*Journal of Chromatography*, 110 (1975) 301–307


CHROM. 8336

# DETERMINATION OF A NEW CEREBRAL VASODILATOR 2,6-DIMETHYL-4-(3-NITROPHENYL)-1,4-DIHYDROPYRIDINE-3,5-DICARBOXYLIC ACID 3-[2-(N-BENZYL-N-METHYLAMINO)]-ETHYL ESTER 5-METHYL ESTER HYDROCHLORIDE (YC-93) IN PLASMA BY ELECTRON CAPTURE GAS CHROMATOGRAPHY

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(Received March 3rd, 1975)

## SUMMARY

A highly sensitive method for the quantitative determination of 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(N-benzyl-N-methylamino)]-ethyl ester 5-methyl ester hydrochloride (YC-93) in plasma is described. After extraction, YC-93 was oxidized to a pyridine analogue with nitrous acid and detected by electron capture gas chromatography. The sensitivity was 2–3 ng/ml, which is sufficient to determine plasma concentrations of YC-93 after oral administration of clinical doses to humans.

## INTRODUCTION

Recently, 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(N-benzyl-N-methylamino)]-ethyl ester 5-methyl ester hydrochloride (YC-93) has been introduced as a new potent vasodilator with preferential effect on the cerebral circulation[1]. The plasma concentration of YC-93 after oral administration to rats and dogs, studied with the radioactive compound, was found to be very low due partly to its small effective dose and partly to its extensive metabolism[2,3]. A highly sensitive assay method was required to determine plasma levels in humans for clinical purposes. The investigation described in this paper is an analytical method for measuring nanogram amounts of YC-93 in plasma.

## EXPERIMENTAL

### *Gas chromatography*

A Model 5700 A gas chromatograph (Hewlett-Packard) equipped with a $^{63}Ni$ electron capture detector (ECD) was used. The column was a glass tube (90 cm × 1.8 mm I.D.) packed with 3% OV-1 on 80–100 mesh AW-DMCS Chromosorb W (Nihon Chromato Works, Tokyo, Japan). The operating conditions were as follows. Col-

umn temperature, 260°; injection port temperature, 300°; detector temperature, 300°; carrier gas, argon–methane (95:5); flow-rate, 40 ml/min. The detector sensitivity was set at range ×32 to ×256 depending on the amount of the sample.

*Mass spectrometry*

Mass spectra were obtained with a Hitachi RMU-7 mass spectrometer. The samples were introduced by direct probe insertion.

*Chemicals*

YC-93, internal standard 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[3-(N-benzyl-N-methylamino)]-propyl ester 5-isopropyl ester hydrochloride (I.S.), authentic compounds of corresponding pyridine analogues and [$^{14}$C]-labelled YC-93 were synthesized in our laboratory[4]. All reagents were guaranteed reagents for analytical work and supplied by Wako (Osaka, Japan). The I.S. solution was prepared by dissolving the compound in distilled water to a concentration of 200 ng/ml.

*Oxidation of dihydropyridine ring to pyridine ring*

The effects of various experimental parameters on the oxidation step in the analytical procedure *viz.*, sodium nitrite and hydrochloric acid concentration, reaction time and temperature, were investigated by gas chromatography. A 1,4-dihydropyridine derivative (100 ng) was added to a 10-ml centrifuge tube and treated with the extraction procedure as described below.

The rate of oxidation, $R$, was calculated as

$$R = \frac{\text{(peak height of pyridine analogue)}}{\text{(peak height of parent compound)} + \text{(peak height of pyridine analogue)}} \times 100\%$$

*Extraction procedure*

Two milliliters of the plasma sample were added to a 10-ml centrifuge tube and 0.5 ml of I.S. solution and 0.5 ml of 2 *N* NaOH were added. The contents of the tube were mixed and extracted with two 2-ml amounts of diethyl ether. The organic layers were combined, placed in a 10-ml centrifuge tube and extracted with 4 ml of 0.05 *N* HCl. To the aqueous layer was added 0.3 ml of a 1% $NaNO_2$ solution and the mixture was kept in a water bath at 45° for one hour. During this procedure, the 1,4-dihydropyridine rings of YC-93 and I.S. were oxidized to pyridine rings. After cooling, the mixture was made alkaline with 0.5 ml of 2 *N* NaOH and extracted with two 2-ml amounts of diethyl ether. The organic layer was evaporated to dryness in a water bath at 45° and 50 $\mu$l of benzene were added to the residue. An aliquot of 3 $\mu$l was injected into the column of the gas chromatograph. The amounts of YC-93 in each sample were calculated by measuring the peak height ratio of the pyridine analogues of YC-93 and I.S. and referring to the standard curve.

