Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 811 (2004) 237–241
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

A sensitive internal standard method for the determination of melatonin in mammals using precolumn oxidation reversed-phase high-performance liquid chromatography

Kenji Hamase, Junzo Hirano, Yuki Kosai, Tatsunosuke Tomita, Kiyoshi Zaitsu*

Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812 8582, Japan

Received 24 May 2004; accepted 18 August 2004
Available online 11 September 2004

Abstract

A sensitive and accurate internal standard method to determine melatonin in mammalian tissues and physiological fluids has been described. This method includes the oxidation of melatonin to a highly fluorescent compound, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA), and the determination of 6-MOQMA by a reversed-phase HPLC system. For the accurate and reliable determination, several melatonin analogs were designed and utilized as the internal standards, and ethyl and isopropyl analogs (having the corresponding alkyl group via the amide bond of melatonin instead of the methyl group) were found to be promising. Using these internal standards, highly accurate and sensitive determination could be accomplished using rat pineal gland samples, and the clear circadian rhythms are demonstrated. This method was also successfully applied to the determination of melatonin in a small amount (20 μL) of human saliva.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Melatonin; Fluorescence detection; Precolumn oxidation; RP-HPLC

1. Introduction

Melatonin is an indole hormone, which is synthesized in the pineal gland from L-tryptophan via serotonin and *N*-acetylserotonin [1,2]. The biosynthesis of melatonin and its release to the blood flow from the pineal gland have clear circadian rhythms [3,4], showing about 10 times higher concentration during nighttime than during daytime. This circadian rhythm of melatonin concentration is controlled by the biological clock in the suprachiasmatic nucleus and the environmental light signals [5,6]; therefore, this hormonal information transmits the environmental day and night cycles to the whole body via the circulation of blood. As higher animals control their behavioral rhythms and tissue activities synchronized to the day and night cycles [7,8], melatonin affects many organs including the central nervous system and endocrine tissues [9–11]. In addition, melatonin is reported to be effective against many diseases such as sleep disorders [12], cancers [13], Alzheimer's disease [14] and depressive syndrome [15]. Therefore, melatonin determination is important for the diagnosis of rhythm disorders, for the research of new biologically active substances, and for the estimation of the effect of medicines.

The aim of the present investigation is to establish a highly sensitive and accurate analytical method that can be used easily and widely for the determination of melatonin in mammalian tissues and physiological fluids. For the determination of melatonin, enzyme-linked immunosorbent assay [16] and radio-immunoassay [17] have been reported. The chromatographic methods using the native fluorescence of melatonin [18] or electrochemical detection [19,20] are also widely used. However, the amounts

* Corresponding author. Tel.: +81 92 642 6596; fax: +81 92 642 6601.
E-mail address: zaitsu@phar.kyushu-u.ac.jp (K. Zaitsu).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.08.027

of melatonin in mammals, especially endogenous amounts, are too small, and the analytical methods with higher sensitivity are needed to determine melatonin in small tissues or small volumes of clinical samples. We have already reported a highly sensitive HPLC method for the determination of melatonin [21–24]. By this method, melatonin is converted to an oxidation compound [24] having strong fluorescence, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA) (Fig. 1), and the sensitivity of this method is about 5–10 times higher than those already reported [16–20]. However, our HPLC method needs skillful techniques to obtain accurate results, and 5-methoxyindole-3-acetic acid (MIAA) has been proposed as the internal standard [23,24], because MIAA shows similar changes in the fluorescence spectra by the oxidation. In the latest report [25], we revealed that the oxidation product derived from MIAA is 6-methoxy-4-quinolone (6-MOQ), indicating that the oxidation pathway from MIAA to 6-MOQ needs additional decarboxylation compared to that producing 6-MOQMA from melatonin. In addition, the retention time of 6-MOQ is shorter than that of 6-MOQMA on the reversed-phase column, although various more hydrophilic interfering substances are observed in the mammalian tissues. Therefore, the more hydrophobic internal standard, which is oxidized via the same pathway as melatonin, is required. In the same report [25], we also revealed that the melatonin analogs having longer alkyl chains are oxidized via the same pathway as melatonin, suggesting that these analogs are suitable internal standards for melatonin determination. Thus, in the present investigation, several melatonin analogs are designed and utilized as the internal standard of melatonin determination, and highly accurate attomole determination could be accomplished. The usefulness of the method is demonstrated using the rat pineal gland and human saliva.

