Journal of Chromatography, 574 (1992) 313–318
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6189

High-performance liquid chromatographic procedure for the determination of tiagabine concentrations in human plasma using electrochemical detection

Linda E. Gustavson* and Sou-yie Chu

Drug Metabolism Department, Abbott Laboratories, D-463, AP9/2, One Abbott Park Road, Abbott Park, IL 60064-3500 (USA)

(First received June 20th, 1991; revised manuscript received October 16th, 1991)

ABSTRACT

A sensitive and precise high-performance liquid chromatographic procedure has been developed for the measurement of tiagabine concentrations in human plasma. Isolation of tiagabine and the internal standard was achieved using solid-phase extraction on disposable C_8 columns. Separation was performed on a C_{18} analytical column using a mobile phase containing sodium octanesulfonate. The effluent was monitored with coulometric electrochemical detection at *ca.* +0.76 V. The workup procedure recovered more than 95% of tiagabine from plasma. Standard curves were linear over the concentration range 0–500 ng/ml. The precision of the method was good: coefficients of variation were typically less than 5% for concentrations as low as 8 ng/ml and although they were higher at concentrations less than 8 ng/ml, they remained within acceptable limits (less than 17%) for concentrations as low as the limit of quantitation (2 ng/ml using a 1-ml plasma sample). The stability of tiagabine in plasma was excellent, with no evidence of degradation after 23 h at room temperature or 2 months at −20°C.

INTRODUCTION

Tiagabine [**I**, (*R*)-N-(4,4-di(3-methyl-2-thienyl)but-3-enyl)nipecotic acid, Fig. 1] is a potent and selective inhibitor of γ-aminobutyric acid (GABA) uptake [1]. The compound is one of a series of nipecotic acid derivatives synthesized by Novo-Nordisk (Bagsvaerd, Denmark). Novo-Nordisk and Abbott Laboratories are jointly beginning development of this compound as a potential anticonvulsant. In order to study the clinical pharmacokinetics of **I** it was necessary to develop an analytical method capable of quantitating the concentrations present in plasma following its administration to human subjects.

Extrapolation of preliminary data obtained in the rat suggested that a very sensitive analytical method would be required to measure concentrations of **I** following oral administration of the

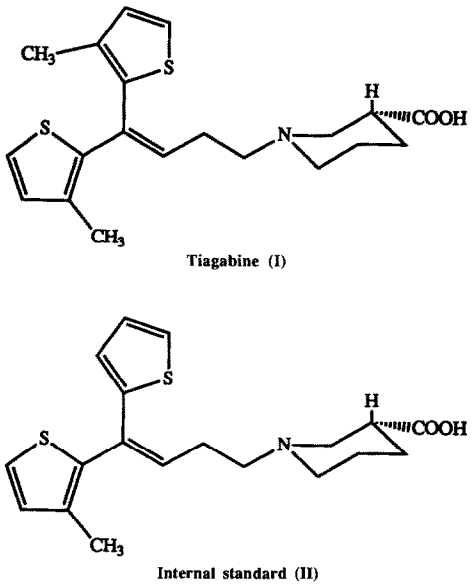

Fig. 1. Molecular structures of tiagabine (**I**) and the internal standard (**II**).

doses of the drug planned for initial clinical studies. Based on our experience with other tertiary amine compounds, including erythromycin derivatives, we decided that the sensitivity of electrochemcial detection offered the best possibility for success.

EXPERIMENTAL

Reagents and chemicals

Tiagabine · HCl (**I** · HCl) and the internal standard (**II**, the monomethyl analogue of **I**; Fig. 1) were provided by Novo-Nordisk. HPLC-grade methanol and acetonitrile, and analytical reagent-grade sodium phosphate (monobasic) and phosphoric acid 85% were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The sodium octanesulfonate was HPLC grade and purchased from Eastman Kodak (Rochester, NY, USA). All water was triple-distilled and passed through a 0.2-mm filter (Gelman Sciences, Ann Arbor, MI, USA).

