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Journal of Chromatography B, 843 (2006) 78–83
JOURNAL OF CHROMATOGRAPHY B
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Sensitive HPLC–APCI–MS method for the determination of cyclovirobuxine D in human plasma

Li Ding [a,*], Jingjing Hu [a], Meng Jiang [b], Ningning Xiong [b]

[a] *Department of Pharmaceutical Analysis, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China*
[b] *Organization for State Drug Clinical Trial, The Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing 210029, China*

Received 27 January 2006; accepted 22 May 2006
Available online 5 July 2006

Abstract

A sensitive high performance liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry (HPLC–APCI–MS) assay for determination of cyclovirobuxine D (CVB-D) in human plasma using mirtazapine as internal standard (I.S.) was established. After adjustment to a basic pH with sodium hydroxide, plasma was extracted by ethyl acetate and separated by high performance liquid chromatography (HPLC) on a reversed-phase C_{18} column with a mobile phase of 30 mM ammonium acetate buffer solution containing 1% formic acid–methanol (48:52, v/v). CVB-D was determined with atmospheric pressure chemical ionisation–mass spectrometry (APCI–MS). HPLC–APCI–MS was performed in the selected-ion monitoring (SIM) mode using target ions at $[M+H]^+$ *m/z* 403.4 for CVB-D and $[M+H]^+$ *m/z* 266.2 for I.S. Calibration curves were linear over the range 10.11–4044 pg/ml. The lower limit of quantification was 10.11 pg/ml. The intra- and inter-run variability values were less than 9.5 and 12.4%, respectively. The mean plasma extraction recovery of CVB-D was in the range of 85.3–92.8%. The method was successfully applied to determine the plasma concentrations of CVB-D in Chinese volunteers.

Keywords: Cyclovirobuxine D; HPLC–APCI–MS

1. Introduction

Cyclovirobuxine D (CVB-D; Fig. 1), an active compound extracted from the Chinese medicinal herb *Buxus microphylla* Sieb. et Zucc. var. *sinica* Rehd. et Wils. [1], is the active component of huangyangning tablet, which is widely used in China for the treatment of arrhythmias, angina pectoris and cardiac insufficiency [1,2]. CVB-D possesses the effect on preventing and treating arrhythmia [3,4]. It can significantly prolong action potential duration in isolated guinea pig atria [3] and papillary muscle [5], enhance myocardial contractive power and have significant positive inotropic action to both auricular muscle and ventricular muscle [5].

The pharmacokinetic profile of CVB-D in humans has not been reported so far. Because the clinical dosage of CVB-D is very low, the concentration of CVB-D in human plasma is at the low pg/ml level. To evaluate the pharmacokinetics of CVB-D in humans, an extremely sensitive method is required. Till now, no article has been reported for determination of CVB-D in human plasma. Recently, Wen et al. developed a liquid chromatography–time of flight–mass spectrometry (LC–TOF–MS) method for determination of CVB-D in dogs [6] and rats [7], in which the lower limit of quantification (LLOQ) was 500 pg/ml in plasma. In this paper, we reported a sensitive HPLC–APCI–MS method that can determine CVB-D plasma concentration in humans as low as 10.11 pg/ml and can be used to evaluate the pharmacokinetics of CVB-D in humans.

2. Experimental

2.1. Materials

CVB-D was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Mirtazapine (internal standard) was a gift from Huayu Pharmaceutical Co. Ltd. (Wuxi, China). The test formulation was huangyangning tablet (each tablet containing 0.5 mg

* Corresponding author. Tel.: +86 25 8327 1289; fax: +86 25 8327 1289.
E-mail address: dinglidl@hotmail.com (L. Ding).

