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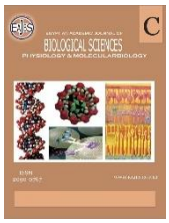

Determination of Esterase Activity on Benzothiazole Based Probe In Biological Samples

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ABSTRACT

Enzyme esterase is an enzyme important for detoxification and activation of drugs found in various body tissues. Here, we introduce and establish A high-performance liquid chromatographic procedure was established to determine esterase activity, relying on the organic probe EBOAc. Experiments show high sensitivity and wide linear range from 0 to 28 U/ml.

INTRODUCTION

Belonging predominantly to liver tissue, esterase holds significance as a key constituent within the serine hydrolase category. Notably, CES participates in the breakdown of different internal and external substances, encompassing esters and amides (Crow *et al*., 2007; Redinbo and Potter, 2005; Soto-Mancera *et al*., 2020; Li et *al*., 2017; Jiang *et al*., 2019; Lan *et al*., 2020; Park *et al*., 2018). For this reason; It has a crucial function in both the activation and detoxification of internal metabolites and external treatments (Shen *et al*., 2021; Satoh and Hosokawa, 2006; Ma *et al*., 2020). The activity of CES is linked to numerous conditions, including fatty, hyperlipidemia, liver disease, atherosclerosis, and liver cancer (Sharma *et al*., 2019; Liu *et al*., 2014; Lv *et al*., 2016). Therefore, it is particularly important to develop an operational procedure to screen for CES activity and content in biological samples. Currently, there are many screening methods such as polymerase chain reaction (PCR), chromatography-based proteomics technology (Feng *et al*., 2020; Dai *et al*., 2021). Nonetheless, these instruments are comparatively intricate, require a significant amount of time, and come with high costs, thereby restricting the widespread adoption of practical application and only able to measure the protein concentration (Chan *et al*., 2012; Du *et al*., 2020; Wang *et al*., 2019).

As it is highly valuable to measure the esterase activity in biological samples; we developed and presented an HPLC method for direct analysis for measuring the esterase activity based on the hydrolysis of EBOAc probe in phosphate buffer. This approach holds advantages over preceding methods, as it eliminates the potential for interference, it does not require specific instruments as GC-MS and takes less time and organic solvents (Lauridsen *et al*., 2001; Koitka *et al*.,2008).

MATERIALS AND METHODS

Equipment: The HPLC system consists of high-pressure gradient pump (P6.1L, Knauer Germany), and a model DAD 2.1L diode array detector (Knauer Germany). The analysis and system control software utilized is Claritychrom, version 7.4.2.107, developed by Dataapex in the Czech Republic, using a model D1357 sample loop (20 µ l) and injector (Knauer Germany). Separation is achieved through the utilization of a C18 column (provided by Knauer Germany) with dimensions of 250*4.6 mm i.d, containing particles that are 5 µm in size and possess an 80 Å pore size, The mobile phase was composed of a mixture of phosphate buffer (pH 6) and acetonitrile with flow rate 1 ml/min. Measurement is done at 400 nm.

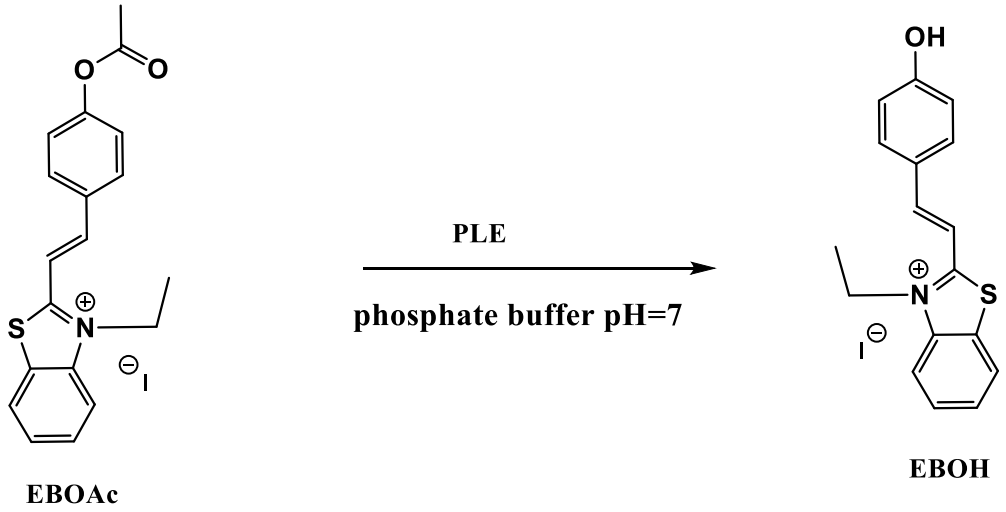
Reagents: Porcine liver esterase was purchased from Research Institute for Liver Diseases (Shanghai) Co. Ltd. As per the information provided by the supplier, the enzyme powder consists of 150 units/mg of protein. Here, one unit signifies the quantity of enzyme required to hydrolyze 1 µmole of substrate per minute. The solvents and their application conditions remain consistent with the previously mentioned details. Both the substrate and product of the enzymatic hydrolysis were synthesized and characterized using the standard identification procedure of organic compounds earlier for this purpose.

