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Flow Injection Spectrophotometric Determination of the Antibiotic Fosfomycin in Pharmaceutical Products and Urine Samples after On-line Thermal-Induced Digestion

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A new flow injection (FI) method for the precise and rapid spectrophotometric determination of the antibiotic fosfomycin (FMC) in urine and pharmaceutical samples is described. The method is based on the on-line quantitative thermal-induced digestion of the analyte prior to injection into the FI system. Ammonium persulfate was used as the oxidation reagent. The resulting orthophosphate ions were determined spectrophotometrically (λ_{max} = 690 nm) using the molybdenum blue approach. Chemical and FI variables that affected on-line oxidation were studied and optimized. The proposed method is very precise (s_r = 1.2% at 1.0 × 10^{-4} mol L^{-1} FMC, n = 12), offers a high sampling rate of 60 h^{-1}, and allows for the determination of the analyte in the range 3.0 × 10^{-6} to 3.0 × 10^{-4} mol L^{-1} with a satisfactory 3σ detection limit of 1.0 × 10^{-6} mol L^{-1}. Application of the proposed method to urine and pharmaceutical samples yielded accurate results with percentage recoveries in the range 96.4–102.5%.

Key Words: **fosfomycin; flow injection; on-line thermal-induced digestion; urine; pharmaceuticals; molybdenum blue.**

Fosfomycin (FMC)[2] (Fig. 1) is a synthetic, broad-spectrum bactericidal antibiotic for oral administration. It has *in vitro* activity against a wide range of gram-positive and gram-negative aerobic microorganisms, which are associated with uncomplicated urinary tract infections. The bactericidal action of fosfomycin is due to its inactivation of the enzyme enolpyruvyl transferase that participates in one of the first steps in bacterial cell wall synthesis. It also reduces adherence of bacteria to uroepithelial cells (1).

The fact that FMC has no UV–Vis absorption or fluorescence properties has led to the development of few analytical methods for its determination. These methods include gas chromatography (GC) (2–4), ion chromatography (IC) (5), HPLC (6), and capillary electrophoresis (CE) (7, 8), after either derivatization or using indirect UV detection. These methods are generally laborious and time consuming, while expensive and complicated instrumentation is required. In addition to the above-mentioned procedures an indirect batch spectrophotometric method has also been reported (9) based on ligand exchange between zirconium and Br-PADAP/FMC. That method is simple and cost effective, but an average time of 20 min is required for the analysis of each sample.

An ideal analytical method for routine analysis and quality assurance should be automated, simple, cost effective, robust, precise, and accurate, and have a high sample analysis frequency. Flow injection (FI) analysis is a well-established analytical technique that fulfills the above-mentioned demands. FI procedures are extremely useful in clinical chemistry and pharmaceutical industry quality control when a lot of samples have to be analyzed.

The developed method is more sensitive than the previously reported methods (2–8) and equally sensitive to a method (9) also previously reported. The proposed method, which is the first FI procedure reporting the determination of FMC, is based on the on-line quantitative thermal-induced digestion of FMC to yield orthophosphate ions according to the reaction

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[2] Abbreviations used: FMC, fosfomycin; FI, flow injection.

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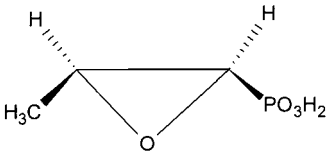

FIG. 1. Chemical structure of fosfomycin.

$$R\text{–}(\text{–}PO_3H_2) + H_2O + S_2O_8^{2-} \rightarrow R\text{–}(\text{–}OSO_3^-) + HSO_4^- + H_3PO_4,$$

where R = C_3H_5O. The orthophosphate ions thus generated were determined using a robust and sensitive molybdenum blue-based FI method originally described by Karlberg and Pacey (10). Ammonium persulfate was used as the oxidizing reagent. The effectiveness of this oxidizing reagent on the on-line digestion of organophosphorus compounds, in both acidic and basic media, is well established (11–13). The incorporation of this on-line digestion step into an FI mode minimized the pretreatment of the samples and offered a rapid and cost-effective procedure for routine determination of the analyte in pharmaceutical products and biological material. Thus, the proposed FI method could be an advantageous alternative to time-consuming chromatographic and batch spectrophotometric methods.

MATERIALS AND METHODS

Reagents and solutions. All chemicals were of analytical reagent grade and were provided by Merck (Darmstadt, Germany) unless stated otherwise and all the solutions were made up with doubly deionized water.

The standard stock solutions of 1.0×10^{-2} mol L^{-1} FMC (Sigma, St. Louis, MO), 200 g L^{-1} ammonium persulfate, and 2.0 mol L^{-1} H_2SO_4 were kept in polyethylene flasks, while working solutions were prepared daily before use.

