HPLC with fluorescence detection of urinary phenol, cresols and xylenols using 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride as a fluorescence labeling reagent†

Kenichiro Nakashima,*[a] Shinobu Kinoshita,[a] Mitsuhiro Wada,[a] Naotaka Kuroda[a] and Willy R. G. Baeyens[b]

[a] *School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852, Japan*
[b] *Laboratory of Drug Quality Control, Faculty of Pharmaceutical Sciences, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium*

Received 16th June 1998, Accepted 4th August 1998

A simple and sensitive HPLC method for the determination of phenolic compounds, *i.e.*, phenol (Phe), cresols (Cres) and xylenols (Xyls), was developed. After a pre-column fluorescence derivatization of these compounds with 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) at 60 °C for 30 min, 11 DIB derivatives were successfully separated within 50 min with an ODS column using CH_3CN–H_2O–CH_3OH (25 + 22 + 53, v/v) as the eluent. The detection limits of DIB derivatives at a signal-to-noise ratio of 3 ranged from 0.15 to 1.09 μM (0.2–1.6 pmol per 20 μl). The precision of the proposed method for both within- and between-day assays of free and total phenol related compounds was satisfactory (RSD < 9.5%). By the proposed method, Phe and *p*-Cre could be detected in normal urine samples, and the calculated concentrations of free Phe and *p*-Cre in unhydrolysed urine samples were 1.5 ± 1.3 and 23.9 ± 24.3 μM and those of total Phe and *p*-Cre in hydrolysed urine samples were 87.3 ± 81.2 and 200.7 ± 195.4 μM (n = 21), respectively.

Introduction

Phenol (Phe) and cresols (Cres) are widely used as disinfectants and in insecticides, herbicides and synthetic fibers. Although Phe and *p*-Cre occur normally in human urine as metabolites of tyrosine by intestinal flora,[1] concentrations above the physiological levels suggest contact with benzene or toluene.[2] *o*-Cre and xylenols (Xyls) are minor phenols in urine, but their levels are known to increase after a heavy exposure to toluene and xylene, respectively.[3] Moreover, phenol related compounds are of interest owing to their carcinogenic[4] and endocrine disrupting effects.[5–7] Therefore, the development of a selective and sensitive method for the determination of phenolic compounds is necessary for environmental, toxicological and clinical studies.

Many methods for determining Phe and Cres in human urine by GC[8–10] and HPLC[11–13] have been developed. A large sample volume (> 1 ml) is required to determine Phes by GC methods owing to their insufficient sensitivity, which is a disadvantage in the case of difficult-to-obtain samples, *e.g.*, infant urine samples. Using HPLC methods, these compounds have been determined directly with either UV,[11] electrochemical[12] and native fluorescence detection[13] or determined with fluorescence detection after fluorescence derivatization.[14] Direct detection methods[11–13] without derivatization are not able to analyse very small amounts of sample owing to the lack of sensitivity. Fluorescence derivatization results in a substantial increase in sensitivity and selectivity compared with direct methods. However, only a few fluorescent derivatization reagents have been developed for this purpose.[14–16]

In a previous paper, we reported the synthesis of a new HPLC–fluorescence derivatization reagent for amines and phenols,[17] *i.e.*, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl), and applied it to the determination of sympathomimetic amines[18] and chlorophenols.[19] In this study, DIB-Cl was applied to determine Phe, Cres and Xyls in urine to confirm its applicability to a wide variety of phenolic compounds.

Experimental

Chemicals

Phe, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-Xyl, *m*-methoxyphenol [internal standard (IS)] and triethylamine (TEA) were obtained from Wako (Osaka, Japan), *o*-, *m*- and *p*-Cres from Tokyo Kasei Kogyo (Tokyo, Japan) and 4-(*N*-phthalimidinyl)benzenesulfonyl chloride (Phisyl-Cl) from Dojindo Laboratories (Kumamoto, Japan). DIB-Cl was synthesized according to our previous method.[17] Water was distilled with an Autostill WG 220 system (Yamato Scientific, Tokyo, Japan) and passed through a Puric-Z purification system (Organo, Tokyo, Japan). Acetonitrile and methanol were of HPLC grade (Wako). The other chemicals used were of analytical reagent grade.

