

## OPTIMIZED CONDITIONS FOR THE ENZYMATIC HYDROLYSIS OF GLUCURONIDATED PHASE II EZETIMIBE METABOLITE IN HUMAN PLASMA

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Received: 27 Apr 2013, Revised and Accepted: 05 Jun 2013

### ABSTRACT

**Objective:** The optimum conditions for the enzymatic hydrolysis of Ezetimibe Phenoxy Glucuronide (EZM-G), one of the major Phase II metabolite of Ezetimibe (EZM) in human plasma, were determined.

**Methods:**  $\beta$ -Glucuronidase enzyme from *Helix Pomatia* source was used. The reaction parameters studied were amounts of enzyme used, temperature, pH and the concentration of substrate upto which the enzyme shows its efficiency in hydrolyzing the glucuronide substrate. EZM was separated from possible interfering components in plasma through the use of HPLC and subsequently monitored with UV detection at 233 nm. The separation was performed on Symmetry shield 100 5C18 (250  $\times$  4.6 mm, 5  $\mu$ m) and the mobile phase consisted of Acetonitrile and 1mM Ammonium Acetate in the proportion 60:40. The EZM production was monitored to assess  $\beta$ -glucuronidase activity.

**Results:** The optimized enzyme concentration was 1457 U/mL of plasma and the incubation was carried out at a temperature of 50 - 55°C using 0.5M sodium acetate buffer adjusted to pH 4.5. Enzyme substrate saturation kinetics was studied in plasma upto 71.52  $\mu$ g/mL of EZM-G. A linear relationship of initial enzyme reaction velocity as a function of peak area of enzyme product was obtained for enzyme activity ranging from 10 to 500 units. Often a plot of the reaction velocity versus the substrate concentration is a hyperbolic curve as described by Michaelis-Menten relationship. However,  $\beta$ -glucuronidase exhibited non-hyperbolic curve.

**Conclusion:** We presume that enzyme is composed of subunits and exhibit cooperative kinetics and that the allosterism plays an important role in the regulation of enzyme activity.

**Keywords:**  $\beta$ -glucuronidase, Ezetimibe Phenoxy Glucuronide, HPLC, *Helix Pomatia*.

### INTRODUCTION

Ezetimibe [1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone] is the first in a new class of cholesterol lowering agents known as cholesterol absorption inhibitors. After oral administration ezetimibe (EZM) is rapidly absorbed and extensively conjugated to Ezetimibe Phenoxy glucuronide (EZMG), which is pharmacologically active in vivo. Thus, EZM and EZMG are the major drug derived compounds detected in plasma, constituting approximately 10-20% and 80-90% of the total drug in plasma, respectively [1, 2].

Although the direct detection of the glucuronide conjugate overcomes the critical limitations of approaches that involve enzymatic cleavage procedures and/or derivatization, indirect methods are still a choice for the routine pharmacokinetic analysis of EZM and its glucuronide in human biological matrices [3]. In case of indirect measurement, the quantification methods for glucuronides involve an enzymatic ( $\beta$ -glucuronidase) or an acid/base hydrolysis of the glucuronide ether bond prior to HPLC analysis [4] as described in Fig. 1. Enzymic hydrolysis is usually preferred due to the fact that although an acid/base hydrolytic procedure is time saving and cost effective; many drugs are destroyed by its extreme conditions [5].  $\beta$ -glucuronidase is used for the enzymatic hydrolysis of glucuronides from urine [6, 7], plasma [8, 9], and other fluids [10] prior to analysis by enzyme immunoassay, mass spectrometry, gas chromatography, high performance liquid chromatography, or other means.

To ensure complete enzymatic hydrolysis, it is necessary to optimize hydrolytic conditions (enzyme concentration, pH, temperature, reaction time and effective substrate concentration). As per Zhai et al.[8] and Andersen et al.[9], typically between 1 and 20 units of glucuronidase is used per  $\mu$ L of plasma for the enzymatic hydrolysis of glucuronides. According to the literature reviewed, approximately, minimum of 5000 U of enzyme is used per 100 - 200  $\mu$ L of plasma, serum, urine or feces, for the hydrolytic conversion of EZM-G [11 - 13]. These methods are being routinely used for the estimation of EZM from human plasma. However, the papers mainly focused on the analytical ability of method and nowhere justification was given for the hydrolytic conditions used.

