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Evaluation of Liquid Chromatographic Behavior of Cephalosporin Antibiotics Using Restricted Access Medium Columns for On-line Sample Cleanup of Bovine Milk

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Microsample deproteinization of bovine milk was carried out on-line using a series of restricted access medium (RAM) bovine serum albumin (BSA) columns: C_8, C_{18}, phenyl, and cyano. The four different columns prepared showed a high percentage of protein exclusion using water as the mobile phase and provided an appropriate retention profile for a series of five cephalosporin antibiotics (cefoperazone, cephacetril, cephalexin, cephapirin, and ceftiofur). Chromatographic conditions such as washing time, buffer pH, and type and percentage of organic modifier were fully evaluated with respect to the protein elution profile and retention of the antibiotic by RAM column. One of these columns was chosen to develop and validate a method for the determination of cefoperazone in bovine milk. The system used in this work was composed of a RAM-BSA phenyl column coupled to a C_{18} analytical column. The standard curve was linear over the range 0.100−2.50 μg/mL. The limits of quantification and detection were 0.100 and 0.050 μg/mL, respectively. The developed method showed high intermediate precision (CV of 2.37−2.63%) and accuracy (90.7−94.3%) with adequate sensitivity for drug monitoring in bovine milk samples.

KEYWORDS: Restricted access media (RAM); column switching; direct injection; cephalosporins; bovine milk

INTRODUCTION

A quick overview of published methods for the analysis of compounds in complex biological samples reveals that the most difficult step is the cleanup or the extraction of a required compound from the matrix (*1*−*4*).

The strategy necessary to analyze exogenous compounds in biological fluids depends greatly upon the nature of the compound and on the bio-matrix.

To reduce sample manipulation, analysts have developed methods that combine sample preparation with the separation methods (*1*−*3*).

Coupled-column separation using restricted access media in the first dimension for the exclusion of macromolecules and retention of micromolecules has been used with great success for a number of biological fluids (*5*−*11*). The number of methods employing these columns for the analysis of exogenous compounds in milk samples is, however, limited (*12*−*17*). Milk is a more complex matrix than plasma and serum due to the presence of a lipid emulsion in addition to the proteins (*4*).

Methods for residual analysis are changing rapidly due to new techniques that are available. Among a variety of classes of compounds that need to be monitored in milk are the antibiotics.

Cephalosporins are β-lactam antibiotics that are effective against a broad spectrum of Gram-positive and Gram-negative bacteria. They are widely used for treatment of clinical mastitis in cows caused by various bacterial infections. Cephalosporins are semisynthetic antibiotics derived from the 7-aminocephalosporanic acid and differ only in the nature of the substituents attached at the 3- and/or 7-positions of the cephem nucleus (**Figure 1**) (*18*).

A number of chromatographic methods using various stationary phases and mobile phases have been described for the determination of cephalosporins in milk (*19*−*26*). All of them, however, required sample preparation prior to the analysis. Different types of sample preparation such as protein precipitation, ultrafiltration, dilution of samples, and liquid−liquid and solid-phase extraction on cartridges have been used. Thus, the possibility of reducing the number of steps in sample preparation for the analysis of cephalosporin antibiotics in bovine milk is an important goal.

This work reports the evaluation of four different types of restricted access medium bovine serum albumin (RAM-BSA) columns for the exclusion of milk proteins and for the analysis of this class of highly polar antibiotics. Among the currently administered cephalosporin antibiotics in agriculture, cefoperazone, ceftiofur, cephalexin, cephacetril, and cephapirin (**Figure 1**) were the five compounds selected for this investigation. **Table 1** shows the maximum residue levels in milk established for

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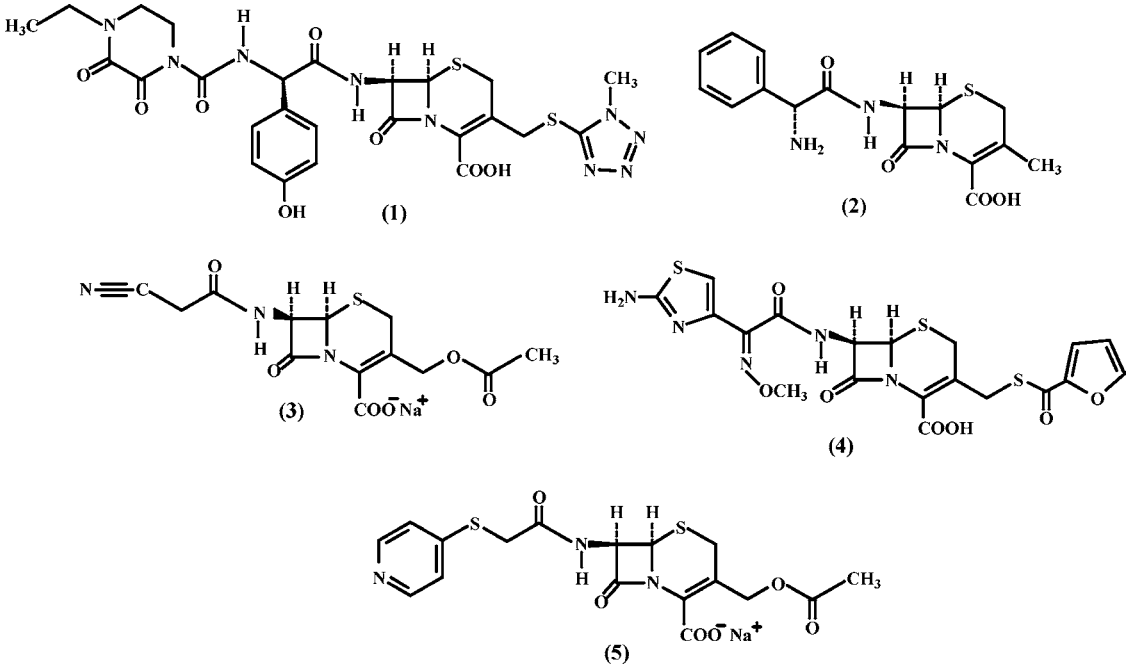

