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Description and validation of an analytical method for the determination of paromomycin sulfate in medicated animal feeds

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A procedure for the extraction of paromomycin from different animal feed matrices (rabbit, chicken, pig feeds) and its subsequent determination *via* a reversed-phase ion-pair HPLC separation coupled with pulsed amperometric detection is described. The procedure optimised in terms of the extracting solvent and the solid phase extraction stationary phase allows the total recovery of the aminoglycoside antibiotic. The criteria used for the validation of the analytical method applied to the cited matrices are the linear dynamic range of the response, the detection limit, the repeatability, the intermediate repeatability and the accuracy. A comparison with a method described in the literature for the bulk analysis of this antibiotic is made.

Paromomycin (PARO) (Fig. 1) is an aminoglycoside antibiotic widely used in human and veterinary medicine.[1] As other antibiotics of the same chemical nature, PARO shows a broad spectrum activity against both Gram-positive and Gram-negative bacteria and for this reason it is added to animal feeds under legal directives. In particular, it is used in the prevention and treatment of different kinds of bacterial enteritis and salmonellosis in many species of brood animals destined for human feeding.[1] As the use of antibiotics would be always very restricted owing to the ability of many pathogens to become resistant to them, the dosage of these compounds in animal feeds must be accurately monitored. Moreover, the quality control of the industrial wastes together with the possible passage of antibiotics into slaughtered flesh makes the availability of a robust and sensitive method of analysis very important.

Methods for the determination of aminoglycoside antibiotics includes microbial methods and chromatographic analyses coupled with derivatisation steps. Microbial methods[2,3] are qualitative in character as they are not able to distinguish among the possible different active compounds and, in particular, are unable to give information on the overall chemical composition of the sample. On the other hand, HPLC analyses with UV detection require the conversion of the amino groups into isoindole derivatives[4] owing to the absence of UV chromophores in the aminoglycoside antibiotics. Several methods have been described for the determination of PARO, including a gas chromatographic method with flame ionisation detection[5] and HPLC separation with UV[6,7] or fluorescence detection.[8] This last method, employing *o*-phthalaldehyde (OPA) and 2-mercaptoethanol as post-column reagents, is in our opinion the most significant, allowing the determination of the two isomers of PARO in standard samples. However, the requirement for derivatisation reactions with toxic reactants makes this method unwieldy. Alternatively, for the determination of neomycin,[9] kanamycin[10] and PARO, very sensitive pulsed electrochemical detection has been employed.[11]

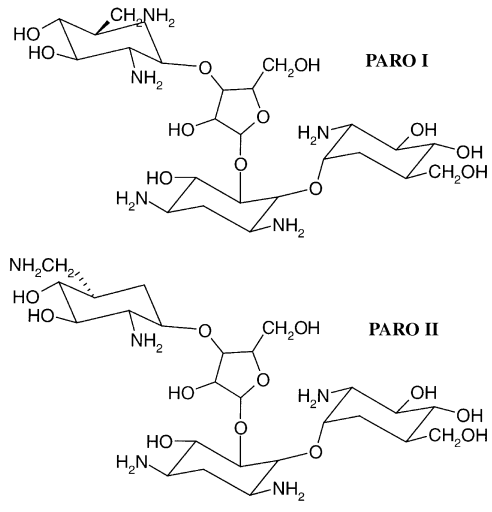

Fig. 1 Structures of paromomycin isomers.

The composition of rabbit, pig and chicken feeds includes, among many organic and inorganic compounds, large amounts of oligo- and polysaccharides which have chemical properties similar to those of the considered antibiotic. As demonstrated by preliminary chromatograms obtained with procedures described in the literature,[6–8,11] these interfering compounds were eluted as a huge chromatographic peak, many starting from the dead volume and expanding for many minutes. Paromomycin was eluted inside this peak and therefore it was not detectable. The procedures reported in the literature are therefore unsuitable for the problem at hand as both the electrochemical detection and the derivatising reactants did not add selectivity to the analysis.

