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RESEARCH ARTICLE

IMPROVEMENT OF "LACTULOSE-MANNITOL RECOVERY RATIO TEST" OF INTESTINAL ABSORPTION BY LC-MS\MS

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ABSTRACT: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has seen enormous growth in clinical laboratories during the last decades due to the fact that it offers analytical specificity superior to that of immunoassays or conventional high performance liquid chromatography. Then, most chemicals and metabolites are now assayed by LC-MS/MS. The "Lactulose\Mannitol recovery ratio (LAMA test)" is a non-invasive gastrointestinal test for the assessment of intestinal absorption and barrier function of the bowel. This article describes an optimized LC-MS/MS method used for the determination of these two sugars, characterized by rapidity, sensitivity, and easy automation.

KEY WORDS: Intestinal absorption, Lactulose, Liquid chromatography-mass spectrometry, Mannitol

INTRODUCTION:

LAMA TEST

The Lactulose\Mannitol recovery ratio (LAMA test) is a powerful noninvasive gastrointestinal test for the assessment of intestinal absorption and barrier function in the bowel.

The intestine is a digestive/absorptive organ for nutrients and an immune and mechanical barrier against bacteria, food antigens, and other macromolecules. Malabsorption and increased intestinal permeability ("leaky gut") slow down gastrointestinal activity leading to chronic imbalances and systemic disorders.

In particular, with the increased intestinal permeability there is an enhanced number of foreign compounds and bacteria antigens entering the bloodstream that lead to auto-immune processes and increased uptake of toxic compounds. As a consequence, the leaky gut is observed in pathologies such as inflammatory Bowel Diseases, Food allergies, inflammatory joint diseases and chronic dermatologic illness.

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Decreased intestinal permeability (Malabsorption), differently, can be due to intestinal infection, ingestion of allergenic foods or toxic chemicals, deficient secretory immunoglobulines IgA, trauma and endotoxemia, use of NSAIDs (non steroidal anti-inflammatory drugs). A proper example of malabsorption is the gluten-sensitive enteropathy[1].

The LAMA test measures the ability of two non-metabolized sugars (Lactulose and Mannitol) to permeate the intestinal mucosa. Specifically, the patient drinks a solution with measured amounts (1 g of Mannitol and 5 g of Lactulose) of these two substances. The degree of intestinal permeability or malabsorption is reflected in the levels of the two sugars recovered in the urine collected over the next 6 hours.

In particular, Mannitol is a monosaccharide that follows the transcellular routes of aqueous pores, reflecting the degree of absorption of small molecules. Lactulose, differently, is a disaccharide that only pass through the intercellular junctional complexes and extrusion zones at the villous tips, reflecting the permeability of large molecules. The LAMA test then compares the permeability of gut for mono and disaccharides through the excretion ratio of the unmetabolized molecules in urine. The strong variation in the administered Lactulose/Mannitol ratio indicates alteration of intestinal absorption. [2] This test is used in clinical practice for the diagnosis of patients with coeliac disease,[3,4] Crohn's disease,[5] atopic dermatitis,[6,7] cow's milk protein intolerance,[8] cystic fibrosis,[9] diarrhea[10] and HIV infection[11].

LC-MS\MS AS METHOD OF DETERMINATION FOR LACTULOSE AND MANNITOL

Mannitol and Lactulose were firstly determined by colorimetric and enzymatic methods.[12,13] However, these techniques were time-consuming and didn't allow simultaneous assay of both sugars. Then, gas chromatography and HPLC (high pressure liquid chromatography) have been proposed. However, gas chromatographic determinations required evaporation and derivatization of the samples before injection [14] while HPLC had poor sensitivity. To overcome this problem, HPLC was associated to an amperometric detector[15] or with a light scattering system.[16] Despite of this, modern approaches for determination of lactulose and mannitol in human urine use ultra high pressure liquid chromatography coupled with mass detector (UHPLC-MS or LC/MS).[17] Liquid chromatography–mass spectrometry is a powerful analytical technique that combines the physical separation capabilities of liquid chromatography with the detection specificity of mass spectrometry.[18] Briefly, liquid chromatography separates the components of the sample according to their chemical-physical characteristics. These elutes are then introduced to the mass spectrometer that creates and detects charged ions. The LC/MS data give information about the molecular weight and the quantity of each component. Such technique has very high sensitivity and is used for the detection, identification or purification of chemicals in complex mixtures.[18] LC/MS significantly expanded the effective analytical use of mass spectrometry to a greater number of organic compounds due to the fact that it is suitable for the analysis of large, polar, ionic, thermally unstable and non volatile compounds[19] along with the ability to combine high analytical specificity with high analytical sensitivity, and short chromatography run-times. Other advantages are easier workflows; higher throughput compared to conventional HPLC or GC-MS, and significantly lowers equipment costs.[20]

MATERIALS AND METHODS:

CHEMICALS

Lactulose and mannitol were obtained from Sigma Chemical Co. (Milan, Italy). LC-MS grade acetonitrile and water were purchased from Carlo Erba (Milan ,Italy).