Figure 1. Chemical structures of cephalosporin antibiotics selected: (**1**) cefoperazone; (**2**) cephalexin; (**3**) cephacetril; (**4**) ceftiofur; (**5**) cephapirin.

Table 1. Maximum Residue Levels of the Five Selected Antibiotics in Accordance with OJ L 224, 18.8.1990 of the Council Regulation (EEC) 2377/90 of June 26, 1990[a]

pharmacologically active substance	marker residue	MRL (μg/kg)
cefoperazone	cefoperazone	50
cephalexin	cephalexin	100
cephacetril	cephacetril	125
ceftiofur	sum of all residues retaining the betalactam structure expressed as desfuroylceftiofur	100
cephapirin	sum of cephapirin and acetyl desacetylcephapirin	60

[a] Data obtained at http://cemu10.fmv.ulg.ac.be/ostc/902377/902377a1.htm (on March 8, 2005).

these compounds in accordance with Council Regulation (EEC) 2377/90 of June 26, 1990.

Furthermore, using direct injection onto one of the chosen RAM columns, a multidimensional high-performance liquid chromatography (HPLC) method was successfully developed and validated to quantify cefoperazone in bovine milk.

MATERIALS AND METHODS

General. Acetonitrile (HPLC grade) was purchased from Mallinckrodt Baker (St. Louis, MO), and water was purified through a Milli-Q system (Millipore, São Paulo, Brazil). Reagents and other chemicals were obtained from the following sources: bovine serum albumin (fraction V powder minimum 98%) and tetrabutylammonium hydrogen phosphate from Sigma (St. Louis, MO); glutaraldehyde and sodium borohydride from Merck (Darmstadt, Germany); and potassium dihydrogen phosphate from Cinética Química (São Paulo, SP, Brazil). The 47 mm diameter, 0.45 μm nylon membranes used to filter all of the mobile phases were also obtained from Millipore. Antibiotics were generously donated as follows: cefoperazone (Pfizer, São Paulo, SP, Brazil); cephacetril (Novartis, São Paulo, SP, Brazil); and cephalexin and ceftiofur (Ouro Fino Produtos Veterinários, Ribeirão Preto, SP, Brazil). Cephapirin was purchased from Sigma (St. Louis, MO). The certificates of analysis of each compound provided the information about purity and identity of the antibiotics used.

Natural pasteurized whole and skimmed milk were obtained from different bakeries in São Carlos (SP, Brazil).

Instrumentation and Columns. The HPLC system consisted of two Shimadzu LC-10ATVP pumps (Kyoto, Japan), with one of the pumps having a valve FCV-10AL for selecting solvent, an autoinjector model SIL 10AVP, a degasser model DGU-14A, a column oven CTO-10A (at 30 °C), an SPD-6AV UV−vis detector (at 270 nm), and an SCL 10AVP interface. A sample valve HPLC 7000 Nitronic EA (Supelco, St. Louis, MO) was used for the automated column switching. Data acquisition was done on Shimadzu Class-VP software.

The analytical column used (150 × 4.6 mm i.d.) (C_{18}-Hypersil, 5 μm, 120 Å) was packed by the ascending slurry method at 7500 psi, using methanol (*9*).

The RAM-BSA columns (100 × 4.6 mm i.d.) were prepared as follows. Octyl (Luna, 10 μm, 100 Å), octadecyl (Hypersil, 10 μm, 120 Å), phenyl (Hypersil, 5 μm, 120 Å), and cyano (Hypersil, 10 μm, 120 Å) silica columns were packed as described for the analytical column. After the columns had been conditioned for ~12 h with methanol at a flow rate of 1.0 mL/min, the immobilization of BSA was carried out in situ according to the Menezes and Felix (*12*) protocol and as published before (*8*, *9*).

Recovery of Milk Proteins and Retention Studies of the Antibiotics by RAM-BSA Columns. The conditions used for milk protein recovery using aliquots (50, 100, and 200 μL) of whole milk type A (top grade milk) were the same as described elsewhere (*8*) for plasma protein and were based on the Bradford method (*27*).

The recovery was calculated from the absorbance ratio of the proteins of the milk reference solutions with the solutions of the proteins of the chromatographed sample milk.

