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SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF TOLOXATONE IN RABBIT CEREBROSPINAL FLUID AND PLASMA

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SUMMARY

A simplified, rapid high-performance liquid chromatographic procedure has been developed for the measurement of toloxatone in rabbit plasma and cerebrospinal fluid. The method involves a single-step extraction of the alkalinized sample with diethyl ether and analysis of the evaporated extract on a C_8 column. Detection was performed by ultraviolet absorbance monitored at 240 nm. The overall run-time of the assay was 8 min at a flow-rate of 1 ml min^{-1}. The sensitivity limit of toloxatone was 70 ng ml^{-1} at a signal-to-noise ratio of 3 · 1 in rabbit cerebrospinal fluid and plasma. The assay has been used to define plasma toloxatone concentration–time profiles and to quantitate cerebrospinal fluid toloxatone levels after a single intravenous injection in rabbits.

INTRODUCTION

The development of selective, reversible monoamine oxidase inhibitors (MAOIs) has led to a renewed interest in these drugs, especially in view of the toxicity of other antidepressants. Toloxatone (TX), 5-(hydroxymethyl)-3-(3-

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methylphenyl)-2-oxazolidinone, is a reversible, selective MAO A inhibitor both in vivo and ex vivo in the rat [1,2] and possesses an antidepressant activity in humans [3–5]. However, the proportions of the two major types of MAO have been shown to vary widely between animal species and between organs from the same species [6]. That is why it seemed interesting to us to study the effects of TX in other species, such as the rabbit, by determination of monoamine metabolites in cerebrospinal fluid (CSF). However, the lack of information about the disposition of the drug in CSF led us first to determine the concentrations of TX in CSF and plasma after a single intravenous (i.v.) injection. For the determination of TX in plasma, two thin-layer chromatographic (TLC) methods [7,8] and a gas chromatographic (GC) method [9] have been reported. However, the TLC methods are either unsuitable for studies with the unlabelled drug [7] or are time-consuming and require a large volume of plasma [8]. The GC method, developed for the measurement of TX at therapeutic concentrations in human plasma, requires derivatization and is unadaptable to low sample volumes. No method has been described for the analysis of this drug in CSF.

This paper reports a simple yet sensitive high-performance liquid chromatographic (HPLC) method using UV detection for the assay of TX in plasma and CSF. This assay has been applied to preliminary pharmacokinetic studies in rabbits after a single rapid i.v. injection.

EXPERIMENTAL

Materials

The following materials were used: toloxatone was supplied by Delalande Labs. (Courbevoie, France); the internal standard, trazodone chlorhydrate, was obtained from UPSA Labs. (Rueil-Malmaison, France); HPLC-grade acetonitrile and reagent-grade potassium dihydrogenphosphate and tetramethylammonium chloride were purchased from Merck (Darmstadt, F.R.G.); reagent-grade diethyl ether, sodium hydroxide and orthophosphoric acid were obtained from Prolabo (Paris, France). The water used in this procedure was deionized using the Milli-Q Reagent Water system (Millipore, Bedford, MA, U.S.A.).

HPLC equipment

Chromatography was performed with a Varian Model 5000 liquid chromatograph (Varian, Sunnyvale, CA, U.S.A.) equipped with a 20-μl universal loop injector (Valco Instruments, Houston, TX, U.S.A.), a Kratos Spectroflow 783 variable-wavelength UV detector (Ramsey, NJ, U.S.A.) and a Varian Model 9176 strip chart recorder (Sunnyvale, CA, U.S.A.). Separation and quantitation were achieved on a reversed-phase system with a C_8 column (LiChrospher

100 CH-8 II, 5 μm, 250 mm×4 mm I.D., Merck) linked to a C_8 precolumn (Hibar LiChroCART, 25 mm×4 mm I.D., Merck).

Chromatographic conditions

The mobile phase was acetonitrile–0.01 *M* phosphate buffer, pH 3.0 (60:40, v/v) containing 0.02 *M* tetramethylammonium, and was filtered before use through a 0.45-μm Millipore filter. The solvent flow-rate was 1.0 ml min^{-1}, and the chromatographic system was operated at ambient temperature (19–22°C). The eluent was monitored by absorbance at 240 nm, and the UV detector was operated from 0.05 to 0.005 a.u.f.s. The recorder chart speed was 0.25 cm min^{-1}.

