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# Determination of 3,4-dihydroxyphenyl glycol in plasma by gas chromatography–mass spectrometry and high-performance liquid chromatography methods

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**Abstract**

Several modifications of GC–MS and HPLC methods for plasma level DHPG have been described. The effects of storage temperature and stabilizing agents on DHPG stability have been studied. The stabilizing agent has been found to play a more important role than low-temperature storage in preventing DHPG from decomposition during sample storage. A specific and sensitive GC–MS method (electron impact) has been established using stable isotope-labeled DHPG as an internal standard. HPLC has been improved by modifying the conditions, resulting in a good separation of DHPG and internal standard from solvent front and other early eluting compounds. Comparison of the GC–MS and HPLC procedures demonstrates a strong correlation between these two methods.

*Keywords:* 3,4-Dihydroxyphenyl glycol; Catecholamines

## 1. Introduction

3,4-Dihydroxyphenyl glycol (DHPG) is formed from the oxidative deamination of norepinephrine (NE) catalyzed by monoamine oxidase after its reuptake into the noradrenergic nerve endings [1,2]. Many studies indicate the importance of DHPG in the metabolic clearance of brain norepinephrine [3–5]. It has been also reported that DHPG formation directly reflects the central nervous system (CNS) noradrenergic activity [6–10]. These findings suggest that determination of DHPG may provide major insight into the biochemical assessment of noradrenergic function.

Therefore, one of the main methods for investigation of central norepinephrine metabolism in man may well be measurement of plasma levels of DHPG. In contrast to the large number of techniques developed for determination of catecholamines, only a few methods have been developed for measuring DHPG in plasma, largely due to its instability during extraction and storage. Current assay methods for DHPG include radioenzymatic procedures [11–13], gas chromatography with mass spectrometry [4,14]

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and liquid chromatography with electrochemical detection [10,15–18]. Radioenzymatic methods are highly sensitive but time-consuming and require the use of radio isotopes. GC–MS and HPLC offer convenient, highly sensitive and specific methods for the measurement of DHPG at relatively low cost. However, the available internal standard used for GC–MS could not give a good correlation curve since it lost two of the three labeled stable isotope atoms during the GC–MS procedure. In HPLC measurement, DHPG was eluted very early, extremely close to a large negative peak or the solvent front and other early eluted compounds. These difficulties, as well as the instability of DHPG during sample storage, still present major analytical problems in the quantitation of DHPG in plasma.

The present study focused on the improvement of the GC–MS and HPLC methods. These findings have led to effective and reliable methods for measurement of DHPG in plasma.

## 2. Experimental

### 2.1. Chemicals and reagents

3,4-Dihydroxyphenyl glycol (DHPG), 2-methyl-3-(3,4-dihydroxyphenyl)alanine ($\alpha$-methyl DOPA), epinephrine (E), norepinephrine (NE), 3,4-dihydroxyphenylalanine (DOPA), dopamine (DA) and Tris base (Trizma) were purchased from Sigma (St. Louis, MO, USA). Sodium metabisulfite, alumina (Brockman, neutral) and sodium 1-heptanesulfonate were from Fisher Scientific (Fair Lawn, NJ, USA). 1-(3,4-dihydroxyphenyl)-1,2-ethane-1,2,2-[$^2H_3$]diol was obtained from MSD Isotopes (St. Louis, MO, USA) and *tert.*-butyldimethylchlorosilane/imidazole (TBDMS/I) was from Alltech (Deerfield, IL, USA).

All the solvents used were HPLC grade. Alumina was purified and activated by the method of Eriksson and Persson [19] and stored at 37°C before use.

### 2.2. Preparation of 3,4-dihydroxyphenyl-2,5,6-[$^2H_3$]glycol

After preliminary experimentation, 16 mg of DHPG was dissolved in 3.5 ml of $^2H_2O$ in an ampoule and sealed following displacement of air by nitrogen. The solution was heated at 120°C for eight days. The reaction solution was diluted 800 times with $^2H_2O$ to ca. 4 ng/$\mu$l and stored at 4°C before using as the internal standard for GC–MS.

