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Short communication

2,5-Dimethyl-1H-pyrrole-3,4-dicarbaldehyde as a precolumn derivatization reagent for HPLC/UV detection of amino acids

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ABSTRACT

The use of 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde as a precolumn derivatization reagent for HPLC analysis of amino acids is proposed. The compound reacts under mild conditions (10 min at ambient temperature) with primary amino groups. The derivatization conditions to obtain quantitative reaction were optimised by considering different parameters (temperature, pH and reagent concentration) using L-Val as the model compound. The synthesized L-Val derivative was characterized by ^{1}H NMR and UV. The derivatives of 19 amino acids were separated by reversed-phase HPLC and detected at λ = 320 nm. The method was applied successfully to the qualitative and quantitative analysis of commercial polyamino acid preparations.

1. Introduction

Amino acids are very important in nutrition, owing to their central role in biochemistry. Dietary supplements have become increasingly popular and convenient sources of free amino acids.

The direct UV or fluorescence detection of these compounds in the chromatographic analysis of amino acids is difficult due to the absence of a strong chromophore and fluorophore. Specific detection approaches have been applied such as amperometric/electrochemical, evaporative light scattering, chemiluminescent nitrogen and mass spectrometry detectors [1–3], but these require expensive instruments, not easily available in common analytical laboratories. The combination of chromatographic separation and pre- or postcolumn derivatization followed by UV or fluorescence detection remains the most convenient and widespread analytical approach to provide improved sensitivity. In particular, precolumn derivatization offers the advantage to increase the hydrophobicity of the amino acids so that they can be retained on the reversed-phase columns. Commercially available derivatization UV and fluorogenic reagents commonly used for precolumn derivatization of amino acids are phenyl isothiocyanate (PITC), 4-nitrobenzoyl chloride, *p*-nitrobenzyl bromide, *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), dansyl chloride (Dns-Cl), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and naphthalene-2,3-dicarboxaldehyde [4–7]. However, the use of these reagents can involve different drawbacks such as limited selectivity and sensitivity, low stability of the derivatives, time-consuming derivatization procedure or need of extraction procedures before the analysis. OPA has the merit to react rapidly under mild conditions with primary amino function, but generally it is used as postcolumn derivatization reagent owing to the instability of its derivatives.

Recently, we have proposed 2,7-dimethyl-3,8-dinitrodipyrazolo[1,5-a:1′,5′-d]pyrazine-4,9-dione (DDPP) and 4,7-phenanthroline-5,6-dione (phanquinone) as new precolumn derivatization reagents for HPLC analysis of amino acids using UV or fluorescence detection [8–11]. Both compounds have been proven to be selective towards amino function giving stable derivatives useful for quality control of commercial formulations. The high sensitivity of the phanquinone method allowed also its application to biological samples. In order to develop further useful reagents, we have focused on the use of 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde (DPD) [12]. Owing to its structural analogy with OPA, it has the intrinsic potentiality to react fast under mild conditions with the amino function, but as far as we know it was not previously studied as an analytical reagent. Therefore, the aim of the present work is to propose DPD as a new derivatizing reagent for HPLC analysis of amino acids.

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2. Experimental

2.1. Materials and solutions

Amino acids: Gly, L-Ile, L-Leu, L-Phe, L-Thr, L-Trp, L-Val, L-Tyr, L-Ala, L-Arg, L-Ser, L-Asn, L-Asp, L-Gln, L-Glu, L-Cys, L-Met, L-His, L-Lys, triethylamine (TEA), methanol and acetonitrile were purchased from Sigma–Aldrich (Milan, Italy). Purified water by a Milli-RX (Millipore, Milford, MA, USA) apparatus was used for the preparation of all solutions and mobile phase. All the other chemicals were of analytical reagent grade. Although commercially available from Sigma–Aldrich's Rare Chemical Library, DPD was synthesized and purified by a simple procedure according to the Vilsmeier reaction [12]. Bakerflex plates (silica gel IB2-F) were used for TLC. Flash chromatography was performed using Aldrich flash chromatography "Silica Gel-60" (E. Merck, 230-400 mesh, Darmstadt, Germany). All solvents and 2,5-dimethylpyrrole used for the DPD synthesis were supplied by Aldrich Chemical Company Ltd. (Milan, Italy).

