

# Metabolic Pathways of Inhaled Glucocorticoids by the CYP3A Enzymes

Chad D. Moore, Jessica K. Roberts, Christopher R. Orton, Takahiro Murai, Trevor P. Fidler, Christopher A. Reilly, Robert M. Ward, and Garold S. Yost

*Department of Pharmacology and Toxicology (C.D.M., J.K.R., C.R.O., T.M., T.P.F., C.A.R., G.S.Y.) and Department of Pediatrics (R.M.W.), University of Utah, Salt Lake City, Utah*

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## ABSTRACT

Asthma is one of the most prevalent diseases in the world, for which the mainstay treatment has been inhaled glucocorticoids (GCs). Despite the widespread use of these drugs, approximately 30% of asthma sufferers exhibit some degree of steroid insensitivity or are refractory to inhaled GCs. One hypothesis to explain this phenomenon is interpatient variability in the clearance of these compounds. The objective of this research is to determine how metabolism of GCs by the CYP3A family of enzymes could affect their effectiveness in asthmatic patients. In this work, the metabolism of four frequently prescribed inhaled GCs, triamcinolone acetonide, flunisolide, budesonide, and fluticasone propionate, by the CYP3A family of enzymes was studied to identify differences in their rates of clearance and to identify their metabolites. Both interenzyme and interdrug variability in rates of metabolism and

metabolic fate were observed. CYP3A4 was the most efficient metabolic catalyst for all the compounds, and CYP3A7 had the slowest rates. CYP3A5, which is particularly relevant to GC metabolism in the lungs, was also shown to efficiently metabolize triamcinolone acetonide, budesonide, and fluticasone propionate. In contrast, flunisolide was only metabolized via CYP3A4, with no significant turnover by CYP3A5 or CYP3A7. Common metabolites included 6 $\beta$ -hydroxylation and  $\Delta^6$ -dehydrogenation for triamcinolone acetonide, budesonide, and flunisolide. The structure of  $\Delta^6$ -flunisolide was unambiguously established by NMR analysis. Metabolism also occurred on the D-ring substituents, including the 21-carboxy metabolites for triamcinolone acetonide and flunisolide. The novel metabolite 21-nortriamcinolone acetonide was also identified by liquid chromatography–mass spectrometry and NMR analysis.

## Introduction

Asthma is a chronic lung disease characterized by recurring episodes of wheezing, shortness of breath, chest tightness, and coughing. These symptoms are caused by bronchial constriction due to hyperreactivity, inflammation, eosinophilic infiltration, and increased mucus production, causing intermittent airway obstruction. Asthma is a multifactorial disease that can be exacerbated by genetics, respiratory infections, allergens, air pollutants, temperature changes, exercise, and stress. Asthma is one of the most prevalent diseases in the world, with an estimated 300 million sufferers (World Health Organization, 2007). Many studies have demonstrated a genetic contribution to asthma, with a 36%–79% heritability risk (Los et al., 1999). The incidence of asthma in the US population alone continues to increase, with 1 in 12 people reported to have asthma in 2009, an increase from 1 in 14 in 2001. This increase comes with an increase in associated annual medical costs from \$48.6 billion in 2002 to \$50.1 billion in 2007 (<http://www.cdc.gov/VitalSigns/Asthma/>).

Inhaled glucocorticoids (GCs) are potent anti-inflammatory agents that are the mainstay of treatment of patients with persistent asthma. Therapeutic agents include triamcinolone acetonide, flunisolide, budesonide, and fluticasone propionate. These compounds bind to the GC nuclear receptors in the relevant airway epithelial cells, which decreases migration and survival of inflammatory cells in the lung, decreases mucus production, and reduces action of proinflammatory cytokines (Barnes, 2011). Although GCs are currently the most effective therapy for controlling asthma, approximately 30% of asthmatics have some degree of steroid resistance or insensitivity (Chan et al., 1998; Szeffler et al., 2002). Current proposed mechanisms explaining GC insensitivity/resistance include defective immune responses that correlate with clinical resistance to GC therapy, genetic abnormalities that result in the inactivation of the GCs, mutations of the GR gene, and molecular mechanisms involving inflammatory cytokines, the exact mechanism of which is unknown (Leung and Bloom, 2003).

Only 2%–10% of the inhaled dose of GCs is deposited in the lungs; the majority of a dose is swallowed and absorbed into systemic circulation (Taburet and Schmit, 1994). GCs are metabolized in the lung and liver by members of the cytochrome P450 (P450) CYP3A family of enzymes. The CYP3A family consists of CYP3A4, -5, -7, and -43, whose expression is differentiated by tissue and age (Koch et al., 2002; Leclerc et al., 2010). CYP3A4 is the predominate isoform found in adult intestine and liver, whereas CYP3A5 is found primarily

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**ABBREVIATIONS:** amu, atomic mass unit; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; GC, glucocorticoid; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; LC/MS, liquid chromatography–mass spectrometry; MS/MS, tandem mass spectrometry; P450, cytochrome P450; RT, retention time.

TABLE 1  
<sup>1</sup>H NMR of flunisolide and Δ<sup>6</sup>-flunisolide in chloroform-*d*<sub>3</sub>

Compound	H <sub>1</sub>	H <sub>2</sub>	H <sub>4</sub>	H <sub>6</sub>	H <sub>7a</sub>	H <sub>7b</sub>	H <sub>11</sub>	H <sub>16</sub>	H <sub>21a</sub>	H <sub>21b</sub>
	<i>ppm</i>									
Flunisolide	7.16, d	6.29, d	6.31, s	5.25, dd, 5.35, dd	1.31, ddd	2.47, ddd	4.49, ddd	5.04, dd	4.14, d	4.64, d
Δ <sup>6</sup> -Flunisolide	7.23, d	6.31, d	6.32, s	—	5.61, d	—	4.54, ddd	5.07, dd	4.17, d	4.65, d

in pulmonary tissue. CYP3A7 is the primary P450 expressed in fetal liver tissue; however, it is silenced within 6–12 months of birth, when CYP3A4 is transcriptionally activated (Wrighton et al., 1988; Schuetz et al., 1994; Lacroix et al., 1997; Leeder et al., 2005). Recent studies suggest that transcripts of CYP3A5 and CYP3A7 are found in adult liver (Hustert et al., 2001; Koch et al., 2002) and CYP3A7 in adult lung (Leclerc et al., 2010), but the lack of specific antibodies has precluded definitive confirmation of expression. Furthermore, GCs induce CYP3A expression, and mRNAs of all three isoforms are induced by dexamethasone in HepG2 liver cells (Krusekopf et al., 2003), and CYP3A5 mRNA is induced up to 4- to 6-fold by dexamethasone, budesonide, and beclomethasone in A549 lung cells (Hukkanen et al., 2003). CYP3A43 is the most recently discovered CYP3A gene and has demonstrated low testosterone hydroxylase activity (Domanski et al., 2001). However, it is only expressed at levels of about 0.1% of CYP3A4 in the liver and is not found in the lung (Domanski et al., 2001; Westlind et al., 2001). Therefore, CYP3A43 was not investigated in this study.

Tissue- and age-dependent expression of the specific members of the CYP3A family of enzymes could lead to differences in the pharmacokinetics of GCs and could affect the therapeutic outcome and toxicities of these compounds. Because the majority of inhaled GCs are swallowed and absorbed systemically, CYP3A4 in the liver is primarily responsible for the efficient systemic clearance. However, in neonatal patients, CYP3A7 is highly expressed in the liver and would play a vital role in systemic clearance. Although only 2%–10% of inhaled GCs remain in the lungs, it is this small portion of the drug at the target site that is most likely responsible for the therapeutic effect of inhaled GCs. Therefore, small changes in the concentration at this site via CYP3A5 or CYP3A7 metabolism could substantially alter the ability of specific GCs to control asthma symptoms. The purpose of this study was to evaluate the relative ability of the CYP3A family of enzymes to metabolize four commonly used inhaled GCs with similar structures, via known P450 pathways, and to identify new metabolites. This information could substantially improve therapeutic outcomes with GCs.

### Materials and Methods

**Chemicals, Reagents, and Treatments.** All GCs (fluticasone propionate, flunisolide, budesonide, and triamcinolone acetonide), internal standard (prednisolone), deuterated chloroform, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), and reagents were purchased from Sigma-Aldrich (St. Louis, MO). GC stocks were prepared in dimethylsulfoxide, internal standard was prepared in 50:50 chloroform/

methanol. All other chemicals for synthesis or analysis were of analytical grade or equivalent and obtained at the highest grade commercially available.

