Hyphenated Liquid Chromatographic Method for the Determination of Colistin Residues in Bovine Tissues

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Abstract

A selective and sensitive high-performance liquid chromatographic (HPLC) method has been developed for the measurement of colistin residues in milk and in four bovine tissues (i.e., muscle, liver, kidney, and fat). The sample treatment consists of protein precipitation using 10% (w/v) trichloroacetic acid, solid-phase purification on C_{18} cartridges, and precolumn derivatization of colistin with *ortho*-phthalaldehyde and 2-mercaptoethanol in borate buffer (pH 10.5). This latter step is performed automatically, and the resulting reaction mixture is injected into a switching HPLC system including a precolumn and an analytical column packed with end-capped LiChrospher RP18 (5 μm). Washing the precolumn and final elution onto the analytical column are conducted using acetonitrile–0.01M phosphate buffer (pH 7.0) mixtures with respective proportions of 65:35 and 68:32 (v/v). Detection is carried out by spectrofluorometry (excitation wavelength, 340 nm; emission wavelength, 440 nm). The retention times of the derivatives corresponding to the two main components of colistin (i.e., polymyxins E2 and E1) are approximately 14 and 18 min, respectively. The structural study of the derivatives corresponding to polymyxins E1 and E2 is carried out by HPLC coupled with electrospray mass spectrometry; data obtained confirms that the derivatization process occurs with the five amino groups of the analytes. Selectivity is obtained in the HPLC system versus other coadministered anti-infective drugs (β-lactams, aminoglycosides, tetracyclines, and sulphonamides) and endogenous compounds. Quantitation is performed using the sum of the peak areas of polymyxin E1 and polymyxin E2 derivatives. Testing linearity affords correlation coefficients greater than 0.990 for calibration curves in the range of 10–500 μL/L for milk, 50–1000 μg/kg for muscle and fat, and 100–1000 μg/kg for kidney and liver. Relative standard deviation values are less than 10% at a concentration of 25 μg/L in milk and 100 μg/kg in tissues (six replicates); recoveries are higher than 60%.

Introduction

Colistin is a multicomponent antibiotic (polymyxins E) that is produced by strains of *Bacillus polymyxa* var. *colistinus*. It consists of a mixture of several closely related decapeptides with a general structure composed of a cyclic heptapeptide moiety and a side-chain acetylated at the *N*-terminus by a fatty acid (Figure 1). Up to 13 different components have been identified (1) that differ from each other by the composition of their amino acids and fatty acid. The two main components of colistin are polymyxins E_1 and E_2; they include the same amino acids but a different fatty acid (6-methyloctanoic acid and 6-methylheptanoic acid, respectively). Colistin exhibits the hydrophobicity of the fatty acid moiety and basic properties (pK_a value is approximately 10) due to the five unmasked γ-amino groups of the 2,4-diaminobutyric acid residues. Colistin, either as sodium methanesulfonate or as sulphate salts, is used in human and veterinary therapies against Gram-negative bacteria.

Previously reported analytical methods devoted to colistin measurement are microbiological (2,3), immunological, (4) and separative (thin-layer chromatography [5], isotachophoresis [6], capillary electrophoresis [7], and highperformance liquid chromatography [HPLC] [1,8,9]). Chromatographic methods are mainly applied to the separation of colistin components in raw materials and to batch-to-batch variation studies in relationship with its potency; all of them include ultraviolet (UV) spectrophotometric detection at a low wavelength, which precludes their use in complex matrices such as tissue samples because of a lack of selectivity and sensitivity. To our knowledge, there has not yet been any report of a separative technique applied to biological samples.

Two nonseparative methods have already been described for the colistin assay in animal tissues: a microbiological method using *Bordetella bronchisepta* as a germ test and applied to calf tissues (limit of quantitation [LOQ], 100–150 μg/kg) (3) and an

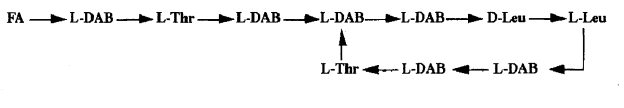

Figure 1. Structure of the main components of colistin (polymyxins E_1 and E_2). FA = fatty acid (6-methyloctanoic acid for polymyxin E_1 and 6-methylheptanoic acid for polymyxin E_2). L-DAB = L-2,4-diaminobutyric acid.

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enzyme immunofluorometric assay in rainbow trout tissues (LOQ, 30 µg/kg) (4).

In the same manner as for the other antibiotics (10–12), suitable analytical methods for the measurement of colistin in edible animal tissues have to be developed in order to comply with regulatory requirements, especially maximum residue limits (MRLs). MRLs established in the European community for colistin in mammals are 50 µg/L in milk; 150 µg/kg in muscle, liver, and fat; and 200 µg/kg in kidney.

The present work is focused on the development of an HPLC system devoted to the measurement of colistin in milk and in four different bovine tissues (i.e., muscle, liver, kidney, and fat) with an LOQ of 10 µg/L in milk, within the range of 50–100 µg/kg in tissues, and with full selectivity with regard to endogenous compounds.

