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Method development for the determination of cefdinir and its related substances by high-performance liquid chromatography

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Abstract

The validation of the HPLC method used for the determination of cefdinir and its related substances is described. The developed method was specific and stability-indicating and provided a linear response with concentration. The system and method precision, expressed as relative standard deviations, were not greater than 1%, and the reproducibilities within and between laboratories were acceptable for the assay method. The procedure can quantitate related substances greater than approximately 0.05% of the principal cefdinir peak.

Keywords: Cefdinir and its related substances, Cephalosporins, Reversed-phase chromatography; Method validation

1. Introduction

Important aspects of HPLC method validation have been reported in many publications [1–5], and validation of analytical procedures has been discussed in the International Conference on Harmonization (ICH). The United States Pharmacopoeia (USP) 23 and 21 Code of Federal Register (CFR) 10.90 specify the various parameters to be evaluated for validating any newly developed method, such as linearity, specificity, accuracy, precision, sensitivity, ruggedness and its stability-indicating nature. In addition, as defined in the British Standard [6], precision under conditions of repeatability (system reproducibility) involves assessment of the variability of the test results, obtained with the same method on identical test material in the same laboratory by the same oeprator using the same equipment within a short interval of time. In contrast, precision under conditions of reproducibility involves assessment of the variability among the test results, obtained with the same method on identical test material in different laboratories with different operators us-

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ing different equipment over a relatively prolonged time interval. Both assessments involve a series of replicate determinations [6]. The sensitivity of the method is a pertinent issue for determining process-related substances contaminating the bulk drug substance and the likely degradation products produced during stability studies. It can be assessed by determination of the quantitation limit, which is a significantly higher level than the minimum determinable level (limit of detection, LOD) of desired analytes [7,8]. Since some of the validation parameters, such as LOD sensitivity and resolution, may change with the operating conditions, the ruggedness of the method must be evaluated by changing various operating conditions. Even though the different terminology has been discussed at the ICH, the terms in the USP were employed in this report.

This paper presents the method validation of well defined chormatographic procedures suitable for the determination of the potency and purity of cefdinir, a new oral cephalosporin antibiotic developed by Fujisawa Pharmaceutical Co. Ltd., and its application to the stability of solid cefdinir under stressed conditions and at room temperature in solution. It also provides actual decision criteria for validation items such as acceptable limits for the y-intercept and so on as a convenient guide for HPLC analysts.

2. Experimental

2.1. Reagents and materials

Cefdinir samples, reference standards, and related substances were provided by the Technological Development Laboratories and Analytical Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. Methanol and dioxane were HPLC grade, water was purified by a Milli-Q system (Millipore Corporation), and all other chemicals were of reagent grade.

2.2. Chromatographic system

A range of equipment was used in the studies to evaluate the ruggedness of the method. The equipment included: auto injectors, Waters WISP 710B, Kyowaseimitsu KST-KMH and Shimadzu SIL-6A with SCL-6A system controller; pumps, Waters 6000A and Shimadzu LC-6A; detectors, Waters 440, Shimadzu SPDs 2A and 6A; automatic data processors, Shimadzu C-R1B, C-R4AX and C-R5A. Citrate (33 mM)–phosphate buffer solution (pH 2.0)–methanol–dioxane (36:4:1, v/v/v) was used as the mobile phase. The mobile phase was filtered and degassed by aspirator before use. Other pertinent HPLC parameters were as follows: detection wavelength, 254 nm; column, TSKgel ODS-80T_M (5 μm, Tosoh); column dimensions, 75 mm × 4.6 mm i.d.; column temperature, 25°C; flow rate was adjusted so that the retention time of cefdinir was about 4 min.

2.3. Sample preparation and quantitation for assay

Cefdinir reference standard (S) and sample (T) were dissolved at 0.2 mg ml^{-1} in phosphate buffer solution (pH 7.0; 0.1 M) containing m-hydroxy benzoic acid (0.7 mg ml^{-1}) as the internal standard. Each solution (5 μl) was injected, and the following equation was used to calculate cefdinir assay values:

$$\text{Purity (\%) of cefdinir} = \frac{Q_T \times C_S}{Q_S \times C_T} \times P$$

where Q_S and Q_T represent the peak area ratio (cefdinir/m-hydroxy benzoic acid) for the cefdinir reference standard and sample respectively. C_S and C_T are the theoretical concentrations of the reference standard and the sample respectively, and P is the purity (% of cefdinir reference standard).