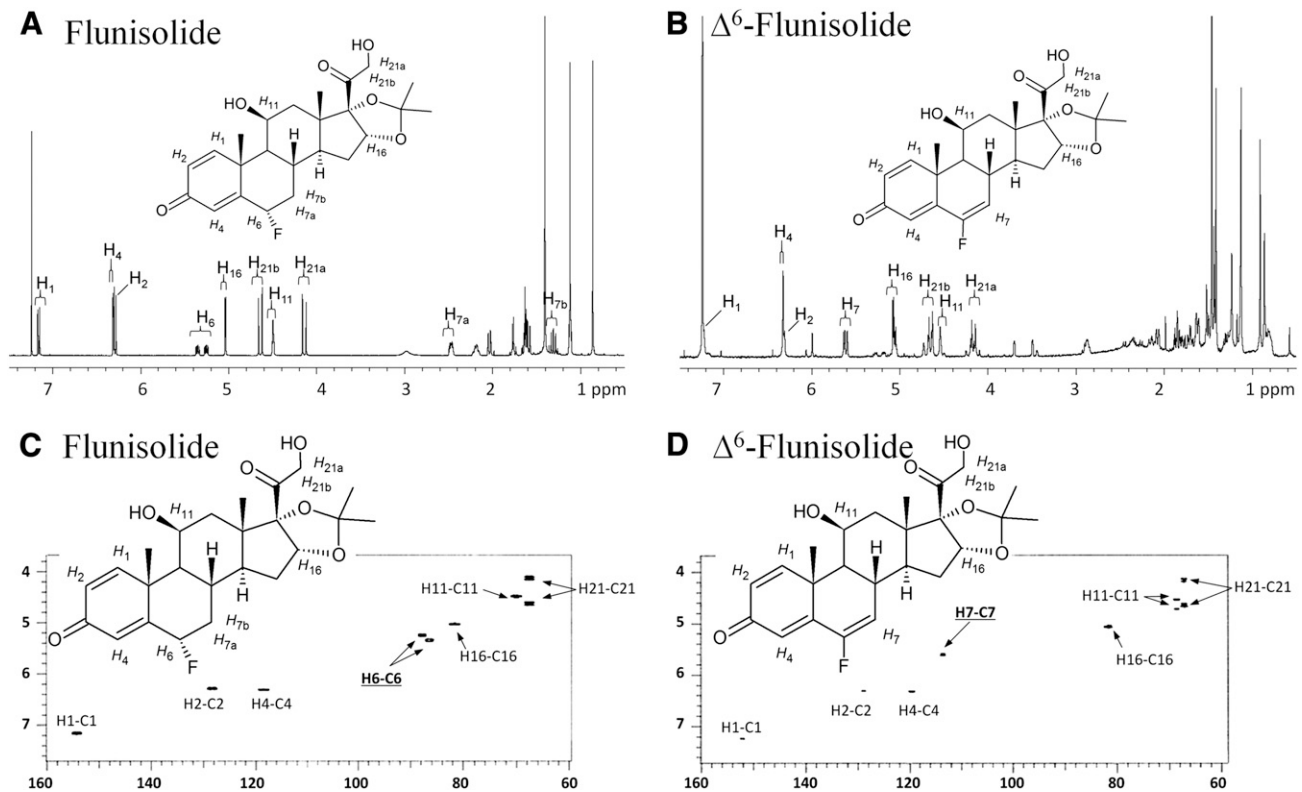
**Instrumentation.** Liquid chromatography–tandem mass spectrometry (LC/MS) was conducted on a Thermo LCQ Advantage Max ion trap instrument equipped with a Finnigan Surveyor LC pump, Surveyor Autosampler, and universal Ion Max source operated with Thermo Xcalibur software version 2.0 (Thermo Fisher Scientific, Waltham, MA). Triamcinolone acetonide, flunisolide, budesonide, fluticasone propionate, and their metabolites were resolved on a 150 × 2 mm Gemini 5-μm C6-Phenyl high-performance liquid chromatography (HPLC) column (Phenomenex, Torrance, CA). The mobile phase consisted of solvent A: acetonitrile; and solvent B: 0.1% formic acid (v/v). The mobile phase was increased from 5% to 27% solvent A for 5 minutes, increasing to 70% over 20 minutes, and finally held at 100% for 5 minutes, with a flow rate of 0.2 ml/min. Selected ion monitoring and tandem mass spectrometry (MS/MS) fragmentation were used to identify each GC compound and their respective CYP3A-dependent metabolites, using previously published material and established P450-dependent mechanisms to predict potential metabolites. Each method also scanned for the internal standard prednisolone (*m/z* 361). Novel GC metabolites were identified and verified by the predicted mass shifts relative to parent compounds, and MS/MS spectra were compared with previously published material and verified using predictive software tools in ChemBioDraw version 11.0.1 (CambridgeSoft, Cambridge, MA) and Mass Frontier 4.0 (HighChem, Bratislava, Slovakia).

**Determination of Rates of Elimination.** Recombinant P450s, containing P450 reductase and cytochrome *b*<sub>5</sub>, were purchased from BD Biosciences (San Jose, CA). In vitro incubations contained 10 pmol P450, 1 μM substrate, 30 mM potassium phosphate buffer (pH 7.4), and 1.3 mM NADPH in a final reaction volume of 0.5 ml. Negative control incubations omitted NADPH. Incubations were initiated by the addition of NADPH and allowed to proceed at 37°C. Aliquots (50 μl) were removed at 0, 2, 4, 6, 10, 15, and 20 minutes. Aliquots from the incubations were added to 2× volume ice-cold acetonitrile containing internal standard (1 nmol prednisolone). Protein was removed by centrifugation at 21,000g for 15 minutes. Supernatant was removed and dried to completion under nitrogen gas and reconstituted in 60 μl of initial mobile phase for analysis via LC/MS. Kinetic curves were generated by plotting the parent/internal standard ratios versus time, and kinetic parameters (half-life [*t*<sub>1/2</sub>]) were calculated by fitting the data to one-phase exponential decay [ $Y = (Y_0 - \text{Plateau})(e^{-k_{\text{obs}}X}) + \text{Plateau}$ ;  $t_{1/2} = 0.69/k_{\text{obs}}$ ] with GraphPad Prism 4.02 (GraphPad Software, La Jolla, CA).

**Identification of GC Metabolites.** Recombinant P450s, containing P450 reductase and cytochrome *b*<sub>5</sub>, were purchased from BD Biosciences. In vitro incubations contained 50 pmol P450, 100 μM substrate, 30 mM potassium phosphate buffer (pH 7.4), and 1.3 mM NADPH in a final reaction volume of 0.5 ml. Negative control incubations omitted NADPH. Incubations were initiated by the addition of NADPH and allowed to proceed at 37°C. Reactions were terminated by the addition of 2× volume of ice-cold acetonitrile. Protein was removed by centrifugation at 21,000g for 15 minutes. GC metabolites were extracted using C-18 Sep-Pak cartridges (Waters, Taunton, MA). The resulting eluate was dried to completion under nitrogen gas and reconstituted in 60 μl of initial mobile phase for analysis via LC/MS.

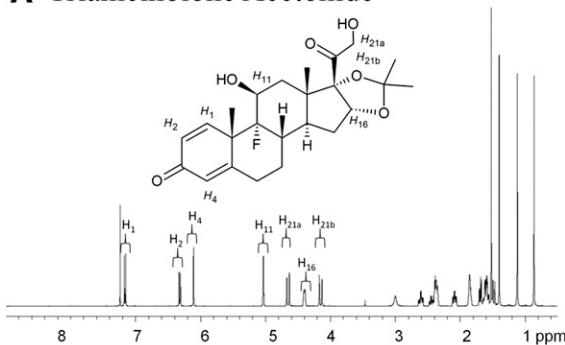
TABLE 2  
<sup>1</sup>H NMR of triamcinolone acetonide and 21-nortriamcinolone acetonide in chloroform-*d*<sub>3</sub>

Compound	H <sub>1</sub>	H <sub>2</sub>	H <sub>4</sub>	H <sub>11</sub>	H <sub>16</sub>	H <sub>21a</sub>	H <sub>21b</sub>
	<i>ppm</i>						
Triamcinolone acetonide	7.16, d, 1H	6.32, d, 1H	6.11, s, 1H	5.03, d, 1H	4.40, d, 1H	4.65, d, 1H	4.15, d, 1H
21-Nortriamcinolone acetonide	7.15, d, 1H	6.33, d, 1H	6.12, s, 1H	5.07, d, 1H	4.42, d, 1H	—	—

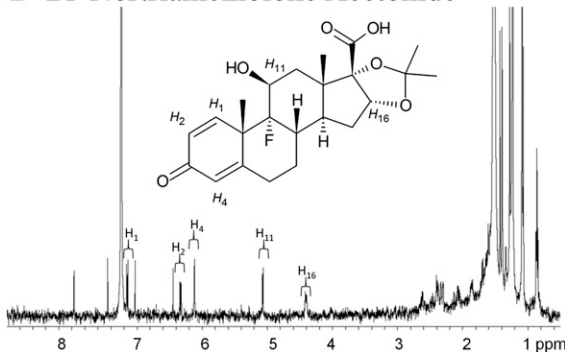


**Fig. 1.**  $^1\text{H}$  NMR spectra of (A) flunisolide and (B)  $\Delta^6$ -flunisolide in chloroform- $d_3$ . HSQC spectra of (C) flunisolide and (D)  $\Delta^6$ -flunisolide in chloroform- $d_3$ , showing key differences between the two compounds.  $\text{H}_6\text{-C}_6$  signals of flunisolide are split due to the coupling with the neighboring fluoride atom.