For the retention studies on the RAM columns, mobile phases composed of 0.01 M potassium dihydrogen phosphate buffer at different pH values (2.5, 6.0, 6.5, 7.0, 7.5, and 8.0) with an appropriate percentage of acetonitrile were investigated. Cefoperazone and ceftiofur were analyzed using 0.01 M phosphate buffer/acetonitrile (70:30 v/v), whereas cephapirin, cephalexin, and cephacetril were analyzed using 0.01 M phosphate buffer/acetonitrile (90:10 v/v) as the mobile phase.

All analyses for retention studies were performed at room temperature. Stock solutions of cefoperazone (50 μg/mL) and ceftiofur (50 μg/mL) were prepared in acetonitrile, and cephacetril (50 μg/mL), cephapirin (50 μg/mL), and cephalexin (50 μg/mL) were prepared in Milli-Q water. A 100 μL aliquot of each solution (50 μg/mL) was injected in duplicate onto the RAM-BSA columns.

Standard Solutions for the Method Validation and Sample Preparation. Stock solutions of cefoperazone (200 μg/mL) were

prepared in acetonitrile. From this stock solution six calibration standards were prepared in acetonitrile at the following concentrations: 25.0, 20.0, 10.0, 5.00, 3.00, and 1.00 μg/mL. Quality control solutions were prepared at 3.60, 15.0, and 22.0 μg/mL. Stock, standard, and quality control solutions were prepared fresh weekly.

To prepare the spiked samples, aliquots (18 μL) of the appropriate standard solution were placed into a series of culture tubes, and the solvents were evaporated under a stream of nitrogen. The dried analytes were reconstituted using a sample of milk (180 μL) that was previously centrifuged at 10000*g* at 20 °C for 10 min, or acetonitrile (for the extraction efficiency evaluations), and then a 0.8 mM solution of tetrabutylammonium phosphate (18 μL) prepared in 0.01 M potassium dihydrogenphosphate buffer (pH 7.5) was added to each tube. The solutions were vortex-mixed for 15 s. Aliquots of 180 μL were transferred to autosampler vials, and 100 μL was injected into the column-switching HPLC system. For the stability assays, the spiked milk samples (thawed to room temperature), and the 0.8 mM solution of tetrabutylammonium phosphate (18 μL) was added to each tube just before analysis. For method development and validation, skimmed milk type A (top grade) was used. The method was also checked in whole milk types A (top grade), B (intermediate grade), and C (inferior grade).

Method Validation. Calibration-spiked skimmed milk standards (2.50, 2.00, 1.00, 0.50, 0.30, and 0.10 μg/mL) were prepared in triplicate. The calibration curve was obtained by plotting the peak area against the concentration of cefoperazone. Analyses of blank skimmed milk were performed daily to evaluate the presence of potential interfering substances.

The extraction efficiency of cefoperazone from spiked skimmed milk samples by the phenyl-BSA column was determined by analyzing quality control samples at three different concentrations: 0.360, 1.50, and 2.20 μg/mL. The peak area ratios of five spiked milk samples at each concentration were compared with those of five injections of standard solutions to derive an extraction percentage.

Inter- and intraday variability of the method was evaluated by replicate analysis at the same three quality control concentrations (0.360, 1.50, and 2.20 μg/mL). Five samples of each concentration were prepared in milk on three nonconsecutive days. The accuracy of the method was evaluated by back-calculation; it was also tested using blinded unknowns, at two different concentrations, which were prepared by a different analyst. The acceptance criteria for the limit of quantification were that the precision and accuracy for three extracted samples were under 20% variability, and the limit of detection was calculated by taking a signal-to-noise ratio of three.

Stability Evaluations. The stability of analytical standard solutions of cefoperazone was evaluated at three quality control concentrations (0.360, 1.50, and 2.20 μg/mL) freshly prepared in triplicate and then stored at 4 and −20 °C. The results of those analyses were compared with those for freshly prepared solutions.

The stability of cefoperazone in milk under long-term and short-term storage was evaluated as follows: Two batches of spiked samples at three quality control concentrations were prepared in bulk (3 mL), analyzed immediately after preparation, and then transferred to three culture tubes and stored at 4 and −20 °C. Each sample was thawed to room temperature before triplicate analysis. This assay was carried out on three separate occasions at intervals of 24 h each. The concentrations of all samples were compared with the results of the first-day analysis. For short-term storage (benchtop) the samples were prepared in triplicate and kept at room temperature for 4 h before analysis. The freeze−thaw stability was evaluated also at the three quality concentrations with samples prepared in bulk (3 mL) and analyzed fresh before storage at −20 °C. Then, the samples were frozen for 24 h and allowed to thaw before analysis. The samples were returned to the −20 °C freezer for another freeze−thaw cycle. This procedure was carried out for two additional cycles.

The stability of samples during the residence time in the autosampler was also assessed. The spiked samples at the three quality control concentrations were prepared and analyzed ($n = 1$) immediately after preparation (time zero). Injections were carried out from the same vials in intervals of 1 h for a period of 24 h. The concentration of all the stability samples was compared to the mean values obtained from the first injection. The stability of the matrix was evaluated at 4 and −20 °C. The chromatograms of the stored milk samples were compared with the chromatograms of freshly prepared milk samples.

