The mucopolysaccharidoses (MPS), a group of lysosomal storage disorders, are caused by the deficiency of specific enzymes that catalyze the stepwise degradation of glycosaminoglycans (GAGs; mucopolysaccharides). There are 10 known enzymes, including 4 glycosidases, 5 sulphatases, and 1 non-hydrolytic transferase involved in the catabolism of chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS), and keratan sulphate (KS). Any enzyme deficiency will block the GAG degradation and result in GAG macromolecules with a specific carbohydrate or sulphated carbohydrate residue accumulating in cells, causing severe dysfunction.

Based on the specific enzyme deficiency, 6 distinct Chinese Medical Journal (Taipei) 2001;64:15-22

Original

Diagnostic Screening for Mucopolysaccharidoses by the Dimethylmethylene Blue Method and Two Dimensional Electrophoresis

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Key Words
dimethylmethylene blue method; glycosaminoglycan; mucopolysaccharidoses; two-dimensional electrophoresis

Background. The diagnosis of a mucopolysaccharidosis (MPS) can be achieved by non-enzymatic screening methods, including two-dimensional electrophoresis (2-D EP), and the dimethylmethylene blue (DMB) method. However, the confirmation of MPS diagnosis is difficult to achieve in Taiwan due to the shortage of a well-established MPS diagnostic service. In this article, we introduce the MPS screening protocol we have already established, and also illustrate the interpretation of 2-DE results for MPS typing determination.

Methods. Thirty-seven patients with different types of MPS were analyzed by 2-D EP and the DMB method. They were 4 with MPS I (Hurler), 15 with MPS II (Hunter), 10 with MPS III (Sanfilippo), 5 with MPS IV (Morquio), and 3 with MPS VI (Maroteaux-Lamy).

Results. The electrophoretic patterns of the affected glycosaminoglycans (GAGs) in different MPS were illustrated, which were the basis of MPS diagnosis. The DMB results showed a significant difference between these diseases, and the dimethylmethylene blue/creatinine (DMB/CRE) ratio of Hunter Syndrome was markedly greater than those of the Sanfilippo, Hurler, and Maroteaux-Lamy Syndrome, successively. The 2-DE is also applicable for MPS prenatal diagnosis. One cell-free amniotic fluid with suspected Hunter Syndrome was analyzed by 2-DE, and the results excluded the possibility of MPS which was confirmed by enzymatic study.

Conclusions. The 2-D EP provides a good separation of urinary GAG, and the DMB method gives an estimation of the GAG concentration in the urine. Both of the methods are specific, sensitive, and easy to perform for MPS screening and diagnosis.

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MPS have been as signed as MPS I (Hurler syn drome), MPS II (Hunter), MPS III (Sanfilippo), MPS IV (Mor quio), MPS VI (Maroteaux-Lamy), and MPS VII (Sly disorder). The di ag no sis of MPS is achieved by 3 se quen tial steps, which are the quan ti ta tive de tection of ex cessive ex cre tion of GAGs in the urine, urine GAG qual i ta tive de tection (2-D EP) to de ter mine the prob a ble MPS type, and fi nally de tection of the spe cific en zyme de fect in leu ko cytes, se rum, plasma, and cul tured cells. Of these, the de ter mi na tion of spe cific en zyme ac tivi ties is the most de fin i tive di ag no sis. It is the main pur pose of this ar ti cle to in tro duce the screen ing pro to cols for MPS that we have al ready es tab lished, and also to pres ent the in ter pre ta tion of 2-D EP re sult for MPS de ter mi na tion.

At pres ent, the most pop u lar first-line tests for MPS are 2-D EP and the DMB method, which are spe cific, sen si tive, and easy to per form. In ad di tion, the 2-D EP is also ap pli ca ble for pre na tal di ag no sis. It is the main pur pose of this ar ti cle to in tro duce the screen ing pro to cols for MPS that we have al ready es tab lished, and also to pres ent the in ter pre ta tion of 2-D EP re sult for MPS de ter mi na tion.

