

Comparison of Ion-Pairing and Ion-Suppressing liquid Chromatographic Methods for the Determination of Pyrimethamine and Ormetoprim in Chicken Feed

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Abstract

A high-performance liquid chromatographic (HPLC) method is developed to simultaneously determine pyrimethamine (PYR) and ormetoprim (OMT) in chicken feed. In the ion-pairing HPLC determination of PYR and OMT, the relation between the retention factor (k') and the concentration of the organic phase (acetonitrile) shows a characteristic curve. The k' value first decreases and then increases slowly with increasing concentrations of acetonitrile, but then increases rapidly when the acetonitrile concentration increases to 90%. Resolutions (R_s) of PYR and OMT decrease gradually when the concentration of organic phase increases. Increasing the concentration of the pairing ion sodium 1-octanesulfonate (PIC B-8) can decrease the k' and R_s values. Optimum values of k' and R_s are obtained using 82% acetonitrile in 0.005M PIC B-8. In ion-suppressing HPLC, varying the concentration of Na_2HPO_4 has little effect on either the k' or R_s values of PYR or OMT at pH 7.5. However, at pH 4.0, k' and R_s decline when the concentration of Na_2HPO_4 increases. In general, ion-pairing HPLC generates more satisfactory results than ion-suppressing HPLC. Using 82% acetonitrile in water containing 0.001M PIC B-8 as the mobile phase, linear calibration curves are obtained in the range from 1 to 5 mg/L of PYR and OMT. Sulfamonomethoxine, sulfadimethoxine, sulfaquinoxaline, trimethoprim, amprolium, clodipol, and nicarbazin do not interfere with the detection of PYR or OMT. The recoveries of PYR from spiked feed at 1 and 5 mg/Kg are 73.0% and 72.0%, respectively, and those of OMT from spiked feed at 3 and 7 mg/Kg are 50.3% and 53.6%, respectively.

Introduction

Although pyrimethamine (PYR) can prevent leucocyto-zoonosis in chickens (1), it is not allowed to be used as a feed additive (2,3). The problem of residual PYR in eggs has caused serious public concern since 1986 in Taiwan. Ormetoprim (OMT) is an analog of PYR and is permitted for use as a feed additive for the

prevention of leucocytozoonosis (2,3). The National Institute for Animal Health is responsible for the testing of PYR and OMT in feed; therefore, a simultaneous determination method for OMT and PYR is urgently needed.

Although spectrophotometry (4,5) and gas chromatography (6) have been used to determine PYR, high-performance liquid chromatography (HPLC) is the most frequently used method in monitoring PYR in edible tissue, plasma, and urine (7-16) and veterinary commercial formulations (17). The methods for the determination of OMT are fluorospectrometry (18) and HPLC (19).

The determination of PYR by reversed-phase HPLC involves either ion-pairing chromatography (7, 8, 12) or ion-suppressing chromatography (9,10). In this study, these two methods are compared in order to choose the best analytical conditions, and a method for the simultaneous determination of OMT and PYR was developed.

Experimental

Instrumentation

The HPLC system consisted of a Shodex (Tokyo, Japan) degasser, a Kratos (Ramsey, NJ) Spectroflow 400 solvent pump, a Kratos Spectroflow 491 sample injector (20- μL fixed volume injections), a Kratos Spectroflow 783 detector (280 nm), an SIC (Tokyo, Japan) chromatogram processor 7000B integrator, a Waters (Milford, MA) RCSS Guard-pak C-18 guard column, and a chromatographic column (Kratos Spheri-5, RP-8, 5 μm , 250 \times 4.6 mm).

Chemicals

PYR and sulfadimethoxine (SDMX) were purchased from Sigma Chemical Company (St. Louis, MO). OMT and sulfamonomethoxine (SMM) were obtained from Daiichi Pharmaceutical Taiwan Ltd. (Taoyuan, Taiwan). Sulfaquinoxaline (SQL) and nicarbazin (NCZ) were purchased from Merck Research Lab

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(Rahway, NJ). Trimethoprim (TMP) was provided by Virbac Laboratories (F-06516 Carros Cédex, France). Amprolium (APL) was provided by K&K Greeff Fine Chemical Ltd. (Croydon, U.K.). Clopidol (CPD) was obtained from the Dow Chemical Company (Midland, MI).

