Nonclinical *In Vitro* Safety Assesment Summary of Hemp Derived (R/S)-Hexahydrocannabinol ((R/S)-HHC)

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Research Article

Keywords: Hemp, Hexahydrocannabinol, Safety, hERG, Cytotoxicity

Posted Date: November 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2299264/v1

Abstract

In pursuit of a more detailed understanding of the naturally rare occurring cannabinoid analogue, Hexahydrocannabinol (HHC), safety becomes a question when possible human consumption is introduced. We explore the safety profile of HHC, using a third-party lab to produce preclinical *in vitro* safety profile data on the cyto-toxicity of cardiomyocytes through hERG, hepatocytes, and lung fibroblasts. hERG[1] (The human Ether Related Gene) is a gene that codes for a protein known as $K_v11.1$, the alpha subunit of a potassium ion channel. This ion channel (simply denoted as “hERG”) is best known for its contribution to the electrical activity of the heart: the hERG channel mediates the repolarizing current in the cardiac action potential, which helps coordinate the heart's beating. Preceding studies of HHC, neglect the base scaffold structure of HHC[2], and test on synthetic derivatives, and various analogs. The purpose of these studies is to demonstrate HHC as a safe cannabinoid for potential human consumption. The preclinical assessments of HHC did not indicate any cardiac safety issues via data from a hERG[3] fast patch assay. HHC also demonstrated no cytotoxicity in the plated human liver hepatocyte cell viability assay. HHC, according to *in vitro* data produced potential cytotoxic effects in human lung fibroblasts when exceeding 10 mM concentrations, when reviewing the data, safe possible human consumption is feasible without complications.

Introduction

The use of cannabinoids dates to ancient China around 2500 BC.[4] These substances, which are found in nature, have been used for recreational and medicinal purposes: to alleviate pain,[5] gastrointestinal (GI) issues[6], multiple sclerosis[7] anxiety,[8] cancer,[9] and more. The explosion of cannabis usage around the world, caused an increasing number of studies to be conducted, to determine the therapeutic benefits of these compounds, in the fight against some of the most debilitating and chronic diseases. Cannabis sativa L. produces over 120 minor cannabinoids[10].

Current and relevant studies of HHC[11] involve substituted groups such as alcohols, and carbonyls which affect the target and binding affinities, and can pose risks that are not widely studied[11], while we focus purely on Hexahydrocannabinol (HHC) which has a methyl at the C9 position rather than other functional groups. Since this cannabinoid (HHC) is found naturally in trace amounts.[12,13] It can be extracted from hemp and cannabis via the conventional isolation methods, but this is an inefficient and costly process. Our group has developed an efficient and robust way to synthesize and isolate (R/S)-HHC starting from D8 and D9-THC while also focusing on the safety study of this rare cannabinoid, including but not limited to cell viability and cytotoxicity studies.

Materials And Methods

Preclinical *in vitro* studies were performed for (R/S)-Hexahydrocannabinol ((R/S)-HHC). The studies were conducted at Charles River Laboratories [Worcester, MA (cell viability); Cleveland, OH (hERG); and Skokie,

IL (Ames)]. All studies were exploratory and carried out under a non-GLP environment. All procedures carried out in the laboratory were reviewed and approved by the Institutional Authorities.

Microbial mutagenesis. The Ames test is one of the most frequently applied tests in toxicology. Almost all new pharmaceutical substances and chemicals used in industry are tested by this assay. The (*Salmonella typhimurium* reverse mutation assay) is a bacterial short-term test for identification of carcinogens using mutagenicity in bacteria as an endpoint. The Ames test detects mutations in a gene of a histidine-requiring bacterial strain that produces a histidine-independent strain. Both direct and indirect (i.e., chemicals that require metabolic activation) mutagens can be identified using the Ames test.

Evaluation of hERG current using patch clamp analysis. One of the most frequent adverse side effects, leading to the failure of drugs, is the cardiac arrhythmias. Such failure is mostly related to the capacity of the drug to inhibit the human ether-à-go-go-related gene (hERG) cardiac potassium channel. Inhibition of the hERG current causes QT interval prolongation resulting in potentially fatal ventricular tachyarrhythmia called Torsade de Pointes. To evaluate anticipated cardiovascular effects, early evaluation of hERG toxicity has been strongly recommended for instance by the regulatory agencies such as U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA).

