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Application of a liquid chromatographic procedure for the analysis of penicillin antibiotics in biological fluids and pharmaceutical formulations using sodium dodecyl sulphate/propanol mobile phases and direct injection

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ABSTRACT

A direct injection liquid chromatography procedure was developed for the simultaneous determination of four penicillin antibiotics (amoxicillin, ampicillin, cloxacillin and dicloxacillin) in pharmaceutical formulations and physiological fluids (urine) using hybrid micellar mobile phases. These antimicrobials are used to treat gastrointestinal and systemic infections. The four penicillins were analysed using a Zorbax C18 reversed-phase column and detected at 210 nm. These antibiotics were separated by an interpretive optimisation procedure based on the accurate description of the retention and shape of the chromatographic peaks. Antibiotics were eluted in less than 16 min with no interference by the urine protein band or endogenous compounds using the mobile phase 0.11 M sodium dodecyl sulphate–6% propanol–0.01 M NaH_2PO_4 buffered at pH 3. The method was validated according to the Food and Drug Administration guideline, including analytical parameters such as linearity ($R^2 > 0.993$), intra- and inter-day precisions (RSD, %: 0.1–4.4 and 1.2–5.9, respectively), and robustness for the four compounds. This method is sensitive enough for the routine analysis of penicillins at therapeutic urine levels, with limits of detection in the 1.5–15 ng mL^{-1} range and limits of quantification of 50 ng mL^{-1}. Recoveries in a micellar medium and a spiked urine matrix were in the 92.4–108.2% and 96–110% ranges, respectively. Finally, the method was successfully applied to determine these antibiotics in urine samples and pharmaceutical formulations.

1. Introduction

Penicillins are β-lactam antibiotics which have been the most widely used antimicrobial drugs for more than 80 years and are still considered one of the most important groups of antibiotics. They are used to treat respiratory tract infections that often result from the encroachment of sensitive bacteria [1]. They are clinically used against Gram-positive and Gram-negative bacteria [2]. The basic structure of penicillins, 6-aminopenicillanic acid, consists in a thiazolidine ring fused to a β-lactam ring with a side chain (e.g., amoxicillin presents a primary amine group in the side chain that does not exist in any other penicillin except epicillin and bacampicillin) [3].

Nowadays, amoxicillin (AMO), an α-amino-substituted β-lactam antibiotic, is the most commonly used antibiotic because of its broad spectrum and low cost. AMO has a moderate polarity (octanol–water partition coefficient, $\log P_{o/w} = 0.87$), and its dissociation constants are $pKa = 2.7$, 7.5 and 9.6 [3,4]. After oral administration, AMO is rapidly absorbed. About 60% of an oral dose is excreted in the urine as an unchanged drug in 6 h, while 20% is excreted as the inactive metabolite, penicilloic acid, in the same period. Ampicillin (AMP) is used to treat infections of the intestinal, urinary and respiratory tracts. AMP exhibits lower antibacterial activity than AMO, has a moderate polarity ($\log P_{o/w} = 1.06$), and its dissociation constants are $pKa = 2.5$ and 7.1 [3,4]. AMP is readily, but incompletely, absorbed after oral administration. About 30% of an oral dose is excreted in the urine as an unchanged drug in 6 h, and about 10% is excreted as penicilloic acid. Cloxacillin (CLO) is a hydrophobic compound ($\log P_{o/w} = 2.5$) with a dissociation constant of $pKa = 2.7$ [3]. It is incompletely absorbed after oral administration and about 35% of an oral dose is excreted unchanged in the urine in 12 h, while around 11% is excreted as penicilloic acid. Finally, dicloxacillin (DIC) has very similar chemical characteristics ($\log P_{o/w} = 2.91$) to CLO [3]. CLO and DIC belong to the penicillinase-resistant penicillin group, while AMO and AMP belong to the broad-spectrum penicillins. Fig. 1 shows the chemical structures of the four antibiotics studied.

Since the discovery of penicillin antibiotics, several analytical techniques have been developed for their analysis. Microbiological assays prove either insensitive and slow or non-quantitative microbiological screening type methods [5]. Colorimetric assays are also non-specific, which therefore limits their frequent application

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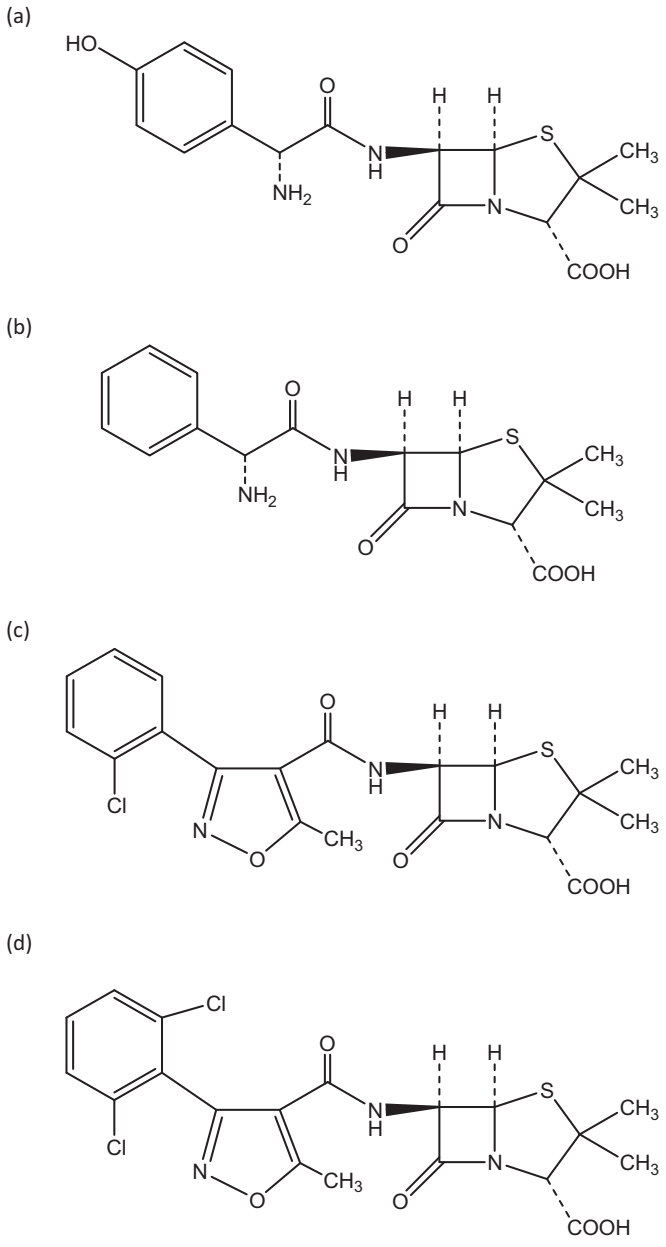

Fig. 1. Chemical structures of amoxicillin (a), ampicillin (b), cloxacillin (c) and dicloxacillin (d).

