Development and Validation of a Robust and Efficient HPLC Method for the Simultaneous Quantification of Levodopa, Carbidopa, Benserazide and Entacapone in Complex Matrices

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ABSTRACT - Purpose: A variety of fixed-dose combination products is used in the therapy of Parkinson Disease. However, to date a proper analytical method applicable for comparative screening of different antiparkinson products was not available. The objective of the present work was thus to develop and validate an analytical method for the simultaneous quantification of levodopa, carbidopa, benserazide and entacapone. The method should be applicable for quantifying samples from drug release experiments with marketed products and prototype formulations performed under compendial and biorelevant test conditions. **Methods:** A fast and robust method applicable for separation and quantification of the four compounds was developed and validated according to International Conference on Harmonization guidelines. Method validation covered applicability to a wide concentration range of all compounds and peak separation in complex sample matrices such as biorelevant dissolution media. **Results:** The compounds were successfully separated by using a gradient elution method on an endcapped LiChrospher 100 RP-18 (250 x 4.6 mm, 5 µm) column coupled with a LiChrospher 100 RP-18 precolumn (4 x 4 mm, 5 µm) at a column temperature of 35.0 °C and a flow rate of 1.50 mL/min. The injection volume was 30 µL and the detection wavelengths were 280 and 210 nm, respectively. For all drug/media combinations the method was linear ($r^2 > 0.999$) for a concentration range corresponding to 1.25 - 125 % label claim (i.e. 200 mg levodopa/entacapone and 50 mg carbidopa/benserazide) released. All other validation parameters were in the specified limits over the same concentration range. **Conclusion:** The new method allows for robust and fast separation of levodopa, carbidopa, benserazide and entacapone without any interference caused by excipients or ingredients of compendial and biorelevant dissolution media and thus presents a valuable tool in both formulation development and *in vitro* drug release screening of numerous fixed-dose combinations of antiparkinson drugs.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative brain disorder in which neurons of the substantia nigra progressively degenerate. As a result, the amount of dopamine available for neurotransmission in the corpus striatum is lowered. The biochemical imbalance manifests with typical clinical symptoms that include resting tremor, rigidity and bradykinesia amongst others. PD develops gradually and while tremor may be the most well-known sign of PD, the disorder also commonly causes stiffness or slowing of movement.

PD can't be cured, but medications can markedly improve symptoms. Since almost 50 years, the most effective mode of symptomatic treatment has been the administration of the l-isomer of 3,4-dihydroxyphenylalanine (levodopa), a dopamine precursor (1). To inhibit the extracerebral decarboxylation of levodopa,

allowing more levodopa to cross the blood-brain barrier to target the striatal dopamine receptors, the drug is typically administered with a peripheral dopa-decarboxylase inhibitor such as carbidopa or benserazide. Since many years the combination of levodopa and a dopa-decarboxylase inhibitor (mostly carbidopa) is thus the primary standard of PD treatment. However, since quite some time, the levodopa/carbidopa combination can be complemented by the administration of entacapone, a selective and reversible inhibitor of the catechol-o-methyl transferase (COMT) which represents another principal levodopa-metabolizing enzyme (2).

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Combination of levodopa with peripheral dopa-decarboxylase and COMT inhibitors extends the elimination half-life and plasma area under the curve of levodopa without affecting the maximal plasma concentration of levodopa (C_{max}) or the time until an oral dose of levodopa reaches its peak plasma concentration (T_{max}). Clinically, these pharmacokinetic effects permit a reduction in the levodopa dose, an increase in periods when the medication is working and symptoms are controlled ("on" time) and, a decrease in periods with reduced mobility ("off" time) in patients that had started to develop motor fluctuations as result of variations in the individual's response to levodopa. Motor benefits can also be seen in stable PD patients. COMT inhibitors are thus an alternative to increasing levodopa doses or adding dopamine agonists to reduce "off" time and enhance motor function in fluctuating PD patients (3).

Today, a variety of dosage forms comprising fixed dose combinations of levodopa/carbidopa, levodopa/benserazide or levodopa/carbidopa/entacapone is on the market. Formulation approaches for these combinations range from immediate release (IR) formulations through delayed release (DR) to extended release (ER) formulations. The variety of products allows an individualized treatment of PD patients in different disease states. Nevertheless, after several years of smooth and stable response to individualized levodopa treatment, most of the patients develop motor fluctuations manifested by "on" and "off" phases (4). Morning akinesia is the most common motor fluctuation in PD (5) since even the administration of modern fixed dose DR and ER combinations cannot prevent a dopaminergic nocturnal decline with insufficient nighttime storage or refresh the dopaminergic system during nighttime and sleep.

A novel dosage form taken at bedtime and providing drug release within the early morning hours might be of essential benefit for patients suffering from advanced PD with pronounced morning akinesia. Based on these considerations we are currently developing an oral formulation intended to provide a fixed dose combination of levodopa, a dopa-decarboxylase inhibitor and potentially also a COMT inhibitor in the early morning hours. To determine the most appropriate release pattern, prototypes of the novel formulation will be subjected to intensive *in vitro* screening applying novel patient-specific *in vitro* test models. *In vitro* drug release profiles of the prototypes will also be compared with those from currently marketed fixed dose combination products including levodopa/carbidopa (e.g. Nacom, MSD Sharp & Dohme GmbH, Germany), levodopa/benserazide (e.g. Madopar, Roche Pharma AG, Switzerland) and levodopa/carbidopa/entacapone (e.g. Stalevo, Orion Corporation, Finland).

