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Identification of Penicillinase-Resistant Penicillins in Human Urine by RPLC Method

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ABSTRACT
In this study, the combined effect of the organic modifier content and mobile phase pH has been used to predict the chromatographic behavior of methicillin, oxacillin, nafcillin, cloxacilin and dicloxacillin. The optimum separation condition of these drugs in acetonitrile-water binary mixture was determined from the mobile phase pH dependence of their retention factor. A Synergy Fusion-RP C_{18} analytical column (150 mm x 4.6 mm I.D., 4 µm) was preferred to carry out the developed method. Chromatographic measurements were done at 30°C with an eluent flow rate of 1 mL min^{-1}. In order to validate the optimized conditions, these drugs were applied successfully in spiked samples of human urine. Valsartan was chosen as an internal standard. The described method was linear over a concentration range of 2-32 µg mL^{-1} and 3-48 µg mL^{-1} for the assay of methicillin, nafcillin, dicloxacillin and oxacillin, cloxacillin respectively. Good linear relationships over the investigated concentration ranges were observed with values of correlation coefficient higher than 0.999 for all of the drugs. The intra-day and inter-day precisions of this method were evaluated with RSD (%) values less than 0,561 and 1,622%, respectively. Optimized assay method was validated according to United States Pharmacopeia (USP) guidelines to confirm linearity, accuracy and precision.
Keywords: combined effect, penicillinase-resistant penicillins, method validation, internal standard, human urine

INTRODUCTION

Penicillins are one of the oldest and most important group of antibiotics widely used for the treatment of infections. The penicillinase resistant penicillins (second generation penicillins) are semisyntheic modifications of natural penicillins that are resistant to bacterial enzyme betalactamase. Their basic structure includes a nucleus consisting of a beta-lactam ring and a side chain. Due to the wide application of this drug family in both human and veterinary

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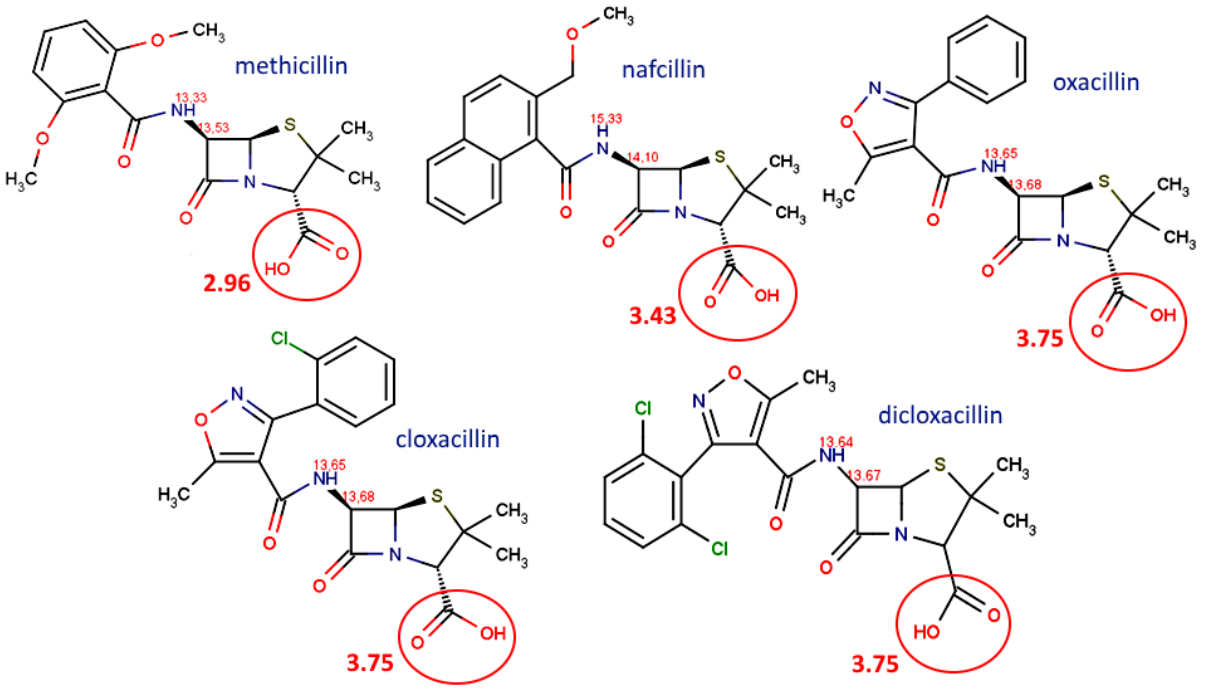

