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Development and validation of a method for quantitative determination of valsartan in human plasma by liquid chromatography-tandem mass spectrometry

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Abstract

A sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the determination of valsartan in human plasma was developed and validated. A 0.5 ml aliquot was extracted using solid-phase extraction in an Empore® high performance extraction disk plate, universal resin 96-well format. The estimated calibration range of the method was 2–2000 ng/ml.

The method was fully validated with intra-day mean accuracy and precision of 94.8–107% and 2.19–5.40% and inter-day mean accuracy and precision of 93.5–105% and 1.87–5.67%, respectively. No significant loss of valsartan in processed samples was confirmed in processed samples for up to 24 h at 10 °C. Sample dilution up to 50-fold with blank human plasma provided acceptable analyses. No interference peaks or matrix effects were observed. No effect of QC sample location results was observed in a 96-well plate. This LC-MS/MS technique was found to improve quantitative determination of valsartan allowing its pharmacokinetic evaluation with clinically relevant doses.

Keywords: Liquid chromatography-tandem mass spectrometry; Valsartan; Human pharmacokinetics; Matrix effects; Inter-subject variability

1. Introduction

Valsartan (*N*-valeryl-*N*[[2′-(1H-tetrazol-5-yl)biphenyl-4-yl] methyl]valine, Fig. 1) is an orally active, potent and specific competitive angiotensin II antagonist acting at the ATI receptor, which mediates all known effects of angiotensin II on the cardiovascular system. Valsartan is widely used in the treatment of hypertension [1]. The drug in unchanged form shows strong pharmacologically activity with high affinity for the ATI receptor. Valsartan is metabolized only to a small extent (ca. 10%) and even its most abundant metabolite (M1) possesses negligible affinity for the ATI receptor (1/200 that of valsartan). In order to determine the relationship between exposure and receptor response, an analytical method for unchanged valsartan with high accuracy is of great importance. The pharmacokinetics (PK) of valsartan in human shows rapid absorption with a peak plasma concentration reached about 2 h after administration and a terminal elimination phase with a half-life of about 7 h [2]. There is considerable individual variability (CV of ca. 30%) in peak plasma concentrations (C_{max}) and area under the curve (AUC) [3]. Therefore, an analytical method for the determination of valsartan in human plasma that is not affected by inter-subject sample deviation is required for appropriate PK evaluation.

Several analytical methods for the determination of valsartan in human plasma by high performance liquid chromatography (HPLC) have been reported [4–7]. These methods utilize a fluorescence detector (FP) after extraction from plasma by solid-phase extraction (SPE) [4,5] or liquid–liquid extraction [6]. They require long chromatographic run times of more than 10 min/sample. Recently, a HPLC/FP analytical method with short analysis run time (ca. 3 min/sample) has been reported [7]. This method, however, has a lower limit of quantification (LLOQ) of only 100 ng/ml. Available PK profiles were only provided for the higher-dose (160 or 320 mg) subjects. In order to eliminate these obstacles, we selected a liquid

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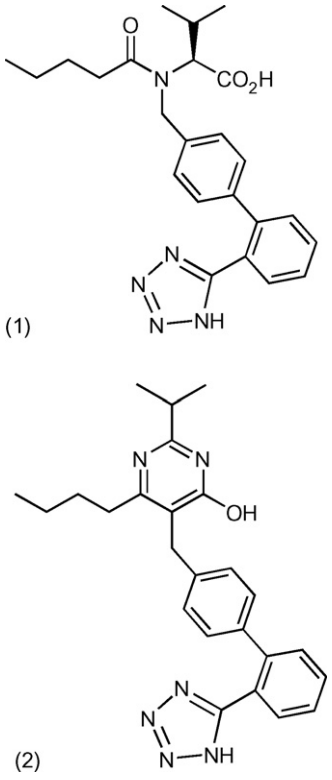

Fig. 1. Chemical structures of (1) valsartan and (2) its analytical internal standard (CGP48791).

chromatography-tandem mass spectrometry (LC-MS/MS) as an analytical instrument. The target features of the method are high sensitivity (<LLOQ of a 5 ng/ml) and short analytical run time (<10 min). For achieving high throughput before LC-MS/MS analysis, solid-phase extraction (SPE) in a 96-well format was also investigated.

