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Comparison of Species-Specific β-Glucuronidase Hydrolysis of Cannabinoid Metabolites in Human Urine

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16. Abstract

Toxicological investigations of fatal aviation accidents include testing for Δ^9-tetrahydrocannabinol (THC), the active component of marijuana, and its major metabolite, 11-nor-9-carboxy-Δ^9-THC (THCCOOH) in postmortem fluids and tissues from deceased pilots. These cannabinoids, as well as others, exist in human urine as their glucuronide conjugates, formed during Phase II metabolism to increase their solubility for excretion. Prior to the analysis of cannabinoids in urine by gas chromatography/mass spectrometry (GC-MS), it is desirable to cleave (hydrolyze) the glucuronic acid to enhance the detection of the unconjugated compounds. In the current study, β-glucuronidases from 3 mollusk species (*Helix pomatia, Patella vulgate,* and *Haliotis rufescens*), 1 bacteria (*Escherichia coli*), and bovine liver were compared for their quantitative efficiency in hydrolyzing the glucuronide bond in spiked, blank urine.

THC and THCCOOH were selected for this project to study the hydrolysis efficiency of the various enzymes on the ester-linked THCCOOH-glucuronide and the ether-bonded THC-glucuronide. An unconjugated metabolite, 11-hydroxy-THC (11-OH-THC), was included to evaluate the effect of the various hydrolysis conditions on its recovery. The hydrolysis variables evaluated were pH, time, and temperature of incubation. Peak area ratio of analyte/deuterated internal standard was used to quantify cannabinoid recovery. All of the enzymes were able to hydrolyze both the THC and THCCOOH glucuronides, but there were significant species-specific differences. The β-glucuronidases from *Haliotis rufescens*, *Escherichia coli*, and *Patella vulgata* produced the greatest recovery of THC and THCOOH during the pH experiments.

While recovery of THCCOOH was acceptable using *Helix pomatia* and bovine liver enzymes, these glucuronidases did not hydrolyze the ether-bonded THC conjugate as efficiently as the other three. Further recovery experiments with *Haliotis rufescens*, *Escherichia coli*, and *Patella vulgata* investigated the effect of incubation time and temperature on the cannabinoid recoveries. Experimental incubation times (hours) were 0.25, 1.0, 4.0, 8.0, and 16 at optimum pH for each enzyme and 37ºC incubation temperature. Experimental temperatures (ºC) were 25, 37, 50, 60, and 90 at optimum pH for each enzyme and a 16 h incubation time. The results demonstrated that β-glucuronidases from *Haliotis rufescens*, *Escherichia coli,* and *Patella vulgata* would provide the best recovery of both ester-linked and ether-linked cannabinoid metabolites with GC-MS analysis. Further research will now analyze actual postmortem urine from THC-positive pilots involved in aviation accidents to identify a more complete metabolic profile of cannabinoids.

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Contents

Comparison of Species-Specific β-Glucuronidase Hydrolysis of Cannabinoid Metabolites in Human Urine

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INTRODUCTION

The global phenomenon of marijuana use and abuse continues to draw the attention of healthcare officials, law enforcement agencies, legislators, and the general public.[1,2] In the United States alone, for example, 5.4 million persons aged 12 or older used marijuana on a daily or almost daily basis in 2012. That same year, the states of Washington and Colorado legalized its recreational use. Other states are considering similar legislative efforts. Internationally, cannabis use is common in many parts of the world, and the lucrative business of cannabis cultivation was demonstrated in Afghanistan, where revenues recently surpassed earnings from the opium poppy harvest.[2,3]

Marijuana is self-administered, usually via inhalation, for the well-studied feelings of euphoria, but it also has a variety of additional pharmacodynamic effects, including decrements in memory and psychomotor performance.[4] As a result of marijuana affecting behavioral and psychomotor functions, forensic toxicology laboratories around the world test for one or more cannabinoids in a variety of biological specimens. The interpretation of the results from postmortem cases relies not only on specific case information (subject behavior, scene investigation, etc.), but also on the pharmacodynamic and pharmacokinetic data available to the toxicologist.

As the primary psychoactive constituent of the cannabis plant, Δ^9-tetrahydrocannabinol (THC) has been the primary focus of years of metabolic research. In humans, THC undergoes Phase I metabolism in the liver via cytochrome P450 (CYP) 2C9 and 2C19 isoenzymes.[5] The predominate metabolites produced in humans are 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH). Phase II metabolism in humans, catalyzed by the UDP-glucuronosyltransferase family of enzymes, produces glucuronide conjugates of THC and its metabolites including 11-OH-THC and THCCOOH. THCCOOH and its glucuronide have been identified as the major metabolic end products of THC metabolism in man.[4] THC and THCCOOH in blood and THCCOOH in urine are most often the compounds of interest when cannabinoid testing is performed. Recent research has brought attention to the usefulness of including 11-OH-THC and other cannabinoids, e.g., cannabidiol, to gain a more comprehensive understanding of the pharmacokinetic profile of cannabis.[6,7]

The detection of cannabinoids in biologic fluids by gas chromatography/mass spectrometry (GC-MS) is a common analytical method for the detection of cannabinoids. It requires extracting the cannabinoids of interest from the biological matrix followed by derivatization to improve detectability. To enhance the recovery of the THC, THCCOOH, and 11-OH-THC by GC-MS, many forensic laboratories hydrolyze the glucuronide conjugates, resulting in increased concentrations of the free forms of these compounds. Hydrolysis prior to extraction improves the chromatographic performance of the cannabinoids and extends the time window for detection of the cannabinoids.

The hydrolysis of the ester-linked glucuronide of THCCOOH is easily accomplished: chemically, with the addition of base to the sample or enzymatically, via incubation with β-glucuronidase. Alkalization is most frequently employed due to cost and time constraints, but this method will only have an effect on the THCCOOH-glucuronide. The ether-bonded glucuronides of THC and 11-OH-THC are not susceptible to alkaline conditions. Therefore, for a more comprehensive analysis of the metabolic pattern of THC by GC-MS, enzymatic hydrolysis with β-glucuronidase is necessary.

