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Journal of Chromatography B, 802 (2004) 247–255
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Systematic study of long-term stability of 3,4-dihydroxyphenylglycol in plasma for subsequent determination with liquid chromatography

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Received 18 June 2002; received in revised form 5 August 2003; accepted 11 August 2003

Abstract

The effect of three storage temperature levels (i.e. +4, −20 and −80 °C) and time intervals from sampling (3, 6 and 9 months) on the degradation of 3,4-dihydroxyphenylglycol (DHPG) and norepinephrine (NE) was investigated in a systematic study. Extracted human plasma samples and acidified standard solutions were stored for long periods (up to 9 months) without the addition of any stabilizing agent. DHPG and NE values, determined using a ion-pair reversed-phase high-performance liquid chromatography method with electrochemical detection of coulometric type (IP-RP-HPLC–CD), remained constant over time in those plasma samples and standard solutions that had been stored at the lowest storing temperature (i.e. −80 °C). The expected degradation was observed at higher temperature levels. Plasma and standard DHPG degradation can, therefore, be prevented by storing samples at a lower temperature than previously suggested with no need to add any stabilizing agent.
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Keywords: Long-term stability; 3,4-Dihydroxyphenylglycol

1. Introduction

The 3,4-dihydroxyphenylglycol (DHPG) is the major free metabolite of the sympathetic neurotransmitter norepinephrine (NE). This metabolite is deaminated, therefore, it is more polar and consequently more soluble than NE in some biological districts (e.g. in the cellular membrane) [1,2]. DHPG is mainly derived from the NE metabolized intraneuronally after its leakage into the axoplasm from storage vesicles. There is also a small quantity of DHPG that originates from the intraneuronal metabolism of recaptured NE (uptake-1) [3,4].

After its re-uptake into the noradrenergic nerve endings, the NE is deaminated by a monoamine oxidase (MAO). The resulting intermediate aldehyde is reduced into DHPG by aldehyde/aldose reductase [2,5]. The intraneuronal metabolism of NE by MAO is the most important metabolic pathway determining the turnover of this neurotransmitter [6].

Plasma levels of DHPG reflect the combined effects of three factors of cytoplasmic NE, namely neuronal uptake, incorporation of NE into storage vesicles and MAO activity. Determining the plasma concentration of DHPG is, therefore, useful to evaluate the level of intraneuronal NE and to assess the efficacy of the neuronal uptake process in terminating the action of NE after its release into the synapse [1,3,4,7]. The measurement of the total production or excretion of NE and its metabolite DHPG, while providing a good measure of neurotransmitter turnover, does not assess exocytotic release of this neurotransmitter. Different studies have shown that the concentration of NE in venous plasma is a more sensitive indicator of α_2-adrenoceptor mediated regulation of NE release than DHPG [8–12]. Some studies have, however, suggested that plasma concentrations of DHPG may be used to monitor the effects of MAO inhibitors [13]. Other studies have suggested that plasma levels of NE and DHPG may provide clinicians with useful information about NE metabolism during antidepressant drug treatment [14].

Several authors have highlighted the importance of simultaneous assay of the plasmatic level of NE and its deaminated

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1570-0232/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2003.08.010

metabolite, DHPG, in the clinical diagnosis of phaeochromocytoma. This condition is a rare form of tumor but its early diagnosis is of considerable importance, since surgical resection is usually followed by a complete return to health. The early diagnosis of phaeochromocytoma by catecholamines assay or by assay of their metabolites is very important, since it brings the prospect of permanent cure to a small but significant proportion of the population who suffers from hypertension [15–17].

The measurement of the plasmatic NE/DHPG ratio is a simple method, which allows one to rule out the presence of phaeochromocytoma in patients with borderline increment of plasmatic concentration of NE [16]. This ratio, however, should be used with caution in the diagnostic evaluation of patients with suspected phaeochromocytoma since the value of this ratio may remain within the normal range in patients suffering from a form of phaeochromocytoma that produces epinephrine (E) [17].

There are several techniques which have been developed for the quantification of catecholamines in plasma, e.g. radioenzymatic assay (RIA) [18], enzyme-linked immunosorbent assay (ELISA) [19], gas chromatography with mass spectrometry (GC–MS) [20] and high-performance liquid chromatography (HPLC) [8,21–23]. The HPLC with electrochemical detection is currently a widely used technique for the quantitation of plasmatic levels of free catecholamines and their metabolites, since this is a more sensitive, cost effective and fast technique [1,9,11,12,22–30]. The development of this sensitive and specific method for the analysis of catecholamines has led to an increased use and reliance on such measurements in the biochemical diagnosis and differentiation of different types of phaeochromocytoma [31].

