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Determination of ramoplanin in human urine by high-performance liquid chromatography with automated column switching

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Abstract

Ramoplanin is a novel glycolipodepsipeptide antibiotic, currently undergoing clinical trials. This method describes the determination of ramoplanin by direct injection of human urine into a coupled-column liquid chromatographic system. An internal-surface reversed-phase column has been used for on-line sample clean-up and enrichment. Analytical separation of ramoplanin and MDL 62 456 used as internal standard, has been achieved on a ABZ+ reversed-phase column with ammonium acetate buffer–acetonitrile–methanol according to a gradient profile. Analytes were detected by their UV absorbance at 270 nm. The limit of quantitation was 0.1 μg/ml urine and the limit of detection was found to be 0.035 μg/ml, corresponding to 13.7 pmol/ml. Linearity was determined in the range 0.1–2 μg/ml. Precision (relative standard deviation) ranged from 0.71 to 8.75% and the accuracy from −9.9 to 11.6%. Different human sources were tested and no interference between analytes and urine constituents was observed. The method is simple and rapid, requiring a total analysis time of 35 min per sample and reaching greater selectivity and accuracy than microbiological assays.

Keywords: Column switching; Ramoplanin; Antibiotics

1. Introduction

Ramoplanin (Fig. 1) is a novel glycolipodepsipeptide antibiotic obtained from the fermentation of *Actinoplanes* strain ATCC 33076 [1–3]. Ramoplanin is active against Gram-positive aerobic and anaerobic microorganisms including coagulase-negative staphylococci with in vitro and in vivo antibacterial activity comparable to reference antibiotics and with greater bactericidal activity than reference antibiotics [2–7]. Ramoplanin inhibits bacterial cell wall synthesis by a mechanism different from vancomycin and teicoplanin or other cell wall synthesis inhibitors [8,9]. A profile of the activity of ramoplanin indicates that it should be clinically useful for topical treatment of the gastrointestinal tract via oral administration. The main clinical indications for oral use, which are currently under investigation, are antibiotic-associated diarrhea, colitis or pseudomembranous colitis caused by *Clostridium difficile,* and selective decontamination of the digestive tract [10]. Ramoplanin drug substance consists of a major component (factor A_2) (Fig. 1) and several strictly related minor components [3]. Although no absorption of ramoplanin from the gastrointestinal tract is expected [4], monitoring of plasma and urine ramoplanin concentrations is necessary. In this paper, the concentration of ramoplanin major component A_2 is determined by direct injection of urine into a coupled-column liquid chromatographic system consisting of a reversed-phase column, as analytical column, and a Pinkerton internal-surface reversed-phase

