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Improvement in precision of the liquid chromatographic–electrospray ionization tandem mass spectrometric analysis of 3′-C-ethynylcytidine in rat plasma

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Abstract

During the development of the liquid chromatography with electrospray ionization–tandem mass spectrometry for the quantitative determination of 3′-C-ethynylcytidine (I) in rat plasma, ion suppression caused by the matrix components was observed for I and its structural analogue, 3′-C-ethylcytidine (II) as the internal standard. In the method initially designed, I/II peak area ratios varied according to the degree of matrix effect, which led to the poor precision of the assay. From the examination of the ion suppression behavior for I and II, it was assumed that this phenomenon is attributed to the difference in the retention time between I and II. Based on this assumption, therefore, the methanol content in the mobile phase was changed from 5 to 25% so as to make I and II the same retention time. As a result of this modification of the initial method, the precision expressed as relative standard deviation was improved from 5.2–16.2 to 2.7–4.2% in intra-assay and from 6.8–14.9 to 3.5–7.2% in inter-assay validations.

Keywords: 3′-C-Ethynylcytidine; Ion suppression; Electrospray ionization

1. Introduction

Liquid chromatography–tandem mass spectrometry (LC–MS–MS), which offers the advantages of sensitivity and specificity, has become an important tool for the quantitative analysis of drugs in biological fluids in pharmacokinetic studies [1]. The utilization of LC–MS–MS allows the development of bioanalytical methods with simple sample preparation and fast chromatographic analysis, because the sample matrix components are rarely detected in selected reaction monitoring (SRM) of analytes. However, the matrix components coeluting with analytes cannot be completely disregarded in the method development using LC–MS–MS just because they are undetectable.

In Kebarle and co-workers work on electrospray ionization (ESI), which is one of the most popular interfaces for LC–MS, it was reported that ionization of analytes was suppressed by other electrolytes coexisting at high concentration [2,3]. As to the bioanalysis using LC–ESI-MS–MS, the ion suppression for analytes is caused by coeluting sample

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matrix components, often affecting the sensitivity, accuracy and precision of the assay [4–9]. In order to overcome this problem, it will be necessary to remove the interfering matrix components with more efficient sample preparation and chromatographic separation.

A novel nucleoside analogue that acts as an RNA synthesis inhibitor, 3′-C-ethynylcytidine (I, Fig. 1), has potent antitumor activity against several human cancer cells [10–12]. As a part of its preclinical pharmacokinetic studies, an LC–ESI-MS–MS method with cation-exchange solid-phase extraction was applied to quantitative analysis of I in rat plasma. During the method, however, ion suppression due to the coeluting sample matrix components was observed for I and its structural analogue, 3′-C-ethylcytidine (II, Fig. 1), as the internal standard. In addition, the variation of the matrix effect adversely affected was found to decrease not only the reproducibility of I and II peak areas but also the reproducibility of I/II peak area ratios, resulting in inadequate precision of the assay in the validation. Although the interfering matrix components should be removed to improve the precision, in practice it was actually difficult to make the present method so efficient as to remove them because of the high polarity of I.

Therefore, the goal of the present study is to improve the precision in some way other than removing the interfering matrix components. As a first approach to the goal, we explored the origin of the variability of I/II peak area ratios according to the degree of sample matrix effect from the viewpoint of the suppression behavior for I and II.

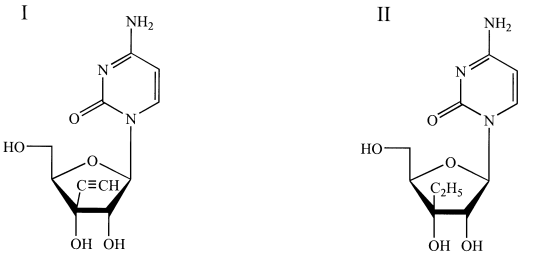

Fig. 1. Structures of 3′-C-ethynylcytidine (I) and 3′-C-ethylcytidine (II).

