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# CAPILLARY ELECTROPHORESIS METHOD FOR THE DETERMINATION OF LACTULOSE AND MANNITOL RATIO FOR ESTIMATING INTESTINAL PERMEABILITY

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## SUMMARY

In this study, a simple, rapid and accurate capillary electrophoresis method has been developed for the determination of lactulose and mannitol in urine for estimating the intestinal permeability. The urine samples were prepared to analyze by using solid phase extraction. For analysis a background electrolyte (BGE) consisted of 6 mmol/L sorbic acid, 1.25mmol/L hexadecyltrimeth ylammoniumbromide (HDTMA), 50 mmol/L LiOH (pH 12.5) was used. Separation was performed at -5 kV over 20 min within the fused capillary column (45 cm, 50 μm I.D.) at 20 °C. The method developed was applied a series of urine sample to measure the lactulose/mannitol concentration rate.

**Key words:** Lactulose, Mannitol, Determination, Permeability, Capillary electrophoresis

## ÖZET

Bu çalışmada intestinal geçirgenliğin incelenmesi amacıyla idrarda laktuloz ve mannitol tayini için basit, hızlı ve doğru bir kapiler elektroforez yöntemi geliştirilmiştir. İdrar örnekleri katı faz özütleme yöntemi ile analize hazırlanmıştır. Analizler için 6 mmol/L sorbik asit, 1.25 mmol/L hekzadesil trimetil amonyum bromür (HDTMA), 50 mmol/ L LiOH içeren pH sı 12.5 e karşılık gelen destek elektrolit kullanılmıştır. Ayrım 45 cm 50 μm iç çaplı fused silika kolonda 20 °C sıcaklıkta -5 kV ayırma potansiyeli kullanılarak 20 dk içerisinde gerçekleştirilmiştir. Geliştirilen yöntem laktuloz / mannitol derişim oranlarını ölçmek için bir seri idrar numunesine uygulanmıştır.

**Anahtar Kelimeler:** Laktuloz, Mannitol, Tayin, Geçirgenlik, Kapiler elektroforez

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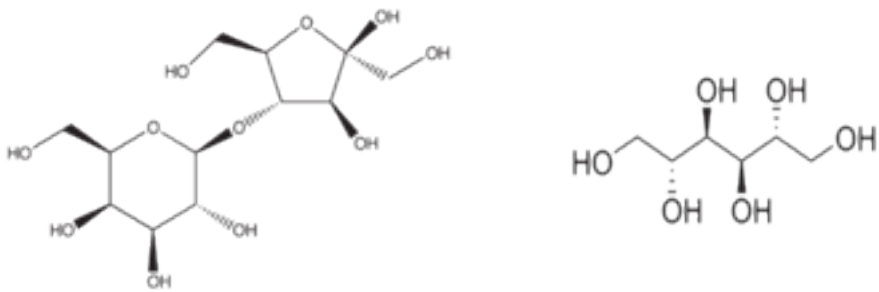
## INTRODUCTION

The pathogenesis of many small intestinal diseases entails interaction between changed intestinal permeability, luminal aggresive factors and mucosal defence mechanisms. Each of these factors can be implicated primarily in the pathogenesis of disease [1].

The test of intestinal permeability was specifically designed to test intestinal barrier function [1]. Intestinal permeability, expression of altered mucosal barrier function, may be assessed noninvasively by measuring urinary excretion after oral administration of test substances [2]. For this test, the ideal permeability probe should be nondegradable, nontoxic, nonmetabolized and water soluble and the measurement should be easy, sensitive, accurate and affordable. The sugar mixtures have the advantage of being nonradioactive and their differantial excretion corrects for pre- and postmucosal factors such as gastric emptying, intestinal dilution and bacterial degradation [2].

The sugar alcohol Mannitol (MAN) (~5-7 Å) permeats the intestinal mucosa via a transcellular pathway through the water-filled pores on the cell membrane, whereas the disaccharide, Lactulose (LAC) (~10-12 Å) uses a paracellular route through the intercellular junctional complexes between adjacent enterocytes and extrusion zones at the villous tip (Figure 1). If the mucosal barrier is damaged as in celiac disease, Crohn' s disease, atopic dermatitis, cow' s milk proteins intolerance, cystic fibrosis, diarrhea, HIV infection and diabetes, the gut is more permeable to intact sugars and proteins [3].




