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Determination of cyanide, in urine and gastric content, by electrospray ionization tandem mass spectrometry after direct flow injection of dicyanogold

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ABSTRACT

A rapid and sensitive electrospray ionization tandem mass spectrometric (ESI-MS-MS) procedure was developed for the determination of cyanide (CN⁻). CN⁻ in biological fluids was reacted with NaAuCl₄ to produce dicyanogold, Au(CN)₂⁻, which was extracted with methyl isobutyl ketone (MIBK). One μL of the extract was injected directly into an ESI-MS-MS instrument. Quantification of CN⁻ was performed by selected reaction monitoring of the product ion CN⁻ at *m/z* 26 that derived from precursor ion Au(CN)₂⁻ at *m/z* 249. CN⁻ could be measured in the quantification range of 10⁻⁷ to 5 × 10⁻⁵ M with the limit of detection at 4 × 10⁻⁸ M using 10 μL of urine within 10 min. A victim's urine and gastric content were diluted with water to 4-fold and 500-fold and measured, respectively.

Key words: Cyanide; Gold; Electrospray ionization; Tandem mass spectrometry, Urine

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1. Introduction

Levels of cyanide (CN^-) in biological fluids can be increased by breathing in HCN gas, taking CN^- salts or nitroprusside and cyanogenic glycosides. It has been found to inhibit the activity of cytochrome oxidase [1-3], and so rapid and decisive determination of CN^- levels is required in the diagnosis of suspected poisoning. Healthy, toxic and fatal levels of CN^- in blood were reported to be 2×10^{-7} , 5×10^{-5} and 10^{-4} M, respectively [1,2].

Thiocyanate (SCN^-), the major metabolite of CN^- , is found in appreciable amounts in blood plasma and saliva [1-3] and reacts similarly to CN^- with some reagents [1-6]. Several studies have attempted to quantify CN^- samples containing both SCN^- and CN^- anions [1-10]. Although a gas chromatographic (GC) method can separate and quantify pentafluorobenzyl derivatives of CN^- and SCN^- respectively, their quantification ranges were at very high concentration ranges of 10 to 100 ppm, i.e., 4×10^{-4} to 4×10^{-3} M [4]. An alternative study determined levels of CN^- using a head space GC by evaporating HCN from the blood leaving SCN^- retained [5]. In some spectrophotometric flow injection methods, the amount of SCN^- was determined by masking CN^- either with nickel ion or formaldehyde, and the amount of CN^- was calculated by subtracting the amount of SCN^- from the total amount of CN^- and SCN^- [6,7]. Mass spectrometry (MS) is a more decisive method than GC and spectrophotometric methods, since it can determine the atomic mass of the analyte. In some MS studies, CN^- in biological fluids was derivatized with organic molecules to eliminate interference from the matrix and was detected by GC-MS and liquid chromatography mass spectrometry (LC-MS) [2,8,9]. These preparations, however, took an appreciable time, 30 min to 2 h, needed for the evaporation of HCN from blood [8,9] and another 30 min for the derivatization of CN^- [2].

The tandem mass spectrometric (MS-MS) determination of CN^- after chelate complex formation has not yet been reported. The strong affinity of CN^- for gold

(Au) enables the instantaneous formation of dicyanogold, $\text{Au}(\text{CN})_2^-$ [11]. Extraction of $\text{Au}(\text{CN})_2^-$ with methyl isobutyl ketone (MIBK) eliminated interfering substances that were contained within the biological fluids. MS-MS detection is an efficient procedure that can determine analytes in crude materials without GC or LC separation. The method was used previously for sensitive determination of chelate complexes of small cations such as Co^{3+} , Cr^{6+} and As^{3+} in biological fluids [12-15]. In the present study, this method has been applied to the small anion, CN^- , for the first time.

2. Materials and methods

2.1 Materials

MIBK of atomic absorption grade, metals of atomic absorption standard and other chemicals of analytical grade were obtained from Wako Pure Chemicals, Osaka, Japan. $\text{K}^{13}\text{C}^{15}\text{N}$ (^{13}C , 99%, ^{15}N , 98%) was obtained from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. Pure water with a specific resistance of 18 M Ω cm was used (Millipore, Bedford, MA, USA). Plasma, urine and blood were obtained from healthy volunteers under permission and used for the control samples.

