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Rapid determination of sulbactam and tazobactam in human serum by high-performance liquid chromatography

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Abstract

A simple and rapid HPLC method for the determination of tazobactam and sulbactam, two β-lactamase inhibitors, in serum for the therapeutic follow-up of patients is described. The effect of the pH of the aqueous mobile phase and column temperature on column efficiency and retention were examined and equations for their dependences were derived. The use of a chromatographic response function showed that methanol–buffer (5:95, v/v) (pH 6.3) as the mobile phase and a 45°C column temperature were optimum values for chromatographic separation. The analytical method was linear from 10 to 200 μg/ml. This assay limit range is sufficient for the analysis of human serum. The limit of detection was 10 μg/ml for sulbactam and 5 μg/ml for tazobactam. The coefficient of variation was less than 5%. The speed at which this assay can be performed makes it especially useful for estimating the levels of these drugs in human serum.

1. Introduction

Several methods have been developed for the determination of tazobactam and sulbactam in biological fluids. The levels of sulbactam in blood, urine and cerebrospinal fluid (CSF) are determined by HPLC [1–5]. A gradient elution HPLC method has been described for the determination of tazobactam in human plasma, serum bile and urine [6,7]. This work concerns a simple and rapid HPLC method for the simultaneous routine determination of sulbactam and tazobactam in human serum using benzoic acid as an internal standard.

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2. Experimental

2.1. Apparatus

The HPLC system consisted of a Model 501 HPLC pump (Waters, Saint Quentin en Yvelines, France), a Rheodyne Model 7125 injection valve (Interchim, Montluçon, France) fitted with a 20-μl sample loop, an L 4000 variable-wavelength UV spectrophotometric detector (Merck, Nogent sur Marne, France) and a Model 4500 diode-array detector (Merck). A Waters Nova Pak column (150 mm × 4.3 mm I.D., 5 μm particle size) was used with the temperature controlled within ±1°C in an Interchim TM N° 701 oven in the range 27–45°C. The mobile phase was phosphate buffer–methanol

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(95:5, v/v). The experiments were carried out at pH 3.00, 3.40, 4.60, 6.20 or 7.00 and a flow-rate of 2 ml/min. The eluent was acidified by addition of phosphoric acid. Lower pH values were not used because of the excessive analysis time obtained with a pH of 3 and a column temperature of 27°C.

2.2. Reagents

Methanol (Carlo Erba, Lyon, France) and phosphate buffer (Prolabo, Paris, France) were of HPLC-grade. Phosphoric acid, phosphate and zinc sulfate were obtained from Merck (Darmstadt, Germany). Sulbactam, benzoic acid and tazobactam were obtained from Pfizer (Orsay, France), Aldrich (Strasbourg, France) and Lederle (Ourlins, France), respectively.

2.3. Sample preparation

A stock standard solution containing 1 mg/ml of sulbactam and tazobactam was prepared in water. Serum standard solutions for calibration were prepared by appropriate dilutions of this stock standard solution with drug-free serum to give concentrations of 200, 100, 50, 25 and 10 μg/ml. To 1 ml of standard serum solution were added 0.4 ml of a solution containing 10 mg/ml of zinc sulfate and 350 μg/ml of benzoic acid as an internal standard. The mixture was shaken for 30 s in a vortex mixer and centrifuged for 5 min at 5500 *g*. A 20-μl volume of the supernatant was injected into the chromatograph.

2.4. Chromatographic response function

The separation of the two compounds must be obtained rapidly, so the objective was to optimize the analysis time and not be too demanding with regard to resolution. This problem was solved by using chromatographic response functions (CRFs) [8–12]. This widely used CRF is defined as

$$\mathrm{CRF} = F_{\mathrm{obj}} + n^{a} + b(T_{\mathrm{A}} - T_{\mathrm{L}}) + c(T_{\mathrm{L}} - T_{0}) \quad (1)$$

where: F_{obj} = objective function expressed in terms of the discrimination factor d_{ij} [13,14] or the resolution factor R_{ij} [15,16] between two adjacent peaks i and j. In this application, F_{obj} is given by

$$F_{\mathrm{obj}} = \Sigma \ln R_{ij} \quad (2)$$

The sum is extended to all the peak pairs of the chromatogram. Other parameters are n = detected peak number, T_{A} = maximum acceptable analysis time, T_{L} = retention time of the last peak, T_0 = minimum acceptable time of the first peak, T_{L} = retention time of the first peak and a, b and c are constants.

