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ORIGINAL RESEARCH PAPER

Separation of ampicillin on polar-endcapped phase: Development of the HPLC method to achieve its correct dosage in cardiac surgery

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ABSTRACT

The accurate, simple, and selective reversed phase high performance liquid chromatography (RP-HPLC) has been established and validated for the determination of an antibiotic ampicillin (AMP) in human blood plasma. The SPE extraction was used for the sample preparation. Chromatographic separation was accomplished by a mobile phase containing 15 mM monopotassium phosphate solution of pH 3.3 and methanol (75:25, v/v) in an isocratic mode at a flow rate of 1.4 mL min^{-1} at 30 °C. The separation was evaluated on a column with a new polar-endcapped C_{18} stationary phase Arion® Polar C18 or well-known phase Luna® Omega Polar C18. Excellent linearity (R^2 0.9998) was shown over range 10–300 mg L^{-1} with mean percentage recovery 90%. Peak shapes were symmetrical in both columns, Arion® Polar C18 and Luna® Omega Polar C18, with asymmetry factor of 1.0 and 1.4, tailing factor of 1.0 and 1.2, and retention factor of 4.6 and 5.6, respectively. The Arion® Polar C18 was almost 1.4-fold more effective than Luna® Omega Polar C18 phase. The LOQ for ampicillin was achieved 10 mg L^{-1} for Luna® Omega Polar C18 and 5 mg L^{-1} for Arion® Polar C18 using 20 µL of a solution containing 0.24 mg mL^{-1} of cephalexin as an internal standard.

A number of articles dealing with the determination of ampicillin is limited, therefore, this study showed the HPLC method suitable for the determination of AMP in human blood plasma from patient who underwent elective cardiac surgical revascularization. In addition, the determination of AMP was also performed for the first time using an Arion® Polar C18 column, which effectively separated AMP from other compounds present in human blood plasma. This new polar-endcapped phase can help in separation of polar antibiotics or other polar compounds, which are unsuccessfully separated on conventional C_{18} column, and thus can help during method development.

KEYWORDS

ampicillin, polar column, separation

INTRODUCTION

Antibiotics have been widely used in human health care and veterinary medicine. The broad range of antibiotic use, their overuse, and incorrect indications have led to the development of bacterial resistance [1]. It is therefore logical that there is a growing demand for more targeted and personalized antibiotic dosing. Currently, a number of methods for the determination of antibiotics in various matrices are available, including high-performance liquid chromatography (HPLC) [2–8] or mass spectrometry [9, 10], or a combination of HPLC with mass spectrometry [5, 11–13] or with electrochemical methods [14–16]. However, number of articles dealing with the determination of ampicillin is limited. Ampicillin is one of antibiotics that works against Gram-positive and Gram-negative aerobic bacteria and anaerobic bacteria [17, 18] (structure shown in Fig. 1). Its prophylactic use is a standard in a cardiac

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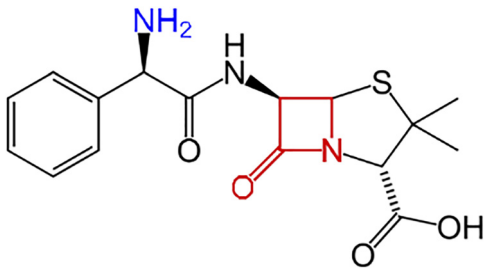

Fig. 1. Chemical structure of ampicillin with the β-lactam ring (marked in red) and polar group (marked in blue)

surgery. For this reason, it is important to develop and improve reliable method for determining ampicillin concentrations in order for a prophylaxis to be effective. In HPLC methods, reversed octadecylsilane (C_{18}) phase columns or octylsilane (C_8) phase columns are usually selected for the separation of antibiotic analytes. Modifications of these stationary phases can lead to better separation of compounds, thus reducing the risk of false positive signals caused by other substances eluting at the same time as the analyte. One of the types of modified C_{18} columns is polar-endcapped stationary phase (Fig. 2) where an addition of a polar functional group [19–21] enables a full interaction with the longer alkyl chains and makes the retention of polar analytes under highly aqueous conditions more reproducible [1, 22, 23]. The C_{18} ligand provides hydrophobic interactions, while the polar-modified surface provides increased retention of polar substances. These phases are also characterized by stability in 100% aqueous conditions [24, 25]. Therefore, the aim of our study was to develop and validate HPLC method for the determination of ampicillin in human blood plasma with the use of column containing a polar-endcapped stationary phase. In addition, the comparison of its separation on two polar-endcapped stationary phases was also the subject of interest. One of such columns is an analytical column containing the Arion® Polar C18 phase that is new on the market. Analyses with the use of the column have not yet been published. The new stationary phase was compared with other polar-endcapped stationary phase Luna® Omega Polar C18 that has been on the market for a long time and has already been used for the determination of other antibiotics in biological samples [8]. For these reasons, this column was chosen as reference. In addition, both column have similar properties, as they have fully porous particles.