Only a few methods have been developed for simultaneous measurements of free plasma NE and DHPG levels, largely due to the instability of the DHPG during extraction and storage of plasma samples. This analyte, similarly to catecholamines, does oxidize very easily in alkaline solutions [8,16,20]. The main difficulties encountered in the assay of DHPG using a reversed-phase HPLC method are due to the fact that DHPG elutes very early, extremely close to a large negative peak or to the solvent front and also close to other early eluting compounds. These difficulties in the assay phase, coupled with the instability of DHPG during sample storage, represent a major analytical problem in the quantitation of the plasmatic levels of DHPG.

A recent study looked at the effect of storage at two different temperature levels, −20 and −70 °C, on DHPG stability and contrasted the results with those achieved by addition of a stabilizing agent. This study demonstrated that the use of a stabilizing agent improved DHPG stability and prevented its degradation over a short time period (i.e. 7 days) to a higher degree than that achieved by sample storage at a low temperature (−70 °C) [20]. Xie et al., therefore, recommended the storage of plasma samples at a temperature of −70 °C with the addition of a small quantity of a stabilizing agent. This study offered some reassurances as to the quality of a method of ion-pair reversed-phase (IP-RP) HPLC with electrochemical detection for the quantitative analysis of free plasma DHPG and its reliability for immediate application in the clinical field.

This study, however, did not deal with the evaluation of DHPG stability over a storage period of several months and did not evaluate whether a further lowering of the storage temperature may prevent oxidation of DHPG avoiding, therefore, the need to add a stabilizing agent.

The present study focused on a systematic evaluation of the stability of free DHPG over a long storage period (i.e. 9 months) at three different temperature levels (+4, −20 and −80 °C) without the addition of a stabilizing agent in extracted samples of human plasma and in acidified standard solutions. A temperature level of −80 °C was chosen as this is the temperature level most often used for long-term preservation of biological samples [32]. As a control condition and to have a stability indicator parameter of the assay [33], the stability of extracted aqueous standard and plasma DHPG preserved for 24 h at a very high temperature (+28 °C) was also assessed.

2. Experimental

2.1. HPLC apparatus and chromatographic conditions

The HPLC system consisted of a model 305 piston pump/10 WTI equipped with a model 806 pulse dampener (Gilson, Italy), a 231 autosampler injector—401 dilutor equipped with rheodyne 7010 valve and a 50 μl loop (Gilson, Italy), a 0.5 μm high-pressure biocompatible inline filter (Gilson, Italy) and a Chromcart Nucleosil 100-C_{18} ion-pair reversed-phase column (125 mm × 4 mm i.d., particle size 5 μm) (Macherey-Nagel/Alfatech, Italy). The system was connected to an ESA 0.22 μm graphite-inline filter and a model 5200A Coulochem II multi-electrode detector equipped with a model 5021 conditioning cell and a model 5011 high sensitivity analytical cell (ESA/Alfatech, Italy). The potential of the electrode of the conditioning cell was set at +400 mV and the potentials of electrodes 1 and 2 of the analytical cell were set at +100 mV (filter 2 s, output 100 mV, offset 0%, R_1: 100 μA) and at −350 mV (filter 10 s, output 100 mV, offset 0%, R_2: 20 nA), respectively. The signal from working electrode 2 was recorded with the 715 HPLC system software, equipped with a model 506B interface (100 mV) (Gilson, Italy) and an Epson LX400 printer (speedchart 10 mm/min), operating in peak-height mode. The CAT-A-PHASE (ESA/Alfatech, Italy) was used as mobile phase, consisting of a phosphate buffer containing methanol and a ion-pairing agent with a final pH of 2.5. This mobile phase was pumped isocratically at 1 ml/min and recycled after equilibration of the column.

2.2. Chemicals and reagents

NE/arterenol (free base), DHPG crystalline, 3,4-dihydroxybenzylamine-hydrobromide (DHBA), Tris (trizma base), ethylene-diamine-tetra-acetic-acid (EDTA, disodium salt), alumina (type WA-4: acid), glutathione free-acid crystalline (reduced form) and ethylene-glycol-tetra-acetic-acid (EGTA) were purchased from Sigma–Aldrich, Italy. Perchloric acid 60% was from Merck (Incofar, Italy). The reagents, HCl 2N and sodium hydroxide 1N, were of RPE grade and were obtained from Antibioticos (Carlo Erba, Italy) as was the water plus for HPLC. Other reagents (glacial acetic acid) and solvents (methanol) of HPLC grade were purchased from Baker (Incofar, Italy).

