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High-performance liquid chromatographic determination of trimebutine and its major metabolite, *N*-monodesmethyl trimebutine, in rat and human plasma

Eun-Hee Joo[a], Woo-Ik Chang[a], Injoon Oh[a], Sang-Chul Shin[a], Han-Kwang Na[b], Yong-Bok Lee[a,*]

[a]*College of Pharmacy and Research Institute of Drug Development, Chonnam National University, 300 Yongbong-Dong, Buk-Gu, Kwangju 500-757, South Korea*

[b]*Department of Clinical Pharmacology, Toxicology Research Institute, Korea FDA, 5 Nokbeon-Dong, Eunpeong-Gu, Seoul 122-020, South Korea*

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Abstract

A rapid, selective and very sensitive ion-pairing reversed-phase HPLC method was developed for the simultaneous determination of trimebutine (TMB) and its major metabolite, *N*-monodesmethyltrimebutine (NDTMB), in rat and human plasma. Heptanesulfonate was employed as the ion-pairing agent and verapamil was used as the internal standard. The method involved the extraction with a *n*-hexane–isopropylalcohol (IPA) mixture (99:1, v/v) followed by back-extraction into 0.1 *M* hydrochloric acid and evaporation to dryness. HPLC analysis was carried out using a 4-μm particle size, C_{18}-bonded silica column and water–sodium acetate–heptanesulfonate–acetonitrile as the mobile phase and UV detection at 267 nm. The chromatograms showed good resolution and sensitivity and no interference of plasma. The mean recoveries for human plasma were 95.4±3.1% for TMB and 89.4±4.1% for NDTMB. The detection limits of TMB and its metabolite, NDTMB, in human plasma were 1 and 5 ng/ml, respectively. The calibration curves were linear over the concentration range 10–5000 ng/ml for TMB and 25–25000 ng/ml for NDTMB with correlation coefficients greater than 0.999 and with within-day or between-day coefficients of variation not exceeding 9.4%. This assay procedure was applied to the study of metabolite pharmacokinetics of TMB in rat and the human.

Keywords: Trimebutine; *N*-Monodesmethyltrimebutine

1. Introduction

Trimebutine (TMB) maleate, 2-dimethylamino-2-phenylbutyl-3,4,5-trimethoxybenzoate hydrogen maleate, possesses antispasmodic actions on the gastrointestinal (GI) tract and is clinically used for treatment of various GI disorders including irritable bowel syndrome and postoperative ileus [1–5].

The metabolic pathways of TMB in various animals were investigated using radiolabelled drug and six metabolites were identified [6,7]. Not only TMB but also its major metabolite, NDTMB, has weak opioid agonistic characteristics (Fig. 1) [8–15]. However little is known about the metabolite kinetics of TMB. The main reason for this paucity of

*Corresponding author. Fax: +82-62-530-2949; e-mail: leeyb@orion.chonnam.ac.kr

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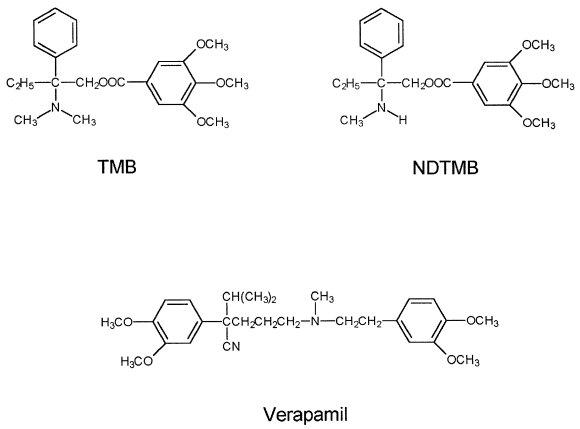

Fig. 1. Chemical structures of trimebutine (TMB), *N*-monodesmethyltrimebutine (NDTMB) and verapamil (I.S.).

information appears to be due to the lack of simultaneous analytical methods applicable to various animal and human studies.

