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Short communication

Simultaneous determination of cefatrizine and clavulanic acid in dog plasma by HPLC

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Abstract

A rapid and specific high-performance liquid chromatographic method was developed and validated for the simultaneous determination of cefatrizine and clavulanic acid in the plasma of beagle dog. The sample pretreatment procedure involved reaction of clavulanic acid with 1,2,4-triazole, which readily produced a derivative with its maximum UV absorption at 314 nm. This derivative was separated in a reverse-phase C-18 column without being interfered by other components present in plasma. Cefatrizine, however, was not derivatized and, therefore determined directly at 269 nm. Sulfanilamide was used as an internal standard. The retention times of sulfanilamide, the derivative, and cefatrizine were, 3.5, 4.9, and 6.0 min, respectively. The assay showed linearity from 2 to 100 μg/ml for cefatrizine and from 1 to 50 μg/ml for clavulanic acid. Precision expressed as R.S.D. ranged from 4.2 to 18.2% for cefatrizine and 5.5 to 15.8% for clavulanic acid. Accuracy ranged from 97.9 to 120% (lower limit of quantitation) for cefatrizine and from 97.7 to 119.2% for clavulanic acid. Extraction efficiencies for cefatrizine, clavulanic acid, and internal standard from dog plasma averaged 79.8 ± 5.8%, 84.8 ± 6.2%, and 89.0 ± 3.8%, respectively. This method was employed successfully to follow the time course of the concentration of cefatrizine and clavulanic acid in beagle dogs following oral administration of cefatrizine and clavulanic acid.

Keywords: Cefatrizine; Clavulanic acid; 1,2,4-Triazole

1. Introduction

Clavulanic acid (Fig. 1), produced by *Streptomyces clavuligerus*, is a potent inhibitor of β-lactamase enzymes which are responsible for the protection of micro-organisms against β-lactam antibiotics [1,2]. Microorganisms which are resistant to certain antibiotics are increasingly posing serious problems in the treatment of infectious diseases [3]. β-Lactam antibiotics are one of the most frequently used antimicrobial agents. However, with the increased prevalence of β-lactamase-producing bacteria, penicillins

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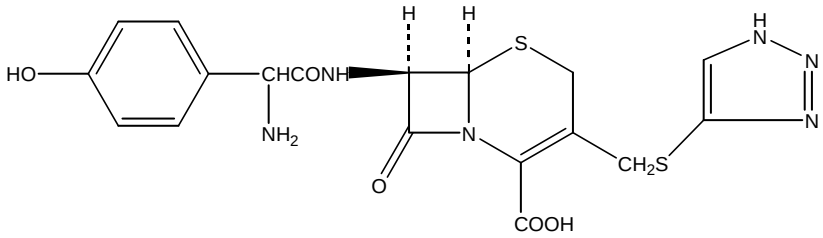
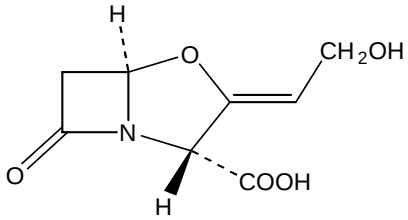


Cefatrizine



Clavulanic acid

Fig. 1. Chemical structures of cefatrizine and clavulanic acid.

and cephalosporins, have become less effective. One modern strategy to cope with these problems is the discovery and development of potent and selective enzyme inhibitors lacking intrinsic antimicrobial activity, such that the antibiotics are protected from the hydrolytic activity of diverse β-lactamases [4–6]. A number of in vitro microbial investigations have revealed that clavulanic acid used in combination with certain penicillins or cephalosporins exhibits marked reduction in minimum inhibitory concentrations against various β-lactamase-producing clinical isolates [5,7,8] with significant broadening of their spectrum against certain bacteria [9,10]. One such formulation is Augmentin, which is comprised of amoxicillin and clavulanic acid which has been reported to give good results in clinical use [11,12]. Cefatrizine (Fig. 1) is an orally active semisynthetic cephalosporin antibiotic with broad-spectrum antibacterial activity [13–15] and similar or superior to the other oral cephalosporins in terms of activity against a wide range of Gram-positive and Gram-negative bacteria [16–19]. Like other β-lactam antibiotics, cefatrizine is degraded and becomes inactive by various types of β-lactamases.

Cefatrizine/clavulanic acid in combination showed a good activity against laboratory strains of Gram-positive and Gram-negative bacteria and exhibited an excellent antibacterial activity not only against β-lactamase-producing strains but extended-spectrum β-lactamase-producing strains [20,21]. Based on these results, new formulations containing cefatrizine and clavulanic acid could be developed to exploit this strongly synergistic effect. However, no methods have been reported for the simultaneous determination of cefatrizine and clavulanic acid in biological samples. The present paper describes an isocratic reversed-phase HPLC method to simultaneously quantify cefatrizine and clavulanic acid in beagle dog plasma employing a combination of published methods [22–24]. Sulfanilamide was used as an internal standard. This method was validated with respect to accuracy, precision, selectivity, and limits of quantitation (LOQ) and detection (LOD) according to Good Laboratory Practice Guidelines [25,26].

