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ARTICLE

An Ultrasensitive LC-APPI-MS/MS Method for Simultaneous Determination of Ciclesonide and Active Metabolite Desisobutyryl-Ciclesonide in Human Serum and Its Application to a Clinical Study

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Background: The development of more efficient drug delivery devices for ciclesonide inhalation products requires an ultrasensitive bioanalytical method to measure systematic exposure of ciclesonide (CIC) and its active metabolite desisobutyryl-ciclesonide (des-CIC) in humans.

Method: Serum sample was extracted with 1-chlorobutane. A reversed-phase liquid chromatography coupled with atmospheric pressure photoionization–tandem mass spectrometry (LC-APPI-MS/MS) method was used for quantification of 1–500 pg/mL for both analytes in a 0.500-mL serum. The analysis time was 4.7 min/injection. CIC-d_{11} and des-CIC-d_{11} were used as the internal standards.

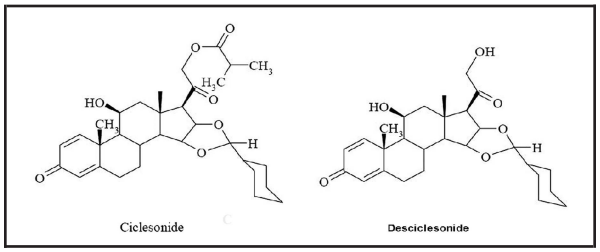
Results: Calibration curves showed good linearity ($r^2 > 0.99$) for both analytes. This novel method was precise and accurate with interassay precision and accuracy of all within 9.6% CV and ± 4.0% bias for regular QC samples. Extraction recovery was approximately 85% for both analytes. Serum samples are stable for 3 freeze–thaw cycles, 24 h at bench top, and up to 706 days at both −20 °C and −70 °C. This method was successfully used to support a pharmacokinetic (PK) comparison between the inhalation suspensions and an inhalation aerosol of ciclesonide in healthy participants. The method robustness was also supported by the good incurred sample reanalysis reproducibility.

Conclusion: APPI, a highly selective and sensitive ionization source, made possible for quantifying CIC and des-CIC with a lower limit of quantification (LLOQ) of 1 pg/mL in human serum by LC-MS/MS. A 10-fold sensitivity improvement from the most sensitive reported method (LLOQ, 10 pg/mL) is essential to fully characterize the PK profiles of CIC and des-CIC in support of the clinical development of the ciclesonide-related medications for patients.

IMPACT STATEMENT

This novel reversed-phase liquid chromatography coupled with atmospheric pressure photoionization–tandem mass spectrometry (LC-APPI-MS/MS) method with superior performance is the first ever method capable of unambiguously monitoring the concentration–time profile of CIC and des-CIC in the clinical study in which an extremely low dose of highly potent ciclesonide medication was given to the patients. The quantification limit of 1 pg/mL allows the accurate estimation of elimination half-life of CIC and des-CIC; therefore, the potential drug accumulation could be well characterized to lower the risk for those patients who suffer from asthma and other relevant diseases.

Ciclesonide (CIC),[3] 2- [(1S,2S,4R,8S,9S,11S,12S, 13R)-6-cyclohexyl-11-hydroxy-9,13-dimethyl-16-oxo-5,7-dioxapentacycloicosa-14,17-dien-8-yl]-2-oxoethyl-2-methylpropanoate, is a non-halogenated glucocorticoid that was developed for treatment of persistent asthma and allergic rhinitis (*1*–*3*). As an inactive prodrug, CIC is administered in an aerosol solution and enzymatically converted by intracellular esterases in the upper and lower airways to its pharmacologically active metabolite, desisobutyryl-ciclesonide (des-CIC). Their chemical structures are as follows:



Des-CIC is highly bound (99%) to plasma proteins, and CIC has low oral and systemic bioavailability, especially when it is administrated as an inhalation suspension formulation. As a result, low serum concentrations of CIC and des-CIC were attained after inhalation, and therefore reliable detection of a very low concentration (e.g., low pg/mL concentration) is necessary in clinical pharmacology studies (*2*, *4*). Otherwise it would be difficult to obtain meaningful pharmacokinetic (PK) data that is required to supporting development of new CIC-related products.

Several analytical methods were published for determining CIC and des-CIC in human serum (*5*–*10*) with thus far the best sensitivity with a lower limit of quantification (LLOQ) of 10 pg/mL (*8*). The reported LC-MS/MS methods had been tried with different ionization techniques such as atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) and atmospheric pressure photoionization (APPI) (*6*, *8*, *9*). APPI is a more recently developed soft ionization technique. Although photoionization works only for a relatively small group of molecules, it has some advantages for the analysis of nonpolar analytes and those analytes hard to protonate or deprotonate (*11*–*16*). For some compounds, APPI may be the only ionization technology for successful LC-MS/MS measurement. The study presented here is one example of this kind because an extremely demanding sensitivity requires a superior specificity of the analyte ionization. APPI uses vacuum ultraviolet radiation (photon emitter), and its ionization efficiency can be enhanced by a dopant such as toluene and acetone. Several reviews on APPI and its applications are available in the literature (*17*–*19*). This presentation describes the development, validation, and application of an ultrasensitive and specific LC-APPI-MS/MS method (LLOQ, 1 pg/mL) for simultaneous quantification of CIC and its metabolite des-CIC in human serum.