DPD solution (about 45 mM) was prepared daily in a mixture A:B (70:30, v/v), where A is methanol and B is borate buffer (pH 9.5; 0.4 M). 2-[(6-Hydroxy[4,7]phenanthrolin-5-yl)imino]propanoic acid used as internal standard (IS) was synthesized as previously described [8]. Then, IS solution (0.25 mg/ml) was prepared by dissolving it in methanol/triethylammonium phosphate buffer (pH 2.5; 0.05 M) 8:92, v/v (Method I), or 20:80, v/v (Method II). Amino acid standard solutions were prepared in water (concentration as calibration ranges). Borate buffer solution (pH 9.5; 0.4 M) was prepared by dissolving boric acid in water and adjusting to pH 9.5 with 0.1 M sodium hydroxide. Triethylammonium phosphate buffer (pH 2.5; 0.05 M) was prepared by dissolving TEA in water and adjusting to pH 2.5 with orthophosphoric acid.

2.2. Equipment

The liquid chromatograph consisted of a PU-1580 pump equipped with the LG-1580-02 ternary gradient unit and a diode-array detector (DAD) model MD-910 (Jasco Corporation, Tokyo, Japan). Data were collected on a PC equipped with the integration program Borwin-PDA. The solvents were degassed on line with a degasser model DG 2080-53 (Jasco Corporation). Manual injections were carried out using a Rheodyne model 7725i injector with 20 μl sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. The ^{1}H NMR spectra were recorded in D_2O on a Varian Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in δ (ppm). UV spectra were recorded on a Hewlett Packard 8453 spectrophotometer (Waldbronn, Germany).

2.3. Synthesis of (S)-2-{[1-(4-formyl-2,5-dimethyl-1H-pyrrol-3-yl)-meth-(E)-ylidene]-amino}-3-methylbutyric acid (DPD derivative of L-Val)

The methanolic solution of DPD (1.72 mmol) and L-Val (1.72 mmol) was stirred at room temperature for 4 h. After removal of the methanol by evaporation under reduced pressure, the residue was purified by flash chromatography on silica gel eluting with methanol. The resultant solid was treated with ethyl acetate to afford a pure product collected by filtration. The obtained solid was characterized as follows: yield = 50%; mp > 300 °C (dec.); ^{1}H NMR, 0.90 (6H, m), 2.29 (2H, m), 2.38 (3H, d), 2.45 (3H, d), 8.33 (1H, s), 9.57 (1H, s, –CHO); UV (methanol) λ = 218 nm (ε = 18,600), λ = 316 nm (ε = 13,000).

2.4. Derivatization reaction

To 50 μl aliquot of the amino acid solution 50 μl of purified water and 40 μl of DPD solution were added. The reaction was carried out in a micro-centrifuge tube (1.5 ml) at ambient temperature for 10 min after 5 s of ultrasonication; then, 300 μl aliquot of IS solution was added. Finally, a 20 μl aliquot was injected into the chromatograph.

2.5. Chromatographic conditions

The HPLC separations were performed at 33 ± 2 °C on a Phenomenex Gemini 5 μm ODS (250 mm × 3.0 mm i.d.) stainless steel column, with a guard column packed with the same stationary phase. Gradient elution conditions were used with a mobile phase consisting of a mixture A:B, where A is methanol and B is triethylammonium phosphate buffer (pH 2.5; 0.05 M), at a flow-rate of 0.32 ml/min. The adopted gradient profile was t = 0 min, 8% A; t = 10 min, 32% A; t = 25 min, 50% A; t = 30 min, 8% A (Method I). Isocratic elution conditions (Method II) were adopted for the analysis of only branched-chain amino acid using a mixture A:B, where A is binary mixture of methanol/acetonitrile 75:25 (v/v) and B is triethylammonium phosphate buffer (pH 2.5; 0.05 M), 26/74 (v/v), at a flow-rate of 0.4 ml/min. Detection at λ = 320 nm was used.

2.6. Analysis of pharmaceutical formulations

2.6.1. Sample preparation

2.6.1.1. Medicine in packets. The contents of five packets were mixed and an amount equivalent to about 12–24 mg of each amino acid was dissolved with 50 ml of water. Sample was subjected to ultrasonication for 10 min at room temperature. Then, 1 ml of solution was diluted to 5 ml with water.

2.6.1.2. Tablets. Twenty tablets were finely ground and an amount of powder equivalent to about 4–20 mg of each amino acid was introduced in a 50 ml volumetric flask filling up to volume with water and subjecting to ultrasonication for 10 min at room temperature. Then, 2.5 ml of solution was diluted to 10 ml with water.