*Standard curve*

A standard curve was prepared by subjecting the control plasma, to which known amounts of YC-93 (2.5–30 ng) had been added, to the above procedure. The

peak height ratio of the pyridine analogues of YC-93 and I.S. was plotted against the concentration of YC-93.

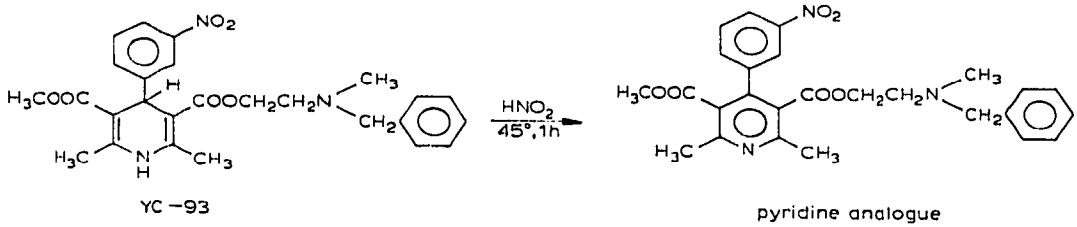
*Extraction recoveries*

Extraction recoveries were determined by adding [$^{14}$C]-labelled YC-93 (20–100 ng, 800–4000 dpm) to the centrifuge tube prior to the addition of plasma, and by measuring the radioactivity of the final ether extracts with a Model 3380 liquid scintillation counter (Packard). The scintillation fluid consisted of PPO (7 g), dimethyl POPOP (0.3 g) and naphthalene (100 g) in 1 l of dioxane.

## RESULTS AND DISCUSSION

A fluorometric method, for the estimation of a similar 1,4-dihydropyridine derivative with a sensitivity in the order of a microgram, has been reported[5]. A study using [$^{14}$C]-labelled YC-93 has revealed that the plasma concentration of intact drug after effective oral dosage in rats and dogs was at the nanogram level[2]. In the present study, an electron capture gas chromatographic method was used to obtain the sensitivity and specificity required for clinical studies.

When nanogram amounts of YC-93 were injected into the column of the gas chromatograph it was partly oxidized to its pyridine analogue resulting in two peaks. Identification of the second peak to be the pyridine analogue was made comparing the retention time of this peak with that of the authentic compound. This led us to include in the assay procedure quantitative oxidation of the dihydropyridine compounds to pyridines. The oxidation method of the 1,4-dihydropyridine ring with nitrous acid has been reported previously[6]. The effects of various experimental parameters on the oxidation step were investigated by gas chromatography. These results revealed that oxidation was most satisfactorily effected by incubating the sample with 0.3 ml of a 1% $NaNO_2$ solution at 45° for one hour in 4 ml of 0.05 *N* HCl (Fig. 1, Tables I–IV). The formation of pyridine derivatives was confirmed by comparison with mass spectrometry and gas chromatography of authentic compounds.




YC-93 pyridine analogue

Fig. 1. Formation of pyridine analogue by oxidation of YC-93.

The pyridine analogue of YC-93 shows a parent peak at *m/e* 477 which is two mass units less than that of the parent compound, indicating the oxidation of the 1,4-dihydropyridine ring to the pyridine ring. The situation was the same with the similarly structured I.S. (Fig. 2).

The chromatograms of YC-93 and I.S. extracted from plasma are shown in Fig. 3. The drug-free control plasma gave no interfering peaks. Retention times of

TABLE I

EFFECT OF $NaNO_2$ CONCENTRATION ON THE OXIDATION OF DIHYDROPYRIDINES TO PYRIDINES

Conditions: 45°, 90 min, 0.02 *N* HCl.

| *Compound* | *Rate of oxidation (%)* | | | | |
|---|---|---|---|---|---|
| | *Amounts of 1% $NaNO_2$ (ml)* | | | | |
| | *0.01* | *0.1* | *0.3* | *0.5* | *1.0* |
| YC-93 | 13.0 | 100.0 | 100.0 | 100.0 | 96.0 |
| I.S. | 16.7 | 100.0 | 100.0 | 100.0 | 100.0 |

TABLE II

EFFECT OF HCl CONCENTRATION ON THE OXIDATION OF DIHYDROPYRIDINES TO PYRIDINES

Conditions: 45°, 90 min, 1% $NaNO_2$ (0.3 ml).