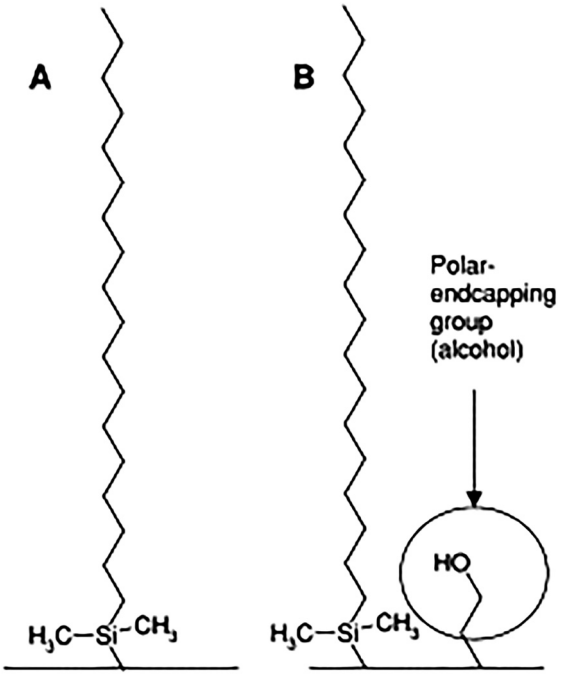

Fig. 2. Conventional C_{18} (A) and polar-endcapped C_{18} (B) stationary phases according to Layne (2002) [19]

MATERIALS AND METHODS

Chemicals and reagents

Ampicillin sodium (purity ≥95%; AMP) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Cephalexin monohydrate (pharmaceutical primary standard grade; CEX) was purchased from Merck (Darmstadt, Germany) and was used as an internal standard. Acetonitrile (ACN), methanol (MeOH), and ultrapure water (all HPLC grade) were purchased from VWR (Radnor, PA, USA). Monopotassium phosphate (KH_2PO_4) and 85% orthophosphoric acid were purchased from Penta (Prague, Czech Republic).

Preparation of solutions

After approval from the research ethics committee, human blood plasma from one patient (man, 76 years old, who underwent elective cardiac surgical revascularization) was used to compare AMP and CEX (i.s.) separations using different columns. For validation studies, human blood plasma from three female and three male donors obtained from the Transfusion Department of the University Hospital Olomouc was mixed and used. The blood plasma was stored at −70 °C. The stock solutions of AMP and CEX were prepared in ultrapure water (HPLC grade) at a concentration of 1 mg mL^{-1}. The solution of CEX was subsequently diluted to a final concentration of 0.24 mg mL^{-1}. The solution of AMP was diluted and added to blood plasma in the following final concentrations: 10, 20, 40, 60, 80, 100, 150, and 300 mg L^{-1} in six parallels from each concentration. Samples prepared in this way were used for a determination of intra-day precision and accuracy of measurement. For inter-day precision and accuracy measurements, blood plasma with AMP at concentrations of 10, 60, and 300 mg L^{-1} was prepared as well. Samples were prepared in three parallels and were measured on different days. Blood plasma with AMP at concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, and 20 mg L^{-1} was prepared for limit of quantification (LOQ) and limit of detection (LOD) measurements. These samples were prepared in six parallels from each concentration and were measured during one day.

For the lipophilicity determination, the solution of AMP was diluted to a 15 mM monopotassium phosphate buffer of pH 3.3 to a final concentration of 100 mg L^{-1}. An amount of 25 µL of the prepared standard sample was injected into HPLC.

Sample preparation

The volume of 100 µL of human blood plasma spiked with AMP (for validation studies) or a plasma sample from a patient (for comparison of different columns) was treated with 20 µL of a solution containing 0.24 mg mL^{-1} of CEX (an internal standard). Subsequently, 50 µL of 1 M orthophosphoric acid and 50 µL of ultrapure water were added to the treated plasma. Further preparation of all samples was performed with 1-mL solid-phase extraction (SPE) cartridges containing 100 mg of DSC-18 phase (Merck, Darmstadt, Germany). The SPE extraction consisted in a conditioning of cartridges with 1 mL of ACN, equilibration of cartridges with 1 mL of ultrapure water, 100 µL of sample loading, washing away of interferences with 1 mL of ultrapure water two times, and finally eluting of AMP and CEX with 1 mL of 70% ACN maintained at 37 °C. The sample obtained from the elution step was evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 100 µL of a mixture containing ultrapure water and MeOH (75:25, v/v). An amount of 25 µL of the prepared sample was injected into HPLC.

Method validation

The analytical performance parameters of HPLC, including linear range, limit of quantification (LOQ), limit of detection (LOD), intra- and inter-day precision and accuracy, were studied with the use of two different columns packed with a polar-endcapped C_{18} stationary phase. An Arion® Polar C18 column (Chromservis, Prague, Czech Republic) and Luna® Omega Polar C18 column (Phenomenex, Torrance, CA, USA) were selected for these validation studies. The calibration curve for AMP was measured in the range of 10–300 mg L^{-1} and each concentration level was measured at least six times. Fundamental chromatographic parameters, such as efficiency (N), retention factor (k), separation factor (α), asymmetry (A_SF), and tailing factor (TF) were calculated according to Ravisankar et al. [26] for a comparison of separation properties of the selected columns.

Determination of lipophilicity from chromatographic parameters

Ampicillin was separated on four different stationary phases (Luna® Omega Polar C18, Arion® Polar C18, LiChrospher® RP-18, and Nucleosil® C18) from different companies using mobile phase containing a 15 mM monopotassium phosphate buffer of pH 3.3 mixed with methanol (in a ratio 10%–35%). The HPLC experiments were performed in isocratic modes. Obtained retention factors (k) were used for the calculation of log k. From these, the retention factor at 100% aqueous mobile phase (log k_w) was extrapolated using the linear Soczewinski–Snyder relationship [27, 28], log k = log kw−Sφ, where log k is the retention factor in a specific mobile phase composition, S is a slope of linear regression of a dependence of log k and φ, and the φ is the volume fraction of the methanol in mobile phase and it is constant for a given solute and a given HPLC system. Then, the chromatographic lipophilicity index φ_0 was obtained by extrapolation. It is defined as the volume percentage of the organic modifier (methanol) required to achieve an equal distribution of a compound between the mobile and stationary phase, corresponding to log k = 0 [29–31].

