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Assessing and Optimising Several Enzymes Used for Drug Analysis in Urine

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Abstract

Glucuronidation can play an important role in the metabolism of some drugs such as paracetamol. In High-performance liquid chromatography, it is difficult to directly detect glucuronide conjugate in urine samples. The detection of paracetamol in the urine specimens requires releasing paracetamol from its glucuronide conjugates by a hydrolysis reaction. To develop and validate a suitable enzymatic hydrolysis method for paracetamol. To identify the optimal hydrolysis conditions, paracetamol 3-D-glucuronide was synthesized. Urine spiked with synthetic paracetamol 3-D-glucuronide was hydrolysed using five β-glucuronidases (5000 unit's/ml urine) from different sources (Helix pomatia, Escherichia coli, Patella vulgate, bovine liver and abalone), with different incubation times of the hydrolysis reactions. The results revealed differences in the reactivity of these enzymes. Enzymes obtained from Patella vulgate and Helix pomatia were more effective in optimizing the hydrolysis procedure than the other three enzymes. However, the enzyme obtained from Patella vulgate was the most effective across different incubation times. The method evaluation showed good linearity (r^2= 0.99), good accuracy (ranging from 93-118 %, with variation of results less than 20%), and good inter-day (1.75%) and intra-day (9.57%) precision. The values of precision and accuracy were acceptable based on the Scientific Working Group for Forensic Toxicology (SWGTOX) guideline. The source of the enzyme and the incubation times are critical factors that influence enzymatic hydrolysis of glucuronide metabolites in urine samples. Therefore, different other factors such as the hydrolysis pH and the incubation temperature should be carefully considered when conducting enzymatic hydrolysis of glucuronide metabolites in urine samples.
Keywords: β-Glucuronidase Enzyme, urine, enzymes

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الملخص

يمكن أن يلعب الجلوكورونيد دورًا مهمًا في استقلاب بعض الأدوية مثل الباراسيتامول. في الكروماتوغرافيا السائلة عالية الأداء، من الصعب الكشف مباشرة عن اقتران الجلوكورونيد في عينات البول. يتطلب الكشف عن الباراسيتامول في عينات البول إطلاق الباراسيتامول من اقتران الجلوكورونيد عن طريق تفاعل التحلل المائي، لتطوير واعتماد طريقة تحلل إنزيمية مناسبة للباراسيتامول، ولتحديد ظروف التحلل المائي المثلى، تم تصنيع الباراسيتامول 3-د-جلوكورونيد. تم تحلل البول المملوء بالباراسيتامول الاصطناعي 3-D-glucuronideباستخدام خمسة glucuronidases-(5000 وحدة / مل من البول) من مصادر مختلفة (Helix pomatia وEscherichia coli وPatella vulgate والكبد البقري وأذن البحر)، مع أوقات حضانة مختلفة للتحلل المائي تفاعلات. كشفت النتائج عن وجود اختلافات في تفاعل هذه الانزيمات. كانت الإنزيمات التي تم الحصول عليها من Patella vulgate و Helix pomatia أكثر فعالية في تحسين إجراء التحلل المائي من الإنزيمات الثلاثة الأخرى. ومع ذلك، كان الإنزيم الذي تم الحصول عليه من Patella vulgate هو الأكثر فعالية عبر فترات الحضانة المختلفة. أظهر تقييم الطريقة خطية جيدة (r2 = 0.99) ودقة جيدة (تتراوح بين 93-118٪ مع اختلاف في النتائج أقل من 20٪) ودقة جيدة بين اليوم (1.75٪) ودقة خلال اليوم (9.57٪). كانت قيم الدقة والدقة مقبولة بناءً على إرشادات الفريق العامل العلمي لعلم السموم الشرعي(SWGTOX) ، ويعتبر مصدر الإنزيم وأوقات الحضانة من العوامل الحاسمة التي تؤثر على التحلل المائي الأنزيمي لمستقلبات الجلوكورونيد في عينات البول. لذلك، يجب مراعاة العوامل الأخرى المختلفة مثل درجة الحموضة ودرجة حرارة الحضانة بعناية عند إجراء التحلل المائي الأنزيمي لمستقلبات الجلوكورونيد في عينات البول.

الكلمات المفتاحية: إنزيم β-Glucuronidase، البول، الإنزيمات

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1. Introduction and Literature review

Drugs are chemical compounds that have many favourable therapeutic effects on the human body. The metabolism process, in particular, plays an important role in determining the effects of drugs on the human body. Both phase I and phase II hepatic reactions play an important role in the metabolism of most drugs. During phase I, metabolism takes place by different chemical processes including oxidation, hydrolysis, and reduction (Kumar and Surapaneni, 2001). These processes convert the chemical compounds in drugs into polar metabolites that can be eliminated easily in the urine. During phase II reactions, the chemical compounds of the drug molecules are converted to other metabolites that have fewer toxic effects on the human body (Guengerich, 2006). Phase II hepatic reaction involves a conjugation reaction in which a covalent link is formed between the functional groups of the chemical compounds in the drug molecules and acetate, amino acids, sulphates, glutathione, or glucuronides. This conjugation reaction results in detoxification of the drug (Zamek-Gliszczynski et al., 2006).