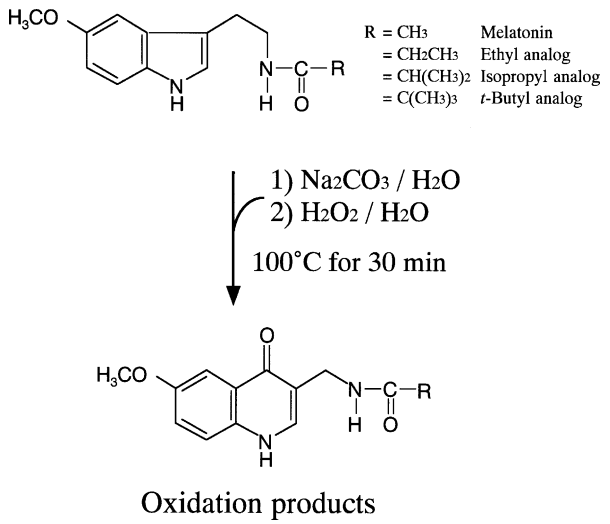

Fig. 1. Oxidation of melatonin and synthesized melatonin analogs.

2. Experimental

2.1. Materials

Melatonin was purchased from Sigma Chemical Company (St. Louis, MO). 5-Methoxytryptamine, propionic anhydride, isobutyric anhydride and pivalic anhydride were obtained from Tokyo Kasei (Tokyo, Japan). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade, trifluoroacetic acid (TFA), sodium carbonate and aqueous hydrogen peroxide (31%, v/v) of guaranteed grade were the products of Wako (Osaka, Japan). Water was purified using a Milli-Q system (Elix 3 and Gradient A10, Millipore, Bedford, MA). All other reagents were of reagent grade and used without further purification.

2.2. Synthesis of melatonin analogs

Melatonin analogs were synthesized in our laboratory by the condensation of 5-methoxytryptamine and acid anhydrides. Briefly, 5-methoxytryptamine (100 mg) was dissolved in 10 mL of MeCN, and 3.4 mL of 2% (w/v) propionic anhydride in MeCN was added, then stirred for 20 min at room temperature. To this mixture, 20 mL of aqueous 20 mM Na_2CO_3 was added, and the product was extracted twice with 20 mL of ethyl acetate. The organic layer was washed with 20 mL of water three times and evaporated. The residue was recrystallized from water, and colorless needles (*N*-[2-(5-methoxyindol-3-yl)ethyl]propanamide, ethyl analog, yield: 97.6 mg) were obtained. For the syntheses of isopropyl analog (*N*-[2-(5-methoxyindol-3-yl)ethyl]-2-methylpropanamide) and *tert*-butyl analog (*N*-[2-(5-methoxyindol-3-yl)ethyl]-2,2-dimethylpropanamide), isobutyric anhydride and pivalic anhydride were used instead of propionic anhydride. The authenticity was confirmed by the ^{1}H-NMR, MS, and elemental analysis.

2.3. HPLC system

The HPLC system consisted of a DG-2080-53 degasser (Jasco, Tokyo, Japan), a PU-2080Plus pump (Jasco), a 7725i injector (Rheodyne, Cotati, CA, USA), a CO-2065Plus column oven (Jasco), an FP-2020Plus fluorescence detector (Jasco) and an 807-IT integrator (Jasco). The analytical column used was a CAPCELL PAK C18 MG (150 mm × 2.0 mm i.d., 40 °C, Shiseido, Tokyo, Japan). The mobile phase was MeCN–TFA–water (15:0.05:85, v/v), and the flow rate was 0.2 mL/min. The fluorescence detection was carried out at 392 nm with excitation at 247 nm. For the determination of melatonin in human saliva, a reversed-phase micro-HPLC system (Nanospace, Shiseido) was used. The analytical column used was a CAPCELL PAK C18 MG S3 (75 mm × 1.0 mm i.d., 40 °C, Shiseido). The mobile phase was MeCN–TFA–water (10:0.05:90, v/v), and the flow rate was 50 μL/min. The fluorescence detection was carried out at 392 nm with excitation at 247 nm.

