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Application of a semipermeable surface column for the determination of amoxicillin in human blood serum

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Abstract

A high-performance liquid chromatography column-switching system for the automated determination of amoxicillin in human serum was developed as a more efficient alternative for the already existing systems with off-line sample pretreatment. The column-switching system consists of a semipermeable surface (SPS) column and an analytical reversed-phase (RP) C18 column. After centrifuging, pure serum samples were injected into the column-switching system. Clean-up, with regard to removal of proteins, was performed on the SPS column. The fraction containing amoxicillin was concentrated on the analytical RP-C18 column. Finally, chromatography and detection were performed with the RP-C18 column using UV detection at 234 nm. The total analysis time was 15 min. The method has proven to be reliable and to be more time- and resource-efficient compared to previously used methods with off-line sample clean-up. It is now used in bioavailability studies for the development of new amoxicillin formulations.

Keywords: Amoxicillin

1. Introduction

Amoxicillin (Fig. 1) is a widely used broad spectrum antibiotic. It belongs to the group of penicillins characterized by a β-lactam group. For the bioanalysis of this compound, several assay methods with off-line sample clean-up have been described [1–8]. However, it was perceived that the efficiency of these methods could be increased by optimisation of the sample clean-up. It was reported that solid-phase extraction is an efficient technique for the clean-up of amoxicillin-containing serum samples [2,3]. However, in these reports solid-phase extraction was not followed by on-line elution to a HPLC column. In a later report, a further step towards automation was made by using the Varian AASP (Advanced Autosampler Sample Processor) as

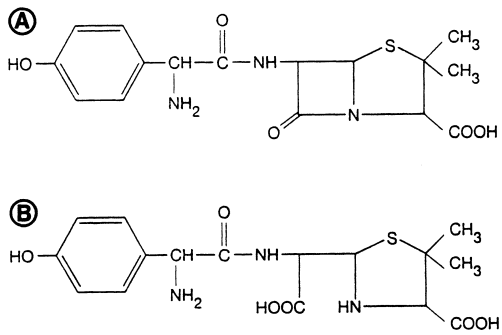

Fig. 1. Structures of (A) amoxicillin and (B) amoxicilloic acid.

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a fast and simple technique for off-line preparation of amoxicillin-containing samples, followed by on-line elution to a HPLC column [8]. A disadvantage of the use of the AASP is the restricted stability of amoxicillin on the RP cartridges and the limited availability and poor support of the AASP system.

In 1985, Regis patented a new type of stationary phase, GFF (Glycine–Phenylalanine–Phenylalanine) [9,10]. This type of stationary phase was called an Internal Surface Reversed Phase (ISRP). ISRP columns are suitable for direct injection of plasma and serum samples without sample pre-treatment. Separation is effected on porous material with a hydrophilic outer surface and a hydrophobic GFF internal surface. Plasma proteins will elute unretained, while small molecules such as drugs will penetrate into the internal surface and interact with the GFF phase according to reversed-phase interactions. Shortly after the introduction of these columns, several applications became available [11–20]. However, due to relatively poor separation characteristics, the limit of quantification of several drugs in plasma was too high for pharmacokinetic purposes. Column switching was found to be a suitable technique to overcome the problem [21,22]. However, in the case of amoxicillin, the retention and the plate number on the ISRP material were poor and the resolution between the amoxicillin peak and other plasma components was insufficient (results from unpublished experiments).

Another type of stationary phase suitable for direct injection is the semipermeable surface (SPS) phase. SPS columns are designed for chromatography of small molecules in the presence of proteins. The SPS packing material has an outer and an inner phase. The outer hydrophilic phase is a polyoxyethylene polymer, covalently bound to the silica surface. The inner phase is also bound to the silica surface, but below the hydrophilic polymer. The inner phase is one of the common reversed phases that is used in chromatography, C18. The outer phase forms a semipermeable surface that prevents large molecules such as proteins from reaching the inner phase. Small molecules penetrate into the semipermeable surface and are retained by the inner phase, whereas proteins are eluted. An SPS column in combination with a column-switching system was used for direct injection of plasma samples containing amide-type local anaesthetics [23].

In the present study the SPS column was explored for direct serum injection in the bioanalysis of amoxicillin-containing samples. The system consisted of a column-switching system and was expected to be fast, simple and reliable in the bioanalysis of amoxicillin.