### A Triamcinolone Acetonide



### B 21-Nortriamcinolone Acetonide



**Fig. 2.**  $^1\text{H}$  NMR of (A) triamcinolone acetonide and (B) 21-nortriamcinolone acetonide in chloroform- $d_3$ . The loss of  $\text{H}_{21a}$  and  $\text{H}_{21b}$  indicates the loss of  $\text{C}_{21}$  to form 21-nortriamcinolone acetonide.

**$\Delta^6$ -Flunisolide and 21-Nortriamcinolone Acetonide Synthesis.** Triamcinolone acetonide or flunisolide (18 mg) was dissolved in 15 ml of dry acetonitrile. DDQ (15 mg) was added, and the reaction mixture was refluxed for 4 hours at  $90^\circ\text{C}$  with consistent stirring. The mixture was allowed to cool to  $25^\circ\text{C}$ , after which glutathione (30.7 mg in 0.48 ml of phosphate-buffered saline, pH 7.4) was added and the mixture stirred for 0.5 hours. The synthesized products were extracted using C-18 Sep-Pak cartridges. The products were eluted off the cartridges with 100% methanol, and the eluate was evaporated to dryness under nitrogen and reconstituted in 1:1 acetonitrile/ $\text{H}_2\text{O}$  (v/v). The product of interest was isolated via HPLC, conducted on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) including an autosampler and a diode-array UV/VIS detector. Chromatography was performed on a Phenomenex Luna  $5\text{-}\mu\text{m}$  C18 ( $250 \times 4.60$  mm) reverse-phase column, with the mobile phase consisting of linear gradient from 20% to 80% acetonitrile over 20 minutes with water as the countercurrent solvent and a flow rate of 1 ml/min. Collected fractions were pooled and lyophilized for 48 hours. Products were solubilized in deuterated chloroform for analysis via NMR on an Inova500 NMR (Agilent Technologies). The chemical shifts of the parent compounds and the synthesized standards are found in Tables 1 and 2.

## Results

**Synthesis of  $\Delta^6$ -Flunisolide and 21-Nortriamcinolone Acetonide.** Dehydrogenation of flunisolide could occur at several places, so to establish the site of desaturation and provide a synthetic standard of this metabolite, flunisolide was chemically oxidized with DDQ to form a dehydrogenated product. Following isolation of the product with  $m/z$  433 ( $-2$  atomic mass units [amu] of parent compound) via HPLC, the  $^1\text{H}$  NMR and the heteronuclear single quantum coherence (HSQC) spectra of both the parent and the dehydrogenated compound were determined (Fig. 1; Table 1). From the flunisolide  $^1\text{H}$  and HSQC spectra, the  $\text{H}_6$  proton was identified by its downfield shift and

TABLE 3

Observed half-lives for clinically relevant inhaled glucocorticoids by CYP3A isoforms in vitro

Drug	Half-Life of Drug (Mean $\pm$ S.E.) in Isoform		
	3A4	3A5	3A7
		<i>min</i>	
Fluticasone propionate	0.86 $\pm$ 0.08	16 $\pm$ 2	58 $\pm$ 9
Flunisolide	16 $\pm$ 1	N.A.	N.A.
Triamcinolone acetonide	12 $\pm$ 1	130 $\pm$ 43	213 $\pm$ 100
Budesonide	11 $\pm$ 1	40 $\pm$ 5	71 $\pm$ 16

N.A., not applicable.

splitting due to coupling to the fluorine (Fig. 1, A and C). The protons H<sub>7a</sub> and H<sub>7b</sub> were located at 1.31 and 2.47 ppm. However, the spectra of the dehydrogenated product showed that the H<sub>6</sub> signal was no longer present. Furthermore, although the protons at 1.31 and 2.47 ppm (H<sub>7a</sub> and H<sub>7b</sub>, respectively) were no longer detected, there was now a doublet at 5.61 ppm (Fig. 1, B and D). This doublet signal is consistent with a single proton on C<sub>7</sub>, which has been shifted downfield because it is on an sp<sub>2</sub> carbon of an alkene. The loss of protons on C<sub>6</sub> and C<sub>7</sub>, and the addition of an alkene at C<sub>6</sub>-C<sub>7</sub>, unambiguously established the structure as  $\Delta^6$ -flunisolide.

Although the original intent of the chemical oxidation of triamcinolone acetonide was to obtain a dehydrogenated product, fortuitously a product with *m/z* 421 was discovered. Because a novel metabolite of *m/z* 421 was observed from the CYP3A-mediated metabolism of triamcinolone acetonide (see *Identification of Metabolites*), this product was collected for analysis by proton NMR. Pure triamcinolone acetonide was used as a comparison with obtain proton NMR data (Table 2). From these data, the doublet signals at 4.15 and 4.65 ppm were assigned to the two protons (H<sub>21b</sub> and H<sub>21a</sub>, respectively) on C<sub>21</sub> (Fig. 2A). The NMR spectrum of the *m/z* 421 product was similar to that of the parent; however, the doublet signals for H<sub>21b</sub> and H<sub>21a</sub> were lost (Fig. 2B). Due to the loss of these protons and the mass decrease of 14 amu from the parent, we identified this chemical oxidation product as 21-nortriamcinolone acetonide.

**Rates of Elimination of GC.** Beginning with an initial concentration of 1  $\mu$ M, the initial clearance rate of each GC was evaluated for each CYP3A isoforms. Despite the structural similarities among these GCs, significant variability between the rates of elimination was observed for each enzyme (Table 3). Furthermore, there was notable variability in the rate of disappearance catalyzed by different members of the CYP3A family (Fig. 3). CYP3A4 was the most efficient at metabolism of all four GCs, and fluticasone propionate had the shortest half-life. CYP3A5 was the second-most-efficient metabolizer of fluticasone propionate and budesonide, and CYP3A7 was the least

