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Studies on intestinal permeability of cirrhotic patients by analysis lactulose and mannitol in urine with HPLC/RID/MS

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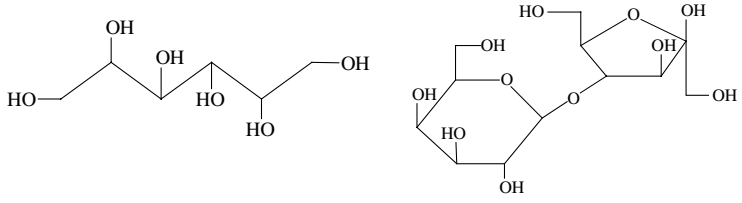
Abstract—The method for separation and determination of lactulose (L) and mannitol (M) in urine was developed by HPLC with a refractive index detector (RID). The linearity ranged from 5 to 1000 μg/mL for L and M, respectively. Recoveries ranged from 93.1% to 97.1%. The intra- and inter-day relative standard deviations of peak area were between 0.8–1.4% ($n = 3$) and 1.4–3.6% ($n = 3$). The limits of detection were obtained with 1.40 μg/mL for L and 1.65 μg/mL for M. The ratios of L/M in the urine samples for the spontaneous ascitic fluid infection (SAI), sterile ascitic fluid (SA) patients, and healthy volunteers (HV) were determined. The results showed well the correlations among the L/M ratio, intestinal permeability (IP) and the illness status of patients, and also indicated lactulose could improve the IP of SAI patients. The peaks of L and M in chromatograms were identified by electrospray ionization/mass spectrometry (ESI/MS), which ensured the accurate measurement of the ratio L/M. This method presented a rapid, accurate, and practical technique for determining IP in clinical practice and investigating the pathology of hepatocirrhosis.

1. Introduction

Change in intestinal permeability (IP) has been widely applied to evaluate and monitor the intestinal mucosa damage, infection, and many other diseases, since its first introduction by Caridis[1] about 30 years ago. For measuring the IP, the dual-sugars probes, mannitol, and lactulose (M and L, Fig. 1), have been commonly used. L and M are absorbed in different regions of the gastrointestinal tract and excreted nonmetabolized in the urine. With L and M orally administered, smaller molecular M is thought to permeate the mucosa mainly via the transcellular pathway (small pores), whereas the larger molecular L entered through the paracellular



Mannitol ($C_6H_{14}O_6$, Mw 182.17) Lactulose ($C_{12}H_{22}O_{11}$, Mw 342.29)

Figure 1. The molecular structure of mannitol and lactulose.

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pathway (large pores). The ratio L/M, the ratio of the excretion percentage L% to M% in urine, is considered as a sensitive, direct, and accurate indicator of IP. Under the infection, damaged, and invasive ways, the width of the intercellular channels enlarged, which leads to the high throughput for the larger molecular L. Hence, the L/M ratios for patients are higher than those for healthy individuals.

To date, most of the investigations[2–12] on IP has been well conducted for healthy individuals or patients suffering from burn, HIV, and post-operation. However the study on the IP of liver patients still remains at a moderately low level. Only a few reports on the determination of L/M and IP for hepatocirrhosis patients with gas chromatography (GC) have been published.[13,14] Spontaneous ascitic fluid infection (SAI) as a new conception in infection ascetics of hepatic cirrhosis,[15] it can result in infection shock, enteron bleeding, viscera eclipse, and finally death. Therefore, the investigations on IP for SAI patients by determining the ratio of L/M will be meaningful in developing new therapy scheme.