Instrumental conditions

All measurements were performed with the Prominence LC–20A HPLC system equipped with a binary solvent manager, a sample manager with a flow-through needle fitted with a 50 µL loop, and an SPD-20A UV-Vis detector (Shimadzu, Kyoto, Japan). Separation of AMP and CEX was performed with a mobile phase containing a 15 mM KH_2PO_4 solution of pH 3.3 and MeOH (75:25, v/v) at a flow rate of 1.4 mL min^{-1}. Both compounds were separated at 30 °C on an Arion® Polar C18 column (250 × 4.6 mm; 5 µm) purchased from Chromservis (Prague, Czech Republic) or on a Luna® Omega Polar C18 column (250 × 4 mm; 5 µm) purchased from Phenomenex (Torrance, CA, USA). The detection of both compounds was performed at 210 nm. The method was validated on these columns which were then compared with each other. HPLC separations were also compared with the following columns: LiChrospher® RP-18 (250 × 4 mm; 5 µm) from Merck (Darmstadt, Germany); and Nucleosil® C18 (250 × 4 mm; 5 µm) from Watrex Praha (Prague, Czech Republic). The LabSolutions software (ver. 5.93; Shimadzu, Kyoto, Japan) was used for instrument control, data acquisition, and processing.

RESULTS AND DISCUSSION

Method optimization

The antibiotics AMP and CEX (i.s.) are polar compounds soluble in water. Therefore, a precipitation of biological sample with MeOH or ACN itself is not suitable because analytes would not separate so well (as we found out by experiments; data not shown). The best results were obtained with SPE extraction using a column with C_{18} phase (the exact procedure is described in the section Sample preparation) where the recovery of both compounds was calculated as 90%. The preparation and separation of AMP and i.s. were optimized on Luna® Omega Polar C18 column.

Ampicillin is a zwitterionic compound with a dissociation constant of 2.5 and 7.3 at 23 °C [32, 33]. Therefore, the mobile phase containing 15 mM monopotassium phosphate of a different pH was tested and, subsequently, its pH of 3.3 was selected to assure a good peak shape during the separation process. The addition of MeOH to the mobile phase enabled good separation of ampicillin and i.s. from other

compounds present in a human blood plasma sample. The resulting mobile phase containing a 15 mM KH_2PO_4 solution of pH 3.3 and MeOH (75:25, v/v) was used for the determination of the antibiotics AMP and CEX (i.s.). These chromatographic conditions were also used for Arion® Polar C18 column and other conventional C_{18} stationary phases for the evaluation of their chromatographic performance. Specifications of column phases used in this study are shown in Table 1.

The aim of our study was to establish the accurate, simple, and selective HPLC/UV method for the determination of AMP and/or other antibiotics in human blood plasma using column with polar-endcapped stationary phase and also show possibly limitation when the HPLC/UV method is used. In our study, columns were tested by method that was developed based on the article by do Nascimento et al. [4] with some modification. The advantage of our method is a smaller sample volume (100 µl instead of 300 µl) and smaller inject volume of sample.

All HPLC methods with UV detection are limited by the fact that another substance may elute at the site of elution of the analyte and may not be visible, it may be hidden behind the analyte. In such a case, it is advisable to take blood from the patient before the antibiotic application as well to verify that no other substances are present in the plasma that could co-elute with AMP and i.s. A more suitable method is certainly the use of mass spectrometry but even in this case we would recommend measuring a blood plasma sample before the application of the antibiotic.

Method validation

Based on optimal sample preparation and chromatographic conditions, the analytical performance parameters of HPLC, including linear range, intra- and inter-day precision and accuracy as well as LOQ and LOD, were studied with the use of selected columns packed with a polar-endcapped C_{18} stationary phase (Arion® Polar C18 or Luna® Omega Polar C18 phases). Data obtained from both columns were compared to determine how much the columns from different companies could have an effect on the validation method. The linear range 10–300 mg L^{-1} was selected for both columns, the LOQ was determined 10 mg L^{-1} for Luna® Omega Polar C18 and 5 mg L^{-1} for Arion® Polar C18. Summary of the data is given in Table 2. In human blood plasma, AMP and i.s. did not show a degradation for 24 h when stored at 4 °C and up to 30 days when stored at −70 °C.

Table 2. Validation parameters of the HPLC method developed for determination of ampicillin in human blood plasma on two different columns

Polar-endcapped stationary phase	Luna® Omega Polar C18	Arion® Polar C18
Calibration curve range (mg L^{-1})	*10–300*	*10–300*
Linear correlation coefficient (R^2)	*0.9998*	*0.9998*
Limit of quantification (mg L^{-1})	*10*	*5*
Limit of detection (mg L^{-1})	*0.5*	*0.5*
Intra-day precision (%)		
10 mg L^{-1}	3.87	1.59
20 mg L^{-1}	2.75	0.84
40 mg L^{-1}	2.27	1.79
60 mg L^{-1}	2.68	1.94
80 mg L^{-1}	6.10	0.97
100 mg L^{-1}	3.85	4.17
150 mg L^{-1}	2.20	1.27
300 mg L^{-1}	2.39	1.26
Inter-day precision (%)		
10 mg L^{-1}	3.65	2.08
60 mg L^{-1}	5.23	3.28
300 mg L^{-1}	5.87	0.55
Intra-day accuracy (%)		
10 mg L^{-1}	87.32	105.34
20 mg L^{-1}	91.84	103.89
40 mg L^{-1}	103.71	102.00
60 mg L^{-1}	104.66	99.53
80 mg L^{-1}	102.07	99.80
100 mg L^{-1}	103.75	97.33
150 mg L^{-1}	105.81	99.13
300 mg L^{-1}	108.70	100.42
Inter-day accuracy (%)		
10 mg L^{-1}	89.90	111.18
60 mg L^{-1}	104.42	111.26
300 mg L^{-1}	103.08	100.08

Table 1. Specifications of used stationary phases

	Arion® Polar C18	Luna® Omega Polar C18	LiChrospher® RP-18	Nucleosil® C18
Column dimension [mm]	250 × 4.6	250 × 4	250 × 4	250 × 4
Particle size [µm]	5	5	5	5
Pore size [Å]	120	100	100	100
Surface area [m^2 g^{-1}]	325	260	350	350
Carbon load [%]	16	9	21	15
Endcapped	yes	yes	yes	yes
pH stability	1.5 to 7	1.5 to 8.5 (10*)	2 to 7.5	2 to 8
100% aqueous mobile phase stability	yes	yes	no	no

Data were obtained from the literature [24, 29–31].
*under isocratic conditions.

