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Note

New assay method for the determination of vinpocetine in human plasma with gas chromatography–mass spectrometry without transesterification caused by solvents

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Vinpocetine belongs to the group of indole alkaloids of the eburnamine type. It is used in the treatment of cerebral dysfunction. Vinpocetine is able to diminish the sequalae of hypoxic and ischaemic attacks and to accelerate recovery. In addition, it has cytoprotective effects [1–3]. In 1987, Hammes and Weyhenmeyer [4] described a gas chromatographic–mass spectrometric (GC–MS) method for the determination of vinpocetine in human plasma. They extracted vinpocetine with *n*-hexane and used apovincaminic acid methyl ester (AVAM) as internal standard (I.S.). The extraction residue was dissolved in methanol. The extraction itself was performed in borosilicate glass vials. However, using an autosampler together with high sample throughput, as required for routine analysis, produced an insufficient reproducibility in our hands.

This paper therefore describes for the first time a validated method for the determination of vinpocetine in human plasma; it also describes the results of the validation, which prove that this method is suitable for use in pharmacokinetic studies.

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EXPERIMENTAL

Instruments and instrumental conditions

The assay was performed with a GC–MS system based on a Hewlett-Packard 5890 gas chromatograph and an MSD 5970 mass-selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). The carrier gas was helium 6.0 (Messer Griesheim, Duisburg, F.R.G.) at a flow-rate of 1.2 ml/min. The injector was a split–splitless injector (Gerstel, Mülheim/Ruhr, F.R.G.), used at 270°C and with a split opening after 0.75 min. The column was an Ultra 1 fused-silica capillary (12.5 m×0.2 mm I.D.) with cross-linked methyl silicone and with a film thickness of 0.33 μm (Hewlett-Packard).

The mass spectra were obtained in the total ion current (TIC) mode. The determinations of vinpocetine and the I.S. were performed in the selected-ion monitoring (SIM) mode: *m/z* 321.1 for vinpocetine and *m/z* 307.1 for AVAM. Other conditions: transferline temperature, 280°C, ion source temperature, 200–250°C; analyser temperature, 280–310°C; ionization voltage, 70 eV; vacuum, $1.33 \cdot 10^{-3}$ Pa; autosampler, 7673A (Hewlett-Packard) with controller; injection volume, 2 μl, rinsing, 2×5 with 4 μl of 2-propanol each; integrator, work station 300/9000 (Hewlett Packard); software, Hewlett-Packard 59 972C, Revision 3.1.1. The GC temperature programme began at 150°C, and increased at 30°C/min to the final temperature of 280°C, which was held for 2 min. The purification phase was 2 min at 300°C. The injection amplitude in the automatic process was 13 min 5 s.

The following instruments were also used: shaker, IKA Vibrax VXR (Janke & Kunkel, Staufen, F.R.G.) with multiple carrier for extraction by rotation–vibration (own development); centrifuge, Varifuge RF (Heraeus Christ, Osterode, F.R.G.); evaporator, own development; pipettes, Eppendorf Comforpette 4700 (Eppendorf-Netheler-Hinz, Hamburg, F.R.G.); pipette tips, Comfortips (Eppendorf-Netheler-Hinz); extraction vials, 15 ml and 4 ml AR-Glas® (Serolab, Aidenbach, F.R.G.); sample vials, Microvials Wheaton, received via Hewlett-Packard.

Chemicals and reagents

Vinpocetine was obtained from Gedeon Richter (Budapest, Hungary), apovincaminic acid methyl ester from Covex (Madrid, Spain) and human pool plasma from the blood bank of the University Hospital (Düsseldorf, F.R.G.). The extracting medium was *n*-hexane of reagent purity for the analysis of residues (Merck, Darmstadt, F.R.G.). The solvents for samples were 2-propanol of reagent purity (Merck), absolute ethanol of reagent purity (Merck) and methanol of reagent purity LiChrosolv® (Merck). A vinpocetine stock solution was made at a concentration of 50 μg/ml in ethanol and stored at 20°C by protecting it from light. An apovincaminic acid methyl ester stock solution was made at a concentration of 50 μg/ml in methanol (solution A) and stored at

20°C by protecting it from light. The AVAM working solution was 10 μl of solution A with 4 ml of methanol: this concentration corresponds to 125 ng/ml, and 20-μl aliquots (2.5 ng) were used.

