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Detection of budesonide in human urine after inhalation by liquid chromatography–mass spectrometry

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Abstract

Budesonide, a corticosteroid frequently used in the treatment of asthma, is most often administered via inhalation. Its use in sports is allowed when medically necessary. A fast, sensitive and accurate LC–MS method was developed and validated for the quantification of budesonide and its major metabolite 16α-hydroxyprednisolone in urine samples after inhalation of a metered dose (Pulmicort-Turbohaler®200).

Sample preparation consists of an alkaline liquid–liquid extraction with ethyl acetate. Analysis was performed using liquid chromatography–tandem mass spectrometry with electrospray ionization (ESI). The method was linear in the range of 5–100 and 0.5–10 ng/mL for 16α-hydroxyprednisolone and budesonide, respectively. The limits of quantification were 5 ng/ml for 16α-hydroxyprednisolone and 0.5 ng/mL for budesonide. The accuracy ranged from 2.2 to 3.5% for 16α-hydroxyprednisolone and from 0.8 to 16.4% for budesonide. After administration of 200 μg of budesonide to five healthy volunteers budesonide could not be detected in any urine sample whereas 16α-hydroxyprednisolone was detectable up to 12 h post-administration.

Keywords: Doping; LC–MS; Corticosteroids; Budesonide; Urine

1. Introduction

Corticosteroids are very powerful anti-inflammatory agents used for the treatment of inflammatory diseases such as asthma. They can cause euphoria [1] and they alleviate pain in general, allowing athletes to perform while they are actually injured. Hence corticosteroids appear on the prohibited list of substances issued by the World Anti-Doping Agency (WADA) [2]. Athletes use budesonide (Fig. 1) mainly for the treatment of excercise induced asthma and it is a frequently administered corticosteroid by inhalation. Budesonide is rapidly metabolised to different metabolites of which 16α-hydroxyprednisolone (Fig. 1) is the major one in man [3]. Hence this metabolite is the primary target compound for the detection of budesonide in doping analysis [4].

Nevertheless, budesonide is still suggested as target compound for its detection in urine [5,6].

Despite structural similarities between budesonide and desonide regarding the acetal moiety at the 16 and 17 positions, 16α-hydroxyprednisolone was not detected as a metabolite of desonide [7].

Although different pharmacokinetic studies [8–10] have been published, few data on the detection of 16α-hydroxyprednisolone and budesonide related to doping analysis is available [4]. The aim of the current study was to determine budesonide and 16α-hydroxyprednisolone in urine after the inhalation of a single dose of budesonide using a Pulmicort®–Turbohaler®200.

2. Experimental

2.1. Chemicals and reagents

Betamethasone and budesonide were a gift from Glaxo-Wellcome (Greenford, United Kingdom), 16α-hydroxyprednisolone was a kind gift from Astra-Zeneca (Lund, Sweden).