[6]. High-performance liquid chromatography (HPLC) is currently the most widely used technique for analysing antibiotics because it combines specificity with sensitivity, reproducibility and cost-effectiveness. Although this technique has been mainly applied to the analysis of amoxicillin, the simultaneous analysis of a range of penicillins has also been developed.

Several HPLC methods have been reported for the determination of AMO [7–20], AMP [11–23], CLO [11–17,19,21,24–26] and DIC [11–16,20,23–27] in biological fluids (serum, plasma, and urine), pharmaceutical formulations, food products of animal origin, among others. UV, fluorometric, electrochemical and, in some cases, mass spectrometry detection have been used. The sample preparation techniques reported for plasma or serum include deproteinisation [12,23] and extraction [8,9,11,13,14,18]. Few methods describe pre-column [15,26] or post-column derivatisation [10] with fluorescent detection, or photochemical degradation [24] with the electrochemical detection of penicillins. Moreover, other methods have been established for the analysis of penicillins in pharmaceuticals [7,12,28,29], biological fluids [29,30] and food products [31–33]. Penicillins have also been analysed by capillary electrophoresis [34–37] and micellar electrokinetic capillary chromatography [38,39].

Drug analysis has been greatly enhanced through HPLC technology. However, the assay of drugs in physiological fluids presents many problems. Frequently, drugs are at a very low concentration, strongly bound to proteins and in a complex matrix where interference from numerous endogenous compounds is expected. The high-molecular-mass proteins in these samples are particularly troublesome since they tend to denature and precipitate in the injection valve or at the column head. This clogs the system, and leads to a rapid degradation of chromatographic performance and an increase in back-pressure system. Several approaches have been adopted to facilitate sample preparation for physiological fluids. One simple approach is to precipitate proteins by organics or sodium hydroxide, or to remove them by ultrafiltration. More often than not, other separation steps are required such as liquid–liquid or solid-phase extraction from the matrix, re-extraction and evaporation. All these procedures are time-consuming, require lots of repetitive work, and possibly allow the introduction of additional sources of error because of the incomplete recovery of drugs. An additional problem of extraction processes that should be considered is the use and disposal of toxic solvents and chemicals, which are dangerous not only to the analyst, but also to the environment. By way of example, Baranowska et al. [15] transferred 0.75 mL of human urine sample to a volumetric flask (10 mL), and then sodium hydroxide was added to adjust the required pH. The solution was then mixed with 1.5 mL acetonitrile and 1.5 mL methanol. Next, it was completed to the mark by adding water. After shaking for 1 min, the sample was transferred to a centrifuge tube and centrifuged for 15 min at 6500 rpm. Finally, the clear supernatant was injected directly into the HPLC column.

Micellar liquid chromatography (MLC), which uses a surfactant solution as the mobile phase and whose concentration is above the critical micellar concentration, is an alternative to conventional HPLC. MLC allows the analysis of complex matrices without the aid of extraction since micelles tend to bind proteins competitively, thus leading to protein-bound drugs and proteins. Proteins are solubilised and harmlessly washed away with the solvent front instead of being precipitated into the column. Several selected studies show the use of MLC with direct injection when applied to the determination of drugs in pharmaceuticals [40], serum [41] and urine [42]. The proposed method permits the direct injection of the sample without the need for an extraction step to precipitate the proteins, thus avoiding analyte loss, time-consuming practices and, consequently, lowering the procedure cost.

The purpose of this study was to develop and validate a new MLC method for the simple, rapid and specific determination and quantification of four penicillins (AMO, AMP, CLO and DIC) in biological fluids and pharmaceutical formulations. The method was validated according to the Food and Drug Administration (FDA) guideline [43]. The objective of developing such a method is to apply it to routine analyses in the quality control process of pharmaceutical samples and in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Amoxicillin and ampicillin were purchased from Sigma (St. Louis, MO, USA). Cloxacillin and dicloxacillin were obtained from MP Biomedicals (Solon, Ohio, USA). Sodium dodecyl sulphate (SDS)

was acquired from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and 1-propanol came from Scharlab (Barcelona, Spain). Hydrochloric acid and ethanol were obtained from J.T. Baker (Deventer, The Netherlands). Ultrapure water was used throughout (Millipore S.A.S., Molsheim, France). The pharmaceuticals were purchased in a local Spanish pharmacy, except Pathocil which was directly supplied by Sandoz (Princeton, New Jersey, USA).

2.2. Instrumentation

The analytical balance used was an AX105 Delta-Range (Mettler-Toledo, Greifensee, Switzerland). A vortex shaker and sonication unit (Selecta, Barcelona) were employed for sample pretreatment. The pH of the solutions was measured with a potentiometer model GLP 22 (Crison, Barcelona) equipped with a combined Ag/AgCl/glass electrode. The chromatographic system (Agilent Technologies, Series 1100, Palo Alto, CA, USA) was equipped with a quaternary pump, thermostatted autosampler tray and column compartments, and a diode-array detector (range 190–700 nm). Several columns were assayed: Zorbax C18, Kromasil C18 and Hypersil Phenyl (Scharlab), all of which were the same size (150 mm × 4.6 mm) and with a 5-μm particle size.

2.3. Preparation of the mobile phase, standard and urine samples

The micellar mobile phases were prepared by dissolving SDS in ultrapure water, and they were buffered with 0.01 M sodium dihydrogen phosphate at pH 3 using diluted hydrochloric acid. Finally, propanol was added to obtain the desired concentration of the organic solvent to be then topped up with ultrapure water to the mark on the volumetric flask. All the mobile phases were filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA).

Individual stock solutions of 50 $\mu g\,mL^{-1}$ of each antibiotic (AMO, AMP, CLO and DIC) were prepared by dissolving each one in a few millilitres of ethanol with the aid of an ultrasonic bath, and were finally topped up with 0.05 M SDS solution at pH 3.

Urine samples were collected in a Urine Collection Cup (BD Vacutainer Systems, Plymouth, UK). For optimisation purposes, urine stock solutions spiked with 50 $\mu g\,mL^{-1}$ of the four penicillins were prepared by diluting urine by a 1:50 factor with 0.05 M SDS at pH 3. In the method validation, different blank urine samples were spiked at different concentrations (e.g., in the calibration curve, accuracy and precision, etc.), and were afterwards diluted to a 1:50 factor prior to the analysis. Thus, a simulation of a real situation is achieved. Solutions were prepared daily, protected from direct light and stored at 4 °C until use.

