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DETERMINATION OF (*E*)-(2,3-DICHLORO-4-METHOXYPHENYL)-2-FURANYLMETHANONE-O-[2-(DIETHYLAMINO)ETHYL]OXIME METHANESULPHONATE (ANP-4364) IN PLASMA USING GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic method has been developed that permits the accurate and specific determination of the antianginal agent ANP-4364 in plasma. ANP-4364 is extracted with *n*-hexane containing ethyl chloroformate and, after a clean-up procedure, derivatized to the trichloroethyl carbamate, which is assayed on a gas chromatograph equipped with an electron-capture detector.

Accurate determinations are possible over a concentration range from 1 to 50 ng/ml of ANP-4364 in plasma with a relative standard deviation of 7.5%. The minimum detectable concentration is 0.5 ng/ml. Plasma levels of ANP-4364 in dogs receiving oral (10 mg/kg) or intravenous (0.1 mg/kg) dosing have been determined.

INTRODUCTION

(*E*)-(2,3-Dichloro-4-methoxyphenyl)-2-furanylmethanone-O-[2-(diethylamino)-ethyl]oxime methanesulphonate (ANP-4364, Fig. 1) is a new antianginal compound that possesses a novel pharmacological profile in experimental animals[1]. In order to study the pharmacokinetics of ANP-4364, a sensitive and specific assay method for the unchanged drug in plasma is necessary.

Hartvig and co-workers[2–5] have reported that the tertiary amines related to imipramine in plasma can be determined as the corresponding trichloroethyl or pentafluorobenzyl carbamate by gas chromatography with electron-capture detection. However, the method requires elimination of the N-dealkylated metabolite, one of the possible metabolites of tertiary amines[6], before carbamate formation. A column chromatographic method[4,5] is used for that purpose, but it is time consuming. A

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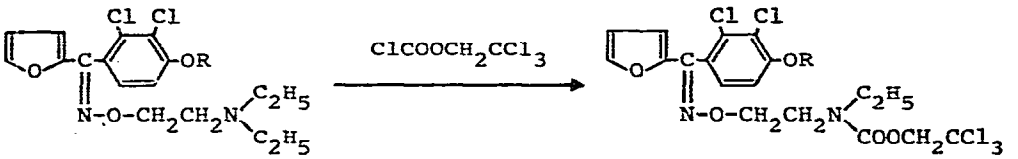

R = CH_3 ANP-4364

= $n\text{-}C_3H_7$ internal standard

Fig. 1. Reaction of ANP-4364 and internal standard with trichloroethyl chloroformate.

simpler method is preferable for the routine determination of the unchanged drug in plasma.

In this paper we describe a gas chromatographic method for the determination of ANP-4364 in plasma, based on the simple removal of N-desethyl ANP-4364, followed by conversion into the trichloroethyl carbamate (Fig. 1). This method can be applied to the determination of similar drugs of the tertiary amine type at nanogram levels in the presence of the dealkylated metabolites.

EXPERIMENTAL

Chemicals and reagents

ANP-4364 was a gift from Albert Rolland S.A. (Chilly-Mazarin, France). (*E*)-(2,3-Dichloro-4-propoxyphenyl)-2-furanylmethanone-O-[2-(diethylamino)ethyl]-oxime oxalate (internal standard) and N-desethyl ANP-4364 were synthesized in our laboratory.

Trichloroethyl chloroformate was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and ethyl chloroformate from Tokyo Kasei Kogyo (Tokyo, Japan). *n*-Heptane (analytical-reagent grade) was washed with concentrated sulphuric acid before use. All other chemicals were of analytical-reagent grade.

A methanolic alkaline solution was prepared by dissolving 2.2 g of potassium hydroxide in a mixture of 75 g of methanol and 22 g of water, and a saturated methanolic alkaline solution was a saturated solution of potassium hydroxide in methanol[4].

All centrifuge tubes, pipettes and flasks were silanized as described previously[7].

Gas chromatography

Gas chromatography was carried out using a JEOL, Model JGC-20KE, gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector. A silanized glass column (100 cm × 2 mm I.D.) was packed with 2% OV-101 on Gas-Chrom Q (80–100 mesh). Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min. The column temperature was 280° and the injector and detector temperatures were 300°.

Examination of the reaction conditions was performed in the same instrument equipped with a flame-ionization detector. The column temperature was 260° and the other conditions were as described above.