When designing the analytical protocol for *in vitro* testing and drug quantification, we wanted to implement an analytical method allowing for the simultaneous quantification of all drugs included in the study. However, when screening the relevant literature, it quickly became clear that even though there is a multitude of valuable methods available for the simultaneous detection of levodopa and dopa-decarboxylase inhibitors, e.g. (6-8), to date no method for the simultaneous detection of levodopa, carbidopa, benserazide and entacapone has been described in the literature. A couple of methods enabling the detection of levodopa, carbidopa and entacapone in a single HPLC run have been described in the recent past (9-11) and were investigated for our purpose.
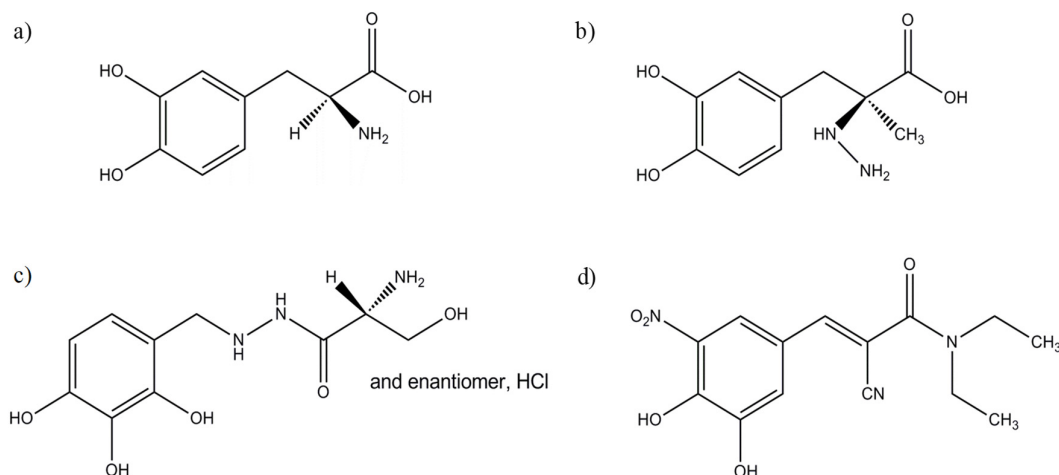

Figure 1: Chemical structures of a) levodopa, b) carbidopa, c) benserazide hydrochloride and d) entacapone

However, it turned out that without modification the respective methods were not applicable for also properly detecting benserazide (9-10). Moreover, one of the methods turned out to not even being applicable for the detecting the drug combination given in the title of the manuscript (11).

The purpose of the present study was thus to develop and validate a robust and efficient HPLC method for the simultaneous quantification of levodopa, carbidopa, benserazide (in the form of its hydrochloride salt) and entacapone (figure 1). The method should be applicable for quantifying samples from drug release experiments performed with prototypes and marketed fixed dose combinations under compendial test conditions as well as from patient-specific *in vitro* experiments comprising a number of biorelevant dissolution media (12). The method would thus need to be robust towards both matrix effects caused by formulation excipients and ingredients of the dissolution media.

MATERIALS AND METHODS

Standards
All active pharmaceutical ingredients (API) used for method development and validation were of analytical grade. Levodopa (batch # SLBB4239V) was purchased from Sigma-Aldrich Corporation (St. Louis, USA). Carbidopa (batch # FC012961550), benserazide hydrochloride (batch # FB181711550) and entacapone (batch # FE226781551) were purchased from Carbosynth Limited (Compton, UK). All APIs were obtained with a certificate of analysis confirming that they met product specification and had a purity > 99 %. Table 1 lists the physicochemical properties of the four APIs that might affect separation. Preferably, the respective data were taken from relevant literature. However, where no or no reliable data were available, the respective structure-based properties where predicted using an online tool (13).

Drug formulations
Four different marketed dosage forms representing the different drug combinations applied in PD treatment were selected to screen method robustness towards matrix effects caused by typical formulation excipients. The selected dosage forms included Madopar Depot (Roche Pharma AG, Switzerland; batch # M2379B01), Nacom (MSD Sharp & Dohme GmbH, Germany; batch # E000146) and Stalevo (Orion Corporation, Finland; batch # 1648258) which were obtained

from the hospital pharmacy of the university medicine in Greifswald. Madopar DR (Roche Pharma AG, Switzerland; batch # B4012B72) was imported from Switzerland via a local pharmacy.

Chemicals
Sodium dihydrogen phosphate dihydrate was purchased from Merck KGaA (Darmstadt, Germany), di-sodium hydrogen phosphate dihydrate was obtained from Fagron GmbH & Co. KG (Barsbüttel, Germany) and hydrochloric acid was from AppliChem GmbH (Darmstadt, Germany). HPLC gradient grade acetonitrile and orthophosphoric acid for HPLC were purchased from VWR Chemicals (Fontenay-sous-Bois, France). Water for mobile phases and sample preparation was prepared in house applying a Milli-Q reference water purification system (Merck KGaA) and filtered through a 0.22 µm polyvinylidene fluoride (PVDF) filter before use. All other compounds were of analytical grade and were purchased commercially.

Equipment
Method development and validation were performed on a Waters high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, USA), consisting of a 1525 series binary pump, an 2707 automatic injector equipped with a 100 µL loop, a thermostated 1500 column compartment and an 2489 UV/Visible detector. System control, data acquisition and integration were accomplished with the Breeze™ 2 software (Waters Corporation).

