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# Determination of apovincaminic acid in human plasma by high-performance liquid chromatography

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**Abstract**

An isocratic high-performance liquid chromatographic method is described for the quantitative analysis of low concentrations of apovincaminic acid (AVA) in blood plasma. AVA, interfering plasma components and primidone (used as the internal standard) were separated on a reversed-phase column of LiChrospher 60 RP-Select B (125 mm × 3 mm i.d.; 5 $\mu$m) (Merck). A UV-Vis detector was used at a wavelength of 254 nm.

Each chromatographic separation was completed in 14 min and the results showed a relative recovery which varied between 95.9 and 116%, a good overall precision (relative standard deviation, 7.00%) and sensitivity over a linear range of 5.00–300 ng ml$^{-1}$ ($R = 0.999$) for AVA in plasma.

The method was applied to the analysis of plasma samples obtained from healthy subjects treated with one single oral dose of 20 mg of vinpocetine. The results indicate the method to be suitable for pharmacokinetic studies.

*Keywords*: High-performance liquid chromatography; Vinpocetine; Apovincaminic acid; Plasma

## 1. Introduction

Vinpocetine is an eburnamenine derivative used as a cerebral vasodilator [1,2], which improves the utilisation of oxygen by the cerebral cells and protects the brain cells against ischaemic anoxia [3]. In addition, vinpocetine was shown to improve erythrocyte fluidity and inhibits platelet aggregation. It affects cerebral and vascular monoaminergic systems, too, and increases cerebral noradrenaline turn-over [4].

After oral administration, vinpocetine is rapidly absorbed and undergoes a marked first-pass effect, during which approximately 75% of the substance is hydrolysed into its main metabolite, apovincaminic acid (AVA) (Fig .1), leading to an absolute bioavailability of 7% in man [5]. Since

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vinpocetine is converted into AVA through the first-pass effect, plasma levels of unchanged vinpocetine are not found shortly after administration and only AVA plasma concentrations can be evaluated.

In the last few years, some sensitive methods involving gas chromatography [6–8] and liquid chromatography [9,10] have been developed for AVA quantitation in human plasma. However, and especially for pharmacokinetic studies, there is a need to improve these methods in terms of ease of sample handling and/or analysis time, as a large number of samples have to be analysed.

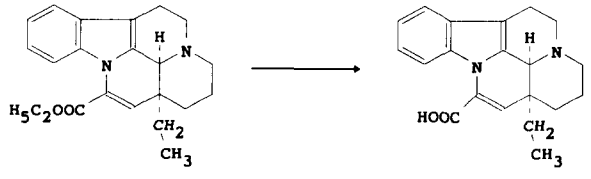
The purpose of the present study was to develop a rapid and sensitive isocratic HPLC method for the quantitative determination of AVA in plasma for use in a comparative bioavailability study after a single oral dose of vinpocetine in different dosage forms, such as conventional tablets, controlled-release tablets and an oral solution.

For the validation of the assay, recommendations from the conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies" were followed [11].

## 2. Materials and methods

### 2.1. Chromatographic conditions

Analysis was performed on a Merck–Hitachi liquid chromatograph equipped with a reversed-phase column of LiChrospher 60 RP-Select B (125 mm × 3 mm i.d.; 5 $\mu$m) (Merck, Darmstadt, Germany) protected by a guard column of LiChrospher 100 RP-8 (5 $\mu$m), 4-4 nm$^2$ (Merck, Darmstadt, Germany).




Vinpocetine Apovincaminic acid

Fig. 1. The metabolic pathway of vinpocetine for the formation of apovincaminic acid.

The solvent was delivered by means of a Merck–Hitachi pump, model L-6000, which was coupled to a Merck–Hitachi automatic injector, model AS-2000 A (Merck, Darmstadt, Germany).

A variable-wavelength UV-Vis detector, Merck–Hitachi, model L-4200, operated at 254 nm and a sensitivity of 0.002 a.u.f.s., was used.

Peak heights were measured by means of a Hewlett Packard integrator, model 3390 A (Avondale, PA, USA) with a chart speed of 0.1 cm min$^{-1}$.

