Journal of Chromatography, 182 (1980) 171—177
Biomedical Applications

CHROMBIO. 516

DETERMINATION OF PEPTIDO-AMINOBENZOPHENONE (2-*o*-CHLOROBENZOYL-4-CHLORO-N-METHYL-N'-GLYCYL-GLYCINANILIDE) BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

TOMOYUKI AGOH, MASAHARU KONISHI and YOSHIO MORI

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

(Received October 26th, 1979)

SUMMARY

A gas—liquid chromatographic method for the determination of peptido-aminobenzophenone (2-*o*-chlorobenzoyl-4-chloro-N-mehtyl-N'-glycyl-glycinanilide, I) in dog plasma was developed. Decomposition of compound I was observed during chromatography. In alkaline medium, compound I in plasma was submitted to ring closure to yield 3-amino-6-chloro-5-(2-chlorophenyl)-1-methylquinolin-2-one (aminoquinolone), and the hexane extract was assayed by gas—liquid chromatography using electron-capture detection. The concentration range of compound I studied was 10—90 ng per 0.5 ml of plasma. Interference from both endogenous and exogenous sources was negligible. The method could be applied to the determination of the plasma level of compound I in dogs following oral administration of a single 5 mg/kg dose.

INTRODUCTION

Peptido-aminobenzophenone (2-*o*-chlorobenzoyl-4-chloro-N-methyl-N'-glycyl-glycinanilide, I) is one of a new series of minor tranquilizers recently developed in our laboratory [1]. Numerous drugs are extensively used as tranquilizers, among them 1,4-benzodiazepines [2].

Peptido-aminobenzophenone is known as a pro-drug of 1,4-benzodiazepine because chlorodiazepam [7-chloro-1,3-dihydro-1-methyl-5-(2-chlorophenyl)-2-H-1,4-benzodiazepin-2-one, II] was identified as a major metabolite in plasma after administration of compound I [3].

In general, as the dosages of minor tranquilizers are low, high sensitivities are required to measure the amounts of these drugs in biological fluids. Pulse polarography [4], fluorimetry [5], radioimmunoassay [6], high-performance liquid chromatography [7] and gas—liquid chromatography (GLC) using

electron-capture detection (EC-GLC) [8] have been used to measure 1,4-benzodiazepines, the most extensively used technique being EC-GLC [9, 10]. The metabolites of compound I in dogs, as shown in Fig. 1, are chlorodiazepam (II), chlorodesmethyldiazepam [7-chloro-1,3-dihydro-5-(2-chlorophenyl)-2H-1,4-benzodiazepin-2-one, III], lorazepam (IV) and an oxalanilic acid derivative [2-(2-chlorobenzoyl)-4-chloro-N-methyloxalanilic acid, V] in urine, and also compounds II and III in plasma [3].

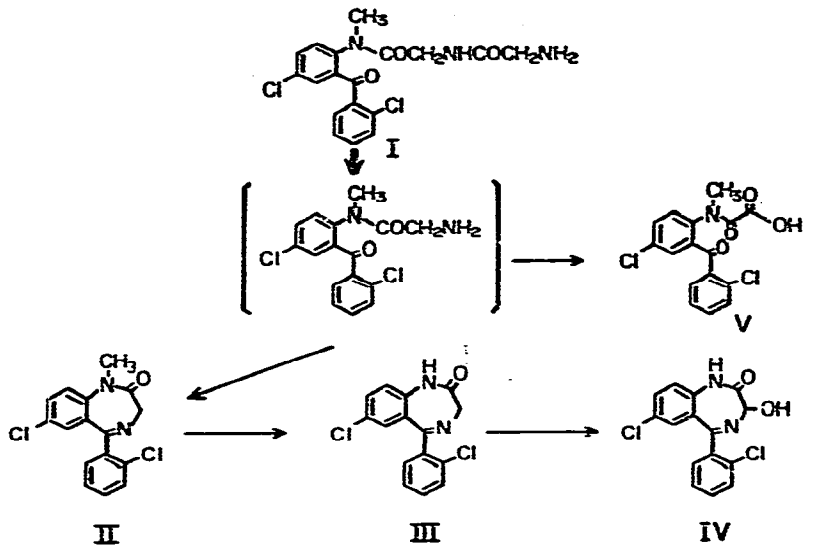


Fig. 1. Postulated metabolic pathway of peptido-aminobenzophenone in dogs [3].