2. Experimental

2.1. Equipments

The chromatographic system consisted of Hitachi D-7000 system manager software (Hitachi, Ibaraki, Japan), Model 7725 injector (Rheodyne, Cotati, CA, USA) fitted with 50-μl sample loop, Model L-7100

pump (Hitachi), D-7000 interface module (Hitachi), and Model L-7450 photodiode array detector (Hitachi). The wavelengths were set at 269 nm for cefatrizine, 314 nm for clavulanic acid, and 313 nm for sulfanilamide (0.005AUFS). HPLC separation was performed on a Crestpak C18S analytical column (150 mm × 4.6 mm i.d.) packed with 5 μm diameter particles (Jasco, Tokyo, Japan).

2.2. Reagents and materials

Clavulanic acid and cefatrizine, used as its potassium salt and its propylene glycol form, respectively, were kindly provided by Dong-A Pharmaceutical Company (Seoul, Korea). Sigma Chemical Co. (St. Louis, MO, USA) was the supplier of 1,2,4-triazole. Other chemicals were of reagent grade or HPLC grade. All the water used was purified by the Milli-Q-Grade water system (Millipore Bedford, MA, USA). An 1,2,4-triazole reagent solution was made as follows: 6.9 g of 1,2,4-triazole was dissolved in 30 ml of water, and the pH of the solution was adjusted to 9.00 ± 0.05 using 4 M NaOH. After diluting to a final volume of 50 ml, the reagent solution was routinely used for the derivatization of clavulanic acid in buffer solutions and in dog plasma samples.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of 0.01 M phosphate buffer and acetonitrile (91.5:8.5, v/v) adjusted to pH 3.5 with phosphoric acid. This mobile phase was filtered through a 0.45-μm HV filter (Millipore), then deaerated ultrasonically prior to use. Derivatized clavulanic acid, sulfanilamide, and cefatrizine were quantified by diode array detection following HPLC separation at 314, 313, and 269 nm, respectively. Elution rate and injection volume were 1 ml/min and 50 μl, respectively. A guard column (Whatman column survival kit) containing identical packing material to that in the analytical column was used. All chromatographic operations were carried out at ambient temperature.

2.4. Preparation of standard solutions

Stock solutions of cefatrizine (0.5 mg/ml), clavulanic acid (0.5 mg/ml), and sulfanilamide (0.5 mg/ml) were prepared, respectively, in distilled water, methanol, and acetonitrile and stored at −80 °C. Standard solutions of cefatrizine and clavulanic acid in distilled water were prepared by spiking appropriate volume of the stock solution to give the final concentrations of 2, 5, 10, 20, 50, and 100 μg/ml for cefatrizine and 1, 2, 5, 10, 20, and 50 μg/ml for clavulanic acid. They were prepared extemporaneously and employed to prepare calibration standards and working solution containing both cefatriazine (25 μg/ml) and clavulanic acid (10 μg/ml). Stock solution of sulfanilamide was diluted with acetonitrile to give the concentration of 7.5 μg/ml.

2.5. Optimization of derivatization conditions

The factors affecting the generation of chromophore by pre-column derivatization of clavulanic acid with 1,2,4-triazole reagent were carefully studied. The effects of pH (6–12), concentration of 1,2,4-triazole reagent (0.5–4 M), temperature (20–40 °C), and duration of reaction (2–20 min) were investigated to optimize the reaction conditions. Working solutions containing both clavulanic acid (10 μg/ml) and cefatrizine (25 μg/ml) were prepared in the buffer solutions and an aliquot of 50 μl was added into an E-tube with 20 μl of internal standard (sulfanilamide, 7.5 μg/ml in acetonitrile). This solution was reacted with 1,2,4-triazole reagent (50 μl) in a water bath. The E-tubes were then cooled in an ice-water bath, and 50 μl of the reaction mixture was directly injected onto HPLC system. Peak area ratios of cefatrizine and clavulanic acid derivative over the sulfanilamide were recorded.

2.6. Analytical procedure

An aliquot of 50 μl of the clavulanic acid or cefatrizine standard solution was added into an E-tube and evaporated under nitrogen gas. To this residue was added 50 μl of plasma. After vortex-mixing for 5 min, 20 μl of internal standard (sulfanilamide, 7.5 μg/ml in acetonitrile) and 550 μl of acetonitrile were added and vortex-mixed for 10 s. The precipitated protein was removed by centrifugation for 3 min at 12,000 × g. The supernatant was transferred to a clean E-tube and treated with 750 μl of chloroform. The mixture was again vortexed and centrifuged for 3 min at 12,000 × g.

The lower organic layer was discarded, and an aliquot of 50 μl taken from aqueous upper layer was transferred into E-tube. To this solution was added 50 μl of 2 M 1,2,4-triazole reagent solution (pH 9.0) and reacted for 10 min at 30 °C. After speed agitation, 50 μl of the reaction mixture was injected directly onto the HPLC system.

