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Development and validation of a liquid chromatographic method for the determination of ascorbic acid, dehydroascorbic acid and acetaminophen in pharmaceuticals☆

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Abstract

A reversed-phase ion pair liquid chromatographic method (RP-LC) for the determination of dehydroascorbic acid (DHA) and ascorbic acid (AA) and also acetaminophen, which is combined in pharmaceuticals, is proposed and validated. AA and acetaminophen were analyzed directly, while DHA was determined after pre-column derivatization with 4,5-dimethyl-1,2-phenylenediamine (DMPD). The derivatization reaction was carried out under mild conditions (10 min at ambient temperature) in the dark in sodium acetate buffer (80 mM; pH 3.7) solution containing EDTA as metal scavenger. The chromatographic separations were performed on a Phenomenex Synergi 4u hydro-RP (150 mm × 4.6 mm) under isocratic elution conditions, using cetyltrimethylammonium bromide (CTAB) as ion-pairing reagent in the mobile phase. Linear responses were observed for each compound. The intra-day precision (R.S.D.) was ≤1.40% and there was no significant difference between intra- and inter-day data. Recovery studies showed good results for all compounds (99.7–101.8%) with R.S.D. ranging from 0.56 to 1.82%. The limits of quantitation were about 40, 50 and 140 pmol for acetaminophen, AA and DHA, respectively. The DHA impurity values found in dosage forms were ≤0.2% of AA.

Keywords: Reversed-phase liquid chromatography (RP-LC); 4,5-Dimethyl-1,2-phenylendiamine (DMPD); Validation; Dehydroascorbic acid (DHA); Ascorbic acid (AA); Acetaminophen; Pharmaceuticals

1. Introduction

Ascorbic acid (vitamin C), a water-soluble vitamin, is essential for the synthesis of collagen and intercellular material [1]. Vitamin C deficiency develops when the dietary intake is inadequate. It is rare in adults, but may occur in infants, alcoholics, or the elderly. Deficiency leads to the development of a well-known syndrome known as scurvy. Vitamin C is administered in the treatment and prevention of deficiency and showed to be useful to acidify urine and in the treatment of many disorders, including Alzheimer's disease, atherosclerosis, cancer and the common cold. Ascorbic acid or sodium ascorbate is used in treating methaemoglobinemia, and also calcium ascorbate or ascorbyl palmitate as antioxidants in pharmaceutical manufacturing and in the food industry [2].

The determination of AA and DHA is difficult owing to a variety of problems such as poor solution stability, DHA low absorbivity and easily coelution of AA and DHA from chromatographic reversed-phase columns, because of their highly polar character. Really, in presence of oxidizing agents AA is degraded reversibly to DHA which is in his turn degraded with continued oxidative stress to 2,3-diketogulonic acid and then to over 50 species containing five or less carbons lacking in anti-scorbutic effect [3,4]. In particular, DHA in solution adsorbs UV light well at the wavelength of 185 nm, which can give interference by overlapping UV absorbance profile with other matrix molecules, but it has little absorbance above the wavelength of 220 nm. This is in contrast to AA that strongly absorbs at the wavelength of 265 nm [5]. To solve the problem of DHA low absorbivity, some authors propose the previous reduction of

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DHA to AA using homocysteine or dithiothreitol. The quantitation of the latter acid allows an indirect estimation of DHA levels [4–14]. Nevertheless, the sample reduction procedures either involved a lengthy manual step prior to chromatography or a post-column reaction. In general, traditional methods, such as titration, spectrophotometry, enzymatic and chemiluminescence measurements require also long analysis times, favouring AA degradation, can give overestimations because of other oxidizable species, or finally are unable to independently measure both AA and DHA. Even if in literature are reported several chromatographic [5,8,15–24] and capillary electrophoresis [5,25] AA and DHA separative procedures, as far as we known there is an only article regarding the determination of DHA as impurity in AA dosage forms (capsules). However, the described LC method requires the use of both UV detector and fluorescence detector [21]. On the other hand, other papers are reported which describe only the quantitation of AA and acetaminophen [26–28].

The derivatization prior [20,21,23,24,29,30] or after [17–19,22] to LC separation constitutes an effective and convenient technique to analyze DHA and to perform selective and sensitive analyses without the need of reduction procedures. In particular, from the used reagents (*o*-phenylenediamine, 2,4-dinitrophenylhydrazine, benzamidine) is reported 4,5-dimethyl-1,2-phenylenediamine (DMPD); it is a fluorogenic reagent and is highly specific for DHA [29,31] especially in comparison to other reagents for carbonylic group such as 2,4-dinitrophenylhydrazine (2,4-DNP), which requires also longer reaction time [30].