This paper describes an analytical procedure for the extraction of PARO from real animal feed matrices and for its quantification by HPLC coupled with pulsed amperometric detection (PAD). A statistical evaluation was carried out to allow the proposal of the reported method as the reference method for the determination of PARO in animal feed matrices.

Experimental

Reagents

For the preparation of solutions, ultra-pure water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) was always

used. Methanol and acetonitrile were of HPLC grade. NaOH, HCl, anhydrous Na_2SO_4, sodium octane sulfonate, sodium decane sulfonate and sodium dodecane sulfonate were supplied by Prolabo (Paris, France) and used without further purification. Paromomycin sulfate and the medicated integrator (trade name Gabbrocoll) were supplied by Sigma (Steinheim, Germany) and Vetem Centralvet (Milan, Italy) respectively.

The eluent solution was obtained by mixing solutions A and B in an 80:20 ratio, solution A being at 3 mmol dm^{-3} $C_{10}H_{21}SO_3Na$ and 0.08 mol dm^{-3} Na_2SO_4 in 0.1% acetic acid and solution B pure CH_3CN. All the solutions were de-aerated with helium.

Apparatus

A Perkin-Elmer (Norwalk, CT, USA) Model 410-LC chromatograph equipped with a 25 μl injection loop was always used. All the chromatograms were recorded with a Perkin-Elmer Model 900 interface and processed with Perkin-Elmer Turbocom version 4.1 software. The analytical column was a C_{18}, 3 μm particle size, 150 × 4.6 mm id Luna column (Phenomenex, Torrance, CA, USA). Electrochemical detection was carried out with an ESA (Chelmsford, MA, USA) Coulochem II amperometric detector equipped with a gold working electrode, a stainless steel counter electrode and an Ag/AgCl, $KCl_{sat.}$ reference electrode. For the post-column addition of NaOH a Perkin-Elmer Model 250 LC pump was used. The mixing coil was 1 m × 0.02 cm id. The sample treatment was performed with Waters (Milford, MA, USA) OASIS-HLB columns containing 60 mg of stationary phase.

Procedures

(a) Extraction of the analyte from the animal feed matrices. A volume of 500 ml of 0.1 mol dm^{-3} HCl was added to 20 g of the animal feed for rabbit, chicken and pig. The mixture was homogenised by stirring for 30 min at room temperature and then for 60 min in an ultrasonic bath. The resulting mixtures were centrifuged at 8000 rpm for 5 min and then filtered on a 0.45 μm polyethylene membrane. The resulting solution was diluted 1 + 1 with further HCl solution if the concentration of PARO was > 100 μg kg^{-1}.

(b) Purification of the extraction solution. The purification of the solution containing the analyte was carried out using the following procedure: (i)1 ml of methanol, 5 ml of water and 5 ml of 0.1 mol dm^{-3} HCl were slowly percolated through an OASIS-HLB column; (ii) the column was dried under vacuum (20 mmHg); (iii) 750 μl of the sample solution were passed down the column and recovered in a 1 ml calibrated flask; and (iv) 250 μl of 0.1 mol dm^{-3} HCl solution were kept for 2–3 min inside the column for washing and then collected in the flask.

(c) Chromatographic analyses. Taking into account the composition of the eluent reported by Olson *et al.*[8] (the only paper which, to our knowledge, reports the chromatographic conditions for the analysis of PARO), the following seven parameters were considered for the resolution of PARO from the matrix by reversed-phase ion-pair chromatography: pH of the eluent, nature of the organic modifier, organic modifier content, temperature, nature of the counter ion, counter ion and Na_2SO_4 concentration. The analytical approach to the problem accounted for the choice of the chromatographic parameters able to delay suitably the elution of PARO and the purification of the animal feed matrix. Concerning the detection system, the pulsed amperometric detection described in ref. 11 was adopted since it allowed the direct detection of the analyte, avoiding any treatment of the sample.

The pH of the eluent was conditioned by the nature of the analyte as the necessity to protonate the molecule of PARO to form an ion pair with a suitable counter ion compelled the use of acidic pH values. The other chromatographic conditions summarised below were established by a systematic investigation described in the Results and discussion section.