2.7. Calibration curves and QC samples

A peak area ratio method was used to calculate the concentration of cefatrizine or clavulanic acid in reference to the internal standard. Standard solutions of clavulanic acid and cefatrizine were used to spike blank dog plasma in order to obtain calibration standards at the concentrations of 2, 5, 10, 20, 50, and 100 μg/ml for cefatrizine and 1, 2, 5, 10, 20, and 50 μg/ml for clavulanic acid. The calibration standards were prepared on a daily basis. Calibration curves were obtained by plotting the peak area ratios of either cefatrizine or clavulanic acid to internal standard sulfanilamide versus the nominal concentrations. Standard curves of clavulanic acid and cefatrizine in plasma samples were constructed on three different days to determine the between-run variability of the slopes and intercepts. The regression equations were obtained by the least-squares method using a regression analysis. QC samples for evaluating the accuracy and precision of the method were prepared at concentrations of 2, 7.5, 50, and 85 μg/ml in plasma for cefatrizine and 1, 4, 25, and 40 μg/ml in plasma for clavulanic acid and stored at about −20 °C until analyzed. Quantification of QC sample concentrations was obtained by interpolation from the equations of the regression lines of the respective calibration curves.

2.8. Linearity

From recorded peak areas, the ratios of the drug to internal standard were calculated. Unweighted least-squares linear regression of the peak area ratio as a function of the theoretical concentrations was applied to each standard curve ($y = ax + b$, where x = concentration (μg/ml), y = peak area ratio, a = slope, and b = intercept). The equation parameters (slope and intercept) of each standard curve were used to obtain concentration values for unknown samples.

Concentrations were back-calculated and compared to the nominal concentrations, and the relative concentration residuals (RCRs) were calculated from Eq. (1), where RC is the interpolated concentration and NC is the nominal concentration:

$$\%\mathrm{RCR} = \frac{100(\mathrm{RC} - \mathrm{NC})}{\mathrm{NC}}. \quad (1)$$

2.9. Specificity

The specificity of the method was investigated by screening six different batches of blank dog plasma to check if endogenous components co-eluted with cefatrizine, clavulanic acid and sulfanilamide.

2.10. Precision and accuracy

The precision and accuracy of this HPLC method were established by repetitive analyses of QC samples in plasma against a calibration curve. Each QC sample with three concentration levels was analyzed six times consecutively within day ($n = 6$) to determine within-run precision and accuracy, and once a day for six successive days ($n = 6$) at four concentration levels to determine between-run precision and accuracy. Accuracy was expressed as the recovery (100 × mean back-calculated concentrations/nominal concentrations), while the precision was given by the between- and within-run relative standard deviations (R.S.D.s).

2.11. Determination of the limits of quantitation and detection

The lower limit of detection (LLOD) is the lowest concentration of analyte in the sample that can be detected but not quantified under the stated experimental conditions [25]. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the analyte in the sample which can be measured with acceptable accuracy and precision under the stated experimental conditions [25,27]. The measured concentration of the proposed LLOQ should lie between 80 and 120% of its theoretical concentration and the R.S.D. should be less than 20% on a day-to-day basis [28,29]. LLOD and LLOQ were derived from multiple measurements in the low concentration range and were determined based on the signal-to-noise

approach [30]. The level of approximately three times greater than the noise level was defined as LLOD and the level of approximately ten times of the noise level was used as LLOQ [27,31], which is the lowest concentration point in the calibration curve [32].

2.12. Extraction efficiency

The extraction efficiency was determined three times at QC concentration levels for cefatrizine and clavulanic acid and at the concentration used during the assay for the internal standard (7.5 μg/ml). The representative peak areas of analytes obtained after derivatization reaction from extracted plasma were compared to those obtained directly after derivatization reaction from chromatographic standard solutions prepared in mobile phase at equivalent concentrations.

2.13. Stability study

The stability of working solution containing both cefatriazine (25 μg/ml) and clavulanic acid (10 μg/ml) was assessed immediately after preparation and 24 h after bench-top storage at room temperature (20 °C) and at 4 °C. The stability of stock solutions was studied at −80 °C over a period of 3 months. The short-term stability was tested with QC samples at concentrations of 7.5 and 85 μg/ml for cefatrizine, and 4 and 40 μg/ml for clavulanic acid in plasma over a time period of 24 h at 4 and 20 °C. The stability of the drug in frozen samples (−80 °C) was determined by periodic analysis over 3 months. Prior to their analysis, frozen samples were brought to room temperature and vortex-mixed well and concentrations were determined using a calibration curve. The freezing-thawing stability of the drug at −80 °C was also confirmed. QC samples at concentrations of 7.5 and 85 μg/ml for cefatrizine and 4 and 40 μg/ml for clavulanic acid were thawed and brought to room temperature for 1 h on each of three consecutive days and subsequently analyzed. A drug was considered stable if more than 90% of the intact drug was retained at the end of the study period [33,34]. Each determination was performed in triplicate and the mean percent of the drug remained were calculated.