2.3. Standard preparation

2.3.1. Primary standard (DHPG and NE)

The primary standard, containing 2 mg/ml of DHPG and 1 mg/ml of NE, were prepared dissolving 100 mg of DHPG and 50 mg of NE together in a 50 ml solution of 80% methanol and 20% 0.1 M perchloric acid and stored, at −80 °C, into a dark glass bottle without the addition of any stabilizing agent. This solution was used for subsequent preparation of a calibration standard curve.

2.3.2. Calibration standard curve

The calibration standard curve included four concentration levels for each analyte: 250, 500, 1000 and 1500 pg/ml for DHPG and 62.5, 125, 250 and 500 pg/ml for NE. These concentration levels were chosen because they reflect those of clinical interest or significance. This standard curve were produced daily to determine the linearity of the peak-height response. From the above primary standard, a working solution containing 2 ng/ml of DHPG and 1 ng/ml of NE was obtained by dilution 1:1,000,000 with water for HPLC. From this solution, subsequent 1:2 dilutions were performed to obtain the four working concentrations. These working solutions were prepared prior to every plasma sample analysis and extracted immediately, together with the plasma samples. One milliliter of HPLC grade water was extracted as level zero to verify the absence of any contaminating substance.

2.3.3. Primary internal standard and working solution

The primary internal standard (IS), containing 1 mg/ml of DHBA, was prepared by dissolving 50 mg of DHBA into 50 ml solution of 80% methanol and 20% 0.1 M perchloric acid and stored, at +4 °C, into a dark glass bottle without the addition of any stabilizing agent.

The working solution, containing 5 ng/ml of DHBA (IS), was prepared prior to every analysis by diluting the primary internal standard 1:100,000 with water for HPLC and by further diluting 1:2 the resulting solution.

2.4. Human plasma sample preparation

Plasma, for this study, was obtained from a healthy 35-year-old male volunteer. The day before giving this venous blood sample he did not eat any food that could alter the level of secretion of these analytes (e.g. bananas, vanilla, coffee, tea). He was also required to fast overnight. An antecubital venous catheter was inserted in his arm and 10 blood samples (12 ml each) were taken after 30 min of supine rest. The blood was directly transferred to pre-chilled polypropylene tubes containing a 200 μl solution 9.5% EGTA–6% reduced glutathione with final pH 6.8 and were placed in ice immediately after collection. The blood samples were then centrifuged at 2000 rpm for 5 min and a plasma pool obtained.

2.5. WA-4 alumina extraction procedure

On the day of analysis, the plasma samples were removed from the freezer and were thawed quickly into bain-marie at 37 °C, then centrifuged at 2000 rpm for 5 min. Twenty-five milligrams of WA-4 alumina, 1 ml of 1 M Tris (pH 8.6) buffer solution, 1 ml of plasma, or standard solution, and 50 μl of 5 ng/ml DHBA (IS) were dispensed into each polypropylene test-tube. All test-tubes were plugged and their content was mixed in a rotation shaker for 10 min, then centrifuged at 3500 rpm for 5 min. After centrifugation, the plugs were removed from all test-tubes and the supernatant was discarded. The alumina precipitate was washed with 2 ml of HPLC grade water, then vortex-mixed for 10 s and centrifuged at 3500 rpm for 5 min. This operation was repeated twice. After centrifugation, the supernatant was discarded and the compounds were eluted with 200 μl of mobile phase (pH 2.5). The content of all test-tubes was, then, vortex-mixed vigorously for 15 s and centrifuged at 3500 rpm for 5 min. A 50 μl aliquot of the resulting eluate was injected into the HPLC system.

2.6. Quantitation

Determinations of the extracted plasma samples were computed based on the peak-height ratio using the internal standard method. Calibration standard curve and quality controls were ran every day of analysis. The chosen calibration model described adequately the linear relationship between response function (peak-height) and concentration of calibration levels for both analytes. Correlation coefficients equal to 1 were found for both the DHPG and the NE.

2.7. Stability in standard solutions and plasma samples

Ten primary standards, containing 2 mg/ml of DHPG and 1 mg/ml of NE, were prepared as previously described and each one was subdivided in four separate aliquots. Three of these aliquots were stored for 9 months at three different temperature levels, i.e. +4, −20 and −80 °C, without

the addition of any stabilizing agent. A working solution, containing 2 ng/ml of DHPG and 1 ng/ml of NE, was then obtained from the remaining aliquot by dilution with mobile phase (pH 2.5). This working solution was injected immediately into the HPLC–CD system and was detected at a sensitivity level of 10 nA. The working solution was injected three times on the same day (morning, afternoon and evening). This multiple injection procedure provided mean height baseline values for each analyte, and was adopted to protect against chromatographic errors due to unforeseeable problems that could affect reliability during HPLC injection. The three aliquots which had been stored at three different temperature levels were then analyzed with a similar procedure after 3, 6 and 9 months.