Results and discussion

Choice of the chromatographic conditions

The selection of the chromatographic operating conditions included the choice of the detector and, to obtain a suitable retention time for PARO, the optimisation of the elution parameters. As regards the detection system, a pulsed amperometric one[11] was adopted since it allowed direct detection without derivatisation of the analyte. PARO gives two peaks [see Fig. 3(c)] relative to its isomers[8] with capacity factors, k', continuously increasing with the decrease of the pH from 7.2 to 4.5 and almost constant at lower pH values (Fig. 2). At pH > 7.2 the analyte was not retained as in the form of completely deprotonated neutral species. These findings led to the use of a 0.1% acetic acid solution, pH ≈ 3.5, which ensured the total protonation of the analyte and at the same time did not make critical the achievement of the basic conditions necessary for amperomeric detection.

Of the most usual organic modifiers employed in HPLC, CH_3OH and CH_3CN, the latter had to be chosen as CH_3OH is electroactive in the potential range used for pulsed amperometric detection. Tests carried out at different temperatures indicated the negligible influence of temperature on the separation. In fact, both the peak width and the retention time in the range 10–40 °C are weakly affected and therefore 40 °C was selected. Since the longer the alkyl chain length the higher is the retention time, the performances of the counter-ions 1-octane-, 1-decane- and 1-dodecanesulfonate were tested. Decanesulfonate (DS) was chosen as its proper concentration is in the millimolar range; to achieve the same retention time of the analyte octanesulfonate required too high a concentration and dodecanesulfonate too low a concentration. Another significant advantage of the use of DS was that the retention times achieved were compatible with the increase in the organic modifier concentration, which, in turn, produced a narrower matrix peak. The elution time of the analyte was finally adjusted by the addition of Na_2SO_4 which competed with DS. Na_2SO_4 also made the peak shape sharper and more symmetric.

The final concentrations of CH_3CN, DS and Na_2SO_4, reported in the Reagents section, were determined neither by a multifactorial experiment nor by a simplex procedure and therefore most probably they represent only a local optimum. However, they allowed the elution of the two peaks of the analyte in the appropriate position in the chromatogram,

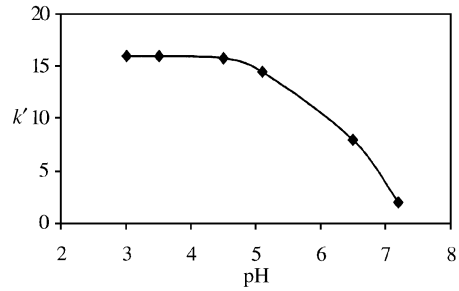

Fig. 2 Variation of the capacity factor, k', of the primary peak as a function of pH. Chromatographic conditions: column, C_{18}; T = 40 °C; eluent, 1.2 mmol dm^{-3} [$C_{10}H_{21}SO_3Na$] and 0.2 mol dm^{-3} [Na_2SO_4] in 1% acetic acid; flow rate, 1 ml min^{-1}; pulsed amperometric detection by post-column addition of 0.3 M NaOH at 0.8 ml min^{-1}.

avoided the interference of other compounds present in the matrix and compressed as much as possible the peak of the matrix close to the dead volume.

Sample treatment

As shown in Fig. 3(a) for a PARO-free rabbit feed sample, even operating with the optimised chromatographic conditions, the real sample requires a previous treatment to reduce the effects of potential interfering compounds. A successful treatment was solid phase extraction using the OASIS HLB columns constituted by a balanced macroporous divinylbenzene–*N*-vinylpyrrolidone copolymer. Other stationary phases were tested, namely Al_2O_3, ion exchangers and C_{18}. The first two types did not give a complete recovery and the third was not suitable for strongly acidic media. The chromatograms obtained before and after the extraction step in the absence and presence of PARO are reported in Fig. 3(b) and 3(c), respectively.

Statistical treatment of data

The reliability of the method was checked by evaluating the following parameters:[12] (i) linearity of the calibration graph; (ii) detection limit; (iii) repeatability; (iv) intermediate repeatability; (v) accuracy (yield of recovery); and (vi) comparison with a reference method.

