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Assay of nicergoline and three metabolites in human plasma and urine by high-performance liquid chromatography–atmospheric pressure ionization mass spectrometry

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ABSTRACT

A method for the simultaneous determination of nicergoline and three of its metabolites in human plasma and urine has been developed using high-performance liquid chromatography–atmospheric pressure ionization mass spectrometry. Nicergoline and its metabolites were extracted from the plasma and urine samples with chloroform and separated on a reversed-phase ODS column. The eluents were led to the atmospheric pressure ionization interface and then analysed in the selected-ion monitoring mode. The detection limits of nicergoline and three of its metabolites were *ca.* 2 ng/ml in plasma and *ca.* 10 ng/ml in urine, at a signal-to-noise ratio of 4.

INTRODUCTION

Nicergoline (10α-methoxy-1,6-dimethylergoline-8β-methanol 5-bromonicotinic acid ester), which is a derivative of semisynthetic ergot alkaloids, was first developed at Farmitalia Carlo Erba (Milan, Italy) [1,2]. It shows vasodilating and α-receptor blocking activity [3], and has been used clinically in Japan for improving blood circulation and brain metabolism for three years.

The adsorption, distribution, metabolism and excretion of nicergoline in animals have been investigated with a tritium-labelled compound [4]. A radioimmunoassay method for the determination of nicergoline in human plasma has been reported [5], but the effect of immunological cross-reaction between the unchanged drug and its metabolites was not studied sufficiently, and the simultaneous determination of nicergoline and metabolites could not be performed.

Recently, there has been interest in the use of high-performance liquid chromatography–mass spectrometry (HPLC–MS) for the analysis of non-volatile organic compounds in various fields. Many groups have reported the determination of new drugs in biological fluids using HPLC–MS systems, including a thermospray method [6–9], a moving-belt method [10,11], a vacuum nebulizing method [12,13], an atmospheric pressure ionization (API) method [14–16], and so on.

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Eckers *et al.* [17] studied crude extracts of ergot fermentation broths by HPLC–MS using a moving-belt interface and a simple form of mass spectrometry–mass spectrometry (MS–MS).

We reported previously the HPLC determination of nicergoline and three of its metabolites in plasma and urine [18]. However, the separation in this method was not complete because of admixture in plasma and urine, and it required a long and complicated procedure for sample preparation. A highly selective assay method for the unchanged drug and three of its metabolites in plasma and urine is required for the pharmacokinetic parameters to be obtained with good precision.

This paper describes the separation and simultaneous determination of nicergoline and three of its metabolites using HPLC with API-MS in the selected-ion monitoring (SIM) mode. This system has a higher selectivity for nicergoline and three of its metabolites than the previous HPLC method. It was applied to a study of the assay of nicergoline and three of its metabolites in plasma after oral administration of nicergoline tablets.

EXPERIMENTAL

Materials

Nicergoline, 10-methoxy-1,6-dimethylergoline-8β-methanol (1-MMDL), 1-hydroxymethyl-10-methoxy-6-methylergoline-8β-methanol (1-OHMMDL), 10-methoxy-6-methylergoline-8β-methanol (MDL) and 10α-methoxy-1,6-dimethylergoline-8β-methanol 5-chloronicotinic acid ester (5-chloronicergoline, internal standard) used as reference standards were synthesized and identified by Farmitalia Carlo Erba. Ammonium acetate was reagent grade, and chloroform and acetonitrile were HPLC grade, all from Katayama Chemicals (Osaka, Japan).

Apparatus

The HPLC–API-MS system used was a Model M-1000 (Hitachi, Tokyo, Japan) attached to a post-accelerator. This system was exclusive for HPLC–MS and was equipped with quadrupole mass spectrometer. The pump unit used was a Hitachi Model L-6200 with low-pressure gradient system, and a Hitachi Model L-4000 UV detector was also used to monitor the UV absorption of eluents. A UV detector was placed between the pump unit and the API interface. Sample solutions were injected with a syringe through a Rheodyne Model 7161 loop injector. The loop volume was 200 μl. Separation was performed on a reversed-phase ODS Cosmosil column (particle size 5 μm, 150 mm $\times$ 4.6 mm I.D.) (Nacalai Tesque, Kyoto, Japan).

