



Liquid chromatography–mass spectrometry method for the quantification of corticosteroids, β 2-adrenoreceptor agonists and anticholinergics in human hair

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This submission includes supplemental material (4 tables and 3 figures).

27 **ABSTRACT**

28 Pharmacological treatments of asthma and chronic obstructive pulmonary disease include
29 medications such as inhaled corticosteroids, long- or short-acting β_2 -adrenoreceptor agonists and
30 anticholinergics. There is an unmet need for the monitoring of adherence and drug exposure to
31 those therapies since poor adherence and/or inhalation technique may impact the control of the
32 disease and the pharmacological strategy. Since plasma therapeutic drug monitoring only reflects
33 the body exposure in the last few hours, the measurement of hair drug concentrations may be of
34 great interest to assess the chronic exposure. A liquid chromatography – tandem mass spectrometry
35 method was therefore developed for the quantification of corticosteroids, β_2 -adrenoreceptor agonists
36 and anticholinergics in human hair. The method was validated according to the European Medicines
37 Agency and Food and Drug Administration guidelines. Sensitivity, accuracy and precision were
38 excellent, allowing the quantification of drugs in the pg/mg range. The method was shown suitable
39 for the analysis of clinical hair samples, demonstrating that it could be used for hair therapeutic
40 drug monitoring in asthma or chronic obstructive pulmonary disease patients.

41

42 **Keywords:** corticosteroids; β_2 -adrenoreceptor agonists; anticholinergics; liquid chromatography-
43 mass spectrometry; hair; therapeutic drug monitoring.

44

1. INTRODUCTION

Asthma affects 3-10% of the European population and is a heterogeneous disease in its clinical manifestations and also in its response to treatment. Conventional pharmacological treatments are classified as relievers (bronchodilators such as short-acting β_2 -adrenoreceptor agonists or anticholinergics) or controllers (inhaled corticosteroids, long-acting β_2 -adrenoreceptor agonists, leukotriene modifiers, oral corticosteroids and anti-IgE treatments). Patients with severe asthma are defined as those requiring treatment with high-dose inhaled (or systemic) corticosteroids in combination with a second controller medication [1]. Approximately 50% of adults and children on long-term therapy for asthma and even less in chronic obstructive pulmonary disease (COPD) patients fail to take medication as directed at least part of the time. In difficult-to-treat asthma, up to 75% patients has insufficient adherence [2-5]. In addition, only 46 to 59% of patients have an efficient inhalation technique, resulting in insufficient drug exposure and participating to poor asthma control [4, 6, 7]. In clinical practice, the effectiveness of inhaled medications on an individual patient can be hopelessly confounded by a lack of objective accurate measure of both intentional and unintentional nonadherence. This lack of information is potentially leading to over-prescription or unnecessary additional treatment, particularly the expensive add-on type 2 biologic therapy in severe asthma. Monitoring the adherence/exposure to those therapies therefore remains an unmet need and new techniques are needed to assess and improve treatment long-term adherence and efficacy. Traditional therapeutic drug monitoring with the measurement of plasma drug concentration has no benefits for asthma and COPD drugs since plasma concentration is a snapshot of the body drug load at a single timepoint and does not allow to assess the chronic exposure, in addition to analytical challenges related to the very low plasma concentrations observed after low-dose inhaled administration. Since drugs present in the bloodstream also reach and stay in the forming hair where their concentrations can be measured and interpreted as an overall chronic exposure, hair analysis could become a valuable tool in the monitoring of inhaled asthma and

