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Short Communication

Determination of apovincaminic acid in serum by means of high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the determination of low concentrations in serum of apovincaminic acid, the main metabolite of vinpocetine, is reported. The assay includes a two-step ion-pair extraction with tetrabutylammonium as counter ion. Recovery is *ca.* 40%. Separation is performed on a narrow-range 5 μm particle size octadecylsilane modified silica packing. Heptanesulphonic acid is the pairing ion in the eluent, and the ultraviolet detection wavelength is 224 nm. Yohimbine serves as the internal standard. The assay is fast, accurate and sensitive quantifying at least 5 ng/ml apovincaminic acid in serum. The method was applied to the analysis of serum samples from aged subjects, treated with a 20-mg dose of vinpocetine.

INTRODUCTION

Vinca minor is a medicinal herb that is one of the most investigated species in phytotherapy [1]. An important group of those isolated alkaloids contains the eburnamenine skeleton. Derivatives of this type are easy to separate and they show pharmacological effects on the heart and specifically on the cerebral nervous system. One of them, vinpocetine (eburnamenine-14-carboxylic acid ethyl ester) has been found to act as a cerebral vasodilator. It was developed by the Hungarian company Gedeon Richter. In Germany it is intended to introduce it on the pharmaceutical market.

In humans vinpocetine is extensively metabolized as shown in Fig. 1. The predominant metabolic route is the hydrolysis of the carboxylate function to apovincaminic acid (1; Fig. 2).

For pharmacokinetic investigations of vinpocetine it is important to determine apovincaminic acid, because there is special interest in the pharmacokinetic behaviour of this metabolite in humans. Also it is necessary to determine the metabolite in drug interaction studies. Apovincaminic acid has been analysed by gas chromato-

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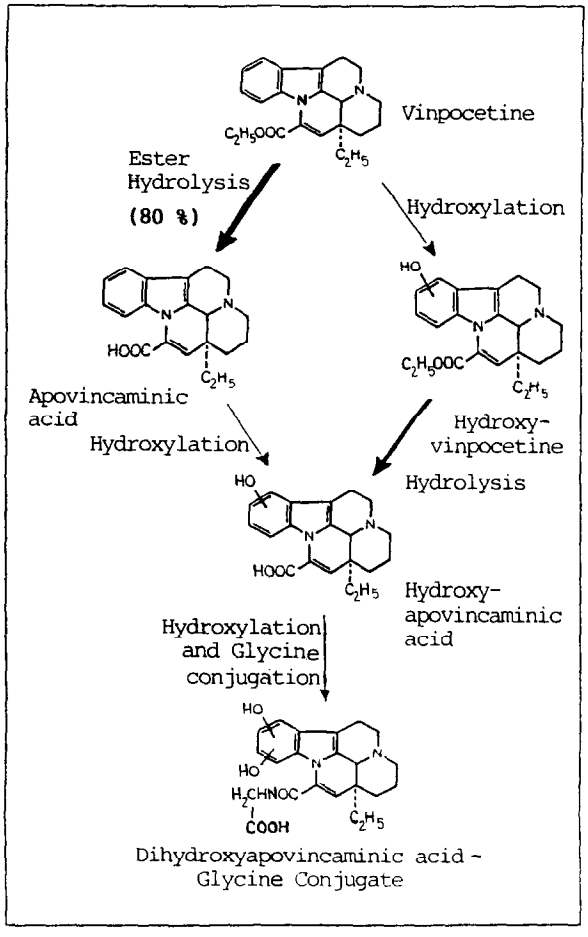

Fig. 1. General scheme of metabolic pathway of vinpocetine. The first step is hydrolysis to apovincaminic acid (investigation of urine samples from rats and dogs).

graphic methods [2,3], radio-thin-layer chromatography [4] and liquid chromatography [5–8]. The most suitable of these assays is that of Kosma *et al.* [8], a simple reversed-phase high-performance liquid chromatographic procedure with UV detection at 254 nm. However, for investigations of humans treated with low doses of vinpocetine, this method lacks specifity and sensitivity in the region below 20 ng/ml. Therefore the method described here has been developed using smaller-particle octadecylsilane-substituted silica packings and ion-pair chromatography, which results in a clearcut separation and a detection limit of less than 5 ng/ml. Yohimbine (II; Fig. 2) was used as the internal standard.

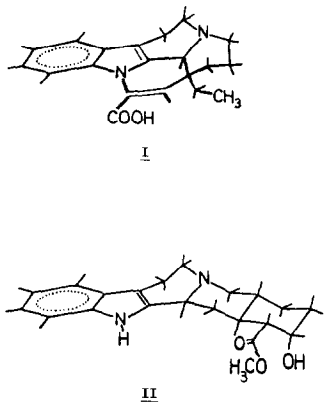

Fig. 2. Structures of apovincaminic acid (I) and yohimbine (II).