API interface

The interface consisted of a nebulizing and vaporizing unit [19]. The temperatures of both units could be controlled independently, and a high sensitivity to samples could be obtained by setting the optimal temperature for each unit in turn.

Vaporized sample and solvent molecules were led into the ion source of the API-MS system, the solvent molecules were ionized by corona discharge, and then the sample molecules and ionized solvent molecules underwent ion–molecule reactions. The corona discharge voltage is 3 kV. The ions produced under atmospheric pressure were led into the ion source housing through the intermediate region at *ca.* 60 Pa, located between the first electrode with a 250-μm aperture and the second electrode with a 400-μm aperture. The intermediate region was evacuated by a mechanical booster pump and a rotary pump. The ion source housing and mass analysis region were evacuated with two diffusion pumps (570 l/s) to *ca.* $1 \cdot 10^{-2}$ and *ca.* $1 \cdot 10^{-4}$ Pa, respectively. The acceleration energy was fixed at 3 keV by the second-electrode potential. The scan range was 10–1000 a.m.u., and the scan speed was 1000 a.m.u. per 1–20 s.

HPLC–API-MS conditions

The mobile phase for the HPLC separations was a mixture of 0.1 *M* ammonium acetate (pH 6) and acetonitrile: 0.1 *M* ammonium acetate–acetonitrile (4:1, v/v) was immediately changed to 0.1 *M* ammonium acetate–acetonitrile (1:1, v/v) after 4 min by a low-pressure gradient system. The flow-rate was 1.0 ml/min. The temperature of the vaporizer giving the strong intensity of measured ions was changed from 320 to 300°C after the peak of 1-MMDL appeared. The temperature of the desolvation region was set to 399°C.

The mass spectrometer was operated in the API mode, the drift voltage was set to 130 V, and the focus voltage was set to 110 V. The multiplier voltage was set to 2.7 kV. The UV detection wavelength was 225 nm.

Analytical procedure

Blood samples were collected in heparinized containers and centrifuged to obtain the plasma, which was stored at −20°C until analysis.

The plasma samples (1.0 ml), after addition of internal standard (100 ng), 0.5 ml of a saturated solution of ammonium acetate and 8 ml of chloroform, were shaken vigorously for 10 min and centrifuged at 1800 *g* for 5 min. Then 6 ml of the chloroform layer were taken and dried under a stream of nitrogen at 50°C. The residue was dissolved in 0.3 ml of 0.1 *M* ammonium acetate–acetonitrile (4:1, v/v), mixed by vortex-mixer for 15 s, and supersonicated for 1 min. A 0.2-ml aliquot of the mixture was injected into the HPLC–API-MS.

The urine samples (0.2 ml), after addition of internal standard (100 ng), 0.3 ml of distilled water, 0.5 ml of a saturated solution of ammonium acetate and 8 ml of chloroform, were shaken vigorously for 10 min, and centrifuged at 1800 *g* for 5 min. Then 6 ml of the chloroform layer were taken through the same procedure as the plasma samples.

Calibration curves

The calibration curves (10, 25, 50, 75 and 100 ng for the plasma samples, 25,

50, 75, 100 and 150 ng for the urine samples) were prepared by addition of nicergoline, 1-MMDL, 1-OHMMDL and MDL to human plasma and urine, respectively. At the same time, the internal standard (100 ng) was added to each sample. The spiked samples were extracted and analysed by the procedure described above. The calibration curves were calculated by the internal standard method for each peak area.

RESULTS AND DISCUSSION

The main metabolites of nicergoline and the metabolic pathway were identified with tritium-labelled compounds in animal studies (Fig. 1) [4]. The previous HPLC method needed 6.0 ml of plasma and a complicated and lengthy sample preparation because of the effects of interfering substances in biological fluids. This HPLC–API-MS method requires only 1.0 ml of plasma and has higher selectivity. Moreover, the sample preparation is simple and rapid.

Sample preparation

A simple and rapid sample preparation for the determination of nicergoline and its metabolites in biological fluids was developed. The established procedure of chloroform extraction and drying produced good specificity and sensitivity in MS detection in the SIM mode. However, it was difficult to monitor these compounds simultaneously by UV detection, 1-OHMMDL in urine could not be determined at all.

5-Chloronicergoline was chosen as the internal standard because it shows similar chromatographic behaviour to nicergoline and its three metabolites, and is not affected by admixture in plasma and urine.

