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FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF REDUCING SUGARS USING Dns-HYDRAZINE AS A PRE-LABELLING REAGENT

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SUMMARY

The fluorescence high-performance liquid chromatography of reducing sugars is described. Dns-hydrazine was used as a fluorescent pre-labelling reagent. Various reducing sugars, such as fucose, glucose, galactose and maltose, were labelled with Dns-hydrazine in trichloroacetic acid–ethanol solution and then chromatographed on LiChrosorb SI 100 column using ethanol–water–chloroform as eluent. Good separations of thirteen reducing sugars involving pentoses, hexoses and disaccharides were obtained within 25 min by stepwise elution system. Calibration graphs are linear in the range from 0.05 to 1.0 nmol for each sugar. The detection limits were about 3–20 pmol of reducing sugar.

INTRODUCTION

In recent years there has been increasing interest in the development of high-performance liquid chromatography (HPLC) for the analysis of sugars. However, the sensitivity of HPLC methods was too low owing to the use of refractive index and UV detectors. In order to increase the sensitivity of HPLC for the analysis of sugars, spectrophotometric pre-column[1,2] and post-column derivatization[3–6] techniques were employed. Recently, more sensitive HPLC methods for sugars were developed by using fluorophotometric post-column derivatization with 2-cyanoacetamide[7,8], ethylenediamine[9] and ethanolamine[10]. Although these methods are sensitive to as little as 1 nmol or less, the reaction temperature must be above 100°C and reagent pumps and a reaction system are required.

Dns-hydrazine (N-dimethylaminonaphthalene-5-sulphonic acid hydrazide) has been suggested as a fluorophotometric pre-labelling reagent for carbonyl compounds[11] and we have applied it to the analysis of oxo-steroids in biological fluids[12–14]. Avigad[15] reported the use of Dns-hydrazine for the fluorimetric detection of reducing sugars employing thin-layer chromatography. Alpenfels[2] developed the fluorescence reversed-phase HPLC of reducing sugars using Dns-hydrazine.

During the course of our study on the fluorescence HPLC of biological substances using Dns-hydrazine, we reported preliminary results for the fluorescence

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HPLC of reducing sugars using Dns-hydrazine as a pre-labelling reagent[16]. In this paper, we give the experimental details and variables of the fluorescence HPLC method using Dns-hydrazine as a pre-labelling reagent for the determination of reducing sugars.

EXPERIMENTAL

Materials

Dns-hydrazine (grade II) was purchased from Sigma (St. Louis, MO, U.S.A.) and various reducing sugars from Daiichi Kagaku Kogyo (Tokyo, Japan) and Wako (Tokyo, Japan). All other chemicals were of the highest grade commercially available. Water was distilled in glass.

Apparatus

A Hitachi 635 high-speed liquid chromatograph and a Hitachi 204 or a Shimadzu RF-500 spectrofluorophotometer equipped with a xenon lamp were used.

Reagent solutions

Dns-hydrazine solution (1.0%, w/v). A 100-mg amount of Dns-hydrazine was dissolved in 10 ml of ethanol, and the solution was stored at 4°C until required for use.

Trichloroacetic acid–ethanol solution (0.5%, w/v). A 0.5-g amount of trichloroacetic acid was dissolved in 100 ml of ethanol.

Stock solutions of sugars (1 mM). Each sugar was dissolved in water and made up to 1 μmol/ml.

Derivatization procedure with Dns-hydrazine

To 50 μl of the sample solution containing 10–100 nmol of reducing sugars were added 100 μl each of trichloroacetic acid–ethanol solution and Dns-hydrazine solution. The mixture was incubated at 50°C for 90 min and then cooled to room temperature. After evaporating the solvent under a stream of nitrogen gas, the derivatized sugar was redissolved by addition of 250 μl of ethanol and an aliquot of the resulting solution was injected into the chromatograph.

Chromatographic conditions

A 250 × 4 mm I.D. stainless-steel column was packed with LiChrosorb SI 100 by means of the slurry technique. For the separation of Dns-sugars the following three mobile phase systems were used: eluent I, 8% (v/v) ethanol–0.5% (v/v) water in chloroform; eluent II, 12% (v/v) ethanol–0.5% (v/v) water in chloroform; and eluent III, 20% (v/v) ethanol–0.6% (v/v) water in chloroform. The column was maintained at 27°C and the flow-rate of eluent was generally 1.5 ml/min, which corresponded to a pressure drop of 120 kg/cm^2 in the column. The effluent from the column was monitored at an emission wavelength of 500 nm with a spectrofluorophotometer using an excitation wavelength of 350 nm.