Chromatography system

The HPLC system consisted of a Model 400 solvent-delivery system (Applied Biosystems, Ramsey, NJ, USA), an Ultrasphere-ODS C_{18} analytical column (Beckman Instruments, San Ramon, CA, USA; 25 cm × 4.6 mm I.D., 5 μm particle size) and a Model 5100A Coulochem coulometric electrochemical detector (Environmental Sciences Associates, Bedford, MA, USA). Samples were injected onto the system using an SP8780 autosampler, and the detector response was monitored on an SP4100 computing integrator (Spectra-Physics, San Jose, CA, USA).

Standard preparation

An initial stock solution of **I** (usually 1 mg/ml) was prepared in methanol. Because the drug was provided as the hydrochloride salt, a proportionate excess (*ca.* 10%) was weighed in the preparation of this solution to account for the presence of the salt. The initial stock solution was serially diluted with drug-free human plasma to provide standards of **I** over the concentration range 2.5–500 ng/ml. Calibrators used to validate the analytical procedure were prepared in a similar manner from a separate weighing of the hydrochloride salt.

A 1 mg/ml stock solution of the internal standard, **II**, was prepared in methanol. The stock solution was diluted 1:1000 with methanol–water (50:50, v/v) to produce the working internal standard solution (1 μg/ml).

Extraction procedure

A 1-ml aliquot of plasma was mixed with 0.2 ml of water and 0.2 ml of working internal standard solution. The plasma mixture was then centrifuged at *ca.* 1000 *g* for 15 min to pellet any large fibrinous materials that might clog the extraction column. Bond Elut C_8 solid-phase extraction columns, 3 ml (Analytichem International, Harbor City, CA, USA), were preconditioned by rinsing with 5 ml of methanol followed by 2 ml of water. The plasma mixture supernatant was decanted onto a preconditioned extraction column and drawn through the column under gentle vacuum. The extraction column was rinsed with 2 ml of water followed by 2 ml of 5% (v/v) methanol–water. The compounds of interest were then eluted from the extraction column with 1 ml of methanol, and the eluent was evaporated to dryness under a gentle stream of air at *ca.* 40°C.

Recovery

The recovery of **I** from dog plasma was assessed using ^{14}C-labelled drug. Approximately 10 000 dpm of radiolabelled **I** were added to 1 ml of drug-free plasma in duplicate. The spiked plasma was processed according to the extraction procedure above. The recovery was calculated as the percentage of the added radiolabel present in the extraction residue. Regular assessments of recovery were performed through comparison of the peak heights obtained by direct injection of a reference solution of **I** with the peak heights from chromatography extracts.

Chromatography

The mobile phase consisted of 37% (v/v) acetonitrile and 10% (v/v) methanol in an aqueous

solution prepared to contain final concentrations of 0.01 *M* phosphoric acid, 0.01 *M* sodium phosphate (monobasic) and 0.005 *M* sodium octanesulfonate. The mobile phase was delivered to the column at a flow-rate of 1.2 ml/min. The extraction residues were reconstituted in mobile phase (normally 0.2 ml), and aliquots (normally 50–60 μl) were injected onto the chromatography system. The electrochemical detector contained an analytical cell with two porous graphite electrodes to monitor the column effluent. The screening electrode was set at $+0.50 \pm 0.04$ V, and the working electrode was set at $+0.76 \pm 0.02$ V. A guard cell was positioned just before the injection valve. The guard cell electrode was set at $+0.95 \pm 0.04$ V.

Standard curve and calculations

Daily standard curves were constructed using the ratios of the observed peak heights of the drug and the internal standard. The equations to calculate unknown concentrations were derived by linear regression of peak-height ratio against concentration, employing the reciprocal of the peak-height ratio as a weighting scheme.

The inter- and intra-day precisions of the assay procedure were assessed at concentrations of **I** in the range 2–200 ng/ml. Five replicate samples were assayed for intra-day precision and eight samples were used for inter-day variability. The precision is expressed as the percentage deviation (+ or −) from the theoretical concentration of the sample.

Stability

The stability of **I** in human plasma was examined at concentrations of 10 and 200 ng/ml. Triplicate samples were assayed at various times following room temperature storage (*ca.* 22°C) for up to 23 h, and following frozen storage (−20°C) for up to 2 months.

