Development and validation of a fully automated LC method for the determination of cloxacillin in human plasma using anion exchange restricted access material for sample clean-up

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Abstract

In the framework of a preliminary investigation on the plasma profile of cloxacillin after oral administration, a simple and rapid LC method was developed for the direct determination of this compound in human plasma. The on-line sample clean-up was carried out using a weak anion exchanger (diethylaminoethyl groups) as restricted access material (RAM). The effects of the washing liquid pH, the ionic strength and the addition of organic modifier to the washing liquid were studied in order to obtain an efficient sample clean-up and a high recovery of cloxacillin. The separation was achieved on octadecylsilica stationary phase using a mobile phase consisting in a mixture of phosphate buffer (pH 4.0; 25 mM) and acetonitrile (72:28, v/v). The UV detection was performed at 215 nm. The most appropriate regression model of the response function as well as the limit of quantitation (LOQ) were first selected during the pre-validation step. These criteria were then assessed during the formal validation step. The LOQ was 50 ng/ml. The method was also validated with respect to analyte recovery, precision, trueness, accuracy and linearity. Finally, it was successfully applied for the analysis of the first plasma samples obtained from patients having taken an oral dose of 500 mg cloxacillin.
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1. Introduction

Cloxacillin [1] is a β-lactam antibiotic of broad spectrum against gram positive and negative bacteria. It is widely used for treatment of several bacterial infections in human and animal diseases.

Recently, several chromatographic methods have been reported for the determination of cloxacillin with other β-lactam antibiotics, especially in food and agricultural fields as residuals [2,3]. However, few LC methods were also described for the determination of cloxacillin in human plasma. In these methods, sample clean-up procedure was carried out using liquid–liquid extraction employing high percentages of organic modifiers [4], protein precipitation by acids or organic solvents followed by back extraction [5] or solid phase extraction (SPE) employing conventional ion-exchange cartridges [6,7] or small C_{18} pre-packed columns [8]. Moreover, UV detection was performed at around 220 nm. Therefore, an efficient sample clean-up enhancing method selectivity in this wavelength range is required.

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The automation of the extraction procedure avoiding manual sample handling allows to decrease analysis run time and increase method precision. Such an automation can be achieved by coupling of a pre-column packed with restricted access material (RAM) to a LC column via the column switching technique. This approach has been widely applied successfully in different analytical fields [9–12] and is of particular interest when the stability of the analyte is poor, as it is the case for cloxacillin.

Due to the use of a RAM sorbent, macromolecules, such as proteins, which are restricted to enter into the pores during the sample clean-up, are flushed out, while the analyte of the interest is retained and consequently transferred to the analytical column. A type of RAM supports, namely RP-4, RP-8 and RP-18 alkyl diol silica (ADS), belonging to the family of the internal surface reversed phase (ISRP) sorbents, has been commonly used for the extraction of different drugs from biological fluids [13–16]. The outer surface of these RAM particles contains hydrophilic and electroneutral diol groups, which prevents the adsorption and denaturation of proteins. Recently, we have tested two new RAM sorbents with internal ion-exchange functionalities for the extraction of basic and acidic compounds from plasma [17–19] prior to their LC determination. A selective and complete extraction of the analytes of interest has been achieved. One of the two supports, namely exchange diol silica-diethylaminoethyl (XDS-DEAE) sorbent, presents the properties of a weak anion exchanger towards acidic compounds, since diethylaminoethyl groups are bonded to the inner surface of the silica particles. The access restriction is obtained due to the mean pore diameter (about 6 nm) and the external surface is biocompatible.

The objective of the present paper is to develop and validate a fully automated method for the determination of cloxacillin in human plasma using this kind of sorbent for on-line sample clean-up coupled to a LC–UV system in order to obtain a simple and fast alternative to the previously reported procedures. The method was fully validated according to the new strategy proposed by the Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of quantitative analytical procedures [20,21]. The validation strategy consists in two steps. The first step, the so called prevalidation step, has mainly permitted the selection of the most appropriate regression model using the accuracy profile as decision tool [20–23]. The second step, representing the validation itself, consists in testing the method selectivity towards endogenous components and the assessment of method precision, trueness and accuracy [20,21,23,24] at different concentration levels over the range investigated as well as the confirmation of the limit of quantitation (LOQ) and the method linearity [20–22]. Finally the method reported was successfully used to perform the quantitative determination of cloxacillin in real human plasma samples and was found to be applicable for the quantification of this compound in future bioequivalence studies.