Figure 1. Chemical structures of investigated compounds

medicine, the analysis of pharmaceutical formulations as well as biological fluids for monitoring its bioavailability, pharmacokinetic profile and efficiency is of utmost importance. The basic structure of penicillin is a thiazolidine ring attached to a β-lactam ring. Five members of this class of antibiotics are determined in the present study: Oxacillin, methicillin, nafcillin, dicloxacillin and cloxacillin (**Figure 1**) [1-2].

Reversed phase liquid chromatography (RPLC) has been the most important branch of high performance liquid chromatography. In RPLC method, the most important aim of the optimization studies is to find the best chromatographic separation conditions. There are several approaches to predict the retention behavior of an ionisable solute as a function of chromatographic conditions. Because of the ionization properties of these types of solutes, the factors generally selected to optimize the chromatographic separation are the pH of the mobile phase and the percentage of the organic solvent of the eluent [3-9]. The effect of these parameters on the chromatographic behavior is somewhat more complex since the variation of organic modifier and analyze these compounds.

Knowledge of the state of ionization of acids is essential in RPLC separations in order to explain changes in retention, peak shape or overloading behaviour of acids. Solute ionization can be estimated from dissociation constant values (pK_a). RPLC technique is used to method development and in the understanding of the physicochemical phenomena acid-base species. The knowledge of the pK_a values of the species is crucial in predicting the influence of pH on retention and selectivity in LC [4]. Thus, a knowledge of the dissociation

constants of the compounds in hydroorganic solvent mixtures can help to advance the analytical method [8].

A search of the literature revealed that a number of reversed phase liquid chromatography (RPLC) method have been developed to determine the individual or several drugs in dosage forms [10] or in biological fluids [11-16]. Nevertheless, no report has been published on the optimization using the combined effect of organic modifier concentration and pH of the mobile phase to separate oxacillin, methicillin, nafcillin, dicloxacillin and cloxacillin.

In this work, the experimental region was selected such a way that the retention factors (k) of the penicillins would stay within the limits $1 < k < 10$. RPLC technique has been applied to the simultaneous determination of these antibiotics in human urine. No method for the simultaneous determination of these drugs has been found in the literature.

EXPERIMENTAL

Chemicals and reagents

All the chemicals used in this study were analytical reagent grade and HPLC grade. Analyte drugs were purchased from Sigma (USA). Acetonitrile (used as organic modifier) and sodium hydroxide (used in pH adjustment) were obtained from Merck (Darmstadt, Germany). Ortho-phosphoric acid used as buffer component of mobile phase was purchased from Riedel-de Haen (Riedel-de Haen, Germany).

Apparatus

The chromatographic equipment used consisted on a Shimadzu HPLC system (Shimadzu Technologies, Kyoto, Japan) equipped with a pump (LC-20AD), a UV Visible detector (SPD-20A), a column oven (CTO-20A) and a degasser system (DGU-$20A_3$). Synergy Fusion-RP C18 analytical column (150 mm x 4.6 mm I.D., 4 µm) was used as stationary phase (Phenomenex®, USA). In order to measure the pH values of the eluents, a Mettler Toledo MA 235 pH/ ion analyser (Schwerzenbach, Switzerland) was chosen. For the standardization of potentiometric system according to the IUPAC rules [17], potassium hydrogen phthalate (0.05 mol kg^{-1}) was used.