2.4. Preparation of rat pineal gland samples

Male Wistar rats (9 weeks of age, SPF, housed under a 12 h light/12 h dark cycle, lights on at 07:00 a.m.) were anesthetized with diethyl ether, and the pineal glands were excised at 13, 17, 21, 01, 05 and 09:00. Each pineal gland was homogenized in 400 μL of MeOH, and centrifuged at 4500 × *g* for 5 min. To a glass vial (screw-cap vial, S-06 with a melamine cap and a Teflon-faced silicone liner, Nichiden-Rika Glass, Kobe, Japan), this supernatant (50 μL), and 10 μL of MeOH containing both 25 nM of ethyl and isopropyl analogs were added, and dried at 100 °C. The residue was thoroughly dissolved in 40 μL of water, and 5 μL of aqueous 2 M Na_2CO_3 and 5 μL of aqueous 50 mM H_2O_2 solutions were then added. The vial was tightly capped and heated at 100 °C for 30 min, and 10 μL of the reaction mixture was injected into the HPLC.

2.5. Preparation of human saliva samples

Human saliva (20 μL) was mixed with 200 μL of MeOH containing 687.5 pM ethyl analog and centrifuged at 4500 × *g* for 5 min. The supernatant (200 μL) was dried at 100 °C, the residue was dissolved in 40 μL of water, and 5 μL of aqueous 2 M Na_2CO_3 and 5 μL of aqueous 50 mM H_2O_2 were added. After the mixture was heated at 100 °C for 30 min, 10 μL of 10% (v/v) aqueous TFA and 140 μL of water were added and applied to the solid phase extraction cartridge (RP-select B, MERCK, Darmstadt, Germany). This cartridge was washed with 10% (v/v) aqueous MeOH (3 mL) and water (3 mL). The oxidation products of the melatonin and ethyl analog were eluted with 500 μL of MeOH, the eluate was then dried at 100 °C. The residue was dissolved in 50 μL of water, and the aliquot (5 μL) was injected into the HPLC.

3. Results and discussion

3.1. HPLC separation of the melatonin analogs and their oxidation products

As the candidates of the novel internal standards for the determination of melatonin, three melatonin analogs (ethyl, isopropyl and *t*-butyl analogs) having longer alkyl chains than melatonin were designed. Fig. 2 shows the chromatogram analyzing these melatonin analogs after the oxidation. By the oxidation, the wavelengths of the fluorescence excitation and emission maxima of all melatonin analogs tested were shifted from 278 (Ex.) and 341 (Em.) to 247 (Ex.) and 392 (Em.) nm, respectively. The fluorescence peak areas of the three peaks corresponding to the oxidation products of ethyl, isopropyl and *t*-butyl analogs are almost the same as that of 6-MOQMA, and the S/N ratios of all melatonin analogs tested became higher by the oxidation. In the previous investigation [25], we reported that the molar absorptivities (ε) at the maximum excitation wavelengths and the fluorescence quantum yields (Φ) of the oxidation products derived from the three melatonin analogs are almost the same as those of 6-MOQMA. Therefore, the present results indicate that the three melatonin analogs were converted to the corresponding oxidation products in the same ratios as in the case of melatonin. The retention times of the oxidation products of ethyl and isopropyl analogs are sufficiently close to that of 6-MOQMA, showing that these two compounds are the promising candidates for the internal standards of the melatonin determination.

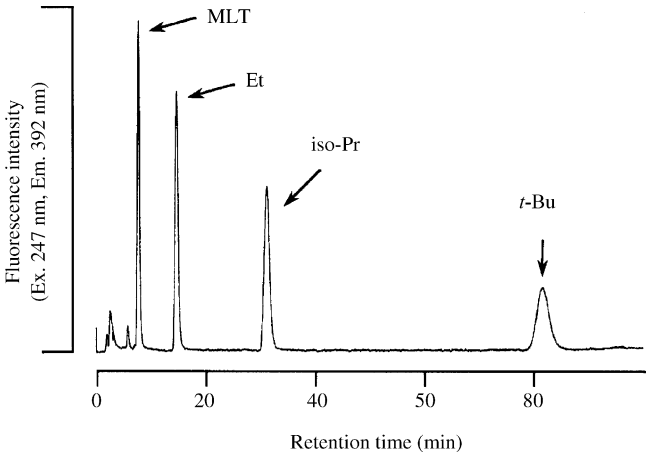

Fig. 2. HPLC separation of the oxidation compounds of melatonin and melatonin analogs. Melatonin and melatonin analogs (100 fmol each) were oxidized and injected. The HPLC conditions are as follows: column, CAPCELL PAK C18 MG (150 mm × 2.0 mm i.d., 40 °C, Shiseido); mobile phase, MeCN–TFA–water (15:0.05:85, v/v); flow rate, 0.2 mL/min. Peaks: MLT, oxidation compound of melatonin (6-MOQMA); Et, iso-Pr and *t*-Bu, oxidation compounds of the ethyl, isopropyl and *tert*-butyl analogs.

