ELSEVIER

Journal of Chromatography B, 707 (1998) 9–15

JOURNAL OF CHROMATOGRAPHY B

Separate determination of human urinary conjugated and unconjugated 3-methoxy-4-hydroxyphenylethyleneglycol

Hirofumi Inagaki*, Kimiko Hirata, Masayasu Minami

Department of Hygiene and Public Health, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113, Japan

Received 4 September 1997; received in revised form 27 November 1997; accepted 2 December 1997

Abstract

A new determination procedure for human urinary 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) was established. In addition to the previously established solid-phase extraction method for unconjugated MHPG, another solid-phase extraction method for conjugated MHPG was developed. Unconjugated MHPG was adsorbed on a Sep-Pak Diol cartridge and selectively recovered by elution with ethyl acetate. The eluate was evaporated and the residue was redissolved and analyzed by reversed-phase high-performance liquid chromatography with fluorimetric detection. Conjugated (sulfate plus glucuronide) MHPG was adsorbed on a Sep-Pak Accell QMA cartridge and quantitatively eluted with 0.2 *M* NaCl. After enzymatic hydrolysis, deconjugated MHPG was extracted using a Sep-Pak Diol cartridge and analyzed in the same manner as unconjugated MHPG. The new method is simple and rapid and can quantitate conjugated and unconjugated MHPG discriminatively. © 1998 Elsevier Science B.V.

Keywords: 3-Methoxy-4-hydroxyphenylethyleneglycol

1. Introduction

3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) is one of the major metabolites of norepinephrine and epinephrine. Human urinary MHPG was reported to mainly originate in central nervous noradrenergic neurons and to reflect the activity of such neurons [1]. Therefore, urinary MHPG is monitored as an indicator of some psychiatric disorders [2–5].

There are many reports that describe methods for the determination of urinary MHPG and most of them employ gas chromatography–mass spectrometry (GC–MS) [4,6,7] or high-performance liquid chromatography–electrochemical detection (HPLC–ED) [8–18]. Although these methods are highly selective and sensitive, respectively, the former requires complex derivatization procedures and the latter needs extensive extraction procedures.

The present report describes a simple and rapid determination procedure for human urinary conjugated and unconjugated MHPG employing high-performance liquid chromatography–fluorescence detection (HPLC–FD). HPLC–FD is employed because it is simple to perform, and the urinary MHPG concentration is sufficiently high to be detected by it. The newly established solid-phase extraction method is highly selective, results in high recovery and enables us to determine urinary MHPG by HPLC–FD.

The amount of conjugated MHPG is determined by subtraction of the amount of unconjugated MHPG

*Corresponding author

0378-4347/98/$19.00
PII S0378-4347(97)00623-3

from that of total (unconjugated plus conjugated) MHPG in nearly all methods previously published. It is considered that this subtraction method magnifies experimental errors and lowers the reliability of the results. Thus, discriminative determination of conjugated MHPG is desired. Our new method described below enabled separate determination of conjugated MHPG.

2. Experimental

2.1. Reagents

MHPG, MHPG-sulfate, isovanillyl alcohol (iso-VAl) and arylsulfatase–β-glucuronidase (Type H-2) were purchased from Sigma (St. Louis, MO, USA). Sep-Pak Vac Accell QMA and Sep-Pak Vac Diol cartridges were obtained from Waters (Milford, MA, USA). The Wakosil-II 5C18 HG column was from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals used were all of analytical grade.

2.2. Apparatus

The solid-phase extraction manifold was purchased from J&W Scientific (Folsom, CA, USA). The SpeedVac Concentrator was obtained from Savant Instruments (Farmingdale, NY, USA) and used with a water jet aspirator. The HPLC system was composed of Model 302 and 305 pumps (Gilson, Villiers le Bel, France), a Model 231-401 autosampler (Gilson), a CTO-6A column oven (Shimadzu, Kyoto, Japan), a RF-535 fluorescence detector (Shimadzu) and a Model 715 system controller/data integrator (Gilson).

2.3. Chromatographic conditions

The column used was a Wakosil-II 5C18 HG (5 μm particle size; 250×4.6 mm I.D.) with a precolumn (30×4.6 mm I.D.). Gradient elution was performed with mobile phase A (50 m*M* sodium phosphate, pH 6.0) and mobile phase B (50% (v/v) acetonitrile in distilled water). The flow-rate was set at 1.0 ml/min and the mobile phase composition was changed from 100% A to 20% B over a period of 30 min. The column was maintained at 45°C. The fluorescence intensity was monitored at 320 nm with an excitation wavelength of 280 nm.