RESULTS AND DISCUSSION

Optimal conditions

Optimal conditions for the formation of the Dns-hydrazones of reducing sugars were examined so as to formulate the analytical procedure. The results were assessed from the peak heights in the chromatogram after the derivatization reaction.

Concentration of trichloroacetic acid and Dns-hydrazine solutions. The reaction of reducing sugars with Dns-hydrazine was catalysed by acid. Trichloroacetic acid was used according to Avigad[15]. The results for some sugars are shown in Fig. 1. The fluorescence response (peak height) decreased almost linearly with increasing trichloroacetic acid concentration and increased with increasing Dns-hydrazine concentration. Similar results were obtained with other sugars. Although the peak height increased with increasing concentration of Dns-hydrazine, the solubility of Dns-hydrazine was low and, moreover, unknown subpeaks appeared in the chromatogram. Therefore, the trichloroacetic acid and Dns-hydrazine concentrations were fixed at 0.5 and 1.0% (w/v), respectively.

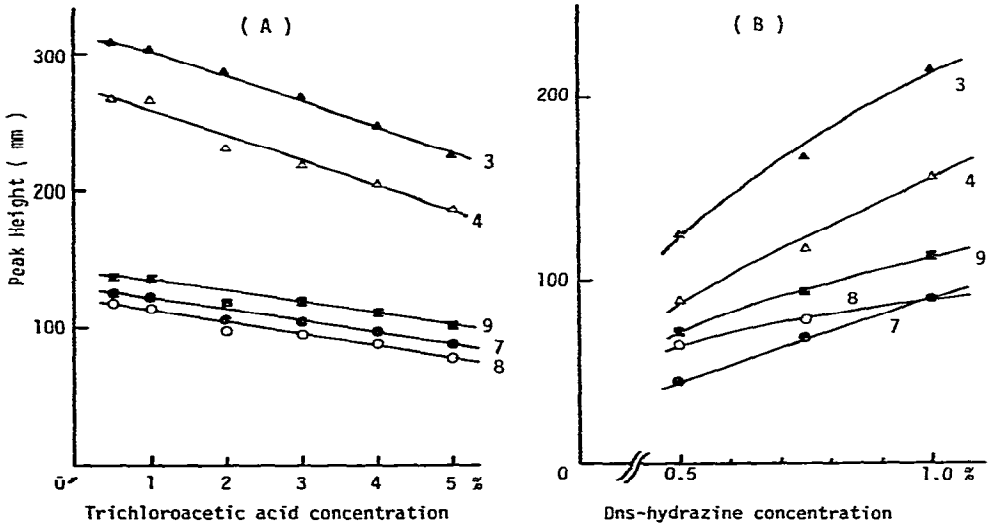

Fig. 1. Effects of (A) trichloroacetic acid and (B) Dns-hydrazine concentration on fluorescence intensity (peak height). 3 = Fucose; 4 = rhamnose; 7 = glucose; 8 = galactose; 9 = mannose.

Reaction temperature and time. Fig. 2 shows the effects of the reaction temperature and time on the fluorescence intensity. The peak heights of reducing sugars reached a maximum in the range 40–50°C, and for glucose at 50°C. With further increases in the reaction temperature, the responses decreased. The effect of the reaction time from 15 to 120 min was also examined. The fluorescence responses of sugars increased with increasing reaction time, except for glucose, and reached a plateau at 45 min. The peak height for glucose increased gradually with increasing reaction time and dit not reach a plateau. Therefore, it was decided to employ a reaction temperature of 50°C and a reaction time of 90 min.