Table 2. Physical–Chemical Properties of Stationary Phases Used for Preparing the RAM Columns[a]

silica (type)	trade name	particle size (μm)	pore size (Å)	pore volume (mL/g)	superficial area (m^2/g)	carbon %	covering (μmol/m^2)
C_8	Luna	10	100		400	13.50	5.50
C_{18}	Hypersil	10	120	0.70	170	10.00	2.84
Ph	Hypersil	5	120	0.70	170	5.00	2.40
CN	Nucleosil	10	100	1.00	350	4.00	1.73

[a] Data supplied from manufacturers.

Table 3. Recoveries of Bovine Milk Proteins Excluded from RAM-BSA Columns, in 5 min Using Water as Mobile Phase

	injection volume of bovine milk					
	50 μL		100 μL		200 μL	
column[a]	av (%)	CV (%)	av (%)	CV (%)	av (%)	CV (%)
C_8-BSA	89.0	1.57	93.0	3.67	93.0	5.72
C_{18}-BSA	99.0	6.24	94.0	2.60	93.0	1.44
Ph-BSA	80.0	3.57	90.0	1.31	94.0	3.75
CN-BSA	100	0.73	86.0	1.47	90.0	4.54

[a] Column size: 10 × 0.46 cm i.d.

RESULTS AND DISCUSSION

Exclusion Efficiency of the Bovine Milk Proteins by the RAM-BSA Columns. There has been growth in the use of column switching to process a large number of samples and also to achieve high selectivity associated with high sensitivity. Recently, two revisions on the application of RAM columns for on-line sample cleanup demonstrate their utility for a number of different biological matrixes (*5*, *28*). The number of reported methods for direct analysis of milk samples is, however, still very small, probably due to the complex nature of the milk matrix (*5*, *12*−*17*, *28*, *29*). To permit direct injection of biological samples, the RAM column must be capable of removing endogenous components with high efficiency. To evaluate the efficiency of exclusion of integral bovine milk proteins by the RAM column, the recovery of proteins from all RAM columns prepared was measured by using Bradford's method (*27*). Despite the differences in the alkyl chains and in the trademark of the silicas used (**Table 2**), all four columns exhibited a high capacity of protein exclusion for the milk proteins. Recoveries in the range of 80−100% were obtained in 5 min using only water as mobile phase at a flow rate of 1 mL/min. These results are presented in **Table 3** and represent injections of 50, 100, and 200 μL of milk. It was not necessary to use organic solvent or buffer to salt-out the milk proteins. Similar performances were already observed in exclusion of plasma proteins from C_8 and C_{18} RAM-BSA columns (*8*, *9*, *30*).

Retention Evaluation for Cephalosporin Antibiotics on RAM-BSA Columns. In a preliminary experiment, the retentions of the series of cephalosporins in all four RAM-BSA columns were evaluated using water as mobile phase. However, the compounds cephacetril, cephapirin, and cephalexin were not sufficiently retained under these conditions. To evaluate the influence of mobile phase pH on the retention of these hydrophilic antibiotics by the RAM columns, the compounds were analyzed at various pH values using 0.01 M phosphate