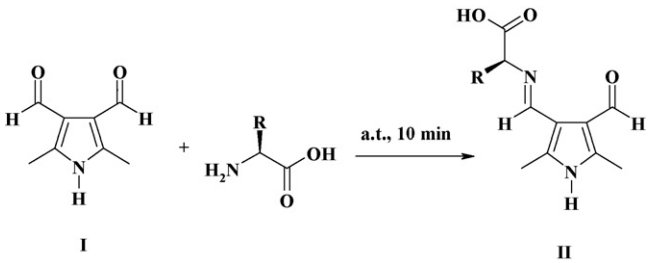
2.6.1.3. Injectable solution. A 1 ml aliquot corresponding to about 0.2–12 mg of each amino acid was drawn from the bottle and diluted to 50 ml of water.

After filtration through a 0.45 μm nylon filter, the final solution of the samples was subjected to the derivatization procedure and the amino acid content was determined by comparison with an appropriate standard solution.

3. Results and discussion

3.1. Derivatization reaction

The derivatization reaction was carried out under mild conditions: 10 min at ambient temperature and at about a 30 molar ratio of DPD (Fig. 1, I) to amino acid in a mixture of water, methanol and borate buffer (pH 9.5; 0.4 M) 71:20:9 (v/v/v). To assure the



I II

Fig. 1. Reaction scheme of amino acids with DPD (I).

Table 1
Effect of the reagent solution composition on the derivatization reaction.

Solvent	Response (%)			
	L-Val	L-Ile	L-Leu	L-Tyr
Methanol/borate buffer (pH 9.5; 0.4 M) 70:30 (v/v)	100	100	100	100
Methanol/borate buffer (pH 9.5; 0.4 M) 30:70 (v/v)	63.9	62.0	58.9	74.4
Methanol/borate buffer (pH 9.5; 0.4 M) 90:10 (v/v)	76.2	77.2	71.1	75.9
Methanol/borate buffer (pH 9.5; 0.4 M) 70:30 (v/v)	87.7	88.9	83.7	86.4
Methanol/borate buffer (pH 9.5; 0.2 M) 70:30 (v/v)[a]	80.8	81.7	73.2	79.4
Methanol/borate buffer (pH 9.5; 0.4 M)/mercaptoethanol 19.6:78.4:2 (v/v/v)[b]	97.8	97.6	87.2	108
Methanol/triethylammonium phosphate buffer (pH 11) 70:30 (v/v)	66.6	67.3	65.2	68.4
Methanol/water 70/30 (v/v)	12.7	12.0	12.6	27.7
Methanol	11.4	10.9	12.3	25.2
Acetonitrile	3.82	2.72	4.04	13.3
Methoxyethanol	4.96	3.67	5.25	17.8
Water	3.24	2.90	2.51	8.21

[a] Derivatization reaction: 50 μl of the amino acid solution, 50 μl of borate buffer (pH 9.5; 0.2 M) and 40 μl of DPD solution.
[b] Reagent solution prepared analogously to described method for reagent OPA [5].

optimum conditions, the parameters (reaction solvents, pH, temperature, time, and reagent/analyte molar ratio) which affect the derivatization reaction were critically evaluated chosen L-Val as an example (Fig. 2A–C). Analogous behaviour was observed also for other amino acids such as L-Leu, L-Ile and L-Tyr. Fig. 2A shows that ambient temperature is optimal while Fig. 2C shows that the optimum DPD/amino acid ratio is 30. It is seen from the curves in Fig. 2B, that the addition of 50 μl of water to the DPD buffer solution is optimal, whereas poor results were obtained with the addition of 50 μl of buffer solutions at different acidic or basic pH values. Derivatization in water without any buffers provided the lowest yield (Table 1). The reaction was found to be essentially quantitative (96%) by comparison with an authentic specimen of synthesized L-Val adduct. The structural characterization data of the adduct suggest that only one of the formyl groups of DPD forms Schiff base (Fig. 1, II) which seems to be activated by the other formyl group. As confirmation, preliminary studies showed that the monoaldehyde had an insignificant reactivity under the derivatization conditions. The cyclization does not seem to occur, contrary to derivatization with OPA reagent; on the other hand, as it can be seen in Table 1 the presence of a co-reagent such as the toxic and unpleasant smell mercaptoethanol is irrelevant for the reaction. DPD showed to be selective to the primary amino functional group, because under the described conditions it did not react with secondary amino group such as L-Pro and other different chemical functions.