EXPERIMENTAL

Reagents and solvents

Apovincaminic acid was a gift from Thiemann Arzneimittel (Waltrop, Germany); yohimbine hydrochloride, triethylamine, tetrabutylammonium hydroxide, and sodium octanesulphonate were purchased from Sigma (Deisenhofen, Germany); sodium hydroxide, potassium dihydrogenphosphate, phosphoric acid and sulphuric acid were obtained from Merck (Darmstadt, Germany). Solvents came from Rathburn (Walkerburn, UK).

Standard solutions

Apovincaminic acid and yohimbine hydrochloride were diluted in methanol to give stock solutions of 100 μg/ml, which were stable for more than 30 days at 5°C. From those an apovincaminic acid working solution of 5 μg/ml in methanol was made for spiking serum calibration standards in the range 5–500 ng/ml. As the internal standard, a solution of yohimbine was prepared in methanol.

Extraction procedure

In a 2.2-ml polypropylene tube (Eppendorf 3812, Hamburg, Germany) 50 μl of 30 m*M* tetrabutylammonium hydroxide were added to 450 μl of serum and 40 ng of yohimbine (40-μl solution, 1 μg/ml). Such samples were extracted with 1.4 ml of chloroform for 10 min. After centrifugation (Eppendorf centrifuge 5415, 11 800 *g*) for 5 min

the upper phase was completely aspirated by means of a Pasteur pipette. The remaining chloroform was overlaid with 500 μl of 50 mM sulphuric acid agitated for 10 min and centrifuged (5 min, 11 800 g). Then 450 μl of the acidic layer were transfered to a new tube (Eppendorf 3810) and brought to pH 9 by the addition of 50 μl of 1 M sodium hydroxide. Back-extraction was carried out by again adding 50 μl of 30 mM tetrabutylammonium hydroxide and 0.7 ml of chloroform, agitating and centrifugation as above. After total removal of the aqueous layer by aspiration, chloroform was evaporated under a stream of nitrogen at 55°C. The dry residue was dissolved in 50 μl of the eluent, vortex-mixed and injected, into the chromatograph.

Chromatographic conditions

Analysis was performed on a modular HPLC system consisting of a Kontron 420 pump with an analytical head (Kontron, Eching, Germany), a Kontron 460 HPLC autosampler, a Nucleosil 120-5 C_{18} column (100 mm × 4 mm I.D.) equipped with a precolumn (30 mm × 4 mm I.D.) of the same material (Macherey & Nagel, Düren, Germany), a Kratos spectroflow 757 variable-wavelength UV detector (Applied Biosystems, Weiterstadt, Germany) set at 224 nm with a sensitivity of 0.005 a.u.f.s. and a D2000 integrator (Merck/Hitachi, Darmstadt, Germany). The flow-rate was 1.1 ml/min at a pressure of 75 bar. The normal injection volume was 20 μl.

The mobile phase was a 10 mM potassium phosphate buffer (pH 2.8) containing 1 mM octanesulphonate, 0.07 vol.% triethylamine and 24% acetonitrile. It was filtered (0.45 μm membrane filter, Sartorius, Göttingen, Germany) and degassed before use.

RESULTS AND DISCUSSION

Chromatography and selectivity

Fig. 3 shows representative chromatograms of extracts from blank serum (a), a blank serum containing yohimbine (40 ng) as internal standard (b) and serum spiked with 8 and 150 ng of apovincaminic acid (c and d). Although the sample preparation and concentration procedure was thorough, there are interfering compounds in the

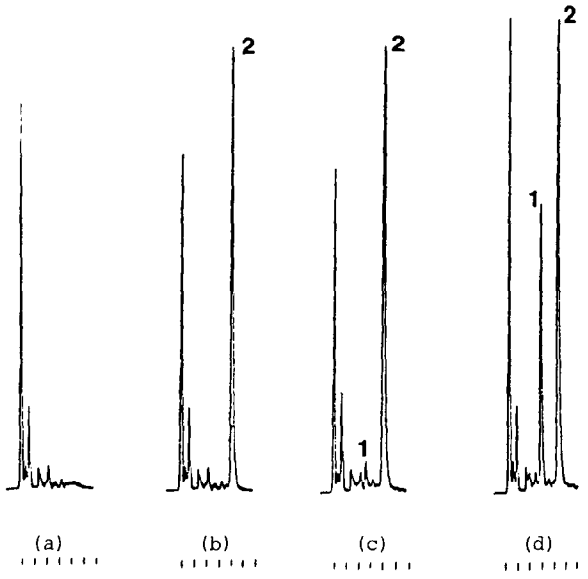

Fig. 3. Ion-pair HPLC separation of apovincaminic acid and yohimbine from a serum matrix. (a) Blank extract; (b) blank extract containing 40 ng of yohimbine; (c) extract containing 8 ng/ml apovincaminic acid and yohimbine (40 ng); (d) extract with 150 ng/ml apovincaminic acid and 40 ng of yohimbine. Peaks: 1 = apovincaminic acid; 2 = yohimbine. Each bar on the x-axis is equivalent to 1 min.

extracts. In order to obtain blank chromatograms free of interfering compounds at the retention time of apovincaminic acid octanesulphonic acid was added to the eluent. This leads to complete separation of the endogenous compounds from apovincaminic acid, which elutes close to yohimbine. Good sharp symmetrical peaks are produced.

