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Liquid chromatographic determination of minocycline in brain-to-plasma distribution studies in the rat

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic procedure was developed for the determination of minocycline in rat plasma and brain and applied to brain-to-blood (plasma) distribution studies. The procedure is based on isolation of the compound and the internal standard (either demeclocycline or tetracycline may be used) from plasma and brain constituents using the Oasis HLB cartridge, with satisfactory recovery and specificity, and separation on a Symmetry Shield RP8 (15 cm×4.6 mm, 3.5 μm) column coupled with a UV detector set at 350 nm. The assay was linear over a wide range, with a lower limit of quantification of 50 ng ml^{-1} or g^{-1}, using 0.2 ml of plasma and about 200 mg of brain tissue. Precision and accuracy were acceptable. In the rat minocycline crossed the blood–brain barrier slowly, achieving mean brain concentrations between 30 and 40% of the equivalent systemic exposure, regardless of the dose and route of administration.

Keywords: Brain-to-plasma distribution; Minocycline

1. Introduction

The semisynthetic second-generation tetracyclines (see Fig. 1 for chemical structures) doxycycline and minocycline have neuroprotective effects in models of global and focal cerebral ischemia [1–3]. In vitro and in vivo in rodents these and some older tetracyclines inhibit the formation of amyloid aggregates, a major neuropathological feature in Alzheimer's disease [4,5]. Minocycline also prevents nigrostriatal dopaminergic neurodegeneration and microglial activation in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease [6,7], and delays disease progression in a transgenic model of Huntington disease [8] and in a mouse model of amyotrophic lateral scrolosis [9,10]. However, while all these studies suggest that tetracyclines, and particularly minocycline might offer a useful pharmacological approach to various neurodegenerative diseases little is yet known on the concentrations reached in the brain and how they relate to blood concentrations in animal models. This information may help in evaluating the relevance of in vitro findings for the in vivo situation and in extrapolating pharmacological data across species. An accurate, precise and sensitive enough analytical procedure to quantitate minocycline in blood and brain is an obvious prerequisite.

Analyses based on relatively simple protein precipitation [11], liquid–liquid [12–14] or solid-phase

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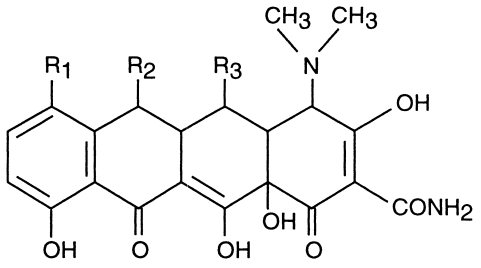

TETRACYCLINE	R_1	R_2	R_3
MINOCYCLINE	- N(CH3)(CH3)	- H	- H
DOXYCYCLINE	- H	- CH_3	- OH
DEMECLOCYCLINE	- Cl	- OH	- H

Fig. 1. Chemical structures of some second-generation tetracyclines.

extraction procedures [15–17] followed by high-performance liquid chromatography with ultraviolet detection (HPLC–UV) have been described for minocycline, but are generally aimed only at analyzing these drugs in body fluids or tissues other than brain. HPLC with mass spectral detection has been used to quantitate minocycline in mouse brain [6] but the application of this method is limited on account of the initial cost of the apparatus. We therefore set up a relatively simple HPLC–UV method for the quantification of minocycline in rat plasma and brain. The method uses an available solid-phase extraction procedure and resolves the compound, the internal standard and endogenous components by an isocratic reversed-phase system. The method was successfully applied in preliminary brain-to-blood partition studies of minocycline in rats.

2. Experimental

2.1. Materials and reagents

Minocycline hydrochloride, demeclocycline hydrochloride, doxycycline hydrochloride and tetracycline hydrochloride were obtained from Sigma–Aldrich (Milan, Italy). Stock solutions were prepared by dissolving tetracyclines in methanol at the concentration of 1 mg ml^{-1}, and were stable for at least 1 week when stored at −20 °C. Working standard solutions were prepared from the stock solutions by dilution with methanol and kept at −20 °C in dark bottles.

