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Research Article

Direct Determination of ECD in ECD Kit: A Solid Sample Quantitation Method for Active Pharmaceutical Ingredient in Drug Product

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Technetium-99m ethyl cysteinate dimer (Tc-99m-ECD) is an essential imaging agent used in evaluating the regional cerebral blood flow in patients with cerebrovascular diseases. Determination of active pharmaceutical ingredient, that is, *L*-Cysteine, *N*, *N*′-1,2-ethanediylbis-, diethyl ester, dihydrochloride (ECD) in ECD Kit is a relevant requirement for the pharmaceutical quality control in processes of mass fabrication. We here presented a direct solid sample determination method of ECD in ECD Kit without sample dissolution to avoid the rapid degradation of ECD. An elemental analyzer equipped with a nondispersive infrared detector and a calibration curve of coal standard was used for the quantitation of sulfur in ECD Kit. No significant matrix effect was found. The peak area of coal standard against the amount of sulfur was linear over the range of 0.03–0.10 mg, with a correlation coefficient (r) of 0.9993. Method validation parameters were achieved to demonstrate the potential of this method.

1. Introduction

To date, technetium-99m ethyl cysteinate dimer (Tc-99m-ECD or bicisate) is one of the most essential single-photon emission-computed tomography (SPECT) imaging agents in hospitals. According to the practice guidelines of the American College of Radiology (ACR) and the European Association of Nuclear Medicine Neuroimaging Committee (ENC), clinical indications of Tc-99m-ECD include evaluating the regional cerebral blood flow (rCBF) in patients with (i) cerebrovascular diseases, (ii) transient ischemic attack, (iii) various forms of dementia, (iv) symptomatic traumatic brain injury, (v) encephalitis, (vi) vascular spasm following subarachnoid hemorrhage, (vii) inflammation, (viii) epileptic foci, and (ix) lacunar infarctions [1, 2].

The indications of Tc-99m-ECD in SPECT brain perfusion imaging of neuropsychiatric disorders and chronic fatigue syndrome have not been fully characterized [1, 2]. However, investigations of the conversion in patients of mild cognitive impairment (MCI) to Alzheimer's disease (AD) [3], the functional compensation mechanism in incipient AD [4], the mechanism for suppression of parkinsonian tremor by thalamic stimulation [5], the mechanism by which thyroid hormone availability affects cerebral activity [6], brain glucose metabolism in hypothyroidism [7], reduction in the bifrontal regions and diffusion-weighted imaging of Creutzfeldt-Jakob disease [8, 9], quantitation and differentiation in patients with Tourette's syndrome [10–12], and abnormal rCBF in patients with Sjögren's syndrome [13] were reported.

For clinical implements, Tc-99m-ECD is obtained by radiolabeling of active pharmaceutical ingredient (API), that is, *L*-Cysteine, *N*, *N*′-1,2-ethanediylbis-, diethyl ester, dihydrochloride (ECD) with Tc-99m. Radiochemical purity (RCP) of Tc-99m-ECD is used for the quality control (QC) purpose [14–16]. Although the characteristics of Tc-99m-ECD, such as *in vivo* kinetics and biodistribution studies in healthy human [15, 17], pharmacological studies in primates [14, 18], uptake, clearance, and brain retention [19–22], biotransformation, metabolites, and stability [14, 21, 23], have

Table 1: Optimized parameters of elemental analyzer for quantitation of ECD in ECD Kit.

Parameters	
Operation mode	CHNS
Combustion temperature (°C)	1150
Reduction temperature (°C)	900
Flush gas/time (sec)	He/10
O_2 dosing time (sec)	120
Column standby temperature (°C)	
CO_2	Ambient temperature
H_2O	Ambient temperature
SO_2	140
Column desorption temperature (°C)	
CO_2	240
H_2O	150
SO_2	220
Carrier gas/Flow rate (mL/min)[(1)]	He/230
Flow rate of O_2 (mL/min)	15
Flow rate of O_2 during combustion (mL/min)	30–35

[(1)] Same as the mass flow control (MFC) TCD flowing gas and flow rate.

been well-investigated, the chemical properties (such as purity and content) of ECD in ECD Kit (Vial A), that is, API in drug product, which might significantly disqualify the efficacy of Tc-99m-ECD have not been much discussed. Moreover, no analytical method for the determination of content and uniformity of ECD in ECD Kit has been published.