2.4. Sample preparation and quantitative determination for related substances

Cefdinir sample was dissolved in phosphate buffer solution (pH 7.0; 0.1 M) at 10 mg ml^{-1}, diluted with the mobile phase to a final concentration of 0.5 mg ml^{-1}, and 5 μl was injected. The following equation was used to calculate the content of related substance:

$$\% \text{ of each related substance} = \frac{A_I}{A_T} \times 100$$

where A_T and A_I reperesent the total peak area and peak area of individual related substances respectively.

2.5. Stability of cefdinir under stressed conditions

Stability of the solid state cefdinir was demonstrated by storing for 6 weeks at 80°C in a capped glass bottle and for 4 weeks under 30 000 luxes in a Petri dish. The following aqueous solutions of cefdinir were stored at 25°C, water solution (0.01 mg ml^{-1}), the 1st fluid solution (0.05 mg ml^{-1}) and the 2nd fluid solution (0.05 mg ml^{-1}), to assess stability. The 1st and 2nd fluids used were those described under Dissolution Test, General Tests in Japanese Pharmacopeia (JP) XII (1991).

3. Results and Discussion

3.1. Optimization of HPLC conditions

In order to establish the HPLC conditions for determination of cefdinir and its related substances (Table 1), a complete resolution among all the compounds was achieved.

Since cefdinir and its related substances have ionizable functions such as carboxyl, hydroxyimino and amino groups, the reversed-phase HPLC mode was suitable to determine them simultaneously. A fully endcapped TSKgel ODS-$80T_M$ was selected due to its high efficiency and suitability for polar molecules compared with other commercially available octadecyl silanized silica gel packing materials. A Short column (4.6 × 75 mm) was employed to reduce the analysis time to less than 20 min and to achieve the resolution of individual related substances from cefdinir.

Key parameters to optimize resolution were the selection of aqueous buffer pH and organic modifier in the mobile phase. The dissociation constants of cefdinir were as follows: $pKa_1 = 1.9$ (carboxylate); $pKa_2 = 3.3$ (amino group in 2-aminothiazole ring); $pKa_3 = 9.9$ (hydroxyimino). The pH solubility profile of cefdinir was U-shaped with minimum solubility around pH 3. Several phosphate, acetate and citrate–phosphate buffers (pH 2–4) were investigated to obtain good peak shapes and this was achieved at pH 2 with citrate–phosphate buffer. Acetonitrile was not suitable for good resolution between compounds VI and VII (Table 1); methanol, however, was satisfactory. Furthermore, the addition of a small quantity of dioxane improved peak sharpness and resolution between related compounds.

In order to obtain a precise and rugged method, several aromatic compounds were selected and tested as an internal standard. Finally, *m*-hydroxy benzoic acid was selected as the internal standard due to its suitable retention time. Conventional reversed-phase chromatography, ion-suppression mode, provided good resolution between the standard mixture and internal standard substance (*m*-hydroxy benzoic acid) within 15 min. (Fig. 1).

Response factor

The measurement of response factors for each impurity determination is important when the calculations are being made on a relative percent basis. Authentic samples of related substances were dissolved in the mobile phase, and their absorption coefficient at 254 nm was determined using a spectrophotometer. As shown in Table 1, the ratios of absorption coefficient of related substances to cefdinir exist in the range between 0.79 and 1.21. The response factors of each related substance were not considered to be significantly different for the determinations at such low concentrations. Since a lot of unknown related substances including degradation products were present in cefdinir as well as other cephalosporins [9,10], it would be difficult to use response factors for the determination of each impurity.

Effect of column temperature

Differences in resolution due to day-to-day change of ambient temperature were observed. The effect of column temperature on resolution, in isocratic mode, for the separation of some penicillins has been reported [11]. In the study reported here, resolution (R_S) between cefdinir and internal standard decreased when the temper-

Table 1
Cefdinir and its related substances

Substances	Chemical structure	Retention time (min)	Absorption coefficient ($E^{1\%}_{1cm}$) at 254 nm[a]
Cefdinir		4.0	406 (1.00)
I		1.1	453 (1.12)
II		2.3	320 (0.79)
III		2.5	391 (0.96)
IV		0.5	493 (1.21)
V		1.5	411 (1.01)
VI		7.2	391 (0.96)
VII		9.4	371 (0.91)
VIII		0.6	436 (1.07)

[a] The values in parentheses represent the ratio to the absorption coefficient of cefdinir and are considered to be a response factor to cefdinir.

ature was decreased from 30°C to 25°C, and at 20°C $R_S = 3.0$, which is still acceptable. The temperature of 25°C was therefore selected for routine use.