The mobile phase consisted of potassium phosphate buffer (pH 2.8; 10 mM)–acetonitrile (86:14, v/v), triethylamine (0.07%) and 1-octanesulfonic acid, sodium salt monohydrate (1 mM). The final apparent pH was 4.7.

The eluent was delivered at a flow rate of 0.4 ml min$^{-1}$.

The mobile phase was filtered by passing through a 0.45 $\mu$m membrane filter (S-Pak filter, Millipore) under vacuum, and was degassed in an ultrasonic bath for 15 min.

### 2.2. Reagents and standards

Purified water (Barnstead E-pure purification system, Barnstead Thermolyne, Iowa, USA) and HPLC-grade acetonitrile (Merck, Darmstadt, Germany) were used throughout .

Triethylamine (Sigma, St. Louis, USA), 1-octanesulfonic acid sodium salt monohydrate (Fluka, Buchs, Switzerland), tetrabutylammonium hydroxide (TBAH) (Sigma, St. Louis, USA), chloroform, sodium hydroxide, sulfuric acid (95–97%), orthophosphoric acid (85%) and potassium dihydrogen phosphate (Merck, Darmstadt, Germany) were of analytical grade.

Primidone (Sigma, St. Louis, USA) was used as internal standard at a concentration of 1 mg ml$^{-1}$ in methanol (stock solution). AVA (Covex, Madrid, Spain) was supplied by Tecnimede (Sacavém, Portugal) and was used as reference substance at a concentration of 1 mg ml$^{-1}$ in methanol (stock solution). The standard solutions were stored protected from light at 4°C.

Control plasmas were prepared from working solutions diluted with blank plasma at concentrations 20, 100 and 300 ng ml$^{-1}$.

Blank plasma was obtained from healthy subjects not undergoing drug therapy.

### 2.3. Sample preparation

In a 16 × 125 mm screw-cap tube with a Teflon lining, 20 μl of methanol containing 20 μg of internal standard (primidone), 50 μl of 30 mM TBAH and 3 ml of chloroform were added to 1 ml of plasma.

The tubes were vigorously shaken for 30 s (Vortex) and then mixed on a reciprocating mixer at 300 strokes per minute for 10 min. After centrifugation (3000 rev $min^{-1}$ for 5 min at 5°C), the aqueous layer was removed by aspiration and 50 mM sulphuric acid (1 ml) was added to the organic phase, followed by another period of shaking and centrifugation. The aqueous layer was transferred to a clean tube, and 120 μl of 1 N sodium hydroxide, 50 μl of 30 mM TBAH and 2 ml of chloroform were added.

The samples were shaken and centrifuged as described above, and after removal of the total aqueous layer, the organic phase was evaporated under a stream of nitrogen (38°C). The dry residue was reconstituted with 100 μl of mobile phase, the mixture was centrifuged (3000 rev $min^{-1}$ for 1 min at 5°C) and the supernatant was injected onto the HPLC system.

On every working day, a calibration curve (six different concentrations) and quality control samples (three different concentrations) were extracted and analysed along with the unknown plasma samples.

### 2.4. Quantitation

Plasma concentrations of AVA in unknown and control samples were determined by using the linear regression equation from daily calibration curves which were constructed by plotting the AVA to internal standard ratio over the concentration range of 5.00–300 ng $ml^{-1}$.

## 3. Results

### 3.1. Chromatography

Fig. 2 shows the chromatograms obtained from (A) blank plasma as well as (B) blank plasma spiked with AVA (100 ng $ml^{-1}$) and the internal standard (20 μg $ml^{-1}$ of primidone) obtained by means of the described methodology .

