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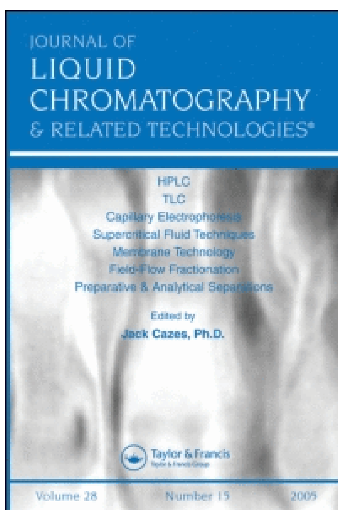

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A Rapid and Sensitive HPLC Method for the Determination of Venlafaxine and O-Desmethylvenlafaxine in Human Plasma with UV Detection

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A Rapid and Sensitive HPLC Method for the Determination of Venlafaxine and O-Desmethylvenlafaxine in Human Plasma with UV Detection

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ABSTRACT

A rapid and sensitive high performance liquid chromatographic (HPLC) method was developed and validated for the analysis of venlafaxine (VEN) and O-desmethylvenlafaxine (ODV) in human plasma. The analytes were extracted from human plasma by using liquid–liquid extraction technique. Citalopram (CTP) was used as the internal standard. A Zorbax XDB C-18 column provided chromatographic separation of analytes followed by UV detection with wavelength at 229 nm. The linear

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range for both analytes was 5 ng/mL to 1000 ng/mL with precision and accuracy of <10%. Total elution time was 12 min.

Key Words: Venlafaxine; ODV; Plasma; Citalopram.

INTRODUCTION

Venlafaxine (VEN), 1-2-(dimethylamino)-1-(4-methoxyphenyl) ethyl-cyclohexanol hydrochloride, is a novel antidepressant. The pharmacologic action of VEN appears to involve the inhibition of neuronal uptake of catecholamines and serotonin. On the basis of clinical trials, this drug appears to lack many of the side effects associated with tricyclic antidepressants.

Venlafaxine has several metabolites, one of which is biologically active. About 1% of VEN is desmethylated to *N*-desmethylvenlafaxine, 1-(2-(methyl-amino)-1-(4-methoxyphenyl) ethyl) cyclohexanol; 16% becomes *N*,O-didesmethylvenlafaxine, 4-(2-(methylamino)-1-(1-hydroxycyclohexyl)ethyl) phenol; and 56% is metabolized to O-desmethylvenlafaxine (ODV), 4-(2-dimethylamino)-1-(1-hydroxycyclohexyl) ethyl) phenol which, unlike the others, has an activity profile on monoamine transporters similar to VEN. Therefore, both VEN and ODV levels in plasma are important pharmaco-kinetic and pharmacodynamic parameters in assessing their efficacy.

Several methods have been reported in the literature for monitoring plasma levels of VEN and ODV.[1–15] The techniques used in these methods include mass spectrometric detection,[1,2] Capillary electrophoresis.[3–9] High performance liquid chromatography (HPLC) with fluorimetric detection,[10,11] HPLC with coulometric detection,[12] and HPLC with UV detection.[13–15]

This paper describes a simple and rapid HPLC method with UV detection for the determination of VEN and ODV in human plasma. This paper also describes the validation of the analytical procedure.

EXPERIMENTAL

Chemicals and Reagents

The reference standards of VEN, and Citalopram HBr (CTP) were obtained from Wockhardt Research Centre (Aurangabad, India). O-Desmethylvenlafaxine was synthesized at Wockhardt Research Centre. High performance liquid chromatography quality water was prepared from an Elga water purification system. High performance liquid chromatography grade methanol, isopropyl alcohol, *n*-hexane, chloroform, and 1-octanesulphonic

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acid sodium salt were purchased from E. Merck Ltd. (Mumbai, India). Analytical reagent (AR) grade orthophosphoric acid, potassium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Qualigen fine chemicals (Mumbai, India). Drug free (blank) heparinized human plasma was obtained from Wockhardt Research Centre (Aurangabad, India).