2. Experimental

2.1. Material and reagents

3′-C-Ethynylcytidine (I) and 3′-C-ethylcytidine (II) were synthesized by Taiho Pharmaceuticals (Tokyo, Japan). Cation-exchange resin, AG 50W-×4 (100–200 mesh, H^+ form) was obtained from Bio-Rad (Hercules, CA, USA). Methanol of HPLC grade, 1 *M* hydrochloric acid of analytical grade and acetic acid and ammonia solution (25% NH_3) of special grade were purchased from Wako Pure Chemical Industries (Osaka, Japan). Purified water from a Milli-Q system (Millipore Japan, Tokyo, Japan) was used.

2.2. Preparation of standard solutions, calibration standards and quality control samples

A stock solution of I was prepared in purified water at a concentration of 100 μg/ml. The stock solution was diluted with purified water to prepare a series of working standard solutions. Calibration standards at 10, 25, 100, 250 and 1000 ng/ml and quality control (QC) samples at 10, 25, 100 and 800 ng/ml were prepared by spiking the appropriate working standard solutions into blank rat plasma. A stock solution of II was prepared in purified water at a concentration of 100 μg/ml. A working internal standard solution at 500 ng/ml for use in sample preparation was prepared by diluting the stock solution of I with purified water.

2.3. Sample preparation

To 0.1 ml of rat plasma, 0.05 ml of the internal standard solution (500 ng/ml II) and 1 ml of 1% acetic acid were added, and the mixture was applied to a cation-exchange cartridge. The cartridge was washed with 4 ml of purified water and then 1.5 ml of 1% ammonia solution. I and II were eluted from the cartridge with 2 ml of 1% ammonia solution. The eluate was evaporated to dryness with a stream of nitrogen gas at about 50°C. The residue was reconstituted in 0.1 ml (initial method) or 0.2 ml (modified method) of purified water, and a 10-μl aliquot was injected into the LC–MS–MS system.

The cation-exchange cartridge for use in the sample preparation was prepared as follows: 0.5 ml of Bio-Rad AG 50W-×4 (100–200 mesh, H^+ form) suspended in 1 *M* hydrochloric acid was packed in an empty cartridge (8 mm I.D.). The packed cartridge was washed with 2 ml of 1 *M* hydrochloric acid, 4 ml of purified water and 1 ml of 1% acetic acid in this sequence.

2.4. LC–ESI-MS–MS analysis

The HPLC system consisted of a Model 616 pump, a Model 717 plus autosampler and a Model 600S controller (Waters, Milford, MA, USA). Chromatographic separation was achieved on an Inertsil ODS-2 (150×2.1 mm I.D., 5 μm) reversed-phased column from GL Sciences (Tokyo, Japan) at a column temperature of 35°C. The mobile phase was composed of methanol–1% acetic acid (5:95, v/v; initial method) and (25:75, v/v; modified method), and the flow-rate was 0.2 ml/min.

A Finnigan TSQ 7000 triple stage quadrupole mass spectrometer (ThermoQuest, San Jose, CA, USA) was used with an electrospray interface at a spray voltage of 4.5 kV in the positive ion mode. Monitored quasi-molecular ion transitions $[M+H]^+$ for I and II in the SRM mode were m/z 268→112 and m/z 272→112, respectively. The heated capillary was maintained at 250°C. Argon was used as the collision gas at a pressure of 0.29–0.33 Pa and the collision energy was set at 25 eV. Nitrogen was served both as the sheath gas at a pressure of 480 KPa and as the auxiliary gas at a flow-rate of 10 units.

2.5. Quantification

Calibration curves were constructed by weighted $(1/C^2)$ least-squares linear regression analysis of I/II peak area ratios versus the concentrations of I. The concentrations in QC samples were calculated from their peak area ratios using the calibration curves. The regression and data analysis were performed using the quantification software Quan Guide compatible with ICIS 8.2 (ThermoQuest).

3. Results and discussion

3.1. Ion suppression

In order to evaluate the sample matrix effect on ion intensities of I and II in the initial method, the peak areas of I and II spiked into the extract from rat blank plasma were compared with the peak areas of intact I and II. The results are shown in Table 1. Both I and II peak areas were lower with the sample extracts than without them, which indicates that ion intensities of I and II were suppressed by the coeluting sample matrix components. Additionally, the ion suppression for I (77.3%) was greater than that for II (59.7%).

