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Development and validation of a liquid chromatographic method for the determination of branched-chain amino acids in new dosage forms

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Abstract

A reversed-phase liquid chromatographic method (RP-LC) is proposed and validated for the analysis of branched-chain amino acids (L-leucine, L-isoleucine and L-valine) in new pharmaceutical formulations. The pre-column derivatization reaction of these amino acids with 2,4-dinitrofluorobenzene (DNFB) has been investigated considering the matrix effect. The compound reacts at 60 °C for 10 min at pH 9 with the amino function, in presence of cetyltrimethylammonium bromide (CTAB), to give adducts that have been separated on a RP amide C16 column and detected at $\lambda = 360$ nm. Linear responses were observed for each derivative. The intra-day precision (R.S.D.) was $\leq$2.22% and there was no significant difference between intra- and inter-day data. Recovery studies showed good results for all amino acids (98.9–100.9%) with R.S.D. ranging from 0.1% to 0.8%. The limit of quantitation was about 20 nmol/mL.

Keywords: Reversed-phase liquid chromatography (RP-LC); 2,4-Dinitrofluorobenzene (DNFB); Validation; Branched-chain amino acids; Pharmaceuticals

1. Introduction

Branched-chain amino acids (BCAAs), namely leucine, isoleucine and valine, are neutral amino acids with interesting and clinically relevant metabolic effects [1]. BCAAs are not only a substrate for protein synthesis, but also modulate several components of synthetic machinery and help to conserve muscle mass. The beneficial effects on nutrition were reported to improve muscle performance, reduce protein loss during bed-rest, favour weight loss in obesity, reduce catabolism in trauma patients and improve clinical outcomes in patients with advanced cirrhosis [2].

The determination of amino acids by high performance liquid chromatography (HPLC) has been dominated by pre- or post-column derivatization methods to improve separation arising from greater compatibility with reversed-phase columns and to improve detection by using highly chromophoric or fluorophoric groups. Typical amino acid reagents where the derivatization chemistry is well understood include *o*-phthaldehyde (OPA), 5-dimethylamino-1-naphthalenesulphonyl(dansyl)chloride (Dns-Cl), fluorescamine, fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC), phenylthiohydantoin (PTH) and 2,4-dinitrofluorobenzene (DNFB, Sanger's reagent) [3–6].

However, the use of these reagents involves a number of drawbacks. OPA is the most reported reagent, having a remarkable reactivity to the primary amino function, but it is used preferably for post-column derivatization, due to the instability of the reaction products. As regards other reagents (e.g. PITC, FMOC-Cl), the selectivity is relatively low and the procedure is lengthy, above all because the excess of reagent has to be removed before the analysis. On the other hand using Dns-Cl, the amino acid adducts can react with excess reagent to form side product mixtures and the steric hindrance, as occurs with the branched-chain amino acids, favours the decomposition [6].

DNFB is a UV derivatization reagent for amine [5–14], but it can react also with phenols, thiols and hydrazino compounds [15,16] introducing a potential interfering. In aqueous borate buffer, DNFB yields yellow coloured products reacting with primary and secondary amines through nucleophilic aromatic substitution reaction. Kinetic studies demonstrated that the reactions of DNFB are catalysed in the presence of micelles of cetyltrimethylammonium bromide (CTAB) [16,17].

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2,4-Dinitrofluorobenzene derivatives have been found to be very stable under common laboratory conditions. This is a real advantage for the routine automated analyses at company level. Other advantages of this tagging reaction is that it proceed in aqueous solution and the excess of the reagent does not interfere. However, a drawback of this reagent is its toxicity; it must be handled with protective gloves. Even if good separations of a number of AA using DNFB are reported in literature, the respective papers are difficult to consult.

The first approach of this work was based on the optimization of the labelling and LC separation conditions of DNFB-derivatives to applied the procedure to quality control of new pharmaceutical dosage forms. At this end, the method was accurately validated from the point of view of specificity, linearity, limit of detection and quantitation, accuracy, precision and stability, according to the approved validation standard operating procedure (SOP) of the pharmaceutical producer.

2. Experimental

2.1. Materials

L-Valine (L-Val), L-isoleucine (L-Ile), L-leucine (L-Leu), L-tryptophan (L-Trp), 1-fluoro-2,4-dinitrobenzene (DNFB) and hexadecyltrimethylammonium bromide (CTAB) were purchased from Fluka AG (Buchs, Switzerland). Boric acid, triethylamine (TEA) and acetonitrile for chromatography (RP-HPLC grade) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All the other chemicals were of analytical reagent grade. Deionized, double distilled water was used for the mobile phase preparation.