Six plasma pool aliquots were extracted and injected immediately into the HPLC–CD system, together with a calibration standard curve and one known plasma sample acting as quality control, and detected at a sensitivity level of 10 nA. The DHPG and NE concentrations obtained from this quantitation were considered as baseline concentrations for subsequent long-term quantitations. The remaining plasma aliquots were immediately transferred into polypropylene cryotubes, without addition of any stabilizing agent, and stored at three different temperature levels, i.e. +4, −20 and −80 °C (18 aliquots for each temperature level) for subsequent long-term analysis with HPLC–CD after 3, 6 and 9 months.

As a control condition and as stability indicator parameter, the stability of the analytes during the assay by HPLC–CD was determined for extracted unpreserved standard and plasma samples stored for 24 h at a temperature level of + 28 °C and compared with the values achieved in the time 0 assays. The percentage of degradation between the two assays was calculated. The stability of DHPG in the extracted standard was assessed at only one concentration level. The lowest concentration level (250 pg/ml of DHPG and 125 pg/ml of NE) of the calibration curve was chosen. Six aqueous standards and six plasma aliquots were extracted and analyzed by HPLC–CD. The chromatograms were obtained with a detector sensitivity level set at 20 nA.

3. Results

3.1. Stability in standard solutions

An analysis of variance with repeated measures was performed on the mean peak-height values obtained from the injections performed at time 0 and those values obtained after 3, 6 and 9 months, at each temperature level.

The analysis on the data obtained from the acidified standard solutions stored at +4 °C showed a significant decrement in DHPG values over time ($F_{(3,27)} = 13490$, $P < 0.0001$). A similar significant decrement in DHPG values over time was also present in the data obtained from the acidified standard solutions stored at −20 °C ($F_{(3,27)} = 134$, $P < 0.0001$). No significant decrement in DHPG values over time was observed in the data obtained from the acidified standard solutions stored at −80 °C ($F_{(3,27)} = 1.309$, n.s.). Table 1 shows the DHPG peak-height values over time at the three different temperature levels.

Table 1
Stability over time of DHPG in unpreserved acidified standard solutions stored at three different temperatures

Temperature (°C)	Time 0	3 months	6 months	9 months
+4	7440 (234)	843 (26)	243 (19)	0 (0)
−20	7440 (234)	6562 (167)	6139 (132)	6312 (118)
−80	7440 (234)	7325 (254)	7379 (155)	7433 (99)

Data are means (±S.D.) of peak-height (mV) obtained from 10 standard solutions.

A further series of analysis of variance was performed on the percentage of degradation of the second and third injection compared to the first injection performed at time 0 and the percentage of degradation observed in the injections performed after 3 and 9 month interval at each temperature level.

The analysis on the data obtained from the solutions stored at +4 °C showed a significant decrease of DHPG peak-height over time ($F_{(1,9)} = 6782.556$, $P < 0.0001$) and a significant effect of time of injection ($F_{(1,9)} = 1133.571$, $P < 0.0001$). In detail, DHPG peak-height was reduced of 89% (±0.53) after 3 months at +4 °C and of 97% (±0.31) after 6 months. After 9 months, 100% degradation of DHPG was observed. A significant interaction was also present between time of injection and degree of decrease of DHPG peak-height ($F_{(1,9)} = 828.907$, $P < 0.0001$).

The analysis on the data obtained from the acidified standard solutions stored at −20 °C showed a similar, although less dramatic, pattern of degradation. There was a significant decrease of DHPG peak-height over time ($F_{(1,9)} = 88.315$, $P < 0.0001$) and a significant effect of time of injection ($F_{(1,9)} = 8.228$, $P < 0.02$). DHPG peak-height was reduced of 12% (±3.45) after 3 months at −20 °C, of 13% (±2.50) after 6 months and of 15% (±2.68) after 9 months. A significant interaction was also present between time of injection and degree of decrease of DHPG peak-height ($F_{(1,9)} = 6.927$, $P < 0.03$).

The analysis on the data obtained from the acidified standard solutions stored at −80 °C showed a completely different pattern. There was no significant decrement of DHPG peak-height over time ($F_{(1,9)} = 1.547$, n.s.) and no significant effect of time of injection ($F_{(1,9)} = 0.446$, n.s.). No significant interaction was observed between time of injection and degree of decrement of DHPG peak-height ($F_{(1,9)} = 1.914$, n.s.).