The main target of this work was to develop a simple and reliable UV-DAD LC method for the determination of AA and its impurity DHA in pharmaceuticals (effervescent tablets), containing also acetaminophen, which is combined in the formulations for its anti-inflammatory effect. The simultaneous separation of all compounds was achieved in standard solution, but dual chromatographic runs were performed because of the high difficulties to determine together the compounds for their remarkable concentration difference in pharmaceuticals. In particular, AA and acetaminophen, were analyzed directly at the wavelength of 245 nm (procedure A), while DHA was determined after derivatization with DMPD at the wavelength of 360 nm (procedure B) (Fig. 1).

Both the procedures were subjected in detail to validation to allow the application of the method in each common laboratory.

2. Experimental

2.1. Materials

Dehydroascorbic acid (DHA), ascorbic acid (AA), sodium ascorbate, 4,5-dimethyl-1,2-phenylendiamine (DMPD), metaphosphoric acid and acetonitrile for chromatography (HPLC grade) were purchased from Sigma–Aldrich (Milan, Italy). The internal standard (IS), 8-hydroxyquinoline, ethylenediaminetetraacetic acid disodium salt (EDTA) and sodium acetate trihydrate were obtained from Carlo Erba (Milan, Italy). Acetaminophen and cetyltrimethylammonium bromide (CTAB) were purchased from Fluka (Milan, Italy). All the other chemicals were of analytical reagent grade. Deionized, double distilled water was used for all solution and mobile phase preparation. The tablet formulation (type I) was provided from E-Pharma Trento S.p.A. (Ravina, Italy), while the other tablets are commercially available (Table 4).

2.2. Solutions

All solutions were prepared freshly and stored at 2–8 °C during the day. Standard solutions of AA and acetaminophen were prepared in mobile phase (concentration under calibration graphs) in presence of 18% (v/v) EDTA (1 mM) in sodium acetate buffer solution (80 mM; pH 3.7). Standard solutions of DHA (concentration under calibration graphs) were obtained dissolving DHA in sodium acetate buffer solution (pH 3.7) containing EDTA. Sodium acetate buffer (80 mM; pH 3.7) was prepared adding to trihydrate sodium acetate solution glacial acetic acid to the desired pH. The 8-hydroxyquinoline (IS) solutions (about 0.10 mg/mL for procedure A, and 0.016 mg/mL for procedure B) were prepared in mobile phase. Reagent DMPD solution (1 mg/mL) was prepared in the buffer solution (pH 3.7) containing EDTA, as described above. CTAB solution (5 mM) in sodium phosphate buffer (40 mM; pH 3.5) was prepared adding to CTAB the appropriate buffer solution slowly to reduce the froth.

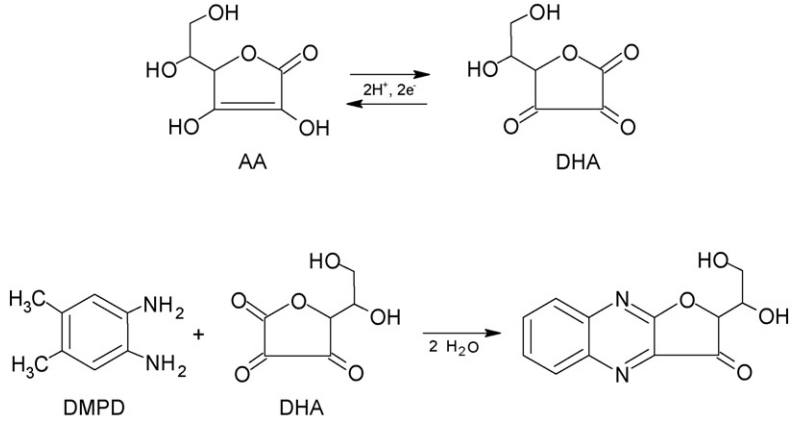

Fig. 1. Chemical structures of AA and DHA and scheme of derivatization reaction of DHA with DMPD.

Sodium phosphate buffer solution (40 mM; pH 3.5) was prepared adding orthophosphoric acid to a sodium phosphate dibasic solution up to the desired pH. Metaphosphoric acid solution (50 mM; pH 3.7) was prepared adding sodium hydroxide (1 M) to an aqueous metaphosphoric acid solution to the desired pH.

2.3. 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). 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 DG 208053 (Jasco, Tokyo, Japan).

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 of DHA with DMPD

To a 300 μL aliquot of DHA solution (reference or sample) were added 300 μL of DMPD solution. The reaction was carried out at ambient temperature for 10 min in the dark in a microcentrifuge tube (2 mL). Then, 600 μL of IS were added and nitrogen was fluxed for 1 min. A 20 μL aliquot of the mixture was injected immediately into the chromatograph or preserved on ice in the dark.