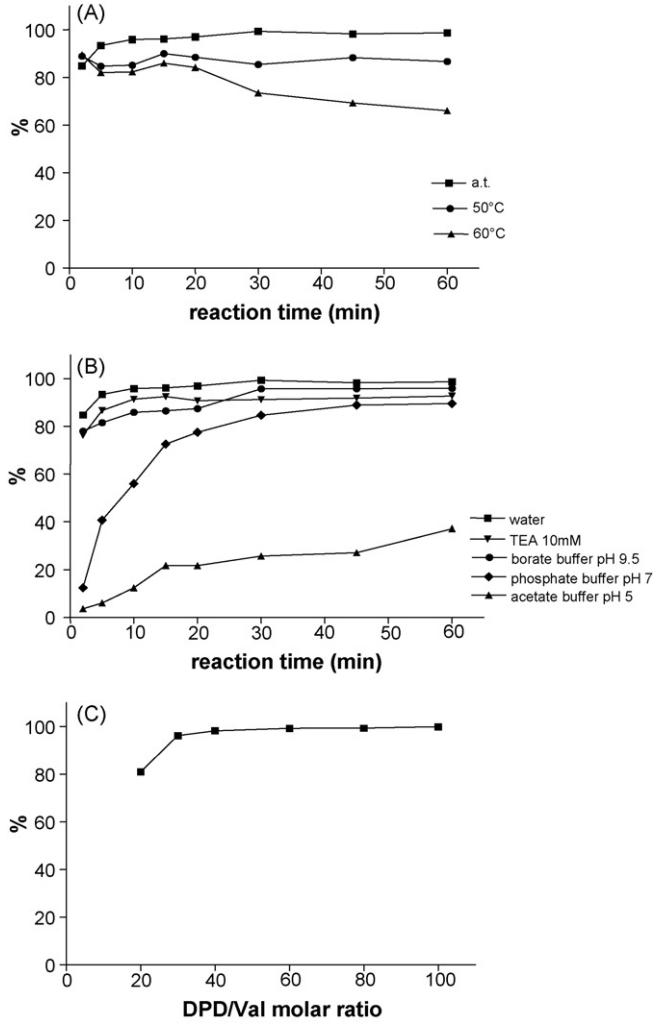


Fig. 2. Effect of (A) temperature, (B) water or different buffer addition to DPD buffer solution and (C) DPD (I) to amino acid molar ratio on the derivatization reaction of L-Val; %, percent yield of reaction.

3.2. Chromatography and detection

The above described chromatographic conditions were optimised by serial experiments using different mobile and stationary phases. Representative chromatograms obtained by gradient elution (Method I) and by isocratic elution (Method II) are reported in Fig. 3. As it is seen, the peak of excess DPD does not interfere with the analysis and the IS shows favourable retention time. The isocratic conditions (Method I) proved to be suitable for a more simple and fast analysis of the branched-chain amino acids (Val, Ile and Leu) in dietary supplements. The derivatization reaction enhances the detectability of all amino acids at a wavelength (λ = 320 nm), where the interference from other compounds is reduced. The limit of detection (LOD; S/N = 3), was found to be in the range of 3.80–10.9 pmol/injection, which allows a reliable quality control of formulations. DPD derivatives of all amino acid reaction mixtures (peak area ratio variation within ±2% of the initial value) proved to be stable for at least 4 h at ambient temperature and at least 24 h at 4 °C.

3.3. Analysis of amino acids in formulations

Under the described chromatographic conditions linear relationship (correlation coefficient ranging from 0.9993 to 0.9999) between peak area ratio (derivatized amino acid to IS) and the analyte concentration (calibration graphs in variable ranges from 0.02 to 5.15 mM) was observed for each compound. The within-run precision (repeatability) of the methods (derivatization and HPLC

Table 2
Results of LC determination of amino acids in commercial formulations.