The reliability of quantitative LC-MS/MS data obtained from determination of drugs in biological matrices can be adversely affected by endogenous components in biological fluids. Matrix effects are a major issue in quantitative bioanalytical examination using LC-MS/MS. Taylor reported that assessment of matrix effects from individual matrix sources was required to support quantitative LC-MS/MS analysis. Inter-subject variability within 15% at each quality control (QC) level (expressed as % relative standard deviation; %R.S.D.) has been proposed as an acceptance criterion [8]. This approach has been used and found practical in our previous study [9]. If an analytical method is compromised by matrix effects, its results may not be reliable. In the present study, matrix effects were also evaluated based on Taylor's approach.

Assessment of intra-well variability in a 96-well format has been reported [10–12]. Likewise, intra-well variability was evaluated in our validation study. The 96-well format SPE was used for simplification of sample preparation procedure.

The purpose of the present study was to develop and validate a simple, accurate, reproducible and sensitive analytical method for the determination of valsartan in human plasma using a 96-well format SPE for the sample preparation procedure and an LC-MS/MS instrumentation without individual or intra-well variability. The analytical method established was to be applied to samples obtained from a PK study in healthy volunteers who received oral dose of valsartan.

2. Experimental

2.1. Chemicals and reagents

Valsartan and CGP48791 (analytical internal standard, IS, Fig. 1) were supplied by Novartis Pharma AG (Basel, Switzerland). Methanol and acetonitrile (HPLC grade) were purchased from Kanto Chemical Co Inc. (Tokyo, Japan) and trifluoroacetic acid (HPLC grade) from PIERCE (Rockford, IL, USA). Water was deionized and purified on a Millipore water purification system (Milford, MA, USA). Human heparinized plasma was purchased from the New Drug Development Research Center (Iwamizawa, Japan).

2.2. Calibration standards (CS), quality control (QC), mock samples and dilution test samples

Stock solutions for CS and QC were prepared separately in methanol. CS samples were prepared by spiking into heparinized human plasma of respective stock solutions of valsartan to yield concentrations of 2, 5, 20, 100, 500, 1000 and 2000 ng/ml. QC samples were prepared at 2, 4, 16, 80, 400 and 1800 ng/ml in the same manner as CS samples. Mock samples were used to ascertain reproducible extraction on the entire 96-well plate. The mock samples were prepared by adding stock solution to blank human plasma aliquots to obtain a final concentration of 80 ng/ml.The results from the mock samples were not used in any other evaluations.

Dilution test samples were prepared by spiking in blank human plasma of respective methanol standard solutions. The concentrations used for the dilution test were 1800 (the highest QC sample), 5000 and 10,000 ng/ml.

All prepared plasma samples were stored at −20 °C and all prepared stock solutions were stored at 4 °C.

2.3. Sample preparation

A 500 μl aliquot of each plasma sample was placed in each well of 96-well collection plates along with 50 μl of IS solution (500 ng/ml in methanol/H_2O, 50/50, v/v) and 250 μl of 2% trifluoroacetic acid. The entire volume of the resulting sample was transferred to a 96-well solid-phase extraction plate (Empore® high performance extraction disk plate, universal resin, 3 M, NJ, USA) that had been pretreated with 100 μl of methanol and 500 μl of 1% trifluoroacetic acid. Each well was rinsed, in order, with 800 μl of 1% trifluoroacetic acid, 800 μl of 1% trifluoroacetic acid/methanol (95/5, v/v), 800 μl of 1% trifluoroacetic acid/methanol (80/20, v/v), and 800 μl of 1% trifluoroacetic acid. The sample was eluted into a 96-well collection plate with 500 μl of a methanol/water solution (90/10, v/v). The eluate was evaporated to dryness under a nitrogen stream at 50 °C. The residue was reconstituted in 100 μl of methanol/0.1% trifluoroacetic acid (50/50, v/v), transferred to a 96-well filter plate

(Unifilter, 0.45 μm polypropylene, Whatman, NJ, USA) and centrifuged at 1900 × *g* for 10 min at 4 °C. The filtrate was collected in a 96-well collection plate and 10 μl volume of the filtered samples were applied to LC-MS/MS.