The UDP-glucuronosyltransferases play an important role in the metabolic pathway in which the glucuronidation reaction for xenobiotic biotransformation occurs. This metabolic pathway also plays an essential role in the excretion endogenous compounds including fat-soluble vitamins, bile acids, steroids, and bilirubin. A hydrolase enzyme, namely β-Glucuronidase, is involved in the hydrolytic cleavage of biomolecules related to drug-glucuronide conjugates (Fisher et al., 2001). The glucuronidation is a metabolic process by which glucuronide conjugate is formed. The pathway of β-Glucuronidase hydrolysis reaction is demonstrated in **Figure 1.** The Glucuronic acid conjugation is mediated by UGTs, making lipophilic molecules much more soluble in water and readily excretable through the kidneys (Ding et al.,2013). During conjugation reactions, the hydrogen atoms in Hydroxyl-, sulfhydryl-, thiol-, aromatic amino- and

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carboxylic acid moieties are replaced by a conjugating agent. In some situations, using this method is not effective with glucuronides that maintain their stability in incubation mixtures and looking for another approach for hydrolysis is needed. Alternatively, chemical hydrolysis could be used to overcome this limitation (Ding et al.,2013).

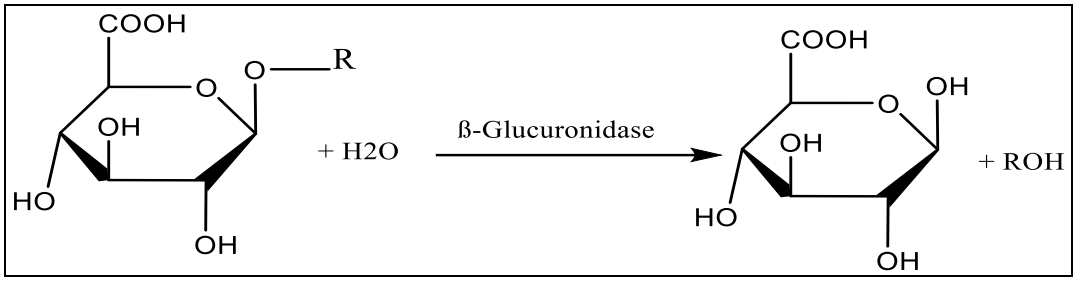

Figure 1: Hydrolysis reaction using β-glucuronidase

1.1 Glucuronide determination

The indirect method for identification and quantification with β-glucuronidase is used only after considering the stability of glucuronides (Roškar and Lušin, 2012). Another important factor in determining the method used for measurement of glucuronides is the availability of authentic glucuronide standards. In forensic toxicology laboratories, glucuronide drug analysis needs specific handling steps to accurately identify the concentrations of analytes in the biological samples.

1.1.1 Sample preparation methods and matrices

Sample preparation is an integral part of the analysis and the analytical information is largely affected by this step during chromatographic and spectroscopic analyses. Preparation of the sample may involve the analytes and/or the matrix of the sample (Vas and Vekey, 2004). The techniques used for sample preparation are used before undergoing sample analysis by chromatographic mass spectrometric methods (Prabu and Suriyaprakash, 2012). These techniques aim to eliminate lipids, proteins, and other unnecessary components of the sample and to keep the target analyte to be easily detected. In addition, sample preparation methods in the laboratory involve eliminating contamination of the instruments that occurs when the samples of

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biological matrices are inserted directly into the instruments (Prabu and Suriyaprakash, 2012), (Juhascik and Jenkins, 2009).

1.1.1.1 Hydrolysis

Hydrolysis occurs in phase I metabolic pathways that often occur in parallel with phase II reactions. As mentioned earlier, phase I reactions involve hydrolysis, reduction, and oxidation to change the structure of the parent compound and increase its polarity to be easily excreted with urine (Kostiainen et al., 2003). In most assays, it is impossible to detect the drug conjugated form. Therefore, the analytical procedure facilitates its detection by conducting hydrolysis to enhance its detection in urine. By hydrolysis of glucuronide, the pharmacologically active compound is regenerated and the concentrations of the parent drug increased. This enzyme is naturally present in various human tissues, including intestines and other body fluids. It is also found in other mammalian species. It plays a major role in hydrolysing glucuronidated conjugates of drugs by the cleavage of the C–O bond (Kerdpin et al., 2006).

Hydrolysis reaction can be performed using enzymes or concentrated acids. Using enzymes for the purpose of conducting hydrolysis will not cause degradation and it usually maintains the stability of the sample under physiological conditions. The β-glucuronidase enzyme can be extracted from various natural sources, such as Escherichia coli bacteria (E. coli), Helix pomatia, and bovine liver (Ding et al., 2013). Enzymatic hydrolysis procedure could be affected by different factors including the pH of the reaction, the enzyme/substrate ratio, hydrolysis time, and temperature (Malik-Wolf et al., 2014). On the other hand, using concentrated chemical when performing hydrolysis may cause extensive degradation in the sample.

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1.1.1.2 Extraction mechanisms

To achieve a low limit of detection (reliably measure the smallest concentration the analyte) for drug compounds and their metabolites, any matrix compound that may interfere with the analytical procedure should be eliminated (Peters et al., 2007). Therefore, in the urine sample, it is important to separate the drug compounds and their metabolites from the interfering components to succeed in the determination of the target analytes during the analytical procedure. These compounds may naturally occur in the urine samples or resulting from the hydrolysis procedure itself.

Liquid/liquid extraction (LLE) of drugs from biological samples is a method used in the laboratories and analytical toxicology to screen for the general unknown. It basically works by separating compounds and classifying them based on their polar or non-polar relative solubility. In addition, using SPE is advantageous over LLE and overcomes the most common limitations of LLE such as the high probability of occurrence of emulsification in samples with surfactant-like compounds (i.e. free fatty acids and proteins), being time consuming, the need for a large amount of the solvent for the clean-up, adsorbing analytes to particulates, and risks of cross-contamination and sample loss when using some solvents (Ding et al., 2013). Some disadvantages of the SPE include being a complex and multi-step technique and the requirement of the sample amount to be large enough to meet the detection limit (Negrusz, 2013). The SPE has different modes of action including mixed-mode, reversed-phase, normal phase or ion-exchange. The mixed mode is based on polymeric sorbents that can enhance the selectivity of the extraction process. Due to its strong hydrophobicity, the C18 is suitable to be used for the extraction of the compounds that are polar. The SPE can easily remove the excess enzyme that can influence the analysis. This will reduce, to a great extent, the fouling the HPLC columns. In SPE, the solid phase replaced the organic solvent and the matrix travels

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through a sorbent bed, and the analytes are completely settled on the solid sorbent (Pufal et al., 2000).

