Investigation of Cholesterol Oxidation Products (COPs) in Liposomal Drug Products

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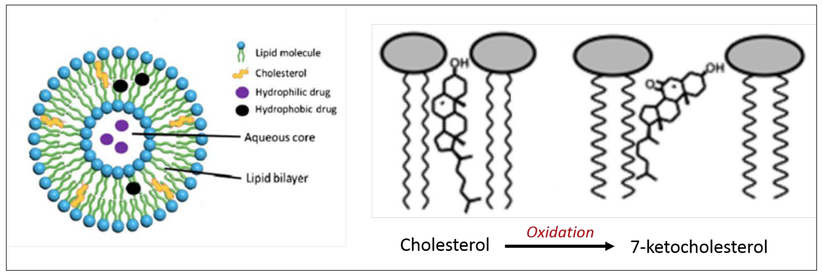
Introduction

Cholesterol is one of the major excipients in a liposomal bilayer and is vulnerable to oxidation to generate cholesterol oxidation products (COPs). During the manufacturing and/or storage of liposome drug products (LDP), the presence of oxygen, heat, light, certain metals, and radicals can accelerate COPs generation. COPs are reported to be associated with serious diseases such as atherosclerosis, cancer and diabetes[1,2].

Once cholesterol is replaced by polar COPs, its orientation will be changed inside the lipids bilayer due to the introduction of hydrophilic groups[3], affecting liposome bilayer integrity and consequently the release of encapsulated drugs.

COPs were rarely monitored in the LDPs due to the lack of sensitivity of traditional analytical methods. We reported here a sensitive and robust LCMS analytical method to quantitate trace amounts of COPs in LDPs.



Biochemistry, 2005, 44 (30), pp 10423–10433

Figure 1. Cholesterol in lipids bilayer and effect on bilayer integrity when oxidized into COPs

COPs Structures

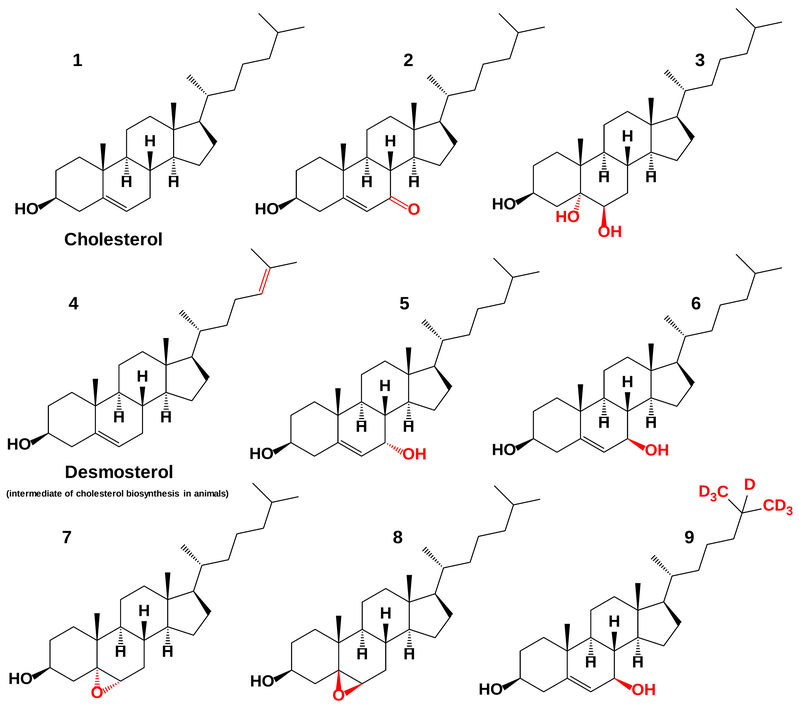


Figure 2. Structures of desmosterol and cholesterol oxidation products

1, Cholesterol **2**, 7-keto-cholesterol **3**, Cholestane-3β,5α,6β-triol

4, Desmosterol **5**, 7α-hydroxycholesterol **6**, 7β-hydroxycholesterol

7, Cholesterol 5α,6α-epoxide **8**, Cholesterol 5β,6β-epoxide **9**, 7β-hydroxycholesterol-D7 (IS)

Methods

Instrumentation:
LC: Agilent 1290 infinity UPLC system
MS: Agilent 6550 QTOF mass spectrometer

Column:
1, RP: Agilent Eclipse plus RRHD C18 column (100×2.1 mm×1.8 µm)
2, NP: Waters Acquity UPLC HSS Cyano column (100×2.1mm×1.8 µm)
RP-HPLC: Mobile Phase: A, 0.1% formic acid ; B, MEOH with 0.1% formic acid
NP-HPLC: Mobile Phase: A, Hexane; B, Isopropanol
MS conditions:
APCI source in Positive mode.
Temperature 250°C, Vaporizer 375°C, N_2 Drying gas flow 14 L/min, Nebulizer 60 psi, Capillary voltage 3,000 V, Nozzle 500 V, Corona 6 µA, Fragmentor voltage 175 V, Skimmer voltage 65 V, Oct RF Vpp 750 V. Acquisition parameters: MS mode range of 100–1,000 *m/z*, acquisition rate at 2 spectra/s. QTOF was tuned under 1700 *m/z* mass range and 2 GHz dynamic mode with high-resolution slicer position.