70 COPD treatments. Recent reports suggest an interest for hair therapeutic drug monitoring in the
71 clinics in other areas: for HIV infection, there is a strong relationship between hair antiviral
72 concentration and virologic suppression and hair drug concentration is correlated to the dose [8-11];
73 the hair imatinib concentration in patients with chronic myeloid leukaemia was also lower in
74 patients with therapeutic failure than in those with optimal response [12] and an interest in hair
75 concentration monitoring was also suggested to assess compliance in epileptic patients [13]. The
76 incorporation of corticosteroid into human hair has already been documented with prednisone and
77 beclometasone in forensic or clinical cases; with endogenous hormones as well as betametasone,
78 methylprednisolone and triamcinolone in doping controls, or with inhaled fluticasone propionate
79 [14-19]. For β_2 -adrenoreceptor agonists, hair concentrations were reported for salbutamol,
80 salmeterol, formoterol and vilanterol in patients with asthma or in asthma-related death [19, 20].
81 With respect to anticholinergics, there were reports with the detection of tiotropium and
82 umeclidinium, each in one patient, but with concentrations below the lower limit of quantification
83 [19]. Although there were numerous liquid chromatography – mass spectrometry methods already
84 published for the quantification of asthma or COPD drugs in human serum, plasma, urine or
85 sputum, methods for hair analysis are much scarcer and either concern endogenous compounds or
86 anabolic steroids [14, 17, 18], oral or intravenous corticosteroids [15, 16, 18] or inhaled drugs with
87 no method validation [15, 19]. The objectives of the present study were therefore to develop and
88 validate according to regulatory agencies guidelines a highly sensitive liquid chromatography –
89 tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantitation of inhaled or
90 oral corticosteroids, β_2 -adrenoreceptor agonists and anticholinergics in human hair. Budesonide
91 metabolites were also included in the method since budesonide is transformed to 16 α -
92 hydroxyprednisolone and 6 β -hydroxybudesonide by liver metabolism and also forms lipophilic
93 intracellular fatty acid esters (oleate, palmitate, linoleate, palmitoleate) in airways [21, 22], which
94 metabolites may have hair incorporation profiles different from their parent drug.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Budesonide, 6 β -hydroxy budesonide, budesonide-d₈, formoterol fumarate, formoterol-d₆ fumarate, tiotropium-d₃ bromide and salbutamol-d₉ were supplied by Santa Cruz Biotechnology (Texas, United States). Fluticasone propionate, ipratropium bromide monohydrate, tiotropium bromide monohydrate, beclomethasone, salmeterol xinafoate, salbutamol and terbutaline hemisulfate were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). Prednisolone was supplied by Hoechst (Höchst, Germany). Budesonide oleate and budesonide palmitate were supplied by AstraZeneca (Cambridge, United Kingdom). LC-MS-grade ammonium carbonate, formic acid and methyl *tert*-butyl ether (MTBE) were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). LC-MS-grade methanol, dichloromethane, chloroform, ammonium hydroxide and water were from Fisher Scientific (Illkirch, France).

2.2. Working, calibration standard and quality control solutions

Stock solution of all compounds and internal standards (IS) (1 g/L) were prepared in methanol. Working solutions for calibration standards (CS) and quality control (QC) were prepared separately by dilution of each stock solution in methanol. Working solution of IS (formoterol-d₆, budesonide-d₈, tiotropium-d₃ and salbutamol-d₉ respectively 100.0, 1000.0, 200.0 and 500.0 ng/mL) was obtained by dilution of each stock solution in methanol. Solutions were stored at -20°C.

2.3. Sample preparation

Hair samples were washed for 2 minutes in hot distilled water (water was heated to boil then applied on hair) and dried at room temperature. Hair was then cut in 1- to 3-millimetres pieces. Twenty milligrams of each sample were weighted into conic 2.0 mL tubes and 10 microliters of IS

working solution were added. Ten microliters of the appropriate working solution were added for calibration standards and control samples (concentrations of each calibration standard are shown in supplementary Table 1). One millilitre of the extraction solvent (methanol/chloroform – 70/30, v/v) was then added; samples were put in ultrasonic bath for 30 minutes then pulverized in a ball mill for 10 min at 50 Hz with a TissueLyser LT (Qiagen, Hilden, Germany). Samples were centrifuged at 13000 rpm for 10 minutes and 800 µL of the supernatant layer were dispensed into conic 1.5 mL tube and evaporated under vacuum at 43°C for 60 minutes. Fifty microliters of mobile phase (methanol/20 mM pH 9.2 ammonium carbonate buffer – 5/95, v/v) were finally added. Samples were vortex mixed for 30 seconds and transferred into injection vials. Each vial was centrifuged at 3000 rpm for 10 minutes at 4°C before LC-MS/MS analysis.

2.4. LC-MS/MS system and analysis procedure

Method optimization was performed for the analytical conditions such as chromatographic column and mobile phase composition, to setup the most sensitive and fastest method. The final chromatography and mass spectrometry parameters were as follows. Chromatography was performed with an UltiMate 3000 Quaternary Rapid Separation Pump (Thermo Scientific Dionex, Villebon, France), using a 1.7 µm Kinetex evo C18 column (150 x 2.1 mm i.d.) (Phenomenex, California, United States) maintained at 40°C in an UltiMate 3000 Rapid Separation Thermostated Column Compartment (Thermo Scientific Dionex). Ten microliters (10 µL) were injected in the chromatographic system with an UltiMate 3000 Rapid Separation Autosampler (Thermo Scientific Dionex) with a sample stack maintained at +8°C. The following mobile phase was used: methanol and 20 mM pH 9.2 ammonium carbonate buffer. The gradient starting composition was 5% methanol, held for 2 min and reaching 100% at 10 min. Then a clean-up step at 100% methanol was run for 5 minutes. Finally, initial conditions were restored and kept for 6 minutes. The flow rate was 300 µL/min and total run time was 21 min. Compounds were ionized with a heated electrospray