Analytical procedure

For each calibration curve and quality control sample an independent stock solution was prepared. To determine the recovery and the intra-assay and inter-assay reproducibilities, the samples were spiked with a stock solution. Depending on the final concentrations of the plasma samples for the calibration curve, the corresponding dilutions (5.00, 2.50, 1.25, 0.63, 0.31, 0.16 and 0.08 ng per 20 μl ethanol) of the stock solution were prepared. Of each dilution, 20 μl/ml plasma were used. In 15-ml AR-Glas universal vials, 20 μl of each stock solution of the I.S. were transferred and evaporated to dryness under a stream of nitrogen. After this, 20 μl of the corresponding vinpocetine calibration solution were added and also evaporated to dryness. Then 1 ml of plasma was added and mixed on a vortex mixer for ca. 10 s.

For plasma samples, 20 μl of the I.S. were pipetted into a 15-ml AR-Glas universal vial and the solvent was evaporated. After this, 1 ml of plasma was added and mixed briefly on the vortex mixer, and then 3 ml of *n*-hexane were added. The extraction was performed on the IKA Vibrax for 5 min. The sample was then centrifuged at 20°C and 4600 *g* for 5 min. The organic phase (upper part) was pipetted as completely as possible into a 4-ml AR-Glas universal vial and evaporated to dryness at 40°C under a stream of nitrogen. The extraction process was repeated once with the prior extracted sample. In order to obtain the maximum concentration of the extract at the bottom of the vial, the walls of the 4-ml AR-Glas universal vial were rinsed with 100 μl of *n*-hexane and the hexane was evaporated. The residue was dissolved in 20 μl of 2-propanol, and the solution was transferred to a 100-μl microvial, which was tightly sealed with a top. A 2-μl aliquot of this solution was injected into GC–MS.

The recovery was determined by analysing 1 ml of a spiked plasma sample as described. The absolute analytical recovery was determined at six different concentrations ($n=5$). For comparison, injections of the same concentrations without plasma were made.

RESULTS AND DISCUSSION

The previously described method [4] shows inadequate reproducibility. This is because of (a) the use of methanol to dissolve the residue during sample preparation and (b) the use of borosilicate glass, which strongly adsorb vinpocetine and has an unpredictable release and resulting carry-over effect. Investigations of the adsorption of vinpocetine to borosilicate glass showed that in one sample at a concentration of 5 ng/ml no vinpocetine could be detected after an incubation period of 20 h at −20°C. AR-Glas universal vials did not

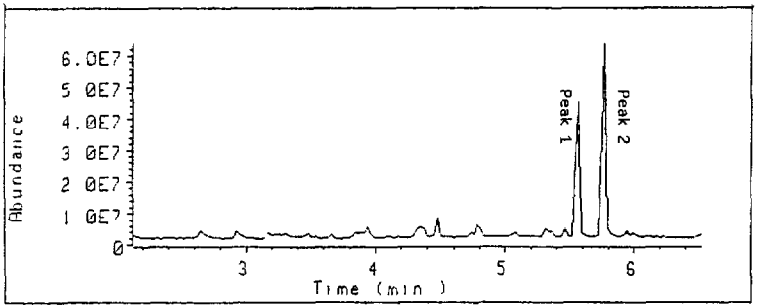

Fig. 1. Total-ion current (TIC) chromatogram of vinpocetine dissolved in methanol; note the appearance of two peaks instead of one.

show this effect. Fig. 1 shows the chromatogram of a solution of vinpocetine in methanol: two peaks (1 and 2) are clearly apparent. In order to identify these peaks, they were both examined by MS, and their spectra were compared with the mass spectra of vinpocetine (dissolved in ethanol) and AVAM (dissolved in methanol) (Fig. 2). (The injection of the pure solvents produced no signals.) Thus peak 1 was identified as AVAM and peak 2 as vinpocetine, which proves that dissolution of the residue in methanol results in transesterification of vinpocetine to AVAM before or during GC. This means that uncontrolled amounts of the I.S. are generated.

