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Original

Effect of Thiotriazinone Impurity on Insoluble Microparticle Generation Associated with Ceftriaxone-calcium Salt Precipitation in Original (Rocephin®) and Japanese Generic Ceftriaxone Products

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Abstract: This study examined the effect of thiotriazinone impurity on the generation of insoluble microparticles (IMPs) associated with ceftriaxone-calcium salt precipitation in original (Rocephin®) and Japanese generic ceftriaxone (A; Sawai, B; Nichi-Iko) products when mixed with Ca^{2+} 4.3 mEq/l. We found that the generation rate of IMPs associated with ceftriaxone-calcium salt precipitation among the three ceftriaxone products tested was in the order of generic (A) < original < generic (B), as assessed by light obscuration particle counting. Typically, after 60 min, one of the generic ceftriaxone (B)-calcium mixtures was highly opaque with numerous aggregates of milky-white precipitates, the original ceftriaxone-calcium mixture exhibited noticeable IMPs, and the second generic ceftriaxone (A)-calcium mixture was transparent. The levels of thiotriazinone contaminants, known to be a major impurity in ceftriaxone products, were determined by HPLC and found to be in the order of generic A > original > generic B. Moreover, the addition of a small amount of thiotriazinone into the generic ceftriaxone (B)-calcium mixture significantly decreased the amount of IMPs, suggesting that the impurity retards ceftriaxone-calcium crystal growth. We thus concluded that the thiotriazinone impurity acts as a suppressive factor of ceftriaxone-calcium salt precipitation, and that the high level of thiotriazinone impurity in the ceftriaxone (B) product could underlie its lowest rate of IMP generation when mixed with calcium. We thus recommend caution regarding the clinical risk of ceftriaxone-calcium compatibility due to impurity contamination in ceftriaxone products.

Key words: ceftriaxone, calcium, insoluble microparticles, light obscuration particle counter, generic products

Introduction

Ceftriaxone is a third-generation cephalosporin antibiotic that was introduced worldwide in the early 1980s. It is commercially available as a highly soluble sodium salt; however, it can form

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a poorly soluble ceftriaxone-calcium salt when combined with calcium ions. Thus, in September 2007, the FDA issued an initial alert to healthcare professionals suggesting that ceftriaxone and calcium-containing products should not be coadministered to any patients within 48 hr in order to prevent possible end-organ damage secondary to ceftriaxone-calcium precipitation[1]. In April 2009, the FDA modified this warning to recommend that ceftriaxone and calcium-containing products may be sequentially administered in patients older than 28 days if the infusion lines are thoroughly flushed between infusions with a compatible fluid[2].

The experimental evidence for ceftriaxone-calcium interactions, which became the basis of FDA's alert and subsequent recommendations, was obtained using the original marketed ceftriaxone sodium preparation, Rocephin®[3]. Recently, Tange *et al*[4] reported some differences in the appearance of IMPs between the original ceftriaxone product and some of the subsequent generic versions when 2% calcium chloride injection solution was added to the product. However, the mechanism underlying the above differences in IMP generation remains to be clarified.

To further investigate such findings[4], in this study we compared the generation of insoluble microparticles (IMPs) over 24 hours among the original ceftriaxone product and two Japanese generic versions, i.e., Rocephin® (Roche) and ceftriaxone for injection (A ; Sawai and B ; Nichi-Iko). To quantitate the IMPs by size and number of particles, we used a light obscuration particle counter and visual observation. Furthermore, Lambert and Conway[5] compared the pharmaceutical qualities of 34 ceftriaxone generic products with Rocephin®, and found that the most common differences among ceftriaxone products were clarity of solution and contamination of thiotriazinone (Ro 11-8390), which is used in the synthesis of ceftriaxone and remains in the manufactured powder as an impurity. Therefore, we also focused on the different quantities of contaminating thiotriazinone among the ceftriaxone products tested in this study, aiming to provide a mechanistic insight into the generation of IMPs in ceftriaxone-calcium mixtures.

Materials and methods

Chemicals

Original (Rocephin®) and generic formulations of ceftriaxone sodium (1 g) for injection were obtained from Chugai (Tokyo, Japan, as a Roche Group), Sawai Pharmaceutical Co., Ltd. (Osaka, Japan), and Nichi-Iko Pharmaceutical Co., Ltd. (Toyama, Japan). Isotonic sodium chloride solution (100 ml) and calcium chloride injection solution (2%) were purchased from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) and tetrahydro-2-methyl-3-thioxo-1,2,4-triazine-5,6-dione (Ro11-8390), hereafter referred to as thiotriazinone, was purchased from Fluorochem Ltd. (Derbyshire, UK).