Chromatographic conditions
Chromatographic separation of the analytes was achieved on an endcapped LiChrospher 100 RP-18 column (Merck KGaA, Darmstadt, Germany) with the dimensions 250 x 4.6 mm and 5 µm particle size. The column was coupled with a LiChrospher 100 RP-18 precolumn of 4 x 4 mm and 5 µm particle size (Merck KGaA). HPLC analyses were carried out by applying the gradient conditions as stated in table 2. Solvent A consisted of a 30 mM phosphate buffer pH 2.50 (20 mM sodium dihydrogen phosphate dihydrate and 10 mM di-sodium hydrogen phosphate dihydrate). The pH of the solvent was adjusted with orthophosphoric acid. Solvent B was composed of a mixture of water that prior to mixing had been adjusted to pH 2.50 with orthophosphoric acid and acetonitrile (50:50, v/v).

Table 1: Physicochemical properties of levodopa, dopa-decarboxylase and COMT inhibitors (the corresponding literature sources are given in brackets)

	levodopa	carbidopa	benserazide	entacapone
molecular weight (g/mol)	197.2 (14)	226.2 (14)	293.7 (14)	305.3 (14)
logP	- 2.4 (15)	- 0.1 (15)	-1.90 (13)	1.63 (13)
pK$_a$	2.3 (15)	3.59 (13)	8.66 (13)	4.5 (15)

Table 2: HPLC gradient conditions

Time (min)	Flow rate (mL/min)	% A	% B
0	1.50	100	0
5	1.50	100	0
10	1.50	0	100
13	1.50	0	100
14	1.50	100	0
17	1.50	100	0

All eluents were filtered through a 0.22 µm polyethersulfon filter (Millipore Express plus, Merck KGaA, Darmstadt, Germany) before use. Experiments were conducted at a flow rate of 1.50 mL/min and the column temperature was maintained at 35.0 °C. The injection volume was 30 µL. Prior to starting a set of experiments the system was equilibrated with solvent A for 30 min. Levodopa, carbidopa and entacapone were detected at a wavelength of 280 nm, benserazide was monitored at 210 nm and 270 nm, respectively. Two different wavelengths were chosen for the detection of benserazide because of the fact that, whereas in some publications benserazide is detected at about 270 nm (7), in the official method of the European Pharmacopoeia benserazide is monitored at 210 nm (14). Our objective was thus to determine which wavelength is the most appropriate one for our purpose.

Preparation of stock solutions and dilutions
The decision for setting the concentration range for the standard dilutions was based on the intended use of the novel HPLC method, i.e. the quantification of antiparkinson drugs in *in vitro* drug release experiments. For the drug release experiments the following marketed dosage formulations were chosen: Madopar Depot (levodopa / benserazide 200 mg / 50 mg), Madopar DR (levodopa / benserazide 200 mg / 50 mg), Nacom (levodopa / carbidopa 200 mg / 50 mg) and Stalevo (levodopa / carbidopa / entacapone 200 mg / 50 mg / 200 mg). The dose strength of each of these dosage forms represents the highest single API dose available in fixed dose combination products, i.e. 200 mg levodopa and entacapone and 50 mg carbidopa and benserazide, respectively. According to the intended use of the method concentrations of the standard stock solutions referred to 125 % of the respective API dose in the formulation dissolved in a media volume of 900 mL which represents a typical volume of dissolution medium used in standard dissolution experiments in USP apparatus 1 or 2 (Basket or Paddle apparatus) (16). Consequently, stock solutions of levodopa, carbidopa and benserazide hydrochloride were prepared by dissolving the corresponding standards (pure APIs) in 0.1 N hydrochloric acid resulting in concentrations of 277.8 µg/mL, 69.4 µg/mL and 79.2 µg/mL (benserazide hydrochloride), respectively. Due to the limited aqueous solubility of entacapone, the entacapone stock solution was obtained by first dissolving 27.78 mg of the drug in 10 mL acetonitrile and then adding water to obtain 100.0 mL stock solution with a concentration of 277.8 µg/mL. Sets of standard dilutions covering a given "working range" for method validation were prepared by diluting the stock solutions with the corresponding solvents. All standard dilutions ("samples") were filtered through a 0.45 µm cellulose acetate membrane filter before injection.

Analytical method validation
Validation of the novel HPLC method was performed according to International Conference on Harmonization (ICH) and United States Pharmacopoeia (USP) validation guidelines (16-17). Parameters examined included linearity and range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity, system suitability and robustness.

The novel HPLC method was mainly developed for quantifying drug load and drug release of ER fixed dose combinations with high drug loads. However, as possible the method should also be applicable in routine testing for instance for testing IR formulations containing

combinations with lower doses of the same APIs. For the latter objective, the method should thus enable to appropriately quantify the APIs over a wide concentration range. Based on these considerations two separate concentration ranges were selected for the validation procedure. Accordingly, the validated concentration ranges for levodopa, carbidopa and entacapone were 1.25 to 12.5 % (0.69 to 6.94 µg/mL for carbidopa and 2.78 – 27.78 µg/mL for levodopa and entacapone) and 12.5 to 125 % (6.94 to 69.4 µg/mL for carbidopa and 27.78 – 277.8 µg/mL for levodopa and entacapone) of the maximum expected analyte concentration represented by complete dissolution of the highest single dose of each drug. Based on the significantly lower intensity of ultraviolet light absorption of benserazide at 270 nm, the validated concentration ranges for both wavelengths (210 nm and 270 nm) were 2.50 to 12.5 % (1.58 to 7.92 µg/mL) and 12.5 to 125 % (7.92 to 79.2 µg/mL) of the target concentration, respectively. The sets of dilutions for each API were examined for a linear relationship by plotting the analyte peak areas of 10 different samples versus the corresponding concentrations followed by least square linear regression and calculation of the slope, intercept and coefficient of determination. Three separate series of calibration standards for each calibration range were prepared to establish linearity.

