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Graphical Abstract

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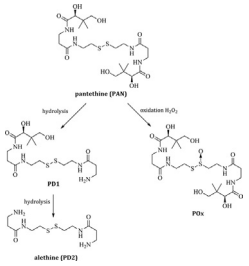
Development and validation of a stability-indicating LC–UV method for the determination of pantethine and its degradation product based on a forced degradation study

Rossana Canavesi, Silvio Aprile, Elena Varese, Giorgio Grosa*



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Highlights

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Development and validation of a stability-indicating LC–UV method for the determination of pantethine and its degradation product based on a forced degradation study

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- Degradation products of pantethine were identified by LC–MS/MS analysis.
- Hydrolytic and oxidative degradation pathways of pantethine were elucidated.
- The LC–UV method was validated and applied for pantethine and its degradation product assays.
- The LC–UV method was applied for determination of preservant system.
- The LC–UV method was applied for analysis of real samples in stability studies.

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Development and validation of a stability-indicating LC–UV method for the determination of pantethine and its degradation product based on a forced degradation study

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ABSTRACT

Pantethine (D-bis-(*N*-pantothenyl-β-aminoethyl)-disulfide, PAN), the stable disulfide form of pantetheine, has beneficial effects in vascular diseases being able to decrease the hyperlipidaemia, moderate the platelet function and prevent the lipid peroxidation. Furthermore, recent studies suggested that PAN may be an effective therapeutic agent for cerebral malaria and, possibly, for neurodegenerative processes. Interestingly, in the literature, there were no data dealing with the chemical stability and the analytical aspects of PAN. Hence, in the present work the chemical stability of PAN was for the first time established through a forced degradation study followed by liquid chromatography tandem mass spectrometry investigation showing the formation of three degradation products of PAN (PD1, PD2 and POx) arising from hydrolytic, thermal and oxidative stresses. Based on these data a stability-indicating LC–UV method for simultaneous estimation of PAN, and its most relevant degradation product (PD1) was developed and validated; moreover the method allowed also the separation and the quantification of the preservative system, constituted by a paraben mixture. The method showed linearity for PAN (0.4–1.2 mg mL^{-1}), MHB, PHB (0.4–1.2 μg mL^{-1}) and PD1 (2.5–100 μg mL^{-1}); the precision, determined in terms of intra-day and inter-day precision, expressed as RSDs, were in the ranges 0.4–1.2 and 0.7–1.4, respectively. The method demonstrated to be accurate and robust; indeed the average recoveries were 100.2, 99.9, and 100.0% for PAN, MHB and PHB, respectively, and 99.9 for PD1. By applying small variations of the mobile phase composition, counter-ion concentration and pH the separation of analytes was not affected. Finally, the applicability of this method was evaluated analyzing the available commercial forms at release as well as during stability studies.

Q2 1. Introduction

Pantethine (D-bis-(*N*-pantothenyl-β-aminoethyl)-disulfide, PAN) is the stable disulfide form of pantetheine, and the precursor of coenzyme A, an essential cofactor involved in ~4% of primary metabolic pathways, playing a central role in the metabolism of fatty acids, polyketide and non-ribosomal synthases [1]. Pantethine has beneficial effects in vascular diseases being able to decrease the hyperlipidaemia, moderate the platelet function and prevent the lipid-peroxidation [2]. Additionally its positive role on cataract and cystinosis diseases was also proved [3,4]. Moreover recent and exciting studies showed that administration of pantethine prevented the cerebral syndrome in *Plasmodium berghei* ANKA-infected mice preserving the blood-brain barrier integrity [5]. These features suggested the possibility that, in combination with anti-parasitic treatment, pantethine may be an effective therapeutic agent for cerebral malaria [5,6]. Finally, the role of pantethine in the treatment of neurodegenerative processes (e.g. Parkinson's disease) has been also reported [7]. Overall, one of the most favourable aspects of pantethine is that this molecule is a well-tolerated therapeutic agent being the frequency of its side-effects very low and mild [8]. Pantethine as active ingredient is currently available in two forms: a highly compressible 55% powder (Pantesin® HF 55) and the 80% liquid

Abbreviations: PAN, pantethine (D-bis-(*N*-pantothenyl-β-aminoethyl)-disulfide); PD1, D-*N*-pantothenyl-*N*′-β-alanyl-bis(β-aminoethyl)-disulfide; PD2, bis-[*N*-(β-alanyl)-2-aminoethyl]-disulfide, alethine; EHB, ethyl-*p*-hydroxybenzoate; MHB, methyl-*p*-hydroxybenzoate; PHB, propyl-*p*-hydroxybenzoate; HBA, *p*-hydroxybenzoic acid; CYS, cystamine dihydrochloride.

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form, the last one generally in combination with a preservative system. While Pantesin® may be advantageously employed in the formulation of tablets or capsules with minimal processing required, the high potency liquid form may be used in the manufacture of soft-gelatin elastic capsules: this is an excellent choice for high-dosage forms and multiple ingredient formulations. It is worth of mention that PAN is considered at the same time, a drug and a dietary supplement depending on the country in which it is commercialized.

Despite increasing number of studies dealing with the biological activities of PAN showing its potential role in the treatment of relevant vascular and neurodegenerative diseases, a comprehensive search of the relevant literature did not yield any results on its chemical stability and its analytical aspects. Hence, no validated stability-indicating LC method for the determination of PAN in drug substance and drug product has been so far published. These features suggested that an investigation on the stability of PAN and the development of a suitable stability-indicating analytical method are demanding in light of the requirements of the current international guidelines [9]. Hence, in the present work we have established for the first time the intrinsic chemical stability of PAN through a forced degradation study and LC–ESI–MS/MS investigation. Based on these data a stability-indicating LC–UV method for simultaneous estimation of PAN, its main degradation product and preservative system was developed. The validation of the proposed method was also carried out and its applicability was evaluated analyzing the available commercial forms.

2. Experimental

2.1. Reagents and chemicals

Pantethine ($C_{22}H_{42}N_4O_8S_2$ Mw: 554.72, >99%), methanol (HPLC grade), potassium phosphate monobasic, sodium phosphate dibasic, concentrated ortophosphoric acid, iron(III) trichloride hexahydrate, copper sulphate pentahydrate, dichloromethane, 30% w/w hydrogen peroxide solution, cystamine dihydrochloride (CYS), methyl-*p*-hydroxybenzoate (MHB), ethyl-*p*-hydroxybenzoate (EHB), propyl-*p*-hydroxybenzoate (PHB), *p*-hydroxybenzoic acid (HBA), sodium 1-pentanesulfonate, poly(ethylene glycol) 400, glycerol, (hydroxypropyl)methyl cellulose, L-leucine β-chloropropionyl chloride, sodium azide and triphenylphosphine were purchased from Sigma-Aldrich (Milano; Italy). Water (HPLC grade) was obtained from Milli-Q RO system. The drug product, Pantethine® soft-gelatin capsules, and the drug substance pantethine working standard (80%) were obtained as gift samples from Pharmafar S.r.l. (Torino, Italy). Pantethine working standard contained water (20%) and a preservative system constituted by a MHB and PHB mixture. The drug product Pantethine® soft-gelatin capsules contained PEG400 and glycerol as excipients. Moreover the preservative EHB is used in the capsule shell manufacturing process. The dietary supplement Pantethine capsules, containing the active ingredient Panthesin® and the excipients ((hydroxypropyl)methyl cellulose and L-leucine, (Allergy Research Group LLC, Alameda, CA, USA) was purchased on-line. The main physico-chemical properties of pantethine, parabens and their related substances were herewith reported: CYS (clog *P*: 0.62, pK_a 8.60) [10,11]; PAN (log *P*: −4.17) [12]; PD2 (bis-(*N*-β-alanyl-β-aminoethyl)-disulfide, alethine) (clog *P* −1.29 ± 0.57, cpK_a 9.24 ± 0.10); HBA (log *P*: 1.58; pK_a: 4.54); MHB (log *P*: 1.96; pK_a: 8.4); EHB (log *P*: 2.47; pK_a: 8.34); PHB (log *P*: 3.04; pK_a: 8.34) [13,14]. cLog *P* and cpK_a values were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02.