2.4. Pretreatment of Sep-Pak Vac cartridges

Sep-Pak Vac Diol cartridges (3 ml, 500 mg gel) were prewashed by sequential application of 3 ml each of methanol, ethyl acetate and *n*-hexane.

Sep-Pak Vac Accell QMA cartridges (3 ml, 500 mg resin) were prewashed with 10 ml of distilled water and equilibrated with 10 m*M* Tris–HCl buffer (pH 8.0).

2.5. Determination of unconjugated MHPG (Fig. 1)

Unconjugated MHPG was determined as previously described using iso-VAl as an internal standard (I.S.) [19]. Briefly, a urine sample was mixed with an equal volume of 2 nmol/ml iso-VAl, then a 200-μl aliquot was applied on prewashed Diol cartridge. After the sample was adsorbed, the cartridge was washed with 1 ml of *n*-hexane and unconjugated MHPG was eluted with 2 ml of ethyl acetate. The eluate was evaporated in a SpeedVac Concentrator, then the residue was dissolved in 1 ml of 50 m*M* sodium phosphate (pH 6.0, mobile phase A) and a 100-μl aliquot was subjected to HPLC analysis.

2.6. Elution profile of MHPG-sulfate

To analyze the elution profile of MHPG-sulfate, 8 nmol of authentic MHPG-sulfate was dissolved in 1 ml of 10 m*M* Tris–HCl buffer (pH 8.0) and applied onto the QMA cartridge. Then, eluents were applied in the following order and fractions were collected; 1 ml of distilled water, 1 ml of 0.1 *M* HCl, five 1-ml aliquots of 0.2 *M* NaCl and three 1-ml aliquots of 0.5 *M* NaCl.

Two hundred and fifty microliters of 2 nmol/ml iso-VAl and 100 μl of arylsulfatase–β-glucuronidase were added to a 250-μl aliquot of the QMA eluate and incubated at 37°C for 2 h. Only for 0.1 *M* HCl eluate, pH was adjusted to 7 by addition of an appropriate amount of 1 *M* Bis-Tris prior to adding the enzyme solution. Then, a 200-μl aliquot was applied onto the Diol cartridge and processed as unconjugated MHPG, as described above.

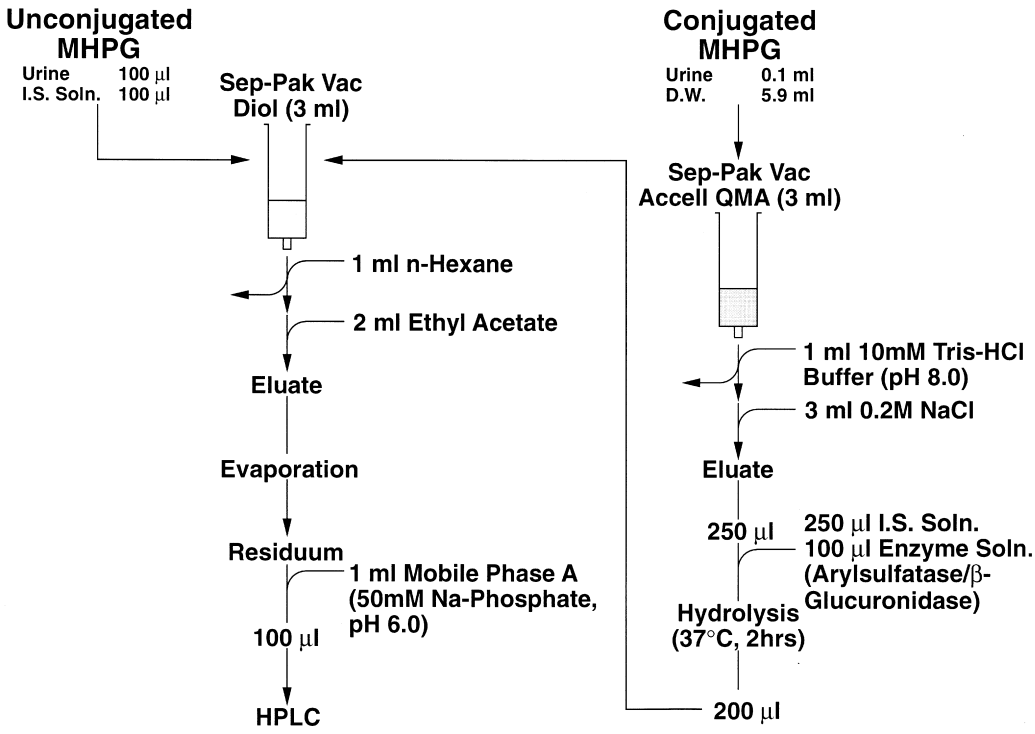

Fig. 1. Schematic presentation of the extraction procedure.