MATERIALS AND METHODS

Chemicals and materials

CIC ($C_{32}H_{44}O_7$; MW, 540.7), des-CIC ($C_{28}H_{38}O_6$; MW, 470.6), and corresponding internal standards

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[3] **Nonstandard abbreviations:** CIC, ciclesonide; des-CIC, desisobutyryl-ciclesonide; PK, pharmacokinetic; LLOQ, lower limit of quantification; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; APPI, atmospheric pressure photoionization; IS, internal standard; HFA, hydrofluoroalkane; ME, matrix effect; AUC, area under the curve; SPE, solid-phase extraction; LLE, liquid–liquid extraction.

(ISs) ciclesonide-d_{11} (CIC-d_{11}, IS1) and desisobutyryl-ciclesonide-d_{11} (des-CIC-d_{11}, IS2) were obtained from Toronto Research Chemicals. Other compounds used for potential interference tests were from USP or Sigma-Aldrich. Acetonitrile (HPLC grade) was from EMD chemicals. Methanol (HPLC grade) was from Mallinckrodt; 1-chlorobutane (GR grade) was obtained from Acros. Acetone (HPLC grade), ammonium acetate (GR grade), acetic acid (GR grade), and dimethyl sulfoxide (HPLC grade) were all from Sigma-Aldrich. The used water was purified with a Milli-Q system (18.2 MΩ · cm) from Millipore. Blank human serum was purchased from Bioreclamation.

Calibration standards and QC samples

Respective stock solutions were prepared by weighing each compound into a volumetric flask and dissolving in methanol to achieve their target concentrations, that is, 1.00 g/L for CIC and des-CIC and 200 µg/mL for each IS. The combined working solutions were created and used to generate the 8-point calibration curve in human serum at 1, 2, 10, 50, 100, 250, 400, 500 pg/mL. Regular QC samples were prepared in human serum with concentrations of 3 (low), 150 (mid), and 380 (high) pg/mL for both analytes (limited to <5% supplementing volume into the matrix). The LLOQ QC samples (1 pg/mL) and dilution QC samples (2000 pg/mL) were also prepared for the method validation use. The combined IS working solution at 10 ng/mL was made by diluting their stock solutions with 1:1 methanol and water. All the stock solutions, supplement solutions, and QC samples were stored at −20 °C. A subset of QC samples were stored at −70 °C for a lower temperature storage stability evaluation. One set of special QC samples were prepared in prehemolyzed matrix and used for hemolysis effect testing.

Serum sample extraction

For extraction, 20 µL of the combined IS working solution was added to 0.500 mL of human serum sample and vortex mixed for approximately 15 s (each IS in serum was approximately 400 pg/mL). Next, 0.50 mL of 200 mmol/L ammonium acetate solution was added to each sample tube and briefly mixed. After that, 2 mL of 1-chlorobutane was added to extract the analytes with a 5-min vortex mixing followed by 5 min of centrifugation at 12000 g. Thereafter, the aqueous phase was frozen in a dry-ice/acetone bath, and the organic phase was then decanted to another clean glass tube. A 50-µL aliquot of DMSO was added to the extract and mixed well. The organic extract was evaporated to complete dryness under a nitrogen stream of approximately 15 psi in a water bath set at 35 °C (approximately 20 min). The residue was reconstituted in 75 µL of 0.1% acetic acid in 1:1 methanol and water. Following a 1-min vortex mixing and a 3-min centrifugation at 1200 g, the resulting sample was transferred into a glass injection vial. Typically, 10 µL of sample was injected for analysis.

LC-APPI-MS/MS analysis

The LC system was a Shimadzu HPLC with LC-20AD pumps and SIL-20AC HT injector. The separation was on a Phenomenex Synergi MAX-RP column (50 × 2 mm, 4 µm, 80 Å) using a gradient mobile phase with a flow rate of 0.60 mL/min unless otherwise specified [mobile phase A, 0.01% acetic acid in water-acetone (90/10, v/v); mobile phase B, 0.01% acetic acid in acetonitrile-acetone (90/10, v/v)] at the following gradient steps: from 0.01 to 1.70 min, 45%–100% B; from 1.70 to 2.30 min, 100% B; from 2.30 to 3.20 min, 100% B, but flow rate from 0.60 to 1.60 mL/min; from 3.20 to 3.30 min, 100% to 45% B, and flow rate from 1.60 to 0.60 mL/min; from 3.30 to 4.70 min, 45% B, and flow rate maintained at 0.60 mL/min. The retention times were 2.25 min for CIC and IS1, and 1.40 min for des-CIC and IS2, respectively. The HPLC was coupled to an API 5000 triple quadrupole mass spectrometer (SCIEX) with APPI source operated in the negative mode. The source temperature was

maintained at 300 °C and the sprayer voltage was set at −800 V. Nitrogen was used as the collision gas at a setting of 4. Multiple reaction monitoring was used for MS/MS transitions of *m/z* 599.2→339.1 for CIC, 529.2→357.2 for des-CIC, 610.4→339.1 for CIC-d_{11} (IS1), and 540.4→357.2 for des-CIC-d_{11} (IS2), respectively. The collision energy was 26 eV for CIC and CIC-d_{11}, and 21 eV for des-CIC and des-CIC-d_{11}. The dwell time was 70 ms for each transition. Peak area ratios of the analyte/IS were used to generate calibration curves and calculate the concentrations of QCs and unknown samples. Data acquisition, peak integration, and concentration calculation were performed with Analyst® 1.4.2 from SCIEX.