### 2.3. HPLC apparatus and chromatographic conditions

The HPLC system consisted of a Model 6000A solvent-delivery system, a Model 710B WISP autoinjector (Waters, Milford, MA, USA) and a Model 5100A Coulochem electrochemical detector (ESA, Bedford, MA, USA). All HPLC separations were carried out using a Supelcosil $C_{18}$ column (250×4.6 mm I.D., 5 $\mu$m particle size) from Supelco (Bellefonte, PA, USA). The analytical column was maintained at 32°C by a Model CH-30 Eppendorf column heater (Bedford, MA, USA). The mobile phase consisted of 0.05 *M* potassium phosphate ($K_2HPO_4$) containing 0.125% EDTA, 0.12% acetic acid, 0.2% acetonitrile and 0.3% THF with final pH 5.2, pumped isocratically at 0.8 ml/min and recycled following column equilibration. The column eluent was monitored through a Model 5011 dual porous graphite electrode cell with the potentials of electrodes 1 and 2 set at +0.05 V and −0.030 V, respectively, vs. the proprietary reference electrode. A Model 5020 guard cell set at +0.35 V vs. the proprietary reference electrode was positioned between the solvent delivery pump and the injector to electrochemically condition the mobile phase. The signal from detector electrode 2 was recorded on Recordal strip-chart recorder series 5000 (Fisher Scientific, Springfield, NJ, USA) following an attenuation set at ×3000 and a response time of 4 s. An unattenuated signal was sent to a Model 3203 data acquisition system (Concurrent Computer, Neptune, NJ, USA) where the data were transformed into peak heights and peak areas for the calculation of the standard calibration curves and, finally, to the computation of unknown samples.

### 2.4. Gas chromatography and mass spectrometry

A HP 59872C RTE-A MS data system (Hewlett-Packard, Sunnyvale, CA, USA) was used to control the HP 5988B GC–MS system and to collect and quantitate the data. The gas chromatograph was a

Hewlett-Packard Model 5890A, equipped with a fused-silica capillary column (30 cm×0.25 mm I.D.) with cross-linked DB-17 as stationary phase and an HP 7633A automatic sampler. The temperatures of injector and the interface between the chromatograph and the spectrometer were both set at 280°C. The column was programmed from 80°C (1 min) to 280°C (3 min) at 30°/min. The samples were injected in the splitless mode and the split valve was opened 1 min after injection. The ion-source temperature was 200°C.

### 2.5. Preparation of plasma samples

Whole blood was collected from patients in balanced oxalate tubes containing 0.5% sodium metabisulfite as an antioxidant, and cooled in iced water until centrifuged at 1500 *g* for 15 min within 1 h of collection. The plasma supernatant was removed and stored at −70°C.

### 2.6. Alumina extraction procedure for HPLC

An aliquot of 50 μl of 5 m*M* sodium metabisulfite solution and 4 ng of internal standard, α-methyl DOPA, in 20 μl of 0.1 *M* acetic acid were added to 1.0 ml of distilled water. Sample plasma (1 ml) was pipetted into the water solution followed by addition of 20 mg of alumina and 1 ml of 1 *M* Tris (pH 8.6) buffer solution. The content of the tube was mixed on a rotation shaker for 15 min. After centrifuging at 1500 *g* for 2 min, the supernatant was discarded. The alumina precipitate was washed twice with 1.5 ml of distilled water and the DHPG was eluted from the alumina twice by 250 μl of 0.1 *M* acetic acid. The combined eluents were evaporated without heat to dryness in a vacuum centrifuge. The residue was dissolved in 80 μl of mobile phase and 60 μl was injected.

### 2.7. Extraction and derivatization procedures for GC–MS

An aliquot of 1 ml of plasma sample containing 50 μl of 5 m*M* sodium metabisulfite solution and 2 ng of internal standard, [$^2H_3$]DHPG, in 20 μl of $^2H_2O$ was extracted by alumina using the same procedure as for HPLC. The residue obtained was dissolved in 50 μl of TBDMS/I and the solution was heated at 80°C for 30 min. After cooling to room temperature, the reaction solution was partitioned between 0.5 ml of distilled water and 0.2 ml of cyclohexane. The cyclohexane layer was evaporated and the residue was re-dissolved in 20 μl of cyclohexane. The injection volume was 2 μl.

### 2.8. Quantitation

Determinations of plasma samples were calculated based on the peak-height and peak-area ratios using the internal standard method. The calibration standards (range of 0.5 to 5.0 ng/ml) and quality controls were run with each day's analysis.

