Development of a direct and validated method of amoxicillin in physiological fluids using micellar liquid chromatography

Maria Rambla Alegre[1], Samuel Carda Broch[1], Josep Esteve Romero[1]*

[1]Àrea de Química Analítica, QFA, Universitat Jaume I, 12071 Castelló, Spain

Keywords: Amoxicillin; Direct injection; Micellar mobile phase; Urine

Correspondence: Josep Esteve-Romero, Àrea de Química Analítica, QFA, ESTCE, Universitat Jaume I, 12071 Castelló, Spain.

Phone: +34 964 728093. Fax: + 34 964 728066. E-mail: josep.esteve@qfa.uji.es

Abbreviations

MLC micellar liquid chromatography

FDA food and drug analysis

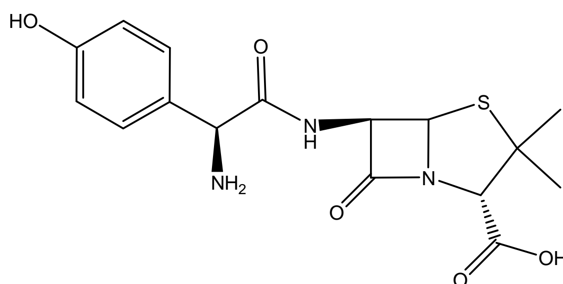
Abstract

A simple and robust method was developed for the routine identification and quantification of amoxicillin by micellar liquid chromatography. Amoxicillin, a *β*-lactamase inhibitor, is one of the most commonly prescribed drugs in the treatment of urine and skin structure infections. In this work, amoxicillin was determined in urine samples without any pre-treatment step in a phenyl column using a micellar mobile phase of 0.10 M sodium dodecyl sulfate and 4% butanol at pH 3. A UV detection set at 210 nm was used. Amoxicillin is eluted at 5.1 min with no interference by the protein band or endogenous compounds. Linearities ($r > 0.9998$), intra- and inter-day precisions were determined (RSD (%) 0.4-2.7% and 0.3-5%, respectively in micellar media, and 0.14-2.6% and 0.13-6%, respectively in urine), and robustness was studied in the validation of the method. LOD and LOQ were 0.06 and 0.4 µg/mL in micellar media and 0.11 and 0.4 µg/mL in urine, respectively. Recoveries in the urine matrix were in the range of 95-110%. The validated method proved to be reliable and sensitive for the determination of amoxicillin in urine samples.

1 Introduction

Amoxicillin (α-amino-*p*-hydroxybenzyl-penicillin) is an aminopenicillin with a broad spectrum, and currently it is the most commonly used antibiotic. It differs structurally from ampicillin only in the addition of a hydroxyl group on the phenyl ring, which makes it susceptible to a variety of degradative processes. Amoxicillin is also commercially available in fixed-ratio combinations with clavulanate potassium salt. Although clavulanic acid has only a

weak antibacterial activity when used alone, the combined use of clavulanic acid and certain penicillins leads to a synergistic effect which expands the spectrum of activity of penicillin against many strains of β-lactamase-producing bacteria [1]. Chemically, amoxicillin is a hydrophylic compound ($log\ P_{o/w}$ = 0.87) whose dissociation constants are $log\ K$ = 2.4, 7.4, 9.6 [2]. Structure of amoxicillin is shown here.



Amoxicillin is used orally to treat lower respiratory tract infections, otitis media, sinusitis, skin and skin structure infections, and urinary tract infections. It is also used orally for the treatment of chancroid and gonorrhea caused by susceptible organisms. In the medical practice, amoxicillin is used in the conventional tablet form with a minimal dose of 250 mg and a maximal dose of 875 mg [1]. After administering orally, amoxicillin is rapidly absorbed with peak serum levels appearing within 1-2 h. About 60% of an oral dose is excreted in urine as an unchanged drug in 6 hours and 20% as the inactive metabolite (penicilloic acid and penamaldic acid) [2].

Several analytical methods have been developed for the determination of amoxicillin. These methods include microbiological assay [3], enzymatic assay [4] and polarography [5]. Most of the described methods refer to the use of high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), which require large volumes of solvents, extraction and derivatizing treatment. GC-MS also involves high costs, thus limiting

accessibility. HPLC methods were developed for more specific assays. Early HPLC methods involved pretreatment with imidazole [6], precolumn [7], and postcolumn derivatization [8]. Later HPLC methods using reversed-phase and UV detection, and column switching or amperometric detection [9-15] has been developed. In order to enhance selectivity and sensitivity, some methods with fluorescence detection are carried out using post-columns [16-17] or pre-columns [19]. Other techniques involve capillary zone electrophoresis [19,20], micellar electrokinetic chromatography [21,22], as well as techniques coupled with flow injection analysis [23]. HPLC separation followed by selective mass spectrometric detection has recently become a method of choice [24-27]. Although these methods are selective, fast and sensitive, they are not suitable for routine clinical analysis because of the requirements they involve and for financial reasons.