Method validation

As this work was completed before year 2018, the method was validated following the Food and Drug Administration guidance issued in 2001 (*20*) with regard to selectivity, sensitivity, linearity, precision, and accuracy. Extraction recovery, matrix effect including hemolysis effect, and sample stability (e.g., freeze/thaw, bench top, extract stability, reinjection viability, and long-term frozen storage, etc.) were also thoroughly evaluated.

Matrix effect was calculated with the formula below:

$$\text{Matrix effect (ME\%)} = \left(1 - \frac{\text{Peak area in post} - \text{extraction spiked sample}}{\text{Peak area in neat solution}}\right) \times 100$$

Hemolysis effect was investigated by measuring the 5% hemolyzed QC samples against the regular calibration curves. The extraction recovery was investigated by comparing the peak areas of the QC samples with those of postextraction-supplemented samples at the same nominal concentrations.

Clinical application

The validated method was applied to measure the concentrations of CIC and des-CIC for a clinical study entitled "A Phase 1, Open-Label, Randomized, 4-Way Crossover Study Evaluating the Pharmacokinetics, Safety, and Tolerability of Ciclesonide Inhalation Suspension 0.25 mg, 0.50 mg, and 1.0 mg compared to ciclesonide inhalation aerosol 160-µg in Healthy Adult Volunteers" under Sunovion Clinical Protocol #SEP070-103. The primary objective of this study was to determine the PK of ciclesonide inhalation suspension as compared to ciclesonide inhalation aerosol. The study enrolled 24 healthy male and nonpregnant/nonlactating female participants, ages 18–50. The inhalation suspension was administered via jet nebulizer whereas the aerosol was given through a hydrofluoroalkane (HFA) metered-dose inhaler. Blood samples were collected for serum PK analysis at predose and 5, 15, 30, 45, 60, and 90 min, and 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after the completion of drug nebulization (sputtering) for the inhalation suspension and after dose for the inhalation aerosol. In total, 1504 serum samples were collected and analyzed by the presented method. The PK analyses were performed with Phoenix WinNonlin® professional software. The major PK parameters obtained included C_{max}, $AUC_{0\text{-last}}$ (area under the curve), t_{max}, and $t_{1/2}$. This clinical protocol was reviewed and approved by Integreview an institutional review board that the PPD Clinical Development recommended and used.

RESULTS

Calibration curves

Freshly prepared standard curves consisted of 8 calibrators over a concentration range of 1.00–500 pg/mL for CIC and des-CIC in matrix. Calibration curves were generated with peak area ratio of analyte/IS vs the analyte concentration with a weighted ($1/x^2$) linear least-squares regression. All standard curves across the entire validation study had a coefficient of determination (r^2) ≥0.9947 for

CIC and ≥0.9953 for des-CIC (the detailed curve data not shown.).

Sensitivity and selectivity

By switching to APPI from ESI or APCI, the interference was eliminated, the noise level was considerably reduced, and the gain in S/N ratio and ultimately sensitivity of the method was significantly improved. In this new method, with the APPI source and our optimized method conditions no interference was detected at the retention times of CIC and des-CIC or the ISs. Shown in Fig. 1 are the representative chromatograms of matrix blank, 1 pg/mL (LLOQ), 10 pg/mL (STD3), and 150 pg/mL (mid-QC) added in human serum obtained during the method validation. This result clearly showed that the APPI produced a clean chromatogram for matrix blank and sufficient S/N ratio at the 1 pg/mL (LLOQ) concentration. The precision and accuracy data for LLOQ QCs, as presented in Table 1, met the acceptance criteria (accuracy within 100 ± 20% and CV ≤20%). Therefore, this method was sensitive enough to reliably measure CIC and des-CIC in human serum down to 1 pg/mL.

Precision and accuracy

The intra- and interassay precision and accuracy data for 3 regular QC concentrations (3 pg/mL, 150 pg/mL, and 380 pg/mL) are also shown in Table 1. The intraassay CVs of CIC and des-CIC were ≤8.1% and ≤6.2%, whereas the interassay CVs were calculated to be ≤9.6% and ≤6.3%, respectively. Intraassay accuracy data were between 93.3% and 109.7% for CIC and 97.3% and 104.0% for des-CIC. Interassay accuracy ranged from 96.0% to 100.3% for CIC and 99.3% to 100.3% for des-CIC.

Extraction recovery

The extraction recovery was determined for CIC and des-CIC at low, mid, and high QC concentrations. For each level, 3 measurements were performed. The mean recoveries for CIC and des-CIC were 85.9% and 84.2%, respectively. The mean recoveries for IS1 and IS2 were found to be 94.9% and 90.2%, respectively. The recovery results are also summarized in Table 2.