Several methods have been developed to assay the sugars including L and M in urine, for example, enzymatic method,[16–18] spectrometry,[19] thin-layer chromatography,[20] GC,[2,13,21–24] and high performance liquid chromatography (HPLC).[3–12,25] Among above methods, HPLC was the most powerful technique for use in clinical analysis of sugars because of its advantages of rapidity, precision, accuracy, and automation. It avoids the sophisticated derivative reaction and time-consuming sample preparation in GC. In this work, using refractive index detector (RID) and mass spectrometer with electrospray ionization as monitors, a practical and reliable HPLC/RID/mass spectrometry (HPLC/RID/MS) has been developed for identification of L and M and determination of the ratio of L/M in SAI patient urines. Using the MS identification for L, M, and possible endogenous glucose in urine, the accurate results of the ratio L/M were obtained. By comparison of the L/M ratios, the correlations among the ratio L/M, IP, and status of an illness were also obviously seen.

2. Instrumental and analysis conditions

Shimadzu HPLC system with RID was used to separate and quantify L and M. The separation was carried out on a Nucleosil NH_2 column (250 × 4.6 mm; id 5 μm) at 35 °C. The mobile phase was CH_3CN/H_2O (70/30, v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL. The identification of the L and M was performed on HPLC–ESI/MS system including an Agilent 1100 series HPLC (Agillent Co., Germany) and an esquire 3000 °C mass spectrometer (Bruker Daltonik Gmbh, Germany). The polarity mode was positive in the full scanning mode (m/z 50–500). The spray temperature was at 300 °C and voltage was at 4.0 kV. Nebulizer pressure was 22 psi. The nitrogen flow rate was 9 L/h. Before the eluate from HPLC was introduced into mass spectrometer, the split of flow rate was 25:1. The softwares for HPLC/MS include Bruker Daltonics ESQUIRECONTROL 5.XX, DATAANALYSIS 2.00 and AGILENT CHEMSTATION A.07.

3. Chemicals and solutions

Unless specified otherwise all chemicals and solvents were of analytical reagent grade and obtained from Beijing Chemical Factory (Beijing, China). Methanol and acetonitrile were of HPLC grade. Water was purified using Milli-Q. Lactulose and mannitol were purchased from Sigma. All solvents and sample solutions used for HPLC were filtered with 0.45 μm, membrane.

The stock solution was prepared by dissolving 100 mg of L and 100 mg of M in 100 mL water to yield 1000 μg/mL for both L and M. The standard solutions were prepared by diluting the stock solution in the mobile phase to produce concentrations at 5, 10, 50, 100, 250 and 500 μg/mL for each L and M.

4. Subjects, sugar ingestion, urine collection, and sample preparation

Thirty-four cirrhosis patients of SAI were randomly divided into group A and B. Group A, 15 persons containing 11 men and 4 women with the average age of 54.8 ± 13.1, was administered with normal antibiotics. Group B, 19 persons including 15 men and 4 women at the average age of 47.2 ± 14.8, was dosed with normal antibiotics and lactulose oral liquids. Group C, 11 cirrhosis patients of SA with 8 men and 3 women at the average age of 52.2 ± 18.2, did not use any antibiotics or lactulose. Group D, 11 healthy individuals including 7 men and 4 women with the average age of 35.3 ± 12.1, was used as control group. The patients of group A, B, and C came from the First Affiliated Hospital of Zhengzhou University, and the volunteers of group D were postgraduates of Zhengzhou University. The study was approved by the Health Department of Henan Province Government. Informed consent was obtained from each subject.

The urine collection was carried out with similar procedures[6,8] with a slight modification. For group A, C, and D, the subjects were fasted overnight between 19:00 p.m. to 8:00 a.m. At 8:00 a.m., the subjects drank a sugar test solution, containing 5 g of M and 10 g of L in 100 mL water. After 30 min, a liberal intake of water was allowed to increase the urine flow. Urine samples within 6 h were collected after administration. One milliliter of 20% (w/v) chlorohexidine as a preservative was added to each collected urine. The total volume was measured and recorded. Ten milliliter urine sample was taken and stored at −20 °C until analysis. For each subject, the amount of each sugar in total urine was determined. The excretion percentage (L% or M%) of each sugar and the ratio of L/M can be calculated. This ratio is used as an index of IP.

The subjects of group B were administrated with normal antibiotics and lactulose for successive 7 days. At the interval of 3 days, the ingestions of L and M and urine collections were performed as the procedures for the subjects of group A, C, and D.