Conventional HPLC methods using aqueous-organic mobile phases for the determination of drugs in physiological fluids with direct injection present many problems. Drugs are often strongly bound to proteins, in a complex matrix, where interference from numerous endogenous compounds is expected. This technique usually requires elaborate sample pretreatment due to the proteins' tendency to denature and precipitate in the injection valve or inside the column, thus, obstructing or clogging the system. Micellar liquid chromatography (MLC) allows the analysis of complex matrices without the aid of extraction. Micelles tend to bind proteins competitively, thereby releasing protein-bound drugs and proteins; rather than precipitating into the column, they are solubilized and washed away harmlessly to elute with the solvent front. Another advantage of using micellar mobile phases is that they are nontoxic, nonflammable, biodegradable and relatively inexpensive in comparison with aqueous-organic solvents [28].

MLC has proved to be useful technique in urine; three selected studies are an example of using micellar mobile phases with direct injection [29-31].

The purpose of the present work is to develop a rapid and sensitive quantitative chromatographic method for the determination of amoxicillin in urine using a new MLC method. The procedure developed herein could also be useful in the area of quality control, routine analysis and pharmacokinetic studies.

2 Experimental

2.1 Apparatus

The pH of the solutions was measured with a Crison GLP 22 (Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. The balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker and sonification unit were from Selecta (Barcelona, Spain). The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, thermostatted autosampler tray and column compartments, and a diode-array detector (range 190-700 nm). Columns were: phenyl, amino and C18. The dimensions of all columns (Scharlab, Barcelona) were 150 mm×4.6 mm, 5 μm particle size.

2.2 Materials and Reagents

Amoxicillin was purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and sodium hydroxide were from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate, propanol, butanol and n-amyl alcohol was from Scharlab. Hydrochloric acid, methanol and ethanol were from J.T. Baker (Deventer, The Netherlands). Ultrapure water was used throughout (Millipore S.A.S., Molsheim, France). The pharmaceuticals Normon (Madrid, Spain) and Alter (Madrid) were purchased in a local pharmacy.

2.3 Standard, samples and mobile phase preparation

The micellar mobile phases were prepared by dissolving SDS in water, which were buffered with sodium dihydrogen phosphate 0.01 M at pH 3 using 0.1 M of hydrochloric acid. Finally, butanol was added to achieve the desired concentration of the organic solvent and then water was added up to the mark-up of the volumetric flask.

A stock solution of 100 µg/mL of amoxicillin was prepared by dissolving the compounds in a few milliliters of ethanol, with the aid of an ultrasonic bath, and was finally filled up with 0.05 M SDS-pH 3. Urine samples were collected in Urine Collection Cup (BD Vacutainer Systems, Plymouth, UK). Spiked urine stock solutions, also containing 100 µg/mL of amoxicillin, were prepared by dilution of 1 mL urine in a factor 1:100 using 0.05 M SDS-pH 3. Solutions were prepared daily. For analysis, stock micellar or urine-micellar solutions were diluted to the desired concentration with the 0.05 M SDS pH 3 or urine: 0.05 M SDS-pH 3 (1:100) solutions, respectively. Analyses of patient urine were performed diluting urine with 0.05

M SDS-pH 3 (1:100), which was injected directly into the chromatographic system. All solutions were filtered through 0.45 µm nylon membranes (Micron Separations, Westboro, MA, USA) before analysis.

2.4 Recommended chromatographic conditions

Separation was performed in a reversed-phase Hypersil phenyl column (150 mm×4.6 mm, 5 µm particle size) thermostated at 25ºC. The mobile phase was 0.10 M SDS, 4% (v/v) butanol, NaH_2PO_4 0.01 M at pH 3. The flow-rate, injection volume and UV wavelength were 1 mL/min, 20 µL and 210 nm, respectively. Under these conditions, the retention time for amoxicillin was 5.1 min. Chromatographic signals were acquired and processed with an Agilent ChemStation (Rev. A.10.01). Michrom software [32, 33] was used for chromatographic data treatment.