Matrix effect: ion suppression/enhancement and hemolysis effect

The matrix effect (ME) was evaluated at low, mid, and high QC concentrations for both analytes in 3 replicates. The test was also conducted for both ISs in 9 replicates at the concentration used in the method, that is, 400 pg/mL. For CIC, an overall mean ME was approximately 30% (36.4%, 36.1%, and 23.3% at the low, mid, and high QC concentrations, respectively). For des-CIC, an overall mean ME was about −40% (−45.1%, −29.1%, and −46.6% at the low, mid, and high QC concentrations, respectively). ME was about 29% for CIC-d_{11} and −51% for des-CIC-d_{11}. ME with a positive value indicates ion suppression; ME with a negative value indicates an enhancement. The above results demonstrated that CIC had about 30% ion suppression, whereas des-CIC had about 40% signal enhancement. Six individual lots of blank human sera were evaluated for the potential ME's lot-to-lot variation. Both analyte channels were showing no detectable interference signal at targeted retention time windows, and the CIC's ion suppression and des-CIC's signal enhancement were also confirmed to be consistent with a CV within 15%. Another matrix-related effect, that is, hemolysis effect, was evaluated with the 5% hemolyzed serum. As the data also summarized in Table 2, the hemolyzed matrix would not have a significant effect on the quantification of CIC and des-CIC. The stable isotope-labeled ISs have compensated all kinds of ME reasonably well.

Sample stability

Various stability assessments were conducted as part of the method validation, and the

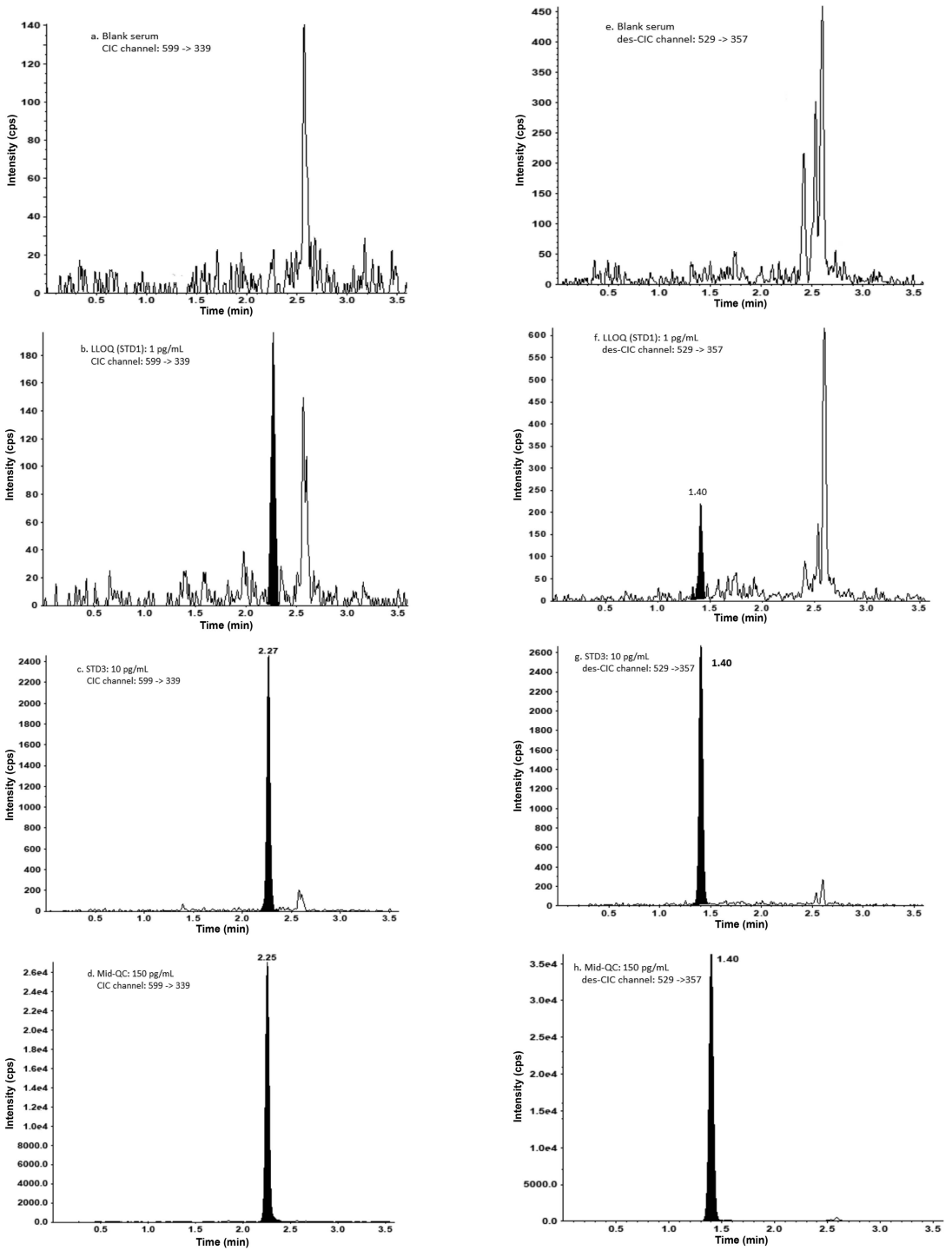

Fig. 1. Representative chromatograms of blank serum, serum with 1 pg/mL (LLOQ), 10 pg/mL (STD3), and 150 pg/mL (Mid-QC) of analytes (*a-d:* CIC channel; *e-h:* des-CIC channel).