Patients' urine samples were injected directly into the chromatographic system after the aforementioned dilution with the surfactant solution. All the samples were filtered directly into the autosampler vials through 0.45 μm nylon membranes before their analysis.

2.4. Chromatographic conditions

Separation was performed in a reversed phase Zorbax C18 column (Scharlab) (150 mm × 4.6 mm, 5-μm particle size) thermostatted at 25 °C. The composition of the selected mobile phase was 0.11 M SDS–6% (v/v) propanol–0.01 M NaH_2PO_4 buffered at pH 3. The flow rate, injection volume and UV wavelength were 1 $mL\,min^{-1}$, 20 μL and 210 nm, respectively. Under these conditions, the complete analysis time was less than 16 min. Chromatographic signals were acquired and processed with an Agilent ChemStation (Rev. B.03.01). The measurement of the peak properties and the optimisation of mobile phase composition were assisted by the Michrom software [44].

2.5. Sample preparation for pharmaceutical formulations determination

The pharmaceuticals analysed were tablets and enteric-coated capsules containing from 250 to 875 mg of the corresponding penicillin and excipients. The average weight per tablet/capsule content was calculated from 10 units. The tablet or capsule content was ground and reduced to a homogeneous fine powder in a mortar. Several portions of this powder were accurately weighed and sonicated in the presence of ethanol (5%, v/v, of the final content) in an ultrasonic bath. A 0.05 M SDS solution at pH 3 was added to favour the extraction of the analyte, for which the ultrasonic bath was used. A dilution was then made with the micellar solution at a final antibiotic concentration of 20 $\mu g\,mL^{-1}$. Since the excipients in the tablets and capsules were not soluble in the micellar medium, sample solutions were filtered through 0.45 μm nylon membranes before being injected into the chromatographic system.

3. Results and discussion

3.1. pH selection of the mobile phase

The structures of the investigated antibiotics are shown in Fig. 1. As this figure depicts, AMO and AMP are cationic under acidic conditions because of the protonation of the amine group, zwitterionic in the neutral medium given the protonation of the amine group and the deprotonation of the carboxylic acid group, and anionic under basic conditions due to the deprotonation of the carboxylic acid group. However, CLO and DIC are neutral at acidic pH values and anionic at basic pH values.

It should be taken into account that the stability of penicillins greatly depends on pH, and their maximum stability tends to be in the region of pH 6.0–7.0 [45]. Thus, the pH of the mobile phase should be in this range. However, the retention factor of the four compounds under study lowered when the pH of the mobile phase increased, and eluted at the dead volume at pH 7 (data not shown) because of the repulsion between the negatively charged antibiotics and the anionic surfactant. Thus, pH 3 was finally selected since the best resolution and adequate retention times were obtained for all four compounds. The next step was to study the stability of the antibiotics in this medium.

3.2. Antibiotics stability

Most penicillins are practically unstable under strong acidic conditions since their molecules are subject to several hydrolytic reactions, while the structural identity of their molecules are maintained under neutral or alkaline conditions. However, and as commented above, only the mobile phases buffered at pH 3 provide an adequate control on the retention of penicillins, and their stability should be checked under these conditions. To assess the stability of penicillins after sample preparation, blank urine samples were spiked with AMO, AMP, CLO and DIC. Stability was investigated at pH 3 at room temperature and at low temperature (4 °C) using the mobile phase 0.11 M SDS–6% propanol. Degradation of AMO, AMP, CLO at pH 3, after two days of storage, was confirmed by the new peaks overlapped with the peaks of the analytes that emerged in the chromatograms. DIC, however, decomposed at only 3 h after preparation. All the solutions were prepared freshly on a daily basis and kept at a low temperature (4 °C) until required. Penicillin solutions were analysed immediately after preparation and at selected time intervals after storage throughout the study period. After 6 h, 24 h and 42 h at 4 °C temperature, the decompositions of AMO, AMP, CLO and DIC were 0%, 1% and 35%; 0%, 0.2% and 18%; 1%, 10% and 30%; and 53%, 74% and 78%, respectively. Furthermore, decomposition

Table 1
Chromatographic parameters (retention factors, *k*, efficiencies, *N* and asymmetry factors, *B*/*A*) obtained for the penicillins in the Kromasil and Zorbax columns.

SDS (M)	Propanol (%, v/v)	AMO			AMP			CLO			DIC		
		k	*N*	*B*/*A*	*k*	*N*	*B*/*A*	*k*	*N*	*B*/*A*	*k*	*N*	*B*/*A*
Kromasil C18													
0.05	2.5	12.0	1100	1.2	15.9	700	1.4	9.4	2000	0.8	20.5	200	1.3
0.05	12.5	6.9	200	1.9	13.1	100	1.4	6.8	200	1.1	10.8	800	1.5
0.1	7.5	3.6	400	1.4	12.5	1000	1.2	6.2	700	1.4	8.2	800	1.4
0.15	2.5	3.8	800	1.2	14.2	1100	1.1	9.6	1000	1.1	12.5	900	1.2
0.15	12.5	2.1	70	2.3	7.8	700	1.5	3.3	300	1.4	4.4	500	1.4
Zorbax C18													
0.05	2.5	6.4	1100	1.1	29.3	2200	1.1	17.1	1900	1.1	23.8	1300	1.2
0.05	7.5	4.3	2600	1.7	21.6	900	0.9	4.3	700	1.3	8.8	900	1.1
0.1	5	2.4	1600	1.8	11.1	500	1.8	5.6	300	1.9	8.0	500	1.7
0.15	2.5	2.1	500	1.4	8.2	800	1.4	5.9	800	1.3	7.8	800	1.3
0.15	7.5	1.7	1300	1.0	6.2	800	1.5	2.8	300	1.1	3.9	400	1.5

between 5% (AMP) and 90% (DIC) was observed at room temperature after 3 h, which was confirmed by a new overlapping peak emerging in the chromatogram. Under such conditions, samples should be analysed within 3 h. Urine samples may be stored for several weeks at −20 °C with no noticeable degradation.

3.3. Chromatographic optimisation: column and mobile phase selection

Three columns (Kromasil C18, Zorbax C18 and phenyl) were tested to select the best analysis conditions. The following characteristics were common for all the columns: pore size (100 Å), particle size (5 μm), and length and internal diameter (150 mm × 4.6 mm). C18 stationary phases are still the most popular and most widely used silica-based packages for reversed-phase liquid chromatography thanks to their ability to separate a wide range of solutes with good resolution, selectivity and column efficiency. Phenyl-type stationary phases have proven useful for the separation of species in which π–π interactions may be exploited during the retention process. This is the particular case of highly aromatic compounds.