EG-GLC could offer a useful method for measuring the amount of compound I and its metabolites, which show high sensitivities for EC-GLC. But differentiation of compound I from these metabolites is necessary with biological samples.

This paper describes a sensitive and selective EC-GLC method for the determination of compound I in dog plasma.

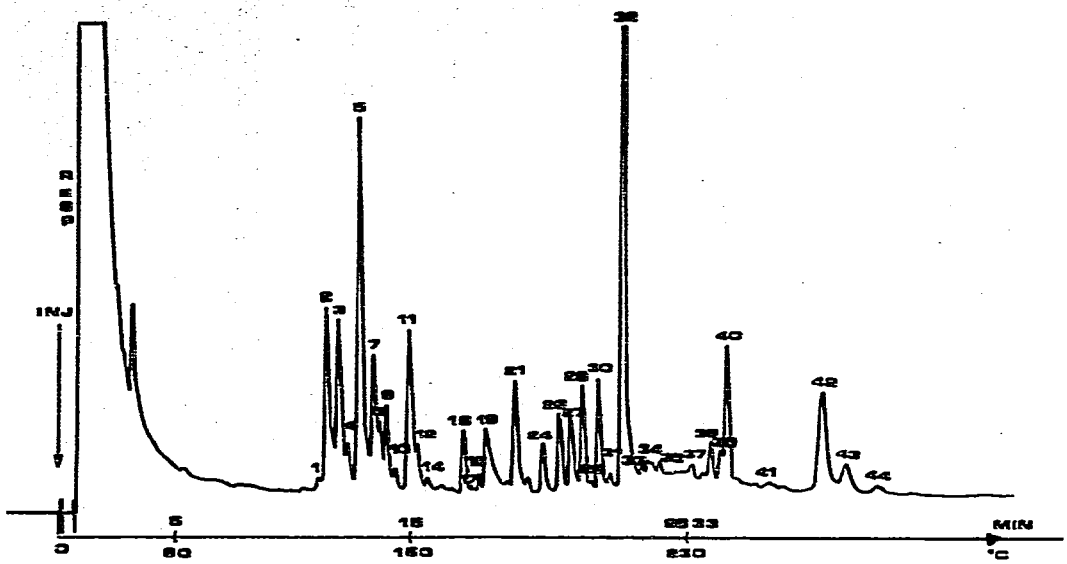
EXPERIMENTAL

Chemicals and reagents

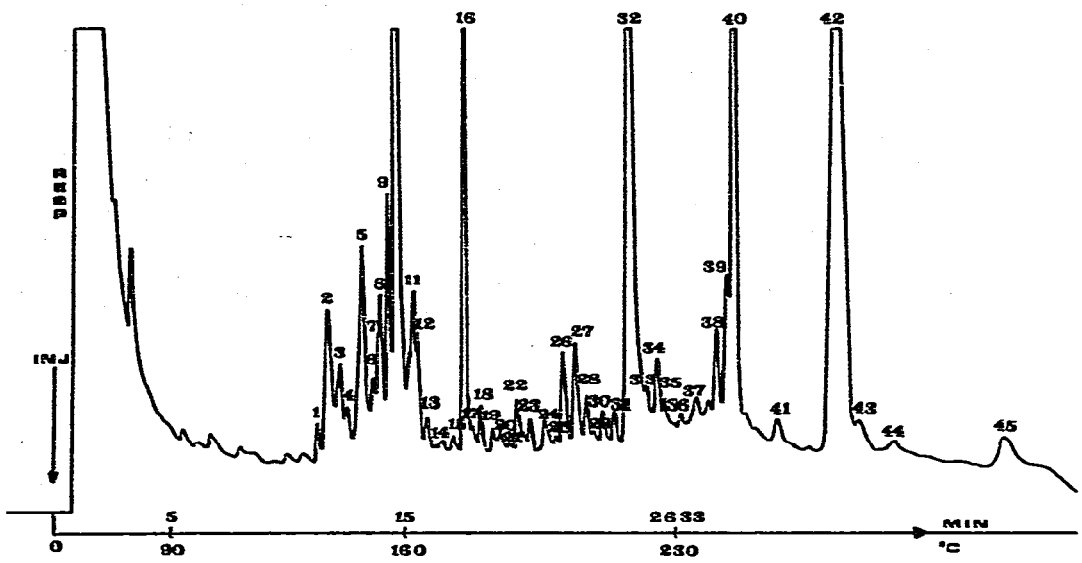
Compound I and other required compounds (II, III, IV and VI) were synthesized in our laboratory. Solvents used were of a special grade for EC-GLC (Wako, Osaka, Japan), while other chemicals were of reagent grade and were used without further purification.