In the proposed method, investigation of the extraction of nicergoline, 1-MMDL, 1-OHMMDL, MDL and the internal standard from aqueous solution using several organic solvents showed that they could be recovered quantitatively with chloroform in the presence of ammonium acetate. It was found that the plasma samples were deproteinized by extraction with chloroform alone. Ammonium acetate did not interfere with the API interface, so it was also used for the salting-out effect.

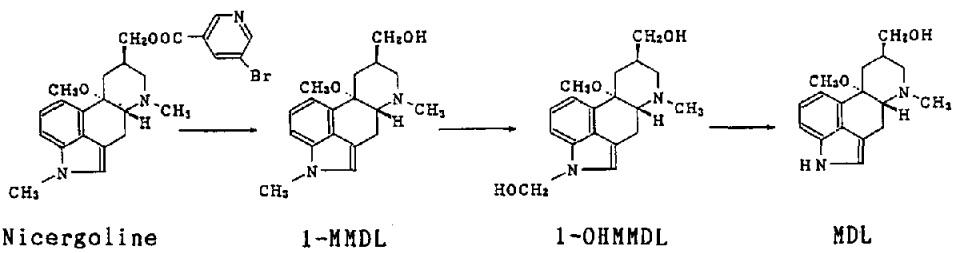

Fig. 1. Structures and proposed metabolic pathway of nicergoline in humans.

It was also found that when the resulting residue was dissolved in water–acetonitrile (1:1, v/v) instead of ammonium acetate–acetonitrile (4:1, v/v), the baseline of the mass chromatograms was disturbed. Also, when phosphoric acid was used instead of ammonium acetate, the API interface rapidly became corroded.

After several investigations, the presented method was established, and 1-OHMMDL in urine, which could not be analysed by the HPLC method, could also be analysed easily.

HPLC–API-MS conditions

Nicergoline, 1-MMDL, 1-OHMMDL and MDL could not be analysed simultaneously by isocratic HPLC, because the nicergoline and internal standard peaks were too broad. First of all, the HPLC conditions were investigated. Ammonium acetate was used as the buffer in mobile phase, because it did not corrode the API-MS system. For the quick separation of nicergoline and three metabolites, gradient elution should be used, and 0.1 *M* ammonium acetate–acetonitrile (4:1, v/v) was employed as mobile phase at a flow-rate of 1 ml/min until MDL appeared (4 min), and then the mobile phase was immediately changed to 0.1 *M* ammonium acetate–acetonitrile (1:1, v/v) for the elution of 1-MMDL, nicergoline and the internal standard. The temperature of the vaporizer and the drift voltage for API-MS were also considered, and the best conditions varied somewhat for each compound. Because nicergoline, as the last-eluted compound, was estimated at low sensitivity, the best conditions for nicergoline were selected.

For the HPLC–API-MS system, the sensitivity strongly depends on the size of solvent particles produced by the interface. To obtain very small droplets a nebulizer is required, so the temperature of the vaporizer should be changed as the ratio of organic solvent in mobile phase changes. However, changing the mobile phase and the temperature of the vaporizer disturbed the baseline of the mass chromatograms, although a small change in the temperature of the vaporizer did not disturb the baseline. After several investigations, the measurement conditions for HPLC–API-MS were established.

The temperature of the vaporizer, the drift voltage and the focus voltage were set to give a strong intensity of the measured ions. Nicergoline and the internal standard, analysed with 0.1 *M* ammonium acetate–acetonitrile (1:1, v/v), were most intense at a vaporization temperature of 300°C, and the metabolites 1-MMDL, 1-OHMMDL and MDL, analysed with 0.1 *M* ammonium acetate–acetonitrile (4:1, v/v), were most intense at 320°C. Therefore the temperature of the vaporizer was changed from 320 to 300°C after the elution of the metabolite 1-MMDL. The drift voltage and the focus voltage were set to 130 and 110 V, respectively. The changes to the mobile phase and the temperature of vaporization did not affect the stability of the baseline of the mass fragmentograms.