2.2. Solutions

Solutions of the reagent DNFB (27 mg/mL) were prepared in acetonitrile, stored at 2–8 °C and protected by the light. The solutions were found to be stable for at least a week. Standard solutions of the amino acids were prepared in water (concentration under calibration graphs). The internal standard (IS, Trp) solutions (2.0 mg/mL for the analysis of effervescent tablets and 2.5 mg/mL both for the soluble granules and sachets) were prepared in water. Triethylammonium phosphate buffer (pH 3; 0.05 M) was prepared adding orthophosphoric acid to an aqueous TEA solution up to the desired pH value. Borate buffer solution (pH 9; 0.1 M) was prepared according to standard methods [3]. CTAB solution (3 mM in borate buffer 0.04 M) was prepared adding borate buffer (0.1 M; pH 9) to a water solution of CTAB to obtain the final solution.

2.3. Equipment

A 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). The data were collected on a PC equipped with the integration program Borwin-PDA. Manual injections were carried out using a Rheodyne model 7125 injector with 20 μL sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. The solvents were degassed on line with a degasser model Gastorr 153 S.A.S. Corporation (Tokyo, Japan). A second liquid chromatograph consisted of an Agilent series 1100 gradient unit, an Agilent series 1100 pumping system and a Agilent series 1100 diode-array detector (DAD) (Agilent, Germany) connected to a personal computer. The data were collected on a PC equipped with the integration program Chemstation Agilent. Automatic injections were carried out using an Agilent series 1100 autosampler (Agilent, Germany).

The derivatization reaction was carried out on a ReactiTherm Heating/Stirring module (Pierce, Rockford, IL, USA) or alternatively in a heater (Piardi, Brescia, Italy). Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication.

2.4. Derivatization procedure

To a 250 μL aliquot of the filtered amino acid (reference or sample) solution were added 500 μL of CTAB solution in borate buffer and 50 μL of DNFB solution. The reaction was carried out at 60 °C for 15 min in a micro-reaction vessel (3 mL) or alternatively for 30 min in heater in a 4 mL vial with solid cap. Then, after cooling in ice the reaction solution was diluted with 2 mL of a mixture of triethylammonium phosphate buffer (pH 3; 0.05 M)–acetonitrile, 25:75 (v/v). Then, 20 μL aliquot of the mixture was injected into the chromatograph or alternatively the vial was inserted in the autosampler of the chromatograph.

2.5. Chromatographic conditions

After chemical derivatization, the LC separations were performed at 33 ± 2 °C on a Supelco Discovery RP amide C16 5 μm (250 mm × 4.6 mm i.d.) stainless steel column. For routine analyses a mobile phase consisting of a mixture of triethylammonium phosphate buffer (pH 3; 0.05 M)–acetonitrile (55:45, v/v) at a flow-rate of 0.8 mL/min was used. UV-diode array detection, setting the wavelength at λ = 360 nm, was used.

2.6. Analysis of pharmaceuticals

2.6.1. Sample preparation

Effervescent tablets. Twenty tablets were finely grinded and an amount of powder equivalent to about 20 mg of L-Val, L-Leu and L-Ile, respectively, was introduced in a 200 mL volumetric flask and dissolved with water. After adding 10 mL of IS solution and 6 mL of 0.7 M NaOH, the solution was sonicated for 10 min at ambient temperature and then filled up to volume with water. Finally, an aliquot of the solution was filtered through a 0.45 μm regenerated cellulose filter.

Soluble granules. The content of twenty sachets was finely grinded and an amount of powder equivalent to about 25 mg of L-Val, L-Leu and L-Ile, respectively, was introduced in a 250 mL volumetric flask and dissolved with water. After adding 10 mL of IS solution and 3 mL of 0.7 M NaOH, the solution was sonicated for 10 min at ambient temperature and then filled up to volume

with water. Finally, an aliquot of the solution was filtered through a 0.45 μm regenerated cellulose filter.

Sachets (*Friliver*®). The content of twenty sachets was finely grinded and an amount of powder equivalent to about 15 mg of L-Val, 15 mg of L-Ile and 30 mg of L-Leu was introduced in a 250 mL volumetric flask and dissolved with water. Then, the solution was treated as described above for "Soluble granules".