Samples such as gastric content, urine and blood were obtained at autopsy from a victim who may have committed suicide by drinking orange juice containing NaCN. White powder in a small glass bottle left near the victim was also examined and found to be NaCN.

2.2 Standard solutions

The 1 M CN^- stock solution was prepared by dissolving KCN into 0.1 M $(\text{CH}_3)_4\text{NOH}$ solution and the 1 M SCN^- stock solution was prepared by dissolving KSCN into water. The concentrations of CN^- and SCN^- stock solutions were determined by using the pyridine-pyrazolone method [16] and were used for one week. The standard solutions of CN^- and SCN^- were prepared before measurement by diluting

the stock solution with water and sample fluids containing both 0.1 M (CH₃)₄NOH and 10⁻⁴ M Na₂S₂O₄, respectively.

2.3 Assay procedures

To 10 μL of sample solution in a tube (Eppendorf AG, Hamburg, Germany), 1 μL of the solution containing 10⁻³ M Na₂S₂O₄ and 1 M (CH₃)₄NOH was added and the pH of the solution was adjusted to 10-13 if necessary. A 1-μL aliquot of 10⁻² M NaAuCl₄ was added, mixed and after 10 s, 10 μL of MIBK was added and mixed for a further 30 s before centrifugation at 5000 g for 30 s.

2.4 Instruments

ESI-MS-MS was performed using a TSQ 7000 LC-quadrupole mass spectrometer (ThermoQuest, Japan) in the negative ion mode. Methanol was flowed as the mobile phase at 200 μL min⁻¹ and the capillary temperature was set at 230 °C. The electrospray voltage was set at -4.5 kV, multiplier voltage at 1.3 kV, and collision voltage at 35 V. Nitrogen was used as the sheath gas (469 kPa) and also as an auxiliary gas (8 units) and argon was used as the collision gas (134 kPa). An aliquot of 1 μL MIBK layer was injected manually into ESI-MS-MS apparatus in the direct flow injection. The characteristic spectrum appeared within 30 s of sample injection and the sample could be injected every 60 s. The quantification in ESI-MS-MS was performed by the integration of the peak area of the product ion at m/z 26.2 ± 0.2, derived from the precursor ion at m/z 248.9 ± 0.3, using a calibration curve made up with spiked matrix samples at different concentrations.

3. Results and discussion

3.1 Suitable pH and solvent for the extraction of Au(CN)₂⁻ from aqueous solution

The signal intensities of Au(CN)₂⁻ produced in 0.1 M (CH₃)₄NOH/HCl solutions at

pH 7, pH 9 and 10-13 were 1, 80 and 100 %, respectively, using MIBK as the extractor for all of these solutions. The low recovery of $\text{Au}(\text{CN})_2^-$ at pH 7 may be explained by the pKa value of HCN, 9.2, that is, CN^- prefers to take HCN form at pH below 9.2.

The signal intensities extracted from $(\text{CH}_3)_4\text{NOH}$ solutions at 0.005 M, 0.01-0.1 M and 0.2 M were 90, 100 and 95 %, respectively. Therefore, 0.1 M $(\text{CH}_3)_4\text{NOH}$ solution was used as the standard aqueous solution. The extraction efficiencies of several organic solvents such as isoamyl alcohol, octanol, hexanol, pentanol, cyclohexanol and diisobutyl ketone were nearly 80, 60, 50, 40, 20 and 20 %, respectively, by taking the efficiency of MIBK as 100%. The blank signal of MIBK was smallest among these solvents and was therefore used as the extractor of the complex.

The observed amount of $\text{Au}(\text{CN})_2^-$ in MIBK extract after 24 h was more than 95 % of the starting amount at room temperature under visible light. Since the $\text{Au}(\text{CN})_2^-$ is a negative ion, it may be dissolved with other cations, such as $(\text{CH}_3)_4\text{N}^+$, Na^+ , K^+ and Au^+ in MIBK. These cations could not be identified in the present work.