3.2. Validation of the melatonin determination using ethyl and isopropyl analogs as the internal standards

Since the pineal gland is the tissue in which melatonin is biosynthesized, the applicability of the ethyl and isopropyl analogs as the novel internal standards was investigated using the rat pineal glands. Both during daytime and during nighttime, we could find no interfering substances in the pineal glands. Therefore, using the ethyl and isopropyl analogs as the internal standards, the calibration curves, within-day precisions and day-to-day precisions of melatonin determination were investigated. Calibration curves of melatonin were constructed by the addition of known amounts of melatonin (25, 100, 500 and 2500 fmol) and the two internal standards (ethyl and isopropyl analogs, 250 fmol each) to the diurnal rat pineal gland samples (50 μL of the supernatant described in Section 2). The calibration curve obtained using ethyl analog as the internal standard is $y = 0.0168x + 0.611$, where x is the amount of melatonin added (injection amount, fmol) and y is the ratio of the peak heights (oxidation product of melatonin/oxidation product of ethyl analog). The equation of the calibration curve obtained using isopropyl analog as the internal standard is $y = 0.0278x + 1.02$. Both of these calibration curves showed good linearity over the range of 5–500 fmol (injection amounts), and the correlation coefficients are 0.9997.

Within-day precision was investigated analyzing the endogenous melatonin in a diurnal rat pineal gland sample five times within a day. The R.S.D. values of the pineal melatonin amounts obtained by five analyses were 0.75 and 2.15%, using ethyl and isopropyl analogs as the internal standards, respectively. These values were much lower than those obtained without the use of internal standards. Day-to-day precisions were also investigated for 5 days, and the obtained R.S.D.s of melatonin amounts in the pineal gland were 2.11% (using the ethyl analog) and 2.62% (using the isopropyl analog). These results indicated that reliable determination of melatonin in mammalian tissues could be performed using either the ethyl or the isopropyl analog as the internal standard. Especially, the ethyl analog would be useful because the retention time of the oxidation product is close to that of 6-MOQMA. However, the present results, showing that both analogs are promising as the internal standards, are valuable because the interfering substances have the variety among the tissues, organs and physiological fluids.

3.3. Application of the method to the biological samples

As the application of the present internal standard method, the circadian changes of endogenous melatonin in the rat pineal gland and the change in the melatonin amount in human saliva after oral administration of the melatonin supplement were demonstrated. The rat pineal glands were excised every 4 h, and the endogenous melatonin was determined. The amounts of endogenous melatonin in the daytime are 1.96–2.02 pmol/pineal gland (13:00–17:00 p.m., using the ethyl analog) and 1.81–1.97 pmol/gland (using the isopropyl analog), which increased during nighttime, and the highest values of 12.07 pmol/gland (using the ethyl analog) and 11.94 pmol/gland (using the isopropyl analog) were obtained at 01:00 a.m. (Fig. 3). The typical chromatograms obtained using the diurnal and nocturnal pineal glands are shown in Fig. 4. The amount of rat pineal melatonin was determined by various methods including immunoassays and LC methods and was reported to be 1–2 pmol/gland during daytime, about 10 pmol/gland during nighttime [16,19–22,26]. The results obtained in the present investigation are consistent with those reported in the literature.