2.7. Determination of conjugated (sulfate plus glucuronide) MHPG (Fig. 1)

A urine sample was diluted 60-fold with distilled water and a 6-ml aliquot was applied onto the prewashed and equilibrated QMA cartridge. After washing with 1 ml of 10 m*M* Tris–HCl buffer (pH 8.0), conjugated (sulfate plus glucuronide) MHPG was eluted with 3 ml of 0.2 *M* NaCl.

Two hundred and fifty microliters of 2 nmol/ml iso-VAl and 100 μl of arylsulfatase–β-glucuronidase were added to a 250-μl aliquot of the QMA eluate and incubated at 37°C for 2 h. Then, a 200-μl aliquot was applied onto the Diol cartridge and processed as described above.

2.8. Determination of total (conjugated plus unconjugated) MHPG

Two hundred and fifty microliters of 2 nmol/ml iso-VAl and 100 μl of arylsulfatase–β-glucuronidase were added to 250 μl of urine and incubated at 37°C for 2 h. Then, a 200-μl aliquot was applied onto the Diol cartridge and processed as described above.

2.9. Urine samples

Urine samples were collected in the morning from seven healthy young adults and were stored at −20°C after the addition of conc. HCl (10 μl to 10 ml urine) until use.

3. Results and discussion

3.1. Determination of unconjugated MHPG

As reported previously [19], unconjugated MHPG was successfully extracted using a Sep-Pak Diol cartridge. The recovery of MHPG from each cartridge was corrected by that of iso-VAl used as an internal standard. Typical HPLC chromatograms of authentic MHPG (Fig. 2A), authentic MHPG after extraction (Fig. 2B) and human urine extract (Fig.

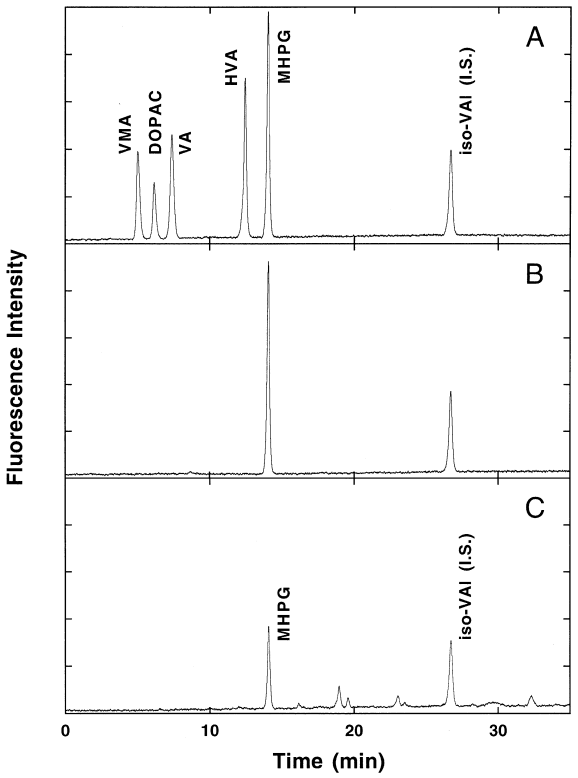

Fig. 2. Typical HPLC chromatograms of authentic MHPG before (A) and after (B) solid-phase extraction with Sep-Pak Vac Diol, and a human urine sample after extraction (C). A standard solution containing 4 nmol/ml each of authentic MHPG, 3,4-dihydroxyphenylacetic acid (DOPAC), vanillic acid (VA) and homovanillic acid (HVA), and 2 nmol/ml of vanillylmandelic acid (VMA) was prepared. The standard solution was mixed with an equal volume of 2 nmol/ml isovanillyl alcohol (iso-VAl; I.S.), diluted five-fold with mobile phase A, and a 100-μl aliquot was injected into HPLC–FD (A). The same standard solution (B) and a urine sample (C) were mixed with I.S. solution, treated with Sep-Pak Diol cartridges, and analyzed by HPLC as described in Section 2.5 and Fig. 1. The MHPG concentration in the urine sample was calculated to be 2.06 nmol/ml (C).