2.4. Liquid chromatography/mass spectrometry

The HPLC system consisted of PU-980 pumps, DG-980-50 degasser, CO-965 column oven and AS-1559 autosampler with a Borwin 1.50 system controller (JASCO, Tokyo, Japan). The HPLC column was a XTerra MS C18, 2.1 mm × 50 mm, 3.5 μm (Waters, Milford, MA, USA) with a preceding guard column (Opti-guard-mini C18, 1 mm × 15 mm, Optimized Technologies, Oregon, OR, USA). The HPLC mobile phase was 0.1% trifluoroacetic acid/methanol/acetonitrile (45/30/25, v/v/v). The flow rate was set at 0.2 ml/min and the column temperature at 50 °C and analysis was performed in the isocratic mode. Total run time was 5 min for each injection. A divert valve was used to discard the LC effluent during the first 2.2 min and the last 0.2 min of each chromatographic run.

The mass spectrometer was a TSQ700 triple quadrupole equipped with an electrospray ionization (ESI) interface (Thermo Fisher Scientific Inc., San Jose, CA, USA). Data were acquired by ICIS (version 8.2.1) and processed by Xcalibur (version 1.0.1). Detection was performed by monitoring the positive ions with selected reaction monitoring (SRM). The theoretical *m/z* values of the precursor $[M+H]^+$ and product ions were set at 436.2 and 291.2 for valsartan and 429.2 and 401.2 for the IS. The following parameters were retained for optimal valsartan detection: spray voltage: 4.5 kV; sheath gas (N_2) pressure: 90 psi; auxiliary gas (N_2): 15 ml/min; capillary temperature: 225 °C; collision energy: −20 V; and collision gas (Ar) pressure: ca. 2.0 mTorr. The scan time for each analyte was set at 1.0 s.

2.5. Validation of the analytical method

The analytical method was validated to determine accuracy, precision, specificity, calibration curve range, and reproducibility according to the FDA guidance for bioanalytical method validation [13].

A seven-point calibration curve was constructed by plotting the peak area ratio (*y*) of analyte to IS versus analyte concentration (*x*). Analysis of CS samples at each concentration was performed in duplicate. Results for blank samples were not used as part of the calibration curve. Slope, intercept and coefficient of determination (r^2) were calculated as regression parameters by weighted ($1/x^2$) linear regression. For determination of intra-day and inter-day data variability, duplicates of reference samples, blank samples, blank samples with IS and CS samples were placed in wells A1 to B8 of 96-well plates. QC samples were randomly placed in B9 to H12 according to a random number table created with Excel 97™ (Microsoft). Any empty wells were filled with mock samples.

Precision and accuracy were evaluated by determining the concentrations in six replicates of each QC sample at six different concentrations on 3 separate days. Each run consisted of CS samples, blank plasma samples with and without the IS in duplicate, and QC samples in six replicates. The analytical range and variability in results between wells in 96-well plate format were evaluated using prepared CS, QC and mock samples as described above. The dilution test was performed with blank human plasma aliquots with concentrations above the calibration range samples (5000 and 10,000 ng/ml) and the highest QC concentration (1800 ng/ml).

Pooled blank human plasma samples with and without the IS were also assayed on each day of analysis. The specificity of the method was investigated by analysing six individual human blank plasma samples. Matrix effects, absolute recovery, process efficiency and inter-subject variability of the method were also investigated by analysing six individual human blank plasma samples. These were evaluated at three valsartan concentrations, 5, 80 and 1800 and 500 ng/ml for the IS, which was used in the assay. The following formulas were used for calculations: matrix effects [(peak area of post-extraction spiked plasma samples)/(mean peak area of neat samples) × 100 − 100], absolute recovery [(peak area of before-extraction spiked plasma samples)/(peak area of post-extraction spiked plasma samples) × 100], process efficiency [(peak area of before-extraction spiked plasma samples)/(mean peak area of neat samples) × 100] and inter-subject variability [%R.S.D.].