2.14. Pharmacokinetic study

This overall analytical procedure has been used to determine concentrations of cefatrizine and clavulanic acid in plasma samples from beagle dogs following co-administration of those drugs orally. Male conditioned beagle dogs (weighing 8–10 kg), purchased from the Sam Youk Animal Facility (Kyounggi-Do, Korea) were housed in a light-controlled room kept at a temperature of 22 ± 1 °C and a humidity of 55 ± 10% (College of Pharmacy, Yeungnam University, Dae-Gu, Korea) with food (Sam Yang Company, Seoul, Korea) and water ad libitum. The experiment was carried out after overnight fasting with free access to water. After administration of both cefatrizine and clavulanic acid at a dose of 20 and 10 mg/kg, respectively, in a capsule, approximately 120 μl of blood was obtained from foreleg vein immediately before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 h after dose. Each blood sample was centrifuged immediately at 4 °C and 50 μl aliquot of each plasma sample was stored at −80 °C until HPLC analysis for cefatrizine and clavulanic acid. A standard method [35] was used to calculate the following pharmacokinetic parameters. The area under the plasma concentration–time curve from time zero to infinity (AUC) were calculated by the trapezoidal rule-extrapolation method. The area from the last data point to time infinity was estimated by dividing the last measured plasma concentration by the terminal rate constant.

3. Results and discussion

3.1. Retention times and specificity

Typical chromatograms of blank beagle dog plasma, plasma spiked with 25 μg/ml of cefatrizine and 10 μg/ml of clavulanic acid, and plasma obtained from a beagle dog 60 min after oral administration of cefatrizine (20 mg/kg) and clavulanic acid (10 mg/kg) in the presence of the internal standard are shown in Fig. 2. Observed retention times were approximately 3.5, 4.9, and 6.0 min for sulfanilamide, clavulanic acid, and cefatrizine, respectively. There was no interference in the peaks of sulfanilamide, clavulanic acid, and cefatrizine during the time intervals.

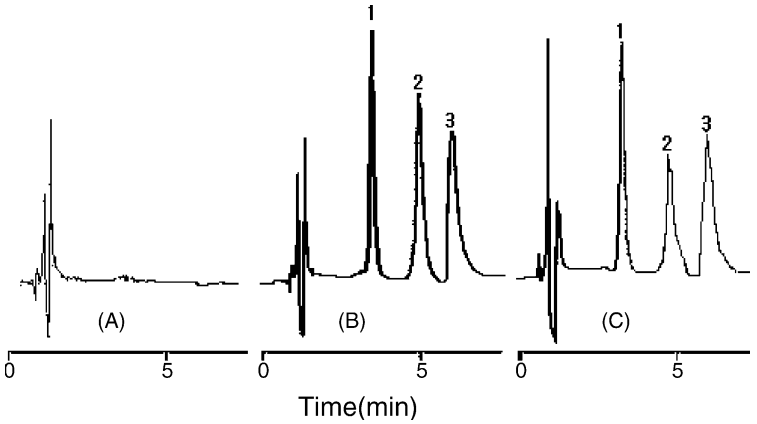


Fig. 2. Chromatograms of blank plasma (A), plasma spiked with 25 μg/ml of cefatrizine and 10 μg/ml of clavulanic acid (B), and plasma obtained from a beagle dog at 60 min after simultaneous oral administration of cefatrizine (20 mg/kg) and clavulanic acid (10 mg/kg) (C) are shown. Peaks 1, 2, and 3 are sulfanilamide (internal standard), clavulanic acid derivative and cefatrizine, respectively. Their retention times were approximately 3.5, 4.9, and 6.0 min, respectively.

3.2. Linearity

The calibration data and calibration curve parameters (Table 1) demonstrate that the calibration curves were linear in the concentration range from 1 to 50 μg/ml for clavulanic acid and 2 to 100 μg/ml for cefatrizine. The linear regression equation is $y = 0.06756x - 0.03917$ with determination coefficient of 0.9991 for clavulanic acid, and $y = 0.01746x + 0.01923$ with determination coefficient of 0.9993 for clefatrizine. Good linearities for both clavulanic acid and cefatrizine were demonstrated in terms of peak area ratios as a function of analyte concentrations. The assay has the necessary sensitivity and linearity to cover the concentration range of clavulanic acid and cefatrizine expected in the beagle dog experiments.

Table 1
Concentrations found from calibration curves

Nominal concentration (μg/ml)	Concentration found (μg/ml)	R.S.D. (%)	RCR (%)
Cefatrizine ($n = 6$)			
2	2.37	15.01	18.67
5	5.46	13.46	9.15
10	9.83	8.21	−1.71
20	20.05	3.25	0.27
50	48.04	4.13	−3.91
100	100.91	3.15	0.91
Clavulanic acid ($n = 6$)			
1	1.19	15.42	19.41
2	2.28	9.10	13.85
5	5.38	4.65	7.61
10	9.79	6.78	−2.11
20	18.95	4.86	−5.25
50	50.41	3.05	0.82

n, number of replicates. RCR, relative concentration residual. R.S.D., relative standard deviation.

For each point of calibration standards, the concentrations were calculated from the equation of the linear regression curves and the R.S.D.s were computed. From 5 to 100 μg/ml for cefatrizine and from 2 to 50 μg/ml for clavulanic acid, R.S.D.s did not exceed 15% (3.05–13.46%); at the lowest concentrations (2 μg/ml for cefatrizine; 1 μg/ml for clavulanic acid), they were 15.01 and 15.42%, respectively. As shown in Table 1, the RCR at the LLOQ (18.67% for cefatrizine and 19.41% for clavulanic acid) were less than 20% and the RCR at the other concentration levels were less than 15%, and thus acceptable [32].