The reagents for the molybdenum blue method were prepared according to the literature (10). The molybdate ion reagent (10 g L^{-1} ammonium heptamolybdate in 35 ml L^{-1} concd H_2SO_4) was stable for several months, while the stannous chloride reagent (0.2 g L^{-1} $SnCl_2$ plus 2 g L^{-1} hydrazinium sulfate in 28 ml L^{-1} concd H_2SO_4) was stable for at least 1 week if was kept refrigerated and protected from light. Both solutions were degassed with purified nitrogen daily prior to their use.

Special attention was paid to clean effectively all the glassware to avoid phosphate contamination. For this reason all glassware were thoroughly washed with a hot 1:1 HCl solution and then rinsed many times with doubly deionized water.

Apparatus. The FI system used was a Tecator 5010 analyzer with a Type III Tecator chemifold. The detector was a Tecator 5023 FIAstar double-beam spectrophotometer, consisting of a 5032 detector controller and a 5023-011 spectrophotometer optical unit. The absorbance of the colored product was monitored at 690 nm through a 1-cm-path-length flow cell with an 18-μL internal volume. The flow system used was 0.5-mm-i.d. Teflon tubing throughout. Tygon pump tubes were used for delivering the aqueous solutions. A Tecator FIAstar 5101 thermostat was used to control the temperature to the proper value for the on-line digestion of the analyte. Two 10-cm-long/1.0-mm-i.d. pieces of expanded PTFE (e-PTFE, Zeus, Orangeburg, NJ) microporous tubing were placed between the digestion coil and the injection valve and before the detector, to ensure the effective debubbling of the digestion mixture prior to injection and of the reaction product prior to detection.

Procedure for aqueous solutions. The optimized FI setup is depicted in Fig. 2. The sample was merged at equal flow rates with the ammonium persulfate stream (R_3) and the mixture was driven through a 250-cm-long/0.7-mm-i.d. digestion coil (DC) which was thermostated at 90°C. A 10-cm-long/1.0-mm-i.d. e-PTFE microporous tube was placed between the digestion coil and the injection valve to remove possible O_2 and CO_2 bubbles formed prior to injection. Two hundred microliters of the final mixture was injected directly into an aqueous carrier stream (C). The sample zone was merged successively with the molybdate (R_1) and the tin(II)/hydrazinium sulfate (R_2) reagent streams. The molybdenum blue product was formed on passage through a 60-cm-long reaction coil (RC_3) and was measured at 690 nm. A second e-PTFE tube was placed before the detector to ensure that no air bubbles entered the flow cell. Possible free orthophosphate ions

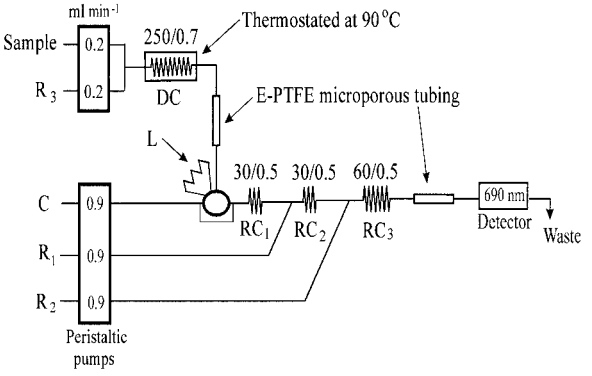

FIG. 2. Optimized FI setup for the determination of fosfomycin. C, carrier stream (water); R_1, molybdate ion stream; R_2, tin ion stream; R_3, ammonium persulfate stream; L, sample loop (200 μL); DC, digestion coil; RC_1, RC_2, RC_3, reaction coils. Numbers above coils denote coil length (cm)/inside diameter (mm) ratio.

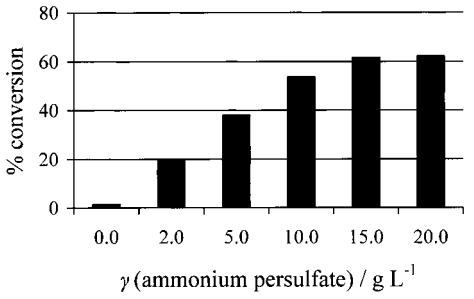

FIG. 3. Effect of the ammonium persulfate mass concentration on the digestion of 2.0×10^{-4} mol L^{-1} fosfomycin; $c(H_2SO_4)$ = 0.2 mol L^{-1}, T = 90°C, sample and digestion reagent flow rates = 0.4 mL min^{-1} each.

existing in the analyzed samples were measured and subtracted in a second run, by replacing the oxidizing reagent (R_3) with water.