The earlier method of analysis for TMB was based on HPLC [16]. The only quantitative measurement of its metabolites in plasma was based on GC–MS using radiolabelled compounds and HPLC with fluorescence detection [6,7]. But this method was inadequate for pharmacokinetic studies of TMB because of its time-consuming extraction procedures and limited reproducibility, particularly for NDTMB when the HPLC method was employed.

A simple, rapid, routine, non-radioactive and simultaneous assay of TMB and NDTMB in plasma was required for further investigations of their pharmacokinetics. We have established an HPLC method applicable to bioassay and available to pharmacokinetics by means of not only simultaneous determination of TMB and NDTMB, but also improving sensitivity. This type of assay using ion-pairing reversed-phase HPLC is the subject of this paper.

2. Experimental

2.1. Chemicals

TMB, TMB maleate and NDTMB were kindly supplied from Sam Il Pharmaceuticals (Seoul, South Korea). Verapamil hydrochloride (internal standard, Fig. 1) and 1-heptanesulfonic acid sodium salt were purchased from Sigma (St. Louis, MO, USA) and *n*-hexane, isopropylalcohol, triethylamine, *tert*.-butyl methyl ether (TBME), anhydrous sodium carbonate, acetic acid and trihydrated sodium acetate were products of Aldrich (Milwaukee, WI, USA). HPLC grade methanol and acetonitrile were obtained from Merck (Rahway, NJ, USA). HPLC grade water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA) and used throughout the study. The other chemicals and organic solvents were of analytical or HPLC grade and used without further purification. The mobile phase components such as acetate buffer, acetic acid, heptane sulfonate, acetonitrile were filtered through a 0.45-μm pore size membrane filter prior to mixing and ultrasonically degassed after mixing. All plasma samples were stored at −20°C after collection.

2.2. Instrumentation

The HPLC system was from Waters (Millipore). It consisted of a Model 501 pump, a Model 484 tunable UV absorbance detector set at 267 nm with 0.001 AUFS and a Model U6K injector. The detection wavelength, 267 nm, was determined by scanning the maximum absorbance wavelength of TMB and NDTMB in the mobile phase with UV spectrophotometer (Uvikon 930, Kontron Instruments, Zürich, Switzerland). Separations were performed on a Nova-Pak C_{18} reversed-phase column (300 mm×3.9 mm I.D., 4-μm particle size) with a Guard-Pak precolumn module and insert (Millipore). Detector output was quantitated on a Model D520A computing integrator (Young In Scientific, Seoul, South Korea).

2.3. Stock solutions and standards

Stock solutions of TMB, NDTMB (0.5 mg/ml) and verapamil, the I.S. (0.3 mg/ml), were prepared in methanol. Working standards of TMB, NDTMB and verapamil were freshly obtained daily by diluting stock solutions with methanol. Working standards (100 μl) were pipetted into 10-ml glass test tubes and evaporated to dryness under a nitrogen stream in a centrifugal evaporator (CVE-100, Tokyo Rikakikai, Tokyo, Japan). Calibration standards were

freshly prepared by dissolving these dried residues with blank plasma to give concentrations of 10 000, 5000, 1000, 500, 200, 100, 50, 20 and 10 ng/ml for TMB, 25 000, 10 000, 2500, 1000, 500, 250, 100, 50 and 25 ng/ml for NDTMB.