## 3. Results and discussion

### 3.1. Extraction recovery from plasma

DHPG had been extracted from plasma with low recovery because of its polar structure. We have evaluated three extraction methods following the previously published procedures, which include ethyl acetate extraction [20], organic solvent extraction following acetylation [4,20] and alumina extraction [15–17]. Of these methods, alumina extraction offered the best recovery with about 25% yield compared with ca. 10% yield for the ethyl acetate extraction and 5% for the acetylation extraction. It has been reported that the low extraction recovery was due to the incomplete desorption of DHPG from alumina [17]. Therefore, factors related to the desorption may be important for obtaining a good recovery. In the previously published alumina extractions [15–17], 1 or 2 ml of plasma was extracted with 20 to ca. 50 mg of alumina and the alumina was washed twice with 2 ml of water followed by elution with 0.2 *M* perchloric acid. We modified the method by eluting the alumina twice instead of once, this increased recovery from ca. 20% to 56%. A recent report [18] has indicated that reducing the amount of alumina from 50 mg to 5 mg can significantly improve the extraction recovery of DHPG from plasma up to 75%. We attempted to repeat this modification without success.

### 3.2. Stability in plasma

DHPG can be very easily oxidized during sample extraction as well as during sample storage. It may decompose even at −20° to ca. −30°C. Antioxidant agents were used during the extraction procedures to minimize this effect. Several stabilizing agents have been reported to be used, such as glutathione [17], ascorbic acid [14,21] and sodium metabisulfite [15]. Since glutathione and ascorbic acid were found to interfere with the HPLC chromatogram in the present study, sodium metabisulfite was used in our work. One milliliter of plasma containing 2 ng of DHPG and 1%, 0.5%, 0.2% or 0% sodium metabisulfite was stored at −70°C for seven days, and another set of the samples was stored at −20°C for the same duration. All samples were extracted and measured identically. The results in Table 1 indicate that the main factor affecting the stability of DHPG is the presence of a stabilizing agent. When 1% or 0.5% sodium metabisulfite was used, the DHPG demonstrated the same stability at −70°C or −20°C. The storage temperature plays an important role only in the absence of the stabilizing agent. Also, DHPG is found to be unstable in working standard solutions. As shown in Table 1, 10% of the DHPG in the working solution was decomposed in seven days when it was stored at −20°C without using any stabilizing agent. Therefore, plasma samples should be stored at −70°C with at least 0.5% stabilizing agent (sodium metabisulfite). The standard working solution should also be stored at −70°C with the stabilizing agent.

Table 1
Recoveries (%)[a] of DHPG after seven days' storage at different temperatures and using different concentrations of stabilizing agent, sodium metabisulfite (SMB)

| $T$ (°C) | Concentration of SMB | | | |
|---|---|---|---|---|
| | 1% | 0.5% | 0.2% | 0% |
| *Plasma solution* | | | | |
| −20 | 98.6 | 94.5 | 87.7 | 29.5 |
| −70 | 100.7 | 93.2 | 82.9 | 84.2 |
| *Standard aqueous solution* | | | | |
| −20 | | | | 91.1 |

[a] The standard solution stored at −70°C and blank plasma were used as relative standard.

### 3.3. Internal standard and GC–MS

The electron impact (EI) mass spectrum of the TBDMS derivative of DHPG is shown in Fig. 1a. The molecular ion ($M^+$), which was calculated to occur at *m/e* 680, was not detected because of the cleavage between the $\alpha$ and $\beta$ side-chain carbon atoms. Therefore, the major fragment at *m/e* 481 from DHPG and that at *m/e* 484 from [$^2H_3$]DHPG were used for the quantitation.

In our initial experiments, the commercially available DHPG deuterium-labeled isomer, 1-(3,4-dihydroxyphenyl)-1,2-ethane-1,2,2-[$^2H_3$]diol, was used as an internal standard. However, using this internal standard, the DHPG-TBDMS derivative did not give a linear calibration curve. The reason for this may be explained by the cleavage of the side-chain bond in the derivatized molecule during GC–MS measurement. Two deuterium atoms were lost because of the cleavage, resulting in the formation of the fragment at *m/e* 482 instead of the expected fragment at *m/e* 484. This fragment has only one deuterium atom so it is very difficult to achieve quantitative separation from that of the DHPG. Clearly, we needed to synthesize an isotope isomer in which the product yielding mass fragments is stable enough during the derivatization and GC–MS measurement.