Fig. 1 shows the comparison of peaks in a chromatogram obtained from an acidified standard solution over time. Fig. 1a shows that the degradation of DHPG peak, which was greater in standards stored at +4 °C, was associated with the appearance of unknown peak, which had a different

retention time from that of the analytes. This unknown peak was absent in the chromatograms obtained from standards stored at −80 °C (Fig. 1c) for 9 months. Fig. 1a–c also shows a great stability of the non-extracted NE across all different temperature levels studied.

The analysis of variance with repeated measures performed on the data obtained from the extracted unpreserved aqueous standards stored at +28 °C for 24 h showed a significant decrement in DHPG values over time ($F_{(1,5)} = 9.956$, $P = 0.0252$) as well as a major significant decrement in NE values ($F_{(1,5)} = 587.216$, $P < 0.000002$) (see Table 2).

3.2. Stability in plasma samples

An analysis of variance with repeated measures was carried out on the mean concentration values obtained from the

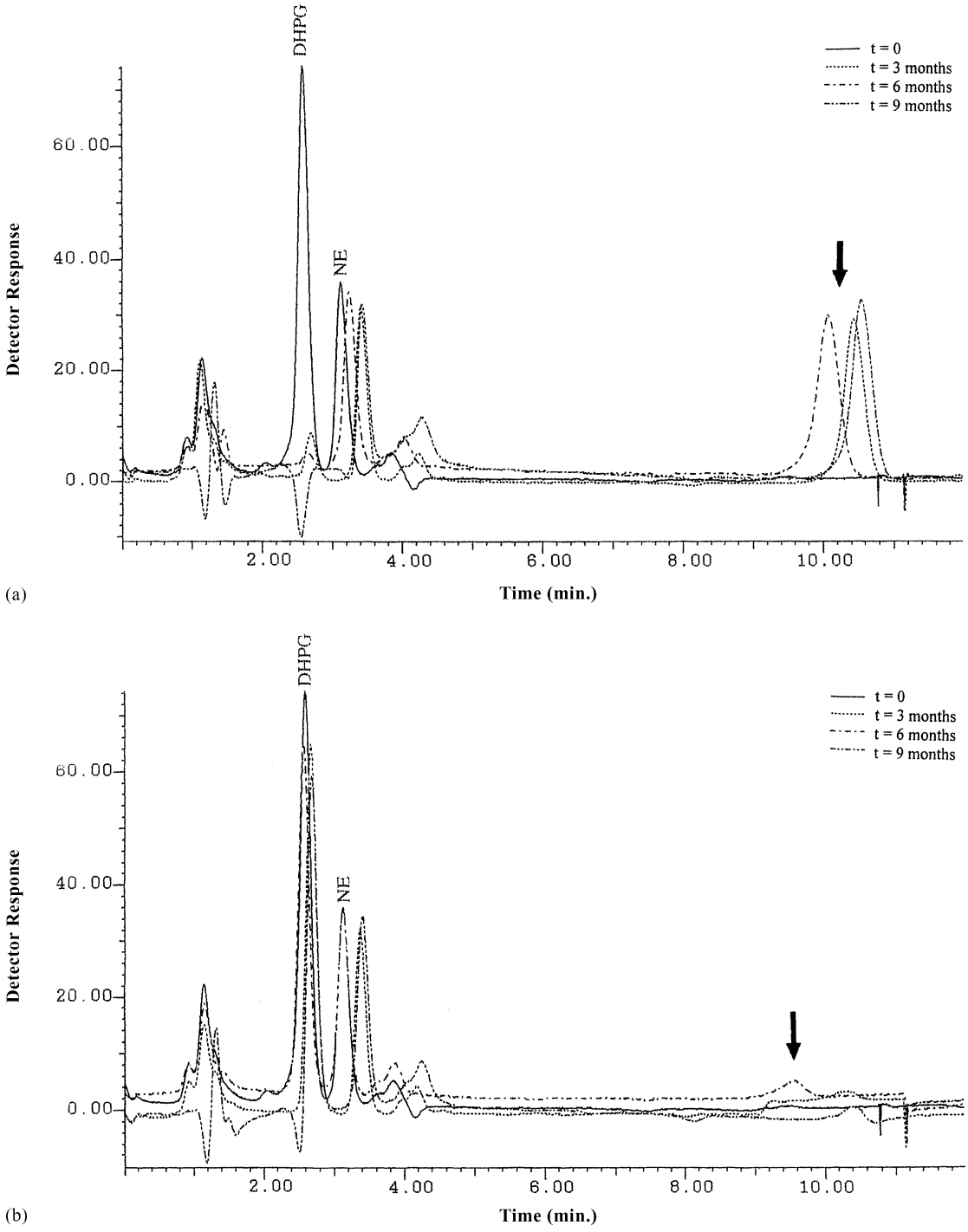

Fig. 1. Comparison between chromatograms obtained from an acidified standard solution, containing DHPG 2 ng/ml and NE 1 ng/ml, stored at +4 °C (a), −20 °C (b) and −80 °C (c) for 9 months. The arrow flags an unidentified peak present at +4 °C (a) and −20 °C (b), temperature levels at which there is more DHPG degradation. These peaks were detected with a sensitivity level set at 10 nA.