The constants $a = 1$, $b = 0.5$ and $c = 1$ were determined empirically to give a function that sharply discriminated manifestly unsatisfactory separations from better separations. Hence the main serum peak did not interfere strongly with the peak of sulbactam. T_0 was chosen as 1 min and T_{L} was set at 2.8 min to give a correct peak integration. The optimum separation conditions are obtained when the CRF reaches its maximum in a very short analysis time.

2.5. Chemometric methodology

A chemometric approach based on the use of experimental design has been used in HPLC to study column efficiency or separation of compound mixtures. Guillaume and Guinchard [13,17] showed the interelationship between the resolution of two solutes, column plate height, linear mobile phase velocity, column temperature and mobile phase composition. Hu and Massart [18] utilized the uniform shell (Doehlert matrix) design for reversed-phase chromatography; this two-factor design required seven experiments and produced a quadratic model with interaction terms. Deming et al. [19] reported several algorithms and experimental designs for the optimization and interpretation of various user-selected chromatographic and analytical parameters. Glajch and Kirkland [20] reported a method developed in reversed-phase, normal-phase, ion-pair and gradient elution

HPLC, including experimental design, numerical separation criteria, peak tracking and software. Wieling et al. [21] optimized the chromatographic selectivity of twelve sulfonamides using mixture designs and multi-criteria decision making. Tucker et al. [22] used a central composite design for the enantiomeric separation of the antifungal drug tioconazole. The determination of aliphatic amines in industrial solution was studied by Vialle et al. [23] using this chemometric methodology. A full 3^2 factorial design (M) was used by Wang et al. [24] to study simultaneously the variations in all the factors. These models can be used for regression analysis: a two factor model takes the form

$$y = \alpha + \beta(\ln \mathrm{pH}) + \gamma(\ln T) + \delta(\ln \mathrm{pH})^2 + \epsilon(\ln T)^2 + \phi(\ln \mathrm{pH})(\ln T) \quad (3)$$

where y is the response studied:

$$y = MP \quad (4)$$

$$y = \begin{pmatrix} y_1 \\ y_2 \\ y_3 \\ y_4 \\ y_5 \\ y_6 \\ y_7 \\ y_8 \\ y_9 \end{pmatrix}$$

$$M = \begin{vmatrix} 1 & -1 & 1 & 1 & 1 & -1 \\ 1 & -1 & -1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & -1 & 1 & 1 & -1 \\ 1 & 0 & -\sqrt{2} & 0 & 2 & 0 \\ 1 & 0 & +\sqrt{2} & 0 & 2 & 0 \\ 1 & -\sqrt{2} & 0 & 2 & 0 & 0 \\ 1 & +\sqrt{2} & 0 & 2 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \end{vmatrix}$$

$$P = \begin{pmatrix} \alpha \\ \beta \\ \gamma \\ \delta \\ \epsilon \\ \phi \end{pmatrix}$$

where y_1, y_2, ..., y_9 are the experimental y values recorded for each pair of temperatures and pH (T_i, pH_i). The matrix M is given in reduced coordinates. The variables were coded to have a variation range from $-\sqrt{2}$ to $+\sqrt{2}$. The parameter vector P was calculated as:

$$P = (M^t M)^{-1}(M^t y) \quad (5)$$

where M^t is the transposed matrix of M.

3. Results and discussion

3.1. Chromatographic separation

Using the chemometric methodology, the effects of mobile phase pH and column temperature on column efficiency represented by the height equivalent to a theoretical plate (H) were studied.