2.4. Extraction procedure

The extraction procedures for the simultaneous determination of TMB and NDTMB were determined after the evaluation of several extraction methods. Two extraction solvents were used in order to compare the extraction efficiency of TMB and NDTMB from plasma. Solvent A was *n*-hexane–IPA (99:1, v/v) and solvent B was TBME. And also, the effect of back-extraction using 0.1 *M* hydrochloric acid was evaluated. Finally, an aliquot of 100 μl of the I.S. (3 μg/ml of verapamil) was pipetted into a series of 10-ml glass test tubes and evaporated to dryness under a nitrogen stream in a centrifugal evaporator. The dried residue was dissolved in an unknown plasma sample. For a rat plasma sample (100 μl), an aliquot of 200 μl of methanol and 500 μl of 5% sodium carbonate were added. For a human plasma sample (500 μl), an aliquot of 1 ml of methanol and 1 ml of 5% sodium carbonate were used. The sample was then extracted with 3 to 5 ml of *n*-hexane–IPA (99:1, v/v) by vortex-mixing for 1 min. After centrifugation at 3000 *g* for 10 min and freezing for 3 min, the supernatant was decanted into a second series of 10-ml glass test tubes. Subsequently, the organic layer was back-extracted with 100 μl of 0.1 *M* hydrochloric acid by vortex-mixing for 1 min and centrifuged to separate the phase at 3000 *g* for 5 min. After freezing for 1 min, the organic layer was decanted. The aqueous layer was then evaporated to dryness under a nitrogen stream in a centrifugal evaporator. The residue was reconstituted in 100 μl of mobile phase by vortex-mixing, of which 10 μl was then injected into the HPLC system. In addition, the stability of TMB, NDTMB and verapamil in 0.1 *M* hydrochloric acid was checked.

2.5. Chromatography

Two mobile phases were compared. Mobile phase A was a mixture (82:18, v/v) of acetonitrile and 0.05 *M* acetate buffer containing 5 m*M* heptanesulfonic acid adjusted to pH 5.5 with acetic acid and mobile phase B was consisted of acetonitrile–methanol–0.04 *M* ammonium bromide–triethylamine (36:24:40:0.1, v/v/v/v, pH 6.4). All separations were performed isocratically at a flow-rate of 1 ml/min at ambient temperature with a column inlet pressure of about 9 MPa.

2.6. Quantitation

Quantitation was based on electronically integrated peak-height ratios of TMB and NDTMB to verapamil using calibration standards covering the expected concentration ranges. Calibration curves were obtained by weighted linear regression (weighting factor: $1/[\text{concentration}]^2$). The unknown concentrations of TMB and NDTMB were calculated by inverse prediction from the calibration equations. Calibration standards and the unknown samples were analysed identically.

2.7. Recovery

Absolute recoveries of control standards (1000, 100 and 10 ng/ml for TMB, 2500, 100 and 25 ng/ml for NDTMB) were determined by assaying these samples ($n=5$) as described above and comparing the peak heights of TMB, NDTMB and verapamil with those obtained from direct injection of the compounds dissolved in mobile phase.

2.8. Precision and accuracy

Within-day precision was determined by analysing control standards (1000, 100 and 10 ng/ml for TMB, 2500, 100 and 25 ng/ml for NDTMB) five times over one day in no fixed order, while between-day precision was determined from the analysis of each control standard (1000, 100 and 10 ng/ml for TMB, 2500, 100 and 25 ng/ml for NDTMB) once on each of five different days. Assay precision (C.V.) was assessed by expressing the standard deviation of the measurements as a percentage of the mean value. The accuracy of the assay was estimated for each of the control standards (1000, 100 and 10 ng/ml for TMB, 2500, 100 and 25 ng/ml for NDTMB) by

comparing the target concentration of TMB and NDTMB with the assayed concentration.

2.9. Stability

To test the stability of basic drugs such as TMB, NDTMB and verapamil in the acidic condition during the back-extraction, they were injected into the HPLC system at 0, 1, 2, 4, 6, 8, 12 and 24 h after they were put into 0.1 *M* hydrochloric acid.

The stability of TMB and NDTMB was determined in each control standard comprising 1000 ng/ml and 100 ng/ml of both compounds in plasma stored at −20°C for three months.

And also, the stability of stock solutions stored in the light-protected glass bottles at 4°C for three months was checked.