Instruments

The HPLC system consisted of a pump (Model Constametric 3500, Thermo Separation products, USA), an autosampler (Model AS 3000, Thermo Separation Products, USA), UV variable wavelength detector (Model UV 1000, Thermo Separation Products, USA), an interface (Model SP4510, Thermo Separation Products, USA), and a data station with PC 1000 software version 3.5.1. The analytical column used was Zorbax XDB-C18, 4.6 mm × 7.5 cm, 5 μm (Agilent Technologies, USA).

Chromatographic Conditions

The mobile phase was a mixture of 62% water containing 0.05 M potassium dihydrogen phosphate, 28% methanol, and 10% isopropyl alcohol. It was filtered and degassed by passing through 0.45 μm membrane filter paper (Millipore, USA). The flow-rate was 1.0 mL/min and the column temperature was 50°C. The total elution time was 12 min. The analytes were detected and quantified by UV detection at a wavelength of 229 nm.

Preparation of Standard Solutions

Stock solutions of VEN and internal standard CTP were prepared in water at free base concentration of 1000 μg/mL. Secondary and working standard solutions were prepared by dilution with water. Stock solution of ODV was prepared by dissolving it in 10 mL of methanol and diluting with water at free base concentration of 1000 μg/mL. Secondary and working standards were prepared by dilution with water.

Extraction Procedure

To a screw capped 15 mL glass test tube, 1 mL plasma and 50 μL of the internal standard (0.8 μg/mL) were added. The mixture was shaken on a

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vortex mixer for 30 sec. The pH of plasma was made alkaline by adding 0.2 mL of 0.5 M disodium hydrogen phosphate and 5 mL mixture of chloroform and *n*-hexane (70 : 30) was added. The mixture was shaken on a vortex mixer for 2 min and centrifuged for 10 min at 3000 rpm. About 4 mL of the organic layer was transferred to another 15 mL glass stoppered centrifuge tube containing 0.4 mL orthophosphoric acid (0.01% v/v). The mixture was shaken on a vortex mixer for 2 min and centrifuged for 10 min at 3000 rpm. The aqueous layer containing the drug was evaporated under a stream of nitrogen for 10 min at ambient temperature to evaporate any dissolved organic solvents. The residual aqueous phase was then transferred to an injection vial and 100 μL of the extract was injected into the chromatographic system.

Specificity and Linearity

The specificity of the method was evaluated with regards to interference due to the presence of endogenous substances in the extracted plasma. For the same, plasma pouches were randomly chosen for experimentation. Six different plasma samples were analysed. To establish the range of linearity for VEN and ODV, the concentrations 5, 10, 25, 50, 100, 200, 300, 500, 750, and 1000 ng/mL of VEN and ODV in plasma were used.

Limit of Quantification

Limit of quantification was defined as the lowest concentration at which the precision expressed by relative standard deviation (RSD) is less than 20% and accuracy expressed by relative difference of the measured and true value was also less than 20%. To determine the limit of quantification, the plasma solutions containing 1, 2, 3, 4, 5, 6, 10, 25, 50, 100, 200, 300, 500, 750, and 1000 ng/mL of VEN and ODV in plasma were prepared and extracted as per the procedure and injected into the chromatographic system. The values were back calculated from the standard curve.

Recovery

The recoveries of VEN, ODV, and CTP in the extraction procedure were determined by comparing the peak areas obtained from an extracted sample spiked with known amounts of VEN, ODV, and CTP with those obtained from the pure compounds of the same concentrations in the solutions. The

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recoveries of VEN and ODV were determined at the concentrations of 10, 100, and 750 ng/mL. The recovery of CTP was determined at 400 ng/mL.

Precision and Accuracy

To assess the accuracy and precision of the method, intra-day and inter-day (day 1, 2, 3, 4, and 5) measurements of VEN and ODV were completed and the relative error (% RE) and relative standard deviation (% RSD) for replicate samples ($n = 5$ for intra-day, $n = 25$ for inter-day) at concentration 5, 10, 100, and 750 ng/mL was determined. Both intra-day and inter-day samples were back calculated from calibration curves.