Other chemicals and solvents, CH_3CN and CH_3OH (Carlo Erba, Milan, Italy), KH_2PO_4 (E. Merck, Darmstadt, Germany) were of analytical-reagent grade and were used without further purification. Water was deionized before use.

Drug-free plasma and brain tissue were obtained from male CD-COBS rats (Charles River, Calco, Italy) and were stored at −20 °C after collection and processing.

2.2. Chromatographic apparatus and conditions

HPLC analysis was done on a Waters system (Milford, MA, USA) equipped with a Wisp-717 sample processor, a Model 510 solvent delivery system and a Model 2487 UV detector set at 350 nm, coupled with a Model C-R6A Chromatopac Shimadzu integrator (Shimadzu, Kyoto, Japan).

Separation was carried out on a Symmetry Shields RP 8 column (15 cm×4.6 mm, 3.5 μm; Waters, Milan, Italy), at room temperature, protected by a Brownlee RP-8 precolumn (Perkin-Elmer, Norwalk, CT, USA). The mobile phase for minocycline analysis was CH_3CN–CH_3OH–0.01 *M* KH_2PO_4 (5:20:72.1, v/v) containing 0.03 m*M* Na_2EDTA and 60% $HClO_4$ (2.9 ml), adjusted to pH 2.5 with 10 *M* KOH. It was then slightly modified for the analysis of doxycycline which required 9% CH_3CN and 30% CH_3OH in 0.01 *M* KH_2PO_4 to be reasonably eluted in about 20 min.

The eluents were filtered through a 0.45-μm filter, degassed before use, and delivered isocratically at a flow-rate of 0.8 ml min^{-1}.

2.3. Extraction

We used 1 ml/30 mg Oasis HLB extraction cartridges (Waters) to clean up plasma samples [15,16]. Before extraction plasma samples (0.2 ml) were mixed with 25 μl of a methanolic solution of the internal standard (I.S.) (10 μg/ml) and 20 μl

H_3PO_4 [15,16], then diluted to 1.5 ml with 0.01 *M* phosphate buffer, pH 7.4. The final solution was vortex-mixed and centrifuged in a Sorval centrifuge for 30 min at 4 °C at 2000 rpm.

Brain tissue was homogenized (5 ml g^{-1}) in 0.01 *M* phosphate buffer, pH 7.4, in ice. To 1 ml of this homogenate the I.S. (25 μl) and H_3PO_4 (20 μl) were added, vortex-mixed and centrifuged at 2000 rpm for 30 min at 4 °C. The precipitate was re-dissolved in 1 ml of 0.01 *M* phosphate buffer, pH 7.4 and centrifuged. The supernatants were combined and processed as described for plasma.

2.4. Data analysis

The precision and accuracy of the method were determined by replicate analyses of quality control (QC) samples containing small, medium and large known amounts of tetracycline, stored at −20 °C. On different days these QC samples were assayed with standard samples and the calculated concentrations were compared (inter-assay variance). Intra-assay variance was checked by replicate analysis of QC samples on the same day.

Daily standard curves with six concentrations over the working range were plotted in duplicate with QC samples injected between the two sets of standards. The relative response factor was computed as the tetracycline to the I.S. peak-height ratio. Calibration lines were constructed by least-squares linear regression on the relative response factors against the nominal concentration of the compound. Concentrations of QC—and unknown—samples were obtained by back-solving the usual equation of the calibration line.

2.5. Animals and treatment

Male CD-COBS rats weighing 200–250 g (Charles River) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., Suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, 12 December 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Rats were given minocycline intravenously at a dose of 25 mg kg^{-1} or orally at doses of 5 to 100 mg kg^{-1} and were killed at various times after dosing by decapitation under deep anesthesia. Blood samples were collected in heparinized tubes, centrifuged and the plasma was stored at −20 °C. Brains were rapidly removed, blotted with paper to remove excess surface blood and stored at −20 °C until analysis. Water and food were freely available throughout the studies.