3. Results and discussion

3.1 pH and column selection

In order to select the best analysis conditions, three columns (C18, phenyl and amino), several mobile phases and pH were checked. In a micellar chromatographic system with the anionic SDS, the stronger attraction of the doubly charged cationic drugs to the surfactant adsorbed in the stationary phase, in relation to the micelles in the mobile phase, increases their

retention at a decreasing pH. The influence of the mobile phase pH on the amoxicillin retention was studied at different pH values (3 and 7) using pure micellar mobile phases of SDS (M): 0.05, 0.10, 0.15 and 0.20. Characteristics are summarized in Table 1. As seen, amoxicillin at pH 7 elutes either near or in the dead volume in all columns, which is the reason why pH 3 was finally chosen.

The modification of the stationary phase by adsorbed surfactant can have profound implications with regard to retention, selectivity and asymmetry in MLC. In the C18 column, the hydrophobic alkyl tail of the SDS is associated with the bonded phase, with the sulfate group oriented away from it [34]. So with its polar head group projecting away from the bonded phase toward the mobile phase, this would greatly affect the polarity of the bonded phase, and would also lead to the formation of an anionic hydrophilic layer, which would explain the high resolution achieved by SDS for hydrophilic compounds. On the other hand, the shorter retention times of compounds are due to an increase in the polarity of the stationary phase which occurred as a result of surfactant adsorption.

While developing the method of polar compounds, such as amoxicillin, and using C18 columns, the octadecyl alkyl chain often collapsed for the aqueous mobile phases. This can lead to poor retention and selectivity as well as too poor reproducibility. This problem can be addressed by the amino columns; these are amine groups bonded to the silica polar stationary phases [35]. Amine bonded stationary phases will be protonated at neutral and acidic mobile phase pH values. The amino columns retain longer polar compounds than non-polar compounds when the mobile phase is polar.

The chromatographic behavior of phenyl columns is well described by hydrophobic and π-π interactions. Phenyl columns have a high retention character because of their high aromatic nature and also due to their spatial circumstances as the molecule is planar, so it enables stronger retention through π-π interactions. In phenyl columns, the preferential retention of aromatic and polar compounds is observed in aromatic phases, and less retention of saturated hydrocarbons is noted compared to alkyl phases.

The retention of amoxicillin on phenyl, amino and C18 columns was studied using pure micellar mobile phases containing SDS (M): 0.05, 0.10, 0.15, 0.20, or SDS (M)–propanol (%): 0.05-2, 0.05-6, 0.10-4, 0.15-2 and 0.15-6. Retention factors, efficiencies and asymmetry factors are shown in Table 1. The usual behavior in MLC with SDS was observed in all the columns and mobile phases. Thus, the retention factors decrease for SDS and alcohol while increasing the concentration of both. On the other hand, efficiencies decreased when surfactant concentration increase conversely efficiencies increased at higher concentrations of the modifier. Retention times, efficiency and asymmetry were checked to decide the optimum column. All columns show an appropriate retention factor for the analysis, good efficiency and asymmetry. A C18 column presents a lower retention factor, but a phenyl column presents higher efficiency. In the amino and C18 columns on the other hand, the amoxicillin peak overlapped with the protein band and the endogenous compounds of the urine matrix in nearly all the mobile phases. For this reason, the phenyl column was selected for further analysis.

3.2 Optimization strategy and mobile phase selection

First of all, SDS was selected for many reasons, other than being the most used surfactant, its high sensitivity and easy desorption and clean up. The use of pure micellar mobile phases originates moderate retention times. The addition of a small amount of organic solvent was seen suitable to decrease the analysis times and to increase efficiencies. The use of hybrid micellar mobile phases of variable concentrations of surfactants and the modifier usually produces changes in the retention factors, efficiencies and asymmetries of the chromatographic peaks. A suitable control of the additives concentration is therefore necessary so that chromatograms show a good resolution and sufficient elution strength.

Once the phenyl column was selected to find the best composition of the mobile phase, amoxicillin was injected in the mobile phases at pH 3, which contained SDS (M) / modifier (%, v/v): SDS/propanol (0.05/2, 0.05/6, 0.1/4, 0.15/2, 0.15/6), SDS/butanol (0.05/2, 0.05/6, 0.75/5, 0.10/4, 0,125/3, 0.125/5, 0.15/2, 0.15/6) and SDS/pentanol (0.05/2, 0.10/4). Retention factors (*k*), efficiencies (*N*) and asymmetry factors (*B/A*) were measured and summarized in Table 2.