β-glucuronidases are available from multiple natural sources, including mollusks, bacteria, and bovine liver. Studies investigating cannabinoids and morphine have shown that the degree of activity for each enzyme is species-specific and is affected by multiple factors, including the concentration of enzyme applied to the sample, the pH of the reaction, incubation temperature, and incubation time.[10-12] The pH for optimal hydrolytic activity for commercially available β-glucuronidases may be obtained from the manufacturers.[8,9] These values, however, were determined by testing the ability of each enzyme to liberate phenolphthalein from phenolphthalein-glucuronide. It has been shown that optimal conditions may differ for other compounds.[12] Based upon previous research performed by Kemp et al., the current study was undertaken to better understand the effect of species specific β-glucuronidases on cannabinoid glucuronide conjugate hydrolysis.[11] Other researchers have compared methods of hydrolysis for a variety of drugs and metabolites, including benzodiazepines and opiates, as well as cannabinoids.[10, 12, 16, 17]

One of the primary missions of the Bioaeronautical Sciences Research Laboratory (BSRL) at the Civil Aerospace Medical Institute is to perform toxicological investigations on pilots fatally injured in aviation accidents.[13] Numerous tests are performed to assist investigators with determining the cause of an aviation accident. As a result, we have a unique opportunity to study the distribution of drugs, including THC, its metabolites, and other cannabinoids, in a wide variety of postmortem fluids and tissues. These data can then be disseminated, not only to the aviation community, but also to the forensic science community, for the purpose of characterizing the patterns of drug use by pilots and its effect on performance.

This research describes the development and optimization of the initial hydrolysis procedure necessary for detecting THC and THCCOOH in postmortem urine. We do not currently analyze for THC in urine, so this procedure will enhance the analytical capabilities of the laboratory while expanding the knowledge of THC metabolism and distribution. β-glucuronidases from a variety of animal species were tested to determine the optimum

pH, temperature, and incubation time necessary for maximizing THC, 11-OH-THC, and THCCOOH recovery. Of particular interest is the effect of changing hydrolysis parameters on the recovery of THC, the primary psychoactive component of marijuana.

This procedure will be applied to future research on other fluids (e.g., vitreous fluid, bile, cerebrospinal fluid) and tissues to investigate the postmortem distribution of cannabinoids in the human body. These types of drug distribution studies are critical for reducing uncertainty in medicolegal death investigations.[14]

METHODS AND MATERIALS

Chemicals and Reagents

Methanolic, deuterated THC, THCCOOH, and 11-OH-THC (100 µg/mL), as well as methanolic, non-deuterated THCCOOH-glucuronide and 11-OH-THC (100 µg/mL) were obtained from Cerilliant Corporation (Round Rock, TX). THC-glucuronide (10 µg/mL) was purchased from ElSohly Labs (Oxford, MS). The reagents glacial acetic acid and hexane were obtained from Fisher Scientific (Pittsburgh, PA), and ethyl acetate was purchased from Varian, Inc. (Palo Alto, CA). Sodium phosphate monobasic and dibasic powders and β-glucuronidase enzymes from limpet (*Patella vulgata;* PV), bacteria (*Escherichia coli;* EC), mollusk (*Helix pomatia;* HP), and bovine liver (BL) were acquired from Sigma Aldrich (St. Louis, MO). β-Glucuronidase from the red abalone sea snail (*Haliotis rufescens;* HR) was obtained from Kura Biotec (Inglewood, CA). The derivatizing agent, *N,O*-Bis(trimethylsilyl) trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA+1%TMCS), was obtained from United Chemical Technologies, Inc. (UCT; Bristol, PA). Drug-free human urine was obtained from UTAK Laboratories, Inc. (Valencia, CA). The pH of solutions was measured with a Thermo Electron Corporation Orion pH meter (model 520A+; Waltham, MA) connected to an Accumet Electrode from Fisher Scientific (Pittsburgh, PA).

Urine, Buffer, and Enzyme Preparation

Drug-free urine was fortified with 50 ng/mL THC-glucuronide (equivalent to 32 ng/mL THC), 100 ng/mL THCCOOH-glucuronide (equivalent to 66 ng/mL THCCOOH), and 50 ng/mL 11-OH-THC. Phosphate buffers, with varying pH ranging from 3.0-7.0 at 0.5 increments, were prepared at a concentration of 0.1 M by mixing prepared stock solutions of 0.2 M sodium dibasic phosphate and 0.2 M sodium monobasic phosphate. Enzymes were prepared in deionized water to achieve an approximate concentration of 5,000 units of enzyme per 200 µL. The enzymes were placed into a snap-cap centrifuge tube and stored frozen (-20˚C).

Enzymatic hydrolysis

The first hydrolysis parameter evaluated was pH. One mL urine samples (already spiked with THC-glucuronide, 11-OH-THC, and THCCOOH-glucuronide, as described above) were placed in 16 x 100-mm disposable glass culture tubes. This was followed by the addition of 1 mL of pH-targeted buffer (pH range: 3.0 to 7.0 in 0.5 increments). If necessary, the samples were adjusted with glacial acetic acid to achieve the desired pH. To each tube, 100 ng of deuterated internal standards (THC-D_3, 11-OH-THC-D_3, and THCCOOH-D_3) and 200 µL (5,000 units) of β-glucuronidase enzyme were added. The enzyme concentration was maintained at 5,000 Fishman units/sample for all experiments, as this enzyme concentration was previously shown to be appropriate for cannabinoid hydrolysis.[10, 19]

Tubes were capped, vortexed, and placed into a heated, shaking water bath for hydrolysis. The samples were incubated at 37˚C for 16 h for pH experiments. All samples were analyzed in triplicate for each pH data point. Recoveries of THC, 11-OH-THC, and THCCOOH were determined using peak area ratios (analyte/deuterated internal standard).

Incubation time and temperature were optimized using the pH that returned the maximum recovery of the cannabinoids with PV (pH 5.0), HR (pH 5.0) and EC (pH 6.0). For the timed experiments, the samples were prepared in triplicate and incubated in a heated (37ºC) water bath with shaking for 0.25, 1, 4, 8, and 16 h. Temperature was evaluated by incubating the samples for 16 h at 25, 37, 50, 60, and 90˚C with shaking. Peak area ratios were used for evaluating recovery and determining the effect of incubation time on hydrolysis.

Liquid-liquid extraction

Extraction of THC, 11-OH-THC, and THCCOOH from urine was accomplished using previously reported method modified for this study.[15] The samples were removed from the water bath at the appropriate time and allowed to cool to room temperature. To the tubes,1 mL of 1M phosphate buffer (pH 4) was added. They were vortexed and 5 mL of hexane: ethyl acetate (5:1) was added. The tubes were placed onto a rotary mixer for 10 min. and then centrifuged at 3,000 rpm for 5 min. The organic layer was transferred to clean conical tubes. To maximize recovery, another 5 mL of hexane:ethyl acetate (5:1) was added to the remaining aqueous layer, followed by mixing and centrifugation. The two extracts were combined in a clean test tube and evaporated to dryness with a gentle stream of nitrogen at 40˚C. Derivatization was performed by adding of 50 µL of BSTFA + 1% TMCS and placing of all tubes into a dry heat block for 20 min at 90˚C. The samples were then transferred to a glass autosampler vial for injection onto the GC-MS.

