detector [15], recoveries and sensitivity were higher than with the other methods. If we had an electrochemical detector we could use it to analyse the amoxicillin in our own body fluids using this type of detector.

This paper evaluates how effective capillary electrophoresis is when applied to biological fluids. Capillary electrophoresis, in the form of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC), is a technique which has been used extensively to analyze pharmaceutical compounds [16–29], but has not been used to analyse amoxicillin in biological fluids.

Penicillin antibiotics, because of the pK_a values of their different functional groups, were analysed by MECC [25–29]. Hows et al. [28] described the simultaneous separation of three types of compounds: sulphonamides, dihydrofolate reductase inhibitors and β-lactam antibiotics using MECC. This paper showed how factorial design is applied to optimise separation of these complex mixtures.

Although SPE of amoxicillin has been used on biological samples [4–8], none of the authors reported the use of off-line SPE followed by capillary electrophoresis analysis.

After evaluating two different types of sample pretreatment, deproteinization with trifluoroacetic acid (TFA) and SPE using C_{18} cartridge, we developed a CZE method using a C_{18} cartridge extraction of amoxicillin and UV detection at 210 nm. We also studied different injection techniques [electrokinetic and field-amplified sample injection (FASI)] to enhance the sensitivity of the method [30–32]. This combination of extraction, injection and detection produces a method that is reliable and easy to perform.

2. Experimental

2.1. Instrumentation

Electrophoretic experiments were performed using a Prince CE System (Lauer, Emmen, Holland) with a UNICAM 4225 UV detector. Data were collected using the software provided with the HP3365 Series II Chemstation (Hewlett-Packard) which was operated under Windows 3.1 (Microsoft). The capillary was fused-silica (70 cm×75 μm I.D.) supplied by Supelco (Bellefonte, USA). A detection window was prepared by burning off the polyimide coating 56 cm from the capillary inlet.

2.2. Reagents and standards

Amoxicillin trihydrate was purchased from Sigma. A standard stock solution of 1000 mg l^{-1} was prepared in water which had been purified by the use of a Milli-Q system (Millipore, Bedford, USA) and stored under refrigeration. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the standard stock solution with Milli-Q quality water.

TFA (Fluka, Buchs, Switzerland), acetonitrile (ACN) (Fisher, Leicestershire, UK), methanol (Merck, Germany) and potassium dihydrogen phosphate (KH_2PO_4) (Fluka) were used to pretreat the sample.

Sodium tetraborate (Fluka), sodium dodecyl sul-

phate (SDS) (Sigma, St. Louis, USA) and HCl (Probus, Barcelona, Spain) were used to prepare the electrophoretic solution.

2.3. Electrophoretic conditions

The electrophoretic solution was prepared by adjusting the pH of 20 m*M* sodium tetraborate solution to 9 with 6 *M* HCl. Before use, the capillary was rinsed with 0.1 *M* NaOH (Probus) (1000 mbar pressurised flow) for 15 min, then with H_2O Milli-Q for 15 min and finally flushed with running buffer for 10 min. Before each analysis, the capillary was flushed with Milli-Q water for 2 min and the run buffer for 3 min successively. The detector was set at 210 nm. Injection was performed hydrodynamically at a pressure of 100 mbar for 1.8 s and the capillary temperature was 30°C. The voltage was 15 kV.

2.4. Pretreatment of the sample

Two types of pretreatment were used.

2.4.1. TFA 3% in ACN

TFA 3% was prepared by dissolving 750 μl TFA 98% in 25 ml of ACN. Four hundred μl of this solution and 100 μl of animal plasma were then mixed in a vortex and centrifuged at 3787 *g* at 0°C for 7 min. Finally, the clear supernatant was separated and filtered through a 0.45 μm nylon filter and stored at 0°C until it was used.

2.4.2. SPE

Waters Sep-Pak cartridges plus (360 mg, C_{18}) were used to carry out SPE [7]. The cartridge was activated with 4 ml of methanol followed by 1 ml 0.02 *M* KH_2PO_4 at a flow-rate of 1–2 ml min^{-1} using a water aspirator as a vacuum source connected to the cartridge pack. One ml of animal plasma sample was diluted with 1 ml of 0.02 *M* KH_2PO_4 and passed through the cartridge. The cartridge was washed with 1 ml of 0.02 *M* KH_2PO_4 and 0.5 ml of water. The amoxicillin was eluted from the cartridge with 2 ml aliquot of water–methanol (85:15).

Pig plasma was used in all experiments.

3. Results and discussion

To develop a method for determining amoxicillin, we first tried MECC because this technique is always used to analyse β-lactams [25–29], so we studied an electrolyte with SDS as the micellar agent with a 20 m*M* borate buffer at pH 9 according to the literature [28]. Fig. 1 shows electropherograms of a standard at 10 mg l^{-1} with different concentrations of SDS: 50 m*M* SDS (Fig. 1a), 75 m*M* SDS (Fig. 1b) and 150 m*M* SDS (Fig. 1c). The results show that analysis time increases and the peak shape was correct in all cases.

