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Journal of Chromatography B, 852 (2007) 436–442
JOURNAL OF CHROMATOGRAPHY B
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# A liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of valsartan and hydrochlorothiazide in human plasma

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Received 26 October 2006; accepted 2 February 2007
Available online 15 February 2007

**Abstract**

A rapid and sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed and validated for simultaneous quantification of valsartan and hydrochlorothiazide in human plasma. After a simple protein precipitation using acetonitrile, the analytes were separated on a Zorbax SB-Aq C18 column using acetonitrile −10 mM ammonium acetate (60:40, v/v, pH 4.5) as mobile phase at a flow rate of 1.2 mL/min. Valsartan and hydrochlorothiazide were eluted at 2.08 min and 1.50 min, respectively, ionized using ESI source, and then detected by multiple reaction monitoring (MRM) mode. The precursor to product ion transitions of *m/z* 434.2–350.2 and *m/z* 295.9–268.9 were used to quantify valsartan and hydrochlorothiazide, respectively. The method was linear in the concentration range of 4–3600 ng/mL for valsartan and 1–900 ng/mL for hydrochlorothiazide. The method was successfully employed in a pharmacokinetic study after an oral administration of a dispersible tablet containing 80 mg valsartan and 12.5 mg hydrochlorothiazide to each of the 20 healthy volunteers.


*Keywords:* Valsartan; Hydrochlorothiazide; LC/MS/MS; Pharmacokinetics

## 1. Introduction

Most cardiovascular events are attributed to high blood pressure. Hence, antihypertensive therapy is to reduce considerably the risk of developing cardiovascular complications that cause a high mortality rate in the patients with hypertension. Hydrochlorothiazide (HCT) is one of the oldest thiazide diuretics, often prescribed in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers [1]. Valsartan (VAL) is a new antihypertensive drug belonging to the family of angiotensin II receptor antagonists [2,3]. More recently, a new combination dosage form of VAL and HCT is indicated in the treatment and management of hypertension. This combination is more effective than either drug alone, and is effective in patients not responding to monotherapy with either agent [1,4]. As a result, it is desirable to develop an analytical method to allow both drugs to be quantified simultaneously in human plasma after a therapeutic dose.

Over the years, many quantification methods for the individual determination of VAL or HCT in biological matrices have been reported. For the determination of VAL, several high-performance liquid chromatographic (HPLC) methods using fluorimetric detection and spectrophotometric detector are available [5–8], while various methods have been investigated for assaying HCT, either individually or in combination with other antihypertensive drugs in plasma, such as HPLC with UV or diode array detector [9–11], and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) [12]. Simultaneous determination of both drugs is highly desirable as this would allow more efficient generation of clinical data and could be more cost-effective than separate assays. There are very few methods appearing in the literature for the simultaneous determination of VAL and HCT in tablets and since these methods were

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doi:10.1016/j.jchromb.2007.02.014

based on HPLC and UV-derivative spectrophotometry [13,14]. The sensitivity was insufficient for pharmacokinetic (PK) studies and the run times were rather long. Unfortunately, to our knowledge, no methods have been reported for the simultaneous quantification of these important antihypertensive drugs in biological matrices.

The coupling of HPLC with MS/MS is a more definitive technique with improved sensitivity, high specificity and selectivity compared to traditional HPLC and GC methods. Therefore, we developed a rapid and sensitive LC/MS/MS method to measure VAL and HCT simultaneously in human plasma using a one-step protein precipitation without evaporation. This validated analytical method has been successfully applied to a pharmacokinetic study of VAL and HCT in healthy volunteers after an oral administration of a dispersible tablet containing 80 mg VAL and 12.5 mg HCT.

## 2. Experimental

### 2.1. Chemicals and reagents

VAL, HCT (purity >99.0%, respectively) and probenecid (internal standard, I.S., purity >99.5%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with molecular structures shown in Fig. 1. Acetonitrile (HPLC-grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA), and heparinized blank (drug-free) human plasma (different batches examined) was obtained from Changchun Blood Donor Service (Changchun, China). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals were HPLC grade. A mixture of acetonitrile −10 mM ammonium acetate (60:40, v/v) adjusted to pH 4.5 using formic acid was used as the isocratic mobile phase and the pH was adjusted in the aqueous solution and not in the mixed organic-aqueous solution.