The HPLC pump unit, the UV detector and the API source were directly connected in our HPLC–API-MS system, therefore the UV and the total ion

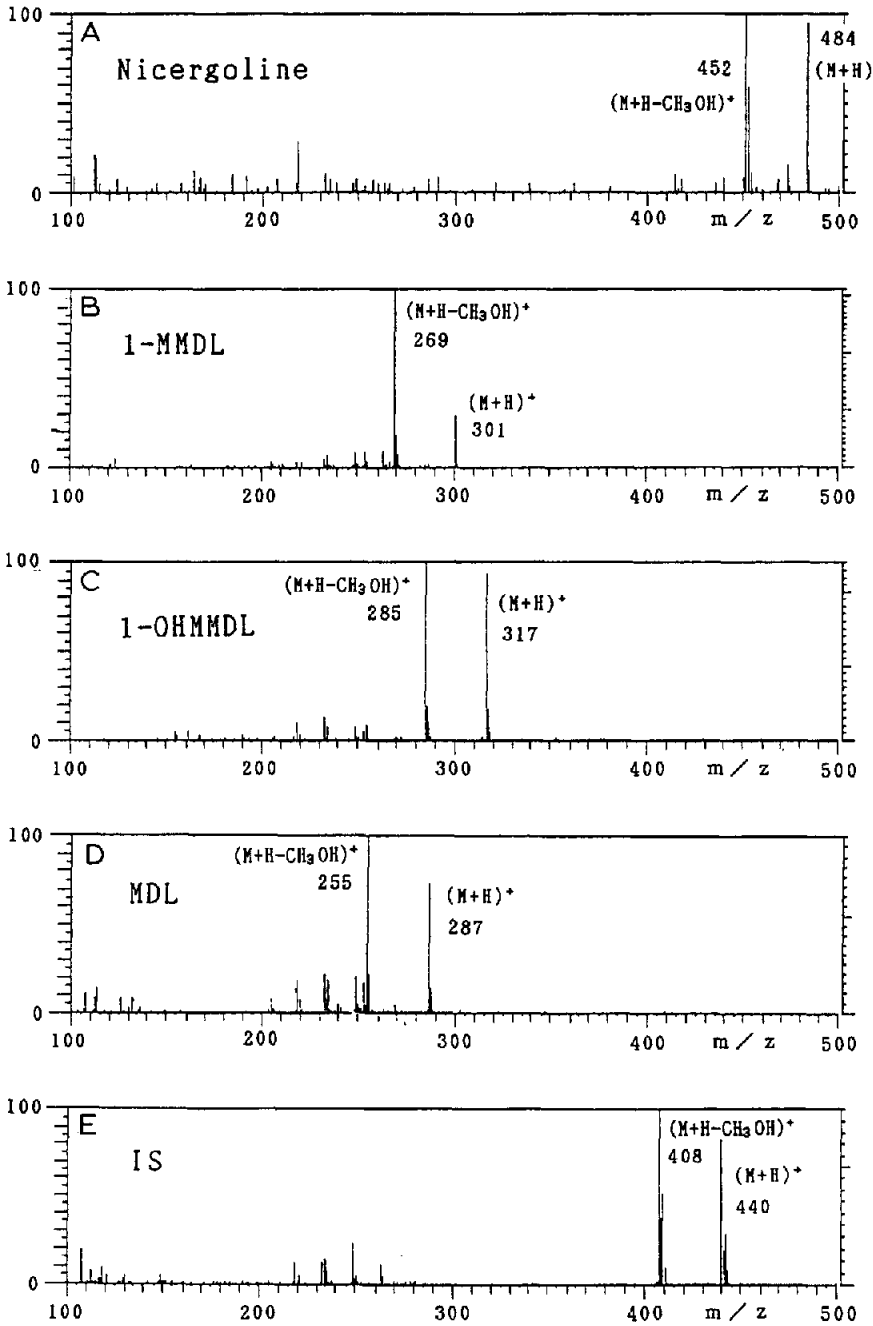

Fig. 2. Mass spectra of nicergoline (A), 1-MMDL (B), 1-OHMMDL (C), MDL (D) and the internal standard (E).

current (TIC) chromatograms were simultaneously obtained. The delay time between the UV detector and the mass spectrometer was *ca.* 0.1 min.

