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Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC–ESI mass spectrometry

Kyung-Hwan Yoon[a], So-Young Lee[a], Won Kim[a], Jong-Sei Park[a], Hie-Joon Kim[b,*]

[a] LabFrontier, KSBC Bldg, Iui-dong, Youngtong-ku, Kyunggi-do 442-270, South Korea
[b] School of Chemistry, Seoul National University, Shillim-dong, Kwanak-ku, Seoul 151-747, South Korea

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Abstract

A simple, fast and sensitive high-performance liquid chromatography (HPLC)–mass spectrometric (MS) method has been developed for simultaneous determination of amoxicillin and clavulanic acid in human plasma using terbutaline as internal standard. After precipitation of the plasma proteins with acetonitrile, the analytes were separated on a C_8 reversed-phase column with formic acid–water–acetonirile (2:1000:100) and detected using electrospray ionization (ESI) mass spectrometry in negative selected ion monitoring (SIM) mode. The method was validated and successfully applied to analysis of amoxicillin and clavulanic acid in clinical studies. The limit of quantitation, 0.12 μg/ml for amoxicillin and 0.062 μg/ml for clavulanic acid, was five times lower than that of the published HPLC–UV method.
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Keywords: Amoxicillin; Clavulanic acid; Electrospray ionization LC–MS

1. Introduction

Amoxicillin ((2*S*, 5*R*, 6*R*)-6-[(*R*)-(−)-2-amino-2-(*p*-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate, $C_{16}H_{19}N_3O_5S{\cdot}3H_2O$, MW 419.46) is an analog of ampicillin, derived from the basic penicillin nucleus, 6-aminopenicillanic acid (Fig. 1). For treatment of infection caused by β-lactamase-producing bacteria that are resistant to amoxicillin when administered alone, it is frequently combined with the β-lactamase inhibitor, clavulanic acid. Clavulanic acid administered as potassium salt (potassium (*Z*)-(2*R*, 5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate, $C_8H_8KNO_5$, MW 237.25) is originally produced by fermentation of *Steptomyces clavuligerus*. It inactivates a wide variety of β-lactamases by blocking the active sites of theses enzymes [1].

Analytical methods for amoxicillin and clavulanic acid include microbiological assay [2], enzymatic assay [3], ultraviolet spectrometry [4], and polarography [5]. High-performance liquid chromatographic (HPLC) methods were developed for more specific assay. Early HPLC methods involved pretreatment of amoxicillin and clavulanic acid with imidazoale [6], precolumn [7] and postcolumn derivatization [8] or ion-pairing [9]. Later HPLC methods using reversed-phase or β-cyclodextrin with ultraviolet (UV) or amperometric detection [10–16] have been developed. Capillary electrophoresis (CE) with UV detection was also used for a simultaneous determination of amoxicillin and clavulanic acid in a pharmaceutical formulation [17].

Recently, HPLC separation followed by selective mass spectrometric detection has become a method of choice. Kaye et al. [18] used HPLC–tandem mass spectrometry in a pharmacokinetic study of an amoxicillin/clavulanate formulation; however, they did not describe the mass spectrometric conditions. Reidiker and Stadler [19] used HPLC–tandem mass spectrometry for determination of amoxicillin in bovine milk,

* Corresponding author. Tel.: +82 2 887 3415; fax: +82 2 874 6690.
E-mail address: hjkim1@snu.ac.kr (H.-J. Kim).

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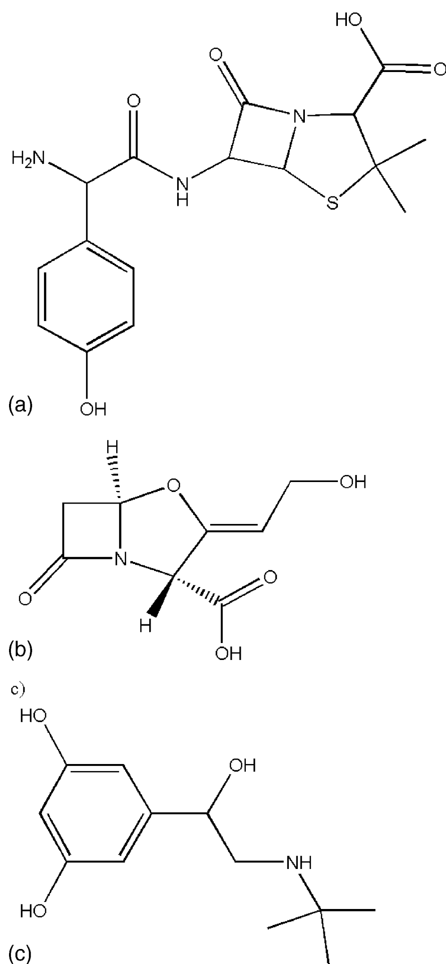