2. Experimental

2.1. Chemical and reagents

Cloxacillin sodium was purchased from Sigma (St. Louis, MO, USA); potassium dihydrogen phosphate, acetic acid and phosphoric acid (85%) were obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile were LiChrosolv LC gradient grade solvents purchased from Merck. The water used in all experiments was purified by means of a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

2.2. Equipment

The LC-integrated sample clean-up system was composed of a model 422 LC pump from Kontron Instruments (Schlieren, Switzerland) (pump 1) and the following units from Merck-Hitachi: a model L-6200 A pump (pump 2), a model AS-2000 A autosampler equipped with a 100 μl injection loop and a model L-4250 UV–vis detector which was monitored at 215 nm.

The LiChroCART (25 × 4 mm, i.d.) pre-column was prepacked with LiChrospher 60 XDS (DEAE/diol) (particle size: 25 μm) supplied as research sample by Merck and was fitted to a Valco model VICI AG six-port switching valve (Valco Europe, Schenkon, Switzerland). Separation was achieved on a Nova Pack C_{18} column (150 mm × 3.9 mm, i.d.; particle size: 4 μm) from Waters (Milford, MA, USA), which was thermostated at 30 ± 0.1 °C in a model L-5025 programmable column oven (Merck).

The different modules were connected through an interface (D-6000, Merck-Hitachi) with an IBM compatible computer (PC-AT; CPU type Pentium) on which the D-7000 HPLC manager software was loaded for the control of the analytical system and data collection. The model 422 pump from Kontron (pump 1) was controlled manually.

The e.noval® software (Arlenda, Belgium) was used to determine the accuracy profiles as well as all the validation results.

2.3. Conditions for sample clean-up and chromatographic separation

The clean-up step of sample matrix was carried out using a washing liquid consisting in a mixture of 0.005% acetic acid (pH 5.0)/methanol (97:3, v/v).

Isocratic separation was performed at 30 °C using a constant flow-rate of 1.2 ml/min. The mobile phase was a mixture of phosphate buffer (pH 4.0; 25 mM) and acetonitrile (72:28, v/v). Before use, the washing liquid and the LC mobile phase were passed through a membrane filter (0.45 μm) and then degassed for 15 min in an ultrasonic bath.

2.4. Standard solutions

2.4.1. Stock solutions

Stock solutions of cloxacillin were prepared at 1 mg/ml in water and stored at $-20\,C^\circ$ until use.

2.4.2. Solutions used for method development

The working solution was made up daily by diluting 1.0 ml of a stock solution with water to obtain a concentration of 50 µg/ml.

2.4.3. Solutions used in the pre-validation step

The stock solution was diluted in order to obtain three solutions at 100, 10 and 1 µg/ml. These intermediate solutions were used to spike free drug plasma samples at five concentration levels covering a range from 50 to 5000 ng/ml. The first concentration level was close to the expected limit of quantitation. Two other series were then prepared from independent stock solutions according to the same protocol. One non-biological calibration curve was also performed for the determination of absolute recovery.

2.4.4. Solutions used in the validation step

Two types of plasma samples were prepared: calibration standards covering the same concentration range as used in the pre-validation step and validation standards [19,20]. Four concentration levels were selected, the lowest one being the limit of quantitation. Each validation sample was analysed four times for three different days.

2.5. Plasma samples

Human plasma samples were obtained from the Blood Transfusion Centre of Liège (Liège, Belgium) and were stored under $-20\,°C$. Before use, the plasma samples were thawed at room temperature and centrifuged at $4500 \times g$ for 10 min. Aliquots were spiked with the diluted standard solutions and these plasma samples were prepared daily.

2.6. Human study

A single dose of 500 mg cloxacillin was given orally to healthy volunteers after overnight fast. Venous blood samples were collected in heparinised vacutainer tubes after 0 (predose), 1, 2, 3, 4, 5, 6 and 7 h. The tubes were centrifuged at $4000 \times g$ for 15 min, the plasma was collected and stored at

$-20\,C^\circ$ until analysis. The concentration of cloxacillin was determined by injecting 100 µl of each sample.