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doi:10.1016/j.jchromb.2006.05.040

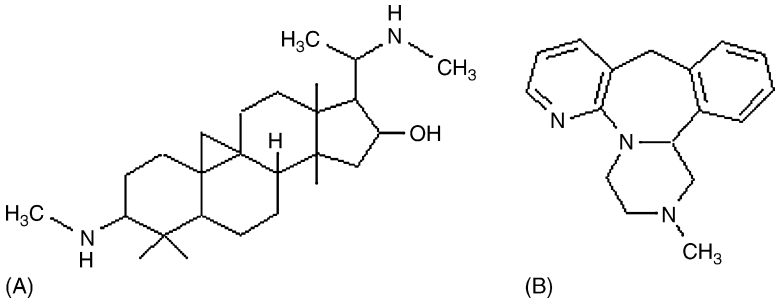

Fig. 1. Chemical structures of cyclovirobuxine D (A) and mirtazapine (B).

CVB-D) provided by Tonghua Jinma Pharmaceutical Incorporated Company (Jilin, China). Methanol was of HPLC grade (Merck, Darmstadt, Germany). Ethyl acetate, ammonium acetate, sodium hydroxide and formic acid were of analytic-grade purity and purchased from Nanjing Chemical Regent Co. (Nanjing, China). Distilled water, prepared from demineralized water was used throughout the study.

2.2. Instrumentation

HPLC–APCI–MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA, USA) with a Hanbon Lichrospher 5-C_{18} column, 5 μm, 250 mm × 4.6 mm i.d. (Jiangsu Hanbon Science & Technology Co. Ltd., China). The HPLC–APCI–MS was controlled by a computer employing the HP ChemStation software (10.02 A) supplied by Agilent.

2.3. HPLC–APCI–MS conditions

The mobile phase was 30 mM ammonium acetate buffer solution containing 1% formic acid–methanol (48:52, v/v) at a flow rate of 1.0 ml/min. The column temperature was maintained at 31 °C. HPLC–APCI–MS was carried out using nitrogen to assist nebulization. A quadrupole mass spectrometer equipped with an APCI source was set with a drying gas (N_2) flow of 4 l/min, nebulizer pressure of 60 psi, drying gas temperature of 350 °C, vaporizer temperature of 350 °C, capillary voltage of 4.0 kV and the positive ion mode. The fragmentor voltage was 150 V. HPLC–APCI–MS was performed in selected-ion monitoring (SIM) mode using target ions at $[M+H]^+$ *m/z* 403.4 for CVB-D and $[M+H]^+$ *m/z* 266.2 for I.S. Fig. 2 shows a typical full-scan APCI mass spectrum of CVB-D and I.S.

2.4. Preparation of standard solutions

Stock solutions of CVB-D (1.011 mg/ml) and internal standard (100.7 μg/ml) were prepared in methanol and stored at −20 °C. Standard solutions of CVB-D with concentrations of 101.1 μg/ml, 10.11 μg/ml, 1.011 μg/ml, 101.1 ng/ml, 10.11 ng/ml and 1.011 ng/ml, were made by serial dilution of CVB-D stock solution with methanol in separate 10 ml volumetric flasks. A solution containing 100.7 ng/ml internal standard was also obtained by further dilution of I.S. stock solution with methanol.

2.5. Sample preparation

A 1 ml aliquot plasma sample was added with 5 ml ethyl acetate after addition of 20 μl I.S. (100.7 ng/ml) solution and 0.1 ml of 1 M sodium hydroxide solution. After vortex mixing for 3 min, the mixture was centrifuged for 10 min at 4000 rpm. The organic phase was separated and evaporated to dryness under a stream of nitrogen in a water bath of 40 °C. The residue was reconstituted with 120 μl of mobile phase and a 30 μl aliquot

