

Comparison of Programmable *versus* Single Wavelength Fluorescence for the Detection of Three Fluoroquinolone Antibacterials Isolated from Fortified Chicken Liver Using Coupled On Line Microdialysis and HPLC

Summary

A recently introduced programmable fluorescence detector was compared with a single wavelength fluorescence detector for quantification of fluoroquinolone (FQ) antibacterial agents, which have widely varying spectral characteristics. The two detectors were connected in parallel to an HPLC system to test their performance characteristics. With single wavelength detection, two FQs, flumequine and oxolinic acid could be detected at an emission wavelength of 368 nm in a single chromatogram while a third FQ, sarafloxacin, was not observed at that wavelength. Similarly, when the detector was optimized for sarafloxacin emission at 440 nm, the other two compounds were undetected. In contrast, all three FQs were quantified at their individual maxima in a single run using the programmable fluorescence detection. The applicability of an HPLC – programmable fluorescence detector, in combination with on-line microdialysis, also was evaluated using chicken liver fortified at low ppb levels with the three FQs. After on-line microdialysis sample clean up, the resultant HPLC chromatograms were free of background interference enabling the programmable detector to optimize the quantitation of the three analytes in a single run. The limit of quantification (LOQ) determined for each FQ was 1.0 ppb and the limit of detection (LOD) was 0.2 ppb, an order lower in magnitude than was obtainable with single wavelength detection.

1 Introduction

To help protect the nation's food supply, the Food Safety Inspection Service (FSIS, USDA) monitors the levels of chemical residues such as antibacterials and pesticides in meat and meat products [1]. Many of the procedures used to monitor these residues are time consuming and solvent intensive. Therefore, FSIS and other regulatory agencies require replacement of many of their current analytical technologies to increase sample throughput and to comply with EPA regulations which require reduction in use of certain organic solvents in Federal laboratories [2]. For these reasons, on-line microdialysis is under investigation in this laboratory as a potential replacement for more traditional solvent intensive isolation and clean up methodologies. On line microdialysis is an automated aqueous-based technique for sample clean up where the analytes of interest are separated from other components in biological matrices by means of a flow through dialysis membrane. The analytes then flow onto a concentrator column and finally into an HPLC system for detection and quantification. On-line microdialysis typically is performed using a commercial ASTED™ XL system (Automated Sequential Trace Enrichment of Dialysates)¹⁾. Analytes isolated using this technol-

ogy include: drugs, food additives, toxins, bioamines, vitamins and pesticides [3–6]. However, there are few references for application of this technology to ppb-ppm levels of chemical contaminants in complex food matrices.

All of the reported applications to date, using HPLC and fluorescence detection in combination with on line microdialysis, have used single wavelength detectors [3, 4, 7, 8]. This combination may pose problems in multi-residue sample clean up and detection when analytes in the dialysate possess widely different fluorescence emission and excitation maxima. In such a situation, complete quantification may require multiple HPLC chromatographic runs at different wavelengths. In an attempt to overcome this problem and to expand its capability to carry out multi-residue analyses, we combined the ASTED XL-HPLC system with a recently introduced programmable wavelength fluorescence detector. Comparison of the performance characteristics of the single vs. the programmable wavelength detectors was carried out using three antibacterial agents, sarafloxacin (SAR), oxolinic acid (OXO), and flumequine (FMQ). Two of these drugs, SAR and FMQ, are members of the fluoroquinolone (FQ) drug class. Oxolinic acid, however, is structurally related but lacks the fluorine group and for the purpose of this paper is considered a FQ (Figure 1). The widely different spectral characteristics of these three drugs provided the basis for the comparison of the performance characteristics of the two detectors.

In addition to completing this comparison, we also assessed the performance of the programmable detector combined with an ASTED XL™ –HPLC system for the sample clean up and analysis of the three FQs isolated from fortified chicken liver. The use of HPLC and programmable fluorescence detection in conjunction with this sample clean up technology demonstrated the potential applicability of such a system for the development of multiresidue isolation and detection methods for these antibacterial agents from biological matrices.