All the solvents and reagents were of analytical grade from E. Merck (Darmstadt, Germany) except the mobile phase, which was of liquid chromatographic grade. Ortho-phosphoric acid was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 5% Isobutanol-benzene was prepared by mixing 5 mL of isobutanol and 95 mL of benzene.

The pairing ion was sodium 1-octanesulfonate (PIC B-8) and was obtained from Waters Corporation.

The carrier and blank feed for layers were provided by Cyanamid Taiwan Corporation (Taipei, Taiwan) and Cargill Taiwan Corporation (Taipei, Taiwan), respectively.

HPLC system suitability test

Ion-pairing chromatography

Stock standard solutions of PYR and OMT were diluted separately with methanol (MeOH) in order to prepare 5-mg/L standard solutions. The effects of different concentrations of acetonitrile (60%, 70%, 80%, 82%, 85%, and 90%), PIC B-8 (0, 0.005, 0.01, and 0.002M), and pH (3.5 and 5.0) on the retention factor (k') and resolution (R_s) were tested to establish the best analytical conditions.

Ion-suppressing chromatography

A mobile phase (acetonitrile- H_2O , 40:60) containing 0.02 or 0.05M Na_2HPO_4 was prepared. Phosphoric acid was used to adjust the pH to 7.5 or 4.0. Standard solutions (5 mg/L) of PYR and OMT were used to test the effect of Na_2HPO_4 and pH on k' and R_s .

Calibration curves

Exactly 25 mg of PYR and OMT were weighed and placed into separate 25-mL volumetric flasks then diluted to 25 mL with MeOH in order to prepare a mixed standards stock solution of 1000 mg/L. The mixtures were sonicated when needed to dissolve the drugs. Appropriate amounts of the previously mentioned stock solutions were diluted with MeOH to prepare working solutions of 1, 3, and 5 mg/L of PYR and OMT. The working standards were injected into the HPLC four times per concentration. Peak areas were measured to obtain the calibration curve.

The mobile phase containing acetonitrile- H_2O (82:18) and 0.001M of PIC B-8 was filtered through a 0.45- μ m pore size membrane. The flow rate was 1.2 mL/min.

Detection of possible interference

The following compounds were chosen to test for their possible interference in the determination of PYR and OMT: (a) SDMX (allowed for use in combination with OMT); (b) APL, CPD, and NCZ (with similar structures and allowed for use); (c) TMP (with a similar structure); and (d) SMM and SQL (other sulfa drugs used for poultry).

Recovery test

Adequate amounts of PYR and OMT were mixed separately with the carrier in order to prepare high concentration of premixes.

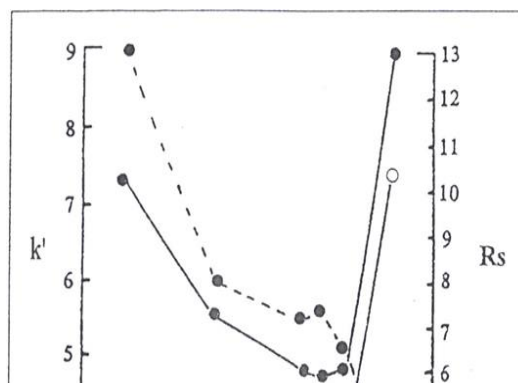
Blank feed for layers was ground and passed through a 20-mesh sieve. The previously mentioned premixes were added to blank feed in order to prepare two concentration of spiked feed samples (one containing 1 mg/Kg PYR and 3 mg/Kg OMT and the other containing 5 mg/Kg PYR and 7 mg/Kg OMT). Triplicates of each spiked feed sample were extracted as follows.