Enzyme Assay: A modified procedure was used based on the previous method (Ajandouz *et al*., 2006) . Porcine liver enzyme powder (20 mg) Was dissolved in 10 ml of deionized water, so the total activity of the enzyme is 300 U/ ml and kept at –20°C up until used. The stock solution of the substrate (1000 µM) was prepared by dissolving proper weight in phosphate buffer (35mM, pH 7). Employing 1.5-mL colored microcentrifuge tubes, these were positioned within a temperature-regulated bath for the experiment, The commencement of the reaction was achieved by blending the enzyme solution (in volumes of 0, 1, 2.5, 5, 7.5, 10, and 15 µL) with the substrate solution (475 µL) and buffer solution (50,49,47.5,45,42.5,40 and 35 µL) so the total volume of the solution is 525 µL, followed by vigorous vortexing (5 s). After periods of time (10 min), the reaction was stopped by adding 150 µL of absolute ethanol and mixed then centrifuged for 10 min. In the blank, the reaction stopped directly after mixing with PLE. Then and after that 20 µL of the solution was injected and analyzed.

Determination of Esterase Activity in Tissue Intestine of Chicken: About 10 cm of tissue intestine was cut from freshly slaughtered chicken; its weight was 1.8 g, the intestine tissue was cleaned and smashed in mortar and pestle, followed by dissolving in phosphate buffer (35mM, pH 7) so the total volume of the solution is 10 ml, different volume of the intestine solution (10, 25, 50,100 and 250 µL) mixed with 475 µL of EBOAc, the blank solution consisted from of 475 µL of EBOAc and 50 µL of phosphate biuffer, and the aforementioned procedure was used.

RESULTS AND DISCUSSION

In order to be able to detect and measure the activity of carboxyl esterase, the enzyme-substrate (EBOAc) and the enzymatic hydrolysis product (EBOH) were earlier synthesized and characterized, their thermal stability, cytotoxicity and photophysical properties already assessed for this purpose.



Scheme- 1: Proposed mechanism of PLE action.

According to scheme-1, the phenolic ester moiety in (EBOAc) served as the target group here for the enzyme detection, its hydrolysis by the action of the PLE would produce the phenolic correspond (EBOH). So as to prove the proposed mechanism and establish the detection conditions (Fig. 1), three HPLC measurments were done for EBOAc, EBOH and a combination of EBOAc and PLE. Since EBOH is a phenolic compound which losing a proton in aqueous media into phenolate with an equilibrium between two forms making the detection process hard, phosphate buffer (pH=6) was used in the mobile phase instead of water which retains EBOH in one form and prevents the auto hydrolysis of EBOAc.

