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Determination of L-dopa, carbidopa, 3-*O*-methyldopa and entacapone in human plasma by HPLC–ED

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ABSTRACT

The aim of the study was the development of analytical methods suitable for the quantification of L-dopa, carbidopa and entacapone in plasma of Parkinsonian patients treated with Stalevo®. The metabolite 3-*O*-methyldopa was also determined to obtain some indications on the pharmacokinetics of L-dopa.

For the simultaneous analysis of L-dopa, 3-*O*-methyldopa and carbidopa, a RP C18 column as the stationary phase and a mixture of methanol and a pH 2.88 phosphate buffer (8:92, v/v) as the mobile phase were used. A feasible plasma pre-treatment based on protein precipitation was implemented, obtaining extraction yield higher than 94% for all the analytes. For the analysis of entacapone a RP C8 column and a mixture of methanol, acetonitrile and a pH 1.90 phosphate buffer as the mobile phase (17.5:22.5:60, v/v/v) were used. A plasma pre-treatment procedure was developed, based on solid phase extraction of entacapone using Oasis HLB cartridges. Extraction yields were good, being always higher than 96%.

Both methods, based on HPLC–ED (V = +0.8 V), have been fully validated. Good linearity was obtained over the following concentration ranges: 100–4000 ng mL^{-1} for L-dopa, 200–10,000 ng mL^{-1} for 3-*O*-methyldopa, 25–4000 ng mL^{-1} for carbidopa and 20–4000 ng mL^{-1} for entacapone. Precision data were satisfactory, being R.S.D.% values lower than 5.64%; accuracy also resulted very good with recovery data higher than 90%. The proposed methods have been successfully applied to the analysis of patient plasma samples and seem to be suitable for therapeutic drug monitoring purposes.

1. Introduction

Parkinson's disease is a progressive, neurodegenerative disorder of the extrapyramidal nervous system. It affects the mobility and control of the skeletal muscular system and is characterized by tremor, rigidity, bradykinesia and postural instability [1].

Levodopa (3-hydroxy-L-tyrosine, LD, Fig. 1a) is the most effective agent in the treatment of Parkinson's disease, but is itself largely inert. In fact, although levodopa is able to cross the blood–brain barrier, not more than 1% of the administered dose can reach the central nervous system due to rapid metabolism by two enzymatic pathways, dopa decarboxylase (DDC) and catechol-*O*-methyltransferase (COMT) [2]. In addition, dopamine released into the circulation by peripheral conversion of LD produces undesirable effects, particularly nausea and hypotension. For these reasons, LD is almost always administered in combination with a peripherally acting inhibitor of DDC, such as carbidopa ((αS)-α-hydrazinyl-3,4-dihydroxy-α-methyl-benzenepropanoic acid, CBD, Fig. 1b), which increases the amount of LD that reaches the brain and reduces the incidence of pheripheral side effects. Unfortunately, the efficacy of chronic administration of LD/DDC inhibitor decreases with time and most patients develop fluctuating responses and dyskinesias [3].

A further improvement in the treatment of Parkinson's disease was obtained during the last decade, with the introduction of COMT inhibitors, such as entacapone ((2E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl-2-propenamide, EN, Fig. 1c), used to stop the LD metabolism to 3-*O*-methyldopa (3-OMD, Fig. 1d), a potentially harmful metabolite of LD.

Nowaday, a formulation containing a combination of levodopa, carbidopa and entacapone is available on the market as oral tablets with the trade name of Stalevo®, approved by FDA since 2003 [4]. The administration of EN together with LD and CBD leads to greater and more sustained plasma levels of LD than those obtained after administration of LD and CBD.

Several papers, based on HPLC with ED detection, have been reported for plasma analysis of LD with its metabolite or LD with CBD or LD alone [5,6–12]. The biological samples pre-treatments are based on protein precipitation using perchloric acid [9,13],

Abbreviations: LD, levodopa; CBD, carbidopa; EN, entacapone; 3-OMD, 3-*O*-methyldopa; IS, internal standard; ED, electrochemical detection; SPE, solid phase extraction.

