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DETERMINATION OF APOVINCAMINIC ACID IN HUMAN PLASMA BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

High-resolution capillary gas–liquid chromatography (GLC) and highly sensitive and selective nitrogen–phosphorus flame ionization detection were applied for the detection of apovincaminic acid (AVA), the main metabolite of the cerebral vasodilator vinpocetine in human plasma.

AVA was recovered from plasma by ion-pair extraction and determined by GLC as its methyl ester after derivatization with diazomethane. An acid–base washing technique was used in order to reduce the contamination of GLC samples by material of endogenous origin to a minimum.

The lowest detection limit of the method is 2 ng of AVA per ml of plasma.

INTRODUCTION

We required to determine the concentration of apovincaminic acid (AVA) in human plasma for studies on the biotransformation of vinpocetine (apovincaminic acid ethyl ester).

Vereczkey and co-workers[1,2] treated Wistar rats with [^{3}H]vinpocetine and found that, both after oral and intravenous administration of the drug, AVA was the main metabolite in urine and the only one in plasma. It was established that, after intravenous administration, the maximum concentration of AVA in plasma almost reaches that of vinpocetine and after oral treatment it exceeds that of the parent drug. From the elimination part of kinetic curves the level of AVA was found to be approximately one order higher than that of vinpocetine.

Our preliminary studies of plasma from patients treated with vinpocetine showed that in humans also a considerable part of the drug is transformed into AVA.

In the present paper we describe a gas–liquid chromatographic (GLC) method, in which a capillary column and a highly sensitive and selective nitrogen–phosphorus flame ionization detector (N–P FID) are applied for detection of AVA in human plasma. Prior to chromatography, AVA is transformed into methyl apovincaminate (AVAM). This technique was used to obtain the time–plasma concentration curve of AVA in a study where the pharmacodynamic effect of vinpocetine was examined in parallel with the pharmacokinetics of the drug and its main metabolite, AVA.

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EXPERIMENTAL

Reagents

Vinpocetine methyl apovincaminate, apovincaminic acid and 9-bromoapovincaminic acid (Br-AVA, internal standard) were obtained from G. Richter (Budapest, Hungary). Tetrabutylammonium hydroxide used as ion-pairing reagent for extraction of AVA was obtained from E. Merck (Darmstadt, G.F.R.). All other reagents were of analytical grade and were purchased from Reanal (Budapest, Hungary). Solvents were purified by distillation before use.

Drug administration and plasma sampling

Patients suffering from cerebrovascular disorders were treated with vinpocetine at a dose of 1 mg per kg body weight. Physiological saline (100 ml) containing the drug was administered by intravenous infusion at a rate of 4 ml/min. Blood (4.5 ml) was pooled at 0, 5, 10, 20, 25, 30, 45, 60 and 90 min and at 2, 4, 6, 8, 10, 12, 24 and 48 h after the start of the infusion, and 0.5 ml of 3.8% (w/v) sodium citrate were added to each sample as anticoagulant. Plasma obtained from blood after rotation at 3000 *g* for 10 min was stored frozen at −10°C until use.

Standard solutions

The concentration of the AVA solution used for preparation of standards was 1.2 μg/ml and that of the internal standard was 9.7 μg/ml. Both substances were dissolved in methanol.

Serum standards

Serum standards for calibration were prepared by measuring 0–250 μl AVA and 10 μl of internal standard solutions in extraction tubes. Methanol was evaporated to dryness by gentle heating, and 1 ml of human plasma was added to each tube.

Diazomethane reagent

A 5.6-g amount of KOH was dissolved in a conical flask in 6 ml of distilled water. Diethyl ether (20 ml) was added and the mixture was cooled to 0°C. Two grams of N-nitroso-N-methylurea were added cautiously in small portions, and the flask was gently shaken and cooled. When the last particles of nitrosomethylurea had disappeared, the ether was decanted into a cold flask containing granules of KOH. The aqueous phase was washed with another 20 ml of ether and the two organic phases were combined. The quantity of reagent thus obtained is adequate for methylation of about 35 samples. The aqueous phase was neutralized by addition of a few millilitres of acetic acid and discarded.