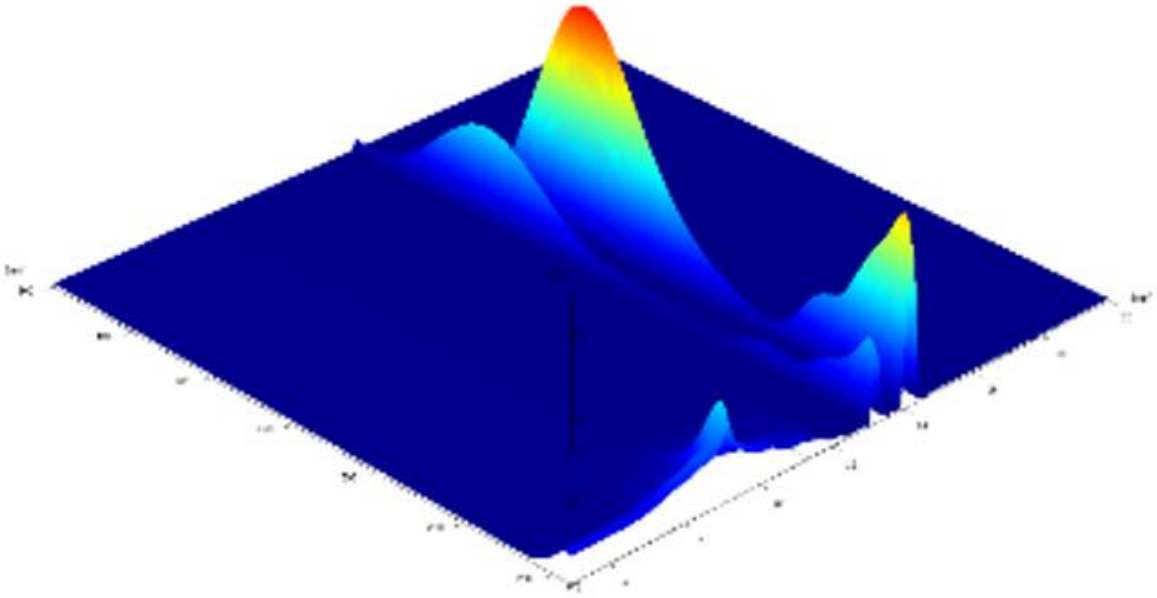


Fig. 1: 3D-absorbtion profile of EBOAc and EBOH**.**

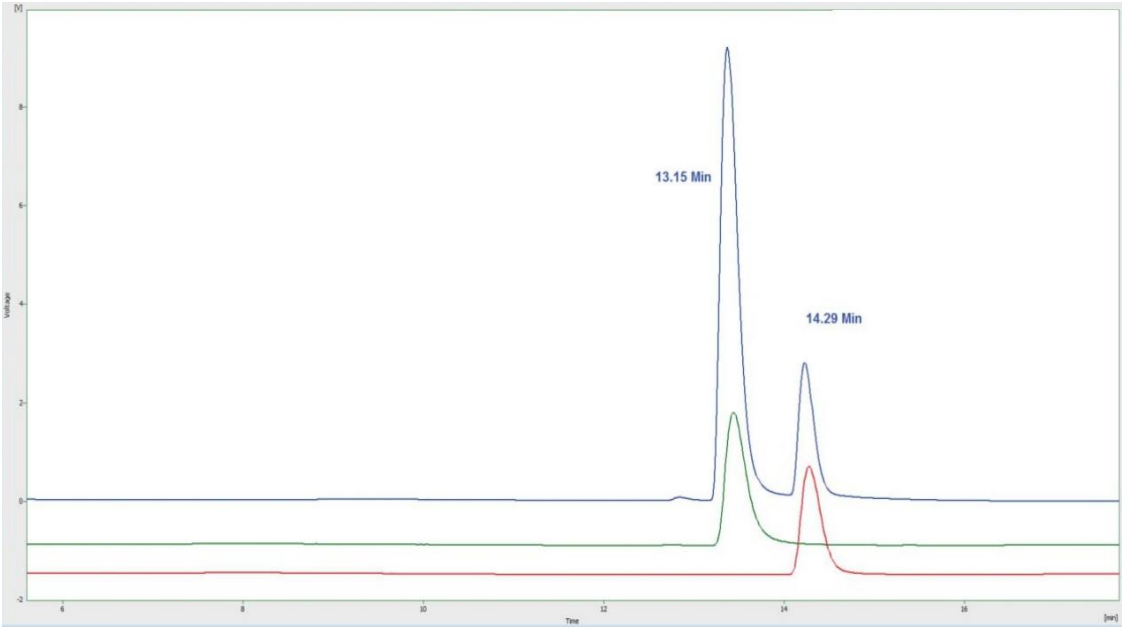


Fig. 2: Plot of intensity vs. retention time of EBOAc and EBOH.

Figure 2 revealed typical chromatograms of the time course elution and conversion of EBOAc to EBOH. EBOH showed only one peak with short retention time (13.15 min) due to polar criteria while the peak of EBOAc appeared at 14.29 min, treatment of EBOAc with PLE then examined the reaction mixture, we found that there are two peaks of both EBOAc and EBOH.

In order to measure the probe response for PLE, different concentrations of esterase enzyme were taken (Fig.3). The chromatographic runs showed that increasing the PLE concentration leads to decline in EBOAc peaks while the peaks of EBOH were in an opposite manner.