Accuracy, expressed as mean absolute recovery and percent relative standard deviation (% RSD), for all analytes was assessed in triplicate for each concentration of the specified ranges.

The precision of the novel method expressed as repeatability (intraday) and intermediate precision (interday) was screened by preparing six individual samples of the lowest (1.25 % or 2.50 %, respectively), medium (12.50 %) and highest (125 %) concentration of the working range for each compound. To evaluate interday precision standards were prepared in the same way and analyzed on three different days. Repeatability and intermediate precision were assessed via absolute recovery and % RSD of the calculated concentrations.

The LOD is a characteristic value for the sensitivity of the method, at which the respective compound is just measurable, whereas the LOQ is the lowest concentration with acceptable linearity, accuracy and precision. The LOD was determined based on signal-to-noise ratio of 3:1, and the lowest concentration of the working range 1.25 % or 2.50 % to 12.5 % were set as the LOQ for

levodopa, carbidopa, entacapone (1.25 %) and benserazide (2.50 %).

Specificity which is an essential part of method validation was assessed as follows: First, a set of standard solutions (API reference material dissolved in a simple solvent or dissolution medium) of the four analytes were prepared with the three possible API combinations available in marketed levodopa fixed-dose combination products. Different standard solutions were prepared using simple solvents (e.g. acetonitrile/water) and the compendial and biorelevant media listed in table 3. Then, the impact of excipients used in the manufacture of the selected marketed dosage forms on the proper assessment of the API peaks was screened. For this purpose, single tablets/capsules of Madopar Depot, Madopar DR, Nacom and Stalevo were placed in separate containers containing 200 mL of one of the media listed in table 3 and the fluid was slightly agitated for one hour. Following sample analysis, the obtained chromatograms were checked for peak area and interference of excipients at the API retention times.

System suitability was examined by determination of tailing factor (T_f), retention factor (k), number of theoretical plates (N), height equivalent to the theoretical plate (HETP), resolution (R_s) of the respective analytes and the reproducibility of peak areas and retention times. In addition, calibration curves of the media listed in table 3 were established for the four analytes and checked for linearity.

To determine the reliability of the proposed HPLC method robustness was evaluated by varying different method parameters. Several parameters were considered critical factors for the analysis. The self-imposed parameter limits set in for these parameters were inspired by other publications (6-7, 18) and own experience. Parameters were studied as follows: The flow of the mobile phase (± 2 %), column temperature (± 5 °C), buffer strength of the aqueous component (± 5 mM) and pH value (± 0.25) for both mobile phase A and mobile phase B were investigated by injecting a series of dilutions with three individual standards for lowest, medium and highest concentrations (of the concentration range discussed in the previous section) of levodopa, carbidopa, benserazide and entacapone in triplicate. The robustness of the method was assessed by absolute mean recovery, % RSD of recovery (precision) and r^2 of the resulting calibration curves.

Table 3: Media used to investigate specificity and system suitability

Buffers/compendial media
Simulated Gastric Fluid *sine pepsinum* (SGF*sp*) pH 1.2
Acetate buffer pH 4.5
Simulated Intestinal Fluid (SIF) pH 6.5
Simulated Colonic Fluid (SCoF) pH 5.8
Biorelevant media
Fasted State Simulated Intestinal Fluid (FaSSIF) V-1 pH 6.5
Fed State Simulated Intestinal Fluid (FeSSIF) V-1 pH 5.0

RESULTS

Method development and optimization

The novel separation method was targeted to demonstrate acceptable chromatographic performance and to be universally applicable on standard HPLC equipment. Following an initial literature research promising HPLC methods for the targeted analytes were selected and checked for their suitability. In the early stages of the present method development, chromatographic methods with isocratic elution were tested with a Zorbax Eclipse XDB-C18 column (250 x 4.6 mm, 5 µm; Agilent Technologies, Inc., Santa Clara, USA). Subsequently, to achieve better selectivity, an endcapped LiChrospher 100 RP-18 (250 x 4.6 mm, 5 µm) was used. To protect the column from potential contamination by components of biorelevant media to be used in future dissolution experiments (e.g. bile compounds, fat droplets etc.), a precolumn was installed. All cited literature methods comprise the use of a 250 mm column. This results in long run times or the need of higher flow rates and is thus time-consuming and cost-intensive. Since the objective was to develop a robust and effective method, in the next stage of method development, promising literature methods were transferred onto a shorter column (LiChrospher 100 RP-18, 125 x 4 mm, 5 µm) and screened for their applicability in detecting the four APIs of interest. However, results from these experiments clearly indicated that with a simple switch to a shorter column with the same or stationary phase, a proper analyte separation was not possible.

