Journal of Chromatography, 464 (1989) 343–352
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 131

DETERMINATION OF AMPHETAMINE-RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE AND FLUORESCENCE DETECTIONS

KAZUICHI HAYAKAWA*, KIMIE HASEGAWA and NORIKO IMAIZUMI
Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920 (Japan)
OSBORNE S. WONG
Oread Laboratories Inc., 1501 Wakarusa Drive, Lawrence, KS 66046 (U.S.A.)
and
MOTOICHI MIYAZAKI
Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920 (Japan)
(First received August 1st, 1988; revised manuscript received November 21st, 1988)

SUMMARY

High-performance liquid chromatography with chemiluminescence detection has been established for the determination of trace levels of amphetamine-related compounds (APs) after fluorigenic derivatization. Bis(2,4,6-trichlorophenyl) oxalate and hydrogen peroxide in acetonitrile was used as a post-column chemilumigenic reagent. As derivatization reagents, dansyl chloride (Dns-Cl), 4-fluoro-7-nitrobenzoxadiazole (NBD-F) and naphtalene-2,3-dicarbaldehyde (NDA) were compared. Dns-Cl was the most suitable of the three for the simultaneous determination of both primary and secondary amino APs. The on-column detection limits for Dns-derivatives were $3 \cdot 10^{-15} - 4 \cdot 10^{-15}$ mol. NDA gave the most sensitive derivatives (cyanobenz[*f*]isoindole, CBI derivatives) with only primary amino APs. The detection limits for CBI derivatives were as low as $2 \cdot 10^{-16}$ mol. The present method was applied to the determination of methamphetamine in human urine. Only diethyl ether extraction was necessary as a clean-up treatment before Dns derivatization, because diethyl ether extracted methamphetamine quantitatively from urine at strong alkaline pH and the extract showed few interfering peaks around the retention time of methamphetamine after the derivatization. Methamphetamine concentrations as low as $1 \cdot 10^{-7}$ *M* in urine were determined after the above treatments.

INTRODUCTION

The abuse of amphetamine-related compounds (APs) has led to thorough studies of their metabolism, distribution and excretion. Many chromatographic methods were developed for the sensitive determination of APs in urine and plasma. Gas chromatography (GC) with either nitrogen selective or flamethermionic detection can detect sub-ng amounts of APs[1,2]. Gas chromatography–mass spectrometry (GC–

0021-9673/89/$03.50

MS) method can detect as little as several pg of APs[3]. High-performance liquid chromatography (HPLC) methods for the determination of APs have an advantage over GC and GC–MS in that the preparations of aqueous samples such as biological fluids are easy. The sensitivities of the HPLC methods reported were not as high as those of GC and GC–MS, although some of them used fluorescence or electrochemical detection[4–6].

HPLC with chemiluminescence detection was reported to be highly sensitive and selective for fluorescent compounds[7]. The chemiluminescence detection limits of several fluorescent compounds were 10 – 100 times lower than those by fluorescence detection. The selectivity of the method reduced clean-up treatments for the determination of polynuclear aromatic hydrocarbons (PAHs) in environmental samples[8–10]. Moreover, both primary and secondary amines were also chemilumigenically detected after derivatization to fluorescent compounds with such reagents as dansyl chloride (Dns-Cl)[11,12]. It was suggested that HPLC with chemiluminescence detection might be useful in the sensitive and selective determination of APs. However, there has been no application of this method to the determination of APs.

The purpose of this study was to find the best derivatizing agent for the determination of APs by HPLC with chemiluminescence detection and to compare the sensitivity of chemiluminescence detection with that of fluorescence detection. Furthermore, the present method was applied to the determination of low levels of methamphetamine in human urine.

EXPERIMENTAL

Chemicals

Acetonitrile, acetone, methanol, diethyl ether and imidazole of reagent grade were obtained from Nakalai (Kyoto, Japan). Bis(2,4,6-trichlorophenyl) oxalate (TCPO) and 30% hydrogen peroxide solution of electronic grade were from Tokyo Kasei (Tokyo, Japan) and Kanto (Tokyo, Japan), respectively. Dns-Cl and naphthalene-2,3-dicarbaldehyde (NDA) were from Wako (Osaka, Japan) and Molecular Probes (Eugene, U.S.A.), respectively. 4-Fluoro-7-nitrobenzoxadiazole (NBD-F) was kindly supplied by Dr. K. Imai, Pharmacy of Branch Hospital, University of Tokyo. Other chemicals used were all commercially available.