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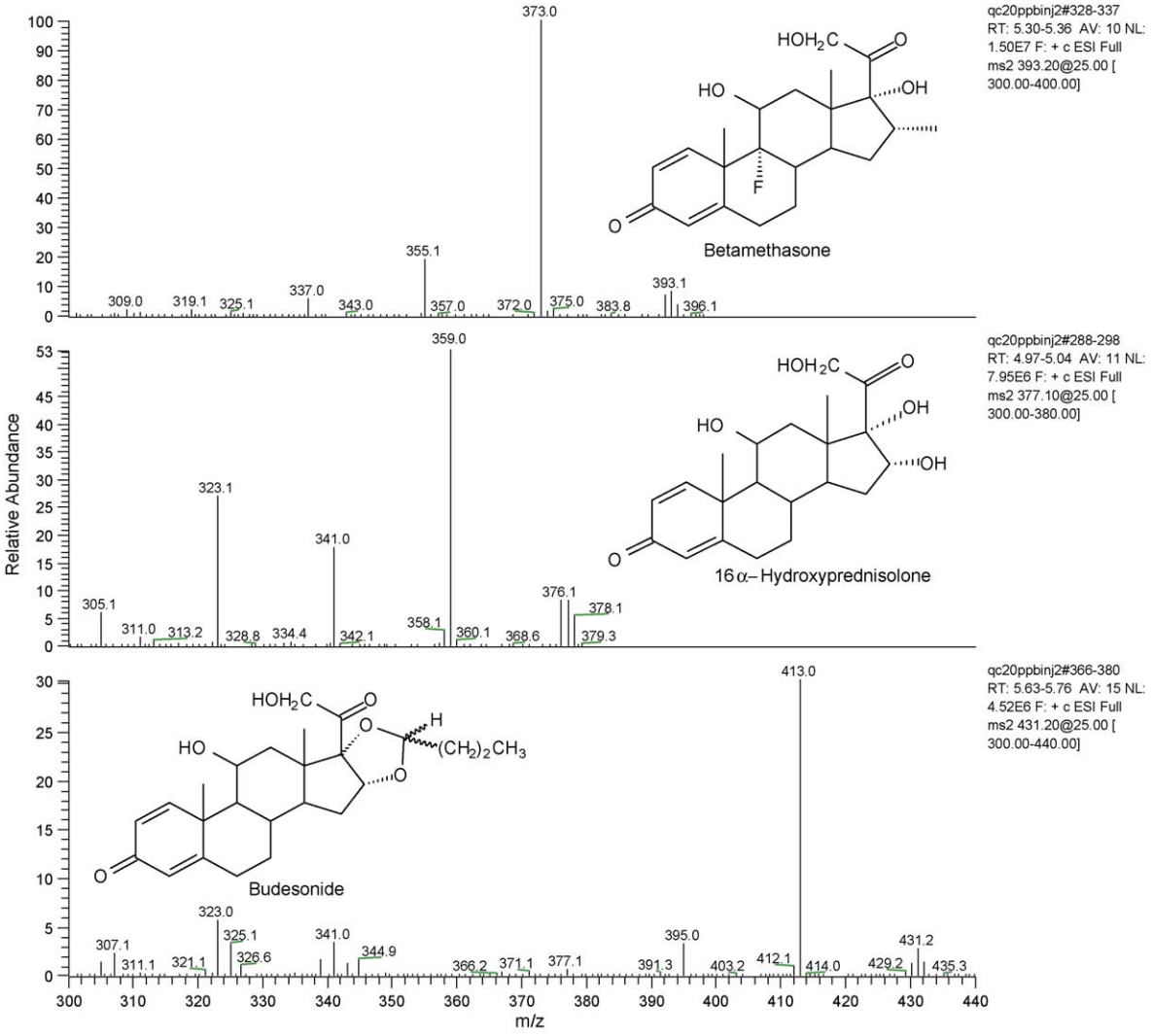

Fig. 1. Positive product ion mass spectra of betamethasone (IS), 16α-hydroxyprednisolone and budesonide.

Analytical grade potassium carbonate, sodium hydrogen carbonate and acetic acid were from Merck (Darmstadt, Germany) and ethyl acetate from Acros (Geel, Belgium). HPLC grade water was obtained from Fischer (Loughborough, United Kingdom) and acetonitrile from Biosolve (Valkenswaard, The Netherlands). Gases used in mass spectrometry were helium (Alphagaz-grade) and nitrogen (LASAL2001-grade) both purchased from Air Liquide (Desteldonk, Belgium).

2.2. Excretion study

The study was performed with five healthy male volunteers aged 24, 26, 29, 33 and 37. The study protocol was reviewed and approved by the ethical committee of the institution (UZGent, Project 2005-160). Each volunteer signed a statement of informed consent and inhaled 200 μg of budesonide during 5 s using a Pulmicort®–Turbohaler®200 (AstraZeneca, Brussels, Belgium). Urine samples were collected before (0 h) and quantitatively 1, 2, 3, 6, 9 and 12 h after intake. Additional samples were taken 24 and 48 h after inhalation. All urine samples were either analyzed directly or stored at −20 °C awaiting analysis. Volume and urinary density were measured and all samples were analyzed in duplicate.