(i) The calibration plot was obtained by injecting five standard solutions (three replicated injections for each solution) ranging between 1.2 and 6.3 mg dm^{-3} of PARO, which is the estimated concentration interval of the analyte extracted from the matrix. The regression parameters of the two lines are reported in Table 1. The ratio between the slopes of the two 'isomer lines', $R = b_{princ.}/b_{sec.} = 8.0 \pm 0.5$, furnished their relative abundance in the sample under the assumption of the same sensitivity. This result agreed very well with that obtained by fluorescence detection ($R = 7.99$).[8] The circumstance that the chromatographic technique is able to separate the two isomers is fundamental to testing the composition of the mixture, but it can offer no improvement of the quantitative analysis if the two isomers exhibit the same biological activity. Under our conditions the quantitative analysis must be performed by using the calibration graph relative to the principal peak.

(ii) The detection limit was calculated with the Hubaux–Vos method[13] and resulted in 0.21 mg dm^{-3} at a level of significance of 5% for both the α and β forms. The limit of quantification was 0.43 mg dm^{-3} under the same conditions.

(iii) The repeatability was calculated by injecting 10 times non-stop a 4.3 mg dm^{-3} PARO solution. The relative standard deviation obtained of 0.97% indicated very good repeatability.

(iv) The intermediate repeatability was evaluated by comparing calibration plots obtained on different days. Referring to the joint confidence region for slope and intercept,[14] the calibration plot was sometimes statistically not equivalent, indicating the necessity for frequent checks of the sensitivity of the detector, as foreseeable for electrochemical detection. Table 2 reports the results relative to three different calibration plots obtained over an interval of 1 week.

(v) When, as in this case, a reference method or a certified standard is not available, the accuracy can be evaluated from the recovery from a 'blank' matrix spiked with a weighed amount of standard. Table 3 reports the recoveries of different amounts of PARO added to blank matrices and evaluated on the basis of a calibration graph. The table is self-explanatory and demonstrates the complete recovery of the analyte from the matrix.

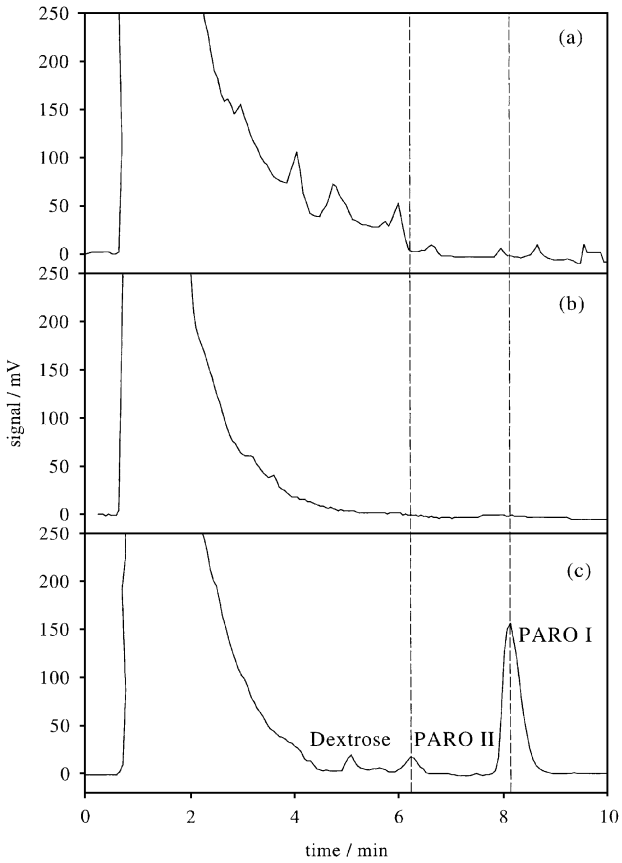

Fig. 3 Chromatograms of an extracted solution from rabbit feed: (a) 'blank' matrix without any treatment; (b) same 'blank' matrix purified with OASIS HLB column; (c) as (b) spiked with a weighed amount of Gabbrocol. Chromatographic conditions are reported in the Reagents section.