Chromatographic procedure

Throughout this study, mobile phase assayed was acetonitrile-water at 40% (v/v), to determine the chromatographic behavior of these compounds. The pH of the mobile phase containing 30 mM o-phosphoric acid was adjusted between 2.5 and 7.0 by the addition of 1M sodium hydroxide. Chromatographic measurements were done at 30 °C with an eluent flow rate of 1 mL min^{-1}. The volume of solution injected into the column was 20 µL for each run. The studied compounds had different optimal wavelengths (for methicillin, oxacillin, cloxacillin, dicloxacillin, valsartan (I.S.) 210 nm, nafcillin 228 nm). For each compound, the

retention time values, t_R, were determined from three different injections. Retention factors were calculated from $k = (t_R-t_0)/t_0$. Separation factor (α) was calculated from k values of two adjacent peaks ($\alpha = k_2/k_1$).

Preparation of standard solutions

In LC analysis, studied compounds were prepared carefully at a concentration of 100 µg mL^{-1} by dissolving it in the mobile phase. All the subsequent dilutions for working standards were prepared with the mobile phase. Sodium hydroxide solution used in pH adjustment was prepared by dilution in water. All stock solutions were stored at 4°C.

Preparation of urine calibration standards

Calibration standards were prepared by spiking urine with the appropriate amount of stock solutions. Different stock solutions were used to prepare calibration standards. Calibration curves were constructed by analyzing a serials of blank human urine, spiked with methicillin, nafcillin, dicloxacillin and oxacillin, cloxacillin at the concentration 2.0, 4.0, 8.0, 16.0, 24.0, 32.0 µg mL^{-1} and 3.0, 6.0, 12.0, 24.0, 36.0, 48.0 µg mL^{-1}, respectively. The concentration of I.S. was maintained at a constant level of 0.8 µg mL^{-1}. Separate standard calibration graphs were constructed for each component by plotting the ratio of the peak area of the drug to that of I.S. against the drug concentration. Preparation of the urine samples were carried out as described under preparation procedure.

Preparation of human urine samples

The urine sample was diluted 20-fold in deionized water in order to avoid matrix effect. 2 mL diluted urine was transferred to a 110 x 30 mm plastic tube and 3 mL acetonitrile was added to the tube. Stocks of human drug-free urine from healthy volunteers were spiked with different amounts of methicillin, oxacillin, nafcillin, cloxacillin, dicloxacillin. To 5 mL of the urine sample, 160 µL of internal standard (valsartan, 0.8 µg mL^{-1}) and different concentrations of studied compounds were added and the solution vortexed. Samples of 10 mL urine were spiked for final concentrations of 8 and 16 µg mL^{-1} for methicillin, nafcillin, dicloxacillin and 12 and 24 µg mL^{-1} for oxacillin, cloxacillin, respectively. The spiked samples were microfiltered through a 0.45 µm filter. A 20 µL volume of each sample was injected into the HPLC system.

Recovery studies from human urine samples

To keep an additional check on the accuracy of this developed method, recovery experiments were performed by adding the known amount of pure drug to pre-analyzed samples of human urine sample. Known amounts of the pure drug and a constant level of an internal standard were added to human urine sample and the mixtures were analyzed. The percent recovery was calculated by comparing the concentration obtained from spiked samples with the actual added concentration. After five repeated experiments, the average recovery percentage of these compounds was calculated for each compound.