During the sample preparation, SPE can achieve various functions including the elimination of the interfering compounds, separation or concentration of the analyte and transfer of analyte into another solvent that is suitable for analysis (Poole, 2003).

1.1.1.3 Analytical methods for metabolite quantification

Liquid chromatography coupled to mass spectrometer or UV detector or other different detectors has become among the most commonly used methods for quantification (Vehovec and Obreza, 2010). Recently, several analytical methods have been developed for glucuronides quantification in biological matrices (Negrusz, 2013). In pharmacokinetic studies, the LC-MS/MS has become a commonly used bioanalysis method which has proved its effectiveness in terms of sensitivity, specificity, speed, and reproducibility. However, HPLC coupled with various detection systems is often used for the analyses of different compounds in many fields and it is considered a prerequisite for correct quantification of metabolites in samples obtained from human matrices (Holčapek et al., 2008). Furthermore, one highly efficient and rapid separation method for metabolites quantification is Capillary Electrophoresis (CE). This method, however, is not widely used because of its low sensitivity (Plenis and Bączek, 2011).

1.2 Example of glucuronide metabolite

The primary toxicity of paracetamol is associated with its metabolism in the liver (Mazer and Perrone, 2008). Both LLE and SPE can be used for the identification and extraction of paracetamol and its metabolites.

1.2.1 General view of Paracetamol

Despite its widespread use, the overdose of Paracetamol or its intake for a long time could result in fatal toxicities (Chandra et al., 2013). Paracetamol toxicity after chronic intake or intake in high doses could cause hepatotoxicity, nephrotoxicity,

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and severe liver diseases (Cohen et al., 2018), (Tittarelli et al., 2017). In both the United Kingdom and the United States, paracetamol toxicity has been the most common cause of hepatic failure and it accounts for a large percentage of drug poisoning incidents. Further, in the UK, paracetamol toxicity is responsible for 150–250 cases of death yearly (Caparrotta et al., 2018). Paracetamol is extensively metabolized in the human body and it undergoes a conjugation, resulting in detecting only small amounts of it in the urine. Therefore, its conjugated metabolites (i.e. glucuronides) are mainly present in urine. The chemical structure of paracetamol is demonstrated in **Figure 2.**

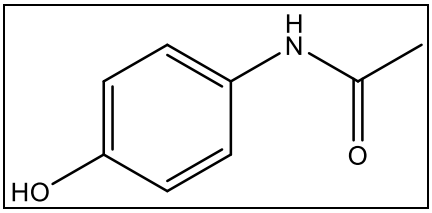

Figure 2: Chemical structure for paracetamol

1.2.2 Pharmacokinetics

For the intravenous infusion, its recommended dose is 650-1000 mg, 4 to 6 times daily, with infusion over 15 minutes (Baselt, 2014). Most of paracetamol is metabolized by the liver, while only a small amount is metabolized by intestine and kidney. Paracetamol metabolism by the liver occurs in three pathways; oxidation of paracetamol is catalysed by the Cytochrome P450 enzymes, glucuronidation is catalysed by UGT, and conjugation with sulfuric acid **(Figure 3).** Oxidation of paracetamol results in producing a toxic product, namely Nacetyl-p-benzoquinone imine (NAPQI). This toxic product is detoxified by the endogenous glutathione (GSH) to be easily excreted with urine. Any deficit in GSH causes an increase in NAPQI concentration in the hepatocyte, causing damage or death of the liver cells that can be fatal (Gum and Cho, 2013). More than 50% of the glucuronide and about 30% of sulphate metabolites are excreted in the urine, while some paracetamol glucuronide appears in the bile with subsequent transport into the intestine (McGill

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and Jaeschke, 2013). Only 3% of the paracetamol is eliminated in the urine unchanged, while about 8% excreted as GSH derived adducts.

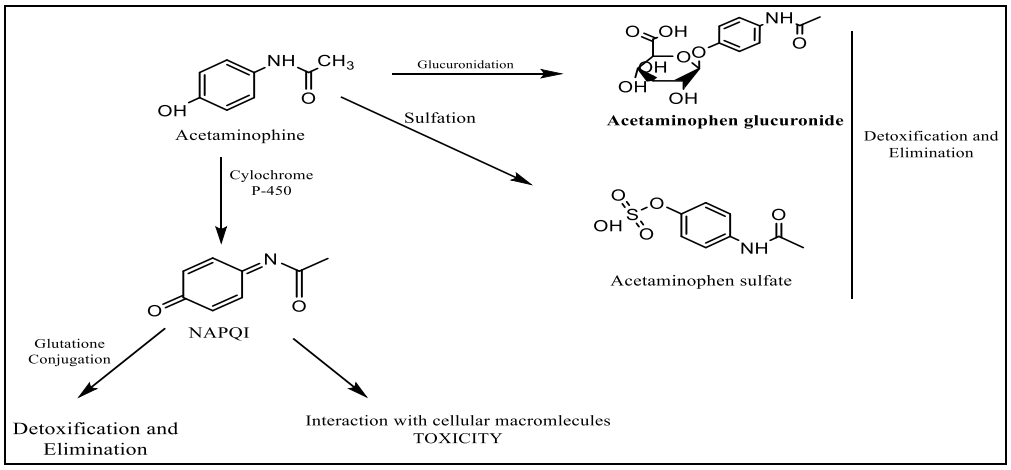

Figure 3: The pathway of acetaminophen metabolism (Bolded metabolite was measured in this study).