APs and their standard solutions

The structures and sources of APs used are listed in Table I. A standard solution ($1.0 \cdot 10^{-4}$ *M*) was prepared by dissolving the appropriate amount of each compound in water.

Apparatus

The HPLC system consisted of two Shimadzu (Kyoto, Japan) LC-6A high-pressure pumps, a 7125 injector with a loop of 20 μl (Rheodyne, Cotati, CA, U.S.A.), an MPLC guard column (30 mm × 4.6 mm I.D.) packed with Spheri-5 RP-18 (Brownlee, Santa Clara, U.S.A.), a separation column (250 mm × 4.6 mm I.D.) packed with Inertsil ODS-2 (Gasukuro Kogyo, Tokyo, Japan), a reaction coil (300 mm × 0.25 mm I.D.), a damper coil (2000 mm × 0.1 mm I.D.), a KZS-1 mixing device (Kyowa Seimitsu, Mitaka, Japan), a Shimadzu RF-530 fluorescence detector,

TABLE I

FORMULAE OF APs USED

Compound	*–R*	*Source*[a]	*Peak number*
Benzylamine	$-CH_2NH_2$	1	2
Phenylethylamine	$-CH_2CH_2NH_2$	1	4
Phenylpropylamine	$-CH_2CH_2CH_2NH_2$	2	5
Phenylbutylamine	$-CH_2CH_2CH_2CH_2NH_2$	2	6
N-Methylphenethylamine	$-CH_2CH_2NH-CH_3$	2	7
Methamphetamine[b]	$-CH_2CH(CH_3)-NH-CH_3$	3	8
Phenylpropanolamine	$-CH(OH)-CH(CH_3)-NH_2$	1	1
Ephedrine[b]	$-CH(OH)-CH(CH_3)-NH-CH_3$	3	3
N-Isopropylbenzylamine	$-CH_2NH-CH(CH_3)_2$	2	9

[a] 1 = Nakarai (Kyoto, Japan); 2 = Aldrich (Milwaukee, WI, U.S.A.); 3 = Dainippon Pharmacy (Osaka, Japan).

[b] Hydrochloride form.

an AC-2220 luminomonitor (spiral flow cell of 60 μl) (Atto, Tokyo, Japan) and two Shimadzu C-R3A integrators. A schematic diagram of the system is shown in Fig. 1.

The fluorescence spectra were measured by a 650-10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

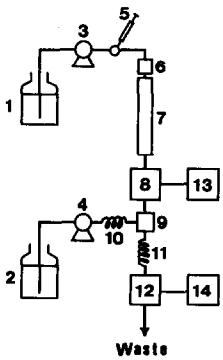

Fig. 1. Schematic diagram of the HPLC system. 1 = Reservoir (eluent); 2 = reservoir (chemilumigenic reagent); 3, 4 = pumps; 5 = injector; 6 = guard column; 7 = separation column; 8 = fluorescence detector; 9 = mixing device; 10 = damper coil; 11 = reaction coil; 12 = luminomonitor; 13, 14 = integrators.

HPLC operating conditions

The mobile phase for HPLC was prepared by dissolving imidazole in acetonitrile–water to a final concentration of $1.0 \cdot 10^{-3}$ *M*. The mixture was adjusted to pH 7.0 with nitric acid before adjusting the final volume. The ratios of acetonitrile–water were 8:2 (v/v) for the separation of CBI (cyanobenz[*f*]isoindole) derivatives, 7:3 for Dns derivatives and 6:4 for NBD derivatives. Each mobile phase was treated with a Fuji Film FR-40 membrane filter (pore size 0.4 μm) and supersonicated before use. The chemilumigenic reagent solution was prepared before use as follows. TCPO (0.112 g) was dissolved in 500 ml of acetonitrile, and 8.6 ml of 30% hydrogen peroxide solution were added. The mixture was supersonicated.

Other conditions were as follows: Flow-rate of the mobile phase, 1.0 ml/min; flow-rate of the chemilumigenic reagent solution, 1.0 ml/min; column temperature, ambient; injection volume, 20 μl; excitation (Ex) and emission (Em) wavelengths of the fluorescence detector, 343 nm (Ex) and 530 nm (Em) for Dns derivatives, 418 nm (Ex) and 483 nm (Em) for CBI derivatives and 470 nm (Ex) and 530 nm (Em) for NBD derivatives. The attenuation of the integrator was adjusted to give similar height of corresponding peaks in the comparison of chromatograms obtained by chemiluminescence and fluorescence detections.