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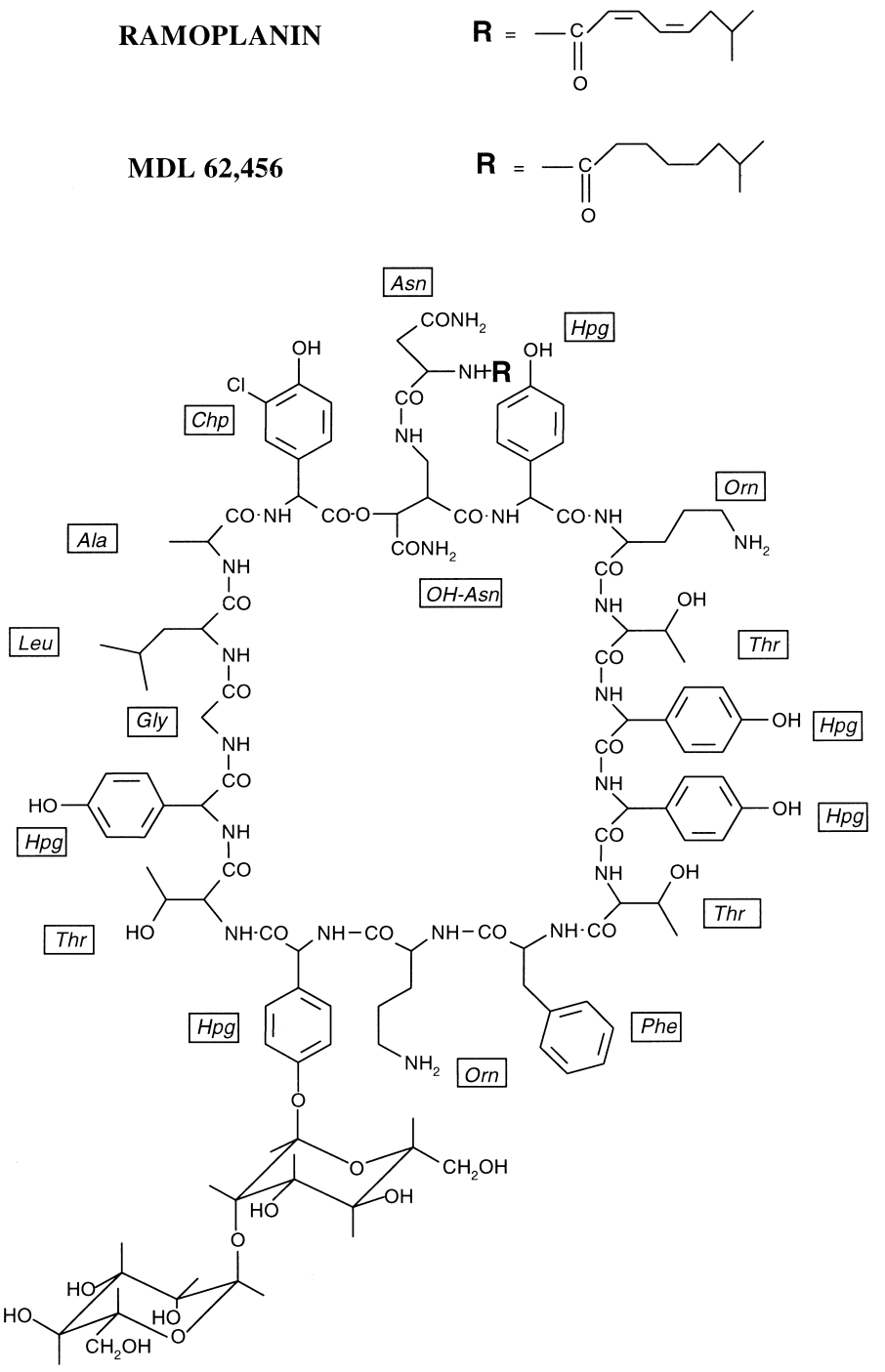

Fig. 1. Structures of ramoplanin factor A_2 and the internal standard.

(ISRP) guard column [11], as sample clean-up and enrichment column.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol, both HPLC grade, were purchased from Carlo Erba (Rodano, Italy), 2-propanol HPLC grade, was from Merck (Darmstadt, Germany) and ammonium acetate, analytical grade, from Fluka (Buchs, Switzerland). Eighteen MΩ water was from Milli-Q System (Millipore, Saint Quentin, France). Acetic acid was from Carlo Erba.

2.2. Equipment

The pH was measured using a PHM 93 reference pH meter (Radiometer, Copenhagen). The liquid chromatographic system was from Gilson (Villiers-le-Bel, France) and consisted of two 305/10WSC pumps equipped with a 805 manometric module and a 811 dynamic mixer, and connected to a 232 autoinjector containing two six-port Rheodyne switching valves and operating with 401 dilutor fitted with a 1 ml syringe; the UV detector was a 116 model (Gilson). Two additional Shimadzu LCAS10 pumps (Shimadzu, Europe) were linked to the autoinjector for sample enrichment and switching column washing. Control of the HPLC system, integration of chromatographic peaks and communication with the complete 232 system was achieved, via a Gilson GSIOC 506/C interface module, using Unipoint system software version 1.65/win32S (Gilson) located in a Vectra VE PC (Hewlett-Packard, Grenoble, France).

