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Automated determination of levodopa and carbidopa in plasma by high-performance liquid chromatography–electrochemical detection using an on-line flow injection analysis sample pretreatment unit

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Abstract

An automated analytical procedure is described for the parallel determination of L-3,4-dihydroxyphenylalanine (levodopa, L-dopa, LD) and the analogous hydrazine compound carbidopa (CD) in dog plasma by ion-pair high-performance liquid chromatography with electrochemical detection (HPLC–ED). After deproteinization of the plasma samples with perchloric acid the catecholamines were extracted from the supernatant by adsorption on a small column filled with alumina. The extraction and redissolution were automatically performed in a flow injection analysis unit (FIA) coupled to the HPLC system. The performance of the whole system was tested on dog plasma samples including specimens taken after oral administration of the anti-Parkinsonism drug Duellin[®], which is a combination tablet of levodopa and carbidopa. © 1997 Elsevier Science B.V.

Keywords: Levodopa; Carbidopa

1. Introduction

Parkinsonism is an ailment of the vegetative neural system. Its symptoms have been efficiently treated by medication increasing the dopamine level in brain. Dopamine cannot be administered directly because it cannot penetrate the blood–brain barrier. Levodopa, on the other hand, can be orally administered and reaches the brain where it is enzymatically decarboxylated to dopamine. This decarboxylation reaction may also occur at some rate before levodopa reaches the brain. To avoid this, levodopa is applied

in combination with an inhibitor of the decarboxylase enzyme such as carbidopa. The widespread use of levodopa has necessitated fast and selective analytical methods for its determination in human and animal body fluids. Table 1 shows a selection of different methods that have been published for the determination of levodopa and/or carbidopa.

The fluorimetric and radiometric techniques have been largely abandoned, mainly because of their uncertain specificity [1]. They have been replaced now by other methods like reversed-phase HPLC with ion-pair formation.

Levodopa analysis in blood samples usually requires some kind of sample pretreatment. Serum or

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Table 1
Selected methods for determination of levodopa and/or carbidopa in body fluids

Method:	Chromatographic		Fluorimetric		Radiometric	
	RP-HPLC	Ion-exchange techniques	GC	Dihydroxyindole reaction		Ethylenediamine condensation
Comments:	Amperometric, coulometric and UV detection	After separation on an ion exchanger are quantitated post-column by reaction with ninhydrin or orthophthalaldehyde Amperometric detectors	Flame ionization detection, electron-capture detection, mass spectrometric detection	After oxidation, cyclization and isomerization in alkaline media, DOPA forms a dihydroxyindole derivative with fluorescent properties	Levodopa can complex with Tb(III) at pH 8.0–8.5 with emission of the characteristic fluorescence	An enzyme is used to transfer a radioactive methyl group to the catecholamine
Literature:	[2] ^a [3–5] [6,8] ^a [9] ^a [10] ^a [11] ^b [12] [13] ^a [14] ^a [15] [16] ^a [17,18]	[19]	[1]	[7] ^a	[20] ^a	[22]

^a Only LD was detected.

^b Only CD was detected.

Those without superscripts are analyses including both LD and CD.

plasma may be deproteinized with perchloric acid, trichloroacetic acid or an organic solvent. The protein-free fraction can be directly injected into the HPLC system. Such samples may shorten the life-time of the precolumn and decrease the efficiency of the analytical column, presumably due to residual proteins [2–4].

Better purification of the samples has been achieved by adding Al_2O_3 to the plasma or the deproteinized plasma. Catecholamines are selectively adsorbed on Al_2O_3 . The sorbent is washed several times and finally the catecholamines are eluted from it with an acidic solution [4–7]. This is a very useful method but it is quite laborious because of the repeated wash steps. Al_2O_3 has been occasionally replaced with other sorbents like strong cation exchanger [1] or a C_{18} phase [8]. These sorbents are less selective than Al_2O_3 and therefore chromatographic interferences are more likely. Liquid–liquid extraction with e.g. *n*-hexanol may also be used but it is even less convenient than the other methods [9].