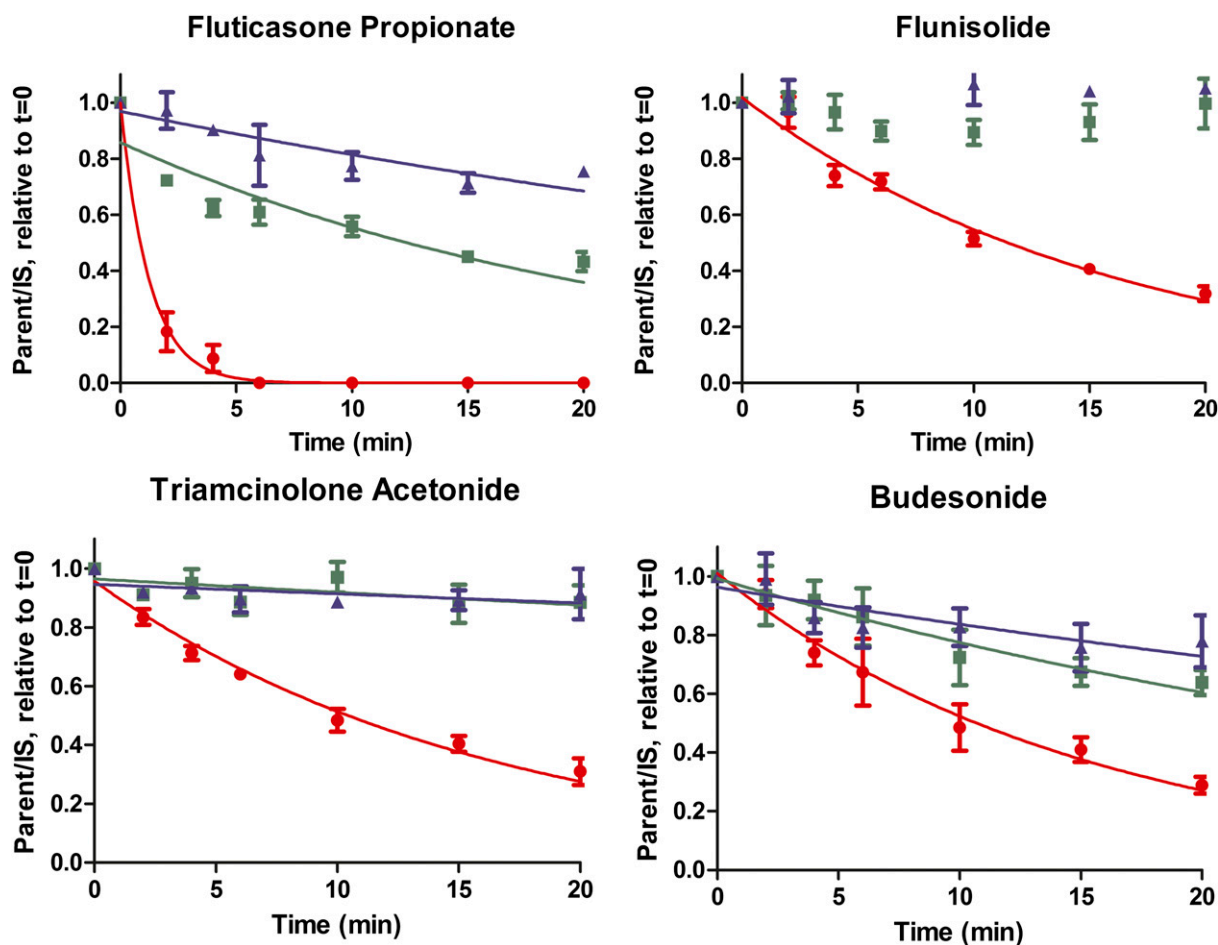
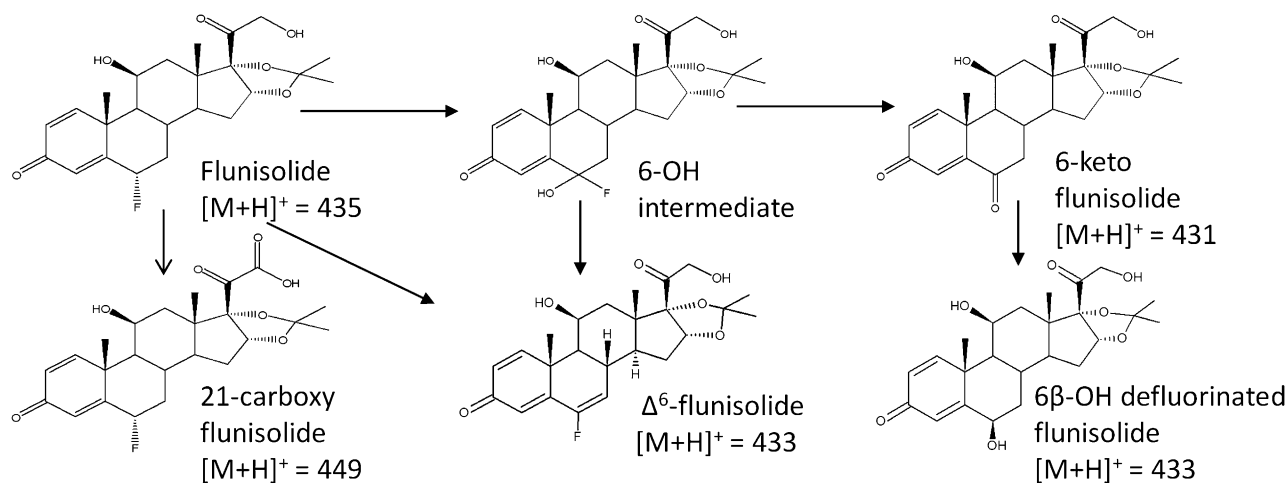


Fig. 3. Plots representing the initial rate of elimination for fluticasone propionate, flunisolide, triamcinolone acetonide, and budesonide by CYP3A4 (red circles), CYP3A5 (green squares), and CYP3A7 (blue triangles). All time points were run in triplicate. IS, internal standard.



Scheme 1. Scheme of CYP3A-mediated metabolism of flunisolide.

efficient enzyme. CYP3A5 and CYP3A7 were poor metabolizers of triamcinolone acetonide. Although flunisolide metabolites were detected for CYP3A5 and CYP3A7 at time points greater than 15 minutes, the initial rates of elimination were negligible.

**Identification of Metabolites.** Incubations with recombinant CYP3A enzymes were used to identify new and previously identified GC metabolites. Teitelbaum et al. (1981) previously demonstrated that flunisolide is metabolized to a 6-keto metabolite ( $m/z$  431), a 6 $\beta$ -hydroxy-defluorinated metabolite ( $m/z$  433), and  $\Delta^6$ -flunisolide ( $m/z$  433) using human liver microsomes (Scheme 1). From our incubations with recombinant CYP3As, four major flunisolide metabolites were identified with  $m/z$  433,  $m/z$  449, and two at  $m/z$  431 (Fig. 4). The 433  $m/z$  metabolite was identified as  $\Delta^6$ -flunisolide, because it had the identical retention time (RT = 20.6 minutes) and MS/MS fragmentation as chemically synthesized  $\Delta^6$ -flunisolide (Figs. 4 and 5). The novel  $m/z$  449 metabolite (RT = 23.1 minutes) was determined to be 21-carboxy flunisolide based on the comparison of the MS/MS fragmentation of the  $m/z$  449 metabolite to that of the parent (Fig. 5). Unfortunately, the core steroid structures of all the GCs tested are very stable and do not fragment to any predictable ions. Therefore, it is difficult to determine the site of oxygenation on these compounds by MS/MS. However, for all of the GCs tested, and others not in this study, the major sites of metabolism occur at the C<sub>6</sub> position or on the D-ring substituents. The  $m/z$  449 metabolite is 14 amu greater than that of the parent, suggesting the formation of a ketone without the loss of the fluorine. The fluorine is at the C<sub>6</sub> position, which was the only

major site of GC metabolism on the core steroid structure. Therefore, oxygenation at this site would most likely result in the loss of the fluorine and the formation of the 6-keto metabolite. Because the fluorine was retained, the ketone must be on one of the D-ring substituents of flunisolide. The fragmentation of the  $m/z$  449 metabolite produced daughter ions at  $m/z$  335 and 353, corresponding to the loss of the acetonide group, while retaining the ketone on the molecule. Together, these data strongly suggest that the ketone is found on the hydroxyacetone group of flunisolide. Furthermore, P450s are known to catalyze the formation of acetic acid from alcohol (Bell-Parikh and Guengerich, 1999), and triamcinolone acetonide has previously been shown to form the same P450-mediated 21-carboxy metabolite (Argenti et al., 2000). Two metabolites with  $m/z$  431 (RT = 15.8 and 17.6 minutes) were also observed, consistent with the formation of a ketone, and the additional loss of HF. From the work of Teitelbaum et al., one of the  $m/z$  431 metabolites is assumed to be 6-keto flunisolide. Again, the lack of fragmentation of the core steroid structure prevents precise identification of the site of oxygenation. However, the daughter ions at  $m/z$  355 and 377 correspond to the loss of acetonide, while retaining the ketone on the molecule (Fig. 5). In addition, the loss of HF on the molecule suggests that oxygenation is occurring on the B-ring of flunisolide. Unfortunately, the MS/MS fragmentations of both  $m/z$  431 metabolites were identical, and so 6-keto flunisolide could not be assigned to either peak. Teitelbaum et al. identified 6 $\beta$ -hydroxy-defluorinated flunisolide in their studies with human liver microsomes. This metabolite was not detected using

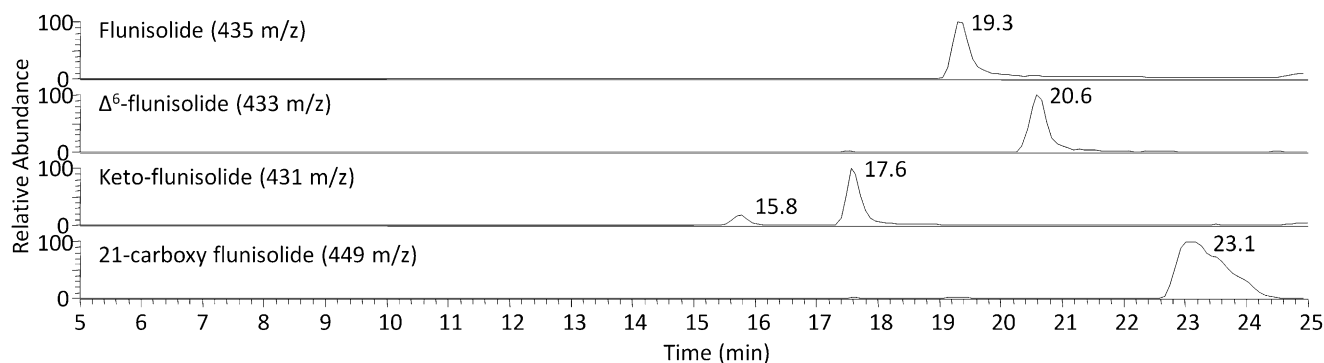


Fig. 4. LC/MS chromatograms of flunisolide and its CYP3A-mediated metabolites.

recombinant CYP3A enzymes, and therefore it was concluded that the oxidation of 6-keto flunisolide to 6 $\beta$ -hydroxy-defluorinated flunisolide is mediated via enzymes other than the CYP3As. The  $\Delta^6$ -flunisolide and 6-keto flunisolide metabolites were probably both formed through a 6-OH, 6-F intermediate, which could lose HF to form the 6-keto metabolite or lose H<sub>2</sub>O to form  $\Delta^6$ -flunisolide (Scheme 1). It is theoretically possible that  $\Delta^6$ -flunisolide was formed directly via P450-mediated dehydrogenation, but our results could not differentiate this pathway from the dehydration of the 6-OH, 6-F intermediate.