To the best of our knowledge, the optimization of conditions for the hydrolysis of EZM-G in human plasma using  $\beta$ -glucuronidase has not been published. In this study, we report the optimization of conditions for the hydrolysis of EZM-G in human plasma using  $\beta$ -glucuronidase from *Helix Pomatia*.

### MATERIALS AND METHODS

#### Chemicals and Reagents

*Helix Pomatia*  $\beta$ -glucuronidase [145700U/mL (Type HP-2)] was obtained from Sigma-Aldrich, Germany. Ezetimibe (99.52%) and Ezetimibe Phenoxy Glucuronide (99.2%) were obtained as gift samples from C. B. Patel Research Centre, India. Acetonitrile and Methanol of HPLC grade was purchased from E-Merck (India). Sodium acetate, Sodium Borate, and Ammonium Acetate of analytical grade were procured from Qualigens. Glacial acetic acid (GAA) and Triethylamine (TEA) of analytical grade were procured from S. D. Fine Chem. Ltd. HPLC grade water, used for dilution, was prepared in-house using 'miniquartz distiller' of Qualigens.

#### Enzymatic Hydrolysis and Extraction

1 mL of sodium acetate buffer of approximate pH was added to 1 mL of plasma sample to maintain the optimum conditions for the enzyme activity. 100  $\mu$ L of enzyme solution in water was added and the mixture was incubated in a water bath. The enzymatic reaction was stopped by adding 0.1M sodium borate buffer (pH 9.80). The amount of enzyme, temperature, pH and time were varied in experiments performed to determine the optimized conditions. The product formed by hydrolysis was recovered from plasma with *tert*-butyl methyl ether. After centrifugation of the solution at 3000 rpm for 5 min, the organic layer was transferred to a centrifuge glass tube and evaporated to dryness under stream of nitrogen. The residues were reconstituted with 100  $\mu$ L of mobile phase and injected onto HPLC for analysis.

#### HPLC determination of cleaved product

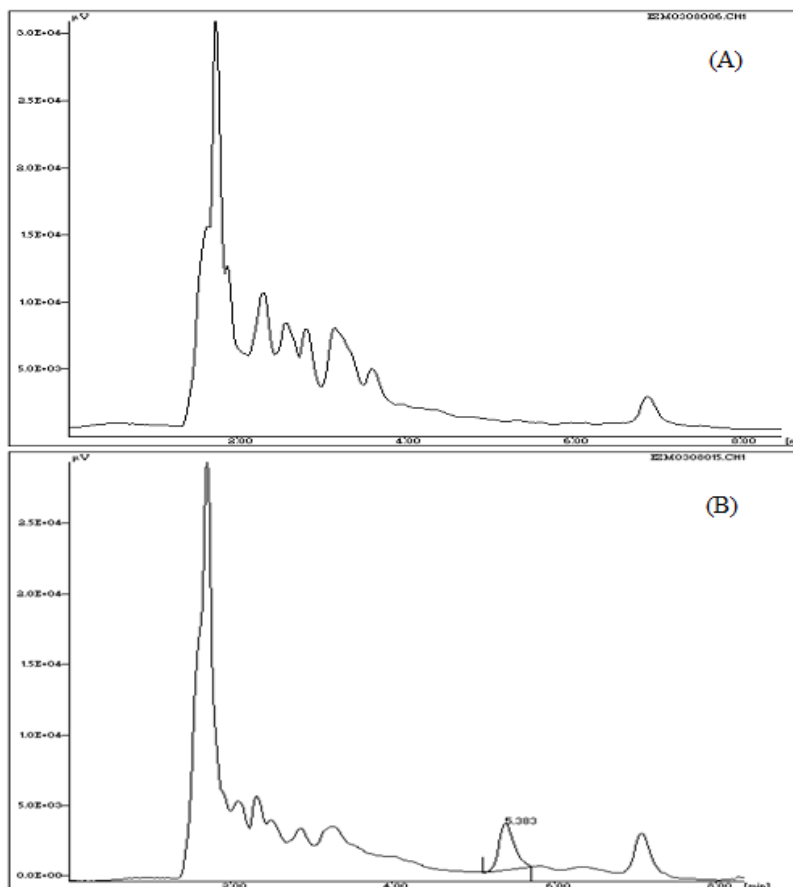
Chromatographic separation was performed on HPLC system (JASCO 1500) equipped with PU-980 pump unit attached to a

manual injector with a 20µL loop, and a UV-1575 detector. The data acquisition was carried on Borwin software version 1.50. The HPLC column used was Symmetry shield 100 5C18 (250 × 4.6 mm) 5 µm. The mobile phase consisted of Acetonitrile and 1mM NH<sub>4</sub>COOCH<sub>3</sub> in the proportion 60:40 v/v. The flow rate was 1ml/min and the eluate was monitored with UV detection at 233nm. The injection volume

was 20 µL and total run time was 12 min. The response obtained for EZM was monitored to check for the activity of enzyme.