TABLE I

RETENTION TIMES AND RELATIVE FLUORESCENCE INTENSITIES OF REDUCING SUGARS

Sugar	*Eluent I*		*Eluent II*		*Eluent III*		*Eluent I → III*	
	*R.T. (min)**	*R.I.***	*R.T. (min)**	*R.I.***	*R.T. (min)**	*R.I.***	*R.T. (min)**	*R.I.***
Glyceraldehyde	4.6	499	3.3	571	–	–	4.5	94
Ribose	4.9	223	3.4	241	–	–	4.8	52
Arabinose	7.2	239	4.2	184	–	–	7.2	81
Xylose	7.8	292	4.1	221	–	–	7.7	96
Fucose	5.3	371	3.2	271	–	–	5.3	194
Rhamnose	6.8	435	3.7	217	–	–	6.7	109
Glucose	14.5	100	6.8	100	3.6	100	13.0	100
Galactose	15.5	68	7.5	73	3.8	85	13.2	109
Mannose	19.0	137	8.2	115	3.8	122	13.4	158
N-Acetylglucosamine	–	–	10.2	10	4.6	38	14.6	48
N-Acetylgalactosamine	–	–	10.2	20	4.8	50	–	–
N-Acetylmannosamine	–	–	14.9	31	6.2	41	15.6	61
Maltose	–	–	–	–	7.4	29	18.6	27
Cellobiose	–	–	–	–	8.6	24	20.0	25
Gentiobiose	–	–	–	–	11.0	23	–	
Lactose	–	–	–		12.2	34	–	

* R.T. = retention time.

** R.I. = relative fluorescence intensity.

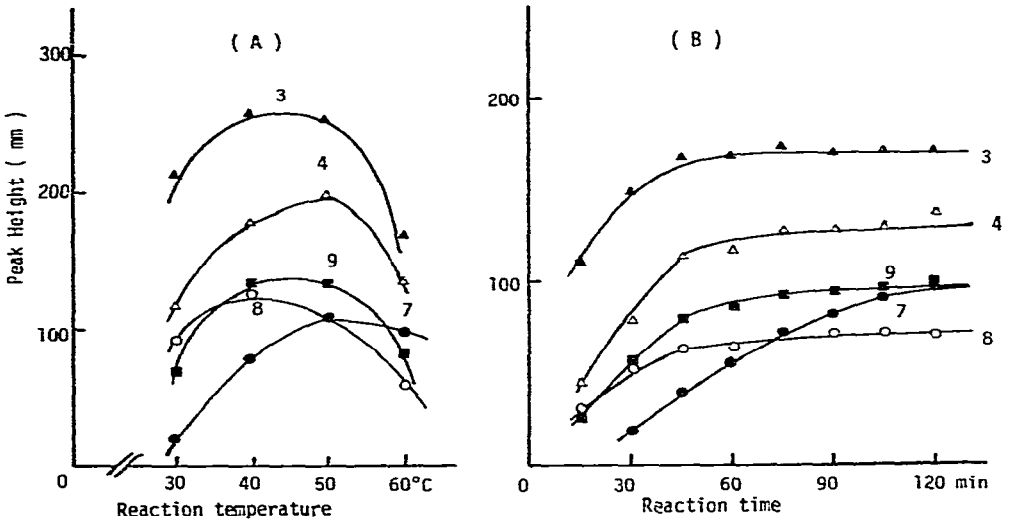

Fig. 2. Effects of (A) reaction temperature and (B) reaction time on fluorescence intensity (peak height). 3 = Fucose; 4 = rhamnose; 7 = glucose; 8 = galactose; 9 = mannose.