Extraction

Methamphetamine hydrochloride was dissolved in human urine at the concentration of $1.0 \cdot 10^{-7}$ *M*. To 2.0 ml of this solution in a test-tube, 2.0 ml of sodium hydroxide solution (2.0% in water) and 2.0 ml of diethyl ether were added successively. The tube was capped and shaken vigorously for 5 min. After centrifuging at 1000 *g* rpm for 10 min, the ether phase was collected. The extraction procedure was repeated twice.

Derivatization

The procedure of Dns derivatization was as follows. To 0.1 ml of the solution of APs (the mixture of $1.0 \cdot 10^{-4}$ *M* of each compound) in a test-tube, 0.4 ml of carbonate buffer (sodium bicarbonate dissolved in water at a concentration of $1.0 \cdot 10^{-2}$ *M*, pH adjusted to 9.0 with sodium hydroxide solution) and 0.5 ml of Dns-Cl solution ($1.0 \cdot 10^{-3}$ *M* in acetone) were added successively. After mixing, the capped tube was kept at 45°C in a water-bath in the dark for 1 h. When methamphetamine in human urine was to be determined, 0.1 ml of a diethyl ether extract was used instead of the amine solution.

The procedure for NBD derivatization was as follows. To 0.1 ml of the solution of APs (as used for Dns derivatization) in a test-tube, 0.4 ml of borate buffer (boric acid dissolved in water at a concentration of $1.0 \cdot 10^{-1}$ *M*, pH adjusted to 8.0 with sodium hydroxide solution) and 0.5 ml of NBD-F ($8.0 \cdot 10^{-2}$ *M* in ethanol) were added successively. After mixing, the capped tube was kept at 60°C in a water-bath in the dark for 1 min.

The procedure for CBI derivatization was as follows. To 0.1 ml of the solution of APs (as used for Dns derivatization) in a test-tube, 0.3 ml of borate buffer (boric acid was dissolved in water at a concentration of $5.0 \cdot 10^{-2}$ *M*, pH adjusted to 9.0 with sodium hydroxide solution), 0.4 ml of acetonitrile, 0.1 ml of sodium cyanide solution ($1.0 \cdot 10^{-2}$ *M* in water) and 0.1 ml of NDA solution ($2.0 \cdot 10^{-3}$ *M* in

methanol) were added successively. After mixing, the capped tube was kept at room temperature in the dark for 2 h.

Each derivatized solution was mixed and diluted in acetonitrile–water (1:1). An aliquot of the solution was injected into the chromatographic system.

RESULTS AND DISCUSSION

In our previous work concerning the determination of PAHs, we found that a mixture of TCPO and hydrogen peroxide was stable enough in acetonitrile to be used by a one-pump post-column chemilumigenic reaction system of HPLC for several hours[13]. The same system was used in the present work. The concentration of acetonitrile in the mobile phase was optimized to give the best resolution in each series of derivatives of APs. In a batchwise operation with the TCPO–hydrogen peroxide solution and the mobile phases described above, chemiluminescence intensities of the three derivatives tested reached their maxima in 2 s and lasted for more than 10 s after mixing. The size of the reaction coil of the present system was adjusted to detect the chemiluminescence between 0.5 and 2.3 s after mixing. The other conditions were the same as used for PAHs.

Dns-Cl, which reacts with both primary and secondary amines, is a popular fluorigenic labelling reagent. It has also been used as a chemilumigenic labelling reagent[11,12]. Fig. 2 shows chromatograms of nine Dns derivatives of APs with chemiluminescence (A) and fluorescence (B) detections by the injection of $1.0 \cdot 10^{-12}$ mol of each derivative. All APs were separately determined by both detection methods in 22

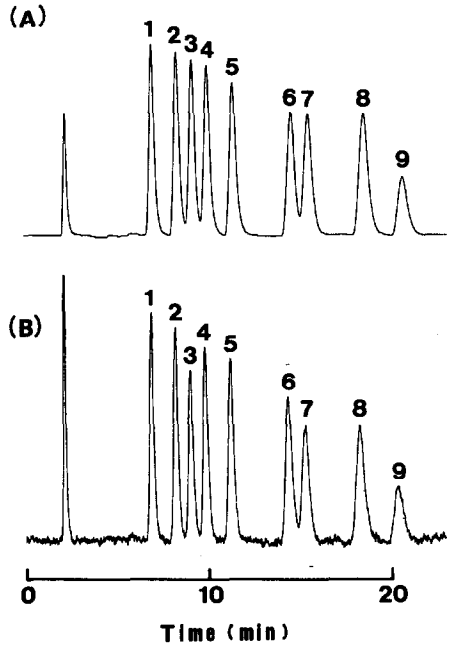

Fig. 2. Typical chromatograms of Dns-APs with chemiluminescence (A) and fluorescence (B) detections. Peak numbers as in Table I. For conditions see text.