Examination of derivatization conditions

Reaction conditions were studied using the following derivatization procedure. To 0.2–1 ml of ANP-4364 base (1 mg/ml) and *n*-octacosane (0.2 mg/ml, internal standard) in *n*-heptane were added 1–30 μl of trichloroethyl chloroformate and about 10 mg of anhydrous sodium carbonate. The mixture was heated in a block heater. A 1-μl volume of the reaction mixture was injected directly into the gas chromatograph.

The effects of solvent and reagent volume and reaction temperature on carbamate formation were studied. Peak-height ratios of unchanged ANP-4364 and carbamate to the internal standard were measured, and the percentage yields of carbamate were calculated.

Assay procedure for ANP-4364 in plasma

To 1 ml of plasma sample were added 1 ml of 2.5 *N* sodium hydroxide solution, 5 ml of *n*-hexane and 0.1 ml of ethyl chloroformate in a glass-stoppered 10-ml centrifuge tube, and the tube was shaken for 10 min. The organic layer (4 ml) was shaken with 5 ml of 0.1 *N* sulphuric acid for 10 min. The aqueous layer (4 ml) was transferred into a glass-stoppered 10-ml centrifuge tube containing 1 ml of 1 *N* sodium hydroxide solution and 1 ml of internal standard solution (39.4 ng), and then shaken with 0.2 ml of *n*-heptane. The organic layer was transferred into another centrifuge tube, and 5 μl of trichloroethyl chloroformate and about 10 mg of anhydrous sodium carbonate were added. The tube, fitted with a condenser, was heated at 110° for 30 min in a block heater.

The reaction mixture was shaken with 1 ml of methanolic alkaline solution for 5 min, 1 ml of water was added and the mixture was shaken for a further 5 min. After centrifugation, the aqueous layer was discarded and 0.5 ml of saturated methanolic alkaline solution was added. The tube was shaken for 1 min, 1 ml of water was added and the mixture was shaken for a further 5 min. A 10-μl aliquot of the organic layer was injected into the gas chromatograph.

Calibration graph

Samples (1 ml) of the control plasma containing 1–50 ng of ANP-4364 were treated as described under *Assay procedure for ANP-4364 in plasma*. Peak-height ratios of ANP-4364 to the internal standard were measured and plotted against the amount of ANP-4364 present.

Animal experiment

Male beagle dogs (8–11 kg) fasted for 16 h were used. ANP-4364 was administered orally and intravenously in doses of 10 and 0.1 mg/kg, respectively, and about 5 ml of blood were drawn by venipuncture 0.5, 1, 2, 4, 6, 8 and 24 h after dosing. Blood samples were centrifuged and plasma samples were kept frozen until taken for analysis.

RESULTS AND DISCUSSION

Reaction conditions

The time course and the yield of the reaction of carbamate formation of ANP-4364 with trichloroethyl chloroformate at 90°, 110° and 130° are shown in Fig. 2. The

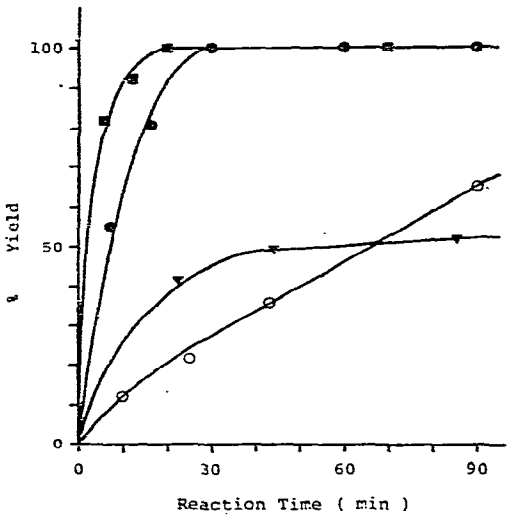

Fig. 2. Effect of reaction temperature on the formation of the trichloroethyl carbamate of ANP-4364. Reaction conditions: 0.2 mg of ANP-4364 in 0.2 ml of *n*-heptane treated with 5 μl of trichloroethyl chloroformate and 10 mg of sodium carbonate. Reaction temperature: ■, 130°; ●, 110°; ▼, 90°; ○, 110°, without sodium carbonate.