The stability of valsartan in extracted plasma samples (4, 80 and 1800 ng/ml) retained on an autosampler (10 °C, 24 h) was investigated. The stability of valsartan in plasma has already been reported (72 h at room temperature, 1 month at −20 °C and three freeze-and-thaw cycles) [4–7].

2.6. Pharmacokinetic study

The method was applied to the determination of plasma concentrations from 24 healthy volunteers following oral administration of 80 mg valsartan. Once-daily 80 mg administration is a clinical regimen for hypertension [1]. Blood samples were collected in heparinized tubes before and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16 and 24 h post-dosing and centrifuged to obtain the plasma fraction. The plasma samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Sample preparation and LC–MS/MS conditions

Since valsartan is a weak acid (pK_a values: 3.90 and 4.73), it exists mainly in non-ionic form in solution at low pH. In this assay, the sample applied to the SPE plate was acidified to lower than pH 2 with a 2% trifluoroacetic acid solution. In non-ionic form, the analyte is retained to the reverse-phase SPE plate enabling sufficient sample clean up (with 1% trifluoroacetic acid/methanol; 95/5 and 80/20, v/v). However, the analyte could easily be eluted with 500 μl of a methanol/water solution (90/10, v/v). Using characterization of the analyte, sufficient absolute recovery from plasma matrix was achieved (63.0–73.0%, Table 1). The evaporated samples were dissolved in 100 μl of methanol/0.1% trifluoroacetic acid (50/50, v/v) for injection onto the LC/MS system.

Table 1
Results of matrix effects, absolute recovery, process efficiency and inter-subject variability for valsartan ($n = 6$, mean ± S.D.) and its analytical internal standard (CGP48791, $n = 18$, mean ± S.D.) in human plasma

	Valsartan concentration (ng/ml)			CGP48791
	5	80	1800	500 ng/ml
Matrix effects (%)	15.4 ± 12.5	11.2 ± 7.36	15.1 ± 6.15	6.22 ± 8.43
Absolute recovery (%)	73.0 ± 7.07	69.5 ± 4.68	63.0 ± 4.75	57.8 ± 5.02
Process efficiency (%)	83.8 ± 7.56	77.1 ± 3.70	72.5 ± 5.51	61.2 ± 4.68
Inter-subject variability (%)	7.11	4.92	3.76	8.69[a]

[a] Expressed as CV% by peak area.

LC separation was performed in isocratic mode. The order of elution of valsartan and the IS could be adjusted by changing the composition of methanol and acetonitrile in the mobile phase. When the methanol content was higher than that of acetonitrile with the same total organic solvent to aqueous phase ratio, valsartan was strongly retained on the reverse phase analytical column. This might have been due to the interaction between the carboxylic group of valsartan (Fig. 1) and the hydroxy group of methanol. We established nearly identical retention times of valsartan and the IS in order to improve accuracy and precision. This investigation on improvement of precision and reproducibility with the modification of mobile phase was reported by Kitamura et al. [14].

Due to the presence of a basic nitrogen in its molecule (Fig. 1), valsartan exhibited favorable sensitivity in positive ion mode detection because of the efficiency of ionization of the analyte. The product ion mass spectrum from $[M+H]^+$ precursor ion (*m/z*: 436.2) with estimated chemical structures of fragment ions is shown in Fig. 2. The intense product ion of *m/z*: 291.2 was selected and the IS product ion (*m/z*: 401.2) was selected in the same manner as for valsartan. Optimization of LC-MS/MS conditions was performed by infusion of the neat solution with HPLC mobile phase. The optimized parameters are indicated in Section 2.