In amoxicillin the pK_a values of the COOH, NH_2 and OH groups are 2.4, 7.4 and 9.6, respectively [4]. For this reason amoxicillin is negatively charged at the pH of the working electrolyte (pH 9) and we could use the CZE technique because of its simplicity. This mode of operation, however, has never been used to analyse a mixture of β-lactam antibiotics. We

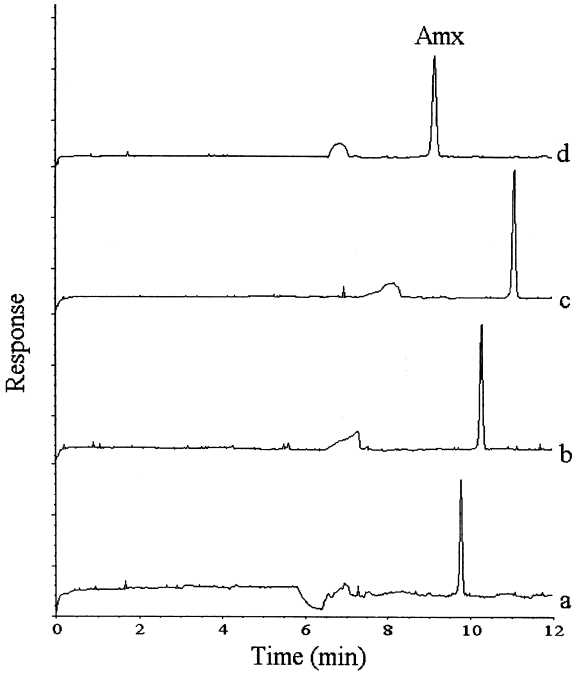

Fig. 1. Electropherograms of 10 mg l^{-1} of amoxicillin from standard solution with 50 m*M* SDS (a), 75 m*M* SDS (b) and 150 m*M* SDS (c) in the running buffer, and without SDS (d). Running buffer: 20 m*M* sodium tetraborate, pH 9. Injection: 100 mbar for 1.8 min. Capillary temperature: 30°C. Separation voltage: 15 kV.

could use CZE because only amoxicillin was analysed. With the same borate buffer but without SDS, the migration time was still less and the peak shapes and sensitivity were similar. Fig. 1d shows the electropherogram obtained in this case. Hence we have developed the study of amoxicillin using borate as running buffer without SDS.

After testing Ohm's law for this running buffer, the highest voltage we could apply was 15 kV since with higher voltages the analysis was not reproducible.

To evaluate linearity we prepared and analysed different solutions of amoxicillin with concentrations ranging from 0.4 to 35 mg l^{-1}. These solutions were injected five times starting with the least concentrated and ending with the most. The area values obtained were successively analysed using ULC (univariate linear calibration) software [33] to evaluate the correlation coefficient (r), relative standard deviation (RSD) within solutions and limit of detection (LOD). Linearity was good, with a correlation coefficient of 0.994. The LOD was calculated by the method of Winefordner and Long [34] using the ULC program with K equal to 6, and the LOD was 0.2 mg l^{-1}. The RSD of the areas was 1.3% and the RSD of the migration time was 0.9% for ten repeated injections of standard solutions of 10 mg l^{-1}. A fresh buffer was used after each sequence of three injections.

To improve LODs, there are different injection modes for capillary electrophoresis [30]. This paper studied electrokinetic injection [30] and FASI [31,32]. In electrokinetic injection, the sample is introduced by applying a voltage across the capillary while one end is immersed in the sample solution and the other buffer. The sample is drawn into the capillary by a combination of electrophoresis and electroosmotic (EOF) flows. When electrokinetic injection is performed by applying different voltages (5–15 kV) for different times (3–15 s), amoxicillin migrates to the cathode because of the EOF developed by the running buffer, but since the difference between EOF and electrophoretic mobility is little, no peak can be detected. On the other hand, the effect of adding salts to the sample matrix was also examined. Addition of NaCl to the sample at a concentration of 100 mM increases the amount of amoxicillin injected. This improvement is due to the increase in the sample equivalent conductance. As a result, the electrophoretic mobility of the amoxicillin in the sample during the injection step decreases. In this case, amoxicillin will be introduced into the capillary not only depending on the individual equivalent conductance but also on the total conductance of the sample solution. In this case, the electrophoretic mobility of amoxicillin decreases when salt is added and consequently, a large quantity will be introduced by electroosmosis. Fig. 2 shows the electropherograms when a standard solution of 10 mg l^{-1} was injected electrokinetically at 15 kV for 0.25 min. without the addition of salt (Fig. 2a) and with the addition of salt (Fig. 2b). Results from this method are similar to those obtained from hydrodynamic injection. This shows that it was not possible to increase sensitivity in this way.

