

In Vitro Metabolism of ¹⁴C-Ciclesonide in Hepatocytes from Mice, Rats, Rabbits, Dogs, and Humans

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Introduction

Ciclesonide (CIC) is a novel and effective inhaled glucocorticoid for treatment of persistent asthma. CIC has low glucocorticosteroid receptor affinity and undergoes conversion via esterases located primarily in the lungs after inhalation dosing to yield its high affinity and active metabolite, desisobutrylyl-ciclesonide (des-CIC). The current investigation was conducted to gain insights into the *in vitro* metabolism of CIC in hepatocytes from these four animal species as compared to humans. Detailed structures and profiles of ¹⁴C/CIC metabolites in mouse, rat, rabbit, dog, and human hepatocyte incubations are proposed and reported herein. It was found that *in vitro* metabolite profiles in mouse, rat, rabbit, and dog hepatocytes were similar to the profiles in human hepatocytes; hence, it would be appropriate to use these animal species for drug safety evaluation of CIC.

Experimental

Hepatocyte Incubations:

Incubations were carried out at 37°C under an atmosphere of 95% air, 5% CO₂, and 95% humidity. The final concentration of CIC was 10 µM in the incubation mixtures. To assist with metabolite characterization and identification, a mixture of ¹⁴C/CIC and non-radioabeled CIC in ca. 1:1 ratio was used in the hepatocyte incubations. Time-course experiments were carried out by removing 0.5-ml aliquots of incubation mixture at 0.5, 1, 2, and 4 h. Reactions were terminated by adding 2 x volume of ice-cold acetonitrile and the samples sonicated and centrifuged (5000 g for 5 min).

High-Performance Liquid chromatography (HPLC):

LC System: Waters 2695 Separations Module
Analytical Column: C18 column, 4.6 x 150 mm, 3 µm
Flow Rate: 1.0 mL/min
Mobile Phase A: CH₃CN
Mobile Phase B: 0.4% HCOOH Aqueous Solution
Mobile Phase C: 0.01M NH₄OAc Aqueous Solution
Gradient: A linear gradient from 0 to 75% A in 40 min followed from 75 to 100% A in 10 min was used. Mobile phases A and B were used for metabolite profiling and positive ion mode LC-MS analysis and mobile phases A and C were used for negative ion mode LC-MS analysis.

Mass Spectrometry:

MS Systems 1 & II: Finnigan MAT TSO-7000 Triple Quadrupole & LCO™ Mass Spectrometers (for metabolite i.d.)
MS System III: PE Sciex API365 Triple Quadrupole LC/MS/MS Spectrometers (for monitoring of unchanged CIC)

Thin Layer chromatography (TLC):

TLC was used for characterization of M6 (hippuric acid)

TLC Plate: Silica gel 60 F₂₅₄ 250 µM

Solvent System for 1st D: Hexane/Isopropanol/HCOOH (50/50/2, v/v/v)

Solvent System for 2nd D: Ethyl Acetate/Isopropanol/H₂O/NH₄OH (40/50/10/2, v/v/v/v)

Tab. 1. Metabolites of ciclesonide characterized/identified by LC/MS in mouse, rat, rabbit, dog, and human hepatocyte incubations

Metabolite ID No.	R. (min)	MW	Structure or Proposed Structure	Source
Ciclesonide (CIC)	~46	340		RH, DH, HH
Des-CIC or M1	~35	470		MH, RH, RHH, DH, DHH, HH
M6	~13	179		RH, RHH, HH
M7a, b, c, d, e	~10.2, 22.5	302		RH, RH, HH, DH, DHH, HH
M8a or M8b	~21, 23.8	302		RH, RH, RHH, DH, DHH, HH
M9a, b, c, d, e, f	23.5, 28.5	486		MH, RH, RHH, DH, DHH, HH
M2	~29	486		MH, RH, RHH, DH, DHH, HH
M9	~22.5	662		DH
M10	~23.7	662		DH
M11	~28.8	646		MH, DH
M12	~30.8	648		DH
M13	~31.3	488		RH
M4	~38.7	472		RH
M14	~26.8	484		MH, RH, RHH, DH, DHH, HH

*Denotes the position of ¹⁴C label. DH, dog hepatocyte incubations; RH, human hepatocyte incubations; MH, mouse hepatocyte incubations; RHH, rabbit hepatocyte incubations; and RHH, rat hepatocyte incubation.

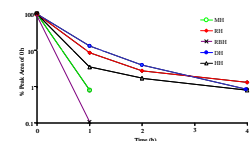


Fig. 1. Time courses of unchanged ciclesonide in cryopreserved mouse (MH), rat (RH), rabbit (RHH), dog (DH), and human (HH) hepatocytes

1 In Vitro Metabolism of CIC in Animal and Human Hepatocytes Figure 1 illustrates the time courses of the unchanged ¹⁴C/CIC in the mouse, rat, rabbit, dog, and human hepatocytes. CIC was rapidly metabolized by animal and human hepatocytes. Unchanged CIC remaining in the mouse, rat, dog, and human hepatocyte incubation mixtures after the first hour were 0.8, 8.5, 13.0, and 3.4%, respectively. CIC was not detected in the rabbit hepatocyte incubation mixture after one hour incubation.