2.6.2. Assay procedure

A 250 μL aliquot of the sample solution was subjected to the described derivatization procedure and the amino acid content in each sample was determined by comparison with an appropriate standard solution.

2.7. Specificity

L-Val, L-Leu, L-Ile (0.1 mg/mL, respectively), sample solutions, each containing L-Trp (IS, 0.1 mg/mL), placebo (a mixture of excipients and other ingredients, except BCAAs) and blank (water) solution were prepared according to the method. All solutions were subjected to the described derivatization procedure.

2.8. Linearity

Placebo solutions spiked of L-Val, L-Leu, L-Ile (concentration ranges in Table 3) and IS were prepared in water. A 250 μL volume of amino acid solutions was subjected to the described derivatization procedure. Triplicate injections for each solution were made and the peak-area ratio of analyte to IS was plotted against the corresponding amino acid concentration to obtain the calibration graphs.

2.9. Precision

Twenty effervescent tablets and the content of twenty sachets were finely grinded and six aliquots were accurately weighted corresponding to the weight of 2/5 and 1/2 for tablets and sachets, respectively. The solutions were prepared according to the method and then derivatized.

2.10. Accuracy

The accuracy of the method was determined as mean recovery on nine solutions containing known amounts of amino acids corresponding to about 75, 100 and 125% of the claimed content, in presence of placebo. The samples were prepared by weighting the amounts reported in Tables 6 and 7, in accordance with the method described above. Each solution was injected twice. The recovery was calculated with respect of the standard solution.

3. Results and discussion

3.1. Derivatization reaction

The derivatization reaction was carried out at 60 °C for 15 min by using a stirring/heating apparatus. To achieve optimum conditions using DNFB at pH 9.0, the derivatization reaction with BCAAs and Trp (IS) in presence of the surfactant CTAB was investigated. In particular, due to the complexity of the matrix containing potential interfering components, standard solutions were compared with reconstituted samples. Relevant, before the derivatization reaction, to have comparable solutions, the pH of the reconstituted sample (pH 3.25) was adjusted with 0.7 M NaOH to the pH value of standard solution (pH 6.7). As it can be seen in Fig. 1a, as regards the reconstituted sample, the reaction is complete after 10 min at 60 °C, while it proceeds more slowly at lower temperatures (40 °C). On the other hand, for the standard solution the reaction is already complete at 40 °C after 10 min. Trp (IS) reacts more rapidly than BCAAs (room temperature for 5 min) (Fig. 1b), probably due to the major effect of the micellar catalysis on the reaction of this amino acid [17]. Under the described conditions the response intensity reaches a plateau at a reagent to amino acid molar ratio of about five for all considered amino acids and further reagent excess does not interfere (Fig. 1c). Alternative reaction procedures to the use of a Reacti-Therm have been investigated and it was found that the reaction can be carried out at 60 °C for 30 min in a heater:

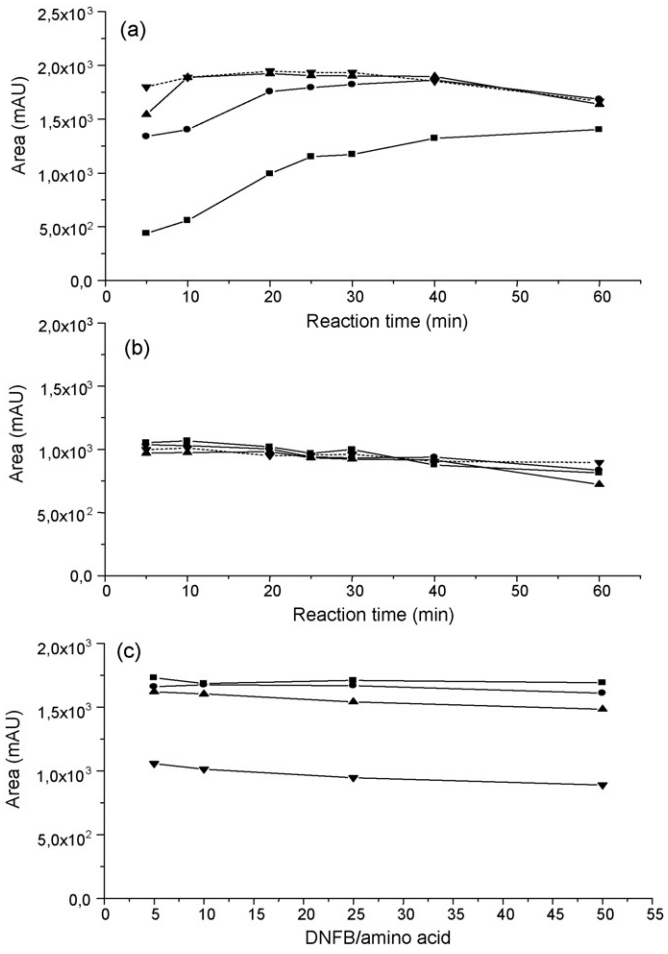

Fig. 1. Influence of the temperature on the derivatization reaction of L-Val (a) and L-Trp (b) with DNFB. Reconstituted sample at ambient temperature (■), 40 °C (●), 60 °C (▲); standard solution at 40 °C (▼). (c) Effect of the reagent to amino acid molar ratio on the derivatization reaction between DNFB and amino acids: L-Val (■); L-Leu (●); L-Ile (▲); L-Trp (▼).

Table 1
Summary of better chromatographic conditions obtained on different tested columns

Column	Mobile phase	t_{rLeu}[a]	t_{rIle}[a]	$R_{sLeu\text{-}Ile}$[b]
Synergi 4 U fusion RP 80A (150 mm × 3.0 mm)	ACN/THF/TEA phosphate buffer (pH 3; 0.05 M) 30/2/68 (v/v/v); flow rate 0.4 mL/min; $T = 33\,^{\circ}C$	40.83	42.18	0.81
Prodigy 5 U ODS3 100A (250 mm × 3.2 mm)	ACN/TEA phosphate buffer (pH 3; 0.05 M) 40/60 (v/v); flow rate 0.4 mL/min; $T = 33\,^{\circ}C$	29.32	29.32	Not resolved peaks
Gemini 5 U ODS 110A (250 mm × 3.0 mm)	ACN/THF/TEA phosphate buffer (pH 3; 0.05 M) 28/14/58 (v/v/v); flow rate 0.8 mL/min; $T = 33\,^{\circ}C$	22.61	23.52	1.05
Synergi hydro-RP (150 mm × 4.6 mm)	ACN:THF (70:30)/potassium phosphate buffer (pH 3; 0.05 M) 35/65 (v/v); flow rate 1 mL/min; $T = 33\,^{\circ}C$	23.00	23.91	1.07
Luna phenyl-hexyl (250 mm × 3.0 mm)	ACN/TEA phosphate buffer (pH 3; 0.05 M) 35/65 (v/v); flow rate 0.4 mL/min; $T = 33\,^{\circ}C$	37.83	39.61	0.82
Synergi MAX-RP (250 mm × 4.6 mm)	ACN:THF (80:20)/TEA phosphate buffer (pH 5; 0.05 M) 24/76 (v/v); flow rate 0.8 mL/min; $T = 33\,^{\circ}C$	49.88	53.43	1.89
Synergi 4 U polar-RP 80A (250 mm × 3.0 mm)	MeOH:THF (90:10)/potassium phosphate buffer (pH 3; 0.05 M) 48/52 (v/v); flow rate 0.4 mL/min; $T = 33\,^{\circ}C$	54.84	58.68	1.55
Synergi 4 U MAX-RP 80A (150 mm × 3.0 mm)	ACN:THF (70:30)/TEA phosphate buffer (pH 3; 0.05 M) 28/72 (v/v); flow rate 0.4 mL/min; $T = 36\,^{\circ}C$	60.68	63.11	0.94
Discovery RP-amide C16 (250 mm × 4.6 mm)	ACN/TEA phosphate buffer (pH 3; 0.05 M) 45/55 (v/v); flow rate 0.8 mL/min; $T = 33\,^{\circ}C$	18.31	19.82	2.25

[a] Retention time (min).
[b] Resolution according to the formula: $1.18 \times (t_{r2} - t_{r1})/(w_2 - w_1)$, where t_{r1}, t_{r2} is the retention time of the first and second peak, respectively, and w_1, w_2 is the peak half height width of first and second peak, respectively.

under these conditions comparable results in terms of reaction reproducibility and yield were obtained.

3.2. Chromatography

In the development of the method to assure an optimum separation of the analytes, potential impurities and IS a resolution ≥2 (value as method acceptability) was fixed according to the official instructions [18,19]. At the same time it is necessary to have short times of chromatographic course to apply the method to routine analysis of large samples at company level.

