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Journal of Chromatography B, 663 (1995) 193–200

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of 3-methoxy-4-hydroxyphenylethylene glycol in urine using reversed-phase liquid chromatography with column switching and electrochemical detection

John Rollag, Tsentao Liu, David S. Hage*

Department of Chemistry, University of Nebraska, 738 Hamilton Hall, Lincoln, NE 68588-0304, USA

First received 12 August 1994; revised manuscript received 5 October 1994

Abstract

A column-switching method was developed for the determination of total 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) in urine. This was performed by first treating samples with β-glucuronidase, followed by extraction with ethyl acetate. The reconstituted extracts were injected onto an HPLC system containing an amperometric detector and tandem Nucleosil C_{18} and C_8 reversed-phase columns connected by a switching valve. The total analysis time for MHPG was 12 min. The limit of detection was 0.18 ng, or 9 μg/l for 20-μl injections of a 1.0-ml reconstituted extract prepared from 1.0 ml of urine. The linear range extended up to 80 mg/l. The within-day precision for a urine sample containing 170 μg/l total MHPG was ± 6% and the day-to-day precision was ± 15%. The average levels determined by this method for total MHPG in normal subjects showed good agreement with previous literature values. This approach could be modified for the determination of free MHPG by using only ethyl acetate extraction for sample pretreatment.

1. Introduction

MHPG (3-methoxy-4-hydroxyphenylethyleneglycol) is a major end-pathway product of norepinephrine metabolism in humans [1]. Norepinephrine is one of the major neurotransmitters in the body. MHPG is produced from norepinephrine through deamination by the enzyme monoamine oxidase and is made primarily in the central nervous system [2]. These characteristics have made MHPG useful as an index of norepinephrine activity in the brain and related regions of the nervous system [3,4]. Applications of MHPG include its use in the study and characterization of various psychiatric disorders (e.g. depression) and as an indicator of possible pharmacological agents which might be useful in treating these disorders (e.g. tricyclic antidepressants) [5–7]. MHPG levels can also serve as an aid in the diagnosis of catecholamine secreting tumors [8,9].

Many current methods for MHPG are performed using urine as the sample matrix [10–29]. MHPG is excreted into urine in both conjugated and unconjugated forms. The concentration of total MHPG in urine ranges from 10 to 2000 μg/l, with about 20% of this being present in the unconjugated, or free, form. Although most previous methods have been developed for the measurement of total MHPG, several recent

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00464-1

studies have also reported procedures for the determination of free MHPG levels [16,21–25].

Previous techniques for quantitating urinary MHPG have been based on either gas chromatography (GC) [19,26] or high-performance liquid chromatography (HPLC) [10–18,20–25,27–29]. One disadvantage of the GC techniques is that they all require derivatization of MHPG prior to analysis [19,26]. HPLC methods based on absorbance or fluorescence detection generally suffer from low sensitivity and usually require derivatization of MHPG in order to obtain acceptable detection limits [10–13,24].

The use of HPLC with electrochemical detection (LC-ED) is one approach that can be used to obtain adequate detection of urinary MHPG without requiring derivatization [14–18,20–24,27–29]. However, current LC-ED methods for MHPG still have a number of disadvantages. For example, many of these techniques use complex extraction procedures or are designed to monitor several compounds in addition to MHPG [14,15,17,18,20–24,27–29]. This has resulted in long analysis times for previous MHPG methods because of the extra time required for sample pretreatment or for separating all of the desired components with good resolution on the HPLC system.

This work examines the development of an HPLC method for MHPG that is both fast and capable of directly measuring this compound in urine. The presented approach is based on the use of LC-ED along with a switching scheme involving two tandem reversed-phase columns. Items that were examined in the optimization of this method included the extraction and elution conditions required for sample pretreatment and analysis. The precision, response, and speed of this method were then evaluated and the results obtained by this technique for normal subjects were compared to those given in the literature.