2 Experimental

2.1 Chemicals and Reagents

Sarafloxacin hydrochloride (SAR) (purity = 88.5%) was obtained from Abbott Laboratories (North Chicago, IL). Oxolinic acid (OXO) (purity = 100%), flumequine (FMQ) (purity = 100%), sodium hydroxide, sodium dibasic phosphate, and sodium mono-basic phosphate were purchased from Sigma (St. Louis, MO). Triethylamine (HPLC-grade) and phosphoric acid

¹⁾ See end of paper for a list of abbreviations.

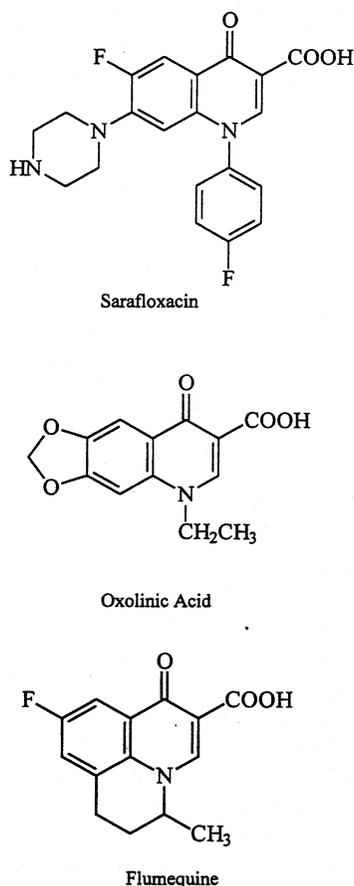


Figure 1. Chemical structures of sarafloxacin, oxolinic acid, and flumequine.

85% (HPLC-grade) were purchased from Fisher Scientific (Fairlawn, NJ). Nanopure water was obtained from a Barnstead NANOpure filter & deionizer unit (Dubuque, IA). All solvents were HPLC-grade obtained from Burdick & Jackson (Muskegon, MI), and all buffered and non-buffered solutions prepared for this study were filtered through a 0.2 μm pore filter prior to use.

2.2 Preparation of Standards

A stock solution of SAR, OXO, and FMQ (100 $\mu\text{g}/\text{mL}$) was prepared by dissolving 10 mg of each drug as received (except SAR, which was corrected for listed purity – 88.5% as free base) in 100 mL of 0.03 M sodium hydroxide. These solutions were stored in amber glass bottles at 4°C when not in use and were stable for at least 3 months [10]. A working solution of 1 $\mu\text{g}/\text{mL}$ was prepared daily by diluting the standard stock solutions with 0.03 M sodium hydroxide. Standard solutions were prepared by diluting the working solutions (1000 ppb) with buffer A (0.1 M sodium phosphate, pH 9.0) to final concentrations for HPLC analysis, of 100, 50, 25, 10, 2, and 0.2 ng/100 μL using the ASTED XL™ dilution program.

2.3 Chromatography and Fluorescence Detection

The ASTED XL was connected to a Rainin HPLC system (Rainin Instrument Co. Inc., Woburn, MA) consisting of two

model HPXL pumps controlled by a Macintosh computer using Dynamax system software V.1.3. Standard solutions, containing the three FQs, were handled by the autosampler component of the ASTED XL™ that loaded the analytes into a 100 μL injection loop. The three FQs then were eluted using a gradient mobile phase of the following composition: (a) 0.025 M phosphoric acid adjusted to pH 2.7 with triethylamine (TEA), and (b) acetonitrile. Both fluids were degassed using an in-line Degasser (Degasys DG-1310, Rainin Instrument Co. Inc., Woburn, MA). The initial mobile phase composition was a ratio of 65:35 v/v buffer/acetonitrile. This ratio was gradually changed to 42/58 v/v buffer/acetonitrile over 9 min, and held for 1 min. The ratio was then gradually changed back to 65/35 v/v buffer/acetonitrile within 5 min, and held for 3 min before the next injection. Analytical separations were achieved on a 250 mm \times 4.6 mm i.d. 5 μm Supelco-sil-ABZ + Plus column (Supelco, Inc., Bellefonte, PA) at a flow rate of 1 mL/min.