3.2. Assay validation

After optimization of analytical conditions, the evaluation of parameters such as linearity,

repeatability, stability of solution and reproducibility was completed for the validation of the method.

Linearity

The solutions of cefdinir were prepared at concentrations from 0.1 to 0.3 mg ml^{-1}, while that of the internal standard was fixed at 0.7 mg ml^{-1}. The relationship between the peak area ratio (Y) and the concentration ratio (X) was studied. A good linearity ($Y = 6.075X + 0.031$, $r = 0.9999$) was demonstrated within the above concentration range, and the intercept was very close to zero. The result indicates that this assay method can be used with a single point standard.

The expression "the calibration curve is usually obtained as a straight line through the original point" is employed in the JP. However, it does not show any clear limitation for the Y intercept. How much difference in the Y intercept from the zero point is actually acceptable in the linearity study for an assay method? Assay methods are usually applied to content uniformity tests, and each dosage unit should be within ± 15% of the labeled claim even though this interpretation is not absolutely identical to the acceptable limit for the content uniformity test in the JP. If an analytical error of 0.5% is allowable at two extremes (85% and 115%) of limits and the error is exclusively derived from the Y intercept, then 3.3% of the Y value for a nominal concentration of standard solution is the acceptable Y intercept. From the above point, the Y interecpt from the zero point should be within 3% of the Y value for a nominal concentration of standard solution in our laboratories.

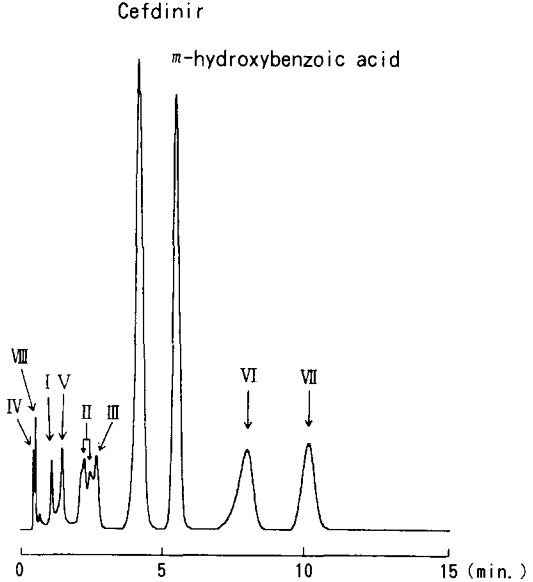

Fig. 1. Typical chromatogram of cefdinir and *m*-hydroxy benzoic acid, spiked with related substances of cefdinir.

Table 2
System and method reproducibilities

Repetition	System reproducibility (peak area ratio)	Method reproducibility [purity (%)]
1	1.2519	97.2
2	1.2467	97.8
3	1.2496	97.3
4	1.2450	97.1
5	1.2448	98.0
6	1.2450	98.6
$\bar{x}$	1.2472	97.7
S.D.	0.00295	0.58
% R.S.D.	0.24	0.59

Repeatability

Precision of the cefdinir assay was characterized by performing six replicate injections of the standard solution for system reproducibility and six replicate assays on the representative sample for method reproducibility. The latter represented the data obtained from six preparations of sample and standard solutions. The precision data obtained by this method are shown in Table 2; RSD values for both system and method reproducibilities were less than 1%.

Stability of solutions

The standard solution containing cefdinir and *m*-hydroxy benzoic acid (internal standard) was stored for 24 h at room temperature (about 25°C) and in a refrigerator (about 4°C), and the remaining percentages of cefdinir and *m*-hydroxy benzoic acid were measured using a freshly prepared standard solution at each test point. The standard solution was stable after storage for 8 h at room temperature and for 24 h in a refrigerator.

Table 3
Assay results of cefdinir[a] (Batch No. CFDN-1) in Analytical Research Laboratories and QC department of the plant

Analytical Research Laboratories				QC department of plant				Statistical analysis[b]
Analyst								
V		W		X		Y		
Day								
a	b	c	d	e	f	g	h	
97.3	97.5	97.5	97.5	97.1	97.5	98.3	97.1	A : 0.5402
97.5	97.1	97.5	97.3	97.3	96.8	96.9	97.4	D : 0.1977
97.2	97.5	97.6	97.0	97.2	97.4	98.1	97.2	A × D : 0.4182
$\bar{x} = 97.4$ R.S.D. = 0.26%		$\bar{x} = 97.5$ R.S.D. = 0.58%		$\bar{x} = 97.4$ R.S.D. = 0.18%		$\bar{x} = 97.4$ R.S.D. = 0.22%		
	$\bar{x} = 97.4$ R.S.D. = 0.46%				$\bar{x} = 97.4$ R.S.D. = 0.20%			
			$\bar{x} = 97.4$ R.S.D. = 0.35%					

[a] Purity (%).
[b] Statistical analyses were conducted by two-way anova using the SAS program. Figures in the statistical analysis column represent P values. When the P value is not more than 0.05, the results show significant difference at the 95% confidence level. A and D mean the results between analyst and day respectively.