Gas—liquid chromatography

A Shimadzu Model 4CMPFE gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 10-mCi ^{63}Ni electron-capture detector was used with a 0.5 m × 3 mm I.D. glass column filled with 3% OV-17 on 100—120 mesh Gas-Chrom Q. The column temperature was kept at 240° after conditioning with a nitrogen

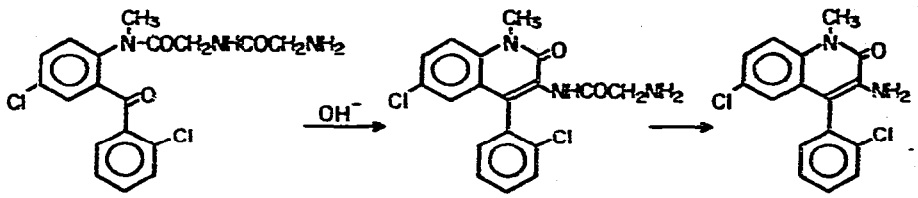


g. 3. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat e-ball tissue. Column and conditions as in Fig. 1.



g. 4. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat art muscle tissue. Column and conditions as in Fig. 1.

possessed good GC properties, which allowed the analysis of compound I. Next, the reaction causing both cyclization and hydrolysis of the glycine moiety to obtain compound VI in one step was investigated. With 5% potassium carbonate in ethanol at 72°, cyclization of compound I to compound VIII was easy, but further conversion into compound VI was incomplete even after 220 min. To complete the reaction, treatment with 1 *N* potassium hydroxide in ethanol was required for 4 h at 75°. Reaction at the higher temperature of 92° with 1 *N* potassium hydroxide in *n*-propanol afforded a considerable reduction in reaction time from 4 to 2 h; the yield was about 85%. With 1 *N* potassium hydroxide in water at 95°, the reaction time was shortened to 30 min, with a yield of 75%.



I VIII VI

To simplify the determination procedure, the assay reaction was performed directly in the plasma solution containing 1 *N* potassium hydroxide without preliminary extraction of compound I. Another merit is that this method prevents sample loss during extraction and enhances the precision of the determination.

Interference

No endogenous plasma components interfered with this assay, as shown in Fig. 2. The inverted peak appearing at the retention time of 6.6 min was assumed to have arisen from endogenous cholesterol.

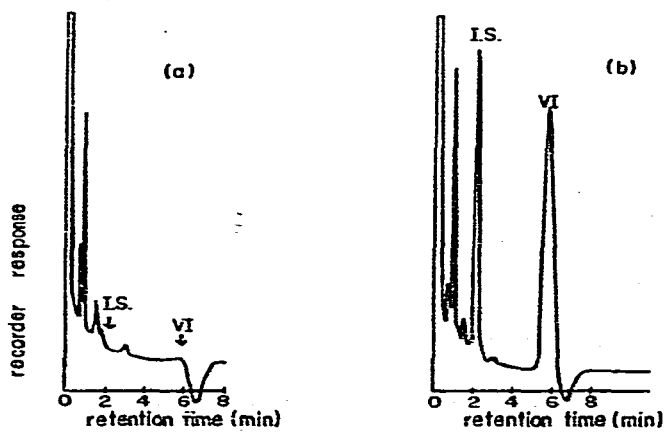


Fig. 2. Gas chromatograms of control dog plasma (a) and of dog plasma after dosing with peptido-aminobenzophenone (b).

flow-rate of 20 ml/min for 48 h at 330°. The detector and injection port temperatures were 300°. The nitrogen flow-rate was 100 ml/min, and the pulse mode was of an 800-μsec frequency with an 8-μsec width.