Preparation of standards

Stock solutions of TX and the internal standard, trazodone (TZ), were prepared by dissolving the appropriate amounts in methanol to make 1 mg ml^{-1} free-base solutions. They were stored in glass volumetric flasks at 4°C. Calibration curves were prepared by diluting appropriate volumes of the stock TX solution in drug-free plasma and CSF to give final concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10 μg ml^{-1} and 0.125, 0.25, 0.5, 1.25, 2.5, 5 μg ml^{-1}, respectively.

Extraction of TX from CSF and plasma samples

An aliquot (50 μl) of plasma or CSF was placed in a 5-ml glass centrifuge tube with a screw cap. Internal standard (50 μl, i.e. 100 ng for plasma and CSF) and 20 μl sodium hydroxide (4 *M*) were added. Each sample was mixed briefly following each addition. The sample was then extracted for 1 min with 750 μl of diethyl ether on a Vortex Genie mixer. After centrifugation (2600 *g*) for 1 min to separate phases, the organic layer was transferred to a 5-ml conical glass tube and evaporated to dryness at 40°C under a stream of nitrogen gas. The residue was dissolved in 50 μl of methanol, and an aliquot (20 μl) was injected directly into the HPLC system.

Quantitation

The standards were extracted daily according to the above extraction procedure. Peak-height ratios of TX to the internal standard, TZ, were used to construct the standard curves. All calibration curves were calculated by a least-squares linear regression analysis of peak-height ratio versus drug concentration. The peak-height ratios of unknown samples were compared with the standard curve in plasma or CSF.

Extraction recovery

The extraction efficiency of TX from stripped plasma and drug-free CSF was determined at concentrations of 0.25, 5 and 10 μg ml^{-1} (plasma) and

0.125, 2.5 and 5 μg ml^{-1} (CSF). The extraction efficiency (%) was calculated as follows:

$$\text{efficiency} = \frac{\text{peak height TZ extracted}}{\text{peak height TX non-extracted}} \times \frac{\text{peak height TX extracted}}{\text{peak height TZ extracted}} \times 100$$

Precision

Low-, medium- and high-quality controls were prepared to contain 0.25, 5 and 10 μg ml^{-1} TX in plasma and 0.125, 2.5 and 5 μg ml^{-1} TX in CSF, respectively. Intra-assay precision was determined by analysing each quality control five times on the same day. Inter-assay precision was determined by analysing one aliquot of each quality control per day for five days over a period of one week.

Rabbit pharmacokinetic study

Plasma and CSF levels of TX were measured after a rapid i.v. infusion through the marginal ear vein of curarized Fauve de Bourgogne rabbits (Elevage Scientifique des Dombes, Romans, France), weighing between 3.0 and 3.6 kg. TX was administered at two doses: 5 and 10 mg kg^{-1}. TX powder was dissolved in physiological saline to concentrations of 5 and 10 mg ml^{-1}. The TX solutions were infused at a rate of 5 and 10 mg kg^{-1} min^{-1}. CSF samples (100–150 μl per 15 min) were continuously collected from the third ventricle [10] at intervals of 0.25 h over a period of 3 h. Blood samples (0.5 ml) were collected into plastic tubes containing lithium heparin at 0, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min after the start of TX infusion, with a femoral arterial catheter. Plasma was obtained by centrifugation for 10 min at 1000 *g* (19–22°C). All biological samples were stored at once after collection at −20°C until analysis by the HPLC procedure.

Pharmacokinetic analysis

Plasma concentration–time data were fitted to a polyexponential equation using a computer program [11] for statistical and pharmacokinetic analysis of data.