The two detectors compared were a single wavelength detector (FL-750 HPLC-Plus spectrofluorimeter and 750-03 Universal lamp power supply, McPherson Instruments, Acton, MA), and a multi-wavelength JASCO FP-920 Intelligent Fluorescence Detector (Jasco International Co., Ltd., Easton, MD). Both detectors were connected in parallel to the HPLC system, however, a switching valve directed the mobile phase to either one alternatively, for comparisons. Detector signals were processed using the Rainin Dynamax HPLC Method Manager Ver.1.3 (Rainin Instrument Co., Emeryville, CA) installed on a Macintosh classic personal computer (Apple Computers Inc., Cupertino, CA).

2.4 Dialysis – ASTED

Microdialysis was performed on the Gilson ASTED XL™ system (Gilson, France), comprised of the following components: autosampler tray (or sample rack); two syringe pumps (model 401C) with 5 mL and 1 mL syringe volumes; flat-bed dialysis block (370 μL donor volume), equipped with a Cuprophan™ (cellulose acetate) membrane (15 kD MWCO); 4.6 mm i.d. \times 5.8 mm trace enrichment cartridge (TEC) containing 10 μm particle size Hypersil™ ODS packing, using a 5 μm frit (Keystone Scientific, Inc., Bellefonte, PA); two Rheodyne 6-port switching valves; and a controller keypad supplied with controller software 722 version V.2.00. The methods used to prepare samples for analyses on the ASTED and the operation of this system, which includes the isolation of analytes from fortified chicken liver, have been reported elsewhere [8].

2.5 Automation

Automation of the ASTED XL could not be achieved when it was combined with the single wavelength detector. However, complete automation of the microdialysis, trace enrichment, and HPLC system was achieved using the programmable detector, by adding a programmable microprocessor controller (ChronTrol Corporation, San Diego, CA) to the system. This controller coordinated and integrated operations between the ASTED XL™, the HPLC software run on the Macintosh, and the programmable detector. It also provided a means to automatically shut down the system when analyses were completed.

3 Results and Discussion

The three FQs chosen as the target analytes in this study are a relatively new class of synthetic antibacterial drugs, some of which have recently been approved for use in food production animals. Flumequine and OXO are commonly used in aquaculture, while SAR was recently approved for use in poultry [9]. Extensive use and misuse of these compounds in both human and veterinary medicine may lead to the emergence and spread of resistant bacterial strains [10]. Because of these factors, rapid sensitive multi-residue methods for members of this class of antibacterials are needed.

The FQs exhibit a high degree of water solubility and fluorescence activity, thus lending themselves to analysis using aqueous-based instrumental techniques such as on-line microdialysis coupled with HPLC and fluorescence detection. Our initial attempts to develop a multiresidue detection method for SAR, OXO, and FMQ using HPLC with single wavelength fluorescence detection were unsuccessful. This result may be attributed to the chemical structures of the three analytes, which have structurally similar FQ backbones (Figure 1), but widely varying emission and excitation maxima. The problem occurring with single wavelength detection of compounds with differing spectral characteristics can be noted from the chromatograms shown in Figure 2. Figure 2a and 2b are two HPLC chromatograms of the three FQ standards at different emission and excitation settings using single wavelength detection. Two of the three drugs in the injected mixture, OXO and FMQ, have similar fluorescence spectra and were detected at their optimal excitation and emission wavelengths of 318 nm and 368 nm, respectively, whereas, the third FQ in the mixture, SAR, could not be detected at these settings. Conversely, when the detector was set at the optimal excitation and emission wavelengths for SAR, of 278 nm and 440 nm, respectively, OXO and FMQ could not be detected (Figure 2b).

To overcome this spectroscopic limitation, we evaluated a new type programmable fluorescence detector in comparison with our single wavelength detector. The two detectors were connected in parallel to the ASTED-HPLC system and their performances were subjected to side by side evaluations. A set of five standard mixtures of the three FQs in decreasing concentrations were injected into the HPLC system alternatively directed to each detector by means of a switching valve. This side by side comparison demonstrated that while multiple analyses were necessary using the single wavelength detector, the programmable fluorescence detector could quantify all three analytes in a single analysis with a linear range of 1–100 ppb (Figure 2c). The response of the programmable detector for the FQ standards at the 2 ppb level is about ten fold greater than the response for the three analytes using the single wavelength detector as is evident from the milli-volt scales in the chromatograms in Figure 2. For the single wavelength detector, the limit of quantification (LOQ) was experimentally determined as 5.0 ppb, and the LOD was 2.0 ppb, based on a determination of the mean value of the matrix blank readings plus three standard deviations of the mean, whereas the LOQ for the programmable detector was 1.0 ppb with an LOD of 0.2 ppb. The peak broadening observed in the two single wavelength chromatograms (Figure 2a and 2b) was not evident in the chromatogram obtained with the programmable detector (Figure 2c). These differences in peak broadening