Formulation	%Found[a] (% RSD)												
	L-Val	L-Ile	L-Leu	L-Lys	L-Phe	L-Met	L-Thr	L-Trp	L-Arg	L-His	L-Ala	L-Ser	Gly	L-Cys
Medicine in packets[b]	99.3 (1.9)	105.2 (1.1)	100.0 (0.6)	-	-	-	-	-	-	-	-	-	-	-
Tablets	104.9 (2.1)	98.4 (2.0)	100.3 (2.4)	113.1 (1.2)	98.3 (0.8)	97.8 (1.8)	96.6 (1.8)	91.0 (3.9)	-	-	-	-	-	-
Injectable solution[c]	98.9 (2.9)	100.3 (1.9)	100.9 (2.2)	107.2 (3.2)	101.0 (2.1)	104.0 (3.0)	99.8 (1.9)	101.5 (2.5)	100.4 (1.9)	99.3 (3.4)	101.5 (3.8)	93.7 (2.2)	95.8 (2.0)	109.7 (3.4)

[a] Mean of five determinations expressed as percentage of the claimed content.
[b] Other ingredients: mannitol, saccharosium, citric acid, natural flavor, sucrose ester, maltodextrin, demethylpolysiloxane.
[c] Other ingredients: L-Pro, magnesium chloride hexahydrate, potassium acetate, sodium bisulfite, water for injection.

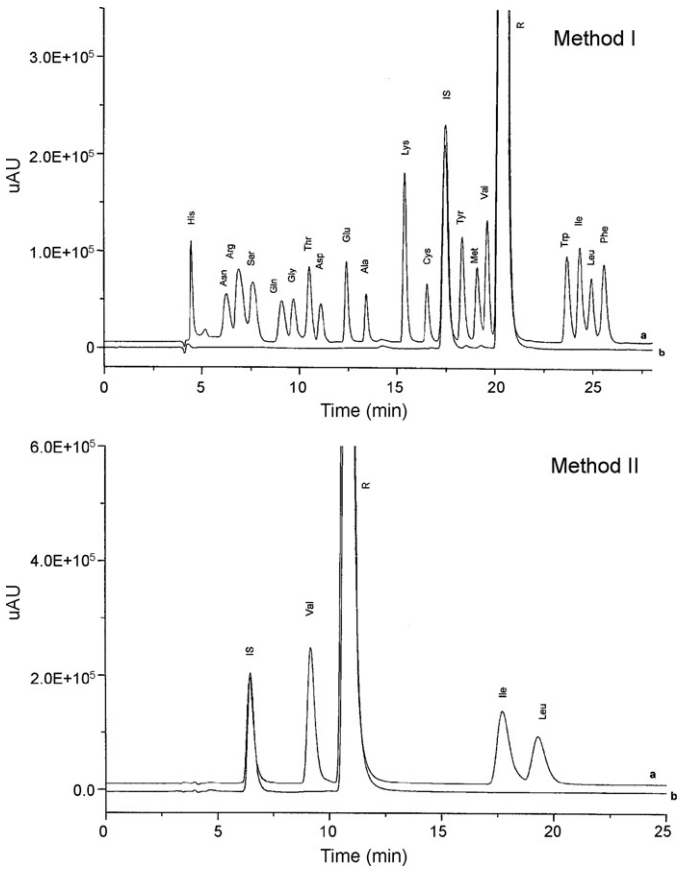


Fig. 3. Representative chromatograms of amino acids obtained by Methods I and II. (a) Amino acids derivatized with DPD; (b) reagent (R) under the reaction conditions as above in the absence of amino acids. For the chromatographic conditions and detection see Section 2.

separation) was satisfactory as indicated by the RSD (Relative Standard Deviation) range (1.4–5.0%) at three levels of concentration (0.2, 0.4, 0.6 mM) for each amino acid; every level was obtained from replicated ($n = 8$) analyses of standard solutions of amino acids. The results obtained from pharmaceutical formulation analysis (Table 2) were found to be in agreement with the claimed content. The other ingredients of the formulations did not interfere with the analysis. The accuracy of the methods was verified by analysing the commercial samples spiked with known amounts (20% of the claimed amino acid content). Quantitative recoveries were obtained in each instance (recovery %: 93.2–109.8%; RSD %: 1.1–4.6, $n = 6$).

4. Conclusions

DPD has proven to be a suitable UV precolumn reagent for the HPLC analysis of amino acids in pharmaceuticals. It showed better selectivity towards the amino group than Dns-Cl, FMOC-Cl, NBD-F and fluorescein isothiocyanate. DPD reacted faster and under milder reaction conditions than DDPP, panquinone and above all than PITC and FMOC-Cl, where the derivatization procedure is lengthy, because the reagent excess has to be removed before analysis. DPD adducts showed good stability in comparison with OPA derivatives, and the derivatization reaction proceeds without a mercapto compound as co-reagent. The simplicity of derivatization reaction, without additional reagents, allows easy manipulation. The proposed method does not need sophisticated and expensive instrumentation, and it can have practical importance for the quality control of amino acid preparations.

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