IMP quantitation by light obscuration particle counting and visual observation

The size and number of IMPs were determined using a light obscuration particle counter, KL-04 (RION Co., Ltd., Tokyo, Japan). The volume of each sample was 4.5 ml, and the mean value of triplicate samples was calculated. Visual observational status (VOS) corresponded to the following : (−) transparent solution without any IMPs ; (+) transparent solution with notice-

able IMPs; (++) moderately opaque, milky white solution with more abundant IMPs; and (+++) highly opaque, milky white solution with numerous aggregates of IMPs. All VOS data were collected and evaluated by three examiners.

Time courses of IMP generation in the original and generic ceftriaxone solutions mixed with $CaCl_2$

We added 1.2 ml of 2% (w/v) calcium chloride solution to 100 ml of the original and generic ceftriaxone solutions for injection, to obtain ceftriaxone solutions (10 mg/ml) with the final calcium ion concentrations of 4.3 mEq/l. The size and number of IMPs generated in these ceftriaxone-calcium mixtures were measured after the addition of $CaCl_2$, using a light obscuration particle counter.

Microscopic assessment

We added 60 µl of 2 % (w/v) calcium chloride solution to 5 ml of the original and generic ceftriaxone (B) solutions (10 mg/ml), resulting in a final calcium ion concentration of 4.3 mEq/l. In another experiment, 4 mg of thiotriazinone (i.e., impurity) was added to the generic ceftriaxone solutions (10 mg/ml), to which 60 µl of 2% (w/v) calcium chloride solution was added, in order to investigate the effect of impurity contamination on IMP generation. Crystallization of insoluble ceftriaxone-calcium salt in the ceftriaxone solutions was monitored at 0, 20, and 30 min after the addition of $CaCl_2$, using an Olympus IX71 inverted light microscope (Olympus Co., Ltd., Tokyo, Japan) connected to a computer (Fujitsu Tokyo, Japan).

Influence of thiotriazinone impurity on the generation of IMP in the generic ceftriaxone (B) solutions mixed with $CaCl_2$

We mixed 4 mg of thiotriazinone, as a simulated impurity, with generic ceftriaxone powder (B) (10 mg/ml), and then dissolved the mixture in 100 ml isotonic sodium chloride solution. Then, 1.2 ml of 2% (w/v) calcium chloride solution was added to obtain a final calcium ion concentration of 4.3 mEq/l. The size and number of IMPs were measured as described above.

High-performance liquid chromatographic separation of thiotriazinone impurities in the original and generic ceftriaxone preparations

The contaminating components derived from thiotriazinone impurities in the original and generic ceftriaxone products were analyzed by high-performance liquid chromatography (HPLC), according to a method described in the 16th edition of the Japanese Pharmacopeia (2011). The HPLC system (Nexera XR, Shimadzu, Kyoto, Japan) used was equipped with an ultraviolet detector (SPD-20A), an integrator, a dual pump (LC-20AT), and a reverse-phase column (TSKgel ODS-80Ts, 5 mm, 4.6 mm × 15 cm) kept at 25℃. The flow rate was 1.0 ml/min, and the wavelength was set at 254 nm.

Statistical analysis

The IMP variables are expressed as mean ± S.D. values (n = 3). The data were compared

among the two groups by an unpaired Student's t-test or among the three products by a one-way analysis of variance (ANOVA) test followed by the post-hoc Dunnett's test, using the statistical software JMP® Pro 11 (SAS Institute Inc., Cary, NC, USA). Differences were considered to be significant at $P < 0.05$.

Results

The size and number of IMPs generated in the original and generic ceftriaxone (A) solutions with Ca^{2+} added to a concentration of 4.3 mEq/l were measured by a light obscuration particle counter at 15 min, 60 min, and 24 hr after Ca^{2+} addition (Table 1). There were significantly fewer total IMPs in the generic ceftriaxone (A) solution at 60 min and 24 hr compared to the original ceftriaxone solution, indicating a faster rate of ceftriaxone-calcium precipitation in the original mixture than in the generic (A) solution.