ionization source and detected with a triple quadrupole Quantiva mass spectrometer (ThermoFisher) using multiple reaction monitoring (MRM). Nitrogen (N2-45 nitrogen generator, VWR International, Fontenay sous bois, France) was employed as sheath and auxiliary gas and argon (Messer, Puteaux, France) was used as a collision gas. The electrospray source was set in the positive ionization mode. Instrument parameters were optimized using a continuous 20 µL/min infusion of each compound (1 mg/L in mobile phase). The source parameters were as follows: ion spray voltage: 4100V; vaporizer temperature: 115°C; sheath and aux gas pressure: 31 and 11 arbitrary units, respectively; ion transfer tube temperature: 350°C; collision gas pressure: 1.5 mTor; Q1 and Q3 resolution: 0.7 FWHM and cycle time: 1s. The ions, transitions and RF lens values selected for the analysis of each compound are detailed in supplementary Table 2. Data acquisition and processing was performed using Chromeleon v 6.80 (Thermo Scientific Dionex) and Xcalibur v3.0.63 (ThermoFisher).

2.5. Method validation procedure

The developed method was validated according to the European Medicines Agency guideline (EMA/CHMP/EWP/192217/2009 Rev.1 Corr.2 - <https://www.ema.europa.eu/en/bioanalytical-method-validation>) and Food and Drug Administration guideline (Bioanalytical Method Validation - Guidance for Industry, May 2018 - <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry>) for the following parameters: selectivity, carry-over, lower limit of quantification, calibration curve, accuracy, precision, matrix effect and stability.

2.5.1. Selectivity and carry over

Blank hair samples from six different donors were used. According to the Fischer-Saller scale, hair colours were light brown (2), dark brown (1) and red (1). Two donors also had grey hair, which is

not included in the Fischer-Saller scale. First, 6 blank samples (without compound nor IS) were processed and compared to samples from the same donors spiked with the internal standards and with each compound at the lower limit of quantification. To evaluate the carry-over, a blank sample was analysed immediately after the highest CS in each run. Assay selectivity and carry-over were defined by a mean area of less than 20% for each compound and less than 5% for the IS in blank samples injected before the lower CS (for selectivity) or just after the highest CS (for carry-over) in comparison to the signal obtained for the LLOQ.

2.5.2. Lower limit of quantification

The LLOQ was defined as the lowest concentration measurable with a signal superior or equal to 5 times the signal of a blank sample, an accuracy between 80% and 120% and a precision with a coefficient of variation of $\pm 20\%$ or less over six measurements. The LLOQ was used as the lowest calibration standard.

2.5.3. Calibration curve

Calibration curves included a blank sample, a zero sample, and seven CS was made each day. Quantitation was achieved by plotting the peak area ratios of each compound to the internal standard versus concentration followed by regression analysis, excluding blank and zero samples. Back-calculated concentrations of at least six CS out of the seven (including the LLOQ) had to be within 85-115% of the nominal concentrations, except for the LLOQ for which the range was 80-120%.

2.5.4. Accuracy and precision

Accuracy (measured value/nominal value) and precision (coefficient of variation (CV)) were determined for the three QC levels and for the LLOQ. Six replicates of each QC level were

processed the same day for the intraday assay, whereas each QC level was processed six times three different days over a period of two weeks for the interday assay. The data was analysed using analysis of variance. For QCs, accuracy had to be within 85-115% of the nominal values and precision of $\pm 15\%$, while values between 80-120% and $\pm 20\%$ were accepted for accuracy and precision of the LLOQ, respectively.

2.5.5. Extraction recovery, matrix effect and overall recovery

Human hair samples from six different donors were used to investigate extraction recovery, matrix effect and overall recovery. Three procedures (A, B and C) were performed on each blank hair sample or matrix-free samples spiked at two QC concentrations (corresponding to the lowest and highest quality control levels) to evaluate these parameters: (A) each compound and IS were spiked in hair samples and the complete sample preparation procedure was carried through; (B) blank matrix samples were prepared using the complete sample preparation procedure, compounds and IS were spiked after the evaporation step and the preparation procedure finished; (C) compounds and IS were spiked in mobile phase and directly injected.

Extraction recovery was defined by the ratio between the peak area obtained in procedure A and the peak area obtained in procedure B. Matrix effect was assessed by calculating the matrix factor, defined as the ratio between the peak area obtained in procedure B and the peak area obtained in procedure C. The IS-normalised matrix factor was calculated by dividing the matrix factor of each compound by the matrix factor of appropriate IS. The CV of this IS-normalised matrix factor had to be less than 15%. Overall recovery was defined as the ratio between the peak area obtained in procedure A and the peak area obtained in procedure C.