Cell viability assay using human lung fibroblasts. The cell viability assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells.

Cell viability assay using plated human hepatocytes. Similar to the lung fibroblasts, the highly sensitive ATP CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the potential of *in vitro* liver toxicity of (R/S)-HHC. The test compound ((R/S)-HHC) and the control (Terfenadine) were tested at a concentration range of 0.05 to 50.0 µM for cell viability (cytotoxicity) in human hepatocytes.

Experimental

(R/S)-Hexahydrocannabinol (HHC): A 5L reactor equipped with a reflux condenser and an addition funnel was purged with argon for 15 minutes. Pd/C (10% by mass) was added to the reaction slowly using a powder funnel under argon. The reactor is then purged with argon for 15 minutes. Ethanol (200 mL) was added slowly to avoid sparking the solvent. A mixture of D8- and D9-THC (100 g, 318 mmol) was dissolved in ethanol (100 mL). The solution is added to the reactor under argon and purged for 15 minutes. Afterwards, the atmosphere of argon is stopped, and an atmosphere of hydrogen (1 bar) is introduced. The reaction is then stirred at 25 °C until complete by HPLC. Upon completion, the reaction is purged with argon for 20 minutes. The reaction mixture is filtered over 1–3-micron filter paper on a Buchner funnel and then the solution is concentrated *in vacuo*. The crude oil is then dissolved in hexane and purified over silica (0 to 5% EtOAc). The fractions of interest are concentrated *in vacuo* and then distilled to afford a colorless to light yellow oil with three compounds of similar m/z ratios. HPLC (C18):

7.940 min, 8.141 min, 8.395 min. LRMS: $[M + 1]^+$ 317. ^{1}H NMR (500 MHz, CD_3CN) δ 0.89, 0.93, 1.18, 1.30, 1.43, 1.53, 1.62, 2.06, 2.38, 2.42, 2.60, 3.06, 6.08, 6.68

The final product was isolated and 99.9% (R/S)-Hexahydrocannabinol ((R/S)-HHC) is shown in Fig. 1. In addition, ^{1}H NMR and GC-MS analysis were preformed to determine the structure of (R/S)-HHC, as illustrated in Fig. 2 and Fig. 3. respectively.

Results And Discussion

Genotoxicity assay

Microbial mutagenesis. Potential of (R/S)-HHC to induce reversion mutations in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA both with and without metabolic activation was evaluated. The plate incorporation screen for (R/S)-HHC was tested in duplicate with strains TA98, TA100, TA1535, TA1537, and WP2 uvrA in the presence and absence of a metabolic activation system (phenobarbital/5,6-benzoflavone-induced rat liver S9 microsomal fraction) at 1, 5, 10, 50, 100, 500, 1000, and 5000 µg/plate. The positive controls without metabolic activation were 2-nitrofluorene (TA98, 2.5 µg/plate), sodium azide (TA100 and TA1535, 1 µg/plate), ICR-191 Acridine (TA1537, 0.5 µg/plate), and 4 nitroquinoline N oxide (WP2 uvrA, 2.0 µg/plate). The positive control with metabolic activation for all strains was 2-aminoanthracene (2.5 µg/plate for Salmonella strains and 10 µg/plate for the E. coli strain). DMSO was used as the vehicle control.

(R/S)-Hexahydrocannabinol was not mutagenic, up to concentrations at ≥ 100 µg/plate in strain TA1535 without metabolic activation, and at ≥ 500 µg/plate in strains TA98, TA100, TA1537, and WP2 uvrA both with and without metabolic activation and in strain TA1535 with metabolic activation. The data from the Ames test suggests that (R/S)-HHC is a non-mutagenic compound.