Modification of the stationary phase by an adsorbed surfactant can have profound implications with regard to retention, efficiency and asymmetry in MLC. In addition, most analytical procedures require the addition of an organic solvent, which lowers retention times and increases peak efficiencies. A short-chain alcohol, such as propanol, butanol or pentanol, is normally employed. In this case, propanol was selected as it allows the complete resolution of the four compounds in an adequate analysis time. The AMO peak and the protein band were fully resolved when propanol was used. However, when butanol or pentanol are employed, an overlapping of the penicillin peaks with those of the urine matrix can be expected. It is important to note that the selection of alcohol is directly related to the polarity of the studied compounds.

The stock standard and urine stock solutions of the four penicillins were injected into the selected columns using hybrid micellar mobile phases at different SDS and propanol concentrations. The retention factors (*k*), efficiencies (*N*) and asymmetry factors (*B*/*A*) obtained are summarised in Table 1. The results of the phenyl column are not shown since several compounds did not elute in most of the mobile phases assayed. Thus, this column was disregarded. It can be observed that the retention factors of the antibiotics decreased when the surfactant and the organic solvent concentrations increased. Thus, two optimisation processes were then carried out: one for the Kromasil C18 column and another for the Zorbax C18 column.

An interpretive optimisation strategy was followed to select the most adequate column and the best surfactant and organic solvent concentrations for the simultaneous analysis of the four penicillins. The experimental design consisting in five mobile phases (four located in the corners of a rectangular factor space and the fifth in its centre) buffered at pH 3 was used to examine the chromatographic behaviour of the four antibiotics. Thus, for the Kromasil column they were injected into the following compositions of SDS (M)–propanol (%): 0.05–2.5, 0.05–12.5, 0.10–7.5, 0.15–2.5 and 0.15–12.5. These concentration ratios were selected for the purpose of avoiding excessive retention times or elution near the void volume. However, the maximum concentration of propanol for the Zorbax column was 7.5% instead of 12.5%, because the elution for all the penicillins was close to the dead time when working at a propanol concentration above 7.5%, which introduces a high error in the predictions of the later modelling step. The chromatographic data obtained (*k*, *N* and *B*/*A*) were processed with the Michrom software [44]. This software enables any changes in the chromatograms to be graphically depicted when the user progressively varies the concentration of the surfactant and organic solvent. The retention of the compounds was modelled according to [46]:

$$k = \frac{K_{AS}(1/1 + K_{AD}\,\varphi)}{1 + K_{AM}((1 + K_{MD}\,\varphi)/(1 + K_{AD}\,\varphi))[M]} \quad (1)$$

where [*M*] and φ are the concentrations of the surfactant and the modifier, respectively; K_{AS} and K_{AM} correspond to the equilibria between the solute in bulk water and the stationary phase or micelle, respectively; K_{AD}, K_{SD}, and K_{MD} measure the relative variation in the concentration of solute in bulk water, stationary phase and micelles, respectively, given the presence of the modifier, as compared to a pure micellar solution (with no modifier).

A global resolution criterion based on the equations developed by Lapasió et al. [47] was used to predict the chromatograms. This criterion measures the non-overlapped fractions for each individual peak and facilitates the understanding of the information obtained in the optimisation process. Different regions of the variable space are often associated with different critical peak-pairs. Thus, the resolution of a multicomponent mixture requires an analysis that involves all the components in the whole variable space. Inspection of the contour maps of global resolution will allow the robustness of the optimum to be evaluated.

Fig. 2a and b show the contour maps of the resolution for the antibiotics when injected into the Zorbax or the Kromasil columns, respectively. They were drawn using the chromatographic data (*k*, *N*, and *B*/*A*) obtained with the five mobile phases indicated above. For the Kromasil C18 column, most of the penicillin peaks overlapped with the urine protein band and/or the endogenous compounds at several mobile phase compositions, giving rise to a poor resolution in almost all the space defined by the variables (Fig. 2b). Only a small region was suitable for the separation of compounds, while complete baseline separation was not possible. Furthermore, several inversions in the elution order of the com-