2.7. On-line sample clean-up and chromatographic separation

The time events of the column-switching valve for on-line sample clean-up and chromatographic separation are indicated in Table 1.

2.7.1. Loading and washing of sample matrix

One hundred microliters of plasma sample were directly injected by the autosampler into the pre-column. The biological matrix was washed out with the washing liquid consisting in a mixture of 0.005% acetic acid (pH 5.0)/methanol (97:3, v/v). The washing liquid was delivered by pump 1 at a flow-rate of 1.0 ml/min for 8 min. During this step, the analytical column was re-equilibrated with the LC mobile phase delivered by pump 2 at a flow-rate of 1.2 ml/min.

2.7.2. Desorption and transfer

After rotation of the switching valve, the pre-column was coupled to the analytical column and the analyte was then desorbed in the back-flush mode and transferred to the top of the analytical column by the LC mobile phase delivered by pump 2 at a flow-rate of 1.2 ml/min.

2.7.3. Conditioning of the pre-column and chromatographic separation

After 2 min, the switching valve was turned back to its initial position allowing the re-equilibration of the pre-column with the washing liquid before the next injection. Simultaneously, the analyte transferred to the analytical column was separated and quantified.

3. Results and discussion

3.1. Method development for sample clean-up

The determination of polar compounds monitored photometrically at low wavelengths is usually difficult in plasma. However, when an efficient sample handling procedure is applied prior to LC analysis, a fraction free from interference can be obtained and a rather good detectability can be achieved. In this work, the use of restricted access material with anion-exchange properties should improve method selectivity, since only negatively ionized compounds can be

Table 1
Time events of the column-switching valve for on-line sample clean-up and chromatographic separation

Step	Process	Switching time (min)	Switching valve coupling	
1	Sample clean-up	0–8	Pre-column–waste	LC column–UV detector
2	Elution and transfer	8–10	Pre-column–LC column–UV detector	
3	Equilibration of the pre-column and LC separation	10–17	Pre-column–waste	LC column–UV detector

retained through electrostatic interactions with the DEAE groups bonded to the inner surface of the support.

Moreover, cloxacillin, as a β-lactam antibiotic, has a limited stability at room temperature and in organic solvents, such as acetonitrile and methanol [25]. Consequently, the coupling of on-line sample clean-up with LC by means of the column switching technique is of particular interest to overcome this drawback.

3.1.1. Effect of the washing liquid pH on the retention of cloxacillin

Since the XDS (DEAE/diol) sorbent presents the properties of a weak anion exchanger towards low molecular mass compounds, the retention of anionic analytes is mainly due to electrostatic interactions. Therefore, the ionization of the DEAE groups and the acidic analytes is mandatory to obtain a complete and selective extraction. In a previous work [18], the sorbent capability for retaining acidic compounds was evaluated and was found to be optimal in a pH range from 4.0 to 6.5, which is also suitable for the ionization of cloxacillin (pK_a: 2.7). By using a washing liquid containing 0.005% acetic acid (pH 5.0), cloxacillin was retained sufficiently. Indeed, the breakthrough volume observed was 25 ml, which allowed to clean-up the sample matrix during a washing time of 8 min without eluting the analyte of interest.

3.1.2. Influence of the addition of organic modifier on the retention of cloxacillin

In the column-switching systems coupling restricted access material to LC, the addition of a limited amount of organic modifier, such as methanol, acetonitrile or 2-propranol, to the washing liquid is very useful to enhance the extraction selectivity, to release the analyte from the binding sites of the plasma proteins and to obtain high recoveries [17–19]. Consequently, the influence of the addition of methanol to the washing liquid composed of 0.005% acetic acid was investigated.