3.2. Selectivity

Assay selectivity was assessed by analysis of drug-free plasma from six individual human subjects and evaluation of the peaks that interfered with valsartan and the IS. Representative chromatograms obtained from blank human plasma and plasma spiked with valsartan and the IS are shown in Fig. 3. No interfering peak was observed in the samples at the retention times of either the analyte or IS. As mentioned in the section above, with adjustment of chromatographic conditions, the retention times of valsartan and the IS were almost the same (3.1 and 3.0 min, respectively, Fig. 3).

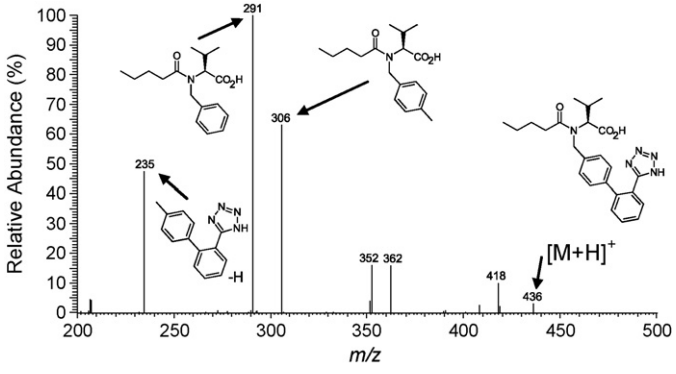

Fig. 2. Product ion mass spectrum of valsartan from $[M+H]^+$ precursor ion (*m/z*: 436.2) and the estimated chemical structures.

3.3. Matrix effects, absolute recovery, process efficiency and inter-subject variability

Matrix effects generally take the form of either ion suppression or ion enhancement, and their magnitude may vary between

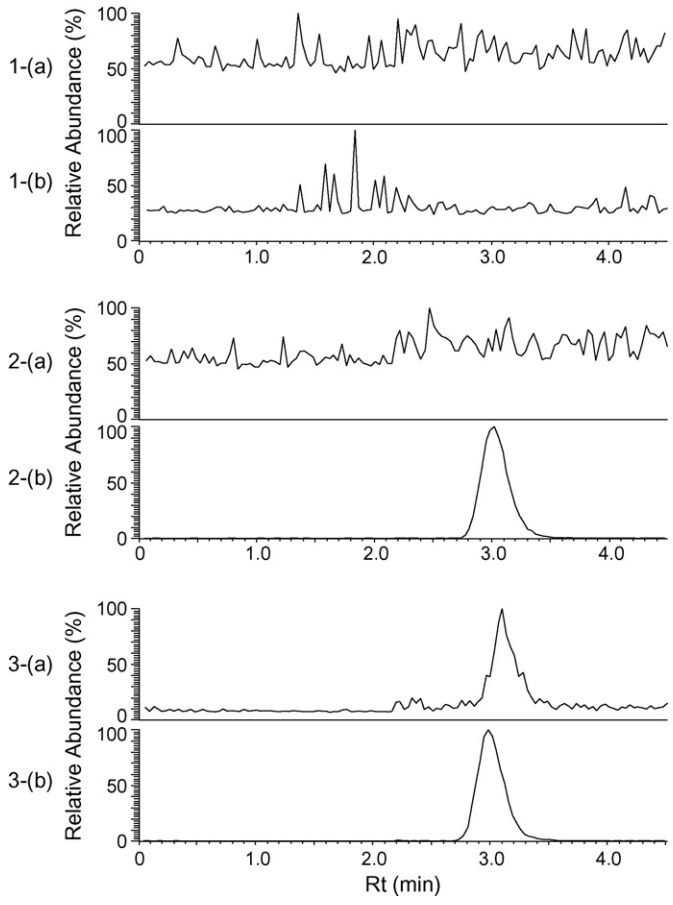

Fig. 3. Representative mass chromatograms from (1) blank sample, (2) blank sample with internal standard (CGP48791) and (3) LLOQ sample with (a) valsartan (*m/z*: 436.2 → 291.2) and (b) internal standard (*m/z*: 429.2 → 401.2) in ESI positive ion and SRM detection mode.