| *Compound* | *Rate of oxidation (%)* | | | |
|---|---|---|---|---|
| | *HCl concentration (N)* | | | |
| | *0.01* | *0.02* | *0.05* | *0.1* |
| YC-93 | 50.0 | 100.0 | 100.0 | 100.0 |
| I.S. | 91.0 | 100.0 | 100.0 | 100.0 |

TABLE III

EFFECT OF REACTION TIME ON THE OXIDATION OF DIHYDROPYRIDINES TO PYRIDINES

Conditions: 45°, 1% $NaNO_2$ (0.3 ml), 0.05 *N* HCl.

| *Compound* | *Rate of oxidation (%)* | | | | | |
|---|---|---|---|---|---|---|
| | *Reaction time (min)* | | | | | |
| | *0* | *10* | *30* | *60* | *90* | *120* |
| YC-93 | 89.0 | 98.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| I.S. | 91.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

TABLE IV

EFFECT OF REACTION TEMPERATURE ON THE OXIDATION OF DIHYDROPYRIDINES TO PYRIDINES

Conditions: 60 min, 1% $NaNO_2$ (0.3 ml), 0.05 *N* HCl.

| *Compound* | *Rate of oxidation (%)* | | | | |
|---|---|---|---|---|---|
| | *Reaction temperature (°C)* | | | | |
| | *3* | *20* | *30* | *45* | *60* |
| YC-93 | 95.2 | 98.1 | 99.2 | 100.0 | 100.0 |
| I.S. | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

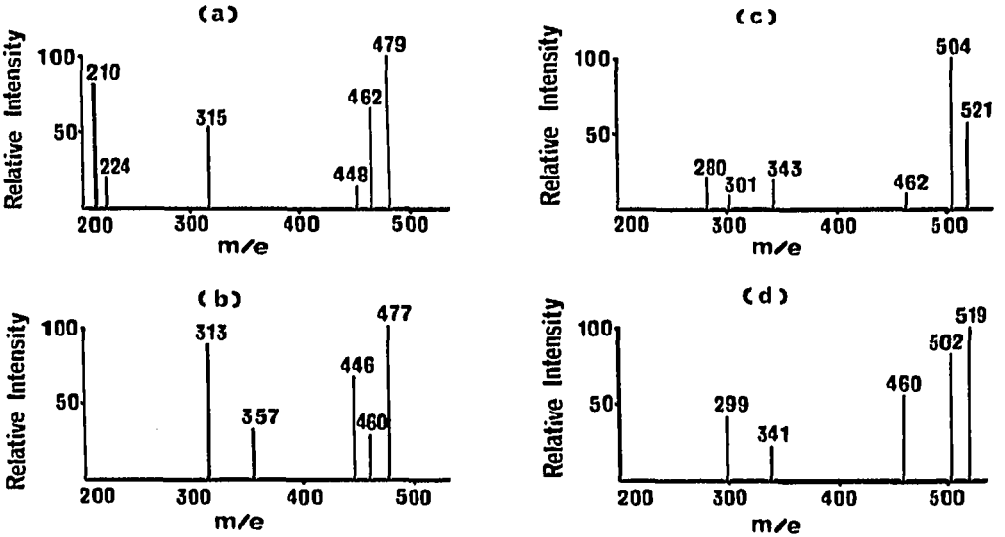



Fig. 2. Mass spectra of YC-93, I.S. and corresponding pyridine analogue after oxidation with $HNO_2$. (a), YC-93 and (b), pyridine analogue; (c) I.S. and (d), pyridine analogue.

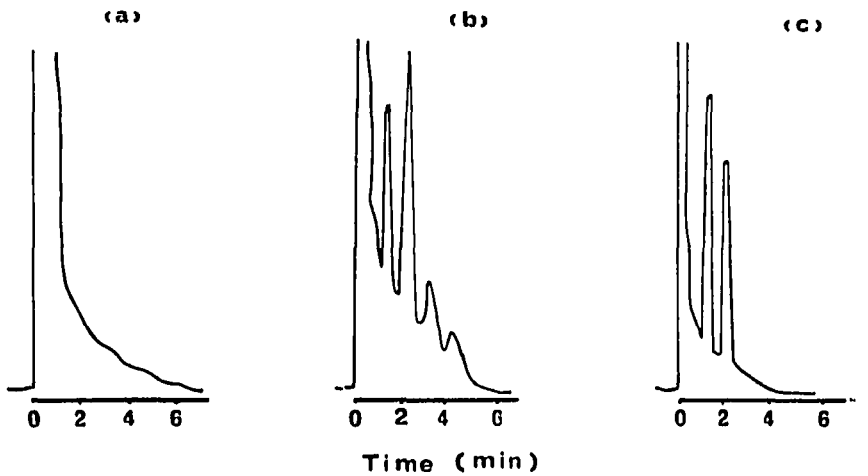



Fig. 3. Chromatograms of YC-93 and I.S. extracted from plasma. (a), control plasma; (b), YC-93 and I.S.; (c) YC-93 and I.S. extracted after oxidation with $HNO_2$.