Ten grams of feed was vortex-mixed with 30 mL of 5% isobutanol-benzene for 5 min and centrifuged at 1105 g for 5 min. The extraction was repeated twice. The upper layers were collected, pooled, and evaporated to 5 mL in a 45°C water bath. The residue was transferred to a separating funnel, vortex-mixed, and extracted with 15 mL of 0.5N HCl. This extraction was repeated twice. The HCl layers were collected and pooled. The acid extract was washed once or twice with 10 mL of benzene (depending on the color of the benzene layer), neutralized with 0.5N NaOH, vortex-mixed, and extracted with 20 mL of 5% isobutanol-benzene. This procedure was repeated twice. The 5% isobutanol-benzene layer was collected, pooled, dehydrated with anhydrous sodium sulfate, and evaporated to dryness in a 45°C water bath. The residue was dissolved in 5 mL of MeOH with the aid of sonication and filtered through a 0.45- μ m Millipore membrane. A 20- μ L volume of the filtrate was injected into the HPLC.

Results

HPLC system suitability test

In ion-pairing chromatography, the effect of the concentration of acetonitrile on the k' of PYR and OMT showed a characteristic curve (Figure 1). The k' of PYR and OMT both decreased as the concentration of acetonitrile increased, with the k' of PYR decreasing at a higher rate than that of OMT. When the proportion of organic solvent was increased to 82% (PYR) or 70% (OMT), the k' value started to increase slowly then rapidly increased at 90% acetonitrile. Resolution changed with a similar pattern to k' but decreased rapidly at 90% acetonitrile. Optimum k' and R_s values were obtained when the ratio of acetonitrile-0.005M PIC B-8 was 82:18. When the mobile phase was acetonitrile- H_2O (82:18) containing 0.005M PIC B-8, the k' values of PYR and OMT were 1.44 and 0.97 respectively. Because of the low k' , the acetonitrile- H_2O ratio was adjusted to 60:40 to test the effect of the PIC B-8 concentration on k' and R_s . The results indicated that k' and R_s decreased as the concentration of the pairing ion PIC B-8 increased (Figure 2). In the absence of PIC B-8, PYR and OMT were retained in the column for 70 min and could not be eluted. However, higher concentrations of PIC B-8 (0.02M) resulted in the splitting of OMT into two peaks. Therefore, PIC B-8 generally should be added at a concentration of not more than 0.005M. The k' value decreased rapidly as the pH of the mobile phase was adjusted



However, at pH 4.0, k' decreased as the concentration of Na_2HPO_4 increased, and the k' value of PYR decreased at a higher rate than that of OMT. No matter whether the concentration of Na_2HPO_4 was 0.02 or 0.05M, the k' value of PYR at pH 7.5 was always greater than that at pH 4.0. The k' value of OMT was high at 0.02M Na_2HPO_4 and pH 7.5. Therefore, ion-suppressing chromatography was not suitable for the simultaneous determination of PYR and OMT, because the k' of OMT was too low and the R_s was too high.

According to these results, ion-pairing chromatography is better than ion-suppressing chromatography for the simultaneous determination of PYR and OMT. The optimum mobile phase for ion-pairing HPLC is acetonitrile- H_2O (82:18) containing 0.001M PIC B-8.

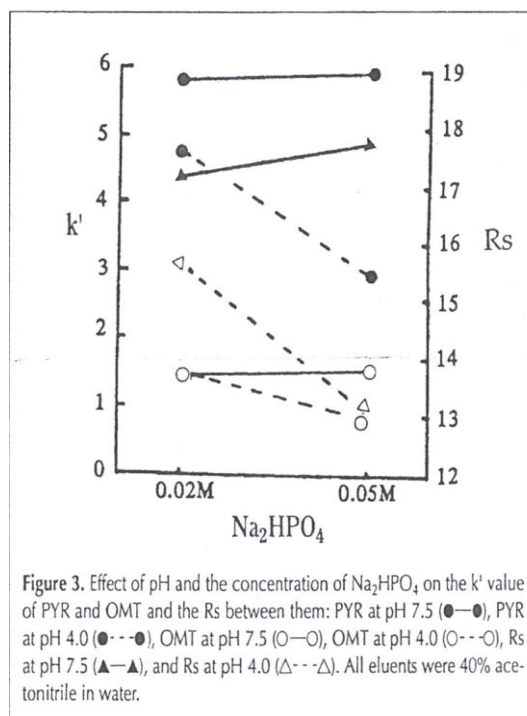


Figure 3. Effect of pH and the concentration of Na_2HPO_4 on the k' value of PYR and OMT and the R_s between them: PYR at pH 7.5 (●—●), PYR at pH 4.0 (○---○), OMT at pH 7.5 (○—○), OMT at pH 4.0 (○---○), R_s at pH 7.5 (▲—▲), and R_s at pH 4.0 (△---△). All eluents were 40% acetonitrile in water.