2.2. Instrumentation and chromatographic conditions

2.2.1. LC–UV analyses (method A)

A Shimadzu HPLC system (Shimadzu Europe GmbH, Duisburg, Germany), consisting in two LC-10AD *Vp* module pumps and a DGU-14-A on-line vacuum degasser, was used. The analyses were carried out on a Luna C18(2) (150 × 4.6 mm I.D., 5 μm d_p; Phenomenex, Torrance, CA, USA) column as a stationary phase. The composition of the mobile phase was: eluant A: sodium 1-pentanesulfonate (8 mM) in aqueous phosphate buffer (pH = 2.5; 20 mM); eluant B: sodium 1-pentanesulfonate (8 mM) in phosphate buffer (pH = 2.5; 20 mM)–CH_3OH (20:80 v/v). The following gradient, at a constant flow rate of 1.5 mL min^{-1}, was used: from 0 to 10 min, the composition was increased from 37.5 to 100% B. From 10 to 12 min the percentage of eluant B was decreased to 37.5% and then maintained 6 min for column equilibration (method A). The eluants A and B were filtered through a 0.45 μm PVDF membrane filter prior the use. A SIL-10AD *Vp* autosampler was used for the injection of samples (20 μL). The SPD-M10A *Vp* photodiode array detector was used to detect PAN and preservatives (MHB and PHB) at 254 nm and the degradation products (PD1) at 210 nm. A LC solution 1.24 software (Shimadzu) was used to process the chromatograms. All the analysis were carried out at room temperature.

2.2.2. Semipreparative LC–UV (method B)

Semipreparative chromatography was performed on a Waters LC system equipped with a model 2487 dual λ absorbance detector, a model 600 controller and a Delta 600 pump system (Waters Corporation, Milford, MA, USA); the separations were carried out on a Luna C18(2) (250 × 10 mm I.D., 5 μm d_p; (Phenomenex) column as a stationary phase. The mobile phase consisted in a mixture of eluant A: H_2O/CH_3OH 80/20 and eluant B: H_2O/CH_3OH 20/80 using the following gradient at a constant flow rate of 4.5 mL min^{-1}: from 0 to 9 min the composition was maintained at 0% B then, from 9 to 9.5 min, increased to 100% B. From 9.5 to 16 min the percentage of eluant B was maintained to 100%. Finally from 16 to 16.50 min the percentage of eluant B was decreased to 0% and then maintained for 6 min for column equilibration. A 7775 Rheodyne valve was used for the injection of samples (100 μL, injected mass: ~35 mg). The detector was set at 254 nm. The Enpower 1 software (Waters Corporation) was used to process the chromatograms. All the separations were carried out at room temperature.

2.2.3. LC–MS/MS analyses (method C)

A Thermo Finningan LCQ Deca XP Plus system equipped with a quaternary pump, a Surveyor AS autosampler, a Surveyor PDA detector and a vacuum degasser was used for LC–MS analysis (Thermo Electron Corporation, Waltham, MA, USA).

The analysis were performed on a Luna C18(2) (150 × 4.6 mm I.D., 5 μm d_p; (Phenomenex) column as a stationary phase maintained at 35 °C. The mobile phase (flow rate 0.8 mL min^{-1}) was composed of eluant A: H_2O (0.1% v/v HCOOH) and eluant B: CH_3OH using a linear gradient: from 30% to 80% solvent B in 10 min, afterward the B fraction was decreased to 30% in 2 min and then maintained for 6 min for the equilibration; the sample injection volume was 10 μL. The eluate was injected into the electrospray ion source (ESI) with a splitting of 40% and the MS and MS/MS spectra were acquired and processed using the Xcalibur® software (Thermo Electron Corporation). The operating conditions of the ion trap mass spectrometer in positive ion mode were the following: spray voltage, 5.30 kV; source current, 80 μA; capillary temperature, 350 °C; capillary voltage, 44.0 V; tube lens offset, −20 V; multipole 1 offset, −8 V; multipole 2 offset, −21.50 V; sheath gas flow (N_2), 55 Auxiliary Units; sweep gas flow (N_2): 8.0 Auxiliary Units. Data were acquired both full scan and MS/MS product ion scan modes using

mass scan range *m/z* 110 to 500, optimizing the collision energy at 32–35%.

2.2.4. ^{1}H,^{13}C-NMR and spectroscopic analyses

^{1}H and ^{13}C experiments were performed on a JEOL Eclipse ECP 300 FT MHz spectrometer (Jeol Ltd. Tokyo, Japan) operating at 7.05 T. Chemical shifts are reported in part per million (ppm). UV–vis and FT-IR experiments were performed on a Lambda 35 (Perkin-Elmer, Waltham, MA, USA) and on AVATAR 370 FT-IR (Thermo Fisher Scientific, Madison, WI, USA) spectrophotometers respectively.

2.3. Chemical stability

2.3.1. Forced degradation study

All degradation studies in solution were done at a PAN (80% working standard) concentration of 1 mg mL^{-1}. For acidic degradation study, PAN was dissolved in 0.1 M HCl and the solution was left in the dark at ambient temperature for 24 h or alternatively for 6 h at 45 °C. The degradation in alkaline conditions was done in 0.01 M NaOH and the solution was left in the dark at ambient temperature for 5 h. To test the stability in neutral solution, PAN was dissolved in water and left at room temperature or at 60 °C for 18 h. For oxidative conditions, the degradation was done in a 1% hydrogen peroxide solution and it was left in the dark at room temperature for 5 h. Moreover, to evaluate the influence of transition metals, PAN solution was treated with 1.5 mM Cu^{2+} or Fe^{3+} at ambient temperature for 3 h. For thermal stress, PAN was placed in a hot air oven maintained at 60 °C for 18 h. Photodegradation studies were carried out, at room temperature, by exposing a thin layer (50 mg) of PAN to daylight and UV–light (366 nm) for 240 and 6 h, respectively.

Before LC–UV (method A) and LC–MS/MS (method C) analyses, acidic and alkaline samples were first neutralized by addition of a suitable amount of sodium phosphate dibasic and concentrated phosphoric acid, respectively, and then diluted by adding an appropriate volume of mobile phase. The samples arising from oxidative, thermal and photolytic stresses, were diluted with an appropriate volume of mobile phase. For comparison purposes a freshly prepared aqueous solution of PAN (1 mg mL^{-1}) was diluted and analyzed as above.

2.3.2. Long-term and accelerated stability studies

The stability study was performed in a Binder-KBF-115 constant climate chamber (Binder GmbH, Tuttlingen Germany). Drug product (Pantethine® soft-gel capsules) packaged in the container closure system used for marketing was stored using the following conditions: long-term study: 25 °C ± 2 °C 60% RH ± 5%, 24 months.