Analysis of the content and uniformity of ECD in ECD Kit is a relevant requirement for the pharmaceutical QC in processes of mass fabrication. In the stability study of Mikiciuk-Olasik and Bilichowski, they demonstrated that ECD decomposed as soon as it was dissolved in phosphate buffer solutions [24]. Our earlier observations also agreed with findings of Verduyckt et al. [25], showing that the composition of ECD Kit is the major obstacle to determine stability of ECD in (non)aqueous solutions.

ECD is the only component which contains sulfur in ECD Kit. Methods for the determination of sulfur, including Eschka method [26], gas chromatography-mass spectrometry (GC-MS) [26], inductively coupled plasma atomic emission spectrometry (ICP-AES) [27], instrumental neutron activation analysis (INAA) [27], X-ray fluorescence [27, 28], and elemental analyzer coupled with a thermal conductivity detector (EA-TCD) [29] or an isotope ratio mass spectrometer (EA-IRMS) [30], have been developed. We here presented a direct solid sample determination method of ECD in ECD Kit without sample dissolution to avoid the rapid degradation of ECD in aqueous solution using elemental analyzer (EA) coupled with a nondispersive infrared detector (NDIR). Method validation parameters were achieved to demonstrate the potential of this method.

2. Experimental

2.1. Materials and Reagents. ECD (purity: 97.53%) was obtained from ABX (Radeberg, Germany). Coal standard (ELTRA coal standard no. 92510-50; C: 76.6%, S: 3.07%) was purchased from ELTRA (Neuss, Germany). All chemicals and reagents were of analytical grade and used as received without further purification.

2.2. Elemental Analyzer. An elemental analyzer (EA) (vario EL cube, Elementar Analysensysteme GmbH, Hanau, Germany), equipped with a microbalance (Mettler-Toledo XP6, Mettler-Toledo GmbH, Giessen, Germany), a nondispersive infrared detector (NDIR), and a thermal conductivity detector (TCD) was employed for the measurement of sulfur. The microbalance was connected to control a personal computer (PC) of the EA for automatic transmission of the sample weight to the PC. The measurement of sulfur was switched to NDIR photometer in operation mode of "CHNS". Since the NDIR detector is sensitive to water vapor, the measured gas was dried with a U-tube filled with Sicapent (phosphorus pentoxide drying agent) before entering the NDIR.

For EA analysis, the samples were sealed in a tin container and were dropped automatically into a combustion tube filled with catalytic material (WO_3 granulate) and maintained at a temperature of 1150°C. As the sample entered the combustion tube, a fixed amount of oxygen was injected into the helium carrier. Time for oxygen dosing was set at 120 seconds. The exothermic oxidation of tin made the samples combust completely. After passing through a reduction tube (silver wool, corundum balls, and copper) at a temperature of 900°C, elements of nitrogen, carbon, sulfur, and hydrogen in the samples were converted into gases of nitrogen, carbon dioxide, sulfur dioxide, and water, respectively. The mixture of gases was separated by gas chromatographic column, and the TCD or NDIR signals of CO_2, H_2O, and SO_2 were recorded. Data were acquired and processed with software from Elementar (vario EL version of 1.3.1., Hanau, Germany). The optimized EA parameters are presented in Table 1. Figure 1 shows the typical EA analytical chromatogram of ECD in ECD Kit.

2.3. Method Development/Validation

2.3.1. Preparation of Standards, QC, and Blank Samples. The preparation of ECD Kit (Vial A) was done according to the procedure of Walovitch et al. [14], which was freeze-dried under an N_2 headspace and contained 0.90 mg ECD, 72 μg $SnCl_2 \cdot 2H_2O$, 360 μg $Na_2EDTA \cdot 2H_2O$, and 24 mg mannitol.

Compositions of ECD calibration standards (Std_{ECD}), blanks (Bk_{Kit}), and QC samples (QC_{ECD}: QC-L, QC-M, QC-H) for method validation were prepared by Isotope Application Division, Institute of Nuclear Energy Research (INER, Taoyuan, Taiwan) and summarized in Table 2. ECD Kit and Kit blank samples were grounded by using an agate mortar for 40 seconds before determination.

