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A Validated HPLC Method for the Simultaneous Determination of Vincamine and its Potential Degradant (Metabolite), Vincaminic Acid: Applications to Pharmaceutical Formulations and Pharmacokinetic Studies

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ABSTRACT

A sensitive and specific HPLC method was developed and validated for the simultaneous determination of vincamine and its potential degradant (metabolite), vincaminic acid**.** Chromatographic separation was achieved on a Spheri-5 RP-C8 (5 μm) (220 × 4.6 mm i.d.) column using a mobile phase composed of acetonitrile and 0.05 M sodium acetate, pH 4.0 (30 : 70, v/v) at a flow rate of 1 mL/min. The UV detector was set at 270 nm and the quantitation of the analytes was based on the peak areas. The method was proven to be accurate and precise with linearity ranges of 0.1 - 50 and 0.4 - 50 μg/mL for vincamine and vincaminic acid, respectively. The limits of detection were 0.03 and 0.08 μg/mL for vincamine and vincaminic acid, respectively. The method was applied to serve two goals. First; stability-indicating assay of vincamine in its pharmaceutical formulations, in addition, the determination of vincaminic acid down to a level of 0.07% in presence of excess of the parent drug. Second; drug monitoring of vincamine and its main metabolite, vincaminic acid in human plasma/urine samples taken from a healthy volunteer treated with 60 mg oral dose of vincamine. The accuracy of the method was satisfactory (recovery > 97%). The overall standard deviation ranged from 1.4 to 2.3%.

Key words: HPLC, vincamine, vincaminic acid, stability-indicating assay, *in-vivo* plasma/urine assays

INTRODUCTION

Vincamine (methyl (3α, 16α)-14,15-dihydro-14ß-hydroxyeburnamenine-14-carboxylate)[1] is an eburnane-type alkaloid obtained from *Vinca minor*. It is claimed to increase cerebral circulation and utilization of oxygen and has been used in a variety of cerebral disorders[1]. Vincamine (VN) is also one of several active ingredients in dietary supplements promoted as smart drugs, cognitive enhancers or nootropics. Several methods have been reported for the determination of VN in pharmaceutical formulations and/or biological samples, including HPLC[2-9], GC[10-13], TLC[8], fluorimetry[14], spectrophotometry[7,8,15-19], ^{1}H-NMR[20] and adsorptive stripping voltammetry[21]. VN has also been determined in *Vinca minor* by TLC[22] and HPLC[23-25] methods.

VN is an ester derivative of eburnamenine nucleus. The drug is susceptible to degradation through hydrolysis to yield vincaminic acid (VA)[8]. Figure 1 shows the chemical structures of VN and its hydrolysis product, VA. Recently, some studies have been interested in developing stability-indicating assays of VN by different analytical methods. These include derivative-ratio spectrophotometry[8], ratio-subtraction spectrophotometry[15],

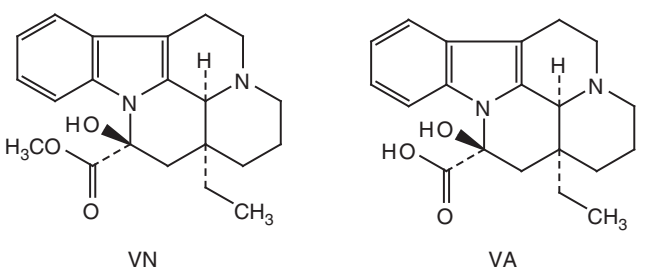

Figure 1. Chemical structures of vincamine (VN) and vincaminic acid (VA).

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chemometric-assisted spectrophotometry[16], TLC[8] and HPLC[8]. All these methods have been focused on the determination of VN in the presence of VA. Shehata *et al*. reported the selective determination of VN without interference from VA up to a ratio of 1 : 9 (VN : VA)[8]. However, a major task that the quality control pharmacists face is not only to analyze the intact drug in the presence of its degradation products, but also to determine the concentrations of the degradation product in bulk raw material and final pharmaceutical preparations. To our best knowledge, none of the previous studies handled this issue. The current study is thus aimed at exploring the strategy to determine the level of VA in VN drug substance and pharmaceutical preparations.

In humans, VN is rapidly absorbed after oral administration and metabolized through ester cleavage to VA. The amount of VN eliminated unchanged in urine is ~ 7%. Radiolabel studies on rats[26], after an oral dose of VN, indicated that VN is hydrolyzed by plasma esterase to VA. The latter is quickly decarboxylated and oxidized to eburnamenine. However, apovincaminic acid, the structurally related analogue of VA and the main metabolite of vinpocetine (semi-synthetic derivative of VN), has been determined in plasma/urine samples obtained from human subjects treated with an oral dose of vinpocetine[27,28].

To our knowledge, detection/determination of VA in human plasma/urine samples has not been reported yet. Most of the previous research[2-5,11-13] have been interested in the quantification of VN only.

The purpose of the present work was to develop a simple and reliable RP-HPLC method with UV-detection for simultaneous determination of VN and its degradant/metabolite, VA. This was in order to serve three goals: (I) establishment of a stability indicating assay method of VN to be applied in pharmaceutical analysis; (II) determination of VA as a potential degradant/impurity of VN, in raw material samples and pharmaceutical formulations; and (III) application to biological analysis; *in-vitro* and *in-vivo* assays of VN and its metabolite, VA in human plasma and urine samples.

MATERIALS AND METHODS

I. *Instrumentation*

The HPLC system used was Perkin Elmer Series 200 (USA), equipped with a quaternary pump solvent delivery system, auto-sampler and UV detector. The system was operated with TotalChrom Workstation Perkin Elmer Chromatography software. The detector was set at 270 nm. Photodiode array absorption spectra were acquired with Agilent 1200 series HPLC system with a multiple wavelength photodiode array detector (PDA). The HPLC system consisted of a quaternary pump and an on-line injector equipped with a 50-µL sample loop, and was operated with Agilent ChemStation Software.

^{1}H-NMR spectra were recorded on JEOL spectrometer (500 MHz, δ ppm) using DMSO-d_6 as solvent. Mass spectra were run on Finnigan SSQ/7000 Mass Spectrometer (70 eV, Thermo Electron Corporation). IR spectra were obtained on a Perkin-Elmer RX I FT-IR system.