Fig. 1. Structures of: (a) amoxicillin, (b) clavulanic acid, and (c) terbutaline.

and De Baere et al. [20] used it for amoxicillin in animal tissue. Both groups used solid phase extraction as part of sample preparation. In this paper, we present a simple, fast, and sensitive analytical method for simultaneous determination of amoxicillin and clavulanic acid in human plasma using HPLC with ESI mass spectrometry in the negative selected ion monitoring mode. The method was optimized, validated and applied to analysis of plasma samples in a pharmacokinetic study involving 30 healthy volunteers.

2. Experimental

2.1. Chemicals

Amoxicillin trihydrate and potassium clavulanate were provided by Kolon Pharmaceuticals Inc. (Seoul, Korea). Terbutaline was a kind gift from Professor Kyung-Ho Kim (College of Pharmacy, Kangwon National University, Korea). Both amoxicillin and clavulanate were 99.9% pure and terbutaline was 99% pure. HPLC grade acetonitrile and dichloromethane were from Burdick & Jackson (Muskegon, MI, USA). Reagent grade formic acid, acetic acid, trichloroacetic acid, perchloric acid and ammonium acetate were from Junsei Chemical Co. Ltd. (Tokyo, Japan). Deionized water was prepared using Aquamax Ultra (Younglin instrument, Anyang, Korea).

2.2. Calibration and assay

Standard stock solutions of amoxicillin (400 μg/ml), clavulanic acid (400 μg/ml) and terbutaline (100 μg/ml) were prepared in deionized water. Drug-free human plasma obtained from healthy male volunteers was stored at −30 °C and used as blank plasma. A solution of amoxicillin (8 μg/ml) and clavulanic acid (4 μg/ml) in blank plasma was prepared and serially diluted two-fold with plasma six times to make calibration solutions.

Quantitative analyses were performed using internal standard. Standard calibration curves were obtained by the chromatographic area ratios of amoxicillin and clavulanic acid against terbutaline. Concentrations of amoxicillin and clavulanic acid were calculated from their area ratio and the calibration curve.

To 200 μl of plasma sample in 1.7 ml polypropylene tube, 400 μl of 5 μg/ml terbutaline solution in acetonitrile, used to precipitate proteins, was added and the solution was vortex mixed for 10 s. After centrifugation at 17,000 × g for 10 min, the supernatant solution was transferred to a new glass test tube. Five hundred μl of dichloromethane was added to the solution and the solution was vortex mixed for 10 s. After centrifugation at 17,000 × g for 5 min, 2 μl aliquot of the aqueous phase was injected into the LC–MS system.

The accuracy, precision, and detection limit of the method were studied by performing five separate analyses per day for five days at seven amoxicillin and clavulanic acid concentrations. Limit of detection (LOD) was determined from signal to noise ratio (S/N) = 3 and lower limit of quantitation (LLOQ) from S/N = 10. Precision and accuracy of LLOQ was within 20% as required by the Korean Food and Drug Administration.

Recovery of amoxicillin and clavulanic acid was evaluated using standard samples dissolved in water instead of plasma. A standard sample containing 4 μg/ml amoxicillin and 2 μg/ml clavulanic acid was prepared three times according to the sample preparation method stated above, and the result was compared with the theoretical recovery.

For clinical test, 30 healthy male volunteers were given a single oral dose of amoxicillin/clavulanic acid (250/125 mg) (Augmentin®). Plasma samples were obtained by centrifuging blood samples collected before and at 20, 40, 60, 80, 100, 120, 140, 160, 180, 210, 240, 300, 360 and 480 min after intake. Plasma samples were stored frozen at −30 °C until analysis. 450 clinical samples were divided into five batches. Each batch included a seven-point calibration curve before clinical samples and two replicates of three-point quality controls after clinical samples. The precision and accuracy of the intermediate samples were evaluated using quality controls.