As shown in Fig. 1, the increase of the proportion of methanol in the washing liquid gives rise to a decrease in
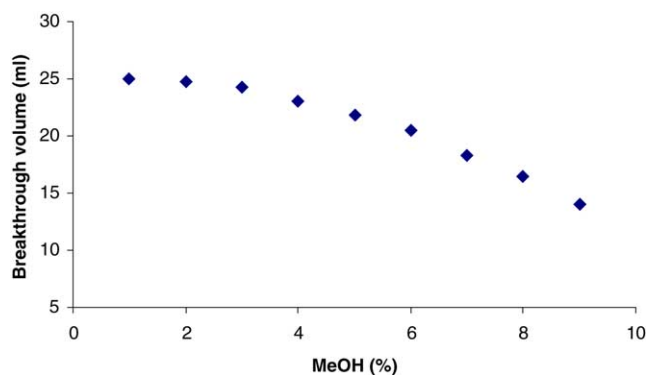

Fig. 1. Effect of the addition of methanol to the washing liquid on the breakthrough volume of cloxacillin. Washing liquid: 0.005% acetic acid/methanol; flow-rate: 1.0 ml/min; injection volume: 100 μl; detection: UV at 220 nm; temperature: 25 °C. Other conditions: see Section 2.

the breakthrough volumes of cloxacillin. However, small changes were observed when the methanol content was lower than 5% (v/v). Consequently, a washing liquid consisting of 0.005% acetic acid and methanol (97:3, v/v) was finally selected.

3.1.3. Efficiency of the washing liquid composition to clean-up the sample matrix

The efficiency of the selected washing liquid to wash out the sample matrix was tested by injecting 100 μl of plasma into the XDS (DEAE/diol) pre-column directly connected to the UV detector monitored at 280 nm. The elimination of the biological matrix could be considered as complete when the detector signal reached the baseline. Under these conditions, a washing time of 8 min was sufficient for sample clean-up and gave rise to no loss of analyte, since its breakthrough time was higher (25 min).

3.1.4. Transfer of cloxacillin from the pre-column to the LC column

The determination of the transfer time of cloxacillin from the pre-column to the LC column was performed by connecting directly the UV detector with the switching valve. Due to the high ionic strength of the LC mobile phase, a time period of 2 min was sufficient to elute cloxacillin in the back-flush mode and to transfer it to the analytical column. The possibility of a carry-over effect was also investigated at this stage of the method development by coupling of the pre-column with the LC column. No carry-over effect was observed under the operating conditions. Ten minutes after sample application, the switching valve returned to its initial position, allowing the sorbent to be re-equilibrated with the washing liquid.

3.2. Pre-validation step

3.2.1. Analysis of the response function, estimation of the range and the LOQ

Before the formal validation phase, an important step consists in the assessment of the relationship between response and concentration in order to avoid serious difficulties in the estimation of other validation criteria [22]. In order to select the most appropriate response function, the SFSTP approach based on the accuracy profile (β-expectation tolerance intervals for total measurement error) of calibration samples has been used [20,21]. The response function is considered as adequate when the accuracy profile is within the acceptance limits – fixed a priori – on all the dosing range of interest. Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria, i.e. 20% according to the Washington conference [26] or FDA document [27].