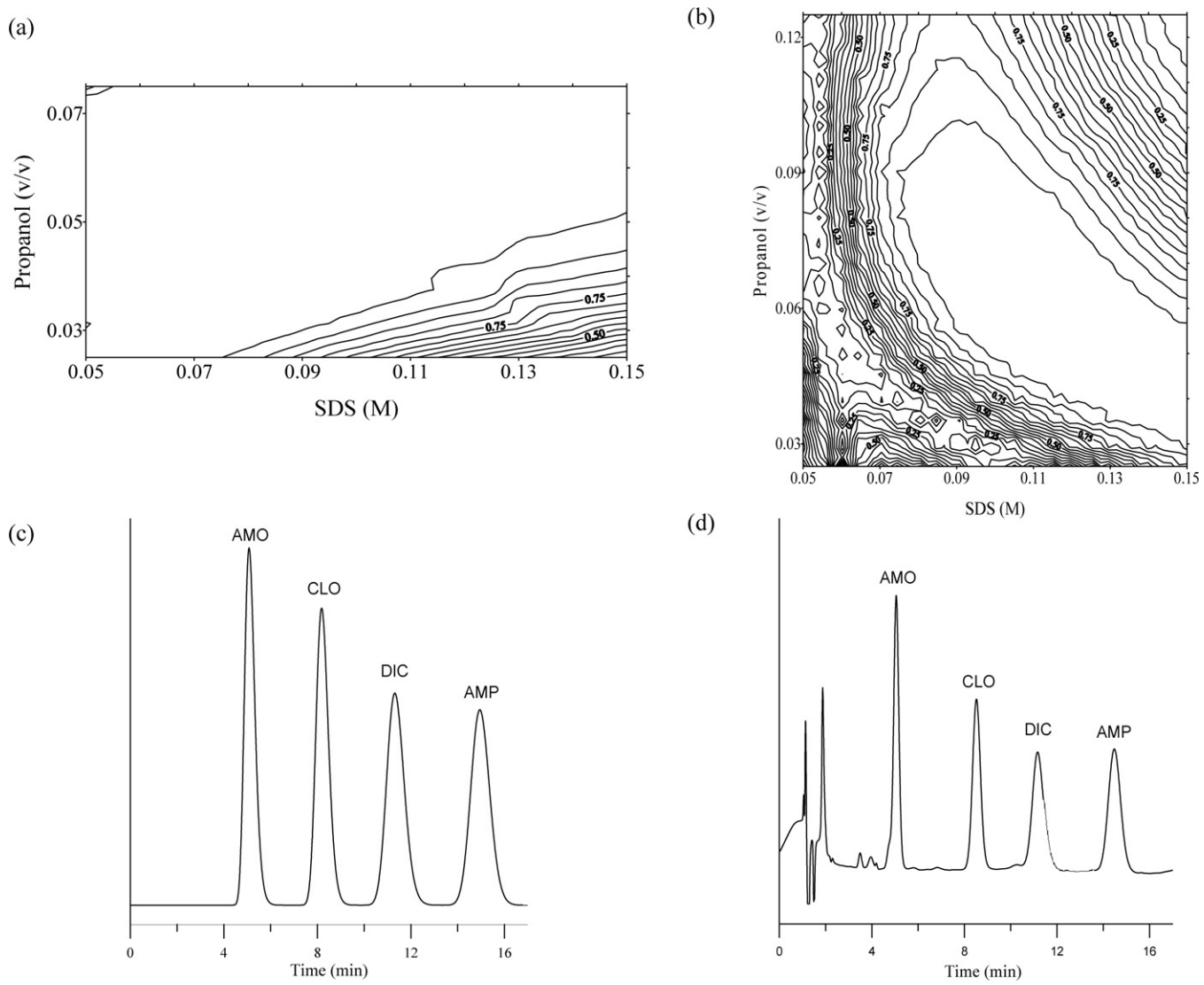

Fig. 2. Contour maps of global resolution for the separation of the four antibiotics using the Kromasil (a) or Zorbax column (b); simulated (c), and real chromatograms (d) for a mixture of AMO, AMP, CLO and DIC (10 μg mL^{-1}). Mobile phase: 0.11 M SDS-6% propanol-pH 3, flow rate: 1 mL min^{-1}, UV detection at 210 nm.

pounds were observed throughout the factor space when both the surfactant and the organic solvent concentrations were changed (e.g., AMO and AMP on the one hand, and CLO and DIC on the other). Regarding the Zorbax C18 column, adequate retention times and efficiencies were obtained for the four compounds with no interferences by the urine matrix. In this case, the elution order of the antibiotics was the same in the whole variable space. Fig. 2a indicates that optimum resolution (values near one) is obtained for a broad region of SDS and propanol concentrations, which is scarcely modified except in two regions, which dramatically decrease due to an overlapping between peaks: (a) in a very narrow space at a low SDS concentration (0.05 M) and a high alcohol concentration (7.5%) where AMO and CLO overlapped; (b) in a space limited for the SDS concentrations between 0.075 M and 0.15 M, and up to 5% propanol, where DIC and AMP fully overlapped, or even a partial overlapping among AMP, CLO, and DIC occurs. Since the Zorbax column provides the best robustness (a major region where the compounds can be fully resolved at the baseline level), it was selected for further work.

A mobile phase of 0.11 M SDS–6% propanol–0.01 M NaH_2PO_4 at pH 3 was selected to analyse the antibiotics since it permitted their determination with analysis times below 16 min with good chromatographic resolution (R = 0.993). No interferences by the protein band or endogenous compounds were observed. The chromatographic parameters (k, N and B/A) obtained with this mobile phase for the antibiotics were: 2.4, 1600 and 1.4; 11.1, 800 and 1.8; 5.6, 800 and 1.8; and 8.0, 800 and 1.7 for AMO, AMP, CLO and DIC, respectively. Fig. 2c shows the simulated chromatogram for the mixture of the four penicillins in the optimum mobile. The agreement between the simulated and experimental chromatograms is quite good (Fig. 2c and d).

3.4. Urine blank behaviour

The background signal of the urine samples, due to the proteins (wide band at the head of the chromatograms) and several endogenous compounds (peaks at diverse retention times), can seriously affect the detection of the antibiotics. However, diluting the urine sample before its injection reduced the width of the protein band and of some endogenous peaks, thus allowing the detection of those drugs that could overlap in the original sample, while also benefiting the column by increasing its useful life.

Several urine blanks were injected directly into the micellar chromatographic system after suitable dilution with a view to assessing the background signal. For all the penicillins, the sensitivity achieved after dilution in a 1:50 factor was adequate for their detection in urine, at least up to 12–14 h post-ingestion of the