Interestingly, fluticasone propionate, which is structurally similar to flunisolide, was reported in a previous study to only be oxidized to

17 $\beta$ -carboxy fluticasone propionate (Scheme 2) (Pearce et al., 2006). However, more recent work using fluticasone furoate has detected defluorinated and several hydroxylated metabolites in plasma and fecal samples (Hughes et al., 2008). Therefore, it was concluded that fluticasone propionate was metabolized by oxidative defluorination and hydroxylation by CYP3A enzymes. Analysis of fluticasone propionate incubations detected the previously reported 17 $\beta$ -carboxy fluticasone metabolite (RT = 19.7 minutes), but no additional metabolites were detected. Interestingly, in the absence of NADPH, incubating fluticasone propionate with either CYP3A supersomes or human liver microsomes did not yield any 17 $\beta$ -carboxy fluticasone propionate. This suggests that esterases do not cleave the thioester of

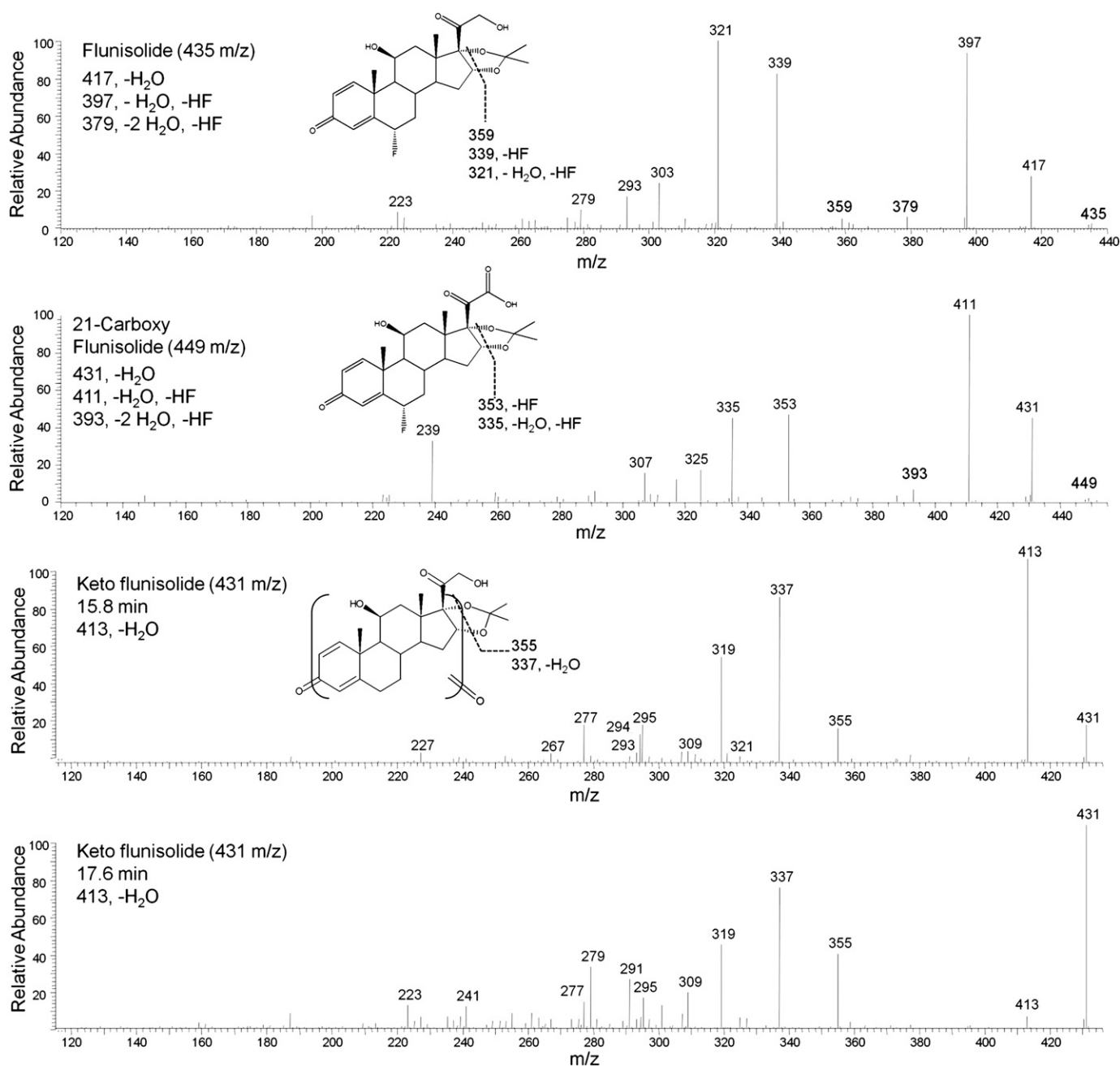
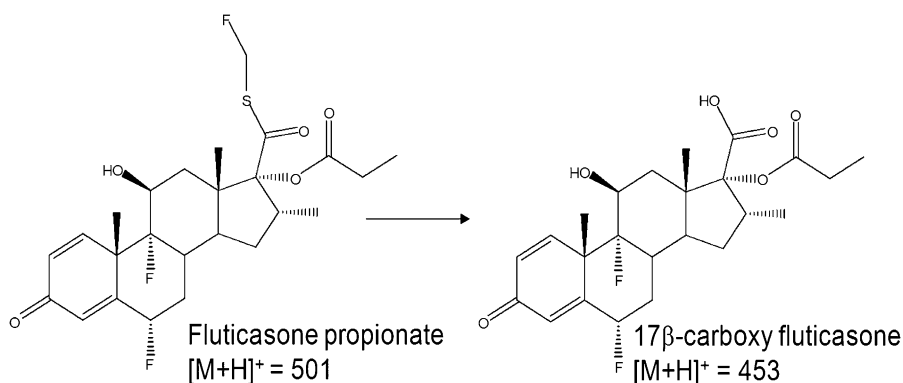


Fig. 5. MS/MS fragmentation spectra of flunisolide and its CYP3A-mediated metabolites.