Reproducibility
Cefdinir bulk material (Batch No. CFDN-1) was assayed by two analysts on different days in the Analytical Research Laboratories, and it was also assayed by two analysts in the QC Laboratories of the plant. Three replicate determinations were conducted by each analyst on each day in each laboratory. As shown in Table 3, reproducibility was characterized by calculating, within and between laboratories and by total RSDs. The RSDs obtained in each case are not greater than 1.0%, which is much lower than the value (not greater then 2%) recommended in the *Pharmacopoeial Forum*. There are no significant differences in the assay results between analyst (A) and/or day (D) from the results of statisitical analysis. Therefore, the reproducibility of the assay within and between laboratories is considered to be acceptable and establishes the ruggedness of the method.

3.3. *Validation of determination of related substances*

The HPLC conditions for cefdinir assay were intended to be applicable to the determination of related substances to achieve both determinations in the same chromatograph. Parameters such as linearity, limit of detection and quantitation and method reproducibility were evaluated for the validation of the method for the determination of related substances in cefdinir.

Linearity
The solutions of cefdinir were prepared at low concentrations from 0.5 to 25 μg ml^{-1} and at high concentrations from 100 to 2000 μg ml^{-1}, and the relationship between peak area (Y) and concentration (X) was observed. Good linearities [high concentration: $Y = 563X - 2780$ ($r =$ 0.9999); low concentration: $Y = 555X - 8.09$ ($r =$

Table 4
Detection limits and the reproducibility of peak areas at around the quantitation limit

Compound	I	II 1st peak	II 2nd peak	III	IV	V	VI	VII	VIII
Detection limit (%)	0.01	0.01	0.01	0.01	0.005	0.01	0.03	0.04	0.004
Reproducibility of peak areas[a]	0.04	0.07	0.04	0.05	0.05	0.06	0.03	0.06	0.06
	0.04	0.07	0.06	0.06	0.05	0.05	0.04	0.05	0.06
	0.04	0.08	0.05	0.06	0.05	0.05	0.04	0.05	0.07

[a] The results represent the percentage calculated on the peak area of cefdinir.

0.9999)] were obtained within the above concentration range, and the intercept was not significantly different from zero.

Limit of detection and quantitation

The signal-to-noise ratio was determined by comparing the peak heights of the known concentrations of each related substance with that of the baseline noise obtained from the blank samples. A signal-to-noise ratio of 3:1 was empolyed, and the results obtained are summarized in Table 4. The detection limits of individual compounds were different, as expected, due to their peak shape, retention time and extinction coefficient.

The limit of quantitation is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. For instrumental procedures, a common approach is to measure the magnitude of background response by analyzing a number of blank samples and to calculate the mean value of this response. In the USP, the mean background response multiplied by a factor, usually 10, provides an estimate of the limit of quantitation. The limit of quantitation can also be validated by repeated analyses of each related substance at known concentration close to the likely limit of quantitation. In the case of impurity determination, however, several analytes should be simultaneously determined with acceptable precision and accuracy. It is considered difficult to settle the quantitation limit of each realted substance individually in the case of a compound containing a large number of such related substances, because a complicated program for quantitation and calculation has to be employed. Therefore the same levels of quantitation limit for each related substance are preferable for routine analysis.

The sample solution prepared by spiking cefdinir reference standard (1.0 mg ml^{-1}) with related substances (0.0005 mg ml^{-1}) was injected three times and peak areas were determined. The results are presented in Table 4. In spite of differences in individual detection limits, each related substance can be determined with acceptable accuracy and precision at around 0.05% of the nominal concentration of cefdinir.

