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DETERMINATION OF APOVINCAMINIC ACID IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for measuring the concentrations of apovincaminic acid and vincaminic acid (internal standard) in blood plasma and urine by high-performance liquid chromatography using ion pair extraction. The main metabolite of vinpocetine, apovincaminic acid, and vincaminic acid were extracted from 1 ml of plasma and urine into chloroform as an ion pair using tetrabutylammonium hydroxide as counter ion. Analysis was carried out on a reversed-phase column of RP-8 with acetonitrile–0.0075 *M* phosphate buffer (28:72) at pH 3.5 as mobile phase. The eluted derivatives were detected by UV absorption at 254 nm. The sensitivity of the method is 20 ng/ml for AVA in plasma and urine samples. The relative recovery of these compounds added to plasma was about 50%.

INTRODUCTION

Apovincaminic acid (AVA) is the main metabolite of vinpocetine (Cavinton, apovincaminic ethyl ester) and is produced by non-specific esterases in living organisms (Fig. 1).

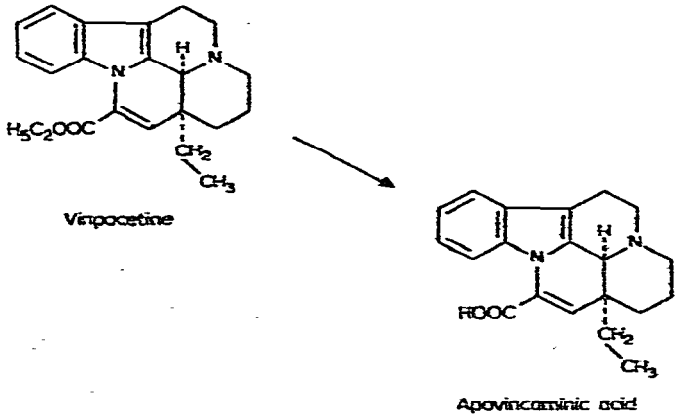

Fig. 1. The biochemical pathway for the formation of apovincaminic acid from vinpocetine by esterases.

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After administration of [^{3}H]vinpocetine, AVA in blood plasma and urine of rats was isolated by thin-layer chromatography (TLC) and identified by mass spectrometry (MS) and infrared (IR) spectroscopy[1]. In order to study the pharmacokinetics of AVA in humans, it was necessary to develop a highly sensitive and specific method of monitoring AVA produced from unlabelled drug in biological samples.

AVA (mol. wt. 322) is a hydrophilic acid, the pK values of the functional groups being 2.4 and 8.3 respectively. The maximum UV absorbance occurs at 260 nm, and does not depend on the pH value. AVA is very soluble in water and methanol; therefore it cannot be extracted with organic solvents from biological media.

In the present paper we report on a method of extraction for AVA and a separation procedure in blood plasma and urine by high-performance liquid chromatography (HPLC). The influence of ionic strength and pH on retention was investigated.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 1081 B high-performance liquid chromatograph, equipped with a fixed-wavelength UV detector (254 nm) and a 3380 S integrator, was employed. The separations were performed on a reversed-phase Hewlett-Packard column (200 × 4.6 mm) of LiChrosorb RP-8 (particle size 10 μm).

Chemicals

Apovincaminic acid and vincaminic acid (VA) were produced by Gedeon Richter (Budapest, Hungary). Tetrabutylammonium hydroxide (40%) and acetonitrile were obtained from E. Merck (Darmstadt, G.F.R.). All other chemicals (p.a. quality) were purchased from Reanal (Budapest, Hungary).

Extraction procedure

A 400-ng amount of vincaminic acid (internal standard) and 0.3 ml of 0.005 M tetrabutylammonium hydroxide (TB) were added to 1 ml of plasma and urine. The samples in test-tubes were extracted with 5 ml chloroform for 5 min. After centrifugation at 3000 g for 10 min at 4°C, the aqueous phase was removed and the chloroform phase was extracted with 1.5 ml of 0.1 N HCl, with shaking for 5 min. The chloroform was discarded and 2 N NaOH was added to the acid phase (to adjust the pH to 8–9) followed by 0.2 ml of 0.005 M TB. The solution was extracted with 2 ml chloroform for 5 min. The chloroform was evaporated in a stream of nitrogen at 50°C in Hewlett-Packard sample vials.

