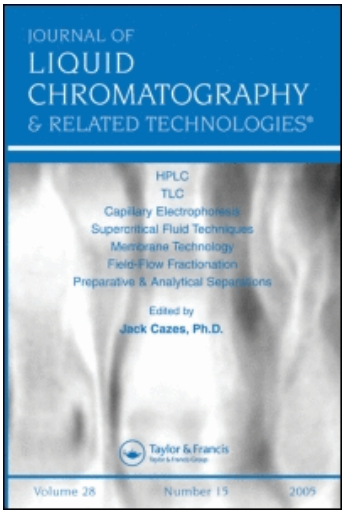

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COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MINOCYCLINE OF NUDE MICE SERUM

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ABSTRACT

A rapid, direct, accurate and sensitive liquid chromatographic assay with on-line extraction for minocycline of nude mice serum is described. Serum was directly injected onto the extraction column where the drug was separated from the serum concomitants using a solid phase extraction procedure based on cation exchange. Using an on-line column switching system, minocycline was quantitatively transferred and separated on the analytical column. Ultraviolet absorption at 352 nm was used for detection. The assay was linear from 0.2 to 10 μg/mL with a correlation coefficient of 0.999. Detection limit was 50 ng/mL. Recovery was 97%.

The method has been developed for the determination of minocycline levels of nude mice serum collected from a study designed to evaluate the potential for tumor anti-angiogenic effects of this drug.

INTRODUCTION

Minocycline is a semisynthetic tetracycline antimicrobial. It is rapidly absorbed from the gastrointestinal tract, is widely distributed in body tissues and fluids, and has a prolonged half-life.[1] Tetracycline derivatives, long used clinically for their antibiotic effects, have recently been shown to have potential benefit in the treatment of cancer.[2,3] Although the mechanism of this antitumor effect has not yet been definitively elucidated, tetracyclines have been shown to inhibit tumor angiogenesis,[4] presumably due to inhibition of collagenase.[5,6]

Our purpose was to develop a simple and sensitive assay for quantitation of minocycline in serum of nude mice as part of a study programmed to investigate the correlations between serum levels and the effects of the drug on human tumors transplanted into nude mice. Previously, the determination of minocycline in biologic specimens has been difficult because of the poor specificity of microbiological and fluorometric assays. High pressure liquid chromatography (HPLC) has become the preferred method of assay, but previous techniques involved complicated and time consuming procedures.[7,8]

We propose a column-switching HPLC method involving direct injection of serum samples into the chromatographic system to simplify the assay and decrease the potential of introducing bias into the results. A similar chromatographic system has been used to determine other drugs in biological fluids[9,10] which gave excellent results.

EXPERIMENTAL

Reagents and Chemicals

Minocycline hydrochloride was purchased from Sigma Chemical Co. (St.Louis, MO, USA). Acetonitrile was HPLC grade and purchased from E. Merck (Darmstadt, Germany).

Both sodium dihydrogenphosphate mono-hydrate and di-sodium hydrogen orthophosphate dihydrate were supplied by Carlo Erba Farmitalia (Milan, Italy). All chemicals were reagent grade.

Analytical grade, filtered water was obtained fresh daily from an Elgastat UHQ PS apparatus (ELGA, High Wycombe, Bucks, UK). Pooled drug-free serum of nuce mice was used to prepare standards.

Apparatus

The liquid chromatograph consisted of a Beckman 126 Programmable Solvent Module (Beckman, Fullerton, CA, USA), a Varian model Vista 5500 pump (Walnut Creek, CA, USA), and a Beckman 166 Programmable Detector Module. The injector was a Rheodyne model 7725i manual injection valve, fitted with a 200 μL sample loop. The chromatograms were integrated with a System Gold Laboratory Data System (Beckman, Fullerton, CA, USA). The coupled-column system was operated by a pneumatic, six-port, automated switching valve (Valco, Schencon, Switzerland) controlled by the HPLC system.

Chromatographic Conditions

The extraction column (50 mm x 4 mm I.D.) was dry-packed with a 40 μm WCX stationary phase obtained from an ion-exchange SPE cartridge (LC-WCX) from Supelco (Bellefonte, PA, USA). The analytical column was an Ultrasphere C_{18} (25 cm x 4.6 mm, particle size 5 μm) from Beckman (Fullerton, CA, USA). Mobile phase 1 consisted of a mixture of 2.5% methanol in 0.01 M phosphate buffer (pH 7.0); mobile phase 2 consisted of 14% acetonitrile in 0.1 M phosphate buffer (pH 3.0). Sample aliquots of 200 μL (mouse serum + mobile phase 1, 1:3, v/v) were injected directly onto the pre-column. Mobile phase 1 was used as washing solvent to eliminate the biological matrix from the extraction column. The flow-rate for both columns was set at 1 mL/min. The effluent from the analytical column was monitored by UV at a wavelength of 352 nm. All analyses were performed at ambient temperature.