To this end a variety of reversed phase column was investigated (Table 1). As a result of that a Supelco Discovery RP amide C16 column was chosen and binary mixture of triethylammonium phosphate buffer–acetonitrile in isocratic elution conditions was used to achieve an adequate separation of derivatized amino acids (Fig. 2). As it can be seen the reagent did not interfere with the analysis. Even if the Leu and Ile separation is critical because these amino acids differ only from methyl group position, using the described column a resolution higher than two was obtained (Table 1). For further information in the detail of Fig. 2 the spectrum of L-Ile adduct is reported.

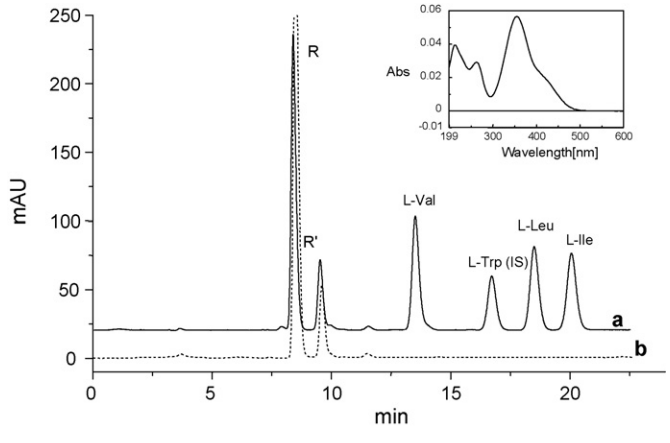

Fig. 2. Representative LC separation at 33 ± 2 °C of: (a) amino acids derivatized with DNFB and (b) reagent under reaction conditions (blank). R, R' = reagent peaks; IS = internal standard. LC conditions: Supelco Discovery® RP amide C16 (250 mm × 4.6 mm i.d.) column with a mixture of triethylammoniun phosphate buffer (pH 3.0; 0.05 M) and acetonitrile, in the ratio 55:45 (v/v), as mobile phase; flow-rate: 0.8 mL/min. UV-DAD detection: λ = 360 nm. Detail: representative UV-DAD spectrum of derivatized BCAA (L-Ile).

3.3. System suitability test

In order to establish that the HPLC system and procedure are capable of proving data of acceptable quality [18,19], some parameters are determined and compared against the specifications set for the method. The parameters of the system suitability test obtained under the developed chromatographic conditions and the related acceptance limits, as defined in the company validation SOP, are reported in Table 2.

3.4. Specificity

For both analyzed formulations the retention times of L-Val, L-Leu, L-Ile and L-Trp standard solution have been compared with the placebo, sample and reagent blank solutions prepared under the same conditions. No interferences with amino acid peaks due to the placebo or blank have been observed (chromatograms in Fig. 3). The method is thus specific for the identification and assay of BCAAs.

3.5. Linearity

The linearity was determined as linear regression with the least-square method on five spiked placebo solutions having the

Table 2
System suitability test

Amino acid	Parameters	Reference	Effervescent tablets	Soluble granules	Acceptance limits (%)
L-Val	t_r (min)[a]	13.6	13.4	13.4	±15
	N (USP)[b]	15,900	11,700	15,200	±30
	N (Eur.P.) [b]	16,700	12,200	16,100	±30
	f_a (10%)[c]	1.24	1.10	1.14	0.8–1.5
	f_a (Eur.P., USP)[c]	1.10	1.06	1.26	0.8–1.5
L-Leu	t_r (min)[a]	18.6	18.4	18.3	±15
	N (USP)[b]	16,800	13,800	16,500	±30
	N (Eur.P.)[b]	17,500	14,200	17,000	±30
	f_a (10%)[c]	1.18	1.09	1.10	0.8–1.5
	f_a (Eur.P., USP)[c]	1.05	1.05	1.06	0.8–1.5
L-Ile	t_r (min)[a]	20.1	19.9	19.8	±15
	N (USP)[b]	17,000	14,200	16,700	±30
	N (Eur.P.)[b]	17,900	14,700	17,400	±30
	f_a (10%)[c]	1.17	1.03	1.09	0.8–1.5
	f_a (Eur.P., USP)[c]	1.05	1.01	1.04	0.8–1.5

[a] Retention time.
[b] Theoretical plate number.
[c] Asymmetry factor.