II. *Chemicals and Reagents*

Vincamine standard (98% purity) was purchased from Sigma-Aldrich. Oxybral® capsules (each labeled to contain 30 mg of VN) and Oxybral® ampoules (each labeled to contain 15 mg of VN) were supplied by Glaxo-SmithKline S.A.E., Egypt. HPLC-grade acetonitrile was purchased from Labscan Ltd., Dublin, Ireland. Other chemicals were all of analytical grade.

III. *Chromatographic Conditions and Analysis*

Chromatographic separation was performed on a Spheri-5 RP-C8 column (Perkin-Elmer BrownLee columns, 220 mm × 4.6 mm i.d., 5 µm particle size). The column temperature was maintained at 25°C.

Separation was achieved with a mobile phase of acetonitrile - 0.05 M sodium acetate (30 : 70, v/v), adjusted to pH 4.0 using glacial acetic acid. The flow rate was set at 1.0 mL/min throughout the run and the UV detector was set at 270 nm. The injection volume was 50 µL. The injections were made in duplicate. The peak areas were measured.

IV. *Degradation Reaction, Isolation and Purification of the Degradation Product, VA*

Five hundred milligram of VN was mixed with 20 mL of 3 M HCl and subjected to hydrolysis by heating in a boiling water bath for 100 min. The resulted solution was cooled and VA was extracted by 3 × 20-mL portions of chloroform. The combined chloroform extract was dried over anhydrous sodium sulphate and evaporated under vacuum to obtain a solid residue (yellow crystalline solid). Crystallization was carried out using methanol.

V. *General Procedure*

Standard stock solutions of VN (reference substance) and VA (isolated and purified substance), 400 µg/mL each, were prepared in methanol and kept in the refrigerator. The solutions were stable for at least two days. Working standard solutions with the concentration ranges of 0.1 - 50 µg/mL and 0.4 - 50 µg/mL for VN and VA, respectively, were prepared by dilution of aliquots of the stock solutions with the mobile phase.

VI. *Preparation of Assay Solutions from Capsules/Ampoules*

To an accurately weighed quantity of the mixed powdered content of Oxybral® capsules, equivalent to 40 mg of VN, 50 mL of methanol were added and stirred

for 10 min followed by filtration through 0.45-µm Millipore membrane filter into a 100-mL volumetric flask. The residue was washed with two 10-mL portions of methanol and the washings were added to the filtrate and diluted to volume with methanol. The resulting solution contained 400 µg/mL of VN. Working assay solutions were prepared by suitable dilutions with methanol.

An accurate volume of the mixed contents of five Oxybral® ampoules, equivalent to 40 mg of VN, was transferred into a 100-mL volumetric flask, mixed and diluted to volume with methanol. The resulting solution contains 400 µg/mL of VN. Working assay solutions were prepared by suitable dilutions with methanol.

VII. *Assay Solutions for the Determination of VA at Trace Level in VN*

Using a set of 10-mL volumetric flasks, working standard solutions of VN were prepared to attain a final concentration range of 5-1000 µg/mL, taking into consideration that such solutions were spiked with VA at different levels (within 10 to 0.05%). The volumes were made up to 10 mL with the mobile phase.

VIII. *Preparation of Plasma/Urine Samples*

Frozen plasma/urine was thawed at room temperature. Separate 1-mL aliquots of plasma/urine were transferred into a set of centrifuge tubes and spiked with suitable amounts of standard VN and VA solutions to cover the concentration range of 0.02 - 0.5 and 0.08 - 1.0 µg/mL for VN and VA, respectively. Protein precipitation was carried out with 1 mL of ethanol, followed by centrifugation for 15 minutes at 4000 rpm. The supernatant of each prepared sample was evaporated to dryness with nitrogen. Separate residues were reconstituted in 200 µL of mobile phase. The same sample handling process was carried out for the linearity, recovery, precision, accuracy and stability tests.

IX. *Preparation of In-vivo Samples and Pharmacokinetic Study*

The method was applied to determine the concentrations of VN and its metabolite, VA, in the plasma/urine samples from a healthy subject, who was treated by a single 60-mg oral dose of VN sustained-release capsules. Venous blood samples were drawn before dosing (0 h) and at 0.5 h, 1 h, 2 h, 3 h, 4 h, and 6 h, after dosing. All blood samples were collected in heparinized tubes and centrifuged at 4000 rpm immediately. The separated plasma samples were stored at -20°C until analysis. Urine samples were collected before dosing (0 h) and at 2 h intervals, up to 8 h and stored at -20°C until analysis. One milliliter aliquot of thawed plasma/urine samples was processed as described in Section VIII above.

RESULTS AND DISCUSSION

I. *Aspects Concerning Hydrolysis of VN*

Previous studies[8,15] on VN hydrolytic degradation indicated that the reaction is acid-catalyzed. The degradation product, vincaminic acid (VA), has been isolated and structurally confirmed by MS analysis. As described in these studies[8,15], hydrolysis was achieved under stress conditions of 2 M HCl/100°C for 7 h. To our knowledge, no study on the kinetics of the degradation, pH-degradation rate profile or the effect of mild conditions of temperature, moisture, *etc.* has been reported.

In the present work, the reaction time was reduced at the expense of acid strength. Three hydrolysis reactions were carried out in 2, 3 and 4 M HCl, at 100°C followed by the chromatographic measurement of the degradation product, VA, which eluted at 5.06 min under the conditions described above. The results indicated that the degradation in 3 M HCl/100°C for 100 min, is adequate for complete hydrolysis (Figure 2). Hence, the degradation product, VA, was prepared and isolated as described in Materials and Methods Section IV.

The structure of the isolated product, VA, was confirmed by IR, ^{1}H-NMR, MS spectroscopy and elemental analysis. All criteria have assigned the structure to be (3α, 16α)-14,15-dihydro-14ß-hydroxyeburnamenine-14-carboxylic acid. The IR spectrum (KBr disk) of VA refers to C = O and OH stretching bands at 1662 cm^{-1} and 3300 cm^{-1}, respectively. The IR spectrum (KBr disk) of VN shows the characteristic ester C = O band at 1749 cm^{-1} while the OH band is missing.