Experimental

Chemicals, reagents, and standards

All chemicals and solvents were of analytical-reagent or HPLC grade and were used without further purification. HPLC-grade water was obtained using a Milli-Q system (Millipore, Saint-Quentin-en-Yvelines, France). *ortho*-Phthalaldehyde (OPA) was purchased from Fluka (Buchs, Switzerland), triethylamine (HPLC grade; purity greater than 99.5%) was from Prolabo (Fontenay/Bois, France), and 2-mercaptoethanol (Merc) was from Merck (Darmstadt, Germany). Bond Elut C_{18} (100 mg) cartridges were obtained from Varian (Les Ulis, France). The different colistin sulphate batches and all other anti-infective substances were obtained from Virbac SA Laboratories (Carros, France).

Stock solutions of colistin were prepared at a concentration of 1 g/L in 0.01M hydrochloric acid and stored at 5°C for up to one month. Further dilutions used for calibration curves were realized in 0.01M hydrochloric acid in the following concentration ranges: 0.1–5.0 mg/L to spike milk and 0.5–10.0 mg/L to spike tissues.

The derivatization reagent was prepared as follows: OPA (80 mg) was dissolved in 15 mL of methanol, and 15 mL of 0.2M borate buffer (pH 10.5) and 0.1 mL of Merc were added. The resulting mixture was homogenized and stored in an amber bottle at room temperature for 12 h before use.

Raw material analysis

The HPLC system consisted of a solvent delivery pump (model P2000, Thermo Separation Products, Les Ulis, France), an injection valve (model 7725i, Rheodyne, Cotati, CA) fitted with a 20-µL loop, an analytical column (125 × 4-mm i.d.) prepacked with 5-µm Nucleosil C_{18} (Macherey-Nagel, Düren, Germany), a column oven (model Croco Cil, Thermo Separation Products) and a spectrophotometric detector (model UV 2000, Thermo Separation Products). All data collection and calculations were performed using an integrator connected to a data station (Chromjet model with "Winner on Windows" software, Thermo Separation Products).

The mobile phase consisted of acetonitrile and a 0.035M triethylamine solution adjusted to pH 2.5 with phosphoric acid and mixed in 17:83 (v/v) proportions. The flow rate was 1.5 mL/min, the column temperature was 35°C, and UV detection was operated at 210 nm. The colistin solutions were prepared at a concentration of 0.5 g/L in HPLC-grade water and directly injected into the HPLC system.

HPLC system coupled with electrospray mass spectrometry

The HPLC system consisted of a solvent delivery pump (model 140A, Applied Biosystems, Foster City, CA), an injection valve (model 7725i, Rheodyne) fitted with a 20-µL loop, and an analytical column (125 × 2-mm i.d.) prepacked with 5-µm Nucleosil C_{18} (Macherey-Nagel). A postcolumn tee with a low dead volume was used as a 1:15 splitter to reduce the flow rate of the eluent entering the electrospray interface of the mass spectrometer (MS) (model VG Bio Q, Fisons Instruments, Arcueil, France). The ionization mode used electrospray in a positive mode (cone energy, 50 V), and data was collected and reprocessed by means of Mass Lynx software (Micromass, Altrincham, UK). Gradient elution was run over 20 min from mobile phase A (acetonitrile–water–trifluoroacetic acid, 60:39.9:0.1, v/v/v) to mobile phase B (acetonitrile–water–trifluoroacetic acid, 80:19.9:0.1, v/v/v) at a flow rate of 0.25 mL/min.

A 1-mL amount of a colistin solution prepared at a concentration of 0.02 g/L in methanol–0.01M hydrochloric acid (50:50, v/v) was mixed with 0.2 mL of the OPA–Merc reagent. After homogenization and a reaction time of approximately 1 min, the mixture was injected into the HPLC system.

Biological sample treatment

Milk and tissue samples were stored at –20°C until analysis. They were left to thaw at room temperature and treated as follows. Milk samples (10 mL) were transferred into 40-mL Teflon screw-capped tubes and fortified with 1 mL of the appropriate spiking solution: 2 mL of a 10% (w/v) trichloroacetic acid (TCA) solution was added. After homogenization and centrifugation at 10,000 *g* for 10 min at 5°C, the supernatant was transferred into a 20-mL volumetric flask; 0.2 mL of 1M sodium hydroxide was added, and the volume was made up with methanol–0.01M hydrochloric acid (50:50, v/v).

Tissue samples were minced and 5.0 g ± 0.1 were weighed in 50-mL Virtis glass vials, then fortified with 0.5 mL of the appropriate spiking solution. After 30 min at room temperature, 20 mL of methanol–10% TCA (50:50, v/v) was added, and the mixture was blended for 10 min using a mixer (model Virtis 45, Gardiner, New York, NY) fitted with U-shaped blades. The blended tissues were transferred into a 40-mL Teflon tube and centrifuged at 10,000 *g* for 10 min at 5°C. The supernatant was transferred into a 25-mL volumetric flask, 0.3 mL of 1M sodium hydroxide was added, and the volume was made up with methanol–0.01M hydrochloric acid (50:50, v/v).