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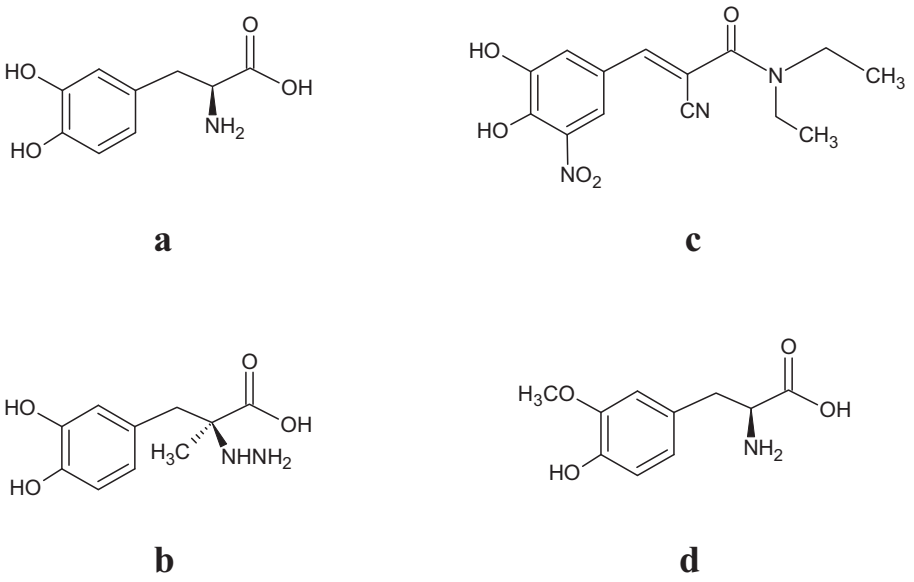

Fig. 1. Chemical structures of LD (a), CBD (b), EN (c) and 3-OMD (d).

methanol containing perchloric acid [5], trichloroacetic acid [7]; solid-phase extraction (SPE) procedures are also employed (C 30 cartridges [12] or alumina [8,11]). To the best of our knowledge, no papers dealing with the assays of LD and EN are reported in literature. Two methods have been published for plasma analysis of patients taking only entacapone, based on HPLC with UV [14] or ED detection [15] using a liquid–liquid pre-treatment. The aim of this research was the development of methods suitable for the plasma analysis of LD, 3-OMD, CBD and EN, in order to carry out the therapeutic drug monitoring (TDM) of patients undergoing therapy with Stalevo®.

TDM of these drugs is useful to monitor pharmacokinetic of Stalevo® and consequently optimize the dosage according to the needs of a specific patient. In fact it is very important to maintain levodopa plasma levels as stable as possible and to keep the dosage the lowest possible throughout the years of treatment in order to minimize motor fluctuations and dyskinesias [16–18]. TDM plays a key role also at the advanced stages of the disease, when the therapeutic window becomes narrower thus increasing the risk of side effects [16].

2. Materials and methods

2.1. Chemicals

Levodopa (LD), 3-*O*-methyldopa (3-OMD), carbidopa (CBD), catechol and promethazine (both used as internal standard, IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (both HPLC-grade), potassium dihydrogen phosphate, EDTA, phosphoric acid (85%, w/w), 1-octanesulphonic acid monohydrate sodium salt (OSA), 0.6 *N* trichloroacetic acid (TCA), hydrochloric acid (37%, w/v), sodium metabisulphite, sodium chloride, potassium chloride and triethylamine, all pure for analysis, were from Sigma–Aldrich (St. Louis, MO, USA). Disodium hydrogen phosphate and bovine serum albumin were produced by Carlo Erba (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, Mass., USA).

2.2. Apparatus

HPLC analyses were performed using a 9002 Varian (Harbor City, CA, USA) HPLC pump. Detection was carried out using an Electrochemical Detector (Antec Decade, DB Leiden, The Netherlands) equipped with a carbon glass electrode (work electrode) and an Ag/AgCl electrode (reference electrode). The data were processed by means of a Varian Star Chromatography 4.0 software.

A Crison (Barcellona, Spain) Basic 20 pH meter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.3. Plasma sample collection

Blood samples were withdrawn both from patients undergoing therapy with Stalevo® (100/25/200 mg of LD/CBD/EN) and healthy volunteers not subjected to any pharmacological treatment and collected into commercially available test tubes containing EDTA as the anticoagulant. Blood samples were immediately centrifuged (1780 RFC, 5 °C, 20 min) and the supernatant plasma fractions were transferred into test tubes and frozen at −20 °C until analysis. For the preliminary assays "reconstituted" plasma was used. It was prepared by dissolving 160 mg of sodium chloride, 23 mg of disodium hydrogen phosphate, 4 mg of potassium dihydrogen phosphate, 4 mg of potassium chloride and 800 mg of bovine serum albumin in 20 mL of ultrapure water and then adjusting the mixture to pH 7.4.