(a) (b)

**Figure 1-** Chemical structure of (a) lactulose and (b) mannitol

Several methods have been reported for the determination of lactulose and mannitol for estimating the intestinal permeability by different techniques. Capillary electrophoresis [3], HPLC/Amperometric/light scattering detection [4-8], gas chromatographic method [9] were used for the determination of the Lactulose/Mannitol ratio. The development of methods for assessing the intestinal barrier function became feasible after the introduction of non-metabolizable oligosaccharides as test substances in the 1970's [10]. The oral administration of sugars and the subsequent measurement of these substances in the urine, denoted the intestinal permeability test, is a non-invasive method that has been used for the assessment of the integrity of the epithelial barrier [11].

In this study a capillary electrophoretic method was presented to estimate the intestinal permeability by measuring lactulose/mannitol rate. The method used for capillary electrophoretic prameters of LAC- MAN analysis was modified from a literature [3]. In this study, easy and cheap LAC-MAN extraction method from urine was developed, by using hand made cartridges for solid phase extraction step.

## MATERIAL AND METHOD

*Chemicals and Reagents*

LAC and MAN standard materials were supplied from Sigma ( St.Louis, MO, USA). All used reagents were of analytical reagent grade. Stock solutions of LAC and MAN were prepared in urine (1mg/ml). LiOH, Sorbic acid and hexadecyltrimethyl ammonium bromide (HDTMA) were purchased from Sigma (St.Louis, MO,USA). Ultra pure water was obtained by using Elga water purification system (UK).

*Instrumentation and Analytical Conditions*

Capillary electrophoretic measurements were carried out using Agilent 3D Capillary Electrophoresis system with DAD detector. A fused silica capillary column with 45 cm, 50 µm I.D. was used for analysis. In this method reversed polarity mode was used and LAC and MAN were monitored by indirect UV detection using sorbic acid (300,2 Ref:254,2) . Separation was performed at -5 kV over 20 min within the capillary column at 20 °C using background electrolyte consisted of 6 mmol/L sorbic acid, 1.25mmol/L HDTMA, 50 mmol/L LiOH (pH 12.5). A washing programme was used

for the first usage of column, of which steps were washing with 1M LiOH for 20 min, 0.1 M LiOH for 20 min and water for 20 min respectively. The calibration curve was prepared by plotting peak height vs. concentration of LAC and MAN.

*Sample Processing*

*Preparation of Standard Solutions and Quality Control Samples*

Urine samples were purified prior to analysis using solid phase extraction. 12 mL urine samples were taken and centrifuged. 9-10 mL supernatant was transferred tubes containing 0.4 g Amberlite IR-120 and 0.6 g amberlite IR-400 resins and shaken for 1-2 min, centrifuged for 10 min 3500 rpm. After centrifugation step 6 mL supernatant were filtered from hand made solid phase column and the final eluat were filtered from 0.45 μm millipore filter, and stored in -80 °C until assayed. Standard solutions and patient samples were prepared by using same procedure.

Hand made cartridges was prepared by using insuline syringe. A piece of glass wool was inserted in syringe and then filled with 0.5 g silicagel 40. A piece of glass wool was inserted on silicagel surface and suppresed to prevent uprising of silicagel particules (Figure 2). The prepared columns conditioned with methanol and equilibrate with water.

*Real Sample Processing*

Urine samples were collected according to following procedure : After 8 h fasting period, 2 g MAN, 5 g LAC and 5 g glucose dissolved in 300 mL distilled water were given to patients. At the end of this time amount of urines were measured and purifiying procedure explained in previous section was applied to samples. Same procedure was applied for preparing standard solutions [5].