Authentic DIB-Phe derivative was prepared as follows: a mixture of DIB-Cl (0.09 g, 0.23 mmol), Phe (0.043 g, 0.46 mmol) and TEA (0.5 ml) in 50 ml of acetonitrile was heated at 60–65 °C for 1 h with stirring. On condensing the reaction mixture to about 10 ml, a white–yellow precipitate was obtained. The crude product was filtered and recrystallized from acetonitrile to give white–yellow crystals; yield 0.06 g, 65%; m.p. 219–223 °C, EI-MS, (*m/z*) 416 [M^+]. Analysis: calculated for $C_{28}H_{20}O_2N_2 \cdot H_2O$, C 77.40, H 5.10, N 6.45; found, C 77.51, H 5.49, N 6.98%.

† Presented at the VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis, Las Palmas de G.C., Spain, May 26–29, 1998.

Apparatus

Mass spectra were measured using a JEOL (Tokyo, Japan) JMS-DX 303 mass spectrometer.

The HPLC system for the determination of DIB derivatives consisted of a CCPD HPLC pump (Tosoh, Tokyo, Japan), a model 7125 injector with a 20 μl sample loop (Rheodyne, Cotati, CA, USA), a Daisopak-SP-120-5-ODS-BP analytical column (250 × 4.6 mm, id, 5 μm) (Daiso, Osaka, Japan), an RF-550 fluorescence detector (Shimadzu, Kyoto, Japan) and an FIB-1 recorder (Tosoh). DIB-Phe derivatives were separated isocratically with CH_3CN–H_2O–CH_3OH (25 + 22 + 53, v/v) as the eluent and monitored at 450 nm with excitation at 340 nm.

Pre-treatment of urine samples and derivatization

Normal urine samples were obtained from healthy volunteers in our department. The urine samples were treated according to the assay procedure shown in Fig. 1. Because phenolic compounds are present in urine as sulfate or glucuronide conjugates in addition to the free forms, they should be hydrolyzed to determine the total concentrations prior to the measurement. In this study, to determine total concentrations of phenolic compounds, portions of 100 μl of urine containing *m*-methoxyphenol as IS (final concentration 100 μM) were hydrolysed with 200 μl of 4 M HCl at 60 °C for 60 min. Preliminary examination of the hydrolysis process of urine samples showed that constant and maximum peak heights for DIB-Phe and DIB-*p*-Cre were obtained on treatment at 60 °C for 60 min. The mixture was cooled to room temperature and neutralized with 200 μl of 4 M NaOH and the resultant solution was used as the hydrolyzed urine sample. Phenolic compounds were extracted with diethyl ether as follows: after 1 ml of 0.1 M phosphate buffer (pH 5.5) and 2.5 ml of diethyl ether had been successively added to 100 μl of the hydrolyzed urine sample, the mixture was vortex mixed for 1 min and centrifuged at 2000 rpm for 5 min at 4 °C. To prevent volatilization of phenolic compounds, 10 μl of 3 M NaOH were added to 1.5 ml of the organic layer and the mixture was vortex mixed and evaporated to dryness with an RD-31 centrifugal evaporator (Yamato Scientific, Tokyo, Japan). For the determination of free phenols, 100 μl of unhydrolyzed urine sample containing the IS (final concentration 10 μM) were used.

To the residue dissolved in 200 μl of CH_3CN, 200 μl of 15 mM DIB-Cl in CH_3CN containing 1.5% TEA were added and the mixture was heated at 60 °C for 30 min. After addition of 10 μl of 3 M HCl and 390 μl of eluent, the reaction mixture was chilled in tap water and passed through a membrane filter (0.45 μm). Aliquots of 20 μl of this solution were injected into the HPLC system.