2.3. Sample treatment

An internal standard (IS) solution (50 μL betamethasone, 1 μg/mL) was added to 5 mL of urine, followed by the addition of 200 mg of a solid buffer containing sodium hydrogen carbonate and potassium carbonate (2:1 w/w) to adjust the pH to 9.2. Liquid–liquid extraction was performed by rolling for 10 min with 4 mL of ethyl acetate. After centrifugation the organic layer was transferred into a new tube and evaporated until dry under oxygen free nitrogen (OFN) at 40 °C. The remaining residue was dissolved in 200 μL of the initial mobile phase, 50 μL was injected into the HPLC-system.

2.4. Instrument parameters

Separation of the compounds was performed on an Omnispher C18 column 50 mm × 3 mm, 3 μm (Chrompack, Antwerp, Belgium), protected with a guard column 10 mm × 2 mm

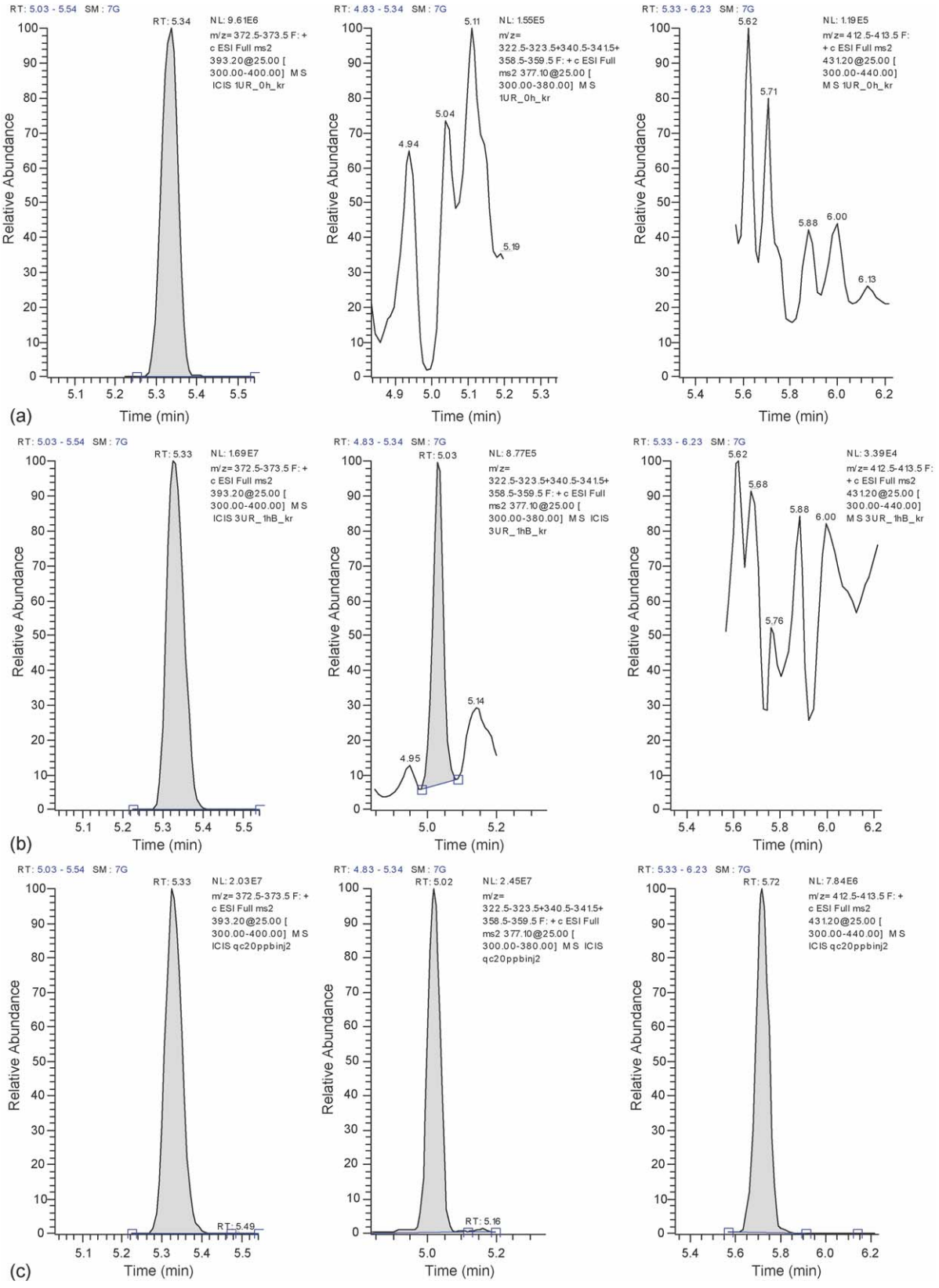

Fig. 2. Ion chromatograms obtained from blank urine (a), urine sample 1 h after inhalation (b), control urine spiked at 20 and 2 ng/mL with 16α-hydroxyprednisolone and budesonide, respectively (c). From left to right: betamethasone (IS), 16α-hydroxyprednisolone and budesonide.