3. Results and discussion

3.1. Chromatography

Minocycline could be recovered selectively and efficiently from plasma and brain homogenate using the 1 ml/30 mg Oasis HLB extraction cartridge [15,16]. The solvent used to elute the samples, CH_3OH—as also suggested by the manufacturer—extracted only a few impurities and no interfering substances from either tissue. Fig. 2 gives examples of chromatograms of extracts from drug-free rat plasma and brain, spiked plasma (B) and brain (D) and plasma of a rat injected i.v. with 25 mg kg^{-1} minocycline. Separation was on the Symmetry Shields RP 8 column with the mobile phase, containing Na_2EDTA and $HClO_4$ to improve chromatography as discussed elsewhere [12,18].

The overall mean recovery, determined by comparing the peak height of minocycline from spiked plasma and brain plasma with those from direct injection of minocycline dissolved in the mobile phase, averaged 89±9 and 81±8% in plasma and brain tissue, respectively. Either demeclocycline or tetracycline may be used as I.S. because both are well separated from minocycline and endogenous substances and their extraction recovery from plasma and brain is similar to that of their semisynthetic derivative (Table 1). Although tetracycline had a slightly shorter retention time (Table 2), we used demeclocycline because it was an acceptable I.S. in the analysis of all derivatives considered in these brain-to-plasma distribution studies.

The second-generation derivative doxycycline showed a much longer retention time than