A Supelcosil LC-ABZ+ (250 mm×4.6 mm I.D., 5 μm) (Supelco, Bellefonte, PA, USA) was the analytical column, while a Pinkerton ISRP GFF II guard cartridge (10 mm×3 mm I.D.) (Regis, Morton Grove, IL, USA) was the switching column.

2.3. Preparation of calibration standards

Individual stock solutions of ramoplanin dihydrochloride and MDL 62 456, internal standard compound (Fig. 1), respectively 100 μg/ml and 216 μg/ml, were prepared in water. For ramoplanin, working standards containing 10 μg/ml, 20 μg/ml, 50 μg/ml, 100 μg/ml were prepared in water. Further dilutions of the working standards were prepared at ramoplanin concentrations of 5 μg/ml, 7.5 μg/ml, 37.5 μg/ml, 75 μg/ml and 90 μg/ml. Five calibration standard concentrations (0.1 μg/ml, 0.2 μg/ml, 1 μg/ml, 1.5 μg/ml, 2 μg/ml) were prepared by spiking blank urine with the working standards adding a constant volume of 20 μl/ml urine. Quality controls were prepared in the same way in order to obtain concentrations of ramoplanin in urine of 0.15 μg/ml, 0.75 μg/ml and 1.8 μg/ml. Thirty μl of the internal standard solution were added to each calibration and quality control sample, corresponding to 6.48 μg internal standard/ml urine.

2.4. Analytical conditions

Two hundred μl of urine samples prepared as previously described were directly injected into the coupled-column LC system. Prior to each analysis, the ISRP column was conditioned with ammonium acetate buffer 25 mM, adjusted to pH 3.3 with acetic acid (1.0 ml/min). In a first step (see Table 1) analytes were injected onto the switching column (Pinkerton ISRP) and washed with the ammonium acetate buffer for 4 min at the same flow-rate. The

Table 1
Column switching time-table

Time (min)	Events
0	Sample injection onto switching column
0–4	Sample washing with washing phase A; flow 1 ml/min
4	Rotate switching valve
4–7	Back-flush elution with gradient elution phases: 20% phase B; flow 1.5 ml/min
7	Rotate switching valve to initial position
10–18	Switching column washing with washing phase B; flow 0.5 ml/min

column eluent passed through the Rheodyne switching valve to waste. The analytical mobile phase consisted of two phases for gradient elution: phase A ammonium acetate buffer 25 m*M*–pH 3.3-acetonitrile (95:5, v/v), and phase B ammonium acetate buffer–acetonitrile–methanol (5:85:10, v/v/v). The initial analytical mobile phase composition was 20% phase B, directed through the analytical column at a flow-rate of 1.5 ml/min. After 4 min the switching valve redirected the flow of analytical mobile phase towards the ISRP column in a backflush mode eluting the sample onto the analytical column. This was continued for 3 min, time needed to complete the switching valve cycle. Then the ISRP column was washed with water–2-propanol (75:25, v/v) for 8 min at a flow-rate of 0.5 ml/min, while gradient program was eluting samples from the analytical column. The gradient program started half a minute after completion of the switching valve cycle. The gradient profile was from 20 to 50% of B in 14.50 min; from 50 to 80% of B in 1 min; 3 min in isocratic conditions (80% of B) and then the percentage of B returned to its initial value (20%) in 1 min. The analytes were detected at 270 nm.The HPLC columns were operated at room temperature. Fig. 2 illustrates the analytical system and the switching valve connections.