Levodopa determination from blood would obviously benefit from an automated sample pretreatment

scheme. Zürcher et al. have developed a HPLC column-switch technique [10]. Deproteinized blood plasma was injected on the first, strong cation exchanger column. The relevant fraction of the eluate was further separated on the second, reversed-phase column. The method is fast, accurate and the system has a high throughput. However, there are also some disadvantages: the chromatographic base line shows instabilities due to column switching, the ageing of the cation exchange column necessitates relatively frequent readjustments and finally a system with two high pressure pumps is needed.

We have automated the simultaneous determination of levodopa and carbidopa in blood plasma by using a low pressure on-line sample pretreatment unit, similar to a flow injection analysis (FIA) set-up. A short column packed with Al_2O_3 was inserted into the low pressure flow line to carry out the cleaning and partial concentration of the analytes. This simple system gave good analytical results without using a second high pressure pump and without baseline fluctuation.

2. Experimental

2.1. Reagents

Fig. 1 shows the structure of the active material levodopa (LD), the stabiliser (carboxylase inhibitor) carbidopa (CD) and the internal standard, α -methyl dopa (MD). Methyl dopa, levodopa and carbidopa were manufactured by Egis Pharmaceutical Ltd. (Budapest, Hungary). All three are catecholamines. This group of compounds is selectively adsorbed on alumina from basic media [5]. The alumina used was aluminium oxide 90 active acidic (activity stage I) from Merck with particle size 0.063–0.200 mm. Sodium disulfite, sodium dihydrogen phosphate dihydrate, ortho-phosphoric acid, octanesulfonic acid sodium salt (Merck, Darmstadt, Germany), perchloric acid (Carlo Erba, Milan, Italy) and EDTA Na_2 (Reanal, Budapest, Hungary) were analytical grade reagents.

Stock solutions of the analytes and internal standard were made up to 0.5 mg/ml with the chromatographic eluent containing also $1.68 \cdot 10^{-3}$ M sodium disulfite as antioxidant. The two streaming solutions

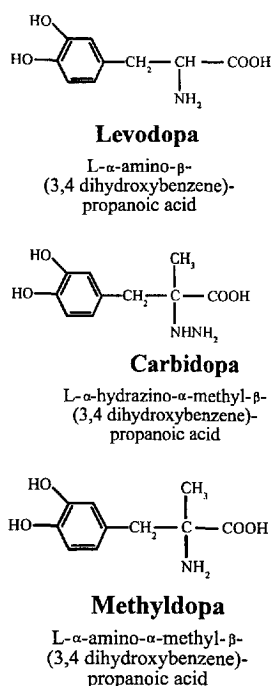


Fig. 1. Structure of levodopa, carbidopa and the internal standard (methyl dopa).

in the FIA system were double distilled water and 0.16 M perchloric acid, respectively. The chromatographic eluent consisted of phosphate buffer–methanol (84:16, v/v). The buffer was composed of 0.07 M NaH_2PO_4 , 0.001 M EDTANa_2 , 0.001 M octanesulfonic acid sodium salt and phosphoric acid to bring the pH to 2.79.

2.2. Sample processing

Blood samples of 4.00 ml volume were collected into 0.50 ml of 3% EDTA anticoagulant using heavy-wall centrifuge tubes with calibration mark at 4.50 ml. The samples were centrifuged at 1500 g. The plasma was transferred to 10-cm Wassermann tubes and stored at -20°C until analysis.

Thawed samples were spiked with internal standard (MD, 100 μl 1 $\mu\text{g}/\text{ml}$ to 900 μl plasma) and deproteinized by mixing with 100 μl 4.0 M perchloric acid. The precipitate was separated by 20 min centrifugation at 1500 g. A 0.75-ml aliquot of the supernatant was transferred into a test tube and the pH adjusted to 8.6 with Tris buffer.