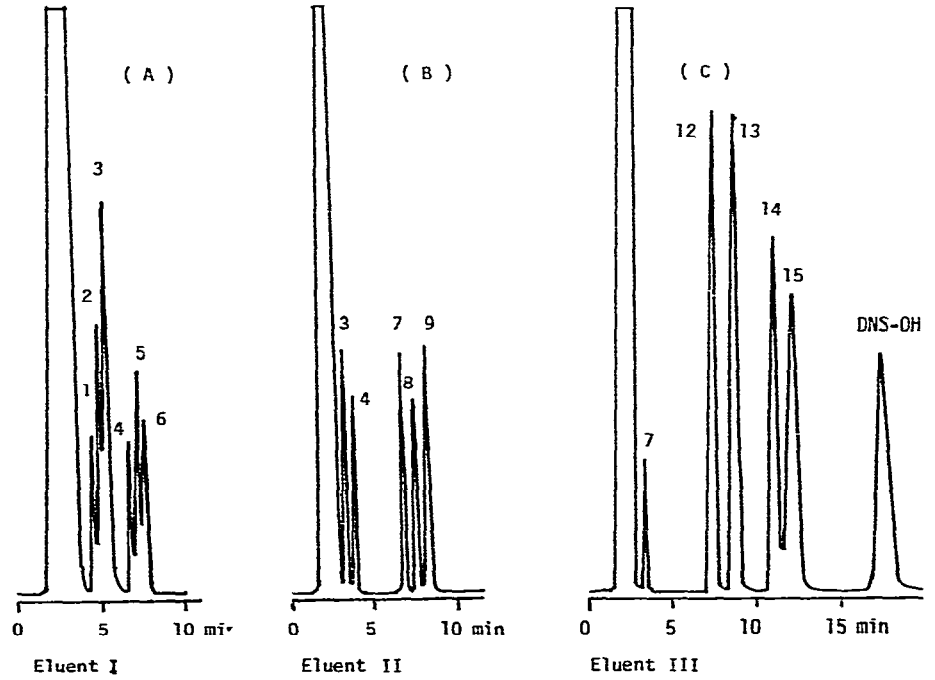

Fig. 3. Separation of fluorescent Dns-hydrazone derivatives of various reducing sugar mixtures. Peaks: 1 = glyceraldehyde; 2 = ribose; 3 = fucose; 4 = rhamnose; 5 = arabinose; 6 = xylose; 7 = glucose; 8 = galactose; 9 = mannose; 12 = maltose; 13 = cellobiose; 14 = gentiobiose; 15 = lactose; DNS-OH = Dns-sulphonic acid. Column: LiChrosorb SI 100 (250 × 4 mm I.D.). Mobile phase, ethanol–water in chloroform: eluent I, 8–0.5% (v/v); eluent II, 12–0.5% (v/v); eluent III, 20–0.6% (v/v). Flow-rate: 1.5 ml/min.

Separation of sugars

Recently, Alpenfels[2] reported a method for the determination of monosaccharides as their Dns-hydrazones by reversed-phase HPLC using a μBondapak C_{18} column and acetonitrile–water as the mobile phase. In previous work[12–14], Dns-hydrazones of various oxo-steroids were rapidly and completely separated by using a normal-phase system in which a microparticulate silica gel column and dichloromethane–ethanol–water as the mobile phase were used. Although the polarities of Dns-hydrazones of reducing sugars are different from those of steroid derivatives, the normal-phase system was chosen for the separation in this study.

Many mobile phase systems were examined in order to obtain the complete separation of Dns-hydrazone derivatives of reducing sugars. The mixtures of ethanol–water–chloroform (eluent I, II and III) were found to be suitable when used with a LiChrosorb SI 100 column. The chromatograms presented in Fig. 3 show good separations of standard mixtures of reducing sugars, including pentose, hexose and disaccharide, by using these eluents. As shown in Fig. 3, the separation conditions used in these analyses do not allow the simultaneous separation of all reducing sugars. Table I gives the retention times of all of the reducing sugars tested, as eluted by the three mobile phase systems and by stepwise elution using eluents I and III. The chromatogram shown in Fig. 4 was obtained by elution successively with eluents I