Instrumentation

The instrument utilized for this study was a 6890 gas chromatograph equipped with a 5973 mass selective detector and a 7683 autosampler (Agilent Technologies; Santa Clara, CA). The column used was a 100% methylsiloxane capillary column with 0.2 mm I.D. x 12 m length x 0.33 µm film thickness. The carrier gas was ultra-high purity helium. The MSD was operated

in electron ionization mode with selected ion monitoring for the trimethylsilyl derivatives of the three analytes and their deuterated internal standards. The GC and MS parameters are listed in Table 1. The total time for the run was 8.0 min.

RESULTS AND DISCUSSION

As a result of Phase II metabolism, THC and its metabolites, both hydroxylated and carboxylated, are excreted in urine primarily as glucuronides and sulfates. Previous research has suggested that as much at 75% of the urinary metabolites of THC are conjugated.[18] The current study was undertaken to optimize the initial glucuronide hydrolysis step prior to subjecting the sample to liquid-liquid extraction and GC-MS analysis. Glucuronidases from five different species (PV, EC, HR, BL, HP) were evaluated. Three hydrolysis parameters were investigated: pH of the incubation solution, incubation temperature, and incubation time. Figures 1 - 9 show the results of the experiments evaluating the effect of these variables on the hydrolysis activity of β-glucuronidase from PV, BL, HP, EC, and HR. As expected, there were differences found in the ability of the species-specific β-glucuronidases to hydrolyze cannabinoid glucuronides.

pH Experiments

A significant difference in the recovery of THC from hydrolysis of the THC-glucuronide, as determined from peak area ratios (analyte/internal standard), was observed between the five enzymes tested. HR, PV, and EC β-glucuronidase demonstrated superior hydrolytic performance compared to BL and HP (Figure 1). Triplicate analyses showed a maximum mean peak ratio of 0.88 for HR at pH 5.0. Maximum mean peak area ratios for EC and PV were 0.77 at pH 6.0 and 0.57 at pH 5.0, respectively. The peak area ratios for BL and HP only reached maximums of 0.23 and 0.14, respectively. These results are consistent with other studies that have demonstrated the superiority of PV, EC, and HR for hydrolyzing

Table 1. GC-MS instrument conditions

Injector	
Flow mode	Constant flow
Injection mode	Splitless
Injection volume	2 µL
Inlet temperature	250˚C
Pressure	13.0 psi
Purge flow	2.0 mL/min
Purge time	0.50 min
Total flow	5.9 mL/min

Oven Parameters		
	Temp (˚C)	Hold time (min)
Initial	150	
Ramp @ 35˚C	200	
Ramp @ 23˚C	250	
Ramp @ 5˚C	255	2.0
Ramp @ 15˚C	270	0.4
Transfer Line	280	

MS Conditions		
	Ions, m/z*	Retention Time
THC	**371**, 386, 303	4.3 min
d_3-THC	**374**, 389, 306	
11-OH-THC	**371**, 459, 474	5.7 min
d_3-11-OH-THC	**374**, 462, 477	
THCCOOH	**371**, 473, 488	6.8 min
d_3-THCCOOH	**374**, 476, 491	

***Quantitation ions are bolded**

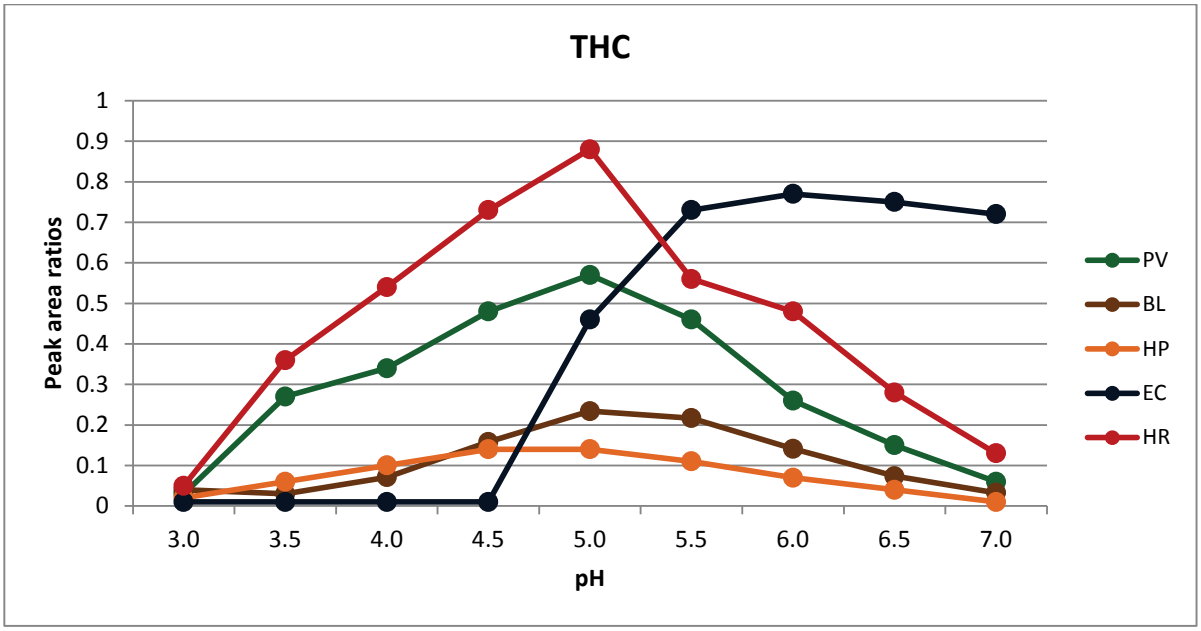

Figure 1. Effect of altering pH on the ability of β-glucuronidase enzymes to hydrolyze THC-glucuronide.

glucuronide conjugates. For example, a 1995 study by Kemp and associates showed that the EC glucuronide hydrolyzed THC-glucuronide more efficiently than either base or HP methods.[11] When compared to other glucuronidases, PV was shown to produce a greater recovery of morphine from morphine-glucuronide.[12] HR was found to be effective in hydrolyzing cannabidiol-glucuronide, benzodiazepines, and synthetic cannabinoids.[10,16]

Interestingly, the EC β-glucuronidase hydrolytic activity was greatly reduced for the lower pH range. Very low mean peak area ratios were found at pH 3.0, 3.5, 4.0, and 4.5. At pH 5.0, the mean peak area ratio jumped to 0.46, climbing to the maximum 0.77 at pH 6.0. In addition, none of the other enzymes showed significant hydrolytic activity for THC-glucuronide at pH 3.0, with area ratios ranging from 0.01 for EC to 0.05 for HR. The inactivity at low pH was also detected with THCCOOH-glucuronide (Figure 3). Greatly reduced recovery of THCCOOH was obtained with EC at pH 3.0, 3.5, and 4.0, improving at pH 4.5. These results would suggest that EC hydrolytic activity is not compatible with the acidic pH range of 3.0 to 4.5. While the cause of this reduction in activity was not conclusively identified, pH can have an effect on the ionization state of amino acids, thus potentially altering the shape of the protein structure leading to altered recognition of the cannabinoid substrate.