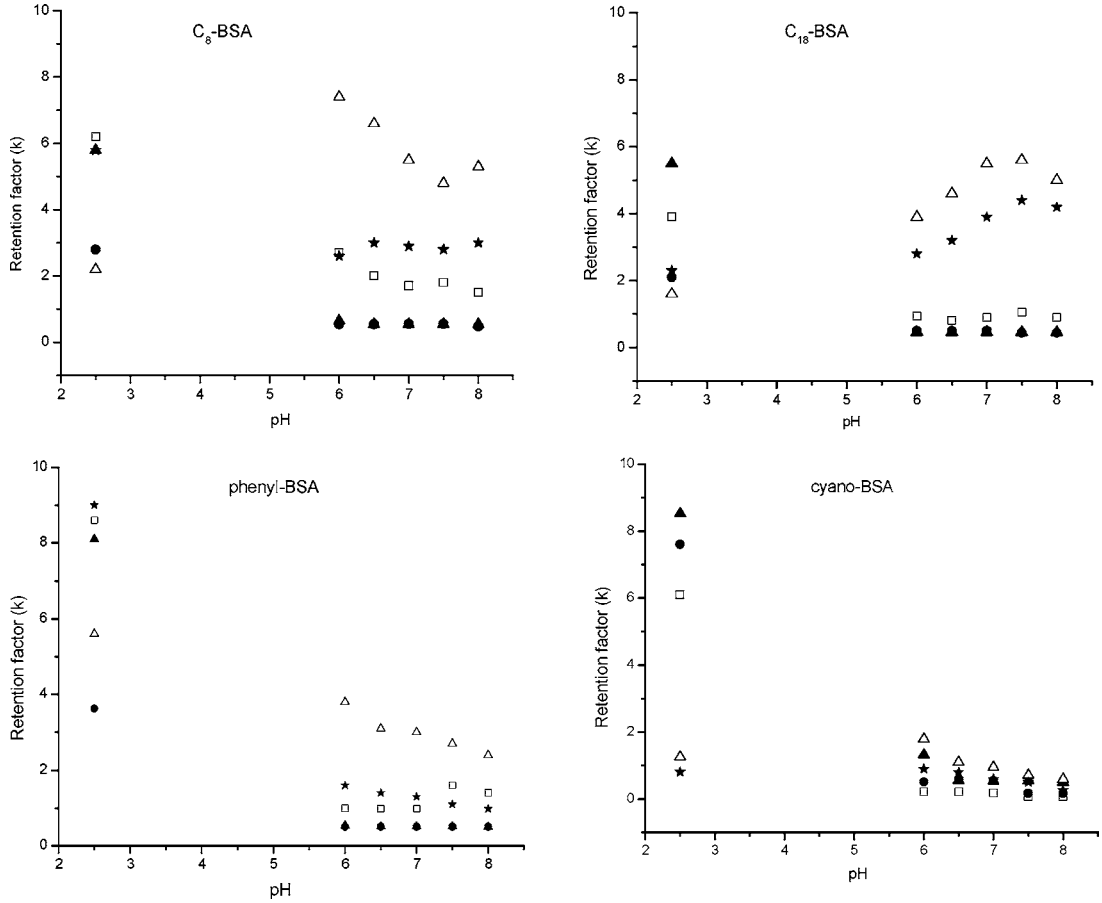

Figure 2. Dependence of retention factors on pH value. Cefoperazone and ceftiofur were analyzed using 0.01 M phosphate buffer/acetonitrile (70:30 v/v), whereas cephapirin, cephalexin, and cephacetril were analyzed using 0.01 M phosphate buffer/acetonitrile (90:10 v/v) as the mobile phase: □, cefacetril; ●, cefoperazone; ▲, ceftiofur; ★, cefalexina; △, cefapirina.

buffer at 1.0 mL/min. The pH values evaluated were 2.5, 6.0, 6.5, 7.0, 7.5, and 8.0 (**Figure 2**). The range from 3.0 to 5.5 was not used to avoid values around the isoelectric point of milk proteins, at which protein precipitation can occur (*31*). Cefoperazone and ceftiofur were analyzed using 0.01 M phosphate buffer/acetonitrile (70:30 v/v), whereas cephapirin, cephalexin, and cephacetril were analyzed using 0.01 M phosphate buffer/acetonitrile (90:10 v/v) as the mobile phase.

The retention factors were strongly influenced by the apparent pH of the mobile phases. The cephalosporins possess a carboxylic acid group and another acidic or basic substituent in their cephem nucleus. In the pH range from 6.0 to 8.0 all cephalosporins are completely ionized, whereas at pH 2.5 the ionization of the acid group is suppressed and, as a result, the retention of the compounds cefoperazone, cephacetril, and ceftiofur was increased on all columns. Cephalexin and cephapirin showed, however, an increase in retention factor on the C_{18}-BSA column above pH 6.0. This was again observed for cephapirin on the C_8-BSA column, but not for cephalexin, suggesting that ionic exchange secondary interactions may be occurring between the ionized analytes and the stationary phases.

All RAM-BSA columns evaluated gave an acceptable retention factor in at least one of the mobile phases used. These results established that it is possible to use any of the BSA columns prepared for the method development and quantification of these antibiotics in biological samples.

Method Development. From the series of antibiotics (**Figure 1**) cefoperazone was selected as the model compound for method development. The phenyl-BSA-RAM column was selected on the basis of the retention factor of cefoperazone on this column and also the peak shape obtained. The C_{18}- and phenyl-BSA columns afforded better symmetric chromatographic bands when compared to the C_8- and cyano-BSA columns for the series of cephalosporins evaluated (**Figure 3**). Columns and experimental conditions that provide symmetrical chromatographic bands are always preferred because this allows accurate transfer of the analyte to the separating column. Although this column had showed the lowest exclusion capacity for milk proteins when water was used as the mobile phase (**Table 3**), suitable protein exclusion was obtained by adjusting the pH of the mobile phase.

On the basis of the data from **Figure 2**, the highest retention factors were obtained using mobile phase at pH 2.5. Thus, the chromatographic exclusion behavior of milk proteins was initially investigated using 0.01 M phosphate buffer at pH 2.5. However, at this pH the exclusion of proteins was inefficient because the elution of the proteins was too long. Different buffer pH values were evaluated, and adequate exclusion was obtained at a pH >6.0, with or without an organic modifier. At these pH ranges the proteins were excluded within 5 min using a flow rate of 1.0 mL/min. The addition of low percentages of organic modifier was desired to inhibit undesired adsorption of lipids and proteins onto the pore surface of the hydrophobic phase. Therefore, the mobile phase for exclusion of milk proteins was chosen as 0.01 M phosphate buffer pH 7.5/acetonitrile (98:2 v/v). Following proteins exclusion, a second mobile phase was needed to elute cefoperazone from the hydrophobic phase. Among the different mobile phase compositions investigated, 0.01 M phosphate buffer pH 7.5/acetonitrile (87:13 v/v) was selected. However, poor reproducibility of retention factors was