min. The noise level by chemiluminescence detection was much smaller than that by fluorescence detection, and the corresponding peaks showed similar heights in the two detection methods. This result indicated that the detection sensitivity of the former method was better than that of the latter in the determination of Dns derivatives of APs. The peak areas of the nine APs were almost constant except for that of N-isopropylbenzylamine which may have a steric effect on the tertiary propyl group next to nitrogen on the derivatization yield or the quantum yield. The constant response is advantageous in the simultaneous determination of metabolites of APs.

NBD-F reacts with both primary and secondary amines to give fluorescent compounds. The reaction is very rapid under mild conditions, which is superior to NBD-Cl[14]. NBD derivatization has not been applied for the HPLC determination of amines with chemiluminescence detection, although the chemiluminescence intensities of NBD-proline have been compared to those of other compounds[15]. Fig. 3 shows chromatograms of NBD derivatives of nine APs with chemiluminescence (A) and fluorescence (B) detections. The separation of phenylpropanolamine and N-methylphenethylamine was not achieved under these conditions. The amount of primary amino APs injected was $1.0 \cdot 10^{-12}$ mol of each, while that of the secondary amino APs was $1.0 \cdot 10^{-11}$ mol of each, because of the poorer response of the latter. The noise level by chemiluminescence detection was much larger than that by fluorescence detection in Fig. 3. The main disadvantage of NBD derivatives was that the sensitivity of APs by chemiluminescence detection was less than that by fluorescence detection.

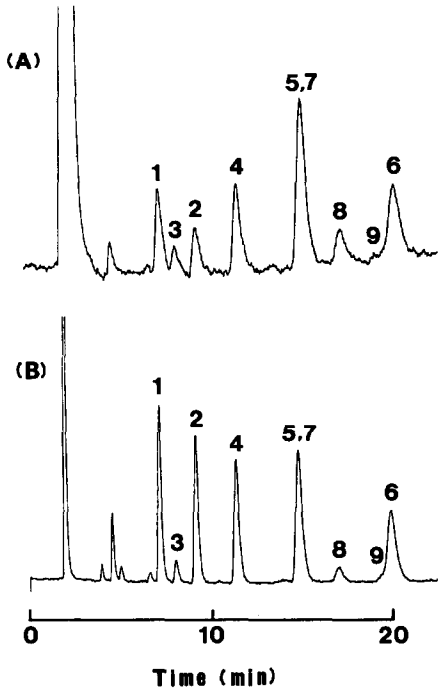

Fig. 3. Typical chromatograms of NBD-APs with chemiluminescence (A) and fluorescence (B) detections. Other details as in Fig. 2.

NDA is a fluorigenic reagent which reacts only with primary amines in the presence of cyanide[16]. Recently, it has been reported that NDA derivatives (also called CBI derivatives) of catecholamines were very sensitive to chemiluminescence detection[17]. Fig. 4 shows chromatograms of CBI derivatives of primary amino APs with both chemiluminescence (A) and fluorescence (B) detections by the injection of $1.0 \cdot 10^{-13}$ mol. Five APs were separately determined in 14 min. The areas of the corresponding peaks for five APs were not very different by each detection mode as shown in Fig. 3. The important fact is that the large signals of the CBI derivatives were observed with a very stable baseline by chemiluminescence detection even with the injection of 1/10 of the amount of Dns derivatives in Fig. 2. This suggests that NDA might be a very useful derivatization agent in the sensitive chemilumigenic determination of such primary amino APs as amphetamine.