As illustrated in Fig. 2, once the pre-validation experiments have been performed, the response function can be determined by applying different regression models and, from
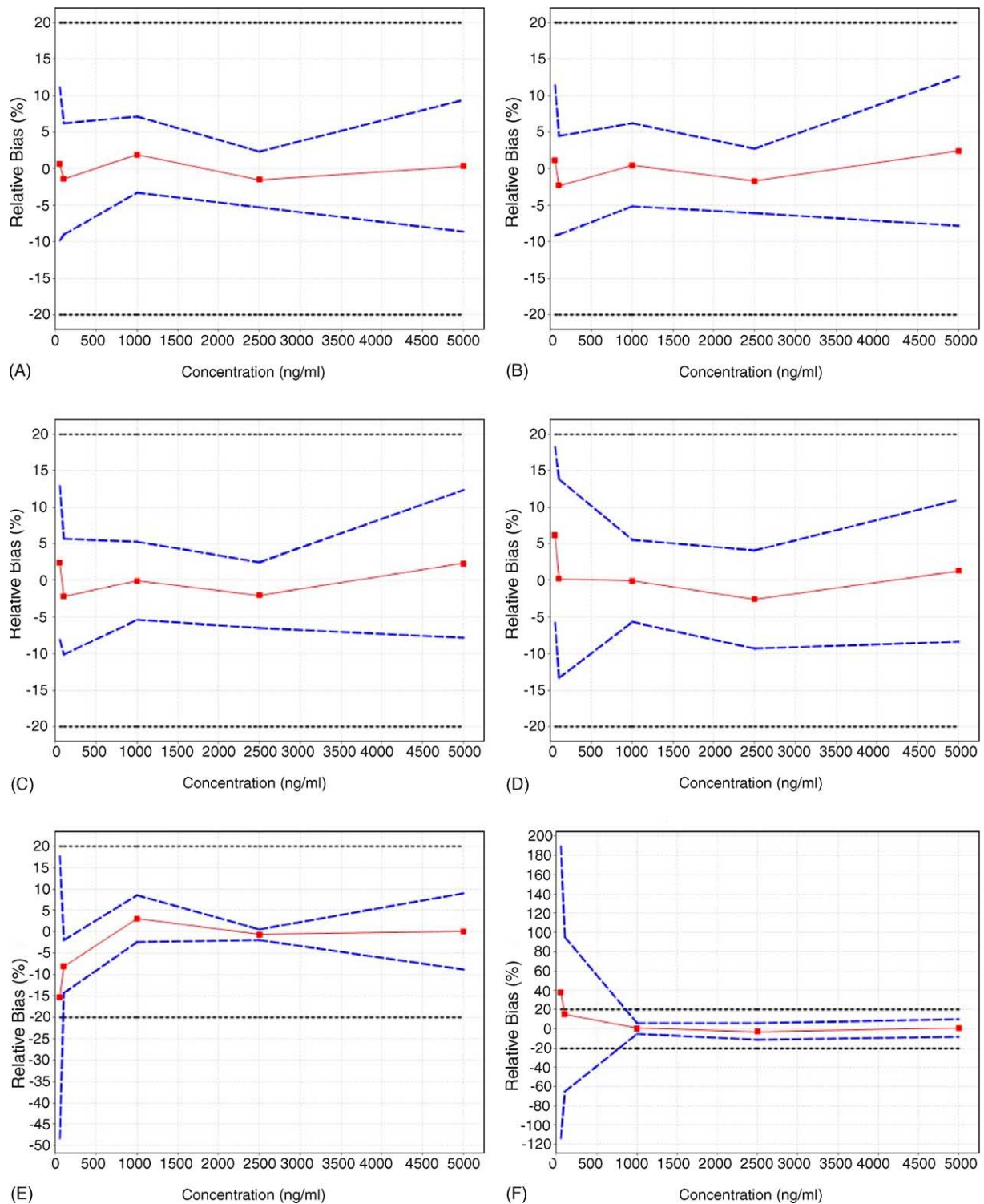
Fig. 2. Accuracy profiles of cloxacillin (concentration ng/ml) using (A) weighted quadratic regression, (B) weighted linear regression model with a weight equal to $1/X^2$, (C) linear regression model after logarithm transformation, (D) linear regression model after square root transformation, (E) quadratic regression, (F) linear regression.

both analytical responses and regression line obtained, selecting the most suitable accuracy profile for the intended use of the analytical method. According to the accuracy profiles obtained (Fig. 2), regression analysis could be performed in the present study using four different regression models: weighted quadratic regression (Fig. 2A), weighted linear regression (Fig. 2B), linear regression after logarithm transformation (Fig. 2C) and linear regression after square root transformation (Fig. 2D). Among these four possibilities, the well-known weighted least-squares model with a weight equal to $1/X^2$ (where X is the theoretical concentration) was selected since it represents the simplest model adequately describing the concentration-response relationship. In addition, by use of the weighted linear regression model, the procedure was able to quantify over the whole range under investigation. Consequently, the concentration range was defined and the LOQ, corresponding to the first concentration level, was estimated.

3.2.2. Absolute recovery

The absolute recovery of cloxacillin at four concentration levels was determined by comparing the peak areas measured after analysis of spiked plasma samples according to the whole procedure with those found after direct injection into the chromatographic system of non-biological samples at the same concentration levels. As shown in Table 2, the analyte recoveries were close to 100% and the extraction efficiency was relatively constant over the considered range according to the relative standard deviation values obtained.