Table 1. Intra- and inter-assay precision and accuracy data of QC samples.

		CIC QC samples, pg/mL					Des-CIC QC samples, pg/mL				
P & A statistics		1.00[a]	3.00	150	380	2000[b]	1.00[a]	3.00	150	380	2000[b]
Run 1	Mean	0.900	2.86[c]	140	358	1970	0.980	2.98	150	377	2050
(*n* = 6)	CV, %	4.4	6.3	3.7	2.5	4.0	8.2	6.0	1.7	2.1	4.7
	Accuracy %	90.0	95.3	93.3	94.2	98.5	98.0	99.3	100.0	99.2	102.5
Run 2	Mean	1.19	2.85	145	379	—	1.02	2.92	148	379	—
(*n* = 6)	CV, %	11.8	8.1	2.3	1.6	—	19.6	6.2	2.8	1.4	—
	Accuracy, %	119.0	95.0	96.7	99.7	—	102.0	97.3	98.7	99.7	—
Run 3	Mean	1.08	3.29	149	375	—	1.03	3.12	150	386	—
(*n* = 6)	CV, %	11.1	6.1	2.7	3.1	—	18.4	5.4	1.2	1.4	—
	Accuracy, %	108.0	109.7	99.3	98.7	—	103.0	104.0	100.0	101.6	—
Interassay	Mean	1.06	3.01	144	370	—	1.01	3.01	149	380	—
(N = 18)	CV, %	15.1	9.6	3.8	3.4	—	15.8	6.3	2.0	1.9	—
	Accuracy, %	106.0	100.3	96.0	97.4	—	101.0	100.3	99.3	100.0	—

[a] LLOQ QCs at 1.00 pg/mL.
[b] A 50-fold predilution with blank serum matrix was applied.
[c] One outlier was excluded from run 1 statistics for CIC only.

results are summarized in Table 2. Bench-top (24 h at room temperature) stabilities were 96.7%–101.3% for CIC and 94.0%–98.3% for des-CIC. Freeze–thaw (3 cycles) stability data were 99.3%–103.2% for CIC and 102.9%–103.7% for des-CIC. Extract stability data, evaluated at room temperature for 119 h, were 100.0%–109.2% for CIC and 100.0%–104.3% for des-CIC. Long-term storage stabilities at −20 °C and −70 °C were demonstrated for up to 706 days; at −20 °C, the measured stability was 91.7%–98.7% for CIC and 88.9%–114.0% for des-CIC; at −70 °C, 102.0%–107.7% for CIC and 99.5%–110.7% for des-CIC. Several earlier time points were also tested, and no stability issue was noticed.

Potential interference tests

Nine individual compounds (with concentration of each based on its maximum serum concentration reported in the literature or product labeling) were prepared in blank human serum and in a mid-QC sample (150 pg/mL CIC and des-CIC) as follows for interference test: 32 µg/mL for acetaminophen, 25 ng/mL for 19-norethindrone, 25 ng/mL for D-(-)-norgestrel, 150 ng/mL for 17α-ethynylestradiol, 150 ng/mL for drospirenone, 4 ng/mL for norgestimate, 8 ng/mL for desogestrel vetranal, 60 ng/mL for β-estradiol, or 50 ng/mL for ethynodiol diacetate. These interference evaluations showed that the measured concentrations of

Table 2. Summary of stability assessments, extraction recovery, and hemolysis effect.

Analyte	Ciclesonide, %	Des-ciclesonide, %
Freeze thaw (3 cycles)	99.3–103.2	102.9–103.7
Bench top (24 h)	96.7–101.3	94.0–98.3
Extracted-sample stability (119 h)	100.0–109.2	100.0–104.3
Hemolysis effect	106.7–109.2	102.4–106.0
LTS (706 days, −70°C)	102.0–107.7	99.5–110.7
LTS (706 days, −20°C)	91.7–98.7	88.9–114.0
Extraction recovery	85.9 (IS1, 94.9)	84.2 (IS2, 90.2)

CIC and des-CIC in all tested samples by the current method were well within 100 ± 15% of their nominal values (data not shown), indicating freedom from interference by these medications; thus, the present method is highly selective and specific. If the method is going to be used for supporting patient studies in the future, other inhaler medications may need to be checked for potential interference.

Clinical application: PK comparison between the inhalation suspensions and inhalation aerosol

This ultrasensitive method allowed the precise determination of CIC and des-CIC concentrations in the PK comparison study described above. Representative chromatograms of a predose and a postdose serum sample together with IS are shown in Fig. 2. During the study, there was no single run failure. For the HFA inhalation aerosol treatment, serum samples from the first 2 time points showed a concentration >500 pg/mL (upper limit of quantification) from the initial analysis, and these samples were reanalyzed with appropriate predilution to obtain valid CIC concentration results. Further, a total of 151 samples (approximately 10% of 1504 samples) selected for incurred sample reanalysis evaluation had 146 (96.7%) for CIC and 149 (98.7%) for des-CIC, results that agreed with the originals within 20%. These results demonstrated solid assay reproducibility and robustness.