Mass fragmentography with SIM

Nicergoline, 1-MMDL, 1-OHMMDL, MDL and the internal standard were completely separated under the given HPLC conditions. Fig. 2A–E shows the mass spectra corresponding to the individual peaks in the TIC chromatograms. The protonated molecular ions $[M + H]^+$ corresponding to each molecular species were observed. However, the characteristic clusters produced by water or ammonium ion attachment to each $[M + H]^+$ were not observed. Nicergoline, 1-MMDL, 1-OHMMDL, MDL and the internal standard produced the fragment ions $[M + H - CH_3OH]^+$ at *m/z* 452, 269, 285, 255 and 408, respectively, as shown in Fig. 2A–E. The fragment ions $[M + H - CH_3OH]^+$, which showed the strongest intensity in the mass spectra, were chosen for an investigation of the quantitative analysis of nicergoline and the three metabolites in plasma and urine by the SIM method. It was found that the impurities in human plasma and urine have no influence on the determination of nicergoline, 1-MMDL, 1-OHMMDL and MDL, as shown in Fig. 3A and B. Nicergoline and its metabolites showed a good selectivity in the SIM mode.

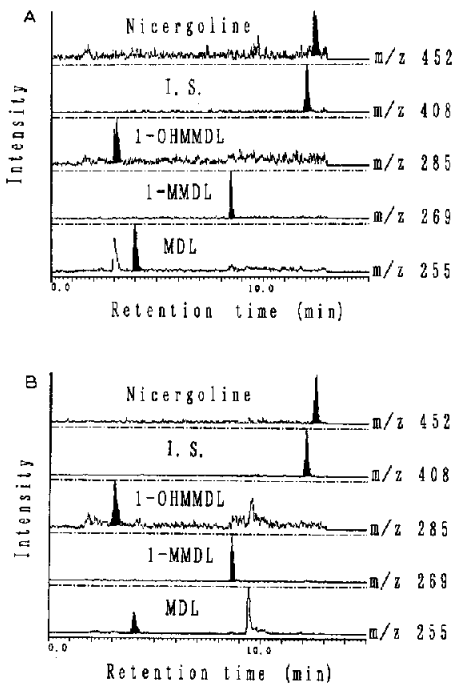

Fig. 3. Mass fragmentograms, obtained by selected-ion monitoring, of nicergoline and its metabolites (1-MMDL, 1-OHMMDL, MDL), added in 25-ng amounts to human plasma (A) and in 50-ng amounts to human urine (B).

Reproducibility

The reproducibility of the assay was evaluated by analysing known amounts (5 and 10 ng in plasma, 50 and 100 ng in urine) of nicergoline, 1-MMDL, 1-OHMMDL and MDL. The coefficient of variation (C.V.) was obtained by calculating the ratio of the peak intensities of nicergoline and three of its metabolites to that of 5-chloronicergoline. The results are given in Table I. The reproducibility of the proposed method was found to be within the acceptable limit.

Linearity and detection limit

The calibration curves were obtained by plotting the ratio of the peak intensity of nicergoline, 1-MMDL, 1-OHMMDL and MDL to that of the internal standard against the concentration. The calibration curves passed through the origin and were linear in the range 10–100 ng/ml of plasma, and 25–150 ng/ml of urine. The detection limits for these compounds were *ca.* 2 ng/ml in plasma and *ca.* 10 ng/ml in urine, at a signal-to-noise ratio of 4.

Application (for clinical samples)

The HPLC–API-MS method with SIM was applied to the determination of nicergoline, 1-MMDL, 1-OHMMDL and MDL in plasma of healthy volunteers

TABLE I

ACCURACY AND REPRODUCIBILITY OF MASS FRAGMENTOGRAPHIC ASSAY

Compound	Concentration added (ng/ml)	Found concentration (ng/ml ± S.D.)	C.V. (%)
Plasma (n = 10)			
Nicergoline	5	4.5 ± 0.61	13.5
	10	9.6 ± 0.87	9.1
1-MMDL	5	4.5 ± 0.46	10.3
	10	9.4 ± 0.93	9.9
1-OHMMDL	5	4.1 ± 0.65	15.9
	10	8.7 ± 1.04	12.0
MDL	5	4.6 ± 0.54	11.8
	10	9.4 ± 0.81	8.6
Urine (n = 7)			
Nicergoline	50	48.1 ± 1.59	3.3
	100	97.3 ± 4.28	4.4
1-MMDL	50	47.7 ± 3.10	6.5
	100	96.4 ± 5.88	6.1
1-OHMMDL	50	45.4 ± 3.31	7.3
	100	93.5 ± 6.26	6.7
MDL	50	47.6 ± 2.71	5.7
	100	98.3 ± 1.67	1.7