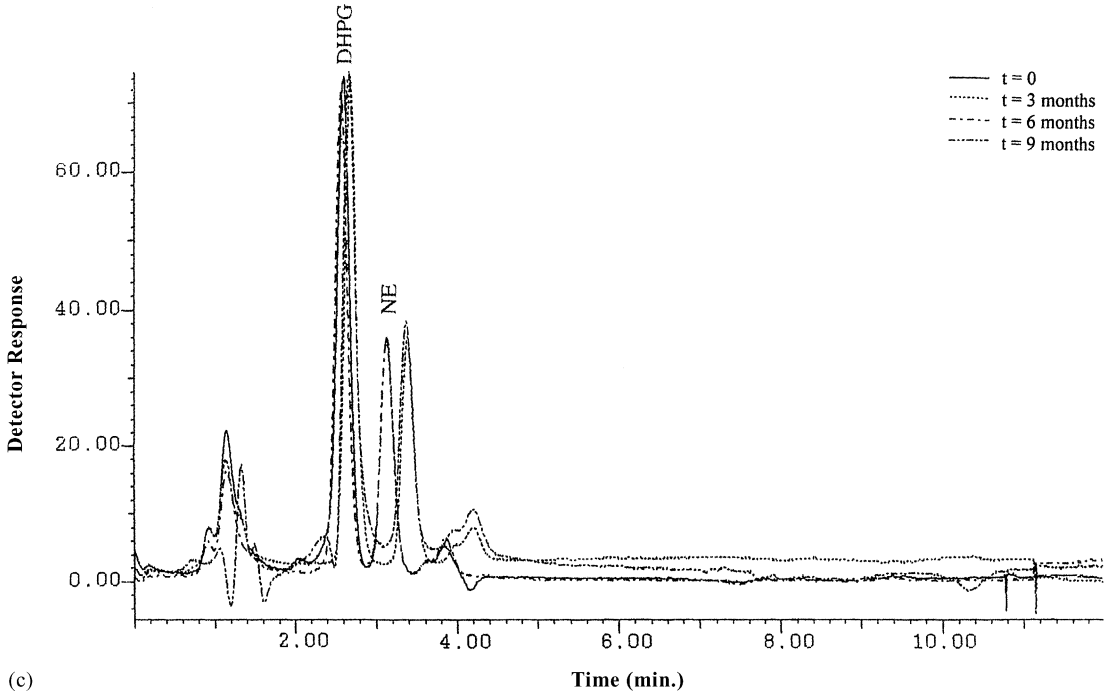

Fig. 1. (*Continued*).

Table 2
Stability of DHPG and NE stored in extracted unpreserved standard solutions (DHPG 250 pg/ml–NE 125 pg/ml) and plasma samples for 24 h at +28 °C

Analyte	Standard solutions ($n = 6$)	Plasma samples ($n = 6$)
DHPG (%)	2 (2.12)	6 (3.83)
NE (%)	59 (5.05)	22 (3.33)

Data are mean (±S.D.) percentages of peak-height degradation.

injections performed at time 0 and those values obtained after 3, 6 and 9 months, at each temperature level.

The analysis on the data obtained from the plasma samples stored at +4 °C showed a significant decrement in DHPG concentration over time ($F_{(3,15)} = 721.426$, $P < 0.0001$). A similar significant decrement in DHPG concentration over time was also present in the data obtained from the plasma samples stored at −20 °C ($F_{(3,15)} = 5.262$, $P < 0.02$). No significant decrement in DHPG concentration over time was observed in the data obtained from the plasma samples stored at −80 °C ($F_{(3,15)} = 0.646$, n.s.). Table 3 show DHPG quantitation in plasma samples over time.

Table 3
Stability over time of DHPG stored in unpreserved plasma samples at three different temperatures

Temperature (°C)	Time 0	3 months	6 months	9 months
+4	639 (10.8)	276 (7.9)	269 (17.2)	259 (24.4)
−20	639 (10.8)	604 (24.7)	612 (19.8)	629 (3.4)
−80	639 (10.8)	634 (12.6)	632 (10.5)	631 (9.2)

Data are mean concentrations (±S.D.) obtained from six replications at each temperature level.