2. Experimental

2.1. Columns

The analytical column used was a 250×4.6 mm I.D. stainless steel pre-packed column containing 5 μm Chromspher C18 (Chrompack, Middelburg, The Netherlands). A Semi Permeable Surface pre-column (10×3 mm, 5 μm particle size) and a Semi Permeable Surface column (250×4.6 mm, 5 μm particle size) (Regis Chemical Company, Morton, Grove, USA) were used for direct injection of serum samples. The pre-column was replaced every 75 samples.

2.2. Chemicals and solvents

Acetonitrile used in preparing the mobile phases was of HPLC quality and was purchased from Baker (Deventer, The Netherlands). Water was collected from a Milli-Q system (Waters, Etten-Leur, The Netherlands). Na_2PO_4 buffer solution was prepared by dissolving 17.8 g of di-sodium hydrogenphosphate dihydrate p.a. (E. Merck, Darmstadt, Germany) in 900 ml of deionized water. After adding 0.45 g of tetrahexylammonium hydrogenphosphate (THAH) (Fluka, Bornem, Belgium), the pH was adjusted to 7.5 with diluted HCl solution. Water was added to make a total volume of 1000 ml. Both the buffer and acetonitrile were degassed by continuous helium sparging. The solvents were mixed on-line by the HPLC pump.

Amoxicillin·$3H_2O$ was provided by Gist-brocades (Delft, The Netherlands). A stock solution was prepared by dissolving 65 mg of amoxicillin·$3H_2O$ in 45 ml of water. After dissolution, water was added to make up to a volume of 50.0 ml. The stock solution was stored at −20°C.

2.3. Instruments

For the circulation of mobile phases, two Spectra Physics SP9900 HPLC pumps (Spectra Physics Analytical, San Jose, USA) were used. The SPS pre-column, the SPS column and the analytical column were placed in a Chrompack SpH 99 column thermostat (Chrompack, Middelburg, The Netherlands). The temperature was maintained at 35°C. A MUST column-switching system (Spark, Holland, The Netherlands) was used to direct the SPS elute fraction containing amoxicillin to the analytical column. Serum samples were injected into the system using a Waters 717 autosampler (Waters Chromatography, Etten Leur, The Netherlands). A Spectra Physics Spectra 200 variable-wavelength UV detector was used to monitor the effluent of the analytical column at a wavelength of 234 nm.

2.4. Chromatographic system

The chromatographic system is presented in Fig. 2. The sample was injected onto the SPS column into a mobile phase of a mixture of 0.1 *M* Na_2PO_4 and acetonitrile (98+2) which was circulated using pump A. The SPS elute was first directed to waste through position 1-2 of the switching valve, while pump B delivered the mobile phase for the analytical column through position 5-6 of the switching valve. The fraction containing amoxicillin was directed to the RP-C18 analytical column by switching the valve in position 1-6 during a time interval of 2–5 min. After transferring the amoxicillin-containing fraction to the analytical column, the valve was turned back and amoxicillin was separated from matrix constituents on the RP-C18 column using a mixture of 0.1 *M* Na_2PO_4 and acetonitrile (85.5+14.5), also containing 0.001 *M* THAH as mobile phase. Final detection of eluted components was performed using the UV detector tuned at 234 nm. To prevent increasing back pressure and decreasing performance of the SPS column, a SPS quard column was used and replaced after each 75 serum injections.

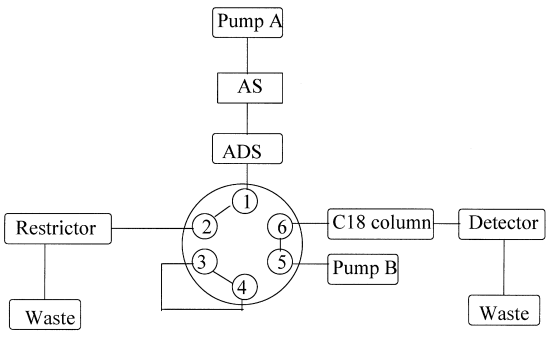

Fig. 2. Chromatographic system.

2.5. Sample collection and preparation

The assay was applied to 1782 blood serum samples of untreated subjects, and of male subjects who received an oral 125 or 1000 mg dose of amoxicillin.