Similar analyses were performed to compare IMP generation in the original and generic (B) ceftriaxone solutions containing 4.3 mEq/l Ca^{2+}, in this case with and without 4 mg thiotriazinone (impurity), at 15, 30, and 60 min after Ca^{2+} addition (Table 2). There was a significantly higher number of total IMPs detected in the generic ceftriaxone (B) solution than in the original mixture at 15 and 30 min after the addition of $CaCl^{2+}$, although at 24 hr after Ca^{2+} addition, the number of IMPs in the generic ceftriaxone (B)-calcium mixture was beyond the upper

Table 1. Number of insoluble microparticles (IMPs) and visual observation status (VOS) in the original and generic ceftriaxone preparations in the presence of 4.3 mEq/l Ca^{2+}

Time	Particle size (μm)	Rocephin®		Ceftriaxone (A)	
		Number of IMPs per ml	VOS	Number of IMPs per ml	VOS
15 min	2.0–10.0	551.1±61.5	(–)	396±79.6	(–)
	10.0–25.0	15.1±4.6		5.6±1.7	
	25.0–50.0	1.4±1.2		0.1±0.2	
	Above 50.0	0.1±0.1		0.1±0.1	
	Total Number	567.7±67.3		401.8±81.5	
60 min	2.0–10.0	865.8±156.5	(+)	399.3±77.8	(–)
	10.0–25.0	78.9±49.6		5.3±2.5	
	25.0–50.0	7.3±3.6		0.0±0.1	
	Above 50.0	1.6±0.9		0.0±0.0	
	Total Number	953.6±210.5		404.6±80.3*	
24 hr	2.0–10.0	4505.4±673.3	(++)	3084.6±611.8	(++)
	10.0–25.0	7176.1±902.1		512.8±244.4	
	25.0–50.0	3419.0±863.4		145.3±103.2	
	Above 50.0	4754.7±1515.5		168.9±191.3	
	Total Number	19855.2±3954.3		3911.6±1150.8*	

* Significantly different ($P < 0.05$) from the original ceftriaxone product (Rocephin®).

Table 2. Influence of thiotriazinone (impurity) on the generation of insoluble microparticles (IMPs) and visual observation status (VOS) in the generic ceftriaxone (B) product mixed with 4.3 mEq/l Ca^{2+}

Time	Particle size (μm)	Ceftriaxone (B)		Ceftriaxone (B) + impurity 4 mg	
		Number of IMPs per ml	VOS	Number of IMPs per ml	VOS
15 min	2.0–10.0	2264.8±411.9	(++)	729.7±126.5	(+)
	10.0–25.0	3392.3±1086.5		143.6±4.8	
	25.0–50.0	2027.4±898.7		28.7±4.5	
	Above 50.0	3237.1±1523.8		16.1±3.7	
	Total Number	10921.6±3920.9*		918.2±139.4#	
30 min	2.0–10.0	2451.5±1209.1	(++)	6414.7±253.5	(++)
	10.0–25.0	4461.1±1739.8		2497.1±70.6	
	25.0–50.0	2558.2±157.0		445.6±17.0	
	Above 50.0	6956.9±1633.1		426.6±65.2	
	Total Number	16427.8±4739.0*		9783.9±406.3#	
60 min	2.0–10.0	†	(+++)	7673.2±1102.0	(++)
	10.0–25.0			7079.3±577.9	
	25.0–50.0			2151.4±73.3	
	Above 50.0			2182.1±439.1	
	Total Number			19085.9±2192.2	

* Significantly different ($P < 0.05$) from the original ceftriaxone product (Rocephin®) listed in Table 1.
\# Significantly different ($P < 0.05$) from the control (without an impurity).
† Not measured (counter overflowed) due to excess number of IMPs.

detection limit. Therefore, the generic (B) mixture showed faster ceftriaxone-calcium precipitation than the original ceftriaxone–calcium mixture. Moreover, the generic ceftriaxone (B) samples with thiotriazinone impurity (4 mg) added showed significantly decreased numbers of larger-sized IMPs when mixed with calcium chloride than the comparable samples without the thiotriazinone addition.

Three independent examiners determined the VOS scores of the ceftriaxone-calcium mixtures, and the averaged scores are listed in Tables 1 and 2. These VOS scores were highly variable among the three ceftriaxone products examined, reflecting differences in the number of larger IMPs (> 50 μm in diameter). Typically, after 60 min, the generic ceftriaxone (B)-calcium mixture was highly opaque with numerous aggregates of milky-white precipitates, while the original ceftriaxone-calcium mixture exhibited noticeable IMP generation, and the other generic ceftriaxone (A)-calcium mixture was transparent.