3.2 Suitable concentration of Au for the formation of $\text{Au}(\text{CN})_2^-$ and that of $\text{Na}_2\text{S}_2\text{O}_4$ for the stabilization of SCN^-

The production of $\text{Au}(\text{CN})_2^-$ attained to the maximum when the molar concentration of Au was only more than four times that of CN^- at 5×10^{-5} M. This may be explained by the very high formation constant of $\text{Au}(\text{CN})_2^-$, $K_f = 10^{39}$ [11]. The concentration of Au was set to be 10^{-3} M for the detection of CN^- at 10^{-7} - 5×10^{-5} M.

SCN^- in plasma was reported to be 3×10^{-5} M [1]. Although ascorbic acid was used to prevent the oxidative conversion of SCN^- to CN^- [5], it can work as a reducing agent in acidic solution. Therefore, reducing power of agents such as $\text{Na}_2\text{S}_2\text{O}_4$, K_2S and Na_2SO_3 were examined in the present alkaline solution, and it was noted that they prevented oxidation of SCN^- to CN^- in the order of $\text{Na}_2\text{S}_2\text{O}_4 > \text{K}_2\text{S} > \text{Na}_2\text{SO}_3$. Only 10^{-6} M CN^- was produced from 10^{-3} M SCN^- in the solution containing $\text{Na}_2\text{S}_2\text{O}_4$ at

10^{-4} M. The signal intensities of $\text{Au}(\text{CN})_2^-$ produced in the solution of $\text{Na}_2\text{S}_2\text{O}_4$ at $0 - 10^{-4}$ M and 10^{-3} M were 100 % and 90 %, respectively. The concentration of $\text{Na}_2\text{S}_2\text{O}_4$ was therefore set to be 10^{-4} M.

3.3 MS and MS-MS spectra

The mass spectrum of $\text{Au}(\text{CN})_2^-$ consisted of two peaks at m/z 249 and 250 having relative abundance of 100 : 3 as shown in Figure 1 (a). The isotopic relative abundance of the signal at m/z 249 : m/z 250 : m/z 251 calculated using the Isotopic Distribution Calculator of Applied Biosystems Japan Limited was 100.00 : 3.00 : 0.03, respectively. Choosing $\text{Au}(\text{CN})_2^-$ at m/z 249 as the precursor ion, selected reaction monitoring was examined by changing the collision voltage from 10 to 50 V. Under these conditions, the observable product ion was only CN^- at m/z 26 as shown in Figure 1 (b). When $\text{Na}^{13}\text{C}^{15}\text{N}$ was used as the source of CN^- , the mass spectrum consisted mainly of a peak at m/z 253 and the product ion spectrum, a peak at m/z 28. The collision voltage of 35 V was chosen for the quantification of CN^- since the product ion CN^- showed the maximum peak area at the voltage.

3.4 Interferences from cations and anions in aqueous solution

Metal ions such as Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Zr, Mo, Ru, Pd, Ag, Cd, W, Pt and Pb at 10^{-4} M, respectively, did not interfere with the detection of CN^- under the present conditions. These ions, however, are well known to form complexes with CN^- in aqueous solution under suitable conditions [10]. When the amount of Au was abundant enough to react with CN^- completely, negative ions such as Cl^- , ClO_3^- , Br^- , NO_2^- , NO_3^- , SO_4^{2-} , CO_3^{2-} , PO_4^- , SCN^- , N_3^- , oxalate and citrate did not interfere with the detection of CN^- under the present conditions when they were added to the solution at 50 times that of CN^- , respectively.

Normal biological fluids contain SCN^- [1-3]. $\text{Au}(\text{SCN})_2^-$ was produced most

efficiently in sodium citrate solution at pH 6.5. The intensity ratio between the signal produced in $(\text{CH}_3)_4\text{NOH}$ solution at pH 12 and that in sodium citrate solution at pH 6.5 was 100 : 1 for CN^- , whereas it was 1 : 100 for SCN^- . The condition for the detection of SCN^- is, however, still under investigation since the signal decreases non-linearly with its concentration below 10^{-6} M.

3.5 Recoveries from urine, plasma and blood

The recovery of CN^- was examined at the concentration of 10^{-5} M by comparing the peak area at m/z 26 extracted from biological fluid with that extracted from 0.1 M $(\text{CH}_3)_4\text{NOH}$ solution. The recoveries from urine, plasma and blood were examined, and found to be 100 %, 10 % and 5 %, respectively. Due to the low recovery from plasma and blood, the precision and accuracy of the present method were examined only for water and urine.