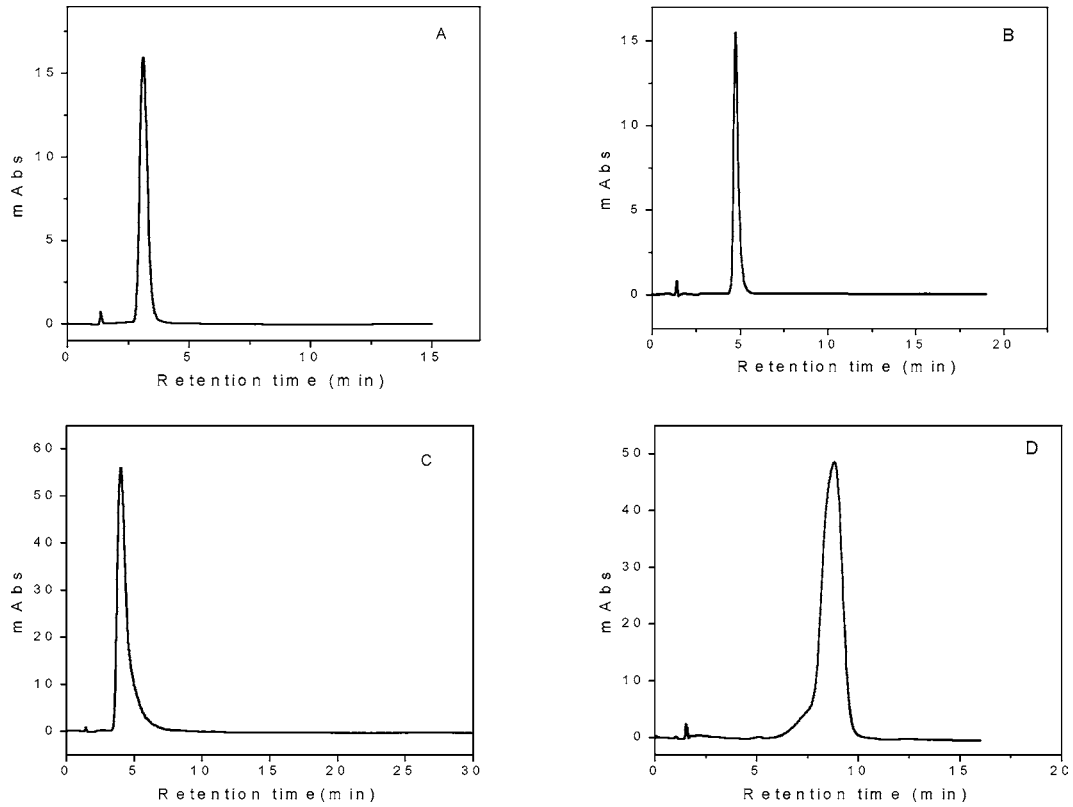

Figure 3. LC-UV chromatogram of cefoperazone in the C_{18}- (**A**), phenyl- (**B**), C_8- (**C**), and cyano-RAM-BSA (**D**) columns.

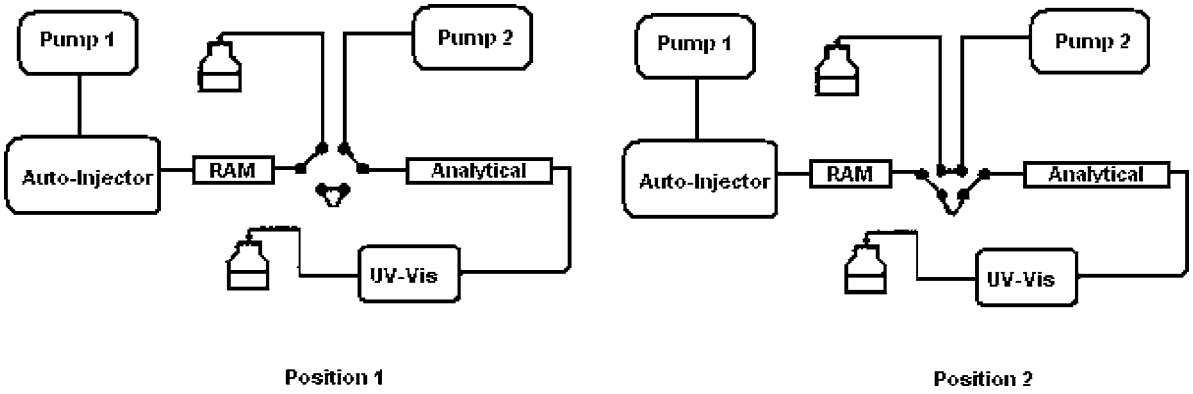

Figure 4. Schematic diagram of the column-switching system.

observed, probably due to the influence of temperature on the ionization of cefoperazone (*32*). To circumvent this problem, the addition of a counterion (TBA^+) in the sample preparation step (*9*) and the use of an oven to control the temperature of the RAM were investigated. The addition of a stronger cationic counterion allowed the formation of a cefoperazone−tetrabutylammonium ion pair, and a temperature of 30 °C provided good reproducibility between a series of consecutive injections.

The retention time of cefoperazone on the C_{18} column was adjusted as a function of pH and also by the percentage of the organic modifier. The best chromatographic condition was obtained with 0.01 M phosphate buffer, pH 5.0/acetonitrile (89:11 v/v).

