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SIMULTANEOUS DETERMINATION OF TRIAZOLO-BENZOPHENONE [2',5-DICHLORO-2-(3-GLYCYLAMINOMETHYL-5-METHYL-4H-1,2,4-TRIAZOL-4-YL)-BENZOPHENONE] AND ITS MAJOR BLOOD METABOLITE, TRIAZOLAM, IN MONKEY PLASMA BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

MASAHARU KONISHI and YOSHIO MORI*

Department of Analytical Chemistry, Shionogi Research Laboratory, Shionogi & Co. Ltd., Fukushima-ku, Osaka 553 (Japan)

and

KENTARO HIRAI

Department of Organic Chemistry, Shionogi Research Laboratory, Shionogi & Co. Ltd., Fukushima-ku, Osaka 553 (Japan)

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SUMMARY

A gas—liquid chromatographic method for the simultaneous determination of triazolo-benzophenone [2',5-dichloro-2-(3-glycylaminomethyl-5-methyl-4H-1,2,4-triazol-4-yl)-benzophenone, TB] and its major blood metabolite, triazolam, 8-chloro-6-(*o*-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-*a*][1,4]benzodiazepine (TZ), in monkey plasma was developed. Decomposition of TB was observed during gas—liquid chromatography. In alkaline medium, TB in plasma was submitted to ring closure reaction to yield triazolo-aminoquinoline, [4-amino-7-chloro-5-(2-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-*a*]quinoline (TAQ), while TZ remained unaffected, and TAQ and TZ in the benzene extract were assayed by gas—liquid chromatography using an electron-capture detector. The concentration ranges studied were from 5 to 40 ng of TB per 0.5 ml of plasma and from 2 to 20 ng of TZ per 0.5 ml of plasma. This method could be applied to the determination of the plasma levels of TB and TZ in monkeys following intravenous administration of a single 0.2 mg/kg dose of TB.

INTRODUCTION

Triazolo-benzophenone [2',5-dichloro-2-(3-glycylaminomethyl-5-methyl-4H-

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1,2,4-triazol-4-yl)-benzophenone, TB] is a member of a new series of sleep inducers recently developed in our laboratory [1], and is expected to be a pro-drug of triazolam (TZ) as well as peptido-aminobenzophenone which was defined as a pro-drug of 1,4-benzodiazepine in previous papers [2—6].

The peptido-aminobenzophenone was assayed in a previous study [5] by a cyclization reaction in alkaline medium in which the peptido-aminobenzophenone was converted into the thermostable aminoquinolone derivative having good chromatographic properties, since the peptido-aminobenzophenone decomposed during gas—liquid chromatography (GLC). But a simultaneous determination of the parent drug, peptido-aminobenzophenone, and its 1,4-benzodiazepine metabolites was not successful because the latter were readily hydrolyzed in the alkaline medium.

In the case of TB, it would be cyclized to 4-amino-7-chloro-5-(2-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-*a*]quinoline (TAQ) in the alkaline medium by the same mechanism as peptido-aminobenzophenone. Further, the simultaneous determination of the parent drug and the metabolite TZ would become feasible, as TZ was stable in the alkaline medium [7].

The metabolism of TZ in several species [8—10] has already been reported and the major blood metabolites in rats and dogs were 1′-hydroxytriazolam (1′-HT) and 4-hydroxytriazolam (4-HT) [9, 10]. Therefore, gas chromatographic differentiation between the above metabolites and TAQ derived from the cyclization of TB would be required. Ultra-micro determinations of TZ have been reported for the radioimmunoassay [11], high-performance liquid chromatography [12], and capillary gas chromatography with electron-capture (EC) detector [13].

This paper describes the simultaneous determination of TB and TZ in monkey plasma by the EC-GLC method in order to investigate the metabolic conversion of TB into TZ following administration of TB.