The results show that the validation of method for the determination of AMP in human blood plasma was successful for both tested stationary phases. The important difference between tested polar phases is LOQ with the use of 20 µL of a solution containing 0.24 mg mL^{-1} of internal standard. The lowest concentration of AMP was measured 5 mg L^{-1} using Arion® Polar C18. In contrast, a concentration of 10 mg L^{-1} was determined using Luna® Omega Polar C18 as the minimum concentration for AMP quantification. Although lower concentrations of AMP can be measured using Arion® Polar C18 compared to Luna® Omega Polar C18 (as results show), it is possible to make other calibration curve for AMP with the use of decreased concentration of the internal standard and thus to achieve the determination of a lower AMP concentration. As we tested it, the LOQ of 2 mg L^{-1} is possible to achieve with the Luna® Omega Polar C18 using 20 µL of 0.03 mg mL^{-1} of internal standard (data not shown). Authors do Nascimento et al. [4] separated AMP on C18 column and measured AMP concentration in the range 0.30–100 mg L^{-1} using 5 µL of solution containing 10 µg mL^{-1} of CEX (i.s.). The LOQ of AMP was 0.3 mg L^{-1} in this case. Even lower concentrations can be achieved by a detection with a mass spectrometry. There is suitable to use a combination of acetic acid, ammonium acetate, and acetonitrile as a mobile phase for the AMP determination [34, 35], where a detection range 0.58–70.05 mg L^{-1} can be achieved. Huang et al. [36] described the use of a combination of acetic acid and acetonitrile as a mobile phase, where detection range of 0.2–500 mg L^{-1} with a quantification limit of 0.2 mg L^{-1} can be possible to achieve.

Our blood plasma sample was obtained from patient who underwent an elective cardiac surgical revascularization. A range of AMP plasma concentration in such patients was 5–230 mg L^{-1} (tested in 38 patients) depending on the sampling time during surgery (data not shown). Therefore, only one calibration curve would be sufficient to determine the AMP concentration with the use of Arion® Polar C18 phase column. Also, a mass spectrometry would be more appropriate not in terms of the sensitivity but the selectivity as was already mentioned in Method optimization.

Evaluation of chromatographic performance of tested stationary phases

Column loading technology is still evolving and therefore the established methods on employed columns may not work on newer columns. Therefore, this study shows what AMP analysis looks like when using the long-known or the new polar C_{18} column. Likewise, analyses using C_{18} columns, which have been on the market for much longer, were compared. Polar-endcapped stationary phases are suitable for the separation of polar compounds because they can retain them for longer time periods than in the case of conventional C_{18} phases [37]. Therefore, two other columns packed with a conventional C_{18} phase (LiChrospher® RP-18 and Nucleosil® C18) were selected for a confirmation that polar-endcapped stationary phases actually retain polar substances longer than conventional C_{18} and are really preferable for the determination of polar compounds. Figure 3 shows the chromatograms of AMP and i.s. in a plasma sample obtained from the same patient. The chromatograms differ only in the use of a stationary phase. Sample preparation, injection into HPLC, composition of mobile phase, and chromatographic conditions were the same for all stationary phases. Chromatographic performance parameters, including N, k, α, A_SF, and TF, were calculated for all selected stationary phases (see Table 3). As results show, peak shapes were symmetrical in all columns. The efficiency of the new and well-known polar-endcapped