2.5. Chromatographic conditions

LC separations were performed at 30 ± 2 °C on a Phenomenex Synergi 4 μm hydro-RP 80 A (150 mm × 4.6 mm i.d.) stainless steel column. For routine analyses a mobile phase consisting of a mixture of CTAB (5 mM) in sodium phosphate buffer (pH 3.5; 0.04 M)-acetonitrile (90:10, v/v) at a flow-rate of 0.8 mL/min was used. UV-diode array detection, setting the wavelength at 360 nm, for DHA analysis and at 245 nm both for AA and acetaminophen, was employed.

2.6. Specificity

DHA, AA, acetaminophen (0.23, 0.14, 0.20 μmol/mL, respectively), sample solutions, each containing IS (0.72 μmol/mL for AA and acetaminophen, respectively, and 0.11 μmol/mL for DHA), placebo (a mixture of excipients and other ingredients, except the analytes) and blank, corresponding to buffer solution (pH 3.7) containing EDTA, were prepared according to the described procedure. The solutions concerning DHA analysis were subjected to the reported derivatization reaction.

2.7. Linearity

AA and acetaminophen standard solutions and placebo solution spiked of both compounds, DHA standard solution and sample solution spiked of DHA (standard addition method) were prepared in the described EDTA/buffer solution. The concentration ranges are reported in Table 1. Triplicate injections for each solution were made directly for AA and acetaminophen, and after the described derivatization procedure for DHA. The peak-area ratio of analyte to IS was plotted against the corresponding DHA, AA and acetaminophen concentration to obtain the calibration graphs. In particular for DHA, the *x*-intercept was then used for calculating the content of impurity in the analyzed sample (type I formulation, batch 1, Table 4) by the standard addition method.

2.8. Analysis of pharmaceuticals

2.8.1. Sample preparation

2.8.1.1. Effervescent tablets. The formulations were analyzed twice owing to the remarkable concentration difference between AA (procedure A) and its impurity DHA (procedure B). Procedure A: 20 tablets were finely grinded and an amount of powder equivalent to about 8–10 mg of AA and 12–16 mg of acetaminophen depending on the formulation was introduced in a 20 mL volumetric flask and dissolved in the described buffer solution (pH 3.7) containing EDTA, under ultrasonication for 5 min till effervescence extinction; the obtained solution was filled up to volume. Then, to a 1 mL aliquot of the solution were

Table 1
Data for calibration graphs ($n = 5$)

Compound	Slope[a]	Confidence interval	*y*-Intercept[a]	Confidence interval	Correlation coefficient	Concentration range (μmol/mL)
DHA[b]	23.72	±0.52	−0.02	±0.07	0.9996	0.03–0.23
DHA[c]	23.83	±0.53	0.77	±0.08	0.9996	0.03–0.25
AA[b]	6.55	±0.11	0.00	±0.02	0.9998	0.07–0.22
AA[d]	6.62	±0.11	−0.01	±0.02	0.9998	0.07–0.22
Acetaminophen[b]	8.68	±0.20	0.01	±0.04	0.9996	0.10–0.30
Acetaminophen[d]	8.78	±0.31	−0.01	±0.07	0.9991	0.10–0.30

[a] According to $y = ax + b$, where x is the analyte concentration and y is the ratio of compound (procedure A) or adduct of reaction (procedure B) peak-area to IS peak-area.
[b] Standard solution.
[c] Analyte spiked in sample solution.
[d] Analyte spiked in placebo solution.

added 1.5 mL of IS solution filling up to volume of 20 mL with mobile phase. Procedure B: 20 tablets were finely grinded and an amount of powder equivalent to about 100 mg of AA was introduced in a 20 mL volumetric flask and dissolved in buffer solution (pH 3.7) containing EDTA under ultrasonication for 5 min till effervescence extinction and then filled up to volume.

An aliquot of each final solution was filtered through a 0.22 μm regenerated cellulose filter.

2.8.2. Assay procedure

As regards the procedure A, a 20 μL aliquot of solution was directly injected into the chromatograph, while for the procedure B a 300 μL aliquot of the sample solution was previously subjected to the described derivatization reaction.