The ^{1}H-NMR spectrum of VA (DMSO, δ ppm, 500 MHz) showed the following characteristic signals: 0.94 (t, 3H, J = 7.7 Hz, CH_2-C$\underline{H}_3$), 1.46-1.50 (m, 2H, C_{18}-H), 1.79 (q, 2H, J = 7.7 Hz, C$\underline{H}_2$-CH_3), 1.87 (s, 2H, C_{15}-H), 1.88-1.93 (m, 2H, C_{17}-H), 2.00-2.10 (m, 2H, C_6-H), 2.90-3.11 (m, 4H, $C_{5,19}$-H), 3.9 (s, 1H, C_3-H), 4.7 (s, 1H, OH), 7.11(t, 1H, J = 7.6 Hz, C_{10}-H), 7.18 (d, 1H, J = 8.4 Hz, C_9-H), 7.3 (d, 1H, J = 8.4 Hz, C_{12}-H), 7.52 (t, 1H, J = 7.0 Hz, C_{11}-H) and 11.3 (broad s, 1H, COOH).

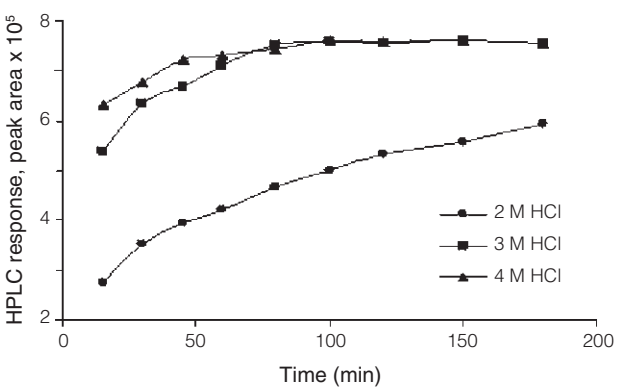

Figure 2. Reaction time course of the hydrolysis of VN (1.0 µg/mL) at 100°C.

The MS spectrum showed diagnostic fragment ions *m/z* 322, (M^+-H_2O, $C_{20}H_{22}N_2O_2$, 53.8%), *m/z* 293 ($C_{18}H_{17}N_2O_2$, 90.0%), *m/z* 248 ($C_{17}H_{16}N_2$, 18.8%), *m/z* 206 ($C_{14}H_{10}N_2$, 43.8%), *m/z* 193 ($C_{13}H_9N_2$, 30.0%), *m/z* 180 ($C_{12}H_8N_2$, 18.8%), *m/z* 154 ($C_{11}H_8N$, 17.5%) and *m/z* 128 ($C_{10}H_8$, 10.0%).

Finally, elemental analysis of VA (molecular formula $C_{20}H_{24}O_3N_2$, M_r 340.40) obtained the percentages, 70.40% and 7.49% for C and H, respectively. The calculated percentages are 70.57% and 7.11% for C and H, respectively.

II. *Method Development and Optimization of Chromatographic Conditions*

Several HPLC methods have been reported for the determination of VN, especially in biological fluids[2-5] and for the separation and determination of VN in combination with piracetam[6,7]. To our knowledge, only one stability-indicating HPLC assay[8] has been recently reported for the determination of VN in the presence of its hydrolytic product, VA, using a C18-column and mobile phase composed of acetonitrile : 0.01 M ammonium carbonate (70 : 30, % v/v). These chromatographic conditions allowed for good separation. However, VN was eluted at a short retention time of 3.6 min.

The present work aimed to develop a RP/HPLC/UV method suitable for the simultaneous determination of VN and VA, which would be of potential importance in monitoring VN and its metabolite, VA in biological fluids, such as plasma and urine. Accordingly, the chromatographic conditions were investigated to achieve effective separation of VN/VA with adequate retention and to assure resolution from plasma/urine interfering peaks.

The chromatographic conditions, especially the composition of the mobile phase (pH and % organic modifier) were optimized. A mobile phase system consisting of acetate buffer and acetonitrile was tested. The effects of acetonitrile content (within 30 to 70%, v/v) and the pH (ranging from 3 to 7) of the acetate buffer on the chromatographic performance were investigated using a standard mixture of VN and VA (1 μg/mL each). The study indicated that a mobile phase consisting of 30% acetonitrile offered the best chromatographic resolution and column efficiency, and advantageously, appropriate retention of VN and VA was obtained with elution times of 7.15 and 5.06 min, respectively.

Although, apparently, pH 7 offers the best resolution and column efficiency, VA is eluted quickly with a retention time of 3.71 min. A mobile phase of pH 4 provided the best retention of VA (retention time of 5.06 min) with suitable resolution, column efficiency and peak symmetry. Accordingly, a mobile phase of pH 4 was chosen as the most appropriate for the current study. A typical chromatogram for the separation of VN and VA using the proposed procedure is shown in Figure 3.

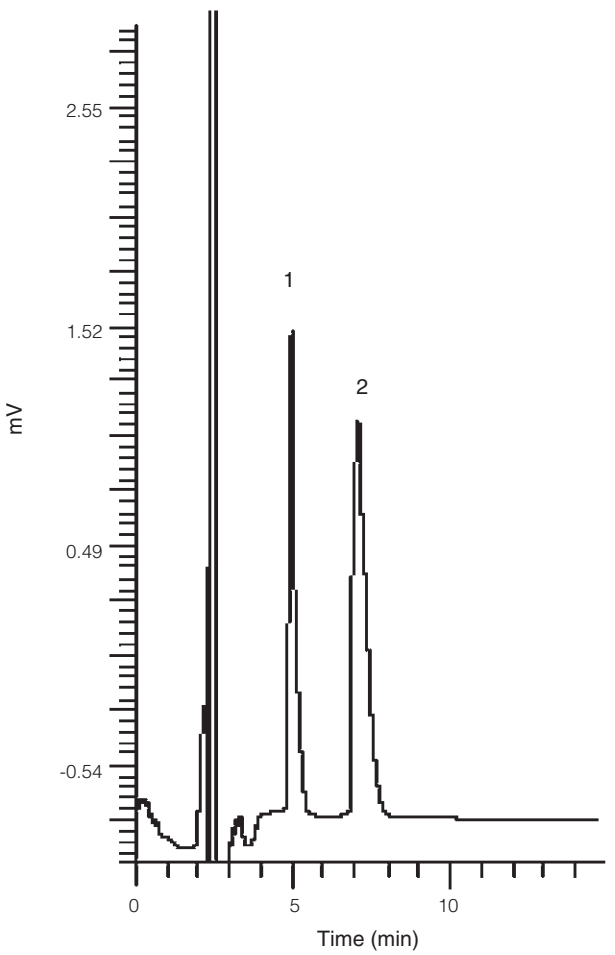

Figure 3. Typical chromatogram of a 50-μL injection of a standard mixture of (1) 1 μg/mL VA and (2) 1 μg/mL VN.