**RESULTS AND DISCUSSION**

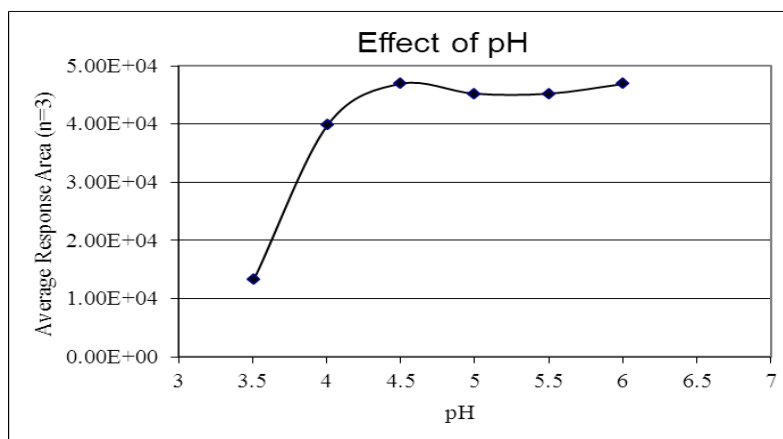
Figure 1 illustrates typical chromatograms obtained from the plasma sample after hydrolysis.



**Fig. 1:** It shows HPLC chromatograms of the plasma extract; (A): Blank Plasma, (B): Spiked Plasma = 250 ng/mL (Retention time of EZM = 5.3 min)

Figure 2 shows the effect of pH on the hydrolysis of EZM-G. Incubations were carried out in triplicate at different pH values to determine the effects of pH on β-glucuronidase activity with 600 ng/mL EZM-G as substrate. 0.1 M sodium acetate buffer with pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 were used. The pH optimum was obtained at 4.5 using 0.5M sodium acetate buffer, with appreciable

hydrolysis activities upto pH 6.0. As per the Sigma-Aldrich, the recommended pH for β-glucuronidase from Helix Pomatia ranges between pH 4 to 5. However, no reduction in the activity of enzyme after pH 5 was observed. The pH range tested was 3.5 to 6. Possibly, the hydrolytic activity might persist beyond the highest pH tested in this study.



**Fig. 2:** It shows effect of pH on the hydrolysis of EZM-G

[Each sample containing 600 ng/mL of EZM-G was incubated for 2 hours at 50 - 55°C containing 25 units/mL of enzyme concentration in plasma]

Figure 3 shows the effect of incubation temperature on the activity of  $\beta$ -glucuronidase. Incubations were carried out in a temperature range of 40 - 60°C with 600 ng/mL EZM-G. It is a very well known fact that, as the temperature increases the rate

of reaction also increases. Enzyme activity increased with increase in temperature and optimum was obtained between 50 - 55°C. Temperature beyond 55°C resulted in precipitation of plasma.