2.4. Analysis of LD, CBD and 3-OMD (Method A)

2.4.1. Solutions

The stock solutions (1 mg mL^{-1}) of LD, CBD and 3-OMD were prepared by dissolving suitable amounts of each pure substance in a mixture consisting of sodium chloride, sodium metabisulphite, hydrochloric acid and water. The internal standard (IS, catechol) stock solution, was 100 μg mL^{-1} in methanol.

The stock solutions were stable for at least 1 month when stored at −20 °C. Working standard solutions were prepared freshly every day by diluting stock solutions with water.

The injection standard solutions were prepared in TCA:water (1:1).

2.4.2. Chromatographic conditions

Isocratic separation was achieved on a Phenomenex (Torrance, CA, USA) C18 column (250 × 4.6 mm i.d., 5 μm). The mobile phase was a mixture of methanol (8%, v/v) and an aqueous solution (92%, v/v) containing 1.45 g L^{-1} of potassium dihydrogen phosphate,

18 mg L^{-1} of EDTA, 90 mg L^{-1} of OSA and 98 mg L^{-1} of potassium chloride, buffered to pH 2.88 with 14 mM phosphoric acid. The solution was filtered through a Millipore (Milford, MA, USA) 0.2 μm nylon filter. The assays were performed at 25 °C with a flow rate of 1.5 mL min^{-1}. The injections were carried out through a 20 μL loop. The potential of the electrochemical cell was set at 800 mV.

2.4.3. Plasma pre-treatment

Real plasma or blank plasma samples were subjected to a protein precipitation procedure using TCA as the precipitant agent. An aliquot of 200 μL of plasma, to which 20 μL of IS working solution (and analyte standard solutions for blank plasma samples) had been added, was placed in a glass tube and 180 μL of TCA (0.6 N) were added. The resulting mixture was vortexed for 5 min and centrifuged for 10 min at 4000 rpm at 5 °C. An aliquot of the supernatant was injected into the HPLC system.

2.4.4. Method validation

2.4.4.1. Linearity, limit of quantification, limit of detection. Aliquots of 20 μL of analyte standard solutions, containing LD, 3-OMD and CBD at six different concentrations and the IS at a constant concentration, were added to 200 μL of reconstituted plasma. The resulting mixtures were subjected to the previously described protein precipitation and the final supernatant was analyzed by HPLC. The analyte/IS peak area ratios were plotted against the corresponding concentrations of the analytes and the calibration curves were obtained by means of the least squares method.

The limit of quantification (LOQ) and the limit of detection (LOD) were calculated according to USP XXVIII Edition [19] and "Cristal City" [20] guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the standard deviation of the baseline noise, respectively.

2.4.4.2. Extraction yield. Aliquots of 200 μL of blank plasma were spiked with 20 μL of standard solutions containing the IS at a constant concentration and LD, 3-OMD and CBD at three different concentrations, corresponding to the lower limit, middle point and upper limit of each calibration curve. The resulting mixtures were subjected to the previously described protein precipitation and the final supernatant was analyzed by HPLC. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

2.4.4.3. Precision. The assays described under "extraction yield" were repeated six times within the same day to obtain repeatability (intraday precision) and six times over different days to obtain intermediate precision (interday precision), both expressed as R.S.D.% values.

2.4.4.4. Accuracy. Accuracy was evaluated by means of recovery assays. An aliquot of 20 μL, containing the analyte standard solutions and the IS at known concentrations, was added to 200 μL of previously analyzed plasma from patients under therapy with Stalevo®. The spiked samples were submitted to the precipitation procedure described above and injected into the HPLC system. Recovery values were calculated according to the following formula: 100 × ([after spiking] − [before spiking])/[added]. The procedure was repeated six times during the same day to obtain mean recovery values.

2.5. Analysis of EN (Method B)

2.5.1. Solutions

Since EN was not commercially available as a pure substance, it was extracted from Comtan® tablets, a commercial formulation containing EN as the single active drug. The extraction was carried out using methanol and the stock solution was obtained at a nominal concentration of 1 mg mL^{-1}.