2.10. Pharmacokinetic studies of TMB

Male Sprague-Dawley rats, weighing 200–250 g and aged 8–10 weeks, were fasted overnight before drug administration. The bolus injection of TMB maleate (10 mg/ml/kg as TMB) was made intravenously to each rat ($n=6$). Blood samples were collected immediately before dosing and up to 2 h postdose, using sodium heparin as the anticoagulant. Plasma was separated by centrifugation (10 000 *g* for 2 min) and immediately frozen at −20°C until analysis.

Six normal healthy male volunteers, weighing 59–78 kg and aged 23–32 years, participated in a pharmacokinetic study of oral TMB after giving written informed consent. They were fasted overnight before drug administration and until the 4-h blood collection. They received a single oral dose of three 100-mg TMB maleate tablets (Modulin®, Jouveinal Laboratories, Fresnes, France). Multiple venous blood samples were drawn into Venoject heparin-containing tubes just prior to and up to 48 h after administration. Plasma was separated and stored as the method described above.

The plasma level–time data of TMB and NDTMB were evaluated by means of a nonlinear regression analysis using PCNONLIN [17]. The relevant pharmacokinetic parameters were calculated according to the procedures described in Ref. [18].

3. Results and discussion

3.1. Mobile phase and internal standard

The chromatograms of TMB and NDTMB in mobile phase A showed more stable base lines and better resolutions than those in mobile phase B using bromide as the ion-pairing counter ion [19]. A base-stable HPLC column using mobile phase A obviates the need to add triethylamine to improve peak shapes [20]. Fig. 2 shows chromatograms using mobile phase A of (A) drug-free rat plasma, (B) rat plasma containing TMB (500 ng/ml), NDTMB (1000 ng/ml) and verapamil, the I.S., and (C) rat plasma taken at 20 min after i.v. administration of TMB maleate (10 mg/ml/kg as TMB) and spiked with verapamil. TMB, NDTMB and verapamil were eluted as sharp, symmetrical peaks after 5.4, 3.4 and 4.2 min, respectively, in areas of the chromatogram free from endogenous peaks. Also, the same results could be obtained from the chromatograms using human plasma.

In the HPLC analysis of TMB by Astier et al.

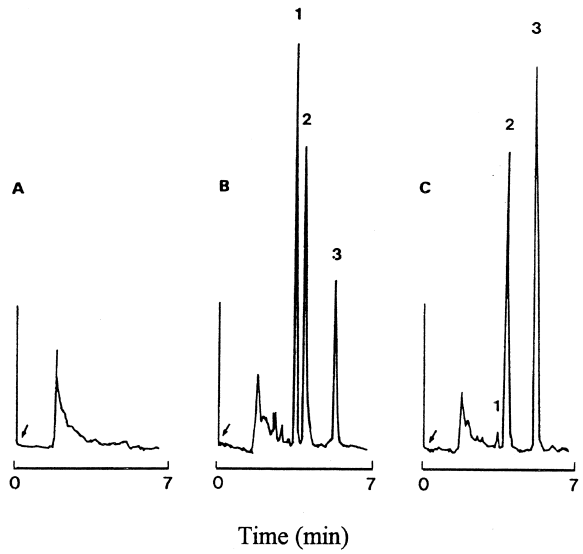

Fig. 2. High-performance liquid chromatograms of (A) blank rat plasma, (B) rat plasma sample containing TMB (500 ng/ml), NDTMB (1000 ng/ml) and verapamil, the I.S. and (C) rat plasma sample taken at 20 min after i.v. administration of TMB maleate (10 mg/ml/kg as TMB) and spiked with verapamil. Peaks: 1=NDTMB (3.4 min), 2=verapamil, the I.S. (4.2 min), 3=TMB (5.4 min), ↙=injection.