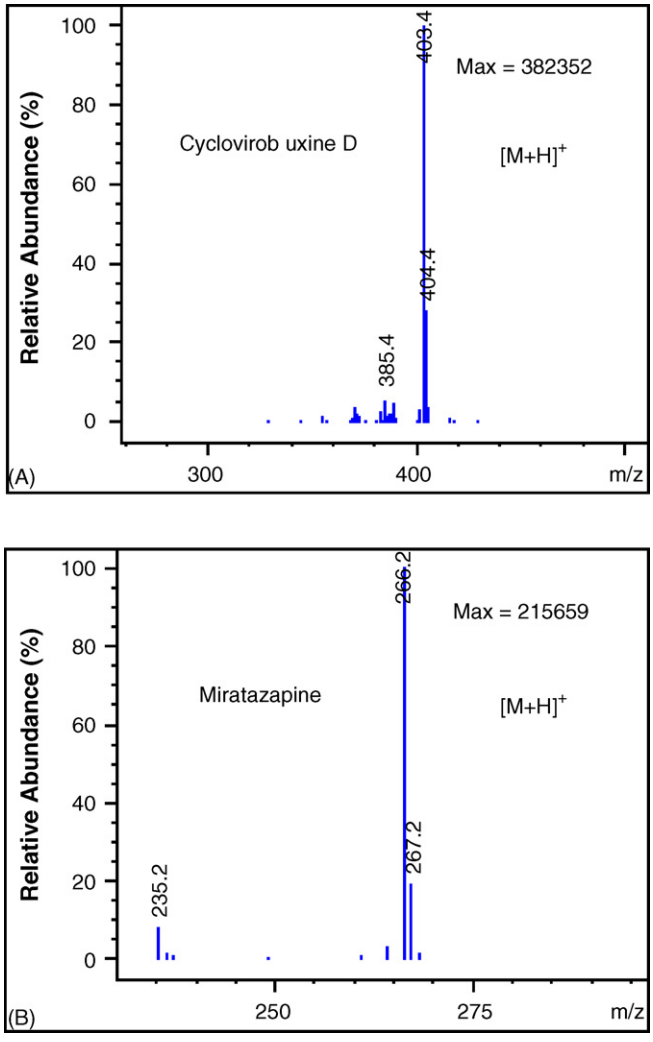

Fig. 2. Mass spectra of the positive ions of CVB-D (A) and I.S. (B) at 150 V fragmentor voltage.

of this solution was injected onto the HPLC–APCI–MS for analysis.

2.6. Preparation of calibration curves and quality control samples

Calibration standards of CVB-D were prepared by spiking appropriate amounts of the standard solutions in 1.0 ml blank plasma obtained from healthy volunteers. Standard curves were prepared in the range of 10.11–4044 pg/ml for CVB-D at concentrations of 10.11, 30.33, 101.1, 303.3, 1011, 2022 and 4044 pg/ml. The calibration curve was prepared and assayed along with quality control (QC) samples. QC samples were prepared in 1.0 ml blank plasma at concentrations of 30.33, 303.3 and 3033 pg/ml for CVB-D and stored at −20 °C.

2.7. Assay validation

2.7.1. Selectivity

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and HPLC–APCI–MS conditions to ensure no interference of CVB-D and I.S. from plasma.

2.7.2. Linearity of calibration curves and lower limits of quantification

Calibration standards of seven CVB-D concentration levels at 10.11, 30.33, 101.1, 303.3, 1011, 2022 and 4044 pg/ml were extracted and assayed. To evaluate the linearity, plasma calibration curves were prepared and assayed on 5 consecutive days. The calibration curve was constructed by plotting the peak area ratios of CVB-D to the I.S. versus the concentrations of CVB-D, using weighted least squares linear regression (weighting factor was $1/C$). The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [8], and it was established using five samples independent of standards.

2.7.3. Precision and accuracy

Validation samples were prepared and analyzed on 3 consecutive days (one run per day) to evaluate the accuracy, intra- and inter-run precisions of the analytical method. The accuracy, intra- and inter-run precisions of the method were determined by analyzing five replicates at 30.33, 303.3 and 3033 pg/ml of CVB-D along with one standard curve on each of 3 days. Assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy is the degree of closeness of the determined value to the nominal true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated using the formula: $\mathrm{RE}\% = (E - T)/T \times 100$.

The QC samples were assayed along with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

2.7.4. Extraction recovery

The extraction recovery of CVB-D was evaluated by analyzing five replicates at 30.33, 303.3 and 3033 pg/ml of CVB-D. Recovery was calculated by comparison of the peak areas of CVB-D extracted from plasma samples with those of injected standards.