2.4. Preparation of reference samples of the degradation products

The reference sample of PD1 was obtained from thermal stress of pantethine and isolated and purified by semipreparative LC–UV (method-B) (Supplemental 1). The reference sample of PD2 (bis-[*N*-(β-alanyl)-2-aminoethyl]-disulfide) was obtained from the synthetic procedure reported in Supplemental 2. The structural characterization of PD1 and PD2 was also reported in Supplemental 1 and 2).

2.5. Preparation of standard and sample solutions

2.5.1. Preparation of standard stock solutions for validation process

The standard solutions of PAN (8 mg mL^{-1}), PD1(1 mg mL^{-1}) and CYS (1 mg mL^{-1}) were prepared using water as solvent. The combined standard solution of preservatives MHB (40 μg mL^{-1}) and PHB (40 μg mL^{-1}) was prepared in a CH_3OH/H_2O 20:80 (v/v) mixture. The standard solution of HBA (4.75 μg mL^{-1}) was prepared by dissolving an appropriate amount (about 47.5 mg) of HBA in 100 mL 95/5 H_2O/CH_3OH mixture; 2.0 mL of the solution were diluted to 200 mL using water as solvent. The stock solutions were properly diluted with water to obtain the working solutions that have been used in the validation protocol.

2.5.2. Preparation of test solutions

2.5.2.1. Test solution for PAN assay. The contents of 20 capsules of the drug product Pantethine® or of the dietary supplement Pantethine were accurately mixed and about 550 mg of the mixture were weighted and transferred into a 250 mL volumetric flask. The volumetric flask was filled to volume with water. After filtration through 0.45 μm PVDF membrane filter, the sample was analyzed by LC–UV (method A).

2.5.2.2. Test solution for degradation product assay. The solution was prepared with the same protocol described above using a 50 mL volumetric flask.

2.6. Validation procedure of method-A

2.6.1. System suitability

The system suitability parameters, resolution (*Rs*), area repeatability and asymmetry factor (*As*) were calculated as previously reported [15].

2.6.2. Selectivity

To assess the method selectivity a combined reconstructed capsule formulation without PAN was prepared using the excipients used for the drug product Pantethine® (PEG400 and glycerol) and for dietary supplement Pantethine ((hydroxypropyl)methyl cellulose and L-leucine). For HPLC analysis the solution was prepared using the same procedure of analytical sample. Moreover to evaluate the chromatographic separation of the analytes PAN, PD1, MHB and PHB from the impurities arising from capsule shell (EHB) or from synthetic (CYS) and degradation (PD2 and HBA) processes, a combined standard solution was prepared and analysed. After LC–UV analyses, resolution factors were calculated.

2.6.3. Robustness

To determine the robustness three parameters were varied: mobile phase pH, counter-ion concentration and the percentage composition of eluant. The influence of the pH of the mobile phase was studied by analyzing the standard mixture of analytes CYS, HBA, PAN, MHB, PHB, EHB and PD1 at three different values: 2.4, 2.5 and 2.6 being 2.5 the reference value. The influence of mobile phase composition was determined by varying the initial (28–30–32%) and final (76–80–84%) percentages of eluant B. Finally the influence of the variation of the counter-ion (sodium 1-pentanesulfonate) concentration was studied in the range 7.5–8.5 mM being 8.0 the reference value. In all cases the effects of the small changes brought to the method have been evaluated by calculating the resolution factors.

2.6.4. Stability of standard and test solutions

To study the stability of the standard and test solutions, analyses at $t = 0$ and after 12 h at room temperature storage were done. Results were expressed as percent of PAN remaining.

2.6.5. Linearity

Linearity of the method was evaluated at five equispaced concentration levels by diluting the standard solutions to give solutions over the range 50–150% of the target concentration for

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PAN and parabens (MHB and PHB) and 0.05–2.0% for degradation product PD1. These were injected in triplicate and the peak areas were inputted into a Microsoft Excel® spreadsheet program to plot calibration curves. To fulfil basic requirements such as homoscedasticity and linearity, the Bartlett test and the lack-of-fit test were, respectively, performed at the 95% significance level.

2.6.6. Precision

Precision was evaluated in terms of repeatability and intermediate precision.

The repeatability was investigated using six separate sample solutions prepared, as reported above, from the freshly reconstructed capsule formulation at 100% of the target level. Each solution was injected in duplicate and the peak areas obtained were used to calculate means and RSD values.

The intermediate precision was checked on four different days, by preparing and analysing in triplicate four separate sample solutions from the reconstructed formulations at the same concentration level of repeatability; the means and RSD values were calculated from peak areas.

2.6.7. Accuracy

To assess accuracy, freshly prepared combined placebo of the pharmaceutical formulations was spiked with various amounts of PAN, MHB and PHB at 50, 100 and 150% concentration levels and PD1 at 0.05, 0.1 and 1.0% concentration levels. Each solution was injected in triplicate and the peak areas were used to calculate means and RSD values and compared with those obtained with standard solutions.

2.6.8. LOD–LOQ

The determination of LOD and LOQ was based on signal-to-noise ratio: this was evaluated by comparing measured signals from samples with known low concentrations of PD1 with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably detected (LOD; signal-to-noise ratio = 3) or quantified (LOQ; signal-to-noise ratio = 10).

2.6.9. Determination of PD1 relative response factor (RRF)

PD1 and PAN solutions at 50 μg mL^{-1} concentration were prepared by dilution of the corresponding standard solutions; the replicated LC–UV (method A) analyses ($n = 6$) were performed at $\lambda_a = 210$ nm and peak areas of PD1 and PAN were integrated. RRF of PD1 was calculated using the following formula RRF = PD1 area/PAN area.

3. Results and discussion

3.1. Forced degradation study and structural characterization of degradation products

The degradation of PAN in neutral, alkaline and acidic solutions and in the presence of other stress conditions (e.g. oxidative, thermal and photolytic) has never been previously reported in the literature. In the present forced degradation study all the stress conditions required by ICH guidelines were included; moreover, in order to avoid unrealistic degradation pathways, the conditions were fixed to obtain about 10–20% degradation of the parent compound. As a matter of fact, in 0.01 M NaOH and 0.1 M HCl the degradation of PAN took place with the formation of the common and more polar degradation product PD1 (tR = 4.45 min) as reported in LC–UV chromatograms (Fig. 1a1 and b1). It is worth of mention that peaks appearing at tR = 7.4 and 10.8 min corresponded to MHB and PHB preservatives. Increasing the reaction time or temperature, the formation of another degradation product, PD2 (tR = 3.15 min) was observed; similarly, the increase of HBA peak (tR = 3.95 min), mainly in the alkaline solution, was also observed (Fig. 1a2). The presence of several amide functions in the structure of PAN suggested that their hydrolysis could cause the formation of different degradation products whose structures were investigated analyzing the reaction mixtures by liquid chromatography tandem mass spectrometry. The extracted ion chromatograms (Supplemental 3) obtained from positive full scan analysis of the alkaline samples revealed the presence of three peaks corresponding to PAN ($m/z = 555$, tR = 6.94 min), PD1 ($m/z = 425$, tR = 2.79 min), and PD2 ($m/z = 295$, tR = 2.25 min) whose MS spectra are reported in Supplemental 4. The degradation products PD1 and PD2 were identified by monitoring the positive ions related to the products arising from the hydrolysis of one or two amide functions, respectively. The positive product ion spectrum of PD1 ($m/z = 425$) depicted in Fig. 2a shows several diagnostic fragment ions at m/z 407 (loss of water), 295 (amide cleavage) and subsequent loss of ammonia (278). On the contrary PD2 ($m/z = 295$) protonated molecule (Fig. 2b) only shows the fragment ion at m/z 278 (loss of ammonia). The peaks appearing at tR = 6.94 min (Supplemental 3, trace B) and 2.88 min (Supplemental 3, trace C) were attributed to artifacts arising from the in-source fragmentation of PAN (peak with tR = 6.94 min) and PD1 (peak with tR = 2.88 min); indeed already during in source charged droplet desolvation and before collision-induced dissociation, PAN underwent both single or double amide cleavage, being present the ions at m/z 425 and 295 in its full scan spectrum. The same occurred for PD1 which can only generate the ion at m/z 295 (see mass spectrum in Supplemental 4). The structure of PD1 was confirmed by the isolation and purification of a reference sample from PAN thermal degradation (Supplemental 1) using semipreparative LC–UV (method B). The purity of PD1 reference sample was 97.8% and its ^{1}H-NMR spectrum showed the presence of the singlet signals at 0.9 and 3.9 ppm referable to the equivalent methyl protons (C3 and C4) and the C5 proton, respectively, belonging to the pantoic acid residue: in particular the analysis of their intensities, done by integration, indicated the presence of only one residue of pantoic acid. These data were confirmed by ^{13}C-NMR spectrum where the signals of only three carbonyl groups were present at 175.5, 173.4 and 171.9 ppm.