A 2.0-mL fraction of the solutions resulting from the precipitation of proteins from milk or tissue samples was passed through a Bond Elut C_{18} cartridge previously activated with 2 mL of methanol and 2 mL of methanol–0.01M hydrochloric acid (50:50, v/v); these steps were performed using a vacuum manifold (Alltech, Templeuve, France) without allowing the

cartridge to run dry. The final elution was performed with 1 mL of methanol–0.01M hydrochloric acid (55:45, v/v), and the eluate was collected directly into an autosampler vial until the cartridge ran dry. The derivatization step was automatically processed using an OPA–Merc reagent–sample mixing ratio of 1 and a reaction time of 1 min before injecting a 200-µL aliquot of the resulting mixture into the HPLC system.

Switching HPLC system and operating conditions

The switching HPLC system and the successive operating steps occurring during the assay are detailed in Figure 2. The switching HPLC system consisted of two solvent delivery pumps (pump A: Eldex A 60-S-2 model, Prolabo; pump B: Spectroflow 400 model, Applied Biosystems) and an autosampler equipped with a reagent addition cassette for automatic derivatization, a 200-µL sample loop, a time-programmable switching valve, and a column oven (model 507, Beckman, San Ramon, CA). Detection was carried out with a fluorescence detector (model Jasco FP-920, Prolabo) equipped with a 150-W xenon short-arc lamp. All data collection and calculation was performed using an integrator connected to a data station (Chromjet model with "Winner on Windows" software, Thermo Separation Products).

The precolumn (30 × 4-mm i.d.), guard column (4 × 4-mm i.d.), and analytical column (125 × 4-mm i.d.) used in-line were packed with end-capped, 5-µm LiChrospher 100 RP-18 (Merck-Clevenot) and kept at 35°C. Fluorescence detection was carried out at an excitation wavelength of 340 nm and an emission wavelength of 440 nm. The selected gain was 1000, and the attenuation was set to 8.

The mobile phase for the washing step of the precolumn was a mixture of acetonitrile–0.01M phosphate buffer (pH 7.0) (65:35, v/v) used at a flow rate of 1.0 mL/min, whereas the mobile phase for the elution step onto the analytical column was in the proportion 68:32 (v/v) at a flow rate of 1.5 mL/min. The duration of the washing and elution steps were 4 and 2 min respectively, and the precolumn was re-equilibrated with the washing mobile phase for 20 min before another run was performed. The precolumn was changed after every 30 injections.

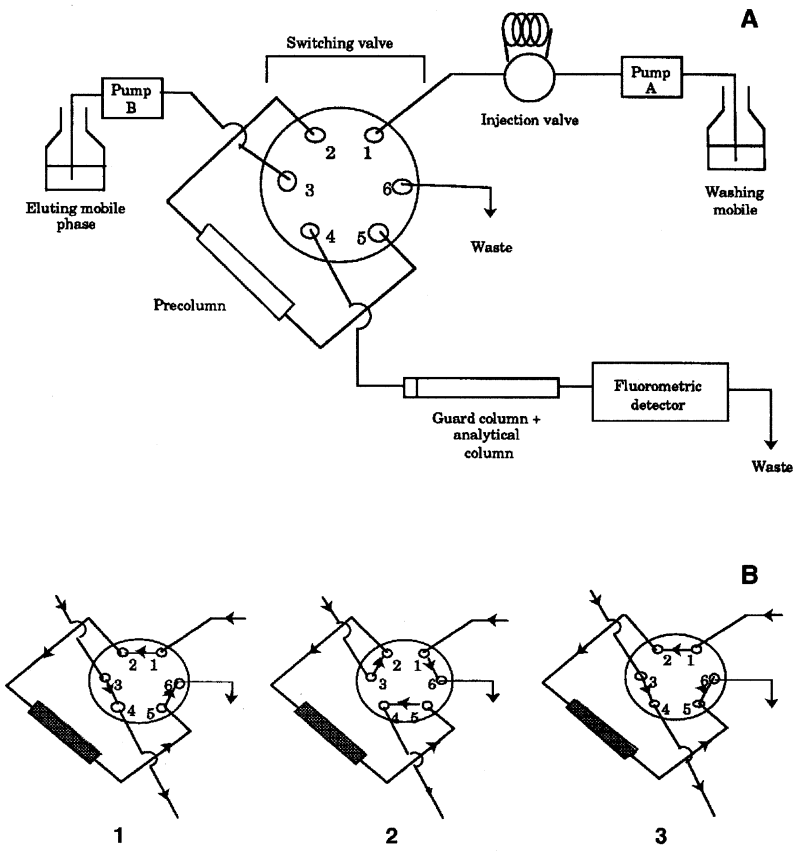

Figure 2. Scheme of the HPLC switching system (A) and the successive steps for the HPLC assay of colistin using precolumn derivatization and a foreflush switching process (B). Step 1: Injection of the reaction mixture (100 µL OPA–Merc reagent and 100 µL sample; 1 min) onto the precolumn and washing step: CH_3CN–0.01M phosphate buffer at pH 7.0 (65:35, v/v); duration, 4 min; flow rate, 1 mL/min. Step 2: Elution onto the analytical column with CH_3CN–0.01M phosphate buffer (68:32, v/v); duration, 2 min; flow rate, 1.5 mL/min. Step 3: Re-equilibration of the precolumn with the washing mobile phase.