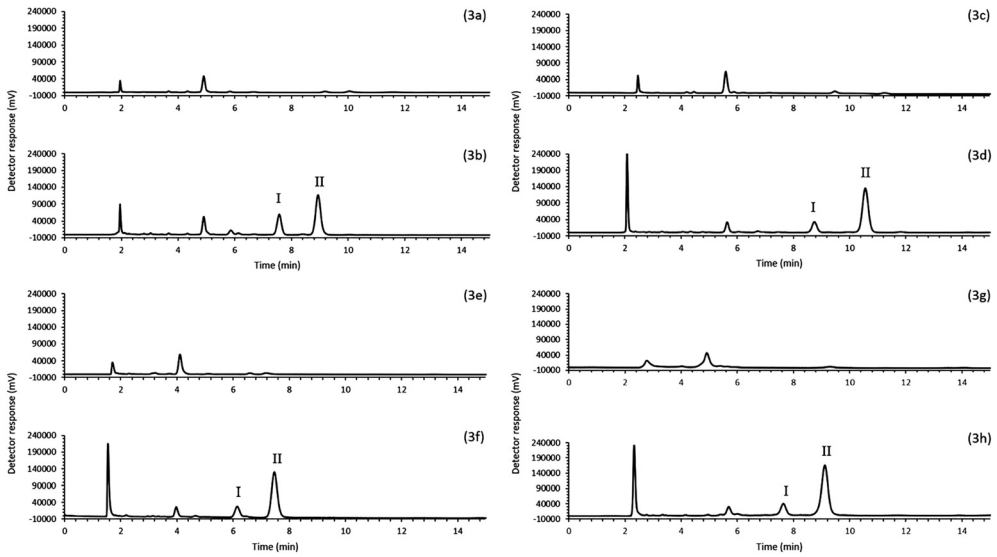

Fig. 3. Representative chromatograms of cephalexin (i.s.; I) and ampicillin (II) in human blood plasma obtained from one patient. The separation was performed using Luna® Omega Polar C18 (3b, blank sample 3a), Arion® Polar C18 (3d, blank sample 3c), LiChrospher® RP-18 (3f, blank sample 3e), and Nucleosil® C18 (3h, blank sample 3g) stationary phases under the same chromatographic conditions (UV detection at 210 nm, a mobile phase containing a 15 mM monopotassium phosphate buffer of pH 3.3 and methanol (75:25, v/v) at a flow rate of 1.4 ml min^{-1}, 25 µl injection into HPLC)

Table 3. Chromatographic performance parameters of selected polar-endcapped C_{18} stationary phases (Luna® Omega Polar C18 and Arion® Polar C18) and conventional reversed phase C_{18} (LiChrospher® RP-18 and Nucleosil® C18) for cephalexin (i.s.; 1) and ampicillin (2) in human blood plasma

	Luna® Omega Polar C18 (250 × 4 mm; 5 µm)	Arion® Polar C18 (250 × 4.6 mm; 5 µm)	LiChrospher® RP-18 (250 × 4 mm; 5 µm)	Nucleosil® C18 (250 × 4 mm; 5 µm)
Efficiency of column for i.s. (N_1) [plates/m]	2694.0	3971.5	2607.2	1702.3
Efficiency of column for ampicillin (N_2) [plates/m]	1890.9	2691.2	1431.5	1237.5
Retention factor for i.s. (k_1)	4.6	3.6	3.0	4.0
Retention factor for ampicillin (k_2)	5.6	4.6	3.9	5.0
Selectivity of column (α)	1.2	1.3	1.3	1.2
Asymmetry factor for i.s. (A_SF_1)	1.2	1.0	1.0	0.7
Asymmetry factor for ampicillin (A_SF_2)	1.4	1.0	1.3	1.0
Tailing factor for i.s. (TF_1)	1.1	1.0	1.0	0.9
Tailing factor for ampicillin (TF_2)	1.2	1.0	1.1	1.0

stationary phase was 2,691 and 1,891 plates/m for AMP and 3,972 and 2,694 plates/m for i.s., respectively. Our study is focused on analytical columns packed with C_{18} particles with a porous surface. Although the length contributes to large number plates, the efficiency of selected columns for AMP and its internal standard was not excellent. The reason is the larger diameter size (4 or 4.6 mm) and the larger particle size (5 µm) in selected columns, as both of which lead to wider peaks. The Arion® Polar C18 was the most effective of all phases tested because its efficiency was higher than 3,000 plates/m for i.s. and almost 2,700 plates/m for AMP (as listed in Table 3). Its efficiency is significantly better than the Luna® Omega Polar C18 phase (almost 1.5-fold higher for i.s. and 1.4-fold higher for AMP). In comparison to conventional C_{18} phases, Arion® Polar C18 was 1.5–2.3 more effective for i.s. and had two times as many plates for AMP.

A more robust separation will be obtained when $2 \leq k \leq 10$, otherwise a gradient is likely a better choice [38]. In all tested phases, the retention factor k for both compounds was $2 < k < 6$ and therefore it seems to be robust in all tested columns. Both measured compounds were successfully separated from each other without using a gradient in all used column. But the separation as such was not excellent on conventional C_{18} phases because AMP co-eluted with some other compound as was observed by comparison with blank samples. In that case, it would be necessary to modify the mobile phase or to use a gradient. It is similar with the Luna® Omega Polar C18 phase where also AMP co-eluted with some other compound. However, there is possibility to evaluate the signal in height because the other compound came out later and had a different retention time than AMP. During the method validation, blank samples were without the co-eluting substance. It appeared with a new batch of human blood plasma that was used for the comparison of patient sample analyses. Therefore, substances, contained in biological samples obtained from different patients, may the column resolution worse with the use of already validated method. In our study, the Arion® Polar C18 phase had the best resolution when the new batch human blood plasma was used. However, it does not have to be the same with other batch blood plasma or plasma of another patient. Therefore, we would recommend to take a blood sample from patient before the antibiotic application to verify that no other substances are present in the plasma that could co-elute with AMP and i.s., as was mentioned above.

At first glance, it might seem that AMP as well as i.s. are retained more on Arion® Polar C18, as shown in Fig. 3. However, an unretained peak came out later on Arion® Polar C18 (1.9 min) compared to the conventional Nucleosil® RP-18 (1.5 min). Therefore, the resulting retention factor for both substances was lower on Arion® Polar C18. The reason is probably due to a smaller surface area and a larger pore size of the new stationary phase compared to Nucleosil® (325 m^2 g^{-1} and 120 Å compared to 350 m^2 g^{-1} and 100 Å, respectively). Therefore, it is also important to select a suitable column for method development based on these properties.