Methods

All the re agents were pre pared in-house, and most of the chem i cals were pur chased from Sigma Co. (St. Louis, MO, USA). Thirty-seven urine sam ples were col lected from MPS pa tients with known di ag no sis on the ba sis of clin i cal symp toms and en zyme de fi ci en cies: four with Hurler, 15 with Hunter, ten with San filippo, five with Mor quio, and three with Maroteaux-Lamy. Mea sure ment of en zyme ac tiv i ties was con fi rmed by Willink Bio chem i cal Ge net ics Unit, Royal Man chester Children Hos pi tal, Pendle bury, Man ches ter, UK. About 10 - 20 mL of urine was re quired for the as say, and the sam ples were stored at 4 °C be fore use. A sol u tion of 4M NaCl, meth a nol, 0.1 M so di um ac e tate buffer to get her, and de ion ized wa ter was added to make 100 mL (stored at 4 and fi l tra tion be fore use). A so lu tion of 4M NaCl, meth a nol, 0.1 M so di um car bon ate, ab so lute meth a nol, 0.1 M bar i um so di um ac e tate, 5% ace tic acid, and pyrid ine : ace tic acid : wa ter buffer (10:1:89 by vol ume) was all re quired to dis so ci ate the MPS-AB com plex. Then, 0.2M so di um chlo ride (0.1 M) were pre cip i tated from urine by the Alcian blue re agent (10 mL) was added to 2 mL cen tri fuged urine, and al lowed to stand at room tem per a ture for 2 hours (or at 4 °C over night). The sam ple was cen tri fuged at 4000 rmp for 15 min utes, and then the super natant was de canted off. In this step, GAGs were pre cip i tated from urine by the Alcian blue re agent (MPS-AB com plex). Then, 0.2M so di um car bon ate (0.2 mL) and meth a nol (0.1 mL) were used to dis solve the MPS-AB com plex. Se quen tially, 0.1M so di um carbonate (0.1 mL) and 0.4 mL wa ter were added to dis so ci ate the MPS-AB. The clear super nat ant was trans fer red to a clean con i cal tube (500 µL) and two low volt age elec tro pho re sis tanks were pur chased from Sar to rius AG (Goettingen, Ger many) and Helena Labo ra tories. (Beaumont, TX, USA), re spec tively.

Alcian blue re agent (10 mL) was added to 2 mL cen tri fuged urine, and al lowed to stand at room tem per a ture for 2 hours (or at 4 °C over night). The sam ple was cen tri fuged at 4000 rmp for 15 min utes, and then the super natant was de canted off. In this step, GAGs were pre cip i tated from urine by the Alcian blue re agent (MPS-AB com plex). Then, 0.2M so di um chlor ide (0.2 mL) and meth a nol (0.1 mL) were used to dis solve the MPS-AB com plex. Se quen tially, 0.1M so di um carbonate (0.1 mL) and 0.4 mL wa ter were added to dis so ci ate the MPS-AB. The clear super nat ant was trans fer red to a clean con i cal tube (5 mL). Finally, a four vol ume of eth a nol was added to re-pre cip i tate the MPS. Af ter dry ing the pre cip i tate at 37 °C, they were ready for sepa ra tion into com ponent GAGs on cell ulose ac e tate sheets by 2-D EP. The GAG pre cip i tate was dis solved with var i ous amounts of wa ter de pend ing on the DMB value in mg/L. A
2 or 3 µL GAG solution was used for 2-D EP. Electrophoresis was carried out in pyridine-acetic acid buffer in the first direction for 1 hour at 50 volts, and in barium acetate buffer in the second direction for 3 hours at 50 volts. The GAGs were visualised by staining with Alcian blue reagent for 1 hour, and then washed with 5% acetic acid to leave urinary GAGs present as blue spots on the celagram.

For identifying GAGs in amniotic fluid by 2-DEP, a cell-free amniotic fluid sample was required for the assay. Ap proximately 3 mL amniotic fluid was placed in a 50 mL culture tube, and Alcian blue reagent was added to a final volume of 50 mL. Refrigerate over night, and treat it in the same manner as for the urine sample. Finally, a 2 µL GAG solution was applied for electrophoresis.

Results

The reference GAG values for the DMB method were determined from 69 healthy control persons (i.e. less than 2 years old, n = 25; 2-17 years of age, n = 24; and adults 18-42 years of age, n = 20). In Table 1, the urine creatinine level was proportional to the age, but inversely proportional to the DMB/CRE ratio. The higher DMB ratio came mostly from the very young group (less than 2-year-old infants), on the contrary, the DMB/CRE ratio was lower and nearly constant in the adult group (older than 18 years old) (Fig. 1). When comparing the DMB/CRE ratio among these 3 age groups by paired Student t test, the differences showed statistically significance (p values < 0.001).

For MPS patients, an elevated DMB ratio, approximately}

| Table 1. The normal reference values of DMB/CRE ratio |
|---------------------------------|-----------------|-----------------|-----------------|
| Detections                      | Age (yr)        |
| Creatinine (mM)                | < 2 (n = 25)    | 2 – 17 (n = 24) | 18 – 42 (n = 20) |
|                                |                 |                 |
| DMB/CRE ratio (mg/mM creatinine)|                 |                 |
| Creatinine (mM)                | 1.05 ±0.49      | 5.19 ±4.06      |
| DMB/CRE ratio (mg/mM creatinine)| 44.6 ±23.7      | 15.3 ±13.0      |
|                                |                 |                 |
|                                | a vs b, p < 0.001; a vs c, p < 0.001; CRE = creatinine; DMB = dimethylmethylene blue.