The retention times were: t_0, 1.12 min; apovincaminic acid, 3.72 min; yohimbine, 5.16 min. The capacity factors (k') were 2.3 and 3.6 for apovincaminic acid and yohimbine, respectively, and the α value for apovincaminic acid was 1.573.

According to the ion-pairing chromatographic conditions, it is clear that no other metabolic compounds will be retained as long as apovincaminic acid because of lower lipophilicity.

Linearity and sensitivity

Serum concentrations of apovincaminic acid are calculated directly from a calibration curve. Such standard graphs from a set of measurements prove the linearity of the method. For concentrations up to 500 ng/ml in all cases there is good linearity, with coefficients of correlation (r)

TABLE I

REPRODUCIBILITY OF THE METHOD FOR APOVINCAMINIC ACID (n = 6)

Theoretical concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	S.E.M.	C.V. (%)	Accuracy (mean) (%)
10	10.7 ± 0.61	0.25	5.7	+7.33
25	25.6 ± 2.07	0.84	8.09	+2.66
50	47.8 ± 4.44	1.81	9.29	−4.33
100	101.1 ± 3.60	1.47	3.56	+0.13
200	201.1 ± 2.31	0.94	1.15	+0.56
300	299.5 ± 5.76	2.35	1.92	−0.18

always better than 0.998. Moreover, the regression lines pass practically through the origin.

The limit of quantitation as a parameter of the sensitivity is defined on the basis of the amount injected, producing a peak approximately three times that of background. By this definition, 4 ng/ml apovincaminic acid can be measured. This is substantiated by the chromatogram in Fig. 3c, which represents a concentration of 8 ng/ml.

Reproducibility and recovery

The reproducibility of the method was determined with human serum samples containing

TABLE II

RECOVERY OF APOVINCAMINIC ACID AND YOHIMBINE

Concentration (ng/ml)	Recovery (mean ± S.D.) (%)	C.V. (%)	n
Apovincaminic acid			
25	40.1 ± 2.9	5.95	4
100	38.1 ± 1.9	3.65	10
500	37.8 ± 4.2	7.12	5
Yohimbine			
40	94.8 ± 6.1	6.53	10

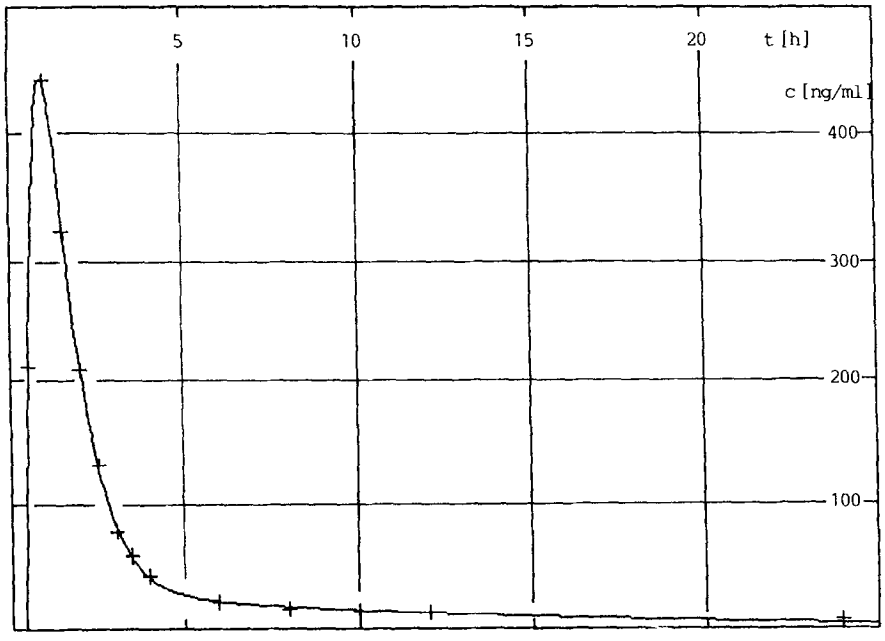

Fig. 4. Plasma concentration–time course of apovincaminic acid in a subject following a single oral dose of 20 mg of vinpocetine.

apovincaminic acid concentrations ranging from 10 to 300 ng/ml. The results are summarized in Table I. From six-fold determinations the imprecision (coefficient of variation, C.V.) varied from 1.15 to 9.29%.

The accuracy was assessed by calculating the percentage deviation between the found and given concentrations. These results are also summarized in Table I.

The final recovery following direct injection of a solution with an identical content of apovincaminic acid is less than 40%; it was consistent in the concentration region studied (Table II). The recovery of yohimbine, which does not dissociate, is nearly complete.

Application

The utility of the assay was demonstrated by analysing apovincaminic acid in serum samples from aged patients treated orally with a 20-mg dose of vinpocetine. Fig. 4 shows a representative plot of serum concentration *versus* time. Levels of apovincaminic acid decline within 1 h to 440 ng/ml and decrease in two phases to a concentrations of less than 10 ng/ml after more than 12 h. This indicates that the described method may be used for pharmacokinetic studies in humans.

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