After these initial set of method screening it was clear that an isocratic separation of the analytes is hardly possible within a reasonable run time. This is a result of the different physico-chemical properties of the APIs given in table 1 and can be explained as follows: Benserazide HCl was detectable with a highly polar, aqueous mobile phase without addition of any organic solvents, whereas entacapone had a stronger affinity to the stationary phase and could only be properly eluted using an eluent containing a certain amount of acetonitrile. Consequently, a gradient method had to be applied. Based on the cited observations (data not shown), theoretically, the gradient separation had to start with a polar mobile phase switching to a more apolar eluent to cover all four compounds. Based on these considerations a phosphate buffer with a pH of 2.50 was chosen as solvent A, while a mixture of water (pH adjusted to 2.50) and acetonitrile (50:50, v/v) was selected as solvent B. Furthermore, the selected eluents were optimized with regard to solvent pH and ionic strength. Suitable injection volume and column temperature were selected and the initial gradient program was also stepwise modified. The final gradient method applying the gradient shown in table 2 combined with an injection volume of 30 µL and a column temperature of 35 °C, was found to be suitable for a good chromatographic separation and selected for subsequent method validation. Figure 2 illustrates a chromatogram of the four target APIs in a mixture of standard solutions of the analytes obtained by applying the final gradient method and using a wavelength of 280 nm for simultaneous UV detection.

Linearity

The linear relationship of analyte concentrations and peak areas is expressed by the coefficient of determination (r^2). Linearity for each of the four compounds could be shown for all calibration curves over the concentration ranges stated earlier since all r^2 values were above 0.999 (see table 4).

Accuracy, precision

Results for accuracy and precision are given in table 4. For every analyte a consistent and high absolute recovery and low % RSD within the acceptance limit of ± 5 % of 100 % drug recovery were demonstrated at all concentrations. The % RSD results for repeatability and intermediate precision were inferior to 2.68 % and thus regarded as acceptable.
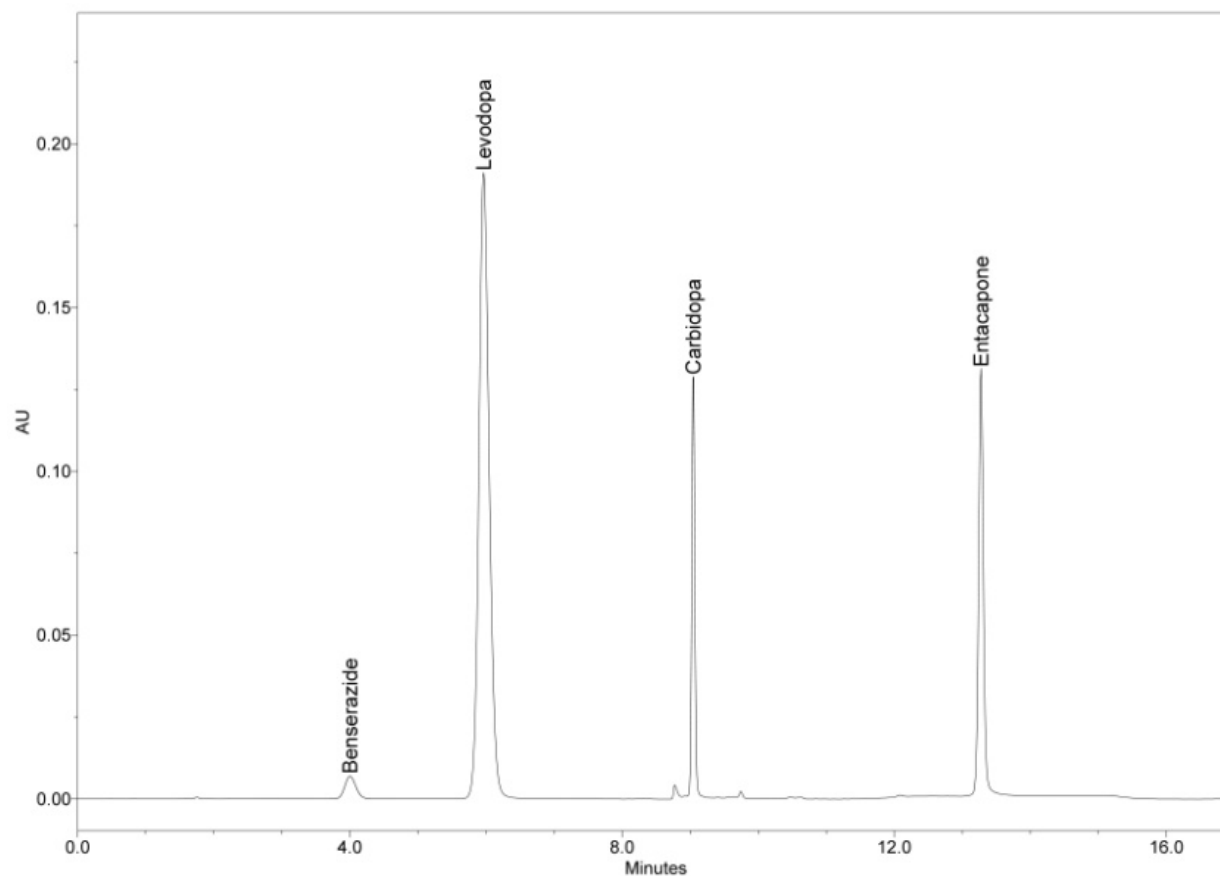
Figure 2: Chromatogram obtained by applying the gradient method given in table 2 and using a detection wavelength of 280 nm.

Specificity

The specificity of the new method was evaluated by first analyzing blank solvents/media and then samples containing a single drug or drug combinations in media of increasing complexity.