III. *Method Validation*

The method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) requirements[29]. The following validation parameters were addressed: system suitability, linearity, range, accuracy, precision, specificity, limits of detection and quantification, solution stability and robustness.

(I) *System Suitability*

System suitability tests were performed to verify the HPLC system performance. The system suitability parameters include retention time, capacity factor, tailing factor, resolution and number of theoretical plates. The system precision was also assessed based on the relative standard deviation (% RSD, n = 6) of the retention times and peak areas of the working standards. The results are listed in Table 1. The system suitability parameters were within the acceptable limits.

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(II) *Linearity*

The linearity range of the HPLC method for determining VN and VA was evaluated by analyzing a series of standards (n = 8) over the concentration levels of 0.1 - 50 and 0.4 - 50 μg/mL, respectively. Table 1 assembles the analytical performance data, including regression equations computed from calibration graphs, concentration ranges, correlation coefficients along with the standard deviation of the intercept (S_a), the slope (S_b), the standard deviation of residuals ($S_{y/x}$) and percentage relative standard deviation of the slope (S_b, %). Correlation coefficient values (> 0.9995) indicated good linearity. Additionally, percentage relative standard deviations of the slope (S_b %) were found to be less than 1.2%.

Table 1. Analytical parameters for the proposed HPLC method

	VN	VA
System suitability parameters		
Capacity factor	2.71	1.61
Number of theoretical plates	1202	5265
Asymmetry	2.18	1.40
Resolution	3.3	
Repeatability-t_R[a]	0.78	1.78
Repeatability-A[a]	0.88	0.64
Validation		
Concentration range (μg/mL)	0.1 - 50	0.4 - 50
Regression data (n = 8)		
a ± Sa[b]	-8890 ± 16200	225 ± 391
b ± Sb[c] (Sb %)[d]	75200 ± 850.05 (1.13)	20300 ± 19.38 (0.10)
r	0.9995	0.9998
Accuracy and Precision		
Mean % recovery [e] (% RSD)	99.2 (1.9)	100.0 (1.8)
Mean % recovery [f] (% RSD)		101.2 (1.2)
LOD (μg/mL) [g]	0.03	0.08
LOQ (μg/mL) [h]	0.09	0.25

[a] % RSD calculated for six replicates for retention time, t_R and peak area, A.
[b] Standard deviation of the intercept.
[c] Standard deviation of the slope.
[d] Percentage relative standard deviation of the slope.
[e] Mean of five determinations of standard mixtures prepared in VN/VA ratios of 1 : 1 to 1 : 25 at three concentration levels.
[f] Mean of five determinations of VA added to capsule simulated solution at three concentration levels (1 - 5 μg/mL).
[g] Limit of detection.
[h] Limit of quantification.

(III) *Specificity*

The specificity of the developed HPLC method for the determination of VN and/or VA in bulk drug, pharmaceutical preparations and plasma/urine samples was investigated by chromatographic analysis of the following: (1) standard solutions of VN and VA, the specificity was proven by the absence of mutual interference of the eluted chromatographic peaks, *i.e.* the peak eluted at the retention time, 7.15 min was selective for VN measurement and 5.06 min for VA measurement (Figure 3); (2) a solution of co-formulated adjuvants commonly used in sustained-release capsules (hydroxypropylmethylcellulose, sodium carboxymethylcellulose and magnesium stearate), the chromatogram of this solution showed no peak throughout the run time of the HPLC method; (3) assay solutions of the capsules and ampoules, the chromatograms of these solutions showed a single peak at 7.15 min due to VN and the spiking of the VN standard into these test solutions resulted in an increase of the peak area at 7.15 min; (4) solutions of treated-drug-free human plasma/urine control samples, the chromatograms of these solutions showed no interfering peaks eluted at/or near the retention times of either VN or VA; (5) solutions of human plasma/urine control samples spiked with VN and VA, the chromatograms of these solutions showed two peaks at 5.06 and 7.15 min for VA and VN, respectively, *i.e.* no variation was observed in their respective retention times, indicating the selectivity of measurements (Figures 4 and 5).

(IV) *Accuracy and Precision*

The accuracy and precision of the developed HPLC method for the determination of VN and/or VA in bulk drug, pharmaceutical preparations and plasma/urine samples were assessed by assays of five determinations over three concentrations covering the specified working range. The first assay involved simultaneous determinations of VN and VA in standard synthetic mixtures within the concentration ranges of 0.1 - 0.5 μg/mL VN and 0.5 - 5.0 μg/mL VA in ratios 1 : 1 to 1 : 25 VN/VA. In the second assay, VA added in known amounts (1 - 5.0 μg/mL) to formulation excipients (hydroxypropylmethylcellulose, sodium carboxymethylcellulose and magnesium stearate) was measured. The accuracy (% recovery) and precision (RSD %) of the method are given in Table 1. All data indicated that the method was highly accurate and precise for the determination of VN and/or VA and no interference was found from the excipients in the formulation. The results implied that VN can be selectively determined in the presence of VA, with good accuracy and precision, up to a ratio of 1 : 25 (VN : VA). The third assay employed the chromatographic analysis to human plasma/urine samples, which were spiked with standard VN and VA over the concentration ranges of 0.02 - 0.5 and 0.08 - 1.0 μg/mL, respectively. The absolute recoveries of VN and VA were calculated according to the calibration data of standard

solutions. The % recovery ranged from 98 to 102% and from 99 to 102% for VN and VA, respectively and the precision (overall SD) ranged from 1.4 to 1.8 and from 1.6 to 2.3, for VN and VA, respectively (Table 2).

(V) *Limits of Detection and Quantification*

The limits of detection (LOD) and quantification (LOQ) were determined at signal to noise ratios of 3 : 1 and 10 : 1, respectively. The data are given in Table 1.

(VI) *Robustness*

The robustness of the proposed HPLC method was tested by investigating the effect of slight variations in the mobile phase (% acetonitrile and pH) on the capacity of the column to retain and resolve VN and VA. Changing the pH of the mobile phase by ± 0.1 pH unit or % acetonitrile by ± 2% (v/v) did not significantly affect the chromatographic performance (k′ and resolution). The % RSD of the measured peak areas after the studied variations did not exceed 2%.