Analyzing urine samples from human subjects after acute exposure to smoked cannabis, a previous study demonstrated that there are species differences in the ability of β-glucuronidases to hydrolyze 11-OH-THC-glucuronide.[11] Since there are no commercial sources for 11-OH-THC-glucuronide, it was impossible to assess the hydrolysis activity of the enzymes for this conjugate with controlled concentrations. Instead, unconjugated (free) 11-OH-THC served as an extraction control to evaluate any effects of the various study conditions on the recovery of this important THC metabolite. The initial experiments evaluating the effect of pH suggested that, while there were some differences between enzymes, they were minimal and there was no effect of the various hydrolysis parameters on free 11-OH-THC recovery (Figure 2). Of note, also seen in subsequent experiments for time and temperature, is that the recovery of the free 11-OH-THC was generally lower for HR across the pH range tested. The recoveries were consistent within species across all pH conditions tested. Mean peak areas for each species were 0.32, 0.28, 0.20, 0.24, and 0.16 for PV, BL, HP, EC, and HR, respectively. HR showed the greatest extraction variability, with a CV of 16.9 across the pH range of 3.0 to 7.0. The reason for the lower and more variable recoveries was not immediately apparent.

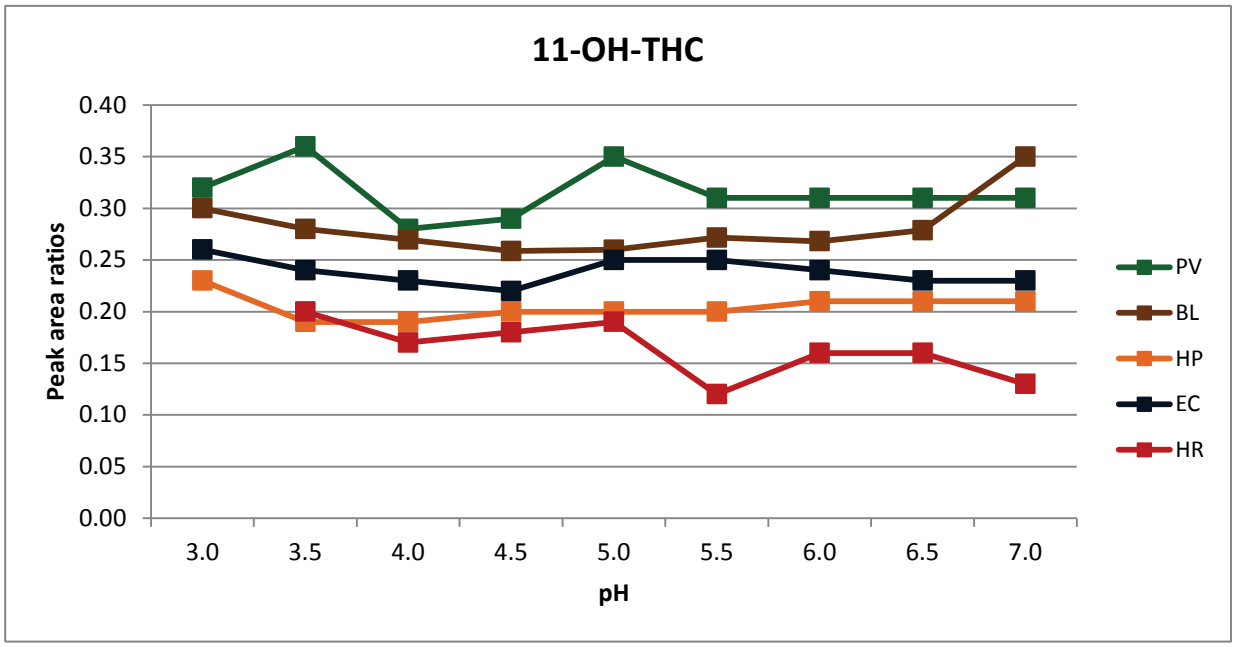

Figure 2. Effect of altering pH and β-glucuronidase enzymes on free 11-OH-THC recovery.

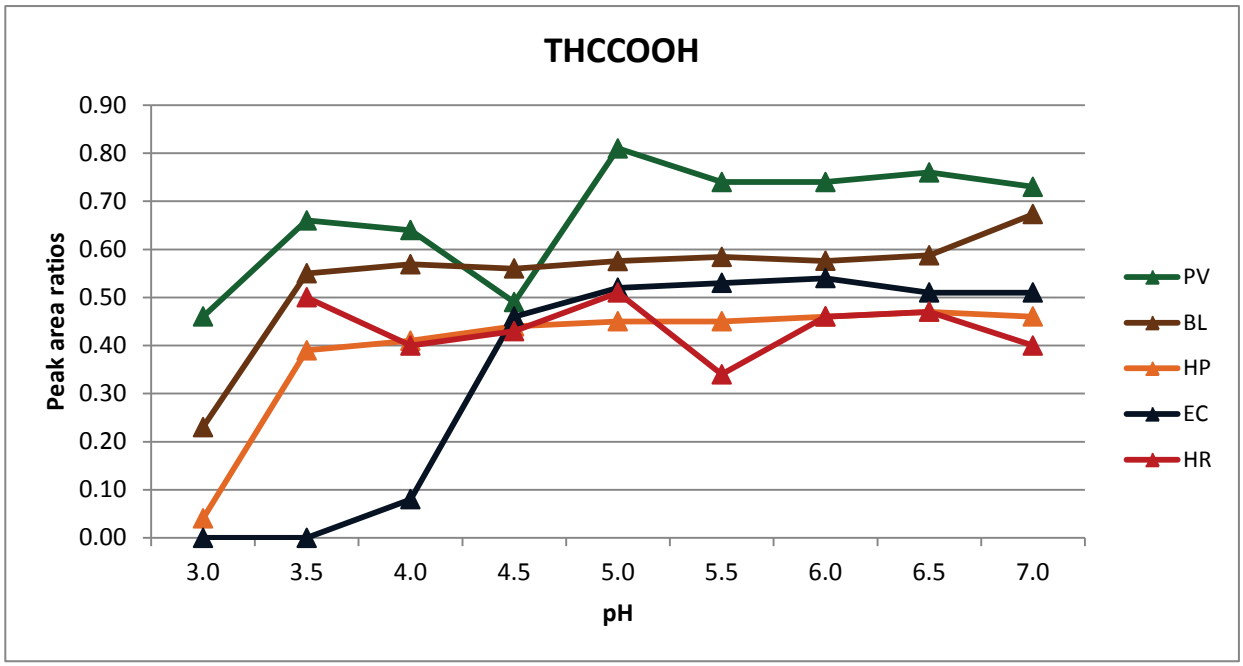

Figure 3. Effect of altering pH on the ability of β-glucuronidase enzymes to hydrolyze THCCOOH-glucuronide.