Human blood samples were collected in plain glass tubes (Venoject, Terumo Europe, Leuven, Belgium) and centrifuged for 10 min between 30 and 45 min after collection. The serum was isolated and stored at −70° C until analysis. On the day of analysis the samples were thawed using cold streaming water. Samples were centrifuged at 2500 *g* for 10 min. The sample was transferred into a glass vial. Finally, 10 μl of the sample was injected into the column-switching system. Calibration samples were prepared by adding 20 μl of a calibration solution of amoxicillin·$3H_2O$ in water to a volume of 200 μl of blank serum. The resulting samples contained amoxicillin·$3H_2O$ concentrations between 0.35 and 21 μg/ml. Weighted least squares regression was used to construct the calibration curve using peak heights, with the square of the reciprocal of the concentration as a weighting factor. Samples with concentrations >21 μg/ml were reanalysed after appropriate dilution.

3. Results and discussion

3.1. HPLC separation

The chromatograms (Fig. 3A and B) obtained from a blank human serum sample and a blank human serum sample spiked with amoxicillin at a level of 0.6 μg/ml show that the amoxicillin signal

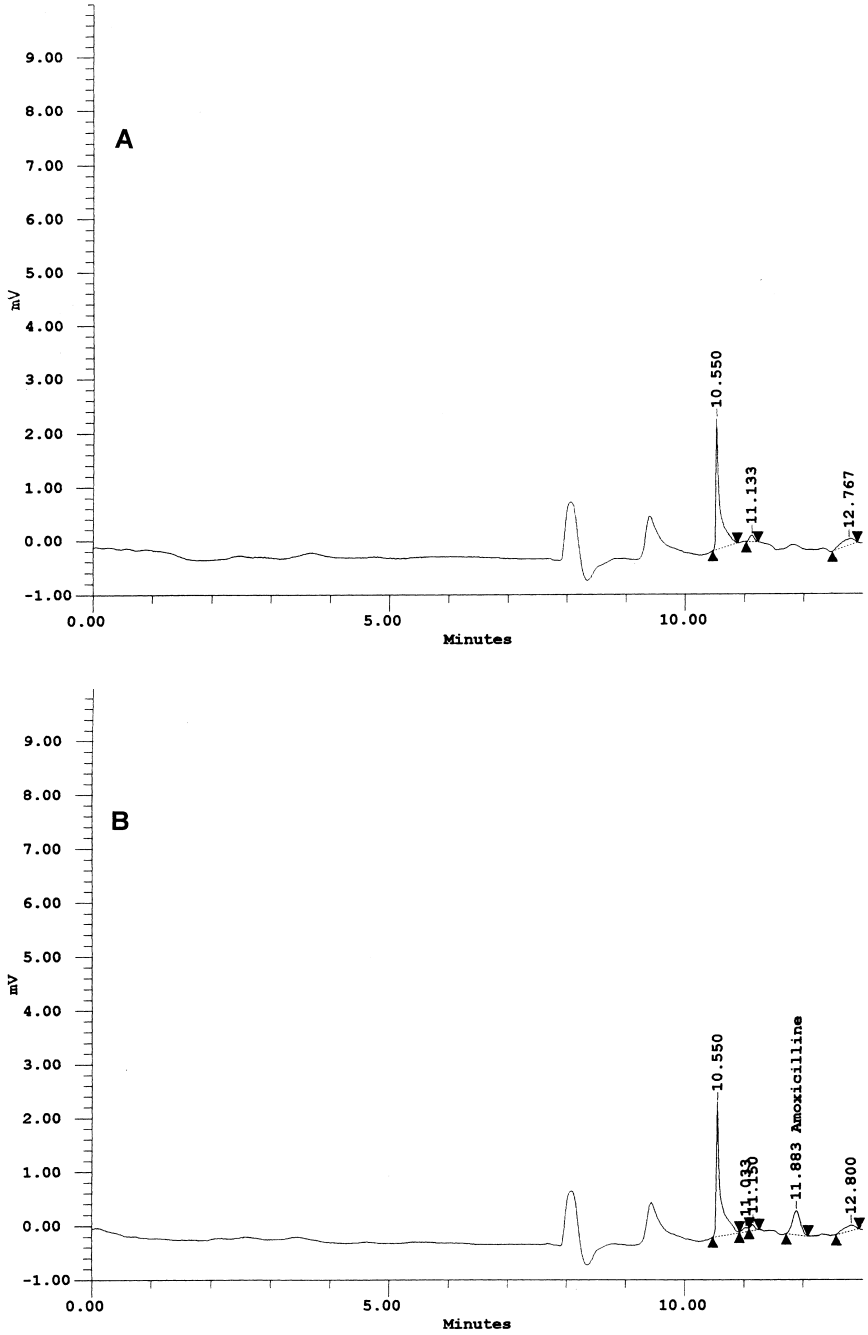

Fig. 3. Chromatograms of (A) a blank human serum sample and (B) a human serum sample spiked with 0.6 μg/ml amoxicillin.

is free of interferences. The retention time of amoxicillin is approximately 11.8 min. Amoxicilloic acid (Fig. 1), the main metabolite of amoxicillin, does not interfere with the amoxicillin assay since this component is not present in the fraction eluted from the SPS column.

3.2. Recovery of amoxicillin

The recovery of amoxicillin was determined at three concentrations by injecting each level six times (three serum batches spiked in duplicate) and comparing the mean peak height with the mean peak height obtained from pure amoxicillin solutions in water. The recovery was not fully complete due to the fact that the volume of the amoxicillin-containing fraction from the SPS column was rather large due to peak tailing. After switching the valve back, a very small amount had not yet been eluted from the SPS column. The recovery of amoxicillin proved to be high and reproducible (Table 1).

3.3. Linearity

The linearity of the calibration graph was tested using a Lack-Of-Fit test [24]. Each level was injected in triplicate in a randomized sequence. The calibration graph proved to be linear between 0.35 and 21.0 μg/ml serum and passed through the origin. The correlation coefficient was at least 0.99. The limit of detection was 0.11 μg/ml.

