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## ASSAY OF HA-966 IN RAT PLASMA BY CAPILLARY GAS-LIQUID CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION

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### SUMMARY

A gas-liquid chromatographic method for the determination of the  $\gamma$ -aminobutyric acid-like drug 1-hydroxy-3-aminopyrrolidone-2 (HA-966) in plasma is described. HA-966 was converted into its diacetyl derivative Ac<sub>2</sub>HA-966 with acetic anhydride. This compound could be suitably eluted from a capillary OV-17 support-coated open tubular column. A sensitive detection method was achieved by making use of nitrogen-phosphorus-selective flame ionization.

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### INTRODUCTION

The experimental drug 1-hydroxy-3-aminopyrrolidone-2 (HA-966) [1], synthesized by Havinga et al. [2] in 1959, has proved to be a valuable agent in the study of central dopaminergic systems [3–6]. Unfortunately little information is available as yet on the fate or metabolism of the drug because of the lack of a suitable analytical method. Hence pharmacokinetics of HA-966 are unexplored and it is even unknown whether HA-966 itself or a metabolite elicits the central depressant effects. In order to improve evaluations of the actions of this drug [4] it was necessary to develop an assay of HA-966 in biological materials. In this article a rapid and sensitive determination of HA-966 by capillary gas-liquid chromatography (GLC) with nitrogen-phosphorus (N-P) selective detection is reported.

### MATERIALS AND METHODS

#### *Apparatus*

A Hewlett-Packard 5830A gas chromatograph equipped with a dual nitrogen-phosphorus flame ionization detector Model 18789A was used. A solid

phase injector was assembled from a Hoke ball valve [7–9]. For the derivatization Pierce Reacti-Vials (Hicol, Rotterdam, The Netherlands) were used. Heating of vials was carried out in a Tecam Dri-Block DB-3 (Salm and Kipp, Breukelen, The Netherlands).  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were recorded with a Jeol-Ps-100 NMR spectrometer equipped with a Jeol-JNM-Pft-100 pulse unit and an EC-100 computer. For  $^{13}\text{C}$ -NMR spectroscopy the Fourier transform mode was used. Mass spectra were measured with an LKB 2091-2130 gas chromatograph–mass spectrometer with a PDP 11 computer system.

#### *Solvents, standards and reagents*

The solvents, analytical grade, were obtained from Baker (Deventer, The Netherlands) and Merck (Darmstadt, G.F.R.). Deionized, glass distilled water was used. PPE-21 and OV-17 were purchased from Chrompack (Middelburg, The Netherlands). Tullanox was from Cabot Corporation (Boston, MA, U.S.A.). Racemic HA-966 was a gift from Organon (Oss, The Netherlands); elemental analysis gave the following percentages: C found 41.38 (calculated 41.37), H 6.94 (6.94), N 27.55 (27.56) and O 23.95 (24.13). Lidocaine was provided by the Rijksmagazijn van Geneesmiddelen (Amsterdam, The Netherlands). Acetic anhydride was from Baker.

#### *Animals*

Male Wistar derived rats (SPF, 225–275 g) were used. The animals were allowed free access to food and water.

#### *Isolation procedure*

To 0.1 ml of rat plasma were added 4  $\mu\text{g}$  of lidocaine in 40  $\mu\text{l}$  of ethyl acetate and 0.3 ml of methanol. After centrifuging for 10 min (1650 g) the supernatant was transferred to a Pierce 1.0-ml Reacti-Vial. To the residue 0.05 ml water and 0.3 ml methanol were added and the mixture was centrifuged. The aqueous methanol supernatants were combined and evaporated at 50°C in a stream of dry nitrogen.

#### *Derivatization procedure*

To the residue thus isolated, 50  $\mu\text{l}$  acetic anhydride were added. The mixture was allowed to react at room temperature for 20 min. Then the solution was evaporated for about 0.5 h at 50°C in a stream of dry nitrogen. The residue was dissolved in 0.4 ml ethyl acetate, transferred to another vial and evaporated at 50°C under nitrogen.