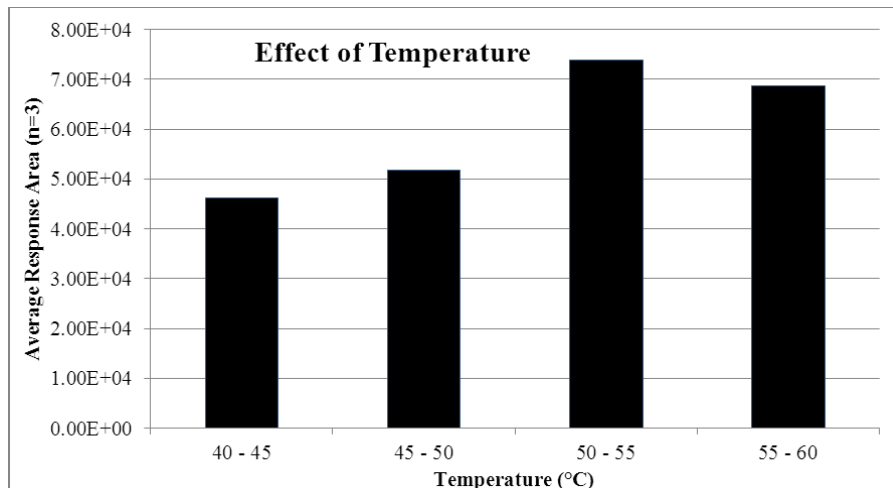


Fig. 3: It shows effect of temperature on hydrolysis of EZM-G

[Each sample containing 600 ng/mL of EZM-G was incubated for 4 hours containing 1457 units of enzyme per mL of plasma at pH 5.0 ('Sigma-recommended pH range': 4.0 - 5.0)]

Figure 4 shows the effect of increase in enzyme concentration with 600ng/mL EZM-G as substrate, incubated in triplicates. The reaction velocity increases with increase in enzyme concentration from 10 to 1457 units enzyme used per mL of plasma later which the steady state is obtained. This indicates that 1457 units enzyme per mL plasma ensures complete hydrolysis of EZM-G as no further increase

in response is obtained with increase in enzyme concentration. It suggests that 1457 units/mL of enzyme concentration in plasma can effectively hydrolyze EZM-G concentrations below 600 ng/mL. The reaction when monitored at 1, 2 3, 4, 5 and 6 hours using the optimized conditions, the reaction attains steady state by the 4<sup>th</sup> hour, later which no significant increase in the response was seen.

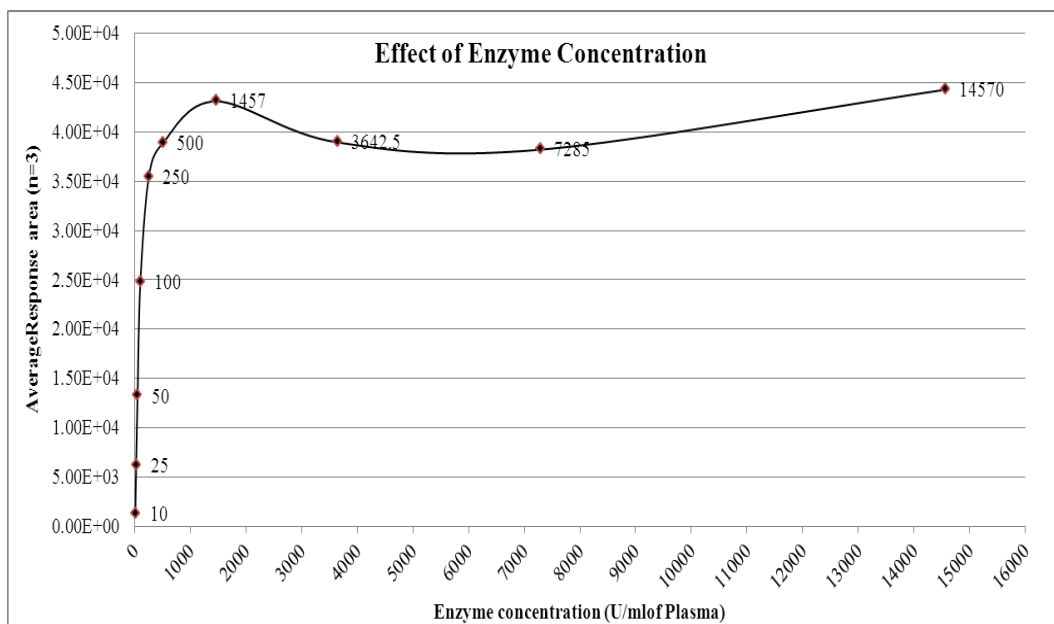


Fig. 4: It shows effect of increasing enzyme concentration on hydrolysis of EZM-G

[Each sample containing 600 ng/mL of EZM-G was incubated for 2 hours at 50 - 55°C using pH 4.5]

The effective concentration of substrate, which can be hydrolyzed using 1457 units/mL of enzyme was checked. The substrate concentration ranging from 35.76 - 13588.90ng/mL was incubated at pH 4.5 kept in a water bath adjusted to temperature 50 -55°C for 4 hours. Figure 5 shows the effect of increasing the concentration of substrate at fixed concentration of 1457 unit/mL of enzyme. A linear curve was obtained in a substrate concentration that was tested

with a correlation greater than 0.9957. The last substrate concentration tested is quite high enough the actual concentrations generally found in volunteers after 10 mg oral dose of EZM administered once daily to healthy human subjects. Thus the enzyme concentration of 1457 units/mL of plasma was found to be effective in a substrate concentration range that was used in this study.