The experimental H values were calculated from the chromatograms. All experiments were repeated three times. The coefficient of variation of the H values was less than 2% in most instances indicating a high reproducibility and good stability of the chromatographic system. The results were processed by computer and the parameters of Eq. 5 were obtained (Table 1). The calculated values of $1/H$ are summarized in Table 2. The fitting of the model to the results is good (98%, 97% and 97% for sulbactam, benzoic acid and tazobactam, respectively). Student's t-test was used to provide the basis for a decision as to whether the model coefficients were significant or not. The results of the test showed that no variable can be excluded from the model. As the signs of the constants α–ϕ were the same, the three-dimensional plots of the three compounds represented by Eq. 3 had identical variations. For example, the surface S corresponding to Eq. 3 was plotted as three-dimensional diagrams for benzoic acid (Fig. 1). Using the experimental design, ln k' values were modelled by a second-order polynomial. The corresponding polynomials are given in Table 3. These models showed that an increase in mobile phase pH or column temperature produced a

Table 1
Values of the coefficients α, β, γ, δ, ϵ, and ϕ and correlation coefficients r for $1/H$ for the three compounds and for the CRF

Independent variable	$1/H$			CRF
	Sulbactam	Benzoic acid	Tazobactam	
α	10.00	16.667	11.236	1.475
β	−0.821	−2.638	−0.637	3.874
γ	−0.390	−0.798	−0.644	0.119
δ	−0.376	−1.418	−0.221	−2.539
ϵ	−0.909	−2.032	−1.096	−0.436
ϕ	+0.449	+1.231	+0.385	+1.078
r	0.986	0.979	0.978	0.987

decrease in the capacity factor k'. The surface S corresponding to the model of benzoic acid was plotted as a three-dimensional diagram (Fig. 2).

For a constant pH of the mobile phase, when the column temperature increased the solute mass transfer from the mobile to the stationary phase increased, producing an increase in column efficiency. Above an optimum temperature, the decrease in the capacity factor with temperature concealed the first phenomenon and produced a decrease in the column plate number. The partial derivative of Eq. 3 against T was equal to zero and was rearranged to obtain the analytical equation relating the optimum temperature to the mobile phase pH:

$$T_{opt} = \exp\left[-\frac{1}{2\epsilon}(\gamma + \phi \ln \mathrm{pH})\right] \quad (6)$$

For mobile phase pHs of 3.00, 5.00 and 7.00, the optimum column temperatures were 29.47, 32.82 and 35.23°C respectively, for benzoic acid. Eq. 6 shows that an increase in mobile phase pH meant that a high column temperature could be used while at the same time preserving the maximum column efficiency (decreased analysis time). With a constant column temperature, for low pH, the solute molecules usually in a non-ionic form were in the non-polar stationary phase for a long period and the solute mass transfer in the mobile phase was weak, producing an increase in peak width. For high pH, the

Table 2
Calculated $1/H$ (mm^{-1}) and CRF values for different experimental conditions

Temperature (°C)	pH	$1/H$			CRF
		Sulbactam	Benzoic acid	Tazobactam	
27	3.40	10.31	17.86	11.49	−4.39
41	3.40	8.77	14.08	9.62	−6.24
27	6.20	7.75	9.90	9.43	1.06
41	6.20	8.00	11.11	9.17	3.56
25	4.58	8.77	13.70	10.00	0.43
45	4.58	7.63	11.49	8.13	0.76
34	3.00	10.42	17.24	11.63	−9.19
34	7.00	8.13	10.20	9.90	1.98
34	4.58	10.00	16.67	11.24	1.47