The PK profiles are presented in Fig. 3. The derived PK parameters are summarized in Table 3. Systemic exposure to CIC and des-CIC were higher after administration of a single-dose of ciclesonide inhalation aerosol (160 µg) than with the inhaled suspensions. The serum C_{max} (2.47 ng/mL) for CIC was achieved rapidly for the 160-µg inhalation aerosol (t_{max} = 5 min, i.e., 0.083 h); the serum C_{max} (0.067 ng/mL) for CIC was achieved at 29 min, that is, 0.484 h, for a 1.0-mg inhalation suspension. Mean C_{max} for des-CIC after the 160-µg inhalation aerosol was 0.399 ng/mL, compared to 0.053 ng/mL for the inhalation suspension 1.0-mg dose. A similar difference in t_{max} was observed for des-CIC (t_{max} = 48 min, or 0.803 h, for 160-µg inhalation aerosol vs 2.21 h for 1.0-mg inhalation suspension). The half-life of CIC for the 160-µg inhalation aerosol was significantly longer than that for the inhalation suspension 1.0-mg dose (8.94 h vs 2.64 h), but the half-life of des-CIC for both the inhalation aerosol and inhalation suspension was similar (9.56 h vs 8.39 h). The $AUC_{0\text{-last}}$ of CIC for the 160-µg inhalation aerosol was about 3.8-fold that for the 1.0-mg inhalation suspension; the $AUC_{0\text{-last}}$ of des-CIC for the 160-µg inhalation aerosol was about 4.5-fold that for the 1.0-mg inhalation suspension. Over the 3 dose levels of inhalation suspension, linear exposure ($AUC_{0\text{-last}}$) was observed for both CIC and des-CIC.

DISCUSSION

Method development

In the literature, solid-phase extraction (SPE) and liquid–liquid extraction (LLE) were exploited for the extraction of CIC and des-CIC from human serum. SPE had been a preferred method (*5*, *7*, *10*), but recently LLE has been more frequently used (*1*, *3*, *6*, *8*, *9*). In general, LLE resulted in a better sensitivity than the SPE methods, likely due to the lipophilic nature of the analytes, which may not bind effectively to the SPE sorbent or may not be fully eluted by the polar elution solvent. The importance of the extraction solvent on quantification was sufficiently discussed (*21*). Diisopropylether and methyl-*tert*-butyl-ether were 2 popularly used solvents for LLE extraction (*1*, *8*, *9*). In the current method, 1-chlorobutane was shown to be a better one than diisopropylether and methyl-*tert*-butyl-ether, which has also been observed by other researchers (*21*). Methyl-*tert*-butyl-ether, 1-chlorobutane, and diisopropylether have