Accuracy expressed as % RE = [(mean/theoretical)−1] ∗ 100. Precision expressed as % RSD = (standard deviation/mean) ∗ 100.

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Data Calculation/Data Acceptability Criteria

Data calculation was accomplished with an online computer system. Calculation of drug concentration in unknown samples was based on a weighted ($1/x^2$) least squares regression of plasma calibrator concentrations against the peak area ratios. The peak area ratios were obtained by dividing the peak area of VEN and ODV by the peak area of the internal standard. Each calibration curve must have a coefficient of determination of at least 0.97. Both the relative error and the relative standard deviation of each level of the linearity curves and quality control samples must be $<15\%$ of the theoretical value except for those at the limit of quantification, which must be $<20\%$.

RESULTS

Chromatography and Specificity

High performance liquid chromatographic profiles for blank human plasma and plasma spiked with VEN, ODV, and CTP are shown in Fig. 1(A) and (B), respectively. The peaks of VEN, ODV, and CTP were well resolved, as shown in Fig. 1(B). No endogenous interfering peaks were found in blank plasma at their retention times [Fig. 1(A)], indicating a good selectivity for the chromatographic method.

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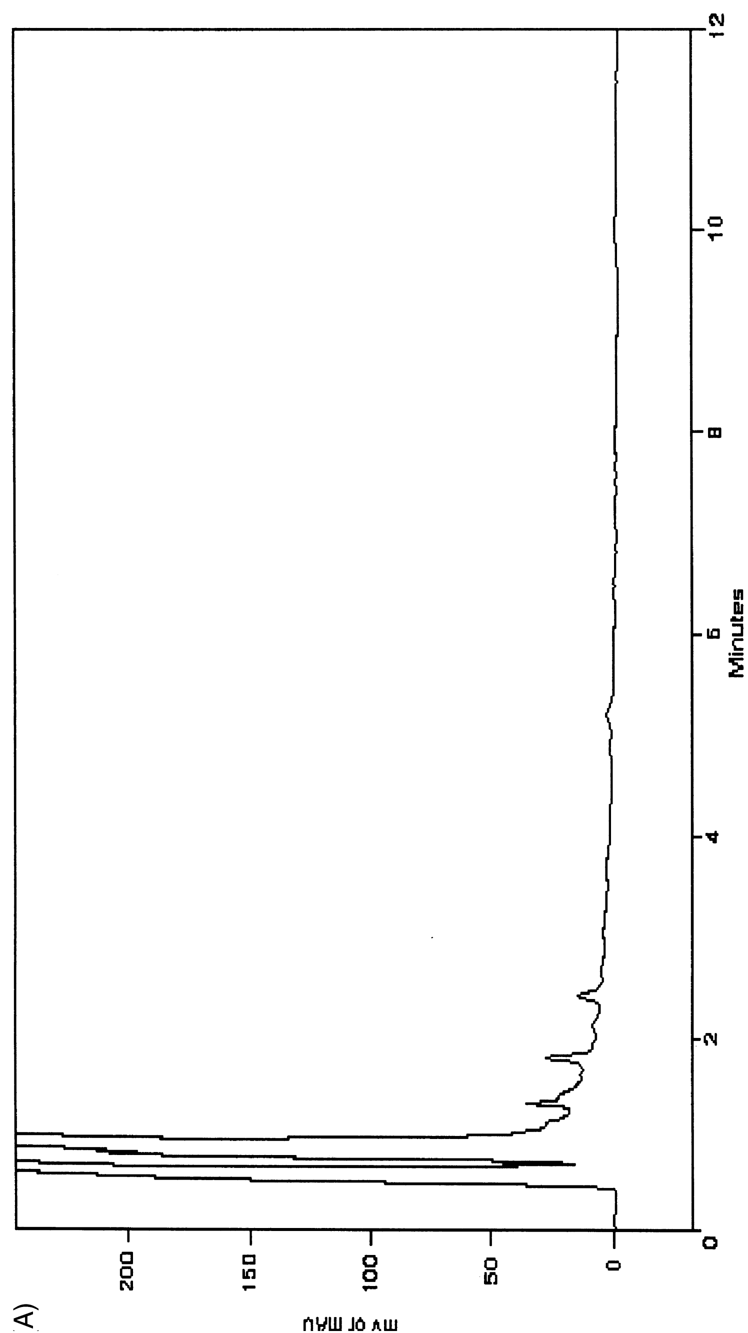