Table 1
Sample matrix effect on ion intensities of I and II

Analyte[a]	Peak area[b]		Ion suppression (100–100×B/A, %)
	Standard: (A)	Sample extract spiked with standard: (B)	
Initial method (methanol content in mobile phase: 5%)			
I	29203	6631	77.3
II	67157	27044	59.7
Modified method (methanol content in mobile phase: 25%)			
I	30171	6673	77.9
II	67315	17556	73.9

[a] The injection amounts of I and II were 1 and 2.5 ng, respectively.
[b] Relative value (n=1).

Table 2
Accuracy and precision of the initial method

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	RE[a] (%)	RSD[b] (%)
Intra-assay ($n=5$)			
800	725.5	−9.3	16.2
100	104.2	4.2	5.2
25	24.3	−2.9	6.9
10[c]	8.5	−14.8	6.1
Inter-assay ($n=5$)			
800	712.7	−10.9	14.9
100	97.9	−2.1	6.8
25	22.5	−9.9	14.3

[a] Relative error.
[b] Relative standard deviation.
[c] Limit of quantitation (LOQ).

3.2. Accuracy and precision of the initial method

Accuracy and precision of the initial method were evaluated in the validation study (Table 2). Accuracy is expressed as relative error (RE), and precision is expressed as relative standard deviation (RSD). REs in the intra-, inter-assay and limit of quantitation (LOQ) were from −9.3 to −4.2%, from −10.9 to −2.1% and −14.8%, respectively. All of the RE data could meet acceptable criteria ($<\pm15\%$ except LOQ and $<\pm20\%$ at LOQ) [13]. On the other hand, RSDs in the intra-, inter-assay and LOQ were from 5.2 to 16.2%, from 6.8 to 14.9% and 6.1%, respectively. The highest RSD data, 16.2% (800 ng/ml, intra-assay) could not meet acceptable criteria ($<15\%$ except LOQ and $<20\%$ at LOQ) [13].

3.3. Comparison of matrix effect among QC samples in the initial method

Regarding 800 ng/ml QC samples ($n=5$) in the intra-assay (Table 2), I and II peak areas and I/II peak area ratios of the samples (QC1 with RE exceeding −30% and QC2–QC5 with RE less than $\pm10\%$) are summarized in Table 3. Both I and II peak areas of QC1, which provided the lower I/II peak area ratio compared to QC2–QC5, were lower than those of QC2–QC5. Considering high and reproducible absolute recoveries of I and II from rat plasma ($87.6\pm5.8\%$ for I and $91.6\pm5.9\%$ for II, $n=15$), the difference in the peak areas between QC1 and QC2–QC5 was attributed to the difference in the amount of coeluting sample matrix components that cause the ion suppression. In addition, the ratio of peak area of QC1 to mean peak area of QC2–QC5 for I was lower than that for II, which means that the ion suppression for I is more pronounced than that for II, just as Table 1 shows. It can therefore be concluded that the variation of I/II peak area ratios according to the degree of matrix effect arises from the difference in the degree of ion suppression between I and II.

The difference in I and II peak areas between QC1 and QC2 was investigated in view of I and II peak profiles. As shown in Fig. 2, both I and II peak profiles on QC1, compared with those on QC2, seemed as if the left side of their peaks was cut off. This phenomenon suggested that the ion suppression for I and II was observed mainly at the anterior part of each peak, that is, the anterior part of the I and II peaks chromatographically overlapped with the forward undetectable peaks of the matrix components that cause the ion suppression. Therefore, the result that the ion suppression for I was greater than that for II can be explained on the assumption that the I peak, which elutes earlier than the II peak, overlaps more broadly with the forward peaks of the interfering matrix components than the II peak does.

As discussed above, the variability of I/II peak area ratios among QC samples in the initial method was attributed to both the variation of the matrix effect among the QC samples and the difference in

Table 3
Comparison of I and II peak areas and I/II peak area ratios among QC samples

Identification of QC sample[a] [RE, %[b]]	I/II peak area ratio	Peak area[c] [ratio of peak area of QC1 to mean peak area of QC2–QC5]	
		I	II
Initial method (methanol content in mobile phase: 5%)			
QC1 [−35.1]	1.179	24588 [0.518]	20853 [0.774]
QC2 [−6.0]	1.707	47725	27955
QC3 [−5.8]	1.712	49233	28764
QC4 [0.8]	1.832	43238	23607
QC5 [−0.5]	1.807	49500	27394
Modified method (methanol content in mobile phase: 25%)			
QC1	2.935	11137 [0.304]	3795 [0.287]
QC2	2.728	37920	13901
QC3	2.697	35818	13278
QC4	2.887	35956	12453
QC5	2.777	36691	13215

[a] 800 ng/ml QC samples in intra-assay (Table 2).
[b] Relative error.
[c] Relative value.

the degree of ion suppression between I and II. Furthermore, it was assumed that this difference in the ion suppression arises from the difference in the retention time between I and II.