For sample preparation, the similar procedures[9] with a slight modification were applied to remove the salts and a small amount of proteins by adding two or three drops of concentrated acetic acid and 0.3 g mixed ion-exchange resin (Doulite MB 5113, BDH) to 3 mL of the thawed urine specimen. The treated urine specimen was centrifuged for 5 min at 3500 rev/min. Then, the supernate was filtered with 0.45 μm membrane for HPLC/RID/MS analysis.

5. Recovery

Analytical recovery of each sugar was examined by adding the sugar to the blank urine from the subject without ingestion of test sugar solution.

6. Data statistic analysis

The results were expressed as mean ± standard deviation ($X \pm \mathrm{SD}$). P value at 0.05 was chosen as the test criterion for significance deviation.

7. HPLC separation

In the published documents, most of the separations for dual-sugars were conducted with NH_2 and ion-exchange columns, and the commonly used detectors were RID and electrochemical detector (ECD). In our research, Nucleosil 250 × 4.6 mm id NH_2 and RID detection were chosen. With the mobile phase of CH_3CN–H_2O (7:3, v/v) at 1 mL/min, the symmetric peaks of M and L were obtained with the retention time at about 8.2 min for M and 12.2 min for L. More importantly, there was no interference observed in blank urine for M and L (Fig. 2).

8. Method evaluation

The linear responses were observed over the concentration range from 5 to 1000 μg/mL for M and L. The linear regression between peak area (A) and concentration (C, μg/mL) yielded the following equations:

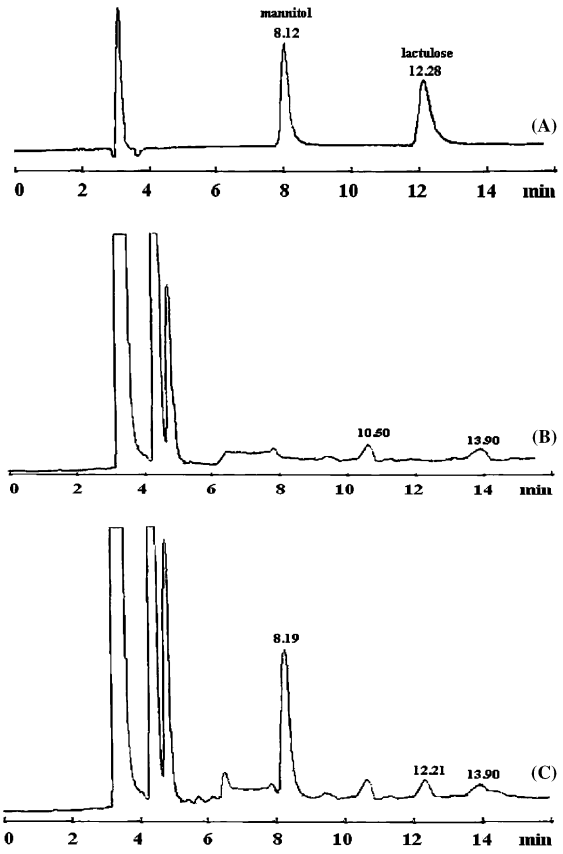

Figure 2. HPLC with RID of dual-sugars (A) and blank urine (B) and urine sample from a SAI patient (C).

for L, $A = 664C - 108$ ($n = 7$, $R^2 = 0.9989$);
for M, $A = 673C - 340$ ($n = 7$, $R^2 = 0.9992$).

By calculating a signal to noise ratio of 2 ($S/N = 2$), the limits of detection (LOD) were obtained with 1.40 μg/mL for L and 1.65 μg/mL for M, respectively.

The reproducibility of the method was obtained by repeated measurement of L and M at concentrations of 20 and 200 μg/mL (spiked in a blank urine). The intra- and inter-day relative standard deviations (RSDs) of peak area ranged from 0.8–1.4% ($n = 3$) to 1.4–3.6% ($n = 3$), respectively. The recoveries were examined by spiking blank urine with three concentrations of L and M (10, 100, and 500 μg/mL). The recoveries were obtained ranging from 93.1% to 97.1% given in Table 1.