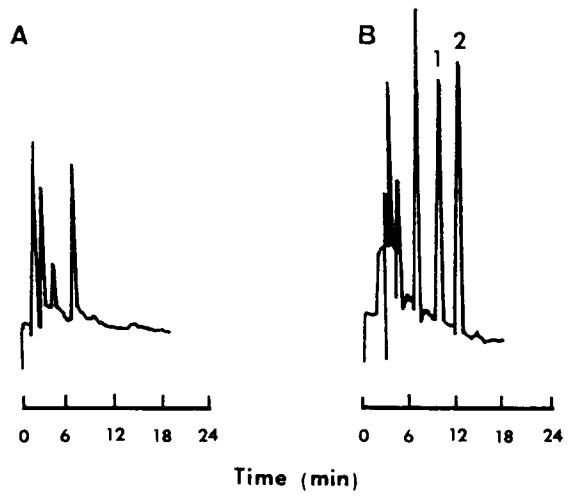



Fig. 2. HPLC of AVA. Peak 1, AVA; peak 2, internal standard. (UV detection wavelength, 254 nm; column, LiChrospher 60 RP-Select B (125 mm × 3 mm i.d., 5 μm); mobile phase, potassium phosphate buffer (pH 2.8; 10 mM)–acetonitrile (86:14, v/v), triethylamine (0.07%) and 1-octanesulfonic acid sodium salt monohydrate (1 mM) (final apparent pH* 4.7)). (A) Blank, extracted plasma; (B) extracted plasma spiked with 100 ng $ml^{-1}$ of AVA and 20 μg $ml^{-1}$ of internal standard.

Fig. 3 shows the chromatograms obtained from one healthy volunteer after oral administration of vinpocetine at two different collection times.

The retention times were apovincaminic acid, 9.74 min; primidone, 12.3 min.

### 3.2. Linearity

The linearity of the method was checked for AVA in plasma (5.00–300 ng $ml^{-1}$). Peak height ratios (referred to the internal standard) and analyte concentrations were found to be linearly related over this range (see Table 1) . Linear regression was used to determine the slope and intercept. The correlation coefficient was 0.99995.

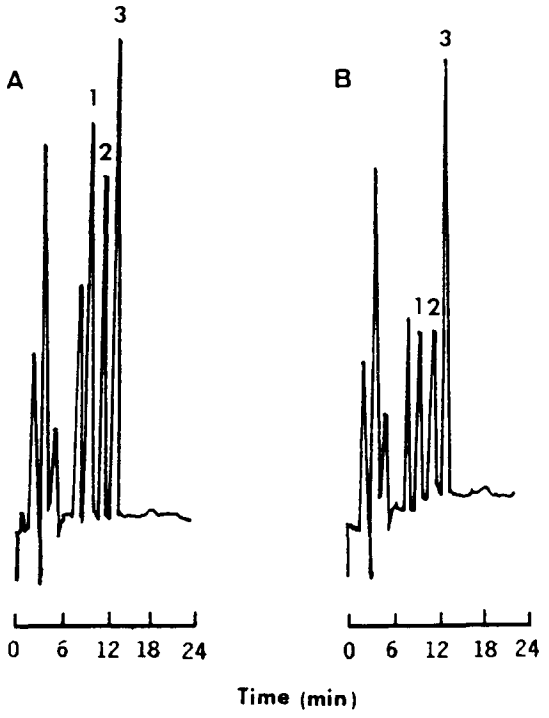



Fig. 3. Chromatograms obtained from one healthy volunteer after the oral administration of vinpocetine at two different collection times: (A) 1.5 h after the oral administration of 20 mg of vinpocetine; (B) 3.0 h after the oral administration of 20 mg of vinpocetine. Peak 1, interfering peak from plasma; peak 2, AVA; peak 3, internal standard.

### 3.3. Between-day precision

The between-day precision of the assay was determined from the analysis of pooled plasma spiked with AVA (20.0, 100 and 300 ng $ml^{-1}$) on each of the 22 days of analysis. The results are summarised in Table 2.