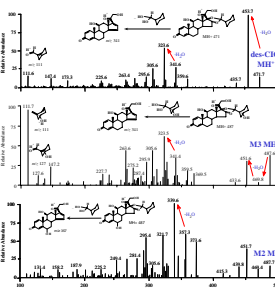


Fig. 2. LC/MS/MS spectra of des-CIC and monohydroxylated des-CIC (M2 and M3)

Results and Discussion

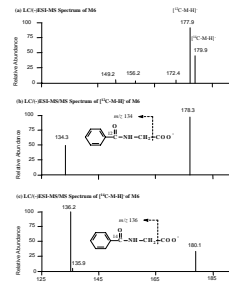


Fig. 3. LC/MS/MS spectra of M6 (hippuric acid)

2 Identification of Metabolites By LC/MS CIC and its metabolites generated by animal and human hepatocytes were separated via HPLC and subjected to mass spectral analysis. The ions with *m/z* values corresponding to CIC metabolites were subjected to collision activated dissociation (CAD)-MS/MS. A total of 13 CIC metabolites, were characterized or identified by LC/MS (Table 1).

Under CAD conditions, facile loss of the cyclohexane moiety from CIC and its metabolites was observed. The resultant characteristic fragment ions are very helpful for the determination of derivatization positions (Figure 2).

Tab. 2. Relative percent distribution of ciclesonide and its metabolites identified in 4-h mouse, rat, rabbit, dog, and human hepatocyte incubations

Metabolite ID No.	Mouse	Rat	Rabbit	Dog	Human
Ciclesonide (CIC)	ND	1.8	ND	2.9	0.4
Des-CIC	47.7	44.4	20.8	1.7	17.8
M6	ND	0.7	1.5	ND	6.4
M7 (a, b, c, and d, e)	1.9	4.9	11.6	20.4	6.4
M8a or M8b	2.9	2.5	2.7	1.6	ND
M9 (a, b, c, d, and e, f)	26.3	20.6	39.1	10.6	25.5
M2	3.5	11.4	5.5	16.8	15.3
M3	ND	ND	ND	3.9	ND
M10	ND	ND	ND	3.4	ND
M11	3.5	ND	ND	14.8	ND
M12	ND	ND	ND	8.8	ND
M13	ND	3.1	ND	ND	ND
M4	ND	1.8	ND	ND	ND
M14	11.1	1.3	5.2	2.0	ND

ND, Non-Detectable

3 Metabolite Profiling

The ¹⁴C-metabolite profiles of CIC in 4-h mouse, rat, rabbit, dog, and human hepatocyte incubation mixtures are depicted in Figure 4. Relative percent distribution of the radioactivity peaks for CIC and its metabolites in 4-h mouse, rat, rabbit, dog, and human hepatocyte incubates is summarized in Table 2.

CIC was rapidly metabolized to des-CIC through deisobutyrylation by carboxylesterases in all animal and human hepatocyte incubations. Des-CIC was readily oxidized to yield mono-hydroxylated (M3 and M2) and di-hydroxylated (M7 and M8) derivatives. Mono- and di-oxidation occurred on the cyclohexane ring (M8 and M3), steroid moiety (M2), or both (M7). Des-CIC, M7, M3, and M2 were found in all animal and human hepatocyte incubation mixtures. Additional metabolites, a keto-derivative of des-CIC (M14), double bond reduction derivatives of des-CIC and M3 (M5 and M13), and glucuronic acid conjugated metabolites (M9, M10, M11, and M12) were found only in animal hepatocyte incubations.

Hippuric acid (HA, M6) was observed in rat, rabbit, and human hepatocyte incubations. HA can be found endogenously in hepatocyte incubation mixtures. Thus, M6 was isolated and purified by HPLC, followed by LC/MS analysis. Molecular ions corresponding to HA were always associated with the radioactivity. The R_p values of [¹⁴C]M6 and its methyl ester in 2D-TLC analysis also agreed with the two reference chemicals. In addition, [¹⁴C-M-H] at *m/z* 180 of the [¹⁴C]HA, derived from C-14 labeled CIC, was clearly observed in the LC/(-)ESI-MS spectrum of M6 (Figure 3). Product ion scans of both [¹⁴C-M-H] at *m/z* 178 and [¹⁴C-M-H] at *m/z* 180 showed an identical characteristic loss of 44 amu (CO₂), consistent with HA. HA found in hepatocyte incubation mixtures could be formed from the cyclohexylmethyl moiety of CIC by cleavage, followed by aromatization of the cyclohexane ring through multiple steps of hydroxylation and dehydration to give benzoic acid, which was further conjugated with glycine. The formation of aromatized molecules from alicyclic precursors has been documented in the literature. For example, the metabolic aromatization of dodecylcyclohexane in rainbow trout has been reported.²

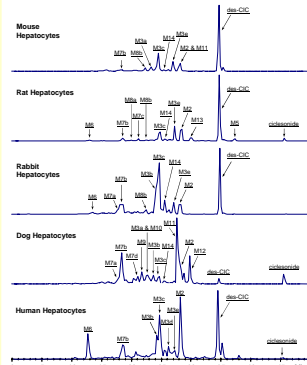


Fig. 4. HPLC radiochromatograms of ciclesonide and metabolites from 10 µM ¹⁴C-ciclesonide incubations with mouse, rat, rabbit, dog, and human hepatocytes for 4 h

Conclusion

Results from this study indicate that CIC and its active metabolite, des-CIC, are extensively metabolized *in vitro* in animal and human hepatocytes and that the metabolite profiles in mouse, rat, rabbit, and dog hepatocytes are similar to the profiles in human hepatocytes. The metabolite profiles of ciclesonide and des-CIC *in vivo* in humans (unpublished data) are also similar to the profiles *in vitro* in human hepatocytes identified in this study.

References

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