2.5. Assay validation

Linearity was determined using calibration standards prepared as described in Section 2.3 (calibration range 0.1–2 μg/ml). Correlation between the spiked and the measured concentrations was tested by linear least-squares regression analysis using the Table Curve program (Jandel, San Rafael, CA, USA). Ramoplanin concentrations against ramoplanin area/internal standard area ratio were used for this purpose. Precision and accuracy were determined analysing quality control samples at three different concentrations: 0.15 μg/ml; 0.75 μg/ml; 1.8 μg/ml. Calibration standards and quality controls were analysed on three different days and in triplicates in order to determine intra- and interday

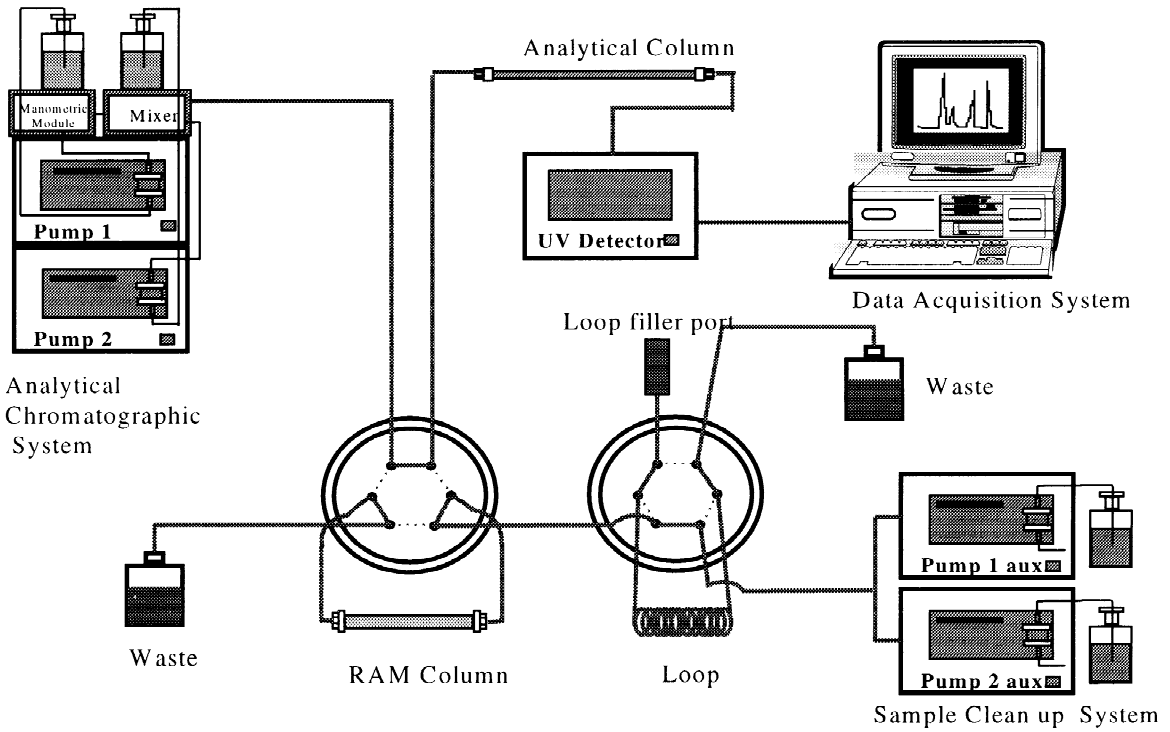

Fig. 2. Scheme of the chromatographic system and of the switching configuration.

precision. Precision was estimated from the relative standard deviation (RSD%).

The limit of quantification (LOQ) precision was determined analysing seven additional samples independently prepared from those used for calibration. The limit of detection (LOD) was defined as the sample concentration of ramoplanin resulting in a peak height of three times the signal-to-noise ratio (S/N).

Specificity of the method was assessed to test matrix influence between different urine samples from healthy donors ($n=6$). Urine samples from healthy donors were collected and analysed directly onto the HPLC system.

2.6. Extraction efficiency

The extraction efficiency was determined by comparing standards, dissolved in 0.25 m*M* ammonium acetate buffer pH 3.3 and directly injected in small volume (50 μl) onto the analytical column, with spiked urine standards injected onto the two-column HPLC system described above. The concentration level used for this purpose was 0.75 μg/ml. The recovery of ramoplanin and of the internal standard were calculated by dividing mean peak areas obtained from the spiked standards ($n=9$) by mean peak areas obtained from the direct standards ($n=3$).

