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Purification of Synthetic Bilirubin Glucuronides by Reversephase HPLC Followed By On Column Solvent Exchange

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Abstract – Impaired bilirubin conjugation, caused by inhibition of UGT1A1, can result in clinical consequences, including jaundice and kernicterus. Thus, Evaluation of the ability of new drug candidates to inhibit UGT1A1- Catalysed bilirubin glucuronidation in vitro has become common Practice. However, the instability of bilirubin and its glucuronides presents substantial technical challenges to conduct in vitro bilirubin glucuronidation assays. Furthermore, because bilirubin can be diglucuronidated through a sequential reaction, establishment of initial rate conditions can be problematic. To conduct these studies need high pure Bilirubin glucuronides. Purification and post purification concentration of synthesized bilirubin glucuronides is often the most difficult part of the synthetic procedure. In this study we describe the purification of synthetic bilirubin glucuronides by reverse phase HPLC, minimizing and reducing expose to light and temperature of pure fraction by on column solvent exchange process.

Keywords: Purification, Bilirubin, On Column Solvent Exchange, Fraction Minimizing.

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INTRODUCTION

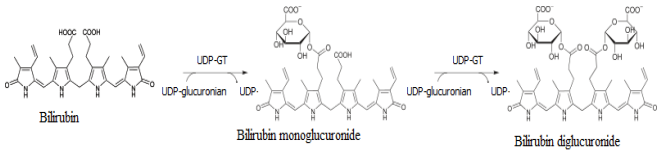
Bilirubin the major degradation product of heme. Bilirubin is a toxic waste product; approximately 250 to 400 mg of bilirubin are produced each day. Glucuronidation of bilirubin, catalysed primarily by UGT1A1, is an obligatory step for bilirubin elimination and takes place in the liver and intestine, In this reaction, a glucuronosyl moiety is conjugated to one of the propionic acid side chains, located on the C8 and C12 carbons of the two central pyrrole rings of bilirubin, resulting in two monoglucuronides (BMGs), which can be further glucuronidated, forming an 8,12-diglucuronide (BDG) is extracted by mammalian liver cells. Unconjugated bilirubin is insoluble in water, and once released in the vascular space, it will bind to plasma albumin with high affinity. The albumin-bound bilirubin is transported to the hepatocytes, where it is mono- (15%) or di-esterified (~85%) with glucuronic acid. The resulted conjugated bilirubin is water-soluble and secreted through the biliary system. None of these pure conjugates are not available and has not been isolated in pure form.

Bilirubin is a reflection of hepatic and biliary system functions, haemolytic disease, transfusion reaction as well as inherited bilirubin metabolism disorders. Considering these clinical Implications of abnormal level of bilirubin, an accurate assessment of bilirubin concentrations in blood is clinically important. Currently, most clinical laboratories measure conjugated (direct) and total bilirubin using a spectrophotometric method. Other methods include enzymatic assay using bilirubin oxidase [1] and vanadate oxidation assay [2].

It is well known that bilirubin is a photosensitive and temperature sensitive substance, undergoing both photo isomerization and photo oxidation. Previous studies have shown that direct bilirubin undergoes Isomerization and hydrolysis of glucuronic acid esters of bilirubin which could art factually inflates the proportion of unconjugated bilirubin present [3].

Purification of Bilirubin glucuronides is often the most difficult part of the process used to obtain the compounds. Several methods are used for isolation and subsequent purification of the post-synthesis mixture (Synthesis process not disclosed).

Standard method for isolation is high-performance liquid chromatography [4, 5],



which enables separation of substances with negligible differences between their chemical structures. In this paper we describe the Reverse phase purification of bilirubin glucuronides. We show, that bilirubin glucuronides can be purified by Reverse phase chromatography, one-step procedure furnishes products of >90% purity. Moreover no need for sophisticated equipment and consumption of mobile phase is minimal.

We postulate that use of on column exchange could be especially beneficial in isolation of bilirubin glucuronides of the various post purification procedures, these purified bilirubin glucuronides are Light and temperature sensitive so evaporation to dryness of aqueous/organic matrices is the most time consuming and represents the bottleneck of the whole purification operation (within our laboratories). During this procedure, the collected target material must be recovered intact from the light, temperature and containing low-volatility solvents (particularly water) and condiments. Precautions against sample decomposition, salt formation, must be taken, because any errors in this step will affect the purity of the final product.