Within-day variability of calibration standards is presented in Table 2. All six validation curves had determination coefficients greater than 0.9983 for cefatrizine and clavulanic acid. The R.S.D. of the slope was 5.10% for cefatrizine and 7.10% for clavulanic acid, showing little day-to-day variability of the slopes. These data clearly demonstrated high reproducibility of the method with excellent linearity.

3.3. Optimization of derivatization conditions

The factors affecting the generation of chromophore by pre-column derivatization of clavulanic acid with 1,2,4-triazole reagent were carefully studied. The effects of pH, concentration of 1,2,4-triazole reagent, temperature, and duration of reaction were investigated to optimize the reaction conditions. Working solutions containing both clavulanic acid (10 μg/ml) and cefatrizine (25 μg/ml) were prepared in the buffer solutions over the pH range of 6–12 and used throughout the optimizing derivatization reaction. Assay samples (50 μl) were reacted with 1,2,4-triazole reagent (50 μl) and 20 μl of sulfanilamide was added and 50 μl of reaction mixture was directly injected onto HPLC.

The formation of chromophore increased with increasing pH up to 9 by the reaction of clavulanic acid and 2 M 1,2,4-triazole reagent at 30 °C for 10 min, above which it decreased (data not shown). Cefatrizine did not degrade in 2 M 1,2,4-triazole reagent solution under pH 9, therefore pH 9 was chosen as an optimal pH for derivatization. The effect of temperature on the reaction of clavulanic acid and 1,2,4-triazole reagent at pH 9 demonstrated maximal efficiency at 30 °C (data not shown). The concentration of 1,2,4-triazole also affected the formation of chromophore in the reaction of the regent and clavulanic acid at pH 9 and 30 °C for 10 min, showing maximal peak area ratio of clavulanic acid derivative over the sulfanilamide at 2 M concentration. Cefatrizine was relatively stable in 2 M concentration of 1,2,4-triazole solution. Incubation time for the reaction was also investigated. At 10 min, the peak area ratio reached maximum and decreased slightly thereafter, probably due to unstable reaction product. Cefatrizine was also shown to be slightly degraded after 15 min reaction that was not significant statistically ($P > 0.05$).

Based on the above results, the optimal conditions for routine use were found to be 2 M 1,2,4-triazole reagent adjusted to pH 9.0 at 30 °C for 10 min.

Table 2
Reproducibility of daily ($n = 3$) calibration curves

Analysis day	Slope	Intercept	Determination coefficient
Cefatrizine			
1	0.0188	0.0122	0.9993
2	0.0176	0.0190	0.9991
3	0.0171	0.0201	0.9983
Mean	0.0178	0.0171	0.9989
S.D. (±)	0.0009	0.0043	0.0004
R.S.D. (%)	5.10	24.856	0.0418
Clavulanic acid			
1	0.0722	−0.0519	0.9990
2	0.0673	−0.0415	0.9992
3	0.0626	−0.0028	0.9999
Mean	0.0674	−0.0321	0.9993
S.D. (±)	0.0005	0.0259	0.0004
R.S.D. (%)	7.10	−80.765	0.0393

n, number of replicates. S.D., standard deviation. R.S.D., relative standard deviation.

3.4. Precision and accuracy

In Table 3, the results for accuracy and within- and between-run precisions for QC samples are presented. The accuracy and precisions were within the acceptable criteria as discussed by Hartmann et al.

Table 3
Assessment of the accuracy and precision

Nominal concentration (μg/ml)	Concentration found (μg/ml)	R.S.D. (%)	Recovery (%)
Cefatrizine			
Within-run ($n = 6$)			
2	2.31	17.10	115.62
7.5	7.85	12.60	104.70
50	51.0	5.27	102.0
85	83.90	4.73	98.67
Between-run ($n = 6$)			
2	2.39	17.40	119.40
7.5	7.89	9.48	105.20
50	48.96	6.77	97.92
85	84.01	4.18	98.83
Clavulanic acid			
Within-run ($n = 6$)			
1	1.17	14.52	116.69
4	4.16	6.24	103.90
25	24.42	8.79	97.68
40	39.25	5.51	98.13
Between-run ($n = 6$)			
1	1.19	15.23	119.15
4	4.29	9.24	107.19
25	24.86	10.15	99.46
40	41.57	7.30	103.91

n, number of replicates. R.S.D., relative standard deviation.

[36] and Hubert et al. [37]. The within-run precision was evaluated by replicate analyses of plasma samples containing clavulanic acid and cefatrizine at four different concentrations. The within-run precision showed R.S.D.s of 4.73–17.1% for cefatrizine and 5.51–14.52% for clavulanic acid. The between-run precision similarly evaluated over six consecutive days at four different concentrations varied from 4.18 to 17.4% (LLOQ) for cefatrizine and 7.30 to 15.23% (LLOQ) for clavulanic acid.

3.5. Extraction efficiency

In plasma, the mean extraction efficiency averaged 79.8 ± 5.8% for cefatrizine and 84.8 ± 6.2% for clavulanic acid, and 89.0 ± 3.8% for the internal standard (Table 4). The extraction efficiency was within the acceptable range with R.S.D. of less than 15% over the range of concentrations measured for cefatrizine and clavulanic acid.

