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High-performance capillary electrophoresis of unsaturated oligosaccharides derived from glycosaminoglycans by digestion with chondroitinase ABC as 1-phenyl-3-methyl-5-pyrazolone derivatives

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ABSTRACT

This paper proposes a new method for simultaneous analysis of unsaturated disaccharides derived from glycosaminoglycans by enzymatic digestion with chondroitinase ABC, based on high-performance capillary electrophoresis (HPCE) of their 1-phenyl-3-methyl-5-pyrazolone derivatives. The O-sulphate group is stable in this derivatization, and this method allows reproducible microdetermination of glycosaminoglycans. This paper also demonstrates the applicability of this method to estimation of urinary chondroitin sulphates. Urinary creatinine as an inherent internal standard could also be estimated by HPCE, though in another mode of separation, *i.e.* ion-exchange electrokinetic chromatography.

INTRODUCTION

Glycosaminoglycans (GAGs) are widely distributed in animal tissues and have a variety of important physiological functions. They are basically composed of hexosamine and uronic acid residues linked alternately. The amino group in the hexosamine residue is either acetylated or sulphated, and the hydroxyl group is partially sulphated. GAGs usually occur together, hence direct analysis requires prior separation. However, there is no ideal method for separation.

However, there are various lyases that cleave the hexosaminide linkages to give oligosaccharides with the unsaturated uronic acid residue at the non-reducing termini. Chondroitinase ABC from *Proteus vulgaris* is a typical example; it cleaves the hexosaminide bonds in chondroitin sulphates A, B, C, D and E, giving 2-acetamideo-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-6S), acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enopyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-diS_D) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-4,6-bis-O-sulpho-D-galactose (ΔDi-diS_E). This lyase also cleaves chondroitin and hyaluronic acid to give 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-D-galactose (ΔDi-0S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-D-glucose (ΔDi-HA), respectively. Since

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analysis of these unsaturated disaccharides allows characterization and determination of GAGs, various methods have been developed for this purpose. Lee *et al.* [1] devised a method based on high-performance liquid chromatography (HPLC) with UV detection, though the sensitivity was not high. Kodama *et al.* [2] improved this method by pre-column conversion of the disaccharides to reductively pyridylaminated derivatives, which are strongly fluorescent. There is another improvement by Toyoda *et al.* [3] based on post-column derivatization with 2-cyanoacetamide. We have also developed a method based on HPLC of 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives. The details will appear elsewhere.

High-performance capillary electrophoresis (HPCE) is a recently developed method for separation which allows high-resolution separation and reproducible microquantification by on-column detection. We have applied this method to carbohydrate analysis and demonstrated its high capabilities [5–8]. Al-Hakim and Linhardt [9] have also reported its application to unsaturated disaccharides derived from a few species of GAGs, but sensitivity was not high because the disaccharides were directly analysed. We present herein an improved HPCE method based on pre-column derivatization with PMP.

EXPERIMENTAL

Chemicals

Authentic specimens of chondroitin sulphate A (whale cartilage), chondroitin sulphate C (shark cartilage), chondroitin sulphate D (shark cartilage), chondroitin sulphate E (squid cartilage) chondroitin (derived from chondroitin sulphate A), hyaluronic acid (pig skin) and unsaturated, sulphated disaccharides (ΔDi-4S and ΔDi-6S) were purchased from Seikagaku Kogyo (Tokyo, Japan) and used as obtained. The authentic samples of ΔDi-diS_D, ΔDi-diS_E, ΔDi-0S and ΔDi-HA were prepared by digestion of chondroitin sulphate D, chondroitin sulphate E, chondroitin and hyaluronic acid, respectively, with chondroitinase ABC. Chondroitinases AC and ABC from *Arthrobacter aurescens* and *Proteus vulgaris*, respectively, were also from Seikagaku Kogyo. PMP was obtained from Kishida Chemicals (Osaka, Japan) and used after recrystallization from methanol. The authentic sample of creatinine was from Wako Pure Chemicals (Osaka, Japan). All other chemicals and solvents were of the highest grade commercially available. Water as solvent was double distilled and filtered through a membrane filter before use.