2. Experimental

2.1. Reagents

The hemipiperazine salt of MHPG, MHPG sulfate, glutathione and *Helix pomatia* β-glucuronidase (type H-2) were from Sigma (St. Louis, MO, USA). The HPLC grade methanol, ACS grade ethyl acetate, and reagents for the creatinine colorimetric assay were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The C_{18} Nucleosil Si-100-3 (3 μm particle size, 100 Å pore size) and C_8 Nucleosil Si-100-5 (5 μm particle size, 100 Å pore size) supports were from Alltech (Deerfield, IL, USA). All other chemicals were of the purest grades available. The Nylon filters were from Amicon (Beverly, MA, USA). The deionized water used in this work was produced in-house by a Nanopure water system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

The chromatographic system consisted of two isocratic single-stroke LC-350 SSI pumps (Scientific Systems, State College, PA, USA) and one LDC/Milton Roy 715 autosampler (Riviera Beach, FL, USA) equipped with a 7125 Rheodyne injection valve (Cotati, CA, USA) and a 20-μl injection loop. Column switching was performed using a 5701 Rheodyne valve equipped with a DVI actuator (Chromtech, Eden Prairie, MN, USA). The switching sequence was controlled by an LDC/Milton Roy GM4000 gradient manager.

Electrochemical detection was performed using an LC-4C electrochemical detector from Bioanalytical Systems (Lafayette, IN, USA). A glassy carbon working electrode was used at a potential of +0.800 V vs. a Ag/AgCl reference electrode. Creatinine measurements were performed on an RA-1000 autoanalyzer from Technicon Industrial Systems (Tarrytown, NY, USA)

The precolumn used in this study was a 100 × 4.6 mm I.D. stainless steel column containing C_{18} Nucleosil Si-100-3 and the analytical column was a 250 × 4.6 mm I.D. stainless steel column containing C_8 Nucleosil Si-100-5. Both columns were downward slurry-packed at 28 MPa (i.e. 4000 psi) using an Alltech HPLC column packer.

2.3. Methods

A primary stock solution containing 100 mg/l MHPG was prepared by dissolving the

hemipiperazine salt of MHPG in 0.10 *M* phosphate buffer (pH 7.0). This solution was stored at 4°C until use. Calibration standards were prepared fresh daily by diluting a portion of the primary standard in 0.20 *M* phosphate buffer (pH 6.0). Pooled controls were prepared from urine obtained from two normal individuals and were stored in 10-ml aliquots at −20°C.

Samples and standards analyzed for total MHPG content were first hydrolyzed by placing 1.0 ml of each sample into a clean 13 × 100 mm borosilicate tube and adding 0.2 ml of 0.20 *M* phosphate buffer (pH 6.0) plus 20 μl of a commercial β-glucuronidase preparation. The amount of added enzyme was equivalent to approximately 2600 units of glucuronidase activity and 100 units of sulfatase activity at pH 5.0. The sample/enzyme mixtures were incubated for 4 h at 60°C. The mixtures were next extracted two times with separate 4.0-ml portions of ethyl acetate and the combined organic fractions were evaporated to near dryness under air at room temperature. The extract was reconstituted in 1.0 ml of 0.10 *M* phosphate buffer (pH 7.0), passed through a Nylon 0.45-μm filter, and injected onto the HPLC system. Samples for the free MHPG analyses were treated in a similar fashion but excluding the step involving β-glucuronidase treatment.

The setup of the column-switching system used in this study is illustrated in Fig. 1. The mobile phase used for sample injection (mobile phase A) was methanol–0.10 *M* phosphate buffer (pH 3.0) (10:90, v/v) applied at a flow-rate of 1.0 ml/min. The solvent used for elution of strongly retained peaks on the C_{18} precolumn (mobile phase B) was methanol–0.10 *M* phosphate buffer (pH 3.0) (40:60, v/v), also applied at a flow-rate of 1.0 ml/min. The time sequence of the HPLC analysis was as follows: at 0.0 min (position 1) mobile phase A was applied to both the precolumn and analytical column and sample was injected; at 2.5 min (position 2) the precolumn was switched to mobile phase B in order to wash off strongly retained analytes, while mobile phase A was still applied to the analytical column for the separation and detection of MHPG; at 10.0 min (position 1) the precolumn

