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Rapid small intestinal permeability assay based on riboflavin and lactulose detected by bis-boronic acid appended benzyl viologens

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ABSTRACT

Background: Although organoboronic acids are efficient high-throughput sugar sensors, they have not been pursued for gut permeability studies. A modification of the lactulose/mannitol assay is described by which small intestinal permeability is assessed at the time of urine collection using a lactulose/riboflavin ratio.
Methods: Volunteers ingested 50 mg riboflavin and either 5 g mannitol or 10 g lactulose. Urine was collected for 6 hrs. Riboflavin was assayed by autofluorescence. Riboflavin was removed by C18 solid phase extraction. Lactulose and mannitol were then assayed using 1,1'-bis(2-boronobenzyl)-4,4'-bipyridinium (4,4'oBBV) coupled to the fluorophore HPTS.
Results: The temporal profile over 6 hrs for riboflavin paralleled mannitol. Riboflavin recovery in urine was 11.1 ± 1.9 % (mean ± SEM, n = 7), similar to mannitol. There was selective binding of 4,4'oBBV to lactulose, likely involving cooperativity between the fructose and galactose moieties. Lower limits of detection and quantification were 90 and 364 μM. The lactulose assay was insensitive to other permeability probes (e.g., sucrose, sucralose) while tolerating glucose or lactose. This assay can be adapted to automated systems. Stability of 4,4'oBBV exceeds 4 years.
Conclusions: Riboflavin measured by autofluorescence combined with lactulose measured with 4,4'oBBV represents a useful new chemistry for rapid measurement of intestinal permeability with excellent stability, cost and throughput benefits.

1. Introduction

Intestinal permeability allows for restrictive leakage of molecules and ions below ~0.4 nm (MW ~250 Da) from the gut lumen into blood circulation. This "paracellular" leakage occurs through tight junctions between epithelial cells comprising the intestinal mucosa and varies between individuals. Other molecules pass through the microvilli of the epithelial cells by "transcellular" transport. Elevated paracellular leakage has been implicated in many human disorders with immunological components, including type 1 diabetes mellitus [1], obesity and type 2 diabetes mellitus [2], inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis), celiac disease and irritable bowel syndrome [3–5], Parkinson's disease [6], environmental enteropathy [7] and cancer [8]. There are comparable associations to laminitis in horses [9]. Relevant to human and veterinary medicine are drug induced lesions and gut toxicity, as for instance by chemotherapy [10] or NSAIDs [11]. Future researchers, drug developers and care givers working with diseases not traditionally within the gastroenterology domain will seek assistance from this discipline due to rising interest to quantify gut permeability.

Gastroenterology units have limited resources to assay large numbers of urine samples for gut permeability to meet the anticipated demand. The classical sugar absorption test for small intestinal permeability uses lactulose to quantify abnormal paracellular leakage [12]. This is referenced against mannitol, giving a lactulose/mannitol ratio. Although mannitol is absorbed by the paracellular route, a low mannitol value in this test is regarded to reflect nutrient deficiency through reduction in nutrient transporters. Neither lactulose nor mannitol has

Abbreviations: ELSD, evaporative light scatter detector; NSAID, non-steroidal anti-inflammatory drug; HPTS, 8-hydroxypyrene-1,3,6-trisulfonate; 4,4'oBBV, 1,1'-bis(2-boronobenzyl)-4,4'-bipyridinium; 4,4'oMBV, 1-benzyl-1'-(2-boronobenzyl)-4,4'-bipyridinium, CV%, coefficient of variation; LLOD, lower limit of detection; LLOQ, lower limit of quantification; RFT2, type 2 riboflavin transporter; S/N, signal to noise ratio; SPE, solid phase extraction, EtOH, ethanol; MeCN, acetonitrile; MeOH, methanol; NAD(P), NAD or NADP; NAD(P)H, NADH or NADPH.

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intrinsic absorbance or fluorescence permitting rapid, direct quantification. Typically, two different HPLC configurations (e.g., ion exchange and reverse phase) are coupled to expensive detectors, such as mass spectrometers [13]. Alternatively, NAD(P)H-coupled enzyme assays are used [14,15], but require considerable time and cost.

Recent years have seen breakthroughs in molecular biology and organic chemistry that should facilitate simpler, faster and less expensive assessment of small intestinal permeability. For instance, riboflavin has been found to be almost entirely absorbed through the type 2 riboflavin transporter (RFT2) at apical epithelial membranes in the small intestine [16]. Loss of RFT2 expression results in severe riboflavin deficiency [17]. Hence, appearance of riboflavin in urine during 6 hrs after oral ingestion reflects absorption at duodenum and jejunum. Riboflavin's intrinsic fluorescence suggests a methodological advantage over mannitol while reflecting absorption of an actual nutrient.

Much work has been done in using organoboronic acids to quantify sugars [18–20]. Focus has been on glucose related measurements, and these have had success in clinics; eight validated HbA1c assays employ organoboranes [21]. However, organoboronic acids are typically more sensitive to fructose and to lactulose, likely due to the fructose moiety. Although less sensitive, they can also bind mannitol. We reasoned that the intestinal permeability test could be simplified by 1) replacing mannitol with riboflavin and 2) removal of riboflavin by C18 solid phase extraction and measuring lactulose with an organoboronic acid coupled to a fluorophore. Benefits include rapid assessment of urine samples and negligible price. Reagents can be stored on ready to use plates in advance of measurement. We therefore synthesized the bis-boronic acid appended viologen 1,1'-bis(2-boronobenzyl)-4,4'-bipyridinium (compound **1**)(4,4'oBBV). For comparison, a mono-boronic analog (compound **3**)(4,4'oMBV) with increased water solubility was also synthesized (Fig. 1). Both can be coupled to fluorophores. These were first used to determine if riboflavin can replace mannitol and then to quantify lactulose.