1.2.3 Determination of Paracetamol glucuronide

One important procedure to be performed is conducting enzymatic hydrolysis (such as using β-glucuronidase) to convert the analyte from the conjugated form to the parent drug. In urine samples obtained for the purpose of paracetamol analysis, the major metabolite of paracetamol that can be quantified is the paracetamol glucuronide (McGill and Jaeschke, 2013). Therefore, there is a need to employ correct and accurate way of drug conjugate quantification in the urine samples. To achieve this purpose, conducting enzymatic hydrolysis is required (Tan et al., 2012).

2. Problem Statement

Usually, the drug conjugates have less pharmacological activities compared with their parent compounds, but some drug conjugates may have pharmacological activities themselves or provide a source of pharmacologically active compounds this is the main problem statement. Because the kinetics of the drug conjugates determine the pharmacological activities of the drugs, it is important to extensively focus on the drug conjugates and their pharmacokinetic behaviours.

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3. Research Questions

1. How and what the methods will be used to evaluate the hydrolysis efficiency of five β-glucuronidase enzymes of their sources, forms (aqueous solution, lyophilized powder, or solid), and concentrations to choose an appropriate enzyme for the hydrolysis reaction?
2. How will be used Optimize the hydrolysis incubation time condition over the five β-glucuronidase enzymes used in the hydrolysis reaction?
3. what the ways and tools used to Validate the quantification method of paracetamol using the most effective enzyme?

4. Research Objectives

To develop and validate a suitable enzymatic hydrolysis method for paracetamol. The main objectives as the following:

1. Evaluate the hydrolysis efficiency of five β-glucuronidase enzymes that are different in term of their sources, forms (aqueous solution, lyophilized powder, or solid), and concentrations to choose an appropriate enzyme for the hydrolysis reaction.
2. Optimize the hydrolysis incubation time condition over the five β-glucuronidase enzymes used in the hydrolysis reaction to identify the most appropriate condition for urinary paracetamol recovery. These incubation times were 60,120 and 180 minutes.
3. Validate the quantification method of paracetamol using the most effective enzyme.

5. Materials and method

a. Materials

For the purpose of this study, a supplier of laboratory chemicals in the UK, namely fisher scientific has supplied us with acetaminophenol, 98% standard, and

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internal standard (ISD) N-(3-Chloro-4-hydroxyphenyl) acetamide, while the Paracetamol β-D-glucuronide was obtained from Sigma-Aldrich. **Figure 4** demonstrates the chemical structure of both Paracetamol β-D-glucuronide and ISD. In addition, Sigma-Aldrich supplied drug-free urine (DFU). A market for laboratory equipment in the UK, namely VWR International Ltd, supplied Na_2HPO_4 (Sodium phosphate dibasic), methanol (MeOH), acetonitrile, glacial acetic acid, NaH_2PO_4 (sodium phosphate monobasic), sodium acetate trihydrate, Orthophosphoric acid. All of these chemicals were HPLC-grade. Regarding the SPE cartridges (Supelclean™ ENVI™-18 SPE Tube) 500 mg cartridges (part number 57063), they were supplied by Sigma-Aldrich Company. An ultrapure water purification system (Water deionizer−Merck Direct QR 3UV) were utilized to prepare the de-ionised water. The HPLC instrument and vials (2 mL) were obtained from Agilent Technologies. The Five β-glucuronidases that are different in term of their type, source, or formulation were supplied from Sigma-Aldrich. These β-glucuronidases are presented in Table 1. Table 2 presents the general laboratory equipment used in this study.

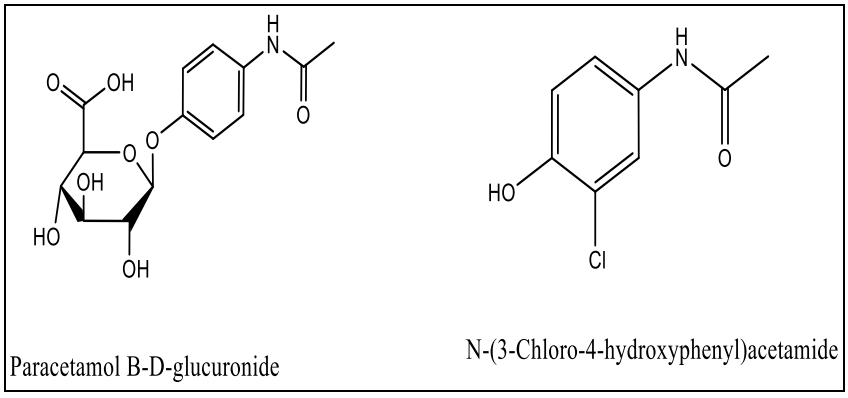

Figure 4 Chemical structure of Paracetamol β-D-glucuronide and internal standard

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Table 1 :β-Glucuronidase, Sources, Forms, and Concentrations

Product No.	Source	Form	Stock concentration
G2174	Limpet (*Patella vulgate)*	Aqueous solution	≥85,000 units/mL
SRE0037	Abalone	Aqueous solution	≥100,000 units/mL
G7017	Helix pomatia	Aqueous solution	≥100,000 units/mL
G7646	Escherichia coli	lyophilized powder	5,000,000 units/g
G0251	bovine liver	solid	1,000,000 units/g