Comparison of DIB-Cl method with Phisyl-Cl method

For comparison, 10 urine samples were spiked with Phe and *p*-Cre at concentrations ranging from 5 to 200 μM per compound

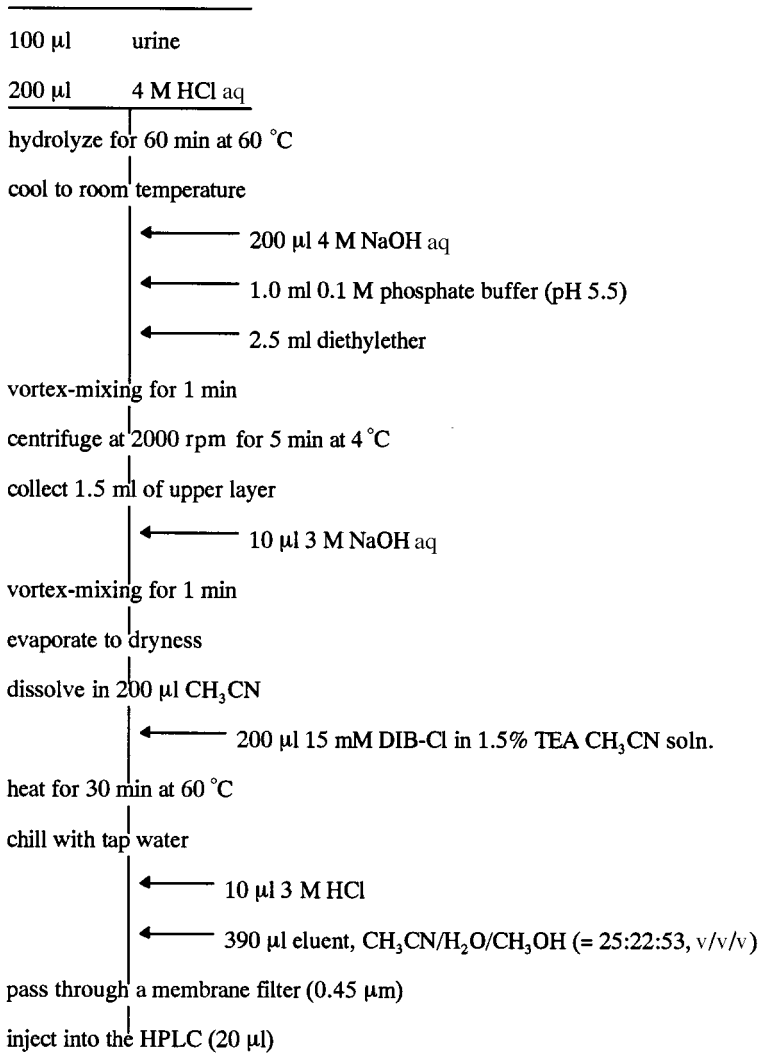

Fig. 1 Assay procedure for determining total Phe and *p*-Cre in urine.

and analysed simultaneously with the proposed DIB-Cl method and also with the Phisyl-Cl method. The phisylation procedure, HPLC conditions and λ_{ex} and λ_{em} values for Phisyl derivatives were as reported in the literature.[14]

Results and discussion

The fluorescence derivatization conditions used were as described in a previous paper;[19] 1.5% TEA was used as a basic catalyst and included in DIB-Cl solution in acetonitrile. The DIB-Cl concentration used was 15 mM, and the reaction temperature and time adopted in this study were 60 °C for 30 min. The peak of authentic DIB-Phe corresponded well with that of the reaction product of DIB-Cl with Phe by co-chromatography. A reaction yield of 97.4% was estimated by comparing the peak heights of authentic DIB-Phe and the reaction product. The DIB-Phe obtained was stable with respect to the peak height for at least 24 h when the reaction mixture was kept in the dark at room temperature.

Eleven DIB derivatives were isocratically separable within 50 min using an ODS column (250× 4.6 mm id) with CH_3CN–H_2O–CH_3OH (25 + 22 + 53, v/v) as the eluent. The retention times of DIB derivatives were very reproducible and gave peaks at 22.4 ± 0.5 min for Phe, 27.2 ± 0.6 min for *o*-Cre, 30.6 ± 0.9 min for *m*-Cre, 31.4 ± 0.9 min for *p*-Cre, 33.6 ± 0.9 min for 2,6-Xyl, 35.7 ± 1.1 min for 2,3-Xyl, 37.6 ± 1.1 min for 2,5-Xyl, 38.9 ± 1.2 min for 2,4-Xyl, 41.0 ± 1.2 min for 3,4-Xyl and 42.6 ± 1.2 min for 3,5-Xyl. The RSDs of the retention times were < 3.1 % (n = 8). Except for *m*- and *p*-Cre, all other compounds could be separated well from each other (resolution > 1.3). A typical chromatogram of DIB derivatives with phenol related compounds is shown in Fig. 2.