Apparatus

HPCE was performed by using a Beckman P/ACE 2000 capillary electrophoresis system, composed of a high-voltage power supply, a pressure-controlled injector, a UV detector equipped with 200-, 214-, 254- and 280-nm cutt-off filters and a data processor. A capillary tube (51 cm × 75 μm I.D. × 375 μm O.D.) of fused silica was mounted on a plastic cassette, wich was maintained at 30°C. Operation by this system was fully automated.

Enzymatic reactions

The method of Saito *et al.* [10] was slightly modified. To an aqueous solution (20 μl) of an authentic specimen of a GAG (10 μg), a mixture of authentic specimens of GAGs (0.5–10 μg each) or a cetylpyridinium chloride (CPC)-precipitated GAG fraction of a urine sample (5.0 ml) was added an aqueous solution (20 μl) of chondroitinase AC or ABC (20 mU), together with 250 m*M* Tris–130 m*M* hydrochloric acid buffer (pH 8.0, 10 μl) containing sodium acetate (2.4%, w/w), sodium chloride (1.5%, w/w) and bovine serum albumin (0.05% w/w), and the mixture was incubated for 15 h at 37°C. The reaction solution was evaporated to dryness *in vacuo*, the residue dissolved in an appropriate volume (50–100 μl) of water, and the solution analysed by HPCE. When necessary, the residue was derivatized with PMP as described below, and the product was subjected to analysis by HPCE.

Derivatization with PMP

The procedure of derivatization was essentially the same as that described in our previous paper [11], but slightly modified. Briefly, an authentic specimen of an unsaturated disaccharide (10 μg), a mixture of authentic unsaturated disaccharides (10 μg each) or a product of enzymatic reaction mentioned above was dissolved in 0.5 *M* sodium hydroxide (30 μl) and a methanolic 0.5 *M* solution of PMP (30 μl) was added. The mixture was allowed to stand at 70°C with the stopper tightly closed, then

neutralized with 0.3 *M* hydrochloric acid (30 μl). Water (200 μl) and ethyl acetate (200 μl) were added to the neutralized mixture, and the whole shaken vigorously. The organic layer was removed and the aqueous layer extracted with the same volume of ethyl acetate. After three more extractions the final aqueous layer was lyophilized, the residue dissolved in water (50 μl), and the solution analysed by HPCE.

Analysis of unsaturated oligosaccharides

HPCE was performed in zone electrophoresis mode. A mixture of authentic specimens of unsaturated disaccharides or a digestion mixture of authentic or urinary GAGs, either directly or derivatized with PMP, was introduced from the anodic end of the capillary tube containing a borate buffer (pH 9.0) prepared by mixing 100 m*M* boric acid and 25 m*M* sodium tetraborate. Analysis was carried out by applying a voltage of 25 kV with UV monitoring at 214 nm.

Collection of urinary GAGs

This was done by the CPC precipitation method described by Di Ferrante and Rice [12]. Briefly, a 5-ml aliquot of a 24-h composite sample of urine was centrifuged at 3000 rpm (1100 *g*) in a 10-ml centrifuge tube, and an aqueous 2.5% solution (100 μl) of CPC was added to the supernatant. After standing overnight at 4°C, the precipitates were collected by centrifugation in a similar manner. The pellet was well vortexed with a saturated solution of sodium chloride in a 95:5 (v/v) ethanol–water mixture (30 μl), and the mixture centrifuged. After repeating this precipitation procedure twice more, the final pellet was subjected to digestion with chondroitinase AC or ABC.

Assay or urinary creatinine

A urine sample was directly introduced to the cathodic end of the capillary tube filled with 100 m*M* borate buffer (pH 9.0) containing polybrene to a concentration of 0.1% (w/v). A voltage of 25 kV was applied, and creatinine and sodium benzoate (internal standard) separated were monitored at 254 nm.

RESULTS AND DISCUSSION

The enzymatic reactions of GAGs with chondroitinase ABC were studied in detail by Saito *et al.* [10]. Under the optimized conditions using Tris–hydrochloric acid buffer (pH 8.0) at 37°C the yields of these disaccharides from chondroitin sulphates A and C reached plateaux in 15 h. On the other hand, a convenient method for derivatization of reducing sugars was established in our laboratory [11]. This method allows quantitative conversion of reducing mono- and oligosaccharides to derivatives which have two PMP groups in a molecule and absorb the UV light strongly.

