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Journal of Chromatography B, 775 (2002) 127–132

JOURNAL OF CHROMATOGRAPHY B

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New and rapid fully automated method for determination of tazobactam and piperacillin in fatty tissue and serum by column-switching liquid chromatography

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Received 5 July 2001; accepted 5 March 2002

Abstract

A sensitive and rapid HPLC assay for determining tazobactam and piperacillin in fatty tissue and serum is described. While the common methods need liquid–liquid extraction before the injection in a automated column switching HPLC, the new method works by direct injection of the filtered tissue extract or diluted serum in a automated column switching HPLC without any other pre-treatment. This was performed by the use of a NH_2-precolumn and enrichment/transfer at different pH-level. During the analyses, the NH_2-precolumn was automatically regenerated with acetonitrile–water. The chromatogram peaks for piperacillin and tazobactam were identified by the retention time and quantified by peak area. The calibration curve was linear between 1 and 16 μg/ml. The quantification limit of tazobactam was about 1 μg/ml in fatty tissue extracts and in diluted serum (calculated for pure serum 2 μg/ml), respectively. For piperacillin it was less. The described procedure allows sample clean-up and determination of the antibiotic within 35 min. The chromatograms with this easy sample treatment had the same quantity of matrix peaks and in contrast to liquid–liquid extraction no loss of piperacillin. Because of the automatically rinsing of the NH_2-precolumn during the chromatographic separation, more than 50 different biological samples could be measured with one NH_2-precolumn without loss of performance. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Tazobactam; Piperacillin

1. Introduction

A HPLC method for the rapid and reliable determination of piperacillin and tazobactam in serum and fatty tissue was developed to study the efficacy of preoperatively administered piperacillin and tazobactam to patients undergoing hand surgery under bloodless conditions. In this case the concentrations of the antibiotics in the tissue, the place of a possible infection, are of interest. Serum concentrations alone cannot determine the risk of infection [1].

Several methods have been described for the determination of piperacillin and tazobactam in serum and other body fluids [2]. For different tissue

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PII: S1570-0232(02)00298-2

and serum a column-switching method has been described [3,4]. All the methods need a deproteinisation of the serum or tissue extracts before injecting into the HPLC system, even when they operate with column switching. Direct injection of serum is usually only performed by using ADS precolumns. The method presented here performs direct injection with a simple NH_2-precolumn by changing the pH levels of the eluents and automatically rinsing the precolumn between the analyses. Deproteinisation with acetonitrile as described in Refs. [2–5] requires also the use of an internal standard, which is not necessary with our method.

2. Material and methods

2.1. Reagents and chemicals

Tazobactam and piperacillin were kindly supplied by Wyeth-Lederle (Münster, Germany). The other antibiotics were purchased from the pharmaceutical industry, except cefuroxim, penicillin G, penicillin V which were obtained from Sigma (Deisenhofen, Germany). Acetonitrile (LiChrosolv®) and sodium dihydrogenphosphate monohydrate were purchased from Merck (Darmstadt, Germany). Formic acid sodium salt was obtained from Sigma. All reagents were at least analytical grade, except for acetonitrile which was gradient grade. HPLC-grade water was generated by using a Milli-Q water-purification system from Millipore (Molsheim, France). Tissue samples were collected from patients suffering from acute severe hand injuries, who were asked to join this study and informed consent was obtained by the Department of Plastic and Hand Surgery, University Medical Center (Freiburg, Germany). The protocol was approved by the Committee of Ethics of the Medical Center of the University of Freiburg.

2.2. Apparatus

The in-line extraction system was constructed with a Knauer Eurospher NH_2 guard column (25×4 mm) and in-line filter (2-μm sieve), Merck (Darmstadt, Germany). The HPLC system consisted of two LC-10AT HPLC pumps, a CTO-10AC column oven, an SPD-10A UV detector and an FCV-12AH 6-Port Valve, controlled by a CBM-10A Module (all items obtained from Shimadzu, Duisburg, Germany). Samples were injected via automatic sample injector SIL 10Avp equipped with a 150-μl sample loop. Chromatography was performed on a Nucleosil C_{18} 250× 4.6 mm, 5-μm analytical column preceded by a guardcolumn CC 8/4 Nucleosil 100-5 C_{18} (Macherey & Nagel, Düren, Germany).