The chromatographic data obtained were treated with Michrom, and the factor of maximum resolution and the minimum analysis time were taken into account. The accurate prediction of the retention behavior, based on a checked model, can speed up the process of finding the optimal composition of the mobile phase for a given compound. The following equation has proved to be adequate to describe the retention of many compounds in MLC with hybrid mobile phases with errors in a range of 2-4% [36]:

$$k = \frac{K_{AS} \dfrac{1 + K_{SD}\varphi}{1 + K_{AD}\varphi}}{1 + K_{AM} \dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}}$$

where *k* is the retention factor, [M] and φ are the concentrations of the surfactant and modifier, respectively; K_{AS} and K_{AM} describe the association equilibria between the solute in bulk water and the stationary phase or micelle, respectively; K_{SD} and K_{MD} are constants that measure the relative variation in the concentration of solute in bulk water and micelles given the presence of the modifier, and refer to a pure micellar solution.

Propanol was not used as an organic modifier as the resolution was good. However, the retention time was not short enough and resulted in a long analysis time as opposed to what was expecting without using a high percentage of alcohol. When pentanol was used, an overlapping of amoxicillin with urine matrix and endogenous peaks took place in almost all the mobile phases. Thus, pentanol does not show any special characteristic to change to a longer carbon chain. Therefore, butanol was selected as the organic modifier which allows for less analysis time and allows the complete resolution. The optimum mobile phase was: SDS 0.1 M – 4% butanol – NaH_2PO_4 at pH 3 and the chromatographic parameters for amoxicillin in this mobile phase (*k*, *N* and *B/A*) were: 2.4, 2940 and 1.1.

3.3 Urine blank behavior

Several urine blanks from males and females were injected directly into a micellar chromatographic system in order to check the background. The profile of the protein band and the endogenous compounds for all the samples were similar to that shown in Figure 1A. No additional peaks were found in subsequent analyses.

3.4 Method Validation

Validation was done following the Food and Drug Analysis (FDA) validation guide [37]. The parameters evaluated were: linearity, detection and quantification limits, precision and accuracy, selectivity, robustness, and the stability of the drug.

3.4.1 Linearity

Calibration curves were constructed using the areas of the chromatographic peaks obtained at seven different concentrations (nine replicates), in the range of 0.4-50 µg/mL in two different matrices: micellar solution and urine-micellar solution (1:100 dilution factor). To study the variability of the calibration parameter, curves were obtained for 5 days over a 2-months period for a different set of standards. The results in the two matrices were similar. The slope and intercept were determined by the method of least squares linear regression analysis, with averages of 0.6525 ± 0.005 and -0.04 ± 0.09, respectively. The regression coefficients (r) were always higher than 0.9998.

3.4.2 Detection and quantification limits

The limit of detection (LOD) for amoxicillin in SDS and urine (n = 10) was determined with the *3s criterion* using a series of 10 solutions containing a low concentration of amoxicillin (0.1 µg/mL). The results were based on the standard deviation of the response and also on the slope of a specific calibration curve containing the analyte. The limit of quantification (LOQ) of amoxicillin in SDS and urine was selected as the lowest concentration used in the calibration curve for which the RSD (%) was ≤ 5.42. LODs of amoxicillin in SDS and urine-SDS were: 0.06 and 0.11 µg/mL, respectively. LOQs in both media were 0.4 µg/mL, according to the FDA validation guide [37].

3.4.3 Precision and Accuracy

The intra and inter-day precisions of the method were determined by analyzing the amoxicillin at seven concentrations in the range 0.4-50 µg/mL in micellar media and in urine-SDS (1:100). The intra-day analysis was determined by injecting these seven test solutions nine times in the same day, while the inter-day analysis was the average of nine measurements of the intra-day values taken on 5 days over a 3-month period performed by different analysts and equipment at the same seven concentrations. The results, expressed as the percentage of the relative standard deviation, for intra- and inter-day values were 0.4-2.7% and 0.3-5%, respectively in micellar media, and 0.14-2.6% and 0.13-6%, respectively, in urine.

3.4.4 Selectivity

Five selected control drug-free human urine samples were processed directly into the chromatographic system and analyzed to determine the extent to which endogenous components may contribute to interfere with the retention time of the drug. No interference for endogenous compounds was found in the physiological matrix studied.