The differences in the retention of AMP on stationary phases can also be explained by lipophilicity parameters. The lipophilicity of drugs is most commonly characterized by their 1-octanol-water partition coefficients (log P) and was proposed by Hansch and co-workers [39, 40]. The parameter log P is traditionally measured by the method of shaking flask [29]; however, other methods for determining drug lipophilicity have already been described [30, 31, 41]. One of the recognized methods uses a chromatography [42] where the lipophilicity parameter log k_w is determined. This method seems more appropriate because the determination of AMP lipophilicity on different stationary phases can explain differences in retention times on these phases. Our study was performed with mobile phase containing 15 mM KH_2PO_4 adjusted to pH 3.3 with changing ratio of methanol as an organic modifier. Graphs in Figs 4a and b show that the dependence of log k on methanol addition in mobile phase is linear in all phases. The log k_w was obtained by extrapolation to zero methanol concentration [27, 28]. The calculated log k_w for AMP was low in all phases (Table 4) which confirms that AMP is a polar compound. As results show, C_{18} phases (Lichrospher® and Nucleosil®) had similar

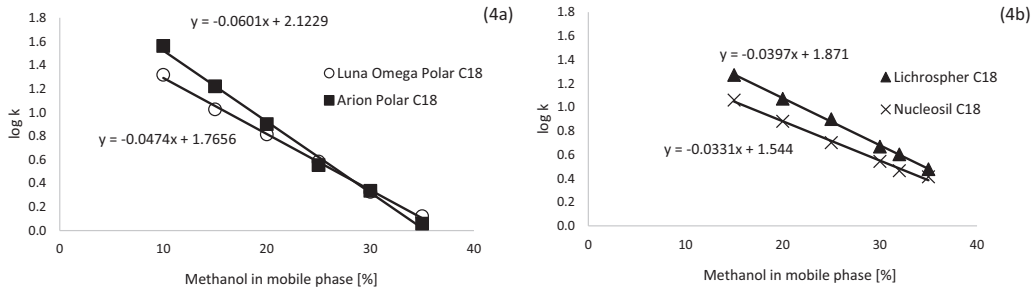

Fig. 4. Plot of log k (retention factor) versus methanol ratio in mobile phase for ampicillin measured on polar-endcapped C_{18} (4a) and conventional C_{18} (4b) stationary phases

Table 4. Regression coefficients and lipophilicity parameters for ampicillin on various stationary phases

Stationary phase	log k_w	φ_0 [%]	S	R^2
Luna® Omega Polar C18	1.76	37	−0.0474	0.9979
Arion® Polar C18	2.12	35	−0.0601	0.9948
LiChrospher® RP-18	1.87	47	−0.0397	0.9986
Nucleosil® C18	1.54	46	−0.0331	0.9950

Log k_w, the retention factor at 100% aqueous mobile phase; φ_0, the volume percentage of methanol required to achieve an equal distribution of ampicillin between the mobile and stationary phase; S, slope of linear regression; R^2, coefficient of determination.

slope of linear regression that was lower compared to polar C_{18} phases. Arion® Polar C18 had markedly higher slope of linear regression than Lichrospher® RP-18, Nucleosil® C18 as well as Luna® Omega Polar C18 phases (1.5-fold, 1.8-fold, and 1.3-fold, respectively). Based on these results, polar C_{18} phases are more selective for the determination of AMP and even the Arion® Polar C18 phase appears as a more selective phase compared to other phases tested, as was observed in the patient sample as well. Table 4 shows that AMP retains longer on Arion® Polar C18 at 100% aqueous mobile phase compared to other column. And also, on Arion® Polar C18, AMP achieves an equal distribution between the mobile and stationary phases faster (according to the lipophilicity index φ_0 shown in Table 4). On the other hand, AMP retains shorter time on Nucleosil® C18 phase at 100% aqueous mobile phase compared to other column, but it achieves an equal distribution between the mobile and stationary phases more slowly. These lipophilicity parameters explain why the AMP retention factor was lower on Arion® Polar C18 compared to Nucleosil® C18.

Arion® Polar C18 phase is characterized by good stability at higher temperatures and its unique production process ensuring high lot-to-lot reproducibility. Its surface is modified by multi-step endcapping that secures good peak shape without fronting and tailing [43]. Our study did not take long enough for us to say that the shape of the peaks would not change over time. We already know that a shape of peaks is changing over time in Luna® Omega Polar C18 (tailing of peaks was observed). However, each column has a certain lifetime, and therefore the change in peak shape must be expected over time.

CONCLUDING REMARKS

This study showed what AMP and CEX analyses look like when using the long-known and new polar C_{18} column. Likewise, analyses using C_{18} columns which have been on the market for much longer, were compared. Testing of these columns can help in the development or modification of methods to achieve better separation, resolution and overall chromatographic performance not only in a medicine but also in a pharmaceutical or food industry.

The accurate, simple, and selective HPLC/UV method has been established and validated for the determination of the antibiotic AMP as a suitable method in a cardiac surgery which does not have to be used only in cardiac surgery. In addition, the new Arion® Polar C18 column has been shown to effectively separate AMP in human blood plasma, the separation was better compared to other tested phases.

Conflict of interest statement: All authors declare no commercial or financial conflict of interest.

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LIST OF ABBREVIATIONS

α	Separation factor
ACN	Acetonitrile
AMP	Ampicillin
A_SF	Peak asymmetry
CEX	Cephalexin
HPLC	High-performance liquid chromatography
k	retention factor
MeOH	Methanol
N	Column efficiency
SPE	Solid-phase extraction
TF	Tailing factor

REFERENCES

1 Blidar, A.; Feier, B.; Tertis, M.; Galatus, R.; Cristea, C. Electrochemical surface plasmon resonance (EC-SPR) aptasensor for ampicillin detection. *Anal. Bioanal. Chem.* **2019**, *411*, 1053–65.

2. Amini, H.; Ahmadiani, A. Sensitive determination of clarithromycin in human plasma by high-performance liquid chromatography with spectrophotometric detection. *J. Chrom. B: Anal. Tech. Biomed. Life Sci.* **2005**, *817*, 193–7.

3. Legrand, T.; Chhun, S.; Rey, E.; Blanchet, B.; Zahar, J. R.; Lanternier, F.; Pons, G.; Jullien, V. Simultaneous determination of three carbapenem antibiotics in plasma by HPLC with ultraviolet detection. *J. Chrom. B: Anal. Tech. Biomed. Life Sci.* **2008**, *875*, 551–6.

