ACTA CHROMATOGRAPHICA, NO. 19, 2007

# DEVELOPMENT AND VALIDATION OF AN HPTLC METHOD FOR DETERMINATION OF MINOCYCLINE IN HUMAN PLASMA

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## SUMMARY

A new high-performance thin-layer chromatographic (HPTLC) method has been established for determination of minocycline in human plasma. Chromatography was performed on aluminium plates coated with silica gel $60F_{254}$; the mobile phase was methanol–acetonitrile–isopropanol–water 5:4:0.5:0.5 (*v*/*v*). Densitometric analysis was performed at 345 nm. The method is rapid (single-step extraction with methanol), sensitive (limit of quantification 15.4 ng per zone), precise ($CV \leq 4.61$ %), accurate (drug recovery 95.08–100.6%), and linear over the range 100–1200 ng per zone. Recovery of minocycline from plasma samples was 95.8 ± 4.5%. The half-life of minocycline in plasma was 9.9 h at 4°C and 6.3 h at 20°C. Minocycline is stable in human plasma for at least two months at −20°C and can tolerate two freeze–thaw cycles with losses <10%. The method was successfully used to determine therapeutic levels of minocycline.

## INTRODUCTION

Minocycline, (4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide, is a tetracycline derivative with a bacteriostatic effect as a result of inhibition of protein synthesis. It is regarded as one of the most effective drugs against chlamydial infections, mycoplasmal infections, brucellosis, and periodontal pathogens [1]. Increased interest in clinical use of minocycline has created a need to determine concentrations of the antibiotic in biological fluids. A sensitive technique with a simple extraction procedure is necessary for the analysis. Techniques currently available are directed toward analysis of tetracyclines in general and not the specific drug minocycline [2,3].

The major obstacle to analysis of minocycline in biological fluids has been its four different dissociation constants in the pH range 1–12. The p$K_a$ values are 5 and 9.5 for the two amine groups and 2.8 and 7.8 for the hydroxyl groups, making chromatography difficult.

Liquid chromatographic–mass spectrometric (LC–MS) and high-performance liquid chromatographic (HPLC) methods described in the literature for quantification of minocycline in biological matrices are inconvenient for routine analysis. For example, LC–MS methods [4] are expensive, time-consuming, and complex, and HPLC methods require either large volumes of sample or long extraction procedures, for example solid-phase extraction [5–8] or liquid–liquid re-extractions [9, 10]. An isocratic reverse phase HPLC method with solid-phase extraction has a limit of quantification of 100 ng $mL^{-1}$ [7]. The most sensitive method has a limit of quantification of 30 ng $mL^{-1}$ but recovery is low [11]. We have previously reported a validated, stability-indicating HPTLC method for determination of minocycline in pharmaceutical dosage forms [12].

The method described below is based on that previously reported by our research group [12] but is now used for analysis of minocycline and determination of its stability in human plasma. The method involves single-step extraction with methanol. This method was validated for accuracy, precision, robustness, selectivity, and limits of quantification and detection and then successfully applied to a pharmacokinetic study.

## EXPERIMENTAL

### Reagents and Solvents

Minocycline was a gift from Ranbaxy Laboratories (Gurgaon, Haryana, India) and certified to contain 99.62% (*w*/*w*) on dry basis. All other chemicals and reagents were of analytical-grade and were purchased from Merck (Worli, Mumbai, India).

For validation of the method, human plasma was collected from healthy human volunteers (Majeedia Hospital, Delhi, India). All samples were frozen at −20°C until analysis.

### Calibration Standards and Quality-Control (QC) Samples

A stock solution of minocycline was prepared by dissolving 150 mg minocycline in 10 mL methanol. Working solutions containing from 1 to 12 mg $mL^{-1}$ were prepared by appropriate dilution of the stock solution with

methanol. Each working solution of minocycline (10 µL) was used to spike plasma (1 mL) to furnish calibration standards containing from 10 to 120 µg $mL^{-1}$. QC sample solutions were prepared at concentrations of 20, 60, and 100 µg $mL^{-1}$. Each solution was divided into 1.0-mL volumes which were immediately frozen at −20°C.

## HPTLC

Chromatography was performed on 20 cm × 10 cm aluminium plates coated with 200-µm layers of silica gel $60F_{254}$ (E. Merck, Germany). Before use the plates were sprayed with 10% (*w*/*v*) aqueous disodium EDTA (ethylene diaminetetraacetic acid) solution, the pH of which had been adjusted to 9.0 with 10% (*m*/*v*) aqueous sodium hydroxide solution. The plates were dried, horizontally, for at least 1 h at room temperature, and then in an oven at 110°C for 1 h, shortly before use.