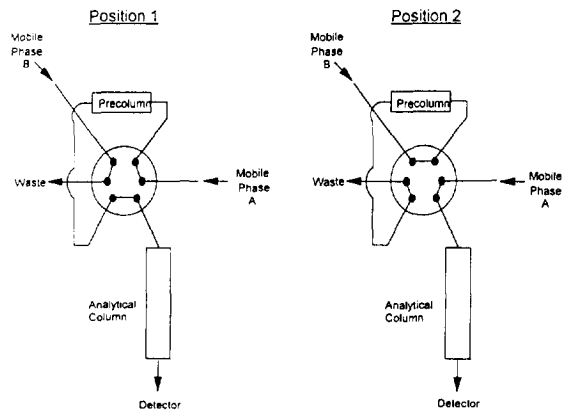

Fig. 1. Column-switching system used for the determination of urinary MHPG. Position 1 was used for sample injection and application of MHPG from the precolumn to the analytical column. Position 2 was used to continue elution of MHPG on the analytical column and wash strongly retained components off the analytical column.

was switched back to mobile phase A and re-equilibrated for 2 min before the next injection.

The extraction recovery of MHPG was determined by using split samples of pooled working standards, with half being injected directly onto the chromatographic system and half being extracted with ethyl acetate prior to injection. The freeze–thaw stability of MHPG was examined by thawing control samples for up to four times prior to extraction. Reinjection stability was determined by extracting and injecting control samples two times at 24-h intervals. The results were then quantitated against the standard curve used for the first sample injection. Samples for the spiked recovery studies were prepared by combining 50 μl of a 100–500 mg/l MHPG solution in 0.10 *M* phosphate buffer (pH 7.0) with 1.0 ml of urine. System carryover was examined by injecting a 10 mg/l MHPG standard or an extracted urine sample followed by the subsequent injection of a blank sample containing only 0.10 *M* phosphate buffer (pH 7.0).

The subjects used in the normal value study were 10 male and 10 female healthy college age volunteers selected randomly from a study performed by Dr. Richard Dienstbier in the Psychology Department at the University of Nebraska (Lincoln, NE, USA). The males participat-

ing in this study had a mean weight of 77 ± 6 kg (i.e. 170 ± 14 LB). The females had a mean weight of 57 ± 4 kg (i.e. 126 ± 8 LB). Urine samples were collected at defined time intervals from these subjects and combined with approximately 0.5 mg of glutathione per ml urine. The samples were then stored at −20°C prior to analysis. The creatinine level in each sample was determined using the Jaffé method [30], as performed on an RA-1000 autoanalyzer.

3. Results and discussion

3.1. Sample pretreatment

Prior to the analysis of total MHPG, all samples were pretreated with the enzyme β-glucuronidase in order to hydrolyze MHPG conjugates back into the free form of the molecule. Direct detection of these conjugates without enzymatic treatment was not possible since these compounds did not give a response at the potential used to monitor the column eluent (i.e. +0.800 V *versus* Ag/AgCl). The conditions required to obtain complete hydrolysis of the MHPG conjugates were initially examined by combining 1.0 ml of a solution containing 500 μg/l total MHPG sulfate in 0.20 *M* phosphate buffer (pH 6.0) with 20 μl of β-glucuronidase and incubating the mixture at 60°C. Aliquots of the reaction mixture were then collected at various times and injected onto the HPLC system. Similar studies were performed with a urine sample containing a total MHPG level equal to 2690 μg/l. The results are shown in Fig. 2.