Figure 3 shows the recovery data for THCCOOH for each change in pH. Maximum peak area ratios for THCCOOH recovered from the hydrolysis of THCCOOH-glucuronide were found with PV (0.81 at pH 5.0). Recovery of THCCOOH remained higher with PV (mean peak areas ranging from 0.73 to 0.76) through pH 7.0. The HR specimens at pH 3.0 were destroyed during analysis, thus, no results were available for this data point. Overall, HR and the other glucuronidases produced similar recoveries of THCCOOH. As with the THC-glucuronide, the EC glucuronidase was far less active in the more acidic environment with little recovery of THCCOOH from pH 3.0 to 4.0. At pH 3.0, the enzymes from all species were reduced in the ability to hydrolyze THCCOOH-glucuronide.

As a result of the initial findings in the pH study and the findings of previous studies outlined above, we concluded that BL and HP would not provide the most efficient hydrolysis of all three cannabinoids studied, particularly THC-glucuronide. They were, therefore, excluded from further testing in this project. PV, EC, and HR were evaluated further in subsequent hydrolysis optimization experiments. The pH of the hydrolysis reaction for each enzyme was set at the pH where the optimal peak area ratio was found (PV, HR at pH 5.0 and EC at pH 6.0). These pH settings were chosen for the purpose of recovering the greatest amount of THC from THC-glucuronide, while simultaneously recovering easily detectable quantities of 11-OH-THC and THCCOOH. Previous research has shown that 11-OH-THC-glucuronide is hydrolyzed in a similar fashion to THC-glucuronide.[11] In addition, THCCOOH-glucuronide is present in significant concentrations in marijuana users and is easily hydrolyzed by the addition of strong base or by enzymatic methods due to the less stable ester bonding of the glucuronide. The selection of hydrolysis parameters, therefore, will not greatly affect detection of this metabolite.

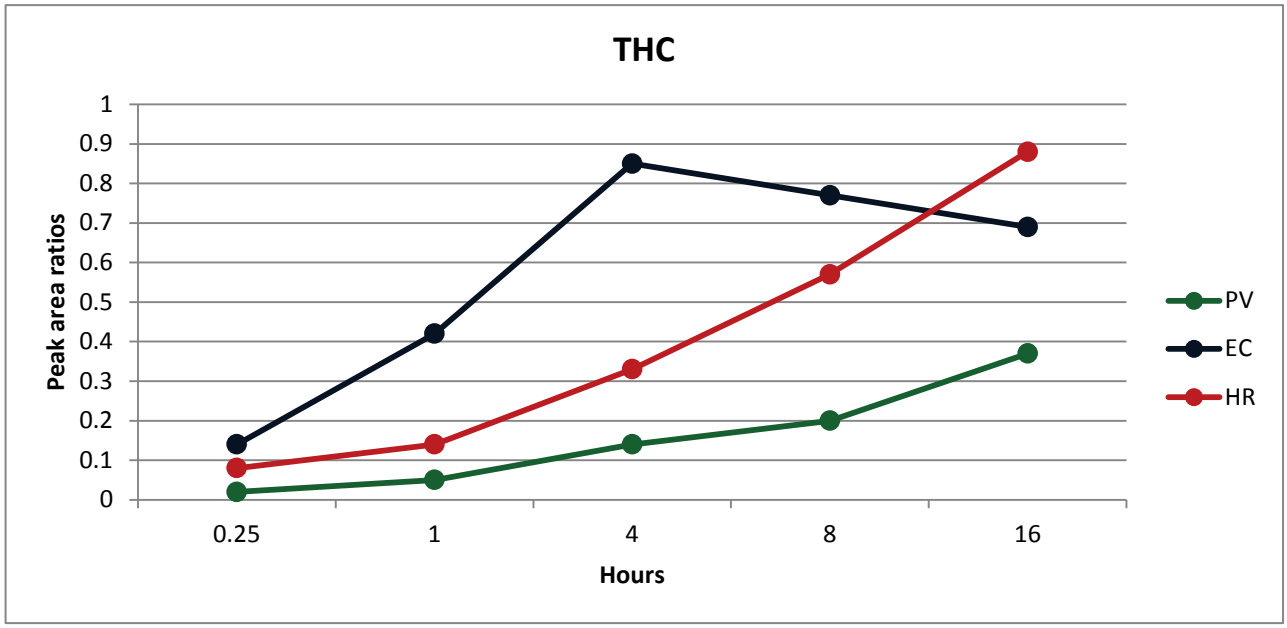

Figure 4. Effect of incubation time (hours) on the ability of β-glucuronidase enzymes to hydrolyze THC-glucuronide.

Incubation Time Experiments

The length of time for optimal hydrolysis activity was evaluated for PV, EC, and HR at 37°C and their optimal pH, as determined by the previous experiments. EC was incubated at pH 6.0, PV and HR at pH 5.0. The hydrolytic activity, as measured by peak area ratios, showed that EC and HR returned similar results for THC recovery, but at different time points (Figure 4). Recoveries of THC were lowest at the shortest hydrolysis time point of 0.25 h (15 min). Recoveries then climbed with longer incubation times. The maximum mean THC ratio using EC (0.85) occurred at the 4-h incubation time point. The maximum with HR (0.88) occurred at 16 h, the longest incubation time measured. The PV maximum peak area ratio of only 0.37 also occurred at the 16-h time point. At the 4-h time point, where the THC recovery ratio was maximal for EC at 0.85, the peak area ratios for PV and HR were only 0.14 and 0.33, respectively. The more rapid 4-h incubation time for EC at 37°C provides a method for completing hydrolysis and extraction of the cannabinoids in a single day, reducing turnaround times for casework.

The THC results with HR, as well as those for PV, were still trending up at 16 h, and it is possible that a higher recovery could occur with a longer incubation time at 37°C (Figure 4). Longer incubation times, however, are more difficult to manage in the laboratory. The 16-h period allowed for overnight incubation and fits well into the routine laboratory schedule. Longer times would be more difficult to schedule and may be disruptive to case turnaround times. The 4-h incubation time with EC (at pH 6.0, 37°C) would allow for hydrolysis and extraction in the same day and data analysis the next day after overnight data acquisition. Interestingly, recovery of THC and THCCOOH from their respective glucuronides, with EC hydrolysis, began to decline after the 4-h time point, with mean peak area ratios ranging from 0.85 to 0.69 for THC and 0.51 to 0.39 for THCCOOH. The reason for this decrease is unclear.