(VII) *Stability Tests*

The stability of standard solutions of VN and VA in methanol, after storing at 4°C for 48 hours, was examined using chromatographic measurements. The recoveries were within 98 to 102%. Freeze-thaw stability was assessed by subjecting plasma/urine samples, spiked with VN/VA at a concentration of 1 μg/mL to three freeze-thaw cycles. Each cycle consisted of removing

Table 2. Accuracy and precision for the HPLC assay of spiked plasma and urine samples

VN		VA	
Spiked (μg/mL)	Mean % Recovery[a] ± SD	Spiked (μg/mL)	Mean % Recovery[a] ± SD
Plasma samples			
0.02	97.92 ± 1.4	0.08	99.9 ± 2.8
0.05	97.9 ± 1.3	0.2	101.2 ± 2.2
0.1	101.7 ± 1.4	0.4	99.1 ± 1.5
0.2	97.7 ± 0.7	0.8	101.0 ± 2.3
0.5	100.9 ± 2.1	1.0	98.9 ± 2.5
	Grand mean (% Recovery): 99.2		Grand mean (% Recovery): 100.0
	Overall SD: 1.4		Overall SD: 2.3
	SAE: 0.32		SAE: 0.51
	Confidence Limit: 0.57		Confidence Limit: 0.89
	Accuracy: 99.2 ± 0.57		Accuracy: 100.0 ± 0.89
Urine samples			
0.02	100.2 ± 2.0	0.08	101.6 ± 1.6
0.05	99.3 ± 2.7	0.2	98.2 ± 1.6
0.1	100.1 ± 1.2	0.4	99.9 ± 2.2
0.2	100.4 ± 1.6	0.8	102.1 ± 1.2
0.5	102.1 ± 1.2	1.0	101.0 ± 1.2
	Grand mean (% Recovery): 100.4		Grand mean (% Recovery): 100.6
	Overall SD: 1.8		Overall SD: 1.6
	SAE: 0.4		SAE: 0.4
	Confidence Limit: 0.71		Confidence Limit: 0.62
	Accuracy: 100.4 ± 0.71		Accuracy: 100.6 ± 0.62

[a] Mean % recovery of five determinations.
SAE: Standard analytical error.

the samples from the freezer, thawing them unassisted to room temperature, keeping at room temperature for 2 h and re-freezing at -20°C. The stability experiment confirmed that plasma/urine samples were stable through three freeze-thaw cycles.

IV. *Applications*

(I) *Determination of VA (Degradation Product/Impurity) at Trace Level in VN*

The validated HPLC method was applied for the determination of VA at trace levels in VN. The mean % recoveries of VA ranged from 98 to 102% (% RSD ranged from 0.8 to 1.3) at starting levels at 10% down to 0.07% (w/w).

(II) *Analysis of Commercial Dosage Forms*

The proposed HPLC method was applied for the determination of VN in commercial dosage forms, including capsules and ampoules. The methods gave satisfactory precision and recovery data, which were in good agreement with the label claims (Table 3). The results were compared statistically with those obtained by the reported derivative ratio spectrophotometric method[8] using Student's *t*-test and the *F*-variance ratio test at 95% confidence level (Table 3). There was no significant difference between the calculated and theoretical values at $p = 0.05$, demonstrating that the proposed method was as accurate and precise as the reported one.

(III) *Detection of VA, Degradation Product/Impurity, in Oxybral® Capsules and Ampoules*

The proposed HPLC method was applied for the detection/determination of VA in VN commercial capsules and ampoules. Solutions of Oxybral® capsules and ampoules were prepared at concentrations ranging from 100 to 1000 μg/mL VN and analyzed for VA content. The results of analysis referred to the presence of VA at a level of 0.075 % (w/w) in Oxybral® capsules. No VA was detected in Oxybral® commercial ampoules.

Table 3. Assay results for the determination of VN in pharmaceutical preparations

Preparations	% Recovery[a] (% RSD)	
	HPLC Method	Reference Method[b]
Oxybral® capsules[c]	99.94 (1.2) t = 0.60 , F = 1.07[e]	99.5 (1.3)
Oxybral® ampoules[d]	100.4 (0.4) t = 1.14 , F = 2.4[e]	100.0 (0.6)

[a] Mean % recovery of five determinations.
[b] Reference (8).
[c] Oxybral® capsules were labeled to contain 30 mg of VN per capsule.
[d] Oxybral® ampoules were labeled to contain 15 mg of VN per 2 mL.
[e] Tabulated *t*-value for $p = 0.05$ and 8 degree of freedom is 2.306; tabulated *F*-value for $p = 0.05$ and $f_1 = f_2 = 4$ is 6.38.

(IV) *Applications to Human Plasma and Urine Samples*

The detection/determination of VA in human plasma/urine samples has not been reported yet. Most of the previous researches have been focused on the quantification of VN only and are based on the extraction clean-up procedure which separates VN only rather than its metabolite, VA. Several HPLC[2-5] and GC[11-13] methods have been reported for the determination of VN in plasma samples. Of the most important points, concisely mentioned were the following: the analytical sensitivity for the determination of concentrations as low as 5 ng/mL VN spiked into control plasma samples has been achieved[2,3]; few studies have been interested in the analysis of plasma samples collected from volunteers treated with VN[2,4,11]; treatment of plasma samples has been based on tedious extraction procedure involving one, two[2] or even three steps[11]; and none of these studies discussed the interference/or determination of VN′s metabolites.

The present work was primarily focused on the employment of the validated HPLC method for the determination of VN and its metabolite, VA, in biological samples (plasma/urine). The specificity, accuracy and precision of the HPLC method had been verified by spiking experiments discussed above (Table 2).

Sample pre-treatment included a clean-up step of protein deposition by ethanol. This was particularly simple, easy to handle and allowed for fast analysis. Moreover, both VN and VA could be measured simultaneously without a tedious extraction procedure. Preliminary experiments were carried out to choose the working solvent, which can be used as a deproteinizing agent of plasma and urine samples. Acetonitrile, methanol and ethanol were examined, of which ethanol was selected as the most appropriate as it showed the maximum % recovery values, as compared to standards containing the same theoretical concentration of the compound being analyzed. The clean-up step, in addition to the appropriate chromatographic conditions, allowed for adequate separation and resolution of VN and VA peaks from plasma/urine peaks (Figures 4 and 5). Furthermore, the reconstitution of residue, obtained from 1 mL of plasma/urine, in 200 μL of mobile phase permitted a pre-concentration step (5-fold). This improved the sensitivity of the quantification of VN and VA to concentration levels of 20 and 80 ng/mL, respectively.