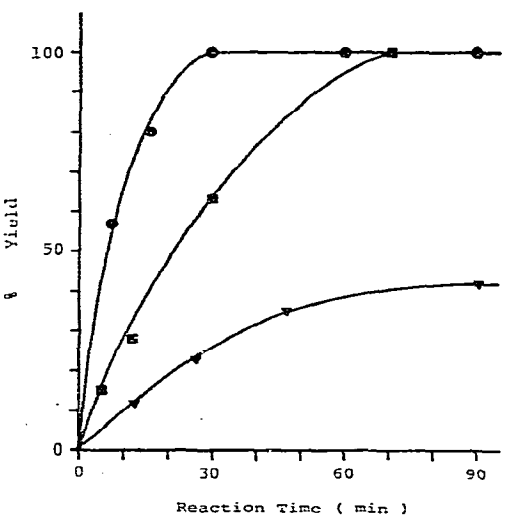

Fig. 3. Effect of solvent volume on the formation of the trichloroethyl carbamate of ANP-4364. Reaction conditions: ANP-4364 in *n*-heptane (1 mg/ml) treated at 110° with 5 μl of trichloroethyl chloroformate and 10 mg of sodium carbonate. Solvent volume: ●, 0.2 ml; ■, 0.5 ml; ▼, 1 ml.

reaction rate increased with increasing reaction temperature; at 110° and 130° the reaction was completed within 30 min, but at 90° it was only 50% complete after 90 min. When sodium carbonate was not present, the yield was less than 70% after 90 min at 110°.

The reaction was also affected by the solvent volume (Fig. 3). The reaction rate increased with decreasing solvent volume and proceeded to completion within 90 min at volumes of 0.2 and 0.5 ml, but at a volume of 1 ml the reaction was only

about 40% complete after 90 min. The reaction was not influenced by the amount of trichloroethyl chloroformate (1–30 μl). As a result of these studies the reaction conditions described under *Assay procedure for ANP-4364 in plasma* were adopted.

Although the reaction of N-dimethyl tertiary amines with trichloroethyl chloroformate has been studied in detail by Hartvig *et al.*[3], that of N-diethyl tertiary amines such as ANP-4364 was not clear, as the reaction rate might be affected by the basicity of the amine owing to the nucleophilic substitution involved. However, no marked differences in the reaction conditions for ANP-4364 and N-dimethyl tertiary amines[3] were found.

Sensitivity of the trichloroethyl carbamate of ANP-4364

The trichloroethyl carbamate of ANP-4364 elicits a very high response on an electron-capture detector. The minimum detectable amount[8], defined as the amount that gives a signal three times the background noise level, was $3.3 \cdot 10^{-16}$ mole/sec, corresponding to 4 pg under the gas chromatographic conditions adopted.

Although the pentafluorobenzyl carbamate of ANP-4364 was found to be 2.5 times more sensitive than the trichloroethyl carbamate, it was not used because a longer reaction time (2 h) was needed and reagent-related substances were insufficiently removed from the reaction mixture.

Selectivity of the method

ANP-4364 is found to be extensively metabolized[9], although the metabolites have not yet been identified. As N-dealkylation is one of the major metabolic pathways of tertiary amines[6], N-desethyl ANP-4364, if it is present, will be converted into the same derivative as that of the unchanged drug. In order to separate the N-dealkylated metabolite from the unchanged drug, a liquid–liquid partition column chromatographic method[4,5] has been reported, but it is time consuming.

In the present method a simple procedure is used to remove the N-dealkylated metabolite alone from the plasma by converting it selectively into an extractable carbamate derivative[10]. The tertiary amine does not react with ethyl chloroformate at room temperature whereas the secondary amine promptly forms the carbamate on extraction under alkaline conditions with an organic solvent containing ethyl chloroformate. Thus, in the organic layer unchanged tertiary amine alone can be back-extracted into the acidic layer. In fact, N-desethyl ANP-4364 did not show any interference on the chromatogram after the entire assay procedure had been performed on a plasma sample to which 500 ng/ml had been added (ten-fold higher concentration of the mean peak level of ANP-4364 in dogs orally treated at 10 mg/kg).

Internal standard

The internal standard should have physical and chemical properties virtually identical with those of the substance to be determined. The internal standard used here, the *n*-propyl ether analogue of ANP-4364, was extracted and derivatized in a similar manner to ANP-4364, but decomposed to some extent in the first extraction step for removing the N-de-ethylated metabolite, owing to its instability under the strongly alkaline conditions adopted. Therefore, the internal standard was added in the second re-extraction step as described under *Assay procedure for ANP-4364 in plasma.*

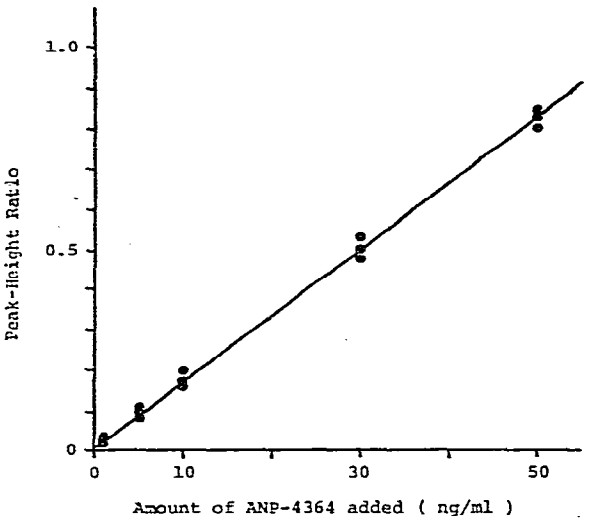

Fig. 4. Calibration graph for ANP-4364 in plasma.