Chromatogram

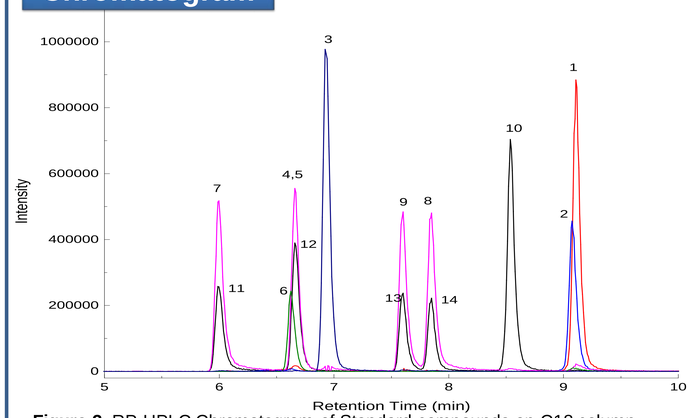


Figure 3. RP-HPLC Chromatogram of Standard compounds on C18 column.
Sample concentration 1 µg/mL in isopropanol.
CHOL (**1**):*m/z* 369.3521 ([M+H-H_2O]$^+$); CHOL-D7 (**2**):*m/z* 376.3950 ([M+H-H_2O]$^+$)
7-Keto (**3**):*m/z* 401.3413 ([M+H]$^+$); 7β-D7 (**6**): EIC *m/z* 392.3921 ([M+H-H_2O]$^+$)
7α (**4**), 7β (**5**), Triol (**7**), 5,6β (**8**), 5,6α (**9**) all share EIC *m/z* 385.3471 ([M+H-H_2O]$^+$)
Desmo(**10**), Triol (**11**) [M+H-3H_2O]$^+$, 7α (**12**), 7β (**12**) [M+H-2H_2O]$^{+\cdot}$ 5,6α (**14**) [M+H-2H_2O]$^{+\cdot}$ 5,6β (**13**), [M+H-2H_2O]$^{+\cdot}$ all share *m/z* 367.3365

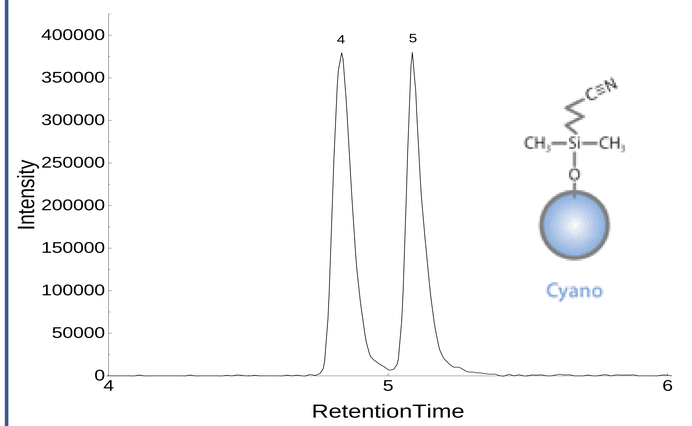


Figure 4. NP-HPLC Chromatogram of 7α, 7β-cholesterol mixture on cyano column.
Sample concentration 1 µg/mL in isopropanol.
7α (**4**), 7β (**5**) isomers share same *m/z* 385.3471 ([M+H-H_2O]$^+$)

Method Validation Results

Accuracy and precision of APCI-LCMS method for the analysis of COPs (m=3, n=5)