Reproducibility

The impurity determination of cefdinir bulk material (Batch No. CFDN-1) was conducted by two analysts on different days in the Analytical Research Laboratories and in the QC Laboratories of the plant. Three replicate determinations were performed by each analyst on each day in each laboratory. The results are summarized in Table 5. There is no significant difference between the results on different days by the statistical evaluation, but there is significant difference between the results of different analysts. This discrepancy among the analysts was due to the slight

Table 5
Content of related substances in cefdinir (Batch No. CFDN-1) determined by Analytical Research Laboratories and QC department of the plant

Content (%) of related substances in cefdinir								Statistical analysis[a]
Analytical Research Laboratories				QC department in plant				
Analyst								
V		W		X		Y		
Day								
a	b	c	d	e	f	g	h	
0.97	0.94	0.86	0.86	0.85	0.67	0.96	0.85	A : 0.0001
0.90	0.99	0.82	0.86	0.86	0.65	0.89	1.02	D : 0.0930
0.84	0.97	0.85	0.86	0.87	0.64	0.92	0.88	A × D : 0.0002

[a] Statistical analyses were conducted by 2-way anova using the SAS program. Figures in the statistical analysis column represent P values. When the P value is not more than 0.05, the result shows significant difference at the 95% confidence level. A and D mean the results between analyst and day respectively.

differences in the peak integration with the automatic data processor, especially for the peaks eluted near the solvent front and the peaks close to the quantitation limit. Peak integration is one of the key issues for good reproducibility of impurity determination, therefore reproducibility between laboratories is expected to be achieved by standardizing the peak integration on automatic data processors.

Mass balance

Mass balance should be considered to evaluate the stability data. Good mass balance is defined as follows: the remaining percentage of active ingredient plus the produce amount (%) of degradation product for the stored sample should be 100%. This concept provides a useful scientific guide for evaluating stability data but it is not achievable in all circumstances. As shown in Table 6, a good mass balance of more than 98% was observed in all samples stored in solid and solution states. Considering the specificity of the method, the established HPLC methods for assay and determination of related substances are thought to be stability-indicating.

4. Conclusions

The reversed-phase HPLC method was developed for determination of cefdinir and its related substances.

Cefdinir was separated from eight related substances on an octadecyl silanized silica gel using a mixture of citrate–phosphate buffer–methanol–dioxane as a mobile phase, Fig. 1. UV detection at 254 nm was employed because the relative absorption of related substances to cefdinir at 254 nm was from 0.8 to 1.2. From the results of the investigation on the effect of column temperature for the separation between cefdinir and its related substances, a controlled column temperature of 25°C ± 5°C was employed.

Table 6
Stability of cefdinir in solid and solution states[a]

Sample			Assay (residual %) (A)	Related substances (%) Content	Increased amount[b] (B)	Mass balance (%) ($A+B$)
Solid state	Initial		100.0	0.89	–	–
	80°C 6W		99.2	1.81	0.98	100.18
	30000 lux[c] 4W		97.7	2.82	1.93	99.63
Solution state[d]	Water	Initial	100.0	1.08	–	–
		25°C 7D	91.9	7.56	6.48	98.38
	1st fluid	Initial	100.0	1.36	–	–
		25°C 3D	89.8	9.61	8.25	98.05
	2nd fluid	Initial	100.0	1.08	–	–
		25°C 7D	95.6	4.87	3.79	99.39

[a] W: weeks, D: days.
[b] Increased amount = content (%) of related substance of stored sample minus that (%) of initial.
[c] The light force for the photo-stability test is a mental halide lamp (Mitsubishi, 30000 lux).
[d] Concentration of solution: water, 0.01 mg ml^{-1}; 1st fluid, 0.05 mg ml^{-1}; 2nd fluid, 0.05 mg ml^{-1}.

After optimizing conditions for the HPLC method, method validation studies were conducted. For the assay method for cefdinir, a good linearity was obtained for 50–150% of the nominal concentration and an acceptable Y intercept was determined to be 3% based on the allowable assay error at the extreme of the limit for content uniformity. The system and method precision, expressed as RSDs, were not greater than 1%, and the reproducibilities within and between laboratories were acceptable for the assay method from the results of statistical analyses of a series of replicate determinations.

For determination of related substances, good linearities were found at both high and low concentrations with identical slopes. In addition, relative detection sensitivity of each related substance against cefdinir varied from 0.8 to 1.2. These results supported the view that a simple peak area percentage method could be employed for the calculation. Even though the limit of detection for each related substance varied due to the peak sharpness, a quantitation limit of 0.05% of the nominal concentration could be employed.

For reproducibility studies, there was significant difference between the results of different analysts. Slight differences in peak manipulation between analysts was observed, indicating that standardization of peak manipulation is a key issue for good reproducibility of impurity determination.

Finally, this HPLC method was applied to stability studies of cefdinir under stress conditions. A good mass balance of more than 98% was observed in all samples, suggesting that this HPLC method is stability-indicating.

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