3.6 Precision and accuracy

Calibration standard solutions of CN^- were prepared by spiking stock solutions to water and control urine at 0, 1×10^{-7} , 1×10^{-6} , 1×10^{-5} and 5×10^{-5} M, respectively. The solutions that did not contain any added Au ion were considered as blanks. In case of water, blanks and the solutions that were not spiked with CN^- (i.e., 0 M) but added with Au ion, gave the same signal. Mass chromatograms of selected reaction monitoring at m/z 26 for water and control urine were examined. Concentrations determined from the peak area (y) were linear to the spiked concentrations (x) (i.e., $y = 1.032x + 0.789$ with a correlation coefficient of 0.999 for water, and $y = 0.996x + 1.453$, with a correlation coefficient of 0.999 for urine). Precision and accuracy were assessed by analyses of water and control urine spiked at 1×10^{-7} , 1×10^{-6} , 1×10^{-5} and

5×10^{-5} M, respectively (Table 1). These samples were analyzed three times a day, as well as on three different days. The coefficient of variation was <13 %, and accuracy was 96–117 % for intra-day and inter-day variations. The limit of quantification of the present method was therefore 10^{-7} M for water and urine. Blanks were measured six times, and their standard deviations (σ) were calculated using the calibration. The limit of detection (LOD) in the present assay was 4×10^{-8} M for water and urine adopting the definition that considers 3σ of blank signals as LOD [12,13].

3.7 Determination of CN⁻ in suspected suicide victim's samples

The victim's gastric content, pH 7, and urine, pH 5, were centrifuged at 5000 g for 120 s, and the upper layers of gastric content and urine were diluted with water up to 500-fold and 4-fold, respectively, since the concentrations of CN⁻ in undiluted samples exceeded the range of quantification. The standard addition method was adopted for the quantification of CN⁻ in the diluted samples, and the results were shown in Figures 2 (a) and (b). Concentrations of CN⁻ in undiluted gastric content and urine were determined three times as $(4.99 \pm 0.22) \times 10^{-3}$ M and $(1.12 \pm 0.12) \times 10^{-5}$ M, respectively.

The concentration of CN⁻ in the present case was compared with those reported in five fatal cases of acute cyanide intoxication [17], where the concentration of CN⁻ in blood was higher in four cases but lower in one case than the fatal concentration, i.e. 10^{-4} M [2]. The concentrations of cyanide as hydrogen cyanide in gastric content reported in cases from 1 to 5 were 26.3, 6.7, 234, 14.7 and 26.5 $\mu\text{g/mL}$, respectively,

and that in our case was 135 $\mu\text{g}/\text{mL}$ that was calculated from the molar concentration observed, 4.99×10^{-3} M. The total amounts of cyanide as hydrogen cyanide in the gastric content reported in cases from 1 to 5 were 0.53, 1.34, 74.9, 9.5 and 2.6 mg, respectively, and that in our case was 25.7 mg that was calculated from the concentration and the volume of gastric content, 190 mL. Therefore, both the concentration and the total amount of cyanide found in our case were secondly highest ones among these six cases.

The white powder in a small glass bottle, found beside the victim, was determined to be reagent grade NaCN. The police did not find out how the victim came to be in possession of the poison. Concentrations of CN^- in the gastric content, urine and white powder were also confirmed using the spectrophotometric method [16,17]. An appreciable difference was observed between the concentration of CN^- of victim's urine and that of control urine, $(1.13 \pm 0.33) \times 10^{-6}$ M ($n=5$).

3.8 Comparison with other methods

Detection at present can be made using only 10 μL of sample whereas previous methods needed 0.2 - 2 mL of sample. The LOD of the present method is 4×10^{-8} M, which is lower than that of the most sensitive methods [1-10]. Previously [2], derivatization of CN^- took 30 min at 55 $^\circ\text{C}$ since CN^- in aqueous phase should be transferred into the organic phase with the aid of a phase-transfer catalyst. In the present method, CN^- reacted with the Au ion completely within 10 s at room temperature. CN^- and Au ions are both dissolved in the aqueous phase and the affinity between CN^- and Au ion is strong as expressed by its high formation constant [11]. Although $\text{Au}(\text{CN})_2^-$ is an inorganic anion, it is extracted with MIBK, a polar organic

solvent, and is ionized easily by ESI and detected sensitively by MS(-MS). After choosing suitable conditions such as pH, metal ion and organic solvent, the present method is applicable for the detection of other water soluble anions that are difficult to be ionized directly from water by mild ionization, e.g., ESI.