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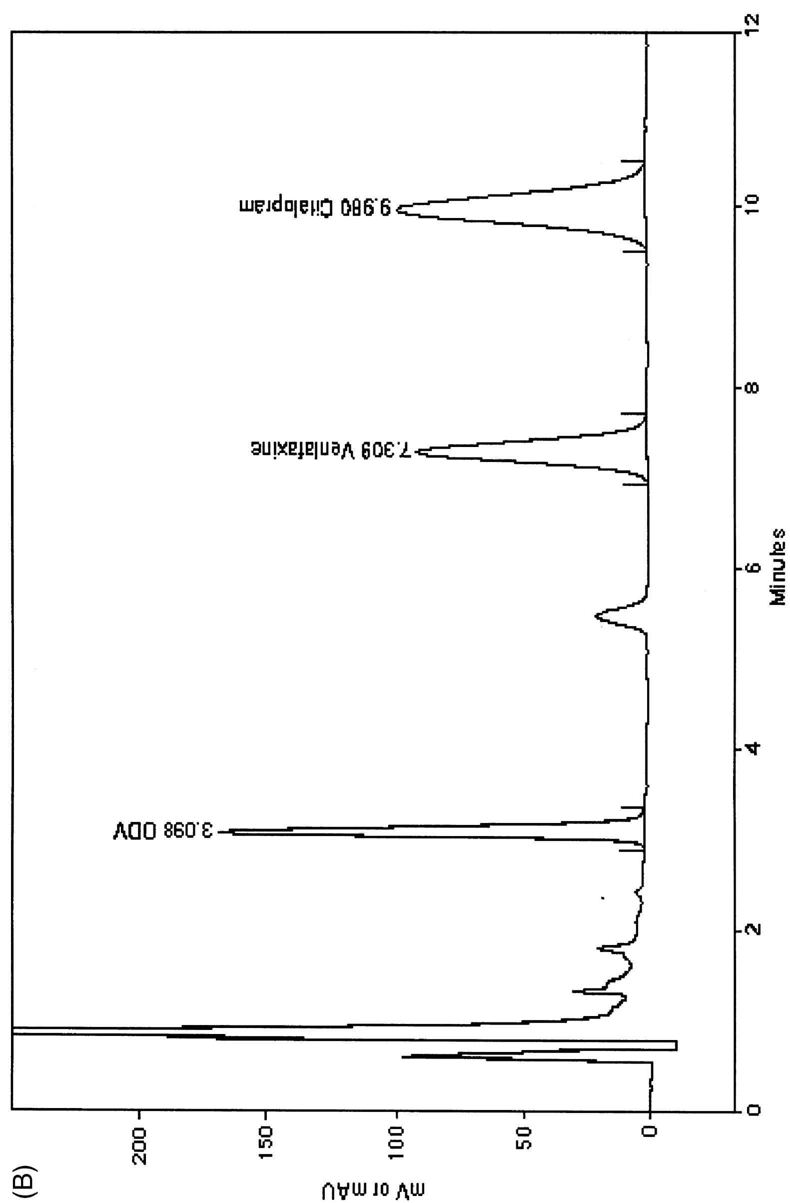

Figure 1. (A) Representative chromatogram of blank human plasma. (B) Representative chromatogram of plasma spiked with VEN (200 ng/mL), ODV (200 ng/mL), and CTP (400 ng/mL).