4. do Nascimento, T. G.; Aragao, C. F. S.; de Medeiros, F. D.; Oliveira, E. D.; Macedo, R. O. Validation of a method for determination of ampicillin in human plasma using LC-DAD. *J. Chrom. Sci.* **2009**, *47*, 749–55.

5. Pickering, M.; Brown, S. Quantification and validation of HPLC-UV and LC-MS assays for therapeutic drug monitoring of ertapenem in human plasma. *Biomed. Chrom.* **2013**, *27*, 568–74.

6. Legrand, T.; Vodovar, D.; Tournier, N.; Khoudour, N.; Hulin, A. Simultaneous determination of eight β-lactam antibiotics, amoxicillin, cefazolin, cefepime, cefotaxime, ceftazidime, cloxacillin, oxacillin, and piperacillin, in human plasma by using ultra-high-performance liquid chromatography with ultraviolet detection. *Antimicrob. Agents Chemother.* **2016**, *60*, 4734–42.

7. Cairoli, S.; Simeoli, R.; Tarchi, M.; Dionisi, M.; Vitale, A.; Perioli, L.; Dionisi-Vici, C.; Goffredo, B. M. A new HPLC-DAD method for contemporary quantification of 10 antibiotics for therapeutic drug monitoring of critically ill pediatric patients. *Biomed. Chrom.* **2020**, *34*, e4880.

8. Fikarova, K.; Horstkotte, B.; Machian, D.; Sklenarova, H.; Solich, P. Lab-In-Syringe for automated double-stage sample preparation by coupling salting out liquid-liquid extraction with online solid-phase extraction and liquid chromatographic separation for sulfonamide antibiotics from urine. *Talanta* **2021**, *221*, 121427.

9. Jariwala, F. B.; Hibbs, J. A.; Zhuk, I.; Sukhishvili, S. A.; Attygalle, A. B. Rapid determination of aminoglycosides in pharmaceutical preparations by electrospray ionization mass spectrometry. *J. Anal. Sci. Tech.* **2020**, *11*, 2.

10. Tian, H. J.; Liu, T.; Mu, G. D.; Chen, F. M.; He, M. Y.; You, S.; Yang, M. L.; Li, Y. L.; Zhang, F. Rapid and sensitive determination of trace fluoroquinolone antibiotics in milk by molecularly imprinted polymer-coated stainless steel sheet electrospray ionization mass spectrometry. *Talanta* **2020**, *219*, 121282.

11. Wagil, M.; Kumirska, J.; Stolte, S.; Puckowski, A.; Maszkowska, J.; Stepnowski, P.; Bialk-Bielinska, A. Development of sensitive and reliable LC-MS/MS methods for the determination of three fluoroquinolones in water and fish tissue samples and preliminary environmental risk assessment of their presence in two rivers in northern Poland. *Sci. Total Environ.* **2014**, *493*, 1006–13.

12. El-bagary, R.; El-Zaher, A. A.; Elkady, E.; Mandour, A. A. Simultaneous determination of ciprofloxacin hydrochloride and metronidazole in spiked human plasma by ultra performance liquid chromatography-tandem mass spectroscopy. *J. App. Pharm. Sci.* **2016**, *6*(3), 41–7.

13. Zhi, S. L.; Zhou, J.; Liu, H. X.; Wu, H. H.; Zhang, Z. L.; Ding, Y. Z.; Zhang, K. Q. Simultaneous extraction and determination of 45 veterinary antibiotics in swine manure by liquid chromatography-tandem mass spectrometry. *J. Chromr. B: Anal. Tech. Biomed. Life Sci.* **2020**, *1154*, 122286.

14. Baikeli, Y.; Mamat, X.; He, F.; Xin, X. L.; Li, Y. T.; Aisa, H. A.; Hu, G. Z. Electrochemical determination of chloramphenicol and metronidazole by using a glassy carbon electrode modified with iron, nitrogen co-doped nanoporous carbon derived from a metal-organic framework (type Fe/ZIF-8). *Ecotoxicol. Environ. Saf.* **2020**, *204*, 111066.

15. Pollap, A.; Baran, K.; Kuszewska, N.; Kochana, J. Electrochemical sensing of ciprofloxacin and paracetamol in environmental water using titanium sol based sensor. *J. Electroan. Chem.* **2020**, *878*, 114574.

16. Yue, X.; Li, Z.; Zhao, S. A new electrochemical sensor for simultaneous detection of sulfamethoxazole and trimethoprim antibiotics based on graphene and ZnO nanorods modified glassy carbon electrode. *Microchem. J.* **2020**, *159*, 105440.

17. Grayson, M. L.; Cosgrove, S. E.; Crowe, S.; Hope, W.; McCarthy, J. S.; Mills, J.; Mouton, J. W.; Paterson, D. L. *Kucers' the use of Antibiotics: A Clinical Review of Antibacterial, Antifungal, Antiparasitic, and Antiviral Drugs*, 7th ed.; CRC Press: Boca Raton, **2018**.

18. Mai, X. L.; Pham, T. V.; Han, G. H.; Kum, S. J.; Woo, S. H.; Kang, J. S.; Woo, M. H.; Na, D. H.; Chun, I. K.; Kim, K. H. Simultaneous determination of ampicillin sodium and sulbactam sodium in powder for injection by HPLC. *Anal. Sci. Technol.* **2019**, *32*, 147–54.

19. Layne, J. Characterization and comparison of the chromatographic performance of conventional, polar-embedded, and polar-endcapped reversed-phase liquid chromatography stationary phases. *J. Chrom. A.* **2002**, *957*, 149–64.