MHPG sulfate was chosen as the substrate for the initial hydrolysis study since it was readily available and it represented the substrate having the lower enzymatic activity in the β-glucuronidase preparation. For example, the enzyme preparation used in this work had an estimated activity of 132 000 units per ml for glucuronide conjugates at pH 5.0 but an activity of only 5000 units per ml for sulfate conjugates under the same conditions. The results in Fig. 2 show that quantitative cleavage of the MHPG sulfate test solution was obtained in about one and a half

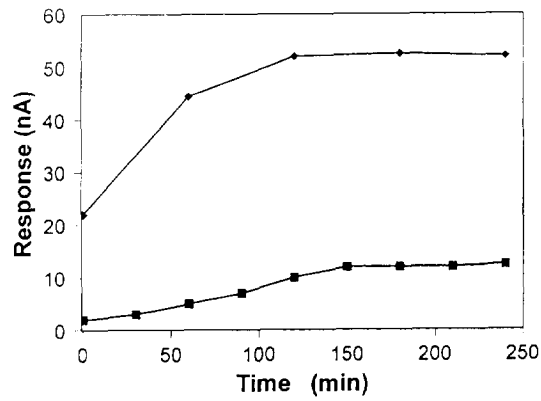

Fig. 2. Effect of incubation time on the hydrolysis of MHPG conjugates in urine (◆) and MHPG sulfate (■) by β-glucuronidase. The experimental conditions are given in the text.

hour under the given incubation conditions. In order to allow for the possible presence of other endogenous substrates in urine, a minimum incubation time of 4 h was used throughout the remainder of this work. As shown in Fig. 2, this time was more than adequate for the complete hydrolysis of MHPG conjugates in the actual urine samples.

After the enzymatic treatment, samples for the total MHPG determinations were extracted in order to remove MHPG from the original sample and β-glucuronidase solution. Extraction was also used in the pretreatment of free MHPG samples without prior addition of enzyme. Ethyl acetate was chosen as the solvent for this extraction since it has been successfully used in a number of previous methods for total MHPG [13–18,20–25,27–29]. Because MHPG is a neutral molecule, its degree of extraction with ethyl acetate was not affected by pH. However, the extraction of other sample components was pH dependent and could be minimized by keeping the aqueous phase buffered at pH 6.0. Table 1 shows the recoveries obtained at pH 6.0 for standard solutions of MHPG using a two-step extraction. The mean recovery of the standards over the range of concentrations tested was 85 ± 4%.

Table 1
Extraction recovery of MHPG standards

Concentration (μg/l)	Recovery (%)[a]
500	90 (± 3)
250	88 (± 5)
100	85 (± 2)
50	82 (± 2)
25	87 (± 3)
10	79 (± 1)

[a] The values in parentheses represent ± 1 S.D. for three trials.

Similar extraction recoveries were obtained for urine samples spiked with various amounts of unconjugated MHPG. For example, the spiked extraction recoveries obtained with a urine samples to which had been added 4.8, 12 or 24 mg/l MHPG were 88 ± 2, 82 ± 4 and 96 ± 4% (mean, 88 ± 7%) when performed in triplicate using both β-glucuronidase hydrolysis and ethyl acetate extraction for sample pretreatment (i.e. the protocol for total MHPG analyses). The spiked recoveries obtained on the same samples after using only ethyl acetate extraction (i.e. the free MHPG protocol) were 93 ± 2, 90 ± 6 and 82 ± 2% (mean, 88 ± 6%).

The extraction recovery and overall precision of the HPLC method developed in this work were both high enough that free and total MHPG levels could be determined without the use of any added internal standard. This is the same approach that has been adapted in previous MHPG methods [10,13–16,18,20,22–25,28]. Some experiments were performed early in this work to examine a number of compounds for use as possible internal standards. These included isoproterenol, 1,2,4-benzenetriol and 1-phenyl-1,2-ethanediol. However, none of these compounds were found to be satisfactory when used in this method. Isoproterenol had a low recovery in the ethyl acetate extraction and 1-phenyl-1,2-ethanediol did not give any measurable response on the electrochemical detector. 1,2,4-Benzenetriol was extracted and did give a detector response, but it also had much stronger retention than MHPG on the reversed-phase columns, thus requiring significantly longer times for the analysis of samples.