The method showed good chromatographic separation of the compounds in standard solutions (API reference material dissolved in a simple solvent), in solutions were the drugs were dissolved in more complex media (API + compendial or biorelevant dissolution medium, see table 3) as well as in samples of the disintegrated/dissolved marketed formulations (fixed dose combination product + compendial or biorelevant dissolution medium). No peak interference of the analytes with blank media, buffer components or excipients of the marketed dosage formulations was observed.

Figures 3 a) and b) show exemplary a chromatogram of a standard solution of a) levodopa and benserazide in 0.1 N HCl and of a mixture of standard solutions of b) levodopa and carbidopa in 0.1 N HCl and entacapone in water/acetonitrile, respectively. The retention times were 4.05 min for benserazide, 6.3 min for levodopa, 9.14 min for carbidopa and 13.15 min for entacapone. Resolution was above 2 in all cases. In figure 3 c) a chromatogram of a standard solution of levodopa and benserazide in FeSSIF V-1 pH 5.0, a biorelevant dissolution medium having a high osmolality and containing a large amount of bile compounds is shown. Figure 3 d) presents a chromatogram of levodopa, carbidopa and entacapone reference material in FeSSIF V-1 pH 5.0 and finally, figures 3 e-f show the chromatograms obtained from samples from a dissolution experiment of Madopar DR (figure 3 e) and Stalevo (figure 3 f) in FeSSIF V-1 pH 5.0. Chromatograms obtained from dissolution experiments of Madopar Depot and Nacom are not shown, but did also show no interferences.
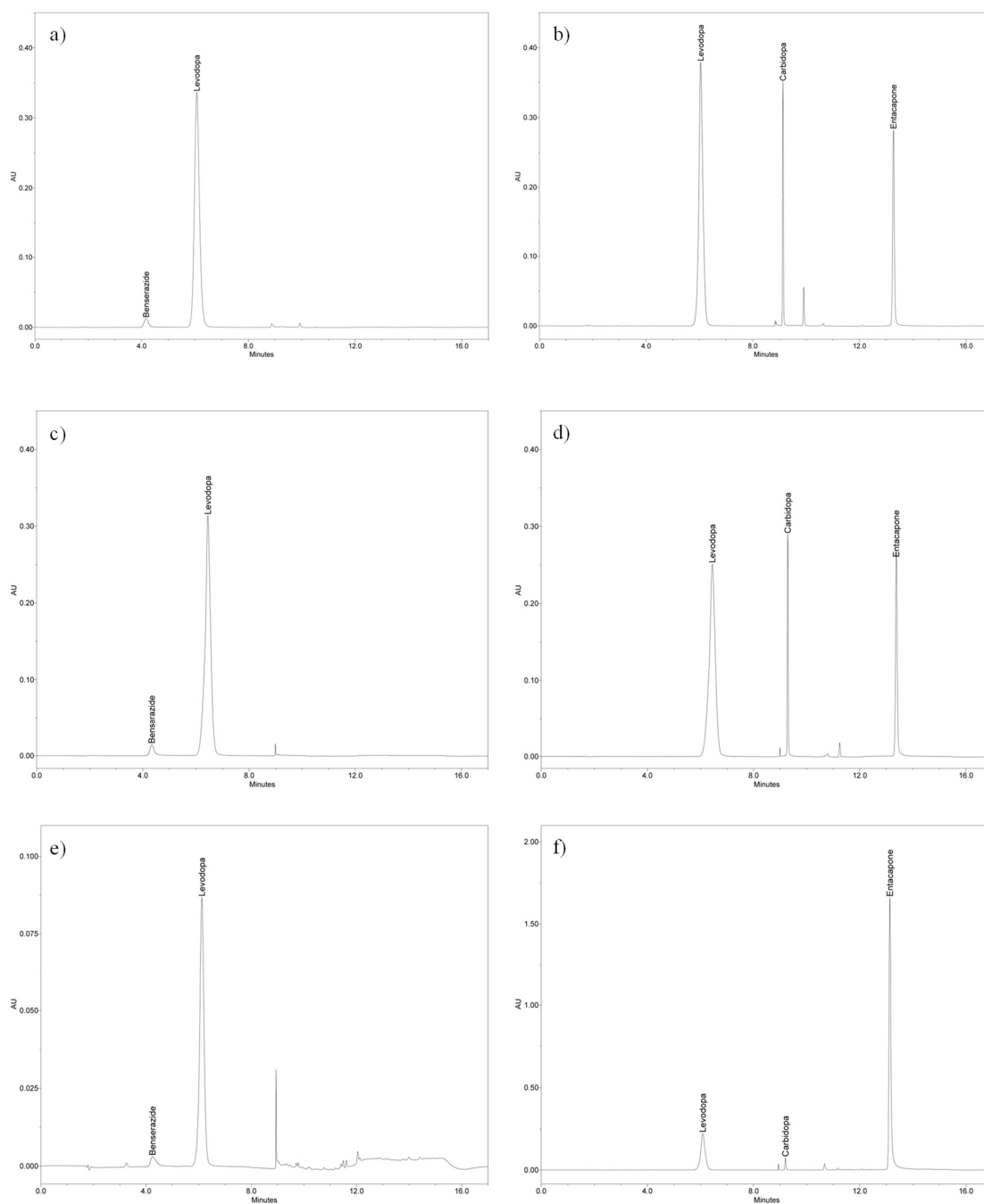
Figure 3: Chromatograms of a) standard solution of levodopa + benserazide in 0.1 N HCl, b) mixture of standard solutions of levodopa + carbidopa in 0.1 N HCl and entacapone in water/acetonitrile, c) standard solution of levodopa + benserazide in FeSSIF V-1 pH 5.0, d) mixture of standard solutions of levodopa + carbidopa and entacapone in FeSSIF V-1 pH 5.0, e) Madopar DR and f) Stalevo after immersion in FeSSIF V-1 pH 5.0 for one hour (280 nm).