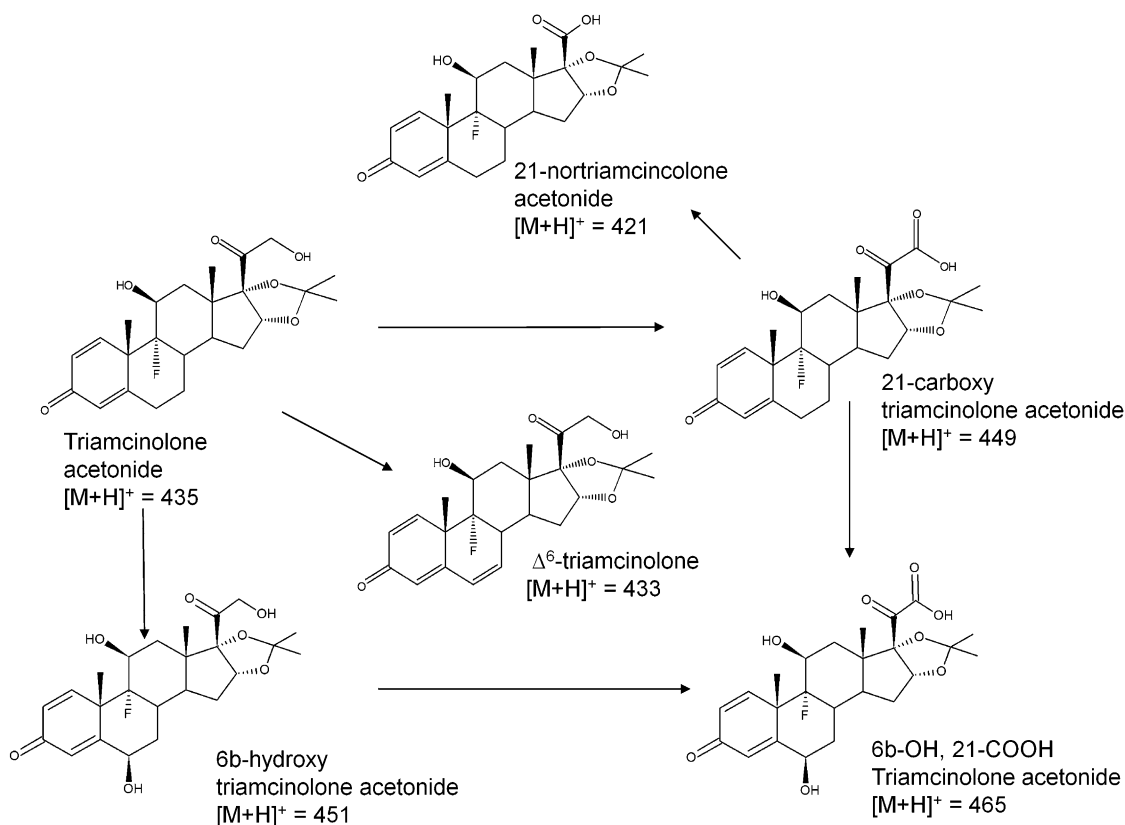


**Scheme 2.** Scheme of CYP3A-mediated metabolism of fluticasone propionate.

fluticasone propionate, but that this hydrolytic process is catalyzed selectively by P450 enzymes.

Previous work by Argenti et al. (2000) demonstrated that after oral administration triamcinolone acetonide (435 *m/z*) was metabolized to the 6β-OH (*m/z* 451) and 21-carboxy metabolites (*m/z* 449) and sequentially oxidized to 6β-OH, 21-carboxy triamcinolone acetonide (*m/z* 465) in human subjects (Scheme 3). From the analysis of triamcinolone acetonide incubated with the CYP3A enzymes, we were able to confirm the presence of all three metabolites (RTs: 6β-hydroxy triamcinolone acetonide = 14.9 minutes; 21-carboxy triamcinolone acetonide = 23.7 minutes; and 6β-OH, 21-carboxy triamcinolone acetonide = 16.4 minutes) (Fig. 6). In addition, two new metabolite peaks with *m/z* 421 and 433 were observed. By means of MS/MS fragmentation, the *m/z* 421 peak (RT = 18.9 minutes) was identified as 21-nortriamcinolone acetonide (Fig. 7). The major daughter ions at

*m/z* 401 and 383 correspond to the loss of HF and the additional loss of water, respectively. The ion at *m/z* 355 corresponded to the loss of the carboxyl group, resulting in a similar fragment from the parent. The ions at *m/z* 343 and 325 were attributed to fragmentation of the acetonide group similar to the parent, with the resulting ions differing by the loss of C<sub>21</sub> (i.e., 14 amu). Furthermore, comparison with the authentic 21-nortriamcinolone acetonide synthetic standard showed identical retention time and MS/MS fragmentation pattern (Figs. 6 and 7). 21-Nortriamcinolone is likely produced by the CYP3A-mediated decarboxylation of 21-carboxy triamcinolone acetonide. Although not a common mechanism, P450s have been shown to catalyze this type of decarboxylation (Fukuda et al., 1994; Komuro et al., 1995). By MS/MS fragmentation, the *m/z* 433 analyte (RT = 19.1 minutes) was determined to be Δ<sup>6</sup>-triamcinolone acetonide (Fig. 7). The *m/z* 433 peak had a similar fragmentation pattern to triamcinolone acetonide,



**Scheme 3.** Scheme of CYP3A-mediated metabolism of triamcinolone acetonide.

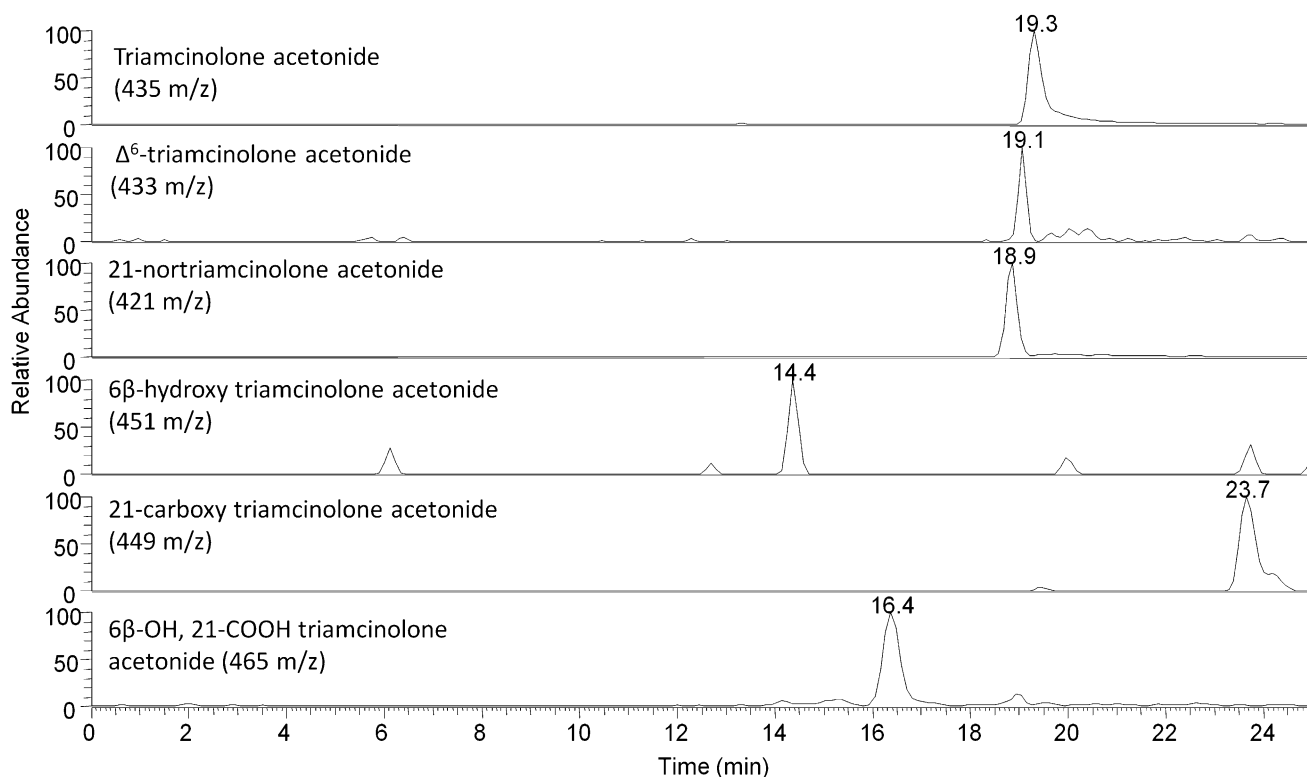


Fig. 6. LC/MS chromatograms of triamcinolone acetonide and its CYP3A-mediated metabolites.

but the major daughter ions were decreased by 2 amu, characteristic of dehydrogenated products.

Budesonide has been extensively studied and showed multiple hydroxylation products by human liver microsomes, including a 6 $\beta$ -hydroxy metabolite, a 23-hydroxy metabolite, and hydroxylation at C<sub>22</sub>, which is probably rearranged to an ester, followed by cleavage to 16 $\alpha$ -hydroxy prednisolone (Scheme 4) (Edsbacker et al., 1987a,b; Jonsson et al., 1995). Budesonide was also efficiently dehydrogenated to  $\Delta^6$ -budesonide. We were able to detect these metabolites from all three CYP3A enzymes. Interestingly, preincubating the recombinant CYP3A supersomes with esterase inhibitors prevented the formation of 16 $\alpha$ -hydroxy prednisolone, leading us to conclude that esterases, and not P450s, are responsible for cleavage of the ester. No novel metabolites were identified from the CYP3As' metabolism of budesonide.

### Discussion

Inhaled GCs are potent anti-inflammatory drugs that have become the mainstay for the treatment of persistent asthma. Despite this fact, approximately 30% of asthmatics have some degree of steroid resistance or insensitivity (Chan et al., 1998; Szeffler et al., 2002). The CYP3A enzymes are the major P450 enzymes known to metabolize these compounds. Therefore, interpatient variability in the metabolism of inhaled GCs could play a role in steroid resistance and insensitivity. Furthermore, GCs have demonstrated the ability to induce CYP3A expression in liver and lung cells (Hukkanen et al., 2003; Krusekopf et al., 2003), which could increase the rate of clearance at the site of action and/or systemically. For these reasons, it is vital to understand the role of CYP3As in the metabolism of inhaled GCs.