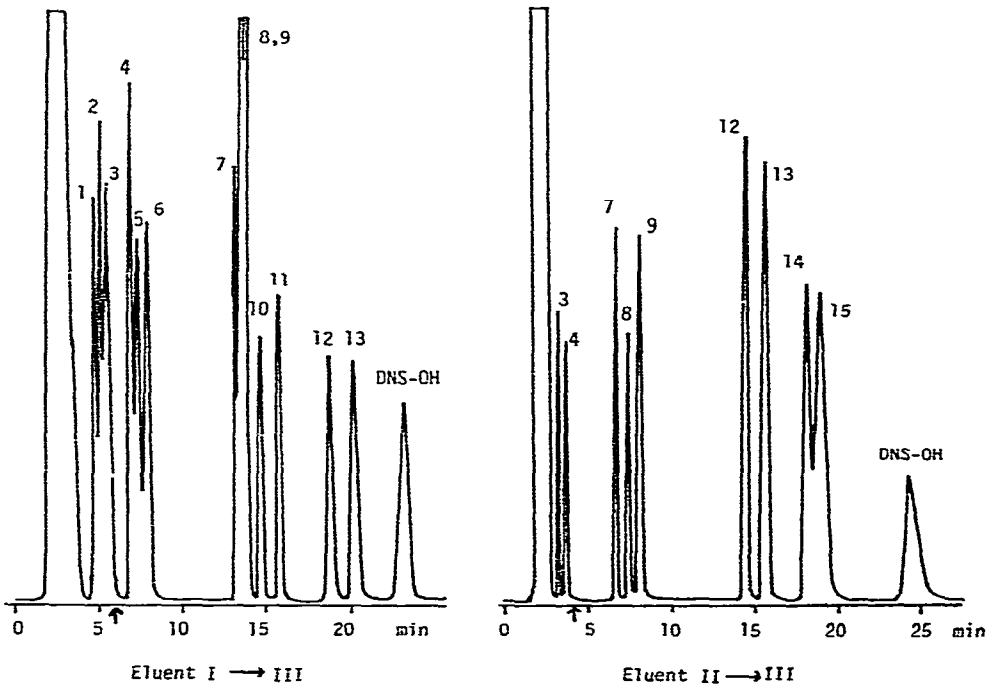

Fig. 4. Chromatograms of Dns-hydrazone derivatives of various reducing sugars by using a stepwise elution system. Peaks: 1 = glyceraldehyde; 2 = ribose; 3 = fucose; 4 = rhamnose; 5 = arabinose; 6 = xylose; 7 = glucose; 8 = galactose; 9 = mannose; 10 = N-acetylglucosamine; 11 = N-acetylmannosamine; 12 = maltose; 13 = cellobiose; 14 = gentiobiose; 15 = lactose; DNS-OH = Dns-sulphonic acid. Mobile phase was changed from I to III or from II to III.

and III, or II and III, with increasing concentrations of ethanol. Under these conditions, thirteen or nine reducing sugars could be separated within 25 min. Such a short separation time is one of advantages of the present method using a normal-phase system.

Calibration graphs and sensitivity

Fig. 5 shows the calibration graphs for sugars separated by using eluents I (A), II (B) and III (C). All of the graphs were linear in the range from 0.05 to 1.0 nmol of

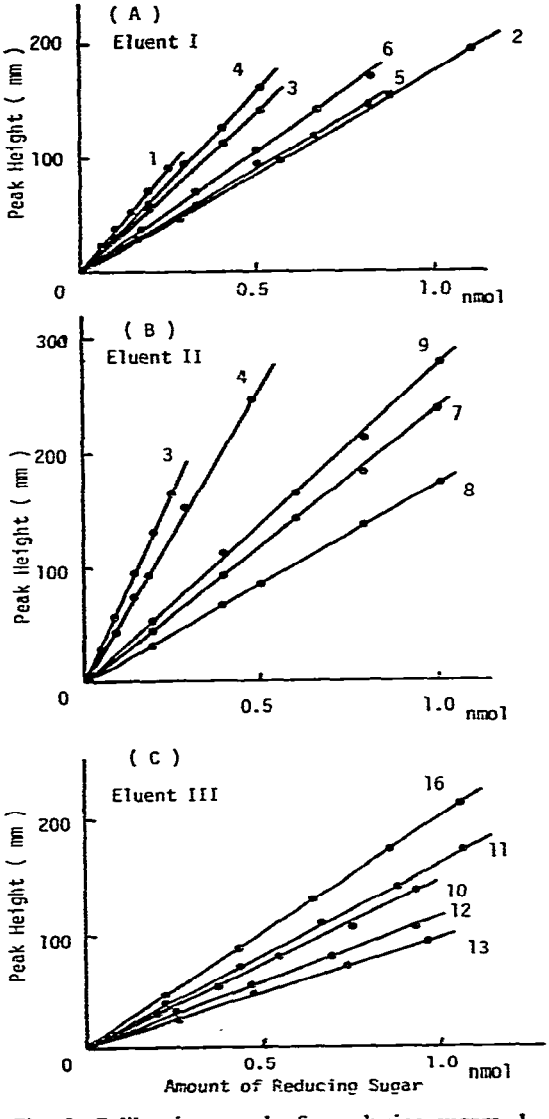

Fig. 5. Calibration graphs for reducing sugars. 1 = Glyceraldehyde; 2 = ribose; 3 = fucose; 4 = rhamnose; 5 = arabinose; 6 = xylose; 7 = glucose; 8 = galactose; 9 = mannose; 10 = N-acetylglucosamine; 11 = N-acetylmannosamine; 12 = maltose; 13 = cellobiose; 16 = N-acetylgalactosamine.

each sugar injected into the chromatograph. The relative response varied over a considerably wide range among the reducing sugars. For example, fucose and rhamnose are more sensitive than glucose, galactose and mannose when using eluent II as the mobile phase. The relative intensities of reducing sugars are listed in Table I, in comparison with glucose, in each elution system. The varying response may reflect varying yields of the derivatization reaction or different polarities of the individual eluents.

The detection limit is estimated to be about 50 pmol from the calibration graphs in Fig. 5. However, the sensitivity of fluorescence HPLC depends on the efficiency of the fluorescence detector, the final injection volume and the polarity of the eluent. When using a Hitachi 204 spectrofluorophotometer as a detector, the limits of detection were in the range 3–20 pmol of reducing sugars, as illustrated in Table II.