The instrumental set-up is presented in Fig. 2. It

consists of two main parts. The dotted frame shows the FIA system used for sample pretreatment. The rest of the system is the HPLC part. The deproteinized and pH adjusted plasma sample was injected using injector 1. A stream of distilled water carried the sample onto the alumina column where the catecholamines were selectively bound while the rest of the sample was eluted through injector 3 to the drain. After a given washing period ($\sim 3\text{--}4$ min) the pneumatically driven injector 2, which was used as a tee valve, was switched and the catecholamines were displaced from the alumina column by 0.16 M perchloric acid. The pneumatic injector 3 was switched from the load to the inject position when the maximum fraction of the FIA peak of the analytes was in its loop (~ 15 s after injector 2 switching). The optimal timing of injection was determined experimentally. This optimum proved to be quite stable as confirmed by control measurements between longer measurement series.

A chromatographic run required 10 min. This time was sufficient for cleaning the alumina column by continued flushing with 0.16 M perchloric acid (4 min), reconditioning the alumina column with a

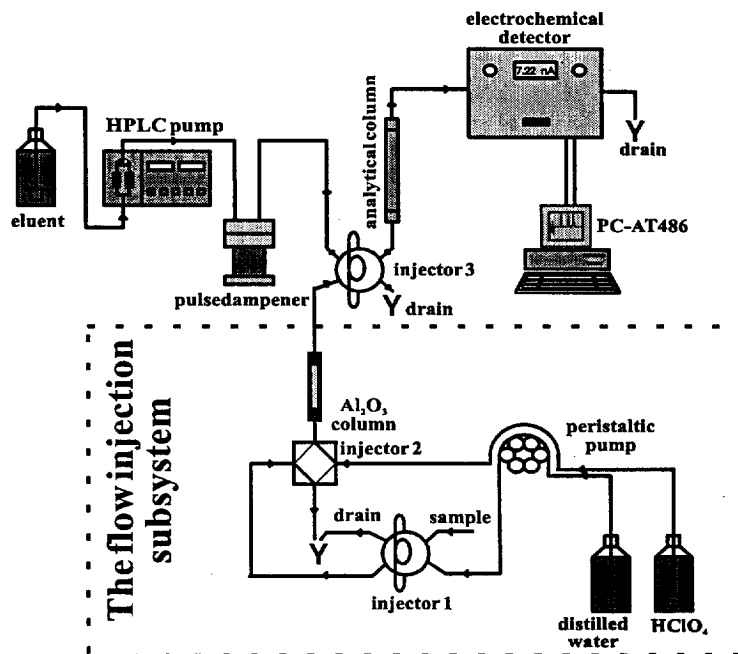


Fig. 2. Instrumental set-up.

stream of distilled water (1 min), loop filling and injection (1 min) and pretreatment of the next sample. Thus the sample pretreatment occurred in parallel with the chromatographic analysis of the previous sample, thus using the chromatographic system at its maximum capacity, i.e. six samples per hour. After the analysis of about 70 samples the performance of the alumina column began to deteriorate. At this point the column had to be refilled and the timing of the injection into the HPLC system was readjusted.

2.3. Equipment

HPLC pump: Beckman 110B solvent delivery system (Beckman Instruments, Berkeley, CA, USA) with an in-house pulsation dampener (flow-rate, 1.0 ml/min). This pump was replaced in the course of our studies by an LKB 2150 (Pharmacia LKB, Bromma, Sweden).

FIA pump: Ismatec (Ismatec, Zürich) Mini-S-540 two channel peristaltic pump (flow-rates: 0.67 and 2.42 ml/min for water and perchloric acid, respectively).

Injectors: No. 1 was a Rheodyne 50 Teflon rotary valve with a 250- μ l loop. Nos. 2 and 3 were both LaborMIM OE-320 (LaborMIM, Budapest, Hungary), pneumatically actuated under computer control. No. 2 was operated as a tee valve. No. 3 had a 100- μ l loop.

HPLC analytical column: ODS Hypersil 200 \times 4.6 mm, 5 μ m (Hewlett–Packard, Palo Alto, CA, USA); precolumn: Upchurch Scientific 20 \times 2 mm guard column filled with Perisorb RP-18, 30–40 mm.