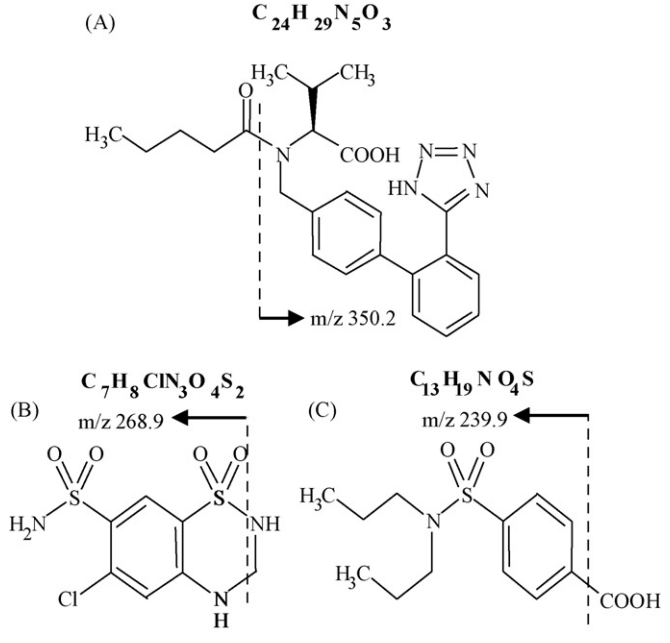


Fig. 1. Chemical structures and observed negatively charged fragment ions of VAL (A), HCT (B), and probenecid (C).

### 2.2. Instrumentation

The LC/MS/MS system consisted an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) equipped with a TurboIonSpray source for ion production. Data acquisition and integration were controlled by Applied Biosystems Analyst Software.

### 2.3. LC/MS/MS conditions

The chromatographic separation was performed on a 150 mm × 4.6 mm, 5 μm Zorbax SB-Aq C18 column maintained at 35 °C using a mobile phase of acetonitrile −10 mM ammonium acetate (60:40, v/v) adjusted to pH 4.5 using formic acid. Degassing was accomplished by sonicating the mobile phase prior to usage. The separation was under isocratic conditions with a flow rate of 1.2 mL/min. The column effluent was split so that approximately 0.6 mL/min entered the mass spectrometer. The electrospray interface heater (IHE) was set to on mode and IonSpray Voltage (IS) was set at −1600 V for negative ionization. The nitrogen curtain gas was adjusted to a constant value of 10 units, Gas 1 (GS1) and Gas 2 (GS2) was set to 35 and 45 units, respectively, and the source temperature (at setpoint) was 500 °C. The mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution. The MRM experiments were conducted by monitoring the precursor ion to product ion transitions in the negative ion mode for VAL from *m/z* 434.2 (Q1) to *m/z* 350.2 (Q3) with DP (Declustering Potential) of −57 eV and CE (Collision Energy) of −25 eV, for HCT from *m/z* 295.9 (Q1) to *m/z* 268.9 (Q3) with DP of −73 eV and CE of −27 eV, and for probenecid from *m/z* 283.9 (Q1) to *m/z* 239.9 (Q3) with DP of −50 eV and CE of −20 eV. The pause time was set at 10 ms and the dwell time at 200 ms.

### 2.4. Preparation of standard and quality control samples

Standard stock solutions of VAL (4 mg/mL) and HCT (1 mg/mL) were prepared by weighing the appropriate amounts of the analytes and dissolving them in acetonitrile:water (80:20, v/v) and refrigerated at 4 °C. The final concentrations of the calibration standards were 4, 12, 40, 120, 400, 1200, and 3600 ng/mL for VAL and 1, 3, 10, 30, 100, 300, and 900 ng/mL for HCT. They were all diluted from stock solution with acetonitrile:water (80:20, v/v) in 10 mL volumetric flasks. Concentrations at 12, 120, and 2880 ng/mL for VAL and 3, 30, and 720 ng/mL for HCT were prepared as quality control (QC) samples. A 5 mg/mL stock solution of probenecid was also prepared in acetonitrile:water (80:20, v/v). This was subsequently diluted with acetoni-

trile:water (80:20, v/v) to obtain a 150 ng/mL working I.S. solution.

### 2.5. Sample preparation

Frozen human plasma samples were thawed in a water bath at room temperature. Hundred microliter of I.S. working solution was added to a 100 μL aliquot of plasma sample. The samples were briefly mixed, and then 300 μL of acetonitrile was added to precipitate proteins. The mixture was vortex–mixed for 1 min and centrifuged for 10 min at 10000 × *g*. The upper clear solution layer was collected, and a 15 μL aliquot of solution was injected into the LC/MS/MS system for analysis.