(Chromsep, Antwerp) using a Surveyor LC-pump and a Surveyor autosampler (both from Thermo, San Jose, USA). The column temperature was kept at 35 °C.

The mobile phase consisted of an aqueous solution of 1% acetic acid (A) and acetonitrile (B). Gradient elution at a flow rate of 0.4 mL/min was as follows: 90% A for 0.25 min, followed by a linear decrease in A to 10% in 1.25 min, isocratic for 4.5 min, followed by an increase in 0.5 min to 90% A which was maintained for 4.5 min before the next injection. The total run time of the method was 11 min.

Detection was carried out using a Thermofinnigan LCQ-Deca® XP Plus-mass spectrometer (Thermo, San Jose, USA) using electrospray ionization (ESI) in positive mode. The ion source voltage was at 5000 V and the sheath gas and the auxilliary gas flow rate were set at 80 and 10 units, respectively. The capillary voltage was 20 V.

For all compounds full scan tandem mass spectrometry was applied. The isolation width was set at 3.0. Activation time and Q value were set arbitrary at 30 ms and 0.250. The collision energy was set at 25% for all compounds.

2.5. Validation

A five-point calibration curve was generated by spiking blank urine with 16α-hydroxyprednisolone and budesonide in triplicate at 5, 10, 20, 50 and 100 ng/mL and 0.5, 1, 2, 5 and 10 ng/mL, respectively. Averages were used to construct the calibration curve.

The area ratios of the product ions of budesonide (m/z 413) and 16α-hydroxyprednisolone (sum of m/z 323, 341, 359) and the product ion of the internal standard (m/z 373) were plotted versus the concentration.

The precision and accuracy of the method were tested at three levels (0.5, 2, 10 for budesonide and 5, 20, 100 ng/mL for 16α-hydroxyprednisolone). Precision was assessed as the percentage relative standard deviation (%R.S.D.) of both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected compound and level. Maximum allowed tolerances for reproducibility and repeatability can be calculated from the Horowitz-equation $\text{R.S.D.}_{\cdot\max} = 2^{(1-0.5\log C)}$ (C = concentration (μg/mL) × 10^{-6}). The maximum allowed tolerances for repeatability and reproducibility are $2/3\text{R.S.D.}_{\cdot\max}$ and $\text{R.S.D.}_{\cdot\max}$, respectively [11].

Accuracy was defined as the difference between the calculated amount and the specified amount for the selected compound and expressed as a percentage [12].

The limit of quantification (LOQ) of the method was defined as the lowest concentration where acceptable reproducibility and accuracy could be guaranteed. The limit of detection (LOD) was defined arbitrarily as 1/2 LOQ.

Selectivity was tested by analysing several structurally related and other routinely screened doping agents, including corticosteroids and anabolic steroids. Concentrations in these mixtures were 1 μg/mL.

Specificity was tested by analysing 10 blank urines as described above to evaluate the presence of matrix interference.

In each batch of excretion urine samples, a blank urine sample, a system blank (aqua bidest) and a quality control sample (spiked at 2 and 20 ng/mL for respectively budesonide and 16α-hydroxyprednisolone) were analyzed concurrently.

2.6. Recovery

Recoveries from ethyl acetate, dichloromethane and diethylether, extraction solvents routinely used in our laboratory, were evaluated. Therefore negative urine samples ($n = 6$) were spiked with 16α-hydroxyprednisolone and budesonide at 5 ng/mL and extracted together with non-spiked negative urine samples ($n = 6$). The extracts of the non-spiked urine samples were then spiked at 5 ng/mL simulating a 100% recovery. Both sets of samples were evaporated and analysed as described. The obtained peak areas of the two sets of samples were compared to evaluate recovery.