HPCE in the zone electrophoresis mode in 100 m*M* borate buffer as carrier gave rather good separation of ΔDi-4S and ΔDi-6S. The order of migra-

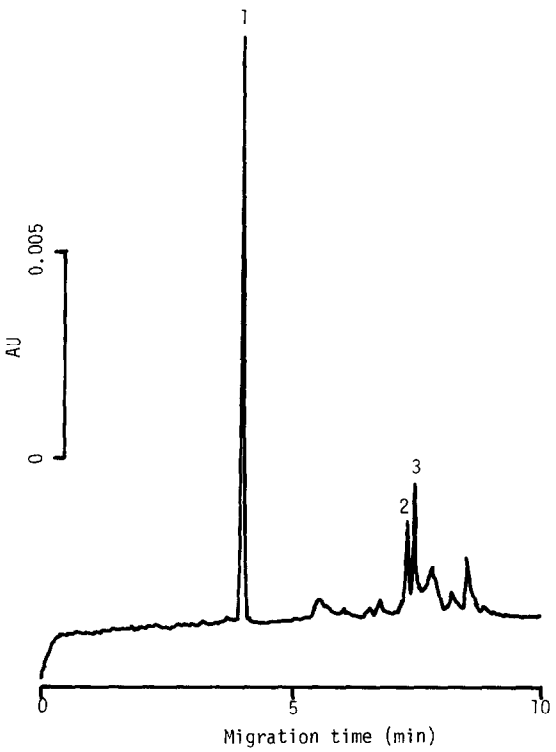

Fig. 1. Direct analysis of a chondroitinase ABC-digested mixture of chondroitin sulphates A and C by zone electrophoresis. Capillary, fused silica (51 cm × 50 μm I.D.); carrier, 100 m*M* borate buffer (pH 9.0); applied voltage, 25 kV; detection, UV absorption at 214 nm. The sample was injected from the anodic end of the tube. Peaks: 1 came from the buffer for enzymatic digestion; 2 = ΔDi-6S; 3 = ΔDi-4S.

tion was ΔDi-6S followed by ΔDi-4S, presumably because of easier complexation of the latter with the borate ion. Although the authentic specimens of these unsaturated disaccharides were well separated, analysis of these disaccharides in a digestion mixture of chondroitin sulphates A and C was interfered with by non-carbohydrate materials, as shown in Fig. 1. In Fig. 1 peaks 2 and 3 correspond to ΔDi-6S and ΔDi-4S, respectively, and the large peak at *ca.* 4 min (peak 1) came from the buffer used for the enzymatic reaction. The product from a mixture of 1.5 g each of the GAG samples was dissolved in water and the solution analysed by HPCE. The unsaturated disaccharides were detected at 214 nm.

Separation and sensitivity were much improved by convesion of these unsaturated disaccharides to the PMP derivatives, as shown in Fig. 2. Fig. 2 also shows separation of the derivatives of other unsaturated disaccharides (ΔDi-diS_D, ΔDi-diS_E, ΔDi-0S and ΔDi-HA) obtained from the corresponding