218 2.5.6. *Stability*

219 The stability of processed samples was studied after storage for 24 hours at +8°C in the
220 autosampler.

221

222 **2.6. Analysis of patient samples**

223 To confirm the suitability of the method for the analysis of clinical samples, it was applied to
224 measure corticosteroids and bronchodilator concentration in hair samples from 6 adult patients
225 participating to a clinical trial (CorticHair, NCT03691961). Those patients had controlled/partly
226 controlled asthma and were receiving a maintenance treatment with budesonide/formoterol at a
227 stable dose (≥ 400 µg/day of budesonide) for at least 2 months. The use of inhaled short acting β_2 -
228 adrenergic receptor agonists was allowed during the study and systemic corticosteroids could be
229 administered if required. Sampling was performed as recommended in the Society of Hair Testing
230 (SoHT) guidelines for drug testing in hair [23]: a strand of hair from the vertex posterior region of
231 the scalp about the width of a thin pencil and with a length equal to or greater than 3 cm was
232 fastened with a string to distinguish the proximal and distal extremities. The hair was cut with clean
233 scissors as close to the scalp as possible, aligned with the root end of the sample clearly identified,
234 secured with an aluminium foil and stored until analysis into an envelope in a dry, dark
235 environment at room temperature, away from direct sunlight.

3. RESULTS AND DISCUSSION

3.1. Sample preparation

3.1.1. Washing optimization

Several solvents for hair washing were compared with the objective of maximizing the removal from drugs which may originate from contamination and be deposited at the external surface of the hair and to minimize the extraction of compounds integrated from the bloodstream to the inside of the hair. Therefore, this optimization step was performed with samples from both treated patients and spiked hair samples, using budesonide as a representative compound. The comparison between dichloromethane, MTBE and hot water resulted in the lowest extraction from incorporated drug and the highest recovery of spiked drug when hot water was used. In order to ensure that the use of hot water for hair washing would not affect the stability of the molecules studied, a triplicate analysis of a solution of all the molecules at the concentration corresponding to the 5th calibration standard was immersed for 2 minutes in hot water before analysis and compared to the same solution kept at room temperature. The area ratio of the chromatographic peaks with and without heating was between 1.02 and 1.08 for all the molecules, indicating that the temperature did not cause any significant degradation.

3.1.2. Extraction method optimization

First, the effect of sample pulverisation was assessed by extracting spiked cut hair samples with or without previous pulverisation. The pulverisation step allowed to increase the peak area of most compounds up to 1.8-fold and was included to the sample extraction procedure. Then, different proportions of the extraction solvent (methanol/chloroform) were compared on hair spiked with all compounds with the ratios 100/0, 70/30, 50/50, 20/80 and 0/100 and peak areas were compared to mobile phase spiked with the same quantities. At this step, the proportions 100/0 and

261 70/30 provided similar, high and reproducible recovery. Samples from patients treated with
262 budesonide/formoterol were used in a last step to compare the recovery with native samples and
263 allowed to conclude that the greatest recovery was observed for the 70/30 ratio.

264

265 **3.2. LC-MS/MS analysis**

266 During chromatographic method development, methanol or acetonitrile were compared as organic
267 modifiers and 20 mM pH 9.2 ammonium carbonate buffer or 0.1% formic acid as aqueous solvents.
268 Two different analytical columns, Acquity BEH C18 1.7 μ m 100 x 2.1 mm i.d. (Waters,
269 Guyancourt, France) and Kinetex Evo C18 1.7 μ m 150 x 2.1 mm i.d. (Phenomenex, Le Pecq,
270 France) were also compared. With respect to the organic modifier, intensities and peak area were
271 greater when methanol was used. For the aqueous solvent, a 2- to 3-fold increase in signal intensity
272 was observed with the ammonium carbonate buffer. The Kinetex Evo C18 column provided the
273 better molecule retention and was therefore retained as the analytical column.
274 With respect to compound detection, multiple reaction monitoring mass spectrometry was used.
275 Source parameters (ion spray voltage, vaporizer temperature, sheath and aux gas pressure, ion
276 transfer tube temperature) were first optimized individually for each molecule, then the best
277 compromise was retained and confirmed to be suitable for all compounds. These final analytical
278 conditions were finally validated on the whole set of compounds and representative chromatograms
279 are shown in Supplementary Figures 1 and 2 for molecules and their internal standards,
280 respectively.