2.3. Analytical procedure

2.3.1. Sample preparation

Serum sample was diluted with the same volume of 12.25 m*M* sodium format buffer (pH 3) and filtered through a 0.45-μm filter (Satorius, Göttingen, Germany) and then injected into HPLC. Fatty tissue samples were taken from patients suffering from acute heavy hand accident injuries. Exsanguination was instigated in the upper extremity and a tourniquet was inflated to 350 mmHg. The patients received an intravenous dose of 2.0 g piperacillin and 0.5 g tazobactam (2.5 Tazobac, Lederle, Wolfratshausen, Germany) for prevention of wound infections during the surgery under bloodless conditions as described in Ref. [1]. Blood was removed from all tissue samples by cleaning with a dry sterile gauze before samples were weighed. The tissue was then placed into sterile polypropylene tubes and stored at −80 °C until analysis. To each gram of tissue sample a minimum of 1 ml of 50 m*M* sodium dihydrogenphosphate buffer (pH 5.0) was added. After 60 s homogenization with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany), the sample was centrifuged at 10 000 rpm for 10 min. The supernatant was filtered through a 0.45-μm filter (Satorius) and injected into the HPLC system. These were the only manual steps in the analytical procedure. Extraction and chromatography of the analytes was carried out automatically by the HPLC integrated extraction system.

2.3.2. HPLC integrated extraction

The HPLC integrated extraction procedure steps were: (i) sample application and fractionation, (ii) transfer of the analyte fraction, and (iii) chromatographic separation.

The mobile phase used for the extraction pro-

cedure contained 12.5 m*M* sodium formate buffer and was adjusted to pH 3.0.

An aliquot (150 μl) of the tissue extract or diluted serum was injected by the autosampler. The mobile phase (low pressure pump set to 0.8 ml/min) transferred the sample to the extraction NH_2-precolumn (Fig. 1A). Piperacillin and tazobactam were retained on this column, while other matrix compounds were discharged to waste together with the eluent. After 8 min, the matrix had been washed out of the NH_2-precolumn and the software time-schedule automatically switched the high-pressure valve B into transfer position, thereby linking the NH_2-precolumn with the HPLC circulation (Fig. 1B). The transfer of both analytes was performed within 1.5 min using 97% phosphate buffer (pH 5) and 3% acetonitrile. During the analysis, valve B was switched to initial position, while at the same time the NH_2-precolumn was rinsed with 50% acetonitrile (Fig. 1C). For the determination of tazobactam the same eluent as for transfer was used. For the determination of piperacillin, after the elution of tazobactam, the eluent was automatically changed to an acetonitrile content of 22%, after another 8 min. During the last 5 min of