RESULTS AND DISCUSSION

Performance of the HPLC system

For optimal UV detection, the wavelength was set at 240 nm, which was approximately the λ_{max} for TX and TZ. Fig. 1 illustrates typical chromatograms of extracts from plasma and CSF of an untreated animal and extracts of plasma and CSF spiked with diluted stock solutions. The analytical peaks of TX and TZ were well resolved and the symmetry was satisfactory. The re-

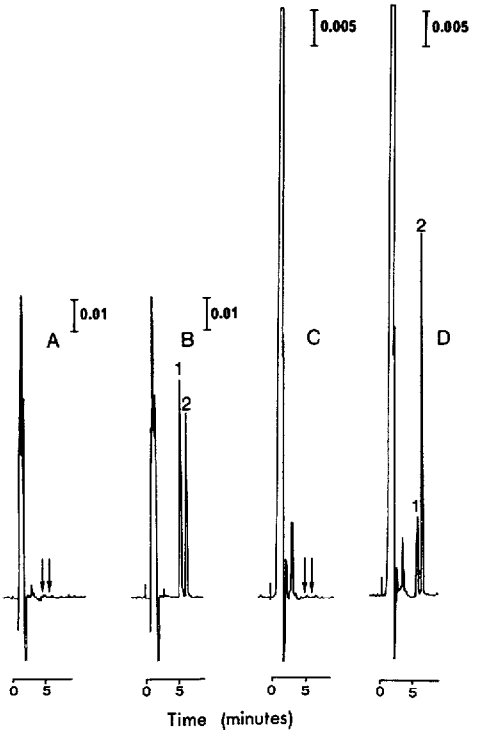

Fig. 1. Chromatograms of extracts (20 μl) from drug-free CSF (A) and plasma (C) samples and from spiked CSF (B) and plasma (D). Peaks: 1 = toloxatone (1.25 μg ml^{-1} in CSF and 312.5 μg ml^{-1} in plasma); 2 = trazodone (internal standard, 100 ng ml^{-1} in plasma and CSF). HPLC conditions are specified in Experimental.

tention times were 4.9 min for TX and 6.0 min for TZ, and their resolution factor was greater than 1 (1.60).

Recovery, precision, calibration and sensitivity

The extraction procedure avoids the uptake of endogenous contaminants as no interfering peak appears in the chromatograms of the drug-free samples (Fig. 1). Table I illustrates the recovery obtained by chromatographing spiked batch plasma and CSF. Recoveries of TX from plasma and CSF were 75%.

Repeated assays of plasma or CSF samples spiked with TX indicated that the reproducibility of the procedure was satisfactory over the calibration range (Table II). Inter-assay coefficients of variation (C.V.) for replicate analysis of 10 and 0.25 μg ml^{-1} plasma samples were 4.64% ($n=5$) and 7.66% ($n=5$), respectively. Similarly, the C.V. values for replicate analyses of 5 and 0.125 μg ml^{-1} CSF samples were 3.21% ($n=5$) and 4.38% ($n=5$), respectively. The intra-assay C.V. values for the same controls ranged between 3.72 and 5.74%, respectively, for plasma, and between 2.59% ($n=5$) and 3.40% ($n=5$), re-

TABLE I

ANALYTICAL RECOVERY OF TOLOXATONE FROM PLASMA AND CSF ($n=5$)

Medium	Concentration (μg/ml)	Extraction efficiency (mean ± S.D.) (%)
Plasma	10	78.6 ± 2.4
	5	78.2 ± 2.0
	0.25	73.4 ± 2.0
CSF	5	73.2 ± 1.3
	2.5	75.9 ± 2.7
	0.125	72.9 ± 3.0

TABLE II

IMPRECISION OF THE ANALYSIS OF TOLOXATONE

Medium	Concentration (μg/ml)	C.V. (%)
Intra-assay imprecision ($n=5$)		
Plasma	10	3.72
	5	3.10
	0.25	5.74
CSF	5	2.59
	2.5	2.74
	0.125	3.40
Inter-assay imprecision ($n=5$)		
Plasma	10	4.64
	5	5.73
	0.25	7.66
CSF	5	3.21
	2.5	3.80
	0.125	4.38

spectively, for CSF. TX calibration curves were linear in the range 0.3–10 μg ml^{-1} in plasma and in the range 0.125–5 μg ml^{-1} in CSF. The regression equations for five calibration curves were $y=666.94x+7.25$ ($r=0.9947$) for plasma and $y=766.88x+28.96$ ($r=0.9983$) for CSF.