2.7. Stability study

In the present study, the stability data of ramoplanin in urine were obtained analysing spiked samples at quality control concentrations, after bench-top storage at room temperature for 24 h, and after one and three freeze–thaw cycles. Each determination was performed in duplicate.

3. Results and discussion

3.1. Liquid chromatographic system

To minimize pretreatment of the sample and to achieve optimum recovery and precision, direct injection of the urine samples is preferred. In the present study a coupled-column system of an internal-surface reversed-phase and a reversed-phase column was used. On the first column analytes of interest are trapped, while hydrophilic endogenous compounds are eluted to waste. Pinkerton ISRP columns have a hydrophobic tripeptide internal surface (glycyl-L-phenylalanyl-L-phenylalanine) and a hydrophilic diolglycine external surface which preferentially retains nonpolar analytes. Additionally the negative charge of the carboxyl group of the amino acids, both at the external and internal surface, provides weak cation-exchange properties and delays elution of positively charged analytes and matrix compounds [11,12]. Starting from these observations, we considered the possibility of selectively trapping into this column ramoplanin and its hydrogenated derivative MDL 62 456, used as internal standard. In fact ramoplanin, even though it has a prevalent hydrophilic behaviour, tends to interact with apolar residues. Moreover at pH 9 ramoplanin possesses two protonated amines [3], making possible a further interaction with ISRP stationary phase through the cation-exchange retention capacity. These suppositions resulted as correct as ramoplanin, when injected onto an HPLC system constituted of only an ISRP guard cartridge directly connected to the UV detector, did not elute from the column even after 30 min (flow-rate 1.5 ml/min), when the mobile phase was constituted only of buffers at different pH values ranging from pH 3 to pH 6.8 (phosphate buffers were used at pH 6–6.8). On the other hand when mobile phase was constituted of buffer–acetonitrile (80:20, v/v), elution was complete in less than 2 min for both ramoplanin and internal standard at a flow-rate of 1.5 ml/min.

This set of experiments clearly demonstrated that both ramoplanin and MDL 62 456 could be selectively and efficiently separated from matrix compounds onto a coupled chromatographic system made of an ISRP guard column and a reversed-phase analytical column.

Fig. 3A shows a chromatogram of an urine sample containing 1 μg/ml ramoplanin, analysed onto the ISRP-column switching device. The peaks of ramoplanin and MDL 62 456 have retention times around 12.45 min. and 14.20 min., respectively. The peaks are clearly separated and well shaped. Fig. 3B shows a chromatogram of a drug free urine sample. From