Table 1 Regression parameters relative to the calibration plot ($y = a + bx$) for PARO for the primary peak, secondary peak and the sum of the two peaks

	a/counts	*b*/counts dm^3 mg^{-1}	s_a/counts[a]	s_b/counts dm^3 mg^{-1}[a]	r^2
Secondary peak	48284	41800	11391	2709	0.950
Primary peak	−163	332905	13324	3169	0.999

[a]

$$s_a = s_y \sqrt{\frac{\bar{x}^2}{\sum_i (x_i - \bar{x})^2} + \frac{1}{N}};$$

$$s_b = \sqrt{\frac{s_y}{\sum_i (x_i - \bar{x})^2}};$$

$$s_y = \sqrt{\frac{1}{N-2} \times \sum_i^N \left[y_i - (a + bx_i)\right]^2}.$$

Table 2 Regression parameters relative to the calibration plot ($y = a + bx$) for PARO of the primary peak obtained in different days

Week	*a*/counts	*b*/counts dm^3 mg^{-1}	s_a/counts	s_b/counts dm^3 mg^{-1}	r^2
1	−163	332905	13324	3169	0.9991
2	3187	384385	35514	5709	0.9993
3	−5485	344367	16894	3169	0.9997

(vi) In order to characterise our procedure more deeply, we made a comparison with the OPA method[8] by means of a paired *t*-test.[15] The comparison was made by analysing with the two procedures three samples extracted independently from four lots of Gabbrocol. The results are summarised in Table 4. The sample of the differences gave $\bar{d}$ = 0.22 as the average, s_d = 0.46 as the standard deviation and $s_{\bar{d}}$ = 0.13 as the standard deviation of the mean of the differences. Since the critical value of *t* for 11 degrees of freedom and with a confidence interval of 95% is $t_{0.05,11}$ = 2.2, greater than the experimental value, 1.65, the two methods are equivalent.

Table 3 Recovery data for PARO from rabbit feed marix

Amount of PARO/mg kg^{-1}	Amount of PARO obtained/mg kg^{-1}	Recovery (%)	Difference (%)	Relative difference (%)
509.8	511.0	100.2	1.2	0.24
539.5	540.9	100.3	1.4	0.26
549.8	546.6	99.4	−3.2	0.58
430.2	428.5	99.6	−1.7	0.40
430.5	427.1	99.2	−3.4	0.79
468.5	462.6	98.7	−5.9	1.26
289.0	288.1	99.7	−0.9	0.31
349.5	351.2	100.5	1.7	0.49
274.5	271.2	98.8	−3.3	1.20
186.5	185.5	99.5	−1.0	0.51
125.5	124.0	98.8	−1.5	1.20
118.5	116.8	98.6	−1.7	1.43
84.4	84.2	99.8	−0.2	0.19
49.5	48.8	98.7	−0.6	1.31
60.2	62.3	103.5	2.1	3.49

Table 4 Comparison between the proposed method and the OPA method.[8] The data are relative to the quantification of PARO present in the medicated commercial product (Gabbrocol)

Lot No.	PAD detection (%)	OPA fluorescence detection (%)	Absolute difference
	14.34	14.43	0.09
1	14.11	14.90	0.02
	14.55	14.55	0
	14.14	14.01	0.13
2	14.01	14.87	0.86
	14.55	14.50	0.05
	14.13	14.11	0.02
3	14.20	14.26	0.06
	14.77	13.90	0.87
	14.38	13.71	0.33
4	14.80	14.51	0.31
	13.96	14.14	0.18

Conclusions

The procedure described in this paper allows the determination of the aminoglycoside antibiotic paromomicyn present in rabbit, pig and chicken animal feed matrices avoiding any derivatisation reaction of the analyte. The combined effect of the sample treatment, extraction of the analyte and the sensitivity of pulsed electrochemical detection allows one to work with dilute solutions, thus further lowering the matrix interference. The statistical analysis of the performance of the method demonstrated its very good reliability and allows us to propose it as the reference procedure for the determination of paromomycin in the considered matrices.

Acknowledgements

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