Table II. Repeatability of Peak Areas of PYR and OMT*

	PYR [†] (%, mean ± RSD)	OMT [†] (%, mean ± RSD)
1 mg/L	28.33 ± 1.4	27.42 ± 0.9
3 mg/L	87.33 ± 0.8	84.36 ± 0.3
5 mg/L	148.16 ± 0.5	143.67 ± 0.8

* The unit of peak area was $10^3 \mu\text{V/s}$.
[†] Each value represents the mean of four injections.

From 5.0 to 3.5. Resolution also decreased accordingly (Table I). Thus, better analytic results were obtained when the pH of the mobile phase remained at 5.0.

The results obtained from ion-suppressing chromatography are listed in Figure 3. At pH 7.5, the concentration of Na_2HPO_4 exerted little effect on the elution of PYR or OMT or on k' or R_s .

Calibration curve

The relative standard deviation (RSDs) of the peak areas of the PYR and OMT standards at the concentrations of 1, 3, and 5 mg/L were 1.4% and 0.8%, 0.5% and 0.9%, and 0.3% and 0.8%, respectively (Table II). The chromatogram in Figure 4 suggested a good separation effect. Linear responses were obtained for PYR and OMT over the range of 1 to 5 mg/L with regression coefficients

(r) of the calibration curves greater than 0.9999. The equations of the calibration curves of PYR and OMT were $y = -1.93 + 29.96x$ and $y = -2.03 + 29.06x$, respectively.

Detection of possible interference

Among the seven possible interfering compounds, SDMX, SMM, SQL, CPD, and NCZ were not retained and could be eluted by solvent, whereas TMP and APL were retained with k' values of 4.51 and 15.56, respectively. The k' values of PYR and

OMT were 6.68 and 4.88, respectively. Although the peaks for TMP and OMT were close, the separation was not affected. Therefore, these seven drugs did not interfere with the detection of PYR and OMT.

Discussion

PYR and OMT are basic drugs with pK_a values of 7.2 and 7.5, respectively. Therefore, PIC B-8 can be used as a counter ion.

Although Merck RP-18 and RP-8 columns (250 × 4.6 mm) have been tested, the Kratos RP-8 column exerted a better performance from the point of avoiding interference from the feed matrix.

In ion-pairing chromatography, it is predictable that k' will decrease as the concentration of organic phase is increased to a certain extent. Therefore, no matter whether ion-pairing chromatography or ion-suppressing chromatography is used, the effect of organic solvent on the k' value of PYR shows a characteristic curve. The results of OMT in ion-pairing chromatography are similar to those of PYR. These observations reflect the behavior of the two drugs during ion-pairing chromatography.

There are two mechanisms in ion-pairing chromatography (20). One is the partition of the drug between the two phases and the other is that the counter ion combines with the sta-

tionary phase and then the ion exchanges with the drug molecule. In this experiment, PYR and OMT were not eluted for 70 min in the absence of counter ions. This suggests that in reversed-phase chromatography, PYR and OMT were strongly partitioned into the stationary phase, resulting in a very high k' value. Therefore, k' increased when the organic phase was increased to certain extent.

The combination between the counter ion and stationary phase strengthened as the concentration of counter ions increased; therefore, the partition between the drug molecule and stationary phase was then decreased. In this condition, the mechanism for elution was mainly through ion exchange (20). In the elution of PYR and OMT, partition was stronger than ion exchange; therefore, PYR and OMT were

retained in the stationary phase, resulting in a decrease of k' (Figure 2).

Although it was reported that the pH of the mobile phase could be reduced to 3.5 in ion-pairing chromatography (7, 8), in this study the result was not satisfactory at pH 3.5 (Table I). The completely different column, organic phase, and test drugs can explain this inconsistency.