Samples were applied to the plates as bands 5 mm wide, 10 mm apart, by means of a Camag (Muttenz, Switzerland) Linomat V sample applicator fitted with a 100-µL syringe. A constant rate of application of 150 nL $s^{-1}$ was used. Linear ascending development, with methanol–acetonitrile–isopropanol (IPA)–water 5:4:0.5:0.5 (*v*/*v*) as mobile phase was performed in a 20 cm × 10 cm twin-trough glass chamber (Camag), with tightly fitting lid, previously saturated with mobile phase vapour for 30 min at room temperature (25 ± 2°C) and relative humidity 60 ± 5%. The development distance was 8 cm. After development the plates were dried in current of air from an air dryer. Densitometric scanning at 345 nm was then performed with a Camag TLC Scanner III in absorbance mode operated by WinCATS software (Version 1.2.0). The source of radiation was a deuterium lamp emitting a continuous UV spectrum in the range 190–400 nm. The slit dimensions were 5 mm × 0.45 mm and the scanning speed 20 mm $s^{-1}$.

## Sample Preparation

Before analysis, plasma samples were thawed at room temperature for approximately 10 min. Plasma calibration standards and QC samples (1 mL) were transferred to vials and mixed with 1.0 mL methanol. After vortex mixing for 1 min, the samples were centrifuged (5 min, >2000*g*). The supernatant was transferred to new tubes and the solvent was evaporated at 37°C under a stream of nitrogen. The residues were dissolved in 100 µL methanol and 1 µL of each sample was applied to the TLC plate to furnish a final calibration range of 100 to 1200 ng per zone. QC samples at final concentrations of 200, 600, and 1000 ng per zone were obtained after application. Each concentration was applied six times to the TLC plate.

## Stability Study

*QC Samples*

For determination of stability, QC samples at three concentrations were used. Plasma was spiked with minocycline then stored at 4°C or 20°C. Stability was assessed after 0.5, 1, 2, 4, and 8 h. The stability of the drug in frozen samples (−20°C) was determined by analysis over a period of two months. Before extraction and analysis samples were brought to room temperature. The degradation rate constant ($k_{obs}$), half-life ($t_{1/2}$), and shelf life ($t_{90}$, i.e. the time when 90% of original concentration of the drug is left) of the drug in plasma were also measured at 4°C and at 20°C.

*Freeze–Thaw Stability*

To determine freeze–thaw stability nine aliquots of each QC sample were stored at −20°C for 24 h. They were then left to thaw completely at room temperature. Three aliquots of each QC sample were analysed after extraction. The other aliquots were returned to −20°C for another 24 h. This cycle was repeated three times.

*Stock Solution*

The stability of the drug in methanol (stock solution) was assessed at 4°C and 20°C. Run-time stability, at room temperature, of processed samples after extraction was determined for QC samples. To test the stability, the samples were analysed immediately after preparation (control) and after a stipulated time period.

## RESULTS AND DISCUSSION

### Specificity

The specificity of the method was investigated by screening five different batches of blank human plasma. Under the conditions described the $R_F$ (0.32) region where minocycline eluted was free from interferences for all the drug-free human plasma tested. Use of methanol enabled rapid extraction, and few impurities and no interfering substances were extracted from plasma. Figure 1 shows chromatograms obtained from extracts of drug-free plasma (A) and a plasma quality-control sample spiked with 600 ng per zone minocycline (B). Use of TLC plates sprayed with a 10% (*w*/*v*) solution of disodium EDTA, pH 9.0, enabled us to prevent formation of minocycline–metal complexes and resulted in improved peak symmetry.