Results and Discussion

Selection of colistin markers

One of the main problems that arises during the development of separative techniques devoted to the assay of multicomponent antibiotics in biological matrices is the difficulty to select one or more representative components as markers for quantitation. As a matter of fact, the assay must be efficient for the measurement of the antibiotic without taking the manufacturing source or production batch of the standard used into consideration.

Moreover, the required LOQ is generally more difficult to reach when the number of components considered in the assay increases. Selecting chromatographic conditions in which most antibiotic components elute as a single peak, as already reported for gentamicin (13), seems to be a convenient approach. However, such a system may be difficult to optimize in some cases because of the important differences between the physicochemical properties of the components.

In the present work, the choice of markers for the colistin assay was first examined. In order to assess the opportunity to measure colistin concentrations in biological matrices on the basis of its two main components (i.e., polymyxins E_1 and E_2), we have studied the variations in their proportion in several batches obtained from different manufacturers.

The isocratic reversed-phase HPLC system developed for these experiments used an

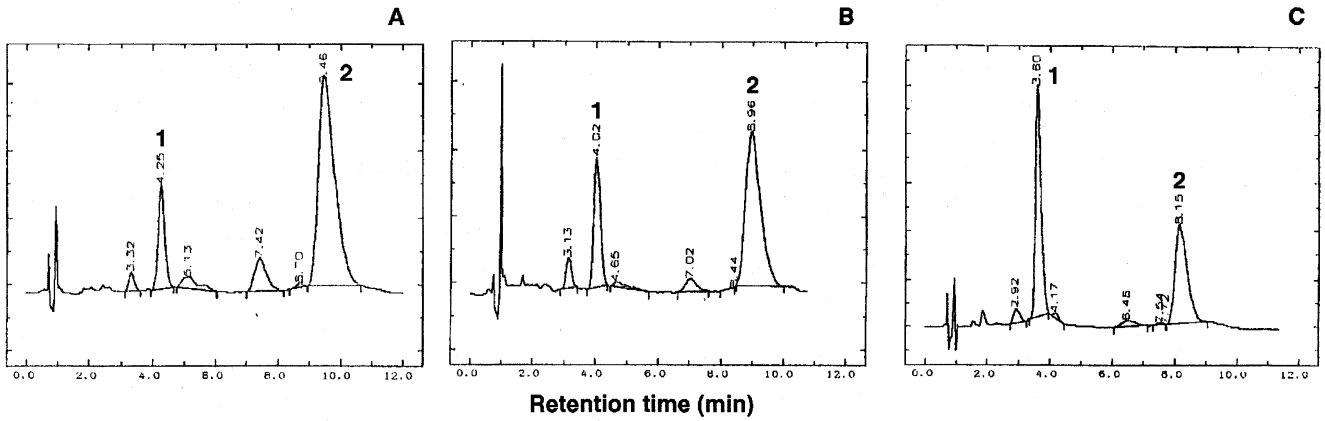

Figure 3. Typical chromatograms corresponding to colistin raw materials from different manufacturers obtained in the HPLC system with UV detection. HPLC operating conditions: Nucleosil C_{18} (5 µm) 125 × 4-mm i.d. eluted with acetonitrile–0.035M triethylamine phosphate at pH 2.5 (17:83, v/v) at a flow rate of 1.5 mL/min and a column temperature of 45°C; UV detection, 220 nm. The concentration of the colistin standard solutions was 0.5 g/L. A, manufacturer 1; B, manufacturer 2; C, manufacturer 3. Peaks: 1, polymyxin E_2; 2, polymyxin E_1.

Table I. Colistin Batch-to-Batch Variations from Different Manufacturers Assayed by HPLC with UV Detection

Manufacturer	Number of batches tested	Proportion (mean % ± SD)* Polymyxin E_2	Polymyxin E_1	Polymyxins $E_1 + E_2$
1	7	15.6 ± 1.1	70.6 ± 0.5	86.2 ± 1.3
2	3	24.4 ± 1.4	64.5 ± 1.7	88.9 ± 0.3
3	1	47.8	43.9	91.7

* Calculated by internal normalization based on the area of the six main peaks.

acidic mobile phase containing triethylamine as a competing agent in order to suppress peak tailing and direct UV detection at 210 nm. Typical chromatograms are shown in Figure 3. The relative amounts of polymyxins E_1 and E_2 and the sum of these two components in the different batches tested were calculated on the basis of a normalization method by taking into account the six main peaks. Values are indicated in Table I. Important variations in colistin composition appear to result from a colistin origin. Quite similar proportions of polymyxins E_1 and E_2 have been found for different batches from the same manufacturer. Moreover, these two components always represent more than 85% of the total peak area. The HPLC assay in biological matrices should therefore be developed in order to carry out quantitation based on the sum of the peak areas of polymyxins E_2 and E_1 or on their corresponding derivatives.