### 2.6. Method validation

To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three separate days. Calibration curves were constructed based on the measurement of the peak–area ratio of the analyte to I.S. Least-squares linear regression was used for curve fitting with $1/x^2$ as the weighting factor. Concentrations of VAL and HCT in the samples were then calculated using the equations from the appropriate calibration curves. QC samples at three concentration levels were analyzed to assess the accuracy and precision of the method. Again, the assays were performed on three separate days, and on each day, six replicates of each analyte at each concentration level were analyzed together with an independently prepared calibration curve (prepared using the same plasma and independently calculated). The accuracy was evaluated by calculating the relative error (RE) and the precision for each QC level was determined as the relative standard deviation (RSD) of the measured concentrations. The intra- and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%. The recovery rate was determined by comparing the mean peak areas of the regularly prepared samples at low, medium, and high QC concentrations (three samples each) with the mean peak areas of the corresponding spike-after-precipitation samples, which represented the 100% recovery. To prepare the spike-after-precipitation samples, blank human plasma was processed according to the sample preparation procedure as described above. The upper clean solution layer was collected, and then the appropriate standard solutions of VAL and HCT were added to give concentrations corresponding to the final concentrations of the pretreated plasma samples.

Matrix effects in the LC/MS/MS method were determined by comparing the mean peak areas of the spike-after-precipitation samples at low, medium, and high QC concentrations (three samples each) with the mean peak areas of the pure samples equally concentrated.

The stability of the analytes has been studied in plasma at room temperature for 6 h. Extracted validation samples at low, middle and high concentrations were kept at room temperature for over 12 h and were then reanalyzed and quantified against freshly made standard curves. Stability in plasma was assessed on storage at −20 °C for 30 days, and the effect of three freeze–thaw cycles was also investigated.

### 2.7. Application of the method

The validated method was applied to determine the plasma concentrations of VAL and HCT from a clinical trial in which 20 healthy male volunteers received an oral dosage (dispersible tablet containing 80 mg VAL and 12.5 mg HCT). Blood samples were collected into heparinized glass tubes before administration and at 0.50, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0, 24.0 and 36.0 h post-dose. Plasma was separated immediately by centrifugation at 3000 × g for 10 min and stored at −20 °C prior to analysis.

## 3. Results and discussion

### 3.1. Sample preparation

Both VAL and HCT have robust MS response. This enabled us to reach the desired limit of quantitation using only protein precipitation in the sample preparation. The simple preparation procedure can save considerable time and simplify the operating process. The selected protein precipitant was acetonitrile, due to its satisfactory efficiency in precipitating and extraction and reduced matrix effects compared to that associated with methanol and trifluoroacetic acid. Different volumes of acetonitrile were evaluated for efficiency of protein precipitation; it was found that three times volume of the plasma can precipitate the plasma proteins completely, and the chromatographic behavior of the analytes was not deteriorated by this procedure. The recovery was high and the analytes were stable under these conditions.

### 3.2. LC/MS/MS conditions

Full-scan mass spectra of each analyte were acquired in both positive and negative ion modes with the infusion of the respective standard solution directly into the ESI source. VAL ionized in both positive and negative modes, and it had higher ionization efficiency in the negative ionization mode, while HCT only ionized in negative mode. Therefore, the negative ionization mode was chosen since both analytes could be sufficiently ionized. The product ion mass spectra of the analytes are shown in Fig. 2. We have chosen MS/MS transitions *m/z* 434.2 → 350.2 and 434.2 → 179.1 for the identification of valsartan and *m/z* 295.9 → 268.9 and 295.9 → 204.9 for hydrochlorothiazide in the preliminary experiment. And MS/MS transitions *m/z* 434.2 → 350.2 and *m/z* 295.9 → 268.9 showed a better response than the others at the same concentrations of valsartan and hydrochlorothiazide, respectively. Therefore, we only monitored the qualifiers (*m/z* 434.2 → 350.2 and *m/z* 295.9 → 268.9) in the final experiment as the sensitivity of other qualifiers were insufficient for pharmacokinetic studies.