The concentration of Na_2HPO_4 exerted little effect on k' at pH 7.5; this pH value is identical to the pK_a of OMT and close to the pK_a of PYR (7.2). At pH 4.0, the increasing ionization of the basic PYR and OMT resulted in a decreased partition into the stationary

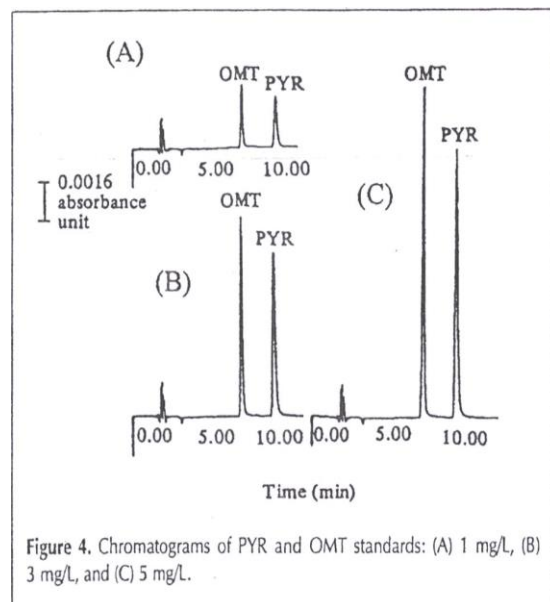


Figure 4. Chromatograms of PYR and OMT standards: (A) 1 mg/L, (B) 3 mg/L, and (C) 5 mg/L.

Table III. Recoveries of PYR and OMT from Medicated Layer Feeds

Spiked (mg/Kg)	PYR			OMT		
	Found (mg/Kg)	Mean ± RSD (%)	%Recovery	Found (mg/Kg)	Mean ± RSD (%)	%Recovery
1	0.778 0.719 0.692	0.730 ± 6.0	73.0			
3				1.49 1.59 1.46	1.51 ± 4.5	50.3
5	3.62 3.59 3.58	3.60 ± 0.6	72.0			
7				3.77 3.52 3.79	3.75 ± 6.0	53.6
Mean			72.5			

OMT were 6.68 and 4.88, respectively. Although the peaks for TMP and OMT were close, the separation was not affected. Therefore, these seven drugs did not interfere with the detection of PYR and OMT.

Recovery test

When blank feed was extracted by the method described, most of the interference could be removed and a stable baseline was

phase. Therefore, the k' value at pH 4.0 was lower than that at pH 7.5.

No matter whether the concentration of Na_2HPO_4 was 0.02M or 0.05M, the analytical result of OMT at pH 4.0 was not satisfactory. This mainly resulted from the low k' (1.54 or 0.97) of OMT, thus the retention time was too short and close to the unretained peak. The unsuitable stationary phase resulted in interference from the feed matrix with the elution of OMT, leading to difficult identification and quantitation errors. The same phenomenon occurred at pH 7.5 (k' was 1.46 or 1.5). Therefore, for the simultaneous determination of PYR and OMT, satisfactory analytical results could not be obtained at either pH 4.0 or pH 7.5 with either 0.02M or 0.05M Na_2HPO_4 . The ideal k' should be from 2 to 6 and the $R_s \geq 1.0$ (21).

Most normal-phase or reversed-phase columns use silica gel as the base, which is stable over the pH range of 2 to 8. In this experiment, pH 7.5 was so near to the stability range of the column that the packing material was unstable, thus poor sep-

with benzene. If the benzene layer showed a pale yellow or slight red color, another wash procedure was conducted to remove the interfering substances. In the final concentration step, the original method was used to evaporate the extract to 1 mL and then inject it into the instrument. However, the evaporation took place in a flask, which made it difficult to measure the correct volume. Therefore, better quantitation was obtained by evaporating to dryness and then dissolving the residue in 5 mL of MeOH. The final filtration clarified the sample. No interfering peak appeared after blank feed was extracted by this modified procedure, and a stable baseline was obtained (Figure 5), allowing for more precise quantitation. Although the repeatability for the quantitation of PYR and OMT were both satisfactory (0.6-6.0% and 4.5-6.0%, respectively), the recovery of OMT was not high enough (50.3-53.6%) and needs to be further improved.