3.2. Chromatographic conditions

Initial separations on the HPLC system were performed by injecting reconstituted sample extracts onto a single C_8 reversed-phase column under isocratic conditions. It was found that a mobile phase of methanol–pH 3.0 phosphate buffer (10:90, v/v) gave good resolution of MHPG from all background peaks; however, several late eluting peaks also appeared in the chromatogram. When using a 250 × 4.6 mm I.D. column and a flow-rate of 1.0 ml/min, MHPG eluted at 3.7 min (i.e. a capacity factor of 3.76). The latest eluting component in the sample came off the column at approximately 35 min, thus adding over 30 min to the total analysis time.

A reversed-phase precolumn and a column-switching scheme were used to reduce the overall analysis time of the HPLC system without sacrificing any resolution of the MHPG peak. In this approach, the sample was first injected onto a tandem arrangement of a C_{18} precolumn and C_8 analytical column in the presence of the 10:90 mixture of methanol–pH 3.0 phosphate buffer. After MHPG had eluted onto the C_8 column, the C_{18} precolumn was switched to a stronger mobile phase for washing off more strongly retained compounds. A 40:60 methanol–pH 3.0 phosphate buffer was used for this purpose. Under these conditions, all of the late-eluting peaks left the precolumn in about 12 min.

By using the switching system to prevent strongly retained compounds from eluting onto the analytical column, the total time between injections was reduced to 12 min. This included 10 min for the separation of MHPG from other components on the analytical column and 2 min for re-equilibration of the precolumn before the next injection. The retention time of MHPG under these conditions was 6.0 min. Typical chromatograms obtained on this system are shown in Fig. 3. Depending on the type of sample pretreatment used, this method could be

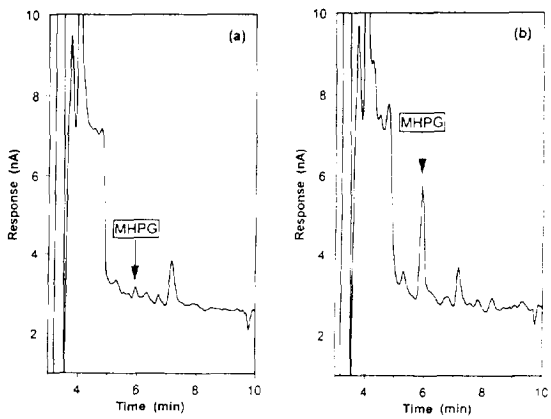

Fig. 3. Chromatograms for an extracted urine sample in the analysis of free MHPG (a) and total MHPG (b). The free MHPG concentration in the sample was 15 μg/l, or 0.24 μg MHPG/mg creatinine. The total MHPG level was 140 μg/l, or 2.21 μg MHPG/mg creatinine.

applied to the analysis of either total MHPG or free MHPG levels in urine (see Fig. 3a,b).

A typical calibration curve obtained on this system for extracted MHPG standards had a slope of 0.0170 ± 0.0002 nA l/μg and an intercept of −0.02 ± 0.03 nA. The correlation coefficient was 0.999 over ten MHPG concentrations in the range of 0 to 70 mg/l. The detection limit of the assay at a signal-to-noise ratio of three was estimated to be 0.18 ng, or 0.7 pmol MHPG. This was equivalent to a level of 9 μg/l for 20-μl injections of a 1.0-ml reconstituted extract prepared from 1.0 ml of urine. The linear range of the curve (i.e. the range of concentrations giving a response within ± 5% of the best-fit line) extended up to about 80 mg/l MHPG. The dynamic range extended over at least four orders of magnitude, or from 9 μg/l to 100 mg/l MHPG.

A control urine sample with a mean total MHPG concentration of 170 μg/l was used to determine the day-to-day and within-day precision of this method. The control was analyzed 162 times during the study with up to six analyses per day over 28 working days. The within-day precision was ± 6% and the day-to-day was ± 15% at this concentration level.