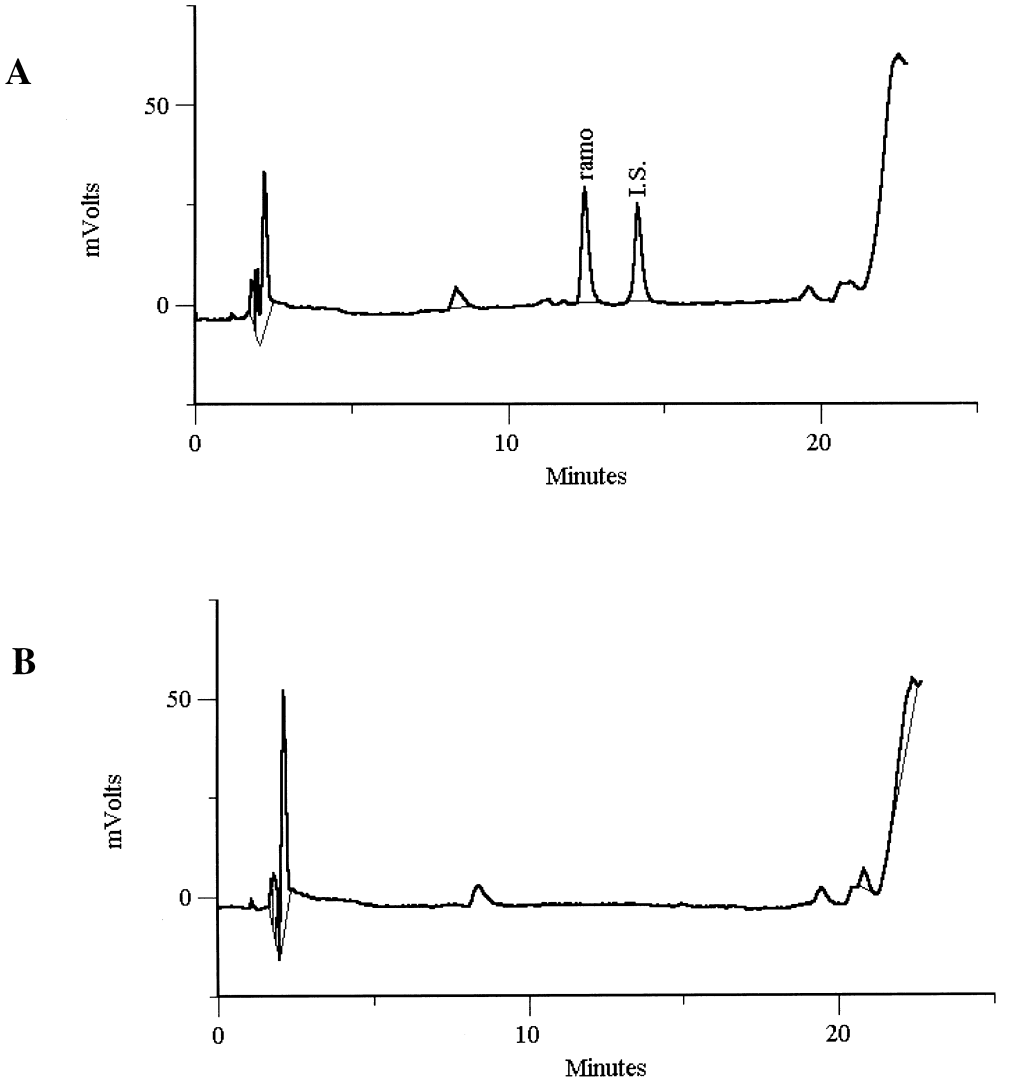

Fig. 3. Chromatograms obtained after a 200 μl injection of human urine. (A) Urine sample spiked with 1 μg/ml ramoplanin and 6.48 μg/ml MDL 62 456. (B) Drug free urine sample.

random urine samples ($n=6$ healthy volunteers), no interference of the matrix was observed.

Mean recovery efficiency was approximately 87% for ramoplanin and 62% for internal standard. Recovery was at maximum degree, 92% for ramoplanin and 68% for internal standard, for at least 30–40 analyses, then it decreased slightly, probably as a consequence of a reduced ISRP column retention capacity.

3.2. Linearity and sensitivity

Ramoplanin area/MDL 62 456 area ratio and ramoplanin concentrations varied linearly ($r^2=$ 0.997–0.995) over the analytical range employed (0.1–2 μg/ml). The range of ramoplanin concentrations chosen is rather low as no significant ramoplanin urine concentrations are expected. The intercept was not significantly different from zero (95%

Table 2
Intra-day precision and accuracy of LOQ; values in μg/ml

	LOQ (0.1 μg/ml)	*n*	Mean (μg/ml)	SD (μg/ml)	RSD (%)	Accuracy (%)
Concentrations found	0.094	7	0.101	0.0061	6.0204	0.8564
	0.098					
	0.107					
	0.098					
	0.096					
	0.102					
	0.110					

confidence limits). The LOQ was set at the lowest standard concentration on the calibration curve (0.1 μg/ml) and the corresponding intraassay RSD ($n=7$) was 6.02%, as described in Table 2. LOD was found to be 0.035 μg/ml.

3.3. Accuracy, precision and stability

Table 3 summarizes the validation data for fresh spiked urine samples. The intraassay precision (RSD%) in urine samples was between 0.71% and 8.75%. Highest RSD values are, as expected, usually associated with the lowest concentrations (0.15 μg/ml). The interassay precision (RSD%), at concentrations of 0.15 μg/ml ($n=9$) was 12.1%. The accuracy of the assay, defined as the mean percentage difference from the nominal value, ranged from −11.6 to 9.3%.