2. Methods

2.1. Preparation of boronic acid viologens 1 (4,4'oBBV) and 3 (4,4'oMBV)

The reaction scheme with compound numbering is shown in Fig. 1a. Synthesis of 4,4'oBBV was as we reported earlier [22]. For 4,4'oMBV, 2-bromomethylphenyl boronic acid was reacted with excess 4,4'-bipyridyl in acetone to afford the mono-substituted 4,4'bipyridyl adduct (compound **2**). Combining excess compound **2** with benzyl bromide in a solvent mixture of MeCN and MeOH yielded 4,4'oMBV (compound **3**) after precipitation from the reaction mixture with acetone. Reagents and conditions were: (i) dimethylformamide, 55 °C, 48 hrs, 90% (compound **1**); (ii) acetone, 25 °C, 2 hrs, 70% (compound **2**); (iii) MeCN, MeOH, 55 °C, 24 hrs, 86% (compound **3**). Chemicals were from Sigma Aldrich (St Louis MO, USA) unless stated otherwise.

Fig. 1b illustrates the molecular mechanism behind the organoborane based fluorescent lactulose assay. The sensing ensemble is comprised of an anionic fluorophore, 8-hydroxypyrene-1,3,6-trisulfonic acid

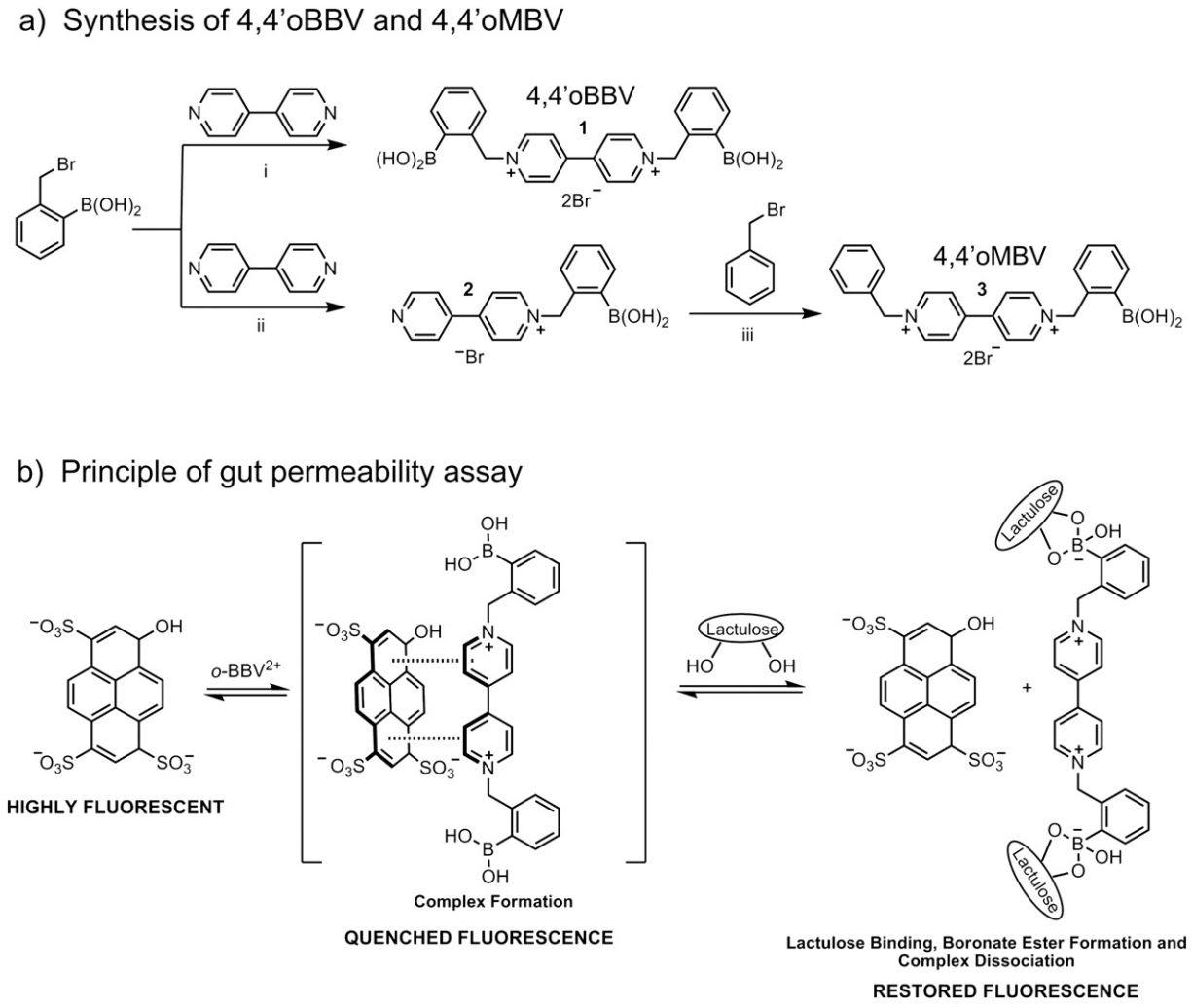

Fig. 1. Synthesis of organoborane sugar sensors (a) and principle of fluorescence assay for urine lactulose or mannitol (b).