Fig. 1 shows the morphology of ceftriaxone crystals examined under an inverted light microscope for the original and generic ceftriaxone (B) solutions following the calcium chloride additions. Importantly, the generated crystals are easily differentiated morphologically from air

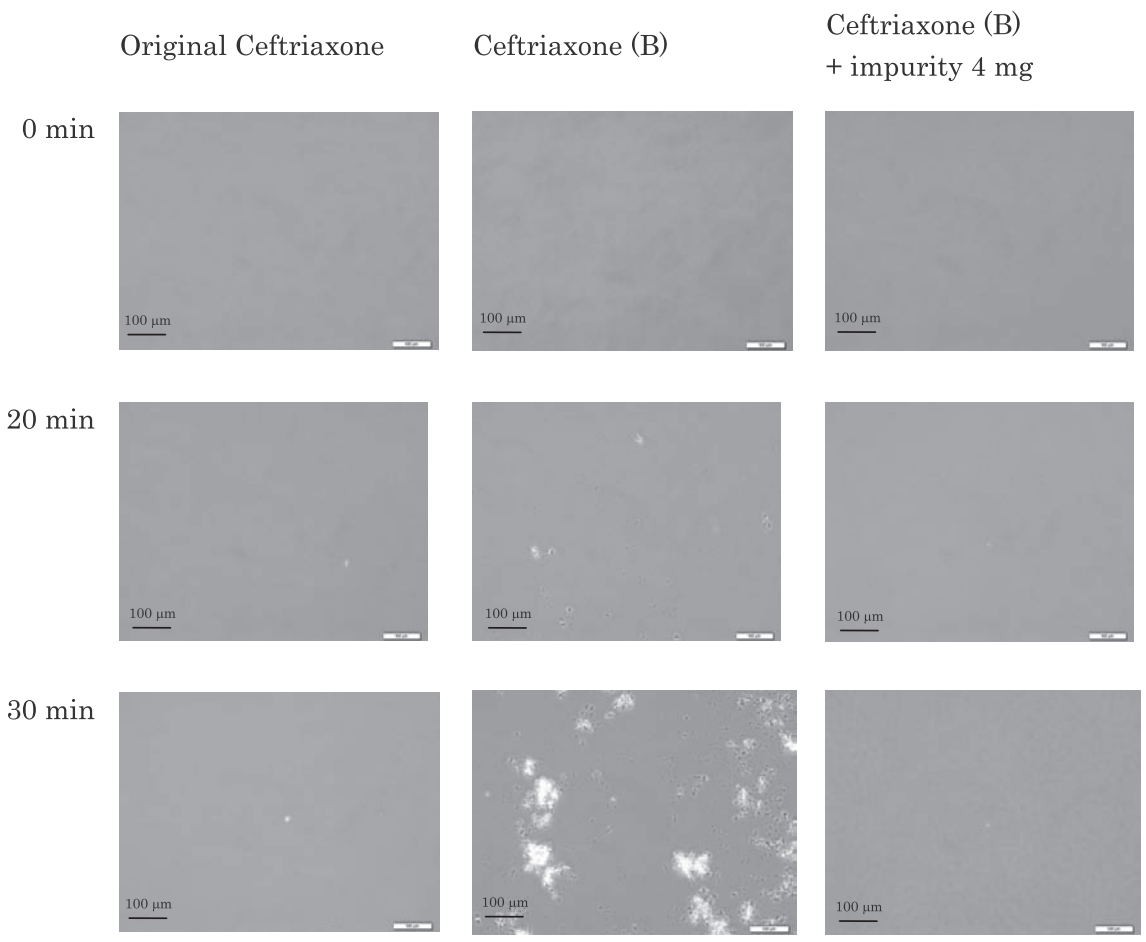

Fig. 1. Microscopic assessment of the size and morphology of ceftriaxone-calcium salt crystallization in the original and generic ceftriaxone (B) solutions (10 mg/ml) mixed with 4.3 mEq/l Ca^{2+} (left two lines of panels) and in the generic ceftriaxone (B) solution (10 mg/ml) mixed with 4.3 mEq/l Ca^{2+} and 4 mg thiotriazinone as an impurity (right line of panels).

bubbles or small dust particles. Ceftriaxone (B) crystals started to appear at about 20 min after the calcium addition, becoming larger with time as numerous crystals aggregated to various sizes $>$ 100 μm. The growth rate of crystals turned out to be much higher in the generic ceftriaxone (B) solution than in the original ceftriaxone solution at approximately 30 min after the calcium addition, supporting the comparison of IMP numbers between the two solutions. The growth of ceftriaxone aggregated crystals was markedly delayed in the generic ceftriaxone (B) mixture with 4 mg of thiotriazinone added compared to the sample without such impurities added. Contaminating amounts of the thiotriazinone impurity in a 1 g ceftriaxone vial were determined by HPLC as 1.35 ± 0.07 mg, 2.92 ± 0.19 mg, and 1.15 ± 0.13 mg ($n = 3$) for the original, generic (A), and generic (B) ceftriaxone products, respectively, giving an impurity scale of generic B $<$ original $<$ generic A. The contaminating amount of thiotriazinone in the original ceftriaxone product (i.e., 0.135 (w/w) % of ceftriaxone in a 1 g vial) is comparable to a previous report[7].