Table 1. Recovery

Known concn (μg/mL)	Detected concn (μg/mL)	Mean concn (μg/mL)	Recovery (%)	RSD (%)
Lactulose				
10	9.20, 9.30, 9.15, 9.38, 9.53	9.31	93.1	1.6
100	92.9, 95.5, 94.6, 92.9, 93.1	93.7	93.7	1.4
500	490.8, 485.5, 475.9, 487.7, 485.6	485.1	97.0	1.2
Mannitol				
10	9.20, 9.10, 9.55, 9.68, 9.13	9.33	93.3	2.8
100	92.9, 94.5, 94.6, 96.9, 97.1	95.2	95.2	1.9
500	493.8, 485.5, 470.9, 488.7, 489.6	485.7	97.1	1.8

9. ESI/MS and HPLC/MS

More recently, HPLC/MS has been extensively used for identification and quantification of drugs in biological matrices. In our study, we applied HPLC/RID to achieve the accurate quantification for L and M in urine specimen. Meanwhile, we also used ESI/MS to identify L and M from the urine matrices. To achieve the aim above, the positive ESI/MS and ESI/MS/MS (ESI/MS^2) of L and M were firstly performed under the adequate conditions and their MS characteristics were elucidated. Their molecular ions and the most abundant ions (with the relative abundance) were summarized in Table 2. For the production ion at m/z 163, [lactulose+H−fructose]$^+$, it was from the cleavage of lactulose shown in Figure 3. The fragment ions at m/z 145, 127, and 97 were attributed to the successive loss of water and formaldehyde from the ion [lactulose+H−fructose]$^+$. It can be seen from Table 2 both L and M were easy formed adducts with sodium ion ([M+Na]$^+$). Thus, in HPLC/MS, the ions [M+Na]$^+$ at m/z 365 and 205 can be used as the specific ions for L and M in the urine.

The HPLC/MS of urine specimen was carried out under the given conditions. Total ion current (TIC, top) and ESI/MS (bottom) of a real urine sample were obtained and shown in Figure 4. As expected, the specific ions at m/z 365 for L and at m/z 205 for M were clearly observed in MS at corresponding retention times. There was no endogenous L or M found in blank urine. However, it is noteworthy that, at the retention time of M, there was endogenous glucose appeared in two specimens of group A. It was identified with its specific ions, [glucose+H]$^+$ at m/z 181 and [glucose+Na]$^+$ at m/z 203 (not shown). The overlap between glucose and M distorts the accurate determination of M. Therefore, in order to avoid the false result, the data for the two specimens with endogenous glucose were excluded during the data statistics.

Table 2. Molecular and product ions and their relative abundance by +ESI/MS and MS^2 spectra of lactulose and mannitol

Substance	Ions	m/z	Relative abundance (%)	MS
Lactulose	[M+Na]$^+$	365	65	+ESI/MS
	[M+H−H_2O]$^+$	325	100	+ESI/MS
	[M+H−$2H_2O$]$^+$	307	8	+ESI/MS
	[M+H−$3H_2O$]$^+$	289	20	+ESI/MS
	[M+H−$4H_2O$]$^+$	271	2	+ESI/MS
	[M+H−fructose]$^+$	163	33	+ESI/MS
	[M+H−fructose−H_2O]$^+$	145	80	+ESI/MS
	[M+H−fructose−$2H_2O$]$^+$	127	35	+ESI/MS
	[M+H−fructose−$2H_2O$−CH_2O]$^+$	97	19	+ESI/MS
[Lactulose+Na]$^+$	[M+Na−H_2O]$^+$	347	100	+ESI/MS^2
	[M+Na−H_2O−CH_2O]$^+$	317	25	+ESI/MS^2
	[M+Na−$4H_2O$−$3CH_2O$]$^+$	203	60	+ESI/MS^2
	[M+Na−$5H_2O$−$3CH_2O$]$^+$	185	7	+ESI/MS^2
Mannitol	[2M+Na]$^+$	387	18	+ESI/MS
	[2M+H]$^+$	365	8	+ESI/MS
	[M+Na]$^+$	205	100	+ESI/MS
	[M+H]$^+$	183	70	+ESI/MS
	[M+H−H_2O]$^+$	165	23	+ESI/MS
	[M+Na−H_2O]$^+$	347	100	+ESI/MS^2
	[M+H−$2H_2O$]$^+$	147	10	+ESI/MS
	[M+H−$3H_2O$]$^+$	129	8	+ESI/MS