Coal calibration standards (Std_{coal}) were freshly prepared daily by weighing 1.00 to 3.50 mg of coal standard. Coal QC samples (QC_{coal}) were prepared in the same way as the coal

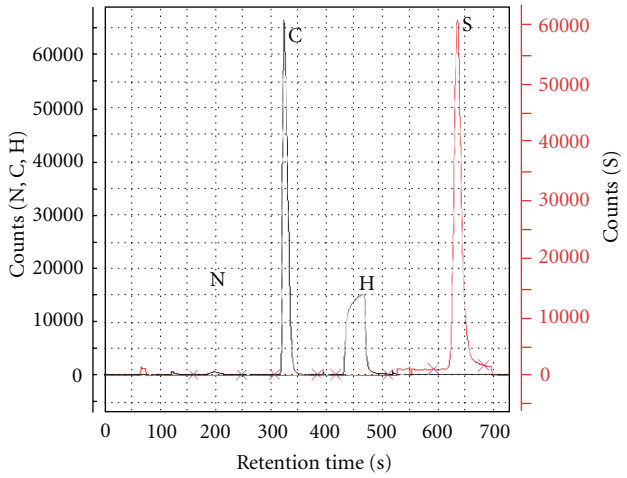

FIGURE 1: Typical elemental analyzer chromatogram of ECD in ECD Kit.

calibration standards by weighing 2.00 ± 0.20 mg of coal standard.

2.3.2. Method Validation. The method was modified and validated according to the International Conference on Harmonization (ICH) guidelines for the validation parameters of analytical method, including specificity, linearity, precision, accuracy, stability, robustness, and system suitability.

Three tin blanks (tin container without sample) and three 7.60 mg Kit blanks (Table 2) were analyzed. Peak areas appeared on the retention time of sulfur were determined to evaluate the specificity (selectivity) of the method in resolution between sulfur and other elements. The calibration curves of five coal standards (1.08 to 3.39 mg) were plotted against the peak areas. The linearity was evaluated by the linear least squares regression method with three coal QC samples determined at concentration of 2.10 mg. The precision of the method was assessed by the same batch of ECD Kit at five concentrations (1.08 to 3.39 mg) and three QC samples determined at concentration of 2.10 mg. Intraday precision (repeatability) and inter-day precision (reproducibility) were evaluated by one analyst within one day and on two different days, respectively. The accuracy was determined by the recovery test. ECD quality control (QC) samples of low (QC-L), medium (QC-M), and high (QC-H) concentration at 0.23, 0.27, and 0.31 mg/vial (nominal weight of ECD per vial of ECD Kit, Table 2) and one coal QC sample at concentration of 2.15 mg were analyzed by the proposed method. Experimental values ($\text{Sulfur(mg)}_{\text{exp}}$ or $\text{Sulfur(\%)}_{\text{exp}}$) were obtained by interpolation to the linear least squares regression equation of a fresh prepared calibration curve (1.08 to 3.45 mg) and compared to the theoretical values ($\text{Sulfur(mg)}_{\text{nominal}}$ or $\text{Sulfur(\%)}_{\text{nominal}}$):

$$\text{Recovery yield (\%)} = \frac{\text{Sulfur(mg)}_{\text{exp}}}{\text{Sulfur(mg)}_{\text{nominal}}} \times 100\%, \quad (1)$$

or

$$\text{Recovery yield (\%)} = \frac{\text{Sulfur(\%)}_{\text{exp}}}{\text{Sulfur(\%)}_{\text{nominal}}} \times 100\%. \quad (2)$$

The bench-top stabilities were examined by analyzing 2.05 ± 0.05 mg of coal standards and 7.52 ± 0.03 mg of ECD Kit samples for three consecutive days. The samples were kept in an autosampler at ambient temperature for EA analysis over this period. Experimental data were obtained by comparing the linear least squares regression equations of calibration curves. The robustness of an analytical method is a basic measurement of its capacity to remain unaffected by small variations in method parameters. In this case, method robustness was evaluated through the effects of dosing time of oxygen, temperatures of combustion tube and reduction tube. The system suitability was assessed by the triplicate analyses of tin blanks and Kit blanks with acceptance criterion of 5,000 counts.