Table 2
Intra-day assay precision and accuracy for valsartan in human plasma ($n = 6$)

Nominal concentration (ng/ml)	Observed concentration (ng/ml, mean ± S.D.)	Precision[a] (%)	Accuracy[b] (%)
2	2.13 ± 0.115	5.40	107
4	4.05 ± 0.191	4.72	101
16	16.7 ± 0.535	3.20	104
80	79.2 ± 1.76	2.22	99.0
400	379 ± 11.0	2.90	94.8
1800	1750 ± 38.3	2.19	97.2

[a] Expressed as %R.S.D.: (S.D./mean) × 100%.
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

Table 3
Inter-day assay precision and accuracy for valsartan in human plasma

Nominal concentration (ng/ml)	Observed concentration[a] (ng/ml, mean ± S.D.)	Precision[b] (%)	Accuracy[c] (%)
2	2.10 ± 0.119	5.67	105
4	3.97 ± 0.209	5.26	99.3
16	16.7 ± 0.445	2.66	104
80	80.3 ± 4.16	5.18	100
400	374 ± 8.53	2.28	93.5
1800	1750 ± 32.8	1.87	97.2

[a] $n = 3$ days with six replicates per day.
[b] Expressed as %R.S.D.: (S.D./mean) × 100%.
[c] Calculated as (mean determined concentration/nominal concentration) × 100%.

sources of plasma. An approach to evaluation of matrix effects, absolute recovery, process efficiency and inter-subject variability has been reported [8,9]. In the present study, we used six different blank plasma samples from healthy subjects at three levels (i.e., each individual plasma was used once at each level) for determining the parameters based on these reports.

The results are shown in Table 1. Matrix effects were in the range of 11.2–15.4% for valsartan and 6.22% for IS. Absolute recovery was in the range of 63.0–73.0% for valsartan and 57.8% for IS. Process efficiency was in the range of 72.5–83.8% for valsartan and 61.2% for IS. Finally, inter-subject variability was in the range of 3.76–7.11% for valsartan (as determined from concentration data) and 8.69% for IS (as determined from peak areas on the mass chromatograms). Although small ion enhancement was observed in matrix effects, the range of the result maintained nearly constant and inter-subject variability also met criterion from the publication [8]. These findings for variability among sources of plasma indicated little or no effect on quantification.

3.4. Linearity of calibration curves and lower limits of quantification (LLOQ)

Weighted ($1/x^2$) least-squares linear regression of ratio of the area of the analyte to that of IS versus concentration was used for calibration. Good linearity was obtained in the range of 2–2000 ng/ml with a coefficient of determination (r^2) at least 0.99. The mean equation (mean ± S.D., $n = 5$) of the calibration curve obtained from seven points was $y = 0.010395416(\pm 0.000482095)x + 0.001602246(\pm 0.000850729)$. The %R.S.D. value for the slopes of the calibration curves was 4.64% ($n = 5$). The LLOQ of 2 ng/ml was determined as the lowest CS and QC concentration that could be measured with a precision less than ±20% and an accuracy of 80–120% from the nominal value (Tables 2 and 3).

3.5. Precision and accuracy

Intra-assay precision and accuracy were assessed by extracting and analysing six replicates of each of the six QC concentrations (Table 2). The intra-assay precision (expressed as %R.S.D.) ranged from 2.19 to 5.40%, while the intra-assay accuracy (expressed as percent of nominal values) ranged from 94.8 to 107%. The inter-assay precision and accuracy were determined by analysing six replicates of each QC concentration in each of three assay runs (total; $n = 18$, Table 3). Method reproducibility exhibited inter-assay precision ranging from 1.87 to 5.67%. Inter-assay accuracy ranged from 93.5 to 105%. We also investigated variability in results among wells in a 96-well plate format using mock samples with a process of randomization and confirmed that there was no difference between each well extract and results for QC samples.