The stock solution of the IS promethazine (1 mg mL^{-1}) was prepared by dissolving suitable amount of the powder in methanol.

The stock solutions were stable for at least 1 month when stored at −20 °C. Working standard solutions were prepared freshly every day by diluting stock solutions with methanol.

2.5.2. Chromatographic conditions

Isocratic separation was achieved on a Phenomenex (Torrance, CA, USA) C8 column (150 × 4.6 mm i.d., 5 μm). The mobile phase, consisting of methanol, acetonitrile, phosphate buffer (pH 1.9, 18 mM) containing 0.25% (v/v) triethylamine (17.5:22.5:60, v/v/v), was filtered through a Millipore (Milford, MA, USA) 0.2 μm nylon filters. The assays were performed at 25 °C with a flow rate of 1.3 mL/min. The injections were carried out through a 20 μL loop. The potential of the electrochemical cell was set at 800 mV.

2.5.3. Sample pre-treatment

Plasma samples were subjected to a solid-phase extraction (SPE) procedure carried out on Waters (Milford, MA, USA) Oasis HLB (30 mg, 1 mL) cartridges by means of a Varian (Harbor City, CA, USA) VacElut apparatus. The cartridges were activated with 2 mL of methanol, conditioned with 2 mL of water and loaded with a mixture of 250 μL of human plasma, 500 μL of water and 50 μL of IS solution. After loading, the cartridges were washed twice with 1 mL of water and dried by applying full vacuum. The analytes were eluted with 1 mL of methanol and the eluate was injected into the HPLC system.

2.5.4. Method validation

2.5.4.1. Linearity, limit of quantification, limit of detection. Aliquots of 50 μL of EN standard solutions at six different concentrations, containing the IS at a constant concentration, were added to 250 μL of blank plasma. The resulting mixtures were subjected to the SPE procedure described above and then injected into the HPLC system. The analyte/IS peak area ratios were plotted against the corresponding concentrations of the analytes and the calibration curves were obtained by means of the least squares method.

The limit of quantification (LOQ) and the limit of detection (LOD) were calculated as described in Section 2.4.4.1.

2.5.4.2. Extraction yield. Aliquots of 250 μL of blank plasma were spiked with 50 μL of standard solutions containing the IS at a constant concentration and EN at three different concentrations, corresponding to the lower limit, middle point and upper limit of the calibration curve. The resulting mixtures were subjected to the SPE procedure and analyzed by HPLC. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

2.5.4.3. Precision. The assays described under "extraction yield" were repeated six times within the same day to obtain repeatability (intraday precision) and six times over different days to obtain intermediate precision (interday precision), both expressed as R.S.D.% values.

2.5.4.4. Accuracy assays. Accuracy was evaluated by means of recovery assays. An aliquots of 50 μL, containing the analyte standard solution and the IS at known concentrations, was added to 200 μL of previously analyzed plasma from patients under therapy with Stalevo®. The spiked samples were submitted to the SPE pro-

Table 1
Linearity parameters.

Analyte	Linearity range (ng mL^{-1})	Equation coefficients ($y = a + bx$)[a]		r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
		a	b			
LD	100–4000	0.1261	0.0103	0.9998	4	8
3-OMD	200–10000	−0.0491	0.0075	0.9996	4	10
CBD	25–4000	−0.0277	0.0072	0.9933	4	10

[a] y = analyte/IS peak area ratio, x = analyte concentration (ng mL^{-1}).

cedure and injected into the HPLC system. Recovery values were calculated as reported in Section 2.4.4.4.

3. Results and discussion

3.1. Preliminary studies

Assays were carried out to simultaneously determine LD (very hydrophilic drug) and EN (very lipophilic drug), but these gave unsatisfactory results, as could be expected from the log P values (−2.31 for LD, 2.50 for EN). Therefore a method for the LD analysis (Method A) and another one for the EN analysis (Method B) had to be developed.

As resulted from preliminary voltammetric assays, the amperometric detector was set at 800 mV for both the methods.