2.3. LC–MS

Agilent (Wilmington, DE, USA) 1100 series HPLC system equipped with 1946D single quadrupole MS was used. Silica-based C_8 column (Zorbax RX C_8, 5 μm, 150 mm × 2.1 mm) from Agilent was used. Eluent was prepared by mixing 2 ml of formic acid with 1000 ml deionized water and 100 ml acetonitrile. Flow rate was 0.4 ml/min. Agilent Chemstation was used for data management.

Electrospray ionization was performed using nitrogen as nebulizing gas at 10 l/min flow rate, 30 psig nebulizing pressure, and 350 °C drying gas temperature. Capillary voltage was set at 3000 V. Fragment voltage applied between capillary outlet and the first skimmer produced fragment ions by in-source collision-induced dissociation by nitrogen. Optimum fragment voltage of 120 V was selected after varying between 100 and 160 V. Negative-ion selected ion monitoring (SIM) mode was used to detect *m/z* 364.1 ([amoxicillin–H]$^-$), *m/z* 136.1 ([a fragment of clavulanic acid]$^-$), and *m/z* 224.1 ([terbutaline–H]$^-$).

3. Results and discussion

3.1. Method optimization and validation

Precipitation of proteins from plasma sample, chromatographic separation, and electrospray ionization followed by mass selective detection were the key steps in this method. Therefore, selection of a solvent system commensurate with these steps was critical.

Initially, simple deproteination by acid was tried to prepare the plasma samples for LC–MS. Trichloroacetic acid or perchloric acid could be used for amoxicillin, but clavulanic acid peak disappeared in these acids probably due to precipitation. Deproteination by acetonitrile gave satisfactory results. Two volumes of acetonitrile were enough to remove proteins from the sample.

At neutral or alkaline pH of the mobile phase, where both amoxicillin and clavulanic acid are deprotonated, the silica-based reversed-phase chromatographic separation is inefficient. Volatile formic acid was added to lower the pH of the mobile phase. Other additives such as acetic acid and ammonium acetate were tested, but best results were obtained