The structure of PD2 was attributed by comparison of its retention time and MS/MS data with those of the synthetic standard obtained in a three-step synthesis starting from cystamine (Supplemental 2). The structure of PD2 reference sample was assigned by ^{1}H- and ^{13}C-NMR analyses. In the ^{1}H-NMR spectrum the lack of diagnostic signals (0.9 and 3.9 ppm) arising from pantoic acid residue was observed, while the triplets from methylenic protons were present at 3.5, 2.9, 2.8 and 2.4 ppm. Finally, as expected, a single signal referable to the symmetrical carbonyl groups (C3 and C8) was present in the ^{13}C-NMR spectrum confirming the structure of alethine for PD2. LC–MS and MS/MS analysis of PAN in acidic solutions showed the formation, even if to a minor extent, of the same degradation products already described for alkaline degradation. In neutral aqueous solution at ambient temperature, PAN was stable, however at 60 °C a degradation product was observed in LC–UV chromatogram (Supplemental 5) being its retention time and mass spectral data identical to those of PD1.

Regarding HBA peak, its presence and its increase in alkaline stressed samples did not appear in relation with PAN degradation. Indeed, even if HBA was also present, as a low-intensity peak, in the chromatograms obtained from other stress conditions, it was not in that of a freshly prepared solution of pantethine working standard (Supplemental 11). Moreover HBA was not observed in the chromatograms obtained from Pantethine hard capsules (Supplemental 13) a paraben-free preparation. Hence, taking into account the preferential formation of HBA in alkaline conditions and the presence of methyl and propylparaben esters in the pantethine working standard and its corresponding drug product, the structure

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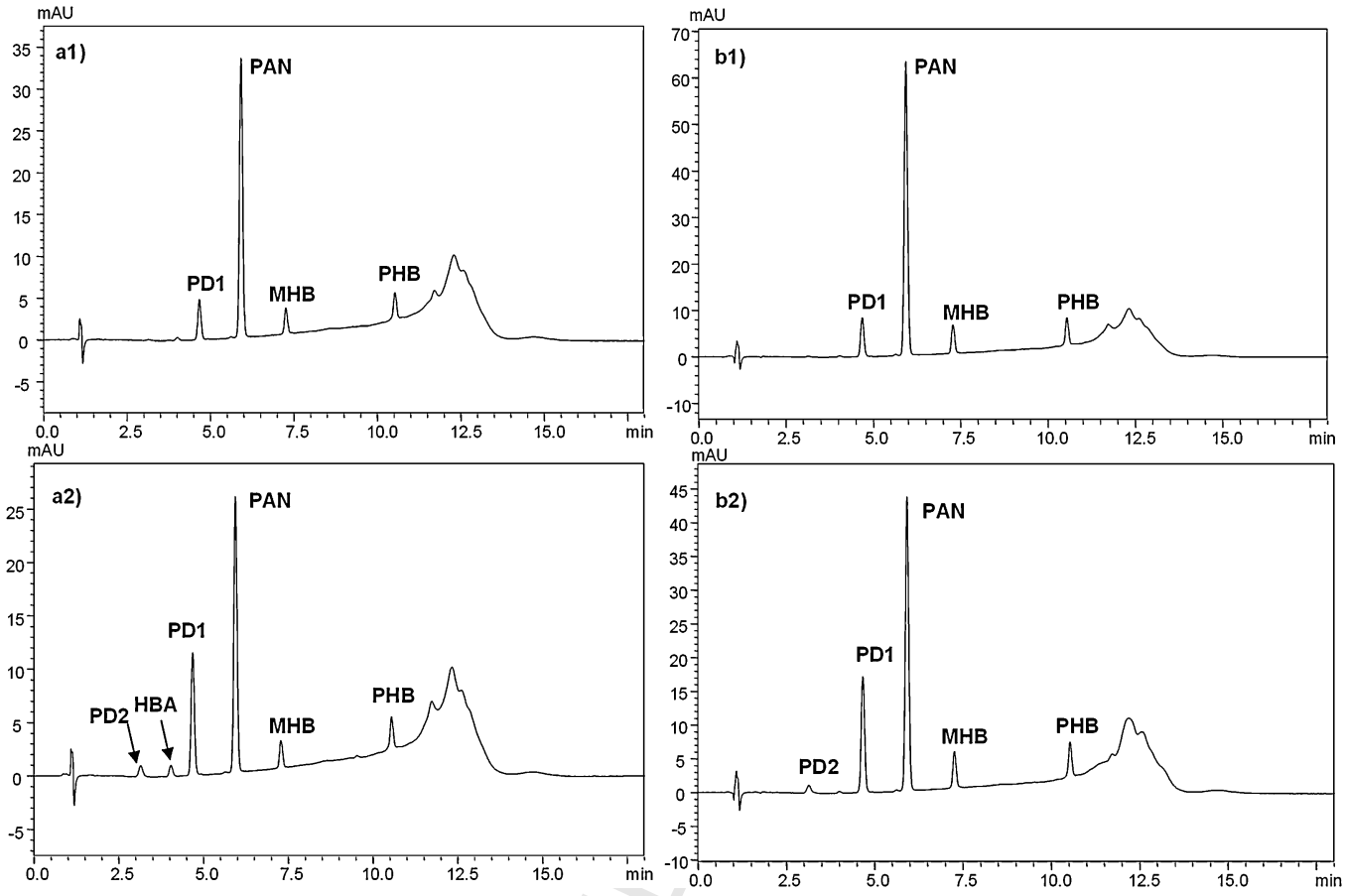

Fig. 1. LC–UV (method A; λ_a = 254 nm) chromatograms of PAN in 0.01 M NaOH solution at room temperature ((a1) t = 5 h; (a2) t = 8 h) and in 0.1 M HCl solution ((b1) t = 25 °C, t = 24 h; (b2) t = 45 °C, t = 6 h).

of *p*-hydroxybenzoic acid for HBA was proposed. The assignment was confirmed by comparing its chromatographic properties with a reference standard of HBA. The oxidative degradation pathway was studied using two different protocols. In the presence of hydrogen peroxide, as reported in LC–UV chromatogram (Fig. 3), a degradation product was formed (POx). As expected, LC–ESI–MS/MS analysis provided a protonated molecule at *m/z* 571, +16 Da with respect to PAN.