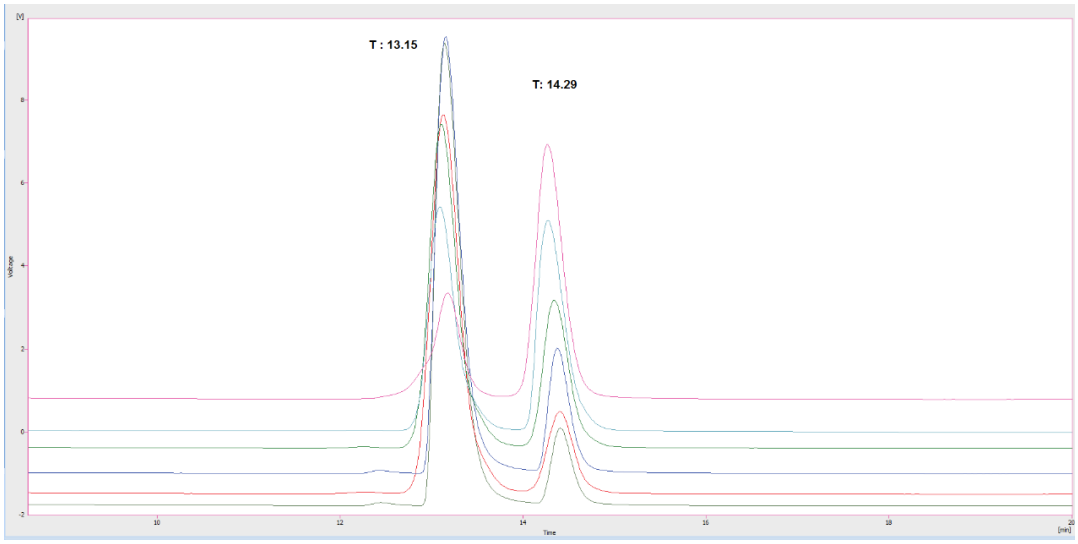


Fig. 3: Plot of intensity vs. retention time of reaction mixture produced by different concentrations of PLE in phosphate buffer.

A standard calibration curve has been built between the ratio of peak areas (A13.15/A14.29) and the concentration of the PLE (**Fig.4**), long range straight relationship was found between 0-28 U/ml, LOD was calculated from the standard deviation of the Y-axes intercept to be 0.2 U/ml.

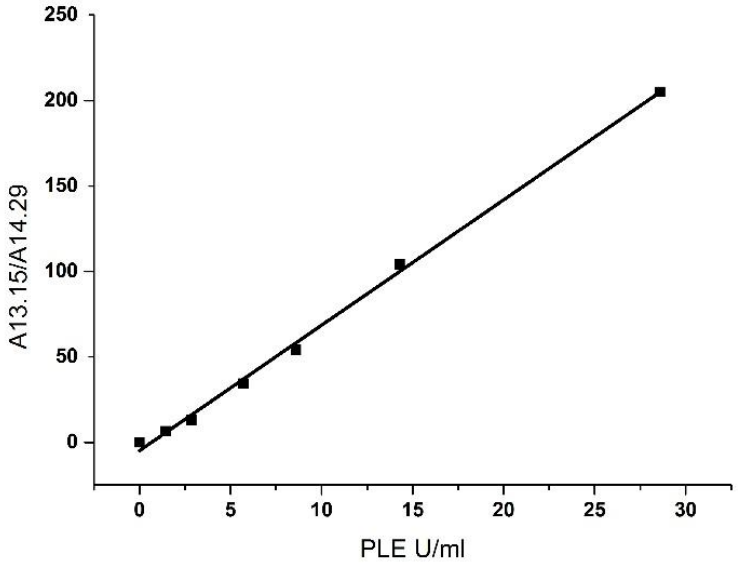


Fig. 4: Plot of standard calibration curve .

Since esterase enzyme can be detected precisely in this method, we have tried to detect the total esterase activity in small intestine of chicken, after formulating the solution of the intestine, five volumes of the small intestine were used with constant amount of the substrate (1000 µM) and the decreased in substrate area were measured.

Table 1: Measurement of total esterase activity in the small intestine of chicken.

Number	Substrate Area	Enzyme added in µL	Enzyme activity U/g
1	24592.572	0	0
2	21567.579	10	3.70
3	17453.849	25	3.60
4	12227.615	50	3.20
5	1750.751	100	3.02
6	331.544	250	

As listed in Table 1 the dilution factor influenced a minor effect on the total esterase activity, also Esterase activity can not detected for the last dilution point and this can be ascribed to excessive depletion of the substrate, the average of the total esterase activity is 3.38±0.32 U/g.

CONCLUSION

A high liquid chromatography procedure was established. The study demonstrated the application of this method for quantifying esterase activity on EBOAc, utilizing readily accessible porcine liver. Within a timeframe of less than 15 minutes, the hydrolysis rate is assessed through the observation of EBOAc depletion and the concurrent emergence of EBOH. The current report describes analytical methods for testing esterase activity on EBOAc hydrolysis with high sensitivity and low detection limit (0.2 U/ml) and long linear range (0-28 U/ml). The result revealed that the esterase activity in biological samples could be calculated, as in the small intestine of chicken to be 3.38±0.32 U/g. The current approach proves valuable for studying esterase activities concerning EBOAc in the small intestine tissue of chicken as a model system that mimics the absorption environment, or any biological or non-biological medium.

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