The comparison between DHPG peak-heights obtained at time 0 and over time in a plasma sample stored at −80 °C and at +4 °C is shown in Fig. 2.

The analysis of variance with repeated measures performed on the data obtained from the extracted unpreserved plasma samples stored at +28 °C for 24 h showed a significant decrement in DHPG values over time ($F_{(1,5)} = 16.805$, $P = 0.0093$) and a major significant decrement in NE values ($F_{(1,5)} = 245.157$, $P < 0.000019$). These data are summarized in Table 2.

4. Discussion

This study has demonstrated that it is possible to prevent DHPG degradation in both standard solutions and plasma samples without the addition of any stabilizing agent by storing the material at a lower temperature level than that previously used.

A few studies have developed methods of simultaneous analysis of DHPG and NE using HPLC–CD. While NE concentration is not altered by the temperature conditions at storage, DHPG concentrations are greatly unstable and their values are affected by temperature conditions. DHPG concentrations may, however, also be affected by the temperature conditions during the extraction procedure [8,16,20]. These difficulties during manipulation and/or storage of samples and standards represent a major problem in the quantitation of this analyte. To overcome these stability problems and to prevent the oxidation of DHPG during manipulation or storage, Xie et al. [20] suggested storage of

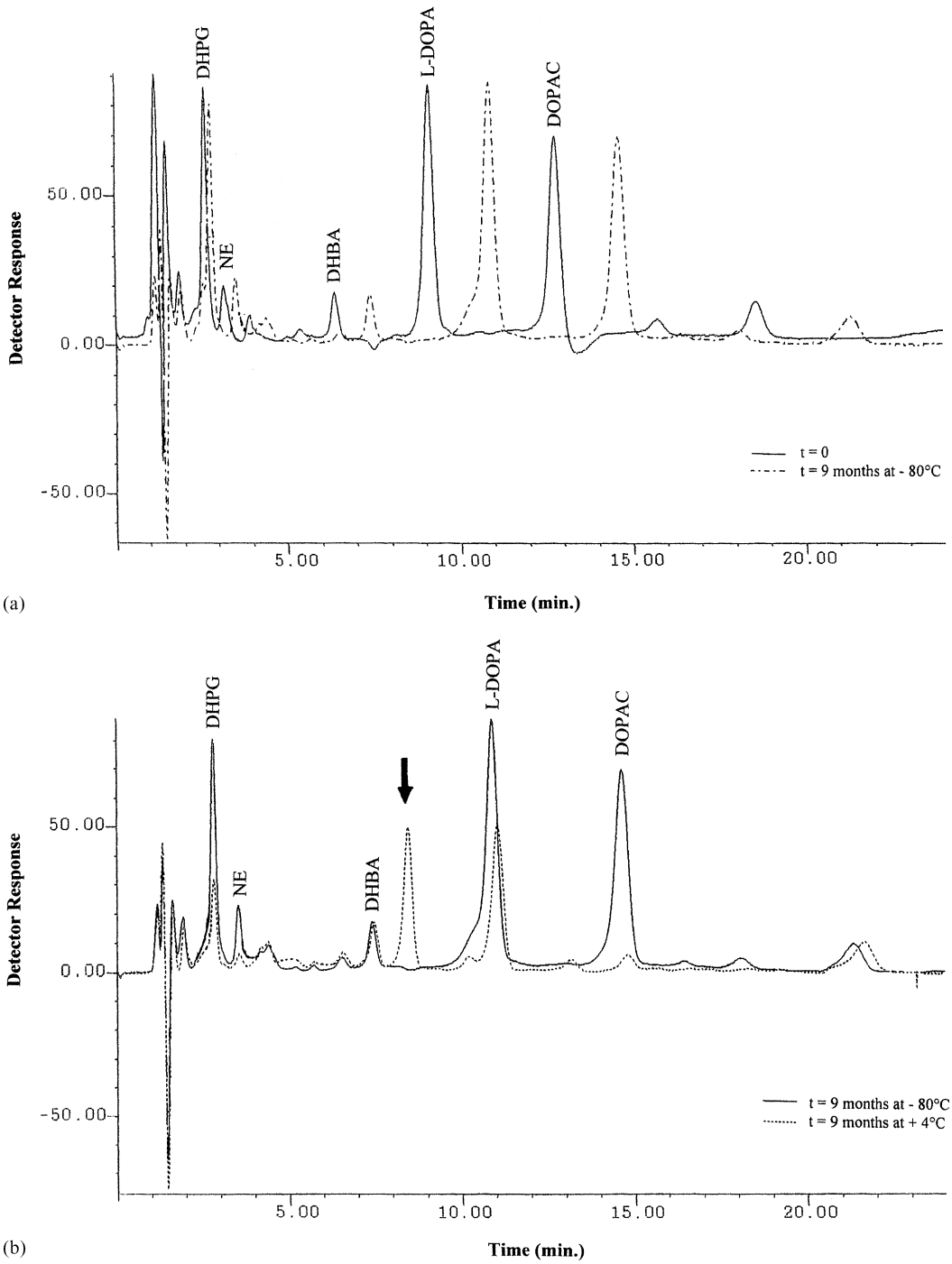

Fig. 2. Comparison between chromatograms obtained, with a detector sensitivity level set at 10 nA, from extracted plasma samples at (a) time 0 and after 9 months of storage at −80 °C and (b) after 9 months of storage at +4 °C and −80 °C. The arrow flags an unidentified peak present at +4 °C (b), temperature level at which there is more DHPG degradation.

samples at a temperature level of −70 °C and with the addition of a small quantity of a stabilizing agent. By following this procedure, these authors claimed that they had achieved stability of DHPG concentrations over a storage period of 7 days. Some stabilizing agents may, however, interfere with the HPLC chromatogram, therefore, whenever possible, their use should be limited. Furthermore, the addition of stabilizing agents or high levels of storage temperature increase deconjugation of catecholamine and metanephrine sulfoconjugates in biological samples, altering, therefore, clinical results [32]. There are no stability problems for the different catecholamines and, even in this study, NE concentration values remained constant in both standard solutions and plasma samples at all three temperature levels. In this study, DHPG stability was achieved without using a stabilizing agent simply by lowering the storage temperature

to −80 °C. These results are in agreement with the findings of Chan et al. who showed that the preservation of biological samples and standards at a very low temperature level prevents degradation of free catecholamines and their metabolites such as metanephrine for at least 3 weeks [32]. The procedure of this study permitted the storage of standard solutions (see Table 1 and Fig. 1c) and plasma samples (see Table 3 and Fig. 2a) without degradation of DHPG for a very long period of time, i.e. 9 months. The possibility of storing material for such a long period of time without degradation of analytes may prove useful when DHPG assay is necessary for research purpose.