The detection limits for Dns-, NBD- and CBI derivatives of five APs are compared in Table II. They are defined as that mass of analyte which provides a signal equal to two times the peak-to-peak noise by using mobile phases corresponding to the three series. The most important result is the high sensitivity of chemiluminescence detection. The detection limits for CBI derivatives were $2 \cdot 10^{-16}$ mol, which is the smallest in the three series. CBI derivatives might be more sensitive to chemiluminescence detection than to fluorescence detection even if excited at 252 nm which gave detection limits 10 times better than those at 418 nm[15]. The disadvantage of CBI derivatives is that they are applicable only for primary amino APs. If both primary and secondary amino APs are to be determined, Dns derivatives give higher sensitiv-

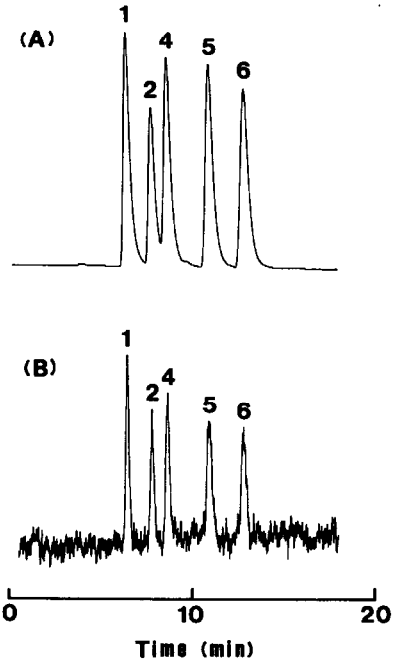

Fig. 4. Typical chromatograms of CBI-APs with chemiluminescence (A) and fluorescence (B) detections. Other details as in Fig. 2.

TABLE II

DETECTION LIMITS FOR DNS-, NBD- AND CBI-APs BY HPLC WITH BOTH CHEMILUMINESCENCE AND FLUORESCENCE DETECTIONS

Derivatization	*Detection method*	*Detection limit[a] of analyte (10^{-15} mol)*				
		PPA	*BA*	*EPH*	*PEA*	*MA*
Dns	Chemiluminescence	4	3	4	4	4
	Fluorescence	50	40	50	50	50
NBD	Chemiluminescence	6000	900	30 000	400	20 000
	Fluorescence	2000	200	4000	200	30 000
CBI	Chemiluminescence	0.2	0.2		0.2	
	Fluorescence	20	30		20	

[a]PPA = Phenylpropanolamine; BA = benzylamine; EPH = ephedrine; PEA = phenylethylamine; MA = methamphetamine. Signal-to-noise ratio = 2.

ity than NBD derivatives. This result is in accord with a report concerning the comparison of NBD, Dns and *o*-phthalaldehyde (OPA) derivatives of aliphatic amines[12]. The detection limits of Dns derivatives were $3 \cdot 10^{-15} - 4 \cdot 10^{-15}$ mol. To date, GC–MS is the most sensitive determination method for APs. The detection limits for heptafluorobutyryl derivatives of amphetamine and methamphetamine by GC–MS were $1.5 \cdot 10^{-14}$ and $1.4 \cdot 10^{-14}$ mol, respectively[3]. The present HPLC method with chemiluminescence detection is more sensitive than GC–MS by a factor of 70 for CBI derivatives and 3.5 for Dns derivatives.

Methamphetamine is a major abuse drug in Japan. A large fraction of methamphetamine administered is excreted in urine as methamphetamine itself[18]. Therefore, the present method was applied to the determination of methamphetamine added in human urine. It was necessary to clean up the urine sample before derivatization in order to reduce interfering peaks. Diethyl ether had the advantages that it easily

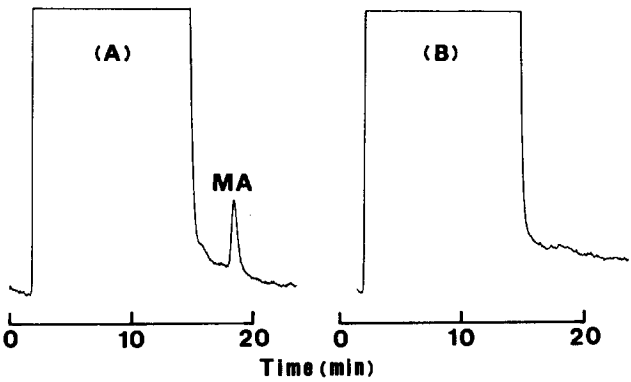

Fig. 5. Typical chromatograms of diethyl ether extracts from methamphetamine-spiked (A) and control (B) human urine samples after Dns-derivatization. The concentration of methamphetamine (MA) was $1.0 \cdot 10^{-7}$ *M* in human plasma. For other conditions see text.