3.6. Limit of quantitation and limit of detection

The LLODs were 0.5 and 1 μg/ml in dog plasma for clavulanic acid and cefatrizine, respectively. The LLOQ for clavulanic acid in plasma was 1 μg/ml and the precision ($n = 6$) at this concentration was 15.23%. The LLOQ for cefatrizine in plasma was 2 μg/ml and the precision ($n = 6$) at this concentration was 18.22%, thus acceptable [28,29].

3.7. Stability

Stock solutions of clavulanic acid and cefatrizine did not reveal any appreciable degradation after 3 months of storage at −80 °C. The stabilities of cefatrizine and clavulanic acid at concentrations tested in working solution were good at both 4 and 20 °C for at least 36 h, with concentration change less than 10%.

When stored at −80 °C for 3 months in plasma, there was no significant change detected in the concentrations of clavulanic acid and cefatrizine with all samples retaining more than 91.2% (Table 5). In plasma samples stored at 4 and 20 °C for 24 h, no significant losses were found for clavulanic acid and cefatrizine, with the recoveries being higher than 90% (Figs. 3 and 4). No significant decrease in concentration of clavulanic acid and cefatrizine in plasma was observed during two freeze-thaw cycles (Table 5). At least two freeze-thaw cycles can be tolerated without losses higher than 10%, whereas after three freeze-thaw cycles a decrease of 15.7% was observed for clavulanic acid at 40 μg/ml. During the stability studies, no additional peaks developed and no changes in the chromatographic pattern were observed in either of the samples.

3.8. Pharmacokinetic study

Fig. 5 shows the mean plasma concentration versus time profiles of cefatrizine (20 mg/kg) and clavulanic

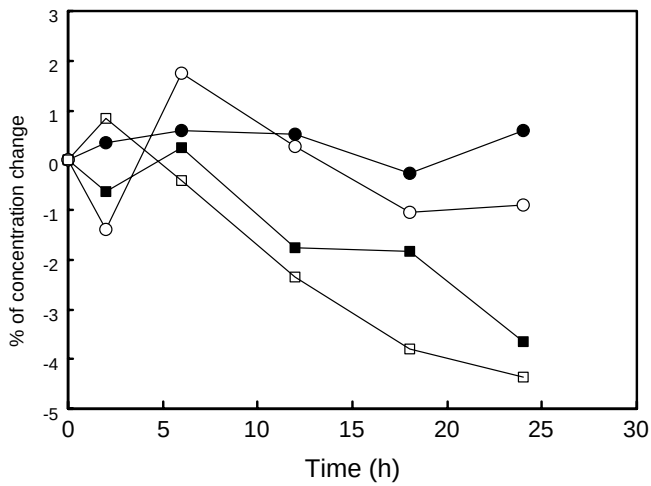


Fig. 3. Stability of cefatrizine in plasma at 4 °C (85 μg/ml, ●; 7.5 μg/ml, ○) and 20 °C (85 μg/ml, □; 7.5 μg/ml, ■).

Table 4
Extraction efficiency from plasma ($n = 3$)

	Cefatrizine (μg/ml)			Clavulanic acid (μg/ml)			Sulfanilamide (μg/ml)
	7.5	50	85	4	25	40	7.5
Recovery mean (%)	79.9	76.3	83.1	85.9	87.3	81.3	89.0
R.S.D. (%)	6.0	11.0	3.0	10.4	6.6	3.9	4.3

n, number of replicates. R.S.D., relative standard deviation.

Table 5
Long-term and freeze-thaw stability of cefatrizine and clavulanic acid in plasma at −80 °C

Drug	Concentration (μg/ml)	Long-term stability		Freeze-thaw stability	
		Storage period (month)	Concentration after storage[a]	Freeze-thaw cycle	Concentration after freeze-thaw cycles[a]
Cefatrizine	7.5	1	102.8 ± 4.9	1	98.7 ± 3.9
		2	100 ± 2.9	2	96.4 ± 4.7
		3	98.5 ± 2.6	3	93.3 ± 2.3
	85	1	98.8 ± 2.4	1	101.6 ± 1.6
		2	96.9 ± 3.4	2	97.8 ± 3.6
		3	97.6 ± 2.0	3	90.7 ± 3.7
Clavulanic acid	4	1	98.3 ± 4.3	1	102.1 ± 3.1
		2	96.2 ± 3.1	2	93.3 ± 3.8
		3	92.0 ± 3.2	3	87.1 ± 5.2
	40	1	101.7 ± 5.4	1	99.3 ± 4.4
		2	97.8 ± 5.0	2	93.0 ± 4.3
		3	91.2 ± 4.4	3	84.3 ± 3.9

[a] Calculated as percentage of the initial concentration and expressed as mean ± S.D. ($n = 3$).