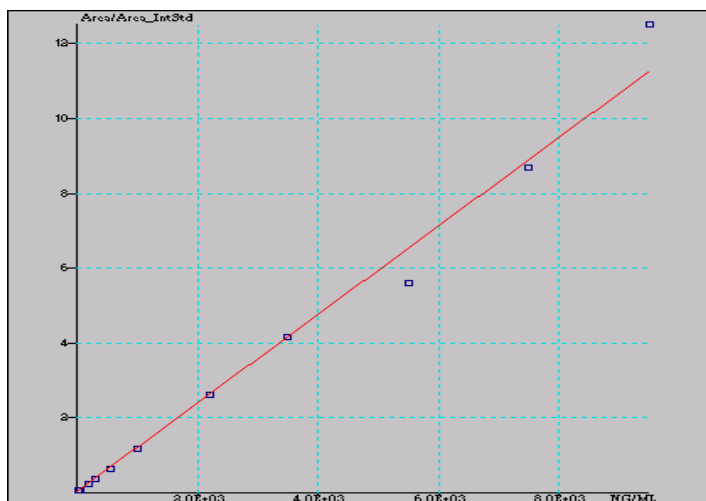


Fig. 5: It shows effect of increasing substrate concentration in the range of 35.76 – 13588.90 ng/mL for the hydrolysis of EZM-G under the optimized conditions

To check the saturation concentration of substrate, the enzyme concentration was lowered to 25 units/mL of plasma and substrate concentration was used ranging from 286.08 – 71520.53 ng/mL. Figure 6 shows that when the substrate concentration ranging from 286.08 – 71520.53 ng/mL was experimented using 25 units/mL of enzyme concentration, keeping other reaction conditions same, in contrast to a typical hyperbolic Michaelis-Menten curve, observed with other artificial

substrate using  $\beta$ -glucuronidase; cleavage of EZM-G showed a sigmoidal curve. The data points were fitted in Eisenthal-Cornish Bowden plot wherein, a tri-phasic plot was obtained (Figure 7). From the figure 6 and 7, it can be inferred that, a small change in substrate concentration will lead to a marked increase in rate of release of EZM from the glucuronide. Further increase in the concentration will not enhance the hydrolysis rate in a proportional manner.

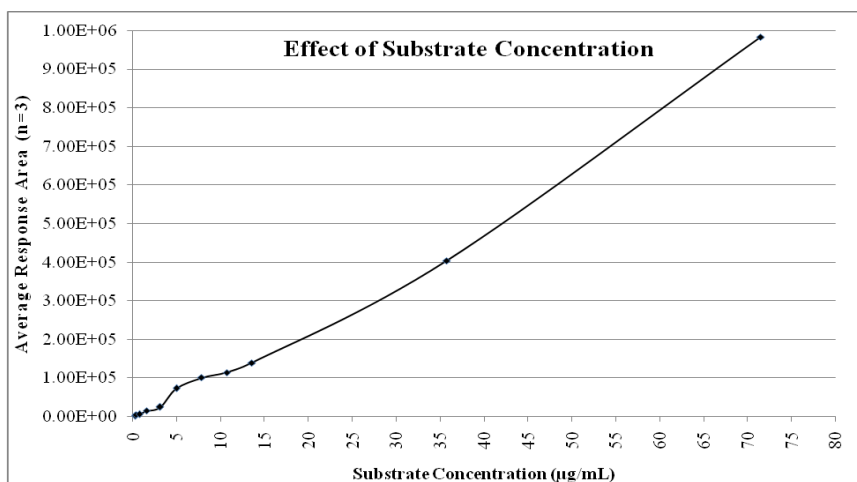


Fig. 6: It shows effect of increasing substrate concentration in the range of 286.08 – 71520.53 ng/mL for the hydrolysis of EZM-G under the optimized conditions