System suitability

Primary parameters to evaluate system suitability such as symmetry factor, retention factor or number of theoretical plates were determined for the lowest, medium and highest concentrations for each analyte and are listed in table 4 (data only shown for the medium concentrations). Levodopa, carbidopa, benserazide and entacapone showed excellent peak symmetry. Moreover, the analyte peaks showed consistent low variability in peak areas and retention times.

Table 4: Results of HPLC method validation with the drugs ordered according to their retention times

Parameter		Values			
	Benserazide		**Levodopa**	**Carbidopa**	**Entacapone**
	210 nm	270 nm			
Retention time	4.05 min	4.05 min	6.3 min	9.14 min	13.15 min
Calibration ranges					
Range 1	1.58 – 7.92 µg/mL	1.58 – 7.92 µg/mL	2.78 – 27.78 µg/mL	0.69 – 6.94 µg/mL	2.78 – 27.78 µg/mL
Range 2	7.92 – 79.2 µg/mL	7.92 – 79.2 µg/mL	27.78 – 277.8 µg/mL	6.94 – 69.4 µg/mL	27.78 – 277.8 µg/mL
Linearity (r^2)					
Range 1	0.9998 / 0.9998 / 0.9996	0.9995 / 0.9998 / 0.9997	0.9996 / 0.9998 / 0.9995	0.9997 / 0.9993 / 0.9995	0.9999 / 0.9997 / 0.9999
Range 2	0.9998 / 0.9998 / 0.9990	0.9998 / 0.9997 / 0.9991	1.0000 / 0.9999 / 0.9999	0.9998 / 0.9999 / 0.9999	0.9997 / 0.9997 / 0.9996
Accuracy					
Mean absolute recovery					
Range 1	98.40 – 101.15 %	98.70 – 102.73 %	95.29 – 103.96 %	97.80 – 104.78 %	97.81 – 102.00 %
Range 2	95.84 – 102.90 %	95.76 – 103.10 %	96.12 – 100.88 %	98.59 – 103.76 %	97.85 – 101.75 %
% RSD					
Range 1	0.30 – 1.39 %	0.23 – 1.21 %	0.12 – 4.35 %	0.19 – 1.56 %	0.02 – 1.41%
Range 2	0.51 – 2.34 %	0.27 – 3.01 %	0.01 – 0.57 %	0.11 – 1.41 %	0.07 – 1.14 %
Precision					
Absolute recovery					
a) low concentration	97.43 – 102.34 %	97.39 – 103.58 %	98.26 – 100.92 %	98.38 – 101.90 %	95.12 – 102.50 %
b) medium concentration	98.41 – 101.63 %	97.68 – 101.85 %	99.47 – 100.48 %	98.81 – 101.29 %	98.26 – 101.86 %
c) high concentration	97.63 – 101.59 %	97.64 – 101.60 %	99.80 – 100.12 %	98.80 – 100.63 %	98.50 – 101.33 %
Repeatability (% RSD)					
a) low concentration	1.09 %	1.33 %	0.71 %	1.09 %	1.64 %
b) medium concentration	1.11 %	1.64 %	0.12 %	0.79 %	1.15 %
c) high concentration	0.66 %	0.66 %	0.10 %	0.25 %	0.97 %
Intermediate precision (% RSD)					
a) low concentration	1.09 % / 2.06 % / 1.29 %	1.33 % / 2.55 % / 1.76 %	0.71 % / 0.83 % / 0.96 %	1.09 % / 1.40 % / 1.06 %	1.64 % / 2.68 % / 1.92 %
b) medium concentration	1.11 % / 1.11 % / 1.01 %	1.64 % / 0.93 % / 1.02 %	0.12 % / 1.13 % / 1.06 %	0.79 % / 0.76 % / 0.83 %	1.15 % / 0.53 % / 0.95 %
c) high concentration	0.66 % / 1.55 % / 1.37 %	0.66 % / 1.55 % / 1.39 %	0.10 % / 0.13 % / 0.09 %	0.25 % / 0.68 % / 0.25 %	0.97 % / 0.76 % / 0.99 %
Limit of detection	0.03 ng/mL	3.95 ng/mL	27.8 ng/mL	13.8 ng/mL	0.01 ng/mL
Limit of quantification	1.58 µg/mL	1.58 µg/mL	2.78 µg/mL	0.69 µg/mL	2.78 µg/mL
System suitability					
Symmetry factor	1.08	1.09	0.91	1.14	1.06
Retention factor	1.39	1.39	2.73	3.96	6.82
Number of plates	3511	3553	9428	458244	219357
HETP	71.21 µm	70.36 µm	26.52 µm	0.55 µm	1.14 µm
Peak areas (% RSD)	0.91 %	1.75 %	0.20 %	0.81 %	0.28 %