Our goal in this study was to investigate initial rates of clearance of the GCs with the CYP3As. The rates of metabolism of the inhaled GCs demonstrated both interenzyme and interdrug variability (Fig. 3; Table 3). CYP3A4 is the most efficient enzyme at eliminating all of the GCs tested. Although CYP3A5 is usually less efficient than CYP3A4, results here demonstrate that CYP3A5 is still an effective metabolizer, while CYP3A7 was the least efficient of the CYP3As. Fluticasone propionate is the most rapidly metabolized of all the GCs with all three enzymes. This is of particular importance because it has recently been demonstrated that fluticasone propionate was an efficient mechanism-based inactivator of CYP3A5, but CYP3A4 and CYP3A7 were inactivated to lesser extents (Murai et al., 2010). Interestingly, both CYP3A5 and CYP3A7 did not significantly metabolize flunisolide. Although CYP3A5 did appear to produce some hydroxylated flunisolide metabolites when incubated for long periods of time (30 minutes or more), the initial rate of metabolism was too slow to measure. Being the predominate lung CYP3A isoform, CYP3A5 activity will have the most profound effect on the GC concentration at the site of action. Furthermore, chronic GC use could result in increased levels of CYP3A5, resulting in more rapid GC elimination and a potential corresponding decrease in drug efficacy, with the exception of fluticasone propionate, which inactivates CYP3A5 and inhibits its own metabolism. This inactivation process could be a potential mechanism through which patients might develop steroid resistance and insensitivity.

In addition to induction, CYP3A5 has been shown to be polymorphically expressed, with significant interracial differences in prevalence (Hustert et al., 2001; Kuehl et al., 2001). It has been demonstrated that people with at least one *CYP3A5\*1* allele express large amounts of enzyme, while the single-nucleotide polymorphisms in *CYP3A5\*3* and *\*6* exhibit alternative splicing and protein truncation. Consequently, those with *CYP3A5\*3* genotype have



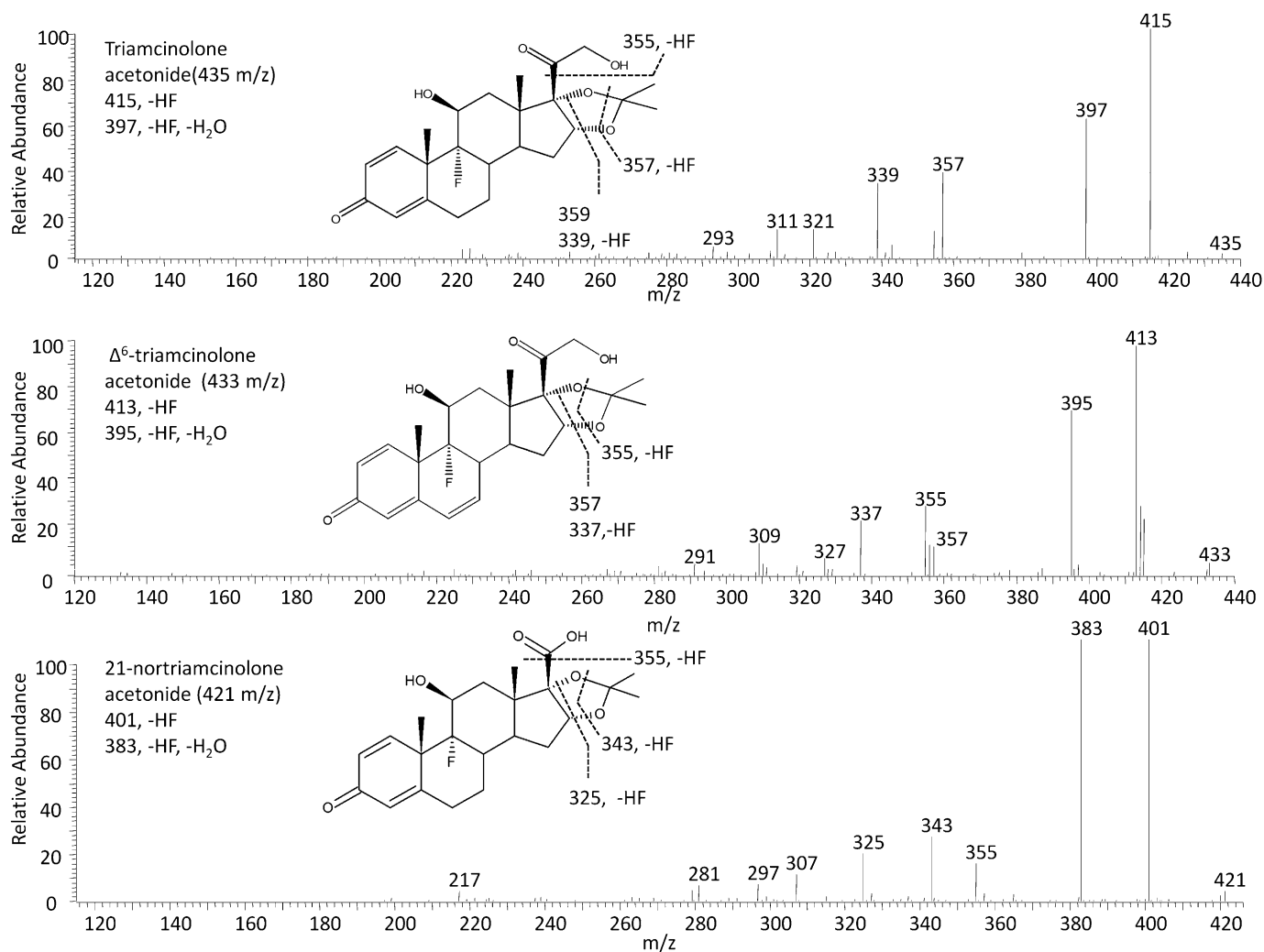


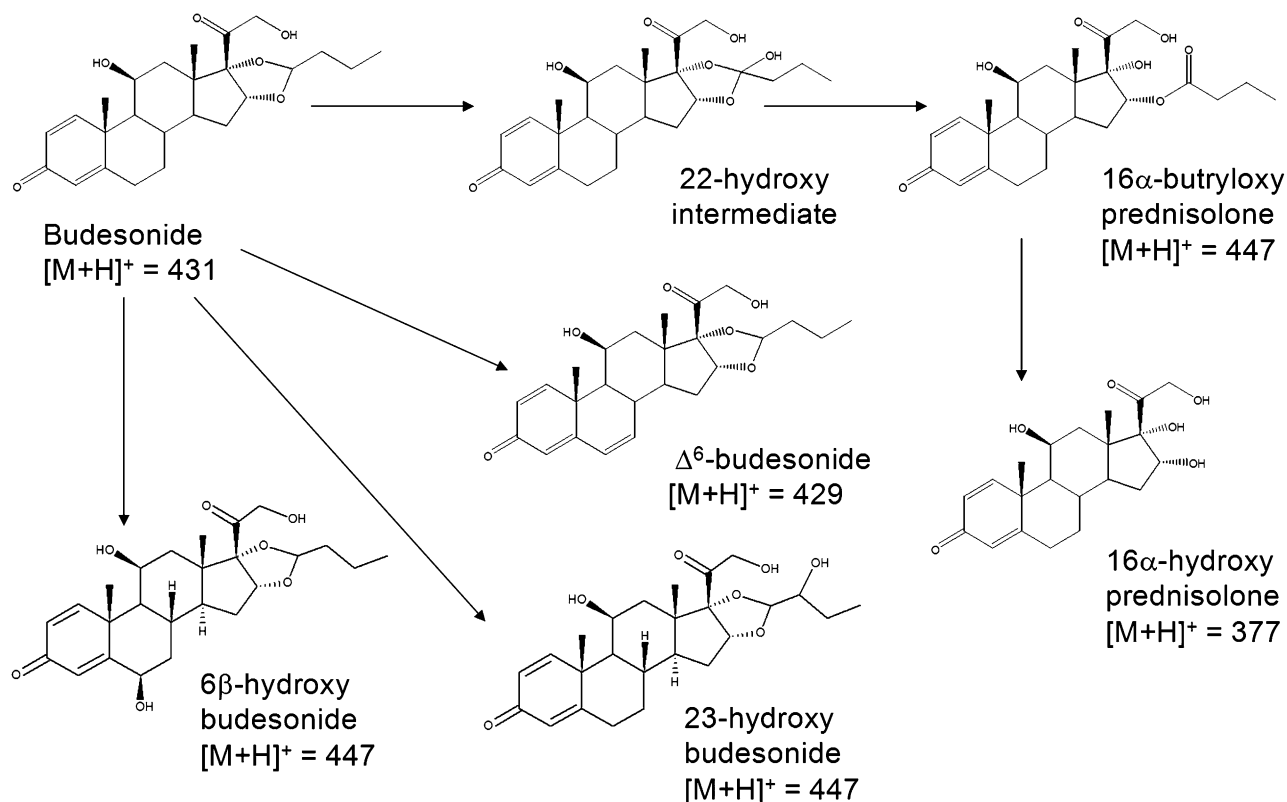
Fig. 7. MS/MS fragmentation spectra of triamcinolone acetonide and its CYP3A-mediated metabolites.

variable drug metabolism compared with the *CYP3A5\*1* populace (Kim et al., 2009). It is likely that those with *CYP3A5\*3* or *\*6* would have decreased metabolism of GCs, and therefore higher levels of GCs at the therapeutic site of action. Conversely, those who are *CYP3A5\*1/\*1* carriers would have increased levels of GC metabolism, which could result in insensitivity to GCs as a result of more efficient clearance in lung cells. Alternatively, flunisolide is not metabolized by CYP3A5 and would not be affected by polymorphic differences in CYP3A5 expression.