System carryover was examined by making alternate injections of a 10 mg/l MHPG standard, or an extracted urine sample, and a sample containing only 0.10 *M* phosphate buffer (pH 7.0). In each set of injections, no detectable levels of MHPG were seen during the analysis of the buffer sample. Based on the limit of detection that had been measured for the HPLC system and the total amount of MHPG that was known to be present in the standard and urine samples, it was determined that there was less than 0.1% carryover for MHPG between consecutive injections.

The long-term stability of the LC-ED method was evaluated by comparing the slopes and intercepts obtained for peak-height calibration curves generated over the course of 28 working days. A total of approximately 1670 standards and samples were analyzed over this period of time. The mean slope obtained during this time was 0.017 ± 0.002 nA l/μg (range, 0.020–0.012 nA l/μg) and the mean intercept was −0.003 ± 0.052 nA (range, −0.150–0.034 nA). The average correlation coefficient was 0.998 (range, 0.989–1.000 for seven levels of standards and 28 calibration curves).

The MHPG samples tested in this study proved to be quite stable to both temperature fluctuations and freeze–thaw cycles. In one freeze–thaw cycle, the change in the total MHPG concentration for the urine control was determined to be only −1.84 ± 0.03% ($n = 6$). In two freeze–thaw cycles, the change was −6.62 ± 0.09% and in three cycles it was −3.9 ± 0.2%. These changes were not significant compared to the within-day or day-to-day variation noted with the method. To test for reinjection stability, control samples were applied onto the HPLC system two times over a 24-h interval. The percent change upon reinjection was 1.3 ± 0.8% for 18 replicate injections. This level of variation was also not significant compared to the precision of the method.

The total MHPG levels for a series of normal volunteers, ten female and ten male, were determined with this method. To correct for variations in urine volume, all results were normalized against the level of creatinine measured in the corresponding urine samples. The results are shown in Table 2. Good agreement was seen

Table 2
Total MHPG concentrations in normal individuals

n	Concentration (mean ± S.D.) (μg/mg cre.)[a]	Reference
Female		
10	1.5 ± 0.3	Present method
7	1.4 ± 0.2	21
43	1.3 ± 0.1	25
Male		
10	1.7 ± 0.4	Present method
8	1.5 ± 0.2	21
46	1.3 ± 0.1	25

[a] The value following each number represents ± 1 S.D. of the mean.

between the results obtained by this method and those reported previously in the literature. The range of values observed in the total MHPG results was 0.22–2.80 μg MHPG/mg creatinine in the males and 0.49–4.94 μg MHPG/mg creatinine in the females, with no significant differences being noted in the average levels obtained in the male vs. female subjects.

4. Conclusions

This study examined the use of tandem reversed-phase HPLC and electrochemical detection for the determination of MHPG in urine. For the analysis of total MHPG, simple enzymatic treatment and extraction steps were all that was required for sample preparation. By using a column-switching scheme, it was possible to obtain the separation of MHPG from other sample components in only 12 min per injection. The limit of detection that was measured for MHPG by this technique was 9 μg/l. The response of this method was linear over almost four orders of magnitude in concentration, or up to 80 mg/l. This covered the range of clinical interest and was adequate for the determination of both free and total MHPG concentrations. In addition, the results for total MHPG in normal subjects were found to have good agreement with previous literature values. The described method had good reproducibility, with within-day and day-to-day precisions of ± 6% and ± 15%, respectively, for a sample containing 170 μg/l total MHPG. Overall, this method was found to be a fast and reliable means for the routine determination of MHPG in urine.

Acknowledgments

This work was supported by the National Institutes of Mental Health under grant No. R01-MH43974. The authors wish to thank Dr. Richard Dienstbier and Jennifer Sloan for their help in obtaining the urine samples used in this study.