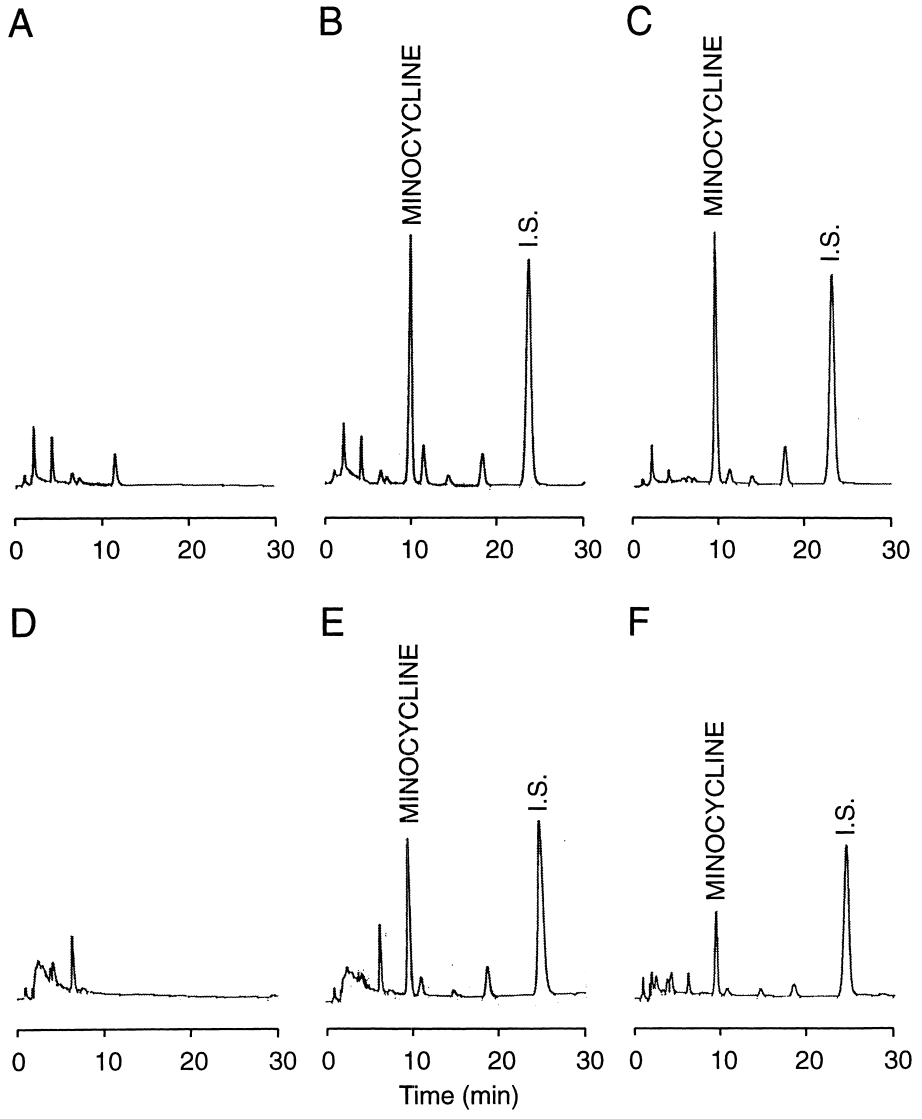

Fig. 2. Chromatograms of extracts from drug-free rat plasma (A) and brain (D) and plasma (B) and brain (E) spiked with 100 ng ml^{-1} or ng g^{-1} minocycline. Also shown are plasma (C) and brain (F) samples from an intravenously injected rat (25 mg kg^{-1} of minocycline hydrochloride). Column: Symmetry Shields RP 8 (15 cm×4.6 mm, 3.5 μm). Mobile phase: CH_3CN–CH_3OH–0.01 *M* KH_2PO_4 (5:20:72.1, v/v) containing 0.03 m*M* Na_2EDTA and 60% $HClO_4$ (2.9 ml), adjusted to pH 2.5 with 10 *M* KOH.

minocycline (Table 2) possibly because of differences in the physicochemical properties [19,20]. It was extracted slightly less from brain tissue than other tetracyclines although recovery from plasma (0.2 ml) was around 90% for all compounds. However, while further optimizing the overall extraction procedure could certainly improve the analysis, the performance was already acceptable with intra-assay and inter-assay variability less than 15% at the limit of quantitation (LOQ) of 125 ng g^{-1}, and even less at higher concentrations (results not shown). Demeclocycline was well separated from doxycycline and

Table 1
Mean recovery of tetracycline derivatives from spiked rat plasma and brain

Drug	Concentration[a] (μg ml^{-1} or g^{-1})	Recovery (%±SD)	
		Plasma	Brain
Minocycline	0.050–2.5	89±9	81±8
Demeclocycline	1.25	95±2	88±3
Doxycycline	0.125–5	87±8	50±4
Tetracycline	0.125–5	91±8	91±7

[a] The drug was added to 0.2 ml of plasma or brain homogenate.

endogenous substances using the same RP-8 column, with a mobile phase with higher organic content (Table 2).

The relationships between the peak-height ratios of minocycline and the I.S. and the amount of the compound added to plasma and brain homogenate were always linear, with the r^2 invariably exceeding 0.995. The slopes of these curves ranged from 0.0099 to 0.0110 for plasma (average regression equation $y=0.0104x+0.0098$) and from 0.0090 to 0.0111 for brain ($y=0.0104x+0.0114$). The lowest calibration standard corresponded to the LOQ (50 ng ml^{-1} or g^{-1} using 0.2 ml of plasma or approximately 200 mg of brain tissue), i.e., the lowest concentration that could be measured with acceptable accuracy and precision as calculated in separate studies (≤20%). This LOQ was already sufficient for pharmacokinetic studies although even lower limits could be reached by increasing the volume of plasma or brain homogenate.

Intra-assay relative standard deviations (RSDs) ranged from 2.4 to 8.9% in plasma and from 2.3 to 17.3% in brain with overall mean accuracy (R.E.), calculated from the deviation of the mean concentration from the nominal value, from −1.7 to 10.7% for plasma and from −1.5 to 1.8% for brain. The inter-day validation results are summarized in Table 3.

Minocycline was stable in plasma and brain homogenate for at least 2 h at room temperature, i.e., approximately the time needed for handling the daily series of biological samples. This compound is stable in biological samples for up to 2 months at −20 °C [16]. Replicate injection of QC samples confirmed that the compound was stable in the automatic sampler for at least 16 h, without the need for refrigeration.

3.2. Brain distribution studies

The mean concentrations of minocycline in plasma and brain as a function of time after intravenous dosing (25 mg kg^{-1}) in male rats is shown in Fig. 3. Plasma concentrations at 8 h were less than one-fifth those at 1 h, and at 24 h they were below LOQ of the analytical procedure.