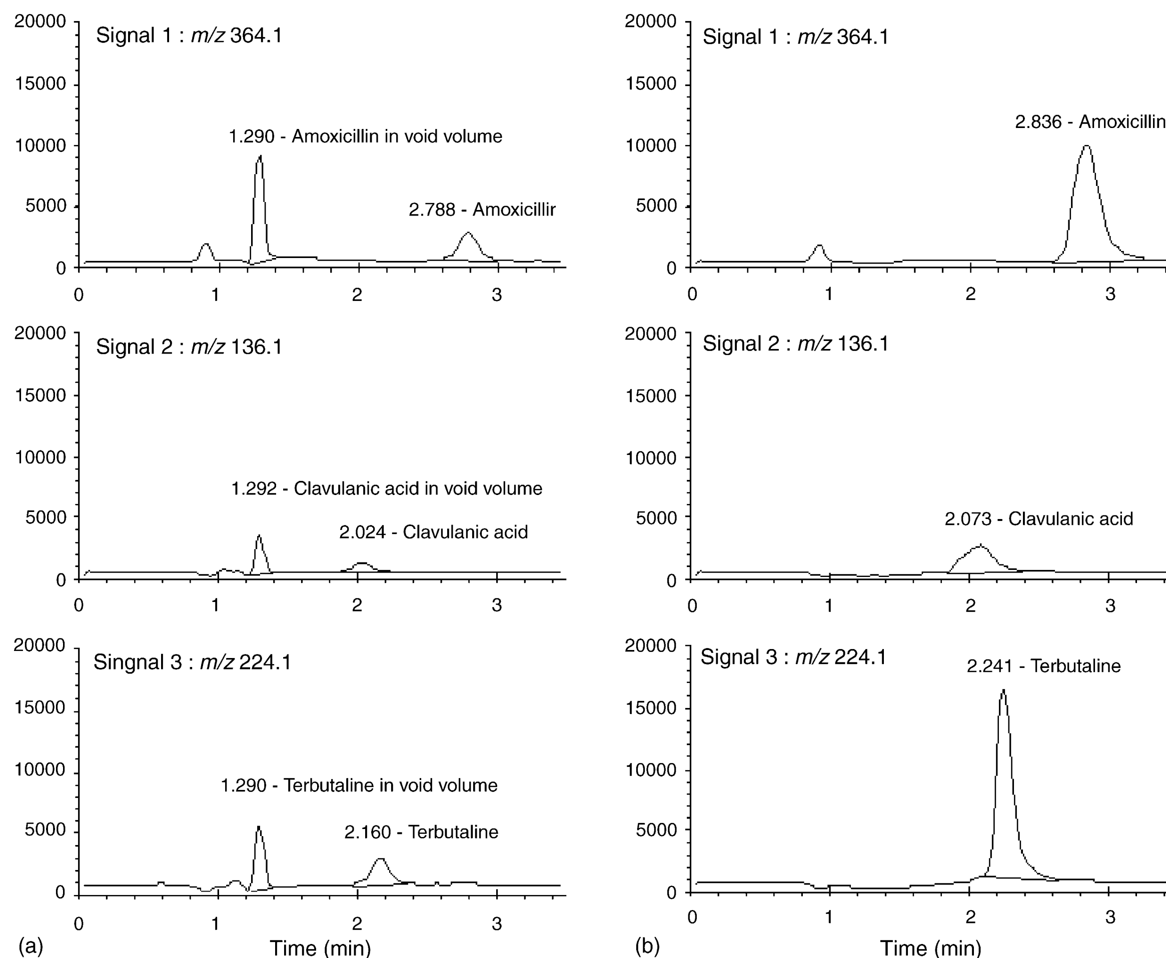

Fig. 2. (a) LC–MS chromatograms showing elution of a portion of the analyte in the void volume due to acetonitrile in the sample solution. (b) Chromatograms showing elution of the analyte as a single peak after removal of acetonitrile with dichloromethane. Peaks were broadened due to larger injection volume.