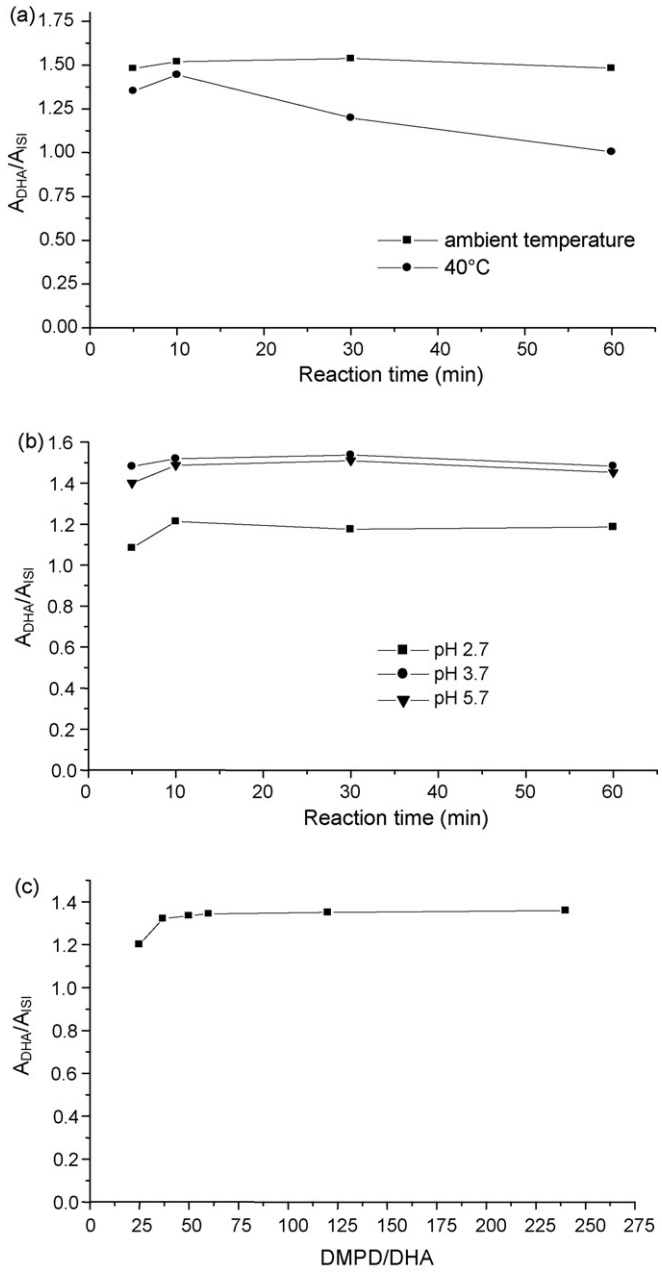

Fig. 2. Effect of the temperature (a), pH (b) and reagent to DHA molar ratio (c) on the derivatization reaction between DMPD and DHA.

DHA, AA and acetaminophen content in each sample was determined by comparison with an appropriate standard solution and for DHA also by the standard addition method.

2.9. Precision

Twenty effervescent tablets were finely grinded and six aliquots corresponding to about 120 mg (procedure A) or 1.2 g (procedure B) of powder were accurately weighted. The solutions were prepared according to the appropriate procedure.

2.10. Accuracy

The accuracy of the method was determined as mean recovery on 9 (procedure A) or 18 (procedure B) solutions, respectively. The solutions contained known amount of AA and acetaminophen corresponding to about 75, 100 and 125% of the claimed content, in presence of placebo, while about 0.1, 0.2 and 0.4% of DHA respect the nominal content of AA. Each solution was injected twice. The recovery was calculated with respect to the standard solutions.

3. Results and discussion

Although the simultaneous separation of DHA adduct, AA and acetaminophen using a standard solution was obtained, the analyte quantitation in dosage forms by using a single run was not realizable, due to the remarkable difference of concentration levels between DHA, present as impurity, and acetaminophen and AA. At the concentration levels necessary for the determination of DHA, unsatisfactory results in terms of peak shape and linearity of response for AA and acetaminophen were obtained. Then, the analysis of DHA and AA in formulations, containing also acetaminophen, was carried out by two chromatographic runs. AA and acetaminophen were determined directly at the wavelength of 245 nm using an appropriately diluted sample

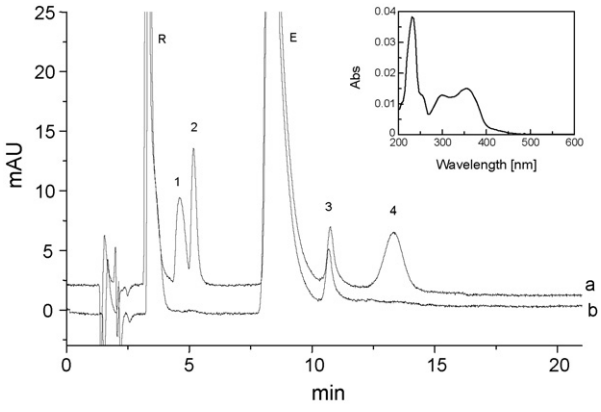

Fig. 3. Representative LC separation at 30 ± 2 °C of: (a) standard mixture of AA (7.8 μmol/mL), acetaminophen (9.2 μmol/mL) and DHA (0.12 μmol/mL) derivatized with DMPD; (b) solvent under derivatization conditions with DMPD (blank). Peak 1 = AA; Peak 2 = acetaminophen; Peak 3 = 8-hydroxyquinoline (IS); Peak 4 = DHA adduct; R = reagent; E = EDTA. UV-DAD detection: λ = 310 nm. Detail: representative UV-DAD spectrum of DHA derivative.

solution, while DHA was analyzed at the wavelength of 360 nm after pre-column derivatization with DMPD to enhance its UV-absorbivity.