3.4.5 Robustness

The robustness of the method was examined by replicate injections (n=6) of standard solution at 10 µg/mL concentration with slight changes made to the chromatographic parameters. Insignificant differences in the peak areas and less variability in the retention time were observed. The results, shown in Table 3, indicate that the selected factors remain unaffected by the slight variations made to these parameters.

3.4.6 Stability

Degradation of amoxicillin, after one week of storage of SDS and urine stock solutions, was confirmed by new peaks emerging in the chromatograms (retention times at 4.58 and 5.30 min); no interferences were found between these peaks and the analyte. All solutions were kept at a low temperature (4ºC) until required, and were used within a week. Amoxicillin was analyzed immediately after preparation and at selected time intervals after storage over the study period. After 24h, 48h and 1 week, 4%, 19% and 64% of decomposition of amoxicillin in urine were observed, respectively.

3.4.7 Analysis of the marketed pharmaceutical formulation

The pharmaceutical formulations analyzed (Normon and Alter) were tablets containing a mixture of amoxicillin (875 mg) and a β-lactamase inhibitor (clavulanic acid, 125 mg). The experimental results of the amount of amoxicillin in tablets, expressed as a percentage of the label claimed by the manufacturer, were in good agreement, therefore suggesting that there was no interference of clavulanic acid and excipients, which are normally present in tablets. The drug content found (mg) ± sd was 878±0.6 (n=6) and 880±0.6 (n=6) for the Normon and Alter drugs respectively.

3.4.8 Determination of spiked urine samples

Amoxicillin recoveries from urine were determined by spiking drug-free urine diluted in a 1:100 factor with 0.05 M SDS at pH 3 with known amounts of the drug at seven different concentrations (0.4-50 μg/mL) within the calibration range (nine replicates for each standard). The spiked samples were processed and analyzed with the developed procedure. The relative (analytical) recovery was calculated by comparing the concentration obtained from the drug-supplemented urine with actual added amounts. The data obtained showed satisfactory recoveries for the amoxicillin, and the results were in the range of 95-110%. Figure 1B shows the chromatogram of a spiked urine sample containing amoxicillin analyzed with the optimum mobile phase.

3.4.9 Drug control in real urine samples

Urinary studies were conducted following the oral administration of a single dose of a conventional tablet containing 875 mg of amoxicillin to volunteers. Urine samples were taken 9 times a day, approximately every 1 h, and the volume was measured. Dilution of urine samples by 1:100 was performed, and the solution was injected directly into the chromatographic system without any other treatment except filtration. No interferences from clavulanic acid were observed since this compound has a poor absorption (λ_{max} = 201 nm in water) in the UV-region. Figure 2 depicts the elimination curve. The maximum concentration excreted in urine was found 4 h after oral administration, and this concentration was 803 µg/mL. The final quantity of amoxicillin eliminated after 8.5 hours as 517 mg, that is, 59% of the dose taken. Figure 1C shows the chromatogram of amoxicillin excreted after 2 h of oral administration in an unchanged form. These results show that pharmacokinetic studies can be performed under the proposed chromatographic conditions.

4 Conclusions

In conclusion, our results indicate that the MLC procedure can be used for the analysis of amoxicillin, a frequently prescribed penicillin, in urine samples, with an analysis time below 6 min.

Validation including linearity, sensitivity, selectivity, accuracy, stability and robustness were developed efficiently with satisfactory results and used for assaying amoxicillin in urine. The

method is sensitive enough to undertake quality control routine analysis and pharmacokinetic studies of the drug if we take into account that the urine samples were injected without any previous treatment to separate or concentrate the analytes.

Acknowledgements

This work was supported by the projects of the Spanish Ministerio Educación y Ciencia (MEC) CTQ 200764473/BQU and the Foundation Caixa Castelló-Bancaixa P1-1B2006-12. Maria Rambla-Alegre also wishes to thank the MEC for the FPU grant.

5 References

[1] American Hospital Formulary Service, Drug Information, American Society of Health-System Pharmacists, Bethesda, MD, **1988**.

[2] Clarke's Analysis of Drugs and Poisons, Pharmaceutical Press, London (UK), **2004**.

[3] Mainz D., Borner K., Koeppe P., Kotwas J., Lode H., *J. Antimicrob. Chemother* **2002**, 50, 699-706.

[4] Hoizey G., Lamiable D., Frances C., Trenque T., Kaltenbach M., Denis J., Millart H., *J. Pharm. Biomed. Anal.* **2002**, 30, 661-666.