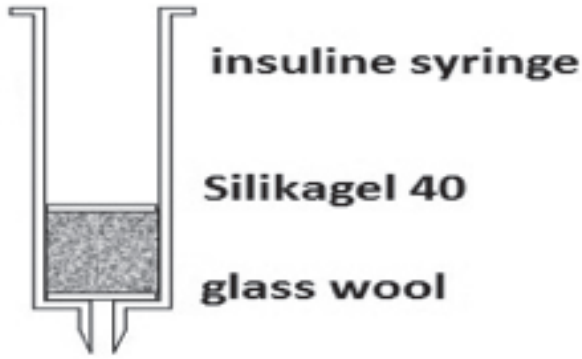



**Figure 2-** Hand Made Amberlite

## RESULTS AND DISCUSSION

LAC and MAN are weak acidic compounds having pKa 10.14 and 13.50 respectively [12- 13]. In capillary zone electrophoretic analysis, only ionised species can be separated and these sugar molecules should be in ionised form for their determination. According to acid- base equation molecule can be ionised form at pH values higher than its pKa value. Thus high pH background electrolyte use to analyse and to stable ionised form of these sugar molecules.

In classic capillary zone electrophoresis system electro osmotic flow (EOF) is composed when studying at pH values higher than pH 7. EOF causes moving of background electrolyte through from anode end of column to cathode end. Due to the EOF, ionised species move to detector and detector observes cation, neutrals and anions respectively. If anions are wanted to observe firstly, one should turn way of EOF to opposite direction. Some materials like HDTMA can be used to achieve this. Thus anions can be observe before neutrals and cations. In consideration of all reason background electrolyte consisted of 6 mmol/L sorbic acid, 1.25 mmol/L HDTMA, 50 mmol/L LiOH (pH 12.5) was used for analysis.

***Specifity and Selectivity***

Six different sources of blank urine samples were analysed for selectivity and specificity. Filtered solutions obtained were measured by the capillary electrophoretic method developed. Electrophoretic separation of LAC -MAN was optimized to provide acceptable resolution, good peak shape and inten-

sity of the response. Figure 3 shows comparison of the electrophoregrams obtained for the extracted blank urine sample and the extracted spiked urine sample respectively.

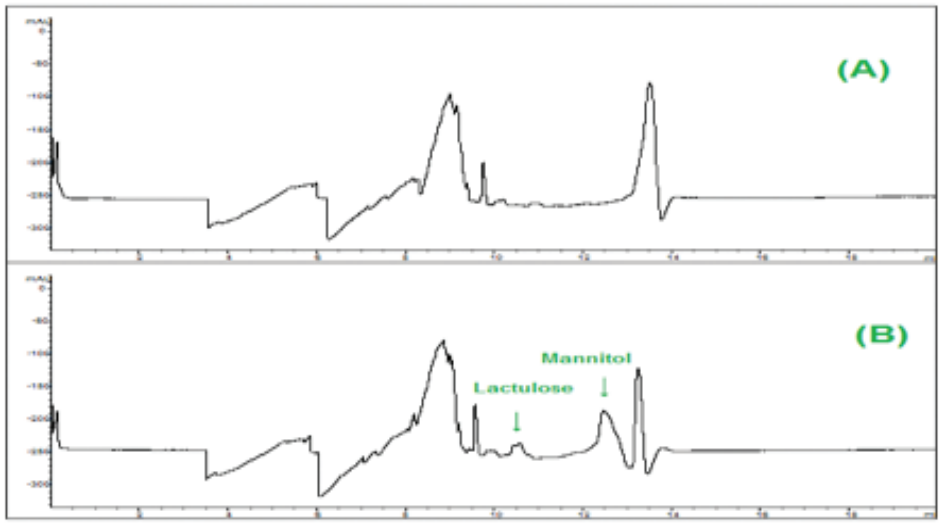



**Figure 3**- Obtained electrophoregrams under optimum conditions: 20° C, -5 kV 20 min, 45 cm 50 mm I.D. Column, 6mmol/L sorbic acid, 1.25 mmol/L hexadecyltrimethylammonium chloride, 50 mmol/L LiOH, DAD Indirect UV measurement (300,2 Ref:254,2) (A) Blank (urine sample) (B) Spiked urine sample containing 0.2 mg/mL Lactulose, 1.0 mg/mL Mannitol

***Linearity and Sensitivity***

The standard calibration curve was linear over the concentration range from 0.02 – 0.40 mg/ mL, 0.30 – 2.00 mg/mL respectively for LAC and MAN. The LOQ was 0.02 mg/mL for LAC and 0.30 mg/ mL for MAN. The calibration curves had regression equations of y=73777x+3.6021 for LAC and of y= 65766x+8.9730 for MAN where y is the peak area and x is the concentrations. Calibration curve parameters obtained of method were given in Table 1.