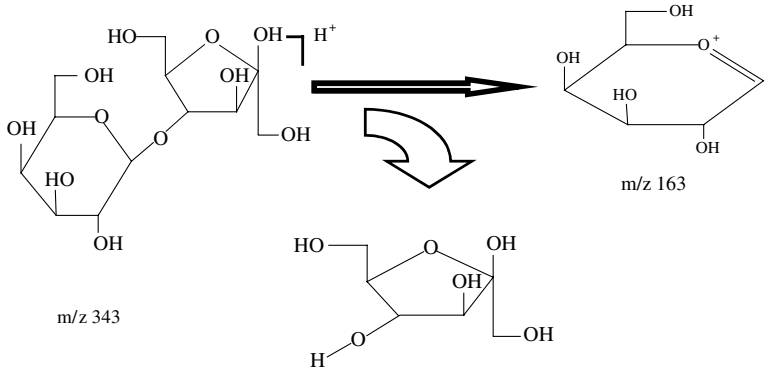

Figure 3. The possible cleavage pathway for ion at m/z 163 by +ESI/MS of L.

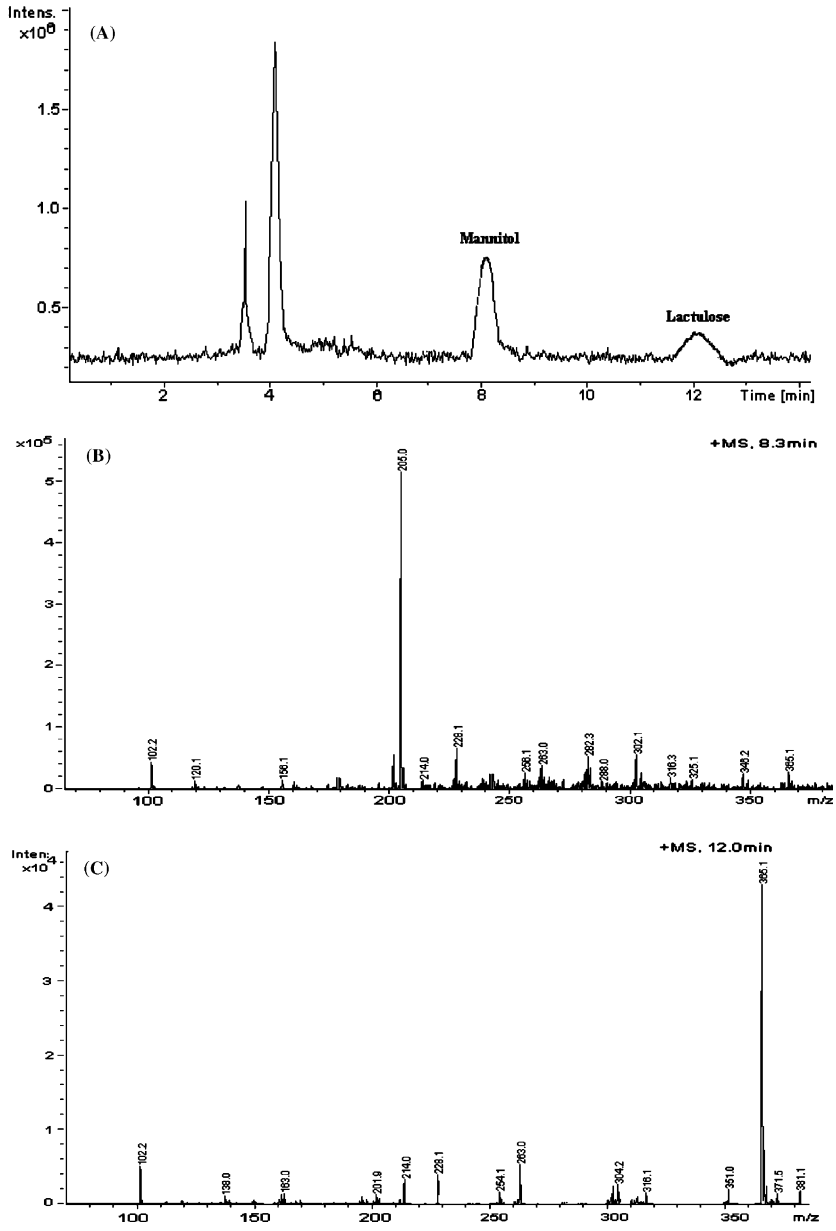

Figure 4. HPLC/MS of urine sample for SAI patient as Figure 2C.

10. Determination of the L/M ratios for subjects

Using the proposed HPLC/RID/MS method, the L%, M%, and L/M ratios for the different subjects of group A, B, C, and D were determined and listed in Tables 3 and 4. Tables 3 and 4 illustrated there was no significant difference among M% values for four groups. However L% and the ratio L/M for SAI and SA patients were obviously higher than those for healthy persons. SAI patients possessed the highest L/M ratios. The results reflected the situation of IP, and IP was consistent with the pathological characteristics for SAI and SA patients. In our research, we also investigated the influence of the different Rx on the ratio of L/M. Table 4 demonstrated that L% and L/M ratio did not distinctively decrease for group A being cured by normal antibiotics for a week. However for group B, a considerable drop of L% and L/M ratios was observed after being dosed together with

Table 3. The results for the subjects of group A, B, C, and D ($X \pm SD$)

Group	Subject	L%	M%	L/M
A+B[a]	32	0.00719 ± 0.0004	$0.0741 + 0.0044$	0.1003 ± 0.0035
C	11	0.00476 ± 0.0006	0.0691 ± 0.0074	$0.0715 + 0.0071$
D	11	0.00177 ± 0.0001	0.0838 ± 0.0070	0.0209 ± 0.0009
P		<0.001	>0.05	<0.001