In Vitro Cardiac Safety Test

Table 1
Effects of (R/S)-HHC on hERG Ion Channel Current

Test Article	IC_{50} (µM)	n	Conc (µM)	Mean % hERG Inhibition	Standard Deviation	Standard Error
(R/S)-HHC	**> 50**	9	6.25	4.8	3.0	1.0
		7	12.5	5.7	2.5	0.9
		3	25	2.3	3.8	2.2
		4	50	6.4	5.4	2.7
Cisapride (positive control)	**< 0.05**	4	0.05	64.1	7.6	3.8

The effect of (R/S)-hexahydrocannabinol on cloned hERG potassium channels (encoded by the KCNH2 gene and expressed in HEK293 cells) was examined using the QPatch II® (Sophion Bioscience A/S, Denmark), an automatic parallel patch clamp system. (R/S)-Hexahydrocannabinol was exposed to hERG at 6.25, 12.5, 25 and 50 µM ($n \geq 3$). The duration of exposure to each test article concentration was a minimum of three (3) minutes. The positive control data confirmed the sensitivity of the test systems to ion channel inhibition.

A summary of the results for (R/S)-hexahydrocannabinol and the positive controls are shown in Table 1 above. The data suggests that (R/S)-HHC does not block HERG-encoded channels expressed in HEK293 cells.

Cytotoxicity

(R/S)-HHC and the control (chlorpromazine) were tested at a nominal concentration range of 0.156 to 50.0 µM for cell viability (cytotoxicity) in human lung fibroblasts. In lung fibroblasts, the IC50 for (R/S)-HHC was 14.4 µM, and the percent cytotoxicity at 50 µM was 74.8%. The control, chlorpromazine, showed an IC50 value of 14.3 µM and 86.4% cytotoxicity at 50 µM. The data is summarized in Table 2.

Table 2
Summary of cell viability (cytotoxicity) in fibroblasts following
hour incubation.

Cell Line	Compound ID	IC_{50} (µM)	Cytotoxicity (at 50 µM)
Fibroblasts	(R/S)-HHC	14.4	74.8%
Fibroblasts	Chropromazine (control)	14.3	86.4%

In human hepatocytes, (R/S)-HHC was non-cytotoxic and the IC_{50} for (R/S)-HHC was not measured, and the percent cytotoxicity at 50 µM was also very low (8.9%) due to its weak response. Terfenadine showed an IC_{50} value of 15.8 µM and 99.9% cytotoxicity at 50 µM. The data is summarized in Table 3. The results indicated that there is no potential for hepatotoxicity, with very weak results in this plated human hepatocyte cell viability assay.

Table 3
Summary of cell viability (cytotoxicity) in human hepatocytes following 48 hours incubation

Compound	Incubation Time	Cell Lines	IC_{50} (µM)	Cytotoxicity (at 50 µM)	Comments
(R/S)-HHC	48 hrs	Plated human hepatocytes	**Not determined**	8.9%	Nontoxic
Terfenadine	48 hrs	Plated human hepatocytes	**15.8**	99.9%	Showed toxicity as expected

Conclusion

(R/S)-HHC derived from Colorado Chromatography Labs, using their patent pending technology demonstrated the following results. (R/S)-HHC was not mutagenic in *in vitro* genotoxic assay (Ames test). Preclinical assessments in the hERG patch clamp study did not indicate that (R/S)-HHC had any cardiac safety issues, which suggests that there does not appear to be an effect of (R/S)-HHC and QTc prolongation. The finding in the cell viability studies demonstrated potential cytotoxic effects of (R/S)-HHC in human lung fibroblasts at > 10 μM concentrations. (R/S)-HHC was not cytotoxic or damaging to human liver cells in *in vitro* human hepatocytes cell viability assay.

The future of this compound can be shifted since there was some slight toxicity to the lung fibroblasts, with directed remediation of molar concentrations as well as possible derivatizing of the future HHC compounds either the virgin HHC compound or derivatized compound could be used as a possible anti-tumor drug and treat certain lung cancers. With this initial cell viability and safety study we are providing evidence that although this compound was made in a lab setting, to increase the production of this rare cannabinoid that is difficult to extract, this compound is safe to consume, and can be opened in the world of research to see what effects and ailments that HHC can treat like other cannabinoids.

Declarations

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Author Contributions

ACC: investigation, writing—original draft, formal analysis. T.T.T.: writing— review and editing, formal analysis. G.A.R.: writing—review and editing, formal analysis, validation. K.P.R.: resources and funding, conceptualization, methodology. W.C.: writing—review and editing, resources and funding, conceptualization, methodology.

Competing Interests

The authors report affiliation in an organization with a financial interest in the subject matter discussed in this manuscript due to participation in business-to-business sales.