281 Since ipratropium and tiotropium are quaternary ammoniums, already carrying a positive charge in
282 the physiological milieu, the precursor ions for the detection of these compounds in the positive
283 ionization mode were therefore $[M]^+$ ions, *i.e.* $m/z = 332.4$ and 392.1 , respectively. For the same
284 reasons, the precursor ion for tiotropium- d_3 was $m/z = 395.2$. On the other hand, all other
285 compounds are bases with secondary amines, hydroxyl groups or both. Consequently, the precursor

ions observed in the positive ionization mode were the protonated $[M+H]^+$ adducts in each case. The most intense MRM transitions were used for the quantification of each compound and IS, whereas two other transitions were used for confirmation for all compounds. One or two confirmation transitions were used for internal standards (supplementary Table 2). For the quantification, budesonide- d_8 was used as an internal standard for the measurement of budesonide, budesonide oleate, budesonide palmitate, 6β -hydroxy budesonide, beclomethasone, fluticasone and prednisolone; formoterol- d_6 was used for the measurement of formoterol, ipratropium and salmeterol; salbutamol- d_9 was used for the measurement of salbutamol and terbutaline; tiotropium- d_3 was used for the measurement of tiotropium.

3.3. Method validation

3.3.1. Selectivity and carry over

The mean ratio between peak areas in samples spiked at the LLOQ and in blank samples was between 8 and 2600 for all compounds ($n = 6$) and 4715, 14127, 86242 and 1868 for budesonide- d_8 , formoterol- d_6 , tiotropium- d_3 and salbutamol- d_9 , respectively ($n = 6$), demonstrating acceptable selectivity for all molecules. With respect to carry-over, the peak area in blank samples injected after the ULOQ fulfilled the acceptance criteria for budesonide, budesonide oleate, budesonide palmitate, 6β -hydroxy budesonide, beclomethasone, fluticasone, prednisolone, salbutamol, terbutaline, ipratropium and tiotropium. For formoterol and salmeterol, the peak area in blank samples injected after the ULOQ were up to 2-fold the peak area observed at the LLOQ, suggesting that carry over may occur. When the injection of 2 supplemental blank mobile phase samples was added to the procedure after the injection of the highest calibration standard, the peak area in the last washing sample was in accordance with the validation criteria for all compounds. Therefore, in case of concentrations of formoterol or salmeterol in patient samples in the highest range of the calibration curve, care has to be taken to inject washing samples to avoid carry-over. For this

method, our procedure systematically includes the injection of a blank mobile phase sample before each new patient to ensure the absence of carry-over from previous samples.

3.3.2. Limit of quantification

The LLOQ for all compounds ranged from 1 to 25 pg/mg, except for 6 β -hydroxy budesonide (150 pg/mg). At the LLOQ, a signal superior to 5 times the signal of a blank sample was obtained for each compound. Accuracy and precision results are available in Table 1 and comply with the validation criteria with intra- and inter-day CV lower than 9 and 20%, respectively, and accuracies in the range 88.8-117.1%. The developed method is also highly sensitive with LLOQ for most compounds in the range 1-5 pg/mg. Previously reported LLOQ values were 100 pg/mg for 10 corticosteroids [15, 16], 160 pg/mg for fluticasone furoate, 80 pg/mg for fluticasone propionate, 0.8 pg/mg for formoterol, 4 pg/mg for salmeterol and 0.4 pg/mg for tiotropium [19] but the LLOQ definition was different from established guidelines or validation not always reported [15, 16, 19].

3.3.3. Calibration curve

The calibration range was defined by a seven-point calibration curve. The visual inspection of the graphs plotting the peak area ratios of each compounds to the internal standard versus concentration suggested the linearity of the response, justifying the choice of an ordinary least square model to fit the data. Since the pattern of residuals plotted as a function of the concentration was heterogeneous, demonstrating heteroscedasticity, a weighted least square regression model was applied with a 1/x weighing factor [24]. Mean fitted equation obtained from six curves and mean least squares linear regression correlation coefficient (r^2) are shown in supplementary Table 3. Back-calculated concentrations were in the range of $\pm 20\%$ for the LLOQ and $\pm 15\%$ for all other calibration standards.

336 3.3.4. Accuracy and precision

337 Intraday and interday precision and accuracies of the QC samples at the 3 concentration levels are
338 summarized in Table 2 and are all within the range of accepted criteria. The developed method is
339 thus highly precise and accurate in a wide range of concentrations. However, since certified
340 reference materials do not exist for hair drug analysis with these classes of compounds, the accuracy
341 measured from authentic hair samples may be different. This method may be useful to compare
342 drug concentrations in different hair segments from the same individual, allowing to assess the
343 month-by-month drug exposure.