The four inhaled GCs tested in this study share the same basic steroid structure, except for the site of fluorination on the B-ring. CYP3A-mediated oxygenation of steroids often occurs at the C<sub>6</sub> on the B-ring. This regiospecific reaction is stereospecific as well, because it produces a hydroxyl group in the  $\beta$ -orientation. Among the GCs tested, CYP3A4 was able to oxygenate the C<sub>6</sub> position of all the compounds except fluticasone propionate. Absence of fluorines (budesonide), fluorination at C<sub>6</sub> (flunisolide), or fluorination at C<sub>9</sub> (triamcinolone acetonide) did not affect hydroxylation of these compounds. Fluticasone propionate is unique as it is fluorinated at both the C<sub>6</sub> and C<sub>9</sub> positions, which may impede C<sub>6</sub> hydroxylation by causing steric impediments in the enzyme active site, which may block binding and catalysis of the C<sub>6</sub> above the heme. Alternatively, the two strong electron-withdrawing fluorines on C<sub>6</sub> and C<sub>9</sub> may impede the

initial abstraction of hydrogen from the C<sub>6</sub> position, preventing subsequent hydroxyl rebound, or subsequent electron abstraction to produce the dehydrogenated product. Furthermore, whereas CYP3A5 and CYP3A7 catalyzed hydroxylation at the C<sub>6</sub> position of triamcinolone acetonide and budesonide, fluticasone propionate was not metabolized at this position. Further study is needed to determine the reason(s) why only the double-fluorinated GC was not hydroxylated at the C<sub>6</sub> position.

In addition to hydroxylation at the C<sub>6</sub> position, P450s were previously found to dehydrogenate GC at the C<sub>6</sub>-C<sub>7</sub> position (Teitelbaum et al., 1981; Edsbacker et al., 1987a). Although  $\Delta^6$ -flunisolide could be formed via a dehydrogenation reaction or loss of water from the 6-OH intermediate (Scheme 1),  $\Delta^6$ -budesonide can only arise from a dehydrogenation reaction. Therefore, we sought to determine if the CYP3As could form dehydrogenated products. Our studies detected CYP3A4-mediated dehydrogenated metabolites of triamcinolone acetonide, flunisolide, and budesonide. CYP3A5- and CYP3A7-mediated dehydrogenated metabolites of triamcinolone acetonide and budesonide were also detected. It was not possible to unequivocally differentiate  $\Delta^6$ -flunisolide formation by a dehydrogenation reaction or from the loss of water through the 6-OH intermediate. The dehydrogenation of flunisolide at the C<sub>6</sub>-C<sub>7</sub> position was confirmed by comparison with an NMR-authenticated synthetic



**Scheme 4.** Scheme of CYP3A-mediated metabolism of budesonide.

standard. Unlike flunisolide, the  $\Delta^6$ -budesonide and novel  $\Delta^6$ -triamcinolone acetonide metabolites could only be formed by a dehydrogenation reaction. Although MS/MS fragmentation could not identify the site of dehydrogenation, it likely occurs at the C<sub>6</sub>-C<sub>7</sub> position because this is the site of hydroxylation, and budesonide and flunisolide are dehydrogenated at this position. Interestingly, only fluticasone propionate did not form a detectable CYP3A-mediated dehydrogenated metabolite. However, if the double fluorination prevents hydroxylation at this site, it would also prevent dehydrogenation by the same process. Conversely, we have shown that fluticasone propionate is an efficient mechanism-based inactivator of CYP3A5, but the inactivating reactive intermediate was not identified (Murai et al., 2010). With the CYP3A-mediated metabolism producing triamcinolone acetonide and budesonide dehydrogenated products at the C<sub>6</sub>-C<sub>7</sub> position, it is reasonable to speculate that CYP3A5 dehydrogenates fluticasone to a reactive intermediate at this vulnerable position. Dehydrogenated products are often unstable and can only be detected by trapping with nucleophiles. The dehydrogenated products that were identified in this study were stable enough to be detected without trapping agents, suggesting that they are not highly electrophilic. Unfortunately, these dehydrogenated metabolites were labile in aqueous solutions, which precluded isolation and identification, except for the relatively stable  $\Delta^6$ -flunisolide product. However, our preliminary studies have discovered NADPH-dependent glutathione adducts with masses corresponding to the parent + glutathione for all the GCs tested (data not shown). These results strongly suggest that the GCs are susceptible to CYP3A-mediated dehydrogenation. Future studies with trapping agents will help to identify dehydrogenated products and elucidate the reactivity of these intermediates.

Other than the fluorination on the B-ring, the most significant structural differences between the inhaled GCs were the modifications found on the D-ring. In addition to the C<sub>6</sub> position, these D-ring modifications were the sites of the majority of the metabolism. The only CYP3A-mediated metabolite of fluticasone propionate was formed by the cleavage of the thioester attached to the D-ring. Interestingly, the adjacent ester moiety was not cleaved by any of the CYP3As. Incubating fluticasone propionate with human liver microsomes, without NADPH, did not produce any fluticasone propionate metabolites. This suggests that neither the thioester nor the carboxylester were cleaved by esterases. Flunisolide, budesonide, and triamcinolone acetonide share a hydroxyacetone moiety on C<sub>17</sub>. However, metabolism on this moiety varied greatly. Both flunisolide and triamcinolone acetonide were metabolized by CYP3A oxygenation at the C<sub>21</sub> position to form similar 21-carboxy metabolites (Figs. 4 and 7). Triamcinolone acetonide was also metabolized to a 21-nortriamcinolone acetonide metabolite, which was confirmed by comparison with an NMR-authenticated standard (Fig. 2). In contrast, the hydroxyacetone moiety of budesonide was not oxidized by any of the CYP3As. We speculated that the carbon chain on the 16,17-butyldenebis(oxy) moiety of budesonide prevents positioning of hydroxyacetone for oxygenation at the C<sub>21</sub> position. The CYP3As are able to oxygenate both C<sub>22</sub> and C<sub>23</sub> of budesonide (Scheme 4), suggesting that the butyldenebis(oxy) moiety prevents the hydroxyacetone moiety from occupying the correct position above the catalytic center.

This study demonstrates both the interenzyme and interdrug variability of the CYP3A metabolism of four commonly prescribed inhaled GCs. Although CYP3A4 was the most efficient metabolizer of all the compounds tested, CYP3A5 was also shown to be an efficient

metabolizer of all the compounds except flunisolide. All the CYP3A isoforms produced previously identified metabolites, and the novel metabolites  $\Delta^6$ -triamcinolone acetonide, 21-carboxy triamcinolone acetonide, 21-nortriamcinolone acetonide, and 21-carboxy flunisolide were identified. These studies will aid in elucidating the mechanisms of steroid insensitivity and resistance via differential metabolism, which is critical to improving the clinical therapeutic use of inhaled GCs.

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#### Authorship Contributions

*Participated in research design:* Moore, Orton, Reilly, Ward, Yost.

*Conducted experiments:* Moore, Roberts, Orton, Murai, Fidler.

*Contributed new reagents or analytic tools:* Moore, Murai, Fidler.

*Performed data analysis:* Moore.

*Wrote or contributed to the writing of the manuscript:* Moore, Orton, Reilly, Ward, Yost.

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**Address correspondence to:** Garold S. Yost, Department of Pharmacology and Toxicology, University of Utah, 30 South 2000 East, Room 201, Salt Lake City, UT 84112. E-mail: gyost@pharm.utah.edu

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