The protonated molecule at *m/z* 571 and its fragment ion at *m/z* 441 (amide cleavage; Fig. 4) indicated the insertion of an oxygen atom in the structure of PAN suggesting the formation of the thiosulfinate derivative. POx appeared quite unstable and this feature prevented its purification and the complete physico-chemical characterization.

Even if hydrogen peroxide was the most used stress condition, the role of transition metals in oxidative stress could be also evaluated, being different their mechanism of reaction [16]; however PAN was found to be stable in other oxidative stress conditions: indeed in Fe^{3+} and Cu^{2+} aqueous solutions PAN did not undergo degradation (data not shown). The photodegradation was studied exposing PAN to day light and 366 nm UV–light without observing the formation of degradation products (data not shown). On the contrary thermal stress condition caused the formation of PD1 as the only degradation product as reported in Supplemental 6.

Data obtained from the forced degradation study allowed to propose the degradation scheme of pantethine reported in Fig. 5. The main degradation pathway is represented by thermal/hydrolytic cleavage of amide functions. In particular, the first degradation step involved one of the terminal amide group with the formation of PD1 and, possibly, pantolactone as the leaving group. The easy dissociation of the terminal amide function could be attributed to the neighbouring group effect of the end-position hydroxyl group. If the reaction time and/or severity of the stress conditions were increased the formation of the symmetrical degradation product, PD2, was observed through the hydrolysis of the other terminal amide function. Finally, another degradation pathway of PAN was represented by the oxidation, possibly involving the formation of pantethine thiosulfinate POx. From a pharmaceutical point of view, the relevance of the degradation products of PAN was not the same; indeed PD2, a secondary degradation product, and POx were never observed in long-term stability studies of PAN and its formulation; on the contrary, PD1 can be effectively considered the indicator of stability for hydrolytic and thermal stresses and it was included in the development and validation of the analytical assay.

3.2. LC–UV method development

Data obtained from forced degradation study allowed to develop a LC–UV assay for determination of PAN, its degradation product PD1 and the preservative system constituted by MHB and PHB. Moreover, to properly assess the selectivity of the method the potential impurity CYS was also included in method development: indeed one of the approaches for the synthesis of PAN is based on the condensation of CYS with pantothenic acid. Similarly, the other degradation product of PAN, PD2 and the degradation product of preservant system HBA were also included in selectivity assessment. Overall, the analytes showed very different structural features as well as physico-chemical properties; hence their chromatographic separation was a challenging task. Indeed, while free primary amino groups were present in PD1, PD2 and CYS, a

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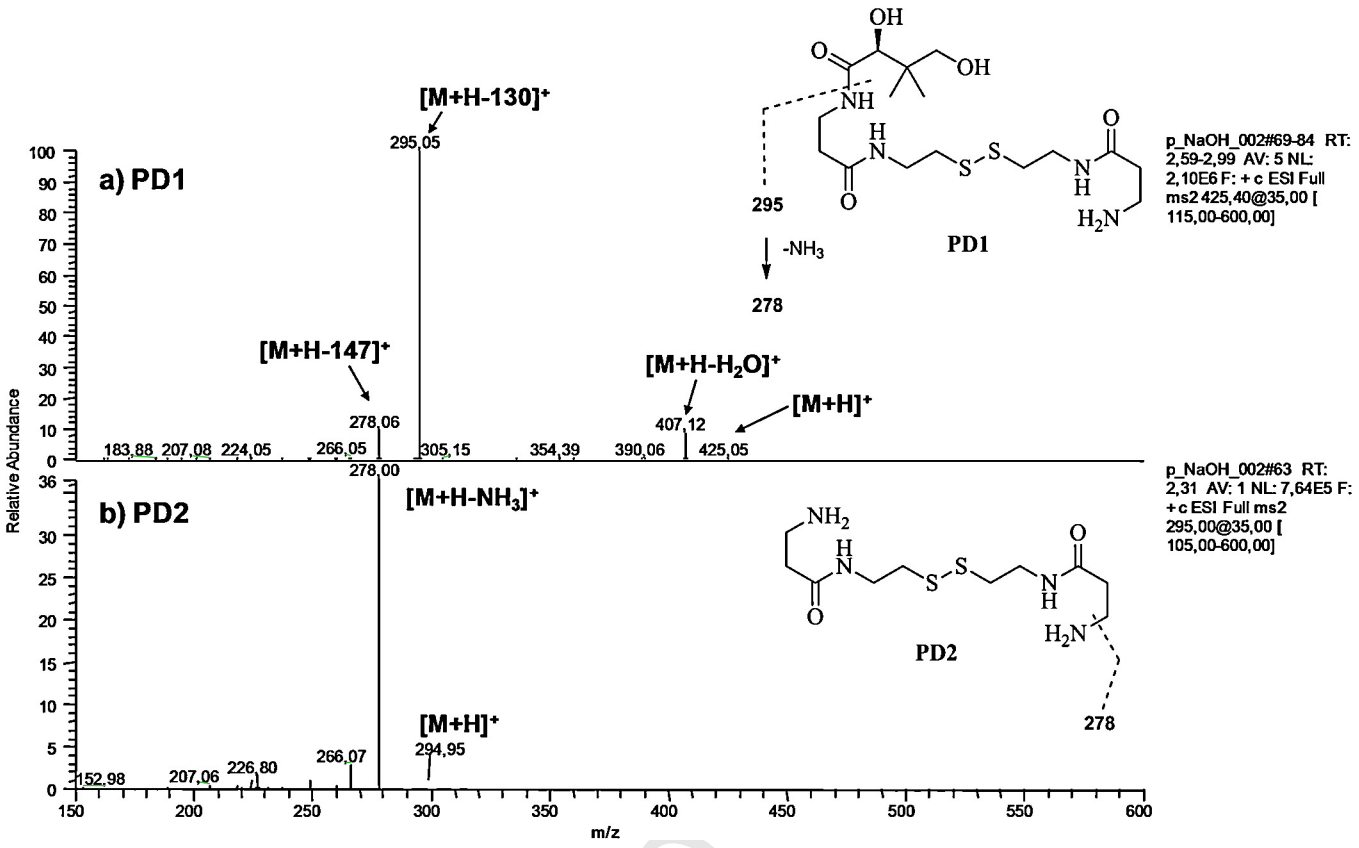

Fig. 2. ESI–MS/MS spectra of PD1 (a) and PD2 (b).