Extraction procedure and derivatization

A 1-ml volume of each plasma sample was placed in an extraction tube containing 97 ng of internal standard (or 970 ng of internal standard if the AVA concentration was expected to exceed 300 ng/ml) and unknowns together with standards were treated with 0.2 ml of 0.05 *M* aqueous tetrabutylammonium hydroxide solution and then extracted with 5 ml chloroform. The organic phase was separated by rotation, transferred into another glass-stoppered tube and evaporated to dryness. The residue was dissolved into 100 μl methanol and 1 ml of diazomethane reagent was added. After 30 min the reaction mixture was evaporated to dryness. A 1-ml volume of 0.1 *N*

HCl and 2 ml of diethyl ether were added and after shaking for a few seconds the organic phase was discarded. The pH of the aqueous phase was adjusted to 11.00 by the addition of 1 ml of 0.2 *M* glycin–NaOH buffer and the compounds of interest were re-extracted with 2 ml of ether. The organic solvent was evaporated to dryness and the residue dissolved in 20 μl ethanol.

GLC

The GLC assay was performed on a Hewlett-Packard 5736A instrument equipped with an Sp 2100 glass capillary column (10 m × 0.25 mm I.D.). The temperatures of the thermostat, injection port and N–P FID were 220, 300 and 300°C, respectively. Nitrogen was used both as a carrier and auxiliary gas. The column pressure was 137 kPa, and the splitting ratio was 1:20. One to three microlitres of sample were injected into the gas chromatograph.

RESULTS AND DISCUSSION

As expected from the character of the AVA molecule, it was not possible to detect this compound in its original form by GLC. According to our earlier experience[3], ethyl and methyl esters of AVA are easily determined in biological fluids by GLC. For this reason we transformed AVA into methyl apovincaminate.

The ion-pairing extraction technique[4] proved to be adequate for recovery of AVA from plasma, but a lot of endogenous material contaminated the chloroform extraction samples and disturbed the analyses. It was therefore necessary to apply an acid–base washing technique for further purification of the methylated samples.

Br-AVA is a suitable internal standard for control of both the extraction and methylation procedures. Under the GLC conditions mentioned above no contamination of endogenous character disturbs the analyses (Fig. 1).

The linear regression curve for the data obtained from analyses of standard samples and used for calibration could be described by $y = 4.12\,x + 0.02$ ($r = 0.997$), where y is the ratio of the peak heights of AVA methyl ester and Br-AVA methyl ester and x is the ratio of concentrations of AVA and Br-AVA in plasma.

A set of standards was run parallel to each batch of unknown samples in order to assure identical extraction and derivatization conditions. The day-to-day coefficient of variation of the slope of the calibration curves was 5.2% ($n = 12$). Coefficients of variation for identical samples ($n = 5$ at each concentration) were 12.3% at 2.4 ng/ml, 3.2% at 24 ng/ml, 4.7% at 120 ng/ml and 5.1% at 1800 ng/ml.

The detector response for methyl apovincaminate in plasma samples obtained after extraction and derivatization is about 50% of that for methanol solutions containing equivalent quantities of methyl apovincaminate standard.

The pharmacokinetic curve in Fig. 2 illustrates the successful application of the method described. Since the detection limit is 2 ng/ml plasma, this GLC technique could be used also for detection of AVA in cases where the vinpocetine dose administered to a patient is much lower than 1 mg/kg.

It should be noted that the GLC analysis can be carried out successfully also on a fused silica capillary column (12 m × 0.2 mm I.D.) coated with OV-101. The temperature of the thermostat must be raised to 250°C in this case. Attempts to perform the assay on a packed column were unsuccessful, since a part of the vinpocetine present in plasma samples passed into the chloroform extract and the resolution obtained was inadequate to separate ethyl apovincaminate from its methyl homologue.