Table 1
Results of linear regression analysis of calibration data

| Parameter | Value |
|---|---|
| Slope, b | 0.01125 |
| Intercept, a | −0.0242 |
| Standard error of slope, Sb | 0.000036 |
| Standard error of intercept, Sa | 0.01266 |
| Range (ng $ml^{-1}$) | 5.00–300 |
| Correlation coefficient | 0.99995 |

Table 2
Between-day precision of the analytical method for the determination of AVA in plasma

| Nominal concentration (ng $ml^{-1}$) | $n$[a] | Mean[b] (ng $ml^{-1}$) | SD[c] | RSD (%) |
|---|---|---|---|---|
| 20.0 | 22 | 21.0 | 3.00 | 14.3 |
| 100 | 22 | 94.5 | 5.66 | 6.00 |
| 300 | 22 | 297 | 18.2 | 6.12 |

[a]Number of plasma samples analysed for each value. Experimental conditions as described in the text.
[b]Mean values of different spiked plasmas analysed on different days.
[c]Standard deviation.

### 3.4. Recovery/accuracy

The extraction efficiency of AVA was determined to be 45.9%. The overall mean recovery was also determined at different AVA concentrations from appropriate spiked blank plasmas ($n$ = 5). The mean value was 101% with relative standard deviations ranging from 3.82% (at 200 ng $ml^{-1}$) to 8.50% (at 10 ng $ml^{-1}$).

### 3.5. Limit of quantitation

The limit of quantitation (LOQ) was calculated on the basis of the analysis of at least five replicates of different concentrations of AVA, taking as the LOQ the lowest concentration value for which an RSD of less than 20% (10) was found. When 1 ml of the sample was used, the LOQ was 5 ng $ml^{-1}$.

### 3.6. Specificity

The specificity of the analytical method was checked by the analysis of six different independent sources of the same biologic matrix, obtained from healthy blood donors.

No endogenous interfering peaks were visible at the retention times of AVA and the internal standard (Fig. 2A). In the chromatographic system described, vinpocetine is not detectable at any presumable significant level.

## 4. Discussion and conclusions

A sensitive HPLC assay was developed for the evaluation of apovincaminic acid from 1 ml of plasma using UV detection at 254 nm. Ion pair extraction using tetrabutylammonium hydroxide proved to be adequate for AVA, but an extensive clean-up of the final extract by successive back extraction into water and the organic phase again had to be developed so that clean chromatograms could be obtained. The HPLC method described is selective and no endogenous interfering peaks were visible in blank plasma (Fig. 2A). Nevertheless, during the development of the method and under initial testing conditions (mobile phase: potassium phosphate buffer–acetonitrile (85:15, v/v) (pH* 6.7)), an interfering substance co-eluted with AVA for a large number of plasma samples (Fig. 4A). A thorough investigation allowed us to establish a relationship between those sample sources and smoking subjects. A more exhaustive analysis led us to conclude that the interfering substance was cotinine, the main metabolite of nicotine. In order to obtain a good separation between AVA and cotinine, variations in the pH and the composition of the mobile phase were made until conditions were obtained under which the method was selective even for smokers (Fig. 4B). The addition of 1-octanesulfonic acid to the eluent leads to a better separation of the endogenous compounds from AVA, and good sharp symmetrical peaks are obtained.

The calibration plot of peak height ratio (AVA/internal standard) is linear over the range 5.00–300 ng $ml^{-1}$ and the precision of the method, calculated from the calibration curve, shows a relative standard deviation (RSD) of 7.00%.

The limit of quantification (LOQ) of the assay is 5.0 ng $ml^{-1}$, which is substantially lower than that reported by Kosma et al. [8] but similar to that reported by Kraus et al. [9].

The utilisation of an automatic injector allows the processing of over 50 samples in one night, which is an advantage in a bioavailability study as a large number of samples have to be analysed .

The method reported here shows good characteristics (selectivity, linearity, sensitivity and precision) and is adequate for the evaluation of AVA in plasma, in pharmacokinetic studies over the collection period, in order to clearly define the absorption and elimination phase of vinpocetine (Fig. 5).