Column Switching Procedure

Figure 1 shows the scheme for the column switching procedure. Serum samples were diluted 1:3, v/v, with phosphate buffer 0.01 M (pH 7.0) to avoid clogging of column frits. Sample aliquots of 200 μl were injected directly into the chromatograph. Sample was brought onto the pre-column by mobile phase 1 (2.5% methanol in phosphate buffer 0.01 M) delivered by pump 1, at a flow-rate of 1 ml/min, and directed to waste, while the pump 2 delivered the mobile phase 2 (14% acetonitrile in phosphate buffer 0.1 M) to the analytical column. The components of the serum matrix were removed whilst minocycline was trapped in the column (Figure 1A). After two minutes the valve was switched and mobile phase 2 from pump 2 eluted the analyte of interest to the analytical column (Figure 1B). The low pH of the mobile phase 2 promotes the elution of

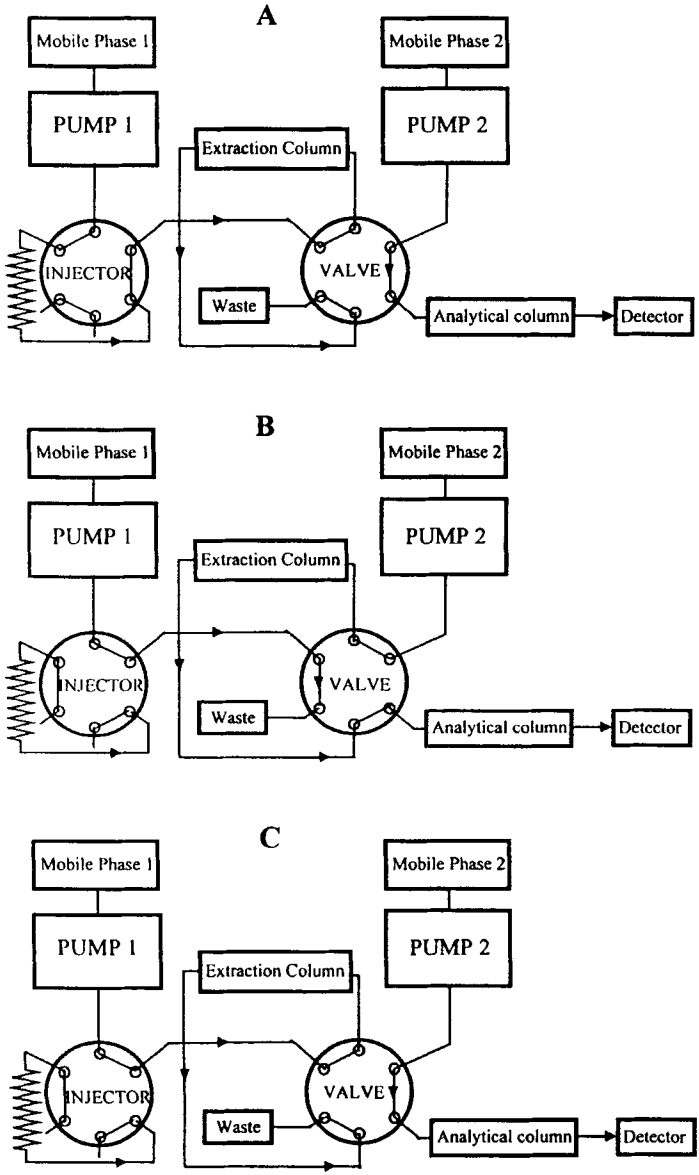

Figure 1. Scheme of column switching.

the analyte from the cation-exchange sorbent of the extraction column. The connection time of the pre-column to the secondary column was 2 minutes. At this stage, the valve was switched to the initial position and pump 1 delivered the mobile phase 1 to the extraction column to prepare it for the next sample, while pump 2 maintained the flow of mobile phase 2 through the analytical column (Figure 1C) where minocycline was separated and detected by UV. The retention time of minocycline was 6.5 minutes and cycle time of one analysis was 10 minutes.

Recovery

Standard solutions were prepared by dissolving minocycline in distilled water and stored in the dark at +4 °C. The drug was found to be stable for up 1 week. Working solutions were prepared daily by dilution of the stock-solutions. Control serum samples were spiked with minocycline standard solutions to reproduce different concentrations in the 0.2 - 10.0 μg/mL range. The percentage of drug recovered for a particular injection was calculated by comparing the peak area obtained from the spiked sample with the value obtained from direct injection onto the analytical column of aqueous solution containing the same concentration of drug.