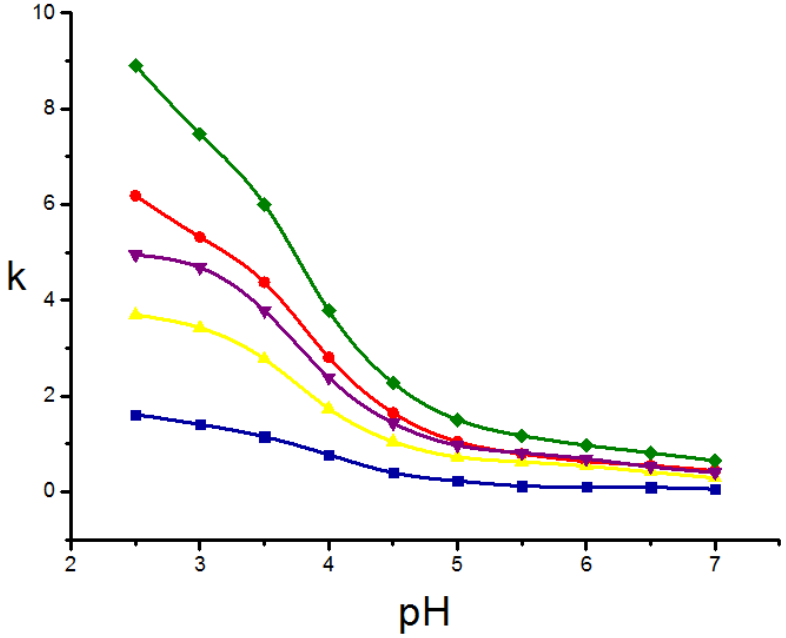

Figure 2. The effect of the mobile phase pH on retention factor values (40%, v/v) of acetonitrile: (■), methicillin; (●), nafcillin; (▲), oxacillin; (▼), cloxacillin; (♦), dicloxacillin. The theoretical results are indicated as continuous lines and the plotted points are experimental results

RESULTS AND DISCUSSION

In this study, the retention behaviors of methicillin, oxacillin, cloxacillin, dicloxacillin and nafcillin were investigated using the combined effect of pH and organic modifier concentration of the eluent. The retention of compounds in reversed phase chromatography depends significantly on the degree of ionization of these compounds and thus on the pH of the mobile phase.

In this study, retention behavior of these compounds as a function of the pH of th mobile phase at a given mobile phase composition has been investigated. The dependence of the retention factors of the compounds on pH value in the mobile phase (40 %, v/v) is given in **Figure 2**. It is noteworthy that better retention versus pH is obtained using the hydroorganic pH values.

The optimization of chromatographic selectivity can be achived by taking into account the ionization constant and retention factors of the ionized and non-ionized forms of the compounds for organic solvent-water binary mixture systems. Synergi Fusion-RP C18 reversed phase HPLC column (150 mm x 4.6 mm I.D., 4 µm) yielded greatly improved efficiencies for studied compounds. In this study, the efficiency, selectivity and retention terms were calculated and summarized in **Table 1** for optimum separation condition. It can be

Table 1. The retention, selectivity and separation factor values for penicillinase-resistant penicillins at 40% at pH 3.5

Compounds	k_2	α	k_2/k_2+1	$\alpha-1+\alpha$	$(1/4)\sqrt{N}$	R_s
Oxacillin/methicillin	2.882	2.405	0.742	0.584	24.533	10.640
Cloxacillin/oxacillin	3.950	1.370	0.798	0.270	26.273	5.665
Nafcillin/cloxacillin	4.563	1.155	0.820	0.134	23.731	2.614
Dicloxacillin/nafcillin	6.283	1.377	0.863	0.274	28.062	6.629
Valsartan(I.S.)/dicloxacillin	8.120	1.292	0.890	0.226	28.368	5.714