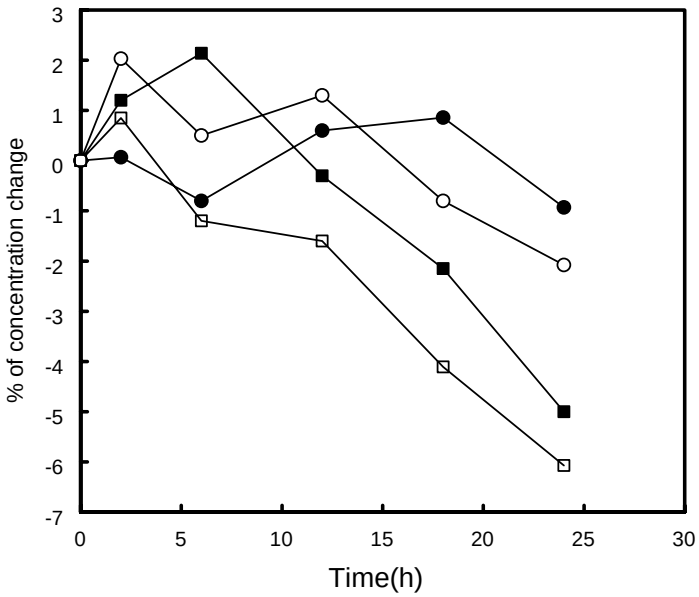


Fig. 4. Stability of clavulanic acid in plasma at 4 °C (40 μg/ml, ●; 4 μg/ml, ○) and 20 °C (40 μg/ml, □; 4 μg/ml, ■).

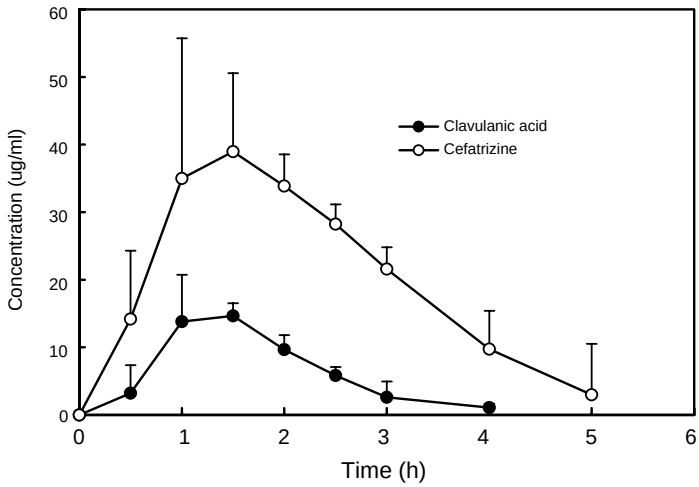


Fig. 5. Plasma concentration–time profiles of cefatrizine and clavulanic acid after oral co-administration to beagle dogs (mean±S.D., $n = 6$).

Table 6
Pharmacokinetic values after oral administration of cefatrizine and clavulanic acid to beagle dogs

Parameters	Cefatrizine (20 mg/kg)	Clavulanic acid (10 mg/kg)
C_{max} (μg/ml)	33.30 ± 10.96	11.90 ± 3.16
T_{max} (h)	1.47 ± 0.25	1.11 ± 0.24
AUC (μg h/ml)	110.77 ± 27.41	32.03 ± 5.09
Half-life (h)	0.80 ± 0.13	0.63 ± 0.11

Values are expressed as mean ± S.D. ($n = 6$).

acid (10 mg/kg) after oral administration to beagle dogs, and some relevant pharmacokinetic parameters are listed in Table 6.

It is hoped that a new antibacterial formulation with cefatrizine and clavulanic acid in combination would be developed employing this HPLC method to determine the pharmacokinetic profiles of cefatrizine and clavulanic acid in beagle dogs used as an experimental model.

4. Conclusions

A reverse-phase HPLC method with ultraviolet detection was developed and validated for the simultaneous quantitation of clavulanic acid and cefatrizine in dog plasma. The results of method validation demonstrated excellent precision and accuracy with acceptable specificity and chromatographic resolution. In addition, all analytes were found highly stable under the conditions of storage, processing and analysis. Good linearity in terms of peak area ratios as a function of analyte concentrations is also demonstrated by the high determination coefficients observed for the regression lines.

The pre-column derivatizing and chromatographic conditions established and validated in the study were simple, reasonable, easily reproducible and sufficiently specific to separate cefatrizine, clavulanic acid and sulfanilamide from other components in plasma samples. To our knowledge, it is the first validated method allowing the simultaneous quantification of cefatrizine and clavulanic acid in dog plasma. The present method is relatively easy to perform and allows to determine simultaneously cefatrizine and clavulanic acid in plasma at the microgram level. Assay performance of the present method was assessed both on the basis of the statistical characteristics of individual calibration curves and from the results of QC samples. Sulfanilamide was regarded as an acceptable internal standard because it exhibits similar extraction and chromatographic properties as the analytes. The LLOQs were 1 and 2 μg/ml in plasma for cefatrizine and clavulanic acid, respectively. The applicability of this newly developed and validated HPLC technique was proved to be satisfactory for pharmacokinetic studies and routine estimation of

clavulanic acid and cefatrizine in beagle dogs, receiving 10 mg/kg of clavulanic acid and 20 mg/kg of cefatrizine as an oral co-administration.

New compounding formulations containing both clavulanic acid and cefatrizine would be developed and tested in beagle dogs employing this assay to improve patient compliance and therapeutic efficacy of cefatrizine.