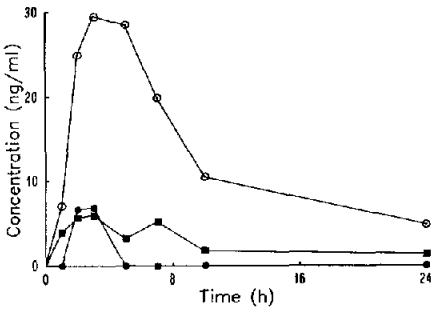

Fig. 4. Plasma concentrations of 1-MMDL (■), 1-OHMMDL (●) and MDL (○) after oral administration of 15 mg of nicergoline.

orally dosed with 15 mg of nicergoline. Typical concentration–time profiles are shown in Fig. 4. The unchanged form of nicergoline was not detected in plasma samples of healthy volunteers. It is apparent that nicergoline is very rapidly metabolized to 1-MMDL, 1-OHMMDL and MDL. The concentration of MDL, one of the activated metabolites, in human plasma *ca.* 3.5 h after oral administration indicates a maximum of *ca.* 30 ng/ml, and the concentrations of 1-MMDL and 1-OHMMDL indicate a maximum of only 5–7 ng/ml.

These results are in good agreement with those obtained with the previous HPLC method. The present method also may be applied to urine samples.

CONCLUSION

It was found that the HPLC–API-MS system with SIM described here can be used for the simultaneous determination of nicergoline and three of its metabolites in biological fluids at the ng/ml level. The selectivity and the rapidity were better than those of the HPLC method and the immunoassay method. The HPLC–API–MS analysis is expected to be useful to studies of the bioavailability and pharmacokinetics after administration of new drugs.

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REFERENCES

1 W. Barbieri, L. Bernardi, G. Bosisio and A. Temperilli, *Tetrahedron*, 25 (1969) 2401.
2 D. T. Mahin and R. T. Lofberg, *Anal. Biochem.*, 16 (1969) 500.
3 G. Arcari, L. Dorigotti, G. B. Fregnan and A. Glasser, *Br. J. Pharmacol.*, 34 (1968) 700.
4 F. Arcamone, A. G. Glasser, J. Grafnetterova, A. Minghetti and V. Nicolella, *Biochem. Pharmacol.*, 21 (1972) 2205.

5 Ch. A. Bizollon, J. P. Rocher and P. Chevalier, *Eur. J. Nucl. Med.*, 7 (1982) 318.
6 K. H. Schellenberg, M. Linder, A. Groeppelin and F. Erni, *J. Chromatogr.*, 394 (1987) 239.
7 G. Karlaganis, E. Peretti and B. H. Lauterburg, *J. Chromatogr.*, 420 (1987) 171.
8 L.-E. Edholm, C. Lindberg, J. Paulson and A. Walhagen, *J. Chromatogr.*, 424 (1988) 61.
9 M. J. Moor, M. S. Rashed, T. F. Kalhorn, R. H. Levy and W. N. Howald, *J. Chromatogr.*, 474 (1988) 79.
10 W. H. McFadden, *J. Chromatogr. Sci.*, 18 (1980) 97.
11 R. D. Smith, J. E. Burger and A. L. Johnson, *Anal. Chem.*, 53 (1981) 1603.
12 K. Matsumoto and S. Tsuge, *Mass Spectrosc.*, 34 (1986) 235.
13 K. Matsumoto, H. Lieu and S. Tsuge, *Fresenius' Z. Anal. Chem.*, 323 (1986) 212.
14 M. Sakairi and H. Kambara, *Anal. Sci.*, 4 (1988) 199.
15 T. R. Covey, D. L. Lee and J. D. Henion, *Anal. Chem.*, 58 (1986) 2453.
16 T. R. Covey, E. D. Lee, A. P. Bruins and J. D. Henion, *Anal. Chem.*, 58 (1986) 1451A.
17 C. Eckers, D. E. Games, D. N. B. Mallen and B. P. Swann, *Anal. Proc.*, 19 (3) (1982) 133.
18 Y. Kawai, Y. Matsumori, T. Suzuki, M. Mizobe and M. Samejima, *Clin. Rep.*, 20 (1986) 520.
19 Y. Kushi, C. Rokukawa, Y. Numajiri, Y. Kato and S. Handa, *Anal. Biochem.*, 182 (1989) 405.