Reduce the time taken for solvent evaporation, avoid salt formation, and reduce exposure to heat and light (while still ensuring that no residual solvent remains), an investing-ation into the removal of water and ammonium acetate additive from the fractions before the evaporation step was initiated. Various criteria were defined, not least that the quality of the final product must be maintained or improved

MATERIAL AND METHODS:

HPLC-grade acetonitrile (Merck, Darmstadt, Germany), Water (Millipore, Bedford, MA), were used in the preparation of the mobile phases. Analytical grade chemicals and solvents used for synthesis and buffer preparation .The HPLC system was fully composed from Waters 4000 Series modules and controlled by Empower software.. Inertsil ODS (5-µm particle size, 100-Å pore size) was purchased from GL sciences (Tokyo, Japan).

INSTRUMENTATION:

Waters Delta Prep 4000 preparative chromatography system with waters prep LC Controller, Waters 2487 Dual λ Absorbance Detector, water fraction collector, Waters Prep Pump. The injector, fraction collector and tubing's were closed with aluminium foil to protect from light. Instrument was controlled using Empower 3 (Waters, Milford, MA).To reload fractions to the column added six port valve before the gradient pump (Figure 1) this valve operated by manually.

PREPARATIVE SEPARATION OF BILIRUBIN GLUCURONIDES

Crude mixture were purified by preparative RP- HPLC using a ODS C18 column (20 × 250 mm) with 10mM ammonium acetate in water (Mobile phase A), Acetonitrile(Mobile phase B) at a flow rate of 18 ml/min with HPLC gradient 90% mobile phase A to 90%mobile phase B over 20minutes .

Preparative system is stabilized with Protocol A (Figure2) by changing solvent selection value in to mobile phase mode. A known accurate weight of Crude (approximately 1.0 g) was dissolved in 7.0ml of mobile phase (50:50). Preparative system is 5min stabilized with initial gradient conditions and 2mL crude solution was injected into the system and eluted with gradient program. Collected required BMG and BDG as fractions in a vessel which is protected from light and surrounded by ice cold water bath maintain a temperature of 5°C .Same process repeated and injected total crude solution and collected Pure BMG and BDG fraction with combination of Aqueous buffer and acetonitrile

MINIMIZING PURE FRACTION AFTER PURIFICATION:

If we use regular process to remove the aqueous buffer from the fraction the purity of fraction may effect because required more expose time to light and temperature [6, 7]. So to eliminate this expose we used on column solvent exchange process in this process pure fraction reloaded on column to minimize the fraction volume to eliminate water, ammonium acetate.

Preparative system mobile phase A is replaced with mille-Q water and mobile phase B is kept constant, ODS C_{18} (20X250mm) column is washed with 18ml/min flow and stabilized with Mobile phase A for 5min, Solvent selection valve is turned to protocol B (Figure3) to load diluted pure BMG directly to column head with same flow, total fraction loaded on the column and solvent selection valve turn back to Protocol A.

An isocratic hold in Mobile phase A for 3 min was followed by an isocratic hold in Mobile phase B for 5min to elute pure BMG in acetonitrile and eluted fraction was collected, in total cycle flow was kept constant. Same process was followed for BDG also.

For a typical fraction volume of 15 mL of both BMG and BDG was collected in amber colour flask, the final fractions was lyophilized to remove water and acetonitrile. Lyophilized pure compounds purity was

recorded with High performance liquid chromatography.

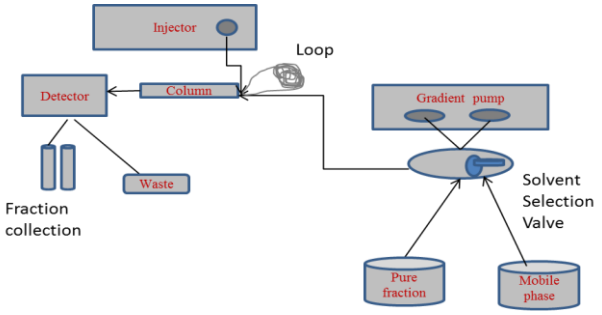


Fig1. Schematic of the modified Waters Instrument configured to perform both preparative separations and on-column solvent exchange using direct-injection and at column-dilution injection protocols