[5] Lyle S.J., Yassin S.S., *Anal. Chim. Acta* **1993**, 274, 225-230.

[6] Matar K.M., Nazi E.M., El-Saved Y.M., Al-Yamani M.J., Al-Suwaveh S.A., Al-Khamis K.I., *J. Liq. Chromatogr. Relat. Technol.* **2005**, 28, 97-107.

[7] Ang C.Y.W., Luo W.H., Hansen E.B., Freeman J.P., Thompson H.C., *J. AOAC International* **1996**, 79, 389-396.

[8] Mascher H.J., Kikuta C., *J. Chromatogr. A* **1998**, 812, 221-226.

[9] Dousa M., Hosmanová R., *J. Pharm. Biomed. Anal.* 2005, 37, 373-377.

[10] Cass Q.B., Gomes R.F., Calafatti S.A., Pedrazolli J., *J. Chromatogr. A* 2003, 987, 235-241.

[11] Aghazadeh A., Kazemifard G., *J. Pharm. Biomed. Anal.* **2001**, 25, 325-329.

[12] Foroutan S.M., Zarghi A., Shafaati A., Khoddam A., Movahed H., *J. Pharm. Biomed. Anal.* 2007, 45, 531-534.

[13] Matar K.M., *Chromatographia* **2006**, 64, 255-260.

[14] Cavazos Rocha N., Vera Cabrera L., Welsh Lozano O., Waksman de Torres N., Salazar Cavazos M.L., J. *Pharm. Biomed. Anal.* **2007**, 43, 1775-1781.

[15] Leroy P., Gayriloff C., Nicolas A., Archimbault P., Ambroggi G., *Int. J. Pharm.* **1992**, 82, 157-164.

[16] Tokumura T., Machida Y., *Int. J. Pharm.* **2001**, 228, 1-4.

[17] Mascher H., Kikuta C., *J. Chromatogr.* **1990**, 506, 417.

[18] Gamba V., Dusi G., *Anal. Chim. Acta* **2003**, 483, 69-72.

[19] Santos S.M., Henriques M., Duarte A.C., Esteves V.I., *Talanta* **2007**, 71, 731-737.

[20] Pajchel G., Pawlowski K., Tyski S., *J. Pharm. Biomed. Anal.* **2002**, 29, 75-81.

[21] Ahrer W., Scherwenk E., Buchberger W., *J. Chromatogr. A* **2000**, 910, 69-78.

[22] Bailón-Pérez M.I., Cuadros-Rodríguez L., Cruces-Blanco C., *J. Pharm. Biomed. Anal.* 2007, 43, 746-752.

[23] González San Miguel H.M., Alpízar Lorenzo J.M., Cerdà Martín V., *Talanta* **2007**, 72, 296-300.

[24] Yoon K-H., Lee S-Y., Kim W., Park J-S., Kim H-J., *J. Chromatogr. B* 2004, 813, 121-127.

[25] Riediker S., Stadler R.H., *Anal. Chem.* **2001**, 73, 1614-1621.

[26] De Baere S., Cherlet M., Baert K., De Backer P., *Anal. Chem.* **2002**, 74, 1393-1401.

[27] Reyns T., De Boever S., De Baere S., De Backer P., Croubels S., *Anal. Chim. Acta* **2007**, 597, 282-289.

[28] A. Berthod, M.C. García-Ávarez-Coque, Micellar Liquid Chromatograpy, Marcel-Dekker, New York, **2000**.

[29] M. Gil-Agustí, M.E. Capella-Peiró, A. Martinavarro-Dominguez, J. Esteve-Romero, *Chromatographia* **2003**, 57, 51-57.

[30] Capella Peiró M.E., Gil Agustí M., Martinavarro Dominguez A., Esteve Romero J., *Anal. Biochem.* 2002, 309, 261-268.

[31] Esteve Romero J., Carda Broch S., Gil Agustí M., Capella Peiró M.E., Bose D., *Trends Anal. Chem.* 2005, 24, 75-91.

[32] Torres Lapasió J.R., García Álvarez Coque M.C., Baeza Baeza J.J., *Anal. Chim. Acta* 1997, 348, 187-196.

[33] Torres Lapasió J.R., Michrom Software, Marcel Dekker, New York, USA, **2000**.

[34] Lavine B.K., Cooper W.T., He Y., Hendayana S., Han J.H., Tetreault J., *J. Colloid and Interf. Sci.* **1994**, 165, 497.

[35] Olsen B.A., *J. Chromatogr. A*, **2001**, 913, 113.