344

345 3.3.5. Extraction recovery, matrix effect and overall recovery

346 The results are summarized in supplementary Table 4. Extraction recovery was in the range 30.4-
347 111.7% for the two QC levels, allowing accurate and precise quantification in a large range of
348 concentrations, as described above. The lowest extraction recoveries were observed for salbutamol
349 and terbutaline, which can be explained by their hydrophilic properties with XLog P of 0.3 and 0.9,
350 respectively, while other compounds have XLogP between 1.6 and 3.9. This highest value is for
351 salmeterol, which also had the highest extraction recovery (101.7-111.7%), showing a direct
352 association between the compound hydrophilicity and the extraction recovery. Tiotropium and
353 ipratropium are also quaternary ammoniums with a positive charge, which may also explain their
354 extraction recovery. The matrix factor was in the range 86.0-107.7% for formoterol,
355 beclomethasone, prednisolone, 6 β -hydroxy budesonide and ipratropium, suggesting that matrix
356 components do not affect the signal for those compounds. For budesonide, fluticasone, salmeterol,
357 budesonide oleate and budesonide palmitate, the matrix factor was in the interval 29.3-59.9%,
358 meaning that a decrease in signal intensity related to matrix components may occur. On the other
359 hand, the signal was modestly increased for salbutamol and terbutaline, with matrix factors between
360 131.8 and 152.6% whereas a huge and unexpected increase in signal intensity was observed for

361 tiotropium, with matrix factors between 4456 and 79410% for all six donors for the 2 QC levels.
362 For all compounds except tiotropium and tiotropium-d₃, the coefficients of variation of the matrix
363 factor was between 9.6 and 35%. Interestingly, the internal standard-normalized matrix factor was
364 between 96.5 and 127.2% for budesonide, budesonide oleate, budesonide palmitate, formoterol,
365 salbutamol, terbutaline, ipratropium and tiotropium, demonstrating that the internal standards
366 appropriately corrected for matrix effects, including for tiotropium. An increase in the internal
367 standard-normalized matrix factor was observed when compared to the non-normalized matrix
368 factor for beclomethasone, prednisolone and 6 β -hydroxy budesonide, suggesting that the use of
369 internal standards others than budesonide-d₈ could be considered. However, all internal standards
370 allowed to reduce the variability since all coefficients of variation of the internal standard-
371 normalized matrix factors were between 1.5 and 14.7% for the two QC levels, meeting all
372 requirements for validation according to the EMA and FDA guidelines. Observations of matrix
373 effect with tiotropium warranted further investigations. There were only few reports of mass
374 spectrometry methods for the measurement of tiotropium in human samples [25-27]. Two methods
375 in human plasma included a method validation according to the US FDA guidelines with the
376 investigation of matrix effect, but either the value of the matrix effect was not reported [27] or only
377 the values of the internal standard-normalized matrix factors [25] were reported. As shown in
378 Supplementary Figure 3, when compared to compounds directly spiked in mobile phase (procedure
379 C), the peak area in samples with matrix spiked at the same concentration before or after extraction
380 increased greatly for tiotropium and tiotropium-d₃ but not for the other molecules. We hypothesized
381 that this matrix effect for tiotropium could be related either to hair components or to solvents used
382 for hair extraction. We thus spiked the compounds in mobile phase and performed the full
383 extraction procedure, which didn't reveal any signal enhancement and allowed to conclude that
384 some hair components may increase the signal of tiotropium. As exposed before, the same
385 phenomenon was observed for tiotropium-d₃ and this deuterated internal standard perfectly

386 corrected the matrix effect, allowing accurate and precise quantification of tiotropium in hair
387 samples.

388

389 3.3.6. Stability

390 The stability of processed QC samples was evaluated by reinjection (with a new calibration curve)
391 of the samples kept at +8°C in the autosampler after 24 hrs and for the 3 QC levels ($n = 6$ for each
392 level). All the differences between the measured and nominal concentrations were between -15%
393 and +15%, showing the stability of processed samples up to 24 hours in the autosampler for each
394 compound.

395

396 3.4. Analysis of patient samples

397 A representative chromatogram for patient analysis is shown in Figure 1 and quantitative values in
398 the samples from the 6 patients are shown in Figure 2A. Budesonide was found in the hair from 5 of
399 the 6 patients at concentrations between 6.1 and 8.5 pg/mg; formoterol was quantified between 1.0
400 and 13.1 pg/mg in the hair from 4 patients, with another patient below the LLOQ (0.9 pg/mg) and
401 salbutamol was detected in the samples from 5 patients also, with only one value above the LLOQ,
402 at 6.4 pg/mg. Observed formoterol concentrations are in line with previous findings for patients
403 receiving ≥ 12 μg formoterol daily [19]. Salbutamol concentrations in the range 27-192 pg/mg were
404 also reported in asthma patients [20], while this is the first report of budesonide concentrations in
405 human hair. Interestingly, the concentrations of budesonide and formoterol in four consecutive hair
406 segments corresponding to a 4-months treatment period at constant dose in one patient were stable
407 over time (Figure 2B).