Calibration curves and detection limits

Calibration curves were prepared with standard phenolic compound-spiked urine samples and good linearity was obtained between the fluorescence intensity as peak height ratios and concentration of phenols up to 12.5 μM for Phe, 100 μM for *p*-Cre and 5.0 μM for the other compounds (r = 0.987) (Table 1).

The detection limits obtained with standard samples ranged from 0.15 to 1.1 μM (0.2–1.6 pmol per 20 μl injection) at a signal-to-noise ratio of 3. The sensitivity of the proposed method is much higher than those of GC (10 μM for Phe and *p*-Cre)[8] and HPLC with native fluorescence detection (3.2 μM for Phe),[3] and comparable to that of HPLC with fluorescence labelling (0.17 and 0.25 pmol per injection; 0.30 and 0.44 μM for Phe and *p*-Cre, respectively).[14]

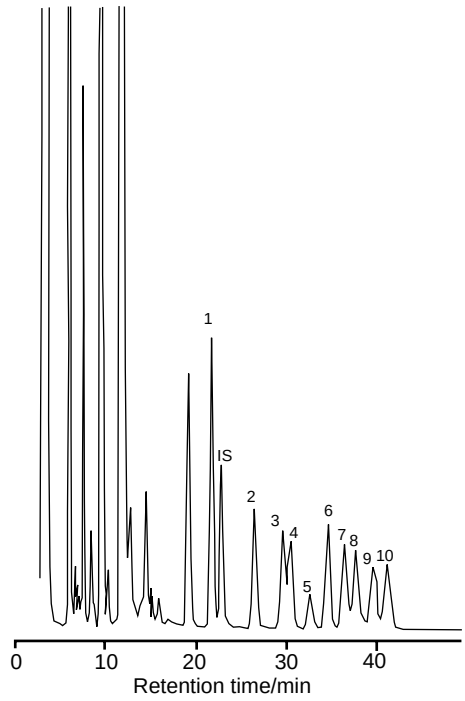

Fig. 2 Typical chromatogram of DIB derivatives of standard phenol related compounds. Sample concentration: Phe 125, IS 100 and the other compounds 50 μM. Other experimental conditions as in the Experimental section. Peaks: 1, Phe; 2, *o*-Cre; 3, *m*-Cre; 4, *p*-Cre; 5, 2,6-Xyl; 6, 2,3-Xyl; 7, 2,5-Xyl; 8, 2,4-Xyl; 9, 3,4-Xyl; and 10, 3,5-Xyl.

Precision and accuracy

Using standard phenolic compound-spiked urine samples, within-day variations were determined by calculating the recoveries of the added standard phenols. For the recovery test with free phenols 7.5 μM of phenols were used, whereas 75 μM of phenols were used for that with total phenols. The recoveries and RSDs obtained were 96.7–103.3% and 2.6–6.9% for free phenols and 94.3–105.9% and 3.1–8.9% for total phenols (n = 6), respectively.

The precisions of between-day assays were determined by analysing the same sample over a period of 1 week. The RSDs obtained for free (n = 6) and total (n = 7) phenols were 2.5–6.7% and 5.2–9.3%, respectively.

The proposed DIB-Cl method was compared with the Phisyl-Cl method. The concentrations of Phe and *p*-Cre in spiked urines obtained by the present method were correlated with those determined by the Phisyl-Cl method. The equations and correlation coefficients (r) between the two methods were y = 0.985x + 4.47 (r = 0.982, n = 10) for Phe, and y = 1.024x + 8.87 (r = 0.961, n = 10) for *p*-Cre, respectively, where y is the concentration (μM) obtained by the Phisyl-Cl method and x is the concentration (μM) obtained by the DIB-Cl method.

Determination of Phe and *p*-Cre in urine

The concentrations of Phe and *p*-Cre in normal urine were determined with the proposed method. Normal urine samples were collected early in the morning before breakfast from volunteers in our department who were consuming ordinary diets.

Typical chromatograms of a hydrolysed normal urine sample and of a hydrolysed urine sample spiked with Phe and *p*-Cre are shown in Fig. 3. DIB derivatives of Phe and *p*-Cre were detected in the hydrolyzed normal urine sample. If the other phenolic

Table 1 Calibration curves and detection limits.