[a] Before dosing any antibiotics or lactulose.

Table 4. The results before and after dosing with normal antibiotics for group A patients, and with normal antibiotics and lactulose for group B patients ($X \pm SD$)

Group A	Subject	L%	M%	L/M
Group A				
Before dosing	12	0.0070 ± 0.0007	0.0735 ± 0.0005	0.1009 ± 0.0068
After dosing	12	0.0060 ± 0.0005	0.0782 + 0.0070	0.0814 + 0.0059
P		>0.05	>0.05	>0.05
Group B				
Before dosing	18	0.0071 ± 0.0006	0.0733 ± 0.0053	0.0985 ± 0.0047
After dosing	18	0.0044 ± 0.0004	0.0732 ± 0.0051	0.0572 ± 0.0042
P		<0.001	>0.05	0.001

normal antibiotics and lactulose oral liquids. These data indicated lactulose played an important role for improving the intestinal function.

11. Conclusion

Modern analytical technique has played a more and more important role in biomedical and medicinal chemistry. The proposed HPLC/RID method had been used to analyze M, L, and the ratios of L/M in urine samples for SAI and SA patients by using NH_2 column and with CH_3CN/H_2O (70/30, v/v) mobile phase. Combining with MS identification, the reliability of the results was ensured. It is more important that L%, M%, and the L/M ratios were first obtained for SAI and SA patients using HPLC/RID/MS method. Moreover, the investigation on relationships among the L/M ratios, IP, and the status of SAI and SA patients would provide the reference for clinical practice.

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