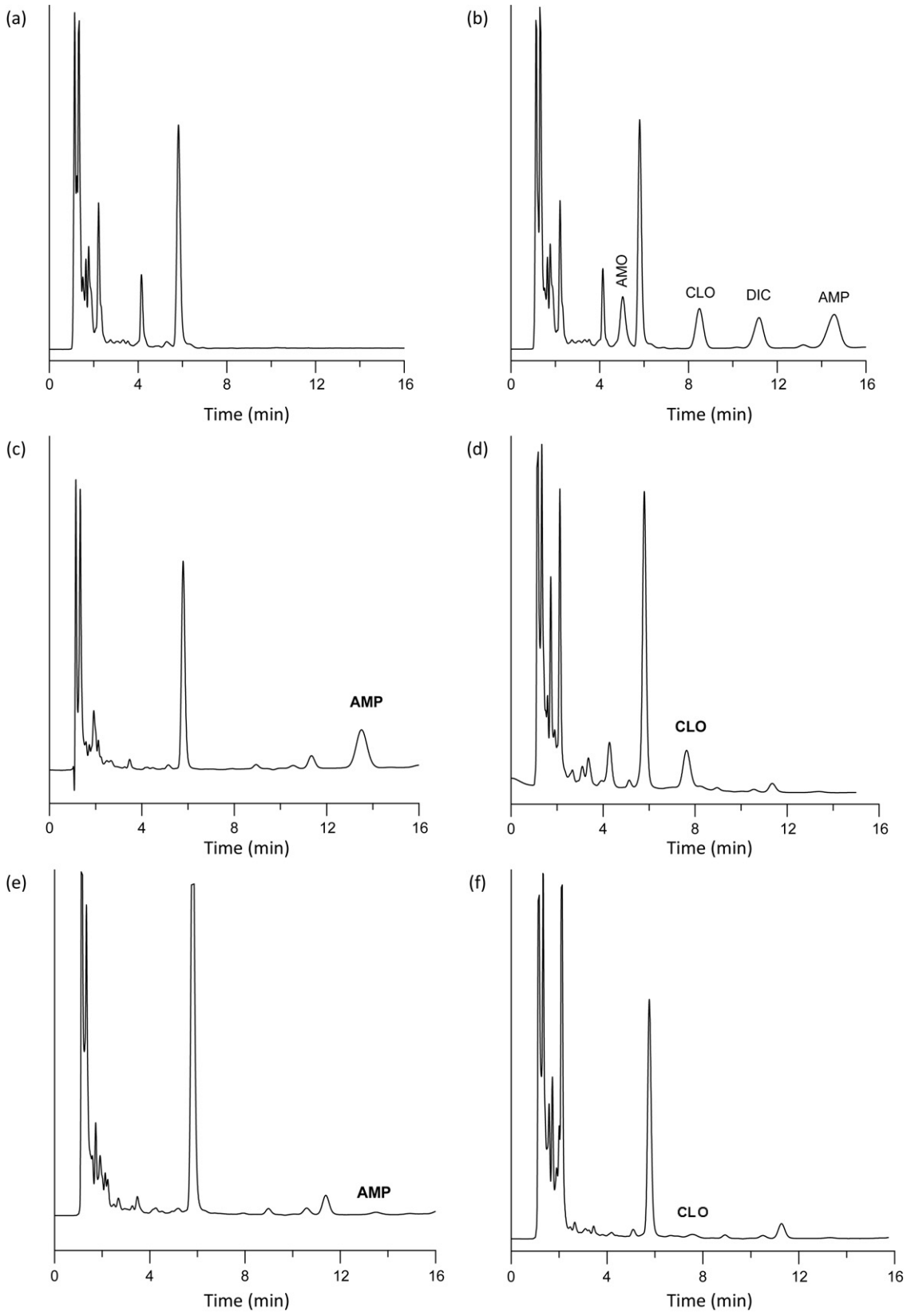

Fig. 3. Chromatograms of (a) urine blank, (b) urine spiked with the four penicillins (10 μg mL^{-1}); (c) AMP and (d) CLO excreted in urine as an unchanged drug 2 h after oral ingestion; and (e) AMP and (f) CLO excreted 12 h after oral ingestion. All the samples were diluted at the 1:50 factor with 0.05 M SDS-pH 3. See Fig. 2 for the chromatographic conditions.

antibiotics. The profiles of both the protein band and the endogenous compounds for all the samples were similar to those shown in Fig. 3a. No additional peaks were found in the subsequent analyses. These results are compatible with routine analysis requirements.

3.5. Method Validation

The Food and Drug Administration (FDA) guideline was followed to validate the method [43]. The parameters evaluated were selectivity, linearity, limits of detection (LOD) and limits of quantification (LOQ), precision and accuracy, recovery and robustness.

3.5.1. Selectivity

Six drug-free urine samples were selected as the controls and processed directly in the chromatographic system after convenient dilution. They were then analysed to determine the extent to which the endogenous components may contribute to interfere with the drug's retention time. No interference by the endogenous compounds at the penicillins' retention times was noted in the physiological matrices studied when the drug-free urine samples were compared with a urine-spiked sample (Fig. 3a and b).

3.5.2. Linearity

Calibration curves were constructed using the areas of the chromatographic peaks obtained at eight different concentrations (six replicates) in the range of 0.05–25 μg mL^{-1} for the four penicillins and in the micellar media (standard calibration curve using only standard solutions) and urine (1:50 dilution factor). Calibration curves were constructed using the same amount of urine (blank), and afterwards different concentrations of the antibiotics were added. Thus, the matrix proportion was the same in the whole linear range studied. To study the variability of the calibration parameters, curves were obtained for 5 days over a 2-month period for a different set of standards. The slopes and intercepts were determined by the least-squares linear regression analysis method. The results in both matrices were similar (relative errors were below 1% in all cases). The correlation found was linear for all the antibiotics in the studied range of concentrations. The adjusted equations, determination coefficients (R^2) and coefficient variations (%) of the calibration slopes for each penicillin in urine are:

Amoxicillin	$y = 0.589x + 0.018$	$R^2 = 0.994$	CV(%) = 1.6
Ampicillin	$y = 0.557x - 0.034$	$R^2 = 0.997$	CV(%) = 1.2
Cloxacillin	$y = 0.436x - 0.031$	$R^2 = 0.994$	CV(%) = 6.7
Dicloxacillin	$y = 0.436x - 0.032$	$R^2 = 0.994$	CV(%) = 1.6

3.5.3. Limits of detection and quantification

The LODs for AMO, AMP, CLO and DIC in micellar and urine samples ($n = 10$) were determined with the *3s criterion* (3s/b) [43] (three times the standard deviation of the lowest concentration solution included in the calibration divided by the slope of the calibration curve) using a series of 10 solutions of a low concentration. The results are based on not only the standard deviation of the response, but the slope of a specific calibration curve containing the analyte. The LODs (ng mL^{-1}) calculated from the standard calibration curve (prepared in SDS) and the urine calibration curve were: 1.5/4, 3.7/4.4, 10.4/10.4, and 11/15 for AMO, AMP, CLO and DIC, respectively. The LOQs for the four compounds in both matrices were selected as the lowest concentration used in the calibration curve (50 ng mL^{-1}).

3.5.4. Precision and accuracy

The intra- and inter-day precisions of the method were determined over a range of 0.5–10 μg mL^{-1} for all four penicillins in micellar media and urine-SDS (1:50 dilution factor) at four different concentrations. The results in both matrices were similar. The intra-day analysis was determined by injecting these test solutions six times on the same day, while the inter-day analyses correspond to the average of five measurements of the intra-day values taken on 5 days over a 3-month period performed by different analysts and equipment at the same four concentrations. Assay precision was assessed by expressing the SD (standard deviation) of the repeated measurements as a percentage of the mean value. Accuracy was investigated by comparing the concentrations of the measured peaks as calculated from the calibration lines by the linear regression analysis versus the nominal (added) concentration. The data show good precision (expressed as relative standard deviation, RSD, %) and accuracy (expressed as relative error, E_r, %) for this method, with values below 6% and 9% for all four analytes in urine-SDS, respectively (Table 2). Thus, these results prove to be good enough for routine analyses.

3.5.5. Robustness

In order to study the robustness of the method, six replicates of the standard solution of each penicillin at 5 μg mL^{-1} were injected into the chromatographic system in a set of mobile phases, where the following parameters were slightly changed: SDS concentration, propanol (%), flow rate, and pH. The results shown in Table 3 indicate that the slight variations to these parameters do not significantly alter the retention factor or the peak area of the compounds under study (average RSD ≈ 6%). As expected, flow rate is the

Table 2
Intra-day ($n = 6$) and inter-day (over a period of five consecutive days) precisions, and accuracy data for the determination of the examined penicillins in spiked urine samples.