### 3.3. Chromatographic conditions

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well

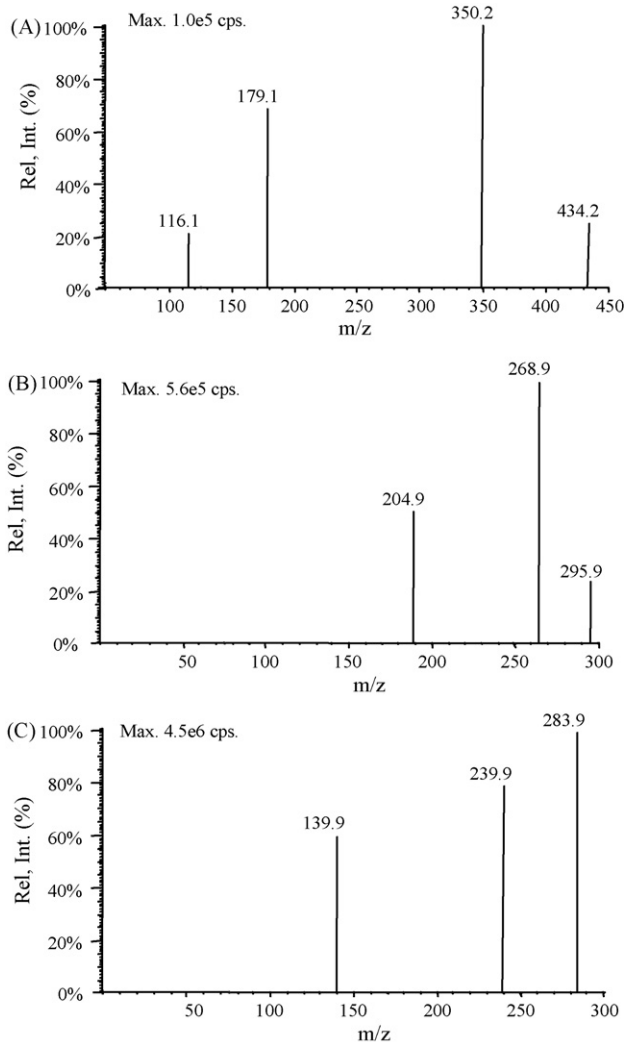


Fig. 2. Full-scan product ion mass spectra of $[M-H]^-$ ions of VAL (A), HCT (B), and probenecid (C). The spectra were recorded under the same conditions as in the analysis using a mobile phase of acetonitrile −10 mM ammonium acetate (60:40, v/v) adjusted to pH 4.5 using formic acid.

as short run time. The inclusion of 10 mM ammonium acetate instead of pure water enhanced MS response without increasing matrix effects. Peak shape was improved by using formic acid to adjust the mobile phase pH to 4.5. Further, improvement in peak shape with reduced cycle time was achieved by increasing the flow rate. A number of $C_{18}$ columns (Diamonsil $C_{18}$, Zorbax extend $C_{18}$, Nucleosil $C_{18}$, and Hypersil $C_{18}$) were evaluated. Zorbax SB-Aq $C_{18}$ gave the best chromatography with a flow rate of 1.2 mL/min and the cycle time was just 2.6 min.

### 3.4. Selection of I.S.

It is necessary to use an I.S. to obtain good accuracy and precision when a mass spectrometer is used as the HPLC detector. Probenecid was adopted as I.S. because of the similarity of its retention time with those of the analytes, and it also ionizes well in the negative ionization mode.

### 3.5. Method validation

#### 3.5.1. Assay selectivity

Selectivity was assessed by comparing the chromatograms for six different batches of blank human plasma with those for the corresponding standard spiked plasma samples. Fig. 3 shows the typical MRM chromatograms of blank, spiked plasma sample with VAL (4 ng/mL), HCT (1 ng/mL) plus I.S. (150 ng/mL), and a plasma sample from a healthy volunteer 1.0 h after an oral administration of a dispersible tablet containing 80 mg VAL and 12.5 mg HCT. There was no significant interference from endogenous substances observed at the retention times of the analytes and the I.S. These results suggested that no considerable endogenous contribution from human plasma interferes with the measurement of the analytes, demonstrating the selectivity and specificity of the MRM technique.