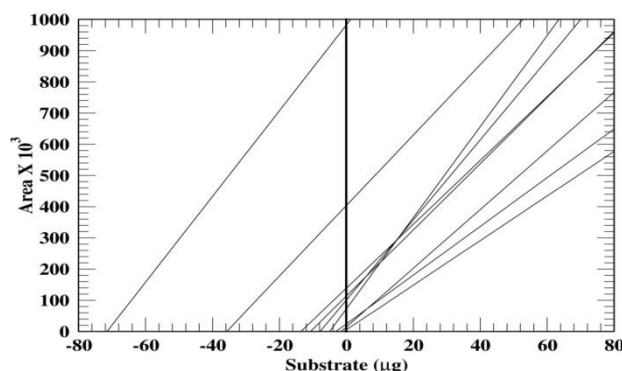


Fig. 7: It shows Eisenthal and Cornish-Bowden Plot of EZM-G concentration ranging from 786.73 - 71520.53 ng/mL

[Data points are plotted on negative x-axis to obtain  $K_m$  value (the distance of point of intersection of all data lines from y-axis) on the positive x-axis]

To ascertain the stability of the diluted enzyme preparation from *Helix Pomatia* in distilled water, the solution of 1457 units/mL was stored at 4°C, using aliquots of this solution for analysis of a fixed concentration of substrate (600 ng/mL) in plasma on successive

days. The results are plotted in figure 8. Enzyme preparation was fairly stable upto 4 days later which reduction in the enzyme efficiency was seen. The amount of product released on 12<sup>th</sup> day was 69.76% of the total product released on 1<sup>st</sup> day.

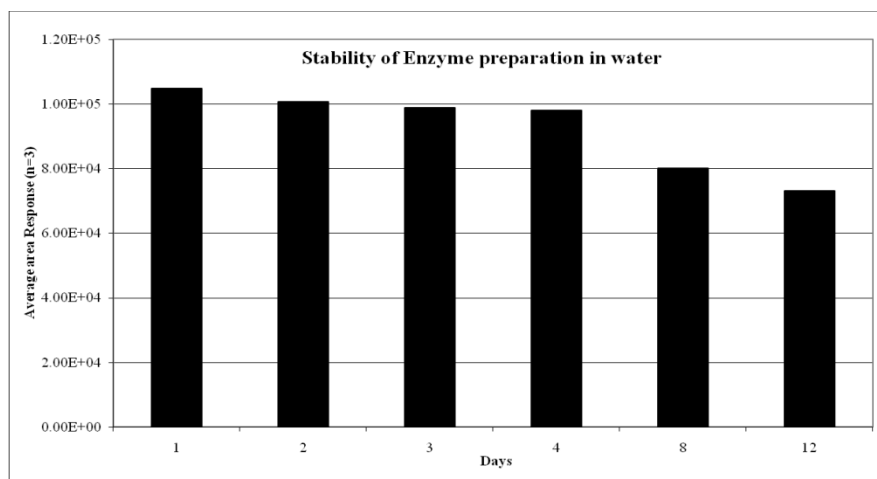


Fig. 8: It shows stability of  $\beta$ -glucuronidase preparation in water over a period of 12 days

[Each sample containing 600 ng/mL of EZM-G was incubated for 6 hours at 50 - 55°C using pH 4.5 buffer, with 1457 units of enzyme per mL of plasma]

According to Dutton et al. [14], the equilibrium point of reaction catalyzed by  $\beta$ -glucuronidase lies far in favor of hydrolysis, thus the effect of product concentration is not checked. The method optimized in this paper for the hydrolytic conversion of EZM-G to its parent form EZM, used 1457 units/mL of enzyme concentration which efficiently extracted substrate concentrations upto 9.5  $\mu$ g/mL. Thus the amount of enzyme used for the hydrolytic conversion of EZM-G, can be reduced further. Since the amount of enzyme used is reduced significantly, the cost of the extraction per tube containing 1 mL of plasma is approximately reduced 5-times as compared to the published papers. Also, the number of samples to be analyzed in commercially available concentrated form of enzyme (~1,00,000 U/mL) by Sigma - Aldrich, increased by 5-fold.

## CONCLUSION

In conclusion, EZM-G can be hydrolyzed using diluted  $\beta$ -glucuronidase from *Helix Pomatia* under the optimized conditions reported in this article so that the EZM is estimated quantitatively from human plasma samples. The method developed uses 1457 units/mL of enzyme concentration per mL of plasma. The reaction mixture has to be set at pH 4.5 and incubated at a temperature of 50 - 55°C for 4 hours to ensure complete hydrolysis of EZM-G. The method has also been successfully applied for the pharmacokinetic studies in 6 healthy human subjects at a single clinical dosage of 10 mg administered orally [15].

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