Chromatographic conditions

The eluent was potassium phosphate buffer–acetonitrile (72:28) at various pH values (3.0, 3.3, 3.5, 3.7, 4.1) and ionic strengths (0.0025, 0.005, 0.0075, 0.01 M). The flow-rate was 0.9 ml/min at a pressure of 40 bar, temperature 29°C. For analytical purpose, 100 μl of extracts prepared from plasma or 10 μl of urine samples were injected.

RESULTS AND DISCUSSION

Extraction

AVA from blood plasma and urine could be extracted as an ion pair into chloroform, using tetrabutylammonium hydroxide as counter ion[2]. We found that 200–400 ng of AVA or VA could be extracted if 0.1 ml of 0.005 *M* TB was added to the samples. Raising the amount of TB solution to 0.5 ml did not influence the peak heights of AVA and VA. Direct extraction gave insufficient prepurification for chromatography, therefore the re-extraction procedure described in the Experimental section was adopted. After this procedure the final recovery of the compounds relative to direct injection of a standard solution was 50%; it was consistent in the concentration region studied.

Chromatography

After acidic purification, three endogenous compounds from plasma still remained which interfered with the AVA and IS peaks. The main problem was therefore the separation of these peaks from AVA and VA. The determination of vincaminic acid derivatives was achieved by the HPLC method[3] using a reversed-phase RP-8 column. The influence of ionic strength and pH on the retention and capacity factor had to be investigated in order to separate AVA and VA from endogenous compounds in plasma and urine. As a result of preliminary experiments, the best chromatographic conditions were chosen for the study of the pharmacokinetics of AVA in humans.

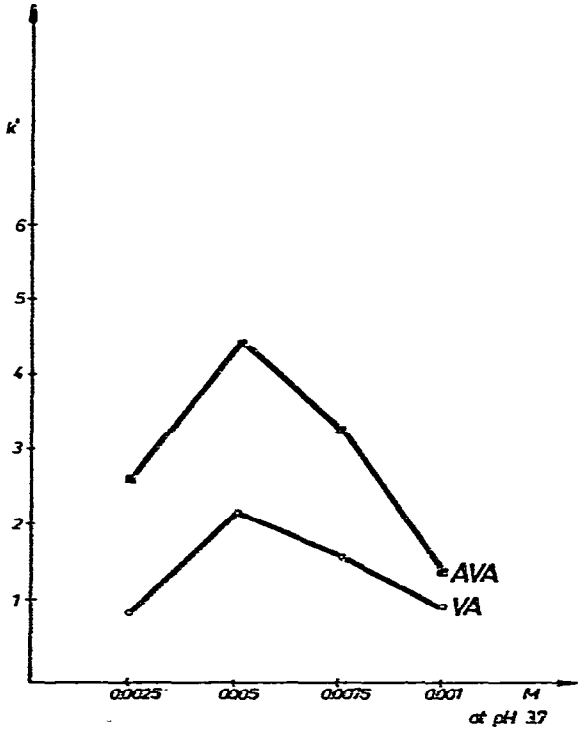

Fig. 2. Relationship between capacity factors of AVA and VA and ionic strength of the buffer.