RESULTS

Chromatography

Fig. 2 presents representative chromatograms from human plasma samples processed using the analytical method described above. Compound **I** has a rentention time of 13–16 min, and the internal standard (**II**) elutes 3–4 min before **I**. Drug-free plasma samples obtained from normal subjects resulted in chromatograms free of interference near the retention times of the **I** and **II** which supports the specificity of the analytical procedure. A group of peaks eluting after **I** was observed in most subjects. Injection intervals of 25–30 min were usually employed to ensure that these late-eluting peaks did not interfere with subsequent chromatograms.

Recovery

Studies using ^{14}C-labelled **I** added to normal dog plasma demonstrated that the work-up procedure recovered *ca.* 97–98% of the tiagabine placed on the extraction columns. Comparisons of peak heights obtained following direct injection of a standard solution of **I** and extracts of fortified human plasma also supported recoveries greater than 95%.

Linearity

Daily standard curves were constructed using linear regression where x was the concentration of the standard and y was the peak-height ratio. A $1/y$ weighting scheme was used. The regressions revealed a linear relationship without systematic bias. The best-fit equation for a representative standard curve was $y = 0.006\,258x - 0.003\,680$. The y-intercept of the regression line was not significantly different from zero. The correlation coefficient was 0.9998.

Precision and accuracy

The data presented in Table I indicate good intra-assay precision for the analytical procedure. Calculated concentrations for the 2 ng/ml samples were determined by linear extrapolation of the standard curve. Coefficients of variation (C.V.) for the analysis of replicate samples at concentrations of 20 ng/ml or more ranged from 0.6 to 8.3%, with most values below 5%. Concentrations of 2 ng/ml had generally higher, but acceptable, C.V. ranging between 2.9 and 14.5%.