Table 2. System suitability parameters for investigated compounds

Parameters	Observed value						Recommended value
	Methicillin	Oxacillin	Cloxacillin	Nafcillin	Dicloxacillin	Valsartan (I.S.)	
Retention time	3.527	6.235	7.952	8.938	11.705	14.659	-
Tailing factor	1.135	1.097	1.078	1.028	1.059	1.054	≤2
Retention factor (k)	1.196	2.883	3.951	4.566	6.289	8.128	>1
Resolution factor (R_s)	-	10.509	5.608	2.597	6.590	5.689	>2
Theoretical plates (N)	4708	9366	10803	8859	12436	12755	>2000
Selectivity factor (α)	-	2.410	1.371	1.155	1.377	1.292	>1
RSD% (for retention time)	0.093	0.046	0.057	0.069	0.079	0.090	≤1
RSD% (for peak area)	0.429	0.404	0.223	0.253	0.516	0.887	≤1

achieved when the acetonitrile content in the mobile phase is 40% (v/v) at pH 3.5, in which all solutes are well separated in an analysis time of about 15 min.

System suitability test is used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. According to US Pharmacopoeia 24th, method <621> [18], system suitability test is an integral part of a liquid chromatographic method. System suitability test was performed by five replicate injections of studied compounds. The results from system suitability test are presented in **Table 2** for each drug. Using the described analytical method, an optimal resolution of the analytes was achieved. A typical chromatogram is shown in **Figure 3**. These examined drugs were all well separated in a total duration of 15 min, with good peak resolutions, sharpness and symmetry.

Calibration graphs were constructed for all compounds. The calibration curves (ratio of peak areas of drug to that of internal standard versus concentration) were linear in human urine in concentration range of 2-32 and 3-48 μg mL^{-1} for methicillin, nafcillin, dicloxacillin and oxacillin, cloxacillin, respectively. The correlation coefficient was always greater than 0.999. **Table 3** lists the calibration curve parameters such as, slope, intercept correlation coefficients, detection limit (LOD) and quantitation limit (LOQ) values.

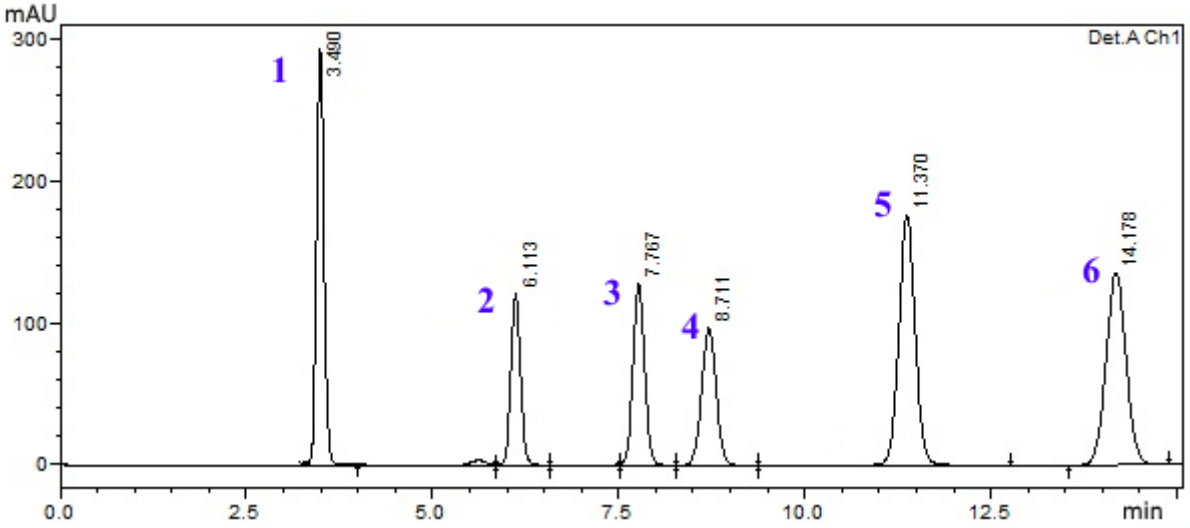

Figure 3. Optimized chromatogram of standard mixture. 1, methicillin (16,67 µg mL^{-1}); 2, oxacillin (16,67 µg mL^{-1}); 3, cloxacillin (16,67 µg mL^{-1}); 4, nafcillin (16,67 µg mL^{-1}); 5, dicloxacillin (16,67 µg mL^{-1}); 6, valsartan (I.S.) (16,67 µgmL^{-1}). Experimental conditions as in chromatographic procedure