**Table 1-** Analytical parameters of developed capillary electrophoretic method for analysis of Lactulose and Mannitol

| Parameter | Lactulose | Mannitol |
|---|---|---|
| Concentration Range (mg/mL) | 0.02 – 0.40 | 0.30 – 2.00 |
| Slope (a) | 73.777 | 65.766 |
| $S_a$ | 2.8489 | 1.4170 |
| Intercept (b) | 3.6021 | 8.9730 |
| $S_b$ | 0.5395 | 0.9790 |
| LOD (mg/mL) | 0.0076 | 0.1400 |
| LOQ (mg/mL) | 0.0200 | 0.3000 |
| Correlation Coefficient ($r^2$) | 0.9945 | 0.9884 |

***Accuracy and Precision***

Intra and inter day accuracy and precision were determined by analysis of three replicates of 3 concentrations including low, medium and high concentration QC samples. The overall precision of the method was expressed as percentage of Relative Standard Deviation and the accuracy of the method was expressed in terms of relative errors. Table 2 gives a summary of the accuracy and precision at LAC-MAN concentration range 0.02-0.04 and 0.30-2.00 mg/mL respectively. This suggests that the method developed was accurate and precise (Table 2).

**Table 2**- Precision and accuracy for the capillary electrophoretic analysis of LAC and MAN

| Concentration known (mg/mL) | Concentration found (mg/mL) | RSD % | RE % |
|---|---|---|---|
| **Lactulose** | | | |
| Intra-day (n=3) | | | |
| 0.02 | 0.02 | 8.10 | 9.65 |
| 0.10 | 0.10 | 1.50 | 2.89 |
| 0.40 | 0.41 | 1.82 | 3.07 |
| Inter-day (n=3) | | | |
| 0.02 | 0.02 | 12.73 | 6.49 |
| 0.10 | 0.09 | 5.29 | 5.98 |
| 0.40 | 0.39 | 3.45 | 1.86 |

| **Mannitol** | | | |
|---|---|---|---|
| Intra-day (n=3) | | | |
| 0.30 | 0.30 | 4.45 | 1.94 |
| 0.80 | 0.78 | 1.45 | 2.55 |
| 2.00 | 2.03 | 0.78 | 1.71 |
| Inter-day (n=3) | | | |
| 0.30 | 0.28 | 9.73 | 6.65 |
| 0.80 | 0.83 | 4.72 | 4.39 |
| 2.00 | 1.97 | 1.70 | 1.60 |

The method developed and validated applied to urine samples from patients and healthy volunteers and measured LAC/ MAN ratio. The method used for capillary electrophoretic prameters of LAC- MAN analysis was modified from a literature [3]. Two different solid phase extraction step were used the method mentioned. Hand made cartridge columns were used in the developed method. These cartridges were prepared easily in a short time and it's cost was very reasonable. However a commercial cartridge was used for sample treatment in other study. It's known that the commercial products are considerably expensive and causes a dependence to companies for researchers. Standart solutions were prepared by using blank urine, this approach was more appropriate for studying in a biological fluid. Due to the urine, some errors especially matrix effects were neglected causing from preparing standart solutions in water media. Thus presented study reflected more realistic results than other published study.

Mannitol is a monosaccharide and passively absorbed through the intestinal mucosa. In contrast, lactulose is a disaccharide and normally not absorbed unless the mucosal barrier is compromised. In the healthy intestine, the mean absorption of mannitol is 14% of the administered dose, whereas the mean absorption of lactulose is less than 1%. The normal ratio of lactulose-mannitol recovered in urine is < 0.03. In this study, LAC/ MAN ratios were found 0.072 and 0.137 in patients urine. LAC and MAN concentrations were below the limit of quantitaion in healthy volunteers.

The capillary electrophoresis method developed and validated can be used for determination of LAC/MAN ratio as a predictor of intestinal permeability.

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