3.4. Modification of the initial method

Equalizing the ion suppression for I and II to each sample should make it possible to minimize the variation of I/II peak area ratios among the samples, irrespective of the variation of the matrix effect. Therefore, based on the above assumption that the ion suppression for I and II will become equal if the I and II peaks coincide chromatographically, we attempted to make I and II the same retention time. For the purpose of achieving such retention behavior, the methanol content in the mobile phase was increased. As shown in Fig. 3, while the I and II peaks did not completely coincide in the case of the mobile phase composed of 5% methanol (the initial

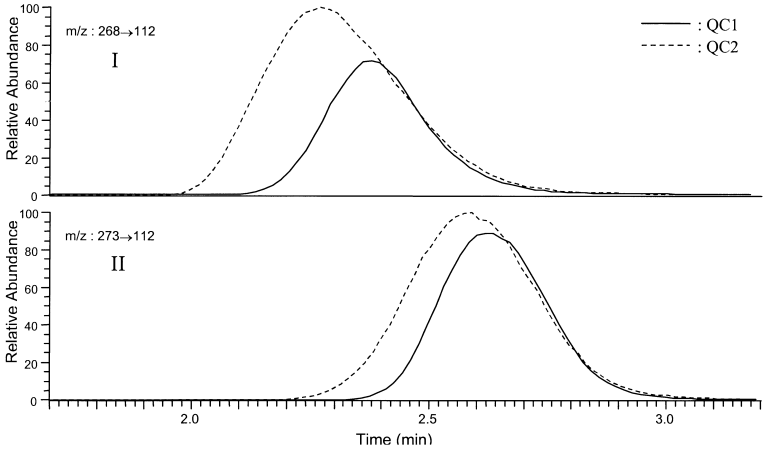

Fig. 2. SRM chromatograms of QC1 and QC2 in the initial method.

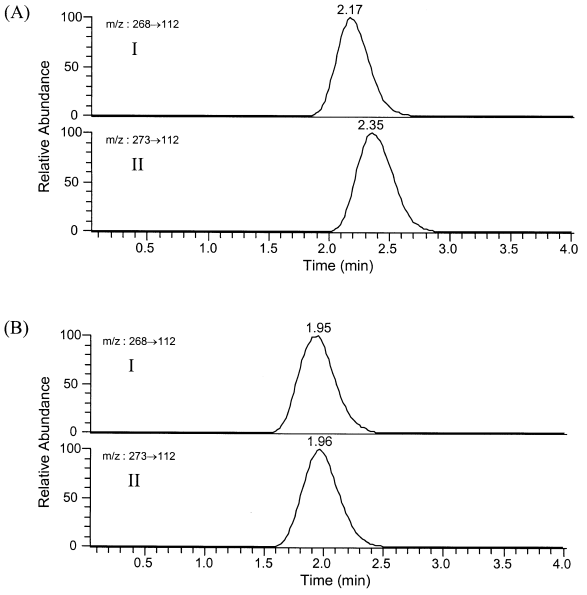

Fig. 3. SRM chromatograms of the standard solutions of I and II using the mobile phases composed of 5% methanol (A) and 25% methanol (B).

method), both peaks were eluted in the approximate void volume (capacity factors of 0.15 and 0.16 for I and II, respectively) and completely coincided by increasing the methanol content to 25%. Additionally, in the use of the mobile phase composed of 25% methanol, no significant difference between the ion suppression for I (77.9%) and that for II (73.9%) was observed (the lower part of Table 1).