Table 3. Statistical evaluation of the calibration data of studied compounds by RPLC

Sample	Linearity range (µg mL^{-1})	Slope	Intercept	S.E. of slope	S.E. of intercept	Correl. coeff.	Detection limit (µg mL^{-1})	Quantitation limit (µg mL^{-1})
Methicillin	2-32	0.457	-0.066	0.003	0.054	0.999	0.647	1.962
Oxacillin	3-48	0.400	-0.060	0.002	0.042	0.999	0.509	1.541
Cloxacillin	3-48	0.494	-0.105	0.003	0.080	0.999	0.789	2.391
Nafcillin	2-32	0.787	-0.116	0.003	0.059	0.999	0.363	1.100
Dicloxacillin	2-32	0.560	-0.065	0.004	0.062	0.999	0.543	1.646

The drug-free human urine, spiked with different concentrations of investigated compounds, were used for precision studies. The precision of the method was estimated by both interday and intraday reproducibilities. The intraday reproducibility was determined by replicate injections. The interday reproducibility of the method was determined by five repeated analysis of samples over three different days. It was found that the relative standard deviations (RSD) calculated from the peak area ratios of each drug versus the I.S. for determinations, both interday and intraday, were all less than 1.70% for the five drugs (**Table 4**).

The accuracy of the method for the analysis of urine samples was determined by recovery experiments. Control urine samples were spiked with two different concentrations of methicillin, nafcillin, dicloxacillin, oxacillin and cloxacillin. The recoveries and relative standard deviations (RSD) were calculated after five replicate analyses are given in **Table 5**.

Table 4. Intra-Day and Inter-Day precision of studied compounds

Compounds	Theoretical Concentration (µg mL^{-1})	Intra-Day measured Concentration Mean (µg mL^{-1})	RSD %	Inter-Day measured Concentration Mean (µg mL^{-1})	RSD %
Methicillin	4	4.072	0.525	4.126	1.622
	16	16.048	0.286	16.068	0.452
Oxacillin	6	6.078	0.432	6.193	0.690
	24	23.911	0.390	24.209	0.935
Cloxacillin	6	6.054	0.252	6.310	1.531
	24	24.131	0.561	24.024	0.761
Nafcillin	4	4.057	0.376	4.040	0.666
	16	16.077	0.353	16.246	1.170
Dicloxacillin	4	4.018	0.433	4.052	0.806
	16	16.061	0.431	16.342	0.976

Table 5. Accuracy and precision of the method for HPLC analysis of investigated compounds in human urine

Compounds	Amount added (µg mL^{-1})	Mean found concentration (µg mL^{-1})	Recovery (% mean± confidence interval)	Standard deviation	RSD (%)
Methicillin	8	7.980	99.746 ± 1.915	0.772	0.774
	16	16.063	100.391 ± 1.050	0.423	0.421
Oxacillin	12	12.066	100.554 ± 1.799	0.725	0.721
	24	24.209	100.870 ± 1.912	0.770	0.763
Cloxacillin	12	12.061	100.508 ± 1.773	0.714	0.711
	24	23.942	99.760 ± 1.258	0.507	0.508
Nafcillin	8	7.991	99.887± 0.893	0.360	0.360
	16	16.047	100.292 ± 0.527	0.212	0.212
Dicloxacillin	8	7.950	99.373 ± 1.094	0.441	0.443
	16	16.132	100.828 ± 0.963	0.388	0.385

Figure 4 shows (1) the chromatogram obtained for blank urine in the optimised experimental conditions and (2) the chromatogram obtained for urine spiked with different concentrations of studied compounds. As a $R_S \geq 1.5$-2.0 is generally accepted as a good resolution between the peak and the closest electing potential interference, these results show that the peaks for five penicillins were resolved from the other components of this biological fluid.