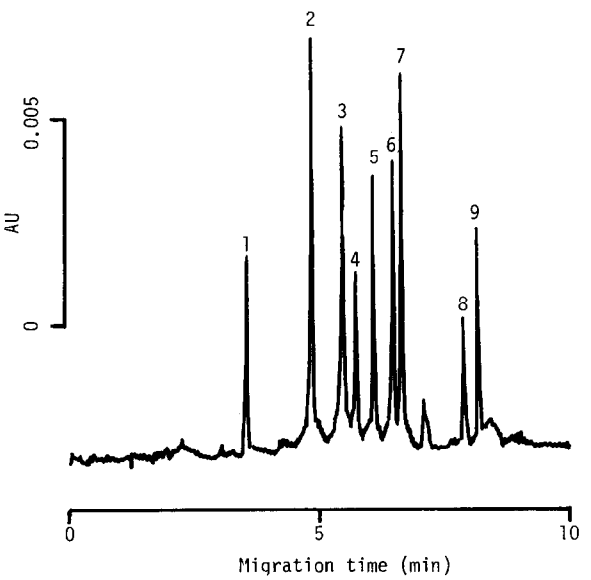

Fig. 2. Analysis of a chondroitinase ABC-digested mixture of chondroitin sulphates A–E, chondroitin and hyaluronic acid by zone electrophoresis after derivatization with PMP. The analytical conditions were as in Fig. 1. Peaks: 1 came from the buffer for enzymatic digestion; 2 = PMP (excess reagent); 3 = PMP derivative of ΔDi-0S; 4 = PMP derivative of ΔDi-HA; 5 = sodium benzoate (internal standard); 6 = PMP derivative of ΔDi-4S; 7 = PMP derivative of ΔDi-6S; 8 = PMP derivative of ΔDi-diS_D; 9 = PMP derivative of ΔDi-diS_E

GAGs. The migration order of ΔDi-4S (peak 6) and ΔDi-6S (peak 7) was reversed compared with that of the intact disaccharides (Fig. 1), presumably owing to change of separation mode. Since the reducing ends were blocked by the PMP groups, complexation was hampered, and as a result these disaccharides were moved by plain zone electrophoresis mode. The limit of detection at 214 nm was at the 10-fmol level as injected amount. The absorption spectra of the PMP derivatives of these disaccharides indicated that the absorbance at this wavelength was approximately one fifth of that at the maximum (245 nm). The peak at *ca.* 6.6 min (peak 4) is that of sodium benzoate as an internal standard, and peaks 3 and 4 are attributable to the PMP derivatives of ΔDi-0S and ΔDi-HA, respectively. The separation of these non-sulphated isomers is obviously the result of the difference of the ease of complexation of the hexosamine residues with the borate ion. Peaks 8 and 9 were assigned to ΔDi-diS_D and ΔDi-diS_E, respectively. Thus, all these disaccharides were well separated from each other by zone electrophoresis as borate complexes.

It is noteworthy that the derivatized products of ΔDi-4S and ΔDi-6S gave no detectable peak as did the PMP derivative of ΔDi-0S. These experimental results indicate that the O-sulphate group was stable in this operation.

The calibration curves of chondroitin sulphates A and C, as observed from the relative responses of ΔDi-4S and ΔDi-6S to sodium benzoate, showed excellent linearity at least for sample amounts in the range 0.5–10 μg. This range corresponds to the 10–400 pg range of injected amount. It is noted that the rate of production of ΔDi-4S from chondroitin sulphate A was considerably lower than that of ΔDi-6S from chondroitin sulphate C. The relative standard deviations (n = 10) of the relative response at the 5-μg level were 3.0% and 2.8%, respectively. The authentic specimen of chondroitin sulphate A gave a small amount of the derivative of ΔDi-6S together with that of ΔDi-4S (ΔDi-4S/ΔDi-6S = 60.5:33.7, in molar proportion based on the assumption that both disaccharides have the same molar absorptivity). Similarly the authentic specimen of chondroitin sulphate C gave the derivative of ΔDi-4S together with that of ΔDi-6S as a minor product (ΔDi-4S/ΔDi-6S = 16.6:82.1). Therefore, the determination of chondroitin sul-

phates A and C in their mixture was corrected by using these proportions.

On the basis of these observations, the GAG fraction of a human urine sample was digested with chondroitinase ABC, the product derivatized with PMP, and the derivative analysed by HPCE. Fig. 3 shows an example of the electropherograms.