Table 2: General laboratory equipment

Equipment	Additional Information / Specification
Microbiological safety cabinet	CLASS I Model: Safe HOOD 165
Water bath	Grant JBN18
Sonicator bath	Grant XUBA3
Balance	Sartorius TE64-0CE Mettler Toledo XPE105 Delta range
Vortex mixer	VWR Analog Vortex Mixer Fisher Scientific Topmix FB15024
PH meter	Hanna instruments PH 213 microprocessor PH meter
Thermometer	Brannan Glass Thermometer
Nitrogen evaporator	Thermo Scientific Reacti-Therm III #TS18826 Evaporation Unit
Gilson pipettes	20-200-1000µL(Bedfordshire, UK)
SPE Vacuum Pump Trap Kit	Supelco / SU57120-U

b. Method

i. Preparation of Glucuronide Standard

For each of the 4-Acetaminophenol and Paracetamol β-D-glucuronide standards, an individual solution was prepared. A concentration of 1mg/mL in de-ionized water was achieved. This concentration (1mg/ml) was achieved by adding 5mg of the 4-

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Acetaminophenol into 5 mL standard volumetric flask. After that, for each drug, the flasks were shaken frequently. The calibration curve was performed with suggested concentrations of calibration including 10, 20, 50, 100 and 200 µg/ml. The blank samples that do not contain the analytes were prepared with (0 µg/ml) and without internal standard.

ii. Preparation of ISD

Ten ml of demineralized water were first obtained. Then 10mg of N-(3-Chloro-4-hydroxyphenyl) acetamide were added to the de-ionized water, resulting in producing a stock solution with a concentration of 1 mg/ml. Then, for each drug, the flasks are shaken a number of times. Finally, a constant amount of (50 µl) of ISD was added to all standards.

iii. Preparation of buffer

1. 1.0 M Acetate buffer (PH5)

The preparation of 1.0 M acetate buffer included diluting an amount of 42.9 g of sodium acetate trihydrate in a volume of 400 mL de-ionized water. Then, an amount of 10.4 ml glacial acetic acid was added to the solution. After adding the glacial acetic acid, the resulting solution was diluted with 500 mL of de-ionized water. Finally, the pH was adjusted to 5.0 ± 0.1 using 1.0 M acetic acid or sodium acetate. The acetate buffer can be used within one month and be stored at 25°C.

2. 100 mM Acetate buffer (PH 5)

The preparation of the 100 mM acetate buffer included diluting an amount of 40 mL 1.0 M acetate buffer in 400 mL de-ionized water. The resulting solution can be stored at 25°C in a plastic or glass container. The solution can be used within one month and it should be stored at 25°C.

3. Phosphate buffer (PH 6)

The preparation of the Phosphate buffer at 0.1 M and pH6 included dissolving an amount of 1.70 g Na_2HPO_4 and 12.14 g of NaH_2PO_4 in a volume of 800

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mL of de-ionized water in a beaker. The resulting solution was then added to a 1000 volumetric flask and de-ionized water was added to the mark. To adequately mix the contents, the volumetric flask was then inverted a number of times. The pH was adjusted to 6.0 ± 0.1 using 100 mM monobasic sodium phosphate to reduce pH or 100 mM dibasic sodium phosphate to increase the PH. The solution can be used within one month and it should be stored at 4°C in glass and observed daily for any contamination.

4. Mobile Phase

The preparation of the mobile phase included using a volume ratio of the following: de-ionized water: Methanol: Acetonitrile: Orthophosphoric acid (500:100:250:0.025). Then, the solution was filtered and sonicated for a sufficient time (15 min).

iv. Working Enzyme stock solution preparation (5000 Fishman Units/mL).

As mentioned earlier, β-Glucuronidase was obtained from different sources including abalone, limpet (Patella vulgata), Helix pomatia, E. coli and bovine liver. These β-Glucuronidases were supplied by Sigma-Aldrich. The solutions of these enzymes were prepared at a concentration of 5000 U/mL in 100mM acetate buffer.

v. Urine sample digestion with β-Glucuronidase

To each urine sample, 5000 units of β –glucuronidase were added and incubated in a heating block which was heated at 65°C. The current study investigated the outcomes of using different incubation times (60 min, 120 min, and 180 min) for different β-glucuronidase enzymes on paracetamol glucuronide and optimizing the analysis procedure.

vi. Solid phase extraction

Considering its advantages in terms of reproducibility, specificity, and the cleanness of the sample, the SPE was used. In the SPE procedure, C18 cartridge was utilized to do the extraction. A volume of 2 mL of MeOH and de-ionized water was used to

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condition the SPE column. Then a volume of 1 mL of 0.1 M phosphate buffer at pH 6 was employed to clean the cartridges and remove extra materials. Then, the sample to be analysed was loaded onto the SPE columns after they were conditioned, and a full vacuum was used for 5 minutes to dry the contents. The columns were eluted using 2 mL of MeOH. Instrumentation

HPLC System (Agilent Technologies, 1200 series) with UV detection was used to analyse all samples in this study. Data collection and identifying peak integration were conducted using the ChemStation (Revision B.04.02) system. The UV-wavelength was selected to be 254nm. The isocratic mode was employed for elution and the separation of analyte was performed using column (Gemini 5µm C18 110A LC column 150×2.0 mm), under degassing by vacuum before being used. The injection volume was 20 µl, column temperature was 25.0°C and the flow rate was 0.5 mL/min.

vii. Data processing

1. Unextracted response of drug in HPLC and Evaluation of the analytical method

To identify the retention time (tR) of drugs and internal standard, drugs with a concentration of 10 µg /ml and internal standard were injected in HPLC. The peaks of all substances have demonstrated different tR in the chromatogram (Appendix 1). The following concentrations (0, 10, 20, 50, 100 and 200 µg/ml) were used to prepare the triplicated samples. This method was employed for the un-extraction of drugs standards and the values of peak area of the drug and ISD were integrated. The peak area ratio (PAR) was calculated using the values of peak area according to **Equation 1**.