Compound	Calibration curve[a]		Detection limit[b] (pmol per injection)
	Regression equation	r	
Phe	y = 0.194x + 0.6	0.994	0.2
o-Cre	y = 0.671x + 2.0	0.987	0.2
m-Cre	y = 0.059x + 0.01	0.995	0.3
p-Cre	y = 0.002x + 0.24	1.000	0.4
2,3-Xyl	y = 0.056x + 0.05	0.996	0.3
2,4-Xyl	y = 0.043x + 0.09	0.998	0.5
2,5-Xyl	y = 0.070x + 0.01	1.000	0.4
2,6-Xyl	y = 0.059x + 0.01	0.966	1.6
3,4-Xyl	y = 0.034x + 0.04	0.999	0.4
3,5-Xyl	y = 0.045x + 0.04	0.999	0.4
IS			0.2

[a] Prepared with urine sample spiked with standard phenols; x, compound concentration in urine (μM); y, peak height ratio (sample/IS). [b] Obtained with standard samples (S/N = 3).

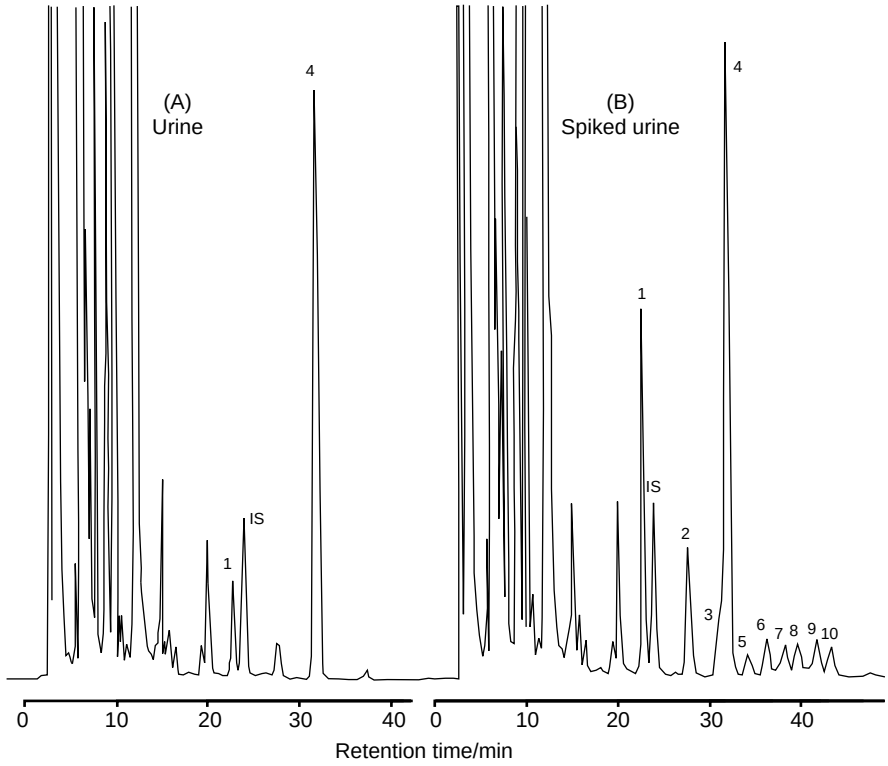

Fig. 3 Chromatograms of DIB derivatives of Phe and *p*-Cre in hydrolyzed urine. Sample concentration: (A) IS 100; (B) Phe 125, IS 100 and others 50 μM. Other experimental conditions as in the Experimental section. Peaks: 1, Phe; 2, *o*-Cre; 3, *m*-Cre; 4, *p*-Cre; 5, 2,6-Xyl; 6, 2,3-Xyl; 7, 2,5-Xyl; 8, 2,4-Xyl; 9, 3,4-Xyl; and 10, 3,5-Xyl.

compounds including Xyls are present in the urines examined, their concentrations must be lower than the detection limit of the present method. This suggests that the volunteers were not heavily exposed to benzene or toluene.