4. CONCLUSION

We describe a method for the simultaneous quantification of corticosteroids, β_2 -agonists and anticholinergics in human hair samples. This method was validated according to the EMA and FDA guidelines and was shown suitable for the analysis of clinical hair samples which revealed the presence of budesonide, formoterol or salbutamol, part of the treatments used during the clinical trial, demonstrating that this method may be used for hair therapeutic drug monitoring of inhaled medicines in asthma or COPD patients in future studies. This method may be particularly suitable for assessing month-by-month exposure by applying segmental analysis since one segment is compared to another segment from the same patient.

5. ACKNOWLEDGEMENTS

None

6. AUTHOR CONTRIBUTIONS

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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8. DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no conflict of interest.

9. TABLES

Table 1: Accuracy and precision data for corticosteroids, β_2 -agonists and anticholinergics in hair LLOQ samples.

	LLOQ				
	Concentration (pg/mg)	Intraday ($n = 6$)		Interday ($n = 6$)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Budesonide	5	5.3	106.1-112.2	6.8	109.1
Formoterol	1	8.7	104.4-115.3	12.4	109.2
Beclomethasone	10	7.1	102.9-112.5	12.3	106.4
Prednisolone	25	4.3	99.8-106.3	8.3	102.3
Fluticasone	25	4.3	90.9-102.4	15.9	95.3
Salmeterol	5	5.8	95.5-102.2	8.7	98.3
Salbutamol	5	6.6	99.2-110.8	15.7	103.2
Terbutaline	5	4.9	93.7-108.9	18.4	101.8
Ipratropium	5	2.6	107.0-116.4	11.1	112.8
Tiotropium*	5	8.3	92.7-96.3	4.8	95.0
Budesonide oleate	5	6.7	101.3-117.1	19.3	107.4
Budesonide palmitate*	5	4.6	98.8-116.0	18.5	106.4
6 β -hydroxy budesonide	150	6.7	88.8-101.6	16.6	95.1

CV and accuracy values are expressed as percentages. *: One (tiotropium) and two (budesonide palmitate) of 18 values were considered as outliers according to Grubbs test and were excluded from the analysis.

439 **Table 2:** Accuracy and precision for corticosteroids, β_2 -agonists and anticholinergics in spiked hair QC samples.

	Low QC					Medium QC					High QC				
	Target (pg/mg)	Intraday		Interday		Target (pg/mg)	Intraday		Interday		Target (pg/mg)	Intraday		Interday	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Budesonide	10	6.0	90.9-99.6	11.3	94.9	75	5.9	90.4-101.4	13.3	95.9	600	2.7	102.3-113.4	13.0	108.8
Formoterol	10	2.4	87.7-96.5	12.4	91.3	75	4.6	90.5-97.2	8.3	93.9	600	7.1	99.6-105.9	7.5	102.6
Beclomethasone	30	7.6	101.7-108.2	8.0	105.6	75	5.4	99.4-112.1	14.1	105.6	600	4.5	102.8-113.7	12.4	108.8
Prednisolone	30	5.3	104.9-110.5	7.3	108.6	75	5.3	102.1-113.0	12.4	107.5	600	9.1	101.9-107.8	6.8	104.8
Fluticasone	30	5.0	89.5-94.3	6.5	92.1	75	5.8	89.6-97.1	10.2	92.8	600	6.5	94.0-106.3	14.4	100.0
Salmeterol	10	6.0	95.3-101.6	8.5	99.2	75	7.0	99.7-112.4	14.2	105.3	600	4.8	105.0-113.4	9.5	109.0
Salbutamol	10	2.2	87.4-92.1	6.9	89.3	75	5.9	108.1-108.2	0.1	108.2	600	5.3	104.2-112.8	10.7	107.3
Terbutaline	10	3.8	86.7-96.3	12.4	91.2	75	8.4	89.5-98.7	12.6	95.6	600	6.0	93.7-99.0	6.7	96.2
Ipratropium	10	9.2	99.1-106	8.7	103.2	75	7.6	103.6-113.7	11.0	108.2	600	6.0	97.8-105.3	8.9	102.0
Tiotropium	10	6.6	97.3-99.5	2.9	98.3	75	3.8	86.7-89.3	3.4	88.1	600	8.5	100.7-107.7	8.6	103.4
Budesonide oleate	10	8.6	93.0-103.9	13.3	99.5	75	9.0	97.5-99.2	2.0	98.2	600	11.7	94.4-105.7	13.4	99.7
Budesonide palmitate	10	8.7	99.2-101.6	3.3	100.1	75	8.7	95.2-97.9	3.8	97.0	600	9.6	96.2-107.4	12.9	101.8
6 β -hydroxy budesonide	250	5.0	98.8-112.8	14.8	106.3	450	4.8	103.1-113.8	12.7	110.2	600	7.9	102.4-113.2	13.4	106.1

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441 Values are expressed as percentages; $n = 6$ for both intraday and interday results.