Fig. 1. The dimethylmethylene blue / creatinine (DMB/CRE) ratio of normal control person and MPS patient (in mg/mmol creatinine). Symbol: ⬤ for normal control person; s for Hurler; ● for Hunter; ■ for Sanfilippo; ♦ for Morquio; and □ for Maroteaux-Lamy.
Fig. 2. The electrophoretic patterns of the affected GAGs in different MPS. (A) Mixture of GAG reference standards; (B) Normal amniotic fluid; (C) Normal control person; (D) Hurler (MPS I); (E) Hunter (MPS II); (F) Sanfilippo (MPS III); (G) Morquio (MPS IV); (H) Moro teaux-Lamy (MPS VI). Key: S = sample application; C = MPS positive control; CS = chondroitin sulphate; HS = heparan sulphate; DS = dermatan sulphate; KS = keratan sulphate; HA = hyaluronic acid.
mately 1.5 to 4 fold the averaged reference values was ob served. The DMB/CRE ratios for each MPS were showed in Fig. 1. In these, the DMB/CRE ratio detected in Hunter’s Syn drome was much greater than others, on the other hand, the ratio obtained from Morquio Syn drome was ran domly scat tered within the normal DMB/CRE dis tri butions.

Two-dimensional electro phoretic anal ysis of uri nary GAG was per formed on a to tal of 54 sam ples, in clud ing 37 MPS pa tients, 3 cell-free amniotic fluid sam ples ob tained from preg nan cies with no known risk for MPS, 1 amniotic fluid sam ple ob tained from a preg nancy at risk for MPS II (ges ta tion for 18 weeks), 12 normal in di vid uals, and 1 lab oratory pre pared mix ture of CS, DS, HS, and KS. GAG was first pre ci tated from the urine and am niotic fluid sam ples, and then 2-3 µL of treated GAG so lu tion was ap plied on cel lu lose ace tate sheet for 2-D EP. The GAG com po nents were iden ti fied when com par ing to the mi grated pat terns of a posi tive con trol sam ple (a MPS II sam ple with CS, DS and HS con tained).

For the lab oratory pre pared mix ture, four prin ci pal GAG com po nents were found. The mi grated ve loc ity from cath ode to an ode at the first one-direction showed the equal move ment of CS and DS, as well as KS and HS, in which the move ment of CS and DS was faster than that of KS and HS. After the second one-direction elec tro phore sis, the move ment of CS was faster than KS, DS, and HS suc cessively (Fig. 2A). For nor mal amniotic fluid sam ple, two dis tinct spots were clearly ob served, which were iden ti fied as CS (mov ing faster) and hyaluronic acid (HA) (Fig. 2B). For the pre na tal di ag no sis, a nor mal elec tro phoretic pat tern of CS and HA was ob served from the am niotic fluid sam ple with a posi tive fam ily his tory of Hunter Syn drome.

For nor mal urine, the only GAG pres ent was CS, dem on strated by a dis crete spot on the celagram (Fig. 2C). Con sidering that dif fer ent GAG are af fected in each MPS phe no type, the elec tro phoretic pat tern would be dif fer ent from one MPS to the other. The elec tro phoretic pat terns were very sim i lar be tween Hurler and Hunter, in which a com posed pat tern of CS, DS and HS was ob served. The ma jor dif fer ence be tween them was an up-widespread por tion of hepa rin ex tend ing from HS in Hunter’s Syn drome (Fig. 2D and 2E). Highly spec ific and def i nite pat terns were ob served in the other MPS types, in clud ing the patterns of CS / HS, and CS / KS de tected in Sanfilippo and Morquio sam ples, re spec tively (Figs. 2F and 2G), and an ex cess DS with very lit tle CS found in Maroteaux-Lamy (Fig. 2H).

**Discussion**

The di ag nos tic screen ing for MPS can be suc cess fully per formed by 2-D EP and the DMB method based on the de tec tion of CS, DS, HS and KS, as well as the total GAG con cen tra tion in the urine. When ever the MPS phe no type is de ter mined, the spe cific MPS en zyme ac tiv ity is then mea sured by dem on strating an en zymatic as say with fluo rogenic, chromogenic or ra dio ac tive sub strates. In or der to spe cify the per for mance of spe cific MPS en zyme matic activity, an ac cu rate and re li able screen ing method for a MPS de ter mi nation is very im por tant and nec es sary.