#### *GLC procedure*

The residue obtained in the derivatization procedure was reconstituted in 100  $\mu\text{l}$  ethyl acetate. A 1- $\mu\text{l}$  aliquot of this solution was used for each solid phase injection on the capillary OV-17 support-coated open tubular (SCOT) column [7–9]. Tullanox was applied as a support material for the capillary column and was subsequently coated twice with PPE-21 and OV-17 [7,8]. The experimental GLC conditions were: column, 10 m  $\times$  0.45 mm I.D., temperatures: oven, 195°C; injection, 280°C; detection, 300°C; flow-rates: carrier gas, helium 2.5 ml/min; auxiliary gas, helium 27.5 ml/min; detection gases:

hydrogen, 3 ml/min; air, 50 ml/min. Representative retention times were: 1-acetoxy-3-acetylaminopyrrolidine-2 ( $\text{Ac}_2\text{HA-966}$ ) 3.4 min, lidocaine 4.0 min.

#### *Calibration curve*

For the construction of a calibration curve amounts of 1, 2, 5, 10, 20 or 50  $\mu\text{g}$  of HA-966 and 4  $\mu\text{g}$  of lidocaine were spiked to 0.1 ml of rat plasma. The samples were processed as described above. The resulting ratios of the peak areas of  $\text{Ac}_2\text{HA-966}$  and lidocaine were plotted against the amounts of HA-966 spiked to the rat plasma (10–500 ng). For the assay of rat plasma samples the calibration curve was determined at least twice daily. Each reference or rat sample was measured at least in duplicate.

#### *Preparation and characterization of $\text{Ac}_2\text{HA-966}$*

Pure samples of  $\text{Ac}_2\text{HA-966}$  (ca. 200 mg) were prepared by heating HA-966 for 1 h in 2 ml acetic anhydride and evaporating the solution for 2 h in a stream of dry nitrogen.  $\text{Ac}_2\text{HA-966}$  was obtained as a colourless oil; it was characterized by  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR. Mass spectrometry (MS) with electron

ionization showed only the  $[\text{M}-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-\text{CH}_2]^+$  fragment with  $m/e$  158. MS with chemical ionization ( $\text{NH}_3$ ) clearly displayed the  $[\text{MH}]^+$  ion with  $m/e$  201, where M denotes  $\text{Ac}_2\text{HA-966}$ .

### RESULTS AND DISCUSSION

Up to now a considerable amount of information is available on the pharmacological action of the GABA-like experimental drug HA-966 (see, for example, refs. 3–6). In order to obtain insight into the actual fate of the drug it was necessary to develop a suitable analytical method. It seemed promising to take advantage of the presence of two nitrogen atoms in HA-966 and apply nitrogen-selective detection in the GLC analysis. Preliminary experiments however indicated that HA-966 in its underivatized form was not suitable for GLC. Therefore it was decided to prepare a derivative of HA-966 with good GLC properties. A second problem offered the isolation of HA-966 from biological material. HA-966 is soluble in rather polar solvents such as water, but only slightly soluble in solvents such as methanol. Since no satisfactory extraction procedure could be developed, a capillary column of high separation power was selected. For the derivatization of HA-966 acylation was expected to lead to a product which could be readily manipulated in GLC (see ref. 7). Trifluoroacetylation proved to be too aggressive, but acetylation yielded promising results. HA-966 reacted smoothly with acetic anhydride under a variety of experimental conditions. Fig.1 presents the course of the reaction; the structural proof of the formation of the compound  $\text{Ac}_2\text{HA-966}$  was given by NMR and MS (see Materials and Methods).

As water is the best solvent for HA-966, which is insoluble in most other solvents, we were not successful in developing an extraction procedure. The removal of protein was sufficient for the correct performance of a GLC analysis after derivatization.

An initial purification step, namely extraction of the evaporated plasma

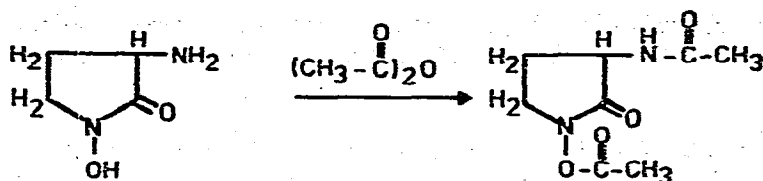


Fig. 1. Conversion of HA-966 with acetic anhydride into  $\text{Ac}_2\text{HA-966}$ .

sample with ethyl acetate, could be omitted. The recovery of the isolation procedure was estimated at 70% (S.D. 10%,  $n = 5$ ) on the basis of absolute peak areas with respect to a sample of derivatized pure HA-966.