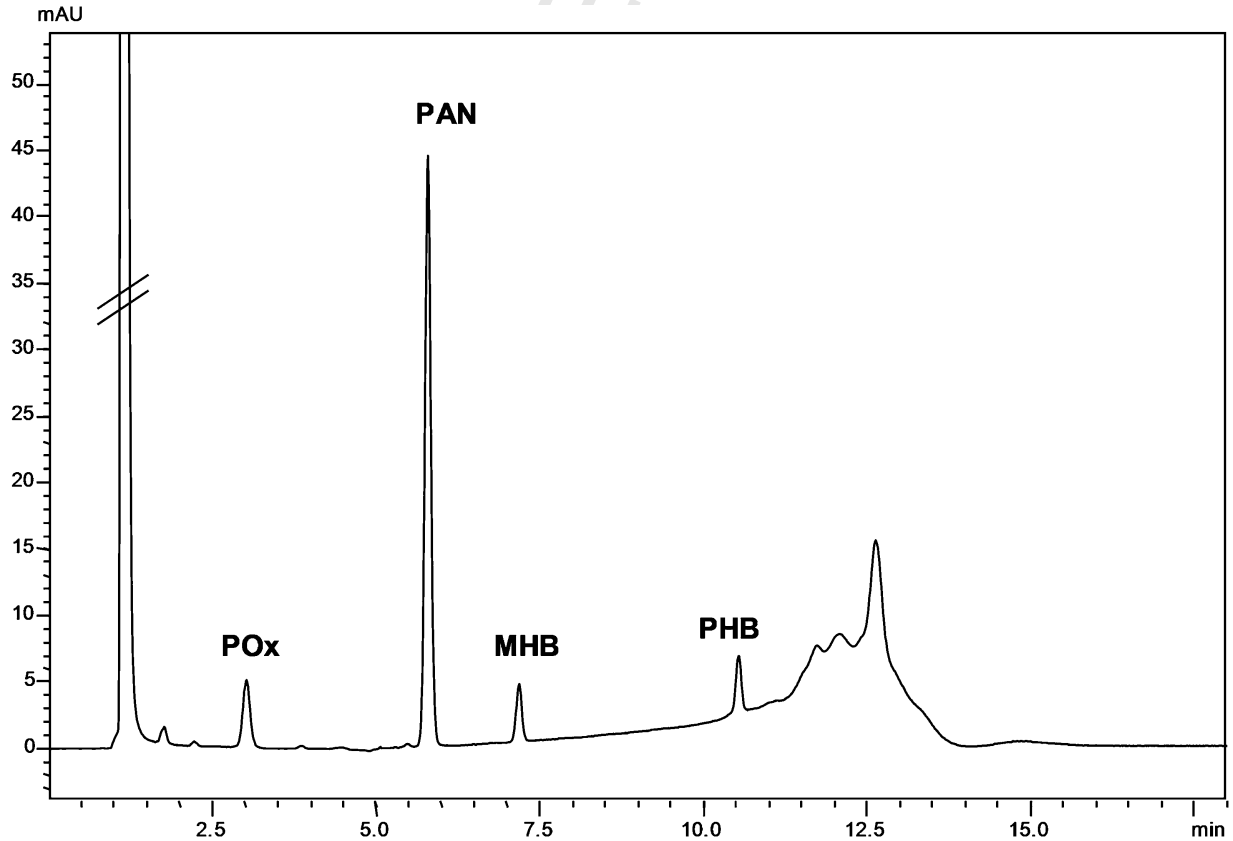

Fig. 3. LC–UV chromatogram of PAN in 1% H_2O_2 solution (*t* = 5h).

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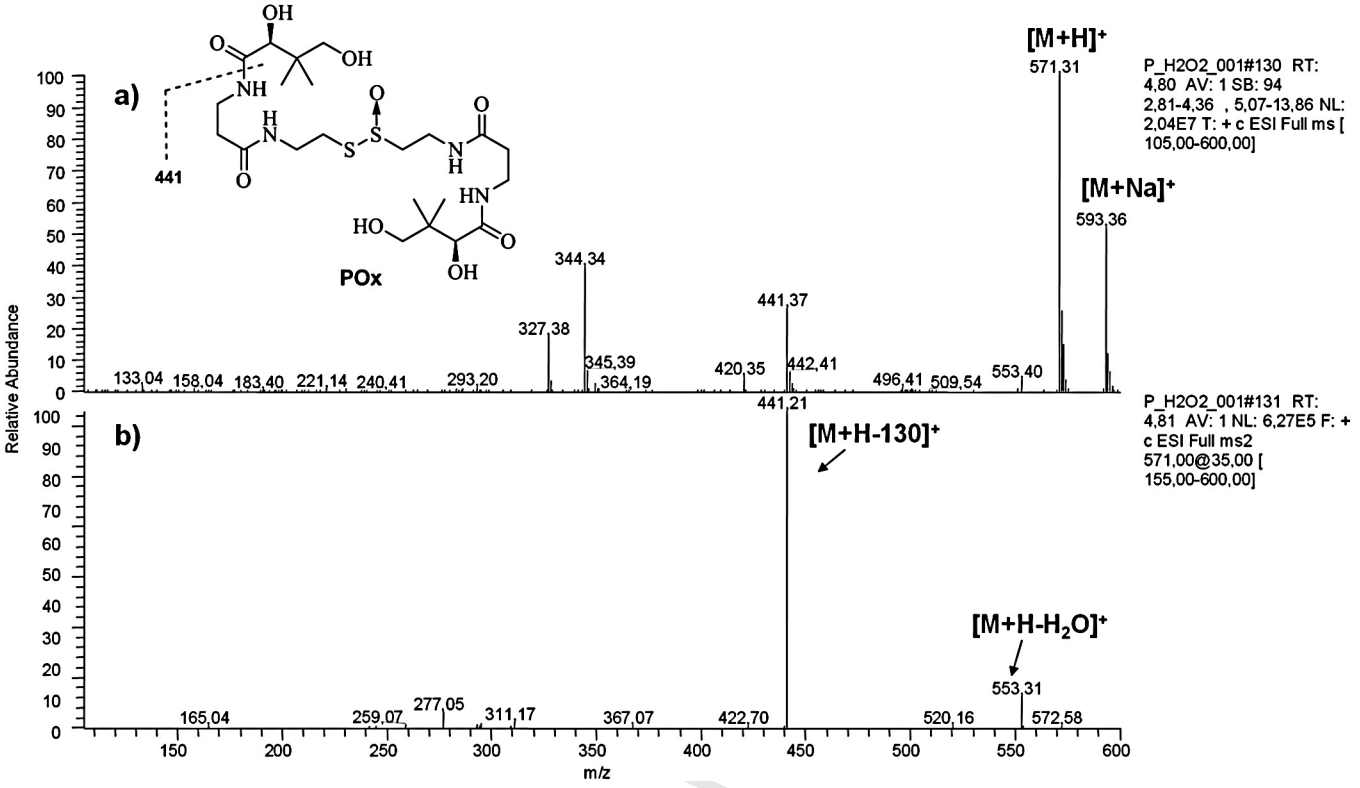

Fig. 4. ESI-MS of Pox: full scan spectrum of the POx (a), and its MS/MS spectrum (*m/z* 571) (b).

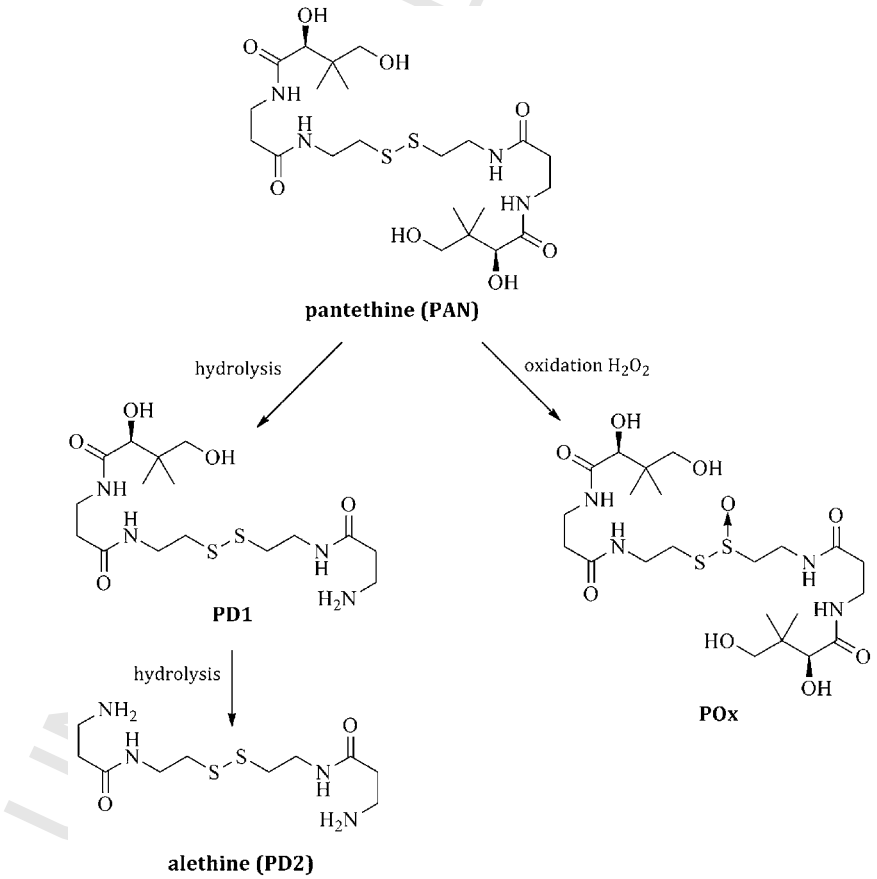

Fig. 5. Degradation pathways of PAN.