EXPERIMENTAL

Chemicals and reagents

TB, TZ, 1′-HT, 4-HT, TAQ and 3-amino-6-chloro-5-(2-chlorophenyl)-1-propylquinolin-2-one (PAQ) were synthesized in our laboratory. The solvents used were of a special grade for EC-GLC (Wako Industries Co., Osaka, Japan) while other chemicals were of reagent grade and used without further purification.

Gas—liquid chromatography

A Shimadzu (Kyoto, Japan) Model 4APE gas chromatograph equipped with a 10 mCi ^{63}Ni electron-capture detector was used with a 0.5 m × 2 mm I.D. glass column filled with 3% OV-17 on 100—120 mesh Gas-Chrom Q. The column temperature was kept at 290°C after conditioning with a nitrogen flow-rate of 20 ml/min for 48 h at 330°C. The detector and injection port temperatures were 320°C. The nitrogen flow-rate was 70 ml/min, and the pulse mode was of a 100-μsec frequency with a 2-μsec width.

The trimethylsilyl (TMS) derivative of 1′-HT was obtained by treatment with bis(trimethylsilyl)acetamide in acetone at room temperature.

TABLE I

RETENTION TIMES AND R_F VALUES OF TRIAZOLO-BENZOPHENONE RELATED COMPOUNDS

	TZ	TAQ	1'-HT	4-HT	PAQ
Retention time (min)	2.75	4.15	2.58*	5.00	1.10
R_F	0.20	0.40	0.16	0.12	0.60

*Retention time of the TMS derivative of 1'-HT.

The retention times and R_F values of the compounds in this assay are listed in Table I.

Thin-layer chromatography

Thin-layer chromatography (TLC) plates of silica gel F_{254} (250 μm; E. Merck, Darmstadt, G.F.R.) were used; the solvent system was chloroform—acetone—ethanol (8:1:1). The chromatograms were visualized under UV light.

Preparation of standard solutions

For preparation of the calibration curve, solutions containing 0.04—0.4 μg/ml TZ, 0.05—0.5 μg/ml TAQ, and 0.15 μg/ml PAQ as an internal standard were prepared in benzene. Samples of 2 μl each were subjected to gas chromatography.

For recovery study, solutions containing both 0.1—0.8 μg/ml TB and 0.04—0.4 μg/ml TZ were prepared in 0.1% aqeuous solution of ethanol; 50 μl of the solutions were added to 0.5 ml of monkey control plasma.

Procedure for determination of TB and TZ in plasma

To a 12-ml centrifuge tube containing 0.5 ml of plasma, 0.5 ml of 2 *M* potassium hydroxide was added and the mixture was heated on a water bath at 91 ± 1°C for 90 min. After cooling, the mixture was extracted with 5 ml of benzene containing 6 ng of the internal standard (PAQ) on a mechanical shaker. The benzene extract was separated and evaporated to dryness in vacuo at 40°C. The residue was dissolved in 0.1 ml of solvent (benzene—acetone, 1:1), and a 5-μl sample was subjected to gas chromatography. Calculations were done using a calibration curve prepared by the peak height method.

Procedure for determination of TZ in plasma

Determination of TZ following its administration was performed without alkaline hydrolysis as follows. To a 12-ml centrifuge tube containing 0.5 ml of plasma, 0.5 ml of water was added and the mixture was extracted with 5 ml of benzene containing 12 ng of the internal standard (PAQ) on a mechanical shaker. The extract was evaporated to dryness in vacuo at 40°C, the residue was dissolved in 0.2 ml of benzene, and a 5-μl sample was subjected to gas chromatography. Calculations were done using a calibration curve prepared by the peak height method.

Animal protocol

Animal experiments were carried out according to the cross-over design of experiment with Latin Square. The male rhesus monkeys used (5.7—7.7 kg) were made to fast overnight before being given the drug.

TB was given intravenously at a dose of 0.2 mg/kg as a 0.04% solution of TB monohydrochloride monohydrate in 0.9% aqueous sodium chloride. TZ was given intravenously to the same four monkeys being used for TB dosing at a dose of 0.2 mg/kg as a 0.04% solution of TZ in 0.9% aqueous sodium chloride containing 40% propylene glycol.