3. Results

3.1. Method Development. Various sulfur forms are presented in coal, that is, pyrite, ferrous sulfate, gypsum, organic sulfur, and elemental sulfur [26, 28, 31]. For direct solid sample analysis of sulfur, effects of matrix, chemical form, and homogeneity of the analyte in sample are relevant to the reliability of analytical results [32–34].

The matrix effect on the determination of sulfur was examined as shown in Table S1 in Supplementary Material available online at doi:10.1155/2011/196238. The average peak area of Kit blanks was ten times higher than that of tin blanks. The linear least squares regression equations of coal standard without and with the existence of Kit blanks were $Y = 1.565 \times 10^{-6}X + 3.174 \times 10^{-3}$ and $Y = 1.547 \times 10^{-6}X + 8.932 \times 10^{-3}$, respectively. No significant differences of linear equations, linearities, and linear ranges were detected. Determination of different concentration ECD standards (0.78 to 1.07 mg, Table 2) in Kit blank using coal for calibration curve were shown in supplemental Table S2. Again, no significant difference of inter-day study coal standard curves was found. Some results of the recovery yields of Std_{ECD} no. 2 and Std_{ECD} no. 4 were outside the acceptance criterion (±5.00%).

3.2. Method Validation. In supplemental Table S1, it is shown that the peak areas on the retention time of sulfur were 248 ± 11 and 2438 ± 642 for tin blanks and Kit blanks, respectively. Data are expressed as average ± SD. Although the peak areas of Kit blanks were higher than those of tin blanks, the areas were approximately half of the acceptance criterion of system suitability (5000 counts).

Standard curves were constructed by plotting peak areas (counts) against the amounts of coal standard and were linear over the range of 1.08 to 3.39 mg (X in weight of sulfur = 0.033–0.104 mg). The linear least squares regression equation of the standard curve in this range was $Y = 1.615 \times 10^{-6}X + 4.747 \times 10^{-3}$, with a correlation coefficient (r) of 0.9993.

Table 2: Preparation and composition of ECD calibration standards, blank, and quality control samples.

Sample	W_{ECD} [1] (mg/vial)	W_{Kit} [2] (mg/vial)	W_S [3] (mg/vial)	$\%W_S$ [4] (%, w/w)
Kit blank (Bk_{Kit})	0.00	25.61	0.00	0.00
ECD Calibration standards				
Std_{ECD} no. 1	0.78	26.27	0.123	0.47
Std_{ECD} no. 2	0.89	26.00	0.140	0.54
Std_{ECD} no. 3	0.97	26.10	0.152	0.58
Std_{ECD} no. 4	1.07	26.04	0.168	0.65
ECD QC samples (QC_{ECD})				
QC-L	0.23	7.27	0.036	0.48
QC-M	0.27	7.23	0.042	0.57
QC-H	0.31	7.19	0.049	0.65

[1] Nominal weight of ECD in ECD Kit.
[2] Total weight of ECD Kit.
[3] Nominal weight of sulfur in ECD Kit.
[4] Percentage of sulfur (%, w/w) in ECD Kit.

Table 3: Precision and accuracy in the analysis of QC samples and ECD in ECD Kit.

Day	Standard curve[1]			Sulfur weight (%)[2]	Recovery yield (%)	
	Dynamic range of sulfur (mg)	Linear least squares regression equation	Correlation coefficient (r)	QC_{coal}	QC_{coal}	QC_{ECD} [3]
1	0.033 –0.104	$Y = 1.615 \times 10^{-6}X + 4.747 \times 10^{-3}$	0.9993	3.13 ± 0.07 (2.25%)	102.08 ± 2.29	—
1	0.031–0.105	$Y = 1.623 \times 10^{-6}X + 1.741 \times 10^{-3}$	0.9989	3.10 ± 0.02 (0.60%)	100.89 ± 0.60	—
2	0.034–0.107	$Y = 1.634 \times 10^{-6}X + 1.034 \times 10^{-3}$	0.9994	3.08 ± 0.04 (1.21%)	100.15 ± 1.21	—
3	0.033–0.106	$Y = 1.576 \times 10^{-6}X + 4.202 \times 10^{-3}$	0.9996	3.18	103.79	102.78 (QC-L) 100.00 (QC-M) 102.08 (QC-H)

[1] Standard curves of coal.
[2] Content percentage of sulfur in coal standard: 3.07% (w/w); data are expressed as average ± SD (%R.S.D.), $n = 3$.
[3] Purity of ECD: 97.53%; compositions of ECD QC samples (QC-L, QC-M, and QC-H) were shown in Table 2.