3.4. Accuracy, precision and limit of quantification

The accuracy and precision of the bioanalytical assay were determined at three serum levels of amoxicillin on 3 days. Accuracy (Table 2) and precision (Table 3) values were acceptable in the described serum concentration range. As can be expected, the precision of the bioanalytical assay improved with increasing serum levels.

The LOQ was defined as the concentration at which the accuracy of the bioanalytical method ranged between 80 and 120% with a maximum precision of 20%. The LOQ of the present method was estimated at 0.35 μg/ml.

Table 1
Mean recovery (±SD) of amoxicillin from human serum (n=6)

Concentration (μg/ml)	Recovery (%)
0.96	94±2.2
3.82	89±3.2
15.9	87±2.8

Table 2
Mean accuracy (±SD, n=9) of amoxicillin assay in human serum

Concentration (μg/ml)	Accuracy (%)		
	Day 1	Day 2	Day 3
0.96	−5±2.9	−3±4.4	15±12.2
3.82	2±2.5	1±1.7	5±2.4
15.9	6±3.0	2±3.1	−2±2.8

3.5. Stability of amoxicillin in serum

The stability of amoxicillin at levels of 1.1 and 17.8 μg/ml in serum was determined at 10°C. After 7 h at 10°C, 95% of amoxicillin was recovered from serum.

4. Conclusion

Due to the high polarity of amoxicillin, sufficient separation from matrix constituents was not possible when using the Semi Permeable Surface column as an analytical column as such, and it was decided to use the column-switching system. In the resulting system, the SPS column displayed good clean-up properties in the bioanalysis of amoxicillin in human serum (Fig. 2A and B). The column-switching system is suitable for automated sample clean-up of human serum samples for amoxicillin. The total chromatographic retention time amounts to 16 min which, together with the extremely limited pretreat-

Table 3
Precision of the amoxicillin assay

Concentration (μg/ml)	SD (n=9) (μg/ml)		
	Day 1	Day 2	Day 3
0.196	0.03	0.04	0.12
3.82	0.10	0.06	0.09
15.9	0.48	0.49	0.45

ment technique, implies a high sample throughput. Over 750 serum samples were analysed without decreasing performance of the SPS column. A maximum of 90 samples (including calibration and quality control samples) could be analysed per day on one system compared to 60 samples/day with a previously used method [8]. Considering the successful implementation of this method, the applicability of this technique will also be explored for other, also less polar, compounds.

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