3. Results and discussion

3.1. Method development

The described HPLC method is an adaptation of a screening method for corticosteroids [13]. By reducing the column length from 100 to 50 mm a reduction of analysis time by a factor of 2 was obtained.

Under the chromatographic conditions described, all compounds eluted as sharp peaks within a short time range. Retention times were 5.01, 5.31 and 5.71 min for 16α-hydroxyprednisolone, betamethasone and budesonide, respectively.

Flow injection analysis was performed to determine the presence of diagnostic ions. For each tested compound a solution of 5 μg/mL was infused at a flow rate of 10 μL/min. In full scan MS, abundant protonated molecular ions were observed for all three compounds. In the MS/MS spectrum of budesonide (molecular weight = 430) one intense product ion was observed at m/z 413 $[MH–H_2O]^+$ and several minor product ions at m/z 395, 341 and 323 (Fig. 1). Despite the low specificity of product ions generated by the loss of water [14] this product ion was used as diagnostic ion for the detection of budesonide. Unlike budesonide, 16α-hydroxyprednisolone (molecular weight = 376) exhibited intensive fragmentation and several intense product ions were observed for this compound, namely m/z 359, 341 and 323 (successive losses of H_2O). To avoid loss of sensitivity due to the intensive fragmentation of this compound the sum of these three ions was used for quantification purposes. For the internal standard, betamethasone (molecular weight = 392) the product ion m/z 373 $[MH–HF]^+$ was used.

3.2. Recovery

Extraction recoveries for the different compounds are given in Table 1. For budesonide relatively small differences were observed between the different solvents. However great differences were observed for 16α-hydroxyprednisolone. Both dichloromethane and diethylether showed poor recovery for

Table 1
Extration recoveries of 16α-hydroxyprednisolone and budesonide[a]

	Recovery (%) ($n=6$)		
	Dichloromethane	Diethylether	Ethyl acetate
16α-Hydroxyprednisolone	12.1 ± 1.2	9.8 ± 1.1	59.5 ± 2.7
Budesonide	85.4 ± 1.2	91.9 ± 0.56	87.7 ± 1.1

[a] Values are presented as mean ± standard deviation ($n=6$), concentrations 5 ng/mL.

16α-hydroxyprednisolone whereas ethyl acetate showed far better recoveries (Table 1). As a consequence ethyl acetate was the preferred extraction solvent.

3.3. Method validation

Using a least square fit, good linearity ($r^2 \geq 0.998$) was observed for 16α-hydroxyprednisolone and budesonide in the range 5–100 ng/mL and 0.5–10 ng/mL, respectively. None of the calibration curves was forced through the origin and for the regression calculation a weighing factor of $1/x$ was used for all data points. The results for precision and accuracy are summarised in Table 2.

As shown in Table 2, these values were not exceeded neither for repeatability nor reproducibility. Deviation of the mean measured concentration from the theoretical concentration (accuracy) for all compounds was below the acceptable threshold of 15 and 20% [12] for all levels in the range of calibration curve.

Regarding the selectivity, interference from other monitored doping agents could not be found. In addition analysis of 10 different blank control urine samples did not result in the detection of interfering substances, proving the specificity of the method.

The limit of quantification (LOQ) of the method was 5 ng/mL for 16α-hydroxyprednisolone and 0.5 ng/mL for budesonide. The limit of detection was 2.5 and 0.25 ng/mL for 16α-hydroxyprednisolone and budesonide, respectively.