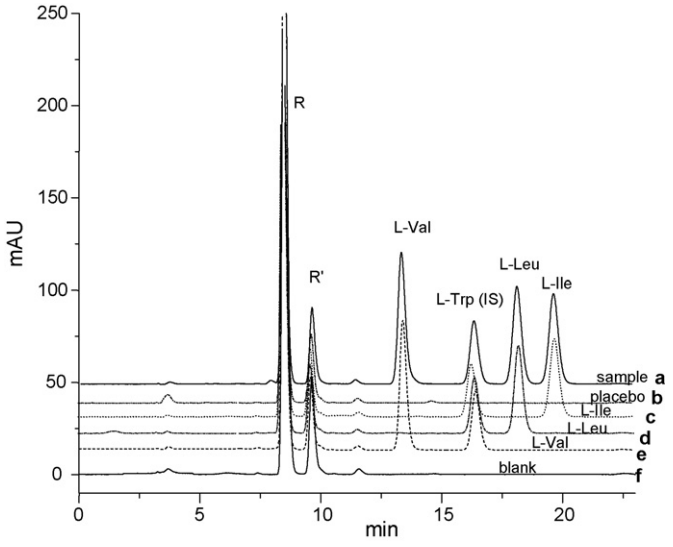

Fig. 3. Overlay of chromatograms of: (a) sample, (b) placebo, (c–e) amino acids and (f) blank. R, R' = reagent peaks; IS = internal standard. Chromatographic conditions as reported in Fig. 2.

following concentration levels: 50, 75, 100, 125 and 150% of the claimed amino acid concentration (corresponding to the range of about 0.05–0.15 mg/mL) as reported in Table 3. Good linearity was found for each amino acid as indicated by the coefficient of determination ≥0.9998.

3.6. Limit of detection (LOD) and quantitation (LOQ)

For the proposed HPLC method LOD and LOQ have been established by the determination of the signal/noise ratios of 3:1 (LOD) and 10:1 (LOQ). The corresponding LOQ values were found to be 4 nmol/mL for L-Val and 6 nmol/mL for L-Leu and L-Ile, while LOQ data were 16 nmol/mL for L-Val and 19 nmol/mL for L-Leu and L-Ile.

3.7. Precision

The precision of the method was expressed as repeatability and was calculated employing six test solutions, each one prepared starting from an homogeneous finished product sample. Besides, the intermediate precision of the method was determined with 12 solutions, prepared changing the parameters *time-analyst*: six solutions were prepared by the analyst

Table 3
Data for calibration graphs ($n = 5$) obtained in the concentration range of 0.05–0.15 mg/mL

Amino acid	Slope[a]	Confidence interval	y-Intercept[a]	Confidence interval	Coefficient of determination, r^2
L-Val[b]	17.99	±0.16	0.02	±0.02	0.9998
L-Val[c]	18.14	±0.10	0.00	±0.01	0.9999
L-Leu[b]	16.09	±0.16	0.02	±0.01	0.9998
L-Leu[c]	16.24	±0.12	−0.01	±0.01	0.9998
L-Ile[b]	17.21	±0.08	0.00	±0.01	0.9999
L-Ile[c]	17.30	±0.10	−0.04	±0.01	0.9999

[a] According to $y = ax + b$, where x is the amino acid concentration and y is the ratio of amino acid peak-area to IS peak-area.
[b] Amino acid spiked in placebo (effervescent tablets).
[c] Amino acid spiked in placebo (soluble granules).

Table 4
Repeatability and intermediate precision (effervescent tablets)

Amino acid	Mean corrected area[a] (S.D.)	R.S.D. (%)	Confidence (%)[b]	mg/tablet (S.D.)	R.S.D. (%)	Confidence (%)[b]
Repeatability ($n=6$)						
Analyst A/day 1						
L-Val	1.83 (0.02)	1.16	0.02 (0.93)	49.32 (0.57)	1.16	0.46 (0.93)
L-Leu	1.61 (0.02)	1.18	0.01 (0.34)	51.04 (0.60)	1.18	0.48 (0.34)
L-Ile	1.58 (0.01)	0.88	0.01 (0.71)	46.52 (0.41)	0.88	0.33 (0.71)
Analyst B/day 2						
L-Val	1.81 (0.04)	2.22	0.03 (1.78)	48.88 (1.08)	2.22	0.87 (1.78)
L-Leu	1.58 (0.03)	1.68	0.03 (1.34)	50.23 (0.84)	1.68	0.68 (1.34)
L-Ile	1.53 (0.33)	1.81	0.03 (1.45)	45.08 (0.82)	1.81	0.65 (1.45)
Intermediate precision ($n=12$)						
L-Val	1.82 (0.03)	1.75	0.02 (0.99)	49.10 (0.86)	1.75	0.49 (0.99)
L-Leu	1.60 (0.03)	1.60	0.01 (0.90)	50.62 (0.81)	1.60	0.46 (0.90)
L-Ile	1.56 (0.03)	1.78	0.02 (1.20)	45.80 (0.97)	1.78	0.55 (1.20)

[a] Amino acid to IS area ratio.
[b] Confidence percentage ($\alpha=0.05$).