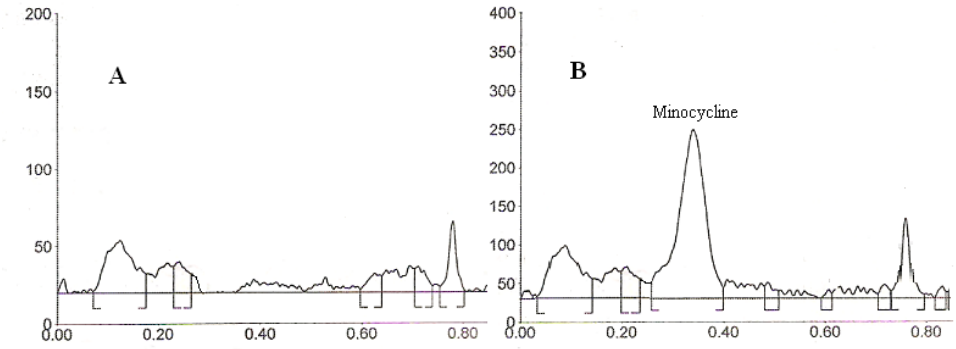


**Fig. 1**

Chromatograms obtained from (A) drug-free human plasma, and (B) human plasma spiked with minocycline at 600 ng per zone. $R_F$ of minocycline, 0.32; detector wavelength, 345 nm

Densitometric analysis of minocycline was performed at 345 nm. Absorbance spectra of minocycline in methanol showed the wavelength of maximum absorbance ($\lambda_{max}$) was 345 nm with an extinction coefficient (log $\varepsilon$) of 4.16, owing to cyclic structure of minocycline. Selection of this wavelength improves specificity and minimizes interferences from plasma that may occur at lower wavelengths.

## Linearity, Precision, and Accuracy

Linear least-squares regression analysis showed there was a good linear relationship ($r^2 > 0.995$) between peak area and concentration in the range 100–1200 ng per zone; the regression equation of the calibration plots was $y = (9.873 \pm 0.042)x + (1832.48 \pm 13.35)$. There was no significant difference between the slopes of standard plots (ANOVA, $P > 0.05$).

Results from determination of intra-day and inter-day precision and accuracy of the assay for QC samples at concentrations of 200, 600, and 1000 ng per zone, on three different days, are summarized in Table I. Intra-day and inter-day precision expressed as coefficient of variation (*CV*, %) were ≤3.49% and ≤4.14%, respectively. Intra-day and inter-day accuracy, as percentage recovery ([mean measured concentration]/[nominal concentration] × 100), were in the range 97.08–99.22%.

## Sensitivity and Recovery

The *LOD*, expressed as 3.3$\sigma$/(slope of the calibration plot), and the *LOQ*, expressed as 10$\sigma$/(slope of the calibration plot) were 5.1 and 15.4 ng

**Table I**

Precision and accuracy of the HPTLC method for determination of minocycline in human plasma ($n = 6$)

| Nominal conc. (ng per zone) | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|
| | Conc. found (ng per zone, mean ± *SD*) | Precision (*CV*, %)[a] | Accuracy (%)[b] | Conc. found (ng per zone, mean ± *SD*) | Precision (*CV*, %)[a] | Accuracy (%)[b] |
| 200 | 196.16 ± 6.85 | 3.49 | 98.08 | 194.16 ± 8.03 | 4.14 | 97.08 |
| 600 | 593.00 ± 6.54 | 1.10 | 98.83 | 591.33 ± 9.75 | 1.65 | 98.55 |
| 1000 | 988.66 ± 10.11 | 1.02 | 98.87 | 992.16 ± 7.65 | 0.77 | 99.22 |

[a]Precision (*CV*, %) = (standard deviation × 100)/(mean concentration found)
[b] Accuracy = (mean concentration found × 100)/(nominal concentration)

per zone, respectively, which indicates the sensitivity of the method is adequate (where $\sigma$ is the standard deviation of the response for the blank sample). In robustness and ruggedness testing the method was found to be reproducible from one analyst to another. The low values of *RSD* (≤0.783%) and *SE* (≤1.775) obtained after small deliberate changes of the conditions (mobile phase composition, mobile phase volume, saturation time, percentage of EDTA solution, pH of EDTA solution, time from application to chromatography, and time from chromatography to scanning) used for the method indicated its robustness. Because of this robustness, use of an internal standard is not required.

Mean recovery of minocycline from plasma using single-step extraction with methanol was 95.8 ± 4.5% for 200, 600, and 1000 ng per zone. Recovery was not statistically different over the range of concentration studied.

## Pharmacokinetic Study

Figure 2 shows concentrations of minocycline in plasma samples collected over 72 h from a healthy volunteer after oral administration of a single 100-mg minocycline tablet. The maximum concentration was approximately 1059 ± 40 ng $mL^{-1}$. Reported minocycline levels after oral administration of a therapeutic dose of 100 mg are 1120 ng $mL^{-1}$ [13], indicating that this method is suitable for therapeutic drug monitoring.