In order to find an adequate GLC column for the analysis of HA-966 as the derivative  $\text{Ac}_2\text{HA-966}$ , several types of capillary columns were tested. Excellent results were obtained with a capillary SCOT OV-17 column, the support material Tullanox first being coated with PPE-21. Chromatograms recorded with this column showed the desired separation of  $\text{Ac}_2\text{HA-966}$  and lidocaine from other compounds present (Fig. 2); peaks were sharp and the sensitivity achieved with N-P-selective detection was very high. Lidocaine proved to be a useful internal standard. Representative examples of chromatograms are given in Fig. 2. The calibration curve in Fig. 3 determined in triplicate from spiked rat plasma samples shows linearity in the range of 10–500 ng per injection.

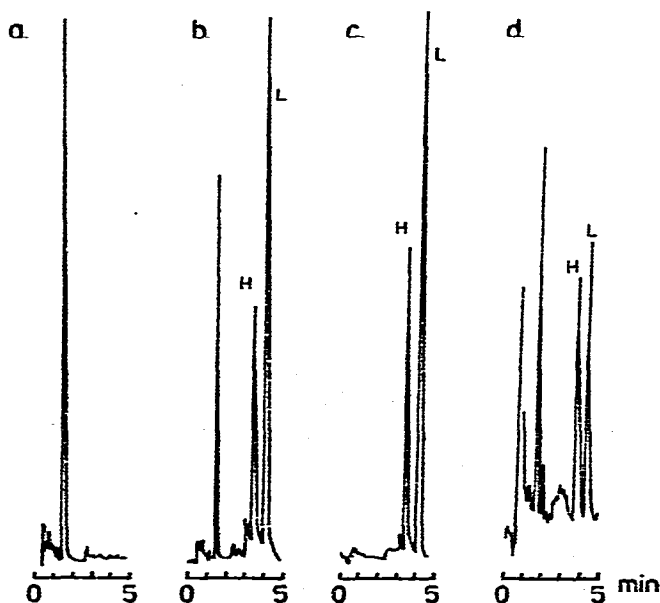


Fig. 2. Representative chromatograms of the assay of HA-966 as  $\text{Ac}_2\text{HA-966}$  on the capillary OV-17 SCOT column with lidocaine as internal standard: (a) blank rat plasma; (b) 20 ng of HA-966, converted into  $\text{Ac}_2\text{HA-966}$ , and 40 ng of lidocaine spiked to rat plasma; (c) reference sample of 20 ng of HA-966, converted into  $\text{Ac}_2\text{HA-966}$ , and 40 ng of lidocaine; (d) 43 ng of HA-966 as  $\text{Ac}_2\text{HA-966}$  and 40 ng of lidocaine in an authentic rat plasma sample. Peaks: H =  $\text{Ac}_2\text{HA-966}$ ; L = lidocaine.