Acknowledgements

The Authors would like to acknowledge Jin Hong of Custom NMR Service for providing professional spectrum analysis, as well as Scott Caudill and Richard Sams of KCA Laboratories for providing high-resolution Mass Spectrometry and Gas Chromatography access.

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Figures

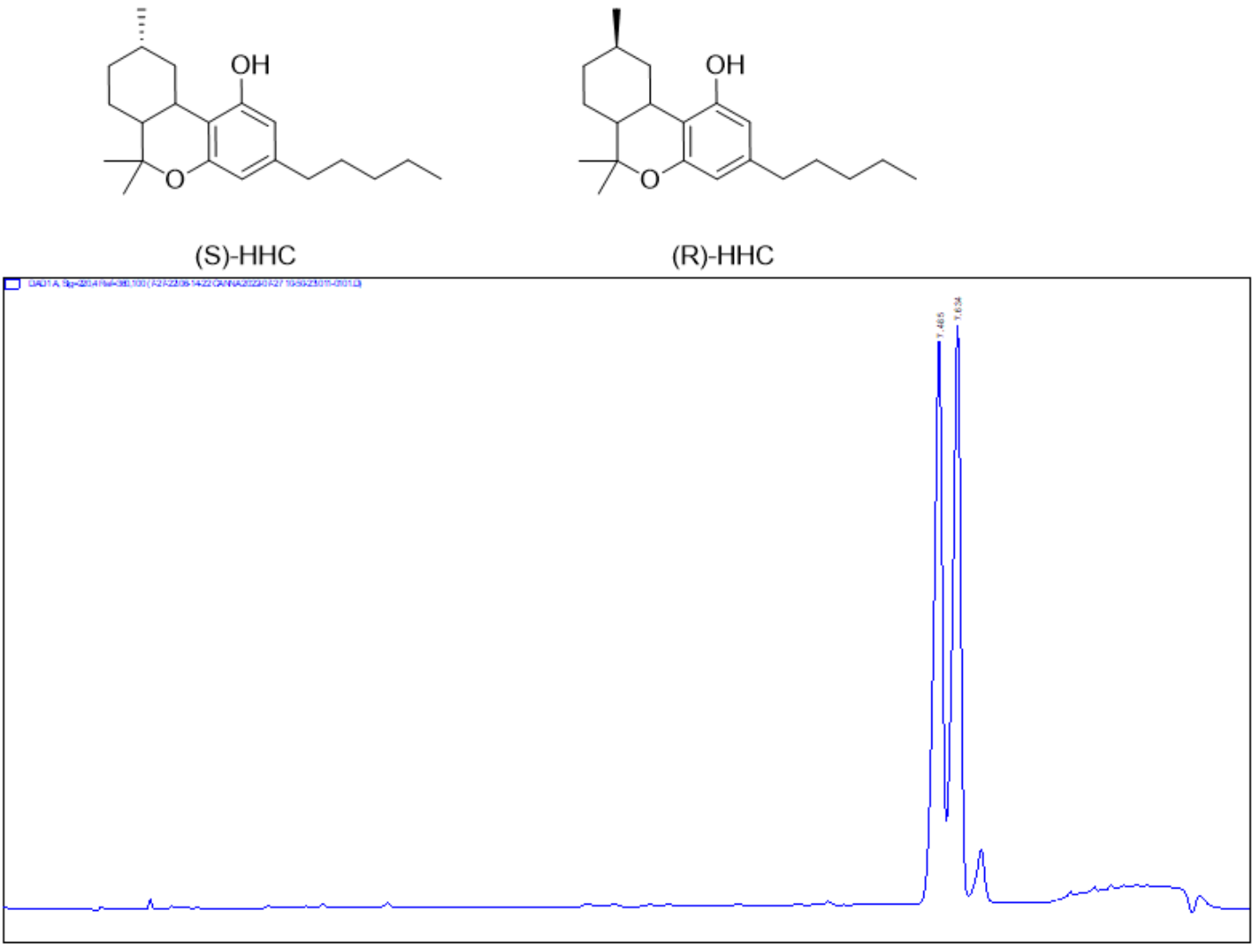

Figure 1

High Performance Liquid Chromatography (HPLC) of (R/S)-HHC

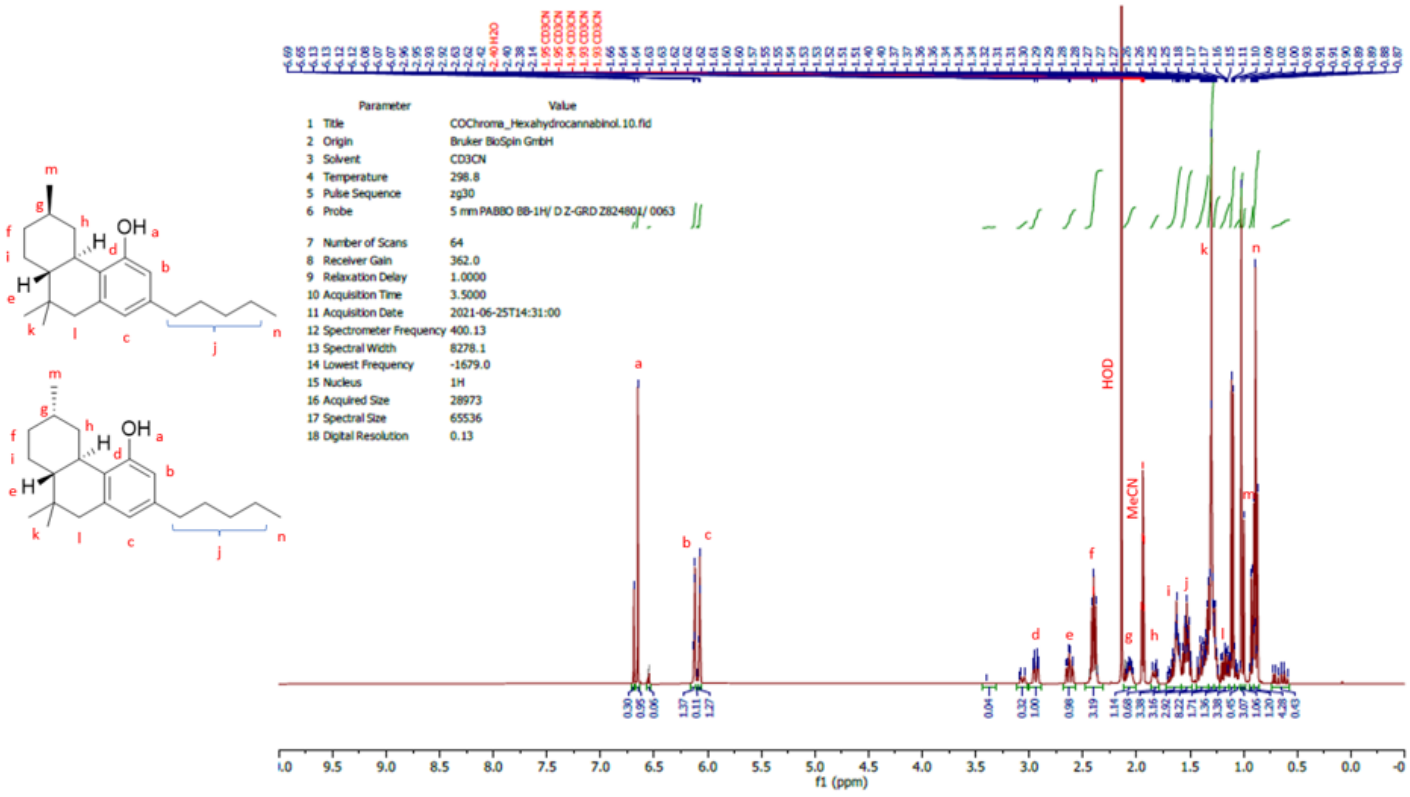