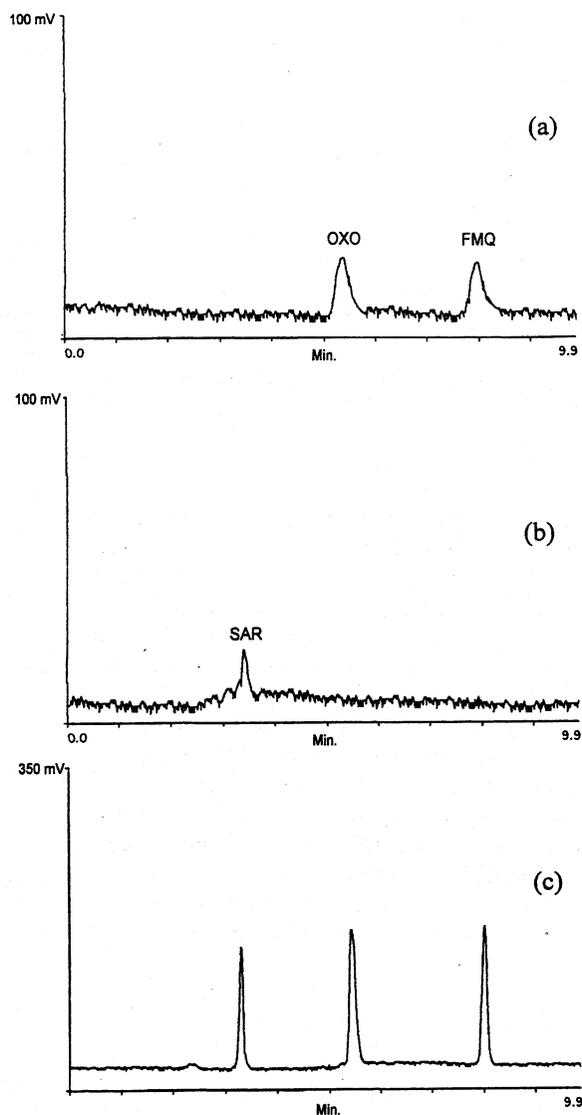


Figure 2. HPLC fluorescence chromatograms of a mixture of SAR, OXO, and FMQ standards with concentrations of 2 ppb each: (a) single wavelength detection λ_{ex} 278 nm, λ_{em} 440 nm; (b) single λ – λ_{ex} 318 nm, λ_{em} 368 nm; (c) programmable wavelength detection – initial conditions: λ_{ex} 278 nm, λ_{em} 440 nm; automatically switched at 5 min to: λ_{ex} 318 nm, λ_{em} 368 nm.

may be attributable in part to the flow-cell characteristics of the two detectors and to the advanced optics design of the programmable detector. In addition, the programmable detector uses a built-in thermistor to monitor optical bench temperature and automatically compensates for temperature variation via feedback through its central processing unit. This feature also may contribute to the universally low background noise observed in the chromatogram in Figure 2c as compared to those obtained with single wavelength detection (Figure 2a and 2b).

The applicability of the programmable detector for multiresidue analysis in conjunction with the ASTED XLTM was tested using chicken liver samples fortified at the 5.0 ppb level with the three FQs. An HPLC chromatogram of the three compounds isolated from chicken liver is shown in Figure 3. This chromatogram

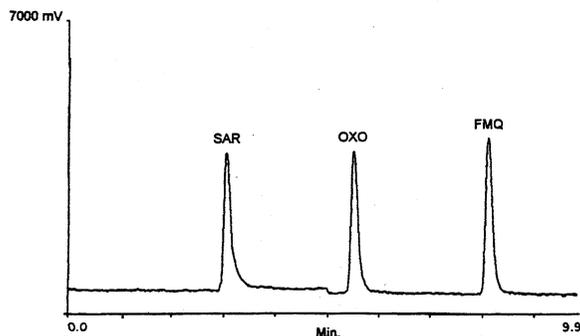


Figure 3. HPLC programmable fluorescence chromatogram of tissue extracts from chicken liver fortified at 5 ppb each with SAR, OXO, and FMQ.