INSTRUMENTS

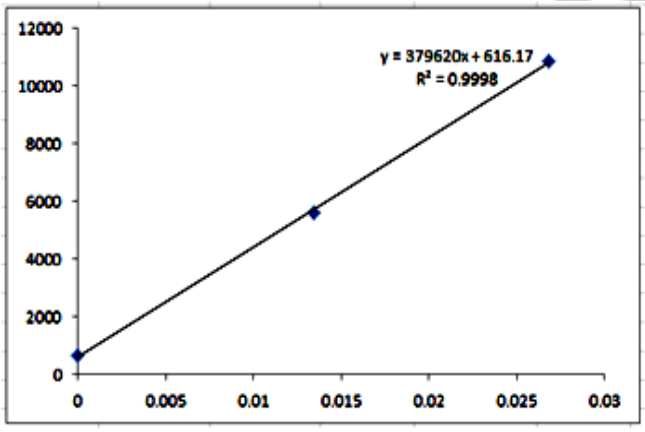
Chromatographic separation was obtained using a UHPLC Nexera XR LC-20AD (Shimadzu corporation. Milan, Italy). Mass detection was performed using a triple quadrupole LCMS-8060 (Shimadzu corporation. Milan, Italy).

PATIENTS PREPARATION

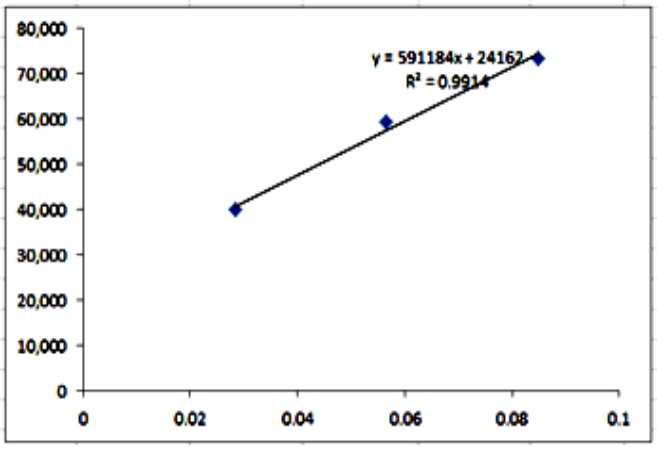
Prior to the analysis, patients are required to drink a solution containing 1 g of lactulose and 5 g of mannitol in 100 mL of pharmaceutical grade water. Urine was collected during the next 6 h. The total volume of urine was measured and an aliquot was analyzed.

SAMPLE PREPARATION

100 microliters of the collected urine are diluted with 400 microliters of a solution acetonitrile\ water 8:2 v\v. Calibration curve in urine is produced adding 100, 200, 300, 400 microliters of lactulose and mannitol standards (in the concentration of µg\ml) in acetonitrile\ water 8:2 regulating the volume of the solvent accordingly. Fig. 1 shows an example of the obtained calibration curve in urine.



(a)



(b)

Fig. 1 Calibration curve of lactulose (a) and mannitol (b) in urine

LC/MS CONDITIONS

Chromatographic separation was performed using a UHPLC Nexera XR LC-20AD (Shimadzu corporation. Milan, Italy) using as mobile phase formic acid 0,001% v/v in water (A) and acetonitrile (B) (isocratic 20:80 A-B). The stationary phase was obtained with a Kinetex reversed phase C18 column (Phenomenex. Bologna, Italy). Mass detection was performed using a triple quadrupole LCMS-8060 (Shimadzu corporation. Milan, Italy) in ESI+ mode. The instrument was optimized automatically by the built-in algorithm to monitor the 181 to 89.10 m/z and 181 to 101.10 m/z transitions for mannitol, 341.2 to 100.90 m/z and 341.20 to 160.90 m/z transitions for lactulose. Data were acquired and processed using LabSolutions software (Shimadzu corporation. Milan, Italy). Fig. 2 shows the obtained spectra.