FIA solid-phase extraction column: two columns were used alternatively. One was made from 30 \times 2.0 mm glass tube. It was filled dry with ~75 mg alumina and equipped with 2- μ m frits at both ends (Supelco filter tips). The other was an Upchurch Scientific guard column 10 \times 2 mm, filled dry with alumina and equipped with 2- μ m frits at both ends. The latter column was easier to handle but otherwise we did not notice particular differences compared with the glass column.

HPLC detector: HP 1049A (Hewlett–Packard) electrochemical detector in amperometric mode, using a glassy carbon working electrode set to +800

mV against the Ag/AgCl/3.3 M KCl (satd. AgCl) reference electrode.

Computer: 486 AT IBM compatible equipped with BORWIN 1.21 for Windows chromatography software (JMBS Developpements, Le Fontanil, France).

Other equipment: Reax 2000 vortex mixer (Heidolph Electro, Kelheim, Germany), 204 centrifuge (Sigma, Osterode am Harz, Germany), Biohit OY automatic pipette and syringes (Hamilton, Bonaduz, Switzerland).

2.4. Animal treatment

One year old, healthy male Beagle dogs, originating from Wobe Beagle Kennel Budapest Hungary, weighing 10.9 kg in average, were treated with one tablet of Duellin[®] 25 mg/250 mg containing 25 mg of carbidopa and 250 mg of levodopa. The animals were fasted for 24 h before treatment and kept under standard environmental conditions.

3. Results and discussion

Fig. 3 shows the chromatograms of a spiked and a blank dog plasma sample. All chromatograms were evaluated for LD/MD and CD/MD peak height ratios. The analytical system was stable, i.e. there was no trend in 16 consecutive parallel measurements of plasma spiked with LD, CD and MD.

Carryover test results with plasma samples show

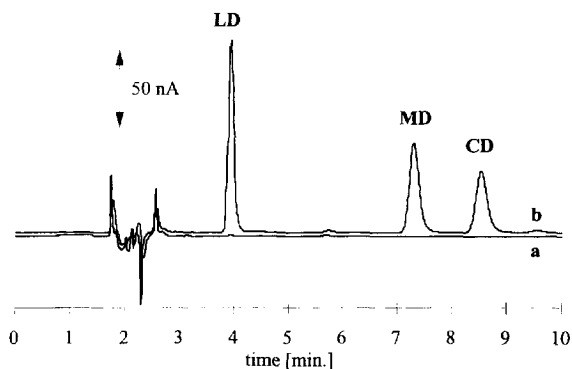


Fig. 3. Chromatogram of a blank (a) and a spiked dog plasma sample (b), the latter containing 500 ng/ml each of levodopa, carbidopa and internal standard. For chromatographic conditions see Section 2.3.

virtually no carryover (less than $10^{-2}\%$ for LD and less than $10^{-4}\%$ for MD and CD). The calibration graphs were linear for levodopa in the range 10–2000 ng/ml plasma and for carbidopa in the range 5–250 ng/ml plasma. The respective statistical detection limits are 0.3 and 1.5 ng/ml (defined by three times the standard deviation of the noise).

The calibration lines were fitted by $1/y^2$ weighting, i.e. the squared deviations from the straight line were divided by the square of the measured (peak height ratio) and the sum of these weighted deviations was minimised. This procedure was justified by showing that the standard deviation of the peak height ratio was directly proportional to the peak height ratio itself. The data in this study came from three replicates of all points on the calibration curve. The respective standard deviations were plotted against the measured peak height ratio. The slope of the straight line fitted to these data showed that the relative standard deviation of the measurements was 1.5% and 3.8% for LD and CD, respectively, in the whole calibration range.