Although the absorptivities of VN and VA at 225 nm is ~ 2-3 folds higher than that at 270 nm, the latter was chosen as the most appropriate for the present work as it

minimized the interference of plasma/urine peaks during VA determination. The % recovery of VA in plasma/urine might reach 130% if 225 nm was chosen for detection.

Plasma/urine samples were taken from a healthy human subject after the oral administration of two Oxybral® capsules (each labeled to contain 30 mg of VN). The concentrations of VN and VA were calculated with reference to the calibration data by peak area measurements. Only VN was observed in the plasma samples. The peak plasma concentration was 102 ng/mL

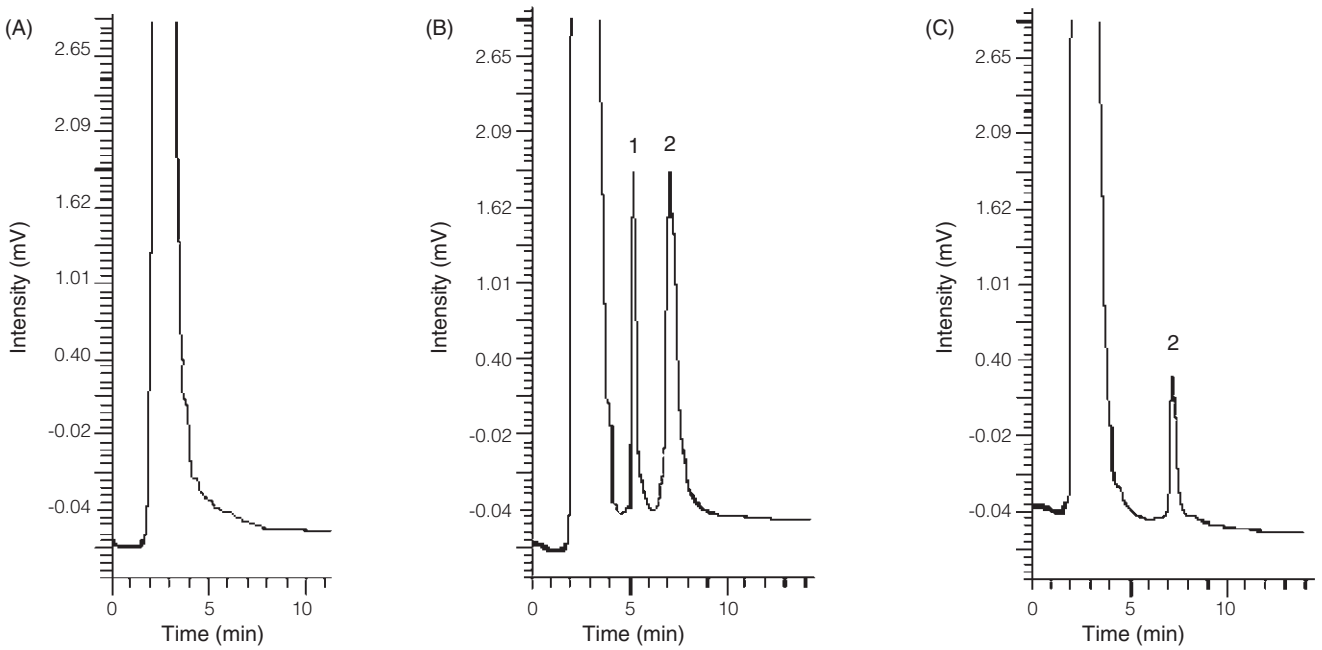

Figure 4. Typical chromatograms of (A) control plasma, (B) plasma sample spiked with VA and VN (1 μg/mL, each) and (C) plasma obtained from the volunteer at 2 h after oral administration of 60 mg of VN. 1 = VA; 2 = VN.

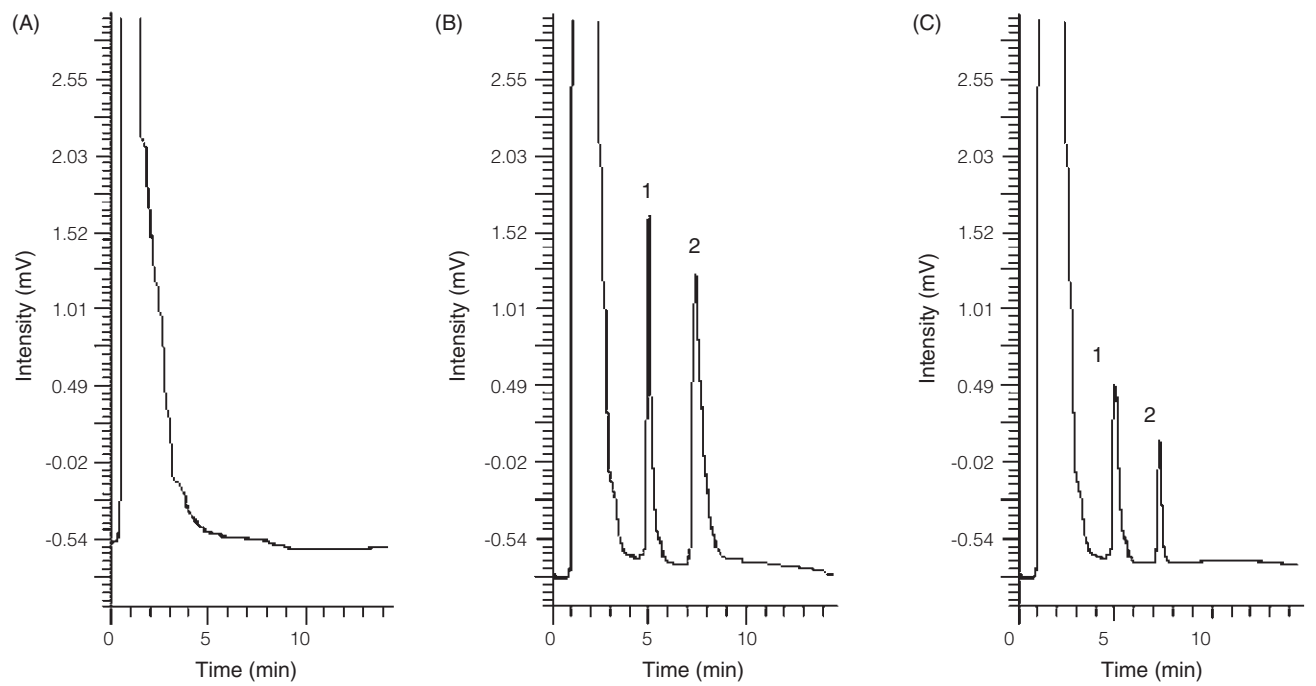

Figure 5. Typical chromatograms of (A) control urine, (B) urine sample spiked with VA and VN (1 μg/mL, each) and (C) urine obtained from the volunteer at 4 h after oral administration of 60 mg of VN. 1 = VA; 2 = VN.