Discussion

The present study confirms a previous report of differences in ceftriaxone-calcium salt precipitation between the original ceftriaxone (Rocephin®) and subsequent generic products[4]. Our further morphological observation of ceftriaxone crystallization under a light inverted microscope (Fig. 1) suggested that the increased IMPs of a larger-size range represented the massive aggregation of ceftriaxone-calcium salt crystals.

Our experiments clearly indicated that IMP generation proceeded over time in all the ceftriaxone products examined. IMPs $>$ 50 μm were first detected at 60 min after the calcium addition for the original product, at 24 hr for the generic ceftriaxone (A) product, and at 15 min for the generic ceftriaxone (B) product. Such differences were clearly reflected in the morphological assessments of solutions and in the VOS scores. Interestingly, the generic ceftriaxone (A) mixed with 4.3 mEq/l Ca^{2+} did not exhibit IMPs $>$ 50 μm at 6 hr after Ca^{2+} addition (data not shown). However, smaller IMPs ($<$ 25 μm) were detected in the three ceftriaxone solutions at all time points examined, indicating that small cores of ceftriaxone-calcium salt were being generated, but not aggregating to a visual size. The i.v. infusion of particulate matter larger than the internal diameter (i.d.) of small blood vessels (e.g., capillaries, i.d. 4–9 μm ; arterioles, i.d. approximately 20 μm) increases the clinical risk of an embolic syndrome[6]. Therefore, the original and two Japanese generic ceftriaxone products examined here are thought to have certain clinical risks, despite the lack of visual signs of ceftriaxone-calcium salt precipitation. In this respect, the FDA's alerts to healthcare professionals suggesting the avoidance of ceftriaxone/calcium coadministration is reasonable. Indeed, the molar concentration of ceftriaxone used for IMP particle counting was 15.1 mM, and that of calcium was 2.15 mM, which correspond to one of the mixing processes used clinically (i.e., ceftriaxone and calcium concentrations possible in the Y-site injection).

Overall, the IMP generation rates associated with ceftriaxone-calcium salt precipitation among the ceftriaxone products tested in this study were in the order of generic (A) $<$ original $<$ generic (B). Moreover, we found that the contaminating amounts of thiotriazinone (impurity) were in the order of generic (B) $<$ original $<$ generic (A), which inversely corresponds to the rate of IMP generation in these products mixed with $CaCl_2$. Despite these orders, the actual differences in impurity content between the original and the generic (B) products were small. Of special note from this study is that only a very small amount of thiotriazinone powder added into the generic ceftriaxone (B) product significantly decreased both the number of IMPs, especially those of larger size, and the visual score of precipitation associated with ceftriaxone-calcium salt. This indicates that low levels of impurity contamination do not cause significant adverse effects in the generic (B) product, based on the growth of ceftriaxone-calcium salt crystals. The data also suggest that a higher number of smaller sized IMPs (2.0–10.0 μm) after 30 min in the impurity-added solution could indicate that the generation of ceftriaxone-calcium salt crystals was suspended at smaller cores and further growth was retarded by addition of the impurity. Generally, an impurity can retard the crystal growth of a major chemical moiety by repeatedly acting

on certain crystallographic faces[8]. Such an impurity may attach briefly to the growing crystal lattice, but soon de-attach as a compound with a more suitable geometry comes in to take its place[9]. Indeed, the present experiments suggested that thiotriazinone, which is used in the ceftriaxone synthesis and remains as a major impurity, acts to suppress the ceftriaxone-calcium salt precipitation, albeit in the allowable range of impurity, i.e., 1% (w/w) of the active moiety as defined by the regulatory agency[10]. Furthermore, the higher contamination of thiotriazinone (impurity) in the generic (A) ceftriaxone product might underlie the fact that it had the lowest rate of IMP generation among the three ceftriaxone products examined when mixed with calcium-containing solution.

Taken altogether, we have provided a mechanistic insight into observed differences in IMP generation among three manufactured products of ceftriaxone sodium when mixed with calcium ions, clarifying earlier findings of similar differences[4]. Based on the present findings, we recommend that the clinical risk of ceftriaxone-calcium compatibility be considered with caution regarding thiotriazinone (impurity) contamination in ceftriaxone products.

Conflict of interest disclosure

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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