To prevent this problem we dissolved the evaporation residue in 2-propanol. Because this solvent is a secondary alcohol, no transesterification effect was observed. The selectivity of the assay can be proved by comparing the chromatogram of a spiked plasma sample with that of blank human plasma (Fig. 3). It can be clearly seen that no interfering peaks occur. In this context it is important that we were able to detect an absolute amount of 450 fg of added vinpocetine. As the blank plasma samples show no vinpocetine signals, they contain less than 450 fg of vinpocetine. The detection limit (defined as three times the baseline noise) for the plasma samples examined was 0.08 ng/ml of human plasma. The linearity of the calibration curve was investigated and shown to be good over the concentration range of 80 pg to 5 ng per ml human plasma. In general, the r values obtained were better than 0.9993. The values of the absolute analytical recovery of vinpocetine and the I.S. were better than 94.8% for vinpocetine and 100% for AVAM. The intra-assay reproducibility was determined by measuring seven different calibration curves. A previously obtained calibration curve was used as reference. All analyses were performed in randomized order. Over the concentration range the intra-assay coefficient of variation (C.V.) was better than 7.09% (5 ng/ml) and 14% (0.08 ng/ml), respectively.

The inter-assay reproducibility was determined by comparing the ratios of

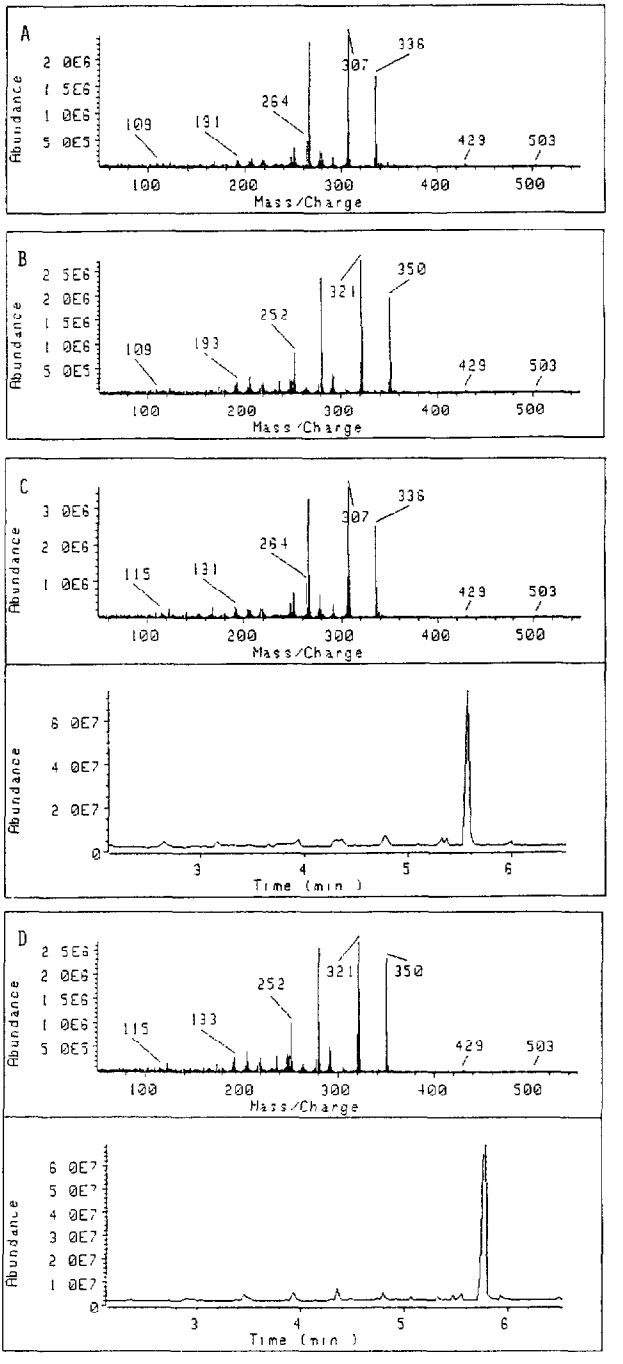

Fig. 2. (A) Mass spectrum of peak 1 in Fig. 1. (B) Mass spectrum of peak 2 in Fig. 1. (C) Mass spectrum and TIC chromatogram of AVAM dissolved in methanol. (D) Mass spectrum and TIC chromatogram of vinpocetine dissolved in ethanol.