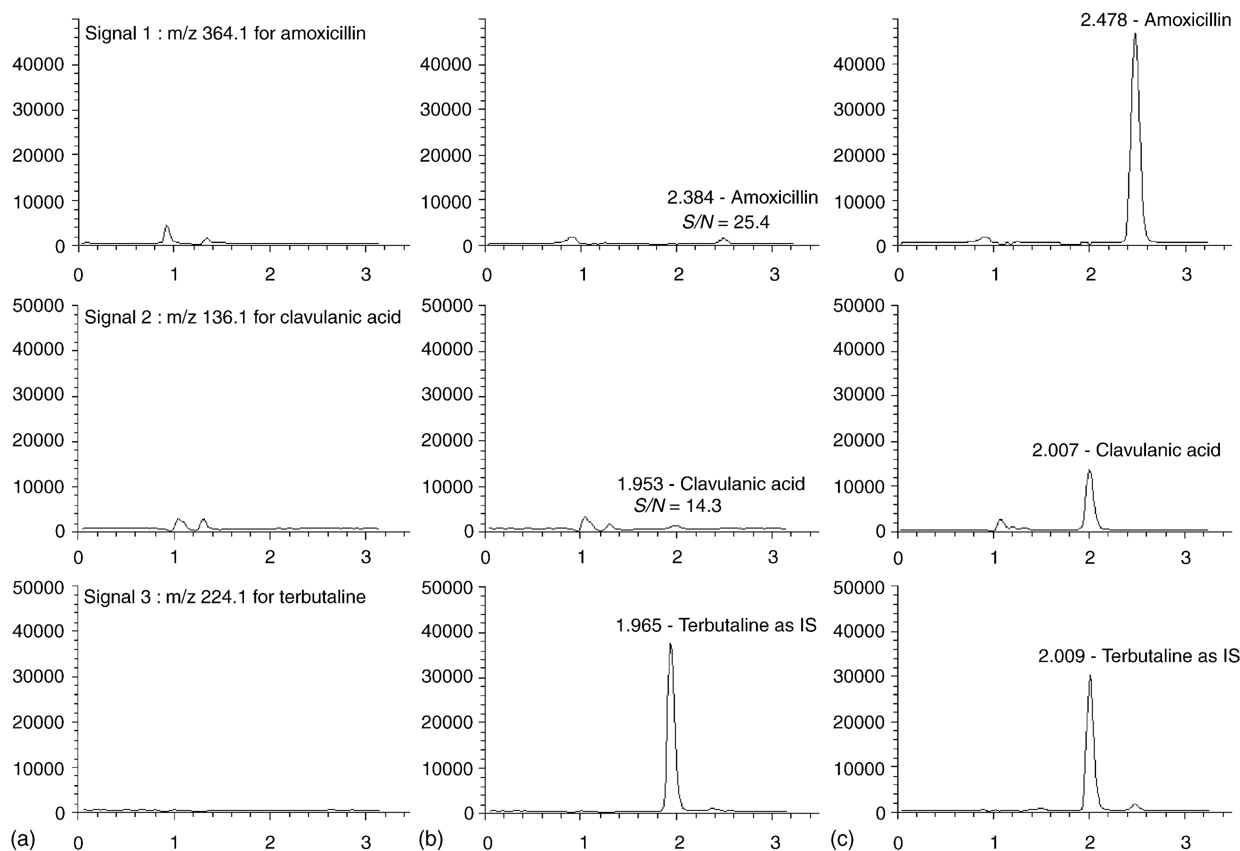

Fig. 3. (a) LC–MS chromatograms from blank plasma. (b) Chromatograms from plasma spiked with 0.125 μg/ml amoxicillin and 0.0625 μg/ml clavulanic acid. (c) Chromatograms from sample plasma, in which amoxicillin and clavulanic acid concentration was 5.5 and 2.1 μg/ml, respectively.

with formic acid. Acetonitrile used in sample preparation was also used in the mobile phase as organic modifier. Methanol could also be used in the mobile phase, but better ESI results were obtained with acetonitrile. Thus, 2 ml of formic acid and 100 ml acetonitrile were added to 1000 ml deionized water to make the eluent. Although terbutaline is not chemically related to the analytes, it was chosen as an internal standard for its stability in both stock solutions and prepared samples. Its chromatographic retention time and peak symmetry were similar to those of the analytes. Terbutaline also showed similar solubility and recovery in sample preparation steps including acetonitrile deproteination and dichloromethane washing. Retention time of amoxicillin, clavulanic acid and terbutaline in the optimized mobile phase was 2.4, 2.0, and 2.0 min, respectively. At terbutaline concentration of 5.0 μg/ml, the chromatographic peak area was similar to that of amoxicillin at its peak concentration in plasma.

Acetonitrile in the sample caused a problem in separation. Even though the injection volume was as little as 2 μl, acetonitrile in the injection volume prevented a significant portion of the analytes from being retained on the column and caused them to be eluted in the void volume (Fig. 2a). In order to reduce the effect of acetonitrile in the sample solution, the sample was diluted with deionized water to make the acetonitrile content the same as in the mobile phase. To maintain the signal intensity, a larger volume was injected and peak broadening occurred (Fig. 2b). Acetonitrile could be extracted with dichloromethane, since acetonitrile dissolves better in dichloromethane than in water. Both amoxicillin and clavulanic acid did not dissolve in dichloromethane. Terbutaline dissolved slightly, but reproducibly. After removal of acetonitrile, the aqueous phase could be injected without any problem (Fig. 3). The analytes thus treated were stable for at least 12 h in solution.

At fragment voltage of 100 V, the default voltage recommended by Agilent, amoxicillin showed a strong signal at m/z 364.1 (Fig. 4), which corresponds to $[M-\mathrm{H}]^-$. Clavulanic acid showed strong signals both at m/z 198.1 for $[M-\mathrm{H}]^-$ and at 397.1 for a dimer ion, $[2M-\mathrm{H}]^-$ (Fig. 5a). Although the dimer ion of clavulanic acid (m/z 397.1) showed strong intensity in a standard sample, it disappeared after preparation of the plasma sample. When m/z 198.1 was used for determination of clavulanic acid, the background ion count was quite high interfering with quantitative analysis of clavulanic acid at low concentrations. The fragment ions of clavulanic acid from in-source collision induced dissociation (CID) were observed at m/z 108.1, 136.1 and 154.1 (Fig. 5b). The m/z 136.1 signal was the strongest when the fragment voltage was 120 V (Fig. 6) and the background ion count was low and stable. Thus, clavulanic acid in plasma could be determined with high sensitivity. At this voltage, m/z 364.1 of amoxicillin still showed a strong signal. Therefore, the fragment voltage was