Table 3 provides the results of repeatability, reproducibility, and accuracy of the proposed method. The Intraday precisions of sulfur weight (%) in coal QC samples were 0.60% to 2.25%. The inter-day precisions of sulfur weight (%) and slope of the calibration curve in coal QC samples were 1.69% and 1.56%, respectively. Average recovery yield of ECD in ECD QC samples was 101.62% ± 1.45% (R.S.D. = 1.42%).

The samples for bench-top stability study were kept in the EA autosampler under ambient environment for a three-consecutive-day experiment (Table 4). Average recovery yields for the determination of sulfur in coal QC samples and ECD in ECD QC samples were 100.88% ± 1.46% (R.S.D. = 1.45%) and 98.93% ± 3.24% (R.S.D. = 3.28%), respectively. The recovery yield of QC_{coal} was approximately 100%. However, recovery yields of QC_{ECD} increased gradually from 96.02%± 2.33% (day 1) to 102.31%± 1.63% (day 3).

The method robustness was evaluated through the effects of dosing time of oxygen, temperatures of combustion tube and reduction tube as shown in Table 5. Optimal dosing time of oxygen, temperatures of combustion tube and reduction tube were 120 sec, 1150°C and 900°C, respectively. No statistically significant difference of linear equations and correlation coefficients were found.

The acceptance criterion of system suitability was assessed by triplicate analyses of the tin blanks and Kit blanks for peak area and was set at 5000 counts.

3.3. Real Sample Analysis. Analytical data of three batch real samples are summarized in Table S3. One in five QC_{coal} samples was outside the acceptance criterion (±5.00%). The determined (experimental) value of ECD by the proposed method gradually increased from 0.934 ± 0.021 mg (batch 1) to 0.984 ± 0.007 mg (batch 3).

4. Discussion

No significant matrix effect of Kit blank on the peak area, linearity of calibration curve, and selectivity of sulfur was found (Table S1). The findings suggest that coal standard (without being spiked into Kit blank) is more convenient and stable (Table S2) than ECD standard to construct the calibration curve.

Table 4: Stability study of QC samples analysis.

Day	Standard curve			Recovery yield (%)[1]	
	Dynamic range of sulfur (mg)	Linear least squares regression equation	Correlation coefficient (r)	QC_{coal}[2]	QC_{ECD}[3]
1	0.032–0.105	$Y = 1.627 \times 10^{-6}X + 4.149 \times 10^{-3}$	0.9994	101.10 ± 0.94	96.02 ± 2.33
2	0.033–0.103	$Y = 1.629 \times 10^{-6}X + 2.390 \times 10^{-3}$	0.9994	99.82 ± 0.90	98.48 ± 1.96
3	0.031–0.105	$Y = 1.609 \times 10^{-6}X + 2.608 \times 10^{-3}$	0.9997	101.72 ± 2.00	102.31 ± 1.63

[1] Data are expressed as average ± SD, $n = 3$.
[2] QC_{coal}: 2.05 ± 0.05 mg of coal QC samples (S = 3.07%, w/w) were analyzed.
[3] QC_{ECD}: 7.52 ± 0.03 mg of ECD QC samples (ECD = 3.61%; S = 0.58%, w/w) were analyzed.

Table 5: Robustness study in the analysis of ECD.