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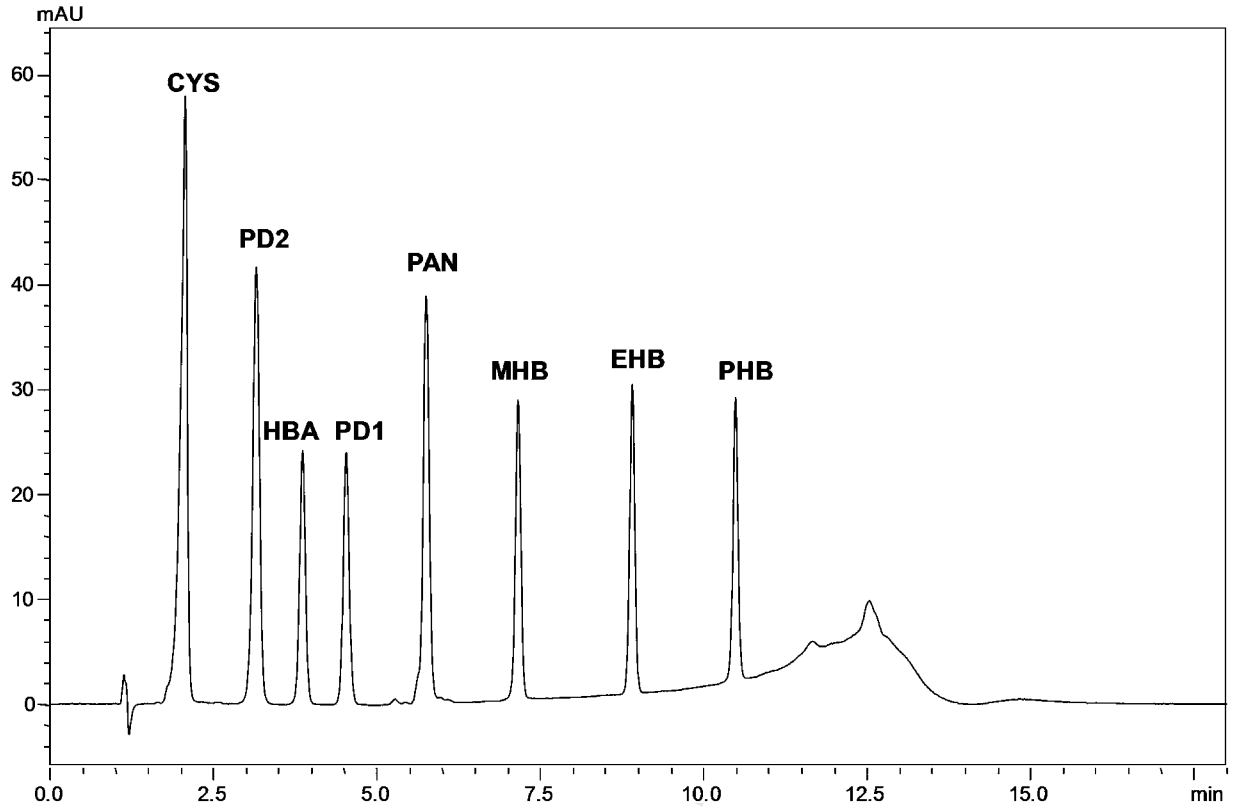

Fig. 6. LC–UV chromatogram (method A, λ_a = 254 nm) of a solution containing CYS, PD2, HBA, PD1, PAN, MHB, EHB and PHB.

carboxylic group was present in HBA structure; moreover, there is a great difference in the lipophilicity of the parabens and the pantethine-related compounds: indeed their log*P* values vary in the wide range −4.17–3.04. During the method development, to select the appropriate chromatographic conditions for separating all the analytes, a number of stationary phases with various isocratic mobile phases, containing acetonitrile or methanol-aqueous phosphate buffers, were evaluated; however all the attempts to achieve the separation of all analytes have failed; in particular, a poor retention of the polar and the charged analytes (e.g. CYS, PD2 and PD1) was observed (data not shown). Taken together these results suggested the inclusion of an appropriate counter-ion and the use of a gradient elution to provide an increase of the lipophilicity of PD2, PD1 and CYS and a baseline resolution of all the analytes in an acceptable analysis runtime. Hence a pH = 2.5 phosphate buffer was chosen to promote the protonation of the amino groups of CYS, PD1 and PD2 and to suppress the ionization of HBA carboxylic group while reducing the ionization of stationary phase silanol functions: these features could mitigate their interactions with the analytes. Alkyl sulfonate sodium salts were used to form the ion-pairs with CYS, PD1 and PD2 being the counter-ion chain length and the concentration the critical factors to ensure the separation of the mixture of the analytes. In particular, sodium pentanesulfonate at 8 mM concentration provided the required method selectivity and robustness. Accordingly, the starting mobile phase was constituted by eluant A: aqueous phosphate buffer (20 mM pH = 2.5, 8 mM sodium 1-pentansulfonate) and eluant B: phosphate buffer (20 mM, pH = 2.5) 8 mM sodium 1-pentanesulfonate) the A:B ratio being 62.5:37.5; the gradient elution was performed at a flow rate of 1.5 mL min^{-1}. The analysis were performed at 254 nm for PAN, MHB and PHB assays; however, for the determination of degradation product (PD1) at 0.1% level of concentration, λ_a = 210 nm was used. Overall, the selected analytical conditions provided chromatograms at λ_a = 254 nm (Fig. 6) and at λ_a = 210 nm (Supplemental 7) with good peak shapes, required for the simultaneous separation of a CYS, PD2, HBA, PD1, PAN, MHB, EHB and PHB mixture with an acceptable time of analysis. It is worth of mention that EHB was included in the method development because of its presence in drug product during stability study: indeed EHB migrates from the capsule shell to the content.

3.3. Method validation

The developed method was validated [17], as described below, for the following parameters: system suitability, specificity, robustness, linearity, precision, accuracy and LOD/LOQ.

3.3.1. Selectivity

Selectivity is the ability of an analytical method to assess unequivocally the analytes in the presence of components that are present in the sample matrix. The chromatogram (Supplemental 8) of the placebo solution constituted by excipient blend (poly(ethylene glycol) 400, glycerol, (hydroxypropyl)methyl cellulose and L-leucine) showed no peak interfering with analytes; moreover the adjacent chromatographic peaks (Fig. 6) CYS/PD2, PD2/HBA, HBA/PD1, PD1/PAN, PAN/MHB, MHB/EHB and EHB/PHB were separated with resolution values greater than 3 as reported in Table 1. Certainly some excess of resolution could be found, in particular between PAN and parabens and between parabens, however the increase of the lipophylicity of PAN and its related substances by using hexane/heptane/octanesulfonate sodium salts did not allow the separation of the analytes. Overall, these data demonstrated that the excipients and the degradation products did not interfere with PAN peak, indicating the selectivity of the method.