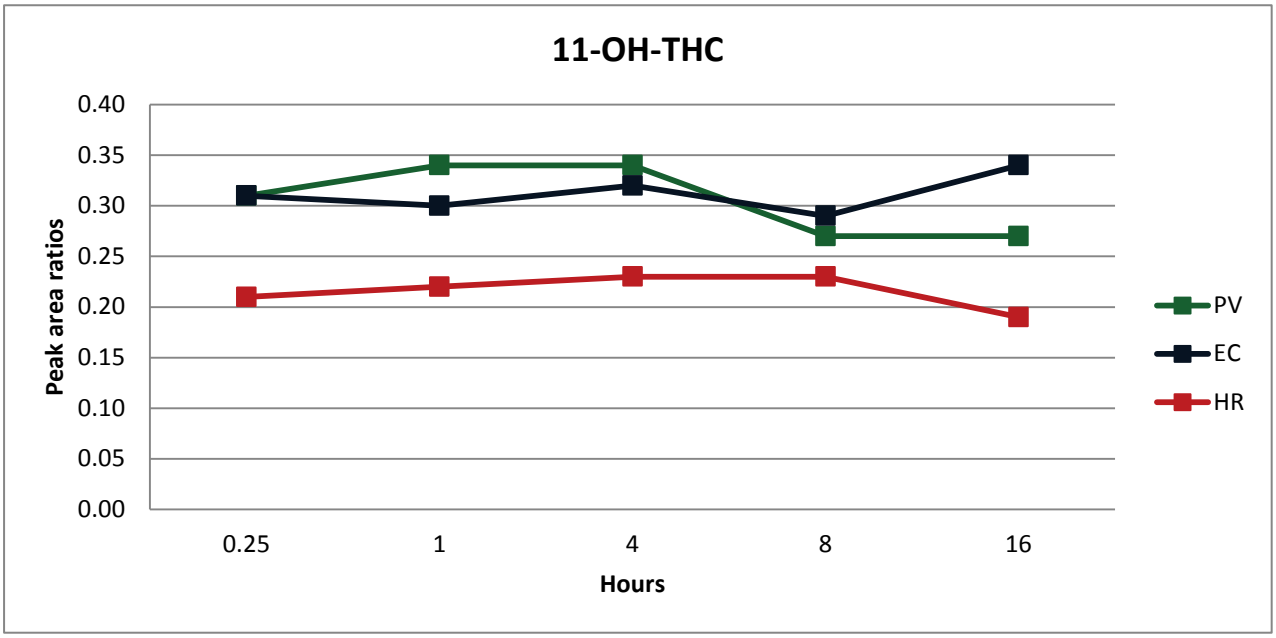

Figure 5. Effect of altering incubation time (hours) and β-glucuronidase enzymes on free 11-OH-THC recovery.

The recovery of 11-OH-THC from spiked blank urine remained consistent for each of the enzymes tested (PV, EC, HR) over the 16-h testing period (Figure 5). As with the pH experiments, the lower recovery of free 11-OH-THC was observed across all time points when HR was used for the hydrolysis reaction. Mean recoveries from triplicate analyses for PC and EC were identical, at 0.31; recovery of 11-OH-THC using HR was somewhat lower, at 0.21.

There was no effect of incubation time, at optimum pH and at 37°C, on the peak area ratios obtained for THCCOOH (Figure 6). Recovery of THCCOOH from hydrolysis of its glucuronide conjugate was stable across all three enzymes tested, ranging from a mean peak area ratio of 0.47 for PV and EC to 0.53 for HR.

Incubation Temperature Experiments

The final hydrolysis parameter evaluated was the temperature of the incubation reaction. The PV, EC, and HR enzymes were tested at their optimal pH as determined in the experiments described above (PV and HR at pH 5.0; EC at pH 6.0). The time of incubation was 16 h. Incubation temperatures (°C) tested were 25, 37, 50, 60, and 90.

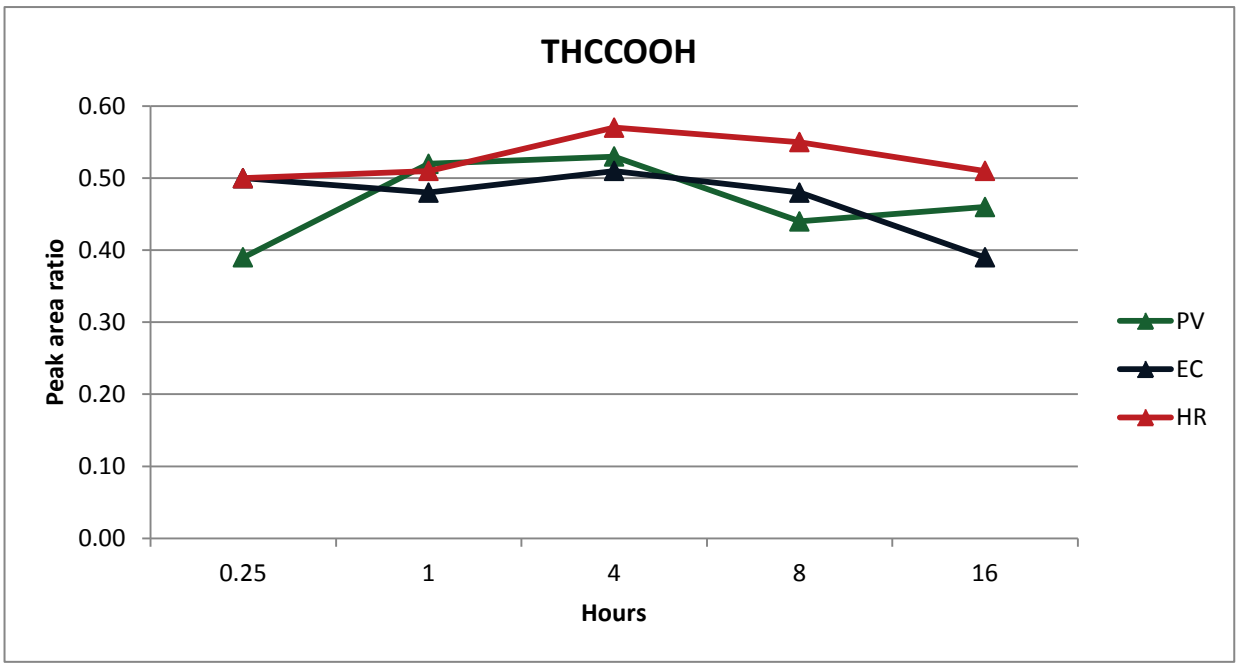

Figure 6. Effect of incubation time (hours) on the ability of β-glucuronidase enzymes to hydrolyze THCCOOH-glucuronide.