[36] García Álvarez Coque M.C., Torres Lapasió J.R., Baeza Baeza J.J., *J. Chromatogr. A* **1997**, 780, 129-148.

[37] Guidance for Industry. Bioanalytical Method Validation. U.S. Department of Health and Human Services. Food and Drug Administration. May **2001**. Rockville, MD, USA.

Table 1

Retention factor, efficiencies and asymmetries of amoxicillin in three different columns

	Phenyl			**Amino**			**C18**		
	k	***N***	***B/A***	***k***	***N***	***B/A***	***k***	***N***	***B/A***
pH 3									
0.05 M	15.9	3140	1.1	16.2	2180	0.87	17.7	820	0.72
0.10 M	7.2	2350	1.1	8.1	1500	0.87	7.97	750	0.82
0.15 M	3.99	1920	1.1	5.2	1400	0.997	5.1	750	0.90
0.20 M	3.1	1530	1.1	3.9	1460	0.995	3.9	750	0.90
pH7									
0.05 M	0.3	1590	1.6	1.8	3260	1.8	0.5	770	1.0
0.10 M	0.3	1570	1.6	1.4	2620	1.4	0.4	770	1.1
0.15 M	0.3	1520	1.2	1.2	2130	1.2	0.4	740	1.0
0.20 M	0.3	1430	1.2	1.2	2100	1.2	0.4	910	1.2
pH3									
0.05 M – 2%[a)]	9.1	3270	1.1	10.3	2010	0.99	9.97	2920	1.1
0.05 M – 6%[a)]	7.4	3660	1.2	7.3	2400	0.80	8.2	3510	1.0
0.10 M – 4%[a)]	4.5	2550	1.1	4.7	1800	0.90	4.6	1790	1.1
0.15 M – 2%[a)]	3.5	1900	1.1	4.8	1870	0.93	3.2	1350	1.1
0.15 M – 6%[a)]	3.2	3100	1.2	3.0	1900	0.94	2.98	1870	1.1

[a)] propanol

Table 2

Retention factors, efficiencies and asymmetry factors of amoxicillin in the method optimization.

SDS (M) – alcohol (%)	***k***	***N***	***B/A***
0.05 – 2[a)]	9.1	3270	1.1
0.05 – 6[a)]	7.4	3660	1.2
0.10 – 4[a)]	4.5	2550	1.1
0.15 – 2[a)]	3.5	1900	1.1
0.15 – 6[a)]	3.2	3100	1.2
0.05 – 2[b)]	7.5	2940	1.2
0.05 – 6[b)]	3.8	3490	1.3
0.075 – 5[b)]	3.5	3310	1.3
0.10 – 4[b)]	2.4	2940	1.1
0.125 – 3[b)]	2.9	2690	1.1
0.125 – 5[b)]	2.2	3190	1.1
0.15 – 2[b)]	2.6	1815	1.2
0.15 – 6[b)]	2.0	2780	1.2
0.05 – 2[c)]	4.6	3450	1.1
0.10 – 4[c)]	2.2	2390	1.4

[a)] propanol, [b)] butanol, [c)] pentanol

Table 3

Robustness evaluation of MLC method

Chromatographic changes	**Level**	**t_R (min)**
A: Flow rate (mL/min)		
0.9	-0.1	5.67
1	0	5.10
1.1	+0.1	4.56
Mean ± SD		5.1±0.6
B: SDS concentration (M)		
0.095	-0.01	5.23
0.1	0	5.10
0.0105	+0.01	5.01
Mean ± SD		5.11±0.11
C: Percentage of butanol in the mobile phase (v/v)		
3.9	-0.1	5.29
4	0	5.10
4.1	+0.1	4.99
Mean ± SD		5.12±0.15
D: pH of mobile phase		
2.9	-0.1	5.31
3	0	5.10
3.1	+0.1	4.92
Mean ± SD		5.11±0.19