To assign the peaks, the eluates corresponding to DIB-Phe and DIB-*p*-Cre were collected and their mass spectra were measured; the molecular ion peaks at *m/z* 416 for DIB-Phe and *m/z* 430 for DIB-*p*-Cre were in good agreement with the theoretical values. A peak at a retention time of about 28 min in Fig. 3(A) was expected for DIB-*o*-Cre. However, this was not observed because the MS data were different to that of DIB-*o*-Cre. The small peak at retention time of about 37 min was unknown and also derived from normal urines. The average concentrations of free and total Phe and *p*-Cre in urine were 1.5 ± 1.3 and 87.3 ± 81.2 μM (n = 21) and 23.9 ± 24.3 and 200.7 ± 195.4 μM (n = 21) respectively. It is known that phenol and *p*-Cre normally exist in the human body as metabolites of tyrosine caused by intestinal flora.[1] The concentrations of phenol obtained with the proposed method are reasonably comparable to those reported by Murray and Adams (Phe, 5.6–184 μM)[20] and Tsuruta *et al.* (Phe, 11.5–382 μM; *p*-Cre, 11.0–987 μM).[14]

Conclusions

DIB-Cl, an easy-to-prepare[17] reagent which is stable[18] in acetonitrile solution for at least 1 month when kept in the dark at room temperature, was previously successfully applied as a fluorescent pre-column reagent for sympathomimetic amines. In the present study, DIB-Cl was also found to be applicable as a fluorescent derivatization reagent for Phe, Cre and Xyls. The proposed method should be useful for toxicological and pharmaceutical studies and for the study of the effects of endocrine disrupting compounds, including some phenolic compounds, on humans, which have recently attracted the attention of many environmental scientists. HPLC studies of endocrine disrupting phenolic compounds are in progress and will be reported elsewhere.

References

1 E. Bone, A. Tamm and M. Hill, *Am. J. Clin. Nutr.*, 1976, **29**, 1448.
2 H. Kontsas, C. Rosenberg and P. Pfaffli, *Analyst*, 1995, **120**, 1745.
3 G. Bienick, *Occup. Environ. Med.*, 1994, **51**, 354.
4 R. K. Boutwell and D. K. Bosch, *Cancer Res.*, 1959, **19**, 413.
5 A. Sato, H. Justicia, J. Wray and C. Sonnenschein, *Environ. Health Perspect.*, 1991, **92**, 167.
6 S. C. Nagel, F. S. vom Saal, K. A. Thayer, M. G. Dhar, M. Boechler and W. V. Welshons, *Environ. Health Perspect.*, 1997, **105**, 70.
7 A. Krishnan, P. Stathis, S. Permuth, L. Tokes and D. Feldman, *Endocrinology*, 1993, **132**, 2279.
8 M. Balikova and J. Koklicek, *J. Chromatogr.*, 1989, **497**, 159.
9 M. Makita, S. Yamamoto, A. Katoh and Y. Takashita, *J. Chromatogr.*, 1978, **147**, 456.
10 L. Weber, *J. Chromatogr.*, 1992, **574**, 349.
11 A. Brega, P. Prandini, C. Amaglio and E. Pafumi, *J. Chromatogr.*, 1990, **535**, 311.
12 W. E. Schaltenbrand and S. P. Coburn, *Clin. Chem.*, 1985, **31**, 2042.
13 B. L. Lee, H. Y. Ong, C. Y. Shi and C. N. Ong, *J. Chromatogr.*, 1993, **619**, 259.
14 Y. Tsuruta, S. Watanabe and H. Inoue, *Anal. Biochem.*, 1996, **243**, 86.
15 P. J. M. Kwakman, D. A. Kamminga and U. A. Th. Brikman, *J. Chromatogr.*, 1991, **553**, 345.
16 C. Pviter, J. F. Bohle, G. J. Jong, U. A. Brinkman and R. W. Frei, *Anal. Chem.*, 1988, 60, 666.
17 K. Nakashima, H. Yamasaki, N. Kuroda and S. Akiyama, *Anal. Chim. Acta*, 1995, **303**, 103.
18 O. Al-Dirbashi, J. Quarnstrom, K. Irgum and K. Nakashima, *J. Chromatogr. B.*, 1998, **718**, 105.
19 M. Wada, S. Kinoshita, Y. Itayama, N. Kuroda and K. Nakashima, *J. Chromatogr. B.*, submitted for publication.
20 K. E. Murray and R. P. Adams, *J. Chromatogr.*, 1988, **431**, 143.

Paper 8/04582H