The other technique we studied to enhance detectability was FASI [31,32]. With this, sample stacking occurred when large volumes of sample were introduced into the capillary and sample matrix was removed by pumping it out of the column using the EOF. The steps in this procedure are the following. First, a large sample plug is hydrodynamically injected into the column in a low-conductivity electrolyte. Second, the polarity is switched and the sample matrix is pushed out of the column. Third, when the sample matrix is almost completely out of the column (which can be determined by monitoring the electric current), the polarity is again reversed. Finally, the separation is performed. We have studied

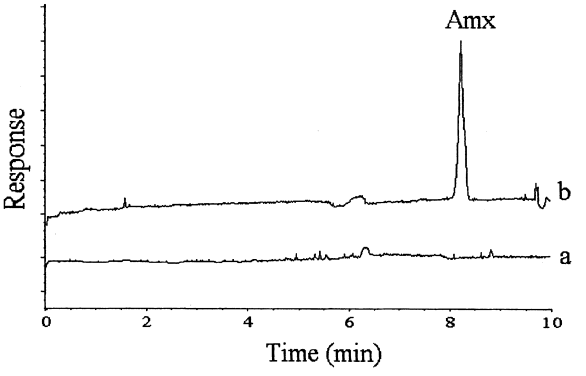

Fig. 2. Electropherograms of 10 mg l^{-1} of amoxicillin from standard solution using electrokinetic injection 15 kV for 0.25 min: (a) without adding NaCl; (b) adding 100 mM of NaCl to the sample. For other conditions see Fig. 1.

two parameters to increase the amount of amoxicillin injected and achieve our objective: length of capillary (70 and 90 cm) and its inner diameter (75 and 100 μm). When both parameters were increased there was an increase in the signal of amoxicillin. We used a standard solution of 10 mg l^{-1}. Results obtained can be observed in Table 1. Results were best for the 90 cm and 100 μm capillary (see Table 1). The sample was introduced hydrodynamically (200 mbar for 1 min), the sample vial was changed to buffer vial and a voltage with a reverse polarity (−10 kV outlet positive) was applied. After 10 min the voltage was stopped, the polarity was switched back to its normal position for CZE (15 kV outlet negative) and CZE separation was began. With this injection mode the LOD obtained was 15 μg l^{-1} using a capillary of 90 cm of length and 100 μm inner diameter.

When plasma was analysed by the HPLC method [8], the sample had to be pretreated in order to decrease the high level of interference. To remove the protein content of plasma we therefore used TFA pretreatment and it was analysed by the CZE method performed in this paper. Fig. 3 shows electropherograms from the analysis of plasma with a standard addition of 50 mg l^{-1}, added to the original sample of plasma, without TFA sample pretreatment (Fig. 3a) and with TFA sample pretreatment (Fig. 3b). The sample therefore had to be pretreated. TFA was used to obtain a selective CZE method. Recovery of amoxicillin from plasma was 75% using the TFA treatment described in the Experimental Section and a standard addition of 50 mg l^{-1} of amoxicillin in a plasma matrix.

After establishing pretreatment, we studied the linear range and the LOD of amoxicillin in this kind of sample. To calculate them we have taken into account the dilution factor of samples, according to the type of pretreatment used. Concentration ranged from 15 to 175 mg l^{-1}, and the LOD obtained was 7.5 mg l^{-1} when the sample was injected hydrodynamically (100 mbar for 1.8 s). We therefore developed studies to enhance sensitivity in the CZE method.

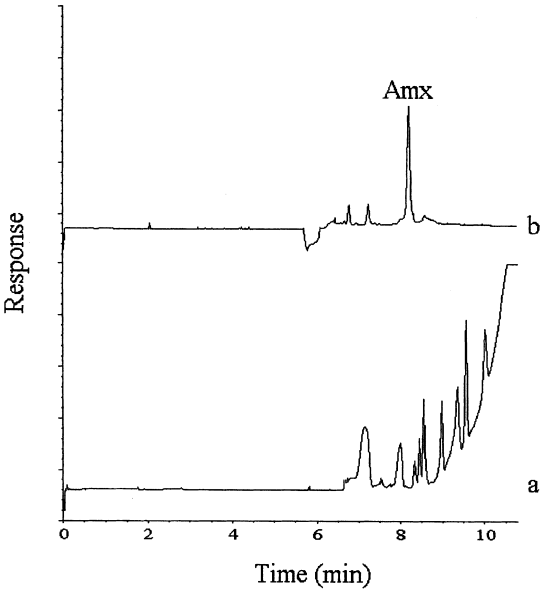

Fig. 3. Electropherogram of spiked plasma sample (50 mg l^{-1} of amoxicillin added to the original sample of plasma) without (a) and with (b) sample TFA pretreatment. For other conditions see Fig. 1.