The column-switching system used for the coupling of the RAM-phenyl-BSA with the C_{18} column is illustrated on **Figure 4**.

To achieve the necessary sensitivity, an injection volume of 100 μL was used. The samples were injected when the valve was in position 1. The sequence time used is listed in **Table 4**. The switching time was set from 9.8 to 13.0 min to transfer the cefoperazone from the RAM to the analytical column. To establish the transference time, the RAM column was initially connected to a UV−vis detector. The elution profile of cefoperazone from the RAM column and the time span used

Table 4. Time Events for the Switching of Columns and of Mobile Phases

time (min)	pump[a]	event	valve position
0.00–5.00	1 (eluent A) 2	milk proteins excluded by RAM column conditioning of the C_{18} column	1
5.01–9.79	1 (eluent B)	elution of cefoperazone from the RAM	1
9.80–13.00	1 (eluent B)	analytes transferred to the C_{18} column	2
13.01–27.00	2	analysis of the cefoperazone	1
13.01–20.00	1 (eluent C)	washing of RAM column	1
20.01–27.00	1 (eluent A)	conditioning of RAM column	1

[a] Pump 1: eluents (A) KH_2PO_4 0.01 M, pH 7.5/CH_3CN (98:2 v/v), (B) KH_2PO_4 0.01 M, pH 7.5/CH_3CN (87:13 v/v), (C) CH_3CN/H_2O/2-propanol (75:15:10 v/v/v); flow rate 1.0 mL/min. Pump 2: eluent KH_2PO_4 0.01 M, pH 5.0/CH_3CN (89:11 v/v); flow rate 1.0 mL/min; λ 270 nm.

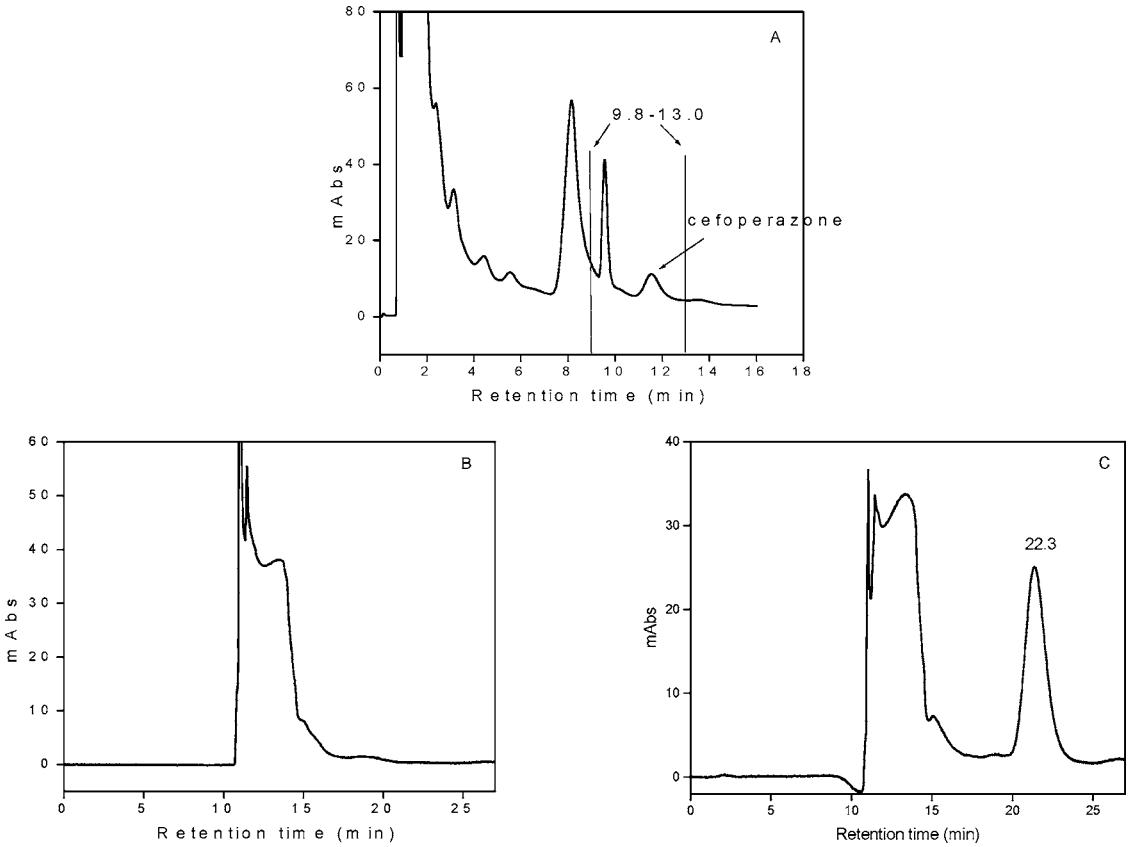

Figure 5. Representative LC-UV chromatograms of bovine milk spiked with cefoperazone at the phenyl-RAM-BSA column (**A**), a drugfree milk (**B**), and a bovine milk spiked with cefoperazone (2.5 μg/mL) (**C**). Conditions for (**B**) and (**C**) as in **Table 4**.

for transferring it to the analytical column are shown in **Figure 5A**. The phenyl-BSA column was cleaned with a mixture of acetonitrile/water/2-propanol (75:15:10 v/v/v).