Conclusion

We have developed a specific and rapid HPLC method for the simultaneous determination of PYR and OMT in feed. Ion-pairing HPLC was better than ion-suppressing HPLC in determining these two compounds. Although the recovery of OMT was somewhat low, it was highly precise ($RSD \leq 6\%$). Therefore, it may be useful for the routine monitoring of these drugs in feed.

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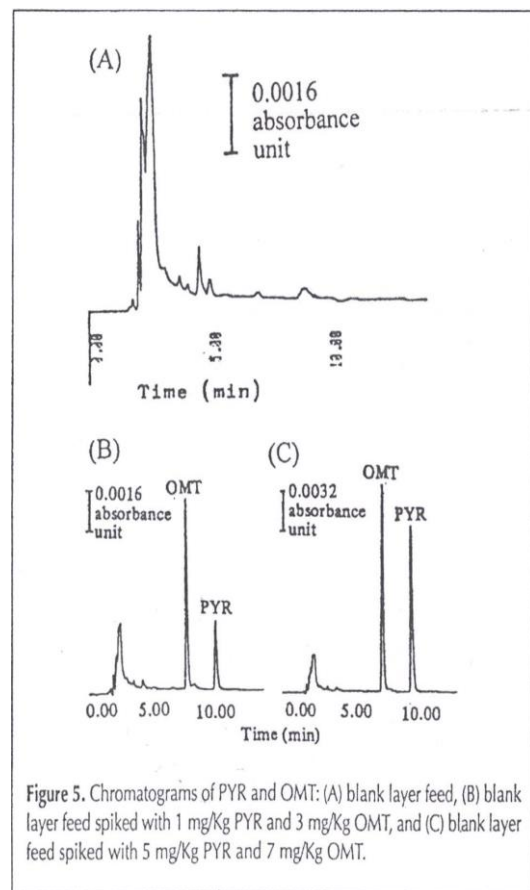


Figure 5. Chromatograms of PYR and OMT: (A) blank layer feed, (B) blank layer feed spiked with 1 mg/Kg PYR and 3 mg/Kg OMT, and (C) blank layer feed spiked with 5 mg/Kg PYR and 7 mg/Kg OMT.

aration resulted. Therefore, the use of silica gel should be avoided.

Good repeatability was demonstrated from the RSDs for the peak areas of PYR and OMT, being $<1.5\%$ and fulfilling the official regulation of 1-2% (21). The high r values of the calibration curve also indicated ideal linearity.

Although a number of published methods (4-6) have been conducted to evaluate the effect of feed pretreatment, satisfactory results for neither PYR nor OMT were obtained in our work. However, some modifications were made to remove interference and increase the accuracy in the quantitation. The modifications are briefly stated as follows. The pooled HCl layer was washed

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配離子與抑制離子化液相色層分析法對雞飼料內 必利美達民及歐美德普之比較

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摘要 利用高效液相色層分析法(HPLC)同時檢驗雞飼料必利美達民(PYR)及歐美德普(OMT)。以配離子 HPLC 分析 PYR 及 OMT，其滯留常數(k')於有機相(acetonitrile)濃度之關係，呈一特殊線型。增加 acetonitrile 濃度時 k' 值先下降而後緩慢上升，但 acetonitrile 濃度增至90%時則快速上升。當有機相增加時，PYR 及 OMT 之解析常數(R_s)緩慢降低。當配離子濃度增加時 k' 及 R_s 值下降。於82% acetonitrile 在0.005M PIC B-8可得最佳 k' 及 R_s 值。於抑制離子化 HPLC，在 pH 7.5時 Na_2HPO_4 之濃度對 PYR 或 OMT 之 k' 及 R_s 值影響不大。但在 pH 4.0時 k' 及 R_s 值下降。一般而言，配離子 HPLC 法較抑制離子之 PYR 及 OMT 可呈線性關係。Sufamonomethoxine，sulfadimethoxine，

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sulfaquinoxaline, trimethoprim, amprolium, clopidol, and nicarbazin 不會干擾 PYR 及 OMT 之檢出。飼料內 1mg/kg 及 5mg/kg 之 PYR 回收率分別為 73.0% 及 72.0%，而 3mg/kg 及 7mg/kg 則分別 50.3% 及 53.6%。