3.2. Method A

3.2.1. Chromatographic conditions

The starting point of this assay was a previous method for the HPLC–ED determination of biogenic amines [21], which are hydrophilic substances as LD. Being the analytes not well separated a C18 column of different length (250 mm instead of 150 mm) and a modified mobile phase (methanol: phosphate buffer, 8:92, v/v instead of 3:97, v/v) were used. The buffer solution pH value of 2.88 was found to be the most appropriate. Under these selected conditions LD, 3-OMD, CBD and catechol (used as the internal standard) were well separated within a 20 min chromatographic run.

3.2.2. Plasma sample pre-treatment

The sample pre-treatment is a crucial step of the analysis to obtain reliable results. Various protein precipitants were tested: sulphosalicylic acid, acetonitrile, methanol and trichloroacetic acid; the latter gave the best results for all the analytes in terms of extraction yield (>94%). In literature another method is reported which uses TCA as precipitant agent [7], but it is applied only to LD and it needs 1 mL of plasma instead of just 200 μL. Fig. 2a shows the chromatogram of a blank plasma sample spiked with the analytes and the IS.

3.2.3. Method validation

Calibration curves were set up on blank plasma spiked with standard solutions. The linearity ranges, the linear regression equations (obtained by means of the least squares method), the correlation coefficients, the limit of quantification (LOQ) and the limit of detection (LOD) for the analytes are reported in Table 1.

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma spiked with different analyte concentrations (Table 2). As one can see, the results were satisfactory: the mean extraction yield was always higher than 94% for the three analytes (80% for the IS), repeatability values were lower than 3.97% and intermediate precision values were lower than 5.64%.

3.2.4. Application to patient plasma samples

After validation, the method was applied to the analysis of LD, 3-OMD and CBD in plasma of some Parkinsonian patients under therapy with Stalevo®. Fig. 3a shows the chromatogram of a plasma sample from a patient taking Stalevo® tablets containing 100 mg of LD and 25 mg of CBD four times a day. The LD plasma level found in this sample was 990 ng mL^{-1} (1710 ng mL^{-1} for 3-OMD and 46 ng mL^{-1} for CBD), which is in the therapeutic range (500–1600 ng mL^{-1}) reported by Kuoppamäki et al. [22].

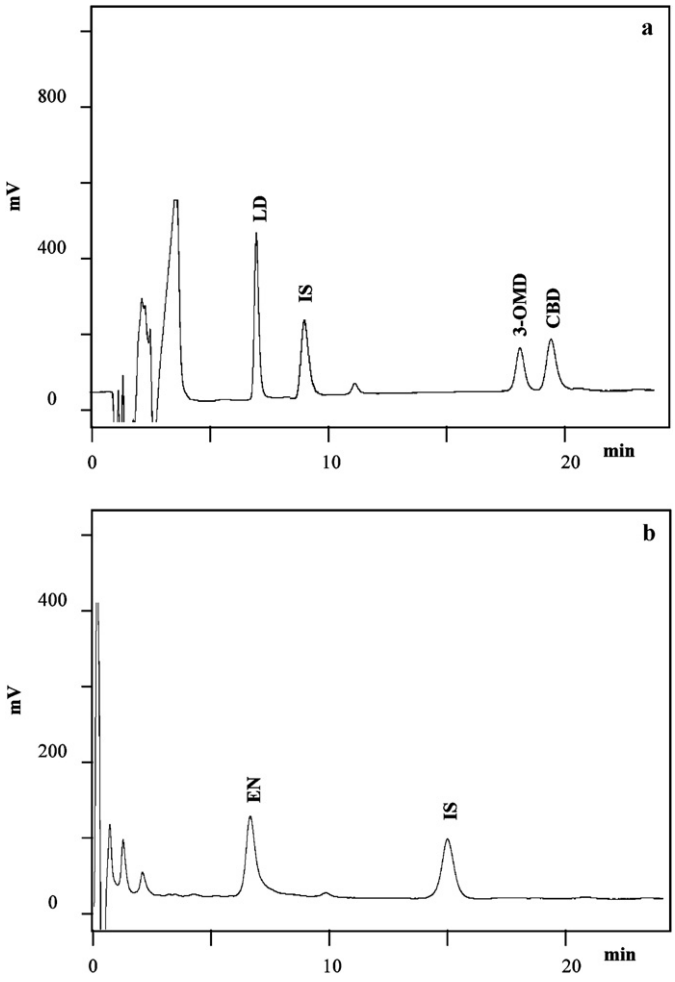

Fig. 2. Chromatograms of a blank plasma spiked with 1000 ng mL^{-1} of LD, 3-OMD and CBD and 100 ng mL^{-1} of the IS (a) and a blank plasma spiked with 100 ng mL^{-1} of EN and 400 ng mL^{-1} of the IS (b).