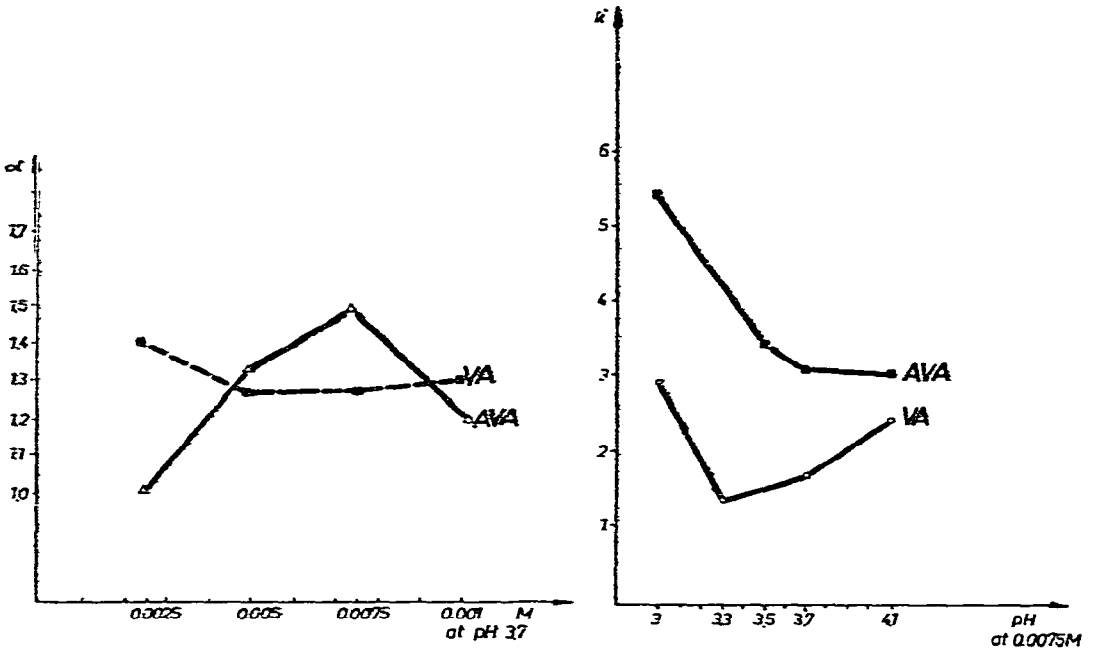

Fig. 3. Influence of ionic strength on the selectivity factor, α, between endogenous compounds and AVA and VA at pH 3.5.

Fig. 4. Influence of pH on the capacity factors of AVA and VA in 0.0075 *M* buffer–acetonitrile (72:28).

At first a methanol–ethanol–water (20:10:70) solution was used for elution, the separation was insufficient. By using acetonitrile–water (30:70) as eluent, the elution order was changed: in the solution containing methanol, AVA was eluted first, while in acetonitrile VA appeared somewhat before AVA. The peak shapes were reasonably good, but a satisfactory separation could not be obtained in either of the above eluents. After considering the physicochemical properties of these compounds, a series of acidic phosphate buffers of different pH values and ionic strengths was prepared.

Effect of ionic strength on retention. At first the ionic strength of the buffer was changed, 0.01, 0.0075, 0.005 and 0.0025 *M* buffer mixed with acetonitrile (72:28) at the same pH 3.7. The variation of capacity factors (k') with ionic strength is shown in Fig. 2. The highest k' values were found between 0.005 *M* (VA, $k' = 2.5$; AVA, $k' = 4.5$) and 0.0075 *M* buffers (VA, $k' = 1.4$; AVA, $k' = 3.8$).

Fig. 3 shows the selectivity factors between VA and AVA and the nearest endogenous peaks in plasma. The best selectivity was obtained between 0.005 and 0.0075 *M* buffers, but VA was not separated sufficiently from the endogenous peaks nearest to it. Therefore different pH values had to be investigated.

Effect of pH on retention. Fig. 4 shows the k' values of AVA and VA at different pH values. At pH $\geq$ 4.1 AVA and VA are not separated from each other[3], but they are well separated at pH $<$ 3.7. On the other hand, separation of these compounds from the nearest endogenous peaks is optimal near pH 3.5 (see Fig. 5).

The following chromatographic conditions were chosen for pharmacokinetic

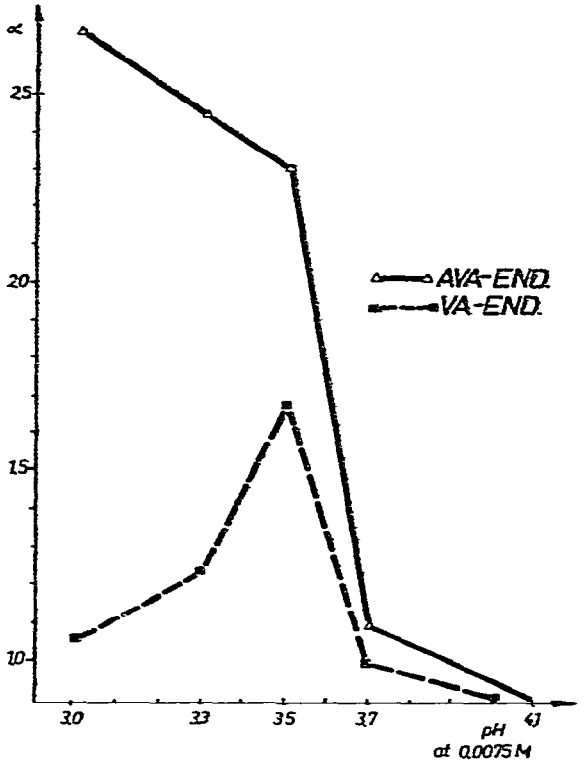

Fig. 5. Effect of eluent pH on the selectivity factor between endogenous compounds and AVA and VA in 0.0075 *M* phosphate buffer–acetonitrile (72:28).