3.3. Validation step

3.3.1. Stability [22]

The stability of cloxacillin in plasma samples placed on the auto-sampler was first determined. After injection of spiked samples at two concentration levels (500 and 5000 ng/ml) in triplicate, no significant degradation of cloxacillin was observed. However, to guarantee the stability of cloxacillin, the samples were directly injected after thawing at room temperature. The stability was also assessed after storage at $-20°$ C for 1 month of plasma samples spiked at 2500 ng/ml. Cloxacillin was determined each week ($n = 3$). The results obtained were comprised between 95 and 105% of the initial value. No significant degradation of cloxacillin was observed.

Table 2
Determination of absolute recovery

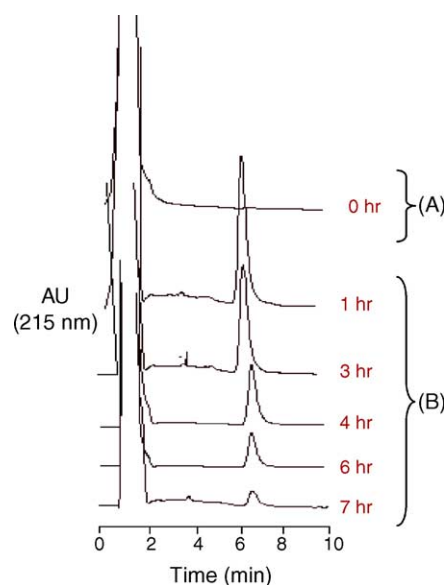| Concentration (ng/ml)	Absolute recovery ($n = 9$)	
	Mean (%)	R.S.D. (%)
50	92.8	4.8
250	96.3	3.5
2500	98.5	2.8
5000	97.4	1.1

Fig. 3. Typical chromatograms obtained after on-line coupling of the XDS (DEAE/diol) pre-column to LC for the analysis of real plasma samples. (A) Chromatogram of a control sample (before oral administration of 500 mg cloxacillin). (B) Chromatograms of dosed samples (from 1 to 7 h after oral administration of 500 mg cloxacillin; hour: 1: 9.6 μg/ml; 3: 6.4 μg/ml; 4: 4.2 μg/ml; 6: 1.5 μg/ml; 7: 0.3 μg/ml). Operating conditions given in Section 2.

3.3.2. Selectivity

The selectivity was studied by analyzing six different sources of plasma [26,27]. No endogenous source of interference was observed at the retention time of the analyte. Typical chromatograms obtained after analysis of a blank plasma and plasma samples containing different concentrations of cloxacillin are illustrated in Fig. 3.

3.3.3. Response function

The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample system [20–22,26,27]. The validation results of the response function are presented in Table 3. As previously mentioned, the weighted linear regression ($1/X^2$) with five concentration levels was used ($m = 5$). During routine analysis, the calibration equation was computed and the concentration of each calibration sample was calculated. If the back-calculated concentration of a calibration sample did not fall within $\pm 15\%$ of the nominal value, the sample was discarded and the equation was recalculated. However, in order that the calibration and the run are valid, no more than two calibration samples were discarded and at least five accepted calibration samples had to be kept.

3.3.4. Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [20,21,24]. As can be seen from the results in Table 3, trueness was expressed in terms of absolute bias (in ng/ml) or

Table 3
Validation results

	Response function ($k=3$, $m=5$, $n=2$) (50–5000 ng/ml)		
	Day 1	Day 2	Day 3
Slope	29.61	28.30	33.53
Intercept	1.312	−33.62	−17.89
r^2	1.000	0.9997	0.9994

	Trueness ($k=3$; $n=4$) Absolute bias: ng/ml (relative bias: %)
50 ng/ml	−0.02 (−0.05)
250 ng/ml	−1.27 (−0.51)
2500 ng/ml	0.93 (0.03)
5000 ng/ml	−6.96 (−0.13)

Precision ($k=3$; $n=4$)		
	Repeatability (R.S.D., %)	Intermediate precision (R.S.D., %)
50 ng/ml	1.5	1.8
250 ng/ml	0.4	0.7
2500 ng/ml	0.5	1.9
5000 ng/ml	0.3	3.4

	Accuracy ($k=3$; $n=4$) β-Expectation tolerance limit in ng/ml (%)
50 ng/ml	47.7–52.3 (−4.5 to 4.5)
250 ng/ml	243.5–254.0 (−2.6 to 1.6)
2500 ng/ml	2283–2720 (−8.7 to 8.7)
5000 ng/ml	4433–5555 (−11.4 to 11.1)