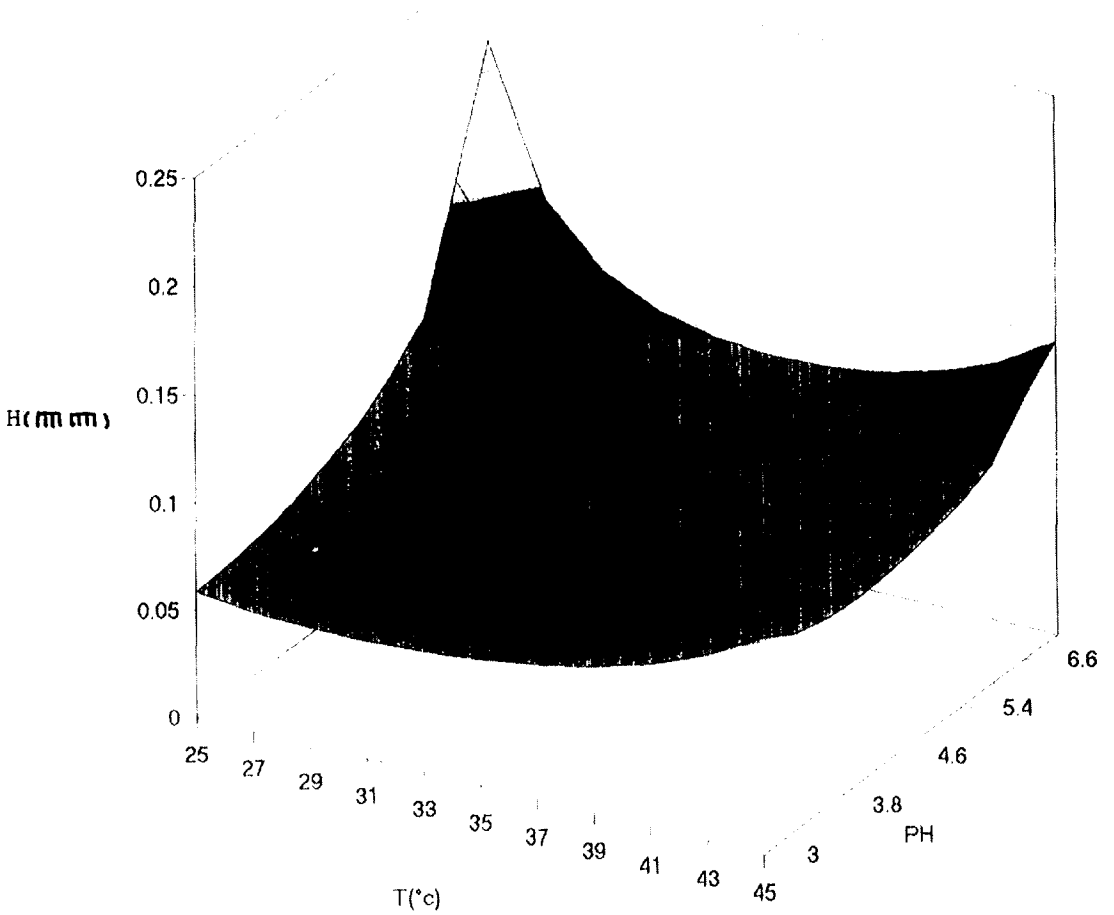

Fig. 1. Three-dimensional plots of Eq. 3 for benzoic acid for the H values.

Table 3
Values of the coefficients α, β, γ, δ, ϵ and ϕ and correlation coefficients r for $\ln k'$ of the three compounds

Independent variable	Sulbactam	Benzoic acid	Tazobactam
α	+0.604	+2.770	+1.449
β	−0.214	−1.248	−0.075
γ	−0.052	−0.050	−0.080
δ	+0.124	−0.217	−0.007
ϵ	−0.011	−0.038	−0.007
ϕ	+0.001	+0.012	−0.002
r	0.988	0.995	0.989

rapid decrease in the capacity factor necessitated a higher plate number and the column efficiency also decreased. Hence, this optimum mobile phase pH was a minimum.