Chromatograms of the direct injection of drug free milk (**Figure 5B**) and a spiked bovine milk sample (**Figure 5C**) show no interferences at the retention time of cefoperazone.

Method validation was carried out according to internationally accepted criteria (*33*) for all of the parameters evaluated.

The calibration curve was obtained with a good linear response at 270 nm from 0.100 to 2.50 μg/mL with a variation coefficient in the range of 0.67−5.14% for the triplicate analysis at each concentration level. The following regression equations and correlation coefficients were obtained: $y = 9.63956 \times 10^{-6}x + 0.08418$ ($r = 0.99843$).

The limits of quantification and detection were 0.100 μg/mL (CV = 1.44%; accuracy = 121%) and 0.050 μg/mL, respectively.

Inter- and intraday precision and accuracy were investigated using three quality control samples analyzed on three nonconsecutive days. The precision determined at each concentration level did not exceed 15% of the coefficient of variation (CV). The accuracy was evaluated by back-calculation and is expressed as the percent deviation between amount found and amount added at the three concentrations examined; all accuracy values were below the 15% variation accepted criterion. These results are shown in **Table 5**.

The extraction and transfer efficiencies (**Table 6**) were determined at the three quality control concentration levels. The results were calculated on the basis of comparison to a solution of cefoperazone in acetonitrile. High recoveries were obtained at the three concentrations examined.

The long-term storage, short-term storage (benchtop), freeze−thaw stability, and autosampler stability were evaluated.

Table 5. Intra- and Interday Accuracy (acc) and Variability (CV) for the Assay of Cefoperazone in Bovine Milk

	first day ($n = 5$)		second day ($n = 5$)		third day ($n = 5$)		pooled ($n = 3$)	
concn (μg/mL)	acc (%)	CV (%)	acc (%)	CV (%)	acc (%)	CV (%)	acc (%)	CV (%)
0.360	86.1	1.30	93.4	2.81	92.5	3.77	90.7	2.63
1.50	91.1	3.29	92.4	2.04	89.2	2.92	90.9	2.75
2.20	95.3	1.89	92.2	2.94	95.5	2.27	94.3	2.37

Table 6. Extraction Efficiencies of the Cefoperazone from Bovine Milk ($n = 5$)

concn added (μg/mL)	extraction efficiency (%)
0.360	99.8
1.50	100
2.20	97.9

Analytical standard solutions were stable for 1 week when stored at 4 or −20 °C. Milk samples spiked with cefoperazone were stable for 48 h when kept at 4 or −20 °C. Samples were stable when stored on the bench at room temperature for 4 h. Autosampler stability was also determined, and all samples were stable over the 24 h period evaluated.

The stability of cefoperazone in milk-spiked samples during three freeze−thaw cycles was evaluated, and the antibiotic was stable for two cycles.

The assay for evaluation of matrix stability showed that the matrix was stable for a 3 day period when stored at 4 or −20 °C. Different batches of milk were also evaluated, and no significant difference was observed between them.

Table 7. Accuracy (acc) and Variability (CV) Obtained in the Duplicate Analyses in the Assay of Cefoperazone in Whole Bovine Milk Type A Samples

sample	concn (μg/mL)	acc (%)	CV (%)
1	0.200	94.3	3.90
2	0.460	92.7	8.23
3	1.10	93.7	3.28
4	1.84	93.1	3.11
5	2.40	91.9	0.420
6	1.50	88.0	0.280
7	1.20	95.7	1.03
8	0.900	90.6	0.470
9	0.680	91.6	4.20
10	0.300	94.2	11.8

Development and validation of the method was performed using skimmed milk type A. This initially was done to avoid the high lipid content of whole milk when compared with skimmed milk. Once the method was validated in skim milk, it was also checked in whole milk.

Ten different samples of whole milk type A spiked with cefoperazone at concentrations unknown to the analyst were analyzed. The samples were prepared in duplicate in concentrations simulating a pharmacological profile. The analysis of these samples was carried out jointly with the analyses of quality control samples (prepared with skimmed milk). The accuracy and precision obtained met the acceptance criteria in all samples evaluated, showing that the method can also be applied to whole milk analysis. The results are described in **Table 7**.

The analysis of eight different types of whole milk (A, B, and C) was performed. The milks selected were trademarks sold at local bakeries in São Carlos, Brazil. Cefoperazone or other interfering compounds with the same retention time as the antibiotic were not found in any of these batches of whole milk.

During all analyses performed (~400 injections) no increase in backpressure was observed; just one RAM-phenyl and one C_{18} analytical column were used to complete the study.

Conclusions. The use of the four BSA-RAM columns described provides the necessary retention profile for the variety of hydrophilic cephalosporin antibiotics evaluated. Their usefulness for microsample deproteinization of bovine milk was illustrated by validating an automated HPLC method with direct injection of bovine milk for the quantification of cefoperazone. This method proved to be precise and accurate with excellent transfer efficiency and good recoveries. The sensitivity of the method is adequate for monitoring drug levels in bovine milk.

LITERATURE CITED

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