The alteration of melatonin concentration in human saliva after oral administration of the melatonin supplement was also investigated. Saliva can be obtained simply and without injury; it is one of the important and useful human clinical samples. In the human saliva, large quantities of interfering substances are present, and the HPLC separation needs longer time than that used for the determination of rat pineal melatonin. Therefore, the isopropyl analog is not suitable as the internal standard because of its long retention time. Using this procedure, the calibration curve of spiked melatonin to the saliva (collected before the administration of the melatonin supplement) was linear from 500 amol to 50 fmol with the correlation coefficient of 0.9998. Within-day precision of melatonin determination was 4.27% (R.S.D., $n = 5$, using the

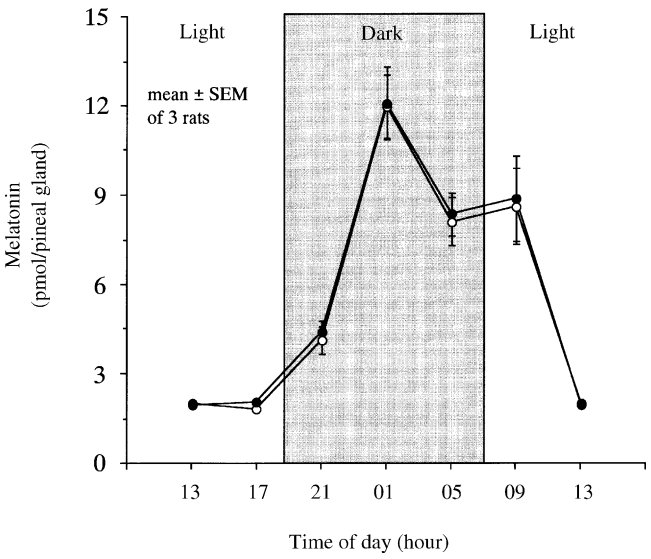

Fig. 3. Circadian changes of melatonin amounts in the rat pineal gland using ethyl and isopropyl analogs as the internal standards. Closed circles represent the values determined using the ethyl analog as the internal standard, and open circles represent those determined using the isopropyl analog. Each value represents the mean ± SEM of three animals. The values of 13:00 were double plotted.

saliva collected 2 h after the administration). Fig. 5 shows the chromatograms obtained before and after the administration of melatonin. No interfering peaks were observed before the administration, and the amount of melatonin increased after the administration; 5.84 pmol/mL saliva of melatonin was observed 2 h after the administration. Since the volume of clinical samples is often limited, and one sample is usually used for the various diagnostic checkups, an analytical method using a lower amount of the sample is preferable. The present method has the advantage that only 20 μL of human saliva was needed for the analysis.

In the present investigation, an internal standard method for the determination of melatonin in mammals has been described. As melatonin determination is often important for purposes such as diagnostic aims and drug discovery, this

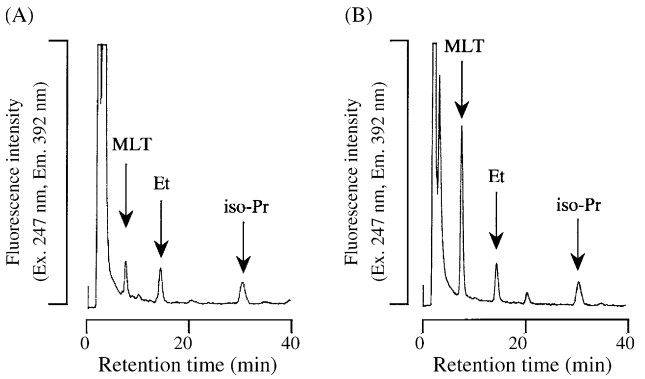

Fig. 4. HPLC separation of the oxidation compounds of rat pineal melatonin and the internal standards. The pineal glands were excised at: (A) 13:00; and (B) 01:00. Peak identities and the HPLC conditions are the same as described in Fig. 2.

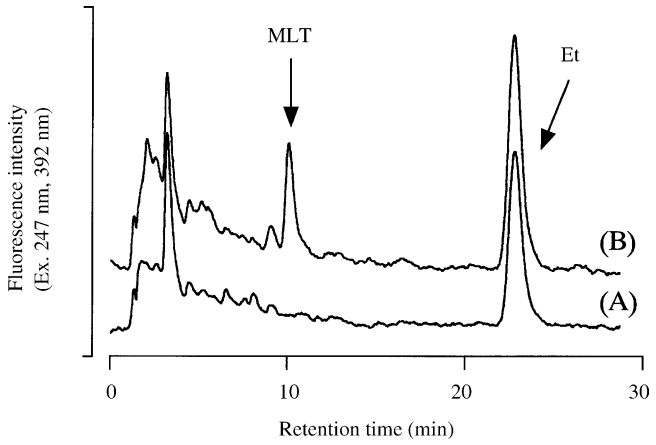

Fig. 5. HPLC separation of the oxidation compounds of melatonin and ethyl analog in human saliva. Human saliva was collected: (A) before and (B) 2 h after the oral administration of the melatonin supplement (3 mg). Peaks: MLT, oxidation compound of melatonin (6-MOQMA); Et, oxidation compound of the ethyl analog. The HPLC conditions are as follows: column, CAPCELL PAK C18 MG S3 (75 mm × 1.0 mm i.d., 40 °C, Shiseido); mobile phase, MeCN–TFA–water (10:0.05:90, v/v); flow rate, 50 μL/min.

method could surely contribute to various clinical and biological investigations.