Linearity ($k=3$; $m=4$; $n=4$) ($N=48$)	
Range (ng/ml)	50–5000
Slope	0.9988
Intercept	0.4194
r^2	0.999

Detection and quantification limits	
LOD	15 ng/ml
LOQ	50 ng/ml

Dilution effect ($n=3$) Factor	Recovery ± S.D. (%)
2 (10000 μg/ml)	101.6 ± 2.1
3 (15000 μg/ml)	102.0 ± 3.3

relative bias (%) and was assessed by means of validation standards in the matrix at four concentration levels ranging from 50 to 5000 ng/ml ($k=3$, $n=4$). Compared to the regulatory requirements fixed [26,27], the trueness of the proposed method was quite acceptable since the bias did not exceed the value of 15% irrespective of the concentration level.

3.3.5. Precision

The precision of the bioanalytical method was estimated by measuring repeatability and intermediate precision at the same concentration levels as those mentioned above. The variance of repeatability and time-dependent intermediate precision as well as the corresponding relative standard devi-

ation (R.S.D.) were calculated from the estimated concentrations [20–22]. The R.S.D. values for repeatability and intermediate precision presented in Table 3 were relatively low, between 0.3 and 3.4%, illustrating the very good precision of the proposed method.

3.3.6. Accuracy

The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result [20–22,24]. The upper and lower β-expectation tolerance limits expressed in ng/ml are presented in Table 3 as a function of the introduced concentrations. As can be seen from the results, the proposed method was accurate, since the different tolerance limits of the bias did not exceed the acceptance limits (±15%) [26,27] for all the concentration levels tested including the lowest one (50 ng/ml).

3.3.7. Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample [20–22]. Consequently, with all the series, a regression line was fitted on the back-calculated concentrations as a function of the introduced concentrations by applying the linear regression model based on the least-squares method. The regression equation is presented in Table 3.

3.3.8. Limit of quantitation

The limit of quantitation (LOQ) of an analytical procedure is the lowest amount of the targeted substance in the sample which can be quantitatively determined under the experimental conditions prescribed with a well defined accuracy, i.e. taking into account the systematic and random errors [20–22]. As the accuracy profile is comprised within the acceptance limits, the LOQ was fixed to 50 ng/ml, i.e. the smallest concentration level investigated. Indeed, precision and trueness were demonstrated at this concentration level (Table 3).

3.3.9. Effect of dilution

Since the amount of cloxacillin in the present preliminary study was not known a priori, the influence of the dilution procedure, which is intended to be used in routine for samples with a concentration higher than the upper limit of the range, has to be checked [22]. In the present study, two dilution factors (2 and 3) were studied before routine analysis and no significant effect was observed (Table 3).

3.4. Application to real samples

The developed procedure was used to determine the plasma profile of cloxacillin after a single oral dose of an immediate release formulation containing 500 mg of cloxacillin. On the basis of the preliminary results, the plot of the plasma concentration of cloxacillin (ng/ml) versus post-dose sampling time (h) is presented in Fig. 4.
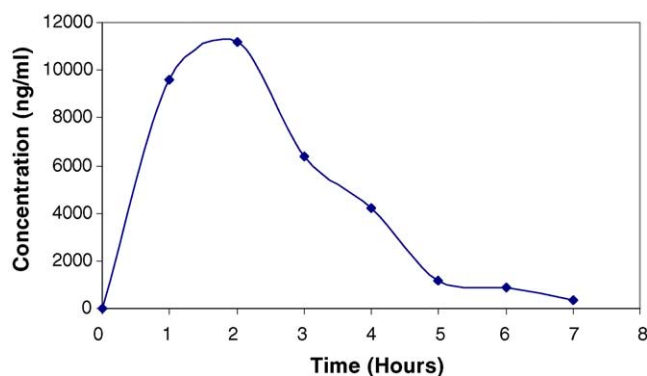
Fig. 4. Plasma concentration–time profile of cloxacillin following the administration of a single oral dose of 500 mg.