3.6. Dilution by blank matrix

Samples above the upper limit of quantification (2000 ng/ml) were reported from the PK profiles (i.e. C_{max} values) [3]. Therefore, dilution tests with blank matrix for the method were performed. Plasma samples containing 1800 (the highest concentration of QC), 5000 and 10,000 ng/ml of valsartan were diluted 2- to 50-fold with blank plasma and analyzed in quadruplicate. The mean accuracy for nominal concentrations ranged

Table 4
Evaluation for dilution with blank human plasma ($n = 4$)

Nominal concentration (ng/ml)	Dilution factor	Observed concentration (ng/ml, mean ± S.D.)	Precision[a] (%)	Accuracy[b] (%)
1,800	2	1760 ± 38.7	2.20	97.5
1,800	5	1800 ± 32.7	1.82	100
5,000	10	4910 ± 68.5	1.40	98.2
5,000	20	5100 ± 84.6	1.66	102
10,000	20	9420 ± 278	2.95	94.2
10,000	50	9540 ± 240	2.52	95.4

[a] Expressed as %R.S.D.: (S.D./mean) × 100%.
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

Table 5
Stability of valsartan in processed samples on an autosampler at 10 °C (24 h, $n = 4$)

Analyte	Concentration (ng/ml)	Stability (24/0 h) (% of nominal)
Valsartan	4	111
	80	109
	1800	99.9
IS (CGP48791)	500	103

from 94.2 to 102% and precision was within 2.95% (Table 4). The results suggested that samples whose concentrations are greater than the upper limit of the standard curve can be assayed to obtain acceptable data.

3.7. Stability tests in processed samples

Stability tests of the analyte were performed using processed samples on the autosampler at 10 °C. Briefly, the overall stability of valsartan was excellent with % nominal values of 99.9–111%, while that of IS was 103% (Table 5). The results indicated that the practical batch size for assay of clinical samples was a maximum of three 96-well plates, based on the run time of analysis (5 min/sample, 288 samples).

3.8. Application of the method

The analytical procedure described was applied to plasma samples obtained from 24 healthy volunteers who were administered after a single oral dose of 80 mg valsartan. A representative concentration-versus-time profile is shown in Fig. 4. The plasma concentration at 2 h after administration was 2580 ± 1370 ng/ml (mean ± S.D.), while that at 24 h was 94.1 ± 57.9 ng/ml. The wide variability (over 50% of CV) is estimated from individual responses to the drug, and not results of analysis, since our established analytical method exhibits high accuracy and precision without individual variability.

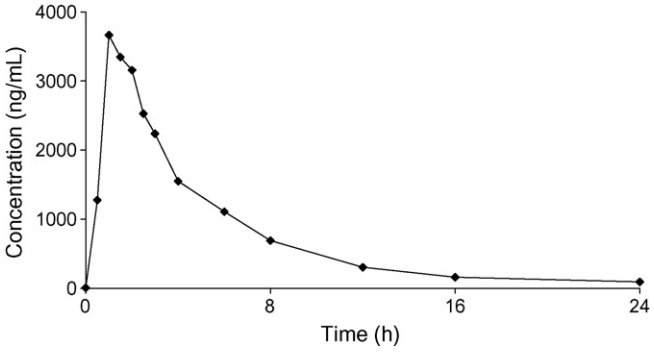

Fig. 4. Representative results showing plasma concentration–time profile for a healthy volunteer following administration of a single 80 mg oral dose of valsartan.

The results for 24 h concentration and ca. 7 h terminal elimination half-life [2] indicate that the LLOQ of the method (2 ng/ml) is applicable to the lowest Japanese clinical dose (20 mg) until 48 h following oral administration.

4. Conclusion

An LC-MS/MS method was developed and validated for the quantitative determination of valsartan in human plasma. The analyte was extracted using 96-well solid-phase extraction plates with no intra-well variability. MS detection was performed with positive SRM and isocratic LC separation was used. The estimated calibration range was 2–2000 ng/ml with practically no interference or matrix effects from endogenous plasma components. Compared to previously reported methods, this method provided higher throughput and much better sensitivity because of its simple sample preparation, short run time, robustness of assay and wide dynamic range.

The method was successfully applied to a clinical investigation of valsartan and proved to be effective in determining plasma concentrations for PK assessment. It is estimated that this method can be used for plasma samples from the lowest clinical dose (20 mg) until 48 h after oral administration.

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