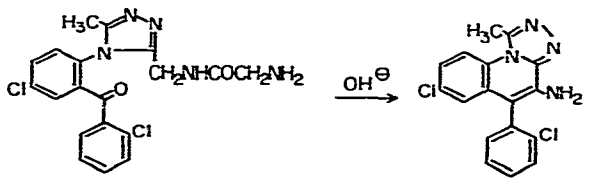
Blood samples (2.5 ml) were collected at 5, 10, 20, 30, 45 min, and 1, 2, 3, 5, 7, 24 and 48 h post-dosing, then immediately centrifuged at 1500 *g* for 15 min. The plasma samples separated were stored at —20° C until analysis.

RESULTS AND DISCUSSION

Derivatization of TB

TB was pyrolized during GLC and gave no remarkable peaks. Therefore, the derivatization of TB into a compound which possessed good chromatographic properties was attempted.

As well as in the analysis of the peptido-aminobenzophenone, when TB was heated in 1 *M* potassium hydroxide solution at 92°C for 1 h, colorless crystals were precipitated. The precipitate was removed by filtration and was subjected to TLC and GLC, which gave a single spot with an R_F value of 0.40 and a single peak with a retention time of 4.15 min, respectively. Furthermore, the mass spectrum of this compound showed a molecular ion peak at *m/e* 342 and was identical to that of authentic TAQ. Thus, TB was found to be converted into TAQ in an alkaline medium, as shown below.



Triazolo-benzophenone (TB) Triazoloaminoquinoline (TAQ)

The reaction conditions were examined further in various alkaline solutions such as 0.1 *M*, 0.5 *M*, 1 *M*, 2 *M*, 3 *M* potassium hydroxide and 5% potassium carbonate. The reaction in 1 *M* potassium hydroxide gave the most satisfactory results with respect to yield and by-product; the result of a time—course study under the same conditions is shown in Fig. 1. The reaction proceeded to completion with a yield of 86% after 90 min. A by-product of the reaction, which appeared on TLC with an R_F value of 0.20 and on GLC with a retention time of 2.75 min, was derived in low yield. This compound possessed the same retention time and R_F value as TZ, and was identified as TZ by mass spectrometry.

To simplify the procedure for the determination of TB in plasma, the above reaction was performed in plasma solution containing 1 *M* potassium hydroxide

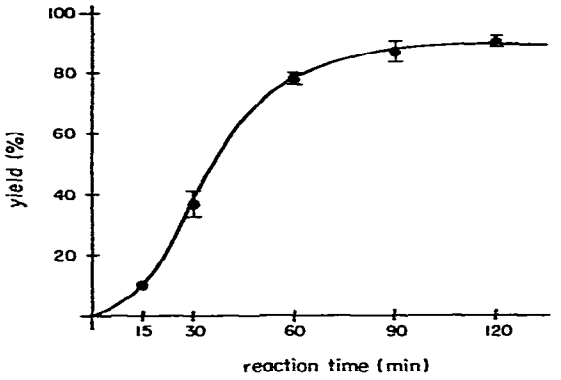


Fig. 1. Time—course for the cyclization reaction of triazolo-benzophenone into triazolo-aminoquinoline in 1 *M* potassium hydroxide solution.

without preliminary extraction of TB according to the same procedure etablished for the analysis of peptido-aminobenzophenone.

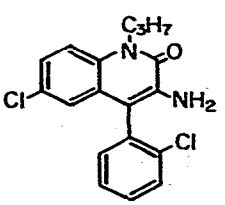
Interference

No endogenous component of plasma interfered with this assay, as shown in Fig. 2. Moreover, 1′-HT and 4-HT, the possible metabolites of TB, and the compounds to be analyzed could be perfectly separated from each other as shown in Table I. In this table, 1′-HT is shown as its TMS derivative because decomposition of the intact compound was observed during GLC. But the by-product, TZ, resulting from the cyclization reaction for the plasma TB interfered with the TZ determination.