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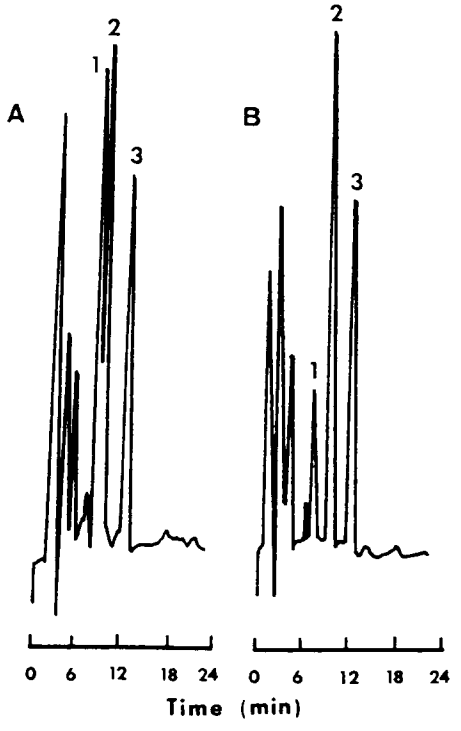


Fig. 4. Effect of mobile phase composition and pH in order to separate cotinine from AVA. (A) Two overlapping peaks (mobile phase, potassium phosphate buffer (pH 2.8; 10 mM)–acetonitrile (85:15, v/v), triethylamine (0.07%) and 1-octanesulfonic acid sodium monohydrate (1 mM) (pH* 6.7)); (B) well-separated peaks under the final conditions (mobile phase, potassium phosphate buffer (pH 2.8; 10 mM)–acetonitrile (86:14, v/v), triethylamine (0.07%) and 1-octanesulfonic acid sodium salt monohydrate (1 mM) (pH* 4.7)). Peak 1, interfering peak from cotinine; peak 2, AVA; peak 3, internal standard.

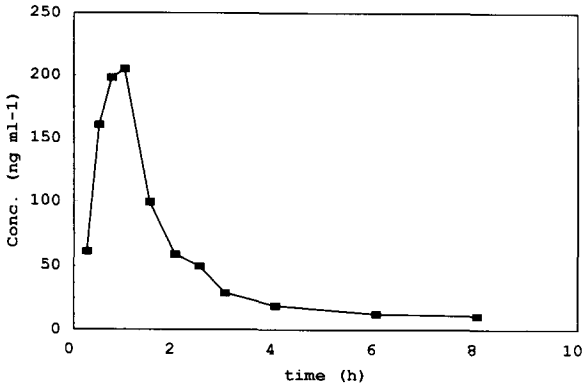


Fig. 5. Concentration vs. time curve of AVA in plasma of a healthy volunteer who was given a single oral dose of 20 mg of vinpocetine in a bioavailability study.

## References

[1] P. Bencsadth and L. Debreczeni, Arzneim. Forsch., 26 (1976) 1920–1923.

[2] S. Fenyes and J. Foldes, Arzneim. Forsch., 26 (1976) 1963–1964.

[3] K. Biró, E. Karpati and L. Szporny, Arzneim. Forsch., 26 (1976) (1918–1920).

[4] B. Kiss, E. Lapis, E. Palosi, D. Groó and L. Szporny, Biochemical and Pharmacological Observations with Vinpocetine, a Cerebral Oxygenator, in A. Wanquier, M. Borgers and W. Amery (Eds.), Protection of Tissues against Hypoxia, Elsevier, Amsterdam, 1982, pp. 305–309.

[5] M. Polgâr and L. Vereczkey, Gas–Liquid Chromatographic Method for Quantification of Vinpocetine in Biological Fluids, in A. Frigerio (Ed.), Chromatography in Biochemistry, Medicine and Environmental Research, Vol. 1, Elsevier, Amsterdam, 1983, pp. 77–81.

[6] M. Polgâr and L. Vereczkey, J. Chromatogr., 241 (1982) 29–32.

[7] W. Hammes and R. Weyhenmeyer, J. Chromatogr., 413 (1987) 264–269.

[8] M. Kozma, P. Pudleiner and L. Vereczkey, J. Chromatogr., 241 (1982) 177–182.

[9] G. Kraus, U. Schulz and A. Lohmann, J. Chromatogr., 573 (1992) 323–327.

[10] M. Polgár, L. Vereczkey and S. Nyáry, J. Pharm. Biomed. Anal., 3 (1985) 131–139.

[11] P. Shah, K. Midha, J. Pharm. Sci., 3 (1992) 309–312.