The partial derivative of Eq. 3 against pH was equal to zero and was rearranged to obtain the analytical equation relating the optimum mobile phase pH to the column temperature T:

$$\mathrm{pH}_{\mathrm{opt}} = \exp\left[-\frac{1}{2\delta}(\beta + \phi \ln T)\right] \quad (7)$$

For column temperatures T of 27, 35 and 45°C, the optimum mobile phase pHs were 2.88,

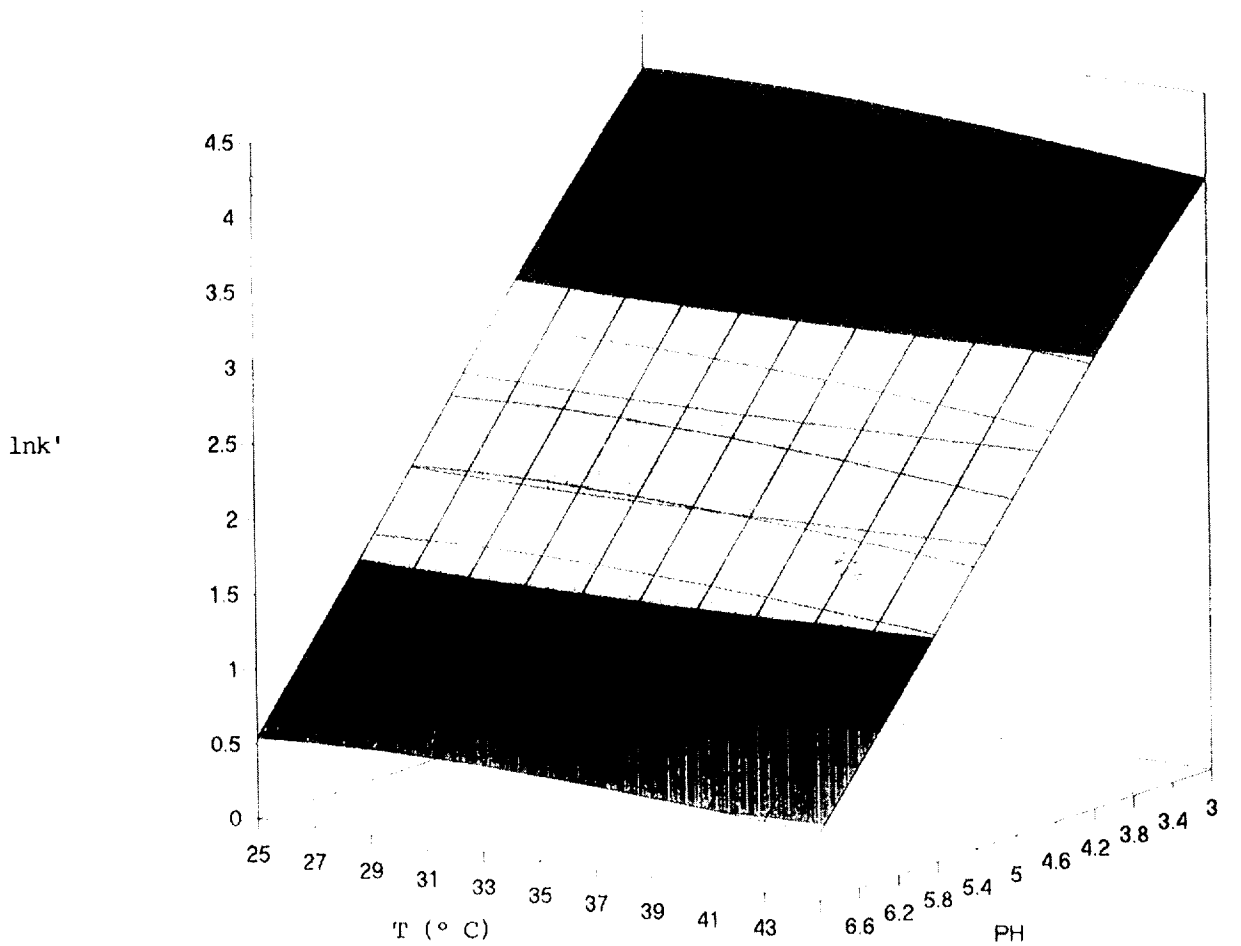

Fig. 2. Three-dimensional plots of Eq. 3 for benzoic acid for the $\ln k'$ values.

3.56 and 4.17, respectively, for benzoic acid. Eq. 7 shows that an increase in column temperature meant that a high mobile phase pH could be used while at the same time preserving the maximum column efficiency (decreased analysis time).