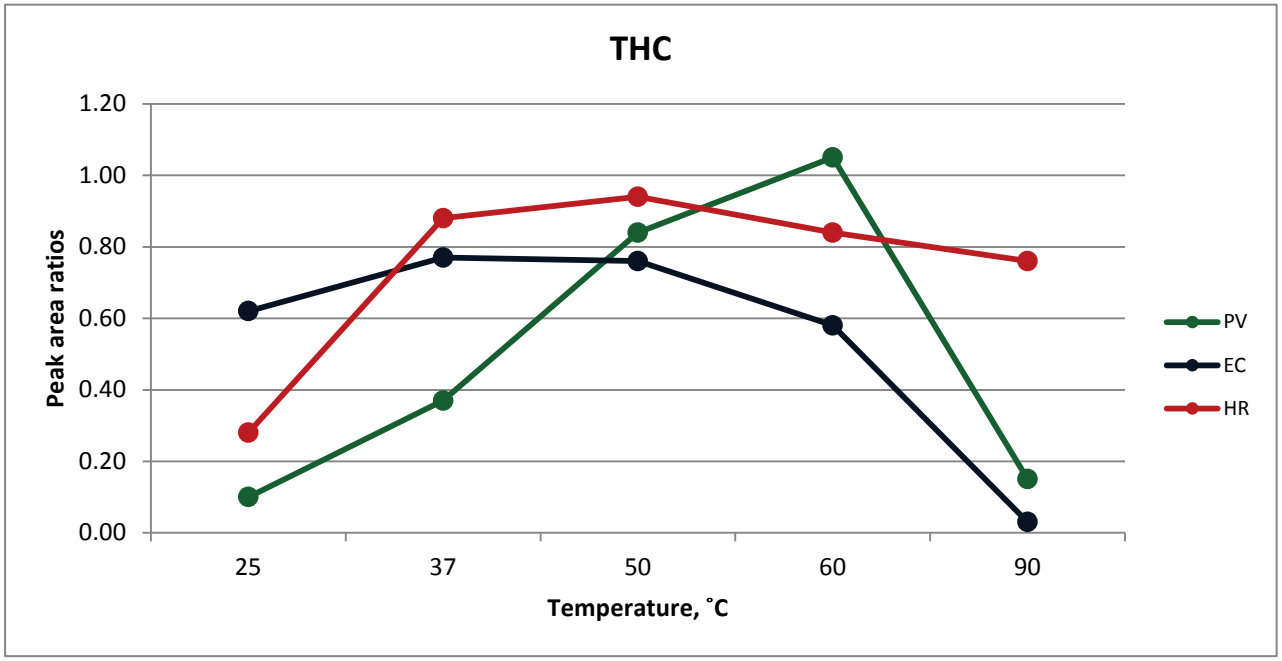

Figure 7. Effect of incubation temperature on the ability of β-glucuronidase enzymes to hydrolyze THC-glucuronide.

As can be seen in Figure 7, temperature had a significant effect on THC recovery. The lowest reaction temperature (25°C) indicated some hydrolytic activity was present for all three enzymes, with the greatest mean peak area ratio produced by EC (0.62). Using the PV glucuronidase, THC recovery steadily increased from a peak area ratio of 0.1 at 25°C to a mean ratio of 1.05 at 60 °C. PV hydrolytic activity on the THC-glucuronide was greatly reduced at the 90°C temperature, as indicated by the low peak area ratio of 0.15. Peak area ratios for THC, using EC, increased from 0.62 at 25°C to 0.77 at 37°C, remaining steady at 0.76 at 50°C, and decreasing to 0.58 at 60°C. HR β-glucuronidase showed similar results to EC in that incubation with the lowest temperature resulted in less recovery of THC. The mean THC/THC-d_3 peak area ratio was 0.28 at 25°C. The ratio increased to 0.94 at 50°C and persisted near that recovery for remainder of the temperature study. The ratios were 0.84 and 0.76 for the 60°C and 90°C incubation temperatures, respectively.

The greatest amount of activity for THC-glucuronide hydrolysis occurred across the range of 37°C to 60°C. The HR enzyme returned a high recovery of THC over a broader range of temperatures. HR was the only enzyme that withstood the highest temperature tested for THC-glucuronide hydrolysis (90°C). PV and EC hydrolysis activity declined significantly at the highest temperature of 90°C. The EC hydrolysis of THC-glucuronide was greatly reduced at 90°C (peak area ratio, 0.03). Future research on the 30°C gap between the last two temperature points to more accurately determine the optimal reaction temperature may prove useful to laboratories choosing to use PV for cannabinoid hydrolysis.

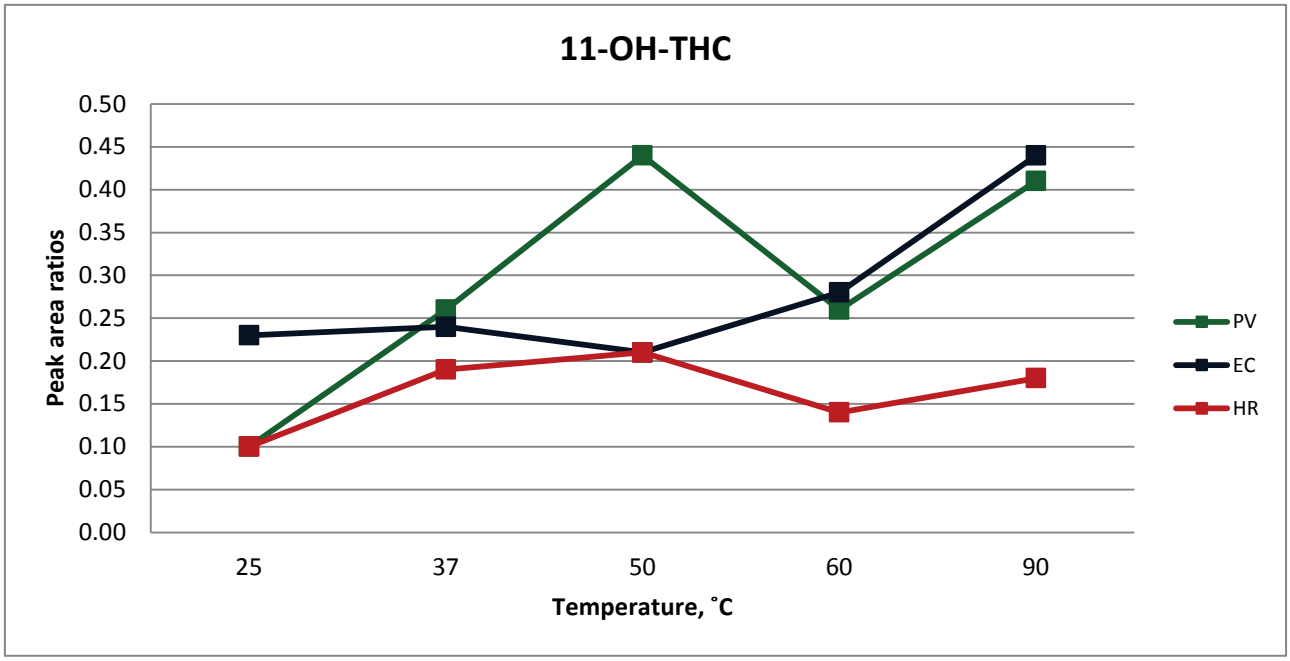

Figure 8. Effect of altering incubating temperature and β-glucuronidase enzymes on free 11-OH-THC recovery.