The residual reconstituted solutions of 800 ng/ml QC samples which have been measured in the intra-assay of the initial method, were re-injected into LC–MS–MS system using the mobile phase composed of 25% methanol (the modified method). Prior to the re-injection, the reconstituted solutions were diluted twofold with purified water for the purpose of reducing the amount of interfering matrix components, which may increase owing to the change of methanol content. Table 3 lists I and II peak areas and I/II peak area ratios of the re-injected QC samples (QC1–QC5) in the modified method. While the difference in the peak areas between QC1 and QC2–QC5 widened further, the peak area ratio of QC1 approximately agreed with the peak area ratios of QC2–QC5. Thus, the chromatographic coincidence of the I and II peaks allowed equalizing the ion suppression for I and II, leading to minimization of the variation of I/II peak area ratios among QC samples.

3.5. Accuracy and precision of the modified method

Accuracy and precision of the modified method, in which the methanol content in the mobile phase was changed from 5 to 25% and the volume of purified water used for reconstituting was changed from 0.1 to 0.2 ml, were evaluated (Table 4). No such anomalous concentration data as observed for the initial method were observed in this validation study.

Table 4
Accuracy and precision of the modified method

Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	RE[a] (%)	RSD[b] (%)
Intra-assay (n=5)			
800	763.9	−4.5	2.7
100	93.6	−6.4	4.2
25	23.9	−4.6	3.5
10[c]	9.1	−9.4	4.5
Inter-assay (n=5)			
800	810.5	1.3	7.2
100	100.1	0.1	3.5
25	24.7	−1.4	3.6

[a] Relative error.
[b] Relative standard deviation.
[c] Limit of quantitation (LOQ).

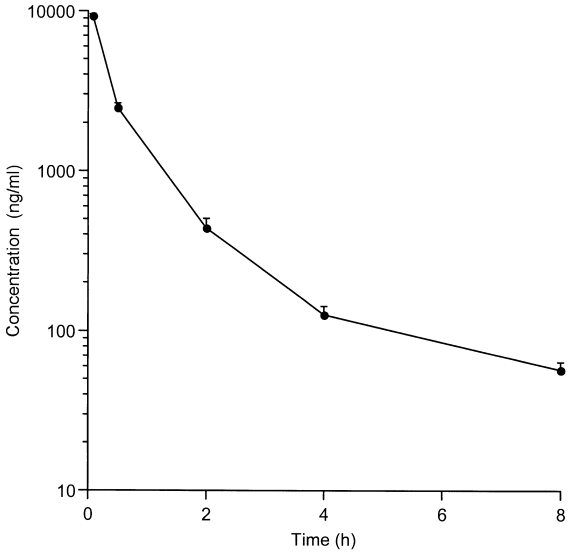

Fig. 4. Mean (+SD) plasma concentrations of I in male rats (n=3) after an intravenous administration of I at 5 mg/kg.

REs in the intra-, inter-assay and LOQ were from −4.5 to −6.4%, from −1.4 to 1.3% and −9.4%, respectively. RSDs in intra-, inter-assay and LOQ were from 2.7 to 4.2%, from 3.5 to 7.2% and 4.5%, respectively. The validation results of the modified method, especially the precision, were quite satisfactory, compared with those of the initial method.

3.6. Application to rat plasma samples

This method was used for the determination of I in rat plasma. Fig. 4 shows the plasma concentration time profile of I in rats after an intravenous administration of I at 5 mg/kg. The plasma concentration of I decreased biphasically in rat.

4. Conclusions

In the bioanalytical method using LC–ESI-MS–MS, the ion suppression for the analyte and/or its internal standard due to the coeluting sample matrix components often adversely affects reproducibility of analyte/internal standard peak area ratios, resulting in the poor accuracy and precision of the assay. One direct solution to the problem is to remove the interfering matrix components with the more efficient sample preparation and chromatographic separation. If the analyte is highly polar such as I, however, in practice it is actually difficult to develop such an analytical method. Another available solution, in this case, is to equalize the ion suppression for the analyte and the internal standard to each sample, enabling minimization of the variation of the peak area ratio among samples without removing the interfering matrix components. The most reliable way to achieve this equalization of the ion suppression is utilization of a stable isotope of the analyte as the internal standard [5–7,14]. And also, allowing that the internal standard is a structural analogue of the analyte, this can be achieved by developing the chromatographic conditions which allow the chromatographic coincidence of coeluting the analyte peak and the internal standard peak, as shown in the present study.

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