## Stability

The stability of minocycline was investigated in human plasma samples. Each experiment was repeated three times and the mean concentration of minocycline was calculated. During storage at 20 and 4°C, a monoex-

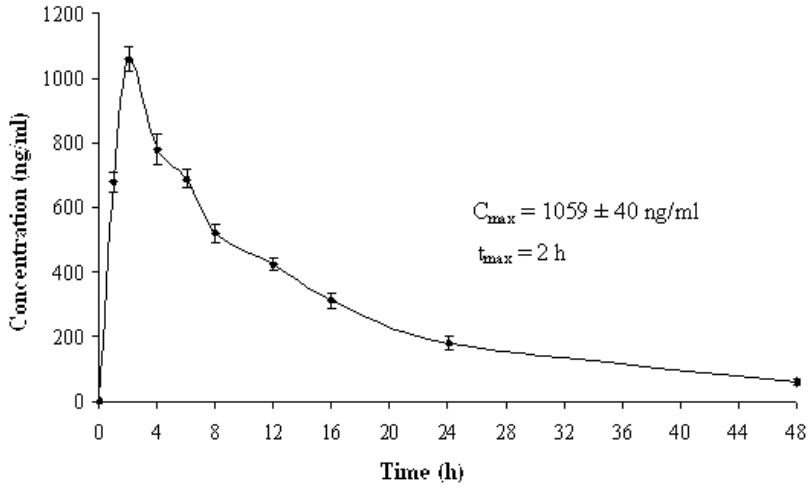


**Fig. 2**

Plasma concentration–time profile for minocycline in the plasma of human volunteers after oral administration of a single 100-mg tablet (the error bar represents standard deviation, $n = 3$)

ponential decline in drug concentration was detected (Table II). The first-order rate constant ($k_{obs}$), half-life ($t_{1/2}$), and shelf-life ($t_{90}$) of the drug in plasma were obtained from the slope of the straight lines at both temperatures (Table III). The half-life was 6.3 h at 20°C and 9.9 h at 4°C.

## Table II

Stability of minocycline in human plasma at 4°C and 20°C ($n = 6$)

| Time (h) | Temperature (°C) | Recovery (%, mean ± *SD*) | | |
|---|---|---|---|---|
| | | 200 ng/zone | 600 ng/zone | 1000 ng/zone |
| 0.5 | 4 | 98.6±1.93 | 98.9±1.75 | 99.3±0.97 |
| 1.0 | 4 | 94.3±3.43 | 94.6±1.44 | 89.6±4.54 |
| 2.0 | 4 | 86.5±2.56 | 83.2±1.78 | 81.2±4.28 |
| 4.0 | 4 | 78.5±0.63 | 78.2±0.89 | 77.9±3.84 |
| 8.0 | 4 | 57.5±2.06 | 57.9±4.23 | 54.5±6.54 |
| 0.5 | 20 | 91.4±1.17 | 98.9±1.55 | 93.1±3.15 |
| 1.0 | 20 | 81.0±2.56 | 84.6±1.44 | 88.3±0.98 |
| 2.0 | 20 | 75.1±0.56 | 78.5±2.26 | 78.0±0.35 |
| 4.0 | 20 | 62.8±2.43 | 64.5±1.66 | 64.7±4.64 |
| 8.0 | 20 | 38.3±2.96 | 40.6±1.63 | 42.8±3.58 |

**Table III**

Kinetic data for degradation of minocycline in plasma

| Temperature (°C) | $k_{obs}$ ($h^{-1}$) | $t_{1/2}$ (h) | $t_{90}$ (h) | $R^2$ for first-order plots |
|---|---|---|---|---|
| 4 | 0.07 | 9.9 | 1.50 | 0.9942 |
| 20 | 0.11 | 6.3 | 0.96 | 0.9917 |

Investigation of long-term freezer stability (−20°C) revealed minocycline was stable in plasma for two months. Average recovery was 95.1%. The freeze–thaw data indicated that a minimum of two freeze–thaw cycles can be tolerated without losses greater than 10%. Stock solutions of minocycline were stable for 2 days at 20°C and for 6 days at 4°C without measurable degradation. Determination of the stability of processed samples at room temperature after extraction revealed no significant losses had occurred after 12 h.

In conclusion, a new, sensitive, and specific assay has been established for analysis of minocycline in plasma. The method validation results indicate the method is sufficiently accurate and precise for pharmacokinetic studies of the drug.

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