Figure 2

^{1}H NMR spectrum of (R/S)-HHC

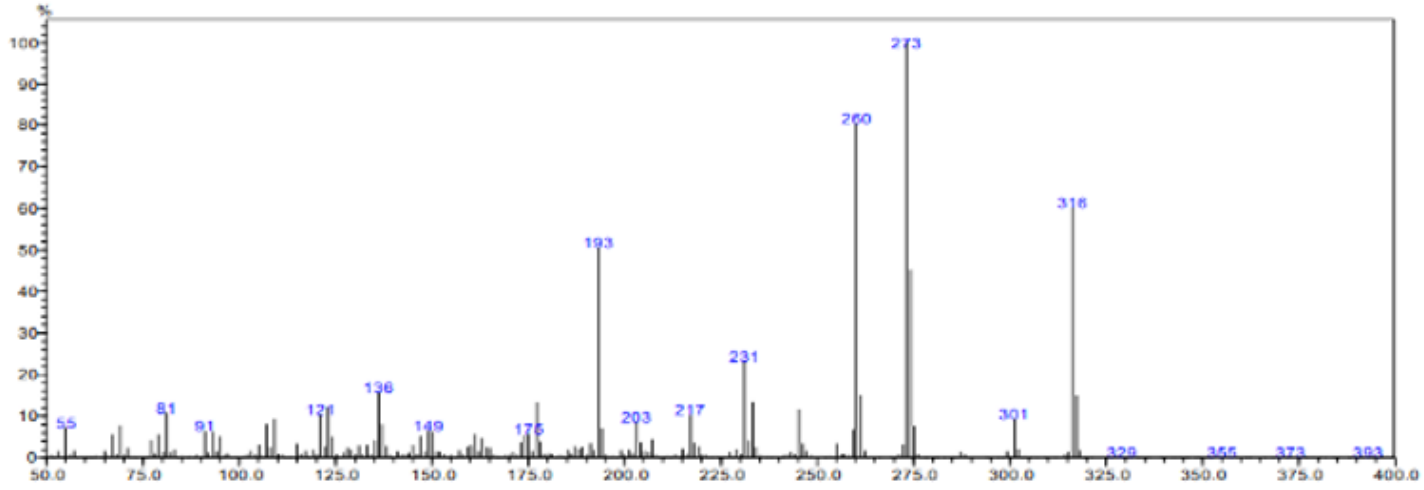

Figure 3

GC-MS data

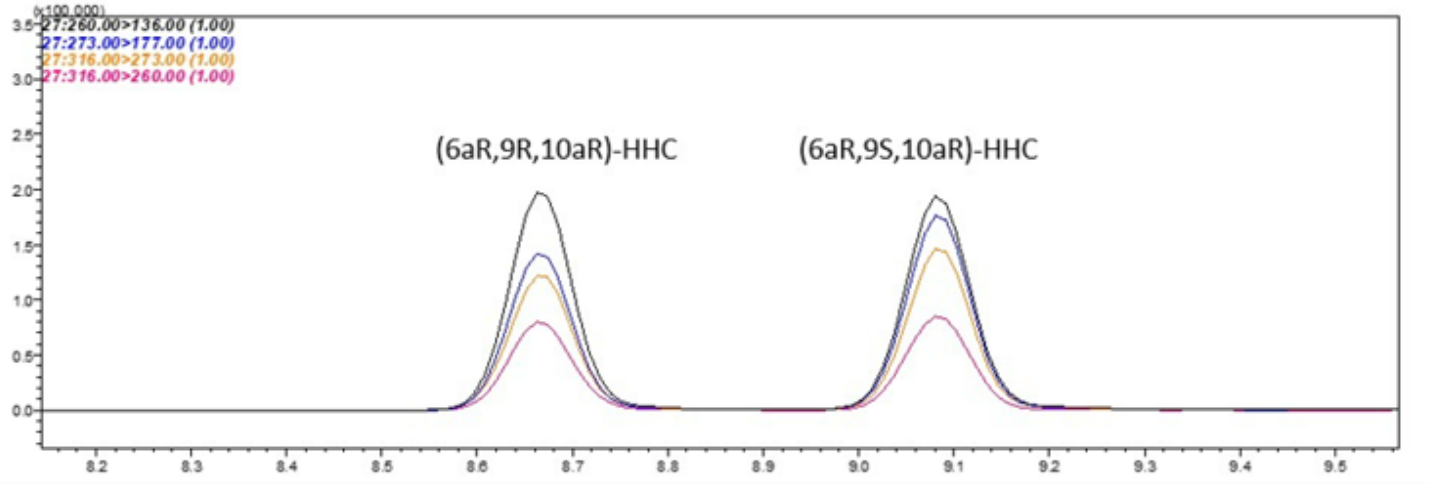

Figure 4

GC-FID analysis

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