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Limit of Quantification

It was found that below 5 ng/mL, the back calculated values failed to meet the acceptance criteria. Hence, 5 ng/mL level was extracted five times and injected. It was found that relative standard deviation was 8.3% and the relative error was 5.0% at this concentration. So 5 ng/mL was established as the lowest limit of quantification.

Standard Curve Characteristics, Precision, and Accuracy

The calibration curves were linear in the studied range. The slope, intercept, and correlation coefficient (mean ± standard deviation) of standard curves ($n = 5$) for VEN was 0.00322 ± 0.00013, 0.00255 ± 0.00338, and 0.99938 ± 0.00033, respectively. Similarly, for ODV, the slope, intercept, and correlation coefficient was 0.00250 ± 0.00015, 0.00614 ± 0.00196, and 0.99916 ± 0.00062, respectively.

The back calculated concentration values for each level of VEN and ODV were obtained from each calibration curve used during method validation. The obtained mean ($n = 5$) back calculated values, as well as, the precision and accuracy estimations for each concentration level are summarized in Tables 1 and 2. The RSD and RE for VEN were 6.3% and −7.46%, respectively, and for ODV were 7.44% and −9.73%, respectively. The precision and accuracy

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Table 1. Standard curve characteristics for VEN.

Target value (ng/mL)	Mean (ng/mL, $n = 5$)	SD	RE (%)	RSD (%)
5	5.03	0.10	0.52	2.08
10	9.97	0.48	−0.32	4.78
25	23.26	1.47	−7.46	6.30
50	53.88	1.23	7.19	2.28
100	103.59	2.69	3.47	2.60
200	198.23	5.00	−0.89	2.52
300	300.61	13.07	0.20	4.35
500	489.64	14.88	−2.12	3.04
750	753.49	30.55	0.46	4.05
1,000	974.56	17.73	−2.61	1.82

Note: SD, standard deviation; RE, relative error; RSD, relative standard deviation.

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Table 2. Standard curve characteristics for ODV.

Target value (ng/mL)	Mean (ng/mL, $n=5$)	SD	RE (%)	RSD (%)
5	4.85	0.24	−3.14	4.94
10	9.95	0.74	−0.50	7.44
25	22.78	0.93	−9.73	4.07
50	53.30	1.77	6.19	3.31
100	102.05	1.83	2.01	1.80
200	207.24	1.10	3.49	0.53
300	295.40	21.82	−1.56	7.39
500	480.39	10.37	−4.08	2.16
750	747.91	32.01	−0.28	4.28
1,000	970.26	11.06	−3.06	1.14

Note: SD, standard deviation; RE, relative error; RSD, relative standard deviation.

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for determination of VEN and ODV was evaluated for replicate analysis of the quality control samples at four different concentrations within the standard curve range (Table 3). Intra-day precision and accuracy for VEN and ODV was found to be less than 12% and inter-day precision and accuracy was less than 8%.

Stability Study

Freeze Thaw Stability

Freeze thaw stability was carried out at three different concentrations (10, 100, and 750 ng/mL). The plasma samples were stored at −40°C and subjected to three freeze-thaw cycles. The results are represented in ng/mL from initial to after third cycle as (mean ± standard deviation). For concentrations of 10 ng/mL, 100 ng/mL, and 750 ng/mL of VEN, the results found were 8.9 ± 0.39, 96.34 ± 2.02, and 732.56 ± 19.58. For concentrations of 10 ng/mL, 100 ng/mL, and 750 ng/mL of ODV, the results found were 9.45 ± 0.34, 93.05 ± 4.71, and 653.59 ± 14.01. The concentrations found were well within the acceptable limit ±15% of the nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

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Table 3. Precision and accuracy for VENL and ODV.