YC-93, I.S. and the corresponding pyridine analogues are 1.4 min, 2.2 min, 3.3 min and 4.4 min, respectively.

A standard curve obtained by the above procedure is shown in Fig. 4. Linear response was obtained up to a plasma concentration of at least 30 ng/ml, the limit of sensitivity being 2–3 ng/ml. The recoveries of added [$^{14}$C]-labelled YC-93 are shown in Table V.

The sensitivity of the above method was high enough to determine plasma concentrations of YC-93 following clinical dosage to humans. The maximum plasma level

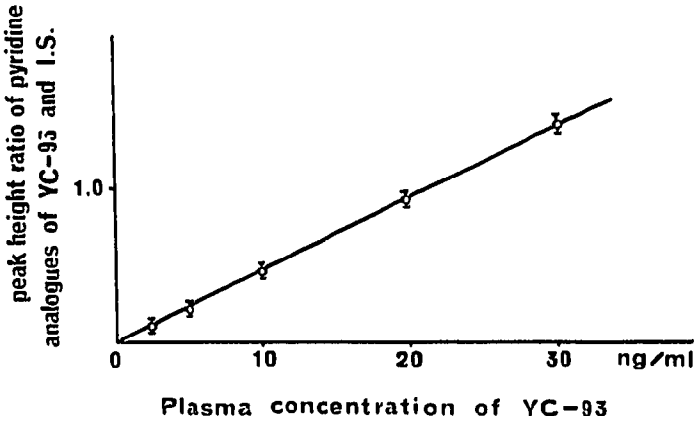



Fig. 4. Standard curve of YC-93. Each point represents the mean ± standard error from five experiments.

TABLE V

RECOVERIES OF [$^{14}$C]-LABELLED YC-93 FROM PLASMA

| *Recovery (%)* | | |
|---|---|---|
| *Plasma concentration of YC-93* | | |
| | *10 ng/ml* | *50 ng/ml* |
| | 83.1 | 89.9 |
| | 76.1 | 81.9 |
| | 76.7 | 93.2 |
| | 79.9 | 92.9 |
| | 78.2 | 90.8 |
| Mean | 78.8 | 89.7 |
| Standard error | 1.3 | 2.1 |

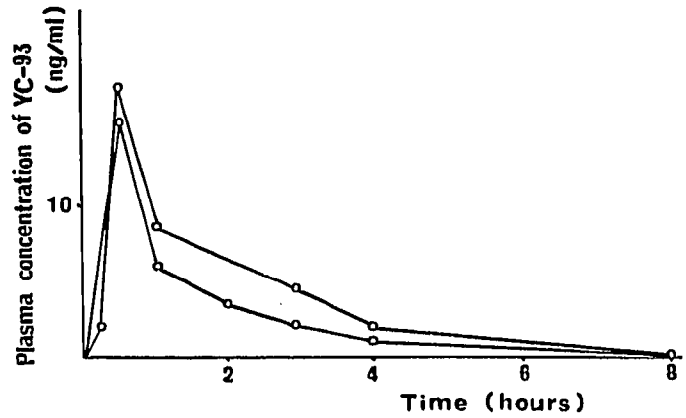



Fig. 5. Plasma concentration of YC-93 after oral administration of 10 mg YC-93 to humans[7].

in two healthy volunteers given 10 mg of oral dose was found to be 16–18 ng/ml (Fig. 5)[7].

Further, when the pyridine analogue was present in the plasma as a metabolite, the values obtained by this method represent the sum of the intact drug and the metabolite. The study using the [$^{14}$C]-labelled compound revealed that, compared with the intact drug, the amount of this metabolite was negligible in rat and dog plasma[2]. However, further study is required to determine the metabolite in human plasma. The method described in this report is much simpler, more specific and far more sensitive than the fluorometric method.

## ACKNOWLEDGEMENTS

We are greatly indebted to Dr. S. Kawahara, Mr. U. Shiobara and Dr. M. Iwanami for their valuable discussions throughout this work, and to Mr. K. Tamazawa for supplying [$^{14}$C]-labelled YC-93. We thank Miss M. Nagasaka for her technical assistance.

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