3.3.2. System suitability

As system suitability tests are an integral part of chromatographic method development and they are used to verify that the

Table 1
System suitability parameters.

Repeatability of peak area[a]		Asymetry factor	Resolution	
PAN	0.4	0.9	CYS/PD2	5.8
PD1	0.9	1.0	PD2/HBA	3.9
MHB	0.2	1.1	HBA/PD1	3.9
PHB	0.4	1.0	PD1/PAN	7.8
			PAN/MHB	8.5
			MHB/EHB	11.4
			EHB/PHB	11.5

[a] The concentrations of analytes were: PAN (0.8 mg mL^{-1}), PD1 (5.0 μg mL^{-1}), MHB and PHB (0.8 μg mL^{-1} each). λ_a = 254 nm for PAN, MHB and PHB and λ_a = 210 nm for PD1.
The figures in parenthesis represent RSD values for six replicates.

system is adequate for the analysis to be performed, resolution, area repeatability and asymmetry factor were the parameters evaluated for the analytes PAN, MHB, PHB and PD1. The suitability of the chromatographic system was demonstrated by comparing the obtained parameter values, reported in Table 1, with the acceptance criteria of the CDER guidance document [18].

3.3.3. Robustness

Even if the high resolution values between the peaks of analytes could confer the required robustness to the method, the role of the most critical method parameters (pH, counter-ion concentration and mobile phase composition) has been evaluated by introducing small but deliberate variations. As reported (Supplemental 9A), the resolution values were always greater than 2.7 also in the case of some potential critical pair of adjacent peaks (e.g. HBA/PD1).

Moreover the standard and test solutions appeared stable over a 12 h time period.

3.3.4. Linearity

Five concentration levels within 50–150% of the target concentration range for PAN, MHB and PHB were considered to study the linearity. For PD1 and PAN at 0.1% level of concentration, a range value of 0.05–2.0% was used. Since the Bartlett test evidenced no significant difference ($p > 0.05$) among the variance values of replicates at different concentration levels the best fit was obtained using an unweighted linear regression model. The linearity was observed in the expected concentration ranges, demonstrating their suitability for analysis. The results of the regression statistics for all the analytes were reported in Table 2. The square of the correlation coefficient ($r^2 > 0.999$) demonstrated a significant correlation between the concentration of analytes and detector response; however it was neither a proof of linearity, nor a useful measure of the calibration variability. Hence the lack-of-fit-test were performed on these data; the significance values ($p > 0.05$) obtained for all analytes indicated that a linear regression model provides a good interpolation of the experimental data. Moreover, the evaluation of residual plot confirmed that underlying assumption like homoscedasticity was met as well as the goodness of fit of the regression model. Finally the confidence interval of the y-intercepts includes zero; moreover the relative residual standard deviations, expressed as percentage, were calculated and used to assess the precision of the regression: all values were <1% except for those corresponding to PD1 and PAN at 0.1% level of concentration (1.9 and 0.9%, respectively).

3.3.5. Precision

The precision of an analytical procedure expresses the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The repeatability (intraday precision) refers to the use of analytical procedure within a laboratory over a short period of time using the same operator with the same equipment. Intermediate precision (interday precision) involves estimation of variations in analysis when a method is used within a laboratory on different days, by different analysts. The results obtained are shown in Supplemental 9B. In all instances RSD values were less than 2%.

3.3.6. Accuracy

Accuracy has been determined by application of the analytical procedure to recovery studies, where known amount of standard was spiked into the placebo. The results of accuracy studies were shown in Supplemental 9C. The method demonstrated to be accurate as the average recoveries were 100.2, 99.9, and 100.0% for PAN, MHB and PHB, respectively, and 99.9 for PD1. Moreover PAN at 0.1% level of concentration showed an acceptable average recovery (100.5%).

3.3.7. LOD–LOQ

LOD and LOQ were determined at 210 nm, by injecting progressively low concentration solutions of PD1 and PAN. PAN and PD1 showed the same values of LOD and LOQ being 0.8 and 2.5 μg mL^{-1}, respectively.

3.3.8. PD1 relative response factor (RRF)

RRF of PD1 was obtained by comparing the peak areas (Supplemental 10) of PD1 and PAN at the same concentration (50 μg mL^{-1}; λ_a = 210 nm); as expected, due to the similarity of their chemical structures RRF value was 1.0.

3.4. Analysis of PAN capsule formulations

LC–UV chromatogram, obtained from Pantethine® capsule sample employed in a long term stability study (t = 24 months; Supplemental 12A) allowed to detect the formation of PD1 as the degradation product of PAN and HBA as the degradation product of the preservatives; on the contrary the presence of PD2 was never

Table 2
Five levels calibration graphs for PAN, MHB, PHB and PD1: unweighted regressions $y = ax + b$; three replicates for each level (n = 15).

	PAN	MHB	PHB	PD1	PAN[a]
Range	0.4–1.2 mg mL^{-1}	0.4–1.2 μg mL^{-1}	0.4–1.2 μg mL^{-1}	2.5–100 μg mL^{-1}	2.5–100 μg mL^{-1}
Slope (a)	482,484	79,677	68,108	10,377.3	10,882.3
Standard error (SE) (a)	1750.3	368.9	300.1	34.9	26.0
Intercept (b)	−2402.8	3.74	138.6	−2832.4	1349.9
(b) C.I.[b]	−5611.3–805.7	−672.6–680.1	−411.6–688.7	−6701.5–1036.7	−1533.6–4233.4
F	75,988.4 $p < 0.01$	46,639.0 $p < 0.01$	51,500.1 $p < 0.01$	88,342.3 $p < 0.01$	174,916.0 $p < 0.01$
r^2	0.9998	0.9997	0.9997	0.9998	0.9997
RRSD[c]	0.5	0.6	0.6	1.9	0.9

[a] Pantethine at 0.1% level of concentration.
[b] 95% confidence interval.
[c] Relative residual standard deviation.

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found during the study. It is worth noting that PD1 increases over the time, during the stability study showing its hydrolytic instability. Moreover the presence of EHB arising from the capsule shell, was also shown. Finally the presence of two unknown impurities (U1 and U2 at ~5.5 and 6.8 min, respectively) was observed in the LC–UV chromatogram (Supplemental 12B). In the case of Pantethine hard gelatine capsule formulation (Supplemental 13A), the expected lack of peaks of parabens was confirmed along the presence of PD1 peak. Furthermore the presence of a third unknown impurity (U3, at ~6.4 min; Supplemental 13B) was also found. It is worth of mention that the unknown impurities (U1–U3) were not observed during the forced degradation study and the peak intensities of U1 and U2 did not change over the time period of the stability study: taking together these features suggest that U1–U3 could arise from synthetic procedures of PAN.

4. Conclusions

In the present work the intrinsic chemical stability of PAN was for the first time investigated by LC–MS/MS technique and a degradation scheme was proposed on the basis of a forced degradation study. The identification and synthesis of the main degradation products allowed the development and validation of a comprehensive stability-indicating LC–UV method for the determination of PAN, its degradation product PD1 and the preservative system in the drug substance and drug product. The complete separation of eight analytes was accomplished in 12 min and the method has been successfully used to perform stability studies of PAN formulations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2014.04.025.

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