illustrates the benefits of automatic wavelength switching possible with programmable fluorescence detection since all three analytes were quantified at their optimal emission and excitation maxima. Figure 3 also demonstrates the potential utility of on-line microdialysis for clean up of biological samples since this chromatogram is free of background interference which could affect quantitation of the analyte peaks. Additionally the trace enrichment feature of the ASTED acts to concentrate the analytes of interest, which are then back-flushed directly into the HPLC system to give increased signal response. Using the combined ASTED-HPLC-programmable fluorescence detection system, overall mean recoveries and their relative standard deviations obtained for SAR, OXO, and FMQ were $95.0 \pm 4.2\%$, $97.0 \pm 4.1\%$, and $88.0 \pm 3.6\%$, respectively. Equivalent recoveries were obtained from chicken liver tissues fortified at the 1, 5, 10, 50, and 100 ppb levels.

4 Conclusion

This study demonstrates the advantages of using programmable fluorescence detection over single wavelength detection for the quantification of ppb level mixtures of FQs. This new type detector provided a higher level of sensitivity and baseline stability than was previously obtainable using fluorescence detection and significantly enhanced the capabilities of the combined automated on-line microdialysis sample clean up technique. In this instance we achieved the isolation and detection of picogram

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Abbreviations

ASTED, Automated Sequential Trace Enrichment of Dialysates; FQ, fluoroquinolone; FMQ, flumequine; OXO, oxolinic acid; SAR, sarafloxacin; TEC, trace enrichment column; HPLC, high-performance liquid chromatography.

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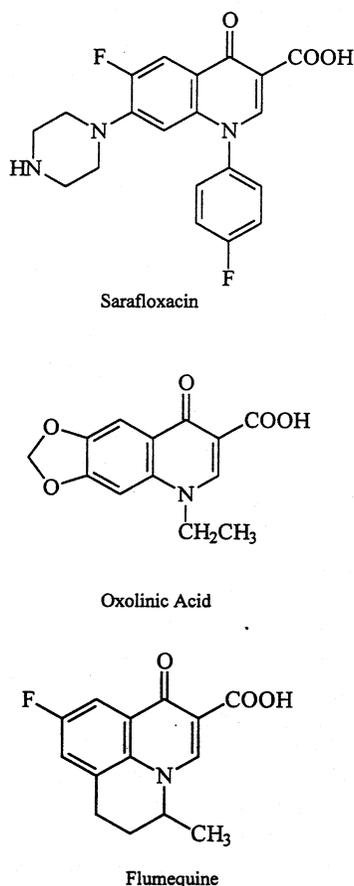


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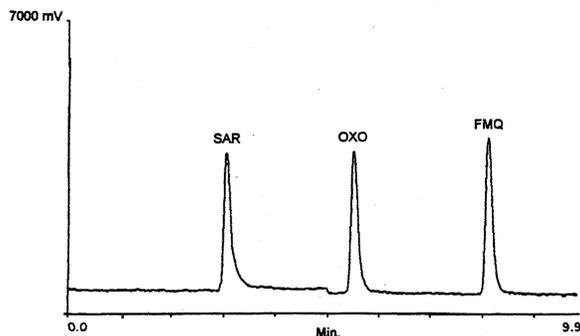


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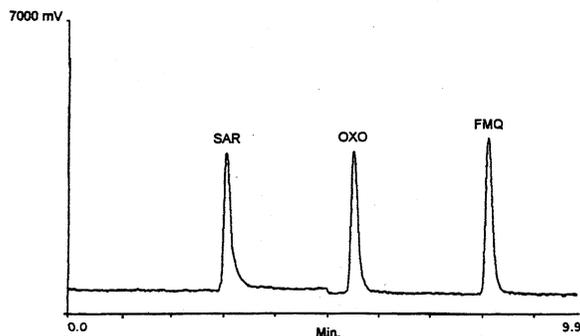


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