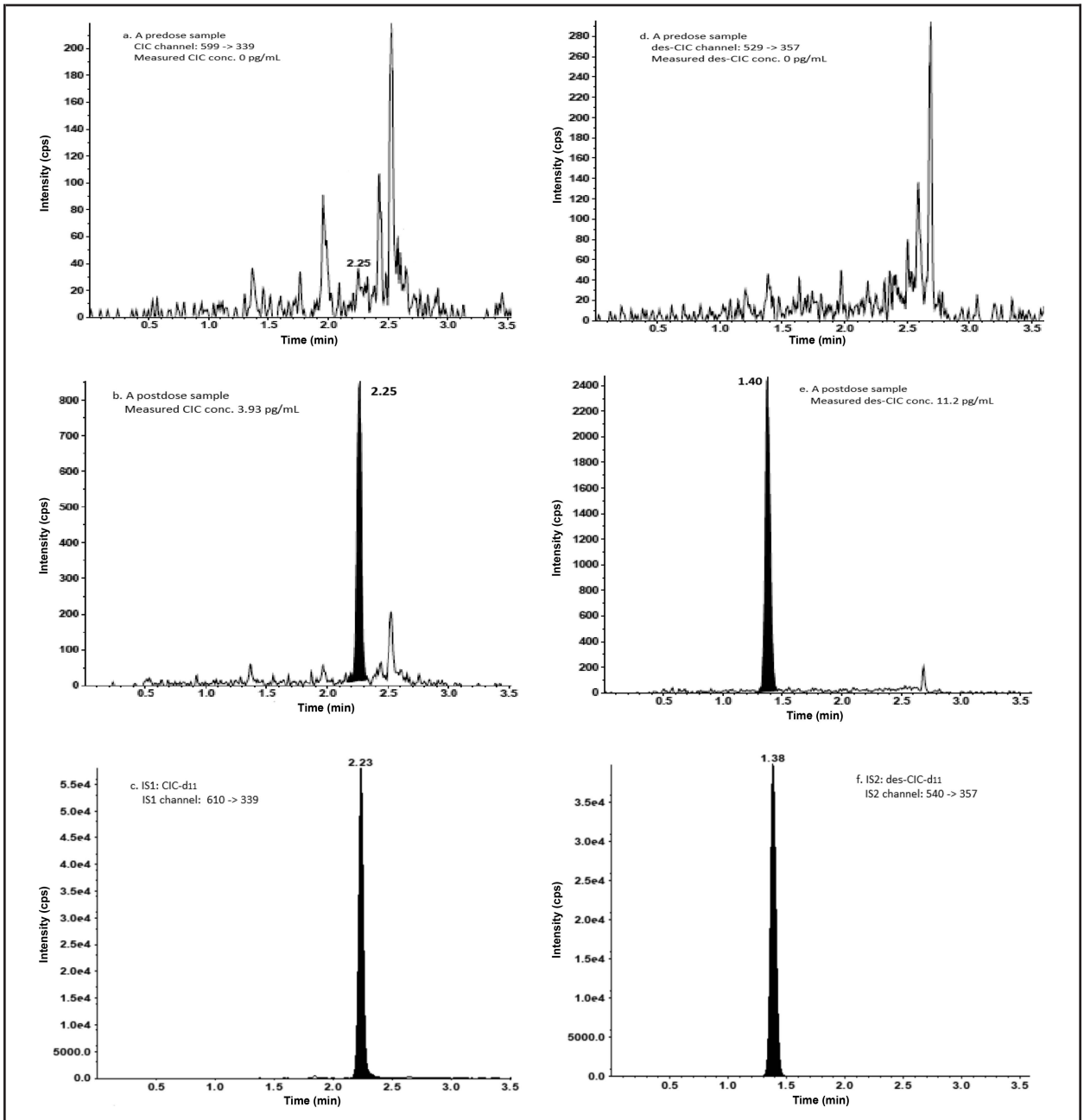

Fig. 2. Representative chromatograms of a predose sample and a postdose human serum sample.

different polarity indices in the order of 2.5, 1, and 1.83, respectively, and a higher polarity index tends to extract more interference components.

Another important observation was the effect of the mode of ionization on quantification. At first, ESI was used to generate gas-phase molecular ions, but significant interference observed at the

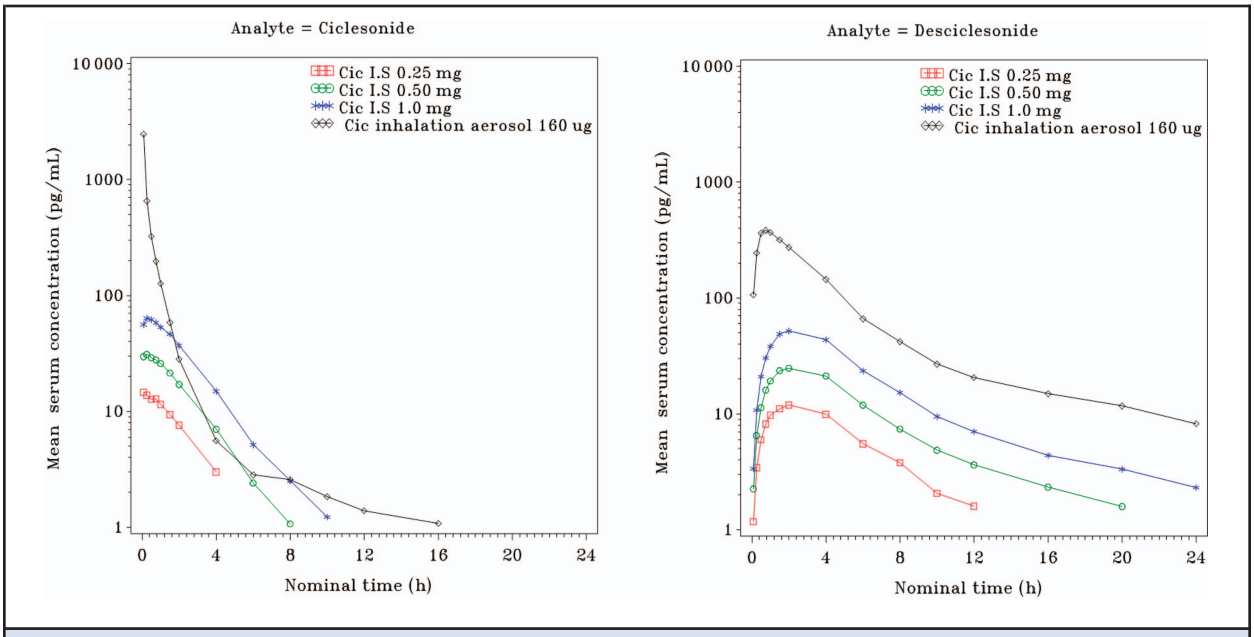

Fig. 3. PK profiles of CIC (left panel) and des-CIC (right panel) in healthy participants for 3 levels of ciclesonide inhalation suspensions (0.25 mg, 0.50 mg, and 1.0 mg) and 1 level of ciclesonide inhalation aerosol (160 µg).

retention time of CIC affected the chromatography and quantification of the parent. When ESI or APCI sources were used, the matrix blank extracts and even the reagent blank extracts would produce a significant interference peak (could be up to several times higher than the LLOQ signal) with the same retention time as CIC. Thus, neither ESI nor APCI source was specific enough for detecting the LLOQ of 1 pg/mL for CIC in human serum. Fortunately, APPI could produce completely a different effect when a clean chromatographic baseline was obtained, and more importantly the interference peak observed when ESI or APCI was used was no longer evident; this unique specificity offered by the APPI source was confirmed with multiple lots of blank matrices and all participants' predose samples collected from the clinical trial. To baseline separate CIC from that of the ESI-detected interference peak will require significantly longer chromatographic time, which may also compromise the detection of des-CIC. This coeluting interference (observed when ESI was used) did not show any effect on the CIC measurement by APPI, possibly because it was at a very low concentration (low pg), and meanwhile the stable-isotope-labeled IS could compensate any coeluting effect from matrix. The use of an APPI source was reported in several methods for CIC and des-CIC (*1*, *3*, *6*, *8*), but none was able to achieve a 1-pg/mL LLOQ.