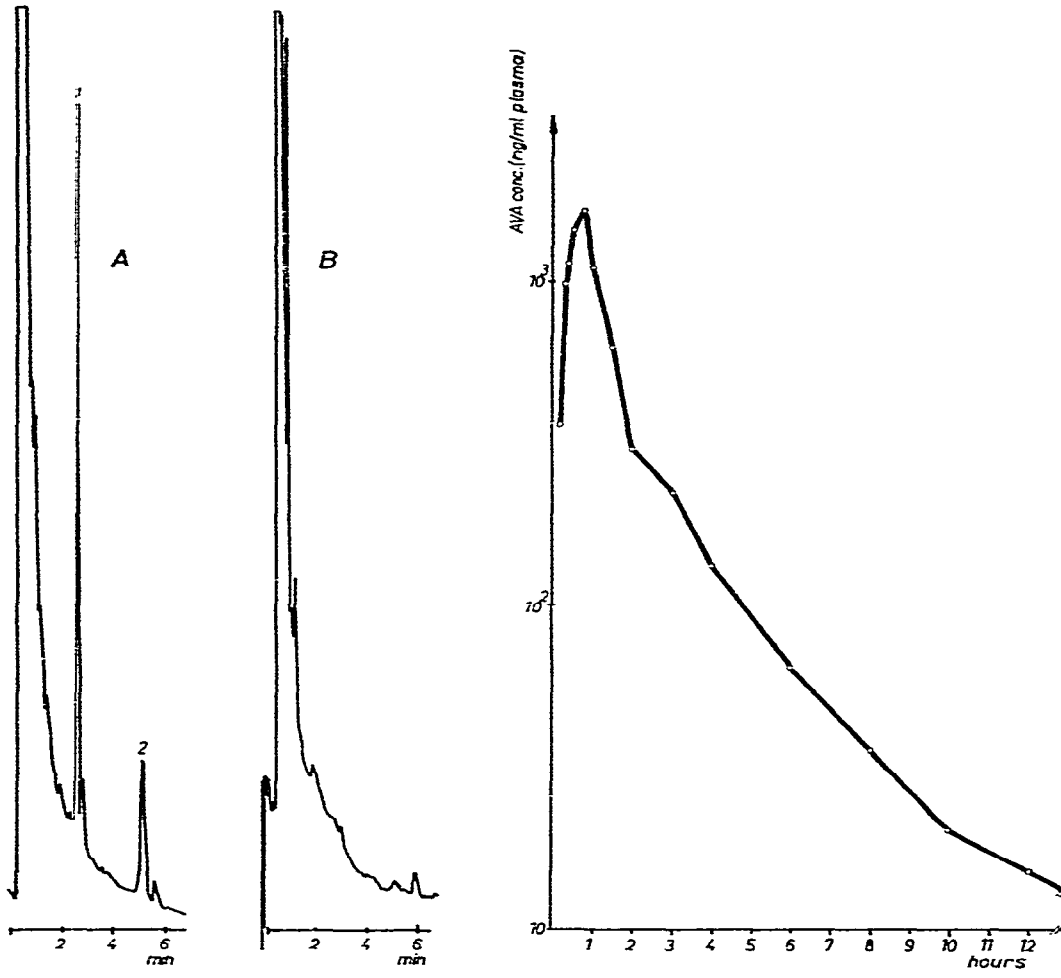

Fig. 1. Chromatograms of plasma extracts: A, plasma containing 120 ng AVA and 97 ng Br-AVA (1 = AVA methyl ester; 2 = Br-AVA methyl ester); B, control plasma.

Fig. 2. Pharmacokinetic curve of AVA after intravenous infusion of vinpocetine at a dose of 1 mg/kg. Duration of infusion: 25 min.

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