[16], procaine hydrochloride was used as the I.S., but NDTMB was not able to be analysed in their chromatographic conditions. In order to select the proper I.S. for the simultaneous quantitation of TMB and NDTMB, the following basic drugs as the candidates for the I.S. were dissolved in mobile phase A and injected into the HPLC system: aminophylline, aniline, caffeine, chlorpheniramine, chlorpromazine, creatinine, desipramine, diazepm, diltiazem, diphenhydramine, fluphenazine, 5-fluorouracil, haloperidol, hydralazine, imipramine, isoniazid, isoproterenol, labetalol, DL-methyl ephedrine, metoclopramide, metoprolol, omeprazole, procainamide, promethazine, propranolol, quinidine, quinine and verapamil. Several basic drugs such as diazepam, diltiazem, diphenhydramine, labetalol and verapamil were selected as the candidates for the I.S.. As the results of the extraction of these candidates (3 μg/ml) by *n*-hexane–IPA (99:1, v/v), the absolute recoveries of diazepam, diltiazem, diphenhydramine, labetalol and verapamil were 48.5±3.6, 61.7±3.2, 3.2±1.1, 33.3±2.7 and 98.5±3.2%, respectively. And the retention times of diazepam, diltiazem, diphenhydramine, labetalol and verapamil were 3.1, 4.4, 6.2, 2.5 and 4.2 min, respectively. Verapamil was selected as the most proper internal standard in this study.

3.2. Determination of extraction procedures

The chromatograms with back-extraction using 0.1 *M* hydrochloric acid after the extraction with each of *n*-hexane–IPA and TBME showed no interference of plasma and represented better resolutions and more stable baselines than those without back-extraction. This back-extraction step was essential to minimize interfering peaks, especially when analysing in the lower nanogram range. The absolute recoveries of TMB (100 ng/ml) for human plasma extracted by each of TBME and *n*-hexane–IPA (99:1, v/v) were 82.7±3.3 and 98.2±1.7%, respectively. Those of NDTMB (100 ng/ml) were 82.5±0.5 and 92.5±4.6%, respectively. And also, it was found that the mean absolute recoveries had no significant differences ($P<0.05$) between rat and human plasma. On the other hand, TBME, a more polar solvent than *n*-hexane, was chosen not only because of its physical properties [21], but also in order to increase the extraction efficiency of NDTMB. But, there was no benefit in using it for the simultaneous extraction of TMB and NDTMB. Therefore, the extraction procedures using *n*-hexane–IPA (99:1, v/v) and back-extraction with 0.1 *M* hydrochloric acid was selected.

3.3. Stability

There were no changes in peak-heights/areas of each of TMB, NDTMB and verapamil in 0.1 *M* hydrochloric acid up to 24 h. It could be confirmed that these basic drugs would not be decomposed during the back-extraction.

TMB and NDTMB in plasma stored at −20°C for three months and stock solutions stored in the light-protected glass bottles at 4°C for three months were stable.

3.4. Linearity

The mean (±SD) regression equations from ten replicate calibration curves on different days for human plasma: $y=(0.00612\pm0.00052)x+(0.31787\pm0.09386)$ for 10–5000 ng/ml TMB and $y=(0.00652\pm0.00073)x+(0.06145\pm0.01383)$ for 25–25 000 ng/ml NDTMB (where, y=peak-height ratio, x=concentration), showed significant linearities ($\gamma=0.9991\pm0.00021$ for TMB, $\gamma=0.9994\pm0.00028$ for NDTMB), with statistically insignificant ($P>0.05$) nonlinear elements in the residual sum of squares, as determined by analysis of variance. And also, the mean regression equations for rat plasma: $y=(0.00131\pm0.00011)x+(0.06595\pm0.02308)$ for 10–5000 ng/ml TMB and $y=(0.00143\pm0.00015)x+(0.01330\pm0.00293)$ for 25–25000 ng/ml NDTMB, showed the same linearities.

3.5. Recovery

The mean absolute recoveries of TMB and NDTMB for human plasma were found to be 95.4±3.1% for TMB and 89.4±4.1% for NDTMB (Table 1). Those for rat plasma were to be 95.2±1.7% and 91.5±4.6%, respectively, but there were no significant differences ($P<0.05$) in the absolute recoveries between rat and human plasma.