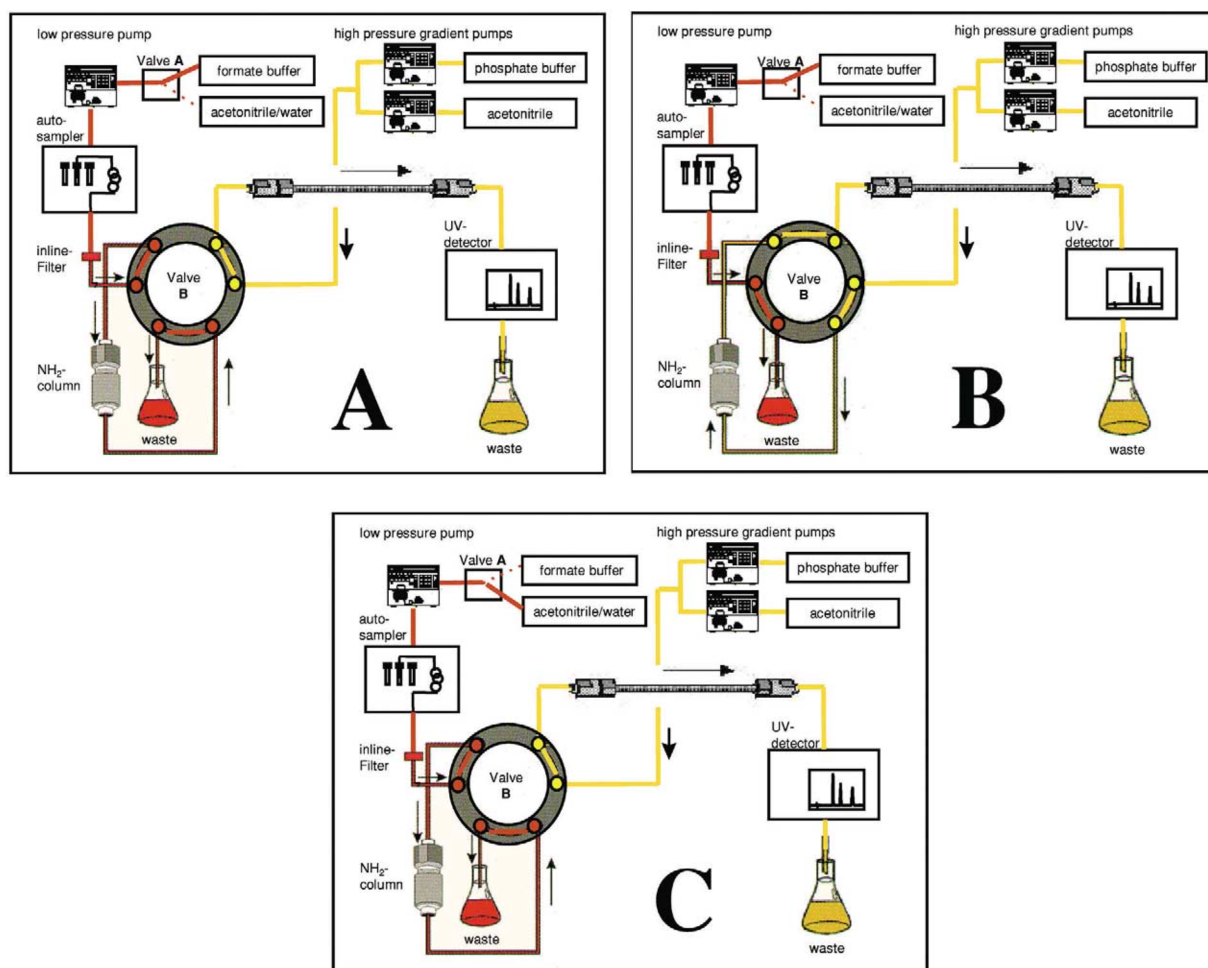

Fig. 1. Scheme of the HPLC integrated sample preparation. (A) Equilibrating the columns and sample application. (B) Transfer of the analyte fraction. (C) Chromatographic separation and rinsing.

the analysis the NH_2-precolumn was re-equilibrated by changing valve A again to format buffer position (Fig. 1A).

2.4. Quantification

Standards to study linearity were made by diluting an aqueous stock solution of piperacillin and tazobactam in a range of 1–16 μg/ml for piperacillin and tazobactam.

All standards were injected into the in-line extraction system as described above. Tazobactam and piperacillin was detected at 210 nm, whereas for some tissue samples the best wavelength for determination of piperacillin was 244 nm [6]. The calibration curve was based on the peak area of each standard plotted versus the nominal antibiotic concentration using least-squares linear regression.

Pooled blank serum was spiked with standard solution of piperacillin–tazobactam to yield concentrations between 1 and 16 μg/ml. Fatty tissue samples were spiked to yield concentrations between 2.25 and 4.5 μg/ml

The chromatogram peaks for piperacillin and tazobactam were identified by the retention time and quantified by peak area. Accuracy of the piperacillin–tazobactam serum and fatty tissue assay was determined by calculating the mean percentage differences between nominal and measured concentrations. The assay precision was characterized by mean value and coefficient of variation (C.V.).

The average quotient of measured concentrations and nominal concentrations indicated the ratios of the recovered antibiotic. The detection and quantification limits were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively.

3. Results and discussion

The analytical method described for piperacillin and tazobactam overcomes time-consuming procedures of sample preparation. While the common methods for serum and also tissues need deproteinisation with acetonitrile, it could be shown that the antibiotics could be well separated from the matrix by direct injection of diluted serum or fatty tissue extracts using a NH_2-precolumn (Fig. 2). The deproteinisation could be performed by an automated system not only by special column packing materials as Lichrospher ADS [7] but also by a simple precolumn as the NH_2-precolumn used. This is of economic interest because the price of the used NH_2-precolumn is about 10 times less than Lichrospher ADS, which is not yet available with a NH_2 surface. One precolumn was used for more than 30 fatty tissue extract samples and 50 serum samples without change in its performance.