A in the day 1, while the other six solutions were prepared by the analyst B in the day 2. The results are reported in Tables 4 and 5. The variance ratio test (F-test) indicated no significant differences between intra- and inter-day data: the calculated F values, $F_{0.05}(5,5)=3.61$, 1.97, 3.97 (effervescent tablets) and 1.16, 4.42, 3.90 (soluble granules) for Val, Leu, Ile, respectively, were smaller than the tabulated F value, $F_{0.05}(5,5)=5.05$.

Finally, the analysis of product representative samples has been performed both in the method development laboratory and the designated laboratory. No statistically significant differences were found between inter-laboratory results.

The results were found in agreement with the nominal content of the formulations. In addition, the method was applied to a commercial product, giving the subsequent values ($n=6$), expressed as percentage of the claimed content: Val 101.2% (R.S.D. = 2.1%), Leu 99.5% (R.S.D. = 1.8%), Ile 97.3% (R.S.D. = 1.5%). Other formulation ingredients (saccharose, mannitol, citric acid, maltodextrin, natural flavour, sucrose ester, citric acid, sorbitol and dimethylpolysiloxane) did not interfere with the analysis.

3.8. Accuracy

The accuracy was based on the recovery of known amounts of analyte, spiking analyte in placebo. Spiked samples were prepared in triplicate at three levels over a range of 75–125% of the target concentration. Quantitative recovery was obtained in each instance (recovery = 98.8–100.9%; R.S.D. = 0.1–0.8%) (Tables 6 and 7).

3.9. Stability of solutions

Injections of final reference and sample solution were performed at different times of preparation and the solution was stored at room temperature, in a vial, inside the autosampler device. The test solution is considered stable in time if $\Delta \leq 1.5\%$ (Table 8).

Table 5
Repeatability and intermediate precision (soluble granules)

Amino acid	Mean corrected area[a] (S.D.)	R.S.D. (%)	Confidence (%)[b]	mg/sach (S.D.)	R.S.D. (%)	Confidence (%)[b]
Repeatability ($n=6$)						
Analyst A/day 1						
L-Val	1.80 (0.03)	1.72	0.02 (1.37)	49.00 (0.84)	1.72	0.67 (1.37)
L-Leu	1.59 (0.02)	0.97	0.01 (0.77)	47.56 (0.46)	0.97	0.37 (0.77)
L-Ile	1.50 (0.01)	0.80	0.01 (0.64)	44.57 (0.36)	0.80	0.29 (0.64)
Analyst B/day 2						
L-Val	1.80 (0.03)	1.86	0.03 (1.49)	48.79 (0.91)	1.86	0.72 (1.49)
L-Leu	1.58 (0.03)	2.04	0.03 (1.63)	47.35 (0.97)	2.04	0.77 (1.63)
L-Ile	1.51 (0.02)	1.58	0.02 (1.26)	44.63 (0.71)	1.58	0.56 (1.26)
Intermediate precision ($n=12$)						
L-Val	1.80 (0.03)	1.72	0.02 (0.97)	48.89 (0.84)	1.72	0.48 (0.97)
L-Leu	1.58 (0.02)	1.53	0.01 (0.87)	47.45 (0.73)	1.54	0.41 (0.87)
L-Ile	1.51 (0.02)	1.22	0.01 (0.69)	44.60 (0.53)	1.22	0.30 (0.69)

[a] Amino acid to IS area ratio.
[b] Confidence percentage ($\alpha=0.05$).