It should be pointed out that the selected parent ions in negative mode of *m/z* 599.2 for CIC and 529.2 for des-CIC were actually their acetic acid-adduct ions. Thus, introduction of a small amount of acetic acid in reconstitution solvent and in the mobile phase was critical, not just for adjusting pH but for a supply of the counterpart to form the acetic acid-adduct ions. Acetone as a dopant was able to further improve the sensitivity of the method. The optimal mobile phases should contain acetic acid (to form acetic acid adducts) and acetone (as the dopant for APPI). The above optimized source conditions were able to fulfill at

Table 3. PK comparison of CIC and des-CIC in human serum for ciclesonide inhalation suspensions (0.25 mg, 0.50 mg, and 1.0 mg) with the ciclesonide inhalation aerosol (160 µg) in healthy participants.

Analyte	Ciclesonide				Desciclesonide			
Dose/formulation	0.25 mg InS[a]	0.50 mg InS	1.0 mg InS	160 µg HFA	0.25 mg InS	0.50 mg InS	1.0 mg InS	160 µg HFA
N	22	22	23	24	22	22	23	24
C_{max}, ng/mL	0.017 (0.011)[b]	0.035 (0.019)	0.067 (0.052)	2.47 (1.03)	0.013 (0.008)	0.027 (0.014)	0.053 (0.039)	0.399 (0.138)
t_{max}, h	0.252 (0.189)	0.419 (0.274)	0.484 (0.286)	0.083 (0.00)	2.23 (0.92)	2.18 (0.81)	2.21 (0.77)	0.803 (0.243)
$t_{1/2}$, h	1.85 (0.65)	1.84 (1.17)	2.64 (2.36)	8.94 (5.62)	7.90 (5.92)	8.36 (3.62)	8.39 (3.39)	9.56 (2.20)
$AUC_{(0\text{-}last)}$, ng×h/mL	0.040 (0.032)	0.091 (0.054)	0.191 (0.160)	0.725 (0.297)	0.085 (0.058)	0.185 (0.102)	0.359 (0.277)	1.63 (0.50)

[a] InS, inhalation suspension; HFA, hydrofluoroalkane inhaler; N, number of participants.
[b] The values in parentheses are SDs of each PK parameter.

least 10-fold increase in sensitivity, that is, lower the LLOQ down to 1 pg/mL for both CIC and des-CIC.

Performance of the LC-APPI-MS/MS assay

Ciclesonide, a glucocorticoid, has been widely used in the treatment of a variety of diseases including asthma and allergic rhinitis (*1–3*). In the past, glucocorticoids were measured from human serum mainly by immunoassays such as RIA or ELISA. Over the past 2 decades, LC-MS/MS has become the method of choice for the analysis of glucocorticoids because the mass spectrometry–based detection has superior specificity and selectivity over the immunoassays (*12–24*). However, sensitivity of MS-based methods was often seen as not equivalent to that of immunoassays, and sometimes when a highly sensitive method was developed, the interference from the matrix, from the solvent, or from the container might appear even with tandem mass spectrometry detection. Therefore, validating and implementing an ultrasensitive LC-MS/MS assay for the measurement of glucocorticoids in clinical development remains quite challenging. The method presented in this work is the most sensitive method to date. This method shows no interference from matrix endogenous or from other common medications. No carryover was observed after a highest calibrator was injected. APPI is a highly specific ionization source for CIC and des-CIC, which could successfully overcome the interference issues from the ESI and APCI sources.

As the understanding of the APPI evolves, there could be some opportunities to further improve the sensitivity of the method. The use of different dopants and ways of adding the dopant could be further optimized. However, a more extensive solvent screening could also be explored because the solvent choice and composition may have a significant effect on sensitivity (*11*, *25–27*). Ion suppression (approximately 30%) may be further reduced for CIC by washing the extract with acid

and base to remove suppressant and potential interferants. After this study was completed, the method was further modified to start with 0.250-mL serum, gaining an additional 2-fold sensitivity improvement. The revised method was also validated and used for monitoring study patients' compliance in a separate study.

Significance of an ultrasensitive assay (LLOQ 1 pg/mL) on CIC-related drug development

As shown in the PK plots (see Fig. 3), with the most sensitive method (LLOQ, 10 pg/mL), only 1 or 2 time points could have measurable des-CIC exposure for the inhalation suspension treatment at a 0.25-mg dose level. Thus, the implementation of a bioanalytical method with an LLOQ of 1 pg/mL or lower for CIC and des-CIC is of great significance in ciclesonide clinical development (*28*, *29*).

In conclusion, we have presented a superior LC-APPI-MS/MS method for the measurement of CIC and des-CIC down to 1 pg/mL concentration, which is essential to fully characterize the clinical PK of CIC and des-CIC for ciclesonide-related drug delivery products to treat asthma patients. For the samples with an exposure >500 pg/mL, measurements could be fulfilled by predilution with blank matrix, which was also validated during the assay validation.

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