Acknowledgements

This work was supported in part by the grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14370785).

References

[1] J. Axelrod, Science 184 (1974) 1341.
[2] L. Tamarkin, C.J. Baird, O.F.X. Almeida, Science 227 (1985) 714.
[3] T. Azekawa, A. Sano, H. Sei, A. Yamamoto, K. Aoi, Y. Morita, Brain Res. Bull. 26 (1991) 413.
[4] S. Ebihara, T. Marks, D.J. Hudson, M. Menaker, Science 231 (1986) 491.
[5] B. Rusak, I. Zucker, Physiol. Rev. 59 (1979) 449.
[6] J. Falcon, Prog. Neurobiol. 58 (1999) 121.
[7] R. Brandstatter, V. Kumar, U. Abraham, E. Gwinner, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 12324.
[8] C. Kopp, E. Vogel, M.-C. Rettori, P. Delagrange, R. Misslin, Physiol. Behav. 70 (2000) 171.
[9] J.D. Glass, G.R. Lynch, Science 214 (1981) 821.
[10] C. Gall, M.L. Garabette, C.A. Kell, S. Frenzel, F. Dehghani, P.M.S. Draeger, D.R. Weaver, H.W. Korf, M.H. Hastings, J.H. Stehle, Nat. Neurosci. 5 (2002) 234.
[11] A.I. Esquifino, P. Castrillon, M.G. Bonacho, E. Vara, D.P. Cardinali, J. Pineal Res. 27 (1999) 15.
[12] A. Miyamoto, J. Oki, S. Takahashi, A. Okuno, Brain Dev. 21 (1999) 59.
[13] P. Lissoni, S. Barni, M. Mandala, A. Ardizzoia, F. Paolorossi, M. Vaghi, R. Longarini, F. Malugani, G. Tancini, Eur. J. Cancer 35 (1999) 1688.
[14] Y. Ohashi, N. Okamoto, K. Uchida, M. Iyo, N. Mori, Y. Morita, Biol. Psychiat. 45 (1999) 1646.
[15] O.T. Dolberg, S. Hirschmann, L. Grunhaus, Am. J. Psychiat. 155 (1998) 1119.
[16] S.M. Yie, E. Johansson, G.M. Brown, Clin. Chem. 39 (1993) 2322.
[17] R.J. Wurzburger, K. Kawashima, R.L. Miller, S. Spector, Life Sci. 18 (1976) 867.
[18] M.H. Mills, M.G. King, N.G. Keats, R.A. McDonald, J. Chromatogr. 377 (1986) 350.
[19] E. Chanut, J.N. Legros, C.V. Botteri, J.-H. Trouvin, J.-M. Launay, J. Chromatogr. B 709 (1998) 11.
[20] R. Vieira, J. Miguez, M. Lema, M. Aldegunde, Anal. Biochem. 205 (1992) 300.
[21] F. Iinuma, K. Hamase, S. Matsubayashi, M. Takahashi, M. Watanabe, K. Zaitsu, J. Chromatogr. A 835 (1999) 67.
[22] K. Hamase, T. Tomita, A. Kiyomizu, K. Zaitsu, Anal. Biochem. 279 (2000) 106.
[23] T. Tomita, K. Hamase, H. Hayashi, K. Zaitsu, Chromatography 22 (2001) 41.
[24] T. Tomita, K. Hamase, H. Hayashi, H. Fukuda, J. Hirano, K. Zaitsu, Anal. Biochem. 316 (2003) 154.
[25] J. Hirano, K. Hamase, H. Fukuda, T. Tomita, K. Zaitsu, J. Chromatogr. A, in press.
[26] T. Harumi, S. Matsushima, J. Chromatogr. B 747 (2000) 95.