extracted methamphetamine from urine at strong alkaline pH[19] and that the extract gave less interfering peaks than other organic solvents tested by GC[20]. Table II shows that Dns-Cl was the best derivatization agent for methamphetamine. Consequently, a diethyl ether extract from urine was directly derivatized by Dns-Cl, and an aliquot of the mixture was injected onto the column. Fig. 5 shows chromatograms of methamphetamine-spiked human urine (A) and control human urine (B) after the treatment described above. Methamphetamine as low as $1.0 \cdot 10^{-7}$ *M* in urine was detected without any interfering peak at the corresponding retention time. Lower detection limits may be obtained by concentration of the ether extracts. The large peak eluted before 15 min interferes with the detection of other analytes which are eluted in 15 min or less, although this peak was almost negligible on diluting the reaction mixture as shown in standard chromatograms. The contribution of the control urine sample to this peak was not significant. It might be decreased by purification of the reagents used or by the extraction of degradation products such as Dns-OH, because it was observed upon injection of the reagent blank. The recoveries of methamphetamine from human urine by the diethyl ether extraction were $99.3 \pm 0.7\%$ when the concentration of methamphetamine was $5.0 \cdot 10^{-7}$ *M*. These results indicate that the present method with diethyl ether extraction and Dns derivatization is promising for the determination of trace levels of methamphetamine in human urine. Optimization of the clean-up and derivatization will increase the practical value of the method, and will be reported elsewhere.

ACKNOWLEDGEMENTS

The authors thank Dr. K. Imai of Pharmacy of Branch Hospital, University of Tokyo for his kind gift of NBD-F and his valuable advice for chemiluminescence detection. The authors also wish to acknowledge the advice of H. Kobayashi and N. Takayama of Ishikawa Prefectural Police Headquarters.

REFERENCES

1 M. Terada, T. Yamamoto, T. Yoshida, Y. Kuroiwa and S. Yoshimura, *J. Chromatogr.*, 237 (1982) 285.
2 M. Terada, S. Yoshimura, T. Yamamoto, T. Yoshida and Y. Kuroiwa, *Eisei Kagaku*, 29 (1983) 143.
3 T. Yamamoto, M. Terada, S. Yoshimura, T. Sato, H. Kitagawa, T. Yoshida, K. Aoki and Y. Kuroiwa, *Eisei Kagaku*, 27 (1981) 331.
4 B. M. Farrell and T. M. Jefferies, *J. Chromatogr.*, 272 (1983) 111.
5 K. Ishikawa, J. L. Martinez, Jr. and J. L. MacGaugh, *J. Chromatogr.*, 306 (1984) 394.
6 N. Takayama, H. Kobayashi and A. Tsuji, *Eisei Kagaku*, 30 (1984) 14.
7 S. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 424.
8 K. W. Sigvardson and J. W. Birks, *Anal. Chem.*, 55 (1983) 432.
9 K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, 56 (1984) 1096.
10 N. Imaizumi, K. Hayakawa and M. Miyazaki, *J. Biomed. Chromatogr.*, submitted.
11 K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.*, 316 (1984) 501.
12 G. Mellbin and B. E. F. Smith, *J. Chromatogr.*, 312 (1984) 203.
13 N. Imaizumi, K. Hayakawa. K. Imai and M. Miyazaki, *Analyst (London)*, in press.
14 Y. Watanabe and K. Imai, *J. Chromatogr.*, 239 (1982) 723.
15 K. Honda, K. Miyaguchi and K. Imai, *Anal. Chim. Acta*, 177 (1985) 111.
16 P. de Montigny, J. F. Stobaugh, R. S. Givens, R. G. Carlson, K. Srinivasachar, L. A. Sternson and T. Higuchi, *Anal. Chem.*, 59 (1987) 1096.

17 T. Kawasaki, O. Wong, K. Imai, T. Higuchi, R. S. Givens, J. F. Stobaugh and T. Kuwana, paper presented at the *10th International Symposium on Column Liquid Chromatography, San Francisco, CA, May 1986*, abstract No. 3511.
18 J. Caldwell, L. G. Dring and R. T. Williams, *Biochem. J.*, 129 (1972) 11.
19 R. C. Driscoll, F. S. Barr, B. J. Gragg and G. W. Moore, *J. Pharm. Sci.*, 60 (1971) 1492.
20 H. Kobayashi and N. Takayama, personal communication, 1988.