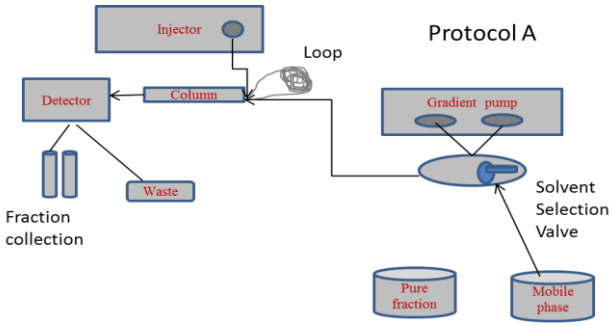


Fig2. In protocol A the modified system performs as regular system the mobile phase is pumped through the solvent section valve.

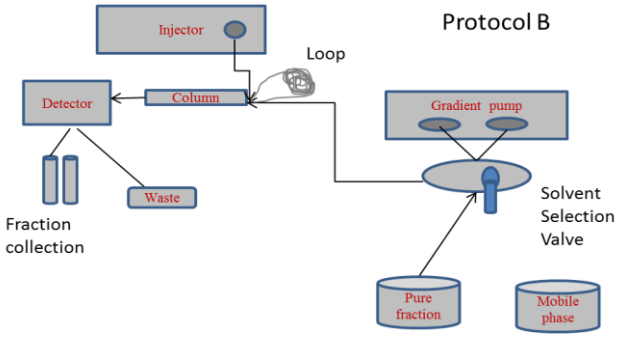


Fig3. In protocol B the modified system performs as on column-Exchange system the pure fraction was pumped through the solvent section valve.

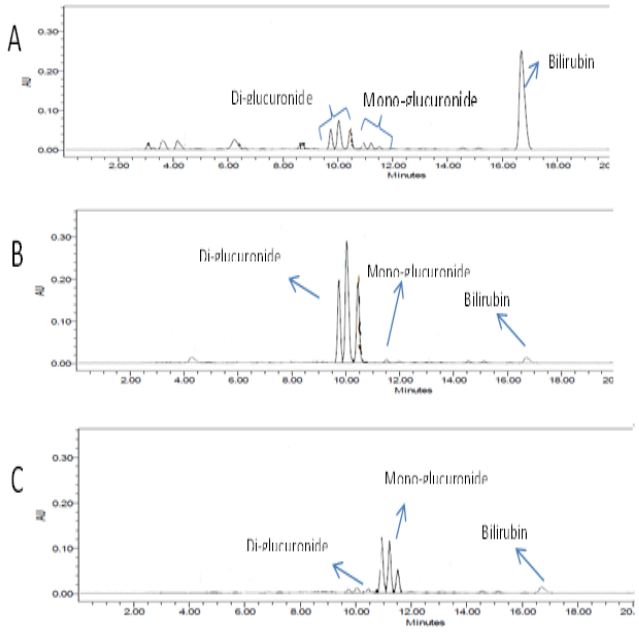


Fig4. HPLC analysis of Bilirubin with its glucoronides and purified individual BDG ,BMG using inertsil ODS (4.6X250)mm,5µ column with mobile phase conditions 10mM ammonium acetate and Acetonitrile with gradient program A) Crude bilirubin glucronides B)92% pure BDG after purification. C) 90% pure BMG after purification.

RESULTS AND DISCUSSION:

The aim of our experiments was to obtain highly pure bilirubin glucoronides relatively quickly. RP-HPLC followed by on column exchange is an easy and inexpensive means of purification and concentration of BDG and BMG. It enables rapid and efficient purification of glucuronides with minimal consumption of organic solvents and eliminates the need for sophisticated equipment.

At the outset of this study, a procedure to reduce the decomposing of these glucuronides potential for salt formation, residual solvent and sample exposure to elevated temperatures and light for extended times during evaporation was envisaged. It was critical that this on-column solvent-exchange procedure did not decrease the quality of the final product or the amount recovered from our automated purification platform. It was also important that the cycle time from sample submission to availability for further testing did not increase. A final requirement was that minimal capital expenditure would be required to achieve this goal.

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