	Target concentration (ng/mL)							
	VEN				ODV			
	5	10	100	750	5	10	100	750
				Day 1[a]				
Mean	5.21	9.49	101.08	765.52	4.92	9.43	102.16	728.23
SD	0.21	0.59	2.94	9.96	0.55	0.89	6.20	45.49
% RE	4.29	−5.14	1.08	2.07	−1.65	−5.70	2.16	−2.90
% RSD	4.02	6.19	2.91	1.3	11.14	9.48	6.07	6.25
				Day 2[a]				
Mean	4.84	9.42	95.61	760.72	4.84	9.27	99.04	734.05
SD	0.24	0.47	3.96	56.40	0.26	0.31	8.20	70.14
% RE	−3.15	−5.81	−4.39	1.43	−3.11	−7.30	−0.96	−2.13
% RSD	4.90	4.94	4.15	7.41	5.33	3.32	8.28	9.55
				Day 3[a]				
Mean	5.55	10.45	105.27	769.78	5.06	9.80	102.96	772.36
SD	0.10	0.67	3.00	20.76	0.14	0.66	4.86	40.80

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% RE	−11.10	−4.47	−5.27	2.64	1.14	−2.04	2.96	2.98
% RSD	1.73	6.41	2.85	2.70	2.71	6.73	4.72	5.28
				Day 4[a]				
Mean	5.13	9.60	99.73	768.38	4.90	9.84	100.21	773.88
SD	0.31	0.73	9.35	59.77	0.31	0.54	10.50	35.53
% RE	2.66	−3.96	−0.27	2.45	−2.10	−1.57	0.21	3.18
% RSD	5.97	7.57	9.37	7.78	6.23	5.52	10.48	4.59
				Day 5[a]				
Mean	4.76	10.04	99.24	711.57	4.76	9.22	98.78	745.30
SD	0.16	1.00	6.92	10.37	0.28	1.01	6.05	8.12
% RE	−4.77	0.44	−0.76	−5.12	−4.77	−7.81	−1.22	−0.63
% RSD	3.45	10.00	6.98	1.46	5.95	10.94	6.13	1.09
				Overall[b]				
Mean	5.10	9.80	100.19	755.19	4.90	9.51	100.63	750.76
SD	0.35	0.76	6.18	41.68	0.32	0.71	6.99	45.19
% RE	2.02	−2.00	0.19	0.69	−2.10	−4.88	0.63	0.10
% RSD	6.85	7.79	6.17	5.52	6.56	7.52	6.95	6.02

[a] $n = 5$ determinations.
[b] $n = 25$ determinations.

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Processed Sample Stability

Processed sample stability was carried out at three different concentrations (10, 100, and 750 ng/mL). The samples were kept in an autosampler at ambient temperature and analysed at regular intervals up to 72 h. The results are presented in Tables 4 and 5. The processed samples were found to be stable at ambient temperature for 72 h.

Recovery

The mean percent recovery of VEN, ODV, and CTP from plasma, following the extraction procedure, was found to be 99.69%, 87.35%, and 73.02%, respectively.

DISCUSSION

Several methods have been reported in the literature for monitoring plasma levels of VEN and ODV.[1–15] The techniques used in these methods include mass spectrometric detection,[1,2] Capillary electrophoresis,[3–9] HPLC with fluorimetric detection,[10,11] HPLC with coulometric detection,[12] and HPLC with UV detection.[13–15] Mass spectrometry and capillary electrophoresis are expensive techniques and would not be available in every laboratory. Fluorimetric and coulometric detectors are specific detectors. Considering the availability of these instruments and the cost involved, we have developed a new method, which utilises a UV detector which is a universal detector.

Also, out of the published methods, in the method utilising fluorimetric detection,[10] recovery of VEN, ODV, and IS was 100, 68, and 53%, respectively. The extraction procedure was tedious and time consuming (overnight freezing of the samples at −20°C required).

Another method[12] utilising coulometric detection quoted the linearity range as 0–200 ng/mL. The reported C_{max} for 75 mg immediate release tablets were 225 ng/mL for VEN and 290 for ODV.[16] If the plasma levels exceed 200 ng/mL, the samples have to be diluted and reanalysed. Considering this, we have established the linearity range for VEN and ODV as 5–1000 ng/mL.