2.7.5. Stability

The stability of CVB-D in plasma was studied under a variety of storage and handling conditions using the low (30.33 pg/ml) and high (3033 pg/ml) QC samples. The short-term temperature stability was assessed by analyzing QC samples that were kept at ambient temperature for 10 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. The QC samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was performed at −20 °C in plasma for 20 days. The stability of stock solutions of CVB-D and the I.S. at −20 °C was also evaluated.

2.7.6. Application

The method described above was applied to the pharmacokinetic study in which plasma concentrations of CVB-D in six healthy Chinese male volunteers were determined up to 120 h after administration of huangyangning tablets containing 2 mg CVB-D. Blood was sampled pre-dose and at 0.5, 1, 2, 3, 4, 5, 6, 7, 9, 12, 24, 48, 72, 96 and 120 h post-dose for determination of concentration of CVB-D. The clinical pharmacokinetic study was approved by the Ethic Committee of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

3. Results and discussion

3.1. Conditions of HPLC

Usually, we prefer choosing a compound that is structurally similar to the analyte or is chemically similar to the analyte as the internal standard. However, for some natural products, such as CVB-D, it is difficult to obtain internal standards that are quite chemically similar to them. In fact, the necessary requirements for a proper internal standard include that it should have similar retention to the analyte, be well resolved from the analyte and other peaks, and mimic the analyte in any sample preparation steps, but does not have to be chemically similar to analyte [9]. CVB-D is a kind of natural alkaloid that has a complicated chemical structure, and it is difficult for us to obtain an internal standard that is quite chemically similar to it. After screening of some alkaloids, we choose mirtazapine as the internal standard in the assay of CVB-D.