References

[1] J.W. Maas and D.H. Landis, *J. Pharmacol. Exp. Ther.*, 147 (1968) 163.
[2] M. Ebadi, *Pharmacology*, 2nd ed., Little, Brown and Company, Boston, MA, 1993, Ch. 1.
[3] P. Blomberry, I.J. Kopin, E.K. Gordon and M.H. Ebert, in E. Usdin, I.J. Kopin and J. Barchas (Editors) *Catecholamines: Basic And Clinical Frontiers*, Vol. 1, Pergamon Press, New York, NY, 1978, p. 1875.
[4] S.M. Shanberg, J.J. Schildkrant, G.R. Breese and I.J. Kopin, *Biochem. Pharmacol.*, 17 (1968) 247.
[5] J.M. Maas, J. Fawcett and H. Dekirmenjian, *Arch. Gen. Psychiatry*, 19 (1968) 129.
[6] J.J. Schildkraut, P.J. Orsulak, A.F. Schatzberg, J.E. Gudeman, J.O. Cole, W.A. Rohde and R.A. Labrie, *Arch. Gen. Psychiatry*, 35 (1978) 1427.
[7] D.M. Gobbin, B. Requin-Blow, L.R. Williams and W.O. Williams, *Arch. Gen. Psychiatry*, 36 (1975) 1111.
[8] S.E. Gitlow, L.B. Dziedzic and L. Strauss, *Cancer*, 32 (1973) 898.
[9] A. Sjoerdsma, D. Engleman, T.A. Waldmann, L.H. Cooperman and W.G. Hammon, *Ann. Int. Med.*, 65 (1966) 1302.
[10] T. Hanai and J. Hubert, *J. Liq. Chromatogr.*, 7 (1984) 1627.
[11] H.K. Jeon, H. Nohta and Y. Ohkura, *Anal. Biochem.*, 200 (1992) 332.
[12] H. Nohta, E. Yamaguchi, Y. Oshkura and H. Watanabe, *J. Chromatogr.*, 493 (1989) 15.
[13] J.T. Taylor, S. Freeman and P. Brewer, *Clin. Chem.*, 27 (1981) 173.
[14] F. Karege, *J. Chromatogr.*, 311 (1984) 361.
[15] R. Alonso, C.J. Gibson and J. McGill, *Life Sci.*, 29 (1981) 1689.

[16] M.H. Joseph, B.V. Kadam and D. Risby, *J. Chromatogr.*, 226 (1981) 361.
[17] J.R. Shipe, J. Savory and M.R. Wills, *Clin. Chem.*, 30 (1984) 140.
[18] D.D. Koch and G. Polzin, *J. Chromatogr.*, 317 (1984) 327.
[19] B.A. Davis, D.A. Durden and A.A. Boulton, *J. Chromatogr.*, 374 (1986) 227.
[20] P.A. Shea and J.B. Howell, *J. Chromatogr.*, 306 (1984) 358.
[21] G. Santagostino, P. Frattini, S. Schinelli, M.L. Cucchi and G.L. Corona, *J. Chromatogr.*, 233 (1982) 89.
[22] C. Julien, C. Rodriguez, J. Sacquet, G. Cuisinaud and J. Sassard, *Clin. Chem.*, 34 (1988) 966.
[23] A.M. Krstulovic, C.T. Matzura, L. Bertani-Dziedzic, S. Cerqueira and S.E. Gitlow, *Clin. Chim. Acta*, 103 (1980) 109.
[24] J.G. Filser, S. Koch, M. Fischer and W.E. Müller, *J. Chromatogr.*, 493 (1989) 275.
[25] K. Abe and R. Konaka, *Clin. Chem.*, 34 (1988) 87.
[26] R. Kobelt, G. Wiesener and R. Kuhn, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 520.
[27] R.T. Brown, J. Oliver, K.L. Kirk and I.J. Kopin, *Life Sci.*, 34 (1984) 2313.
[28] M. Yoshiura, T. Iwamoto, K. Iriyama and T. Kuwana, *J. Liq. Chromatogr.*, 10 (1987) 3141.
[29] P. Moleman and J.J.M. Borstrok, *J. Chromatogr.*, 227 (1982) 391.
[30] M. Jaffé, *Z. Physiol. Chem.*, 10 (1886) 391.