Table 6
Accuracy (effervescent tablets)

Amino acid	Level (%)	Spiked amount[a] (mg)	Theoretical (μg/mL)	Found (μg/mL)	Recovery (%)	Mean recovery, ($n = 3$ (%))	Mean recovery, ($n = 9$ (%))	R.S.D. (%)
L-Val	75	15.06	75.30	74.53	99.0	100.0	99.9	0.6
		15.10	75.50	76.05	100.7			
		15.37	76.85	76.98	100.2			
	100	20.45	102.25	101.05	98.8	99.3		
		20.36	101.80	101.14	99.4			
		20.65	103.25	102.96	99.7			
	125	25.58	127.90	127.92	100.0	100.5		
		25.24	126.20	126.59	100.3			
		25.91	129.55	131.18	101.3			
L-Leu	75	15.30	76.58	75.90	99.1	99.2	99.6	0.8
		15.73	78.73	77.98	99.0			
		15.63	78.23	77.86	99.5			
	100	20.41	102.15	102.80	100.6	100.5		
		20.79	104.05	103.87	99.8			
		20.08	100.50	101.50	101.0			
	125	25.76	128.93	127.63	99.0	99.0		
		25.23	126.28	124.60	98.7			
		25.45	127.38	126.68	99.5			
L-Ile	75	15.00	74.25	73.36	98.8	100.0	100.2	0.4
		15.19	75.19	75.30	100.1			
		15.26	75.54	76.37	101.1			
	100	20.39	100.93	101.06	100.1	99.9		
		20.06	99.30	99.04	99.7			
		20.26	100.29	100.02	99.7			
	125	25.39	125.68	126.26	100.5	100.7		
		25.82	127.81	128.69	100.7			
		25.31	125.28	126.49	101.0			

[a] Placebo amount about 1200 mg.

Table 7
Accuracy (soluble granules)

Amino acid	Level (%)	Spiked amount[a] (mg)	Theoretical (μg/mL)	Found (μg/mL)	Recovery (%)	Mean recovery, ($n = 3$ (%))	Mean recovery, ($n = 9$ (%))	R.S.D. (%)
L-Val	75	18.79	75.16	76.21	101.4	100.9	100.9	0.4
		18.85	75.40	75.98	100.8			
		19.03	76.12	76.40	100.4			
	100	25.08	100.32	100.91	100.6	101.3		
		25.13	100.52	101.97	101.4			
		25.64	102.56	104.60	102.0			
	125	31.96	127.84	127.30	99.6	100.6		
		31.15	124.60	125.95	101.1			
		34.33	137.32	138.95	101.2			
L-Leu	75	18.87	75.56	74.75	98.9	98.8	98.9	0.1
		19.11	76.52	75.72	99.0			
		18.74	75.03	73.91	98.5			
	100	25.11	100.54	99.22	98.7	99.0		
		25.73	103.02	102.46	99.5			
		25.68	102.82	101.60	98.8			
	125	31.25	125.12	123.59	98.8	98.9		
		31.26	125.16	124.18	99.2			
		33.50	134.13	132.59	98.8			
L-Ile	75	18.83	74.57	75.21	100.9	99.6	99.7	0.1
		18.74	74.21	73.49	99.0			
		19.02	75.32	74.51	98.9			
	100	25.78	102.09	103.92	101.8	99.9		
		25.37	100.46	99.47	99.0			
		25.35	100.39	99.18	98.8			
	125	30.97	122.64	121.49	99.1	99.7		
		30.85	122.17	123.16	100.8			
		31.18	123.47	122.56	99.3			

[a] Placebo amount about 2000 mg.

Table 8
Stability of reference and sample final solutions

Solution	Time (h)	A_{Val}/A_{SI}	Δ (%)[a]	A_{Leu}/A_{SI}	Δ (%)[a]	A_{Ilel}/A_{SI}	Δ (%)[a]
Reference	0	1.83	1.0	1.65	0	1.73	1.5
	24	1.85		1.65		1.76	
Effervescent tablets	0	1.86	1	1.66		1.63	0.3
	24	1.88		1.64	−1.4	1.64	
Soluble granules	0	1.67	−0.6	1.52		1.45	0.7
	24	1.66		1.51	−0.9	1.46	

[a] According to formula: Δ (%) = ((corrected area ($t = x$) − corrected area ($t = 0$))/corrected area ($t = 0$)) × 100, where corrected area is amino acid to IS area ratio.

All solutions are stable for at least 24 h. Besides the stability of the analytical solutions was considered; the reagent and reference solutions have been found stable for at least 7 and 3 days, respectively.

Solutions stability, intermediate precision and applicability of the method in different laboratories (university and company) provide an indication of the method ruggedness and robustness.

4. Conclusions

DNFB has been confirmed to be a useful pre-column labelling reagent for the quality control of amino acid dosage forms. The HPLC proposed method was found to be simple, rapid, specific, linear, reliable and robust, allowing the determination of BCAAs in new effervescent dosage forms with very complex composition, without the necessity of preliminary extraction procedures.

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