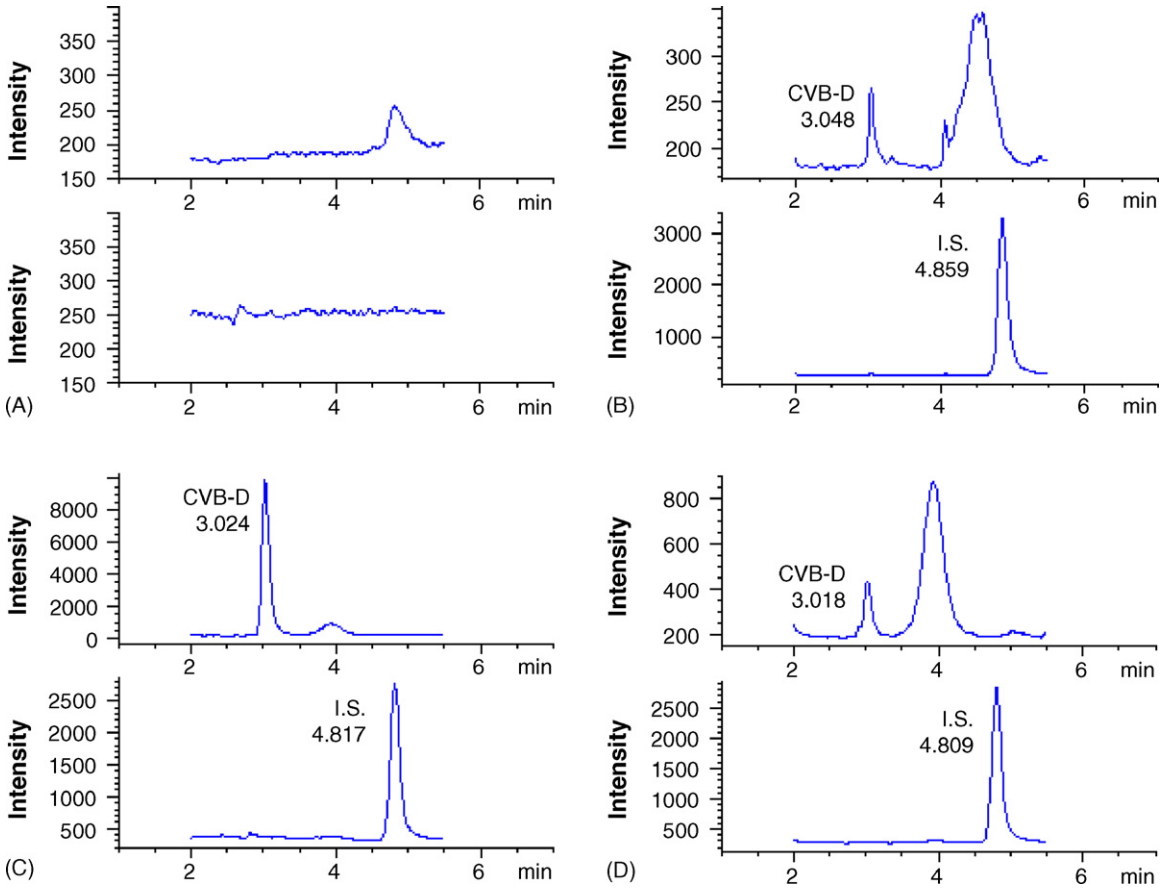

Fig. 3. Typical SIM mass chromatograms of blank plasma (A), LLOQ for CVB-D in plasma (10.11 pg/ml) and I.S. (B), plasma spiked with CVB-D (4044 pg/ml) and I.S. (C) and plasma obtained from a volunteer at 4 h after orally administration of 2 mg huangyangning tablets, the plasma concentration of CVB-D was estimated to be 101.2 pg/ml (D).

In optimizing the chromatographic conditions, the concentration of the ammonium acetate buffer solution used in the mobile phase was investigated. CVB-D and I.S. are all alkaloids. Because of their alkalinity, CVB-D and I.S. would easily appear as tailing peaks in their chromatograms. To suppress the tailing phenomena of chromatographic peaks of CVB-D and I.S., the ammonium acetate buffer solution was adopted in the mobile phase of the HPLC. The test results showed that the mobile phase of 10 mM ammonium acetate solution could not suppress the tailing problem of the peaks of CVB-D and I.S. effectively, but as the concentration of ammonium acetate in the mobile phase was increased, the peak shapes of CVB-D and I.S. were improved. The concentration of 30 mM ammonium acetate solution could sufficiently resolve the tailing problem, and made the chromatographic peaks sharp and symmetric. Further experiment results showed that acidifying the mobile phase with formic acid could not only improve peak shapes of CVB-D and I.S., but also increase the MS sensitivity to CVB-D and I.S. So, a concentration of 1% formic acid was finally added into the ammonium acetate solution of the mobile phase. In addition, by increasing the ratio of the ammonium acetate buffer in the mobile phase, the retention and separation of CVB-D were improved. The acceptable retention and separation of CVB-D was obtained by using an elution system of 30 mM ammonium acetate buffer solution containing 1% formic acid–methanol (48:52, v/v) as the mobile phase. Representative selected-ion chromatograms are shown in Fig. 3. Typical retention times were about 3.0 min for CVB-D and 4.8 min for I.S.

3.2. Conditions for APCI–MS

Because CVB-D is a weak base and has proton affinity, positive ion mode was adopted in the LC–MS. Usually, electrospray ionization (ESI) is used for medium- to high-polarity analytes and atmospheric pressure chemical ionization (APCI) is used for low- to medium-polarity analytes. The APCI process begins with gas-assisted nebulization into a hot, typically 250–400 °C, vaporizer chamber that serves to rapidly evaporate the spray droplets, and it is more suitable for the ionization of the mobile phase that has a high ratio of water phase and is more tolerant to the presence of buffers in the mobile phase stream than is ESI. But, sometimes we cannot easily decide which ionization technique is the best, and we need to investigate the compound's relative merits under the two different ionizations [10]. So, both APCI and ESI sources were evaluated for assay development in positive ion mode. APCI produced greater sensitivity and exhibited less interference than we were able to achieve with

Table 1
Mean inter-day back-calculated standard and standard curve results ($n = 5$)