Quantification

Calibration standards were prepared by spiking control serum at concentrations of minocycline ranging from 0.2 to 10.0 μg/mL. Each spiked serum standard was injected eight times. These samples were processed as described above. Peak areas were plotted *versus* minocycline concentrations and the resulting calibration curve was used to calculate the drug concentrations of the unknown samples.

RESULTS

Optimization

Various experiments were conducted to establish the optimal conditions. In order to optimize the extraction process, C_8 and C_{18} stationary phases were tested for retention and elution. Serum was deproteinized with trichloroacetic acid. Both C_8 and C_{18} gave a satisfactory retention of the drug, but elution of the analyte was difficult. The compound of interest exhibited a broad tailing

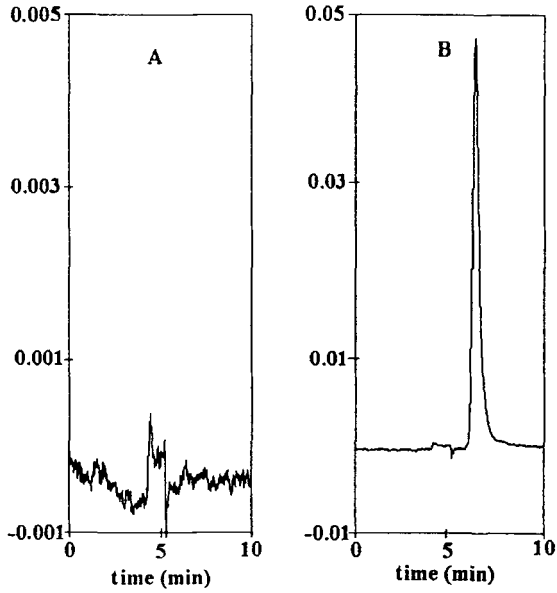

Figure 2. Chromatograms of (A) drug-free serum of nude mice and (B) serum spiked with 5 μg/mL minocycline.

peak, and many interfering substances were present. To enhance the chromatographic profile, a weak cationic exchanger was tested. The packing chosen, a WCX (carboxylic acid, Na^+ counterion), was obtained from a solid phase extraction cartridge from Supelco. Because of its pKa value of 4.8, the functional group is negatively charged at neutral pH and it can bind amino groups as those of the minocycline molecules. In fact, the dimethylamino group at C-4 has a pKa value of 9.3[11] and, at neutral pH, it exhibits a positive charge. The column was washed for two minutes with 2% methanol in 0.01 M phosphate buffer (pH 7.0) that washed off most unwanted material and did not remove the drug.

After retaining the isolate, the pre-column can be washed with up to 5 mL of mobile phase without displacing the isolate from the packing. The analyte can then be eluted from the extraction column with an acidic buffer. Phosphate buffer, 0.1 M, pH 3.0, containing 14% acetonitrile, eluted minocycline by suppressing ionization of the tertiary amine at C-4 of the molecule. In this way, we obtained peaks better resolved from the extraction column using a concentration of organic solvent still optimal for the subsequent separation in the analytical column. In preliminary experiments, it was found that the

Table 1

Linearity, Precision, and Accuracy for Minocycline*

Nominal (μg/ml)	Actual value (mean ± SD, n=8) (μg/ml)			Precision %	Accuracy %
0.2	0.209	±	0.005	2.39	+4.50
0.5	0.472	±	0.017	3.60	-5.60
1.0	1.047	±	0.045	4.30	+4.70
2.0	1.930	±	0.098	5.08	-3.50
5.0	4.890	±	0.120	2.45	-2.20
10.0	10.230	±	0.230	2.25	+2.30

*$Y = (3.67 \pm 0.09)X + (-0.22 \pm 0.15)$

concentration of acetonitrile at 14% gave the optimum separation with a reasonable chromatographic time. The connection time of the pre-column to the analytical column was optimized by stepwise reduction until the peak area of minocycline started to decrease. To avoid clogging column frits, serum was diluted with phosphate buffer.

The equilibration of both the pre-column and the analytical column after an analysis can be achieved in a few minutes so that total analysis time was 10 minutes.

Representative chromatograms from blank and spiked serum of nude mice with minocycline are shown in Figure 2. In the chromatograms, no peak was present that might interfere with the determination of minocycline, thus demonstrating the clean-up efficiency.