Accordingly, the yield of the by-product TZ from the cyclization reaction was estimated from the specific peak height ratio which was obtained by dividing the peak height of TZ by that of TAQ. The specific peak height ratio obtained was 0.086 as the mean (n = 6, C.V. = 3.0%) at the TB concentration of 40 ng per 0.5 ml of plasma. Consequently, the net plasma levels of TZ in the presence of TB were estimated by subtracting 8.6% of the peak height of TAQ from that of TZ.

Choice of an internal standard

Because of the strong alkalinity of the reaction medium, stability of the internal standard is the foremost requirement. Estazolam and PAQ were selected as they possessed adequate GLC properties. PAQ was considered the preferable internal standard, considering the interferences by the metabolites of TB which might appear at longer retention times than that of PAQ in GLC.



N-Propylaminoquinolone (PAQ)

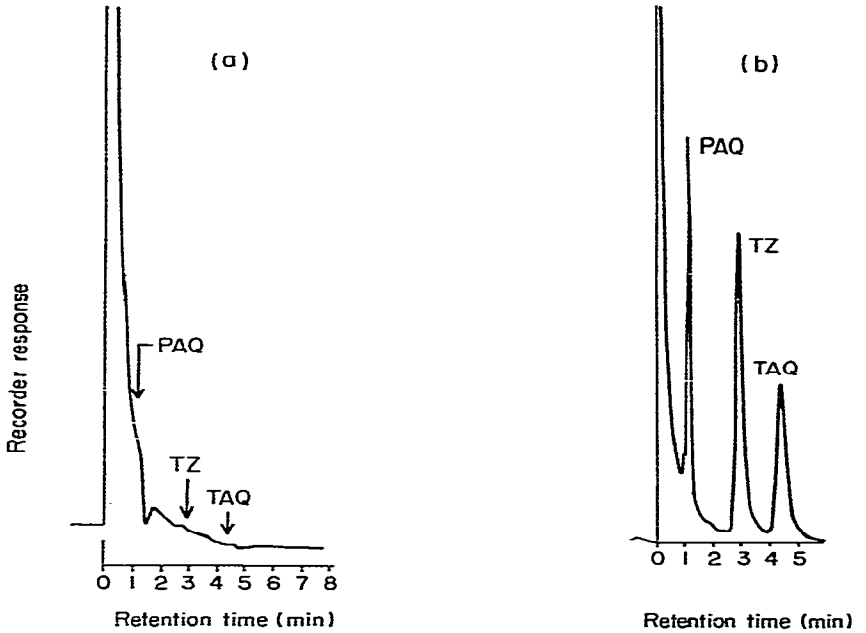


Fig. 2. Gas chromatograms of (a) control plasma and (b) sample plasma.

Calibration curve

The ratios of the peak height of TZ and TAQ to that of PAQ were plotted against the concentrations of TZ and TAQ in the range of 0.04—0.4 μg/ml and 0.05—0.5 μg/ml, respectively. The calibration curve obtained gave satisfactory linearity for each of the two compounds.

Extraction solvent

The recoveries of TZ and TAQ were examined with benzene, ethyl acetate and *n*-hexane, and were 102%, 88% and 37% for TZ and 100%, 96% and 88% for TAQ, respectively. Although ethyl acetate gave sufficient recoveries, the peaks and the baseline were affected by a small amount of water dissolved in the extract. *n*-Hexane gave an unsatisfactory recovery for TZ. Accordingly, benzene was selected as the extraction solvent.

Metabolites

General 1,4-benzodiazepines such as diazepam were readily hydrolyzed by alkali [5] and acid [14], but the metabolite TZ remained unaffected. The recovery of TZ following the cyclization reaction under the assay conditions was 97% as the mean in the range 0.2—1.0 μg/ml TZ.