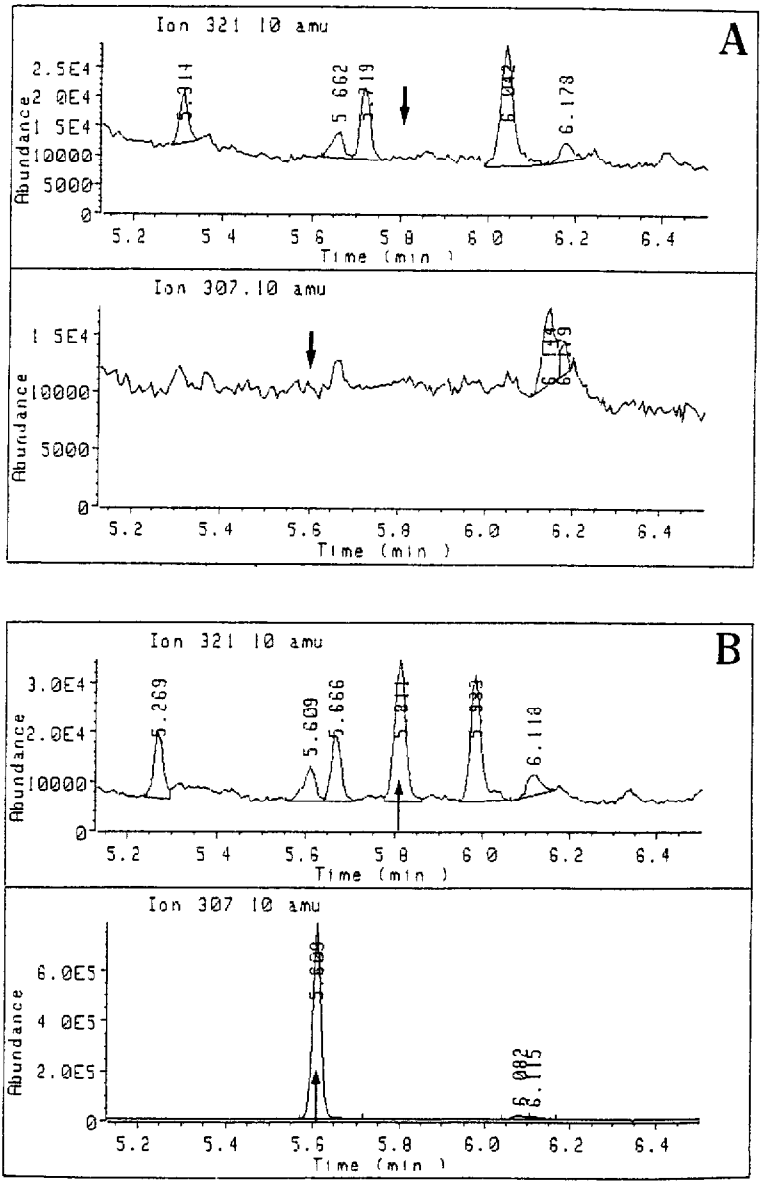

Fig. 3. Selected-ion monitoring (SIM) mode chromatogram (ion 321.1=vinpocetine; ion 307.1=AVAM) of (A) extracted blank human plasma and (B) extracted spiked plasma sample (80 pg/ml).

the calibration curves on fourteen different days. Over the concentration range the inter-assay C.V. was better than 11.26% for 5 ng/ml and 19.9% for 0.08 ng/ml. Thus the intra-assay and inter-assay reproducibilities and the preci-

sion of the method are acceptable. Over more than eight months, neither plasma nor calibration samples showed any sign of deterioration. Plasma samples were kept at −20 °C until use and protected from light. The stock solutions of vinpocetine and the I.S. were stable for at least fourteen days. No interferences were observed in either pooled plasma or plasma samples of more than 100 healthy volunteers and patients.

The method described here for the analysis of vinpocetine in human plasma is thus selective and sensitive. In particular, the transesterification effect observed when methanol is used is eliminated by the use of 2-propanol, and the use of AR-Glas eliminates adsorption effects. The method can therefore be described not only as efficient, but also as inexpensive for the extreme concentration range examined. The high throughput makes it most useful for routine analyses in pharmacokinetic studies.

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