A deuterium–hydrogen atom exchange reaction occurring on the benzene ring of DHPG was attempted. The exchange under acidic conditions (10% HCl) failed since it was found that the reaction did not occur at temperatures below 120°C while the compound decomposed at higher temperatures (>150°C). A milder condition using a neutral solvent ($^2H_2O$) was, therefore, tried. Sixteen milligrams of DHPG in 3.5 ml of $^2H_2O$ was heated at 120°C for eight days, giving the labeled compound, 3,4-dihydroxyphenyl-2,5,6-$^2H_3$-glycol, with isotopic yield >99% and chemical yield 67%. The compound and its purity were confirmed by GC–MS as shown in Fig. 1b.

Using the standard solutions with a concentration range of 0.5 to 5.0 ng/ml, a linear calibration curve ($r$>0.9990) with an intercept of 0.08 ng was obtained for the plot of the ratio of proton to deuterium ($H/^2H$) fragment peak heights and peak areas vs. the concentrations for DHPG. The minimum detection

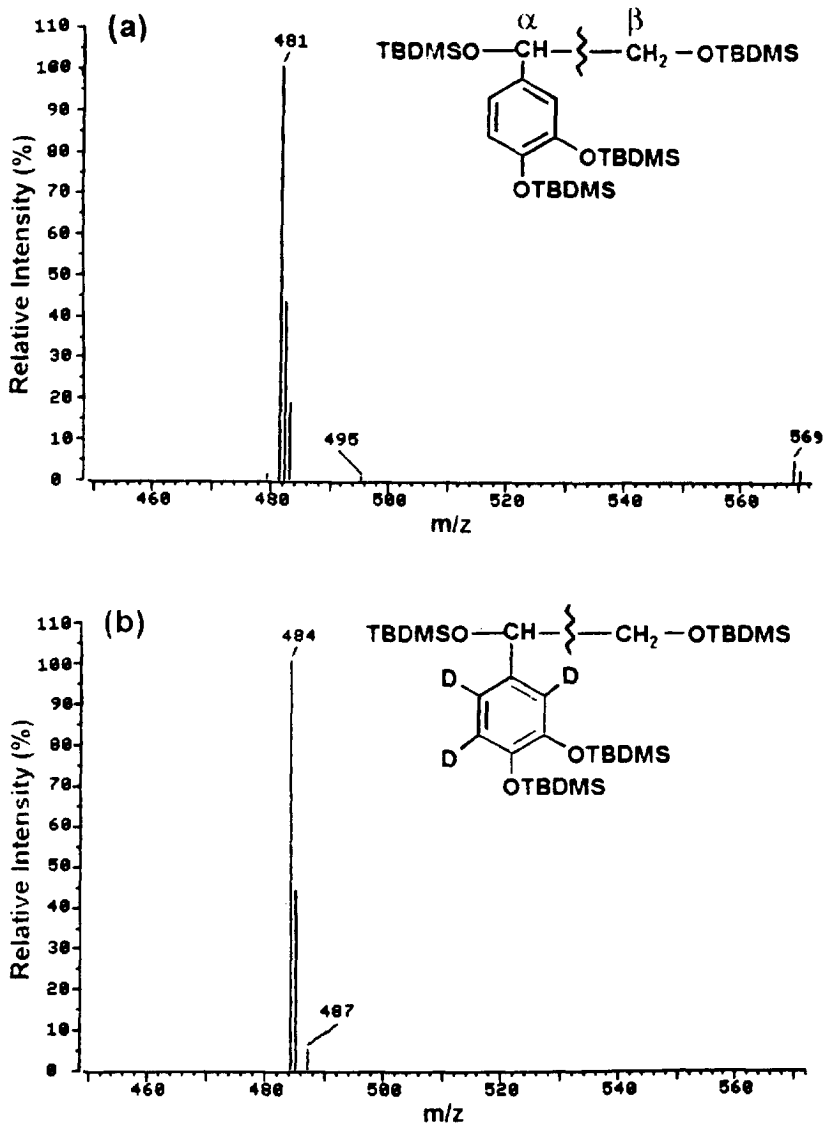


Fig. 1. GC–MS (SIM) for (a) DHPG and (b) synthesized $^{2}H_{3}$-labeled DHPG.

limit was 0.02 ng for the injected derivative from 1 ml of plasma sample. The within-assay precision of this method was determined by using ten replicates of blank plasma containing 0.5, 1.0 and 5.0 ng/ml of DHPG. The coefficients of variation (C.V.s) were 8.1%, 5.3% and 4.4%, respectively. Likewise, the inter-assay precision was determined by testing the plasma containing the three levels of DHPG on three separate days, giving C.V.s of 8.5%, 5.9% and 5.3%, respectively.