The peaks of the PMP derivatives of both ΔDi-4S (peak 6) and ΔDi-6S (peak 7) are distinctly detected, and the amounts of chondroitin sulphates A and C can be determined from the relative responses of these disaccharides to sodium benzoate (peak 5). The minor peak at *ca.* 6 min (peak 3) is attributable to the PMP derivative of ΔDi-0S. Peaks 1 and 2 arose from the buffer used for the enzymatic reaction and the excess reagent, respectively. In parallel with this analysis the GAG fraction of the same urine sample was digested with chondroitinase AC from *Arthrobacter aurescens*, and the digestion mixture was derivatized with PMP. Analysis of the final product gave essentially the same electropherogram (not shown) as that in Fig. 3, and the responses of the ΔDi-4S and ΔDi-6S peaks relative to that of sodium benzoate were almost identical to those obtained with chondroitinase ABC. Chondroitinase ABC cleaves the hexosaminide linkage in chondroitin sulphate B along with those in chondroitin sulphates A and C, whereas chondroitinase AC cleaves the hexosaminide linkages only in chondroitin sulphates A and C. Since chondroitin sulphate B has the sulphate group attached to the 4-position of the galactosamine residue, like chondroitin sulphate A, the yield of ΔDi-4S obtained with chondroitinase ABC should be larger than that obtained with chondroitinase AC, if the GAG fraction contained chondroitin sulphate B. The result obtained from selected urine samples indicates that they did not contain detectable amounts of chondroitin sulphate B.

Since the concentrations of urinary substances vary greatly depending on physical and dietary conditions, they are usually expressed as a ratio relative to an appropriate inherent standard. Creatinine is the substance most widely used for this purpose. Although this compound is usually assayed by a colorimetric method using the reaction with picric acid [13], it can also be easily assayed by HPCE. Fig. 4 shows separation of creatinine from other inherent substances in urine using borate buffer (pH 9.0) containing polybrene, as carrier, as detected at

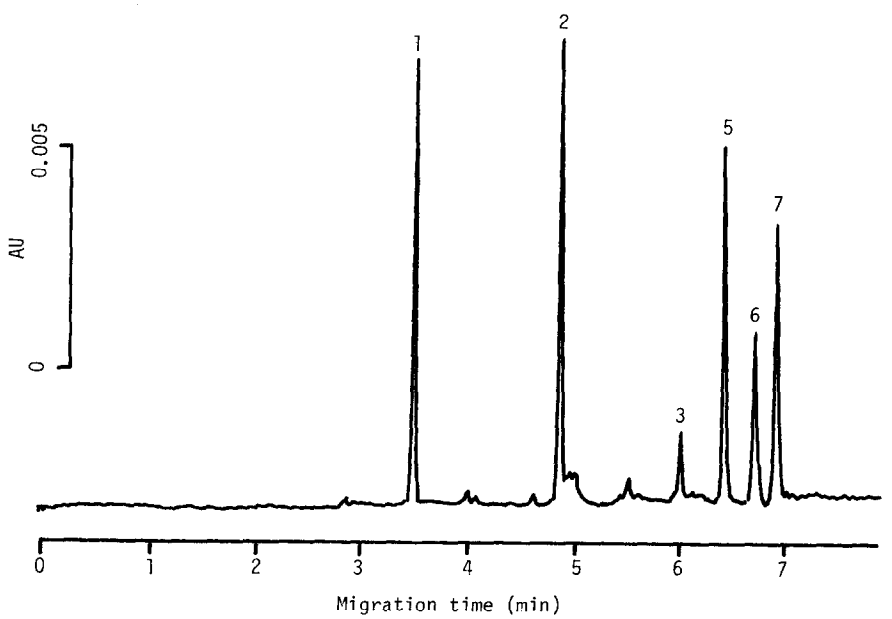

Fig. 3. Analysis of the PMP derivatives of unsaturated disaccharides derived from the GAG fraction of a urine sample digestion with chondroitinase ABC by zone electrophoresis. The analytical conditions were as in Fig. 1. Peak assignment is the same as that in Fig. 2.