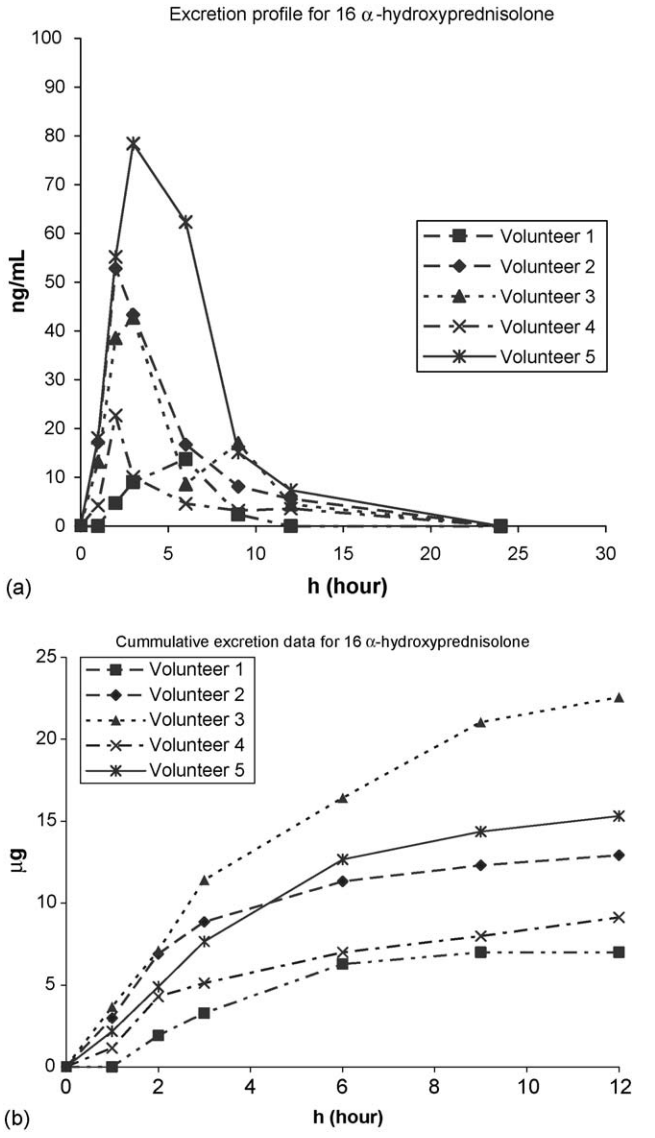

Fig. 3. Urinary concentrations (a) and cumulative excretion (b) of 16α-hydroxyprednisolone after the inhalation of a single dose of budesonide (200 µg).

3.4. Excretion urine samples

Budesonide could not be detected in any of the post administration urine samples which is in accordance with the intensive and fast metabolism [7].

16α-Hydroxyprednisolone could already be detected 1 h after inhalation, except in subject 1. Chromatograms of a blank urine, a quality control urine and an excretion urine sample are presented in Fig. 2. The urinary excretion profiles for 16α-hydroxyprednisolone are shown in Fig. 3. Maximum urinary concentrations of 16α-hydroxyprednisolone were obtained 2–3 h after inhalation, excepting subject 1 (T_{max}: 6 h).

Table 2
Accuracy, repeatability, reproducibility and tolerance limits of the LC–MS method at three concentrations including the lowest point of the calibration curve for 16α-hydroxyprednisolone and budesonide

	Concentration (ng/mL)	Accuracy (%), $n=18$	Repeatability (%), $n=6$	Reproducibility (%), $n=18$	R.S.D.$_{max}$ (%)	2/3R.S.D.$_{max}$ (%)
16α-Hydroxyprednisolone	5	3.5	7.1	8.5	32	21
	20	1.3	10.4	10.1	25	19
	100	2.2	3.4	5.8	23	15
Budesonide	0.5	16.4	5.2	4.9	50	33
	2	−3.2	9.5	7.5	41	27
	10	0.8	1.6	6.6	32	21

The maximum urinary concentrations ranged between 10 and 79 ng/mL. These large differences are at least partially caused by differences in urine volumes. In four out of five subjects 16α-hydroxyprednisolone was detectable until 12 h post-administration.

Cumulative excretion data (Fig. 3) indicate that between 3 and 13% of the administered dose is excreted as 16α-hydroxyprednisolone. These variations can be due to a poor use of the turbohaler in accordance with studies reporting a 37% recovery of budesonide from the inhalation device after application [8]. However, other reasons including poor resorption, cannot be excluded.

4. Conclusions

A quantitative LC–MS method has been developed and validated for the detection of 16α-hydroxyprednisolone and budesonide in urine.

Administration studies showed that 16α-hydroxyprednisolone is the target compound in urine for the detection of budesonide application by inhalation.

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