An internal standard was not necessary because there was no loss of piperacillin and tazobactam during sample diluting and filtration. In contrast to this, we found that during the deproteinisation procedure used by Kinzig et al. [3], there was a loss of piperacillin between 10 and 100% (depending on concentration), which had to be compensated by internal standard. To determine the accuracy of the method, the recovery of piperacillin and tazobactam in spiked tissue extract and serum samples was determined. The results are shown in Table 1. Interference with other antibiotics was tested with a concentration of 5 μg/ml. There was no interference with cefuroxim, ciprofloxacin, doxycyclin, imipenem, meropenem, ofloxacin, sulfamethoxazol,

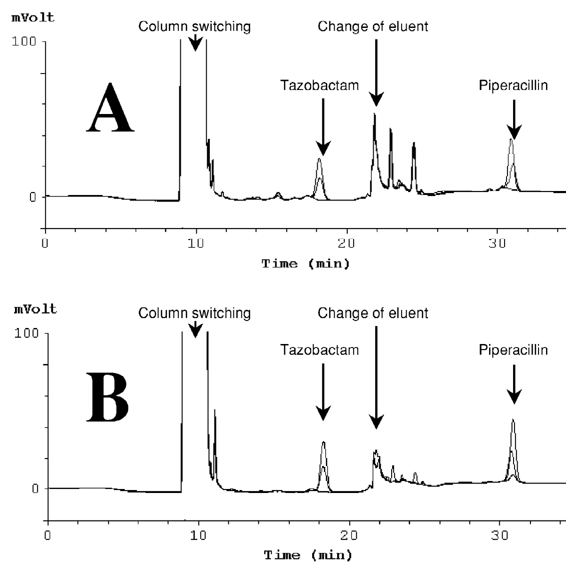

Fig. 2. (A) Chromatograms of spiked serum (2.25 and 4.5 μg/ml) versus blank serum. (B) Chromatograms of spiked fatty tissue extract (2.25 and 4.5 μg/ml) versus fatty tissue extract with low remaining amounts of piperacillin. Real blank fatty tissue extract was not available.

Table 1
Recovery

	Concentration (μg/ml)	Piperacillin		Tazobactam	
		Mean recovery from nominal	R.S.D (%)	Mean recovery from nominal	R.S.D (%)
Fatty tissue[a]	1	101.5% (n=6)	8.3	101% (n=6)	11.3
	2	102.7% (n=3)	9.5	102% (n=3)	3.3
	4	96.9% (n=3)	2.8	101% (n=3)	1.8
	8	99.7% (n=3)	2.0	102% (n=3)	1.3
	16	100.1% (n=3)	1.5	100% (n=3)	1.3
Serum[b]	1	102.6% (n=6)	2.6	101.1% (n=6)	6.3
	2	101.3% (n=3)	3.3	95.9% (n=3)	11.3
	4	99.5% (n=3)	1.8	97.7% (n=3)	3.5
	8	99.8% (n=3)	0.4	98.7% (n=3)	0.5
	16	100.0% (n=3)	1.0	100.6% (n=3)	1.2

[a] Calculated on the basis of addition to fatty tissue extract.
[b] Calculated on the basis of addition to pool serum.

Table 2
Detection and quantification limits

Sample matrix	Piperacillin		Tazobactam	
	Detection limit S/N 1:3 (μg/ml)	Quantification limit S/N 1:10 (μg/ml)	Detection limit S/N 1:3 (μg/ml)	Quantification limit S/N 1:10 (μg/ml)
Serum	0.3	1.0	0.7	2.0
Fat tissue extract	0.3	1.0	0.3	1.1
Millipore water	0.2	0.5	0.2	0.5

trimethoprim, vancomycin. penicillin G, penicillin V and sulbactam can also be determined with the method. Penicillin G and piperacillin cannot be determined at the same time because of interference.

3.1. Quantification and statistics

Accuracy was satisfying in all calibration curves of serum, fatty tissue extract and millipore water (all r^2>0.999). The assays detection and quantification limits are shown in Table 2.

4. Conclusion

The presented assay for the determination of piperacillin and tazobactam in serum and tissue samples is sufficiently fast, sensitive and reliable for drug-monitoring in clinical routine. It allows the automated determination of the antibiotics in serum and fatty tissue samples without preliminary deproteinisation and without special column materials such as Lichrospher ADS. It is also suitable for pharmacokinetic studies in these biological fluids. The detection limits of the assays allow determination of antibiotic levels down to the MIC90 of the most relevant pathogens [8].

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