20. Chamseddin, C.; Jira, T. Evaluation of the chromatographic performance of conventional, polar-endcapped and calixarene-bonded stationary phases for the separation of water-soluble vitamins. *Chromatographia* **2013**, *76*, 449–57.

21. Coym, J. W. Comparison of retention on traditional alkyl, polar endcapped, and polar embedded group stationary phases. *J. Sep. Sci.* **2008**, *31*, 1712–8.

22. Aghaei, A.; Jazi, M. E.; Mlsna, T. E.; Kamyabi, M. A. A novel method for the preconcentration and determination of ampicillin using electromembrane microextraction followed by high-performance liquid chromatography. *J. Sep. Sci.* **2019**, *42*, 3002–8.

23. Veloso, W. B.; Ribeiro, G. A. C.; da Rocha, C. Q.; Tanaka, A. A.; da Silva, I. S.; Dantas, L. M. F. Flow-through amperometric determination of ampicillin using a copper electrode in a batch injection analysis system. *Measurement* **2020**, *155*, 107516.

24. Phenomenex. https://phenomenex.blob.core.windows.net/documents/6340a07c-e563-4342-87b0-305468f0c8f6.pdf (accessed July 23, 2021).

25. O'Gara, J. E.; Walsh, D. P.; Phoebe, C. H.; Alden, B. A.; Bouvier, I. S. P.; Iraneta, P. C.; Capparella, M.; Walter, T. H. Embedded-polar-group bonded phases for high performance liquid chromatography. *LC GC N. Am.* **2001**, *19*(6), 632–42.

26. Ravisankar, P.; Anusha, S.; Supriya, K.; Kumar, U. A. Fundamental chromatographic parameters. *Int. J. Pharm. Sci. Rev. Res.* **2019**, *55*(2), 9, 46–50.

27. Soczewiński, E.; Wachtmeister, C. A. The relation between the composition of certain ternary two-phase solvent systems and RM values. *J. Chromatogr. A.* **1962**, *7*, 311–20.

28. Snyder, L. R.; Dolan, J. W.; Gant, J. R. Gradient elution in high-performance liquid-chromatography .1. theoretical basis for reversed-phase systems. *J. Chromatogr.* **1979**, *165*, 3–30.
29. Valko, K.; Slegel, P. New chromatographic hydrophobicity index (phi-0) based on the slope and the intercept of the log-k' versus organic-phase concentration plot. *J. Chromatogr.* **1993**, *631*, 49–61.
30. Nasal, A.; Siluk, D.; Kaliszan, R. Chromatographic retention parameters in medicinal chemistry and molecular pharmacology. *Curr. Med. Chem.* **2003**, *10*, 381–426.
31. Klose, M. H. M.; Theiner, S.; Varbanov, H. P.; Hoefer, D.; Pichler, V.; Galansk, M.; Meier-Menches, S. M.; Keppler, B. K. Development and validation of liquid chromatography-based methods to assess the lipophilicity of cytotoxic platinum(IV) complexes. *Inorganics* **2018**, *6*, 14.
32. Pubchem. https://pubchem.ncbi.nlm.nih.gov/compound/Ampicillin (accessed July 22, 2021).
33. Danelon, C.; Nestorovich, E. M.; Winterhalter, M.; Ceccarelli, M.; Bezrukov, S. M. Interaction of zwitterionic penicillins with the OmpF channel facilitates their translocation. *Biophys. J.* **2006**, *90*(5), 1617–27.
34. Colin, P.; De Bock, L.; T'Jollyn, H.; Boussery, K.; Van Bocxlaer, J. Development and validation of a fast and uniform approach to quantify beta-lactam antibiotics in human plasma by solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry. *Talanta* **2013**, *103*, 285–93.
35. El-Najjar, N.; Jantsch, J.; Gessner, A. The use of liquid chromatography-tandem mass spectrometry for therapeutic drug monitoring of antibiotics in cancer patients. *Clin.Chem. Lab. Med.* **2017**, *55*, 1246–61.
36. Huang, C. R.; Gao, J.; Miao, L. Y. Simultaneous determination of flucloxacillin and ampicillin in human plasma by ultra performance liquid chromatography-tandem mass spectrometry and subsequent application to a clinical study in healthy Chinese volunteers. *J. Pharm. Biomed. Anal.* **2012**, *59*, 157–61.
37. Majors, R. E.; Przybyciel, M. Columns for reversed-phase LC separations in highly aqueous mobile phases. *LC GC Eur.* **2002**, 2–7.
38. Dolan, J Count the cost, Part II: increasing resolution by increasing retention. *LC GC N. Am.* **2017**, *35*(4), 240–5.
39. Hash, C.; Fujita, T. p-σ-π analysis. A method for the correlation of biological activity and chemical structure. *J. Am. Chem. Soc.* **1964**, *86*(8), 1616–26.
40. Leo A.; Hansch C.; Elkins D. Partition coefficients and their uses. Chem. Rev. **1971**, *71*, 525-+.
41. Vallaro, M.; Ermondi, G.; Caron, G. Chromatographic HILIC indexes to characterize the lipophilicity of zwitterions. *Eur. J. Pharm. Sci.* **2020**, *145*, 8.
42. Grover, M.; Gulati, M.; Singh, B.; Singh, S. RP-HPLC determination of lipophilicity of 22 penicillins, their correlation with reported values and establishment of quantitative structure-log K-W relationships. *Qsar. Comb. Sci.* **2005**, *24*, 639–48.
43. Chromservis. https://www.chromservis.eu/p/arion-polar-c18-hplc-column-5-0-m-250-mm-4-6-mm?lang=EN (accessed July 23, 2021).