To increase the sensitivity of the CZE method a FASI technique was applied but the sample was not preconcentrated and focused in the column. This studies were made with a capillary of 90 cm and 100 μm. The problem may be due to an organic phase (ACN) in the injected sample following TFA pretreatment. We removed this organic phase by heating the sample. As ACN is difficult to remove, we used cool acetone for pretreatment. The organic phase was then removed, and FASI could be performed. The sample pretreatment was not optimised by replacing ACN with acetone, however, because this is out of the scope of this paper.

Table 1
The normalised results obtained using the FASI method

Hydrodynamic injection	Field-amplified sample injection (FASI)		
L_T: 70 cm I.D.: 75 μm	L_T: 70 cm I.D.: 75 μm	L_T: 90 cm I.D.: 75 μm	L_T: 90 cm I.D.: 100 μm
1	8.9	12.8	35.2

Another type of pretreatment, SPE, was proposed for removing the matrix of the sample [7]. Applying SPE to the analysis of β-lactam antibiotics in biological fluids is not very common. Many β-lactam antibiotics contain both amine and carboxylic acid groups, and are charged at any pH except at the isoelectric point. This would apparently make it difficult for them to be extracted efficiently by nonpolar C_{18} cartridges. The present application of a SPE for amoxicillin demonstrates the potential usefulness of this approach in the extraction of other amphoteric β-lactam antibiotics from biological fluids by solid-phase cartridges. Fig. 4a shows an electropherogram of a plasma sample after SPE using a FASI technique to introduce the sample in the capillary. In this case the analysis time is longer because with the FASI optimised conditions the capillary is 90 cm long and has an internal diameter of 100 μm. This is larger than when the analysis was performed without the FASI technique (70 cm×75 μm). Electropherograms from ten different samples were similar and there was no evidence of interfering substances. Fig. 4b shows an electropherogram of a spiked plasma sample after SPE. The recovery of amoxicillin was determined by comparing the peak area of the extracted samples with the peak area of the same point on the calibration graph and was found to be 90%±1.3% (n=5). This recovery is higher than when TFA pretreatment was used. The linear range of amoxicillin in plasma samples was from 10 to 70 mg l^{-1}. Pretreating the sample in this way makes it possible to apply the FASI injection mode to enhance the sensitivity of the method, since no ACN was used to elute the sample, instead methanol was used. Using the same conditions for FASI injection previously optimised and with the same capillary dimensions, sensitivity increased fourfold, which was significantly higher than with a TFA pretreatment. The LOD was 280 μg l^{-1}, higher than when FASI was applied to standard samples when the LOD was 15 μg l^{-1}.

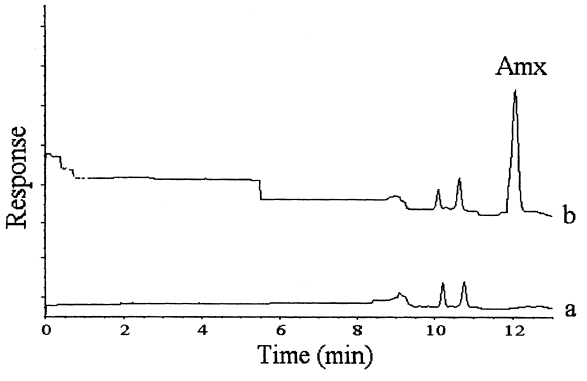

Fig. 4. Electropherograms of a blank plasma sample (a) and a spiked plasma sample (20 mg l^{-1} of amoxicillin added to the original sample of plasma) (b) after SPE. Capillary: 90 cm, 100 μm. Injection: FASI technique (conditions in the text). For other conditions see Fig. 1.

This difference probably arises because the RSD values are higher than when the plasma samples were analysed. The RSD of the areas, calculated with a standard addition of 20 mg l^{-1}, was 14% for ten repeated injections.

The LOD was similar to that of a previous report [8] in which HPLC and SPE were used to monitor amoxicillin in plasma samples. This showed that CZE may be a valuable alternative technique to HPLC for analysing amoxicillin in plasma samples. The LOD was also similar to that obtained when different β-lactams were analysed in formulations by CZE [27] and MECC [26].

Other important reasons for the success of this assay are the clean background and high recovery with SPE and the fact that we can analyse the extract by CZE with the FASI injection technique to enhance detectability. Another factor which contributes to the high degree of sensitivity when analysing plasma samples is the 210 nm UV light used to monitor amoxicillin.

4. Conclusions

This investigation has shown that analysing amoxicillin in animal plasma samples by CZE and introducing the sample into the capillary by the FASI technique after SPE with C_{18} cartridges, is an alternative to HPLC methods with a similar LOD.

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