Added C (pg/ml)	Found C (pg/ml)					Mean	S.D.	R.S.D. (%)	RE (%)
	I	II	III	IV	V				
Mean inter-day back-calculated standard results									
10.11	9.457	10.17	10.24	12.93	11.36	10.83	1.4	12.9	7.1
30.33	33.23	33.79	32.74	27.30	30.95	31.60	2.6	8.2	4.2
101.1	104.9	95.39	100.0	91.80	98.12	98.04	4.9	5.0	−3.0
303.3	305.9	277.3	286.8	275.1	285.5	286.1	12.2	4.3	−5.7
1011	939.4	1049	981.8	1012	932.7	983.0	49.1	5.0	−2.8
2022	1944	1974	1991	2009	2023	1988	30.7	1.5	−1.7
4044	4173	4076	4115	4088	4131	4117	38.2	0.9	1.8
Standard curve results									
Slope ($\times 10^{-3}$)	0.7690	0.7790	0.7286	0.7543	0.7359	0.7534	0.0213	2.8	
Y-intercept	−0.0011	0.0040	0.0024	−0.0007	0.0004	0.0010	0.0022	NA	
r	0.9991	0.9996	0.9997	0.9996	0.9994	0.9995	0.0002	0.0	

Note: Calibration curves were weighted 1/concentration. R.S.D., relative standard deviation; RE, relative error; NA, not applicable; *n*, number of calibration curves.

ESI. Thus, APCI in positive ion mode was adopted for the assay of CVB-D.

In order to determine the optimal fragmentor voltage, the intensities of CVB-D protonated molecular ion $[M+H]^+$ *m/z* 403.4 were compared at fragmentor voltages of 30, 50, 70, 90, 110, 130, 150, 170, 200 and 250 V in the SIM mode. The highest sensitivities could be obtained by using a 150 V fragmentor voltage. Fig. 4 shows the intensity of CVB-D (101 μg/ml) at different fragmentor voltages.

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank, a spiked plasma sample with CVB-D (10.11 pg/ml) and I.S., a spiked plasma sample with CVB-D (4044 pg/ml) and I.S., and a plasma sample from a healthy volunteer. There was no significant interference from endogenous substances observed at the retention times of the analytes.

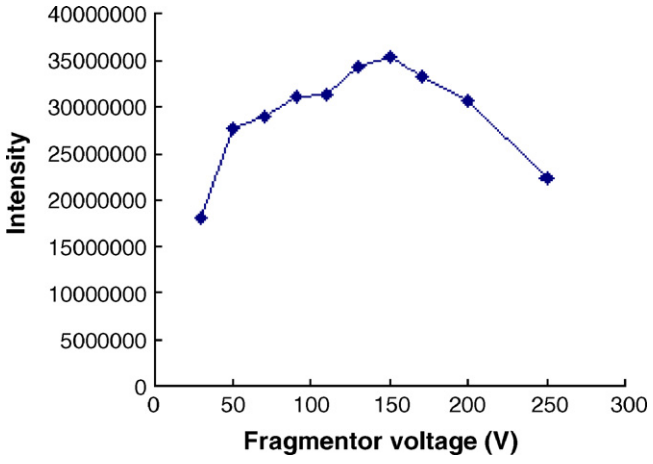

Fig. 4. The intensity of cyclovirobuxine D (101 μg/ml) at different fragmentor voltages.

Table 2
Accuracy and precision for the analysis of LLOQ ($n = 5$)

Added C (pg/ml)	Found C (pg/ml)	Mean (pg/ml)	R.S.D. (%)	RE (%)
10.11	9.42	9.84	12.1	−6.8
10.11	9.58			−5.2
10.11	10.14			0.3
10.11	8.42			−16.7
10.11	11.66			15.3

Note: R.S.D., relative standard deviation; RE, relative error; *n*, number of replicates.

3.3.2. Calibration curve and sensitivity

Five calibration analyses were performed on 5 consecutive days and the back-calculated values for each level were recorded (see Table 1). The relative standard deviation (R.S.D.%) at each level varied from 0.9 to 12.9 and the relative error (RE%) varied from −5.7 to 7.1. The relative standard deviation (R.S.D.%) of the five slopes was 2.8 and the mean correlation coefficient (*r*) of the five calibration curves was 0.9995. The calibration curves did not exhibit any non-linearity within the chosen range. The back-calculated results showed good day-to-day accuracy and precision. The LLOQ for CVB-D in plasma was 10.11 pg/ml. The data of LLOQ is shown in Table 2.