Analyte	Added (μg mL^{-1})	Intra-day			Inter-day		
		Found (mean ± SD) (μg mL^{-1})	E_r (%)	Intra-day RSD (%)	Found (mean ± SD) (μg mL^{-1})	E_r (%)	Inter-day RSD (%)
AMO	0.5	0.522 ± 0.007	4.4	1.3	0.517 ± 0.008	3.4	1.5
	1	0.955 ± 0.021	4.5	2.2	0.94 ± 0.03	6.0	3.2
	5	4.89 ± 0.19	2.2	3.9	4.94 ± 0.08	1.2	1.6
	10	10.36 ± 0.06	3.6	0.6	10.6 ± 0.4	6.0	3.8
AMP	0.5	0.525 ± 0.018	5.0	3.4	0.534 ± 0.013	6.8	3.6
	1	0.926 ± 0.018	7.4	1.9	0.918 ± 0.011	8.2	1.2
	5	5.22 ± 0.23	4.4	4.4	5.3 ± 0.3	6.0	5.7
	10	9.82 ± 0.06	1.8	0.6	10.2 ± 0.6	2.0	5.9
CLO	0.5	0.519 ± 0.010	3.8	2.0	0.533 ± 0.019	6.6	3.6
	1	0.933 ± 0.016	6.7	1.7	0.96 ± 0.03	4.0	3.1
	5	4.62 ± 0.07	7.6	1.5	4.84 ± 0.23	3.2	4.8
	10	10.827 ± 0.012	8.3	0.1	10.4 ± 0.5	4.0	4.8
DIC	0.5	0.518 ± 0.011	3.6	2.1	0.541 ± 0.020	8.2	3.7
	1	0.93 ± 0.03	7.0	3.2	0.97 ± 0.03	3.0	3.1
	5	5.28 ± 0.04	5.6	0.7	5.3 ± 0.3	6.0	5.7
	10	10.63 ± 0.11	6.3	1.0	10.61 ± 0.14	6.1	1.3

Table 3
Robustness evaluation of the developed MLC method.

Chromatographic changes	Level	AMO		AMP		CLO		DIC	
		t_R (min)	Area	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area
A: Flow rate (mL/min)									
0.9	−0.1	5.2	3.02	14.6	3.11	8.4	1.96	11.1	1.3
1	0	4.6	3.10	13.1	3.11	7.5	1.92	10.1	1.5
1.1	+0.1	4.6	2.94	11.9	3.11	7.3	2.13	9.2	1.4
Mean ± SD		4.8 ± 0.3	3.02 ± 0.08	13.2 ± 1.4	3.1 ± 0.0	7.7 ± 0.6	2.00 ± 0.11	10.1 ± 1.0	1.42 ± 0.12
RSD (%)		6.3	2.6	10.6	0.0	7.8	5.5	9.9	8.4
B: SDS (M)									
0.105	−0.005	4.7	2.88	13.5	3.16	8.7	1.82	11.2	1.6
0.11	0	4.6	3.10	13.1	3.11	7.5	1.92	10.1	1.5
0.115	+0.005	4.5	2.88	13.1	3.30	7.3	1.67	9.5	1.6
Mean ± SD		4.63 ± 0.11	2.99 ± 0.06	13.23 ± 0.23	3.15 ± 0.04	7.8 ± 0.7	1.81 ± 0.12	10.3 ± 0.9	1.37 ± 0.03
RSD (%)		2.4	2.0	1.7	1.3	9.0	6.6	8.7	2.2
C: Propanol (%, v/v)									
5.9	−0.1	4.4	3.09	13.0	3.16	7.6	1.98	10.0	1.56
6	0	4.6	3.10	13.1	3.11	7.5	1.92	10.1	1.55
6.1	+0.1	4.5	2.96	13.1	3.22	7.2	1.93	9.5	1.63
Mean ± SD		4.51 ± 0.12	2.99 ± 0.06	13.07 ± 0.06	3.14 ± 0.03	7.43 ± 0.21	1.95 ± 0.06	9.9 ± 0.3	1.37 ± 0.03
RSD (%)		2.7	2.0	0.5	1.0	2.8	3.1	3.0	2.2
D: pH									
2.9	−0.1	5.2	3.08	13.6	3.07	7.1	1.99	10.0	1.54
3	0	4.6	3.10	13.1	3.11	7.5	1.92	10.1	1.55
3.1	+0.1	4.2	3.04	11.4	3.02	7.3	1.96	8.6	1.48
Mean ± SD		4.7 ± 0.5	3.07 ± 0.03	12.7 ± 1.2	3.07 ± 0.04	7.3 ± 0.2	1.85 ± 0.12	9.6 ± 0.8	1.34 ± 0.07
RSD (%)		10.6	1.0	9.4	1.3	2.7	6.5	8.3	5.2

parameter that modifies the retention times or peak areas to the greatest extent (average RSD ≈ 8.7%) because the conditions of this parameter were changed by ±10%, and this variation could be significant for some of the studied compounds. A variation of ±5% to the flow rate conditions could prove more appropriate, and would originate lower RSD values.

3.5.6. Analysis of the marketed pharmaceutical formulations

Calibration curves were constructed as shown in Section 3.5.2. Ten commercial pharmaceuticals containing AMO, AMP, CLO and DIC were analysed, most of which are prescribed in Spain (Table 4). Ten samples of each pharmaceutical formulation were analysed, and six replicate injections were performed to obtain average antibiotic concentration values. Table 4 provides the declared and found contents, together with the label claim percentages, which are around 102.2%, while the residual standard deviations fall in the 0.07–1.8% range. Excellent accuracy (0.4–5.2%) and precision (0.1–2.4%) are observed for all the drug formulations. The results are in agreement with the manufactures' declared contents. The excipients were non-soluble, and were removed from the solution by filtration to prevent them from interfering in the analysis.

3.5.7. Determination of spiked biological samples

Recoveries of the four penicillins were determined by spiking the drug-free urine samples (1:50 factor dilution) with solutions of 0.05 M SDS at pH 3 containing known amounts of the drug at four different concentrations (0.5, 1, 5 and 10 μg mL^{-1}). For comparative purposes, recoveries were also obtained in the SDS solutions. Spiked samples were processed and analysed following the procedure described above. Absolute recovery was measured by comparing the peak area of the spiked urine matrices with the non-spiked urine samples. The mean recovery rates for AMO were 100.2% in SDS and 104.6% in urine; for AMP, 100.2% in SDS and 102% in urine; for CLO, 99.0% in SDS and 99.2% in urine; and finally for DIC, 102.1% in SDS and 104.2% in urine. The data obtained show satisfactory recoveries for all four penicillin antibiotics.