Five replicate injections per sample were made in all instances.

Determination of FMC in pharmaceuticals and urine by the proposed FI method. An accurately weighed amount of a selected pharmaceutical formulation was dissolved in doubly deionized water. The resulting solution was analyzed without any other pretreatment using the above-described FI procedure for aqueous solutions.

Urine samples from healthy members of the laboratory were spiked with FMC in a wide concentrations range. In addition, a urine sample was collected from one of the authors 2 h after receiving the usual therapeutic dose of the formulation containing 3.0 g of the analyte. All urine samples were 100-fold diluted in doubly deionized water and analyzed using the above-described FI procedure for aqueous solutions.

RESULTS AND DISCUSSION

Preliminary Studies

Preliminary experiments were focused on whether the analyte could be digested under flow conditions. The manifold used was similar to that depicted in Fig. 2, with the difference being that the flow rates of the sample and oxidant streams were kept equal at 0.4 mL min^{-1} each. A mixture of 10 g L^{-1} ammonium persulfate and 0.2 mol L^{-1} H_2SO_4 was used as the oxidation reagent. The experiments showed that under the above-mentioned conditions and at a temperature of 90°C, ca. 53% conversion was achieved at a 2.0×10^{-4} mol L^{-1} FMC level.

It should also be noted that the originally proposed molybdenum blue-based FI method for the determination of orthophosphates (10) was modified in terms of simplicity. A single carrier stream was used instead of two, while all streams were kept at equal flow rates (0.9 ml min^{-1}).

Optimization of Chemical and FI Variables

The various chemical and FI variables that affect the on-line digestion were studied and optimized at a fixed FMC amount concentration of 2.0×10^{-4} mol L^{-1} using the univariate approach, with the exception of the effect of the sample oxidant flow rates, which were studied at three amount concentrations (2.0, 3.0, and 4.0×10^{-4} mol L^{-1} FMC). The digestion coil was 250 cm long and 0.7 mm in i.d. and the flow rates of the sample and oxidant and the digestion temperature were as in the preliminary studies (0.4 ml min^{-1} each and 90°C, respectively).

The effect of ammonium persulfate mass concentration on digestion was studied in the range 0–20 g L^{-1} in 0.2 mol L^{-1} H_2SO_4 in all cases. The experimental results are depicted in Fig. 3. As can be seen, maximum and constant percentage conversion (ca. 60%) was achieved above 15 g L^{-1} ammonium persulfate. A mass concentration of 20 g L^{-1} was therefore chosen as optimal.

The effect of the amount concentration of H_2SO_4 in the digestion mixture was studied in the range 0–0.5 mol L^{-1}. The experimental results shown in Fig. 4 indicate that maximum percentage conversion (ca. 85%) was achieved in the range 0–0.05 mol L^{-1} H_2SO_4. We therefore chose not to use H_2SO_4 in further experiments. It should also be noted that acid amount concentrations above 0.3 mol L^{-1} caused the appearance of "double peaks" due to the formation of a low pH gradient in the center of the injected zone, suppressing the molybdenum blue reaction.

The effect of the sample and oxidation reagent flow rates is a critical variable, as it influences the time that the digestion is allowed to proceed in the digestion coil. As mentioned above, the study was performed with three FMC amount concentrations, namely, 2.0, 3.0, and 4.0 mol L^{-1}. The sample-to-persulfate flow rate ratio was kept at 1:1 in all cases. The experimental results shown in Fig. 5 clearly demonstrate that using a flow rate of 0.2 mL min^{-1} for each stream, 100% conversion and, thus, maximum determination range

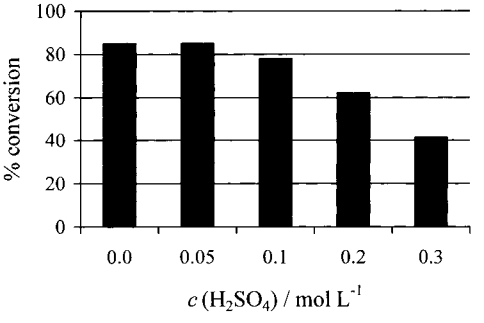

FIG. 4. Effect of the H_2SO_4 amount concentration on the digestion of 2.0×10^{-4} mol L^{-1} fosfomycin; γ(ammonium persulfate) = 20 g L^{-1}, T = 90°C, sample and digestion reagent flow rates = 0.4 mL min^{-1} each.

were achieved up to a FMC amount concentration of 3.0×10^{-4} mol L^{-1}. The value of 0.2 ml min^{-1} was therefore chosen as optimal.