Table 1
Absolute recoveries of TMB and NDTMB for human plasma

Concentration (ng/ml)	Recovery (mean±SD, $n=5$) (%)	
	TMB	NDTMB
2500	N.A.[a]	89.3±2.5
1000	97.8±3.4	N.A.
100	98.2±1.7	92.5±4.6
25	N.A.	86.4±5.1
10	93.3±4.1	N.A.

[a] Not available.

These high, reproducible recoveries of TMB and NDTMB as shown in Table 1 were able to increase their assay sensitivities.

3.6. Limit of quantitation

The criteria used to estimate the limit of quantitation (LOQ) were maximal within-day and between-day coefficients of variation of 20% and a mean deviation from the nominal concentration also less than 20%. The LOQ for human plasma was estimated to be 1 ng/ml for TMB and 5 ng/ml for NDTMB when signal-to-noise (S/N) ratio was 3 and that for rat plasma was 2 and 10 ng/ml, respectively. So, if larger volumes of human plasma were used, the LOQ was substantially decreased in this HPLC assay.

3.7. Precision and accuracy

Precision and accuracy were investigated by replicate analyses of control standards for human plasma (Table 2), and in all cases the within-day and between-day precision was acceptable at a coefficient of variation of 9.4%, or less. In addition, the accuracies of TMB and NDTMB were within 6.0 and 5.6%, respectively, when assay results were compared with target concentrations across this range. And also, the precision and accuracy of both drugs for rat plasma were found to be within 10% and were apparently not significantly different ($P<0.05$) from the corresponding values in human plasma. So, it is expected that the current method will be applicable to the metabolite kinetics of TMB for human and rat.

3.8. Application

The mean plasma profiles of TMB and NDTMB after intravenous bolus administration of TMB maleate (10 mg/ml/kg as TMB) to six rats and single oral administration of three 100-mg TMB maleate tablets to six normal healthy male volunteers, are shown in Figs. 3 and 4, respectively. The TMB kinetics followed a biexponential pattern as pointed out in Ref. [16]. The mean (±SD) terminal half-life, total body clearance, volume of distribution at steady state and residence time of TMB in rats were 43.5 (±3.8) min, 90.0 (±2.6) ml/min/kg,

Table 2
Precision and accuracy of HPLC analysis of TMB and NDTMB

TMB				NDTMB			
Target (ng/ml)	Found (Mean±SD)	C.V. (%)	Accuracy[a] (%)	Target (ng/ml)	Found (Mean±SD)	C.V. (%)	Accuracy[a] (%)
Within-day (n=5)							
1000	1012.5±54.7	5.4	1.3	2500	2438.6±229.2	9.4	−2.5
100	98.4±5.0	5.1	−1.6	100	103.9±4.7	4.5	3.9
10	10.6±0.9	8.5	6.0	25	26.4±1.8	6.8	5.6
Between-day (n=5)							
1000	1019.4±80.5	7.9	1.9	2500	2432.9±138.7	5.7	−2.7
100	104.2±5.6	5.4	4.2	100	98.4±5.1	5.2	−1.6
10	9.9±0.24	2.4	−1.0	25	26.1±1.9	7.3	4.4

[a] Defined as: [(measured conc.−target conc.)/target conc.]×100%.