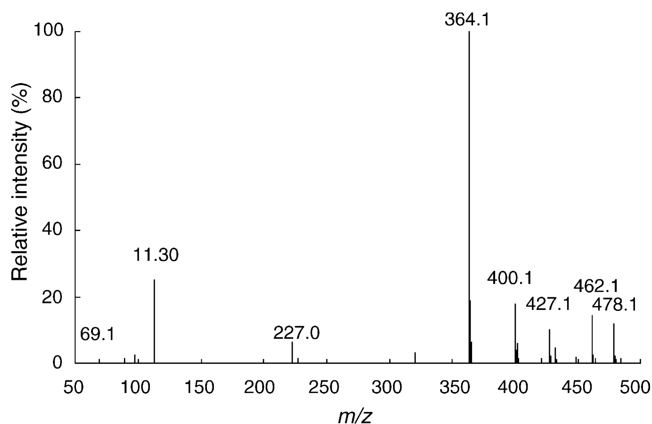

Fig. 4. Negative-ion mass spectrum of amoxicillin obtained using electrospray ionization at fragment voltage of 100 V.

fixed at 120 V and amoxicillin, clavulanic acid, and terbutaline were monitored at *m/z* 364.1, 136.1, and 224.1, respectively.

Fig. 2 shows representative chromatograms, obtained under the optimized conditions using SIM, of blank plasma, plasma spiked with the analytes, and sample plasma from human subjects. All peaks showed good symmetry and high intensity. Using a 150 mm × 2.1 mm column and isocratic elution, all analytes were eluted within 3 min. No other interfering peaks were present near the analyte peaks. This observation is in sharp contrast with the published results using UV detection at 220 nm [14] and clearly illustrates the advantage of monitoring the selected ions. Using UV detection, a longer column (250 mm × 4 mm) was necessary, and even with gradient elution 10 min was required to elute the analytes.

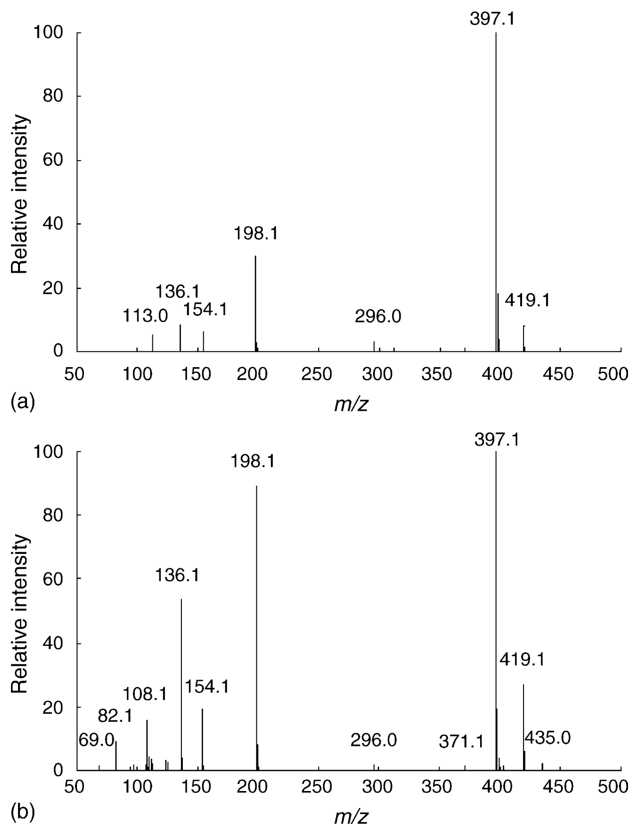

Fig. 5. Negative-ion mass spectrum of clavulanic acid obtained using electrospray ionization at fragment voltage of 100 V (a) and 120 V (b).