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Figure captions

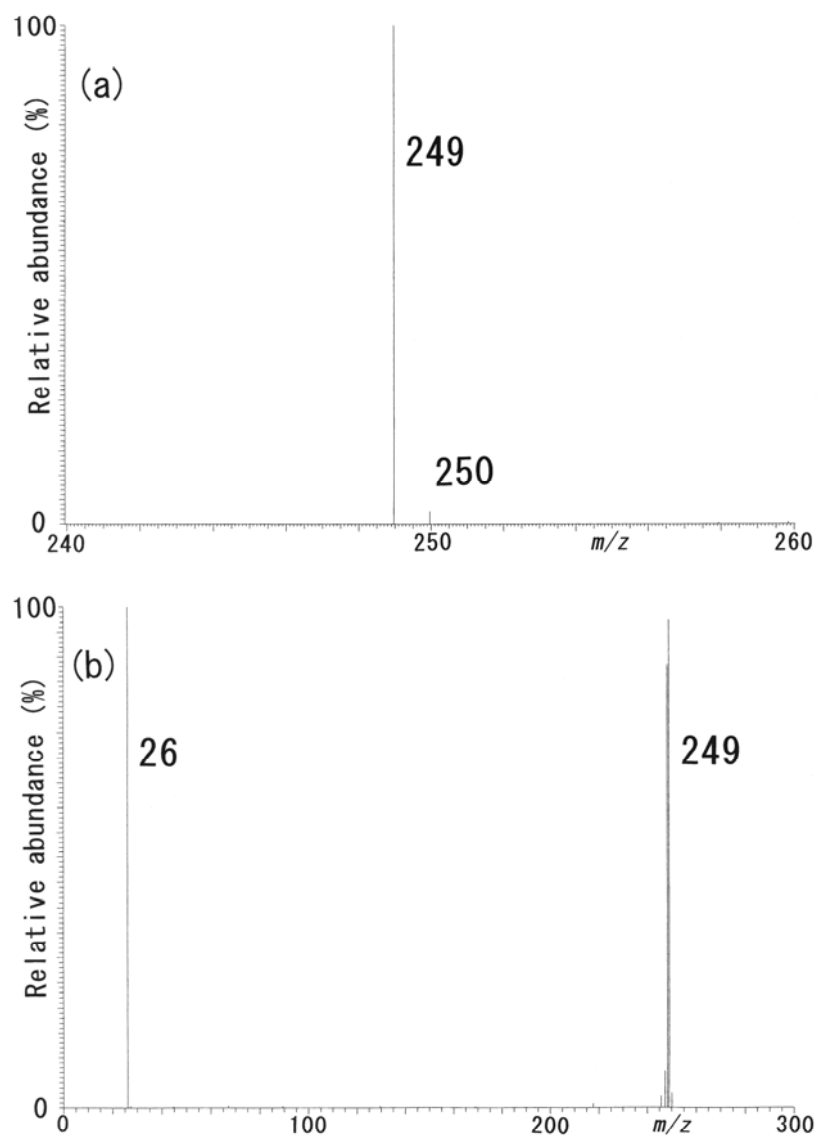


Figure 1: Mass spectrum of methyl isobutyl ketone extracted from 10^{-5} M CN^- aqueous solution (a), and its product ion spectrum at collision voltage of 45 V from the precursor ion $\text{Au}(\text{CN})_2^-$ at m/z 249 (b).