3.1. Derivatization reaction of DHA with DMPD

The easy AA oxidation in aqueous solutions depends on a variety of factors such as temperature, light, pH, presence of metal traces and molecular oxygen. In order to prevent oxidation of AA to DHA and subsequent degradation of DHA and DHA derivative, each solution of DHA and reagent was prepared freshly in buffer solution (pH 3.7) containing EDTA, previously subjected to ultrasonication for 15 min, fluxed under current of nitrogen to remove molecular oxygen and stored at 2–8 °C in the dark.

Moreover, to avoid forming further degradation products, the derivatization reaction of DHA with DMPD [29,31] was carried out in mild conditions (room temperature; 10 min) in the dark. In the development of the method, several parameters including time, temperature, reaction solvent, pH and reagent to DHA molar ratio were considered and the reaction course state was graphically reported. As it can be seen in Fig. 2a, the reaction was complete after 10 min at room temperature, while at higher temperature (already at 40 °C) the results were unsatisfactory, probably owing to the easy DHA decomposition to another chemical species. Similar results in the range of pH 3.7–5.7 (Fig. 2b) were found. Moreover, pH 3.7 was chosen because the acidic ambient is favourable to DHA stability [14], even if pH 2.7 was found not advantageous. Under the described conditions, the response intensity reaches a plateau at a reagent to DHA molar ratio of about 35 and further reagent excess does not interfere (Fig. 2c). Metaphosphoric acid solution was considered as reaction ambient, because it is frequently used for the extraction of AA and DHA from complex matrices. It seems to provide the most efficient extraction in food samples because it prevents oxidation of AA better than other acids, even if it may cause serious analytical interactions with silica-based column materials, e.g., C18 or NH_2 bonded phases. These interactions may result in drifts in the baseline and retention time [22]. However, sodium acetate buffer solution has showed to dissolve more easily the reagent and to give higher reaction yields.

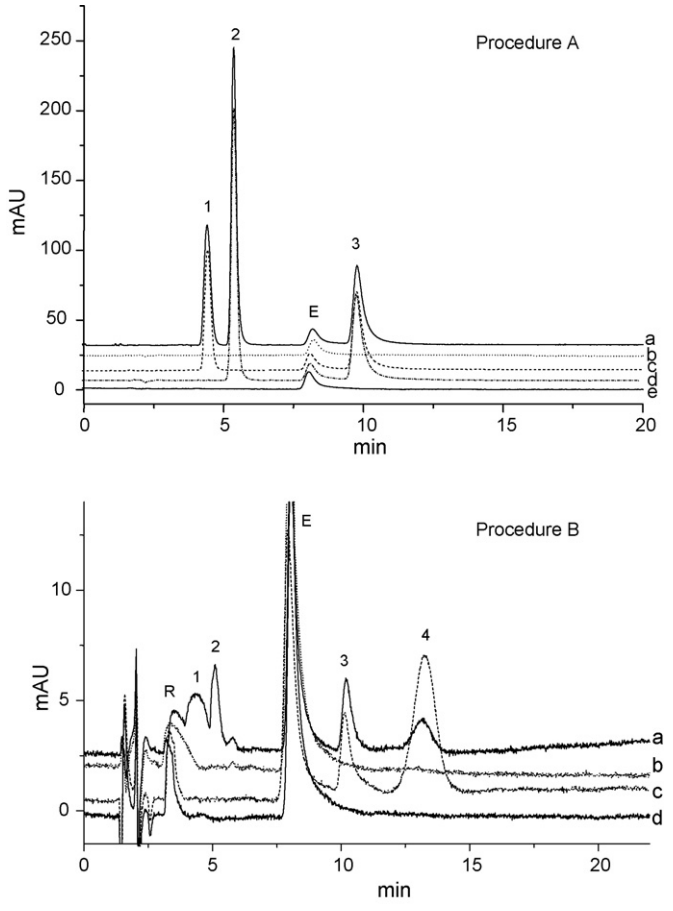

Fig. 4. Overlay of chromatograms. Procedure A: (a) sample, (b) placebo, (c) AA, (d) acetaminophen and (e) solvent (blank). UV-DAD detection: λ = 245 nm. Procedure B: (a) sample, (b) placebo, (c) DHA and (d) solvent under derivatization conditions with DMPD (blank). Peak 1 = AA; Peak 2 = acetaminophen; Peak 3 = 8-hydroxyquinoline (IS); Peak 4 = DHA adduct; R = reagent; E = EDTA. UV-DAD detection: λ = 360 nm.