3.3.3. Assay precision and accuracy

Table 3 summarizes the intra- and inter-run precision and accuracy for CVB-D evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this

Table 3
Accuracy and precision for the analysis of CVB-D in human plasma (in prestudy validation, three runs, five replicates per run)

Added C (pg/ml)	Found C (pg/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	RE (%)
30.33	29.61	9.5	12.4	−2.4
303.3	286.2	6.2	10.1	−5.6
3033	3079	5.5	8.4	1.5

Note: R.S.D., relative standard deviation; RE, relative error.

Table 4
Stability data of CVB-D in human plasma under various storage conditions ($n = 3$)

Storage conditions	Added C (pg/ml)	Found C (pg/ml)	Inter-run R.S.D. (%)	RE (%)
Room temperature for 10 h	30.33	31.35	7.7	3.4
	3033	2982	5.1	−1.7
Three freeze–thaw cycles	30.33	28.78	10.2	−5.1
	3033	3054	7.2	0.7
20 days at −20 °C	30.33	28.97	6.5	−7.2
	3033	3021	6.1	−0.4

Note: R.S.D., relative standard deviation; RE, relative error; *n*, number of replicates.

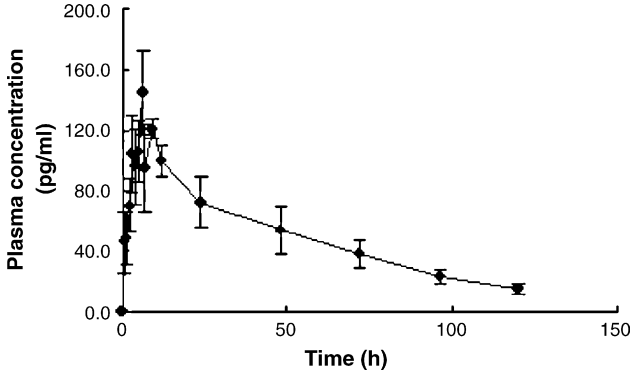

Fig. 5. Mean CVB-D plasma concentration–time profile in six healthy volunteers after oral administration of 2 mg CVB-D tablets.

assay, the intra-run precision was 9.5% or less, and the inter-run precision was 12.4% or less for each QC level of CVB-D. The results above demonstrate that the values are within the acceptable range and the method is accurate and precise.

3.3.4. Extraction recovery

The ethyl acetate was chosen as the extraction solvent for its higher extraction efficiency to the two target compounds. The extraction recoveries of CVB-D and I.S. could be promoted by raising the pH of the plasma samples with sodium hydroxide, and so 0.1 ml of 1 M sodium hydroxide was added to 1.0 ml plasma sample before extraction. The recovery of CVB-D, determined at three concentrations of 30.33, 303.3 and 3033 pg/ml were 85.3 ± 0.5, 87.3 ± 3.0 and 92.8 ± 0.9% ($n = 5$), respectively.

3.3.5. Stability

The stability of CVB-D was studied under a variety of storage and handling conditions. The results (see Table 4) showed that no significant degradation occurred at ambient temperature for 10 h and during the three freeze–thaw cycles for CVB-D plasma samples. CVB-D in plasma at −20 °C was stable for at least 20 days. The stock solutions of CVB-D and I.S. at −20 °C were stable for at least 1 month. The results were obtained by a comparison with freshly prepared solutions, and the percentage concentration deviations were within ±5%.

3.4. Application

The assay was successfully applied in the pharmacokinetic study in which plasma concentrations of CVB-D in six healthy Chinese volunteers were determined up to 120 h after administration of tablets containing 2 mg CVB-D. The maximum CVB-D plasma concentration (C_{max}) was 151.8 ± 18.0 pg/ml and the time to C_{max} was 6.3 ± 1.6 h. The mean plasma concentration–time curve of CVB-D is shown in Fig. 5.

4. Conclusion

The assay achieved good sensitivity and specificity for the determination of CVB-D in human plasma. No significant interferences caused by endogenous compounds are observed. This simple and sensitive assay is suitable for pharmacokinetic studies of CVB-D in human subjects.

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