It should be pointed out that the results in Sections 3.5.4 and 3.5.7 were obtained from two different and independent sets of experiments. Accuracy and precision were calculated using a batch of urine-spiked solutions with the penicillins, and the recovery data were obtained from a completely new batch, both prepared on different days. Intra- and inter-day precisions and accuracy values were performed and obtained over 5 days during a 3-month period, and recoveries were calculated on the same day by injecting the corresponding solutions several times and then calculating the average. The same concentrations were used in these sections. Sometimes accuracy and recovery are obtained from the same experiment, but we preferred to perform these studies independently to prove the usefulness of the developed procedure.

3.5.8. Drug control in real samples

Finally, the procedure was applied in pharmacokinetic studies of urine samples to demonstrate its usefulness. Urinary studies were conducted following the oral administration of three independent

Table 4
Precision and accuracy data from analysing the penicillins in drug formulations (n = 6).

Pharmaceutical (laboratory)	Composition (mg)	Found (mg)	Label claim (%)	E_r (%)	RSD (%)
Amoxicillin Normon (Laboratorios Normon, Spain)	Amoxicillin (875), excipients (tablet)	878.4 ± 0.6	100.4	0.4	0.1
Amoxicillin Ratiopharm (Ratiopharm España, Spain)	Amoxicillin (750), excipients (tablet)	786.4 ± 2.2	104.9	4.9	0.3
Amoxicillin Cinfa (Laboratorios Cinfa, Spain)	Amoxicillin (500), excipients (capsule)	517.5 ± 0.9	103.5	3.5	0.2
Amoxicillin Ardine (Laboratorio Reig Jofré, Sapin)	Amoxicillin (500), excipients (capsule)	496 ± 3	99.2	0.8	0.6
Amoxicillin Sandoz (Sandoz farmacéutica, Spain)	Amoxicillin (500), excipients (tablet)	516.3 ± 1.1	103.3	3.3	0.2
Ampicillin Britapen (Laboratorio Reig Jofré)	Ampicillin (500), Excipients (capsule)	526 ± 11	105.2	5.2	2.1
Ampicillin Gobemicina (Laboratorio Normon)	Ampicillin (500), Excipients (capsule)	512 ± 9	102.4	2.4	1.8
Cloxacillin Anaclosil (Laboratorio Reig Jofré)	Cloxacillin (500), Excipients (capsule)	505 ± 9	101	1.0	1.8
Cloxacillin Orbenin (GlaxoSmithKline, Spain)	Cloxacillin (500), Excipients (capsule)	508 ± 12	101.6	1.5	2.4
Pathocil (Sandoz Inc., USA)	Dicloxacillin (250), Excipients (tablet)	251.4 ± 1.6	100.6	0.6	0.6

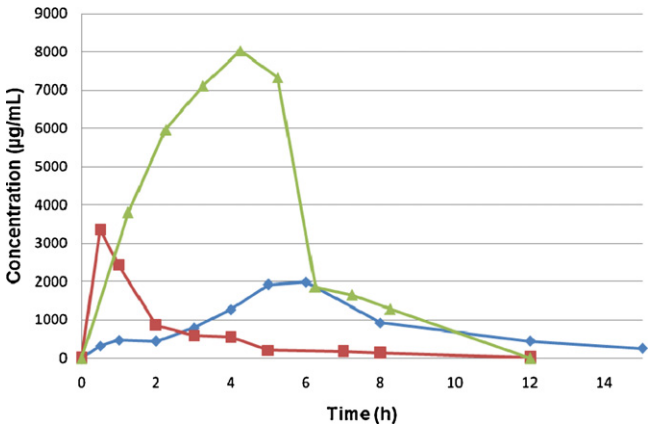

Fig. 4. Study of the urinary excretion of some penicillins after oral administration to healthy volunteers: AMO (▲), AMP (♦) and CLO (■). See Fig. 2 for the chromatographic conditions.

single doses of tablets containing 875 mg of AMO, 500 mg of AMP and 500 mg of CLO, respectively, to healthy volunteers. A sample was collected immediately before administering the drug to be used as a blank. Other urine samples were collected ten times a day, at appropriate times, and were refrigerated at +4 °C until analysed. Urine samples were diluted by the 1:50 factor, and injected directly into the chromatographic system with no treatments other than filtration. Fig. 4 shows the variation of the AMO, AMP, and CLO concentrations during urinary excretion, as determined in this work. The maximum AMO, AMP and CLO concentrations excreted in urine were found at 4 h, 6 h and 0.5 h after oral administration, respectively. The final quantity of AMO, AMP and CLO excreted after 6 h was 500 mg, 194 mg and 202 mg; that is, 57%, 39% and 40% of the ingested dose, respectively. These urinary excretion results are in agreement with bibliographical reports [3]. Fig. 3c and d reveal the chromatograms of AMP and CLO excreted 2 h after oral administration, while Fig. 3e and f depict the chromatograms of AMP and CLO excreted 12 h after oral administration. These results indicate that excretion studies can be performed under the proposed chromatographic conditions.

4. Conclusions

The MLC procedure described herein is useful to quantify four penicillins (amoxicillin, ampicillin, cloxacillin and dicloxacillin) with a total analysis time below 16 min. Compared to other methods developed for the determination of penicillins in urine samples, micellar mobile phases are less flammable, less expensive, less toxic, biodegradable, and can co-solubilise hydrophobic and hydrophilic analytes in this kind of matrices. The elution of hydrophobic and hydrophilic analytes in the same MLC run is possible without a gradient elution. One advantage of this procedure is the possibility of injecting urine samples directly into the chromatographic system with no previous treatment other than homogenisation, dilution and filtration, thus avoiding tedious extractions from matrices due to the solubilisation of proteins by the surfactant's micelles and monomers. The use of an interpretative optimisation strategy in MLC also makes it a more efficient and reliable mobile phase selection. The LOD values are in agreement with those reported in the literature and help to monitor these compounds in urine at therapeutic levels. However, urine was directly injected without a pre-treatment step, thus avoiding tedious extractions and possible sample loss. Validation was efficiently performed according to the FDA guideline with satisfactory results in the selectivity, linearity, precision, accuracy, recovery and robustness studies. This procedure also allows pharmacokinetic studies to be conducted. Pharmaceutical formulations containing penicillins were analysed and obtained good claim percentages (around 100%). The results reveal that the procedure is sensitive enough for the routine analyses of antibiotics in both biological and pharmaceutical applications.

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