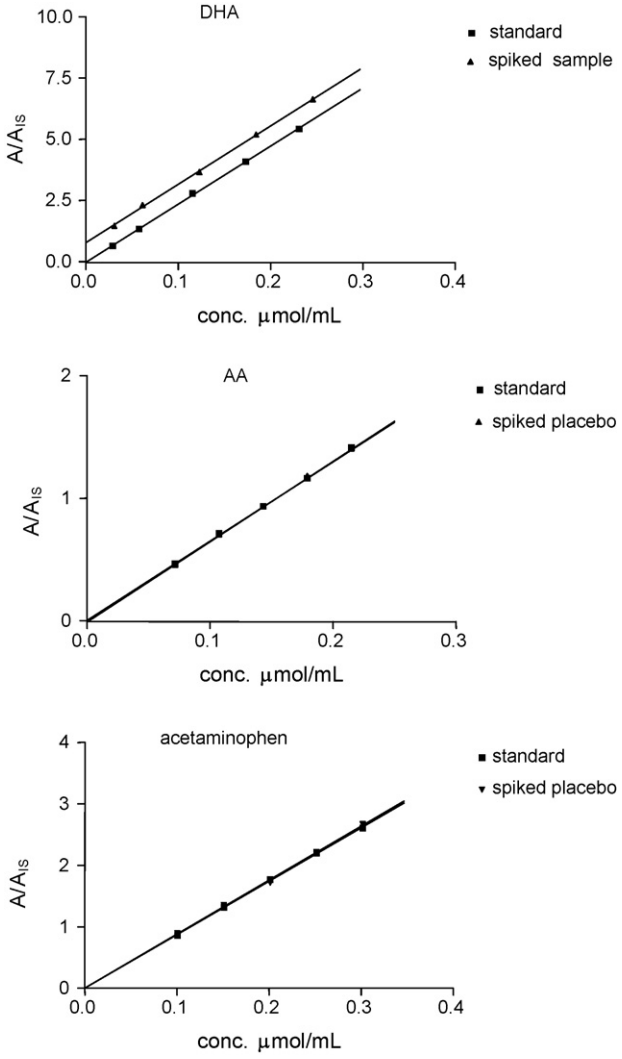

Fig. 5. Calibration graphs of DHA at λ = 360 nm, AA and acetaminophen at λ = 245 nm.

The stability both of the DHA starting solution and DHA solution obtained after derivatization with DMPD was investigated. The solution is considered stable in time if the peak area ratio variation is within ±1.5% of the initial value. DHA solution have been stable for 8 h and the response intensity decrease of 2.8% after 24 h and 20% after 78 h, while the DHA derivatization solution showed a good stability till 3 h, that allows an enough reasonable work time.

3.2. Chromatography

In particular, during the phase of the optimization of analyte chromatographic separation, two RP-columns were compared: Prodigy ODS column and Synergi hydro-RP, a column of new typology. Prodigy™ columns are columns already present on the market for some years. They are made with high purity HPLC silica phases and stable at pH range (2–9) and represent an advance in the analysis of basic, acidic and amphoteric compounds. Unlike conventional C18 columns, Synergi hydro-RP's C18 bonded phase is endcapped with a proprietary polar group to provide high retention both of hydrophobic as well as extremely polar compounds via polar interactions, hydrogen bonding or electrostatic interactions. The high (475 m^2/g) 4-mm silica surface area combined with a dense bonded phase coverage allows for substantial interaction between the sample analyte and the bonded phase yielding overall a very retentive C18 phase. At the same time, several mobile phases were investigated to evaluate the effect of composition and pH on the compound separation. As results of these experiences, using Synergi hydro-RP's column, a mobile phase constituted from a mixture of phosphate buffer (pH 3.5) and acetonitrile as organic modifier in presence of CTAB was found suitable to obtain an adequate separation of all analytes. CTAB allowed to increase the retention time (t_r) of AA and to obtain a better repeatability of t_r for each compound. As it can be seen in Fig. 3, in the described chromatographic conditions the simultaneous separation of all analytes and IS in short time was achieved. Besides, the reagent and EDTA peaks did not interfere with the analysis. For further information, in the detail of Fig. 3 the UV-DAD spectrum of DHA derivatized with DMPD is reported.

3.3. Specificity

For all analyzed formulations, the retention times of analytes and IS in standard solution have been compared with those in placebo, sample and reagent blank solutions prepared under the same conditions. No interferences with the analyte peaks due to placebo or blank have been observed (Fig. 4). On the basis of that, the method results specific for the quali-quantitative analysis of DHA, AA and acetaminophen.