2C) are shown. The peaks of MHPG and iso-VAl were well separated and no interfering peaks were observed. In addition, the extraction procedure was selective for MHPG, and other catecholamine metabolites, such as VMA and HVA, were not recovered.

To validate the assay procedure, a recovery experiment was carried out using two independent urine samples. The recoveries of 1 nmol/ml of MHPG added to the urine samples were 97.6 and 112.8%. When 4 nmol/ml of MHPG was added, the recoveries were 101.4 and 113.3%.

3.2. Solid-phase extraction of conjugated MHPG using a Sep-Pak Accell QMA cartridge

In preliminary experiments using *p*-nitrophenyl sulfate and *p*-nitrophenyl β-D-glucuronide, these conjugated compounds adsorbed onto the Sep-Pak Accell QMA cartridge. In addition, glucuronide could be eluted by either 0.1 *M* HCl or 0.2 *M* NaCl, whereas sulfate could be eluted only by 0.2 *M* NaCl (data not shown).

Therefore, adsorption/elution experiments were performed with authentic MHPG-sulfate. As shown in Fig. 3, MHPG-sulfate was adsorbed onto the Sep-Pak Accell QMA cartridge and not eluted with 0.1 *M* HCl but with 0.2 *M* NaCl. This result was similar to that of Karasawa et al. [20] using QAE-Sephadex A-25 as an ion-exchange resin. Authentic MHPG-glucuronide was not tested because of its unavailability. However, it was considered that it could be eluted with 0.2 *M* NaCl and this was demonstrated as described below.

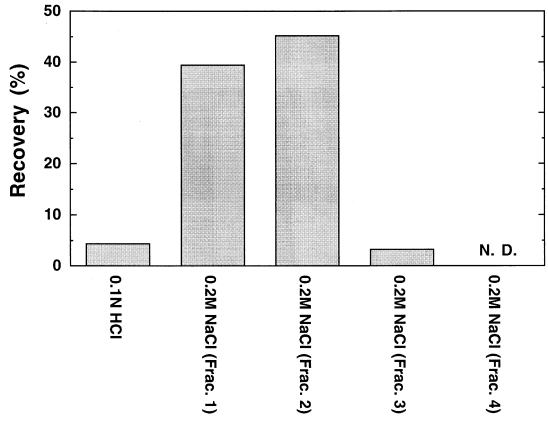

Fig. 3. Elution profile of authentic MHPG-sulfate using the Sep-Pak Vac QMA cartridge. Eight nanomoles of authentic MHPG-sulfate was dissolved in 1 ml of 10 m*M* Tris–HCl buffer (pH 8.0), and applied onto the cartridge. Then, eluents were sequentially applied and fractions were collected. Finally, the concentration of MHPG-sulfate in each fraction was determined as described in Section 2.6. The recoveries of applied authentic MHPG-sulfate in each fraction are shown. MHPG-sulfate was not detected in fractions after 0.2 *M* NaCl (Frac. 4). N.D., not detected.

3.3. Determination of conjugated MHPG

According to the results of adsorption/elution experiments, 0.2 *M* NaCl was chosen as an eluent for the elution of conjugated MHPG from the Sep-Pak Accell QMA cartridge. Urine samples were diluted before application onto the Sep-Pak QMA cartridge in order to lower their ionic strength and to promote the quantitative adsorption of conjugated MHPG. The procedure is summarized in Fig. 1.

The duration of enzymatic hydrolysis was determined separately with some human urine samples. When 100 μl of arylsulfatase–β-glucuronidase were added to 250 μl of urine, the amount of liberated MHPG reached a plateau after 2 h incubation at 37°C (data not shown). Therefore, the incubation time was set to 2 h.

When the 0.2 *M* NaCl eluate was passed through a Sep-Pak Diol cartridge without enzyme treatment, no MHPG was detected. This indicated that conjugated MHPG was separated from unconjugated MHPG which was probably not retained on the Sep-Pak QMA cartridge.

Fig. 4A and Fig. 4B are typical HPLC chromatograms of QMA-extracted, enzymatically hydrolyzed and Diol-extracted authentic MHPG-sulfate and human urine, respectively. Some unidentified peaks appeared following the enzyme treatment, but none of them interfered with the peaks of MHPG or the internal standard.