Derivatization of colistin

Because colistin exhibits poor UV absorbance and no native fluorescence properties, derivatization reactions have to be envisaged. Such an approach has previously demonstrated its efficiency for the measurement of antibiotic residues in edible tissues (12,13). The most usual fluorogenic reagent to label primary amino groups is OPA; fluorescamine and naphthalene 2,3-dicarboxaldehyde (NDA) have also been used for this purpose. OPA reacts with an aliphatic primary amino group in the presence of a nucleophilic substance such as a thiol to give the corresponding 1-alkylthio-2-alkyl-substituted isoindole adduct, which exhibits high molar absorbance, fluorescence, and electrochemical properties (13–15). This reaction has been widely used for the derivatization of amino acids (14) and amino drugs (13,15). However, when this reaction is applied to the derivatization of amino β-lactams (16) or peptides (17,18), it seems to be far less efficient, especially with regard to the fluorescence yield; the most frequently reported reason is a quenching process due to a carbonyl group in a position close to the amino group involved in the reaction. Fluorescamine (17,19) and NDA in the presence of cyanide (18) can be alternative reagents for these analytes because their derivatives exhibit fluorescence properties less sensitive to the environmental structure close to the fluorophore. In the present work, OPA reaction with colistin was tested, and such a problem was not observed because the primary amino (γ-NH_2) groups reacting with OPA and Merc were located at such a distance from the carbonyl groups of peptidic bonds that no interference occurred during the fluorescence process.

Derivatization of colistin with OPA–Merc was first conducted in a postcolumn mode using the HPLC system previously developed to study the antibiotic composition. A high fluorescence level was observed in the eluent, probably due to the fact that triethylamine contains impurities that react with OPA and Merc. No other competing base was tested, and a precolumn derivatization approach was further investigated.

The derivatization reaction was first optimized by studying the methanol proportion needed in the reaction mixture in the range 0–50% (v/v); at least 20% was necessary to obtain reproducible results. The formation rate and the stability of the derivatives were then examined (Figure 4). The optimal delay period between addition of the reagent and injection time in order to obtain a maximal signal was in a short range, between 1 and 2 min.

The reaction automation using a dedicated autosampler was then studied. The apparatus presently available could only operate derivatization in a reagent–sample solution ratio of 1, and a loop volume of 200 µL was selected. Repeatability of the reaction was tested with colistin standard solutions at a con-

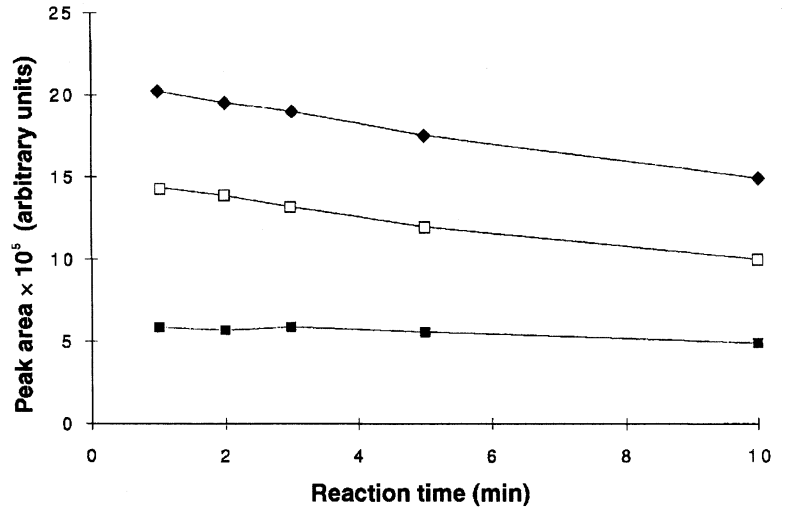

Figure 4. Time dependence for the formation rate and stability of the derivatives obtained from the reaction of colistin with the OPA–Merc reagent. The concentration of the colistin standard solution was 100 µg/L. □, area of polymyxin E_1 adduct; ■, area of polymyxin E_2 adduct; ◆, area of polymyxin E_1 + polymyxin E_2 adducts.