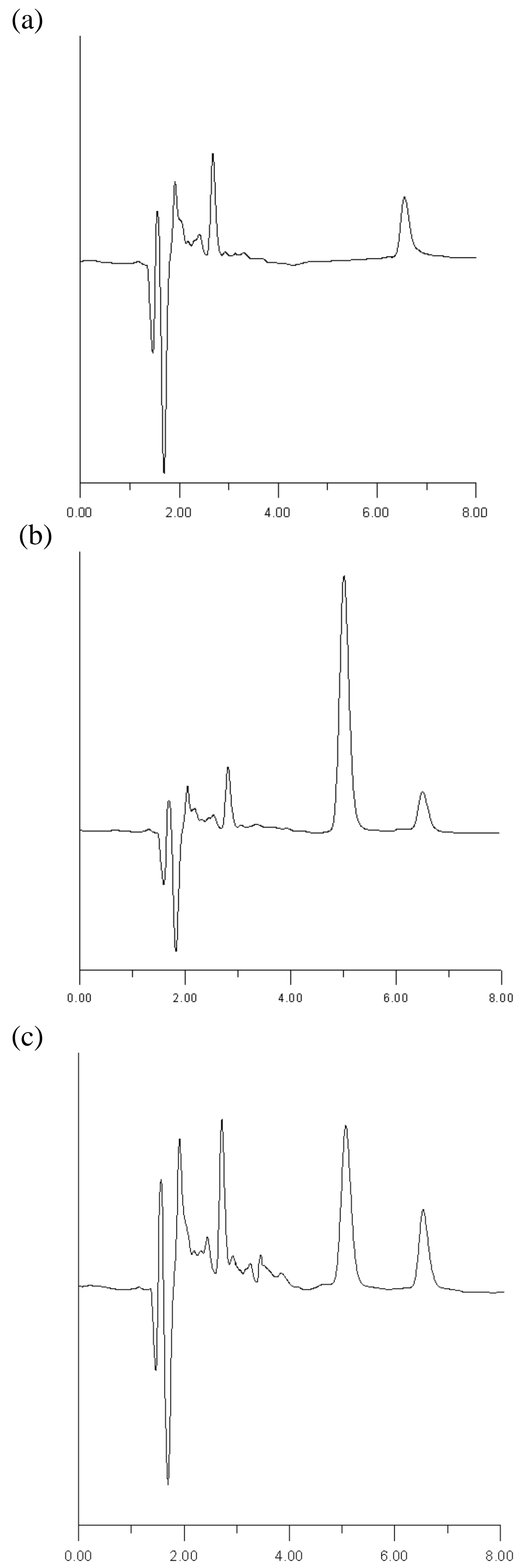


Figure 1. Chromatograms of urine blank (a), urine spiked (15 µg/mL) (b) and amoxicillin excreted (6 µg/mL) in urine as unchanged drug 2 h after oral ingestion (c). Mobile phase: 0.1 M SDS-4% butanol-pH 3.

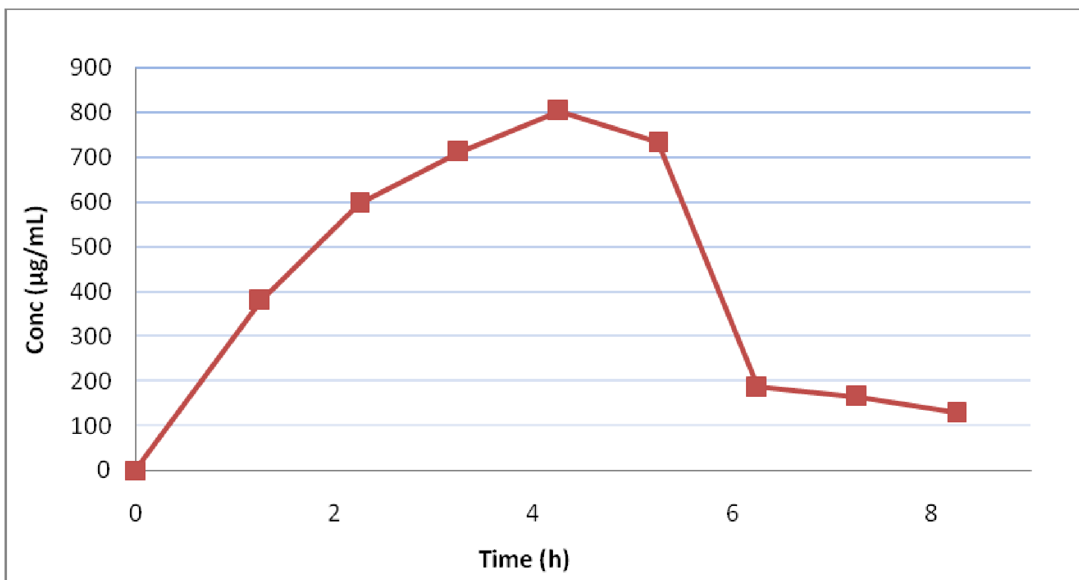


Figure 2. Elimination curve of amoxicillin in urine