Finally, the effect of digestion temperature was studied in the range 25–90°C, under the optimal variables described above. No conversion of the analyte was observed below 50°C, while quantitative conversion was achieved above 80°C. A temperature of 90°C was chosen as optimal, so that small variations do not influence the oxidation reaction and, thus, the precision and the accuracy of the method.

Features of the Proposed FI Method

Using the FI setup shown in Fig. 2 and under the optimal conditions described above, a FMC calibration graph was obtained that was linear over the range 3.0×10^{-6} to 3.0×10^{-4} mol L^{-1} and was described by the equation

$$A = 0.002(\pm 0.003) + [0.347(\pm 0.011)] \times 10^{4} c(\mathrm{FMC}),$$

where A is the absorbance as measured by the detector, and c(FMC) is the amount concentration of the analyte, with a relative standard deviation of $s_r = 1.2\%$ (at 1.0×10^{-4} mol L^{-1} FMC, $n = 12$), a correlation coefficient of $r = 0.9996$, and a 3σ detection limit of 1.0×10^{-6} mol L^{-1} ($n = 10$). Reproducibility studies before, after, and within the analysis of the samples were always performed and in all cases the results obtained were similar to the relative standard deviation of the method.

All standards were run in five replicate injections ($n = 5$).

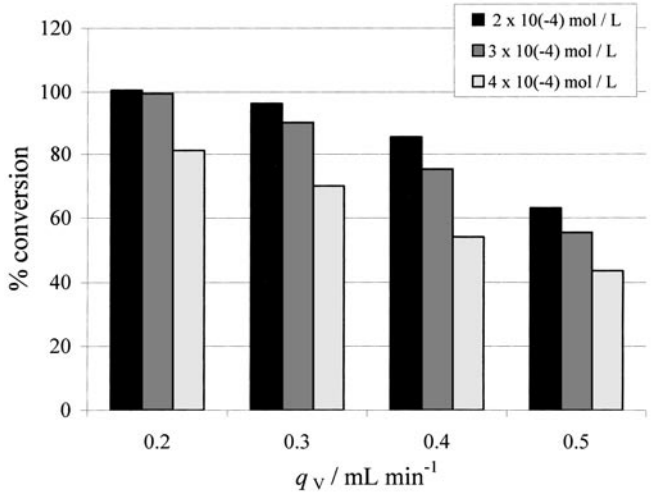

FIG. 5. Effect of the sample and ammonium persulfate reagent flow rates on the digestion of 2.0, 3.0, and 4.0×10^{-4} mol L^{-1} fosfomycin; γ(ammonium persulfate) = 20 g L^{-1}, $T = 90$°C.

TABLE 1

Determination of Fosfomycin in Urine Samples by the Proposed FI Method

Urine sample[a]	FMC added (mg L^{-1})	FMC found[b] (mg L^{-1})	Recovery (%)
Spiked I	300.0	289.3	96.4
Spiked II	500.0	493.5	98.7
Spiked III	750.0	763.2	101.8
Spiked IV	1000.0	1025.0	102.5
Spiked V	1250.0	1215.8	97.3
Urine 2 h[c]	—	680.5	
Urine 2 h[c]	500.0	1164.0	96.7

[a] One hundred-fold dilution before analysis.
[b] Mean of five results.
[c] Urine samples collected 2 h after oral administration of a usual drug dose.

Analysis of Real Samples

The proposed FI method was applied to the determination of FMC in a pharmaceutical preparation for the treatment of cystitis (Monurol), in spiked urine samples, and in a urine sample collected 2 h after oral receipt of the usual dose of the drug (3.0 g FMC). The pharmaceutical preparation was found to contain 0.368 g FMC g^{-1} (labeled value, 0.375 g FMC g^{-1}). Urine samples free of the analyte were spiked with FMC in the concentration range usually found in urine a few hours after administration of the drug Monurol (706 ± 466 mg L^{-1} FMC 2–4 h after administration of the drug) (1). The experimental results of the urine analysis are shown in Table 1. Recoveries of the analyte from the spiked samples were very satisfactory (96.4–102.5%), while the FMC found in a urine sample collected 2 h after oral administration of the usual therapeutic dose of Monurol was within the expected values mentioned above (1).

CONCLUSIONS

The proposed method is the first FI procedure reporting the determination of the antibiotic fosfomycin. The method offers high sensitivity, robustness, a sampling rate of 60 h^{-1}, and minimum sample pretreatment prior to analysis. It is advantageous over chromatographic methods in terms of simplicity, cost efficiency, and analysis time. Application of the developed method to pharmaceutical and urine samples produced very precise and accurate results.

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