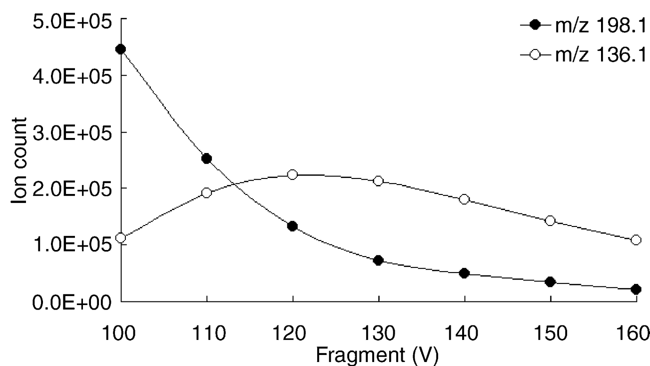

Fig. 6. Ion counts of *m/z* 136.1 and 198.1 signals at different fragment voltages.

A good linear relationship between peak area ratio and concentration was established between 0.125 and 8 μg/ml for amoxicillin and between 0.0625 and 4 μg/ml for clavulanic acid. Average of five determinations was used for each concentration. Following equations were obtained from linear regression: $y = 0.29239x\,(\pm 0.00812) + 0.00197\,(\pm 0.00739)$; $R^2 = 0.99990\,(\pm 0.00056)$, where y is the peak area ratio (amoxicillin/terbutaline) and x is the concentration (μg/ml) for amoxicillin, and $y = 0.36518x\,(\pm 0.00833) - 0.00642$ (± 0.00397); $R^2 = 0.99970\,(\pm 0.00123)$ for clavulanic acid.

The LOD, 0.04 μg/ml for amoxicillin and 0.02 μg/ml for clavulanic acid, was determined as the concentration of analyte at signal to noise ratio (S/N) of 3. And the LLOQ, 0.12 μg/ml for amoxicillin and 0.062 μg/ml for clavulanic acid, was determined at S/N = 10 and from precision and accuracy within 20%. The LLOQ by the HPLC–UV method was 0.615 and 0.313 μg/ml for amoxicillin and clavulanic acid, respectively [14]. Kaye et al. [18] reported LLOQ of 0.05 μg/ml, using a 50 μl aliquot, for both analytes by the HPLC–tandem mass spectrometric method. Considering that our injection volume was only 2 μl, the limit of quantitation of our method appears adequate.

Within-assay and between-assay precision and accuracy determined in human plasma for four concentration sets of amoxicillin and clavulanic acid are summarized in Table 1. All results were within acceptable range of R.S.D. (%) and accuracy (%) for bio-analytical applications.

Recovery of amoxicillin and clavulanic acid was evaluated. As both analytes are soluble in water and insoluble in dichloromethane, they are expected to be recovered with high yield. Actually, 97.5(±2.3)% of amoxicillin and 95.6(±4.2)% of clavulanic acid were recovered.

Although clavulanic acid is known to be highly unstable in blood, Choi et al. [21] showed that no significant change was detected in the concentration of clavulanic acid when

Table 1
Intra- and inter-day precision and accuracy of amoxicillin and clavulanic acid in human plasma

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	R.S.D. (%)	Accuracy[a] (%)
Amoxicillin			
Intra-day ($n = 5$)			
0.125	0.122 ± 0.003	2.59	98.0
0.25	0.261 ± 0.004	1.39	104.6
1	1.038 ± 0.014	1.31	103.8
4	4.136 ± 0.066	1.59	103.3
Inter-day ($n = 5$)			
0.125	0.114 ± 0.014	12.28	91.2
0.25	0.246 ± 0.013	5.40	98.3
1	1.026 ± 0.046	4.48	102.6
4	4.131 ± 0.117	2.83	103.3
Clavulanic acid			
Intra-day ($n = 5$)			
0.0625	0.055 ± 0.001	1.94	88.0
0.125	0.116 ± 0.002	1.65	92.8
0.5	0.517 ± 0.023	4.35	103.4
2	2.129 ± 0.039	1.83	106.4
Inter-day ($n = 5$)			
0.0625	0.056 ± 0.006	10.62	89.9
0.125	0.124 ± 0.007	5.92	99.3
0.5	0.503 ± 0.020	4.02	100.6
2	2.019 ± 0.104	5.14	106.0

[a] Calculated as ((mean observed concentration/nominal concentration) × 100).

stored at −80 °C for 3 months in plasma. We also found no significant reduction in the concentration of both amoxicillin and clavulanic acid when stored at −30 °C for 2 weeks in plasma. Stock and working solutions of amoxicillin, clavulanic acid and terbutaline were all stable at room temperature for at least a week (data not shown).