While maximum sensitivity was not a goal of the present study, the detection limit of this assay (defined at a signal-to-noise ratio of 3:1) was 70 ng ml^{-1} for TX in plasma and CSF. It could be further improved by extraction of larger sample volumes.

Rabbit pharmacokinetic study

The use of a small amount of biological fluid for analysis allowed us to obtain many plasma samples without drastically changing the total blood volume and was adapted to the low volume of CSF samples (average flow-rate of 10 μl min^{-1} for continuous sampling).

Representative profiles are shown in Fig. 2. TX was detected in plasma for only 3 h following bolus i.v. administration of 5 and 10 mg kg^{-1} in five rabbits. At 5 min the mean values of the TX concentration were 15.0 ± 2.2 μg ml^{-1} (TX, 10 mg kg^{-1}) and 6.5 ± 1.6 μg ml^{-1} (TX, 5 mg kg^{-1}), which rapidly declined to 0.31 ± 0.15 μg ml^{-1} (TX, 10 mg kg^{-1}) and 0.24 ± 0.05 μg ml^{-1} (TX, 5 mg kg^{-1}), at 180 min. Pharmacokinetic analysis of the data is consistent with a two-compartment open model. The terminal half-lives were similar: 41.5 ± 4.7 min (TX, 10 mg kg^{-1}) and 57.3 ± 24.2 min (TX, 5 mg kg^{-1}), but the mean residence times were different: 52.2 ± 5.1 min (TX, 10 mg kg^{-1}) and 71.9 ± 24.2 min (TX, 5 mg kg^{-1}). The claim that values of 41.5 and 57 min for terminal half-lives are similar could only be as a result of the large standard deviation estimated for the 5 mg kg^{-1} dose.

The mean C_{max} values in CSF were 4.7 ± 0.6 μg ml^{-1} (TX, 10 mg kg^{-1}) and 2.8 ± 0.4 μg ml^{-1} (TX, 5 mg kg^{-1}) during the first 15 min after i.v. infusion. Afterwards, drug concentrations decreased progressively and concurrently with the plasma concentrations to 0.19 ± 0.02 μg ml^{-1} (TX, 10 mg kg^{-1}) and 0.15 ± 0.01 μg ml^{-1} (TX, 5 mg kg^{-1}). Thus, TX traverses the blood–brain barrier with no difficulty, possibly by the passive transfer mechanism.

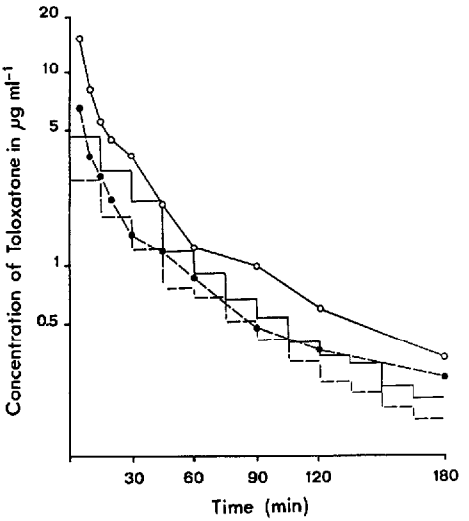

Fig. 2. Plots of log concentration versus time for TX in five healthy rabbits following an i.v. injection of TX at a dose of 5 mg kg^{-1} (●, plasma; ---, CSF) and at a dose of 10 mg kg^{-1} (○, plasma; —, CSF).

CONCLUSIONS

The present HPLC method has the following advantages over the other methods. First, the assay uses UV detection and requires only a small amount of biological fluid (50 μl of plasma or CSF). Second, the extraction procedure is simple, fast and allows a sufficient purification of the extract. Third, this technique is rapid because the chromatography is completed in less than 8 min.

The lower limit of detection for TX is 70 ng ml^{-1} (signal-to-noise ratio = 3) in plasma and CSF. But a sensitivity as low as this reported with GC determination or TLC with densitometric quantitation could be probably obtained by extraction of larger sample volumes. The reproducibility of the procedure was satisfactory over the calibration range. The intra-assay and inter-assay imprecisions were less than 5 and 10%, respectively. Thus, the assay could be adapted and modified for study in patients so that a more rational approach to the dosage regimen, after monitoring plasma TX concentrations, may result.

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