#### 3.5.2. Linearity of calibration curves and LLOQ

The assay was linear over the concentration ranges of 4–3600 ng/mL for VAL and 1–900 ng/mL for HCT. All calibration curves were weighted according to the $1/x^2$ weighting scheme. Typical equations of the calibration curves were as follows:

$$\text{VAL}: \quad y = 0.0174x + 0.0106, \quad r = 0.9982$$

$$\text{HCT}: \quad y = 0.00272x + 0.00114, \quad r = 0.9988$$

where $y$ represents the ratio of analyte peak area to that of the I.S., and $x$ represents the plasma concentration of the analyte. Good linearity was shown in the stated concentration ranges. The LLOQs were determined to be 4 ng/mL for VAL and 1 ng/mL for HCT, which were sufficient for clinical PK studies following oral administration.

#### 3.5.3. Precision and accuracy

Table 1 summarizes the intra- and inter-day precision and accuracy values for VAL and HCT. Intra-day precision and accuracy were calculated from the analysis of six samples at each QC point on three separate days. Inter-day precision and accuracy were calculated from the pooled data from 3 days. Three QC points, of concentrations 12, 120, and 2880 ng/mL for VAL and 3, 30, and 720 ng/mL for HCT, were selected to validate the method. The precision was calculated by using the RSD

Table 1
Accuracy and precision for the analysis of VAL and HCT in human plasma (in pre-study validation, $n = 3$ days, six replicates per day)

| | Concentration (ng/mL) | | RSD (%) | | RE (%) |
|---|---|---|---|---|---|
| | Spiked | Mean calculated | Intra-day | Inter-day | |
| VAL | 12.00 | 12.05 | 2.62 | 5.85 | 0.38 |
| | 120.0 | 124.0 | 1.96 | 3.01 | 3.58 |
| | 2880 | 2654 | 1.92 | 2.21 | −7.85 |
| | 3.00 | 2.97 | 4.81 | 7.12 | −0.90 |
| HCT | 30.0 | 29.9 | 4.55 | 6.23 | −0.19 |
| | 720 | 735 | 5.52 | 3.91 | 2.05 |