Equation 1: Peak Area Ratio

$$\mathrm{PAR} = \left(\frac{\text{Analyte peak area}}{\text{Internal standard peak area}}\right)$$

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viii. Optimize hydrolysis using β-glucuronidase from various species

To examine the hydrolysis effectiveness of β-glucuronidase enzymes obtained from various sources, the ability of hydrolysis methods to regenerate the parent drug from the glucuronide standard was investigated. This procedure was conducted for all β-glucuronidase enzymes at one concentration (10 µg/ml) and examined the hydrolysis efficiency of Paracetamol β-D-glucuronide. All the five enzymes involved in the study were previously presented in Table 1, with samples run at a temperature of 65°C and the following incubation times (60,120,180 minutes). Then, the samples were extracted using the SPE method and analysed by HPLC-UV.

c. Results and discussion

The outcomes of the experiment were summarized in figure 4. The results of the experiment revealed a difference in hydrolysis efficiency between the five enzymes examined. Among the five enzymes, Patella vulgate and Helix pomatia were more effective at hydrolysis than the other three enzymes. Variation between two β-glucuronidase enzymes (Patella vulgate and Helix pomatia) also was determined by applying t-test. At 60 and 180 minutes, there was no significant difference result, p-value = 0.15 and 0.09 respectively. However, at 120 minutes, a significant difference was noted, p-value = 0.01. For most of the enzymes examined, rapid regeneration of the parent drug from the glucuronide metabolite needed an incubation time of 180 minutes. However, the optimal incubation time was also observed for some enzymes before this point in time. Overall, β-glucuronidase from Limpets (Patella vulgate) had the best conversion across all incubation times.

The ultimate goal of the method optimization is to gain a maximum enzymatic hydrolysis rate. Considering the role of the enzyme source can play in the hydrolysis reaction, the selection of the appropriate source of enzyme should be considered an important parameter that needs to be optimized in the analytical method. Meanwhile, incubation durations for different types of enzymes or enzymes obtained from

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various sources should be optimized as they can influence the rate of hydrolysis and its activity. Previous research suggested that enzymes from different sources demonstrate different activities in the hydrolysis reaction. This was supported by Elsohly et al. (2005) who examined the effects of β-glucuronidase enzymes obtained from different sources on the hydrolysis reaction. In this study, urine spiked with buprenorphine-3-b-D-glucuronide and hydrolysed using enzymes from E. coli, Helix pomatia, and patella vulgate. The results of the study indicated a differences between these enzymes on their effects on buprenorphine-3-b-D-glucuronide (Elsohly et al., 2005).

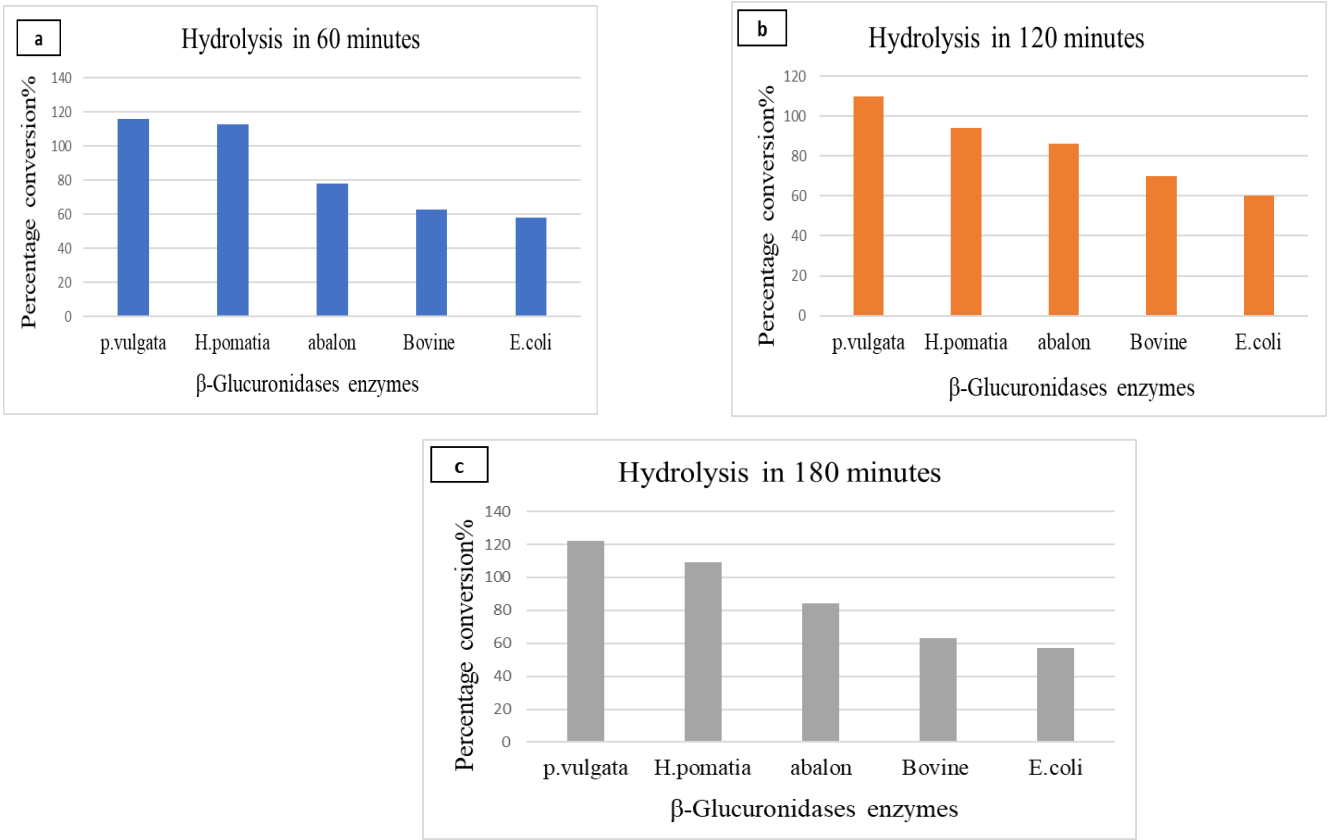

Figure 4: Effect of incubation times on hydrolysis efficiency of different β-glucuronidase enzymes