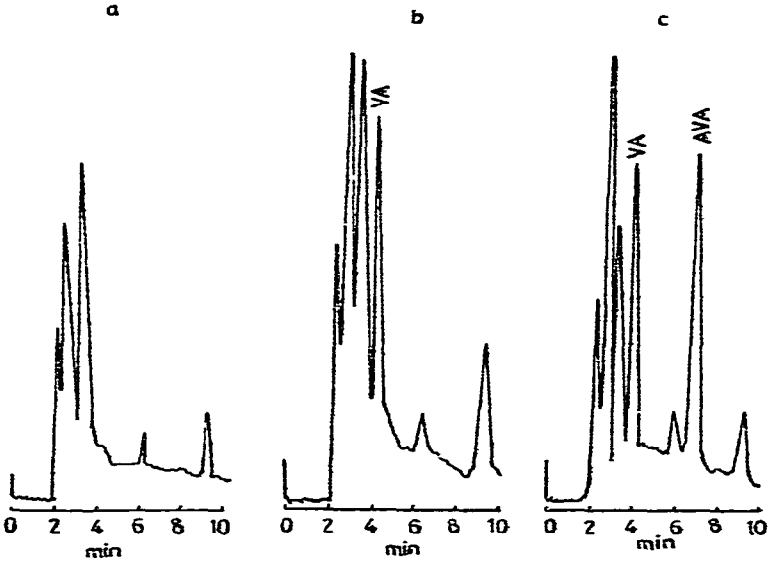

Fig. 6. Chromatograms of human plasma extracts: a, blank plasma extract; b, plasma and vincaminic acid extract; c, plasma extract after *per os* administration of 15 mg vinpocetine (Cavinton).

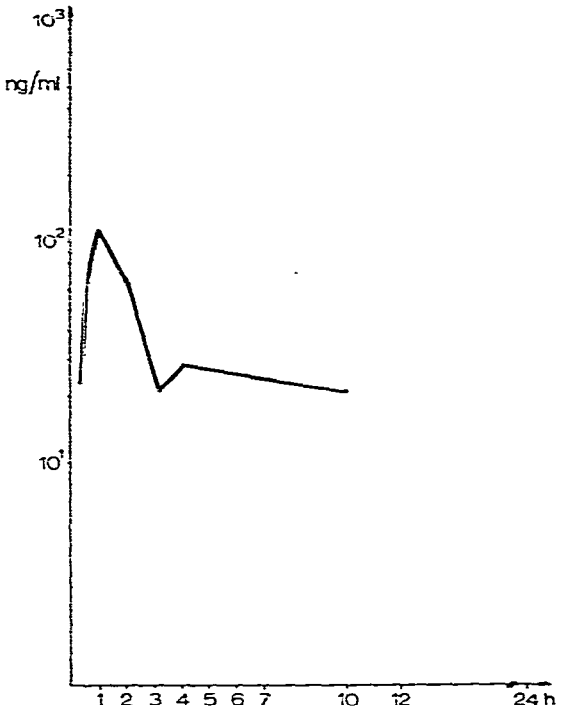

Fig. 7. Human plasma AVA concentration *vs.* time curve after oral administration of 15 mg vinpocetine.

investigations *in vivo*: eluent, 0.0075 *M* phosphate buffer–acetonitrile (72:28) at pH 3.5; flow-rate 0.9 ml/min at a pressure of 40 bar; temperature, 29°C.

Fig. 6 shows chromatograms of AVA and VA and a plasma extract. The method can be used with success for the analysis of AVA in humans and animals. Fig. 7 shows the mean plasma concentration *vs.* time curve of AVA in five healthy volunteers after *per os* administration of 15 mg vinpocetine (Cavinton) using the method described. The unknown amounts of AVA were evaluated by measuring the peak heights relative to that of the internal standard. AVA and VA were identified by their relative retention times. The reproducibility of the determination of AVA in our parallel GLC[4] and HPLC investigations was within 10%. In urine collected over 24 h, 1.5 mg of AVA were detected, 20% of the administered dose.

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