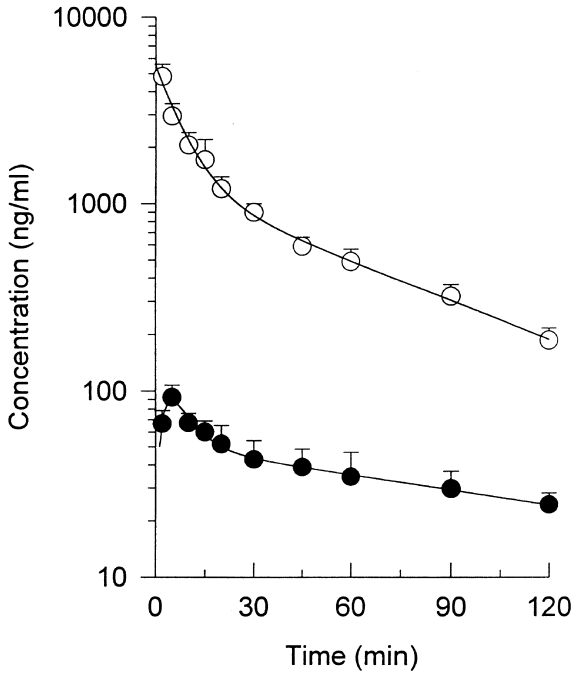

Fig. 3. Mean arterial plasma concentration–time profiles of TMB and NDTMB after femoral vein administration of TMB maleate (10 mg/ml/kg as TMB) to rats (n=6). Each solid line was calculated by PCNONLIN program. Vertical bar represents the standard error of the mean. Key: ○=TMB, ●=NDTMB.

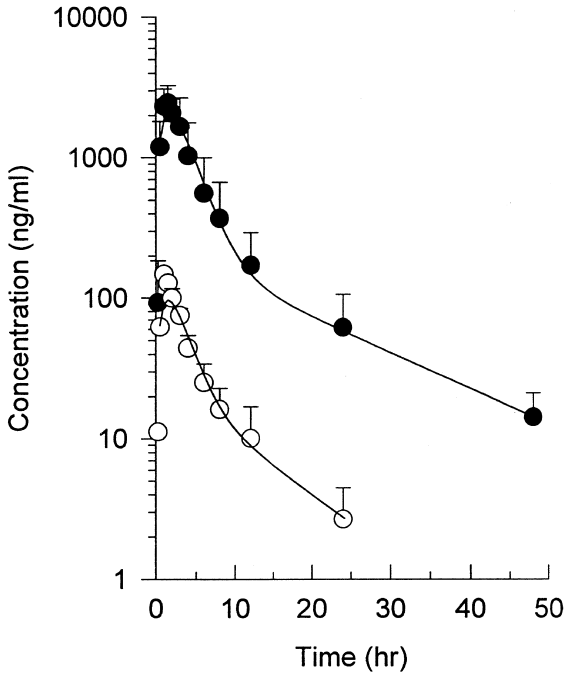

Fig. 4. Mean venous plasma concentration–time profiles of TMB and NDTMB after oral administration of three 100-mg TMB maleate tablets to humans (n=6). Each solid line was calculated by PCNONLIN program. Vertical bar represents the standard error of the mean. Key: ○=TMB, ●=NDTMB.

4242 (±262) ml/kg and 47.2 (±3.1) min, respectively. The mean (±SD) terminal half-life of NDTMB and ratio of area under the plasma concentration–time curves (AUC) of NDTMB formed from TMB to AUC of TMB in rats were 113.4 (±20.9) min and 0.078 (±0.009), respectively. On the other hand, the mean (±SD) terminal half-life and AUC of TMB in the human were 443.1 (±124.1) min and 33864 (±4704) ng/ml/min, respectively. But, the terminal half-life of TMB reported by Astier et al. [16] was 164.4 min. It was thought that the lower part of the terminal phase of TMB could be detected because the LOQ of the present HPLC method was much lower than the previous HPLC method [16]. The mean (±SD) terminal half-life of NDTMB and AUC ratio of NDTMB to TMB in the human were 707.4 (±176.9) min and 21.8 (±3.3), respectively. It is of interest to note that the terminal half-lives of TMB and NDTMB in the human were much longer than those in rat and that AUC ratio of NDTMB to TMB was dependent on the administration route and species.

4. Conclusion

We have described a simple, robust, accurate and precise HPLC method for the simultaneous determination of TMB and its major metabolite, NDTMB, within the plasma concentration range observed in the human and rat. It has been currently used in this laboratory for investigating the metabolite kinetics of TMB following intravenous and chronic oral TMB therapy in the human and rat. The present method has been applied successfully in a study of the metabolite kinetics of TMB and these results will be presented elsewhere.

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