Plasma data points could be satisfactorily fitted to a monoexponential equation. The mean total body clearance averaged 238 ml h^{-1} kg^{-1} which, in conjunction with a relatively small volume of distribution of 1.04 l, resulted in a mean elimination $t_{1/2}$

Table 2
Retention time of tetracycline derivatives on the Symmetry Shields RP 8 reversed-phase column (15 cm×4.6 mm, 3.5 μm)

Mobile phase (CH_3CN–CH_3OH–KH_2PO_4, v/v)[a]	Tetracycline derivative	Retention time (min)
5:20:72.1	Minocycline	10.1
	Demeclocycline	22.1
	Tetracycline	13.5
9:30:58.1	Demeclocycline	7.9
	Doxycycline	18.5

[a] Containing 0.03 m*M* Na_2EDTA and 60% $HClO_4$ (2.9 ml), adjusted to pH 2.5.

Table 3
Summary of quality control samples results for minocycline analysis in rat plasma and brain

Added[a] (ng ml^{-1} or g^{-1})	Found (ng ml^{-1} or g^{-1})	RSD (%)	R.E. (%)
Plasma			
50	55.3±4.9	8.9	10.7
250	257.6±11.5	4.5	3.1
1250	1229.0±29.6	2.4	−1.7
Brain			
50	50.9±8.8	17.3	1.8
250	246.2±8.5	3.5	−1.5
1250	1273.4±29.8	2.3	1.7

[a] To 0.2 ml of plasma or approximately 200 mg of brain tissue.

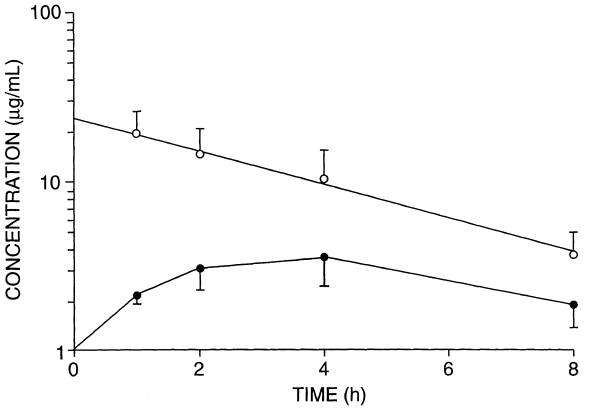

Fig. 3. Mean plasma and brain concentration–time curves of minocycline after intravenous injection (25 mg kg^{-1}) in male rats. Each point is the mean±SD of three rats for plasma (open symbols) and brain (closed symbols).

of 3 h. In man too the total body clearance of minocycline is low; it appears to be due partly to excretion of unchanged drug and partly to oxidation to inactive metabolites [19]. The volume of distribution of this drug in man (80–115 l) [19] is close to that we calculated here for rats.

Minocycline brain concentrations rose gradually up to 4 h after injection (25 mg kg^{-1}) amounting to about 35% of the plasma concentrations. The mean brain-to-plasma distribution ratio, in terms of area under the curve ($AUC_{0-8\ h}$), averaged 0.3. Minocycline has been found in the brain of mice after oral dosing [6] but no attempt was made to correlate brain concentrations with the simultaneous plasma concentrations. In dog brain it reaches higher concentrations than other tetracycline derivatives [21]. In human cerebrospinal fluids it achieves levels between 25 and 30% of serum concentrations after oral administration [22], and is thus the tetracycline that diffuses best through the blood–brain barrier, possibly because of its greater lipophilicity [19,20]. In our preliminary studies in rats the brain concentrations of tetracycline amounted to only about 13% of the plasma concentrations whereas the brain-to-plasma concentration ratio for doxycyline averaged 0.31, 4 h after intravenous injection (25 mg kg^{-1}).

It remains to be seen whether these distribution ratios reflect the true extent of brain-to-blood partition since the plasma protein binding of tetracyclines was not determined in these studies in rats. In man plasma protein binding ranges from 70 to 80% for minocycline and from 80 to more than 90% for doxycycline [19–21] and it is therefore possible that in rodents the brain uptake ratio based on total plasma concentrations underestimates the uptake of the unbound drug which is actually available for brain distribution.