Method accuracy was evaluated by means of recovery studies. Known amounts of analyte standard solutions (i.e. 500 ng mL^{-1} for LD, 1000 ng mL^{-1} for 3-OMD, 100 ng mL^{-1} for CBD) and IS (100 ng mL^{-1}) were added to previously analyzed plasma. Mean recovery values found were 102%, 96% and 94% for LD, 3-OMD and CBD, respectively.

Method selectivity was evaluated by injecting into the HPLC system standard solutions of some catecholamines and metabolites (dopamine, serotonin, norepinephrine, homo-vanillic acid, hydroxyindolacetic acid, 4-hydroxy-3-methoxyphenylethylene glycol and 3,4-dihydroxyphenylacetic acid) and benserazide, another dopa

Table 2
Extraction yield and precision data (Method A).

Analyte	Concentration added (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD%)[a]	Intermediate precision (RSD%)[a]
LD	100	95.3	3.84	5.64
	1000	99.1	2.79	4.46
	4000	100.1	2.24	2.69
3-OMD	200	98.0	3.79	5.38
	1000	99.8	2.67	5.08
	10,000	100.0	1.91	4.29
CBD	200	94.3	3.97	4.34
	1000	95.7	3.94	4.06
	4000	96.0	2.21	2.47
IS	100	80.0	2.30	5.40

[a] $n = 6$.

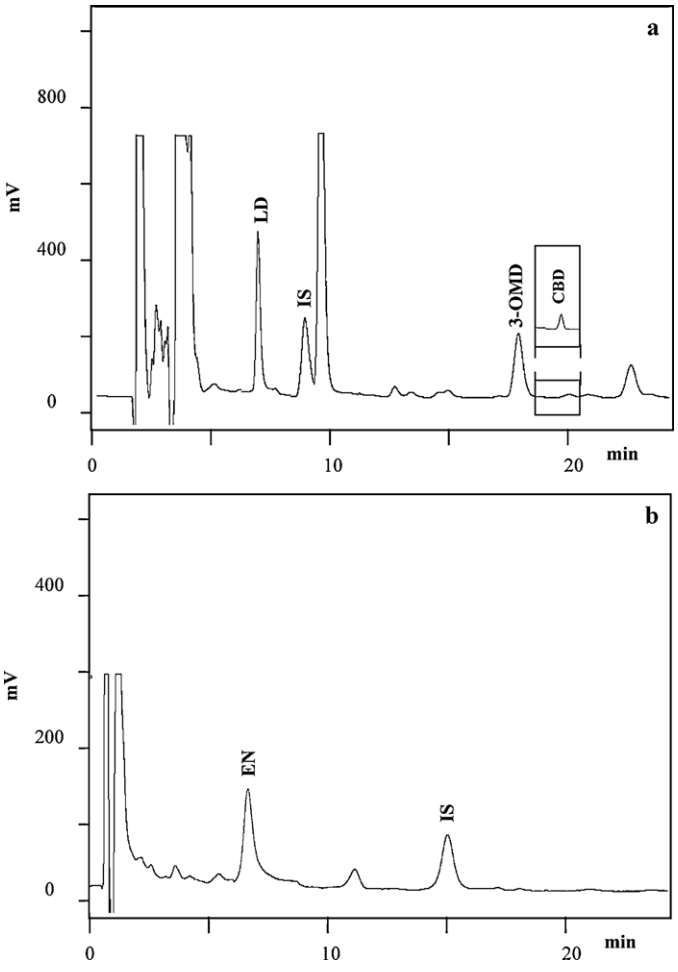

Fig. 3. Chromatograms of a plasma sample from a patient taking Stalevo (100/25/200). 4 times a day: determination of L-dopa, carbidopa and 3-*O*-methyldopa (a), determination of entacapone (b).

decarboxylase inhibitor. Only dopamine could interfere with the detection of LD, but this does not seem to be a problem, since endogenous dopamine plasma levels are lower than the LOD of the proposed method. Benserazide did not give interference, showing a retention time of 13.2 min; on the contrary it could be used as the IS as a good alternative to catechol. In fact the extraction yield resulted very satisfactory (>90%).