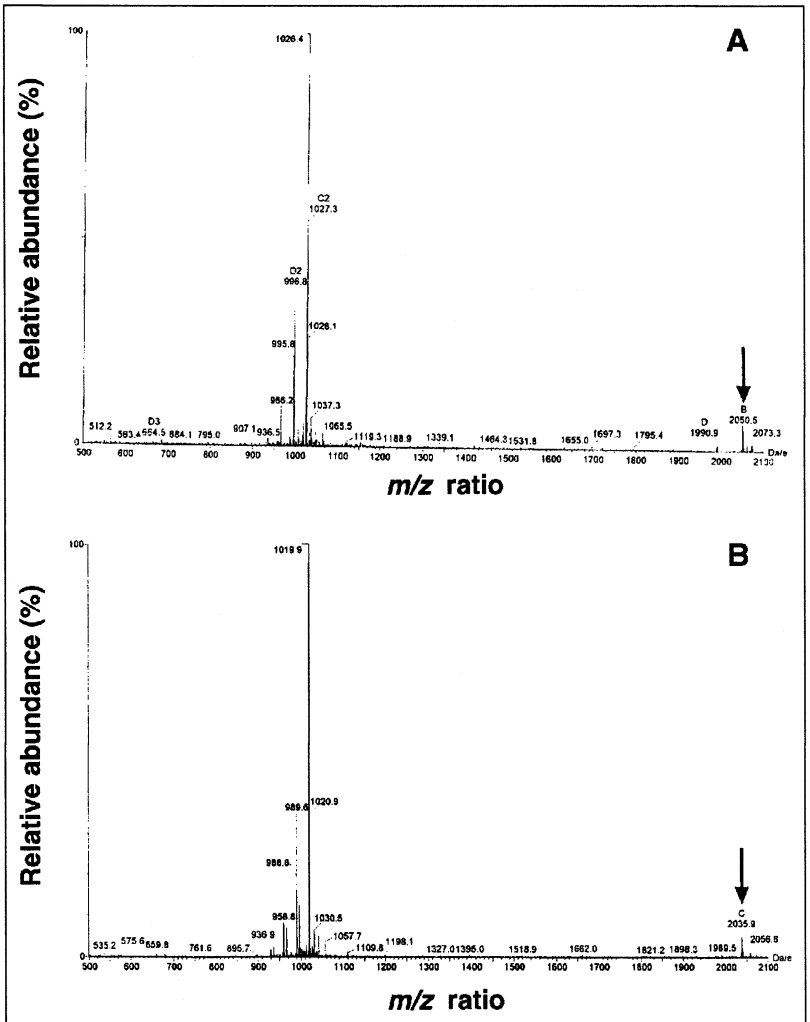

Figure 5. Mass spectra of the derivatives of the main components of colistin obtained by HPLC–electrospray MS. Arrows indicate the protonated molecular ion $(M+H)^+$ of polymyxin E_1 (A) and polymyxin E_2 (B) derivatives.

centration of 100 µg/L with a reaction time of 1 min; the resulting relative standard deviation (RSD) values were less than 3% (five replicates).

Elution profiles of colistin derivatives in a reversed-phase HPLC system coupled with spectrofluorometric detection exhibited two main peaks. A relationship between these derivatives and polymyxins E_1 and E_2 was established; peak fractions corresponding to each native polymyxin separated in the reversed-phase HPLC system coupled with UV spectrophotometric detection were collected and submitted to derivatization and HPLC analysis with spectrofluorometric detection. Only one peak was observed for each fraction, and the same elution order was obtained for native polymyxins E_1 and E_2 and for their corresponding derivatives in the two reversed-phase HPLC systems: polymyxin E_2, because of its less hydrophobic fatty acid moiety, eluted before polymyxin E_1.

Because this derivatization approach looks convenient (an LOQ of 10 µg/L could be reached using solutions of colistin standards from different manufacturers; the signal-to-noise ratio was 11, and the RSD was 5.3% with five replicates), the assay development was further continued by successively studying the structure of the obtained derivatives and the biological sample treatment.

Structural study of colistin derivatives by HPLC–MS

In order to assess that the derivatization reaction with OPA and Merc occurs with the five unmasked amino groups of the colistin components considered in the present work (i.e., polymyxins E_1 and E_2), their corresponding derivatives were analyzed using HPLC–MS. Different approaches have already been reported for the MS of the *N*-substituted 1-alkyl-isoindole derivatives. Macroscale synthesis of drug derivatives was followed by direct introduction into the MS proceeding with chemical ionization (15). Gas chromatography–MS with electron-impact detection was used for the structural study of *n*-aliphatic amine derivatives, but the silylation of the polar hydroxyl group of Merc was required before the chromatographic step (20). More recently, HPLC–MS with thermospray ionization has been reported for the diastereoisomeric isoindole derivatives of amino acids (21). However, this ionization mode is used mainly in the case of compounds with low molecular weights (MWs). High MWs (greater than 2,000) and instability of the derivatives presently examined require the use of HPLC–MS with electrospray as the ionization mode.

Chromatographic conditions were developed

for this purpose, especially in order to facilitate the ionization process. Mobile phases containing acetonitrile, water, and a volatile component (i.e., trifluoroacetic acid) instead of phosphate buffer and a gradient elution were used. Two well-resolved peaks at retention times of 9.7 and 10.9 min, corresponding to polymyxin E_2 and E_1 derivatives, respectively, were observed. The mass spectra (Figure 5) indicate the presence of the protonated molecular ion $(M+H)^+$ at respective *m/z* values of 2050.5 and 2035.9 for the derivatives of polymyxins E_1 and E_2; the calculated MWs correspond to the expected exact mass of these adducts: 2048.9 and 2034.9, respectively. These MWs were similar to the values deduced from the base peaks (*m/z* 1026.4 and 1019.9) corresponding to $(M+2H)^{2+}$ with relative errors of 0.09 and 0.14%, respectively. It was therefore clearly demonstrated that the five amino groups of the main colistin components were converted into isoindole groups when reacting with OPA and Merc.