4. Conclusions

A simple and selective LC method based on the column switching technique has been developed for the on-line determination of cloxacillin in human plasma by means of a pre-column packed with anion exchange restricted access material for sample clean-up. The column switching technique was of particular interest since the stability of cloxacillin is poor. Moreover, due to the use of a new type of restricted access material with diethylaminoethyl groups bonded to the inner surface of the porous sorbent (XDS-DEAE support), a selective sample clean-up was obtained allowing to achieve a rather good detectability of cloxacillin at a low wavelength in UV detection (50 ng/ml).

The procedure was then fully validated and successfully applied to a preliminary study for the determination of cloxacillin plasma levels after a single oral dose of an immediate release formulation containing 500 mg of this compound. In conclusion, the proposed method could be used for routine analysis and bioequivalence studies of different cloxacillin dosage forms.

Acknowledgement

References

[1] European Pharmacopoeia, Directorate for the Quality of Medicines of the Council of Europe, Strasbourg, France, fourth ed., Supplement 4.6, Monograph 01/2004:0929.

[2] Y. Ito, Y. Ikai, H. Oka, H. Matsumoto, T. Kagami, K. Takeba, J. Chromatogr. A 880 (2000) 85–91.

[3] L.K. Sorensen, B.M. Rasmussen, J.O. Boison, L. Keng, J. Chromatogr. B 694 (1997) 383–391.

[4] A.B. Jamaludin, G. Sarwar, M.A. Rahim, M.K. Rahman, J. Chromatogr. 490 (1989) 234–246.

[5] B. Pérez, C. Prats, E. Castells, M. Arboix, J. Chromatogr. B 698 (1997) 155–160.

[6] S. Lihl, A. Rehorek, M. Petz, J. Chromatogr. A 729 (1996) 229–235.

[7] F. Jehl, Ph. Birckel, H. Monteil, J. Chromatogr. 413 (1987) 109–115.

[8] C.T. Hung, J.K. Lim, A.R. Zoest, J. Chromatogr. 425 (1988) 331–341.

[9] T.C. Pinkerton, J. Chromatogr. 544 (1991) 13–23.

[10] M. Beth, K.K. Unger, M.P. Tsyurupa, A.V. Davankov, Chromatographia 36 (1998) 351–358.

[11] Z. Yu, D. Westerlund, K.-S. Boss, J. Chromatogr. B 704 (1997) 53–62.

[12] J. Haginaka, Trends Anal. Chem. 10 (1991) 17–22.

[13] K.-S. Boos, A. Rudolphi, S. Vielhauer, A. Walfort, D. Lubda, F. Eisenbeiß, Fresenius J. Anal. Chem. 352 (1995) 684–690.

[14] K.-S. Boos, C.-H. Grimm, Trends Anal. Chem. 18 (1999) 175–180.

[15] S. Vielhauer, A. Rudolphi, K.-S. Boos, D. Seidel, J. Chromatogr. B 666 (1995) 315–322.

[16] A. Rudolphi, S. Vielhauer, K.-S. Boos, D. Seidel, I.M. Bäthge, H. Berger, J. Pharm. Biomed. Anal. 13 (1995) 615–623.

[17] P. Chiap, O. Rbeida, B. Christiaens, Ph. Hubert, D. Lubda, K.-S. Boos, J. Crommen, J. Chromatogr. A 975 (2002) 145–155.

[18] O. Rbeida, B. Christiaens, P. Chiap, Ph. Hubert, D. Lubda, K.-S. Boos, J. Crommen, J. Pharm. Biomed. Anal. 32 (2003) 829–838.

[19] O. Rbeida, B. Christiaens, Ph. Hubert, D. Lubda, K.-S. Boos, J. Crommen, P. Chiap, J. Chromatogr. A 1030 (2004) 95–102.

[20] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. (2004), in press.

[21] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma Pratiques 13 (2003) 101–138.

[22] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135–148.

[23] B. Boulanger, P. Chiap, W. Dewe, J. Crommen, Ph. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753–765.

[24] ISO 5725-1, Application of the statistics—accuracy (trueness and precision) of the results and methods of measurement, Part 1: General principles and definitions, International Organization for Standardization (ISO) Geneva, Switzerland.

[25] K.L. Tyczkowska, R.D. Voyksner, A.L. Aronson, J. Chromatogr. 594 (1992) 195–201.

[26] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309–312.

[27] Guidance for industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), May 2001.