3.2. Clinical test

After validation, 450 study samples (30 individuals × 15 time intervals) were processed in five batches, each including a seven-point calibration curve and six quality controls. A batch of 105 samples including calibration and quality controls required about an hour for sample preparation and about 8 h for analysis. Two batches were analyzed in a day, and all batches were analyzed in 3 days. Results of the quality controls included in the sample batches, summarized in Table 2, show that the method is reliable.

Table 2
Summary of sample batch quality controls of amoxicillin and clavulanic acid

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	R.S.D. (%)	Accuracy (%)
Amoxicillin ($n = 10$)			
0.250	0.245 ± 0.010	3.91	97.8
1.00	0.984 ± 0.032	3.30	98.4
4.00	3.952 ± 0.117	2.96	98.8
Clavulanic acid ($n = 10$)			
0.0625	0.062 ± 0.001	1.46	99.5
0.250	0.250 ± 0.006	2.44	101.1
1.00	1.014 ± 0.040	3.94	101.4

Table 3
Pharmacokinetics results of amoxicillin and clavulanic acid obtained from 30 healthy male volunteers given a single oral dose of amoxicillin/clavulanic acid (250/125 mg), Augmentin®

	Amoxicillin	Clavulanic acid
C_{max} (μg/ml)	4.4 ± 1.7	1.4 ± 0.7
AUC_{8h} (μg/ml h)	11.7 ± 3.2	3.0 ± 1.5
AUC_{inf} (μg/ml h)	11.9 ± 3.3	3.0 ± 1.5
T_{max} (h)	1.54 ± 0.45	1.44 ± 0.54
$t_{1/2}$ (h)	1.36 ± 0.27	1.24 ± 0.52

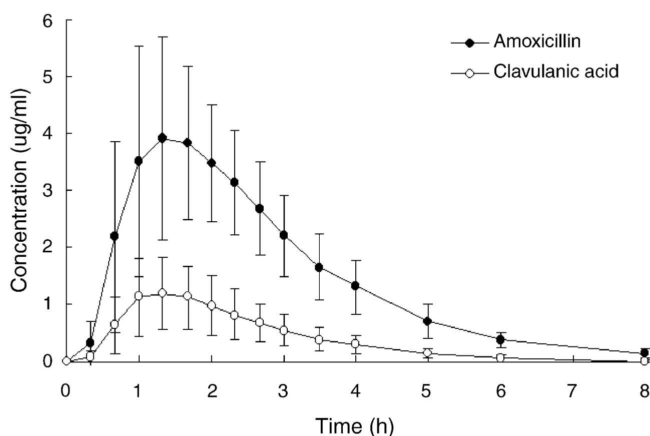

Fig. 7. Pharmacokinetic profile of amoxicillin and clavulanic acid in plasma in 30 healthy male volunteers after oral administration of a amoxicillin/clavulanic acid (250/125 mg) tablet. Each point represents a mean concentration of 30 volunteers.

The pharmacokinetic results for amoxicillin and clavulanic acid in plasma obtained from 30 healthy male volunteers are summarized in Table 3 and Fig. 7. After 8 h, the concentration of amoxicillin and clavulanic acid decreased to 0.130 and 0.065 μg/ml, respectively, which were near the limit of quantitation for both analytes. The area under curve (AUC) at 8 h was almost the same as that for infinite time (Table 3). Therefore, the limit of quantitation of our method was adequate for a pharmacokinetic study. Overall, the pharmacokinetic profile was comparable to the published results [1,14,18].

In summary, in the present ESI LC–MS method the aqueous phase is injected directly into the LC–MS system after precipitation of the proteins from plasma. Reversed-phase separation and mass selective detection takes less than 3 min. Simultaneous determination of amoxicillin and clavulanic acid in human plasma is achieved with high reproducibility and reliability.

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