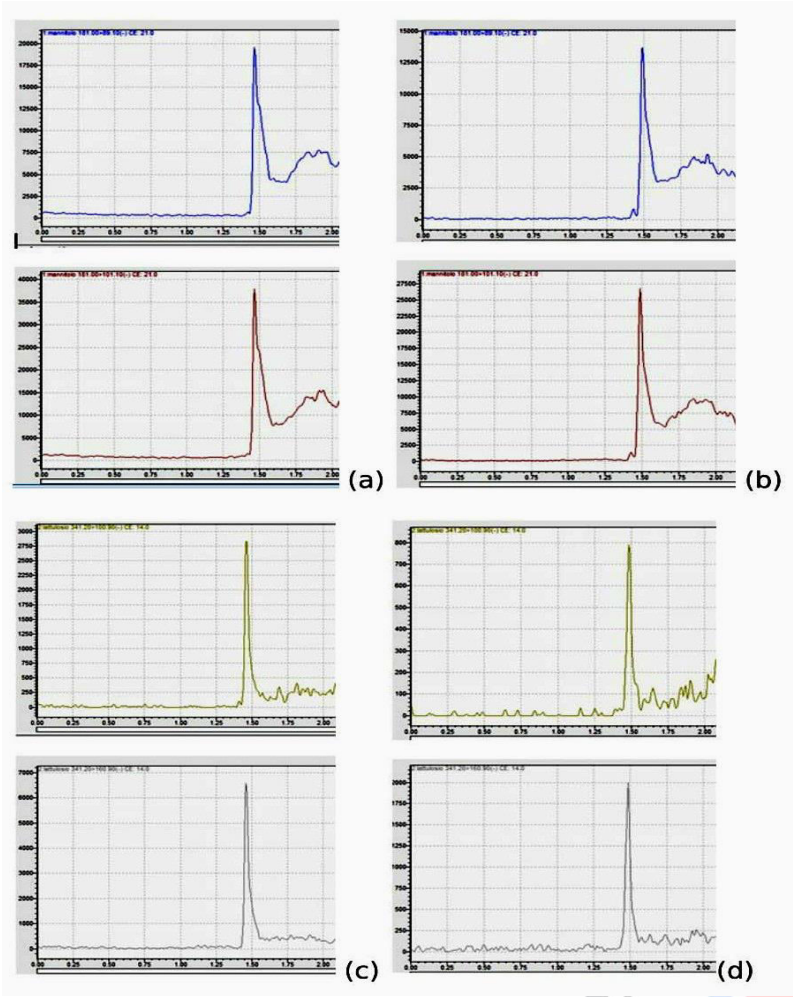


Fig. 2 (a) chromatograms of mannitol with standard addition. (b) chromatograms of mannitol without standard addition. (c) chromatograms of lactulose with standard addition. (d) chromatograms of lactulose without standard addition

RESULTS AND DISCUSSIONS:

The optimized LC-MS\MS method is routinely used in the toxicology analytical laboratory of AMES group (Casalnuovo di Napoli, Italy). In Fig. 3 is shown the utilized medical report.

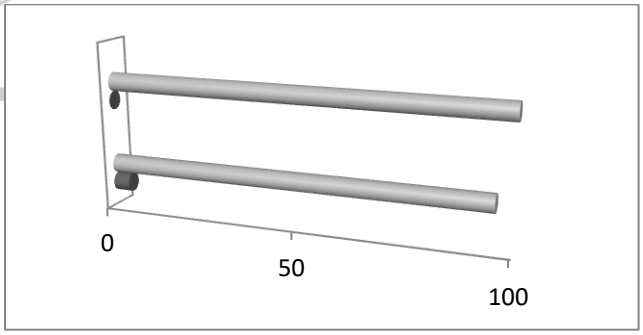
INTESTINAL PERMEABILITY EVALUATION (I.P.)

The analysis is performed following the administration of a solution containing 1g of Mannitol and 5g of Lactulose.

NAME SURNAME, --/--/---- (date of birth)

	RESULT	MEASURING UNITS	REFERENCE RANGE
LACTULOSE (method LC-MS/MS)	0.--	mg/mL	
MANNITOL (method LC-MS/MS)	0.--	mg/mL	
I.P. EVALUATION	0.---	Lactulose\Mannitol ratio	< 0.03

	RESULT	**REFERENCE RANGE**
% LACTULOSE RECOVERY	0.--	0.20 – 0.45 %
% MANNITOL RECOVERY	-.--	10-25 %



Responsible analyst
Dr. Raffaele Conte

Fig. 3 Medical report for LAMA test (in use at AMES group)

The great innovation of the LAMA test relates to the fact that bowel diseases are difficult to investigate; intestinal anomalies often require invasive examinations because noninvasive tests are almost always nonspecific for the diagnosis of diseases involving damage of the intestinal mucosal. Instead, the described method has the advantages of being rapid, precise, and accurate. The use of LC-MS eliminates any interfering substance in the chromatograms and does not require additional purification.

Data collected in AMES revealed that the excretion ratio of lactulose/mannitol found in healthy subjects (mean ± SD) is 0.02±0.005. These values are comparable with those reported by other authors who use similar protocols[21]. In conclusion, this technique is efficient and easy to automate for the study of intestinal permeability in clinical practice.

CONCLUSION:

The old methods for lactulose\mannitol ratio analysis require time-consuming sample preparation and purification steps before the chromatographic separation. Moreover, the detection limits of these method are often high. Differently, the described LAMA test is rapid, sensitive, and easy to automate for the study of intestinal permeability in clinical practice.

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