3.4. Linearity

The linearity was determined as linear regression with least-square method on standard solution and spiked placebo solution for AA and acetaminophen and on fortified sample solution of DHA in the type I formulation of complex matrix. Concentration levels were 50, 75, 100, 125, 150% of the claimed analyte concentration, corresponding to the range of about 0.07–0.22 μmol/mL for AA and 0.10–0.30 μmol/mL for acetaminophen (Table 1). On the other hand, the DHA levels were 0.1, 0.2, 0.4, 0.6, 0.8% of the nominal content of AA, corresponding to the DHA concentration range of about 0.03–0.2 μmol/mL. Good linearity was found for each compound as indicated by the coefficient of determination ≥0.9991. As regards AA and acetaminophen, the standard and placebo slope and y-intercept are not significantly different. The overlapping of the calibration curves (Fig. 5) attests that the matrix did not interfere with the compound analysis, while for DHA the calibration graph of standard and spiked sample (standard addition graph) solutions are not superimposable. In fact, the slope values are the same, but the intercept value is significantly different from the 0 value, due clearly to the amount of DHA present in the formulation.

Table 2
Repeatability and intermediate precision (effervescent tablets)

Compounds	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/day1						
DHA	0.74 (0.01)	1.40	0.01 (1.12)	0.32 (0.00)	1.40	0.00 (1.12)
AA, sodium salt	0.93 (0.01)	0.56	0.00 (0.45)	281.27 (1.40)	0.56	1.12 (0.45)
Acetaminophen	1.81 (0.01)	0.66	0.01 (0.53)	310.94 (2.05)	0.66	1.64 (0.53)
Analyst B/day 2						
DHA	0.72 (0.01)	1.14	0.01 (0.91)	0.31 (0.00)	1.14	0.00 (0.91)
AA, sodium salt	0.92 (0.01)	0.65	0.00 (0.52)	278.47 (1.61)	0.65	1.29 (0.52)
Acetaminophen	1.78 (0.01)	0.52	0.01 (0.42)	305.28 (1.58)	0.52	1.27 (0.42)
Intermediate precision ($n=12$)						
DHA	0.73 (0.01)	1.88	0.01 (1.06)	0.32 (0.01)	1.88	0.00 (1.06)
AA	0.92 (0.01)	0.78	0.00 (0.44)	279.87 (1.94)	0.78	1.10 (0.44)
Acetaminophen	1.80 (0.02)	1.11	0.01 (0.63)	308.11 (3.43)	1.11	1.94 (0.63)

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

Table 3
Accuracy

Compound	Level (%)	Spiked amount[a]	Theoretical[b] (μg/mL)	Found (μg/mL)	Recovery (%)	Mean recovery[c] (%)	R.S.D. (%)	Mean recovery (%, $n = 9$)	R.S.D. (%)
AA, sodium salt	75	8.41	21.02	21.12	100.5	100.7	0.87	100.4	0.82
		8.60	21.50	21.86	101.7				
		8.74	21.85	21.85	100.0				
	100	11.65	29.12	29.43	101.0	100.3	1.00		
		11.56	28.90	28.71	99.5				
		11.19	27.98	28.06	100.3				
	125	14.10	35.25	35.11	99.6	100.2	1.06		
		14.21	35.52	35.34	99.5				
		14.04	35.10	35.58	101.4				
Acetaminophen	75	9.07	22.68	22.98	101.3	100.4	1.65	101.1	1.10
		9.12	22.80	22.45	98.5				
		9.17	22.92	23.22	101.3				
	100	12.06	30.15	30.52	101.2	101.7	0.56		
		12.04	30.10	30.58	101.6				
		11.99	29.98	30.67	102.3				
	125	15.09	37.72	37.89	100.4	101.2	0.70		
		15.06	37.65	38.19	101.4				
		15.33	38.32	39.02	101.8				
DHA	0.1	5.50	11.60	11.55	99.6	100.4	1.82	100.6	1.50
			11.62	11.40	98.1				
			11.58	11.60	100.2				
			11.63	11.60	99.7				
			11.61	11.76	101.3				
			11.64	12.04	103.4				
	0.2	11.30	17.68	18.08	102.3	101.8	0.66		
			17.68	17.86	101.0				
			17.68	17.89	101.2				
			17.72	18.22	102.8				
			17.74	18.04	101.7				
			17.68	18.00	101.8				
	0.4	20.60	27.01	26.59	98.5	99.7	1.12		
			26.99	27.05	100.2				
			27.00	27.30	101.1				
			27.03	26.79	99.1				
			26.95	27.15	100.7				
			26.97	26.61	98.7				

[a] mg in about 120 mg of placebo (procedure A); μg/mL in about 1200 mg of sample (procedure B).
[b] Spiked amount (procedure A); spiked amount + content of the sample (procedure B).
[c] $n = 3$ (procedure A) and $n = 6$ (procedure B).