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

TABLE I

RETENTION TIMES IN EC-GLC OF COMPOUNDS RELATED TO COMPOUND I

	II	III	IV	VI	Diazepam
Retention time (min)	3.9	5.1	1.7	6.0	2.0

Preparation of standard solutions

Aminoquinolone. Solutions containing 6—80 ng/ml of compound VI and 60 ng/ml of diazepam were prepared in toluene.

Internal standard. A solution containing 60 ng/ml of diazepam in toluene was used.

Peptido-aminobenzophenone. Ethanolic solutions in the concentration range 0.2—1.8 μg/ml were used.

Procedure for determination of compound I in plasma

A 12-ml centrifuge tube containing 0.5 ml of plasma, 0.4 ml of distilled water and 0.1 ml of 10 *N* potassium hydroxide solution was gently shaken, then heated on a water bath at 95 ± 1° for 60 min. After cooling, the mixture was extracted twice with 7 ml and 5 ml of *n*-hexane on a mechanical shaker. The *n*-hexane extracts were combined in flasks containing 1 ml of the standard solution of diazepam, then the solvent was evaporated to dryness in vacuo at 40°. The residue was dissolved in 1 ml of toluene and a 2-μl sample was subjected to gas chromatography. Calculations were done using a calibration curve prepared by the peak height ratio method.

RESULTS AND DISCUSSION

Derivatization of compound I

Compound I was pyrolyzed during GLC to give 2,5'-dichloro-2-methylaminobenzophenone (VII), chlorodiazepam (II) and aminoquinolone (VI) in low yield. Therefore, the following derivatizations of compound I were performed in order to find a suitable compound for analysis by GLC.

Both the carbamate derivative of compound I obtained by reaction with chloromethylformate and the acetyl derivative with acetic anhydride were pyrolyzed during GLC and gave no noticeable peaks.

Reduction of compound I with sodium borohydride produced the benzhydrol derivative in an unsatisfactory yield. Thus, when compound I was heated in ethanol in the presence of potassium carbonate, ring closure leading to the glycylaminoquinolone derivative [6-chloro-5-(2-chlorophenyl)-4-glycylamino-1-methylquinolin-2-one, VIII] occurred, but decomposition of compound VIII was observed during GLC. However, aminoquinolone (VI) obtained after hydrolysis of VIII, as shown below, was thermostable and

Recovery studies

Solutions containing 10, 30, 60, and 90 ng per 0.5 ml of compound I were prepared by adding 50 μl of an ethanolic solution of compound I to 0.5 ml of heparinized dog plasma. Each solution was analyzed according to the procedure described above. The mean percentage recovery of compound I, calculated from a total of 18 analyses, was 75.5% (S.D. = 7.9%).

Metabolites

In the assay procedure described above, the peaks due to the metabolites II and III disappeared due to alkaline hydrolysis of the 1,4-benzodiazepine ring and, consequently, simultaneous determination of the unchanged drug and the metabolites can not be performed. The metabolites should be measured by an alternative procedure, which will be reported in our next paper [11].

Plasma levels of compound I in dogs

The plasma levels of compound I were determined in dogs following a single oral dose of 5 mg/kg, as shown in Fig. 3. The plasma level of compound I reached a peak of 0.15 μg/ml 1 h after administration and decreased rapidly with a half-life of about 1.2 h. The fact that the plasma levels of compound I were quite low suggested that the conversion of compound I into some metabolites, II, III and/or others, was extensive. Further studies on the metabolism and excretion of compound I will be discussed in our next paper [11].