### *3.4. HPLC measurements*

Compared with radioenzymatic and GC–MS methods, HPLC is relatively simple and less expensive. However, DHPG, because of its polar characteristics, was very difficult to separate from the solvent front and other early eluting compounds or followed a large negative peak in the chromatogram by the previously published methods [10,15–18]. This requires constant adjustment of the mobile

phase to make quantitation possible. In this paper, we describe improvement in the separation of DHPG through omitting ion-pairing agents that were used in the previous methods. The effect of one of the agents, sodium heptanesulfonate (SHS) was investigated. The presence of SHS significantly prolonged the retention times of each component in plasma with the exception of DHPG. In the presence of 0.01% SHS, a very good separation of DHPG, α-methyl DOPA, NE, E, DA and DOPAC was achieved but DOPA, a major component in plasma was found to overlap with the target compound DHPG. The retention times increased significantly by increasing the concentration of SHS to 0.05%, resulting in a tediously long chromatogram and broad peaks. Using a mobile phase without SHS resulted in a clean chromatogram. The target compound DHPG and internal standard α-methyl DOPA can be adequately separated from the major plasma components and the solvent front peak (Fig. 2).

Using α-methyl DOPA as the internal standard, the standard curve for DHPG was obtained with high linearity ($r$>0.9980) and low intercept (0.041 ng). The detectable limit for the injected sample from 1 ml of plasma was 0.15 ng. The within-assay and inter-assay precisions were determined exactly as for GC–MS method. The C.V.s for within-assay were 8.1%, 8.6% and 4.8%, and those for inter-assay were 9.3%, 9.5% and 6.6%, respectively.

### 3.5. Comparison of the GC–MS and HPLC methods

The agreement of the GC–MS and the HPLC assays was investigated by using the same volumes of the same plasma samples from clinics. Nine tubes containing 0.8 ml of plasma were extracted by alumina together with the respective internal standard, i.e., α-methyl DOPA for HPLC and [$^2H_3$]DHPG for GC–MS. The results found with the two methods are shown in Table 2.

In conclusion, the present study emphasized the crucial necessity of a stabilizing agent for DHPG during sample storage. Also, the modified GC–MS and HPLC assay methods allow the accurate and reliable measurement of plasma DHPG. The GC–MS method can provide a more sensitive and specific