Spiked urine samples, kept at room temperature for 24 h resulted to be stable. There was no apparent freezing effect after one and three cycles of freezing and thawing.

4. Conclusions

An automated method for the determination of ramoplanin in human urine by column-switching HPLC has been developed. The method is simple and rapid, requiring a total analysis time of 35 min

Table 3
Intra- and inter-day precision and accuracy of quality control samples; values in μg/ml urine

	Conc. added	Concentrations found			*n*	Mean (μg/ml)	SD (μg/ml)	RSD (%)	Accuracy (%)
Day 1									
	0.15	0.137	0.135	0.135	3	0.136	0.001	0.748	−9.289
	0.75	0.663	0.698	0.715	3	0.692	0.023	3.892	−7.742
	1.8	1.755	1.773	1.791	3	1.773	0.018	0.994	−1.493
Day 2									
	0.15	0.135	0.127	0.150	3	0.137	0.012	8.750	−8.493
	0.75	0.732	0.685	0.701	3	0.706	0.024	3.402	−5.868
	1.8	1.691	1.684	1.668	3	1.681	0.012	0.714	−6.614
Day 3									
	0.15	0.173	0.153	0.176	3	0.167	0.013	7.753	11.643
	0.75	0.655	0.687	0.687	3	0.676	0.018	2.723	−9.864
	1.8	1.667	1.603	1.659	3	1.643	0.035	2.114	−8.713
Inter-day									
	0.15				9	0.147	0.018	12.097	−2.047
	0.75				9	0.691	0.024	3.480	−7.825
	1.8				9	1.699	0.061	3.612	−5.607

per sample. LOD was found to be 0.035 μg/ml, that, considering ramoplanin's high molecular mass (2554.1), corresponds approximately to 13.7 pmol/ml. Compared to microbiological assays the method demonstrates greater accuracy and precision and above all satisfactory selectivity that cannot be achieved with commonly used bioassays.

References

[1] B. Cavalleri, H. Pagani, G. Volpe, E. Selva, F. Parenti, J. Antibiot. 37 (1984) 309–317.
[2] R. Pallanza, M. Berti, R. Scotti, E. Randisi, V. Airioli, J. Antibiot. 37 (1984) 318–324.
[3] R. Ciabatti, B. Cavalleri, in: M.E. Bushell, U. Graefe (Eds.), Progress in Industrial Microbiology: Bioactive Metabolites from Microorganisms, Elsevier, New York, 1989, pp. 205–219.
[4] F. de Lalla, B. Romeo, R. Nicolin, G. Pellizzer, R. Merati, E. Riva, Can J. Infect. Dis. 6 (1995) 425C.
[5] M.D. O'Hare, G. Ghosh, D. Felmingham, R.N. Gruneberg, J. Antimicrob. Chemother. 25 (1990) 217–220.
[6] C.C. Johnson, S. Taylor, P. Pitsakis, P. May, M.E. Levison, J. Antimicrob. Chemother. 36 (1992) 2342–2345.
[7] L.A. Collins, G.M. Eliopoulos, C.B. Wennerstein, J. Ferraro, J.R.C. Moellering, Antimicrob. Agents Chemother. 37 (1993) 1364–1366.
[8] P.E. Reynolds, E.A. Somner, Drugs Exp. Clin. Res. 16 (1990) 385–389.
[9] E.A. Somner, P.E. Reynolds, Antimicrob. Agents Chemother. 34 (1990) 413–419.
[10] F. Bivasco, E. Manso, P.E. Varaldo, Antimicrob. Agents Chemother. 35 (1991) 195–197.
[11] S.E. Cook, T.C. Pinkerton, J. Chromatogr. 368 (1986) 233–248.
[12] S.C. Ruckmick, B.D. Hench, J. Chromatogr. 565 (1991) 277–295.