Optimum conditions corresponding to the highest column efficiency for the three compounds require a long analysis time ($\geq$10 min). For rapid routine analysis this is too long. The chromatographic response function (CRF) gives a good resolution between the three peaks ($R_s \geq$ 1.5) and a short analysis time ($T_a \leq 3$ min). Calculated values of CRF from Eq. 1 for the nine experiments are given in Table 2. The coefficient of multiple determination r corresponding to the CRF was equal to 0.98. The parameter estimates generated for the regression model are given in Table 1. The CRF for all the pH–T combinations investigated to separate the three compounds is shown in Fig. 3 with a maximum for a mobile phase pH of 6.30 at a column temperature of 45°C. This value is on the border of the parameter space examined in the experimental design. An experiment carried out at 50°C showed that the method was within the range. The maximum value of CRF was 3.80. The corresponding retention times for sulbactam, benzoic acid and tazobactam were 1.40, 1.90 and 2.40 min, respectively. This CRF op-

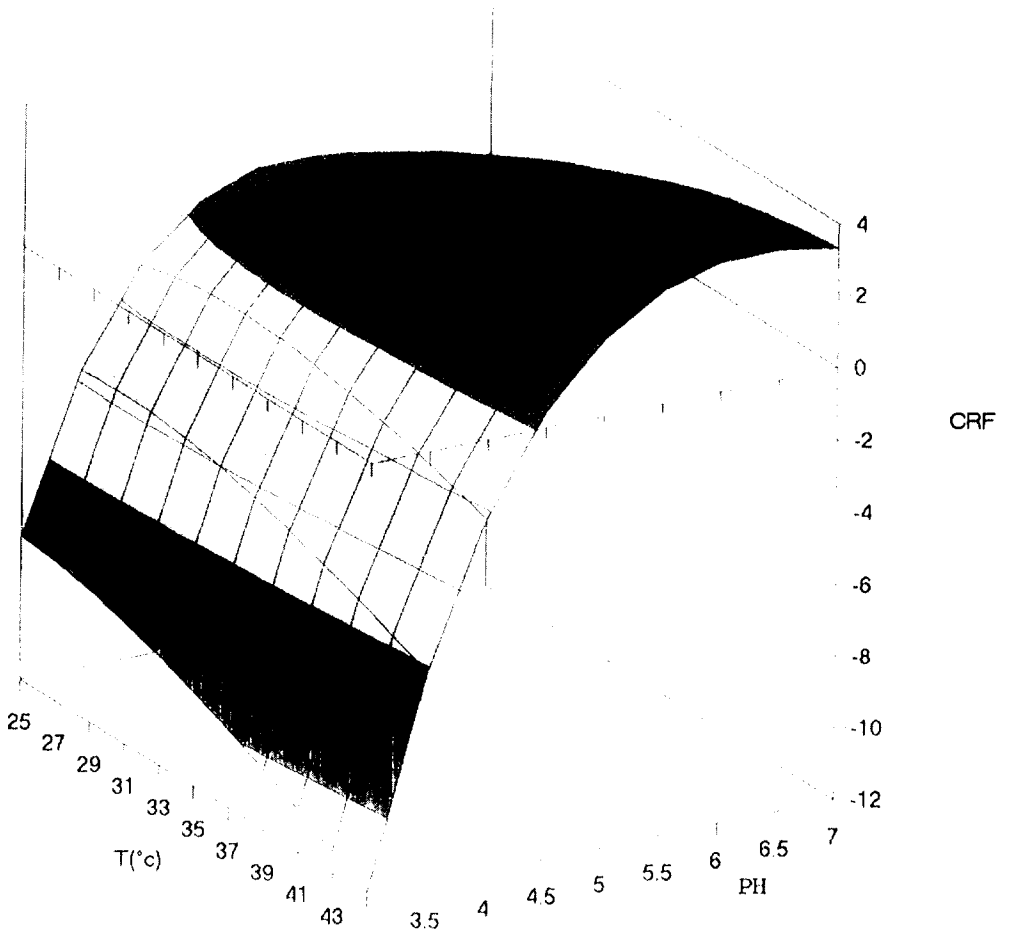

Fig. 3. Three-dimensional plots of Eq. 3 for the CRF values.

timization procedure improved both the analysis time and the resolution with a limited number of experiments and shows advantages over other optimization methods. A typical chromatogram of a sample containing 70 μg/ml of sulbactam, benzoic acid and tazobactam is shown in Fig. 4.