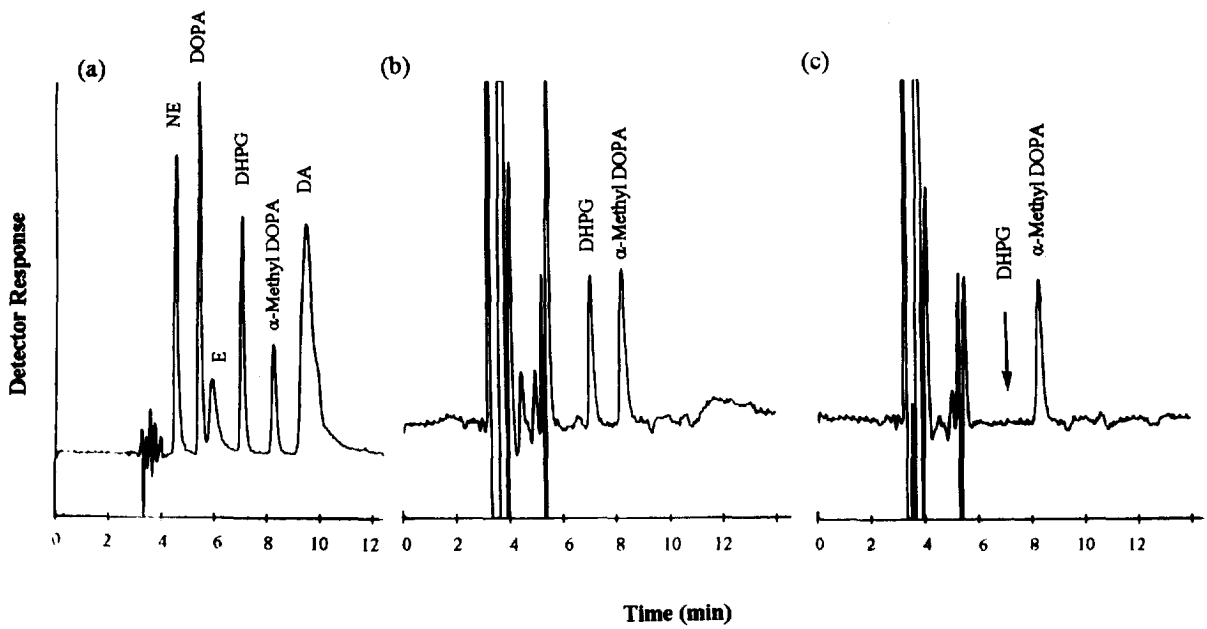


Fig. 2. Chromatograms obtained by HPLC from: (a) a working solution containing DHPG, α-methyl DOPA, NE, DOPA, E and DA in a respective 2 ng concentration; (b) an extract of 1 ml of patient plasma containing 4 ng of internal standard α-methyl DOPA (the concentration of DHPG in the sample is about 1.5 ng/ml); (c) a blank plasma sample with 4 ng of α-methyl DOPA.

Table 2
Replicates for DHPG concentration of patient plasma samples measured by HPLC and GC–MS methods

| HPLC (ng/ml) | GC–MS (ng/ml) |
|---|---|
| 1.16 | 1.20 |
| 1.22 | 1.25 |
| 1.42 | 1.44 |
| 0.97 | 0.98 |
| 1.30 | 1.25 |
| 0.49 | 0.45 |
| 1.00 | 0.93 |
| 1.38 | 1.38 |
| 0.50 | 0.50 |

GC–MS = 1.025·HPLC − 0.031 ($r$=0.995); paired-$t$=0.434 ($p$> 0.05), no significant difference.

measurement for validation, while the HPLC method is simple and relatively inexpensive.

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## References

[1] P.P. Li, J.J. Warsh and D.D. Godse, Prog. Neuropsychopharmacol., 5 (1981) 531.
[2] T.J. Verbeuren and P.M. Vanhoutte, Naunyn-Schmiedeberg's Arch. Pharmacol., 318 (1982) 148.
[3] C. Braestrup, M. Nielsen and J. Scheel-Kruger, J. Neurochem., 23 (1974) 569.
[4] J.J. Warsh, D.D. Godse, S.W. Cheung and P.P. Li, J. Neurochem., 36 (1981) 893.
[5] P.P. Li, J.J. Warsh and D.D. Godse, J. Neurochem., 41 (1983) 1065.
[6] J.J. Warsh, P.P. Li, D.D. Godse and S.W. Cheung, Life Sci., 29 (1981) 1303.
[7] G.A. Smythe, J.E. Bradshaw and R.F. Vining, Endocrinology, 113 (1983) 1062.
[8] J.F. Pardal, A.R. Granata, A. Barrio and A.L. Gimeno, Naunyn-Schmiedeberg's Arch. Pharmacol., 313 (1980) 27.
[9] B. Scatton, Life Sci., 31 (1982) 495.
[10] G. Jackman, J. Snell, H. Skews and A. Bobik, Life Sci., 31 (1982) 923.
[11] J.L. Izzo and D. Greulich, Life Sci., 33 (1983) 483.
[12] C.A. Baker and G.A. Johnson, Life Sci., 29 (1981) 165.
[13] N.D. Vlachakis, E. Kogosov, S. Yoneda, N. Alexander and R.F. Maronde, Clin. Chim. Acta, 137 (1984) 199.
[14] D.C. Jimerson, S.P. Markey, J.A. Oliver and I.J. Kopin, Biomed. Mass Spectrom., 8 (1981) 256.
[15] L.G. Howes, S. Miller and J.L. Reid, J. Chromatogr., 338 (1985) 401.
[16] G. Eisenhofer, D.S. Goldstein, R. Stull, H.R. Keiser, T. Sunderland, D.L. Murphy and I.J. Kopin, Clin. Chem., 32 (1986) 2030.
[17] B.-M. Eriksson and B.-A. Persson, J. Chromatogr., 386 (1987) 1.
[18] C. Holmes, G. Eisenhofer and D.S. Goldstein, J. Chromatogr. B, 653 (1994) 131.
[19] B.-M. Eriksson and B.-A. Persson, J. Chromatogr., 228 (1982) 143.
[20] S. Takahashi, D.D. Godse, J.J. Warsh and H.C. Stancer, Clin. Chim. Acta, 81 (1977) 183.
[21] D. Hugh, A. Grennan, M.A. Abugila and C. Weinkove, Clin. Chem., 33 (1987) 569.