Recovery

The column-switching procedure gave nearly complete recovery of minocycline from serum. Absolute recovery of the drug was calculated by comparing the peak areas after direct injections of minocycline with those of equal amounts of analyte "extracted" from serum. Values obtained were 97% (n=8) for the concentrations that were examined.

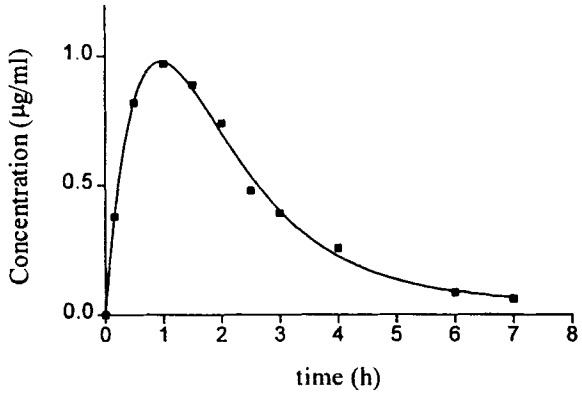

Figure 3. Serum concentration-time curve of minocycline from a nude mouse after 10 mg/Kg oral administration.

Linearity, Precision, Accuracy, and Sensitivity

The calibration curves obtained were linear over the working interval 0.2 - 10.0 µg/mL of minocycline. The regression line obeyed the equation $Y = (3.67 \pm 0.09)X + (-0.22 \pm 0.15)$; the correlation coefficient being $r^2 = 0.999$. The accuracy was +4.50% for the lower limit of quantitation and +2.30% for 10 µg/mL. Values for precision (coefficient of variation) at these concentrations were 2.39 and 2.25%, respectively. The results obtained are summarized in Table 1. The detection limit with 50 µL of serum was 50 ng/mL. This limit corresponds to a serum concentration resulting in a peak height equal to 3 times the signal-to-noise ratio.

Application

The procedure described above was employed to determine the serum concentrations of minocycline after oral administration to 5 nude mice. The pharmacokinetic parameters (means ± SD) calculated in this study, terminal half-life ($t_{1/2b}$), area under the serum concentration-time curve (AUC), absorption rate constant (Ka), serum clearance (Cl_s), volume of distribution of the central compartment (V_1) and mean residence time in the central compartment (MRT_1) are given in Table 2. Figure 3 shows a typical serum profile of minocycline obtained after oral administration of a 10 mg/Kg dose to a nude mouse.

Table 2

Pharmacokinetic Parameters of Minocycline after Oral Administration of 10 mg/Kg*

$t_{1/2b}$ (h)	AUC (mg h/L)	K_a (h)	V_1 (L/Kg)	Cl_s (ml/min Kg)	MRT_1 (h)
4.90 ±1.22	3.20 ±0.09	1.15 ±0.01	3.20 ±0.06	52.06 ±1.49	1.03 ±0.02

*Means ± S.D.

DISCUSSION

Only a few HPLC techniques have been published which analyze minocycline in biological fluids. They involve complicated and time-consuming procedures which also increase the potential of introducing a bias in the results. Previous methods have necessitated ethyl acetate extraction and evaporation,[7] ion-exchange systems, or complex mobile phases and gradient elution.[8] Birmingham's method[12] requires time-consuming sample preparation and the use of an internal standard, which prolongs analysis time. Moreover the sensitivity of the method is lower than that obtained with the proposed technique.

Classical preparation techniques involve numerous steps, which can cause loss of the compounds of interest. In recent years, an increasing number of HPLC methods incorporating on-line sample clean-up by solid phase extraction, using column switching, have been developed. With column-switching techniques, the manual sample preparation steps are drastically reduced, or even eliminated. As a result, the accuracy and precision that can be obtained in the determination of drugs in biological fluids is generally improved Additionally, the time required for processing the samples with this latter technique is greatly shortened, and the selectivity that can be achieved is comparable with, and sometimes better than, that obtained with traditional sample pre-treatment. There are additional advantages of column-switching over classical procedures for sample clean-up. Good precision and accuracy can be achieved without the need for an internal standard. Moreover, sample clean-up by column switching protects light-sensitive analytes from light during the analysis. The proposed method has all these advantages.

CONCLUSIONS

In conclusion, the proposed method is extremely simple and rapid since it requires no extraction or cleanup steps and no internal standard. The diluted sample is directly injected onto the chromatographic column. The assay offers appreciable accuracy and precision. The selectivity of the method, in regard to endogenous compounds, is very satisfactory; no interference was found. High sensitivity can be achieved in a relatively short analytical time. Moreover the method is relatively inexpensive.

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