The inter-assay precision was determined using

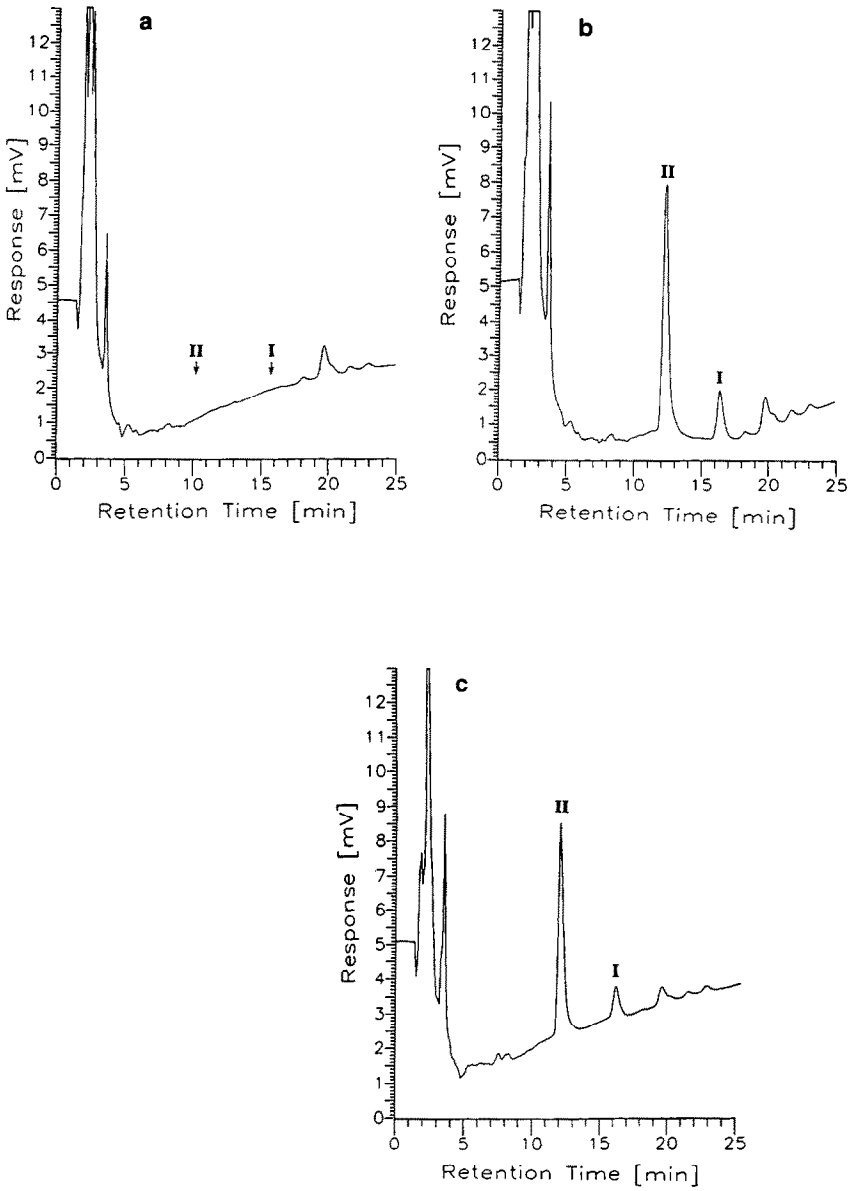

Fig. 2. Representative chromatograms from human plasma samples. (a) Drug-free human plasma: the retention times of tiagabine (**I**) and internal standard (**II**) are indicated. (b) Drug-free human plasma fortified with 50 ng/ml **I** and with **II**. (c) Plasma collected from a healthy male subject 6 h after a 10-mg oral dose of **I** · HCl: the concentration was determined to be 45 ng/ml.

eight replicate measurements of calibrators at concentrations of 8 and 160 ng/ml. Results for the 8 ng/ml calibrators were 8.03 ± 0.33 (4.1%) [mean ± S.D. (C.V.)] and results for the 160 ng/ml calibrators were 160 ± 4.3 (2.7%). These data, along with the overall precision data presented in Table I, revealed good precision for the determination of plasma concentrations of 8–200 ng/ml with all C.V. values less than 5%. For concentrations of 2 ng/ml, the C.V. was higher (16.5%).

The data in Table I and representative calibrator results also demonstrate the accuracy of the assay based on the good agreement between add-

TABLE I

PRECISION AND ACCURACY OF THE PROCEDURE FOR MEASURING CONCENTRATIONS OF I IN HUMAN PLASMA

Concentration added (ng/ml)[a]	Concentration calculated (ng/ml)	Deviation from added concentration (%)
Day 1[b]		
2.00	2.38 ± 0.07 (2.9)	19.0
20.0	20.4 ± 0.13 (0.6)	2.0
200	201 ± 1.7 (0.8)	0.5
Day 2[b]		
2.00	2.39 ± 0.35 (14.5)	19.5
20.0	21.0 ± 0.51 (2.4)	5.0
200	203 ± 2.4 (1.2)	1.5
Day 3[b]		
2.00	1.74 ± 0.09 (5.3)	−13.0
20.0	21.0 ± 1.7 (8.3)	5.0
200	207 ± 4.4 (2.1)	3.5
Overall[c]		
2.00	2.20 ± 0.36 (16.5)	10.1
20.0	20.8 ± 1.0 (4.9)	4.0
200	204 ± 4.0 (2.0)	1.8

[a] Pooled drug-free human plasma fortified with I.
[b] Mean ± S.D. (C.V. %); $n = 5$.
[c] Mean ± S.D. (C.V. %); $n = 15$.

TABLE II

STABILITY OF I IN HUMAN PLASMA

Storage at 20°C		Storage at −17°C	
Time (h)	Concentration (ng/ml)[a]	Time (days)	Concentration (ng/ml)[a]
0	10.2 ± 0.67	0	10.2 ± 0.67
	212 ± 8.6		212 ± 8.6
4	9.51 ± 0.28	28	9.88 ± 0.35
	201 ± 2.5		198 ± 2.1
23	9.59 ± 0.25	61	10.1 ± 0.52
	213 ± 3.8		215 ± 5.3

[a] Mean ± S.D.; $n = 3$ at each time point. Added concentrations were 10 and 200 ng/ml.

ed and calculated concentrations. For concentrations of 20 ng/ml or more, deviations were ±5% or less. Deviations were greater for concentrations of 2 ng/ml, with values ranging from +19.5% to −13.0%.