In all blank dog plasma samples a small peak was observed at the retention time of levodopa. Such a peak was not observed with non-serum blanks and therefore it could not result from reagent contamination. We attribute these peaks to endogenous levodopa. The concentration calculated from these peaks was between 1 and 5 ng/ml in different blank samples and since this value was very low compared to levels obtained after tablet administration, (i.e.

less than 3 parts per thousand of the measured concentration) we did not further investigate the identity of this chromatographic peak. This observation means, however, that the analytical detection limit for exogenous LD can only reach the statistical limit (see above) if the endogenous value is measured before administration and used for correction.

The efficiency of analyte (and internal standard) extraction in the present procedure is separately determined by the efficiency of the manual deproteinization step and the FIA sample pretreatment step, respectively. The efficiency of the manual deproteinization step was 27.3%. This means that 27.3% of the analytes (and internal standard) was injected into the FIA system. The losses arose at the separation of the precipitated proteins and in the loop filling procedure (where about 60% of remaining sample is used to wash the loop). The losses in the FIA system arose because the width of the FIA peak produced by the perchloric acid washing of the alumina column is $\sim 200\ \mu\text{l}$ while the HPLC injector picks up only the central $50\ \mu\text{l}$ of this peak. The resulting efficiency as determined by experiment was around 40%. The overall extraction efficiency of the whole procedure was thus relatively low, yet – as seen from the reproducibility and calibration data presented – the overall precision and linearity remained quite satisfactory. If the method were to be applied in a situation where higher recovery is required one could apply partial loop filling (with a large loop) in the FIA system. This would increase

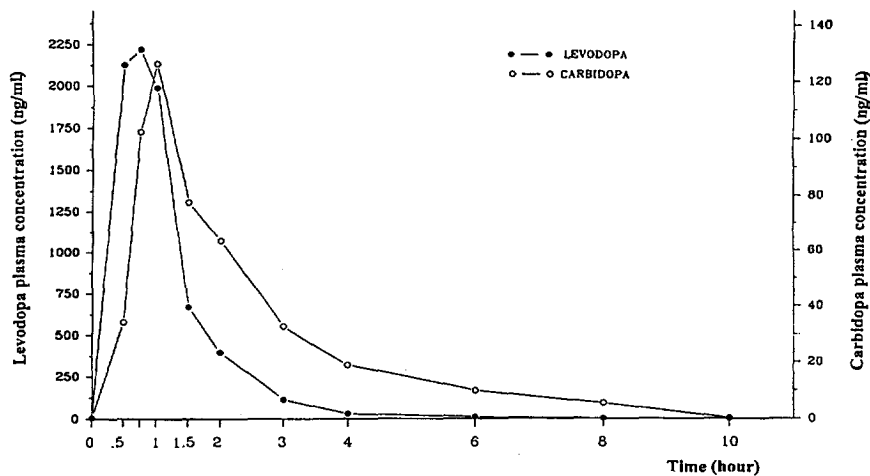


Fig. 4. Pharmacokinetic curve of levodopa and carbidopa in dog after oral administration of Duellin[®] (250/50 mg tablet).

the efficiency by a factor of ~2.5. The HPLC injector loop volume might also be increased albeit to the detriment of good peak separation.

In vivo experiments were carried out on healthy Beagle dogs. Blood samples were collected for the pharmacokinetic study at different times after oral administration of Duellin®. The respective pharmacokinetic curves are shown in Fig. 4. The slope and the smoothness of these curves indicate the adequateness of the analytical procedure. Preliminary data show that the new bioanalytical method can be used also for the determination of carbidopa and levodopa in micro pig and human plasma samples.

4. Summary

Levodopa and carbidopa have been measured with good linearity and precision, sufficiently low detection level and low carryover. Despite the wide and substantially different plasma concentration ranges of the two components the analyses could be carried out simultaneously. These features make the method useful for pharmacokinetic investigation. Manual sample pretreatment was kept at a minimum (de-proteinization). The rest of the sample pretreatment was carried out on a rather simple flow injection type set-up (FIA) which was combined on-line with HPLC. Thus the whole automated system behaved for the operator as a simple HPLC system with sample injections following each other at intervals determined only by the time requirement of the chromatographic separation.

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