Temperature appeared to have an effect on 11-OH-THC recovery, as measured by peak area ratios (Figure 8). Free 11-OH-THC was relatively stable using EC with peak area ratios of 0.23, 0.24, 0.21, and 0.28 at the hydrolysis temperatures of 25°C, 37°C, 50°C, and 60°C, respectively. Interestingly, at 90°C the recovery of 11-OH-THC appeared to increase, as indicated by a peak area ratio of 0.44. Mean peak area ratios for 11-OH-THC with PV varied from 0.1 at 25°C to 0.44 at 50°C, showing significant variability between the hydrolysis incubation temperatures. Results with HR, including the 90°C temperature, showed more stability for 11-OH-THC recoveries, but the ratios were smaller across the temperature range studied. The peak area ratios were 0.1, 0.19, 0.21, 0.14, and 0.18 at 25°C, 37°C, 50°C, 60°C, and 90°C, respectively. We noted that, for all three enzymes, the concentration of 11-OH-THC demonstrated more variability over the course of the experiments. Mean coefficients of variation (%CV) were 10.1 (range: 5.3 – 16.9), 8.5 (range: 6.2 – 11.5), and 35.5 (range: 26.8 – 46.5) for the pH, time, and temperature experiments, respectively. Further research is needed to elucidate the reason for this variability in 11-OH-THC recovery.

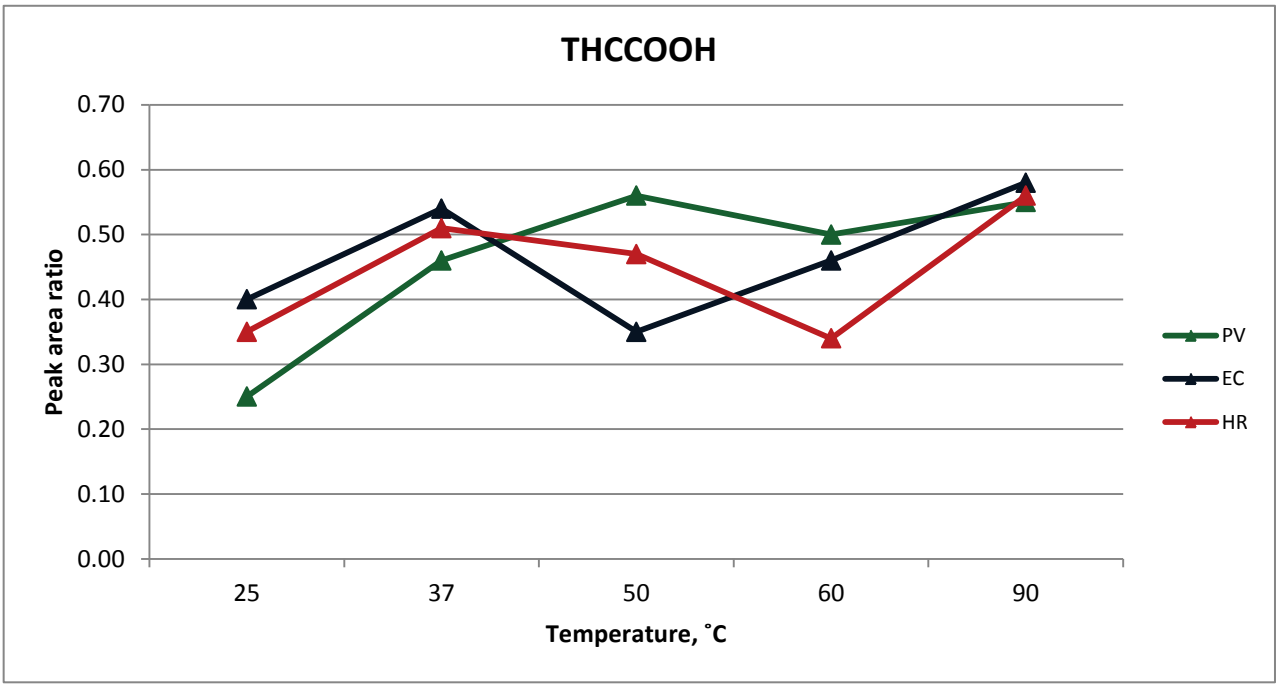

Figure 9. Effect of incubation temperature on the ability of β-glucuronidase enzymes to hydrolyze THCCOOH-glucuronide.

As shown in Figure 9, there was no significant temperature effect on the recovery of THCCOOH from THCCOOH-glucuronide. Post-hydrolysis concentrations of THCCOOH were relatively stable across the temperature range studied. Following incubation with PV, THCCOOH peak area ratios increased from a low of 0.25 at 25°C to a high ratio of 0.56 at 50°C. The ratios remained steady for the 60°C and 90°C (0.50 and 0.55, respectively). EC produced peak area ratios ranging from a low of 0.35 peak area ratio at 50°C to a high of 0.58 at 90°C. The results for THCCOOH using HR were consistent with those of both EC and PV with peak area ratios of 0.35, 0.51, 0.47, 0.34, and 0.56 at 25°C, 37°C, 50°C, 60°C, and 90°C, respectively. These results are consistent with the previous study by Kemp et al., which demonstrated that at optimal pH, THCCOOH-glucuronide is easily hydrolyzed by multiple methods as a result of the less stable ester bond.[11]

PV, EC, and HR were more effective than BL or HP in hydrolyzing ether-bonded THC-glucuronide. We concluded that, under the conditions of this study, BL and HP were not the ideal enzyme choice for hydrolyzing the ether-bonded glucuronides (e.g., THC and 11-OH-THC) but would provide acceptable recoveries for the less stable ester-bonded glucuronides (e.g., THCCOOH). Results with PV, EC, and HR suggest that these enzymes would provide an efficient means of hydrolyzing the broadest range of cannabinoid glucuronides at their optimum incubation time, temperature, and pH.

Future research evaluating hydrolysis methods will focus on actual cases from aviation accidents to confirm the findings from the current study in real cases. The expectation is that this work will result in an analytical method that will effectively evaluate a broad array of cannabinoids and their metabolites in biological fluids and tissues.

CONCLUSIONS

Five biological sources of β-glucuronidase were tested for their capability to hydrolyze THC-glucuronide and THCCOOH-glucuronide under three varying incubation conditions (pH, incubation time, incubation temperature). All five of the glucuronidases studied hydrolyzed the conjugated cannabinoid metabolites, but there are definite species-specific differences in their ability to do so. In addition, pH, incubation time, and incubation temperature had a significant effect on each of the enzymes tested. For example, extremes of pH and temperature had a negative effect on the hydrolysis activity of the PV, EC, and HR β-glucuronidases in this study.

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