Possible interferences from other drugs were evaluated. Commonly used drugs that were tested and that did not interfere with the assay included piperacillin and cefaclor.

3.2. *Linearity, assay validation and detection limits*

The calibration graphs were linear from 10 to 200 μg/ml for sulbactam and tazobactam. The equations determined from five different concentrations (experiments repeated twice) were as follows:

$$\text{sulbactam:} \quad y = 7.221 \cdot 10^{-3} + 6.737 \cdot 10^{-3} x \quad (r = 0.9954) \tag{8}$$

$$\text{tazobactam:} \quad y = -2.133 \cdot 10^{-1} + 1.881 \cdot 10^{-2} x \quad (r = 0.9950) \tag{9}$$

where y is the peak-area ratio of sulbactam or tazobactam to benzoic acid, x is the concentration of sulbactam or tazobactam in μg/ml and r is the correlation coefficient. The precision and accuracy for the calibration graphs were determined by varying the standard solutions. The

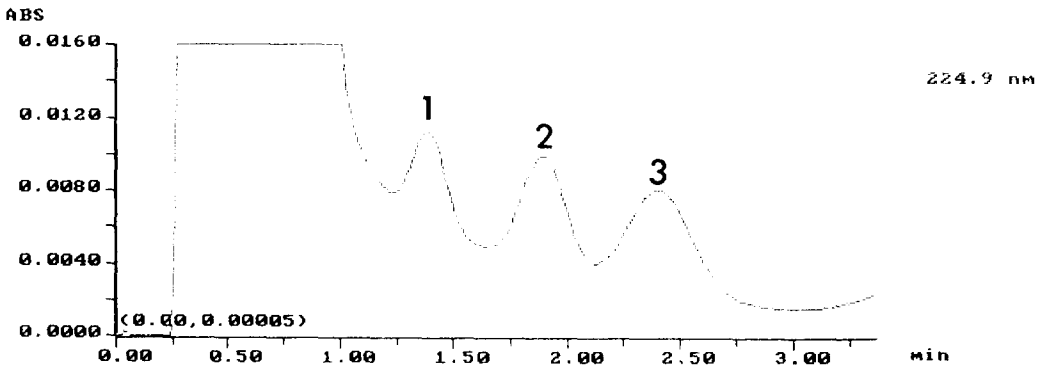

Fig. 4. Typical chromatogram of human serum spiked with 70 μg/ml of drugs and the internal standard under the optimum conditions: mobile phase, phosphate buffer–methanol (95:5, v/v); pH, 6.30; flow-rate, 2 ml/min; column temperature, 45°C. Peaks: 1 = sulbactam; 2 = benzoic acid; 3 = tazobactam.

within-day coefficient of variation was less than 5% ($n = 6$). This results indicates good precision for the assay. Table 4 gives the detailed intra-day precision and accuracy results. The detection limit was 10 μg/ml for sulbactam and 5 μg/ml for tazobactam.

4. Conclusion

This approach to the determination of tazobactam and sulbactam in human serum provided an appropriate combination of mobile phase pH and column temperature with a limited number of experiments. The results demonstrate the need to control the column temperature to obtain a short analysis time. The typical run time was less than 3 min.

Table 4
Within-run reproducibility of the assay for drugs in serum ($n = 6$; nominal concentration = 100 μg/ml)

Compound	C.V. (%)	Accuracy[a] (%)
Sulbactam	4.07	105.35
Tazobactam	3.84	97.55

[a] Accuracy (%) = (observed concentration/nominal concentration) · 100.

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