Cmpds	Spiked Conc. (ng/mL)	Intraday Measured Value^n Mean ± SD	Accuracy (%)	Precision (%)	Interday Measured Value^m Mean ± SD	Accuracy (%)	Precision (%)
CHOL	50	54.1 ± 0.2	108.2	0.4	52.3 ± 1.8	104.5	3.4
	400	441.1 ± 7.5	110.3	1.7	446.1 ± 7.5	111.5	1.7
	800	862.5 ± 13.1	107.8	1.5	860 ± 7.6	107.5	0.9
7Keto-CHOL	50	51.3 ± 0.3	102.6	0.5	49.3 ± 2.5	98.6	5.1
	400	402.3 ± 7.6	100.6	1.9	409.3 ± 11.1	102.3	2.7
	800	791.6 ± 8.4	99	1.1	784.2 ± 8.1	98	1
7α-CHOL	50	47.2 ± 3.3	94.4	6.9	46.8 ± 0.6	93.7	1.2
	400	369 ± 22.4	92.2	6.1	383.3 ± 13.1	95.8	3.4
	800	751.2 ± 14.1	93.9	1.9	776.2 ± 58.2	97	7.5
7β-CHOL	50	47.4 ± 2.3	94.8	4.8	48.6 ± 1.4	97.1	2.9
	400	377.3 ± 22.6	94.3	6	388.6 ± 9.8	97.1	2.5
	800	767.9 ± 14.9	96	1.9	787.9 ± 21.4	98.5	2.7
Triol-CHOL	50	52.8 ± 2.1	105.5	4	49.6 ± 3.9	99.2	7.9
	400	399.7 ± 13.4	99.9	3.3	399.4 ± 21.3	99.9	5.3
	800	834.6 ± 16.7	104.3	2	809 ± 22.3	101.1	2.8
5,6α-CHOL	50	49.4 ± 5.3	98.8	10.6	47.3 ± 1.9	94.6	4.1
	400	400.9 ± 17.5	100.2	4.4	399.3 ± 12.1	99.8	3
	800	785.3 ± 12	98.2	1.5	796.7 ± 19.5	99.6	2.4
5,6β-CHOL	50	51.9 ± 1.6	103.8	3.1	50 ± 2	100.1	4
	400	397.9 ± 5.2	99.5	1.3	413.1 ± 15.8	103.3	3.8
	800	808.4 ± 18.4	101.1	2.3	816.4 ± 7	102	0.9
Desmo	50	49.7 ± 0.9	99.4	1.8	49 ± 1	98.1	2.1
	400	402.4 ± 4.8	100.6	1.2	413.6 ± 12.1	103.4	2.9
	800	806.3 ± 10.3	100.8	1.3	803.6 ± 5.3	100.4	0.7

Analysis of Cholesterol and Liposome Drug Products

COPs and desmosterol in liposomal drug products (Average ± SD, unit: %cholesterol*10^{-4}, n=3)

	7α	7β	7-Keto	Desmo	5,6α	5,6β	Triol
Cholesterol	0± 0	0± 0	0± 0	0± 0	0± 0	0± 0	0± 0
DP1	412± 49	93± 14	116± 14	121± 29	0± 0	0± 0	36± 5
DP2	336± 100	372± 43	249± 14	2062± 186	0± 0	271± 20	77± 4
DP3	890± 58	1363± 59	581± 36	2123± 89	0± 0	228± 34	94± 19
DP4	1972± 857	1357± 36	948± 50	2392± 136	121± 31	673± 71	136± 34
DP5	2773± 26	1819± 26	1478± 114	2668± 40	73± 13	457± 18	154± 12

Analysis of Desmosterol in Cholesterol from Different Sources

Desmosterol concentration was detected in the bulky cholesterol materials (n=3)

Vendor	Biological Source	Manufacturing	Storage	Stabilizer	Detected ng/mL	%	RSD %
Vendor1	Ovine wool	NA	-20	NA	321.49	1.47	0.05
Vendor2	Wool Grease	GMP	RT	a-Tocopherol	499.16	2.54	0.03
Vendor3	Plant Derived	GMP	-20	NA	0.00	0.00	0.00
Vendor4	NA	NA	refrigerate	NA	108.35	0.49	0.10
Vendor5	Sheep wool grease	GMP	RT	a-Tocopherol	551.19	2.66	0.01

USP Acceptance Criteria: Desmosterol NMT 3% in bulky cholesterol material; NA means not applicable

Summary and Conclusion

1. Report of an UPLC-APCI-QTOF method for quantitation of COPs and desmosterol in LDPs.
2. Separation of enantiomeric forms of 7α and 7β-hydroxycholesterol achieved on cyano column.
3. COP impurities were detected in LDPs but not in cholesterol raw materials.
4. Desmosterol impurity was detected in both LDPs and USP/NF cholesterol raw materials.
5. Impact of these COPs on liposome drug product safety and efficacy warrants further investigation.

References

1, *Chemistry and Physics of Lipids*. 2016, 199:144-160 2, *Trends in Endocrinology & Metabolism*. 2017, 28(7):485-496
3, Biochemistry, 2005, 44 (30), pp 10423–10433

Acknowledgements

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