Parameter		Standard curve of coal[1]		QC_{coal}[2]	
		Linear least squares regression equation	Correlation coefficient (r)	Sulfur weight (%)	Recovery yield (%)[3]
Dosing time (sec)	90	$Y = 1.544 \times 10^{-6}X + 3.394 \times 10^{-3}$	0.9992	3.14 ± 0.06	102.36 ± 2.58
	120	$Y = 1.615 \times 10^{-6}X + 4.747 \times 10^{-3}$	0.9993	3.13 ± 0.07	101.91 ± 2.30
	150	$Y = 1.604 \times 10^{-6}X + 7.508 \times 10^{-4}$	0.9999	3.05 ± 0.10	99.17 ± 2.84
Temperature of combustion tube (°C)	1120	$Y = 1.605 \times 10^{-6}X + 8.215 \times 10^{-4}$	0.9997	3.13 ± 0.06	102.01 ± 1.85
	1150	$Y = 1.615 \times 10^{-6}X + 4.747 \times 10^{-3}$	0.9993	3.13 ± 0.07	101.91 ± 2.30
	1180	$Y = 1.586 \times 10^{-6}X + 1.126 \times 10^{-3}$	0.9985	3.03 ± 0.03	98.68 ± 1.14
Temperature of reduction tube (°C)	850	$Y = 1.621 \times 10^{-6}X + 9.226 \times 10^{-5}$	0.9997	3.12 ± 0.06	102.01 ± 1.75
	900	$Y = 1.615 \times 10^{-6}X + 4.747 \times 10^{-3}$	0.9993	3.13 ± 0.07	101.91 ± 2.30
	950	$Y = 1.649 \times 10^{-6}X - 1.288 \times 10^{-3}$	0.9996	3.00 ± 0.02	97.97 ± 0.87

[1] Standard curves were constructed by the coal concentration range of 1.01 to 3.49 mg.
[2] Data are expressed as average ± SD, $n = 3$.
[3] Recovery yield (%) = $\text{Sulfur(mg)}_{exp}/\text{Sulfur(mg)}_{nominal} \times 100\%$.

In this investigation, background peak area of sulfur is attributed to the sample moisture and usage of EA tubes such as Sicapent tube, combustion tube, and reduction tube. Although the background peak area of sulfur is variable, the proposed method has sufficient selectivity (resolution) to the sulfur determination.

The system suitability can be simply assessed by background peak areas of tin blanks and Kit blanks. Background of coal standard and ECD Kit can be deducted by tin and Kit blanks, respectively. Although samples of multiple batches can be assayed within one single day, background peak area of each batch should be determined separately. Each analytical batch should consist of tin blanks, Kit blanks, coal QC samples, calibration coal standards, and unknown samples.

Coal standards are grounded and dried under 110~120°C for at least 2 hours before determination and prepared for the standards curve freshly.

The number of QC samples (in multiples of three) depends on the total number of samples in a batch. Table S3 demonstrates that triplicate QC samples analyses are necessary to ensure quality of the assay for a batch within 10–20 samples. Acceptance criterion is suggested to set at least 67% (2 out of 3) of QC samples, which should be within ±5% of their respective nominal value, and 33% of the QC samples may be outside ±5% of nominal value.

Nominal content of ECD in each ECD Kit vial is 0.900 ± 0.135 mg/vial, which is equal to the weight of sulfur in the range of 0.033–0.104 mg/vial. Therefore, one-third to half of content of ECD Kit was suggested to sample for EA analysis.

The observation of three-day stability study of ECD Kit in Table 4 (recovery yields of QC_{ECD} increased gradually) is difficult to explain, but it might be related to the degradation of ECD in ECD Kit due to the moisture. For example, an intermolecular sulfur-sulfur bonding compound was found in our preliminary forced degradation study.

In Table 5, the results of method robustness evaluation further support the optimal conditions of Table 1. Additionally, the results of method validation in Tables 3, 4, and 5 indicate the potential of this method in pharmaceutical QC.

However, this method is limited to QC analysis of "fresh prepared" ECD Kit, where purity of ECD should be determined prior to mass fabrication processes. Based on the test specification in practice guidelines of the American College of Radiology (ACR) and the European Association of Nuclear Medicine Neuroimaging Committee (ENC), the radiochemical purity (RCP) determinations of Tc-99m-ECD should be performed on each vial prior to injection and can also be used to verify the quality of ECD Kit [1, 2].

5. Conclusion

Since the composition of ECD Kit may cause degradation of ECD as soon as it is dissolved in (non)aqueous solutions, the best way to adopt for the quantitation is highly restricted to

a method of direct solid samples analysis. This investigation provides a method for the intended purpose, for example, routine QC of chemical manufacturing. ECD is one of the diamino dithiol (DADT) derivatives to form stable complexes with radiorhenium or radiotechnetium. Therefore, this method can be also a useful tool to investigate the QC quantitation and properties of thiol-contained derivatives. Finally, this research not only enhances our understanding of ECD Kit about its stability but also raises some questions that require further investigation, especially the degradation pathways, degradation compounds of ECD in ECD Kit and a more stable ECD Kit, formulation design.

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