TABLE II

DETECTION LIMITS OF REDUCING SUGARS

Sugar	*Detection limit (pmol)**	*Sugar*	*Detection limit (pmol)**
Glyceraldehyde	5	Galactose	5
Ribose	10	Mannose	3
Arabinose	6	N-Acetylglucosamine	10
Xylose	5	N-Acetylmannosamine	8
Fucose	3	Maltose	19
Rhamnose	5	Cellobiose	20
Glucose	5		

* Stepwise elution: Eluent I → III.

Recently, the post-labelling fluorescence HPLC methods for sugars using 2-cyanoacetamide[7,8], ethylenediamine[9] and ethanolamine[10] have been reported, with detection limits of 0.01, 1.0 and 0.3 nmol, respectively. The highest sensitivity, reported by Honda *et al.*[8], was obtained when a purified sample of 2-cyanoacetamide reagent was used. Although all of these methods are highly sensitive, the reaction temperature has to be high, *viz.*, 100°C for the 2-cyanoacetamide method and 140–150°C for the ethylenediamine and ethanolamine methods.

Application

A urine sample, which was pre-treated according to the procedure reported by D'Amboise *et al.*[5], was assayed by the present method. The chromatogram is shown in Fig. 6. Fucose, ribose, xylose (or arabinose) and glucose were found in the chromatogram using eluent II. Phytolaccasaponin E, containing one molecule each of xylose and glucose, was assayed by the present method after hydrolysis. Xylose and glucose were found in the resultant chromatogram and their ratio was nearly 1:1. Glucose and other reducing sugars in wine, vinegar, Japanese wine and soy sauce could be assayed by the proposed method after clean-up with an ion-exchange resin and charcoal. Detailed studies of applications will be reported in the near future.

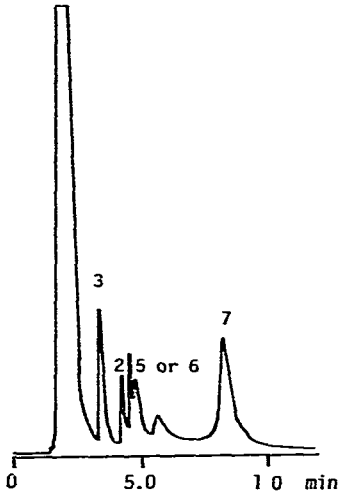

Fig. 6. Typical chromatogram of urine sample. Peaks: 2 = ribose; 3 = fucose; 5 = arabinose (or 6 = xylose); 7 = glucose. Eluent II was used as mobile phase and the peaks were identified by comparing their retention times with those of a standard mixture.

REFERENCES

1 S. Honda and K. Kakehi, *J. Chromatogr.*, 152 (1978) 405.
2 W. F. Alpenfels, *Anal. Biochem.*, 114 (1981) 153.
3 M. Sinner and J. Puls, *J. Chromatogr.*, 156 (1978) 197.
4 M. D'Amboise, D. Noel and T. Hanai, *Carbohydr. Res.*, 79 (1980) 1.
5 M. D'Amboise, T. Hanai and D. Noel, *Clin. Chem.*, 26 (1980) 1348.
6 S. Honda, M. Takahashi, Y. Nishimura, K. Kakehi and S. Ganno, *Anal. Biochem.*, 118 (1981) 162.
7 S. Honda, Y. Matsuda, M. Takahashi and K. Kakehi, *Anal. Chem.*, 52 (1980) 1079.
8 S. Honda, M. Takahashi, K. Kakehi and S. Ganno, *Anal. Biochem.*, 113 (1981) 130.
9 K. Mopper, *Anal. Chem.*, 52 (1980) 2018.
10 T. Kato and T. Kinoshita, *Anal. Biochem.*, 106 (1980) 238.
11 R. W. Frei and J. F. Lawrence, *J. Chromatogr.*, 83 (1973) 321.
12 T. Kawasaki, M. Maeda and A. Tsuji, *J. Chromatogr.*, 163 (1979) 143.
13 T. Kawasaki, M. Maeda and A. Tsuji, *Yakugaku Zasshi*, 100 (1980) 925.
14 T. Kawasaki, M. Maeda and A. Tsuji, *J. Chromatogr.*, 226 (1981) 1.
15 G. Avigad, *J. Chromatogr.*, 139 (1977) 343.
16 M. Takeda, M. Maeda and A. Tsuji, *23th Meeting on Liquid Chromatography, Kyoto*, 1980, Abstracts, p. 59.