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

LOD was determined considering the signal/noise ratio of 3:1, while for LOQ 10:1. The LOD values were 15, 12.5 and 60 pmol for AA, acetaminophen and DHA, respectively, while LOQ data were 50, 40 and 140 pmol for AA, acetaminophen and DHA, respectively.

3.6. Precision

The precision of the method was expressed as repeatability and intermediate precision. The repeatability was calculated both on standard and sample solutions employing in the latter case 6 test solutions, each one prepared starting from an homogeneous finished product sample. The results of within-run precision (repeatability) obtained from replicate analysis ($n = 8$) of a standard solution of AA (25 μg/mL) and acetaminophen (30 μg/mL) for procedure A and DHA (12 μg/mL) for procedure B, were satisfactory, as indicated by the R.S.D. value: 0.45, 0.78 and 0.64%, for AA, acetaminophen and DHA, respectively. The intermediate precision of the method was determined on the sample with 12 solutions, prepared changing the parameters time-analyst: 6 solutions were prepared by the analyst A in the day 1, while the other 6 solutions were prepared by the analyst B in the day 2. The results of precision determined on the sample (type I formulation) are reported in Table 2. 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) = 1.67, 1.30, 1.55 for acetaminophen, AA and DHA, respectively, were smaller than the tabulated F value, $F_{0.05}$ (5,5) = 5.05. Moreover, the analysis of preparation representative samples has been performed in two different laboratories. No statistically significant differences were found between inter-laboratory results.

The intermediate precision and applicability of the method in different laboratories in addition to the solution stability provide an indication of the method ruggedness and robustness.

3.7. Accuracy

The accuracy was calculated on the recovery of known amounts of analyte, spiking analyte in placebo (procedure A) or sample (procedure B). Spiked samples were prepared in triplicate at three levels over a range of 75–125% of the target concentration of AA and acetaminophen, while for DHA at three levels corresponding of 0.1, 0.2, 0.4% of AA. Quantitative recovery was obtained in each instance (98.7–101.8%; R.S.D. ≤1.82%) (Table 3).

3.8. Analysis of pharmaceuticals

The results of some new (type I) and commercial (type II and III) formulations (Table 4) were found in agreement with the nominal content and within the fixed range of USP (90.0–110.0%) for several dosage forms [32]. Other formulation ingredients did not interfere with the analysis. As it can be seen DHA impurity (calculated as percentage w/w respect to AA) is present at level ≤0.2% of AA in each formulation. Besides, the results of the type I formulation (batch 1) obtained using the reference method were comparable with those obtained with the standard addition method. The Student's t-test value at a 95% confidence level for 6 degrees of freedom ($t_{calculated} = 2.432$) did not exceed the tabulated value of $t = 2.477$, indicating no significant difference between the methods. The variance ratio F-test value calculated for $P = 0.05$ and $f_1 = 4, f_2 = 2$ ($F_{calculated} = 16.16$) did not exceed the tabulated value of $F = 19.25$, again indicating that was no significant difference between the precision of two analytical procedures.

Table 4
Results for the LC determination of acetaminophen, AA and DHA in pharmaceutical formulations

Effervescent tablets		%Found[a] (R.S.D.)		
		Acetaminophen	AA[b]	DHA
Type I	Batch 1	101.46 (0.53)	99.30 (0.88)	0.11 (1.15)
	Batch 2	105.64 (0.84)	97.51 (1.16)	0.19 (1.60)
	Batch 3	102.26 (0.49)	102.82 (1.16)	0.10 (0.54)
Type II[c]		100.19 (0.81)	97.66 (0.72)	0.13 (0.65)
Type III[d]		103.14 (0.59)	100.45 (0.67)	0.23 (1.79)

[a] Mean of five determinations expressed as a percentage of the claimed content, for AA and acetaminophen, and as percentage w/w of DHA to AA.
[b] Sodium ascorbate in type I formulation and ascorbic acid in type II and III formulations.
[c] Other ingredients: sodium bicarbonate, citric acid, mannitol, fumaric acid, sodium saccharin, lemon flavor; polyvinylpyrrolidone, sodium docusate.
[d] Other ingredients: sodium bicarbonate, potassium bicarbonate, sorbitol, citric acid, sodium benzoate, sodium docusate, polyvinylpyrrolidone.

4. Conclusions

The UV-DAD HPLC validated method has proved to allow a reliable quality control of AA and acetaminophen formulations with complex composition and can be applied in common analytical laboratories, not requiring a sophisticated instrumentation. The procedures are simple and the analyses were performed by mild conditions in absence of preliminary extraction methodologies or laborious step of sample pre-treatment. The fluorogenic reagent DMPD has been showed to be suitable also for the UV determination of DHA in pharmaceutical formulations at very low levels (≤0.2% respect to AA), without the necessity of more sensitive detectors.

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