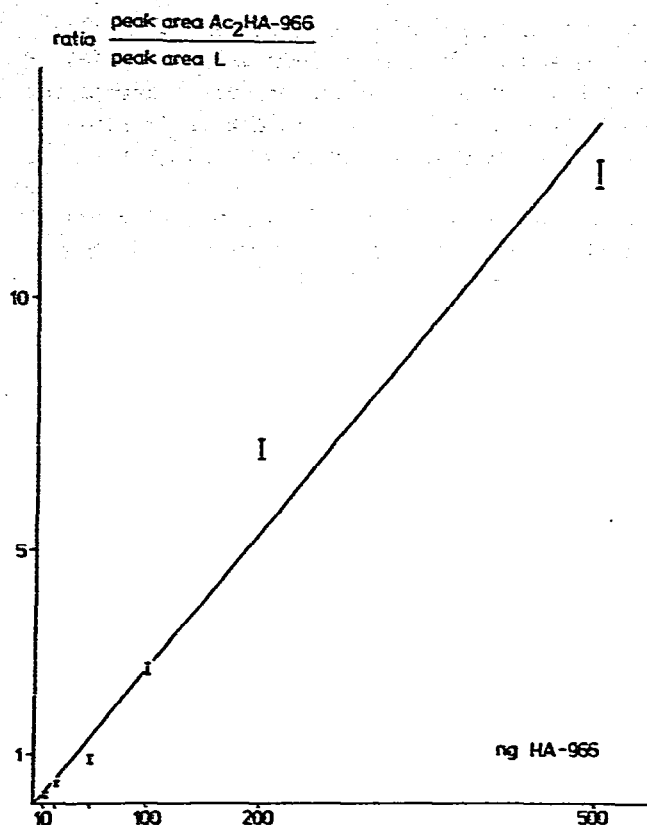


Fig. 3. Calibration curve for the determination of HA-966 as the derivative Ac<sub>2</sub>HA-966 in the range of 10–500 ng measured from plasma extracts. The ratio of the peak areas of Ac<sub>2</sub>HA-966 and the internal standard lidocaine (L) is plotted against the amount of HA-966 ( $n=3$ , correlation coefficient, 0.999).

TABLE I

PHARMACOKINETIC DATA OF HA-966 INTRAPERITONEALLY ADMINISTERED TO RATS

Data calculated according to a one-compartment model;  $n = 5$ .

Dose	100 mg/kg
$t_1$	$40.2 \pm 4.8^*$ min
$\bar{C}_0$ extrap.	$111 \pm 29$ $\mu$ g/ml
$V$	$226 \pm 62$ ml
$Cl_{tot}^{**}$	$4.0 \pm 1.3$ ml/min
$AUC^{***}$	$66 \cdot 10^2 \pm 20 \cdot 10^2$ $\mu$ g/ml $\cdot$ min
$Cl_{tot}^{\S}$	$3.9 \pm 1.3$ ml/min

\*Standard deviation.

\*\* $Cl_{tot} = k \cdot V$ .

\*\*\*Calculated by the trapezoidal rule.

$\S Cl_{tot} = \text{dose}/AUC$ .

For the GLC method described a small amount of plasma of only 0.1 ml was needed. A further advantage is the rather low detection limit which was found to be about 5 ng of HA-966 on a fresh column. When before starting the analysis, some blank plasma samples were injected, an additional decrease of the detection limit to 1 ng was reached, probably owing to deactivation of the column.

As an illustrative example the plasma decay of HA-966 in a male Wistar rat after an intraperitoneal injection of HA-966 (100 mg/kg) is presented in Fig. 4. Some pharmacokinetic data for HA-966 are given in Table I.

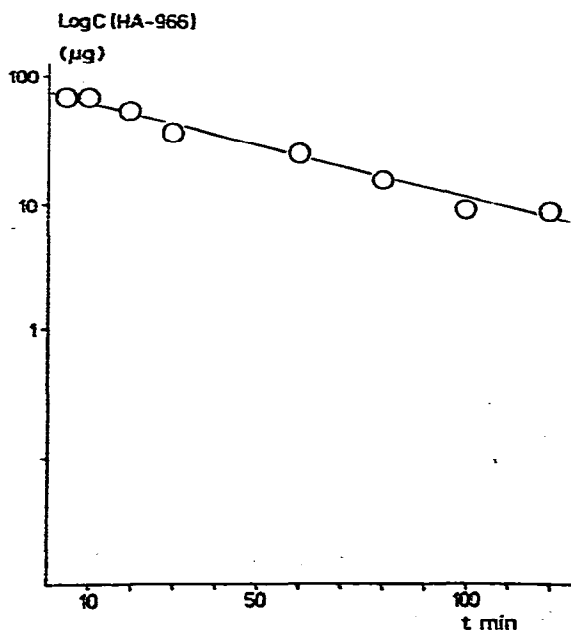


Fig. 4. Plasma decay of HA-966 in a male Wistar rat after intraperitoneal injection of 100 mg/kg of HA-966.

In conclusion it can be stated that HA-966 can be determined in a rapid and sensitive way. The pharmacokinetics of HA-966 in relation to its pharmacodynamics are under current research.

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