In the three previously published HPLC methods with UV detection, the first method[13] has quoted the limit of quantification for VEN and ODV as 200 ng/mL. The second method[14] was used to determine VEN only and not ODV in human serum. The limit of quantification for VEN was 25 ng/mL.

The third method[15] has quoted the limit of quantification as 10 ng/mL. The procedure involves a total analysis time of 100 min for one sample, which includes shaking, centrifugation, and chromatographic runs. In comparison, in

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Table 4. Processed sample stability for VEN.

Sample conc. (ng/mL)	Initial		After 24 h		After 48 h		After 72 h	
	Measured	% RE	Measured	% RE	Measured	% RE	Measured	% RE
10	10.35	3.50	10.68	6.80	10.42	4.20	10.22	2.20
100	103.19	3.19	105.58	5.58	106.55	6.55	106.03	6.03
750	769.72	2.63	787.62	5.02	779.84	3.98	781.35	4.18

Note: RE, relative error.

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Table 5. Processed sample stability for ODV.

Sample conc. (ng/mL)	Initial		After 24 h		After 48 h		After 72 h	
	Measured	% RE	Measured	% RE	Measured	% RE	Measured	% RE
10	9.19	−8.10	8.96	−10.40	8.93	−10.70	9.69	−3.1
100	100.41	4.10	102.79	2.79	103.73	3.73	102.89	2.89
750	738.59	−1.52	746.69	−0.44	742.66	−0.98	741.51	−1.13

Note: RE, relative error.

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the newly developed method, only 46 min are required for analysing one sample. Also the limit of quantification established is 5 ng/mL. Thus, the current method reduces the analysis time of each sample by approximately 50% and is more sensitive than the published method.

In this study, we have developed a specific, accurate, and precise analytical method for the simultaneous determination of VEN and ODV in human plasma. The limit of quantification for VEN and ODV, each, has been established as 5 ng/mL. The total elution time was about 12 min. The newly developed method is more rapid and sensitive than previously published HPLC methods with UV detection. The inter-day precision expressed as %RSD for VEN and ODV was less than 8%. The inter-day accuracy expressed as %RE for VEN and ODV was between −2.10% and 2.02%. The intra-day precision expressed as %RSD for VEN and ODV was less than 11.5%. The intra-day accuracy expressed as %RE for VEN and ODV was between −11.10% and 11.14%.

Adjusting the solvent composition, mobile phase, pH, and column length optimised the presently described chromatographic method. In the present extraction procedure, recovery of VEN was approximately 100% over the nominal concentration of 10, 100, and 750 ng/mL. The recovery of ODV and CTP was approximately 87% and 73%, respectively. Increasing the composition of hexane in the extraction solvent resulted in an increase in the recovery of ODV, however, the recovery of VEN and CTP decreased. Trace amounts of organic solvents in the aqueous phase (0.01% v/v orthophosphoric acid) resulted in interfering peaks in close proximity of ODV. Evaporation of the aqueous phase for 10 min was, therefore, necessary to remove the traces of organic solvents.

The C_{max} values of VEN and ODV for 75 mg VEN immediate release tablets are 225 ng/mL and 290 ng/mL. The C_{max} values of VEN and ODV for 150 mg VEN extended release capsules are 150 ng/mL and 260 ng/mL.[16]

In the present method, we have established a linearity range of 5–1000 ng/mL. This linearity range covers the C_{max} values of the above mentioned strengths of VEN. Hence, this method can be applied for quantifying the levels of VEN and ODV in vivo, when either of the strengths is administered to the subjects.

CONCLUSION

A HPLC method for the determination of VEN and ODV in human plasma has been developed and validated. It has been shown to be rapid, accurate, precise, and sensitive. The statistical analysis of the precision and

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accuracy data demonstrates that the method is suitable for quantifying the two analytes during clinical trials and/or pharmacokinetic studies to a limit of quantification of 5 ng/mL.

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