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References

[1] D. Greenwood, F. O'Grady, P. Baker, J. Antimicrob. Chemother. 5 (1979) 539–547.
[2] C. Reading, P. Hepburn, Biochem. J. 179 (1979) 67–76.
[3] H.C. Neu, Science 257 (1992) 1064–1073.
[4] M.D. Reed, Ann. Pharmacother. 32 (1998) s17–s21.
[5] Y. Yang, B.A. Rasmussen, D.M. Shlaes, Pharmacol. Ther. 83 (1999) 141–151.
[6] E.B. Grant, D. Guiadeen, E.Z. Baum, B.D. Foleno, H. Jin, D.A. Montenegro, E.A. Nelson, K. Bush, D.J. Hlasta, Bioorg. Med. Chem. Lett. 10 (2000) 2179–2182.
[7] S.M.H. Qadri, Y. Ueno, B.A. Cunha, Chemotherarpy 42 (1996) 334–342.
[8] F. Luzzaro, M. Perilli, G. Amicosante, G. Lombardi, R. Belloni, A. Zollo, C. Bianchi, A. Toniolo, Int. J. Antimicrob. Agents 17 (2001) 131–135.
[9] M. Turck, J. Antimicrob. Chemother. 22 (Suppl. A) (1988) 45–62.
[10] P.A. Todd, P. Benfield, Drugs 39 (1990) 264–307.
[11] G.K. McEvoy (Ed.), AHFS Drug Information 95®, American Society of Health-System Pharmacists, MD, USA, 1995, pp. 292–297.
[12] L.A. Miller, K. Ratnam, D.J. Payne, Curr. Opin. Pharmacol. 1 (2001) 451–458.
[13] Y. Kanazawa, T. Kuramata, K. Matsumoto, Jpn. J. Antibiot. 36 (1983) 709–716.
[14] N.A. Carlone, A.M. Cuffini, M. Forno Pizzoglio, G. Cavallo, Drugs Exp. Clin. Res. 11 (1985) 447–451.
[15] P.J. Santella, B. Tanrisever, Drugs Exp. Clin. Res. 11 (1985) 441–445.
[16] J. Verhaegen, L. Verbist, Acta Clin. Belg. 47 (1992) 377–386.
[17] M. Miyazaki, O. Ogushi, A. Kajiki, M. Takamoto, T. Ishibashi, Jpn. J. Antibiot. 34 (1981) 955–958.
[18] K. Ueda, H. Tsuto, T. Yamamoto, Y. Minami, M. Muraoka, T. Tsushima, T. Morihana, K. Nagata, K. Nakao, K. Shimada, Jpn. J. Antibiot. 34 (1981) 1092–1097.
[19] G. Cremonesi, V. Tarantino, M. Stura, M. Ciampini, Clin. Ther. 9 (1987) 263–266.
[20] S.M. Song, J.H. Lee, N.Y. Lee, Kor. J. Clin. Microbiol. 2 (1999) 194–198.
[21] S.H. Choi, J.Y. Kim, G.W. Kim, W.B. Kim, M.J. Shim, K.W. Lee, Y.S. Chong, J. Appl. Pharmacol. 7 (1999) 44–53.
[22] E. Crombez, G. Van Der Weken, W. Van Der Bossche, P. De Moerloose, J. Chromatogr. A 173 (1979) 165–173.
[23] J. Haginaka, H. Yasuda, T. Uno, J. Chromatogr. 377 (1986) 269–277.
[24] J. Martín, R. Méndez, J. Liq. Chromatogr. 11 (1988) 1697–1705.
[25] United States Pharmacopeia 23, Section 1225, Validation of Compendial Methods, United States Pharmacopeia Convention, Inc., Rockville, MD, 1995.
[26] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3–10.
[27] G.W. Peng, W.L. Chiou, J. Chromatogr. B 531 (1990) 3–50.
[28] A. Parra, J. Garcia-Villanova, V. Rodenas, M.D. Gomez, J. Pharm. Biomed. Anal. 12 (1994) 653–657.
[29] H. Mahgoub, F.A. Aly, J. Pharm. Biomed. Anal. 17 (1998) 1723–1728.
[30] H. Rosing, Y.W. Man, E. Doyle, A. Bult, J.H. Beijnen, J. Liq. Chromatogr. Related Technol. 23 (2000) 329–354.
[31] International Conference on Harmonization, Note for Guidance on Validation of Analytical Procedures: Methodology, Committee for Proprietary Medical Products, CPMP/ICH/281/95, Approval 18 December 1996.
[32] S. Braggio, R.J. Barnaby, P. Grossi, M. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375–388.
[33] H. Hill, Proceedings of Analytical Validation: A Practical Approach to Meet Current Regulatory Requirements, Management Forum, London, 1994.
[34] J. Wieling, G. Hendricks, W.J. Tamminga, J. Hempenius, C.K. Mensink, B. Oosterhuis, J.H.G. Jonkman, J. Chromatogr. A 730 (1996) 381–394.
[35] L. Shargel, A.B.C. Yu, Applied Biopharmaceutics and Pharmacokinetics, 3rd. ed., Prentice-Hall, New York, 1993, pp. 47–76.
[36] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowall, J. Pharm. Biomed. Anal. 17 (1998) 193–218.
[37] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135–148.