This systematic study of the stability of standard and plasma DHPG provides further evidence that the temperature level is a determining factor in sample storage for long-term preservation of free catecholamines and their free metabolites in extracted plasma samples (Fig. 2).

One further interesting finding of this study is the observation that both standard and plasma DHPG degrade at high temperature levels (i.e. +4 °C) over time. This degradation is associated with the appearance of an unknown peak, that eluates later compared with the DHPG and the NE, whose peak-height grows proportionally to the progressive reduction of the DHPG peak-height (Figs. 1a and 2b). Since this unknown peak is also observed in chromatograms of non-extracted standards it might be suggested that this represents a product of DHPG degradation [33].

Another analytical problem that needs to be addressed when performing DHPG assay is its stability during the extraction procedure and whether stability is affected by high temperature such as +28 °C in extracted aqueous standard solutions and plasma samples. In this study, we controlled for this factor by looking at the percentage of recovery of the extraction method and the precision and accuracy of the quantitation procedure. The percentage of recovery of DHPG, from both standard solutions and plasma samples, was equal to 74 ± 3.85% [34]. This value is comparable to those achieved by other authors (71% [13]; 75% [23]; 74% [2]) and in some instances higher (54–56% [8,20]). Intra-assay DHPG precision, expressed as percentage coefficient of variation, was 1.70, whereas inter-assay precision was 1.46; DHPG accuracy, expressed as percentage bias, was −2.48. These values are within the acceptable range of measures that permits one to consider a bioanalytical method valid [35]. We feel confident, therefore, that the results of our systematic study of long-term stability of DHPG are a reliable measure of real stability rather than an artifact of a method with low degree of validity.

As for whether high temperature affects stability, the instability of both standard and plasma DHPG in alumina eluate expressed by the percentage of degradation observed at a temperature level of +28 °C supports previous evidence that high temperature levels are a detrimental factor for the stability of other catecholamine metabolites such as metanephrine [32] or dihydroxyphenylacetic acid (DOPAC) [23]. The present study has also highlighted that high temperature levels are even more detrimental for the extracted NE than for DHPG stability (see Table 2).

Acknowledgements

The authors thank Professor Annalena Venneri, University of Hull, UK, for her advice on data analysis and amendment of the English.

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