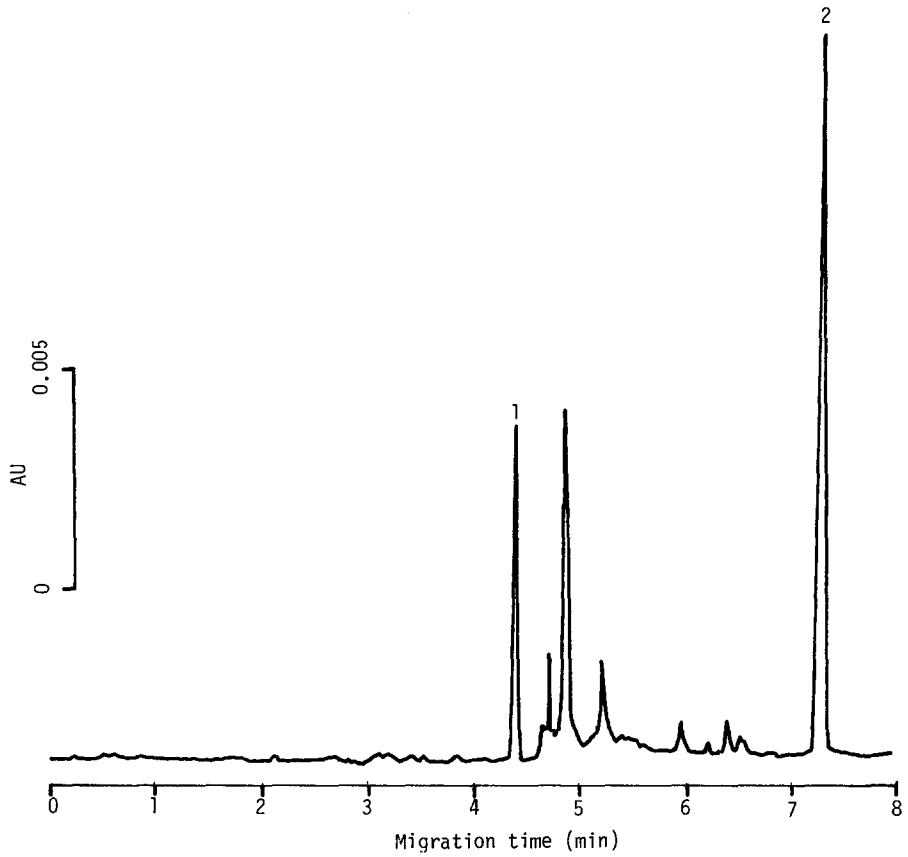

Fig. 4. Direct analysis of urinary creatinine by ion-exchange electrokinetic chromatography. Capillary, fused silica (51 cm × 50 μm I.D.); carrier, 100 m*M* borate buffer (pH 9.0) containing polybrene (0.1%, w/w); applied voltage, 15 kV; detection, UV absorption at 254 nm. Intact urine was injected from the cathodic end of the capillary tube. Peaks: 1 = sodium benzoate (internal standard); 2 = creatinine.

254 nm. In this system electro-osmotic flow was toward the anode, and separation based on the difference of the magnitude of interaction with the moving molecules of polybrene (ion-exchange electrokinetic chromatography mode) was realized. Under these conditions the relative response of creatinine to sodium benzoate (internal standard) was almost linear, at least in the range 0.1–5 mg/ml of creatinine (figure not shown), and urinary creatinine could be readily estimated by using this calibration curve without any prior clean-up procedures.

We could obtain the concentrations of urinary chondroitin sulphates A and C, together with that of creatinine, by HPCE as mentioned above. The following are a few examples of the ratios of chondroitin sulphates A and C to creatinine (μg/mg of creatinine). Sample 1 (male, 23 years old): A, 1.27; C, 0.59. Sample 2 (male, 23 years old): A, 0.84; C, 0.34. Sample 3 (male, 54 years old): A, 1.09; C, 0.50. Such values are consistent with the reported values [14]. The presence of Δ Di-0S in the digest of urinary GAGs in a normal subject has been noted in the literature [15], possibly arising from chondroitin or low-sulphate-content chondroitin sulphate, but the concentration was not reported. In the above cases the production of Δ Di-0S from samples 1, 2 and 3 was 8.9%, 13.8% and 9.3%, respectively, of total unsaturated disaccharides. Although Δ Di-HA was not detected in the present samples, some metabolic disorders such as Hurler's disease and Morquio's disease will give high values of Δ Di-HA, as suggested by Kodama *et al.* [2]. In such cases the present

method will be useful, since separation of ΔDi-HA from ΔDi-0S is good by the present method.

We are accumulating data on clinical samples. Discussion in relation to phatological conditions will be published elsewhere.

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