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11. FIGURE LEGENDS

Figure 1: Chromatograms obtained during the analysis of a patient hair sample.

Representative LC-MS/MS chromatograms of salbutamol, budesonide and internal standard (budesonide-d₈, formoterol-d₆ and salbutamol-d₉) obtained after the analysis of an asthmatic patient treated with budesonide, formoterol and salbutamol.

Figure 2: Patient samples analysis. Hair drug concentrations in single hair segments from 6 patients participating to a clinical trial (A) or in 4 consecutive segments of one brown-hair patient treated daily with 800 µg budesonide / 12 µg formoterol (B). Open symbols represent values below the lower limit of quantification, which are therefore purely indicative.

Figure 1

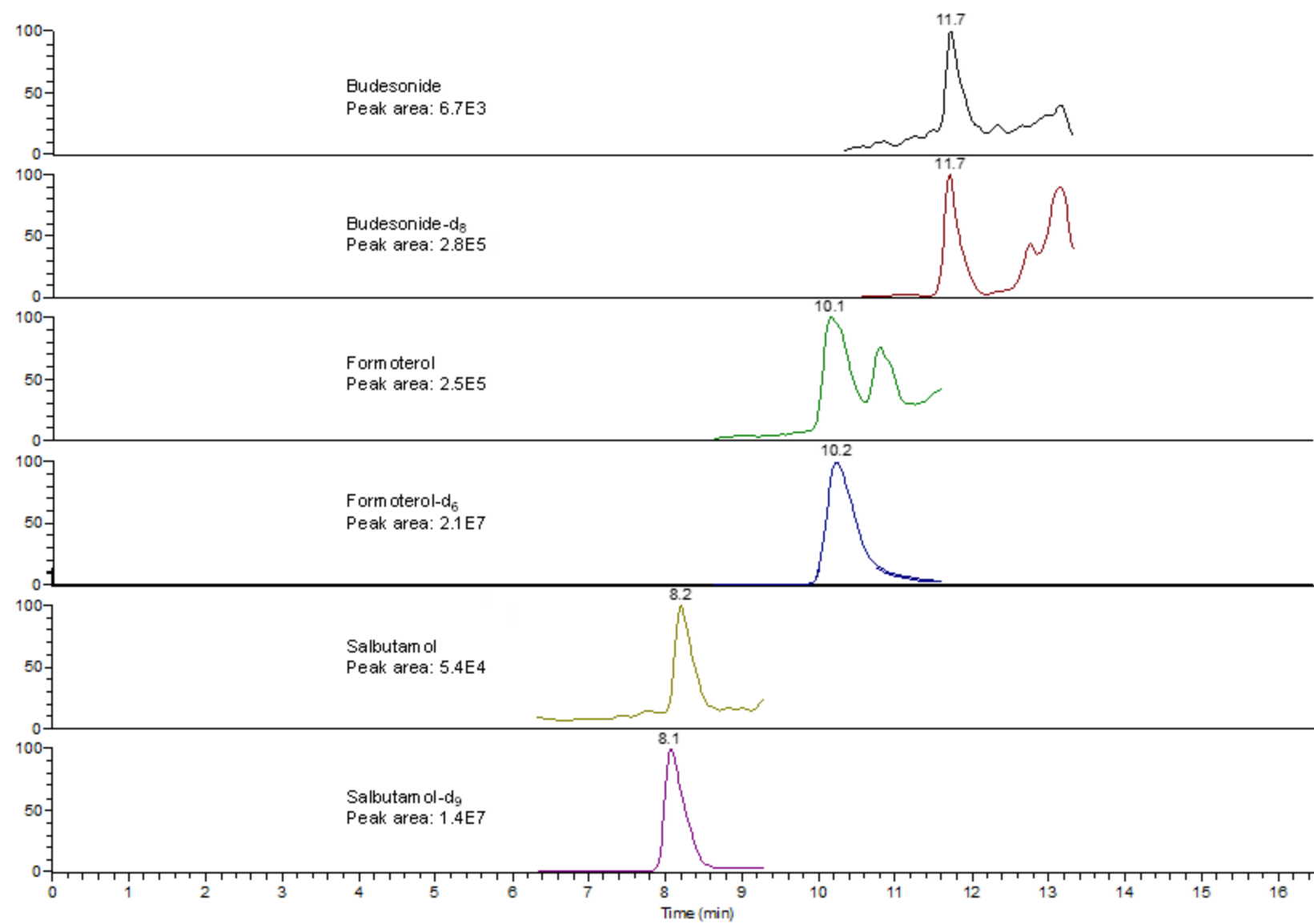


Figure 2