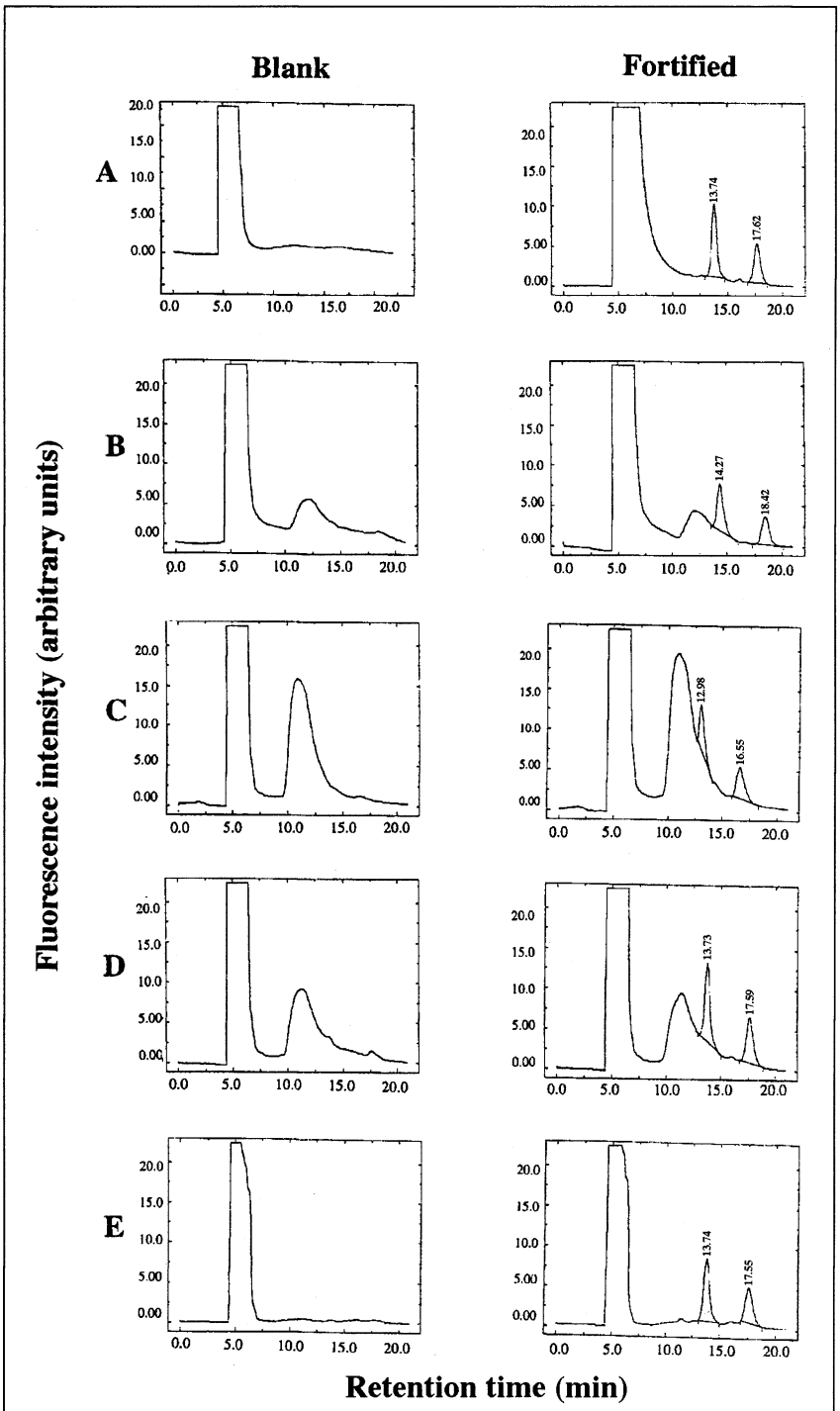

Figure 6. Typical chromatograms corresponding to milk, muscle, kidney, and fat samples. A, milk; B, muscle; C, liver; D, kidney; E, fat. Samples were fortified with 25 µg/L of colistin for milk and 100 µg/kg for tissues. HPLC operating conditions were the same as in Figure 2.

Purification of biological samples

Purification of samples before HPLC analysis of peptide-related drugs is usually realized by multistep procedures combining protein precipitation with different solid-phase extraction techniques. Although immunoaffinity appears to be a promising way of purifying pharmacological active peptides (18,22), antibodies are often not easily available, and their linkage to a solid matrix is often a homemade operation. All these operations are time-consuming and may suffer from a lack of reproducibility. Solid-phase extraction using ion exchange (17) or a C_{18} cartridge (23) is therefore still the most frequently used approach.

The present methodology for the treatment of milk and solid tissues was started by a homogenization step in TCA that provided protein precipitation and breakage of drug-protein bonds (the reported binding proportion of this drug to bovine plasma proteins is approximately 55% [24]); no additional operation was needed to eliminate lipid compounds in milk fat; addition of methanol to TCA solution was necessary in the case of solid tissues in order to improve recoveries of colistin.