Fig. 4A illustrates the relationship between

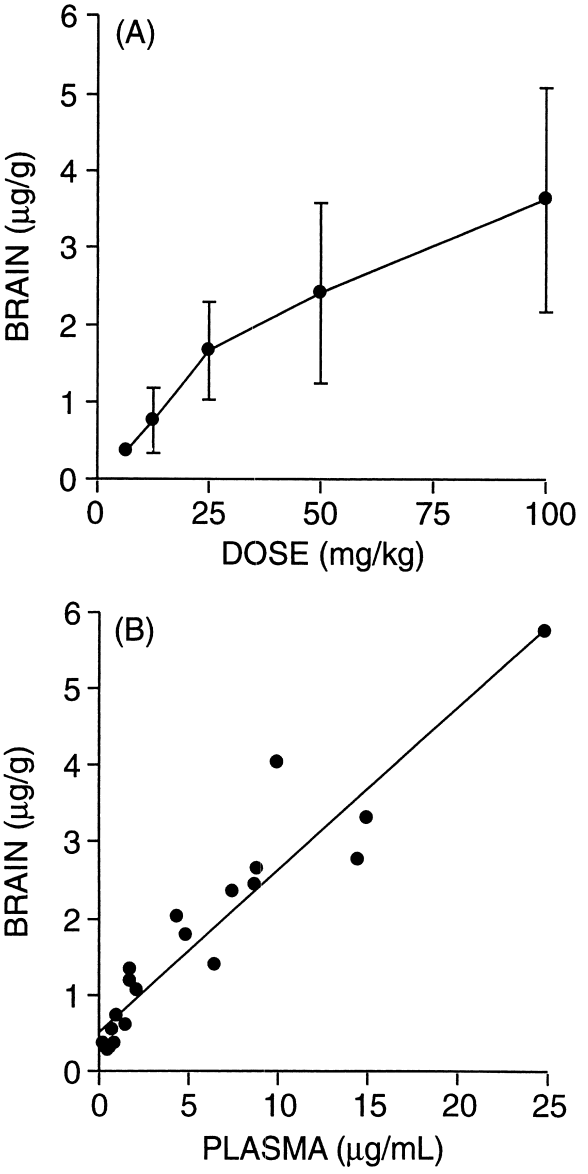

Fig. 4. The relationships between minocycline dose and brain concentrations (A) and between minocycline plasma and brain concentrations (B).

minocycline dose and brain concentrations 4 h after oral dosing (5–100 mg kg^{-1}). The mean brain concentrations appeared to be dose proportional between 5 and 25 mg kg^{-1} but slightly less than proportional at higher doses. This may reflect dose-related variability in the rate or extent of minocycline absorption from the rat gastrointestinal tract because the concentrations in brain were highly correlated with those in the systemic circulation ($[\text{Minocycline}]_{\text{brain}} = 0.21 \cdot [\text{Minocycline}]_{\text{plasma}} + 0.51$) (Fig. 4B). This linear relationship further suggests that minocycline passes through the blood–brain barrier by passive diffusion, with plasma concentrations consistently reflecting the concentration of the drug in the central nervous system.

4. Conclusions

Interest in tetracyclines and particularly second generation derivatives has been increasing in recent years in the light of their potential pharmacological use and utility in investigations of various neurodegenerative diseases. The availability of analytical methods for determining these drugs in central nervous system tissues of experimental animals may therefore help clarify the relationship between their concentrations at the site of action and concomitant effects. Although other methods are available to quantitate minocycline and related drugs in brain tissue [6,20] the one described can be easily used in most laboratories, being based on a simple solid–liquid extraction procedure, followed by reversed-phase HPLC–UV. This is sensitive and precise enough for analysis in plasma and brain as evidenced in these studies in rats given a wide range of intravenous and oral minocycline doses. It can be adapted to plasma and brain concentration measurements of other tetracycline derivatives and thus has the potential for various pharmacological applications.

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