3.3. Method B

3.3.1. Chromatographic conditions

Since clozapine and EN logP are similar (2.7 and 2.5, respectively), preliminary assays were performed using the same chromatographic conditions developed to determine clozapine in human plasma [23]. The retention time of EN resulted too long, so the composition of the mobile phase was modified increasing the amount of acetonitrile (from 17.50% to 22.50%) and decreasing the percentage of methanol (from 20.00% to 17.50%). Under these modified conditions, at a flow rate of 1.3 mL min^{-1}, the analyte and promethazine, used as the internal standard, showed good chromatographic peaks at a retention time of 7.0 and 15.0 min, respectively.

3.3.2. Plasma SPE pre-treatment

In an attempt to use a unique sample pre-treatment, the extraction of EN from the plasma matrix with the same protein precipitation procedure used for LD was tested. Being the absolute recoveries unsatisfactory (40% for EN and 13% for the IS), a solid-phase extraction (SPE) procedure using HLB cartridges was implemented, obtaining good results in term of purification of the biological matrix and extraction yield following the procedure described (Section 2.5.3). The chromatogram of a blank plasma spiked with EN and the IS subjected to the SPE procedure is shown in Fig. 2b.

3.3.3. Method validation and application to plasma samples

Calibration curves were set up on blank plasma spiked with standard solutions. Good linearity ($r^2 = 0.9993$) was found over the 20–4000 ng mL^{-1} concentration range. The linear regression equation was $y = 0.01x - 0.0343$, where y is the ratio between the area of EN and that of the IS and x is the EN concentration expressed as ng mL^{-1}.

The LOQ was 20 ng mL^{-1} and the LOD was 10 ng mL^{-1}.

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma spiked with different EN concentrations. The results (see Table 3) were very good: the mean extraction yield was always higher than 96% and the precision data were satisfactory.

Selectivity was evaluated by injecting into the HPLC system several standard solutions of drugs that could be co-administered with Stalevo® and precisely clozapine and metabolites, risperidone, pramipexole and amantadine. None of the tested compounds interfered with EN detection.

After validation, the method was applied to some plasma samples of Parkinsonian patients under therapy with Stalevo® (EN dosage 200 mg).

Table 3
Extraction yield and precision data (Method B).

Analyte	Concentration added (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD%)[a]	Intermediate precision (RSD%)[a]
EN	20	96.5	3.66	3.73
	400	97.2	3.54	3.63
	4000	98.9	2.94	3.02
IS	400	99.5	1.35	1.62

[a] $n = 6$.

The chromatogram of a plasma sample from the same patient already analyzed for LD, taking Stalevo® four times a day, is reported in Fig. 3b; the EN plasma level was found to be 173 ng mL^{-1}.

The accuracy was also satisfactory as resulted by recovery studies. Known amounts of EN standard solution (50 ng mL^{-1}) and IS (400 ng mL^{-1}) were added to previously analyzed plasma. Mean analyte recovery was found to be 90%.

4. Conclusion

Two HPLC–ED methods for the quantification of LD, 3-OMD, CDB and EN in plasma of patients under therapy with Stalevo®, a recent medication used in the treatment of Parkinson's disease, have been developed. The proposed methods are rapid, precise and accurate. The implemented sample pre-treatment procedures (i.e. a protein precipitation for LD, 3-OMD and CBD and a solid-phase extraction for EN) gave very good extraction yields.

When compared to another HPLC–ED method which detects LD, 3-OMD and CBD [5], the proposed chromatographic method shows better precision (5.64% vs 6.7%, express as R.S.D.%) and higher reliability, as it assesses accuracy, which is not reported in the cited article. Moreover it uses a faster protein precipitation procedure; in fact it does not require evaporation to dryness and subsequent dissolution in mobile phase as the previous reported method [5].

If compared to the only two reported papers which make use of liquid–liquid extraction [14,15], the developed SPE procedure for EN allows to obtain higher extraction yield (96% instead of 60% [14] or 88% [15]) using smaller volumes of plasma (250 μL instead of 1 mL) and avoids the employment of polluting and potentially toxic organic solvents.

The proposed methods have been successfully applied to the analysis of plasma of some patients undergoing therapy with Stalevo® and seem to be promising for therapeutic drug monitoring (TDM) purposes.

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