The selection of further purification steps in order to obtain higher selectivity for colistin quantitation was carried out by combining different "off-line" and "on-line" extraction processes. Initial experiments concerning solid-phase purification were realized "off-line" using a cationic exchange technique previously reported for aminoglycosidic antibiotics (13). It afforded a low selectivity level and poor recoveries (approximately 25%).

The use of C_{18} cartridges gave better recoveries, but the resulting chromatographic profiles corresponding to milk and tissue extracts showed a large peak of endogenous compounds that partially co-eluted with the polymyxin E_2 derivative. An additionnal solid-phase extraction process was therefore preferentially developed "on-line" in order not to increase the time of sample handling;

Table II. Validation Parameters for Colistin Assay in Spiked Bovine Tissues by HPLC with Spectrofluorometric Detection

Matrix	RSD (%)* (six replicates)	Concentration range (µg/L or µg/kg)	Regression line equation†	Correlation coefficient	Recoveries‡ (%) ± SD (six replicates)
Milk	3.2	10–250	$y = 24{,}486{,}671x - 377{,}453$	0.995	61.7 ± 2.0
Muscle	3.4	50–1000	$y = 9{,}468{,}820x - 22{,}317$	0.996	78.2 ± 2.7
Liver	6.5	100–1000	$y = 9{,}647{,}994x - 14{,}200$	0.992	62.1 ± 4.0
Kidney	9.5	100–1000	$y = 8{,}288{,}987x + 379{,}498$	0.996	71.9 ± 6.8
Fat	8.4	50–1000	$y = 7{,}557{,}648x - 136{,}780$	0.991	71.1 ± 6.0

* Calculated for milk and tissue samples fortified at 25 µg/L and 100 µg/kg, respectively. RSD values recommended for concentrations in the range of 10–100 µg/kg < 20% (10).
† Where x is the colistin concentration and y is the sum of peak areas (arbitrary units) corresponding to polymyxin E_1 and E_2 derivatives.
‡ Calculated for milk and tissue samples fortified at 25 µg/L and 100 µg/kg, respectively.

it consisted of a switching system operating in a fore-flush mode. The following parameters were examined for the switching optimization.

Packing material

The highly hydrophobic styrene–divinylbenzene copolymer did not afford better resolution between endogenous compounds and polymyxin E_2 derivatives than a C_{18} silica with a high carbon content (22%; LiChrospher RP18 end-capped); this latter was therefore retained.

Precolumn length

A length of 30 rather than 10 mm was preferred in order to obtain a reproducible selectivity over a large number of sample injections. At least 30 assays could be performed without alteration of the performances. The precolumn was changed when the number of theoretical plates for each peak fell below 12,000 plates/m.

Washing and eluting mobile phases

A slight decrease in the acetonitrile content (3%) of the washing mobile phase versus the eluting mobile phase was necessary to obtain the required selectivity degree, which also provided fast re-equilibration before the next injection.

Washing step duration

The duration of the washing step (4 min) was optimized in order to avoid loss of colistin; an elution time of 2 min was enough to provide full recovery of colistin derivatives from this precolumn.

The two solid-phase extraction steps rely on the same principle (i.e., reversed-phase partition on C_{18} silica). However, colistin is involved in the two successive purification steps under different forms. In the first, colistin is in its native form, which is much more polar than in the second step, in which colistin is derivatized by five hydrophobic structures. This fact can explain why the combination of these two operations affords a high degree of selectivity for colistin components in the biological matrices. Moreover, no late-eluting peak was observed in the reported operating conditions for milk nor the tissue samples, which allow a rate of sample injections of three per hour.

Assay validation

The developed HPLC assay was selective against other co-administered anti-infective substances: β-lactams (amoxicillin, ampicillin, cephalexin, and cloxa-cillin), aminoglycosides (kanamycin and neomycin), tetracyclines (oxytetracycline), sulphonamides (sulphamerazine, sulphamidine, sulphame-thoxypyridazine, and sulphadime-thoxine), and trimethoprim. Selectivity was obtained with regard to endogenous compounds of the different bovine tissues studied. Figure 6 shows typical chromatograms obtained for milk, muscle, kidney, liver, and fat sample blanks and fortified with colistin.

Linearity of the method was tested for concentrations of colistin ranging from 10 to 500 µg/L in milk, 50 to 1000 µg/kg in muscle and fat, and 100 to 1000 µg/kg in kidney and liver. The main validation parameters are reported in Table II. The higher sensitivity observed for milk could be explained by a lower sample diluting factor than for solid tissues (1:2 versus 1:5).

Conclusion

To our knowledge, the described method is the first report of an HPLC assay of colistin in biological matrices, and it appears to be valuable for monitoring residues of this drug in edible tissues with regard to MRLs. Selectivity was obtained for the two main colistin components selected as markers in the different tested matrices, and the assay could be performed regardless of the origin of the colistin used as standard. Validation parameters fulfilled the requirements for drug residues in edible tissues (10). The method could be adapted to other biological matrices and to other cyclic polypeptide antibiotics such as polymyxins B, provided that minor changes in sample treatment and mobile phase composition are introduced.

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Manuscript accepted July 17, 1997.