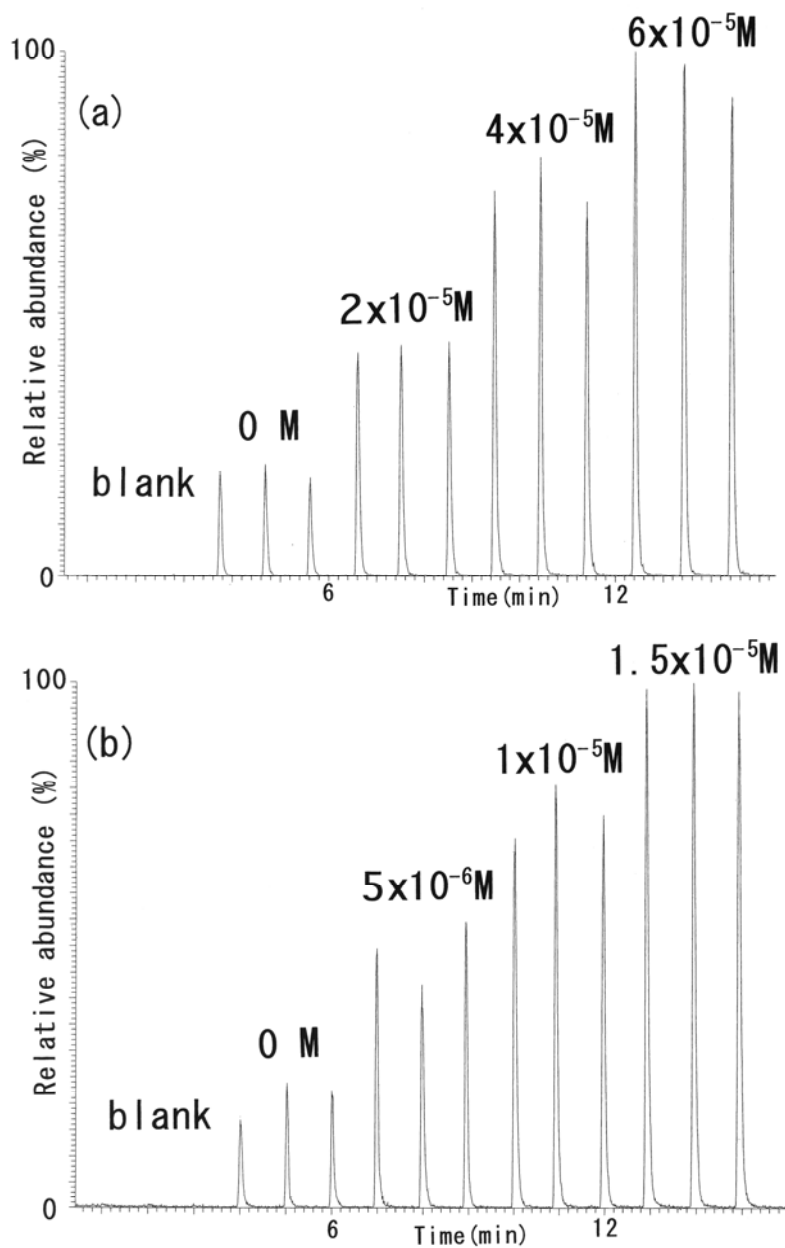


Figure 2: Mass chromatograms of selected reaction monitoring at m/z 26 for the quantification of CN^- in the victim's gastric content diluted 500-fold (a), and those in victim's urine diluted 4-fold (b), respectively. Blank indicates sample that Au ions were not added. Samples were injected three times for each spiked CN^- concentration.

Table 1

Intra-day (3 times) and inter-day (3 days) variations of CN^- values determined by the present method. The observed values and coefficient of variations (C.V.) for CN^- spiked at $1 \times 10^{-7} - 5 \times 10^{-5}$ M into water and urine, respectively.

Spiked [CN^-] (M)	Intra-day				Inter-day			
	water		urine		water		urine	
	Observed [CN^-] (M)	C.V. (%)	Observed [CN^-] (M)	C.V. (%)	Observed [CN^-] (M)	C.V. (%)	Observed [CN^-] (M)	C.V. (%)
1×10^{-7}	1.17×10^{-7}	12.0	1.12×10^{-7}	13.3	1.09×10^{-7}	6.4	1.14×10^{-7}	7.3
1×10^{-6}	0.96×10^{-6}	4.2	1.14×10^{-6}	8.3	1.14×10^{-6}	4.0	1.17×10^{-6}	6.6
1×10^{-5}	0.98×10^{-5}	3.6	0.99×10^{-5}	1.3	1.00×10^{-5}	2.8	0.96×10^{-5}	3.7
5×10^{-5}	4.81×10^{-5}	5.4	4.99×10^{-5}	2.4	5.06×10^{-5}	2.2	4.95×10^{-5}	1.8