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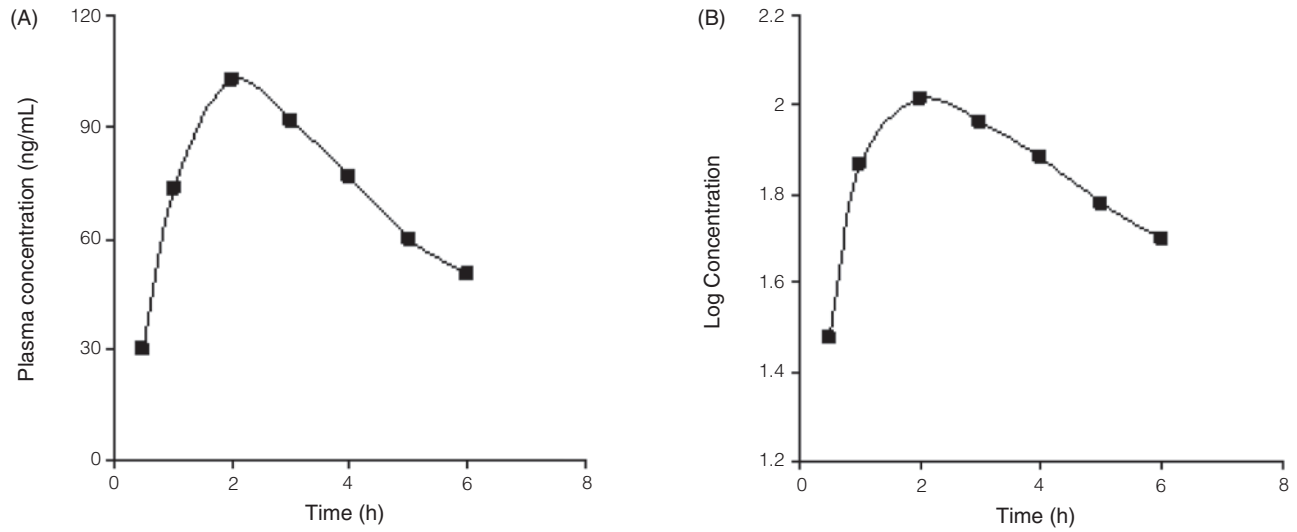

Figure 6. (A) Plasma concentrations and (B) Log plasma concentrations of VN after oral administration of two Oxybral ® capsules (60 mg of VN).

Table 4. The amounts of VN and its metabolite (VA) found in the urine of a healthy volunteer after oral administration of two Oxybral® capsules

Time (h)	Amount of VN excreted (μg)	Amount of VA excreted (μg)
2	1.1	1.5
4	3.6	5.9
6	3.7	9.3
8	3.4	11.1

at 2 h. Figure 6A displays the profile of plasma concentration of VN *versus* time. The elimination constant, K′, derived from the log plot (Figure 6B) was found to be 0.18 h^{-1}, and accordingly, the half life time of VN was 3.85 h. The data obtained was closely related to that reported on sustained-release formulations[11]. Both VN and VA were observed in the urine samples. The amounts of VN and VA excreted were listed in Table 4.

Figure 4 displays the chromatograms of (A) control plasma, (B) control plasma sample spiked with VN and VA, 1 μg/mL each, and (C) plasma sample taken at 2 h. No signal appeared in the blank plasma at the retention times of VN and VA (A). Also, the chromatographic peaks of VN and VA, spiked into plasma sample (B) appeared at the same retention times as those of standards. Analysis of plasma sample collected at 2 h revealed the VN peak only. Figure 5 shows the chromatograms of (A) control urine, (B) control urine sample spiked with VN and VA, 1 μg/mL of each, and (C) urine sample collected at 4 h. No signal appeared in the blank urine at the retention times of VN and VA (A). Also, the chromatographic peaks of VN and VA spiked into urine sample (B) showed the same retention times as those of standards. Analysis of urine sample collected at 4 h revealed both VN and VA peaks.

The identity of VA (metabolite) observed in urine samples was confirmed through peak enrichment in the spiking experiments of the assay solution (urine) with VA standard. No shift in the retention time was observed. Moreover, confirmation of the identity of VA was done using HPLC-system coupled with photodiode array detector to supply the absorption UV spectra for the peaks eluted for VA standard solution and urine assay solution. Figure 7 displays the photodiode array absorption spectra of VA standard solution and urine assay solution. The wavelengths of the absorption maxima and minima were identical for both. This finding confirmed the identity of VA as a metabolite of VN, excreted in human urine.

CONCLUSIONS

The HPLC method established in this work provided a simple, specific, accurate and precise way for determining VN and/or VA. Being a separation method, the developed procedure presented selectivity advantage over non-selective spectrophotometric stability-indicating reports for VN[8,15,16]. For the linearity ranges, the proposed method was more sensitive and reliable than the previous stability-indicating methods[8,15,16]. The proposed HPLC method provided a worthy addition to the scientific literature of VN in view of the following points: (a) direct and fast analysis, (b) it offers advantageous application in monitoring and simultaneous determination of VN and VA in human plasma/urine samples, following a simple and fast sample clean-up step. No analytical method has been reported for the

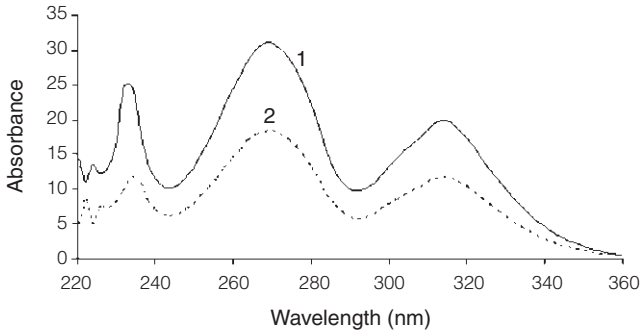

Figure 7. (1) Absorption spectra of standard VA solution and (2) urine assay solution after 4 h obtained by HPLC system coupled with photodiode array detector.

determination of VA in human plasma/urine samples (*in-vivo*), (c) the identity of VA, as a metabolite of VN observed in human urine samples, was confirmed using UV spectra acquired by HPLC-photodiode array system, (d) determination of VA as an impurity/degradant in VN samples down to 0.07% level, and (e) the proposed procedure was validated and proved suitable for the estimation of VN in commercial pharmaceutical formulations.