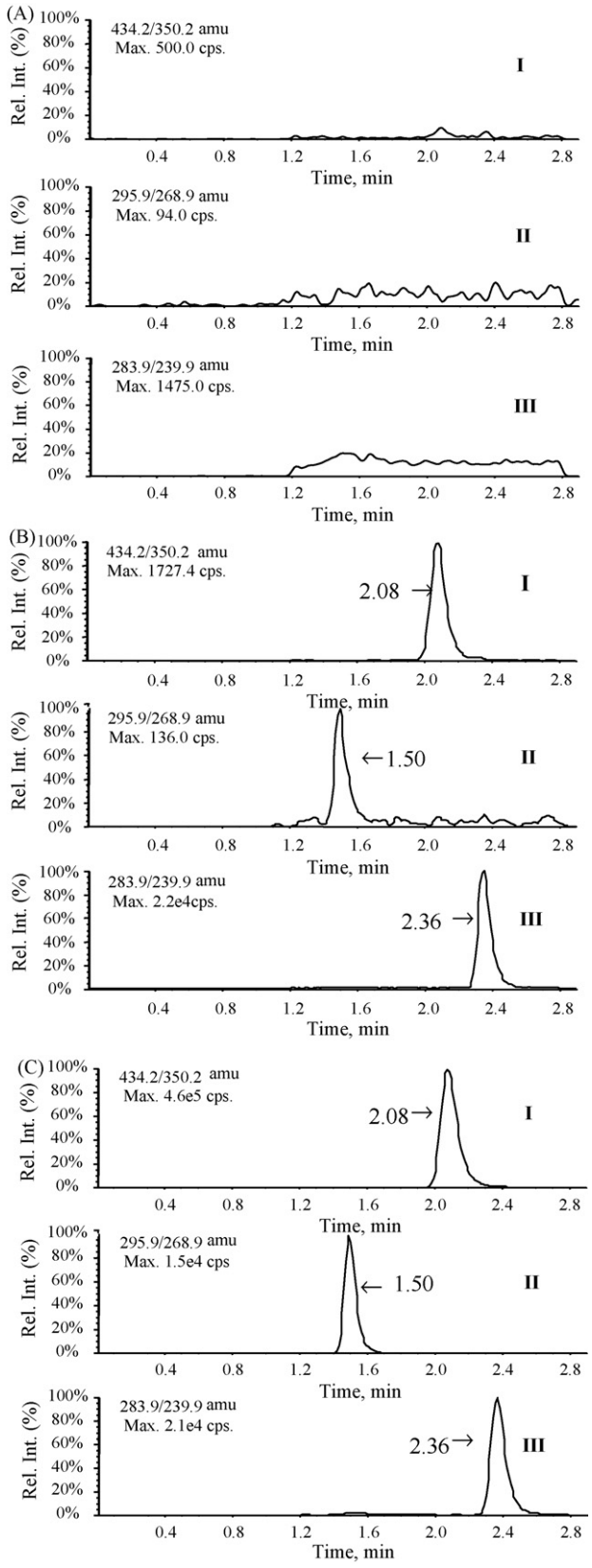


Fig. 3. Representative LC-MRM chromatograms for VAL (I), HCT (II), and I.S. (probenecid, III) in human plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with VAL (4.0 ng/mL), HCT (1.0 ng/mL), and I.S. (150 ng/mL); and (C) a volunteer plasma sample 1.0 h after an oral administration of a dispersible tablet containing 80 mg VAL and 12.5 mg HCT.

and the accuracy was evaluated using the RE. In this assay, the intra- and inter-day precision were 5.85% or less for each QC level of VAL and 7.12% or less for each QC level of HCT. The accuracy was within ±7.85% for VAL and within ±2.05% for HCT. The above values were within the acceptable range, and the method was thus judged to be suitably accurate and precise.

### *3.5.4. Recovery, matrix effect and stability*

The mean recovery rates of VAL in the protein precipitation with acetonitrile were 75.4, 76.9, and 74.7% at concentrations of 12, 120, and 2880 ng/mL, while, the mean recovery rates of HCT were 82.8, 86.3, and 85.6% at concentrations of 3, 30, and 720 ng/mL, respectively. Mean recovery for the internal standard (150 ng/mL) was 98.9 ± 3.5%.

The matrix effect is a noticeable problem while determining compounds in human plasma. To evaluate the possibility of ion suppression or enhancement in the present experiments, chromatographic peak areas of VAL and HCT from the spike-after-precipitation samples at low, middle and high concentration levels were compared to those obtained from the standard solution at the same concentrations in neat solvent. The percent nominal concentrations and corresponding RSD determined were 79.0 ± 0.7%, 80.5 ± 0.8%, and 83.8 ± 1.0% at 4, 40, and 2880 ng/mL for VAL and 30.4 ± 1.1%, 31.0 ± 0.6%, and 35.5 ± 1.1% at 1, 10, and 720 ng/mL for HCT, respectively, after evaluating three different lots of plasma at each concentration level. The same evaluation was performed for the I.S. and the percent nominal concentration was 114.4 ± 2.7%. The results indicate that ion suppression or enhancement from plasma matrix was consistent for this analytical method and would not interfere the measurement of the analytes.

Table 2 summarizes the data from the short-term (the analytes in human plasma and the analytes after extracting and reconstitution), freeze/thaw, and long-term stability tests for VAL and HCT. The short-term test indicated reliable stability under the collection conditions of the blood samples and the experimental conditions of the analytical runs. The results of the freeze/thaw stability test indicated that the analytes were stable in human plasma for three cycles when stored at −20 °C and thawed to room temperature. The findings from the long-term test indicated that storage of plasma samples containing VAL and HCT at −20 °C was adequate when maintained for 30 days. Thus, no stability-related problems are expected during the routine analyses for the PK study. In addition, standard stock solutions of VAL and HCT were shown to be stable for at least 24 h at 24 °C and 20 days at 4 °C.

### *3.6. Applying the method to a clinical PK study with healthy volunteers*

This validated analytical method was used to study the PK profiles of VAL and HCT in human plasma after an oral administration of a dispersible tablet containing 80 mg VAL and 12.5 mg HCT. The plasma concentration time profiles are illustrated in Fig. 4. The corresponding mean PK parameters of VAL and

Table 2
Stability data of VAL and HCT during the routine analyses (three samples each concentration)