The DMB method for uri nary GAG es ti ma tion is ref er en tial, and it cor re lates closely with the MPS di ag no sis by 2-D EP. The DMB/CRE ra tio var ied from one MPS to the other. In our study, the DMB/CRE ra tio ob tained from Hunter’s Syn drome was de fi nitely higher than that of Sanfilippo, Hurler, which the aver aged urine creatinine lev els of iden ti fied MPS pa tients of each type ap peared no sig nif i cant dif fer ences (i.e. 5.28 ±3.67, 6.53 ±0.98, 5.29 ±3.3, 5.34 ±0.53, and 6.57 ±1.33 mM, re spec tively to the above phe no types). The higher DMB/CRE ra tio in Hunter syn drome might be greatly re lated with the youn ger age of the pa tients at the time for study. The clin i cal fea tures of MPS may pres ent from birth to late child hood or even early adult hood de pend ing on the se ver ity of the MPS phe no types. In our study, the age of on set of Hunter Syn drome ranged from 1 to 9 years old (most investigated pa tients were less than 5 years old), which was much youn ger than that of Sanfilippo, Hurler, which the aver aged urine creatinine lev els of iden ti fied MPS pa tients of each type ap peared no sig nif i cant dif fer ences (i.e. 5.28 ±3.67, 6.53 ±0.98, 5.29 ±3.3, 5.34 ±0.53, and 6.57 ±1.33 mM, re spec tively to the above phe no types). The higher DMB/CRE ra tio in Hunter syn drome might be greatly re lated with the youn ger age of the pa tients at the time for study. The clin i cal fea tures of MPS may pres ent from birth to late child hood or even early adult hood de pend ing on the se ver ity of the MPS phe no types. In our study, the age of onset of Hunter Syn drome ranged from 1 to 9 years old (most investigated pa tients were less than 5 years old), which was much youn ger than that of Hurler (8 - 14 years old), Sanfilippo (5 - 10 years old), Morquio (5 - 15 years old), and Maroteaux-Lamy (8 - 16 years old).

The 2-DEP method is re li able, spe cific, and sen sitive. It pro vides very good sep a ra tion of GAG com po nents, and thus makes the in ter pre ta tion eas ier. One di men sional thin-layer cel lu lose chro ma tography used
to be the method of choice for biochemica l classification of MPS, how ever, it is no lon ger used in many laboratories be cause of the tri mma te procedure and the equivocal separation of GAG contents. When performing the 2-D EP for MPS diagn osis, there are three critical steps to in sure quality re sults. First, one needs to in sure the best re covery of GAG from the urine. A poor GAG iso lation will re sult in an indefi nite pat tern, which could pos si bly lead to misdiagnosis. Sec ond, an ade quate vol ume of GAG so lu tion must be ap plied to the gel. The ap plied vol ume was 1 - 3 µL depend ing on the DMB value in mg/L. Ap plying more or less will influence the inter pretation by in fe rior sepa ration of GAG con tents, or a poor res olu tion of GAG ap pearances. Third, one needs to den on strate a posi tive con trol sam ple as a ref er ence for GAG iden ti fi cation and inter pretation. A posi tive control sample was only used in the sec ond one-direction EP. The pos i tive control sam ple was pre pared from Hunter pa tients, whose ur in contained CS, DS and HS com ponents. With out a posi tive con trol pat tern as a ref er ence there will only be con fu sion in the in ter pretation. An ar ti fi cial stan dard mix ture (in-house pre pared with CS, DS, HS, and KS) is not ap pro pri ate to be used as a posi tive con trol be cause of a in fe rior sepa ra tion of KS and HS.

Prenatal diagn osis of MPS is ap pli ca ble by 2-D EP, how ever, a final con firmation is ab solutely re quired by per form ing a mo lecular anal y sis or an en zymatic assay of that specific en zyme ac tiv ity in the amniotic fluid. In our study, a mo lecular anal y sis was also re con firmed by us ing poly mer ase chain re ac tion (PCR) to am plify the known iduronate sul phatase (IDS) gene de fect fol low ing re stric tion en zyme di ges tion. The re sult of re stric tion frag ments anal ysis showed that only a half of the PCR prod uct was cleaved. Con clu sively, we de duced that the proband was a female car rier, which was very much cor re sponding with our 2-D EP re sult.

By re view ing 2-D EP and the DMB method, both of them are well-es tablished, and the meth ods are de fin i te, sen si tive, and easy to per form for MPS screen ing and diag no sis.

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