Recovery studies

Solutions containing both 5—40 ng of TB and 2—20 ng of TZ were prepared in 0.5 ml of heparinized monkey plasma. Each solution was analyzed according to the procedure described above. The man percentage recoveries of TB and TZ, calculated from a total of 27 analyses, were 67.5% (S.D. = 4.3) and 112% (S.D. = 7.5), respectively.

Plasma levels of TB and TZ in monkeys

The plasma levels of TB and TZ were determined in monkeys following a single intravenous dose of 0.2 mg/kg, as shown in Fig. 3. The plasma level of the intact drug TB was 0.2 μg/ml at 5 min after dosing (0.3 μg/ml as the mean for the four monkeys), declined rapidly by apparent first-order elimination with a half-life of 20 min (16 min as the mean), and was below the detection limit of the assay at about 1 h post-dosing. The plasma level of the metabolite TZ was immediately detectable after dosing and disappeared more slowly than TB with apparent first-order elimination kinetics with a half-life of 68 min (67 min as the mean). The plasma half-lives of TB and TZ after intravenous 0.2 mg/kg dosing of TB are shown in Table II. The values were obtained from estimation by the least-squares method of each β-phase of the plasma concentration—time curve.

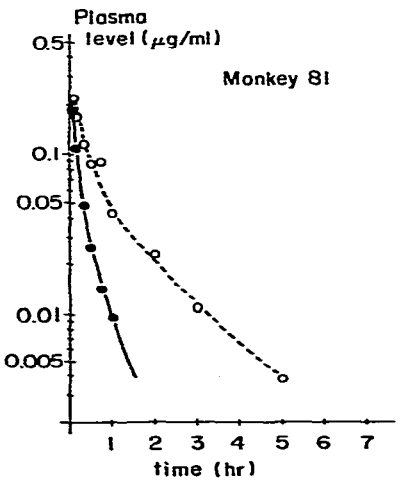


Fig. 3. Plasma levels of triazolo-benzophenone (•) and triazolam (○) following 0.2 mg/kg intravenous administration of triazolo-benzophenone to a monkey.

TABLE II

PLASMA HALF-LIVES OF TRIAZOLO-BENZOPHENONE AND TRIAZOLAM IN MONKEYS FOLLOWING 0.2 mg/kg INTRAVENOUS ADMINISTRATION OF TRIAZOLO-BENZOPHENONE

Monkey No.	Plasma half-life (min)	
	TB	TZ
79	16	77
81	20	68
92	12	48
99	14	74
Mean (S.D.)	16 (3)	67 (13)

On the other hand, the area under the plasma concentration—time curve (AUC) of TZ following the intravenous administration of TZ was significantly larger ($P < 0.05$) than that after TB dosing (Table III). However, the AUC ratio of the latter to the former, calculated from AUC values normalized to an equimolar dose basis, was 0.82. Therefore, the conversion of TB into TZ was at least 82%. Thus, TB seemed to be rapidly metabolized to TZ with high efficiency.

TABLE III

AREA UNDER THE PLASMA CONCENTRATION—TIME CURVES (AUC) OF TRIAZOLAM AFTER INTRAVENOUS 0.2 mg/kg DOSE OF TRIAZOLO-BENZOPHENONE OR TRIAZOLAM

Values are expressed as $\mu g \cdot h \cdot ml^{-1}$. AUC was estimated by the trapezoidal rule. TB/TZ ratios were calculated from AUC values normalized on an equimolar dose basis.

	Monkey No.				Mean (S.D.)
	79	81	92	99	
TB	0.195	0.176	0.118	0.166	0.164 (0.033)
TZ	0.247	0.353	0.324	0.223	0.287 (0.062)*
TB/TZ	1.09	0.685	0.501	1.02	0.824 (0.279)

*$P < 0.05$.

CONCLUSION

The determination of TB after the cyclization reaction with alkali proved to be applicable to general peptido-aminobenzophenone as described in the previous paper [5]. In addition, simultaneous determination of the parent drug TB and the metabolite TZ was successful. This method should be useful in further studies on the metabolism of TB.

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