REFERENCES

1. Sweetman, S. C. 2007. "Martindale: The Complete Drug Reference". 35th ed. pp. 2186. The Pharmaceutical Press. London, U.K.
2. Dal Bo, L., Ceriani, G. and Broccali, G. 1992. Determination of vincamine in human plasma by high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. 573: 158-162.
3. Smyth, M. R. 1986. Determination of vincamine in plasma by high-performance liquid chromatography with voltammetric detection. Analyst 111: 851-852.
4. Dubruc, C., Caqueret, H. and Bianchetti, G. 1981. Determination of vincamine in human plasma using automated high-performance liquid chromatography. J. Chromatogr. 204: 335-339.
5. Pietta, P., Rava, A. and Catenacci, E. 1981. High-performance liquid chromatographic determination of vincamine. J. Chromatogr. 210: 149-153.
6. El-Saharty, Y. S. I. 2008. Simultaneous determination of piracetam and vincamine by spectrophotometric and high-performance liquid chromatographic methods. J. AOAC Int. 91: 311-321.
7. El-Gindy, A., Sallam, S. and Abdel-Salam, R. A. 2006. Liquid chromatography and chemometric-assisted spectrophotometric methods for the simultaneous determination of vincamine with piracetam and diflunisal with naproxen. Bull. Fac. Pharm. Cairo Univ. 44: 279-298.
8. Shehata, M. A. M., El Sayed, M. A., El Tarras, M. F. and El Bardicy, M. G. 2005. Stability-indicating methods for determination of vincamine in presence of its degradation product. J. Pharm. Biomed. Anal. 38: 72-78.
9. Amato, A., Cavazzutti, G., Gagliardi, L., Profili, M., Zagarese, V., Chimenti, F., Tonelli, D. and Gattavecchia, E. 1983. Determination of vincamine by high-performance liquid chromatography with dual-wavelength ultraviolet detection. J. Chromatogr. 270: 387-391.
10. Sriewoelan, S. and Bres, J. 1985. Gas chromatographic determination of vincamine using semi-capillary column. Acta Pharm. Indones. 10: 46-49.
11. Michotte, Y. and Massart, D. L. 1985. Capillary gas chromatographic determination of vincamine in plasma. J. Chromatogr. 344: 367-371.
12. Devaux, P., Godbille, E. and Viennet, R. 1979. Quantitative determination of vincamine in human plasma by gas chromatography-mass spectrometry. Recent Dev. Mass Spectrom. Biochem. Med. 2: 191-203.
13. Kinsun, H. and Moulin, M. A. 1977. Gas chromatographic method for the determination of vincamine in blood. J. Chromatogr. 144: 123-126.
14. Iven, H. and Siegers, C. P. 1977. Fluorometric assay and pharmacokinetics of vincamine in rats. Arzneimittelforschung 27: 1248-1254.
15. El-Bardicy, M. G., Lotfy, H. M., El-Sayed, M. A. and El-Tarras, M. F. 2008. Smart stability-indicating spectrophotometric methods for determination of binary mixtures without prior separation. J. AOAC Int. 91: 299-310.
16. El-Sayed, M. A. 2007. Stability indicating chemometric methods for determination of vincamine. Bull. Fac. Pharm. Cairo Univ. 45: 27-37.
17. Ganescu, I., Papa, I., Ganescu, A., Bratulescu, G. and Cirtina, D. 2002. Thiocyanatochromic complexes in analytical chemistry. Vincamine determination. Acta Chim. Slov. 49: 181-185.
18. Ganescu, I., Mircioiu, C., Papa, I., Ganescu, A., Aldea, V., Chirigiu, L. and Barbu, A. 2001. Thiocyanatoplatinum complexes in analytical determination of vincamine. Farmacia (Bucharest, Romania) 49: 62-68.
19. Bayer, J. 1972. Collective UV-spectrophotometric determination of vincamine and vincine. Acta Pharm. Hung. 42: 249-253.
20. Aboutabl, E. A., El-Azzouny, A. A. and Afifi, M. S. 1998. PMR assay of vincamine and formulations. Pharm. Acta Helv. 73: 193-197.
21. Beltagi, A. M. 2008. Development and validation of an adsorptive stripping voltammetric method for the quantification of vincamine in its formulations and human serum using a Nujol-based carbon paste electrode. Chem. Pharm. Bull. 56: 1651-1657.
22. Chen, Q., Miao, J. and Ma, J. 1995. Determination of vincamine in tissue culture of Vinca minor L. by TLC scanning method. Zhongguo Yaoke Daxue Xuebao 26: 350-352.
23. Proksa, B. and Grossmann, E. 1991. High performance

liquid chromatographic determination of alkaloids from Vinca minor L. Phytochem. Anal. 2: 74-76.

24. Li, P., Yu, W., Xu, S. and Zhou, Y. 1991. Application of photodiode array detector in analysis of vincamine by HPLC. Fenxi Ceshi Tongbao 10: 42-45.
25. Li, P., Yu, W., Zhang, R. and Zhou, Y. 1990. Determination of vincamine in Vinca minor and Vinca major by reversed-phase high performance liquid chromatography with the photodiode array detector. Sepu 8: 139-142.
26. Vereczkey, L., Tamas, J., Gzira, G. and Szporny, L. 1980. Metabolism of vincamine in the rat in vivo and in vitro. Arzneimittelforschung 30: 1860-1865.
27. Maya, M. T., Pais, J. P., Araujo, H. M. and Morais, J. A. 1996. Determination of apovincaminic acid in human plasma by high-performance liquid chromatography. J. Pharm. Biomed. Anal. 14: 617-622.
28. Chen, J., Cai, J., Tao, W., Mei, N., Cao, S. and Yiang, X. 2006. Determination of apovincaminic acid in human plasma by high-performance liquid chromatography using solid-phase extraction and ultraviolet detection. J. Chromatogr. B 830: 201-206.
29. ICH, Q2A (R1), 2005. Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonisation, Geneva, November 2005. http://www.ich.org/LOB/media/MEDIA417.pdf.