In order to determine the recovery of the conjugated MHPG from the Sep-Pak QMA cartridge, authentic MHPG-sulfate was added to urine and analyzed by the present method. Two independent urine samples were used. In one urine sample, the recoveries of 7.88 and 31.51 nmol/ml of authentic MHPG-sulfate added to the urine were 105.6 and 105.7%, respectively. In another urine sample, 8.05 and 32.21 nmol/ml were added and the recoveries were 99.6 and 101.9%, respectively. They were all near 100%, confirming that the method is quantitative.

It was not shown whether MHPG-glucuronide is recovered simultaneously with MHPG-sulfate, because authentic MHPG-glucuronide is not available. Therefore, the recovery of MHPG-glucuronide from the Sep-Pak QMA cartridge was determined using endogenous MHPG-glucuronide in human urine.

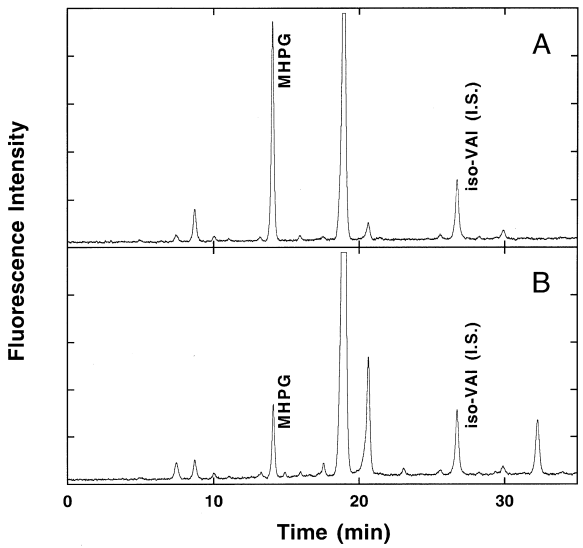

Fig. 4. HPLC chromatograms obtained from authentic MHPG-sulfate (A) and human urinary conjugated MHPG (B) by the present procedure. Authentic MHPG-sulfate (149.6 nmol/ml) (A) and a urine sample (B) were diluted, treated with Sep-Pak QMA cartridges, enzymatically hydrolyzed, treated with Sep-Pak Diol cartridges, and analyzed by HPLC–FD as described in Section 2.7 and Fig. 1. Urinary concentration of conjugated MHPG was calculated to be 48.0 nmol/ml (B).

Unconjugated, conjugated and total MHPG in seven human urine samples were measured separately and the recovery of endogenous conjugated MHPG was calculated (Table 1). The recovery of conjugated MHPG was calculated as (amount of conjugated MHPG)/[(amount of total MHPG)−(amount of unconjugated MHPG)]. The mean recovery was 98%, and this again indicated that conjugated MHPG is extracted quantitatively. In addition, the results showed that MHPG-glucuronide was also recovered quantitatively in the Sep-Pak QMA eluate, because total MHPG is composed of unconjugated MHPG, MHPG-sulfate and MHPG-glucuronide, and total amount of MHPG minus amount of unconjugated MHPG was equivalent to the amount of conjugated MHPG recovered in the Sep-Pak QMA eluate. Furthermore, the presence of a substantial amount of MHPG-glucuronide in the QMA eluate was shown using arylsulfatase-free β-glucuronidase instead of arylsulfatase–β-glucuronidase (data not shown).

As shown in Table 1, the concentrations of

Table 1
Unconjugated, conjugated and total MHPG concentrations in seven human urine samples

	Concentration (mmol/mol creatinine)			Recovery (%) $(U+C)/T$
	Unconjugated MHPG (U)	Conjugated MHPG (C)	Total MHPG (T)	
Sample 1	0.154	1.314	1.535	95.6
Sample 2	0.113	1.341	1.534	94.8
Sample 3	0.068	1.459	1.450	105.3
Sample 4	0.085	1.691	1.730	102.7
Sample 5	0.123	1.169	1.354	95.4
Sample 6	0.116	0.942	1.071	98.8
Sample 7	0.070	2.415	2.541	97.8
Mean	0.104	1.476	1.602	98.6
S.D.	0.031	0.475	0.461	4.0