Stability

The data assessing the stability of **I** in plasma at room temperature and while frozen are presented in Table II. There was no evidence of degradation of **I** in plasma at concentrations of 10 or 200 ng/ml at room temperature for up to 23 h or while frozen for up to 2 months.

Clinical pharmacokinetics

The described analytical method was used to determine the plasma concentrations of I obtained during a single ascending dose tolerance

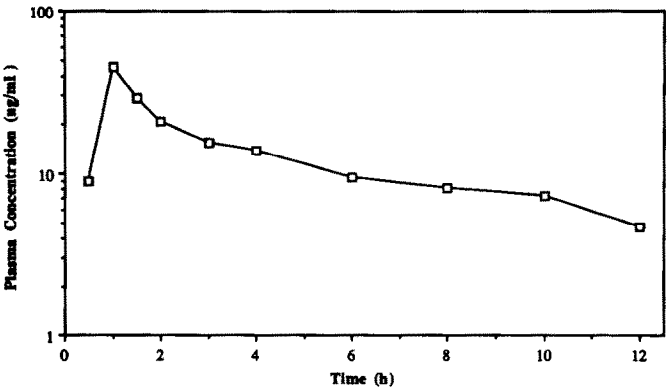

Fig. 3. Representative plasma concentration–time profile for **I** in a healthy male subject following a 2-mg oral dose of **I** · HCl.

study of **I** · HCl carried out in healthy male subjects. Fig. 3 shows a representative plasma concentration–time curve following a single 2-mg dose of **I** · HCl in one of these subjects. The maximum plasma concentration of 45.2 ng/ml occurred at the 1-h sampling time. The elimination half-life was 6.9 h.

DISCUSSION

Attention to certain specific details was found to be important in assuring the best possible recovery of **I** and **II** using this analytical procedure. First, the extraction column must not be allowed to dry out between preconditioning and the addition of the plasma mixture. The column should receive an additional rinse with 1 ml of water if more than 5 min has elapsed since the completion of preconditioning. Second, the vacuum used to draw the plasma mixture and subsequent rinses through the column should not exceed *ca.* 3.5 kPa. Finally, once the plasma has been placed on the extraction column, rinsing and elution should take place without delay. Extraction columns that were allowed to dry out, a vacuum that was too strong, and delays in rinsing and elution all appeared to result in decreased recovery of the compounds from plasma.

The recovery studies using ^{14}C-labelled **I** in dog plasma have not been repeated using human plasma as a matrix. However, comparisons of peak heights following direct injection of a standard solution containing I and injection of an extract of human plasma confirmed that a recovery of greater than 95% would be expected. This is consistent with the results of the ^{14}C recovery studies using dog plasma.

Numerous standard curves similar to the representative curve discussed above have been generated during the development of this assay procedure. Correlation coefficients are generally greater than 0.999, demonstrating excellent linearity over a wide concentrations range (2.5–500 ng/ml). The precision and accuracy of this method were excellent for all concentrations greater than 8–10 ng/ml. The variability increased and the accuracy decreased for lower concentrations, but the precision and accuracy remained acceptable for concentrations as low as 2 ng/ml. The acceptability of these data led to the assignment of 2 ng/ml using a 1-ml plasma sample as the quantitation limit for this assay procedure.

The described analytical method appears to be suitable for the measurement of concentrations of **I** in the plasma samples that will be generated during pharmacokinetic studies planned for the clinical development of this potential antiepileptic agent. The method is precise, simple and sensitive enough to reliable quantitate concentrations of **I** in human plasma following the lowest dose (2 mg) of **I** · HCl currently planned for the clinical development of this drug.

ACKNOWLEDGEMENTS

The authors thank Sheila T. Bunnell and Richard A. Finley for their technical assistance.

REFERENCE

1 C. Braestrup, E. B. Nielsen, U. Sonnewald, L. J. S. Knutsen, K. E. Anderson, J. A. Jansen, K. Frederiksen, P. H. Andersen, A. Mortensen and P. D. Suzdak, *J. Neurochem.*, 54 (1990) 639.