Calibration graph

The calibration graph obtained with 1–50 ng of ANP-4364 in 1 ml of plasma is shown in Fig. 4. The graph was rectilinear and passed through the origin. The precision of the method was 7.5% (relative standard deviation) and the minimum detectable concentration was 0.5 ng/ml in plasma.

Fig. 5 shows a typical chromatogram of a plasma sample containing 30 ng/ml of ANP-4364 and a chromatogram of blank plasma containing internal standard alone.

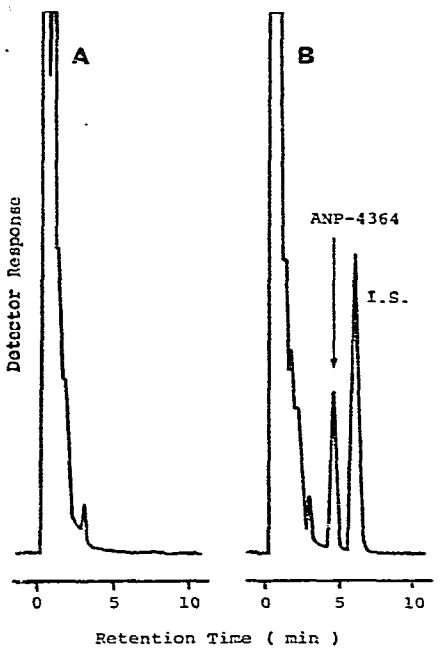

Fig. 5. Typical chromatograms of ANP-4364 in plasma. (A) Control plasma; (B) ANP-4364 corresponding to a plasma concentration of 30 ng/ml. Gas chromatographic conditions as described in the text.

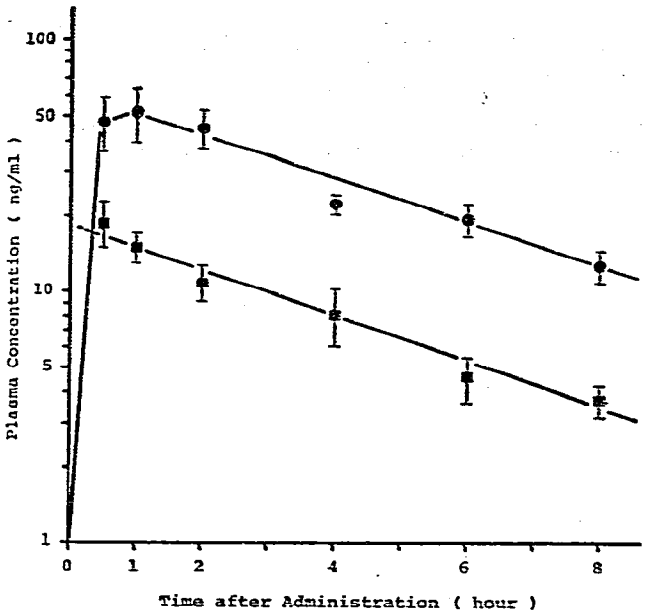

Fig. 6. Plasma levels of ANP-4364 in dogs following single oral (●) or intravenous (■) administration. Plotts are mean values for four dogs ± S.E. Dose: ●, 10 mg/kg; ■, 0.1 mg/kg.

Determination of ANP-4364 in dog plasma

Plasma levels of ANP-4364 following oral (10 mg/kg) and intravenous (0.1 mg/kg) administration are shown in Fig. 6. The drug levels in orally treated dogs were maximal 1 h after dosing with a mean peak level of 50 ng/ml, followed by a first-order decrease with a half-life of 3.6 h, suggesting rapid absorption and moderate elimination. In intravenously treated dogs, plasma levels were nearly one third of the oral plasma level after a 10-fold lower dosing, but showed a similar elimination half-life of 3.1 h. By 24 h after either dosing, ANP-4364 was not detected in plasma.

The gas chromatographic method described would be specific and sensitive enough for the determination of unchanged drug in plama following a low dose of ANP-4364 and would therefore permit pharmacokinetic studies of ANP-4364 in man and experimental animals.

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