| Storage conditions | Drug | Concentration (ng/mL) | | RSD (%) | RE (%) |
|---|---|---|---|---|---|
| | | Spiked | Mean calculated | | |
| Freezing for 30 days at −20 °C (in human plasma) | VAL | 12.0 | 11.7 | 1.33 | −2.25 |
| | | 120 | 122 | 0.90 | 1.47 |
| | | 2880 | 2555 | 1.75 | −11.27 |
| | | 3.0 | 2.7 | 2.71 | −9.90 |
| | HCT | 30.0 | 30.8 | 1.98 | 2.59 |
| | | 720 | 705 | 2.55 | −2.05 |
| Three freeze/thaw cycles (in human plasma) | VAL | 12.0 | 12.0 | 0.90 | −0.33 |
| | | 120 | 119.2 | 0.49 | −0.64 |
| | | 2880 | 2617 | 1.29 | −9.13 |
| | | 3.0 | 2.8 | 0.60 | −6.28 |
| | HCT | 30.0 | 27.2 | 0.98 | −9.28 |
| | | 720 | 677.8 | 0.47 | −5.87 |
| Stability at room temperature for 6 h (in human plasma) | VAL | 12.0 | 11.8 | 2.10 | −1.94 |
| | | 120 | 123.0 | 1.67 | 2.50 |
| | | 2880 | 2873 | 0.53 | −0.23 |
| | | 3.0 | 3.1 | 5.09 | 2.22 |
| | HCT | 30.0 | 29.5 | 6.01 | −1.67 |
| | | 720 | 715 | 1.84 | −0.69 |
| Stability at room temperature for 12 h (after extracting and reconstitution) | VAL | 12.0 | 11.2 | 1.54 | −7.06 |
| | | 120 | 116.3 | 6.57 | −3.06 |
| | | 2880 | 2680 | 0.67 | −6.96 |
| | | 3.0 | 2.8 | 2.67 | −7.20 |
| | HCT | 30.0 | 27.5 | 1.18 | −8.24 |
| | | 720 | 689 | 1.56 | −4.29 |

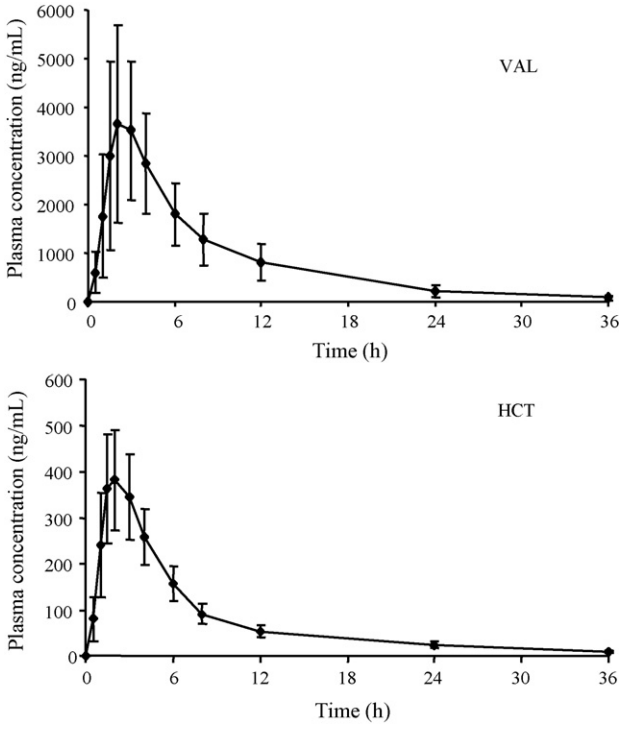


Fig. 4. Mean plasma concentration–time curves for VAL and HCT after an oral administration of a dispersible tablet containing 80 mg VAL and 12.5 mg HCT to each of 20 healthy volunteers. Error bars represent standard deviation.

Table 3
Mean pharmacokinetic parameters for VAL and HCT after an oral administration of a dispersible tablet containing 80 mg VAL and 12.5 mg HCT to each of 20 healthy volunteers

| Parameters | VAL | HCT |
|---|---|---|
| $T_{max}$ (h) | 2.5 | 2.0 |
| $C_{max}$ (ng/mL) | 4245 | 426.6 |
| $AUC_{0-\infty}$ (ng × h/mL) | 30903 | 2837 |
| $T_{1/2}$ (h) | 6.9 | 8.9 |

HCT are summarized in Table 3. The results show that this simple and selective method for the simultaneous determination of VAL and HCT in human plasma was readily applicable to the clinical PK study with combinational drug therapy containing VAL and HCT, or to plasma concentration monitoring for patients suffering from hypertension.

## 4. Conclusions

A robust, sensitive and high throughput LC/MS/MS method has been developed for the simultaneous extraction and quantification of VAL and HCT in human plasma. The major advantages of this method are the simple sample preparation, the short run time for high throughput analysis, and the efficiency obtained by analyzing two drugs simultaneously, which are all important characteristics when dealing with large batches of samples. This method has been successfully applied to clinical PK studies of VAL and HCT in healthy volunteers.

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