CONCLUSION

This work represents the first study dealing with the simultaneous chromatographic determination of Penicillinase-resistant penicillins fixed acetonitrile percentage. In this study, the experimental model of combined pH/organic modifier was successfully applied to

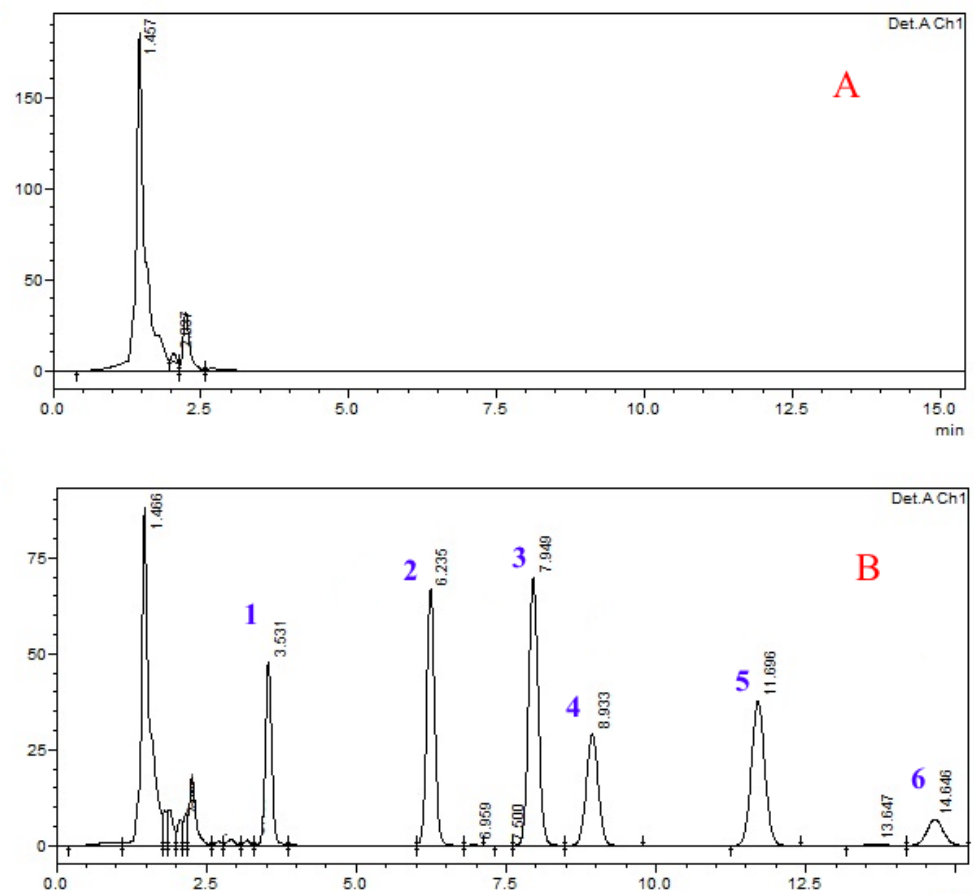

Figure 4. Typical chromatograms for blank human urine (A), human urine spiked with methicillin, dicloxacillin, nafcillin at 8 µg mL^{-1}, oxacillin, cloxacillin at 12 µg mL^{-1} and valsartan at 0.8 µg mL^{-1} (B)

describe the retention factors in different pH and fixed organic modifier condition. A sensitive and effective RPLC method was developed for the simultaneous determination of methicillin, nafcillin, dicloxacillin, oxacillin and cloxacillin in human urine by reversed phase high-performance liquid chromatography with ultraviolet detection. The method was validated showing satisfactory data for all the method validation parameters tested. Validation of the method for the quantification of these compounds in urine showed that the method had high sensitivity and accuracy with µg.mL^{-1} level measurement. The good validation criteria results of the method allowed its use in the quantification of these compounds.

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