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6. Method validation

After the method development work, it is needs to prove the validity of this method. Method validation is a process of testing a particular analytical method to confirm the suitability of the employed analytical procedure used for its intended purpose (Shabir, 2003). Procedures for method validation include specific experimental tests used to judge the accuracy, precision, quality, the limit of quantification (LOQ), the limit of detection (LOD), linearity, and recovery. There are various guidelines used for validation of the analytical method such as the guidelines published by the UK and Ireland Association of Forensic Toxicologists (UKIAFT) and the Scientific Working Group for Forensic Toxicology (SWGTOX) (Elliott et al., 2018), (Toxicology, 2013). The validation parameters in these guidelines include accuracy, precision, limit of quantification, limit of detection, linearity, and recovery. In the current study, to measure each parameter, the analysis was conducted in triplicates. The validation parameters are defined as follows:

Linearity: Analytical method linearity is the ability of the test to yield results that are directly and positively correlated with the concentration or number of analytes in samples. This can be judged by the calibration curve and values of the correlation coefficient (R^2) between X and Y, where higher R^2 value indicating better linearity. The minimum acceptable R^2 value for most applications is 0.99. However, in some circumstances R^2 value of 0.98 is acceptable based on the UKIAFT guidelines. In addition, evaluate the range of the calibration by calculating the value of each calibrator against the curve (Elliott et al., 2018).

Bias and precision: the accuracy (bias) is the degree of agreement between the results of the test and the accepted reference value (% deviation). This can be performed by dividing the value of the concentration obtained by the test by the accepted reference value for different concentrations (Equation 2) (González and Herrador, 2007). At each concentration, the maximum accepted bias is ± 20%

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(Toxicology, 2013). In a bioanalytical method validation, precision is defined as the level of agreement of the test results obtained from multiple samples that have the same concentration.

Equation 2: Accuracy

$$\text{accuracy\%} = \left(\frac{\text{mean conc.}}{\text{actual conc.}}\right) \times 100$$

Precision is estimated by the coefficient of variation (% CV), which is determined by the mean and standard deviation of the test result obtained for each concentration (Equation 3). At each concentration, the maximum accepted coefficient of variation is ± 20% (Toxicology, 2013).

Equation 3: Coefficient of variation

$$\%\text{CV} = \left(\frac{\text{SD}}{\text{mean}}\right) \times 100$$

Two different types of precision calculation are conducted during method validation for quantitative procedures. These two different types are the intra-day (Within-run) and inter-day (between run). The calculation of intra-day precision (Equation 4) is conducted by identifying the relative standard deviations (RSD) within runs, while the calculation of inter-day precision (Equation 5) is conducted by identifying the relative standard deviations (RSD) on different days (Toxicology, 2013).

Equation 4: Intra−day precision calculation (RSD)

$$RSD\ \% = \left(\frac{\text{Standard deviation of a single run of samples}}{\text{Mean calculated value of a single run of sample}}\right) \times 100$$

Equation 5: Inter−day precision calculation (RSD)

$$RSD\ \% = \left(\frac{\text{Standard deviation of grand mean for each concentration}}{\text{Grand mean of each concentration}}\right) \times 100$$

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Specificity is the ability of an analytical method to differentiate the analyte of interest from other substances in the sample, including its related components or compounds. Examples of these substances are other active ingredients, matrices, degradation products, impurities, excipients, and placebo ingredients. Specificity confirms that a peaks response observed is due to one component (no peak coelutions). During method validation, specificity is judged by the ability to differentiate the analyte of interest from other substances in the sample or by performing a comparison with known reference materials.

LOD and LOQ are defined using several terms. The LOD is referred to the smallest concentration of an analyte in a sample that can be reliably distinguished from blank matrix and its detection can be feasible by the analytical method. The LOQ can be defined as the lowest concentration of analyte in a sample that can be reliably quantified with suitable accuracy and precision (Lappas and Lappas, 2015), (Shah et al., 2000). LOD and LOQ can be estimated using different methods. The estimation of LOD is commonly performed by signal to noise ratio (S/N). For estimation of the detection limit, an S/N value of three is generally considered acceptable. It can be estimated by employing linear calibration curve, with a standard deviation of the *y* intercept (*sy*) and the average slope of calibration curve (Avg_m) (Toxicology, 2013). This approach is helpful when the method has no background noise. The detection is estimated using the following Equation:

Equation 6:LOD

$$LOD = \frac{3.3sy}{Avgm}$$

Similarly, the LOQ can be estimated using different methods. When the method shows background noise, it can be determined based on a signal to noise ratio of 10. Alternatively, the following Equation (Equation 7) can also be used for its

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estimation. The symbol “б” appears in the equation refers to the standard deviation of the response, while the symbol “S” represents the calibration curve slope.