unconjugated and conjugated MHPG determined by the present method were 0.104 ± 0.031 and 1.476 ± 0.475 mmol/mol creatinine (mean$\pm$S.D.), respectively. These values can also be expressed as 0.243 ± 0.055 and 3.638 ± 1.571 mg/l (mean$\pm$S.D.), respectively, or 0.314 ± 0.094 and 4.449 ± 1.430 mg/24 h (mean$\pm$S.D.) (when daily creatinine excretion is assumed to be 1.5 g), respectively. All of these values are higher than those in previous reports. For example, Bourdeaux et al. [14] reported that the concentrations of unconjugated and conjugated (sulfate plus glucuronide; they were determined separately) MHPG were 0.12 and 3.09 mg/l (mean), respectively. Filser et al. [11] reported values of 0.158 and 1.779 mg/24 h (mean), respectively. It was considered that the oxidative degradation of unconjugated and deconjugated MHPG during the ethyl acetate extraction procedure might be the main reason why our values were higher. The recovery of authentic MHPG by ethyl acetate extraction was low due to oxidative degradation during the shaking in our preliminary experiments, as described previously [19]. The present solid-phase extraction procedure using the Sep-Pak Diol cartridge includes no shaking process and thus minimized the oxidative degradation of MHPG.

In the present method, MHPG-sulfate and MHPG-glucuronide are not determined separately due to the unavailability of highly active, β-glucuronidase-free arylsulfatase. If it becomes available, it will be possible to determine MHPG-sulfate and MHPG-glucuronide separately, by treatment of the QMA eluate with respective enzymes.

Finally, the present method is more rapid than those described previously. For example, solid-phase extraction of one sample with a Sep-Pak Diol cartridge was completed within 5 min. It took about 70 min for the analysis of unconjugated MHPG for one sample. Moreover, extraction and evaporation steps were completed within 2 h even with analysis of 20 samples. In the case of conjugated MHPG, an additional 10 min was required for Sep-Pak QMA extraction and 2 h for enzymatic hydrolysis.

The present determination procedure for unconjugated and conjugated MHPG is simpler and more rapid than those described before, and sufficiently sensitive to analyze human urine samples. It will be useful to studies of the physiological significance of urinary MHPG.

References

[1] J.W. Maas, D.H. Landis, J. Pharmacol. Exp. Ther. 147 (1968) 163.
[2] M.S. Buchsbaum, G. Muscettola, F.K. Goodwin, Neuropsychobiology 7 (1981) 212.
[3] I.J. Kopin, Psychopathology 17 (1984) 83.
[4] H. Agren, Psychiatry Res. 6 (1982) 185.
[5] J.J. Schildkraut, P.J. Orsulak, A.F. Schatzberg, J.E. Gudeman, J.O. Cole, W.A. Rohde, R.A. LaBrie, Arch. Gen. Psychiarty 35 (1978) 1427.
[6] G. Muscettola, W.Z. Potter, E.K. Gordon, F.K. Goodwin, Psychiatry Res. 4 (1981) 267.
[7] S. Murray, T.A. Baillie, D.S. Davies, J. Chromatogr. 143 (1977) 541.
[8] R. Alonso, C.J. Gibson, J. McGill, Life Sci. 29 (1981) 1689.
[9] J.R. Shipe, J. Savory, M.R. Wills, Clin. Chem. 30 (1984) 140.
[10] R.T. Brown, J. Oliver, K.L. Kirk, I.J. Kopin, Life Sci. 34 (1984) 2313.

[11] J.G. Filser, S. Koch, M. Fischer, W.E. Muller, J. Chromatogr. 493 (1989) 275.
[12] M.H. Joseph, B.V. Kadam, D. Risby, J. Chromatogr. 226 (1981) 361.
[13] D.D. Koch, G. Polzin, J. Chromatogr. 317 (1984) 327.
[14] R. Bourdeaux, B. Capolaghi, C. Lacroix, C. Younos, P.R. Lehr, J. Chromatogr. B 665 (1995) 45.
[15] A.M. Krstulovic, C.T. Matzura, L. Bertani-Dziedzic, S. Cerqueira, S.E. Gitlow, Clin. Chim. Acta 103 (1980) 109.
[16] P. Moleman, J.J. Borstrok, J. Chromatogr. 227 (1982) 391.
[17] G. Santagostino, P. Frattini, S. Schinelli, M.L. Cucchi, G.L. Corona, J. Chromatogr. 233 (1982) 89.
[18] P.A. Shea, J.B. Howell, J. Chromatogr. 306 (1984) 358.
[19] K. Hirata, H. Inagaki, M. Minami, Yakugaku Zasshi 116 (1996) 813.
[20] T. Karasawa, K. Furukawa, M. Shimizu, J. Neurochem. 30 (1978) 1525.