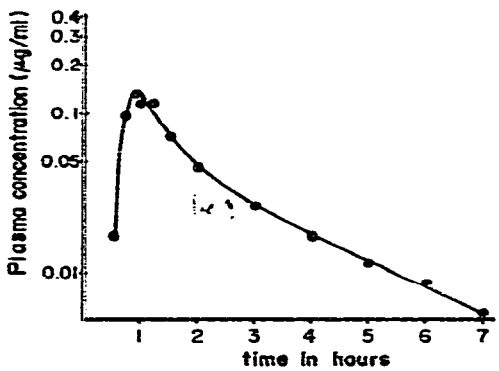


Fig. 3. Plasma concentrations of compound I in a dog after a single 5 mg/kg oral dose of compound I.

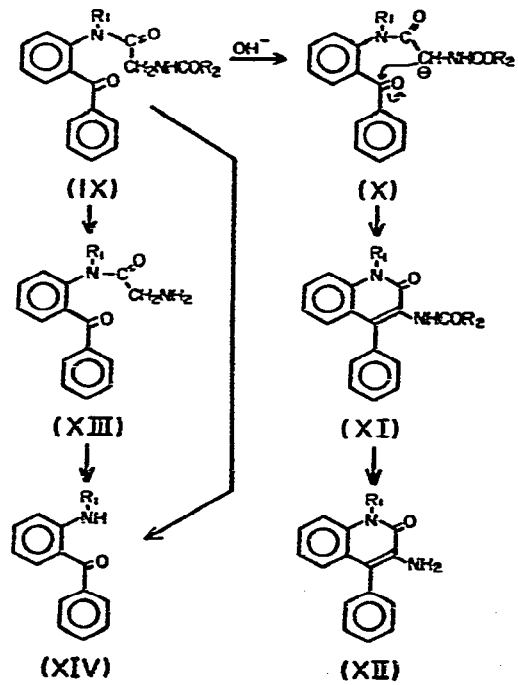


Fig. 4. Mechanism of the cyclization reaction of general peptido-aminobenzophenones.

Recovery of compounds II and III from plasma containing 2 μg of each compound after the assay reaction was 5% and 7%, respectively, and no interference was observed because they were subjected to alkaline hydrolysis to form aminobenzophenone derivatives having smaller retention times.

Compounds VI and VIII, possible metabolites after administration of compound I, were not found in the plasma samples; their presence would lead to serious errors in the determination.

Choice of an internal standard

Stability of the standard is the foremost requirement in the internal standard method. Because of the strong alkalinity of the reaction medium, a hydrocarbon, 9,10-diphenylanthracene, was tentatively selected as the internal standard, but it caused deterioration of the column within a moderate period of use. Thus, it was replaced by diazepam. Diazepam showed good chromatographic properties and could be separated from all metabolites and endogenous components in plasma. However, as diazepam was hydrolysed by alkali during extraction, it had to be added to the extract fractionated. Accordingly, extraction was performed twice in order to minimize the experimental errors.

Calibration curve

When the ratio of the peak height of compound VI to that of diazepam was plotted against the concentration of compound VI in the range 6—80 ng/ml, the calibration curve obtained gave a satisfactory linearity: $r = 0.999$, C.V. = 3.4%.

Sensitivity

Sensitivities for EC-GLC of compounds related to compound I in this assay are shown in Table II. High electron-capture potency was seen in compound VI as well as in 1,4-benzodiazepine metabolites II and III. In this assay, the limit of detection for compound I was 9 ng per 0.5 ml of plasma.

TABLE II

SENSITIVITIES IN EC-GLC OF COMPOUNDS RELATED TO COMPOUND I

Compound	Molecular weight	Minimum detectable amounts (pg/injection)	Sensitivity (ampere per sec per mole)
II	319	2.0	32
III	305	3.0	15
VI	319	5.0	7
Diazepam	283.5	3.0	31

Extraction solvent

The recovery of compound VI examined with dichloromethane, *n*-hexane, ethyl acetate and benzene, was 99, 97, 93 and 92%, respectively. Considering the interference of endogenous substances and contamination, *n*-hexane, the most non-polar solvent, was selected as the extraction solvent.