Equation 7:LOQ

$$LOQ = \left(\frac{10б}{S}\right)$$

Recovery is related to the extraction efficiency. It represents the ratio of peak area response of an analyte from an extracted sample to the detector response of the analyte from an unextracted sample with the same amount of analyte that was added to the extracted sample. It can be calculated using Equation 8 (Shah et al., 2000). Recovery should not be reproducible more than ±15%.

Equation 8:Extraction recovery

$$\text{Recovery } \% = \left(\frac{Peak\ area\ ratio\ of\ extracted\ standards}{Peak\ area\ ratio\ of\ unextracted\ standards}\right) \times 100$$

a. Results and discussion

i. Linearity method

Six concentrations of the standard in DFU were prepared with and each of them was extracted three times in different days. The calibration graph was drawn. As listed in 3.2.8.1, the concentration ranges including blank sample were the same. In addition, three quality controls (QCs) at concentrations of 15 (QC Low), 75 (QC Mid) and 150 (QC High) µg /ml were prepared. Figure 5 represents the calibration curve of concentration of the target analyte (X-axis) against the peak area ratio (y-axis). The curve represents a strong positive linear relationship (R^2> 0.99).

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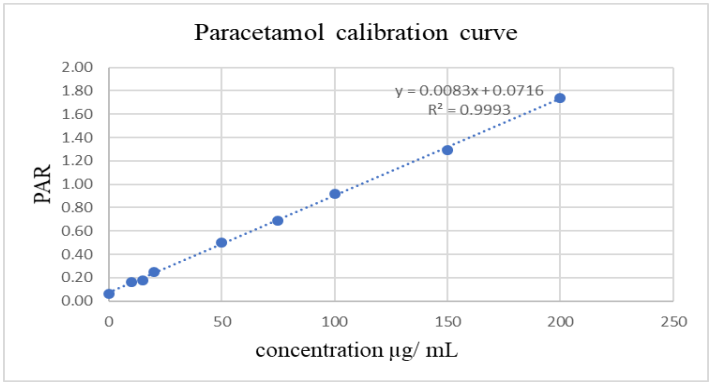

Figure 5: Calibration curve of Paracetamol using hydrolysis enzyme from limpets (*Patella vulgate)*

ii. Accuracy and precision method

The accuracy and precision were calculated for each calibrator point and quality control. Table 3 and Table 4 represents the outcomes of accuracy, intra- and inter-day precision evaluation. The relative standard deviations values did not exceed the acceptable limit identified by the Scientific Working Group for Forensic Toxicology (all of them were in the range ±≤20%).

Table 3:Accuracy results

Calibrator (µg/ml)	Mean calculated conc. (µg/ml)	Accuracy (%)	Standard deviation	%CV
10	11.8	118	0.4	3.8
20	22.6	113	0.5	2.0
50	51.6	103	0.2	0.4
100	102.0	102	0.8	0.8
200	199.2	100	0.4	0.2

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Low QC (15)	14.0	93	0.3	2.2
Mid QC (75)	74.5	99	0.8	1.0
High QC (150)	150.0	100	6.4	4.2

Table 4: Inter-day and intra-day precision and accuracy of HPLC method for paracetamol

Concentration spiked (µg/ml)	**Mean**	**SD**	**RSD%**	**Accuracy%**
Intra-day (n=5)				
15	14.3	0.25	1.75	95
75	70.7	0.04	0.05	94
150	146.1	0.06	0.04	97
Inter-day (n=5)				
15	13.0	1.25	9.57	87
75	71.9	2.03	2.82	96
150	147.6	1.01	0.69	98

iii. Determination of Limit of Detection and Quantitation

Based on the SWGTOX Guideline, LOQ and LOD were estimated using the Standard Error of the Y-Intercept. Urine was spiked to yield concentrations of 10, 20, 50, 100, 200 µg/ml. At each of the five concentrations, triplicates were analysed. Microsoft Excel (2016 software) was used to calculate the equation of the line for each drug. The LOD and LOQ were estimated using equations 6 and 7, respectively (Table 5).

Table 5: Limits of Detection and Quantitation

Parameter	µg/ml
LOD	0.9
LOQ	2.8

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iv. Specificity

Spiked samples were used to identify assays specificity. The procedure is performed by confirming that the method results are not influenced by the presence of excipients or impurities, in comparison with blank sample and sample containing both paracetamol and dihydrocodeine (DHC). When comparing the two chromatograms for blank and mixture sample, the method response was shown to paracetamol only.

v. Recovery

The recovery of the method was determined by employing triplicate spiked urine samples. The samples included three different concentrations (15, 75 and 150 µg/ml). The analysis of the samples was performed in one day using SPE. A constant amount of the ISD was added for each sample. The sample preparation was applied using SPE. The ISD was added prior to the evaporation step for unextracted samples (Table 6)

Table 6: Percentage extraction recoveries

Concentration (n=3) µg /mL	%Recovery
15	105
75	94
150	106

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7. Conclusion

When analysing glucuronide metabolite, there are several important factors that should be considered to optimize hydrolysis using β-glucuronidase. Examples of these factors are the source of enzyme and its concentration, incubation time and temperature, and hydrolysis PH. However, the current study revealed that the hydrolysis incubation times was the most critical factor to be considered when doing enzymatic hydrolysis of glucuronide metabolites in urine samples. The source of enzyme can be important in some cases, as the hydrolysis process of some glucuronide metabolites was optimized with a specific β-glucuronidase enzyme. Nevertheless, most of the optimization studies suggest that optimal hydrolysis conditions are largely compound dependent. The current study has important recommendations for future research. Considering the role that the PH of the reaction can play in optimizing enzymatic hydrolysis procedure, there is a need to investigate the ability of the five β-glucuronidase enzymes to detect paracetamol in urine samples using different pH values. Finally, the current study used five types of β-glucuronidase enzymes.

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