Table 4. Continued…

Retention times (% RSD)	0.09 %	0.07 %	0.07 %	0.03 %	0.00 %
Robustness (abs. mean recovery)					
Flow rate					
a) 1.47 mL/min	99.10 % (1.39 % RSD)	98.95 % (0.82 % RSD)	100.00 % (0.09 % RSD)	99.07 % (1.95 % RSD)	98.97 % (0.15 % RSD)
b) 1.53 mL/min	98.96 % (0.38 % RSD)	98.42 % (0.49 % RSD)	99.76 % (0.03 % RSD)	99.20 % (1.41 % RSD)	99.15 % (0.17 % RSD)
Temperature					
a) 30 °C	98.46 % (0.85 % RSD)	98.55 % (0.94 % RSD)	99.40 % (0.04 % RSD)	101.92 % (1.60 % RSD)	100.50 % (0.44 % RSD)
b) 40 °C	99.21 % (0.48 % RSD)	99.21 % (0.30 % RSD)	100.37 % (0.23 % RSD)	99.20 % (0.61 % RSD)	100.24 % (0.40 % RSD)
Ionic strength					
a) 25 mM	99.41 % (1.17 % RSD)	100.10 % (0.63 % RSD)	100.92 % (1.01 % RSD)	101.80 % (1.55 % RSD)	99.65 % (0.88 % RSD)
b) 35 mM	99.09 % (0.50 % RSD)	99.40 % (0.37 % RSD)	99.51 % (0.73 % RSD)	100.30 % (0.72 % RSD)	99.40 % (0.94 % RSD)
pH value					
a) 2.25	101.16 % (0.92 % RSD)	101.12 % (1.91 % RSD)	100.20 % (0.62 % RSD)	100.89 % (0.63 % RSD)	98.83 % (1.89 % RSD)
b) 2.75	99.41 % (1.19 % RSD)	98.74 % (3.22 % RSD)	99.46 % (0.70 % RSD)	100.77 % (1.07 % RSD)	100.79 % (0.53 % RSD)

In all cases the coefficient of determination of the calibration curves prepared in the different media types applied in the study was above 0.999, indicating that the method was suitable for samples with simple or rather complex matrices.

Robustness

Data of the robustness study indicate that linearity, absolute mean recovery and precision of the developed method remain unaffected by small changes of critical method parameters. The corresponding results are given in table 4 (data only shown for medium concentrations). Variations of temperature, flow rate, ionic strength and pH value did not affect the recovered amount of the analytes. Absolute mean drug recovery for all compounds was within 95 – 105 % and the % RSD was below 4.32 %. The resulting calibration curves showed good linearity, i.e. the coefficients of determination were above 0.999 in all cases.

DISCUSSION

An analytical method to be applied in formulation screening, biopredictive dissolution testing and quality control of fixed-dose combination products should be robust, efficient and reliable. In the course of developing prototype formulations of fixed-dose combination products containing two or more antiparkinson drugs, it became obvious that a method fulfilling the above-mentioned criteria and that can be applied for the simultaneous quantification of levodopa, carbidopa, benserazide and entacapone had not yet been described in the literature. A detailed literature research revealed that a few robust and efficient HPLC methods for the parallel detection of levodopa and carbidopa have been published over the last decades, e.g. (6-8). These methods mainly differ in the detection method applied, but in principle would have been applicable for our purpose.

However, an initial screening of these methods in our lab indicated that the methods were not directly applicable for detecting all four antiparkinson drugs of interest. Selective and reliable HPLC methods for detecting more than two of the compounds within a single run were published by Ribeiro *et al.* (9) and Vemic *et al.* (10). However, the cited methods did not cover the complete range of APIs studied in our experiments and the overall run times of the methods were quite long. The method for the simultaneous analysis of levodopa, carbidopa and entacapone published by Vemic *et al.* (10) had for instance a total run time of 40 min, even though a relatively short 150 mm C-18 column was applied and thus was not regarded as efficient. Another published "liquid chromatographic method for the estimation of levodopa, carbidopa and entacapone in combined dosage forms" (11) reporting the application of a 250 x 4.6 mm, 5 µm C-18 column, a mobile phase consisting of a pH 4.0 phosphate buffer:methanol 60:40 (v/v), i.e., a polar eluent, a column temperature of 25 °C and a flow rate of 1.0 mL was also screened for our purpose. That method turned out to lack reproducibility due to the following reasons: The stock solutions could not be prepared in the given concentrations because of the limited solubility of the APIs and besides that, the indicated concentrations of the stock solutions were untypically high for a HPLC method. Following injection of a standard solution containing lower concentrations of levodopa, carbidopa and entacapone and exactly following the test protocol proposed by Thahaseen *et al.* (11), the dead volume of the column turned out to be much higher than the one given in the respective paper and also the retention times of the peaks were much higher. For these reasons, the cited method was not regarded as reliable or productive, respectively. Consequently, we had to develop a novel method. Using a 250 x 4.6 mm, 5 µm C-18 column with our optimized gradient separation method, we were able to obtain an excellent chromatographic separation of levodopa, carbidopa, benserazide and entacapone in the presence of complex matrices such as a variety of formulation excipients and ingredients of biorelevant dissolution media within a relatively short overall run time. The method was properly validated according to ICH guidelines and represents a robust, efficient and reliable method that can be applied in screening the uniformity of content and drug release of fixed-dose combination products containing two or more of the four antiparkinson drugs.

CONCLUSION

A RP HPLC method for an effective separation of levodopa, carbidopa, benserazide and entacapone was developed and validated. The method allows a fast and robust quantification without any interference caused by formulation excipients or ingredients of compendial and biorelevant dissolution media. The novel method thus presents a valuable tool in both formulation development and *in vitro* drug release screening of numerous fixed-dose combinations of antiparkinson drugs.

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